BIOCHEMICAL EFFECTS OF DIFFERENT PHENOLIC COMPOUNDS ON *OREOCHROMIS MOSSAMBICUS* (PETERS)

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By

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To God Almighty

Declaration

I hereby do declare that the thesis entitled, **"BIOCHEMICAL EFFECTS OF DIFFERENT PHENOLIC COMPOUNDS ON *OREOCHROMIS MOSSAMBICUS* (PETERS)"** is an authentic record of research work done by me under the supervision and guidance of **Dr. BABU PHILIP**, Professor, Dept. of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology for the degree of **Doctor of Philosophy** in Biochemistry and that no part thereof has been presented for the award of any other degree in any University.

Kochi- 16
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Certificate

This is to certify that the thesis entitled **"BIOCHEMICAL EFFECTS OF DIFFERENT PHENOLIC COMPOUNDS ON *OREOCHROMIS MOSSAMBICUS* (PETERS)"** is an authentic record of research work carried out by **Mrs. Remya Varadarajan** under my supervision and guidance in Dept. of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology in partial fulfilment of the requirements for the degree of **Doctor of Philosophy in Biochemistry** and no part thereof has been presented before for the award of any other degree, diploma or associateship in any university.

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LIST OF ABBREVIATIONS AND NOTATIONS

ACP	-	acid phosphatase
ALP	-	alkaline phosphatase
ALT	-	alanine amino transferase
ANOVA	-	Analysis of variance
APs	-	alkylphenols
AR	-	analytical reagent
AST	-	aspartate amino transferase
ATPase	-	adenosine triphosphatase
CAT	-	catalase
CD	-	conjugated diene
CoA		coenzyme A
dl	-	decilitre
$^{\circ}C$	-	degree celsius
EPA	-	Environmental Protection Agency
$g.l^{-1}$	-	gram per litre
GDH	-	glutamate dehydrogenase
g	-	gram
GPx	-	glutathione peroxidase
GSH	-	reduced glutathione
GSSG	-	oxidised glutathione
GST	-	glutathione-S- transferase
H_2O_2	-	hydrogen peroxide
$^{\cdot}OH$	-	hydroxide radical
H_2SO_4	-	sulphuric acid
HCl	-	hydrochloric acid
h	-	hour
HP	-	hydroperoxide
IU	-	International Unit
$L^{\cdot}$	-	lipid radical
LC_{50}	-	lethal concentration causing 50 % mortality

LDH	-	lactate dehydrogenase
$logK_{ow}$	-	logarithm of the octanol-water partition coefficient
$LOO^{\cdot}$	-	lipid peroxyl radical
LOOH	-	lipid hydroperoxide
LSI	-	lysosomal stability index
MDA	-	malondialdehyde
µg	-	microgram
µM	-	micromolar
mg l^{-1}	-	milligram per litre
min	-	minute
ml	-	millilitre
mm	-	millimoles
M	-	molarity
NADH	-	reduced nicotinamide adenine dinucleotide
NAD	-	nicotinamide adenine dinucleotide
nm	-	nanometer
N	-	normality
NPEOs	-	nonylphenolethoxylates
O_2^{-}	-	super oxide radical
OH^{-}	-	hydroxyl ion
OPs	-	octylphenols
pK_a	-	negative logarithm of acid dissociation constant
ppm	-	parts per million
ppt	-	parts per thousand
%	-	percentage
$^1O_2$	-	singlet oxygen
SD	-	standard deviation
SOD	-	superoxide dismutase
SPSS	-	Statistical Package for the Social Sciences
UDP	-	uridine diphosphate
wt	-	weight

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Chapter 1

GENERAL INTRODUCTION & REVIEW OF LITERATURE

Contents

1.1 General Introduction

The unique physical and chemical properties of water have allowed life to evolve in it. The following quote from Szent-Gyorgyi (1958) illustrates this point of view: "Life originated in water, is thriving in water, water being its solvent and medium. It is the matrix of life." Water pollution is significant only when it influences living or biological systems either directly or indirectly. In a broad sense, it can be depicted as a normal consequence of the growth of organisms including man in or near the aquatic habitat. The presence of toxic pollutants in aquatic ecosystems poses a serious threat to environmental health.

Industrialization and growth of human population have led to a progressive deterioration in the quality of the earth's environment. Urban, agricultural and industrial activities release xenobiotic compounds that may pollute the aquatic habitat. Schwrzenbach *et al*. (2006) reported that about 300 million tons of synthetic compounds seep annually into water systems (rivers, lakes and sea). Industrial processes generate a variety of molecules that may pollute air and water systems due to negative impacts on ecosystems and humans (toxicity, carcinogenic and mutagenic properties). To improve the quality of aquatic ecosystems, it is necessary to know how the rivers and lakes are impaired and what factors caused the environmental deterioration. Pollution of water sources due to chemicals plays a primary role in the destruction of ecosystems but chemical analyses alone may not suffice to describe the adverse effects of the complex mixtures of chemicals present at contaminated sites. The potential utility of biomarkers for monitoring both environmental quality and the health of organisms inhabiting in the polluted ecosystems has received increasing attention during the last years. (Lopes *et al*., 2001; de la Torre *et al*., 2005; Mdegela *et al*., 2006; Minier *et al*., 2006).

The aquatic environment is particularly sensitive to the toxic effects of contaminants since a considerable amount of the chemicals used in industry, urbanization, and in agriculture enter marine and other aquatic environments. The

stressors in the environment exert their adverse effects at the organismal level leading to impaired physiological functions in aquatic organisms. Xenobiotics are potentially harmful to fish by inducing tissue damage in gill, kidney and liver (Ahmad *et al*., 2004), growth retardation (Gad and Sadd, 2008), genotoxicity (Aas *et al*., 2000), reproductive disturbances (Maradonna *et al*., 2004), tissue bioaccumulation (Rice *et al*., 2000; Hellou and Leonard, 2004). Since the second half of the last century, the environment has been contaminated by numerous xenobiotics; amongst these phenolics are of special concern. Hence they are good models of widespread xenobiotics to study with, in the field of environmental research.

1.1.1 Phenolic compounds

Phenolic compounds may be defined as any compounds with aromatic nucleus bearing a hydroxyl group directly linked to the aromatic nucleus. This definition would include di- and trihydric phenols, hydroxybenzoic acids, nitro-, chloro- amino-, methoxy-, phenoxy- and alkyl-phenols. Also included are some of the hydroxy derivatives of condensed aromatic nuclei such as naphthols. Degradation products from pesticides such as 2,4-D (2,4-dichlorophenoxy acetic acid), 2,4,5-T (2,4,5-trichlorophenoxy acetic acid), TFM (3-trifluoromethyl-4-nitrophenol) and Carbaryl (1-naphthyl-methylcarbamate) are also included. Phenolic compounds have been shown to be toxic to aquatic life at parts per million levels and several phenolics have the ability to impart tastes and odours' to drinking water supplies and edible aquatic life at parts per billion levels. Many phenolics are more toxic than pure phenol, but its toxicity is often used as a guide to the toxicity of other phenolics to fishes when no other data are available. The toxicity of phenolic compounds varies widely between fish species and under varying environmental conditions. Phenol and its derivatives are common substances present in industrial wastewaters and in non-specific pesticides, herbicides, bactericides and fungicides (Gupta *et al*., 1983).

Phenolic compounds are environmentally important due to their extensive use in various industries, presence in wastewaters and their potential toxicity. These lipophilic compounds have numerous industrial applications, which enhance the risk to the environment and to human health (Bradbury *et al.*, 1989). Industrial concerns and cities are delivering a heavy load of these substances which are integral part of the cleaning procedure. The presence of phenols in aquatic environment is undesirable because of their toxicity to aquatic organisms. Among the different phenolic compounds, phenols and cresols are widely used organic solvents. These solvents are widely used for extracting, dissolving or suspending materials such as fats, waxes and resins that are not soluble in water. These compounds have been identified in water- soluble fractions of oil since they are potential degradation products of aromatic hydrocarbon metabolism.

Phenolic compounds are used in the manufacture of many agricultural pesticides (Gimeno *et al*., 2005). They can also be introduced into the environment through degradation of natural substances (Davı and Gnudi, 1999) and industrial activities (e.g., dyes, plastics, pharmaceuticals and explosives) (Hoffsommer *et al*., 1980; Gutes *et al*., 2005). Phenolic compounds can cause toxicity, with bioaccumulation effects in animals and plants (Davı and Gnudi, 1999). Their inhalation and ingestion may be dangerous for human health; causing systemic damage to the nervous system (Meyer, 1989). Quantification of phenols in water has become increasingly important because of their toxicity for humans and aquatic organisms (Gutes *et al*., 2005). Creosote is classified as a hazardous substance for occupational exposure (Deichmann and Keplinger, 1981; Chemwatch, 2006).

Establishing the origin of phenolic compounds (anthropogenic or natural) is difficult because they can come into the aquatic ecosystems from the outside (primary pollution) or accumulate as a result of the functioning of all units of the food web (secondary pollution). The composition and concentration of these compounds in natural waters are dependent upon the balance of self-depuration

and secondary pollution. There are over 300 chemicals in creosote, and the most toxic are phenols, cresols and polycyclic aromatic hydrocarbons (PAHs) (Agency for Toxic Substances and Disease Registry (ATSDR), 2002). By their toxicological and organoleptic properties, phenolic compounds markedly differ from each other. It is believed that volatile low-molecular weight phenols (monophenol, 3 isomers of cresol, 6 isomers of xylenols, guaiacol, thymol) are the most toxic, and maximum permissible concentrations (MPCs) were set for each of them. Phenolic compounds taken for the present investigation include phenol, m-cresol and 4-nonyl phenol.

(i) Phenol

Phenol is among the first compounds described as toxic by the Environmental Protection Agency - United States (EPA-US), and due to its relevance as an ecotoxin it has been maintained in the priority list. Current national recommended water quality criteria from EPA-US advises phenol concentrations lower than 300 μg/l in order to protect aquatic organisms and a concentration of 1μg/l to prevent the tainting of fish flesh. Two MPC levels were set for the sum of volatile phenols: 0.001 mg/l for domestic and drinking water disinfected with chlorine or for chlorinated waste waters; 0.1 mg/l for natural waters which were not chlorinated, but, in this event, the organoleptic properties of water such as colour, odour, and taste serve as the limiting factor.

Phenols are a group of biologically active compounds with an extremely wide distribution and a well-known chemistry. Phenol is produced as a waste product of many industrial activities and, as such, appears in industrial effluents that contaminate aquatic ecosystems. As it adversely affects the aquatic biota, phenol is one of the 129 specific priority chemicals that are considered toxic under the 1977 Amendments to the Clean Water Act and for which the U. S. Environmental Protection Agency (EPA) has issued water quality criteria (EPA, 1979a, b; Babich *et al*., 1981). In addition, phenol is produced commercially and, as a potential occupational chemical hazard, the U. S. Occupational Safety and

Health Administration (OSHA) has set a safety standard for phenol that is based on toxicological data from laboratory animals and human beings.

As a pure substance, phenol is used as a disinfectant, for the preparation of some cream and shaving soap for its germicidal and local anesthetic properties, in veterinary medicine as an internal antiseptic and gastric anesthetic, as a peptizing agent in glue, as an extracting solvent in refinery and lubricant production, as a blocking agent for blocked isocyanate monomers, as a reagent in chemical analysis and as a primary petrochemical intermediate. Its largest use (35%) is to produce phenolic resins like phenol–formaldehyde resins (Bakelite) which are low-cost thermosetting resins applied as plywood adhesive, construction, automotive and appliance industries. By reaction with acetone it may also be converted into bisphenol A, a monomer for epoxy-resins (28%). It is also used to produce cyclohexanone and cyclohexanone–cyclohexanol mixtures by selective catalytic hydrogenation. Cyclohexanone is later converted into its oxime and further to caprolactam, the monomer for nylon 6 (16% of phenol applications). The mixture cyclohexanone–cyclohexanol is oxidized by nitric acid to adipic acid, one of the monomers for the production of nylon-66. Phenol is also used to produce polyphenoxy and polysulphone polymers, corrosion-resistant polyester and polyester polyols. Phenol may be converted into xylenols, alkylphenols, chlorophenols, aniline, and other secondary intermediates in the production of surfactants, fertilizers, explosives, paints and paint removers, textiles, rubber and plastic plasticizers and antioxidants, and curing agents and so on. Phenol is also a building block for the synthesis of pharmaceuticals, such as aspirin.

Phenol is a metabolite of a widely used organic solvent-benzene. Benzene is lipophilic, hydrophobic, nonpolar, and can pass through membranes readily because the center of the lipid bilayer (the fatty acids tails) is nonpolar (Butters, 2008). For benzene to exert its toxicity, it must first be metabolized in the liver by the activity of cytochrome P450 2E1 (CYP2E1) to form benzene oxide, which can rearrange non enzymatically to form phenol. Powley and Carlson (2001) reported

that phenol is mainly metabolized by the subfamily CYP2E1; nevertheless other families such as CYP2F2 could also be involved. Phenol can either be conjugated to a sulphate or glucuronide or be hydroxylated to catechol, hydroquinone and 1, 2, 4-benzenetriol. These polyphenolic metabolites travel to the bone marrow, where they are oxidized to highly toxic quinines by myeloperoxidase and produce hematotoxic and leukemogenic effects (Smith *et al*., 2000). The metabolism of phenol in fish yields the known phenyl conjugates (phenyl sulphate and phenyl glucuronide) and quinol sulphate (Nagel, 1983). At concentrations < 1 μgl^{-1} considered a non-toxic concentration; phenols can have an adverse effect on the taste and odour of water and fish (Pocurull *et al*., 1995).

The main source of polluting phenols is anthropogenic activities, such as petrochemical, pharmaceutical and textile industries, and as constituents of resins, dyes, paints, non-specific insecticides, herbicides, bactericides and fungicides (Gupta *et al*., 1983). Also, because of its antiseptic properties, phenol is commonly used in hospitals as disinfectant, throat lozenges and mouthwashes. It can also result from natural processes, such as biotransformation of benzene (Bruce *et al.,* 1987), tyrosine synthesis and reactions in the digestive system of vertebrates (Tsuruta *et al*., 1996). Phenolic compounds are known to enter water bodies via sewage waters from wood-processing plants, petroleum refineries, coal producers, and chemical plants. However, quite a variety of phenolic compounds are formed as a result of secondary pollution of natural aquatic ecosystems, i.e. in the process of vital activity of aquatic organisms, during microbiological degradation and transformation of allochthonous and autochthonous organic compounds that are formed in the water column as well as in bottom sediments (Kondrat'eva, 2001). In surface waters, phenolic compounds occur in free, dissolved state and, being capable of condensation and polymerization reactions, form humus like complexes and polyaromatic compounds. The phenol concentrations in aquatic ecosystems depend on the season and differ in the surface and near-bottom waters. Phenolic compounds differ in chemical inertness and resistance to microbial degradation. Therefore, some of them are readily

oxidized in the aquatic environment or metabolized by microbial communities, while others remain unchanged for a long time or, accumulating in a body of water and bring a real threat to its inhabitants.

Coconut husk retting is the basic process involved in the manufacture of coir. This small scale industry practiced in the backwaters leads to deterioration of water quality. Retting is basically a biological process involving the pectinolytic activity of micro-organisms present in the retting grounds, especially bacteria and fungi, liberating large quantities of organic substances in to the medium (Jayasankar, 1985). According to Prabhu (1957) large chunks of phenol, pectin, cellulose and tannin are released from the husk in to the medium during different stages of retting. Studies by Paulmurugan *et al*. (2004) showed that pH values in the retting zone where on the acidic side. Husk retting results in the production of organic acids into the medium and this in turn lowers the pH. In their study a low level of 1.26 mg/l of dissolved oxygen was observed in the retting zone of the Kadinamkulam Kayal, a major retting zone in Kerala. The oxygen present in the aquatic medium gets utilized by the bacteria inhabiting the area for the decomposition of large quantities of coconut husk used for retting activity. Thus the retting zones appeared as dark turbid areas resulting in the production of a foul odour. The anaerobic situation created in the area may lead to the depletion of living resources such as plankton, benthos and nekton. Mass mortality of the fishes in the retting grounds of Kerala was reported earlier (Nandan and Aziz., 1995). Jayasankar and Bhatt (1966) found *Pseudomonas* sp. and *Micrococcus* sp. as the microflora associated with the leaching of polyphenols.

(ii) Cresol

Cresol is popularly used as a disinfectant. U.S. Environmental Protection Agency recommends a maximum permissible concentration of 0.1 ppm m-cresol in water for fish and wildlife. EPA has classified *o*-cresol, *m*-cresol, and *p*-cresol as Group C, possible human carcinogens (USEPA, 1999). It is a well-known environmental pollutant, toluene metabolite, uremic toxicant and accidental

poisoning product (Chiu *et al*., 2005). Cresols, monomethyl derivatives of phenol, exist as three isomers (*ortho*, *meta*, and *para*) and are produced commercially by chemical synthesis or by distillation from petroleum or coal tar (Kirk-Othmer, 2004). Cresols are of natural or synthetic origin. Commercially cresol is a mixture of the *ortho, meta* and *para* isomers of cresol, in which the m-isomer predominates. The mixture is derived from coaltar or petroleum (ACGIH, 1991). All cresol isomers have a strong phenolic odour (Griem, 2000). Cresol solutions are used in household cleaners and disinfectants, under the trade name Lysol. Volumes of U.S. production and import are in the hundreds of millions of lbs/year (ATSDR, 2006). Cresol mixtures condensed with formaldehyde are important for modifying phenolic resins. However the m-isomer content is critical to the mixture because m-cresol is the most reactive of the three isomers. Crude cresol (commercial grade) contains approximately 20% o-cresol, 40% m-cresol, and 30% p-cresol. *m*-cresol is used to produce certain herbicides, as a precursor to the pyrethroid insecticides, to produce antioxidants, and to manufacture the explosive, 2,4,6-nitro-*m*-cresol.

Cresols have a wide variety of uses including the manufacture of synthetic resins, tricresyl phosphate, salicylaldehyde, coumarin, and herbicides. Cresols also serve as components of degreasing compounds in textile scouring and paintbrush cleaners as well as fumigants in photographic developers and explosives. Cresols also function as antiseptics, disinfectants, and parasiticides in veterinary medicine. An approximate breakdown of cresol and cresylic acid use is 20% phenolic resins, 20% wire enamel solvents, 10% agricultural chemicals, 5% phosphate esters, 5% disinfectants and cleaning compounds, 5% ore floatation, and 25% miscellaneous and exports. Cresols are also formed from the atmospheric photo oxidation of toluene. m-cresol is an effective ingredient of lysol used as a strong disinfectant and anti-parasites` agent in fish farming and agriculture.

Cresol isomers are used individually or in mixtures in the production of disinfectants, preservatives, dyes, fragrances, herbicides, insecticides, explosives, and

as antioxidants used to stabilize lubricating oil, motor fuels, rubber, polymers, elastomers, and food. Mixtures of cresols are used in wood preservatives and in solvents for synthetic resin coatings, degreasing agents, ore floatation, paints, and textile products. Cresols occur naturally in oils of some plants and are formed during combustion of cigarettes, petroleum-based fuels, coal, wood, and other natural materials (International Programme on Chemical Safety, (IPCS), 1995). Various foods and beverages contain cresols (Suriyaphan *et al*., 2001; Zhou *et al*., 2002: Kilic and Lindsay, 2005; Guillén *et al*., 2006;) and cresols have also been detected in air, sediment, soil, surface and groundwater, primarily near point sources (McKnight *et al*., 1982; Bezacinsky *et al*., 1984; Jay and Stieglitz, 1995; Nielsen *et al*., 1995; Jin *et al*.,1999; Schwarzbauer *et al*., 2000; Thornton *et al*., 2001; Atagana *et al*., 2003; Tortajada-Genaro *et al*., 2003; Morville *et al*., 2006). High production and distribution of cresols in the environment indicate the potential for widespread exposure to humans. However, levels of exposure certainly vary among individuals depending on their occupation, lifestyle and location. In humans, cresols or their metabolites are detected in tissues and urine following inhalation, dermal, or accidental and intentional oral exposure (Green, 1975; Yashiki *et al*., 1990; Wu *et al*., 1998; IPCS, 1995). Cresols are also detected in humans following absorption of other phenolic chemicals, e.g. toluene (Woiwode and Drysch, 1981; Dills *et al*., 1997; Pierce *et al*., 2002).

(iii) Alkylphenols

Surfactants are synthetic organic chemicals used in detergents, household cleaning products and in the food, mining, oil and textile industries. Surfactants are ubiquitous and in untreated effluents, certain classes of surfactants can be present in sufficient concentrations to constitute toxicity problems to aquatic organisms (Ankley and Burkhard, 1992). The effects of anionic surfactants to aquatic species have been more frequently studied in the past than those of nonionic and cationic surfactants (Lewis and Suprenant, 1983). Anionic surfactants have been the most widely used, but the importance and use of nonionic and cationic surfactants has increased (Lewis and Suprenant, 1983;

Huber, 1984; Dorn *et al*., 1993). There is therefore an obvious need for more toxicity studies in these groups.

Alkylphenols (APs), particularly nonylphenols (NPs) and to a lesser extent octylphenols (OPs), are extensively used for the production of alkyphenolpolyethoxylates (NPEOs), a class of non-ionic surfactants that has been largely employed for more than 40 years in textile and paper processing and in the manufacture of paints, coatings, pesticides, industrial detergents, cosmetics and spermicidal preparations, as well as various cleaning products. NPs are also used in the manufacturing processes of many plastics and as monomers in the production of phenol/formaldehyde resins. Commercially produced NPs are predominantly 4-nonyl phenol and this compound is often selected as a model for NPs.

Nonyl phenol (NP) is not a single chemical compound. Instead, the term is used to refer to a family of compounds all of which have a central aromatic (or benzene) ring and a nine carbon side chain. 4-nonyl phenol, in which the side chain is attached to the carbon directly opposite the hydroxyl group (OH; oxygen and a hydrogen atom), is the most common member of this family, making up over 90 percent of commercial nonyl phenol. In addition, the nine carbon side chain can have many different shapes; a branched side chain is more common than a side chain with all nine carbons in a straight line. Similar compounds with side chains with different numbers of carbon atoms are grouped together as alkyl phenols. Surfactants related to nonyl phenol but with additional groups of atoms called ethylene oxide units are called nonyl phenol ethoxylates. Alkyl phenol ethoxylates is another commonly-used term used to group the nonyl phenol ethoxylates with some closely related compounds that have carbon side chains of different lengths. Once released into the environment, nonyl phenol ethoxylates break down into nonyl phenol, nonyl phenol monoethoxylate, nonyl phenol diethoxylate and other related compounds. These breakdown products are called "biorefractory" because they are persistent in the environment.

Nonylphenol (NP) is a by-product of alkylphenol polyethoxylates (APEs) found in many products including detergents, plastics, emulsifiers, pesticides, and industrial and consumer cleaning products (Talmage, 1994). The annual worldwide production of APEs exceeds 500,000 metric ton (Renner, 1997), with an estimated 60% of this production ending up in the bodies of water around the world. Researchers have identified NP as the most critical metabolite of APEs mainly due to three major reasons, namely: its resistance to biodegradation, its ability to bioaccumulate and its toxicity (Ahel *et al*., 1994a, b; Tyler *et al*., 1998). As a consequence of APEs use in a variety of products, NP is quite common in rivers, estuaries and other aquatic environments that receive sewage discharges or are near offshore oil platforms (Brendehaug *et al*., 1992; Isobe *et al*., 2001; Ying *et al*., 2002; Ashley *et al*., 2003; Jonkers *et al*., 2003). Hale *et al*. (2000) reported that NP released into sewage effluent reached concentrations of up to 12 mg/l in the USA. Another important source of AP contamination is the degradation products of surfactants, such as alkylphenol polyethoxylates (APEOs), including para-substituted nonyl- and octylphenols (4-NP and 4-OP) (Giger *et al*., 1984; Ahel *et al*., 1987). APEOs are commonly used in production of paints, cleaning agents, plastics and pesticides, and are therefore major constituents of waste water from some chemical industries (Krogh *et al*., 2003). The use of APEOs on offshore installations as detergents and as additives in production processes is now banned in the Norwegian sector of the North Sea and is in the process of being phased out in other sectors, but they are still widely used in the USA and Asia (Renner, 1997; Lye, 2000).

In the present investigation a baseline attempt to investigate and assess the toxicities of three different phenolic compounds viz, phenol, m-cresol and 4-nonylphenol on fresh water - adapted euryhaline teleost *Oreochromis mossambicus* (Peters) has been carried out. *O. mossambicus* selected for the present study fulfils most of the criteria listed for a standard fish. They are found in abundance in the rivers, lakes and backwaters of Kerala. They have been described as 'miracle fish' owing to their bio-economic advantage such as quick growth, fewer bones, tasty flesh, good market acceptance, faster rate of

reproduction, acceptability to wide range of environmental alterations, ready acceptance of artificial feed, direct assimilation of blue green algae (Jhingran, 1984) and effectiveness in controlling growth of harmful insects and weeds.

The following criteria have been listed by Adelman and Smith (1976) for the choice of a standard fish.

(a) It must have a constant response and have neither high nor low sensitivity to a broad range of toxicants tested under similar conditions.

(b) It must be available throughout the year.

(c) A constant size group of that species should be available throughout the year.

(d) It should be easy to collect, transport and handle.

(e) The adults should be small enough so that acute or chronic tests can be conducted without undue difficulties in maintaining the recommended loaded densities.

(f) It should be possible to breed the species in laboratory.

(g) It should complete its life cycle within one year or less.

Since the cichilid fish, tilapia satisfies almost all the conditions it is widely used in toxicological studies.

Organisation of the Thesis

The thesis is divided into **7** chapters with the following objectives.

(a) To study the metabolic changes on exposure to different phenolic compounds by investigating selected metabolic parameters and enzymes involved in important metabolic pathways.

(b) To assess and evaluate the effects of different phenolic compounds on antioxidant enzymes and lipid peroxidation.

(c) To study the effect of exposure of phenolic compounds on branchial ATPases, serum ions and haematological parameters.

(d) To determine the effect of different phenolic compounds on stability of biological membranes.

(e) To examine the histopathological changes in gills, liver and kidney on exposure to different phenolic compounds.

1.2 Review of literature

Oil and its refined products consist of 75% short and long hydrocarbon chains (Neff, 1979) and are perhaps the most complex and variable mixtures to evaluate toxicologically. The short chains are volatile, remaining less time in the aquatic environment, but have a high toxic potential for aquatic life (Brauner *et al*., 1999). The water soluble fraction (WSF) of crude oil contains a mixture of polyaromatic hydrocarbons (PAH), phenols, and heterocyclic compounds, containing nitrogen or sulphur (Saeed and Mutairi, 1999). Although the more toxic compounds are volatile, fish can quickly absorb part of the WSF with adverse consequences to biological organization (Collier *et al*., 1996). The components of crude oil dissolved in the water have been considered as an important determinant of the petroleum toxicity in accidental spills (Saeed and Mutairi, 1999). Dauble *et al*. (1983) recorded that coal liquid dispersion, of which phenol is one of the major constituents, caused a complete inhibition of spawning in fathead minnows and rainbow trout. A road tanker accident in June, 1993 and the resultant phenol spillage into the Peechi reservoir, (Kerala state, South India) affected the drinking water supply in central Kerala (Rajasekharan and Sherief, 1998).

The total concentration of phenol and alkylphenols (APs) in water varies with production field, and ranges between 0.6 and 10 mg/l (Brendehaug *et al*., 1992; Roe, 1998; Utvik, 1999). Due to their relatively high water solubility, phenol together with C_1–C_3 APs constitute more than 95% of total phenols in

produced water whereas APs with a higher degree of alkylation (butyl through heptyl, C_4–C_7) are present in lower concentrations, 2–237 µg/l (Brendehaug *et al*., 1992; Roe, 1998; Boitsov *et al*., 2004). Although there is a high degree of dilution around offshore installations, the sum of C_1–C_4 APs has been determined in concentrations up to 140 ng/l in surrounding waters (Riksheim and Johnsen, 1994). Generally, water solubility and degradation rate of APs decrease with increasing degree of alkylation, whereas the bioaccumulation factor increases (McLeese *et al*., 1981; Freitag *et al*., 1985; Tollefsen *et al*., 1998). Both acute and chronic effects of alkylphenols on marine species are highly dependent on molecular structure and degree of alkylation (McLeese *et al*., 1981; Holcombe *et al*., 1984; Choi *et al*., 2004).

1.2.1 Physiological, behavioural and biochemical studies on exposure phenolic compounds

Mason-Jones (1930) investigated the toxicity of a wide range of substances found in tar, using experimental animals such as, perch, yearling trout and trout fry and described the symptoms produced by phenol and the cresols. At higher concentrations there was a very characteristic rapid loss of the sense of balance; the fishes showed a wild, dashing movement and turned on its side; the gill covers, at first widely opened, then they were tightly closed; the respiratory movements became irregular and feeble, and before dying the fish turned turtle. Ellis (1937) tested the toxicity of phenol in goldfish and stated that it produced a paralysis of neuromuscular mechanisms. A detailed discussion of the pharmacology of phenol and the cresols is given by Edmunds and Gunn (1936); their action on fishes is not discussed but it is stated that in frog phenol causes fibrillary twitching in the muscles followed by tonic convulsions and then a complete paralysis of the central nervous system.

Cresols are known respiratory irritants in animals and humans (ATSDR, 2006). Further, Vernot *et al*. (1977) determined that technical grade cresol (and individual isomers) was corrosive to the skin of rabbits. Burns and fatalities have been recorded in humans accidentally or intentionally exposed to cresol-

containing products (Green, 1975; Yashiki *et al*., 1990; Monma-Ohtaki *et al*., 2002; ATSDR, 2006).

Negative impacts of phenol on reproduction in aquatic animals have been reported for fish (Ghosh, 1983) and invertebrates such as gastropods (Kordylewska, 1980), prawns (Law and Yeo, 1997), and sea urchins (Anderson *et al*., 1994). Reduction of egg production was observed in a copepod (*Acartia clausi*) after exposure to phenol (0.5mg l^{-1}) for 8 days (Buttino, 1994). Au *et al.* (2003) found that chronic exposure to phenol at 0.1 mg l^{-1}could lower the quality of sperm and reproductive success in sea urchins, which may threaten the survival of these ecologically important species. Brauner *et al*. (1999) found that the exposure of a facultative air breather, *Hoplosternum littorale*, to 12.5, 25, and 37.5% of the water soluble fraction (WSF) of Urucu crude oil, affected gas exchange and ion regulation.

Phenol is highly lipophilic and the absorption of its chloro derivatives occurs through passive diffusion of nonionic forms (Kishino and Kobayashi, 1995). They are commonly found in the marine environment and in fish tissues (Mukherjee *et al*., 1990) where they induce acute or chronic toxicities. Their actions are multiple and often antagonistic. They are immunotoxic (Taysse *et al*., 1995), genotoxic (Jagetia and Aruna, 1997) and carcinogenic (Tsutsui *et al*., 1997). Because of its lipophilicity, phenol has a potential for accumulating along the trophic chain. Therefore, phenol not only presents a threat to natural environment, but also to human health. Phenol intoxication must be considered in the fish rearing systems. Phenol is created in natural conditions in animals and human from tyrosine and its derivatives in the digestive system (Tsaruta *et al*., 1996). Exposure to 100 mg phenol/m^3 for 15 days significantly affected the central nervous system of rats (Dalin and Kristofferson, 1974).

The use of biochemical approaches has been advocated to provide an early warning of potentially damaging changes in stressed fish. In toxicological studies changes in enzymes activities often directly reflect cell damage in specific organs

(Casillas *et al*., 1983). Fish exposed to pentachlorophenol showed increase in oxygen consumption (Crandall and Goodnight, 1962; Peer *et al*., 1983; Kim *et al*., 1996) and reductions in stored lipids and growth (Holmberg *et al*., 1972; Webb and Brett, 1973; Hickie *et al*., 1989; Samis *et al*., 1994). Phenol metabolism is of particular interest because it is a major oxidized metabolite of benzene, a known animal carcinogen. It has been studied extensively as a model compound for absorption and biotransformation in vertebrates, and its metabolites are known to be readily excreted in the urine (McKim Jr. *et al*., 1993). Phenolic compounds are generally concentrated through the food chain due to their accumulation in lipids (Mukherjee *et al*., 1990).

In rainbow trout hepatic microsomal biotransformation of phenol into hydroquinone and catechol has been observed (Kolanczyk and Schmieder, 2002). Moreover, the presence of phenyl sulphate and phenyl glucuronide in bream, goldfish, guppy, minnow, perch, roach, rudd and tench (Layiwola and Linnecar, 1981; Nagel and Urich, 1983) suggests that several freshwater fishes may have enzymes for conjugating phenol. Particularly noteworthy is the fact that biotransformation could result in more toxic compounds. In carp, *Cyprinus carpio*, comparative studies with phenol, hydroquinone and catechol showed that hydroquinone is the most immunotoxic compound (Taysse *et al*., 1995). Saha *et al*. (1999) reported that chronic (1 month) low level exposure (2.85 to 4.11 mg l^{-1}) to water-borne phenol under laboratory conditions decreased food consumption (~27%), growth (~45%), and fecundity (~45%). Phenol can be more toxic to fish than bacteria and unicellular green algae (Tisler and Zagorc-Koncan, 1997). These effects at the organismal level can be the result of many actions of phenol beyond those referred above. For example, Dunier and Siwicki (1993) reported that phenol causes suppression of fish immune system. It can also cause substantial changes in the composition of plasma membrane phospholipid (Kotkat *et al*., 1999).

Moreover, phenol and its derivatives can cause many alterations on the metabolism of fish (Holmberg *et al*., 1972; Dalela *et al*., 1980; Gupta *et al*., 1983; Reddy *et al*., 1993). Many enzymes of intermediary metabolism of fish are affected by exposure to phenol. Gupta *et al*. (1983) found both ALT and AST activities altered in different tissues by a wide range of phenolic compounds. Other enzymes such as succinate dehydrogenase, lactate dehydrogenase, acetyl cholinesterase and glutamate dehydrogenase were found to respond to phenol intoxication in the brain and white muscle of *Channa punctatus* (Reddy *et al*., 1993). Other reports have pointed out other enzymes susceptible to phenol such as alkaline and acid phosphatases (Dalela *et al*., 1980), superoxide dismutase (SOD) and catalase (Roche and Bogé, 1996). The known toxic effects of phenol in fish are wide and multiple. It can cause several alterations in energy metabolism (Hori *et al*., 2006).

Roche and Boge (2000) studied *in vivo* effects of phenolic compounds (phenol and OH-phenols) on a marine fish (*Dicentrarchus labrax*). The results showed that OH-phenols treated fish showed metabolic disorders such as hypoglycemia, low blood urea nitrogen level (BUN) and decrease of alkaline phosphatase activity. Chan *et al*. (2005) found that environmental toxicants such as *o* and *m*-cresol showed inhibition of cyclooxygenase activity, platelet aggregation and thromboxane B_2 production. In rats, after gastric intubation direct absorption of cresol by stomach and small intestine into blood stream has been reported (Morinaga *et al*., 2004).

Parvez *et al*. (2006) studied the effect of paper mill effluent on the gill ATPases in freshwater fish *Channa punctatus* and it was found that inhibition of total ATPase, ouabain-insensitive ATPase, and Na^+, K^+-ATPase activity occurred, with maximum impairment in Na^+, K^+-ATPase activity. Dong *et al*. (2009) investigated the effect of pentachlorophenol (PCP) in primary cultures of hepatocytes of freshwater crucian carp (*Carassius carassius*) as an *in vitro* model. It was revealed that Ca^{2+}, Mg^{2+}-ATPase activity and ATP content were declined, and the intracellular Ca^{2+} was increased by PCP.

The most intensively studied APs with respect to chronic effects are 4-t-OP and various isomers of 4-NP due to the estrogenic activities of these compounds (White *et al*., 1994; Jobling *et al*., 1996; Lech *et al*., 1996) and the large amounts released from degradation of APEOs. Routledge and Sumpter (1997) found that the estrogenic effect of alkylphenols is dependent on position (para > meta > ortho), branching (tertiary > secondary = normal) and size of alkyl group, with 4-t-OP reported as the most potent estrogen.

The alkylphenols, which exhibit moderate hydrophobicity and limited biodegradation potential (Ahel *et al*., 1994; Nimrod and Benson, 1996), are known to bioaccumulate and cause acute toxicity to algae, clams, shrimp, crustaceans and fish (McLeese *et al*., 1979, 1981; Saarikoski and Viluksela, 1982; Granmo *et al*., 1989; McCarty *et al*., 1993; Tollefsen *et al*., 1998). Some of these chemicals have also been reported to interact with intracellular and extracellular estrogen binding proteins (Knudsen and Pottinger, 1999) and cause interference with reproductive functions and normal developmental of fish (Jobling *et al*., 1996; Ashfield *et al*., 1998; Gimeno *et al*., 1998; Seki *et al*., 2003). It has been shown that exposure to low-level doses of nonylphenol inhibits ATP synthesis in mitochondria (Bragadin *et al*., 1999). Evans *et al*. (2000) found that marine gastropods exposed to nonylphenols can induce male sexual characteristics in females.

1.2.2 Antioxidant responses on exposure to phenolic compounds

Phenolics are frequently considered as reactive oxygen species-generating agents leading to major cell damage, such as oxidation of membrane polyunsaturated lipids (Pradhan *et al*., 1990). Some of them are scavengers for free radical species, while others are considered as reactive oxygen species generating agents (Winston, 1991). Bukowska *et al*. (2007) showed that 3-(dimethylamino) phenol increased the level of free radicals and changed the properties of the cell membrane, caused strong oxidation of haemoglobin and also changed the activity of glutathione peroxidase, catalase, superoxide dismutase and acetylcholinesterase in human erythrocytes.

Avci *et al*. (2005) investigated the possible effects of the waste water contamination of a petroleum industry on the oxidant/antioxidant status of muscle and liver tissues from fish in the Kizilirmak River, Kirikkale, Turkey. Results obtained suggest that some contaminants from the petrochemical industry cause oxidation in fish muscle tissues by impairing the antioxidant system.

Research by Zhang *et al*. (2004) showed that the activities of catalase (CAT) and selenium-dependent glutathione peroxidase (Se-GPx) and the content of oxidized glutathione (GSSG) were increased significantly on the whole compared to control group in freshwater fish, *Carassius auratus* on long-term exposure to 2, 4-dichlorophenol. Achuba and Osakwe (2003), observed elevated levels of lipid peroxidation, superoxide dismutase and catalase activities in all tissues examined in catfish, *Clarias gariepinus* exposed to petroleum (oil in water dispersions). Li *et al*. (2007) studied the hydroxyl radical generation and oxidative stress in liver of *Carassius auratus* by injecting intraperitoneally, different doses of 2,4,6-trichlorophenol. Results showed that under the effects of 2,4,6-trichlorophenol, the generation of free radical increased significantly, whereas the activities of antioxidant enzymes such as CAT, SOD and GST decreased. A decreased GSH/GSSG ratio and a significantly increased MDA content were also observed which indicated that *C. auratus* was subjected to oxidative stress and damage.

Heinz body haemolytic anaemia and hyperbilirubinemia under the influence of phenol have been also reported (WHO, 1994). Bukowska *et al*. (2000) studied the effects of exposure to different concentrations of phenoxyherbicides and their metabolites in human erythrocytes, with particular attention to catalase. The results showed that 4-chloro-2-methylphenoxyacetic acid (MCPA), 2,4-dimethylphenol (2,4-DMP) and 2,4-dichlorophenoxyacetic acid (2,4-D) did not affect CAT activity, but 2,4-dichlorophenol (2,4-DCP) and 2,4,5-trichlorophenol (2,4,5-TCP) decrease its activity, the latter being the more inhibitory.

Bukowska and Kowalska (2004) studied phenol and catechol induced prehaemolytic and haemolytic changes in human erythrocytes in human blood

cells *in vitro*. They found that both compounds induced methaemoglobin formation, glutathione depletion and conversion of oxyhaemoglobin to methaemoglobin, which is associated with superoxide anion production and formation of ferryl haemoglobin, hydrogen peroxide or hydroxyl radicals. 3-dimethylamino phenol strongly oxidizes haemoglobin (Vick and Von- Bredow, 1996). Bukowska and Kowalska (2003) suggested that the intensity of haemoglobin oxidation by phenolic derivatives may be presented in the order of decreasing potency: catechol > 3-dimethylamino phenol > 2, 4-dimethylophenol > 2, 4-dichlorophenol > phenol.

