Evaluation of Stress Reducing Capacity of Selected Anaesthetics for the Live Transportation of Green Chromide *Etroplus suratensis*

Thesis submitted to

Cochin University of Science and Technology

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in

Fisheries Management

Under the Faculty of Marine Sciences

by

Sindhu M.C

SCHOOL OF INDUSTRIAL FISHERIES
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
KOCHI – 682016

February, 2015

Evaluation of Stress Reducing Capacity of Selected Anaesthetics for the Live Transportation of Green Chromide *Etroplus suratensis*

Ph.D. Thesis under the Faculty of Marine Sciences

Author

Sindhu M.C
Research Scholar
School of Industrial Fisheries
Cochin University of Science and Technology
Kochi - 682016
Email: sindhin@gmail.com

Supervising Guide

Dr. A. Ramachandran
Professor
School of Industrial Fisheries
Cochin University of Science and Technology
Kochi - 682016
Email: rahul_rama@hotmail.com

School of Industrial Fisheries
Cochin University of Science and Technology
Kochi - 682016

February, 2015

Dedicated to

The Small Scale Fish Farmers of Kerala

SCHOOL OF INDUSTRIAL FISHERIES
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
KOCHI – 682016

Dr. A. Ramachandran Ph: 9447062400
Professor email:rahul_rama@hotmail.com

Certificate

This is to certify that this thesis entitled **"Evaluation of Stress Reducing Capacity of Selected Anaesthetics for the Live Transportation of Green Chromide *Etroplus suratensis*"** is an authentic record of research work carried out by Mrs. Sindhu M.C. under my supervision and guidance in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the School of Industrial Fisheries, Cochin University of Science and Technology, Kochi-682 016. No part of this thesis has been presented for the award of any other Degree or Diploma or associate ship in any University.

Dr. A. Ramachandran
(Supervising Guide)

Kochi - 682016
February, 2015

SCHOOL OF INDUSTRIAL FISHERIES
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
KOCHI – 682016

Dr. A. Ramachandran Ph: 9447062400
Professor email:rahul_rama@hotmail.com

Certificate

This is to certify that this thesis entitled **"Evaluation of Stress Reducing Capacity of Selected Anaesthetics for the Live Transportation of Green Chromide *Etroplus suratensis*"** is an authentic record of research work carried out by Mrs. Sindhu M.C under my supervision and guidance. I also certify that all the relevant corrections and modifications are suggested by the audience during the pre-synopsis Seminar and recommended by the Doctoral committee of the candidate have been incorporated in the thesis.

Dr. A. Ramachandran
(Supervising Guide)

Kochi - 682016
February, 2015

Declaration

I, SINDHU M.C, do hereby declare that the present work entitled **"Evaluation of Stress Reducing Capacity of Selected Anaesthetics for the Live Transportation of Green Chromide *Etroplus suratensis*"** is an authentic work done by me under the guidance and supervision of Dr. A. Ramachandran, Professor and supervising guide, School of Industrial Fisheries, Cochin University of Science and Technology, Kochi-16. The work presented in the thesis has not been presented for any other degree or diploma earlier.

Sindhu M.C

Kochi - 682016
February, 2015

Acknowledgements

At the outset I wish to thank God, Almighty for his grace and blessing for making this work a successful one.

I would like to express my sincere gratitude to my supervisor, Professor Dr.A Ramachandran, who deserves thanks for his contribution, positive attitude, new ideas and guidance.

I would like to thank my research committee members, Dr. Saleena Mathew, Dr. Harikrishnan for their guidance throughout my work.

I also wish to thank our Director, Professor Dr.K.T .Thomson, and Dr.Mini Sekharan for their guidance.

I would also like to thank Dr. Sudhir, Professor and Head, Department of Ayurveda college, Government of Kerala, Thripunithura.India and Dr. Anil at Government Ayurveda College, Puthiyakavu; Thripunithura, for helping me to study the possible application of plant anesthetics as a tool for transportation of live fishes.

I am also very thankful for the guidance and selfless invaluable analytical assistance of Professor Angel Mathew (Research and Analytical wing UGC), at the Great Maharajas College who also provided selfless invaluable assistance with experimental design and statistical analysis towards completion of the thesis.

Of course, none of this would have been fulfill without the tireless support of Dr. M.Mukundan, the former Director, CIFT, INDIA and Dr.K.S. Purushan, former Dean, College of Fisheries, Panangad, Kochi,… whose are the grate passionate for aquaculture and provided critical reading of the manuscripts, which I am very grateful for.

Thanks to Dr. Sasi Menon, Sajil, Sinu, Sini, Soumya, at the Faculty of Biochemistry, DDC International, Kochi for assistance with blood sampling and analysis.

At KUFOS, I am very grateful to Dr. Manjusha who selflessly gave her time, advice, and support over the years which helped me immensely to finish this thesis promptly.

Also, I am grateful to the library in charges of the School of Marine Sciences at the University of Cochin, Mr.Manuel for their constant encouragement, especially, Thadevus sir is also an ornamental fish hobbyist and culturist, who give practical ideas in shaping my research design.

I am indebted to Marson, Aqua farm Puthuvyppu, Fish farmer and exporter who supplied the plenty of juveniles Etroplus suratensis, for the entire length of my study. It would have been impossible for me to complete the work without his support.

I am grateful to my colleagues Soumya Subrade, Liya Jayalal, Antony, Asha, Anupama, Anjaly, Dayana were always there to help me when ever I was in need. I thank them wholeheartedly.

I sincerely thank staff, School of Industrial Fisheries for their timely helps.

I record my thanks to Mr. Binoop (Indu photos & Graphics) for his perfect sense of commitment which helped a great deal in the layout and design aspect of my thesis.

My brothers Biju and Shaiju were always keen about my research and I thank them for their curiosity.

I owe my father (Late), mother and mother in law for their love, support and confidence throughout the past years.

I am deeply obliged to Nandu and Kunju for their understanding, who were adjust with the thick and thin research days of their Amma.

Last, but certainly not least, I thank my family for their unfailing support and love throughout this journey, especially my husband, Gopinath without whose love, encouragement and understanding, this work would never have been materialized!

And after everything else but not least, all the little fish that gave (?) their life to science—thank you!!

Sindhu M. C

Contents

General Introduction .... 01 - 27

a). Kerala and State Fish "Karimeen" (*Etroplus suratensis*) 16
b). Transportation of juveniles of *Etroplus suratensis* 22
c). Justification 24
d). Scope of the study 25
e). Objectives of the study 26

Review of Literature .... 29 - 47

a). Transportation of Fishes 29
b). Water Quality 30
c). Stress 33
d). Scope of the study 35

Chapter 1

Acute toxicity of selected anaesthetics on juveniles of *Etroplus suratensis* correlates with certain water quality parameters .... 49 - 154

1.1 Introduction 49
1.2 Materials and Methods 53
  1.2.1 Juveniles of *Etroplus suratensis* (Bloch 1790) 53
1.3 General protocol 55
  1.3.1 Specimen collection and acclimatization 55
1.4 Anaesthetic agents 56
  1.4.1 Preparation of anaesthetics agents 56
    1.4.1.1 Preparation of clove oil (*Syzygium aromaticum*) 56
    1.4.1.2 Preparation of cinnamon oil (*Cinnamomum zeylanicum*) 57
    1.4.1.3 Preparation of Cassumunar ginger (*Zingiber cassumunar* Roxb) 58
    1.4.1.4 Preparation of Tobacco leaf extracts (*Nicotiana tobaccum*) 59
    1.4.1.5 Preparation of Tricaine methanesulfonate (MS-222) 60
    1.4.1.6 Preparation of Hypothermic condition 61
1.5 Experimental set up for anaesthetization of fish 63
  1.5.1 Experimental design 63
    1.5.1.1 Determination of acute toxicity (96 h LC_{50}) test 63
    1.5.1.2 Mortality change in pattern of the fish during 24, 48, 72, and 96 h 64

1.5.1.3 Determination of water quality parameters 65
1.5.2 Post treatment survival 65
1.6 Statistical analyses 66
1.7 Results 66
1.7.1 Effect of clove oil 67
1.7.2 Effect of Cinnamon oil 74
1.7.3 Effect of *Zingiber cassumunar* Roxb 81
1.7.4 Effect of Tobacco leaves extract (*Nicotiana tobacum*) 87
1.7.5 Effect of MS-222 (Tricaine methanesulfonate) 94
1.7.6 Effect of Hypothermic condition 100
1.8 Discussion 106
1.8.1 Effect of clove oil 106
1.8.2 Effect of Cinnamon oil 115
1.8.3 Effect of Cassumunar ginger extracts (*Zingiber cassumunar* Roxb) .. 122
1.8.4 Tobacco leaves extract (*Nicotiana tobacum*) 128
1.8.5 Effect of MS-222 137
1.8.6 Effect of Hypothermic condition 143
1.9 Summary 149

Chapter 2

Behavioural assays and safest level of selected anaesthetics during bath administration in different concentrations on juveniles of *Etroplus suratensis*.... 155 - 233

2.1 Introduction 155
2.2 Materials and Methods 166
2.2.1 Fish and experimental conditions 166
2.2.2 Experiments 166
2.2.2.1 Effect of ethanol as an anaesthetic 166
2.2.2.2 Investigation of the safest level and behavioral assays during the exposure of different concentration of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS222 and hypothermia 167
2.2.2.3 Video Recording System 169
2.2.3 Post treatment survival 170
2.3 Statistical analyses 170
2.4 Results 170
2.4.1 Effect of Ethanol as an anaesthetic 170
2.4.2 Effect of Clove oil as an anaesthetic 171

2.4.3 Effect of Cinnamon oil *(Cinnamom zeylanicum)* as an anesthetic 175
2.4.4 Effect of *Zingiber casumunar* (Cassumunar Ginger) as an anaesthetic 178
2.4.5 Effect of Tobacco leaf extracts (*Nicotiana tobaccum*) as an anaesthetic 181
2.4.6 Effect of MS222 as an anaesthetic 184
2.4.7 Effect of Hypothermic condition as an anaesthetic 187
2.4.8 Overall desirability functions of six anaesthetics 189
2.5 Discussion........ 195
2.5.1 Effect of Clove oil as an anaesthetic 195
2.5.1.1 Behavioural induction 195
2.5.1.2 Recovery from anaesthesia 198
2.5.2 Cinnamon........ 201
2.5.2.1 Behavioural induction 201
2.5.2.2 Behavioural recovery 204
2.5.3 *Zingiber casumunar* Roxb (Cassumunar Ginger) 206
2.5.3.1 Behavioural induction 206
2.5.3.2 Behavioural recovery 209
2.5.4 Tobacco leaf extract 211
2.5.4. 1 Behavioral induction 211
2.5.4. 2 Recovery from anaesthesia 215
2.5.5 MS222 (TMS or Tricane methanesulphonate)........ 217
2.5.5.1 Induction of anesthesia........ 217
2.5.5. 2 Behavioural recovery........ 221
2.5.6 Hypothermia (as described by the *AVMA Guidelines on Euthanasia*) 225
2.5.6.1 Induction behavior 225
2.5.6.2 Recovery from hypothermia 228
2.5.7 Overall desirability function at different doses of the six anaesthetic agents........ 231
2.5.8 Limitations of the study 232
2.6 Summary 232

Chapter 3

Haematological studies of juveniles of *Etroplus suratensis* exposed to optimum concentration of selected anaesthetics during 24 and 48 hours .... 235 - 260

3.1 Introduction 235
3.2 Materials and Methods........ 238
3.2.1 Fish and experimental conditions........ 238

3.3 Haematological analysis....................238
3.3.1 Blood sampling procedures....................239
3.4 Statistical analysis....................240
3.5 Post treatment survival....................240
3.6 Results....................241
3.7 Discussion....................248
3.7.1 Clove oil....................250
3.7.2 Cinnamon oil....................252
3.7.3 Cassumunar ginger extract....................253
3.7.4 Tobacco leaf (*Nicotiana tobaccum*)....................253
3.7.5 MS222....................255
3.7.6 Hypothermia....................256
3.8 Summary....................259

Chapter 4

The stress reducing capacity of optimum concentration of selected anaesthetics during 24 and 48 hours of exposure of *Etroplus suratensis*.......... 261 - 307

4.1 Introduction....................261
4.2 Materials and Methods....................264
4.2.1 Fish and experimental conditions....................264
4.3 Biochemical analysis of stress indices....................265
4.3.1 Blood sampling procedures....................266
4.3.2 Blood analytical procedure....................267
4.3.2.1 Estimation of Plasma Cortisol, Glucose and Lactate....................267
4.4 Post treatment survival....................268
4.5 Statistical analysis....................268
4.6 Results....................268
4.6.1 Clove oil....................268
4.6.2 Cinnamon oil....................273
4.6.3 Cassumunar ginger extract....................277
4.6.4 Tobacco leaves extract....................281
4.6.5 MS222 (Tricane methanesulphonate)....................285
4.6.6 Hypothermia....................289
4.7 Discussion....................293
4.7.1 Clove oil....................293
4.7.2 Cinnamon oil....................296
4.7.3 Cassumunar ginger extract....................297
4.7.4 Tobacco leaf extract....................297
4.7.5 MS222....................298

4.7.6 Hypothermia 303
4.8 Summary 307

Chapter 5

Combined anaesthetic effects of optimum concentration of clove oil and hypothermia on juveniles of *Etroplus suratensis* in closed bag transport during 24 and 48 hours .... 309 - 332

5.1 Introduction 309
5.2 Materials and Methods 315
5.2.1 Fish and experimental conditions 315
5.2.2 Experimental designs 316
5.3 The sedative and anaesthetic effect of clove oil, hypothermia and the combination of optimum levels of clove oil and hypothermia 317
5.3.1 The sedative and anaesthetic effects of clove oil on juvenile *Etroplus suratensis* 317
5.3.2 The sedative and anaesthetic effects of hypothermia on juvenile *Etroplus suratensis* 317
5.3.3 The sedative and anaesthetic effects of combined clove oil anaesthesia and hypothermia combinations on juvenile *Etroplus suratensis* 317
5.3.4 Post treatment survival 317
5.3.5 Statistical analyses 318
5.4 Determination of biochemical analysis of stress indices of combinations of clove oil anaesthesia and hypothermia on juvenile *Etroplus suratensis in* closed bag transport during 24 and 48hrs 318
5.4.1 Fish and experimental conditions 318
5.4.2 Experimental designs 318
5.4.3 Biochemical analysis of stress indices 319
5.4.4 Post treatment survival 319
5.4.5 Statistical analysis 320
5.5 Result 320
5.5.1 The sedative and anaesthetic effects of clove oil on juvenile *Etroplus suratensis* 320
5.5.2 The sedative and anaesthetic effects of hypothermia on juvenile *Etroplus suratensis* 321

5.5.3 Sedative and anaesthetic effects of combined clove oil anaesthesia and hypothermia combinations on juvenile *Etroplus suratensis* 323
5.5.4 Biochemical analysis of stress indices 324
5.5.4.1 Sugar 324
5.5.4.2 Lactate 326
5.5.4.3. Cortisol 327
5.6 Discussion 329
5.7 Summary 331

Summary and Conclusions .... 333 - 338

References .... 339 - 397

Appendices .... 399 - 407

Publications .... 409 - 413

List of Tables

Table 1.A. Trimmed Spearman-Karber Method for Estimating LC_{50} on exposure of different concentrations of clove oil 68

Table 1.A.1. Lethal concentrations of clove oil with upper and lower limit of 95% confidence intervals...68

Table 1.B. Trimmed Spearman-Karber Method for Estimating LC_{50} on exposure of different concentrations of cinnamon oil 75

Table 1.B.1. Lethal concentrations of cinnamon oil with upper and lower limit of 95% confidence intervals 75

Table 1.C. Trimmed Spearman-Karber Method for Estimating LC_{50} on exposure of different concentrations of cassumunar ginger extracts *(Zingiber cassumunar* Roxb*)*................................82

Table 1.C.1. Lethal concentrations of cassumunar ginger extract (*Zingiber cassumunar* Roxb) with upper and lower limit of 95% confidence intervals 82

Table 1.D. Trimmed Spearman-Karber Method for Estimating LC_{50} on exposure of different concentrations of tobacco leaf extract 88

Table 1.D.1. Lethal concentrations of tobacco leaf extract with upper and lower limit of 95% confidence intervals.................................89

Table 1.E. Trimmed Spearman-Karber Method for Estimating LC_{50} on exposure of different concentrations of MS-222 (Tricaine methanesulfonate)...95

Table 1.E.1. Lethal concentrations of tobacco leaf extract with upper and lower limit of 95% confidence intervals.................................95

Table 1.F. Trimmed Spearman-Karber Method for Estimating LC_{50} on exposure of different concentrations of hypothermia.............101

Table 1.F.1. Lethal concentrations of tobacco leaf extract with upper and lower limit of 95% confidence intervals...............................101

Table 2.1 Summary statistics of induction and recovery times at different doses of clove oil for *Etroplus suratensis* (Mean ±SEM) 174

Table 2.2 Summary statistics of induction and recovery times at different doses of cinnamon oil for *Etroplus suratensis* (Mean ±SEM)...177

Table 2.3 Summary statistics of induction and recovery times at different doses of cassumunar ginger extract for *Etroplus suratensis* (Mean ±SEM) 180

Table 2.4 Summary statistics of induction and recovery times at different doses of tobacco leaf extract for *Etroplus suratensis* (Mean ±SEM) 183

Table 2.5 Summary statistics of induction and recovery times at different doses of MS222 for *Etroplus suratensis* (Mean ±SEM) 186

Table 2.7 Average values of overall desirability function at different doses of the six anesthetic agents for *Etroplus suratensis* (Mean ±SEM) 189

Table 3.1 Haematological indices of juveniles of *Etroplus suratensis* exposed to optimal concentrations of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS222 and hypothermia during 24 and 48 hours 242

Table 4.1 Plasma glucose levels of *Etroplus suratensis* exposed in optimum concentration of clove oil (1.7mg/ L) during 1, 24 & 48 h 270

Table 4.2 Plasma lactate level of *Etroplus suratensis* exposed in optimum concentration of clove oil (1.7mg/ L) during 1, 24 & 48 h 271

Table 4.3 Plasma cortisol levels of *Etroplus suratensis* exposed in optimum concentration of clove oil (1.7mg/ L) during 1, 24 & 48 h 272

Table 4.4 Plasma glucose level of *Etroplus suratensis* exposed in optimum concentration of cinnamon oil (0.50 mg/ L) during 1, 24 & 48 h 274

Table 4.5 Plasma lactate level of *Etroplus suratensis* exposed in optimum concentration of cinnamon oil (0.50 mg/ L) during 1, 24 & 48 h 275

Table. 4.6 Plasma cortisol level of *Etroplus suratensis* exposed in optimum concentration of cinnamon oil (0.50 mg/ L) during 1, 24 & 48 h 276

Table 4.7 Plasma glucose level of *Etroplus suratensis* exposed in optimum concentration of cassumunar ginger extract (1.30 mg/ L) during 1, 24 & 48 h 278

Table 4.8 Plasma lactate level of *Etroplus suratensis* exposed in optimum concentration of cassumunar ginger extract (1.30 mg/ L) during 1, 24 & 48 h 279

Table 4.9 Plasma cortisol level of *Etroplus suratensis* exposed in optimum concentration of cassumunar ginger extract (1.30 mg/ L) during 1, 24 & 48 h 280

Table 4.10 Plasma glucose level of *Etroplus suratensis* exposed in optimum concentration of Tobacco leaves extract (6 mg/ L) during 1, 24 & 48 h 282

Table 4.11 Plasma lactate level of *Etroplus suratensis* exposed in optimum concentration of Tobacco leaves extract (6 mg/ L) during 1, 24 & 48 h 283

Table.4.12 Plasma cortisol level of *Etroplus suratensis* exposed in optimum concentration of Tobacco leaves extract (6 mg/ L) during 1, 24 & 48 h 284

Table.4.13 Plasma glucose level of *Etroplus suratensis* exposed in optimum concentration of MS222 (52.2mg/ L) during 1, 24 & 48 h 286

Table.4.14 Plasma lactate level of *Etroplus suratensis* exposed in optimum concentration of MS222 (52.2mg/ L) during 1, 24 & 48 h 287

Table.4.15 Plasma cortisol level of *Etroplus suratensis* exposed in optimum concentration of MS222 (52.2mg/ L) during 1, 24 & 48 h 288

Table.4.16 Plasma glucose level of *Etroplus suratensis* exposed in optimum concentration of Hypothermia (16°C/L) during 1, 24 & 48 h 290

Table.4.17 Plasma lactate level of *Etroplus suratensis* exposed in optimum concentration of Hypothermia (16°C/l) during 1, 24 & 48 h 291

Table.4.18 Plasma cortisol level of *Etroplus suratensis* exposed in optimum concentration of Hypothermia (16°C/l) during 1, 24 & 48 h 292

Table 5.1 Mean ± SEM of induction and recovery times at different doses of hypothermia, clove oil and the combinations of clove oil and hypothermia and overall desirability values 324

Table 5.2 Plasma sugar levels of *Etroplus suratensis* exposed in different doses of hypothermia, clove oil and the combinations of clove oil and hypothermia during 1, 24 & 48 h 326

Table 5.3 Plasma lactate levels of *Etroplus suratensis* exposed in different doses of hypothermia, clove oil and the combinations of clove oil and hypothermia during 1, 24 & 48 h 327

Table 5.4 Plasma cortisol levels of *Etroplus suratensis* exposed in different doses of hypothermia, clove oil and the combinations of clove oil and hypothermia during 1, 24 & 48 h 328

List of Figures

Fig.1.A.1 Change in pattern of temperature (°C) at different concentration of clove oil during 96 h duration 71

Fig.1.A.2 Change in pattern of dissolved oxygen concentration (mg/L) at different concentration of clove oil during 96 h duration 71

Fig.1.A.3 Change in pattern of pH at different concentration of clove oil during 96 h duration 72

Fig.1.A.4 Change in pattern of turbidity (μ) at different concentration of clove oil during 96 h duration 72

Fig.1.A.5 Change in pattern of NH_3 (mg/L) at different concentration of clove oil during 96 h duration 73

Fig.1.A.6 Change in pattern of NO_2- (mg/L) at different concentration of clove oil during 96 h duration 73

Fig.1.A.7 Change in pattern of NO_3- (mg/L) at different concentration of clove oil during 96 h duration 74

Fig.1.B.1 Change in pattern of temperature (°C) at different concentration of cinnamon oil during 96 h duration.......................... 77

Fig.1.B.2 Change in pattern of pH at different concentration of cinnamon oil during 96 h duration... 78

Fig.1.B.3 Change in pattern of DO at different concentration of cinnamon oil during 96 h duration... 78

Fig.1.B.4 Change in pattern of turbidity at different concentration of cinnamon oil during 96 h duration... 79

Fig.1.B.5 Change in pattern of NH_3 at different concentration of cinnamon oil during 96 h duration... 79

Fig.1.B.6 Change in pattern of NO_2- at different concentration of cinnamon oil during 96 h duration... 80

Fig.1.B.7 Change in pattern of NO_3- at different concentration of cinnamon oil during 96 h duration... 80

Fig.1.C.1 Change in pattern of temperature (°C) at different concentration of *Zingiber cassumunar* Roxb extract during 96 h duration 84

Fig.1.C.2 Change in pattern of pH at different concentration of *Zingiber cassumunar* Roxb extract during 96 h duration 84

Fig.1.C.3 Change in pattern of DO at different concentration of *Zingiber cassumunar* Roxb extract during 96 h duration 85

Fig.1.C.4 Change in pattern of turbidity at different concentration of *Zingiber cassumunar* Roxb extract during 96 h duration 85

Fig.1.C.5 Change in pattern of NH_3+ at different concentration of *Zingiber cassumunar* Roxb extract during 96 h duration 86

Fig.1.C.6 Change in pattern of NO_2- at different concentration of *Zingiber cassumunar* Roxb extract during 96 h duration 86

Fig.1.C.7 Change in pattern of NO_3- at different concentration of *Zingiber cassumunar* Roxb extract during 96h duration 87

Fig.1.D.1 Change in pattern of temperature (°C) at different concentration of tobacco leaf extract (*Nicotiana tobacum*) during 96 h duration 90

Fig.1.D.2 Change in pattern of pH at different concentration of tobacco leaf extract (*Nicotiana tobacum*) during 96 h duration 91

Fig.1.D.3 Change in pattern of DO at different concentration of tobacco leaf extract (*Nicotiana tobacum*) during 96 h duration 91

Fig.1.D.4 Change in pattern of turbidity at different concentration of tobacco leaf extract (*Nicotiana tobacum*) during 96 h duration 92

Fig.1.D.5 Change in pattern of NH_3+ at different concentration of tobacco leaf extract (*Nicotiana tobacum*) during 96 h duration 92

Fig.1.D.6 Change in pattern of NO_2- at different concentration of tobacco leaf extract (*Nicotiana tobacum*) during 96 h duration 93

Fig.1.D.7 Change in pattern of NO_3- at different concentration of tobacco leaf extract (*Nicotiana tobacum*) during 96 h duration 93

Fig.1.E.1 Change in pattern of temperature at different concentration of MS-222 (Tricaine methanesulfonate) during 96 h duration 97

Fig.1.E.2 Change in pattern of pH at different concentration of MS-222 (Tricaine methanesulfonate) during 96 h duration 97

Fig.1.E.3 Change in pattern of DO at different concentration of MS-222 (Tricaine methanesulfonate) during 96 h duration 98

Fig.1.E.4 Change in pattern of turbidity at different concentration of MS-222 (Tricaine methanesulfonate) during 96 h duration 98

Fig.1.E.5 Change in pattern of NH_3+ at different concentration of MS-222 (Tricaine methanesulfonate) during 96 h duration 99

Fig.1.E.6 Change in pattern of NO_2- at different concentration of MS-222 (Tricaine methanesulfonate) during 96 h duration 99

Fig.1.E.7 Change in pattern of NO_3- at different concentration of MS-222 (Tricaine methanesulfonate) during 96 h duration 100

Fig.1.F.1 Change in pattern of pH at different concentration of hypothermia during 96 h duration 103

Fig.1.F.2 Change in pattern of DO at different concentration of hypothermia during 96 h duration 103

Fig.1.F.3 Change in pattern of turbidity at different concentration of hypothermia during 96 h duration 104

Fig.1.F.4 Change in pattern of NH_3^+at different concentration of hypothermia during 96 h duration 104

Fig.1.F.5 Change in pattern of $N0_2$- at different concentration of hypothermia during 96 h duration 105

Fig.1.F.6 Change in pattern of NO_3^- at different concentration of hypothermia during 96 h duration 105

Fig.2.1 Non-linear regression analyses showing the effects of gradients of clove oil concentrations on the induction behavior and recovery of *Etroplus suratensis*. 174

Fig.2.2 Non-linear regression analyses showing the effects of gradients of cinnamon oil concentrations on the induction behavior and recovery of *Etroplus suratensis* 177

Fig. 2.3 Non-linear regression analyses showing the effects of gradients of cassumunar ginger extract (Zn) concentrations on the induction behavior and recovery of *Etroplus suratensis* 180

Fig.2.4 Non-linear regression analyses showing the effects of gradients of tobacco leaf extract concentrations on the induction behavior and recovery of *Etroplus suratensis* 183

Fig. 2.5 Non-linear regression analyses showing the effects of gradients of MS222 concentrations on the induction behavior and recovery of *Etroplus suratensis* 186

Fig.2.6 Non-linear regression analyses showing the effects of gradients of hypothermia concentrations on the induction behavior and recovery of *Etroplus suratensis* 189

Fig.2.7.1 Average values of overall desirability function at different doses of clove oil for *Etroplus suratensis* 192

Fig.2.7.2 Average values of overall desirability function at different doses of cinnamon oil for *Etroplus suratensis* 192

Fig.2.7.3 Average values of overall desirability function at different doses of cassumunar ginger extract for *Etroplus suratensis* 193

Fig.2.7.4 Average values of overall desirability function at different doses of tobacco leaf extract for *Etroplus suratensis* 193

Fig. 2.7.5 Average values of overall desirability function at different doses of MS222 for *Etroplus suratensis* 194

Fig. 2.7.6 Average values of overall desirability function at different doses of hypothermia for *Etroplus suratensis* 194

Fig.3.1 Haematocrit (HCT %) of *Etroplus suratensis* exposed in optimum concentration of certain anaesthetics during 24 & 48 h 243

Fig.3.2 Hemoglobin (Hb (g)) of *Etroplus suratensis* exposed in optimum concentration of certain anaesthetics during 24 & 48 h 243

Fig.3.3 PCV (%) of *Etroplus suratensis* exposed in optimum concentration of certain anaesthetics during 24 & 48 h............ 244

Fig.3.4 RBC (million) of *Etroplus suratensis* exposed in optimum concentration of certain anaesthetics during 24 & 48 h............ 244

Fig.3.5 MCV (mu) of *Etroplus suratensis* exposed in optimum concentration of certain anaesthetics during 24 & 48 h............ 245

Fig.3.6 MCH (pg) of *Etroplus suratensis* exposed in optimum concentration of certain anaesthetics during 24 & 48 h............ 245

Fig.3.7 MCHC (%) of *Etroplus suratensis* exposed in optimum concentration of certain anaesthetics during 24 & 48 h..................246

Fig. 4.1 Plasma glucose level of *Etroplus suratensis* exposed in optimum concentration of clove oil (1.7mg/ L) during 1, 24 & 48 h 270

Fig. 4.2 Plasma lactate level of *Etroplus suratensis* exposed in optimum concentration of clove oil (1.7mg/ L) during 1, 24 & 48 h 271

Fig.4.3 Plasma cortisol level of *Etroplus suratensis* exposed in optimum concentration of clove oil during 1, 24 & 48 h................272

Fig.4.4 Plasma glucose level of *Etroplus suratensis* exposed in optimum concentration of cinnamon oil (0.50 mg/ L) during 1, 24 & 48 h 274

Fig. 4.5 Plasma lactate level of *Etroplus suratensis* exposed in optimum concentration of cinnamon oil (0.50 mg/ L) during 1, 24 & 48 h 275

Fig. 4.6 Plasma cortisol level of *Etroplus suratensis* exposed in optimum concentration of cinnamon oil (0.50 mg/ L) during 1, 24 & 48 h 276

Fig. 4.7 Plasma glucose level of *Etroplus suratensis* exposed in optimum concentration of cassumunar ginger extract (1.30 mg/ L) during 1, 24 & 48 h...278

Fig. 4.8 Plasma lactate level of *Etroplus suratensis* exposed in optimum concentration of cassumunar ginger extract (1.30mg/ L) during 1, 24 & 48 h..279

Fig. 4.9 Plasma cortisol level of *Etroplus suratensis* exposed in optimum concentration of cassumunar ginger extract (1.30mg/ L) during 1, 24 & 48 h 280

Fig.4.10 Plasma glucose level of *Etroplus suratensis* exposed in optimum concentration of Tobacco leaves extract (6 mg/ L) during 1, 24 & 48 h 282

Fig.4.11 Plasma lactate level of *Etroplus suratensis* exposed in optimum concentration of Tobacco leaves extract (6 mg/ L) during 1, 24 & 48 h 283

Fig.4.12 Plasma cortisol level of *Etroplus suratensis* exposed in optimum concentration of Tobacco leaves extract (6 mg/ L) during 1, 24 & 48 h 284

Fig.4.13 Plasma glucose level of *Etroplus suratensis* exposed in optimum concentration of MS222 (52.2mg/ L) during 1, 24 & 48 h 286

Fig.4.14 Plasma lactate level of *Etroplus suratensis* exposed in optimum concentration of MS222 (52.2mg/ L) during 1, 24 & 48 h 287

Fig.4.15 Plasma cortisol level of *Etroplus suratensis* exposed in optimum concentration of MS222 (52.2mg/ L) during 1, 24 & 48 h 288

Fig.4.18 Plasma cortisol level of *Etroplus suratensis* exposed in optimum concentration of Hypothermia (16°C/l) during 1, 24 & 48 h 290

Fig. 5.1 Plasma glucose level of *Etroplus suratensis* exposed in optimum concentration of different doses of hypothermia, clove oil and the combinations of clove oil and hypothermia during 1, 24 & 48 h..326

Fig. 5.2 Plasma lactate level of *Etroplus suratensis* exposed in optimum concentration of different doses of hypothermia, clove oil and the combinations of clove oil and hypothermia during 1, 24 & 48 h..327

Fig. 5.3 Plasma cortisol level of *Etroplus suratensis* exposed in optimum concentration of different doses of hypothermia, clove oil and the combinations of clove oil and hypothermia during 1, 24 & 48 h 328

List of Abbreviations and Notations

ALT	-	Alanine aminotransferase
ANOVA	-	Analysis of variance
APHA	-	American Public Health Association
AST	-	Aspartate aminotransferase
ATP	-	Adinosine triphosphate
AVMA	-	American Veterinary Medical Science Association
CaCO3	-	Calcium carbonate
CIBA	-	Central Institute of Brackish water Aquaculture
CIN	-	Cinnamon oil
CL	-	Clove oil
CNS	-	Central nervous system
Co_2	-	carbon dioxide
°C	-	degree celsious
DMPBD	-	3, 4-dimethoxyphenyl butadiene
DO	-	Dissolved oxygen
DOA	-	death on arrival
°F	-	Fahrenheit
FDA	-	*Food and Drug Administration*
FIRMA	-	State fisheries resource management society
FRP	-	Fiberglass reinforced plastic
g	-	Gram
GRAS	-	Generally recognized as safe analysis of variance
h	-	Hour
Hb	-	Hemoglobin
Hb	-	Haemoglobin
HPI	-	Hypothalamus pituitary axis
Ht	-	Haematocrit
I_1	-	First stage of induction
I_2	-	Second stage of induction
I_3	-	Third stage of induction
IACUC	-	*Institutional Animal Care and Use*

ICAR	-	Indian council of agricultural research
Karimeen	-	pearlspot
Kg	-	kilogram
KUFOS	-	*Kerala University of Fisheries and Ocean Studies*
L	-	Litre
$L.L^{-1}$	-	Litter per litter
L^{-1}	-	Per litre
LC_{50}	-	Lethal concentration, 50%
LD_{50}	-	Lethal dose, 50%
LDPE	-	Low density polyethylene bags
MCH	-	Mean corpuscular haemoglobin
MCHC	-	Mean corpuscular haemoglobin concentration
MCV	-	Mean corpuscular volume
mg / kg / day	-	Milligram per kilogram per day
mg/L	-	Milligram per litre
ml	-	millilitre
mL^{-1}	-	Per millilitre
MOCA	-	Methoxycinnamaldehyde
MS-222 (TMS)	-	Tricaine methanesulphonate
MT	-	Metric tones
NAIP	-	National Agricultural Innovation *Project*
$NaNO_2$	-	Sodium nitrite
ng	-	Nano gram
NH_3^+	-	Ammonia
NH_3NO_3	-	Ammonium nitrate
NO_2^-	-	Nitrite
NO_3^-	-	Nitrate
O_2	-	Oxygen
OECD	-	Organization for Economic Co-operation and Development
PCV	-	Packed cell volume
pH	-	hydronium ion concentration
Ppm	-	Parts per million
R_1	-	First stage of recovery

R_2	-	Second stage of recovery
R_3	-	Third stage of recovery
RBC	-	erythrocyte count
RSCB	-	Romanian Society for Cell Biology
TB	-	Tobacco leaf extract
TEC	-	Total erythrocyte count
UNEP	-	U*nited nations environment programme*
WBC	-	total white blood cell count
ZN	-	Cassumunar ginger extract
µM	-	Micrometer
µ	-	Micron
µ/L	-	Micron per Litter

.....ꕤ.....

General Introduction

Contents

a). *Kerala and State Fish "Karimeen" (Etroplus suratensis)*
b). *Transportation of juveniles of Etroplus suratensis*
c). *Justification*
d). *Scope of the study*
e). *Objectives of the study*

The recession and globalization have caused setbacks to the developmental activities in many developing and undeveloped countries. To achieve the 'Millennium developmental goals' it will require higher efforts. Globally South Asian countries show the slowest economic growth and have experienced the biggest setback. India is one such country struggling with a high level of poverty and poverty related issues. Food production and its even distribution are believed to be the most prominent tool to fight world poverty, as it will provide economic growth and employment. Aquaculture is an important contributor to India's food basket and economy with a reported farm-gate value of Rs 76,699 crore during 2012-13 agricultural censuses by the Department of Animal Husbandry, Dairying and Fisheries, Ministry of Agriculture, Government of India, and New Delhi (Annual Report, 2012-2013).

Transport of live aquatic organisms which is more than a century old, perhaps started in the 1870's (Norris et al, 1960). Live fish transportation is an essential practice in aquaculture particularly in rural areas of developing countries representing the only means of supplying fry to small scale aqua culturists (Taylor and Ross, 1988). Very often, large numbers of fry, fingerlings, juveniles and adult fish are being transported from the hatchery to fish farms, fish farms to market, processors and consumers. Live fish command large economic importance in the fresh fish market than dead and iced fish. Medina Pizzali (2001) observed that live fish in the Kolkata market was usually sold at higher prices than dead fish and most consumers were prepared to pay premium prices for live fish, which is considered as the best guarantee of freshness, quality, and intrinsic characteristics of its flesh (better texture and delicate flavour) in comparison with fresh/chilled seafood. Various government and private agencies undertake transport of live fish for commercial live fish market or for artificial propagation of game (Peer Mohamed and Devaraj, 1997). The transporters of live food fish, notably those who carry carps, probably are still second in the total weight of live fish including ornamental fish. In terms of the range of species and distances shipped, tropical fishes stand first in live fish transport (Peer Mohamed and Devaraj, 1997). Like carps, live shrimps, lobsters, crabs etc., are being shipped. Live transportation has become one of the most significant practices in the shrimp industry that constantly endeavour’s to find ways to enhance the final product value (Salin and Vadhyar, 2001). Japanese Kuruma shrimp *Penaeus japonicus* is traditionally transported in live condition. Much research has gone into increasing the marketability of cultured and wild captured shrimp by transporting them live in order to preserve the maximum

freshness. Fresh and live fish are the main products being shipped, though live lobsters are perhaps the seafood with the longest history of shipment. There is an increase in the demand for crabs as a consumer's delight both in India and abroad. An important molluscan product which is gaining ground as a delicacy is the edible oyster which can survive out of water for several days if carefully handled and kept moist and cool (Peer Mohamed and Devaraj, 1997).

The major constraint to the development of live marketing of fish is the lack of information on how to handle the products after they are caught, right through the point of direct sale to customers (Pramod et al., 2010). The main objective of live transportation is to transport as many fish as possible with minimal loss and at economic costs (Peer Mohamed and Devaraj, 1997). This activity involves hauling a large number of fish in small quantities of water, which can result in extensive deterioration of water quality. The successful transportation of live fish needs to consider various factors such as optimum number and size of fish, variation in the enroute temperature, duration of transportation and above all, the requirements of oxygen (Pandit and Ghosh, 2012).

There are scores of prevailing practices of live transportation as a means for increasing the acceptability of the different species based on the conditions of live packing and the packing media. But, the traditional mode of transport was in open earthen pots and metal containers (Jhingran, 1975; Amend et al., 1982; Berka, 1986). There are three general methods of transporting live fish. First, they may be transported without any water at all under certain circumstances, except for being kept damped. The second method, which will be referred to as the "tank method" is to transport them in

containers of various types open to the atmosphere. The third method, which may be called the "plastic-bag method", is to transport fish in a closed bag partly filled with water, and with pure oxygen in the space over the water (Lim et al., 2003).

During the transportation of live fish in plastic bags fish health is likely to be affected by changes in various water quality parameters. The parameters to be considered are temperature, dissolved oxygen, pH, carbon dioxide, ammonia, and the salt balance of the fish's blood. The rate of change of each parameter will be affected by the weight and size of fish to be transported and the duration of transport (Berka, 1986). High water temperatures, turbulence, and reduced oxygen levels during transport may add to the general stress associated with fish harvest and transport. In addition, water temperature and oxygen levels may impact other water quality parameters (ammonia, nitrite, pH, and CO_2) that could influence the level of stress experienced by fish during transport (Amend et al., 1982; Erikson et al., 1997; Patterson et al., 2003). During transportation, fish metabolism is three times higher than the normal (Froese, 1998). Low oxygen capacity of water and the accumulation of metabolic end products such as ammonia and carbon dioxide are often noticed in the closed transporting system.

The duration of the transport period varies according to the distance to be covered and the methods being used. Accompanying the growth of the aquaculture industry, there is an increase in the demand for appropriate methods for live fish transportation, over extended periods of time i.e. >8 hours). In many cases the grow-out farms/ponds for farmed fish (ponds, cages, net pens, etc.) are distant from the hatchery or the nursery location that supplies the juveniles.

In reality the transportation of fish seed from a hatchery to far and wide locations within a country and even abroad requires high expenditure. On the farm, transportation time is usually very short for a few minutes up to 30 minutes at room temperature (28°C). Beyond the farm, transport time is usually longer, varying from a few hours to one or two days (Berka, 1986) at 22°C in sealed polythene bags filled with water and over- saturated with oxygen. This necessitates the transportation of large quantities of fish by land, sea, and air freight (Harmon, 2009). Shipping costs can be substantial, depending on the quantity of the aquatic media within which fish must be shipped (Guo et al., 1995; Lim et al., 2003; Paterson et al., 2003). Therefore, it is in the best interest of buyers and sellers to transport fish in ways that minimize shipping costs while maximizing fish survival (Norris et al., 1960; Lim et al., 2003; Harmon, 2009).

The transport/logistics inevitably involved stress the animals, causing post-transport mortality, and exporters are expected to compensate customers for losses exceeding 5% death on arrival (DOA) industry standard (Lim et al., 2003). A stressed fish will have increased metabolic rate, which gives increased metabolic load that will in turn impair the water quality parameters. Transportation stress also adversely affects the health and appearance of fishes as well as reduces their post- transport marketability due to fin erosion, scale loss, and erythema etc., (Crosby, 2008). As a warranty for the buyer, the industry standard states that the exporters are expected to compensate for a loss that exceeds 5 % death of arrival (DOA) (Lim et al., 2003). High DOA results in low quality products and low economic benefit for the exporter (Pramod et al., 2010). Herein the transportation process, ideally the fish should arrive in good physiological conditions to meet the criteria demanded

by the buyer (Carneiro et al., 2002). Like ornamentals (Tlusty et al., 2005; Watson, 2000) high post-transport mortality from rural communities is a common problem in live fish transportation due to absence of equipment, holding facilities and undeveloped infrastructure in combination with poor handling techniques. To supply a resistant and healthy fish, it is necessary to establish proper handling management that will avoid handling-related stress.

Several techniques have been developed and perfected for the maintenance of live fish in captivity, packing and transportation to distant places by road, sea and air, using various techniques to minimize stress and increase survival of the fish before, during, and after the transportation period (Carmichael et al., 1984; Weirich and Tomasso, 1991; Weirich et al., 1992; Gomes et al., 2003a; Harmon, 2009). Air shipment of live aquatic products has increased rapidly during the last few years. There are many obvious advantages for fish traders to air-transport live selected high-value fishery products (Peer Mohamed and Devaraj, 1997). The techniques include starving fish before packing (Phillips and Brockway, 1954; Nemato, 1957), lowering the temperature of transport water (Phillips and Brockway, 1954; Norris et al., 1960; Lim and Chua, 1993), addition of anaesthetics (Takashima et al., 1983; Teo and Chen, 1993), chemicals or drugs (Ling et al., 2000), and removal of metabolites or cooling the water in an attempt to reduce the metabolic rate of the fish (Ross and Geddes, 1979; Hersh, 1984; Ross and Ross, 1984; Frose, 1985; Yamamitsu and Itazawa, 1988; Putro, 1989; Teo and Chen, 1993; Chow et al., 1994).

There is a definite time limit for transportation of fish without water, but for very hardy species this can be a substantial period. According to Peer Mohamed and Devaraj (1997), on the west coast of North America, the long-

jawed mud sucker, *Giffichthys mirabilis*, is flown in moist moss from the lagoons of Mexico to California (Peer Mohamed and Devaraj, 1997). The Mummichog, *Fundulus heteroclitus,* of the Atlantic coast is transported for bait in a similar, fashion. McDonald (1984) demonstrated that carp would survive long periods of time in limited amounts of water. Various salmonids (Cuerrier, 1952), northern pike and the yellow pike perch (Schultz, 1956) have been transported in crushed ice or moss with ice. These fishes were all relatively large from one to several kilos in weight. They were immobilized by tranquilizing substances like urethane or MS 222 and packed with less stocking density.

Anaesthetizing with chemicals has been used for the transportation of live fish in recent times. Sedation of fish brings about practical benefits such as reduction in overall stress on the-fish, decrease in metabolic rates, oxygen consumption, carbon dioxide production and excretion of toxic wastes, control in excitability of the fish and thereby reduction in the metabolic rates, swimming activity and chances of physical injury, reduction in the time required for handling them (Peer Mohamed and Devaraj, 1997).

A number of anaesthetics, including Tricaine methanesulfonate (MS-222), benzocaine, 2-phenoxyethanol, quinaldine sulphate, metomidate, and lidocaine have been used in juvenile fish transportation (Guo et al., 1995; Myszkowski et al., 2003; Park et al., 2009; Pramod et al., 2010) which are hazardous, expensive and not very effective (Munday and Wilson, 1997; Erdmann, 1999). The most inexpensive method of tranquilizing fish is the use of cold water (5 to 10°C water) as a transporting medium without any chemical tranquilizer. But this method is impracticable in tropical and

subtropical regions because of difficulty in getting and maintaining cold water during transport. If cold water as a transporting medium is not available, and then chemical tranquilizers should be used before transporting larger fish and brood fish (Peer Mohamed and Devaraj, 1997). Though these anaesthetics are effective, only tricaine has been permitted to use in food fish anaesthesia mainly due to environmental and health risks (Marking and Meyer, 1985).

Because of the many drawbacks of current anaesthetics there is need for an alternative anaesthetic (Ross and Ross, 1999) that is effective, convenient for use, low cost, available to third world countries, good margin of safety for fish and is nontoxic to humans and the environment. Due to concern about animal welfare and potential suffering caused to aquaculture fish, derivation of the term “Good anaesthetic” (Ashley, 2007) is in practice. “Green anaesthetic” **(**Ramanayaka and Atapattu, 2006) viz., Plant extracts are potential sources of new anaesthetics with low environmental and health risks and have long been used by indigenous tribes of South America and almost every other continent as part of their arsenal of fishing tools **(**Power et al., 2010).

The present work attempts to assess the stress reducing capacity of selected anaesthetics for the live transportation of Green chromide (*Etroplus suratensis*).The natural anaesthetics selected for this study were; clove oil (*Eugenia aromatica*), cinnamon oil (*Cinnamomum zeylanicum*), Cassumunar ginger **(***Zingiber cassumunar*) extract, tobacco leaf (*Nicotiana tobaccum*) extract, in comparison with the chemical anaesthetic MS-222 (Tricaine methanesulphonate) and the physical anaesthetic cold (hypothermia) condition are the most used within the Asian aquaculture sector, and are generally considered effective and safe in use.

Clove oil (Plate1.1) recently has become a commonly used anaesthetic that can serve as an alternative to Tricaine methanesulfonate in commercial (non-food) fish and fish industries in the United States and Japan (Hikasa et al., 1986). Clove oil is considered to be a potential fish anaesthetic (Woody et al., 2002). This oily substance is distilled from buds, leaves and stems of the clove tree (*Eugenia aromatica*). The main chemical ingredient of clove oil is eugenol (70-98%; Taylor and Roberts, 1999), which is reported to possess high antibacterial and antifungal activity (Karapmar and Aktug, 1987; Briozzo et al., 1989). It is non-carcinogenic and non-mutagenic (Nagababu and Lakshmaiah, 1992). Eugenol has been successfully used as an anaesthetic in rabbit fish (Soto and Burhanuddin, 1995); gold fish, crucian carp (Endo et al., 1972) and Indian major carps (Farid, 1999).

Cinnamomum zeylanicum (Plate1.2) is one of the oldest herbal medicines known, having been mentioned in Chinese manuscripts as long as 4,000 years ago. True cinnamon (*C. zeylanicum*) is among 300 species of Cinnamomum that belong to the Lauraceae family. It is often used for medicinal purposes due to its unique properties. The essential oil from *Cinnamomum zeylanicum* bark is rich in trans-cinnamaldehyde with antimicrobial effects against animal and plant pathogens, food poisoning and spoilage bacteria and fungi (Mastura et al., 1999). Until now, more than 300 volatiles were found as constituents of essential oils of cinnamon. Major compounds present in cinnamon stem-bark oil and cinnamon root-bark oil are cinnamaldehyde (75%) and camphor (56%), respectively. Senanayake and colleagues (1978) identified 53 constituents along with the major component eugenol (81-84.5%) in cinnamon leaf oil. The main properties of cinnamon are astringent, warming, stimulating, carminative, antiseptic, sedative, antifungal,

antiviral, blood purifying, and aiding digestion. All of these properties of cinnamon make it a good medicinal plant.

Cassumunar ginger (*Zingiber cassumunar* Roxb) (Plate1.3) commonly known as plai, is widely used in folklore remedies as a single plant or as component of herbal recipes in Thailand and many Asian countries for the treatments of conditions, such as: inflammation, sprains and strains, rheumatism, muscular pain, wounds and asthma, cough and respiratory problems, and as a mosquito repellent, a carminative, a mild laxative, muscle relaxant and an antidysenteric agent, (Wanauppathamkul, 2003; Pithayanukul et al., 2007). *Zingiber cassumunar* has local anaesthetic activity similar to iodocaine on nerve action potential of sciatic nerve (Ansary, 2009). The essential plai oil, distilled from rhizome extracts, has proven to be extremely useful for human health. Plai oil has a pale amber color, cool scent and a green peppery odor. Plai oil has anti-inflammatory effect and exhibits antimicrobial activity (Wasuwat et al., 1989; Giwanon et al., 2000; Pithayanukul et al., 2007; Tripathi et al., 2008), Active chemicals of plai oil have been identified as sabinene (25-45%) γ-tepinene (5-10%), α-tepinene (2-5%), terpinen 4-ol (25-45%), and (E)-1-(3,4-dimethoxyphenyl butadiene) (DMPBD) (1-10%) (Wanauppathamkul, 2003). DMPBD, as a pure compound isolated from plai, has shown anti-inflammatory activity (Ozaki et al., 1991; Jeenapongsa et al., 2003). Terpinen-4-ol and sabinene were found as the major constituents of plai oil and their antimicrobial activities were reported in comparison with commercial terpinen-4-ol (Wasuwat et al., 1989; Giwanon et al., 2000). The rhizome oil of plai was found to exhibit high activity against dermatophytes and yeasts (Pithayanukul et al., 2007). Plai is an important medicinal plant and there are many regions where plai is cultivated in Thailand.

Tobacco (Plate1.4) is the common name for the plant *Nicotiana tobacum*. It is a native of tropical and subtropical America but it is now commercially cultivated worldwide (Knapp, 2004) and Brazil is the largest producer of tobacco leaves. Tobacco contains the following phytochemicals: Nicotine, Anabasine (an alkaloid similar to the nicotine but less active), Glucosides (tabacinine, tabacine), 2,3,6-Trimethyl-1,4-naphthoquinone, 2-Methylquinone, 2-Napthylamine, Propionic acid, Anatalline, Anthalin, Anethole, Acrolein, Anatabine, Cembrene, Choline, Nicotelline, Nicotianine and Pyrene and they are generally recognized as being a narcotic. The active ingredient of the plant used, is the nicotine (Hassal, 1982). Nicotine (C_5H_4N)-CH-$(CH_2)_3$-N-(CH_3) is made up of pyridine and pyriolidine ring. This property makes it useful as narcotics, molluscicides, piscicides and pesticides (Aleem, 1983; Agbon et al., 2002). Nearly all the nicotine is produced in the root and transported to the leaves for storage. It is soluble in water, alcohol, chloroform, ether, kerosene and some fixed oils (Vogue, 1984). Tobacco leaf dust has been used as an effective insecticides and treatment of predators/pest in the water (pond) since it is completely biodegradable (Aleem, 1987; Tobor, 1990).

Tricaine methanesulfonate (MS-222) (Plate1.5) (alternative names: tricaine, Finquel™, metacaine, ethyl m-amino benzoate methanesulphonate) is the most common anaesthetic agent used on fish and is a benzocaine derivative (Marking and Meyer, 1985). It is a water soluble powdered substance (solubility 1 g/0.8 ml), which is typically buffered with sodium bicarbonate to reduce its acidic properties. Its two parts are methane sulphonic acid (a strong acid) and ethyl m-amino benzoate (a weak base) which causes solutions of MS-222 to be acidic. However, this anaesthetic agent is regarded as a carcinogenic and also a 21-day withdrawal period is

required if the fish is intended for human consumption (Kolanczyk et al., 2003; Rombough, 2007). Yoshimura et al., 1981 reported that it is not mutagenic. Additionally, MS- 222 is relatively expensive and frequently unavailable in many countries due to several international restrictive rules regarding importation of this chemical. It is the only FDA-approved anaesthetic for use in fish intended for use as food, with a required 21-day withdrawal period prior to human consumption.

Hypothermia (cold shock) (Plate1.6) is known to reduce the stress in fish handling; either by itself or in combination with chemical anaesthetics (Rodman, 1963; Hovda and Linley, 2000; Ross and Ross, 1999). Cold shock can be defined as an acute decrease in ambient temperature that has the potential to cause a rapid reduction in body temperature, resulting in a cascade of physiological and behavioural responses. The basic principle behind the technology of live transportation is temperature induced cold anaesthetization. The water temperature is brought down to a limit at which the metabolic rate of the animal is reduced to a minimum, so that its storage and transport in this condition does not affect any apparent increase in metabolic rate. In mammals, exposure to cold temperatures may result in anaesthesia (Martin, 1995). Other teleost fish actually lack receptors that respond to cold and likely do not experience pain associated with cold (Ashley et al., 2006). The movements of the cold-anaesthetized shrimp are minimum, there is no stress caused by vibrations, noise and light; weight loss is usually negligible, and the animals produce no excreta because there is no feed intake and metabolism (Schoemaker, 1991). Although these studies provide valuable insights into cold exposure in other poikilotherms, one cannot draw direct correlations specific to fish, especially tropical species,

from these data. Although cold-shock stress has traditionally been viewed as a cause of sub lethal and lethal effects on fish and fish populations, a recent shift in research has focused on the application of cold-shock stress as a powerful tool in fisheries science (Donaldson et al., 2008). It has seen applications as a short-term anaesthetic (Hovda and Linley, 2000), as a means to alter embryonic sex ratios (Craig et al., 1996) and most commonly as an agent in the induction of polyploidy (Peruzzi et al., 2007). Cold shock is more common than warm shock in experiments with warm-water fish, such as *O. aureus* (Donaldson et al., 2008).

Plate 1.1 a. Clove (*Syzygium aromaticum*)

b. Flower bud (WHO/PLIM)

c. Clove oil

Plate 1.2. a. *Cinnamomum zeylanicum*

b. *Cinnamomum zeylanicum* stem bark

c. Cinnamon oil

Plate1.3 a. Cassumunar ginger **b.** Cassumunar ginger extract

Plate1.4 a. Tobacco leaf **b.** Tobacco leaf extract

Plate1.5 Tricaine methanesulfonate (MS-222)

Plate1.6 Hypothermia (cold shock)

a. Kerala and State Fish "Karimeen" (*Etroplus suratensis*)

Biologically the Green chromide, *Etroplus suratensis* (plate 1.7) is undoubtedly the most ideal estuarine fish species of commercial importance. It possesses certain requisite qualities essential for aquaculture such as good body weight, growth rate, and high adaptability for food, tasty and nutritive flesh (Mukundan and James, 1978) and good market price (Joseph, 1980;

Jayaprakash and Phil, 1980; Rattan, 1994). It is widely distributed and extensively found along the east and south-west coasts of Peninsular India and Srilanka (Padmakumar et al., 2012). Among the two cichlid species indigenous to Asia, the pearlspot (*Etroplus suratensis,* Cichlidae), popularly known as "Karimeen" in malayalam, is a widely cultured species in the Indo-pacific region and is known to breed in confined waters (Hora and Pillay, 1962). Commercial culture of pearlspot in the different agro climatic regions of India have been described by Jhingran and Natarajan, (1973); Thampy, (1980); Sumitra et al., (1981). Being euryhaline, the species could easily be acclimatized to fresh waters indicating its suitability in pond aquaculture (Devaraj et al., 1975). However, the successful culture of any endemic species depends on the efficient management of the farming system. In India though it is found in the southern states, the fish is cherished and used as a delicious food mainly in Kerala. *Etroplus suratensis* is also maintained elsewhere as aquarium fish because of its illuminant green colour with dark spot (Rattan, 1994). With that intention, small individuals are utilized heavily for the export trade. It is proven that the life span of pearl spot in aquarium tanks is more than 8 years. Even though it was officially announced as the "State Fish of Kerala" (National Bureau of Fisheries and Genetic Resources) only in the year 2010, karimeen is the most influential fish in the lifestyle of Kerala (Shyam et al., 2013) from time immemorial.

Plate 1.7 *Etroplus suratensis*

The green chromide, *Etroplus suratensis* is commonly found in the native ecosystems of lakes of Kerala (Vembanadu, Alappuzha), western rivers of Karnataka and the lakes of Andhra Pradesh. Though it is a brackish water fish, it is also found in freshwater reservoirs, lakes and rivers. Even though the fish can grow well in fresh water also, its breeding is limited. In brackish water the fish is a year round breeder and the peak breeding season coincide with the South-West and North-East monsoon seasons when the salinity is low and breeding during other months is limited (Padmakumar et al., 2012). But in artificial ponds or hatcheries where the water qualities can be controlled, regular year-round breeding and seed production of ‘‘Karimeen’’ is possible by appropriate breeding technologies.

Fishermen in Kerala, who have been affected by the trawler ban and bad weather, are trying to make a living by operating in the backwaters where all-time favourite fish, Karimeen is available throughout the year. They fetch anywhere between ` 450-600/kg. Recently Kerala Government has declared

Pearl spot as “State Fish of Kerala” and the state has celebrated the year 2010-2011 as the “The Year of Karimeen” (Vikas, 2012) for creating awareness about the need for conservation and commercial production potential in Kerala. The present annual production of 2,000 MT is found to be insufficient to meet the ever-increasing demands for “Kerala Karimeen” (Pearl spot) among the “natives and foreigners” in the country. Because of its delicacy, shape and beauty, the pearl spot is considered an excellent table fish. It can be farmed under extensive and intensive farming in freshwater, brackish water eco-systems and in homestead ponds in the backyard of houses which can serve as an occupation for poverty alleviation and high valued fish production in states like Kerala (CIBA).

It is estimated that annual production of 10,000 MT would be required to meet the present requirement. This situation mobilized the farmers to initiate Pearl spot culture using wild caught seeds in different parts of the state. At present, the seeds (fry’s/fingerlings) required for the culture in backyard ponds, tanks, artisanal cages etc. are collected from wild. Successful induced breeding of this fish has not been reported so far mainly because of the complex breeding behaviour of the fish. Over exploitation of indigenous Pearl spot seeds from wild resulted in the depletion of standing stock in recent times. The population of wild pearl spot began to decline in the mid-1970s and continued to decline through the mid-1990s. Now the wild production of pearl spot is reported to have declined to 250 tonnes from 1500 tonnes in 10 years (Padmakumar et al., 2002). As a result of the population decline, pearl spot have been listed as threatened species (Anon, 2013a) under the provisions of the Endangered Species Act, 1973.

Various Committee reports submitted to Government and Central and State Research Institutes also pointed out that the Pearl spot fishery of Kerala is in declining phase. During 1960s, the Pearl Spot fishery of Kerala contributed 1,252 tonnes and it reduced to 200 tonnes in 2002 (Anon, 2013 b) and the share of Pearl Spot in the total inland fish production has declined from 10% of total inland catches in 1990-91 to a further low of about 6% in 2002-03 (Karimeen varsham, 2010-2011). The maximum size and weight of Pearl Spot catches nowadays is also showing a diminishing trend. A series of interventions like the reclamation of shallow stretches of the lake into "padasekharams", construction of embankments, impoundments, spillways and barrages during last century, all oriented to facilitate and intensify rice cultivation have altered the ecology of the dwelling places of Pearl Spot. The disappearance of the once luxuriant, mangrove formations in and around the backwaters of Kerala consequent to ecosystem changes and its correlation with the poor breeding recruitment of Pearl spot is often cited to indicate the direct inter relationship of such fringe vegetation on estuarine fisheries; especially the indigenous fish varieties. With the boom of backwater tourism, the demand for Karimeen, the high valued food fish in Vembanad, is on the increase. Since the indiscriminate exploitation of this most valuable species is to the maximum, any further increasing pressure to exploit this species shall lead to a total disappearance of this species from our waters. This is evident from the decline in average size of this species in catches. Factors believed to contribute to the decline of pearl spot runs include mortality of emigrating juveniles near the industrial areas of Aluva and Chalakkudy on the Periyar rivers, predation on juveniles and adults in the estuary by growing avian populations and high conception, and decreased survival resulting from

cyclic changes in weather and oceanic conditions (Anon, 2013b). Another reason for reducing the production of this species is the lack of availability of healthy breeders for the production of healthy fingerlings. Apart from this, highly polluted mud and water and the burrowing habit of pearl spot have compounded its healthy survival and propagation (Padmakumar et al., 2012). Thus, there is a vital need to produce seeds in captive conditions and supply of the seed to the farmers.

Because of the new initiative, the production is expected to go up to 5,000 tonnes in a year. Certain state and central government institutes like FIRMA, ICAR, CIBA has developed an innovative technology for easy propagation of Pearl spot by breeding under environmental controlled conditions. CIBA has successfully developed a captive brood stock of pearl spot fish in cages from diverse genetic pool (from Pulicat and Muttukadu in Tamil Nadu and Kumarakam in Kerala) and established a simple and effective facility for consistent seed production of this fish species (Source: NAIP Sub-Project on Mass Media Mobilization, DKMA with inputs from CIBA, 2011-2012). An average of 1200 juveniles can be obtained from a pair of parent fishes. The "Matsya Keralam" Scheme has taken up a massive programme for the farming of brackish-water fishes on large-scale increasing the demand for the quality pearl spot fish juveniles. As a beginning, on 2nd July 2011, the hatchery produced seeds of pearl spot were supplied to women self help group fish farmers from Kerala.

The CIBA, Chennai has been approached by the Kerala Government to extend its knowledge and skill with regard to this technology for seed production and farming technology of pearl spot fish, as well as their similar

other programmes to promote fish production in the state. Efforts are also being made to promote pearl spot culture in derelict inland saline wetlands of Karnataka.

b. Transportation of juveniles of *Etroplus suratensis*

Pearl spot is one of the prime high value candidate fish species presently used for brackish water and fresh water fish farming ventures in Kerala. Similarly the hike in the price of this species is mainly due to the scarcity for consumption. So it cannot be seen even on the tables of middle income groups in Kerala. Hence extensive farming of this fish species is very important, but is not being done mainly due to the non-availability of sufficient seed. Seed is the most important input both in agriculture and aquaculture. However, the larviculture of this species remains as bottle neck, failing to achieve economically viable results in commercial aquaculture operations.

Plate1.7.a Juveniles of *Etroplus suratensis*

In the live juvenile fish industry of Kerala, an acceptable percentage of mortality following transportation is equal to 3-4 % of the shipment (Marson peter, Marson Farms, personal communication). Mortalities during and after transportation events are presumably caused by osmoregulatory dysfunction or stress-mediated diseases (Crosby, 2008). To minimize physical damage and death; the fishes must be handled carefully throughout transportation (Ross and Ross, 1999; Francis-Floyd, 1995; Crosby et al., 2006a).

The value of juvenile fishes (plate1.7.1) is directly affected by their marketability (appearance, behaviour and activity level) and survival (Crosby, 2008). Marketability of fishes may be affected by handling (e.g., trapping and netting) and physical abrasion that result in both scale loss and frayed fins prior to, during, and after transportation. Not only can transportation affect marketability, but also survival (Crosby et al., 2006a). Juvenile fishes from Puthuvyppu (Govt. hatchery), Vypin (private hatchery) and KUFOS hatchery are transported state-wide to wholesale facilities, retail facilities, and hobbyists. Juveniles are typically shipped in plastic bags (double) that contain water and oxygen gas at a ratio of approximately 60% oxygen gas to 40% water by volume (Crosby et al., 2006b). The ratio of oxygen gas to water may vary by fish species and size of bag used. The plastic bag is then placed into polystyrene shipping box which provides thermal protection (Ross and Ross 1999, Lim et al., 2003). Ice or heat packs may be used depending on the season and species of fish being shipped. The polystyrene boxes are then placed into a labelled outer cardboard box for transportation (Crosby et al., 2006a).

It is reported that post-transportation mortalities among hatchery reared pearl spot in the state are very high. Although the death rate varies greatly,

the average loss occurring from one to seven days following liberation has been estimated to be ten percent of the fish transported. Here is the importance of application of anaesthetics for the transportation of juveniles of *Etroplus suratensis* simulated in-transit motion, density of fish transported, and pre-hauling starvation period has apparent influence on the magnitude of delayed deaths. Certain environmental conditions are perhaps associated with delayed mortalities. Central institute of fresh water aquaculture has reviewed several studies conducted over the past 20 years on problems related to live fish shipment.

c. Justification

It is very important to select cost effective, natural, ecofriendly sedatives / anaesthetics which can support longer duration of transportation for the growing industry of fish farming. The prohibitive cost (200 US Dollars per 500g MS-222) and non-availability of synthetic anaesthetics is a good reason why aqua culturists must look for available indigenous plant materials that can be used as fish sedatives to reduce cost.

In the case of *Cinnamomum cassia*, and *Zingiber cassumunar*, there are no available data on the possible use of these plants as fish sedatives.

The study is basically important in fisheries development especially in the handling of live fish during tagging, stripping for gametes, weighing and in transport. It is expected that this study would provide solutions to frequent fish losses resulting from excessive stress and strain during aqua cultural operations and transportation.

Etroplus suratensis is a common fauna in the tropical freshwaters of several Asian countries where it is widely used in aquaculture in several Asian countries, hence its selection for this investigation. Approximately 200,000 juveniles of *Etroplus suratensis* are traded nationally and internationally. It is logistically impossible to mark or separate individual fish within such large numbers for observation without affecting their behaviour, so any behavioural study on a commercial transport of *Etroplus suratensis* must be based on group behaviours. With anaesthetization, juveniles of pearl spot take up more of the water column and begin sedating behaviour. Therefore this provides an opportunity to assess fish welfare during transportation.

This is an attempt made for the first time to study the juveniles of *Etroplus suratensis* with different anaesthetics at different concentrations. Clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS-222 and cold water were used to identified the best anaesthetics which have the following characteristics like convenience for use, low cost, available to third world countries, good margin of safety for fish and is nontoxic to humans and the environment. Anaesthetic's application also allows shipments of greater density of fish (high biomass relative to water volume) over extended periods of time (>8 hours) (Lim et al., 2003).

d. Scope of the Study

The scope of the study is to investigate the hypothesis that transportation of juveniles of *Etroplus suratensis* under appropriate anaesthetic sedation would:

1) Reduce plasma cortisol, lactate, and blood glucose levels (major stress indices).
2) Improve overall marketability (i.e., appearance, behaviour, activity level) of transported fishes.

e. Objectives of the study

The intention of this study is to develop and improve techniques for live transportation of juveniles of *Etroplus suratensis.* To be able to deliver a healthy and resistant fish of high value, it is necessary to establish proper handling strategies that will take care of the fish. Anaesthetics may be a useful tool, as they have the potential to both reduce physical injuries and perception of the stressor during handling and transport procedures. To find the efficacy and stress-reducing capacity of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS-222 and cold anaesthesia on the *Etroplus suratensis,* and the chosen study objectives are as follows:

1) Behavioural assays and efficacy of clove oil, cinnamon oil, Cassumunar ginger extract, tobacco leaf extract, MS-222 and cold condition (hypothermia) during bath administration in different concentrations on juveniles of pearl spot (*Etroplus suratensis*).
2) Acute toxicity studies of different concentrations of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS-222 and hypothermia on fingerlings of *Etroplus suratensis* in correlation with certain water quality parameters
3) To study certain haematological indices during exposure at optimal concentrations of clove oil, cinnamon oil, cassumunar ginger extract,

tobacco leaf extract, MS-222 and hypothermia during 24 and 48 hours of exposure of juveniles of *Etroplus suratensis*

4) To determine the stress reducing capacity of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS-222 and cold on plasma biochemical profile during 24 and 48 hours of exposure of juveniles of *Etroplus suratensis*.

5) To study the combined effects of optimum concentrations of clove oil anaesthesia and hypothermia and maximum packing density on juvenile *Etroplus suratensis* in closed bag transport during 24 and 48hrs.

.....ꝏ.....

Review of Literature

Contents

a). *Transportation of Fishes*
b). *Water Quality*
c). *Stress*
d). *Scope of the study*

a. Transportation of fishes

There is a plethora of scientific literature devoted to fish physiology and the effects of alterations in water quality, temperature, salinity, pH, ammonia, and the use of anaesthetics during transportation. Several agency and independent work groups have reviewed the efficacy of the transportation program. Transportation and handling procedures consist of several potential stressors, such as capture, on-loading, transport, unloading, temperature differences, water quality changes and stocking (Iversen et al., 1998, 2003, 2005; Finstad et al., 2003; Portz et al., 2006; Ashley, 2007). Monitoring physiological parameters during stressful operations, like transportation can provide valuable data for the establishment of adequate management practices, even for situations where there is no fish mortality (Sulikowski et al., 2005). For successful fish handling and transportation, a stronger effort towards the animal well-being is more desirable than surveying for fish mortality (Gomes et al., 2003a). The first factor of transportation is the initial health status of the fishes. Transportation of unhealthy animals may result in increased mortality during transport or after arrival at the destination

(Wedemeyer, 1996). Many fish are so stimulated by handling and transportation that they readily accumulate dangerous levels of lactic acid in their blood (Black, 1958). Prior to transportation, fishes may be treated prophylactically with chemotherapeutants to increase post-transport survival (Lim et al., 2003; Crosby et al., 2006b). Ideally diagnostic tests should be performed to identify and document specific pathogens before any treatment. The use of chemotherapeutants without an accurate disease diagnosis may increase production costs. In addition, inappropriate prophylactic drug treatments may harm fish. Moreover, inappropriate use of any antibiotic can increase microbial resistance (Khachatourians, 1998; Cabello, 2006).

b. Water quality

According to Portz et al. (2006), however, there are many water quality information sources for long term and intensive culture of fishes (Pickering, 1981; Adams, 2002), but sparse information related to short term holding of fish in confinement. Temperature, dissolved oxygen, ammonia, nitrite, nitrate, salinity, pH, carbon dioxide, alkalinity and hardness in relation to aluminium and iron species are the most common water quality parameters affecting physiological stress (Stefansson et al., 2007). Thermal stress occurs when the water temperature exceeds the optimal temperature range, with energy demanding stress responses, and potential decrease in individual survivorship (Elliott, 1981; Portz et al., 2006). Most fish can gradually acclimate to normal temperature changes but rapid changes in temperature, as may happen under fish loading and transportation, may result in thermal stresses or lethal conditions (Portz et al., 2006). It is well known that the excitability caused by handling, low ambient oxygen (hypoxic level and below), warm water temperature (30°C and above) increase the metabolic rates

(rate of O_2 consumption, CO_2 output and NH_3 excretion) in aquatic animals including fishes (Peer Mohamed, 1974). Furthermore, fishes may not survive the additional oxygen demand required to sustain basal metabolism due to increased oxygen consumption from digestion and transportation stress. In addition, stress due to handling and transportation may increase oxygen consumption up to 20%. Low dissolved oxygen concentrations lead to respiratory stress, tissue hypoxia, and possible mortality (Wedemeyer, 1996).

Another consequence of metabolism is the production of carbon dioxide. As carbon dioxide levels accumulate during respiration of fishes, the pH of the shipping water declines (Wedemeyer, 1996). In addition, carbon dioxide is highly soluble and can easily diffuse across the gills, lowering the blood pH (Moran et al., 2008). The resultant blood acidosis decreases the affinity of oxygen (O_2) to bind with hemoglobin (Hb) by weakening the Hb-O_2 bond (Wedemeyer, 1996). Tissues become hypoxic when shipping water pH is lower than 6.5 and carbon dioxide levels are greater than 30–40 mg/L (Wedemeyer, 1996; Ross and Ross, 1999) which are common shipping water physico-chemical conditions. Indeed, Moran et al., (2008) reported that juvenile yellowtail kingfish *Seriola lalandi* had a 30% decrease in hemoglobin (Hb) concentration when exposed to simulated transport for 5 hours and 8 or 50 mg/L carbon dioxide. The rate of excretion of nitrogen is related to the rate of metabolism. Peer Mohamed and Devaraj (1997) reported that as with other products of metabolism, a large fish of a particular species produce less nitrogen per unit weight than do small ones. In addition, they are somewhat more resistant to the toxicity of ammonia. Gerking (1955) found that at 25°C, blue gills weighing 25 g excreted nitrogen at a rate of approximately 500 mg / kg / day, whereas the rate for fish weighing 100 g was only 120 mg / kg / day.

There are several factors that affect how fishes are packed and include time since last meal, packing materials (e.g., bags, boxes, etc.), packing density, and shipping water additives. A common practice is to withhold feed from the fishes for 1–2 days prior to transport to allow the digestive tract to be purged (Wedemeyer, 1996; Lim et al., 2003) as digestion may increase oxygen consumption by up to 50% (Wedemeyer, 1996a). This practice also aids in maintenance of shipping water quality by reducing carbon dioxide and waste production (Wedemeyer, 1996; Ross and Ross, 1999; Lim et al., 2003). Likewise the short term crowding stress occurs commonly in aquaculture practices; possess characteristics of acute as well as chronic stress with long-term compromised immune systems, resulting in disease or death (Portz et al., 2006). Therefore, optimal densities at loading and in transport tanks should always be taken care of regardless of profitability or convenience (Ellis et al., 2002; Portz et al., 2006). Optimal densities are species specific and are affected by behavioural requirements for physical space (Wedemeyer, 1996) and total length of time in transport (Lim et al., 2003). Additionally, transportation densities may be limited by potential adverse changes in water physic-chemical parameters (Lim et al., 2003). Freight is a major component of the cost of transportation of fishes (ornamental); therefore, fishes are densely packed into shipping bags with minimal water volume to reduce the overall freight weight of the shipment (Kaiser and Vine, 1998; Lim et al., 2003). Like ornamental fishes, the live fishes are frequently packed into bags with 40% water to 60% oxygen gas, but this ratio may vary depending on the species being transported (Crosby et al., 2006b).

However, with the ever-increasing variety of species being cultured for both the ornamental and food fish markets, there is no "standard" shipping

methodology that applies to all species (Emata, 2000). Nearly all aspects of fish transportation is aimed at reducing the metabolic costs of the fish while supplying the necessary elements for survival in a confined space (Durve, 1975; Weirich et al., 1992; Guo et al., 1995; Gomes et al., 2003b; Paterson et al., 2003; Colburn et al., 2008; Harmon, 2009). Fish farmers also need to be conscious of "batch variability" when it comes to transport fish, as variations in genetic makeup, feeding regime, culture conditions, or size distribution can all have marked impacts on the overall success of live fish transport. The difference between shipping success and failure typically comes down to the small variations between shipping methods and the physiological tolerance levels of the species being transported (Pennell, 1991; Weirich et al., 1992; Chow et al., 1994; Paterson et al., 2003; Pavlidis et al., 2003; Harmon, 2009).

According to Pickering (1981) these management procedures as crucial as they are, produce some level of disturbances, which can elicit a stress response leading to decreased fish performance (Maule and Shreck, 1990), alterations of the peripheral leukocyte distribution, such as heterophilia and lymphocytopaenia (Ellsaesser and Clem 1986, Ainsworth et al., 1991; Gabriel et al., 2007) increased susceptibility to diseases (Pickering and Pottinger 1985; Maule et al., 1989) and in extreme, cases leads to mortality (Akinrotimi et al., 2007).

c. Stress

Transportation of fishes can be a substantial cause for stress. Stress is defined as the physiological change that occurs in response to an imposed demand on an organism that aids in the maintenance of homeostasis (Barton, 1997). Overall the stress load will affect fishes physiological

system, causing reduced growth, inhibits reproduction and suppresses its immune function. Eventually the fish will be exhausted and is likely to incur disease and die (Barton, 2002, Bonga, 1997, Barton and Iwama, 1991, Portz et al., 2006, Davis, 2010, Adams, 1990, Crosby et al., 2006). Its increased focus on stress physiology as studies show that stress has effect upon other hormones: in male songbird, the testosterone level was reduced by 37 - 52 % in response to acute stress (Deviche et al., 2010), in red-sided garter snake it was demonstrated that increased glucocorticoids inhibit melatonin synthesis (Lutterschmidt and Mason, 2010) and in rainbow trout prolactin levels were reduced up to 60% when subjected to chronic stress (Pottinger et al., 1992). The physiological stress reaction follows the same basic pattern in all vertebrates; initiated with elevated levels of catecholamine and corticosteroids. In some species concentration of corticosteroids will fluctuate with annual rhythms, as it may control other body processes (Davis and Parker, 1986). Acute stress factors such as handling and transportation cause significant increases in plasma cortisol levels, a biological indicator of stress, as demonstrated in American shad (*Alosa sapidissima)* (Shrimpton et al., 2001), coho salmon (*Oncorhynchus kisutch*) (Avella et al., 1991), rainbow trout (*Oncorhynchus mykiss*) (Woodward and Strange 1987; Pickering and Pottinger, 1989), brown trout (*Salmo trutta*) (Pickering and Pottinger, 1989), hybrid striped bass (*Morone saxatilis*x, *Morone chrysops*) (Davis and Griffin, 2004), and Nile tilapia (*Oreochromis niloticus*) (Barcellos et al., 1999). The increased concentration of circulating catecholamine and cortisol will in turn cause physiological changes on blood and tissue, referred to as the secondary response. Catecholamine will cause the increased ventilation rate and blood flow for increased oxygen uptake

and consumption, as well as initiate glycogenolysis (Portz et al., 2006). When plasma cortisol levels in fishes are elevated, blood flow and pressure are increased, oxygen demand and gill perfusion are increased, and hepatic gluconeogenesis is stimulated (Norris and Hobbs, 2006). Additionally, oxygen consumption can increase up to 20% (Wedemeyer, 1996). These physiological adaptations increase the chances of fish survival (Wedemeyer, 1996). However, studies have indicated that even small increases in plasma cortisol levels can have an immunosuppressive effect that may lead to an increased incidence of disease and mortality (Brown, 1993; Wedemeyer, 1996). Even gentle handling of fishes is a significant stress that may result in physiological changes such as an increase in plasma cortisol and blood glucose levels. Traditionally, freshwater and marine fish have been transported in both open and closed systems (Amend et al., 1982; Berka, 1986), using techniques to minimize stress and increase survival of the fish before, during, and after the transportation period (Carmichael et al., 1984; Weirich and Tomasso, 1991; Weirich et al., 1992; Gomes et al., 2003a; Harmon, 2009). In general a stressed fish will have increased metabolic rate, which gives increased load of metabolic products that in turn will give bad quality water. This is often the case in fish transported from rural communities due to the absence of equipment, holding facilities and undeveloped infrastructure in combination with poor handling techniques. To supply a resistant and healthy fish, it is necessary to establish proper handling and management that will avoid handling-related stress.

d. Anaesthetics

The use of anaesthetics has been shown to assist in the handling of fish species in aquaculture systems, reducing many of the negative impacts of

stress (Munday and Wilson, 1997; Ross and Ross, 1999; Ortuno et al., 2002; Wagner et al., 2002; Pirhonen and Schreck, 2003). Pickering (1993) proposed sedation or mild anaesthesia as a stress-ameliorating measure during handling and transportation of fish. The anaesthetics lower the metabolic activity of fish, which facilitates the transport of more fish in a given quantity of water for a long time. In recent times anaesthetizing chemicals have been used in the transporting medium of fish seeds and adult fish. It has also been proved that anaesthetics make the otherwise time consuming work of handling, weighing, marking, tagging, fin clipping, stripping and operative procedures much easier and also lower the mortality of fish due to handling and transport (Saxena, 1986). The choice of anaesthetics is often dependent on considerations such as availability, cost-effectiveness, ease of use, nature of the study and user safety (Cho and Heath, 2000; Mylonas et al., 2005). However, the increased concern for fish health and product quality makes the use of anaesthetics inevitable to reduce the stress during handling and transportation procedures. Before recommending the use of a particular anaesthetic, a range of stress-response indices must be measured to assess its efficacy (Pramod et al., 2010). To date, much of the information on the use of anaesthetics in fish has been derived from studies on salmonids (Pickering, 1992; Iversen et al., 2003; Pirhonen and Schreck 2003; Iversen et al., 2009) and other temperate species (Mattson and Ripple, 1989). Only the liquid and solid anaesthetics, especially those, which are readily soluble in water, are useful in this field. Very little published information is available on sedation of tropical cultured fish species (Lindsay and Geddes, 1979; Basavaraja and Antony, 1997). Especially information on use of thiopentone - sodium, xylodac, lignocaine and sodium

chloride as anaesthetics/sedative agents are scanty (Saxena, 1986; Johnson and Metcalf, 1982).

Ross et al., (1993) reported that administration of anaesthetics reduced the effect of stress during handling and hauling of fish. Different handling procedures demand different anaesthetic approaches. For instance light anaesthesia (sedation), which is defined as reduced activity and reactions to external stimuli, is sufficient for procedures such as transport or weighing of fish. Full anaesthesia can be defined as loss of consciousness and reduced sensing of pain, loss of muscular tones and reflexes and is needed when surgical procedures are applied (McFarland, 1959).

Anaesthetizing the fish is often useful during handling procedures to reduce trauma and injury (Neiffer and Stamper, 2009). ‘Anaesthesia’ means loss of sensation or insensibility (Ross and Ross, 2008), and can be introduced to fish through physical or chemical techniques. Physical anaesthetics are applied through electric tension or refrigeration (Brattelid, 1999b), while chemical anaesthetics are based on immersing the fish in a water solution containing a chemical agent. These techniques will cause general anaesthesia as they affect the fish sensitivity, equilibrium and consciousness. Mostly this is introduced through ‘inhalation anaesthesia’ where the active agent mixed in the water is ventilated through the fish gills (minor through the skin). The agent will pass the blood-brain barrier and have an effect upon the fish central nervous system (CNS) (Brattelid, 1999b, Ross and Ross, 2008). The chemical agent interacts with membrane components and will cause blockage or depression of nerve impulses (Ross and Ross, 2008). This lead to loss of mobility, equilibrium and muscle reflexes (Brattelid, 1999b).

Anaesthetic treatment may reduce the fish's perception of the stress and thus prevent the nervous input to the CNS (Woods et al., 2008, Brattelid, 1999b). This is desirable because it will block or reduce the cortisol synthesis. Cortisol elevation is known to depend upon the intensity and duration of the stressor, and may be detrimental to the fish as the cascade of physiological changes may persist for days or weeks. However, improper dosages and anaesthetic drugs may have undesirable side effects upon the fish and may self induce unnecessary stress. It is therefore necessary to find the anaesthetic and dosage that is appropriate and have desirable effects on the fish (Carter et al., 2011). An appropriate anaesthetic and the dosage will provide a smooth and rapid anaesthesia for a time period followed by recovery (Woods et al., 2008), and should not cause any undesirable side-effects. In addition, the anaesthetic agent should provide a satisfying blockage upon the hypothalamus pituitary (HPI) axis, in order to prevent cortisol elevation when anaesthesia subsides (Brattelid, 1999a).

The degree of chemical blockage upon the nervous system varies according to chemical agent, dosage and duration (Burka et al., 1997, McFarland and Klontz, 1969). McFarland (1959) was the first to classify this chemical effect into stages based on behavioural signs (Table 1). The anaesthetic effect ranged from 'sedation', giving a calming effect, to 'surgical anaesthesia', giving full immobilization. The basic procedure for introducing anaesthesia in fish is divided into three phases; introduction, maintenance and recovery (McFarland and Klontz, 1969; Ross and Ross, 2008). The depth of the introduced anaesthesia will vary according to dosage and duration. In order to not traumatize and stress the fish, the introduction phase should last for a few minutes. However, too rapid introduction is neither desirable as it

will harm and kill the fish. The most desirable anaesthesia is set to be achieved within 3 minutes (Ross and Ross, 2008; Marking and Meyer, 1985). In some procedures like transportation or surgery, it will be necessary to maintain anaesthesia. It should be kept in mind that different species will have different tolerance to dosage and duration of anaesthetic drugs. Maintenance of deep anaesthesia for a few minutes is likely to cause death from ventilation and circulatory arrest. Flaring and spasms of the opercula function as warning signals to medullary collapse (McFarland and Klontz, 1969; Ross and Ross, 2008).

Table 1 Stages of anaesthesia; modified from (McFarland and Klontz, 1969; Burka et al., 1997)

Stage	Description	Behaviour sign
0	Normal	Active swimming patterns; reactive to external stimuli; normal equilibrium; normal muscle tone.
1	Light sedation	Reduced swimming activity; slight loss of reactivity to visual and tactile stimuli.
2	Light narcosis	Slightly loss of equilibrium
3a	Deep narcosis	Total loss of equilibrium; decreased muscle tone; reactivity to strong tactile stimuli; decreased respiratory rate
3b	Surgical anaesthesia	Total loss of reactivity; total loss of muscle tone; low respiratory rate
4	Medullary collapse	Respiration creases, cardiac arrest; death ensures

Chung (1980) classified anaesthetic effect into four different stages: first stage is where the fish is normal, reacts to external stimuli normally, swimming and opercular movements are normal. The second stage is where the fish is in a state of light anaesthesia, it becomes sluggish, has weak equilibrium, it swims partially and opercular movement is also partial while

the third stage is where the fish is in a stage of deep anaesthesia, exhibits loss of movement and very weak equilibrium with partial opercular movement. The 4th stage, which is characterized by the total loss of equilibrium, opercular movement, this in a few minutes leads to heart failure. The second and third stages are of great relevance as the fish is then insensitive to pain. The choice of anaesthetics for fish must be based on the species, the size of fish and the duration of operation, water temperature and chemistry, exposure time, good safety margin (Lemm, 1993). The time to introduce anaesthesia depends on both biotic and abiotic factors. Age, lipid content, size and metabolism are biological factors that will affect the anaesthetic effect. The anaesthetic can also have different effects within the same species due to biological differences like sex, life-stage and season (Brattelid, 1999b).

Recovery from anaesthesia will occur when the fish is immersed in freshwater. The anaesthetic agent is then excreted through the gills. As with the introduction of anaesthesia, recovery is also divided into different stages based on behavioural sign (Table 2). The recovery should be attained within few minutes to prevent stress and harmful effects on the fish (Woods et al., 2008). The most desirable recovery is set to be retained within 5 minutes (Marking and Meyer, 1985; Ross and Ross, 2008). Higher concentrations and longer exposure time of the anaesthetic correspond with longer recovery time (McFarland and Klontz, 1969). After anaesthetic procedure the fish is recommended to be under closer observation for 24-72 hours, as death can occur (Ross and Ross, 2008).

Table 2 Stages of recovery; modified from (Hikasa et al., 1986)

Stage	Behaviour sign
1	Reappearance of opercula movement; weak muscle tone visible
2	Reappearance of swimming activity, but still loss of equilibrium
3	Partial recovery of equilibrium
4	Full recovery of equilibrium; reaction in response to visual and tactile stimuli; still stolid behavioural response
5	Total behaviour recovery; normal swimming activity

Use of anaesthetic is well established within the aquaculture sector for food fish during handling, transport, confinement, vaccination, grading, etc. There are several different chemical drugs that can immobilize fish, but not all are described as safe and effective for use on fish. However, the wide variety in anatomy, physiology and behaviour in the fishes, make the anaesthetic treatment potential harmful (Neiffer and Stamper, 2009), however, there are some publications which emphasize on the anaesthetic efficacy on some species (Bircan- Yildirim et al., 2010; Young, 2009; Grush et al., 2004; Kaiser and Vine, 1998). Marking and Meyer (1985) listed up six criteria for an ideal anaesthetic; permit the reasonable duration of exposure, produce anaesthesia within 3 minutes or less, allow recovery within 5 minutes or less, cause no toxicity to fish at treatment levels, present no mammalian safety problems and leave no tissue residues after a withdrawal time of 1 hour or less.

The chemical properties of anaesthetics may depend upon environmental factors like temperature, pH, salinity, chemical additives and oxygen content (Burka et al., 1997). Lipid soluble anaesthetics may depend upon temperature or solvent for resolution, and some anaesthetic will in turn have effect upon water parameters. Fish is a poikilotherm animal and temperature will affect

its biological functions. Both temperature and pH will affect gill perfusion, which in turn affects uptake and clearance rate of the anaesthetic agent (Ross and Ross, 2008; Burka et al., 1997). To avoid undesirable effects on the fish, the anaesthetic treatment is recommended to be carried out in water close to the fish biological optima (Brattelid, 1999b).

There are a variety of anaesthetic agents such as tricaine methane sulfonate (i.e., MS-222), quinaldine, metomidate hydrochloride, and clove oil that has been used in shipping water to alleviate transportation related stress; however, it is important to note that there are no drugs currently approved by the U. S. Food and Drug Administration (FDA) for transporting fishes. The shipping water may be treated such that the fishes are shipped under sedation, a stage of anaesthesia. During transport, anaesthetics should only lightly sedate fish, not anaesthetize them, to avoid interfering with osmoregulation or gas exchange (Forteath 1993). The stress response may be minimized if the anaesthetic takes effect quickly (Robertson et al., 1988; Ross and Ross 1999). Tricaine Methanesulfonate (TMS) is absorbed by the fish and its effects are cumulative over time (Crosby et al., 2006c). Additional TMS may need to be added to sedate all fish (Brown, 1993), but too much TMS may over anaesthetize fish, leading to ventilatory arrest (Ross and Ross, 1999). Metomidate has been shown to suppress parts of the biochemical pathway blocking cortisol synthesis (Ross and Ross, 1999). Quinaldine is inexpensive, effective, and undetectable 24 h after exposure; it is more potent at for sedation higher temperatures and in hard water (Ross and Ross, 1999). Hypno is a proprietary registered product for sedation containing quinaldine (Crosby et al., 2006c). However, there may be problems associated with the use of these anaesthetic agents. For example, sedation with MS-222 or

quinaldine may cause an initial excitatory response that results in increased plasma cortisol levels; and clove oil have a slow induction time (Barton and Peter, 1982; Robertson et al., 1987; Ross and Ross, 1999). Hypothermia (cold anaesthesia) known to reduce the stress in fish handling, either by itself or in combination with chemical anaesthetics (Rodman, 1963; Hovda and Linley, 2000; Ross and Ross 1999). Yoshikawa et al. (1989) showed that carp, previously acclimated to 23°C, would be safely held at 5 °C for 5 h, and achieved sedation at 8-14° C for 24h.

Clove oil is the best-known herbal product used as a local analgesic and it has long been employed to obtain transient relief from toothache (Ghelardini et al., 2001). In Indonesia, it has been used as a topical anaesthetic for tooth aches, headache and joint pain (Soto and Burhanuddin, 1995). Clove bud oil, which obtained by distillation method is a clear, colorless to yellow mobile liquid, becoming browner with age or contamination with iron or copper, with a strong characteristic sweet and spicy clove odor, and a warm, almost burning and spicy flavor (Weiss, 1997). Moreover, this oil consists of three components: eugenol (70-90%), eugenyl acetate (17%) and caryophyllene sesquiterpenes (mainly β-caryophyllene) 5 – 12% (Vernin et al., 1994). The other constituent of clove bud oil is β-caryophyllene (Walter, 1972). This component has a local anaesthetic activity similar to eugenol as reported by Ghelardini et al. (2001). They compared β-caryophyllene with caryophyllene and found that the former has a strong local anaesthetic action when administered in rabbits. Clove oil has been used as a mild anaesthetic since antiquity and its effectiveness as an anaesthetic in dentistry is well known (Ross and Ross, 1999). Clove oil is readily available and is inexpensive compared to MS-222 (Keene et al., 1998). The primary constituent

of clove oil, eugenol, is similar in structure to Tricaine metanesulfonate and 2-phenoxyethanol (Varner, 2000). The anaesthetic effects of eugenol have been studied to varying degrees on *Medaka oryzias latipes* (Temminck and Schlegal, 1846), gold fish *Carassius auratus* (L.) and crucian carp *C. carassius* (L.) (Endo et al., 1972 as cited by Keene et al., 1998). Hikasa et al., (1986) showed that it gave effective anaesthesia in adult common carp (*Cyprinus carpio*) at 25 to 100 ppm. Soto and Burhanuddin (1995) studied the use of clove oil as a tool of sedation for measuring length and weight of rabbit fish (*Siganus lineatus*).

Cinnamon (*Cinnamomum zeylanicum*) which is native to India and Sri Lanka (Ceylon) Vaibhavi and Jakhetia et al., (2010) and now it is cultivated in many tropical countries, including Mexico as one of the most important medicinal plants. Cinnamon contains 0.5 to 1.0% volatile oil composed mainly of cinnamyldehyde (50.5%), eugenol (4.7%), cinnamic acid, methoxycinnamaldehyde (MOCA) and cinnamyl acetate (8.7%) (Charu Gupta et al., 2008). Research interest has focused on the cinnamon that possesses antispasmodic, anti-ulcer, sedative, hypothermic, antifungal, antibacterial, antiviral, antipyretic, lipolytic, anaesthetic, cytotoxic, hypolipidemic, antiplatelet properties and also stimulates the immune system that may be useful adjuncts in helping to reduce the risk of cardiovascular disease and cancer (Cralg, 1999). Cinnamon (*Cinnamomum zeylanicum*) bark also contains eugenol, but its use as an anaesthetic has not been explored (Power et al., 2010). Eugenol content of the leaf oil is antiseptic and anaesthetic (Khare, 2007).

Tobacco is the common name for the plant *Nicotiana tobacum.* It is a native of tropical and subtropical America, but it is now commercially

cultivated worldwide (Knapp, 2004). Tobacco contains the following phytochemicals: Nicotine, Anabasine (an alkaloid similar to the nicotine but less active), Glucosides (tabacinine, tabacine), 2,3,6-Trimethyl-1,4-naphthoquinone, 2-Methylquinone, 2-Napthylamine, Propionic acid, Anatalline, Anthalin, Anethole, Acrolein, Anatabine, Cembrene, Choline, Nicotelline, Nicotianine and Pyrene and they are generally recognized as being narcotic **(**Agokei and Adebisi, 2010). This property makes it useful as narcotics, mulluscicides, piscicides, an anaesthetic and pesticide (Aleem, 1983; Agbon et al., 2002). Agokei and Adebisi (2010) reported that the tobacco extracts acted as an anaesthetic in Nile tilapia, *Oreochromis niloticus.* Detailed studies on the use of tobacco as an anaesthetic for juveniles of *Etroplus suratensis* is not available and it would appear that experimental studies on this subject are rare.

The most common synthetic anaesthetic agent used on fish is Tricaine Methanesulfonate (MS-222) (Marking and Meyer, 1985) and is the only anaesthetic verified by the U.S. Food and Drug Administration (FDA). It occurs as a white crystalline powder directly applied to the water. However, this anaesthetic agent is regarded as a carcinogen and also a 21-day withdrawal period is required if the fish is intended for human consumption (Kolanczyk et al., 2003; Rombough, 2007). Additionally, MS- 222 is relatively expensive and frequently unavailable in many countries due to several international restrictive rules regarding import of this chemical. Although a number of studies have described the physiological responses of fish to sedate and immobilizing doses of MS-222, only a few studies have reported on responses to higher, lethal concentrations of MS-222 or other anaesthetics. A few studies using higher concentrations of MS-222 were 125 mg L^{-1} (Laidley and Leatherland, 1988); 150mg L^{-1} (Holloway et al.,

2004) have evaluated changes in blood chemistry at the time of induction of deep anaesthesia, 2-3min after the initiation of exposure.

In recent years, a shift in research has occurred where response to cold shock is measured in terms of sub lethal effects to fishes rather than just mortality. The cold-shock response may be a beneficial tool for fisheries science (e.g. for induction of polyploidy) and future cold-shock research may reveal other novel opportunities. Hypothermia is also known to reduce the stress in fish handling, either by itself or in combination with chemical anaesthetics (Rodman, 1963; Hovda and Linley, 2000; Ross and Ross, 1999). Application of hypothermia or cold shock has been reported as a short-term anaesthetic (Hovda and Linley, 2000), as a means to alter embryonic sex ratios (Craig et al., 1996) and, most commonly, as an agent in the induction of polyploidy (Peruzzi et al., 2007). The basic principle behind the hypothermia for live transportation of fishes is cold temperature induced anaesthetization. The water temperature is brought down to a limit at which the metabolic rate of the animal is reduced to a minimum, so that its storage and transport in this condition does not affect any apparent increase in metabolic rate. Yoshikawa et al., (1989) showed that carp, previously acclimated to 23°C, would be safely held at 5°C for 5 h, and achieved sedation at 8-14°C for 24h. The movements of the cold-anaesthetized shrimp are minimum, there is no stress caused by vibrations, noise and light; weight loss is usually negligible, and the animals produce no excreta because there is no feed intake and metabolism (Schoemaker, 1991).

The present work attempts to assess the stress reducing capacity of certain anaesthetics such as clove oil (*Syzygium aromaticum*), cinnamon oil

(*Cinnamomum cassia*), Cassumunar Ginger (*Zingiber cassumunar*) extract, tobacco leaf (*Nicotiana tobaccum*) extract, MS-222 (Tricaine methanesulphonate) and cold (hypothermia) anaesthesia on the experimental organism selected for this study, namely Green chromide (*Etroplus suratensis*, Bloch,1790), and to probe into the behavioural, toxicological, haematological and biochemical responses of the organism.

.....ꙮꙮ.....

