Effect of spice oleoresins in microbial decontamination and their potential application in quality stabilization of Tuna (*Euthynnus affinis*) during storage

Thesis submitted to

Cochin University of Science and Technology

In partial fulfilment of the requirements for the degree of

Doctor of Philosophy

By

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Kochi – 682016

October 2012

Reverence for the Lord is the foundation of true wisdom

Psalm 111:10

Dedicated to my dear Husband for his whole hearted
support and encouragement,
to my Dad for his unconditional love,
to my Mother for her sleepless nights,
to my dear Teacher for being
a constant source of inspiration
and my Heavenly father for all these blessings.

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CERTIFICATE

This is to certify that the Doctoral Thesis entitled **"Effect of spice oleoresins in microbial decontamination and their potential application in quality stabilization of Tuna *(Euthynnus affinis)* during storage"** ***is an authentic record of research work carried out by Ms.Dhanya.P.R, under my supervision and guidance at the School of Industrial fisheries, Cochin University of Science and Technology, in partial fulfilment of the requirements for the degree of Doctor of philosophy of the Cochin University of Science and Technology and that no part thereof has been submitted before for any degree.***

Kochi-16 ***Prof. (Dr.) Saleena Mathew***
16-10-12

DECLARATION

This is to certify that this thesis entitled **"Effect of spice oleoresins in microbial decontamination and their potential application in quality stabilization of Tuna *(Euthynnus affinis)* during storage"** ***is a bonafide record of research carried out by me under the supervision and guidance of Dr. Saleena Mathew, Professor, School of Industrial Fisheries, Cochin University of Science and Technology, in partial fulfilment of the requirements for the PhD degree of Cochin University of Science and Technology and that no part of it has previously formed the basis for award of any degree, diploma, associateship, fellowship or other similar recognition in any University or Institution.***

Kochi-16 ***Dhanya.P.R***

16-10-12

ACKNOWLEDGEMENT

There are a number of people without whom this thesis might not have been written, and to whom I am greatly indebted. First of all, I bow before my Heavenly Father for giving me the health and strength for completing this thesis.

I register my heart felt admiration to my dear teacher, Dr. Saleena Mathew, Professor, School of Industrial Fisheries, for her keen interest, skillfull guidance and timely helps rendered during the period of research, which led to the completion of this manuscript. I offer my gratitude and appreciation to her for the deft ways in which she lovingly challenged and supported me through out the whole of this work - knowing when to push and when to let up.

I am greatly indebted to my mother, Ms.Elizabeth C.M, who has been a source of encouragement and inspiration to me throughout my life. A very special thank you for nurturing and bearing me through the months of writing. And I also thank my father, Mr.Rajan K. Pulikkottil, for patiently answering the innumerable questions I had asked during childhood, for encouraging me to ask more and for opening my eyes to the beauty of science. I also thankfully recall all the sleepless nights you and my husband have spent outside the lab, waiting for my arrival and the interest you took in arranging the samples required for my study.

My heartfelt gratitude to my best friend and husband, Bijoy Joseph, who remains willing to engage with the struggle and ensuing discomfort of having a partner engaged in research. I thank God for your unconditional love and practical & emotional support during the ups and downs of our lives. I wish to thank my in- laws, Mrs. & Mr. T.V.Joseph, for encouraging me to continue my studies and all the mental support they provided through out these years.

I am grateful to Dr. A. Ramachandran, Director, School of Industrial Fisheries, for providing me with necessary facilities and to Cochin University of Science and Technology for awarding the financial support as JRF & SRF for the successful completion of the Ph.D programme. I take this opportunity to thank all my teachers at the School of Industrial Fisheries, especially, Dr. John Mohan, for his invaluable suggestions rendered during this period. Words fail to express my appreciation and indebtness to Dr.P.K.Surendran, Joint Director & Head of Division (Retd.), CIFT, for always finding the right solutions for my research problems and leading me on from blind ends. I would also like to thank Dr. A.V. Saramma for helping me with the presentation of microbiological results.

I express my hearty thanks due to Dr.Chempakam.B (Principal scientist & Head of Division) and Dr. Sushama (Librarian), Indian Institute of Spices Research (IISR), Calicut, for giving me an opportunity to collect literature in their prestigious institute. I owe my sincere thanks to Dr. Bright Singh and Dr. Renjith Kanjoor for providing the pure culture of pathogenic bacteria

necessary for the investigation and to the management and Staff of M/s Synthite chemicals Kolenchery, for providing me with oleoresin samples. A special thanks to Mr. V. A. Joseph & Sibin.P.B. for arranging the fish samples and for following all the instruction regarding its transportation. I register my sincere thanks to Mr. Nabajyothi Biswas for helping me with the installation of SPSS software and Mr.Baiju.K.K. for his timely assistance regarding the analysis of the data. I also thank Dr. Shibu.M.Eapen and Mr. Melbin Baby for all their cooperation in getting the SEM results in time.

Loving thanks to my friends / learning partners, Dr.Manjusha Pramod, Mr.Abhilash.S, Mr.Prasanth Varavoor and Mrs.Pearly Tony who played such important roles along the journey, as we mutually engaged in making sense of the various challenges we faced and in providing encouragement to each other at those times when it seemed impossible to continue. I would also like to thank Ms.Shubhasree Shankar, Dr.Saritha Thomas, Mr.Bijoy.V.M, Mr.John Chembian, Dr.Beena Sulochanan, Mr.Shanavaz, Ms.Dana.E.B and Dr.Prajith for their camaraderie and support during my tenure.

With much gratitude, I hereby acknowledge the sincere co-operation and help rendered by the technical and administrative staff of the school of Industrial fisheries. I would like to express a special thanks to Bindhu chechi for inspiring me to face any trouble with courage; Pilla chetta for helping me out with the technical troubles of autoclaves and Jayasankar Sir, Tony Sir, Susan Madam & our Alby chetta for taming the paper tigers. I am grateful to the staff of the Library in the lake side campus, CUSAT; Spices Board, Ernakulam; IISR, Calicut and CIFT, Cochin, for their kind assistance during the period of literature collection.

I take this opportunity, to thank all my colleagues at St.Albert's College Ernakulam, especially, Dr. Benno Pereira, for hastening me to complete the thesis and Dr. Ajith Thomas John for offering a helping hand for proof reading. I also express my sincere thanks to Ms.Renjini.P.K - friend, 'sister', colleague and Mr. Jose Emmanuel.L, my role model as a teacher, for their invaluable presence in my life.

Thanks are due to Mr.Anilajen and also my dear juniors Ms. Liya Jayalal& Mr. Antony.V.T for extending a helping hand in the editing of synopsis slides and Ms.Liji Thomas, Mr.Jithu Paul Jacob & Ms.Sri Kala for proof reading the manuscript. I owe a thanks to my neighbour Ms.Vijaya Narayanan and her children Shruthi and Shankar for helping me get 'unstuck' with this thesis on many occasions by their refreshing talks and gardening experiments. I take this opportunity to thank all my relatives for their love & prayers and apologize for not mentioning each one of them personally. Once again, I thank God the Divine, who continues to make the impossible possible through his gracious interventions and blessings. *Dhanya.P.R*

CONTENTS

List of Figures

List of Tables

List of Plates

Abbreviations

ANOVA	**Analysis of variance**
BA	**Biogenic amine**
BP	**Baird Parker medium**
BSA	**Bismuth sulphate agar**
cfu	**Colony forming units**
DW	**Distilled water**
HDB	**Histidine decarboxylating bacteria**
HEA	**Hektoen's Enteric Agar**
HFP	**Histamine food poisoning**
HPLC	**High pressure liqiud chromatography**
IQF	**Individually quick frozen**
MIC	**Minimum inhibitory concentration**
SEM	**Scanning electron microscope**
SPSS	**Scientific Package of Social Science**
T7	**Tergitol 7**
TCA	**Trichloroacetic acid**
TCBS	**Thiosulphate citrate bile salt**
TGA	**Tryptone glucose agar**
TPA	**Texture profile analysis**
TPC	**Total plate count**
TSI	**Triple sugar iron agar**
WPS	**Water phase salt**
XLD	**Xylose Lysine Desoxycholate**

Chapter 1

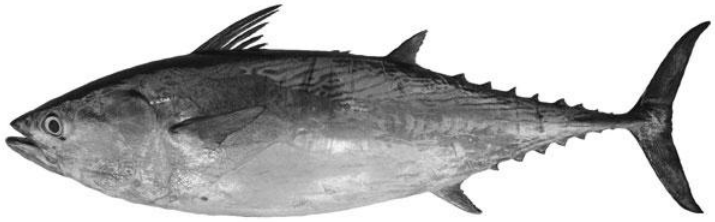
Introduction

Chapter 2

Spices as Antimicrobial Agents- A Review

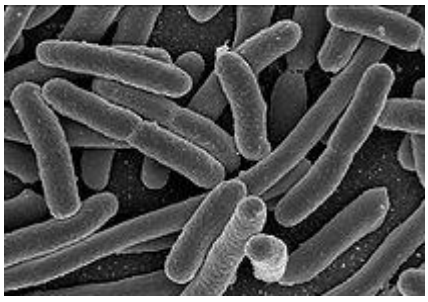
Chapter 3

Microbial profile of tuna



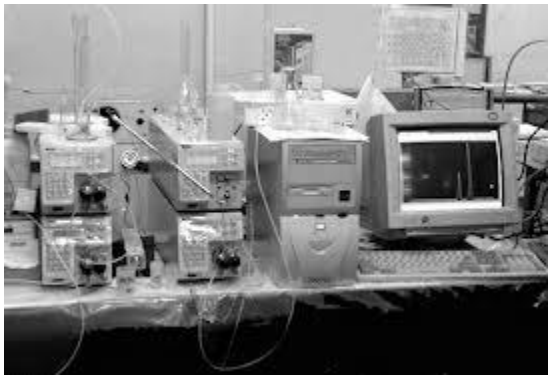
Chapter 4

Antibacterial Effect of Spice oleoresins



Chapter 5

Effect of spice oleoresins on biogenic amine formation



Chapter 6

Effect of spice oleoresins on texture and sensory attributes of tuna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Chapter 7

Effect of spice oleoresins on tuna during frozen storage

Chapter 8

Summary and Conclusion

References

Appendix

Appendix A

4.1. ANOVA between spoilage bacteria and spice treatment

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	690.987[a]	24	28.791	11.996	.000
Intercept	12008.013	1	12008.013	5003.339	.000
Bacteria	208.320	4	52.080	21.700	.000
Treatment	276.187	4	69.047	28.769	.000
Bacteria * Treatment	206.480	16	12.905	5.377	.000
Error	120.000	50	2.400		
Total	12819.000	75			
Corrected Total	810.987	74			

a. R Squared = .852 (Adjusted R Squared = .781)

4.2. Significant difference between individual treatments in the spoilage bacterial count (Post hoc tukey test)

	Micrococcus	*Pseudomonas*	*Bacillus*	*Aeromonas*	*Lactobacillus*
Micrococcus		.000	.144	.000	.954
Pseudomonas	.000		.087	.028	.001
Bacillus	.144	.087		.000	.474
Aeromonas	.000	.028	.000		.000
Lactobacillus	.954	.001	.474	.000	

*The mean difference is significant at the 0.05 level.

4.3. ANOVA between bacteria and minimum inhibitory concentration of spices

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	6.891[a]	23	.300	71905.957	.000
Intercept	12.177	1	12.177	2922507.000	.000
Concentration	2.321	5	.464	111425.400	.000
Bacteria	2.008	3	.669	160605.667	.000
Concentration * Bacteria	2.562	15	.171	40992.867	.000
Error	.000	48	4.1676		
Total	19.068	72			
Corrected Total	6.891	71			

a. R Squared = 1.000 (Adjusted R Squared = 1.000)

4.4. Post hoc tukey test for bacteria and minimum inhibitory concentration of spices

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
E.coli	8.00	.1867*	.00068	.000	.1849	.1885
	9.00	-.2333*	.00068	.000	-.2351	-.2315
	10.00	.1594*	.00068	.000	.1576	.1613
S.flexneri	7.00	-.1867*	.00068	.000	-.1885	-.1849
	9.00	-.4200*	.00068	.000	-.4218	-.4182
	10.00	-.0272*	.00068	.000	-.0290	-.0254
S.typhi	7.00	.2333*	.00068	.000	.2315	.2351
	8.00	.4200*	.00068	.000	.4182	.4218
	10.00	.3928*	.00068	.000	.3910	.3946
V.cholerae	7.00	-.1594*	.00068	.000	-.1613	-.1576
	8.00	.0272*	.00068	.000	.0254	.0290
	9.00	-.3928*	.00068	.000	-.3946	-.3910

b

4.5. ANOVA between TPC and spice treatment in fish media

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.284	7	.326	487.383	.000
Within Groups	.011	16	.001		
Total	2.295	23			

4.6. ANOVA between *E.coli* and spice treatments in fish media

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	32.427	7	4.632	12.329	.000
Within Groups	6.012	16	.376		
Total	38.438	23			

4.7. Post hoc tukey test for individual treatments in the *E.coli* count

	Control	Chlorine	Cardamom	Clove	Garlic	Oregano	Rosemary	Turmeric
Control		.000*	.020*	.000*	.007*	.007*	.980	.752
Chlorine	.000*		.235	1.000	.520	.520	.001	.003
Cardamom	.020*	.235		.235	.999	.999	.114	.329
Clove	.000*	1.000	.235		.520	.520	.001*	.003*
Garlic	.007*	.520	.999	.520		.323	.039*	.131
Oregano	.422	.009*	.646	.009*	.323		.908	.999
Rosemary	.980	.001*	.114	.001*	.039*	.039*		.997
Turmeric	.752	.003*	.329	.003*	.131	.131	.997	

*The mean difference is significant at the 0.05 level

4.8. ANOVA between treatments in fish media inoculated with *S.typhi*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	31.765	7	4.538	233.711	.000
Within Groups	.311	16	.019		
Total	32.076	23			

4.9. Significant difference between individual treatments in the *S.typhi* count

	Control	Chlorine	Cardamom	Clove	Garlic	Oregano	Rosemary	Turmeric
Control		.814	.000	.000	.842	.911	.993	.688
Chlorine	.814		.000	.000	1.000	.193	.387	.086
Cardamom	.000	.000		1.000	.000	.000	.000	.000
Clove	.000	.000	1.000		.000	.000	.000	.000
Garlic	.842	1.000	.000	.000		.212	.417	.096
Oregano	.911	.193	.000	.000	.212		1.000	1.000
Rosemary	.993	.387	.000	.000	.417	1.000		.977
Turmeric	.688	.086	.000	.000	.096	1.000	.977	

4.10. ANOVA between treatments in fish media inoculated with *V.cholerae*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11.872	7	1.696	31311.077	.000
Within Groups	.001	16	.000		
Total	11.873	23			

d

4.11. Significant difference between individual treatments in the *V. cholerae* count (Post hoc tukey test)

	Control	Chlorine	Cardamom	Clove	Garlic	Oregano	Rosemary	Turmeric
Control		.000	.000	.000	.000	.000	.000	.000
Chlorine	.000		1.000	1.000	1.000	1.000	1.000	1.000
Cardamom	.000	1.000		1.000	1.000	1.000	1.000	1.000
Clove	.000	1.000	1.000		1.000	1.000	1.000	1.000
Garlic	.000	1.000	1.000	1.000		1.000	1.000	1.000
Oregano	.000	1.000	1.000	1.000	1.000		1.000	1.000
Rosemary	.000	1.000	1.000	1.000	1.000	1.000		1.000
Turmeric	.000	1.000	1.000	1.000	1.000	1.000	1.000	

Appendix B

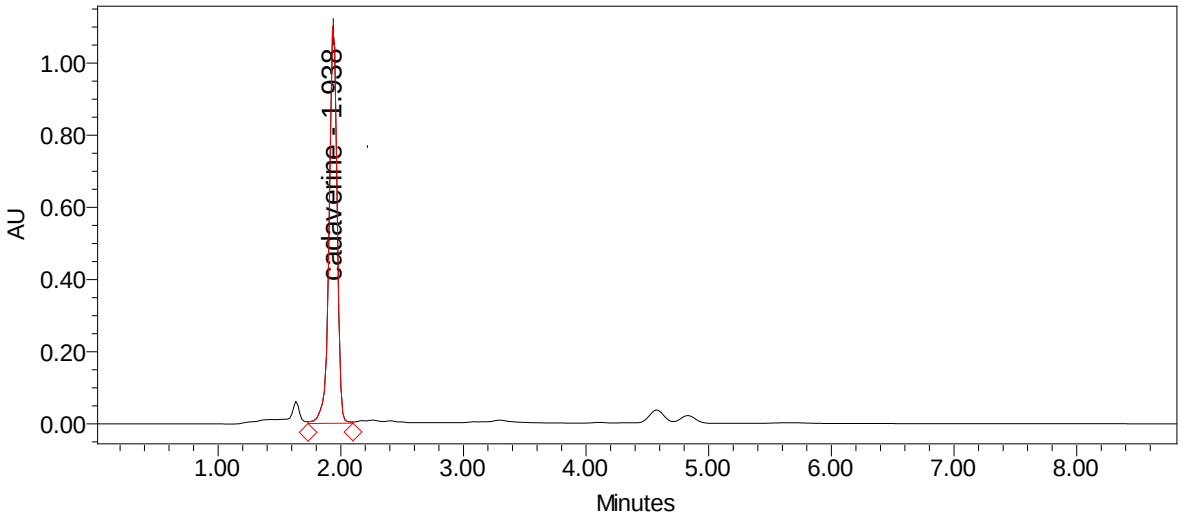

Fig 5.1. HPLC peak for Standard Cadaverine

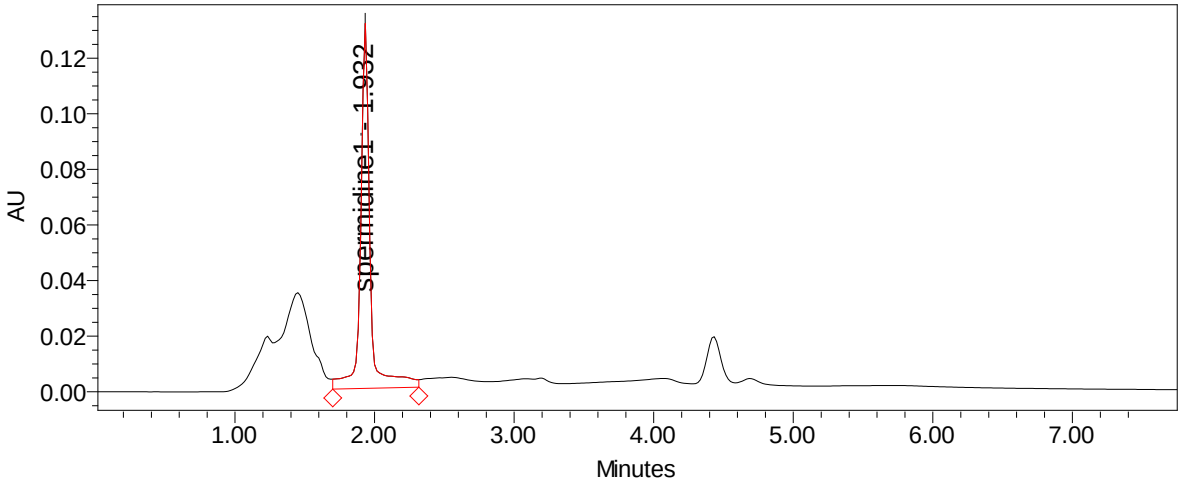

Fig 5.2. HPLC peak for Standard spermidine

f

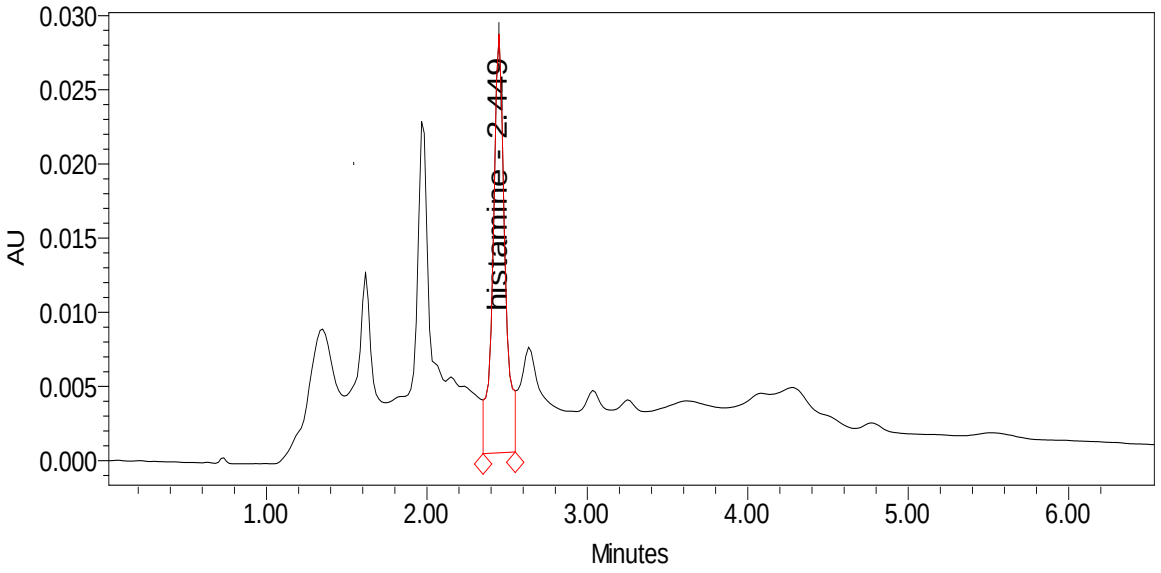

Fig 5.3. HPLC peak for Standard Histamine

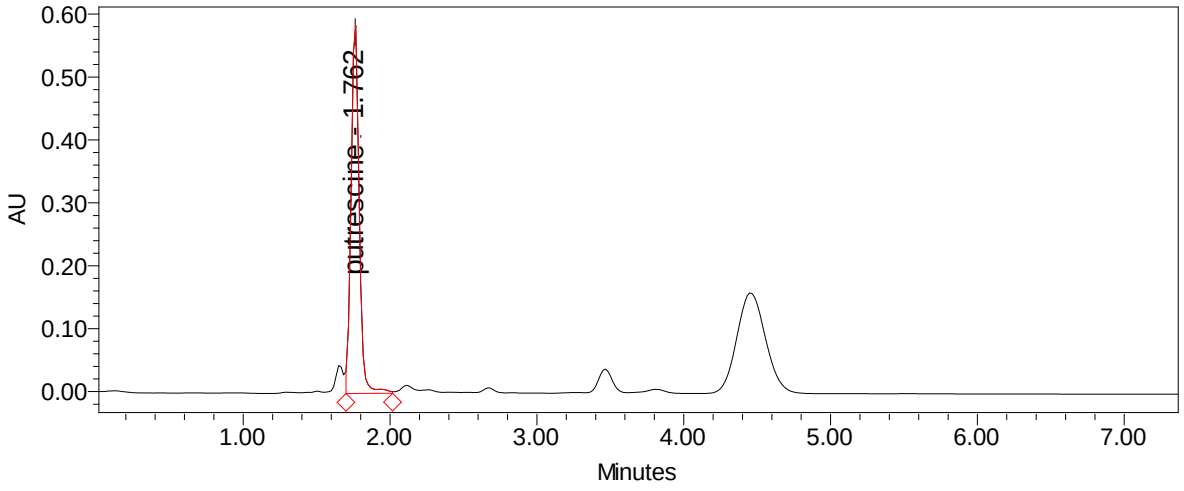

Fig 5.4. HPLC peak for Standard putrescine

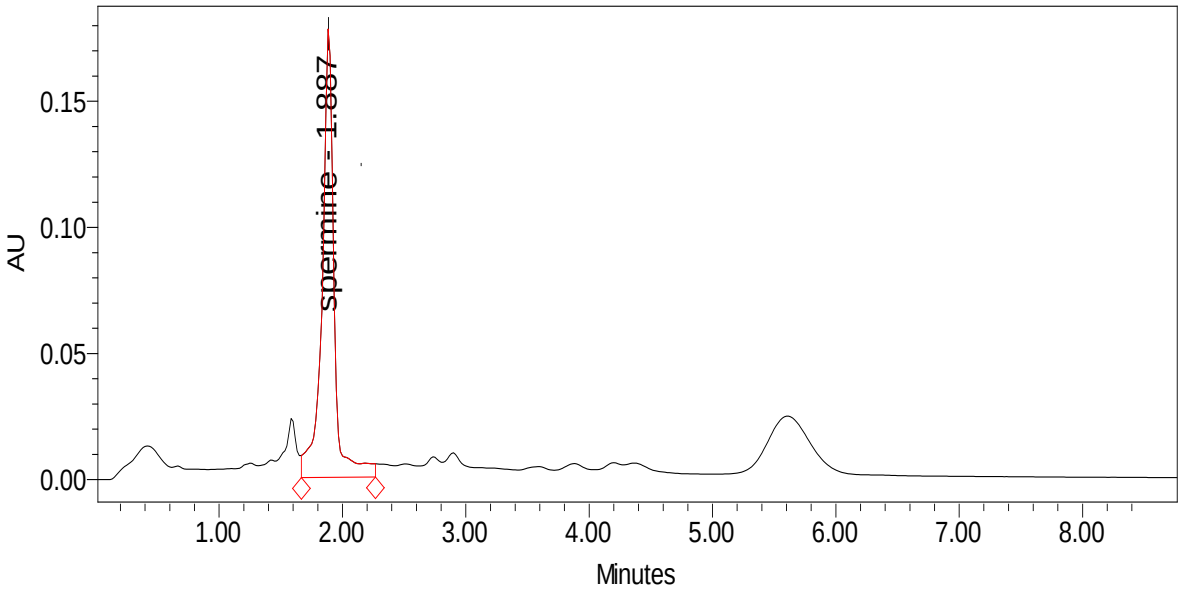

Fig 5.5. HPLC peak for Standard spermine

5.1. ANOVA between HDB bacteria and treatments

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3.6069	31	1.1638	2.5097	.000
Intercept	1.1489	1	1.1489	2.4768	.000
Bacteria	2.7739	3	9.2438	1.9938	.000
Treatment	2.2568	7	3.2237	6949702.190	.000
Bacteria * Treatment	6.0778	21	2.8947	6240741.370	.000
Error	296.787	64	4.637		
Total	4.7549	96			
Corrected Total	3.6069	95			

a. R Squared = 1.000 (Adjusted R Squared = 1.000)

h

5.2. Tukey HSD for HDB bacterial count and duration of storage

(I) VAR00004	(J) VAR00004	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	4.00	-54.3917*	.62164	.000	-56.0315	-52.7519
	8.00	-1028.7667*	.62164	.000	-1030.4065	-1027.1269
	24.00	-12736.4333*	.62164	.000	-12738.0731	-12734.7935
4.00	1.00	54.3917*	.62164	.000	52.7519	56.0315
	8.00	-974.3750*	.62164	.000	-976.0148	-972.7352
	24.00	-12682.0417*	.62164	.000	-12683.6815	-12680.4019
8.00	1.00	1028.7667*	.62164	.000	1027.1269	1030.4065
	4.00	974.3750*	.62164	.000	972.7352	976.0148
	24.00	-11707.6667*	.62164	.000	-11709.3065	-11706.0269
24.00	1.00	12736.4333*	.62164	.000	12734.7935	12738.0731
	4.00	12682.0417*	.62164	.000	12680.4019	12683.6815
	8.00	11707.6667*	.62164	.000	11706.0269	11709.3065

Based on observed means. * The mean difference is significant at the 0.05 level.

5.3. ANOVA for histamine content

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2.98111	31	9.6179	1.9258	.000
Intercept	3.38311	1	3.38311	6.7729	.000
Between treatments	1.02511	7	1.46510	2.9328	.000
Between time period	7.60510	3	2.53510	5.0758	.000
Treatment vs Time	1.19611	21	5.6939	1.1408	.000
Error	3196.667	64	49.948		
Total	6.36411	96			
Corrected Total	2.98111	95			

a. R Squared = 1.000 (Adjusted R Squared = 1.000)

5.4. Multiple Comparisons between time period and histamine production

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 hour	4th hour	-35031.9583*	2.04018	.000	-35037.3400	-35026.5767
	8th hour	-56954.3333*	2.04018	.000	-56959.7150	-56948.9517
	24th hour	-75651.4583*	2.04018	.000	-75656.8400	-75646.0767
4th hour	0 hour	35031.9583*	2.04018	.000	35026.5767	35037.3400
	8th hour	-21922.3750*	2.04018	.000	-21927.7567	-21916.9933
	24th hour	-40619.5000*	2.04018	.000	-40624.8817	-40614.1183
8th hour	0 hour	56954.3333*	2.04018	.000	56948.9517	56959.7150
	4th hour	21922.3750*	2.04018	.000	21916.9933	21927.7567
	24th hour	-18697.1250*	2.04018	.000	-18702.5067	-18691.7433
24th hour	0 hour	75651.4583*	2.04018	.000	75646.0767	75656.8400
	4th hour	40619.5000*	2.04018	.000	40614.1183	40624.8817
	8th hour	18697.1250*	2.04018	.000	18691.7433	18702.5067

*. The mean difference is significant at the 0.05 level.

j

5.5. Correlation between bacterial growth and histamine production

		Bacterial count	Histamine
Bacterial count	Pearson Correlation	1	.836**
	Sig. (2-tailed)		.000
	N	15	15
Histamine	Pearson Correlation	.836**	1
	Sig. (2-tailed)	.000	
	N	15	15

**. Correlation is significant at the 0.01 level (2-tailed).

5.6. Sum of biogenic amines (Histamine, Putrescine and cadaverine)

Hours	Control	Cardamom	Chlorine	Clove	Garlic	Oregano	Rosemary	Turmeric
1	2.531	2.378	1.116	1.4779	1.4825	2.0184	1.0185	4.158
4	8.1882	3.165	1.655	2.015	1.656	1.7347	1.431	4.158
8	11.8013	4.9105	2.038	3.446	3.5396	4.5939	3.345	6.2728
24	19.27	17.3597	24.414	4.068	4.1382	4.8227	4.596	16.303

Appendix C

6.1 Performa 1 for hedonic scaling (Texture)

The response to the properties of the material on the first bite:

Initial characteristics:

1. Wateriness: The release of water on compression: this is the initial response and is to be distinguished from juiciness - Scale points: 1, much less water released: 5 neither much nor less: 10, much more water released.
2. Firmness: The force required to compress the material between the molars or between the tongue and palate – Scale points: 1, much softer and less consistent; 5, neither soft nor firm; 10, much firmer and more consistent.
3. Elasticity: The ability of the material to return to its original shape after deformation. It is judged by compressing the substance slightly between the molars or between the tongue and the palate and noting to what extent the material returns to its original shape- Scale points:1,muchmore plastic;5,neither much nor less cohesive;10, much more cohesive.
4. Cohesiveness: The extent to which a material can be deformed before it rupture-Scale point: 1, much more cohesive; 5, neither much nor less cohesive; 10, much more cohesive.

The response to the properties of the material after chewing a few times:

Secondary characteristics:

1. Hardness: Resistance to breakdown on chewing to a state, suitable for swallowing-Scale points: 1, much more tender: 5, neither much nor less tender; 10, much tougher.
2. Juiciness: The sensation of a progressive increase of free fluids in the oral cavity during mastication-Scale points: 1, much drier; 4, neither much nor less juicier; 7, much juicier.

6.2. Performa for hedonic scaling (sensory)

Date ……Name……

Sample code	Odour	Appearance	Taste/Flavour	Overall acceptability scoring

6.3.ANOVA for cookloss between treatments

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	6	.000	2.044	.127
Within Groups	.001	14	.000		
Total	.001	20			

6.4. ANOVA for Protein lost during cooking

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.005[a]	13	.000	20.210	.000
Intercept	.149	1	.149	8144.867	.000
treatments	.002	6	.000	20.792	.064
duration	.002	1	.002	117.561	.000
treatments * duration	.000	6	6.2205	3.403	.012
Error	.001	28	1.8285		
Total	.154	42			
Corrected Total	.005	41			

a. R Squared = .904 (Adjusted R Squared = .859)

6.5. ANOVA for TPA between different concentrations and treatments

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	27.921[a]	13	2.148	.647	.794
Intercept	309200.892	1	309200.892	93167.810	.000
concentration	.368	1	.368	.111	.742
treatment	25.189	6	4.198	1.265	.305
concentration * treatment	2.364	6	.394	.119	.993
Error	92.925	28	3.319		
Total	309321.738	42			
Corrected Total	120.846	41			

a. R Squared = .231 (Adjusted R Squared = -.126)

6.6. ANOVA for hardness

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.018	4	.005	150.500	.000
Within Groups	.000	5	.000		
Total	.018	9			

6.7. Correlation bet gumminess and cohesiveness

		Gumminess	Cohesiveness
Gumminess	Pearson Correlation	1	1.000**
	Sig. (2-tailed)		.000
	N	10	10
Cohesiveness	Pearson Correlation	1.000**	1
	Sig. (2-tailed)	.000	
	N	10	10

**. Correlation is significant at the 0.01 level (2-tailed).

n

6.8. Multiple Comparisons for hardness

Tukey HSD

(I) VAR00005	(J) VAR00005	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
27.00	28.00	-.04000*	.00548	.004	-.0620	-.0180
	29.00	.06500*	.00548	.000	.0430	.0870
	30.00	-.04500*	.00548	.002	-.0670	-.0230
	31.00	.03500*	.00548	.007	.0130	.0570
28.00	27.00	.04000*	.00548	.004	.0180	.0620
	29.00	.10500*	.00548	.000	.0830	.1270
	30.00	-.00500	.00548	.881	-.0270	.0170
	31.00	.07500*	.00548	.000	.0530	.0970
29.00	27.00	-.06500*	.00548	.000	-.0870	-.0430
	28.00	-.10500*	.00548	.000	-.1270	-.0830
	30.00	-.11000*	.00548	.000	-.1320	-.0880
	31.00	-.03000*	.00548	.014	-.0520	-.0080
30.00	27.00	.04500*	.00548	.002	.0230	.0670
	28.00	.00500	.00548	.881	-.0170	.0270
	29.00	.11000*	.00548	.000	.0880	.1320
	31.00	.08000*	.00548	.000	.0580	.1020
31.00	27.00	-.03500*	.00548	.007	-.0570	-.0130
	28.00	-.07500*	.00548	.000	-.0970	-.0530
	29.00	.03000*	.00548	.014	.0080	.0520
	30.00	-.08000*	.00548	.000	-.1020	-.0580
32.00	27.00	.04500*	.00548	.000	-.0950	-.0530
	28.00	.04310	00548	.017	-.0070	-.0420
	29.00	.0036	00548	.014	-.0180	-.0570
	30.00	.04500	00548	.002	-.0940	-.0670
	31.00	.03000	00548	.007	-.0370	-.0970

*. The mean difference is significant at the 0.05 level.

6.9 ANOVA for odour

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	35.450	6	5.908	590.825	.000
Within Groups	.140	14	.010		
Total	35.590	20			

6.10. ANOVA for appearance

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	23.872	6	3.979	11.461	.000
Within Groups	4.860	14	.347		
Total	28.732	20			

6.11. P value of Multiple Comparisons between appearance and treatment

	Clove	Cardamom	Garlic	Turmeric	Oregano	Rosemary	Control
Clove		.995	.003	.232	.002	1.000	.095
Cardamom	.995		.001	.084	.001	.977	.031
Garlic	.003	.001		.258	1.000	.005	.527
Turmeric	.232	.084	.258		.151	.314	.997
Oregano	.002	.001	1.000	.151		.003	.345
Rosemary	1.000	.977	.005	.314	.003		.134
Control	.095	.031	.527	.997	.345	.134	

p

6.12. ANOVA for taste

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	14.118	6	2.353	137.259	.000
Within Groups	.240	14	.017		
Total	14.358	20			

6.13. Multiple Comparisons between taste and treatment (posthoc tukey test)

	Clove	Cardamom	Garlic	Turmeric	Oregano	Rosemary	Control
Clove		1.000	.000	1.000	.000	1.000	.960
Cardamom	1.000		.000	1.000	.000	1.000	.960
Garlic	.000	.000		.000	.232	.000	.000
Turmeric	1.000	1.000	.000		.000	1.000	.995
Oregano	.000	.000	.232	.000		.000	.000
Rosemary	1.000	1.000	.000	1.000	.000		.960
Control	.960	.960	.000	.995	.000	.960	

Appendix D

7.1. ANOVA moisture

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1347.193	6	224.532	.962	.484
Within Groups	3266.019	14	233.287		
Total	4613.213	20			

7.2. ANOVA for protein loss

		Sum of Squares	df	Mean Square	F	Sig.
protein	Between Groups	2.763	6	.461	6.568	.002
	Within Groups	.982	14	.070		
	Total	3.745	20			

7.3. ANOVA pH 0^{th} month

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.937	7	.277	454.826	.000
Within Groups	.010	16	.001		
Total	1.947	23			

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7.4. Multiple Comparisons for pH 0th month

	Control	Clove	Garlic	Turmeric	Chlorine	Rosemary	Oregano	Chlorine
Control		.000	.000	.000	.000	.000	.000	.000
Clove	.000		.001	.010	.000	.010	1.000	.933
Garlic	.000	.001		.000	.000	.877	.002	.000
Turmeric	.000	.010	.000		.000	.000	.004	.090
Chlorine	.000	.000	.000	.000		.000	.000	.000
Rosemary	.000	.010	.877	.000	.000		.026	.001
Oregano	.000	1.000	.002	.004	.000	.026		.713
Chlorine	.000	.933	.000	.090	.000	.001	.713	

7.5. ANOVA pH 6th month

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.818	7	.117	105.796	.000
Within Groups	.018	16	.001		
Total	.835	23			

7.6. Multiple Comparisons for pH 6th month

	Control	Clove	Garlic	Turmeric	Cardamom	Rosemary	Oregano	Chlorine
Control		.394	.000	.000	.460	.004	.603	.810
Clove	.394		.000	.000	1.000	.232	1.000	.994
Garlic	.000	.000		.946	.000	.000	.000	.000
Turmeric	.000	.000	.946		.000	.000	.000	.000
Cardamom	.460	1.000	.000	.000		.191	1.000	.998
Rosemary	.004	.232	.000	.000	.191		.127	.065
Oregano	.603	1.000	.000	.000	1.000	.127		1.000
Chlorine	.810	.994	.000	.000	.998	.065	1.000	

7.7. ANOVA 6th month moisture

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.488	7	.498	9.334	.000
Within Groups	.854	16	.053		
Total	4.342	23			

7.8. Multiple Comparisons for 6th month moisture

	Control	Clove	Cardamom	Oregano	Garlic	Turmeric	Rosemary	Chlorine
Control		.805	1.000	.032	.930	.000	.679	.710
Clove	.805		.778	.396	1.000	.002	1.000	1.000
Cardamom	1.000	.778		.029	.914	.000	.648	.679
Oregano	.032	.396	.029		.252	.156	.523	.492
Garlic	.930	1.000	.914	.252		.001	.999	1.000
Turmeric	.000	.002	.000	.156	.001		.004	.003
Rosemary	.679	1.000	.648	.523	.999	.004		1.000
Chlorine	.710	1.000	.679	.492	1.000	.003	1.000	

7.9. ANOVA for Thaw drip between duration and treatments

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.780[a]	15	.052	211.847	.000
Intercept	16.877	1	16.877	68744.522	.000
Duration	.520	1	.520	2119.824	.000
Treatment	.165	7	.024	95.847	.000
Duration * Treatment	.095	7	.014	55.279	.000
Error	.008	32	.000		
Total	17.665	48			
Corrected Total	.788	47			

a. R Squared = .990 (Adjusted R Squared = .985)

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7.10. ANOVA between treatment and bacterial counts each month

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	172.014[a]	47	3.660	447.441	.000
Intercept	3323.302	1	3323.302	406292.964	.000
VAR00001	23.247	7	3.321	406.017	.000
VAR00002	132.424	5	26.485	3237.923	.000
VAR00001 * VAR00002	16.343	35	.467	57.085	.000
Error	.785	96	.008		
Total	3496.101	144			
Corrected Total	172.799	143			

a. R Squared = .995 (Adjusted R Squared = .993)

1.1. Introduction

Food microbial spoilage and poisoning is a problem that has not yet been brought under adequate control, despite the range of preservation techniques available these days. All foods begin to spoil as soon as they are harvested or slaughtered and microorganisms cause a large share of the spoilage. It also results from chemical changes within the food itself due to natural processes such as enzyme action or oxidation. Food preservation systems such as heating and refrigeration can be used to reduce the risk of outbreaks of food poisoning. However, these techniques have associated adverse changes in organoleptic characteristics and loss of nutrients. Within the disposable arsenal of preservation techniques, the food industry keeps on investiging more on new preservation techniques due to the increased consumer demand for tasty, nutritious, natural and easy-to-handle food products. Improvements in the cold distribution chain have made international trade of perishable foods possible, but refrigeration alone cannot assure the quality and safety of all perishable foods.

In many foods, antimicrobials are required to maintain food quality, ensure safety and extend shelf life. A number of compounds are approved by international regulatory agencies for the use as direct food antimicrobials. Chemicals commonly added to food to prevent spoilage include benzoic acid, sorbic acid, and sulfur dioxide. Antioxidants such as butylated hydroxyanisole and ascorbic acid prevent compounds in food from combining with oxygen to produce inedible changes. The use of chemical additives has not been without controversy. The spread of often unnecessary and sometimes harmful chemical additives to food during the late 1800s led to governmental regulation in both England and the United States.

Today, in spite of modern improvements in food production techniques, food safety is still an increasingly important public health issue. It has been estimated that as many as 30% of people in industrialized countries suffer from a food borne disease each year. In 2000 at least two million people died from diarrhoeal disease worldwide (WHO, 2002a). Therefore, there is still a need for new methods of reducing or eliminating food borne pathogens, possibly in combination with existing methods. At the same time, Western society appears to be experiencing a trend of 'green' consumerism (Smid and Gorris, 1999), desiring fewer synthetic food additives and products with a smaller impact on the environment. Moreover, the World Health Organization has already called for a worldwide reduction in the consumption of salt in order to reduce the incidence of cardio-vascular disease (WHO, 2002b). If the level of salt in processed foods is reduced, it is possible that other additives will be needed to maintain the safety of foods. Hence, there is scope for new methods of making food safe which have a natural or 'green' image (Rasooli, 2007).

An increasing number of consumers prefer minimally processed foods, prepared without chemical preservatives. Many of these ready-to-eat and novel food types represent new food systems with respect to health risks and spoilage association. Against this background, and relying on improved understanding and knowledge of the complexity of microbial interactions, recent approaches are increasingly directed towards possibilities offered by biological preservation.

Throughout the development of both Western and Eastern civilization, plants, plant parts, and derived oils and extracts have functioned as sources of food and medicine, symbolic articles in religious and social ceremonies, and remedies to modify behavior. Taste and aroma not only determine what we eat but often allow us to evaluate the quality of food and, in some cases, identify unwanted contaminants. The principle of self-limitation taken together with the long history of use of natural flavor complexes in food argues that these substances are safe under intended conditions of use.

Until recently, spices have been studied most from the viewpoint of their flavor and fragrance only for flavoring foods, drinks and other goods. However, spices and their components are gaining increasing interest because of their relatively safe status, their wide acceptance by consumers, and their exploitation for potential multi-purpose functional use. It has long been recognized that some essential oil in spices have antimicrobial properties which contributes to the self-defense of these plants against infectious organisms. These have been reviewed in the past but, the relatively recent enhancement of interest in 'green' consumerism has lead to a renewal of scientific interest in these substances. Besides antibacterial properties, spices or their components have been shown to exhibit antiviral, antimycotic, anti oxidative, antitoxigenic, antiparasitic and insecticidal properties. These characteristics are possibly related to the function of these compounds in plants. The antibacterial properties of essential oil and their components are exploited in such diverse commercial products as dental root canal sealers, antiseptics and feed supplements for lactating sows and weaned piglets. It is therefore scientifically sound to evaluate the impact of spices on food and food products safety.

The antibacterial action of spices will be most effective in reducing the risk factor associated with the consumption of uncooked food or food items that had not gone through any kind of processing. These food products are perishable by nature and require protection from spoilage during their storage and distribution to give them desired shelf-life. Because food products are now often sold in areas of the world far distant from their production sites, the need for extended safe shelf-life for these products has also expanded. Among the raw unprocessed food, raw seafood items impose a greater threat compared to fruits and vegetables.

Recently, It was reported that tuna product is the source of the *Salmonella bareilly* outbreak that has sickened 116 across 21 states in USA. Of the 53 outbreak victims interviewed, 43 (81 percent) reported eating sushi in the week preceding their illnesses. Of

the 43 who recalled eating sushi, 39 (91 percent) ate sushi that contained tuna (The Wall Street Journal, 2012). In addition to this many species of marine finfish from the families Scombridae and Scomberesocidae primarily tuna, mackerel, sauries and other dark-fleshed finfish, which contains a high amount of free histidine, are the major culprits in histamine food poisoning.

Despite of all these havocs, tuna, commonly called as 'the chicken of the sea', are of high demand in the world market. Presently, nearly 80 nations harvest tuna from the oceans of the world. Tunas are among the largest and most specialized and commercially important of all fishes. They are the fourth major internationally traded fish resource and contribute 7.6% of the international fish trade in value terms (Thomas, 2008). The Indian Ocean contributes 19% of the world tuna catch. Chilled tuna is the highest unit value earning item, while larger quantities of tuna are exported in frozen form. For tuna, Japanese and USA markets determine quality and thus price. For instance the most discriminating Japanese Sushi bars look for tuna with a bright red colour, firm texture, translucency and fatty content. Some of the major tuna products include canned tuna, smoked tuna and flavoured light tuna. The flavouring is done mostly with lemon, pepper, tomato, onion and chilly.

Maintaining the quality of the tuna is a process that can never be reversed or improved. For fresh tuna loins frozen, the speed of the processing again determines the quality. Once thawed the firmness of the tuna next to microbiological and chemical parameters give indications of the quality of frozen loins. In poor quality tuna, the muscle tissue peels like paper. A good quality tuna steak due to processing and raw material characteristics should have a Total plate count of 10000-50000.

Preservation of tuna by chilling temperatures is only a temporary method and freezing is currently the best way to preserve tuna or any other fish for a longer time.

Freezing kills most organisms but not the psychrophiles and the spores. Most of these survive freezing and grow during thawing. Salting and drying method of fish preservation is an age old custom. Sodium chloride serves both as a chemical preservative as well as binds the moisture. This may, however, not affect the halophiles which sometime cause discolouration during drying. Other chemicals used in fish preservation to retard microbial activity include sodium and potassium nitrite and nitrate, benzoate and sorbate. A dip in antibiotic solutions has been tried and found' successful but these days it is not encouraged.

It is in this situation that the importance of natural antimicrobials in maintaining the quality of the tuna comes in. If spices are released in tuna products at a controlled rate to deliver effective inhibitory concentrations over extended periods it can thereby extend shelf-life. Consumer acceptance of the product is the limiting factor here. But, raw sashimi grade tuna is normally consumed with a pungent extract from roots of a plant called wasabi. Flavoured tuna products is also available in the market which utilizes spices to impart flavour. This fact opens up an opportunity to explore the antibacterial effect of spices on tuna. This study is intended to evaluate the possible applications of spice oleorsins in protection of tuna from bacterial spoilage. The effect of clove, turmeric, cardamom, oregano, rosemary and garlic in increasing the shelf–life is investigated.

1.2. Major objectives

- **To isolate and identify the spoilage and pathogenic bacteria present on tuna.**
- **To study the antimicrobial effect of spice extracts on pathogenic and spoilage bacteria present on tuna.**
- **To study the effect of spice treatment on the bacterial cells.**
- **To study the effect of spice extracts on inhibition of biogenic amine formation in fresh fish**
- **To study the textural characteristics of treated and cooked samples of tuna.**
- **To study the effect of spices on the quality and storage stability of tuna.**

2.1. Introduction

Food preservation for future consumption is one of the oldest practical arts. It enabled to man settle down in one place and put an end to the never ending hunt for fresh food. Techniques for drying foods date back to ancient times, when fruits and vegetables were dried in the sun. The dehydrated foodstuffs would not support microorganisms and therefore did not spoil without the presence of water.

Each culture preserved their local foods by using the basic preservation techniques. The Chinese were using salt and spices, by 1000 BC, to create a sterile environment for different food products. In the present scenario, new food products are being introduced into the market with a lot of additives.The producers of all food products aims at a longer shelf lives and greater assuarance of freedom from foodborne pathogenic organisms. These cannot be achieved without the help of preservatives. Now a days, chemical preservatives are commonly added to prevent spoilage. However, consumers have developed suspicion to the use of chemical additives because of their potential toxicity.

The search for new substances to be used in food preservation is hampered by regulatory restrictions and a great deal of time and money may be required to develop a new chemical preservative and get it approved. Such obstacles provide new opportunities for those seeking alternative routes for food preservation. In such a situation, there is considerable interest in the possible use of natural alternatives as food additives. They can be used to prevent the growth of food borne pathogens or to delay the onset of food spoilage. More over use of natural antimicrobials will produce 'green' labels, for the resulting products. Many naturally occurring compounds, such as phenols and organic acids have been considered in this context.

2.2. Plant extracts and their major components

Plants are poorly exploited sources of alternative antimicrobial agents whose structure and modes of action may well differ from those derived from the more well known sources (Mitscher, 1975). Among natural preservatives from plants, spices and condiments are most important in improving the palatability of meat and fish products. Moreover, they are used widely in the food industry as flavours and fragrances. Spices also exhibit useful antimicrobial and antioxidant properties (Edris, 2012). Many plant-derived antimicrobial compounds have a wide spectrum of activity against bacteria, fungi and mycobacteria and this has led to suggest that they could be used as natural preservatives in food (Farag *et al*., 1989a).

Components present in intact plants include alkaloids, dienes, flavonols, flavones, glycosides, lactones, organic acids, phenolic compounds and protein like compounds (Lopez-Malo *et al*., 2000). Post infection inhibitors may include isothiocyanates, phenolic compound, phytoalexins and sulfoxides. Compounds from spices and their essential oils are of greatest potential as food antimicrobials.

2.3. Antibacterial activity

Many spices and herbs and extracts possess antibacterial activity due to the essential oil fraction (Deans and Ritchie, 1987). Thus the essential oil of oregano, thyme, sage, rosemary, clove and coriander exhibit antibacterial activity to food borne pathogens (Salmeron *et al*., 1990; Paster *et al*., 1990; Farag *et al*., 1989a; Aureli *et al*., 1992; Stecchini *et al*., 1993).

Gram positive bacteria, Gram negative bacteria ,yeasts and moulds (Mandeel *et al*., 2003; Ayar *et al*., 2004; Lee *et al*., 2008; Mishra and Sree, 2008; Tayung and Rath, 2008) are all affected by wide range of essential oils. Well known examples include the essential

oils from bay, blackpepper, caraway, clove, turmeric, coriander, cumin, red chilli, fennel, nutmeg, majoram, licorice, garlic, lemon, onion, orange, oregano, rosemary, thyme, sage, cardamom and ginger (Mandeel *et al*., 2003; Ayar *et al*., 2004; Nolan and Labbe, 2004; Al-Turki *et al*., 2008; Rasooli *et al*., 2008; Farooke *et al*., 2009).

Inhibitory action of thyme, mint and laurel ground leaves and their extracts on *S. aureus, S. typhimurium* and *V. parahaemolyticus* was reported by Aktug and Karapinar (1986). Several studies showed that cinnamon, clove, pimento, thyme, oregano and rosemary had strong and consistent inhibitory effect against several pathogen and spoiling bacteria (Shelef *et al*., 1980; Aureli *et al*., 1992).

Pandit and Shelef (1994) tested the antilisterial effect of 18 spices and observed significant inhibitory effect of rosemary ($\geq$ 5% w/v) and clove ($\geq$ 1% w/v) on *L. monocytogenes*. Outara *et al*. (1997) also reported antimicrobial activity of many spices. He classified their activities as strong, medium, or weak. According to him, *Brochothrix thermosphacta* was inhibited by cinnamon, clove, garlic and rosemary essential oil (1/100 v/v); *Serratia liquefaciens* by cinnamon, clove, garlic, pimento and rosemary essential oil (1/100 v/v); *Carnobacterium piscicola* by cinnamon, clove, pimento and rosemary essential oil (1/100 v/v); *Lactobacillus sake* by cinnamon, clove, black pepper, pimento and rosemary essential oil (1/100 v/v) and *L. curvatus* by black pepper and pimento essential oil (1/100 v/v).

Arora and Kaur (1999) studied the antimicrobial activity of garlic, ginger, clove, black pepper and ground green chilli and their aqueous extracts on human pathogenic bacteria including *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Shiguella flexneri* and found that all tested bacteria were sensitive to ground garlic and its extract. Moreover, garlic extract showed considerable cidal effect on *S. typhi*.

The effectiveness of cardamom, anise, basil, coriander, rosemary, parsley, dill and angelica essential oil for controlling the growth and survival of pathogenic and saprophytic microorganisms was reported by Elgayyar *et al*. (2001). The results showed inhibitory property for oregano, basil and coriander essential oil, which presented minimum lethal concentration (v/v) ranging between 8 and 50ppm for *Pseudomonas aeruginosa* and *Stahylococcus aureus*. The inhibitory effect of methanolic extracts of seven Turkish spices on *E. coli* 0157:H7 was assayed by Sagdic *et al*. (2003a). In their study oregano (1.0, 1.5 and 2.0 v/v) had showed prominent results as bactericidal in both paper disc and agitated liquid culture assay.

Medicinal plants used by tribes all over the world are being analysed for their antimicrobial potential by scientists. It is established by scientists that even little known plants like rosinweeds inhibit bacterial strains of *Staphylococcus aureus* and *Escherichia coli*. It is also proved that the crude extracts of *Dorsenia barteri* have bactericidal effects (Mbaveng *et al*., 2008). The individual components of essential oil are also evaluated for their influence on airborne microbes and the average reduction of 59.4% germ was noted for some components like terpineol (Krist *et al*., 2008).

Exact mechanism of antibacterial action of spices and derivatives is not yet clear (Lanciotti *et al*., 2004). Some of the major hypothesis regarding the action of spices are: a) perturbation of membrane permeability consequent to its expansion and increased fluidity causing the inhibition of membrane embedded enzymes (Cox *et al*., 2000); b) hydrophobic and hydrogen bonding of phenolic compounds to membrane proteins, followed by partition in the lipid bilayer (Juven *et al*., 1994); c) membrane disruption (Caccioni *et al*., 2000); d) destruction of electrons transport systems (Tassou *et al*., 2000); e) cell wall perturbation (Odhav *et al*., 2002).

Generally, gram-negative bacteria have been reported to be more resistant than Gram-positive to essential oils antimicrobial effect because of their cell wall lipopolyssaccharide (Russel, 1991). Cell wall lipopolyssacaride may prevent that essential oils active compounds reach the cytoplasmic membrane of Gram-negative bacteria (Chanegriha *et al*., 1994).

2.4. Antifungal activity

Antifungal activity of spices and its derivatives has been studied regarding viable cells count, mycelial growth and mycotoxins synthesis. The inhibitory effect of various concentrations of mint, sage, bay, anise and ground red pepper (0.5, 1.0, 2.0, 4.0, 8.0, 16.0% w/v) on the growth of *Aspergillus parasiticus* and its aflatoxin production was analysed by Karapinar (1985). He reported that only thyme presented significant delay on the fungal growth up to 10 days at 2.0%.

Akgul and Kivanç (1988) studied antifungal activity of selected Turkish spices on some foodborne fungi. They reported that oregano ground (1.0, 1.5, 2.0 % w/v) and its essential oil (0.05%, 0.025%) showed inhibitory effect on *Aspergillus flavus, A. niger, Geotrichum candidum, Mucor spp.* and *Penicillium roqueforti*. Oregano essential oil exhibited higher inhibitory effect than sorbic acid.

Thyagaraja and Hosono (1996) assayed the antifungal activity of chilli, coriander, pepper, cumin and asafoetida in inhibiting food spoilage moulds and asafoetida showed promising results in inhibiting the fungal growth. Adam *et al*. (1998) tested the antifungal activity of essential oils from oregano, sage, lavender and mint on human pathogen fungi and found inhibitory action on *Malassezia furfur, Trichophyton rubrum* and *Trichosporum beigelii*.

Basilico and Basilico (1999) studied the inhibitory effect of oregano, mint, basil, sage and coriander on the mycelial growth of *Aspergillus ochraceus* and its ochratoxin synthesis. The results showed that oregano (750 ppm) completely inhibited the fungal growth and ochratoxin A synthesis upto 14 days at 25°C. Basil (750 ppm) was effective to inhibit the mycelial growth up to 7 days.

The sensitivity of yeasts to spices aqueous extracts was assayed by Arora and Kaur (1999). They found that garlic and clove extract were able to inhibit *Candida acutus, C. albicans, C. apicola, C. catenulata. C.inconspicua*, *C.tropicalis*, *Rhodotorula rubra, Sacharomyces cerevisae* and *Trignopsis variabilis* and in some cases strong cidal effect was observed. Grohs and Kunz (2000) observed that mixtures of ground spices (2 and 5% w/v) were effective in inhibiting the growth of *Candida lipolytica*.

Elgayyar *et al*. (2001) observed that anise essential oil was highly inhibitory on *Aspergillus niger, Geotrichum* and *Rhodotorula*, although it was not active on bacteria. Benkeblia (2004) observed inhibitory effect of onion essential oil at concentrations 50, 100, 200, 300 and 500ml/L on *Fusarium oxysporum, Aspergillus niger* and *Penicillium cyclopium*.

Juglal *et al*. (2002) studied the effectiveness of nine essential oils to control the growth of mycotoxins-producing moulds and noted that clove, cinnamon and oregano were able to prevent the growth of *Aspergillus parasiticus* and *Fusarium moniliforme*. It was reported that clove markedly reduced the aflatoxin synthesis in infected grains. These findings could be useful for rural communities to prevent the synthesis of fungal toxins in contaminated grains by simple measures.

Little information on spices and derivatives action on/in the fungal cell is known in order to promote fungistatic or fungicide effect. Inhibitory action of natural products on mould involves cytoplasm granulation, cytoplasmic membrane rupture and inactivation or

inhibition of intercellular and extracellular enzymes. These biological events could take place separately or concomitantly culminating with mycelium germination inhibition (Cowan, 1999). Also, Brull and Coote (1999) reported that plant lytic enzymes act in the fungal cell wall causing breakage of α-1,3 glycan, α-1,6 glycan and chitin polymers

2.5. Food preservation using Spices

Controlling the growth of spoiling and pathogenic food-related microorganisms helps in achieving food conservation. By suppressing one or more essential factors for microbial survival, microbial control in food could be assured (Horace, 1982). This is possible by adding suitable substances like weak organic acids, hydrogen peroxide, chelators and organic biomolecules. Applying physical (temperature, packaging) and/or chemical procedures (pH, oxide-reduction potential, osmotic pressure) is also advisable for reducing bacteria (Brull and Coote, 1999). The microorganisms could be killed or made unviable by these procedures.