1.2.3 Histopathological studies on exposure to phenolic compounds

Cresol intoxication associated haemorrhagic changes in different organs, such as lung, epicardium, kidney, pancreas as well as bronchus has been observed in several studies (Labram and Gervais, 1968; Green, 1975; Clayton and Clayton, 1982; ATSDR, 1992; OEHHA, 2003). Long-term animal treatment with phenol results in changes of skin, liver, lung and kidney (Bruce *et al*., 1987). Exposure to sub lethal levels of phenolic wastes has been noted to evoke a variety of lesions such as gill necrosis, degenerative changes in the muscles, and various inflammatory degenerative and necrotic changes in heart, liver, and spleen (Waluga, 1966; Kristoffersson *et al*., 1974; Nemcsok and Borros, 1982; Benedeszky *et al*., 1984; Post, 1987). Sub acute toxicity of the nonylphenol on fish was investigated in laboratory toxicity tests with rosy barb (*Puntius conchonious*) by Bhattacharya *et al*. (2008). The results showed that NP caused alteration of the structure in gills, liver and kidney as evidenced by the hyperplasia of epithelium and the fusion of secondary lamellae in the gills, the disappearance of the cell membrane and the cell necrosis in the liver as well as haemorrhages in the kidney.

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Chapter 2

METABOLIC ALTERATIONS IN *OREOCHROMIS MOSSAMBICUS* ON EXPOSURE TO DIFFERENT PHENOLIC COMPOUNDS

Contents

2.1 Introduction

Homeostasis refers to the state of an organism in which its internal environment is maintained in a stable and constant condition. The physiological processes that maintain this equilibrium form a complex and dynamic system. The maintenance of homeostasis is critical to sustain life and changes in the environment can represent a threat to this equilibrium (Charmandari *et al*., 2005), and can lead to an array of physiological responses often referred to as stress response. Several factors (stressors) can challenge this equilibrium. In fish, for example, changes in water quality, exposure to pollutants, handling, and changes in stocking density have been shown to cause stress (Roche and Bogé, 1996; Vijayan *et al*., 1997; Barton, 2002; Iwama *et al*., 2004; Urbinati *et al*., 2004).

Generally, the responses to stressors are divided into primary, secondary and tertiary responses. The primary response is neuroendocrine and is the result of a stimulation of the hypothalamic-chromaffin axis and the hypothalamic- pituitary interrenal (HPI) axis. In response to stress two main classes of hormones, catecholamines and corticosteroids are released by the chromaffin and interrenal cells respectively (Wendelaar-Bonga, 1997). Secondary responses usually are defined as the many-fold immediate actions and effects of these hormones at blood and tissue level, including increases in cardiac output, oxygen uptake, and mobilization of energy substrates and disturbance of hydromineral balance. Tertiary responses extend to the level of the organism and population leading to inhibition of growth, reproduction, immune response and reduced capacity to tolerate subsequent or additional stressors. Of the three stages of stress, the primary and secondary stages are perhaps the easiest to monitor in the laboratory.

Changes brought about by a stressor could be metabolic in nature, affecting molecular and cellular components such as enzymes or impairing functions such as metabolism, immune response, osmoregulation, and hormonal regulation (Barton and Iwama, 1991). Biomarkers are defined as changes in biological responses (ranging from molecular through cellular and physiological responses to

behavioural responses) which can be related to exposure to or toxic effects of environmental chemicals (Peakall, 1994). Since the interaction between toxicants and biomolecules is the first step in the generation of toxic effects (preceding cellular and systemic dysfunction),the understanding of biochemical alterations induced by the exposure to pollutants may contribute to the prediction of toxic effects that may occur later at higher levels of biological organization. Moreover, the use of biochemical biomarkers may allow early interventions with the objective of protecting wild populations exposed to chemical agents (Newman, 1998).

Several studies have shown that changes in fish energy metabolism may occur to overcome toxic stress. In fact, under chemical stress (i.e. hypoxia due to intense exercise, excess of nutrients and organic matter) the attempt to enhance the supply of energy from anaerobic sources may be essential (Begum and Vijayaraghavan, 1999). Moreover, organic compounds that interfere with the aerobic metabolic pathway altering the mitochondrial structure and causing disturbances on enzymatic activities and metabolites (e.g. affecting the translocation of protons across the mitochondrial membrane, and consequently the cellular respiration) may also lead to impaired levels of energy metabolism (Nath, 2000). The response is characterized by a switch from an anabolic to a catabolic state, thereby providing the fish with the necessary resources to avoid or overcome the immediate threat, and has evolved as an adaptive response to short-term or acute, stresses. The exposure of fish to sub-lethal concentrations of contaminants can disturb homeostasis and impose considerable stress on physiological systems.

Biochemical constituents and certain enzymes have been explored as potential biomarkers for a variety of different organisms because these parameters are highly sensitive and conserved between species and are less variable. Their advantages are that biochemical and enzyme activities tend to be more sensitive, less variable, highly conserved between species, and often easier to measure as stress indices (Agrahari *et al*., 2007). Biomarkers using aquatic species are

important for detecting stressor components such as the presence of pollutants and changes in environmental factors. Enzyme activities are considered as sensitive biochemical indicators before hazardous effects occur in fish and are important parameters for testing water and the presence of toxicants. Such a biochemical approach has been advocated to provide an early warning of potentially damaging changes in stressed fish (Casillas *et al.,* 1983). Enzymes are attractive as indicators because they are more easily quantified than other indicators, such as changes in behaviour. The tissue specific response depends upon the metabolic requirements of the tissue in question. The analysis of marker enzymes such as lactate dehydrogenase, transaminases and phosphatases serve as specific indications of water-pollution-induced changes in the enzyme activity of fish.

Carbohydrates are generally used as energy supply particularly in cases of stress. It is well known that the sugars serve as energy reserve for the metabolic process. Carbohydrates are considered to be the first among the organic nutrients degraded in response to stress conditions imposed on an animal. Chemical stress causes rapid depletion of stored carbohydrates primarily in liver and other tissues (Jyothi and Narayan, 2000).

Cortisol, the principal glucocorticoid in teleosts, is secreted by the interrenal tissues (analogous to the adrenal cortex) dispersed in the head kidney region. The main secretagogue for cortisol is adrenocorticotropic hormone (ACTH) released from the anterior pituitary. ACTH release, in turn, is controlled by corticotropin releasing factor (CRF) produced by the hypothalamus (Pickering and Pottinger, 1995; Mommsen *et al*., 1999). Gills, intestine and liver are important targets for cortisol in fish. These organs reflect the two major actions of cortisol in fish: regulation of the hydromineral balance and energy metabolism. In this respect, cortisol combines actions in fish comparable to those of the mineralocorticoid aldosterone and the glucocorticoids in the terrestrial vertebrates. Accordingly, a role for cortisol in the control of several processes such as intermediary metabolism, ionic and osmotic regulation, growth, stress, and immune function

was repeatedly demonstrated in teleost fish (McCormick, 1995; Wendelaar-Bonga, 1997; Mommsen *et al*., 1999).

Cortisol and glucose have been consistent indicators of stressors such as handling, thermal shock and transportation. However, it has been shown that toxicants can impair the endocrine system (Hontela, 1997) and therefore affect the classical cortisol and glucose stress responses. It is a widely accepted fact that carbohydrate deposits in tissues like liver and muscle provide the immediate energy requirements in teleost fishes under different kinds of stress. The effects of the stress of environmental pollution on carbohydrate metabolism in fish tissues are not always proportionate to the toxicity of the pollutant and they probably depend on the type and degree of changes produced by the pollutant in other activities of the fish-both behavioural and metabolic.

One of the important functions of the liver and, to a lesser extent, of the kidney cortex is to provide glucose during conditions of starvation. Glucose is formed from gluconeogenic precursors in both tissues, and in the liver also from glycogen. Glucose- 6-phosphatase (G-6-Pase) is an enzyme which catalyses the reaction causing the hydrolysis of glucose- 6-phosphate formed either through glycolysis or gluconeogenesis, to glucose and phosphate in a characteristic manner. Since this enzyme plays a role in the final stage of gluconeogenesis, its physiological functions or properties merit attention. G-6-Pase thus plays a critical role in blood glucose homeostasis.

The lactate dehydrogenase (LDH) activity is a marker for tissue damage in fish (Ramesh *et al*., 1993), muscular harm (Balint *et al*., 1997) and hypoxic conditions (Das *et al*., 2004) and serves as a good diagnostic tool in toxicology.

Aminotransferases are widely acknowledged for their significance in protein metabolism by virtue of their ability to regulate both the synthesis and degradation of amino acids. Changes in their activities, whether induced by endogenous or exogenous factors, are often associated with changes in many other metabolic

functions and may thus represent widespread alterations in the organism's physiological state. Aminotransferases such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) catalyse the reactions of transamination of alanine, glutamic and aspartic acids. They couple the protein, carbohydrate and fat metabolism and tricarboxylic acid cycle under altered physiological, pathological and induced environmental stress conditions (Murugesan *et al*., 1999). AST activity is less specific for liver damage. Although both are gluconeogenic enzymes and both use pyridoxal phosphate as a coenzyme, the synthesis of ALT is more strongly inhibited by pyridoxine deficiency than the synthesis of AST. Extent of damage to mitochondria is another factor controlling AST activity (Diehl *et al*., 1986).

Alkaline phosphatase (ALP) is a brush border enzyme, which catalyses dephosphorylation of many molecules including nucleotides, proteins and alkaloids at alkaline pH. It is well known that phosphatases are involved in carbohydrate metabolism, growth and differentiation, protein synthesis, synthesis of certain enzymes, secretory activity and transport of phosphorylated intermediates across the cell membranes. Hydrolysis of phosphoesters, phosphate transferase activity, protein phosphatase activity, phosphate transport, modulation of organic cation transport and involvement in cell proliferation have been suggested as possible functions of ALP (Sarrouilhe *et al*., 1992).

Acid phosphatase (ACP) is a lysosomal enzyme that hydrolyses the phospho-esters in acidic medium. Inhibition and induction of these biomarkers is a good approach to measure potential impacts of environmental pollutants on organisms (El-Shehawi *et al*., 2007).

Glutamate dehydrogenase is a mitochondrial enzyme containing zinc. It catalyses the oxidative deamination of L-glutamic acid to α-ketoglutarate, a member of the TCA cycle.

Several reasons prompted an examination of the neuroendocrine and biochemical stress responses of *O. mossambicus* exposed chronically to different

phenolic compounds. First, the paradigm of the neuroendocrine stress response is well documented in teleosts, and generally yields a consistent pattern for xenobiotic stressors. Second, fish are exposed to dissolved pollutants via an extensive respiratory surface. The high bioavailability of many chemicals in water, in combination with a variety of highly sensitive perceptive mechanisms in the integument, typically generates an integrated stress response in fish in addition to toxic effects. The ability of fish to mount an appropriate stress response, and the negative consequences associated with chronic stress is of both evolutionary and ecological significance. Thus, the objective of this work was to detect alterations in energy metabolism of *O. mossambicus* induced by the exposure to phenolic compounds (1/10 of LC-$_{50}$/96 h) to gain a better understanding of the organismal effects of these chemicals and the adaptive metabolic responses of this species.

2.2 Materials and methods

2.2.1 Phenolic compounds used for the study

Analar monohydric phenol (C_6H_5OH, MW-94.11) and m-cresol ($CH_3C_6H_4OH$, MW -108.14) purchased from Sisco Research Laboratories (SRL), India were used. The nominal concentrations needed were prepared from fresh stock solutions.

(i) Chemical and physical properties of phenol

Systematic name - Phenol

Molecular formula- C_6H_5OH

Molecular weight (g/mol) - 94.11

Melting point- 40.9°C

Boiling point- 181.7°C

p*K*a = 9.89

(ii) Chemical and physical properties of m-cresol

Systematic name- 3-methylphenol

Molecular formula- C_7H_8O

Molecular weight (g/mol) - 108.14

Melting point- 11.8°C

Boiling point- 202°C

p*K*a =10.99

2.2.2 Experimental animal

Systemic position of the experimental animal, *Oreochromis mossambicus* employed in this study is as follows (Fig 2.1).

Fig 2.1 *Oreochromis mossambicus*

Kingdom	:	Animalia
Phylum	:	Chordata
Class	:	Teleostomi
Order	:	Perciformes
Family	:	Cichilidae
Genus	:	*Oreochromis*
Species	:	*mossambicus*
Common name	:	Tilapia

2.2.3 Experimental design

(i) Collection and maintenance of test fish

O. mossambicus (15±3g) were collected from the culture ponds of Kerala Agricultural University, Puduvypu and brought to the laboratory in large aerated tanks. In the laboratory, they were kept in large tanks where a continuous and gentle flow of tap water was maintained. The tap water had dissolved oxygen content of 7.8 ppm, hardness below detectable amounts, pH 7.0 ± 0.37, temperature 26 ± 3^0C and salinity 0 ppt (parts per thousand). They were fed on a commercial diet *ad libitum* and were acclimated in tanks for a month before the experiment.

(ii) Experimental design for lethal toxicity study

LC_{50} determination was carried out by following semi-static acute toxicity test. For the experiment, 6 fishes were transferred to large experimental tubs, each containing 18 litres of dechlorinated tap water. Eight phenol concentrations from 27 mg l^{-1} (no mortality) to 34 mg l^{-1} (100 % mortality) were chosen for the final 96-hour test to determine the 50 % lethal concentration (LC_{50}). For m-cresol eight concentrations from 19 mg l^{-1} (no mortality) to 26 mg l^{-1} (100 % mortality) were chosen for the final 96-h test to determine the 50 % lethal concentration (LC_{50}). Fishes transferred to tanks containing no toxicants served as control. Water in the control tanks and water and toxicant in the experimental tanks were renewed daily to remove the debris, taking care to give minimum disturbance to the fish. The fishes were not fed during the entire exposure period. Fishes were checked for mortality at every 24 hours interval.

The LC_{50} levels and 95% confidence limits were calculated using Probit analysis (Finney, 1971). The lethal toxicity experiments were repeated wherever necessary.

(iii) Experimental design for sub-lethal toxicity studies

For conducting the biochemical study, *O. mossambicus* (15 ± 3g) were taken in two separate tanks which contained desired concentrations of toxin, $1/10^{th}$

of LC_{50} value of phenol and m-cresol. Six replicates were kept for each experiment. The experimental animals were dosed for 21 days. Daily the contents in the tanks were replaced with the same concentrations of toxicant so as to avoid any possible degradation of constituents of toxicant. During the experimental period of 21 days the animals were fed on the same diet so as to avoid the effects of starvation on normal physiological processes. Any other factors likely to influence the toxicity were nullified by maintaining suitable controls in tanks that contained no toxicant.

2.2.4 Preparation of tissue samples for the study.

After the experimental period (21days) the fishes were killed by pithing (by damaging the brain and severing the spinal cord between the head and trunk region using a sharp needle) and the tissues such as liver, gills, kidney and muscle were removed from its body, wiped thoroughly, using blotting paper to remove blood and other body fluids. Then they were washed in ice cold 0.33 M sucrose and again blotted dry and the desired amounts of the tissue were weighed and used.

2.2.5 Preparation of serum samples

Blood was drawn from the common cardinal vein using 1 ml syringe. The blood collected was then kept at room temperature for 30 minutes to separate the serum. The serum thus obtained was then subjected to centrifugation at 3000 rpm for 3 minutes. The serum separated was then stored at -20°C until assayed.

2.2.6 Parameters investigated

2.2.6.1 Estimation of serum cortisol

The level of serum cortisol was estimated by electrochemiluminescence immunoassay (ECLIA).

Reagents

Elecsys cortisol reagent kit, cat. No. 11875116 was used for the assay.

(a) Streptavidin coated microparticles, 0.72 mg/ml; binding capacity: 470 ng biotin/mg particles.

(b) Anti-cortisol-Ab-biotin, Biotinylated polyclonal anti-cortisol antibody (ovine) 90 ng/ml; MES buffer 100mmol/L pH 6.

(c) Cortisol derivative (Synthetic) labelled with ruthenium complex 25 ng/ml; danazol 20 µg/ml; MES buffer 100 mmol/L, pH 6.

Procedure

To 20µl of serum sample, cortisol-specific biotinylated and a ruthenium complex labelled cortisol derivative were added. It was incubated at 37°C for 9 minutes. Streptavidin coated microparticles were added and was incubated at 37°C for 9 minutes. This forms complex which gets bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture was then aspirated into the measuring cell where the microparticles were magnetically captured onto the surface of the electrode. Unbound substances were then removed with ProCell. Voltage was applied to the electrode which induced chemiluminescent emission. It was measured by a photomultiplier. Results thus obtained were determined via a calibration curve which was instrument specific generated by a 2-point calibration and a master curve provided via the reagent barcode. The results obtained were then expressed as µg/dl.

2.2.6.2 Estimation of total carbohydrate

Total carbohydrate was estimated by the method of Carrol *et al.* (1956).

Reagents

(a) TCA: 10%.

(b) Anthrone Reagent: Dissolved 200 mg anthrone in 100 ml ice-cold 95 % H_2SO4.

(c) Glucose (stock) standard: 100 mg of glucose was dissolved in 100 ml distilled water.

(d) Working standard: 10 ml of the stock was diluted to 100 ml with distilled water.

Procedure

0.5% homogenate of liver and 10% homogenate of gills, kidney and muscle tissues were prepared in 10% TCA and centrifuged at 1000 × g for 15 minutes. To 0.2 ml supernatant, 5 ml of anthrone reagent was added and boiled for 15 minutes. The tubes were cooled and the absorbance was read at 620 nm in a spectrophotometer against a reagent blank. The standards were also treated similarly. The values were expressed as mg of glucose / g wet wt. of tissue.

2.2.6.3 Assay of Glucose 6-phosphatase (EC 3.1.3.9)

Glucose 6-phosphatase was assayed according to the method of King (1965 b).

Reagents

(a) Citrate Buffer: 0.1 M, pH 6.5.

(b) Substrate: Glucose-6-phosphate, 0.1 M in distilled water.

(c) Ammonium molybdate reagent: Added 25 g of ammonium molybdate to 200 ml distilled water. To 300 ml 10 N H_2SO_4, added molybdate solution and diluted to 1 litre with distilled water.

(d) Amino naphthol sulphonic acid (ANSA): Ground 0.2 g of ANSA with 1.2 g of Na_2SO_3 and 1.2 g of sodium bisulphite ($NaHSO_3$). Kept the mixture in the freezer. At the time of use, dissolved 0.25 g in 10 ml distilled water.

(e) TCA: 10%.

(f) Phosphorus standard: 35.1 mg of KH_2PO_4 was dissolved in 100 ml double distilled water. Working standard was prepared by taking 1ml of the stock and diluted to 10 ml with double distilled water.

Procedure

10 % homogenate of liver tissue was prepared in 0.33 M sucrose solution and centrifuged at 11,000×g for 30 minutes in a refrigerated centrifuge. The supernatant

obtained was again centrifuged for 60 minutes at 10,500×g and the supernatant was discarded. The pellet was suspended in ice-cold 0.33 M sucrose solution and homogenized in a glass-Teflon homogenizer. The homogenate obtained was used as the enzyme source. The incubation mixture in a total volume of 1ml contained 0.3 ml of buffer, 0.5 ml of substrate and 0.2 ml of enzyme preparation. The incubation was carried out at 37°C for 60 minutes. Arrested the reaction by the addition of 1 ml of 10% TCA and centrifuged. The phosphorus content of the supernatant was estimated by the method of Fiske and Subbarow (1925).The enzyme activity was expressed as µg of inorganic phosphorus liberated / min / mg protein.

2.2.6.4 Estimation of Blood Glucose

Blood Glucose was estimated by the method of Sasaki *et al*. (1972)

Reagents

(a) Ortho toluidine boric acid reagent: This reagent consists of 2.5 g of thiourea and 2.4 g of boric acid in 100 ml solvent, consisting of a mixture of water, acetic acid (AR) and ortho toluidine in the ratio of 10:75:15.

(b) Standard glucose: 100 mg of glucose was dissolved in 0.1% benzoic acid. 10 ml of the above solution was diluted to 100 ml to give 100 µg of glucose per ml.

Procedure

To 0.2 ml of blood, 0.8 ml of 10 % TCA was added. The contents were mixed well. The tubes were centrifuged at 1000×g for 5 minutes. 0.5 ml of supernatant was taken. To this 2.0 ml of ortho toluidine reagent was added. The tubes were then heated in a boiling water bath for 15 minutes. The standards were also treated in the same manner along with the reagent blank. The values were expressed as mg glucose / dl.

2.2.6.5 Assay of Lactate Dehydrogenase (LDH) (EC 1.1.1.27)

Lactate Dehydrogenase was assayed according to the method of King (1965a).

Reagents

(a) 0.1M glycine buffer

(b) Buffered substrate: Dissolved 2.76 g of lithium lactate in 125 ml of glycine buffer containing 75 ml of 0.1 N NaOH to adjust the pH to 10. Prepared this just prior to use.

(c) 0.4 N NaOH.

(d) Dissolve 5.0 mg of NAD^+ in 1.0 ml of distilled water. Prepared this just before use.

(e) 2, 4- Dinitrophenyl hydrazine (DNPH) reagent: Dissolved 200 mg of DNPH in 85 ml of concentrated HCl and made up to 1litre with distilled water.

(f) Standard pyruvate solution: Dissolved 11.01 mg of sodium pyruvate in 10 ml of distilled water. Diluted this further to 100 ml with distilled water and prepared fresh each time.

Procedure

To 1.0 ml of the buffered substrate, added 0.2 ml of sample and incubated at 37° C for 15 minutes. After adding 0.2 ml of NAD^+ solution, continued the incubation for another 30 minutes and then added 1.0 ml of DNPH reagent. Incubated the mixture for a period of 15 minutes at 37°C. Then added 7.0 ml of 0.4 N NaOH solution and measured the colour developed at 520 nm in a spectrophotometer. Treated the standards also in the same manner along with blank. The enzyme activity was expressed as μ moles of pyruvate liberated / h / mg protein.

2.2.6.6 Estimation of Pyruvate

Pyruvate was estimated by the method of Friedman and Haugen (1943).

Reagents

(a) TCA: 10%.

(b) 2,4- Dinitrophenyl hydrazine reagent(DNPH): 0.2 %.

(c) 2.5 N NaOH.

(d) Pyruvate standard: Dissolved 125 mg of sodium pyruvate in 10 ml of 0.1 N H_2SO_4 and diluted to 100ml with 0.1 N H_2SO_4 .

Procedure

5% homogenate of liver and 10% homogenate of gills, kidney and muscle tissues were prepared in 10% TCA and centrifuged at 1000× g for 15 minutes. To 2.0 ml of supernatant, 0.5 ml of 0.1% 2, 4-DNPH reagent was added and the tubes were kept at room temperature for 5 minutes and 3.0 ml of 2.5 N NaOH solution was added. After 10 minutes the absorbance was read at 540 nm in a spectrophotometer against a reagent blank. The blank consisted of 2.0 ml of 10% TCA, 0.5 ml of 0.1% 2, 4-DNPH and 3.0 ml of 2.5 N NaOH solutions. Treated the standards also in the same manner. The values were expressed as μ moles of pyruvate / g wet wt. of tissue.

2.2.6.7 Assay of Alanine aminotransferase (ALT) (EC 2.6.1.2)

Alanine aminotransferase was assayed by the method of Mohun and Cook (1957).

Reagents

(a) Buffered substrate (0.1 M phosphate buffer, pH 7.4; 0.2 M DL- alanine; 2mM 2-oxoglutarate).

(b) 2, 4 – Dinitro phenyl hydrazine (DNPH).

(c) 0.4 N NaOH.

(d) Standard pyruvate : Dissolved 11.01 mg of sodium pyruvate in 100 ml of distilled water.

(e) 0.33 M Sucrose.

Procedure

10% homogenate of gills, liver, heart, kidney and muscle were prepared in 0.33 M sucrose solution and centrifuged at 1000× g for 15 minutes. The

supernatant obtained was used as the enzyme source. Pipetted out 1ml buffered substrate into 'test' and 'control'. Added 0.2 ml of the enzyme source into the 'test' and incubated the tubes at 37 ° C for 60 minutes. After incubation, 0.2 ml enzyme was added to the control. 1ml of 2, 4 – DNPH reagent was added and kept at room temperature for 20 minutes. The reaction was stopped by the addition of 10 ml of 0.4 N NaOH, vortexed and kept at room temperature for 5 minutes. The absorbance was measured at 540 nm in a spectrophotometer against a reagent blank. The ALT activities were expressed as µ moles of pyruvate liberated / h / mg protein.

2.2.6.8 Assay of Aspartate aminotransferase (AST) (EC 2.6.1.1)

Aspartate aminotransferase was assayed by the method of Mohun and Cook, 1957.

Reagents

(a) Buffered substrate (0.1 M phosphate buffer, pH 7.4; 1.0 M aspartic acid; 2mM 2-oxoglutarate).

(b) 2, 4 – Dinitro phenyl hydrazine (DNPH)

(c) 0.4 N NaOH

(d) Standard pyruvate : Dissolved 11.01 mg of sodium pyruvate in 10 ml of distilled water. Dilute this further to 100 ml with distilled water and prepare fresh each time.

(e) 0.33 M Sucrose.

Procedure

10% homogenate of gills, liver, heart, kidney and muscle were prepared in 0.33 M sucrose solution and centrifuged at 1000× g for 15 minutes. The supernatant obtained was used as the enzyme source. Pipetted out 1ml buffered substrate into 'test' and 'control'. Added 0.2 ml of the enzyme source into the 'test' and incubated the tubes at 37 °C for 60 minutes. After incubation, 0.2 ml enzyme was added to the control. 1ml of 2, 4 – DNPH reagent was added and kept

at room temperature for 20 minutes. The reaction was stopped by the addition of 10 ml of 0.4 N NaOH, vortexed and kept at room temperature for 5 minutes. The absorbance was measured at 540 nm in a spectrophotometer against a reagent blank. The AST activities were expressed as µ moles of pyruvate liberated / h / mg protein.

2.2.6.9 Assay of Alkaline phosphatase (ALP) (EC 3.1.3.1)

Alkaline phosphatase was assayed by the method of King and King (1954).

Reagents

(a) Substrate: Disodium phenyl phosphate (10 mmol/L).

(b) Buffer: Sodium carbonate – bicarbonate buffer (100 mmol/L).

(c) Buffered substrate: Mixed equal volumes of substrate and buffer, this had a pH of 10.

(d) Stock phenol standard: 100 mg% in 0.1 N HCl. Working standard: 1mg %

(e) Sodium Hydroxide (NaOH): 0.5 N.

(f) Sodium Bicarbonate ($NaHCO_3$): 0.5 N.

(g) 4 – Aminoantipyrine: 6g/L in water.

(h) Potassium ferricyanide: 24g/L in water.

Procedure

10% homogenate of gills, liver, heart, kidney and muscle were prepared in 0.33 M sucrose solution and centrifuged at 1000×g for 15 minutes. The supernatant obtained was used as the enzyme source. Pipetted out 1ml buffered substrate into 'test' and 'control'.and incubated for a few minutes at 37 $^{\circ}$ C. Then added 50µl of enzyme source to the 'test'. Again incubated at 37°C for 15 minutes. Added 0.8 ml of NaOH and 1.2 ml of $NaHCO_3$ to both the tubes. Then added 50µl of enzyme source to the 'control'. This was followed by the addition of 1 ml of 4 – aminoantipyrine and 1 ml of potassium ferricyanide to both the tubes. Read the

absorbance at 520 nm. Treat the blank and standards also similarly. The values were expressed as mg of phenol liberated /min / mg protein.

2.2.6.10 Assay of serum Acid Phosphatase (ACP) (EC 3.1.3.2)

Acid Phosphatase was assayed by the method of King (1965).

Reagents

(a) Citrate buffer: 0.1M, pH 4.9. (mix 37.5 ml 0.1 M citric acid with 62.5 ml of 0.1 M trisodium citrate and adjust the pH to 4.9.

(b) Substrate: 0.01 M disodium phenyl phosphate solution.

(c) Folin -Ciocalteu Phenol reagent: Diluted with distilled water in a ratio of 1:10.

(d) 15% sodium carbonate in water.

(e) Standard of phenol: 1g pure phenol in 100 mmol / l HCl.

Procedure

Mixed 1.5 ml of citrate buffer, 1.0 ml of substrate and 0.2 ml of serum together. Incubated the reaction mixture at 37°C for 15 minutes. Terminated the reaction by the addition of 1.0 ml of Folin & Ciocalteu's reagent. Incubated the controls without enzyme source and added the enzyme source after the addition of Folin –Ciocalteu Phenol reagent. Then added 1.0 ml of 15% sodium carbonate solution and incubated for a further 10 minutes at 37 ° C. Read the blue colour developed at 640 nm against a blank. A set of graded volumes of phenol standards were also run simultanously. The activity of the enzyme was expressed as mg of phenol liberated / min / mg protein.

2.2.6.11 Assay of Glutamate dehydrogenase (GDH) (E.C.1.4.1.3)

Glutamate dehydrogenase was assayed by the method of Plummer (1995).

Reagents

(a) Sodium phosphate buffer (0.1 M, pH 7.4).

(b) 2-oxoglutarate (0.15 M), prepared in phosphate buffer and pH adjusted to 7.4.

(c) Ammonium acetate (0.75 M), prepared in phosphate buffer and pH adjusted to 7.4.

(d) EDTA (30mM), prepared in phosphate buffer and pH adjusted to 7.4.

(e) NADH (2.5 mg/ml in phosphate buffer, prepared fresh).

(f) Triton X-100.

Procedure

10% homogenate of gills and 5% homogenate of liver, kidney and muscle were prepared in 0.33 M sucrose solution and centrifuged at 1000×g for 15 minutes. The supernatant obtained was used as the enzyme source. The reaction mixture consisted of 2.1 ml phosphate buffer, 0.2 ml enzyme source, 0.1 ml NADH, 0.2 ml Ammonium acetate, 0.2 ml EDTA and 0.1 ml Triton X-100. The above mixture was equilibrated at room temperature for 10 minutes. Started the reaction by adding 0.1 ml of 2-oxoglutarate and the rate of change of extinction at 340 nm with time were noted. Molar Extinction Coefficient of NADH is 6.3×10^3 litres mol^{-1} cm^{-1}. The enzyme activity was calculated as micromoles of NADH oxidized / minute / mg protein.

2.2.6. 11 Estimation of Protein

Protein was estimated by the method of Lowry *et al*. (1951).

Reagents

(a) Alkaline Copper Reagent.

Reagent A: 2% Na_2CO_3 in 0.1 N NaOH.

Reagent B: 0.5% $CuSO_4 .5H_2O$ in sodium potassium tartrate.

The alkaline copper reagent was made by mixing 50 ml of reagent A and 1 ml of reagent B.

(b) Folin- Ciocalteu Phenol Reagent

Folin- Ciocalteu Phenol Reagent is commercially available which is diluted with distilled water in the ratio 1:2.

(c) 0.1 N NaOH

(d) 10 % TCA

(e) Protein (stock) standard solution: 100 mg % Bovine serum albumin in 0.1 N NaOH.

Working standard: 10 ml of the stock was diluted to 100 ml with distilled water.

Procedure

Pipetted out 0.2 ml of tissue homogenate to the test tube and added 1 ml of 10 % TCA. The tubes were centrifuged at 5000 × g for 10 minutes. The supernatant was discarded and the precipitate was dissolved in 1 ml of 0.1 N NaOH. Added 5 ml of alkaline copper reagent and kept for 10 minutes at room temperature. After 10 minutes added 0.5 ml Folin- Ciocalteu Phenol Reagent and kept in dark for 30 minutes. The absorbance was read at 620 nm against a reagent blank. A set of graded volumes of protein standard were also run simultaneously. The values are expressed as mg of protein / g wet wt. of tissue.

2.2.7 Statistical Analysis

The statistical analysis was carried out using the software SPSS 13.0 package. Two-way analysis of variance (ANOVA) was carried out to compare between phenolic compound treated groups and also between tissues. If significant differences were revealed by the ANOVA test, Tukey's test was used to further elucidate which tissues and treatments were significantly different. One-way ANOVA followed by Tukey's test was also carried out for the comparison between different treatments in each tissue. For determining the significant difference between different treatments in blood and serum parameters one-way ANOVA followed by Tukey's test was done. Significance level (P value) was set at 0.05 in all tests.

2.3 Results

2.3.1 Lethal Toxicity Study

The calculated LC_{50} value for phenol and m-cresol exposure in *O. mossambicus* over periods of up to 96 hours is 31.25 mg l^{-1} and 22.2 mg l^{-1} respectively. The LC_{50} levels and 95% confidence limits were calculated using Probit analysis (Finney, 1971). The results are presented in table 2.1.

Table 2.1 96 h LC_{50} value for phenol and m-cresol in *O. mossambicus.*

Phenolic compound	**Acute Toxicity Range (mg l^{-1}) 95% confidence limit**		**Median LC_{50}(mg l^{-1})**
Phenol	31.59	30.91	31.25
m-Cresol	22.43	22	22.2

2.3.2 Serum cortisol

One-way ANOVA followed by Tukey's test showed that there was significant decrease in cortisol ($P<0.05$) in both the treated groups compared to control (Fig 2.1 and Table 2.1). Among the treated groups phenol treated group showed the least cortisol level.

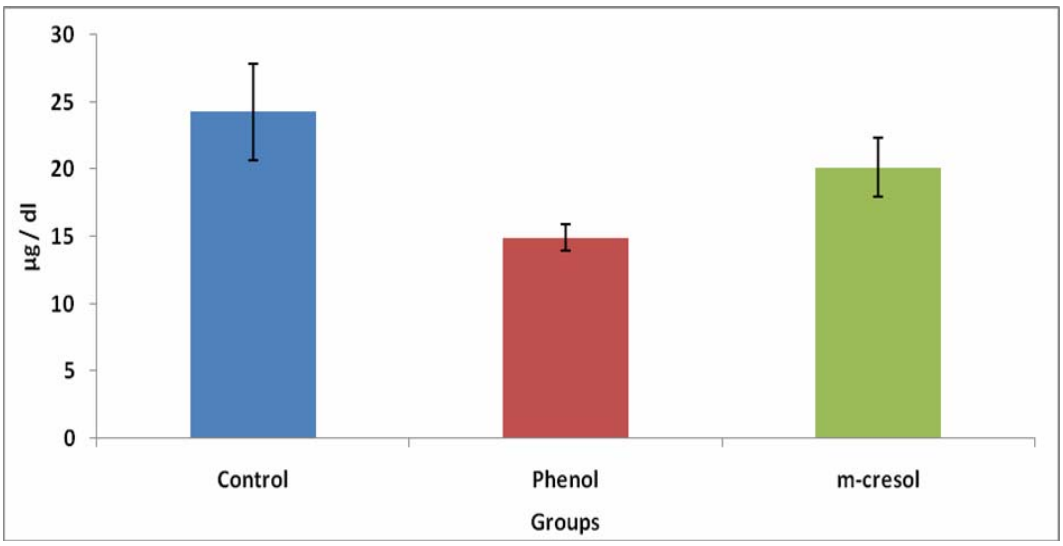

Fig 2.2. Effect of different phenolic compounds on cortisol in *O. mossambicus.*

Table 2.2 Effect of different phenolic compounds on cortisol in *O. mossambicus.* Values in the same row with different upper case letters vary significantly (P<0.05) between treatment groups.

Cortisol level	Control	Phenol	m-cresol
	24.28^{C}	14.92^{A}	20.14^{B}

- Values are expressed as µg / dl.
- Each value represents the mean ± S.D of six separate experiments.

2.3.3 Total carbohydrate

In the present study total carbohydrate in different tissues of *O. mossambicus* treated with different phenolic compounds showed significant variations (P<0.05) compared to control group (Fig 2.2 and Table 2.2). A statistically significant decrease in total carbohydrate (P<0.05) was observed in liver and muscle of both the treated groups compared to control. Among the tissues kidney showed a statistically significant elevated carbohydrate level (P<0.05) in both the treated groups compared to control. No significant variation was observed in gills of both the treated groups compared to control.

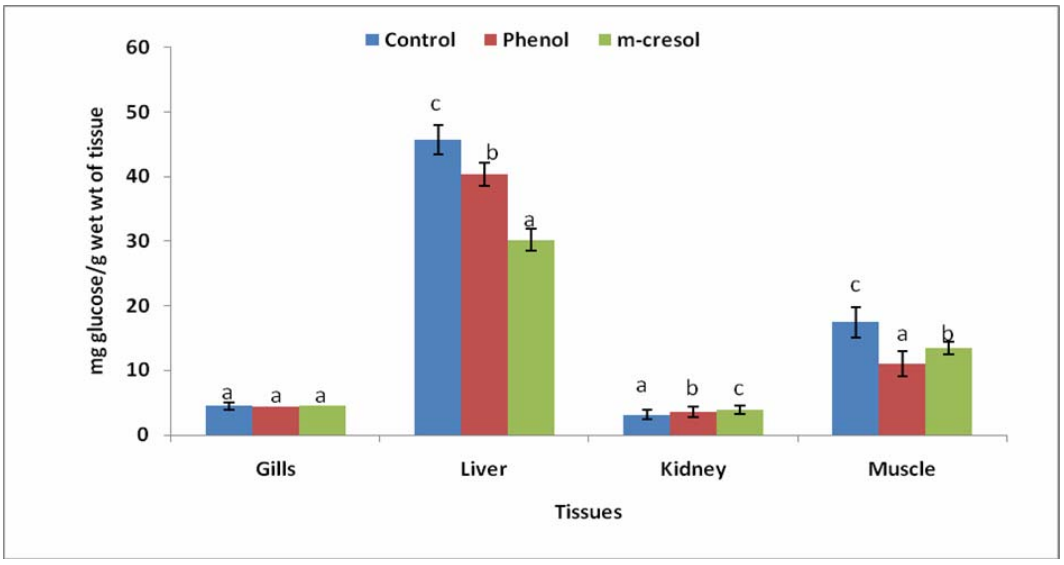

Fig. 2.3 Effect of different phenolic compounds on total carbohydrate in *O. mossambicus.* Each bar diagram represents mean ± S.D. On each set of bars, values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA).

Table 2.3 Effect of different phenolic compounds on total carbohydrate (mean ± S.D) in *O. mossambicus.* Values in the same column with different upper case letters vary significantly (P<0.05) between tissues and values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups (Two-way ANOVA).

Tissues	Total carbohydrate		
	Groups		
	Control	Phenol	m-cresol
Gills	$_{c}4.46 \pm 0.49^{B}$	$_{b}4.34 \pm 0.01^{B}$	$_{a}4.48 \pm 0.02^{B}$
Liver	$_{c}45.66 \pm 2.23^{D}$	$_{b}40.30 \pm 1.78^{D}$	$_{a}30.22 \pm 1.76^{D}$
Kidney	$_{c}3.11 \pm 0.70^{A}$	$_{b}3.62 \pm 0.81^{A}$	$_{a}3.81 \pm 0.64^{A}$
Muscle	$_{c}17.45 \pm 2.39^{C}$	$_{b}11.04 \pm 1.91^{C}$	$_{a}13.4 \pm 0.96^{C}$

- Values are expressed as mg / g wet wt of tissue.
- Each value represents the mean ± S.D of six separate experiments.

2.3.4 Glucose-6-phosphatase

One-way ANOVA followed by Tukey's test showed that there was significant decrease (P<0.05) in glucose-6-phosphatase activity in both the treated groups compared to control (Fig 2.3 and Table 2.3). Among the treated groups phenol treated group showed the least activity.