Chapter 1

Acute toxicity of selected anaesthetics on juveniles of *Etroplus suratensis* correlates with certain water quality parameters

Contents

1.1 Introduction
1.2 Materials and methods
1.3 General protocol
1.4 Anaesthetic agents
1.5 Experimental set up for anaesthetization of fish
1.6 Statistical analyses
1.7 Results
1.8 Discussion
1.9 Summary

1.1 Introduction

The toxicity of certain plant extracts on fish has been reported (Ufodike and Omoregie, 1994; Onusiruika and Ufodike, 1994; Aguigwo, 1998). The toxicity and effect of anaesthetics are of special interest since they are frequently used in research and routine aquaculture procedures to immobilize fish and minimize their stress responses (King et al., 2005). Toxicity refers to the degree at which a substance is being harmful, destructive or poisonous to life (Boyd and Lichtkoppler, 1979). There are numbers of terms that are

associated with toxicity one among which is acute toxicity. The measures which can be used to reflect the levels of toxicity are LC_{50} and LD_{50}. The former refers to the concentration of a chemical or toxicant that can kills 50% of a sample population. This measure is generally used when exposure to a chemical while the animal is breathing. The latter refers to the dose of chemical or toxicant which kills 50% of a sample population when the exposure is by swallowing, through skin contact or by injection (Johnson and Finley, 1980). LC_{50} is a useful tool because it can predict the effects of a potential toxin in aquaculture systems (Claude E. Boyd, 2005). LC_{50} data can also help define maximum allowable toxicant concentrations (Hamilton et al., 1977). Generally, 24–48 h exposure is required for maximum accumulation of toxicants in fish (Huey et al., 1980; Eddy et al., 1983; Aggergaard and Jensen, 2001). As expected, the lethal concentration LC_{50} declines after 24 h. The Change in pattern of decline is very low by the time the exposure has reached 96 h. Thus the relevant duration for short-term toxicity testing is probably 24 to 96 h as is the case for many toxicants (Lewis and Morris, 1986). Other common durations are 24, 48, and 72 h of exposure. As a general rule, the longer the exposure, the lower the LC_{50}. If the exposure is long enough, an asymptotic LC_{50} value can be obtained. The asymptotic LC_{50} is not time-dependent (Hamilton et al., 1977).

Naturally occurring toxicity can result from low dissolved-oxygen concentrations and high concentrations of ammonia, carbon dioxide, nitrite, or hydrogen sulfide. Toxicity also can result from contamination of culture systems by drugs or chemicals for disease management, pesticides and heavy metals, or industrial chemicals (Claude, 2005). There are differences in toxicity for the anaesthetics at water quality variables. For many a fish, the

skin is a respiratory organ, responsible for up to 30% of oxygen uptake in some species (Bruecker and Graham, 1993); most marine species in particular have well-vascularized skin capable of significant gas exchange (Ishimatsu and Itazawa, 1993). Younger fish, regardless of species, tend to have thinner and less scaled skin, which permits greater oxygen uptake (Myskowski et al., 2003). And fish species that start out as larvae lacking gills require skin respiration until differentiation of gill lamellae is complete (Oikawa et al., 1994). Skin is also a route for immersion drug uptake (and presumably drug excretion) and in some species may actually be more efficient than other respiratory organs—in the electric eel (*Electrophorus electricus*), for example, quinaldine uptake across the skin was higher than through the gills (Brown et al., 1972).

The use of essential oils extracted from plants has proved to be a feasible alternative to chemical anaesthetics during fish handling and transportation (Kaiser et al., 2006; Pálic et al., 2006; Simões and Gomes, 2009). The toxicity of most plant extracts varies depending on the type and the animal species involved. This is due in part to the phytochemical composition of the extract and also due to the very great variation in susceptibility between individual animals. Zebra fish, carp, cat fish and guppy showed comparable tolerance to acute toxicity of clove oil (Doleželová et al., 2011). Cinnamon (*Cinnamomum zeylanicum*) bark also contains eugenol, but its use as an anaesthetic has not been explored. There are no scientific reports available in the existing literature on LC_{50} (96 h) and toxic effect of *Zingiber cassumunar* Roxb. *Clarias gariepinus* indicated that mortality occurred after 96 h in graded concentrations of 100, 80 and 60 % of the graded extract of tobacco leaf (*Nicotiana tobacum*) (Aguigwo, 1998). The

toxicity of MS-222 has been reported to decrease with fish age in zebrafish (Rombough, 2007). Hypothermia can act as a lethal factor when its effect is to destroy the integrity of the organism (Fry, 1947).

It is critical to monitor water quality in order to reduce anaesthetic morbidity and mortality (Harms, 1999). The influence of other environmental conditions (temperature, pH, etc.,) on the toxicity of anaesthetics has also been investigated (Park et al., 2008; Zahl et al., 2009). In aquatic body, toxicants present above the normal level i.e., at lethal concentrations bring about mortality of fish and also increase the change in pattern of oxygen consumption in survived fish (Tilak et al., 2007). Assuming aeration, DO, pH, and temperature are appropriate, the greatest concern is ammonia concentrations. Ammonia toxicity is greater in more alkaline water (Harms, 1999). Besides, knowledge of how water quality influences anaesthesia or sedation helps limit complications (Neiffer and Stamper, 2009). Nitrite is a natural component of the nitrogen cycle in ecosystems, and its presence in the environment is a potential problem due to its well documented toxicity to animals (Lewis and Morris, 1986; Jensen, 2003).

Although juveniles of *Etroplus suratensis* transportation is not new, information on the toxicity of fishery chemicals to juveniles of *Etroplus suratensis* is limited. Wellborn (1969) and Hughes (1971) determined the toxicity of several compounds used in striped bass culture. They concluded that striped bass is more sensitive to chemicals than most freshwater fishes. The present study was conducted to determine the sensitivity of juveniles of *Etroplus suratensis* to six anaesthetics that are commonly used in culture or that have been proposed for such use. The anaesthetics included plant

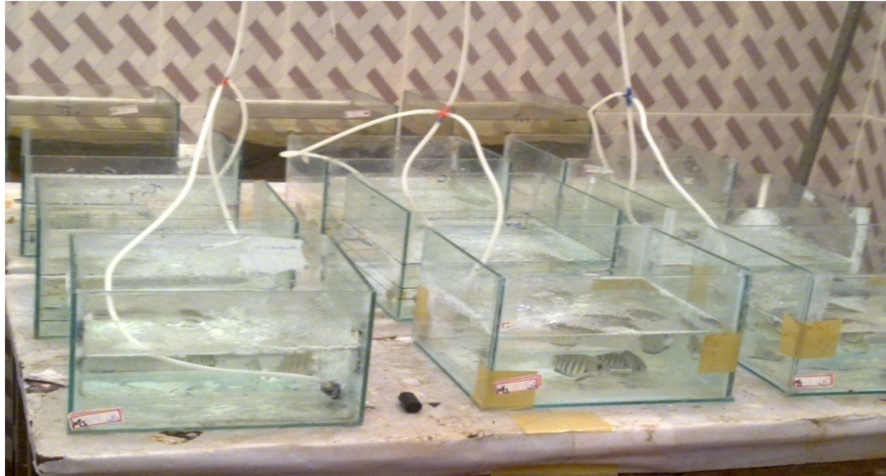
anaesthetics, chemical anaesthetics and physical anaesthetics. They also determined the effects of selected levels of water temperature, turbidity and pH on the toxicity of used anaesthetics.

Therefore the aims of present work were (1) to evaluate the levels for acute toxicity of different concentrations of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS-222 and cold for 96 h exposures to juveniles of *Etroplus suratensis* and also to observe the toxic effects on mortality rate and (2) to evaluate different concentrations of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS-222 and hypothermic condition (cold) were tested on juveniles of *Etroplus suratensis* in the anaesthetic exposed water of different temperatures, dissolved oxygen, turbidity, PH, ammonia, nitrite and nitrate to determine if changes in water characteristics affect sensitivity to anaesthetics in juveniles of *Etroplus suratensis* or induces the mortality of juveniles of *Etroplus suratensis* .

1.2 Materials and Methods

1.2.1 Juveniles of *Etroplus suratensis* (Bloch 1790)

Class: Actinopterygii
Order: Perciformes
Family: Cichlidae
Genus: *Etroplus*
Species: *E.suratensis* (Bloch 1790)
Common name: Green chromide, Pearl spot
Local name: Karimeen



Etroplus suratensis, the largest among the three indigenous cichlids, is a native of peninsular India, occurring primarily in Kerala and Southern Karnataka; the other species being *Etroplus maculatus* and *Etroplus canarensis*. *E. maculates* occurs in all backwaters of Kerala while *E. canarensis* (Bloch) is restricted to the coastal wetlands of Karnataka. The family Cichlidae comprises over 700 species of fishes that occur in freshwater as well as brackish water habitats.

Cichlids are oval shaped (disc like form) spiny-rayed fishes distinguished externally by the presence of only one nostril on each side of the head. Most species are usually short snouted, deep with a large head and eyes, fairly large scales, and a strong jutting jaw with well-developed lips. The colour of the body is grayish-green on both sides with 6 to 8 yellowish oblique dark bands, a dark spot at base of pectoral fin and also many scales on sides with a pearly spot. Specimens from salt waters have a deep purple colour and bands are almost black. Fingerlings possess a conspicuous ocellus on the dorsal fin. The dorsal fin is long based and single, the front part being spiny and usually

larger than the soft rear part. The anal fin usually consists of at least three spiny rays and soft after part. In almost all cichlids, the dorsal and anal fins are pointed among males, while it is rounded in females. The lateral line is seen as two parts, the first part extends from the gill cover to just below the soft rayed part of the dorsal, and the second part looks as if it has been broken off and replaced at a lower level.

They have fruitful production, profuse breeding in confined conditions and their unique parental care which demand a lot of free space. Within the family, their reproductive behavior (pair formation) varies from monogamous biparental care of eggs and fry (spawning and caring for the young), to mouth brooding by the female as observed among tilapias. Normally, cichlids are divisible into two groups: the substrate guarders and mouth brooders. Among cichlid group, *Etroplus* is the only genus endemic to India and are herbivorous in nature.

1.3 General protocol

1.3.1 Specimen collection and acclimatization

Juveniles of green chromide *Etroplus suratensis* size classes; (2.078 ±0.15g and 4.0±0.1cm) used for the experiment were obtained from a commercial fish farm located in Ernakulam, Kerala, India during June and December of 2010, 2011 and 2012. Fishes were transported using aerated polythene bag to the laboratory situated 35km away from the farm site. The live specimens were held and acclimatized for one month in rectangular light blue background fiberglass reinforced plastic (FRP) tanks (capacity, 1000 L) at a juvenile density of 5 fish L^{-1} in an enclosed system supplied with a continuous flow of aerated fresh water. Fish were fed with fresh water plants and algae and pellet feed (Higashimaru Co., Ltd; Japan). The tanks were

cleaned daily and specific water quality parameters were measured twice a week using a handheld meter and submersible electrode (Microprocessor Water and soil analysis kit, Model 1160 E1, Environmental and Scientific Instruments, Industrial Area, phase-11, Panchkula, India).

1.4 Anaesthetic agents

The natural anaesthetic agents used were clove oil (*Syzygium aromaticum,* Universal Oleoresins, India), cinnamon oil (*Cinnamomum zeylanicum,* Universal Oleoresins, India), Cassumunar Ginger (raw material) **(***Zingiber cassumunar* Roxb***)*** extract, tobacco leaf (raw material) extract (*Nicotiana tobaccum*). Both raw materials were collected from Ayurveda College, Thripunithura; Kerala, and were botanically identified by Dr. Sudhir, Professor and Head, Department of Ayurveda College, Government of Kerala, Thripunithura, India. The quality of the plants was ascertained as per Ayurvedic Pharmacopoeia of India (2004) by determining alcohol soluble extractive and water soluble extractive values.

The chemical anaesthetic used was MS-222 (tricaine methanesulphonate, HiMedia, India). The physical anaesthetic parameter used was set at hypothermic condition of 12±1°C, 16±1°C, 18±1°C, 22±1°C. Doses of the anaesthetic agents were prepared freshly within a few minutes prior to anaesthetic induction experiments. Specific control live specimens without any anaesthetic treatment were also set during experimentation.

1.4.1 Preparation of anaesthetics agents

1.4.1.1 Preparation of clove oil (*Syzygium aromaticum*)

Essential oil of clove (*Syzygium aromaticum*) was purchased from Universal Oleoresins (India). Major component of clove oil is eugenol (70-90%)

having the anaesthetic effect (Woody et al., 2002). The hydrophobic trait of clove oil does not allow using it directly in the water. Hence, it was first diluted in ethanol (one part clove oil: 10 ethanol) and a stock solution was made before the experiment. To reduce the amount of photo degradation, the clove–oil stock solution was kept in an amber colored bottle at approximately 19-20°C.

The acclimated fingerlings were randomly allocated to glass aquaria (22 x 22 x 15). Dechlorinated tap water from storage tank was used and the parameters, viz., temperature, dissolved oxygen, pH, conductivity, NO_2^-, NO_3^- and NH_3^+ were monitored in each aquarium, with the aid of portable water quality analysis kit. Different concentrations of test solution prepared from the stock solution of clove oil pharmaceutical grade were assayed: 0.10, 0.17, 0.23, 0.30 and 0.33 mg/L. The volumes of each test solution used in the bioassay per replicate in all treatments were 3 litres. The dosage was arrived after several preliminary investigations. Trials with appropriate controls in each experiment. The different concentrations were introduced in five aquaria with three replicates for each treatment. Ten fish were put into each aquarium for the acute bioassay test, which lasted for 48 h. Fresh preparations of the test solutions were introduced into the aquarium for various physiological and biochemical assays.

1.4.1.2 Preparation of cinnamon oil (*Cinnamomum zeylanicum*)

Essential oil of cinnamon was purchased from Universal Oleoresins (India). Major constituent of the leaf oil is eugenol (28–98%) which also used as sedative (Cralg, 1999). The hydrophobic trait of cinnamon oil does not allow using it directly in the water. Hence, it was first diluted in ethanol (one

part cinnamon oil: 10 ethanol) and a stock solution was made before the experiment. To reduce the amount of photo degradation, the cinnamon oil stock solution was kept in an amber colored bottle at approximately 19-20°C.

The acclimated fingerlings were randomly allocated to glass aquaria (22 x 22 x 15). Dechlorinated tap water from storage tank was used and these parameters: temperature, dissolved oxygen, pH, conductivity, NO_2^- , NO_3^- and NH_3^+ were monitored in each aquarium, with the aid of portable water quality analysis kit. Different concentrations of test solution prepared from the stock solution of cinnamon oil pharmaceutical grade were assayed: 0.5, 1, 1.5, and 1.7 mL^{-1}. The volume of each test solution used in the bioassay per replicate in all treatments was 3 liters. The concentrations were arrived at after several preliminary investigations. There was also a control. These concentrations were introduced in five aquaria with three replicates for each treatment. Ten fish were put into each aquarium for the acute bioassay test, which lasted for 48 hthe presents. Fresh preparations of the test solutions were introduced into the aquarium for various physiological and biochemical assays.

1.4.1.3 Preparation of Cassumunar ginger (*Zingiber cassumunar* Roxb)

The plant rhizome of *Zingiber cassumunar* Roxb were purchased from the Ayurvedic shop at Thripunithura, Ernakulam district, Kerala and was identified by Dr. Sudhir, Professor and Head, Department of Ayurveda College, Government of Kerala, Thripunithura, India. The quality of the plants was ascertained as per Ayurvedic pharmacopoeia of India (Vol I to IV (2004), by determining alcohol soluble extractive and water-soluble extractive values. Dried rhizome of *Zingiber cassumunar* was finely powdered in a mechanical mixture. The powdered rhizome about 100 g was extracted using

700ml of ethanol (80%) using a modified Soxhlet distillation unit for about 8 h. The isolated extract of stock solution was kept in an amber colored bottle at approximately 19-20°C.

The acclimated fingerlings were randomly allocated to glass aquaria (22 x 22 x 15). Dechlorinated tap water from storage tank was used and these parameters: temperature, dissolved oxygen, pH, conductivity, NO_2^-, NO_3^- and NH_3^+ were monitored in each aquarium, with the aid of portable water quality analysis kit. Different concentrations of test solution prepared from the stock solution. The volume of each test solution used in the bioassay per replicate in all treatments was 3 litres. The isolated extract of stock solution in each treatment was 8.3 mL^{-1}, 6.6 mL^{-1}, 5 mL^{-1}, 4 mL^{-1}, and 3.3 mL^{-1}. The concentrations were arrived at after several preliminary investigations. There was also a control. These concentrations were introduced in five aquaria with three replicates for each treatment. Ten fish were put into each aquarium for the acute bioassay test, which lasted for 48 h. Fresh preparations of the test solutions were introduced into the aquarium for various physiological and biochemical assays.

1.4.1.4 Preparation of Tobacco leaf extracts (*Nicotiana tobaccum*)

The leaves of tobacco (*Nicotiana tobaccum*) were purchased at a local market in Thripunithura, Ernakulam district, Kerala and were identified by Dr.Sudhir, Professor and Head, Department of Ayurveda College, Government of Kerala, Thripunithura, India. The quality of the plants was ascertained as per Ayurvedic pharmacopoeia of India by determining alcohol soluble extractive and water-soluble extractive values. The collected samples were sun dried for 7 days to a constant weight, grounded in to powder with the aid

of electric blender, sieved and then stored in a sealed plastic container until required. The concentrations of tobacco used were calculated as 50%, 96 h LC_{50} (96 h LC_{50} of tobacco leaf dust on *Etroplus suratensis* as obtained from preliminary investigation). The stock solution was prepared by boling of 100 gm powdered leaves with 800 ml of distilled water in a beaker, finally reduced to a volume of 200 ml of solution and strained through filter paper. The isolated extracts were stored at 19°C in airtight containers. The different concentrations were introduced into 15 sets of aquaria.

The acclimated fingerlings were randomly allocated to glass aquaria (22 x 22 x 15). Dechlorinated tap water from storage tank was used and these parameters: temperature, dissolved oxygen, pH ,conductivity, NO_2^-, NO_3^- and NH_3^+ were monitored in each aquarium, with the aid of portable water quality analysis kit. Different concentrations of test solution prepared from the stock solution. The volume of each test solution used in the bioassay per replicate in all treatments was 3 litres. The concentrations of the water extract of tobacco dust in each treatment were 8.3 mL^{-1}, 6.6 mL^{-1}, 5 mL^{-1}, 4 mL^{-1}, and 3.3 mL^{-1}. The concentrations were arrived at after several preliminary investigations. There was also a control. These concentrations were introduced in five aquaria with three replicates for each treatment. Ten fish were put into each aquarium for the acute bioassay test, which lasted for 48 h. Fresh preparations of the test solutions were introduced into the aquarium for various physiological and biochemical assays.

1.4.1.5 Preparation of Tricaine methanesulfonate (MS-222)

The chemical name for MS-222 is tricaine methanesulfonate (Sigma Chemicals, St Louis, MO, USA). It is readily soluble in water, therefore

prepared at different concentrations of MS-222 (35, 40, 45, 50, 52.5, 75, 100 and 102 mg/L) solution and were directly poured into the experimental tank.

The acclimated fingerlings were randomly allocated to glass aquaria (22 x 22 x 15). Dechlorinated tap water from storage tank was used and the parameters such as temperature, dissolved oxygen, pH, and conductivity, NO2-, NO3- and NH_3^+ were monitored in each aquarium, with the aid of portable water quality analysis kit. Different concentrations of test solution was prepared from the stock solution. The volume of each test solution used in the bioassay per replicate in all treatments was 3 litres. The concentrations of the water-soluble MS-222 in each treatment were 35, 40, 45, 50, 52.5, 75, 100 and 102 mg/L. The concentrations were arrived at after several preliminary investigations. There was also a control. These concentrations were introduced in five aquaria with three replicates for each treatment. Ten fish were put into each aquarium for the acute bioassay test, which lasted for 48 h. Fresh preparations of the test solutions were introduced into the aquarium for various physiological and biochemical assays.

1.4.1.6 Preparation of Hypothermic condition

The effects of hypothermia were assessed by keeping in twelve low density polyethylene bags (LDPE of 22 × 60 cm size) in a thermostatically controlled chilling unit (Rotek Instruments, Chest type model, temperature range 0-30°C, M/S.B and C Instruments, Kerala) at different temperature, viz., 22±1, 19±1, 15±1°C; optimum number of fish (tenfish /3 L of water) over 24 h and 48 h respectively. The temperature of the packed water was adjusted to the desired level using the thermostat of chilling unit. For acclimation of juvenile *Etroplus suratensis* to 22±1°C, the temperature was

gradually reduced from 28°C at a gradient of 3°C h^{-1} in order to prevent the temperature shock and induced mortality. Induction was accessed after 15min when the temperature reached to 22±1°C. Recovery time was also recorded. The fish were re-acclimated to 23°C over 23 min in fresh water and recovery was monitored at 1, 2, 4, 8,16, 32, 64, 94 and 120 min survival was recorded at the end of the trial.

After obtaining optimum hypothermal transportation temperature, fishes were ready to pack in low-density polyethylene bags (LDPE) of 22 × 60 cm size. The optimal packing densities of fishes were adopted and transferred into each polyethylene bag filled with 3 L water and the optimal hypothermal temperature. It was then inflated with medical-grade oxygen and the top of the bag was tied and made airtight. A control group with the room temperature 27 ±1°C was also similarly maintained. All treatments were in triplicate. The experiment was conducted at different hypothermal temperatures of 22±1, 19±1, 15±1°C, to simulate the air shipment conditions for 24 and 48 h. The fish were observed carefully at 30-min intervals and their behavioural responses were noted. The maximum hypothermic temperature providing sedation, but the lowest mortality at the end of 48 h was chosen as the optimal dose for maximum transportation. During the experimental period water temperature was monitored. The temperature measurements were done in a controlled bag using a thermometer, while the dissolved oxygen (DO) and pH were monitored using a handheld meter and submersible electrode (Microprocessor Water and soil analysis kit, Model 1160 E1. Environmental and Scientific Instruments, Industrial Area, phase-11, Panchkula, India). Total ammonia, nitrogen, nitrite and nitrate (Spectroquant^{R}

NOVA 60) were measured before and after 24 and 48 h of the experiment (Appendix 1.1).

1.5 Experimental set up for anaesthetization of fish

The fish were starved for 48 h prior to experiment. The experimental glass tanks of 5L capacity (22×22×15cm) were acclimated for a minimum of three days with fully aerated 3L of fresh water. The entire experimental set-up was located indoors, and the fish were maintained under adequate aeration using air blowers for a photoperiod of 12 h L: 12 h D, maintained using a fluorescent bulb (100 W Philips build) providing a light intensity ~800 lx at the water surface. Physico-chemical parameters such as DO, Temperature, pH and Turbidity were measured.

1.5.1 Experimental design

The experimental design involved introducing Juveniles of green chromide *Etroplus suratensis* (4-6cm) into different tanks containing appropriate doses of anaesthetic agents of clove oil (*Syzygium aromaticum)*, cinnamon oil (*Cinnamomum cassia*), cassumunar ginger **(***Zingiber cassumunar*) extract, tobacco leaf (*Nicotiana tobaccum*) extract, MS-222, hypothermic condition (cold) and control.

1.5.1.1 Determination of Acute Toxicity (96 h LC_{50}) test

In determining the relative toxicity of a new chemical to aquatic animal, an acute toxicity test is first conducted and estimated the median lethal concentration (LC_{50}). The LC_{50} is the concentration estimated to produce mortality in 50% of a test population over a specific time (Summerfelt and Smith, 1990; Ross and Ross, 1999). The acute lethal

toxicity of clove oil, cinnamon oil, Cassumunar ginger extract, tobacco leaf extract, MS-222 and hypothermic condition (cold) to juvenile fishes of Pearl spot was determined following the methodology for static test (Rand and Petrocelli, 1985; Parrish, 1985; APHA, 1998).

Preliminary experimental tests were carried out to determine suitable concentration of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS-222 and cold that will not result in outright mortality of fish. For static bioassays the experimental protocol were described as in chapter, section 1.3. Experimental set up and design for anaesthetization of fish were as described in section 1.5 and 1.5.1. Different concentrations of each anaesthetics prepared were as described as in this chapter, section 1.4.1 (1.4.1.1, 1.4.1.2, 1.4.1.3, 1.4.1.4, 1.4.1.5, 1.4.1.6) and were used for the biostatic assays. Each concentration had replicate in series. Two glass aquaria without the extract served as the control experiment. Fresh preparations were introduced into the experimental media on a daily basis. The physio-chemical parameters of the water of various experimental media were monitored every 24 h. For each batch of experiment, 10 fingerlings of Green chromide (*Etroplus suratensis*) (mean weight 1.078 ±0.15-5.373±0.51) were introduced. The following experiments were then conducted to determine: 1. Effect of acute toxicity (96 h LC_{50}) of different concentrations of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS-222 and hypothermic condition (cold).

1.5.1.2 Mortality Change in pattern of the fish during 24, 48, 72 and 96 h

Methods of conducting acute bioassays as described in UNEP (1989) report were employed for the acute toxicity investigation. The exposure

period lasted 96 h (4 days) during which period the fish were not fed. The fish were observed at 3h intervals for the higher concentrations until complete mortality occurred. At lower concentration, they were observed every 6 h. Dead fish were removed immediately from the experimental set-up.

The water quality parameters of the experimental tanks were observed at every sampling time according to APHA (1998) procedures and the experimental protocol were described as in chapter 1, section 1.3.

1.5.1.3 Determination of water quality parameters

The levels of water quality parameters like temperature, dissolved oxygen, pH, turbidity, ammonia, nitrite and nitrate during 96 h (24, 48 and 96 h of induction were monitored every 24-h. Water quality parameters like temperature was monitored with a digital thermometer (-50°C to 200°C range; Superfit, India). Dissolved oxygen (range 0 – 20 ppm, accuracy ±0.01), pH (range 1.0-15.0, accuracy ±0.01) and total turbidity (range 0-20ppm, 0-200ppm, accuracy ± 2% of range ±1 digit) was measured using a handheld meter and submersible electrode (Microprocessor Water and soil analysis kit, Model 1160 E1. Environmental and Scientific Instruments, Industrial Area, phase-11, Panchkula, India). Total ammonia (range 0.20-8.00 mg/L NH_3-N), nitrite (range 0.005-1.00 mg/L NO_2-N) and nitrate (range 0.2-20.0 mg/L NO_3-N.0) were measured using a Spectroquant^R NOVA 60, Photometer (Merck, Frankfurter, Darmstadt Germany) before and after 24, 48, 72 and 96 h of the experiment (Appendix 1.1).

1.5.2 Post treatment survival

After 48 h of experiment, the remaining fishes in the experimental tanks containing were slowly transferred with the help of a handled net into

the Fiber Reinforced Plastic tanks containing aerated water for 1 h. Separate tanks were maintained for all the sets of experimental groups for observing post-transport mortality for seven days after simulated transport. The water temperature in the tanks was 28±1°C with an average dissolved oxygen level of 12 mg/L and the fishes were fed with pelleted feed.

1.6 Statistical analyses

To calculate the LC_{50} of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS-222 and cold, the Trimmed Spearman-Karber method was applied, with lower and upper 95% confidence interval end points (Hamilton et al., 1977).

Water quality data are reported as mean ± SE. The mean values of different tanks were compared by using the ANOVA analysis. The differences were considered to be significant at $p<0.05$. The level of water quality parameters were expressed as graphical summary in accordance with each concentration of anaesthetic. All statistical analyses were performed using IBM SPSS STATISTICS 20.0 (Statistical Data Analysis and Scientific Research centre, UGC, Statistics Department; Mahathma Gandhi University, Kottayam, Kerala) and the level of significance (α) for all tests was 0.05.

1.7 Results

All the six anaesthetics tested, in higher doses, were toxic leading to mortality. The results of the mortality rates (Trimmed Spearman-Karber Method., Hamilton et al., 1977) of the fingerlings of Green chromide (*Etroplus suratensis*) exposed to clove oil, cinnamon oil, cassumunar ginger

extract, tobacco leaf extract, MS-222 and hypothermia (cold) are presented in Tables 1.A.and1.A.1 respectively.

1.7.1 Effect of clove oil

It was observed that fishes were exposed at different concentration of clove oil (0.10, 0.17, 0.23, 0.30, and 0.33) and the cumulative mortality rates (%) for clove oil are presented in Table 1.A. No mortality was observed in the group exposed to lower concentrations (0.10) within the first 24 h of exposure. For all the clove oil concentrations tested in this experiment, the mortality rate was always higher at 0.23, 0.30 and 0.33 mg/L (Table 1.A) during 96 h duration. Depending on the duration of exposure, the mortality rate at each concentration differed. The mortality rate at 0.23 mg/L was lower than 0.30 mg/L (Trimmed Spearman-Karber method; Hamilton et al., 1977). Cent percent survival rates were observed at the lowest concentration of 0.17 mg/L. The cumulative mortality rate (table 1.A) indicated that mortality change in pattern of the test fish and concentrations of clove oil are positively correlated. This shows that the mortality change in pattern of the fish increased with increase in the concentrations of clove oil. Particular lethal concentrations of clove oil with upper and lower limit of 95% confidence intervals for each lethal concentration for *Etroplus suratensis* are shown in Table 1.A.1. No significant difference between LC_{50} values for *Etroplus suratensis* was found when applying the Trimmed Spearman-Karber Method.

The LC_{50} of fingerlings of Green chromide (*Etroplus suratensis*) exposed to various concentrations of clove oil for 24 h was 0.32 mg/L with lower and upper confidence limits of 0.29 and 0.34 mg/L, for 48 h was 0.23 mg/L with lower and upper confidence limits of 0.25 and 0.35 mg/L, for

72 h was 0.23 mg/L with lower and upper confidence limits of 0.25 and 0 35 mg/L and for 96 h was 0.24 mg/L with lower and upper confidence limits of 0.23 and 0.25 mg/L respectively.

Table 1.A. Trimmed Spearman-Karber Method for Estimating LC_{50} on exposure of different concentrations of clove oil

Concentration	Cumulative Mortality (%)			
	Time (h)			
	24	48	72	96
Control	0	0	0	0
0.10	0	0	0	0
0.17	0	0	0	0
0.23	0	0	0	44
0.30	44	54	56	75
0.33	56	63	88	100

Table 1.A.1. Lethal concentrations of clove oil with upper and lower limit of 95% confidence intervals

Time	LC_{50}	95% confidence interval	
		Lower	Upper
24	0.3162	0.2909	0.3438
48	0.2963	0.2495	0.3517
72	0.2898	0.279	0.3011
96	0.2433	0.2322	0.2549

During each exposure period (24, 48, 72 and 96 hthe presents) of the acute toxicity test for clove oil, it was observed that the mean values of all water quality parameters were significantly different ($P < 0.05$). Water quality varying among replicates for all tested variables (DO, pH, temperature, turbidity, ammonia, nitrate and nitrite) was compared to the control values;

they were still within acceptable limits (Mackereth, 1963). The results of the mean values (mean±SE) of variables are presented in Appendix 1.1.

During the determination of 96 h LC_{50} (24, 48, 72 and 96 h) of clove oil, there is no significant change in temperature of the exposed anaesthetic water at concentrations of 0.10 mg/L, 0.17 mg/L, 0.23 mg/L, 0.30 mg/L and 0.37 mg/L respectively (Fig 1.A.1). Each concentration showed a slight variation in temperature rate than that of control. The overall mean temperature in exposure containers for 96 h LC 50 was 27.50 ± 0.13°C for 10min, 27.67 ± 0.133°C for 24 h, 27.23 ± 0.04°C for 48 h, 29.82 ± 0.09°C for 96 h (range, 27-30°C; Appendix 1.1). All measured water temperatures in exposure containers were within the limits specified in the study protocol.

The overall mean pH measurements in exposure containers for 96 h LC_{50} was 6.71 ± 0.0613 for 10min, 5.01 ± 0.38 for 24 h, 7.03 ± 0.12 for 48 h, 5.24 ± 0.29 for 96 h (range, 5-7; Appendix 1.1). There was no substantial difference in pH measured in each concentration (0.10, 0.17, 0.23 and 0.30 mg/L) of exposure containers, indicating that addition of clove oil concentration did not affect water pH during 10min, 24 and 48 h duration. But during 96 h at 0.37 mg/L there was slight increase (7.85) in pH than control (7.41) (Fig.1.A.2).

The dissolved oxygen concentration in the exposed anaesthetic water showed slight increase when compared with the control during 10min, 24 h, 48 h and 96 h at 0.10 mg/L. But at 0.17 mg/L, the dissolved oxygen concentration increased during 96 h duration (10min, 24 h, 48 h and 96 h). At 0.23 mg/L also having the same result of increasing dissolved oxygen concentration. Similarly at 0.30 and 0.37 mg/L showed significant increase in

dissolved oxygen consumption rate (Fig 1.A.3). The overall mean DO measurements in exposure containers for 96 h LC_{50} was 5.98 ±0.37 for 10 min, 6.78 ± 0.05 for 24 h, 7.26 ± 0.15 for 48 h, 5.46 ± 0.23 for 96 h (range, 4-7; Appendix 1.1). All measured DO concentrations in the exposure containers were above the minimum recommended by Piper et al., (1982) for transporting juvenile *Etroplus suratensis.*

Turbidity showed a slight decrease during 10 min and 24 h at the concentration of 0.10 mg/L, 0.17 mg/L, and 0.23 mg/L respectively. But at the concentration of 0.30 and 0.37 mg/L, slight increase was observed in 48 and 96 h (Fig.1.A.4). In the case of NH_3^+ there is no significant change in each concentration during 10 min. During 24 h, the change in pattern of NH_3^+ showed a slight decrease from control 0.10, 0.17, 0.23 mg/L respectively, but at 0.37 mg/L, it showed a significant increase (0.16 mg/L) than control (0.10 mg/L). During 48 and 96 h the change in pattern of NH_3^+ (0.09 mg/L) showed an equivalent result with the control (0.10 mg/L) (Fig.1.A.5). At 10min duration there was no change in the pattern of NO_2- (0.00) with the control (0.00) at each concentration. During 24, 48 and 96 h, the NO_2-rate showed significant increase with the control at each concentration (Fig.1.A.6). NO_3- rate does not change at each concentration with the control during 10min duration. During 24 h each concentration and control showed the same result (0.10 mg/L). During 48 h duration NO_3-rate (0.09 mg/L) showed significant increase with the control (0.05 mg/L) (Fig.1.A.7). But at 96 h the NO_3- rate decreased significantly with control at each concentration.

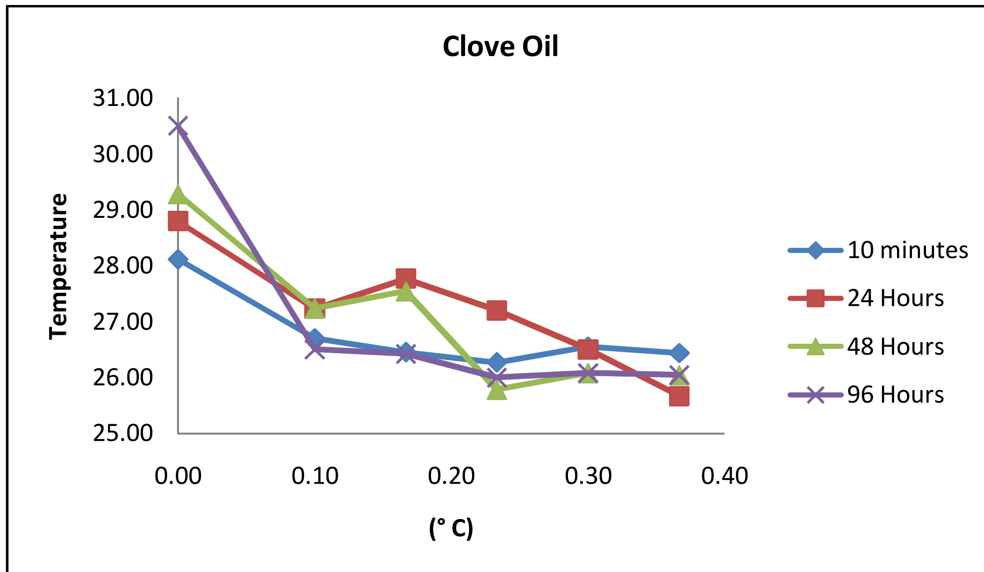


Fig.1.A.1 Change in pattern of temperature (° C) at different concentration of clove oil during 96 h duration

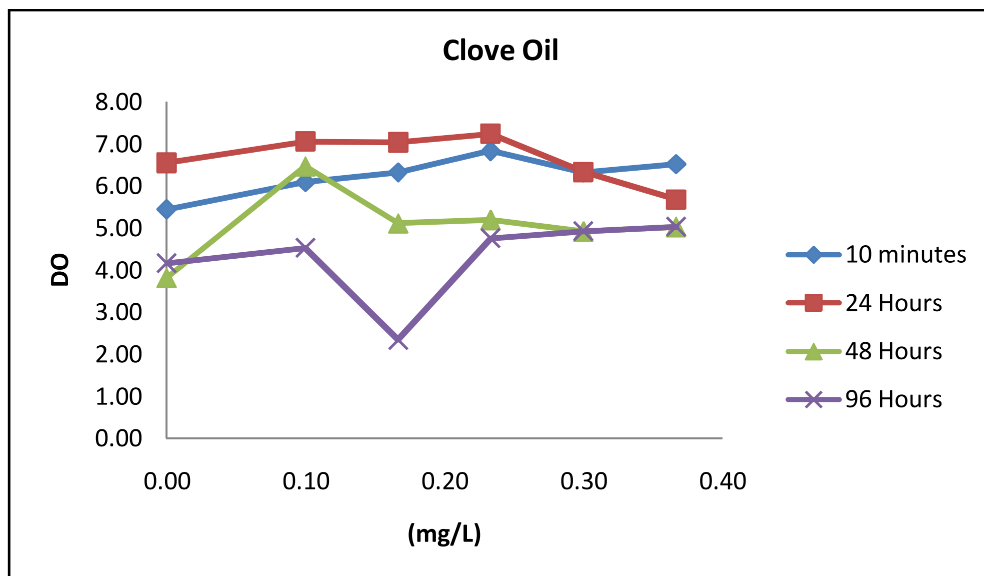


Fig.1.A.2 Change in pattern of dissolved oxygen concentration (mg/L) at different concentration of clove oil during 96 h duration

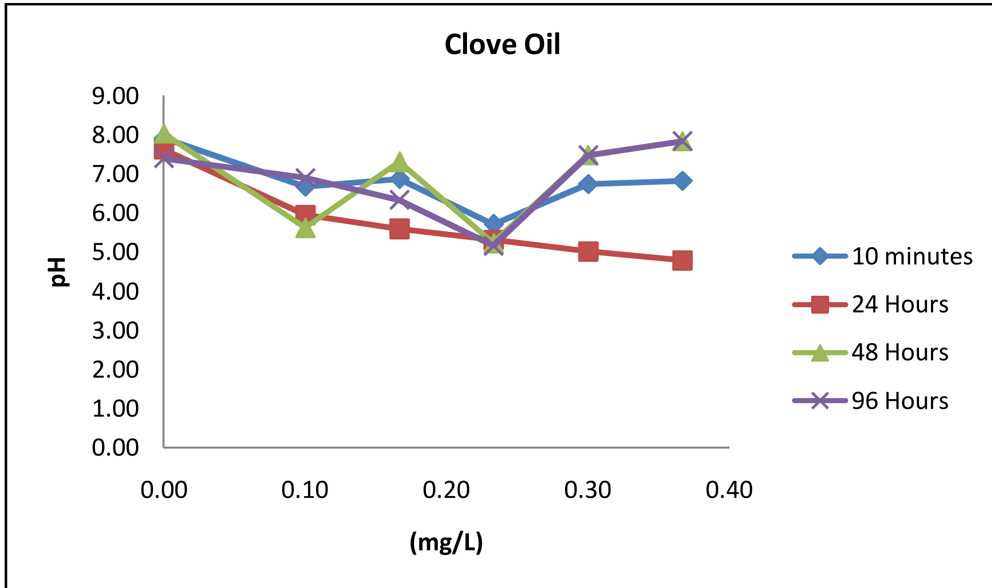


Fig.1.A.3 Change in pattern of pH at different concentration of clove oil during 96 h duration

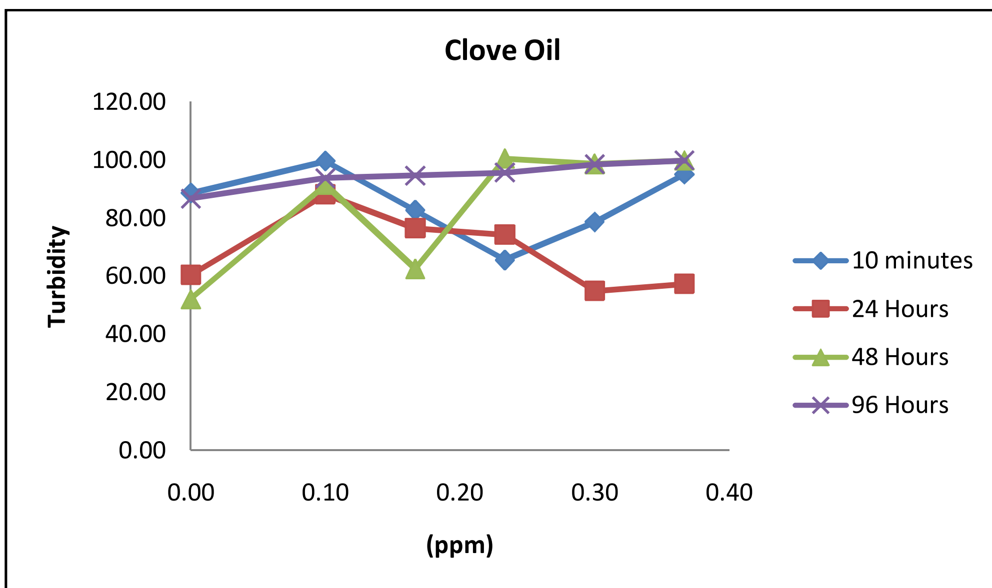


Fig.1.A.4 Change in pattern of turbidity (μ) at different concentration of clove oil during 96 h duration

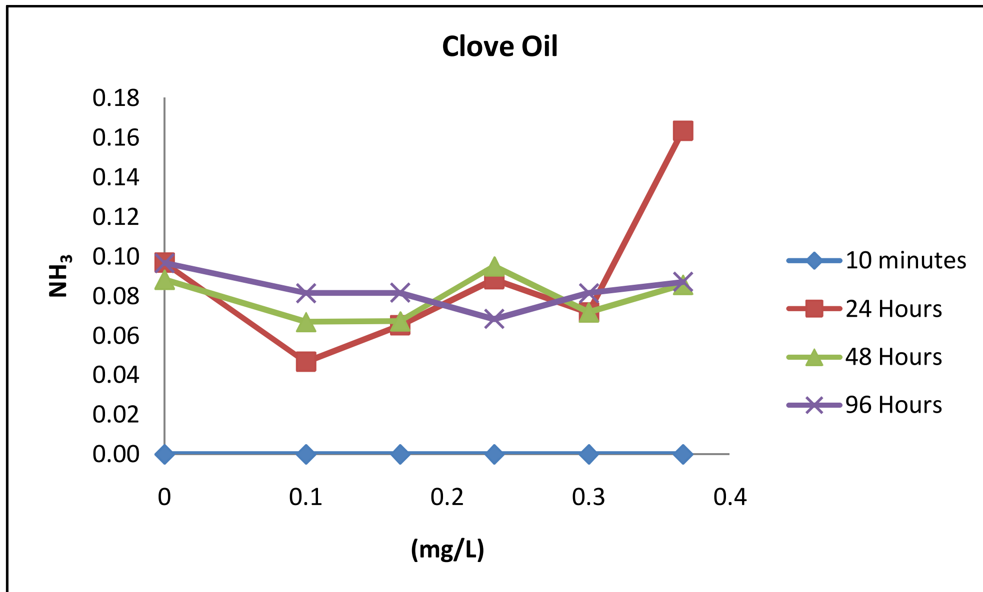


Fig.1.A.5 Change in pattern of NH_3 (mg/L) at different concentration of clove oil during 96 h duration

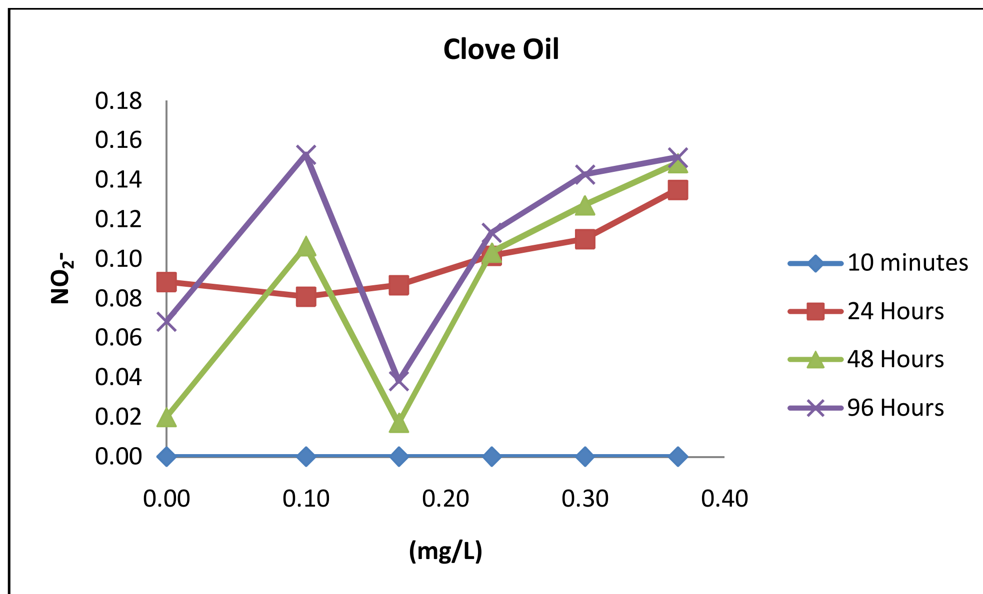


Fig.1.A.6 Change in pattern of NO_2- (mg/L) at different concentration of clove oil during 96 h duration

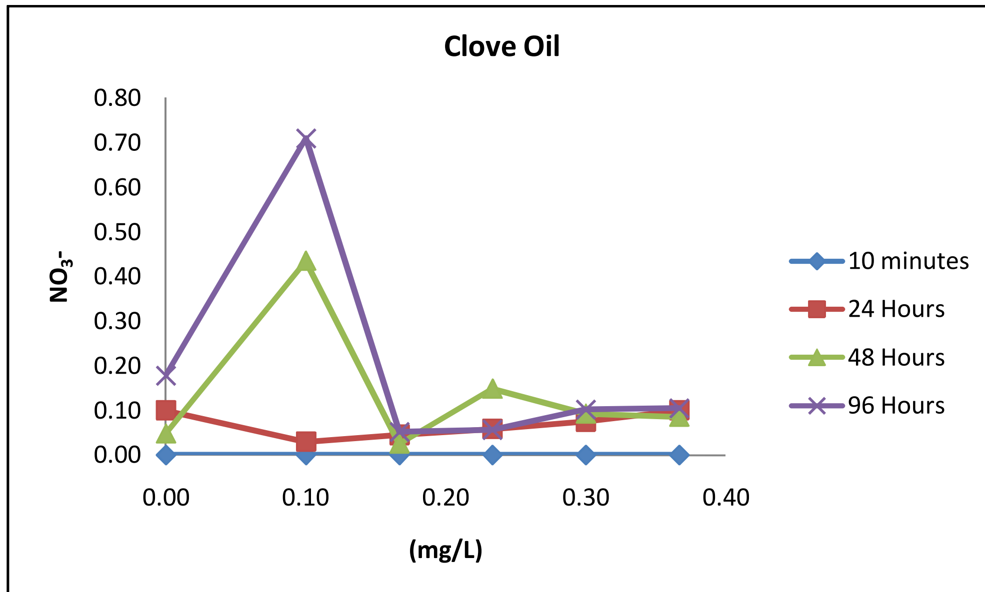


Fig.1.A.7 Change in pattern of NO_3- (mg/L) at different concentration of clove oil during 96 h duration

1.7.2 Effect of Cinnamon oil

The arithmetical representation of the mean mortality change in pattern of 96 h LC_{50} of cinnamon oil is presented in table 1.B. It was observed that fish were exposed at different concentration of cinnamon oil (0.33, 0.50, 0.57, 0.60, and 0.67) and the cumulative mortality rates (%) values for cinnamon oil is also presented in Table 1.B. No mortality was observed in the group exposed to lower concentrations (0.33, 0.50 mg/L) within 96 h of exposure.

For all the cinnamon oil concentrations tested in this experiment, the mortality rate was always higher at 0.67 mg/L (Table 1.B). Cinnamon oil, toxicity was higher at 96th h in the concentration of 0.57, 0.60 and 0.67 and the mortality rate increased with increase in concentration. The mortality rate at 0.57 mg/L was lower than that of 0.60 and 0.67 mg/L (Trimmed Spearman-

Karber method, Hamilton et al., 1977). Cen percent survival rate was observed at the lowest concentration of 0.33 and 0.50 mg/L. The cumulative mortality rate (table 1.B) indicated that mortality change in pattern of the test fish and concentrations of cinnamon oil are positively correlated. This showed that the mortality change in pattern of the fish increased with increase in the concentrations of cinnamon oil. Particular lethal concentrations of cinnamon oil with upper and lower limit of 95% confidence intervals for each lethal concentration for *Etroplus suratensis* are shown in Table 1.B.1. No significant difference between LC_{50} values for *Etroplus suratensis* was found when applying the Trimmed Spearman-Karber Method.

Table 1.B. Trimmed Spearman-Karber Method for Estimating LC_{50} on exposure of different concentrations of cinnamon oil.

Concentration	**Cumulative Mortality (%) Time (h)**			
	24	**48**	**72**	**96**
Control	0	0	0	0
0.33	0	0	0	0
0.50	0	0	0	0
0.57	0	0	42	50
0.60	0	44	63	75
0.67	0	63	75	88

Table 1.A.1. Lethal concentrations of cinnamon oil with upper and lower limit of 95% confidence intervals

Time	**LC_{50}**	**95% confidence interval**	
		Lower	**Upper**
24			
48	0.6224	0.5757	0.6729
72	0.5845	0.5661	0.6036
96	0.5681	0.5554	0.5811

During each exposure period (24, 48 and 96 h) of the acute toxicity test for cinnamon oil, it was observed that the mean values of all water quality parameters were significantly different ($P < 0.05$).Water quality varied among replicates for all tested variables (DO, pH, temperature, turbidity, ammonia, nitrate and nitrite) compared to the control values. The result of the mean values (mean±SE) of variables are presented in Appendix 1.1

During the determination of 96 h LC_{50} (24,48and 96 h) of cinnamon oil, there is no significant change in temperature of the exposed anaesthetic water at concentrations of 0.10, 0.17, 0.23, 0.30 and 0.37 mg/L respectively (Fig 1.B.1) with control. The pH decreased in all treatments of different concentration (0.33, 0.50, 0.57 and 0.60 mg/L) with the control and 0.67 mg/L) during 10min, 24h and 48 h. During 96 h duration (Fig.1.B.2) it showed a slight increase in pH. At 0.33, 0.50, and 0.57mg/L the DO concentration showed a slight increase with the control during 10 min. During 24 h the DO showed a significant decrease with the control (Fig.1.B.3). At 48 h duration there was no significant change with the control and treatments (Fig.1.B.3). During 96 h all treatments showed a slight decrease with the control (Fig.1.B.3). Turbidity decreased with the control in all treatment during 10 min and 24 h. At 48 h, turbidity showed a slight increase with the control and is quiet reversal during 96 h (Fig.1.B.4). After 10 min exposure of anaesthetic concentration (0.33, 0.50, 0.57, 0.60, and 0.67mg/L) $NH3^{+}$ remained at 0 which was same with control treatment. During 24, 48 and 96 h of all treatments it did not show any significant change with the control (0.10 mg/L) (Fig.1.B.5). After 10 min, in all concentrations of cinnamon the NO_2- rate was same with the control result

(0). During 24 h duration NO_2-showed a decreased state (0.02 mg/L) than that of control (0.09). There was no significant change in NO_2- rate between the control and all treatments during 48 h and 96 h (Fig.1.B.6). There was not any significant change in NO_3- rate in the control and different concentration within 10min NO_3- rate in all concentrations showed decreased rate than control during 24 and 48 h. (Fig.1.B.7). During 96 h there is significant change in all treatments (0.03, 0.05, 0.05, 0.06 and 0.67 mg/L) and control (0.18mg/L).

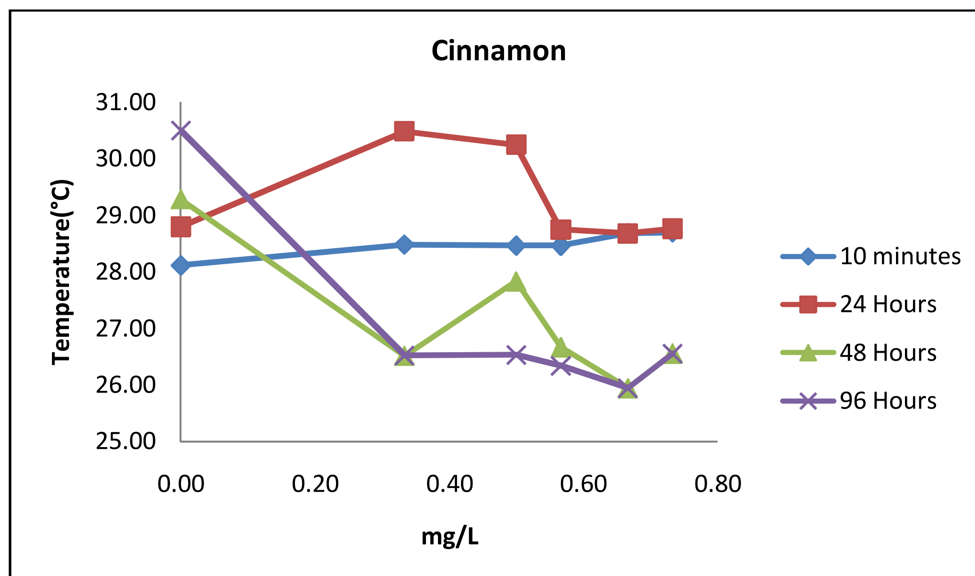


Fig.1.B.1 Change in pattern of temperature (°C) at different concentration of cinnamon oil during 96 h duration

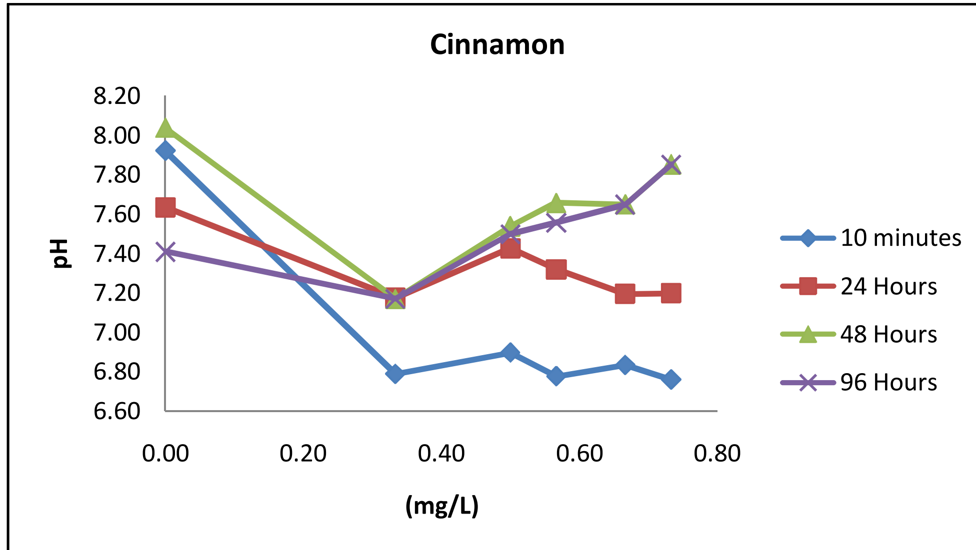


Fig.1.B.2 Change in pattern of pH at different concentration of cinnamon oil during 96 h duration

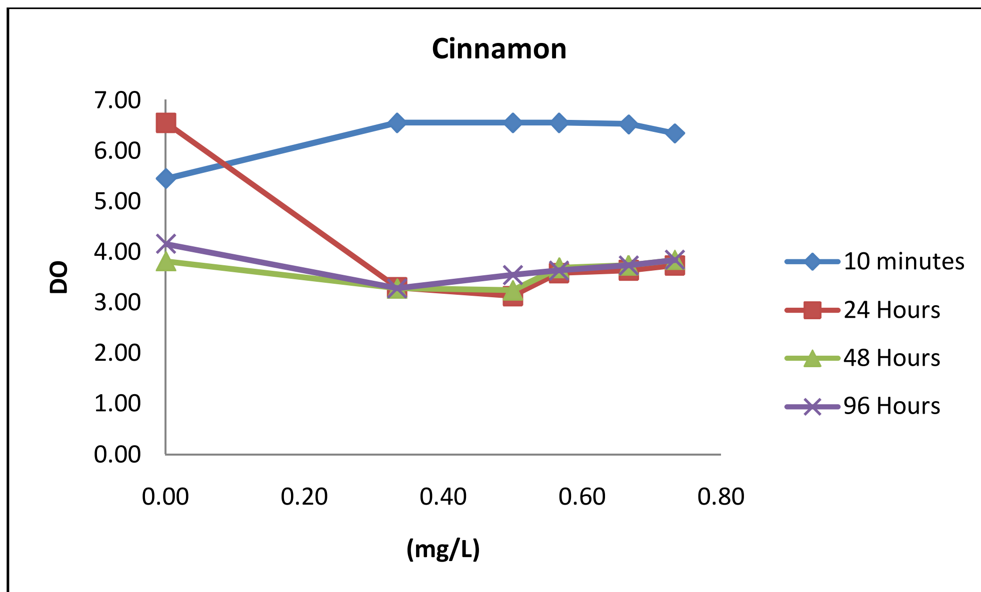


Fig.1.B.3 Change in pattern of DO at different concentration of cinnamon oil during 96 h duration

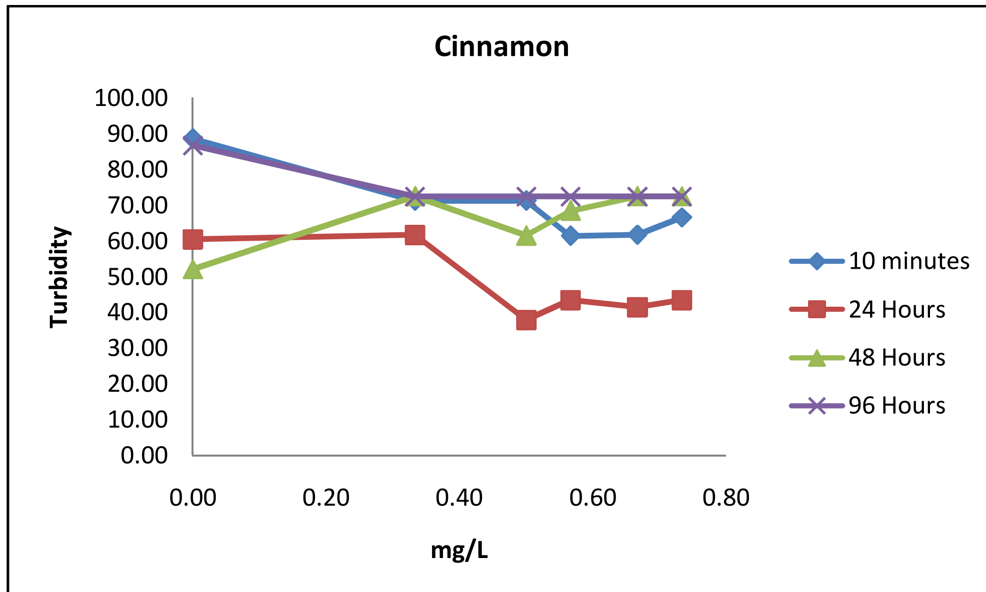


Fig.1.B.4 Change in pattern of turbidity at different concentration of cinnamon oil during 96 h duration

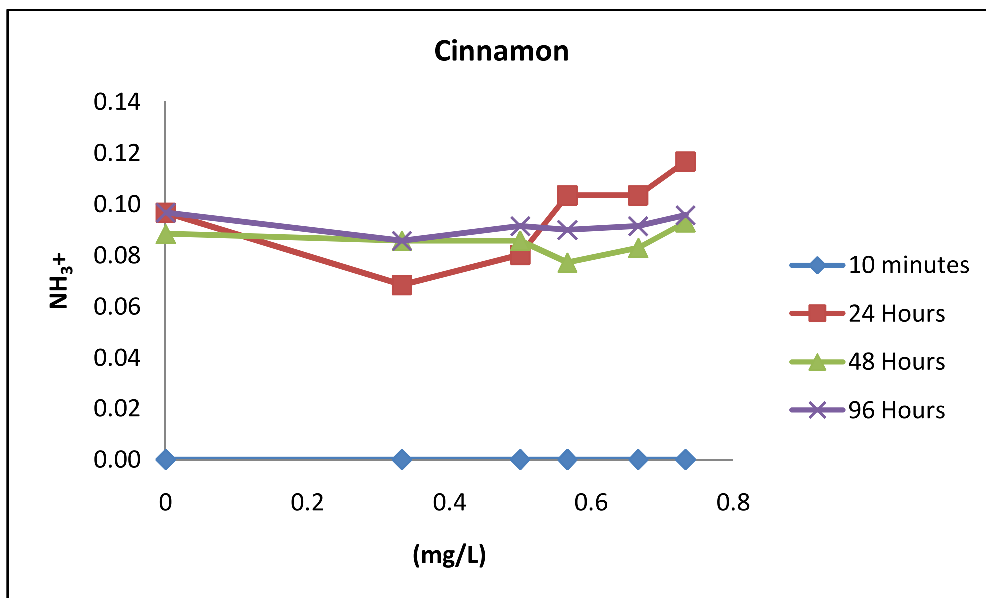


Fig.1.B.5 Change in pattern of NH_3 at different concentration of cinnamon oil during 96 h duration

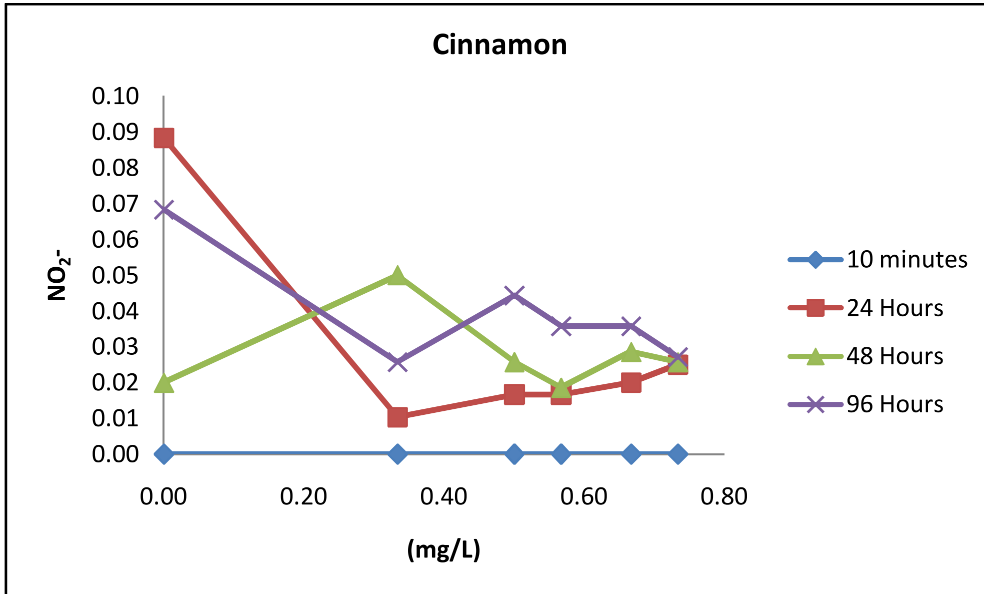


Fig.1.B.6 Change in pattern of NO_2- at different concentration of cinnamon oil during 96 h duration

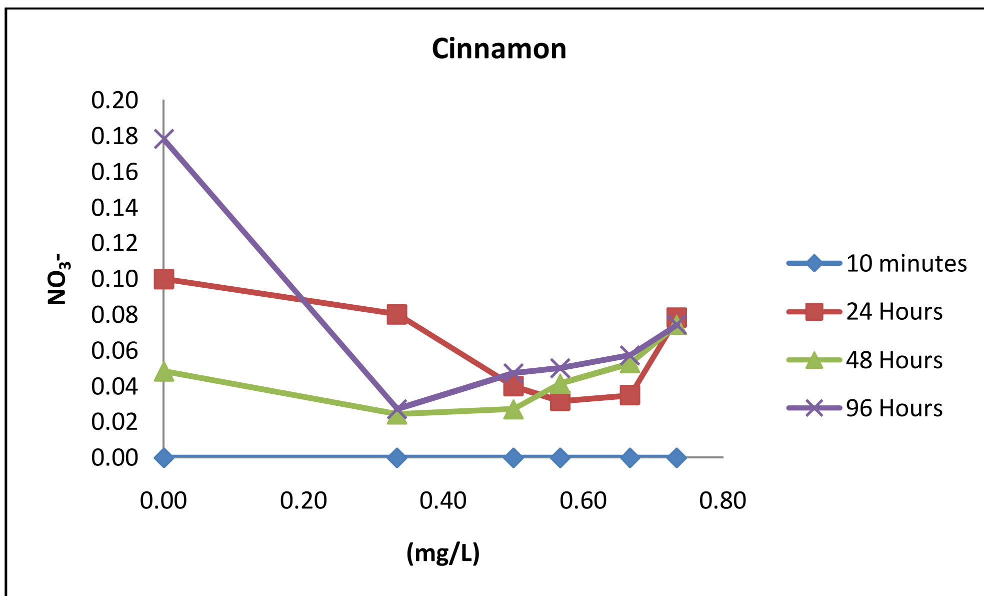


Fig.1.B.7 Change in pattern of NO_3- at different concentration of cinnamon oil during 96 h duration

1.7.3 Effect of *Zingiber cassumunar* Roxb

The results observed for fish exposed at different concentration of *Zingiber cassumunar* Roxb extract, (0.50, 0.70, 1.30, 1.50, 1.70 mg/L) and the cumulative mortality rates (%) is presented in Table 1.C. The diagrammatic representation of (bar diagram) of the mean mortality change in pattern of 96 h LC_{50} of *Zingiber cassumunar* Roxb extract is presented in Figure 1.C. No mortality was observed in the group exposed to lower concentrations (0.10mg/L) within the first 24 h of exposure. For all the *Zingiber cassumunar* Roxb extract concentrations tested in this experiment, the mortality rate was always higher at 1.50, 1.60 and 3.0 mg/L respectively (Table 1.C) during 96 h duration. Depending on the duration of exposure period, the mortality rate at each concentration differed. The mortality rate at 0.50 mg/L was lower than 0.60 and 3 mg/L (Trimmed Spearman-Karber method, Hamilton et al., 1977). Cen percent survival rate was observed at the lowest concentration of 0.50 and 0.70 mg/L. The cumulative mortality rate (table 1.C) indicated that mortality change in pattern of the test fish and concentrations of *Zingiber cassumunar* Roxb extract are positively correlated. This shows that the mortality change in pattern of the fish increased with increase in the concentrations of *Zingiber cassumunar* Roxb extract. Particular lethal concentrations of *Zingiber cassumunar* Roxb extract with upper and lower limit of 95% confidence intervals for each lethal concentration for *Etroplus suratensis* are shown in Table 2.C.1. No significant difference between LC_{50} values for *Etroplus suratensis* was found when applying the Trimmed Spearman-Karber Method.

Table 1.C. Trimmed Spearman-Karber Method for Estimating LC_{50} on exposure of different concentrations of cassumunar ginger extracts *(Zingiber cassumunar* Roxb*)*

Concentration	Cumulative Mortality (%)			
	Time (h)			
	24	48	72	96
Control	0	0	0	0
0.50	0	0	0	0
0.70	0	0	0	0
1.33	0	0	6	19
1.50	38	50	63	75
1.60	38	50	63	75
3.00	50	63	100	100

Table 1.C.1. Lethal concentrations of cassumunar ginger extract (*Zingiber cassumunar* Roxb) with upper and lower limit of 95% confidence intervals

Time	LC_{50}	95% confidence interval	
		Lower	Upper
24			
48	1.7464	1.3251	2.3016
72	1.6273	1.5393	1.7203
96	1.4689	1.3823	1.561

During each exposure period (24, 48 and 96 h) of the acute toxicity test for *Zingiber cassumunar* Roxb extract, it was observed that the mean values of all water quality parameters were signifycantly different ($P < 0.05$).Water quality varying among replicates for all tested variables (DO, pH, temperature, turbidity, ammonia, nitrate and nitrite) compared to the control values were still within acceptable limits (Mackereth, 1963). The results of the mean values (mean±SE) of variables are presented in Appendix 1.1.

There was no significant change in all treatments and control for temperature during 10 min, 24h, 48h and 96 h duration (Fig 1.C.1). pH

showed a slight decrease in all concentration (0.50, 0.70, 1.30, 1.50 and 1.70 mg/L) than control during 10min of treatment.There was no significant change in control and all the test concentrations during 24 h treatment. During 48 h treatment there was significant increase in pH in all concentration, compared with control. During 96 h, pH showed a slight increase in all concentration with control (Fig.1.C.2). After 10 min a slight increase in DO in all treatments were seen compared with control. During 24 h duration there was a slight variation in DO with control (6.56 mg/L) in treatments of 0.50, 0.70 and 1.30 but in treatment 0.50 and 0.70 mg/L there was significant increase in DO concentration (8.57 and 7.33 mg/L) respectively with control (6.56 mg/L). During 48 h, the changes in pattern of DO were same with that control and in all treatments. During 96 h duration the concentration change in pattern of DO showed a slight decrease in all treatments with control (Fig.1.C.3). During 10 min and 24 h duration, turbidity showed a decreased rate with control. During 48 h a slight variation noticed. But during 96 h the turbidity showed a significant decrease (Fig.1.C.4). After 10min the change in pattern of NH_3 in control as well as in all treatment was zero. During 24, 48 and 96 h a significant decrease was noticed in all treatment with control (Fig.1.C.5). After 10min the NO_2- rate was zero in control as well as in all concentration. During 24 h a significant decrease in all treatment was seen compared with control (0.09 mg/L). During 48 h a tendency to increase the NO_2- was seen than control with increase in concentration of *Zingiber cassumunar* Roxb extract. During 96 h, the change in pattern of NO_2- increased with increase in concentration (Fig.1.C.6). NO_3- rate remained same in all treatments and controls (0). During 24 h a sudden increase in NO_3- level with increase in concentration of

Zingiber cassumunar Roxb extract than control (0.10 mg/L) was noticed. During 48 and 96 h a significant decrease in all treatment and in control (Fig.1.C.7) was observed.

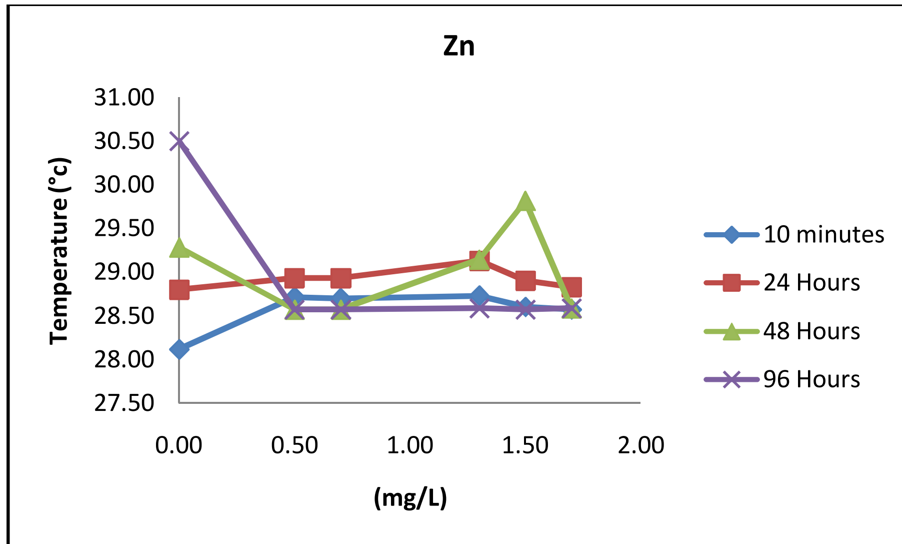


Fig.1.C.1 Change in pattern of temperature (°C) at different concentration of *Zingiber cassumunar* Roxb extract during 96 h duration

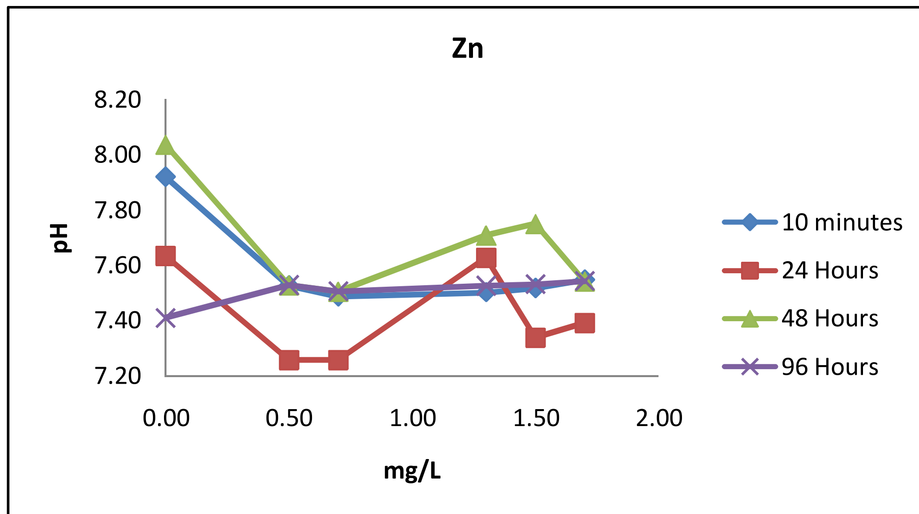


Fig.1.C.2 Change in pattern of pH at different concentration of *Zingiber cassumunar* Roxb extract during 96 h duration

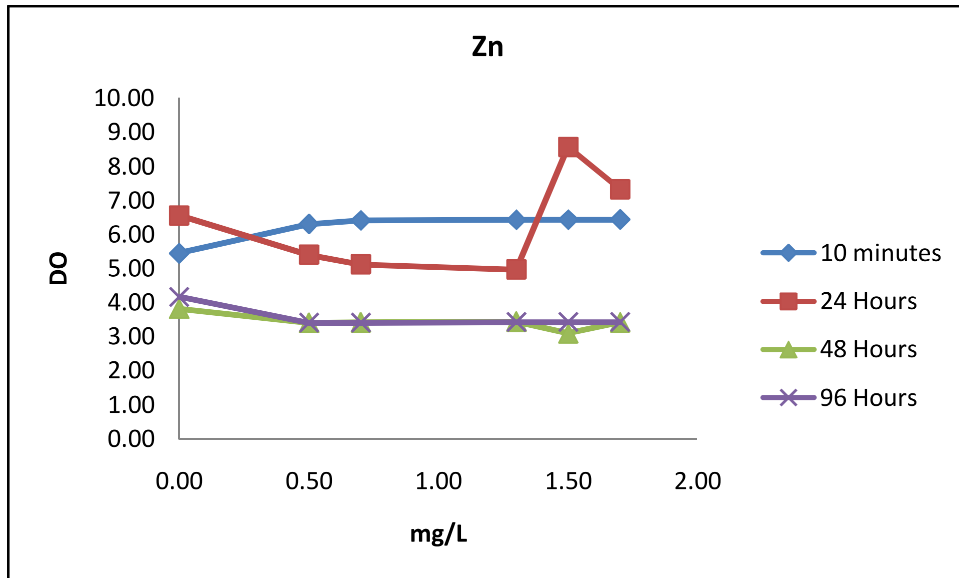


Fig.1.C.3 Change in pattern of DO at different concentration of *Zingiber cassumunar* Roxb extract during 96 h duration

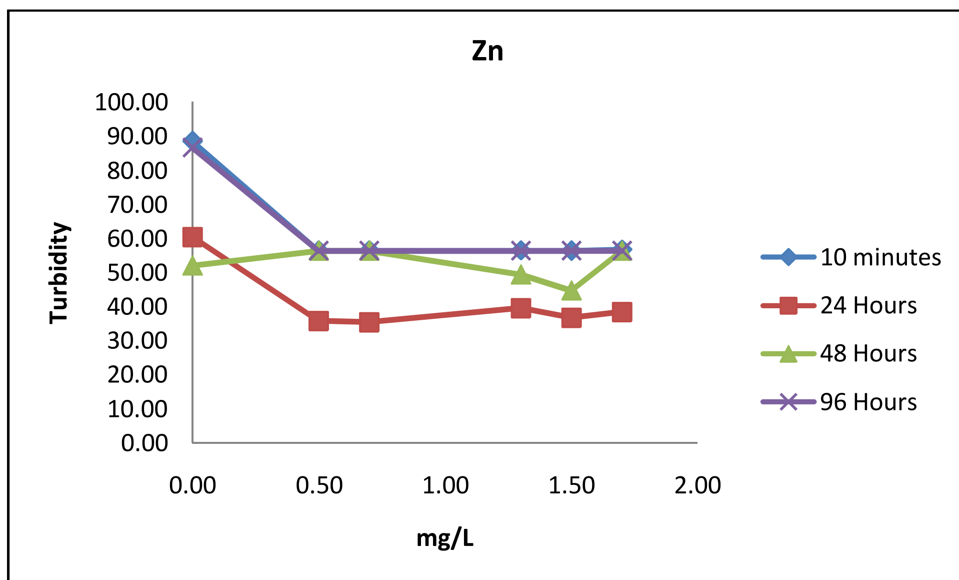


Fig.1.C.4 Change in pattern of turbidity at different concentration of *Zingiber cassumunar* Roxb extract during 96 h duration

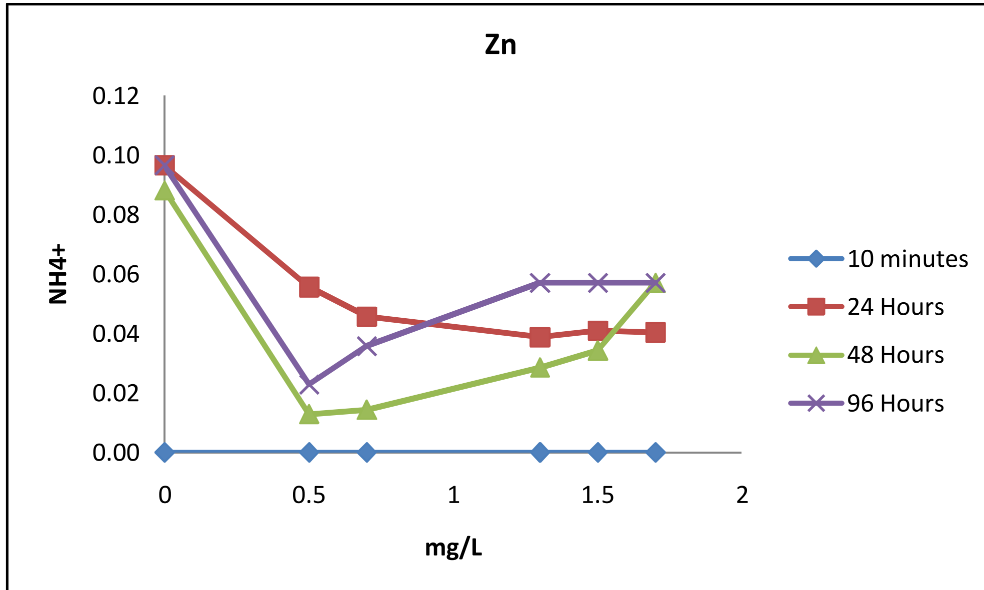


Fig.1.C.5 Change in pattern of NH_3+ at different concentration of *Zingiber cassumunar* Roxb extract during 96 h duration

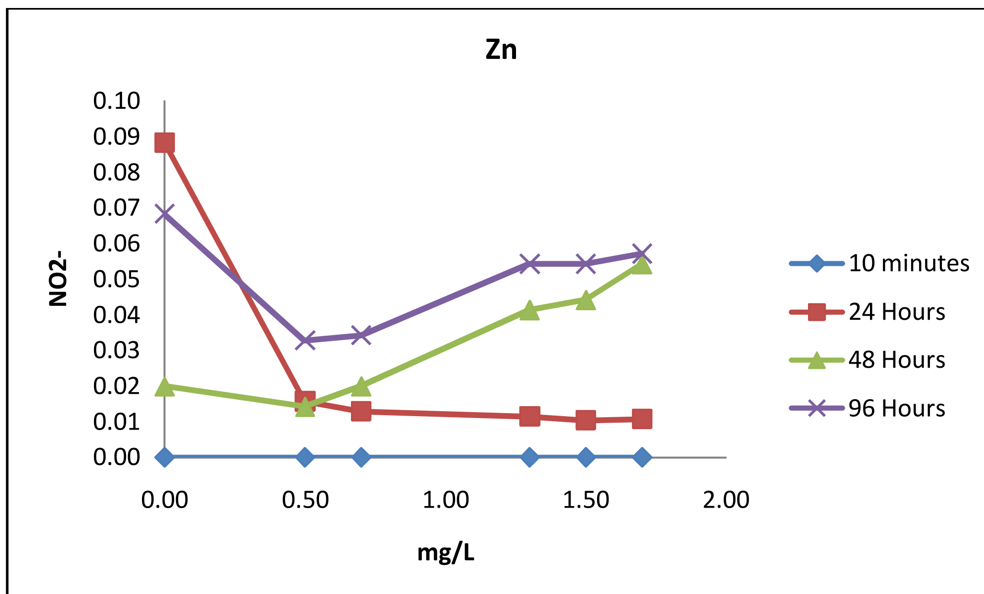


Fig.1.C.6 Change in pattern of NO_2- at different concentration of *Zingiber cassumunar* Roxb extract during 96 h duration

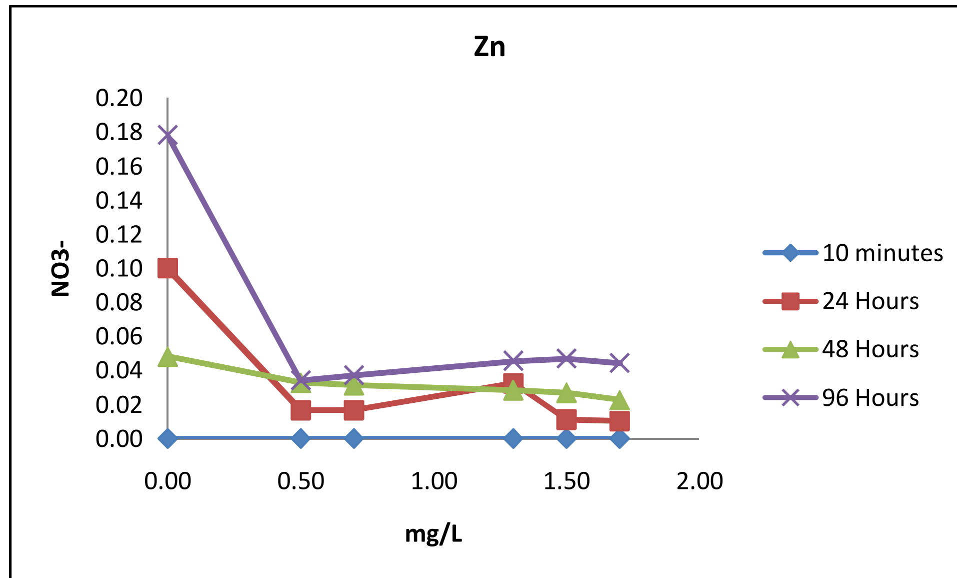


Fig.1.C.7 Change in pattern of NO_3- at different concentration of *Zingiber cassumunar* Roxb extract during 96h duration

1.7.4 Effect of Tobacco leaves extract (*Nicotiana tobacum*)

The data pertaining to fishes exposed at different concentration of tobacco leaf extract (*Nicotiana tobacum*) (2, 5, 6, 7 and 8 mg/L) and the cumulative mortality rates (%) for tobacco leaf extract (*Nicotiana tobacum*) is presented in Table1.D. For all the tobacco leaf (*Nicotiana tobacum*) extract concentration tested in this experiment, the mortality rate was always higher at 7 and 8 mg/L (Table 2.D) during 96 h duration. Depending on the duration of the present result, the mortality rate at each concentration differed. The mortality rate at 6 mg/L was lower than 7 and 8 mg/L (Trimmed Spearman-Karber method; Hamilton et al., 1977). 100% survival rate was observed at the lowest concentration of 2 and 5 mg/L.

The cumulative mortality rate (table 1.D) indicated that mortality change in pattern of the test fish and concentrations of tobacco leaf extract (*Nicotiana tobacum*) are positively correlated. This showed that the mortality change in pattern of the fish increased with increase in the concentrations of tobacco leaf extract (*Nicotiana tobacum*). Particular lethal concentrations of tobacco leaf extract (*Nicotiana tobacum*) with upper and lower limit of 95% confidence intervals for each lethal concentration for *Etroplus suratensis*, are shown in Table 1.D.1. No significant difference between LC_{50} values for *Etroplus suratensis*, was found when applying the Trimmed Spearman-Karber Method.

The LC_{50} of fingerlings of Green chromide (*Etroplus suratensis*) exposed to various concentrations of tobacco leaf extract (*Nicotiana tobacum*) for 24 h was 0.32 mg/L with lower and upper confidence limits of 0.29 and 0.34 mg/L, for 48 h was 0.23 mg/L with lower and upper confidence limits of 0.25 and 0.35 mg/L, for 72 h was 0.23 mg/L with lower and upper confidence limits of 0.25 and 0.35 mg/L and for 96 h was 0.24 mg/L with lower and upper confidence limits of 0.23 and 0.25 mg/L respectively.

Table 1.D. Trimmed Spearman-Karber Method for Estimating LC_{50} on exposure of different concentrations of tobacco leaf extract

Concentration	Cumulative Mortality (%)			
	Time (h)			
	24	48	72	96
Control	0	0	0	0
2	0	0	0	0
5	0	0	0	0
6	0	0	8	19
7	38	50	63	75
8	50	63	100	100

Table 1.D.1. Lethal concentrations of tobacco leaf extract with upper and lower limit of 95% confidence intervals

Time	LC_{50}	95% confidence interval	
		Lower	Upper
24			
48	7.1343	6.2155	8.1889
72	6.7447	6.5868	6.9064
96	6.5094	6.3449	6.6782

During each exposure period (24, 48 and 96 h) of the acute toxicity test for tobacco leaf extract (*Nicotiana tobacum*), it was observed that the mean values of all water quality parameters were significantly different ($P < 0.05$). Water quality varying among replicates for all tested variables (DO, pH, temperature, turbidity, ammonia, nitrate and nitrite) compared to the control values; and were within acceptable limits (Mackereth, 1963). The results of the mean values (mean±SE) of variables are presented in Appendix 1.1

During the determination of 96 h LC_{50} (24, 48 and 96 h) of tobacco leaf extract (*Nicotiana tobacum*), there is no significant change in temperature of the exposed anaesthetic water at concentrations of 2, 5, 6, 7 and 8 mg/L respectively (Fig 1.D.1). Each concentration showed a slight variation in temperature than that of control. During 10 min pH decreased with control. All treatments during 48 h showed a slight decrease in pH with control. In all concentrations during 96 h there was slight increase in pH than control (Fig 1.D.2). After 10 min slight variation was noticed in DO level at lower concentration, but the level increased with increase in concentration. During 24 h, there is no significant difference between control and other concentration (2, 5, 6, 7 and 8 mg/L). During 48 h the DO was seen to significant increase than

control. During 96 h DO showed a slight variation between different concentration and control (Fig 1.D.3). All concentration of tobacco leaf extract showed high significant change in turbidity than that of control during 10min, 24h, 48h and 96 h (Fig 1.D.4). The change in pattern of $NH3^+$ was zero as well as in control and all other concentrations after 10 min. During 24 and 48 h NH_3^+ decreased than control, but during 48 h at high concentration (8 mg/L) the NH_3+ concentration increased (1.24 mg/L) than control (1.10 mg/L) (Fig 1.D.5). Similarly during 96 h the NH_3^+ (1.63 mg/L) rate significantly increased than control (1.10mg/L). After 10 min NO_2- rate was zero. NO_2- rate increased with increase in concentration during 24 h. During 48 and 96 h the change in pattern of NO_2- increased with increase in concentration than control (Fig 1.D.6). After 10 min NO_3- rate was zero at all concentration and control. After 24 h the change in pattern of NO_3- decreased from that control. But during 48 h, the NO_3- increased with increase in concentration. During 96 h there was no significant difference between control and all concentration (Fig 1.D.7).

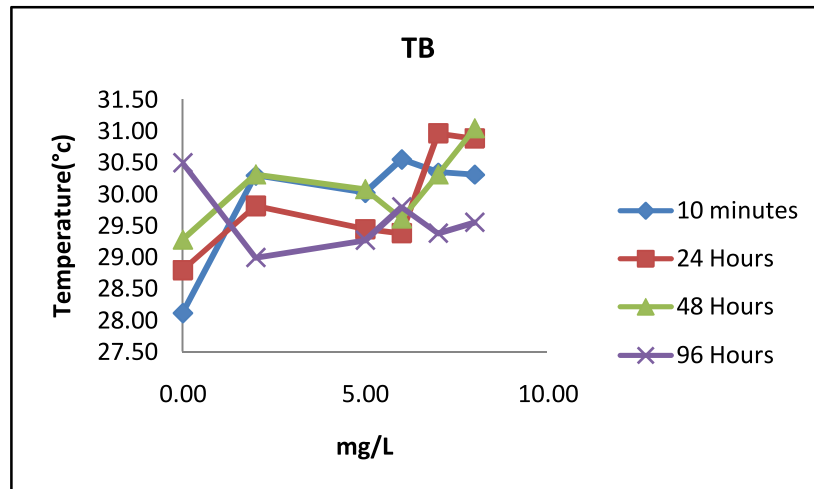


Fig.1.D.1 Change in pattern of temperature (°C) at different concentration of tobacco leaf extract (*Nicotiana tobacum*) during 96 h duration

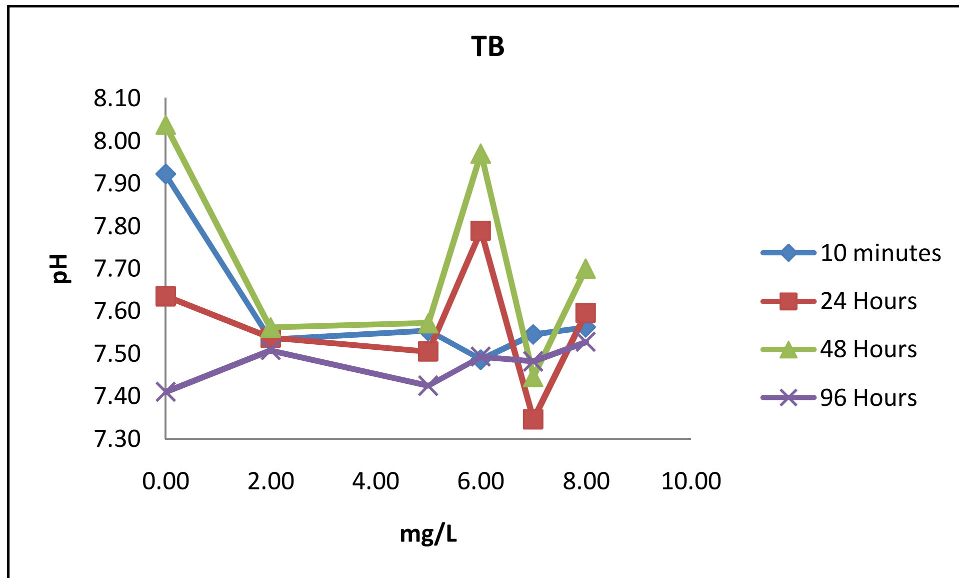


Fig.1.D.2 Change in pattern of pH at different concentration of tobacco leaf extract (*Nicotiana tobacum*) during 96 h duration

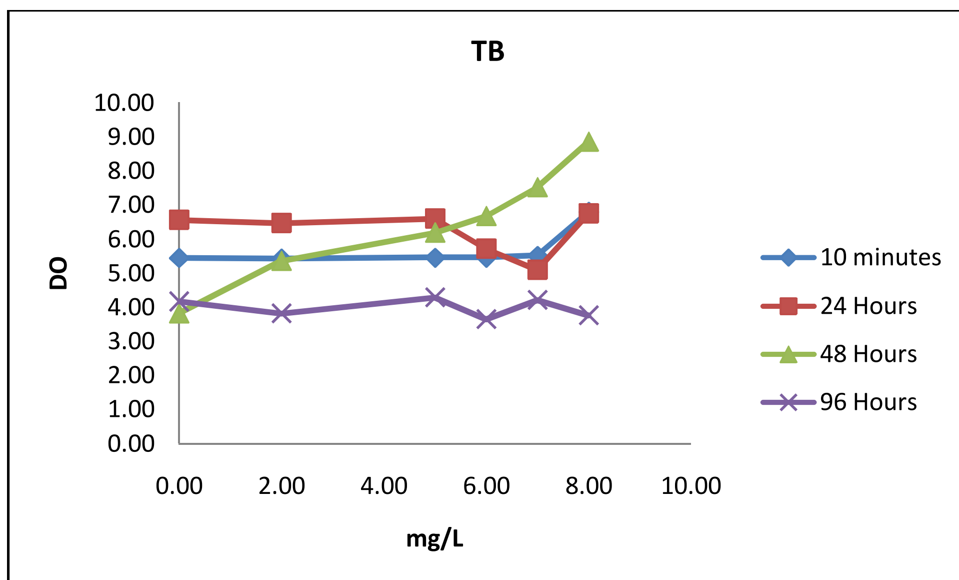


Fig.1.D.3 Change in pattern of DO at different concentration of tobacco leaf extract (*Nicotiana tobacum*) during 96 h duration

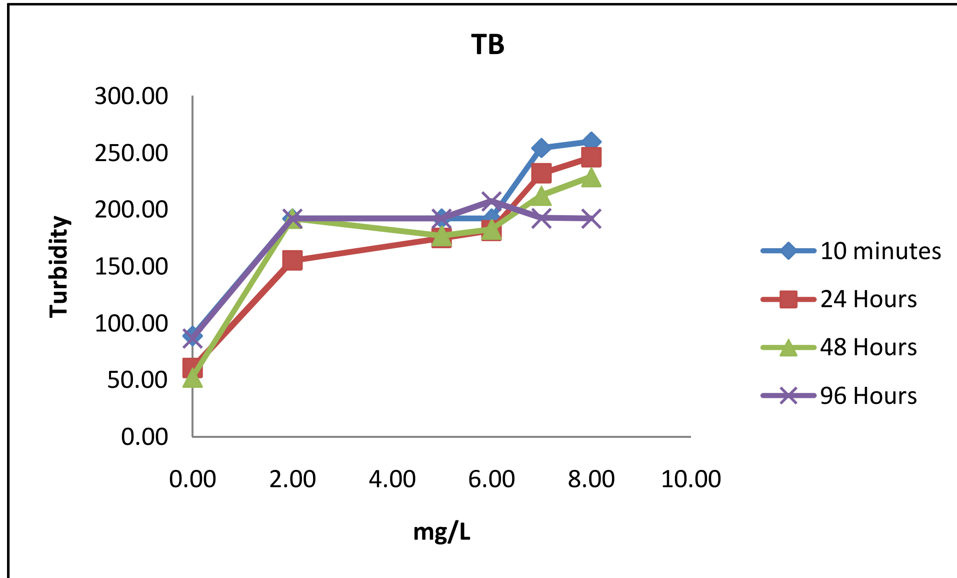


Fig.1.D.4 Change in pattern of turbidity at different concentration of tobacco leaf extract (*Nicotiana tobacum*) during 96 h duration

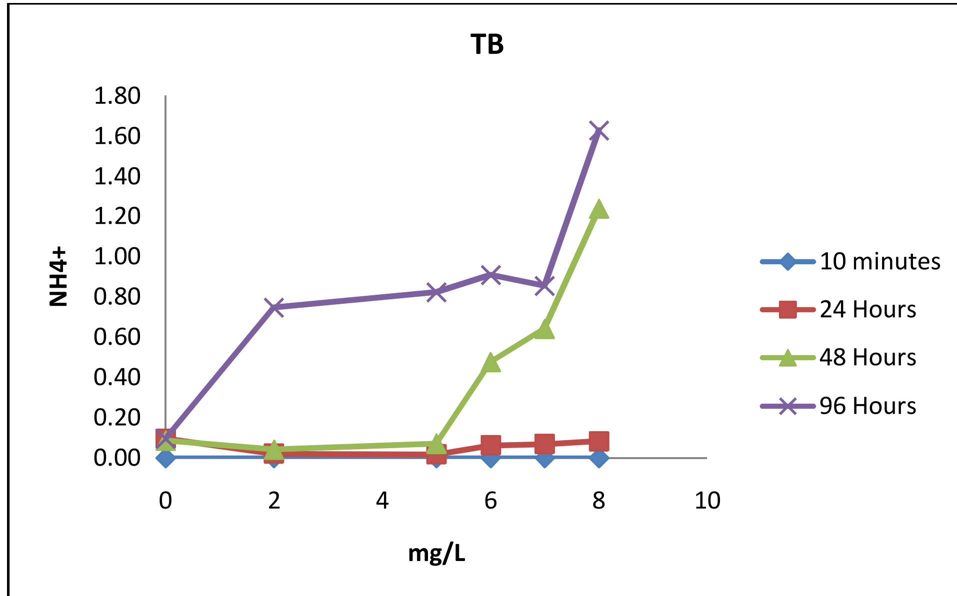


Fig.1.D.5 Change in pattern of NH_3+ at different concentration of tobacco leaf extract (*Nicotiana tobacum*) during 96 h duration

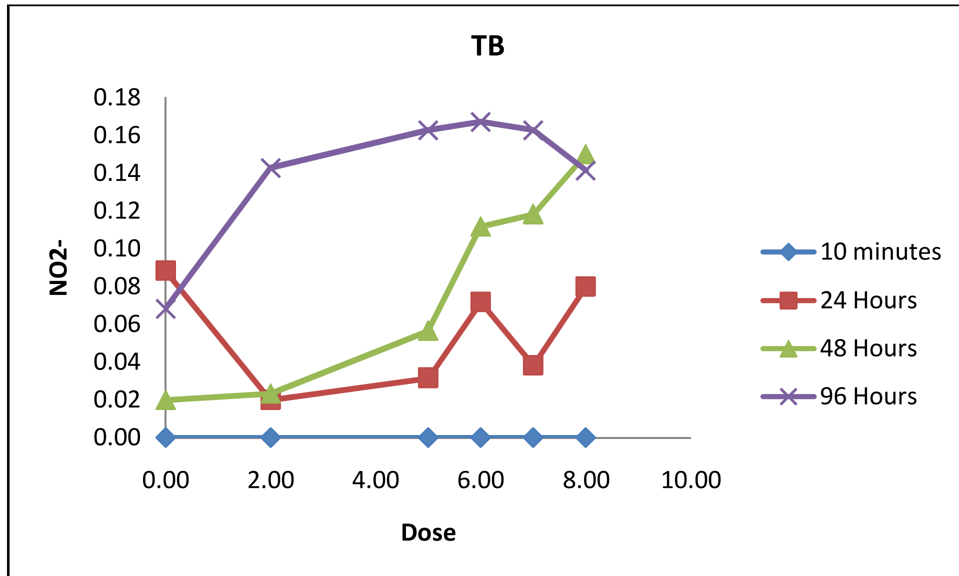


Fig.1.D.6 Change in pattern of NO_2- at different concentration of tobacco leaf extract (*Nicotiana tobacum*) during 96 h duration

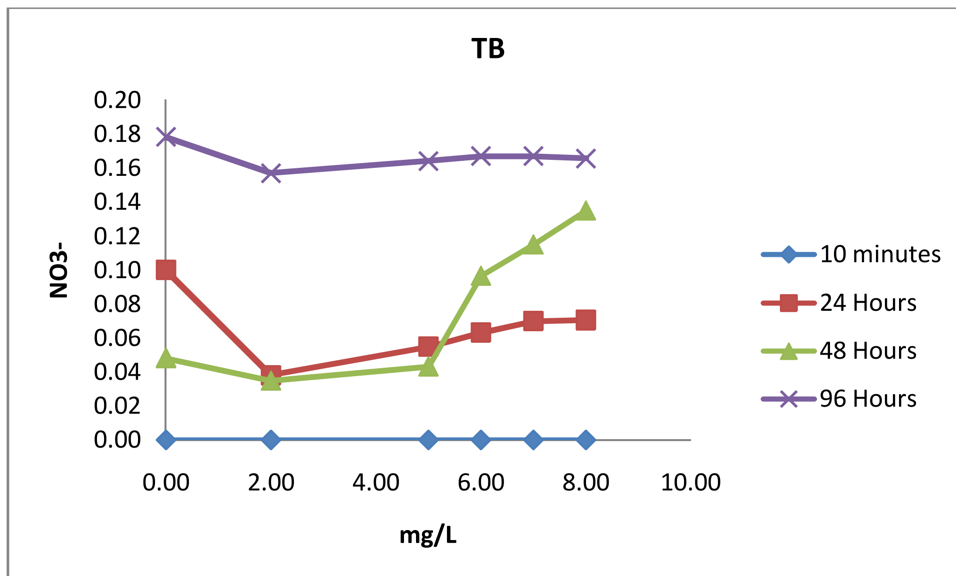


Fig.1.D.7 Change in pattern of NO_3- at different concentration of tobacco leaf extract (*Nicotiana tobacum*) during 96 h duration

1.7.5 Effect of MS-222 (Tricaine Methanesulfonate)

The data in fishes exposed at different concentration of MS-222 (Tricaine methanesulfonate) (45, 50, 75 and 100 mg/L) and the cumulative mortality rates (%) for MS-222 (Tricaine methanesulfonate) is presented in Table1.E.For all the MS-222 (Tricaine methanesulfonate) concentrations tested in this experiment, the mortality rate was always higher at 75 and 100 mg/L (Table 1.E) during 96 h duration. Depending on the duration of hthe presents, the mortality rate at each concentration differed. The mortality rate at 53 mg/L was lower than 75 and 100 mg/L (Trimmed Spearman-Karber method, Hamilton et al., 1977). Cen percent survival rate was observed at the lowest concentration of 45 and 50 mg/L.The cumulative mortality rate (table 1.E) indicated that mortality change in pattern of the test fish and concentrations of MS-222 (Tricaine methanesulfonate) are positively correlated. This shows that the mortality change in pattern of the fish increased with increase in the concentrations of MS-222 (Tricaine methanesulfonate). Particular lethal concentrations of MS-222 (Tricaine methanesulfonate) with upper and lower limit of 95% confidence intervals for each lethal concentration for *Etroplus suratensis*, are shown in Table 1.E.1. No significant difference between LC_{50} values for *Etroplus suratensis* was found when applying the Trimmed Spearman-Karber Method.