There has been increasing concern by the consumers about foods free or with lower level of chemical preservatives because these could be toxic (Bedin *et al*., 1999). Concurrently, consumers have also demanded for foods with long shelf life and absence of risk of causing foodborne diseases. This perspective has put pressure on the food industry for progressive removal of toxic chemical preservatives and adoption of natural alternatives to obtain its goals concerning microbial safety. This resulted in increasing search for new technologies for use in food conservation systems, which include combined effect of underlethal procedures, alternative antimicrobial compounds, combination of conventional and alternative antimicrobials (Brull and Coote, 1999).

Resistance to classic antimicrobial agents

Uncontrolled use of chemical antimicrobial preservatives has been an inducing factor for appearance of microbial strains more and more resistant to classic antimicrobial agents. Difficulty to control the microbial survival, as shown by isolation of multi-resistant strains, has been reported all over the world. Fifty years of increasing use of chemicals antimicrobials have created a situation leading to an ecological imbalance and enrichment of multiples of multi-resistant pathogenic microorganisms (Levy, 1997). Antibiotic resistance in foodborne pathogens is a fact, though substantial qualitative and quantitative differences have been observed (Teuber, 1999a,b). Formation of resistant foodborne pathogens to a variety of antimicrobials have become a major health concern (Kiessling *et al*., 2002) and it could decrease the successful application of control measures on spoilage and pathogen microorganisms and leads to use of less safe, ineffective or expensive alternatives (Levy, 1997).

Changes in/on the antimicrobial target, inactivation by enzymes, changes in cellular permeability, antimicrobial active efflux and over production of target enzymes have been common mechanisms of antimicrobial resistance (McKeegan *et al*., 2002). Brull and Coote (1999) have reported microbial resistance for some antimicrobials used in food conservation like weak-organic acids, hydrogen peroxide, chelators and small organic biomolecules.

Spices as antimicrobial agent in food

The successful story of microbial chemocontrol lies in the continuous search for new antimicrobial substances to control the challenge posed by resistant strains (Notermans and Hoogenboon, 1992). Essential oils present in spices provide a gold mine in this aspect. Rosemary and its essential oil were useful for preservation of pork sausage during storage at 5°C for 50 days. Spices mixtures were able to inhibit the growth of various meat-spoiling microorganisms (*Bacillus subtillis*, *Enterococcus* spp., *Staphylococcus* spp., *E. coli* K12

and *Pseudomonas fluorescens*) providing stabilizing effect on colour and smell of fresh portioned pork meat (Grohs and Kunz, 2000).

Effect of spice combinations including cumin (*Cuminum cyminum*), coriander (*Coriandrum sativum*), mustard (*Brassica juncea*), black pepper (*Pipper nigrum*) and lemon (*Citrus aurantifolia*) on *V. parahaemolyticus, S. aureus, S. typhi* and *E. coli* count in fish sauce showed that the spices mixtures were able to exert static effect on all assayed bacteria when in interaction with an initial inoculum of 1.0×10^4 cfu/ml, except on *S. typhi* (Al-Jedah *et al*., 2000).

Leuschner and Zamparini (2002) studied the growth and survival of *E.coli* O157 and *Salmonella enterica* serovar *enteridis* in mayonnaise in the presence of garlic, ginger, mustard and ground clove. Garlic (1% w/v) showed bacteriostatic and clove (1% w/v) showed bactericidal effect towards *S. enterica* and *E. coli* O157. These results are significant since *E. coli* 0157 and *S. enterica* serovar *enteridis* are emerging as foodborne pathogens that have significant impact on the food industry. Moreover, these bacteria present various undesirable attributes of virulence that in combination make them cause some of the most serious threats for food safety (Proctor and Davis, 2000).

2.6. Active components in spice extracts

2.6.1. Clove

Clove (*Syzygium aromaticum* L.) is one of the most ancient and valuable spices of the Orient. It is a member of the family *Myrtaceae*. The clove of commerce is its dried unopened flower buds.

Botany

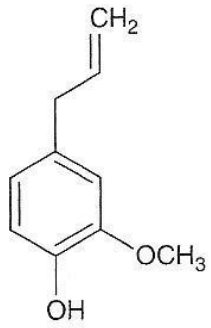
Clove is a medium-sized tree, which grows to a height of 10-20m, which can live up to 100 years or more. The bark is grey; the leaves are elliptical in shape and fragrant with crimson flowers. The flowers are hermaphrodite with a fleshy hypanthium surrounded by sepals. The fruit is a purple drupe, about 2.5 cm long (Fig. 2.1).

Fig 2.1. Dried clove buds

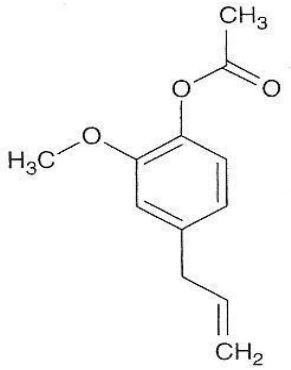
Chemistry

Good quality clove buds constitute 15-20% of essential oil (Gopalakrishnan and Narayanan, 1988; Pino *et al*., 2001; Zachariah *et al*., 2005). The oil is dominated by eugenol

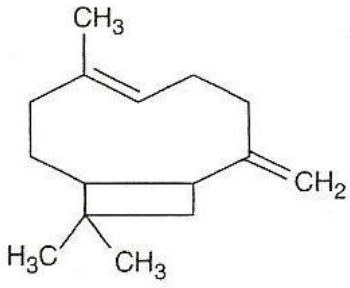
(70-85%), eugenyl acetate (15%) and β-caryophyllene (5-12%), which together make up 99% of the oil. The oil also include methylamylketone, methylsalicylate, alpha–and beta-humulene, benzaldehyde and chavicol. The minor constituents like methyl amyl ketone, methylsalicylate etc., are responsible for the characteristic pleasant odour of cloves. Clove contain 10-13% tannin. Eugeniin, ellagitannin, maslinic acid, triterpene and oleanolic acid were isolated from cloves by researchers (Nonaka *et al*., 1980; Narayanan and Natu, 1974; Brieskorn *et al*., 1975). The chemical structures of major compounds are given in Fig. 2.2.



Eugenol



Eugenyl acetate



Caryophyllene

Fig. 2.2. Chemical structures of major constituents in clove bud

Uses

Medicinal and Pharmacological Uses

India's traditional Ayurveda healers have used clove since ancient times to treat respiratory and digestive ailments. Like many culinary spices, cloves help to relax the smooth muscle lining of the digestive tract and eating cloves is said to be aphrodisiac. Aqueous extract of clove flower bud inhibits immediate hypersensitivity in rats by inhibition of histamine release from mast cells in *vivo* and *in vitro* (Kim *et al*., 1998).

Cloves are one of Mother Nature's premium antiseptics. A few drops of the oil in water can stop vomiting and an infusion relieves nausea. In traditional Chinese medicine, it is used to treat indigestion, diarrhea, hernia, ringworm and other fungal infections. It is used as a kidney tonic, to warm the body, increase circulation and as a digestive aid. They are also used for flatulence, hiccups, stomach chills, fever, toothache and cholera. The medieval German herbalists used cloves as part of an antigout mixture. Clove oil is an active ingredient in several mouthwash products and a number of over-the-counter toothache pain-relief preparations. It is also used to disinfect root canals.

Antimicrobial Activity

Clove exhibits potent antimicrobial activity against *Bacillus subtilis, Escheriachia coli* and *Saccharomyces cerevisiae* (De *et al*., 1999). Essential oils from clove and eugenol show various degrees of inhibition against *Aspergillus niger, S.cerevisiae, Mycoderma sp., Lactobacillus acidophilus* and *B. cereus*, as estimated by the paper disc agar diffusion method (Meena and Sethi, 1994). The oil also inhibits the growth of *Fusarium verticilloids* (Veluti *et al*., 2004). Clove oil (1% v/w) inhibits *Listeria monocytogenes* in chicken frankfurters (Mytle *et al*., 2006). It has excellent antimicrobial properties and is used in food preservation (Smith Palmer *et al*., 2001).

Essential oil of clove is effective against *Streptococci, Staphylococci and Pneumomocci* bacteria. The volatile oils of clove exhibited considerable inhibitory effects and antibacterial activity against several genera of bacteria, including animal and plant pathogens and food poisoning and spoilage bacteria (Deans and Ritchie, 1987; Dorman and Deans, 2000).

Clove kills intestinal parasites and exhibits broad antimicrobial properties, thus supporting its traditional use as a treatment for diarrhea, intestinal worms and other digestive ailments. Clove essential oil is strongly antimicrobial, antiseptic, haemostatic and anti-inflammatory. Because of its strong antiparasitic action, clove is also included in Dr. Huda Clark's protocol for elimination of parasites from the digestive system. It has also been found that a 0.05% solution of eugenol is sufficient to kill *B. tuberculosis*. Clove oil showed antimicrobial activity against some human pathogenic bacteria resistant to certain antibiotics (Arora and Kaur, 1999; Lopez *et al*., 2005).

Antifungal activity

Clove oil and eugenol were reported to possess significant antifungal activity against rye bread spoilage fungi (Suhr and Nielsen, 2003). Clove oil shows antifungal activity against the fungi belonging to *Eurotium, Aspergillus* and *Penicillium* species, commonly causing deterioration of bakery products (Guynot *et al*., 2003). Eugenol possesses antifungal activity against *Cladosporium herbarum, Penicillium glabrum, P. expansum* and *A. niger (*Martini *et al., 1996;* Kong *et al., 2004).*

Clove bud oil causes inhibition of both mycelial growth and aflatoxin production of *A. parasiticus* (Farag *et al*., 1989b; Gowda *et al*., 2004). Clove oil, at concentrations > 100 µg/ml, resulted in reduction in the aflatoxin production in liquid cultures (Sinha *et al*., 1993).

Clove oil inhibits the growth and production of fumonisin B1 by *F. proliferatum* (Veluti *et al*., 2003).

Other effects of clove

Clove is a potent antiviral agent and eugenin isolated from clove buds showed antiviral activity against *Herpes simplex* virus at a concentration of 10 microlitre/ml (Chaieb *et al*., 2007). Clove has other important properties like antioxidant activity (Gulcin *et al*., 2004; Jirovetz *et al*., 2006), antithrombotic activity (Srivastava, 1990)**,** anticancerous activity (Zheng *et al*., 1992), antipyretic effect (Feng and Lipton, 1987), anti-inflammatory activity (Ghelardini *et al*., 2001) etc. The spice clove and its value added products are used extensively for flavouring food and confectionery. Clove oil has many industrial and pharmacological applications. Most of the studies conducted so far pertain to the clove volatiles. Their importance as antiviral and antibacterial agents can be utilized in food industries.

2.6.2. Cardamom

Small cardamom, popularly known as 'Queen of spices', is the dried fruit (Fig.2.3) of the tall perennial herbaceous plant, *Elettaria cardamomum*, belonging to the family Zingiberaceae. In India, cardamom is cultivated in the southern states of Kerala, Karnataka and Tamil Nadu.

Botany

It is a shade loving plant cultivated at an altitude of 600 to 1200 m above Mean Sea Level. It requires an annual rainfall of 1500 to 4000 mm and a temperature range of 10 to 35°C. Cardamom is indigenous to the evergreen rainforests of western ghats of Southern India. The genus belongs to the natural order Scitaminae, family Zingiberaceae under monocotyledons.

Fig 2.3. Cardamom pods

Chemistry

The early work of several authors, summarized by Guenther (1975) and Iyer *et al*. (2009), showed the presence of 1,8-cineole, d-alpha-terpineol, terpinyl acetate, limonene, sabinene and borneol. The first detailed analysis of the volatile oil of cardamom was reported by Nigam *et al*. (1965). 1,8-cineole and alpha-terpinyl acetate are the major components in the cardamom volatile oil (Fig.2.4). Besides the usual terpene hydrocarbons and alcohols as minor compounds and the dominance of 1,8-cineole and alpha terpeneacetate, it is significant that methyl eugenol also has been identified (Lawrence, 1979).

The basic cardamom aroma is produced by a combination of the major components. The percentages of the main components given by Lawrence (1979) are as follows: alpha-pinene(1.5%), beta-pinene(0.2%), sabinene(2.8%), myrcene(1.6%), alpha phellandrene (0.2%), limonene (11.6%), 1,8-cineole (36.3%), gamma-terpinene(0.7%), p-cymene(0.1%), terpinolene(0.5%), linalool (3.0%), linalyl acetate (2.5%) and terpinen-4-ol(0.9%).

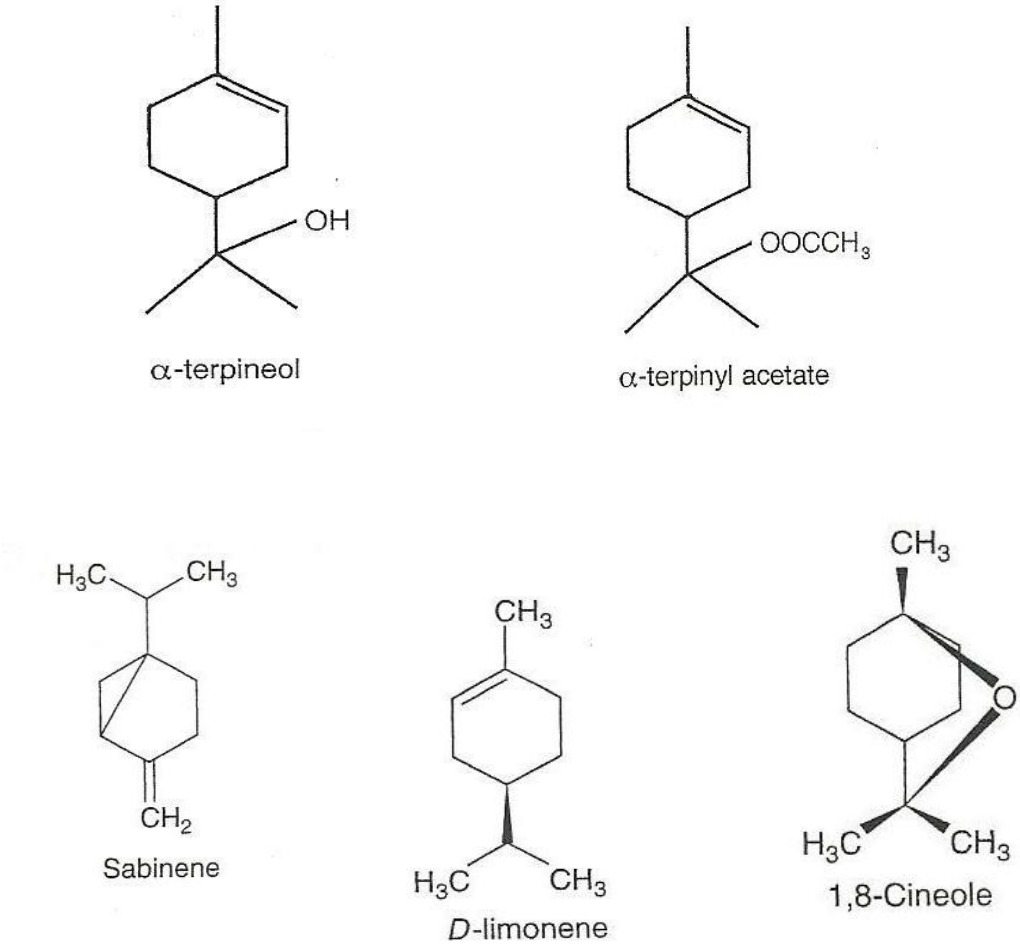


Fig 2.4. Chemical structures of major components in cardamom essential oil

Uses

Medicinal and Pharmacological properties

Cardamom essential oil traditionally was used as a tonic to the digestive system. Cardamom oil may relieve spasm, possibily making it beneficial for colitis, irritable bowel syndrome, indigestion and cramps.It may be of benefit where the digestive system is

affected by the nervous tension. In addition, cardamom oil can relieve nausea and may be useful for morning sickness in pregnancy.

Cardamom is a strong tonic and stimulant, is stomachic and carminative and, to a lesser degree, is listed as a neuromuscular antispasmodic.It is also reported as anti-inflammatory and analgesic (Al-Zuhair *et al*., 1996) and is effective against post-operative nausea and vomiting.

Antimicrobial activity

Extract of cardamom seed displays a variable degree of antimicrobial activity on different microorganisms. Assays indicate that cardamom seed has inhibitory activity on *Mycobacterium smegmatis, Klebsiella pneumoniae, Staphylococcus aureus, Enterococcus faecalis,Micrococcus luteus* and *Candida albicans* (Agaoglu *et al*., 2005). However no inhibitory activity was observed against *Pseudomonas aeruginosa*.The anti-microbial effect of the oil was tested against nine bacterial strains, one fungus and one yeast.The oil was 28.9% as effective as phenol,with minimal inhibitory concentration of 0.7mg/ml (Badei *et al*., 1991; Kubo *et al*., 1991).

The antimicrobial activity of spice extracts including cardamom was investigated by Mishra and Behal (2010). They reported that the crude aqueous and alcoholic extract showed broad antimicrobial activity against bacteria and fungi. Antimicrobial activity against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeroginosa* by volatile components of cardamom was observed by Kubo *et al*. (1991).

Other properties

Antioxidant effect (Badei *et al*., 1991), antiinflammatory activity (Al-Zuhair *et al*., 1996) and insecticidal activity (Jacobson, 1989; Shayya *et al*., 1991; Huang *et al*., 2000) were also reported. Cardamom seeds are used widely for flavouring purposes in food and as

carminative. Despite their numerous application in the cooking styles of Srilanka, India and Iran, 60% of the world production is exported to Arab countries and North Africa, where the largest percentage is used to prepare coffee.

2.6.3. Garlic

Garlic (*Allium sativum* L.*)* is the second most widely cultivated Allium. Garlic is in demand all around the world both in fresh form and also in dehydrated form. Egypt tops the list in production (25,366 Kg/ha).

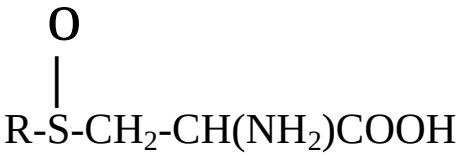
Botany

Garlic is a frost-hardy bulbous perennial erect herb of 30-100 cm in height, narrow flat leaves and bears small white flowers and bulbils (Janick, 1979). The shape of garlic is smooth, round and solid for its entire length unlike onion which is hollow. Many plants of garlic do not produce flower stalks. The bulb consists of 6-35 smaller bulblets called cloves and is surrounded by a thin white or pinkish papery sheath (Fig. 2.5)(Bose and Som, 1986).

Fig. 2.5. Garlic bulbs

Chemistry

Garlic or any other Allium is characterised by the remarkable sulphur- containing compound present in it which gives distinctive smell and pungency. Uninjured bulb of garlic contains a colourless, odourless water soluble amino acid 'Alliin' which include the presence of the volatile flavour compounds. These precursors are of the general name S-Alk(en)yl cysteine sulphoxide. The general structure of the flavour precursor is



When the tissue is damaged, the flavour precursors react under the control of the enzyme alliinase (S-alk(en)yl-L-cysteine sulphoxide Lyase) to release the highly reactive sulphenic acids In garlic, alliinase catalyses the formation of allicin (Fig.2.6), which gives fresh garlic its characteristic smell.

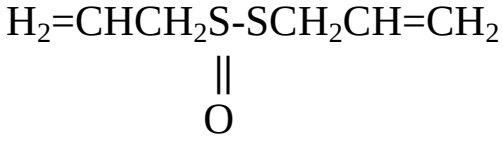


Fig. 2.6. Chemical structure of allicin

Uses

Medicinal properties

Garlic has been used as an excellent carminative, a nerve tonic and an antiseptic agent in Hindu medicine for centuries (Aman, 1969). Cholesterol lowering properties were reported by numerous studies (Reuter and Sendl,1994; Han *et al*., 1995; Adler and Holub, 1997). These studies have reported an average 10% reduction in total serum cholesterol. Evidence suggests that these effects are due to allicin or allicin –derived compounds (Yeh and Yeh, 1994; Gebhardt and Beck, 1996).

Garlic significantly lowers blood pressure (Silagy and Neil, 1994; Das *et al*., 1995a and 1995b). Garlic has an influence on platelet aggregation, an important factor in cardiovascular disease (Han *et al*., 1995; Batirel *et al*., 1996). It also has an effect on the blood coagulation and fibrinolytic activity which are factors in the development of thrombosis (Han *et al*., 1995; Breihtaupt-Grogler *et al*., 1997).

Garlic also has antioxidant properties which are helpful in preventing cancer and cardiovascular diseases (Horie *et al*., 1992; Phelps and Harris, 1993). Garlic has antibiotic properties and has been used to treat wounds when other antibiotics were not available (Fenwick and Hanley, 1985; Han *et al*., 1995).

Antimicrobial properties

Allicin, one of the active principles of freshly crushed garlic homogenates, has a variety of antimicrobial activities. Allicin in its pure form was found to exhibit i) antibacterial activity against a wide range of Gram-negative and Gram-positive bacteria,

including multidrug-resistant enterotoxicogenic strains of *Escherichia coli*; ii) antifungal activity, particularly against Candida albicans; iii) antiparasitic activity, including some major human intestinal protozoan parasites such as *Entamoeba histolytica* and *Giardia lamblia*; and iv) antiviral activity. The main antimicrobial effect of allicin is due to its chemical reaction with thiol groups of various enzymes, e.g. alcohol dehydrogenase, thioredoxin reductase, and RNA polymerase, which can affect essential metabolism of cysteine proteinase activity involved in the virulence of *E. histolytica* (Ankri and Mirelman, 1999).

The antibacterial effect of a home-made raw garlic extract and commercial garlic tablets alone and in combination with antibiotics or omeprazole was determined against clinical isolates of *Helicobacter pylor* by Jonkers *et al*. (1999). MIC values of raw garlic extract and three types of commercial garlic tablets ranged from 10,000 to 17,500 mg/L.

Food processing

Garlic is used practically all over the world for flavouring various dishes. In America about 50% of the entire output of fresh garlic is dehydrated and sold to food processors for use in manyonnaise products, salad dressings, tomato products and in several meat preparations. Raw garlic is used in preparations of garlic powder, garlic salt, garlic vineager, garlic cheese croutons, potato chips, garlic bread, garlicked meat tit-bits and garlicked bacon etc., which have been boosted in the American market. Spray-dried garlic products including garlic preparations are also available in the market.

In India and other Asian countries it is used in several food preparations notably in pickles, curry powders, curried vegetables, meat preparations and tomato ketch up, etc. There has been increasing demand for garlic by food industries for garlic powder as a condiment. Oil of garlic has now been appreciated as a valuable flavouring agent, for use in all kinds of meat preparations, soups, canned foods and sauces (Pruthi, 1987).

2.6.4. Oregano

It belongs to the genus Origanum.The name is derived from the Greek words oros, mountain and hill, and ganos, ornament. The members of the genus are mainly distributed around the Mediterranean region and 35 out of 43 species occur exclusively in the East Mediterranean (Peter, 2004).

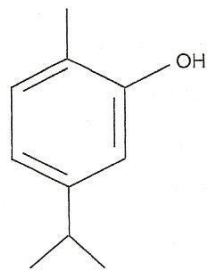
Botany

Oregano is generally considered as a perennial herb, with creeping roots, branched woody stems and opposite, petiolate and hairy leaves. The flowers are in corymbs with reddish bracts, a two-lipped pale purple corolla and a five-toothed calyx. In moderate climates, the flowering period extends from late June to August. Each flower produces, when mature, four small seed-like structures. The foliage (Fig.2.7) is dotted with small glands containing the volatile or essential oil that gives the plant its aroma and flavour (Simon *et al*., 1984).

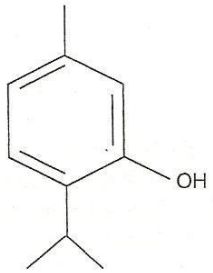
Fig. 2.7. Foliage of oregano

Chemical structure

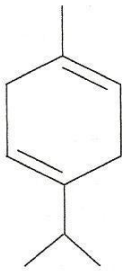
Although abundant chemical compounds have been isolated from oregano, the most important group is its volatile oils, basically terpenoids. Oregano species are rich in phenolic monoterpenoids such as carvacol and thymol (Fig.2.8). It contains a number of compounds such as gamma-terpinene, *p*-cymen, thymol and carvacol. Methyl ethers, thymol and carvacol acetates and compounds such as p-cymene,p-cymen-8-ol,p-cymen-7-ol,thymoquinone and thymohydroquinone are also present (Skoula and Harborne, 2002).



Carvacol



Thymol



γ-terpinene

Fig. 2.8. Chemical structures of major components in oregano

Other chemical groups that are commonly detected in Origanum species are acyclic monoterpenoids such as geraniol, geranyl acetate, linalool, linalyl acetate and beta-myrcene; bornane-type compounds such as camphene, camphor, borneol and isobornyl acetate.

Uses

Main uses in Medicine

There are various reports on the traditional medicinal uses of European oregano. It has been used as a folk remedy against colic, coughs, headaches, nervousness, toothaches and irregular menstrual cycles. Many of the studies confirmed benefits of oregano for human health and its use for the treatment of a vast list of ailments, including respiratory tract disorders such as cough, as an oral antiseptic,in urinary tract disorders and in dermatological affections (alleviation of itching, healing crusts, insect stings), viral infections and even cancer (Baricevic and Bartol, 2002).

Antimicrobials properties

In conjunction with the antioxidant properties of the herb, there are abundant reports on the microbial inhibitory effects of oregano essential oil or its components. These effects are generally classified either as antifungal or antibacterial. It is found that there is a relationship between the chemical structure of the most abundant essential oil components and their antifungal and anti-aflatoxigenic potency, which is, strongly correlated with the concentration of the essential oil or active ingredient and pH of the testing medium *in vitro* (Deans and Svoboda, 1990; Baricevic and Bartol, 2002). Phenols are believed to be the most potent antimicrobials, followed by alcohols, ketones, ethers and hydrocarbon (Charai *et al*., 1996)

In more practical terms ground oregano (at 2% concentrations) was found to possess a strong antifungal potential against several food-contaminating moulds, such as *Alternaria alternate* Keissler, *Fusarium oxysporum* Schlecht, *Pencillium citrinum, P.roqueforti, P.patulum,Aspergillus flavus and A.parasiticus* (Azzouz and Bullerman, 1982; Schmitz *et al*., 1993).

Although the antibacterial properties of oregano extracts are far less documented, Hammer *et al*. (1999) found that *Oreganum vulgare* (Australian origin) yielded one of the most potent antibacterial agents among 52 investigated essential oils, which considerably inhibited the growth of all tested microorganisms. Other reports (Biondi *et al*., 1993; Izzo *et al*., 1995) demonstrated the inhibitory effects of oregano extracts against a number of Gram positive (such as *Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative bacteria (such as *Proteus vulgaris* and *Escherichia coli*). These activities have been mainly attributed to thymol and carvocol. However,as also shown for the antifungal properties of the species, it seems more appropriate to combine the antimicrobial efficacy of different food-preservative compounds, creating synergistic effects, such as those reported by Paul and Smid(1999) for carvacrol and nisin (a bactericidal peptide, used as a biopreservative in certain foods) against *Bacillus cereus* and *Listeria monocytogenes in vitro*.

Food processing

Oregano is used in meat, sausages, salads, stewings, dressings and soups. The food industry uses oregano oil and oregano resin both in foods and in beverages and also in cosmetics. It is the most common spice for pizza. Along with black pepper, it is a common ingredient of dressings and a good substitute for table salt.

Apart from its dietary value, oregano is an effective antioxidant additive in different types of foods, such as mayonnaise and French dressing (Chipault *et al*., 1956; Nakatani and Kikuzaki, 1987; Baratta *et al*., 1998). This property is usually attributed to the high carvacol

content of the spice (Tsimidou and Boskou, 1994), although additional compounds, such as flavonoids may also be responsible (Vekiari *et al*., 1993).

2.6.5. Turmeric

Turmeric, *Curcuma longa* L., has been attributed a number of medicinal properties in the traditional system of medicine for treating several common ailments (Srivastava *et al*., 1985; Raghunath and Mithra,1982; Dash,1987). It belongs to the genus Curcuma, which consists of several plant species with underground rhizomes and roots. The name derives from the Latin term *terra merita*, meaning 'meritorious earth', referring to the colour of ground turmeric, which resembles a mineral pigment. In many languages, turmeric is named simply as 'yellow root' (Fig.2.9).

Botany

Turmeric is an erect perennial herb with thick and fleshy rhizomes and leaves in sheaths, characteristic of the family Zingiberaceae. The plant reaches a height of about 1m. Leaves are alternate, obliquely erect or subsessile. The leaf number ranges from 7-12. The leaf length ranges from 30-45 cm with a breadth of between 14 and 16 cm, with the petiole equalling the blade. The inflorescence is a cylindrical, fleshy, central spike of 10-15 cm, arising through the pseudostem.

Fig. 2.9. Turmeric root

Chemistry

Turmeric, dried and cured, generally yields 1.5% to 5.0% volatile oil. Turmeric owes its aromatic taste and smell to the oil present in the rhizome. Analysis of the oil, obtained by steam distillation of the powdered rhizome, followed by fractional distillation and derivatization, shows that the components are a mixture of predominantly sesquiterpene, ketones and alcohol (Kelkar and Rao,1933).The residue on steam distillation yields mainly sesquiterpene alcohols.

Besides these major components, they have also identified a mixture of low-boiling terpenes, d-sabinene.alpha-phellandrene, cineole, borneol and zingibeene in substantial amounts (25%). The main coloured substances in the rhizomes are curcumin (1,7-bis (4-hydroxy-3-methoxy propenyl)-1, 6-heptadiene-3, 5-dione) and two related demethoxy compounds, demethoxy curcumin and bis-demethoxy curcumin (Fig.2.10).

Uses

Medicinal and pharmacological uses

The rhizome extracts of turmeric,apart from the ethanolic and methanolic ones, have been examined for their biological activities and have been in use for centuries. Anti-inflammatory activity was established by Srimal and Dhawan (1985). Antioxidant effect is reported by many scientists (Sharma, 1976; Reddy and Lokesh, 1992; Subramonian *et al*., 1994; Ruby *et al*., 1995; Sreejayan and Rao, 1994; Masuda *et al*., 2002).

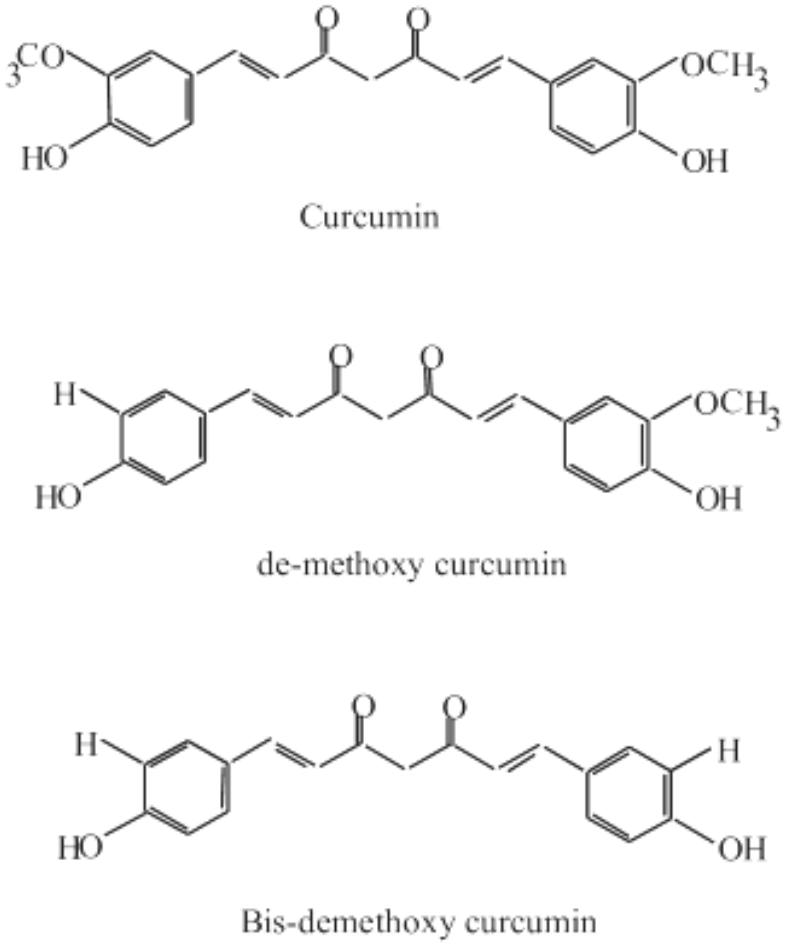


Fig. 2.10. Chemical structures of major components of turmeric

Curcumin also has the potential to prevent oxidative damage to the arterial wall (Soni and Kuttan, 1992).The anticancer action of curcumin has been studied in a standard model of radiation induced tumour in rat mammary gland (Inano *et al*., 2000). An interesting property of curcuminoids is their anti-HIV effect,which has been demonstrated during in-vitro and in vivo experiments,including a limited number of human studies (Lin *et al*.,1994).

Antimicrobial activity

Curcuminoids have also been shown to exhibit antimicrobial properties.The antimicrobial effects of alcoholic extract of turmeric, curcumin and oil from turmeric have been studied by Banerjee and Nigam (1978). Extracts from turmeric as well as active principles from curcuminoids, were found to inhibit the growth of numerous Gram positive and Gram negative bacteria, fungi and intestinal parasite, *Entamoeba histolytica*. The ethanol extract of turmeric have been reported to have anti-amoebic activity against *E.histolytica in vitro* (Dhar *et al*., 1968). Curcumin at concentrations of 2.5 -50.0 mg/100 ml inhibited *in vitro* growth of *Staphylococcus aureus* (Shankar and Srinivasamurthy, 1979).

Curcumin also inhibits the *in vitro* production of aflatoxins – toxins produced by the mould *Aspergillus parasiticus*, which may grow and contaminate poorly preserved foods and is a potent biological agent causing injury to the liver, often resulting in liver cancer (Madhyastha and Bhat, 1985; Polasa *et al*., 1992; Jayaprakasha *et al*., 2001).

2.6.6. Rosemary

Rosemary (*Rosemarinus officinalis* L.) belongs to the family *Lamiaceae*. Rosemary is a native of Mediterranean region and numerous cultivars and wild forms are available in Mediterranean countries (Giugnolinini, 1985). It is also grown in Algeria, China, Middle East and to a limited extent in India.

Temperate climate is suitable for the cultivation of Rosemary. The word rosemary is derived from the Latin word ‘rosemarinus’, meaning ‘sea dew’. It was also called ános’by the ancient Greeks, meaning the flower of excellence or ‘libanotis’ for its smell of incense (Giugnolinini, 1985).

Fig. 2.11. A twig of Rosemary plant

Botany

It is a dense, evergreen, hardy, perennial aromatic herb of 90-200 cm height with small (2-4cm) pointed, sticky and hairy leaves. The upper surface of the leaf is dark green whereas it is white below. Leaves are resinous, branches are rigid with fissured bark and stem is square, woody and brown. Pale blue small flowers appear in cymose inflorescence (Fig.2.11). The leaves, flowering tops and twigs yield an essential oil and oleoresin valued in traditional medicine, modern medicine and aroma therapy as well as in the perfumes and flavour industries.

Chemistry

The composition of rosemary oil is 1,8-cineol (30-40%), camphor (15-25%), borneol (16-20%), bornyl acetate (upto 7%), alpha-pinene (25%) and other terpenes. 1,8-cineol and camphor are the major components in the essential oil of rosemary (Fig.2.12).

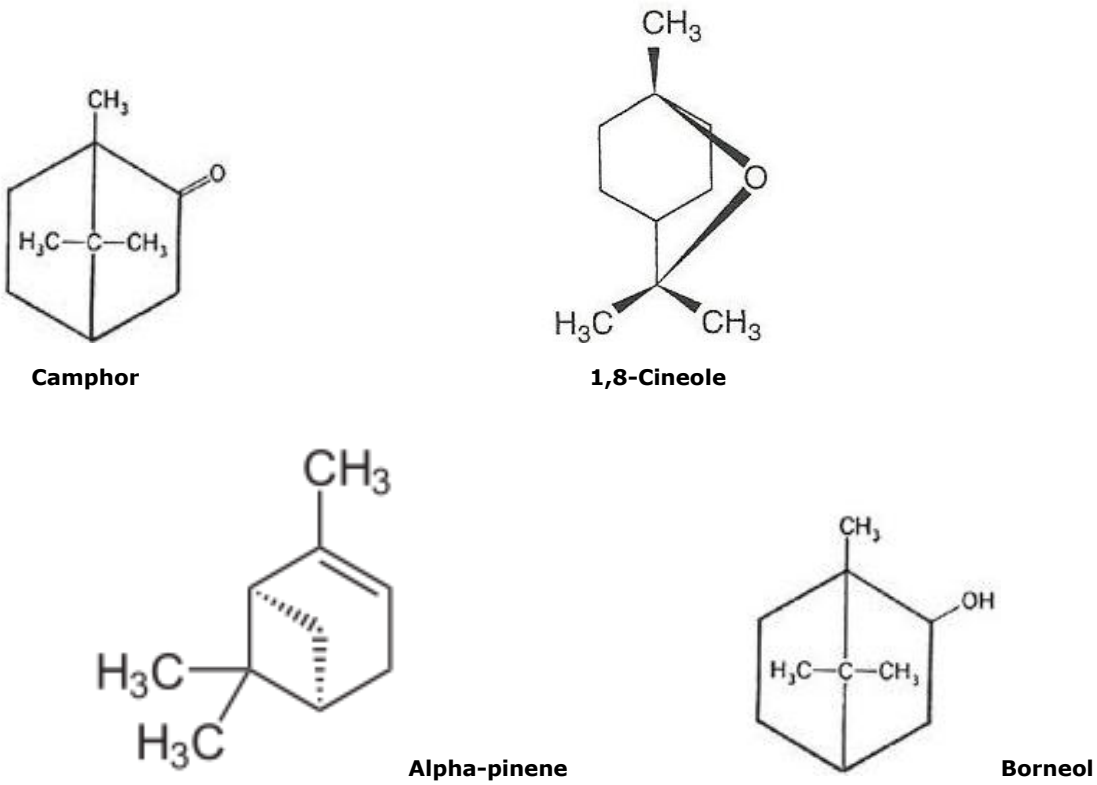


Fig. 2.12. Chemical structures of major constituents of rosemary

Uses

Medicinal properties

Rosemary is credited as a carminative, antidepressant, antispasmodic, antimicrobial, anti-inflammatory agent, carcinogen blocker and liver detoxifier, antirheumatic and abortifacient. It has an emerging potential as a source of anticancer molecules and bioavailability enhancer of cancer drugs (Jones, 2002; Plouzek *et al*., 1999)

Liquid deodorized extract of rosemary and oily extracts of mixture of herbs such as rosemary, thyme, sage and oreganum inhibited human immuno deficiency virus (HIV) infection at a very low concentration. Carnosol and carnosic acid were found to top the main active constituents of the extracts (Aruoma *et al*., 1996). Rosemary oil is useful in controlling dandruff, promoting hair growth and controlling greasy hair. Flower, calyx and leaves of rosemary are used in potpuri, tussie-mussies, herb pillows,etc. (Bonar,1994).

Antimicrobial properties

Antibacterial activity of the essential oil of rosemary against an array of bacterial and fungal species including *Listeria monocytogenes* and *Aspergillus niger* have been reported by Faliero *et al*. (1999) and Baratta *et al*. (1998). Gram positive bacteria such as *Staphylococcus aureus* and *S.epidermid* have been found to be more susceptible to rosemary oil than other Gram negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* (Pintore *et al*., 2002). Rosemary leaves are found to be the source of antibacterial molecules. The main compounds responsible for the antimicrobial activity are α-pinene, bornyl acetate, camphor and 1,8-cineole (Daferera *et al*., 2000; 2003; Pintore *et al*., 2002).

Ouatara *et al*. (1997) investigated the antibacterial activity of selected essential oils against some food spoilage organisms and found that rosemary essential oil was one among the most active antimicrobial . Similar results were obtained by Valero and Salmeron (2003) for the antibacterial activity of rosemary essential oil against *Bacillus cereus* strains grown in carrot broth.

Food processing

Rosemary is a most effective herb with a wide range of uses in food processing. In Europe and the USA, rosemary is commercially available for use as an antioxidant, though not technically listed as a natural preservative or antioxidant (Yanishlieva and Heinonen,

2001). Rosemary has potential application in the suppression of warmed over flavour (Valenzuela and Neito, 1996). The main antioxidant principles in rosemary are carnosic acid, 12-methoxy carnosic acid and carnosol as well as the antioxidative diterpenes such as epirosmarinol, isorosmanol, rosemaridiphenol, rosemariquinone and rosemarinic acid (Richheimer *et al*., 1996).

The antioxidant properties of rosemary are attributed to its ability to scavenge superoxide radicals, lipid antioxidation, metal chelating, etc. Extracts and essential oil of rosemary can be used to stabilized fats, oils and fat containing foods, butter etc. against oxidation and rancidity (Pokorny *et al*., 1998; Zegarska., 1996) and fermented meat product, etc. (Korimova *et al*., 1998).

Rosemary leaves and flowering tops are used in lamp roast, mutton preparations, marinades, bouquet garni, with baked fish, rice salads, egg preparations, dumplings, apples, summer wine cups and fruit cordials (Bonar, 1994). As rosemary extract is known to have antioxidant properties, it is of use in bakery, beverages, savoury foods, for retarding rancidity in fats and oils, preventing flavour degradation, etc.

Other properties

An active compound effective against the plant pathogen *Streptomyces scabies*, under laboratory studies, has been isolated from the leaves of rosemary (Takenaka *et al*., 1997). Rosemary is known to possess insect repellent properties. Comparative laboratory studies on the effect of dusting different herbal powder, including rosemary powder, on stored grains of wheat and French beans against *Sitophilus granarius* and *Acanthoscelides obtectus* revealed that grainwheat can be very effectively protected against *S.granarius* with the dust of rosemary (Kalinovic *et al*., 1997).

2.7. About this thesis

Unprocessed seafood harbor high number of bacteria, hence are more prone to spoilage. In this circumstance, the use of spice in fish for reduction of microorganism can play an important role in seafood processing. Many essential oils from herbs and spices are used widely in the food, health and personal care industries and are classified as GRAS (Generally regarded as safe) substances or are permitted food additives. A large number of these compounds have been the subject of extensive toxicological scrutiny. However, their principal function is to impart desirable flavours and aromas and not necessarily to act as antimicrobial agents. Given the high flavour and aroma impact to plant essential oils, the future for using these compound as food preservatives lies in the careful selection and evaluation of their efficacy at low concentrations but in combination with other chemical preservatives or preservation processes. For this reason they are worth of study alone or in combination with processing methods in order to establish if they could extend the shelf-life of foods.

In this study, the effect of the spices, clove, turmeric, cardamom, oregano, rosemary and garlic in controlling the spoilage and pathogenic bacteria is investigated. Their effect on biogenic amine formation in tuna especially, histamine, as a result of bacterial control is also studied in detail. The contribution of spice oleoresin in the sensory and textural parameters is investigated using textural profile analysis and sensory panel. Finally, the potential of spices in quality stabilization and in increasing the shelf–life of tuna during frozen storage is analysed.

3.1. Introduction

Fish is an important part of a healthy diet since they contain high quality protein. It is also a highly perishable commodity. They are also prone to contamination at various stages of handling and processing (Gananambal and Patterson, 2005). Contamination is a very important aspect as this is the mode that most unwanted microorganisms may be transmitted onto seafood and other food products. There is substantial evidence that seafoods are high on list of foods associated with outbreaks of food borne diseases (Huss *et al*., 2003).In raw fish, spoilage takes place mainly due to enzymatic, microbial and chemical action. Among these three, fish spoilage due to bacteria gains greater concern as a health hazard. Bacteria are present on the surface slime, skin, gills and intestine of fish. In dead fish, bacteria begin to invade the tissues causing spoilage and production of undesirable compounds leading to food safety issues.

3.1.1. Initial bacterial flora

The subsurface flesh of live healthy fish is usually sterile and does not contain any bacteria or other organisms. But, microorganisms colonize the skin, gills and the gastrointestinal tract of fish. The geographical location, the season and the method of harvest are the reasons deciding the number and diversity of microbes. The natural fish microflora tends to reflect the microbial communities of the surrounding waters. Fish harvested from eutrophic and warm waters will present higher bacterial numbers than fish from clean and cold waters. But, potential human pathogens may be present in both cases (Arias, 2009).

Gram-negative genera including: *Acinetobacter, Flavobacterium, Moraxella, Shwenella* and *Pseudomonas* dominates the autochthonous bacteria flora of fish. *Vibrio, Photobacterium* and *Aeromonas spp.* are also common aquatic bacteria, and typical of fish flora. Huss (1995) observed that Gram-positive organisms such as *Bacillus, Micrococcus, Clostridium, Lactobacillus* and *coryneforms* can also be found in varying proportions on fish.

The initial microflora of fish can contain human pathogenic bacteria, posing a concern for seafood borne illnesses (Davies *et al*., 2001). These pathogens can be divided into two groups: organisms naturally present on the fish and those that although not autochthonous to the aquatic environment, are present there as a result of contamination or are introduced to the fish during harvest, processing or storage. However, number of pathogenic organisms in fresh raw fish tends to be low and risk associated with the consumption of seafood is low. In addition, potential pathogenic bacteria is outgrown by indigenous spoilage bacteria during storage. Shelf life depends on the initial microflora on the fish, potential contaminants added during handling, processing, and conditions of storage (Arias, 2009).

3.1.2. Sources and routes of contamination

Unwanted microorganisms may access fish processing environments through raw material, personnel or mobile equipment such as forklifts, through leakage and openings in buildings, or through pests and some pathogens may even become established in the processing plant and form niches where they can survive for long periods of time (Reij *et al*., 2003). Many of these microorganims occur naturally in aquatic and general environments, and may be transmitted onto seafood before capture, during and after processing. Also, contamination through air can occur through dust particles or via aerosols which are formed especially when contaminated surfaces, floors or drains are sprayed with high pressure-jets, resulting in formation of droplets that can be suspended in the air (Aantrekker *et al*., 2003). Water is also a vehicle for transmission of many agents of diseases (Kirby *et al*., 2003).

Liston (1980) estimated the total number of microorganisms to vary enormously from a normal range of 10^{5}-10^{7} cfu (colony forming units)/cm^{2} on the skin surface. Contamination of fish products through contaminated surfaces has also been observed in many cases (Reij *et al*., 2003). Unclean, insufficiently or inadequately cleaned processing equipment have been identified as a source of bacterial contamination in processed seafood. Containers, pumps or tanks used for holding or transporting unprocessed raw materials, have

occasionally been used for processed products without any cleaning and disinfection (Morgan *et al*., 1993; Hennessy *et al*., 1996 and Llewellyn *et al*., 1998). It is therefore necessary that equipment in the processing establishment, coming in contact with food, be constructed in such a way as to ensure adequate cleaning, disinfection and proper maintenance to avoid the contamination (CAC, 1997a).

Transfer of microorganisms by personnel, particularly from hands, is of vital importance (Chen *et al*., 2001; Montville *et al*., 2001 and Bloomfield, 2003). During handling and preparation, bacteria are transferred from contaminated hands of workers to food and subsequently to other surfaces (Montville *et al*., 2002). Low infectious doses of organisms such as *Shigella* and pathogenic *Escherichia coli* have been linked to hands as a source of contamination (Snyder, 1998). Poor hygiene, particularly deficient or absence of hand washing has been identified as the causative mode of transmission (Reij *et al*., 2003). Proper hand washing and disinfection has been recognized as one of the most effective measures to control the spread of pathogens, especially when considered along with the restriction of ill workers (Adler, 1999 and Montville *et al*., 2001).

3.1.3 Bacterial indicators

Various bacteria are found in the digestive tracts and feaces of animals and humans. Some of these bacteria, i.e. faecal coliforms, *E.coli* (the predominant group of the faecal coliform group), and *Enterococcus* spp., are used as hygiene indicators (Frahm and Obst, 2003). Indicator microorganisms are microorganisms or a group of microorganisms indicative for the possible presence of pathogens whose presence in given numbers points to inadequate safety in processing (Mossel *et al*., 1995). In general, they are most often used to assess food sanitation (Jay, 1992).

There is no universal agreement on which indicator microorganism is most useful, nor are there federal regulations mandating a single standard for bacterial indicators. Thus, different indicators and different indicator levels identified as standards are used in different states, countries, and regions. Today, the most commonly measured bacterial indicators are

total coliforms (TC), faecal coliforms (FC), and enterococci (EC). More recently, *E.coli* (a subset of the FC group) and EC were established as prefered indicators (Noble *et al*., 2003).

Food plants and many other institutions require sanitary conditions in order to prevent microbial contamination. The continuous evaluation of these environments is particularly important in order to assure the safety and quality of products, and the number of microbial cells contaminating food surfaces must be determined for this assessment (Yamaguchi N *et al*., 2003). Many methods have been developed to detect microorganisms, and although some methods of analysis are better than others, every method has certain inherent limitations associated with its use (Jay, 1992).

3.1.4. Spoilage

Food spoilage is considered as any change that renders the product unacceptable for human consumption (Sivertsvik *et al*., 2002). Spoilage of fish starts upon death due to autoxidation, autolytic activity and metabolic activities of microorganisms present in the fish.

Microbial food spoilage occurs as a consequence of either microbial growth in a food or release of microbial extracellular and intracellular enzymes into the food environment. Some of the detectable parameters associated with spoilage of different types of foods are changes in color, odor, and texture; formation of slime; accumulation of gas; and accumulation of liquid. Between initial production and final consumption, different methods are used to preserve the qualities of foods, which include the reduction of microbial numbers and growth. Yet, microorganisms grow and cause spoilage, which for some foods like fish could be relatively high.

3.1.5. Significance of bacteria in spoilage

Raw and processed foods normally contain many types of microorganisms capable of multiplying and causing spoilage. Bacteria, because of their shorter generation time, are

in a favourable position over moulds to cause rapid spoilage of foods. Therefore, among the three microbial groups, the highest incidence of spoilage, especially rapid spoilage, of processed foods is caused by bacteria, followed by yeasts and molds. Due to this reason the initial microbial load and the percentage of spoilage bacteria in it decide the shelflife of any product.

The objective of the present investigation is to determine the bacterial flora of tuna (*Euthynnus affinis*) with a view to asses their percentage distribution. The change in total microbial load and bacterial profile during spoilage was observed. The spoilage potential of the spoilage bacteria was also analysed. The study is essential for isolating the bacterial culture needed for the antimicrobial screening test.

3.2. Materials and Methods

3.2.1. Materials

3.2.1.1. Raw material

Fresh tuna (*Euthynnus affinis*) was procured from Vypeen harbour, Kochi. The size of the fish was about 60-70cm and weighed 1-1.5Kg. The fish were aseptically packed in sterile polythene covers and delivered to the laboratory in insulated boxes containing ice for maintaining the sample under conditions which preserved the original bacterial flora as completely as possible.

The fish was beheaded, gutted and cut into chunks of 2cm x1 cm x1cm (l x b x h) using sterile knife under aseptic conditions. Care was taken not to cross contaminate the sample from the surrounding area. These fish chunks were used for further studies. The samples were analysed within 1 hr of sample collection for obtaining total plate count of fresh tuna. The tuna chunks were kept at ambient temperature for 48 hrs and also at 4° C for 10 days to record plate count of spoiled fish.

3.2.1.2. Bacteriological Media

For microbiological examination of fish, standard culture media were used (Himedia and SRL brands). For detection and characterization of pathogens like *Salmonella* and *Vibrio cholera*, standard culture media described in the Bacteriological Analytical Manual (USFDA, 2001) were used. Media for *E.coli* and *Staphylococcus* were used as described in the Laboratory Manual for Microbiological examination of Seafood (Surendran *et al*., 2006). Composition of the media, diluents, reagents and indicators used in the study are given below.

Composition of Media/Reagents/Indicators

A. Media preparation

Alkaline Peptone Water

Peptone	10g
Sodium Chloride	5g
Disilled water (DW)	1L

pH: 9.1±0.1

Dissolved the ingredients in 1 liter distilled water (DW) and adjusted pH to 9.1±0.1. Distributed 225ml in 500ml conical flask and autoclaved at 121ºC for 15 minutes.

Baird-Parker Medium (BP)

Tryptone	10g
Beef extract	5g
Yeast extract	1g

Sodium pyruvate	12g
Glycine	12g
$LiCl.6H_2O$	5g
Agar	15g
Distilled Water	1L

Dissolved the ingredients in 1 liter distilled water (DW) and adjusted pH to 7.0±0.2. Distributed 100ml qualities in flasks. Sterilized at 121ºC for 15 minutes. Before pouring in to plates, added the following per 100ml of the molten and cooled (45ºC) medium.

1. Sterile egg yolk; 5ml of 50% egg yolk
2. Sterile potassium tellurite: 1ml of 1% solution.

Allowed the plates to set. Dried at 56ºC in an incubator for 45 minutes, cooled to room temperature (RT) before use.

Bismuth Sulphite Agar (BSA)

Peptone	10g
Beef extract	5g
Glucose	5g
K_2HPO_4	4g
$FeSO_4$	0.3g
Bismuth Sulphite indicator	8g
Agar	15g
DW	1 L

pH: 7.6±0.2. Dissolved the required ingredients for 500ml DW in a 1 litre flask to avoid boiling over of the contents. Mixed thoroughly and heated with agitation. Boiled for one minute to obtain a uniform suspension. Cooled to 50ºC, mixed well and poured into plates to get a thick layer. Dried at 56ºC for 45 minutes. Prepared plates one day before use and stored in dark.

Hektoen's Enteric Agar (HEA)

Protease peptone	12.0g
Yeast extract	3.0g
Lactose	12.0g
Sucrose	12.0g
Salicin	2.0g
Bile salt no.3	9.0g
NaCl	5.0g
Sodium thiosulphate	5.0g
Ferric ammonium citrate	1.5g
Acid Fuchsin	0.1g
Bromothymol blue	0.065g
Agar	15g
Distilled water	1 L

pH : 7.1±0.2. .Dissolved by gentle heating. Poured into plates and dried before use.

Hugh & Leifson Glucose O/F medium (H&L)

Peptone	1g
NaCl	0.5g
K_2HPO_4	0.4g
Dextrose	1g
Agar	0.3g
Distilled water	100ml

pH: 7.1±0.1. Dissolved and adjusted pH to 7.1. Then added 1ml of 0.1% solution of phenol red indicator. Dispensed in 8 ml quantities in 150x 12 mm tubes and sterilized at 121ºC for 20 minutes.

Lactose broth

Beef extract	3g
Peptone	5g
Lactose	5g
Distilled water	1 L

pH : 6.9±0.1. Dissolved the ingredients in DW, dispensed in 225ml quantities in 500ml conical flask, pluged with non-absorbent cotton and sterilized at 115 ºC for 20 minutes.

Methyl Red Voges-Ptoskauer Medium(MRVP)

Peptone	0.5g
D-glucose	0.5g
K_2HPO_4	0.5g
Distilled water	100ml

pH: 6.9±0.1. Dissolved the ingredients in 100ml distilled water. Dispensed in 4 ml quantities in 100x12mm test tubes. Sterilized at 115 ºC for 20 minutes .

Normal Saline(NS)

NaCl	8.5g
Distilled water	1 L

Dispensed in flask (95ml) / tubes (9.5ml). Sterilized at 121 ºC for 15 minutes.

Nutrient Agar

Peptone	10g
Beef extract	3g

NaCl	5.0g
Agar	15.0g
Distilled water	1L

pH: 7±0.1. Heated to dissolve and adjusted pH. Distributed in tubes for slants or in 100ml volumes in conical flasks for plating. Autoclaved at 121°C .

Nutrient Broth

Peptone	10g
Beef extract	3g
NaCl	5.0g
Distilled water	1L

pH 7±0.1. Heated to dissolve, adjusted pH. Distributed in 225ml quantities in 500ml conical flasks. Autoclaved at 121°C for 15 minutes.

Selenite Cystine Broth

Tryptone	5g
Lactose	4g
Na_2HPO_4	10g
L-Cystine	0.1g
Sodium biselenite	4g
Distilled water	1 L

pH:7.1 +/- 0.1. Dissolved sodium biselenite in 1 L distilled water and added the other ingredients. Warmed to dissolve, dispensed in test tubes in 10ml quantities. Sterilized by steaming in an autoclave for 15 minutes.

Sugar fermentation Media

Peptone	10g
Sodium chloride	5g
Distilled water	1L

pH:7.2±0.1. Dissolved ingredients in 1L of distilled water. Added 10ml of Andrade's indicator. Adjusted pH. Added the required sugar to 1% level. Dissolved and distributed in small test tubes (100x12mm) containing inverted Durhams tubes. Sterilized at 115ºC for 20 minutes.

Tergitol-7 Agar(T-7)

Peptone	10g
Yeast extract	6g
Beef extract	5g
Lactose	20g
Tergitol-7	0.1g
Bromothymol blue	0.05g
Agar	15g
Distilled water	1L

pH:7.2±0.2. Dissolved and distributed in 100ml quantities in flasks. Sterilized at 115ºC for 20 minutes. Before pouring the plates, after melting, added 0.25ml of 1% solution of sterilized Triphenyl Tetrazolium Chloride per 100ml media. Dried the plates at 56ºC for 45 minutes and cooled to room temperature.

Tetrathionate broth

a) Base

Beef extract	0.9g

Peptone	4.5g
Yeast extract	1.8g
NaCl	4.5g
$CaCO_3$	25g
Sodium thiosulphate	40.7g
Distilled water	1L

pH:8.2±0.2. Dissolved the ingredients in distilled water and heated to boil. Cooled below 45 °C and added 20ml of iodine solution. Mixed well and distributed in test-tube in 10ml quantities.

b) Iodine solution

Iodine crystals	6g
Potassium Iodide	5g
Distilled water	20ml

Grinded iodine and KI in a mortar and added water to dissolve. Added the iodine solution to the basal media.

Thiosulphate Citrate Bile Salt Sucrose Agar (TCBS)

Yeast extract	5g
Peptone	10g
Sucrose	20g
Sodium thiosulphate	10g
Sodium Citrate Dihydrate	10g
Sodium chloride	3g
Ox-gall	5g
Sodium chloride	10g
Ferric citrate	1g

Bromothymol blue(BTB)	0.04g
Thymol blue (TB)	0.04g
Agar	15g
Distilled water	976ml

pH:8.6+/-0.1. Weighed all ingredients except the dyes. Added distilled water. Heated to boiling with agitation to obtain complete solution. Added 20ml 0.2% BTB and 4ml 0.1% TB. The solution was heated to boiling point once again . Cooled to 45° C. Poured 15-20ml into petriplates and allowed to set. It was dried at 56°C for 45 minutes.