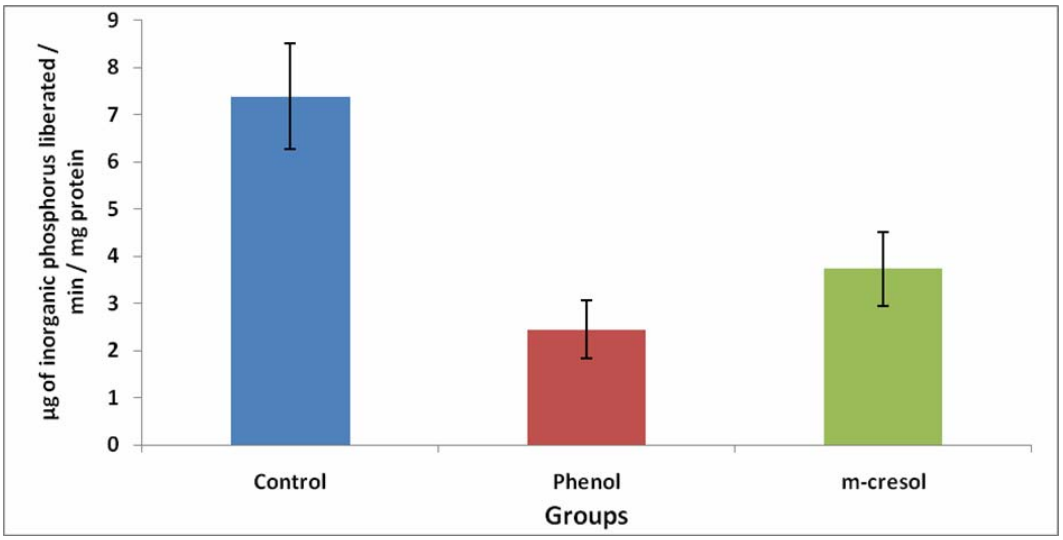

Fig 2.4 Effect of different phenolic compounds on glucose-6-phosphatase activity in *O. mossambicus.*

Table 2.4 Effect of different phenolic compounds on glucose-6-phosphatase activity (mean ± S.D) in *O. mossambicus.*

Groups	Control	Phenol	m-cresol
Glucose-6-phosphatase activity	7.38 ± 1.11^{C}	2.44 ± 0.60^{A}	3.72 ± 0.77^{B}

- Values are expressed as µg of inorganic phosphorus liberated/min/mg protein.
- Values in the same row with different upper case letters vary significantly ($P<0.05$) between treatment groups.
- Each value represents the mean ± S.D of six separate experiments.

2.3.5 Blood glucose

One-way ANOVA followed by Tukey's test showed that there was significant decrease in blood glucose ($P<0.05$) in both the treated groups compared to control (Fig 2.4 and Table 2.4).

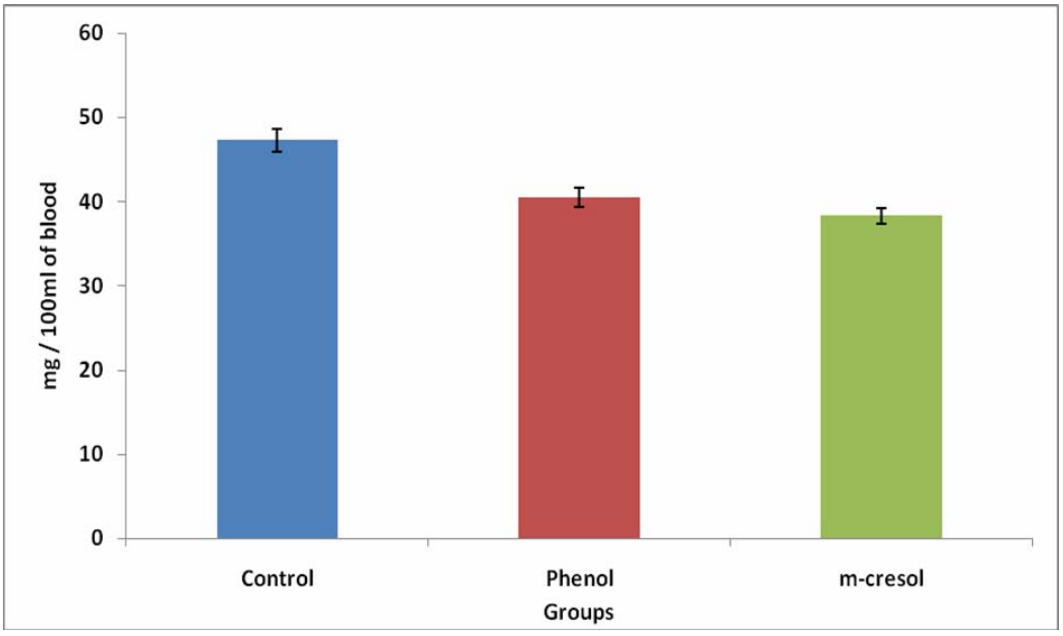

Fig 2.5 Effect of different phenolic compounds on blood glucose level in *O. mossambicus.*

Table 2.5 Effect of different phenolic compounds on blood glucose level (mean ± S.D) in *O. mossambicus.*

Groups	Control	Phenol	m-cresol
Blood glucose level	47.30 ± 1.32^{C}	40.50 ± 1.1^{B}	38.29 ± 0.95^{A}

- Values are expressed as mg/dl.
- Values in the same row with different upper case letters vary significantly ($P<0.05$) between treatment groups.
- Each value represents the mean ± S.D of six separate experiments.

2.3.6 Pyruvate

Two-factor ANOVA followed by Tukey's test showed that there was significant variation ($P<0.05$) in pyruvate level between treatments and also between tissues (Fig 2.5 and Table 2.5). Gills, liver and kidney of both the treated groups showed a significantly increased pyruvate level ($P<0.05$) compared to control. In both the treated groups muscle showed a significantly decreased pyruvate level ($P<0.05$) compared to control.

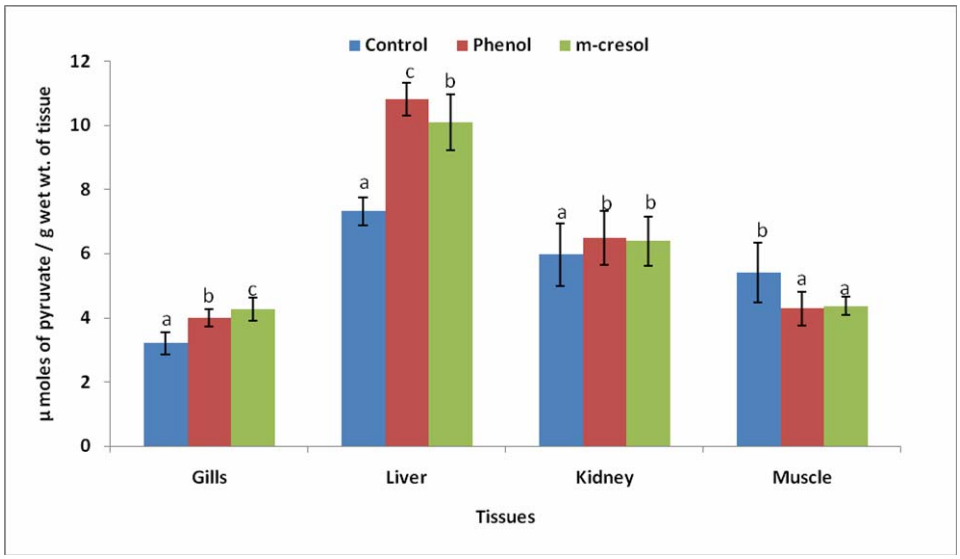

Fig 2.6 Effect of different phenolic compounds on level of pyruvate in *O. mossambicus.* Each bar diagram represents mean ± S.D. On each set of bars, values with different lower case letters vary significantly ($P<0.05$) in each tissue on different treatments (One-way ANOVA).

Table 2.6 Effect of different phenolic compounds on level of pyruvate (mean ± S.D) in *O. mossambicus.* Values in the same column with different upper case letters vary significantly (P<0.05) between tissues and values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups.

Tissues	Pyruvate		
	Groups		
	Control	Phenol	m-cresol
Gills	$_{a}3.19 \pm 0.34^{A}$	$_{c}3.98 \pm 0.27^{A}$	$_{b}4.27 \pm 0.36^{A}$
Liver	$_{a}7.31 \pm 0.42^{D}$	$_{c}10.81 \pm 0.50^{D}$	$_{b}10.08 \pm 0.86^{D}$
Kidney	$_{a}5.95 \pm 0.98^{C}$	$_{c}6.49 \pm 0.84^{C}$	$_{b}6.38 \pm 0.77^{C}$
Muscle	$_{a}5.39 \pm 0.93^{B}$	$_{c}4.27 \pm 0.53^{B}$	$_{b}4.35 \pm 0.28^{B}$

- Values are expressed as µmoles of pyruvate / g wet wt of tissue.
- Each value represents the mean ± S.D of six separate experiments.

2.3.7 Lactate dehydrogenase

LDH activity in different tissues of *O. mossambicus* treated with different phenolic compounds showed significant variations (P<0.05) compared to control (Fig 2.6 and Table 2.6). In the phenol treated group, tissues such as liver, kidney and muscle showed significantly elevated activity (P<0.05) compared to control. Among the tissues of m-cresol treated group the gills and muscle showed a significantly elevated activity (P<0.05) and the liver and kidney showed a significantly decreased activity (P<0.05) compared to control.

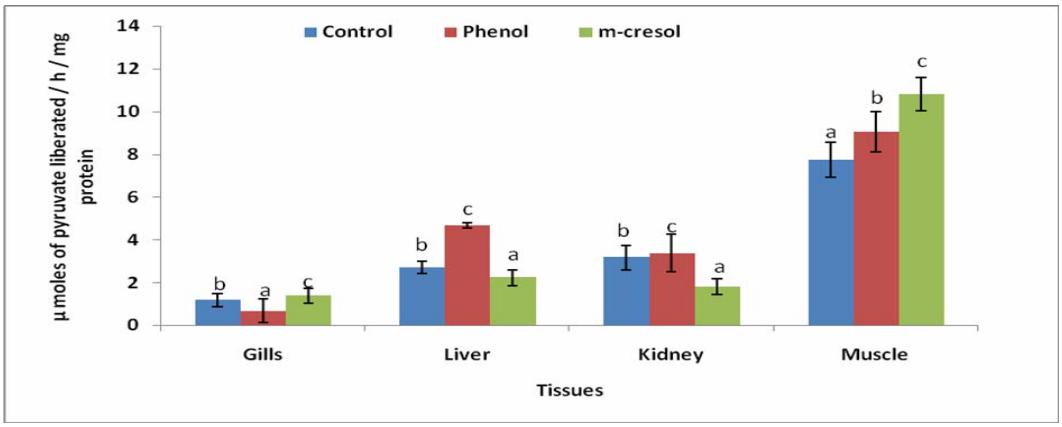

Fig 2.7 Effect of different phenolic compounds on lactate dehydrogenase activity in *O. mossambicus.* Each bar diagram represents mean ± S.D. On each set of bars, values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA).

Table 2.7 Effect of different phenolic compounds on lactate dehydrogenase activity (mean ± S.D) in *O. mossambicus.* Values in the same column with different upper case letters vary significantly (P<0.05) between tissues and values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups.

Tissues	LDH activity		
	Groups		
	Control	Phenol	m-cresol
Gills	$_{a}1.16 \pm 0.31^{A}$	$_{c}0.67 \pm 0.55^{A}$	$_{b}1.37 \pm 0.33^{A}$
Liver	$_{a}2.71 \pm 0.27^{C}$	$_{c}4.68 \pm 0.13^{C}$	$_{b}2.23 \pm 0.37^{C}$
Kidney	$_{a}3.17 \pm 0.57^{B}$	$_{c}3.37 \pm 0.87^{B}$	$_{b}1.81 \pm 0.35^{B}$
Muscle	$_{a}7.74 \pm 0.82^{D}$	$_{c}9.04 \pm 0.92^{D}$	$_{b}10.81 \pm 0.79^{D}$

- Values are expressed as µmoles of pyruvate liberated / h / mg protein.
- Each value represents the mean ± S.D of six separate experiments.

2.3.8 Alanine aminotransferase

Two-factor ANOVA followed by Tukeys test showed that there was significant elevation in ALT activity (P<0.05), in both the treated groups compared to control (Fig 2.7 and Table 2.7). Liver and kidney of phenol treated group showed significantly elevated activity (P<0.05) compared to m-cresol treated group. Gills and muscle of m-cresol treated group showed significantly elevated activity compared to phenol treated group.

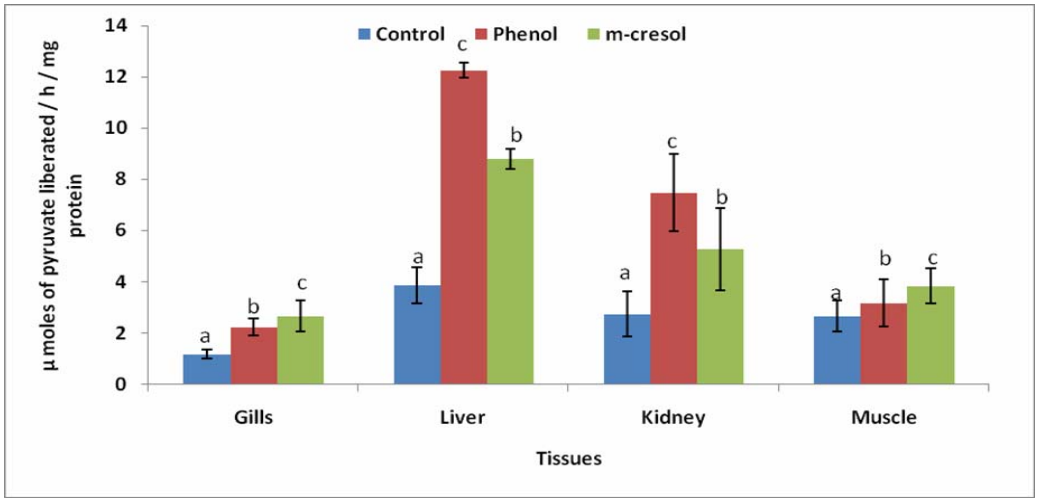

Fig 2.8 Effect of different phenolic compounds on ALT activity in *O. mossambicus.* Each bar diagram represents mean ± S.D. On each set of bars, values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA).

Table 2.8 Effect of different phenolic compounds on ALT activity (mean ± S.D) in *O. mossambicus.* Values in the same column with different upper case letters vary significantly (P<0.05) between tissues and values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups.

Tissues	ALT activity		
	Groups		
	Control	Phenol	m-cresol
Gills	$_{a}1.18 \pm 0.16^{A}$	$_{c}2.24 \pm 0.32^{A}$	$_{b}2.66 \pm 0.61^{A}$
Liver	$_{a}3.85 \pm 0.71^{D}$	$_{c}12.24 \pm 0.28^{D}$	$_{b}8.79 \pm 0.38^{D}$
Kidney	$_{a}2.74 \pm 0.87^{C}$	$_{c}7.48 \pm 1.51^{C}$	$_{b}5.27 \pm 1.61^{C}$
Muscle	$_{a}2.66 \pm 0.60^{B}$	$_{c}3.17 \pm 0.92^{B}$	$_{b}3.84 \pm 0.68^{B}$

- Values are expressed as µmoles of pyruvate liberated / h / mg protein.
- Each value represents the mean ± S.D of six separate experiments.

2.3.9 Aspartate aminotransferase

AST activity was found to be significantly elevated (P<0.05) in tissues such as liver, kidney and muscle of both the treated groups compared to control (Fig 2.8 and Table 2.8). In the phenol treated group liver and kidney showed significantly elevated AST activity (P<0.05) compared to m-cresol treated group. Gills of both the treated groups did not show any significant variation compared to control.

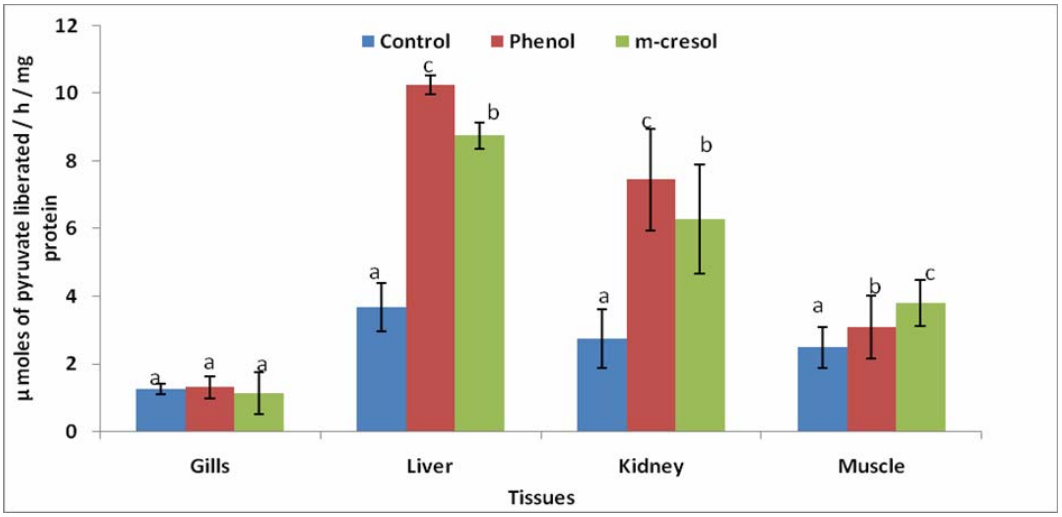

Fig 2.9 Effect of different phenolic compounds on AST activity in *O. mossambicus.* Each bar diagram represents mean ± S.D. On each set of bars, values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA).

Table 2.9 Effect of different phenolic compounds on AST activity (mean ± S.D) in *O. mossambicus.* Values in the same column with different upper case letters vary significantly (P<0.05) between tissues and values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups.

Tissues	AST activity		
	Groups		
	Control	Phenol	m-cresol
Gills	$_{a}1.26 \pm 0.16^{A}$	$_{c}1.31 \pm 0.32^{A}$	$_{b}1.14 \pm 0.61^{A}$
Liver	$_{a}3.68 \pm 0.71^{D}$	$_{c}10.24 \pm 0.28^{D}$	$_{b}8.76 \pm 0.38^{D}$
Kidney	$_{a}2.75 \pm 0.87^{C}$	$_{c}7.44 \pm 1.51^{C}$	$_{b}6.28 \pm 1.61^{C}$
Muscle	$_{a}2.50 \pm 0.60^{B}$	$_{c}3.08 \pm 0.92^{B}$	$_{b}3.80 \pm 0.68^{B}$

- Values are expressed as µmoles of pyruvate liberated / h / mg protein.
- Each value represents the mean ± S.D of six separate experiments.

2.3.10 Alkaline phosphatase

Statistical analysis showed significant variations in ALP activity (P<0.05) in all the treated groups compared to control (Fig 2.9 and Table 2.9). Gills, kidney and muscle of both the treated groups showed significantly elevated ALP activity (P<0.05) compared to control. Liver of m-cresol treated group showed a significantly elevated activity (P<0.05) whereas the liver of phenol treated group did not show any significant variation compared to control.

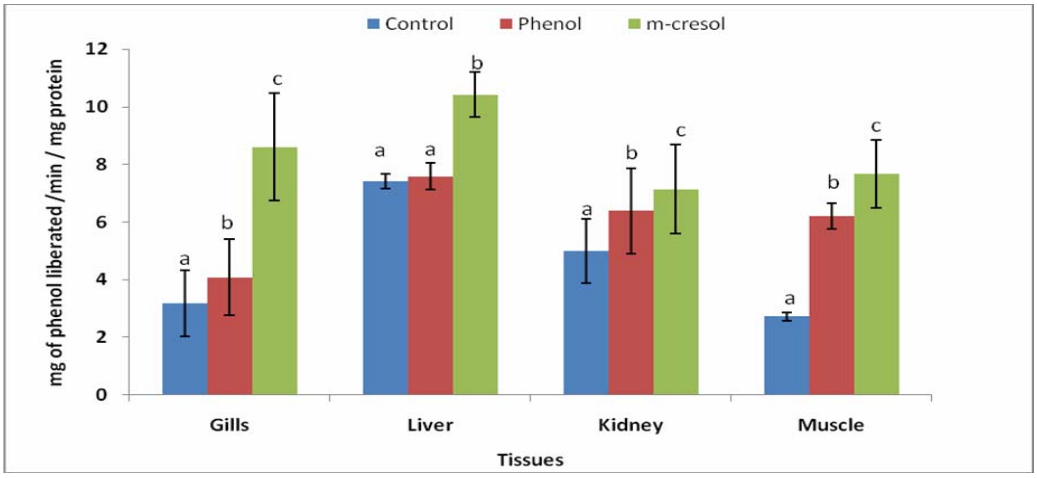

Fig. 2.10 Effect of different phenolic compounds on ALP activity in *O. mossambicus.* Each bar diagram represents mean ± S.D. On each set of bars, values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA).

Table 2.10 Effect of different phenolic compounds on ALP activity (mean ± S.D) in *O. mossambicus.* Values in the same column with different upper case letters vary significantly (P<0.05) between tissues and values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA).

Tissues	ALP activity		
	Groups		
	Control	Phenol	m-cresol
Gills	$_{a}3.18 \pm 1.13^{A}$	$_{b}4.08 \pm 1.31^{A}$	$_{c}8.60 \pm 1.86^{A}$
Liver	$_{a}7.42 \pm 0.25^{D}$	$_{b}7.58 \pm 0.45^{D}$	$_{c}10.42 \pm 0.77^{D}$
Kidney	$_{a}4.98 \pm 1.10^{C}$	$_{b}6.38 \pm 1.47^{C}$	$_{c}7.13 \pm 1.54^{C}$
Muscle	$_{a}2.72 \pm 0.13^{B}$	$_{b}6.20 \pm 0.45^{B}$	$_{c}7.68 \pm 1.17^{B}$

- Values are expressed as mg of phenol liberated / min / mg protein.
- Each value represents the mean ± S.D of six separate experiments.

2.3.11 Serum acid phosphatase

One-way ANOVA followed by Tukey's test showed that there was significant increase in serum acid phosphates activity (P<0.05) in both the treated groups compared to control (Fig 2.10 and Table 2.10).

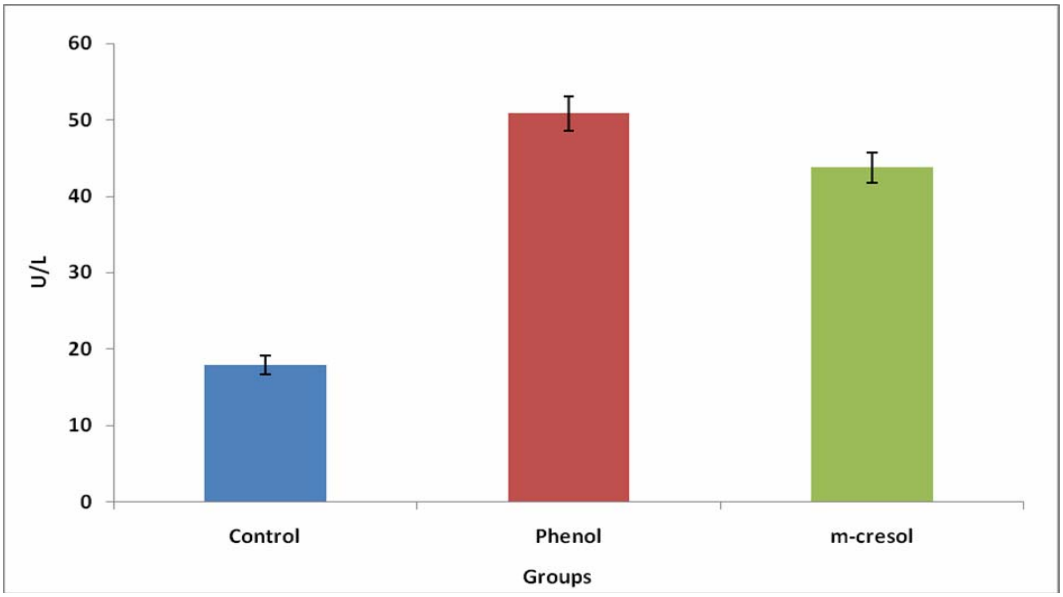

Fig. 2.11 Effect of different phenolic compounds on serum ACP activity in *O. mossambicus.*

Table 2.11 Effect of different phenolic compounds on serum ACP activity in *O. mossambicus.*

Serum ACP activity	Control	Phenol	m-cresol
	18 ± 1.21^{A}	51 ± 2.2^{C}	44 ± 2^{B}

- Values are expressed as U/L.
- Each value represents the mean ± S.D of six separate experiments.

2.3.12 Glutamate dehydrogenase

GDH activity was found to show statistically significant variations ($P<0.05$) in all the treated groups compared to control (Fig 2.11 and Table 2.11). In the phenol treated group, tissues such as gills, liver and kidney showed a statistically significant elevated activity ($P<0.05$) compared to control. Both gills and liver of the m-cresol treated group showed a statistically significant elevated activity ($P<0.05$) compared to control. Muscle of both the treated groups showed a statistically significant decreased activity ($P<0.05$) compared to control.

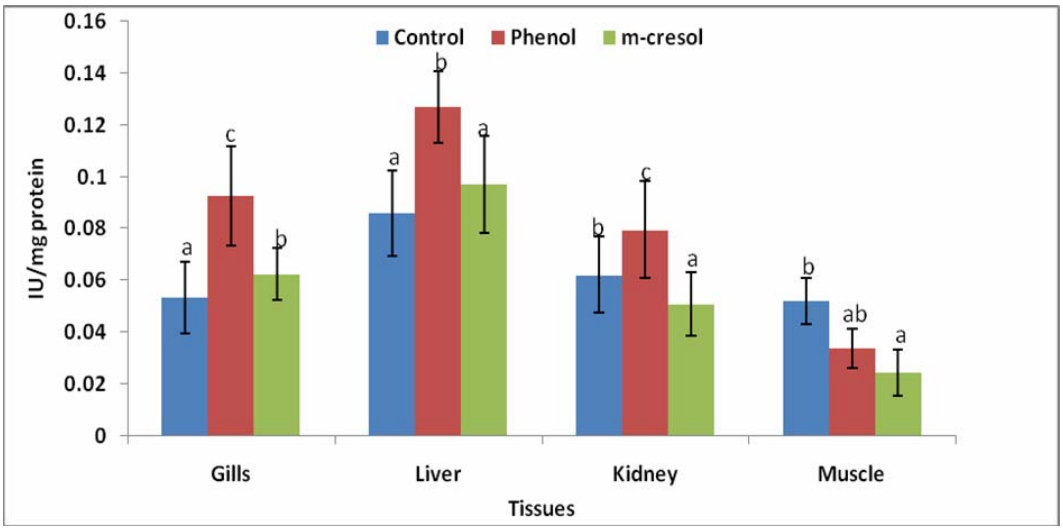

Fig 2.12 Effect of different phenolic compounds on GDH activity in *O. mossambicus.* Each bar diagram represents mean ± S.D. On each set of bars, values with different lower case letters vary significantly ($P<0.05$) in each tissue on different treatments (One-way ANOVA).

Table 2.12 Effect of different phenolic compounds on GDH activity (mean ± S.D) in *O. mossambicus.* Values in the same column with different upper case letters vary significantly (P<0.05) between tissues and values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups.

Tissues	GDH activity		
	Groups		
	Control	Phenol	m-cresol
Gills	$_{a}0.053 \pm 0.013^{B}$	$_{b}0.092 \pm 0.019^{B}$	$_{a}0.062 \pm 0.009^{B}$
Liver	$_{a}0.086 \pm 0.016^{C}$	$_{b}0.126 \pm 0.013^{C}$	$_{a}0.097 \pm 0.018^{C}$
Kidney	$_{a}0.062 \pm 0.014^{B}$	$_{b}0.079 \pm 0.018^{B}$	$_{a}0.051 \pm 0.012^{B}$
Muscle	$_{a}0.052 \pm 0.008^{A}$	$_{b}0.033 \pm 0.007^{A}$	$_{a}0.024 \pm 0.008^{A}$

- Values are expressed as IU / mg protein.
- Each value represents the mean ± S.D of six separate experiments.

2.3.13 Total protein

Statistically significant decreased protein level (P<0.05) was observed in liver and muscle of both the treated groups compared to control (Fig 2.12 and Table 2.12). Gills and kidney of both the treated groups showed a significantly elevated protein level (P<0.05) compared to control.

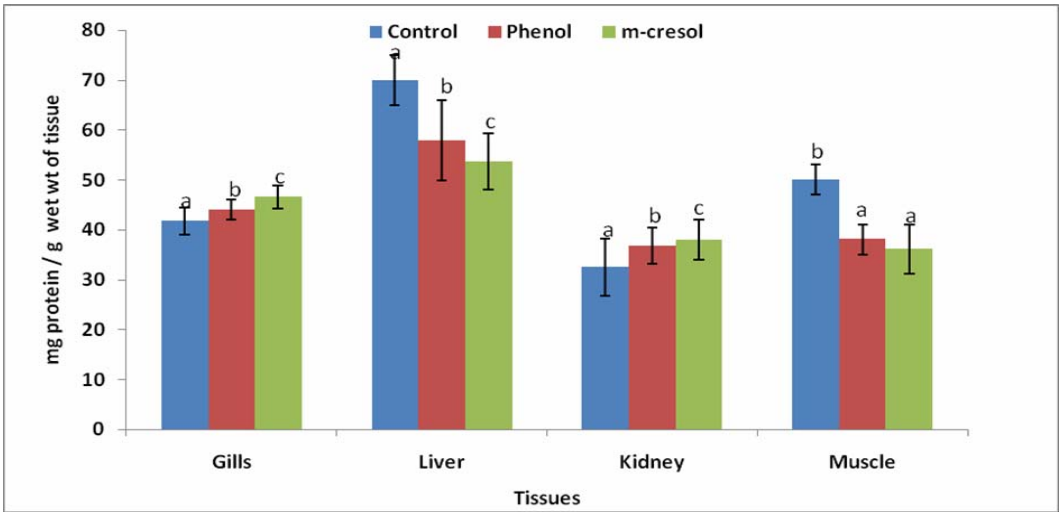

Fig. 2.13 Effect of different phenolic compounds on total protein level (mean ± S.D) in *O. mossambicus.* Each bar diagram represents mean ± S.D. On each set of bars, values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA).

Table 2.13 Effect of different phenolic compounds on total protein level in *O. mossambicus.* Values in the same column with different upper case letters vary significantly (P<0.05) between tissues and values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups.

Tissues	Total protein level		
	Groups		
	Control	Phenol	m-cresol
Gills	$_{b}41.76\pm 2.68^{C}$	$_{a}44.03\pm 1.99^{C}$	$_{a}46.61\pm 2.37^{C}$
Liver	$_{b}70.00 \pm 5.00^{D}$	$_{a}57.98 \pm 8.0^{D}$	$_{a}53.66 \pm 5.66^{D}$
Kidney	$_{b}32.53 \pm 5.73^{A}$	$_{a}36.84 \pm 3.54^{A}$	$_{a}38.03 \pm 4.00^{A}$
Muscle	$_{b}50.09 \pm 3.03^{B}$	$_{a}38.09 \pm 2.98^{B}$	$_{a}36.12 \pm 4.89^{B}$

- Values are expressed as mg protein / g wet wt of tissue.
- Each value represents the mean ± S.D of six separate experiments.

2.4 Discussion

Relatively little is known about the effects of low concentrations of phenolic compounds on the normal physiological functions of freshwater fishes. Phenolic compounds present in sub-lethal concentrations in water might enter into the blood stream of *O. mossambicus* through the gills or the mucus epithelium of the mouth and finally be distributed in different organs of the body which in turn affects various metabolic pathways.

In the present study, decreased cortisol level was observed in both phenol and m-cresol treated fishes compared to control. There are several studies which provided evidence that the capacity to raise plasma cortisol is impaired in fish exposed to organic pollutants (Aluru *et al*., 2004) and metals (Brodeur *et al*., 1997; Norris, 2000; L´evesque *et al*., 2002). As cortisol is involved in the regulation of physiological functions that helps the animal to cope with stress, inhibition of the pituitary–interrenal axis will impair the ability of the animal to cope with stressors (Vijayan *et al*., 1997). Hontela *et al*. (1992) proposed that prolonged exposure to pollutants may lead to hyperactivity, and as a result in the

exhaustion of the pituitary-interrenal axis. The lack of cortisol response suggests that, similar to other xenobiotics phenol and m-cresol can act as an endocrine disruptor and as such impair steroidogenesis. It is not known how phenolics can affect cortisol production. However there are some possible explanations. First, it might be possible that one of the primary steps in the steroid hormone synthesis pathway was compromised. Cholesterol is the substrate for steroid synthesis (Mommsen *et al*., 1999), particularly the non-esterified cholesterol (Mukherjee *et al*., 1991). It has been demonstrated in carp (*Cyprinus carpio*) that chronic exposure to water-borne phenol (8 mg l^{-1}) caused significant accumulation of non-esterified cholesterol in both tissues and serum by days 15 and 30 of exposure, respectively (Mukherjee *et al*., 1991). This accumulation was due to the inability of the steroidogenic tissues to synthesize steroids. Second, it has also been shown that xenobiotics can inhibit the transport of cholesterol to the mitochondria (Hontela, 1997; Walsh *et al*., 2000). Lastly, xenobiotics can affect the action of the adrenocorticotropic hormone (ACTH), which stimulates the synthesis of cortisol by the interrenal cells in fish (Wendelaar-Bonga, 1997). It might be possible that the concentration tested was sufficient to affect steroidogenesis and compromise the cortisol response.

Several studies have corroborated the impairment in the cortisol synthesis and secretion due the action of chemicals. Gravel and Vijayan (2006) studied the impacts of three pharmaceuticals (acetaminophen, ibuprofen and salicylic acid) in rainbow trout and supported the hypothesis that these pharmaceuticals disrupt steroidogenesis in fish interrenal tissue. These findings were also tested *in vitro* and observed that salicylic acid produced a depression of ACTH stimulation in cortisol secretion and a lower gene expression of steroidogenic acute regulatory (StAR) protein, which is involved in steroidogenesis of cortisol (Hontela, 2006); the same author also stated that StAR protein may be sensitive target of many environmental pollutants, ranging from pesticides to pharmaceuticals. Also, the expression of StAR and P450scc decreased in fish exposed to xenobiotics because they bind aryl hydrocarbon–receptor (AhR), a cytosolic induced transcription

factor, with a consequent depression of steroidogenic enzyme activity and finally altering the cortisol production and secretion (Aluru *et al.,* 2005). Therefore many pollutants halt cortisol secretion and even if the fish is under stress this will probably not be reflected in cortisol response.

As cortisol is an important metabolic hormone in fish, any alteration in its dynamics associated with exposure to contaminants could have a significant impact on the ability of the animal to mount a physiological response, thereby attenuating the chances of coping with subsequent natural or anthropogenic stressors. Fish exhibiting an impaired cortisol stress response may be at a disadvantage in coping with environmental stressors.

Total carbohydrate content was found to be decreased in liver and muscle of both the treated groups compared to control. Chemical stress causes rapid depletion of stored carbohydrates primarily in liver and other tissues (Jyothi *et al*., 2000). However, there exist in fish tissues detoxification mechanisms which convert the lipid-soluble compounds into water-soluble metabolites that can be readily excreted (Varanasi and Malins, 1977; Malins and Hodgins, 1981). Such systems are of special significance during continuous exposure, as in the present study, where the process of depuration by simple diffusion cannot be expected to be properly effective due to the constant presence of pollutants in the medium. Hence detoxification mechanisms become active and the hepatic synthesis of detoxifying enzymes requires high energy levels which might be derived from carbohydrate metabolism, for driving the various enzyme-mediated reactions. UDP-glucuronic acid is an important carbohydrate derivative. Phenolics are often excreted as glucuronyl derivatives by conjugating with UDP-glucuronic acid. UDP-glucuronyltransferases (UGTs) are one of the phase II enzymes that catalyse this conjugation. UGTs are induced by a variety of natural and synthetic compounds and play a key role in catalyzing the conjugation and potential excretion of different xenobiotics in fish (Clarke *et al*., 1992a). On exposure to both the phenolic compounds fishes showed behavioural changes such as intense

and frequent avoidance reactions, consisting chiefly of agitated, erratic and violent swimming bouts. Thus the more extensive breakdown of stored carbohydrates in the muscle may be due to the greater physical activity of the organism.

Inhibition of glucose-6-phosphatase activity was found in the liver of both the treated groups compared to control. Inhibition of glucose-6-phosphatase activity may be a reflection of damage to the microsomal membrane as the enzyme is localized exclusively in the membranes of the endoplasmic reticulum. The blood glucose levels have been used as indicators of stress in fish. In the present investigation a significantly decreased blood glucose level was observed in both the treated groups compared to control. This shows that blood glucose homeostasis was not maintained on exposure to phenolics. This may be due to the lack of cortisol response and decreased glucose-6-phosphatase activity.

On exposure to phenolic compounds gills, liver and kidney showed an elevated pyruvate level compared to control. This might be due to the higher glycolysis rate, which is the only energy-producing pathway for the animal when it is under stress conditions. The end product of the glycolytic pathway is pyruvate. Pyruvate occupies an important junction between various metabolic pathways. It may be decarboxylated to acetyl CoA which can enter the TCA cycle or it may be utilized for fatty acid synthesis. Pyruvate may be carboxylated to oxaloacetate which can be used for gluconeogenesis. Muscle of both the treated groups showed a decreased pyruvate level compared to control.

Lactate dehydrogenase is an enzyme recognized as a potential marker for assessing the toxicity of a chemical. LDH is an anaerobic enzyme involved in the conversion of pyruvate to lactate in glycolysis. The LDH in the liver and kidney of fishes treated with phenol showed an elevated activity compared to control. Cohen *et al*. (2001) have reported a similar increase in LDH activity in juvenile Australian Bass and *Macquaria novemaculeata* in response to two different crude oil spills. The increase in LDH activity also suggests a significant increase in the conversion of pyruvate to lactic acid, thereby leading to the accumulation of lactic

acid. Compared to control a significant decrease in LDH activity in liver and kidney of m-cresol treated fishes and in gills of fishes treated with phenol was observed. This may be due to increased tissue damage. Similar results were obtained when *O. mossambicus* were exposed to sub-lethal concentrations of organophosphorus insecticide (Rao, 2006). Stimulation of LDH in muscle of *O. mossambicus* on exposure to phenolic compounds suggests that the final product of glycolysis - pyruvate was preferentially used to produce lactate. Lactate formed is an important gluconeogenic substrate which can be used to cope with the high and rapid demand of energy due to stress.

Several reports revealed decreased LDH activity in tissues under various toxic conditions (Tripathi *et al*., 1990; Mishra and Shukla, 2003). LDH is an important glycolytic enzyme in biological systems and is inducible by oxygen stress. Therefore, the activity of several regulatory enzymes may be altered in order to meet the required energy demands under toxic stress (Mayer *et al*., 1989), including the activity of lactate dehydrogenase (LDH), which sustains the continued process of glycolysis under anaerobic conditions (Diamantino *et al*., 2001). Several reports revealed decreased LDH activity in tissues under various toxic conditions (Tripati and Shukla, 1990; Mishra and Shukla, 2003). The level of LDH was found to be increased in the gills and decreased in the liver, kidney and muscles in the monocrotophos exposed fish (Agrahari and Gopal, 2009).

Compared to control, ALT and AST activities were found to be highly elevated in all the tissues of fishes treated with phenol and m-cresol compared to control. The highest activity was observed in liver followed by kidney and muscle. In fish, one of the primary energy currencies is amino acids. Transaminases like alanine aminotransferase and aspartate aminotransferase play an important role in the conversion of amino acids to keto acids like pyruvate and oxaloacetate, which could be used as intermediates in Kreb's cycle or directed into the gluconeogenic pathway. ALT is cytosolic whereas AST has both cytosolic and mitochondrial forms. Under normal conditions there is a baseline

activity of these enzymes. But when the organism is subjected to stress, the levels of these enzymes are significantly increased in order to meet the increase in ATP demands.

ALT is an enzyme frequently used in the diagnosis of damage caused by pollutants in various tissues such as liver, muscle, and gills (de La Torre *et al*., 1999, 2000). This enzyme is known to play a key role in mobilizing L-amino acids for gluconeogenesis and function as links between carbohydrate and protein metabolism under altered physiological, pathological and induced environmental conditions (Nichol and Rosen, 1963; Knox and Greengard, 1965; Victor, 1985). Elevation in the levels of AST and ALT in different tissues of *O. mossambicus* can be considered as a response to the stress induced by phenolic compounds to generate keto acids like α-ketoglutarate and oxaloacetate for contributing to gluconeogenesis and/or energy production necessary to meet the excess energy demand. Significant elevations in AST activity was recorded in *Cyprinus carpio* exposed to copper sulphate (Karan *et al*., 1998). Elevations in ALT activitiy were noticed in *C. carpio* and *Oreochromis niloticus* exposed to cadmium (de La Torre *et al*., 2000; De Smet and Blust, 2001; Almeida *et al*., 2002). ALT activity of *Carassius auratus* liver was stimulated by low concentrations of ytterbium (Guo *et al*., 2002).

Similar type of observation was also observed by Janice *et al*. (1979), when American oysters and brown shrimps were exposed for a chronic period to crude oil. The exposure to phenol caused an increase of both ALT and AST activities in *Notopterus notopterus* (Gupta *et al*., 1983). The amino acids through transamination and deamination reactions might have supplied necessary keto acids to act as precursors for the maintenance of carbohydrate metabolism to meet the energy requirements during pollutant stress.