Table 1.E. Trimmed Spearman-Karber Method for Estimating LC_{50} on exposure of different concentrations of MS-222 (Tricaine methanesulfonate).

Concentration	Cumulative Mortality (%) Time (hthe presents)			
	24	48	72	96
Control	0	0	0	0
45	0	0	0	0
50	0	0	0	0
53	0	0	0	4
75	0	19	50	75
100	50	63	100	100

Table 1.E.1. Lethal concentrations of tobacco leaf extract with upper and lower limit of 95% confidence intervals

Time	LC_{50}	95% confidence interval	
		Lower	Upper
24			
48	92.1092	86.0894	98.55
72	73.7175	70.4335	77.1546
96	67.4407	64.7235	70.27

During each exposure period (24, 48 and 96 h) of the acute toxicity test for MS-222 (Tricaine methanesulfonate), it was observed that the mean values of all water quality parameters were significantly different ($P < 0.05$). Water quality varying among replicates for all tested variables (DO, pH, temperature, turbidity, ammonia, nitrate and nitrite) compared to the control values; were within acceptable limits (Mackereth, 1963). The results of the mean values (mean±SE) of variables are presented in Appendix 1.1.

There was no significant difference between temperatures of anaesthetic exposed water (MS-222) and control, during 10 min, 24h, 48h and 96 h treatment (Fig.1.E.1). There was no significant difference between controls and all other concentration of MS-222 after 10min. There was slight variation in pH during 24 h between control and all other concentrations of MS-222. During 48 h there was slight decrease in pH concentration and other treatment. During 96 h there was no significant change between control and all other concentration (Fig.1.E.2). During 10 min, 24 h, 48 h and 96 h treatment, the DO rate increased with increase in concentration (45, 50 53, 75 and 100 mg/L) (Fig.1.E.3).There was no significant change in turbidity during 10 min, 24 h, 48 h and 96 h between control and all treatments (Fig.1.E.4). During 10 min the control and all other treatment was at zero level. During 24 h, the lower concentration (45, 50 and 53 mg/L) showed decreased value (0.06 mg/L) of NH_3+ than control (0.10 mg/L), but it increased (0.11mg/L) with increase in concentration (75 and 100 mg/L). In 48 and 96 h the value of NH_3+ increased with increase in concentration (45, 50 53, 75 and 100 mg/L) than control (Fig.1.E.5). NO_2- rate was zero at all concentration level after 10min. During 24 h, 48 h and 96 h the NO_2- increased in all treatments with increase in concentration than control (Fig.1.E.6). Change in pattern of NO_3- at zero level was noticed in control as well as in all treatment. During 24 h, the NO_3- rate decreased with control and all treatment concentration. During 48 h the change in pattern of NO_3- of control and all other treatment has not significant difference (0.05 mg/L). But during 96 h the change in pattern of NO_3- decreased than control (Fig.1.E.7).

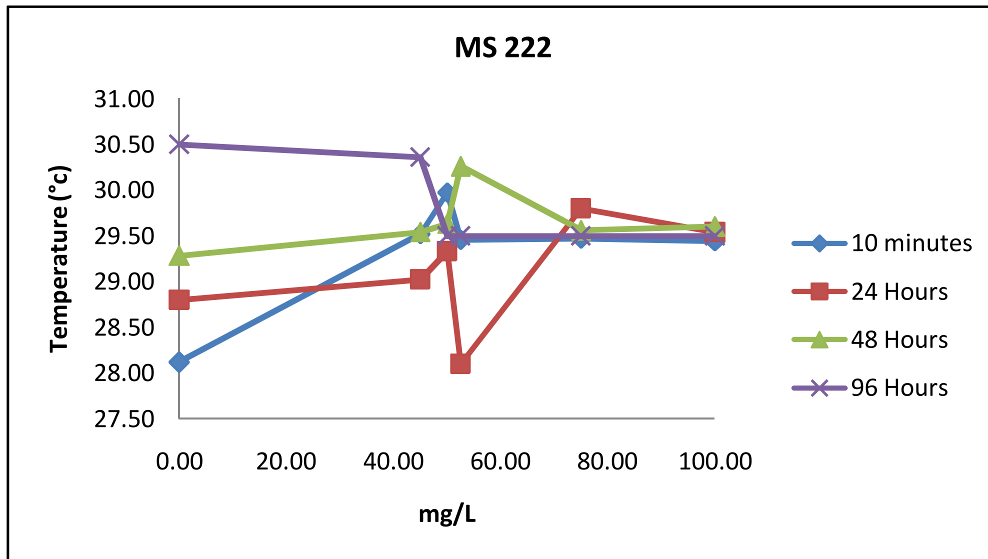


Fig.1.E.1 Change in pattern of temperature at different concentration of MS-222 (Tricaine methanesulfonate) during 96 h duration

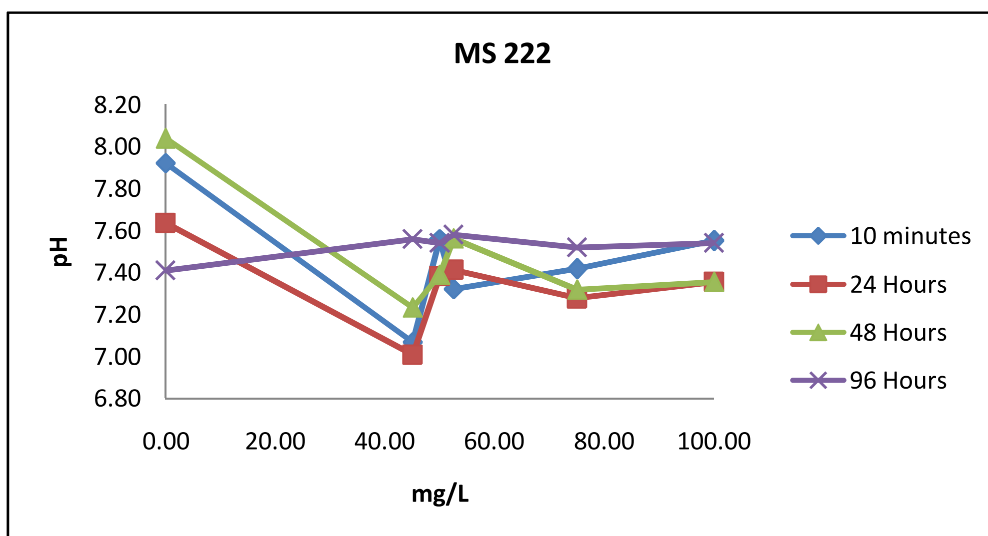


Fig.1.E.2 Change in pattern of pH at different concentration of MS-222 (Tricaine methanesulfonate) during 96 h duration

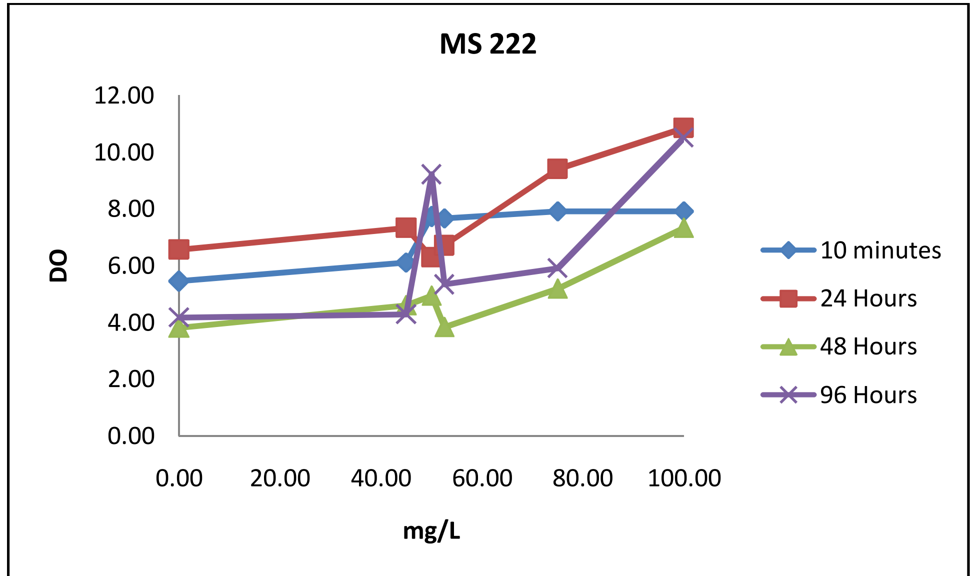


Fig.1.E.3 Change in pattern of DO at different concentration of MS-222 (Tricaine methanesulfonate) during 96 h duration

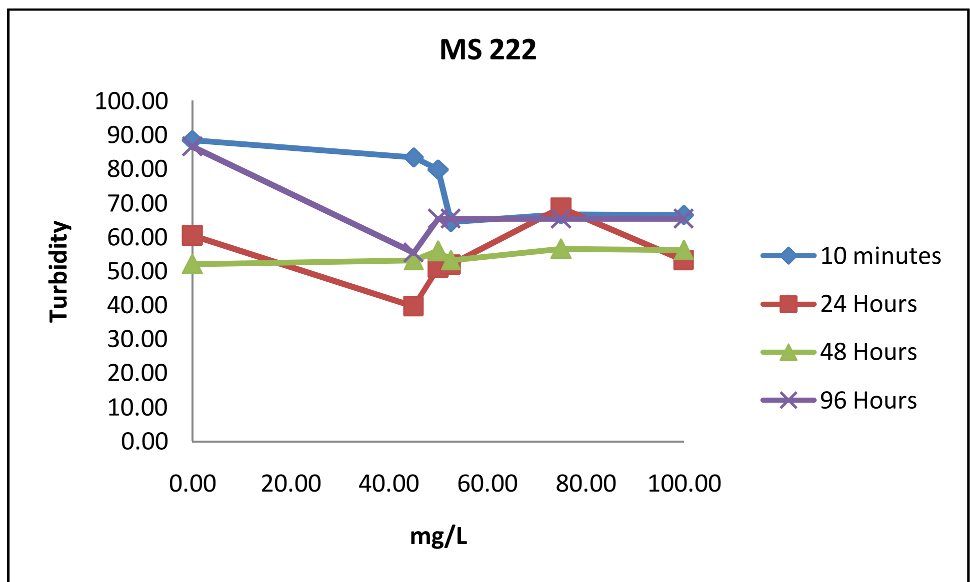


Fig.1.E.4 Change in pattern of turbidity at different concentration of MS-222 (Tricaine methanesulfonate) during 96 h duration

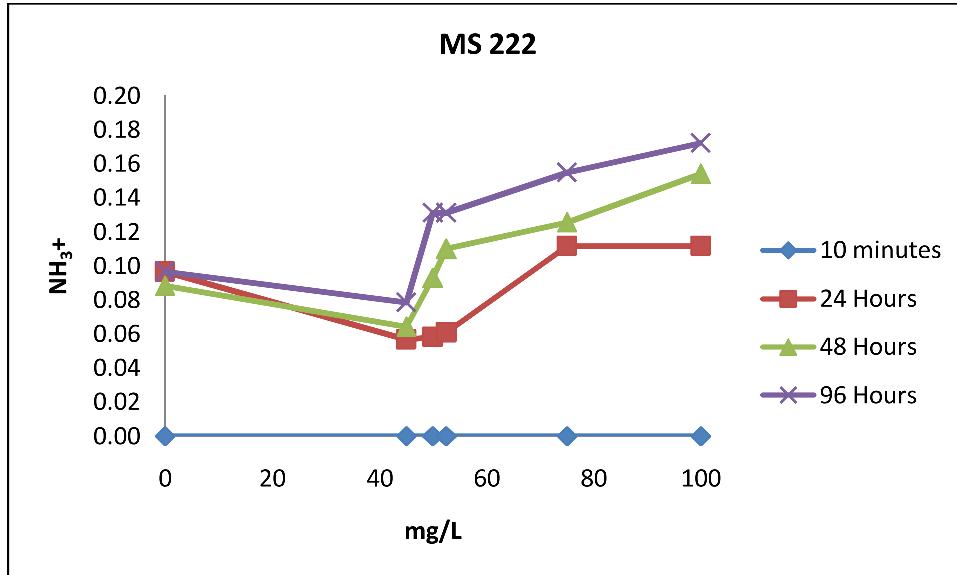


Fig.1.E.5 Change in pattern of NH_3+ at different concentration of MS-222 (Tricaine methanesulfonate) during 96 h duration

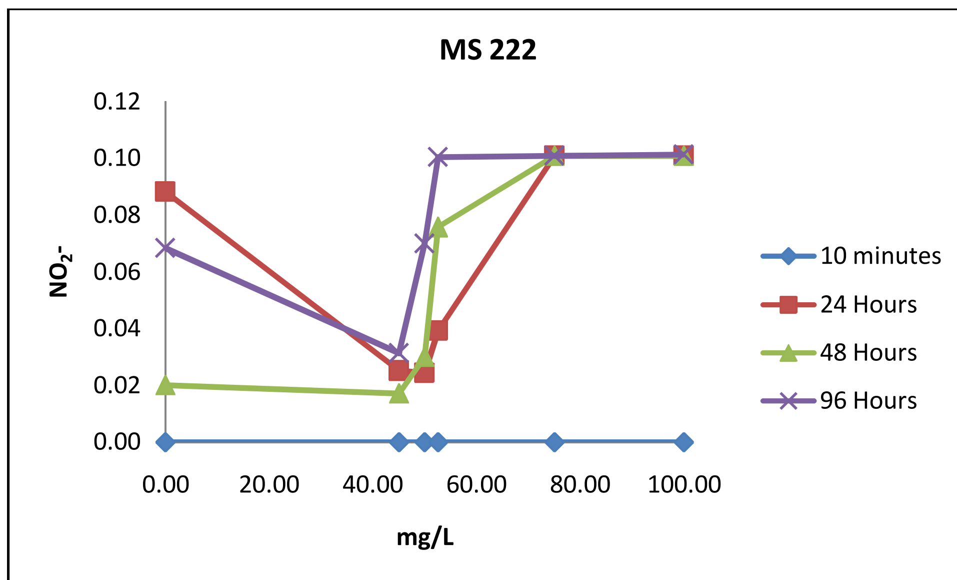


Fig.1.E.6 Change in pattern of NO_2- at different concentration of MS-222 (Tricaine methanesulfonate) during 96 h duration

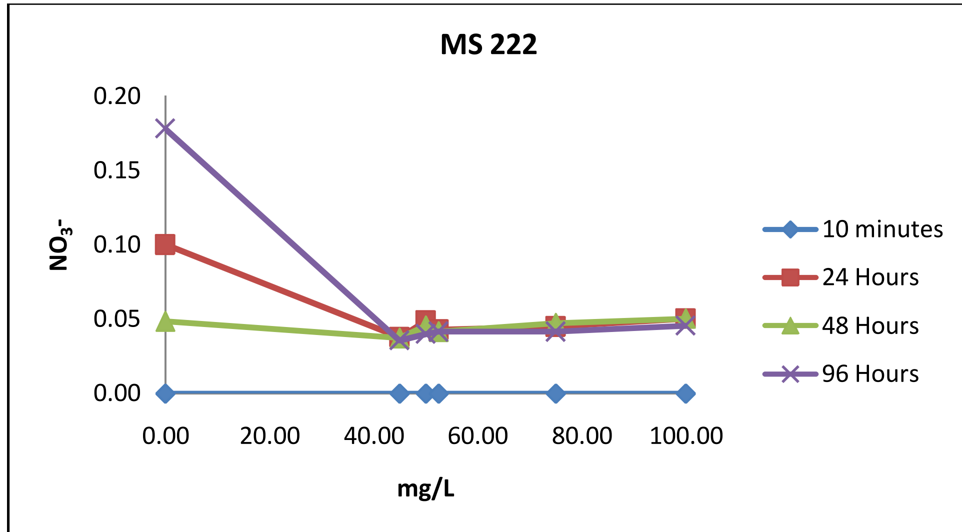


Fig.1.E.7 Change in pattern of NO_3- at different concentration of MS-222 (Tricaine methanesulfonate) during 96 h duration

1.7.6 Effect of Hypothermic condition

The observation of fishes exposed at reduced temperature levels of hypothermia (8, 12, 16, 18 and 22°C) and the cumulative mortality rates (%) for hypothermia presented in Table 1.F. For all the hypothermia levels (8, 12, 16, 18 and 22°C/L) tested in this experiment, the mortality rate was always higher at 8 and 12°C (Table 1.F) during 96 h duration. Depending on the duration of hthe presents, the mortality rate at each level differed. The mortality rate at 16°C/L was lower than 8 and 12°C/L (Trimmed Spearman-Karber method, Hamilton et al., 1977).100% survival rate was observed at the reduced temperature of 18 and 22°C. The cumulative mortality rate (table 1.F) indicated that mortality change in pattern of the test fish and reduced levels of hypothermia are negatively correlated. This shows that the mortality change in pattern of the fish increased with decrease in temperature levels. Particular lethal concentrations of hypothermia with upper and lower

limit of 95% confidence intervals for each lethal concentration for *Etroplus suratensis* are shown in Table 1.F.1. No significant difference between LC_{50} values for *Etroplus suratensis* was found when applying the Trimmed Spearman-Karber Method.

Table 4.F. Trimmed Spearman-Karber Method for Estimating LC_{50} on exposure of different concentrations of hypothermia

Concentration	Cumulative Mortality (%) Time (the presents)			
	24	**48**	**72**	**96**
Control	0	0	0	0
22	0	0	0	0
18	0	0	0	0
16	0	0	17	46
12	38	58	81	100
8	100	100	100	100

Table 1.F.1. Lethal concentrations of tobacco leaf extract with upper and lower limit of 95% confidence intervals

Time	LC_{50}	95% confidence interval	
		Lower	**Upper**
24	11.7172	12.2744	11.1425
48	12.5581	13.0992	11.9995
72	13.9886	14.5061	13.4538
96	15.5341	15.9416	15.1147

During each exposure period (24, 48 and 96 h) of acute toxicity test for hypothermia, it was observed that the mean values of all water quality parameters were significantly different ($P < 0.05$).Water quality varying among replicates for all tested variables (DO, pH, temperature, turbidity, ammonia, nitrate and nitrite) compared to the control values; were within

acceptable limits (Mackereth, 1963). The results of the mean values (mean±SE) of variables are presented in Appendix 1.1.

In the case of hypothermic condition temperature has no significant effect on the water quality. But reduction in temperature has severe effect on the survivability of the organism. pH decreased with reduction in temperature in all hypothermic level of anaesthesia (8, 12, 16, 18 and 22°C/L) (Fig.1.F.1). According to the reduction in temperature, pH rate increased than control during 24, 48 and 96 h. After 10 min DO rate increased with reduction in temperature and reach at 10.55 mg/L than control (5.45 mg/L). DO rate during 24 and 48 h show the similar pattern of increased tendency with reduction in temperature. During 96 h, the change in pattern of DO decreased significantly at 22, 18, 16 °C/L (2.04, 0.69 and 0.78 mg/L) with control (5.46 mg/L) (Fig.1.F.2). In all hypothermic level of anaesthesia (8, 12, 16, 18 and 22°C/3L) the turbidity decreased significantly with control (Fig.1.F.3). During 24, 48 and 96 h, change in pattern of NH_3+ reduced with reduction in temperature than control and at lowest level of hypothermia (8°C/L), the change in pattern of NH_3+ was zero (Fig.1.F.4). The change in pattern of NO_2- decreased with reduction in temperature and became zero, during 24, 48 and 96 h duration in all treatment than control (Fig.1.F.5). Similarly the change in pattern of NO_3- decreased with reduction in temperature and became zero, during 24, 48 and 96 h duration in all treatment than control (Fig.1.F.6).

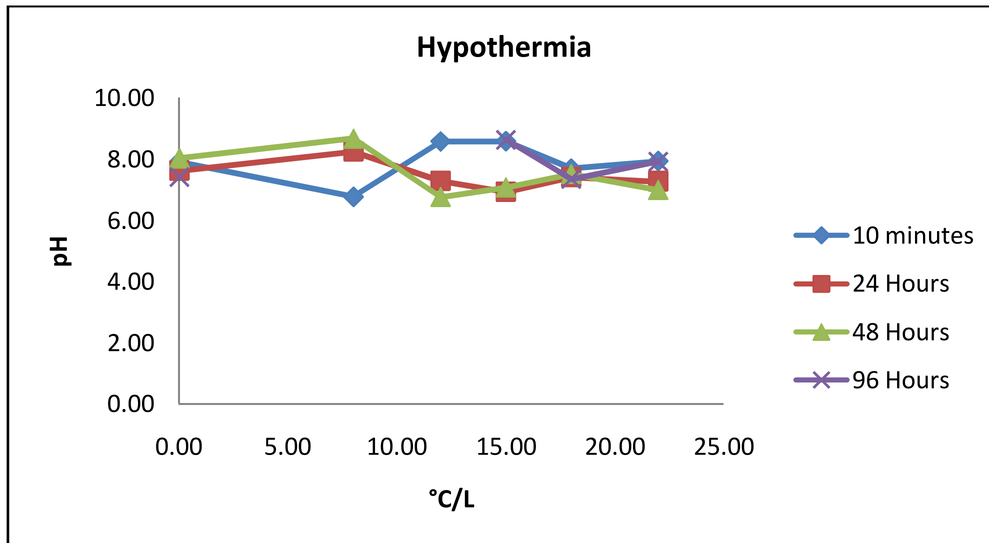


Fig.1.F.1 Change in pattern of pH at different hypothermic conditions during 96 h duration

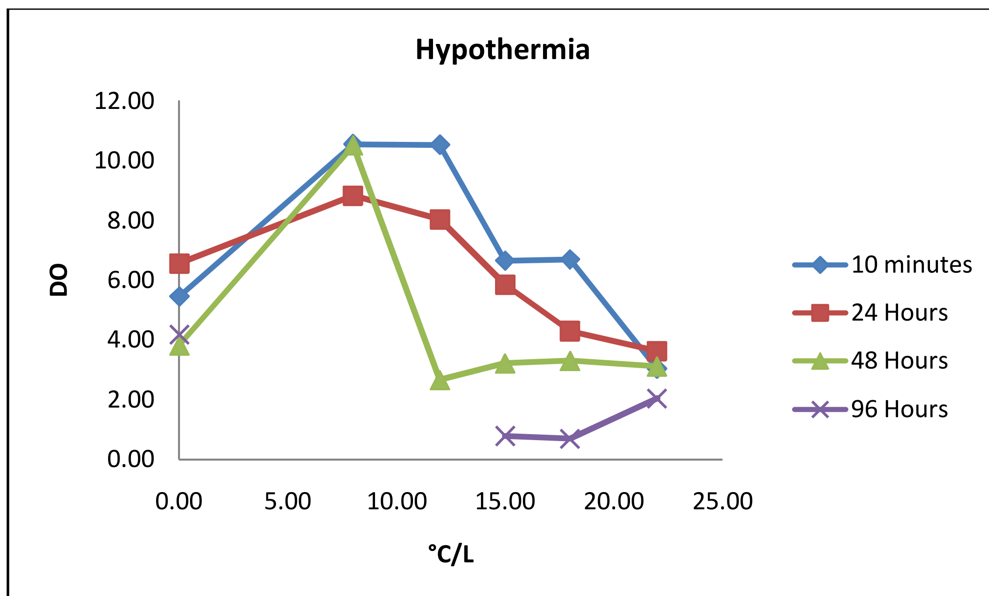


Fig.1.F.2 Change in pattern of DO at different hypothermic conditions during 96 h duration

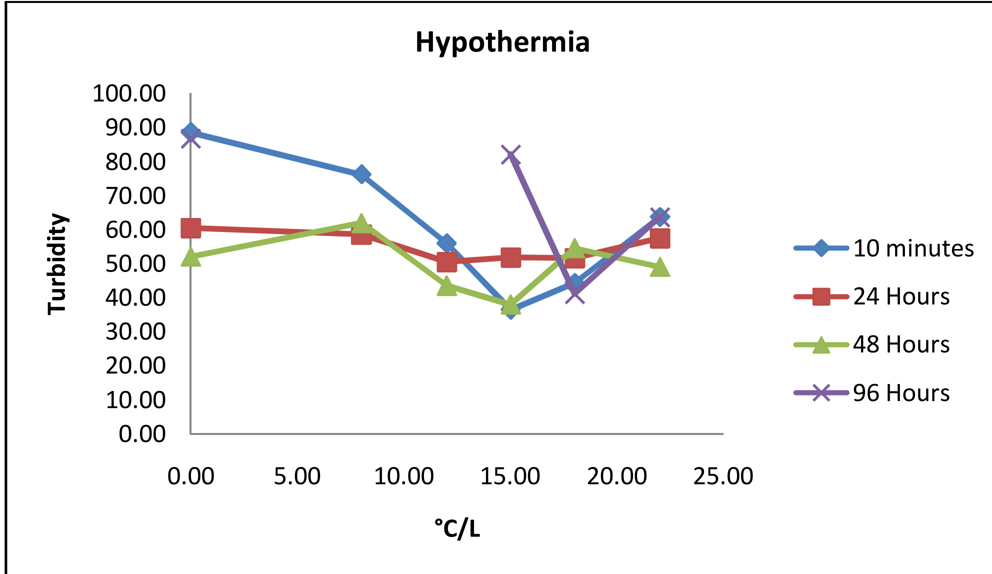


Fig.1.F.3 Change in pattern of turbidity at different hypothermic conditions during 96 h duration

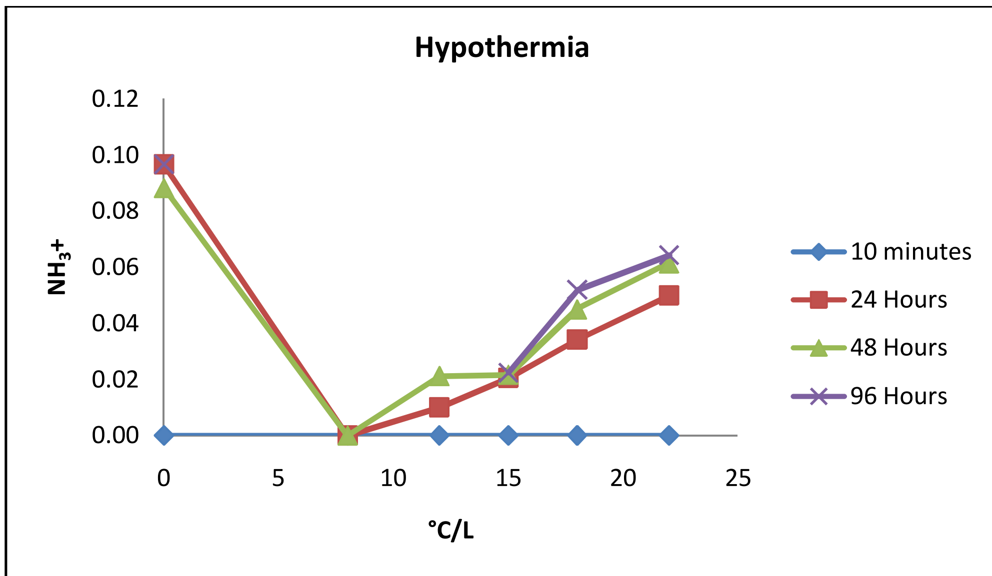


Fig.1.F.4 Change in pattern of NH_3^+at different hypothermic conditions during 96 h duration

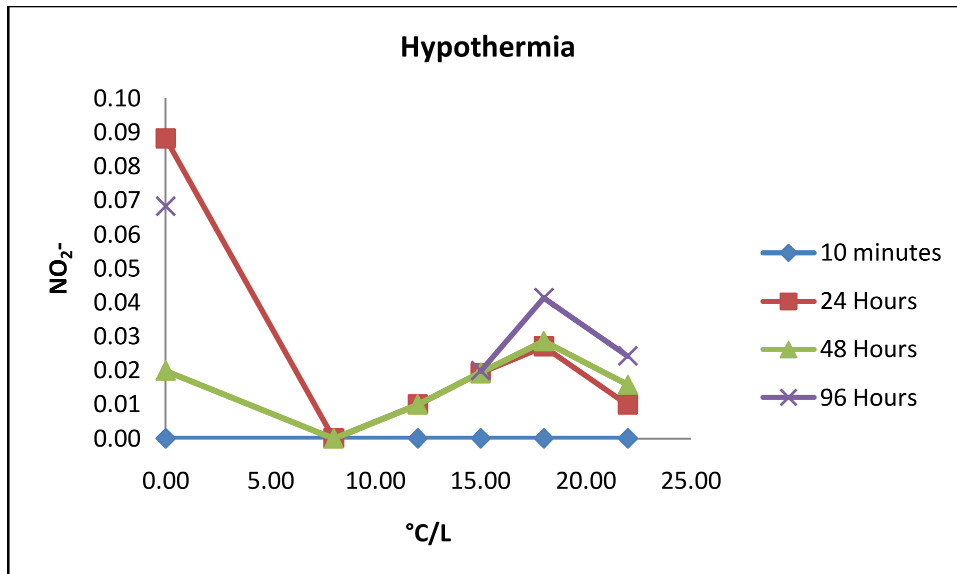


Fig.1.F.5 Change in pattern of $N0_2$- at different hypothermic conditions during 96 h duration

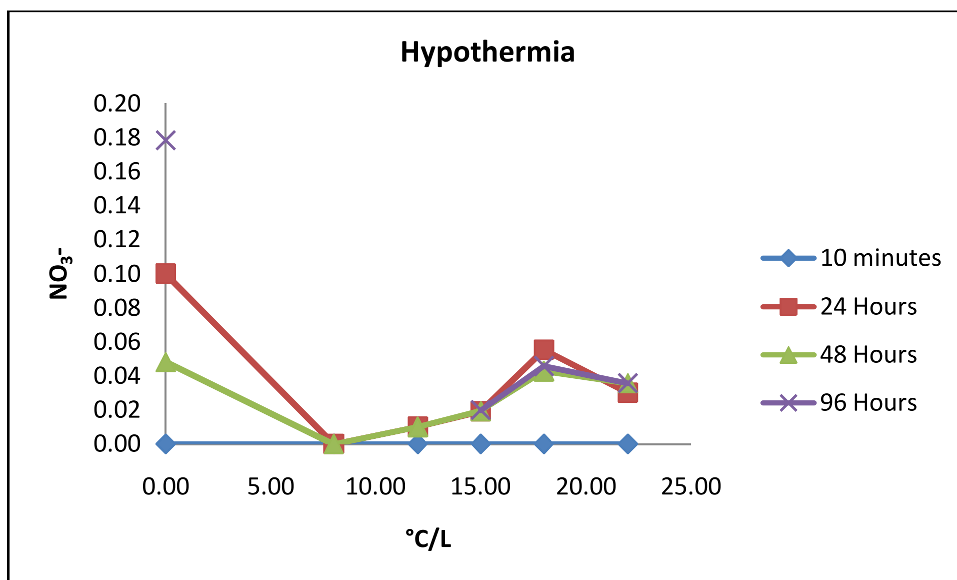


Fig.1.F.6 Change in pattern of NO_3^- at different hypothermic conditions during 96 h duration

1.8 Discussion

At higher doses all the six anaesthetics tested (clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS-222 and cold) all of them were toxic leading to mortality of *Etroplus suratensis* juveniles. Conventional toxicological studies involve the determination of LC_{50} (or LD_{50}) for a given xenobiotic, while chronotoxicity investigations determine these parameters taking into account the existence of circadian changes in host tolerance (Dridi et al., 2005).

1.8.1 Effect of clove oil

In the present study we tested the acute toxic effects of clove oil on juveniles of *Etroplus suratensis.* The present data indicate that juveniles of *Etroplus suratensis* are very sensitive to clove oil. Grush et al. (2004) studied the anaesthetic effects and acute toxicity of clove oil in one-month old zebra fish. No mortality was observed in any of the clove oil anaesthesia concentrations in the 24, 48 and 72 h measurement. Mortality of fish and of the 0.23 mg/L treatment group, after 12 and 24 h of transportation was only 0% (Table 1.A). Mortality increased with duration of exposure time. In the 0.33 mg/L clove oil treatment, the mortality at 24 h of transportation (56 %) was significantly higher than the values observed for the other concentrations used. The Lake Victoria cichlid, *Haplochomis obliquidens* (Hilgendorf, 1888), anaesthetized with 18 ¼ $L.L^{-1}$ of clove oil also revealed higher mortality rates after long transportation times (48 h) (Kaiser et al., 2006). Accumulated mortality, measured 96 h after transportation presented a similar tendency as compared to the mortality after the end of the experimental period, and was significantly higher in fish experimented in

0.30 and 0.33 mg/L clove oil for 24 h. Total mortality for control fish and for fish anaesthetized with 0.23 clove oil was only 2%. The determination of clove oil acute toxicity is important not only for its usage in fish (Walton et al., 1997). In the present results, the concentration of 0.30 and 0.33 mg/L was highly toxic to juveniles of *Etroplus suratensis,* even during 24 h the survival rate being only 50%. Although the recommended concentrations of clove oil for the short-term immobilization of fish range from 40 up to 100 mg/L (Keene et al., 1998; Waterstrat, 1999), acute toxicity values of clove oil expressed at 10 min LC_{50} were 74.3 $mg \cdot l^{-1}$ for carp (Velíšek et al., 2005b) and 76.70 mg/L for the European catfish (Velíšek et al., 2006). Another possibility for the different LC_{50} values stated by different authors is the variability of the clove oil composition used. Zebra fish, carp, catfish and guppy showed comparable tolerance to acute toxicity of clove oil (Doleželová et al., 2011). The embryo toxicity of clove oil for zebra fish embryos according to the OECD No. 212 method (OECD No. 212: Fish, short term toxicity test on embryo and sac fry stages) yielded a 168 h LC_{50} value of 15.6 mg/L (Macova et al., 2008). The 96 h LC_{50} obtained in this study was 21 $mg \cdot l^{-1}$, which is comparable to the present result, obtained in juveniles of *Etroplus suratensis.* A comparable sensitivity to clove oil was reported by Velíšek et al., (2005b) in common carp (*Cyprinus carpio*) with 96 h LC_{50} value of 18.1 $mg \cdot l^{-1}$ and in European catfish (*Silurus glanis L.*) with an 96 h LC_{50} value of 18.4 $mg \cdot l^{-1}$ (Velíšek et al., 2006). In juveniles of *Etroplus suratensis*, the clove oil LC_{50} during 96 h, was 0.2433 mg/L, 72 h, was 0.2898 mg/L, 46 h, was 0.2963 mg/L, and 24 h, was 0.3162 mg/L. This difference might be the result of several factors, such as anaesthetic absorption, distribution, excretion and metabolism (Hooven et al., 2009).

Analysis of 95% confidence interval of 96 h LC_{50} values of clove oil for juveniles of *Etroplus suratensis* showed highly significant variations with concentration of clove oil (Table1.A.1). On the other hand, a lower lethal concentration 96 h LC_{50} of 14.1 $mg \cdot l^{-1}$ was reported in rainbow trout (*Oncorhynchus mykkis*) by the same author (Velíšek et al., 2005a). Keene et al., (1998) obtained a similar result in rainbow trout considering that the estimated 96 h LC_{50} for eugenol (active form of clove oil) was found to be approximately 10 $mg \cdot l^{-1}$. However, in the present result, the 96 h LC_{50} value of lower concentrations of 0.10, 0.17 and 0.23 mg/L of clove oil found in the study suggests that it is relatively harmless for fish. Barton and Helfrich (1981) reported that use of lower concentrations of the anaesthetic should provide much wider safety margins for anaesthesia. Roubach et al., (2005) found that exposure of tambaqui (*Colossoma macropomum*) to 65 $mg \cdot l^{-1}$ of eugenol for up to 30 min did not cause fish mortality. There was no mortality in tambaqui at doses of 135 $mg \cdot l^{-1}$ (exposure duration was not reported). The rainbow trout is the most sensitive fish species, with LC50 values for clove oil of 81.1 $mg \cdot l^{-1}$ for 10 min (Velíšek et al., 2005a) or 65 $mg \cdot l^{-1}$ for an exposure time of 30 min (Keene et al., 1998). Taylor and Roberts (1999) determined the median lethal concentration for 10-min exposure for rainbow trout at 250 $mg \cdot l^{-1}$, which means about 3 × higher than the result obtained by Velíšek et al., (2005a). Comparable values were reported by the same authors for Chinook salmon (*Oncorhynchus tshawytscha*) at 62 $mg \cdot l^{-1}$, higher for coho salmon (*O. kisutch*) at 96 $mg \cdot l^{-1}$ and for white sturgeon (*Acipenser transmontanus*) at 526 $mg \cdot l^{-1}$. However, when lethal concentrations are used but the gills are artificially ventilated, fish can be kept alive for a longer period (Brown, 1987). The change in pattern of anaesthetic elimination

during recovery also increases with artificial ventilation (Kiessling et al., 2009).

It was suggested that when used in fish, clove oil should be administered in anaesthesia baths at concentrations between 25–100 mg/L, according to the genus and size of the fish (Hikasa et al., 1986; Walsh and Pease, 2002; Hamackova et al., 2004). In their study of juvenile rainbow trout, Keene et al. (1998) reported LC_{50} for the 8 to 96 h period at approximately 9 ppm (10 $mg \cdot l^{-1}$). The values were in good agreement with 96 h LC_{50} of 0.24 mg/L for juveniles of *Etroplus suratensis* found in the present study. On comparing the present experimental results (0.24 mg/L for 96 h LC_{50}) and the recommended concentrations for fish anaesthesia, it was clear that although the concentration of clove oil is low compared with the other similar studies of other fishes, the effect of a clove oil immersion bath on juvenile of *Etroplus suratensis* could result in higher mortality.

The juveniles of *Etroplus suratensis* used for the present experiments showed a diurnal activity pattern and therefore anaesthetic absorption through the gills might have also been higher. Thus, for a given concentration, toxicity would be greater during the day since not only the swimming activity would be higher but also the respiration and metabolic activities, as observed in fish species exposed to the pesticide lindane (Walton et al., 1997).

We also determined the effects of selected levels of anaesthetics water temperature, turbidity, pH and DO. There were differences in toxicity for the anaesthetics at water quality variables. The toxicity of many chemicals can be affected by differences in water quality (Bills et al., 1993). Low dissolved oxygen level, pH, ammonia and nitrite etc., are known to be toxic to fish

(Lemmer, 1996; Reinberg, 1991), and special attention must be paid to whether the anaesthetic exposure may induce to increase their toxicity during live transportation of juvenile of *Etroplus suratensis*. However, in fish, as opposed to mammals, there is a lack of research dealing with the differential toxicity of xenobiotics depending upon the water quality parameters.In the present study viz., clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS-222 and hypothermic conditions were used as fish anaesthetics to determine their effects on juvenile *Etroplus suratensis* at different levels of water quality. The present data indicate that juveniles of *Etroplus suratensis* respond differently tested to the anaesthetics with respect to the variations in water quality parameters.

Velíšek et al., (2005) reported that acute toxicity of clove oil to fish from the point of view of the use of clove oil as an anaesthetic and also the possible contamination of the water environment by such anaesthetic. In the present study a range of LC_{50}'s from 24 to 96 h (0.31 to 0.24 mg/L) and water contamination risk was recorded for *Etroplus suratensis* under varying conditions of temperature, pH , DO, turbidity, ammonia, nitrate and nitrite. Weyl et al., (1996) and Hamaakova et al., (2001) stated that the most important factor influencing efficacy in fish is temperature, i.e. the higher the temperature, the higher the efficiency of the anaesthetic for fish. Water temperature was similar across all concentration treatments in the first 10 min and 24 h exposure of clove oil (26-27°C) (Appendix 1.1). Svobodová et al. (1987) reported that using 2-phenoxyethanol for rainbow trout at the concentration of 0.30 $ml \cdot l^{-1}$ is that the 10 min LC_{50} values were recorded for water temperature of 15°C. Under such conditions, 10 min LC_{50} values will probably be higher. Collectively the present result indicate that during the 96 h

LC_{50} test the range of water temperature was at 27-30°C (Mean ±SE). This higher temperature may induce the mortality during 96 h LC_{50} test at 0.23, 0.30 and 0.33 mg/L. Throughout the experiment, temperature variation was under 1°C.

Sensitivity to anaesthetics may also be influenced by fish health and physical condition. Sensitivity to anaesthetics is also influenced by oxygen concentrations in the sense that oxygen deficit enhances the anaesthetic efficiency (Svobodová et al., 1987). In the present result the DO level in anaesthetized water were within the range of 6-7 mg/L during 10 min, 7-6 mg/L during 24 h, 6- 4 mg/L during 48 h, and 5-2 mg/L during 96 h. Oxygen levels were higher in the 6 h transportation time followed by a continuous decrease in the parameter, up to 24 h transportation time in all groups. Indeed, it was observed that from anaesthetizing fish in clove oil at higher concentrations (i.e., N 40 mgl^{-1}) suggest that heart rate (cardiac output) decreases after prolonged anaesthesia (Cooke, unpublished data). Peştean et al., (2011) in Annals of RSCB, Vol. XVI, reported that in aquaculture, anaesthetics are used during transportation to prevent physical injury and reduce metabolism (DO consumption and excretion). At the end of the transportation experiment, dissolved oxygen was significantly higher in the control group as compared to the groups transported using clove oil. Determination of oxygen consumption by the fish is useful for assessment of lethal effects and is one of the important indicators which reflect physiological state of animal (Tilak et al., 2007). A pronounced consumption of oxygen was seen in fish treated with the clove oil in the 96 h measurements. This may be linked with the hyperactivity in fish and decrease in DO probably increases the mortality rate during 96 h. Gomes et al. (2009) reported that fish

anaesthetized with clove oil at concentrations between 9 and 18 $mg.L^{-1}$ (at a density of 50 fish/L maximum) transportation times should be 24 h, since oxygen levels in this experimental configuration reached values near the critical survival threshold.

Variations in water turbidity probably affect the toxicity of clove oil to *Etroplus suratensis* (Appendix 1.1). The 96 h toxicity of clove oil was not affected by variation in water turbidity, but as water pH decreased, the toxicity increased nearly 10% during 96 h. pH decreased with water temperature which was more toxic to juveniles of *Etroplus suratensis*. Inoue et al. (2005) also reported that pH decreased after 4 h of transportation of juvenile matrinxã (*Brycon cephalus,* Gunther) anaesthetized with 5 $mg.L^{-1}$ clove oil, probably due to the increase in CO_2 values. At the end of the transportation experiment, pH was significantly higher in the control group as compared to the fish anaesthetized with 18 $mg.L^{-1}$ clove oil. Present data indicate that the responses of Etroplus suratensis to these compounds are similar to that of most especially other species. 96 h LC_{50}'s reported for rainbow trout, which ranged from 0.879 to 1.73 mg/L for varying conditions of water temperature, turbidity and pH (Marking and Bills, 1975). According to Gomes et al. (2009) increases in water quality parameters values are imputable mainly to the escalation of ammonia levels in the water. Ammonia measurements in the control group did not diverge significantly across the different clove oil concentrations tested. It is believed that the use of anaesthetics in fish transport may reduce the fish activity and the ammonia excretion through the gills (Inoue et al., 2005). In the treatments with the anaesthetic, ammonia concentration was markedly higher in the 24 h transportation period (Appendix 1.1) at 0.33 mg/L concentration. Water

temperature was constant avoiding another variant of ammonia toxicity during the exposure of clove oil in *Brycon cephalus* during transportation (Inoue et al., 2005). The pH influences the toxicity of several substances including total ammonia, which is present in the water as two forms: un-ionized (NH_3 – toxic to the fish, it diffuses easily across the gills) and ionized ($NH^{4+)}$. At low pH, un-ionized ammonia represents a small portion of the total ammonia (Boyd, 1982). Although in the present study the levels of total ammonia increased during transportation (Table 2), the levels of ionized ammonia were high (due to the increase in the pH) and probably were toxic to the fish. Furthermore, water temperature was constant avoiding another variant of ammonia toxicity.The ammonia values at 24 h of transportation (10-11 $mg.L^{-1}$) were below the lethal concentration threshold for Nile tilapia juveniles (Benli and Köksal, 2005). How ever these values were similar to the values reported for *Etroplus suratensis*. In a study on rainbow trout (Vedel et al., 1998), when the desired nitrite and ammonia concentrations were achieved by adding dissolved $NaNO_2$ and NH_3NO_3, the combined nitrite and ammonia exposure resulted in high mortality at the highest exposure concentrations (600μM NO_2^- and 18 μM NH_3). The NO_2^- level in the entire concentration group increased than the control group during 24 h (0.08-0.14), 48 h (0.11-0.15) and 96 h (0.11-0.15) treatment. But there was significant difference between the control and experimental groups during 96 h experimental period. This possibly will lead to increase the mortality change in pattern of juveniles of *Etroplus suratensis*. The NO_3^- level of all experimental group also showed an increasing pattern with control during 24, 48 and 96 h treatment. This might increase the mortality change in pattern of juveniles of *Etroplus suratensis* during 96 h clove oil treatment. An elevated

ambient nitrite concentration is a potential problem for freshwater fish since nitrite is actively taken up across the gills in competition with chloride. Nitrite is a well-known toxicant for fish as well as a disrupter of multiple physiological functions including ion regulatory, respiratory, cardiovascular, endocrine and excretory processes. Oxygen can affect nitrite toxicity because nitrite reduces the oxygen-carrying capacity of blood. A reduction in oxygen supply in the external medium will exacerbate the oxygen supply problem in fish. Bowser (2001) showed that an oxygen concentration of 5 mg/L, in the presence of nitrite, was not sufficient for channel catfish even though channel cat fish normally tolerate oxygen concentrations below this value. Temperature, which influences tissue oxygen demand, could also be expected to affect nitrite toxicity. Over a relatively small range (22–30°C), Colt and Tchobanoglous (1976) showed no significant relationship between nitrite toxicity and temperature. In the study of Huey et al., (1984), channel cat fish kept at 30°C in the presence of 0.91 mg/L nitrite-N over a period 24 h developed methaemoglobin concentrations almost twice as high as those of fish held at 10°C. Huey et al. (1984) also found that the fish kept at 30°C showed a more rapid return to background haemoglobin levels in the absence of nitrite. A higher amount of oxygen in water at lower temperatures and lower metabolic rates of fish at lower temperatures might render nitrite a less potent toxin at lower temperatures. However, it is also assumed that lower temperatures reduce the efficiency of detoxification mechanisms (Lewis and Morris, 1986).

Consequently, clove oil would provide better water quality for transport, and larger amounts of fish could be transported in the same container (Kubtiza, 1998; Kubitza, 2003). However, the results of the water analysis in

this study demonstrated that during transport the water quality deteriorated in all treatments, and the anaesthetic addition did not attenuate the water deterioration as expected.

1.8.2 Effect of Cinnamon oil

In the present study the acute toxic effects of cinnamon oil (*Cinnamomum zeylanicum*) on juveniles of *Etroplus suratensis* were tested. The present data indicated that juveniles of *Etroplus suratensis* are very sensitive to cinnamon oil. There is no mortality during 24 h LC_{50} in juveniles of *Etroplus suratensis* at different concentrations (Table 1.B) which has not any comparable results. Small amounts of cinnamon have been used for thousands of years as a spice without any reports of side effects (Hanafi et al., 2010). By contrasts, no reliable data are available on the effects of the administration of high concentration of cinnamon oil on lower vertebrate like fish, frog etc. Budavari et al. (1989) have reported acute toxicity of Cinnamon in the animals is very low i.e. Benzaldehyde (LD_{50} orally, 1300 mg/kg rat), cinnamaldehyde (LD_{50} orally, 2220 mg/kg rat), linalool (LD_{50} orally, 2790 mg/kg rat), and salicylaldehyde (LD_{50} orally, 520 mg/kg rat). Morozumi (1978) found that its toxigenicity is low. Toxicological data on ingredients of **c**innamon oil according to material safety data Sheet: Oral (LD_{50}): Acute: 2800 mg/kg [Rat]. 2670 mg/kg [Mouse]. Dermal (LD_{50}): Acute: 320 mg/kg [Rabbit]. In juveniles of *Etroplus suratensis*, the cinnamon oil LC_{50} during 96 h was 0. 57 mg/L, 72 h was 0.58 mg/L; 48 h was 0.62 mg/L. This difference might be the result of several factors, such as anaesthetic absorption, distribution, excretion and metabolism according to Hooven et al., (2009).

Mortality of fish at the 0.33 and 0.55 mg/L treatment group, after 12 and 24 h of transportation was only 0% (Table1.B.1). Total mortality for control fish and for fish anaesthetized with 0.57 mg/L of cinnamon oil was only 1% for 48 h duration. An extended period of exposure could increase the toxicity of anaesthetics (Vrskova and Modra, 2012). Present study results also prove that mortality increased with duration of exposure time. In the 0.67 mg/L cinnamon oil treatment, the mortality at 48 h of transportation (63%) was significantly higher than the values observed for the other concentrations used. Mortality, measured 96 h after the end of the experimental period was significantly higher in fish experimented in 0.60 and 0.67 mg/L cinnamon oil for 72 and 96 h.

Analysis of 95% confidence interval on 96 h LC_{50} values of cinnamon oil for juveniles of *Etroplus suratensis* showed no significant variations with concentration of cinnamon oil. Mitul et al. (2013) reported that oral administration of aqueous and alcoholic extracts of *Cinnamomum zeylanicum* bark in mice for determination of toxicity studies were observed for up to 24 and 72 h. Animals did not show any mortality or toxic symptoms but showed signs of slight sedation and perspiration at a dose up to 5000 mg/kg body weight. Similarly ethanol extract of *Cinnamomum zeylanicum* was also tested at 0.5, 1.0, and 3 g/kg and results showed no acute toxicity in mice (Shah et al., 1998). But the coumarin content in cinnamon also has potentially toxic effects on the liver in humans (Ghosh et al., 1997). Cinnamon is used as a spice in food material in Asia so its safety is quite obvious. Khunoana (2011) reports that besides all health benefits of cinnamon, the plant contains a toxic compound, coumarin which impacts badly on animals resulting in death, and little information on its toxicity to human beings has been documented. In

contrast, *C. zeylanicum* has shown to contain a lesser content of coumarin and thus it may be possible that Ceylon cinnamon could be used in higher doses without toxic effects for longer durations (Ouattara et al., 1997; Rychlik et al., 2008). However, in the present study the 96 h LC_{50} value of lower concentrations of 0.33, 0.50 and 0.57 mg/L of cinnamon oil suggests that it is relatively harmless to fish. Keene et al. (1998) obtained a similar result in rainbow trout considering that the estimated 96 h LC_{50} for eugenol (active form of cinnamon leaf oil 60-86%) was found to be approximately 10 $mg \cdot l^{-1}$. The IACUC review proposed euginol use as an anaesthetic on a case by case basis in fish confined to the laboratory and will not be used for human or animal food. In fish, anaesthetics are absorbed and excreted mainly though gills (Locke, 1969; Hunn and Allen, 1974; Houston and Corlett, 1976; Ferreira et al., 1984). Eugenol and its compounds and metabolites are quickly removed from the blood bed and tissues of fish (Fisher et al., 1990), and the presence of these substances in muscle tissues of fish or other animals is not considered toxic or mutagen (Liu and Gibson, 1977; Maura et al., 1989; Fisher et al., 1990; Philips, 1990; Zheng et al., 1992). The efficacy and safety range of euginol varies according to age, size and species of fish in addition to the concentration and purity of the euginol. Barton and Helfrich (1981) reported that use of lower concentrations of the anaesthetic should provide much wider safety margins for anaesthesia. Roubach et al. (2005) found that exposure of tambaqui (*Colossoma macropomum*) to 65 $mg \cdot l^{-1}$ of eugenol for up to 30 min did not cause fish mortality. There was no mortality in tambaqui at doses of 135 $mg \cdot l^{-1}$ (exposure duration was not reported). However, when lethal concentrations are used, but the gills are artificially ventilated, fish can be kept alive for a longer period (Brown, 1987). The Change in pattern of

anaesthetic elimination during recovery also increases with artificial ventilation (Kiessling et al., 2009).

The determination of cinnamon oil acute toxicity is important for its usage in fish, since there are no scientific reports available in the existing literature on the toxic effect and LC_{50} (96 h) of cinnamon oil (*Cinnamomum zeylanicum*) on fishes. In the present study reveals that at the concentration of 0.60 and 0.67mg/L of cinnamon oil toxic to juveniles of *Etroplus suratensis,* even during 48 h the survival rate was only 44 and 63% respectively. Hanafi et al., (2010) was reported the clinical signs of toxicity in the group of rabbit treated with EO 2.5% and suggested that less concentration of *Cinnamomum zeylanicum* bark oil could be safer for treating animals.

The size and life cycle status of anaesthetized fish is also recognized as a factor influencing the concentration of anaesthetic needed to induce anaesthesia within an acceptable time (Rombough, 2007). The juveniles of *Etroplus suratensis* used for the present experiments showed a diurnal activity pattern and therefore anaesthetic absorption through the gills might have also been higher. Thus, for a given concentration, toxicity would be greater during the day since not only the swimming activity would be higher but also the respiratory and metabolic activities, as observed in fish species exposed to the pesticide lindane (Walton et al., 1997). Besides, aqueous and alcoholic extracts of *Cinnamomum zeylanicum* has been notably used against snake bite in folklore medicine by traditional healers around the world (Mitul et al., 2013).

In the present study the effects of selected levels of water temperature, pH, DO, turbidity, ammonia, nitrate and nitrite and on the toxicity of

different concentration of cinnamon oil exposure was determined. Special attention needs be paid to understand whether the anaesthetic exposure may increase their toxicity during live transportation of juveniles of *Etroplus suratensis.* However, in fish, as opposed to mammals, there is a lack of research dealing with the toxicity of different concentrations of cinnamon oil exposure of fish depending upon the water quality parameters. There were differences in toxicity for the cinnamon oil concentration on water quality variables in the present results. The toxicity of many chemicals can be affected by differences in water quality (Bills et al., 1993). Low dissolved oxygen level, pH, ammonia and nitrite etc., are known to be toxic to fish (Lemmer, 1996; Reinberg, 1991).The present data indicated that juveniles of *Etroplus suratensis* responded to the different concentration of cinnamon oil exposure and variations in water quality in the same way as other fish. Velíšek et al., (2005) reported that the acute toxicity of clove oil to fish is investigated from the point of view of clove oil use as an anaesthetic, but it may also be possible for contamination of the water environment by such anaesthetics. Both the clove oil and cinnamon oil contained euginol concentrations and the water contamination risks at 96 h LC_{50} values were analysed. In their study of juvenile rainbow trout, Keene et al., (1998) reported LC_{50} for the 8 to 96 h period at approximately 9 ppm (10 $mg \cdot l^{-1}$) of clove oil. The values were in good agreement with 96 h LC_{50} of 0.24 mg/L for juveniles of *Etroplus suratensis* found in the present study. A range of LC_{50}'s of different concentration of cinnamon oil from 0.62 mg/L (48 h), 0.58 mg/L (72 h), 0.57 mg/L (96 h) was recorded for *Etroplus suratensis* under varying conditions of temperature, pH, DO, turbidity, ammonia, nitrate and nitrite. Weyl et al., (1996) and Hamaakova et al., (2001) stated that the

most important factors influencing efficacy of anaesthetics in fish is temperature, i.e. the higher the temperature, the higher the efficiency of the anaesthetic for fish. Water temperature was similar across the cinnamon oil concentration treatments in the first 10min experimental periods (Appendix 1.1). In the 24 h experimental period, water temperature was same across the groups treated with different cinnamon oil concentrations, but this temperature was significantly higher than the value measured at first 10 min (Appendix 1.1). Collectively the present study indicates that during the 96 h LC_{50} tests the range of water temperature was at 25-30°C (Mean ±SE).This higher temperature may induce the mortality during 96 h LC_{50} test at 0.33, 0.50, 0.57, 0.60, 0.67 mg/L concentrations. Throughout this experiment, temperature variation was within 1-2°C at 48 and 96 h duration.

In the present study the DO (Dissolved Oxygen) level in anaesthetized water was maintained within the range of 6-7 mg/L during 10 min, 7-6 mg/L during 24 h, 3- 4 mg/L during 48 h and 96 h. Sensitivity to anaesthetics is also influenced by oxygen concentrations in the sense that oxygen deficiency enhances the anaesthetic efficiency (Svobodová et al., 1987). Oxygen levels were higher in the first 10 min experimental period followed by a continuous decrease in the parameter, up to 96 h duration in all groups. Towards the end of the 96 h experimental period, dissolved oxygen in all the concentration was significantly lower than the control group using cinnamon oil. A pronounced consumption of oxygen was seen in fish treated with the cinnamon oil during 24, 48 and 96 h measurements. This may be linked with the hyperactivity in fish and decrease in DO probably increases the mortality rate during 96 h (Svobodová et al., 1987). But in the present study, as water pH decreased, the toxicity increased nearly to 10% during 96 h and was more

toxic to juveniles of *Etroplus suratensis.* At the end (96 h) of the experiment, pH showed a slight increase in the experimental group than the control. However, it showed a slight decrease than control during 10 min, 24 and 48 h cinnamon oil treatment (0.33, 0.50, 0.57, 0.67 and 0.73 mg/L). The present data indicate that the response of *Etroplus suratensis* to these compounds is supposed to increase the water acidity which may be toxic to juveniles of *Etroplus suratensis.* Inoue et al. (2005) also reported that pH decreased after 4 h of transportation in the case of the juvenile *Brycon cephalus* (Gunther) anaesthetized with 5 $mg.L^{-1}$ clove oil, which almight be probably due to the increase in CO_2 values.

Variations in water turbidity, probably affect the toxicity of cinnamon oil in the case of *Etroplus suratensis* (Appendix 1.1). The present study tested *Etroplus suratensis* in fresh water of turbidity (range, 70-100 μ/L) during 96 h LC_{50}. The 96 h toxicity of clove oil was affected by variation in water turbidity, during entire experimental period (96 h) the level turbidity decreased than that of control. 96-h LC_{50}'s was also reported for rainbow trout, which range from 0.879 to 1.73 mg/L for varying conditions of water temperature, turbidity and pH (Marking and Bills, 1975).

According to Gomes et al., (2009), increase in water quality parameters values are imputable mainly to the escalation of ammonia levels in the water. Ammonia measurements in the control group did not diverge significantly across the different cinnamon oil concentrations tested. In all the treatments with the cinnamon oil, ammonia concentration was markedly lower than control during the 24h experimental period (Appendix 1.1).The ammonia values at 24 h of transportation (10-11 $mg.L^{-1}$) were below the lethal

concentration threshold for Nile tilapia juveniles (Benli and Köksal, 2005). However, these values were similar to the values reported for *Etroplus suratensis* during 48 and 96 h cinnamon oil treatment, but this value might be lethal to juveniles of *Etroplus suratensis.*

The NO_2^- level of all the concentration was lower than the control group during 24 h and 96 h treatment. But there was no significant difference between the control and experimental groups during 48 h experimental period. The NO_3^- level showed an increasing pattern with the control during the 24, 48 and 96 h treatment with different concentration of cinnamon oil. An elevated ambient nitrite concentration is a potential problem for freshwater fish, since nitrite is actively taken up across the gills in competition with chloride. Nitrite is a well-known toxicant for fish as well as a disrupter of multiple physiological functions including ion regulatory, respiratory, cardiovascular, endocrine and excretory processes (Lewis and Morris, 1986; Jensen, 2003). During the 24, 48 and 96 h the change in pattern of NO_3^- decreased than control values. This possibly will lead to increase the mortality change in pattern of juveniles of *Etroplus suratensis*.

1.8.3 Effect of Cassumunar ginger extracts (*Zingiber cassumunar* Roxb)

Studies on the acute toxic effects of cassumunar ginger extract (*Zingiber cassumunar* Roxb) on juveniles of *Etroplus suratensis* indicate that juveniles of *Etroplus suratensis* are not vastly sensitive to cassumunar ginger extract (*Zingiber cassumunar* Roxb). By contrast, no reliable data are available on the effects of the administration of high concentrations of cassumunar ginger extract (*Zingiber cassumunar* Roxb) on lower vertebrate like fish, frog etc. There is no mortality during 24 h LC_{50} in juveniles of

Etroplus suratensis which has not any comparable results. In juveniles of *Etroplus suratensis*, different concentration of cassumunar ginger extract (*Zingiber cassumunar* Roxb) LC_{50} during 96 h was 1.46 mg/L, 72 h was 1.62 mg/L, 48 h was 1.74 mg/L, and 24 h was 0 mg/L. This difference might be the result of several factors, such as anaesthetic absorption, distribution, excretion and metabolism (Hooven et al., 2009). Mortality of fish and of the 0.50 and 0.70 mg/L treatment group, after 24 h of transportation was only 0% (Table1.B). Mortality increased with duration of exposure time. In the 3mg/L of cassumunar ginger extract (*Zingiber cassumunar* Roxb) treatment, the mortality at 24 h of experiment (50%) was significantly higher than the values observed in the other concentrations used. The Lake Victoria cichlid, *Haplochomis obliquidens* (Hilgendorf, 1888), anaesthetized with 18 ¼$L.L^{-1}$ of clove oil also revealed higher mortality rates after long transportation times (48 h) (Kaiser et al., 2006). Accumulated mortality, measured 96 h after an experimental period presented a similar tendency and was significantly higher in fish experimented in 0.60 and 3 mg/L cassumunar ginger extract (*Zingiber cassumunar* Roxb*)*. Total mortality of control fish and for fish anaesthetized with 1.33 mg/L of cassumunar ginger extract (*Zingiber cassumunar* Roxb) was only 6 % during 72 h. In the present result, at a concentration of 1.60 and 3 mg/L was highly toxic to juveniles of *Etroplus suratensis,* even during 24 h. The survival rate was 30-50%, although the recommended concentrations of cassumunar ginger extract (*Zingiber cassumunar* Roxb*)* for the short-term immobilization of fish range less than 1.70 mg/L. Another possibility for the different LC_{50} values stated by different authors is the variability of the cassumunar ginger extract (*Zingiber cassumunar* Roxb*)* used.The value of 0.70 mg/L of cassumunar

ginger extract (*Zingiber cassumunar* Roxb*)* found in good agreement with 96 h LC_{50} for anaesthetization of juveniles of *Etroplus suratensis*. When we compare the present experimental results (0.70mg/L for 96h LC50) and the recommended concentrations for fish anaesthesia, it is clear that this concentration of cassumunar ginger extract (*Zingiber cassumunar* Roxb) was low, which could lower the mortality rate on juveniles of *Etroplus suratensis*. An extended period of exposure could increase the toxicity of anaesthetics (Vrskova and Modra, 2012).

Analysis of 95% confidence interval on 96 h LC_{50} values of cassumunar ginger extract (*Zingiber cassumunar* Roxb*)* for juveniles of *Etroplus suratensis* showed highly significant variations with concentration. Barton and Helfrich (1981) reported that use of lower concentrations of the anaesthetic should provide a much wider safety margin for anaesthesia.There was not any comparable values reported. However, when lethal concentrations are used, but keeping gills artificially ventilated, fish can be kept alive for a longer period (Brown, 1987). The change in pattern of anaesthetic elimination during recovery also increases with artificial ventilation (Kiessling et al., 2009).

The juveniles of *Etroplus suratensis* used for the present experiments showed a diurnal activity pattern and therefore anaesthetic absorption through the gills might have also been higher. Thus, for a given concentration, toxicity would be greater during the day not only on account of the swimming activity but, the respiratory and metabolic activities, as observed in two fish species exposed to the pesticide lindane (Walton et al., 1997). The efficacy and safety range of cassumunar ginger extract (*Zingiber cassumunar*

Roxb) varies according to age, size and species of fish in addition to the concentration and purity.

The present study also analysed the effects of selected levels of water temperature, pH, DO and turbidity on the toxicity of cassumunar ginger extract (*Zingiber cassumunar* Roxb). There were significant differences in toxicity for the anaesthetic at water quality variables. The toxicity of many chemicals can be affected by differences in water quality (Bills et al., 1993). Low dissolved oxygen level, pH, ammonia and nitrite etc., are known to be toxic to fish (Lemmer, 1996; Reinberg, 1991), and special attention must be paid as to whether the anaesthetic exposure may induce their toxicity during live transportation of juveniles of *Etroplus suratensis*. However, in fish, as opposed to mammals, there is a lack of research dealing with the differential toxicity of xenobiotics depending on the water quality parameters. The present data indicate that juveniles of *Etroplus suratensis* respond to the anaesthetics and variations in water quality in the same way as other fish. However, there are no scientific reports available in the existing literature on LC50 (96 h) and toxic effect of *Zingiber cassumunar* Roxb.

A range of LC 50's from 24 to 96 h of different concentrations (0.50, 0.70, 1.33, 1.50, 1.60 and 3 mg/L) cassumunar ginger extract (*Zingiber cassumunar* Roxb) were recorded in the case of *Etroplus suratensis* under varying conditions of temperature, pH , DO, turbidity, ammonia, nitrate and nitrite. Collectively the present results indicate that during the 96 h LC50 test, the range of water temperature was at 28-30°C (Mean ±SE). Weyl et al., (1996) and Hamaakova et al., (2001) stated that the most important factor influencing efficacy in fish is temperature, i.e. the higher the temperature, the

higher the efficiency of the anaesthetic for fish. Svobodová et al., (1987) reported that using 2-phenoxyethanol for rainbow trout at the concentration of 0.30 $ml \cdot l^{-1}$ for 10min LC_{50} values were recorded for a water temperature of 15°C. This higher temperature may induce the mortality during 96 h LC50 test at 0.50, 0.70, 1.33, 1.50, 1.60 and 3 mg/L. Water temperature was similar across the control as well as in different concentration treatments during the first 10 min, 24 h, 48 h and 96 h experimental periods. Throughout this experiment, temperature variation was under 1ºC.

During 24, 48 and 96 h of the experimental period, pH decreased than the control value and assumed that were more toxic to juveniles of *Etroplus suratensis*. But as water pH decreased, the toxicity increased nearly 10% during 96 h . Inoue et al*.,* (2005) also reported that pH decreased after 4 h of transportation of juvenile matrinxã (*Brycon cephalus,* Gunther) anaesthetized with 5 $mg.L^{-1}$ clove oil, probably due to the increase in CO_2 values. At the end of the experiment, pH was significantly higher in the control group as compared to the fish anaesthetized with different concentrations of cassumunar ginger extract (*Zingiber cassumunar* Roxb).

In the present study the DO level in anaesthetized water within the range of 3-7 mg/L during the 96 h of entire experimental period. Dissolved oxygen levels in all concentration were seen significantly higher in the first 10 min than control value followed by a continuous decrease, up to 24 h experimental time. During 48 and 96 h of the experimental period, dissolved oxygen was significantly higher in the control group as compared to the experimental groups using cassumunar ginger extract (*Zingiber cassumunar* Roxb). Sensitivity to anaesthetics is also influenced by oxygen concentrations

in the sense that oxygen deficit enhances the anaesthetic efficiency (Svobodová et al., 1987). A pronounced consumption of oxygen was seen in fish treated with the cassumunar ginger extract (*Zingiber cassumunar* Roxb) in the 96 h measurements. This may be linked with the hyperactivity in fish and decrease in DO probably increases the mortality rate during 96 h. Since oxygen levels in this experimental configuration reached values near the critical survival threshold.

Variations in water turbidity probably affect the toxicity of cassumunar ginger extract (*Zingiber cassumunar* Roxb) to *Etroplus suratensis* (Appendix 1.1). We tested *Etroplus suratensis* in the fresh water of turbidity (range, 52-100 µ/L) during 96 h LC50 was tested. The 96 h toxicity of cassumunar ginger extract (*Zingiber cassumunar* Roxb) was not affected by variation in water turbidity and was always lower than control values in all treatments. 96-h LC50's reported for rainbow trout, which range from 0.879 to 1.73 mg/L for varying conditions of water temperature, turbidity and pH (Marking and Bills, 1975). The present data indicated that the responses of *Etroplus suratensis* to these compounds were similar to that of most other species.

Ammonia measurements in the entire experimental group always remained in lower rate, which did not diverge significantly across the control group concentrations tested. In all the treatments with the anaesthetic, ammonia concentration was markedly lower than control during the 24, 48 and 96 h of experimental period (Appendix 1.1). According to Gomes et al. (2009) increases in water quality parameters values are imputable mainly due to the escalation of ammonia levels in the water. The ammonia values at 24 h

of transportation (10-11 mg.L^{-1}) were below the lethal concentration threshold for Nile tilapia juveniles (Benli and Köksal, 2005). However, these values were similar to the values reported for *Etroplus suratensis*. The NO_2^- level in the entire concentration group decreased than the control group during 24 h. During the 48 and 96 h of treatment there was an increasing tendency noticed with increase in concentration, which might lead to increase the mortality changes in pattern of juveniles of *Etroplus suratensis*. The NO_3^- level of all experimental group also showed a declining pattern with the control during 24, 48 and 96 h treatment.

1.8.4 Tobacco leaves extract (*Nicotiana tobacum*)

In the present study, we tested the acute toxic effects of tobacco leaf extract (*Nicotiana tobacum*) on juveniles of *Etroplus suratensis.* The present data indicate that juveniles of *Etroplus suratensis* are very sensitive to tobacco leaf extract (*Nicotiana tobacum*). In the present study no mortality was observed in lower concentrations (2 and 5mg/L) of the tobacco leaf extract (*Nicotiana tobacum*) anaesthesia concentrations in the 24, 48, 72 and 96 h measurement. Mortality of fish and in the 6.21 mg/L treatment group, after 12 and 24 h of transportation was only 0% (Table 1.C). Mortality increased with duration of exposure time. In the higher concentration of 7 and 8 mg/L tobacco leaf extract (*Nicotiana tobacum*) treatment, the mortality at 24 h of transportation (38 and 50 %) was significantly higher than the values observed for the lower concentrations used (2,5 and 6 mg/L). Similar studies on an acute toxicity of tobacco (*Nicotiana tobacum*) leaf dust were carried out on *Oreochromis niloticus* by Agbon et al. (2002). The extract was found to be toxic with 48 h LC_{50} value of 109.6 mg/L. This value is far higher than that estimated in this study in which juveniles of *Etroplus suratensis* was

found to be sensitive at a concentration of 7.13 mg/L, thereby indicating that juveniles of *Etroplus suratensis* were less resistant to tobacco toxicity than *O.niloticus*. Mortality, measured 96 h after experimentation presented a similar tendency as compared to the mortality after the end of the experimental period, and was significantly higher in fish experimented in 7 and 8 mg/L tobacco leaf extract (*Nicotiana tobacum*) for 24 h. Total mortality for control fish and for fish anaesthetized with 2 and 5 mg/L tobacco leaf extract (*Nicotiana tobacum*) was only 0 %. The determination of tobacco leaf extract (*Nicotiana tobacum*) acute toxicity is important for its usage in fish. In the present result, at the concentration of 7 and 8 mg/L of tobacco leaf extract (*Nicotiana tobacum*) was highly toxic to juveniles of *Etroplus suratensis* and even during 24 h the survival rate was only 50%. In juveniles of *Etroplus suratensis*, the tobacco leaf extract (*Nicotiana tobacum*) LC_{50} during 96 h was 6.50 mg/L, 72 h was 6.70 mg/L , 48 h was 7.13 mg/L, and 24 h was 0 mg/L. This difference might be due to the result of several factors, such as anaesthetic absorption, distribution, excretion and metabolism (Hooven et al., 2009). A comparable acute toxicity test of *Clarias gariepinus* exposed to acute concentrations (25.00, 20.00, 15.00, 10.00, 5.00 and 0.00 mg/L) of tobacco leaf dust during the 96 h exposure period revealed 100 percent mortalities in 25 mg/L and 20 mg/L concentration of tobacco leaf dust during the 96h exposure (Adamu and Kori-Siakpere, 2011). However, 15.00 mg/L concentrations revealed 75 percent mortality after 96 hs. It is suggested that when used in fish, the better concentration of tobacco leaf extract (*Nicotiana tobacum*) be administered in anaesthesia baths at concentrations between 6.21- 8.18 mg/L, because this concentration causes better anaesthetic effects and lower mortality rate up to 96 h of the

experimental period. Barton and Helfrich (1981) reported that use of lower concentrations of the anaesthetic should provide much wider safety margin for anaesthesia. The acute toxicity test of tobacco leaf dust on the African catfish *Clarias gariepinus* is dose dependent. As the concentration of the tobacco leaf dust increases the change in the pattern of the mortality of *Clarias gariepinus* also increases, which is directly proportional (Adamu and Kori-Siakpere, 2011). This statement is in good agreement with 96 h LC_{50}'s of 2,5,6,7 and 8 mg/L of tobacco leaf extract (*Nicotiana tobacum*) in the case of juveniles of *Etroplus suratensis* found in the present study. In comparison, between the present experimental results and the recommended concentrations for fish anaesthesia, it is clear that the concentration of tobacco leaf extract (*Nicotiana tobacum*) is low. Otherwise the effect of higher concentration on juveniles of *Etroplus suratensis* could result in higher mortality.

The juveniles of *Etroplus suratensis* used for the present experiments showed a diurnal activity pattern and therefore anaesthetic absorption through the gills might have also been higher. Thus, for a given concentration, toxicity would be greater during the day, not only on account of higher swimming activity but also the respiratory and metabolic activities, as observed in fish species exposed to the pesticide lindane (Walton et al., 1997). However, when lethal concentrations are used the gills are artificially ventilated; fish can be kept alive for a longer period (Brown, 1987).

Analysis of 95% confidence interval on 96 h LC_{50} values of tobacco leaf extract (*Nicotiana tobacum*) for juveniles of *Etroplus suratensis* showed highly significant variations with concentration of tobacco leaf extract (*Nicotiana tobacum*). The 96 h LC_{50} by probit analysis of juveniles of Green

chromide (*Etroplus suratensis*) exposed to various concentrations of tobacco leaf extract (*Nicotiana tobacum*), for 48 h LC_{50} with lower and upper confidence limits of 6.21 and 8.18 mg/L, for 72 h with lower and upper confidence limits of 6.58 and 6.90 mg/L and for 96 h with lower and upper confidence limits of 6.34 and 6.67 mg/L respectively. A comparative study reported by Adamu and Kori-Siakpere (2011) revealed that the 96 h LC_{50} value was 10.96 mg/L, r^2 value of 0.68 with 95% lower and upper confidence limit of 7.50 and 16.00 mg/L respectively. In *Clarias gariepinus* (Burchell), 48 h LC50 estimated by probit analysis, acute exposure was found to be 626.0 mg/L Omoniyi et al. (2002). According to Olufayo and Jatto (2011) the LC_{50} at the end of 96 h was 1.35 g/L when Nile tilapia (*Oreochomis niloticus*) juveniles were exposed to tobacco (*Nicotiana tobaccum*) leaf dust. However, in the present result, the 96 h LC_{50} value of lower concentrations of 2,5,6,7 and 8 mg/L of tobacco leaf extract (*Nicotiana tobacum*) found in the study suggested that it is relatively harmless for fish.

The effects of selected levels of water temperature, pH, DO and Turbidity on the toxicity of tobacco leaf extract (*Nicotiana tobacum*) were determined to assess the differences in toxicity for the anaesthetic at water quality variables. The toxicity of many chemicals can be affected by differences in water quality (Bills et al., 1993). Low dissolved oxygen level, pH, Ammonia and nitrite etc., are known to be toxic to fish (Lemmer, 1996; Reinberg, 1991), and special attention must be paid to whether the anaesthetic exposure may induce to increase their toxicity during live transportation of juveniles of *Etroplus suratensis*. However, in fish, as opposed to mammals, there is a lack of research dealing with the differential toxicity of xenobiotics depending upon the water quality parameters. The

present data indicate that juveniles of *Etroplus suratensis* respond to the anaesthetics and variations in water quality in the same way as other fish.

Since there are no scientific reports available in the existing literature on LC_{50} (96 h) and toxic effect of tobacco leaf extract (*Nicotiana tobacum*), the present study on the usage and dose determination of tobacco leaf extract (*Nicotiana tobacum*), that are used as a fish anaesthetic to determine their effects on *Etroplus suratensis* at different levels of water quality is of great significance in the ornamental and commercial fish culture/aquaculture. Mean values of water quality parameters of the different lethal concentrations (96 h LC_{50}) of tobacco leaf extract (*Nicotiana tobacum*) and control media to which the test fish *O. niloticus* were exposed over the 96 h exposure period is as presented in Appendix 1.1. The present data indicate that juveniles of *Etroplus suratensis* respond to the tobacco leaf extract (*Nicotiana tobacum*) and variations in water quality in the same way as other fish. The variation in the reported result of monitoring parameters could be associated to the exposure period and the level of tobacco leaf dust concentrations (Omoniyi et al., 2002). Adamu and Kori-Siakpere (2011) reported that the mean values of the water quality parameters of the different sub lethal concentrations of tobacco (*N. tobaccum*) leaf dust and control media to which the test fish hybrid catfish was exposed over the 14 days exposure period. The points of view of water contamination risks, 96 h LC_{50} values are used. (Adamu and Kori-Siakpere, 2011) water quality parameters such as temperature, dissolved oxygen, free carbon dioxide; pH, alkalinity and conductivity are the parameters that are paramount to the many factors that affect fish health, growth and reproduction (Camus et al., 1998; Hill, 1995). However, Richards (1977) reported that the main cause of mortality in aquarium fish was the

inadequate maintenance of the water environment. In this study the monitored parameters were noted to be significantly different from the control test after 96 h LC_{50} exposure period, which invariably meant that tobacco leaf dust had an impact on the water chemistry. In the present study a range of LC $_{50}$'s of tobacco leaf extract (*Nicotiana tobacum*), from 24 to 96 h (2,5,6,7 and 8 mg/L) was recorded for *Etroplus suratensis* under varying conditions of temperature, pH , DO, Turbidity, ammonia, nitrate and nitrite. According to Omoniyi et al. (2002) sub lethal effects of tobacco leaf dust on the haematological parameters of the *Clarias gariepinus* revealed increased and decreased difference in the monitored water quality parameters. Weyl et al. (1996) and Hamaakova et al. (2001) stated that the most important factor influencing efficacy in fish is temperature, i.e. the higher the temperature, the higher the efficiency of the anaesthetic for fish. Collectively the present result indicates that during the 96 h LC_{50} test, there was no significant difference in temperature between the control and other concentration group and hence the effects of temperature on this study could be negligible. Range of water temperature was at 27-30°C (Mean ± SE) (Appendix 1.1) throughout the experiments.

Sensitivity to anaesthetics is also influenced by oxygen concentrations in the sense that oxygen deficit enhances the anaesthetic efficiency (Svobodová et al., 1987). In the present result the DO level in anaesthetized water within the range of 5-6 mg/L during 10min, 5-7 mg/L during 24 h, 5-8 mg/L during 48 h, 3-5 mg/L during 96 h. Oxygen levels were increased with increasing temperature in the 10 min experimental time. As of the 24 h experimental time there was no significant difference in dissolved oxygen concentration between the control and experimental groups. But at 48 h the dissolved oxygen

concentration was significantly increased than the control group and revealed that fish consume less amount of oxygen from the anaesthetic water. At the end of the experimental period (96 h), dissolved oxygen in the entire experimental group was significantly lower than the control group using tobacco leaf extract (*Nicotiana tobacum*). A pronounced consumption of oxygen was seen in fish treated with the tobacco leaf extract (*Nicotiana tobacum*) in the 96 h measurements. This may be linked with the hyperactivity in fish and decrease in DO probably increases the mortality rate during 96 h. Olufayo and Jatto (2011) reported that in the case of Nile tilapia (*Oreochomis niloticus*) the monitored water quality parameters such as temperature, pH and dissolved oxygen was significantly decreased while total alkalinity and conductivity increased significantly in the exposed media, compared to the control test. Konar (1970) reported accumulation of mucus in the gills reduces respiratory activity in fish. The inability of the gill surface to actively carry out gaseous exchange might be responsible for the recorded mortalities (Omoniyi et al., 2002). Omoniyi et al. (2002) reported that the acidic condition of the water had resulted in the decrease in the level of dissolved oxygen, free carbon dioxide and temperature with a corresponding increase in the values of total alkalinity and conductivity. Omoniyi et al. (2002) had also reported a decrease in the temperature, dissolved oxygen with an increase in conductivity values, respectively. The strange behaviour exhibited by the fish may be as a result of the respiratory impairments due to the effect of Nicotine on the gills which may reduce respiratory activity in fish and the inability of the gill surface to actively carry out gaseous exchange, might be responsible for the recorded mortalities which is shown to be dependent on the concentration of the tobacco extracts in the bioassay (Adamu and Kori-Siakpere, 2011).

Variations in water turbidity probably affect the toxicity of tobacco leaf extract (*Nicotiana tobacum*) to *Etroplus suratensis* (Appendix 1.1). The present studies showed that *Etroplus suratensis* in the fresh water of turbidity (range, 54-100 µ/L) during 96 h LC_{50} of the entire experimental group for *Etroplus suratensis* was significantly higher than the control. According to Adamu (2009), the values of total alkalinity and conductivity in the exposed media were found to be significantly ($p<0.01$) increased as the concentrations of tobacco (*Nicotiana tobaccum*) leaf dust increased, compared to the control test on enzymatic activities of Heteroclarias (a hybrid of *Heterobranchus bidorsalis* and *Clarias gariepinus*). The 96 h toxicity of tobacco leaf extract (*Nicotiana tobacum*) was affected by variation in water turbidity, but as water pH decreased, the toxicity increased nearly 10% during 96 h to juveniles of *Etroplus suratensis*. Inoue et al. (2005) also reported that pH decreased after 4 h of transportation of juvenile matrinxã (*Brycon cephalus,* Gunther) anaesthetized with 5 $mg.L^{-1}$ clove oil, probably due to the increase in CO_2 values. At the end of the experimental period (96 h), pH was significantly lower in the entire experimental group as compared with control group. Noga (1996) and Richards (1977) recommended the pH for fresh water fish to be 6.5 to 8.5, the value of pH in the highest concentration of tobacco leaf extract was found to be intermediate in all the concentration. Thus the significant decrease in pH value during 96 h as the concentrations of tobacco leaf extract increased revealed that the toxicant resulted in acidic condition. This was supported by the findings of Omoniyi et al. (2002) who reported acidic condition in water of *Clarias gariepinus* exposed to tobacco leaf dust. The decline in pH with time could be due to the production of acidic metabolites (Delyan et al., 1990). According to Adamu (2009) the value of temperature

and free carbon dioxide, pH and dissolved oxygen were found to significantly ($p<0.05$) and ($p<0.01$) decrease as the concentrations of tobacco leaf dust increased on enzymatic activities of Heteroclarias (a Hybrid of *Heterobranchus bidorsalis* and *Clarias gariepinus*). The present data indicate that the response of *Etroplus suratensis* to these compounds is similar to that of most other species treated with tobacco leaf extract (*Nicotiana tobacum*). Marking and Bills (1975) reported 96-h LC_{50}'s for rainbow trout, which range from 0.879 to 1.73 mg/L for varying conditions of water temperature, turbidity and pH. According to Gomes et al. (2009), increases in water quality parameter values are imputable mainly to the escalation of ammonia levels in the water. Ammonia measurements in the control group did not diverge significantly with the different tobacco leaf extract (*Nicotiana tobacum*) concentrations tested. In the treatments with the anaesthetic, ammonia concentration was markedly higher in the 24, 48 and 96 h h transportation period (Appendix 1.1) at 2, 5, 6, 7 and 8 mg/L concentration. The ammonia values at 24 h of transportation (10-11 $mg.L^{-1}$) were below the lethal concentration threshold for Nile tilapia juveniles (Benli and Köksal, 2005). However, these values were similar to the values reported for *Etroplus suratensis*. The NO_2^- level in the entire concentration group increased than the control group during 24 h (0.02-0.08 mg/L), 48 h (0.02-0.15 mg/L) and 96 h (0.14-0.17 mg/L) treatment. But there was a significant difference between the control and experimental groups during 96 h experimental period. This possibly will lead to increase the mortality changes in pattern of juveniles of *Etroplus suratensis*. The NO_3^- level of all experimental group also showed an increasing pattern with the control during 24, 48 and 96 h treatment. This might increase the mortality change in pattern of juveniles of *Etroplus suratensis* during 96 h tobacco leaf

extract (*Nicotiana tobacum*) treatment. According to Svobodova and Kolarova (2004), Svobodova et al., (2005a) the increase in nitrite concentration in water may result in mass fish mortality.

1.8.5 Effect of MS-222

In the present study we tested the acute toxic effects of MS-222 on juveniles of *Etroplus suratensis.* The present data indicate that juveniles of *Etroplus suratensis* are very sensitive to MS-222. In juveniles of *Etroplus suratensis*, the MS-222 LC_{50} during 96 h was 67.44 mg/L, 72 h was 73.71 mg/L and 48 h was 92.10 mg/L. These values are similar to those reported by Marking (1967) for MS-222 for several fish species. The present data indicate that concentrations of anaesthetics used for other fish should also be safe for use on *Etroplus suratensis*.

No mortality was observed in the concentration of MS-222 of 45, 50 and 53 mg/L in the 24, 48 and 72 h measurements on juveniles of *Etroplus suratensis*. Mortality of control fish and of the 45 mg/L treatment group, after 12 and 24 h of transportation was only 0% (Table1.E). Mortality increased with duration of exposure time. The Lake Victoria cichlid, *Haplochomis obliquidens* (Hilgendorf, 1888) anaesthetized with 18 ¼ $L.L^{-1}$ of clove oil also revealed higher mortality rates after long transportation times (48 h) (Kaiser et al*.,* 2006). Accumulated mortality of MS-222 after the end of the experimental period (96 h LC50) was significantly higher at the concentration of 75 and 100 mg/L. In the present result at the 100 mg/L MS-222 treatment, the mortality at 24 h of transportation (50 %) was significantly higher than the values observed for the other concentrations used. This difference in mortality might be the result of several factors, such as anaesthetic absorption,

distribution, excretion and metabolism (Hooven et al., 2009). In the present result, the concentration of 75 and 100 mg/L highly toxic to juveniles of *Etroplus suratensis,* even during 24 h the survival rate was only 50%. Another effect of high concentrations of MS-222 seems to be heart failure, since this anaesthetic affects ion transport, blocking Na+ and K+ conductance (Frazier and Narahashi, 1975). Furthermore, recent investigations in Nile tilapia (*Oreochomis niloticus*) have demonstrated the absence of genotoxic activity induced by MS-222, under both *in vivo* and *in vitro* conditions (Barreto et al., 2007).

Analysis of 95% confidence interval on 96 h LC50 values of MS-222 for juveniles of *Etroplus suratensis* showed highly significant variations with concentration of MS-222. However, in the present result, the 96 LC_{50} values of lower concentrations of 45, 50 and 53 mg/L of MS-222 found in the study suggests that it is relatively harmless to fish. In gilthead sea bream, MS-222 did not depress humoral or cellular immune responses (Ortuño et al., 2002). Barton and Helfrich (1981) reported that use of lower concentrations of the anaesthetic should provide much wider safety margin for anaesthesia. The toxicity of MS-222 has been reported to decrease with fish age in zebra fish (Rombough, 2007). However, when lethal concentrations are used, but the gills are artificially ventilated, fish can be kept alive for a longer period (Brown, 1987).The change in pattern of anaesthetic elimination during recovery also increases with artificial ventilation (Kiessling et al., 2009).

On compare the present experimental results (67.44 mg/L for 96 h LC50) and the recommended concentrations (53 mg/L) for 48 h fish anaesthesia, it is clear that although the concentration of MS-222 is low

compare with the concentrations of other fishes. That value is in good agreement with 96 h LC50 of 53 mg/L for juveniles of *Etroplus suratensis* found in the present study. The juveniles of *Etroplus suratensis* used for the present experiments showed a diurnal activity pattern and therefore anaesthetic absorption though the gills might have also been higher. Thus, for a given concentration, toxicity would be greater during the day since not only the swimming activity would be higher, but also the respiration and metabolic activities, as observed in two fish species exposed to the pesticide lindane (Walton et al., 1997). Ortuño et al., (2002) reported that MS-222 toxicity and effectiveness in gilthead sea bream were higher at ML than at MD and, consequently the time needed to induce anaesthesia by means of a sub lethal concentration was shorter during the day. The efficacy and safety range of MS-222 varies according to age, size and species of fish (Gilderhus and Marking, 1987). MS-222 has a large safety margin between the effective, maximum safe and euthanasia concentrations, which is desirable for the field research or with novice users (Carter et al., 2011).

The efficacy and stress associated with MS-222 vary with species, age, life history, body size, and sex (Gilderhus and Marking, 1987; Stehly and Gingerich, 1999; Tsantilas et al., 2006; Carter et al., 2011) as well as water physicochemical parameters, such as salinity, turbidity, pH, oxygen levels, and water temperature (Mylonas et al., 2005; Zahl et al., 2011; Carter et al., 2011). The influence of other environmental conditions (temperature, pH, etc.) on the toxicity of anaesthetics has also been investigated (Park et al., 2008; Zahl et al., 2009). Acute toxicity of 2-phenoxyethanol to fish is investigated from the aspect of 2-phenoxyethanol use as an anaesthetic, and of the risk of water contamination with anaesthetizing baths (Velisek et al.,

2007). Likewise we also determined the effects of selected levels of water temperature, turbidity, pH, DO and on the toxicity of MS-222. There were differences in toxicity for the MS-222 at water quality variables. The present data indicate that juveniles of *Etroplus suratensis* respond to the anaesthetics and variations in water quality in the same way as other fish. The toxicity of many chemicals can be affected by differences in water quality (Bills et al., 1993). Low dissolved oxygen level, pH, ammonia and nitrite etc., are known to be toxic to fish ((Lemmer, 1996; Reinberg, 1991)), and special attention must be paid to whether the anaesthetic exposure may induce to increase their toxicity during live transportation of juveniles of *Etroplus suratensis*. Acute toxicity of MS-222 to fish is investigated from the point of view of MS-222 use as an anaesthetic, but also possible for contamination of the water environment by such anaesthetic. The points of view of water contamination risks, 96 h LC_{50} values are used.

In the present study range of LC $_{50}$'s from 24 to 96 h (45, 50, 53, 75 and 100 mg/L) was recorded for *Etroplus suratensis* under varying conditions of temperature, pH , DO, turbidity, ammonia, nitrate and nitrite. Collectively the present result indicates that during the 96 h LC_{50} test the range of water temperature was at 29-30°C (Mean ±SE). Throughout the 96 h experimental period, water temperature was significantly similar across the groups treated with different concentration of MS-222. In the present study the results indicate that during the 96 h LC_{50} test, there was no significant difference in temperature between the control and other concentration group and hence the effects of temperature on this study could be negligible. Throughout this experiment, temperature variation was below 1ºC. It should be observed that MS-222 becomes toxic in seawater exposed to sun (Bell, 1987). Weyl et al.,

(1996) and Hamaakova et al. (2001) stated that the most important factor influencing efficacy in fish is temperature, i.e. the higher the temperature, the higher the efficiency of the anaesthetic for fish.

At the end of the experiment (96 h), pH was significantly higher in the experimental group as compared to the control. But as water pH increased, the toxicity increased nearly 10% during 96 h to juveniles of *Etroplus suratensis*. The present data indicate that the response of *Etroplus suratensis* to these compounds is similar to that of most other species. 96-h LC_{50}'s reported for rainbow trout, which range from 0.879 to 1.73 mg/L for varying conditions of water temperature, turbidity and pH (Marking and Bills, 1975). But according to Wedemeyer (1970) MS-222 gives an acid solution and a dosage of 75 mg/L can cause the pH to fall to 4.0 in soft water. According to Stoskpok and Posner (2008) the addition of MS-222 and Benzocaine lowers the pH of water to which is added, to as low as 5. TMS is acidic and can produce respiratory stress by lowering the pH of the fish's blood (Summerfelt and Smith, 1990) depending on the TMS dose and alkalinity. Aerated well water used in this preliminary study did not need to be buffered, given the water's high buffering capacity; the pH of the water was 8.5 and did not change with the addition of either TMS or quinaldine over the duration of the experiment.

Sensitivity to anaesthetics is also influenced by oxygen concentrations in the sense that oxygen deficit enhances the anaesthetic efficiency (Svobodová et al., 1987). In the present result the DO level in anaesthetized water within the range of 6-8 mg/L during 10 min, 7-10 mg/L during 24 h, 4-7 mg/L during 48 h and 4-10 mg/L during 96 h. Oxygen levels were higher in the 6 h

transportation time, followed by a continuous decrease in the parameter, up to 24 h transportation time in all groups. At the end of the experimental period (96 h), dissolved oxygen was significantly higher in the experimental group as compared to the control groups using MS-222. A pronounced consumption of oxygen was seen in the control group throughout the 96 h measurements. There was a sufficient amount of DO in all concentration during 96 h experimental period and hence the effects of DO on this study could be negligible. Collectively the present results indicate that during the 96 h LC_{50} test, there was a significant difference in DO between the control and other concentration group.

In the present study tested the fresh water of turbidity (range, 54-100 µ/L) during 96 h LC_{50}. Turbidity showed a decreasing tendency in all concentration groups among the control. The 96 h toxicity of MS-222 was not affected by variation in water turbidity (Appendix 1.1). According to Gomes et al. (2009) increases in water quality parameter values are imputable mainly to the escalation of ammonia levels in the water. An ammonia measurements in the control group diverge significantly across the different MS-222 concentrations tested. In the treatments with the anaesthetic, ammonia concentration was markedly higher in the 24 h transportation period (Appendix 1.1) at 75 and 100 mg/L concentration. Above all the ammonia values at 24, 48 and 96 h of experimental duration were of an increasing tendency to increase in concentration. Probably this may lead to increase the mortality rate. The NO_2^- level in the entire concentration group showed an increasing tendency throughout the experimental time (96 h) than the control group. But there was significant difference between the control and experimental groups during 96 h experimental period this perhaps force to raise the mortality change in

pattern of juveniles of *Etroplus suratensis*. The literature dealing with long-term toxicity of sub lethal nitrite concentrations corresponding to 10% of 96 h LC_{50} suggests that such a concentration should not be detrimental to freshwater fish. Neither growth suppression nor tissue damage was observed (Wedemeyer and Yasutake, 1978; Colt et al., 1981). The NO_3^- level of all experimental groups showed a declining pattern with the control during 24 and 96 h treatment. But at 48th h of the experimental group showed the value of $NO3^-$ similar with the control group.