Triple Sugar Iron Agar(TSI)

Peptone	20g
Yeast extract	3g
Beef extract	3g
Lactose	5g
Sucrose	10g
Glucose	1g
Ferric citrate	0.3g
Sodium thiosulfate	0.3g
Phenol red(0.2% solution)	12ml
Agar	12g
Distilled water	988ml

pH:7.4±0.2. Added all ingredients except phenol red to distilled water. Mixed and heated to boiling to dissolve. Cooled to 50-60°C and adjusted pH to 7.4±0.2. Added phenol red. Filled one-third of the tubes (150x18mm) and pluged it with non-absorbent cotton. Sterilized at 115°C for 20 minutes. Cooled the tubes in slanted position overnight to obtain a 2.5 cm deep butt.

Tryptone Broth (Indole Medium)

Tryptone	1g
NaCl	0.5g
Distilled water	100ml

pH:7.1±0.1. Distributed 5ml quantities in 100 x 12mm tubes. Sterilized at 121°C for 15 minutes.

Tryptone Glucose Agar(TGA)

Tryptone	0.5g
Beef extract	0.3g
NaCl	0.5g
D-glucose	0.1g
Agar-agar	1.5g
Distilled water	100ml

pH:7.1±0.1. Sterilized at 121°C for 15 minutes.

Xylose lysine desoxycholate Medium (XLD)

Yeast extract	3g
L-Lysine hydrochloride	5g
Lactose	7.5g
Xylose	3.75g
Sodium desoxycholate	1g
NaCl	5g
Sodium thiosulphate	6.8g
Ferric Ammonium Citrate	0.8g
Phenol red	0.08g

Agar	15g
Distilled water	1 L

pH:7.4±0.2. Heated with agitation till boiling. Cooled to 50°C, poured into plates. Dried at room temperature for 2 hours. Used on the day of preparation.

Modified Nivens medium: (Niven *et al*., 1981).

Glucose	0.5 %
Tryptone	0.5 %
Yeast extract	0.5 %
NaCl	0.5 %
$CaCO_3$	0.1 %
Agar	2.0 %
Histidine hydrochloride	1.0 %
Saline	0.9 %
Peptone water	0.1%

Dissolved the ingredients proportionately for the required volume by gentle heating and sterilized by autoclaving at 121°C for 15 minutes at 15 lbs pressure.

B. Reagents

1.Kovac's cytochrome oxidase reagent

N:N:N:N.Tetramethyl-p-phenylene diamine hydrochloride	100g
DW	10ml

Dissolved the above ingredients. Impregnated filter paper with the reagent and drained. Kept the test paper in a refrigerator away from light.

2.Kovac's Indole reagent

p-dimethyl amino benzaldehyde 5g
N-Butyl alcohol 75ml
Dissolved and added 25ml concentrated HCl. Filtered and kept in amber coloured bottle overnight before test.

Voges Prousker test reagents

Solution A

α-naphthol 0.25g
Alcohol 5ml
Dissolved α-naphthol in 5 ml alcohol.

Solution B

KOH 2g
Distilled water 5ml
Dissolved 2g KOH in 5 ml distilled water.

Preparation of sterile egg yolk(50%)

Took 5 hen's eggs, washed free of dirt with soap and water. Wiped, dried and kept immersed in alcohol in a 1 L beaker for 2 hours. Drained off the alcohol into a bottle. Took out the egg, made a small opening at one end using a sterile scalpel and poured out all the egg white carefully. Carefully broke the shell a little more and transferred the egg yolk into a sterile conical flask. Added an equal volume of sterile normal saline. Agitated well and allowed to stand. Pipetted 5 ml each of the egg yolk saline into sterile test tubes, pluged with sterile cotton and kept in refrigerator (at 5-8°C).

C.Staining Solutions

Crystal violet for Gram's stain

Solution A

Crystal violet	2g
Ethyl alcohol	20ml

Solution B

Ammonium oxalate	0.8g
Distilled water	80ml

Mixed solutions A&B, filtered and kept overnight before use.

Gram's iodine(For Gram's staining)

KI	2g
Iodine crystals	1g
Distilled water	300ml

Grinded iodine and KI together in a glass mortar; dissolved and filtered.

Safranine(For Gram's stain)

Safranine	1g
Ethyl alcohol	40ml
Distilled water	360ml

Dissolved safranine in alcohol, added distilled water and filtered.

d. Indicator solutions

1.Andrade's indicator(For sugar fermentation)

Acid fuchsin	0.5g
Distilled water	100ml
1N NaOH solution	16ml

Dissolved acid fuchsin in distilled water. Added NaOH solution. Kept overnight.

2.Bromocresol purple indicator

Bromocresol purple	0.2g
Alcohol	10ml
Distilled water	90ml

Dissolved bromocresol purple in 10 ml alcohol. Added 90ml distilled water to the solution.

3.Methyl Red indicator

Methyl red	50mg
Alcohol	150ml
Distilled water	100ml

Dissoved Methyl red in alcohol and diluted with distilled water and filtered.

4.Potassium tellurite solution

Potassium tellurite	1g
Sterile distilled water	100ml

Steamed for 30 minutes in a water bath.

3.2.2. Methods

The fish samples were analyzed for microbiological parameters qualitatively as well as quantitatively as per methods (USFDA 2001, Surendran *et al*, 2006).

3.2.2.1. Sampling of fish for TPC

For spread plating method, agar plates had to be poured and dried in advance. Melted one flask of TGA in a water bath, cooled one flask of the medium to 45°C; poured into petri dishes (about 15-18 ml each). Allowed to set. Dried the surface of the medium in a laminar flow chamber for 45 minutues.

Sampling

Asceptically cut 10g of skin with muscle of the fish sample into a sample dish. Macerated with 90 ml diluent (normal saline) in a sterile mortar (10^{-1} dilution). Pipetted 1ml of the supernatant to 9 ml diluent and mixed well (10^{-2} dilution). From the 10^{-2} dilution pipetted 1ml of the supernatant to 9 ml diluent and mixed well (10^{-3} dilution). Prepared 10^{-4} and 10^{-5} dilution in the above manner.

Spread plating

Arranged the pre set TGA plates in 3 rows in duplicates and labeled appropriately. Inoculated 0.5ml each of the 10^{-3}, 10^{-4}, 10^{-5} dilutions on the surface of the respective plates. It was spread on the surface of each plate using a sterile bent glass rod. Between spreading operations of each plate, the rod was sterilized by dipping in alcohol and flaming. After 30 minutes, incubated the plates at 37°C for 48 hrs. After 48hrs of incubation, the colonies developed in each plate were counted using a Quebec colony counter. The colony counts of duplicate plates agreed within 10% limit and the counts between decimal dilutions agreed decimally. TPC/g sample was calculated using the relation: TPC/g sample = average count x

dilution factor x 10/w, where w = weight of the sample. Only those plate counts which fell between 30 -300 were statistically acceptable.

3.2.2.2. Isolation of bacterial cultures from TPC plates

From a suitable TPC plate,on which the colonies were well apart and the counts fell preferably between 30 and 60, colonies were picked into a suitable liquid medium using a sterile platinum loop. Care was taken to pick the different colonies in the same proportion of their relative occurrence in the plate. Labeled the tubes and incubated at 37°C.

Purification of bacterial isolates

Pre-set plates of TGA were made as described earlier. Loopful of bacterial culture grown in Tryptone broth was streaked at random across the first quarter on the surface of the TGA plate. Sterilized the loop. From the edge of the streaks on the first quarter, streaked many lines to the second quarter. Sterilized loop, continued as above to the third quarter and similarly to the fourth quarter. Incubated at 37°C for 24 - 48 hrs. Picked well isolated colony to a Nutrient agar slant, labelled and incubated . This pure culture was used for further studies for identification.

3.2.2.3. Identification of the spoilage bacteria

The following tests are done for the identification:

1. Gram's staining and microscopy
2. Motility
3. Test for catalase
4. Pencillin sensitivity
5. H&L glucose O/F reaction
6. Cytochrome oxidase test
7. Fermentation of glucose

8. Pigmentation
9. Growth at zero NaCl level.

Staining and microscopy

Only young cultures are used for staining. Usually 16-24hr cultures are considered young. A speck of young culture was emulsified with a drop of sterile water in the middle of a sterile glass slide and spread uniformly. Fixed by passing the slide 3-4 times through the blue flame of Bunsen burner. Flooded the smear with Gram's crystal violet stain for 1 minute. Washed with water. Flooded with gram's iodine for 1 minuteand again washed with water. Destained dropwise with addition of alcohol until washings were free from violet colour. Washed once again and counter stained with Safranine for 1 minute. Dried in air and observed the cells under microscope (100X objective). The colour, shape, size and arrangement of the cells were noted. Purity of the culture was also checked. In case of the cells being Gram +ve rods, it was further examined for spore formation

Pencillin sensitivity

Prepared pre-set antibiotic agar plates and dried the surface at 56°C for 45 minutes. Cooled to room temperature. Divided each plate into 4 quarters by drawing lines on the bottom so that one plate could be used for 4 cultures. A little of the culture was smeared over about 4 cm^2 area in each quarter. A filter paper disk impregnated with pencillin (each disk contains 2.5 IU pencillin) was placed on the surface of each smear. Plates are incubated without inverting for 18-24 hrs. Examined for clear zones of inhibition around the discs. Cultures showing clear zones of inhibition are sensitive to 2.5 IU pencillin.

Cytochrome oxidase test

Smeared a little of the young culture on the test paper (already impregnated with Kovac's cytochrome oxidase reagent). Development of a blue colour in a few seconds indicated a positive test.

H&L glucose O/F reaction test (Hugh / Leifson's oxidative verses fermentative reaction test using glucose as substrate)

Using a platinum wire, a little of the culture was stab-inoculated into the H&L glucose O/F medium, in such a way that at least 2 cm long column of the medium at the bottom of the tube remained uninoculated. Incubated for 18-24 hrs and observed changes. A colour change into yellow indicated acid production from glucose. A deepening of red colour of the medium indicated an increase in pH to alkaline level.

Growth of bacteria along the line of inoculation and an yellow colour throughout the medium indicated fermentative reaction (fermentative with acid but no gas). If gas bubbles were seen trapped in the medium, reaction was fermentative with gas production (Fermentative with gas; FAG)

If yellow colour appeared only at the top part of the medium, the reaction was oxidative. Sometimes, a deep pink colour developed near the top surface, indicating the change of medium to alkaline (Alkaline top). In both cases, the reaction was nonfermentative (NF).

Motility of bacteria

A small drop of distilled water was placed in the middle of a cover slip, a speck of young culture from agar slant was emulsified with it. A cavity slide was taken, the margin of the cavity was smeared with a little paraffin wax. The slide was inverted on the cover slip in such a way that the cover slip got attached to the slide and on turning upside down, the culture drop hanged into the cavity. Observed under high power objective (45X).

Catalase test

Placed a speck of young culture on a clean glass slide and flooded with 2 drops of 30% H_2O_2. Evolution of gas from the culture indicated positive test for catalase.

Spore formation

Took a dust free, dirt free and oil free microscopic glass slide. A speck of young culture was emulsified with a drop of sterile water in the middle of the glass slide and spread uniformly. Dried in air. Added a drop of malachite green stain. Fixed by passing the slide 3-4 times through the blue flame of Bunsen burner. Care was taken not to char the smear. Vegetative cells were then decolourized with water and stained pink with safranin counter stain.

3.2.2.4. Identification of Pathogenic bacteria

Identification of *E.coli*

Sampling was done as described in 3.2.2.1. From the 10^{-1} dilution, pipetted out 0.5 ml on the surface of previously dried Tergitol -7 Agar plates. After 24 hours, yellow, circular, flat, non-mucoid colonies appeared

Bio-chemical tests for confirmation

Indole test

Inoculated a little of the culture to tryptone broth and incubated at 37 °C for 48 hrs. Tested for indole production using Kovac's reagent. A red or pink colour at top indicated positive test. Indole forms a red dye with p-dimethyl amino benzaldehyde of the Kovac's reagent.

Methyl red test

Inoculated each culture into 2 tubes of MRVP medium and incubated at 37° C for 48hrs. Into one tube, added methyl red indicator. A red colour indicated positive MR test.

Voges Proskauer test

In a small test tube took 1 ml of 48 hour bacterial culture grown in MRVP medium, added 0.6ml solution A and 0.2ml solution B (Refer 3.2.1.2.b). Shook well, put a small crystal of creatinine. Shook and allowed to stand upto 4 hours. Eosine pink colour indicated a positive VP test.

Citrate utilization test

Streaked a little of the culture to Simmon's citrate agar slants and incubated at 37 °C for 48 hrs. Growth indicated by a change in the colour of the medium from green to blue indicated a positive test for citrate utilization by bacterial culture.

Identification of *Salmonella typhi*

Transferred 25g of sample to 225ml of lactose broth and incubate at 37 °C for 24±2hrs. Transferred 1 ml each from above to 9ml selenite cysteine broth and 9ml Tetrathionate broth and incubate both the tubes at 37 °C for 24±2hrs. Streaked a 3mm loopful from the incubated Selenite cysteine broth on Hektoen Enteric Agar (HEA), Bismuth sulphite Agar (BSA), Xylose Lysine Deoxycholate Agar (XLD). Also repeated the streaking from the incubated tetrathionate broth to HEA, BSA, XLD Agars. Incubated the plates at 37 °C for 24±2hrs. Examined the plates for suspicious Salmonella colonies.

Selected the suspicious colonies from each selective agar and inoculated into Triple Sugar Iron Agar (TSI) and incubated at 37 °C for 24± 2hrs. Typical Salmonella cultures produced an alkaline slant (red) and an acidic butt (yellow) with the production of H_2S (blackening of the agar) and gas.

Cultures giving typical reaction of *Salmonella* on TSI were confirmed for Salmonella by the following biochemical tests.

Oxidase test

Fermentation of glucose

Fermentation of sucrose
Fermentation of mannitol
Fermentation of inositol
Fermentation of arabinose
Lysine decarboxylation.

Identification of *Vibrio cholerae*

Transferred 25g of sample to 225ml alkaline peptone water and incubate for 6 hrs at 37° C. Streaked one loopful from the surface growth of the above to TCBS Agar plates. Also transferred 1ml to 9ml alkaline peptone water and incubated for 18hrs at 37°C and then streaked one loopful from second enrichment to another TCBS plate for 18-24 hours at 37 °C.

Transferred the suspected colonies (yellow flat and smooth colonies with opaque centres and transparent peripheries, 2-3 mm diameter) from both TCBS plates to Kigler Iron Agar (KIA) slants and incubated at 37°C for 18 hrs. *Vibrio cholerae* gave an acidic butt (yellow) butt and alkaline (pink) slant with no gas and no hydrogen sulphide (no black colour).

The following biochemical tests were done to confirm for *Vibrio cholerae*

Fermentation of Lactose
Fermentation of sucrose
Fermentation of Salicin
Fermentation of Dulcitol
Indole production
Urease test
Lysine Iron Agar

Identification of *Staphylococcus aureus*

Sampling was done as described in 3.2.2.1. 10^{-1} dilution was inoculated on Baird Parker Agar plate. Black colonies surrounded by clear zone was the characteristic of staphylococcus colonies on this agar. Coagulase test was performed to confirm *Staphylococcus aureus*.

3.2.2.5. Determination of spoilage index

1 gm of minced tuna was taken in a sterile test-tube and 1ml of normal saline was added to it. The desired bacteria was inoculated into the above from a 24 hrs culture .Three parameters namely, odour, ammonia formation and hydrogen sulphide production were monitored at 4 different temperatures (0° C, 4° C,20°C, 27°C).

Ammonia formation

A drop of the fish sample medium was put on Whatman No.1 filter paper. Added a drop of concentrated NaOH solution to it followed by a drop of Nessler's reagent. A chocolate brown colour indicated the presence of ammonia.

Hydrogen sulphide Formation

Whatman No.1 filter paper was dipped in lead acetate solution and held at the mouth of the test tube using a cotton plug. The presence of H_2S was indicated by the filter paper turning black in colour.

3.3. Result and Discussion

3.3.1. Bacterial quality of tuna

Table 3.1 gives the plate count of fresh and spoiled tuna. The total bacterial counts (TPC) of fresh tuna was in the range of 10^5 -10^6 cfu/g. Significant difference ($p<0.05\%$) was found in the microbial number of fresh and spoiled samples. There was an increase of 2 log cycles in the overall count on TGBA plates in spoiled fish compared to that of the fresh one. Fish flesh starts visibily to spoil when bacterial level rises above 10^7 organisms/g. This is often refered to as spoilage detection level.

Table 3.1 Total plate count of fresh and spoiled tuna at 28±2°C(cfu/gm)

Sl.no	Month	No.of samples	TPC(cfu/gm)	
			Fresh Tuna	Spoiled Tuna
1	Jan	5	$2.50\pm0.12 \times 10^5$	$2.10\pm0.05 \times 10^7$
2	Feb	4	$2.21\pm0.07 \times 10^5$	$2.90\pm0.21 \times 10^7$
3	Mar	4	$2.20\pm0.23 \times 10^6$	$8.90\pm0.06 \times 10^7$
4	Apr	5	$3.10\pm0.16 \times 10^5$	$4.20\pm0.31 \times 10^7$
5	May	4	$2.80\pm0.29 \times 10^5$	$2.86\pm0.12 \times 10^7$
6	Jun	4	$2.23\pm0.08 \times 10^5$	$2.50\pm0.11 \times 10^7$
7	Jul	5	$3.40\pm0.11 \times 10^5$	$3.62\pm0.09 \times 10^7$
8	Aug	4	$2.62\pm0.08 \times 10^5$	$2.00\pm0.04 \times 10^7$
9	Sept	5	$2.48\pm0.15 \times 10^5$	$2.94\pm0.14 \times 10^7$
10	Oct	5	$1.90\pm0.02 \times 10^6$	$2.32\pm0.08 \times 10^7$
11	Nov	4	$2.50\pm0.14 \times 10^5$	$3.70\pm0.21 \times 10^7$
12	Dec	5	$5.80\pm0.05 \times 10^6$	$2.10\pm0.11 \times 10^8$

The given values are expressed as mean±standard deviation

3.3.2. Isolation and Identification of bacterial cultures

From a suitable TPC plate, on which the colonies were well apart and the counts fell between 30 and 60, colonies were picked into nutrient agar slants using a sterile loop. The different colonies were picked in the same proportion of their relative occurrence in the plate. The colony morphology of the selected colonies were studied and the isolated bacterial colonies were subjected to detailed biochemical investigations. The critical biochemical characteristics of the bacterial cultures are presented in Table 3.2.

Based on the distribution of characteristic colonies on the culture plate and the identification of the representative colony, the relative distribution of the various bacterial groups obtained are given in Fig. 3.1. The percentage composition of bacterial flora of fresh tuna (*Euthynnus affinis*) indicated higher Gram –ve organisms (52.81%) whereas Gram +ve organisms accounted for 47.19%. *Micrococcus spp.* was dominant among all the isolated cultures. Among Gram negative bacteria *Pseudomonas* was dominant followed by *Vibrio* spp. Among the isolated bacteria, *Pseudomonas spp.* and *Bacillus spp.* were found in psychrotrophic conditions in previous studies (Singh and Venkataramana, 1998).

Following aerobic storage of tuna at 4°C for 10 days, the population reached 10^7 cells/g, with relative levels of *Pseudomonas* spp. being 97% and all others 3%. Many of the bacterial species present initially could grow at the storage condition of fish, but *Pseudomonas* spp. had the shortest generation time. As a result, initially even though they constituted only 18% of the population, after 10 days, they became predominant. This dominance is assumed to be attributable exclusively to their rapid growth at chill temperature.

Table 3.2. Biochemical characteristic of the bacterial culture isolated from tuna

Culture no.	Gram's staining	Catalase test	Motiliy	H&L glucose O/F reaction test	Pencillin sensitivity test	Cytochrome oxidase test	Identification
TN1	+	+	-ve	-ve			*Micrococcus*
TN2	+	+	-ve	+			*Staphylococcus*
TN3	+	+	-ve	-ve			*Arthrobacter*
TN4	+	-ve	-ve	-ve			*Lactobacillus*
TN5	+,spore forming	+	-ve	-ve			*Bacillus*
TN6	-ve	+	+ve	-	-ve	+	*Pseudomonas*
TN7	-ve	+	+ve	+	-ve	+	*Vibrio*
TN8	-ve	+	-ve	+	-ve	+	*Aeromonas*
TN9	-ve	+	-ve	+	-ve	-ve	*Enterobacteriac-eae*

Scheme of identification as per Surendran *et al*. (2006)

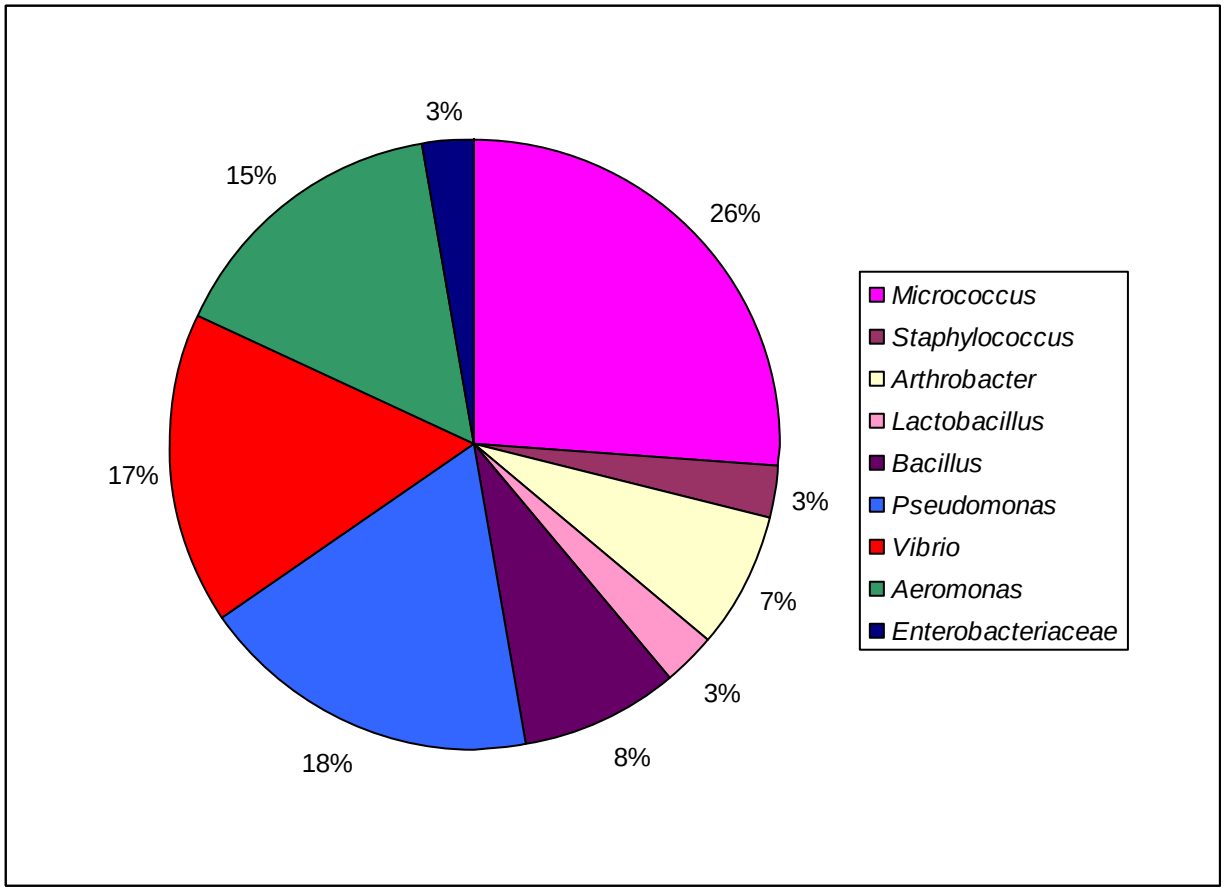


Fig 3.1. Relative distribution of bacterial flora present on tuna

3.3.3. Isolation of Pathogenic bacteria

A total of 54 tuna samples, consisting of 4-5 samples per month were tested for the presence of pathogenic bacteria. Sampling was done as described in 3.2.2.1. From the 10^{-1} dilution of macerated fish, pipetted out 0.5 ml each on the surface of previously dried Tergitol-7, XLD, HEA, BSA, TCBS agar plates. The agar plates showing characteristic colonies were subjected to confirmatory tests.

Bacterial quality parameters like total plate count (TPC), as well as the presence of pathogens like *E.coli*, *S.aureus*, *Salmonella*, *Vibrio cholerae* are presented in Table. 3.3. The total bacterial counts (TPC) of tuna was more or less in the range of 10^5 cfu/g, except a few samples where the total plate counts (TPC) were still higher at 10^6cfu/g. Most of the samples had the presence of *S.aureus*. It was present in 20.4% of the tested samples. *Salmonella* was detected in 2 and *E.coli* in 5 out of 54 samples (3.7% and 9.2%, respectively). Presence of *Vibrio cholerae* was limited to one sample (1.85%).

3.3.4. Determination of the spoiling potential of the bacterial culture in fish media

Spoilage organisms convert many nitrogen compounds into off smelling volatile bases. Non-protein compounds present in fish are good substrate for spoilage organisms. To study the spoilage potential of selected bacteria, 10% fish media was made by adding distilled water to minced fish and then autoclaving at 121ºC, 15 lbs. The desired bacteria was inoculated into the above from a 24 hour culture. Three parameters namely, odor, ammonia formation and hydrogen sulphide production were monitored at 4 different temperatures (28±2°C, 8±2°C , 0±2°C, -20±2°C). The results are given in Fig 3.2.

Table 3.3. Bacterial quality of fresh tuna collected from Cochin

Sl.no	Month	No.of samples	TPC (cfu/gm)	No.of positive results			
				E.coli	*S.aureus*	*V.cholerae*	*Salmonella*
1	Jan	5	$2.50\pm0.12 \times 10^5$	ND	1	ND	ND
2	Feb	4	$2.21\pm0.07 \times 10^5$	ND	N.D	ND	ND
3	Mar	4	$2.20\pm0.23 \times 10^6$	1	3	ND	1
4	Apr	5	$3.10\pm0.16 \times 10^5$	ND	1	ND	ND
5	May	4	$2.80\pm0.29 \times 10^5$	ND	2	ND	ND
6	Jun	4	$2.23\pm0.08 \times 10^5$	2	1	1	ND
7	Jul	5	$3.40\pm0.11 \times 10^5$	ND	1	ND	1
8	Aug	4	$2.62\pm0.08 \times 10^5$	ND	ND	ND	ND
9	Sept	5	$2.48\pm0.15 \times 10^5$	ND	ND	ND	ND
10	Oct	5	$1.90\pm0.02 \times 10^6$	1	1	ND	ND
11	Nov	4	$2.50\pm0.14 \times 10^5$	1	ND	ND	ND
12	Dec	5	$5.80\pm0.05 \times 10^6$	ND	1	ND	ND

The given values are expressed as mean±standard deviation

There was significant difference in the production of odor, ammonia and hydrogen sulphide at room temperature and at 0°C. No significant difference was found in odour production and ammonia production at room temperature and 8°C but, production of hydrogen sulphide was delayed at 8°C. At -20°C, none of the parameters analyzed were detected for a 90 day period.

The result implies that microorganisms actively degrade the fish muscle at ambient temperature. Refrigerated temperature (8°C) can only delay the onset of spoilage by 1 day. Maximum spoilage potential was observed at 28±1°C. Storage at 0°C and -20°C showed the minimum potential for spoilage. The pattern was similar for all the spoilage bacteria tested including the dominant *Pseudomonas spp.* A higher initial load of the spoilage bacteria and the presence of other microorganisms can further lower the shelf-life of the product. Hence,

extending the shelf-life of tuna products can be attained only by controlling the initial load of spoilage microorganisms. Potential use of plant extracts for attaining this goal is analysed in the coming chapters.

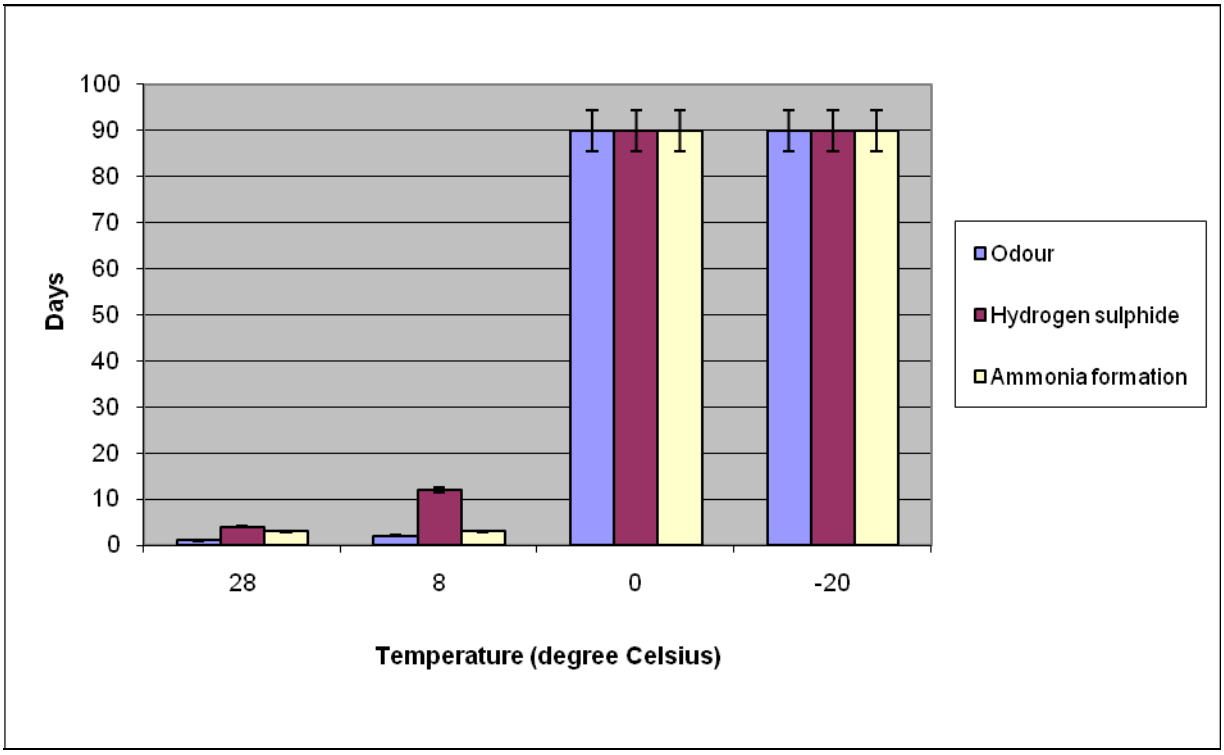


Fig. 3.2. Shelf-life of sterile fish media spiked with spoilage bacteria

3.4. Discussion

3.4.1. Total plate count

Bacterial growth is the main cause of fish spoilage; therefore, it is logical to use bacteria numbers as an index of quality. For high quality fresh fish, the number of bacteria present on the surface vary from 3 to 4 log cfu/g. On gills, counts are normally 1 or 2 orders higher, and intestinal counts can reach 9 log cfu/g (Sikorski, 1990). Increased microbial populations on fish usually result from rapid growth of *Alteromonas* and *Pseudomonas*, which are gram-negative spoilage bacteria (Liston, 1980; APHA, 1992). Spoilage microflora produce enzymes that cause proteolysis, deamination, and decarboxylation resulting in

accumulation of unpleasant metabolites and loss of taste substances (Connell and Shewan, 1980; Huss, 1988; Sikorski, 1990; Sikorski *et al*., 1994). Arannilewa *et al*. (2006) found that the total coliform count range in fish was between 3.0 x 10^3 - 7.5 x 10^6 with increasing values, as the duration of storage increases.

Microbiological criteria for food defines the acceptability of a product or a food lot based on the absence or presence or number of microorganisms, including parasites and/or quantity of their toxins/metabolites per unit mass, volume, area or lot (CAC, 1997b; EC, 1997). The most widely accepted microbiological criteria for chilled and frozen raw fish are those set for aerobic plate counts (APC) at 25°C and *E.coli* proposed by the International Commision on Microbiological Specifications for Foods (ICMSF). An increase of APC to levels in excess of 10^6 cfu/g is usually indicative of inadequate refrigeration, long storage under refrigeration or one of the former prior to freezing. USFDA, EU and BIS have stipulated maximum permissible limits for bacterial parmeters in fresh fish for human consumption (USFDA, 2001). Generally the upper limit fixed for TPC is below 5 x 10^5 cfu/g, *E.coli*,20/g, *S.aureus* 100/g and *Salmonella typhi* and *Vibrio cholerae* to be absent in 25 grams. According to this criteria, the tuna available in Cochin market is not of premium quality. But, the occurance of pathogens like *Salmonella typhi* and *Vibrio cholerae* is below 4%. The total plate count also shows that the tuna is of moderate freshness.

3.4.2. Microbial flora in fish

The percentage composition of bacterial flora of fresh tuna (*Euthynnus affinis*) indicated higher Gram –ve organisms. Among Gram negative bacteria Pseudomonas was dominant followed by Vibrio spp. Jeyasekaran *et al*., (2006), has also reported that *Aeromonas, Pseudomonas and Vibrio* spp were the bacterial microbiota associated with fresh raw seafood. Ristori *et al*. (2007) isolated *Aeromonas spp* and pathogenic *Vibrio spp* from fishes. Among the isolated bacteria, *Pseudomonas spp* and *Bacillus spp* were found in psychrotrophic conditions in previous studies conducted by Singh and Venkataramana (1998).

Storage of tuna at 4°C for 10 days increased the population of microorganisms to 10^7 cells/g. The relative levels of *Pseudomonas* spp.shot up considerably with respect to all others genera. *Pseudomonas* spp. are important spoilage organisms in many chilled food products , such as milk (Reddy *et al*., 1969), chicken (Pittard *et al*., 1982) and fish (Miller *et al*., 1973a,b), in which they become the dominant flora during chilled storage. This dominance is assumed to be attributable exclusively to their rapid growth at chill temperature. Other bacterial species present initially could grow at the chilled storage, but *Pseudomonas* spp. had the shortest generation time. As a result, they became predominant during the storage.

3.4.3. Pathogenic bacteria

Salmonella was detected in 3.7% of the tuna samples tested. Nambiar and Surendran (2003) have also reported the incidence of S*almonella* in 17.3% of the samples from retail fish markets of Cochin. *E.coli* was detected in 5 samples. *S.aureus* was present in 11 out of the 54 samples tested. Presence of *Vibrio cholerae* was limited to one sample. Heinitz *et al*. (2000) found that 10% of imported and 2.8% of domestic raw seafood was positive for *Salmonella, Enterococcus sp* and *Aeromonas sp*, fecal and total coliform. The presence of Salmonella *spp* from the external surface of tilapias were shown by Morales *et al*. (2004). The investigation carried out by Varma *et al*. (1989) reported the presence of *Vibrio cholerae* non-01 in 37.6% of the samples analysed. Similar results were reported by Mathew *et al*. (1988) who carried out the analysis of fresh fish samples for *Vibrio cholerae* in seafoods and environments of Mangalore. 45% of the samples analysed by Sindhu and Surendran (2006) had *Staphylococcus aureus* indicating unhygienic handling and abuse of temperature in Cochin area.

Thampuran *et al*. (2005) reported the microbial quality of the tilapia and indicated that all tissue samples except muscle tissues were contaminated with fecal coliform, where *Escherichia coli* was the most common contaminant and was often encountered in high numbers. The presence of *E. coli* as well as verotoxigenic *E. coli* O157:H7 in fish meal was

investigated by several workers (Ayulo *et al*., 1994; Hwang *et al*., 2004; Thampuran *et al*., 2005 and Ristori *et al*., 2007).

3.4.4. Spoiling potential of the bacterial culture

Spoilage is a natural phenomenon that eventually leads to the decomposition of a food substratum. The spoilage of fish is also an ecological phenomenon that encompasses a series of changes in constituents of fish muscle through natural processes (Nollet and Toldra, 2009). Spoilage organisms convert many nitrogen compounds into off smelling volatile bases which produces foul smell during spoilage. The initial stages of fish spoilage characterized by the loss of characteristic odour are mainly due to autolytic degradation, while final stages of quality deterioration is characterized by softening of flesh texture along with production of off flavour due to microbial activity (Connell, 1995; Gill, 1992). Non-protein compounds present in fish are good substrate for spoilage organisms. The free amino acid pool in the muscle of the fish is readily utilized by typical spoilage organisms by the process of deamination. This results in the formation of ammonia which is the primary compound produced during decomposition of fresh fish (Lakshmanan, 2002). Ammonia is the main component in the total volatile nitrogen (TVN) fraction which often is used as a quality indicator for fresh fish. Thus, high amount of ammonia in these species is an indication of spoilage.

Chemical examination of spoiling fish muscle has shown that organoleptically the most important constituents are the volatile sulphur compounds such as hydrogen sulphide, dimethylsulphide and methylmercaptan. Esters of lower fatty acids such as acetic, propionic, butyric and hexanoic acids are also produced. Volatile sulphur compounds influence the organoleptic characters, especially odours, in spoiling fish. Spoilage associated with H_2S can be detected at even a lower microbial load (Lakshmanan, 2002).

Maximum spoilage potential was observed at 28±1°C. Storage at 0°C and -20°C showed the minimum potential for spoilage. By far the most effective way of reducing the rate of whole fresh fish spoilage is temperature control (Huss,1994; Huss,1995). Fish spoils

as a result of the chemical, biochemical and microbiological reactions taking place within and on the fish. All the chemical reaction rate kinetics (and thereby microbial growth) are temperature-dependent; the lower the temperature of storage, the slower the spoilage processes proceed.

Another important factor is the initial load of *Pseudomonas* spp. present on the sample. Psychrotolerant Gram-negative bacteria like *Pseudomonas* spp., grow on chilled fish. *Pseudomonas* spp. mediated spoilage is characterized by fruity, oniony and faecal odours from the production of ketones, aldehydes, esters and non-hydrogen sulphide sulphur-containing compounds such as methyl sulphide (Miller *et al*., 1973b; Vogel *et al*., 2005). Members of the genus are able to produce pigments, and proteolytic and lipolytic enzymes that may affect the quality of fresh and more especially, processed fish products.

The food with a relatively higher initial loads of spoilage bacteria and a storage condition that favours rapid growth will spoil more rapidly than a food with a low initial load of microbes with longer generation time. To reduce microbial spoilage of a food, one needs to aim at achieving both the initial load and longer generation time of spoilage microorganisms during storage. It has to be recognized that the mere presence of 10^7cells/g with out growth (eg. from massive initial contamination) will not immedietly cause the food to lose its acceptance quality; but such food will spoil rapidly making it unsafe for consumption.

3.5. Conclusion

The present study clearly shows that the tuna caught from the Indian Ocean are not spoiled ,but are contaminated with pathogens and spoilage bacteria. The total bacterial counts (TPC) of fresh tuna was in the range of 10^5 cfu/g. Most of the samples had the presence of *S.aureus*. It was present in 20.4% of the tested samples. Presence of *Vibrio cholerae, Salmonella, E.coli* were limited to 1.85%, 3.7% and 9.2%, respectively.

Fish flesh starts

ed visibily to spoil at bacterial levels above 10^7 organisms/g and this is often known as spoilage detection level. The percentage composition of bacterial flora of fresh tuna (*Euthynnus affinis*) indicated higher Gram negative organisms compared to Gram positive. Among Gram negative bacteria *Pseudomonas* spp. was dominant followed by *Vibrio* spp. Following aerobic storage of tuna at 4°C for 10 days, the population reached 10^7 cells/g, with relative levels of *Pseudomonas* spp. 97% and all others 3%. Many of the bacterial species present initially could grow at the storage condition of fish, but *Pseudomonas* spp. had the shortest generation time. As a result, initially even though they constituted only 18% of the population, after 10 days, they became predominant. Proper sanitary care is needed to reduce the contamination and so care should be taken during processing and preservation to ensure the safety of the consumer.

Maximum spoilage potential was observed at 28±1°C. Storage at 0° C and -20°C showed the minimum potential for spoilage. The pattern was similar for all the spoilage bacteria tested including the dominant *Pseudomonas spp*. To reduce microbial spoilage of a food, one needs to aim at achieving both low initial load and longer generation time of spoilage microorganisms during storage.

Faecal pollution in marine environment poses a potential health hazard if fish caught from such regions were consumed without adequate processing. The spoilage and pathogenic bacteria isolated from tuna was used for screening the antimicrobial activity of spice extracts and the method of analyzing antimicrobial activity and the results are given in the next chapter.

4.1. Introduction

Emergence of psychrotrophic food-borne pathogens has been a main concern in ready to cook or processed products. Based on this fact, re-evaluation of food preservation methods is unavoidable matter. Therefore, the introduction of new or improved methods that comply with current needs like, chilled products with low levels of chemical preservatives is essential. A large number of antibiotics and chemical preservatives have been tried earlier to check the growth of psychrophilic flora of fish stored in ice and to extend its shelf life for a reasonably longer period (Anand and Setty, 1981). Some of the chemical preservatives used were propyl-hydroxy-4-benzoate, methyl-para hydroxyl benzoate, o-chlorobenzoic acid, sodium hypochlorite, polyethylene glycol etc. Sodium hypochlorite is the prominent one among these. Only marginal activity was reported by sodium hypochlorite (20ppm) against six genera of bacteria isolated from marine fish (Anand and Setty, 1981). Recently, consumers have started losing faith in the chemical preservatives due to their reported side effects and toxicity problems. The net result is increasing pressure on food manufacturers to either completely avoid chemical preservatives in their food products or to adopt more 'natural' alternatives for the maintenance or extension of a product's shelf life. In this context, the food industry and food research have driven towards the use of 'natural' ingredients or biopreservation.

Uncontrolled use of chemical antimicrobial preservatives has been an inducing factor for appearance of microbial strains more and more resistant to classic antimicrobial agents. Isolation of multi-resistant strains, has been reported all over the world and it is difficult to control their survival. Fifty years of increased use of chemical antimicrobials have created an ecological imbalance and enrichment of multiples of multi-resistant pathogenic microorganisms (Levy, 1997). Antibiotic resistance in food borne pathogens is a reality, though substantial qualitative and quantitative differences have been observed (Teuber, 1999 a and b). Strains of resistant food borne pathogens to a variety of antimicrobials have become a major health concern (Kiessling *et al*., 2002) and it could decrease the successful application of control measures on spoilage and pathogen microorganisms, many times leading for use of less safe, ineffective or expensive alternatives (Levy, 1997). Brull and

Coote (1999) have reported microbial resistance for some antimicrobials used in food conservation such as weak-organic acids, hydrogen peroxide, chelators and some small organic biomolecules.

The successful story of microbial chemocontrol lies in the continuous search for new antimicrobial substances to control the resistant strains. Biopreservation opens a new vista in this area. Biopreservation often implies the use of *Lactobacillus*, their metabolic products or both to improve safety and quality of foods (Montville and Winkowski, 1997). The use of other antimicrobial compounds of plant, animal or microbial origin is also considered in biopreservation (Ray, 1992). Many studies indicate that in some plants there are many substances such as peptides, alkaloids and essential oils. These plants are potentially significant therapeutics against human pathogens including bacteria (El astal *et al*., 2005).

4.1.1. Antimicrobial compounds of plant origin

Although more than 1300 plants have been reported as potential sources of antimicrobial agents (Wilkins and Board, 1989), such alternative compounds have not been sufficiently exploited in foods to date. Components present in intact plants include alkaloids, dienes, flavonols, flavones, glycosides, lactones, organic acids, phenolic compounds, and protein like compounds (Lopez-Malo *et al*., 2000). Of greatest potential as food antimicrobials are compounds from spices and their essential oils.

4.1.2. Spices as food antimicrobials

In recent times, there has been increasing interest in discovering new natural antimicrobials (Sagdiç *et al*., 2003a). Plant products with antimicrobial properties have obtained emphasis for a possible application in food production in order to prevent bacterial and fungal growth (Lanciotti *et al*., 2004). Plant products are characterized for a wide range of volatile compounds. Some of them are important flavor quality factors (Utama *et al*., 2002).

Besides, plant volatiles have been generally recognized as safe (GRAS) (Newberne *et al*., 2000). Systematic screening for biological interactions between microorganisms and plant products has been a major source of new and effective antimicrobial substances. They can have different action ways on/in the microbial cell when compared to other conventional antimicrobials (Souza *et al*., 2005). Plants synthesize many compounds with complex molecular structures by a secondary metabolism and some of them have been related with antimicrobial properties. Some of these secondary metabolites are alkaloids, flavonoids, isoflavonoids, tanins, cumarins, glycosides, terpens and phenolic compounds (Simões *et al*., 1999).

Being plant natural foodstuffs, spices appeal to consumers who tend to question the safety of synthetic additives (Farag *et al*., 1989a; Sagdic *et al*., 2003a,b). Antimicrobial properties of spices have been documented in recent years and interest continues to the present (El-Shami *et al*., 1985; Akgul and Kivanç, 1988; Cosentino *et al*., 1999; Dorman and Deans, 2000; Ristori *et al*., 2002; Radhakrishanan and Velusamy, 2003). Still little information is available emphasizing the preservative and antimicrobial role of spices in the prevention of foods and of the microbial action.

Spices are recognized to stabilize the foods by controlling the microbial action. This could be observed when spices show initially high microbial charge and as the time progresses, the microbial growth become progressively slower or it is eventually totally suppressed (Kizil and Sogut, 2003). Antimicrobial activity of spices depend on several factors, which includes: a) kind of spice, b) composition and concentration of spice, c) microbial species and its occurrence level, d) substrate composition and e) processing conditions and storage (Shelef, 1983; Farag *et al*., 1989a)

Spices have been defined as plant substances from indigenous or exotic origin, aromatic or with strong taste, used to enhance the taste of foods (Germano and Germano, 1998). Spices include leaves (bay, mint, rosemary, coriander, laurel, oregano), flowers (clove), bulbs (garlic, onion), fruits (cumin, red chilli, black pepper), stems (cinnamon), rhizomes (ginger) and other plant parts (Shelef, 1983). Although, spices have been well known for their medicinal, preservative and antioxidant properties, they have been currently

used with primary purpose of enhancing the flavor of foods rather than extending shelf-life (Aktug and Karapinar 1986, Ristori *et al*., 2002).

Active compounds in spices have been included in class of naturally occurring food preservatives and their inclusion in foods is allowed by food production regulator offices (Brull and Coote, 1999). Several scientific reports describe the inhibitory effect of spices on a variety of microorganisms, although considerable variation for resistance of different microorganisms to a given spice and of the same microorganisms to different spices has been observed (Akgul and Kivanç, 1988).

Gould (1995) has emphasized the possible use of spices and derivatives like alternatives for inclusion in a new perspective of food conservation called "natural antimicrobial system", which could use the synergistic effect of antimicrobial compounds from animal, plant and/or microbial origin. This will create an inhospitable environment for microbial survival in foods. Recent studies done by Tijjani *et al*. (2011, 2012) and Zhang *et al*. (2009) also proves that medicinal plants have important chemical compounds with pharmacological value.

The objective of research work in this chapter was to examine the antibacterial activity of spice extracts against pathogens isolated from seafood and type culture obtained from MTCC.

4.2. Materials and Methods

A.Materials

4.2.1. Raw material

Fresh tuna (*Euthynnus affinis*) was procured from Vypeen Harbor, Kochi. The size of the fish was about 60-70cm and weighed 1-1.5Kg.The fish was washed, beheaded, gutted and cut into chunks of 2cm x1 cm x1cm (l x b x h) without any delay. These fish chunks were used for further studies.

4.2.2. Spice oleoresins

Initial antimicrobial screening was done on ethanol extracts of the spices prepared in the laboratory. For obtaining the ethanol extracts 100g of shade dried plant parts ground into fine powder was taken and soaked in 150-ml ethanol. It was kept for 48hrs with intermittent shaking, filtered and evaporated the ethanol out of the filtrate completely. The extract was diluted in 1ml ethanol and used for the initial screening of antimicrobial activity. The later studies were limited to six spices namely, Garlic, Cardamom, Turmeric, Oregano, Rosemary and Clove based on the initial study and literature review. Spice oleoresins were used for further investigation to ensure standard characteristics. These were obtained from M/s Synthite Chemicals, Kolenchery.

4.2.3.Bacterial cultures

For this study pathogenic bacteria isolated from tuna and type cultures obtained from NACCH, School of Environmental studies of Cochin University of Science and Technology were used. The type cultures used were MTCC 3906 *Vibrio cholerae* (0139), MTCC 45 *Escherichia coli*, MTCC 1457 *Shigella flexneri* .

4.2.4. Bacteriological Media

For microbiological examination, standard culture media, namely, Nutrient agar, Nutrient broth, Bismuth Sulphite Agar (BSA), Hektoen's Enteric Agar (HEA), Tergitol-7 Agar(T-7), Thio-sulphate Citrate Bile Salt Sucrose Agar (TCBS) and Xylose lysine desoxycholate Medium (XLD) were used (Himedia brand). Composition of the media is as per Section 3.2.1.2.

Composition of Mueller Hinton Agar

Peptone	10.0g
NaCl	10.0g
Agar	15.0g

DW 1L

pH 7.2±0.1.Distributed in 100ml quantities in conical flasks.Sterilize at 121°C for 15 minutes.

Preparation of fish media

Tuna meat was minced with 5 times its weight of distilled water. One ml each of this meat slurry was transferred to sterilized test-tubes (15cm x 18mm dia) and sterilized by steaming in an autoclave for 30 minutes. The tubes were cooled and used as media for growth studies of pathogenic bacteria in the presence of spice extracts.

Paper disks

Whattman No.1 filter papers were used for making 6mm paper disks. These disks were placed in a sterile Petri dish, covered in brown paper and autoclaved at 121° C at 15 lbs pressure for 15 minutes. Later it was oven dried. These disk were used for antimicrobial screening.

4.2.5. Reagents for sample preparation (Scanning Electron Microscope)

Gluteraldehyde solution (2.5%)

Analytical grade 25% gluteraldehyde was purchased. 10 ml of 25% gluteraldehyde was diluted to 2.5% using 90 ml distilled water.

Sterile seawater

Dissolved 0.5g of NaCl into 1L distilled water. It was autoclaved at 121° C at 15lbs pressure for 15 minutes. Used after reaching room temperature.

Acetone series

An acetone series of 70-100% was prepared for dehydrating the SEM sample.

B.Methods

4.2.6. Antimicrobial activity screening

Antimicrobial susceptibility test of the isolated organisms was done by disc diffusion method using the Kirby-Bauer technique (Bauer *et al.,* 1966) and as per modifications of Islam *et al.,* (2008). All tests were performed on Mueller-Hinton agar. The surface was lightly and uniformly inoculated by cotton swab. Prior to inoculation, the swab stick was dipped into bacterial suspension having visually equivalent turbidity to 0.5 McFarland standards. The swab stick was then took out and squeezed on the wall of the test tube to discard extra suspension. The swab was streaked in at least three directions over the surface of the Mueller-Hinton agar to obtain uniform growth.

Allowed the plates to dry for five minutes. Using sterile forceps, placed the disks on the plate. Incorporated the antimicrobial agent using micropipette under aseptic conditions. Gentamycin disks were used as standard. Incubated the plates within 15 minutes after applying the disks. Following overnight incubation at 37°C, measured the diameter of the zone of growth inhibition around each disk to the nearest whole mm. Results were recorded .

4.2.7. Minimum inhibitory concentration (MIC)

Preparations of plates

100 ml Mueller-Hinton medium of each flask was autoclaved and allowed to cool at 50°C in water bath. Required amount of antimicrobial agent (0.10% to 0.80%) was added to each flask, mixed thoroughly and the media was poured immediately on the plate.

Inoculum preparation for MIC test (Islam *et al*., 2008)

Inocula were obtained from an overnight agar culture of the test organism. Inoculum for the MIC test was prepared by taking at least three to five well-isolated colonies from an agar plate culture. The top of each colony was touched with a sterile loop and the growth was transferred into a tube containing 4 to 5 ml of normal saline. The broth culture was incubated at 35°C until it achieved the turbidity of the 0.5 McFarland standards. The turbidity of the actively growing broth culture was adjusted with sterile broth to obtain turbidity comparable to that of the 0.5 McFarland standards.

Turbidity standard for MIC inoculum preparation

To standardize the inoculum density for a susceptibility test, $BaSO_4$ turbidity standard, equivalent to a 0.5 McFarland standards was used. A 0.5 McFarland standard was prepared as described in Islam *et al*. (2008). To make the turbidity standard, 0.5 ml of 1.175% barium chloride solution was added to 99.5 ml of 1% sulfuric acid solution and mixed well. A small volume of those turbid solutions was transferred to a screw-capped tube of the same type as used for preparing the control inocula and stored in the dark at room temperature.

Inoculation and incubation of the medium

Agar surface of the plates containing different concentration of antimicrobial agent and the control plate without antimicrobial agent were spot inoculated with a 2µl suspension. Inoculation was done from the plate containing lowest concentration of antimicrobial and the control plate was inoculated lastly. Inoculated agar plates were allowed to stand until the inoculum spot were completely absorbed and after then it was incubated at 35°C for overnight.

Interpretation of results

The MIC represents the minimum concentration of antimicrobial at which there is complete inhibition of growth. In reading the end points, a barely visible haze of growth or a single colony is disregarded. Media impregnated with ethanol and later inoculated with bacteria was kept as control. The MICs were determined as the lowest concentration of extract inhibiting the visible growth of each organism on the agar plate.

4.2.8. Disposal of the used culture plates and other items

All the used culture plates and other articles used for the microbiological experiments were disposed off very carefully. All the microorganisms present were killed by sterilization in an autoclave at 15 lbs for 30 minutes. The pipettes used for pipettting of live bacterial cultures and slides used for the experiment with live culture were disinfected by keeping immersed in 10% phenol overnight before washing.

4.2.9. Bacterial preparation for SEM

An aliquot of 10ml culture was centrifuged at 8000rpm in a refrigerated centrifuge for 15 minutes. The pellets were washed with sterile seawater of 0.5% salinity and fixed in 2.5% gluteraldehyde prepared in sterile seawater at 4°C overnight. Subsequently the suspension was washed repeatedly with seawater and dehydrated. the dehydration was done through an acetone series of 70-100%, centrifuged and supernatant removed. After dehydration with 100% acetone, it was kept overnight in a dessicator. The sample was sputter-coated with 25nm platinum. Observations were made on a JEOL model JSM-6390 LV microscope.

4.3. Results and discussion

4.3.1. Antibacterial activity of spices

Initial screening of antimicrobial activity was done using ethanol extracts of selected spices. The activity was tested against MTCC 45 *Escherichia coli* and spice extracts which did not show any significant inhibition of the bacterial culture and those that developed fungal growth during storage was eliminated from further study. Due to the high antimicrobial activity, garlic, clove, cardamom, oregano, rosemary and turmeric were selected for further investigations.

4.3.2. Effect of spice oleoresins on bacteria

The effect of natural antimicrobial agents on the bacterial species isolated from tuna and pathogenic bacteria obtained from MTCC collection was observed on Mueller-Hinton agar as described in Section 4.2.6. The diameter of the zone of inhibition of each spice against specific bacteria is presented in the Fig.4.1-4.5. Control plates were also kept to confirm the viability of the bacteria and for comparing results (Plate 4.7). Data obtained in this study was analysed statistically using SPSS (Scientific Package of Social Science) version 17.0. Two way ANOVA test was used to compare differences in means of inhibitory zones of different pathogenic bacteria and amount of spice used for producing the inhibition. This was followed by Tukey post-hoc analysis to determine specifically which treatment showed significant difference from control.

Two way ANOVA showed that there was significant difference in the inhibitory zones of bacteria among the different spice treatments (Appendix A: 4.1). The results showed that all spice oleoresins tested exhibited different degrees of antibacterial activity against the tested organisms and the activity differed significantly ($p \leq 0.05$) from that of control.

Among the pathogenic bacteria analysed, 5µl of the undiluted oleoresin of clove produced 20mm clear inhibition zone against *Escherichia coli* (MTCC 45) in the disc diffusion method (Fig 4.1). 3-4 µl of clove oleoresin showed moderate activity of 16-17mm. Activity of turmeric was nil for *E.coli* at all the concentrations used in the analysis (activity equallent to 6mm is taken as nil). Oregano, cardamom and rosemary exhibited moderate activity where as activity of garlic was weak. The results were similar for the five pathogenic bacteria analysed. Antibacterial activity against *Vibrio cholerae* (0139) is presented in Fig 4.2 and activity against *S.typhi* is depicted in Fig 4.3.

The pattern of inhibition of *S.typhi* by various spices was almost similar to the one shown by *E.coli*. *S.aureus* was the most resistant bacteria among the tested species (Fig 4.4). Though clove produced the maximum inhibitory zone for *S.aureus* (15±1.4mm), it was comparatively less than those generated for *V.cholerae* (16±2mm) and *E.coli* (20±2mm) by the same spice extract. *S.aureus* was susceptible to garlic and turmeric at 3µl and 4µl respectively though they showed negligible activity (< 9mm). Cardamom, turmeric and oregano expressed good activity toward *Shigella flexnerii* (MTCC 1457). As in the other cases, clove generated the highest inhibition zone for this pathogen also(Fig 4.5.).

Figure 4.1 to 4.5 and Plates 4.1 to 4.6 clearly indicates that as the concentration of spice increases the zone of inhibition increases. This is because of the presence of higher amount of antibacterial component with increasing concentration.

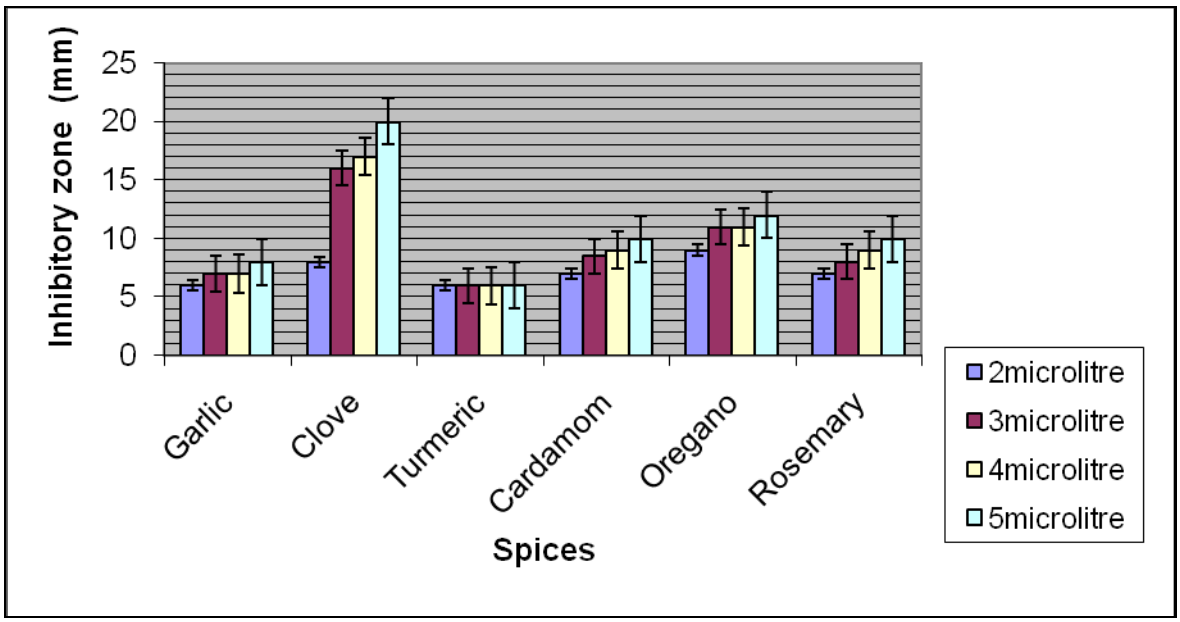


Fig.4.1. Inhibitory zones of *E.coli* at various volumes of spice extracts.