Alkaline phosphatase and acid phosphatase catalyses the hydrolysis of monophosphate esters and has a wide substrate specificity. The activity of ALP has been significantly elevated in all the tissues (gills, liver, kidney and muscle)

treated with phenol and m-cresol compared to control. Increased ALP activity may be due to pathological processes such as liver impairment, kidney dysfunction and bone disease (Barse *et al*., 2006). Phosphatases play major roles in the moulting physiology of many fishes (Ezhilarasi, 1982). Serum acid phosphatase showed an elevated activity compared to control. An elevation in ACP activity suggests an increase in lysosomal mobilization and cell necrosis due to the toxicity of phenolics. This increase also suggests the supply of phosphate group for energy metabolism. This shows an adverse impact on metabolism, which may lead to negative impact on growth, health and reproduction. Degeneration and necrosis induced in hepatic parenchymatous cells by these toxicants may cause release of acid phosphatase in the serum. Alterations in ALP and ACP activities in tissues and serum have been reported in pesticide treated fish (Palanivelu *et al*., 2005). Increase in the levels of ALP and AST has been shown to reflect liver damage, whereas an elevation in the ALP activity may be indicative of renal and liver damage (Gill *et al*., 1990; Bhattacharya *et al*., 2005).

GDH activity was found to be elevated in almost all tissues treated with phenol compared to control. This increased activity may have helped in funneling more α-ketoglutarate into TCA cycle for more energy generation. Whereas in fishes treated with m-cresol tissues such as liver and gills showed almost constant activity but kidney and muscle showed a decreased activity compared to control. An inhibition of GDH activity in gills, brain, kidney and liver of fishes exposed to toxicants was observed by Ghosh (1985).

Fishes exposed to sub-lethal concentrations of different phenolic compounds showed alterations in protein content in different tissues compared to control. Gills and kidneys of both the treated group showed increased protein content compared to control. Liver and muscle of both the treated groups showed decreased protein content compared to control. The reduction in protein content indicates that under stress conditions the tissue protein may undergo proteolysis, which may have resulted in the production of free amino acids which can be used

in the tricarboxylic acid cycle for energy production. This would lead to an increased free amino acid pool (Bayne *et al*., 1981) which can be used for ATP production by transamination reactions or by gluconeogenic pathway. The tissue protein is metabolised to produce glucose by the process of gluconeogenesis and it is utilized for energy production under stress conditions (Elumalai and Balasubramanian, 1999). The decrease in protein content under stress induced by phenolic compounds may be attributed to the utilization of amino acids in various catabolic reactions. The depletion of protein content may also be due to the rapid utilization of tissue protein as the food utilization decreases when the animals are under stress conditions. Yadav *et al*. (2007) has reported that the animals exposed to chemicals obtain extra energy requirement from the tissue protein. The depletion of cellular proteins might be caused by one or more of the following factors: inhibition of amino acid incorporation, breakdown of proteins into amino acids and diffusion out of the cells. Badawy *et al*. (1969) established that the inhibition of RNA synthesis precedes inhibition of protein synthesis and that necrosis occurs later than these two events. The decline in protein content may be related to impaired food intake, the increased energy cost of homeostasis, tissue repair and the detoxification mechanism during stress (Neff, 1985). Another reason that can be attributed for the decrease in proteins under toxic stress may be due to formation of lipoproteins, which are utilised for repair of damaged cell and tissue organelles.

Therefore, the sum of these alterations can have a significant effect on energy metabolism. In conclusion, the present work indicates that phenolic compounds causes considerable changes in intermediary metabolism and is likely to induce tissue damage in *O. mossambicus*. The causes for these alterations appear to be the result of high energy demands.

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Chapter 3

EFFECTS OF DIFFERENT PHENOLIC COMPOUNDS ON ANTIOXIDANT ENZYMES AND LIPID PEROXIDATION IN *OREOCHROMIS MOSSAMBICUS*

Contents

3.1 Introduction

Oxidative stress and oxidative damage to fundamental biomolecules and to antioxidant defenses of organisms is an established field in environmental toxicology and ecotoxicology (Kelly *et al*., 1998; Regoli *et al*., 2002a, b).The biochemistry of 'reactive oxygen species' (ROS) is an important field with practical implications. This is because oxygen is an essential component of living organisms and the formation of reactive oxygen intermediates seems to be common in aerobically metabolizing cells. In addition to aerobic metabolism-encompassing electron-transfer chains and certain enzyme activities, environmental sources such as air pollutants, photochemical smog, industrial chemicals, ionizing radiations, as well as metabolism of xenobiotics contribute to the cellular steady-state concentration of ROS. Further, reactive species are formed as a response to diverse stimuli by specialized physiological reactions: the formation of oxyradicals during respiratory burst and the release of the endothelium-derived releasing factor, identified as nitric oxide are such examples. Regulated production of free radicals in higher organisms and maintenance of ''redox homeostasis'' are essential for the physiological health of organisms (Ames *et al*., 1993). But during these metabolic processes, a small proportion (2–3%) of free radicals may escape from the protective shield of antioxidant mechanisms, causing oxidative damage to cellular components.

Other endogenous sources of ROS within cells are several oxidizing enzymes, such as tryptophan dioxygenase, xanthine oxidase and cytochrome P450 reductase which can produce $O_2^{\cdot-}$, while enzymes such as guanyl cyclase and glucose oxidase generate H_2O_2 (Vigo-Pelfrey, 1990). Cytochrome P450 involvement in the production of ROS is of additional interest in toxicology because it is involved in the metabolism of xenobiotics (Fridovich, 1978).

3.1.1 The Oxygen Radical Cascade

The oxygen activation follows a series of electron transfer reactions and the pertinent ones are as follows.

O_2+e- → O_2^{-}

O_2^{-} +H^+ → HO_2

HO_2+ H+ e- → H_2O_2

H_2O_2+ e- → OH+OH-

The endogenous sources for production of O_2^{-} ranges from small to large autooxidizable molecules such as catecholamines, ubihydroquinone and oxidoreductases such as haemoproteins and flavin enzymes. Thus O_2^{-} is generated in virtually all sub cellular compartments including cytosol, mitochondria, endoplasmic reticulum, nuclei etc.

General harmful effects of reactive oxygen species in cell are: oxidations of polyunsaturated fatty acids in lipids (lipid peroxidation), damage of DNA, oxidations of amino acids in proteins, oxidative inactivation of specific enzymes by oxidation of co-factors.

3.1.2 Lipid Peroxidation

The term lipid peroxidation is broadly defined as the formation of lipid radical, which would then react with molecular oxygen in the form of a chain of reactions resulting in the breakdown of polyunsaturated fatty acids (PUFA). This reaction sequence is known alternatively as lipid peroxidation or "oxidative deterioration of polyunsaturated lipids". Lipid peroxidation is a free radical mediated chain reaction that results in the oxidative degradation of unsaturated lipids especially PUFA, such as C20:4 and C22:6 which occurs in the phospholipids of most biological membrane assemblies. Biological membranes rich in unsaturated fatty acids are in close contact with oxygen rich metal ion containing fluid. Therefore membrane lipids are highly susceptible to peroxidative damage (Ray and Akhtar, 2002).

Initiation of lipid peroxidation in a membrane or free fatty acid is due to the attack of any species that has sufficient reactivity to abstract a methylene hydrogen atom from the diene portion of an unsaturated fatty acid (Fig. 3.1) and forms a lipid

radical. Reaction of the lipid free radical with molecular oxygen leads to the formation of a lipid hydroperoxy radical ($L00^{\cdot}$). The lipid hydroperoxy radical could then react along either of the two pathways. It could: - abstract a methylene hydrogen from a neighbouring unsaturated fatty acid forming a lipid hydroperoxide and a second lipid radical or undergo an intermolecular–cyclisation to form a five membered lipid endoperoxide radical. These lipid radicals formed would then react with molecular oxygen and with other unsaturated lipids resulting in the extraction of methylene hydrogen and continuation of the radical chain reactions (Ray and Akhtar, 2002). Breakdown of lipid hydroperoxides and endoperoxides leads to formation of more than twenty known products of lipid peroxidation (Fridovich, 1983).

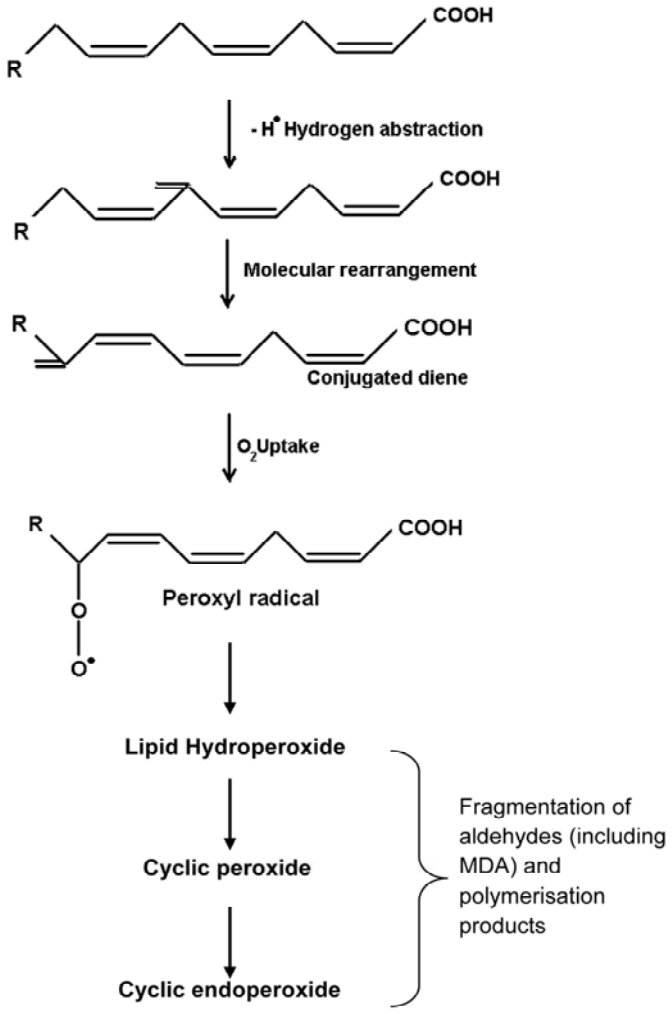

Fig.3.1 Formation of Conjugated diene (CD) and lipid hydroperoxide (HP) following hydrogen abstraction from a polyunsaturated fatty acid.

The resulting lipid hydroperoxides can easily decompose into several reactive species including lipid alkoxyl radicals, aldehydes (e.g. malondialdehyde, OHC–CH_2– CHO), alkanes, lipid epoxides, and alcohols. Most of these products are toxic and active mutagens (Esterbauer *et al*., 1990., Porter *et al*., 1995; D'Ischia *et al*., 1996). The only mechanism, which produces malondialdehyde (MDA) in biological systems, is lipid peroxidation. MDA is not the major product of lipid peroxidation, but a typical degradation product. Lipid peroxidation products may form DNA adducts giving rise to mutations and altered patterns of gene expression (Marnett, 1999). MDA reacts with nitrogenous base of DNA to form DNA adducts (Fujimoto *et al*., 1984). Uncontrolled lipid peroxidation leads to the disruption of phospholipid membranes and to cell lysis. Peroxides also interact with macromolecules such as sulphydryl groups of proteins and bases of nucleic acids abrupting normal function and causing mutation (Slater and Benedetton, 1981). Additionally peroxides and free radial are produced by the immune system to mediate response to a foreign organism. Peroxidized membranes become rigid and lose permeability and integrity.

It is evident then that 'oxidative stress is a chain event, and a single initiating event caused by a prooxidant may cascade into a widespread chain reaction that produces many deleterious products in concentrations many magnitudes greater than the initiator (Ahmad, 1992). This is exemplified by the fact that thousands of PUFA molecules may be destroyed by a lipid peroxidation chain reaction initiated by a single initiator free radical (McCord, 1985). It is imperative that in order to prevent this vicious chain reaction, the O_2 radical cascade to $^{\cdot}O_2^{-}$ and H_2O_2 must be attenuated, and the peroxides converted to innocuous metabolites. The prevention of lipid peroxidation is an essential process in all the aerobic organisms, as lipid peroxidation products can cause DNA damage. On the other hand, hydrogen peroxide has been implicated as an intracellular messenger that affects cellular processes including protein phosphorylation, transcription and apoptosis (Choi *et al*., 1998).

DNA in cellular nuclei is another key cellular component that is particularly susceptible to oxidative damage by ROS (Cerutti, 1985). The polyanionic nature of DNA provides a useful substrate for infiltration through membranes and adherence of metal cations, thus facilitating the formation of $HO^{\cdot}$ adjacent to these critical biological targets (Halliwell and Aruoma, 1991). Additionally, the heterogeneity of DNA molecules allows for $HO^{\cdot}$ attacks, including the nucleobases and the sugar–phosphate backbone (Buxton *et al*., 1988). Hydroxyl radicals react with nucleobases approximately five times faster than with the nucleic acid backbone (Cadet *et al*., 1997). Other hydroxyl radical attacks can be directed towards the sugar–phosphate backbone of DNA, causing different lesions, including apurinic sites where the base has been removed, fragmentation of deoxyribose with single-strand breaks, and oxidation of the sugar moiety (Dizdaroglu *et al*., 1975; Breen and Murphy, 1995).

Protein oxidation reactions involve various propagating radicals and ROS and the results are oxidative modifications of amino acid side chains, reactive oxygen species mediated peptide cleavage, reactions of peptides with lipids and carbohydrate oxidation products and formation of carbonyl derivatives of proteins. Oxidatively modified proteins accumulate during ageing. Of the various indices of protein oxidation, protein carbonyl formation is the best studied (Stadtman and Berlett, 1999). There are a variety of pathways through which protein carbonyls are formed (Levine *et al*., 2000). Experimental studies showed that oxidative damage to proteins by ROS leads to the accumulation of oxidatively modified forms of enzymes which are implicated in ageing (Wolff and Dean, 1986). This accumulation during ageing reflects a loss in the capacity of the organisms to degrade oxidised proteins, with subsequent effects on the transcriptional and translational fidelity mechanisms (Duikan *et al*., 2000).

The aquatic environment receives daily substantial amounts of environmental pollutants that have the potential to cause oxidative stress in aquatic organisms through free radical and ROS mechanisms. The uptake of these

pollutants by aquatic organisms can occur from sediments, suspended particulate matter with toxic properties and food sources. Exposure to these contaminants will depend on the particular dietary and ecological lifestyles of the aquatic organisms. Current knowledge and recent advances of oxidative toxicity by xenobiotics in aquatic organisms provide a fertile field for aquatic toxicology studies (Livingstone, 1998). Aquatic organisms were chosen as test species because of their filtration capacity and sensitivity to oxidative damage from concerning chronic exposure or sub-lethal concentrations. Aquatic organisms can provide model systems for investigation of how ROS damage cellular components, how cells respond, how repair mechanisms ameliorate this damage and how oxidative stress can lead to disease (Di Giulio *et al*., 1989; Livingstone *et al*., 1994). Aquatic organisms are more sensitive to exposure and toxicity compared to terrestrial organisms including mammals and in this respect they may provide experimental data for evaluation of subtle effects of oxidative stress, mutagenicity, and other adverse effects of pollutants (Lackner, 1998). Some aquatic organisms can provide better models for linking malignant neoplasms with carcinogenic pollutants (Malins *et al*., 1988). Environmental toxicity studies on aquatic organisms have focused primarily on redox cyclic compounds (quinones, aromatic hydrocarbon-quinones, nitropyrene, lindane, paraquat, nitrobenzoic acid, etc.) and their effects on subcellular fractions (microsomes) of the major organs of biotransformation using the adult or larval stages (catfish, rainbow trout, flounder, mussels, etc.) (Garcia Martinez and Livingstone, 1995; Lemaire and Livingstone, 1997; Sjo lin and Livingstone, 1997).

Biological systems have developed during their evolution adequate enzymatic and non- enzymatic antioxidant mechanisms to protect their cellular components from oxidative damage. Consequently, all aerobic organisms possess elaborate defense mechanisms to prevent the formation of toxic forms of oxygen and to remove peroxides formed.

Aquatic organisms metabolize organic xenobiotics by phase I metabolism which produces reactive oxygen species (ROS) as by-products (Livingstone, 1991). Oxidative stress occurs when reactive oxygen species (ROS), such as superoxide ion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) and singlet oxygen ($^1O_2$) react with lipids, proteins or nucleic acids resulting in several biochemical injuries (Yu and Anderson, 1997; Pinchuk and Lichtenberg, 2002; Valvanidis *et al*., 2006). Detoxification of ROS is one of the prerequisites of aerobic life (McCord, 2000), and many defenses have evolved providing an antioxidant system which is able to prevent, intercept and repair damages. It consists of non-enzymatic ROS scavengers such as: ascorbic acid, reduced glutathione, α- tocopherol, flavonoids, β-carotene and urate, and also of an enzymatic system that includes superoxide dismutase, glutathione peroxidase, catalase, NADPH quinine oxidoreductase, DT-diaphorase, epoxide hydrolase, glucose-6-phosphate dehydrogenase and a few conjugation enzymes (Sies, 1991; Valvanidis *et al*., 2006).

Oxidative stress develops when the levels of antioxidants are lowered or when production of reactive oxygen species (ROS) exceeds the capacity of the cell to dispose of them. ROS are produced by the univalent reduction of dioxygen to superoxide anion, which in turn disproportionates to H_2O_2 and O_2 spontaneously or through a reaction catalyzed by superoxide dismutase (SOD).

$$2\ {}^{\cdot}O_2^{\cdot -} + 2\ H^+ \longrightarrow H_2O_2 + O_2$$

SOD (EC 1.15.1.1) was first discovered by McCord and Fridovich in 1969. The SOD family consists of four metalloforms; two forms containing copper and zinc, one form containing manganese and another form containing iron. Cu,ZnSOD is found in the cytosol of most eukaryotic cells (Fridovich,1975). A different form of Cu,ZnSOD is found in extracellular fluids, where it is called ECSOD (Marklund,1984; Marklund *et al.*,1985). MnSOD is located in the mitochondrial matrix as well as in bacteria, while FeSOD is present in many aerobic bacteria (Fridovich, 1974). In eukaryotic cells, three forms of SOD are known to exist: Cu,ZnSOD, EC-SOD, and MnSOD.

Catalase: CAT (EC 1.11.1.6) is one of the oldest known enzymes; it was named by Loew in 1901 (Percy, 1984). The enzyme catalyzes the reaction:

$$2\ H_2O_2 \longrightarrow 2H_2O + O_2$$

Most aerobic cells contain this enzyme. In animals, CAT is present in all major body organs, being especially concentrated in liver and erythrocytes. At the sub cellular level, CAT is found mostly in peroxisomes (80%) and cytosol (20%). The usual form of CAT consists of four protein subunits, each containing a heme (Fe(lll)-protoporphyrinl) group bound to its active site.

Glutathione peroxidase: GPx (EC 1.1 1.1.9) was first described in 1957 by Mills. The enzyme catalyzes the oxidation of GSH to GSSG at the expense of H_2O_2.

$$2GSH + ROOH \rightarrow GSSG + ROH + H_2O$$

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$$

By its selenium (Se) dependency, GPx can be divided into two forms: Se-dependent GPx and Se-independent GPx. Se-dependent GPx is a tetramer with very high activity toward both H_2O_2 and organic hydroperoxides. It contains one residue of selenocysteine per mole at each of the active sites and is found in both cytosol (70%) and mitochondria (30%). To recycle GSSG, the cell utilizes the enzyme NADPH-dependent GSH reductase, the NADPH being supplied to the reaction by glucose-6-phosphate dehydrogenase (Bachowski *et al*., 1997).

The Se-independent GPxs are the GSH- S-transferases (GST, EC 2.5.1.18). They were originally observed in the catalysis of the first step in the formation of the mercapturic acids (Booth, 1961).The enzymes are dimers with at least 7 different forms of subunits and 8 isoenzymes. The enzymes have relatively low activity towards organic hydroperoxides but none at all towards H_2O_2. They have multiple functions, but are mainly involved in the biotransformation of xenobiotics (Jakoby, 1978) and detoxification of carcinogens (Smith, 1977). The intracellular distribution was found to be both cytosolic and mitochondrial.

Glutathione transferases are a family of detoxifying enzymes with broad and overlapping substrate specificities towards carcinogenic, mutagenic, toxic and pharmacologically active compounds (Boyland and Chasseaud, 1969). Among the substrates are many reactive intermediates formed via the P450 system (Chasseaud, 1978), as well as compounds formed during lipid peroxidation (Ahlin, 1985). Glutathione S-transferases (GST) play an important role in the detoxification and excretion of xenobiotics by catalyzing the conjugation of the tripeptide glutathione (GSH) with the xenobiotic in the phase II of the biotransformation process promoting its elimination from the organism (Leaver *et al*., 1992). This enzyme has been used in laboratory and field studies as a biomarker for several contaminants (Almar *et al*., 1998; Fenet, 1998).

Glutathione (γ-_-glutamyl-cysteinyl-glycine) is a tripeptide that is mainly present in cells in its reduced form (GSH), which basically acts as an intracellular reductant and nucleophile. It functions in the synthesis of proteins and DNA, amino acid transport, maintenance of the thiol-disulphide status, free radical scavenging, signal transduction, as an essential cofactor of several enzymes, as a non-toxic storage form of cysteine, and as a defence against oxidizing molecules and potentially harmful xenobiotics such as metals (Pena-Llopis *et al*., 2001; Elia *et al*., 2003).

The depletion of dissolved oxygen concentration of waters polluted with phenolic wastes (Phipps *et al*., 1981) leads to formation of free radicals, especially superoxide ($O_2^{\cdot-}$**)** which acts by oxidizing various cellular substrates, especially unsaturated fatty acids, which are very susceptible to free radical damage (Rady, 1993). In living organisms, it is necessary to limit the DNA damage caused by reactive species with antioxidants in order for them to survive. Most components of cellular structure and function are likely to be the potential targets of oxidative damage and the most susceptible substrates for auto oxidation are polyunsaturated fatty acids of the cell membrane, which undergo peroxidation rapidly. This may lead to muscle degradation, impairment of the nervous system, haemolysis, general deterioration of the cellular metabolism and eventual cell death.

Many environmental pollutants, such as phenol, may cause oxidative stress in aquatic organisms by inducing ROS production (Sayeed *et al*., 2003; Oruc *et al*., 2004). In biological systems, the balance between both endogenous and exogenous pro-oxidant factors versus antioxidant defenses can be used to assess oxidative damage induced by different classes of chemical pollutants (Valvanidis *et al*., 2006). Changes of activity of antioxidant enzymes may depict a change in the ROS within the cells. Therefore, these enzymes can be used as biomarkers for oxidative stress (Roche and Boge, 2000; Valvanidis *et al*., 2006). Aquatic organisms are usually more sensitive than terrestrials and may be better experimental subjects to evaluate subtle effects of oxidative stress (Ahmad *et al.,* 2000; Valvanidis *et al*., 2006).

In the present study biomarkers of oxidative stress in the gills, liver, kidney and muscle of *O. mossambicus* exposed to different phenolic compounds for 21 days were experimented. The oxidative stress biomarkers studied included the non-enzymatic antioxidant glutathione and antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase and glutathione-S-transferase. Also indicators of lipid peroxidation such as malondialdehyde, hydroperoxide and conjugated dienes were estimated.

3.2 Materials and methods

Collection, maintenance, acclimatization and experimental design and preparation of tissue samples were the same as explained in detail in chapter 2, section 2.2.

3.2.1 Parameters investigated

3.2.1.1 Assay of superoxide dismutase (SOD) (E.C.1.15.1.1)

Superoxide dismutase in different tissues was determined using the method of Kakkar *et al.* (1984).

Reagents

0.33 M sucrose, n-butanol , 0.052 M sodium pyro-phosphate buffer (pH 8.3), 0.0025 M Tris-HCl buffer (pH 7.4), 186 μM phenazine methosulphate (PMS), 300 μM Nitro blue tetrazolium (NBT), 780 μM NADH and glacial acetic acid.

Procedure

Weighed samples of tissues were homogenised in 0.33 M sucrose and subjected to differential centrifugation under cold conditions to obtain the cytosol fraction. Before estimating the activity, an initial purification was done by precipitating the protein from the supernatant with 90% ammonium sulphate and this fraction was then dialysed against 0.0025 M Tris- HCl buffer (pH 7.4). The supernatant was used as the enzyme source. Assay mixture contained 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of PMS, 0.3 ml of NBT, 1.3 ml of distilled water and 0.1 ml of the enzyme source. The tubes were kept at 30°C for one minute and then 0.2 ml of NADH were added and incubated at 30°C for 90 seconds and the reaction was stopped by the addition of 1 ml of glacial acetic acid. Reaction mixture was shaken vigorously with 4.0 ml of n-butanol. The mixture was allowed to stand for 10 minutes and centrifuged. The upper butanol layer was removed. Absorbance of the chromogen in butanol was measured at 560 nm against n-butanol blank. A system devoid of enzyme served as control. One unit of enzyme activity is defined as the enzyme concentration required to inhibit chromogen production by 50% in one minute under the assay conditions and specific activity is expressed as units / mg protein.

3.2.1.2 Assay of Catalase (CAT) (E.C.1.11.1.6)

Catalase level in different tissues was determined using the method of Maehly and Chance (1955).

Reagents

0.01M phosphate buffer (pH 7.0), 30mM H_2O_2.

Procedure

The estimation was done spectrophotometrically following the decrease in absorbance at 230 nm. The reaction mixture contained 0.01 M phosphate buffer, 30 mM hydrogen peroxide and the enzyme extract prepared by homogenizing the tissue in phosphate buffer and centrifuging at 5000 rpm. Specific activity was

expressed as International Units / mg protein. 1 1U = change in absorbance / min/ extinction coefficient (0.021).

3.2.1.3 Assay of Glutathione peroxidase (GPx) (E.C. 1.11.1.9)

Glutathione peroxidase in different tissues was estimated by the method of Rotruck (1973).

Reagents

0.4 M Tris buffer (pH 7.0) 10mM sodium azide solution, 10% Trichloro acetic acid (TCA) , 0.4 mM Ethylene diamine tetra acetic acid (EDTA), 0.2 mM Hydrogen peroxide (H_2O_2) , 2 mM glutathione solution (GSH).

Procedure

Weighed samples of different tissues were homogenized in a known volume of Tris buffer. To 0.2 ml of Tris buffer, 0.2 ml EDTA, 0.1 ml sodium azide and 0.5 ml tissue homogenate were added and mixed well. To this mixture 0.2ml of GSH followed by 0.1 ml H_2O_2 solution were added. The contents were mixed and incubated at 37°C for 10 minutes along with a control containing all reagents except tissue homogenate. After 10 minutes the reaction was arrested by the addition of 0.5 ml of 10% TCA. Tubes were centrifuged and the supernatant was assayed for GSH. The values are expressed as μg of GSH / min/ mg protein.

3.2.1.3 Assay of Glutathione–S-transferase (GST) (E.C.2.5.1.18)

Glutathione-S-transferase in different tissue was determined using the method of Beutler *et al*. (1986).

Reagents

0.5 M phosphate buffer (pH 6.5), 25 mM of 1-chloro-2, 4-dinitro benzene (CDNB) in 95% ethanol, 20 mM glutathione (GSH)

Procedure

All the tissues were homogenized in 0.5 M phosphate buffer. The reaction mixture contained 200μl phosphate buffer, 20 μl CDNB and 680 μl distilled

water. Then the tubes were incubated at 37°C for 10 minutes and added 50 µl of GSH. After mixing well, added 50µl of tissue extract to the tube. Increase in absorbance was noted at 340nm for 5 minutes in a UV–visible spectrophotometer. Values are expressed in µmoles of CDNB complexed / min/ mg protein. The extinction coefficient between CDNB –GSH conjugate is 9.6 mM^{-1} cm^{-1}.

3.2.1.4 Estimation of total reduced glutathione (GSH)

Total reduced glutathione was estimated by the method of Ellman (1958).

Reagents

DTNB (0.6 Mm) in 0.2 M phosphate buffer (pH-8.0), TCA 5%, standard glutathione.

Procedure

Precipitated protein in the homogenates of gills, liver, kidney and muscle with 0.1 ml 5% TCA and 0.4 ml distilled water. Mixed the contents well for complete precipitation of proteins and centrifuged. To 0.5 ml clear supernatant, added 2.5 ml of 0.2 M phosphate buffer and 50 µl of DTNB. Read the absorbance at 412 nm against a blank containing all the reagents. A series of standards were run along with blank treated in a similar manner to determine the glutathione content. Values were expressed as nmoles/100 g wet tissue.

3.2.1.5 Estimation of Conjugated dienes (CD)

The concentration of conjugated dienes was estimated according to the method of Retnagal and Ghoshal (1966).

Procedure

Membrane lipids were extracted and evaporated to dryness as described for the iodometric assay for hydroperoxides. The lipid residue was dissolved in 1.5 ml of cyclohexane and the absorbance at 233 nm was determined against a cyclohexane blank. Molar extinction coefficient of conjugated dienes is 2.52 x 10^4 M^{-1} cm^{-1}.

3.2.1.6 Estimation of Hydroperoxides (HP)

Hydroperoxides was estimated by method of Mair and Hall, 1977.

Reagents

Potassium iodide, 0.5% cadmium acetate

Procedure

1ml of the tissue homogenate of the different tissues was mixed thoroughly with 5 ml of chloroform: methanol (2:1) followed by centrifugation at 1000×g for 5 minutes to separate the phases. 3 ml of the lower chloroform layer was recovered using a syringe and placed in a test tube and dried in a 45°C water bath. 1 ml of acetic acid: chloroform (3:2) mixture followed by 0.05 ml of potassium iodide was quickly added and the test tubes were stoppered and mixed. The tubes were placed in the dark at room temperature for exactly 5 minutes followed by the addition of 3 ml of cadmium acetate. The solution was mixed and centrifuged at 1000 × g for 10 minutes. The absorbance of the upper phase was read at 353 nm against a blank containing the complete assay mixture except the tissue homogenate. Molar extinction coefficient of hydroperoxide is 1.73×10^4 M^{-1} cm^{-1}.

3.2.1.7 Estimation of Malondialdehyde

Malondialdehyde was estimated by the method of Niehaus and Samuelson, 1958.

Reagents

TCA-TBA-HCl reagent: 15% (w/v) Trichloro acetic acid, 0.375% (w/v) Thiobarbituric acid (TBA) in 0.25 N HCl. 0.1 M Tris-HCl buffer (pH7.5).

Procedure

The tissue homogenate of different tissues were prepared in Tris-HCI buffer and was combined with thiobarbituric acid reagent and mixed thoroughly and heated for 15 minutes in a boiling water bath. It was then cooled and centrifuged for 10 minutes at 600×g. The absorbance of the sample was read spectrophotometrically at 535 nm against a reagent blank that contained no tissue extract. The extinction

coefficient for malondialdehyde is 1.56×10^5 M^{-1} cm^{-1}. The values are expressed as millimoles / 100g wet wt of tissue.

3.2.1.8 Estimation of Protein

Protein was estimated by the method of Lowry *et al*. (1951), (Chapter 2, Section 2.2.6.11).

3.3 Statistical analysis

The statistical analysis was carried out using the software SPSS 13.0 package. Two-way analysis of variance (ANOVA) was carried out to compare between different phenolic compounds treated groups and also between tissues. If significant difference were revealed by the ANOVA test, Tukey's test was used to further elucidate which tissues and treatments were significantly different. One-way ANOVA followed by Tukey's test was also carried out for the comparison between different treatments in each tissue. Significance level (P value) was set at 0.05 in all tests.

3.4 Results

3.4.1 Superoxide Dismutase (SOD)

Two-factor ANOVA followed by Tukey's test showed that there was significant ($P<0.05$), (Fig 3.1 and Table 3.1) variation in SOD activity between treatments and also between tissues. Between the treated groups, both phenol and m-cresol treated groups showed significant variation in SOD activity and also with the control. SOD activity was found to be significantly ($P<0.05$) elevated in gills, liver and kidney of *O. mossambicus* treated with phenol compared to control and among these tissues liver showed the maximum activity, whereas the fishes treated with m-cresol showed significantly elevated activity in liver, kidney and muscle compared to control. A significantly ($P<0.05$) decreased activity compared to control was shown by gills treated with m-cresol and muscle treated with phenol.

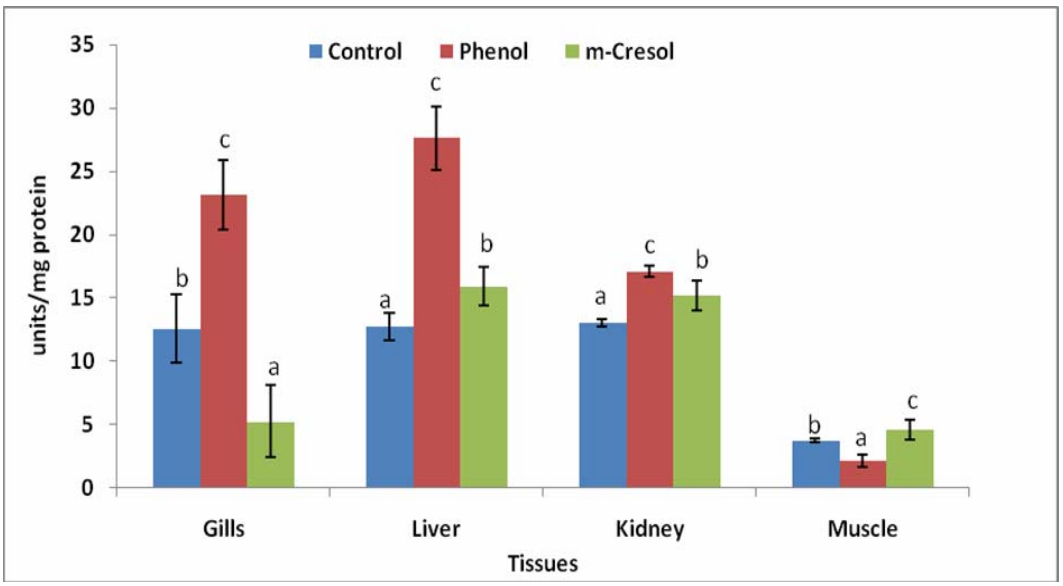

Fig. 3.1 Effect of different phenolic compounds on SOD activity in *O. mossambicus.* Each bar diagram represents mean ± S.D. On each set of bars, values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments.

Table 3.1 Effect of different phenolic compounds on SOD activity in *O. mossambicus*. Values in the same column with different upper case letters vary significantly (P<0.05) between tissues and values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups.

Tissues	SOD activity		
	Groups		
	Control	Phenol	m-cresol
Gills	$_{b}12.57\pm2.73^{B}$	$_{c}23.11\pm2.76^{B}$	$_{a}5.24\pm2.86^{B}$
Liver	$_{b}12.70\pm1.10^{D}$	$_{c}27.60\pm2.47^{D}$	$_{a}15.90\pm1.54^{D}$
Kidney	$_{b}13.0\pm0.25^{C}$	$_{c}17.10\pm0.45^{C}$	$_{a}15.21\pm1.17^{C}$
Muscle	$_{b}3.73\pm0.13^{A}$	$_{c}2.11\pm0.45^{A}$	$_{a}4.60\pm0.77^{A}$

- Values are expressed as units/mg protein. One unit is defined as the amount of enzyme which gives 50% inhibition of formazon formation / minute.
- Each value represents the mean ± S.D of six separate experiments

3.4.2 Catalase (CAT)

In the present study catalase activity in different tissues of *O. mossambicus* treated with different phenolic compounds showed significant variations ($P < 0.05$), (Fig 3.2 and Table 3.3) compared to control group. Tukey's test showed significant difference between phenolic compounds treated groups and also with the control. Highest CAT activity was found in the liver of fishes treated with m-cresol. On treatment with both phenol and m-cresol gills, liver and kidney showed significantly elevated CAT activity compared to control. Comparison between groups treated with different phenolic compounds revealed that there was significant increase ($P<0.05$) in CAT activity in all tissues compared to control except in muscle. Muscle showed a statistically significant decreased activity compared to control.

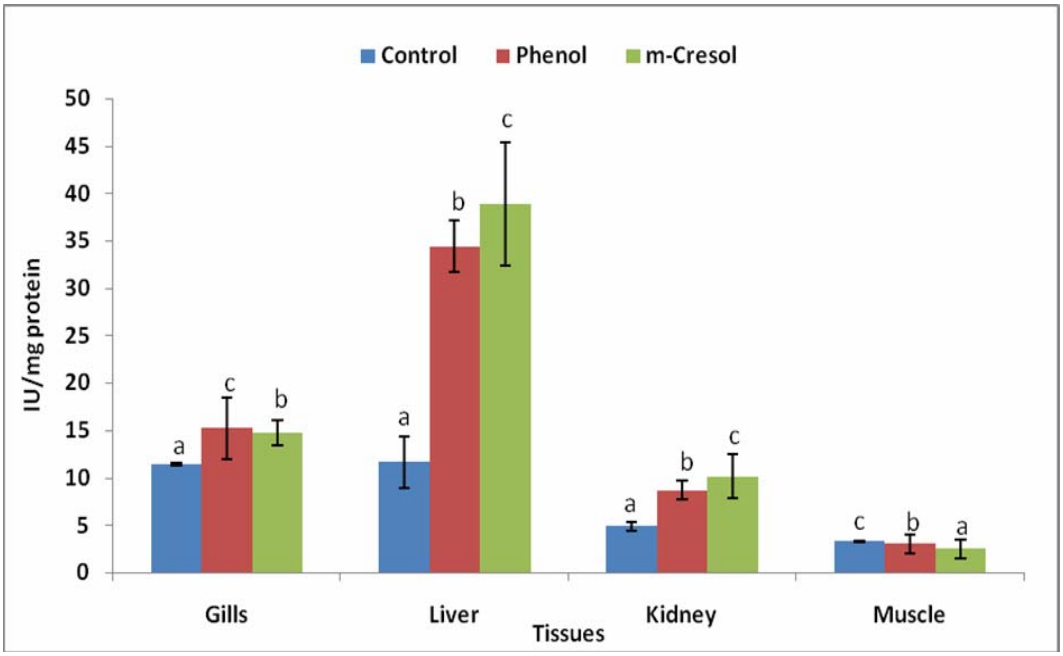

Fig 3.2. Effect of different phenolic compounds on CAT activity in *O. mossambicus*. Each bar diagram represents mean ± S.D. On each set of bars, values with different lower case letters vary significantly ($P < 0.05$) in each tissue on different treatments.

Table 3.2. Effect of different phenolic compounds on CAT activity in *O. mossambicus*. Values in the same column with different upper case letters vary significantly (P<0.05) between tissues and values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups.

Tissues	CAT activity		
	Groups		
	Control	Phenol	m-cresol
Gills	$_{a}11.07\pm0.10^{C}$	$_{b}15.06\pm3.22^{C}$	$_{c}14.44\pm1.32^{C}$
Liver	$_{a}11.50\pm2.76^{D}$	$_{b}34.34\pm2.74^{D}$	$_{c}38.87\pm6.49^{D}$
Kidney	$_{a}4.81\pm0.48^{B}$	$_{b}8.62\pm0.98^{B}$	$_{c}9.86\pm2.31^{B}$
Muscle	$_{a}3.24\pm0.11^{A}$	$_{b}2.97\pm0.99^{A}$	$_{c}2.53\pm0.98^{A}$

- One IU = Change in absorbance at 230 nm / min, Extinction Coefficient = 0.021
- Each value represents the mean ± S.D of six separate experiments

3.4.3 Glutathione peroxidase (GPx)

Glutathione peroxidase activity showed an overall significant change (P<0.05) in experimental groups of animal (Fig 3.3and Table 3.4) compared to control. Tukey's test showed significant difference between phenolic compounds treated groups and also with the control. Statistical analysis between tissues showed that GPx activity was found to show a statistically significant (P<0.05) decreased activity in liver and kidney of the treated groups compared to control. Whereas gills treated with phenol showed a decreased GPx activity compared to control. On treatment with both phenol and m-cresol muscle showed a significantly (P<0.05) elevated activity compared to control.