1.8.6 Effect of Hypothermic condition

In the present study, we tested the acute toxic effects of lowering temperature (hypothermia) on juveniles of *Etroplus suratensis.* The present data indicate that juveniles of *Etroplus suratensis* are very sensitive to hypothermia. In the present study the 96 h LC_{50} value of hypothermic conditions of 16, 18 and 22°C suggests that it is relatively harmless for fish. In contrast, anaesthesia water temperatures of −1.5, −3.0, −4.5, and −6.0°C on adult pink salmon Oncorhynchus gorbuscha had no effect on survival, nor was there a significant difference in survival between the experimental groups and the control (Hovda and Linley, 2000). In the present work no mortality was observed in the concentration of hypothermia of 22, 18 and 16°C up to 48 h measurement on juveniles of *Etroplus suratensis*. Optimal temperatures ranges, as well as upper and lower lethal temperatures, vary widely between and among species and are dependent on genetics, developmental stage and thermal histories (Beitinger et al., 2000; Somero, 2005). Within a range of non-lethal temperatures, fishes are generally able to cope with gradual temperature changes that are common in natural systems (e.g. diel variation, tidal activity, currents and seasonal cooling). In the

present study after 96 h of experiment, mortality of control fish and of the 22 and 18°C treatment group was only 0% (Table1.F) and of 16°C the mortality was 17% during 72 h and 46% during 96 h. In the present result accumulated mortality of hypothermia after the end of the experimental period (96 h LC_{50}) was significantly higher (100%) at the concentration levels of 12 and 8°C than the values observed for the other concentrations used. In the present result, the concentration of 12 and 8°C/L highly toxic to juveniles of *Etroplus suratensis,* even during 24 h the survival rate was only 60 %. Similar effects was recorded by Ross et al, (2007) on *Menidia estor* that rapid temperature reduction from 24 to 21°C or 18°C had no significant sedative effect but at 15°C swimming ceased in all fish after 2 min and 80% had lost touch sensitivity after 4min. Hypothermia was used alone, stable sedation of *M. estor* was induced at 15 and 12°C, with no mortalities At 12°C, swimming ceased and touch sensitivity was suppressed immediately, resulting in a form of deep sedation. At 8°C but there was also some loss of equilibrium and 65% of the fish ceased opercular movements after 4 min. Recovery was uneventful, requiring progressively longer from lower temperatures and mortality was the result. (Ross et al., 2007). According to Donaldson et al. (2008a) cold shock that reduces body temperatures to the lower limit of an organism's thermal range can result in severe sub lethal disturbances and mortality. Mortality increased with duration of exposure time. The 96 h LC_{50} obtained in juveniles of *Etroplus suratensis* by Trimmed Spearman-Karber Method was 15.53°C and 48 h was 12.55°C. The present data indicate that concentrations of hypothermia used for other fish should also be safe for use on *Etroplus suratensis*. Hypothermia was used alone; stable sedation of *M. estor* was induced at 15 and 12°C, with no mortalities (Ross et al., 2007).

The magnitude of the cold-shock response is dependent on both the change in pattern of temperature decrease and the magnitude of change in relation to thermal tolerance limits (Crawshaw, 1977; Tanck et al., 2000; Van den Burg et al., 2005).

Analysis of 95% confidence interval on 96 h LC_{50} values of hypothermia for juveniles of *Etroplus suratensis* showed slight significant variations with different concentrations of hypothermia. The values are in good agreement with 96 h LC_{50} of 15.53°C/L for juveniles of *Etroplus suratensis* found in the present study. On compairing the present experimental results (15.53°C/L for 96 h LC_{50}) and the recommended concentrations (16°C/L) for 48 h fish anaesthesia, it is clear that the concentration of hypothermia is comparatively high with the concentrations of other fishes. Cold temperatures within lethal limits, during the later developmental stages, may not reduce the probability of survival (Alderdice and Velsen, 1978, Hubert and Gern, 1995). This trend was demonstrated with coho salmon (*Oncorhynchus kisutch*) (Walbaum) embryos by Tang et al. (1987) who demonstrated that mortality approached 100% at temperature tolerance limits above 14°C and below 1.3°C, but abrupt temperature changes within these extremes resulted in little increased mortality.

The present study determined the effects of selected parameters of water, like temperature, turbidity, pH, DO, ammonia, nitrite and nitrite on the toxicity of hypothermia. There were differences in toxicity for the hypothermia at water quality variables. The present data indicate that juveniles of *Etroplus suratensis* respond to the hypothermia and variations in water quality in the same way as other fish. According to the father of fish environmental

physiology, F.E.J. Fry, temperature as well as heat (sensustricto) can influence fishes in multiple ways (Currie et al., 1998). Among these, temperature can 'act as a lethal factor when its effect is to destroy the integrity of the organism' (Fry, 1947). The influence of other environmental conditions (temperature, pH, etc.) on the toxicity of anaesthetics has also been investigated (Park et al., 2008; Zahl et al., 2009). The toxicity of many chemicals can be affected by differences in water quality (Bills et al., 1993). Low dissolved oxygen level, pH, ammonia and nitrite etc., are known to be toxic to fish (Lemmer, 1996; Reinberg, 1991), and special attention must be paid as to whether the hypothermic exposure may induce to increase their toxicity during live transportation of juveniles of *Etroplus suratensis*. Acute toxicity of hypothermia to fish is investigated from the point of view of hypothermia use as an anaesthetic, but also possible for contamination of the water environment by such anaesthetic. The points of view of water contamination risks, 96 h LC_{50} values are used.

A range of LC_{50}'s from 24 to 96 h of exposures of hypothermic concentrations of 22, 18, 16, 12 and 8°C/L was recorded for *Etroplus suratensis* under varying conditions of temperature, pH, DO, turbidity, ammonia, nitrate and nitrite. Weyl et al., (1996) and Hamaakova et al., (2001) stated that the most important factor influencing efficacy in fish is temperature, i.e. the higher the temperature, the higher the efficiency of the anaesthetic for fish. But in the case of hypothermia, collectively the present result indicate that during the 96 h, the LC_{50} range of hypothermia was at 15.53°C/L (Mean ±SE) (Appendix1.1). Above this lower temperature may induce the mortality during 96 h LC_{50} test. Through out the 96 h experimental period, it was better to select the hypothermic level of 16-18°C/L. Collectively

the present result indicate that during the 96 h LC_{50} test, there was significant difference in temperature between the control (28±1°C) and other concentration group and hence the effects of temperature on this study could be considerable. Water temperatures can exceed 32°C and DO levels are generally less than 4 ppm during transportation (Bosworth et al., 2004). Through out this experiment, temperature variation was under 4°C.

In the present result the DO level in different hypothermic condition showed decreasing pattern with increasing water temperature and also inversely proportional to the exposure time. DO level were higher in the 10 min experimental period followed by a continuous decrease in the parameter, up to 24 h transportation time in all groups. The combination of increased water temperature, which decreases oxygen solubility (Wetzel, 1983), and vice versa. At the end of the experimental period (96 h), dissolved oxygen was significantly lower in all experimental groups as compared to the control groups using hypothermia. A pronounced consumption of oxygen was seen in control group through out the 96 h measurements. There was sufficient amount of DO in all concentration up to 48 h experimental period, but during 96 h, 100% mortality will be the result. Collectively the present results indicate that during the 96 h LC_{50} test, there was significant difference in DO between the control and other concentration group of hypothermia. In addition, water temperature and oxygen levels may impact other water quality parameters (ammonia, nitrite, pH, and CO_2) that could influence the level of stress experienced by fish during transport (Amend et al., 1982; Erikson et al., 1997; Patterson et al., 2003). Sensitivity to anaesthetics may also be influenced by fish health, physical condition (Velisek et al., 2007) and

also influenced by oxygen concentrations in the sense that oxygen deficit enhances the anaesthetic efficiency (Svobodová et al., 1987).

But as water pH decreased, the toxicity increased nearly 10% during 96 h. At the first 24, 48 and 96 h pH increased with increase in hypothermic condition. Also pH was seen directly proportional to the exposure time. At the end of the experiment (96 h), pH was significantly higher in the experimental group as compared with control. Increase in pH might be responsible for high mortality rate in higher levels of hypothermia (12 and 8°C) on 96 h. The present data indicate that the response of *Etroplus suratensis* to these compounds is similar to that of most other species.96 h LC_{50}'s reported for rainbow trout, which range from 0.879 to 1.73 mg/L for varying conditions of water temperature, turbidity and pH (Marking and Bills, 1975).

The studies indicated that in the case of *Etroplus suratensis* in fresh water of turbidity (range, 54-100 µ/L) during 96 h $LC_{50,}$ in all the hypothermic tested level, turbidity showed a decreasing tendency with control. Above all, there was no significant difference between the control and all hypothermic levels. So the 96 h toxicity of hypothermia was not affected by variation in water turbidity.

According to Gomes et al. (2009), increases in water quality parameter values are imputable mainly to the escalation of ammonia levels in the water. Ammonia measurements in the hypothermic group diverge significantly with the control. In the treatments with the hypothermia, ammonia concentration was markedly lower in the entire experimental period (Appendix 1.1). Above all, the ammonia values at 24, 48 and 96h of experimental duration were of

an increasing tendency with increase in concentration and exposure duration. Because of nil significance among the control, concentration (hypothermia) and experimental duration, the probability of NH_3+ as reason for increase the mortality rate was neglected. The NO_2^- level in the entire hypothermic group showed decreasing tendency with increase in hypothermic condition. There was no significant difference of NO_2 level among the experimental group through out experimental duration (96 h) and hence the effects of NO_2^- on this study could be negligible. The NO_3^- level of all experimental groups showed a declining pattern with control during the entire treatment period (96 h). There was no significant difference of NO_3^- level among the experimental group throughout experimental duration (96 h) and hence the effects of NO_3^- on this study could be negligible.

1.9 Summary

In conclusion, clove oil is an efficient anaesthetic for routine fish handling procedures for juveniles of *Etroplus suratensis* that require anaesthesia for up to 24 h at the concentration of 0.29 mg/L. According to these findings, juveniles of *Etroplus suratensis* showed the highest sensitivity to clove oil at higher concentrations (0.30 and 0.33 mg/L), and comparable tolerance to acute toxicity of clove oil. The appropriate clove oil concentration to induce surgical anaesthesia for 48-h is 0.25 $mg.L^{-1}$, while for biometry procedures the best concentration of the anaesthetic is between 0.25 and 0.35 mg/L. As for transportation procedures, this concentration should be avoided for juveniles of *Etroplus suratensis*, as this induces a greater osmorregulatory disturbance and mortality rate. The present study showed a lower sensitivity of juveniles of *Etroplus suratensis* to higher concentration of clove oil

(0.30 and 0.33mg/L). An extended period of exposure could increase the toxicity of anaesthetics (Vrskova and Modra, 2012). The present result indicates that the risk to juveniles of *Etroplus suratensis* is high when they are exposed to anaesthetic concentrations of 0.3O and 0.33mg/L clove oil for 96 h.

Also, the clove oil apparently does not exert any toxic effect (Ross and Ross, 2008). The IACUC review proposed Clove oil use as an anaesthetic on a case by case basis in fish that will not leave the laboratory and will not be used for human or animal food. The efficacy and safety range of clove oil varies according to age, size and species of fish in addition to the concentration and purity of the clove oil (Ross and Ross, 2008).

Similarly cinnamon oil is an efficient anaesthetic for transportation of juveniles of *Etroplus suratensis*. Cinnamon bark (*Cinnamomum zeylanicum*) also contains eugenol, but its use as an anaesthetic has not been explored. Eugenol is considered to be a noncarcinogenic, nonmutagenic (Maura et al., 1989), "generally recognized as safe" (GRAS) substance by the FDA. When we compare the present experimental results (0.56 mg/L for 96 h LC_{50}) and the recommended concentrations for fish anaesthesia, it is clear that the concentration of cinnamon oil is low compared with the concentrations of euginol in other fishes. Even though the margin of safety is narrow for cinnamon oil, it is better to use the lower concentration (0.57mg/L) for 48 h transportation (personal observation). According to these findings, juveniles of *Etroplus suratensis* showed the highest sensitivity to cinnamon oil like clove oil at higher concentrations (0.60 and 0.67 mg/L), and comparable tolerance to acute toxicity of cinnamon oil. The appropriate cinnamon oil

concentration to induce surgical anaesthesia for 48 h is 0.57 mg/L, while for biometry procedures the best concentration of the anaesthetic is between 0.57 and 0.67 mg/L. As for transportation procedures, this concentration should be avoided for juveniles of *Etroplus suratensis*, as this induce a greater osmoregulatory disturbance and mortality rate. The present study showed a higher sensitivity of juveniles of *Etroplus suratensis* to higher concentration of cinnamon oil (0.60 and 0.67 mg/L). The present result indicate that reduced the risk of transporting juveniles of *Etroplus suratensis* with lower concentration of cinnamon oil (>0.57 mg/L) is high when they are exposed to anaesthetic concentrations of 0.3O and 0.33 mg/L cinnamon oil for 24 h. Since there are no scientific reports available in the existing literature on LC_{50} (96 h) and toxic effect of cinnamon oil on fishes. Therefore, the present study was undertaken to investigate the LC_{50} (96 h) of cinnamon oil *in-vivo* test models in fingerlings of *Etroplus suratensis.*

In the case of cassumunar ginger extract (*Zingiber cassumunar* Roxb) is an efficient anaesthetic for routine fish farming and handling procedures for juveniles of *Etroplus suratensis* that require anaesthesia for up to 24 h at the concentration of <1.33 mg/L, up to 48 h at the concentration of 1.32 mg/L, up to 72 h at the concentration of 1.53 mg/L and up to 96 h at the concentration of 1.38 mg/L. According to these findings, juveniles of *Etroplus suratensis* showed the highest sensitivity to cassumunar ginger extract (*Zingiber cassumunar* Roxb*)* at higher concentrations (0.60 and 3 mg/L), and comparable tolerance to acute toxicity of cassumunar ginger extract (*Zingiber cassumunar* Roxb). The appropriate cassumunar ginger extract (*Zingiber cassumunar* Roxb) concentration to induce surgical anaesthesia for 48 h is 1.32 $mg.L^{-1}$, while for biometry procedures the best concentration of the anaesthetic

is between 1.32 and 2.30 mg/L. As for transportation procedures, this concentration should be avoided for juveniles of *Etroplus suratensis*, as this induce a greater osmorregulatory disturbance and mortality rate. The present result indicate that the risk to juveniles of *Etroplus suratensis* is high when they are exposed to anaesthetic concentrations of 0.6O and 3 mg/L cassumunar ginger extract (*Zingiber cassumunar* Roxb) for 96 h.

Similarly lower concentration of tobacco leaf extract (*Nicotiana tobacum*) is insufficient to act as an efficient anaesthetic for routine fish farming and handling procedures for juveniles of *Etroplus suratensis* that require anaesthesia for up to 24 h at the concentration less than 6.21 mg/L. According to these findings, juveniles of *Etroplus suratensis* showed the highest sensitivity to tobacco leaf extract (*Nicotiana tobacum*) at higher concentrations (7 and 8 mg/L), and showed no comparable tolerance to acute toxicity of tobacco leaf extract (*Nicotiana tobacum*). The appropriate tobacco leaf extract (*Nicotiana tobacum*) concentration to induce surgical anaesthesia for 48-h in between 6.21-8.18 $mg.L^{-1}$. As for transportation procedures, higher concentration should be avoided for juveniles of *Etroplus suratensis*, as this induce a greater osmoregulatory disturbance and mortality rate. The present study showed a higher sensitivity of juveniles of *Etroplus suratensis* to higher concentration of tobacco leaf extract (*Nicotiana tobacum*) (7 and 8 mg/L). An extended period of exposure could increase the toxicity of anaesthetics (Vrskova and Modra, 2012). The present result indicate that the risk to juveniles of *Etroplus suratensis* is high when they are exposed to anaesthetic concentrations of 7 and 8 mg/L tobacco leaf extract (*Nicotiana tobacum*) for 96 h. However, the active ingradient of tobacco leaves is nicotine, which constitute between 2-5% of the dry leaves (Hassaall, 1982).

Thus there is a need to study the nicotine effects at sub-lethal concentrations on some enzymatic activities of *Etroplus suratensis* in a static bioassay system after the 48 or 96 hexposure period.

MS-222 one of the most widely used efficient anaesthetic for routine live fish management procedures and also for transportation of juveniles of *Etroplus suratensis* that require anaesthesia for up to 48 h at the optimum concentration of 53 mg/L. According to these findings, juveniles of *Etroplus suratensis* showed the highest sensitivity to MS-222 at higher concentrations (75 and 100 mg/L), and comparable tolerance to acute toxicity of MS-222. The appropriate MS-222 concentration to induce surgical anaesthesia for 48 h is 53 mg/L. As for transportation procedures, the higher concentration should be avoided for juveniles of *Etroplus suratensis*, as this induces a greater osmoregulatory disturbance and mortality rate. The present study showed a lower sensitivity of juveniles of *Etroplus suratensis* to lower concentration of MS-222 (45 and 50mg/L). An extended period of exposure could increase the toxicity of anaesthetics (Vrskova and Modra, 2012). The present result indicate that the risk to juveniles of *Etroplus suratensis* is high when they are exposed to anaesthetic concentrations of 75 and 100 mg/L MS-222 for 96 hs.

In consideration of hypothermia has been used as a physical sedative for juveniles of *Etroplus suratensis* that require anaesthesia for up to 48 h at the optimum hypothermic concentration of 16-18°C/L. According to these findings, juveniles of *Etroplus suratensis* showed the highest sensitivity to hypothermia at higher concentrations (12 and 8°C/L), and comparable tolerance to acute toxicity of hypothermia. The appropriate hypothermic condition to induce surgical anaesthesia for 48 h is 12-13°C/L. When the

temperature was reduced further to11°C, the fish became stressed, exhibiting tachyventilation, darker body colour and partial loss of equilibrium. Although there was some degree of acclimation to this lower temperature, it would not be advisable to cool to this extent for transportation (Ross et al., 2007). As for transportation procedures, the higher concentration should be avoided for juveniles of *Etroplus suratensis*, as this induce a greater osmoregulatory disturbance and mortality rate. The present study showed a lower sensitivity of juveniles of *Etroplus suratensis* to lower concentration of 18 and 22°C/L. An extended period of exposure could increase the toxicity of anaesthetics (Vrskova and Modra, 2012). The present result indicate that the risk to juveniles of *Etroplus suratensis* is high when they are exposed to high hypothermic condition of 12 and 8°C for 24 h, 48 h and 96 h. The present work is a pioneer study focusing on the (96 h LC_{50}) toxicity of hypothermia in juveniles of *Etroplus suratensis*.

.....ꕤ.....

Chapter 2

Behavioural assays and safest level of selected anaesthetics during bath administration in different concentrations on juveniles of *Etroplus suratensis*

Contents

2.1 Introduction
2.2 Materials and Methods
2.3 Statistical analyses
2.4 Results
2.5 Discussion
2.6 Summary

2.1 Introduction

Behaviour of anaesthetized fish

The behaviour of animals after the administration of anaesthetics shares many similarities with anaesthetic use in humans (Pizza and Moal, 1998). Anaesthetics are widely used by fish biologists because of the negative effects that handling has on a fish physiology and behavior when they are not anaesthetized (Summerfelt and Smith, 1990; Anderson et al., 1997; Cooke et al., 2004). In mammals, the loss of nerve function starts as a loss of the senses of pain, temperature, touch, and the loss of equilibrium and perception, followed by loss of skeletal muscle tone (Rang et al., 2003). Although the

loss of nerve function is not as well documented in fish, predictable behavioral changes during anaesthesia induction are documented and used to gauge the level of anaesthesia being experienced by the fish (McFarland 1959; Summerfelt and Smith 1990; Ross and Ross 2008).

The behavior of anaesthetized fish is different, in some respects, from that of non anaesthetised fish (McFarland 1960). At first, the chemical causes irritation which seems to increase in intensity with concentration. Anaesthetics reduce activity in fish and general anaesthesia occurs which ends in a total loss of consciousness (Öğretmen and Gökçek, 2013). Shortly thereafter they lose equilibrium without entering a pronounced stage of sedation (Schoettger and Julin, 1966). Reflex activity is lost entirely and skeletal muscle tone is also reduced (McFarland, 1960). Overdose or overexposure during treatments reduces breathing and results in low oxygen saturation in blood and ultimately in respiration and circulation disorders (Tytler and Hawkins, 1981).

Bath-administered anaesthetics aid in fish transport by lowering metabolism through decreasing activity, Oxygen consumption and excretion of waste products. (McFarland 1959; Solomon and Hawkins 1981; Robertson et al., 1987; Forteath 1993; Ross and Ross 1999). An ideal bath anaesthetic enters through the gills into the respiratory system and blocks reflex actions in less than 15 min with a recovery tie of 5 min or less (Summerfelt and Smith 1990). During transport, anaesthetics should only lightly sedate fish, not anaesthetize them, to avoid interfering with osmoregulation or gas exchange (Forteath, 1993). Among the various factors affecting sedation level in fish are temperature, fish species, and size.

Anaesthetics used as sedatives dull sensory perception without complete loss of equilibrium, decrease oxygen consumption and excretion of metabolic products (Ross and Ross, 1999). The ideal level of sedation for fish transport is referred to as deep sedation and includes loss of reactivity to external stimuli, decrease in metabolic rate, but maintenance of equilibrium (McFarland, 1959). This level of anaesthesia is consistent with stage 2 anaesthesia as described by Summerfelt and Smith (1990) (Appendix-1). Anaesthesia progresses rapidly to total loss of equilibrium (stage 11) and then slows. At this level the fish are relatively motionless and may rest upright, inverted, or on their sides on the bottom of the container. They can be handled gently, but striking the container or squeezing the caudal peduncle or fin induces strong reflex movements (Schoettger and Julin, 1966). Fish can be maintained in total loss of equilibrium, (stage 2) for relatively long periods, depending on concentration, before the onset of loss of reflex and medullary collapse. Thus, loss of equilibrium is best suited for evaluating the efficacy of anaesthetics. If fish are too heavily sedated, lose equilibrium, and cease swimming, they may die from suffocation if they all settle to the bottom, or experience mechanical injury from hitting the tank walls (Schoettger and Julin,1966). Practical concentrations of chemicals to produce desirable anaesthesia in fish have been defined under field conditions by several workers (Meister and Ritzi, 1958; Thompson, 1959). Klontz (1964) outlined 14 methods used to anaesthetize fish. However, during induction, hypoventilation (Houston et al., 1971; McFarland and Klontz, 1969) and a decrease in oxygen consumption (Baudin, 1932a; Blahm, 1961; Dixon and Milton, 1978; Ross and Ross, 1984) are observed as a result of an aesthesia.

When fish returned to anaesthetic-free fresh water, cardiac output and heart rate typically increased (Cooke et al., 2004). Behavioral recovery followed similar patterns to physiological recovery. Coupled with the variation in depth of anaesthesia, we observed substantial differences in physiological disturbance and behavior during transportation. In addition, behavioral and physiological recovery rates varied with level of anaesthesia (Cooke et al., 2004). Although recovery times were variable across anaesthetic concentrations, there was a strong relationship between recovery time and anaesthetic concentration (Summerfelt and Smith, 1990). Recovery time varied significantly among different anaesthetic concentrations, which in general increases was observed with the dose. Furthermore, at low-level of an aesthesia, the recovery time is rapid, and the behavioral recovery also fast relative to anaesthetized at other levels or no anaesthetized controls (Ross and Ross, 1984). Early in recovery, hyperventilation and an increase in oxygen consumption due to oxygen debt and/or stress often occurs (Keys and Wells, 1930; Summerfelt and Smith, 1990). The rate of anaesthetic elimination during recovery also increases with artificial ventilation (Kiessling et al., 2009).

For the correct usage of an anaesthetic, it is important to establish its ideal dose, since inappropriate dosages can lead to undesired effects and also an eventual fish mortality (Shepherd and Bromage,1992).The ideal dosage is also important from an economic viewpoint, since anaesthetics are expensive and inadequate dosages might induce unnecessary economic losses (Roubach et al., 2001). Choosing an appropriate anaesthetic depends mainly on its effectiveness in immobilizing fish with good recovery rates (Gilderhus and Marking, 1987; Burka et al., 1997). One of the criteria that proper anaesthetic

in fish to meet is its safety at treatment concentrations (Marking and Meyer, 1985). The recommended treatment concentrations vary according to fish species, fish size, exposure time, bath quality and temperature (Doleželová et al., 2011). An ideal anaesthetic should possess several attributes such as non-toxic, inexpensive, simple to administer and result in rapid induction and calm recovery (Treves-Brown, 2000). The size and life cycle status of anaesthetized fish is also recognized as a factor influencing the concentration of anaesthetic needed to induce anaesthesia within an acceptable time (from a welfare point of view) (Rombough, 2007). It is often advisable to identify the lowest effective concentration of different anaesthetics in a specified species, as the responses to the same anaesthetic may vary considerably among different species (Pawar et al., 2011).

In this context, plant materials such as clove oil is being used in the fish laboratories, because it has been used for centuries as typical anaesthetic for humans (Woody et al., 2002). Previous work has characterized the dose response to clove oil for a number of salmonids including brown trout *Salmo trutta*, (Hoskonen and Pirhonen., 2004a, 2004b) sockeye salmon *Oncorhynchus nerka* (Woody et al., 2002), rainbow trout *O. mykiss* (Anderson et al., 1997; Keene et al., 1998; Taylor and Roberts 1999; Hoskonen and Pirohnen 2004a, 2004b), and Atlantic salmon *S. salar* (Chanseau et al., 2002). Most studies have assessed high clove oil concentrations that result in deep sedation, loss of equilibrium, and loss of reflex activity. While these high concentrations and levels of sedation may be ideal for some fish culture applications and for invasive procedures such as surgery associated with implanting radio or sonic transmitters (Prince and Powell, 2000), there are instances where moderate sedation is more desirable than deep sedation, such

as tag insertion during field sampling. Several studies have also compared the physiological effects of using clove oil versus more conventional anaesthetics. Clove oil generally compares favorably with other common anaesthetics such as tricaine methanesulfonate (MS 222) or Quinaldine for induction and recovery times (Anderson et al., 1997). Many aqua culturists and clinicians add the clove oil directly to water baths to achieve the desired effect. Some authors (e.g., Wagner et al., 2003; Larissa et al., 2011) have suggested that low concentrations of clove oil may facilitate fish transport, but at present there is only one preliminary study that actually examines low levels of clove oil. Cooke et al., (2000) evaluated the response of adult rainbow trout transported using four clove oil concentrations by activity radio telemetry. The authors stated that clove oil showed promise for this purpose, but most of the concentrations tested resulted in total or partial loss of equilibrium and thus hyper activity or hypo activity.

Cinnamon which is native to India and Sri Lanka (Ceylon) and now it is cultivated in many tropical countries and is considered as one of the most important medicinal plants. The scientific name for cinnamon is *Cinnamomum zeylanicum which* belongs to the family *Lauraceae*; its medicinal parts include the outer bark, inner bark, leaves, and essential oil. The active principles in those parts are the volatile oils (cinnamaldehyde, eugenol, cinnamic acid, and weitherhin), mucilage, diterpenes, and proanthocyanidins (Soliman and Badeaa, 2002). Most of the cinnamon extracts are safe and having little side effects. Their essential oil contains both antifungal and antibacterial activity that can be used as antibiotics and to prevent food spoilage due to bacterial contamination (Dragland et al., 2003). It is also possesses anti-diabetic property (Broadhurst et al., 2000). Cinnamon oil was

noted to cause an initial period of hyperactivity, which was followed by reduced activity, similar to that found with low concentrations of clove oil. Cinnamon bark (*Cinnamomum zeylanicum*) also contains eugenol, but its use as an anaesthetic has not been explored (Pawar et al., 2011). Eugenol (2-methoxy-4-2-propenyl phenol), a very effective anaesthetic for fish and considered by the US Food and Drug Administration as a generally recognized safe (GRAS) compound. For these reasons, the present study was conducted to evaluate the ability of cinnamon oil as anaesthetic for juveniles of *Etroplus suratensis* with different dose determination patterns.

No literature on use of *Cinnamomum zeylanicum* as an anaesthetic for fish in India are available and it would appear that experimental studies on this subject are rare.

Zingiber cassumunar Roxb is used in folk medicine for the treatment of conditions such as inflammation, Sprains, rheumatism, muscular pain, wounds and asthma, and as a mosquito repellant, a carminative, a mild laxative and an antidysenteric agent, cough and used as a cleansing solution for skin diseases (Oliveros, 1996). The main active chemical constituents of the rhizome oil are sabinene (27-34%), γ-terpinene (6-8%), α-terpinene (4-5%), terpinen-4-ol (30-35%), and (E)-1-(3,4- dimethoxyphenylbutadiene (DMPBD) (12-19%) (Pongprayoon et al., 1997). Cassumunar Ginger (*Zingiber cassumunar)* has local anaesthetic activity similar to iodocaine on nerve action potential of sciatic nerve (Ansary, 2009). For these reasons, the present study was conducted to evaluate the ability of *Zingiber cassumunar* Roxb as anaesthetic for juveniles of *Etroplus suratensis* with different dose determination patterns. At present there is no literature on use of *Zingiber*

cassumunar as an anaesthetic for any fish in India and it would appear that experimental studies on this subject are rare.

Tobacco is the common name of the plant *Nicotiana tobacum* and to a lesser extent *N. rustica*. Tobacco contains the following phytochemicals: Nicotine, Anabasine (an alkaloid similar to the nicotine but less active), Glucosides (tabacinine, tabacine), 2,3,6-Trimethyl-1,4-naphthoquinone, 2-Methylquinone, 2-Napthylamine, Propionic acid, Anatalline, Anthalin, Anethole, Acrolein, Anatabine, Cembrene, Choline, Nicotelline, Nicotianine and Pyrene and they are generally recognized as being narcotic. This property makes it useful as narcotics, mulluscicides, piscicides, an anaesthetic and pesticide (Aleem, 1983, Agbon et al., 2002). According to Agokei and Adebisi (2010) the alcoholic extract of tobacco leaf has a lower effective dose and a comparable recovery time with aqueous extract on *O. niloticus* and also has sequential progression through the various stages of anaesthesia with increasing dose and time as the patterns of typical fish anaesthetic. Jegede and Olanrewaju (2012) revealed that *Heterobranchus bidorsalis* fingerlings exposed to *N. tobaccum* exhibit marked behavioural changes like erratic swimming, hyperventilation, vertical swimming motions and settling at the bottom which demonstrated a sensitive indicator of physiological stress in fish. For these reasons, the present study was conducted to evaluate the ability of *Nicotiana tobacum* as anaesthetic for juveniles of *Etroplus suratensis* with different dose determination patterns. At present there is no literature on use of tobacco as an anaesthetic for fish in India and it would appear that experimental studies on this subject are rare.

Tricaine methanesulfonate (TMS), known also as MS-222, ethyl 3-aminobenzoate methanesulfonic acid, tricaine mesilate, Aqualife TMSTM (Syndel, Qualicum Beach, BC Canada) metacaine, methanesulfonate, FinquelTM (Argent Chemical laboratories, Redmond, WA, USA), or Tricaine-STM (Western Chemical, Inc., Ferndale, WA, USA), is classified as an ester-type synthetic local anaesthetic and is commonly used in the fisheries industry (Sato et al., 2000). Local TMS (Tricaine methanesulfonate) (MS222) injections are ineffective because the drug is eliminated too quickly to induce anaesthesia (Allen and Hunn 1986; Malmstrøm et al., 1993; Burka et al., 1997). Therefore, for the vast majority of procedures involving fish, TMS is administered by immersion in an anaesthetic bath and, when appropriate, followed by continuous irrigation of the gills with anaesthetic solution. Fish immersion or gill irrigation in TMS provides continual uptake of the anaesthetic during exposure. For this reason, induced fish should be monitored to prevent overdosing or deeper stages of outline methods and precautions for administration and in anaesthesia (Carter et al., 2011). Tricaine methanesulfonate is absorbed by the fish and its effects are cumulative over time. Although classified as a local anaesthetic, TMS acts systemically when absorbed through the gills and skin of fish (e.g., scale less fish with well-vascularised skin) in an anaesthetic bath (McFarland, 1959; Hunn and Allen, 1974; Ferreira et al., 1984). During deeper anaesthesia in TMS, the fish behavior progressively changes and its physiological effects are very potential for compromising fish health. Additional TMS may need to be added to sedate all fish (Brown, 1993), but too much TMS may over anaesthetize fish, leading to ventilatory arrest (Ross and Ross, 1999). Even though, MS-222 (Tricaine methanesulphonate) is the most frequently used and preferred anaesthetic for fish (Ross and Ross, 2008).

In another study, it was found that 40 and 20 mg L^{-1} of MS-222 and benzocaine, respectively, were optimal in *P. filamentosus* to impart light sedation, above which significant loss of equilibrium and mortality resulted (Pramod et al., 2010). Although benzocaine (ethyl amino benzoate) is an effective fish anaesthetic with the desirable characteristics of rapid induction and recovery times (Ross and Ross, 2008).

Temperature controls and limits all physiological and behavioural parameters of ectotherms (Fry, 1947). Rapid decreases in water temperature may result in a number of physiological, behavioural and fitness consequences for fishes termed 'cold shock' (Donaldson, 2008). Tertiary responses initiated by cold shock on individuals as a whole like changes in growth and development rates, disease resistance and behavioural modifications (Mazeaud et al., 1977; Wendelaar Bonga, 1997; Barton, 2002). Behavioural modifications include changes in microhabitat use, abundance and distribution, feeding, predation, migration and spawning behaviours. Rapid cooling affords several advantages as the ability to euthanize many animals simultaneously, minimization of handling of individual animals (which is necessary when using injectable agents or decapitation), minimal risk of operator error when preparing the euthanasia bath, and reduction in occupational health and safety risk to personnel associated with chemical and physical methods of euthanasia (Wilson, 2009). Wedemeyer (1997) found that by reducing the hauling water by 10°C, most warm water species will reduce oxygen consumption by 50%. In practice, the temperature of transport water lowered to 22°C for packing ornamental fish of tropical origin. At this temperature, fish are less active but swim normally. This packing temperature is also very close to that in the cargo hold (21-22°C) of most aircraft. Fish of temperate

origin such as koi and gold fish can tolerate a much lower temperature and are therefore packed at 15-18°C (Hersh, 1984; Lim et al., 2007). Cooling the water by 5–7°C is a widely used protocol in many salmonid transports (Wedemeyer, 1996). However, caution must be used in the cooling process to ensure that there is not too much of a gradient difference between the holding water temperatures and the hauling temperatures as an abrupt change in temperature itself could be a stressor (Harmon, 2009).

The objective of this study is to examine the behavioral assessment and safest level during exposure at different concentrations of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS222 and cold as a calming agent for fish transportation and handling using *Etroplus suratensis* (*Etroplus suratensis*) as a model. In particular, we were interested in identifying the concentration of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS222 and cold that resulted in deep sedation for fish, while permitting the maintenance of equilibrium. This level of sedation has been determined to be optimal for fish transport and general handling (McFarland, 1959; Berka, 1986). Our comprehensive approach examined the behavioral responses of fish to a gradient of concentrations. Behavioral assessments involved visual observations of period of induction and recovery, as well as video graphic observations. Observations of the physiological effects of anaesthesia and anaesthetics on whole fish not only show how the whole organism reacts, but they also give clues as to which organs and systems are affected. Taken together, this study represents one of the first assessments of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS222 and cold for transporting fish, and is one of the first behavioral assays of fish responses to hauling.

2.2 Materials and Methods

2.2.1 Fish and experimental conditions

Collection, maintenance, acclimatization and experimental designs and preparation of samples were the same and explained in details in chapter 1 section 1.1, 1.2, 1.3, 1.3.1, 1.4, 1.4.1, 1.4.2, 1.4. 3 and 1.5.

2.2.2 Experiments

2.2.2.1 Effect of ethanol as an anaesthetic.

We used ethanol as a solvent in the preparations of clove oil, cinnamon oil, and cassumunar ginger in the present experiments and conducted a short experiment to determine whether there were any anaesthetic effects of ethanol on the juveniles of *Etroplus suratensis.* Although the present experiment was conducted in a water temperature of 28°C, when we used the above plant materials without any solvent, the oil required vigorous shaking and produced an oily layer on the water surface. When we used ethanol as a solvent, we received a completely dissolved mixture without an oily layer that was very easy to use for experimental purposes.

Juvenile fishes of *Etroplus suratensis* were exposed for 15 min to various concentrations of ethanol: 5, 10,15,20,25 ml/L (table 2). The desired concentrations were obtained by adding ethanol to test tanks containing water. Ten fishes of same size were individually placed into the glass tanks (22 x 22 x15 cm) at each concentration. Behavioral changes and induction and recovery times were noted. The purpose of this experiment was to make sure that, in subsequent experiments, the clove oil, cinnamon oil; cassumunar ginger would be the ingredients acting as an anaesthetic on the fishes.

2.2.2.2 Investigation of the safest level and behavioral assays during the exposure of different concentration of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS222 and hypothermia

Juvenile fishes of *Etroplus suratensis* size classes; (2.078 ± 0.15 - 5.373 ± 0.51gm) and (4.0 ± 0.1- 6 ± 0.1 cm) were exposed to aquaria containing 3L of water and different concentrations of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS222 and cold (table-2) at the water temperature of 28 °C. Fish were fasted for 24 h before the experiments and were transferred to an acclimation glass tanks two hours prior to the experimental performance. During the experiments, single fish was quietly scooped and transferred from the acclimation tank and immersed to the treatment tank (22 x 22 x15 cm) containing lowest anaesthetic concentrations of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS222 and cold with 3L of freshwater. To evaluate the time required for anaesthesia induction, 10 juveniles of *Etroplus suratensis* at a time were exposed for each concentration tested, and each juvenile was used only once (Schoettger and Julin, 1967). The assays were performed from lowest to the uppermost concentration to ensure no residual effects from the glass adsorption. Fresh solutions were prepared for each concentration and tanks were thoroughly cleaned and filled with aerated fresh water between trials. The maximum observation time was 10 min. The air supply to the anaesthetic bath was disconnected immediately before introduction of the fish so that behavior could be clearly observed during the induction period. Aeration was provided in the course of experiments, and physical and chemical water conditions were same in the acclimation tank. All experiments were performed in five times. The time lapsed for introduction

of the different anaesthetic stages followed (Appendix-1) Table 1. Total induction times and behavioural changes were recorded with a video recording system when the fishes reached stage 6 (Appendix-1) (Table 1). After induction, juveniles were transferred to anaesthetic-free aquaria to measure anaesthesia recovery time. Once stage 6 was deemed to have been reached, the test fish was immediately taken out, by hand, dried, weighed, and then placed in a recovery tank. Recovery was held transferring fish to 5L glass tanks (22 x 22 x15 cm) provided with anaesthetic free fresh water (27±1°C) and fish assumed as recovered (stage III of recovery). When the equilibrium was re-established and they started to swim horizontally (Iwama and Ackerman, 1994) and this was considered the start of the recovery period. Total recovery was reached when the fish regained the upright position and started moving in the container. Times to regain the upright position and behavioural changes were also measured and recorded. Each fish was used only once and then transferred to the revival aquaria containing only aerated freshwater and there they were monitored for one week to observe post-treatment survival. Each trial was repeated five times with different individuals for each concentration.

The induction time for fish to reach anaesthetic stage II, recovery times and behavioral changes were recorded and measured in seconds with a stopwatch and the behavior of the fish was observed and analyzed according to the phases described in (Appendix-1) Table 1. Induction and recovery times are expressed as means (min ± SEM). In this study, the focus was on the time needed for the fish to reach phase 3 and 4 of anaesthesia and respectively the period needed for recovery. Water temperature was controlled to approximately 28°C up to the completion of all experiments. To

eliminate or systematize sampling errors, it was set to carry out and record all the induction times and recovery times very accurately.

Behavioral assessments involved visual observations of anaesthesia induction and recovery time, as well as video graphic observations during treatments. Durations for each stage of anaesthesia were recorded as the interval from initial exposure to the anaesthetic until the end of each stage of anaesthesia. Durations for total recovery also were recorded, beginning with reintroduction of the fish to anaesthesia-free fresh water. The stages of anaesthesia and recovery were monitored as outlined by Stoskopf (1993) (Appendix 2).

2.2.2.3 Video Recording System

To monitor and examine the behavioral responses of fish during treatments, a high resolution 0 Lux underwater video camera with infrared illumination (OLYMPUS-14 MP) was positioned inside each tank (Cooke and Bunt, 2004), and a video cassette recorder (SRT 7072, Sanyo, Tokyo) was used to record fish behavior for subsequent analyses. Online records were acquired in movies of 1 min length (600 frames), saved on the computer and later analyzed for speed activity (mm/sec) using the software Image Pro-Plus®. During the experiments, the pre-treatment behavior of individual fish was recorded for 10 min before anaesthesia. During treatment, each fish was videotaped for at least 10 min, of which a 60-s period was used for transcription. A series of response variables were transcribed during playback on a monitor at between normal and 1/10th speed after collection of data. After a recovery period of 5 min, post-treatment behavior was recording every 10 min (10 movies of 600 frames each) at intervals of 30 min after recovery (three sections of records were done).

2.2.3 Post treatment survival

Etroplus suratensis reared in post-treatment tanks recovered well after the anaesthetic experiment. No mortality was observed during post-treatment period.

2.3 Statistical analyses

Differences in behavioural induction and recovery were plotted versus the concentrations of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS222 and cold for all treatments. Non-linear regression and analysis of variance (ANOVA) are used to establish the relationship between dosage and induction time, as well as dosage and recovery time. Mean induction (time from stage I_1 to I_3) and total recovery times (time from stage R_1 to R_3) were compared among treatment groups. Since the induction time and recovery time have opposite effects on the concentration of the anaesthetic, we use the concept of desirability functions to find the effective concentration (Derringer and Suich, 1980). All statistical analyses were performed using IBM SPSS STATISTICS 20.0 (Statistical Data Analysis and Scientific Research centre, UGC, Statistics Department; Mahatma Gandhi University, Kottayam, Kerala) and the level of significance (α) for all tests was 0.05. Non-linear regression analyses are used to establish the relationship between dosage and induction time, as well as dosage and recovery time.

2.4 Results

2.4.1 Effect of Ethanol as an anaesthetic

Juvenile fishes of *Etroplus suratensis* were exposed to an ethanol concentration of 5, 10, 15, 20, 25 ml/L for 15 minutes and exhibited regular behavior. Therefore, it was concluded that ethanol used as a solvent for clove oil, cinnamon oil and cassumunar ginger extract, has no sedative effect on fishes.

Investigation of the safest level and behavioral assays during the exposure of different concentration of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS222 and hypothermia

In the present work, the behavioural response to all anaesthetics changed with dose. The mass or total length of fish did not vary across the gradient of concentrations (Regressions, PN0.05) or among the six categorical concentrations (ANOVA, PN0.05) for fish used for behavioural analyses.

A gradual decrease of reaction to external stimuli and an increase of pigmentation and opercular rate were found in the middle and high dose. The highest dose of clove oil (0.37 mg/L), cinnamon oil (0.73 mg/1), cassumunar ginger extract (3 mg/ 1), tobacco leaf extract (8 mg/ 1), MS222 (100 mg/ 1), and cold (8±1°C) could be described as deep sedation or a complete loss of equilibrium was observed (Stoskopf, 1993) (Appendix 2). At higher doses quick recovery occurred as in the unanaesthetized group behaviour.

2.4.2 Effect of Clove oil as an anaesthetic

The various anaesthetic concentration used in behavioral assays vary across the gradient of concentrations (Non linear regression Analyses, PN 0.05) or among the six categorical concentrations (ANOVA, PN 0.05). Significant differences (P<0.05) in the induction and recovery stages at different concentration levels of clove oil were identified. Induction times generally decreased significantly with increasing doses of clove oil concentration evaluated. The maximum depth of anaesthesia increased significantly as clove oil concentration increased (Regression, (R^2=0.9963); Fig.2.1). Most fish achieved either stage 2 or stage 3 anaesthesia, which is indicative of

partial loss of equilibrium. Stage 2 anaesthesia is regarded as an ideal value for fish transport and general handling. Control fish exhibited no indication of anaesthesia (Stage 0). When examined on a categorical basis, there was a consistent increase in stage of anaesthesia for each increasing clove oil concentration category (ANOVA, $P<0.05$; Non- linear regression analysis; Table 2.1). Non-linear regression analyses are used to establish the relationship between dosage and induction time, as well as dosage and recovery time (Fig.2.1). The time required to reach the maximal and stable stage of anaesthesia also varied significantly in a nonlinear manner and was best described by a 3rd order polynomial equation. Induction times decreased significantly with increasing concentrations for clove oil. A significant negative correlation is observed between anaesthetic concentration and induction time for Clove oil (r=-0.929, $P<0.05$), whereas scatter plot yields a cubic relationship. The regression equation for induction time and concentrations (c) of the anaesthetic agent clove oil is,

$$I_3 = -42670\, c^3 + 34366\, c^2 - 9492.6\, c + 995.56 \quad (R^2=0.9963)$$

Five minutes after the introduction of the clove oil, fish shifted in to the clove oil free fresh water, the behavioral recovery times varied extensively by concentration. On the other hand, recovery times increased with increasing concentrations of Clove oil ($P<0.05$) (Table 2.1). A significant positive correlation is observed between anaesthetic concentration and recovery time for Clove oil (r=0.936, $P<0.05$), whereas scatter plot yields a cubic relationship. The regression equations for recovery time and concentrations for clove oil is $R_3 = -21094\, c^3 + 13349\, c^2 - 1905.1\, c + 113.28$ ($R^2=0.9454$) (fig.2.1).

When examined on a categorical basis, the lowest concentration categories achieved significantly lower stages of anaesthesia than the highest categories (ANOVA, P<0.05; Non- linear regression analysis; Table 2.1). Similarly, basal behavioural variables (i.e., induction time, recovery time and maximum stage of anaesthesia) vary across the gradient of concentrations (Regression, PN0.05) or among the five categorical concentrations (ANOVA, (P<0.05) (Table 2.1). Overall mean basal behavioural variables during experiments conducted at 28 ± 3°C were I_3 347.3 ± 4.6, R_3 37.9 ± 6.6 for 0.10mg/L, I_3 170.6 ± 3.1, R_3 58.1 ± 2.7 for 0.17mg/L, I_3 109.6 ±1.7, R_3 143.7 ± 1.8 for 0.23mg/L, I_3 88.7 ± 1.3, R_3 162.9 ± 2.4 for 0.30, I_3 31.9 ± 1.1, R_3 172.3 ± 0.7 for 0.37mg/L. Although it was indicating that basal behavioural variables varied with treatment, there was sufficient individual variation when we transformed individual values in minutes for the seconds. Behavioural responses during movement were quite changeable across a gradient of clove oil concentrations. When initially exposed to clove oil, fish experienced a brief erratic movement (seconds) through increases in gill movement. Both behavioural induction and behavioural recovery exhibited an inverse pattern relative to clove oil concentration. (Regressions, r=0.936, P<0.05; Fig. 2.1) and differed among the clove oil concentrations (ANOVA's, (P<0.05) (Table 2.1). The lowest category that incorporated concentration of 0.17mg/L consistently had within the minimum behavioural induction and recovery time (I_3 170.6 ± 3.1, R_3 58.1 ± 2.7) than the other concentrations (ANOVA, P<0.05; Non- linear regression analysis; Table 2.1).

Recovery time varied significantly among clove oil concentration categories, increasing with the higher categories (Fig. 2.1). The only departure from this pattern was the 0.10 mg/L category where recovery times

were significantly faster (37.9 ± 6.6: ~1min) than all other categories (143.7±1.8 to 172.3 ± 0.7: ~2 min 39 sec to 2 min 87 sec) including the category range (0.10, 0.17, 0.23, 0.30 and 0.37 mg/L, ANOVA, P<0.05; Non-linear regression analysis; Table 2.1).

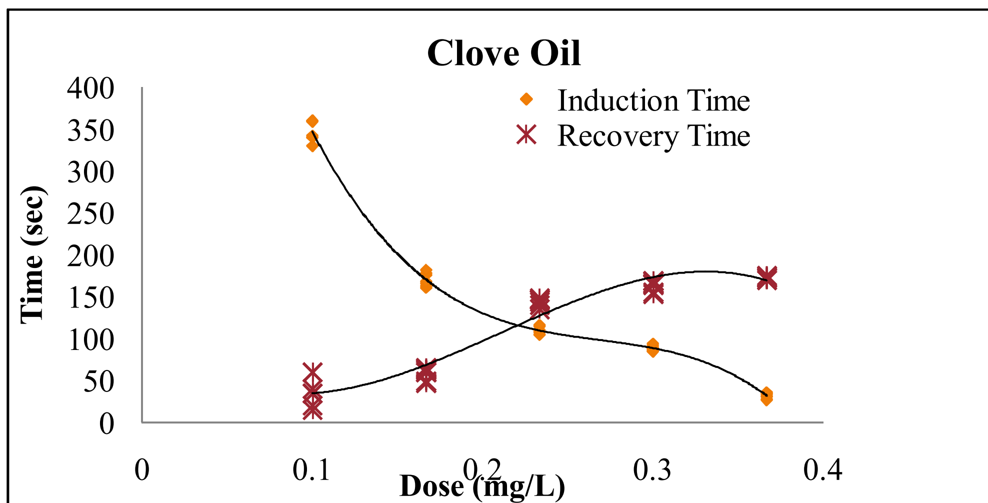


Fig.2.1 Non-linear regression analyses showing the effects of gradients of clove oil concentrations on the induction behavior and recovery of *Etroplus suratensis.*

Table 2.1 Summary statistics of induction and recovery times at different doses of clove oil for *Etroplus suratensis* (Mean ±SEM)

	Clove oil concentrations (mg/L)				
Stages	**0.10**	**0.17**	**0.23**	**0.30**	**0.37**
I_1	177.9 ±2.7	106.4 ± 5.1	60.0 ± 3.7	43.7 ±1.3	13.1 ± 0.5
I_2	271.3 ± 8.6	129.3 ± 5.9	87.7± 1.9	69.3 ±0.9	22.7 ± 2.9
I_3	347.3 ± 4.6	170.6 ± 3.1	109.6 ±1.7	88.7 ± 1.3	31.9 ± 1.1
R_1	16.1 ± 2.5	41.1 ± 0.4	114.0 ± 2.1	112.4 ± 1.3	121.7 ± 1.1
R_2	23.0 ± 3.4	48.7 ± 1.4	123.4 ± 3.7	142.7 ± 1.2	146.3 ± 0.6
R_3	37.9 ± 6.6	58.1 ± 2.7	143.7 ± 1.8	162.9 ± 2.4	172.3 ± 0.7

- Values are expressed in seconds
- Average of six values in each group

2.4.3 Effect of Cinnamon oil *(Cinnamom zeylanicum)* as an anaesthetic

Summary statistics of induction and recovery times at different doses of cinnamon oil for *Etroplus suratensis* is given in the Table1.2 that gives the values Mean ± SEM. Induction times decreased significantly with increasing concentrations for cinnamon oil. The maximum depth of anaesthesia increased significantly as cinnamon oil concentration increased. Non-linear regression analyses are used to establish the relationship between dosage and induction time, as well as dosage and recovery time (Fig.2. 2).

After the introduction of the fish in to the cinnamon oil free fresh water, the behavioral recovery times varied extensively by concentrations. A significant negative correlation is observed between anaesthetic concentration and induction time for cinnamon oil (r=-0.913, P<0.05), whereas scatter plot yielded a cubic relationship. The regression equation for induction time and concentrations (c) of the anaesthetic agent cinnamon oil is, I_3= $-6023.1c^3 + 10344c^2 - 5918.8c + 1280.6$($R^2$=0.9857).

On the other hand, recovery times increased with increasing concentrations of cinnamon oil (P<0.05) (Table 1.2). A significant positive correlation is observed between anaesthetic concentration and recovery time for cinnamon oil (r=0.952, P<0.05), whereas scatter plot yielded a quadratic relationship. The regression equations for recovery time and concentrations for cinnamon oil is,

$$R_3 = 501.44c^2 - 315.92c + 125.85 \qquad (R^2=0.9828).$$

When examined on a categorical basis, the lowest concentration categories achieved significantly lower stages of anaesthesia than the highest

categories (ANOVA, P<0.05; Non- linear regression analysis; Table 2.2). Similarly, basal behavioural variables (i.e., induction time, recovery time and maximum stage of anaesthesia) vary across the gradient of concentrations (Regression, PN0.05) or among the five categorical concentrations (ANOVA, (*P*<0.05) (Table 2.2). Overall mean basal behavioural variables during experiments conducted at 28±3°C were I_3 243 ± 1.9, R_3 76.7 ± 1.7 for 0.33mg/L, I_3 154.9± 2, R_3 91.9 ± 2.1 for 0.50 mg/L , I_3 151.7±1.5, R_3 107.4 ± 0.8 for 0.57 mg/L , I_3 148.1 ± 1.5, R_3 141.3 ±1.5 for 0.67, $I_3$127.7± 1.9, R_3 162.0 ± 1.2 for 0.73 mg/L. Although there was indicating that basal behavioural variables varied with treatment, there was sufficient individual variation that we transformed individual values in minutes for the seconds. Behavioural responses during movement were quite changeable across a gradient of cinnamon oil concentrations. When initially exposed to cinnamon oil, fish experienced a brief erratic movement (seconds) through increases in gill movement. Both behavioural induction and behavioural recovery exhibited an inverse pattern relative to cinnamon oil concentration. (Regressions, r=0.936, *P*<0.05; Fig. 2.2) and differed among the cinnamon oil concentrations (ANOVA's, (*P*<0.05) (Table 2.2). The second lowest category that incorporated concentrations of 0.50 mg/L - 0.57 mg/L consistently had within the minimum behavioural induction and recovery time (I_3 154.9± 2, R_3 91.9 ± 2.1, I_3 151.7±1.5, R_3 107.4 ± 0.8) than the other concentrations (ANOVA, P<0.05; Non- linear regression analysis; Table 2.2).

Recovery time varied significantly among cinnamon oil concentration categories, increasing with the higher categories (Fig. 2.2). The only departure from this pattern was the 0.33 to 0.50 mg/L category where recovery times were significantly faster (76.7±1.7 to 91.9 ± 2.1; ~1min 27 sec

to 1min 53 sec) than all other categories of 0.67 mg/L to 0.73 mg/L (range of 141.3 ±1.5 to162.0 ± 1.2; ~2 min 35sec to 2 min 7 sec) including the category range (0.33,0.50,0.57,0.67 and 0.73 mg/L, ANOVA,P<0.05;Non- linear regression analysis; Table 2.2).

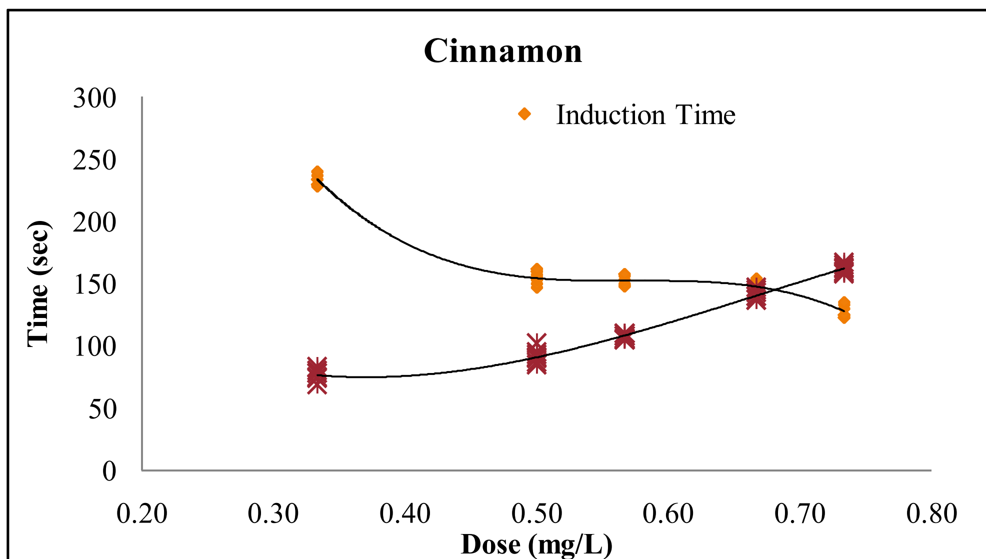


Fig.2.2 Non-linear regression analyses showing the effects of gradients of cinnamon oil concentrations on the induction behavior and recovery of *Etroplus suratensis*

Table 2.2 Summary statistics of induction and recovery times at different doses of cinnamon oil for *Etroplus suratensis* (Mean ±SEM)

	Cinnamon oil concentrations (mg/L)				
Stages	**0.33**	**0.50**	**0.57**	**0.67**	**0.73**
I_1	42.1±1.0	38.3± 1.0	35.9 ±1.9	35 ±1.3	34.4± 1.6
I_2	98.1±1.0	89.7± 1.0	81.3± 1.3	75.9 ±1.5	72.4± 0.8
I_3	243 ± 1.9	154.9± 2	151.7±1.5	148.1 ± 1.5	127.7± 1.9
R_1	62.3±1.2	69 ± 2.2	72.7± 1.0	74 ± 1.2	80.1± 0.6
R_2	70 ± 1.4	81.9 ± 1.0	92.4± 1.4	97.3± 1.5	103.9 ± 1.2
R_3	76.7 ± 1.7	91.9 ± 2.1	107.4 ± 0.8	141.3 ±1.5	162.0 ± 1.2

- Values are expressed in seconds
- Average of six values in each group

2.4.4 Effect of *Zingiber casumunar* (Cassumunar Ginger) as an anaesthetic

In the case of cassumunar ginger extract, induction times decreased significantly with increasing concentration. Summary statistics of induction and recovery times at different doses of cassumunar ginger extract for *Etroplus suratensis* is given in the Table 1.3, that gives the values Mean ± SEM. Non-linear regression analyses are used to establish the relationship between dosage and induction time, as well as dosage and recovery time (Figure 2.3).

A significant negative correlation is observed between anaesthetic concentration and induction time for cassumunar ginger extract (r=-0.940, P<0.05), whereas scatter plot yielded a cubic relationship. The regression equation for induction time and concentrations (c) of the anaesthetic agent cassumunar ginger extract is,

$$I_3 = -352.77c^3 + 1198.3c^2 - 1353.5c + 687.54 \quad (R^2=0.9860)$$

When examined on a categorical basis, the lowest concentration categories achieved significantly lower stages of anaesthesia than the highest categories (ANOVA, P<0.05; Non- linear regression analysis; Table 2.3).

On the other hand, recovery times increased with increasing concentrations of cassumunar ginger extract (P<0.05). A significant positive correlation is observed between anaesthetic concentration and recovery time for cassumunar ginger extract (r=0.991, P<0.05), whereas scatter plot yielded a linear relationship. The regression equations for recovery time and concentrations for cassumunar ginger extract is,

$$R_3 = 65.118c + 100.44 \quad (R^2=0.9826).$$

When examined on a categorical basis, the lowest concentration categories achieved significantly lower stages of anaesthesia than the highest categories (ANOVA, P<0.05; Non- linear regression analysis; Table 2.3). Similarly, basal behavioural variables (i.e., induction time, recovery time and maximum stage of anaesthesia) vary across the gradient of concentrations (Regression, PN0.05) or among the five categorical concentrations (ANOVA, (*P*<0.05) (Table 2.3). Overall mean basal behavioural variables during experiments conducted at 28±3°C were I_3 266.9 ± 0.9, R_3 131.6 ± 1.6 for 0.50 mg/L, I_3 205.0 ± 2.1, R_3 147.6 ± 1.3 for 0.70 mg/L, I_3 181.7 ±1.3, R_3 187.1 ± 0.8 for 1.30 mg/L, I_3 156.1 ± 1.3, R_3 197.3 ± 0.8 for 1.50 mg/L, I_3 148.1 ± 0.6, R_3 206.9 ± 0.9 for 1.60 mg/L. Although it was indicating that basal behavioural variables varied with treatment, there was sufficient individual variation that we transformed individual values in minutes for the seconds. Behavioural responses during movement were quite changeable across a gradient of cassumunar ginger concentrations. When initially exposed to cassumunar ginger, fish experienced a brief erratic movement (seconds) through increases in gill movement. Both behavioural induction and behavioural recovery exhibited an inverse pattern relative to cassumunar ginger concentration. (Regressions, r=0.936, P<0.05; Fig.2.3) and differed among the cassumunar ginger concentrations (ANOVA's, (*P*<0.05) (Table 2.3). The second lowest category that incorporated concentration of 1.30 mg/L consistently had within the minimum behavioural induction and recovery time (I_3 181.7 ±1.3, R_3 187.1 ± 0.8; I_3 ~3 min 02sec, R_3~3 min 11 sec) than the other concentrations (range (ANOVA, P<0.05; Non-linear regression analysis; Table 2.3).

Recovery time varied significantly among cassumunar ginger concentration categories, increasing with the higher categories (Fig. 2.3). The only

departure from this pattern was the 0.50 to 0.70 mg/L category where recovery times were significantly faster (131.6 ±1.6 to147.6 ±1.3; ~2 min 19 sec to 2 min 46 sec) than all other categories (range of ~3 min 11 sec to 3 min 44 sec) including the category range (0.50, 0.70, 1.30, 1.50 and 1.60 mg/L, ANOVA, P<0.05; Non- linear regression analysis; Table 2.3).

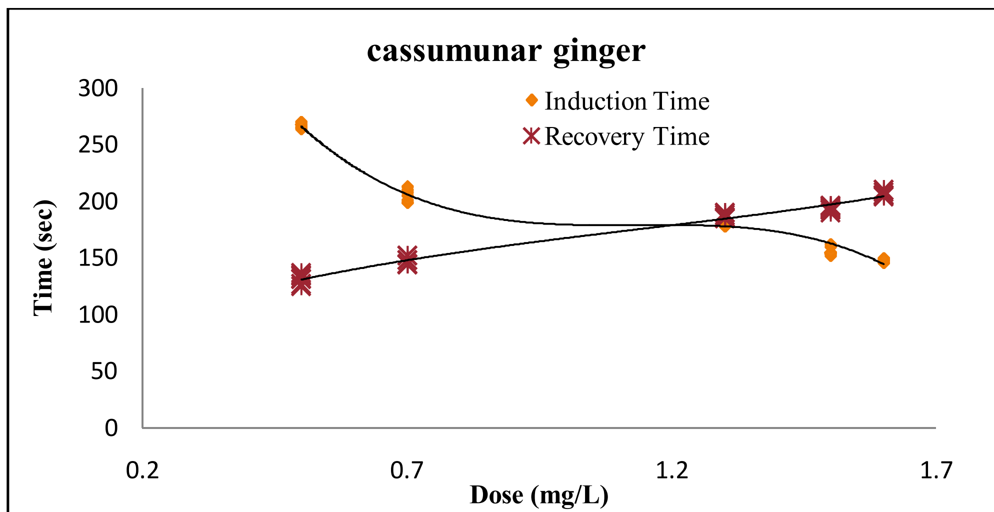


Fig. 2.3 Non-linear regression analyses showing the effects of gradients of cassumunar ginger extract (Zn) concentrations on the induction behavior and recovery of *Etroplus suratensis*

Table 2.3 Summary statistics of induction and recovery times at different doses of cassumunar ginger extract for *Etroplus suratensis* (Mean ±SEM)

	Cassumunar ginger extract concentrations (mg/L)				
Stages	**0.50**	**0.70**	**1.30**	**1.50**	**1.60**
I1	88.1±15	55.4± 1.6	37.4 ±0.5	34.9 ±1.1	28.3± 1.1
I2	153.3±1.5	143.9± 1.2	127.4± 0.8	106.1 ±0.9	96.0± 0.7
I3	266.9±0.9	205.0± 2.1	181.7±1.3	156.1 ± 1.3	148.1± 0.6
R1	83.1±0.7	112.9± 1.0	124.4± 2.0	136.7 ± 1.1	151.0± 1.5
R2	123.3 ± 1.0	136.9 ± 0.8	144.9±0.8	175.1± 1.4	180.9 ± 1.2
R3	131.6 ± 1.6	147.6± 1.3	187.1 ± 0.8	193.7 ±0.8	206.9 ± 0.9

- Values are expressed in seconds
- Average of six values in each group

2.4.5 Effect of Tobacco leaf extracts (*Nicotiana tobaccum*) as an anaesthetic

Summary statistics of induction and recovery times at different doses of Tobacco leaf extract for *Etroplus suratensis* is given in the Table 2.4, which gives the values Mean ± SEM. Induction times decreased significantly with increasing concentrations for tobacco leaf extract.

Non-linear regression analyses are used to establish the relationship between dosage and induction time, as well as dosage and recovery time (Figure 2.4). A significant negative correlation is observed between anaesthetic concentration and induction time for tobacco leaf extract (r=-0.934, P<0.05), whereas scatter plot yields a quadratic relationship. The regression equation for induction time and concentrations (c) of the anaesthetic agent tobacco leaf extract is,

$$I_3=12.351\, c^2-182.46\, c + 807.29 \qquad (R^2=0.9921).$$

When examined on a categorical basis, the lowest concentration categories achieved significantly lower stages of anaesthesia than the highest categories (ANOVA, P<0.05; Non- linear regression analysis; Table 2.4).

A significant positive correlation is observed between anaesthetic concentration and recovery time for tobacco leaf extract (r=0.977, P<0.05), whereas scatter plot yielded a quadratic relationship. On the other hand, recovery times increased with increasing concentrations of tobacco leaf extract (P<0.05). The regression equations for recovery time and concentrations for tobacco leaf extract is,

$$R_3= -5.0086\, c^2+93.61\, c-158.25 \qquad (R^2=0.9953)$$

When examined on a categorical basis, the lowest concentration categories achieved significantly lower stages of anaesthesia than the highest categories (ANOVA, $P<0.05$; Non- linear regression analysis; Table 2.4). Similarly, basal behavioural variables (i.e., induction time, recovery time and maximum stage of anaesthesia) vary across the gradient of concentrations (Regression, PN 0.05) or among the six categorical concentrations (ANOVA, ($P<0.05$) (Table 2.4). Overall mean basal behavioural variables during experiments conducted at 28 ± 3°C were I_3 494.4 ± 1.4, R_3 9.7 ± 0.8 for 2 mg/L, I_3 187.0 ± 1.6, R_3 177.6 ± 0.8 for 5 mg/L, I_3 167.9 ± 1.1, R_3 232.4 ± 0.9 for 6 mg/L, I_3 148.4 ± 1.6, R_3 249.3 ± 2.1 for 7 mg/L, I_3 128.4 ± 1.6, R_3 269.3.9 ± 2.1 for 8 mg/L. Although it was indicating that basal behavioural variables varied with treatment, there was sufficient individual variation that we transformed individual values in minutes for the seconds. Behavioural responses during movement were quite changeable across a gradient of tobacco leaf extract concentrations. When initially exposed to tobacco leaf extract, fish experienced a brief erratic movement (seconds) through increases in gill movement. Both behavioural induction and behavioural recovery exhibited an inverse pattern relative to tobacco leaf extract concentration. (Regressions, $r = 0.936$, $P<0.05$; Fig. 2.4) and differed among the tobacco leaf extract concentrations (ANOVA's, ($P<0.05$) (Table 2.4). The lowest category that incorporated concentration of 6 mg/L consistently had within the minimum behavioural induction and recovery time (I_3 167.9 ±1.1, R_3 232.4 ± 0.9; I_3~ 3 min 19 sec, R_3~4 min 27 sec) than the other concentrations(range (ANOVA, $P<0.05$; Non- linear regression analysis; Table 2.4).

Recovery time varied significantly among tobacco leaf extract concentration categories, increasing with the higher categories (Fig. 2.4). The

only departure from this pattern was the 2 to 5 mg/L category where recovery times were significantly faster (~0.16 sec to 3 min 36 sec) than all other categories (range of ~4 min 27 sec to 4 min 48 sec) including the category range (2,4,6 and 8 mg/L, ANOVA, P<0.05; Non- linear regression analysis; Table 2.4).

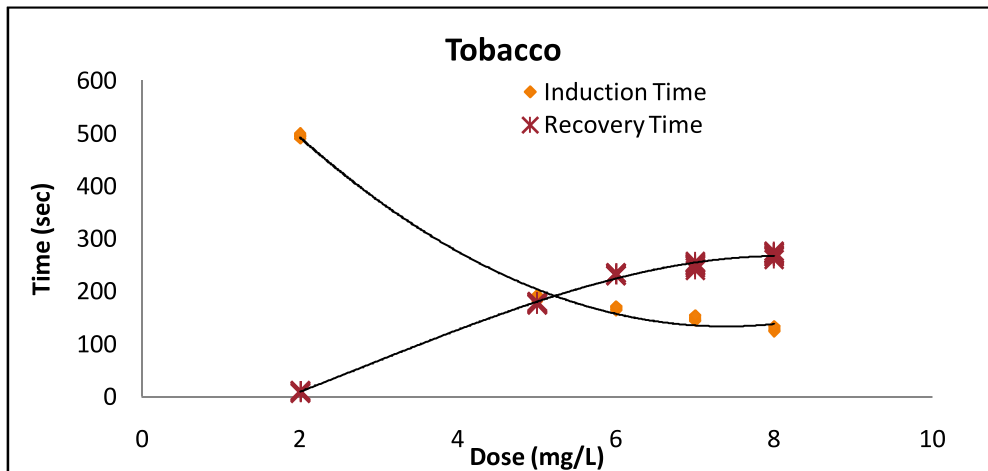


Fig.2.4 Non-linear regression analyses showing the effects of gradients of tobacco leaf extract concentrations on the induction behavior and recovery of *Etroplus suratensis*

Table 2.4 Summary statistics of induction and recovery times at different doses of tobacco leaf extract for *Etroplus suratensis* (Mean ±SEM)

	Tobacco leaf extract concentrations (mg/L)				
Stages	**2**	**5**	**6**	**7**	**8**
I1	317.3±0.8	120.3±1.5	114.1±1.8	113.4±1.0	106.1±1.8
I2	408.9±1.4	138.0±2.0	126.6±0.9	117.0±1.0	112.7±1.8
I3	494.4±1.4	187.0±1.6	167.9±1.1	148.4±1.6	128.4±1.6
R1		42.9±1.4	82.4±1.3	103.0±0.9	203.0±1.5
R2		92.0±0.6	112.3±0.8	212.3±0.8	221.7±1.4
R3	9.7±0.8	177.6±0.8	232.4±0.9	249.3±2.1	269.3±2.1

- Values are expressed in seconds
- Average of six values in each group

2.4.6 Effect of MS222 as an anaesthetic

Summary statistics of induction and recovery times at different doses of MS 222 for *Etroplus suratensis* is given in the Table 2.5, which gives the values Mean ± SEM. Induction times decreased significantly with increasing concentrations for MS 222. There was a clear linear pattern of decreasing induction time with increasing concentration of MS222, with the longest induction times for fish in the group exposed to 45mg l^{-1} (287.6±0.8 seconds) and the shortest for fish exposed to 100mg of MS222 l^{-1} (43.4 ±2.7seconds.). All fish exposed to 45 mg of tricaine methanesulfonate (MS222) reached the maximum value for induction of 287.6 seconds, indicating that none of the fish exposed to this concentration of tricaine methanesulfonate was induced.

Non-linear regression analyses are used to establish the relationship between dosage and induction time, as well as dosage and recovery time (Figure 2.5). A significant negative correlation is observed between anaesthetic concentration and induction time for MS 222 (r=-0.971, P<0.05), whereas scatter plot yields a cubic relationship. The regression equation for induction time and concentrations (c) of the anaesthetic agent MS 222 is,

$$I_3 = -0.0042\,c^3 + 0.7165\,c^2 - 42.457\,c + 1013 \qquad (R^2=0.9715)$$

A significant positive correlation is observed between anaesthetic concentration and recovery time for MS 222 (r=0.903, P<0.05), whereas scatter plot yielded a cubic relationship. On the other hand, recovery times increased with increasing concentrations of MS 222 (P<0.05). The regression equations for recovery time and concentrations for MS 222 is,

$$R_3 = -0.0037\,c^3 - 0.569\,c^2 + 28.85\,c - 236.98 \qquad (R^2=0.9958)$$

When examined on a categorical basis, the lowest concentration categories achieved significantly lower stages of anaesthesia than the highest categories (ANOVA, P<0.05; Non- linear regression analysis; Table 2.5). Similarly, basal behavioural variables (i.e., induction time, recovery time and maximum stage of anaesthesia) vary across the gradient of concentrations (Regression, PN0.05) or among the six categorical concentrations (ANOVA, (*P*<0.05) (Table 2.5). Overall mean basal behavioural variables during experiments conducted at 28 ± 3°C were I_3 287.6 ± 0.8, R_3 217.7 ± 1.4 for 45 mg/L, I_3 217.4 ± 0.9, R_3 229.4 ± 1.3 for 50 mg/L, I_3 172.3 ±0.8, R_3 249.1 ± 1.5 for 53 mg/L, I_3 127.7 ± 0.8, R_3 260.9 ± 1.4 for 75 mg/L, I_3 43.4 ± 2.7, R_3 371.4 ±1.6 for 100 mg/L. Although it was indicating that basal behavioural variables varied with treatment, there was sufficient individual variation that we transformed individual values in minutes for the seconds. Behavioural responses during movement were quite changeable across a gradient of MS222 concentrations. When initially exposed to tobacco leaf extract, fish experienced a brief erratic movement (seconds) through increases in gill movement. Both behavioural induction and behavioural recovery exhibited an inverse pattern relative to MS222 concentration. (Regressions, *r*=0.936, *P*<0.05; Fig. 2.5) and differed among the MS222 concentrations (ANOVA's, (*P*<0.05) (Table 2.5). The lowest category that incorporated concentration of 53 mg/L consistently had within the minimum behavioural induction and recovery time (I_3 172.3 ± 0.8, R_3 249.1 ± 1.5; I_3~3 min 27 sec, R_3~4min 15 sec) than the other concentrations range (ANOVA, P<0.05; Non- linear regression analysis; Table 2.5).

Recovery time varied significantly among MS222 concentration categories, increasing with the higher categories (Fig. 2.5). The only

departure from this pattern was the 45 to 53 mg/L category where recovery times were significantly faster (~4 min 02 sec to 4 min 22sec) than all other categories (range of ~4 min 34 sec to 6 min 19 sec) including the category range (45, 50, 53, 75 and 100 mg/L, ANOVA, P<0.05; Non- linear regression analysis; Table 2.5).

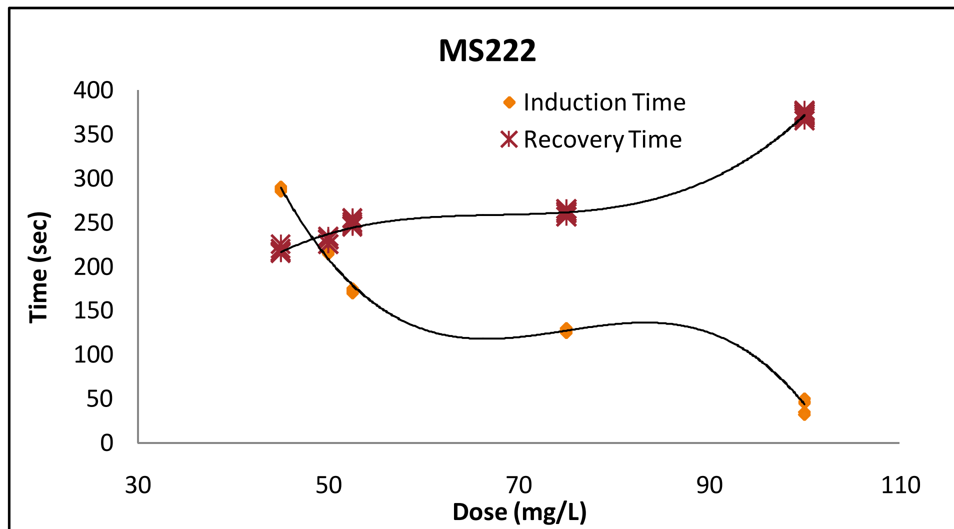


Fig. 2.5 Non-linear regression analyses showing the effects of gradients of MS222 concentrations on the induction behavior and recovery of *Etroplus suratensis*

Table 2.5 Summary statistics of induction and recovery times at different doses of MS222 for *Etroplus suratensis* (Mean ±SEM)

	MS 222 concentrations (mg/L)				
Stages	**45**	**50**	**53**	**75**	**100**
I1	156±1.3	84.9±0.9	72.0±1.1	43.0±2.8	-
I2	217.7±0.8	193.1±2.4	134.9±1.3	79.7±1.6	-
I3	287.6±0.8	217.4±0.9	172.3±0.8	127.7±0.8	43.4±2.7
R1	111.6±2.2	117.9±0.7	117.6±2.8	156.0±1.6	240.0±2.1
R2	149.4±1.6	162.4±3.1	181.9±1.5	197.4±0.8	309.0±1.7
R3	217.7±1.4	229.4±1.3	249.1±1.5	260.9±1.4	371.4±1.6

- Values are expressed in seconds
- Average of six values in each group

2.4.7 Effect of Hypothermic condition as an anaesthetic

Summary statistics of induction and recovery times at different doses of hypothermia for *Etroplus suratensis* is given in the Table 2.6 that gives the values Mean ± SEM. Induction times decreased significantly with increasing concentrations for *hypothermia*. On the other hand, recovery times increased with increasing concentrations of hypothermia (P<0.05). Non-linear regression analyses are used to establish the relationship between dosage and induction time, as well as dosage and recovery time (Figure 2.6). A significant positive correlation is observed between anaesthetic concentration and induction time for hypothermia (r=0.987, P<0.05), whereas scatter plot yielded a quadratic relationship. The regression equation for induction time and concentrations (c) of the anaesthetic agent hypothermia is,

$$I_3=1.1116c^2-8.5536c + 8.7143 \qquad (R^2=0.9937)$$

A significant negative correlation is observed between anaesthetic concentration and recovery time for hypothermia (r=-0.983, P<0.05), whereas scatter plot yields a quadratic relationship. The regression equations for recovery time and concentrations for hypothermia is,

$$R_3= -1.0446c^2+8.5c+231.57 \qquad (R^2=0.9863)$$

When examined on a categorical basis, the lowest concentration categories achieved significantly lower stages of anaesthesia than the highest categories (ANOVA, P<0.05; Non- linear regression analysis; Table 2.6). Similarly, basal behavioural variables (i.e., induction time, recovery time and maximum stage of anaesthesia) vary across the gradient of concentrations (Regression, PN 0.05) or among the six categorical concentrations (ANOVA, (P<0.05)

(Table 2.6). Overall mean basal behavioural variables during experiments conducted at different hypothermic conditions were I_1 66.14 ± 6.54, R_1 58.28 ± 1.80 for 18 ± 1°C , I_3 156.4 ± 3.2, R_3 100.1 ± 4.5 for 16 ± 1°C, I_3 66.1 ± 0.6, R_3 183.1 ± 0.7 for 12 ± 1°C and I_3 11.4 ± 1.0, R_3 232.7 ± 0.6 for 8 ± 1°C. Although it was indicating that basal behavioural variables varied with treatment, there was sufficient individual variation that we transformed individual values in minutes for the seconds. Behavioural responses during movement were quite changeable across a gradient of hypothermic concentrations. When initially exposed to hypothermia, fish experienced a consistent movement (seconds) through decrease in gill movement. Both behavioural induction and behavioural recovery exhibited an inverse pattern relative to hypothermic condition (Regressions, r=0.936, P<0.05; Fig. 2.6) and differed among the hypothermic concentrations (ANOVA's, (P<0.05) (Table 2.6). The lowest category that incorporated concentration of 16±1°C consistently had within the minimum behavioural induction and recovery time (I_3 156.4 sec ±0.2, R_3 100.1 ± 4.5sec; I_3 ~3min, R_3 2 min 6sec) than the other concentrations range (ANOVA, P<0.05; Non- linear regression analysis; Table 2.6).

Recovery time varied significantly among hypothermic concentration categories, increasing with the higher categories (Fig. 2.6). The only departure from this pattern was the18 ±1°C to 22 ±1°C category where recovery times were significantly faster (58.28±1.80sec to 0) including the category range (22±1°C,18±1°C,16±1°C,12±1°C and 8±1°C mg/L, ANOVA, P<0.05; Non- linear regression analysis; Table 2.6).

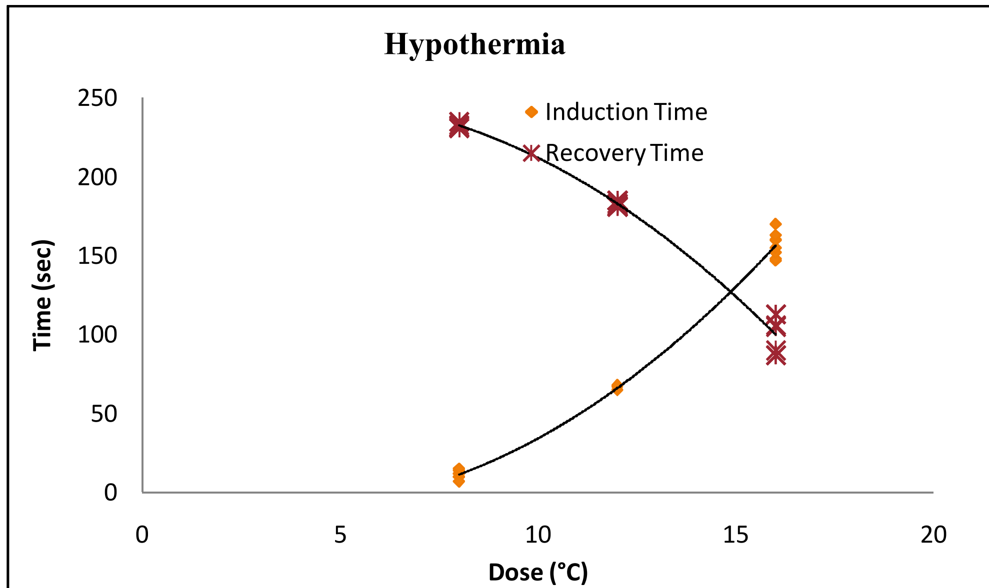


Fig.2.6 Non-linear regression analyses showing the effects of gradients of hypothermia concentrations on the induction behavior and recovery of *Etroplus suratensis*

Table 2.6 Summary statistics of induction and recovery times at different doses of hypothermia for *Etroplus suratensis* (Mean ±SEM)

	Hypothermia concentrations				
Stages	**8±1°C**	**12±1°C**	**16±1°C**	**18±1°C**	**22±1°C**
I1		5.3±0.8	69.6±2.3	66.14±6.54	nil
I2		62.6±0.2	78.9±2.1	nil	
I3	11.4±1.0	66.1±0.6	156.4±0.2	nil	
R1	181.6±2.0	81.9±2.1	73.6±0.5	58.28±1.80	nil
R2	207.0±0.9	167.0±0.9	79.9±1.9	nil	
R3	232.7±0.6	183.1±0.7	100.1±4.5	nil	

- Values are expressed in seconds
- Average of six values in each group

2.4.8 Overall desirability functions of six anaesthetics

According to Stoskopf (1993) (Appendix 2) the lowest effective concentration is the concentration that produces general anaesthesia within

3 minutes and allows the recovery within 5 minutes. Since the induction time and recovery time have opposite effects on the concentration of the anaesthetic we use the concept of desirability functions to find the lowest effective concentration.

The basic idea of the desirability function approach is to transform a multiple response problem into a single response problem by means of mathematical transformations. For each response Y_i, a desirability function d_i (Y_i) assigns numbers between 0 and 1 to the possible values of Y_i, with $d_i = 0$ representing a completely undesirable value of Y_i and $d_i = 1$ representing a completely desirable or ideal response value. The individual desirability is then combined using the geometric mean, which gives the overall desirability:

$$D = (d_1 d_2 ... d_k)^{1/k},$$

Where k denoting the number of responses. Note that if any response Y_i is completely undesirable ($d_i = 0$), then the overall desirability is zero.

Depending on whether a particular response Y_i is to be maximized, minimized, or assigned a target value, different desirability functions d_i can be used. A useful class of desirability functions was proposed by Derringer and Suich (1980).

If a response is to be minimized, the individual desirability is defined as

$$d_i = \left| \frac{Y_i - U_i}{a_i - U_i} \right|, \ a_i \le Y_i \le U_i$$

and $d_i = 0$ for $Y_i > U_i$, where a_i is the smallest possible value for the response Y_i and U_i is the value above which the response is considered to be undesirable.

Since the induction time (I_3) and the recovery time (R_3) are to be minimized, the corresponding desirability functions are defined as

$$d_1 = \left|\frac{I_3 - 180}{0 - 180}\right|, \; 0 \le I_3 \le 180, \; d_1 = 0 \text{ for } I_3 > 180 \text{ and}$$

$$d_2 = \left|\frac{R_3 - 300}{0 - 300}\right|, \; 0 \le R_3 \le 300, \; d_2 = 0 \text{ for } R_3 > 300.$$

Now, the overall desirability function is $D = \sqrt{d_1 d_2}$.

For finding out the best anaesthetics among the six anaesthetic agents i.e., clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS222 and hypothermia used in this experiments we found the average values of overall desirability functions of induction and recovery times at different doses for *Etroplus suratensis* (Table 2.7 (1,2,3,4,5,6)).

The following graphs show the average values of overall desirability function at different doses of the six anaesthetic agents

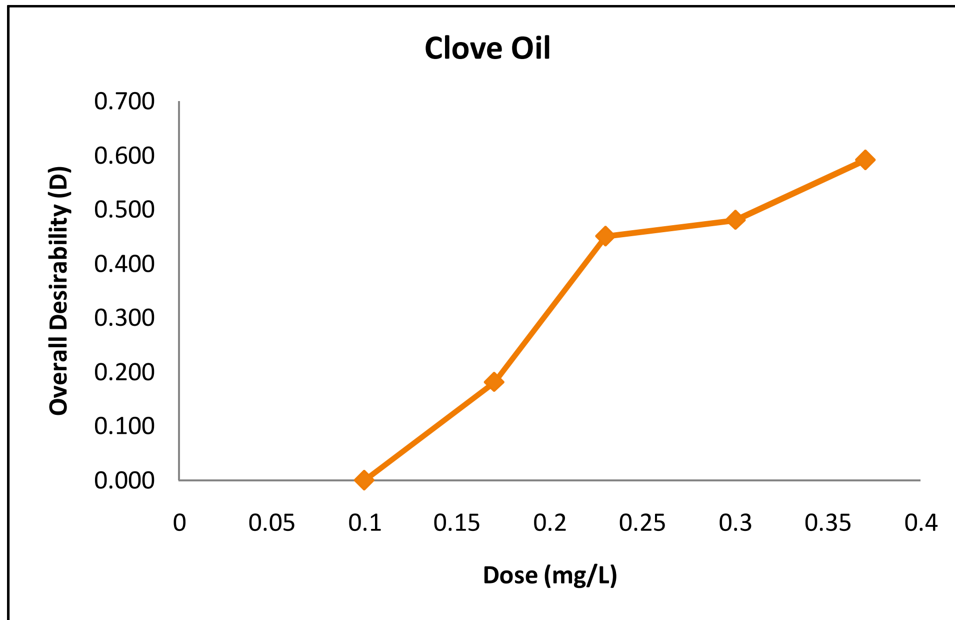


Fig.2.7.1 Average values of overall desirability function at different doses of clove oil for *Etroplus suratensis*

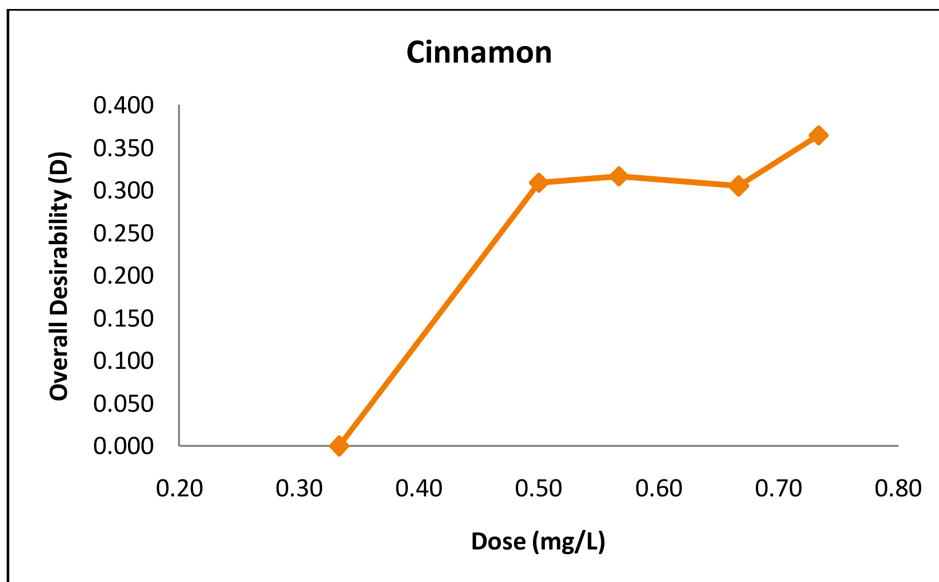


Fig.2.7.2 Average values of overall desirability function at different doses of cinnamon oil for *Etroplus suratensis*

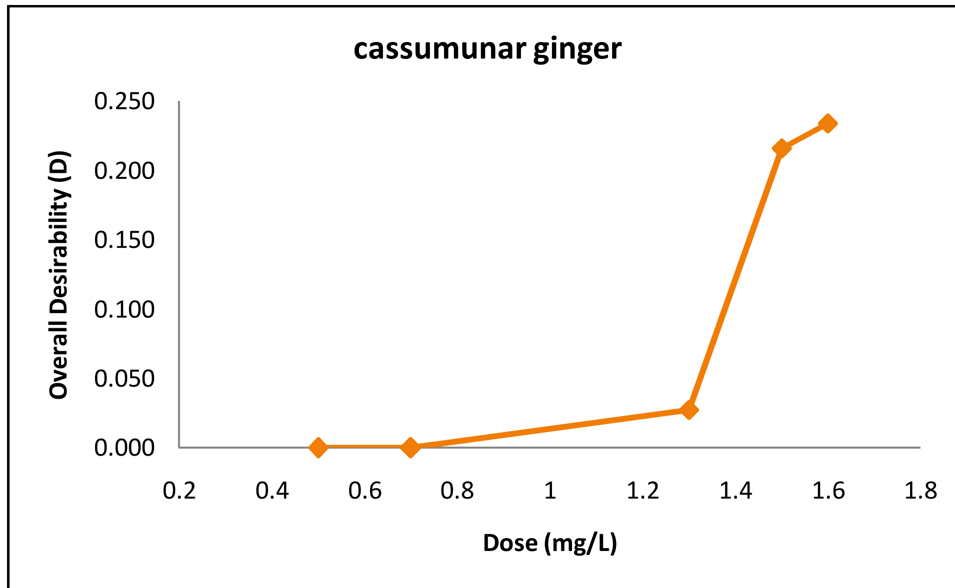


Fig.2.7.3 Average values of overall desirability function at different doses of cassumunar ginger extract for *Etroplus suratensis*

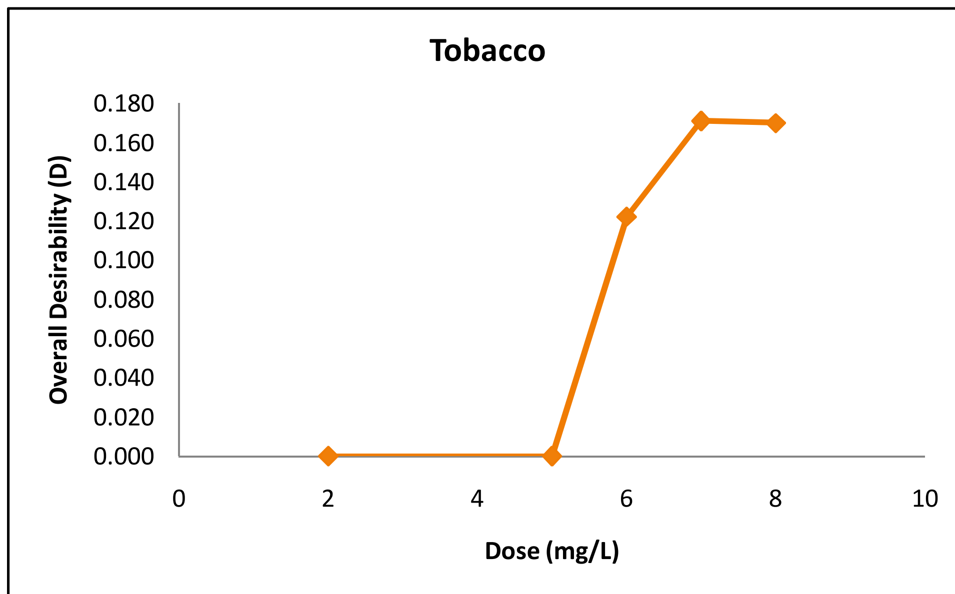


Fig.2.7.4 Average values of overall desirability function at different doses of tobacco leaf extract for *Etroplus suratensis*

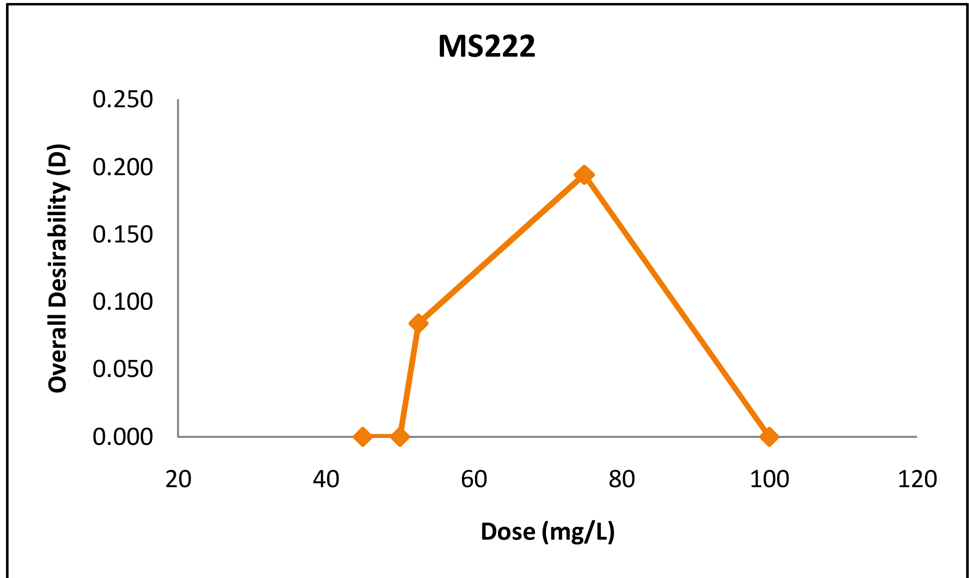


Fig. 2.7.5 Average values of overall desirability function at different doses of MS222 for *Etroplus suratensis*

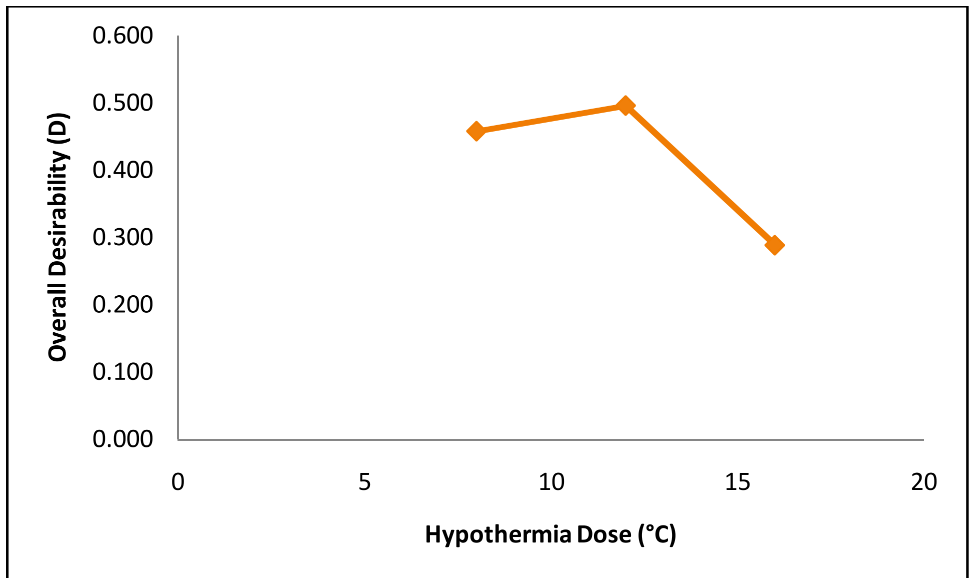


Fig. 2.7.6 Average values of overall desirability function at different doses of hypothermia for *Etroplus suratensis*

Table 2.7 Average values of overall desirability function at different doses of the six anaesthetic agents for *Etroplus suratensis* (Mean ±SEM)

Desirability function					
clove oil mg/L	0.1	0.17	0.23	0.3	0.37
Desirability	0.000±0.000	0.181 ± 0.041	0.451± 0.003	0.481±0.001	0.592±0.004
cinnamon oil	0.33	0.5	0.57	0.67	0.73
Desirability	0.000±0.000	0.309±0.011	0.317±0.008	0.305± 0.006	0.365±0.005
zn cassumunar	0.5	0.7	1.3	1.5	1.6
Desirability	0.000±0.000	0.000±0.000	0.027 ±0.013	0.216±0.006	0.2342±0.001
TB	2	5	6	7	8
Desirability	0.000±0.000	0.000±0.000	0.122±0.005	0.171±0.002	0.170±0.004
MS222	45	50	53	75	100
Desirability	0.000±0.000	0.000±0.000	0.084±0.004	0.194±0.002	0.000±0.000
hypothermia	8	12	16		
Desirability	0.458±0.002	0.496±0.001	0.289±0.019		

2.5 Discussion

This study evaluated the use of different concentrations of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS222 and hypothermia as a tool for sedating fish thereby reducing stress for handling and transportation in aquaculture. Individual induction and recovery times for *Etroplus suratensis* exposed to a range of different concentrations of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS222 and cold during experiments were recorded. All lines presented are 3rd order polynomials with 95% confidence intervals.

2.5.1 Effect of Clove oil as an anaesthetic

2.5.1.1 Behavioural induction

In the present study, results indicate that low levels of clove oil can be used to induce anaesthesia ranging from subtle calming to complete immobilization and loss of equilibrium. Similar work has been reported by

McFarland (1960); Piper et al. (1982) that light anaesthesia permits fish to maintain equilibrium, swimming activity, and breathing can be effective for mitigating stress associated with fish handling and fish transport.