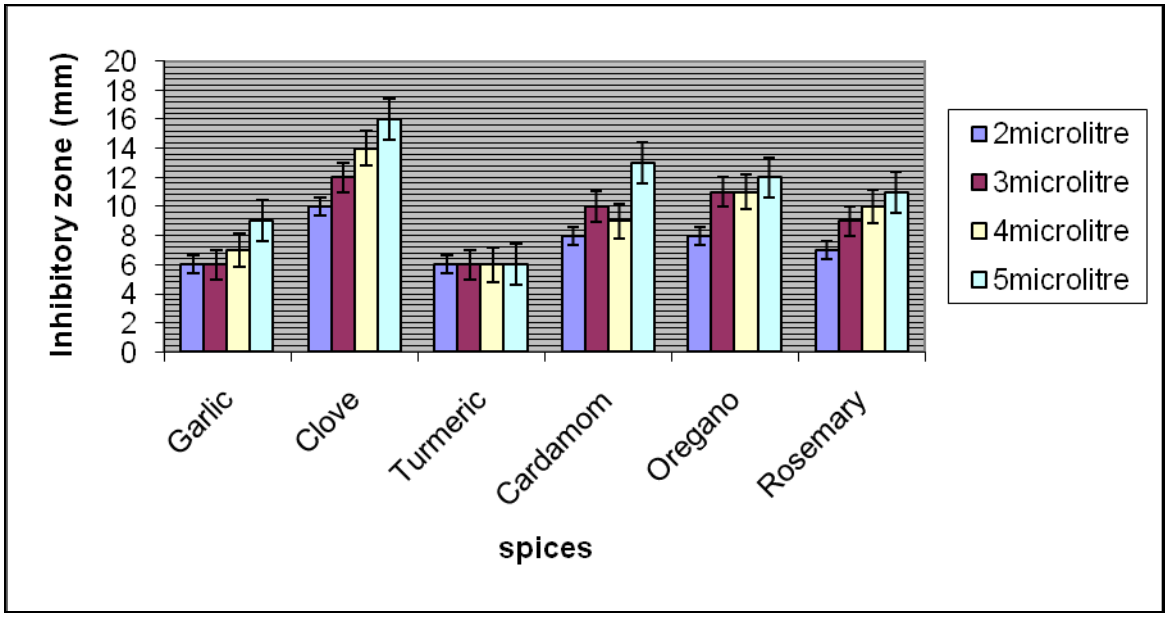


Fig.4.2. Inhibitory zones of *V.cholerae* at various volumes of spice extracts.

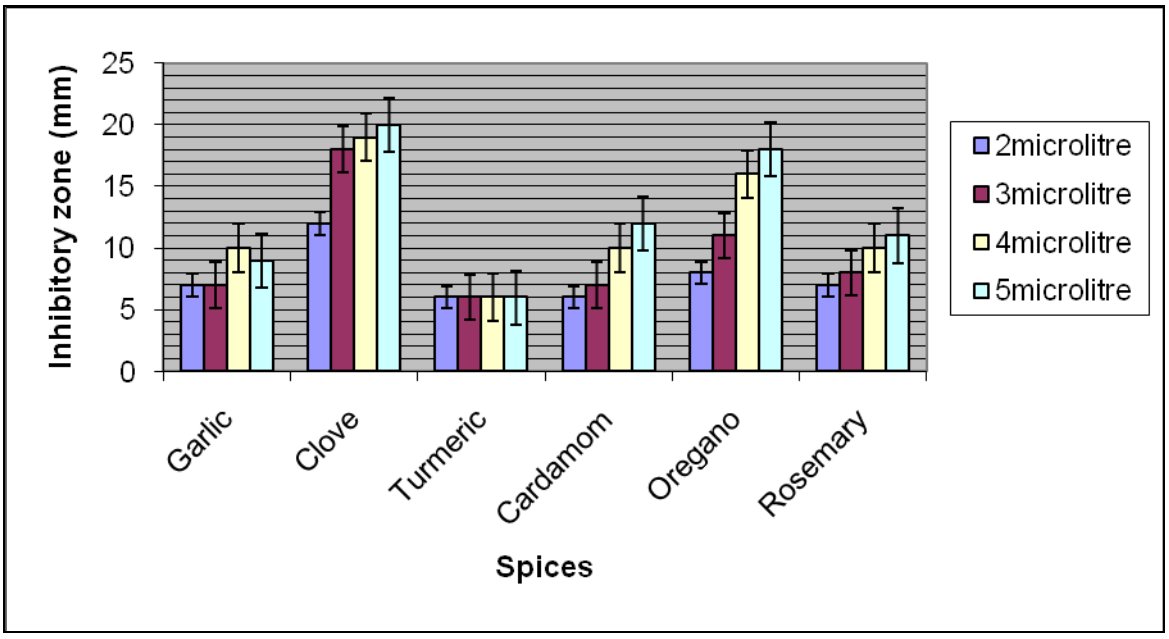


Fig.4.3. Inhibitory zones of *S.typhi* at various volumes of spice extracts.

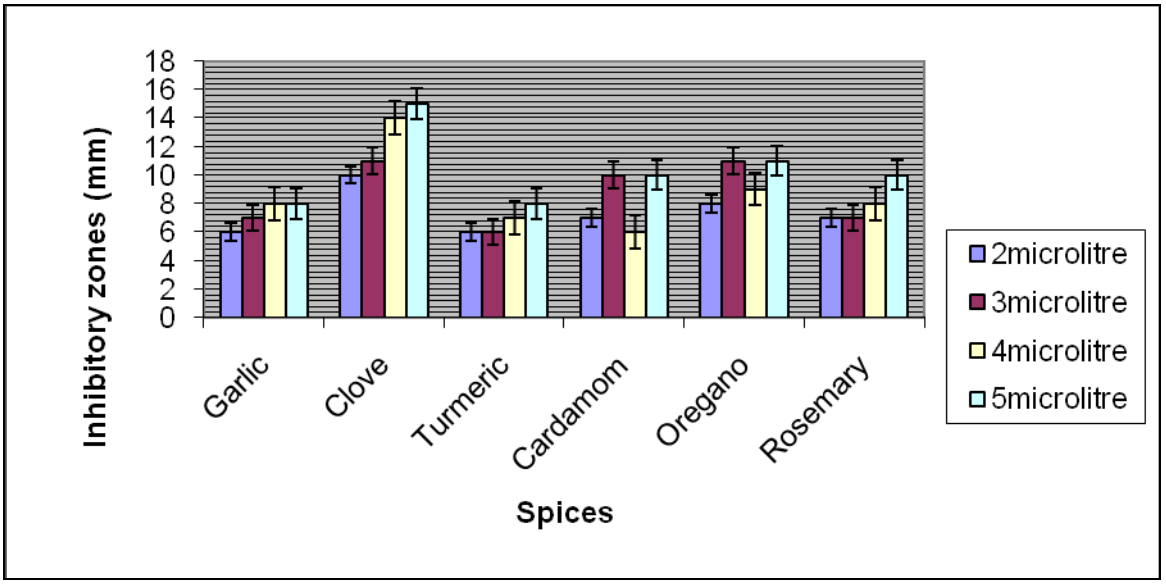


Fig.4.4. Inhibitory zones of *S.aureus* at various volumes of spice extracts

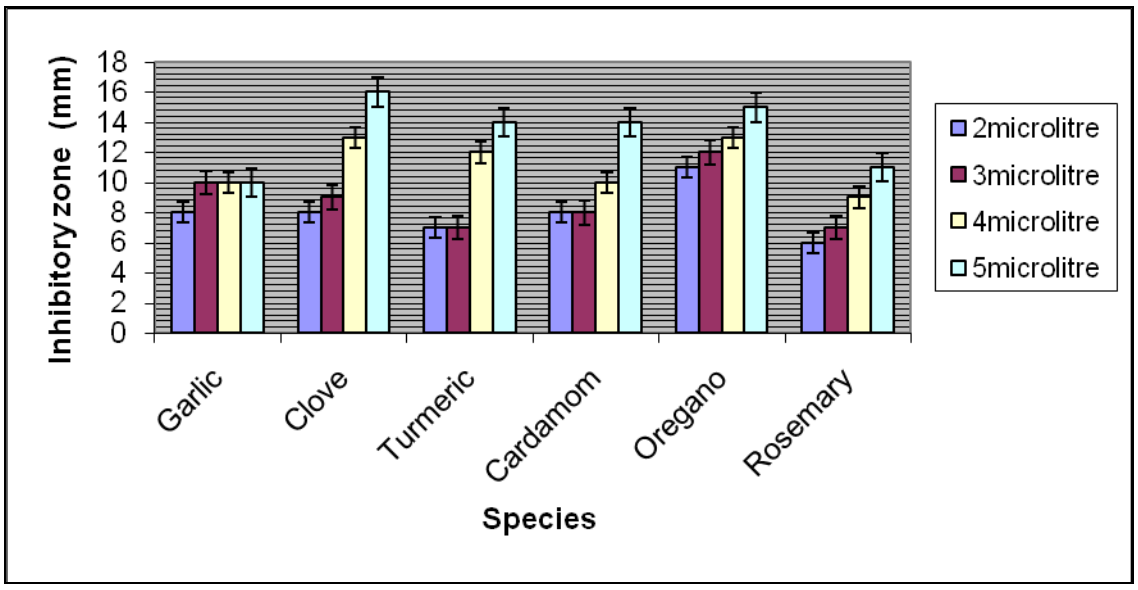
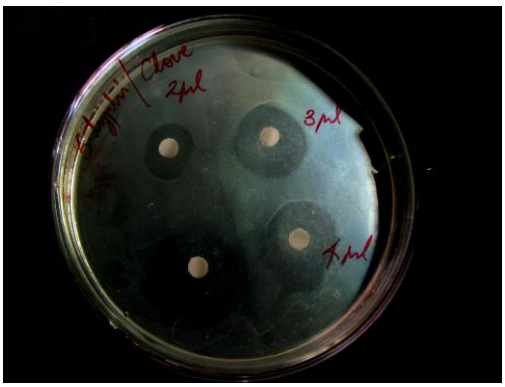


Fig.4.5. Inhibitory zones of *S.flexnerii* at various volumes of spice extracts.

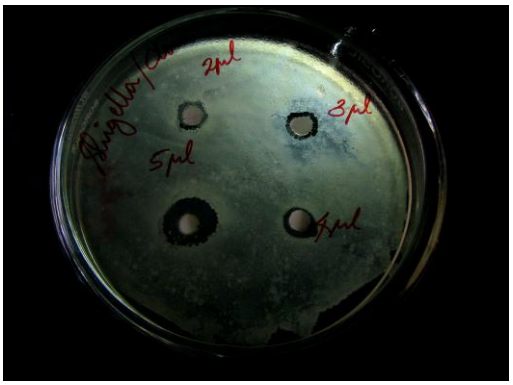
Table 4.1 summarizes the antibacterial activity of spice extracts against pathogenic bacteria. Under the test conditions, all bacterial strains showed some degree of susceptibility towards each spice. The extracts were effective against both Gram-positive and Gram-negative microorganisms. Clove and oregano appeared more active, exerting greater inhibitory activity against bacterial strains. Table 4.2 compares the inhibitory zones of spoilage bacteria isolated from tuna. ANOVA between spoilage bacteria and treatments showed that there is significant difference between the inhibition zone produced by the treatments and bacteria used for the study (Appendix A: 4.2.). Inhibitory zone measurements reveal that bacterial strains isolated from tuna was more resistant to the spice extracts compared to the pathogenic strains. The inhibitory zones for pathogens were 6-20 mm and that of bacterial strains from tuna was 7-18mm. Turmeric showed excellent activity towards the spoilage bacteria though the activity was limited for *E.coli,V.cholerae* and *S.typhi*.

The post hoc tukey test conducted to analyse the behaviour of different spoilage microorganism against spices revealed that *Lactobacillus* vs *Bacillus* (0.474), *Lactobacilus* vs *Micrococcus* (0.954) and *Bacillus vs Micrococcus* (0.144) had p value greater than 0.05 (Appendix A: 4.2).

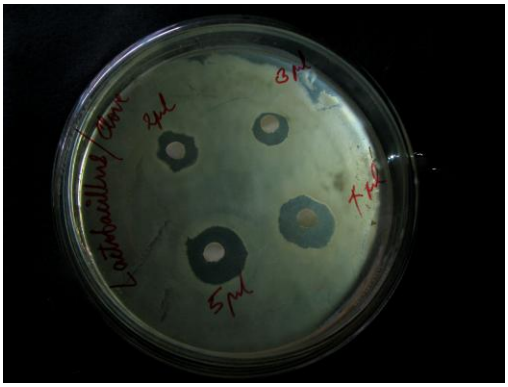


4.1 (a)

4.1 (b)

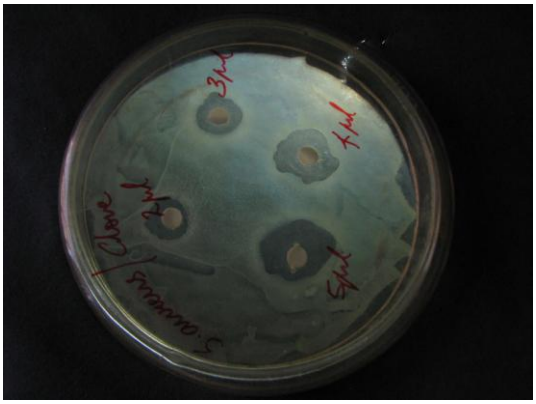


4.1 (c)

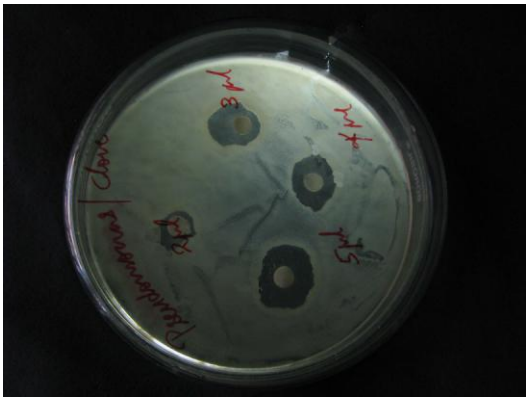


4.1 (d)

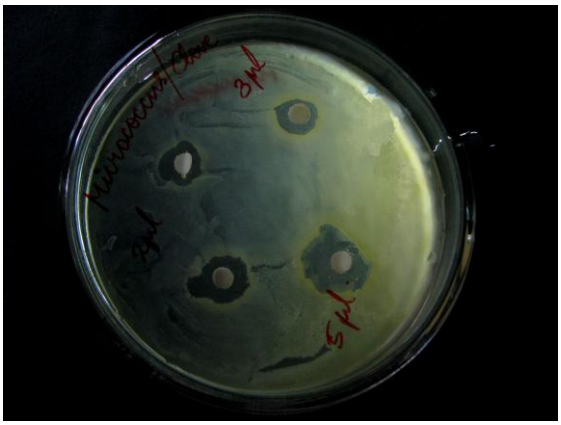
4.1 (e)



4.1 (f)



4.1 (g)

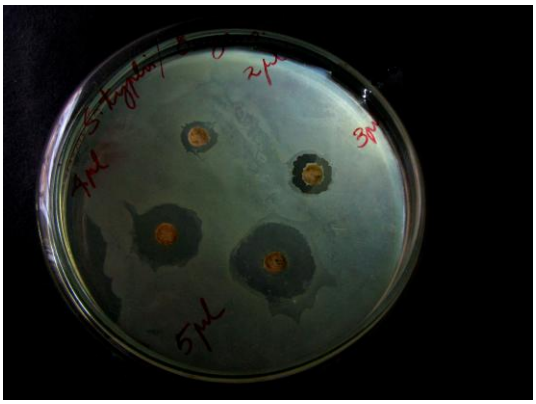


4.1 (h)

4.1(i)

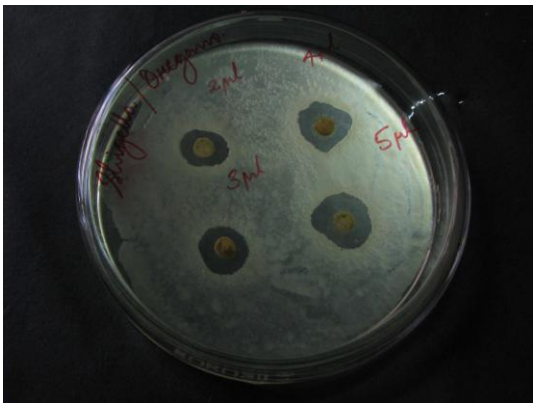
4.1(j)

Plate 4.1. Inhibitory zones produced by clove on bacteria. a - *Salmonella typhi*, b - *Vibrio cholerae*, c - *Shigella flexneri*, d - *Lactobacillus spp.*, e - *Aeromonas spp.*, f - *Staphylococcus aureus*, g - *Pseudomonas spp.*, h - *Micrococcus spp*, i - *Escherichia coli.* and j - *Bacillus spp*.



4.2.(a)

4.2 (b)



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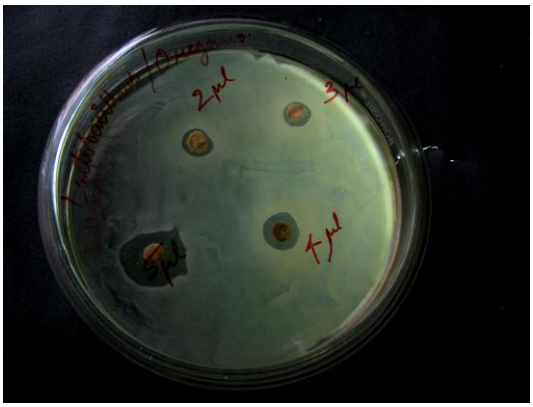

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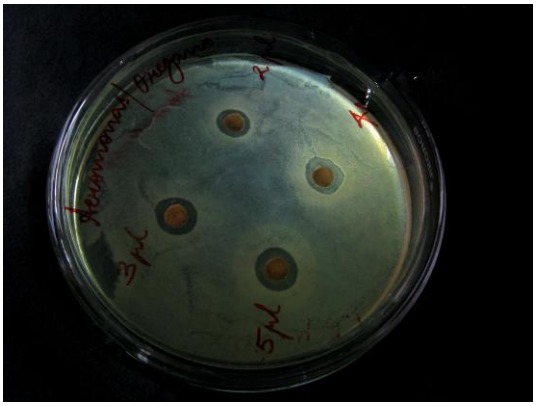

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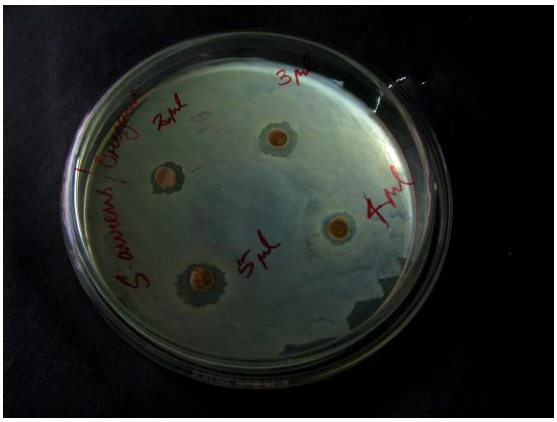

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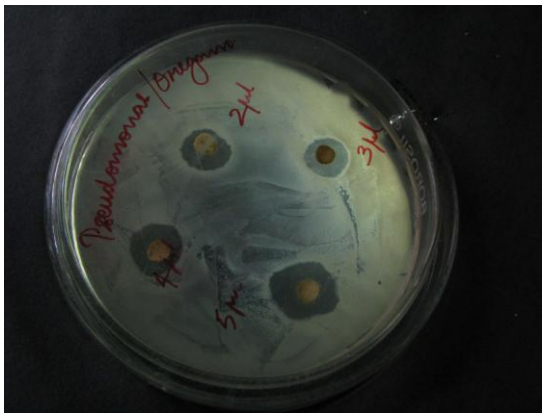

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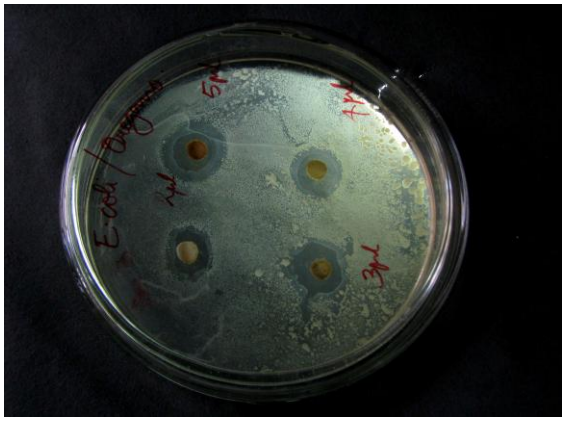

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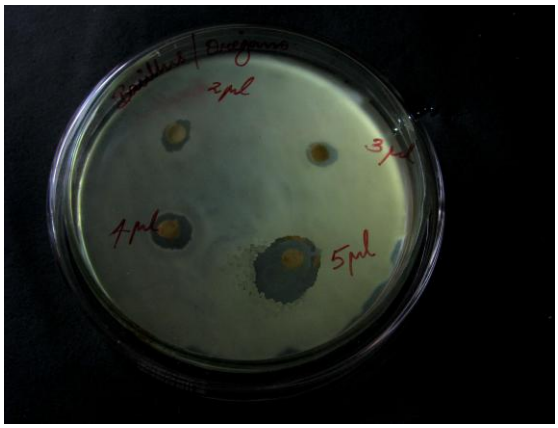
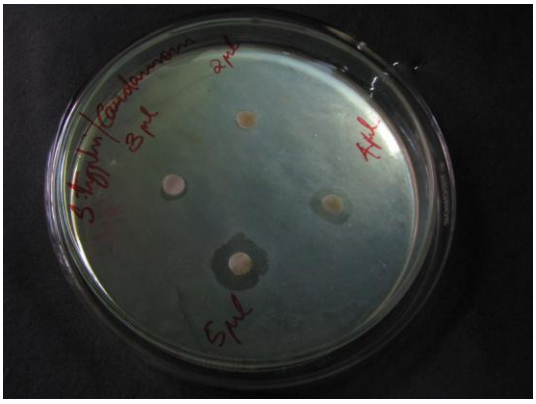


4.2 (j)

Plate 4.2. Inhibitory zones produced by oregano on bacteria. a - *Salmonella typhi*, b - *Vibrio cholerae*, c - *Shigella flexneri*, d - *Lactobacillus spp.*, e - *Aeromonas spp.*, f - *Staphylococcus aureus*, g - *Pseudomonas spp.*, h - *Micrococcus spp*, i - *Escherichia coli.* and j - *Bacillus spp*.



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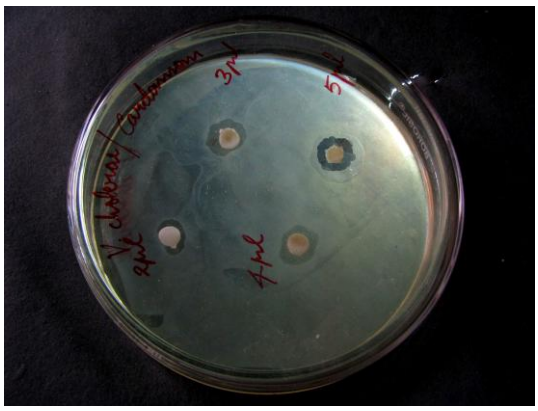

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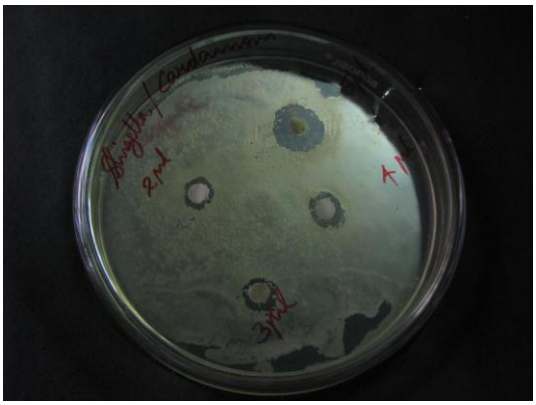

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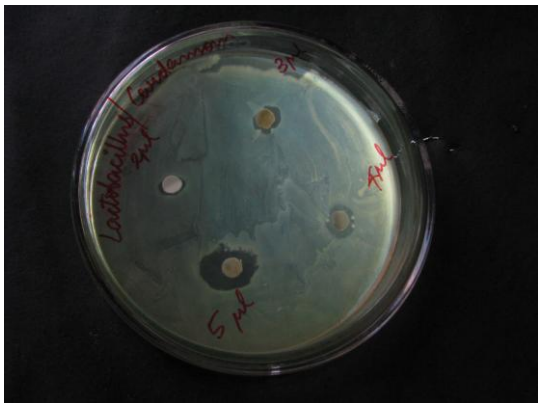

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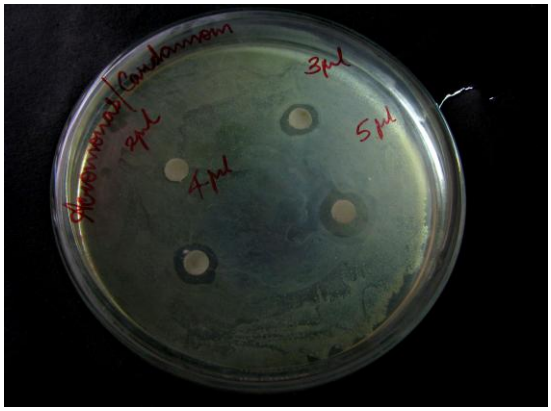

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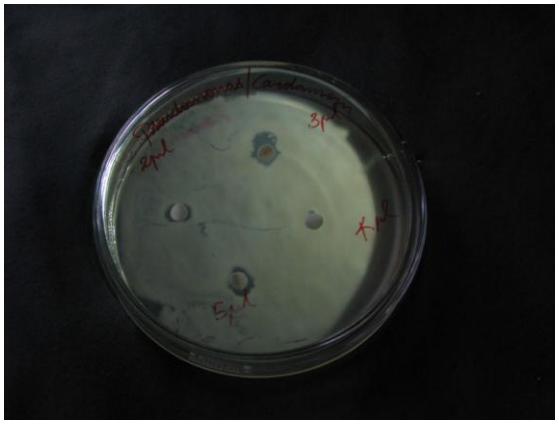

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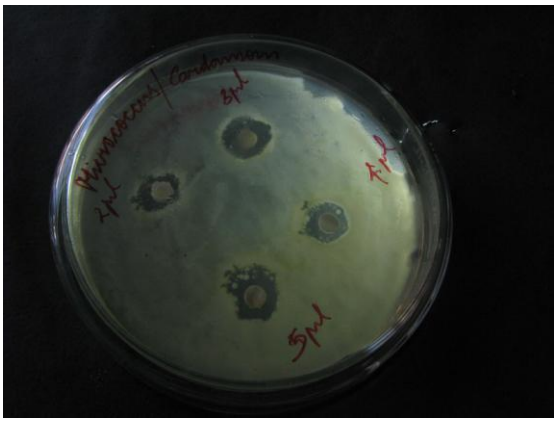

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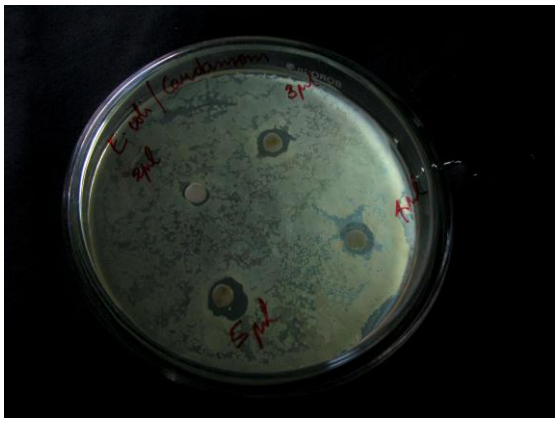

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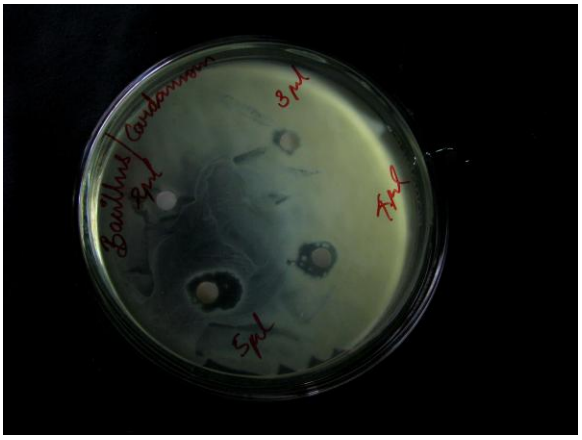
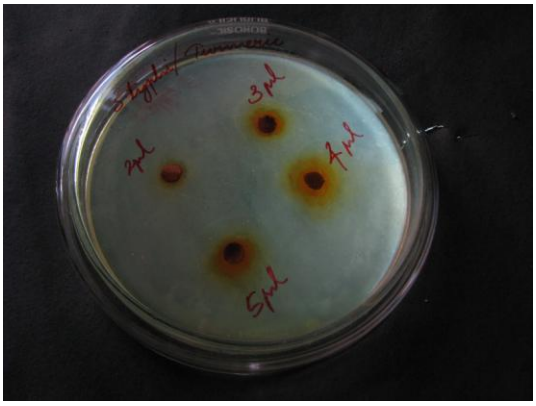


4.3 (j)

Plate 4.3. Inhibitory zones produced by cardamom on bacteria. a - *Salmonella typhi*, b - *Vibrio cholerae*, c - *Shigella flexneri*, d - *Lactobacillus spp.*, e - *Aeromonas spp*., f - *Staphylococcus aureus*, g - *Pseudomonas spp.*, h - *Micrococcus spp*, i - *Escherichia coli.* and j - *Bacillus spp*.



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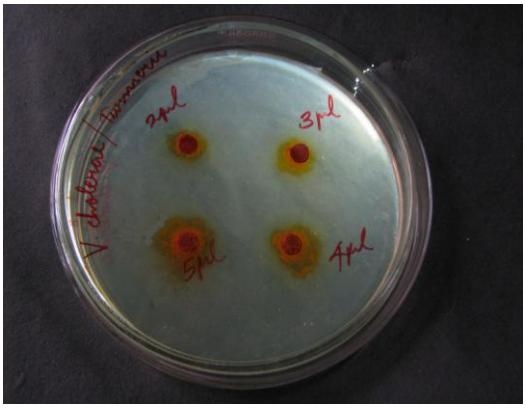

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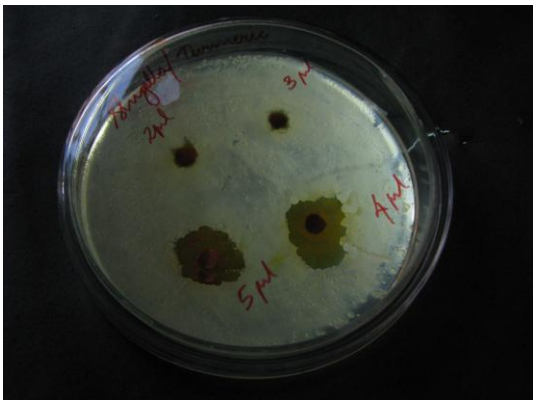

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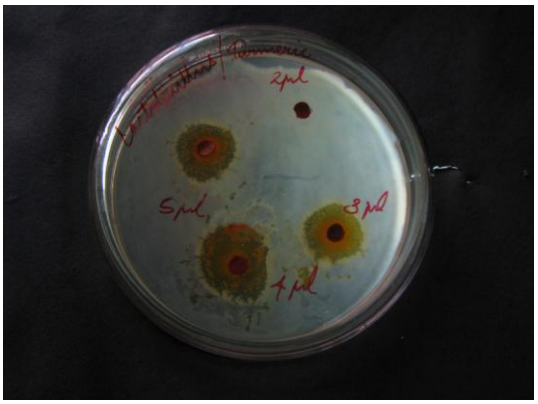

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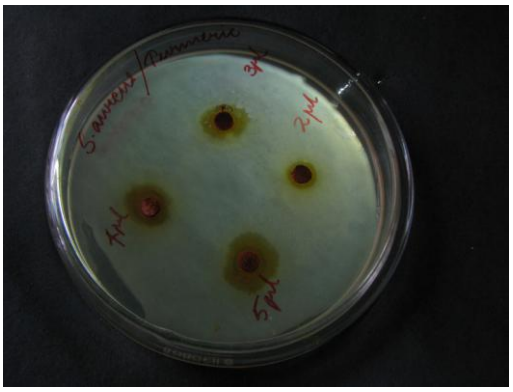
4.4 (e)



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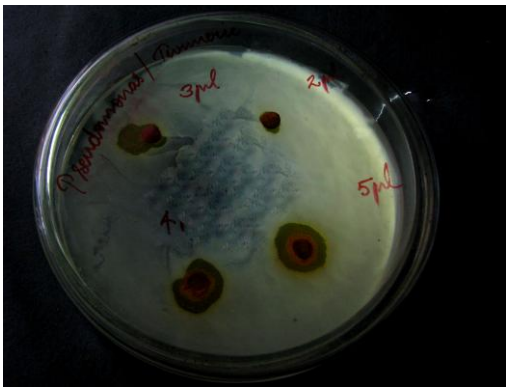

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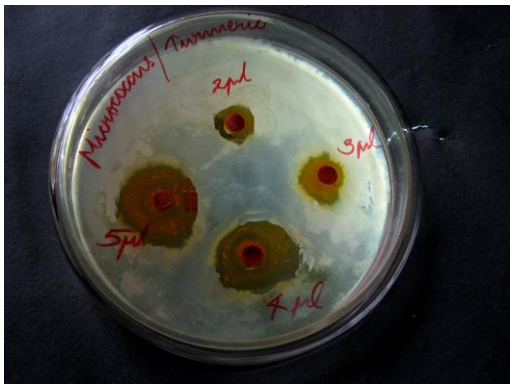

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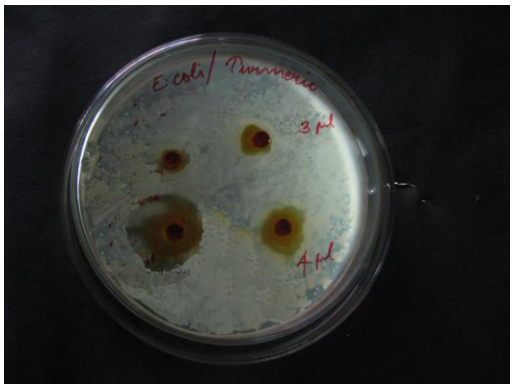

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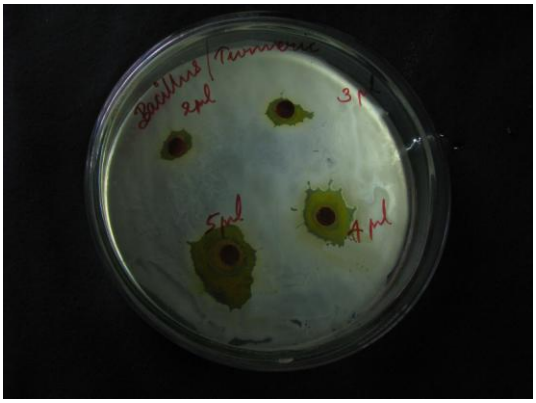
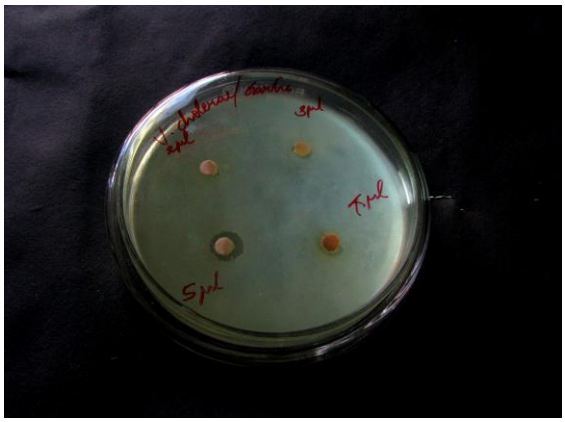


4.4 (j)

Plate 4.4. Inhibitory zones produced by turmeric on bacteria. a - *Salmonella typhi*, b - *Vibrio cholerae*, c - *Shigella flexneri*, d - *Lactobacillus spp.*, e - *Aeromonas spp.*, f - *Staphylococcus aureus*, g - *Pseudomonas spp.*, h - *Micrococcus spp*, i - *Escherichia coli.* and j - *Bacillus spp*.

4.5 (a)



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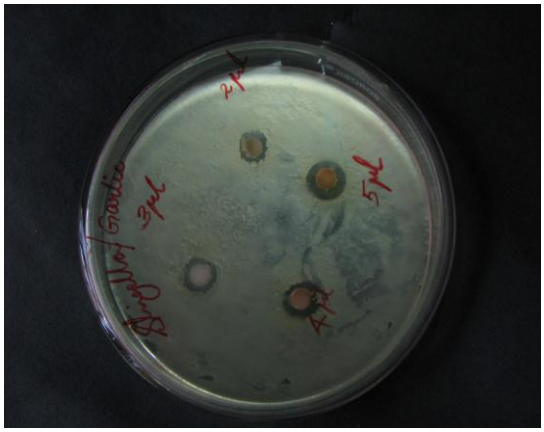

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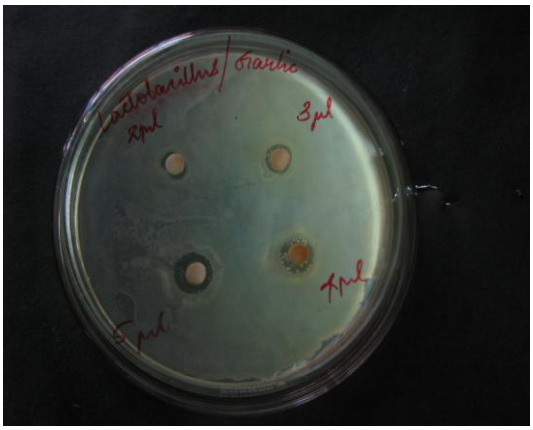

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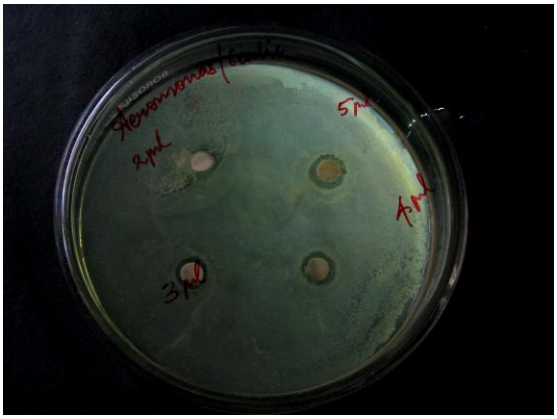
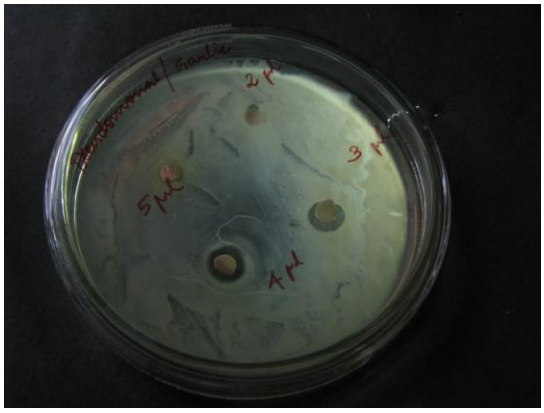


4.5 (e)

4.5 (f)



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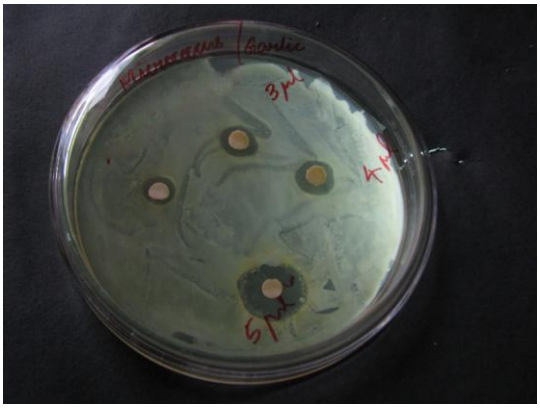

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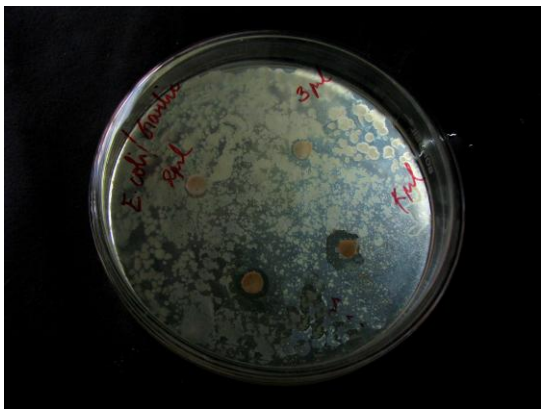

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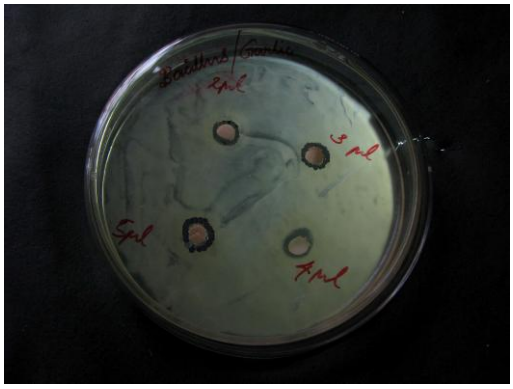
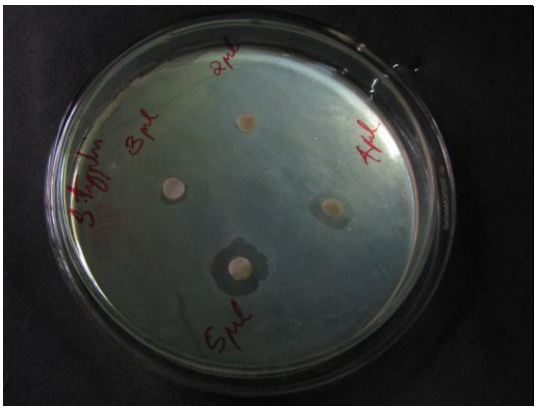
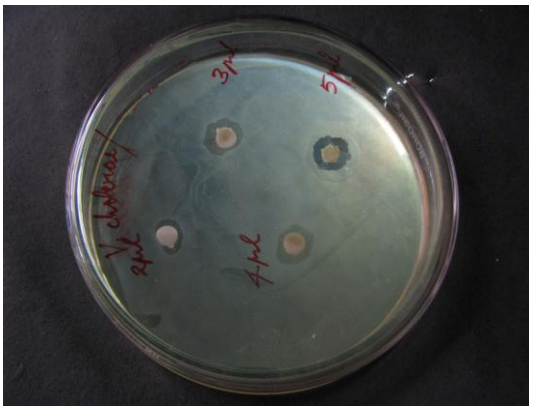


4.5 (j)

Plate 4.5. Inhibitory zones produced by garlic on bacteria. a - *Salmonella typhi*, b - *Vibrio cholerae*, c - *Shigella flexneri*, d - *Lactobacillus spp.*, e - *Aeromonas spp.*, f - *Staphylococcus aureus*, g - *Pseudomonas spp.*, h - *Micrococcus spp*, i - *Escherichia coli.* and j - *Bacillus spp*.



4.6 (a)



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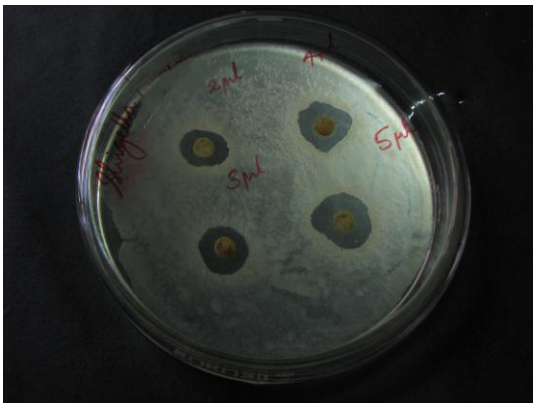

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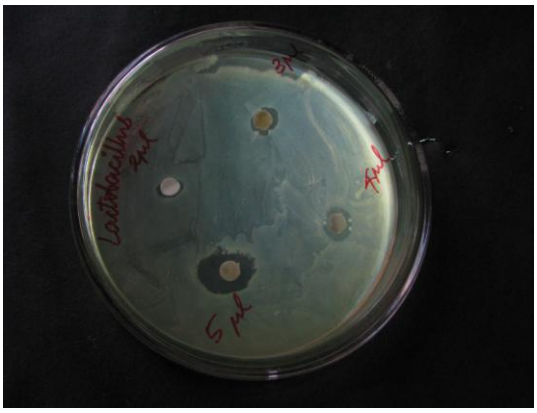

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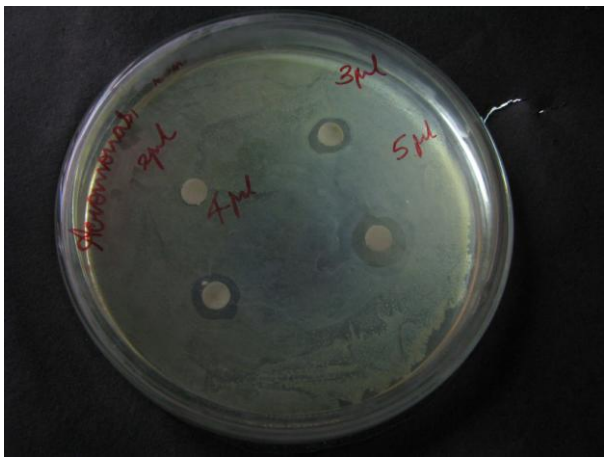

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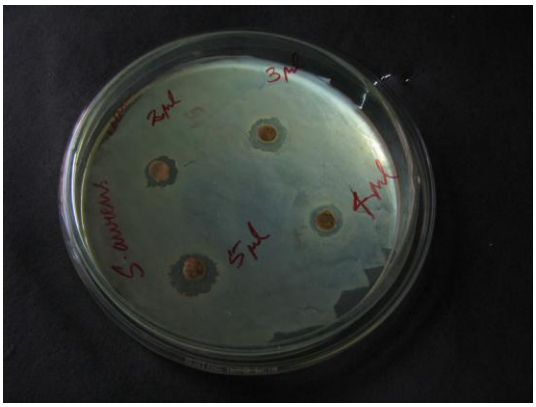

4.6(f)

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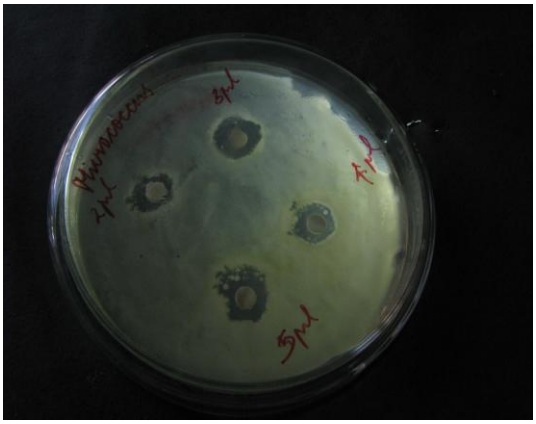

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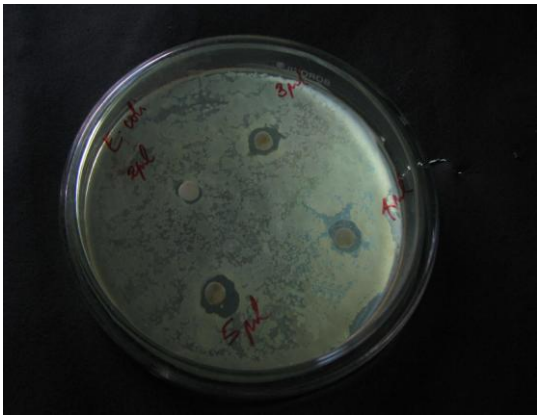
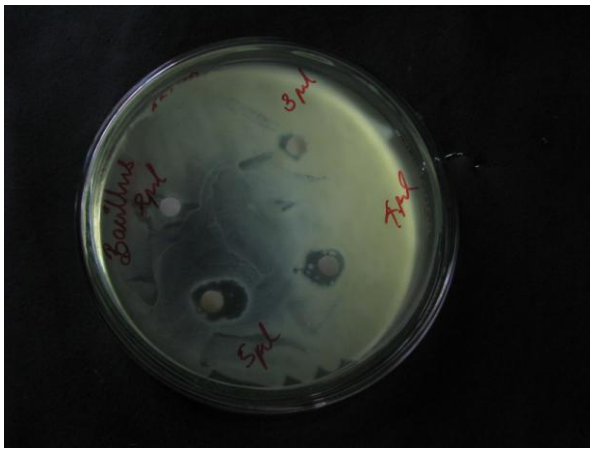
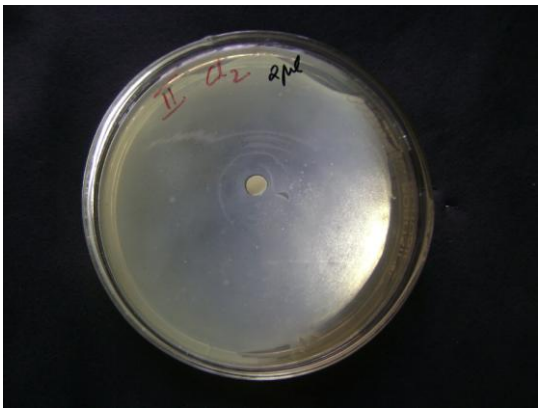


4.6(i)



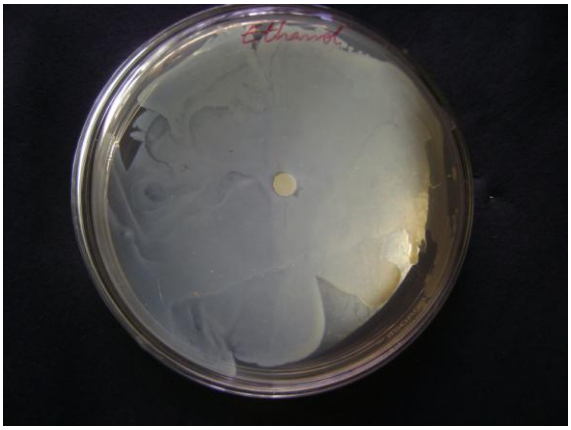
4.6(j)

Plate 4.6. Inhibitory zones produced by rosemary on bacteria. a - *Salmonella typhi*, b - *Vibrio cholerae*, c - *Shigella flexneri*, d - *Lactobacillus spp.*, e - *Aeromonas spp*., f - *Staphylococcus aureus*, g - *Pseudomonas spp.*, h - *Micrococcus spp*, i - *Escherichia coli.* and j - *Bacillus spp*.



4.7 (a)

4.7 (b)



4.7 (c)

Plate 4.7. Control plates. a – chlorine, b – negative control and c- ethanol.

This explains that there is no significant difference between the susceptibility of these bacteria towards the tested spices. Table 4.2 shows that activity of *Pseudomonas* and *Aeromonas* ranged from 8 to 12mm and 7 to 13 mm, respectively, whereas, those of *Lactobacillus*, *Bacillus* and *Micrococcus* is in a higher range of 10-16mm, 8-18mm, 11-16.5mm, respectively. Since these three bacterial genera (*Lactobacillus*, *Bacillus* and *Micrococcus*) belong to the Gram positive group, the difference in degree of sensitivity to the spice extracts may be a reflection of the Gram reaction of the organism.

Table 4.1. Comparision of Inhibitory zones of pathogenic bacteria against spice extracts(5µl)

	Inhibitory Zone in mm				
Treatments	***E.coli***	***V.cholerae***	***S.typhi***	***S.aureus***	***S.flexnerii***
Garlic	8.0±0.00	9.0±1.00	9.0±1.00	8.0±0.50	10.0±1.2
Clove	20.0±2.00	16.0±2.00	19.0±1.00	15.0±1.40	16.0±1.7
Turmeric	6.0±0.00	6.0±0.00	6.0±0.00	8.0±1.00	14.0±0.5
Cardamom	10.0±1.00	13.0±1.50	11.0±0.00	10.0±1.00	14.0±1.0
Oregano	10.0±0.43	12.0±0.94	17.0±2.00	11.0±0.62	13.0±1.0
Rosemary	10.0±0.62	11.0±0.30	11.0±1.00	10.0±0.46	1.0±1.4

The given values are expressed as mean±standard deviation, n = 3.

Table 4.2. Comparision of Inhibitory zones of bacterial strains isolated from tuna against spice extracts(5µl)

Treatments	Inhibitory Zone in mm				
	Micrococcus	***Pseudomonas***	***Bacillus***	***Aeromonas***	***Lactobacillus***
Garlic	16.0±1.00	9.0±0.35	8.0±0.0	8.0±0.60	10.0±1.00
Clove	16.5±1.50	15.0±0.80	18.0±1.4	13.0±1.00	15.0±1.15
Turmeric	15.0±2.00	12.0±1.11	15.0±0.5	7.0±0.00	16.0±2.00
Cardamom	11.0±1.20	8.0±0.13	12.0±1.0	11.0±0.54	13.0±2.00
Oregano	13.0±1.14	12.0±0.56	17.5±1.5	9.0±1.00	14.5±1.04
Rosemary	11.0±0.50	11.0±1.04	12.0±0.42	11.0±1.00	13.0±0.56

The given values are expressed as mean±standard deviation, n = 3.

4.3.3. Minimum inhibitory concentration of spice extracts

The MICs were determined as the lowest concentration of extract inhibiting the visible growth of each organism on the agar plate. The experiment was done as described in Section 4.2.7. The presence of one or two colonies were disregarded. The results were statistically analysed to understand whether there is any significant difference between the spices used and the MIC produced (Appendix A: 4.3). Two way ANOVA test explained that there was considerable difference between various treatments. Secondary analysis showed that the results also varied significantly between the different pathogens used for the study (Appendix A: 4.4).

Table 4.3. Minimum inhibitory concentration of spice extracts on pathogenic bacteria

	Minimum inhibitory concentration (MIC)					
Pathogen	clove	garlic	turmeric	cardamom	rosemary	oregano
S.typhi	0.20%	>0.8%	>0.8%	0.80%	>0.8%	0.40%
E.coli	0.12%	0.40%	0.40%	0.80%	0.80%	0.12%
V.cholerae	0.12%	0.80%	0.24%	0.12%	0.12%	0.12%
S.flexnerii	0.12%	0.12%	0.80%	0.24%	0.12%	0.12%

The MIC of 6 spice extracts obtained by agar dilution method are depicted in Table 4.3. Bacterial strains showed an MIC ranging from 0.12 % to 0.8%. *Salmonella typhi*, the resistant bacteria among the tested pathogens, was susceptible to clove at a concentration of 0.2%. The spice with the widest spectrum of activity was found to be clove (*Syzygium aromaticum)*. Activity of oregano was comparable to that of clove except in the case of *S.typhi*. Cardamom and Rosemary had almost a similar pattern of inhibition.

V.cholerae showed the highest sensitivity towards the spices. *E.coli* was susceptible to 0.12% of clove extract. Oregano also generated an MIC of 0.12% towards *E.coli*. *Shigella flexnerii* was susceptible to cardamom and turmeric at 0.24% and 0.80%, respectively. But, the antimicrobial activity was recorded at 0.12% for all other spice used in this investigation against *S.flexnerii*. This study confirms that spice extracts possess *in vitro* antibacterial activity. These spices can be used as a potent inhibitor of pathogens in food systems and thereby increase the shelf life and decrease the possibilities of food poisoning and spoilage in processed foods.

4.3.4. Activity of spices on raw fish media

There as been relatively few studies of the antimicrobial action of essential oils in model food systems and in real foods. One way ANOVA (Appendix A: 4.5) showed that there is significant difference between treatments and in the resultant total plate count. The effect of spice extracts on raw fish media is depicted in Table 4.4. Clove treated samples showed an 88.22 % reduction in the total plate count compared to the untreated control samples. Turmeric showed the least activity but still was successful in killing 31.93 % of the viable bacterial load. Rest of the spices had a reduction rate above 50%. One of the striking factor is that the antimicrobial activity of all the spices is considerably reduced in fish media compared to their effect in Mueller-Hinton agar.

Table 4.4 Total plate count of fish sample plated on TGA containing 0.2% of the spice extract.

Treatments(0.2%)	TPC	% reduction in count
Control	2.13×10^6	
Garlic	8.4×10^5	60.56
Clove	2.51×10^5	88.22
Turmeric	1.03×10^6	31.93
Cardamom	1.45×10^6	51.64
Oregano	2.73×10^5	79.18
Rosemary	8.2×10^5	61.51
Chlorine	9.8×10^5	53.99

4.3.5. Effect of spice extracts on growth of pathogenic bacteria in fish media

Fish media were prepared as explained in Section 4.2.4. The tubes containing fish media were cooled and used as media for growth studies of pathogenic bacteria in the presence of spice extracts. The inoculum was prepared as described in Section 4.2.7. Known quantities of the spice extract (0.2%) was added followed by inoculation of known number of the test culture suspension. 0.2% of the spice extracts were used since that was the MIC produced by the most efficient oleoresin through the *in vitro* experiment. The test-tube was mixed well and allowed to act for 10 minutes. The control tubes were also kept which consisted of fish media inoculated with pathogens. Spice extracts was not added into the control tubes. At the end of 10 minutes, the contents were transferred to a mortar, diluted with 9 ml of sterile Normal saline. Appropriate dilutions were plated on the respective media and counts were taken.