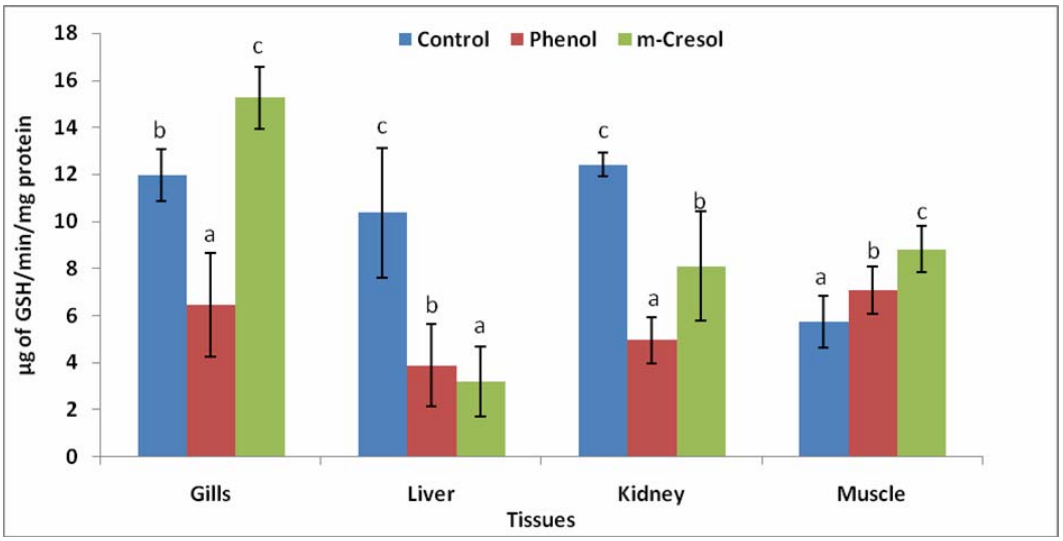

Fig.3.3 Effect of different phenolic compounds on GPx activity in *O. mossambicus.* Each bar diagram represents mean ± S.D. On each set of bars, values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments.

Table 3.3 Effect of different phenolic compounds on GPx activity in *O. mossambicus.* Values in the same column with different upper case letters vary significantly (P<0.05) between tissues and values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups.

Tissues	GPx activity		
	Groups		
	Control	Phenol	m-cresol
Gills	$_{c}11.98\pm1.10^{D}$	$_{a}6.45\pm2.22^{D}$	$_{b}15.25\pm1.32^{D}$
Liver	$_{c}10.36\pm2.76^{A}$	$_{a}3.89\pm1.74^{A}$	$_{b}3.2\pm1.49^{A}$
Kidney	$_{c}12.42\pm0.48^{C}$	$_{a}4.95\pm0.98^{C}$	$_{b}8.11\pm2.31^{C}$
Muscle	5.72 ± 1.11^{B}	$_{a}7.07\pm0.99^{B}$	$_{b}8.82\pm0.98^{B}$

- Values are expressed as µg of GSH/min/mg protein.
- Each value represents the mean ± S.D of six separate experiments.

3.4.4 Glutathione-S-transferase (GST)

In the present study, glutathione-S-transferase activity in different tissues of *O. mossambicus* treated with different phenolic compounds showed significant variations (P<0.05), (Fig 3.2 and Table 3.5) compared to control group. Tukey's

test showed significant difference among the phenolic compounds treated groups and also with the control. Among the tissues treated with different phenolic compounds highest GST activity was seen in liver. Both kidney and muscle showed significantly ($P<0.05$) decreased GST activity compared to control. Significant differences were found in GST activity between the phenol and m-cresol treated groups and also with the control.

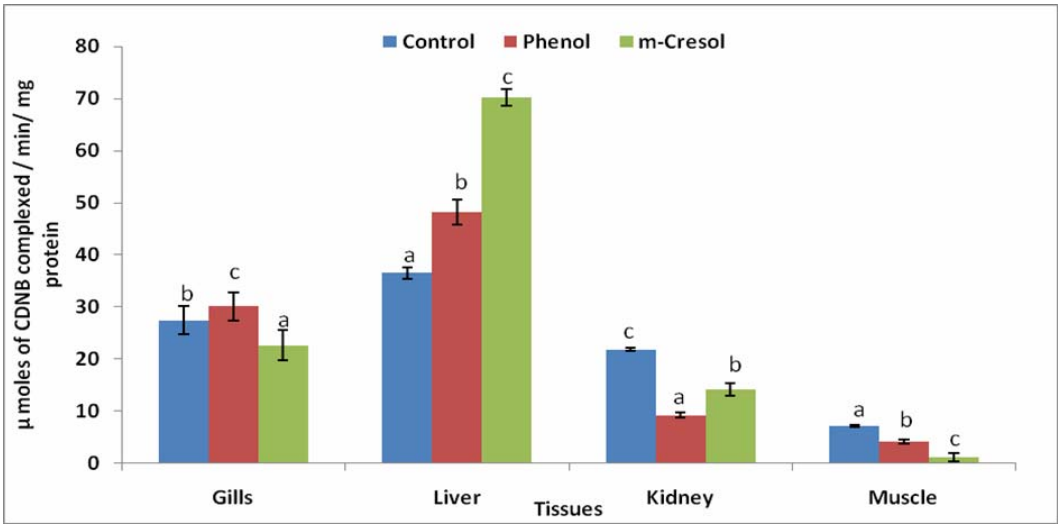

Fig. 3.4 Effect of different phenolic compounds on GST activity in *O. mossambicus.* Each bar diagram represents mean ± S.D. On each set of bar, values with different lower case letters vary significantly ($P<0.05$).

Table 3.4 Effect of different phenolic compounds on GST activity in *O. mossambicus.* Values in the same column with different upper case letters vary significantly ($P<0.05$) between tissues and values in the same row with different lower case letters vary significantly ($P<0.05$) between treatment groups.

Tissues	GST activity		
	Groups		
	Control	Phenol	m-cresol
Gills	$_{b}27.41\pm2.73^{C}$	$_{c}30.11\pm2.76^{C}$	$_{a}22.62\pm2.86^{C}$
Liver	$_{b}36.58\pm1.10^{D}$	$_{c}48.20\pm2.47^{D}$	$_{a}70.2\pm1.54^{D}$
Kidney	$_{b}21.82\pm0.25^{B}$	$_{c}9.21\pm0.45^{B}$	$_{a}14.24\pm1.17^{B}$
Muscle	$_{b}7.13\pm0.13^{A}$	$_{c}4.12\pm0.45^{A}$	$_{a}1.21\pm0.77^{A}$

- Values are expressed in µmoles of CDNB complexed / min/ mg protein.
- Each value represents the mean ± S.D of six separate experiments.

3.4.5 Total reduced Glutathione (GSH)

Two-factor ANOVA followed by Tukey's test showed that there was significant (P<0.05), (Fig 3.5 and Table 3.6) variation in total reduced glutathione content between treated groups and between tissues treated with different phenolic compounds. There was statistically significant (P<0.05) different changes in the GSH level among the treated groups and between the treated groups and the control. Among the tissues, gills, liver and muscle showed significantly (P<0.05) elevated activity compared to control but the kidney in both the treated groups showed statistically significant (P<0.05) reduced activity compared to control.

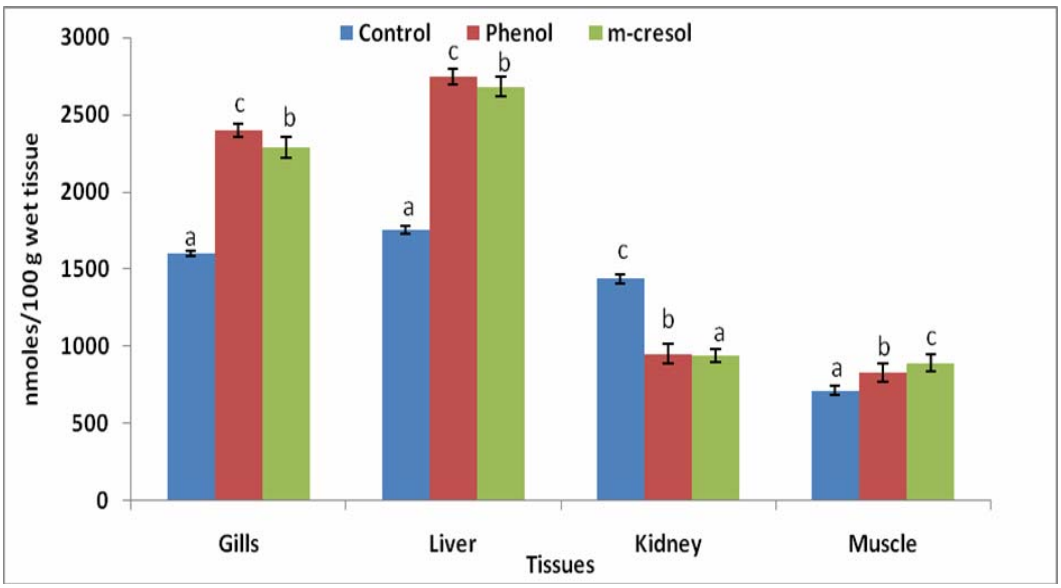

Fig. 3.5 Effect of different phenolic compounds on total reduced GSH content in *O. mossambicus.* Each bar diagram represents mean ± S.D. On each set of bar, values with different lower case letters vary significantly (P<0.05).

Table 3.5 Effect of different phenolic compounds on total reduced GSH content in *O. mossambicus.* Values in the same column with different upper case letters vary significantly (P<0.05) between tissues and values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups.

Tissues	GSH content		
	Groups		
	Control	Phenol	m-cresol
Gills	$_{a}1601.5\pm20.1^{C}$	$_{c}2401\pm40.2^{C}$	$_{b}2290.3\pm69.3^{C}$
Liver	$_{a}1757.8\pm25.7^{D}$	$_{c}2745.8\pm52.7^{D}$	$_{b}2681.3\pm61.4^{D}$
Kidney	$_{a}1436.8\pm30.4^{B}$	$_{c}952.6\pm64.9^{B}$	$_{b}937.4\pm42.3^{B}$
Muscle	$_{a}716.1\pm27.1^{A}$	$_{c}830.2\pm60.9^{A}$	$_{b}892.2\pm56.9^{A}$

- Values were expressed as nmoles/100 g wet tissue.
- Each value represents the mean ± S.D of six separate experiments.

3.4.6 Conjugated dienes (CD)

Conjugated diene level in all the phenolic compounds treated groups was significantly (Fig 3.6 and Table 3.7) (P<0.05) different when compared to control. Among the tissues gills, liver and muscle showed a statistically significant elevated CD level in both the treated groups compared to control whereas the kidney in both the treated groups showed a statistically significant (P<0.05) reduced level compared to control.

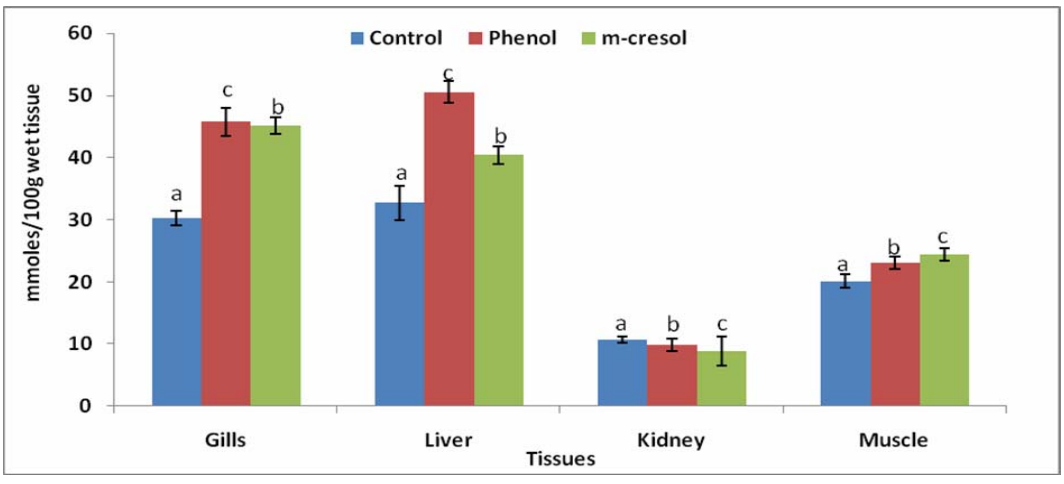

Fig. 3.6 Effect of different phenolic compounds on level of CD in *O. mossambicus.* Each bar diagram represents mean ± S.D. On each set of bar, values with different lower case letters vary significantly (P<0.05).

Table 3.6 Effect of different phenolic compounds on level of CD in *O. mossambicus.* Values in the same column with different upper case letters vary significantly (P<0.05) between tissues and values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups.

Tissues	CD level		
	Groups		
	Control	Phenol	m-cresol
Gills	$_{a}30.26\pm1.1^{C}$	$_{c}45.78\pm2.2^{C}$	$_{b}45.08\pm1.3^{C}$
Liver	$_{a}32.71\pm2.7^{D}$	$_{c}50.51\pm1.7^{D}$	$_{b}40.37\pm1.4^{D}$
Kidney	$_{a}10.72\pm0.4^{A}$	$_{c}9.91\pm0.9^{A}$	$_{b}8.84\pm2.3^{A}$
Muscle	$_{a}20.11\pm1.1^{B}$	$_{c}23.11\pm0.9^{B}$	$_{b}24.44\pm0.9^{B}$

- Values are expressed as mmoles/100g wet tissue.
- Each value represents the mean ± S.D of six separate experiments.

3.4.7 Hydroperoxides (HP)

The level of hydroperoxides in the groups treated with both the phenol and m-cresol showed statistically significant (P<0.05) (Fig 3.6 and Table 3.8) difference between them and also with the control group. Tissues such as gills, liver, kidney and muscle showed statistically significant (P<0.05) elevated levels compared to control. Among the tissues the highest level of hydroperoxide was seen in liver.

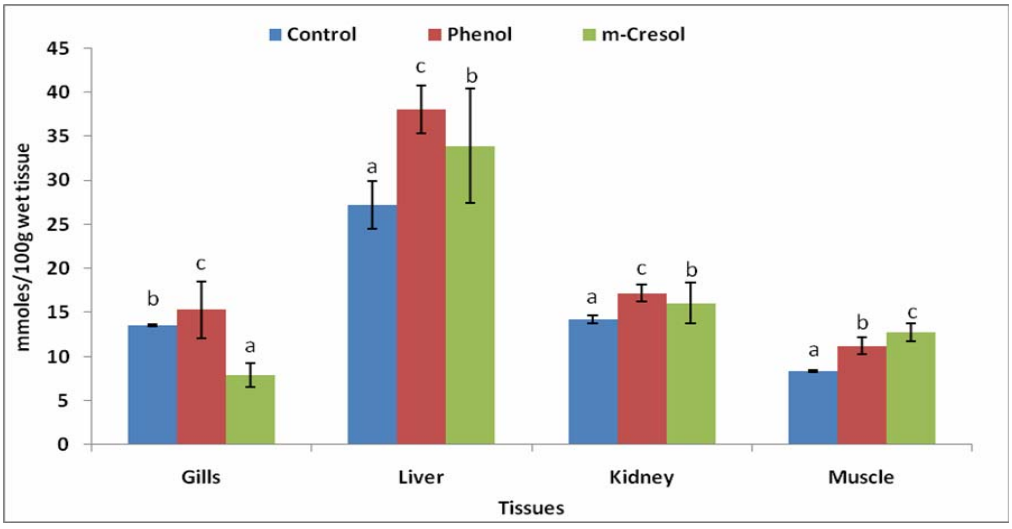

Fig.3.7 Effect of different phenolic compounds on level of HP in *O. mossambicus.* Each bar diagram represents mean ± S.D. On each set of bar, values with different lower case letters vary significantly (P<0.05).

Table 3.7 Effect of different phenolic compounds on level of HP in *O. mossambicus.* Values in the same column with different upper case letters vary significantly (P<0.05) between tissues and values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups.

Tissues	HP level		
	Groups		
	Control	Phenol	m-cresol
Gills	$_{a}13.5\pm0.10^{B}$	$_{c}15.33\pm3.2^{B}$	$_{b}7.91\pm1.32^{B}$
Liver	$_{a}27.21\pm2.76^{D}$	$_{c}38.07\pm2.7^{D}$	$_{b}33.90\pm6.49^{D}$
Kidney	$_{a}14.21\pm0.48^{C}$	$_{c}17.22\pm0.98^{C}$	$_{b}16.08\pm2.31^{C}$
Muscle	$_{a}8.31\pm0.11^{A}$	$_{c}11.24\pm0.99^{A}$	$_{b}12.73\pm0.98^{A}$

- Values are expressed as mmoles/100g wet tissue.
- Each value represents the mean ± S.D of six separate experiments.

3.4.8 Malondialdehyde (MDA)

No significant difference (Fig 3.8 and Table 3.9) in MDA level was found in gills, kidney and muscle among the treated groups. Among the tissues, statistically significant (P<0.05) elevated MDA level was found in gills and liver compared to control.

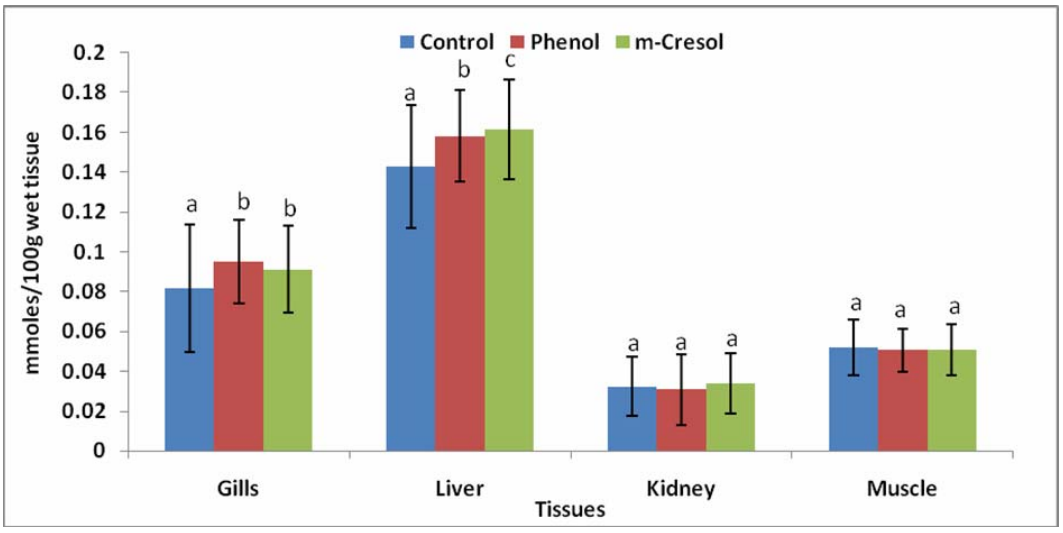

Fig.3.8 Effect of different phenolic compounds on MDA level in *O. mossambicus.* Each bar diagram represents mean ± S.D. On each set of bar, values with different lower case letters vary significantly (P<0.05).

Table 3.8 Effect of different phenolic compounds on MDA level in *O. mossambicus.* Values in the same column with different upper case letters vary significantly (P<0.05) between tissues and values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups.

Tissues	MDA level		
	Groups		
	Control	Phenol	m-cresol
Gills	$_{a}0.081\pm0.032^{C}$	$_{b}0.095\pm0.021^{C}$	$_{b}0.091\pm0.022^{C}$
Liver	$_{a}0.142\pm0.031^{B}$	$_{b}0.158\pm0.023^{B}$	$_{b}0.161\pm0.025^{B}$
Kidney	$_{a}0.032\pm0.015^{A}$	$_{b}0.030\pm0.018^{A}$	$_{b}0.033\pm0.015^{A}$
Muscle	$_{a}0.052\pm0.014^{D}$	$_{b}0.050\pm0.011^{D}$	$_{b}0.050\pm0.013^{D}$

- Values are expressed as mmoles/100g wet tissue.
- Each value represents the mean ± S.D. of six separate experiments.

3.5 Discussion

The antioxidant defense mechanism of *O. mossambicus* was responsive to the exposure of different phenolics. Xenobiotics such as phenol, are metabolized by the multienzymatic system cytochrome P450 (CYP) (Andersson and Förlin, 1992). Sometimes biotransformation processes lead to increase of toxicity of individual compounds by the formation of electrophilic metabolites that may bind and damage DNA or enzymes. The enzymatic bioactivation of phenolics catalyzed by cytochrome P450 leads to the formation of products such as hydroquinones, catechols and benzoquinones. The metabolites formed can cause increased generation of reactive oxygen species (ROS) or oxidative stress. Aerobic organisms have developed through evolutionary processes antioxidant defense mechanisms designed to prevent cellular damage from ROS.

In the present study, almost all the tissues treated with phenol and m-cresol for 21 days in *O. mossambicus* showed significantly elevated SOD and CAT activity compared to control. SOD is the first enzyme to respond against oxygen radicals (McCord and Fridovich, 1969) and is the one that offers the greatest

response to oxidative stress (Winston and Di Giulio, 1991). The tissue specific increase in SOD activity showed the following trend for fishes treated with phenol: kidney > gills > liver whereas the muscle showed a significantly decreased SOD activity compared to control. On treatment with m-cresol, tissues such as liver, kidney and muscle showed a significantly elevated activity whereas gills showed a significantly decreased activity compared to control. Changes in the levels of superoxide dismutase have been detected in fishes exposed to various degrees of oxygen tension (Lushchak *et al*., 2001) and environmental perturbations (Achuba, 2002). Superoxide dismutase is inducible in mammals and microorganisms and the level of the enzyme increases with an increased need of protection against toxic oxygen radicals (Fridovich 1974; Trostler *et al*., 1979). Mn-containing superoxide dismutase and Cu/Zn dependent superoxide dismutase are involved in the general defense system against natural or chemically induced production of reactive oxygen species (Fridovich, 1986). Catechol increases the reduction of O_2 and this may have resulted in an increased SOD activity. Also catechol reduces the dismutation of O_2, and thus leads to the production of larger amounts of H_2O_2. Thus for the detoxification of increased H_2O_2 generated a significantly elevated CAT activity was observed in gills, liver and kidney of fishes treated with both the phenolics whereas muscle showed a significantly decreased CAT activity compared to control in both phenol and m-cresol treated groups. An increased generation of H_2O_2 may have occurred due to several reasons such as oxygen depletion, (Penning *et al*., 1996), dismutation reaction of $O_2^{\cdot-}$ catalyzed by increased SOD activity.

The elevated CAT activity observed may be for the detoxification of increased H_2O_2 formed from different reactions. Therefore, the SOD-CAT system provides the first defense against oxygen toxicity. Perhaps a peroxisomal proliferation may have also occurred as they are cell organelles that play key roles in multiple cell functions (Mannaerts and Van Veldhoven, 1993) especially in the metabolism of ROS (Singh, 1996). The most abundant peroxisomal enzyme is CAT and the proliferation may have resulted in elevated CAT activity. Increase of

SOD and CAT in liver is reported in some fish species under oxidative stress (Bainy *et al*., 1996; Sayeed *et al*., 2003; Güll *et al*., 2004; Zhang *et al*., 2004; Nam *et al*., 2005; Wilhelm-Filho *et al*., 2005). Considering the results for each tissue in both treated groups, it was found that liver showed the highest SOD and CAT antioxidant activity, both enzymes appearing to have an important role in combating the sequential generation of superoxide radical ($O_2^{\cdot}$-) and hydrogen peroxide (H_2O_2) from the intense metabolic activity characteristic of this tissue. The significant increase in catalase and superoxide dismutase activities in gills, liver and kidney examined may represent an adaptive response to protect the fish from free radical toxicity induced by phenolic compounds.

GPx glutathione peroxidase activity, a seleno-enzyme that neutralizes ROS such as organic and hydrogen peroxides (Matés, 2000) activity in gills, liver and kidney of fishes treated with phenol and m-cresol showed a significantly decreased activity compared to control. Whereas muscle in both treated groups showed a significantly enhanced activity compared to control. CAT and GPx activities are fundamental to remove hydrogen peroxide from cytoplasm, however, only the GPx activity was decreased in *O. mossambicus* exposed to both the phenolics. In theory, reduced enzymatic activity implies that some ROS are not being quenched, thus predisposing cells to oxidative stress. The low GPx activity might be due to a direct phenol inhibition of enzyme synthesis or due to increased generation of hydroperoxide which may have inhibited the enzyme activity. Also catechol toxicity is mainly associated with damage to the protein and generation of hydrogen peroxide, which is capable of causing further damage (Barreto *et al*., 2009). Significantly elevated GPx activity in muscle shows that an induction in glutathione peroxidase activity has occurred in this tissue.

GST is a multicomponent enzyme involved in the detoxification of many xenobiotics, which plays an important role in protecting tissues from oxidative stress (Fournier *et al*., 1992). GST was found to be strongly inhibited in kidney and muscle on exposure to different phenolic compounds. GST activity was found

to be highly elevated in liver on exposure to phenolics, since liver plays an important role in the detoxification of xenobiotics and in elimination by conjugating them with glutathione. GST-mediated conjugation may be an important mechanism for detoxifying peroxidised lipid breakdown products, which have a number of adverse biological effects when present in high amounts. Induced GST activity indicates the role of this enzyme in protection against the toxicity of xenobiotic-induced lipid peroxidation (Leaver and George, 1998). Many studies analyzing GST in liver of fish exposed to different insecticides showed an enzymatic induction (Andersson *et al*., 1985; Rodriguez *et al.,* 1991; Leaver *et al*., 1992; Scott *et al*., 1992). However, inhibition of GST activity has also been reported in gills of mosquito fish exposed to carbofuran (Rondon *et al.*, 2005). Thus, it is possible that the enzyme is regulated *in vivo* by, for instance, thiol-disulphide interchange and proteolysis or by some other mechanism. Since reactive metabolites of foreign compounds are substrates for glutathione transferase, an attractive idea would be that these metabolites modify the microsomal glutathione transferase covalently, thereby increasing the enzyme activity by which these reactive metabolites are eliminated through conjugation. This would allow the cell to adjust rapidly to exposure to reactive compounds. The microsomal metabolism of phenol to species which will bind to proteins is most likely catalyzed by P450 monooxygenases (; Sawahata *et al*., 1983; Wallin *et al*., 1985). These enzymes are probably the major targets for the covalent binding of phenol. It is likely that the electrophilic metabolites benzoquinone and 2- hydroxybenzoquinone conjugate with the sulphydryl group of the enzyme, thereby activating the enzyme (Irons, 1981). In summary, microsomal glutathione transferase can be activated by reactive metabolites of phenol and m-cresol, and is caused by covalent binding of the metabolites to the enzyme.

GSH is the major cytosolic low molecular weight sulphydryl compound that acts as a cellular reducing and a protective reagent against numerous toxic substances including most inorganic pollutants, through the –SH group (Stryer, 1988). Gills,

liver and muscle showed elevated GSH level when treated with phenolics. Among the tissues, GSH level was found to be highest in liver compared to other tissues which may be due to an adaptive mechanism to slight oxidative stress through an increase in its synthesis which can be provided for the increased GST activity. However, a depletion of GSH was observed in kidney which shows that severe oxidative stress may suppress GSH levels due to loss of adaptive mechanisms and the oxidation of GSH to GSSG. During scavenging the ROS, GSH is oxidized and forms glutathione-protein mixed disulphides; hence, the cell's ability to reduce or synthesize GSH is the key to how effectively the cell can manage the oxidative stress. Total glutathione will be a prospective biological index to indicate exposure to contaminants (Stein *et al*., 1992). Due to its function in resisting the reactive oxygen toxicity, the changing degree for total glutathione can serve as markers of exposure to pollutants which disturb the piscine oxyradicals.

The conjugated diene level was found to be elevated in liver, kidney and muscle of both the treated groups and also in gills treated with phenol. CD is the initial peroxidative product and is an accurate indicator of lipid peroxidation and its elevated level indicated that lipid peroxidation has been initiated. An increased hydroperoxide level was observed in liver, kidney and muscle of both the treated groups which may be due to decreased GPx activity observed in these tissues. This maybe because GPx catalyzes the reduction of H_2O_2 derived from oxidative metabolism as well as peroxides from oxidation of lipids and is considered the most effective enzyme against lipid peroxidation (Winston and Di Giulio, 1991). Being more polar than parent lipids, hydroperoxides perturb membrane structure/function and can be deleterious to cells (Girotti, 1998). An increased MDA level was observed in both gills and liver on exposure to different phenolics indicating that elevated antioxidant enzyme activities were not efficient enough to prevent lipid peroxidation in these tissues. Significant oxidative damage and lipid peroxidation should theoretically occur if antioxidant defenses were overwhelmed by ROS production (Kappus, 1987; Halliwell and Gutteridge, 1989; Winston and

Di Giulio, 1991). In addition to changes in the antioxidant defense system, one of the hallmarks of oxidative stress is damage to biological macromolecules such as the phospholipids of cell membranes (Shi *et al*., 2004). MDA is a major oxidation product of peroxidized polyunsaturated fatty acids and increased MDA content is an important indicator of lipid peroxidation. Taken as a whole, our data seems to implicate phenolic compounds as a potent mediator of free radical generation in fish.

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Chapter 4

EFFECTS OF DIFFERENT PHENOLIC COMPOUNDS ON BRANCHIAL ATPases, SERUM IONS AND HAEMATOLOGICAL PARAMETERS IN *OREOCHROMIS MOSSAMBICUS*

Contents

4.1 Studies on Branchial ATPases and serum ions

4.1.1 Introduction

Fishes are primary aquatic vertebrates using gills for respiration. The gill surface is the principal organ for oxygen exchange between the fish and its environment. Under certain conditions, it is also the site for uptake of environmental contaminants from water. Fish are able to uptake and retain different xenobiotics dissolved in water via active or passive processes. Hence they can be used to detect and document pollutants released into their environment. So these extrinsic stressors could influence branchial activities and jeopardise the homeostatic control of the body fluids (Wendelaar-Bonga and vander Meij, 1989). In teleosts, the most important route of elimination for neutral, water-soluble, low molecular-weight chemicals is across the gills. Pollutants can affect the respiratory and osmoregulatory function of gills. (Mallat, 1985). Most stressors affect branchial structure and as a result hydromineral balance. This is one of the main reasons for the high vulnerability of fish to water pollutants.

The penetration of toxic products by direct route (from contaminants present in the water) depends on crossing the branchial barrier or the cutaneous layers. The presence of scales and mucus considerably reduces the accessibility of the exogenous molecules to the epidermal layer. Whereas the anatomical and physiological features of fish gills that promote efficient exchange of respiratory gases also contribute to uptake of xenobiotic compounds directly from water - namely a thin membrane separating blood and water, large surface area and high rates of counter-current blood (perfusion) and water (ventilation) flow. Its complexity and constant contact with the external environment make the gill the first target to waterborne pollutants (Mallatt, 1985; Perry and Laurent, 1993). In fact, pollutants not only enter the organism through the gills, but also exert their primary toxic effects on the branchial epithelium (Playle *et al*., 1992) which in turn may influence general gill functions.

The lamellar structure of the fish gill is central to the functions of gas exchange, osmotic and ionic regulation, acid-base balance regulation, excretion of nitrogenous wastes, passive uptake and elimination of natural and anthropogenic lipophilic compounds. The structural complexity of the gill apparatus reflects these multiple functions (Franchini *et al*., 1994; Ribelles *et al*., 1995).

The gills consist of four branchial arches each bearing pairs of primary filaments upon which rows of secondary lamellae are situated. The filaments and lamellae are covered by epithelial cells (i.e. pavement cell (PVC), chloride cell (CC) and mucus cell, supported by a complex system of blood vessels (Laurent, 1984). Chloride cells, the ion-transporting cells in gills of fish, play an important role in the maintenance of ionic balance in these animals (Perry, 1997). Hence, the gill epithelium provides an extensive surface of contact with the environment to facilitate ion transport and gaseous exchange. But because of the highly vascular structure of this epithelium, it is also a primary target for waterborne toxicant. Counter current blood flow to different parts of the gill is under hormonal and neural control and enables the functional surface area of the gill to be regulated to meet the exchange needs of the fish. Following chronic or acute exposure to different pollutants, alterations in gill tissue can occur. These changes represent a response to stressors that have often been interpreted as non-specific (Mallat, 1985; Evans, 1987).

ATPases play an important role in maintenance of functional integrity of plasma membrane and in several intracellular functions and are considered to be a sensitive indicator of toxicity (Yadwad *et al*., 1990). They hydrolyze adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and inorganic phosphate (Pi). In this process, the energy released becomes available for cation transport (Kultz and Somero, 1995). Therefore they are enzymes concerned with immediate release of energy and are responsible for a large part of basic metabolic and physiological activities. ATPase activity can be taken as meaningful indicator of cellular activity and forms a useful toxicological tool (Rahman *et al*., 2000).

ATPase, in various ion dependent forms, is a membrane-bound enzyme and is responsible for the transport of ions through the membrane and thus regulates cellular volume, osmotic pressure and membrane permeability (Kundu *et al*., 1992). Detection of ATPase inhibition could prove to be an important index for tolerable levels of a large group of environmental contaminants (Ozcan *et al*., 2002).

The well known membrane bound ATPases are Na^+ K^+-ATPase, Mg^{2+}-ATPase and Ca^{2+}-ATPase. Na^+K^+-ATPase transports Na^+ and K^+ and play a central role in whole-body osmoregulation purposes (Sancho *et al*., 2003). Mg^{2+}-ATPase enzyme is found in association with Na^+K^+-ATPase in fish, related to the transport of Mg^{2+} across the gill epithelium, and is also essential for the integrity of the cellular membrane and for the stabilisation of branchial permeability (Reddy *et al*., 1991). Mg^{2+}-ATPase is responsible for transepithelial regulation of Mg^{2+} ions. Ca^{2+}-ATPase present in the gill membranes are probably involved in Ca^{2+} transport (Fenwick, 1976). Mg^{2+} and Ca^{2+} are essential to maintain the integrity of the cellular membrane, the intracellular cements and for the stabilisation of branchial permeability (de la Torre *et al*., 2000).

Adenosine triphosphate (ATP), the major 'energy currency' in tissues, can be generated by either aerobic or anaerobic pathways. Aerobic metabolism, in which oxygen acts as the electron acceptor, is by far the most energetically efficient mechanism for ATP production and it is therefore, advantageous to the fish to maintain oxidative pathways wherever possible. Freshwater teleosts continuously take up sodium ions from the strongly hypotonic environment in order to compensate for the diffusional loss of the cation.

In general, the gills play a much greater role in the absorption and excretion of xenobiotics by fish. Gill is the main organ for osmotic regulation in teleosts and chloride cells are the sites of ion transport across gill epithelium. Chloride cells have all the characteristic features of ion-transporting cells (Berridge and Oschman, 1972). The features include: abundance of mitochondria and extended tubular system where ion transporting enzymes such as Na^+K^+-ATPase, Ca^{2+}-ATPase and Na^+,Ca^{2+}-

exchangers are located, (Karnaky *et al*., 1976; Flik *et al.,* 1985*a*; Perry and Flik, 1988; Verbost *et al,* 1994), and contact with both blood and water. A positive correlation has been found between chloride cell density and Ca^{2+} influx in the opercular membrane of a variety of fish (McCormick *et al*., 1992; Marshall *et al*., 1995).

Na^+K^+-ATPase is an important enzyme which plays a central role in whole body osmoregulation (Alam and Frankel, 2006). They provide energy for the active transport of Na^+ and K^+ across the cell membrane and also affect the transepithelial movements of cations in gills. It is found in abundance in special epithelial ionocytes commonly referred to as chloride cells (CC) or mitochondria rich cells (MRC). These cells generally compose <10% of the epithelium (Wilson and Laurent, 2002). Both ultracytochemical (Hootman and Philpott, 1979) and biochemical (Sargent *et al.,* 1975) studies have shown that the chloride cells contain the highest levels of Na^+K^+-ATPase of all branchial cells. Maintaining this Na^+ and K^+ gradient is required for the uptake of metabolites such as glucose and amino acids into cells, regeneration of transmembrane potential during nerve excitation, for muscle stimulation, maintenance of osmotic equilibrium in cells and control of transcellular ion movement.

Na^+K^+-ATPase plays a pivotal role in the gills of both marine and freshwater teleosts (Heath, 1987; Evans *et al*., 2005). This enzyme uses the chemical energy from the hydrolysis of ATP for transferring 3 Na^+ ions out of the cell and 2 K^+ ions into the cell to maintain the ionic balance. This enzyme is a membrane bound protein primarily responsible for maintaining the transmembrane Na^+ and K^+ gradients, giving a measurement of the ion-balance disturbances and the ability to osmoregulate the $Na^+ K^+$-channel movements under chemical stress (Mayer *et al*., 1989). Osmoregulatory disruption by metals and other chemicals (e.g.detergents, organochlorines and hydrocarbons) raised several questions about the possible involvement of the brain and endocrine tissues to explain this altered function (Heath, 1995). Na^+K^+-ATPase is also very important

for physiological activities including several cellular functions like ionic regulation, Ca^{2+}concentration, membrane potential, cytoplasmic enzyme activity and muscle contraction (Larsson *et al*., 1985; Diaz *et al*., 1998). Inhibition of Na^+ K^+-ATPase by xenobiotics may produce adverse effects in the organism.

Concentrations of individual ions and total osmolarity in blood plasma are physiological variables that have been used as indicators of the effects of pollution on fish (Abel, 1989). Electrolytes of body fluids have various functions - the most important of which are to contribute a majority of the osmotically active particles, to provide buffer systems and mechanisms for the regulations of pH (acid–base balance). In addition, they provide proper ionic balance for normal neuromuscular irritability and tissue functions. In the regulation of osmolarity of a system, sodium, potassium and calcium ions play significant roles to keep the hyper osmotic properties of freshwater fishes. In freshwater fish the physiological regulations of major electrolytes are very sensitive to environmental stressors and are commonly altered in response to pollutants including pesticides (McDonald *et al*., 1989).

Interaction of environmental pollutants with ATPases, however evoked a good deal of interest. Because inhibition of this enzyme occurs before gross osmoregulatory dysfunction, this would point the use of ATPases as an early warning of pollutant-induced damage to the ionic and osmoregulatory system (Stagg *et al*., 1992). The interest in understanding the physiological mechanisms associated with fish responding to environmental stressors has been growing. The gill being the dominant ionoregulatory organ and ATPases the important driving force in active transepithelial transport, important branchial ATPases such as Na^+K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase activities in crude gill homogenates were assayed. Serum ion levels reflect the dynamic equilibrium established between the fish and the ambient environment by virtue of the various osmo and iono regulatory processes acting at the gills, kidney, intestinal sites etc. As indicators of ion regulation, serum Na^+ and K^+ ion levels were also measured.

4.1.2 Materials and methods

Collection, maintenance, acclimatization and experimental design were the same as explained in detail in chapter 2, section 2.2.

4.1.2.1 Extraction of the enzyme

10% gill homogenate prepared in 0.33 M sucrose was centrifuged at 3000×g for 15 minutes and the supernatant so obtained was centrifuged at 12,000×g for 30 minutes. The supernatant was again subjected to further centrifugation at 35,000×g for 30 minutes. The pellet so obtained corresponded to heavy microsomal fraction (Davis, 1970). The pellet was then resuspended in cold 0.33 M sucrose and used as the enzyme source.

4.1.2.2 Parameters Investigated

4.1.2.2.1 Assay of Na^+K^+-ATPase

Na^+K^+-ATPase activity was assayed according to the method of Bonting (1970).

Reagents

(a) 184 mM Tris-HCl buffer, pH-7.5.

(b) 50 mM MgSO4.

(c) 50 mM KCl.

(d) 600 mM NaCl.

(e) 1 mM EDTA.

(f) 40 mM ATP.

(g) 10 % TCA

Procedure

1.0 ml of Tris-buffer and 0.2 ml of each of the reagents were mixed together. The reaction mixture was incubated at 37°C for 10 minutes and the reaction was started by the addition of 0.1 ml of homogenate. The assay medium was incubated for 15 minutes. After incubation the reaction was arrested by the

addition of 1.0 ml of 10% TCA. The enzyme source was added to control tubes after the addition of 10% TCA. The contents were centrifuged and the phosphorus content in the supernatant was estimated by the method of Fiske and Subbarow (1925). The enzyme activity is expressed as μmoles of Pi liberated/min/mg protein.

4.1.2.2.2 Assay of Ca^{2+}ATPase

Ca^{2+}-ATPase activity was assayed according to the method of Hjerton and Pan (1983).

Reagents

(a) 125 mM Tris-HCl buffer

(b) 50 mM $CaCl_2$.

(c) 10 mM ATP.