In the present study, the induction times decreased significantly with the increasing concentrations of clove oil, ($P<0.05$). In the case of clove oil the results are in agreement with previous studies in teleost fish (Mattson and Riple, 1989; Hseu et al., 1998; Mylonas et al., 2005; Gullian and Villanueva, 2009; Weber et al., 2009; Heo and Shin, 2010). Coupled with this variation in depth of anaesthesia, we observed substantial differences in physiological disturbance and behavior during transportation. However, the present results clearly identified a range of clove oil concentrations that are optimal for fish handling and transport. Specifically, concentrations of clove oil ranging from 0.17 to 0.37 mg l^{-1} yielded rapid and stable stage 3 anaesthesia (Appendix 2; Stoskopf, 1993). In our study, we observed that interaction rates between fish were highest for unanaesthetized controls. During transport, fish can become injured from physical interactions with each other or from abrasion or concussion with the tank walls (McFarland, 1959). In a preliminary study, Cooke et al. (2000) used low levels of clove oil and monitored activity in adult rainbow trout during transport and determined that fish which loose equilibrium may expend significant energy attempting to correct them. During transport, fish anaesthetized at stage 3 level exhibited reduced activity and interaction, but were able to maintain equilibrium, swimming capacity, and avoid physical damage resulting from collision with the tank walls. These findings are reliable with the belief that stage 3 is efficient for minimizing fish injure during transport. Furthermore, the magnitude of anaesthesia was low, the induction time was too long, and the behavioral

recovery was fast relative to *Etroplus suratensis* anaesthetized at other levels, or unanaesthetized controls. We discuss our findings in the context of using low concentrations of clove oil for fish handling and transportation.

Although there are a number of factors including water temperature (Hama´cˇkova´ et al., 2001; Walsh and Pease, 2002), fish size (Woody et al., 2002), and gender (Woody et al., 2002) that may affect induction time, our experience with using clove oil to anaesthetize *Barilius bakeri* for a number of surgical procedures (Sindhu, 2009) indicates that at higher concentrations, induction of *Barilius bakeri* is rapid (Sindhu and Ramachandran, 2013) and is same in the case of juveniles of *Etroplus suratensis* (Sindhu, personal observations). For example, at similar water temperatures, largemouth bass that were both smaller (Cooke et al., 2003a) larger (Cooke et al., 2003b) exposed to 60 mg l^{-1} required less than 300 s to reach stage 5 anaesthesia. Stage 3 anaesthesia appears relatively easy to achieve compared to stage 4 anaesthesia. Stage 3 involves loss of partial equilibrium and most fish either maintain equilibrium and stay at stage 2 or lose equilibrium completely and progress to stage 4 (Stoskopf, 1993). Fish exposed to high levels of anaesthesia in our study (0.30–0.37 mg/L) spent much of their time sitting on the bottom, often on their side or upside down. At the higher end of concentrations that yielded stage 2 anaesthesia, induction was rather rapid, requiring less than 5 min. This timing is more consistent with the rapid induction times previously noted among many studies of clove oil. This also provides support that 0.23 to 0.37 mg/L is an effective concentration for rapidly inducing stage 3 anaesthesia. The duration of time required to reach a stable level of anaesthesia was longer than previously documented when clove oil was used at higher concentrations in other species (e.g., white

sturgeon, 100 mg l^{-1}, 246 s, Taylor and Roberts, 1999; rainbow trout, 30 mg l^{-1}, 3.7 min, Prince and Powell, 2000; red pacu, 50 mg l^{-1}, 290 s, Sladky et al., 2001; sockeye salmon, 50 mg l^{-1}, 84 s, Woody et al., 2002; Atlantic salmon, 50 mg l^{-1}, 360 s, Iversen et al., 2003). Other researchers that have used low concentrations of clove oil indicated protracted induction times relative to higher concentrations. Although someone else reports the low values, (e.g., Atlantic salmon, 10 mg l^{-1}, 720 s to reach stage 3, Iversen et al., 2003; white sturgeon, 10 mg l^{-1}, 180–260 s to unreported stage, Taylor and Roberts, 1999; coho salmon *Oncorhynchus kisutch* and Chinook salmon *Oncorhynchus tshawytscha*, 10 mg l^{-1}, 240 s to unreported stage, Taylor and Roberts, 1999).

2.5.1.2 Recovery from anaesthesia

Recovery time will, however, depend upon chemical, dosages and exposure time. On the other hand, in the present study results showed that recovery times increased with increasing concentrations of anaesthetic in fingerlings of *Etroplus suratensis.* Behavioral and physiological recovery rates varied with level of anaesthesia (Cooke et al., 2004). Behavioral recovery followed similar patterns to physiological recovery. Cardiac recovery times were used as an indicator of physiological recovery and fish exposed to anaesthesia generally exhibited increased recovery time with increasing concentrations of clove oil (Cooke et al., 2004). Fish exposed to higher concentrations, yielding deeper levels of anaesthesia, exhibited slower behavioral recovery. Higher concentrations will introduce faster anaesthesia than lower concentrations, but will hence correspond with longer recovery time (Hveding, 2008; Gomes et al., 2001; Hoskonen and Pirhonen, 2004a). McFarland and Klontz (1969) argued that the recovery time was proportional to the concentration and exposure time of the anaesthetic. This is connected

to the increased drug accumulation, which has shown to be in accordance with the study on mullet fingerlings (Durve, 1975). It was observed that the concentration of 0.10 mg/L clove oil was not sufficient to sedate the juveniles of *Etroplus suratensis,* while 30 % of the juveniles reached the stage. During the next dose (0.17mg/L) most fish achieved either stage 2 or stage 3 anaesthesia, which is indicative of partial loss of equilibrium within 170.6 ±3.1 (~3min and 24sec) and completely recovered within 58.1±2.7(~1 min).

In the present results the concentration of 0.23mg/L, it induce anaesthesia within the desirable time of 109.6 ± 1.7(~2min 22 sec) and the recovery time of 143.7± 1.8(~2 min 4sec) according to the criteria of Stoskopf, 1993-Appendix 2). Cooke et al. (2004) reported that fish at lower concentrations of clove oil (i.e., 2.5–9 mg l^{-1}) recovered in ~60 min, even more quickly than unanaesthetized control fish (~75 min). Prolonged recovery with increased anaesthetic dosage has been reported in sockeye salmon (Woody et al., 2002) and cobia (Gullian and Villanueva, 2009). In particular, those fish that reached level 4 and 5 anaesthesia (Appendix1; Summerfelt and Smith, 1990) required between 10 and 30 min to recover behaviorally (Cooke et al., 2004). It was observed that the next concentration (0.30 mg/L) gave considerably longer recovery for juveniles (162.9±2.4; ~3 min 12sec).This period is substantially shorter than recovery times reported for other fishes at higher concentrations (sockeye salmon, 50 mg l^{-1}, 330 s, Woody et al., 2002; rainbow trout, 30 mg l^{-1}, 294 s, Prince and Powell, 2000; white sturgeon, 50 mg l^{-1}, 186 s, Taylor and Roberts, 1999). However, decreasing recovery times with an increase in concentration of clove oil and 2-phenoxyethanol for European sea bass and gilthead sea bream has been reported by Mylonas et al. (2005). In aquaculture settings, recovery of that

duration would be problematic, particularly if fish were being stocked to supplement a fishery (Cooke et al., 2004). Fish would be highly susceptible to predation and displacement by flow or currents during prolonged recovery so transport at these deep levels of sedation (i.e., N stage 2) would be undesirable. Furthermore, the magnitude of anaesthesia was low, the recovery time was rapid, and the behavioral recovery was fast relative to *Etroplus suratensis* anaesthetized at other levels, or unanaesthetized controls. Behavioral recovery was more rapid for low concentrations than controls (Cooke et al., 2004). The present results shows that with increasing clove oil concentrations, sedation and anaesthesia induction times were reduced, but recovery times exhibited the opposite pattern with fish experiencing more rapid recovery when exposed to lower clove oil concentrations. The recovery times were significantly lower in clove oil, in lowest concentration ($P<0.05$). These results are in agreement with those found in other species anaesthetized with eugenol or clove oil (Endo et al., 1972; Hikasa et al., 1986; Munday and Wilson, 1997; Keene et al., 1998; Woody et al., 2002; Iversen et al., 2003; Hoskonen and Pirhonen., 2004a). Because of longer recovery time, especially in high concentrations of clove oil is not advisable for juvenile *Etroplus suratensis* in short time applications to reduce working time and labour. When combined with the reduced oxygen demand during transport (inferred from lower cardiac output), ease of handling, and reduced interaction with conspecifics during transport, the use of the low levels of clove oil appears to be more favorable than transporting unanaesthetized fish (Cooke et al., 2004).

Pawar et al. (2011) put forward that, with the highest concentration the fish is not contact with the anaesthetic for long, which allow faster recovery. Also, differences in the physiological responses of fish to the anaesthetic

agents also influence this trend (Weber et al., 2009). According to Marking and Meyer (1985) the anaesthetic agent is considered effective if it produces a complete induction within 180 s and recovery with 300 s for fish. In this study, application of clove oil at safe concentration of 0.17 mg/L resulted in quick induction, total immobilization and fast recovery in *Etroplus suratensis* juveniles. Although higher concentrations of clove oil achieved shorter induction times, above mentioned concentrations were effective and presented a good margin of safety when compared against the above efficacy criteria. On the other hand, the clove oil used in the experiment contained 67% of eugenol and the eugenol used in the experiment was pure (99%). Except the lowest concentration of clove oil (0.1mg/L), there was modest significant differences during induction time of *Etroplus suratensis* ($p>0.05$).

2.5.2 Cinnamon

2.5.2.1 Behavioural Induction

During the present study, the induction times decreased significantly with the increasing concentrations of cinnamon oil, ($P<0.05$). Light anaesthesia that permits fish to maintain equilibrium, swimming activity, and breathing can be effective for mitigating stress associated with fish handling and fish transport (McFarland, 1960; Piper et al., 1982). Together, the results pointed out that low level of cinnamon oil can be used to induce anaesthesia ranging from slight calming to complete immobilization and loss of equilibrium. Throughout cinnamon oil treatment the results are in agreement with previous studies in platy fish (Power et al., 2010). Within our consequences cinnamon oil (*Cinnamomum zeylanicum*) (0.5mg/L) also had a fairly rapid action and totally direct effect and sedated fish within three minutes (154.9± 20; ~2min 58sec) of exposure. It was observed that,

cinnamon oil concentration above 0.50 mg/L induced stage 3 of anaesthesia in *Etroplus suratensis* less than 3 min, while lower concentration (0.33mg /l) was not sufficient to induce anaesthesia for juveniles, at the same time as only 20 % of the juveniles reached the stage. A positive effect was determined by a rapid induction to stage 3 of anaesthesia (Stoskopf, 1993) (Appendix 2). During the induction to the next dose (0.50 mg/L) 75% of the juveniles undergo sedation (stage 2 or 3). At the concentration of 0.57 mg/L (151.7 ± 1.5), the juveniles shows the induction time to some extent similar with 0.50 mg/L (154.9 ± 2), but the juveniles took longer recovery time (107.4 ± 0.8) than 0.57mg/L (91.9 ± 2.1) and did not have damage to the fish at this concentration. So this concentration 0.50 mg/L was indicating for induction to stage 3 of anaesthesia.This was supported by the result of Roubach et al. (2005) that exposure of tambaqui (*Colossoma macropomum*) to 65 $mg \cdot l^{-1}$ of eugenol was sufficient to induce an anaesthetic state, and recovery time was similar for dosages up to 100 $mg \cdot l^{-1}$. Exposure to 65 $mg \cdot l^{-1}$ for up to 30 min did not cause fish mortality. There was no mortality in tambaqui at doses of 135 $mg \cdot l^{-1}$ (exposure duration was not reported). While during the next concentrations (0.67 to 0.73 mg/L) gave considerably faster induction time for juveniles (148.1 ± 1.5 to 127.7 ± 1.9), it is noticed that this concentration was effective to make harm to the fishes.

Eugenol (4-allyl-2-methoxyphenol), the active principle, makes up 28-98% of cinnamon leaf oil, *Cinnamomum zeylanicum (*The Ayurvedic Pharmacopoeia of India Vol. I to IV). Vidal et al. (2007) reported that eugenol had strong anaesthetic effect on *L. macrocephalus* and a dose of 37.5 mg/L of eugenol was recommended for the fast and safe anaesthesia of piavuçu juveniles (*n* = 72). In a similar investigation, benzocaine and

eugenol, at 50 ppm, induced fast anaesthesia and recovery (3 min and 5 min, respectively) (Okamoto et al., 2009). Induction was rapid but recovery was very slow even at low concentrations (Filiciotto et al., 2012). Eugenol (20 μl/L) was investigated in sub-adult and post-larvae of white shrimp (*Litopenaeus vannamei*) and determined to be effective at inducing anaesthesia after 6 h (Parodi et al., 2012).

However, the present results clearly identified a range of cinnamon oil concentrations that are optimal for fish handling and transport. Specifically, concentrations of cinnamon oil ranging from 0.50 to 0.73 mg l^{-1} yielded rapid and stable stage 3 anaesthesia (Appendix 2; Stoskopf, 1993). A significant positive correlation is observed between the results of clove oil and cinnamon oil, but cinnamon oil needs much more concentration to anaesthetize the same size range of fingerlings than clove oil. Cinnamon oil compared with clove oil had a significantly faster induction time to sedation at its lower concentrations (0.10mg/L versus 0.33mg/L) (6min 18 sec versus 4 min 05 sec, $P < 0.05$). Power et al. (2010) reported that after preliminary testing (0.67–67 μg/L), the concentration of cinnamon oil needed to produce reversible anaesthesia was optimized. A comparison of clove oil (67 μg/L) versus cinnamon oil (67 μg/L) revealed a significantly longer time to sedation (125 ± 19 versus 235 ± 24 sec, $P = 0.02$), although no significant difference in decline in activity was noted. This was supported by our data which revealed that with increasing cinnamon concentrations, sedation and anaesthesia induction times were reduced. But recovery times exhibited the opposite pattern with fish experiencing more rapid recovery when exposed to lower cinnamon concentrations. The recovery times were significantly longer in cinnamon than clove oil ($P<0.05$).

2.5.2.2 Behavioural recovery

In the present study recovery times increased with increasing concentrations of anaesthetic in fingerlings of *Etroplus suratensis* (*Etroplus suratensis).*This study supports the earlier findings of Cooke et al., 2004 that the behavioral and physiological recovery rates varied by level of anaesthesia. Similar results by Hveding (2008), Gomes et al. (2001), Hoskonen and Pirhonen (2004 a) etc., reported that at higher concentrations will introduce faster anaesthesia than lower concentrations, but will hence correspond with longer recovery time. Pawar et al. (2011) put forward that, with the highest concentration the fish is not in contact with the anaesthetic for long, which allow faster recovery. Also, differences in the physiological responses of fish to the anaesthetic agents also influence this trend (Weber et al., 2009). Recovery time will, however, depend upon chemical, dosages and exposure time. In this study, application of cinnamon oil at a safe concentration of 0.50 to 0.57 mg/L resulted in quick induction (154.9 ± 2 to 151.7 ± 1.5), total immobilization and fast recovery (91.9 ± 2.1 to 107.4 ± 0.8) in *Etroplus suratensis* juveniles. Above mentioned concentrations were effective and presented a good margin of safety when compared against the above efficacy criteria. At the lower concentration of 0.33 mg/L of cinnamon oil was not sufficient to quick recovery (243 ± 1.9). Except the lowest concentration of cinnamon oil (0.33 mg/L), there was modest significant differences between recovery time of *Etroplus suratensis* ($p>0.05$). According to Marking and Meyer (1985), the anaesthetic agent is considered effective if it produces a complete induction within 180 s and recovery with 300 s for fish. McFarland and Klontz (1969) argued that the recovery time was proportional to the concentration and exposure time of the anaesthetic.

This is connected to the increased drug accumulation, which has shown to be in accordance with the study on mullet fingerlings (Durve, 1975). Although in the present results at higher concentrations of cinnamon oil (0.67 to 0.73) achieved longer recovery times (141.3 ± 1.5 to 162.0 ± 1.2), were not desirable. Fish exposed to higher concentrations, yielding deeper levels of anaesthesia, exhibited slower behavioral recovery (Cooke et al., 2004). Furthermore, the magnitude of anaesthesia was low, the recovery time was rapid, and the behavioral recovery were fast relative to *Etroplus suratensis* anaesthetized at other levels, or unanaesthetized controls. In aquaculture settings, recovery of that duration would be problematic, particularly if fish were being stocked to supplement a fishery (Cooke et al., 2004). Fish would be highly susceptible to predation and displacement by flow or currents during prolonged recovery so transport at these deep levels of sedation (i.e., N stage 2) would be undesirable.

Although behavioral recovery was more rapid for low concentrations than controls (Cooke et al., 2004). The present results show that with increasing cinnamon oil concentrations, sedation and anaesthesia induction times were reduced, but recovery times exhibited the opposite pattern with fish experiencing more rapid recovery when exposed to lower cinnamon oil concentrations. This was supported by our data which revealed that with increasing cinnamon concentrations, sedation and anaesthesia induction times were reduced. But recovery times exhibited the opposite pattern with fish experiencing more rapid recovery when exposed to lower cinnamon concentrations. The recovery times were significantly longer in cinnamon than clove oil ($P<0.05$). These results are in agreement with those found in other species anaesthetized with eugenol or clove oil (Endo et al., 1972;

Hikasa et al., 1986; Munday and Wilson, 1997; Keene et al., 1998; Woody et al., 2002; Iversen et al., 2003; Hoskonen and Pirhonen, 2004a). Because of longer recovery time especially in high concentrations of cinnamon oil is not advisable for juvenile *Etroplus suratensis* in short time applications to reduce working time and labor. When combined with the reduced oxygen demand during transport (inferred from lower cardiac output), ease of handling, and reduced interaction with conspecifics during transport, the use of the low levels of cinnamon oil appears to be more favorable than transporting unanaesthetized fish (personal observation). The dose of cinnamon or clove oil required for anaesthesia in the present study was significantly lower than those reported in previous studies (range 4–150 mg/L) using aquaculture species, but this is probably a consequence of the anaesthetic endpoint chosen, species, water temperature, and other factors previously described to influence anaesthesia (Mylonas, 2005; Santilas, 2006). The result of the present study indicates that cinnamon is effective as a fish anaesthetic. But previously reported as clove oil, cinnamon oil offer a safe alternative to current anaesthetics, sedating fish in a reversible fashion within 347.3 ± 4.6 sec and 243 ± 1.9 sec at its lower concentrations for clove and cinnamon oils, respectively.

2.5.3 ***Zingiber casumunar*** **Roxb** (Cassumunar Ginger)

2.5.3.1 Behavioural induction

Collectively, the present results indicate that low levels of cassumunar ginger can be used to induce anaesthesia ranging from subtle calming to complete immobilization and loss of equilibrium. Coupled with this variation in depth of anaesthesia, we observed substantial differences in physiological disturbance and behavior during transportation. The present results show that

at the concentration of 0.5 mg/L cassumunar ginger extract was not sufficient to induce anaesthesia for juveniles of *Etroplus suratensis*, while at the concentration of 0.7mg/L only 60 % of the juveniles *Etroplus suratensis*, reached the stage 3. Light anaesthesia that permits the fish to maintain equilibrium, swimming activity, and breathing can be effective for mitigating stress associated with fish handling and fish transport (McFarland, 1960; Piper et al., 1982). In the present study, the induction times decreased significantly with the increasing concentrations of cassumunar ginger, ($P<0.05$). In addition, behavioral and physiological recovery rates varied with level of anaesthesia (Cooke et al., 2004). However, it was clearly identified a range of cassumunar ginger extracts concentrations that are optimal for fish handling and transport.

Specifically, concentrations of cassumunar ginger extract ranging from 0.50 to 1.60 mg/L yielded rapid and stable stage 3 anaesthesia (Appendix 2; Stoskopf MK, 1993). In the present study, it was observed that interaction rates between fish were highest for unanaesthetized controls. During transport, fish may become injured from physical interactions with conspecifics or from abrasion or concussion with the tank walls (McFarland, 1959). During transport, fish anaesthetized at stage 3 level exhibited reduced activity and interaction, but were able to maintain equilibrium, swimming capacity, and avoid physical damage resulting from collision with the tank walls. These findings are reliable with the belief that stage 3 is efficient for minimizing fish injure during transport. Furthermore, the magnitude of anaesthesia was low, the induction time was too long, and the behavioral recovery was fast relative to *Etroplus suratensis* anaesthetized at other levels, or unanaesthetized controls. We discuss our findings in the context of using low concentrations of cassumunar ginger extract for fish handling and transportation.

However, it was observed the sedative traits and unbalance for the juveniles which transferred to the next dose (1.30 mg/L), while 75 % of the juveniles of *Etroplus suratensis*, struggled with the balance (stage 2 or 3). Here the duration of time required to reach a stable level of anaesthesia was longer. Although there are a number of factors including water temperature (Hama´cˇkova´ et al., 2001; Walsh and Pease, 2002), fish size (Woody et al., 2002), and gender (Woody et al., 2002) that may affect induction time. The concentration of 1.30 mg/L was chosen as the most satisfying concentration, as it induces anaesthesia within the desirable time for juveniles according to the criteria of Stoskopf, 1993-Appendix 2 (R_3 187.1 ± 0.8). Our experience while using cassumunar ginger extract to anaesthetize *Etroplus suratensis* for a number of procedures indicates that at higher concentrations, induction of *Etroplus suratensis* is moderately slow (Sindhu, personal observations). The concentration of 1.30 mg/L induced anaesthesia (181.7 ± 1.3) in juveniles, while the juveniles took longer induction time than the next dose1.50mg/L (156.1 ± 1.3). The next concentration (1.5mg/L) gave considerably rapid induction for juvenile *Etroplus suratensis* (156.1 ± 1.3). Stage 2 anaesthesia appears relatively easy to achieve compared to stage 3 anaesthesia. Stage three involves loss of partial equilibrium and most fish either maintain equilibrium and stay at stage 2 or lose equilibrium completely and progress to stage 4. Fish exposed to high levels of anaesthesia in our study (1.50–1.60 mg/L) spent much of their time lying on the bottom, often on their side or upside down. At the higher end of concentrations that yielded stage 2 anaesthesia, induction was rather rapid, requiring less than 5 min. This timing is more consistent with the rapid induction times.

2.5.3.2 Behavioural recovery

In the present study, results show, recovery times increased with increasing concentrations of anaesthetic in fingerlings of *Etroplus suratensis.* McFarland and Klontz (1969) argued that the recovery time was proportional to the concentration and exposure time of the anaesthetic. This is connected to the increased drug accumulation, which has shown to be in accordance with the study on mullet fingerlings (Durve, 1975). Recovery time will, however, depending upon chemical, dosages and exposure time. It was observed that the concentration of 0.50 mg/L cassumunar ginger extract was not sufficient to sedate the juveniles of *Etroplus suratensis,* while 20 % of the juveniles reached the stage. During the next dose (0.70 mg/L) most fish achieved either stage 2 or stage 3 anaesthesia, which is indicative of partial loss of equilibrium and completely recovered within 147.6 ± 1.3(~2 min 46 se). It was also observed that the concentration of 1.30 mg/L, induces anaesthesia within the desirable time of 181.7 ± 1.3 (~3min 02 sec) and the recovery time of 187.1 ± 0.8(~3 min 12sec) according to the criteria of Stoskopf, 1993-Appendix 2). In the present results the next concentration (1.50 mg/L) gave considerably longer recovery for juveniles of *Etroplus suratensis* (93.7 ±0.8; ~3 min 22sec). Prolonged recovery with increased anaesthetic dosage has been reported in sockeye salmon (Woody et al., 2002) and cobia (Gullian and Villanueva, 2009). In particular, those fish that reached level 4 and 5 anaesthesia (Appendix1; Summerfelt and Smith, 1990) required between 10 and 30 min to recover behaviorally (Cooke et al., 2004). However, increasing recovery times with an increase in concentration of cassumunar ginger extract was noticed in *Etroplus suratensis* (personal observation). In aquaculture settings, recovery of that duration would be

problematic, particularly if the fish were being stocked to supplement a fishery (Cooke et al., 2004). Fish would be highly susceptible to predation and displacement by flow or currents during prolonged recovery. So transport at these deep levels of sedation (i.e., N stage 2) would be undesirable. Furthermore, the magnitude of anaesthesia was low, the recovery time was rapid, and the behavioral recovery was fast relative to *Etroplus suratensis* anaesthetized at other levels, or unanaesthetized controls. The present results show that with increasing cassumunar ginger extract concentrations, sedation and anaesthesia induction times were reduced, but recovery times exhibited the opposite pattern with fish experiencing more rapid recovery when exposed to lower cassumunar ginger extract concentrations. However behavioral recovery was more rapid for low concentrations than controls (Cooke et al., 2004). The recovery times were significantly lower in cassumunar ginger extract, in lowest concentration ($P<0.05$). Because of longer recovery time, especially in high concentrations of cassumunar ginger extract it is not advisable for juvenile *Etroplus suratensis* in short time applications to reduce working time and labour. When combined with the reduced oxygen demand during transport (inferred from lower cardiac output), ease of handling, and reduced interaction with conspecifics during transport, the use of the lower levels of cassumunar ginger extract appears to be more favorable than transporting unanaesthetized fish (Cooke et al., 2004).

In this study, application of cassumunar ginger extract at safe concentration of 1.30 mg/L resulted in quick induction, total immobilization and fast recovery in *Etroplus suratensis* juveniles. Although higher concentrations of cassumunar ginger extract achieved shorter induction times, above mentioned concentrations were effective and presented a good margin

of safety when compared against the above efficacy criteria. Except for the lowest concentration of cassumunar ginger extract (0.50 mg/L), there was modest significant differences between induction times of *Etroplus suratensis*) (p>0.05).

The present study deals with the investigation of the anaesthetic efficacy of rhizome of cassumunar ginger (*Z. cassumunar* Roxb). Regarding *Z. cassumunar* Roxb, there is no work about the behavioural changes in *Etroplus suratensis* available in our country. The present result shows that higher concentrations of cassumunar ginger extraction are needed to induce sedation with shorter induction times.

2.5.4 Tobacco leaf extract

2.5.4. 1 Behavioral induction

According to Agokei and Adebisi, 2010 tobacco appears to meet five of the eight criteria used to define an ideal anaesthetic (Marking and Meyer, 1985). In the present study, the induction times decreased significantly with the increasing concentrations of tobacco leaf extract, (P<0.05). At lower concentrations (< 5.00 mg/L) no observable changes in the fish took place or complete anaesthesia (no opercular movement) was not achieved in the groups ranging from 1 to 5mg/L within 5 min treatment. Collectively, present study indicates that low levels of tobacco leaf extract (6 mg/L) can be used to induce anaesthesia ranging from subtle calming to complete immobilization and loss of equilibrium. Light anaesthesia that permits fish to maintain equilibrium, swimming activity, and breathing can be effective for mitigating stress associated with fish handling and fish transport (McFarland, 1960; Piper et al., 1982). In a preliminary study, Cooke et al., (2000) used low

levels of clove oil and monitored activity in adult rainbow trout during transport and determined that fish which loose equilibrium may expend significant energy attempting to correct them. Coupled with this variation in depth of anaesthesia, we observed substantial differences in physiological disturbance and behavior during transportation. In addition, behavioral and physiological recovery rates varied with level of anaesthesia (Cooke et al., 2004). However, the present study clearly identified a range of tobacco leaf extract concentrations that are optimal for fish handling and transport. Specifically, concentrations of tobacco leaf extract ranging from 2 to 8 mg/L yielded rapid and stable stage 3 anaesthesia (Appendix 2; Stoskopf MK, 1993). The range of Tobacco leaf extract (*Nicotiana tobaccum*) (1.6-8 mg/L) used to anaesthetize *Etroplus suratensis* was equivalent to or greater than the level of anaesthesia recommended for Tobacco leaf extract anaesthesia in Nile tilapia (*Oreochromis niloticus)* (Agokei and Adebisi, 2010). During transport, fish can become injured from physical interactions with conspecifics or from abrasion or concussion with the tank walls (McFarland, 1959). In our study, we observed that interaction rates between fish were highest for unanaesthetized controls. The crude alcoholic leaf extract of Tobacco (*Nicotiana tobaccum*) was found to be a potent anaesthetic for fingerlings of *Etroplus suratensis* at 6.00 mg/L within 167.9 ±1.1 sec. At this stage loss of reactivity to stimuli and to loose equilibrium. During transport, fish anaesthetized at stage 3 level exhibited reduced activity and interaction, but were able to maintain equilibrium, swimming capacity, and avoid physical damage resulting from collision with the tank walls. These findings are reliable with the belief that stage 3 is efficient for minimizing fish injure during transport. Furthermore, the magnitude of anaesthesia was low, the

induction time was too long, and the behavioral recovery was fast relative to Etroplus suratensis anaesthetized at other levels, or unanaesthetized controls. We discuss our findings in the context of using low concentrations of tobacco leaf extracts for fish handling and transportation.

In the present results the duration of time required to reach a stable level of anaesthesia to fingerlings of *Etroplus suratensis* was 169.7± 1.1 s (~2.49min) and 148.4 ±1.6 s (~2.47min) correspondingly for 6 and 7 mg/L. This is equaling with the results of when the tobacco leaf extract was used at higher concentrations (for 6 mg/L 2.4 ± 0.54 min and for 7 mg/L 2 ± 0.55 min) in Nile tilapia (*Oreochromis niloticus*) (Agokei and Adebisi, 2010). Our experience while using the tobacco leaf extract to anaesthetize fingerlings of *Etroplus suratensis* indicates that at higher concentrations, induction is rapid (Sindhu, personal observations). Although there are a number of factors including water temperature (Hama´cˇkova´ et al., 2001; Walsh and Pease, 2002), fish size (Woody et al., 2002), and gender (Woody et al., 2002) that may affect induction time. For example, at similar water temperatures, largemouth bass that were both smaller (Cooke et al., 2003a) larger (Cooke et al., 2003b) exposed to 60 mg l^{-1} required less than 300 s to reach stage 5 anaesthesia. Stage 2 anaesthesia appears relatively easy to achieve compared to stage 3 anaesthesia. Stage three involves loss of partial equilibrium and most fish either maintain equilibrium and stay at stage 2 or lose equilibrium completely and progress to stage 4. Fish exposed to high levels of anaesthesia in the present study (7–8 mg/L) spent much of their time lying on the bottom, often on their side or upside down. At the higher end of concentrations that yielded stage 2 anaesthesia, induction was rather rapid, requiring less than 3min. This timing is more consistent with the rapid induction times

previously noted among many studies of tobacco leaf extract. Similarly, high efficacy was achieved using the concentration of 8 mg/L in 128.4±1.6 seconds where minimal opercular movement was attained and also significantly ($p<0.05$) longer recovery time (269.3±2.1). This also provides support that 7 to 8 mg/L is an effective concentration for rapidly inducing stage 2 anaesthesia. Other researchers that have used low concentrations of tobacco leaf extract indicated protracted induction times relative to higher concentrations., although Agokei and Adebisi (2010) reports that the sequential progression through the various stages of anaesthesia of tobacco leaf extract with increasing dose and time and the recovery of anaesthetized fish. All followed the patterns of typical fish anaesthetic (McFarland, 1960; Marking and Meyer, 1985).

It was observed that anaesthetic time was influenced by dose concentration and the results of this research are in line with the findings of anaesthetics used on fish as Jennings and Looney (1998), Kaiser and Vine (1998), Smith et al., (1999), Ross and Ross (1999), Edwards et al., (2000), Prince and Powell (2000), Hovda and Linley (2000), Roubach et al., (2001), Gomes et al., (2001), Kazun and Siwicki (2001), Sandodden et al., (2001), Browser (2001), Ortuno et al., (2002), Walsh and Pease (2002), Woody et al., (2002) and Wagner et al., (2003). Agokei and Adebisi (2010) reported a dosage of 4 or 4.5 g/L of the aqueous preparation and a dose of 6 or 7 ml/L of the alcoholic extract sufficed for quick anaesthetization of tilapia, more so, as over 75% of the fish recovered well within 10 min of removal from anaesthetizing solution. This time is enough to perform most routine fish handling procedures like retrieving gametes, length and weight measurements, and tagging and sex determination. The results of the current study vary from

that of Sado (1985) where the effective dose required to induce anaesthesia using quinaldine in three species of tilapia including *O. niloticus* was 50 g/L compared with the effective dose of tobacco (using either of the tobacco preparations) needed to anaesthetize the same species was much higher. Also the period of anaesthesia was long (over 12 h) as compared with that of the tobacco preparations. These observations agree well with the findings of Konar (1970) where a concentration of 5 g/L of nicotine (the active ingredient in tobacco) elicited a high degree of excitability and eventual stupor within 5 - 10 min of exposure. Recovery time in the above experiment was however very long, spanning 4 - 6 days.

2.5.4. 2 Recovery from anaesthesia

In the present study, the recovery time increased with increasing concentrations of anaesthetic in fingerlings of *Etroplus suratensis.* It was observed that the concentration of 2 mg/L tobacco leaf extract was not sufficient to sedate the juveniles of *Etroplus suratensis*. During the next dose (5 mg/L) most fish achieved either stage 2 or stage 3 anaesthesia, which is indicative of partial loss of equilibrium within 187.0 ± 1.6 (~3min and 11s) and completely recovered within 177.6 ± 0.8 (~2 min 96 s). According to Cooke et al., 2004 behavioral recovery was more rapid for low concentrations than controls. It was observed that the concentration of 6 mg/L, induces anaesthesia within the desirable time of 167.9 ± 1.1 (~2min 79 s) and the recovery time of 232.4 ± 0.9 (~3 min 87s) according to the criteria of Stoskopf, 1993-Appendix 2). In the next concentration 7 mg/L gave considerably longer recovery for juveniles (249.3 ±2.1; ~4min 15 s). This period is substantially longer than recovery times reported for other doses. Fish exposed to higher concentrations, yielding deeper levels of anaesthesia,

exhibited slower behavioral recovery. Higher concentrations will introduce faster anaesthesia than lower concentrations, but will hence correspond with longer recovery time (Hveding, 2008, Gomes et al., 2001, Hoskonen and Pirhonen, 2004b). McFarland and Klontz (1969) argued that the recovery time was proportional to the concentration and exposure time of the anaesthetic. This is connected to the increased drug accumulation, which has shown to be in accordance with the study on mullet fingerlings (Durve, 1975). However, decreasing recovery times with an increase in concentration of tobacco leaf extract for Nile tilapia (*Oreochromis niloticus)* has been reported by Agokei and Adebisi (2010).

Furthermore, the magnitude of anaesthesia was low, the recovery time was rapid, and the behavioral recovery was fast relative to *Etroplus suratensis* anaesthetized at other levels, or unanaesthetized controls. The present study showed that with increasing concentration of tobacco leaf extract, sedation and anaesthesia induction times were reduced, but recovery times exhibited the opposite pattern with fish experiencing more rapid recovery when exposed to lower tobacco leaf extract concentration. The recovery times were significantly lower in tobacco leaf extract, in lowest concentration ($P<0.05$). These results are in agreement with those found in Nile tilapia (*Oreochromis niloticus)* anaesthetized with the tobacco leaf extract (Agokei and Adebisi, 2010). Because of longer recovery time, especially in high concentrations of tobacco leaf extract it is not advisable for juvenile *Etroplus suratensis* in short time applications to reduce working time and labor. In aquaculture settings, recovery of that duration would be problematic, particularly if the fish were being stocked to supplement a fishery (Cooke et al., 2004). Fish would be highly susceptible to predation and

displacement by flow or currents during prolonged recovery so transport at these deep levels of sedation (i.e., N stage 2) would be undesirable. Malstrom et al., (1993) recommended a dosage of 2.5 g/L for quick anaesthetization of halibut from, which fish recover in 11 min. Using dosages as low as these did not effect anaesthesia in tilapia, using any of the tobacco preparations. Agokei and Adebisi (2010) reported recovery times using 4/4.5 g/L or 6/7 ml/L of the tobacco preparations, however compares favorably with the recovery time when administering MS – 222 as in the afore mentioned investigations. The present results are in parallel with the results obtained by Agokei and Adebisi (2010) where complete anaesthesia of *Etroplus suratensis* was achieved in 3-8 min.

In many countries, the use of fish anaesthetics is a matter of concern as there are no specific laws regulating their use (Pawar, 2011). Clove oil, 2-phenoxyethanol and eugenol have been extensively used as an anaesthetic agent in aquaculture of freshwater and marine fishes. Further studies on different life stages, gender, reproduction state and sizes, followed by assessments of the effects of anaesthetics on haematological profile and respiration rate will advance our understanding of anaesthesia of *Etroplus suratensis*.

2.5.5 MS222 (TMS or Tricane methane sulphonate)

2.5.5. 1 Induction of anaesthesia

Although a number of studies have described the physiological responses of fish to sedate and immobilizing doses of MS-222, only a few studies have reported on responses to higher, lethal concentrations of MS-222 or other anaesthetics (Congleton, 2006). The few studies using

higher concentrations of MS-222 (125 mg L_1, Laidley and Leatherland, 1988; 150mg L_1, Holloway et al., 2004) have evaluated changes in blood chemistry at the time of induction of deep anaesthesia, 2-3min after the initiation of exposure (Congleton, 2006). Differential exposure time and dosage of TMS can induce stages of anaesthesia in fish corresponding to differing states of narcosis or the level of sedation with changes in neural functioning that initiates in the peripheral neural system. The rate of decline for neural function, as well as the level to which it declines, varies primarily with the anaesthetic dosage due to the rapid diffusion of TMS across the gill (Hunn and Allen, 1974). As neural function decreases, fish exhibit predictable changes in behavior that can be used to gauge the current level of anaesthesia (McFarland, 1959).

In the present study, it was observed that interaction rates between fish were highest for unanaesthetized controls and during anaesthetization; it can avoid physical damage of fishes resulting from collision with the tank walls. During transport, fish can become injured from physical interactions with conspecifics or from abrasion or concussion with the tank walls (McFarland, 1959). It was clearly identified a range of TMS concentrations (45 -100 mg /L) that is optimal for fish handling and transport. Light anaesthesia that permits fish to maintain equilibrium, swimming activity, and breathing can be effective for mitigating stress associated with fish handling and fish transport (McFarland, 1960; Piper et al., 1982). Collectively, the present results indicate that low levels of TMS (45-53mg L) can be used to induce anaesthesia ranging from subtle calming to complete immobilization and loss of equilibrium. For minor handling procedures (e.g., measurements, blood samples) or transport, lower dosages (15–30 mg of TMS/L to water for

Salmonidae) result in tranquilization and light sedation (Stages 1–2), this can be held for long periods (Schoettger and Julin, 1967). In the present results the concentration of 45 mg/L TMS was not sufficient to sedate the juveniles of *Etroplus suratensis,* while 30 % of the juveniles reached the stage. During the next dose (50 mg/L) most fish achieved either stage 2 or stage 3 anaesthesia, which is indicative of partial loss of equilibrium within 217.4 ±0.9 (~3min and 62s). Summerfelt and Smith (1990) derived 6 Stage, scales. In the present results we selected up to the Stage 3. During transport, fish anaesthetized at stage 3 levels exhibited reduced activity and interaction, but were able to maintain equilibrium, swimming capacity, partial loss of muscle tone, hyperactive behavior such as erratic swimming and increased opercular rate and reaction only to strong tactile or vibrational stimuli. These findings are reliable with the belief that stage 3 is efficient for minimizing fish injure during transport. In the present study, at the concentration of 53 mg/L, it induce anaesthesia within the desirable time of 172.3 ± 0.8 (~2min 87 s). According to Iwama and Ackerman (1994) the optimal TMS dosage to induce anaesthesia varies between 75 mg/L and 100 mg/L of TMS to water.

There are a number of factors including water temperature (Hama´cˇkova´ et al., 2001; Walsh and Pease, 2002), fish size (Woody et al., 2002), and gender (Woody et al., 2002) that may affect induction time. For example, at similar water temperatures, largemouth bass that were both smaller (Cooke et al., 2003a) larger (Cooke et al., 2003b) and exposed to 60 mg l_1 required less than 300 s to reach stage 5 anaesthesia. Exact dosages for salmonids vary with anaesthesia stage targeted, species, body size, health, age and life stage, and water quality (Summerfelt and Smith

1990; Ross and Ross 2008). In addition, the tolerances of salmonids for specific dosages vary between stocks and/or sexes (Marking, 1967; Schoettger and Julian, 1967; Houston and Corlett, 1976; Burka et al., 1997). Induction and recovery times are inversely correlated with body weight, especially for salmon (Burka et al., 1997; Houston and Corlett, 1976). Water quality parameters, such as temperature, pH, salinity, and hardness, can affect metabolic rate, acid–base regulation, and osmoregulation and ion regulation (Schoettger and Julin, 1967; Heisler, 1988; Iwama et al., 1989; Perry and Gilmour, 2006). These factors can also affect the pharmacodynamics of TMS (Marking, 1967; Ohr, 1976).

In the present result using with TMS to anaesthetize *Etroplus suratensis* for a number of surgical procedures indicates that at higher concentrations (60-85 mg/L), induction of *Etroplus suratensis* is rapid (Sindhu, personal observations). In the present work Stage 3 anaesthesia appears relatively easy at higher doses. Stage three involves loss of partial equilibrium and most fish either maintain equilibrium and stay at stage 3 or lose equilibrium completely and progress to stage 4 (Summerfelt and Smith, 1990) (Appendix1).

Juveniles of *Etroplus suratensis* exposed to high levels of anaesthesia (75– 100 mg/L) spent much of their time lying on the bottom, often on their side or upside down. At the higher concentration (75 to 100 mg /L), induction was rather rapid (127.7 ± 0.8 to 43.4 ± 2.7s) and requiring less than 3 min that yielded stage 2 anaesthesia. Major procedures require higher doses (60–100 mg/L of TMS to water) too quickly (within 4 min) induce deep anaesthesia levels (Stages 4–5; Schoettger and Julian, 1967; Hunn and Allen, 1974; Summerfelt and Smith, 1990). For invasive procedures, such as

intracoelomic transmitter implantation, the fish must be in a deep level of anaesthesia (C Stage 4) to be rendered completely immobile. Several authors have suggested that an ideal anaesthetic should induce Stage 4 anaesthesia quickly (in under 3 min), while allowing for quick recovery (less than 5 min; Marking and Meyer, 1985; Bell, 1987; Iwama and Ackerman, 1994). Higher doses will induce and maintain Stage 4 anaesthesia quickly. However, the risk for adverse side effects would increase if fish were not able to be processed in timely manner (Marking and Meyer 1985; Bell 1987; Iwama and Ackerman, 1994).This timing is more consistent with the rapid induction times previously noted among many studies of TMS. This also provides support that 50 to (52.5)53 mg / L is safe and effective concentration for rapidly inducing stage 3 anaesthesia. Other researchers that have used low concentrations of TMS indicated protracted induction times relative to higher concentrations.

2.5.5. 2 Behavioural recovery

In the present results at the concentration of 45 mg/L TMS was not sufficient to sedate the juveniles of *Etroplus suratensis.* During the next dose (50 mg/L) most fish achieved either stage 2 or stage 3 anaesthesia, and completely recovered within 229.4 ± 1.3 (~3 min 82 s). The recovery times were significantly lower in TMS, in lowest concentration ($P<0.05$), although behavioral recovery was more rapid for low concentrations than controls (Cooke et al., 2004). These results are in agreement with those found in other species anaesthetized with TMS (Hveding, 2008; Gomes et al., 2001; Hoskonen and Pirhonen, 2004a; Perry and Gilmour 2006; Iwama et al., 1989; Heisler 1988; Schoettger and Julin 1967). The present results at the concentration of 53 mg/L, the recovery time of juveniles of *Etroplus*

suratensis is 249.1± 1.5 (~4 min 15s) according to the criteria of Stoskopf, 1993-Appendix 2. Prolonged recovery with increased anaesthetic dosage has been reported in sockeye salmon (Woody et al., 2002) and cobia (Gullian and Villanueva, 2009). In particular, those fish that reached level 4 and 5 anaesthesia (Appendix1; Summerfelt and Smith, 1990) required between 10 and 30 min to recover behaviorally (Cooke et al., 2004). In the present results the higher concentrations (75-100 mg/L) gave considerably longer recovery for juveniles (260.9 ± 1.4; ~4 min 34 s – 371.4 ± 1.6; ~6 min 19 s). In the present study, it was observed that when fish exposed to anaesthesia generally exhibited increased recovery time with increasing concentrations of TMS (personal observation). Fish exposed to higher concentrations, yielding deeper levels of anaesthesia, exhibited slower behavioral recovery (Cooke et al., 2004). Higher concentrations will introduce faster anaesthesia than lower concentrations, but will hence correspond with longer recovery time (Hveding, 2008, Gomes et al., 2001, Hoskonen and Pirhonen, 2004a). McFarland and Klontz (1969) argued that the recovery time was proportional to the concentration and exposure time of the anaesthetic. This is connected to the increased drug accumulation, which has shown to be in accordance with the study on mullet fingerlings (Durve, 1975). Recovery time will, however, depending upon chemical, dosages and exposure time. On the other hand, the present results show recovery times increased with increasing concentrations of anaesthetic in fingerlings of *Etroplus suratensis.*

In aquaculture settings, recovery of that duration would be problematic, particularly if the fish were being stocked to supplement a fishery (Cooke et al., 2004). Fish would be highly susceptible to predation and displacement by flow or currents during prolonged recovery so transport at these deep levels

of sedation (i.e., N stage 2) would be undesirable. Furthermore, the magnitude of anaesthesia was low, the recovery time was rapid, and the behavioral recovery was fast relative to *Etroplus suratensis* anaesthetized at other levels, or nonanaesthetized controls. The present results show that with increasing TMS concentrations, sedation and anaesthesia induction times were reduced, but recovery times exhibited the opposite pattern with fish experiencing more rapid recovery when exposed to lower TMS concentrations. Because of longer recovery time, especially in high concentrations of TMS is not advisable for juvenile *Etroplus suratensis* in short time applications to reduce working time and labor. When combined with the reduced oxygen demand during transport (inferred from lower cardiac output), ease of handling, and reduced interaction with conspecifics during transport, the use of the lower levels of TMS appears to be more favorable than transporting unanaesthetized fish(personal observation).

Pawar et al., 2011 put forward that, with the highest concentration the fish is not contact with the anaesthetic for long, which allow faster recovery. Also, differences in the physiological responses of fish to the anaesthetic agents also influence this trend (Weber et al., 2009). According to Marking and Meyer (1985), the anaesthetic agent is considered effective if it produces a complete induction within 180 s and recovery with 300 s for fish.

In the present study, the induction times decreased significantly with the increasing concentrations of TMS, ($P<0.05$). Coupled with this variation in depth of anaesthesia, in the present study observed substantial differences in physiological disturbance and behavioural changes during transportation. Changes in induction and recovery times occur due to the decay of TMS

within the induction bath as it becomes metabolized or diluted with the physical movement of fish into and out of the induction bath (Schoettger and Julin 1967; Burka et al., 1997; Summerfelt and Smith 1990). Furthermore, the magnitude of anaesthesia was low, the induction time was too long, and the behavioral recovery was fast relative to *Etroplus suratensis* anaesthetized at other levels, or nonanaesthetized controls. We discuss our findings in the context of using low concentrations of TMS for fish handling and transportation.

In this study, application of TMS at a safe concentration of 50 mg/L resulted in quick induction, total immobilization and fast recovery in *Etroplus suratensis* juveniles. Although higher concentrations of TMS achieved shorter induction times, above mentioned concentrations (45-60 mg/L) were effective and presented a good margin of safety when compared against the above efficacy criteria. Except the lowest concentration of TMS (45 mg /L), there were modest significant differences between induction time of *Etroplus suratensis* ($p>0.05$). MS-222 toxicity and effectiveness in gilthead sea bream were higher at ML than at MD and, consequently the time needed to induce anaesthesia by means of a sub lethal concentration was shorter during the day, while the recovery time was longer (Ortuño et al., 2002). Potential day/night differences in cell permeability might also have an influence, although this should be studied further and corroborated in the sea bream. However, when lethal concentrations are used, but the gills are artificially ventilated, fish can be kept alive for a longer period (Brown, 1987). The rate of anaesthetic elimination during recovery also increases with artificial ventilation (Kiessling et al., 2009).

2.5.6 Hypothermia (as described by the *AVMA Guidelines on Euthanasia*)

2.5.6.1 Induction behavior

Collectively, In the present study results indicate that at different levels of hypothermia (22±1°C to 8±1°C) can be used to induce anaesthesia ranging from subtle calming to complete immobilization and loss of equilibrium. Temperature is one of the most important environmental factors, as it determines the distribution, behaviors and physiological responses of animals (Chou et al., 2008). However, rapid increases or decreases in ambient temperature may result in sub lethal physiological and, or behavioural responses (Donaldson et al., 2008). Pickering (1993) proposed sedation or mild anaesthesia as a stress-ameliorating measure during handling and transportation of fish. Hypothermia is also known to reduce the stress in fish handling, either by itself or in combination with chemical anaesthetics (Rodman, 1963; Hovda and Linley, 2000; Ross and Ross, 1999). In addition, rapid cooling generated fewer indicators of distress (Wilson and Bunte, 2009). The behaviours like rapid opercular movements and erratic swimming could be indicators of distress, but they are observed so frequently with chemical anaesthesia in the fish that they typically are considered as behavioral responses during some stages of anaesthesia. (Wilson and Bunte, 2009). Hypothermia has been used successfully as a sedative in several species (Solomon and Hawkins, 1981; Yokohama et al., 1989). Rapid cooling affords several advantages as a method of zebra fish euthanasia (Wilson and Bunte, 2009) were subjected 190 adult pink salmon *Oncorhynchus gorbuscha* to water temperatures of −1.5, −3.0, −4.5, and −6.0°C to evaluate the potential of hypothermia for anaesthesia (Hovda and Linley, 2000). In carp,

previously acclimated to 23 °C, would be safely held at 5 1C for 5 h, and achieved sedation at 8-14° C for 24h (Yoshikawa et al., 1989).

In the present study at hypothermic condition of 22±1°C there is no sedative effect. In juvenile of *M. estor* rapid temperature reduction from 24 to 21 °C or 18 °C had no significant sedative effect, but at 15°C swimming ceased in all fish after 2 min and 80% had lost touch sensitivity after 4min (Ross et al., 2007).

But at a next concentration of 18±1°C, it can induce only stage1 of anaesthesia within 66.14 ± 6.54 (~1min). Light anaesthesia that permits fish to maintain equilibrium, swimming activity, and breathing can be effective for mitigating stress associated with fish handling and fish transport (McFarland, 1960; Piper et al., 1982). During the next decreasing condition of hypothermia (16±1°C) the induction time (stage3) is 156.4 ± 0.2 (~2min 60s). In the present study, the induction times decreased significantly with the increasing concentrations of hypothermia, ($P<0.05$). In the case of hypothermia the results are in agreement with previous studies in juvenile of *M. estor* (Ross et al., 2007), adult pink salmon *Oncorhynchus gorbuscha* (Hovda and Linley, 2000). Coupled with this variation in depth of anaesthesia, we observed substantial differences in physiological disturbance and behavior during transportation. In addition, behavioral and physiological recovery rates varied with level of anaesthesia (Cooke et al., 2004). However, the present results clearly identified a range of hypothermia concentrations that are optimal for fish handling and transport. Specifically, concentrations of hypothermia ranging from 22±1°C to 8±1°C yielded rapid and stable stage 3 anaesthesia (Appendix 2; Stoskopf, 1993). During transport, fish anaesthetized at stage

3 level exhibited reduced activity and interaction, but were able to maintain equilibrium, swimming capacity, and avoid physical damage resulting from collision with the tank walls. These findings are reliable with the belief that stage 3 is efficient for minimizing fish injure during transport. Furthermore, the magnitude of anaesthesia was low, the induction time was too long relative to *Etroplus suratensis* anaesthetized at other levels, or nonanaesthetized controls. We discuss our findings in the context of using low concentrations of clove oil for fish handling and transportation.

Our experience with using hypothermia to anaesthetize *Etroplus suratensis* for a number of procedures indicates that at higher concentrations, induction of *Etroplus suratensis* is rapid (Sindhu, personal observations). Fish exposed to high levels of hypothermic condition in our study (12±1°C to 8±1°C) spent much of their time lying on the bottom, often on their side or upside down. At the higher end of concentrations that yielded stage 2 anaesthesia, induction was rather rapid, requiring less than 3 min (66.1±0.6 to 11.4±1.0). This timing is more consistent with the rapid induction times previously noted among many studies of hypothermia. At 12 °C, swimming ceased and touch sensitivity was suppressed immediately, resulting in a form of deep sedation (Ross et al., 2007). A similar effect was recorded at 9 °C but there was also some loss of equilibrium and 65% of the fish ceased opercular movements after 4min. This reduced to only 30% after 90min, suggesting acclimation to the lower temperature. Recovery was uneventful, requiring progressively longer from lower temperatures. This also provides support that 16 ±1°C is an effective concentration for slowly inducing stage 3 anaesthesia for juveniles of *Etroplus suratensis*. Stage 3 involves loss of partial equilibrium and most fish either maintain equilibrium and stay at stage 2 or

lose equilibrium completely and progress to stage 4. When hypothermia was used alone, stable sedation of juvenile of M. estor was induced at 15 and 12 1C, with no mortalities and full recovery after about 8min (Ross et al., 2007). Other researchers used low concentrations of hypothermia indicated protracted induction times relative to higher concentrations. The time to each anaesthetic stage (sluggishness, loss of movement, and complete anaesthesia) declined with decreasing temperature, but did not differ significantly between sexes (Hovda and Linley, 2000).In the present results it is observed that times from exposure to the water bath until animals lost the ability to swim and lost the righting reflex (stage3). In hypothermia the duration of time required to reach a stable level of anaesthesia was shorter than previously documented anaesthetics. Although there are a number of factors including water temperature (Hama´cˇkova´ et al., 2001; Walsh and Pease, 2002), fish size (Woody et al., 2002), and gender (Woody et al., 2002) that may affect induction time.

2.5.6. 2 Recovery from hypothermia

In the present results the concentration of 22±1°C hypothermia was not sufficient to sedate the juveniles of *Etroplus suratensis,* while 50 % of the juveniles reached the stage. During the next concentration (18±1°C) most fish achieved either stage 3 anaesthesia, which is indicative of partial loss of equilibrium, and completely recovered within 58.28±1.80 (~1 min) and is considered as the suitable hypothermic stage. In the present results of the concentration of 16±1°C, it recovered with in 100.1± 4.5 (~1 min 66s) according to the criteria of Stoskopf, 1993-Appendix 2). In the present results the next concentration (12±1°C) gave considerably longer recovery for juveniles (183.1±0.7; ~3 min 05s). It was observed that fish exposed to

increasing levels of hypothermic anaesthesia, exhibited slower behavioral recovery. Fish exposed to anaesthesia generally exhibited increased recovery time with increasing concentrations of hypothermia (Ross et al., 2007). Higher concentrations will introduce faster anaesthesia than lower concentrations, but will hence correspond with longer hypothermia (rapid temperature reduction from 22±1°C, 18±1°C, 16±1°C, 12±1°C, 8±1°C), yielding deeper levels of recovery time (Hveding, 2008; Gomes et al., 2001; Hoskonen and Pirhonen, 2004a). On the other hand, the present results show, recovery times increased with increasing concentrations (increasing hypothermic condition) of anaesthetic in fingerlings of *Etroplus suratensis* .Time to recovery was also influenced by temperature and was directly related to the time to complete anaesthesia (Hovda and Linley, 2000). McFarland and Klontz (1969) argued that the recovery time was proportional to the concentration and exposure time of the anaesthetic. Recovery time will, however, depending upon chemical, dosages and exposure time. At 8±1°C the recovery time is slightly longer than other hypothermic conditions (232.7±0.6; ~3min 87s). Prolonged recovery with increased anaesthetic dosage has been reported in sockeye salmon (Woody et al., 2002) and cobia (Gullian and Villanueva, 2009). In particular, those fish that reached level 4 and 5 anaesthesia (Appendix1; Summerfelt and Smith, 1990) required between 10 and 30 min to recover behaviorally (Cooke et al., 2004). When hypothermia was used alone, stable sedation of *M. estor* was induced at 15 and 12°C, with no mortalities and full recovery after about 8min. (Ross et al., 2007). In the present results complete recovery at 18±1°C and 16±1°C, but at 12±1°C and 8±1°C, it was very hardy to survive the juveniles of *Etroplus suratensis*, requiring progressively longer recovery time. Recovery was

uneventful, requiring progressively longer from lower temperatures (Ross et al., 2007). There was some degree of recovery with time, even during application of the treatment, suggesting a degree of acclimation to these temperatures. When the temperature was reduced further to11°C, the fish became stressed, exhibiting tachyventilation, darker body colour and partial loss of equilibrium. Although there was some degree of acclimation to this lower temperature, it would not be advisable to cool to this extent for transportation (Ross et al., 2007). The magnitude of anaesthesia was low, the recovery time was rapid, and the behavioral recovery was fast relative to *Etroplus suratensis* anaesthetized at other levels, or nonanaesthetized controls. The present results show that with increasing hypothermia concentrations, sedation and anaesthesia induction times were reduced, but recovery times exhibited the opposite pattern with fish experiencing more rapid recovery when exposed to lower hypothermia concentrations. The recovery times were significantly lower in hypothermia, at the lowest concentration ($P<0.05$). These results are in agreement with those found in other species anaesthetized with hypothermia (Hovda and Linley, 2000; Ross et al., 2007; Wilson and Bunte, 2009). Because of longer recovery time, especially in high concentrations of hypothermia is not advisable for juvenile *Etroplus suratensis* in short time applications to reduce working time and labor. In aquaculture settings, recovery of long duration would be problematic, particularly if the fish were being stocked to supplement a fishery (Cooke et al., 2004). Fish would be highly susceptible to predation and displacement by flow or currents during prolonged recovery so transport at these deep levels of sedation (i.e., N stage 2) would be undesirable, although behavioral recovery was more rapid for low concentrations than

controls (Cooke et al., 2004). When combined with the reduced oxygen demand during transport (inferred from lower cardiac output), ease of handling, and reduced interaction with conspecifics during transport, the use of the lower levels of hypothermia appears to be more favorable than transporting unanaesthetized fish. Hovda and Linley (2000) conclude that hypothermia is effective for short-term anaesthesia of Pacific salmon *Oncorhynchus* spp. for spawning but note that its application for freshwater stenohaline species may be problematic because of the physiological effects induced by cold shock and exposure to high salinity. Wilson and Bunte (2009) demonstrated that rapid cooling result in more rapid, less distressful, and more effective euthanasia than other anaesthetic agents.

2.5.7 Overall desirability function at different doses of the six anaesthetic agents

For finding out the best anaesthetics among the six anaesthetic agents i.e., clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS222 and hypothermia used in these experiments we found the average values of overall desirability functions of induction and recovery times at different doses for *Etroplus suratensis* (Table 1.7).

Clove oil for 0.23mg/L (0.451 ± 0.003), 0.30 mg/L (0.481 ± 0.001), 0.37 mg/L (0.592 ± 0.004). Hypothermia for 8°C/L (0.458 ± 0.002), 12°C/L (0.496 ± 0.001), 16°C/L (0.489 ± 0.001).The highest desirability value is obtained from clove oil at the concentrations of 0.37 mg/L, 0.30 mg/L, 0.23mg/Land for hypothermia at conditions of 12°C/L, 16°C /L, and 8°C/L. The minimum induction time and minimum recovery time attained for those doses are clove oil and hypothermia

2.5.8 Limitations of the study

In higher doses, the mortality rate is very high. Even though the induction and recovery times are within the limits of specified standards, the other anaesthetics do not perform well (table 1.7).

2.6 Summary

As of the above discussion, it is clear that the present results clearly identified a range of concentrations of anaesthetics that are optimal for fish handling and transport. There have been few controlled, systematic investigations of efficacy and physiologic effects for certain anaesthetics as piscine anaesthetic, and there is a need for more complete and concise ranges of safe and effective concentrations or dosages. Our research clearly illustrates the positive effects arising from anaesthesia. Our study result provided novel insights in to the efficacy of and utility of clove oil for fish transport. Clove oil concentrations used in our study were much lower and we observed no empirical evidence of gill collapse or damage and are optimal for fish handling and transport. At lower concentration fish that recover more rapidly have increased metabolic scope for engaging in other activities such as feeding, movement, predator avoidance, or preparation for successive stressors. We also hypothesized that application of hypothermia in *Etroplus suratensis* would result anaesthetization within a shorter time in minimal signs of distress. It can euthanize many animals simultaneously with minimum risk of handling and operation error when preparing the euthanasia bath and reduction in occupational health and safety risk to personnel associated with chemical and physical methods of euthanasia. The behavioural changes like rapid opercular movements and erratic swimming as

signs of distress after exposure to an ice–water bath never occurred. Based upon the positive results of our study using clove oil with hypothermia to transport *Etroplus suratensis* coupled with the growing body of literature cited we suggest that clove oil with hypothermia should be an effective alternative transport anaesthetic. Our study focused on the use of clove oil with hypothermia for fish transportation. The concentrations required to induce anaesthesia identified as optimal for fish transport should also be effective for the general handling of cultured fish for grading, marking, enumerating, inspection, and gamete stripping. This study is the first to identify euthanasia methods for *Etroplus suratensis*, (as described by the AVMA Guidelines on Euthanasia) and the outcome will be important in assisting institutional animal care and use committees and researchers in determining the most appropriate method of euthanasia for *Etroplus suratensis*. Further work will also be needed to determine its utility for large-scale operation. The results of this study comprise a refinement to *Etroplus suratensis* euthanasia techniques and provide more information on techniques necessary for *Etroplus suratensis* studies for the laboratory animal and biomedical research community.

…..ꙮ…..

Chapter 3

Haematological studies of juveniles of *Etroplus suratensis* exposed to optimum concentration of selected anaesthetics during 24 and 48 hours

Contents

3.1 Introduction
3.2 Materials and Methods
3.3 Haematological analysis
3.4 Statistical analysis
3.5 Post treatment survival
3.6 Results
3.7 Discussion
3.8 Summary

3.1 Introduction

Haematological indices are important parameters for the evaluation of fish physiological status. Kocabatmaz and Ekingen (1978) reported that, blood tissue reflects physical and chemical changes occurring in organism; therefore detailed information can be obtained on general metabolism and physiological status of fish in different groups of age and habitat (Ibrahim Orun and Umit Erdemll, 2002). Fish live in very intimate contact with their environment, and are therefore very susceptible to physical and chemical changes which may be reflected in their blood components (Wilson and

Taylor, 1993; Mc Leavy, 1973). In fish, exposure to chemicals or pollutants can induce either increases or decreases in haematological levels (Saliu and Salami, 2010). Studies have shown that when the water quality is affected by toxicants, any physiological change will be reflected in the values of one or more of the haematological parameters (Van, 1986). Thus water quality is one of the major factors, responsible for individual variations in fish haematology. However, in the fish, these parameters are more related to the response of the whole organism, that is, to the effect on fish survival, reproduction and growth (Saliu and Salami, 2010).

Blood cell responses are important indicators of changes in the internal and/or external environment of animals (Saliu and Salami, 2010). A number of haematological indices such as haematocrit (Ht), haemoglobin (Hb), total erythrocyte count (TEC) and so on are used to asses the functional status and oxygen carrying capacity of blood stream (Shah and Altindag, 2004). The count of red blood cells is quite a stable index and the fish body tries to maintain this count within the limits of certain physiological standards using various physiological mechanisms of compensation (Saliu and Salami, 2010). Evaluation of the haemogram involves the determination of the total erythrocyte count (RBC), total white blood cell count (WBC), haematocrit (PCV), haemoglobin concentration (Hb), erythrocyte indices (MCV, MCH, MCHC), white blood cell differential count and the evaluation of stained peripheral blood films (Campbell, 2004). Thrombocytes have been described as the most abundant blood cells after erythrocytes.

Anaesthesia is achieved by placing the fish into an anaesthetic solution that is absorbed through the gills and enters the arterial blood, from where it

acts on the central nervous system (Ross and Ross, 1999). It was demonstrated that anaesthetic concentrations and period of exposure to anaesthetic agents affect blood parameters; particularly stress indicators (Auperin et al., 1997; Holloway et al., 2004; Heo and Shin, 2010; Hoseini et al., 2010). Anaesthetics may affect blood parameters and hemolised tissues (McKnight, 1966). Other researchers have shown susceptibility of certain blood parameters to anaesthetic type and anaesthesia protocol (Auperin et al. 1997; Holloway et al., 2004; Hoseini et al., 2010). The use of haematological techniques is gaining importance for toxicological research, environmental monitoring and assessment of fish health conditions (Shah and Altindag, 2004). Blood parameters are considered patho-physiological indicators of the whole body and therefore are important in diagnosing the structural and functional status of fish exposed to toxicants (Adhikari and Sarkar, 2004; Maheswaran et al., 2008). It should be noted that although the mechanisms of fish physiology and biochemical reaction to xenobiotics have not been investigated enough, it is obvious that species differences of these mechanisms exist. Thus, it is necessary to evaluate the effects of each anaesthetic agent on certain blood parameters in different fish species. Haematological analyses also provide quick screening methods for assessing the health of fish and can be used to determine the incipient lethal concentration of a toxicant (McLeavy, 1973).

The aim of this study was to assess changes in haematological profile of juvenile fishes of Pearl spot (*Etroplus suratensis*) after 24 and 48 h exposure of effective concentration viz., clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS222 and hypothermia (cold)

3.2 Materials and Methods

3.2.1 Fish and experimental conditions

Collection, maintenance, size class, acclimatization and experimental designs and preparation of samples were the same and explained in details in chapter 1 section 1.1, 1.2, 1.3, 1.3.1, 1.4, 1.4.1, 1.4.2, 1.4. 3 and 1.5.

3.3 Haematological analysis

For the haematological profile tests, groups of juvenile fishes of Pearl spot (*Etroplus suratensis*) size classes; (2.078 ±0.15g and 4.0±0.1cm) were used. A total of 96 fish from each of six experimental groups were divided into four groups and examined: Control 1 (controls examined in parallel with Experiment I), Experiment I (24 h exposure of anaesthesia at optimum concentrations of six anaesthetics), Experiment II (48 h exposure of anaesthesia at optimum concentrations of six anaesthetics) and Control II (controls examined in parallel with Experiment II). Those concentrations were based on the result from previous experiment. Each treatment was carried out in three replicates. All groups of fishes treated and packed with optimum concentration anaesthetics. The packing system involved Fifty four LDPE bags (37.5x20cm) were used. Double polyethylene bags, one slipped into another, were used to insure against water loss from perforations or leakage. The anaesthetic was vigorously stirred into the packaging water before the fish were put in. All sets of experimental bags were then flattened to the water surface to expel the air, inflated with medical grade oxygen gas, secured airtight and sealed with rubber bands and finally, put it in the Styrofoam box lined with a 1cm polystyrene sheet for insulation.. Four ice cube packs were also put in the space between bags in Styrofoam box. The

Styrofoam box was left in the laboratory to the thermostatically controlled chilling unit (Rotek Instruments, Chest type model, temperature range 0-30°C, M/S.B and C Instruments, Kerala) for keeping the transportation condition. This unit maintains the temperature of 22 ± 1°c for the test period i.e. 24 and 48 hr and also maintains the temperature required for the hypothermic anaesthetic condition. After the end of simulated-transport of 24 and 48 h, the bags were opened for haematological and water quality analysis (following the above-mentioned procedure) to determine the fish physiological changes made by the use of anaesthetics. Blood samples and water samples were taken, and fish were transferred into the fiberglass tanks. Any dead fish were separated and counted with subsequent calculations of mortality levels. The survived fish were reared and observed for mortality and health condition for 7 days.

3.3.1 Blood sampling procedures

Heparinised injection needles were used to take blood samples from heart of fish to stabilize the blood samples, aqueous solution of heparin sodium salt at 0.01 ml per 1 ml blood was used (Svobodová et al., 1991). Owing to insufficient amount of blood, the haematocrit determination for each experimental schedule was done on pooled samples in triplicate in sterile heparanized vials. Each triplicate was duplicated thus obtaining six samples per treatment. Blood samples were taken at least 40 seconds after collecting the fish from the water bath. Blood-filled heparanized microhaematocrit capillary tubes were centrifuged at 12000 for 5 minutes using a microhaematocrit centrifuge (Hermle model Z320) and the haematocrit (Hct) values were read directly. The haemoglobin concentration

was measured by the cyanmethaemoglobin method (Blaxhall and Daisley, 1973) at a wavelength of 540nm. Concurrently, the Total Red Blood Cell (RBC), White Blood Cells (WBC) and Mean Corpuscular Volume (MCV) were obtained by employing a Coulter-model T540 cell counter. The Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC) were calculated using the methods described by Dacie and Lewis (1966). MCH was calculated in picograms/cell = (Hb/RBC) x 10 and MCHC = (Hb 100mg blood/Hct) x 100. The packed cell volume (PCV) was determined employing microhaematocrit centrifugation method. Similar procedures were adopted for the control (untreated) groups belonging to the respective treatment periods. Blood sampling was completed in less than 2 minutes; the entire autopsy procedure was performed in less than 4 minutes to minimize the risk of stressful condition.

3.4 Statistical analysis

Significant differences among treatments were analyzed using one way analysis of variance (Snedecor and Cochran, 1967). Scheffe's multiple comparison tests were used to determine differences between treatments means. Results were considered statistically significant when $p<0.05$.

3.5 Post treatment survival

After 48 hours of experiment, the experimental bags containing the remaining fishes with good aeration were put into Fiber Reinforced Plastic tanks containing aerated water for 1 h and after that the fishes were allowed to come out slowly from the bags. Separated tanks were maintained for all the sets of experimental groups for observing post-transport mortality for seven days after simulated transport .The water temperature in the tanks was

28±1°C with an average dissolved oxygen level of 12mg/ L and the fishes were fed with pelleted feed.

3.6 Results

Results of the effect of optimal concentrations of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS222 and hypothermia on haematological parameters are shown in Table 3.1.The haematological indices tested were haematocrit (Hct %), hemoglobin (Hbg), packed cell volume (PCV%), red blood corpuscle (RBC/mm^3), mean corpuscular volume (MCVμ3), mean corpuscular hemoglobin (MCHpg), mean corpuscular hemoglobin concentration (MCHC%).

Results of the present investigation showed certain haematological parameters (Hct, Hb, RBC, MCV, MCH, PCV and MCHC) in the blood of *Etroplus suratensis,* during prolonged exposure (24 and 48 h) to the optimum concentrations of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS222 and hypothermia (Table 3.1).As a result of progressive exposure to the optimum concentration of the anaesthetics for 24 h, *Etroplus suratensis* showed significant decrease in Hct, Hb, RBC, MCV, MCH, PCV and MCHC values (Fig.3.1-3.7) than the control except in hypothermic values. But there were significant difference noticed between the haematocrit levels of all anaesthetic groups and control during 48h (Table 3.1).

Table 3.1 Haematological indices of juveniles of *Etroplus suratensis* exposed to optimal concentrations of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS222 and hypothermia during 24 and 48 hours

parameters	Hrs	Control (Mean ± SE)	Clove (Mean ± SE)	CIN (Mean ± SE)	Zn(Mean ± SE)	TB (Mean ± SE)	MS (Mean ± SE)	Hypo (Mean ± SE)	p-value
Hct%	24	0.028±0.004	0.059±0.008	0.034 ±0.007	0.066±0.006	0.056±0.007	0.051±0.003	0.052±0.003	**0.000**
Hct%	48	0.042±0.005	0.047±0.004	0.027 ±0.002	0.048±0.003	0.049±0.006	0.034±0.003	0.049±0.003	**0.000**
Hbgm	24	3.45±1.061	3.282±0.344	2.478 ±0.480	4.369±0.402	3.817±0.409	3.345±0.259	3.617±0.228	0.315
Hbgm	48	3.0666±0.357	3.298±0.284	1.9687 ±0.11	3.335±0.184	3.574±0.415	2.412±0.218	3.425±0.22	**0.000**
pcv%	24	10.323±3.166	9.8583±1.03	7.434 ±1.435	13.107±1.206	11.454±1.222	10.035±0.776	5.6118±0.392	**0.027**
pcv%	48	9.1998±1.072	9.9±0.852	5.9061 ±0.34	10.001±0.549	10.722±1.246	7.236±0.653	10.275±0.665	**0.000**
RBC	24	1.143±0.352	1.091±0.115	0.822 ±0.158	1.453±0.134	1.269±0.136	1.111±0.087	1.206±0.075	0.307
RBC	48	1.022±0.119	1.098±0.096	0.655 ±0.038	1.1105±0.061	1.142±0.171	0.8006±0.073	1.143±0.074	**0.003**
MCV	24	90.45±0.163	90.263±0.07	90.294 ±0.08	90.213±0.079	90.201±0.048	90.396±0.116	89.792±0.052	**0.000**
MCV	48	90.024±0.007	90.249±0.13	90.214 ±0.09	90.099±0.054	90.207±0.107	90.462±0.1243	89.84±0.065	**0.001**
MCH	24	30.193±0.023	30.111±0.03	30.096 ±0.03	30.067±0.025	30.088±0.026	30.129±0.038	29.92±0.033	**0.000**
MCH	48	30.006±0.002	30.081±0.04	30.091 ±0.04	30.033±0.018	30.083±0.042	30.166±0.044	29.92±0.033	**0.000**
MCHC	24	33.344±0.05	33.263±0.03	33.33 ±0	33.307±0.023	33.307±0.023	33.33±0	33.372±0.011	0.079
MCHC	48	33.33±0	33.33±0	33.33 ±0	33.33±0	33.33±0	33.33±0	33.33±0	1.000

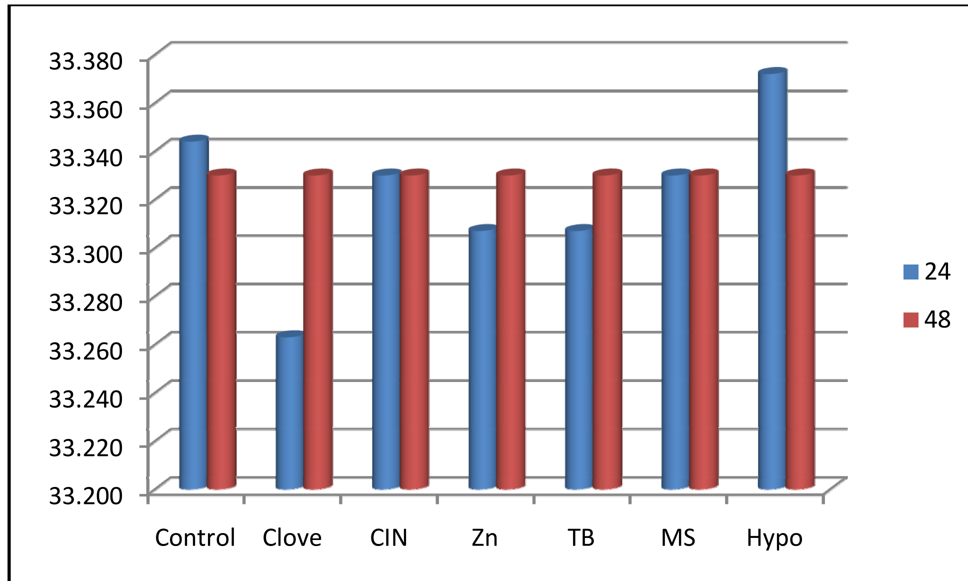


Fig.3.1 Haematocrit (HCT %) of *Etroplus suratensis* exposed in optimum concentration of certain anaesthetics during 24 and 48 h

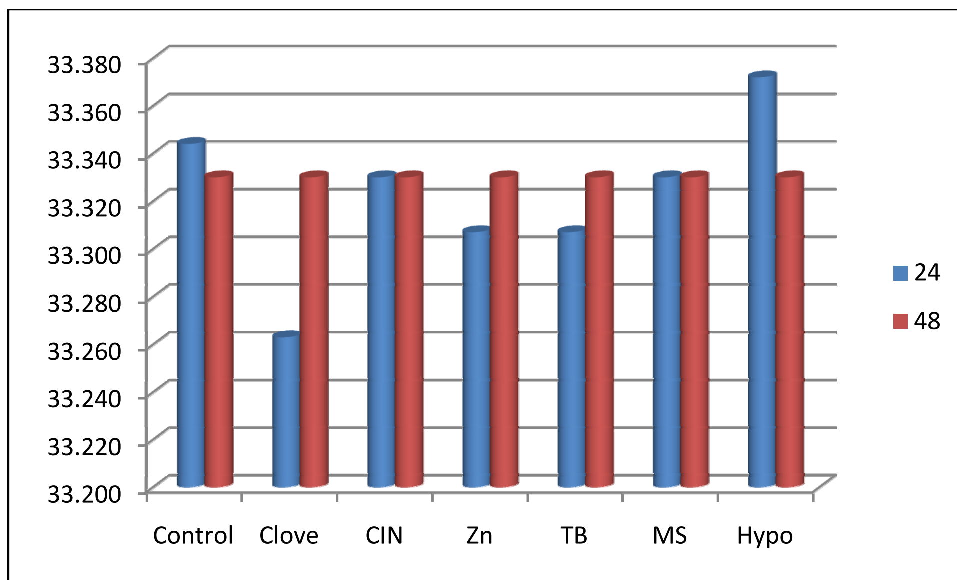


Fig.3.2 Hemoglobin (Hb (g)) of *Etroplus suratensis* exposed in optimum concentration of certain anaesthetics during 24 and 48 h

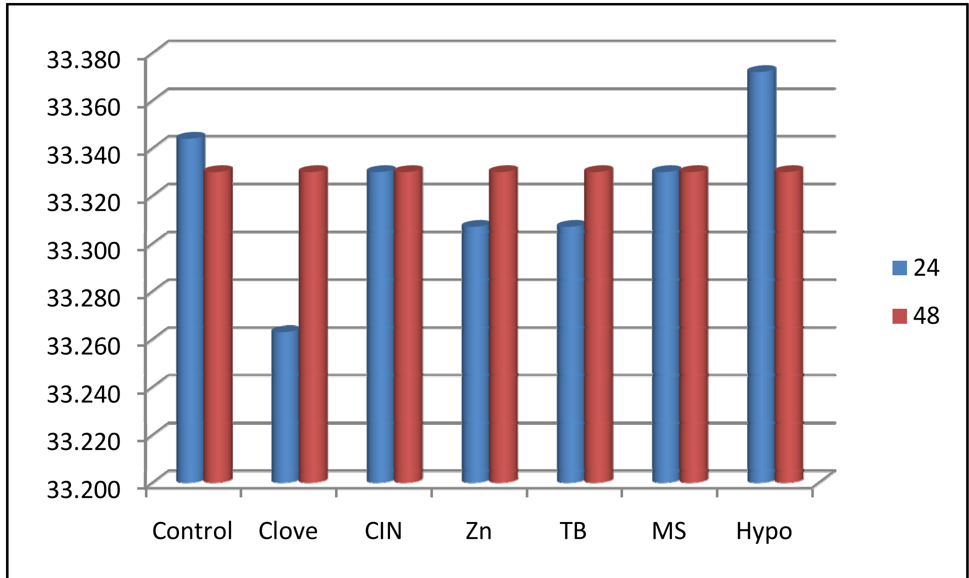


Fig.3.3 PCV (%) of *Etroplus suratensis* exposed in optimum concentration of certain anaesthetics during 24 and 48 h

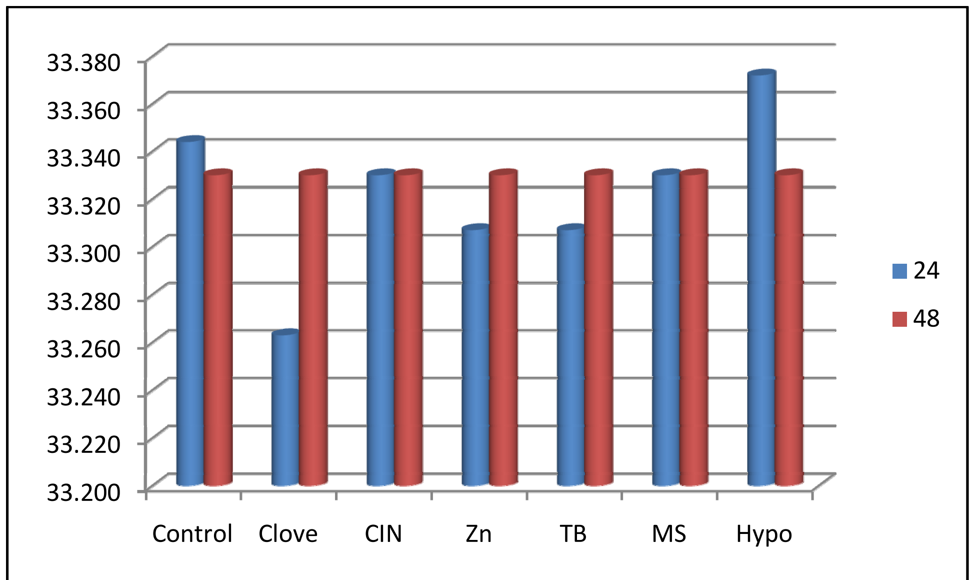


Fig.3.4 RBC (million) of *Etroplus suratensis* exposed in optimum concentration of certain anaesthetics during 24 and 48 h

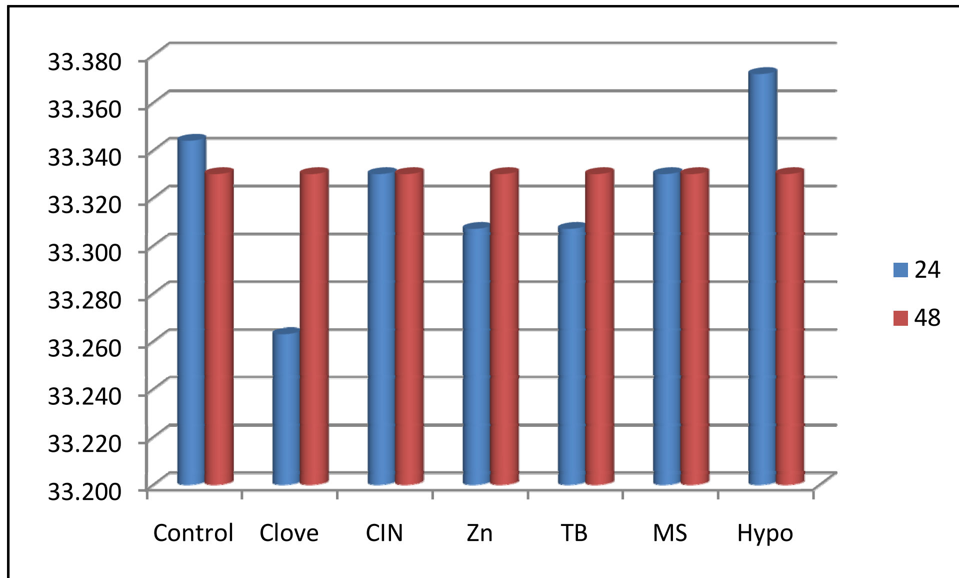


Fig.3.5 MCV (mu) of *Etroplus suratensis* exposed in optimum concentration of certain anaesthetics during 24 and 48 h

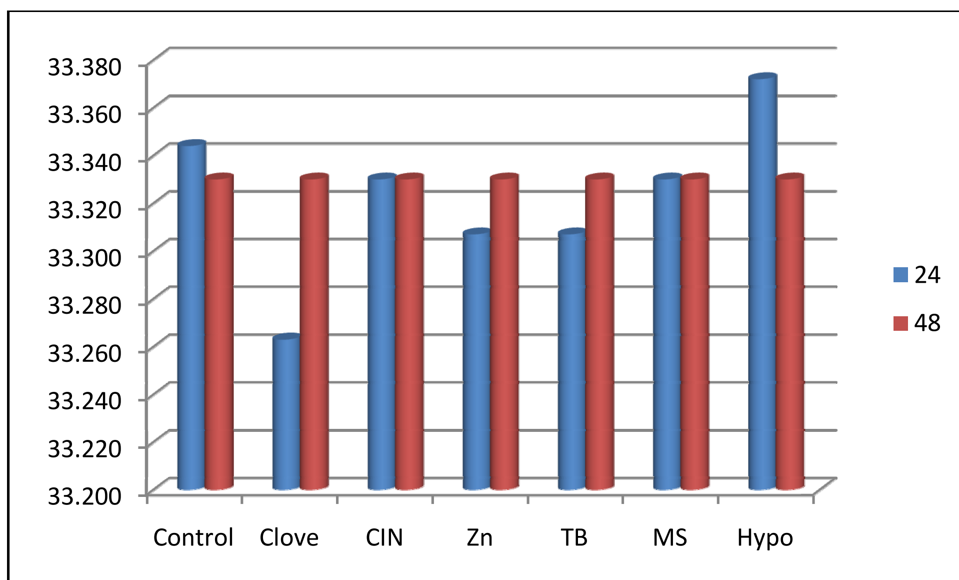


Fig.3.6 MCH (pg) of *Etroplus suratensis* exposed in optimum concentration of certain anaesthetics during 24 and 48 h

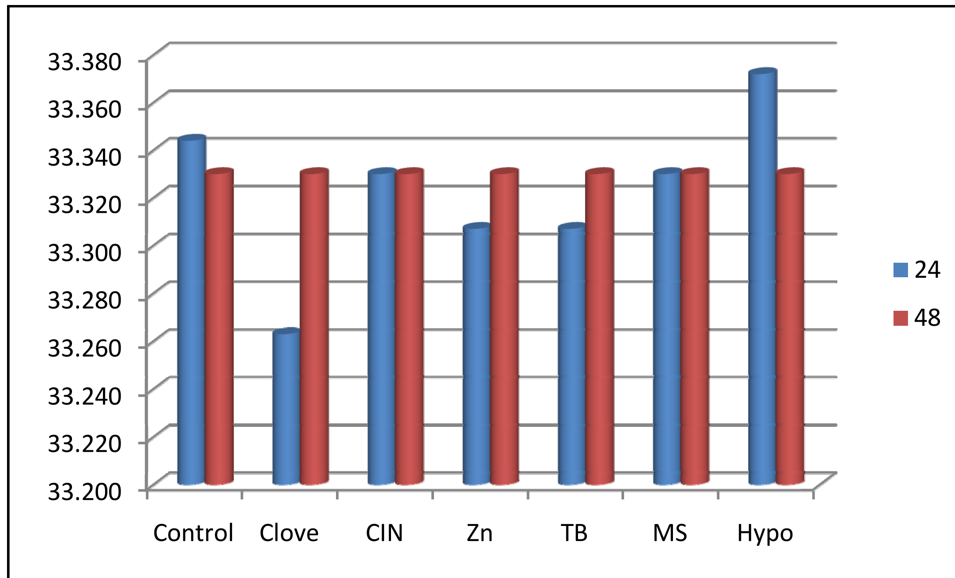


Fig.3.7 MCHC (%) of *Etroplus suratensis* exposed in optimum concentration of certain anaesthetics during 24 and 48 h

The results of Analysis of variance (ANOVA) procedure for haematological parameters are summarized in table 3.1.Since the p-value greater than significance level 0.05, the differences in mean values of different haematological parameters are not statistically significant. Further comparison by one way analysis of variance shows that differences between the values of different anaesthetic groups and control are not statistically significant. That means there is a significant difference between the haematological indices (Hct, Hb, RBC, MCV, MCH, PCV and MCHC values) of control and other experimental groups during 48h (Fig.3.1-3.7). The mean p values of Hct of all the anaesthetic groups and exposure time (24 and 48 h) is less than 0.05 ($p < 0.05$) the mean value for each concentration and control vary significantly. From the table it can be seen that the recorded value increased with exposure time in clove oil, cassumunar ginger extract, tobacco leaf

extract. At the same time level of Hct remains constant in cinnamon oil, MS222 during 48 h with the control which is reversible in the case of hypothermia. One way analysis of variance shows that there is no significant difference among haemoglobin levels during 24hr ($p>0.05$) which was reverse in 48hr ($p<0.05$). The mean p values of PCV of all the anaesthetic groups and exposure time (24 and 48 h) is less than 0.05 ($p <0.05$) the mean value for each concentration and control varies significantly. In the case of RBC level the mean p values are greater than 0.05 ($p>0.05$). There were no significant difference between all the anaesthetic groups and control during 24hr. But during 48hr the mean p value is less than 0.05. There were significant difference in RBC level among all the anaesthetic groups and control. Similarly there were significant difference in MCV ($p<0.05$) and MCH ($p<0.05$) values of all the anaesthetic groups and control during 24 and 48h. In the case of MCHC level the mean p values are greater than 0.05 ($p>0.05$). There were no significant difference among all the anaesthetic groups and control during 24hr and 48h.

There was strong correlation between the exposure time and haematological indices tested, viz., haematocrit (Hct %), hemoglobin (Hb g), packed cell volume (PCV %), red blood corpuscle (RBC /mm^3), mean corpuscular volume (MCVμ3), mean corpuscular hemoglobin (MCH pg), mean corpuscular hemoglobin concentration (MCHC%).The mean p values of Hct ($p <0.05$) for 24 h and 48h, Hb ($p >0.05$) for 24 h and ($p <0.05$) for 48 h, PCV ($p <0.05$) for 24 h and 48 h, RBC ($p >0.05$) for 24 h and ($p <0.05$) for 48 h, MCV ($p <0.05$) for 24 h and 48 h, MCH ($p <0.05$) for 24 h and 48 h, MCHC ($p >0.05$) for 24 h and 48 h levels.

Hypothermia

[Hct] in control fish was 0.028 ± 0.004 % for 24hr and0.042±0.005 % for 48hr and this rose to 0.052 ± 0.003 % for 24hr and 0.049±0.003 % for 48 hr in cold acclimated fish. The Hb of cold acclimated fish (3.617 ± 0.228 g L-1 for 24hr and 3.425±0.222 g L-1 for 48hr) was elevated in comparison with control fish (3.450 ± 1.061 g L-1(24hr) and 3.067±0.357 g L-1(48 hr) P<0.05). In the present work the control fish was compared with the MCHC values of 33.344 ±0.046 % (24hr) and 33.330 ±0.000% (48hr) with the cold acclimated fish 33.372±0.011 (24hr) and 33.330±0.000 (48 hr).

3.7 Discussion

The results of the present investigation showed certain haematological parameters in the blood of *Etroplus suratensis,* during prolonged exposure (48h) to the optimum concentrations of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS222 and hypothermic condition (Table 3.1).When anaesthetic was used in scientific research, anaesthetics as well as the protocol of anaesthesia might change fish blood parameters, particularly stress indicators. Here the results showed that different anaesthetic exposure (24 and 48hr) affects some blood parameters which were in agreement with study on Beluga (Hoseini et al., 2010). WBC and differential leukocyte count are factors which might change during stress (Wedemeyer et al., 1990). Previous studies have demonstrated that effect of stress on WBC and differential leukocyte count are inconsistent. Barcellos et al. (2004) reported change in WBC and differential leukocyte count in *Rhamdia quelen* (Quoy and Gaimard) following acute as well as chronic stress; however Montero et al. (1999) reported no significant changes in these parameters due to chronic stress in *Sparus aurata* (Linnaeus).

The present result showed that the haematological level of the hypothermic group (16°C) was significantly higher than that of other anaesthetic groups and control. (Fig.3.1-3.7). Control levels of haematological parameters in the present study were near the values reported by Mohammadi zarejabad et al. (2009) and Falahatkar et al. (2009) in Beluga. In the present study there was no significant difference in 24hr Hb level, RBC level and 24 and 48hr MCHC levels among different anaesthetic groups with control. But there was significant difference in Hct, PCV, MCV and MCH count among different anaesthetic groups with control. It might be due to long periods of stress caused by anaesthesia (maximum 48hr). But Hb and RBC level showed significant differences with the control group during 48hr.This might be due to the value of haematocrit, relative and actual count of monocytes had returned back to normal within 24 hours (Velisek et al., 2007).

In the present study there were significant difference noticed between the haematocrit levels of all anaesthetic groups and control during 24 and 48h (Fig.3.1-3.7). In the present study, the experimental groups of anaesthesia did not cause an elevation of the percent haematocrit level than the control except in hypothermia during 24hr of exposure of *Etroplus suratensis.* Similar results were reported in another study using a similar concentration of clove oil anaesthesia in sea bream (Tort et al., 2002).There was no significant difference in the haematocrit levels of fish anaesthetized with clove oil or benzocaine (Bressler and Ron, 2004). Similarly, in the present study the haemoglobin levels of all anaesthetic groups showed significant difference during 48hr. But there is no significant difference among haemoglobin levels during 24hr. Since many anaesthetics including eugenol have inhibitory effects on respiratory system, oxygen carrying capacity of the blood should

be increased to combat eugenol effects (Wendelaar Bonga, 1997). This condition leads to increase in Hct, Hb and RBC levels with control during 48hr (Hoseini et al., 2010). In the present result there were significant difference noticed in the PCV values among all anaesthetic groups and control during 24 and 48h. In the case of RBC level there were no significant difference among all the anaesthetic groups and control during 24hr. But during 48hr there were significant difference in RBC level among all the anaesthetic groups and control. Following these changes, haematological indices (MCV, MCH and MCHC) might change because they are related to Hct, Hb and RBC (Hoseini et al., 2010). Similarly in the present result there were significant difference in MCV and MCH of all the anaesthetic groups and control during 24 and 48h. But MCHC levels in all anaesthetic groups and control showed no significant differences.

In the present work there was lower haematological levels (Hct, Hb, RBC, PCV, MCV, MCH and MCHC) than the control at 24hr except in hypothermia and comparatively equal with control in Hct, Hb, RBC, PCV, MCV, MCH and MCHC levels during 48hr. Thus, it seems in constant period of exposure, higher concentrations of anaesthetics might be more stressful in fishes than lower ones (Hoseini et al., 2010). This recent observation suggests period of exposure is an important factor causing stress response in Beluga (Hoseini et al., 2010) which was in agreement with the present study on *Etroplus suratensis*.

3.7.1 Clove oil

In the present study, the clove oil anaesthesia did not cause an elevation of the percent haematocrit level than the control. However, Velisek et al.

(2005a, b) reported no significant effects of clove oil exposure on haematological parameters in common carp, *C. carpio* (L.) and rainbow trout, *Oncorhynchus mykiss* (walbaum). Mohammadi Zarejabad et al. (2009) showed increase in Hct, Hb and RBC and no change in MCV, MCH and MCHC levels as a result of exposure to different concentrations of clove solution over a constant period (10 min). On the other hand, previous works on anaesthesia showed no significant change in WBC and differential leukocyte count after clove solution (Mohammadi Zarejabad et al., 2009) or clove oil (Velisek et al., 2005a, b) exposure. The authors found higher Hct, Hb and RBC levels in fish exposed to higher concentrations of clove solution. Strong positive correlation between induction time and Hct, Hb and RBC levels further suggests that anaesthesia with low concentrations of clove solution over a long period is stressful compared to anaesthesia with high concentrations over short period. The results are supported by previous study on effect of clove solution on serum biochemical parameters in Beluga (Hoseini et al. 2010). Likewise, Auperin et al. (1997) and Heo and Shin (2010) reported similar results in Tilapia, *Oreochromis niloticus* (Linnaeus) and common carp, *C. carpio* (L.) anaesthetized with 2-phenoxyethanol and benzocaine, respectively. Mohammadi Zarejabad et al. (2009) showed there are no irreversible effects on haematological parameters of Beluga, using clove solution. Thus, it seems in constant period of exposure, higher concentrations of clove solution might be more stressful in Beluga than lower ones Hoseini et al. (2010). This recent observation suggests that period of exposure is an important factor causing stress response in Beluga Hoseini et al. (2010) which were in agreement with the present study on *Etroplus suratensis*.

3.7.2 Cinnamon oil

In cinnamon treated group, anaesthesia did not cause an elevation of the percent haematocrit level with control rather than Hb (24 h) and MCHC (24 and 48 h). In the present study the Hct, Hb, RBC, MCV, MCH and MCHC levels in cinnamon treated groups showed no significant increase than control group. The Hct and haemoglobin concentration of fish exposed to the highest concentration of eugenol did not always increase (Sladky et al., 2001). This may have been a function of the brief exposure to the anaesthetic bath, which may have been of insufficient duration to reliably affect the dynamics that would alter Hct (Sladky et al., 2001). Blood plasma profile showed an improvement in haemoglobin (HB), red blood cell (RBCS), haematocrite (PCV), total protein, and total lipids, while there was a decrease in creatinin, urea, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and glucose in fish fed 1% cinnamon in Nile tilapia (Ahmad et al., 2011). In this study the results of HB, RBCs, and PCV analysis shows that blood parameters were highest in Nile tilapia fed 1% cinnamon compared to the other treatments (Ahmad et al., 2011). Since many anaesthetics (clove oil and cinnamon oil) including eugenol have inhibitory effects on respiratory system, oxygen carrying capacity of the blood should be increased to combat eugenol effects. This condition leads to increase in Hct, Hb and RBC levels. Following these changes, haematological indices (MCV, MCH and MCHC) might change because they are related to Hct, Hb and RBC (Wendelaar Bonga, 1997).

Similarly there was also insignificant decrease of red blood cells counts in all cinnamon oil treated groups but again in normal physiological range which shows similarity with the result of Rafeeq et al. 2013 reported that

there was insignificant decrease in hemoglobin concentrations in all treated groups but it was in normal physiological range. These results are compatible with another study reported to show no significant difference in packed cell volume and hemoglobin concentration between *Cinnamomum zeylanicum* as compared to control groups of male rats after 6 weeks of study period (Chukwadi et al., 2011).

3.7.3 Cassumunar ginger extract

In cassumunar ginger extract treated group, anaesthesia causes an elevation of certain haematological parameters with control rather than Hct (24 and 48 h), Hb (48 h) PCV (24 and 48 H), RBC (48 h) MCV and MCH (24 and 48 h). In the present study there are significant differences in the levels of Hb (24 h), RBC (24 h) and MCHC (24 and 48 h) (Table 3.1). There is no comparative literature in juveniles of *Etroplus suratensis* treated with cassumunar ginger extract anaesthesia causes an elevation of certain haematological parameters

3.7.4 Tobacco leaf (*Nicotiana tobaccum*)

The effect of the tobacco leaf dust extract on some haematological parameters of *Etroplus suratensis* shown in (Table 3.1) revealed significant difference ($p<0.05$) between the control and treatment group except for the mean cell haemoglobin concentration (MCHC). Olufayo, and Jatto, 2011 reported in his study of “Haematological Response of Nile Tilapia (*Oreochomis Niloticus*)” that juveniles exposed to tobacco (*Nicotiana tobaccum*) leaf dust, has effects on the blood parameters of the test fish and consequently the biodiversity of the organisms. Similar results were reported in another study using dry tobacco (*Nicotiana tobaccum*) leaves aqueous

extracts in *Oreochromis niloticus* (Omoniyi et al., 2002). Experiment conducted using dry tobacco (*Nicotiana tobaccum*) leaves aqueous extracts on some haematological indices of *Oreochromis niloticus was* found to have an inverse relationship with the heamatolgical indices assessed. In the present work there was significant difference among the dry tobacco (*Nicotiana tobaccum*) leaves extracts and control in the level of Hct, PCV, Hb (24hr), RBC (24hr), MCV and MCH. Statistical analysis using ANOVA revealed that there was a significant difference ($P<0.05$) in the value of red blood count (RBC) and haemoglobin (Hb) in *Oreochromis niloticus* (Omoniyi et al., 2002). Similar reduction of haematological indices was reported by Musa and Omoregie (1999) when *C. gariepinus* was treated with sub-lethal doses of malachite green. Omoregie et al. (1994) had earlier reported similar observations when they subjected *O. niloticus* to sub-lethal concentrations of formalin. After 48h in anaesthetized groups treated with dry tobacco (*Nicotiana tobaccum*) leaves it was observed that all fishes are in paled condition. The reduction in these blood parameters is an indication of anaemia caused by exposure to the extract of tobacco leaf dust. Also, haematological indices indicated that the fish became anaemic and the severity of this condition was directly proportional to the tobacco dust concentrations (Omoniyi et al., 2002). This anaemic response could be as a result of destruction of erythrocyte or inhibition of erythrocyte production (Wintrobe, 1978) or haemodilution as reported by Sampath et al. (1993). The statistically significant ($p<0.05$) decrease in values of the haematological parameters has been reported (Olufayo, and Jatto, 2011).When juveniles of Nile Tilapia (*Oreochromis niloticus*) exposed to tobacco (*Nicotiana tobaccum*) leaf dust, the haematological analysis of the blood revealed

significant haematological changes; the intensity of haematology damages increased with increasing concentrations and exposure to tobacco leaf dust.