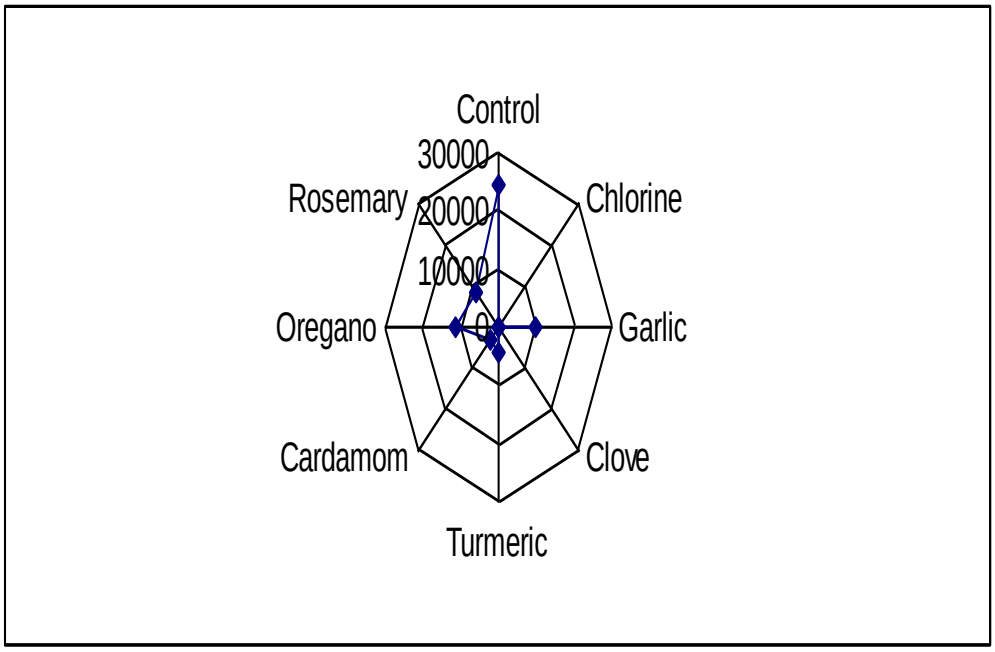


Fig 4.6 Viable count of *E.coli* in fish media (Radar displays changes in values relative to the central point)

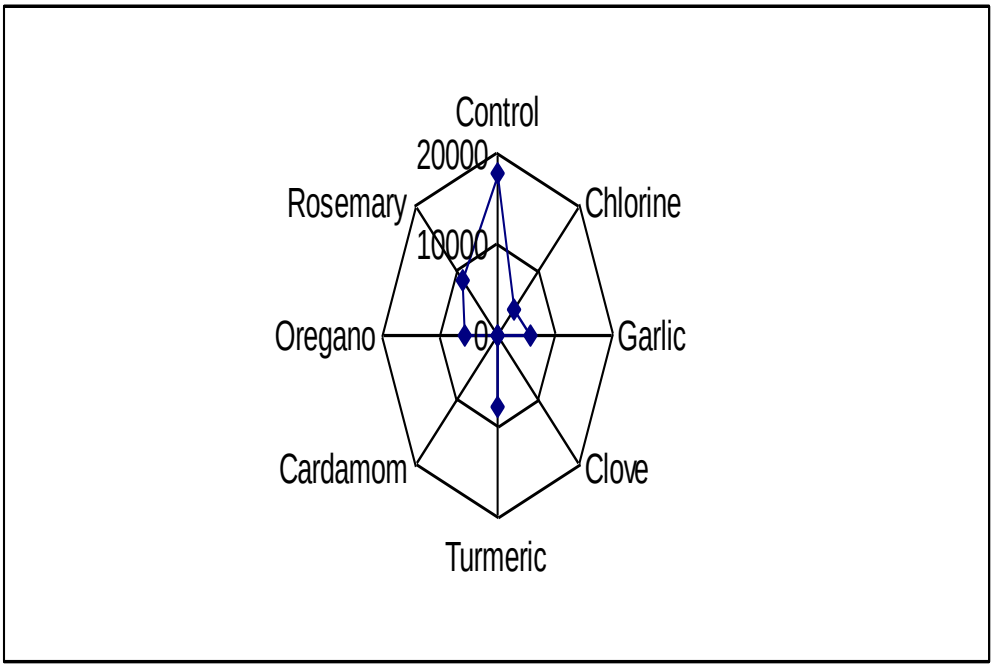


Fig 4.7.Viable count of *S.typhi* in fish media (Radar displays changes in values relative to the central point)

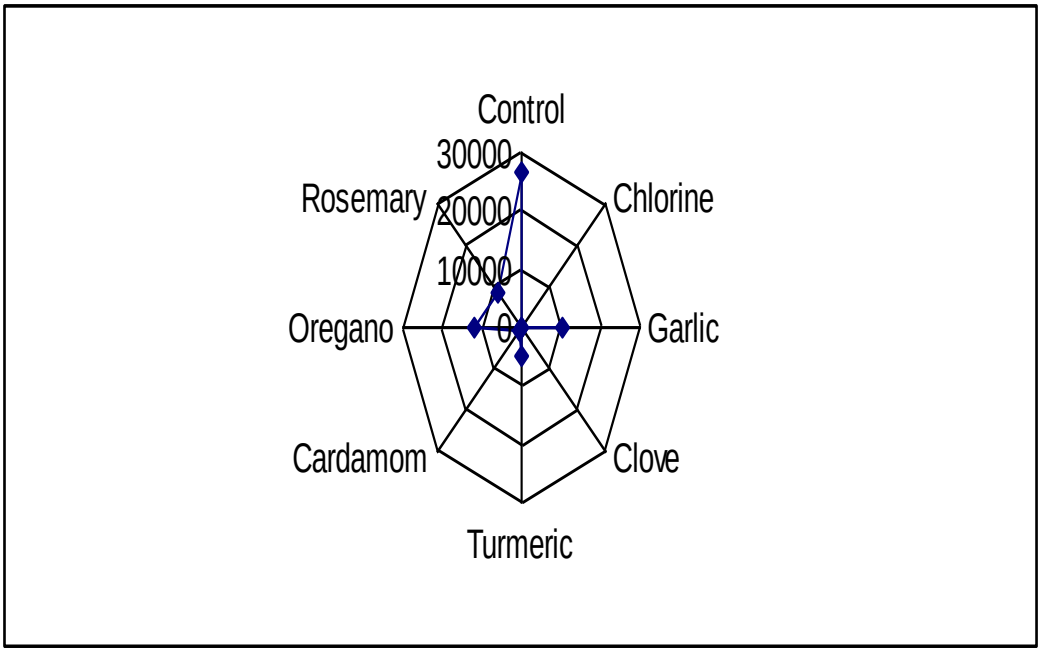


Fig 4.8. Viable count of *S.flexnerii* in fish media (Radar displays changes in values relative to the central point)

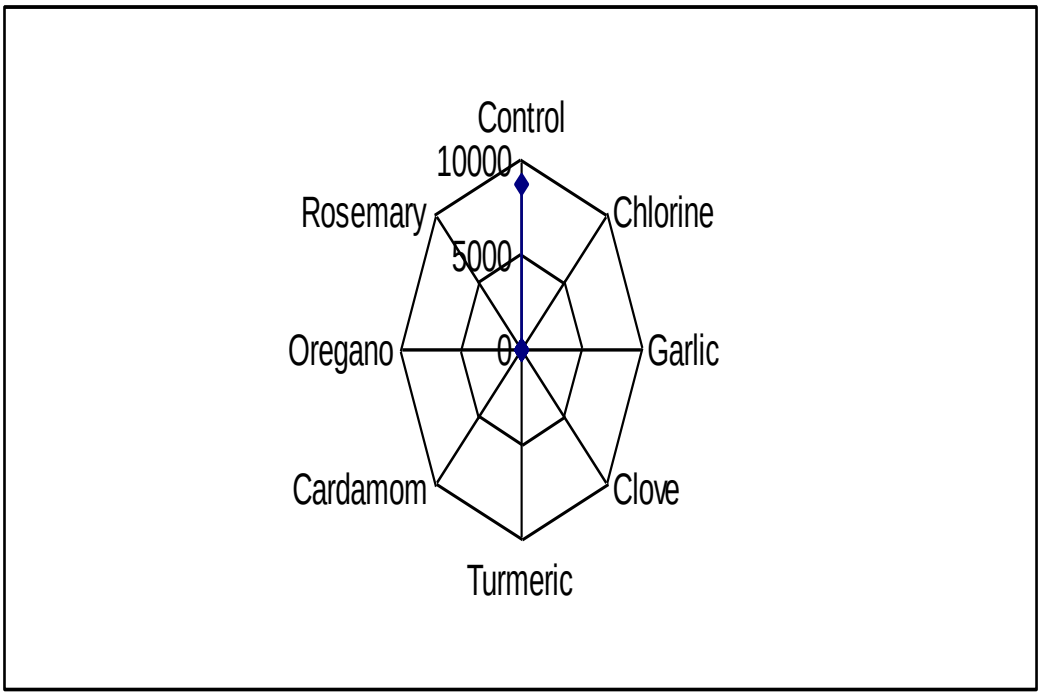


Fig 4.9. Viable count of *V.cholerae* in fish media (Radar displays changes in values relative to the central point)

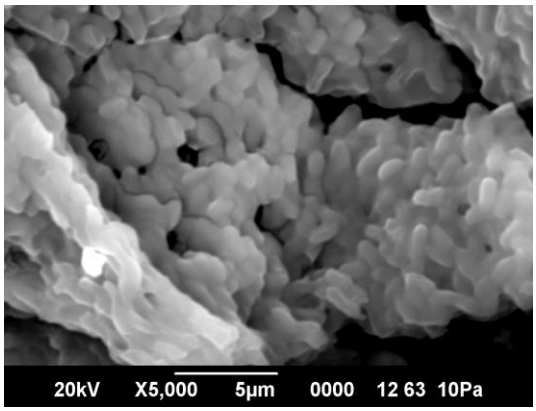
On exposure to spice extracts, *E.coli* within fish media showed a sharp reduction in viable cell count after 24 hrs when compared to that of control samples (Fig 4.6). One way ANOVA of the data shows that there is significant difference between the treatments (Appendix A: 4.6). Post hoc analysis (Appendix A: 4.7) reveals that apart from turmeric ($p=0.752$), rosemary ($p=0.980$) and oregano (0.422) all other spice treatments differed significantly from control ($p<0.05$). There was 3 log reduction in *E.coli* count for cardamom treated sample. None of the pathogenic bacteria was detected in 0.2% clove treated tuna sample.

The inhibition pattern of *S,typhi* is shown in Fig 4.7. Statistical analysis shows variation in mean values of control and other treatments (Appendix A: 4.8). When compared to control, there was only l log reduction in viable cell count for all spices except clove and cardamom. The tukey test (Appendix A: Table 4.9) conducted for detailed analysis explains that there isn't significant difference between rosemary, garlic, turmeric and oregano treatment with that of control ($p>0.05$).

Complete destruction of the microbe was achieved by clove in fish media inoculated with *S.typhi*. One way ANOVA (Appendix A: 4.10) performed for estimating variance in mean value of viable *Vibrio cholerae* resulted in $p<0.05$. Secondary analysis (Appendix A: 4.11) proves that *V.cholerae* was susceptible to all treatments and after 24 hrs of spice treatment it was not detectable in the TCBS plates except the control sample (Fig 4.9). In the case of *S.flexnerii* (Fig 4.8), oregano and garlic showed reduced activity. But, all other spice oleoresins exhibited good antimicrobial action against this bacteria. One of the noteworthy fact was that oregano, whose activity was next only to clove, became less effective in inhibiting pathogenic bacteria in the food system.

4.3.6. Electron microscopic analysis of bacterial cells

Electron microscopy allows structures to be studied at higher magnification than those used in light microscopy. The samples of bacterial cells for scanning electron microscopy was prepared as described in Section 4.2.9. The specimen was chemically fixed in aldehyde, dehydrated in a series of acetone dilutions of increasing concentration and sputter-coated with 25nm platinum. Later, observations were made on an scanning electron microscope (SEM). The SEM analysis observes the surface of the sample. Plate 4.8(1a) shows a cluster of *Salmonella typhi* fixed with gluteraldehyde and observed by SEM. The typical rod shaped bacteria can be seen in this plate. Bacterial cells measures 1.32-1.65μm and is intact (Plate 4.8[2c]). The growth in nutrient broth with 0.2% spice extract resulted in deformed cells. Antimicrobial treated bacterial cells are shown in Plates 4.8(2a) to 4.11. All the treated samples had pit formation suggesting localized collapse of cell wall. Least clumping of cells was showed by garlic treated samples (Plate 4.10:5c). The bacterial shape is retained as such by this treatment though the viability of cell is found to be reduced in the *in vitro* studies done earlier. The cells are damaged beyond recognition in oregano treated samples (Plate 4.10). The damage caused to the bacterial cells by the spice oleoresins confirms their antimicrobial property.



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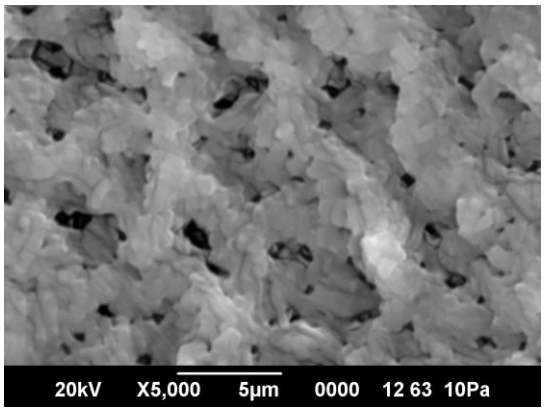

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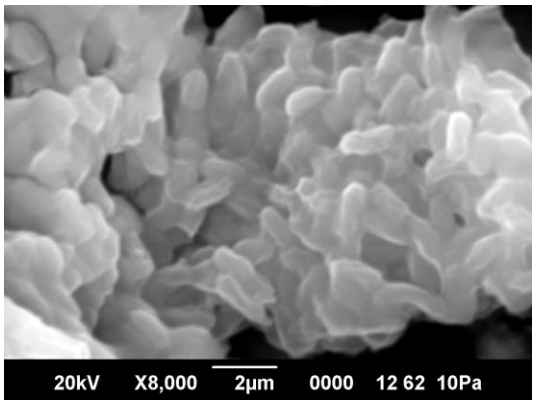

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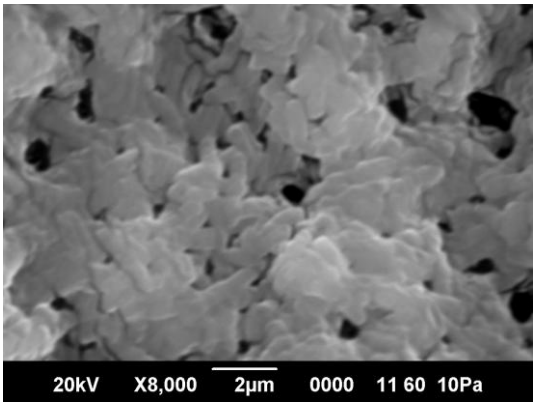

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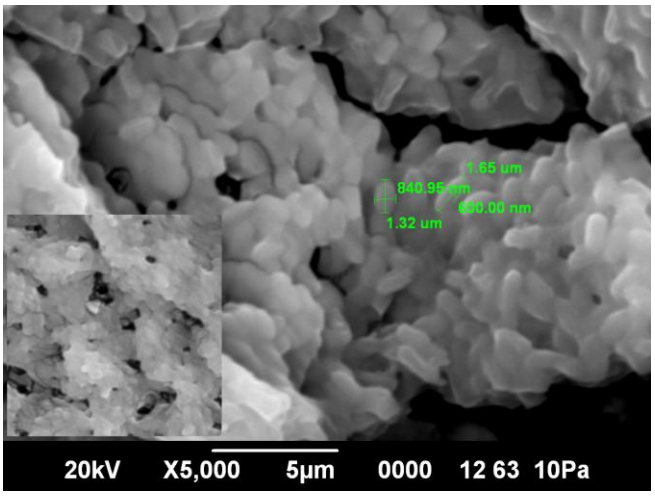
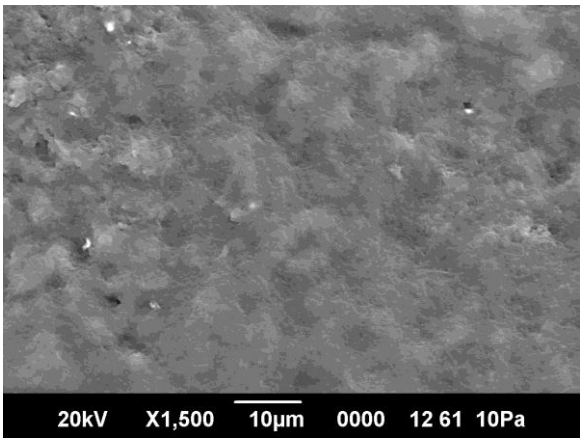


2 (c)

Plate 4.8. SEM images of *Salmonella typhi* (1a-Control at 5000X magnification, 1 b-Control at 8000X, 2a- Clove treated bacterial cells at 5000X, 2b- Clove treated cells at 8000X, 2c- Comparision of control and treated sample [treated sample in the inset] with dimensions)



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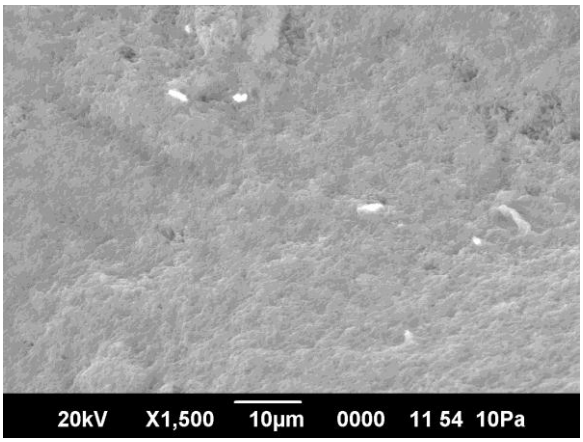

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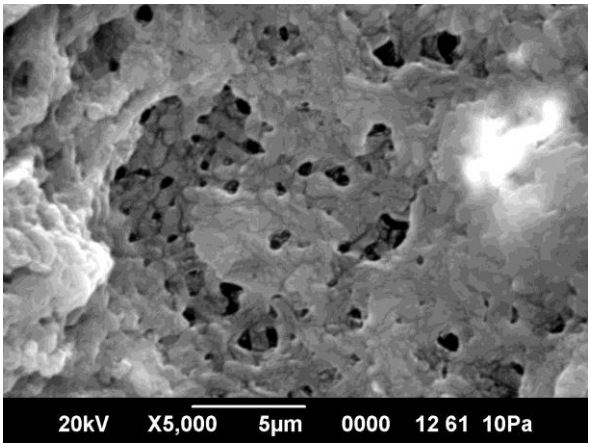

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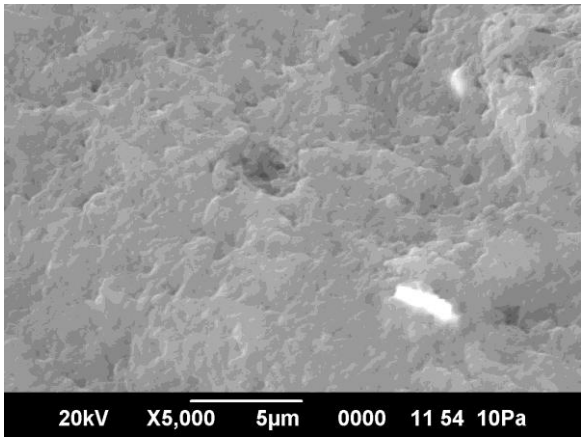

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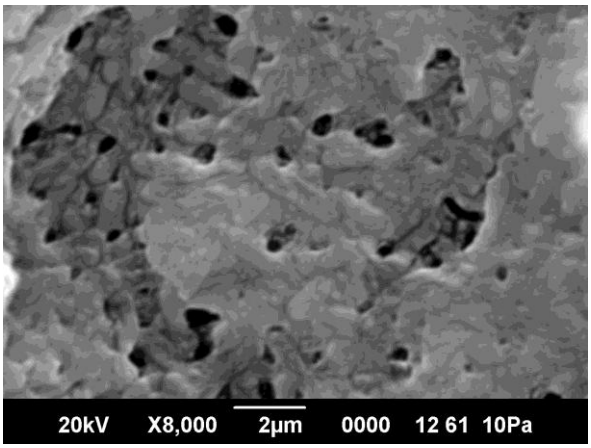
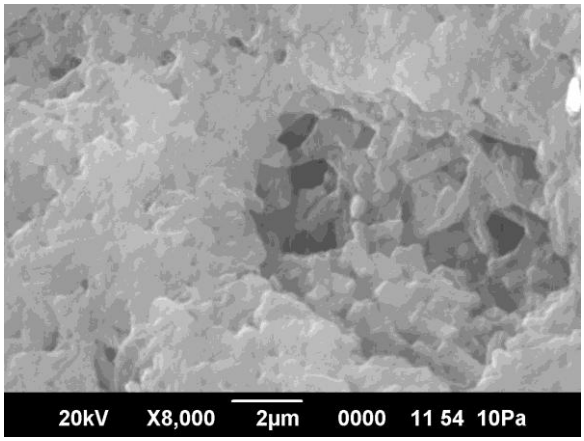
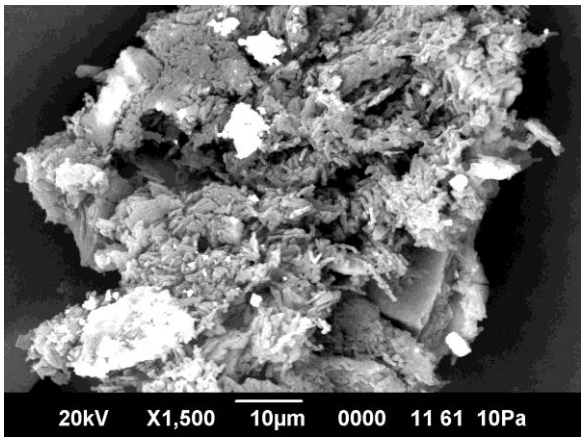


3(c)



4 (c)

Plate 4.9. SEM images of *Salmonella typhi* treated with turmeric (3a-c) and cardamom (4a-c) (a- 1500X magnification, b- 5000X magnification, c - 8000X magnification).



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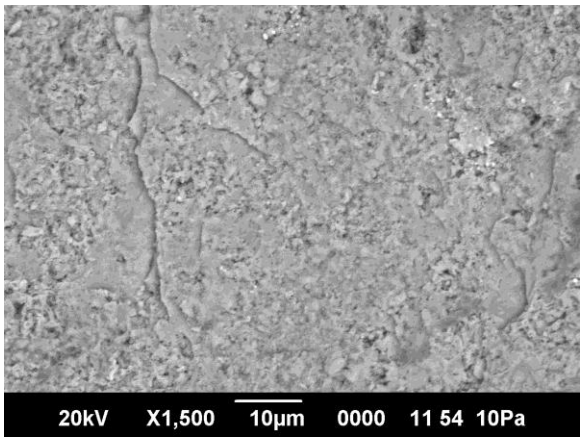
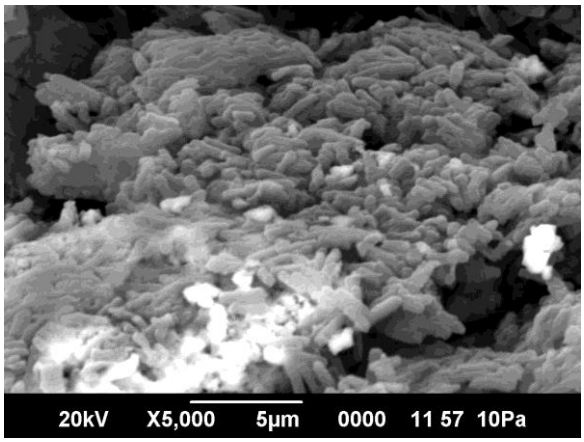


6 (a)



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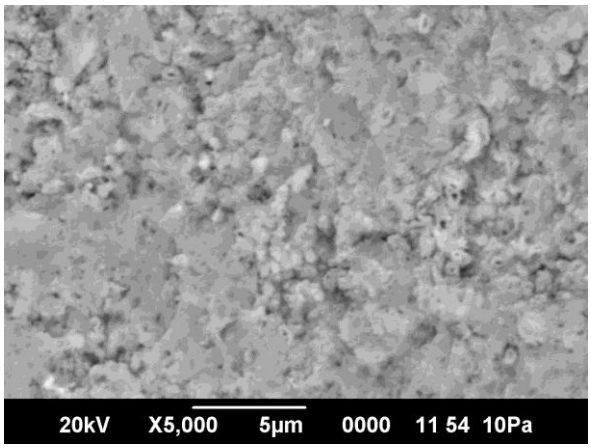

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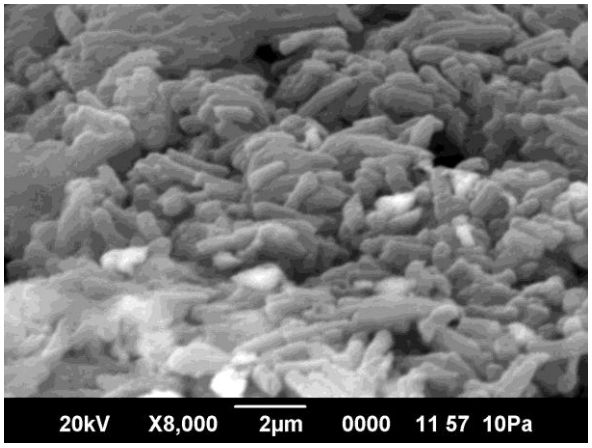
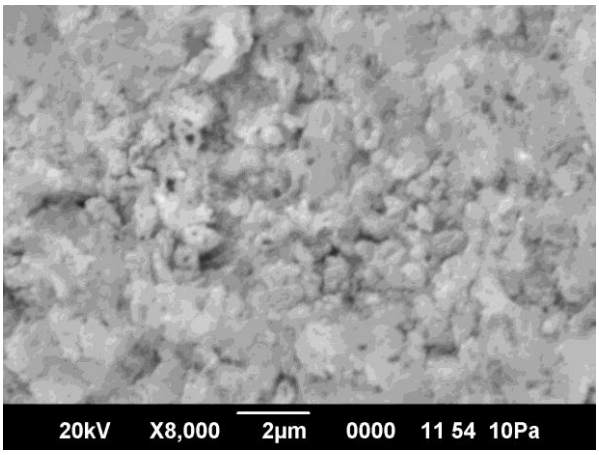
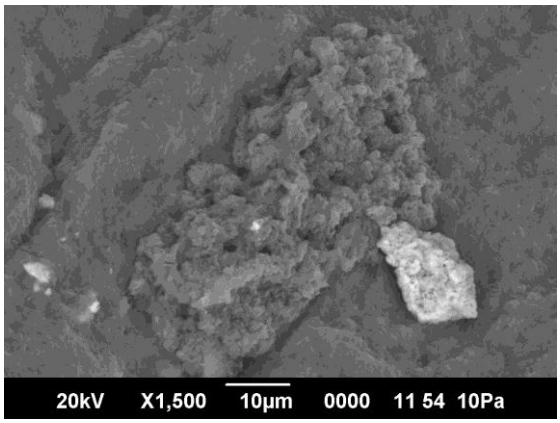


5 (c)

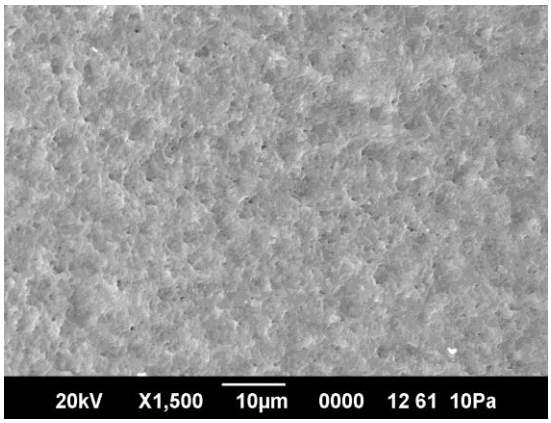


6 (c)

Plate 4.10. SEM images of *Salmonella typhi* treated with garlic (5a-c) and oregano (6a-c) (a- 1500X magnification, b- 5000X magnification, c - 8000X magnification).



7 (a)



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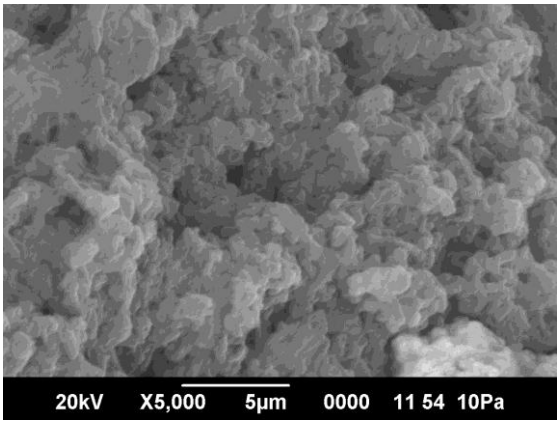

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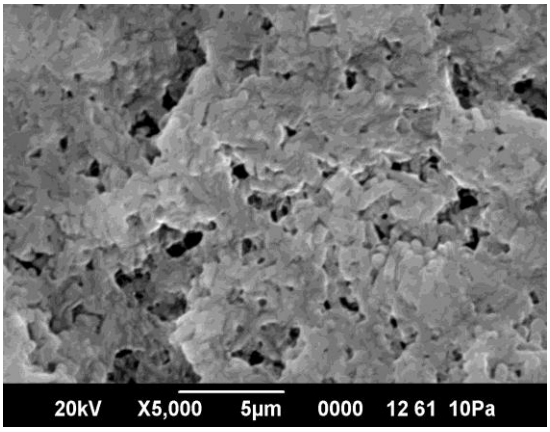

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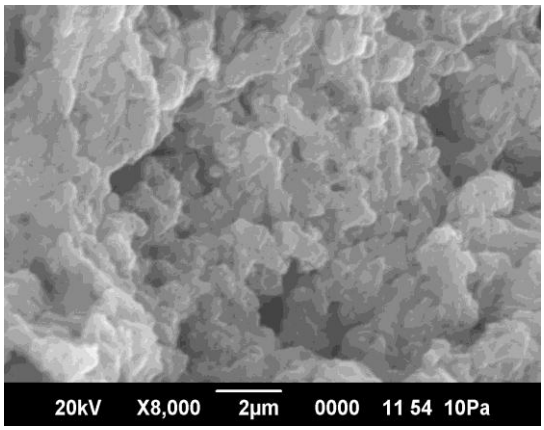

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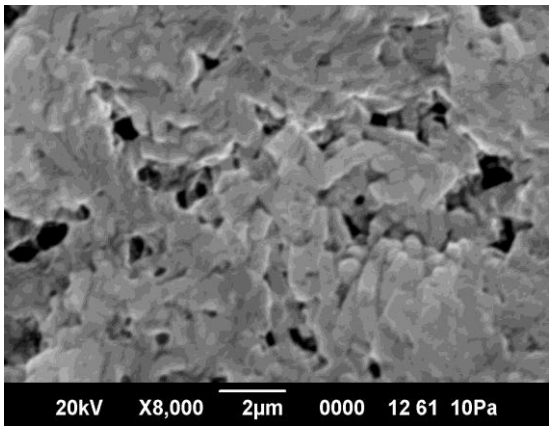

8(c)

Plate 4.11. SEM images of *Salmonella typhi* treated with rosemary (7a-c)and chlorine(8a-c) (a - 1500X magnification, b-5000X magnification, c-8000X magnification).

The mode of action of essential oil in spices is concentration dependent. Low concentration inhibit enzymes associated with energy production while higher amounts may precipitate proteins. 0.2% of spices has resulted in protein coagulation in the tested samples as seen in the SEM images. However it is uncertain whether membrane damage is quantitatively related to the amount of active antimicrobial compound to which the cell is exposed, or the effect is such that, once small injuries are caused, the breakdown of the cell followed.

4.4. Discussion

Seafood harbours a wide variety of bacterial species and they are the major cause of spoilage of most seafood products. Their growth and metabolism results in the formation of amines, sulfides, alcohols, aldehydes, ketones and organic acids with unpleasant and unacceptable off-flavours. Microbial activity is retarded during storage but does not inhibit the spoilage of fish during storage conditions. Hence, the control of microbial growth is the primary step towards ensuring seafood safety.

4.4.1. Effect of spice oleoresins on bacteria

Deans and Ritchie (1987) had found that clove shows antibacterial properties against 23 genera of bacteria. The lowest MIC (minimum inhibitory concentration) of 0.125% was observed in clove among the spices tested by Yutaka *et al*. (2006). In the present study, the MIC of clove against *V.cholerae*, *S.flexnerii* and *E.coli* was found to be 0.12% though the MIC of *S.typhi* was set a little higher at 0.20%. Hao *et al*. (1998a, 1998b) reported inhibitory effect of eugenol, active principle of clove essential oil, on *L. monocytogenes* in cooked beef and poultry at 5°C and 15°C. Karapinar and Aktug (1987) noted inhibitory effect of eugenol, thymol, menthol and anethole (volatile compounds found in several spices) at 50 and 500µg/mL concentrations on *Salmonella typhimurium*, *S. aureus* and *V. prahaemolyticus*.

Essential oils of clove, coriander and nutmug were effective in inhibiting the growth of *Aeromonas spp*. A marked reduction of this organism also occurred in inoculated samples of cooked, non cured pork treated with clove (Stecchini *et al*., 1993). 15% of the total bacterial population isolated from tuna was found to consist of *Aeromonas*. Hence, antimicrobial agents which are effective on this species will contribute in decontaminating the fish media more effectively.

E.coli was found to be susceptible to 0.12% of clove extract during the investigation. This is in agreement with the potent antimicrobial activity of clove reported against *Escherichia coli* (De *et al*., 1999). Clove showed the highest inhibitory effect in an investigation carried out by Pokhrel *et al*. (2012) while, coriander, ginger and turmeric showed no inhibitory effect in the case of crude ethanolic extracts. The constituents of these spices mainly phenols, alcohols, aldehydes, ketones, ethers and hydrocarbons are reported to exhibit the antimicrobial activity.

Oregano also generated an MIC of 0.12% towards *E.coli* . The action of oregano is due to damage in membrane integrity, which further affects the pH homeostasis and equilibrium of inorganic ions (Lambert *et a*l, 2001). It is reported that cranberry and oregano, in synergistic combination with lactic acid, can inhibit *V. parahaemolyticus* in seafood systems. Such a strategy can be used for enhancing food safety in food industry. Helander *et al*. (1998) assayed the effect of carvacrol, (+) carvone, thymol and trans-cinnamaldehyde on *E. coli* O157:H7 and *S. typhimurium* and reported that carvacrol and thymol decreased the intracellular ATP content of *E. coli* cells while the extracellular ATP simultaneously increased. This indicated disruptive action of these compounds toward cytoplasmic membrane. Since carvacol and thymol is the major component of oregano, the same reason is responsible for the inhibitory action shown by the spice towards the bacteria.

Activity of garlic was lower than that of clove, cardamom etc. But, still it had reduced 60.56 % of the initial bacterial load in fish media (Table 4.4).The antibacterial activity exhibited by garlic is mainly due to the presence of allicin. It is reported by many

workers that allicin completely inhibits a variety of Gram-positive and Gram-negative bacteria. It is postulated that the antimicrobial activity of garlic is reported due to the inhibition of succinic dehydrogenase via the inactivation of thiol group. Dellaquis and Mazza (1998) described antimicrobial properties of isothiocyanate derived from onion and garlic. For isothiocyanates, it was hypothesized that they inactivated extracellular enzymes through the oxidative cleavage of disulphide bonds (Brull and Coote, 1999). Dellaquis and Mazza (1998) purposed that the formation of reactive thiocyanate radical could mediate the antimicrobial property.

Assays performed by Agaoglu *et al*. (2005) indicate that cardamom seed has inhibitory activity on *Staphylococcus aureus*, *Micrococcus luteus* and many other bacterial strains. Curcuminoids have also been shown to exhibit antimicrobial properties. In this study, turmeric is seen to have significant effect on spoilage bacteria. The antimicrobial effects of alcoholic extract of turmeric, curcumin and oil from turmeric have been studied by Banerjee and Nigam (1978). Extracts from turmeric as well as active principles from curcuminoids were found to inhibit the growth of numerous Gram positive and Gram negative bacteria, fungi and intestinal parasite, *Entamoeba histolytica*. Curcumin at concentrations of 2.5 -50.0mg/100ml is reported to inhibited *in vitro* growth of *Staphylococcus aureus* (Shankar and Srinivasamurthy, 1979).

Antibacterial activity of the essential oil of rosemary against an array of bacterial and fungal species including *Listeria monocytogenes* and *Aspergillus niger* have been reported by Faliero *et al*. (1999) and Baratta *et al*. (1998). Gram positive bacteria such as *Staphylococcus aureus* and *S.epidermid* have been found to be more susceptible to rosemary oil than other Gram negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* (Pintore *et al*., 2002). *S. aureus* is said to have developed resistant varieties (Methycelene Resistant *Staphylococcus aureus*) in hospitals. Antibacterial action of plant extract as been tested even in the case of antibiotic resistant microorganisms. Their application in surgical and wound dressing application has been investigated by Chinta *et al*. (2012) and found successful.

Antimicrobial and synergistic activity of ingredients of betel leaf, betel nut, cardamom, clove and fennel seeds was tested against microbial population of oral cavity and four enteric pathogens namely *Staphylococcus aureus, Salmonella typhi, Escherichia coli and Shigell flexneri*. It was found that the bacteria investigated showed susceptibility against the tested extracts (Ghanwate and Thakare, 2012). In addition to antimicrobial properties some spice extracts like oregano, rosemary and thyme exhibit antioxidant properties also (Kačániová *et al*., 2012) which can be an additional advantage in processed lipid rich foods and fatty fishes like mackerel (Sulochanan, 2008).

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4.4.2 Activity of spices on food system

There as been relatively few studies of the antimicrobial action of essential oils in model food systems and in real foods. Most of the studies have focused on the activity *in vitro*, and only very few authors have documented their antimicrobial activity on food products (Bajpai *et al*., 2009). The present study focused on the action of spices on fish flesh.

All spices showed marked reduction in total plate count from that of untreated control. One of the striking factor is that the antimicrobial activity of all the spices is considerably reduced in fish media. The effect is much evident in oregano when compared to its performance in Mueller-Hinton agar. The efficacy of spice extracts *in vitro* is often much greater than *in vivo* or *in situ*, i.e. in foods (Nychas and Tassou, 2000). Growth of *E.coli*, *Salmonella*, *Staphylococcus* was inhibited by oregano extract in agar media and broth cultures (4.3.2). However, the antimicrobial action of this spice in a food system seemed to vary much. It as been reported that the type of oil or fat present in a food can affect the antimicrobial efficacy of spices. Skandamis (2001) has found a reduction in the antimicrobial activity of thyme in full fat cheese. In his experiment, in full fat cheese clove was the only spice which reduced the bacterial count. In marine organisms like tuna, lipid is the second largest biochemical constituent. The interference of lipid molecules can be one of the reason for the reduced activity of oregano and other spices in fish media.

According to the Center for Food Safety and Applied Nutrition in Washington (2001), most fish related food borne illness are traced to *Salmonella, Staphylococcus spp., Escherichia spp. a*nd *Vibrio spp*. There have been relatively few studies of the antimicrobial action of essential oils in model food systems and in real foods. Nychas and Tassou (2000) had found that the efficacy of essential oils *in vitro* is often much greater than in foods. Antimicrobial activity of 14 spice extracts against pathogenic and spoilage bacteria in fresh pork and ham slices showed that individual extracts of clove, rosemary, cassia bark, liquorice was the best inhibitor against the pathogens (Huiyun *et al*., 2009)

Ayar *et al.* (2001) stated that herb extracts and their combinations exhibited antimicrobial effects in butter. The essential oil of herbs is reported to inhibit bacteria present in butter by Farag *et al*. (1990) and also by Zegarska and Rafalowski (1997). The antimicrobial effects of green tea and rosemary added to food as antagonists to food borne pathogens were determined in laboratory media and oriental-style rice cakes. Levels of *Bacillus cereus* and *Staphylococcus aureus* were significantly reduced in rice cakes stored for 3 days at room temperature (Lee *et al,* 2009). Deb and Joshi (2007) had found spices to be useful in decreasing microbial load of sprouts.

Sterile reconstituted full-cream milk inoculated with 10^{3} -10^{4} CFU/g of *B.subtilis* and *E.coli,* followed by addition of oregano, marjoram, sage and licorice extracts reduced the bacterial growth by 2.5 log cycle and 0.5 log cycle, respectively after one day storage (Al-Turki *et al,* 2008). Chicken meat patties treated with essential oils of garlic, clove and cinnamon and inoculated with *Staphylococcus aureus* (MTCC 3103) was studied by Babu *et al*. (2012). The samples were stored at refrigeration temperature and the results revealed that essential oil were effective in reducing the bacterial counts. According to these studies, spices are found to be effective in extending the shelf life of food products.

4.4.3. Electron microscopic analysis of bacterial cells

Scanning electron microscopic images showed pit formation in spice treated bacterial samples. Exact mechanism of antibacterial action of spices and derivatives is not yet clear (Lanciotti *et al*., 2004). But, pit formation suggests localized collapse of cell wall.

Many hypothesis have been given regarding the activity of spices. Hydrogen bonding of phenolic compounds to membrane proteins followed by partition in the lipid bilayer was suggested as the reason for susceptibility of microbes towards the spices by Juven *et al*. (1994). Other hypothesis given by workers involve: i) perturbation of membrane permeability consequent to its expansion and increased fluidity causing the inhibition of membrane embedded enzymes (Cox *et al*., 2000); ii) membrane disruption (Caccioni *et al*., 2000); iii) destruction of electrons transport systems (Tassou *et al*., 2000) and iv) cell wall perturbation (Odhav *et al*., 2002). Generally, gram-negative bacteria have been reported to be more resistant than Gram-positive to essential oils antimicrobial effect because of their cell wall lipopolyssaccharide (Russel, 1991). This is evident in the increased activity of tested spice extracts towards *Micrococcus*, *Bacillus* and *Lactobacillus* when compared to Gram negative species, *Pseudomonas* and *Aeromonas*. Cell wall lipopolyssacaride may prevent essential oils active compounds reaching the cytoplasmic membrane of Gram-negative bacteria (Chanegriha *et al*., 1994).

Essential oils damage the structural and functional properties of membranes (Ultee *et al*., 1999, 2000, 2002). Carvacol, an active component of many essential oils, has been shown to destabilize the cytoplasmic and outer membranes and act as 'proton exchanger', resulting in a reduction of the pH gradient across the cytoplasmic membrane (Lambert *et al*., 2001; Helander *et al*.,1998). The collapse of the proton motive force and depletion of the ATP pool eventually lead to cell death (Ultee *et al*., 2002).

Gill and Holley (2004) had reported that effects on energy generation of bacterial cells play a major role in the activity of eugenol at bactericidal concentrations. The mode of action of essential oil is concentration dependent. At lower concentrations, enzymes associated with energy production is inhibited while higher amounts precipitate proteins. It is uncertain whether membrane damage is quantitatively related to the amount of active antimicrobial compound to which the cell is exposed. It is possible that, once small injuries are caused, the breakdown of the cell follows (Judis, 1963). It has been hypothesized that the inhibition involves phenolic compounds, because these compounds sensitize the phospholipid bilayer of the microbial cytoplasmic membrane causing increased

permeability, unavailability of vital intracellular constituents (Juven *et al*., 1994; Kim *et al*., 1995) and/or impairment of bacterial enzymes systems (Wendakoon and Sakaguchi, 1995).

4.5.Conclusion

A recap of several notable recent food poisoning outbreaks illustrats the diverse pathways to food borne illness and demonstrated the need for constant vigilance by both individuals and the food industry. A large number of antibiotics have been tried earlier as preservatives to check the growth of psychrophilic flora of fish stored in ice and to extend its shelf life for a reasonably longer period. Addition of antimicrobial chemical preservatives can better protect the meat and fish from microorganisms. Because of the awareness among the consumers, they are preferring the food without any chemical preservatives. This is especially a worrying issue for the seafood exporters. The use of spice oleoresin as an antimicrobial agent in controlling the spoilage and pathogenic bacteria present on fish has been studied in this chapter. Under the test conditions, all bacterial strains showed some degree of susceptibility towards each spice. The spice extracts were effective against both gram-positive and gram-negative microorganisms. Clove and oregano appeared more active, exerting greater inhibitory activity against bacterial strains. Spoilage bacteria isolated from tuna was more resistant to the spice extracts compared to the pathogenic strains.

Bacterial strains showed an MIC ranging from 0.12 % to 0.8%. *Salmonella typhi* was susceptible to clove at a concentration of 0.2%. The spice with the widest spectrum of activity was found to be clove (*Syzygium aromaticum).* Activity of oregano was comparable to that of clove except in the case of *S.typhi*. Cardamom and Rosemary had almost a similar pattern of inhibition zones and the activity was above 10 mm for all the pathogenic species tested by disk diffusion method.

In model food systems with fish as the growth medium, all spices showed marked reduction in total plate count from that of untreated control. Clove treated samples showed an 88% reduction in the total plate count compared to the untreated control samples. Rest of the spices had a reduction rate above 50%. Fish media inoculated with pathogens also

showed a sharp reduction in viable cell count after 24 hrs when compared to that of control samples. None of the pathogenic bacteria was detected in 0.2% clove treated tuna sample.

In case of *S.typhi*, there was only l log reduction in viable cell count for all spices except clove. *Vibrio cholerae* was susceptible to all treatments and after 24 hrs of spice treatment it was not detectable in the TCBS plates except the control sample. *S.flexnerii* was also susceptible to the activity of spices. Thus, all other spice oleoresins exhibited promising antimicrobial action. Statistical analysis showed that the control had a significant difference ($p<0.05\%$) from the microbial number in fish media treated with clove.

On SEM analysis the untreated control showed rod shaped bacterial cells of *Salmonella typhi* measuring 1.32-1.65μm long. The growth in nutrient broth with 0.2% spice extract resulted in deformed cells. All the treated samples had pit formation suggesting localized collapse of cell wall Least clumping of cells was showed by garlic treated samples. In this case, bacterial shape is retained as such. The cells were damaged beyond recognition in Cardamom and Oregano treated samples.

In summary, spice oleoresins have potent activity against spoilage and pathogenic bacteria present on tuna. Clove exhibited highest activity at the minimum concentration (0.2%) in *in vitro* studies and in fish media. Use of spices could increase the shelf life and decrease the possibilities of food poisoning and spoilage in processed foods. The possibility of controlling histamine formation by controlling histamine forming bacteria is evaluated in the next chapter.

5.1. Introduction

Biogenic amines (BA) are organic bases of low molecular weight that possess biological activity . They can be formed and degraded as a result of normal metabolic activity in animals, plants and microorganisms. According to Zhai *et al*. (2012), BA are formed in foods by microbial decarboxylation of the corresponding amino acids or by transamination of aldehydes and ketones by amino acid transaminases. Biogenic amines such as histamine, cadaverine and putrescine are formed from free amino acids namely histidine, tyrosine, tryptophan, ornithine and lysine respectively. Spermidine and spermine arise from putrescine (Zarei et al., 2011). The amines are important agents of food intoxication and indicators of fish spoilage. Putrescine and cadaverine are good indices of spoilage of marine fish (Mietz and Karmas, 1978; Yamanaka *et al*., 1986; Taylor and Sumner, 1987).

Histamine has been implicated in the toxicity of scombroid and even non-scombroid fishes (Taylor, 1986). Formation of histamine in tuna, sardine, horse mackerel and anchovy is reported by many workers (Wendakoon *et al*., 1990; Mendes, 1999; Yamanaka *et al*.,1986; Marrakchi *et al*.,1990; Okuzumi *et al*.,1990 and Veciana *et al*., 1990).

5.1.1. Biogenic amines in food

Low concentrations of biogenic amines are a natural characteristic of a number of foodstuffs such as fruits and vegetables. They are present as natural metabolic products or intermediates. In food and beverages, they are formed by enzymes of raw material or are generated by microbial decarboxylation of amino acids during aging and storage (Santos, 1996). Free biogenic amines shape the typical taste of mature foods and are precursors of certain aroma compounds (Moret *et al*., 2005; Santos, 1996). The most important biogenic amines occurring in foods and beverages are histamine, β-phenylethylamine, tyramine, tryptamine, putrescine, cadaverine, spermine and spermidine (Bodmer *et al*. 1999, Shalaby, 1996 and Suzzi and Gardini, 2003).

The concentrations of biogenic amines vary extensively not only between different food varieties but also within the varieties themselves (Bodmer *et al*., 1999). In particular, biogenic amines are produced in foods where high levels of proteins are present. 16-18% of protein content is present in fishes and quantitatively it is the second major component (Viswanathan, 2002). Hence, fish is a good target site for biogenic amine formation. During fermentation or spoilage, the protein breakdown products, peptides and amino acids formed act as precursors for amine formation (Bodmer *et al*., 1999; Vinci and Antonelli , 2002).

Presence of biogenic amines is reported in a wide range of food products including fish and fish products (Bardócz, 1995; Bodmer *et al*., 1999; Santos, 1996). Histamine fish poisoning (HFP) is an intoxication that can be caused by consumption of different types of marine finfishes. Tuna and mackerel are the most common fish associated with the poisoning. It is a common characteristic of these seafoods that, at some stage between catch and consumption, specific bacteria grow to high concentrations and form histamine and other biogenic amines in the products.

5.1.2. Factors affecting amine formation

The formation of biogenic amines in foods depends on several factors. Important among them are temperature, time of storage, pH, oxygen supply, muscle type, effect of pre processing and different processing steps like salting, smoking, irradiation etc. The use of antimicrobial agents also has influence on the formation of biogenic amines.

a) Temperature

With respect to the influence of temperature on the synthesis of biogenic amines, there are different views. Santos *et al*. (1986) opined that storage temperature did not significantly influence maximum tyramine content in anchovies though refrigeration temperatures delayed the start of the production. Disagreeing with the above information, Diaz *et al*. (1992) found that histamine and tyramine concentrations increased with the time and storage temperature of chihua cheese. Putrescine biosynthesis by *Enterobacter cloacae*

was detected at 20°C after 24 hour of incubation but not at 10°C. *Klesbsiella pneumoniae* showed less extensive cadaverine production at 10°C compared to 20°C. Further it is reported that histamine production slowed at 10°C and nearly terminated at 5°C. This is attributed to the slow growth of histamine producing bacteria at low temperatures. No histamine was formed by *Pseudomonas morganii, Pseudomonas vulgaris, or Hafnia* strains after one month of incubation at 1°C (Halasz *et al*., 1994). Similarly, Klausen and Lund (1986) reported that amine contents were temperature dependent and were two to twenty times higher at 10°C compared to that at 2°C in both mackerel and herring. In most cases it is proven that there is direct relationship between biogenic amine formation and time and temperature of storage. So it can be inferred that temperature abuse in highly perishable food items is the main cause of biogenic amine toxicity.

Amine concentrations are unaffected by cooking, with the exception of spermine, which decreased during heat treatment of cooked ground beef at 200°C for 2 hours. Histamine is thermally stable during the cooking process (Luten *et al*., 1992). This has great significance in the thermal processing of foods like canning where if the raw material used contains biogenic amines, the final product will also be containing the same amines without much quantitative changes.

b) pH

The pH level is an important factor influencing amino acid decarboxylase activity. Santos *et al*. (1986) found a higher tyramine level in mackerel when the pH was low. The conversion of histidine to histamine by *Klebsiella pneumonie* isolated from skipjack tuna has an optimum pH of 4. Amino acid decarboxylase activity was stronger in an acidic environment, the optimum pH being between 4 and 5.5 (Teodorovic *et al*., 1994). In such an environment bacteria are more strongly encouraged to produce the amino acid decarboxylase enzymes, as a part of their defense mechanisms against the acidity (Halasz *et al*., 1994; Teodorovic *et al*., 1994).

c) Influences of constituents

The amine formation in fishes is also influenced by the constituents in the fishes. In the case of red muscle, more histamine is produced since it contains more histidine. Fishes like tuna and mackerel is more comparable to white muscle fish such as rockfish (Flick *et al*., 2001).

d) Effect of Processing

In smoking process, hot smoking produces less histamine than cold smoking process. Depending on gutting, production of biogenic amines also changes. Whole ungutted fish has more production rate of histamine than fillet of gutted fish (Arnold and Brown, 1978). Anti microbial agents such as sorbic acid, citric, malic and succinic acids have a diminishing effect on synthesis of biogenic amines (Kang and Park, 1984).

5.1.3. Physiological and functional activities of biogenic amines (BA).

Most of the biological functions of BA are attributed to their polycationic nature. As polycations, they bind non-covalently to negatively charged phospholipids and many types of proteins that directly modulate membrane permeability and play an important role in the maintenance of membrane integrity and in other functions (Srivastava and Smith, 1982).

The polyamines, putrescine, spermidine, and spermine are among the most ubiquitous organic compounds found in nature, and they exist as polycations at physiological pH. Thus, it is not surprising that these simple molecules can intereact with a wide variety of cellular constituents, such as RNA – DNA, nucleotides, proteins, and other acidic substances. Polyamines have been shown to interact with the cellular lipid bilayer and have also been shown to promote membrane fusion.

Histamine possesses a powerful biological function, serving as a primary mediator of the immediate symptoms noted in allergic responses (Stratton *et al*., 1991). The activity of histamine is not limited to allergic, peptic and neurologic functions, but extends to other processes related to wound healing, circulatory disease, immunology, oncology and

infectious disease (Ohtsu, 2012). Recently, more than 25 rare diseases related to histamine physiopathology have been identified using a computationally assisted text mining approach. Putrescine, cadaverine and agmatine have been identified as potentiators that increase the toxicity of histamine to human by depressing histamine oxidation (Arnold and Brown, 1978; Halasz *et al*., 1994).

Amines were also investigated as possible mutagenic precursor, since some amines may act as precursor for other compounds capable of forming nitrosamines, which are carcinogenic to various animals including human (Shalaby, 1996). Putrescine and cadaverine on heating are converted to pyrrolidine and piperidine respectively, from which N-nitroso-pyrrolidine and N-nitroso-piperdine are formed. Therefore, while technological processes of food such as salting and smoking induce nitrosamine formation, cooking (frying) enhances their formation (Doyle *et al*., 1993). Tyramine, which leads to the formation of mutagenic compounds like 3- diazotyramine, induces oral cavity cancer in rats. Secondary amines such as agmatine and polyamines such as spermine and spermidine can also produce carcinogenic N-nitrosoamines in fish, meat and vegetable products.

5.1.4. Effect of storage on production of biogenic amines.

Studies have shown that the formation of histamine in Indian mackerel was not significant upto a period of 10 hour at ambient temperature (26°C) reaching an average value of only 7.51 mg/100 gm, and increased significantly thereafter (Vijayan and Balachandran, 1996). Studies by Veciana *et al*., (1996) reported that high contents of biogenic amines in semi-preserved anchovies was influenced by the hygenic quality of the raw material processed and the storage temperature during shelf life. According to Pacheco *et al*. (2000), both endogenous and microbial deterioration processes of sardine muscle could be controlled by storing at 0°C.

Studies by Rossi *et al*. (2002) revealed that cadaverine could be used either alone or together with histamine as part of quality control programme in skip-jack and big eye tuna. Du *et al*. (2002) confirmed that the change of tuna quality was affected by the bacterial

numbers found in the fillets while the increase in aerobic bacteria can serve as a useful indicator of the overall tuna quality, and the presence of a possible histamine or biogenic amine hazard. The increase in histamine producing bacteria contributes to the rapid increase of histamine or biogenic amines contents and health hazard especially to tuna fillets stored at 22°C.

According to Ferrario *et al*.(2012), although histamine level was less than 10 ppm in the tuna samples analyzed, many samples showed high total viable bacterial and enterobacterial counts that reached dangerous levels after temperature abuse for short periods of time. 30.5% of the 141 enteric bacteria isolated from tuna samples by his team were positive and potentially able to produce histamine. In the investigation conducted by Rahimi *et al*.(2012), 30 of 43 canned tuna fish samples (69.8%) had the presence of histamine in concentration between 17 to 210 mg/100g. Histamine in 18.6% canned tuna fish samples from two manufactories were higher than the tolerance limit of histamine contents (50mg Histamine/100g).

5.1.5. Biogenic amines as spoilage indicators

When decomposition progresses in a fish, the amount of biogenic amines also increases (Yamanaka and Matsumbo, 1989). As with other biological indicators such as TMA, DMA, TVB-N and K value, amines are also used as a potential freshness index in fish and shellfish. Yamanaka and Matsumbo (1989) reported that formation of putrescine, cadaverine and histamine and loss of spermidine and spermine were observed as decomposition of tuna progressed. He concluded that these amines might serve as quality indicators of tuna. Shakila *et al.* (2003) reviewed and found that histamine alone is not considered as a reliable indicator of decomposition as concentration of its precursor histidine vary greatly in scombroid and nonscombroid fish. The amines, which increase with storage time at normal temperature, are agmatine; histamine, cadaverine, tyramine etc. For these reasons Mietz and Karmas (1978) suggested a freshness index using different amines. According to them Freshness Index = (ppm cadaverine + ppm putrescine + ppm histamine)/

(1+ppm spermine + ppm spermidine). They reported that this formula has a simple mathematical design and as decomposition progresses, histamine, putrescine and cadaverine rise in their values, while spermidine and spermine fall. Later, Valle *et al*. (1996) modified it and gave the term Quality Index (Ql) or Biogenic Amine Index (BAI). So, Ql or BAI = (Histamine (ppm) + cadaverine (ppm) + Putrescine (ppm) / (Spermidine (ppm) + Spermine (ppm)). Good agreement has been observed between spoilage index and sensory quality.

5.1.6. Histamine food poisoning

Histamine food poisoning (HFP) is common and occurs world wide. Thus, control of the factors that allow seafood to cause HFP deserves to be improved. HFP during 1990s caused 32% of all seafood borne incidents of human disease in England and Wales. The situation is similar in USA where HFP caused 38% of all seafood-borne human disease outbreaks. These outbreaks accounted for 18% of all the people that became ill after consumption of sea food. HFP have been responsible for 8% of all the food-borne disease and 1.2% of all the people that became ill due to food consumption in USA (Mc Lauchlin *et al*., 2006). Between 1990 and 2003 HFP accounted for 7.5% of all food borne diseases outbreaks and 38% of all seafood related diseases reported to U.S. Centers for Disease Control and Prevention (Dewaal *et al*., 2006).