(d) 10 % TCA

Procedure

0.1 ml each of all the reagents was added and mixed well. Then 0.1 ml of distilled water and 0.1 ml enzyme was added and mixed well. The reaction mixture was incubated for 15 minutes at 37°C. After incubation, the reaction was arrested by the addition of 1.0 ml of 10% TCA. The enzyme source was added to control tubes after the addition of 10% TCA. The contents were centrifuged and the phosphorus content in the supernatant was estimated by the method of Fiske and Subbarow (1925). The enzyme activity is expressed as μmoles of Pi liberated/min/mg protein.

4.1.2.2.3 Assay of Mg^{2+}-ATPase

Mg^{2+}-ATPase activity was assayed according to the method of Ohnishi *et al*. (1982).

Reagents

(a) 375 mM Tris-HCl buffer, pH-7.6.

(b) 25 mM $MgCl_2$.

(c) 10 mM ATP.

(d) 10 % TCA

Procedure

The assay was initiated by the addition of 0.1 ml homogenate to an incubation medium containing 0.1 ml of distilled water and 0.1 ml each of the reagents. The reaction mixture was incubated for 15 minutes at 37°C. After incubation, the reaction was arrested by the addition of 1.0 ml of 10% TCA. The enzyme source was added to control tubes after the addition of 10% TCA. The contents were centrifuged and the phosphorus content in the supernatant was estimated by the method of Fiske and Subbarow (1925). The enzyme activity is expressed as μmoles of Pi liberated/min/mg protein.

4.1.2.2.4 Estimation of phosphorus

Phosphorus was estimated by the method of Fiske and Subbarow (1925).

Reagents

(a) Ammonium molybdate reagent: Added 25 g of ammonium molybdate to 200 ml distilled water. To 300 ml 10 N H_2SO_4, added molybdate solution and diluted to 1 litre with distilled water.

(b) Amino naphthol sulphonic acid (ANSA): Ground 0.2 g of ANSA with 1.2 g of Na_2SO_3 and 1.2 g of sodium bisulphate ($NaHSO_3$). Kept the mixture at 4°C. At the time of use, dissolved 0.25 g in 10 ml distilled water.

(c) Phosphorus standard: 35.1 mg of KH_2PO_4 was dissolved in 100 ml double distilled water. Working standard was prepared by taking 1.0 ml of the stock and diluting to 10 ml with double distilled water.

Procedure

Graded volumes of phosphorus standard solution were pipetted out into a series of test tubes and made up to 1ml using distilled water. Suitable aliquots of the enzyme source were pipetted out in to a series of test tubes and made up to

1ml using distilled water. Added 1.0 ml of ammonium molybdate reagent to all the tubes. It was mixed well and allowed to stand at room temperature for 10 minutes. Then added 0.1ml ANSA and incubated at room temperature for 10 minutes. The blue colour developed was read at 680 nm against a blank containing all the reagents.

4.1.2.2.5 Estimation of serum ions

Serum ions were estimated by flame photometry.

Standards

Mixed standards were prepared by using the following two stock standards.

(a) Stock standard for sodium: 1000 mEq/l: It was prepared by dissolving 5.85 g of analar grade sodium chloride in distilled water and diluted to 100 ml.

(b) Stock standard for potassium: 100 mEq/l: It was prepared by dissolving 0.74 g of analar potassium chloride in distilled water and diluted to 100 ml.

Mixed working standards were prepared as follows:

(a) Sodium/ potassium: 120/2.0 mEq/l: It contained 120 mEq of sodium and 2.0 mEq of potassium per litre of distilled water. It was prepared by mixing 12 ml of stock standard A and 2.0 ml of stock standard B in 86 ml of distilled water.

(b) Sodium/ potassium: 140/4.0 mEq/l: It was prepared by mixing 14 ml of stock standard A and 4.0 ml of stock standard B in 82 ml of distilled water.

(c) Sodium/ potassium: 160/6.0 mEq/l: It was prepared by mixing 16 ml of stock standard A and 6.0 ml of stock standard B in 78 ml of distilled water.

Procedure

Diluted 0.1 ml of serum with 10ml of distilled water. 0.1 ml each was taken from all the three working standards and each one diluted with 10ml of distilled

water in three separate beakers. The air compressor was put on and a pressure of 12 lb/sq.inch was adjusted. Gas flame was adjusted till the flame is divided into five sharp cones. Proper filters were selected for the simultaneous determination of sodium and potassium. Zero adjustment was done by using distilled water. Working standards 1, 2 and 3 were introduced one by one and the display showed almost accurate concentration for both sodium and potassium. Then the diluted serum sample was introduced and the readings for sodium and potassium were noted.

4.1.3 Statistical analysis

The statistical analysis was carried out using the software SPSS 13.0 package. One-way analysis of variance (ANOVA) was done followed by Tukey's test in order to determine the significant difference between different treatments. All the data were presented as mean ± S.D and the differences were regarded as statistically significant when $P<0.05$.

4.1.4 Results

4.1.4.1 Branchial ATPases

In the present study, statistical analysis revealed that there was significant variation ($P<0.05$) in the activity of branchial ATPases in *O. mossambicus* exposed to sub-lethal concentrations of different phenolic compounds. The results obtained are shown in Table 4.1and Figures 4.1, 4.2 and 4.3. Comparison between different treatments revealed that there was significant decrease ($P<0.05$) in ATPase activities of both phenol and m-cresol treated groups compared to control. Statistical analysis also revealed that phenol treated group showed the least activity among the treated groups.

4.1.4.2 Serum ions

Statistical analysis revealed significant variation ($P<0.05$) in serum ion levels in *O. mossambicus* exposed to sub-lethal concentrations of different phenolic compounds compared to control (Table 4.2). Serum Na^+ ion (Fig 4.4) level showed a significantly decreased ($P<0.05$) level in both the treated groups

compared to control. Significantly elevated ($P<0.05$) serum K^+ ion level (Fig 4.5) was observed in both the treated groups compared to control.

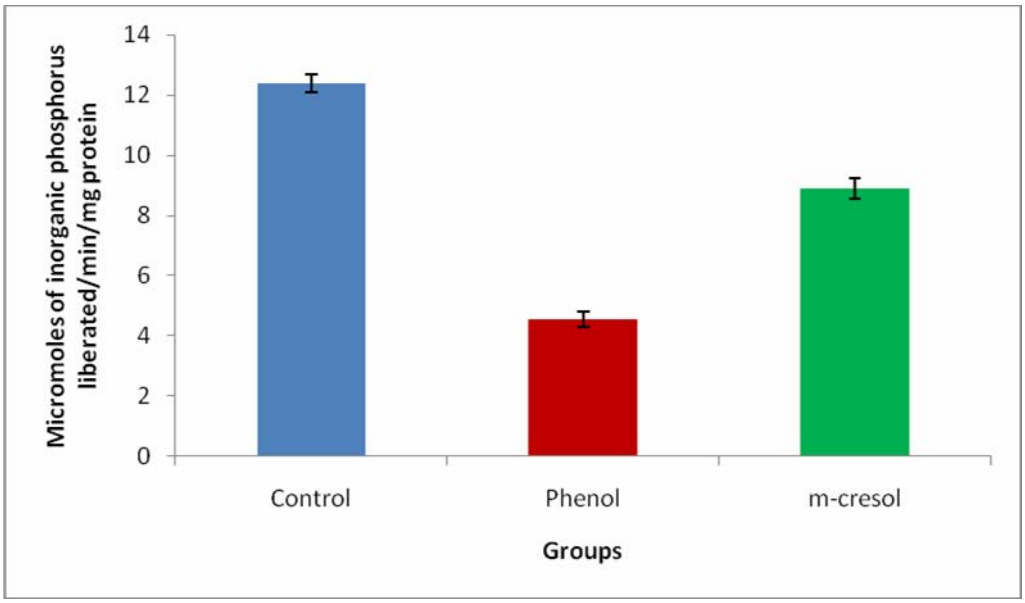

Fig.4.1 Branchial Na^+K^+- ATPase activity in *O. mossambicus* on exposure to different phenolic compounds. Each bar diagram represents mean ± S.D of six separate experiments.

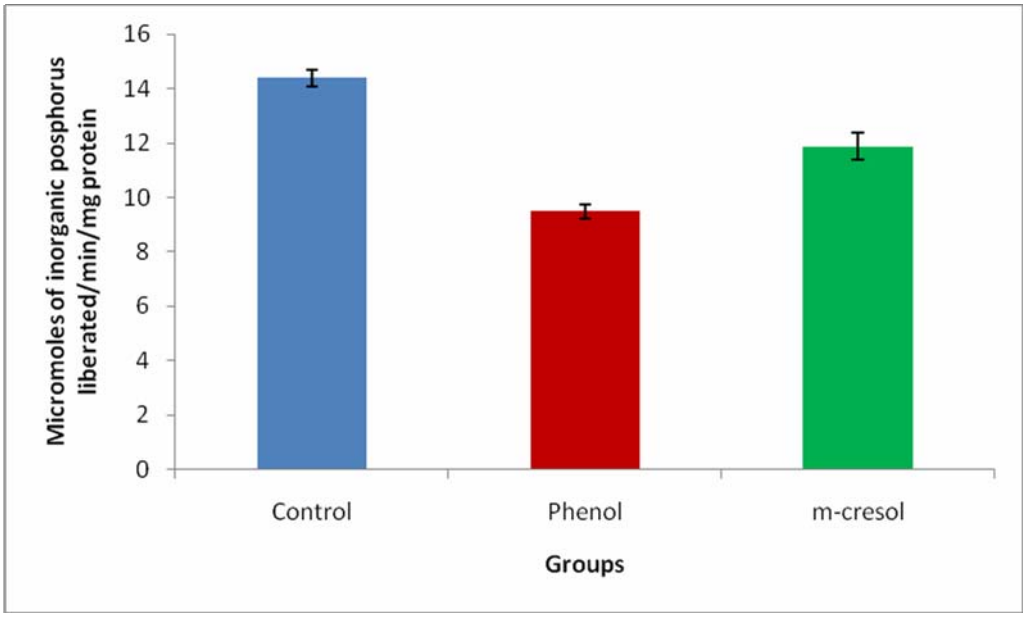

Fig.4.2 Branchial Ca^{2+}-ATPase activity in *O. mossambicus* on exposure to different phenolic compounds. Each bar diagram represents mean ± S.D of six separate experiments.

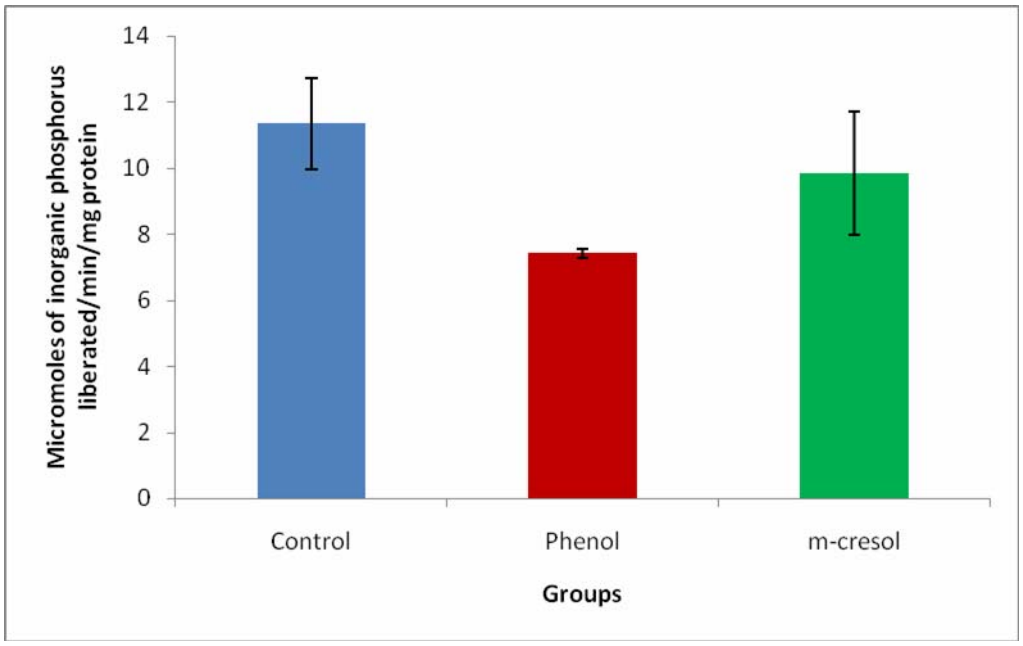

Fig. 4.3 Branchial Mg^{2+}- ATPase activity in *O. mossambicus* on exposure to different phenolic compounds. Each bar diagram represents mean ± S.D of six separate experiments.

Table 4.1 Effect of different phenolic compounds on branchial ATPases activity in *O. mossambicus*. Values in the same row with different lower case letters vary significantly ($P<0.05$) between treatment groups.

Branchial ATPases	Control	Phenol	m-cresol
$Na^{+}K^{+}$-ATPase activity	12.38 ± 0.27^{c}	4.53 ± 0.24^{a}	8.89 ± 0.34^{b}
Ca^{2+}-ATPase activity	14.49 ± 0.54^{c}	9.66 ± 0.50^{a}	12.51 ± 0.78^{b}
Mg^{2+}-ATPase activity	11.35 ± 1.37^{c}	7.42 ± 0.13^{a}	9.85 ± 1.86^{b}

- Values are expressed as µmoles of inorganic phosphorus liberated/min/mg protein.
- Each value represents the mean ± S.D of six separate experiments.

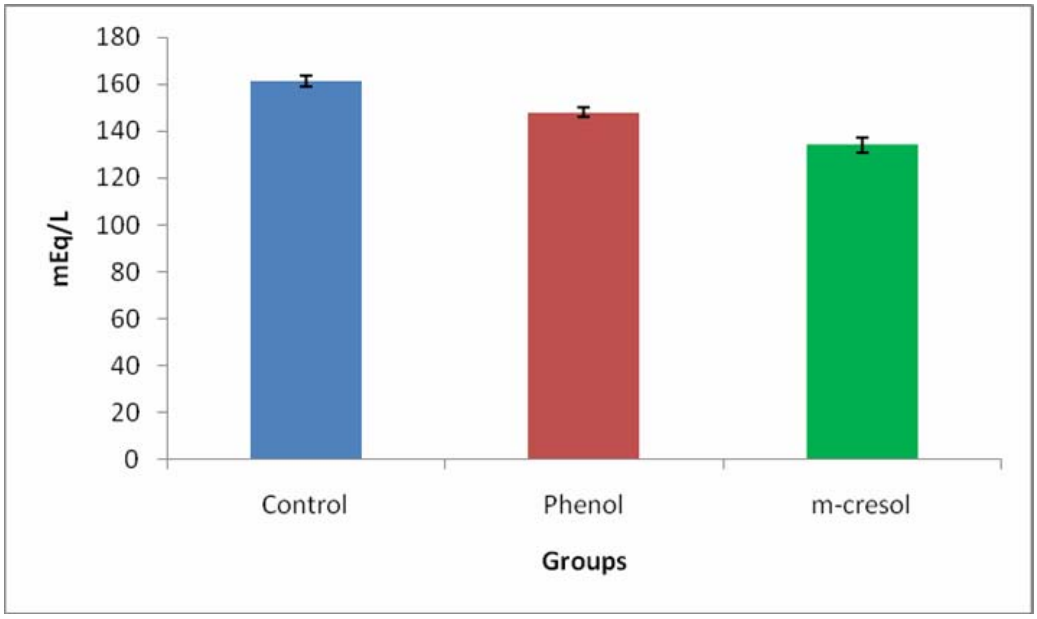

Fig. 4.4 Serum Na^+ ion level in *O. mossambicus* on exposure to different phenolic compounds.

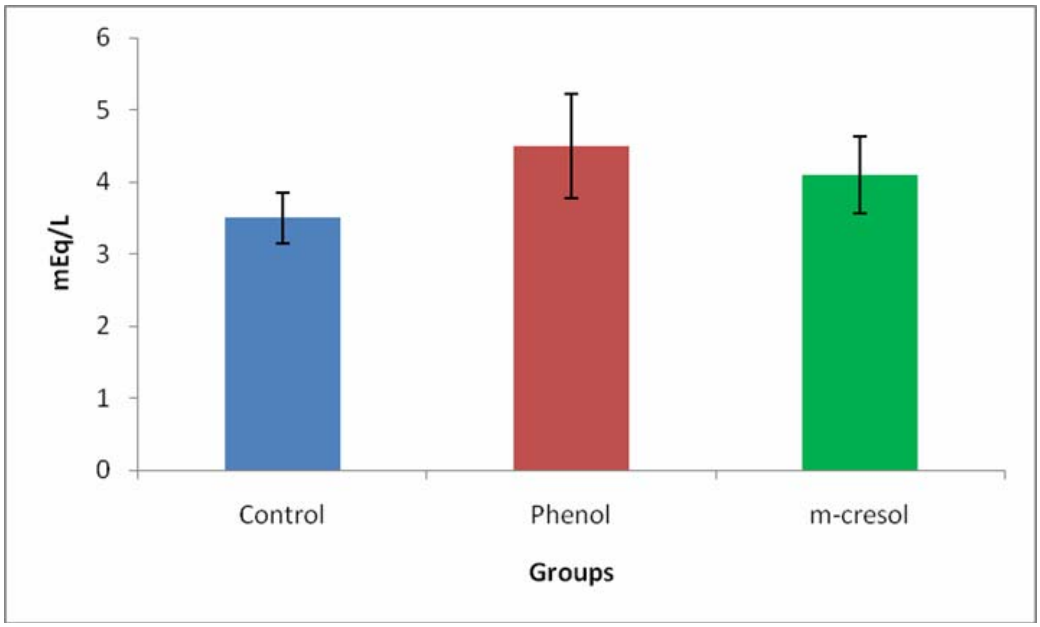

Fig. 4.5 Serum K^+ ion level in *O. mossambicus* on exposure to different phenolic compounds.

Table 4.2 Effect of different phenolic compounds on branchial ATPases activity in *O. mossambicus*. Values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups.

Serum Ions	Control	Phenol	m-cresol
Na^+	161.25 ± 2.11^c	148 ± 2.04^b	134 ± 2.97^a
K^+	3.5 ± 0.34^a	4.53 ± 0.73^c	4.13 ± 0.53^b

- Values are expressed as mEq/L.
- Each value represents the mean ± S.D of six separate experiments.

4.1.5 Discussion

In the present study, on exposure to sub-lethal concentration of both phenol and m-cresol, branchial ATPases which are important membrane bound proteins showed decreased activity compared to control. Also the serum ion levels showed significant variations compared to control thus affecting the ionic homeostasis.

Branchial Na^+K^+-ATPase activity in both the phenolic compound dosed groups showed a decreased activity compared to control. Phenol treated fishes showed decreased activity compared to m-cresol treated group. There are several findings which show that Na^+K^+-ATPase plays a central role in the whole body ion regulation. Thus, any toxicant that interferes with ionic homeostasis may be reflected as altered Na^+K^+-ATPase activity which was found to be decreased in the present investigation. Xenobiotics can also alter Na^+K^+-ATPase activity by disrupting energy-producing metabolic pathways or interacting directly with the enzyme (Alam *et al*., 2006).

As the primary link between environmental change and physiological response, the neuroendocrine system is a critical part of osmoregulatory adaptations (McCormick, 2001). Corticosteroids are believed to regulate Na^+K^+-ATPase in the teleost gill (Butler and Carmichael 1972, Forrest *et al*., 1973, Madsen 1990) both through increase in the numbers of chloride cells and the level of enzyme in each cell. Also glucocorticoids generally enhance the expression of enzymes such as ATPases, ion pumps and chloride cells (Thomas *et al.*, 1999). Cortisol can increase the cellular

differentiation of chloride cells and stimulate branchial Na^+K^+-ATPase activity (McCormick, 1995). But in the present study, a decreased cortisol level was observed when treated with phenolics which may have resulted in decreased ATPase activities. Glucocorticoid receptor gene expression in chum salmon (*Oncorhynchus keta*) chloride cells (Uchida *et al*., 1998), support a direct effect of cortisol on chloride cell function in fish (McCormick, 1995).

Decreased Ca^{2+}-ATPase and Mg^{2+}-ATPase activities were observed in both the treated groups compared to control. Inhibition of Mg^{2+}-ATPase activity by phenolic compounds may reduce ATP production as this enzyme has been reported to be involved in oxidative phosphorylation (Racker *et al*., 1975). A reduction in the activity of Ca^{2+}-ATPases indicated the interaction of phenolic compounds with the microsomal and basolateral Ca^{2+} transporting ATPases (Chris and Wong, 2000). The decreased Ca^{2+}-ATPase activity may have affected the ability to maintain the calcium homeostasis (Milhaud *et al*., 1977). Lipid peroxidation which is indicated by high malondialdehyde level in gills of both the treated groups was also found to be higher compared to control. As both these ATPases play an important role in integrity of cellular membrane and stabilisation of branchial permeability, lipid peroxidation maybe one of the reasons for decreased ATPase activity observed. A change in the permeability characteristics of gills may also have resulted in the decreased activity of ATPases.

The inhibition of Na^+ K^+-ATPase in gills probably disturbs Na^+, K^+ pump, resulting in an erratic entry of Na^+ into the cell along the concentration gradient and the water molecule follows along the osmotic gradient. This process may cause swelling of the cell and finally membrane ruptures (Ozcan *et al*., 2002). Also in the present investigation, histopathological studies showed pathological abnormalities (Chapter 6) in gills such as disruption of the secondary lamellae, epithelial desquamation and necrosis and fusion of secondary lamellae in exposed animals on exposure to both the phenolics which may also have affected the branchial ATPase activity.

The freshwater animals compensate their renal and surface loss of ions, mainly sodium and chloride, by absorbing these ions from the external medium through specialised surface structures. Gills of freshwater fish contain the machinery for the active transport of the electrolytes. Toxic substances may cause damage to gill tissues, thereby reducing the oxygen consumption and disturbing the osmoregulatory function of aquatic organisms. Potassium is the main cation of the intracellular fluid and it is also an important constituent of the extracellular fluid. Ion uptake from water is required to maintain internal acid–base balance and ionic equilibrium between blood and tissues for those ions that are continuously lost by diffusion across permeable parts of the external body surface.

In the present study, a decrease in serum sodium ion levels and an increase in potassium ion levels in the groups exposed to low dose suggest that phenolic compounds can affect osmoregulation. A reduction in the major electrolyte sodium may be due to histological alterations of gills or disturbances in the membrane permeability due to toxicity of phenolics. On exposure to phenolics, altered gill permeability was observed which could have impaired the flux of ions. Freshwater fish tends to have a passive efflux of ions (loss) and a passive influx of water through the gill epithelium (McDonald and Milligan, 1997). To cope with the change in blood osmolarity, they have two main strategies: active uptake of ions through the gill using the Na^+K^+-ATPase and production of large volumes of diluted urine in the kidney, which can also actively uptake ions (Eddy, 1981; Marshall and Grosell, 2005; Iwama *et al*., 2005). But in the present investigation branchial Na^+K^+-ATPase activity was impaired which may have affected the ionic homeostasis. de la Torre *et al*. (1999) have shown that the inhibition of this enzyme by monocrotophos prevents the buildup of high ion concentrations in the extracellular spaces resulting in a blockage of the movement of internal harmful extra ions towards the external medium via the leakage junctions.

Shifts in the hydromineral balance may be a consequence of the action of pollutants on organs involved in osmoregulation, on the endocrine system, on

metabolism or on active transport processes. Usually, after exposure to a single stressor, freshwater fish respond by increasing the efflux of ions through the gills (McDonald and Milligan, 1997). Since freshwater fish take up most of the ions necessary for homeostasis from the water via their gills, the drop of plasma electrolytes is apparently caused by an increased efflux of ions across these organs and an impairment of active ion uptake by the chloride cells of the gill (Wendelaar- Bonga and Lock, 1992). A reduction in the plasma electrolyte level has two important causes. First, there is an elevated passive efflux of ions across the gills due to more or less non-selective branchial permeability to water and ions. This may lead to haemodilution by enhanced osmotic uptake of water across the gills and to passive diffusional ion loss. Second, the inhibition of active ion uptake by the chloride cells of the gills which may further contribute to the negative ion balance of the blood. Many toxic substances including petroleum hydrocarbons cause osmoregulatory disturbances in teleosts (Englehardt *et al*., 1981). Elevated plasma cortisol may be necessary to compensate for the hydromineral imbalance caused by phenolic compounds which was also found to be decreased in the present investigation on exposure to phenolics.

Thus, ATPases are very sensitive to chemical interaction and can be used as reliable biomarker for the toxicity studies of phenolic compounds. In the present study, it has been found that exposure to phenolic compounds caused decreased activity of Na^+ K^+-ATPase in gills. This could be due to the effect of phenolic compounds on cell membrane because of their strong affinity for interaction with membrane lipids causing inhibition of membrane-bound ATPases activity by affecting enzyme complex (Mishra *et al*., 1998). For maintenance of water and ion homeostasis, a strict control of membrane permeability to water and ions, the maintenance of appropriate transepithelial electrical potentials, and the presence of efficient ion-transport mechanisms are essential. Suhel *et al*. (2005) has observed decreased gill ATPase activities in the freshwater fish *Channa punctata* (Bloch) exposed to a diluted paper mill effluent. Chromium compounds were reported to inhibit ATPases, bringing about a failure of osmoregulatory

mechanisms (Thaker *et al*., 1996). In addition to osmoregulatory mechanisms, active transport mechanisms for the absorption of nutrients and essential ions may also have been affected by the inhibition of ATPases. However, it is not clear how the gill ATPases compensate for ionic regulation in the face of exposures to environmental pollutants.

The foregoing results showed that the branchial functioning was impaired and hence the ionic homeostasis in *O. mossambicus* was affected on exposure to sub-lethal concentrations of both phenol and m-cresol.

4.2 Studies on haematological parameters

4.2.1 Introduction

Fish blood is being studied increasingly in toxicological research and environmental monitoring as a possible indicator of physiological and pathological changes in fishery management and disease investigations (Mulcahy, 1975). Alteration in physiological and biochemical parameters of toxicant treated fish has recently emerged as an important tool for water quality assessment in the field of environmental toxicology. This is because blood in the gill has direct contact with the water medium and any unfavourable change in the water could be reflected in the circulatory system. These studies could be used to indicate the health status of fish as well as water quality. Blood chemistry has long been a helpful diagnostic tool in pathological, toxicological and general clinical tests. Fish blood parameters are suitable biomarkers for evaluating the potential risk of chemicals (Roche and Boge, 1996). Fish live in very intimate contact with their environment, and are therefore very susceptible to physical and chemical changes which may be reflected in their blood components.

Blood being the medium of intercellular and intracellular transport, which comes in direct contact with various organs and tissues of the body, the physiological state of an animal at a particular time is reflected in its blood. Freshwater teleost fishes maintain their normal physiological process and their body fluid homeostasis with the help of ion/osmoregulatory processes (Hwang and Lee,

2007). Typically haematological parameters are non-specific in their responses towards chemical stressors. Nevertheless, they may provide important information in effect assessment studies for eg. by providing an indication as to the general physiology and health status of the organism under investigation (Beyer, 1996).

Haematological parameters can provide satisfactory information on the physiological response of fish to environmental stressors for two major reasons, namely, the close association of the circulatory system with the external environment and the ease of availability of fish blood (Houston, 1997; Lohner *et al*., 2001; Cazenave *et al*., 2005). The use of primary haematological indices such as blood haemoglobin (Hb), packed cell volume (PCV) and red blood cell count (RBC count) in assessing sub-lethal concentrations of two different phenolic compounds are considered. Changes in the erythrocyte count or in haemoglobin values following chronic stress are useful as indicators of blood volume changes (haemodilution or haemoconcentration) that have occurred.

4.2.2 Materials and Methods

Collection, maintenance, acclimatization and experimental design were the same as explained in detail in chapter 2, section 2.2.

4.2.2.1 Collection of blood

Blood was drawn directly from the cardinal vein using 1 ml plastic insulin syringe containing 0.2% EDTA as the anticoagulant.

4.2.2.2 Parameters investigated

(i) Estimation of Haemoglobin (Cyanmethaemoglobin method)

Blood was drawn from the common cardinal vein using 1 ml plastic syringe and used for the estimation.

Procedure

0.2 ml of blood was mixed with 5 ml of Drabkin's diluent solution and allowed to stand for 5 minutes for the formation of cyanmethaemoglobin.

Absorbance was measured 540 nm against a reagent blank which consisted of 5 ml of Drabkin's diluent solution. Using haemoglobin standard, a standard calibration curve was prepared from which the values of haemoglobin can be calculated as g/dl.

(ii) Determination of other haematological parameters

The blood was analyzed using cell counter automated analyser (Celltak marketed by Pan Company in India) for the haematological parameters such as PCV (packed cell volume) and RBC (red blood cell) count.

4.2.3 Statistical Analysis

The statistical analysis was carried out using the software SPSS 13.0 package. One-way analysis of variance (ANOVA) was done followed by Tukey's test in order to determine the significant difference between different treatments. All the data were presented as mean ± S.D. and the differences were regarded as statistically significant when $P<0.05$.

4.2.4 Results

4.2.4.1 Haematological parameters

In the present study, statistical analysis revealed that there was significant variation ($P<0.05$) in the haematological parameters in *O. mossambicus* exposed to sub-lethal concentrations of different phenolic compounds. The results obtained are shown in Table 4.2 and Figures 4.6, 4.7 and 4.8. Comparison between different treatments revealed that there was significant increase ($P<0.05$) in haemoglobin content, packed cell volume and RBC count in both phenol and m-cresol treated groups compared to control. Statistical analysis also revealed that m-cresol treated group showed the highest level among the treated groups.

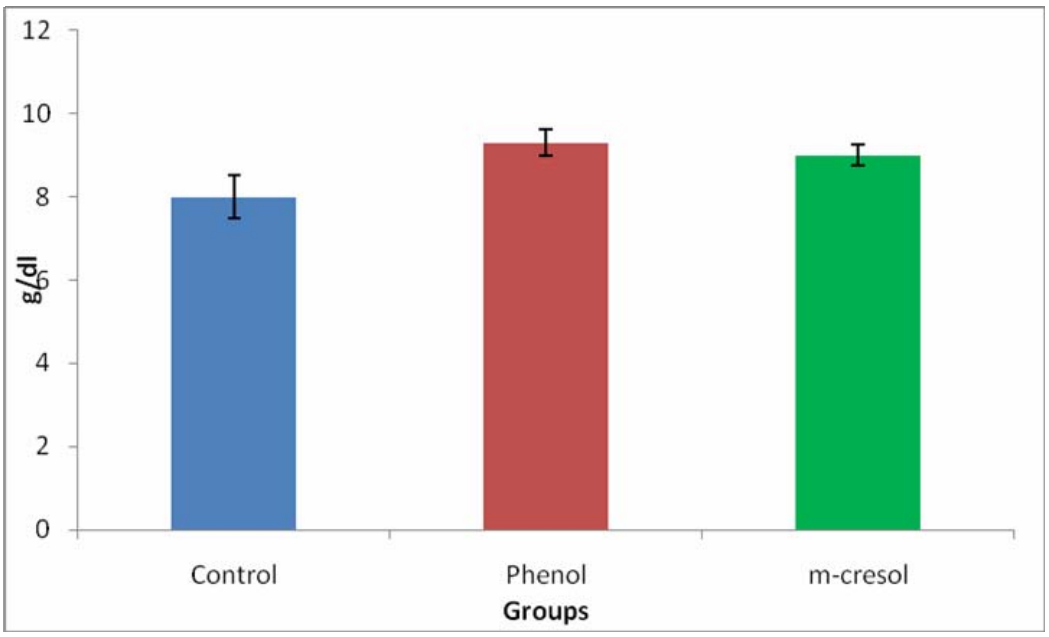

Fig. 4.6 Haemoglobin level in *O. mossambicus* on exposure to different phenolic compounds.

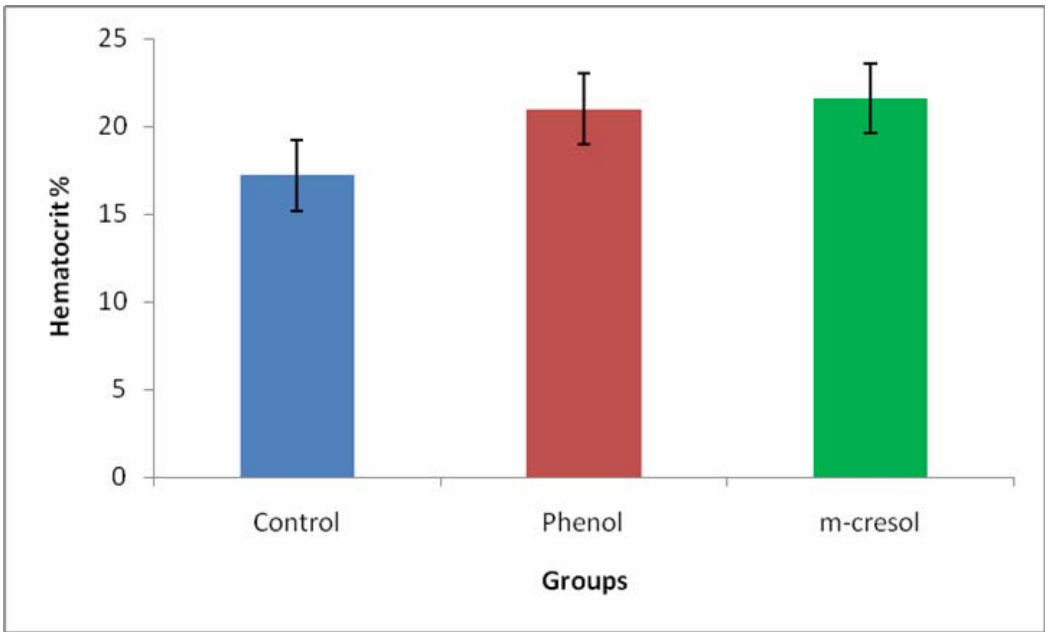

Fig 4.7 Packed Cell Volume in *O. mossambicus* on exposure to different phenolic compounds.

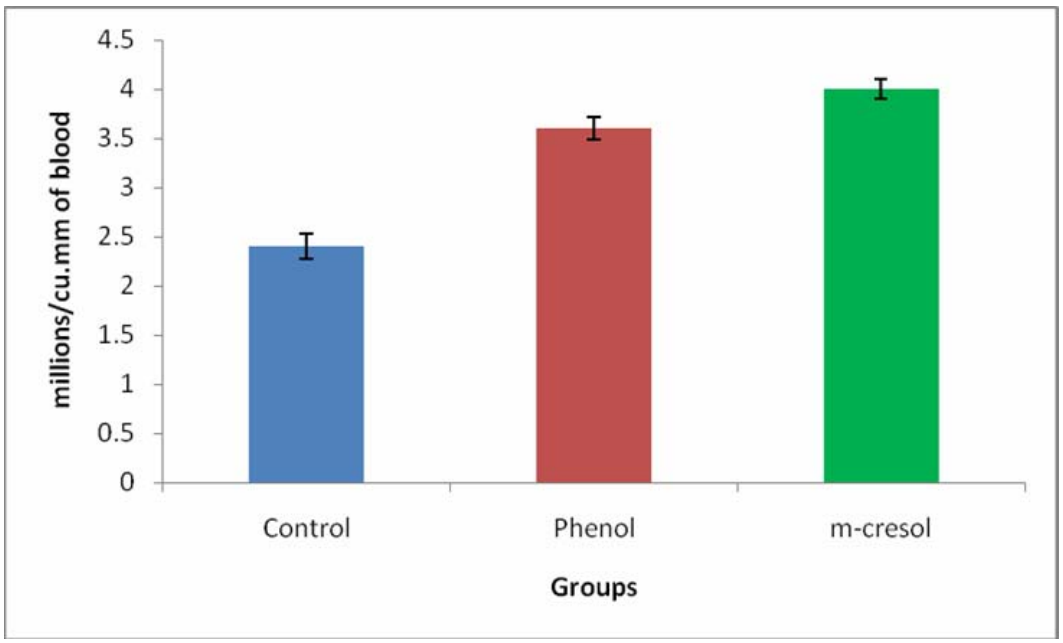

Fig.4.8 Total RBC count in *O. mossambicus* on exposure to different phenolic compounds.

Table 4.2 Effect of different phenolic compounds on haematological parameters in *O. mossambicus*. Values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups.

Haematological parameters	Control	Phenol	m-Cresol
Haemoglobin (g/dl)	8.0±0.511[a]	9.3±0.321[b]	9.0±0.25[b]
Packed cell volume (%)	17.2±0.93[a]	21±0.7[b]	21.6±0.87[b]
Total RBC count (millions/cu.mm of blood)	2.4±0.13[a]	3.6±0.11[b]	4.0±0.09[c]

- Each value represents the mean ± S.D of six separate experiments.

4.2.5 Discussion

In the present study, exposure of *O. mossambicus* to sub-lethal concentrations of phenol and m-cresol resulted in significant haematological alterations. Blood is a pathophysiological reflector of the whole body and

therefore, blood parameters are important in diagnosing the structural and functional status of fish exposed to toxicants (Adhikari *et al*., 2004).

On exposure to both the phenolic compounds, an increased red blood cell count and haematocrit was observed. The increased erythrocyte number and haematocrit value have been shown in many species exposed to chronic hypoxia. In these conditions, it appears that the spleen and maybe the liver may reactivate the erythropoiesis to compensate the demand due to the increased oxygen transport to peripheral tissues (Rifkind, 1980). Erythropoiesis, whereby the number of red blood cells in the circulation is increased is in fact a mechanism through which fish might compensate for poor oxygen uptake in prevailing hypoxic conditions (Wepener *et al*., 1992). Another mechanism by which fish might compensate for poor oxygen uptake during hypoxic conditions is via the release of a large number of mature red blood cells in the general circulation. This is thought to be stimulated by β-adrenergic action on the haemopoietic tissues, which contract and release stored mature red cells (Wepener *et al*., 1992). This mechanism might, however, compensate for short-term variations in oxygen concentration in blood or water (Nespolo and Rosenmann, 2002).

Similar results were found in reports of acute intoxication by dichlorvos on *Clarias batrachus* (Benarji, 1990), by trichlorfon on *Piaractus mesopotamus* (Tavares, 2004) and by quinalphos on *O. mossambicus* (Sampath, 1993). It has been shown that the erythrocyte number and haemoglobin levels may vary with oxygen requirement (Hubrec *et al.*, 2000; Tavares *et al.*, 2004). Therefore, the increase of packed cell volume in *O. mossambicus* is likely to be due to either increased metabolic demand or gill damages resulting in impairment of oxygen transport, or both. In fact, we also have observed drastic changes of the gill ultra-structure in the present investigation on exposure to phenolics.

An increased level of haemoglobin was observed in both the groups exposed to phenolic compounds when compared to control. Two reasons can be attributed to the increased level of haemoglobin. One is, the presence of phenolic

compounds in water creates a high oxygen demand and the compensation by the organism for low dissolved oxygen content is by synthesizing more haemoglobin for binding more oxygen. Another reason that can be attributed is; phenolic compounds or their toxic metabolites are oxidized to free radicals within erythrocytes and induce haemolysis of the erythrocyte membrane. As a consequence, haemoglobin is released which was shown as increased haemoglobin level. The released haemoglobin induces a multitude of toxic effects, summarized by Everse and Hsia (1997). The effects include nephrotoxicity through the formation of Hb dimers, formation of cross-linked haemoglobin, which induce haem oxygenase and liberation of the haem moiety. Other effects such as, cell damage, peroxidation, induction of phagocytosis and liberation of iron may also occur. The iron liberated itself can serve as a prooxidant and increases the risk of bacterial infections.

Iron liberation from oxidatively modified haemoglobin or myoglobin when drastic oxidizing conditions such as hydrogen peroxide or lipid hydroperoxides were applied was reported by Gutteridge (1986), Harel *et al.* (1988) and Rice-Evans *et al.* (1993). In the present investigation also an increased level of lipid hydroperoxides was observed on exposure to phenolic compounds. Since iron ions play an important role as redox catalysts (Fenton reaction, Haber-Weiss), iron liberation from erythrocytes will increase the total prooxidant effect of the xenobiotic.

The physiological function of haemoglobin is to transport oxygen to the tissues; this process depends on the ability of the ferrous form (Hb^{2+}) to reversibly bind molecular oxygen. However in the presence of xenobiotics, oxyhaemoglobin (Oxy-Hb) is able to turn to methaemoglobin (Met-Hb) (the Hb^{3+} form), which is unable to transport oxygen. This conversion is associated with superoxide anion production (Mishra and Fridovich, 1972) and products such as hydrogen peroxide or hydroxyl radicals, which may be derived from superoxide anion itself.