3.7.5 MS222

The effect of MS222 on some haematological parameters of *Etroplus suratensis* shown (Table 3.1) revealed significant difference ($p<0.05$) between the control and treatment group. There were significant differences in Hct, PCV, MCV and MCH levels during 24 and 48 h. Sladky et al., 2001 reported that haematocrit and hemoglobin concentration changed in parallel, because hemoglobin was calculated from the Hct value. When treatment groups were combined and values for fish exposed to tricaine methanesulfonate, the hemoglobin concentration and Hct significantly increased in red pacu (Sladky et al., 2001). On comparison with those for fish exposed to eugenol, hemoglobin concentration and Hct significantly increased when fish were anaesthetized. Molinero and Gonzalez, 1995 reported that MS 222 increased Hb and Hct, in gilthead sea bream. Haematocrit and hemoglobin concentrations did not differ significantly among anaesthetic concentrations within each anaesthetic. Similarly the effects of 2-phenoxyethanol on the haematological profile of common carp for 10-min exposure at the concentration of 0.30 ml·l-1 caused a significant ($p<0.01$) increase of the haematocrit value relative and actual count of monocytes immediately after anaesthesia (Velisek et al., 2007).

In the study reported here, Hct and haemoglobin concentration increased with anaesthetic exposure despite collection of small blood samples. According to Soivio et al. (1977): Iwama et al. (1989); Yoshikawa et al. (1991) haematocrit values often are difficult to interpret in fish when

multiple blood samples are collected over time because of the potential for a decline in Hct as a result of acute blood loss. Because fish were exposed to a maximum anaesthetic duration of 600 seconds, the effect on Hct over longer periods and for collection of multiple blood samples was not evaluated. The mechanism of action contributing to an increase in Hct is undetermined, but the rapidity of the response seems to support a hypothesis of splenic contraction, causing an increase in RBC volume.

3.7.6 Hypothermia

Results of the present investigation showed certain changes in haematological parameters of the blood of *Etroplus suratensis,* during prolonged exposure (48h) to the optimum concentrations of hypothermia (Fig.3.1-3.7). In the present work the hypothermic condition (acclimated to lower temperature) changed the haematological indices of *Etroplus suratensis.* This might be due to the action of lowering the water temperature (hypothermia) on blood tissues as a result of viability of the cell affecting the behaviors and physiological responses of fish. A number of studies have reported that temperature has an influence on haematological and metabolic processes (Donaldson et al., 2008; Chou., 2008; Lermen et al., 2004) but factors such as photoperiod, salinity and developmental stage and body size can pose challenges in interpreting haematological and metabolic parameters following an acute temperature decrease (Sun et al., 1995; Ban, 2000). Low temperature also determines the distribution, behaviors and physiological responses of animals (Chou, 2008). Blood serum chemistry and lymphocyte and neutrophil counts were differentially affected by low temperature compared with transport-induced stress in catfish (Ellsaesser and Clem, 1986; Bly and Clem, 1991).

The observed results were the hypothermic concentration of all haematologcal parameters (Hct, Hb, PCV, RBC, MCV, MCH, and MCHC) showing high variation during 24 and 48 h of experimental period. If anaesthesia might be stressful for fish it could modulate stress hormone release (Hoseini and Ghelichpour, 2011). Releasing of the catecholamine is primary stress response causing erythrocytes to swell and spleen releasing new erythrocyte to blood (Wendelaar Bonga, 1997). This will consequently lead to increase in Hct and RBC as well as hemoglobin levels. A significant increase in Hct was evident in cold acclimated fish compared with control fish ($P<0.05$), (Fig. 3.1). The present investigation which might also be due to the release of new erythrocyte to blood. At lower temperature the concentration of oxygen in the water is reduced, and it could therefore be necessary for the fish to increase Hct to improving the oxygen carrying capacity of the blood as reported by Burton, 1997; Begg and Pankhurst, 2004. In addition, hypoxic exposure reduced swimming performance by 43 % in cold-acclimated fish but by only 30 % in the warm-acclimated group (21-23°C) and therefore lowers the Hct warm-acclimated group of trout (Jones, 1971). Decline in Hct as observed in the present investigation can also be due to the high swimming performance in control fish. However, an earlier studies with rainbow trout demonstrated anaemic fish that were cold-acclimated (8-10°C). This showed a 40 % lower haematocrit (Hct) than non-anaemic fish at the same temperature, whereas warm-acclimated (21-23°C) anaemic fish showed a 67% decrease in Hct (Jones, 1971). Jones (1971) reported that there was no significant relationship between Hct and C/crit in either warm- or cold acclimated fish. It could be suggested that this increase in Hct is an indication that cold acclimated fish are stressed.

Specifically, Zarate and Bradley (2003) found that haematocrit and leucocrit were poor indicators of temperature stress due to their high variability among individuals. Current evidence indicates that some haematological and metabolic responses to cold temperature stress are highly variable (Lermen et al., 2004) and may not be sensitive indicators of cold-shock stress.

The increase in Hb and RBC level of cold acclimated fish observed in this study in comparison with control fish might be due to release new erythrocyte to blood (Wendelaar Bonga, 1997) which compared favorably with earlier studies (Wells et al., 1984; Wells et al., 1989; Ryan, 1995). Similar results also occurred on comparing of warm with cold acclimated fish ($P<0.05$) (Donaldson et al., 2008). In the current study elevated Hb was associated with increased Hct in cold acclimated fish (Baker et al., 2005). In Antarctic fish, there is a general trend for a reduction in Hct to offset the effects of increased blood viscosity which result at low temperatures (Wells et al., 1980).

Comparison with both cold acclimated and control fish caused a significant increase in MCHC as observed in this study and might be due to cellular shrinking. Acclimation to both cold and warm conditions caused an increase in MCHC compared with fresh fish; this might be an indication of cellular shrinking (Donaldson et al., 2008). However, the MCHC values obtained for fresh fish are much lower than those reported by previous authors (Wells et al. 1989; Franklin et al., 1993; Ryan, 1995; Lowe and Davison, 2005), and are more likely to indicate that fresh fish were stressed (particularly when Hct results are considered), and this was associated with cellular swelling (therefore decreased MCHC). Other authors have also

reported cell swelling occurring in acutely sampled fish (Wells et al., 1990). The MCHC values obtained for cold and warm acclimated fish are closer (although still somewhat lower) to those reported by previous authors for fish in an unstressed resting state (Wells et al., 1989; Franklin et al., 1993; Lowe and Davison, 2005), so it is considered that rather than the values for these acclimation groups being elevated, the values obtained for control fish were low. Because there is no significant difference between the MCHC of warm and cold acclimated fish.

3.8 Summary

This study evaluated the effects of optimal concentrations of clove oil (0.17 mg/ L), cinnamon oil (0.50 mg/ L), cassumunar ginger extract (1.30 mg/ L, tobacco leaf extract (6mg/ L), MS222 (52.2 mg/ L) and hypothermia (16 mg/ L) on haematological parameters in *Etroplus suratensis*. Samples were collected during the exposure period of 24 and 48 h to check the haematological parameters. The results showed that there were significant difference noticed between the haematocrit levels of all anaesthetic groups and control during 24 and 48h. The end result showed that the haematological levels of the clove oil treated fish showed significantly lower haematological values among all the anaesthetic group and control during 24 h. During 48 h all the haematological parameters will be same in control and all other anaesthetic group except in hypothermic concentration (16°C). It was clearly proved that in hypothermic condition (16°C) all the haematological parameters were very high during 24 and 48 h exposure period. It is concluded that *Etroplus suratensis* should be anaesthetized with clove oil (0.17 mg/ L) for 24 and 48 h duration to cause no haematological responses. To reach this, use of

other anaesthetics at higher concentrations is not suitable over a short period compared to lower concentration of clove oil because they cause high fluctuations in haematological levels which in turn points out the low immunity level of the fish.

.....ഇൽ.....

Chapter **4**

The stress reducing capacity of optimum concentration of selected anaesthetics during 24 and 48 hours of exposure of *Etroplus suratensis*

Contents

4.1 Introduction
4.2 Materials and Methods
4.3 Biochemical analysis of stress indices
4.4 Post treatment survival
4.5 Statistical analysis
4.6 Results
4.7 Discussion
4.8 Summary

4.1 Introduction

Fish culturists and fish biologists make use of blood chemistry indices for evaluation of fish stress responses, nutritional condition, reproductive state, tissue damage due to handling procedures and health status (McDonald and Milligan, 1992; Wagner and Congleton, 2004; Congleton and Wagner, 2006). The stress response of fish is similar to that of mammals (Schreck, 1981; Wendelaar Bonga, 1997). Stress-related changes in blood chemistry occur within seconds or minutes after fish are disturbed (Mazeaud and Mazeaud, 1981; Gingerich and Drottar, 1989), so that precautions must be

taken to ensure that blood-sampling procedures do not alter the indices of interest. When confronted by a stressor, the fish responds by secreting catecholamine, the so-called “fight or flight” hormones and corticosteroids such as cortisol, the “stress” hormone. This primary hormonal response is almost immediate, and is detected in the blood within minutes. These in turn trigger secondary stress responses, which include changes in metabolic activity, osmoregulation, immunosuppression, and at the cellular level, up regulation of heat shock proteins (Mazeaud et al., 1977; Barton and Iwama, 1991). Responses at the whole-animal level such as changes in growth rate or behaviour are considered tertiary stress responses. Blood glucose and serum cortisol concentrations are commonly used as stress indicators in fish studies because they have proven to be reliable endocrine and secondary indicators for many stressors to fish and are easily measurable parameters (Schreck, 1981). Several studies demonstrated the influence of anaesthetics on the magnitude of corticosteroid and hyperglycemic responses of fish to stress (Iwama et al., 1989; Ortuno et al., 2002a).

Anaesthetics are frequently used in fishery studies and aquaculture to minimize stress response, preventing its negative impact on performance and reducing physical injury during handling procedures (Wedemeyer, 1997). While anaesthesia benefits the fish by minimizing the impact of greater stressors, it is also inherently stressful and its effectiveness depends on the procedure used (Iwama et al., 1989). Different anaesthetic agents can cause diverse changes in physiological parameters (Bressler and Ron, 2004). Several anaesthetics sedate fish facilitating several stressful procedures (Iversen et al., 2003; Pirhonen and Schreck, 2003). Relatively high concentrations (150-200mg L^{-1}) of tricaine methanesulphonate (MS- 222),

which bring about an irreversible deep plane of anaesthesia within a few minutes, have been reported to be effective in preventing changes in plasma cortisol, a primary stress-response indicator (Davis et al., 1982; Barton et al., 1985; Piazza and Moal., 1998), and are recommended for prevention of stress-related changes in blood chemistry (Wedemeyer et al., 1990). Some anaesthetics reduce or block the activation of the hypothalamic-pituitary-inter renal (HPI) axis associated with stressors and thus decrease or prevent the release of the stress hormone cortisol to the bloodstream of fish (Hoskonen and Pirhonen, 2006). Lower, sedating or immobilizing doses of anaesthetic may elicit rather than prevent stress responses (Strange and Schreck, 1978; Smit et al., 1979; Iwama et al., 1989; Thomas and Robertson, 1991).

Haematological tests and analysis of serum constituents have proved to be useful in the detection of the stress and metabolic disturbances (Aldrin et al., 1982). Cortisol is generally used as an endocrine stress indicator in fish (Martinez-Porchas et al., 2009; Bolasina, 2006; Crosby et al., 2006; Barton and Iwama, 1991). Cortisol is documented to have a delayed concentration peak 30 to 60 minutes after stress (Iwama, 2004), and as well the magnitude and extent of cortisol concentration usually reflect the intensity and duration of the stressor (Barton and Iwama, 1991). Haematological parameters are closely related to the response of the animal to the environment, an indication that the environment where fishes live could exert some influence on the haematological characteristics (Gabriel et al., 2004).

Clove oil has received favorable reviews as an alternative anaesthetic for a variety of fish species (Keene et al., 1998; Cho and Heath, 2000). Eugenol (2-methoxy-4-2-(2-propenyl)-phenol), the active component of

clove oil and cinnamon oil. *C. zeylanicum* lowered blood glucose, reduced food intake, and improved lipid parameters in diabetes-induced rats (Priyanga et al., 2012). Also, as fish anaesthetic (Agokei and Adebisi, 2010) haematological indices indicated that the fish became anemic and the severity of this condition was directly proportional to the tobacco dust concentrations (Omoniyi et al., 2002). MS-222 (tricaine methanesulphonate) is the most frequently used and preferred anaesthetic for fish (Ross and Ross, 2008). Hypothermia is also known to reduce the stress in fish handling, either by itself or in combination with chemical anaesthetics (Rodman, 1963; Hovda and Linley 1999; Ross and Ross, 1999).

The use of an anaesthetic in juveniles of *Etroplus suratensis* transportation would be an alternative to reduce the stress response that is certainly unavoidable, but it claims for new strategies to improve this species management. To date, there is no research focusing on the effects of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS222 and cold solution on juveniles of *Etroplus suratensis* haematological parameters, when it is used for blood sampling. Therefore, in this study we examined the effects of optimum concentration of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS222 and cold on the stress response of *Etroplus suratensis* subject to transportation.

4.2 Materials and Methods

4.2.1 Fish and experimental conditions

Collection, maintenance, acclimatization and experimental designs and preparation of samples were the same and explained in details in chapter 1 section 1.1, 1.2, 1.3, 1.3.1, 1.4, 1.4.1, 1.4.2, 1.4. 3 and 1.5.

4.3 Biochemical analysis of stress indices

For the biochemical profile tests, Groups of juvenile fishes of Pearl spot (*Etroplus suratensis*) size classes; (2.078 ±0.15g and 4.0±0.1cm) were used. A total of 96 fish from each of six experimental groups were divided into four groups and examined: Control 1 (controls examined in parallel with Experiment I), Experiment I (24 h exposure of anaesthesia at optimum concentrations of six anaesthetics), Experiment II (48 h exposure of anaesthesia at optimum concentrations of six anaesthetics) and Control II (controls examined in parallel with Experiment II). Those concentrations were based on the result from previous experiment. Each treatment was carried out in three replicates. All groups of fishes treated and packed with optimum concentration of anaesthetics. The packing system involved Fifty four LDPE bags (37.5x20cm) were used. Double polyethylene bags, one slipped into another, were used to insure against water loss from perforations or leakage. The anaesthetic was vigorously stirred into the packaging water before the fish were put in. All sets of experimental bags were then flattened to the water surface to expel the air, inflated with medical grade oxygen gas, secured airtight and sealed with rubber bands and finally, put it in the Styrofoam box lined with a1cm polystyrene sheet for insulation. Four ice cube packs were also put in the space between bags in Styrofoam box. The Styrofoam box was left in the laboratory to the thermostatically controlled chilling unit (Rotek Instruments, Chest type model, temperature range 0-30°C, M/S.B and C Instruments, Kerala) for keeping the transportation condition. This unit maintains the temperature of 22 ± 1°c for the test period i.e. 24 and 48 hr. After the end of simulated-transport of 24 and 48 h, the bags were opened for haematological and water quality analysis (following

the above-mentioned procedure) to determine the fish physiological changes made by the use of anaesthetics. Blood samples, water samples were taken, and fish were transferred into the fiberglass tanks. Any dead fish were separated and counted with subsequent calculations of mortality levels. The survive fish were reared and observed for mortality and health condition for 7 days.

4.3.1 Blood sampling procedures

For biochemical analysis of stress indices, Groups of juvenile fishes of Pearl spot (*Etroplus suratensis*) size classes; (2.078 ±0.15g and 4.0±0.1cm) were used. A total of 40 fish from each of six experimental groups were divided into four groups and examined: Control 1 (before the anaesthetic administration), Experiment I (after 1 h exposure of anaesthesia), Experiment II (after 24 h exposure of anaesthesia at optimum concentrations of six anaesthetics), Experiment III (after 48 h exposure of anaesthesia at optimum concentrations of six anaesthetics) and Control II (controls examined in parallel with Experiment II). The fish were anaesthetized for 1 h, 24 h and 48 h by optimum concentrations of six anaesthetics the concentration of 0.45 ml/3 L of clove oil, 1.5 ml/3 L of cinnamon oil, 4.5 ml/3 L of cassumunar ginger, 12 ml/3L of tobacco leaf extract, 1.5 ml/3 L of MS222 and 18±1°C/3L of cold water (packed condition).Blood samples were also taken to verify the effects of these anaesthetics on plasma biochemical parameters commonly used as an indicator of stress (Wedemeyer et al., 1990). The blood samples, from ventral aorta, (five fish per sampling) were taken at time 0 (designated for each tank at the time of exposure to anaesthetic agent), 24 and 48 h after exposure. Two blood samples were taken from each fish into 1.5 ml heparinized (Heparin LEO, 25 000 IE/mL) syringes (BD plastic pack™:

1 mL, Madrid, Spain) cannula (BD Microlance™ 3: blue: 0.6 x 25 mm and yellow: 0.3 x 12 mm, Fraga, Spain) micro-tubes. The first into a 0.2% NaF/EDTA treated tube to obtain plasma by centrifugation (Mikro 22R, Hettich Zentrifugen) at 4500xg for 10 min at 4°C for and stored at-20°C for the determination of glucose and lactate concentrations (Redding et al., 1984; King and Pankhurst., 2003).; the second into a tube without anticoagulant, in order to obtain serum to determine concentrations of cortisol. Both plasma and serum were frozen (-20°C) for later analysis. This experiment was performed in triplicate. The samples were transferred to the Clinical pathology Laboratory, Doctors Diagnostic Centre International, Cochin Kerala, for plasma biochemical analysis.

4.3.2 Blood analytical procedure

4.3.2.1 Estimation of Plasma Cortisol, Glucose and Lactate

Plasma cortisol, glucose and lactate were analyzed at the Doctors diagnostic research centre; Kochi, India. Plasma cortisol was measured by a fully validated direct enzyme immunoassay (EIA) as outlined in Carey and McCormick (1998). Glucose was evaluated by the hexokinase and glucose-6-phosphate dehydrogenase enzymatic method (Stein, 1963; McCormick and Bjornsson, 1994). Plasma lactate concentrations were determined by reduction of nicotinamide adenine dinucleotide with lactate dehydrogenase as described by Marbach and Weil (1967; see Carey and McCormick, 1998). Plasma cortisol, glucose, and lactate assays were run on a thermomax micro plate reader using SOFT max software (Molecular Devices, Menlo Park, CA, USA) at DDC International, Kochi

4.4 Post treatment survival

Independent sample t-test was used to determine differences between treatment means and control. Significant differences among hypothermal treatments were analyzed using one-way analysis of variance (Snedecor and Cochran, 1967). Results were considered statistically significant when $p<0.05$.

4.5 Statistical analysis

After 48 hours of experiment, the experimental bags containing the remaining fishes with well aeration were put into Fiber Reinforced Plastic tanks containing aerated water for 1 h and after that the fishes were allowed to come out slowly from the bags. Separated tanks were maintained for all the sets of experimental groups for observing post-transport mortality for seven days after simulated transport. The water temperature in the tanks was 28±1°C with an average dissolved oxygen level of 12mg/ L and the fishes were fed with pelleted feed.

4.6 Results

4.6.1 Clove oil

The blood glucose concentration of the anaesthetized fish (*Etroplus suratensis)* was significantly higher than that of the control fish exposed in optimum concentration of clove oil (0.17mg/ L) during 1, 24 and 48 h (Fig.4. 1). There was significant difference in glucose concentration between fish anaesthetized with clove oil and control fish ($p>0.05$). When fish were moved to the induction tank after anaesthetic administration, the mean plasma glucose concentration of the fish was shown to be 21.67 ±

3.59 mg dL^{-1} at 1 h of the exposure of clove oil while the control fish showed 18.33±2.95 mg dL^{-1} the mean plasma glucose concentration was shown to be 32.0 ± 3.61 mg dL^{-1} similar to that of the control group (P>0.05). The plasma glucose concentration increased by 29. 83 ± 3.88 mg dL^{-1} in 24 h, and control increased by 28.67 ± 3.67 mg dL^{-1} in 2 h (P>0.05). The plasma glucose concentration after 48 h showed a less decrease of 27.00 ± 0.89 mg dL^{-1} in clove oil concentration but was still higher than that of the control group 23.83±3.03 (P>0.05).

Plasma lactate level of the anaesthetized fish were comparatively same with that of control fish during 1 h (p>0.05). During 24 h treatment the lactate level of fish anaesthetized with clove oil was significantly less than that of control fish (p>0.05). But during 48 h the plasma lactate level of clove oil treated fish was significantly higher than that of control fish (p<0.05). (Fig.4.2).

Plasma cortisol levels of the anaesthetized fish were significantly lower than that of the control fish during 1 h (p>0.05). However during 24 h the cortisol level of fish anaesthetized with clove oil was significantly higher than that of control fish (p<0.05). But during 48 h the clove oil treated fish showed significant lower cortisol value than that of control fish (p<0.05) (Fig.4.3).

Table 4.1 Plasma glucose levels of *Etroplus suratensis* exposed in optimum concentration of clove oil (1.7mg/ L) during 1, 24 and 48 h

	CL		**Control**		**p-value**
Time	**Mean**	**SE**	**Mean**	**SE**	
1	21.67	3.59	18.33	2.95	0.490
24	29.83	3.88	28.67	3.67	0.831
48	27.00	0.89	23.83	3.03	0.339

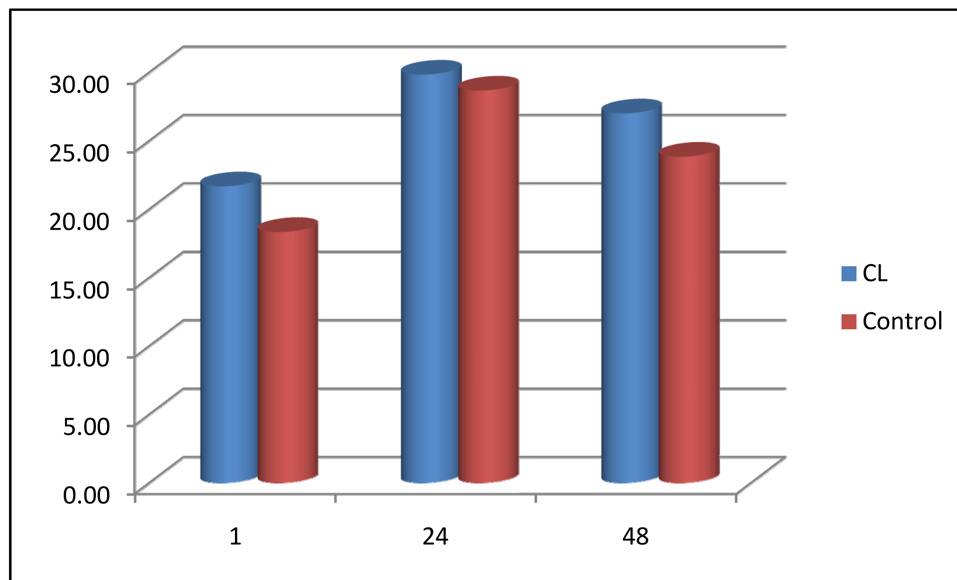


Fig. 4.1 Plasma glucose level of *Etroplus suratensis* exposed in optimum concentration of clove oil (1.7mg/ L) during 1, 24 and 48 h

Table 4.2 Plasma lactate level of *Etroplus suratensis* exposed in optimum concentration of clove oil (1.7mg/ L) during 1, 24 and 48 h

	CL		**Control**		**p-value**
Time	**Mean**	**SE**	**Mean**	**SE**	
1	15.17	0.31	15.17	0.79	1.000
24	18.17	0.98	20.00	1.03	0.227
48	20.67	0.61	17.00	1.06	0.014

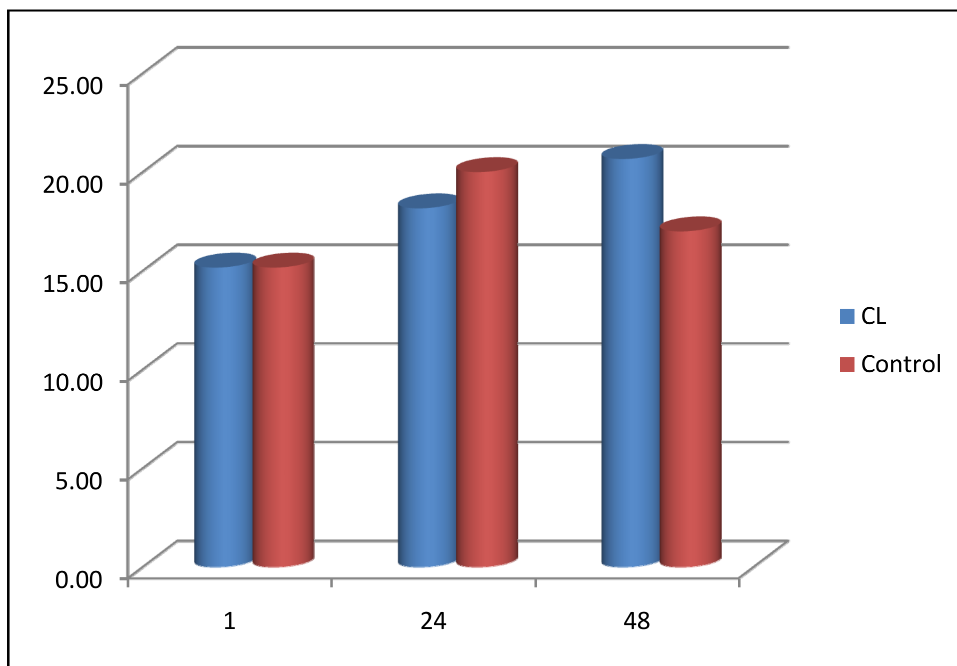


Fig. 4.2 Plasma lactate level of *Etroplus suratensis* exposed in optimum concentration of clove oil (1.7mg/ L) during 1, 24 and 48 h

Table 4.3 Plasma cortisol levels of *Etroplus suratensis* exposed in optimum concentration of clove oil (1.7mg/ L) during 1, 24 and 48 h

Time	CL		Control		p-value
	Mean	SE	Mean	SE	
1	0.442	0.049	0.817	0.291	0.233
24	1.965	0.268	0.342	0.038	0.000
48	0.192	0.025	0.588	0.064	0.000

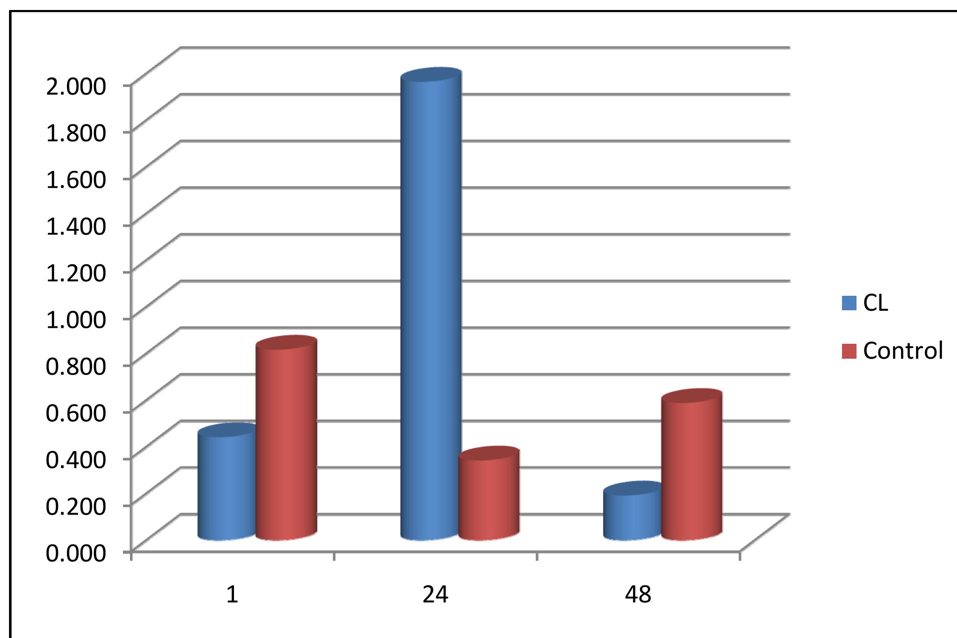


Fig.4.3 Plasma cortisol level of *Etroplus suratensis* exposed in optimum concentration of clove oil during 1, 24 and 48 h

4.6.2 Cinnamon oil

The blood glucose concentration of the anaesthetized fish (*Etroplus suratensis)* was significantly higher ($p>0.05$) than that of the control fish exposed in optimum concentration of cinnamon oil (0.50 mg/ L) during 1 h (Fig.4. 4). There was similarity in glucose value between fish anaesthetized with cinnamon oil and control ($p>0.05$) during 24 h treatment. Again the glucose values of treated fish become significantly increased with that of control ($p>0.05$) during 48 h. Plasma lactate level of the anaesthetized fish were lower than that of control fish during 1 h ($p<0.05$). The lactate level of fish anaesthetized with cinnamon oil was comparatively same with that of control fish ($p>0.05$) during 24 h treatment. But during 48 h the plasma lactate level of cinnamon oil treated fish was significantly higher than that of control fish ($p>0.05$). (Fig.4.5). Plasma cortisol levels of the anaesthetized fish exposed in cinnamon oil were significantly lower than that of the control fish during 1 h ($p>0.05$). However during 24 h the cortisol level of fish anaesthetized with cinnamon oil was significantly higher than that of control fish ($p<0.05$). But during 48 h the cinnamon oil treated fish showed significant higher cortisol value than that of control fish ($p>0.05$) (Fig.4.6).

Table 4.4 Plasma glucose level of *Etroplus suratensis* exposed in optimum concentration of cinnamon oil (0.50 mg/ L) during 1, 24 and 48 h

Time	CN		Control		p-value
	Mean	SE	Mean	SE	
1	21.17	1.60	16.50	4.22	0.326
24	23.83	1.49	23.83	3.72	1.000
48	30.83	2.07	27.00	3.04	0.322

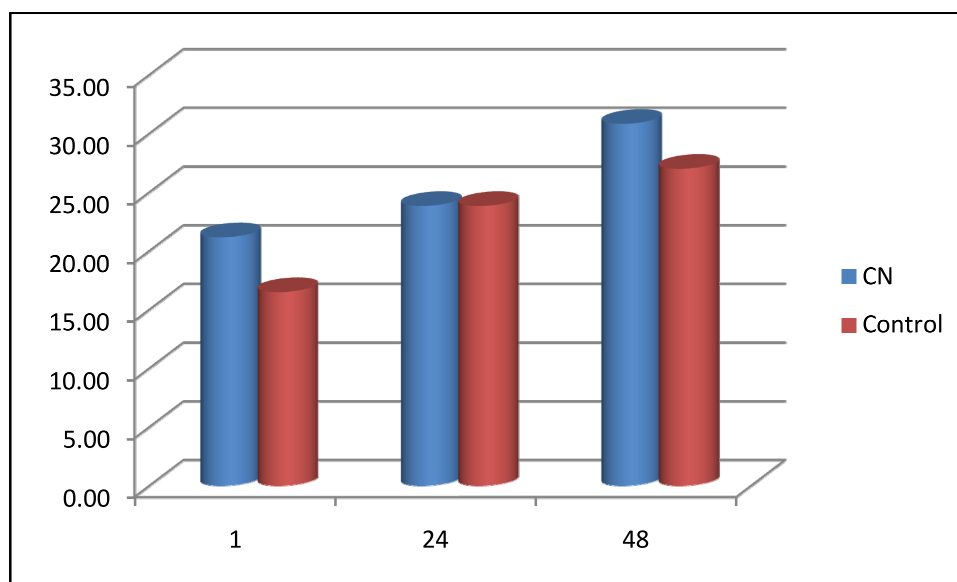


Fig.4.4 Plasma glucose level of *Etroplus suratensis* exposed in optimum concentration of cinnamon oil (0.50 mg/ L) during 1, 24 and 48 h

Table 4.5 Plasma lactate level of *Etroplus suratensis* exposed in optimum concentration of cinnamon oil (0.50 mg/ L) during 1, 24 and 48 h

Time	CN		Control		p-value
	Mean	SE	Mean	SE	
1	14.33	0.84	25.83	4.69	0.036
24	19.50	1.20	19.83	1.01	0.837
48	20.00	0.58	18.67	1.28	0.365

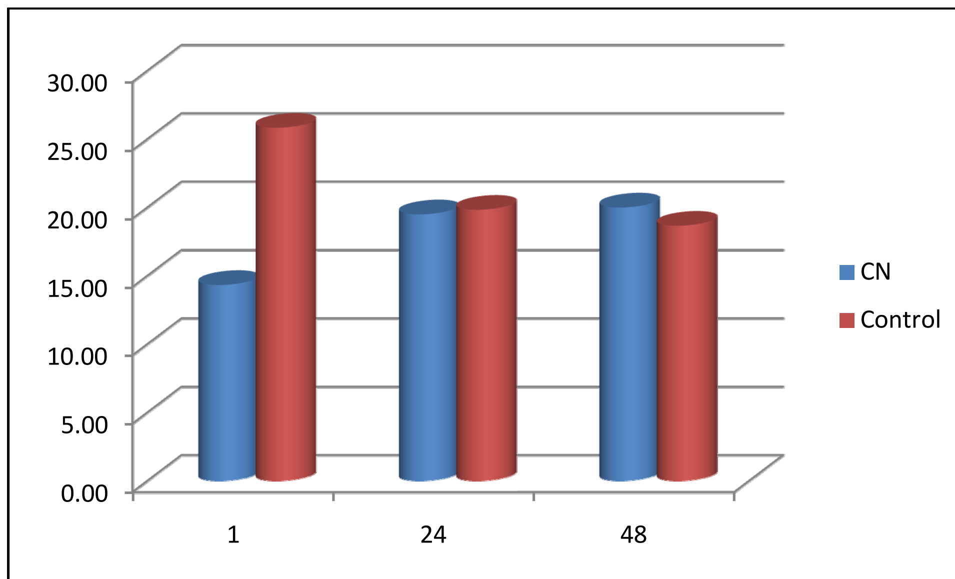


Fig. 4.5 Plasma lactate level of *Etroplus suratensis* exposed in optimum concentration of cinnamon oil (0.50 mg/ L) during 1, 24 and 48 h

Table. 4.6 Plasma cortisol level of *Etroplus suratensis* exposed in optimum concentration of cinnamon oil (0.50 mg/ L) during 1, 24 and 48 h

Time	CN		Control		p-value
	Mean	SE	Mean	SE	
1	0.748	0.327	0.995	0.340	0.613
24	0.868	0.182	0.402	0.049	0.033
48	0.893	0.208	0.522	0.167	0.194

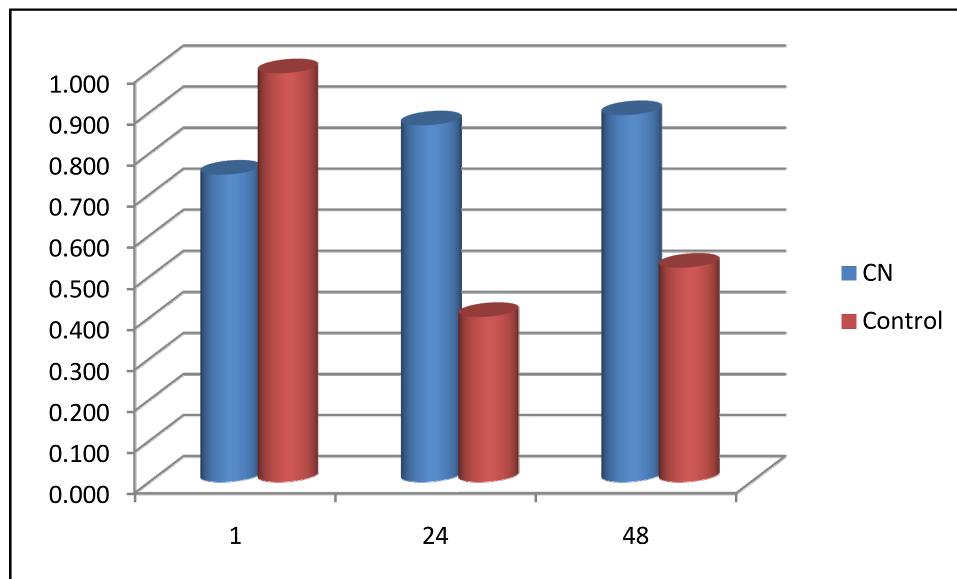


Fig. 4.6 Plasma cortisol level of *Etroplus suratensis* exposed in optimum concentration of cinnamon oil (0.50 mg/ L) during 1, 24 and 48 h

4.6.3 Cassumunar ginger extract

The blood glucose concentration of the anaesthetized fish (*Etroplus suratensis)* was significantly higher ($p<0.05$) than that of the control fish exposed in optimum concentration of cassumunar ginger extract (1.30 mg/L) during 1 h (Fig.4.7). The plasma glucose value of fish anaesthetized with cassumunar ginger extract (1.30 mg/L) was higher than that of control ($p>0.05$) during 24 h treatment. During 48 h the glucose values of treated fish become significantly increased with that of control ($p<0.05$). Plasma lactate level of the anaesthetized fish were significantly lower than that of control fish during 1 h ($p>0.05$). The lactate level of fish anaesthetized with cinnamon oil was lower than that of control fish ($p>0.05$) during 24 h treatment. But during 48 h the plasma lactate level of cinnamon oil treated fish and control fish shows similarity with each other ($p>0.05$) (Fig.4.8). Plasma cortisol levels of the anaesthetized fish exposed in cinnamon oil were significantly higher than that of the control fish during 1 h ($p<0.05$). However during 24 h the cortisol level of fish anaesthetized with cinnamon oil was significantly higher than that of control fish ($p<0.05$). But during 48 h the cinnamon oil treated fish showed significant higher cortisol value than that of control fish ($p<0.05$) (Fig.4.9).

Table 4.7 Plasma glucose level of *Etroplus suratensis* exposed in optimum concentration of cassumunar ginger extract (1.30 mg/ L) during 1, 24 and 48 h

Time	Zn		Control		p-value
	Mean	SE	Mean	SE	
1	28.67	1.45	17.17	4.09	0.024
24	28.50	2.60	27.67	3.39	0.849
48	36.33	0.56	24.50	2.74	0.002

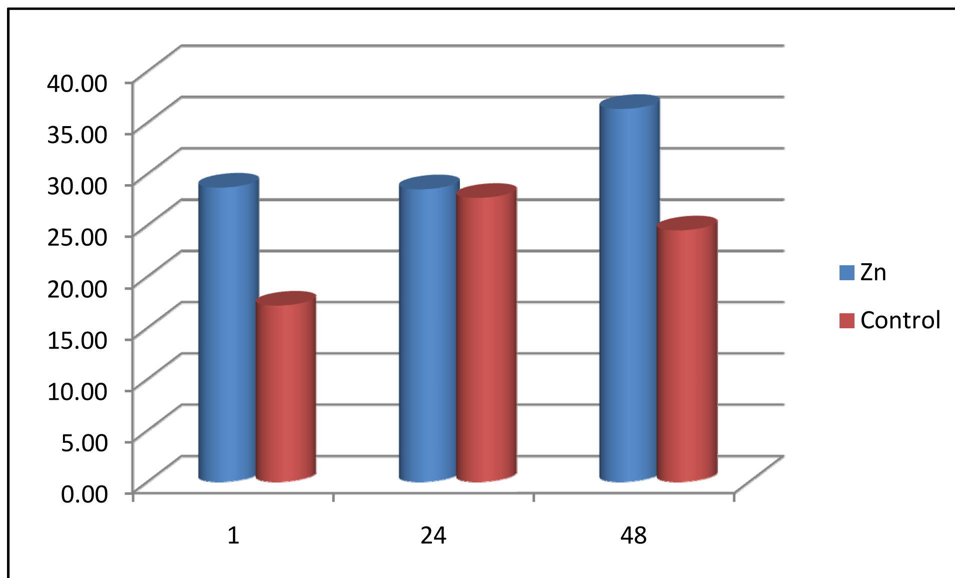


Fig. 4.7 Plasma glucose level of *Etroplus suratensis* exposed in optimum concentration of cassumunar ginger extract (1.30 mg/ L) during 1, 24 and 48 h

Table 4.8 Plasma lactate level of *Etroplus suratensis* exposed in optimum concentration of cassumunar ginger extract (1.30 mg/ L) during 1, 24 and 48 h

Time	Zn		Control		p-value
	Mean	SE	Mean	SE	
1	15.67	1.28	19.00	3.12	0.346
24	18.33	1.05	23.50	2.58	0.093
48	18.17	0.60	18.50	1.26	0.816

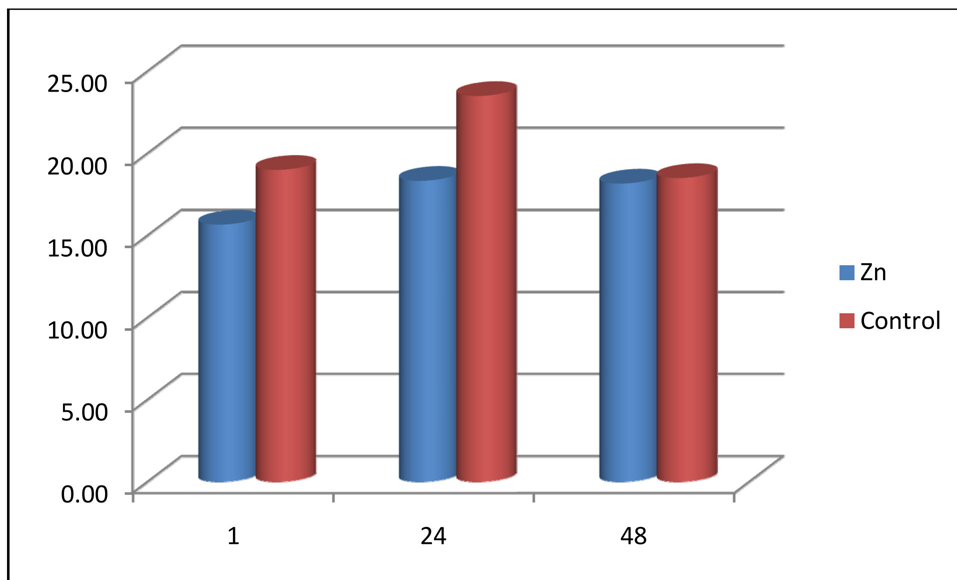


Fig. 4.8 Plasma lactate level of *Etroplus suratensis* exposed in optimum concentration of cassumunar ginger extract (1.30mg/ L) during 1, 24 and 48 h

Table 4.9 Plasma cortisol level of *Etroplus suratensis* exposed in optimum concentration of cassumunar ginger extract (1.30 mg/ L) during 1, 24 and 48 h

Time	Zn		Control		p-value
	Mean	SE	Mean	SE	
1	1.868	0.458	0.455	0.156	0.015
24	5.152	1.703	0.275	0.060	0.017
48	2.392	0.468	0.407	0.102	0.002

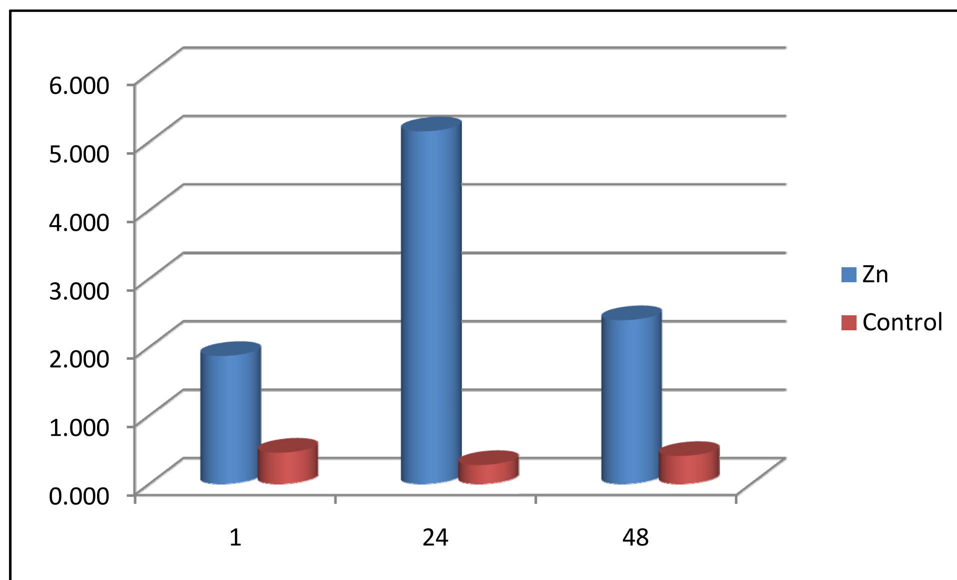


Fig. 4.9 Plasma cortisol level of *Etroplus suratensis* exposed in optimum concentration of cassumunar ginger extract (1.30mg/ L) during 1, 24 and 48 h

4.6.4 Tobacco leaves extract

The blood glucose concentration of the anaesthetized fish (*Etroplus suratensis)* was significantly higher ($p>0.05$) than that of the control fish exposed in optimum concentration of tobacco leaves extract (6 mg/L) during 1 h (Fig.4.10). The plasma glucose value of fish anaesthetized with tobacco leaves extract (6 mg/ L) was same with that of control ($p>0.05$) during 24 h treatment. During 48 h the glucose values of treated fish become significantly higher than that of control ($p>0.05$). Plasma lactate level of the anaesthetized fish were significantly lower than that of control fish during 1 h ($p<0.05$). The lactate level of fish anaesthetized with tobacco leaves extract was lower than that of control fish ($p>0.05$) during 24 h treatment. But during 48 h, the plasma lactate level of fish treated with tobacco leaves extract comparatively higher than that of control fish ($p>0.05$) (Fig.4.11). Plasma cortisol levels of the anaesthetized fish exposed in tobacco leaves extract were significantly lower than that of the control fish during 1 h ($p>0.05$). However during 24 h the cortisol level of fish anaesthetized with tobacco leaves extract was significantly higher than that of control fish ($p>0.05$). But during 48 h the tobacco leaves extract treated fish showed significant lower cortisol value than that of control fish ($p>0.05$) (Fig.4.12).

Table 4.10 Plasma glucose level of *Etroplus suratensis* exposed in optimum concentration of Tobacco leaves extract (6 mg/ L) during 1, 24 and 48 h

Time	TB		Control		p-value
	Mean	SE	Mean	SE	
1	18.67	0.49	14.83	3.60	0.316
24	23.17	3.40	23.50	3.84	0.949
48	30.83	2.43	27.00	3.04	0.348

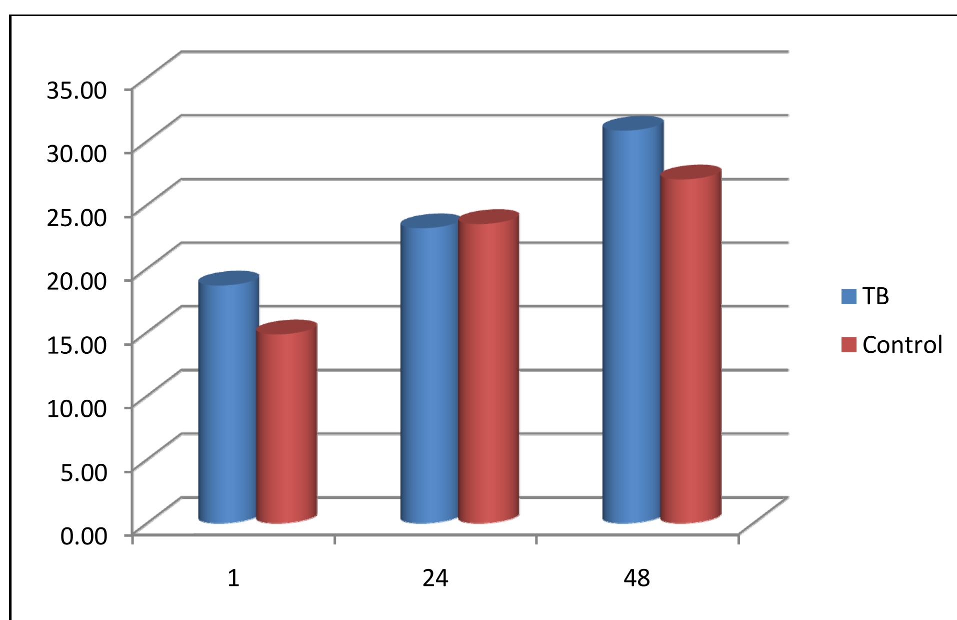


Fig.4.10 Plasma glucose level of *Etroplus suratensis* exposed in optimum concentration of Tobacco leaves extract (6 mg/ L) during 1, 24 and 48 h

Table 4.11 Plasma lactate level of *Etroplus suratensis* exposed in optimum concentration of Tobacco leaves extract (6 mg/ L) during 1, 24 and 48 h

Time	TB		Control		p-value
	Mean	**SE**	**Mean**	**SE**	
1	14.83	0.48	26.00	4.61	0.037
24	16.67	1.31	19.00	1.29	0.233
48	19.67	0.95	18.67	1.28	0.546

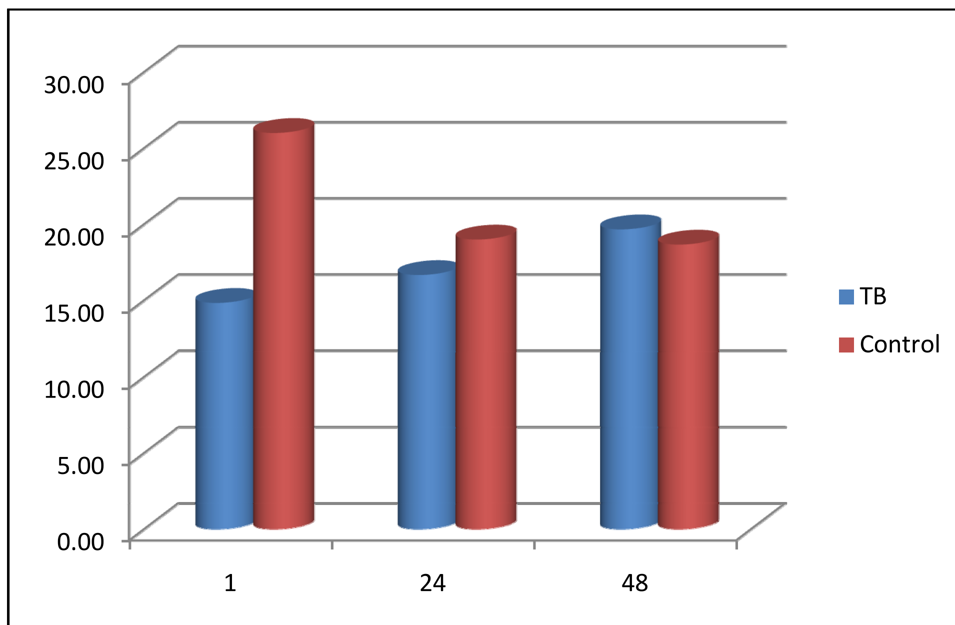


Fig.4.11 Plasma lactate level of *Etroplus suratensis* exposed in optimum concentration of Tobacco leaves extract (6 mg/ L) during 1, 24 and 48 h

Table.4.12 Plasma cortisol level of *Etroplus suratensis* exposed in optimum concentration of Tobacco leaves extract (6 mg/ L) during 1, 24 and 48 h

Time	TB		Control		p-value
	Mean	SE	Mean	SE	
1	0.285	0.026	0.705	0.272	0.156
24	0.485	0.124	0.395	0.055	0.522
48	0.173	0.061	0.522	0.167	0.079

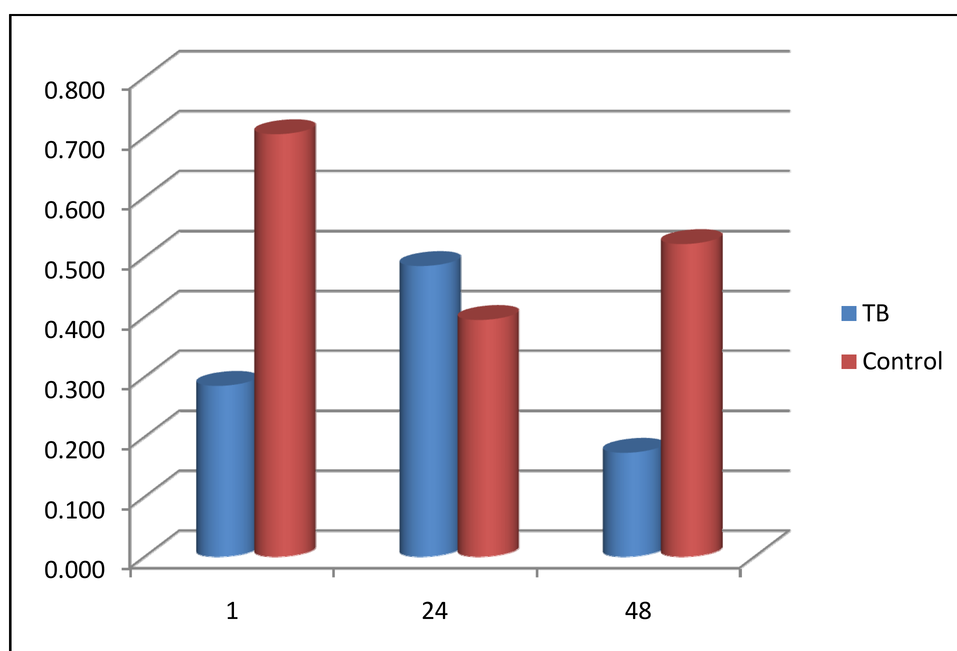


Fig.4.12 Plasma cortisol level of *Etroplus suratensis* exposed in optimum concentration of Tobacco leaves extract (6 mg/ L) during 1, 24 and 48 h

4.6.5 MS222 (Tricane methanesulphonate)

The blood glucose concentration of the anaesthetized fish (*Etroplus suratensis)* was significantly higher ($p<0.05$) than that of the control fish exposed in optimum concentration of Tricane methanesulphonate (52.2mg/ L) during 1 h (Fig.4.13). The plasma glucose value of fish anaesthetized with Tricane methanesulphonate (52.2 mg/ L) was comparatively higher than that of control ($p>0.05$) during 24 h treatment. During 48 h the glucose values of treated fish become significantly higher than that of control ($p>0.05$). Plasma lactate level of the anaesthetized fish were significantly lower than that of control fish during 1 h ($p>0.05$). The lactate level of fish anaesthetized with Tricane methanesulphonate was significantly lower than that of control fish ($p<0.05$) during 24 h treatment. But during 48 h, the plasma lactate level of fish treated with tobacco leaves extract comparatively lower than that of control fish ($p>0.05$) (Fig.4.14). Plasma cortisol levels of the anaesthetized fish exposed in Tricane methanesulphonate were significantly lower than that of the control fish during 1 h ($p>0.05$). However during 24 h the cortisol level of fish anaesthetized with Tricane methanesulphonate was significantly higher than that of control fish ($p<0.05$). But during 48 h the Tricane methanesulphonate treated fish showed comparatively similar values with that of control fish ($p>0.05$) (Fig.4.15).

Table.4.13 Plasma glucose level of *Etroplus suratensis* exposed in optimum concentration of MS222 (52.2mg/ L) during 1, 24 and 48 h

Time	MS		Control		p-value
	Mean	SE	Mean	SE	
1	30.00	0.37	17.33	4.73	0.024
24	25.50	0.22	23.83	3.72	0.664
48	31.83	2.40	30.17	2.32	0.628

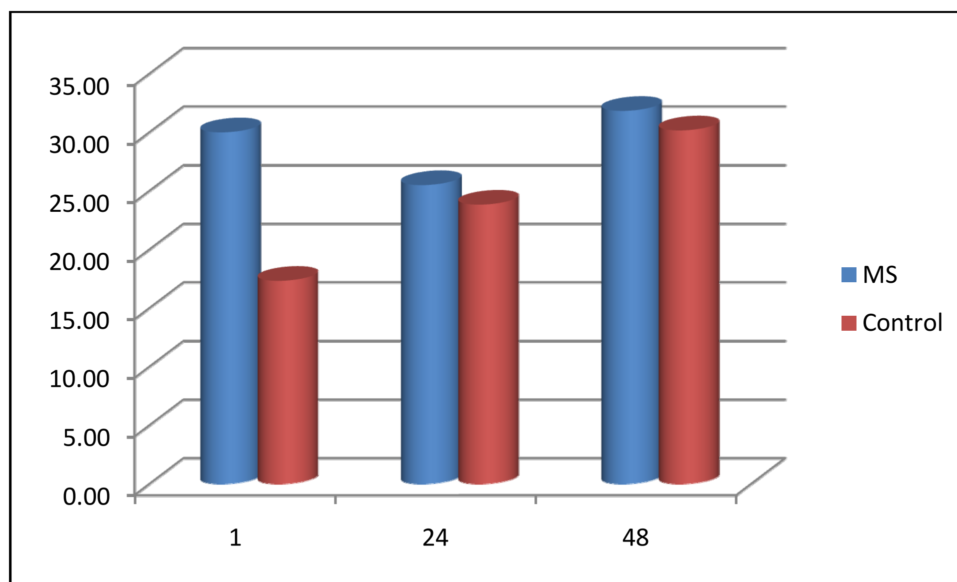


Fig.4.13 Plasma glucose level of *Etroplus suratensis* exposed in optimum concentration of MS222 (52.2mg/ L) during 1, 24 and 48 h

Table.4.14 Plasma lactate level of *Etroplus suratensis* exposed in optimum concentration of MS222 (52.2mg/ L) during 1, 24 and 48 h

Time	MS		Control		p-value
	Mean	SE	Mean	SE	
1	20.00	0.86	25.83	4.69	0.249
24	16.67	0.61	20.00	1.03	0.020
48	16.17	1.80	18.67	1.28	0.284

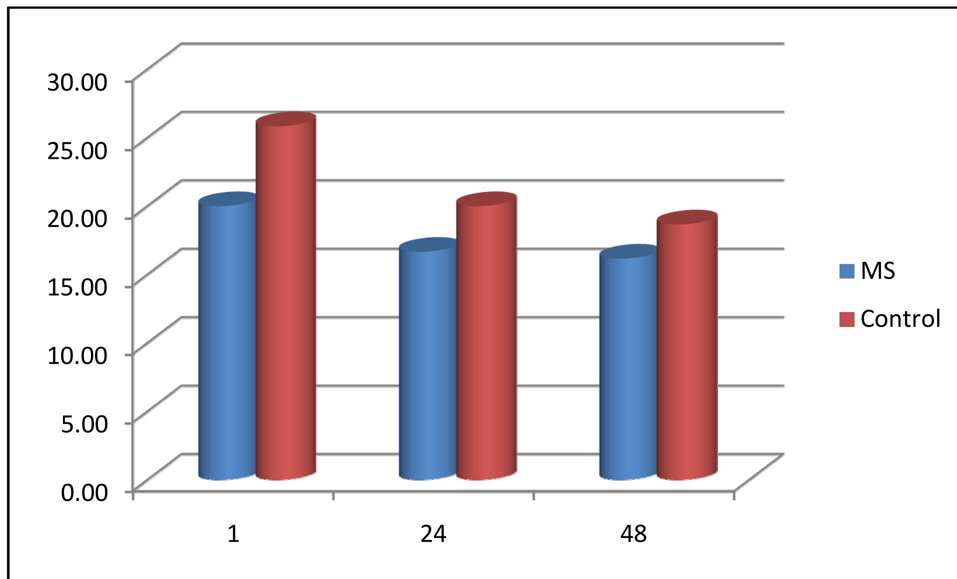


Fig.4.14 Plasma lactate level of *Etroplus suratensis* exposed in optimum concentration of MS222 (52.2mg/ L) during 1, 24 and 48 h

Table.4.15 Plasma cortisol level of *Etroplus suratensis* exposed in optimum concentration of MS222 (52.2mg/ L) during 1, 24 and 48 h

Time	MS		Control		p-value
	Mean	SE	Mean	SE	
1	0.537	0.020	0.753	0.263	0.431
24	4.163	0.019	0.417	0.058	0.000
48	0.582	0.074	0.562	0.165	0.914

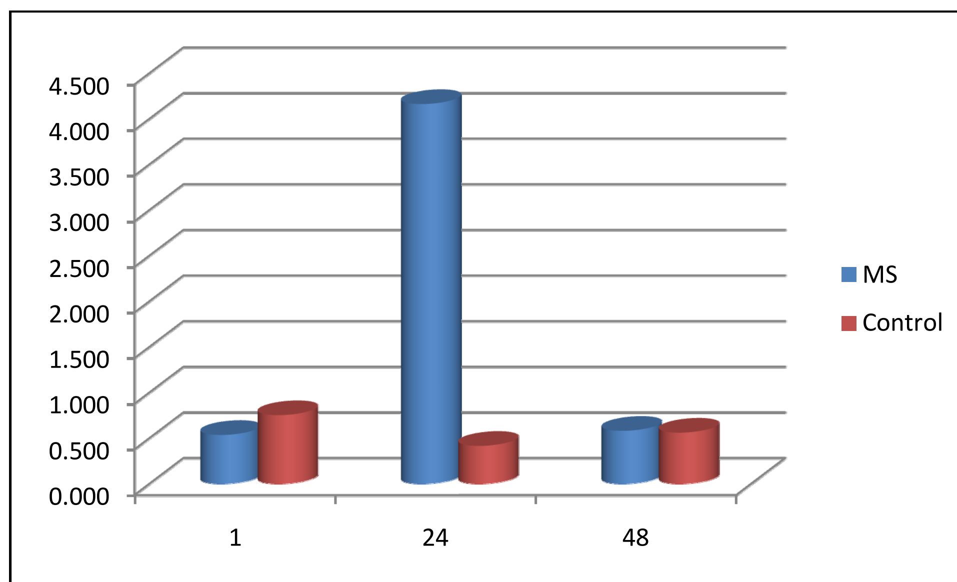


Figure.4.15 Plasma cortisol level of *Etroplus suratensis* exposed in optimum concentration of MS222 (52.2mg/ L) during 1, 24 and 48 h

4.6.6 Hypothermia

The anaesthetized fish (*Etroplus suratensis*) at 16°C showed significantly higher (p>0.05) plasma glucose value than that of other conditions of 18,22 and 32 °C during 1 h (Fig.4.16). The plasma glucose value of fish anaesthetized with 16°C was comparatively lower than that of 18,22 and 32°C during 24 (p<0.05). During 48 h the glucose values of treated fish become significantly lower than that of control (p>0.05). Plasma lactate level of the anaesthetized fish at 16°C were significantly higher than that of 18,22 and 32°C fish during 1 h (p>0.05). The plasma lactate level of fish anaesthetized with 16°C was significantly higher than that of other conditions (p<0.05) during 24 h treatment. But during 48 h, the plasma lactate level of fish treated with 16°C comparatively lower than that of other treatment (p<0.05) (Fig.4.17). Plasma cortisol levels of the anaesthetized fish exposed in 16, 18 and 22°C were significantly lower than that of the fish at 32°C fish during 1 h (p<0.05). However during 24 h the cortisol level of fish anaesthetized with 16°C was significantly lower than that of other treatment of 18,22 and 32°C (p<0.05). Similarly during 48 h the 16°Ctreated fish showed comparatively lower values with that of 18,22 and 32°C (p<0.05) (Fig.4.18).

Table.4.16 Plasma glucose level of *Etroplus suratensis* exposed in optimum concentration of Hypothermia (16°C/L) during 1, 24 and 48 h

	16		18		22		32		p-value
Time	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
1	24.67	2.50	20.17	0.48	21.50	2.33	19.00	0.82	0.162
24	22.67	1.12	26.33	2.54	24.83	2.83	33.00	1.21	0.013
48	25.00	2.73	29.33	1.09	28.17	3.36	27.50	3.13	0.719

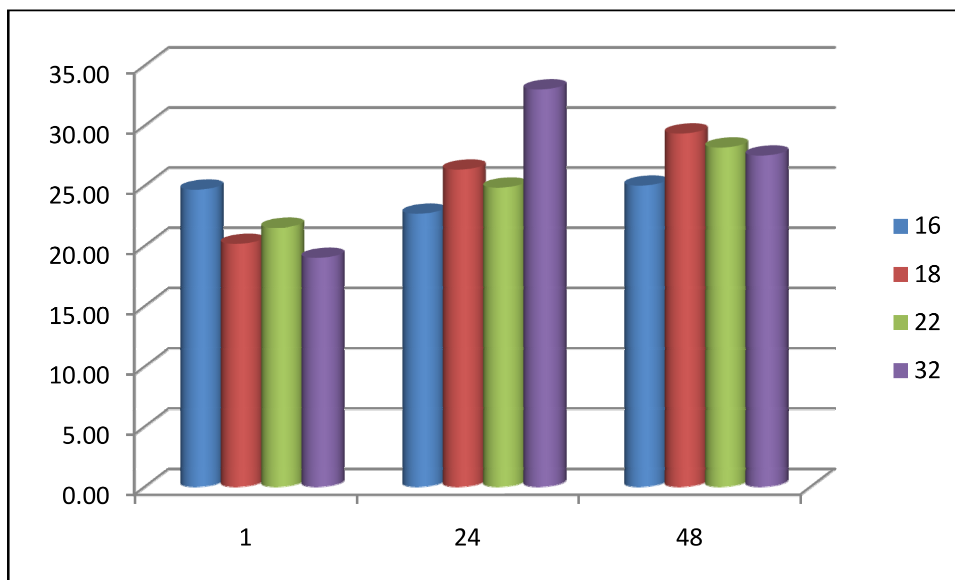


Fig.4.16 Plasma glucose level of *Etroplus suratensis* exposed in optimum concentration of Hypothermia (16°C/l) during 1, 24 and 48 h

Table.4.17 Plasma lactate level of *Etroplus suratensis* exposed in optimum concentration of Hypothermia (16°C/l) during 1, 24 and 48 h

Time	16		18		22		32		p-value
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
1	15.50	0.81	13.50	0.43	14.00	0.89	14.17	0.54	0.242
24	17.00	0.63	17.00	0.82	16.50	1.18	21.33	0.42	0.001
48	12.50	0.67	13.33	1.17	15.50	0.67	18.17	1.35	0.004

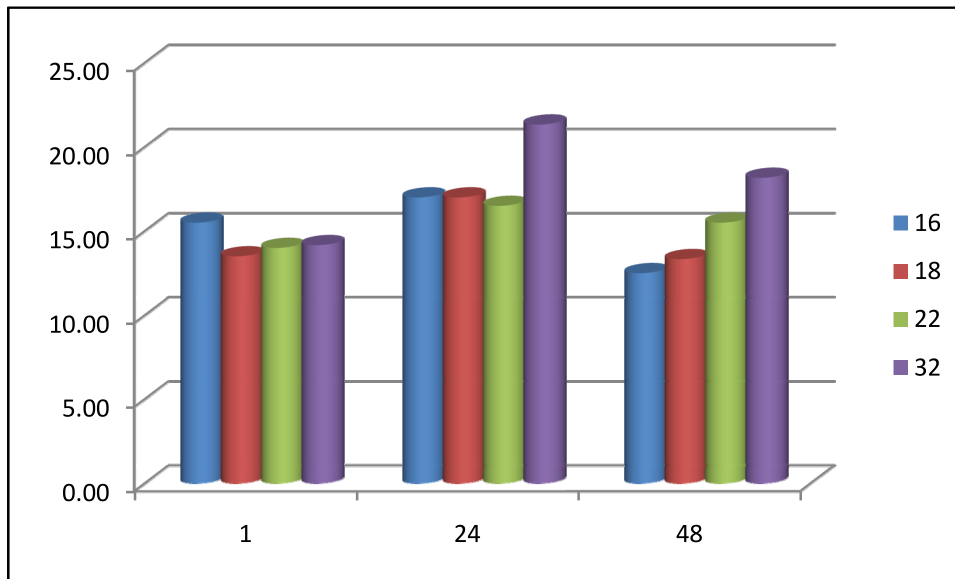


Fig.4.17 Plasma lactate level of *Etroplus suratensis* exposed in optimum concentration of Hypothermia (16°C/l) during 1, 24 and 48 h

Table.4.18 Plasma cortisol level of *Etroplus suratensis* exposed in optimum concentration of Hypothermia (16°C/l) during 1, 24 and 48 h

Time	16		18		22		32		p-value
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
1	0.037	0.009	0.042	0.005	0.027	0.005	0.383	0.054	0.000
24	0.020	0.004	5.022	0.678	5.768	0.781	0.280	0.038	0.000
48	0.033	0.003	2.427	0.505	4.423	0.872	0.608	0.144	0.000

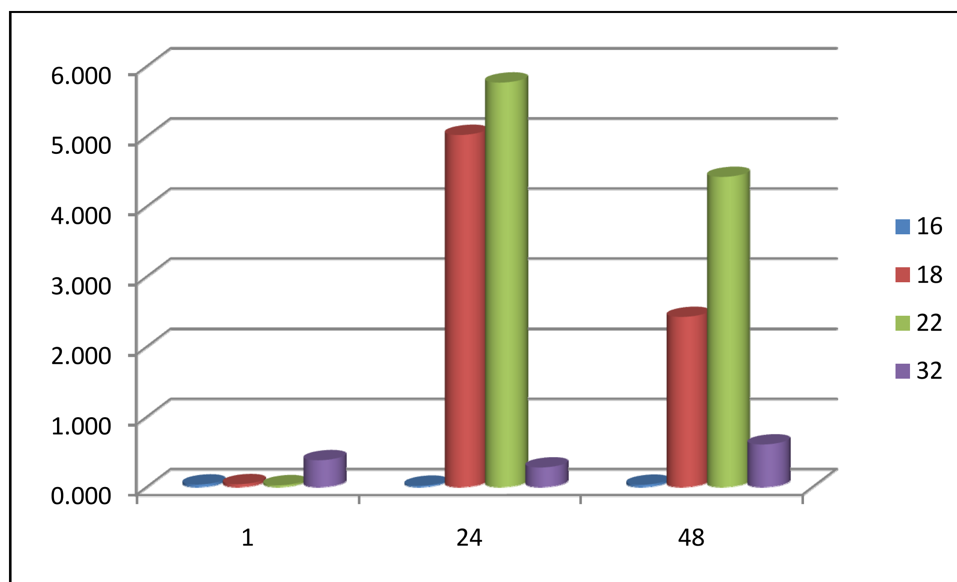


Fig.4.18 Plasma cortisol level of *Etroplus suratensis* exposed in optimum concentration of Hypothermia (16°C/l) during 1, 24 and 48 h

4.7 Discussion

4.7.1 Clove oil

In the present study indicated that exposure of anaesthetics reducing distinct stress responses in the *Etroplus suratensis*, thus emphasize the necessity of reducing the stress responses in fish during transportation. However, Hoseini et al. (2010) showed changes in Beluga's serum biochemical parameters (cortisol, glucose and lactate) as a result of anaesthesia with clove solution using different protocols.

In the present study clove oil induced a stress response in the fish, indicated by the increase in blood glucose level and serum cortisol concentration. The rise of serum cortisol in this study is coincident with the increase in blood glucose. This well- known pattern of hyperglycemia after stress has been shown to result from catecholamine and corticosteroids released into the blood and have been reported in other research (Anderson et al., 1991; Ortuno et al., 2002b). The glucose value and serum cortisol concentration of the control fish in this study were typical for sparids (Rotllant and Tort, 1997; Ortuno et al., 2002b). Anaesthesia with low concentrations of clove solution over a long period is stressful compared to anaesthesia with high concentrations over short period. The results are supported by previous study on effect of clove solution on serum biochemical parameters in Beluga (Hoseini et al., 2010).

Clove oil induced a faster increase in plasma glucose levels compared with the control fish. Inoue et al. (2005) demonstrated that clove oil alleviated most of the measured aspects of the stress response when compared with transported juvenile matrinxã with no anaesthetic. Plasma

cortisol and plasma glucose are recognized as useful indicators of stress in fish (Schreck, 1982). Clove oil shows notable increases in plasma cortisol and plasma glucose levels were reported in kelp grouper *Epinephelus bruneus* (Park et al., 2008). In (Fig.4.3) the plasma glucose concentration showed an increase during 1, 24 and 48 h.Velisek et al. (2005a, b) reported a significant increase in blood plasma glucose immediately after a 10-min clove oil anaesthesia which returned to normal 24 h later and also detected an increase in glucose concentration in rainbow trout (*O. mykiss*) following clove oil anaesthesia, which was in agreement with the current study. Iverzen et al. (2003) found no change in the concentration of glucose in Atlantic salmon (*Salmo salar*) following clove oil anaesthesia. Changes in plasma glucose concentrations may reflect a stressing condition in fish (Hattingh, 1976).

In Fig 4.3 the plasma cortisol concentration of anaesthetized *Etroplus suratensis* increased after 24 h and decreased with that of control during 48 h. According to Park et al. (2008) the plasma cortisol concentration of anaesthetized kelp grouper did not return to normal until 48 h. Barton and Iwama (1991) stated that 'Usually, phenomenon that plasma cortisol concentration of fishes rises by stress is first order reaction, phenomenon that plasma glucose concentration rises is result of second-order first order reaction by hormone rise reaction by stress'. This trend has been reported in the grey mullet, *Mugil cephalus* (Chang and Hur 1999). Iversen et al. (2003); Small (2004); Woody et al. (2002); Keene et al. (1998) etc., reported that the cortisol response can be prevented when fish are exposed to high doses of clove oil. For instance, Atlantic salmon did not elicit cortisol response when exposed to 20-100 mg of clove oil/ L during a 30 min exposure (Iversen et al., 2003). Cat fish also responded in a similar way when exposed to 100 mg of

clove oil/L (Small, 2004). Study with Atlantic salmon demonstrated that exposure to 10 mg/ L of clove oil prevented the cortisol response only during the first 10 min of exposure.

In the present study it was observed that the attenuation on the cortisol response in 0.17mg/L of clove oil anaesthetized fish than that of control during the 1 h transport, after that, plasma cortisol levels significantly increased. Iversen et al. (2003) observed attenuation on the cortisol response in 30 min exposure after that, plasma cortisol levels significantly increased, but the values were lower (attenuated response) than fish not exposed to clove oil and comparatively our results showed the same tendency. The mechanism of how clove oil affects the cortisol response is not known. Iversen et al. (2003) speculated that clove oil may block the transmission of sensory information to the hypothalamus, and therefore high concentrations of anaesthetics prevent the activation of the hypothalamus-pituitary-inter renal (HPI) axis more effectively than lower concentrations. So the cortisol response may be prevented (Iversen et al., 2003).

In the present study the plasma cortisol concentration later decreased while the plasma glucose concentration increased from that of control unlike that reported by Chang and Hur (1999). Such different results seem to be caused by different kinds of species and stresses imposed on fish (Park et al., 2008). In addition, cortisol and glucose levels were only correlated with each other for the social stressor (Barreto and Volpato, 2006). This effect provides some evidence corroborating the classification of stressors proposed by Moreira and Volpato (2004) as they differ from each other by the presence of the psychological component in the social stressor.

In the present study, after exposure to an anaesthesia concentration of 0.17 mg L^{-1} at 22 °C, it took 3 days for the plasma cortisol and plasma glucose concentrations to return to the levels seen before exposure. The anaesthetic effect of clove oil in fish can last for several hours after relocating fish to recovery tanks in this study.

As well the lactate showed a slight increase while the juveniles of *Etroplus suratensis* exposed at 0.17mg L^{-1} of clove oil during 1, 24 and 48 h. Atlantic salmon only showed slight increase in plasma lactate levels 30 min after exposure to 10 mg of clove oil/ L, and the time of increase was related to the higher stages of anaesthesia reached at that time (3a: total loss of equilibrium – fish usually turn over but retain swimming ability) (Iversen et al., 2003). Fish transport in plastic bags containing clove oil prevented plasma lactate rise that is usually seen when oxygen is not available for aerobic cell metabolism (Iversen et al., 2003). It usually takes place after stressful events that involve elevated muscular activity like burst swimming or severe exercise (Barton et al., 1998). In this study, the dropping of lactate response was probably due to the apparent lower muscular activity in fish exposed to clove oil. However, the decreased muscular activity, likely did not interfere with the respiration and ventilation ratios. Decreases on these parameters are frequently associated with anaesthetics used in high concentration, which causes the decreased availability of oxygen to the cells, and therefore eliciting the increase of plasma lactate, a by-product of the anaerobic metabolism (Barton et al., 2002).

4.7.2 Cinnamon oil

In the present study the treated fish showed increase in glucose level during 1, 24 and 48 h with that of control while the fish exposed in 0.50 mg/L

of cinnamon oil. Similarly the lactate and cortisol level also showed an increasing tendency. There was not any work for supporting this result in India on *Etroplus suratensis* by exposing of 0.50 mg/ L of Cinnamon oil.

4.7.3 Cassumunar ginger extract

In the present study the glucose level of treated fish showed an increasing tendency during 1, 24 and 48 h with that of control while the fish exposed in 1.30 mg /L of cassumunar ginger extract. But in the case of plasma lactate, the values showed decreasing tendency with that of control during 1, 24 and 48 h. Similarly, the plasma cortisol level also showed an increasing tendency with that of control value. There was not any supporting work with this result in India on *Etroplus suratensis* by exposing of 1.30 mg/L of casuminar ginger extract.

4.7.4 Tobacco leaf extract

In the present study the plasma glucose level showed significant increase with that of control during 1, 24 and 48 h exposure. There was significant ($p<0.01$) decrease in the liver and kidney glucose values with significant ($p<0.01$) increase in the serum glucose values as the concentrations of tobacco leaf dust increased in hybrid catfish (*clarias gariepinus* and *heterobranchus bidorsalis*) (Adamu and Siakpere, 2011). Glucose is an important diagnostic tool of carbohydrate related disorder. It has been proven to be reliable endocrine and physiological indicator of the relative severity of many acute stresses to fish (Soenges et al., 1992). Hyperglycemia is associated with stressful situations (Fletcher, 1975). Physiological/biochemical responses may be compromised, becoming detrimental to the fish's health and well being at which point the fish is termed distressed (Barton and Iwama, 1991).

In the present study the serum lactate level showed a significant decrease with that of control during 1, 24 and 48 h. The activity of serum lactate dehydrogenase significantly ($p<0.01$) decreased, while liver and kidney lactate dehydrogenase were noticed to decreasing significantly ($p>0.05$) when the *Heteroclarias* exposed to sub lethal concentrations of tobacco (*Nicotiana tobaccum*) leaf dust after the 14-days exposure period (Adamu, 2009). But the plasma cortisol level of juveniles of *etroplus suratensis* showed decreasing tendency along the 1, 24 and 48 h of exposure period in 6 mg/L Tobacco leaf extract.

4.7.5 MS222

In the present study the plasma glucose level showed significantly increased value than that of control for 1, 24 and 48 h. Mousavi et al., 2011 reported that Plasma glucose concentrations increased 1 h after exposure and then decreased 1 h later, this pattern was observed in both the MS-222 and eugenol groups. The highest and intermediate doses of both anaesthetics produced a significantly higher level of glucose compared with the unanaesthetized group at times 0, 2 and 6 (Molinero and Gonzalez, 1995).

Plasma catecholamine and cortisol levels are indices of stress response in fish (Gamperi et al., 1994) and acute stressors cause a rapid increase in these hormones, which in turn increase blood glucose levels through rapid breakdown of glycogen (Barton and Iwama, 1991). Pramod et al. (2010) reported that MS222 anaesthetizing *P.filamentosus* before transport resulted in significantly lower plasma cortisol and glucose levels, indicating effective sedation. Although the plasma cortisol and blood glucose levels increased initially in the anaesthetized groups, compared with the unanaesthetized

controls. The MS-222-treated group recovered faster from the stress as evidenced from the reduced levels of plasma cortisol and blood glucose indices in this group. The initial elevation in cortisol and glucose levels is probably due to the handling stress during the capture of the fish for experiment (Pramod et al., 2010). Molinero and Gonzalez (1995) reported that the intermediate and highest doses of MS 222 or 2-phenoxyethanol produced a higher increase of cortisol level than transport without anaesthetic and a lower dose of MS 222 or 2-phenoxyethanol.At time 0, post-2 hr confinement, cortisol plasma levels of all confined gilthead sea bream were increased in relation to the baseline level (Molinero and Gonzalez, 1995).

Another of the more usual secondary stress response indicators is blood lactic acid (Fraser and Beamish, 1969; Pickering et al., 1982). Plasma lactate levels can be influenced by exercise during fish capture and transport, lactate levels may continue to rise for some hours after exercise, and it has been suggested that this may be a factor in the mortality following transport (Black and Barret, 1957; Black, 1958). Leach and Taylor (1980) indicated that the increased level of lactate may have a functional role in sustaining elevated glucose levels in response to stress as a readily available energy source. But in the present study the plasma lactate level, showed a decreasing tendency during the exposure of 53 mg/ L of MS222 for1, 24 and 48 h. Significant alterations in lactate levels were noted for all treatments with MS222 (anaesthetized and unanaesthetized) and at all times, in gilthead sea bream (Molinero and Gonzalez., 1995). Molinero and Gonzalez, (1995) reported that, the lowest levels of lactate were found in the first capture group at the end of transport in all treated fish. Anaesthetized fish had lower plasma lactate levels than the unanaesthetized fish. Anaesthesia results in a lower

external stimuli response, probably a lower muscular activity and, as a consequence, lower lactate plasma levels. When muscular activity was restored or exercise was stimulated by capture, lactate was produced. Exercise is more intense with a lower density of fish, and this could explain the higher variability in the plasma lactate level of the controle group of fish. Exhaustive exercise in teleost fish results in an accumulation of lactate within the working muscle (Milligan and Wood, 1987a, b). In the present work, the decrease in plasma lactate concentration correlated with the anaesthetic concentration and consequently, the metabolic sense of lactate plasma decrease is also clear. The pattern of lactate accumulation in the blood is species dependent and apparently correlated with the fish ecology (Milligan and Wood, 1986a, b).

In the present study the plasma cortisol levels were lower than the control during 1 h. Crosby et al. (2006) reported that Plasma cortisol levels were significantly lower than the untreated control in three spot gourami after a handling stressor and treatment with one of four anaesthetics—tricaine methanesulfonate (TMS; 60 mg/ L), metomidate (0.8 mg/ L), quinaldine (5mg/ L), and Hypno (0.14 mg/ L)—or salt (NaCl; 3 g/L). Pankhurst and Sharples (1992) ; Ryan (1995) ; Sumpter (1997) ; Grutter and Pankhurst (2000) suggests that basal or resting cortisol levels have similar magnitude (1–12 ng /mL);) involving a variety of species from radically different natural environments and taxonomic families—including those from freshwater and saltwater, tropical, temperate, and Antarctic. Post stress cortisol levels typically range from 40 to 200 ng/mL (Pickering and Pottinger, 1989) and can exceed 1,000 ng/mL in some species (Barton and Iwama, 1991). For example, in the tropical marine black eye thicklip wrasse *Hemigymnus*

melapterus, basal cortisol levels are were less than 5 ng/mL (Grutter and Pankhurst 2000). Wedemeyer et al., (1990) found that normal cortisol levels in healthy rainbow trout *Oncorhynchus mykiss* juveniles fed Oregon Moist Pellet diet and held in soft water (100 mg/ L CaCO3) at 108 °C ranged from 0 to 30 ng/mL; in coho salmon *O.kisutch* juveniles held under the same conditions, cortisol ranged from 0 to 40 ng/mL.

But in the present study the level of cortisol were increasing from the control for 24 h. As induction time increases, cortisol concentrations increase along with associated haematological components such as glucose, lactate, sodium, and potassium concentrations in freshwater fish (Hattingh 1977; Sovio et al., 1977; Strange and Schreck, 1978; Wedemeyer et al., 1990; Sladky et al., 2001). In addition to elevating catecholamine levels, TMS exposure increases the level of circulating cortisol (Iwama et al., 1989; Molinero and Gonzalez 1995; Mommsen et al., 1999). Similarly, Wagner et al. (2002) observed that when adult rainbow trout were anaesthetized with MS-222 or CO_2, the cortisol level returned to the initial level within 7 and 24 h after handling. Wood ward and Strange (1987) found that confining rainbow trout in a net for 12 h caused cortisol levels to rise to over 155 ng/mL. Laidley and Leather land (1988) also noted that 12–14 min after a disturbance, plasma cortisol levels were significantly greater than resting levels in the rainbow trout. In fact, exposure to sedative doses of anaesthetic (MS 222) produces an increased cortisol level, whereas deep sedation after capture, followed by transport without an anaesthetic blocked the cortisol response (Robertson et al., 1988). In addition to elevating catecholamine levels, TMS exposure increases the level of circulating cortisol (Iwama et al., 1989; Molinero and Gonzalez 1995; Mommsen et al., 1999). Davis and

Parker (1986) found that numerous species of warm water fish found in the families Polyodontidae, Clupeidae, Cyprinidae, Catostomoidae, Ictaluridae, Moronidae and Centrarchidae all had elevated cortisol levels (59 to 250 ng/mL) following a 2-h transport. Red drum *Sciaenops ocellatus* after capture and loading had high cortisol levels (100 ng/mL) within 15–30 min from the onset of the stressor (Robertson et al., 1988). Therefore, the use of an anaesthetic is beneficial in keeping cortisol levels lowered, presumably mitigating handling stress Anaesthetics used as sedatives dull sensory perception without complete loss of equilibrium, decrease oxygen consumption, and decrease excretion of metabolic products (Ross and Ross, 1999). Some anaesthetics such as TMS and quinaldine have an initial excitatory effect on fish and can lead to increased cortisol levels (Barton and Peter, 1982; Davis et al., 1982; Robertson et al., 1987). Nevertheless, if fish are sedated quickly, the cortisol response can be blocked (Barton and Iwama, 1991). Cortisol levels increase as exposure time in TMS increases. TMS sedation treatment concentrations as low as 25 mg/L (Wagner et al., 2002) can increase cortisol levels in the rainbow trout (Barton and Peter, 1982) and striped bass (Davis et al., 1982).

The results of our preliminary study shows that anaesthetics are useful in handling tropical fish to keep stress, as manifested by cortisol levels, minimized. TMS is widely used in the industry, and further experimentation with this anaesthetic would provide useful information for tropical fish producers. Although cortisol concentrations in fish given the TMS treatment were not lower than those in controls, additional research using TMS as an osmoregulatory aid in conjunction with an anaesthetic may prove rewarding.

4.7.6 Hypothermia

Temperature is one of the most important environmental factors, as it determines the distribution, behaviors and physiological responses of animals (Chou, 2008). Low environmental temperature can also act through other mechanisms, such as stress (Donaldson et al., 2008). (Donaldson et al., 2008 reported that many studies have found that temperature has an influence on haematological and metabolic processes, but factors such as photoperiod, salinity and developmental stage and body size can pose challenges in interpreting these parameters following an acute temperature decrease (Sun et al., 1995; Ban, 2000). Current evidence indicates that some haematological and metabolic responses to cold temperature stress are highly variable (Lermen et al., 2004) and may not be sensitive indicators of cold-shock stress. On the other hand, rapid temperature reductions per second (i.e. cold shock) may result in primary and secondary stress responses in fish, including elevated plasma levels of cortisol and catecholamines, suggesting that the physiological responses to hypothermia are highly context- and species-specific (Barton et al., 1985; Chen et al., 2002; Donaldson et al., 2008; Foss et al., 2012; Hyvärinen et al., 2004; Tanck et al., 2000).

In the present study the different level of hypothermia affects the biochemical parameters of juveniles of *Etroplus suratensis.* In catfish, blood serum chemistry and lymphocyte and neutrophil counts were differentially affected by low temperature compared with transport-induced stress (Ellsaesser and Clem, 1986; Bly and Clem, 1991; Jones, 1971).

In the present study the plasma glucose level showed an increasing pattern with increasing of temperature (16, 18, 22 and 32°C) according with

the duration of hours. But at 16°C there was not any significant increase during 1, 24 and 48 h. Contrary to results from a cold-shock study using tilapia *Oreochromis niloticus* (L.) by Sun et al. (1992, 1995), Tanck et al. (2000) did not observe a significant increase in plasma glucose. This may be because of the timing of blood sampling, as Sun et al. (1992) detected hyperglycemia 24 h after the onset of the experimental procedure. The results of the current study are consistent with the findings, with control acclimated fish *Etroplus suratensis* showing no significant difference in plasma glucose concentration compared with cold acclimated fish. Both groups of fish demonstrated a decrease in plasma glucose concentration compared with fresh fish and this probably results from the non-feeding protocol for acclimating fish. However, as no change in condition index was apparent between fresh fish and acclimated fish (both cold and control), it is considered that fish were not detrimentally affected by this decreased plasma glucose concentration. Plasma glucose measurements obtained in the current study are in close agreement with those from previous studies (Lowe and Davison, 2005).

In the present study the plasma lactate levels were showed an increasing tendency at different temperature conditions (16, 18, 22 and 32°C) with that of increasing of hours (24 and 48 h). Hyvarinen et al. (2004) studied the effect of temperature reduction on the stress response and recovery time of brown trout *Salmo trutta* following exhaustion with a trawl swimming simulation. After 10 min of treatment, levels of blood cortisol, lactate and glucose were higher for fish exposed to extreme cold after swimming relative to those that were only exercised. Among the different temperature levels, 32°C showed the highest plasma lactate level. Lermen et al. (2004) suggest

that plasma concentration of lactate, which can increase with both activity and stress independently, is of less use than primary stress indicators such as cortisol for the measurement cold-shock stress. Suski et al. (2006) found that reduced temperature resulted in elevated lactate concentrations, impaired replenishment of white muscle energy stores and elevated plasma cortisol concentrations relative to largemouth bass *Micropterus salmoides* recovered at ambient water temperatures. These authors conclude that, similar to the conclusions suggested by Galloway and Kieffer (2003) and Hyvarinen et al. (2004), rapid transfer of fish to cool water reduces the activity of channels, pumps and enzymes that clear lactate and replenish energy stores. Galloway and Kieffer (2003) examined cold shock relative to metabolic recovery from exhaustive exercise in juvenile Atlantic salmon *Salmo salar*. They measured muscle phosphocreatine (PCr), ATP, lactate, glycogen, glucose, pyruvate, and plasma lactate and plasma osmolarity during rest and at 0–4 h following exhaustive exercise at 12° C. The authors found that the recovery of metabolites such as muscle PCr, ATP and plasma lactate that took 2–4 h in the control fish (at 18° C) was delayed in fish that experienced a cold shock (6° C) measured at 0, 1, 2 and 4 h post-exercise.

The present study the plasma cortisol levels showed a decreasing tendency in order to decreasing the temperature level of 32, 22, 18 and 16°C. A study by Chen et al. (2002) found that cold shock modulates catecholamine and cortisol concentrations in tilapia *Oreochromis aureus* (Steindachner) subjected to cold-shock treatments where temperatures decreased from 25 to 12°C over either 15 or 30 min. Tanck et al. (2000) evaluated the cold-shock response of *C.carpio* that were exposed to rapid temperature drops of 7, 9 and 11°C from an initial acclimation temperature of 25°C. Plasma cortisol levels

were positively correlated to the magnitude of temperature decreases. During 1 h period the cortisol level is higher than that of 24 and 48 h. Barton and Peter (1982) exposed fingerling rainbow trout *Oncorhynchus mykiss* (Walbaum) to a rapid temperature decrease (from 10–11 to 1°C) and found that plasma cortisol levels increased within 30 min and were maintained up to 4 h after exposure. However, our results in carp showed an increase in the level of plasma cortisol 2 h after an abrupt change in water temperature from 20 to 12 °C (Le Morvan et al., 1995). Tanck et al. (2000) reported that peak levels of plasma cortisol were recorded 20 min after the initial temperature reduction rather than after 60 min when the final low temperature was reached in *C. carpio*. Even exposure to a 1°C cold shock resulted in a large increase in plasma cortisol levels after 4 h and levels recovered after 24 h. Seth et al. (2013) reported that Plasma cortisol values increased significantly from 27.5±3.5 before to 47.0±6.2 ng ml^{-1} at 30 min of hypothermic exposure of artic char. However, Lermen et al. (2004) found that a varying temperature regime did not have a significant effect on cortisol levels in the South American silver catfish *Rhamdia quelen* (Quoy and Gaimard). The lack of a cortisol response may be explained by the fact that the temperature regimes occurred over a relatively gradual 12 h period, resulting in fish acclimating to low temperatures without inducing stress (Donaldson et al., 2008).

Chen, et al. (2002) reported the elevated levels of plasma catecholamines, which are known to increase during acute hypothermia in arctic char. a study by Chen et al. (2002) found that cold shock modulates catecholamine and cortisol concentrations in tilapia *Oreochromis aureus* (Steindachner) subjected to cold-shock treatments where temperatures decreased from 25 to 12° C over either 15 or 30 min.

4.8 Summary

The most efficient dosage of the anaesthetic drugs tested was chosen to be 0.16mg/ L of clove oil and 16°C of hypothermic condition for size (2.078 ±0.15g and 4.0±0.1cm) classes of *Etroplus suratensis*. Under the introduction treatment of cassumunar ginger extract, Tobacco leaf extract and MS 222, the fish expressed external stress signs (as assessed by plasma cortisol values). There was no such observation for fish during clove oil and hypothermia treatment. The chosen anaesthetic dosage of clove oil and hypothermia for 24 and 48h caused no mortality, indicating high safety margin for *Etroplus suratensis*. Although cinnamon oil and MS-222 gave a mortality rate of 30 % for juveniles of *Etroplus suratensis* exposed for 24h, giving cinnamon oil and MS-222 a lower safety margin for juveniles of *Etroplus suratensis.* None of the three anaesthetics seem to satisfy needs for prolonged sedation of fry because of high mortality rate recorded and dysfunctional signs observed for some of the surviving fish.

Observation during the exposure indicates insufficient blockage on the CNS as the fry (especially treated with the lower dosages of clove oil and hypothermia) showed hyperactive response to external stimuli. *Zingiber cassumunar* extract and tobacco leaf extract treatment is believed to self-induce an increased cortisol concentration. Whether clove oil and hypothermia block at any level in the HPI-axis is still unknown, but it is believed that MS-222 reduces or alleviates the stress response in juveniles of *Etroplus suratensis*. This study concluded that the anaesthetic treatment of clove oil and hypothermia seems to reduce the stress response, while *zingiber cassumunar* and tobacco leaf extract seems to self-induce an increase in plasma cortisol concentration.

…..ꕥ…..

Chapter **5**

Combined anaesthetic effects of optimum concentration of clove oil and hypothermia on juveniles of *Etroplus suratensis* in closed bag transport during 24 and 48 hours

Contents

5.1 Introduction
5.2 Materials and Methods
5.3. The sedative and anaesthetic effect of clove oil, hypothermia and the combination of optimum levels of clove oil and hypothermia
5.4 Determination of biochemical analysis of stress indices of combinations of clove oil anaesthesia and hypothermia on juvenile Etroplus suratensis in closed bag transport during 24 and 48hrs
5.5 Result
5.6 Discussion
5.7 Summary

5.1 Introduction

In India the aquaculture industry is increasingly turning towards the culture of high value species. Accompanying the growth of the aquaculture industry, there is an increase in the transportation of live fish over extended periods of time (>8 hours). In many cases the grow-out sites for farmed fish (ponds, cages, net pens, etc.) are distant from the hatchery or nursery location

that supplies the juvenile fish. This necessitates the transportation of mass quantities of fish by land, sea, and air freight (Harmon, 2009). Shipping costs can be substantial, as a result of the aquatic media within which fish must be shipped (Guo et al., 1995; Lim et al., 2003; Paterson et al., 2003). Therefore, it is in the best interest of buyers and sellers to transport fish in ways that minimize shipping costs while maximizing fish survival (Norris et al., 1960; Lim et al., 2003; Harmon, 2009). Traditionally, freshwater and marine fish have been transported in both open and closed systems (Amend et al., 1982; Berka, 1986), using techniques to minimize stress and increase survival of the fish before, during, and after the transportation period (Carmichael et al., 1984; Weirich and Tomasso, 1991; Weirich et al., 1992; Gomes et al., 2003; Harmon, 2009). There is a plethora of scientific literature devoted to fish physiology and the effects of alterations in water quality, temperature, salinity, pH, ammonia, and the use of anaesthetics during transportation of fish. However, with the ever-increasing demand for the variety of species being cultured for both the ornamental and food fish markets, there is no "standard" shipping methodology that applies to all species (Emata, 2000). Nearly all aspects of fish transportation are aimed at reducing the metabolic costs of the fish while supplying the necessary elements for survival in a confined space (Durve, 1975; Weirich et al., 1992; Guo et al., 1995; Gomes et al., 2003; Paterson et al., 2003; Colburn et al., 2008; Harmon, 2009). Fish farmers also need to be conscious of "batch variability" when it comes time to transport fish, as variations in genetic makeup, feeding regime, culture conditions, or size distribution can all have marked impacts on the overall success of live fish transport. The difference between shipping success and failure typically comes down to the small variations between shipping methods and the physiological

tolerance levels of the species being transported (Pennell, 1991; Weirich et al., 1992; Chow et al., 1994; Paterson et al., 2003; Pavlidis et al., 2003; Harmon, 2009).

However, as the aquaculture industry and the market for live ornamental and food-fish has grown, advancements in fish packaging and shipping methodologies have progressed to allow for shipments of greater density (high biomass relative to water volume) over extended periods of time (>8 hours) (Lim et al., 2003). The shipping biomass levels reported for selected species of live tropical fish greatly exceed those utilized for shipments of juvenile cichlids. Colburn et al. (2008) suggest that there were significant mortalities at this biomass and they did not recommend exceeding 20 kg/m3 for shipping live juveniles of fin fish. However, the Colburn et al. (2008) methodology fails to determine optimal shipping parameters for juvenile cobia, such as optimal salinity and biomass.