Histamine when formed in seafood is relatively stable and not inactivated by freezing or heating such as normal cooking, hot smoking or even canning (Arnold and Brown,1978; Taylor,1986; Lehane and Olley, 2000; Flick *et al*., 2001; FDA/CFSAN, 2001; Kim *et al*., 2003). Histamine in small amounts is not toxic for humans as it is metabolized prior to reaching the blood circulation. The enzymes histamine-N-methyltransferase (HMT), monoamine oxidase (MAO) and diamine oxidase (DAO or histaminase) transform histamine to less toxic metabolites that are excreted in urine and faeces. The enzymes are found primarily in the small intestine and liver of humans (Taylor, 1986). However if the normal histamine metabolism is reduced or very large amounts of histamine are consumed, then the concentration of histamine in the blood increases. It results in cutaneous, neurological and

gastrointestinal symptoms. The symptoms are mainly rashes, utricaria, flushing, headache, diarrhea and vomiting.

5.1.7.Regulations

Even though problems related to biogenic amines are of universal prevalence, very few countries impose limits on biogenic amines particularly on histamine. These limits are determined based on the type of micro flora, their capabilities, condition of spoilage, risk on gastrointestinal diseases, occurrence of amine oxidase inhibitor drugs etc. For this, hazard action level (HAL) or defect action level (DAL) has also been set.

Recently, the Food and Drug Administration (FDA) (21CFR123) established a guidance level for histamine of 5 mg / 100 g (50 ppm) for assuring the safe consumption of scombroid or scombroid-like fish and recommended the use of other data to judge fish freshness, such as the presence of other biogenic amines associated with fish decomposition (FDA, 1996). A maximum average histamine content of 10 mg / 100 g (100 ppm) has been established in the European Community (EC) for acceptance of tuna and other fish belonging to the *Scombridae* and *Scomberesocidae* families (Veciana *et al*., 1997). The EC has suggested that in the future a maximum of 300 ppm for total biogenic amines in fish and fish products may be an appropriate legal limit. In addition to this, cadaverine and putrescine could be used as freshness indices for fish and shellfish respectively (Shakila *et al*., 2003). Sims *et al*. (1992) suggested 0.5 mg/kg as threshold limit of cadaverine in fish.

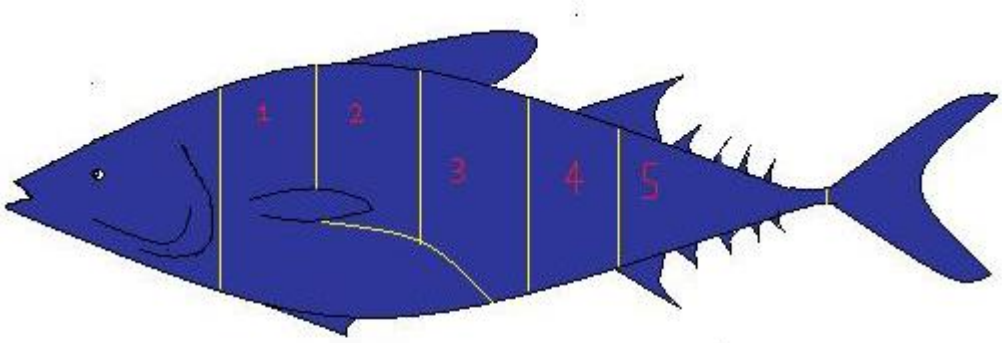
It is important to note, however, that there may be a type of poisoning that does not arise from high levels of histamine. Thus, a low histamine level may not be absolute assurance of a safe product. It may be more appropriate to say that the absence of decomposition in the fish renders it a safe product. As such, a safe product would have no evidence of spoilage including odors of decomposition, high levels of histamine, and other amines such as cadaverine. The present chapter reflects the antimicrobial effect of spice oleoresins in reducing bacteria responsible for biogenic amine formation and the ability to suppress histamine production at ambient temperature. In this study the changes in other

significant biogenic amines namely, putrescine, cadaverine, spermine and spermidine during storage of tuna at ambient temperature is also recorded.

5.2. Materials and methods

5.2.1. Preparation of the fish samples and storage conditions

Tuna (*Euthynnus af*finis) were purchased at the day of capture from a local fishing ground located on the Arabian Sea, Vypeen, Cochin. The average weight and length of fish were 1-1.5Kg and 60-70cm, respectively. The fish were packed in insulated styrofoam box containing ice and delivered to the laboratory, 6 hour postcapture. The fish was beheaded, gutted and cut into chuncks of 2cmx 1 cm x 1cm (l x b x h). According to Frank *et al*. (1981), the concentration of histamine can vary considerably even between different portions of a single fish (Fig. 5.1). Therefore, in order ascertain uniform histamine level, samples from the dorsal region close to head portion were taken in all cases.



Histamine mg/kg	1020	70	16	45	29

Fig 5.1. Within – fish variation in histamine concentrations within a fish (Frank *et al*., 1981)

This fish chunks were divided in to 8 groups. Each group of fish was subjected to dip treatments of spice extract (0.2%) of clove, cardamom, garlic, oregano, rosemary and turmeric. A commercial antimicrobial, chlorine was also used. Control sample was not subjected to any sort of treatments. The duration of dip treatment was 10 minutes at room temperature. The treated samples of fish were arranged on a plastic tray and stored at 28°C ± 2°C. Three randomly chosen fish chunks were immediately sampled (day 0), while the rest were kept at ambient temperature for 24 hours. After 1, 4, 9 and 24 hours, three randomly chosen fish samples were removed from the lot and analyzed in triplicate for the presence of histamine forming bacteria and also biogenic amines.

5.2.2. Biogenic amine analysis

5.2.2.1. Reagents

Biogenic amines standards (Putrescine dihydrochloride, cadaverine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, histamine dihydrochloride and agmatine sulphate) were purchased from Sigma-Aldrich, UK. HPLC grade Acetonitrile was used as solvent A and deionized ultrapure Millipore water was used as solvent B (RiOs and Elix Millipore Water Purification System).

5.2.2.2. Preparation of standard amine solution

Putrescine dihydrochloride (182.9 mg), cadaverine dihydrochloride (171.4 mg), spermidine trihydrochloride (175.3 mg), spermine tetrahydrochloride (172.0 mg), histamine dihydrochloride (165.7 mg) and agmatine sulphate (175.4 mg) were dissolved separately in 10ml HPLC grade water. A composite standard comprising all the above biogenic amines were also used. The final concentration of free base for each amine was 10 mg/ ml solution.

5.2.2.3. Sample preparation

Fish muscle (5 g) from all the treated and control samples were taken from the dorsal part of the fish fillet without skin and transferred to a centrifuge tube. The sample was homogenized with 20 ml 6% TCA (trichloroacetic acid) for 3 min, centrifuged at 12 000 g

for 10 min at 4°C and filtered through Whatman No.1 filter paper. This was made up to 50 ml with distilled water and was stored at -30°C until futher analysis.

5.2.2.4. Derivatization procedure: (Ozogul, 2002)

A stock solution was prepared by dissolving 2% benzoyl chloride in acetonitrile to enhance the reaction with amines. For derivatization of standard amine solutions, 50µl was taken from each free base standard solution (10 mg/ml) and 2 ml of TCA extract for fish samples. One ml of 2M sodium hydroxide was added, followed by 1 ml benzoyl chloride (2%) and vortex mixed for 1 min. The reaction mixture was left at room temperature (24°C) for 30 min. The benzoylation was stopped by adding 2 ml of saturated sodium chloride solution and the solution was extracted two times with 2 ml of diethyl ether. The upper organic layer was transferred into a clean tube and evaporated to dryness in a stream of nitrogen. The residue was dissolved in 500µl of acetonitrile and 20µl aliquot was injected for HPLC analysis.

5.2.2.5. Apparatus

Quantitative determination of the biogenic amines was conducted using a Waters HPLC system with a Binary pump model M 515, a 600 Gradient mixer solvent delivery system, a dual λ absorbance UV/VIS detector model 2487 and a C 18 Symmetry column (5 µM particle size, 4.6 mm id x 250 mm length column) with a flow rate of 1.5 ml/min. The equipment is provided with column oven, and a manual injector. Data analysis was performed using EMPOWER 2 chromatography software.

5.2.2.6. Chromatographic conditions

Chromatographic separation made use of continuous gradient elution with acetonitrile (eluant A) and HPLC grade water (eluant B). The gradient started at 80% acetonitrile and was decreased to 20% and finally increased to 80% in 16 min. The total separation time was less than 7 min and the gradient was run for 20 min to ensure full separation. HPLC gradient profile for separation of benzoyl derivatives of biogenic amines is as shown in Table 5.1. Detection was monitored at 254 nm.

A calibration curve for each of the amines in the range of 0-100µg/ml was prepared. Correlation of peak areas of individual amines standards and composite standards with known concentration was calculated after injecting each of the standard amine solutions.

Table 5.1. HPLC gradient profile for separation of biogenic amines.

	Time (min)	Flow rate ml/min	Acetonitrile (%A)	Deionized Millipore Water (% B)	Curve
1	-	1.5	80	20	
2	10	1.5	80	20	6
3	15	1.5	20	80	6
4	16	1.5	20	80	6
5	20	1.5	80	20	6

Data obtained in this study was analysed statistically using SPSS (Scientific Package of Social Science) version 17.0.

5.2.3. Analysis of Histamine Forming Bacteria

5.2.3.1. Reagents

Glucose (0.5 %), Tryptone (0.5 %), Yeast extract (0.5 %), NaCl (0.5 %), CaCO3 (0.1 %), Agar (2.0 %), Histidine hydrochloride (1.0 %), Saline (0.9 %), Peptone water (0.1%).

5.2.3.2. Plate preparation using Modified Nivens medium: (Niven et al., 1981)

Dissolved the required ingredients for 500ml DW in a 1 litre flask to avoid boiling over of the contents. Mixed thoroughly by gentle heating and sterilized by autoclaving at 121°C for 15 minutes at 15 lbs pressure. Cooled to 50 ºC, mixed well and poured into plates to get a thick layer.

5.2.3.3. Sampling

Asceptically cut 10g of skin with muscle of the fish sample into a sample dish. Macerated with 90 ml diluent (Normal Saline) in a sterile mortar(10^{-1} dilution) and serially diluted. A 1ml aliquot was taken for enumeration of histamine producing bacteria and mixed with Modified Niven's medium. Allowed the plates to set. Plates were incubated at 25°C for 2 days on the Modified Niven's medium. Purple colonies with or without halos were regarded as positive histamine formers. The test was carried out in triplicates.

5.3.Result and Discussion

5.3.1. Histidine decarboxylating bacteria (HDB)

To reduce histamine formation it is essential to inhibit growth of the specific bacteria that plays an important role in the production of the compound. The histamine forming bacteria formed in the samples during the 24 hours after treatment with spice extracts is ploted in the Fig.5.2. Statistical analysis for identifying the difference in means of bacterial count during the study period was done using SPSS software. ANOVA for histidine decarboxylating bacteria for treated samples showed significant difference between treatments ($p \leq 0.05$) and between time intervals ($p \leq 0.05$) (Appendix B: 5.1).The number of bacteria formed in spice treated and untreated samples showed a similar trend until 4^{th} hour of storage (Fig 5.2). After a duration of eight hours, the number of histamine producing bacteria present in all the samples shot up significantly. The control sample had a significantly higher number of histamine producing bacteria than the spice extract treated samples at all stages of the study ($p \leq 0.05$). This difference was evident very clearly after the eight hour of storage at 28±2°C and continued to the 24^{th} hour. This was confirmed by Post hoc tukey test (Appendix B: 5.2).

Analysis of variance explained that there is significant difference between the treatments too. From the graphical representation it can be seen that after storage for 24 hours, clove treated samples showed the least colony forming units of histidine

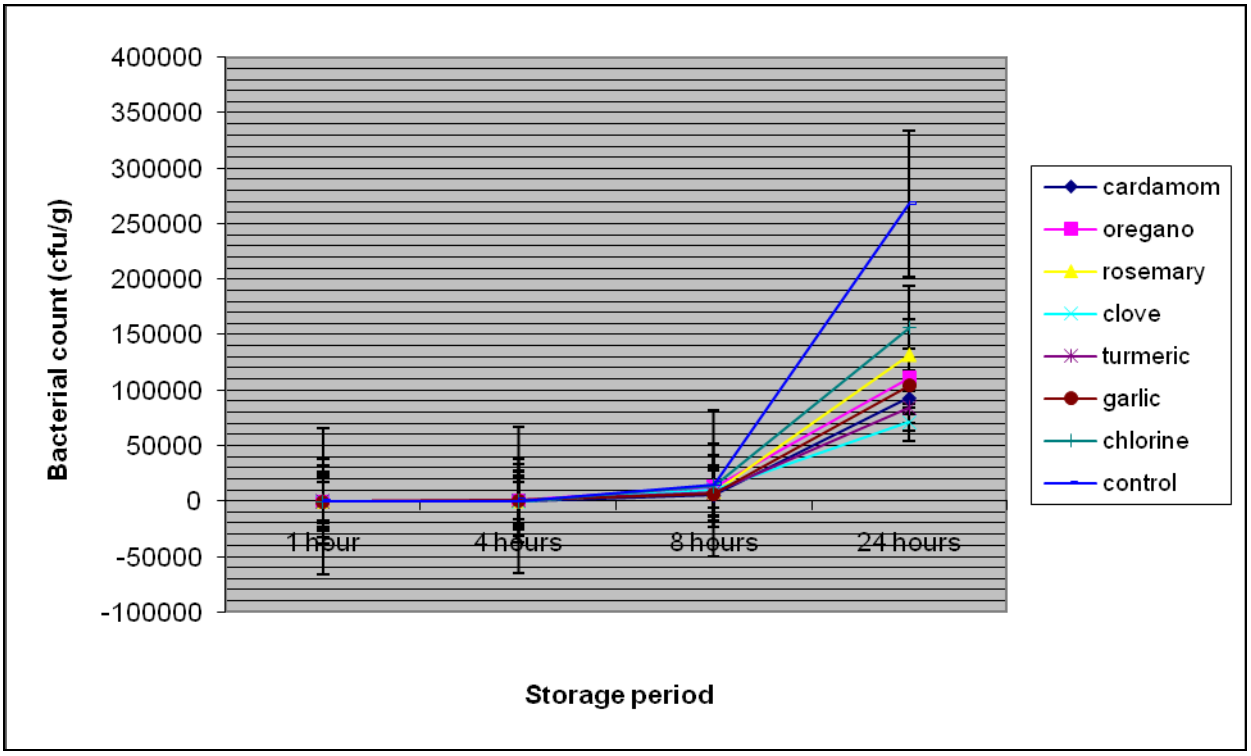


Fig.5.2. Histidine decarboxylating bacteria formed in treated tuna samples during storage at ambient temperature.

decarboxylating bacteria (HDB) compared to others. Control sample produced the highest number of HDB followed by chlorine, the common chemical antimicrobial used in seafood industry. The bacterial count in the ascending order is as follows: clove < turmeric < cardamom < garlic < oregano < rosemary < chlorine < control.

5.3.2. Biogenic amine formation

For estimating the amount of biogenic amines formed in the tuna chunks during the 24 hour storage at ambient temperature, fish muscle (5 g) from all the treated and control samples were taken from the dorsal part of the fish fillet without skin and samples were prepared as described in Section 5.2.2.3. Standard amine solutions were also made as explained in Section 5.2.2.2. The samples as well as standards were loaded in HPLC to estimate the amount of amines produced.

Initially, individual biogenic amine standards were run in the C-18 column and their retention values recognized (Appendix B: Fig 5.1-5.5) followed by a composite standard solution including a mixture of all standards (Fig 5.3). These chromatograms were used as reference in identifying the amine production in the fish sample.

Fig. 5.4. shows the difference in biogenic amines produced in tuna samples (without treatment) during the initial stage (0^{th} hour) of the experiment and after 24 hours storage at room temperature. The area given by a particular peak is directly proportional to the amine produced. It is evident that there is a drastic increase in the putrescine, cadaverine and histamine during the storage period. Spermine and spermidine have a retention time of 2.186 and 2.099 minutes, respectively, in the composite standard run through C-18 column (Fig 5.3). They produced a negligible peak compared to putrescine, cadaverine and histamine at 24^{th} hour of the experiment. A comparision between the chromatograms of 24^{th} hour control sample and 24^{th} hour treated samples is shown in Fig 5.5 to 5.11. In all the treated samples, the area of the peak is lower than that of control.

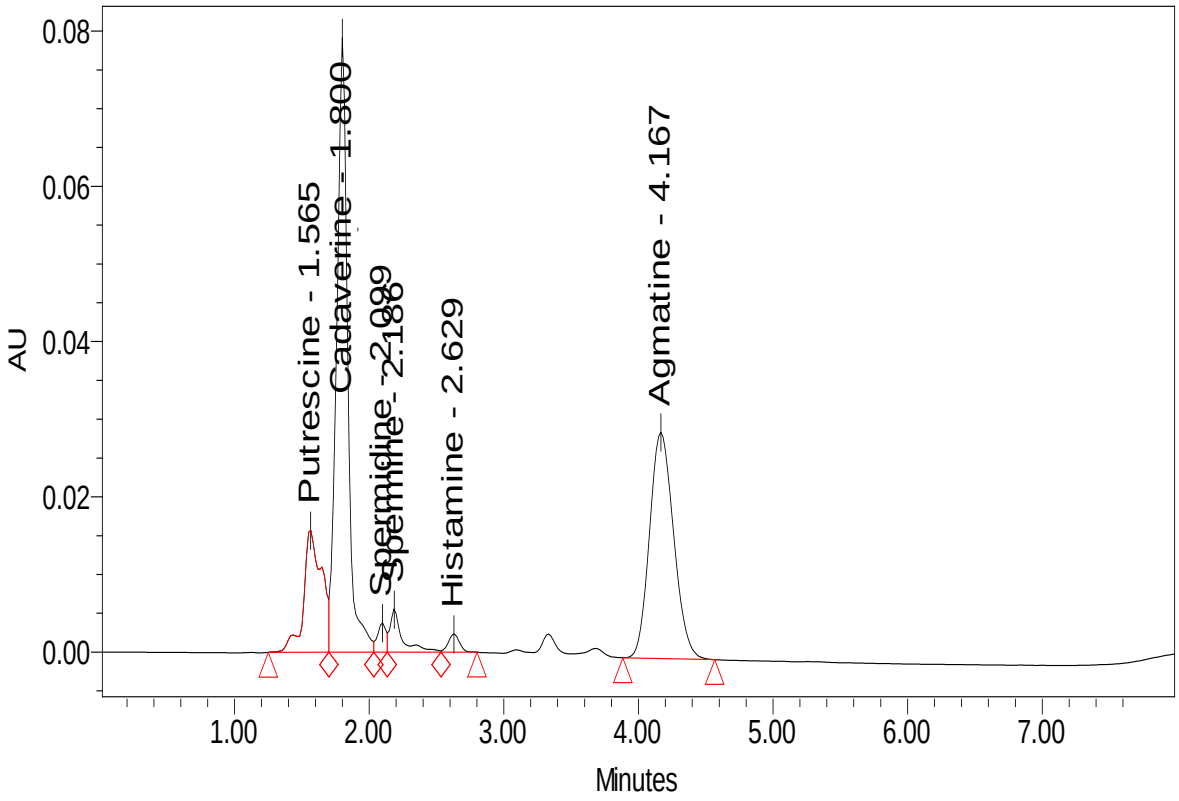


Fig. 5.3. Composite standard of biogenic amines

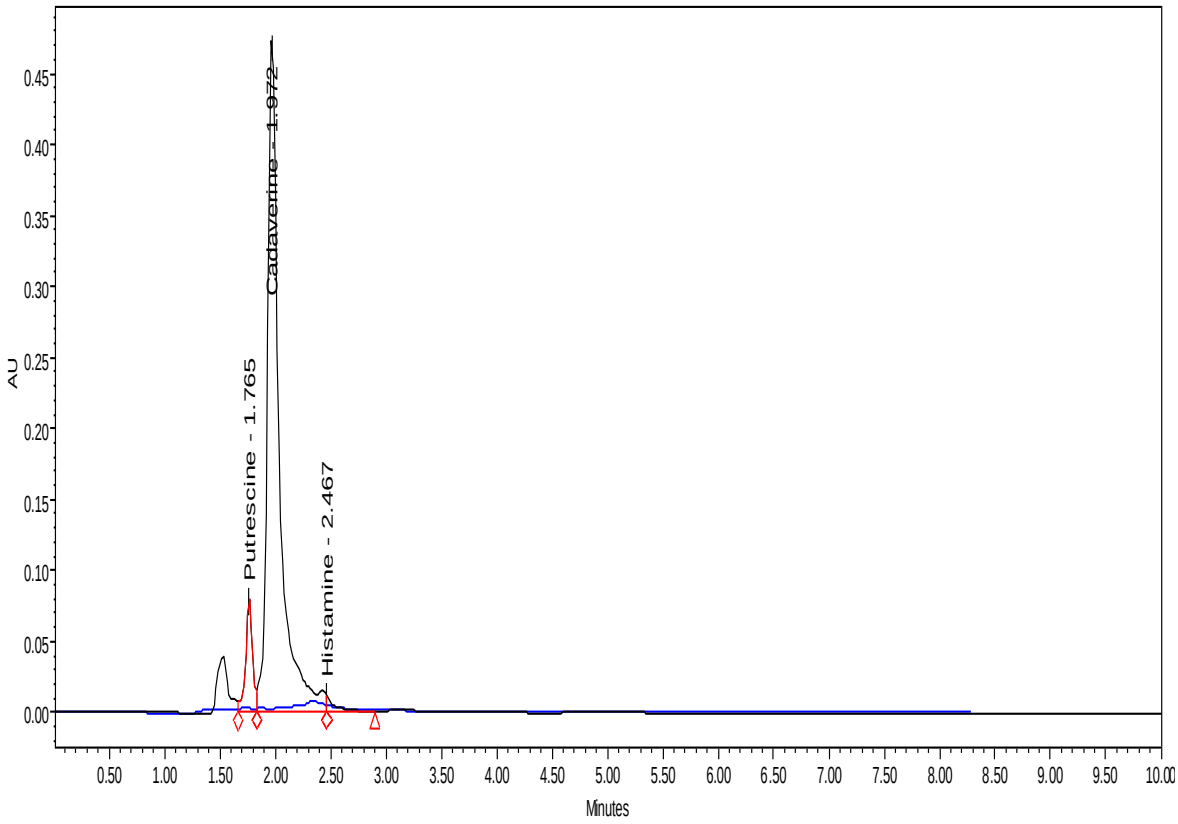


Fig 5.4. Comparison of biogenic amines formed during 0hr and 24 hr in control samples of tuna stored at 28±2°C

---- zero hour control

---- 24th hour control

.

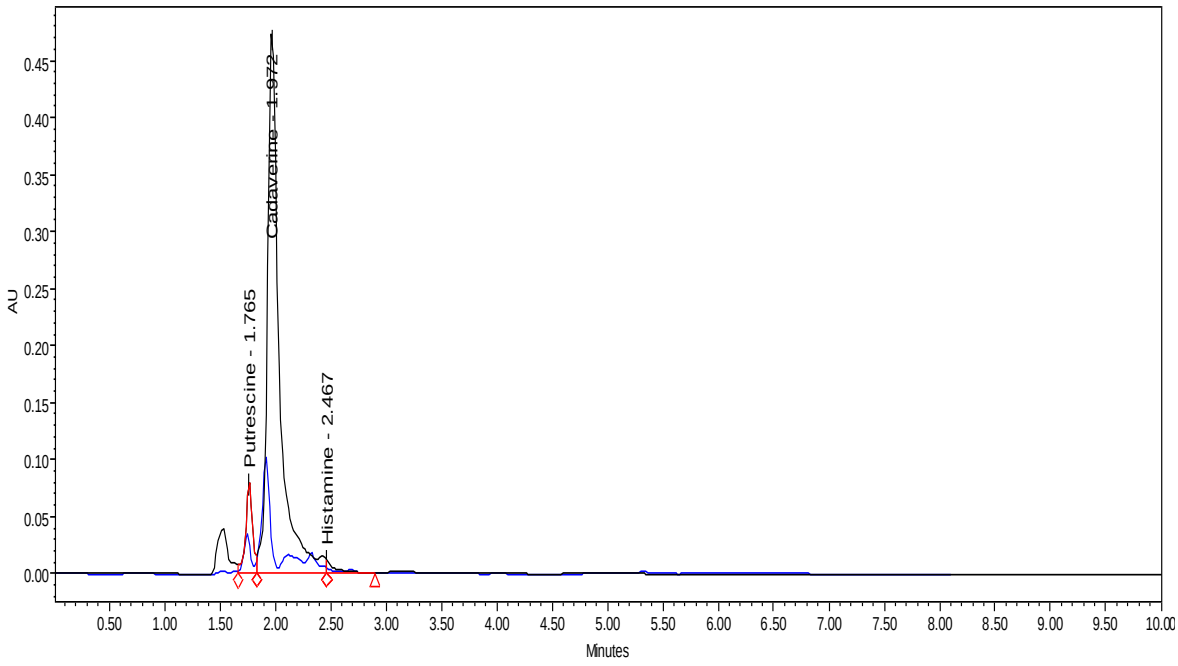


Fig 5.5. Biogenic amines formation in control and chlorine treated samples on storage for 24hrs at ambient temperature (--- 24th hour chlorine treatment , --- 24th hour control)

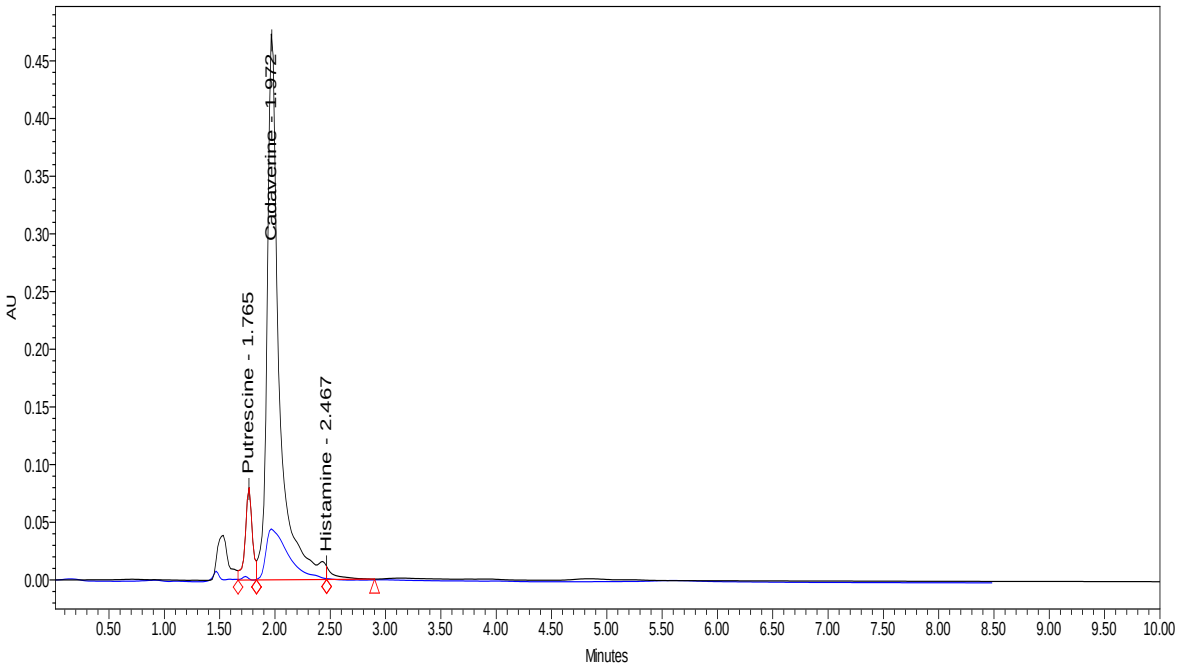


Fig 5.6. Biogenic amines formation in control and clove treated samples on storage for 24hrs at ambient temperature (--- 24th hour clove treatment , --- 24th hour control).

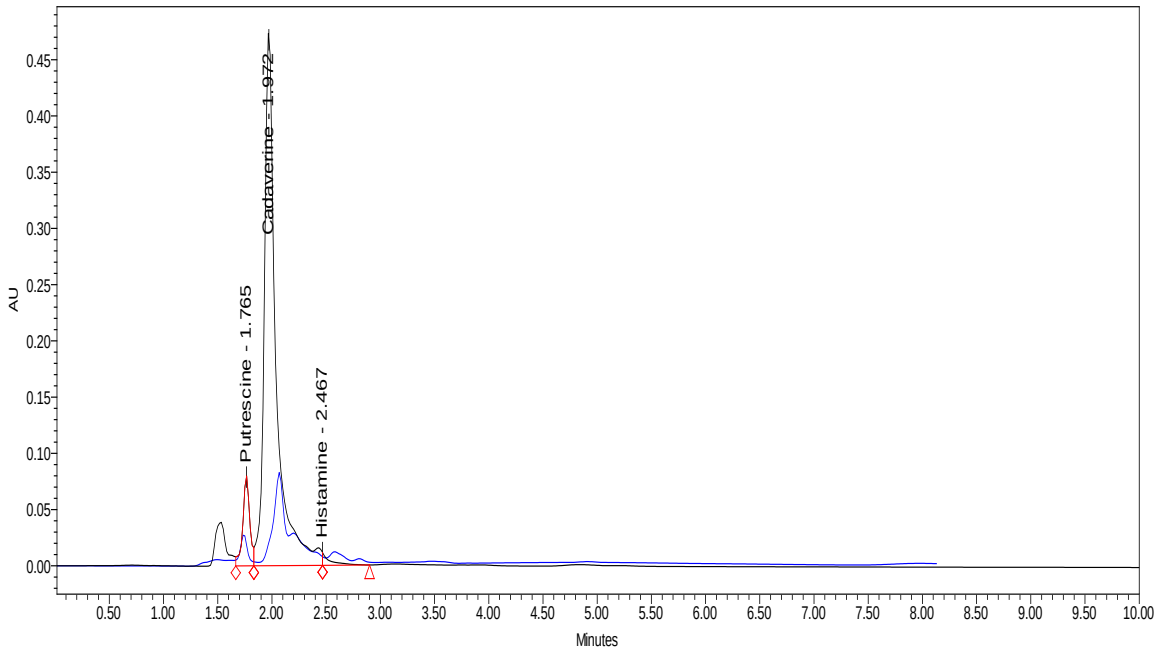


Fig 5.7. Biogenic amines formation in control and oregano treated samples on storage for 24hrs at ambient temperature (--- 24th hour oregano treatment , --- 24th hour control).

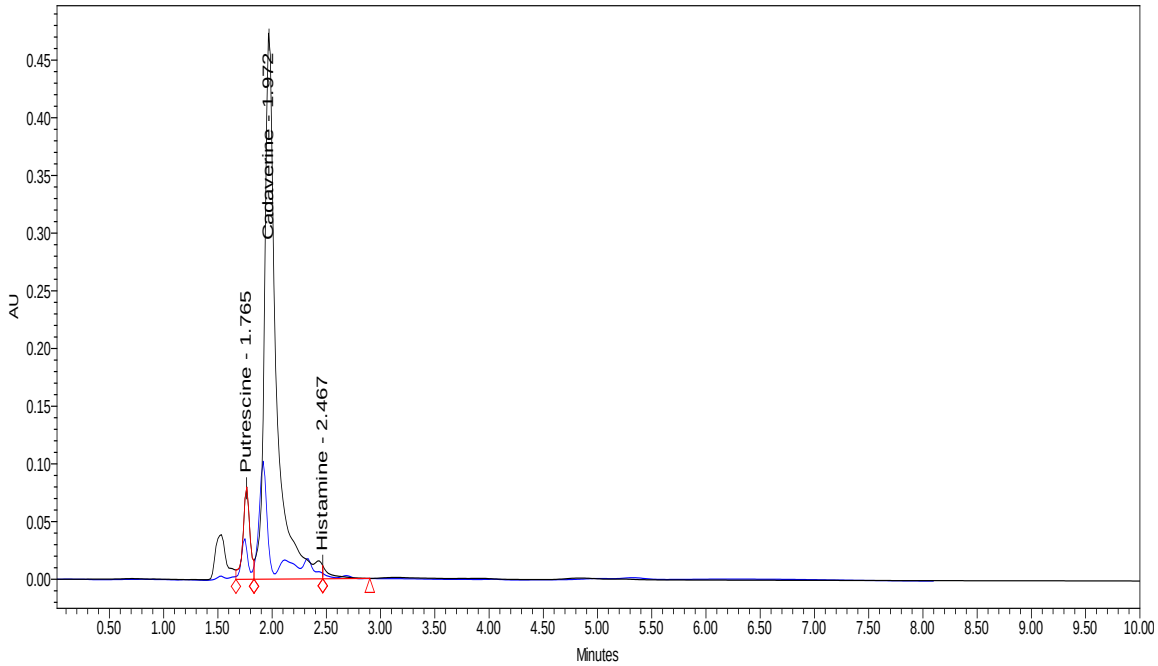


Fig 5.8. Biogenic amines formation in control and rosemary treated samples on storage for 24hrs at ambient temperature (--- 24th hour rosemary treatment , -- 24th hour control).

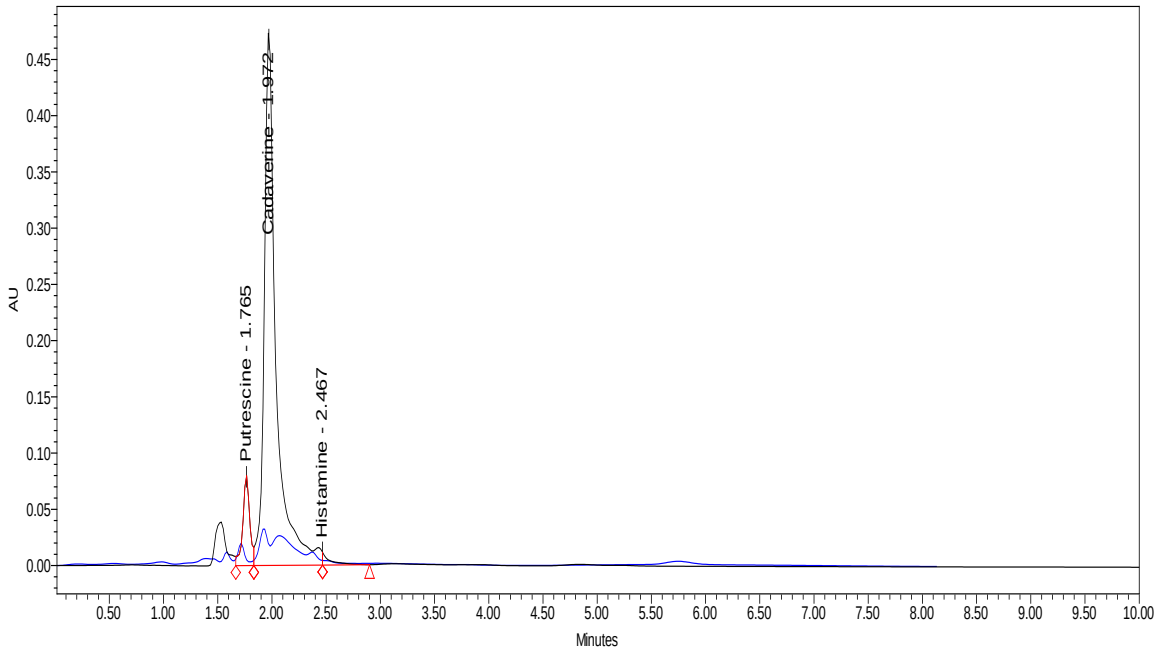


Fig 5.9. Biogenic amines formation in control and garlic treated samples on storage for 24hrs at ambient temperature (--- 24th hour garlic treatment , --- 24th hour control).

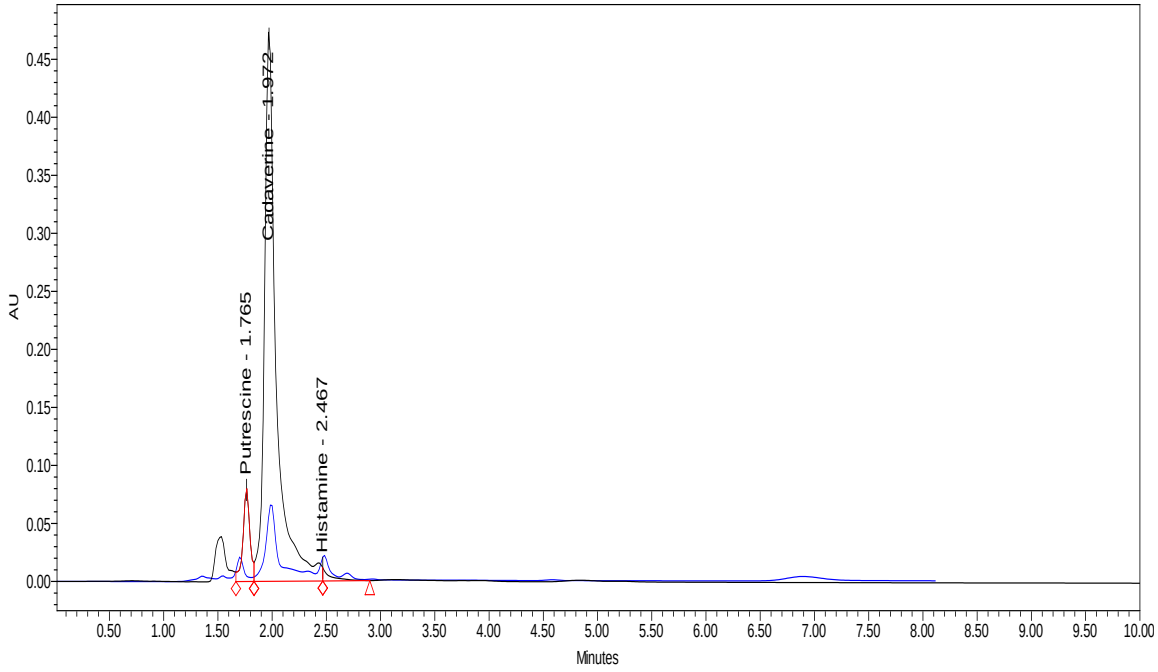


Fig 5.10. Biogenic amines formation in control and turmeric treated samples on storage for 24hrs at ambient temperature (--- 24th hour turmeric treatment , --- 24th hour control).

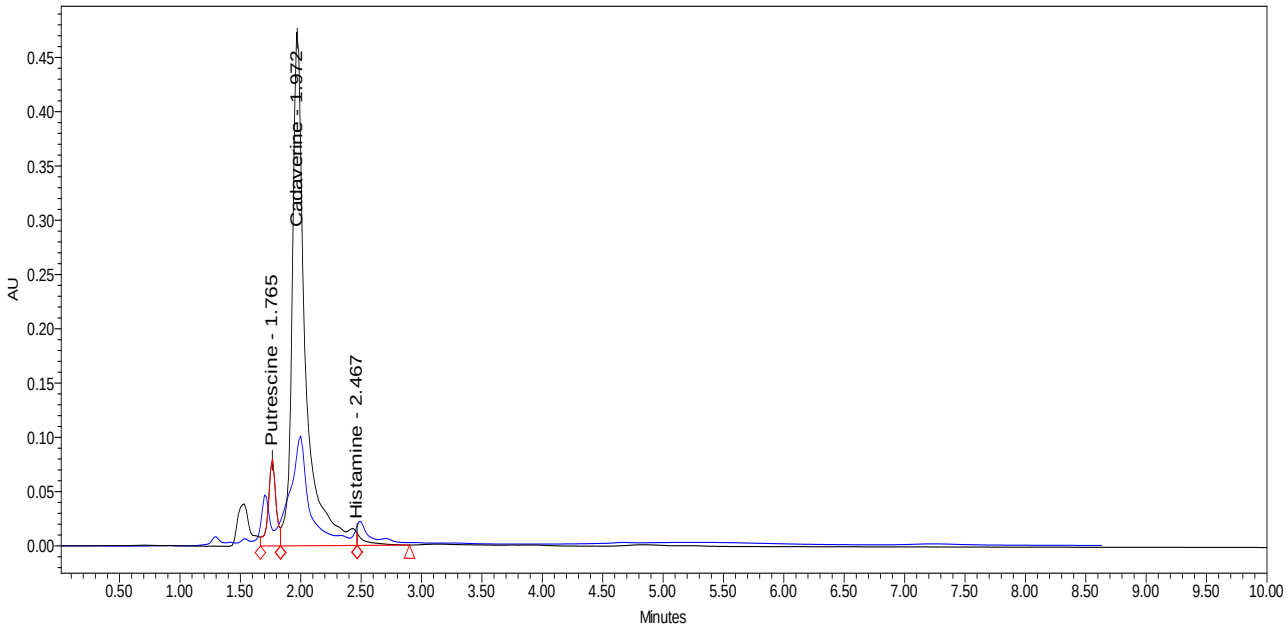


Fig.5.11.Biogenic amines formation in control and cardamom treated samples on storage for 24hrs at ambient temperature (--- 24th hour cardamom treatment , --- 24th hour control).

Table 5.2. Histamine content in spice extract treated tuna stored at 28 ± 2 °Cfor 24 hours (*mg/100g)

Stor-age hrs	Control	Chlorine	Cardamom	Clove	Garlic	Oregano	Rosemary	Turmeric
0	1.48±0.05	1.48±0.05	1.48±.05	1.48±0.05	1.48±0.05	1.48±0.05	1.48±.05	1.48±.05
1	2.34±0.2	1.08±0.08	2.34±0.5	1.45±0.3	1.43±0.62	1.97±0.61	0.96±0.8	3.98±0.6
4	8.05±1.1	1.59±0.45	3.07±0.7	1.93±0.58	1.63±0.79	4.45±0.64	1.37±0.3	5.31±0.4
8	11.5±1.09	1.82±0.39	4.72±1.3	3.33±0.44	3.44±0.34	4.47±0.27	3.24±0.8	6.09±0.7
24	18.2±0.83	23.22±1.7	16.99±1	3.91±0.8	3.99±0.85	4.595±0.73	4.41±0.5	16.05±1

* values are given as Mean±SD.

The values obtained by the analysis were subjected to two way ANOVA to understand whether there is any variance in mean values of histamine produced between treatments and time period (Appendix B : 5.3). Significant difference was observed between the treatment and time period at $p<0.05$. Secondary analysis also establish that the values differ significantly ($p<0.05$) between each time period of storage (Appendix B: 5.4). The amount of histamine produced in control and treated samples during the test period is tabulated in Table 5.2.

The histamine production in the treated and untreated samples stored in boxes at 28 ± 2 °C did not show any significant difference after the first hour of the experiment as seen in Table 5.2. But, sample analysed after the fourth hour gave a higher value of histamine for the untreated tuna stored at 28 ± 2 °C than spice extract treated tuna. A strong positive correlation ($r = 0.836$) was also seen between the amount of histamine produced and the HDB count (Appendix B: 5.5). This explains that bacterial count is directly propotional to histamine content. For this reason, when the number of viable bacteria increases, amount of histamine also goes up. Therefore, the reduction in amine production in treated samples with various spices can be explained by their strong antimicrobial action (Chapter 4).

A mean histamine reduction of 6.5mg/100gm in spice treated samples was observed from the control after 8 hour duration. Except cardamom and turmeric other oleoresin treated samples showed was only negligible increase (average amount =0.416mg/100gm) in histamine content between the 8^{th} and 24^{th} hours storage. The control samples experienced an increase of 6.7 mg/100gm at the above periods. The defect action level was attained by 4 hour of storage in the control. No sample treated with spices exceeded the rejection limit of histamine (50ppm) upto 8^{th} hour. In clove, garlic, oregano and rosemary, the samples remained below the permitted limit of histamine even after 24 hours.

Among the spice treated samples, the tuna chunks dipped in cardamom and turmeric produced a result closer to the control sample. Histamine content in clove, garlic and rosemary treated sample varied significantly from the other treatments with clove giving

the lowest value of histamine. The level of histamine was over 18.2 mg in control samples stored for 24 hours at 28 ± 2 °C and 3.91 mg in clove treated samples. Chlorine treated tuna samples were also analysed since, it is used as a commercial antimicrobial in the seafood industry. Though it produced excellent results till the eighth hour after treatment of the samples, the build up of histamine in the later hours showed a sharp increase from that of the spice oleoresin treated samples. The volatile nature of chlorine may be the reason for this abrupt increase in histamine content after the 8th hour.

Table 5.3. Changes in Putrescine content in treated and untreated samples of tuna stored at ambient temperature (*mg/100g)

Storage period (Hours)	Control	Chlorine	Cardamom	Clove	Garlic	Oregano	Rosemary	Turmeric
1	0.047 ±0.01	0.013 ±0.002	0.016 ±0.01	0.022 ±0.005	0.027 ±0.01	0.016 ±0.01	0.024 ±0.01	0.033 ±0.01
4	0.081 ±0.01	0.035 ±0.01	0.046 ±0.01	0.055 ±0.01	0.015 ±0.01	0.022 ±0.01	0.025 ±0.01	0.023 ±0.01
8	0.095 ±0.02	0.064 ±0.01	0.046 ±0.01	0.040 ±0.006	0.032 ±0.01	0.028 ±0.01	0.029 ±0.01	0.028 ±0.01
24	0.115 ±0.013	0.359 ±0.01	0.076 ±0.01	0.052 ±0.01	0.057 ±0.009	0.045 ±0.01	0.050 ±0.011	0.029 ±0.005

*values are given as Mean±SD

In this experiment, the concentrations of putrescine and cadaverine also increased during the storage period of tuna. The concentrations of cadaverine and putrescine increased more rapidly than spice extract treated tuna stored for 24 hours. There is a significant difference ($p < 0.05$) in concentrations of these amines between untreated tuna and spice treated tuna throughout the storage period. Table 5.3 and 5.4 shows the concentration of

putrescine and cadaverine, respectively, at ambient temperature. The storage periods were extended well beyond the accepted time for edibility in order to give a full picture of the production of these amines from the fresh fish to the putrid state.

Freshly caught tuna had a cadaverine content of 0.14mg/100gm and putrescine content of 0.047mg/100gm. The concentration of these biogenic amines were typically much lower than the concentration of histamine. Interestingly, the concentration of histamine was propotional to the concentration of cadaverine as well as to the sum of concentrations of histamine, putrescine and cadaverine (Appendix B: 5.6). Studies have shown that the levels of cadaverine in toxic or decomposed fish are generally several times greater than the levels of putrescine. In this experiment also the cadaverine content formed at the end of 24 hour storage was 9 times greater than that of putrescine content in the control sample.

Table 5.4. Change in Cadaverine content in treated and untreated samples of tuna stored at ambient temperature (mg/100g)

Storage period (Hours)	Control	Chlorine	Cardamom	Clove	Garlic	Oregano	Rosemary	Turmeric
1	0.144 ±0.04	0.023 ±0.01	0.022 ±0.01	0.006 ±0.01	0.025 ±0.01	0.005 ±0.01	0.037 ±0.01	0.150 ±0.01
4	0.057 ±0.02	0.030 ±0.01	0.049 ±0.01	0.030 ±0.01	0.011 ±0.01	0.013 ±0.006	0.036 ±0.01	0.154 ±0.01
8	0.206 ±0.01	0.154 ±0.01	0.144 ±0.01	0.076 ±0.013	0.067 ±0.01	0.096 ±0.008	0.079 ±0.003	0.155 ±0.01
24	0.955 ±0.011	0.840 ±0.02	0.294 ±0.01	0.106 ±0.007	0.091 ±0.01	0.183 ±0.01	0.143 ±0.01	0.224 ±0.01

*values are given as Mean±SD

5.4. Discussion

5.4.1. Histamine forming bacteria

In our study, tuna flesh is used as a substrate for bacterial growth and the histamine decarboxylating bacteria formed in the course of storage was enumerated using Niven's media. In the previous chapter a detailed microbial analysis of tuna was done and bacterial species namely, *Pseudomonas, Staphylococcus* and bacteria in the family *Micrococcaceae* and *Enterobacteriaceae (Escherichia* and *Salmonella)* were isolated and identified. These bacteria are known to possess amino acid decarboxylases (Galgano *et al*., 2009; Karovičová and Kohajdová, 2005; Marino *et al*., 2000; Suzzi and Gardini, 2003). This enzyme is responsible for producing biogenic amines in food products contaminated with these bacteria. The spice extracts used in the experiment had already shown strong antibacterial activity against these bacteria present on tuna. The decrease in HDB count in the spice treated samples is a result of reduction in the viable count of *Pseudomonas, Staphylococcus* and other bacteria belonging to *Micrococcaceae* and *Enterobacteriaceae.*

The increase in bacterial number over time can proportionately raise the amount of amines formed because amine production has been recognised as a defense mechanism of microorganisms against an acidic environment (Karovičová and Kohajdová, 2005; Suzzi and Gardini, 2003).The fish as a substrate for growth of this bacteria can readily provide an slightly acid environment during its postmortem changes. Tkachenko *et al*. (2001) suggested that some strains, with amino acid decarboxylase activity, could overcome or reduce the effects of temperature, NaCl, and other biological and chemico-physical factors that induce stress responses in the cells, with the production of some biogenic amines.

The importance of using measures focused on the hygienic quality of both raw material and processing units to avoid the development of aminogenic contaminant bacteria and in turn, to reduce biogenic amines content, is well known. However, proper hygiene

may not be enough to avoid some biogenic amines formation and other technological measures must be applied (Latorre-Moratalla *et al*., 2010).

The histamine producers include both mesophilic and psychrotolerant species. Strong histamine producers which include mesophiles do not produce toxic concentrations of histamine below 7-10°C. These bacteria include the Gram-negative *Morganella morganii, Hafnia alvei, Raoultella planticola, Raoultella ornithinolytica, Klebsiella oxytoca, Citrobacter braaaki, C.freundii, Enterobacter aerogenes, Proteus vulgaris, Pr.mirabilis, P.damselae subsp damselae, Serratia fonticola* and Gram-positive *Staphylococcus epidermis and Tetragenococcus muriaticus* (Taylor *et al*.,1978; Lopez-Sabater *et al*., 1994; Kimura *et al*.,2001; Kim *et al*., 2001, 2002; Kanki *et al*., 2002; Takahashi *et al*., 2003).

The present method used for controlling histamine food poisoning mainly target mesophilic species. The control measures used are mostly maintaining a low temperature (0-4°C) with the help of ice cubes and chill rooms at all levels of food processing. But, during frozen storage , the major factor in the histamine production will be psychrotolerant bacteria. *Pseudomonas spp*. are reported as important spoilage organisms in many chilled food products, such as milk (Reddy *et al*.,1969), chicken (Pittard *et al*.,1982) and fish (Miller *et al*.,1973 a,b), in which they become the dominant flora during chilled storage. This dominance is assumed to be attributable exclusively to their rapid growth at chill temperature. *Pseudomonas spp.* and *Bacillus spp.* were found in psychrotrophic conditions in previous studies (Singh and Venkataramana, 1998). It is also reported that these bacteria possess amino acid decarboxylases (Galgano *et al*., 2009; Karovičová and Kohajdová, 2005; Marino *et al*., 2000; Suzzi and Gardini 2003). In the present experiment, 18% of the initial bacterial flora on tuna was *Pseudomonasspp.* which implies the risk of low temperature maintenance alone for keeping histamine formation under control.

Photobacterium phosphoreum and *Morganella psychrotolerance* are also strongly histamine producing psychrotolerant species (Takahashi *et al*.,2003; Kanki *et al*.,2004; Emborg *et al*.,2005, 2006; Emborg and Dalgaard, 2006; Dalgaard *et al*.,2006).

M.psychrotolerans and *P.phosphoreum* can grow at 0°C but not at 37°C and their temperature optimum is 20-30°C (Dalgaard *et al*.,2008). The presence of such bacteria cannot be controlled by the existing control measures like maintaining the product at <5°C before frozen storage. Hence, histamine formation during extended storage of fish at low temperature must not be disregarded. This aspect is not given due care in the present condition. Merely reducing the temperature of the product to be consumed will not help in managing the histamine food poisoning. It seems more promising to reduce growth of the strongly histamine producing bacteria in products of the relevant marine finfish.

In short, it can be stated that toxic concentrations of histamine in seafood are produced by bacteria and high concentrations of strongly histamine producing bacteria are required for this. Therefore, it is possible to control histamine formation by limiting the contamination and by reducing the growth of these bacteria. In the present study, the spice treatment given to the tuna prior to storage ensured the reduction in the number of histamine forming bacteria. Its use will help in bringing down the risk of histamine food poisoning even under storage conditions by keeping the psychrophilic and mesophilic population in check.

5.4.2. Biogenic amines formation

The treatment of tuna with spice extracts have resulted in decreased production of histamine with respect to the untreated samples. Studies show that extract of clove flower bud inhibits immediate hypersensitivity in rats by inhibition of histamine release from mast cells in *vivo* and *in vitro* (Kim *et al*., 1998). This property might be responsible for the reduced histamine production in clove treated samples. The inhibitory activity of the essential oil component and other natural antimicrobials on histidine decarboxylase is a possibility that needs further research.

Chlorine is used as an commercial antimicrobial in the seafood industry. Though it produced excellent results till the eighth hour after treatment of the samples, the build up of histamine content in the later hours had a sharp increase from that of the spice oleoresin

treated samples. The volatile nature of chlorine may be the reason for this abrupt increase in histamine content after the 8th hour. This information is supported by a study conducted by Baranowski *et al*. (1990). In their study, post-harvest antimicrobial treatments of fish did not show much promise in inhibiting histamine information. Fishes (*Mahi mahi*) were incubated in seawater and in seawater containing 100 ppm of sodium hypochlorite or chlorine dioxide. But, neither histamine formation nor quality loss was inhibited. Moreover, the histamine content in sodium hypochlorite treated samples (2340ppm) were higher than the control (1,230ppm) sample after 18 hours of incubation.

Prediction of histamine formation in seafood, as a function of time and temperature, can be used to avoid storage conditions that result in toxic products. Mathematical models can therefore contribute to reduce histamine food poisoning (HFP). Development of models for histamine-producing bacteria is important to improve the usefulness of the approach for management of HFP practice. Multiplication of bacteria is much faster at ambient temperatures. Storage of seafood below 2°C or 4.4°C prevents histamine formation by mesophilic bacteria and reduces the rate of histamine formation by psychrotolerant bacteria. However, psychrotolerant bacteria can produce toxic concentrations of histamine in chilled seafood at 2°C to 4.4°C.

From experiments conducted to study the effect of NaCl on histamine production, it was found that high concentrations of NaCl inhibit the growth of bacteria and thereby histamine formation in seafood (Emborg and Dalgaard, 2006). Use of histamine formation by NaCl to control histamine formation requires firstly that histamine is not formed in the fish raw material prior to the addition of salt. Secondly, the addition of NaCl must be sufficient to reduce growth of relevant histamine forming bacteria. With mackerel at 20°C, 1-2% salt slightly stimulated histamine formation, whereas histamine formation at toxic level was delayed from one to two days by 3% NaCl and from one to four days by 4% NaCl (Yamanaka *et al*.,1985). For vaccum packed cold-smoked tuna at 5°C, histamine formation by *P.phosphoreum* was prevented by 4.4%±0.8% water phase salt (WPS). But, one drawback is that 3% of NaCl will make the fish too salty for consumption. In the present

study it was found that we could reduce the histamine formation by applying 0.2% of spice extracts. The effect of this concentration of spices on the textural properties of tuna and its consumer acceptance is further studied and the result is presented in the next chapter.

Histamine alone may not be the culprit in the toxicity of scombroid fishes. There were instances were people became ill due to consumption of seafood with less than 500mg of histamine/kg (Emborg and Dalgaard., 2007). Human subjects given up to 67.5 mg histamine orally did not produce any subjective or objective symptoms of histamine poisoning (Granerus, 1968). However, Sjaastad (1966) reported that 36 mg or more of histamine administered to subjects subsequently developed symptoms associated with histamine toxicity. Symptoms appeared also with tuna sandwiches containing 100, 150, and 180 mg doses of histamine. Generally, high histamine levels are able to cause a toxic response, but subsequent research has indicated that other factors may also be responsible. A study by Clifford *et al*. (1991) was conducted on mackerel fillets associated with an outbreak of scombrotoxicosis. Statistical analysis failed to detect any differences in amine content between fillets which were shown to be scombrotoxic and those failing to induce symptoms of poisoning. They also failed to establish any significant relationships between the amine doses of both the samples. But it has also been found that 180mg histamine resulted in severe headache and flushing (Motil and Scrimshaw,1979; van Gelderen *et al*.,1992). Thus, available data from challege studies with human volunteers suggest that pure histamine cannot always explain the toxicity of histamine-containing seafood.

Two different hypotheses to explain the apparently low toxicity of pure histamine have been discussed in scientific literature. It was proposed that seafood may contain a mast cell degranulator and that this compound could be cis-urocanic acid. The degarnulator should cause a release of histamine from mast cells in human intestinal tissue, and HFP would then be due to indigenous histamine (Arnold and Brown,1978; Taylor,1986; Clifford *et al.*,1991; Ijomah *et al*.,1991; Lehane and Olley,2000). The other hypothesis is older and suggests that oral toxicity of histamine in seafood can be potentiated by different compounds including other biogenic amines (Kawabata *et al*., 1955; Taylor,1986).Cadaverine and

putrescine have been shown to potentiate the toxicity of histamine (Chu & Bjeldanes, 1981; Taylor & Sumner, 1986; Stratton *et al*., 1991).

A prior study by the investigators (Mietz and Karmas 1977) reported that high quality tuna had cadaverine and putrescine values ranging from 0.24 - 5.32 and 0 - 1.84 ppm (0.02mg-0.5mg/100gm and 0-0.0184mg/100gm), respectively. When cadaverine was administered through stomach catheters simultaneously with histamine, peroral toxicity was observed in the guinea pigs (Bjeldanes *et al*., 1978). Klausen and Lund (1986) reported that at 10 °C the high cadaverine contents of mackerel in comparison with herring could be responsible for mackerel often being implicated in scombroid poisoning and not herring, since histamine levels were similar in both. Cadaverine and putrescine, as well as other diamines, have been suggested to facilitate the transport of histamine through the intestinal wall and to increase its toxicity (Fernandez-Salguero and Mackie, 1987).

Numerous experiments with laboratory animals (cats, dogs, guinea pigs, pigs, rabbits and rats) have demonstrated that various compounds can inhibit the normal histamine metabolizing enzymes and thereby increase the oral toxicity of histamine. Data from human volunteer studies are limited but tuberculosis patients taking the drug isoniazid, which is known to inhibit histamine metabolizing enzymes have increased sensitivity to histamine in seafood (Uragoda and Kottegoda, 1977; Miki *et al*., 2005). This supports the hypothesis that compounds which inhibit histamine degradation increase the oral toxicity of histamine. However, van Gelderen *et al*. (1992) found that 22mg of cadaverine and 18mg of putrescine was unable to potentiate the oral toxicity of histamine.

A variety of microorganisms are able to produce biogenic amines. The production of cadaverine and putrescine by microorganisms is not surprising since the covalent linking of cadaverine and putrescine to the peptidoglycan is necessary for normal microbial growth (Suzuki *et al*., 1988). It may not contribute to significant amounts of putrescine and cadaverine in food samples. Neverthless, a negligible decrease of these amines can occur in the presence of preservatives.