Eyer *et al.* (1975) and Riley (1984) reported that xenobiotics such as phenolic compounds are able to oxidize Oxy-Hb to Met-Hb in a so-called co-oxidation reaction in which the haem oxygen serves as the active oxidant that oxidizes both the ferrous haem centre of haemoglobin and the reducing xenobiotic (R–H):

$$Hb^{2+}O_2 + R–H \rightarrow [Met–Hb^{3+}–O–O^{2-}] + H+ + R\bullet$$

The unstable $Met–Hb^{3+}–O–O^{2-}$ complex (Gasyna, 1979) immediately stabilizes to secondary products that are dependent on the nature of the respective xenobiotic R–H: in the case of phenolic compounds (Stolze and Nohl, 1991, 1992), the transient formation of a compound l type ferryl haemoglobin has been postulated.

$$[TyrH\ Met–Hb^{3+}–O–O_2^{-}] + H^{+} \rightarrow [Tyr\bullet\ Hb_4^{+}=O_2-] + H_2O$$

The highly reactive free radical intermediates were found to attack -SH groups on the haemoglobin molecule, at position β-93 (Maples *et al.*, 1990) or on the constituents of the erythrocyte membrane. (Feix and Butterfield, 1980; Wyse *et al.*, 1989a, 1989b; Hensley *et al.*, 1993; Butterfield *et al.*, 1994).

Apart from the decrease in oxygen binding capacity, the Met-Hb generators irreversibly destroy the haem proteins with which they interact, thereby releasing metabolites that may affect thiol-dependent bioactivities and functional membrane processes. The blood responses seemingly indicate adaptation to hypoxic conditions arising from prooxidants, gill degradation and perhaps oxygen-level fluctuations. The results also show that homeostatic mechanisms were in motion to favour adaptation.

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Chapter 5

EFFECTS OF DIFFERENT PHENOLIC COMPOUNDS ON STABILITY OF BIOLOGICAL MEMBRANES

Contents

5.1 Erythrocyte membrane stability studies

5.1.1 Introduction

From the ecotoxicological point of view, interactions between toxic products and biological membranes are based on two essential aspects: first, crossing of these barriers, which is a precondition for the accessibility of these contaminants into the cell or organism; second, the perturbations brought about at the level of structure and membrane functions that present a threat to the life of the cell.

Erythrocytes represent a good model for the study of membrane stability since their lysis releases the protein haemoglobin which can be readily measured spectrophotometrically. Also its structural and functional simplicity makes it a convenient cellular model that is especially suitable for toxicity studies. The osmotic stability of erythrocytes correlates with their resistance to lysis in response to variation in solvent tonicity and can be determined by measurement of haemoglobin release after a fixed period of incubation of blood in solutions with decreasing concentrations of NaCl (Jain, 1973). Cell membranes must combine the opposing properties of fluidity and stability in order to function effectively (Cribier *et al*., 1993). A degree of fluidity is essential to allow signaling and transport. However, excessive fluidity can compromise stability and vice versa. Membrane stability represents the capacity of this biological complex to maintain its structure under chaotropic conditions such as oxidative stress (Van-Ginkel and Sevanian, 1994), hypotonicity, pH extremes, heat and the presence of solutes (such as ethanol, urea and guanidine) (Timasheff, 1998).

Haemolysis can arise from biochemical, immunologic, physical and/or chemical disturbances in the blood and more specifically from extrinsic factors such as immune disorders, infection, drugs, toxin exposure and mechanical red cell trauma (Hoffbrand *et al*., 2001). The erythrocyte membrane consists of a lipid bilayer including membrane spanning proteins, hydrophilic sugar residues (sialic acid and galactose) on the external side and a protein meshwork (spectrin) on the

cytoplasmic side of the lipid bilayer, which is connected to transmembrane proteins (band 3 proteins and glycophorin) by noncovalent binding (by ankyrin and band 4.1 proteins respectively). In addition to the mechanical stability necessary to survive the shear stress during the passage through small blood vessels, erythrocyte membrane has vital functions including cell-surface receptors, signal transducers and immunological functions (Hensley *et al*., 1993).

Sub-lethal effects of phenol on the phospholipid fatty acid composition of carp erythrocyte plasma membrane by Hossam, (1999) proved the concept that in an aquatic animal (fish) the toxicity of phenol, which is related to intercellular O_2 generation, affects membrane lipids directly, presumably by its direct action on the enzymes influencing fatty acid metabolism.

The acute toxicity of the alkylphenols (APs) increases in general with the hydrophobicity of the chemicals (McLeese *et al*., 1981) and the most toxic APs affect aquatic organisms such as fish at mg/l concentrations (McLeese *et al*., 1981; Tollefsen *et al*., 1998). Although most chemicals cause either polar or non-polar narcosis, several chemicals display greater toxicity than that can be predicted alone by a narcosis mode of action. These chemicals are believed to cause specific uncoupling of oxidative phosphorylation and lead to metabolic inhibition in organisms (Schultz *et al*., 1986). Nonylphenol and certain alkyl phenol ethoxylates have been proposed to cause alterations in the mitochondrial membrane permeability and thus cause metabolic inhibition (Argese *et al*., 1994).

Erythrocytes have been recognized as targets of many xenobiotic compounds; as a consequence, toxicity endpoints related to erythrocyte functions have been widely used in recent years to assess the adverse effects of various toxicants and to investigate the mechanisms of their action by means of suitable *in vitro* systems. In this backdrop, an attempt has been made to study the effects of different phenolic compounds (phenol, m-cresol and 4-nonylphenol) under *in vitro* conditions on the stability of erythrocyte membrane.

5.1.2 Materials and methods

In vitro studies were carried out to investigate the effects of different phenolic compounds on the stability of erythrocyte membrane. The phenolic compounds selected for the present study included phenol, m-cresol and 4-nonylphenol. The physical and chemical properties of both phenol and m-cresol were explained in detail in chapter 2, section 2.2.1.

5.1.2.1 *4-nonylphenol*

In the present study, the range of nonylphenol concentrations selected were 0.15 ppm, 0.25 ppm and 0.35 ppm and these sub lethal concentrations were chosen in accordance with various environmentally observed values (Naylor *et al*., 1992; Blackburn and Waldock, 1995; Hale *et al*., 2000).

Chemical and physical properties of 4-nonylphenol

Systematic name – 4-nonylphenol

Molecular formula- $C_{15}H_{24}O$

Molecular weight (g/mol) - 220.35 g/mol

Melting point- 43-45 °C

Boiling point- 180-181 °C

p*K*a – 10.28

5.1.2.2 *In vitro* erythrocyte membrane stability studies

Collection of blood: Blood was collected from the experimental animal *O. mossambicus* of size 15 ± 3 g from the cardinal vein in plastic syringes containing sodium citrate as the anticoagulant.

Preparation of stock erythrocyte suspension

A stock suspension of erythrocyte was prepared from fresh blood collected and was suspended in centrifuge tubes containing isotonic saline. The tubes were then centrifuged at 1000×g for 10 minutes. The upper layer containing plasma and buffy coat was removed and the pellet was resuspended in isotonic saline. The

pellet was washed three times with isotonic saline and then centrifuged at 3000×g for 8 minutes. The pellet obtained after centrifugation was again suspended in isotonic saline and was used as stock erythrocyte suspension.

Reagents

(a) Isotonic solution - 154 mM NaCl in 10 mM sodium phosphate buffer (pH, 7.4).

(b) Hypotonic solution – 50 mM NaCl in 10 mM sodium phosphate buffer (pH, 7.4).

Procedure

Different volumes of stock RBC suspension were mixed with distilled water to haemolyse the cells and centrifuged at 1000×g for 5minutes. The absorbance of the supernatant was read at 540 nm against distilled water as blank. The dilution giving a suitable absorbance for 100% haemolysis was selected. Also a suitable volume of blood giving a suitable absorbance for 100% haemolysis was noted.

The experiment was done with three different phenolic compounds as described below.

(a) To 0.2 ml of the RBC stock suspension in a centrifuge tube, 5 ml of isotonic saline were added and incubated for 30 minutes at room temperature. Then the tubes were centrifuged at 1000×g for 5 minutes. The absorbance of the supernatant was read at 540 nm. This gives the absorbance of the 'blank' (B).

(b) To 0.2 ml of the RBC stock suspension in a centrifuge tube, 4.5 ml of distilled water were added and incubated for 30 minutes at room temperature. To this 0.5 ml of phenolic compounds was added (such that the final concentration was 1/10, 1/5 and 1/3 of LC_{50} value of the phenolic compounds). Then it was centrifuged at 1000xg for 5 minutes. The absorbance of the supernatant was read at 540 nm. This gives the absorbance corresponding to 100% haemolysis (H).

(c) To 0.2 ml of the RBC stock suspension in a centrifuge tube, 4.5 ml of hypotonic saline was added and incubated for 30 minutes at room

temperature. Then 0.5 ml of the phenolic compounds was added (such that the final concentration was 1/10, 1/5 and 1/3 of LC_{50} value of the phenolic compounds). Then the tubes were immediately centrifuged at 1000×g for 5 minutes. The absorbance of the supernatant was read at 540 nm. This gives the absorbance of control (C).

(d) An acetone control was also run for the experiments using 4-nonylphenol.

(e) To 0.2 ml of the RBC stock suspension in a centrifuge tube, 4.5 ml of hypotonic saline and 0.5 ml of phenolic compounds were added (such that the final concentration was 1/10, 1/5 and 1/3 of LC_{50} value of the phenolic compounds) and incubated for 30 minutes at room temperature. Then the tubes were centrifuged at 1000×g for 5 minutes. The absorbance of the supernatant was read at 540 nm. This gives the absorbance corresponding to the 'test' (T).

Calculations

% Haemolysis in the control (X) = (C-B) / (H-B) × 100

% Haemolysis in the test (Y) = (T-B) / (H-B) × 100

% labilisation by test =(Y-X) / X × 100

5.1.3 Statistical analysis

The statistical analysis was carried out using the software SPSS 13.0 package. One-way analysis of variance (ANOVA) was done followed by Tukey's test in order to determine the significant difference between different treatments. The results obtained as percentage haemolysis after exposure to different sub-lethal concentrations of phenolic compounds were analysed statistically using one-way ANOVA followed by Tukey's test. Significance level (P value) was set at < 0.05 in all tests**.**

5.1.4 Results

5.1.4.1 Erythrocyte membrane stability studies

In the present study, statistical analysis revealed that there was significant variation ($P<0.05$) in the % haemolysis on exposure to different sub-lethal

concentrations of phenolic compounds in *O. mossambicus*. The results obtained are shown in Table **5.1** and Figures **5.1, 5.2** and **5.3**.

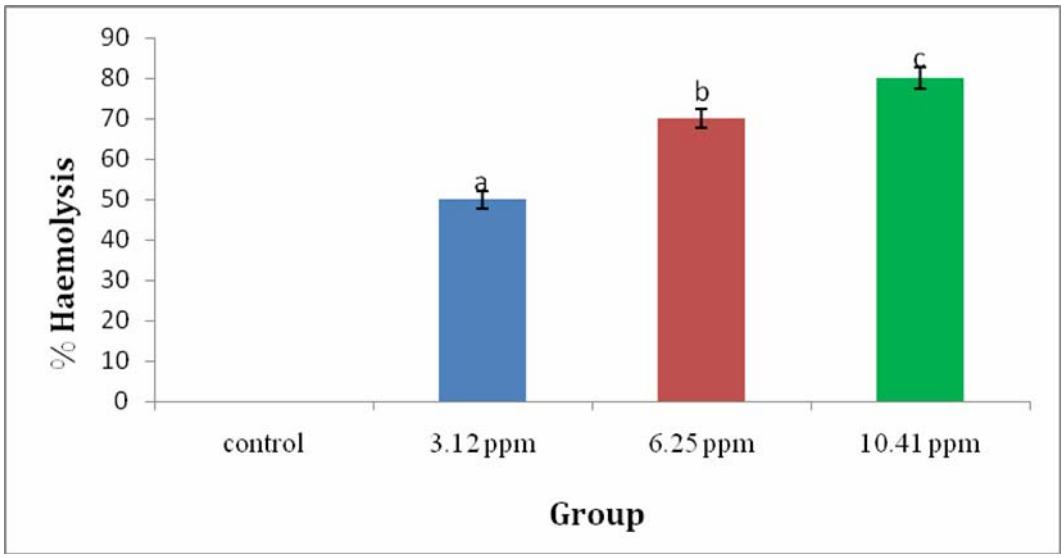

Fig. 5.1 Percentage haemolysis in *O. mossambicus* on exposure to different concentrations of phenol (*in vitro*). Each bar diagram represents mean ± S.D of six separate experiments. On each bar, values with different lower case letters vary significantly (P<0.05) at different sub-lethal concentrations of phenol (One-way ANOVA).

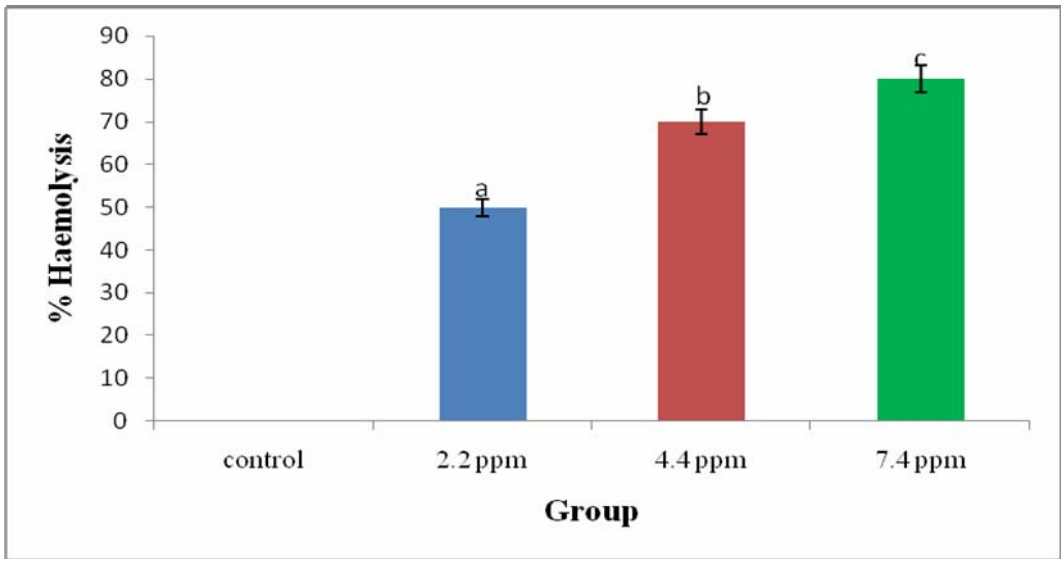

Fig. 5.2 Percentage haemolysis in *O. mossambicus* on exposure to different concentrations of m-cresol (*in vitro*). Each bar diagram represents mean ± S.D of six separate experiments. On each bar, values with different lower case letters vary significantly (P<0.05) at different sub-lethal concentrations of phenol (One-way ANOVA).

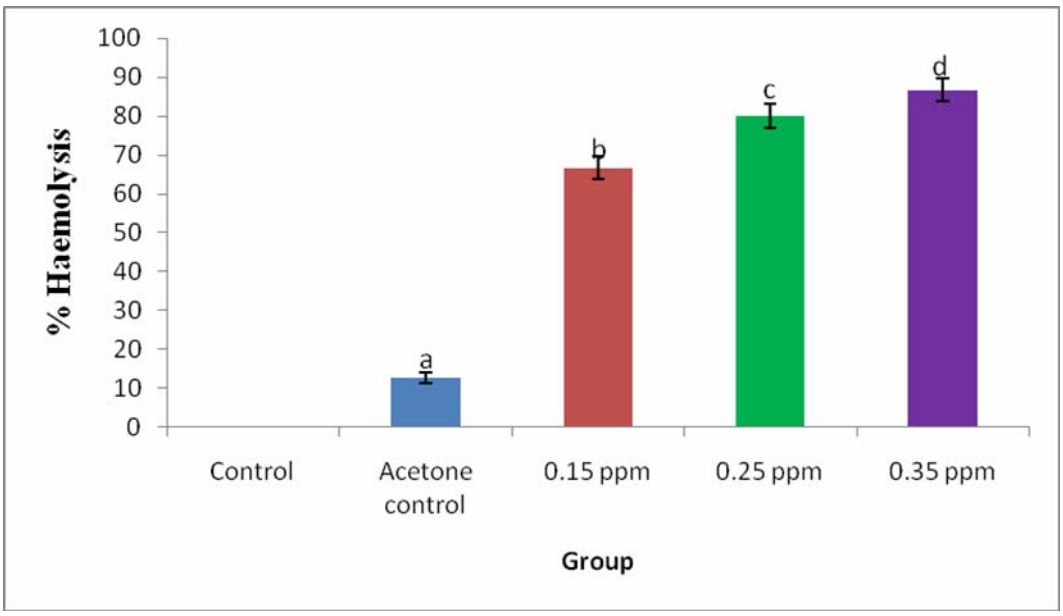

Fig. 5.3 Percentage haemolysis in *O. mossambicus* on exposure to different concentrations of 4-nonylphenol (*in vitro*). Each bar diagram represents mean ± S.D of six separate experiments. On each bar, values with different lower case letters vary significantly ($P<0.05$) at different sub-lethal concentrations of phenol (One-way ANOVA).

Table 5.1 Percentage haemolysis in *O. mossambicus* on exposure to different concentrations of phenolic compounds (*in vitro*).

Groups	Concentrations	% Haemolysis
Control	0 ppm	0
Phenol	3.12 ppm	49.98 ± 2.19
	6.25 ppm	70 ± 2.35
	10.41 ppm	80 ± 2.78
m-cresol	2.2 ppm	50 ± 1.96
	4.4 ppm	75 ± 2.84
	7.4 ppm	87.5 ± 3.12
4-nonylphenol	Acetone control	12.7 ± 1.32
	0.15 ppm	66.7 ± 2.84
	0.25 ppm	77.9 ± 3.12
	0.35 ppm	86.7 ± 3.01

- Each value represents the mean ± S.D of six separate experiments

5.1.5 Discussion

The red blood cell membrane is the most popular model membrane system due to both its availability and the large amount of information available. *In vitro* studies carried out in the present investigation showed a direct effect of the phenolic compounds on the erythrocyte membrane which has resulted in strong, membrane-destabilising effect.

The compact structure of biological membrane derives from weak non-covalent bonds between lipids, proteins and water, determined by hydrophobic, electrostatic and Van der Waals interactions and by hydrogen bonding. Any chemical compound which interferes with these interactions can alter the membrane structure and, consequently, slow down or completely inhibit the membrane processes. Thus the haemolytic effect has been attributed to different phenomena. These include: blockage of sulphydryl groups present on the outside of the membrane while those inside the cells are relatively unaffected (Jacolyn, 1971; MacGregor and Clarkson, 1974); or disruption of the linkage between band 3 and bands 2.1 and 4.2 in the membrane proteins (Ralston and Crisp, 1981).

The activity of a chemical compound to an organism depends on several physical, chemical and biological factors, among which interactions are possible (Bradbury, 1994). McFarland (1970) expressed chemical toxicity as the result of two preceding events. The first event is the penetration of a chemical compound from the environment to the site of action in the organism. The second event is the interaction between the chemical compound and the site of action. Phenols are of interest to environmental toxicologists, which has led to the development of quantitative structure–activity relationships (QSAR) models. Several molecular descriptors are widely used in toxicology and among these the important ones are: the negative logarithm of the acid dissociation constant (*pKa*) and the logarithm of the octanol/water partition coefficient (log *Kow*). The increase of hydrophobocity and the value of log Kow, and the decrease of *pKa* value result in more effective membrane penetration by xenobiotics and, thus, enhance their toxicity (Dani *et al*., 2004).

Some phenolic compounds have high *pKa* (where *K*a is the first dissociation constant) values and others have relatively small *pKa* values. Schultz *et al*. (1998) suggested that phenolic compounds can be categorized either as polar narcotics (which have high *pKa* values) or uncoupling agents by their *pKa* values. Phenolic compounds taken for the present investigation were phenol, m-cresol and 4-nonylphenol which have *pKa* values 9.89, 10.99 and 10.28 respectively. Phenols with *pKa* values > 8.0 exhibit polar narcosis, whereas compounds with *pKa* values < 6.5 are uncoupling agents. Narcosis can be defined as the reversible state of arrested activity of protoplasmic structures resulting from exposure to the xenobiotic (Schultz, 1989) and narcotic compounds are deemed electrophilically unreactive. The mode of action of polar narcosis is not well characterized, but it is assumed to be a non-specific disruption of the functions of the biological membranes causing progressive lethargy, unconsciousness and death (Veith and Broderius, 1987; Oberg, 2004). Compounds with a narcosis mechanism exhibit baseline toxicity or toxicity associated with hydrophobicity, and compounds with other mechanisms have toxicity higher than the baseline toxicity (Verhaar *et al*., 1992).

In fact, log *Kow* (octanol-water partition coefficient) is found to be a significant descriptor of toxicity for the whole group of phenols (Ren and Schultz, 2002). In the present work, the hydrophobicity (log *Kow*=5.44) of the 4-nonylphenol showed a positive and significant relationship with loss of membrane integrity. Steric effects of the alkylated ring structure have been proposed to affect the *in vitro* cytotoxicity of mono- and di-alkylated phenols (Selassie *et al*., 2002). Alkylphenols and other chemicals with acidic hydrogen donating functional groups such as anilines and halogenated phenols have previously been reported to cause higher toxicity than that could be predicted by non-polar narcosis in fish and consequently being grouped as polar narcosis (Schultz *et al*., 1986; Veith and Broderius, 1987; Veith and Broderius, 1990). The findings in the present study are consistent with the assumption that alkylphenols cause toxicity through a polar narcosis mode of action.

Reactive oxygen species formation in erythrocytes on exposure to xenobiotics initiates oxidative processes. The consequence of oxidative stress is enhanced lipid peroxidation of cell membrane, aggregation of membrane proteins, an increase of its permeability, an outflow of potassium from cell and enhanced binding of their own immunoglobin G (Bartosz, 2003). These changes cause accelerated removal of erythrocytes from blood and thus reduction of their life, which leads to anaemia (Bradshaw *et al*., 1995; Giardina *et al*., 1995; Bartosz, 2000). Lipid peroxidation, resulting from the binding of phenolic compounds to polyunsaturated fatty acids in the erythrocyte membrane may have resulted in haemolysis. In carp, lipid peroxidation resultant from sub-lethal effects of phenol is also found in phospholipid composition of erythrocyte membranes with subsequent alterations in membrane fluidity and permeability (Kotkat *et al*., 1999). A direct action of a phenolic compound butylated hydroxyanisole (BHA) on the integrity of the erythrocyte membrane was observed by Nohl and Stolze (1998) leading to haemolysis independent of the formation of prooxidant species.

The effects of the investigated xenobiotics on the erythrocyte membrane can be summarized as a clear effect of all xenobiotics on the lipid phase of the erythrocyte membrane affecting the membrane fluidity that may have resulted in strong membrane-destabilising effect, eventually leading to haemolysis.

5.2 Lysosomal membrane stability studies

5.2.1 Introduction

Lysosomes are membrane bound organelles, which degrade many membranes and organelles that have outlived their usefulness to the cell; they also degrade proteins and particles taken up by the cell. Lysosomes vary in size and shape and several hundred may be present in a typical cell. Lysosomes contain acid hydrolases that work only at acidic pH values. To enable the enzymes to function, the inside of lysosomes is maintained at about pH 4.8 by a hydrogen ion pump in the lysosomal membrane. The acid pH helps to denature proteins and make them accessible to the action of the lysosomal hydrolases, whose structures

resist acid denaturation. The lysosomal membrane protects the cytosol, and therefore the rest of the cell, from the degradative enzymes within the lysosome. The enzymes are inactive at neutral pH values of cells and most extracellular fluids.

Lysosomal perturbations have been widely used as early indicators of adverse effect to various factors, including pollutant exposure (Moore, 2002; Galloway *et al*., 2004; Moore *et al.*, 2004). Consequently, lysosomal function can be used across a range of animals, including annelids, molluscs, crustaceans and fish to detect responses to environmental stress (Cajaraville *et al*., 2000; Kohler *et al*., 2002; Hwang *et al.*, 2002; Galloway *et al*., 2004; Hankard *et al*., 2004). Lysosomes are highly conserved multi-functional cellular organelles present in almost all cells of eukaryotic organisms from yeast to humans except in plants. Their function in the cellular economy includes the degradation of redundant or damaged organelles (e.g., mitochondria and endoplasmic reticulum) and longer lived proteins as part of autophagic cellular turnover (Klionsky and Emr, 2000). Lysosomes are also involved in the digestion of materials ingested by endocytosis and phagocytosis (i.e. intracellular digestion). Lysosomal reactions are involved in normal physiological responses as well as many cell injury and disease processes; these include augmented sequestration and autophagy of organelles and proteins (Moore, 1990; Klionsky and Emr, 2000; Moore, 2002; Cuervo, 2004).

The functional stability of the lysosomal membrane is a good indicator of lysosomal integrity and has been used widely to measure responses to environmental perturbation in fish and molluscs (Hwang *et al*., 2002; Kohler *et al*., 2002; Moore, 2002; Allen and Moore, 2004; Moore *et al*., 2004). Lysosomes are also remarkable for the vast and diverse array of chemicals and pharmaceuticals that they can sequester and accumulate (Rashid *et al*., 1991; Moore *et al*., 2002; Moore *et al*., 2004). These range from metal ions such as iron, copper and mercury, transuranics, asbestos, polycyclic aromatic hydrocarbons (PAHs), heterocyclics, anti-psychotic drugs to nanoparticles, to name but a few (de Duve *et al*., 1974;

Mayernick, and Giam, 1985; Moore, 1985; Nott and Moore, 1987; Rashid *et al*., 1991; Moore *et al*., 1997; Panyam and Labhasetwar, 2003; Gould, 2004; Howard, 2004). Adverse lysosomal reactions appear to provide useful biomarkers that are diagnostic for cell injury and putative indicators for further pathology (Moore, 1990). Exposure to many contaminants, both metals and organic xenobiotics, can result in increased radical generation and the intralysosomal environment is already a site of oxyradical production (Moore *et al*., 1996; Livingstone, 2001). The resulting oxidative damage to membranes, proteins (e.g. carbonyls) and DNA will undoubtedly contribute to decreased protein synthesis, cell injury and patho-physiological dysfunction (Winston *et al*., 1991; Kirchin, *et al*., 1992; Krishnakumar *et al*., 1994; Winston *et al*., 1996; Regoli, 2000; Domouhtsidou and Dimitriadis, 2001; Livingstone, 2001; Kalpaxis *et al*., 2004). Lysosomes are able to accumulate and sequester a wide range of both organic and inorganic compounds as well (Allison, 1969; Moore, 1985).

Intracellular digestion would appear to be the main role of lysosomes but this only acts as a central theme in a broad spectrum of physiological functions which includes regulation of secretory processes, cellular defense mechanisms, cell death, protein and organelle turnover, accumulation and sequestration of xenobiotics and mediation of target tissue-specific hormone functions. There are also indications that lysosomes may be involved in part of the mechanism of regulation of hexose and amino acid transport into cells by degradative inactivation of the carriers by lysosomal proteases as well as in the process of steroidogenesis. Many of these stressors induce alterations in the latency of lysosomal hydrolytic enzymes which can lead to disturbances in the normal physiological functions of lysosomes. Xenobiotic-induced alteration of the permeability or fluidity of the lysosomal membranes will inturn affect the normal processing or turnover of intracellular proteins and organelles (autophagy), as well as the intracellular digestion of pinocytosed food (heterophagy). The mechanism of such interference could well involve the process of fusion of the different components of the lysosomal-vacuolar system, namely primary lysosomes derived

from the Golgi or GERL (Golgi-associated endoplasmic reticulum giving rise to lysosomes) and phagosomes (both hetero- and auto-types).

This study has been undertaken for an improved understanding of interaction between xenobiotics and the lysosomal system and their subsequent effects on lysosomal physiology. The lysosomal stability test was transferred to fish liver with the aim of testing responsive and practicable methods for biological-effects monitoring. Response in fish liver reflected as the injury (membrane destabilisation) of the lysosomal system was investigated.

5.2.2 Materials and methods

Collection, maintenance, acclimatization, dosing of the experimental animal and preparation of tissue samples were the same as explained in detail in chapter 2, section 2.2.

5.2.2.1 Assay of subcellular acid phosphatase activity - *in vivo* study

Liver (10%) tissue was homogenised in ice-cold 0.33M sucrose buffer and the homogenate was centrifuged at 600×g for 10 minutes in a high speed refrigerated centrifuge. The sediment of nuclei, unbroken cells and plasma membrane corresponding to nuclear fraction was separated. The supernatant was again centrifuged at 15,000×g for 30 minutes. The resulting sediment corresponds to lysosomal fraction. Both the nuclear fraction and lysosomal fraction were resuspended in citrate buffer containing 0.2% Brij-35. The 15,000×g supernatant (soluble fraction) was diluted with an equal volume of double strength buffer. The activity of acid phosphatase was determined in all these fractions (Plummer, 1987).

5.2.2.2 Rate of release of acid phosphatase from the lysosome rich fraction of liver - in vitro study

Liver (10%) tissue from control fishes were homogenised in cold isotonic sucrose at 0°C. The homogenate was centrifuged at 600×g for 10 minutes in a high speed refrigerated centrifuge. The sediment of nuclei, unbroken cells and

plasma membrane (nuclear fraction) was separated. The supernatant was again centrifuged at 15000×g for 30 minutes. The lysosomal fraction was obtained. The lysosomal pellet was washed, centrifuged at 15,000×g for 10 minutes and again suspended in 0.33M sucrose. A definite volume of this suspension was incubated at room temperature and aliquots were withdrawn at various time intervals of 0, 15, 30 and 45 minutes. The retrieved fractions were stored immediately at $0^{\circ}C$ (control). In order to study the effect of phenolic compounds on the lysosomal membrane, a definite volume of the lysosomal suspension (Test) was incubated in presence of 0.5 ml of toxin such that the final concentration in tubes were 1/10, 1/5 and 1/3 of LC_{50} value of different phenolic compounds. Here also aliquots were withdrawn at time intervals of 0, 15, 30, and 45 minutes. Both the control and the test aliquots were centrifuged at 15000×g for 30 minutes to separate the unbroken lysosomes and acid phosphatase activity released into the supernatant was determined. Total activity in the lysosomes-rich fraction was estimated after adding citrate buffer containing Brij-35 after appropriate dilution.

5.2.2.3 Determination of acid phosphatase (ACP) activity (Anon, 1963)

0.5 ml of p-nitro phenyl phosphate (400 mg %) was mixed with an equal volume of 0.1 M citrate buffer of pH 4.8. The enzyme source was added and incubated for 30 min at room temperature. At the end of 30 minutes, reaction was stopped by the addition of 4 ml of 0.1 N NaOH. The absorbance of the solution was measured at 410 nm in a UV-visible spectrophotometer. The amount of p-nitro phenol released by the acid phosphatase per hour per mg protein gives the specific activity. Protein was estimated by the method of Lowry *et al*. (1951).

5.2.3 Statistical analysis

Statistical analysis was carried out using the software SPSS 13.0 package. One-way ANOVA followed by Tukey's test was carried out to compare the acid phosphatase activities in different subcellular fractions after treatment with different phenolic compounds. Two-way ANOVA was carried out for the

comparison of acid phosphatase activities at different time intervals and different concentrations of phenolic compounds. Significance level (P value) was set at 0.05 in all tests.

5.2.4 Results

5.2.4.1 Subcellular acid phosphatase activity

One-way ANOVA showed that there was an overall significant change ($P<0.05$) in the acid phosphatase activity in the nuclear, lysosomal and soluble fractions of the hepatic tissue of the different phenolic compounds dosed groups compared to control. A significant increase ($P<0.05$) in acid phosphatase was observed in nuclear and soluble fraction of the phenolic compounds treated groups compared to control. In the phenolic compounds dosed groups the acid phosphatase activity in the lysosomal fraction was significantly ($P<0.05$) lower compared to control but the soluble fraction activity was highly increased. This clearly indicates damage to the lysosomal membrane on exposure to phenolic compounds.

The ratio of acid phosphatase activity in the lysosomal fraction to that in the soluble fraction can be termed as lysosomal stability index (LSI). LSI was found to be 1.18 for phenol treated group and for the m-cresol treated group it was found to be 0.819 whereas for control LSI was 1.696.

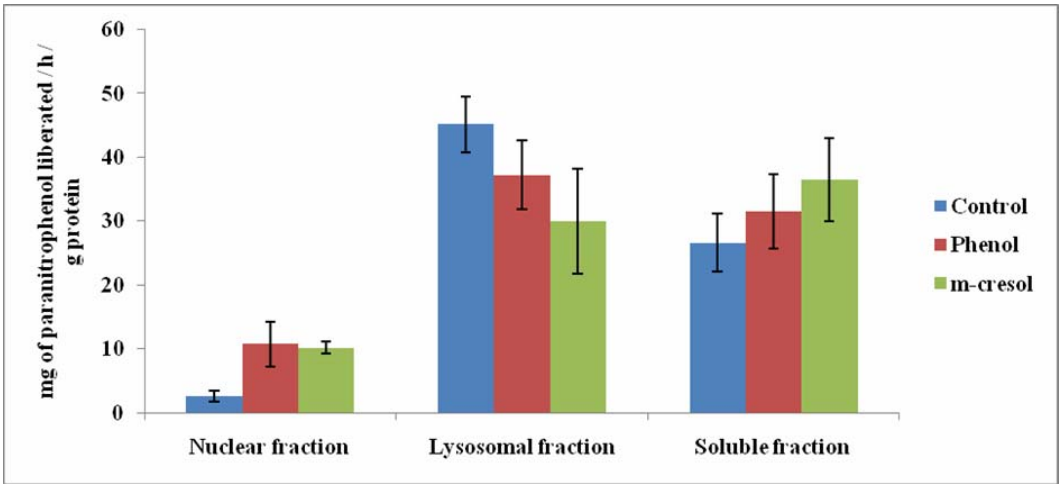

Fig. 5.4 Effect of different phenolic compounds on the subcellular acid phosphatase activity - *in vivo* in *O. mossambicus.* Each bar diagram represents mean ± S.D.

Table 5.2 Effect of different phenolic compounds on the subcellular acid phosphatase activity - *in vivo* in *O. mossambicus.* Values in the same column with different upper case letters vary significantly (P<0.05) between different fractions.

Groups	Acid phosphatase activity in hepatic tissue			
	Nuclear fraction	**Lysosomal fraction**	**Soluble fraction**	**Ratio of Lysosomal to soluble activity (LSI)**
Control	2.65 ± 0.86^{A}	45.20 ± 4.36^{C}	26.64 ± 4.50^{A}	1.696 ± 0.69^{C}
Phenol	10.73 ± 3.45^{B}	37.25 ± 5.36^{B}	31.54 ± 5.75^{B}	1.18 ± 0.93^{B}
m-cresol	10.19 ± 0.90^{B}	29.95 ± 8.25^{A}	36.53 ± 6.46^{C}	0.819 ± 1.27^{A}

- Activities are expressed as mg of paranitrophenol liberated / h / g protein in each fraction.
- Each value represents the mean ± S.D of six separate experiments.

5.2.4.2 Lysosomal enzyme release assay of acid phosphatase (ACP) - *in vitro*

Two-way ANOVA showed an overall significant change in the ACP release (P<0.05) on exposure to different phenolic compounds at different time intervals in the experimental groups compared to control (Table 4.2, 4.3, 4.4). Two factor ANOVA revealed that there was an overall significant change (P<0.05) between time intervals and also between different concentrations of phenolic compounds. Subsequent comparison by Tukey's test showed that there was an overall significant change (P<0.05) between different concentrations with the control and also among themselves. Similarly a significant increase (P<0.05) was noted in the rate of release of ACP with different time intervals when compared with zero time and also among other time intervals. *In vitro* studies showed that as the time interval increases the rate of release of acid phosphatase also significantly (P<0.05) increased.

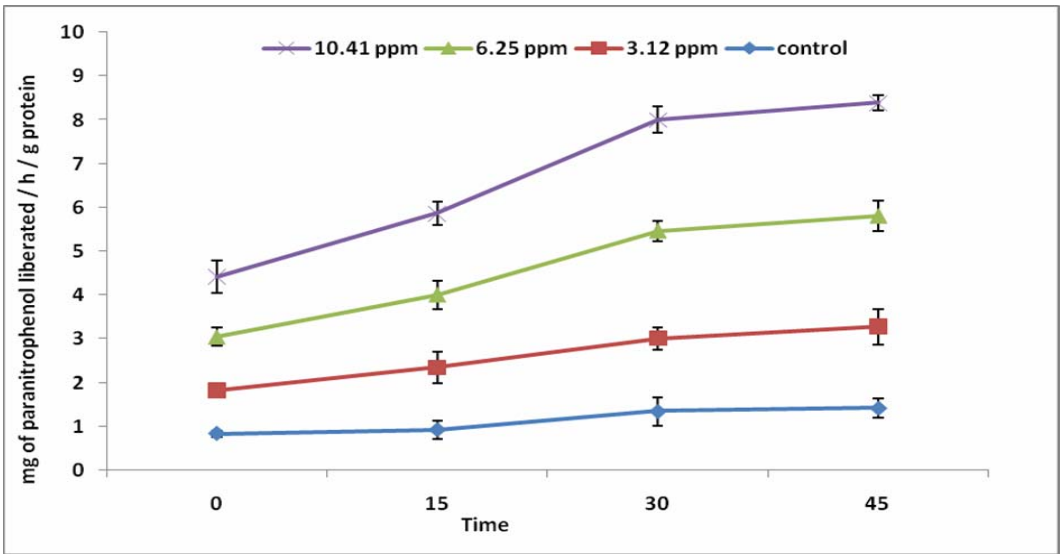

Fig. 5.5 Hepatic lysosomal enzyme release assay (acid phosphatase) *in vitro* – phenol.

Table 5.3 Lysosomal enzyme release assay (acid phosphatase) *in vitro* – phenol. Values in the same column with different upper case letters vary significantly (P<0.05) between time intervals and values in the same row with different lower case letters vary significantly (P<0.05) between different concentrations of phenol.

Time (in minutes)	Lysosomal ACP activity			
	Control	3.12 ppm	6.25 ppm	10.41 ppm
0	$_{a}0.838 \pm 0.07^{A}$	$_{b}0.99 \pm 0.03^{A}$	$_{c}1.21 \pm 0.21^{A}$	$_{c}1.37 \pm 0.36^{A}$
15	$_{a}0.924 \pm 0.21^{A}$	$_{b}1.42 \pm 0.35^{A}$	$_{c}1.66 \pm 0.32^{A}$	$_{c}1.85 \pm 0.27^{A}$
30	$_{a}1.34 \pm 0.32^{B}$	$_{b}1.68 \ 0.26^{B}$	$_{c}2.45 \pm 0.23^{B}$	$_{c}2.53 \pm 0.30^{B}$
45	$_{a}1.42 \pm 0.23^{B}$	$_{b}1.87 \pm 0.4^{B}$	$_{c}2.54 \pm 0.35^{B}$	$_{c}2.58 \pm 0.18^{B}$

- Acid phosphatase activity is expressed as mg of paranitrophenol liberated / h / g protein in each fraction.
- Each value represents the mean ± S.D of six separate experiments.