One of the key factors for the possible management and restoration of populations is the development of aquaculture techniques (Orbe Mendoza et al., 2002). In modem live-fish transportation technique, the use of sedatives finds an important place. The anaesthetics lower the metabolic activity of fish, which facilitates the transport of more fish in a given quantity of water for a long time. In recent times anaesthetizing chemicals have been used in the transporting medium of fish seeds and adult fish (Das and Goswami, 2003). Only the liquid and solid anaesthetics, especially those, which are readily soluble in water, are useful in this field. Currently the main substance used is clove oil, extracted from the leaves and buds of the tree *Eugenia caryophyllata* (Linnaeus). The active principle is eugenol which concentration in clove oil is

between 70 and 90%. Clove oil is considered an appropriate anaesthetic for fish because of its low costs, simple obtaining, and considerable anaesthetic efficiency. Also, the substance apparently does not exert any toxic effect. Clove oil has been extensively used in several fish species, and the results show that the substance is a good economic alternative to the chemicals normally used in fish anaesthesia (Ross and Ross, 2008).

Hypothermia has been suggested to be beneficial from an animal welfare perspective as it reduces crowding stress and may reduce physiological stress responses (Erikson et al., 2006; Skjervold et al., 2001); Yoshikawa et al., (1989) showed that carp, previously acclimated to 23°C, would be safely held at 5°C for 5 h, and achieved sedation at 8-14°C for 24h. However, future studies will have to reveal whether a longer hypothermic exposure, which might occur during live transport of char in ice water, results in more pronounced primary stress responses (Seth et al., 2013).

Hypothermia is also known to reduce the stress in fish handling, either by itself or in combination with chemical anaesthetics (Rodman, 1963; Hovda and Linley, 1999; Ross and Ross, 1999). Anaesthesia, sedation and transportation of the Atherinopsid, *Menidia estor*, were investigated using benzocaine, hypothermia and combinations of the two (Ross et al., 2007).

The chromides or the pearl-spots (Family: Cichlidae) form an important group among the brackish water fishes of the tropics. Species of *Etroplus,* especially *E. suratensis* (pearl spot) being the largest, have many desirable features which make them ideal fishes for aquaculture. In Kerala, the fish culture has traditionally been based upon this valuable native species, the Pearl spot (*Etroplus suratensis*). Its wide salinity tolerance, ability to breed in

confined waters, fast rate of growth, good body weight, tasty flesh, highly adaptable feeding habits, robust and sturdy body and good market price are some of the favourable characteristics for selection of this fish as a candidate species for brackish water aquaculture (Keshava and Mohan, 1988). Unfortunately pollution (Periyar), exotic species introductions and over-fishing have caused marked declines in many of these native fish populations up to the point of extinction of some species like *Etroplus maculates, Etroplus suratensis, Lissa parcia* etc., (Anon). Further more the unpracticed and unauthorized fry capture from the wild also escort the way of high mortality. The fishery for the Pearl spot, (Anon), from Lake Vembanadu and Kochi in Kerala state, has been critically affected and is currently in trouble. However, the larviculture of the species remains an industry bottleneck, with many offshore aquaculture operators failing to achieve economically viable results in this portion of commercial aquaculture operations. With this increase has come an intensified effort to identify the ideal husbandry, care, and management parameters for this species.

Though, Kerala produces 2000 tonnes of Karimeen (*Etroplus suratensis*) annually, it is not sufficient to meet the rising demand (Shyam et al., 2013). Fecundity of pearl spot is low and has been estimated to be around 3000-6000; hence a successful hatchery production of seed is difficult (Bhaskaran, 1946). However the juvenile chromides have been raised successfully at the Kerala University of Fisheries and Ocean Studies (KUFOS), Central Marine Fisheries Research Institute (CMFRI) (Experimental Hatchery) and Central Institute of Brackish water Aquaculture (CIBA), Chennai in commercial quantities (i.e. sufficient to stock numerous net pen operations).

Various quantities of these fish have been shipped to aquaculture operators throughout India and abroad using protocols whereby fish are shipped at an average biomass of 200-250 numbers in 5-6 L of fresh water transport boxes at 20-22°C with virtually 30 % mortality over 24-36 hours.

Recently, there have been numerous studies on basic and applied aspects of the culture of this species in terms of temperature and salinity effects on survival and growth (Samuel, 1969; Thampy, 1980; Wetherall et al., 1987; Bindu, 2006), feeding structures and habits (Joseph, 1980; Jayaprakash and Phil, 1980; Rattan, 1994; Padmakumar, 2003) and a number of other advances (Anikuttan, 2004; Sobhana, 2006). One of the limiting factors for the management of Pearl spot (*Etroplus suratensis*) is their high susceptibility to stress, which causes high mortalities when handled (personal observation).

Temperature of aquatic environment is important for ensuring survival, distribution and normal metabolism of fish, failure to adapt to temperature fluctuations is generally ascribed to the inability of fish to respond physiologically with resultant mortality, which is related to changes in the metabolic pathways (Forghally et al., 1973). Pearl spot (*Etroplus suratensis*) are considered eurythermic animals as they are adaptable to a wide range of temperatures and salinity. The most commonly used maintenance temperature for pearl spot is 28.5 °C (83 °F), although temperatures between 24 and 30 °C (75 and 86 °F) have been recommended. Following periods of acclimation, pearl spot can tolerate a much broader temperature range (Shyam et al., 2013). However; acute exposure to temperatures below their thermal neutral zone can cause death in pearl spot (in any fish) due to their inability to quickly acclimate. This natural phenomenon has been used as a method of euthanasia

in pearl spot (in any fish), but the AVMA Guidelines (2007) on Euthanasia and the report Recognition and Alleviation of Pain and Distress in Laboratory Animals from the Institute for Laboratory Animal Research (1992) both state that hypothermia (also referred to as rapid cooling) is unacceptable as a method of euthanasia for fish. Although these reports provide no scientific explanation regarding why rapid cooling is considered unacceptable, some speculate that ice crystal formation occurs in tissues during rapid cooling.

During culture, juvenile *Etroplus suratensis* necessarily require to be transported from hatcheries to their final on growing systems and for this a reliable, controlled transportation system needs to be developed. Rapid cooling affords several advantages as a method of *Etroplus suratensis* euthanasia. The purposes of this study were A; to assess the sedative and anaesthetic effect of juveniles of Pearl spot (*Etroplus suratensis*) in closed bag transport during 24 and 48 hours of bath administration in clove oil, hypothermia and the combination of optimum levels of clove oil and hypothermia

B; to assess the stress in reducing capacity of clove oil, hypothermia and the combination of optimum levels of clove oil and hypothermia on juveniles of Pearl spot (*Etroplus suratensis*) in closed bag transport during 24 and 48 hours of bath administration.

5.2 Materials and Methods

5.2.1 Fish and experimental conditions

Collection, maintenance, acclimatization of samples and general protocol were the same and explained in details in chapter 1 section 1.2, 1.3, 1.3.1.

5.2.2 Experimental designs

The experimental groups were divided into two sets. The first set was treated with the combination of hypothermia and clove oil concentration (0.10 mg/L clove oil at 18±1°C). The second set contained treated fish with anaesthetic combination of hypothermia and clove oil concentration (0.10 mg/L at 16 ±1°C). The concentrations were based on the result from previous experiment. Each sets contained 10 juvenile fishes and each treatment was carried out in three replicates.

All sets of fishes treated and packed with optimum concentration anaesthetics. The packing system involved 12 LDPE bags (37.5x20cm) were used. Double polyethylene bags, one slipped into another, were used to insure against water loss from perforations or leakage. The anaesthetic was vigorously stirred into the packaging water before the fish were put in. All sets of experimental bags were then flattened to the water surface to expel the air, inflated with medical grade oxygen gas, secured airtight and sealed with rubber bands and finally, put it in the Styrofoam box lined with a1cm polystyrene sheet for insulation. Four ice cube packs were also put in the space between bags in Styrofoam box. The Styrofoam box was left in the laboratory to the thermostatically controlled chilling unit (Rotek Instruments, Chest type model, temperature range 0-30°C, M/S.B and C Instruments, Kerala) for keeping the transportation condition. This unit maintains the temperature of 18 ± 1°C and 16 ± 1°C for the test period for behavioural analysis. After the end of test period, the fishes were transferred for the recovery treatments were the same and explained in details in chapter 2 section 1.5.1.b.2.

5.3 The sedative and anaesthetic effect of clove oil, hypothermia and the combination of optimum levels of clove oil and hypothermia

5.3.1 The sedative and anaesthetic effects of clove oil on juvenile *Etroplus suratensis*

Collection, maintenance, acclimatization and experimental designs and preparation of samples were the same and explained in details in chapter 1 section 1.2.1, 1.3, 1.3.1, 1.4.1.1

5.3.2 The sedative and anaesthetic effects of hypothermia on juvenile *Etroplus suratensis*

Collection, maintenance, acclimatization and experimental designs and preparation of samples were the same and explained in details in chapter 1 section 1.2.1, 1.3, 1.3.1, 1.4.1.1, 1.4.1.6.

5.3.3 The sedative and anaesthetic effects of combined clove oil anaesthesia and hypothermia combinations on juvenile *Etroplus suratensis*

Collection, maintenance, acclimatization and preparation of samples were the same and explained in details in chapter 1 section 1.2, 1.3, 1.3.1, 1.4.1.1, 1.4.1.6.

5.3.4 Post treatment survival

At the end of the trials, the bags of fish from the treatments were each placed in fiber reinforced plastic tanks (L) in the laboratory with well aerated water at 28°C. After 20 min, when temperature had equilibrated, the animals were released into the tanks. Separated tanks were maintained for all the sets of experimental groups for observing post-transport mortality for seven days after simulated transport. The water temperature in the tanks was 28 ± 1°C

with an average dissolved oxygen level of 12 mg/L and the fishes were fed with pelleted feed.

5.3.5 Statistical analyses

Mean ± SEM was used to determine the differences between the induction and recovery times at different doses of hypothermia, clove oil and the combinations of clove oil and hypothermia and overall desirability during 1, 24 and 48 h. Mean induction (time from stage I_1 to I_3) and total recovery times (time from stage R_1 to R_3) were compared among treatment groups. All statistical analyses were performed using IBM SPSS STATISTICS 20.0 (Statistical Data Analysis and Scientific Research centre, UGC, Statistics Department; Mahathma Gandhi University, Kottayam, Kerala) and the level of significance (α) for all tests was 0.05.

5.4 Determination of biochemical analysis of stress indices of combinations of clove oil anaesthesia and hypothermia on juvenile *Etroplus suratensis in* closed bag transport during 24 and 48hrs.

5.4.1 Fish and experimental conditions

Collection, maintenance, acclimatization and experimental designs and preparation of samples were the same and explained in details in chapter 1 section 1.1, 1.2, 1.3, 1.3.1, 1.4, 1.4.1, 1.4.2, 1.4. 3 and 1.5.

5.4.2 Experimental designs

There were four set of studies in each group of simulated transport based on the above experimental results, duration of transport and packing density of fishes (table). The packing system involved twelve LDPE (37.5x20cm) double polyethylene bags, one slipped into another, were used to insure against water

loss from perforations or leakage. The first and second sets were conducted with 24 and 48 hr simulated transport of juvenile pearl spot (*Etroplus suratensis*) without the clove oil at 18 ± 1°C and 22 ± 1°C respectively. The third and forth sets were conducted with 24 and 48 hr simulated transport of juvenile pearl spot (*Etroplus suratensis*) with anaesthetic combination of optimum level of hypothermia and clove oil concentration (clove 0.10 at 18 ± 1°C and clove 0.10 at 22 ± 1°C). Those concentrations were based on the result from previous experiment. Each sets contained 100 fishes and each treatment was carried out in three replicates. After 24 and 48 h experimental conditions the blood samples were taken out for the biochemical analysis of stress indices.

5.4.3 Biochemical analysis of stress indices

Experiment materials and methods were the same and explained in details in chapter 4; Experiment.1

5.4.4 Post treatment survival

After 48 hours of experiment, the experimental bags containing the remaining fishes with well aeration were put into Fiber Reinforced Plastic tanks containing aerated water for 1 h and after that the fishes were allowed to come out slowly from the bags. Separated tanks were maintained for all the sets of experimental groups for observing post-transport mortality for seven days after simulated transport .The water temperature in the tanks was 28 ± 1°C with an average dissolved oxygen level of 12 mg/L and the fishes were fed with pelleted feed.

5.4.5 Statistical analysis

Mean ± SEM was used to determine differences between the stress indices of *Etroplus suratensis* exposed in different doses of hypothermia, clove oil and the combinations of clove oil and hypothermia during 1, 24 and 48 h. All statistical analyses were performed using IBM SPSS STATISTICS 20.0 (Statistical Data Analysis and Scientific Research centre, UGC, Statistics Department; Mahathma Gandhi University, Kottayam, Kerala) and the level of significance (α) for all tests was 0.05.

5.5 Result

5.5.1 The sedative and anaesthetic effects of clove oil on juvenile *Etroplus suratensis*

In the present work, the sedative and anaesthetic effects of clove oil (0.10 and 0.17 mg/L) on juveniles of *Etroplus suratensis* showed the behavioural responses with dose. A gradual decrease of reaction to external stimuli and differences in pigmentation and opercular rate were (personal observation) found in clove oil (0.10 and 0.17 mg/L) categorical doses.

Summary statistics (Mean ± SEM) of induction and recovery stages at different concentration levels of clove oil (0.10 and 0.17 mg/L) on juveniles of *Etroplus suratensis* were identified. Induction times generally decreased significantly with increasing doses of clove oil were evaluated. The maximum depth of anaesthesia increased significantly as anaesthetic concentration increased (Table 5.1). Most fish achieved either stage 2 or stage 3 anaesthesia, which is indicative of partial loss of equilibrium. Stage 2 anaesthesia is regarded as an ideal value for fish transport and general handling. Control fish exhibited no indication of anaesthesia (Stage 0). When examined on a

categorical basis, there was a consistent increase in stage of anaesthesia for each increasing of clove oil concentration category (Mean ± SEM; Table 5.1). The time required to reach the maximal and stable stage of anaesthesia also varied significantly. Induction times decreased significantly with increasing concentrations for clove oil (0.10 and 0.17 mg/L) (Mean ± SEM; Table 5.1).

No sedation or anaesthesia was induced at lower levels of clove oil (0.06 and 0.09 mg/L) after 15 min of exposure but light sedation was obtained at 0.10 mg/L at 5 min 78 sec and deep sedation at 0.17 mg/L at 2 min 75 sec (Table 5.1). Induction became more rapid as the dose increased. After only 2 min at 0.10 mg/L, swimming and touch sensitivity was reduced in some fishes and this increased to 100 % after 5min when a deep surgical anaesthesia was attained (Table 5.1). At higher doses, anaesthesia was rapid and deep (reference: chapter 2), but resulted in some deaths after 60 min of exposure.

Five minutes after the introduction of the clove oil, fish shifted in to the clove oil free fresh water, the behavioral recovery times varied extensively by concentration. On the other hand, recovery times increased with increasing concentrations of Clove oil (Mean ± SEM; Table 5.1). Recovery was rapid and uneventful at 0.10 mg/L within 37.9 ± 6.6 (~1min) becoming progressively longer as the dose rate increased. At 0.17 mg/L, the recovery time was 58.1 ± 2.7sec (1min) (Mean ± SEM; Table 5.1).

5.5.2 The sedative and anaesthetic effects of hypothermia on juvenile *Etroplus suratensis*

Summary statistics of induction and recovery times at different doses of hypothermia for *Etroplus suratensis* is given in the Table 5.1, which gives the

values Mean ± SEM. Induction times decreased significantly with increasing concentrations for *hypothermia*.

On the other hand, recovery times increased with increasing concentrations of hypothermia (Mean ± SEM; Table 5.1). When examined on a categorical basis, the lowest concentration categories achieved significantly lower stages of anaesthesia than the highest categories (Mean ± SEM; Table 5.1). Similarly, basal behavioural variables (i.e., induction time, recovery time and maximum stage of anaesthesia) vary across the gradient of concentrations. Overall mean basal behavioural variables during experiments conducted at different hypothermic conditions were I_1 66.14 ± 6.54, R_1 58.28 ± 1.80 for 18±1°C, I_3 156.4 ± 3.2, R_3 100.1 ± 4.5 for 16 ± 1°C (Mean ± SEM; Table 5.1). Behavioural responses during movement were quite changeable across a gradient of hypothermic concentrations. When initially exposed to hypothermia, fish experienced a consistent movement (seconds) through decrease in gill movement. Both behavioural induction and behavioural recovery exhibited an inverse pattern relative to hypothermic condition (Mean ± SEM; Table 5.1) and differed among the hypothermic concentrations (Mean ± SEM; Table 5.1). Rapid temperature reduction from 24 to 21°C or 18°C had no significant sedative effect but at 22°C swimming ceased in all fish after 1min 31sec and 80% had lost touch sensitivity after 3min (Mean ± SEM; Table 5.1). At 12 °C (chapter:2), swimming ceased and touch sensitivity was suppressed immediately, resulting in a form of deep sedation (Fig. 2c). A similar effect was recorded at 9 °C but there was also some loss of equilibrium and 65% of the fish ceased opercular movements after 4min (Fig. 2d) and recovery was uneventful, requiring progressively longer from lower temperatures (chapter: 2).

The lowest category that incorporated concentration of 16 ± 1°C consistently had within the minimum behavioural induction and recovery time (I_3 156.4 sec ± 0.2, R_3 100.1 ± 4.5sec; I_3 ~3min, R_3 2 min 6sec) (Mean ± SEM; Table 5.1). Recovery time varied significantly among hypothermic concentration categories, increasing with the higher categories (Mean ± SEM; Table 5.1). The only departure from this pattern was the18 ±1°C to 16 ±1°C category where recovery times were significantly faster (58.28 ± 1.80 sec to 0) (Mean ± SEM; Table 5.1).

5.5.3 Sedative and anaesthetic effects of combined clove oil anaesthesia and hypothermia combinations on juvenile *Etroplus suratensis*

In the present work, the sedative and anaesthetic effects of combinations of clove oil and hypothermia (0.10 mg/L at 18 ± 1°C) and (0.10 at 22 ± 1°C) on juveniles of *Etroplus suratensis* showed that behavioral response to all anaesthetics changed with dose (Mean ± SEM; Table 5.1). The combined hypothermia and anaesthesia produced a consistent response in which touch sensitivity was lost first, followed by swimming, equilibrium and finally opercular movements. Induction times generally decreased significantly with increasing doses of the combinations of the two were evaluated. The maximum depth of anaesthesia increased significantly as anaesthetic concentration increased (Mean ± SEM; Table 5.1). At 18°C, 0.10 mg/L clove oil suppressed touch sensitivity and reduced swimming activity and lost equilibrium at 1min and 91sec (Mean ± SEM; Table 5.1). This effect was considerably greater than that obtained with either treatment alone, although induction was less rapid and the depth of sedation achieved was less (Mean ± SEM; Table 5.1). At 22°C, 0.10 mg/L, swimming was suppressed from an early stage and very few fish lost equilibrium even after

1min76 sec (Mean ± SEM; Table 5.1). At 18°C, 0.10mg/L and 22°C, 0.10 mg/L the effect deepened with loss of opercular movement and equilibrium in100% of the fish after about 15 min. The recovery times increased with increasing concentrations of the combinations of the anaesthetics (clove oil and hypothermia).

The recovery time at 22°C, 0.10 mg/L, was 2min which is less than that of 18°C, 0.10 mg/L (1min 39sec). Recovery from all treatments at 22°C, 0.10 mg/L, was uneventful, becoming progressively longer as the dose rate increased (Mean ± SEM; Table 5.1).

Table 5.1 Mean ± SEM of induction and recovery times at different doses of hypothermia, clove oil and the combinations of clove oil and hypothermia and overall desirability values

Concentrations	I_1	I_2	I_3	R_1	R_2	R_3	D
22±1°C	69.6±2.3	78.9±2.1	156.4±3.2	73.5±0.5	79.9±1.9	100.1±4.5	0.29±0.02
18±1°C	66.1±2.5			58.3±0.7			0.00±0.00
Clove 0.10	177.9±2.7	271.3±8.6	347.3±4.6	16.1±2.6	23.0±3.4	37.9±6.6	0.00±0.00
Clove 0.17	106.4±5.1	129.2±5.9	165.1±7.5	41.1±0.4	48.7±1.4	58.1±2.7	0.21±0.06
Clove 0.10 + 18±1°C	78.6±2.3	110.7±3.6	115.1±0.9	58.3±0.7	73.7±0.8	83.6±0.8	0.51±0.00
Clove 0.10 + 22±1°C	66.1±2.5	78.3±1.7	106.1±4.9	84.3±1.0	110.7±2.4	117.3±2.1	0.50±0.01

- Values are expressed in seconds
- Average of six values in each group represents the Mean ± SEM

5.5.4 Biochemical analysis of stress indices

In the present work, the biochemical analysis of stress indices of concentrations of 18 ± 1°C, 22 ± 1°C, combinations of clove oil and hypothermia (0.10 mg/L at 18 ± 1°C) and (0.10 mg/L at 22 ± 1°C) on juveniles of *Etroplus*

suratensis showed the blood glucose concentration of the anaesthetized fish (*Etroplus suratensis).* At the concentration of 0.10 mg/L+ 22 ± 1°C, the blood glucose level showed significant higher level than that of other treatments exposed in optimum concentration of 18 ± 1°C, 22 ± 1°C and 0.10 mg/L at 18 ± 1°C during 1h (Fig.5.1). During 24 h the concentration of 0.10 mg/L at 18 ± 1°C showed an increased rate than other concentrations. After the experimental period (48 h) it was clearly analyzed that the concentrations of 18 ± 1°C and 22 ± 1°C without clove oil showed higher blood glucose level (Fig.5.1) than the other concentrations of 0.10 mg/Lat 18 ± 1°C and 0.10 mg/L+ 22 ± 1°C (Table 5.2).

Similarly the plasma lactate level was minimum at 18 ± 1°C and maximum at 0.10 mg/L at 8 ± 1°C during 1 h experiment period (Table 5.2). During 24 h experimental period, the concentration of 22 ± 1°C showed the minimum lactate level and the concentration of 0.10 mg/L at 22 ± 1°C showed the maximum lactate level. During 48 h experimental period, 18 ±1°C showed the minimum and 0.10 mg/L at 18±1°C, showed the maximum lactate level (Fig.5.2).

The cortisol levels were higher at the concentration of 0.10 mg/L at 22 ± 1°C and 0.10 mg/L at 18 ± 1°C respectively and lower at 22 ± 1°C during 1 h. The 24 h cortisol levels were higher at 22 ± 1°C and lower at 0.10 mg/L at 22 ± 1°C. During 48 h experimental period, the cortisol level higher at 22 ± 1°C and lower at the concentration of 0.10 mg/L at 18 ± 1°C (Fig.5.3).

5.5.4.1 Sugar

Table 5.2 Plasma sugar levels of *Etroplus suratensis* exposed in different doses of hypothermia, clove oil and the combinations of clove oil and hypothermia during 1, 24 and 48 h

Concentrations	Time		
	1	**24**	**48**
18°C	20.17±0.48	26.33±2.54	29.33±1.09
22°C	21.50±2.33	24.83±2.83	28.17±3.36
0.10mg/L clove oil+ 18°C	21.83±1.99	29.83±3.88	27.00±0.89
0.10mg/L clove oil+ 22°C	22.17±1.87	27.67±3.48	26.00±0.63

- Values are expressed in mg dL^{-1}
- Average of six values in each group represents the Mean ± SEM

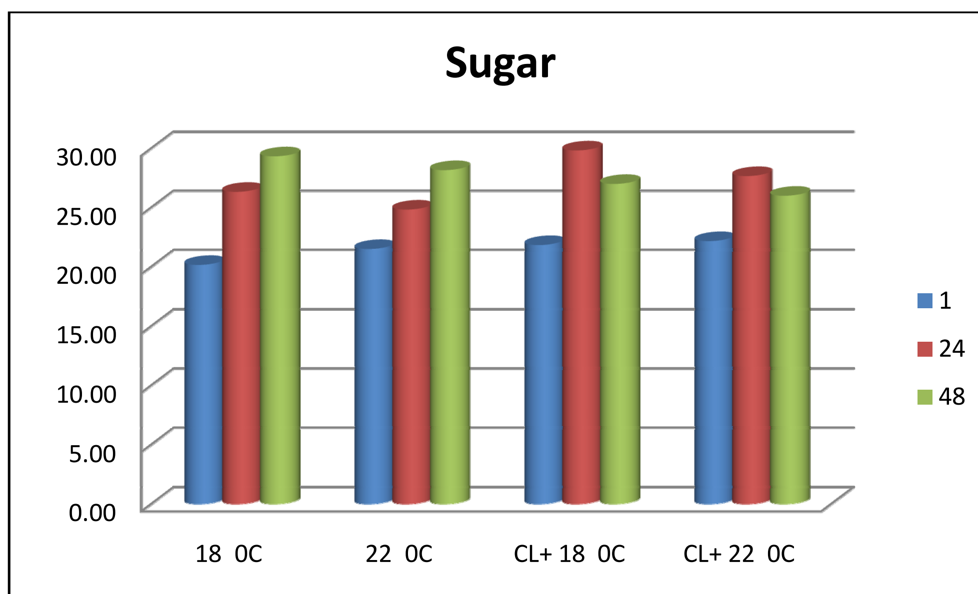


Fig. 5.1 Plasma glucose level of *Etroplus suratensis* exposed in optimum concentration of different doses of hypothermia, clove oil and the combinations of clove oil and hypothermia during 1, 24 and 48 h

5.5.4.2 Lactate

Table 5.3 Plasma lactate levels of *Etroplus suratensis* exposed in different doses of hypothermia, clove oil and the combinations of clove oil and hypothermia during 1, 24 and 48 h

Concentrations	Time		
	1	**24**	**48**
18°C	13.50±0.43	17.00±0.82	13.33±1.17
22°C	14.00±0.89	16.33±1.05	15.50±0.67
CL+ 18°C	15.17±0.31	18.17±0.98	20.67±0.61
CL+ 22°C	15.00±0.26	18.50±0.96	19.33±1.05

- Values are expressed in mg dL^{-1}
- Average of six values in each group represents the Mean ± SEM

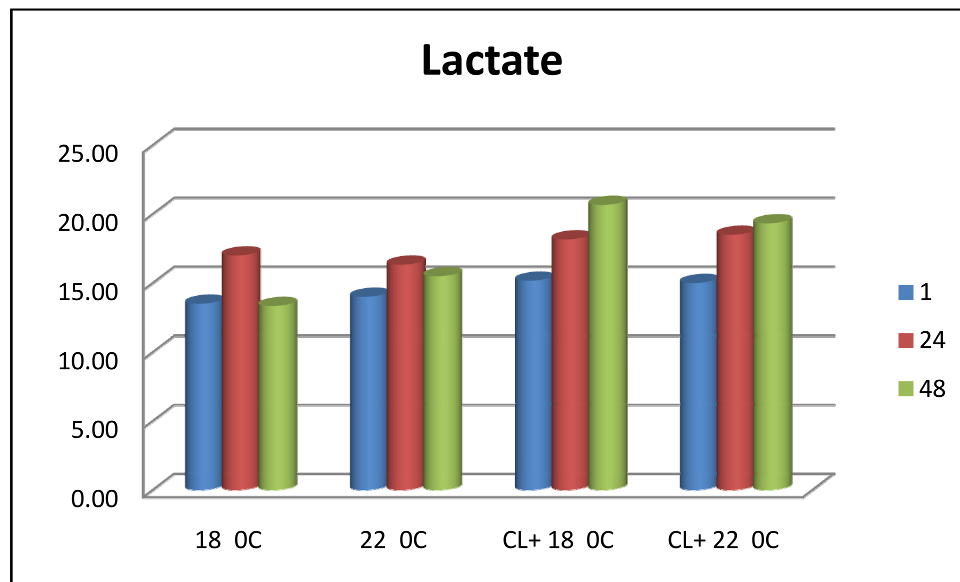


Fig. 5.2 Plasma lactate level of *Etroplus suratensis* exposed in optimum concentration of different doses of hypothermia, clove oil and the combinations of clove oil and hypothermia during 1, 24 and 48 h

5.5.4.3. Cortisol

Table 5.4 Plasma cortisol levels of *Etroplus suratensis* exposed in different doses of hypothermia, clove oil and the combinations of clove oil and hypothermia during 1, 24 and 48 h

Concentrations	Time		
	1	**24**	**48**
18°C	0.04±0.00	5.02±0.68	2.43±0.51
22°C	0.02±0.00	5.74±0.79	4.42±0.87
CL+ 18°C	0.45±0.06	0.98±0.28	0.02±0.00
CL+ 22°C	0.48±0.08	0.39±0.06	0.19±0.03

- Values are expressed in µg dL^{-1}
- Average of six values in each group represents the Mean ± SEM

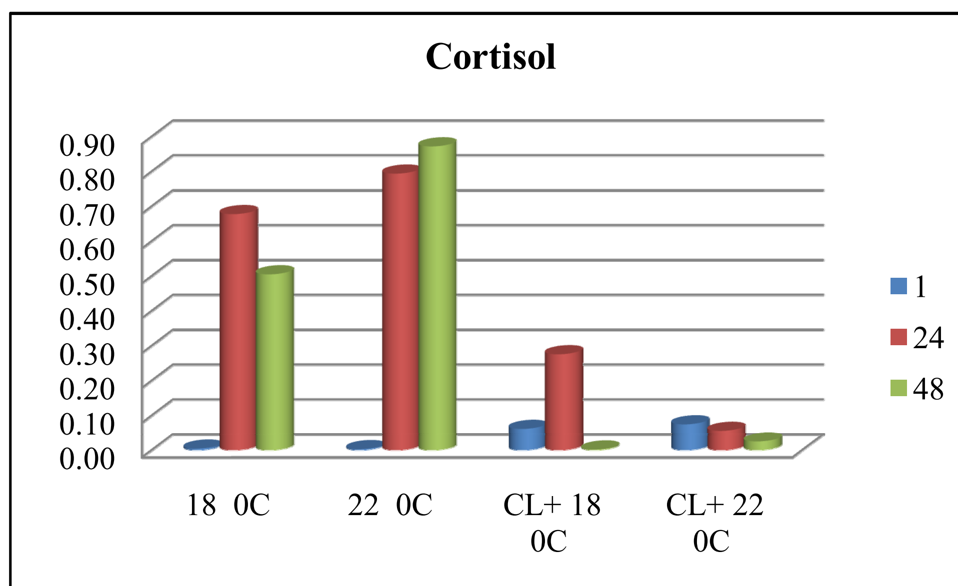


Fig. 5.3 Plasma cortisol level of *Etroplus suratensis* exposed in optimum concentration of different doses of hypothermia, clove oil and the combinations of clove oil and hypothermia during 1, 24 and 48 h

5.6 Discussion

The optimum dose rates of clove oil for sedation of juvenile *Etroplus suratensis* were 0.10 and 0.17 mg/L. At concentrations of 0.17 mg/L, deep anaesthesia was produced. These higher doses produced hemorrhages on the head and fins, leading to death. The effective dose range for *Etroplus suratensis* is thus in the lower range of clove oil anaesthesia is 0.10 mg/L, which is similar with the result of Ross and Ross, 1999 found for a range of fish species, and the dose rates for sustained sedation are about half of the maximum dose. Low levels of clove oil (5 to 9 mg/L) yielded rapid induction and maintenance of stage 2 anaesthesia in sub adult largemouth bass and was effective for mitigating the effects of fish transport stress (Cooke et al., 2004). Hypothermia has been used successfully as a sedative in several species (Solomon and Hawkins, 1981; Yokohama et al., 1989). Rapid cooling affords several advantages as a method of zebra fish euthanasia (Wilson et al., 2009). These include the ability to euthanize many animals simultaneously, minimization of handling of individual animals (which is necessary when using injectable agents or decapitation), minimal risk of operator error when preparing the euthanasia bath, and reduction in occupational health and safety risk to personnel associated with chemical and physical methods of euthanasia. When hypothermia was used alone on *Etroplus suratensis*, stable sedation was induced at 22 ± 1°C and 18 ± 1°C, and full recovery after about 1-2 min with no mortalities. Ross et al., 2007 reported that when hypothermia was used alone, stable sedation of *C. estor* was induced at 15 and 12 °C, with no mortalities and full recovery after about 8min. When the temperature was reduced further to 22 ± 1°C, the fish became stressed, exhibiting tachyventilation, darker body colour and partial loss of equilibrium. Although

there was some degree of acclimation to this lower temperature, it would not be advisable to cool to this extent for transportation. Thus the hypothermic condition of 18 ± 1°C is effective for sustained sedation for transportation of *Etroplus suratensis.*

There was a synergistic effect between clove oil and hypothermia, with the optimal combination being at 18 ± 1°C and 0.10 mg/L clove oil. Rose et al., (2007) also reported the synergism when benzocaine and hypothermia were combined; with the optimal combination being at15°C and 12 mg/L benzocaine. In the present study deep sedation was achieved without mortalities during or after transportation for 24-48 h in contrast to the control in which there were numerous mortalities within 24-30 h. Recovery from these combined treatments was rapid. In a similar study with tilapia, the best combination achieved was 18°C and 20-25 mgL^{-1}, the difference being that tilapia are more resistant to benzocaine anaesthesia (Ross and Ross, 1999) and were cooled from a higher initial temperature. Ross et al., (2007) reported that successful transportation was possible over 3.5 and 8.5 h using combined benzocaine and hypothermia with the optimal combination being at15°C and12 mgL^{-1}benzocaine. Wilson et al., (2009) reported that rapid cooling and unbuffered MS222 immersion as methods of euthanasia in zebra fish.

In the present work the plasma sugar level was very high at 18°C than other three levels of 22°C , combination being at 18 ± 1°C with 0.10 mg/L clove oil and combination being at 22 ± 1°C with 0.10 mg/L clove oil during 48 h. The initial elevation in cortisol and glucose levels is probably due to the handling stress during the capture of the fish for experiment (Pramod et al., 2009).The plasma glucose level were very low at the combination of 22 ± 1°C

with 0.10 mg/L clove oil during 48 h. Further more there is not any relevant literature available in India and abroad relating the plasma glucose level and the combination of hypothermia with clove oil.

Likewise the lactate levels were very high at the combination of 18 ± 1°C with 0.10 mg/L clove oil and very low at 18°C during 48 h treatments. Leach and Taylor (1980) indicated that the increased level of lactate may have a functional role in sustaining elevated glucose levels in response to stress as a readily available energy source. Furthermore there is not any relevant literature available in India and abroad relating the plasma lactate level and the combination of hypothermia with clove oil.

The plasma cortisol levels were very high at 22°C and low at 18 ± 1°C with 0.10 mg/L clove oil during 48 h treatment. On the other hand, rapid temperature reduction/second (i.e. cold shock) may result in primary and secondary stress responses in fish, including elevated plasma levels of cortisol and catecholamine, suggesting that the physiological responses to hypothermia are highly context- and species-specific (Barton et al., 1985; Chen et al., 2002; Donaldson et al., 2008; Foss et al., 2012; Hyvärinen et al., 2004; Tanck et al., 2000). Furthermore there is not any relevant literature available in India and abroad relating the plasma cortisol level and the combination of hypothermia with clove oil.

5.7 Summary

From the above discussion, it is clear that the combination of hypothermia and clove oil (18±1°C and 0.10 mg/L clove oil) is very effective for the transportation of juveniles of *Etroplus suratensis* by reducing the stress

hormones. Glucose, lactate and cortisol concentrations in blood plasma are the most commonly used biochemical markers of stress. Under the light of this study present biochemical alteration of cortisol level in *Etroplus suratensis* is highly reduced at 18 ± 1°C with 0.10 mg/L clove oil.

Our findings demonstrate that combination of hypothermia and clove oil (18 ± 1°C and 0.10 mg/L clove oil) is less stressful and more effective euthanasia than clove oil alone or any other anaesthesia. Hypothermia is an effective euthanasia agent advocated by the AVMA guidelines on euthanasia, (2007). The results of this study comprise a refinement to Green chromide euthanasia techniques and provide more information on sedation techniques necessary for the live fish transportation studies.

.....ഇൽ.....

Summary and Conclusions

Aquaculture is the fastest growing food-producing sector worldwide and has recently been awarded increasing interest and priority in India. However, an increased awareness has grown among the public, as well as government, of the need to secure the welfare and health of farmed fish. For example, a major current challenge for the industry is to find ethically acceptable methods to handle, immobilize during transportation from rearing site to farm. A bottleneck in the continued development of live fish transportation techniques and refinement of existing techniques has been to find reliable physiological markers of stress in fish in order to identify and quantify steps in these processes that may cause stress and suffering and thereby lead to impaired welfare.

The present study is specifically focused on one of the most important aquaculture species; Green chromide (*Etroplus suratensis*) the state fish of Kerala. In order to assess the welfare of juveniles of Green chromide (*Etroplus suratensis*) during commercial transport from hatchery to farm site, it is necessary to reduce stress responses in fish. For this purpose, we have primarily used selected anaesthetics (clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS-222 and cold) during 24-48 hours

commercial transportation enabling sufficient survivability. The objectives in this chapter proved the importance of plant anaesthetics a by scrutinize its toxicity which is capable to control the erratic behavioural, haematological and biochemical stress indices and there by increase the water quality of the packing system. Here also specifies the importance of physical anaesthetic (hypothermia) which also controls the stress indices for the maximum packing density.

The present study on toxicological effects of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS-222 and cold on juveniles of *Etroplus suratensis* were conducted under laboratory conditions using the static bioassays and continuous aeration. The aim was to develop an effective anaesthetic from an indigenous plant material that will be available at low cost to aqua culturists and which would be non-toxic to the fish and consumers. The active ingredients and their compositions in the plant anaesthetics (clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract) as well as chemical (MS-222) and physical anaesthetics (hypothermia) during 96 hours acute toxicity (LC_{50}) on juveniles of *Etroplus suratensis* were investigated. Data obtained from this investigation were subjected to 95% confidence intervals and analysis of variance (ANOVA) at 0.05 level of significance. No mortality was observed in the present study group exposed to lower concentrations of selected anaesthetics within the first 24 hours of exposure. For the selected anaesthetic concentrations tested in this experiment, the mortality rate was always higher at higher concentrations during 96 h duration. Depending on the duration of hours, the mortality rate at each concentration differed. The cumulative mortality rate indicated that mortality rate of the test fish and concentrations of selected anaesthetics are

positively correlated. During each exposure period (24, 48, 72 and 96 hours) of the acute toxicity test for selected anaesthetics, it was observed that the mean values of all water quality parameters were significantly different ($P < 0.05$). This study has shown that clove oil is less toxic, highly effective, cost efficient and safe anaesthetic for juveniles of *Etroplus suratensis* use in aquaculture and laboratory research settings at concentrations not more than 0.10 mg L^{-1}.

The present study reveals that the use of low concentrations of certain plant extracts and oils (clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract) that shows anaesthetic property to achieve sedation through behavioural assessments for fish handling and transportation, compared to the synthetic anaesthetic (MS-222) and physical (hypothermia) anaesthetic was also evaluated along with plant anaesthetics. Clove oil gave the best induction and recovery times. Cinnamon oil compared with clove oil had a significantly longer time to sedation. Cassumunar Ginger (*Zingiber cassumunar* Roxb) showed some anaesthetic properties but was less effective due to longer induction and recovery times. Tobacco leaves (*Nicotiana tobaccum*) showed some anaesthetic properties but were toxic. It is concluded that anaesthetic properties of clove are comparable with the recommended criteria for being an effective anaesthetic. Although MS-222 (tricaine methanesulphonate) is an effective fish anaesthetic with the desirable characteristics of rapid induction and recovery times, but it has less margin of safety and also has 21 days of withdrawal period. The study also proved that application of hypothermia in *Etroplus suratensis* would result anaesthetization within a shorter time in minimal signs of distress. During transportation the induction and recovery rate and the fresh condition of the

live fish indicate the good physiological parameters, demonstrating the benefits of live transportation of fishes.

Behavioural assessments through video monitoring systems are becoming regular in live fish transport. Video monitoring systems is a convenient and non-invasive tool for assessing the behaviour of transported fish; therefore in the present work attempted to come up with easily measured criteria that were comparable to physiological measurements. It is important to determine, however, what the behaviour is for a given group of juveniles as there can be differences in behaviour depending upon strain and rearing conditions.

Results of the present investigation revealed certain haematological parameters (Hct, Hb, RBC, MCV, MCH, PCV and MCHC) in the blood of *Etroplus suratensis,* during prolonged exposure (24 and 48 h) to the optimum concentrations of clove oil, cinnamon oil, cassumunar ginger extract, tobacco leaf extract, MS-222 and cold (hypothermia) showed significant difference with the control group.

As predicted, primary stress (as assessed by plasma cortisol values) was greatest immediately following truck transport. However, primary and secondary stresses were only moderate when compared with other work cited on fish and stress physiology. Because stress responses immediately before and during harvest have been shown to affect product quality (Robb 2001), one can assume anything that increases the stress response before harvest will likely have a similar increased negative effect on the end product quality and therefore should be avoided if possible. Among the anaesthetic treatments investigated in the present study (clove oil, cinnamon oil, cassumunar ginger

extract, tobacco leaf extract, MS-222 and cold) the cassumunar ginger extract and tobacco leaf extract were not effective in consistently reducing the stress responses of juveniles of *Etroplus suratensis* during the 24-48 h simulated transport. All treatment groups showed stress responses for each of the parameters measured. Use of clove oil during 24-48 h transport indicated no clear advantage over water quality. Treatment with cinnamon oil does not give good margin of safety, and thus its use cannot be justified. Hypothermia treatment actually indicated less stress responses compared to other treatment, as indicated by significantly lower cortisol, glucose, and lactate responses. Use of MS-222 resulted in more severe stress responses (haematocrit and cortisol) compared to other treatments and control. But during the combinations of clove oil and hypothermia, it likely indicates a deleterious rather then beneficial effect on end product quality after 24-48 h transportation. Overall, use of anaesthetics during transport of juveniles of *Etroplus suratensis* provided clear advantage, and in the case of combination of anaesthetics viz., clove oil with hypothermia (0.10 mg l^{-1} at 18°C) may have resulted in additional gain of product quality because of a less stress response.

Although all six anaesthetic agents evaluated were effective and presented a good margin of safety, clove oil with hypothermia proved to be most effective in the juveniles of *Etroplus suratensis*. Due to the investigation of different anaesthetic induction and recovery stages, as well as the identification of the lowest effective concentrations of each anaesthetic agent, the findings of the present study has potential significance with regards to pearl spot (*Etroplus suratensis*) husbandry, stress, survival, transport and revenue. In particular the chemical anaesthetic are not reachable for invasive

fisheries research procedures and aquaculture procedures due to the high cost, in availability, hardy to use, lethal to fish and less margin of safety.

Based upon the positive results of our study using clove oil with hypothermia to transport *Etroplus suratensis* coupled with the growing body of literature cited we suggest that clove oil with hypothermia should be an effective alternative transport anaesthetic. Our study focused on the use of clove oil with hypothermia for fish transportation. The concentrations required to induce anaesthesia identified as optimal for fish transport should also be effective for the general handling of cultured fish for grading, marking, enumerating, inspection, and gamete stripping. This study is the first to identify euthanasia methods for *Etroplus suratensis*, (as described by American Veterinary Medical Association [Internet] (2007) AVMA guidelines on euthanasia, 2007) and the outcome will be important in assisting institutional animal care and use committees and researchers in determining of the most appropriate method of euthanasia for *Etroplus suratensis*. Further work will also be needed to determine its utility for large-scale operation. The results of this study comprise a refinement to *Etroplus suratensis* euthanasia techniques and provide more information on techniques necessary for *Etroplus suratensis* studies for the laboratory animal and biomedical research community. The work described here has, for the first time, provided a systematically derived system of safe, long distance, live transportation of this species and others of the genus *Etroplus*. These species are delicate and easily stressed and this system opens further the opportunities for their culture during in large scale operation.

.....ഇൽ.....

References

Adams, S. M. (1990) Status and use of biological indicators for evaluating the effects of stress on fish. Am. Fish. Sym. 8: 1-8

Adams, S.M. (2002) Biological indicators of aquatic ecosystem stress. 644 p, Am. Fish. Soc. Bethesda, MD

Adamu, K. M. (2009) Sublethal effects of tobacco (*Nicotiana tobaccum*) leaf dust on enzymatic activities of Heteroclarias (a Hybrid of *Heterobranchus bidorsalis* and *Clarias gariepinus*) of Jordan. J. Biol. Sci. 2: 4

Adamu, K.M. and Kori-Siakpere, O. (2011) Effects of sublethal concentrations of tobacco (*Nicotiana tobaccum*) leaf dust on some biochemical parameters of hybrid catfish (*Clarias gariepinus* and *Heterobranchus bidorsalis*). Braz. Arch. Biol. Technol. 54 (1): 183-196

Adhikari, S., Sarkar, B. (2004) Effects of cypermehrin and carbofuran on certain haematological parameters and prediction of their recovery in fresh water teleost, *Labeo rohita* (Ham). Ecotoxicol. Environ. Safety. 58: 220-226

Agbon, A.O., Omoniyi, I.T. and Teko, A. A. (2002) Acute toxicity of tobacco (*Nicotiana tobaccum*) leaf dust on *Oreochromis niloticus* and haematological changes resulting from sublethal exposure. J. Aquat. Sci. 17(1): 5-8

Aggergaard, S. and Jiensen, F.B. (2001) Cardiovascular changes and phiysiological response during nitrite exposure in rainbow trout. J. Fish Biol. 59: 13–27

Agokei, O.E., and Adebisi, A.A. (2010) Tobacco as an anesthetic for fish handling procedures. J. Medicinal Plants Res. 4(14): 1396-1399

Aguigwo, J.N. (1998) Studies on acute toxicity of cassava leaf extract on the African Catfish, *Clarias anguillaris*. J. Aqua. Sci. 13: 29-32

Ahmad, M.H., El Mesallamy Amani, M.D., Fatma Samir, F., and Ahran, F. (2011). Effect of Cinnamon (*Cinnamomum zeylanicum*) on Growth Performance, Feed Utilization, Whole-Body Composition, and Resistance to *Aeromonas hydrophila* in Nile Tilapia. J. Appli. Aquaculture. 23: 289–298

Ainsworth, A.J., Dexiang, C., Waterstrat, P.R. (1991) Changes in peripheral blood leukocyte percentages and function of neutrophils in stressed channel catfish. J. Aquat. Anim. Health. 3: 41-47

Akinrotimi, O.A., Ansa, E.J., Owhonda, K.N., Edun, O.M., Onunkwo, D.N., Opara, J.Y., Anyanwu, P.E. and Amachree, D. (2007) Variations in oxygen carrying capacity of *Sarotherodon melanotheron* blood in different acclimation media. J. Anim.Vet. Advances. 6(8): 932-937

Alderdice, D.F. and Velsen, F.P.J. (1978). Relation between temperature and incubation time for eggs of Chinook salmon (*Oncorhynchus tshawytscha*). J. Fisheries Res. Board of Canada. 35: 69–75

Aldrin, J.F., Messager, J.L., Laurencin, F.B., (1982) La Biochimie Clinique en Aquaculture. Interet et Perspective. CNEXO, Actes Colloq. 14: 291- 326

Aleem, S.O. (1983) Studies on escape response and survival of the gastropod mollusk. *Tympanotonus fuscatus* exposed to tobacco waste, Post-graduate Diploma Thesis, African Regional Aquaculture Centre, Port-Harcourt, Nigeria

Aleem, S.O. (1987) An assessment of tobacco waste for control of the gastropod *Tympandonus fuscavis* (Linnaeus) in brackish water fish pond. Aquaculture. 73: 19-25

Allen, J.L. and Hunn J.B (1986) Fate and distribution of some drugs used in aquaculture. Vet. Human. Toxicol. 28: 21–24

Amend D.F., Cory, T.R., Goven, B.A., Johnson, K.A., McCarthy, D.H. (1982) Transportation of fish in closed systems: Methods to control Ammonia, CO2, pH, and bacterial growth. Trans. Am. Fish. Soc. 111: 603-611

American Veterinary Medical Association [Internet] (2007) AVMA guidelines on euthanasia, 2007 update. http://www.avma.org/issues/animal_welfare/euthanasia.pdf [Cited June 2008]

Anderson, D.E., Reid, S.D., Moon, T.W. and Perry, S.F. (1991) Metabolic effects associated with chronically elevated cortisol in rainbow trout (*Oncorhynchus mykiss*). Can, J. Fish Aquat. Sci. 48: 1811-1817

Anderson, W.G., McKinley, R.S., Colavecchia, M. (1997) The use of clove oil as an anesthetic for rainbow trout and its effects on swimming performance. North Am. J. Fish. Manage. 17: 301-307

Anikuttan, K.K. (2004) Pathology of aflatoxicosis and Heavy metal toxicity in pearl spot *Etroplus suratensis* (Bloch). Ph.D thesis.129 p, Central Institute of Fisheries Education, Mumbai

Annual Report (2012-2013) Department of Animal Husbadry, Dairying and Fisheries. Ministry of Agriculture, Government of India, New Delhi

Anon (2013a) Endangered delicacy of Kerala-Report. 17th August 2013, The New Indian Express

Anon (2013b) The Hindu, 10th February, (2013)

Ansary, P.Y. (2009) Classification of anesthetics and related ayurvedic drugs.Paper presented at National seminar on anesthesia.Souvenir–Sammohini.Kerala Govt.Ayurveda College, Thripunithura, Kerala, 19-22

APHA, AWWA, WEF (1998) Standard methods for the Examination of Water and Wastewaters, 20th Edition. American Public Health Association, Washington, DC

Ashley, P.J., Sneddon, L.U. and Mc Crohan, C.R. (2006) Properties of corneal receptors in a teleost fish. Neuro. Sci. Lett. 410: 165–168

Ashley, P.J. (2007) Fish Welfare: Current Issues in Aquaculture. Appl. Anim. Behav. Sci. 104: 199-235

Auperin, B., Baroiller, J.F., Ricordel, M.J., Fostier, A. and Prunet, P. (1997) Effect of confinement stress on circulating levels of growth hormone and two prolactins in freshwater adapted Tilapia (*Oreochromis niloticus*). Gen. Comp. Endocrinol. 108: 35–44

Avella, M., Schreck, C.B. and Prunet, P. (1991) Plasma prolactin and cortisol concentrations of stressed coho salmon *Oncorhynchus kisutch*, in fresh water or salt water. Gen. Comp. Endocrinol. 81: 21–27

Baker, D.W., Wood, A.M., Litvak, M.K. and Kieffer, J.D. 2005. Haematology of juvenile *Acipenser oxyrinchus* and Acipenser brevirostrum at rest and following forced activity. J. Fish Biol. 66: 208–221

Ban, M. (2000) Effects of photoperiod and water temperature on smoltification of yearling sockeye salmon (*Oncorhynchus nerka*). Bulletin of the National Resources Center. 3: 25–28

Barcellos, L.J/G., Kreutz, L.C., De Souza, C., Rodrigues, L.B., Fioreze, I., Quevedo, R.M., Cericato, L., Soso, A.B., Fagundes, M., Conrad, J., Lacerda, L.A. and Terra, S. (2004) Hematological changes in jundia (*Rhamdia quelen quoy* and *Gaimard pimelodidae*) after acute and chronic stress caused by usual aquacultural management, with emphasis on immuno- suppressive effects. Aquaculture. 237: 229–236

Barreto, R.E. and Volpato, G.L. (2006) Ventilatory frequency of Nile tilapia subjected to different stressors. J Exp. Anim. Sci. 43: 189–196

Barreto, R.E., Gontijo, A.M.M.C., Alves-de-Lima, R.O., Raymundi, V.C., Pinhal, D., Reyes, V.A.V., Volpato, G.L. and Salvadori, D.M.F. (2007) MS222 does not induce primary DNA damage in fish. Aquaculture. Int. 15: 163–168

Barton, B. A. (2002) Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. Inte. Comp. Biol. 42(3): 517-525

Barton, B., Morgan, J.D., Vijayan, M.M. (2002) Physiological and condition-related indicators of environmental stress in fish. In: Biological indicators of aquatic ecosystem stress (Adams, S.M. Eds), pp. 111-148, American Fisheries Society, Bethasda

Barton, B., Rahn, A.B., Feist, G., Bolling, H. and Schreck, C.B. (1998) Physiological stress responses of the freshwater chondrostean paddlefish (*Polyodon spathula*) to acute physical disturbances. Comp. Biochem. Physiol. A. 120 (2): 355-363

Barton, B.A. (1997) Stress in finfish: past, present and future a historical perspective. In: Iwama, G.K., Pickering, A.D., Sumpter, J.P., Schreck, C.B. (Eds.), pp 1–33, Fish Stress and Health in Aquaculture. Society for Experimental Biology Seminar Series, vol. 62. Cambridge University Press, Cambridge, England

Barton, B.A. and Helfrich, H. (1981): Time-dose responses of juvenile rainbow trout to 2-phenoxyethanol. Prog. Fish. Cult. 43: 223-231

Barton, B.A. and Iwama, G.K. (1991) Physiological changes in fish from stress in aquaculture with emphasis on the response and effects on corticosteroids. Annual Review of Fish Diseases. 1: 3-26

Barton, B.A. and Peter, R.E. (1982) Plasma cortisol stress response in fingerling rainbow trout, *Salmo gaidneri* (Richardson), to various transport condition, anesthesia and cold-shock. J. fish biol. 20: 39-51

Barton, B.A., Weiner, G.S. and Schreck, C.B. (1985) Effect of prior acid exposure on physiological responses of juvenile raibow trout, *Salmo gairdneri* to acute handling stress. Can. J. Fish. Aquat. Sci. 42: 710-717

Basavaraja, N. and Antony, J.M. (1997) Rearing of spawn and fry of *Cyprinus carpio* on conventional and supplementary feed. Ind. J. Fish. 44(2): 165-170

Baudin, L. (1932a) Action de la tricaine sur la consommation d'oxygene de *Carassius auratus*.Compt. Rend. Soc. BioI. 109: 731-733

Begg, K. and Pankhurst, N.W. (2004) Endocrine and metabolic responses to stress in a laboratory population of the tropical damselfish *Acanthochromis polyacanthus*. J. Fish Biol. 64: 133-145

Beitinger, T.L., Bennett, W.A. and McCauley, R.W. (2000) Temperature tolerance of North American freshwater fishes exposed to dynamic changes in temperature. Environ. Biol. Fish. 58: 237–275

Bell, G.R. (1987) An outline of anesthetic and anesthesia for salmonids, a guide for fish culturists in British Columbia. Canadian Technical Report of Fisheries and Aquatic Sciences No. 1534

Benli, A.Ç.K. and Köksal, G (2005) The acute toxicity of ammonia on Tilapia (*Oreochromis niloticus* L.) larvae and fingerlings. Turk J. Vet. Anim. Sci. 29: 339-344

Berka, R. (1986) The transport of live fish. A review. 52 P, FAO European Inland Fisheries Advisory Commission (EIFAC) Technical paper No.48

Bhaskaran, V.K. (1946) Bionomics and life history of Etroplus *suratensis* (Bloch) with special reference to stocking tanks. Proc. Indian Sci. Congr. 33(3):128

Bills, T.D., Marking, L.L. and Howe, G.E. (1993) Sensityvity of juvenile striped bass to chemicals used in aquaculture- Technical report series. 192p, U.S.Fish and Wildlife service/Resource publication

Bindu, L. (2006) Captive breeding protocols of two potential cultivable fishes, *Etroplus suratensis* (Bloch) and *Horabagrus brachysoma* (Gunther) endemic to the Western Ghat region, Kerala. 215 p, Ph.D. Thesis, Mahatma Gandhi University, Kerala. India

Bircan-Yildirim, Y., Genc, E., Turan, F., Cek, S. and Yanar, M. (2010) The anaesthetic effects of quinaldine sulphate, muscle relaxant diazepam and their combination on convict cichlid, *Cichlasoma nigrofasciatum* (Gunther, 1867) Juveniles. J. Anim. Vet. Advan. 9 (3): 547-550

Black, E. C. (I958) Hyperactivity as a lethal factor in fish. J. Fish. Res. Bd. Can. 15: 573-586

Black, E.C. and Barrett. I. (1957) Increase in levels of lactic acid in the blood of cutthroat and steelhead trout following handlingand live transportation. Can. Fish Cult. 20: 13-24

Blahm, T. H. (1961) Effect of tricaine methanesulfonate on oxygen consumption of juvenile sockeye salmon. Trans. Am. Fish. Soc. 90: 226-227

Blaxhall, P.C. and Daisley, K.W. (1973) Routine haematological methods for use with fish blood. J. Fish Biol. 5: 771 - 781

Bly, J. E. and Clem, L.W. (1991) Temperature-mediated processes in teleost immunity: in vitro immunosuppression induced by in vivo low temperature in channel catfish. Vet. Immunol. Immunopathol. 28: 365–377

Bolasina, S. N. (2006) Cortisol and hematological response in Brazilian codling *Urophycis brasiliensis* (Pisces, Phycidae) subjected to anesthetic treatment. Aquacult. Inter. 14: 569-575

Bonga, S. E. W. (1997) The stress response in fish. Physiological Reviews. 77(3): 591-625

Bosworth, B.G., Small, B.C. and Mischke, C. (2004) Effects of transport water temperature, aerator type and oxygen level on channel catfish *Ictalurus punctatus* fillet quality. J. World Aquacult. Soc. 35: 412-419

Bowser, P.R. (2001) Anaesthetic options for fish. In: Recent advances in veterinary anaesthesia and analgesia: Companion Animals. (Gleed, R.D. and Ludders, J.W., Eds), pp 223-241, New York International Veterinary Services

Boyd, C. E. (1982) Water quality management for pond fish culture, 317 p, Elsevier, Amsterdam

Boyd, C. E. and Lichtkoppler, F. (1979) Water quality management in fish pond fish culture.In: International Center for Aquaculture and Agricultural Experiment Station. 30p, Auburn University Research and Development Series no.22

Brattelid, T. (1999a) Stress og biorytmer. In: Forsøksdyrlære for fiskeforskere (Brattelid, T.Eds), Forsøksdyravdelingen, Norges veterinærhøyskole

Brattelid, T. (1999b). Anestesi, håndtering, kirugiske inngrep og avlivning av fisk. In: Forsøksdyrlære for fiskeforskere (Brattelid, T.Eds), Forsøksdyravdelingen, Norges Veterinærhøyskole

Bressler, K. and Ron, B. (2004) Effect of anesthetics on stress and the innate immune system of gilthead seabream (*sparus aurata*). The Israeli Journal of Aquaculture – Bamidgeh. 56(1): 5-13

Briozzo, J., Nunez, L., Chirife, J., Herszage, L., D' Aquino, M. (1989) Antimicrobial activity of clove oil dispersed in a concentrated sugar solution. J. Appl. Bact. 66: 69-75

Broadhurst, C.L., Polansky, M.M. and Anderson, R.A. (2000) Insulin-like biological activity of culinary and medicinal plant aqueous extracts in vitro. J. Agric. Food Chem. 48(3): 849-52.66:69-75

Brown, E. A. B., Franklin, J. E., Pratt, E. and Trams, E.G. (1972) Contributions to the pharmcology of quinaldine uptake and distribution in the shark and comparative studies. Comp. Biochem. Physiol. 42A: 223-231

Brown, L.A. (1987) Recirculation anaesthesia for laboratory fish. Lab Anim. 21: 210–215

Brown, L.A. (1993) Anesthesia and restraint. In: Fish Medicine (Stoskopf, M.K., Eds), pp 79-90, WB Saunders, Philadelphia

Bruecker, P. and Graham, M. (1993) The effects of the anesthetic ketamine hydrochloride on oxygen consumption rates and behaviour in the fish *Heros (Cichlasoma) citrinellum* (Günther 1864). Comp. Biochem. Physiol. 104C: 57-59

Budavari, S.B., O'Neil, M. J., Smith, A. and Heckelman, P.E. (1989) The Merck Index. Merck and Co, Rahway, N.J

Burka, J.F., Hammel, K.L., Horsberg, T.E., Johnson, G.R., Rainnie, D.J. and Spears, D. J. (1997) Drugs in salmonid aquaculture–a review. J. Vet. Pharm. Therap. 20: 333–349

Cabello, F.C. (2006) Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Environ. Microbiol. 8: 1137–1144

Campbell, T.W. (2004) Hematology of lower vertebrates. In: Proceedings of the 55th Annual Meeting of the American College of Veterinary Pathologists (ACVPC) & 39th Annual Meeting of the American Society of Clinical Pathology (ASVCP), ACVP and ASVCP, USA

Camus, A.C., Burrow, R.M., Hemstreet, W.G., Thure, R.L. and Hawke, J.P. (1998) Aeromonas Bacterial infections – Motile Aeromonas septicemia. Southern Regional Aquaculture Centre Publication No.478

Carey, J.B., McCormick, S.D., 1998. Atlantic salmon smolts are more responsiveness to acute handling and confinement stress than parr. Aquaculture. 168: 237-253

Carmichael, G.J., Tomasso, J.R., Simco, B.A. and Davis, K.B. (1984) Characterization and alleviation of stress associated with hauling largemouth bass.Trans. Am. Fish. Soc. 113: 778-785

Carneiro, P.C.F., E.C. Urbinati and M.L. Martins. (2002) Transport with different benzocaíne concentrations and its consequences on hematological parameters and gill parasite population of matrinxã *Brycon cephalus* (Gunther, 1869) (Osteichthyes, Characidae). Acta Science. 24: 555-560

Carter, K.M., Woodley, C. M. and Brown, R. S. (2011) “A review of tricaine methanesulfonate for anesthesia of fish.” Review of Fish Biology and Fisheries. 21(1): 51–59

Chang, Y. J. and Hur, J.W (1999) Physiological responses of grey mullet *Mugil cephalus* and Nile tilapia *Oreochromis niloticus* by rapid changes in salinity of rearing water. J. Korean. Fish. Soc. 32(3): 310-316

Chanseau, M., Bosc, S., Galiay, E. and Oules, G. (2002) The use of clove oil as an anesthetic for Atlantic salmon smolts (*Salmo salar* L.) and comparison of its effect with those of 2-phenoxyethanol. Bulletin Francais de la Peche et de la Pisciculture. 365/366: 579-589

Charu Gupta., Amar, P., Garg, Ramesh, C., Uniayal, and Kumari, A. (2008) Comparative analysis of the antimicrobial activity of cinnamon oil and cinnamon extract on some food borne microbes. Afr. J. Microbiol. Res. 2: 247-251

Chen, W., Sun, L., Tsai, C., Song, Y. and Chang, C. (2002) Cold-stress induced the modulation of catecholamines, cortisol, imunoglobulin M, and leukocyte phagocytosis in Tilapia. Gen. Comp. Endocrin. 126: 90-100

Cho, G.K. and Heath, D.D. (2000) Comparison of tricaine methanisulphonoate (MS 222) clove oil anesthesia effects on the physiology of juvenile Chinook salmon *Onchorhynchus tshawytcsha* (Walbaum). Aquacult. Res. 31: 537-546

Chou, M.Y., Hsiao, C.D., Chen, S.C., Chen, I.W., Sian-Tai Liu, S.T. and Pung-Pung Hwang, P.P. (2008) Effects of hypothermia on gene expression in zebrafish gills: upregulation in differentiation and function of ionocytes as compensatory responses. J. Exp. Biol. 211: 3077-3084

Chow, P.S., Chen, T.W. and Teo, L.H. (1994) Physiological responses of the common clown fish *Amphrion ocellaris* (Cuvier) to factors related to packaging and long distance transport by air. Aquaculture. 127: 347-361

Chukwudi, N.H, Simeon, O. and Chinyere, A.J. (2011) Analysis of some biochemical and haematological parameters for Mucuna pruriens (DC) seed powder in male rats. Pak. J. Pharm. Sci. 24(4): 523-6

Chung, K.S. (1980) Cold Anaesthesia of tropical fish. Bulletin of the Japanese Society for Scientific Fisheries. 46:391

Claude, E.Boyd. (2005) LC50 Calculations Help Predict Toxicity In sustainable aquaculture practices.Global aquaculture advocates. 84-87

Colburn, H.R., Walker, A.B. and Berlinsky, D. L. (2008) Factors affecting survival of cobia, *Rachycentron canadum*, during simulated transport. J. World Aquacult. Soc. 39 (5): 678-683

Colt, J. and Tchobanoglous, G. (1976) Evaluation of shortterm toxicity of nitrogenous compounds in the channel catfish, *Ictalurus punctatus*. Aquaculture. 8: 209–224

Colt, J., Ludwig, R., Tchobanoglous, G. and Cech, J.J. (1981) The effect of nitrite on the short-term growth and survival of channel catfish *Ictalurus punctatus*. Aquaculture. 24: 111–122

Congleton, J.L. (2006) Stability of some commonly measured blood-chemistry variables in juvenile salmonids exposed to a lethal dose of the anaesthetic MS-222. Aquac. Res. 37: 1146-1149

Congleton, J.L. and Wagner, T. (2006) Blood-chemistry indicators of nutritional status and food intake in juvenile salmonids. J. Fish Biol. 69: 473-490

Cooke, S.J. and Bunt, C.M. (2004) Construction of a junction box for use with an inexpensive, commercially available underwater video camera suitable for aquatic research. North Am. J. Fish. Manage. 24: 253–257

Cooke, S.J., Graeb, B.D.S., Suski, C.D., Ostrand, K. (2003a) Effects of suture material on incision healing, growth and survival of juvenile largemouth bass implanted with miniature radio transmitters: case study of a novice and experienced fish surgeon. J. Fish Biol. 62, 1360–1388

Cooke, S.J., Ostrand, K.G., Bunt, C.M., Schreer, J.F., Wahl, D.H. and Philipp, D.P. (2003b) Cardiovascular responses of largemouth bass to exhaustive exercise and brief air exposure over a range of water temperatures. Trans.Am. Fish. Soc. 132: 1154– 1165

Cooke, S.J., Schreer, J.F., Wahl, D.H. and Philipp, D.P. (2000) Locomotory dynamics as indicators of stress in fish remote measures utilizing activity transmitters. In: Stress in Fish. Proceedings of the International Congress on the Biology of Fish. (Barton, B., Pottinger, T., Iwama, G. and MacKinlay, D., Eds), pp 77– 86, University of Aberdeen, Scotland

Cooke, S.J., Suski, C.D., Ostrand, K.G., Tufts, B.L. and wahl, D.H. (2004) Behavioral and physiological assessment of low concentrations of clove oil anesthetic for handling and transporting largemouth bass (*Micropterus salmoides*). Aquaculture. 239: 509-529

Craig, J. K., Foote, C. J. and Wood, C. C. (1996) Evidence for temperature-dependent sex determination in sockeye salmon (*Oncorhynchus nerka*). Can. J. Fish. Aquatic Sci. 53: 141–147

Cralg, W.J. (1999) Health-promoting properties of common herbs. Amer. J. Clin. Nutr. 70(3): 4915-4995

Crawshaw, L. I. (1977) Physiological and behavioural reactions of fishes to temperature change. J. Fish. Res. Board. Canada. 34: 730–734

Crosby, T. C. (2008) Metomidate hydrochloride as a sedative for transportation of selected ornamental fishes, 152 p, M.S. Thesis, University of Florida, Gainesville

Crosby, T. C., Hill, J. E., Martinez, C. V., Watson, C. A. and Yanong, R. P. E. (2006a) On-Farm transport of ornamental fish. University of Florida, Institute of Food and Agricultural Sciences (IFAS), Fact Sheet FA-119

Crosby, T. C., Hill, J. E., Martinez, C. V., Watson, C. A. and Yanong, R. P. E. (2006b) Preparation of ornamental fish for shipping. University of Florida, Institute of Food and Agricultural Sciences (IFAS), Fact Sheet FA-120

Crosby, T.C., Hill, J.E., Watson, C.A. and Yanong, R.P.E. (2006c) Effects of tricane methane sulphonate, hypno, metomidate, quinaldine, and salt on plasma cortisol levels following acute stress in three spot Gourami *Trichogaster trichopterus*. J. Aquat. Anim. Health.18: 58-63

Cuerrier, J. P. (1952) Transfer of anesthetized adult lake trout by means of aircraft. Can. Fish. Culturist. 13: 1 - 4

Currie, R.J., Bennett, W.A. and Beitinger, T.L. (1998) Critical thermal minima and maxima of three freshwater game-fish species acclimated to constant temperatures. Environ. Biol. Fishes. 51: 187–200

Dacie, J. V. and Lewis. K. (1966) Practical Haematology. 3rd Eds, 435 p G. A. Churchill, London

Das, K.S., Goswami, U.C. (2003) Evaluation of new locally available anesthetics for potential use of fish seed transportation. Fish.Technol. 40 (2): 101-104

Davis, K. B. and Parker, N.C. (1986) Plasma corticosteroid stress response of fourteen species of warmwater fish to transportation. Tran. Am. Fish.Soc. 115: 495–499

Davis, K.B. and Griffin, B.R. (2004) Physiological responses of hybrid striped bass under sedation by several anesthetics. Aquaculture. 233: 531-548

Davis, K.B., Parker, N. C. and Suttle, M. A. (1982) Plasma corticosteroids and chlorides in striped bass exposed to tricaine methanesulfonate, quinaldine, etomidate, and salt. Prog. Fish Cult. 44: 205–207

Davis, M. W. (2010) Fish stress and mortality can be predicted using reflex impairment. Fish and Fisheries. 11(1): 1-11

Delyan, U., Harder, H. and Hopner, T.H (1990) Hydrocarbon biodegradation in sediments and soils. A Systematic Examination of Physical and Chemical Conditions Part II. pH Values. Wisseschaft Technik Sci Tech. 43: 337-342

Derringer, G.C. and Suich, R. (1980) Simultaneous optimization of several response variables. J. Quality Tech. 12(4): 214

Devaraj, K.V., Shantharam, B. and Shetty, H.P.C. (1975) A comparative study of the food of juveniles of the pearl spot *Etroplus suratensis* (Bloch) collected from estuarine and fresh waters. Mysore Univ. J. Agri. Sci. 9: 479-481

Deviche, P. J., Hurley, L. L., Fokidis, H. B., Lerbour, B., Silverin, B., Sabo, J. and Sharp, P. J. (2010) Acute stress rapidly decreases plasma testosterone in a free-ranging male songbird: Potential site of action and mechanism. Gen. Comp. Endocrinol. 169(1): 82-90

Dixon, R.N., and Milton, P. (1978) The effects of the anaesthetic quinaldine on oxygen consumption in an intertidal teleost *Blennius pholis* (L.). J. Fish BioI. 12: 359-369

Doleželová, P., Mácová, S., Plhalová, L., Pištěková, V. and Svobodová, Z. (2011) The acute toxicity of clove oil to fish *Danio rerio* and *Poecilia reticulate.*Acta Vet. Brno. 80 (3): 305–308

Donaldson, M.R., Cooke, B. S. J., Patterson, D.A. and Macdonaldk, J.S. (2008). Cold shock and fish. J. Fish Bio. 73: 1491–1530

Dragland, S., Senoo, H. and Wake, K. 2003. Several culinary and medicinal herbs are important sources of dietary antioxidants. Nutr. 133(5): 1286-1290

Dridi, D., Boughattas, N.A., Aouam, K., Reinberg, A. and Ben Attia, M. (2005) Circa dian time dependent differences in murine tolerance to the antihistaminic agent loratadine.Chronobiol. Int. 22: 499–514

Durve, V.S. (1975) Anaesthetics in the transport of mullet seed. Aquaculture 5: 53 –63

Edwards, S., Burke, C., Hindrum, S. and John, S. (2000) Recovery and growth effects of anaesthetic and mechanical removal on greenlip (*Haliotis lae vigata*) and blacklip (*Haliotis rubra*) abalone. J. Shellfish Res. 19: 510 – 513

Eddy, F.B., Kunzlik, P.A. and Bath, R.N. (1983) Uptake and loss of nitrite from the blood of rainbow trout *Salmo gairdneri* Richardson, and Atlantic salmon, *Salmo salar* L. in fresh water and in dilute sea water. J. Fish Biol. 23: 105–116

Elliott, J. M. (1981). Some aspects of thermal stress on freshwater teleosts. In: Stress and Fish (Pickering, A. D. Eds), pp 209-245, Academic Press, London

Ellis, T., North, B., Scott, A.P., Bromage, N.R., Porter, M. and Gadd, D., 2002. The relationships between stocking density and welfare in farmed rainbow trout. J. Fish Biol. 61: 493–531

Ellsaesser, C.F. and Clem, L.W. (1986) Haematological and immunological changes in channel catfish stressed by handling and transport. J. Fish Biol. 28: 511–521.7(6): 719-727

Emata, A.C. (2000) Live transport of pond-reared milkfish *Chanos chanos* broodstock. J. World Aquac. Soc. 31(2): 279-282

Endo, T., Ogishima, K., Tanaka, H. and Ohshima, S. (1972) Studies on the anesthetic effect of euginol in some fresh water fishes. Bulletin of the Japanese Society of Scientific fisheries. 38: 761-767

Erdmann, M.V. (1999) Clove oil: an 'eco-friendly' alternative to cyanide use in the live reef fish industry? Live Reef Fish Information Bulletin. 5: 4-7

Erikson, U., Hultmann, L. and Steen, J.E. (2006) Live chilling of Atlantic salmon (*Salmo salar*) combined with mild carbon dioxide anaesthesia. I. Establishing a method for large-scale processing of farmed fish. Aquaculture. 252: 183-198

Erikson, U., Sigholt, T. and Seland. A. (1997) Handling stress and water quality during the live transportation and slaughter of Atlantic salmon (*Salmo salar*). Aquaculture. 149: 243-252

Falahatkar, B., Poursaeid, S., Shakoorian, M., Barton, B (2009) Responses to handling and confinement stressors in juvenile great sturgeon Huso huso. J. Fish. Biol. 75: 784–796

Farid, S.M. (1999) Use of clove oil as an anaesthetic for Indian major carps. MS Thesis, Department of Aquaculture, Faculty of Fisheries, Bangladesh AgriculturalUniversity, Mymensingh

Ferreira, J. T., Smith, G. L. and Schoonbee, H. J. 1984. The effect of the anaesthetic benzocaine-hydrochloride on freshwater quality. Water Sa. 5: 123–127

Ferreira, J.T., Schoonbee, H. J. and Smit, G.L. (1984) The anesthetic potency of benzocaine-hydrochloride in three freshwater fish species. South Afr. J. Zool. 19 (1): 46-50. ISSN: 0254-1858

Filiciotto, F., Buscaino, G., Buffa, G., Bellante, A., Maccarrone, V. and Mazzola, S. (2012) Anaesthetic qualities of eugenol and 2-phenoxyethanol and their effect on some haematological parameters in farmed European sea bass (*Dicentrarchus labrax* L.). J. Anim. Vet. Adv. 11: 494–502

Finstad, B., Iversen, M. and Sandodden, R. (2003) Stress-reducing methods for releases of Atlantic salmon (*Salmo salar*) smolts in Norway. Aquaculture. 222: 203–214

Fisher, I.U., Von uhruh, G.H. and Denger, H.J. (1990) The metabolism of eugenol in man. Xenobiotica. 20: 209-222

Fletcher, G.L. (1975) The effects of capture 'stress' and storage of whole blood on red blood cells, plasma proteins, glucose and electrolytes of the winter flounder (*Pseudopleuronectes americanus*). Can. J. Zool. 53: 197-206

Forghaly, A.M., Ezzat, A.A. and Shabana, M.B. (1973) Effect of temperature and salinity changes on the blood characteristics of *Tilapia zilli* in Egyptian littoral lakes. Comp Biochem. Physiol. 46a: 183-193

Forteath, N. (1993) Transportation of live fish. Austasia Aquaculture. 7: 51–55

Foss, A., Grimsbo, E., Vikingstad, E., Nortvedt, R., Slinde, E. and Roth, B. (2012) Live chilling of Atlantic salmon: physiological response to handling and temperature decrease on welfare. Fish. Physiol. Biochem. 38: 565-571

Francis-Floyd, R. (1995) The use of salt in aquaculture. University of Florida, Cooperative extension service. Retrieved from Fact Sheet Vol. 86, 1–3. http://edis.ifas.ufl.edu/vm007. on February 2009

Franklin, C.E., Davison, W., McKenzie, J.C. (1993) The role of the spleen during exercise in the Antarctic teleost, Pagothenia borchgrevinki. J. Exp. Biol. 164: 381-386

Fraser, J.M., and F.W.H. Beamish. 1969. Blood lactic acid concentrations in brook trout, Salvelinus fontinalis, planted by air drop. Trans. Am. Fish. Soc. 98(2): 263-267

Frazier, D.T. and Narahashi, T. (1975) Tricaine (MS-222): effects on ionic conductances of squid axon membranes. Eur. J. Pharmacol. 33 (2): 313-317

Froese, R. (1998) Insulating properties of Styrofoam boxes used in transporting live fish. Aquaculture. 159: 283-292

Fros, R. (1985) Improved fish transport in plastic bags. ICLARM, Newsletter. 10: 8-9

Fry, F.E.J. (1947) Effects of the environment on animal activity. Univ. Toronto Studies Biol. Series No. 55, Publ. Ont. Fish. Res. Lab. 68: 1–62

Gabriel, U.U., Anyanwu, P.E. and Akinrotimi, A.O. (2007) Blood characteristics associated with confinement stress in black chin tilapia. *Sarotherodon melanotheron*. J. Fish. Intl. 2(2): 186-189

Gabriel, U.U., Ezeri, G.N.O. and Opabunmi, O.O. (2004) Influence of sex, source, health status and acclimation on the haematology of *Clarias gariepinus* (Burch, 1822). Afr. J. Biotech. 3(9): 463-467

Galloway, B.J. and Kieffer, J.D. (2003) The effects of an acute temperature change on the metabolic recovery from exhaustive exercise in juvenile Atlantic salmon (*Salmo salar*). Physiological and Biochemical Zoology. 76: 652–662

Gamperi, A.K., Vijayan, M.M. and Boutilier, R.G. (1994) Experimental control of stress hormones levels in fishes: techniques and applications. Rev. Fish Biol. Fish. 4: 215-255

Gerking, S.D. (1955) Endogenous nitrogen excretion of bluegill sunfish. Physiol. Zool. 28: 283 – 289

Ghelardini, C., Galeotti, N., Di Cesare Mannelli, L., Mazzanti, G. and Bartolini, A. (2001) Local anaesthetic activity of β-caryophyllene. I1 Farmaco. 56: 387–389

Ghosh, P., Markin, R.S., Sorrell, M.F. (1997) Coumarin-induced hepatic necrosis. Am. J. Gastroenterol. 92: 348–9

Gilderhus, P.A. and Marking, L.L. (1987) Comparative efficacy of 16 anesthetic chemicals on rainbow trout. N. Am. J. Fish. Manage. 288-293

Gingerich, W.H. and Drottar, K.R. 1989. Plasma catecholamines concentrations in rainbow trout (*Salmo gairdneri*) at rest and after anaesthesia and surgery. Gen. Comp. Endocrinol. 73: 390-397

Giwanon, R., Thubthimthed, S., Rerk-am, U. and Sunthorntanasart. T. (2000) Antimicrobial activity of terpinen-4-ol and sabinene. Thai J. Pharm. Sci. 24 (S): 27

Gomes, L.C., Chippari-Gomes, A.R., Lopes, N. P., Roubach, R. and Araujo-Lima, C. (2001) Efficacy of benzocaine as an anesthetic in juvenile tambaqui *Colossoma macropomum*. J.World Aquac. Soc. 32 (4): 426-431

Gomes, L.C., Araujo-Lima, C.A.R.M., Roubach, R., Chippari-Gomes, A.R., Lopes, N.P., 2003a. Effect of fish density during transportation on stress and mortality of juvenile tambaqui *Colossoma macropomum*. J. World Aquac. Soc. 34: 76–8

Gomes, L.C., Roubach, R., Cavero, B.A.S., Pereira-Filho, M., Urbinati, E.C. (2003b) Transport of pirarucu *Arapaima gigas* juveniles in plastic bag. Acta Amazonica. 33: 631–636

Gomes, L.C.; Brinn,R.P., J.L. Marcon,J.L., Dantas, L.A., F.R. Brandão, F.R.,J.S. Abreu, J.S., P.E.M. Lemos,P.E.M., Mccomb, D.M and Baldisserotto, B. (2009) The benefits of using probiotic EfinolÒL during transportation of cardinal tetra *Paracheirodon axelrodi* (Schultz) in Amazon. Aquac. Res. 40: 157-165

Grush, J., Noakes, D. L. and Moccia, R. D. (2004) The efficacy of clove oil as an anesthetic for the zebrafish *Danio rerio* (Hamilton). Zebrafish. 1: 46–53

Grutter, A.S. and Pankurst, N.W. (2000) The effects of capture, handling, confinement and ectoparasite on plasma levels of cortisol, glucose and lactate in the coral reefish *Hemigymnus melapterus*. J. Fish Biol. 57: 391-401

Gullian, M. and Villaneuva, J. (2009) Efficiency of tricaine methanesulphonate and clove oil as anesthetics for juvenile cobia *Rachycentron canadum*. Aquac Res. 40 (7):852-860

Guo, F.C., Teo, L.H., and Chen, T.W. (1995) Effects of anesthetics on the water parameters in a simulated transport experiment of platy fish, *Xiphophorus maculatus* (Gunther). Aquac Res. 26: 265-271

Hamaakova, J., Sedova, J.M., Pjanova, S.V. and Lepiaova, A. (2001) The effect 2-phenoxyethanol, clove oil and Propiscin anaesthetics on perch (*Perca fluviatilis*) in relation to water temperature. Czech. J. Anim. Sci. 46: 469-473

Hamackova, J., Lepicova, A., Kozak, P., Stupka, Z., Kouril, J. and Lepic, P. (2004) The efficacy of various anaesthetics in tench (*Tinca tinca*) related to water temperature. Vet. Med. Czech. 49(12): 467-472

Hamilton, M.A., Russo, R.C., and Thurston, R.V. (1977) Trimmed spearman karber method for estimating media lethal concentration in toxicity bioassays. Environ. Sci. Technol. 11: 714–718

Hanafi, E.M., Maghraby, N.A., Ramadan, M.M., Mahamoud, M.A. and El-Allawy, H.M. (2010) Aromatherapy of *Cinnamomum zeylanicum* bark oil for treatment of scabies in rabbits with emphases on the productive Performance. American –Eurasian J.AGRIC & Environ.Sci.

Harmon, T.S. (2009) Methods for reducing stressors and maintaining water quality associated with live fish transport in tanks: A review of the basics. Reviews in Aquaculture. 1: 58-66

Harms, C.A. (1999) Anesthesia in fish. In: Zoo and Wild Animal Medicine, Current Therapy 4 (Fowler, M.E. and Miller, R.E., Eds), pp 158-163, Philadelphia, WB Saunders

Hassal, K.A. (1982) The chemistry of pesticides, 372 p, Macmillan press, London

Hattingh, J. (1976) Blood sugar as an indicator of stress in the freshwater fish, *Labeo capensis* (Smith). J. Fish Biol. 10: 191-195

Hattingh, J., 1977. Bloodsugar as an indicator of stress in the freshwater fish *Labeo capensis* (Smith). J. Fish Biol. 10: 19l

Heisler, N. (1988) Acid-base regulation. In: Physiology of elasmobranch fishes (Shuttleworth, T.J. Eds), pp215-252, Springer, Berlin

Heo, G.J. and Shin, G. (2010) Efficacy of benzocaine as an anaesthetic for Crucian carp (*Carassius carassius*). Vet. Anaesth. Analg. 37: 132-135

Hersh, G. (1984) Gold fish without water. Tropical fish hobbyist. 535: 16-24

Hikasa, Y., Takase, K., Ogasawara, T. and Ogasawara, S. (1986) Anesthesia and recovery with tricaine methanosulfonate, eugenol and thiopental sodium in the carp *Cyprinus carpio*. Japan J. Vet. Sc. 48: 341– 351

Hilgendorf, F. M. (1888) Fische aus dem Victoria-Nyanza (Ukerewe-See), gesammelt von dem verstorbenen Dr. G. A. Fischer. Sitzungsber. Ges. Naturf. Freunde Berlín 1888: 75-79

Hill, A.B. (1995) The environment and disease, Association or Causation? Proc. of the Royal Soc. of Med. pp 295-300

Holloway, A.C., Keene, J.L., Noakes, D.G. and Moccia, R.D. (2004) Effects of clove oil and MS 222 on blood hormone profiles in rainbow trout Oncorhynchus mykiss, Walbaum. Aquaculture. 35(11): 1025-1030

Hooven, L.A., Sherman, K.A., Butcher, S. and Giebultowicz, J.M. (2009) Does the clock make the poison? Circadian variation in response to pesticides. PLoS ONE 4: e6469

Hora, S.L. and Pillay, T.V.R. (1962) Hand-book on the fish culture in the Indo-Pacific region. FAD Fish. BioI. Tech. 14: 124

Hoseini, S.M. and Ghelichpour, M. (2011) Efficacy of clove solution on blood sampling and hematological study in Beluga Huso huso (L.) Fish. Physiol. Biochem. DOI 10.1007/s10695-011-9529-5

Hoseini, S.M., Hosseini, S.A. and Jafar Nodeh, A. (2010) Serum biochemical characteristics of Beluga *Huso huso* (L.), in response to blood sampling after clove powder solution exposure. Fish Physiol. Biochem. doi: 10.1007/s10695- 010-9458-8

Hoskonen P, Pirhonen J 2006. Effects of repeated handling, with or without anaesthesia, on feed intake and growth in juvenile rainbow trout *Oncorhynchus mykiss* (Walbaum). Aquac Res 37: 409-415

Hoskonen, P. and P. Pirhonen. (2004b) The effect of clove oil sedation on oxygen consumption of six temperate-zone fish species. Aquaculture Research. 35:1002–1005

Hoskonen, P., and Pirhonen, J. (2004a) Temperature effects on anaesthesia with clove oil in six temperature-zone fishes. J. Fish Biol. 64: 1136-1142

Houston, A.H. and Corlett, J.T. (1976) Specimen weight and MS-222. J. Fish. Res. Board. Can. 33: 1403–1407

Houston, A.M., Madden, J.A., Wood, R.J., and Miles, H.M. (1971) Variation in the blood and tissue chemistry of Brook trout, *Salvelinus fontinalis* subsequent. Journal of the Fisheries Research Board of Canada, 28 (5): 635-642

Hovda, J. and Linley, T.J. (2000) The potential application of hypothermia for anesthesia in adult pacific salmon. N. Am. J.of Aquac. 62: 67-72

Hseu, J.R., Yeh, S.L., Chu, Y.T., and Ting, Y.Y. (1998) Comparison of efficacy of five anaesthetic goldlined seabream *Sparus sarba*. Acta. Zool. 9: 35-41

Hubert, W. A. and Gern, W. A. (1995) Influence of embryonic stage on survival of cutthroat trout exposed to temperature reduction. The Progressive Fish-Culturist. 57: 326–328

Huey, D.W., Beitinger, T.L., Wooten, M.C. (1984) Nitriteinduced methaemoglobin formation and recovery in channel catfish (*Ictalurus punctatus*) at three acclimation temperatures. Bulletin of Environmental Contamination and Toxicology. 32: 674–681

Huey, D.W., Simco, B.A. and Criswell, D.W. (1980) Nitrite induced methaemoglobin formation in channel catfish. Transactions of the American Fisheries Society, 109: 558–562

Hunn, J.B. and Allen, J.L. (1974) Movement of drugs across the gills of fishes. Ann. Rev. Pharmacol. 14: 47–55

Hveding, P. (2008). Efficacy of three anesthetics in the himalayan carp Snow-trout (*Schizothorax plagiostomus*) captured in Khimti Khola river in the easteren mid-hills of Nepal. Master M.sc, Norwegian University of Science and Technology (NTNU)

Hyvärinen, P., Heinimaa, S. and Rita, H. (2004) Effects of abrupt cold shock on stress responses and recovery in brown trout exhausted by swimming. J. Fish Biol. 64: 1015–1026

Inoue, L.A.K.A., Afonso, L.O.B., Iwama, G.K. and Moraes, G. (2005) Effects of clove oil on the stress response of matrinxã (*Brycon cephalus*) subjected to transport. Acta Amazonica. 35(2): 289 – 295

Ishimatsu, A. and Itazawa Y. (1993) Anatomy and physiology of the cardiorespiratory system in air-breathing fish *Channa argus*. In: Advances in Fish Research (Singh, B.R., Eds), pp 55-70, Delhi: Narendra Publishing House, Delhi

Iversen, M., Eliassen, R. A. and Finstad, B. (2009) Potential benefit of clove oil sedation on animal welfare during salmon smolt, *Salmo salar* L. transport and transfer to sea. Aquac. Res. 40: 233-241

Iversen, M., Finstad, B. and Nilssen, k. J. (1998) Recovery from loading and transport stress in Atlantic salmon (*Salmo salar* L.) smolts. Aquaculture. 168: 387-394

Iversen, M., Finstad, B., McKinley, R.S. and Eliassen, R.A. (2003) The efficacy of metomidate, clove oil, Aqui-Sk and Benzoak R as anesthetics in Atlantic salmon (*Salmo salar* (L) smolts, and their potential stress-reducing capacity. Aquaculture. 221: 549– 566

Iversen, M., Finstad, B., McKinley, R.S., Eliassen, R.A., Carlsen, K.T., Evjen, T. (2005) Stress responses in Atlantic salmon (*Salmo salar* L.) smolts during commercial well boat transports, and effects on survival after transfer to sea. Aquaculture. 243: 373–382

Iwama, G. and Ackerman, A. (1994) Anesthetics. In: Analytical techniques in biochemistry and molecular biology of fishes (Hochachka, P. and Mommsen., Eds), 3 (1): 1-15, Elsevier Science, Amsterdam

Iwama, G. K. (2004) Stress in Fish. AquaNet Workshop on Fish Welfare, Campbell River, B.C. Canada

Iwama, G.K., McGeer, J.C. and Pawluk, M.P. (1989) The effects of five fish anesthetics on acid-base balance, hematocrit, blood gases, cortisol, and adrenaline in rainbow trout. Can. J. Zool. 67: 2065–2073

Jayaprakash, V. and Phil, M. (1980) Culture possibilities of pearl spot *Etroplus suratensis* in Kerala. Seafood Export J. 12(11): 13-15

Jeenapongsa, R., Yoovathaworn, K., Sriwatanakul, K.M., Pongprayoon, U. and Sriwatanakul, K. (2003) Anti-inflammatory activity of (*E*)-1-(3,4-dimethoxyphenyl) butadiene from *Zingiber cassumunar* Roxb. J. Ethnopharmacol. 87: 143-148

Jegede, T. and Olanrewaju, B. (2012) Piscicidal effect of tobacco (*Nicotiana tobaccum*) leaf dust on African giant catfish (*Heterobranchus bidorsalis*) fingerlings. Agri. Biol. J. N. Am. 3(11): 435-438

Jennings, C.A., and Looney, G.L. (1998) Evaluation of two types of anaesthesia for performing surgery on striped bass. N. Am. J. Fish. Manage. 18: 87 –190

Jensen, F.B. (2003) Nitrite disrupts multiple physiological functions in aquatic animals. Comp. Biochem. Physiol. Part A. 135: 9–24

Jhingran, V. G. (1975) Fish and Fisheries of India. Hindustan Publishing Corporation (India), New Delhi

Jhingran, V.G. and A.V.Natarajan. (1973) Role of Chilka lake fisheries in the development of coastal aquaculture in Eastern India. In: Coastal Aquaculture in the Indo-pacific Region (T.V.R.Pillay (Eds), pp 109-115, Fishing News (Books) Ltd., London

Johnson, W.W. and Finley, M.T.(1980) Handbook of Acute Toxicity of Chemicals to Fish and Aquatic Invertebrates. Resource Publication 137. US Department of Interior, Fish and Wildlife Service, Washington, DC

Johnson, D.L. and Metcalf, M.T. (1982) Causes and controls of freshwater drum mortalities during transportation. Transactions of the American Fisheries Society. 111: 58-62

Jones, D.R. (1971) The effect of hypoxia and anemia on the swimming performance of rainbow trout (*Salmo gairdneri*) J. exp. Biol. 55: 541-551

Joseph, M.M. (1980) Brackish water finfish and shell fish resources of Karnataka India. In: Proceedings seminar on some aspects of Inland aquaculture in Karanataka. 17-26

Kaiser, H. and Vine, N. (1998) The effect of 2-phenoxyethanol and transport packing density on the post-transport survival rate and metabolic activity in the goldfish, *Carassius auratus*. Aquarium Sciences and Conservation. 2 (1):1

Kaiser., Brill, H.G., Cahill, J., Collett, P., Czypionka, K., Green, A., Orr, K., Pattrick, P., Scheepers, R., Stonier, T., Whitehead, M.A., & Yearsley, R. (2006) Testing clove oil as an anaesthetic for long-distance transport of live fish: the case of the Lake Victoria cichlid *Haplochromis obliquidens*. J. Appl. Ichth. 22: 510-514

Karapmar, M. and Aktug, S.E (1987) Inhibition of foodborne pathogens by thymol, eugenol, methanol and anethole. Inter. J. Food Microbiol. 4: 161–166

Karimeen Varsham, (2010), Report. 5 p, Department of fisheries, Govt. of Kerala Karuppannan, N.V. (1972) Studies on the locomotory metabolism of *Tilapia mossambica* (Peters), Ph. D. Thesis, Madurai Kamaraj University, Madurai, India

Kazun, K. and Siwicki, A.K. (2001) Propiscin - a safe new anesthetic for fish**.** Archiwum Rybactwa Polskiego/Archives of Polish Fisheries. Olsztyn. 9 (2): 183-190

Keene, J.L., Noakes, D.G., Moccia, R.D. and Soto, C.G. (1998) The efficacy of clove oil as an anesthetic for rainbow trout, *Onchorhynchus mykiss* (Walbaum). Aquac. Res. 29: 89-101

Keshava, S. J. P. and Mohan, J. M. (1988) Feeding habits of the pearlspot, *Etroplus suratensis* (Bloch) in the Nethravati-Gurpur Estuary. In: Proceedings of the first Indian Fisheries Forum, Asian Fisheries Society. Indian Branch, Mangalore, pp. 203-206

Keys, A.B., and Wells, N.A. (1930) Amytal anesthesia in fish. Jour. Pharmacol and Exper. Therap. 30: 115-127

Khachatourians, G. (1998) Agricultural use of antibiotics and the evolution and transfer of antibiotic-resistant bacteria. Can. Med. Assoc. J. 159: 1129–1136

Khare, C.P. (2007) Indian Medicinal Plants an Illustrated Dictionary, 836 p, Springer Science, Business Media, LLC

Khunoana, S. (2011) Identification, characterization and quantification of the active and toxic compounds of two cinnamon species.M.Tech Thesis. URI: http://hdl.handle.net/10210/5042 (Accessed 04 October 2011)

Kiessling, A.A., Johanson, D., Zahl, I.H. and Samuelsen, O.B. (2009) Pharmacokinetics, plasma cortisol and effectiveness of benzocaine, MS-222 and isoeuginol measured in individual dorsal aorta-cannulated Atlantic salmon (*Salmo salar*) following bath administration. Aquaculture. 286: 301-308

King, H.R., Pankhurst, N.W., Watts, M. and Pankhurst. P.M. (2003) Effect of elevated summer temperatures on gonadal steroid production, vitellogenesis and egg quality in female Atlantic salmon. J. Fish Biol. 63: 153-167

King, W.V., Hooper, B., Hillsgrove, S., Benton, C. and Berlinsky, D.L. (2005) The use of clove oil, metomidate, tricaine methanesulphonate and 2-phenoxyethanol for inducing anaesthesia and their effect on the cortisol stress response in black sea bass (*Centropristis striata* L.). Aquacult. Res. 36: 1442–1449

Klontz, George, W. (1964) Anesthesia of fishes. In: Experimental Animal Anesthesiology, 13p, Brooks Air Force Base

Knapp, E. (2004) Nomenclatural changes and a new sectional classification in Nicotiana (Solanaceae) Taxon. 53(1): 73-82

Kocabatmaz, M.E. and Ekingen, G. 1978. Bes tatlisu baligi türünde bazi hematolojik normlar üzerine ön çalismalar. Firat Üniversitesi Veterinerlik Fakültesi Dergisi. 4: 223-232

Kolanczyk, R.C., Fitzsimmons, P.N., McKim, J. M., Erickson, R. J. and Schmieder, P. K. (2003) Effects of anesthesia (tricaine methanesulfonate, MS222) on liver biotransformation in rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 64: 177-184

Konar, S.K. (1970) Nicotine as a fish poison. Prog. Fish Cult. 32(3): 103-104

Kubitza, F. (1998) Técnicas de transporte de peixes vivos, 44p, Aqua and Imagem. Campo Grande MS

Kubitza, F. (2003) Amenizando as perdas de alevinos após o manejo e transporte. Panorama da Aqüicultura. 13: 15-25

Laidley, C.W. and Leatherland, J.F. (1988) Cohort sampling, anaesthesia and stocking-density e¡ects of plasma cortisol, thyroid hormone, metabolite and ion levels in rainbow trout *Salmo gairdneri* Richardson. J. Fish Biol. 33: 73-88

Larissa, N. S., Danielle, C.L., Andrea, T.M. and Levy C.G. (2011). Efficacy of clove oil as anaesthetic in handling and transportation of Nile tilapia *Oreochromis niloticus* (Actinopterygii: Cichlidae) juveniles. *Zoological,* 28(3): 285-290

Le Morvan, C., Troutaud, D. and Deschaux, P. (1995) Effects of temperature on carp leukocyte mitogen-induced proliferation and non-specific cytotoxic activity. Dev.comp.Immunol.19: 87–95

Leach, G. J. and Taylor, M. H. (1980). The role of cortisol in stressinduced metabolic changes in *Fundulus heteroclitus*. Gen. Comp. Endocnnol. 42: 219- 277

Lemm, C.A. (1993) Evaluation of anaesthetics on stripped bass, 196 p, Resource Publication National Fisheries Centre, Leetown, Keerneysville, W V

Lemmer, B. (1996) The clinical relevance of chronopharmacology in therapeutics. Pharmacol. Res. 33: 107–115

Lermen, C.L., Lappe, R., Crestani, M., Vieira, V.P., Gioda, C.R., Schetinger, M.R.C., Baldisserotto, B., Moraes, G. and Morsch, V.M. (2004) Effect of different temperature regimes on metabolic and blood parameters of silver catfish Rhamdia quelen. Aquaculture 239: 497–507

Lewis, W.M. and Morris, D.P. (1986) Toxicity of nitrite to fish: A Review. Trans. Am. Fish. Soc. 115: 183–195