The risk related with the compounds which can inhibit the normal histamine metabolizing enzyme is still not properly worked out. With laboratory animals this has been observed for agmatine, cadaverine, putrescine and a combinations of these compounds. Despite all uncertainties reported, histamine levels above 500 - 1,000 mg / kg (500 - 1,000 ppm) are considered potentially dangerous to human health based on the concentrations found in food products involved in histamine poisoning (Ten *et al*., 1990). The spice oleoresin treatment is found to reduce the production of these biogenic amines along with histamine. Hence, whether or not the toxicity of biogenic amine is proved, use of spices will help in reducing HDB formation and as a result help in reducing the risk of histamine food poisoning.

5.5. Conclusion

Using measures focused on the hygienic quality of both raw material and processing units to avoid the development of aminogenic contaminant bacteria and in turn, to reduce biogenic amines content, is usually practised. However, proper hygiene may not be enough to control biogenic amines formation and other technological measures must be applied.

The histamine producers include both mesophilic and psychrotolerant species. The present method used for controlling histamine food poisoning mainly target mesophilic species. But, during frozen storage , the major cause for histamine production will be psychrotolerant bacteria. This aspect is not given due care. Mostly in such cases, merely reducing the temperature of the product to be consumed will not help in managing the histamine food poisoning. At present it seems more promising to reduce growth of the strongly histamine producing bacteria in products of the relevant marine finfish.

Based on the experiments in this chapter, it can be concluded that the treatment with spice oleoresins could delay biogenic amine formation in tuna. Clove, Garlic, Oregano and Rosemary treated tuna had significant difference from control sample in their histamine inhibition property. This reduction in biogenic amine is directly proportional to the reduction

in histamine forming bacteria in the spice treated samples. Spices are of great importance to the food industry. The inhibitory ability of these spices to arrest toxic amine formation can be used beneficially and hence, their application on fish can be strongly recommended. Since spices are usually used for flavouring purposes, its consumer acceptability is already proved. Still, the effect of spices on the texture and sensory properties of tuna flesh needs to be analysed. The methods used for the sensory and textural analysis and the corresponding results are explained in the next chapter.

6.1 Introduction

Texture of a food product along with its sensory properties plays an important role in consumer acceptance of a product. Firmness is a critical factor in determining the acceptability of raw fish products (Veland and Torrissen, 1999). Musculature of fish contains a great number of muscles that, depending on their anatomical location and activity exhibit structural and compositional differences that lead to different functional properties and processing abilities. Both intrinsic and extrinsic factors of muscle tissue affect the rheological characteristics. Myofibrillar protein and collagen that comprise 70 – 80% of the total protein content control the structure and the specific rheological properties of muscle tissue. Post mortem textural changes are caused by physiochemical changes in the myofibrillar proteins and changes in the extra cellular spaces. Texture is also affected by the pattern of arrangement of structural components and the changes occurring during processing techniques employed. Time-temperature profile also plays a significant role in affecting the textural characteristics of the muscle tissue.

Texture is a complex sensory experience and is also a multi-faceted concept describing the physical properties of foodstuff related to mouth feel and quality. Mouth feel means feelings associated with the process of mastication, salivation, touching with tongue and swallowing the food. Textural variations are complex and changes with moisture, size, temperature, state of surface and structures of foodstuffs. The rheological properties like elasticity, viscosity, visco-elasticity etc. are ideal for evaluation, if these parameters could be correlated with one's mouth feel. Textural judgments are solicited after visual and non-oral examination of food. Flavor and texture of muscle are delicate factors influencing sensory preferences of consumers. Thus, their evaluation is a critical factor in seafood products, as improper processing might lead to poor quality and rheological characteristics, reducing their economic value.

6.1.1. Muscle proteins and textural properties

Most textural properties of seafood products are due to the composition and structure of the muscle proteins (Goll *et al*., 1977). Proteins (sarcoplasmic and myofibrillar) alter human perception of fish quality by enzymatic reaction (by the production of sensory compounds like nucleotides and volatile amine compounds) and by direct changes in protein structure that alter tissue properties like juiciness, toughness, gel forming ability and water holding capacity. Chewiness is another function of hardness, cohesiveness and springiness of food (Bourne, 1979). It is another important criterion affected by protein structural changes. Toughness is the most critical quality parameter of tissue. Muscle toughness is a complex property and depend upon the two structural proteins namely connective tissue and myofibrillar protein that give the tissue its mechanical property. Each of the structural components of the connective tissue makes a distinct contribution to the overall toughness of the meat. Hatae *et al*. (1986) observed an inverse correlation between collagen content and tenderness of raw fish muscle. Proteolysis also alters the association of the muscle fibers and their interaction between protein and water molecules that might account for tenderness and rheological changes in the muscle (Dunajski, 1979).

From the quality control point of view it would be desirable to have a simple, nondestructive instrumental method to measure texture in raw fish that could be related to the perceived texture of the final cooked product. The important parameters that are measured includes fracture force, adhesion, hardness etc. Fracture and adhesion may seem very distinct processes, but both depend similarly on the energetic state of surfaces and elasticity of the solids involved, not on any critical value of force or stress (Kendall, 2001). This struggle is mirrored in the human mouth when solid foods are being eaten. When foods are first ingested into the mouth, the major objective is surface creation via the fracture of particles. This surface exposure releases tastes and, later on in the gut, allows gut enzymes to digest food faster.

The elastic behavior of foods is extremely important in determining both fracture and adhesion. In foods with approximate linear stress-strain relationships, the gradient of stress-strain, measured at an appropriate rate, provides value for the Young's modulus of a food. Such measurements are well documented in the food science literature (Bourne, 2002). The essential property for fracture is that which describes the work required to fracture a solid particle, called its toughness. In itself, toughness only provides an estimate of the resistance to crack formation in a food particle during specific mechanical tests designed to measure that quantity. In any practical situation, the elastic response of the solid is also important. If the stress-strain relations of foods are distinctly nonlinear, then more complex fracture models have to be introduced (Purslow, 1991). The property that induces surfaces to stick together is called the work of adhesion (Kendall, 2001). It behaves identically, but oppositely, to that of toughness and is again defined in terms of the work required to separate adhering surfaces, divided by the extent of their surfaces.

6.1.2. Neurophysiology of the oral processing of food

The most important elements for perceiving surface texture are the ability to sense finger force and displacement. There are two particular types of sensory receptors embedded in the skin pads under the finger tips that can help. Meissner's corpuscles lie in the skin itself just beneath the epidermis, while Pacinian corpuscles are rather more deeply set. The former are almost always called 'touch' receptors and the latter, 'pressure' sensors. However, because the skin is very soft, both are restricted to judgments about fine movements or pressures because they become so compressed as pressure rises beyond a certain limit (Peleg, 1980). For a greater sensory range, more distantly organized receptors, either in the muscles or joints, must help out. Of these, the sensory receptors in bony joints seem important only at the limits of movement (Clark and Horch, 1986). Within the muscles, neuromuscular spindles act as important finger-position sensors (Ferrell and Smith, 1988), while Golgi tendon organs, located where fleshy muscle fibres join the collagenous tendon fibres that often attach muscles to bone, respond to force (Crago *et al*., 1982).

There are sensory receptors in the jaw joint, which act like those in fingers and influence jaw movements of very large amplitude (Klineberg, 1980). There are also

abundant spindles in jaw closing muscles, which are certainly important in sensing the fine position of the jaw when it is elevated and the teeth are acting against food. The mouth is much more specialized than hand. Displacements are probably monitored continuously while the jaw is closing, but forces probably are not.

When foods are first ingested into the mouth, the major objective is surface creation via the fracture of particles. This surface exposure releases tastes and, later on in the gut, allows gut enzymes to digest food faster. However, the food fragments that build up in the mouth after a number of chewing cycles can easily get lost around the mouth unless there is some way to keep them together. Saliva release triggered by biting helps food particles adhere together to form a bolus (Hutchings and Lillford, 1988; Lillford, 1991). A bolus can be swallowed in a single action, so clearing the mouth of most food debris.

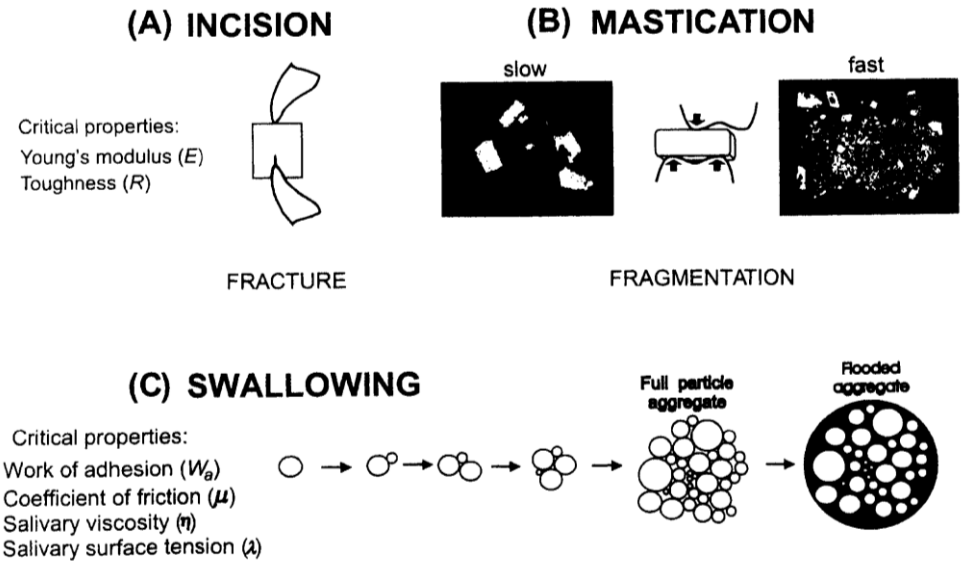


Fig 6.1. Important steps during the intake of food (Lucas *et al*., 2004)

Food particles stick not just to themselves, but to the oral mucosa (the soft tissue lining of the mouth). Critical factors that determine whereas food particles aggregate or either stick around the mouth are the work of adhesion in food-food and food-mucosal

interfaces. The frictional resistance that needs to be overcome to displace food particles from the mucosa using the tongue is another vital factor (Prinz and Lucas, 2000). Assuming linear elastic behaviour in foods, combinations of the toughness of food particles and their Young's moduli largely determine the fracture response of foods either to incision involving fracture of particles by the incisors (Fig.6.1A) or mastication (Fig. 6.1B). This process is followed by swallowing of food (Fig. 6.1C).

6.1.3. Sensory analysis

Descriptive sensory tests are amongst the sophisticated tools in the arsenal of the sensory scientist (Lawless and Heymann, 1998) and involve the detection and description of both the qualitative and quantitative sensory components of a consumer product by trained panel of judges (Meilgaard *et al*., 1991). The qualitative aspects of a product include aroma, appearance, flavour and texture of a product; which distinguish it from others. Sensory judges then quantify these product aspects in order to facilitate description of the perceived product attributes. A major strength of descriptive analysis is its ability to allow relationships between descriptive sensory and instrumental or consumer preference measurements to be determined. Descriptive sensory analyses are also used for quality control, for the comparison of product prototypes, to understand consumer responses in relation to products sensory attributes and for sensory mapping and product matching. It is also used to investigate the effects of ingredients or processing variables on the final sensory quality of a product and to investigate consumer perceptions of product.

All descriptive methods require a panel with some degree of training or orientation. In most cases panelists are required to have a reasonable level of sensory acuity. Of the utmost importance to the overall success of the project is the commitment and motivation of the panelists. They are needed to attend the training or evaluation sessions to be of value to the programme (Murray *et al*., 2001).

6.1.4. Texture analysis

Muscle texture could be measured by studying the rheological properties and by observing some of the physical and chemical parameters related to the texture. Texture of fish muscle could be measured by different organoleptic and analytical procedures. Later techniques include cell fragility tests, changes in protein solubility and water binding capacity (Hamm, 1975). Soft flesh leads to reduced consumer acceptability (Ando, 1999). Fillet texture can be measured using trained taste panels or a variety of instrumental methods (Hyldig and Nielsen, 2001). Instrumental texture analysis when performed under standardised conditions may provide more precision and repeatability relative to taste panels (Veland and Torrissen, 1999). Instrumental texture analysis analyses a group of sensory properties perceptible by means of mechanical and tactile receptors from mechanical, geometrical and surface properties of food (Szczesniak, 1963a, 2002; Bourne, 2002). Various instrumental techniques to measure texture have been developed involving puncture, compression, shear and tensile techniques.

Studies of the rheological properties yield parameters more closely related to the sensory evaluations. Mechanical methods are suitable for quantifying mechanical texture namely hardness, springiness, cohesiveness, toughness (firmness); chewiness, and (stiffness) resistance to mastication (Szezesniak, 1963b). Protein quality was found to influence strain to failure more than rigidity and water content influences rigidity more than strain to failure (torsion). Good correlations have been reported by Sawyer *et al*. (1984) between sensory hardness and chewiness of cooked fish samples from the families *Lutjanidae* and *Scorpaenidae***,** and the maximum shear stress, obtained from a puncture test on cooked samples. Sensory methods are evidently not suitable in most countries. Sensory texture of cooked fish has been analysed by different methods, including the Texture Profile Analysis (TPA), developed by Brandt *et al.* (1963) for all kinds of foods. The parameters considered in this profile are useful for defining fish texture (Cardello *et al*., 1982). The instrumental TPA method, consisting of a double compression of a sample, provides some empirical parameters, related to sensory concepts, that have been proven to be appropriate for

measuring fish texture (Schubring and Oehlenschlager, 1997**;** Barroso *et al*., 1998)**,** including that of tuna.

Davey and Gilbert (1974) investigated the effect of cooking temperature on protein - protein interactions, enzyme hydrolysis and textural quality and observed that at temperatures between 55ºC and 85ºC tenderizing takes place that could be due to alkaline protease activity. It is also indicated that the texture of cooked meat was affected by gelatin derived from the muscle collagen.

6.1.5. Effect of spices on texture and sensory parameters

In all cultures unique flavor principles have been important in preparation of traditional meals. The flavors not only provide nutritional value but enhance the pleasure of eating by making the meal more exciting and complete (Reinbach *et al*., 2007). During past few decades, non-meat additives have been widely utilized in meat products to reduce products costs and improve the functionality of the products. These additives include vegetable proteins, dietary fibers, herbs and spices, and probiotics, and they can increase the nutritional value and provide benefits to human health (Zhang, 2010).

Spices are known to enhance and improve the flavour and colour of foods (Ekanem and Solomon, 1997; Ekanem and Achirnewhu, 1998). They vary in their effects in that the aroma and flavour principles in spices are based on the essential oil contained in them. The ethanolic extract of the spices were also found to have potential for use in the development of antimicrobial drugs in a study conducted by Shamsuddeen *et al*. (2009).

Investigations have shown that ginger possess effective tenderization properties when added to meat products (Thomson *et al*., l973; Ziaudin *et al*., 1995). Naveena and Mendritta (2001) studied the tenderizing effect of ginger on sheep meat and found that samples with ginger were more juicer than control. Sediek *et al*. (2012) showed that it is possible to produce safe and high-quality fresh sausage using natural antioxidants source as ginger extract (1.0%) to improve the quality and stability of frozen sausages.

The objective of the present study is to determine whether the selected spice treatments on tuna have any enhancing effect on textural quality parameters. The effect of spices on textural changes in fish/fish products has not been studied in detail. Hence an attempt is made to investigate the effect of spice treated samples on the textural parameters and organoleptic qualities on thermal treatment. The effect of spice combinations on the textural and sensory properties of the product is also studied.

6.2 Materials and Methods

6.2.1. Raw material collection and sample preparation

Fresh whole tuna (*Euthynnus affinis*) was collected as described in 3.2. It was divided in to 8 groups. Each group of fish was subjected to dip treatments of the specific spice extracts (0.2%) namely, clove, cardamom, garlic, oregano, rosemary and turmeric. A commercial antimicrobial, chlorine (2ppm) was also used. Control sample was not subjected to any sort of treatments. The duration of dip treatment was 10 minutes with two different concentrations of spice extract (0.2% and 0.1%). In case of spice combination, the individual spices were added in such a way that the final spice concentration was not more than 0.2%. The dip treated samples were stored in a refrigerator at 4ºC for one hour.

6.2.2. Cooking Procedure

The cooking was done according to the procedures of Stoneham *et al*. (2000) and Verlinden *et al*. (2000). The uniform sized fish fillets were wrapped in 5mm thick aluminum foil and placed in a wire-mesh basket. It was immersed in a thermostatic water bath, maintained at $100 \pm 1°C$ and cooked for 20 min. After cooking, the samples were immediately cooled in water containing ice for about 3 min and subsequently equilibrated at room temperature prior to conducting the mechanical tests as well as sensory analysis. The cook loss was also calculated.

6.2.3. Electrophoretic analysis of cook water

Single dimension SDS/PAGE was performed on a vertical slab gel electrophoresis system according to Laemmli (1970) to analyse proteins lost in cook water in treated and control tuna samples. The running gels were 10% and sample wells were made using a 20 well template. Sample solubilized in 0.1N NaOH were digested with an equal volume of SDS in sample buffer at 100°C for 5 to 10 minutes. The gels were stained with CBB R-250 (coomassie brilliant blue R-250). A mixture of 7% acetic acid and 20% methanol in water was used as destain.

Protein present in the cook water was also estimated quantitatively by Lowry's method (Lowry *et al*., 1951).

6.2.4. Texture analysis

Instrumental texture profile analyses of samples were done using Texture Analyzer (Lloyds UK Instruments) according to Bourne, (1978). During measurement, a small flat-faced cylindrical probe of 50mm diameter compressed a bite-size sample of fish of standard size and shape (2 cm^3) placed on the base-plate. This was compressed and decompressed two times by a platen attached to the drive system. The test speed and trigger force were standardized to 15 mm/min and 0.5 N respectively. It imitated the action of the human mouth. From the force-time curve various textural parameters like hardness, cohesiveness, springiness and stiffness were evaluated since they were statistically significant. Five replicates of measurements were taken for each sample. The maximum force required for the first and second compression denoted the hardness I and hardness 2 and the ratio of the area under the second cycle of compression curve to the area under first cycle compression curve determined as the cohesiveness (Bourne, 1978).

Fig.6.1 shows a typical TPA curve generated by the texturometer. The height of the peak on the first compression cycle (F2) is defined as hardness1 and the second peak (F3) is defined as the hardness2 (Fig.6.2.). Fracturability was defined as the force of the significant break in the curve on the first bite (F1). The ratio of the positive force areas under the first and second compressions (A1/A2) was defined as cohesiveness. The negative force area of

the first bite (A3) represented the work necessary to pull the compressing plunger away from the sample and was defined as adhesiveness. The distance that the food recovered its height during the time that elapsed between the end of the first bite and the start of the second bite was defined as the springiness. Two parameters were derived by calculation from the measured parameters: Gumminess was defined as the product of Hardness x Cohesiveness and Chewiness was defined as the product of Gumminess x Springiness. Texture Analyzer (Lloyds UK Instruments) gives both a force- time and force- distance ratio.

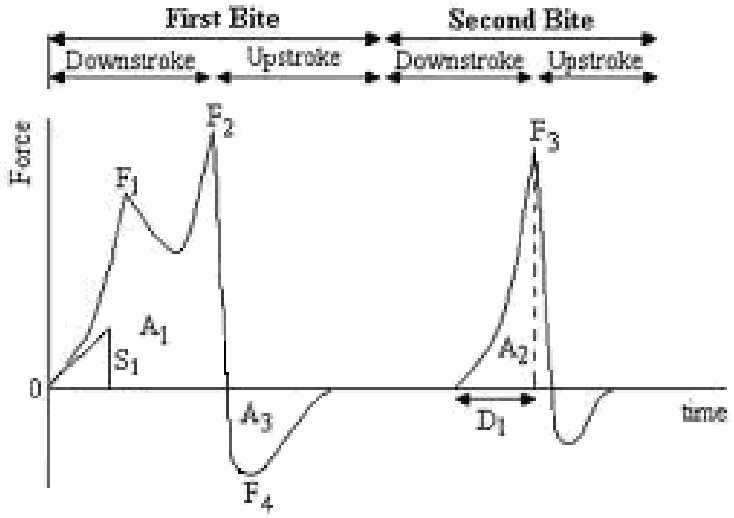


Fig 6.2. Texture profile analysis curve

6.2.5. Organoleptic evaluation

Selection of Panelists.

6 panelists were selected from the staff and post graduate students from CUSAT, Cochin, India based on their interest, availability, articulation, previous experience in sensory evaluation and familiarity in eating fish. A prescreening exercise was done in which the panelists were evaluated for normal sensory acuity through basic taste test, odor and intensity ranking tests (using the hardness scale) as described by ASTM (1981a, b).

The panelists who passed the prescreening test were selected for further training and were trained.

The panelists judged the samples individually. A dorsal piece of each sample wrapped in aluminium foil was served in a dish coded with random three-digit numbers. Consecutive samples were served after a one-minute rest. Mineral water was served to clean the mouth between samples and to eliminate residual mouth-coating effects. Tests were carried at around 28°C, with normal white fluorescent illumination.

Each panelist was given a sensory texture profile evaluation form to evaluate the texture attributes of cooked tuna. The different textural properties evaluated were firmness (force required to compress the material between the molars or between the tongue and palate), elasticity (rubbery mouth-feeling), cohesiveness (extent to which a material could be deformed before it ruptures), juiciness (feeling of liquid in the mouth after chewing 3 to 4 times) and hardness (force required for biting through the sample). The sensory panel also recorded the sensory descriptions of the sample (odour, appearance, flavour/taste and overall acceptability scoring) using 7-point hedonic scale. Five replicates of each sample were considered. The selected characteristics were tested as defined by Jowitt, (1974). The Performa 1 and 2 for the sensory evaluation is given in Appendix C 6.1 and 6.2. In order to check the consistency and reproducibility of their ratings, the panelists were made to assess the products 3 weeks later without being informed that they are rating the same products that they assessed before. Good, reproducible and consistent results were obtained.

6.2.6. Statistical analysis

Analysis of variance (ANOVA) was carried out using the generalized linear model procedure. The difference of means between tests was resolved by using the least significant difference. The level of significance was set at $p< 0.05$ (Snedecor and Cochran, 1989).

Two-way ANOVA (sample and treatment) was applied to the instrumental TPA data. Minimum significant differences were calculated by Tukey's honestly significant test (P <

0.05). Data obtained by the sensory evaluation were also subjected to statistical analysis using SPSS (Scientific Package of Social Science) version 17.0.

6.3. Results

The 0.2% spice extracts of clove, cardamom, garlic, oregano, rosemary and turmeric were found to be effective in controlling bacterial growth and histamine production in the previous chapters. Consumer acceptability of the tuna samples treated with spices was checked by texture profile analysis with a 50 Newton load probe and the data was supplemented with sensory analysis. Cook loss occurring for the treated samples, the amountof protein lost in cookwater and the molecular weight of the lost proteins were also analysed. The experimental data collected were subjected to statistical analysis using two factor ANOVA. Significant difference was calculated at 0.05% level and significant effects were identified.

6.3.1. Cook loss

Table 6.1 shows the cook loss of treated samples cooked at 100^{o}C. There was no significant difference ($p<0.05$) between the spice treated samples and untreated control cooked at 100^{o}C after dip treatment in 0.2% concentration for 10 minutes (Appendix C: 6.3). Control samples had a mean yield of 87.08±1.4 g. Though there was no significant difference in yield ($p<0.05$) among the spice treated samples, there was a marginal decrease in the yield of turmeric treated samples.

During cooking, water soluble sarcoplasmic proteins in the range of 40 KDa to 70 KDa are also lost along with the cook water. Amount of protein lost in the cook water was estimated using Lowry's method. The results are illustrated in Fig 6.3. ANOVA for protein content in treated samples and control did not show any significant change ($p<0.05$) though the mean value was higher for control (Appendix C: 6.4).

Table 6.1. Cook loss of treated samples cooked at 100°C

Sl.no	Treatments	Yield (%)	Cook loss (%)
1	Control	87.08±1.4	12.92±1.4
2	Chlorine	87.91±1.1	12.09±1.1
3	Clove	86.95±1.02	13.46±1.02
4	Cardamom	87.51±1.07	12.49±1.07
5	Garlic	85.02±1.22	14.98±1.22
6	Turmeric	84.55±1.27	15.45±1.27
7	Oregano	86.89±2.6	13.11±2.6
8	Rosemary	85.78±2.1	14.22±2.1

*values are given as Mean±SD

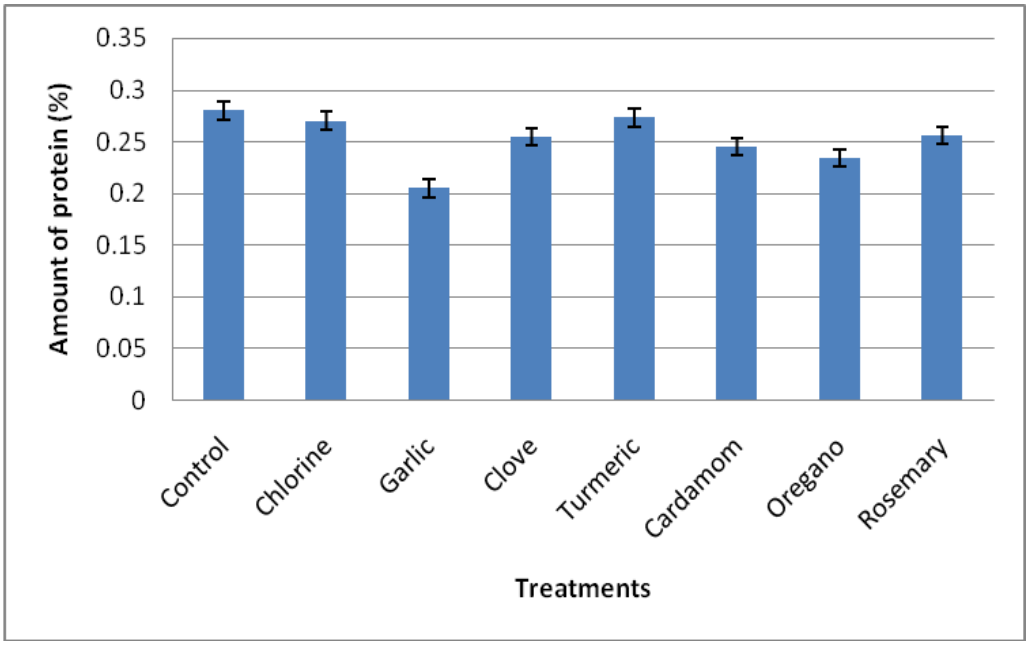


Fig.6.3. Amount of protein lost in cook water in spice treated tuna samples.

6.3.2. SDS-PAGE

SDS-polyacrylamide gel electrophoresis was performed for assessing the effect of spice treatment in the proteins present in cook water. The gels were visualized after staining with commassie brilliant blue. The broad range molecular markers (HMWM 205 KDa to 3 KDa) consisted of the following proteins : Myosin (205 KDa), Phosphorylase B (97.4 KDa), Bovine serum albumin (66 KDa), Ovalbumin (43 KDa), Carbonic anhydrase (29 KDa), Soyabean trypsin inhibitor (20.1 KDa), Lysozyme (14.3 KDa), Aprotinin (6.5 KDa) and Insulin (3 KDa). Plate 6.1 shows the electrophoretogram of proteins present in the cook water of control and spice treated tuna muscles in 10% SDS-PAGE gel. Comparision of the protein bands in the gels with their Rf values showed a similar pattern and hence the bands were compared visually. The corresponding molecular weights of the protein bands are mentioned on the plate.

Cook water of the treated clove samples had fewer protein bands compared to control samples. Rosemary and cardamom treated samples showed narrow banding compared to that of other spices. The protein band in the range of 20.1KDa remained intact in the control and clove treated samples while the protein band of 29 KDa remained intact only in control sample. This may be due to the cleavage of higher molecular weight proteins into lower molecular proteins by the action of spices.

Additional bands were observed to appear between the range 29-43KDa for cardamom and garlic treated samples. This could be the result of protein aggregates formed by the addition of spice. Consequently the protein band toward the origin became thicker with the protein having formed aggregates that were too large to enter the gel during electrophoresis. The low molecular weight bands (20.1 to 3 KDa) which are present in all the treatments including control, is found absent in garlic treated tuna.

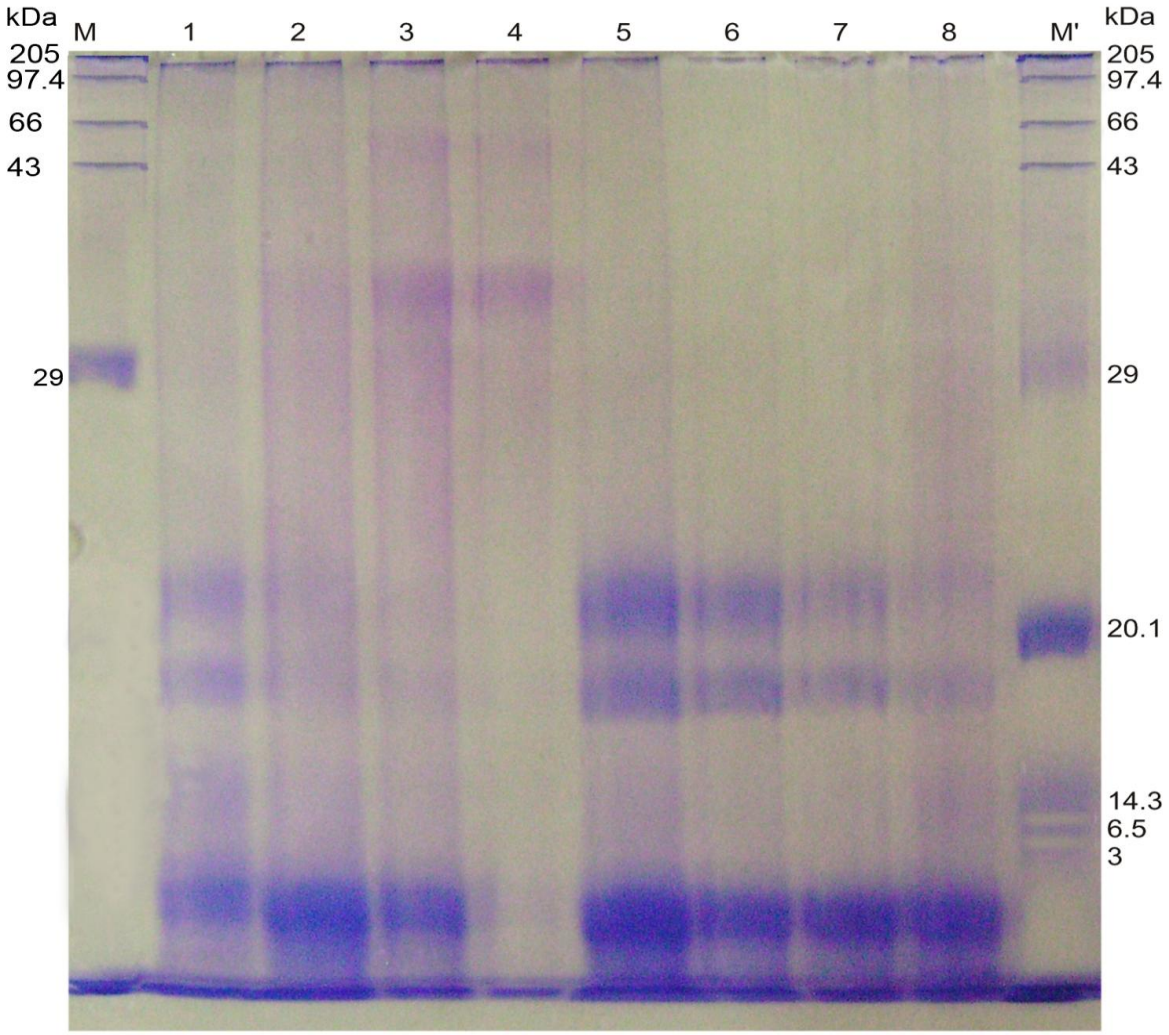


Plate 6.1. Electrophoretic analysis of cook water of treated tuna samples

(M- High range marker, 1-Chlorine, 2-Rosemary, 3-Cardamom, 4-Garlic, 5-Turmeric, 6-Oregano, 7-Clove, 8-Control, M'- Protein marker Broad range)

6.3.3. Texture Profile Analysis (TPA)

A comparative analysis of the texture profile of the control tuna with those of tuna chunks treated with spice extracts were done to determine the variability of the texture profile attributes. The experimental data collected were subjected to statistical analysis using two factor ANOVA.

Samples treated with 0.2% concentration of spice extracts showed higher hardness values than those treated with 0.1% concentration (Table 6.2 and 6.3). But, there was no significant difference between concentrations ($p<0.05\%$) in the cohesiveness, springiness, guminess, chewiness and stiffness values (Appendix C:6.5). Hence, the later investigations were limited only to 0.2% spice treatment. This concentration is already proved to be optimum for keeping bacterial population in check (Chapter 4: Section 4.3).

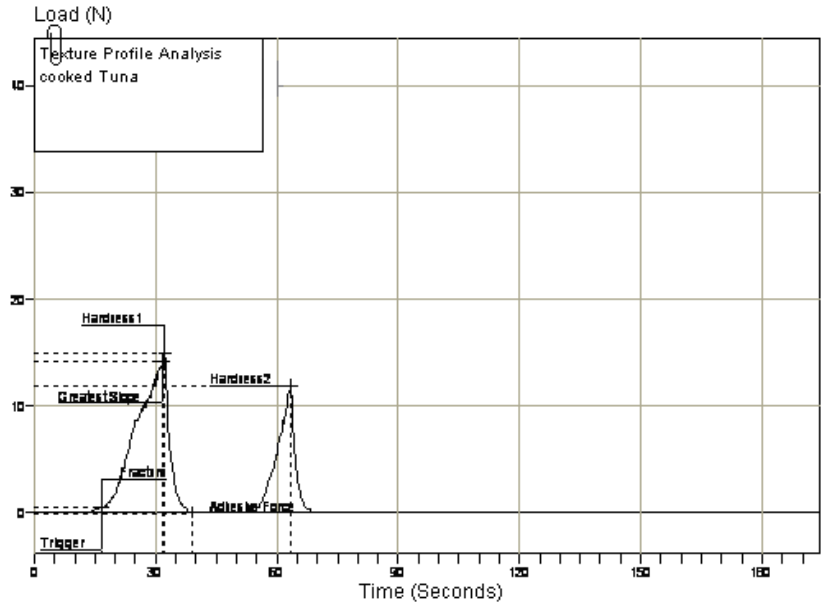


Fig. 6.4. Texture profile analysis (TPA) curve for tuna

Table 6.2. Effect of 0.2% spice treatment and cooking at 100° C on texture of tuna meat

Treatment	Hardness1 (kgf)	Cohesiveness	Springiness (mm)	Gumminess (kgf)	Chewiness (kgf.mm)	Stiffness (kgf/mm)
Control	0.75±0.03	0.256±0.054	2.73±0.071	0.28±0.006	0.65±0.07	0.35±0.056
Chlorine	0.88±0.08	0.238±0.015	2.53±0.013	0.21±0.001	0.73±0.05	0.81±0.029
Clove	1.25±0.08	0.278±0.039	3.20±1.04	0.35±0.09	1.12±0.04	0.34±0.005
Cardamom	0.78±0.06	0.289±0.049	2.65±0.026	0.22±0.07	0.60±0.14	0.36±0.001
Garlic	0.79±0.13	0.257±0.008	2.16±0.04	0.25±0.02	0.63±0.07	0.26±0.001
Turmeric	1.12±0.09	0.323±0.085	3.28±0.13	0.32±0.006	1.07±0.01	0.25±0.040
Oregano	0.89±0.04	0.252±0.011	2.56±0.07	0.10±0.002	0.64±0.01	0.28±0.045
Rosemary	0.71±0.04	0.275±0.072	2.83±0.03	0.19±0.003	0.64±0.08	0.18±0.022

*values are given as Mean±SD

Table 6.3.Effect of 0.1% spice treatment and cooking at 100°C on texture of tuna meat.

Treatments	Hardness1 (kgf)	Cohesiveness	Springiness (mm)	Gumminess (kgf)	Chewiness (kgf.mm)	Stiffness (kgf/mm)
Control	0.75±0.024	0.29±0.051	3.17±1.086	0.23±0.037	0.74±0.038	0.31±0.005
Chlorine	0.86±0.056	0.33±0.017	2.38±1.020	0.32±0.003	0.76±0.031	0.65±0.042
Clove	1.06±0.05	0.25±0.070	3.50±0.026	0.27±0.039	0.95±0.094	0.29±0.017
Cardamom	0.71±0.054	0.33±0.056	3.13±0.020	0.31±0.017	0.97±0.06	0.64±0.06
Garlic	0.77±0.086	0.30±0.053	2.70±0.028	0.27±0.013	0.73±0.034	0.39±0.007
Turmeric	1.08±0.099	0.28±0.017	3.06±0.075	0.36±0.091	1.13±0.227	0.27±0.065
Oregano	0.86±0.061	0.28±0.070	2.63±0.046	0.24±0.086	0.65±0.050	0.25±0.09
Rosemary	0.70±0.074	0.28±0.005	2.88±0.068	0.24±0.017	0.16±0.023	0.18±0.061

*values are given as Mean±SD

From the TPA, the dominant textural parameters for cooked tuna were identified as springiness and hardness for both the concentrations. The influence of spices in the hardness value may be due to moisture lost in the samples treated with different spices. The statistically analyzed results of the TPA of 0.2% concentration spice treated samples are summarized in Appendix C: 6.6, 6.7 and 6.8). ANOVA analysis revealed that spice treatment has significant influence on hardness alone. This implies that spice addition level are critical for the hardness of the tuna sample. However, all other parameters like springiness, stiffness ($p=0.936$), chewiness ($p=0.255$) and cohesiveness ($p=0.155$) did not show any considerable difference between the treatments.

6.3.4. Organoleptic evaluation

The panel evaluation of the samples for the suitability of flavour and taste are given in Fig. 6.5a-d and 6.6a-b. Using a scale of 1 to 7, with 7 being extremely like and 1 being extremely dislike, the panel rated control tuna samples 4.3 for overall acceptability. Addition of spices significantly influenced the sensory properties of the tuna (Appendix C: 6.9). On comparision of the the organoleptic score of samples treated with 0.2% and 0.1% extracts, a lower value was obtained for odour of samples treated with 0.2%, irrespective of the various spices used.

The tuna chunks with cardamom introduced at 0.2% of its weight showed an increased intensity of fragrance compared to other spice treatments and that of control at 5% confidence level. The intensity of odour rating of 0.1% oregano treated samples did not differ from their intensity in the control. 0.2%Garlic treatment was rated at 2 by the sensory panel due to its pungent aroma. All other spices was rated above the control revealing that addition of these spices improved aroma of the experimental samples (Fig 6.5a).

The post hoc test conducted to identify the difference between treatments in contributing to the score pattern for ‘appearance’ revealed that cardamom differed significantly from that of control ($p<0.05$). Cardamom treatment varied with garlic and oregano at $p=0.001$ (Appendix C: 6.10 and 6.11). The rating of turmeric in appearance came

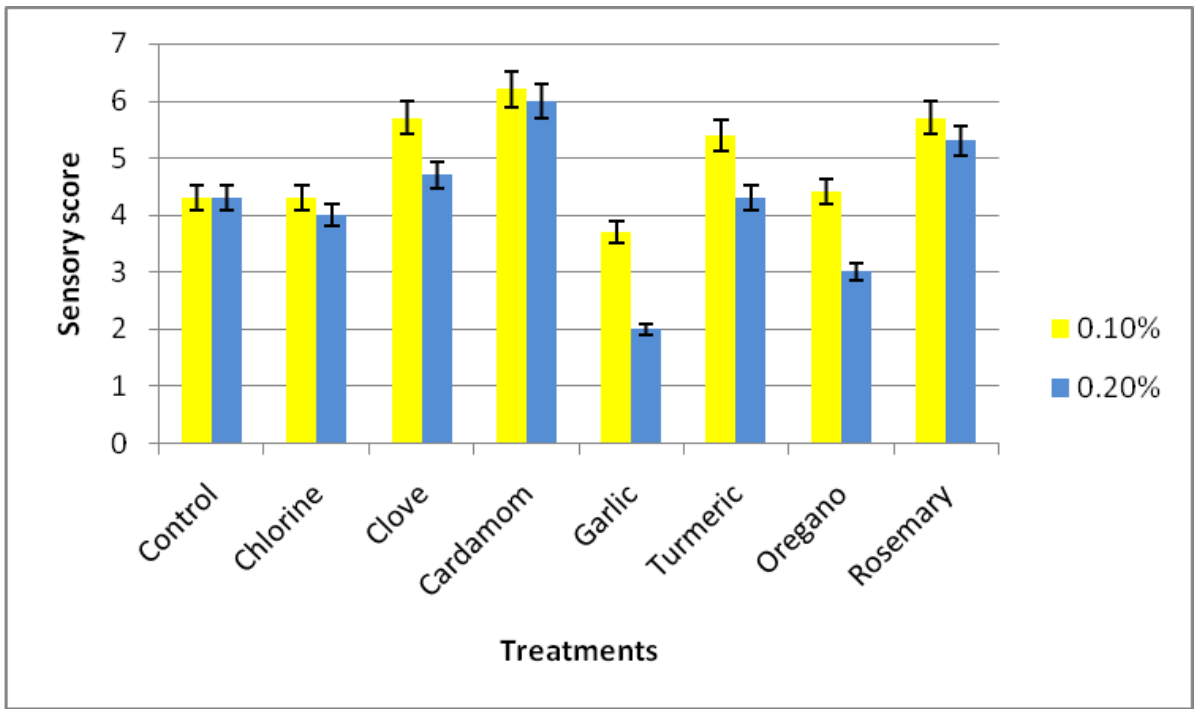


Fig. 6.5a. Effect of different concentration of spice treatment on odour of tuna samples

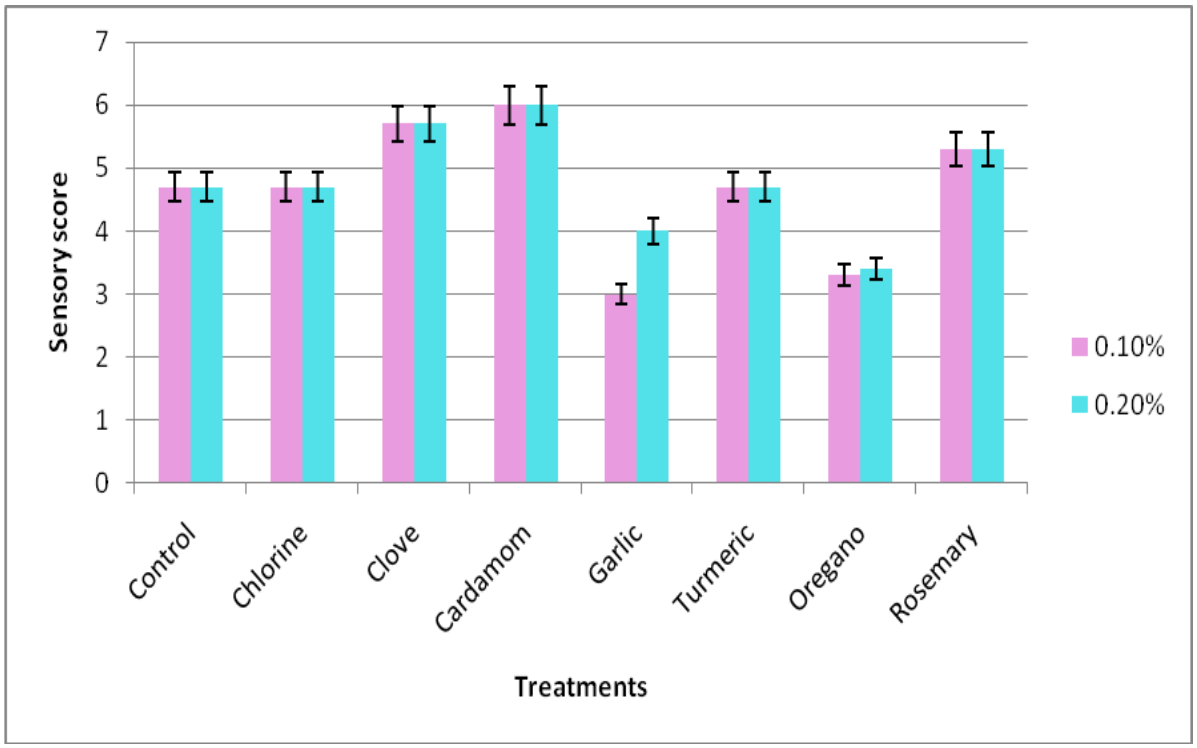


Fig. 6.5b. Effect of different concentration of spice treatment on appearance of tuna

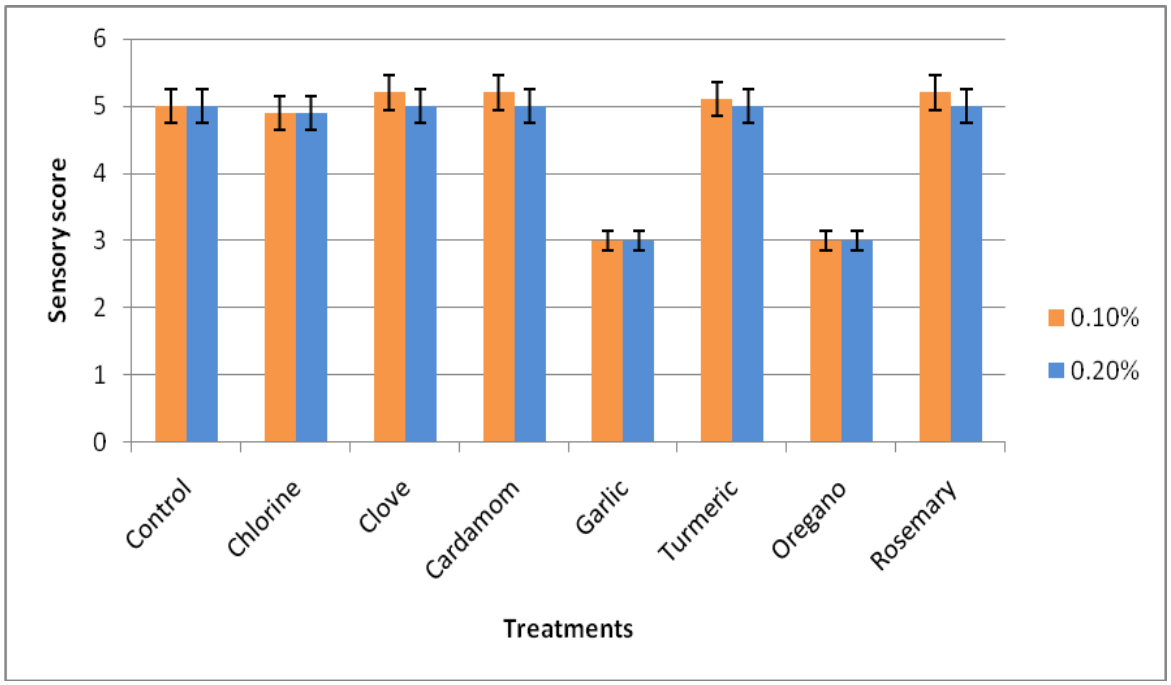


Fig. 6.5c. Effect of different concentration of spice treatment on taste of tuna samples

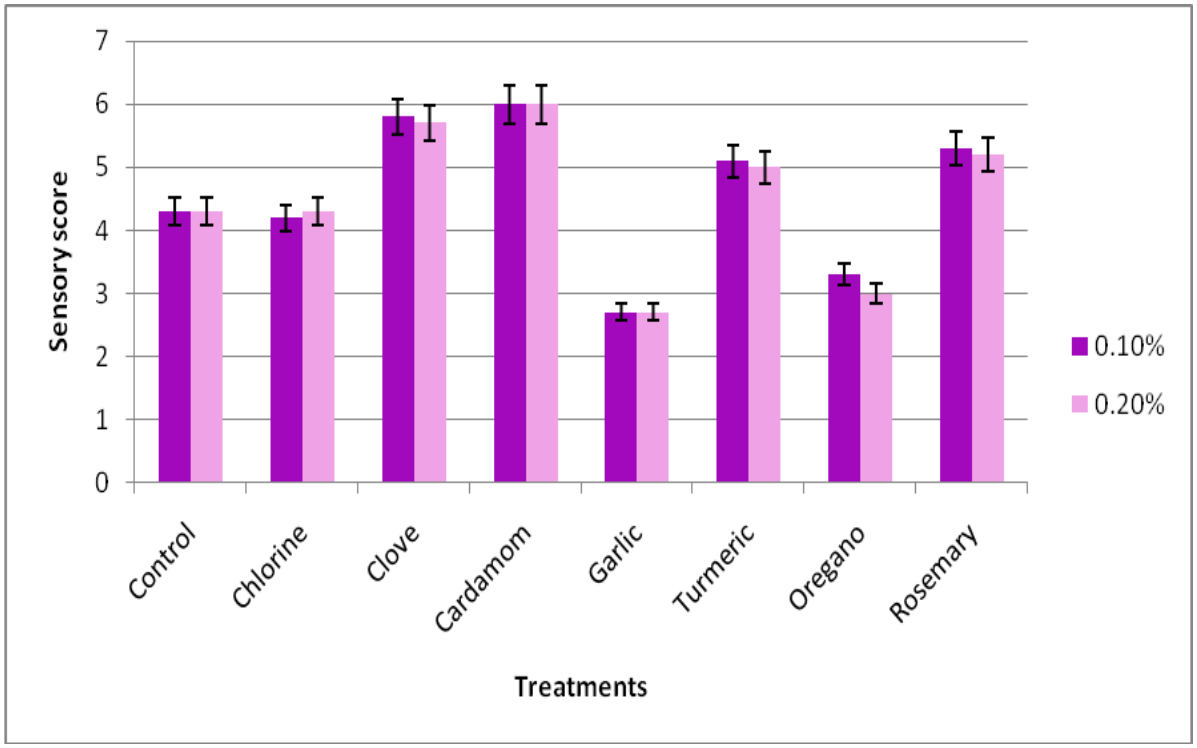


Fig. 6.5d. Effect of different concentration of spice treatment on overall score of tuna

down to 4.7 due to the yellow stain produced by curcumin (Fig.6.5b). There was no difference in the rating for 'taste' between clove, cardamom, turmeric & rosemary treated tuna and from that of control. Tukey test conducted for taste as a sensory parameter showed that control differed significantly from tuna treated with oregano and garlic (Appendix C: 6.12 and 6.13). As in other sensory parameters oregano and garlic had the least score in this case too (Fig 6.5c).

The clove treated sample which showed good results in the previous experiments did not receive the highest consumer acceptance. Interestingly, the highest over all score of 6 was obtained by cardamom treated tuna. Garlic and oregano treated sample were least preferred by the panelists due to their pungency. Rosemary and clove (0.2%) was also well received with an overall rating of 5.3 and 5.7, respectively. The intensity of sensory parameters for turmeric treated samples did not show much variation from the control value.

Among the treatments, cardamom, rosemary and clove showed higher values compared to control and their order of rating is as follows: Cardamom > Clove > Rosemary > Turmeric > Control> Oregano > Garlic (Fig 6.5d). Taking into consideration the data presented above, it may be assumed that addition of spices even in the negligible amount of 0.2% resulted in improvement of taste and aroma features of the experimental samples. The attributes determining the highest degree the sensory profiles of the tuna meat turned out to be taste and aroma notes.

Correlation between the various sensory parameters are presented in Table 6.4. The highest correlation was found between overall score and taste (0.907) at 0.01% confidence level. It was followed by odour (r = 0.0887) and appearance (r = 0.876). These results proves that taste is the chief component in determining the overall score of the product followed by aroma. Though all parameters had a high positive correlation with each other the least value was given by taste and appearance (r = 0.746).

Table 6.4. Pearson Correlations between sensory parameters

Parameters	Odour	Appearance	Taste	Overall
Odour	1	0.768**	**0.825****	**0.887****
Appearance	0.768**	1	0.746**	0.876**
Taste	0.825**	0.746**	1	**0.907****
Overall	0.887**	0.876**	0.907**	1

**Result is significant at 0.001 confidence level

The sensory analysis of the spice treated tuna revealed that among the selected spices, cardamom, clove and rosemary are the most accepted ones. Hence, the effect of combination of these spices on the sensory attributes were investigated. The total concentration of spices was kept below 0.2% in all combinations. The panel evaluation of the sample subjected to a treatment of spice combination led to the results as illustrated in Fig 6.6a and 6.6b.

Spice blending significantly influenced the sensory properties of the tuna. Effect of blending on the fragrance, appearance and taste is depicted in Fig 6.6a. It can be understood very clearly from the graphical representation that all the combinations produced a better result for fragrance than control. The highest rating for odour was given by the combination of all the three spices (cardamom, rosemary and clove) in 1:1:1 ratio and clove and cardamom blending in 1:1 ratio. Rosemary and cardamom combination was also well received by the members of the panel and it scored a 5 on the hedonic scale.

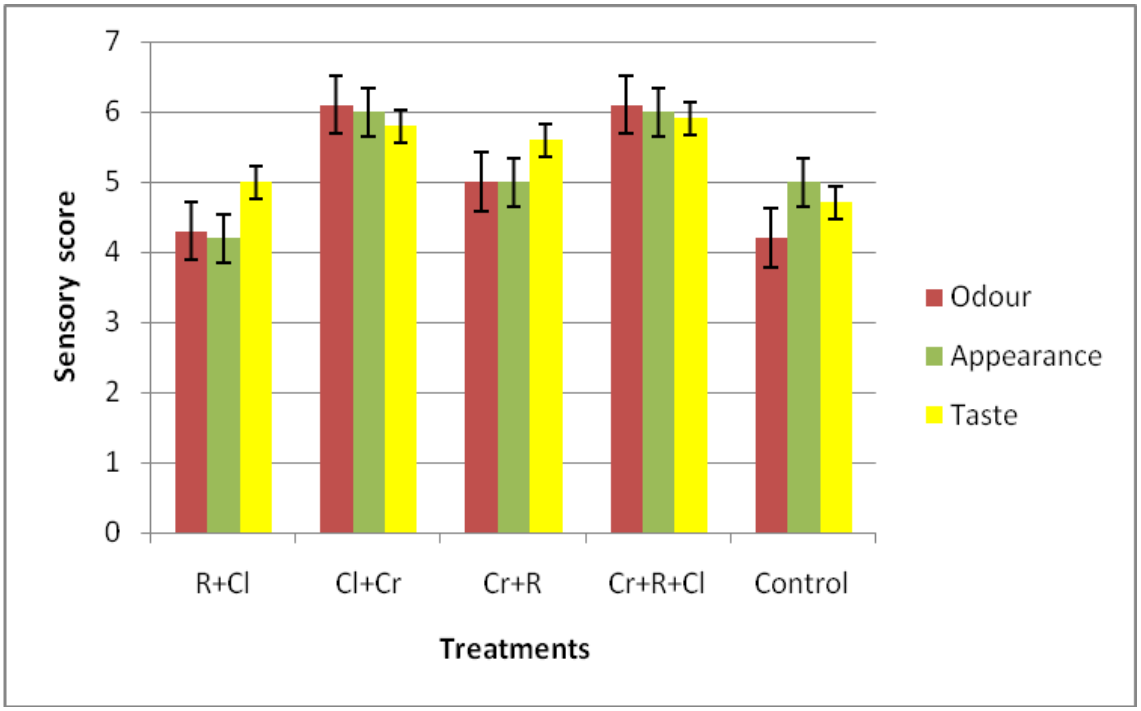


Fig. 6.6a. Effect of spice combination treatment (0.2%) on odour of tuna samples

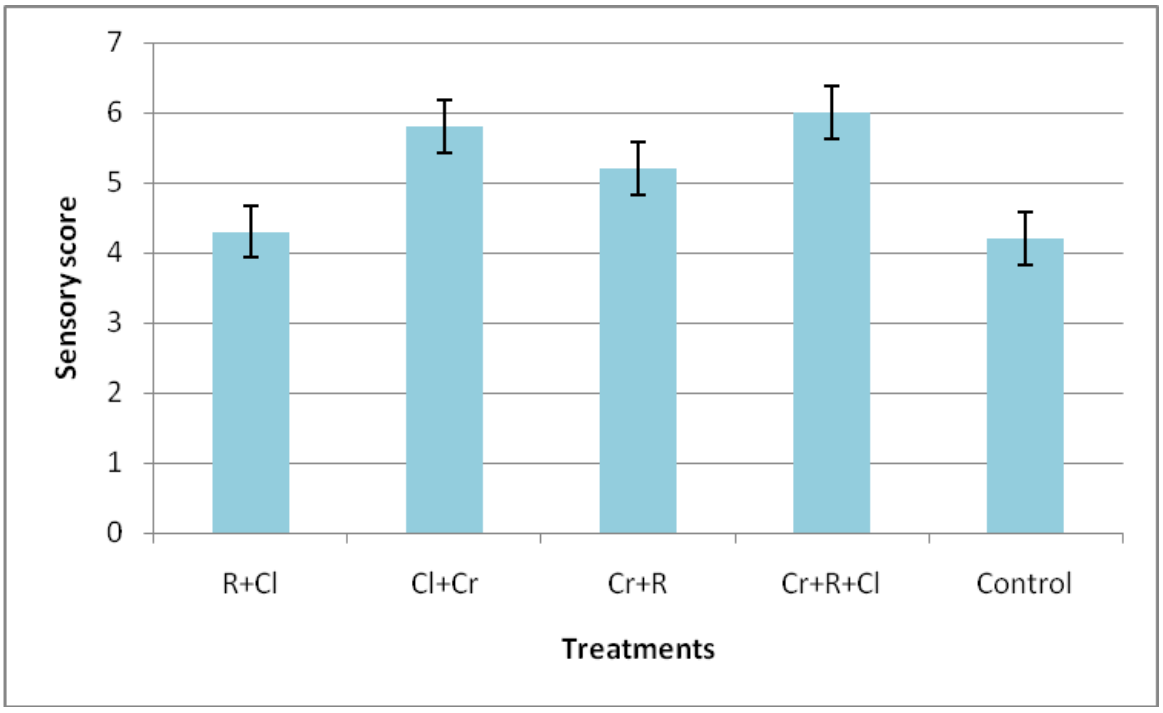


Fig. 6.6b.Effect of spice combination treatment (0.2%) on overall score of tuna samples

R+Cl=rosemary+cardamom(1:1),Cl+Cr=clove+cardamom(1:1),Cr+R=cardamom+rosemary (1:1) and Cr+R+Cl=cardamom+rosemary+clove(1:1:1).

Rosemary and clove blending had a rating lower than the control tuna sample with respect to appearance. This discepancy could not be explained but, all other combinations had sensory score equal to or higher than 5. The results of sensory panel scaling for taste opens an opportunity for evolution of recipes to improve the characteristics and overall acceptability of the product. All the experimental combinations had a far higher score (≥5) for taste compared to control (4.7). This is also reflected in the overall score due to the high positive correlation existing between taste and overall acceptability (Fig.6.6b).