Lim L.C. and Chua L.H. (1993) Transportation of ornamental fish for export - the Singapore experience. In: AQUARAMA Conference Proceedings, pp 24-26, Expoconsult, Singapore

Lim, L.C., Dhert, P., and Sorgeloos, P. (2003) Recent developments and improvements in ornamental fish packaging systems for air transport. Aquacult.Res. 34: 923-935

Lim, L.C., Dhert, P.H., Sorgeloos, P. (2007). Preparation for transport: fish packaging technology: 25-45.In: International Transport of Live Fish in the Ornamental Aquatic Industry (Fossa, S.A., Bassleer, G.M.O., Lim, C. and Ploeg, A., Eds), 126pp, Ornamental Fish International OFI Educational publication 2, OFI, Maarssen, the Netherlands

Lindsay, G. Ross and John, A. Geddes. (1979) Sedation of warm-water fish species in Aquaculture Research. Aquaculture. 16: 183–186

Lindsay, R.B., R.K. Schroeder, and K.R. Kenaston. 1999. Spring Chinook salmon in the Willamette and Sandy rivers. Oregon Department of Fish and Wildlife, Fish Research Report F-163-R-03, Annual Progress Report, Portland

Ling K.H., Chew W.Y., Lim Y.Q., Koh C.H. and Lim L.C. (2000) New approaches to quality enhancement of guppy and angel fish during transportation (abstract). In: Abstract Book of First AVA Technical Seminar, pp 6-7. Agri-food and VeterinaryAuthority of Singapore, Singapore

Liu, E.H. and Gibson, D.M. (1977) Visualization of peroxidase isozymes with eugenol, a noncarcinogenic substrate.Anal. Biochem. 79: 597-601

Locke, D.O. (1969): Quinaldine as an anaesthetic for brook trout, lake trout and Atlantic salmon, Investigations in Fish Control. 124 p, US Fish and Wildlife Service, Washington, DC

Lowe, C.J. and Davison, W. (2005) Plasma osmolarity, glucose concentration and erythrocyte responses of two Antarctic nototheniid fishes to acute and chronic thermal change. J. Fish Biol. 67: 752–766

Lutterschmidt, D.I. and Mason, R.T. (2010) Temporally distinct effects of stress and corticosterone on diel melatonin rhythms of red-sided garter snakes (*Thamnophis sirtalis*). Integr. Comp. Biol. 50: E261-E261

Macova, S., Dolezelova, P., Pistekova, V., Svobodova, Z., Bedanova, I. and Voslarova, E. (2008) Comparison of acute toxicity of 2-phenoxyethanol and clove oil to juvenile and embryonic stages of *Danio rerio*. Neuroendocrinology Letters. 29: 680–684

Mackereth, F.J.H. (1963) Some methods of water analysis for limnologists. Freshw. Biol. Assoc. Publ. 21: 70

Maheswaran, R., Devapaul, A., Velmurugan, B. and Ignacimuthu, S. (2008) Haematological studies of freshwater fish *Clarias batrachus* (L.) exposed to merguric chloride. Inter. J. Integr. Biol. 2(1): 49 – 54

Malmstrøm, T., Salte, R., Gjøen, H.M. and Linseth, A. (1993) A practical evaluation of metomidate and MS-222 as anaesthetics for Atlantic halibut (*Hippoglossus hippoglossus* L.). Aquaculture. 113: 331-338

Marbach, E.P. and Weil, M.H. (1967) Rapid enzymatic measurement of blood lactate and pyruvate. Clin. Chem. 13:314

Marking, L.L. (1967) Toxicity of MS-222 to selected fishes. In: Investigations in fish control. Resource Publication 18, U.S. pp 3-10, Department of the Interior, Bureau of Sport Fisheries and wildlife, Washington, DC

Marking, L.L. and Bills, T.D. (1975) Toxicity of potassium permanganate to fish and its effectiveness for detoxifying antimycin.Trans. Am. Fish. Soc. 104: 579-583

Marking, L.L. and Meyer, F. P. (1985) Are better anesthetics needed in fisheries? Fisheries.10: 2–5

Martin, B.J. (1995) Evaluation of hypothermia for anesthesia in reptiles and amphibians. ILAR. J. 37: 186–190

Martinez-Porchas, M., Martinez-Cordova, L.R. and Ramos-Enriquez, R. (2009) Cortisol and glucose: Reliable indicators of stress? Pan. Am. J. Aquat. Sci. 4: 158-178

Mastura, M., Nor Azah, M.A., Khozirah, S., Mawardi, R. and Manaf, A.A. (1999) Anticandidial and antidermatophic activity of Cinnamomum species oils. Cytobios. 98: 17-23

Mattson, N.S., Riple, T.H. (1989) Metomidate, a better anaesthetic for cod (*Gadus morhua*) in comparison with benzocaine, MS-222, chlorobutanol and phenoxyethanol. Aquaculture.83:89-94

Maule, A.G. and Shreck, C.B. (1990) Changes in number of leucocytes in immune organs of juvenile cohosalmon after acute stress of cortisol treatment. J. Aquacult. Anim. Health.2:298-304

Maule, A.G., Tripp, A.G. and Kaattari, S.L. (1989) Stress alters immune function and disease resistance in Chinook salmon (*Oncorhynchus tshawytscha*). J. Endocr. 120:135-142

Maura, A., Pino, A. and Ricci, R. (1989) Negative evidence in vivo DNA-damaging, mutagenic and chromosomal effects of eugenol. Mutation Res; 227: 125–129

Mazeaud, M.M. and Mazeaud, F. (1981) Adrenergic responses to stress in fish. In: Stress and Fish (Pickering, A.D. Eds.,) pp 49-75, Academic Press Inc., London, England

Mazeaud, M.M., Mazeaud, F. and Donaldson, E.M. (1977) Primary and secondary effects of stress in fish: some new data with a general review. Trans. Am. Fish.Soc. 106: 201–212

Mc Leavy, D.L. (1973) Effect of 12hr and 25 days exposure to kraft pulpmill effluent on the blood and tissue of juvenile Coho Salmon. Canada. J. Fish. Res. Bd. 30: 395-400

McCormick, S.D. and B.T. Bjornsson (1994) Physiological and ¨ hormonal differences among Atlantic salmon parr and smolts reared in the wild and hatchery smolts. Aquaculture 121: 235–244

McDonald, D.G. and Milligan, C.L. (1992) Chemical properties of the blood. In: Fish Physiology (Hoar, W.S., Randall, D.H. and Farrell, A.P., Eds), pp 56-135, Academic Press, NewYork, USA

McDonald, M. (1984) Report on the distribution of carp during the season of 1882. U.S. Comm. Fish, and Fisheries; Commissioners Rept. 1982 (38): 915-942

McFarland, W.N. and Klontz, G.W. (1969) Anesthesia in fishes. In: Federation Proceedings 28(4): 1535-1540

McFarland, W.N. (1959) A study of the effects of anaesthetics on the behaviour and physiology of fishes. Publ. Inst. Mar. Sci., Univ. Tex. 6: 23–55

McFarland, W.N. (1960) The use of anaesthetics for the handling and the transport of fishes. Calif. Fish Game. 46: 407 – 431

McKnight, I.M.A. (1966) Hematological study on the mountain white fish *Popium willasemi* South. Fish. Reb. 23: 45-64

Medina Pizzali, A.F. (2001) Low cost fish retailing equipment and facilities in large urban areas of Southeast Asia. FAO Fisheries Tech. Paper. T405. FAO of UN. Rome

Meister, Alfred, L., Charles, F. and Ritzi. (1958) Effects of chloretone and MS-222 on eastern brook trout. Prog. Fish-Cult. 20(3) 104-110

Milligan, C. L. and Wood, C. M. (1986 b) Tissue intracellular acid-base status and the fate of lactate after exhaustive exercise in the rainbow trout. J. exp. Biol. 123: 123-144

Milligan, C. L. and Wood, C. M. (1986a) Intracellular and extracellular acid-base status and H+ exchange with the environment after exhaustive exercise in the rainbow trout. J. exp. Biol. 123: 93-121

Milligan, C.L., Wood, C.M., 1987a. Regulation of blood oxygen transport and red cell pH after exhaustive activity in rainbow trout (*Salmo gairdneri*) and starry flounder (*Platichthys stellatus*). J. Exp. Biol. 133: 263–282

Milligan, C.L., Wood, C.M., 1987b. Muscle and liver intracellular acid–base and metabolite status after strenuous activity in the inactive, benthic starry flounder *Platichthys stellatus*. Physiol. Zool. 60: 54 – 68

Mitul, P., Handral, M., and Swaroop, T.V.S.S. (2013). Anti-venom activity of *Cinnamomum zeylanicum* extracts against *Naja kaouthia* snake venom. Inter. J. Pharmaceut. Chem. Sci. 2 (3): 1302-1310

Mohammadi zarejabad, A., Bastami, K.D., Sudagar, M. and Pourali Motlagh, S. (2009) Hematology of great sturgeon (*Huso huso* Linnaeus, 1758) juvenile exposed to clove powder as an anesthetic. Comp. Clin. Pathol. doi: 10.1007/s00580- 009-0886-8

Molinero, A. and Gonzalez, J. (1995) Comparative effects of MS 222 and 2-phenoxyethanol on gilthead sea bream (*Spatusaurata* L.) during confinement Camp. Biochem. Physiol. IIIA (3): 405-414

Mommsen, T.P., Vijayan, M.M. and Moon, T.W. (1999) Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Rev. Fish Biol. Fish. 9: 211-268

Montero, D., Izquierdo, M.S., Tort L., Robaina, L. and Vergara, J.M. (1999) High stocking density produces crowding stress altering some physiological and biochemical parameters in gilthead seabream (*Sparus aurata*) juveniles. Fish. Physiol. Biochem. 20: 53–60

Moran, D., Wells, R.M.G. and Pether, S.J. (2008) Physiological responses of the Asian sea bass, *Lates calcarifer* to water quality deterioration during simulated live transport: acidosis, redcell swelling, and levels of ions and ammonia in the plasma. Aquac. Res. 39: 1399-1407

Moreira, P.S. and Volpato, G.L. (2004). Conditioning of stress in Nile tilapia. J. Fish. Biol. 64: 961-969

Morozumi, S. (1978) Appl. Envir. Microbiol. 36 (l 4): 577-583

Mousavi, S.M.E., Majdi Nasab, E., Yavari, V., Rajabzadeh Ghatrami, E. and Razi Jalali, M. (2011) Effects of two anaesthetic regimes, MS-222 and eugenol, on plasma biochemical profile in Barbus sharpeyi. Comp. Clin. Pathol. DOI 10.1007/s00580-011-1189-4

Mukundan, M.K., and James, M.A. (1978) Nutritional quality of some food fish. Fish.Technol. 15: 85-87

Munday, P.L. and Wilson, S.K. (1997) Comparative efficacy of clove oil and other chemicals in anaesthetization of *Pomacentrus amboinesnsis*, a coral reef fish. J. of Fish Bio. 51: 931-938

Musa, S.O. and Omoregie, E. (1999) Haematological changes in the mudfish, *Clarias gariepinus* (Burchell) exposed to malachite green. J. Aquat. Sci. 14: 37 - 42

Mylonas, C.C., Cardinaletti, G., Sigelaki, I., and Polzonetti-Magni, A. (2005) Comparative efficacy of clove oil and 2-phenoxyethanol as anaesthetics in aquaculture of European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus auratus*) at different temperatures. Aquaculture. 246: 467-481

Myszkowski L, Kamiński R, Wolnicki J. 2003. Response of juvenile tench *Tinca tinca* (L.) to the anaesthetic 2-phenoxyethanol. J. Appl. Ichthyol. 19:142-145

Nagababu, E. and Lakshmaiah, N. (1992) Inhibitory effect of euginol on non enzymatic lipid peroxidation in rat liver mitochondria. Bioche. Pharmacol. 43: 2393-2400

NAIP Sub-Project on Mass Media Mobilization, DKMA with inputs from CIBA. (2011-2012)

Neiffer, D. L. and Stamper, M. A. (2009). Fish sedation, anesthesia, analgesia, and euthanasia: considerations, methods, and types of drugs. Ilar Journal. 50 (4): 343-360

Nemato C.M. (1957) Experiments with methods for air transport of live fish. Prog. Fish-Cult.19: 147-157

Noga, E.J. (1996) Fish Disease: Diagnosis and Treatment, 367 p, Mosby Yearbook, Inc. Weslin Industrial Drive; St. Louis, Missouri

Norris, D.O., Hobbs, S.L. (2006) The HPA axis and functions of corticosteroids in fishes. In: Fish Endocrinology (Reinecke, M., Zaccone, Kapoor, B.G., Eds), 2: 721–765, Science Publishers, Enfield, New Hampshire

Norris, K.S., Brocato, R., Calandrino, F. and McFarland, W.N. (1960) A survey of fish transportation methods and equipment. Calif. Fish Game. 46: 5-33

Öğretmen, F. and Gökçek, K. (2013) Comparative efficacy of three anesthetic agents on juvenile African Catfish *Clarias gariepinus* (Burchell, 1822). Turk. J. Fish. Aquat. Sci. 13: 51-56. http://dx.doi.org/10.4194/1303-2712-v13_1_07

Ohr, E.A. (1976) Tricane methanesulphonate–l, pH and its effects on anaesthetic potency. Comp. Biochem. Physiol. 54: 13–17

Oikawa, S., Takeda, T. and Itazawa Y. (1994) Scale effects of MS-222 on a marine teleost, *Porgy pagrus major*. Aquacult. 121: 369-379

Okamoto, M.H., Tesser, M.B., Louzada, L.R., dos Santos, R.A. and Sampaio, L.A.(2009) Benzocaine and eugenol as anaesthetics for pompano juvenile *Trachinotus marginatus*. Cienc. Rural. 39: 866–870

Oliveros, M.B. (1996) Preformulation studies on terpinen-4-ol from *Zingiber purpureum* Rosc, Proceedings of the 2nd Symposium on the Family Zingiberaceae. pp 180-186, Guangzhou, Zhongshan University Press

Olufayo, M.O. and Jatto, I.A (2011) Haematological Response of Nile Tilapia (*Oreochromis niloticus*) Juvenile Exposed to Tobacco (*Nicotiana tobaccum*) leaf dust. Proceedings of the Ninth In ternational Symposium on Tilapia in Aquaculture. Shanghai, China. 60-64

Omoniyi, I., Agbon, A.O. and Sodunke, S.A. (2002) Effect of lethal and sub-lethal concentrations of tobacco (Nicotiana Tobaccum) leaf dust extract on weight and hematological changes in *Clarias gariepinus* (Burchell). J. Appl. Sci. Envir. Manage. 6(2): 37 - 41

Onusiriuka, B.C. and Ufodike, F.B. (1994). Acute toxicity of water extracts of Akee apple, *Bligha sapida* and Sausage plant *Kigella africana* on African catfish *Clarias gariepinus.* J. Aquat. Sci. 9: 35-41

Orbe Mendoza, A.A., Acevedo Garcia, J. and Lyons, J. (2002) Lake Patzcuaro Fishery management plan. Rev. Fish Biol. Fish. 12: 207-217

Ortuno, J., Esteban, M.A. and Meseguer, J. (2002 a) Effects of four anesthetics on the innate immune response of gilthead sea bream (*Sparus aurata* L). Fish and Shellfish Immunology. 12 (1): 49-59

Ortuno, J., Esteban, M.A. and Meseguer, J. (2002 b) Lack of effect of combining different stressors on innate immune responses of sea bream (*Sparus aurata* L).Vet. Immunol. Immunopathol. 84(1-2): 17-27

Ouattara, B., Simard, R.E., Holley, R.A., Piette, G.J. and Bégin, A. (1997) Antibacterial activity of selected fatty acids and essential oils against six meat spoilage organisms. Int. J. Food Microbiol. 37: 155–62

Ozaki, Y., Kawahara, N. and Harada.M. (1991) Anti-inflammatory effect of *Zingiber cassumunar* Roxb. and its active principles. Chem. Pharm. Bull. 39: 2353-2356

Padmakumar, K.G. (2003) Open water Fish sanctuaries. Kerala Calling. 23: 34–36

Padmakumar, K.G., Bindu, L. and Manu, P.S. (2012) *Etroplus suratensis* (Bloch), the State Fish of Kerala. J. Biosci. 37(6): 925–931, Ind. Acad. Sci.

Padmakumar, K.G., Krishnan, A., Radhika, R., Manu, P.S.and Shiny, C.K. (2002) Openwater fishery interventions in Kuttanad, Kerala, with reference to fishery decline and ecosystem changes. In: Riverine and Reservoir Fisheries, Challenges and strategies (Boopendranath, M.R., Meena Kumari, B., Joseph, J., Sankar, T.V., Pravin, P., and Edwin, L., Eds), pp 15-24, Society of Fisheries Technologists (India), Cochin

Pálic, D., Herolt, D.M., Andreasen, C.B., Menzel, B.W. and Roth, J.A. (2006) Anaesthetic efficacy of tricaine methanesulfonate, metomidate and eugenol: Effects on plasma cortisol concentration and neutrophil function in fathead minnows *Pimephales promelas* (Rafinesque, 1820). Aquacult. Res. 254: 675-685

Pandit, D.N. and Ghosh, T.K. (2012) Optimization of Packing conditions for live transportation of *Anabas testudineus* and *Channa punctatus*. Fish. Technol. 49: 59 - 63

Pankhurst, N.W. and Sharples, D.F. (1992) Effects of capture and confinement on plasma cortisol concentrations in the snapper *Pagrus auratus*. Australian Journal of Marine and Freshwater Research. 43:345–356

Park, I-S., Park, M.O., Hur, J.W., Kim, D.S., Chang, Y.J., Kim, Y.J., Park, J.Y. and Johnson, S.C. (2009) Anesthetic effects of lidocainehydrochloride on water parameters in simulated transport experiment of juvenile winter flounder, *Pleuronectes americanus*. Aquaculture. 294: 76–79

Park, M.O., Hur, W.J., Im, S.Y., Seol, D.W., Lee, J., Park, I.S. (2008) Anaesthetic efficacy and physiological responses to clove oil-anaesthetized kelp grouper *Epinephelus bruneus*. Aquacult. Res. 39: 877–884

Parodi, T.V., Cunha, M.A., Heldwein, C.G., De Souza, D.M., Martins, A.C., Garcia, L.D.O., Junior, W.W., Monserrat, J.M., Schmidt, D. and Caron, B.O. (2002) The anesthetic efficacy of eugenol and the essential oils of *Lippia alba* and *Aloysia triphylla* in post-larvae and sub-adults of *Litopenaeus vannamei* (Crustacea, Penaeidae). Comp. Biochem. Phys. C. 155: 462–468

Parrish, P.R. (1985) Acute toxicity test. In: Fundamentals of Aquatic Toxicity (Rand, G. M. and Petrocelli, S.R., Eds), pp 31-57, Hemisphere Publishing Corporation, Washington, and D.C

Patterson, B. D., Rimmer, M. A., Keike, G. M. and Semmens, G. I. (2003) Physiological rcsponses of the Asian sea bass *Lates calcarifer* to water quality deterioration during simulated live transport: acidosis, red-cell swelling, and levels of ions and ammonia in the plasma. Aquaculture. 2 I8: 717- 728

Pavlidis, M., Angellotti, L., Papandroulakis, N., Divanach, P. (2003) Evaluation of Transportation procedures and fry performance in red porgy (*Pagrus pagrus*) fry. Aquaculture. 218: 187-202

Pawar, H.B., Sanaye, S.V., Sreepada, R.A., Harish, V., Suryavanshi, U. and Ansari, Z.A. (2011) Comparative efficacy of four anaesthetic agents in the yellow seahorse *Hippocampus kuda* (Bleeker, 1852). Aquaculture. 311: 155-161

Peer Mohamed, M. (1974) Influence of hypoxia on fish metabolism and activity, Ph. D. Thesis, Madurai Kamaraj University, Madurai, India

Peer Mohamed, M. and Devaraj, M. (1997) Transportation of live finfishes and shellfishes.CMFRI Spl. Publ. 66: 1-43

Pennell, W. (1991) British Columbia salmon farming manual: Fish transportation handbook, 20 p Province of British Columbia, Ministry of Agriculture and Fisheries

Perry, S.F. and Gilmour, K.M. (2006) Acid-base balance and CO_2 excretion in fish: unanswered questions and emerging models. Resp. Physiol. Neuro. 154: 199–215

Peruzzi, S., Kettunen, A., Primicerio, R. and Kauric, G. (2007) Thermal shock induction of triploidy in Atlantic cod (*Gadus morhua* L.). Aquacult. Res. 38: 926–932

Pestean, C., Oana, L., Miclaus, V., Ognean, L., Cristina cernea, Florina lancu. (2011, Vol.XVI) Observations regarding anesthesia and some of the hematological parameters in *Carassius gibelio, Cyprinus carpio* and *Oncorhyncus mikiss.* Annals of the Romanian Society for Cell Biology. 1: 234-241

Phillips, A.M. and Brockway, D.R. (1954) Effect of starvation, water temperature, and sodium amytal on the metabolic rate of brook trout. Prog. Fish-Cult. 16: 65-GB

Phillips, D.H. (1990) Further evidence that eugenol does not bind to DNA in vivo. Mutat. Res. 243: 23-26

Piazza, P.V. and Moal Le, M. (1998) The role of stress in drug self-administration. Trends. Pharmacol. Sci. 19: 67–74

Pickering, A.D. (1981) Stress and fish, 367 p, Academic Press, New York

Pickering, A. D. (1993) Endocrine-induced pathology in stressed salmonid fish. Fisheries Res. 17: 35– 50

Pickering, A.D., Pottinger, T.G. (1985) Acclimation of the brown trout *Salmo trutta* L. to the stress of daily exposure to malachite green. Aquaculture. 44: 145

Pickering, A.D. (1992) Rain bow trout husbandry: management of the stress response. Aquaculture. 100: 125-139

Pickering, A.D. and Pottinger, T.G. (1989) Stress responses and disease resistance in salmonid fish: effects of chronic elevation of plasma cortisol. Fish Phy. and Bioche. 7: 253–258

Pickering, A.D., Pottinger, T.G. and Christie, P. (1982) Recovery of the brown trout, *Salmo trutta* L., from acute handling stress: a time course study. J. Fish Biol. 20: 229–244

Piper, R.G., McElwain, I.B., Orme, L.E., McCraren, J.P., Fowler, L.G. and Leonard, J.R. (1982) Fish Hatchery Management, 517 p, U.S. Fish and Wildlife Service, Washington, DC

Pirhonen, J. and Schreck, C.B. (2003) Effects of anesthesia with MS-222, clove oil and CO2 on feed intake and plasma cortisol in steelhead trout (*Oncorhynchus mykiss*). Aquaculture. 220: 507–514

Pithayanukul, P., Tubprasert, J. and Wuthi- Udomlert, M. (2007) In Vitro Antimicrobial Activity of *Zingibercassumunar* (Plai) Oil and a 5% Plai Oil Gel. Phytotherapy Research. 21: 164-169

Pongprayoon, U., Tuchinda, P., Claeson, P. and Sematong, T. (1997) Topical antiinflammatory activity of the major lipophilic constituents of the rhizome of *Zingiber cassumunar*. Part 1. The essential oil. Phytomedicine 3: 319-22

Portz, D.E., Woodley, C.M. and Cech, C.C. Jr. (2006) Stress-associated impacts of short-term holding on fishes. Rev. Fish Biol. Fish. 16: 125–170

Pottinger, T.G., Prunet, P. and Pickering, A. D. (1992) The effects of confinement stress on circulating prolactine levels in rainbow-trout (*Oncorhynchus-mykiss*) in freshwater. General and Comparative Endocrinology. 88 (3): 454-460

Power, D.M., Fuentes, J. and Harrison, A.P. (2010) A noninvasive monitoring device for anesthetics in fish. Animal Physiology. 2: 17–23

Pramod, P.K., Ramachandran, A., Sajeevan, T.P., Sunesh Thampy., and Srinivas, S. Pai. (2010) Comparative efficacy of MS-222 and benzocaine as anaesthetics under simulated transport conditions of a tropical ornamental fish *Puntius filamentosus* (Valenciennes). Aquaculture Research. 41(2): 309-314

Prince, A. and Powell, C. (2000) Clove oil as an anesthetic for invasive field procedures on adult rainbow trout, North Am. J. Fish. Manage. 20: 1029–1032

Priyanga, R., Sanja, P., Mangala, G., Eranga, A., Nuwan, G., Sirimal, P., Kamal, P., Dilani, L. and Prasad, K. (2012) Effects of *Cinnamomum zeylanicum* (Ceylon cinnamon) on blood glucose and lipids in a diabetic and healthy rat model. Pharmacognosy Res. 4(2): 73–79

Putro, S. (1989) Dry ice - possible use in fresh and five fish handling. Infofish Int. 4(89): 24-25

Rafeeq, A.K., Muhammad, A., Bushra, S. and Mansoor, A. (2013) Acute and sub chronic toxicity of *Mucuna pruriens*, *Cinnamomum zeylanicum*, *Myristica fragrans* and their effects on hematological parameters. Australian Journal of Basic and Applied Sciences. 7(8): 641-647

Ramanayaka, J.C. and Atapattu, N.S.B.M. (2006) Fish anaesthetic properties of some local plant material. Tropical Agricultural Research & Extension. 9: 143-148

Rand, G.M. and Petrocelli, S.R. (1985) Fundamentals of aquatic toxicology: Methods and applications. Washington: Hemisphere Publishing. ISBN 0-89116-382-4

Rang, H.P., Dale, M.M., Ritter, J.M., Moore, P.K (2003) Chapter 43: local anesthetics. In: Pharmacology, 5th edn (Rang, H.P., Dale, M .M, Ritter, J.M and Moore, P.K., Eds), Churchill Livingstone, Edinburgh, Scotland

Rattan, P. (1994) Ecobiology of Pearl Spot (*Etroplus suratensis* Bloch) in Goa Waters, 401 p, Ph. D. Thesis, Goa University

Redding, J.M., Schreck, C.B., Birks, E.K. and Ewing, R.D. (1984) Cortisol and its effects on plasma thyroid hormone and electrolyte concentrations in fresh water and during seawater acclimation in yearling coho salmon *Oncorhynchus kisutch*. General and Comparative Endocrinology. 54: 146–155

Reinberg, A.E. (1991) Concepts of circadian chronopharmacology. In: Temporal control of drug delivery (Hrushesky, W.J.M., Langer, R. and Theeuwes, F., Eds), pp 102-115, Ann. NY Acad. Sci.

Richards, R. (1977) Disease of aquarium fish. Vet. Rec. 101(1): 166-167

Robertson, L., Thomas, P., and Arnold, C.R. (1988) Plasma cortisol and secondary stress responses of cultured red drum (*Sciaenops ocellatus*) to several transportation procedures. Aquaculture. 68: 115–130

Robertson, L., Thomas, P., Arnold, C.R. and Trant, J.M. (1987) Plasma cortisol and secondary stress responses of red drum to handling, transport, rearing density, and a disease outbreak. Progressive Fish-Culturist 49: 1–12

Rodman, T.D. (1963) Anesthetizing and air-transporting young white sturgeons. The Progressive Fish-Culturist. 22: 71-78

Rombough, P.J. (2007) Ontogenetic changes in the toxicity and efficacy of the anaesthetic MS222 (tricaine methanesulfonate) in zebrafish (*Danio rerio*) larvae.Comp. Biochem. Physiol. A Mol. Integr. Physiol. 148: 463–469

Ross, G.L. and Ross, b. (2008) Anaesthetic and sedative techniques for aquatic animals, 222 p, Oxford, Blackwell Science, 3rd Ed

Ross, L.G. & Ross, B. (1984) Anesthetic and sedative techniques for fish, 45p, Institute of Aquaculture Handbook, University of Stirling

Ross, L.G. and Geddes, J.A. (1979) Sedation of warm water fish species in aquaculture. Aquaculture. 16: 183-186

Ross, L.G. and Ross B. (1999) Anesthetic and sedative techniques for aquatic animals, 2nd edn, 159 p, Institute of Aquaculture, University of Stirling, Scotland, Blackwell Science, Oxford, UK

Ross, L.G., Blanco, J.S., Palacios, C.M., Racotta, I.S. and Cuevas, M.T. (2007) Anaesthesia, sedation and transportation of juvenile *Menidia estor* (Jordan) using benzocaine and hypothermia. Aquac. Res. 38: 909-917

Ross, R.M., Backman, T.W.H. and Bennett, R.M., Evaluation of the anesthetic metomidate for the handling and transport of juvenile American shad (1993) Progr. Fish Culturist. 55 (4):236–243

Rotllant, J. and Tort, L. (1997) Cortisol and glucose responses after acute stress by net handling in the sparid red porgy previously subjected to crowding stress. J. Fish Biol. 51: 21-28

Roubach, R., Gomes, L.C. and Val AL. Fonseca FAL. (2005) Eugenol as an efficacious anaesthetic for tambaqui (*Colossoma macropomum*). Aquac. Res. 36 (11): 1056-1061

Roubach, R., Gomes, L.Val.A. (2001) Safest level of tricaine methanesulphonate (MS222) to induce anesthesia in juveniles of Matrinxa *Brycon cephalus*. Acta Amazonia. 31(1): 159-163

Ryan, S.N. (1995) The effect of chronic heat stress on cortisol levels in the Antarctic fish *Pagothenia borchgrevnki*. Experientia. 51: 768–774

Rychlik, M. (2008) Quantification of free coumarin and its liberation from glucosylated precursors by stable isotope dilution assays based on liquid chromatography-tandem mass spectrometric detection. J Agric.Food Chem. 56: 796–801

Sado, E.K. (1985) Influence of the anesthetic quinaldine on some tilapias. Aquaculture 46: 55-62

Salin, K.R. and Jayasree Vadhyar, K. (2001) Cold-anaesthetization and live transportation of Penaeid shrimps. Fish. Technol. 38(2): 71 – 76

Saliu, J. K. and Salami, A.S. (2010) Haematological studies of *Oreochromis niloticus* exposed to diesel and drilling fluid in Lagos., Nigeria. Inter. J. Biodiv. Conserv. 2 (5): 130-133

Sampath, K., Velamnial, S., Kennedy, I.J. and James R. (1993) Haematological changes and their recovery in *Oreochromis mossambicus* as a function of exposure period and sub-lethal levels of Ekalus. Acta Hydrobiologica. 35: 73 – 83

Samuel, C.T. (1969) Problems and prospects of the estuarine fisheries of Kerala. First All India Symposium on Estuarine Biology, Tambaram, Madras

Sandodden, R., Finstad, B. and Inversen, M. (2001) Transport stress in Atlanthic Salmon (*Salmo salar* L.) anaesthesia and recovery. Aquac. Res. 32 (2): 87-90

Sato, T., Kitayama, S., Mitsuhata, C., Ikeda, T., Morita, K. and Dohi, T. (2000) Selective inhibition of monoamine neurotransmitter transport by synthetic local anesthetics. Naunyn. Schmiedeberg's Arch. Pharmacol. 361: 214–220

Saxena, R. K. (1986) Effect of anesthetics in traval sodium on fingerlings and hatchings of fish. In Proc. Nat. Aead. Sci. India, 56(8), Ill: 211- 218

Schoemaker, R. (1991) INFOFISH Technical Hand Book 3: 17

Schoettger. and Arnold, M. Julin. (1966) Efficacy of MS-222 as an anesthetic on four salmonids. In: Investigations in Fish Control. 13-15 p, U.S. Bureau of Sport Fisheries and Wildlife Resource Publication

Schreck, C.B. (1981) Stress and compensation in teleostean fishes: responses to social and physical factors. In: Stress in Fish (Pickering, A.D. Eds), pp 295-321, Academic Press, London

Schultz, F.H. (1956) Transfer of anesthethized pike and yellow walleye. Can. Fish. Cuiturist.18: 1-5

Senanayake, U.M., Lee, T.H. and Wills, R.B.H (1978) Volatile constituents of cinnamon (*Cinnamomum zeylanicum*) oils. J. Agric. Food Chem. 26: 822-824

Seth, H., Axelsson, M., Sundh, H., Sundell, K., Kiessling, A. and Sandblom, E. (2013) Physiological responses and welfare implications of rapid hypothermia and immobilization with high levels of CO_2 at two temperatures in Arctic char (*Salvelinus alpinus*). Aquaculture. 402-403: 146-151

Shah, S.L. and Altindag, A. (2004) Haematological parameters of tench (*Tinca tinca* L.) after acute and chronic exposure to lethal and sublethal mercury treatments. Bull. Environ. Contam. Toxicol. 73: 911 – 918

Shah, A.H., Al-Shareef, A.H, Ageel, A.M. and Qureshi, S. (1998) Toxicity studies in mice of common spices, *Cinnamomum zeylanicum* bark and *Piper longum* fruits.Plant foods for Human Nutrition. 52: 231-239

Shepherd, J. and Bromage, N. (1992) Intensive Fish Farming, 416 p, Blackwell Scientific Publications, Oxford, England

Shrimpton, J. M., Zydlewski, J. D. and McCormick, S. D. (2001) The stress response of juvenile American shad to handling and confinement is greater during migration in freshwater than in seawater. Trans. Am. Fish. Soc. 130(6): 1203–1210

Shyam., Salim, S., Ignatius Boby., Suresh, V. K., Pushkaran, K.N., Salini, K. P. and Abhilash, P.R. (2013) Economic Analysis on the Hatchery Technology and growout of Pearl Spot (*Etroplus suratensis*). Journal of Fisheries, Economics and Development. 14 (1): 1-20

Simões, L.N. and Gomes, L.C. (2009) Eficácia do mentol como anestésico para juvenis de tilápia-do-nilo (*Oreochromis niloticus*). Arquivo Brasileiro de Medicina Veterinária e Zootecnia 61: 613-620

Sindhu, M.C. (2009) Clove oil as an anesthetic for transportation of blue hill trout *Barilius bakeri* (Day) M.Phil Thesis, Cochin University of Science and Technology, Kerala, India, 362 p

Sindhu, M.C. and Ramachandran, A. (2013) Acute toxicity and optimal dose of clove oil as anaesthetic for blue hill trout *Barilius bakeri* (Day). Fish.Technol. 50: 280-283

Skjervold, P.O., Fjæra, S.O., Braarød Østby, P. and Einen, O. (2001) Live-chilling and crowding stress before slaughter of Atlantic salmon (*Salmo salar*). Aquaculture. 192: 265- 280

Sladky, K., Swanson, C., Stoskopf, M., Loomis, M., Lewbart, G. (2001) Comparative efficacy of tricaine methanesulfonate and clove oil for use as anesthetic in red pacu (*Piaractus brachypomus*). Am. J. Vet. Res. 62(3): 337-342

Small, B. C. (2004) Effect of isoeugenol sedation on plasma cortisol, glucose, and lactate dynamics in channel catfish *Ictalurus punctatus* exposed to three stressors. Aquaculture. 238: 469-481

Smit, G. L., Schoonbee, H. J. and Barham, W. T. (1979) Some effects of the anesthetic MS 222 on fresh water. South Afr. J. Sci. 73(11): 351-352

Smith, D.A., Smith, S.A. and Holladay, S.D. (1999) Effect of previous exposure to tricaine methanesulfonate on time to anesthesia in hybrid tilapias. J. Aquatic. Anim.Health. 11(2): 183-186

Snedecor, G.W. and Cochran, W.G. (1968) Statistical Methods, 593 p, Oxford and IBH Publishing Co.

Sobhana, K.S., George, K.C., Sanil, N.K., Surendran, K.K.and Paul Raj, R. (2006) Immunosuppressive action of nuvan (dichlorovos) in the banded pearl spot, *Etroplus suratensis* – evaluation of effect on haematology and humoral immune response. J. Mar. Biol. Ass. India. 48 (1): 29–34

Soenges, J.L., Rey, P., Rozas, G., Andres, M.D. and Aldegunde, M. (1992) Effects of cortisol and thyroid hormone treatment on the glycogen metabolism of selected tissues of domesticated rainbow trout *Oncorhynchus mykiss*. Aquaculture. 101: 317-328

Soivio, A., Nyholm, K. and Huhti, M. (1977) Effects of anaesthesia with MS 222, neutralized MS 222 and benzocaine on the blood constituents of rainbow trout *Salmo gairdneri*. J. Fish. Biol. 10: 91–101

Soliman, K.M. and Badeaa, R.I. (2002) Effect of oil extracted from some medicinal plants on different mycotoxigenic fungi. Food Chem. Toxicol. 40(11): 1669-1675

Solomon, D.J., and Hawkins, A.D. (1981) Fish capture and transport. In: Aquarium systems (Hawkins.A.D. Eds), pp 197–221, Academic Press, London

Somero, G.N. (2005) Linking biogeography to physiology: evolutionary and acclamatory adjustments of thermal limits. Frontiers in Zoology 2: 1–9

Soto, C.and Burhanuddin, S. (1995) Clove oil as a fish anesthetic for measuring length and weight of rabitfish (*Siganus lineatus*). Aquaculture. 135: 149-152

Stefansson, S., Bjerknes, V. Bjørn, P.A., Bæverfjord, G., Finn, R.N., Finstad, B., Fivelstad, S., Handeland, S., Hosfeld, C.D., Kristensen, T., Kroglund, F., Nilsen, T., Rosseland, B.O., Rosten, T., Salbu, B., Teien, H-C., Toften, H. and Åtland, Å. (2007) Fysiologiske egenskaper ved rogn, yngel og smolt. I: Bjerknes, V., Liltved, H., Rosseland, B.O., Rosten, T., Skjelkvåle, B.L., Stefansson, S., og Åtland, Å. (red.) Vannkvalitet og smoltproduksjon, Kapittel 3, side 94-124, Juul forlag, ISBN 978-82- 8090-018-0

Stehly, G.R. and Gingerich, W.H. (1999) Evaluation of AQUI-S (efficacy and minimum toxic concentration) as a fish anaesthetic/sedative for public aquaculture in the United States. Aquacult. Res**.** 30: 365 – 372

Stein, M.W. (1963) D-glucose determination with hexokinase and glucose-6-phosphatedehydrogenase. In: Methods in Enzymatic Analysis (Bergmeyer, H. U. Eds), pp117-122, Academic Press, New York

Stoskopf, M. (1993) Anesthesia. In: Aquaculture for Veterinarians (Brown, L. Eds), pp 161-167, Pergamon Press, Oxford

Stoskopf, M. and Posner, L.M. (2008) Anesthesia and restraint of laboratory fish. In: Anesthesia and analgesia in laboratory animals (Fish, R.E et al., Eds), pp 519-533, Academic Press USA

Strange, R.J. and Schreck, C.B. (1978) Anesthetic and handling stress on survival and cortisol concentration in yearling Chinook salmon (*Oncorhynchus tshawytscha*). J. Fish. Board Can. 35: 345–349

Sulikowski, J. A., Fairchild, E. A., Rennels, N. and Howell, W. H. (2005) The effects of tagging and transport stress on juvenile winter flounder *Pseudopleuronectes americanus*: implications for successful stock enhancement. J. World Aquacult. Soc. 36(1): 148–156

Sumitra Vijayaraghavan, Krishnakumari, L., Gopinath, V.J. and Dhawan, R.M. (1981) Aquaculture of pearlspot *Etroplus suratensis* in an estuarine pond: Environmental characteristics, primary production, growth and cost benefit ratio. Indian J. Mar. Sci., 10: 82-89

Summerfelt, R.C. and Smith, L.S. (1990) Anesthesia, surgery, and related techniques. In: Methods for fish biology (Shreck, C.B., and Moyle, P.B., Eds), pp 213-272, American Fish Society. Bethseda

Sumpter, J.P. (1997) The endocrinology of stress. In: Fish stress and health in aquaculture (Iwama, G.K., Pickering, A.D., Sumpter, J.P., and Schreck, C.B., Eds), pp 95-118, Cambridge University Press., Cambridge., UK

Sun, L.T., Chen, G.R. and Chang, C.F. (1992). The physiological responses of tilapia exposed to low temperatures. J. Thermal. Biol. 17: 149–153

Sun, L.T., Chen, G.R., and Chang, C.F. (1995) Acute responses of blood parameters and comatose effects in salt-acclimated tilapias exposed to low temperatures. J.Thermal. Biol. 20: 299–306

Suski, C.D., Killen, S.S., Kieffer, J.D., and Tufts, B. L (2006) The influence of environmental temperature and oxygen concentration on the recovery of largemouth bass from exercise: implications for live-release angling tournaments. J. Fish Biol. 68(1): 120-136

Svobodova Z., Pravda D., Palackova J. (1991): Unified methods of haematological examination of fish. Research Institute of Fish Culture and Hydrobiology, Vodnany, Edition Methods No.22:31

Svobodova, Z. and Kolarova, J. (2004) A review of the diseases and contaminant related mortalities of tench (*Tinca tinca* L.). Veterinarni Medicina. 49: 19–34

Svobodova, Z., Machova, J., Hamackova, J., Huda, J. and Kroupova, H. (2005a): Nitrite poisoning of fish in aquaculture facilities with water-recirculating systems: Acta Veterinaria Brno, 74, 129–137

Svobodová, Z., Gelnerová, J., Justyn, J., Krauper, V., Máchová, J., Simanov, L., Valentová, O., Vykusová, B. and Wohlgemuth, E. (1987) Toxicology of aquatic animals, 231p, SZN Praha, (In Czech)

Takashima, Y., Wan, Z., Kasai, H. and Asakawa, O. (1983) Sustained anesthesia with 2-phenoxyethanol in yearling rainbow trout**.** J. of Tokyo University of Fisheries. 69 (2): 93-96

Tanck, M.W.T., Booms, G.H.R., Eding, E.H., Wendelaar Bonga, S.E.and Komen, J. (2000) Cold shocks: a stressor for common carp. J. Fish Biol. 57: 881–894

Tang, J., Bryant, M.D. and Brannon, E.L. (1987) Effect of temperature extremes on the mortality and development rates of coho salmon embryos and alevins. Prog. Fish-Cult.49:167–174

Taylor, N.I. and Ross, l.G. (1988) The use of hydrogen peroxide as a source of oxygen for the transportation of live fish. Aquaculture. 70: 183-192

Taylor, P.W. and Roberts, S.D. (1999) Clove oil: an alternative anesthetic for aquaculture. N. Am. J. Aquac. 61: 150–155

Teo, L.H. and Chen, T.W. (1993) A study of metabolic rates of *Poecilia reticulate* (Peters), under different conditions. Aquac. Fish. Manage. 24: 109-117

Thampy D.M. (1980) Culture of *Etroplus suratensis* (Bloch). In: Summer Institute of Brackishwater Capture and Culture Fisheries. CIFRI, Barrackpore

The Ayurvedic Pharmacopoeia of India, Part I., Vol I to IV (2004), (API), Ministry of Health, Govt. of India, New Delhi

Thomas, P. and Robertson, L. (1991) Plasma cortisol and glucose stress responses of red Drum (*Sciaenops ocellatus*) to handling and shallow water stressors and anesthesia with MS-222, quinaldine sulphate and metomidate. Aquaculture. 96: 69-86

Thompson, R.B. (1959) Tricaine methanesulfonate (MS-222) in transport of cutthroat trout. Prog. Fish-Cult. 21(2): 96

Tilak, K.S., Veeraiah, K. and Prema Raju, J.M. (2007) Effects of ammonia, nitrite and nitrate on hemoglobin content and oxygen consumption of freshwater fish, *Cyprinus carpio* (Linnaeus). J. Envi.Biol. 28(1): 45-47

Tlusty, M., Dowd, S., Weber, S., Cooper, R., Chao, N.L. and Whittaker, B. (2005) Shipping Cardinal Tetras from the Amazon–understanding

stressors to decrease shipping mortality. Ornamental Fish Int. J. 48: 21-23

Tobor, J.G. (1990) The Fishing Industry in Nigeria: Status and potential for self sufficiency in fishing production, NIOMR, Technical Paper, No.54, 26 p, Nigeria Institute for Oceanography Research, Lagos, Nigeria

Tort, L., Puigcerver, M., Crespo, S., Padro´s, F. (2002) Cortisol and hematological response in sea bream and trout subjected to the anesthetics clove oil and 2-phenoxyethanol. Research. 33: 907–910

Treves-Brown, K.M. (2000) Anaesthetics in applied fish pharmacology. Kluwer Academic Publishers, Dordrecht, Netherlands: 206-217

Tripathi, P., Dubey, N.K. and Shukla, A.K. (2008) Use of some essential oils as post-harvest botanical fungicides in the management of grey mould of grapes caused by *Botrytis cinerea*. World J. Microbiol. Biotechnol. 24: 39-46

Tsantilas, H., Galatos, A.D., Athanassopoulou, F., Prassinos, N.N. and Kousoulaki, K. (2006) Efficacy of 2-phenoxyethanol as an anaesthetic for two size classes of white sea bream *Diplodus sargus* L., and sharp snout sea bream *Diplodus puntazzo*. Aquaculture. 253: 64–70

Tytler, P. and Hawkins, A.D. (1981) Vivisection, anesthetics and minor surgery. In: Aquarium System (A.D. Hawkins. Eds), pp 247-278, Academic Press, New York

Ufodike, E.B.C., and Omoregie, E. (1994) Acute toxicity of water extracts of barks of *Balanites aegyptiaca and Kigelia africana* to *Oreochromis niloticus* (L). Aquac. Fish. Manage. 25: 873-879

UNEP (United Nations Environmental Programme) (1989) Estimation of the lethal toxicity of pollutants in marine fish and invertebrates. Ref. Methods Mar. Poll. Studies. 43: 27

Vaibhavi Jakhetia, Rakesh Patel, Pankaj Khatri, Neeraj Pahuja, Sunil Garg, Anupriya Pandey, Sonu Sharma. (2010) Cinnamon: A Pharmacological Review, J.Adv.Sci.Res. 1(2):19-23

Van den Burg, E.H., Peeters, R.R., Verhoye, M., Meek, J., Flik, G. and Van der Linden, A. (2005) Brain responses to ambient temperature fluctuations in fish: reduction of blood volume and initiation of a whole-body stress response. J. Neurophysiol. 93: 2849–2855

Van, V.J. H. J. (1986) The effects of toxicants on the haematology of *Labeo umbratus* (Teleostei; cyprinidae). Comparative Biochem. Physiol. 83C: 155-159

Varner, P.W. (2000) Anesthetics. In: Encyclopedia of Aquaculture (R.R. Stickney., Eds), pp 33 – 38, John Wiley and Sons Inc., New York

Vedel, N.E., Korsgaard, B., Jensen, F.B. (1998) Isolated and combined exposure to ammonia and nitrite in rainbow trout (*Oncorhynchus mykiss*): effect on electrolyte status, blood respiratory properties and brain glutamine/ glutamate concentrations. Aquat. Toxicol. 41: 325–342

Velíšek, J., Svobodová, Z., Piačková, V. (2005a) Effect of clove oil anaesthesia on rainbow trout (*Oncorhynchus mykiss*). Acta Vet Brno. 74: 139-146

Velíšek, J., Svobodová, Z., Piačková, V., Groch, L. and Nepejchalová, L. (2005b) Effects of clove oil anaesthesia on common carp (*Cyprinus carpio* L.). Vet Med – Czech. 50: 269-275

Velisek, J., Wlasow, T., Gomulka, P., Svobodova, Z. and Novotny, L. (2007) Effects of 2-phenoxyethanol anaesthesia on sheatfish (*Silurus glanis* L) Veterinarni Medicina, 52 (3): 103–110

Velisek, J., Wlasow, T., Gomulka, P., Svobodova, Z., Novotny, L. and Ziomek, E. (2006) Effects of clove oil anaesthesia on European catfish (*Silurus glanis* L.). Acta Veterinaria Brno 75: 99–106

Vernin, G., Vernin, E., Metzger, J., Pujol, L. and Párkányi, C. (1994) GC/MS analysis of clove essential oil, pp 483 – 500. In G. Charalambous, ed. Spices, Herbs and Edible Fungi. Elsevier Science B.V., Netherlands

Vidal, L.V.O., Furuya, W.M., Graciano, T.S., Schamber, C.R., Dos Santos, L.D. and Soares, C.M. (2007) Eugenol concentrations for deep anesthesia and acute toxicity in piavuçu (*Leporinusmacrocephalus*) juveniles. Acta Sci. 29: 357–362

Vikas, P.A. (2012) Karimeen krishi: sadhyadakal.Kerala karshakan. June-2012: 64-65

Vogue, E. (1984) Tobacco Encylopaedia, Tobacco Journal International Federal Republic of Germany.

Vrskova, D., and Modra, H. (2012) Evaluation of the developmental toxicity of 2-phenoxyethanol and clove oil anaesthetics using the frog embryo teratogenesis assay: Xenopus (FETAX). Veterinarni Medicina. 57 (5): 245–250

Wagner, E., Arndt, R. and Hilton, B. (2002) Physiological stress responses, egg survival and sperm motility for rainbow trout broodstock anesthetized with clove oil, tricaine methanesulfonate or carbon dioxide. Aquaculture. 211: 353–366

Wagner, G.N., Singer, T.D., & McKinley, R.S. (2003) The ability of clove oil and MS-222 to minimize handling stress in rainbow trout (*Oncorhynchus mykiss* Walbaum). Aquac. Res. 34: 1139– 1146

Wagner, T. and Congleton, J.L. (2004) Blood chemistry correlates of nutritional condition, tissue damage, and stress in migrating juvenile

chinook salmon (*Oncorhynchus tshawytscha*). Cana. J. Fish. Aquatic Sci. 61:1066-1074

Walsh, C.T. and Pease, B.C. (2002) The use of clove oil as an anesthetic for the long finned river eel *Anguilla reinhardtii* (Steindachner). Aquac. Res. 33: 627– 635

Walter, R.H. (1972) β-caryophyllene in native clove bud oil. Phytochem 11: 405 – 406

Walton, W.J., Brown, K.L. and Lydy, M.J. (1997) Diurnal fluctuations in toxicity in two fish species: *Gambusia affinis* and *Notropis ludibundis*. Bull. Environ. Contam. Toxicol. 59: 414–421

Wanauppathamkul, S. (2003) Plaitanoids.1 Eds., 40 p, The Innovation Development Fund, National Science and Technology Development Agency, Ministry of Science and Technology, Bangkok

Wasuwat, S., Wanisorn, P., Mahintorntep, B., Kuwaono, K. and Sanghirun. S. (1989) Studies on antimicrobial and antifungal activities of terpinen-4-ol extracted from *Zingiber cassumunar* Roxb. Research Project No. 30-32/Rep.No.1. 46 p.Thailand Institute of Scientific and Technological Research

Waterstrat, P.R. (1999) Induction and recovery from anesthesia in channel catfish *ictalurus punctatus* fingerlings exposed to clove oil. J. World Aquac.Soc. 30 (2): 250-255

Watson, I. (2000) The role of the ornamental fish industry in poverty alleviation. NRI Report No. 2504.66pp

Weber, R.A., Peleterio, J.B., Garcia Martin, L.O. and Aldegunde, M. (2009) The efficacy of 2- phenoxyethanol, metomidate, clove oil and MS-222 as anaesthetic agents in the Senegalese sole (*Solea senegalensis* Kaup, 1858). Aquaculture. 288: 147-150

Wedemeyer, G. (1970a) The role of stress in the disease resistance of fishes. Spec. Publ. Am. Fish. Soc. 5: 30-35

Wedemeyer, G. (1997) Effects of rearing conditions on the health and physiological quality of fish in intensive culture. In: Fish stress and health in aquaculture (Iwama, G.K., Pickering, A.D., Sumptre, J.P. and Schreck, C.B., Eds), pp 35-71, Cambridge University Press, Cambridge, UK

Wedemeyer, G. A., Barton, B.A., and McLeay, D.J. (1990). Stress and acclimation. In: Methods for fish biology (Schreck. and Moyle, P.B. Eds), pp 451–489, American Fisheries Society, Bethesda, Maryland

Wedemeyer, G.A. (1996) Physiology of Fish in Intensive Culture Systems, 232 p, Chapman and Hall, New York

Wedemeyer, G.A., Yasutake, W.T. (1978) Prevention and treatment of nitrite toxicity in juvenile steelhead trout (*Salmo gairdneri*). Journal of the Fisheries Research Board of Canada. 35: 822–827

Weirich C.R. and Tomasso J.R. (1991) Confinement- and transport-induced stress on red drum juvenile: effect of salinity. Prog. Fish-Cult. 53: 146-149

Weirich C.R., Tomasso J.R. and Smith T. I. J. (1992) Confinement and transport-induced stress in white bass *Morone chrysops*, striped bass *M. saxatilis*, hybrids: effects of calcium and salinity. J. World Aquac. Soc. 23: 49-57

Weiss, E.A. (1997) Essential Oil Crops. University Press Cambridge, London

Wells, R.M. G. and Weber, R. E. (1990) The spleen in hypoxic and exercised rainbow trout. J. Exp.Biol. 150: 461–466

Wells, R. M. G., Ashby, M.D., Duncan, S. J. and Macdonald, J. A. (1980) Comparative study of the erythrocytes and haemoglobins in nototheniid fishes from Antarctica. J. Fish Biol. 17: 517–527

Wells, R. M. G., Grigg, G. C., Beard, L.A. and Summers, G. (1989) Hypoxic responses in a fish from a stable environment: Blood oxygen transport in the Antarctic fish Pagothenia borchgrevinki. J. Exp. Biol. 141: 97-111

Wells, R.M.G., Tetens, V. and Devries, A.L. (1984) Recovery from stress following capture and anaesthesia of antarctic fish: haematology and blood chemistry. J. Fish Biol. 25:567-576

Wendelaar, Bonga. S. E. (1997) The stress response in fish. Physiol. Rev. 77(3): 591-625

Wetherall, J.A., Polovina, J.J. and Raston, S. (1987) Estimating growth and mortality in steady state fish stocks from length - frequency data. ICLARM Conf. Proc. 13: 53 – 74

Wetzel, R.G. (1983) Limnology, second edition. Saunders College Publishing, New York, New York, USA

Weyl, O., Kaiser, H. and Hecht, T. (1996) On efficacy and mode of 2-phenoxyethanol as an anesthetic for gold fish, *Carassius auratus* (L.), at different temperatures and concentrations. Aquacult. Res. 27: 757-764

Wilson, J.M., Bunte, R.M. and Carty, A.J. (2009) Evaluation of rapid cooling and Tricaine methanesulfonate (MS222) as methods of euthanasia in Zebrafish (*Danio rerio*). JAALAS. 48(6): 785–789

Wilson, R. W. and Taylor, E.W. (1993) The physiological responses of freshwater rainbow trout, *Onchorynchus mykiss*, during acute exposure. J. Comp. Physiol. 163b: 38-47

Wintrobe, M.M. (1978) Clinical Haematology, 488 p, Kimpton, London, U.K

Woods, L.C., Theisen, D. D. and He, S. Y. (2008) Efficacy of aqui-S as an anesthetic for market-sized striped bass. N. Am. J. Aquac. 70 (2): 219-222

Woodward, C.C., and Strange, R. J. (1987) Physiological stress responses in wild and hatchery-reared rainbow trout. Trans. Am. Fish. Soc. 116: 574–579

Woody, C.A., Nelson, J. and Ramstad, K. (2002) Clove oil as an anesthetic for adult sockeye salmon: field trials. J. Fish Bio. 60: 340– 347

Yamamitsu, S., Itazawa, Y. (1988) Effects of an anesthetic 2-phenoxyethanol on the heart rate, ECG and respiration in carp**.** Nippon Suisan Gakkaishi/ Bull. Jap. Soc. Scient. Fish. 54(10): 1737-1746

Yokohama, Y., Yoshikawa, H., Ueno, S. and Mitsuda, H. (1989) Application of CO_2 anesthesia combined with low temperature fro long-term anesthesia in carp. Bull. Jap. Soc. Scient. Fish. 55: 1203-1209

Yoshikawa H., Ishida Y., Ueno S. and Mitsuda, H. (1989): Changes in depth of anesthesia of the carp anaesthetized with a constant level of carbon dioxide. Bull. Jap. Soc. Scient. Fish. 54: 457–462

Yoshikawa, H., Yokoyama, Y. and Ueno, S. (1991) Changes of blood gas in carp (*Cyprinus carpio*) anesthetized with carbon dioxide. Comp. Biochem. Physiol. 98: 431–436

Yoshimura, H., Nakamura, and M. and Koeda, T. (1981) Mutagenicity screening of anesthetics for fishes. Mutation Res. 90: 119–124

Young, M. J. (2009) The efficacy of the aquatic anaesthetic AQUI-S (R) for anaesthesia of a small freshwater fish *Melanotaenia australis*. J. Fish Biol. 75(7): 1888-1894

Zahl, I.H., Kiessling, A., Samuelsen, O.B. and Hansen, M.K. (2009) Anaesthesia of Atlantic cod (*Gadus morhua*) — effect of pre-anaesthetic sedation, and importance of body weight, temperature and stress. Aquaculture. 295: 52–59

Zahl, I.H., Kiessling, A., Samuelsen, O.B. and Hansen, M.K. (2011) Anaesthesia of Atlantic Halibut (*Hippoglossus hippoglosus*) – effect of pre-anaesthetic sedation and importance of body weight and water temperature. Aquac. Res. 42 (9): 1235–1245

Zarate, J. and Bradley, T.M. 2003. Heat shock proteins are not sensitive indicators of hatchery stress in salmon. Aquaculture. 223: 175-187

Zheng, G., Kenney, P.M. and Lam, L.K.T. (1992) Sesquiterpenes from clove (*Eugenia caryophyllata*) as potential anticarcinogenic agens. J. Nat. Prod. 55**:** 999-1003

.....ജ്വ.....

Appendices

Appendix 1

Stages of anaesthesia induction and recovery from Summerfelt and Smith (1990)

Stage of anaesthesia	Descriptor	Behaviour exhibited
0	Normal	Reactive to external stimuli; operator rate and muscle tone normal
1	Light sedation	Slight loss of reactivity to external visual and tactile stimuli; opercular rate slightly decreased ; equilibrium normal
2	Deep sedation	Total loss of reactivity to external stimuli except strong pressure; slight decrease in opercular rate; equilibrium normal
3	Partial loss of equilibrium	Partial loss of muscle tone; swimming erratic; increased opercular rate ; reactive only to strong tactile and vibrational stimuli
4	Total loss of equilibrium	Total loss of muscle tone and equilibrium; slow but regular opercular rate ; loss of spinal reflexes
5	Loss of reflex reactivity	Total loss of reactivity; opercular movements slow and irregular; heart rate very slow ; loss of all reflexes
6	Medullary collapse	Opercular movements cease; cardiac arrest usually follows quickly
Stage of recovery		
1		Reappearance of opercular movement
2		Partial recovery of equilibrium with partial recovery of swimming motion
3		Total recovery of equilibrium
4		Reappearance of avoidance swimming motion and reaction in response to external stimuli; but still behavioural response is stolid
5		Total behavioural recovery; normal swimming

Appendix 2

Behavioral criteria used for evaluating stages of anaesthesia and recovery in *Etroplus suratensis*
Stages of anesthesia I Onset of erratic opercular movement II Partial loss of equilibrium; continued efforts to right itself III Total loss of equilibrium; no efforts to right itself IV Induction; total loss of voluntary movement and reactivity V Medullary collapse; total cessation of opercular movement
Stages of recovery I Reappearance of opercular movement II Partial recovery of equilibrium; efforts to right itself III Full recovery of equilibrium; successful righting IV Response to external stimuli (tapping on glass of aquarium) V Behavioral recovery; normal swimming activity

*Modified from Stoskopf MK. Clinical pathology. In: Stoskopf MK, ed. Fish medicine. Philadelphia: WB Saunders Co, 1993; 81.

Appendix 3

Water quality parameters Mean SE & p-value of all anesthetics in different concentrations

TEMPERATURE

Anesthetic	Dose	10 Minutes		24 Hours		48 Hours		96 Hours	
		Mean	Std. Error	Mean	Std. Error	Mean	Std. Error	Mean	Std. Error
None	0.00	27.50	0.13	27.67	0.133	27.23	0.04	29.82	0.09
Control	0.00	28.12	0.20	28.80	0.513	29.28	0.54	30.50	0.50
Clove	0.10	26.70	0.07	27.23	0.524	27.23	0.73	26.50	0.07
	0.17	26.45	0.03	27.77	0.408	27.54	0.47	26.42	0.01
	0.23	26.27	0.17	27.20	0.503	25.79	0.05	26.00	0.14
	0.30	26.55	0.08	26.50	0.121	26.08	0.12	26.08	0.12
	0.37	26.43	0.02	25.67	0.071	26.04	0.16	26.04	0.16
Cinnomon	0.33	28.48	0.07	30.48	0.070	26.51	0.08	26.51	0.08
	0.50	28.47	0.06	30.25	0.355	27.83	0.84	26.53	0.05
	0.57	28.47	0.06	28.75	0.314	26.67	0.28	26.34	0.13
	0.67	28.68	0.07	28.68	0.070	25.94	0.24	25.94	0.24
	0.73	28.70	0.05	28.77	0.042	26.56	0.02	26.56	0.02
Zn	0.50	28.71	0.07	28.93	0.089	28.57	0.03	28.57	0.03
	0.70	28.70	0.05	28.93	0.089	28.57	0.04	28.57	0.04
	1.30	28.73	0.05	29.13	0.541	29.14	0.44	28.59	0.04
	1.50	28.60	0.04	28.90	0.109	29.81	0.45	28.57	0.03
	1.70	28.57	0.02	28.83	0.081	28.59	0.03	28.59	0.03
TB	2.00	30.30	0.30	29.82	0.105	30.32	0.35	29.00	0.00
	5.00	30.03	0.18	29.45	0.022	30.08	0.41	29.27	0.18
	6.00	30.56	0.38	29.38	0.416	29.62	0.34	29.80	0.38
	7.00	30.35	0.13	30.97	0.295	30.32	0.35	29.39	0.25
	8.00	30.32	0.35	30.88	0.300	31.05	0.35	29.56	0.37
MS	45.00	29.52	0.57	29.02	0.320	29.54	0.12	30.36	0.06
	50.00	29.97	0.19	29.33	0.316	29.63	0.10	29.50	0.00
	52.50	29.46	0.06	28.10	0.604	30.26	0.45	29.50	0.00
	75.00	29.47	0.06	29.80	0.170	29.56	0.04	29.50	0.00
	100.00	29.44	0.06	29.54	0.043	29.60	0.08	29.50	0.00

pH

Anaesthetic	Dose	10 minutes		24 Hours		48 Hours		96 Hours	
		Mean	Std. Error	Mean	Std. Error	Mean	Std. Error	Mean	Std. Error
None	0.00	6.71	0.06	5.01	0.38	7.03	0.12	5.24	0.29
Control	0.00	7.92	0.18	7.64	0.38	8.04	0.32	7.41	0.12
Clove	0.10	6.68	0.02	5.96	0.20	5.63	0.07	6.91	0.00
	0.17	6.88	0.08	5.60	0.30	7.31	0.54	6.34	0.13
	0.23	5.72	0.05	5.32	0.06	5.24	0.03	5.17	0.02
	0.30	6.75	0.03	5.02	0.17	7.48	0.02	7.48	0.02
	0.37	6.83	0.09	4.80	0.02	7.85	0.01	7.85	0.01
Cinnomon	0.33	6.79	0.03	7.18	0.02	7.17	0.02	7.17	0.02
	0.50	6.90	0.05	7.43	0.14	7.54	0.03	7.50	0.00
	0.57	6.78	0.16	7.32	0.12	7.66	0.10	7.56	0.01
	0.67	6.83	0.06	7.20	0.02	7.65	0.00	7.65	0.00
	0.73	6.76	0.15	7.20	0.02	7.85	0.00	7.85	0.00
Zn	0.50	7.53	0.02	7.26	0.01	7.53	0.02	7.53	0.02
	0.70	7.49	0.02	7.26	0.01	7.51	0.03	7.51	0.03
	1.30	7.50	0.02	7.63	0.13	7.71	0.10	7.53	0.03
	1.50	7.52	0.02	7.34	0.03	7.75	0.09	7.53	0.02
	1.70	7.55	0.01	7.39	0.00	7.54	0.02	7.54	0.02
TB	2.00	7.53	0.01	7.54	0.02	7.56	0.02	7.51	0.00
	5.00	7.55	0.02	7.51	0.02	7.57	0.03	7.42	0.07
	6.00	7.49	0.04	7.79	0.08	7.97	0.13	7.49	0.04
	7.00	7.55	0.05	7.35	0.01	7.45	0.01	7.48	0.04
	8.00	7.56	0.02	7.60	0.03	7.70	0.05	7.53	0.02
MS	45.00	7.07	0.01	7.01	0.15	7.23	0.10	7.56	0.00
	50.00	7.56	0.00	7.38	0.27	7.39	0.15	7.54	0.00
	52.50	7.32	0.01	7.41	0.25	7.56	0.18	7.58	0.00
	75.00	7.42	0.01	7.28	0.26	7.32	0.16	7.52	0.00
	100.00	7.55	0.01	7.36	0.17	7.36	0.13	7.54	0.00
Hypothermia	8.00	6.78	0.05	8.26	0.30	8.68	0.00		
	12.00	8.58	0.03	7.30	0.04	6.77	0.07		
	15.00	8.59	0.06	6.94	0.11	7.08	0.19	8.64	0.01
	18.00	7.70	0.08	7.42	0.21	7.52	0.17	7.36	0.00
	22.00	7.94	0.00	7.28	0.22	7.00	0.08	7.92	0.02

DISSOLVED OXYGEN

Anaesthetic	Dose	10 minutes		24 Hours		48 Hours		96 Hours	
		Mean	Std. Error	Mean	Std. Error	Mean	Std. Error	Mean	Std. Error
None	0.00	237.37	91.15	350.35	86.28	76.58	3.40	74.18	1.89
Control	0.00	88.64	1.37	60.48	12.04	52.11	3.67	86.78	8.18
Clove	0.10	99.57	4.58	88.20	7.00	91.40	5.65	93.83	2.35
	0.17	82.72	3.61	76.61	6.50	62.52	9.69	94.69	0.13
	0.23	65.65	0.42	74.40	6.87	100.48	3.13	95.70	0.12
	0.30	78.69	4.41	54.95	0.56	98.61	0.03	98.61	0.03
	0.37	95.07	0.27	57.37	1.91	99.87	0.04	99.87	0.04
Cinnomon	0.33	71.30	0.39	61.71	0.63	72.48	0.04	72.48	0.04
	0.50	71.30	0.39	37.87	2.00	61.51	7.05	72.42	0.03
	0.57	61.47	0.47	43.49	2.81	68.46	3.94	72.43	0.04
	0.67	61.78	0.42	41.50	0.34	72.45	0.03	72.45	0.03
	0.73	66.58	0.83	43.35	0.54	72.49	0.03	72.49	0.03
Zn	0.50	56.46	0.01	35.94	0.15	56.46	0.01	56.46	0.01
	0.70	56.52	0.04	35.53	0.12	56.47	0.00	56.47	0.00
	1.30	56.46	0.01	39.63	2.97	49.47	3.78	56.47	0.00
	1.50	56.45	0.01	36.76	1.03	44.82	4.13	56.46	0.01
	1.70	56.76	0.04	38.55	0.09	56.47	0.00	56.47	0.00
TB	2.00	192.21	0.00	155.40	0.84	192.21	0.00	192.21	0.00
	5.00	192.21	0.00	174.90	7.07	177.12	13.22	192.34	0.03
	6.00	192.21	0.00	181.58	26.31	182.75	13.29	207.36	18.03
	7.00	254.44	0.98	232.21	0.00	212.29	0.02	192.50	0.00
	8.00	260.00	2.14	246.19	0.02	228.87	2.11	192.40	0.00
MS	45.00	83.59	3.48	39.69	2.28	53.26	5.96	55.46	0.01
	50.00	80.00	5.34	50.98	3.25	55.95	6.57	65.42	0.00
	52.50	64.47	0.83	51.99	2.24	53.16	2.10	65.40	0.00
	75.00	66.71	2.24	68.71	3.28	56.62	4.91	65.43	0.00
	100.00	66.57	2.09	53.22	5.83	56.28	5.91	65.42	0.00
Hypothermia	8.00	76.31	5.13	58.55	4.38	62.00	0.00		
	12.00	56.00	0.00	50.52	3.27	43.58	2.46		
	15.00	36.53	0.53	51.77	6.02	38.06	1.18	82.12	4.29
	18.00	44.34	1.23	51.60	3.66	54.60	2.76	41.12	0.00
	22.00	63.73	1.34	57.40	3.03	49.13	5.74	63.73	3.75

Turbidity

Anaesthetic	Dose	10 minutes		24 Hours		48 Hours		96 Hours	
		Mean	Std. Error	Mean	Std. Error	Mean	Std. Error	Mean	Std. Error
None	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Control	0	0.00	0.00	0.10	0.00	0.09	0.00	0.10	0.00
Clove	0.1	0.00	0.00	0.05	0.00	0.07	0.00	0.08	0.00
	0.1667	0.00	0.00	0.07	0.00	0.07	0.02	0.08	0.00
	0.2333	0.00	0.00	0.09	0.01	0.10	0.01	0.07	0.00
	0.3	0.00	0.00	0.07	0.01	0.07	0.01	0.08	0.00
	0.3667	0.00	0.00	0.16	0.02	0.09	0.00	0.09	0.00
Cinnomon	0.3333	0.00	0.00	0.07	0.01	0.09	0.00	0.09	0.00
	0.5	0.00	0.00	0.08	0.01	0.09	0.00	0.09	0.00
	0.5667	0.00	0.00	0.10	0.01	0.08	0.00	0.09	0.00
	0.6667	0.00	0.00	0.10	0.01	0.08	0.00	0.09	0.00
	0.7333	0.00	0.00	0.12	0.01	0.09	0.00	0.10	0.00
Zn	0.5	0.00	0.00	0.06	0.00	0.01	0.00	0.02	0.00
	0.7	0.00	0.00	0.05	0.00	0.01	0.00	0.04	0.00
	1.3	0.00	0.00	0.04	0.00	0.03	0.01	0.06	0.00
	1.5	0.00	0.00	0.04	0.00	0.03	0.01	0.06	0.00
	1.7	0.00	0.00	0.04	0.00	0.06	0.00	0.06	0.00
TB	2	0.00	0.00	0.02	0.01	0.04	0.00	0.75	0.00
	5	0.00	0.00	0.02	0.01	0.07	0.01	0.82	0.01
	6	0.00	0.00	0.06	0.01	0.48	0.26	0.91	0.00
	7	0.00	0.00	0.07	0.01	0.64	0.15	0.86	0.02
	8	0.00	0.00	0.08	0.00	1.24	0.16	1.63	0.02
MS	45	0.00	0.00	0.06	0.00	0.06	0.00	0.08	0.00
	50	0.00	0.00	0.06	0.00	0.09	0.01	0.13	0.00
	52.5	0.00	0.00	0.06	0.00	0.11	0.01	0.13	0.00
	75	0.00	0.00	0.11	0.00	0.13	0.01	0.15	0.00
	100	0.00	0.00	0.11	0.00	0.15	0.01	0.17	0.00
Hypothermia	8	0.00	0.00	0.00	0.00	0.00	0.00		
	12	0.00	0.00	0.01	0.00	0.02	0.00		
	15	0.00	0.00	0.02	0.00	0.02	0.00	0.02	0.00
	18	0.00	0.00	0.03	0.00	0.05	0.01	0.05	0.00
	22	0.00	0.00	0.05	0.00	0.06	0.00	0.06	0.00

NH_4

Anaesthetic	Dose	10 minutes		24 Hours		48 Hours		96 Hours	
		Mean	Std. Error	Mean	Std. Error	Mean	Std. Error	Mean	Std. Error
None	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Control	0	0.00	0.00	0.10	0.00	0.09	0.00	0.10	0.00
Clove	0.1	0.00	0.00	0.05	0.00	0.07	0.00	0.08	0.00
	0.1667	0.00	0.00	0.07	0.00	0.07	0.02	0.08	0.00
	0.2333	0.00	0.00	0.09	0.01	0.10	0.01	0.07	0.00
	0.3	0.00	0.00	0.07	0.01	0.07	0.01	0.08	0.00
	0.3667	0.00	0.00	0.16	0.02	0.09	0.00	0.09	0.00
Cinnomon	0.3333	0.00	0.00	0.07	0.01	0.09	0.00	0.09	0.00
	0.5	0.00	0.00	0.08	0.01	0.09	0.00	0.09	0.00
	0.5667	0.00	0.00	0.10	0.01	0.08	0.00	0.09	0.00
	0.6667	0.00	0.00	0.10	0.01	0.08	0.00	0.09	0.00
	0.7333	0.00	0.00	0.12	0.01	0.09	0.00	0.10	0.00
Zn	0.5	0.00	0.00	0.06	0.00	0.01	0.00	0.02	0.00
	0.7	0.00	0.00	0.05	0.00	0.01	0.00	0.04	0.00
	1.3	0.00	0.00	0.04	0.00	0.03	0.01	0.06	0.00
	1.5	0.00	0.00	0.04	0.00	0.03	0.01	0.06	0.00
	1.7	0.00	0.00	0.04	0.00	0.06	0.00	0.06	0.00
TB	2	0.00	0.00	0.02	0.01	0.04	0.00	0.75	0.00
	5	0.00	0.00	0.02	0.01	0.07	0.01	0.82	0.01
	6	0.00	0.00	0.06	0.01	0.48	0.26	0.91	0.00
	7	0.00	0.00	0.07	0.01	0.64	0.15	0.86	0.02
	8	0.00	0.00	0.08	0.00	1.24	0.16	1.63	0.02
MS	45	0.00	0.00	0.06	0.00	0.06	0.00	0.08	0.00
	50	0.00	0.00	0.06	0.00	0.09	0.01	0.13	0.00
	52.5	0.00	0.00	0.06	0.00	0.11	0.01	0.13	0.00
	75	0.00	0.00	0.11	0.00	0.13	0.01	0.15	0.00
	100	0.00	0.00	0.11	0.00	0.15	0.01	0.17	0.00
Hypothermia	8	0.00	0.00	0.00	0.00	0.00	0.00		
	12	0.00	0.00	0.01	0.00	0.02	0.00		
	15	0.00	0.00	0.02	0.00	0.02	0.00	0.02	0.00
	18	0.00	0.00	0.03	0.00	0.05	0.01	0.05	0.00
	22	0.00	0.00	0.05	0.00	0.06	0.00	0.06	0.00

NO_2^-

Control	0.00	0.00	0.00	0.09	0.00	0.02	0.00	0.07	0.01
Clove	0.10	0.00	0.00	0.08	0.00	0.11	0.01	0.15	0.00
	0.17	0.00	0.00	0.09	0.00	0.02	0.01	0.04	0.01
	0.23	0.00	0.00	0.10	0.00	0.10	0.01	0.11	0.00
	0.30	0.00	0.00	0.11	0.01	0.13	0.01	0.14	0.01
	0.37	0.00	0.00	0.14	0.01	0.15	0.01	0.15	0.01
Cinnomon	0.33	0.00	0.00	0.01	0.00	0.05	0.03	0.03	0.00
	0.50	0.00	0.00	0.02	0.00	0.03	0.00	0.04	0.00
	0.57	0.00	0.00	0.02	0.00	0.02	0.00	0.04	0.00
	0.67	0.00	0.00	0.02	0.00	0.03	0.00	0.04	0.00
	0.73	0.00	0.00	0.03	0.00	0.03	0.00	0.03	0.00
Zn	0.50	0.00	0.00	0.02	0.00	0.01	0.00	0.03	0.00
	0.70	0.00	0.00	0.01	0.00	0.02	0.00	0.03	0.00
	1.30	0.00	0.00	0.01	0.00	0.04	0.01	0.05	0.00
	1.50	0.00	0.00	0.01	0.00	0.04	0.01	0.05	0.00
	1.70	0.00	0.00	0.01	0.00	0.05	0.00	0.06	0.00
TB	2.00	0.00	0.00	0.02	0.01	0.02	0.00	0.14	0.01
	5.00	0.00	0.00	0.03	0.01	0.06	0.01	0.16	0.00
	6.00	0.00	0.00	0.07	0.01	0.11	0.02	0.17	0.00
	7.00	0.00	0.00	0.04	0.01	0.12	0.02	0.16	0.00
	8.00	0.00	0.00	0.08	0.01	0.15	0.01	0.14	0.01
MS	45.00	0.00	0.00	0.03	0.00	0.02	0.00	0.03	0.00
	50.00	0.00	0.00	0.02	0.00	0.03	0.00	0.07	0.01
	52.50	0.00	0.00	0.04	0.00	0.08	0.01	0.10	0.00
	75.00	0.00	0.00	0.10	0.00	0.10	0.00	0.10	0.00
	100.00	0.00	0.00	0.10	0.00	0.10	0.00	0.10	0.00
Hypothermia	8.00	0.00	0.00	0.00	0.00	0.00	0.00		
	12.00	0.00	0.00	0.01	0.00	0.01	0.00		
	15.00	0.00	0.00	0.02	0.00	0.02	0.00	0.02	0.00
	18.00	0.00	0.00	0.03	0.00	0.03	0.01	0.04	0.00
	22.00	0.00	0.00	0.01	0.00	0.02	0.00	0.02	0.00

NO_3^-

Anaesthetic	Dose	10 minutes		24 Hours		48 Hours		96 Hours	
		Mean	Std. Error	Mean	Std. Error	Mean	Std. Error	Mean	Std. Error
None	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Control	0.00	0.00	0.00	0.10	0.00	0.05	0.01	0.18	0.01
Clove	0.10	0.00	0.00	0.03	0.01	0.44	0.21	0.71	0.18
	0.17	0.00	0.00	0.05	0.00	0.03	0.01	0.05	0.01
	0.23	0.00	0.00	0.06	0.00	0.15	0.09	0.06	0.00
	0.30	0.00	0.00	0.08	0.01	0.09	0.01	0.10	0.00
	0.37	0.00	0.00	0.10	0.00	0.09	0.00	0.10	0.00
Cinnomon	0.33	0.00	0.00	0.03	0.00	0.02	0.00	0.03	0.00
	0.50	0.00	0.00	0.04	0.01	0.03	0.01	0.05	0.01
	0.57	0.00	0.00	0.03	0.01	0.04	0.01	0.05	0.01
	0.67	0.00	0.00	0.04	0.01	0.05	0.00	0.06	0.00
	0.73	0.00	0.00	0.08	0.00	0.07	0.00	0.07	0.00
Zn	0.50	0.00	0.00	0.02	0.00	0.03	0.00	0.03	0.00
	0.70	0.00	0.00	0.02	0.00	0.03	0.00	0.04	0.00
	1.30	0.00	0.00	0.03	0.02	0.03	0.00	0.05	0.00
	1.50	0.00	0.00	0.01	0.00	0.03	0.00	0.05	0.00
	1.70	0.00	0.00	0.01	0.00	0.02	0.00	0.04	0.00
TB	2.00	0.00	0.00	0.04	0.01	0.04	0.01	0.16	0.00
	5.00	0.00	0.00	0.06	0.00	0.04	0.00	0.16	0.00
	6.00	0.00	0.00	0.06	0.01	0.10	0.02	0.17	0.00
	7.00	0.00	0.00	0.07	0.01	0.12	0.02	0.17	0.00
	8.00	0.00	0.00	0.07	0.01	0.14	0.02	0.17	0.00
MS	45.00	0.00	0.00	0.04	0.00	0.04	0.00	0.04	0.00
	50.00	0.00	0.00	0.05	0.00	0.05	0.00	0.04	0.00
	52.50	0.00	0.00	0.04	0.00	0.04	0.00	0.04	0.00
	75.00	0.00	0.00	0.04	0.00	0.05	0.00	0.04	0.00
	100.00	0.00	0.00	0.05	0.00	0.05	0.00	0.05	0.00
Hypothermia	8.00	0.00	0.00	0.00	0.00	0.00	0.00		
	12.00	0.00	0.00	0.01	0.00	0.01	0.00		
	15.00	0.00	0.00	0.02	0.00	0.02	0.00	0.02	0.00
	18.00	0.00	0.00	0.06	0.03	0.04	0.00	0.05	0.00
	22.00	0.00	0.00	0.03	0.00	0.04	0.00	0.04	0.00

.....ഇൽ.....

Publications

List of Publications

1) Sindhu, M.C. and Ramachandran, A. (2013) Acute toxicity and optimal dose of clove oil as anaesthetic for blue hill trout *Barilius bakeri* (Day). Fish.Technol. 50: 280-283

2) Sindhu, M.C. and Ramachandran, A. (2013) Cold chain management– An essential component of the ornamental fish industry. Fishing Chimes.32 (11): 29-31

Article published in popular journals

Fishery Technology 50 (2013) : 280 - 283

Research Note

Acute Toxicity and Optimal Dose of Clove Oil as Anaesthetic for Blue Hill Trout *Barilius bakeri* (Day)

M. C. Sindhu* and A. Ramachandran
School of Industrial Fisheries, Cochin University of Science and Technology, Fine Arts Avenue, Cochin - 682 016, India

Anaesthetics have long been used in handling and transportation of live fish to reduce stress and mortality and to tranquilize fish for biological and aquaculture research (Park et al., 2003; Park et al., 2004; Hur et al., 2005). According to Summerfelt & Smith (1990) and Woolsey et al., (2004) the optimal levels of anesthesia have been determined for fish transportation and general handling. Quite a number of anaesthetics have been used or evaluated for use in aquaculture, but issues such as toxicity, safety, and cost are frequently limiting factors (Ross & Ross, 1999). Though these anaesthetics are effective, only tricaine has been permitted to use in food fish anesthesia mainly due to environmental and health risks. An ideal anaesthetic should meet the following criteria: simple administration, rapid induction of anesthesia, maintenance of the anesthesia state and rapid recovery, effectiveness at low concentrations, a wide range between effective and toxic concentrations, low tissue residues, and low cost (Marking & Meyer, 1985).

There have been few controlled systematic investigations of efficacy and physiologic effects for many of these anaesthetics, and there is a need for more complete and concise ranges of safe and effective concentrations or dosages of anaesthetic agents for fish (Gilderhus, 1987). This has resulted in renewed interest to develop 'green anaesthetic' with low environmental and health risks (Ramanayaka & Atapattu, 2006). A range of crude products and purified compounds are widely used as fish sedatives *viz*., clove, wintergreen, or spearmint oils, quinaldine (Neiffer & Stamper, 2009; Danner et al., 2011).

Clove oil has been indicated as an alternative to traditional anaesthetics such as metomidate, quinaldine, and tricaine methanesulphonate because it is a natural oil and safe to use (Sladky et al., 2001). In the last several years, clove oil has been recognized as an effective anaesthetic for sedating fish for a number of invasive and noninvasive fisheries management and research procedures. Many authors in different fish species (Keene et al., 1998; Taylor & Roberts, 1999; Grush et al., 2004; Hamáèková et al., 2004; Roubach et al., 2005; Velíšek et al., 2005ab, 2006; Cunha & Rosa, 2006; Perdikaris et al., 2010) have investigated the efficacy and advisability of clove oil for fish anesthesia. The efficacy is conditioned by environmental and biological factors (Ross & Ross, 1999). The main chemical content of the clove oil is eugenol (70 to 98%) (Velozo, 2002) and has multiple uses mainly in dentistry and medicine as an antiseptic, analgesic and anaesthetic agent (Curtis, 1990; National Toxicology Program, 2002). Many aquaculturists and clinicians add it directly to water baths to achieve the desired effect. It is also an organic substance safe for both environment and user (McGovern-Hopkins et al., 2003). Therefore, it could be a promising anaesthetic agent in aquaculture and fish handling. In the international ornamental fish market, the indigenous fish blue hill trout *Barilius bakeri* (Day) is considered as medium preferred fish and belongs to the family Cyprinidae. In the present study, we assayed the potential of clove oil to anaesthetize *Barilius bakeri* in the course of handling and determine the lethal concentration of clove oil for *Barilius bakeri.*

Received 03 October 2012; Revised 14 March 2013; Accepted 07 April 2013

* *E-mail: sindhin@gmail.com*

© 2013 Society of Fisheries Technologists (India)

Indian blue hill trout *Barilius bakeri* (mean mass and standard length 3.75 ± 1.5 g; 101 ± 1.3 mm) were collected from Chalakkudy river system, and were acclimatized in laboratory for two weeks in square FRP tanks (1000 l) under natural photoperiod and light conditions. The tests were conducted in dechlorinated tap water (dissolved oxygen 7.3 mg l^{-1}, pH 6-7, temperature 27p C, and hardness 152 ppm). The fishes were fed thrice daily with commercial pellet feed and feeding was stopped 48 h prior to the experiment. The stock solution of clove oil (Universal Oleoresins India) was dissolved in 95% ethanol at a ratio of 1:10 (Cho & Heath, 2000). Two sets of experiments were conducted. Eight blue hill trout were used for each concentration and for the control group. In all experiments, diluted clove oil was added directly to the water and the water was vigorously aerated for the experiments. Dilution and experimental protocol were replicated three times.

The first set of experiment was to find out acute toxicities (24 h LC_{50} test) as per Litchfield & Wilcoxon (1949). This experiment was conducted triplicate in glass aquarium containing 5 l of aerated freshwater with different concentrations of clove oil *viz*., 2, 4, 6, 8 and 10 mg l^{-1} (the concentration lethal to 50% of test animals after 24 h) along with control. Eight fish samples randomly placed in each aquarium. Fish and its behaviour, total mortalities, water temperature, pH and oxygen saturation were monitored (Microprocessor water & soil analysis kit, Model 1160 E1. Environmental and Scientific Instruments, India) and recorded throughout the test and the experiment continued for 24 h under the same conditions to find out the 24 h LC_{50} of *Barilius bakeri.*

The second set of experiments was to find out the optimal dose of clove oil determined by the concentration range of clove oil. The expected concentration was the lowest level that causes 100% mortality and the highest level that causes 0% mortality. Eight fish were exposed to each of different concentrations of clove oil of 2, 4, 6, 8 and 10 mg l^{-1}. Each concentration had three replicates. Experiment was conducted in glass aquarium containing 5 L of water with continuous aeration. A logarithmic spaced series of clove oil concentrations between the highest concentration that killed no fish and the lowest concentration that killed all fish within 24 h were used as the optimal dose. During the 24 h test period, changes in physiological parameters of fish and fish mortality figures were recorded after which the fish were moved to fully aerated fresh water.

Based on tests, the 24 h lethal concentration (LC_{50}) of clove oil was determined as 8 mg l^{-1}. 100% mortality rate was observed at clove oil concentrations of 10 and 12 mg l^{-1} (Fig. 1). It was observed that *Barilius bakeri* exposed to 12 mg l^{-1} died within 2 h of exposure. Velíšek et al. (2005a) reported that the determination of acute toxicity of clove oil is important for its usage in fish anaesthesia, appropriate treatment concentration for anaesthetic baths and also for possible contamination of the water environment by such anaesthetic (Velíšek et al., 2005a). Velíšek et al. (2005a) reported that the rainbow trout is the most sensitive fish species, with LC_{50} values for clove oil of 81.1 mg l^{-1} for 10 min. Acute toxicity values of clove oil expressed as 10 min LC_{50} were 74.3 mg l^{-1} for carp (Velíšek et al., 2005b) and 76.70 mg l^{-1} for the European catfish (Velíšek et al., 2006). Comparable values were reported by the same authors for Chinook salmon (*Oncorhynchus tshawytscha*) at 62 mg l^{-1}, higher for coho salmon (*O. kisutch*) at 96 mg l^{-1} and for white sturgeon (*Acipenser transmontanus*) at 526 mg l^{-1}. Keene et al. (1998) reported that the 0.5 – 96 h LC_{50} of clove oil for rainbow trout (20 g in weight ; 12 cm in fork length) was 65 – 9 ppm. In a study conducted by Taylor & Roberts (1999), the 10 min LC_{50} of clove oil for juvenile white sturgeon, chinook and coho salmon were 526, 62 and 96 ppm, respectively.

Safe and optimal anaesthetic dosage required for inducing sedation with least mortality after 24 h for *Barilius bakeri* was determined as 4 mg l^{-1}. The

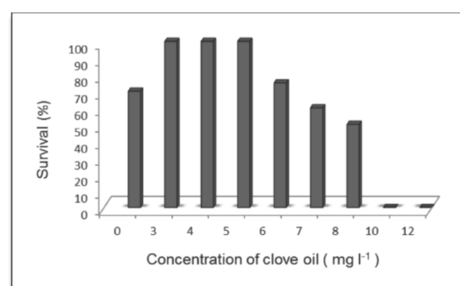


Fig.1. 24 h LC_{50} of *Barilius bakeri*

highest concentration of clove oil that caused 0% mortality was 5 mg l^{-1}. The lowest concentration of clove oil that caused 100% mortality was 10 mg l^{-1}. According to Logarithmic method: 10; 8; 6 & 4 mg l^{-1} of clove oil was plotted against percentage survival on logarithmic-probability paper to obtain the expected death (percentage survival) (Fig. 1). The optimal dose calculated as per the method of Gilderhus & Marking (1987), was carried out to test the ability of clove oil as a good anaesthetic with the same species. Endo et al. (1972) showed that clove oil is effective in Crucian carp (*Carassius carassius*) and Hikasa et al. (1986) showed that it provides effective anaesthesia in common carp (*Cyprinus carpio*) at 25-100 mg l^{-1}. Soto & Burhanuddin (1995) found a dose of 100 mg l^{-1} to be effective in rabbit fish. One of the criteria that proper anaesthetic in fish should meet is its safety at treatment concentrations (Marking & Meyer, 1985). Roubach et al. (2005) found that exposure of tambaqui (*Colossoma macropomum*) to 65 mg l^{-1} of clove oil was sufficient to induce an anaesthetic state. The anaesthetic properties and dosages of clove oil have been tested on different fishes (Griffiths, 2000). Prince & Powell (2000) recommended a concentration of 30 mg l^{-1} clove oil for effective and safe anaesthesia of adult rainbow trout. The optimal dose of clove oil in the range of 250–300 mg l^{-1} at water temperature of 18°C was reported by Park et al. (2008).

Stone (1999) and Kildea et al. (2004) have assessed the advantages and disadvantages of clove oil as a fish anaesthetic. Most of them have reported it to be safe and effective anaesthetic but it has a narrow margin of safety. Care must be taken when using high concentrations of clove oil for induction, because ventilatory failure may occur rapidly. A combined view of the findings leads to the recommendation that, for the size of blue hill trout used in the present study, clove oil, with fast induction and rapid recovery times offers a manageable, safe anaesthetic alternative, which can be useful in aquaculture and fish handling activities. In future clove oil would be in use as an anaesthetic in Indian blue hill trout for long distant transportation.

Acknowledgements

The first author is grateful to the Director, School of Industrial Fisheries (SIF), Cochin for the necessary facilities and kind permission to publish this paper.

References

Cho, G.K., and Heath, D.D. (2000) Comparison of tricaine methanesulphonate (MS-222) and clove oil anaesthesia effects on the physiology of juvenile Chinook salmon Oncorhynchus tshawytscha (Walbaum). Aquacult. Res. 31: 537-546

Cunha, F.E.A, and Rosa I .L. (2006) Anaesthetic effects of clove oil on seven species of tropical reef teleosts. J. Fish. Biol 69: 1504-1512

Curtis, E.K. (1990) In pursuit of palliation: oil of cloves in the art of dentistry. Bull. Hist. Den. 38: 9-14

Danner, G.R., Muto, K.W., Zieba, A.M., Stillman., C.M., Seggio, J.A. and Ahmad S.T. (2011) Spearmint (carvone) oil and wintergreen (methyl salicylate) oil emulsion is an effective immersion anaesthetic of fishes. J. Wildl. Manage. 2: 146-155

Endo, T., Ogishima, K., Tanaka, H. and Ohshima, S. (1972) Studies on the anaesthetic effect of eugenol in some freshwater fishes. B. Jpn. Soc. Sci. Fish. 38: 761-767

Gilderhus, P.A. and Marking, L.L. (1987) Comparative efficacy of 16 anaesthetic chemicals on rainbow trout. N AM J. Fish. Manage. 288-293

Griffiths, S. P. (2000) The use of clove oil as an anaesthetic and method for sampling intertidal rockpool fishes. J. Fish. Biol. 57: 1453-1464

Grush, J., Noakes, D.L.G., Moccia R.D. (2004) The efficacy of clove oil as an anaesthetic for the zebrafish, *Danio rerio* (Hamilton). Zebra fish. 1: 46-53

Hamáèková, J., Lepièová, A., Kozák, P., Stupka, Z., Kouøil, J., Lepiè, P. (2004) The efficacy of various anaesthetics in tench (*Tinca tinca* L.) related to water temperature. Vet Med – Czech. 49: 467-472

Hikasa, Y., Takase, K., Ogasawara, T. and Ogasawara, S. (1986) Anaesthesia and recovery with tricainemethanesulfonate, eugenol and thiopental sodium in the carp, *Cyprinus carpio*. Jpn. J. Vet. Sci. 48: 341-351.

Hur, J.W., Park, I.S., Kho, K.H. and Chang, Y.J. (2005) Changes of hematological characteristics of cultured sweetfish (Pleoglossus altivelis) by anaesthetic transport. Ocean and Polar Res. 27: 59-65

Keene, J.L., Noakes, D.G., Moccia, R.D., and Soto, C.G. (1998) The efficacy of clove oil as an anaesthetic for rainbow trout, *Onchorhynchus mykiss* (Walbaum). Aquacult. Res. 29: 89-101

Kildea, M.A., Allan, G.L., Kearney, R.E. (2004) Accumulation and clearance of the anaesthetics clove oil and AQUI_S from the edible tissue of silver perch (Bidyanus bidyanus). Aquaculture, 232: 265-277

© 2013 Society of Fisheries Technologists (India) ***Fishery Technology*** 50 : 280-283

Litchfield, J. T. and Wilcoxon, F. (1949) A simplified method for evaluating dose effects experiments. J. Pharm, Exp. Therap. pp 96-99

McGovern-Hopkins, K., Tamaru, C.S., Takeshit, G. and Yamamoto, M. (2003) Procedural Guide for the Artificial Insemination of the Lyretail Swordtail (*Xiphophorus helleri).* University of Hawai'i Sea Grant College Program School of Ocean and Earth Science and Technology; available online at http://www.soest.hawaii.edu/SEAGRANT (accessed April 2009)

Marking, L.L. and Mayer, F. (1985) Are better anaesthetics needed in fisheries? Fisheries 10 (6): 2-5

National Toxicology Program (2002) Capturado em 23 mai, 2002. Online. Disponível na Internet http://ntp-server.niehs.nih.gov/ (Accessed on 23 May 2002)

Neiffer, D.L. and Stamper, M.A. (2009) Fish sedation, anaesthesia, analgesia, and euthanasia: considerations, methods, and types of drugs. ILAR J 50: 343-360

Park, I.S., JO, J.H., Lee, S.J., Kim, Y.A., Park, K.E., Hur, J.W., YOO, J.S. and Song, Y.C. (2003) Anaesthetic effect of lidocaine hydrochloride-sodium bicarbonate and MS-222 on the greenling (Hexagrammos otakii). J. Korean Fish Soc. 36: 449-453

Park, I.S., Hur, J.W., Song, Y.C., Im, J.H. and Johnson S.C. (2004) Anaesthetic effect of lidocaine hydrochloride-sodium bicarbonate on the winter flounder, *Pleuronectes americanus.* Ocean and Polar Res. 26: 475-480

Park, M.O., Hur, W.J., Im, S.Y., Seol, D. W., Lee, J. and Park, I.S. (2008) Anaesthetic efficacy and physiological responses to clove oil-anaesthetized kelp grouper *Epinephelus bruneus* Aquacult. Res. 39: 877-884

Perdikaris, C., Nathanailides, C., Gouva, E., Gabriel, U.U., Bitchava, K., Athanasopoulou, F., Paschou, A. and Paschos (2010) Size-relative effectiveness of clove oil as an anaesthetic for rainbow trout (*Oncorhynchus mykkis* Walbaum, 1792) and goldfish (*Carassius auratus* Linnaeus, 1758). Acta. Vet. Brno. 79: 481-490

Prince, A. and Powell, C. (2000) Clove oil as an anaesthetic for invasive field procedures on adult rainbow trout. N AM J. Fish. Manage. 20: 1029-1032

Ramanayaka, J.C. and Atapattu, N.S.B.M. (2006) Fish anaesthetic properties of some local plant material. Trop. Agricult. Res. Exten. 9: 2006

Ross, L.G. and Ross, B. (1999) Anaesthetic and Sedative Techniques for Aquatic Animals. 2nd edn., 76p, Blackwell, London

Roubach, R., Gomes, L.C., Fonseca, F.A.L. and Val, A.L. (2005). Eugenol as an efficacious anaesthetic for tambaqui, *Colossoma macropomum* (Cuvier). Aquacult. Res. 36: 1056-1061

Sladky, K.K. (2001) Comparative efficacy of tricaine methanosulfonate and clove oil for use as anaesthetics in red pacu (*Piaractus brachipomus*). Am. J. Vet. Res . 62: 337-342

Soto, C. and Burhanuddin, S. (1995) Clove oil as a fish anaesthetic for measuring length an weight of rabitfish (*Siganus lineatus*). Aquaculture. 135: 149-152

Stone, D. and Tostin, N. (1999) Clove Bud Oil a Big Yawn for Silver Perch, pp 30-34, Fish NSW Magazine, Spring

Summerfelt, R.C. and Smith, L.S. (1990) Anaesthesia, surgery, and related techniques. In: Methods for Fish Biology (Shreck, C.B. and Moyle, P.B., Eds), pp 213-272, American Fish Society, Bethseda

Taylor, P.W. and Roberts, S.D. (1999) Clove oil: an alternative anaesthetic for aquaculture. N. AM. J. Aquacult. 61**:** 150-155

Velíšek, J., Wlasow, T., Gomulka, P., Svobodová, Z., Novotný, L. and Ziomek, E. (2006) Effects of clove oil anesthesia on European catfish (*Silurus glanis* L.). Acta. Vet. Brno. 75: 99-106

Velíšek, J., Svobodová, Z., Piaèková, and V. (2005a) Effetc of clove oil anaesthesia on rainbow trout (*Oncorhynchus mykiss*). Acta. Vet. Brno. 74: 139-146

Velíšek, J., Svobodová, Z., Piaèková, V., Groch, L. and Nepejchalová, L. (2005b) Effects of clove oil anaesthesia on common carp (*Cyprinus carpio* L.). Vet. Med – Czech. 50: 269-275

Velozo, E. (2002) Histoquímica do cravo. Universidade Federal da Bahia. Homepage. Online. Disponível na Internet (Accessed on 22 March 2002)

Woolsey, J., Holcomb, M. and Ingermann, R.L. (2004) Effect of temperature on clove oil anaesthesia in steelhead fry. N.AM.. J. Aquacult. 66: 35-41

© 2013 Society of Fisheries Technologists (India) ***Fishery Technology*** 50 : 280-283