Cardamom, rosemary and clove in equal ratio was the most accepted experimental sample with the highest overall score of 6 followed by Clove-Cardamom and later Cardamom-Rosemary (Fig 6.6b). In the earlier experiment conducted with individual spice treatment, cardamom treated tuna chunks had the maximum rating. Hence, there is a possibility that the aromatic compounds 1,8-cineole and alpha-terpinyl acetate, which are the major components in the cardamom volatile oil, is responsible for the higher ranking of the products.

6.4 Discussion

6.4.1. Cook loss and electrophoretic analysis

Fish is rarely eaten raw but is usually cooked or processed in different ways before consumption. In India, fish is generally marketed as fresh, chilled or frozen and it is consumed primarily in traditional recipes which includes lot of spices as ingredient. Among the spices, turmeric powder and ginger-garlic paste are most commonly used in the fish preparations. The use of other spices namely, rosemary, oregano, cardamom and clove to enhance the taste of cooked tuna were explored in this study and found to be acceptable by the sensory panel. During cooking, chemical and physical reactions take place that improve or impair the nutritional value of foods (Finot, 1997; Bognar, 1998). This effect is also dependent on the type of cooking (Nath *et al.*, 1996).

Cooking causes interaction of muscle protein fraction, enzyme hydrolysis and changes in the connective tissue thus affecting the nutritional properties of the muscle tissue. Sarcoplasmic and myofibrillar proteins are denaturated and coagulated during the process and H-bonds, which are involved in the secondary and tertiary structures of proteins, are broken. This results in unfolding of the native configuration. The extent of these changes depends on the temperature and duration and also affects the yields and physical properties of the fishery product. March (1984) and Deman (1999) had found that cooking of fish causes solubilization of proteins and hence leads to loss of proteins from the final product. Low solubility of collagen plays a positive role in maintaining the texture and morphology of the cooked fish. In this study there was an average yield of 86.64% after thermal treatment of the tuna sample and no significant difference in the cook loss was found between the spice treatments and control.

SDS-PAGE has been used for identification of different muscle proteins and their subunits in fresh muscle and also to estimate the effects of storage and processing on the stability of proteins (Bechtel and Parrish, 1983). In the present experiment the effect of spice extracts of clove, cardamom, rosemary, oregano, garlic and turmeric on the sarcoplasmic protein present in cook water was analysed by SDS-PAGE. The content of the ~20.1KDa protein fraction increased for turmeric, oregano and clove treated fish compared to that of control. It was probably obtained from breakdown of a larger protein such as myosin (205kDa). In support to this finding, extensive proteolysis was revealed in the eletrophoretic pattern of muscle proteins treated with ginger extract in a study conducted by Naveena *et al*. (2004). Cook water of the clove treated tuna samples had fewer protein bands compared to control samples. Comparing clove treated and control samples, the protein band in the range of 20.1KDa remained intact in the control and clove treated samples while the protein band of 29 KDa remained intact only in control sample. This can be due to the cleavage of higher molecular weight proteins into lower molecular proteins by the action of spices. Preferential degradation of myofibrils in the I-bands as well as of the collagen as a result of treatment with ginger was reported by Lee *et al*. (1986a) and Thomson *et al*. (1973), respectively.

Additional bands were observed to appear between the range 29-43KDa for cardamom and garlic treated samples. This could be the result of protein aggregates formed by the addition of spice. Consequently the protein band toward the origin became thicker with the protein having formed aggregates that were too large to enter the gel during electrophoresis.

Based on the differences in the physico-chemical properties, proteins are classified as sarcoplasmic and fibrillar proteins. The sarcoplasmic proteins form approximately 20-30% of the total proteins (Dyer and Dingle, 1961). They are generally soluble in water and buffers of low ionic strength. Most of these are low molecular weight proteins in the range of 40KDa to 70 KDa. The fibrillar proteins constituting salt soluble and insoluble fractions contribute about 60-80% of the total proteins that have molecular weight in the range of 400-600 KDa. The protein lost during cook loss are water soluble and it was seen that only faded banding was present in the 43-66KDa range, implying that only negligible loss of sarcoplasmic protein occurred during cooking of tuna. The basic aim of the fish processor and food technologist is to control the changes in the functional properties of tissue protein, and thus to preserve and improve the quality of the meat. Cooking tuna does not contribute much to the loss of sarcoplasmic proteins. But, loss of smaller peptides <29KDa is evident from the electrophorogram.

6.4.2. Sensory and Textural parameters

Consumer acceptance is the key for the success of functional foods in the market (Zhang *et al*., 2010). Spices are known to enhance and improve the flavour and colour of foods (Ekanem and Solomon, 1997; Ekanem and Achirnewhu, 1998) and hence contribute to the consumer acceptance of a food product. They vary in their effects in that the aroma and flavour principles in spices are based on the essential oils contained in them. It would be assumed that the oils present in the spices may have produced some modification of the flavour, taste and colour thus making the meat samples generally accepted by the panelists.

Eluyode and Akpa (2007) in their study found that the release of more flavour components occurs due to increased fat solubilization and it contributed to the improved meat flavour. The 'patterning theory of spice' considers the suitability of a spice and certain raw materials to be the result of a synthesis that occurs in the mouth. Flavour preference is evaluated using the senses of taste and of smell. If a spice and an ingredient tasted together are well received, it follows that these two can be combined in cooking (Takemasa and Hirasa, 1998).

Spices are sometimes used for the purpose of deodorizing or masking the smell of raw materials. Fish smell can be deodorized by soaking it in spices or lemon juice/vinegar. The deodorizing mechanism for this phenomenon involves alkali trimethylamine, one of the odorous compounds, being neutralized by the acid to become a nonvolatile compound (Takemasa and Hirasa, 1998). Spices having this kind of deodorizing activity include those having volatile pungent compounds, such as wasabi or mustard. Wasabi is commonly used for masking the smell of raw fish. Therefore raw fish products like sashimi / sushi is often consumed along with wasabi sauce. If the spices showing high antimicrobial properties are used for this masking effect, it can enhance the consumer acceptability as well as increase safety level of seafood.

Kikuchi *et al*.(1968) evaluated the masking of spices sensorilly by adding a spice solution with phased concentration to trimethylamine solution. According to their report, the essential oils of spices are most effective in suppressing the odour of trimethylamine. Trimethyl amine levels in a fish is used as a reverse indicator of seafood freshness because it increases as freshness is lost (Shimizu and Hibiki, 1957). Suppression of the odour of trimethylamine can result in increased sensory score of seafood. In a similar study, the deodorizing effect of rosemary was found to be six times stronger than sodium copper chlorophyllin. Sodium copper chlorophyllin is a chemical deodorizer and is often used in breath-freshening chewing gum and toothpaste. The effective deodorizing compounds of rosemary is carnosol and rosemanol that also have antioxidant function. The spices in the Labiatae family, including rosemary, are most effective at deodorizing because they have

both a chemical and sensational deodorizing function and enhance the deodorizing/masking effect very effectively.

Cardamom, which showed maximum individual sensory score, is often used to flavour Eastern dishes. Rosemary and oregano are commonly used in Western cooking for deodorizing purposes. Both of them belonging to the Labiatae family are routinely used in Mediterranean countries such as Italy and Greece. Rosemary is found to have a high suitability to meat. Most spices suitable to meat have a deodorizing function as well. All the spices used in the experiment namely garlic, oregano, turmeric, clove, cardamom and rosemary were found to be a suitable ingredient in seafood by a detailed survey conducted by Takemasa and Hirasa (1998).

Apart from the sensory enhancement, addition of these spices has other advantages also. The spice clove and its value added products are used extensively for flavouring food and confectionery. Cardamom essential oil was traditionally used as a digestive tonic. Rosemary is commercially available for use as an antioxidant in Europe and the USA (Yanishlieva and Heinonen, 2001).

Extracts and essential oil of rosemary can be used to stabilized fats, oils and fat containing foods etc. against oxidation and rancidity (Pokorny *et al*., 1998; Zegarska., 1996). Rosemary leaves and flowering tops are used in lamp roast, mutton preparations, marinades, with baked fish, egg preparations and fruit cordials (Bonar, 1994).

Kharb and Ahlawat (2010) conducted a study to find out the effect of precooking and spice mix on chemical and physico-chemical properties of dehydrated spent hen meat mince during storage at ambient temperature (27±2°C). Raw and precooked meat mince (with and with out spice mix) were dehydrated at 60°C for 12 hrs and 9 hrs. On the basis of their findings it was concluded that pre-cooked spent hen meat mince treated with 2% spice mix retains the most desirable physico-chemical properties up to 60 days of storage at ambient temperature(27±2°C). In this experiment also the physico-chemical properties did not alter significantly from that of control sample.

Statistically, there are very few cases where only one kind of spice is used for cooking. In most cases, a spice is used in combination with one or more spices. Even a pleasant flavoured spice can have a medicine like smell or taste if used in excess. But, such an aroma or flavour of one spice can be reduced by blending with other spices. This will help in maintaining the antimicrobial effect and produce good sensory values. Each spice has a typical flavour, the quality of which cannot be changed when only that spice is used for cooking, although the strength of its flavour can be controlled by adjusting the amount to be added. However, if a spice is grouped with other spices, the total combination will have a more delicate flavour than when each is used individually. This is called spice-blending effect (Takemasa and Hirasa, 1998). However, Shamsuddeen (2009) reported that individually the extracts of the spices are more active against the microorganisms than when they were combined. This finding limits their antibacterial activity in the product, though the sensory properties are found to be amplified in combined use of spices during the course of the investigation.

Leistner (1978) had introduced the hurdle concept and he stated that the microbial safety, stability, sensorial, and nutritional qualities of foods are based on the application of combined preservative factors that microorganisms present in the food are unable to overcome. Using an adequate mix of hurdles is not only economically attractive; it also serves to improve not only microbial stability and safety, but also the sensory and nutritional qualities of a food (Leistner, 2000). The present study revealed that apart from the microbial safety ensured by the use of hurdles like spice, it also enhances the sensory acceptability of the product. This technology can be used for making new products and for reducing energy-consuming hurdles like refrigeration or toxic chemical preservatives. Moreover, globalization have led to development of new and exciting food styles, where exotic flavors from all over the world are combined with more traditional foods in new and exciting ways (Reinbach *et al*., 2007). Addition of spices not only provide food safety from microbes but also enhance the pleasure of eating by making the meal more exciting, balanced and complete.

6.5. Conclusion

Cook loss was negligible for all samples cooked at 100^{o}C irrespective of the dip treatment given. But, marginal decrease in the yield (3%) has been shown by turmeric treated samples. The SDS-PAGE gels for control and spice treated tuna muscles revealed that rosemary and cardamom treated samples showed a narrow banding compared to that of other spices. The protein band in the range of 20.1KDa remained intact in the control and clove treated samples while the protein band of 29 KDa remained in tact only in control sample.

Samples treated with 0.2% concentration showed significantly high values for hardness than those treated with 0.1% concentration. However, all other parameters like springiness, stiffness, chewiness, cohesiveness did not show any considerable difference between the two concentrations. Since there no significant difference in the textural properties between concentrations used in the study, only 0.2% spice concentration was pursued for frozen storage studies because 0.2% spice had the additional advantage of keeping bacterial population in check.

The panel evaluation of the sample for the suitability of flavour and taste led to the conclusion that addition of spices significantly influenced the sensory properties of the tuna. Interestingly, the highest consumer acceptance occurred for cardamom. Garlic and oregano treated sample had a negative impact on the panelists. Rosemary and clove was also well received by the members of the panel. Evaluation of the sample for the suitability of spice blending proved that rosemary and cardamom blending was well received by the members of the panel. Other combinations also followed close in the sensory score.

To conclude, though spices show good antimicrobial properties, their contribution to textural parameters seems to be limited but, it significantly influenced the sensory properties of the tuna Sensory score for cardamom was highest followed by clove and rosemary. The experiment also proved that spice combination plays an important role in increasing the product acceptability. The changes occurring in spice treated tuna during frozen storage is undertaken in the next chapter.

7.1. Introduction

The most popular method of long term preservation of tuna or any fish is frozen storage. Prolonged frozen storage is known to lead to the deterioration in the quality of fish and shell fish items. Quality changes in fish include protein denaturation and textural changes due to the interaction of proteins with oxidized lipids, resulting in toughening of texture. The effect of spice treatment in combination with frozen storage on the quality of tuna is studied in this chapter.

7.1.1. Frozen storage of fish

Freezing is a commonly employed method for the preservation and maintenance of the nutritional quality of fish by retarding the biochemical and microbiological reactions in the tissue. The freeze induced physico-chemical changes in the colloidal structure of fish protein create several technological problems like exudation of drip from thawed fish. The toughness of fish muscle increases with prolonged storage and results in economic loss and reduced consumer acceptability.

Studies on mackerel (*Rastrelliger kanagurta*), individually quick frozen (IQF) and blocks frozen (BF) and stored at -23ºC showed that block frozen mackerel had higher frozen storage shelf-life than IQF samples based on sensory evaluation (Nair *et al*., 1976). Investigations carried out by Garg *et al*., (1982) on ghol *(Pseudosciaena diacanthus)* fillets frozen stored at -18°C remained in a highly acceptable condition up to 20 weeks and later acceptability steeply declined. Chinnamma *et al*., (1995) observed a proportional extension of shelf life in quick frozen mackerel (*Rastrelliger kanagurta)* as the storage temperature was lowered. Several researchers have carried out frozen storage studies in fish and fishery products and found that it could be stored up to nine months in frozen storage with only slight changes in odor, taste and texture (Singh *et al*., 2004; Singh and Balange, 2005).

7.1.2. Effects of freezing on microorganisms

7.1.2.1. Changes in the microbial environment during freezing

Most water in foods usually contains more or less complex blend of solutes. The presence of solutes in water depresses the temperature at which freezing can initiate, usually to a temperature between -1°C and -3°C (Bogh and Jul.,1985). Once ice crystals start forming in the solution, the concentrations of solutes in the remaining liquid water increases (Berg, 1968). On freezing the water available for the microorganisms for maintaining their metabolic activities decreases. The availability of water in a food, frozen or otherwise can be expressed as its water activity (a_w), which is the ratio of the water vapour pressure of food to that of pure water at the same temperature (Leistner and Russell, 1991). For any frozen food, a_w will be that of ice at the same temperature.

As freezing progresses in a food, microorganisms that are free to move in the liquid phase concentrate in the remaining unfrozen solution (Calcott, 1978). Such planktonic organisms in freezing foods are therefore exposed not only to low temperatures and low water activities but also to increasing solute concentration and perhaps to substantial pH changes (Mazur, 1966). However, in many foods some microorganisms may be attached to or localized within or between immobile solid elements of the food.

7.1.2.2. Growth of microorganisms in frozen foods

Microorganisms that can grow at chiller temperature are referred to as psychrophilic or psychrotrophic, depending on the temperature range within which they can grow (Olson and Nottingham, 1980). The former term is applied to organisms that does not tolerate warm temperatures and are mainly derived from cold environments. The latter term has been

applied to organisms responsible for the spoilage of chilled foods, and usually have maximum temperatures for growth between 30°C and 35°C (Kraft, 2000).

The minimum temperature for growth of a bacteria is regarded as a characteristic feature, but the lowest temperature at which growth occurs is usually higher when an organism is subjected to other stresses in addition to low temperature (Mc Meekin *et al*., 1993). The minimum temperature for growth in the absence of other stresses has been determined for some organisms capable of growth at freezing temperature by cultivating the organisms in supercooled media. The growth of *Vibrios* at -4°C (Sasajima, 1974), and the growth of *Bacilli* at -7°C (Larkin and Stokes, 1968), have been established using this technique. Yet, in practice, microorganisms in frozen foods will almost inevitably be exposed to osmotic stress or desiccation and to inhibiting concentration of some solutes as well as to low temperatures. Moreover, even when the growth of various organisms is possible, the rate of growth of some may be slow as to render any increase in their number or size inconsequential for the safety or storage stability of the product.

Temperature gradients commonly exist within refrigerated facilities and temperatures can rise during periodic defrosting of refrigeration equipment (Jul, 1984). Consequently, if frozen foods are stored in commercial facilities with refrigeration equipment operating at temperatures close to -10°C, some of the products may be exposed to higher temperatures at which microorganisms may grow to cause spoilage. Measurement of sensory, chemical and physical changes have shown that deterioration of fish quality continues to some extent during frozen storage (Haard, 1992). This results in undesirable changes associated with lipids and proteins (Abdalla *et al*.,1989).

7.1.2.3. Limitations of freezing on microbial growth

Foods are usually frozen to prevent their spoilage by microorganisms and to preserve their desirable eating qualities (Hendley, 1993). Generally, freezing at rates that preserve

desirable eating qualities is likely to prevent any substantial growth of pathogenic organisms and pathogens cannot grow at temperatures now usually maintained for frozen foods. But viruses, bacterial spores and sexual spores of fungi are likely to be preserved by freezing, irrespective of the composition of food and the rates of freezing and thawing. Other microorganisms are likely to be damaged by freezing, but the extent to which freezing and subsequent frozen storage reduces the number of any organism may be trivial if the population is in a resistant physiological state or stage of the life cycle. Thus, it cannot be safely assumed that freezing inactivates all the microorganism that may be present in a food in large numbers. Substantial reductions in the numbers of viable organisms of concern would have to be validated for specific foods, if the freezing alone is to be recognized as the decontaminating treatment for a food (Gill, 2012).

7.1.3. Effect of spices on growth of microorganisms in food

Spices are indispensable components of Indian cuisines since ancient times. These are used in minuscule amounts to impart flavour, taste and aroma in food preparation to improve their palatability (Rahman and Gul, 2002; Nair and Chanda, 2006). Spices are also used for stabilizing quite a few food items from deterioration (Kizil and Sogut, 2003). Spices are considered as source rich in bio-active antimicrobial compounds (Lia and Roy, 2004). The typical Indian spices and herbs like cumin, black cumin, mustard, fenugreek, ajowain, curry-leaf, nutmeg and henna are usually used in curries, pickles, sauces etc. These spices are also known to have some ethno-medicinal or anti-microbial properties (Singh *et al*., 2002). Plants traditionally used for medicinal purpose in different parts of the world have been screened for possible antimicrobial action by several workers (Bonjar, 2004). Antibacterial activities of extracts of different plants against various microorganisms have been reported by many scientists (Sagdic and Ozcan, 2003; Nair and Chanda, 2006; Shan *et al*., 2007; Chaudhury and Tariq, 2008; Gutierrez *et al*., 2008). But there are little reports on

some of the Indian spices and herbs (Singh *et al*., 2002; Arora and Kaur, 1999; Romson *et al*., 2011).

Spices are considered as rich source of bio-active antimicrobial compounds (Das *et al*., 2012). Moreover, the use of synthetic preservatives in food have caused an increasing perception by consumers that synthetic compounds may lead to negative health consequences. This has led to a reduced acceptance for their use in foods. Plant derive spices are generally used in foods for flavoring and medicinal purposes. However, a number of studies have demonstrated that compounds existing in many spices also possess antimicrobial activity (Elamathy and Kanchana, 2012). Israti *et al*. (2011) found that the addition of thyme, marjoram and horseradish in marinade resulted in more pronounced decrease of total mesophilic aerobic bacteria compared to the marinade without spices and seasoning plants. The results of that study indicate that marination with marinades consisting of lime-tree honey, spices and seasoning such as thyme (*Thymus vulgaris),* marjoram (*Majorana hortensis*), garlic (*Allium sativum*) and horseradish (*Armoracia rusticana*) can be used as an effective and natural preservation method.

It is already proved by systematic investigations in earlier chapters that the selected spice extracts of namely, clove, cardamom, garlic, oregano, rosemary and turmeric inhibits pathogenic and food-spoiler microorganisms. The inhibitory effect of these spices on histamine decarboxylating bacteria, their role in reduction of biogenic amine production and sensory enhancement implies that they can be used for fish preservation. There is no report on the synergistic effects of spice treatment and freezing on microbes. The objective of the present study is to evaluate the combined effect of selected spices treatment and frozen storage on the quality stabilization of tuna. The physicochemical parameters and microbiological changes are monitored to understand the variation in quality of tuna after spice treatments.

7.2. Materials and Methods

7.2.1. Sample Preparation

The procured fish was divided into 8 lots and given a dip treatment in 0.2% oleoresin of six spices namely, clove, cardamom, garlic, oregano, rosemary and turmeric as well as 2ppm chlorinated water separately for a duration of 10 minutes. The experimental setup was maintained at chill-room temperature. A control without any treatments was also run simultaneously. Each treatment were further divided into sufficient portions and were packed in sterile polythene covers of 200 gauge. Then, the samples were frozen in an airblast freezer and stored at -20°C in a deep freezer (Siemens vertical model). Sampling was done immediately and further at regular intervals of one month for a period of 6 months. Microbiological changes, drip-loss, water retention, protein loss during thaw drip and pH were studied in the samples. In all the above samplings, triplicates were taken at every stage.

7.2.2. Analysis of Biochemical parameters

7.2.2.1. Determination of Proximate composition

The moisture, protein and fat contents of tuna were determined according to the AOAC (1995) method.

7.2.2.2. pH

10 gm of the sample was blended with 90 ml distilled water and the pH of the resultant suspension was measured using a digital pH meter (Cyber Scan pH-500 MERCK) after caliberating with standard buffers of pH 4.0, 7.0, 9.0 (SIGMA) (AOAC,1995).

7.2.2.3. Changes in proteins present in thaw drip during storage

The thaw drip from the stored samples for one month and six months were collected by thawing the samples at room temperature. The drip collected was analysed for the protein fraction by polyacrylamide gel electrophoresis (PAGE). Sample preparation and assembly of the apparatus were done as mentioned in Section 6.3.3. Protein present in the thaw drip was also estimated quantitatively by Lowry's method (Lowry *et al*., 1951).

7.2.2.4. Microbiological analysis

The microbial status of the treated and control tuna samples frozen stored for six months were evaluated by determinig the total viable count as per methods of USFDA (2001) and Surendran *et al*. (2006). The sample preparation and analytical procedures are mentioned in Chapter 3: 3.2.2. Data obtained was analysed using SPSS version 17.0.

7.3. RESULTS

7.3.1. Physicochemical analysis

The proximate composition of spice treated and control samples are given in Table 7.1. Statistical analysis was carried out to estimate the Analysis of Variance for the experiment (Appendix D: 7.1, 7.2). Moisture content of the samples did not differ significantly after the treatment with spices (p=0.484) whereas the protein content showed difference between treatments (p=0.002). Turmeric treated tuna samples differed from the control value significantly. The results of the physicochemical and microbiological analys of spice treated tuna samples stored at -20°C are shown in Tables 7.2–7.4 and Fig. 7.1. The data are expressed as the mean of triplicates.

Table 7.1.Proximate analysis of spice treated tuna (%)

Treatment	Moisture	Crude Protein	Fat	Ash
Control	77.96±1.00	23.50±0.53	1.54±0.06	0.48±0.03
Clove	77.00±0.56	23.65±0.39	0.93±0.33	0.47±0.07
Cardamom	77.40±0.08	22.75±1.10	0.93±0.51	0.44±0.14
Oregano	76.52±1.20	22.81±0.90	0.89±0.76	0.48±0.09
Garlic	77.30±0.37	23.25±0.33	0.93±0.33	0.42±0.11
Turmeric	76.22±0.41	22.50±0.42	1.67±0.40	0.41±0.08
Rosemary	76.96±0.77	22.8±0.53	0.77±0.30	0.45±0.14

*values are given as Mean±SD

Table 7.2 shows the pH of the fresh and frozen samples of tuna treated with different spice oleoresins. On post mortem the pH shows variation with the fish species, catching ground and season. The initial pH of the tuna was found to be slightly acidic. Researchers have reported that post mortem pH is low in heavily feeding fish. The pH ranged between 6.26-6.00 in the fresh tuna. Statistical analysis using One way ANOVA for the first month of frozen stored samples showed a significant difference ($p<0.05$) between pH of control and treated tuna samples (Appendix D: 7.3).

Table 7.2. Variation in pH of the frozen samples of spice treated tuna

Treatments	Months of frozen storage			
	0	2	4	6
Control	6.26±0.24	5.55±0.55	5.77±0.86	5.52±0.20
Chlorine	6.08±0.34	5.60±0.34	5.62±0.34	5.56±0.31
Clove	6.03±0.82	5.66±0.41	5.46±0.26	5.59±0.58
Cardamom	5.24±0.34	5.53±0.07	5.73±0.48	5.63±0.50
Oregano	6.01±0.53	5.51±0.48	5.62±0.36	5.57±0.54
Garlic	5.92±0.82	5.88±0.25	5.54±0.44	5.98±0.66
Turmeric	6.11±0.85	5.85±0.19	5.84±0.66	6.03±0.59
Rosemary	5.96±0.6	5.60±0.36	5.54±0.30	5.65±0.09

*values are given as Mean±SD

Post hoc tests of the data revealed that control pH differed from all spice treated tuna samples during 0^{th} month (Appendix D: 7.4). Among treatments, clove and oregano treated tuna samples showed similar pH whereas, garlic and rosemary treated experimental samples had similarity ($p = 0.877$). In all the cases, that is, in spice treated samples and in control, the pH showed slight;y lower value after 2 months of frozen storage. Statistical analysis of six month stored frozen tuna is given in Appendix D: 7.5. Multivariate analysis of the same data revealed that at the end of six months, control, clove, cardamom and oregano showed homogenous results whereas, garlic and turmeric differed from control (Appendix D: 7.6).

Table.7.3.The percentage retention of moisture in frozen samples of spice treated tuna

Treatments	Months of frozen storage (%)+++			
	0	2	4	6
Control	77.96±2.26	77.08±0.65	75.83±0.86	74.54±1.20
Chlorine	76.02±2.56	75.12±2.74	74.51±2.19	73.82±2.36
Clove	77.00±2.82	76.49±0.91	75.04±0.26	74.10±0.58
Cardamom	77.40±1.64	76.14±0.97	75.71±1.48	74.47±0.62
Oregano	76.52±1.53	75.85±1.48	74.97±0.50	73.95±1.54
Garlic	77.3±2.82	76.53±0.25	75.11±1.44	74.17±0.66
Turmeric	76.22±1.85	75.48±1.19	74.39±1.66	73.14±0.99
Rosemary	76.96±1.6	75.89±0.36	75.19±0.90	74.02±1.04

*values are given as Mean±SD

The changes in water retention of experimental samples were recorded for six months of frozen storage and the results are presented in Table 7.3. There was an average reduction of 3% in the moisture content between the initial (0 month) and final (6months) storage of all samples. There was no significant difference between the moisture content of the clove, cardamom, garlic, rosemary treated and control samples ($p< 0.05$) over the 6 months of storage. But, moisture content of turmeric treated tuna differed significantly from that of control (Appendix D: 7.7, 7.8).

7.3.2. Qualitative analysis using SDS-PAGE

Plate 7.1 show the electrophoretic pattern by SDS-PAGE of thaw drip of tuna subjected to different spice treatments. Lane at the left extreme (M) end and right extreme end (M')show the broad range protein marker and high range protein marker, respectively. Visual comparision of protein bands in the sample lanes with the R_f values of protein markers showed protein bands ranging from 29 KDa to 3.5KDa.

The electrophoretic pattern of thaw-drip protein is very similar in all the spice treated samples. The pattern also closely resembles to that of control (untreated sample). No distinct differences in the number of bands was observed but, there was slight variation in the intensity of the bands among the spice extract treated samples. Thus, the treatments did not appear to have any observable effect on the denaturation of proteins in the concentration range of spice extract used for treatment.

In the electrophoretic pattern of thaw drip only low molecular weight proteins were present (4 bands with molecular weight less than 29 kDa), whereas, in the electrophoretic analysis of cook -water a wide range of protein bands (66KDa-3KDa) were observed (Plate 6.1). The low molecular weight bands similar to thaw-drip electrophoretic pattern were more intense in the cook- water pattern (<3 KDa). Additional bands in high molecular weight range was also seen in the latter but, they were less intense. All the bands in oregano and turmeric treated sample showed more intensity compared to others.

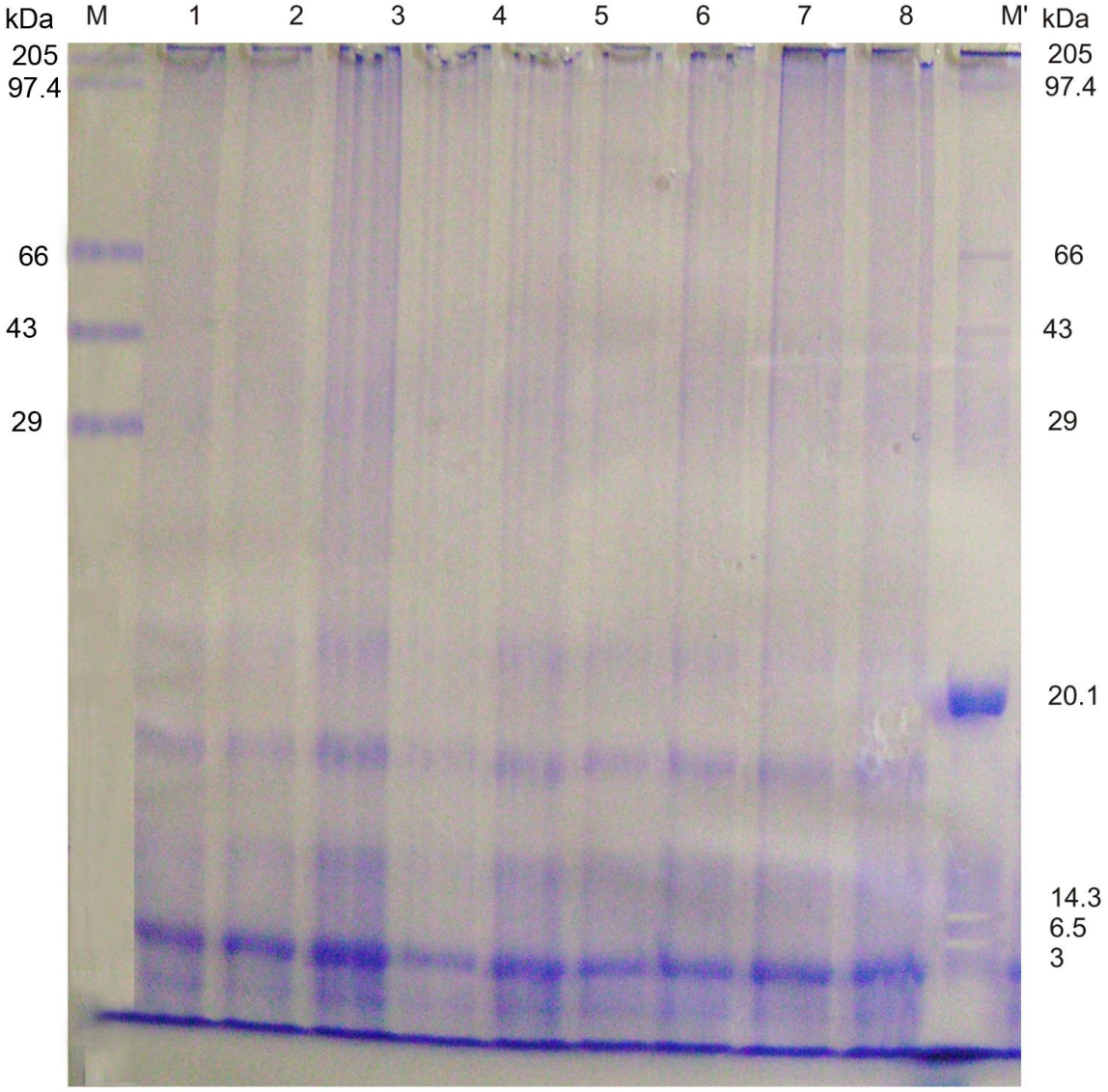


Plate 7.1. Electrophoretic pattern of thaw drip of tuna subjected to different spice treatments.

M-High range marker; 1-Control; 2-Clove; 3-Turmeric; 4-Garlic; 5-Oregano; 6-Cardamom; 7-Rosemary; 8-Chlorine;M'-Broad range marker.

7.3.3. Quantitative analysis of protein loss

The difference in the amount of protein present in thaw drip in spice treated and untreated tuna samples were compared between initial and final months of storage. That data is given in Table 7.4. The drip loss was more in 6 month frozen stored samples in comparison with one month stored samples and the changes in drip loss show similar pattern irrespective of the treatment given. Statistical analysis using SPSS 17.0 showed that the protein lost in thaw drip varied between treatments and duration of frozen storage at 5% level of confidance (Appendix D: 7.9). Tukey test performed to analyse the variation more specifically, revealed that turmeric treated tuna sample had significantly higher thaw drip from that of control.

Table.7.4. Protein lost during thaw-drip in spice treated tuna stored at -20°C.

Treatment	Drip loss (%)	
	Storage 1 month	Storage 6 months
Control	0.459±0.029	0.75±0.011
Chlorine	0.490±0.074	0.69±0.029
Garlic	0.492±0.012	0.68±0.074
Clove	0.513±0.037	0.595±0.026
Turmeric	0.594±0.061	0.905±0.022
Cardamom	0.516±0.050	0.585±0.046
Oregano	0.491±0.057	0.598±0.074
Rosemary	0.414±0.068	0.590±0.089

*values are given as Mean±SD

Protein leaching was highest in turmeric treated samples stored for 6 months (0.9g/100g) (Table 7.4). This was found to be more than that of control sample (0.75g/100g).

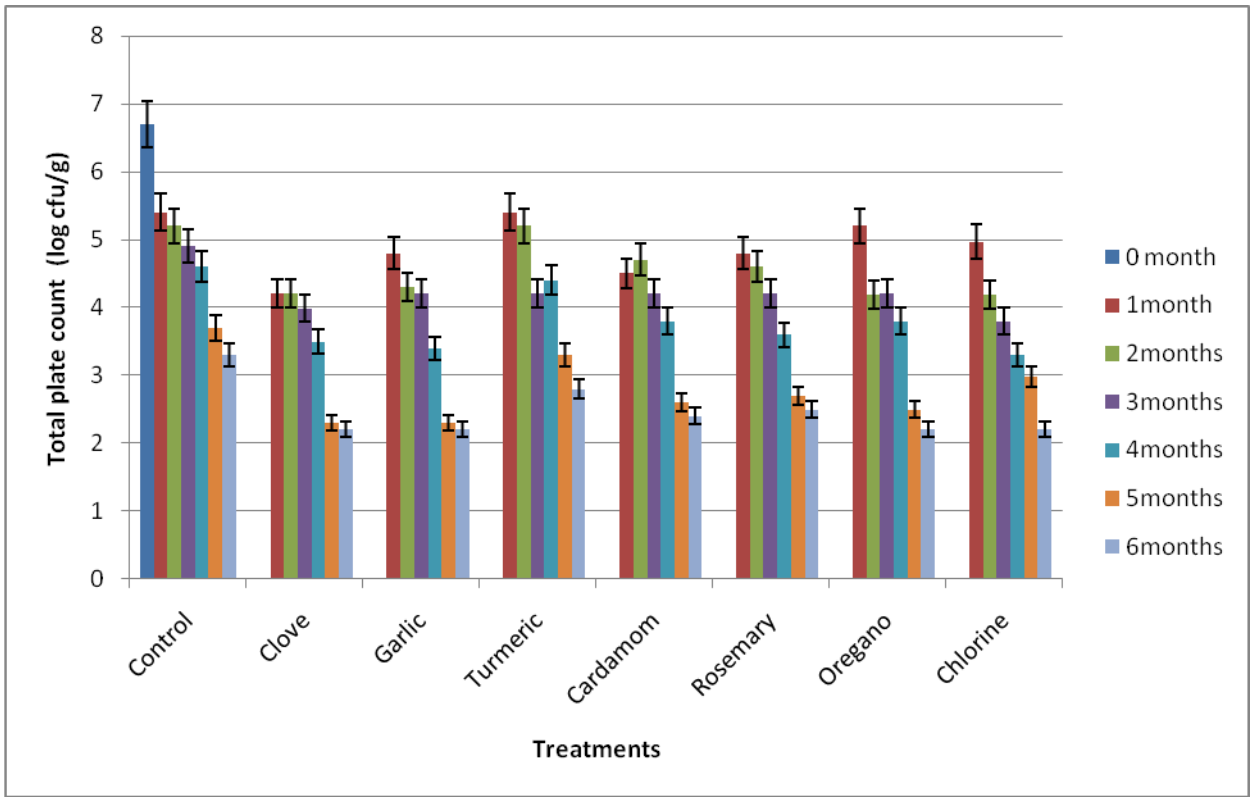


Fig 7.1. The bacterial load of the spice treated tuna samples during frozen storage.

Garlic treated sample did not show any significant difference from control sample but, all other treatments showed lower (0.592±0.0006g/10g) values with significant difference from that of control.

7.3.4. Microbiological analysis

Fig.7.1. illustrates the variation in microbial load of the frozen tuna samples treated with spices over a period of six months. Statistical analysis showed significant difference in the bacterial count at all stages of storage ($p<0.05$) in the control as well as treated samples. Univariate analysis of variance for total plate count revealed significant difference between treatments and time period in log cfu values (Appendix D: 7.10). The population of microorganism was influenced by the addition of spice extracts. The number of the bacterial cells decreased in all the treated samples during the storage period as seen in Fig. 7.1. The initial load of 6.6 log/g bacteria in control reduced to 4.7 log/g towards fourth month of storage whereas, a reduction of 4.2 log/g was attained in clove treated samples within the second month. Between the spices, all had significant differences in bringing down the microbial count. The effect of spices on the bacterial population is as follows: Clove > garlic > cardamom > rosemary > chlorine > oregano > turmeric > control

7.4. Discussion

7.4.1. Physicochemical analysis

The initial pH value of treated samples was lower than their corresponding control samples (Table 7.2); no significant differences of pH were observed in the sixth month period among control and spice treated samples. Rostamzad *et al*. (2011) had found that expressible moisture and pH value of ascorbic acid treated Persian sturge (*Acipenser persicus*) fillets during frozen storage were significantly lower than those in control samples

($p<0.05$). According to other researches, frozen storage did not have significant effect on pH changes during storage period (Aubourg *et al*., 2004). The slightly acid nature of the spice extracts would have contributed to the initial low pH in the treated samples.

Water holding capacity in meat tissue is strongly related to myofibrillar proteins. Decrease in moisture retention is a sign of reduction of water holding capacity due to denaturising of proteins (Suvanich *et al*., 2000). This phenomenon leads to reduction of flavour agents and nutrition value (Reddy and Srikar,1991). In present study moisture content showed a gradual decrease for all samples during the course of frozen storage (Table 7.3). There was no significant difference between the moisture content of the treated and control samples ($p< 0.05$) over the 6 months of storage except turmeric. In the case of cook-loss also there was a marginal decrease in the yield of turmeric treated samples compared to other treatments. The use of curcumin from melon variety of fruits of *Curcumis trigonus* is reported to have proteolytic activity on both collagen and actomyosin (Bawa and Jayathilakan, 2010). Hydrolyzing effect of cucumin on myofibrillar and connective tissue is also well documented (Kumar and Berwal, 1998; Mendiratta *et al*., 2003; Naveena *et al*., 2004). Curcumin is also the major component in turmeric and its activity may be the reason for the variation shown by turmeric during the study.

Though there was no significant difference in the moisture content between control and spice treated tuna, the mean values were lower for spice treated samples. Comparison of ascorbic acid treated and control Persian sturge (*Acipenser persicus*) fillets by Rostamzad *et al*. (2011) revealed that moisture of ascorbic acid treated samples at 6th month of storage was lower than control samples ($p<0.05$). Similar results were reported by Pourashouri *et al*. (2009) on wells catfish and Garner *et al*. (2002) on channel catfish fillets.

Moisture loss is mainly due to sublimation of ice crystals. This leads not only to weight reduction, but also quality loss due to enhancement of oxidative changes, protein

denaturation and color changes (Tejada, 1979). When such changes are irreversible, freeze-burn starts to occur. The present experiment proves that spices used in this study, with the exception of turmeric, does not have any noticeable effect on moisture loss or moisture retention of tuna samples.

7.4.2. Qualitative and quantitative analysis of protein loss

Several quantitative analytical techniques have been utilized to study the structure of protein molecule with its amino acid sequences. Electrophoresis is one of the mostly preferred methodology for the quantitative and qualitative fractionation of the muscle protein. The most common technique used for the separation of protein samples is polyacrylamide gel electrophoresis (PAGE) in the presence of strong ionic detergent such as sodium dodecyl sulphate (SDS). This provides separation of denatured protein subunit partially on the basis of charge but principally on the basis of molecular size. The resolution and sensitivity is balanced by choice of the amount of the gel matrix.

The electrophoretic pattern of thaw-drip protein in tuna was very similar in all the spice treated samples. The pattern also closely resembled that of control (untreated sample). No distict differences in the number of bands was observed but, there was slight variation in the intensity of the bands between the spice extract treated samples. Thus, the treatments did not appear to have any observable effect on the denaturation of proteins in the range of spice extract used for treatment.

Sarcoplasmic proteins are generally soluble in water and buffers of low ionic strength. They are low molecular weight proteins in the range of 40 KDa to 70 KDa. The fish processor and food technologist aims to control the changes in the functional properties of tissue protein, and consequently hopes to preserve and improve the quality of the fish/meat. In the present study only low molecular weight (<29kDa) bands constituting

smaller peptides/proteins were shown. Qualitative analysis of protein revealed that the protein lost during thawing was of similar molecular weight for all treated and untreated samples. Therefore, it is evident that spice treatment itself does not effect the functional properties of tissue protein.

The difference in the protein present in thaw-drip of spice treated and untreated tuna samples showed that drip loss was more in 6 month frozen stored samples in comparison with one month stored samples. Protein leaching was highest in turmeric treated 6 month stored tuna sample. This may be due to the proteolytic activity of curcumin reported by other scientists (Bawa and Jayathilakan, 2010).

Determination of drip from frozen fish after thawing indicated the rate of protein alteration during frozen storage (Mills, 1975). Increase in this parameter is generally related to changes in myofibrillar proteins, since the water-binding capacity of the myofibrillar fraction is reduced by denaturation and aggregation. The moderately low drip loss and its small fluctuations between spice treated samples and control suggest that no notable alterations in the proteins occurred during the 6 months frozen storage (-20°C).

7.4.3. Microbiological stability

The total plate count of the control is showing a marked decrease over the six month duration of storage. During frozen storage, water available for the microorganisms for maintenance of their metabolic activities also decreases. For any frozen food, a_w will be that of ice at the same temperature (Leistner *et al.*,1981). Microorganisms in frozen foods are therefore exposed not only to low temperatures but also decreasing water activities and increasing solute concentration (Mazur, 1966). Although fish typically contain 70-80% of water, the exact percentage depends on the species. At -18°C around 90% of the water will turn to ice. The fish tissue holds an increasing proportion of liquid water and a decreasing

proportion of ice as its temperature rises. At -10°C, approximately 84% of the water is present as ice and 76% at -7°C and 70% at -3°C (Frank, 1985). Unavailability of free water makes the survival of microorganisms unfavourable.

When the microorganisms growing at relatively warm temperatures are subjected to a rapid, substantial decrease in temperature, within or beyond the growth temperature range, the cells can be injured with intracellular metabolites and proteins being lost from the cell (Mackey, 1984). Microorganisms held at temperatures below their minima for growth may progressively loose viability (Christophersen, 1968). Usually loss of viability when growth cannot occur is more rapid at higher temperatures (Ingram and Mackey, 1976). Thus organisms that can grow in foods, but cannot grow at temperatures below 0°C may be adversely affected by the temperature alone when they experience storage temperature of -18°C to -20°C. This is the main reason for reduction in TPC of the frozen stored samples.

Statistical analysis showed significant difference in the bacterial count at all stages of storage ($p<0.05$) in the control as well as treated samples. The total plate count (TPC) at the third month of storage in the control sample is attained by the first month in the tuna sample undergoing spice treatments. Oiye *et al*., (2012) found that rosemary spice can substitute nitrite pickling salt to produce organoleptically acceptable sausages. The lowest microbial count of sausage with 0.5% rosemary spice compared with other samples was attributed to the bactericidal effects of rosemary spice which is reported to take place when the spice is used at this level. Plant extracts such as thyme, garlic, clove and cinnamon could inhibit the growth of spoilage and/or pathogen fungi species, mainly *Aspergillus* sp., *Penicillium* sp., *Mucor* sp., and *Cladosporium* sp.(Özçakmak and Öztürk, 2012).

Examination showed that chilled steak samples treated with the a mixture of five spices namely, cinnamon, clove, star anise, picklyash peel and common fennel remained fresh after 9 days of storage (Wang *et al*., 2012). The investigation carried out by Elamathy

and Kanchana (2012) showed the antimicrobial activity of spice extracts against four common meat spoilage and pathogenic bacteria. The results showed that individual extracts of clove contained strong antimicrobial activity compared to other spices used. This suggests that antimicrobial effect of the spices have a significant role in the reduction of bacterial count apart from the freezing effect. In the present investigation, the significant variation of total plate count of spice treated tuna from the control is explained only by the antimicrobial action . The highest reduction of total plate count by clove extract is also explained by its highest antimicrobial activity compared to other spices.

During frozen storage, the number of viable organisms in foods can continue to decline, but at rates which not only may be much slower than those that occur during freezing but which also decrease with time (Speck and Ray, 1977). After extended periods of storage, the number of some organisms in frozen foods may be essentially stable (Christopherson, 1968), but the number of others may continue to decline to levels at which they cannot be detected (Kraft *et al*., 1963). The microorganisms that remain essentially stable belong to the psychrophilic or cold loving group. The significant decrease in the total plate count of treated samples from that of control after the first month of storage is mainly due to targeting the psychrophilic group.

Some Gram positive bacteria form spores within their cells (endospores) which can survive conditions that destroy the vegetative cells. Thirteen genera of spore formig bacteria have been described (Berkeley and Ali, 1994). Of these, members of the genera *Bacillus* and *Clostridium* are the most important with respect to safety and spoilage of foods. Spores are far more resistant than the parent organisms to a wide range of environmental stress (Gould, 1983). Bacterial spores can be expected to retain viability in frozen foods. The presence of *Bacillus spp*. was detected in tuna during the microbiological analysis (Table 3.2). Its spore forming ability was also identified by malachite green staining. The reduction in the *Bacillus*

spp. and as a consequence its resistant spores may be the second major reason for the reduction of bacterial count in spice treated tuna.

Currently chlorine monopolizes the seafood industry as the antimicrobial agent. In the present study also, the antimicrobial action of chlorine is undeniable in fish processing and low price of this chemical is an additional advantage. But, toxicological studies are underway which arises suspicion to its frequent usage. Only 2ppm chlorine level is approved by goverment agencies (EIA) in the water used for cleaning seafood. But, Thampuran *et al*. (2006) had reported that on headless shrimp with shell-on, 7ppm chlorine was required to destroy 10^3 cfu/g of *Vibrio cholerae* within 10 minutes. Their data indicate that chlorine level has to be adjusted depending on the nature of material and microorganisms present in seafood. Hence, spice treatment, though costly, will help in creating 'green label' and will provide with supplementary advantage of sensory enhancement. It can be used to decontaminate and flavour high priced products like sashimi grade tuna which costs approximately £ 40.80/Kg (Rs.3441.35) in the world market. And for sushi and sashimi, freshness and flavour of the product is of prime importance while price is only secondary to the buyer. This effect of spice treatment on such products can be taken up for further studies.

7.5. Conclusion

Traditionally the people of India have a long-standing practice of using wide variety of herbal products in treatment of diseases or as preservatives in foods. Spices are indispensable components of Indian cuisines since ancient times and are considered as rich source of bio-active antimicrobial compounds. The effect of spice treatment on the microbial load and biochemical parameters of tuna was analysed in this chapter.

The pH of the fresh tuna ranged between 6.26-6.00. The thaw-drip was higher at 6 months storage period in comparison to one month storage, irrespective of the treatments given. No difference between treatments was observed on the electrophoresis patterns of thaw drip. The patterns were identical to those of control sample. Thus, the treatments did not appear to have any observable effect on the fish proteins during frozen storage in the range of spice extract used. Drip loss from treated tuna samples after 6 months of frozen storage also did not show any significant difference from control except turmeric. Protein leaching was highest in turmeric treated 6 month stored tuna sample.

The total plate count of the spice treated samples showed a marked decrease over the six month duration of storage. Statistical analysis showed significant difference in the bacterial count at all stages of storage ($p<0.05$) in the control as well as treated samples.The reduction of viable cells was more in treated samples. The results of the present study suggests that apart from the freezing, antimicrobial effect of the spices also have a significant role in the reduction of bacterial count. The highest reduction of total plate count by clove extract is also explained by its highest antimicrobial activity compared to other spices.

Currently chlorine monopolizes the seafood industry as the antimicrobial agent. Toxicological studies are underway which arises suspicion to its frequent usage. Studies indicate that chlorine level has to be adjusted beyond the 2ppm level used in seafood and the exact amount of chlorine usage will depend on the nature of material and microorganisms present. For this reason, spice treatment although costly will help in creating ‘green label’, provide safety and have added advantage of sensory enhancement.

The potential use of naturally originated preservatives in practical application by food industries is disclosed in this study. It can be used to decontaminate and flavour high priced products like sashimi grade tuna, especially, because it is consumed raw. No

preventive measures are taken against pathogens in such cases apart from looking for the freshness of the product. Moreover, the reduced production of biogenic amines in spice treated tuna chuncks will further enhance its safety.

8.1. Summary and Conclusion

Despite the wide range of preservation techniques available these days, food spoilage and poisoning is not yet brought under adequate control. Today, in spite of modern improvements in food production, around 30% people in industrialized countries are suffering from food borne diseases. Hence, the food industry needs to reach a more advanced state to meet the challenge of providing extraordinary stability in today's complex foods. The use of chemical preservatives for attaining this goal have led to government regulations due to to their many adverse effects. Moreover, consumer thrust is towards green consumerism which has renewed an interest in natural food stabilizers. This requires better understanding of the available natural antimicrobial options, their range of activity, mechanism of action, sources, applications where they work best and those where they do not. The present study analysed the inhibitory effect of the selected spices viz clove, cardamom, rosemary, garlic, oregano and turmeric on biogenic amines, their role as a potent antimicrobial agent and quality stabilizer of tuna.

Tuna and tuna products are of great significance in the world market and currently, fishermen of nearly 80 nations harvest tuna from the world over. Unprocessed tuna harbor high numbers of bacteria, hence are more prone to spoilage. At some stage between catch and consumption of tuna, histidine decarboxylating bacteria grow to high concentrations. They form histamine and other biogenic amines in the products. Morover, human pathogenic bacteria like *Vibrio cholerae*, *Salmonella typhi* etc can also be part of the initial microflora of fish, posing a concern for seafood borne illnesses. In this circumstance, the use of natural antimicrobials in tuna for reduction of microorganism can play an important role in minimizing histamine formation and in turn reduce spoilage and food poisoning.

Chapter 1 gives the general introduction about the purpose of the study. The current statistics of the food borne diseases and the shift of consumer trend towards green labeled products is discussed in this chapter. The need for identifying a suitable natural antimicrobial is also stressed.

In Chapter 2 - Spices as Antimicrobial Agents- A Review, Chemistry of active constituents of spices with special emphasis on the antimicrobial constituents of six selected spices namely, rosemary, cardamom, garlic, oregano, turmeric and clove with chemical structures of the active components are given. Eugenol is the active constituent found in clove. Cardamom is dominated by 1, 8-cineole and alpha terpene acetate. Curcumin is the main component in turmeric. Carvacol and thymol are the active constituents in oregano. Important antimicrobial components in rosemary is camphor and 1, 8-cineole. Pharmacological and food processing uses of the selected spices are also described.

Chapter 3, Microbial profile of tuna, deals with the microbial load of fresh and spoiled tuna. The different strains of bacteria from tuna flesh is isolated and identified in this chapter. The total bacterial counts (TPC) of tuna was analysed as the preliminary step. It was in the range of 10^5 cfu/g. The percentage composition of bacterial flora of tuna (*Euthynnus affinis*) indicated 52.81% Gram negative organisms whereas Gram positive organisms accounted for 47.19%. Among Gram negative bacteria *Pseudomonas* spp. was dominant followed by *Vibrio* spp.

Antibacterial Effect of Spice Oleoresins is described in Chapter 4. Spice oleoresins showed significant antibacterial effect on isolated bacteria. Bacterial strains showed an minimum inhibitory concentration ranging from 0.12 % to 0.8%. Clove inhibited all organisms at $\leqslant$0.2%. The electron micrographic images indicated disruptive action of these compounds towards cytoplasmic membrane. Studies on the antimicrobial action of essential oils in model food systems revealed that clove treated samples showed an 88% reduction in the total plate count compared to the untreated control samples. In summary this study

confirms that spice extracts possess *in vitro* antibacterial activity in the order clove > oregano > cardamom > rosemary > garlic > turmeric.

The effect of spices oleoresins on biogenic amine formation in tuna during the post mortem changes occurring at ambient storage conditions was studied in Chapter 5. Fresh tuna was subjected to a dip treatment of 0.2% for 10 minutes with each spice extract. Sampling was done at regular intervals of 0 hours, 4 hours, 8hours, and 24 hours after dip treatment. The above treated samples of fish were also subjected to bacteriological analysis for enumerating histamine-forming bacteria. Analysis of biogenic amines in spice treated tuna stored at room temperature was done using a Waters HPLC system with a Binary pump model M515. Data analysis was performed using EMPOWER 2 chromatography software. A mean histamine reduction of 6.5mg/100gm in spice treated samples was observed from the control sample after 8 hour duration. With the exception of cardamom and turmeric there was only negligible increase (0.416mg/100gm) in histamine content between the 8^{th} and 24^{th} hours sample in spice oleoresin treated tuna. The control samples experienced an increase of 6.7 mg/100gm at the corresponding stage. The defect action level was attained by 4 hour of storage in the control. None of the samples treated with spices exceeded the rejection histamine limit of 50ppm upto 8^{th} hour. In clove, garlic, oregano and rosemary, the samples remained below the legal limit of histamine even after 24 hours.

Chapter 6, titled 'Effect of spice oleoresins on texture and sensory attributes of tuna' deals with the analysis of various parameters of texture profile in treated samples of tuna. Tuna chunks were subjected to dip treatments with two different concentration of spice (0.2% and 0.1%) for 10 minutes. The dip treated samples, stored for one hour at refrigerated temperature, were cooked at 100ºC and the cook loss assessed. The cooked samples were subjected to analysis of various texture parameters namely hardness, springiness, cohesiveness and gumminess. Results show that the samples with 0.2% and 0.1% concentration and dip treatment for 10 minutes did not show much variation in the textural parameters. But, sensory analysis score was better for 0.1% spice treated samples.

Among the six spices selected for the study, cardamom had maximum sensory score followed by clove and rosemary. Hence, the sensory enhancement by using a combination of these three spices was also studied and it was found to have positive impact on the sensory panel.

In the last chapter , titled 'Effect of spice oleoresins on quality stabilization of tuna during frozen storage' describes the role of spices on the quality of frozen storage of tuna. In the fourth chapter, 0.2% spice is proved to be minimum inhibitory concentration for the most effective spice for controlling bacterial load. Though a lower concentration of spice treatment produced good sensory scores, the antimicrobial effect was higher for 0.2% and in addition 0.1% and 0.2% concentration did not show any significant variation in textural qualities. Hence, 0.2% was the used for the frozen storage studies in this chapter. Dip treatment of tuna chunks were done at 0.2% of concentration spices oleoresin. The samples, after proper packaging, were stored in a cold store at -20ºC. Samples were drawn at regular intervals for a period of 6 months for analysis of various indices. The various parameters analysed were moisture, pH, Total plate count, Electrophoretic pattern of thaw drip proteins and protein content of thaw drip. ANOVA was carried out by using the generalized linear model procedure. The study revealed that all spice treated samples showed lower levels of microbial load on frozen storage up to 6 months confirming its antimicrobial property. Clove, cardamom and rosemary were proved to possess potent antimicrobial activity from the study. The electrophoretic analysis of thaw drip from the treated and control samples did not show much variation. This confirms the fact that spice extracts can be made use of for effective prevention of bacteria without much variation in the biochemical characteristics of tuna meat.

Even though strong antimicrobial activities of many plant extracts and spices have been reported, the need for novel natural antimicobial is obvious and food industries continue to look for them. Spices need to be evaluated at concentration accepted by the senses and with all interfering and synergistic compounds present. Based on the sensory

preferences, antimicrobial property and also their effect on biogenic amine formation, clove, cardamom and rosemary have proven to be the most effective antimicrobial to be used on tuna. This implies that consumption of raw fish products like sashimi grade tuna along with the natural antimicrobials can promote the quality of fishes and improves their flavour and fragrance.

Multitarget food preservation has emerged in relation to hurdle technology, based on the fact that, at times, different hurdles in food have not just an additive effect on microbial stability, but a synergistic one. This approach affords a non-agressive but more effective preservation of foods by the application of multiple soft treatments that disturb homeostasis and metabolic exhaustion and avoid stress reactions by bacteria. Hence, it is more effective to employ different small-intensity preservation factors than one large-intensity preservation factor because the combined use of several preservation factors may produce a synergistic effect. In this context, freezing food products treated with spice extracts can provide synergistic effect and it is proved in Chapter 7.

In recent times, demand has increased for a wide range of processed fishery products. Challenging lifestyles, require products with longer shelf lives and use of preservatives has become essential. Many countries have strict regulatory controls on use of chemical preservatives. New toxicology data impose some caution in the use of the popular synthetic antimicrobials used like sodium hypochlorite and chlorine dioxide. Chlorine added to our drinking water supply and in seafood processing plants is fast becoming a serious health problem world-wide. Chlorine is not used because it is the most diligent, or even the safest method of disinfecting our water but, primarily because it is the cheapest way. The biogenic amine formation of spice treated samples have shown a much lower value than chlorine treated samples. Even the antimicrobial property of spices is seen to be superior to chlorine treated sample in relation to the histamine forming bacteria. Moreover, the addition of spices having anti-carcinogenic, anti-inflammatory and more importantly, antioxidant properties can add to the health benefits of the consumer. In this

context, natural products appear as healthier and safer than chemical antibacterial agents and the present study will be of immense significance regarding this subject.

8.2. Future Prospects

The future research and development in food preservatives should contribute to solving the food preservation and food safety problems in the areas of culture of fish, handling, processing, trade and distribution of fishery products. Risk analysis for food borne pathogens is a new emerging discipline and according to this the main objectives of food preservation are to prolong shelf life and to guarantee safety of the consumer.

A more systematic research approach in hurdle technology should be called for to recognize the needs of the industry. The continuing need for foods that are minimally processed, taste fresh, but have long shelf-life and contain only natural preservatives opens a new vista for processed fish products. The safety and flavour enhancement of chilled products and also raw fish products like sashimi grade tuna can be taken up for future research.

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