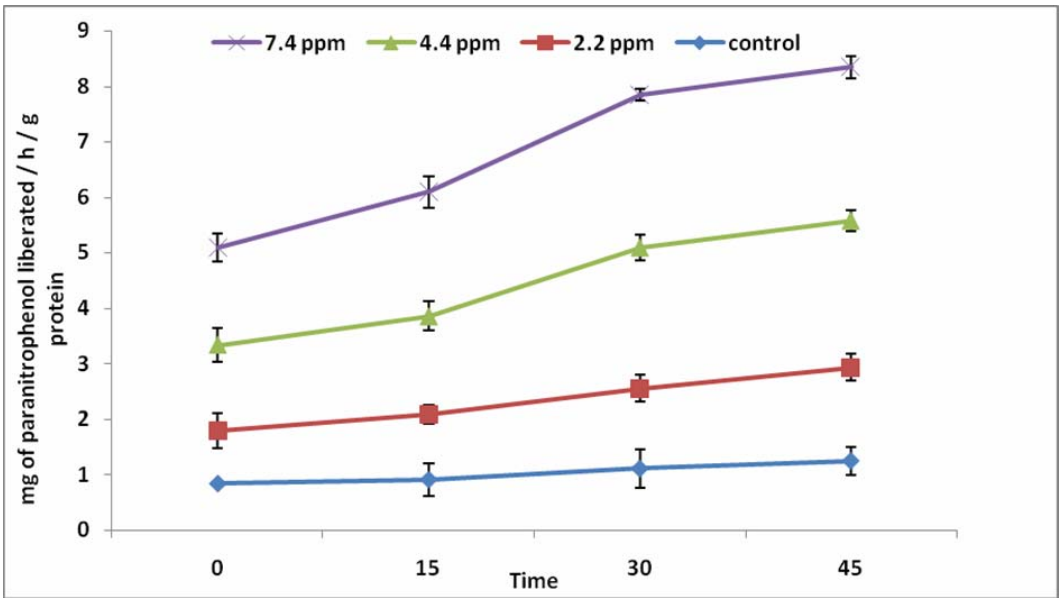

Fig. 5.6 Hepatic lysosomal enzyme release assay (acid phosphatase) *in vitro* – m-cresol.

Table 5.4 Lysosomal enzyme release assay (acid phosphatase) *in vitro* – m-cresol. Values in the same column with different upper case letters vary significantly (P<0.05) between time intervals and values in the same row with different lower case letters vary significantly (P<0.05) between different concentrations of m-cresol.

Time (in minutes)	Lysosomal ACP activity			
	Control	2.2 ppm	4.4 ppm	7.4 ppm
0	$_{a}0.838 \pm 0.07^{A}$	$_{a}0.912 \pm 0.37^{A}$	$_{b}1.11 \pm 0.31^{A}$	$_{c}1.25 \pm 0.24^{A}$
15	$_{a}0.952 \pm 0.35^{B}$	$_{a}1.17 \pm 0.16^{B}$	$_{b}1.44 \pm 0.26^{B}$	$_{c}1.68 \pm 0.28^{B}$
30	$_{a}1.54 \pm 0.34^{C}$	$_{a}1.76 \pm 0.23^{C}$	$_{b}2.53 \pm 0.22^{C}$	$_{c}2.64 \pm 0.10^{C}$
45	$_{a}1.75 \pm 0.25^{C}$	$_{a}2.24 \pm 0.29^{C}$	$_{b}2.75 \pm 0.19^{C}$	$_{c}2.76 \pm 0.20^{C}$

- Acid phosphatase activity is expressed as mg of paranitrophenol liberated / h / g protein in each fraction.
- Each value represents the mean ± S.D of six separate experiments.

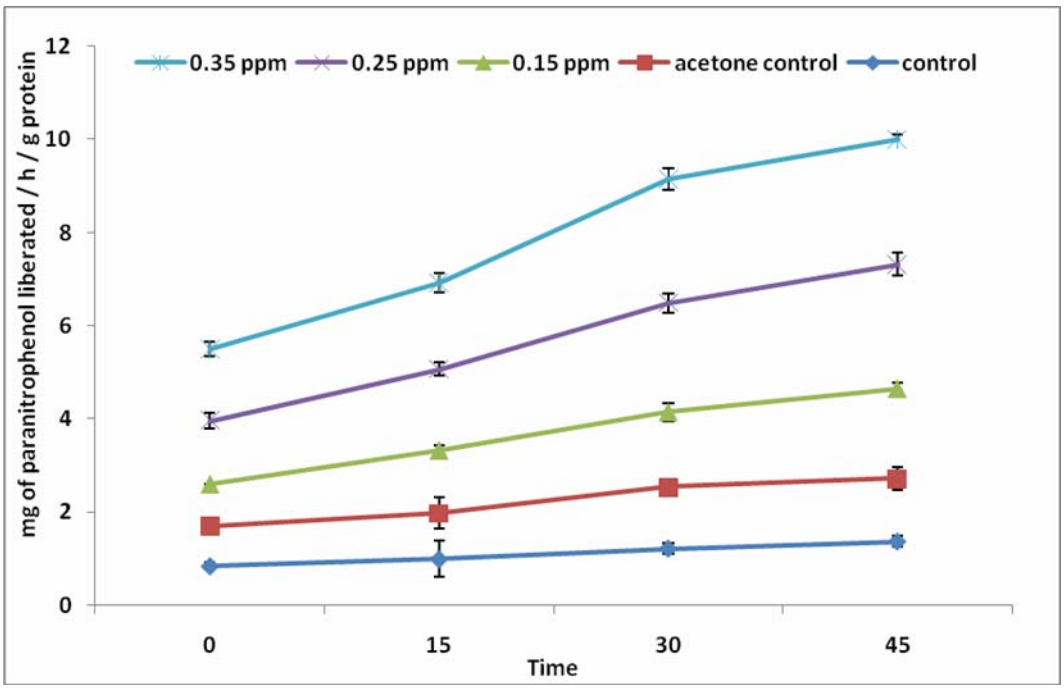

Fig. 5.7 Hepatic lysosomal enzyme release assay (acid phosphatase) *in vitro* – 4-nonylphenol.

Table 5.5 Lysosomal enzyme release assay (acid phosphatase) *in vitro* – 4-nonylphenol. Values in the same column with different upper case letters vary significantly (P<0.05) between time intervals and values in the same row with different lower case letters vary significantly (P<0.05) between different concentrations of 4-nonylphenol.

Time (in minutes)	Lysosomal ACP activity				
	Control	**Acetone control**	**0.15 ppm**	**0.25 ppm**	**0.35 ppm**
0	$_{a}0.838 \pm 0.07^{B}$	$_{a}0.861 \pm 0.10^{B}$	$_{b}0.894 \pm 0.02^{B}$	$_{c}1.36 \pm 0.17^{B}$	$_{c}1.54 \pm 0.15^{B}$
15	$_{a}0.99 \pm 0.38^{A}$	$_{a}0.987 \pm 0.32^{A}$	$_{b}1.34 \pm 0.12^{A}$	$_{c}1.75 \pm 0.15^{A}$	$_{c}1.85 \pm 0.21^{A}$
30	$_{a}1.21 \pm \pm 0.12^{BC}$	$_{a}1.26 \pm 0.14^{BC}$	$_{b}1.61 \pm 0.19^{BC}$	$_{c}2.34 \pm 0.20^{BC}$	$_{c}2.65 \pm 0.24^{BC}$
45	$_{a}1.37 \pm 0.11^{C}$	$_{a}1.35 \pm 0.24^{C}$	$_{b}1.92 \pm 0.13^{C}$	$_{c}2.67 \pm 0.25^{C}$	$_{c}2.67 \pm 0.10^{C}$

- Acid phosphatase activity is expressed as mg of paranitrophenol liberated / h / g protein in each fraction.
- Each value represents the mean ± S.D of six separate experiments.

5.2.5 Discussion

Lysosomal perturbations observed during this study reflected a clear gradient from the control. The results obtained in the present investigation showed that on exposure to different phenolic compounds leakage of lysosomal marker enzyme acid phosphatase occurred. Injury of the lysosomal membrane by the phenolic compounds may have led to leakage of the hydrolytic lysosomal enzymes into the cytoplasm leading to disturbance of cell functions and resulting in degeneration and possibly in neoplasia. All these changes reflect overloading or damage of the lysosomal digestive and detoxifying system. Lysosomal response indicated as the injury of this central cell compartment, resulted in severe liver lesions.

Lysosomal enzymes released into the cytosol presumably cause changes in the membrane fluidity resulting in increased fusion rates. Lowe et *al.* (1981) found that an increase in lysosomal volume accompanied by the formation of pathologically enlarged lysosomes was directly associated with membrane destabilisation in the digestive gland of mussels exposed to oil-derived contaminants. The significant negative correlation between the lysosomal stability and the extension of liver lesion indicates that the lysosomal stability test clearly reflects the overcharge and breakdown of the detoxifying capacity of liver (Kohler, 1989b). The assessment of lysosomal membrane stability in the digestive gland of marine mussels and snails proved to be a highly sensitive measure for the functional state of the cell (Moore, 1985). As in mammals, the fish liver is the central organ for the accumulation and detoxification of organic and inorganic contaminants. Earlier ultra structural studies in flounder liver evidenced severe alterations of the lysosomal system in relation to the contaminant burden (Kohler *et al*., 1986; Kohler, 1989a, 1990).

In vitro studies were carried out by taking labilisation measurements at intervals from 0, 15, and 30 up to 45 minutes. With increase in time and concentration of phenolic compounds, the rate of release of the lysosomal marker

enzyme acid phosphatase increased, this showed a decrease in membrane stability. The lysosomal membrane stability has been proved to be a useful index of cellular conditions and correlates significantly with physiological conditions of organisms. Lysosomal damage is well established as a biomarker of stress in a wide range of vertebrates (Tabata *et al*., 1990) and many agents such as various disease conditions, stress, hormones and drugs can induce destabilising alterations in lysosomes.

The depletion of dissolved oxygen concentration of waters due to the presence of phenolic compounds leads to formation of free radicals, especially superoxide (O_2^-), which acts by oxidizing various cellular substrates, especially unsaturated fatty acids in phospholipids of biological membranes, which are very susceptible to free radical damage. Malondialdehyde, the major oxidation product of peroxidised polyunsaturated fatty acids was found to be higher in liver in all phenolic compounds treated groups compared to control (chapter 3). Peroxidised membranes become rigid and lose permeability and integrity. Cumulative effects of lipid peroxidation have been implicated as underlying mechanisms in numerous pathological conditions in humans (atherosclerosis, haemolytic anaemia, ischemia etc.) and other organisms (Steinberg, 1997). In general, the overall effects of lipid peroxidation are decrease in membrane fluidity and increase in the leakiness of the membrane.

Also in the present investigation the histopathological examination (chapter 6) showed changes in the liver on exposure to both phenol and m-cresol. The most evident change observed in the hepatocytes was necrosis. Cortisol, the principal glucocorticoid hormone which plays an important role in maintaining the stability of biological membranes was found to be decreased (chapter 2) on treatment with different phenolic compounds. Thus it can be inferred that necrosis, lipid peroxidation and decreased cortisol response may have resulted in the labilisation of the hepatic lysosomal membrane under *in vivo* conditions. However, it was also noted that the concentration of phenolic compounds tested evoked adverse effects

on cellular functions resulting in metabolic alterations. This suggests that effect on cellular metabolic functions was one of the causes of cytotoxicity of these chemicals and that the disruption of membrane integrity may be a secondary effect.

The lysosomal tests clearly reflect the breakdown of the adaptive capacity of the fish liver to toxic injury. All the results obtained in both *in vivo* and *in vitro* studies showed that the tested compounds affected the lysosomal membrane stability and resulted in the disruption of cellular homeostasis to the point where membrane integrity was compromised. To conclude, lysosomal membrane stability is a predictive indicator for cell injury and pathology and supporting evidence indicates that this parameter is generic in animals.

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Chapter 6

HISTOPATHOLOGICAL CHANGES IN THE GILLS, LIVER AND KIDNEY TISSUES OF *OREOCHROMIS MOSSAMBICUS* ON EXPOSURE TO DIFFERENT PHENOLIC COMPOUNDS

Contents

6.1 Introduction

Health of aquatic organisms cannot be measured directly. Instead, only indicators of health can be measured and in turn used to assess the ‘‘health’’ status. Histology and histopathology can be used as biomonitoring tools or indicators of health in toxicity studies as they provide early warning signs of disease (Meyers and Hendricks, 1985). Histopathological alterations are biomarkers of effect of exposure to environmental stressors, revealing prior alterations in physiological and/or biochemical function (Hinton *et al*., 1992). Fish is a suitable indicator for monitoring environmental pollution because they concentrate pollutants in their tissues directly from water and also through their diet, thus enabling the assessment of transfer of pollutants through the trophic web (Fisk *et al*., 2001; Boon *et al*., 2002). Due to being exposed to pollutants, major structural damages may occur in their target organs, histological structure may change and physiological stress may occur. This stress causes some changes in the metabolic functions. The changes in the functions are initiated with the changes in the tissue and cellular level. Although qualitative data are used in most cases to study the pathologies the environmental pollutants cause, quantitative data show better reactions of the organisms to pollutants (Jagoe, 1996).

Histopathological investigations have long been recognized to be reliable biomarkers of stress in fish for several reasons (Teh *et al*., 1997; van der Oost *et al*., 2003). The gill surface is more than half of the entire body surface area. In fish the internal environment is separated from the external environment by only a few microns of delicate gill epithelium and thus the branchial function is very sensitive to environmental contamination. Gills are the first organs which come in contact with environmental pollutants. Paradoxically, they are highly vulnerable to toxic chemicals because firstly, their large surface area facilitates greater toxicant interaction and absorption and secondly, their detoxification system is not as robust as that of liver (Mallatt, 1985; Evans, 1987). Additionally, absorption of

toxic chemicals through gills is rapid and therefore toxic response in gills is also rapid. Gills have frequently been used in the assessment of impact of aquatic pollutants in marine as well as freshwater habitats (Haaparanta, *et al*., 1997; Athikesavan *et al*., 2006; Craig *et al*., 2007; Fernandes *et al*., 2007; Jimenez-Tenorio *et al*., 2007). Therefore, lesions in gill tissues can be the start of imbalance of the physiological and metabolic processes of fish.

The liver was examined because it plays a primary role in the metabolism and excretion of xenobiotic compounds with morphological alterations occurring in some toxic conditions (Rocha and Monteiro, 1999). In fish, as in higher vertebrates, the kidney performs an important function related to electrolyte and water balance and the maintenance of a stable internal environment. The kidney excretes nitrogen-containing waste products from the metabolism such as ammonia, urea and creatinine. Following exposure of fish to toxic agents such as pesticides, tissue alterations have been found at the level of the tubular epithelium and glomerulus (Teh *et al*., 1997). Hence, fish serve as excellent bioassay animal for toxicological impact studies and has been widely used for this purpose.

6.2 Materials and methods

Collection, maintenance, acclimatization and experimental design were the same as explained in detail in chapter 2, section 2.2.

6.2.1 Preparation of tissue samples

O. mossambicus were exposed to sub-lethal concentration ($1/10^{th}$ of LC_{50}) of two different phenolic compounds such as phenol and m-cresol for 21 days. After the experimental period the fishes were killed by pithing (by damaging the brain and severing the spinal cord between the head and trunk region using a sharp needle) and the tissues such as liver, gills, and kidney were removed from its body, wiped thoroughly, using blotting paper to remove blood and other body fluids. The tissues were then immediately fixed in 10 % neutral buffered formalin for 24 hours.

6.2.2 Steps involved in histological procedures

The major steps involved in histopathological analysis are fixation, tissue processing, decalcification, section cutting and staining (Raphael, 1976).

(i) Fixation

Fixation is the process of preserving, hardening and preventing postmortem changes in the tissues.

Reagents

10 % neutral buffered formaldehyde solution pH 7.0.

To 100 ml of 37-40 % formaldehyde solution, 900 ml distilled water, 4 g NaH_2PO_4 and 6.5 g of Na_2HPO_4 was added and the pH was adjusted to 7.0.

Procedure

Tissues were placed in fixative immediately after removal from the body. Tissue blocks were then cut to thickness of about 5 mm so that the fixative could readily penetrate throughout the tissue in a reasonably short time. The volume of fixative employed was 15-20 times that of the tissue to be fixed. The duration of fixation was 24 h. They were then washed in running tap water overnight and was then stored in 70% alcohol.

(ii) Tissue processing

This step involves dehydration, clearing and infiltration of the tissue with paraffin. Dehydration using 50-70 % dilution of alcohol prevents distortion that would occur to the tissues. Clearing helps in bringing about miscibility between alcohol and paraffin. The tissue was then impregnated and embedded with molten paraffin.

Reagents

Ethyl alcohol, xylene and paraffin.

Procedure

The following time schedule was used to make paraffin wax blocks for histological studies.

(a) Tissues were washed overnight in running water.

(b) A sudden change of the tissues from aqueous medium to alcohol concentrations of 30 %, 50 % and 70 % was carried out.

(c) The tissues were stored in fresh 70 % alcohol. At this stage tissue can be stored until further processing.

(d) Tissues were then dehydrated by transferring them sequentially to 70 %, 80 %, 90 %, 95 % alcohol for one hour each.

(e) Transferred to absolute alcohol (2 changes) for one hour each.

(f) Placed the tissues in 1:1 mixture of absolute alcohol and xylene for 30 minutes.

(g) Tissues were then placed in acetone for complete dehydration for one hour.

(h) Cleared in xylene until the tissues became translucent.

(i) Tissues were transferred to a mixture of xylene and paraffin wax and left overnight.

(j) Infiltrated the tissues in 2-3 changes of molten paraffin wax of melting point 60-62°C for 1 h each.

(k) Embedded in paraffin wax of melting point 60-62°C.

The blocks were trimmed and sections of 3-4 µm thickness were cut with a rotary microtome.

(iii) Decalcification

Decalcification is the term applied to organic tissues which have been infiltrated with calcium salts. These salts were removed to assure that the specimen is soft enough to allow section cutting.

Reagents

10 % EDTA

Procedure

Gill tissues were cut into small pieces with fine saw. After sufficient fixation, pieces were placed in decalcifying solution containing 10% EDTA. Stirring and heating hastens decalcification. They were then suspended in the upper 1/3rd of fluid during decalcification, so that calcium salts sink to the bottom of the container. Since the decalcifying solution contains acid, the gill tissues were washed thoroughly to remove acid from subsequent processing.

Section cutting

Sections were cut at 5 µm thickness and were floated in a water bath between 38-49°C. The sections from water were then mounted on clean glass slides smeared with Mayer's egg albumin. They were then dried on a hot plate at about 50°C for 30 minutes. The sections on the slides were kept ready for staining.

(iv) Staining procedure using Haematoxylin and Eosin (Luna, 1968)

Reagents

(a) Mayer's Haematoxylin stain: dissolved 50 g of potassium alum in 1liter of water without heating. To this 1 g of haematoxylin was added. Then 0.2 g of sodium iodate, 1 g of citric acid and 50 g of chloral hydrate were added. It was then shaken until all the components got completely dissolved in solution. The final colour of the stain obtained was reddish violet which can be stored for a month.

(b) Stock eosin solution (1%): Dissolve 1g of Eosin Y (water soluble) in 20 ml distilled water. This was made up to 100 ml with 95 % alcohol.

(c) Working eosin solution: Diluted 1 part of the stock eosin solution with 3 parts of 80 % alcohol. 0.5 ml of glacial acetic acid was added for every 100 ml of stain.

Procedure

The slides containing the section were processed serially as follows:

(a) The slides were transferred to xylene: absolute alcohol (1:1) (xylol) and were subjected to two changes for 5 minutes each.

(b) They were hydrated by passing through a descending series (95%, 90 %, 80 %, 70 %, 50 % and 30 %) of alcohol for 5 minutes each.

(c) The slides were washed in running tap water for 5 minutes.

(d) They were stained using haematoxylin for 10 minutes.

(e) The stained slides were washed in running tap water for 10 minutes.

(f) The slides were counter stained by keeping in Eosin working solution ranging from 15 seconds to 5 minutes.

(g) The stained slides were dehydrated by passing them through an ascending series (30 %, 50 %, 70 %, 80 %, 90 % and 95 %) of alcohol for 3 minutes each.

(h) They were subjected to 2 to 3 dips of 95% alcohol in which two changes were provided.

(i) They were followed by 100% alcohol. Two changes were provided for 1 to 2 minutes each.

(j) The slides were then placed in acetone. Two changes were provided for 3 minutes each.

(k) The slides were dipped in xylene: absolute alcohol (1:1). Two changes were provided for 3 minutes each.

(l) Finally the slides after clearing with xylene (2 changes) were mounted in DPX medium. They were examined under microscope (Leica DM/LS type) with camera attachment and were photographed at both high as well as low power resolutions. The nuclei stained blue and cytoplasm in various shades of pink.

6.3 Results

The general histological examination indicated incidence of damage in tissues of *O. mossambicus* after exposure to $1/10^{th}$ LC_{50} concentration of phenol and m-cresol for 21 days. Histopathological changes were studied in gills, liver and kidney tissues in both control and treated groups.

Gills

No histopathological abnormalities were observed in the gill of the control fish. The structural details of the gill of control *O. mossambicus* are shown in plate 6.1a and 6.1c. The gill is made up of double rows of filaments from which arise perpendicularly the lamellae. The lamellae are lined by squamous epithelium composed of pavement and non differentiated cells. Below that epithelium are lamellar blood sinuses separated by pillar cells. Between the lamellae, the filament is lined by a thick stratified epithelium constituted by several cellular types, such as chloride, mucus and pavement cells. In the phenol treated group the changes observed were architectural loss, necrosis, desquamation of epithelial layer, hyperplasia and telangiectesis (Plates 6.1b, d and e). On exposure to m-cresol the most significant changes observed were lamellar necrosis, lamellar shortening, telangiectesis and lamellar clubbing (Plates 6.1f, g and h).

Liver

Liver of teleosts is a bilobed gland comprising of two tissue compartments, the parenchyma and stroma. The parenchyma comprising of hepatocytes and the stroma comprising of hepatopancreas, bile duct, blood vessels and connective tissue. The parenchymatous cells forming hepatic cords lie irregularly and get separated by blood sinusoids. Hepatocytes are polygonal cells with a prominent spherical central nucleus and a densely stained nucleolus. Each sinusoid consists of an outer peripheral connective tissue and an inner lining of endothelial cells.

In the control group, the liver exhibited a normal architecture with hepatocytes presenting a homogenous cytoplasm and a large central or sub central

spherical nucleus (Plate 6.2a). The important histopathological changes observed in the phenol treated groups were pyknotic nuclei and clear cell foci (Plates 6.2b and c). In the m-cresol treated group the changes observed were pyknotic nuclei and necrosis (Plate 6.2d).

Kidney

Histopathological abnormalities were not observed in the kidney tissue of the control fish. The structural details of the kidney of control *O.mossambicus* are shown in plate 6.3a. The histological results show that the organism was negatively affected at tissue level on exposure to both phenol and m-cresol. Important changes observed in the phenol treated groups were glomerular congestion, tubular architectural loss and pyknotic nuclei (Plates 6.3b and c). Most significant change observed in m-cresol treated group was vacuolation of tubular epithelium and necrosis (Plate 6.3d).

6.4 Discussion

The results from the present study suggest that the histopathological lesions observed in the organism are due to exposure to phenolic compounds. Histopathological characteristics of specific organs express condition and represent time-integrated endogenous and exogenous impacts on the organism stemming from alterations at lower levels of biological organization (Chavin, 1973). Therefore, histological changes occur earlier than reproductive changes and are more sensitive than growth or reproductive parameters and, as an integrative parameter, provide a better evaluation for the health of the organism than a single biochemical parameter (Segner and Braunbeck, 1988).

The damage of gills of fish exposed to the sub-lethal concentrations of phenolic compounds was severe. Extensive architectural loss was observed in the gills of phenol treated group. Richmonds and Dutta (1989) divided the commonly reported gill lesions into two groups: (1) the direct deleterious effects of the irritants and (2) the defense responses of the fish. The observed lamellar necrosis

and complete desquamation of the gill epithelium are direct responses induced by the action of phenolic compounds.

Another important histopathological change observed in the phenol treated group was hyperplasia. Morphologically, hyperplasia refers to an increase in the number of normal cells that constitute a given tissue. Gill alterations such as hyperplasia of the epithelial cells can be considered adaptive, since they increase the distance between the external environment and blood, serving as a barrier to the entrance of contaminants. Hyperplasia observed maybe the fish's response (1) to ward off or block something that irritates its tissues, whether externally or internally, or (2) to quickly heal an injured or irritated site. Hyperplasia, however, may play a role in the early stages of neoplasia. Gill hyperplasia might serve as a defensive mechanism leading to a decrease in the respiratory surface and an increase in the toxicant-blood diffusion distance. Increased mucus production and fusion of lamellae were obvious on exposure to both the phenolic compounds. Mucus cells contain mucins, polyanions composed of glycoproteins that can be effective in trapping toxicants and aid in the prevention of toxicant entry into the gill epithelium (Perry and Laurent, 1993). Extensive epithelial desquamation was also observed in the phenol treated group. It is well known that changes in fish gill are among the most commonly recognized responses to environmental pollutants (Mallatt, 1985; Laurent and Perry, 1991; Au, 2004). After acute exposure to hexavalent chromium, *Channa punctatus* exhibited marked degenerative changes in the histology of gills, kidney and liver tissues (Mishra and Mohanty, 2008).

The gills of both phenol and m-cresol treated group exhibited lamellar telangiectesis (localised dilation of blood vessel). This appearance of the secondary lamellae results from the collapse of the pillar cell system and breakdown of vascular integrity with a release of large quantities of blood that push the lamellar epithelium outward (Alazemi *et al*., 1996). Shortening and clubbing of ends of the secondary gill lamellae and clubbing of adjacent lamellae were well marked in the m-cresol treated group. Complete lamellar fusion may

have reduced the total surface area for gas exchange. Otherwise, they increase the distance of the water-blood barrier, which together with epithelial lifting and the increase in mucus secretion may drastically reduce the oxygen uptake.

As fish gills are critical organs for their respiratory and osmoregulatory functions, the injuries in gill tissues observed as a result of exposure to phenolic compounds may have reduced the oxygen consumption and resulted in the disruption of the osmoregulatory functions of the fish. As gills are the major site of osmotic and ionic regulation in fish, any change in gill morphology may result in perturbed osmotic and ionic status which was observed as decreased branchial ATPases activity (chapter 4) in the present investigation. Also the histopathological alterations could be attributed to increased peroxidative damage to gill membrane in fishes exposed to phenolic compounds (chapter 3). It is important to stress that lamellar fusion and disappearance of secondary lamellae can lead to a notable reduction in the respiratory surface, which consequently can hinder gas exchanges (Rajabanshi and Gupta 1988; Poleksic and Mitrovic-Tutundzic, 1994). The defense responses will take place at the expense of the respiratory efficiency of the gills and eventually, the respiratory impairment must outweigh any protective effect against pollutant uptake.

We have observed significant deformations in liver on exposure to both the phenolic compounds. Liver being the main organ of various key metabolic pathways, toxic effects of chemicals usually appear primarily in the liver. This, in turn, provides important data on the chemical's toxicity and mode of action. Also it is a principal site of detoxification based on the fact that in teleosts it is the major site of cytochrome P450 which inactivates some chemicals and activates others. Furthermore, nutrients derived from gastrointestinal absorption are stored in hepatocytes and released for further metabolism by other tissues (Moon *et al*., 1985), bile synthesized by hepatocytes aids in the digestion of fatty acids (Boyers *et al*., 1976) and carries conjugated metabolites of toxicants (Gingerich, 1982) into the intestine for excretion or enterohepatic recirculation, and the yolk protein

vitellogenin is synthesized within the liver (Vaillant *et al*., 1988). Many organic compounds induce toxicopathic lesions in the liver of fish species. Stressor-associated alterations of hepatocytes may be found in the nucleus or cytoplasm or both.

An important observation in the current study on exposure to phenol was clear cell foci which exhibited an altered staining pattern. Focal lesions are precursors to the development of hepatocellular neoplasm indicating a reduced capacity to metabolize xenobiotics. Myers *et al*. (1990) suggest that there are strong and consistent associations among all of the putatively preneoplastic foci of cellular alteration (basophilic, eosinophilic, and clear cell foci), between focal lesions and the different types of neoplasms, and among the various neoplasm types. Hepatocellular foci of altered hepatocytes have been suggested as an early stage in the stepwise formation of hepatic neoplasia and as such provide an excellent example of a histopathological biomarker for contaminant exposure (Hinton *et al*., 1992). Histologic examination of mummichog (*Fundulus heteroclitus*) from a creosote-contaminated site in the Elizabeth River, Virginia, revealed high incidences of hepatic neoplastic lesions (Vogelbein *et al*., 1990). Stehr *et al*. (2003) observed that on chemical contaminant exposure English sole (*Pleuronectes vetulus*) in Vancouver Harbour, Canada showed toxicopathic liver lesions such as neoplasms, preneoplasms, specific degeneration/necrosis and non-neoplastic proliferative lesions.

Another important change observed in the liver of treated groups was necrosis. Necrosis, which is a passive mode of cell death shows that the capacity to maintain homeostasis was affected. Thus occurrence of necrosis may be one of the important reasons for decreased lysosomal membrane stability (chapter 5) observed leading to the leakage of lysosomal marker enzyme acid phosphatase to the soluble fraction. Also the increased level of the important marker enzyme ALT in liver (chapter 2) indicates the stress induced by the phenolic compounds in this tissue.

In both the phenolic compounds treated groups shrunk and pyknotic nuclei were observed in liver. Pyknotic nuclei observed indicate that the cells became hypofunctional. Pyknosis results in irreversible condensation of chromatin in the nucleus of a cell. Acute toxic injury usually includes cloudy swelling or hydropic degenerations and pyknosis, karyorrhexis and karyolysis of nuclei (Hawkes, 1980; Hinton and Lauren, 1990; Hinton *et al*., 1992; Visoottiviseth *et al*., 1999; Jiraungkoorskul *et al*., 2003) Cloudy swelling, bile stagnation, focal necrosis, atropy and vacuolization have been reported in the *Corydoras paleatus* exposed to methyl parathion (Fanta *et al*., 2003). Cengiz and Unlu (2006) reported hypertrophy of hepatocytes, increase of kupffer cells, circulatory disturbance, narrowing of sinusoids, pyknotic nuclei, fatty degeneration and focal necrosis in the liver of *Gambusia affinis* exposed to deltamethrin. The cellular degeneration in the liver may be also due to oxygen deficiency as a result of gill degeneration and/or to the vascular dilation and intravascular haemolysis with subsequent stasis of blood (Mohamed, 2001).

The kidney is a highly dynamic organ in most of the vertebrates. Kidney receives about 20% of the cardiac output. Any chemical substances in the systemic circulation are delivered in relatively high amounts to this organ. Thus a nontoxic concentration of a chemical in plasma could become toxic in the kidney. The kidney of the fish receives largest proportion of postbranchial blood, and therefore renal lesions might be expected to be good indicators of environmental pollution (Ortiz *et al*., 2003). In the present study the most evident changes observed in the kidney of phenol treated groups were glomerular congestion, pyknotic nuclei and renal tubular architectural loss. m-cresol treated group showed histopathological alterations such as necrosis and vacuolation of tubular epithelial cells. It was also observed that in both the treated groups epithelial cells have become swollen and basophilic.

Heavy metal-induced alterations of interrenal cells were demonstrated in several other species (Norris *et al*., 1997; Hontela, 1998; Levesque *et al*., 2003)

which may be due to the stress impact of metals in this endocrine component. Elsan treatment in *Channa punctatus* resulted in a significant decrease in the dimension of Bowman's capsule and glomerulus, and the tubules lost their regular shape due to precipitation of cytoplasm and karyolysis (Banerjee and Bhattacharya, 1994). Hypertrophy of renal cells, changes in the nuclear structure, formation of vacuoles, necrosis and degeneration of renal components were noticed on the renal cells of *Cyprinus carpio* exposed to malathion and sevin (Dhanapakiam and Premlatha, 1994). Dass and Mukherjee (2000) reported dilation of tubules, necrotic changes characterized by karyorrhexis and karyolysis at the nuclei of affected cells of *Labeo rohita* exposed to hexachlorocyclohexane. The exposure of fish to toxic agents such as pesticides and heavy metals induces histological alterations in several components of the trunk kidney (Kendall, 1975; Kirubagaran and Joy, 1988; Ortiz *et al*., 2003; Velmurugan *et al*., 2007). Cengiz (2006) observed lesions in the kidney tissues of fish exposed to deltamethrin, characterized by degeneration in the epithelial cells of renal tubule, pyknotic nuclei in the hematopoietic tissue, dilation of glomerular capillaries, degeneration of glomerulus, intracytoplasmatic vacuoles in epithelial cells of renal tubules with hypertrophied cells and narrowing of the tubular lumen. Ayas *et al*. (2007) observed histopathological changes in liver and kidney of three different fish species having different feeding habits in Sariyar Reservoir, Turkey, contaminated with organochlorine pesticide residues. They noticed characteristic changes such as mononuclear cell infiltration, congestion and nuclear pyknosis in liver and kidney.

As a conclusion, the findings of the present histological investigations demonstrate a direct correlation between exposure to phenolic compounds and histopathological disorders observed in several tissues. All the histopathological observations indicated that exposure to sub-lethal concentrations of phenolic compounds caused destructive effect in the gills, liver and kidney tissues of *O. mossambicus*. It is important to stress that phenolic compounds are biotransformed in the liver of fish by phase I and phase II reactions. In phase I,

reactions of oxidation, reduction and hydrolysis catalysed by CYP 450 system occur, whereas phase II involves the conjugation of the phase I products with the endogenous molecules, such as glutathione, sulphate or glucuronic acid (Andersson and Forlin, 1992; Siroka and Drastichova, 2004). The activation in fishes frequently depends on oxidative metabolism catalyzed mostly by microsomal cytochrome P-450-dependent mixed-function oxidases. However, CYP-catalyzed biotransformation may also activate nontoxic procarcinogens to potent carcinogens or even to toxic metabolites (Yan and Caldwell, 2001). The metabolites get distributed throughout the organism by the bloodstream, causing even greater damage. The observed abnormal behaviour and altered histopathology of vital organs demonstrate the severe adverse effects to exposure of phenolic compounds in *O. mossambicus*.

The current study reinforces the application of histopathology as a powerful tool for monitoring anthropogenic contamination within aquatic environments. Whilst links between such pathologies and contaminants are not definitive, such surveillance provides a useful insight into individual, population and overall ecosystem quality. When these pathological endpoints are assessed in conjunction with other parameters such as parasite community structure, sediment and water chemistry, enzyme responses, bile metabolite levels and molecular damage indices, a clearer picture of the complex interactions between anthropogenic and natural environmental modifiers will emerge.

Plate 6.1 Histopathological changes observed in the gill tissue of *O. mossambicus* *on* exposure to different phenolic compounds.

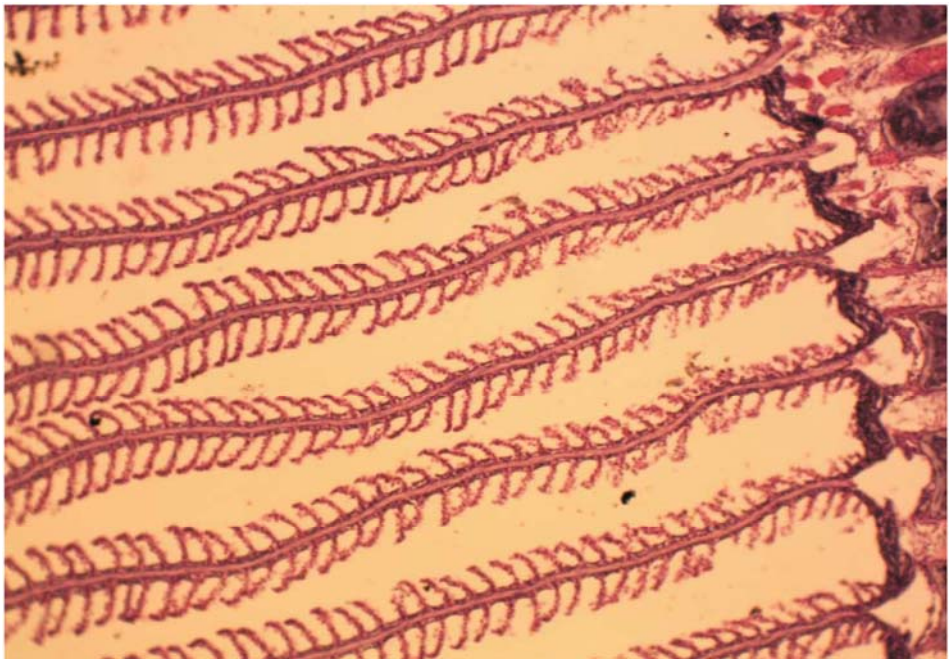

Plate 6.1a Photomicrograph of control gills of *O. mossambicus* showing normal gill architecture with primary lamellae and secondary lamellae (20X).

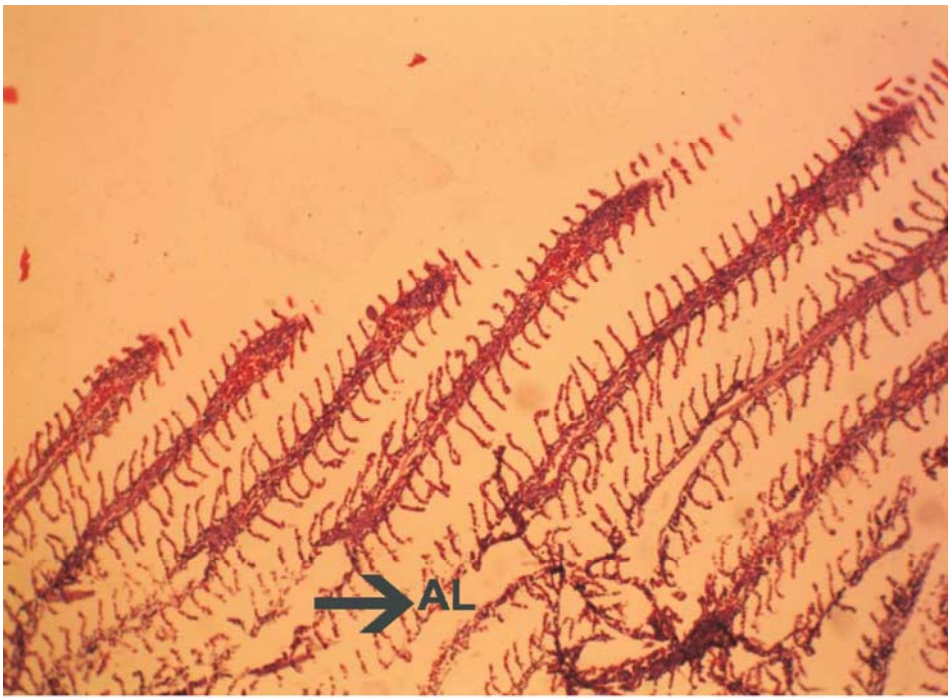

Plate 6.1b Photomicrograph of gills of *O. mossambicus* treated with phenol showing complete architectural loss (20X).

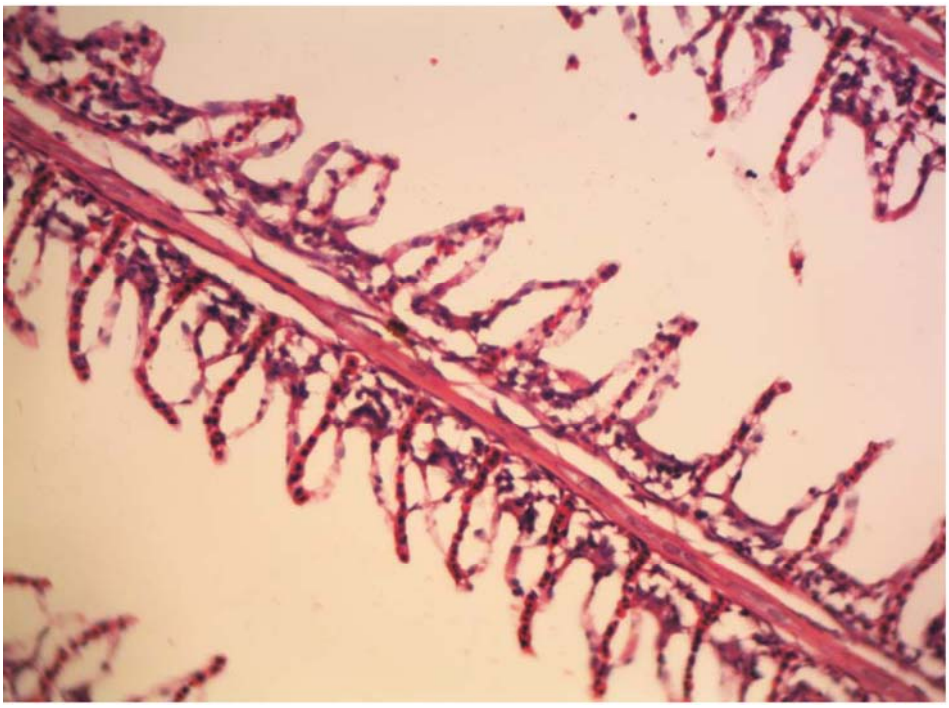

Plate 6.1c Photomicrograph of control gills of *O. mossambicus* showing normal gill architecture with primary lamellae and secondary lamellae (40X).

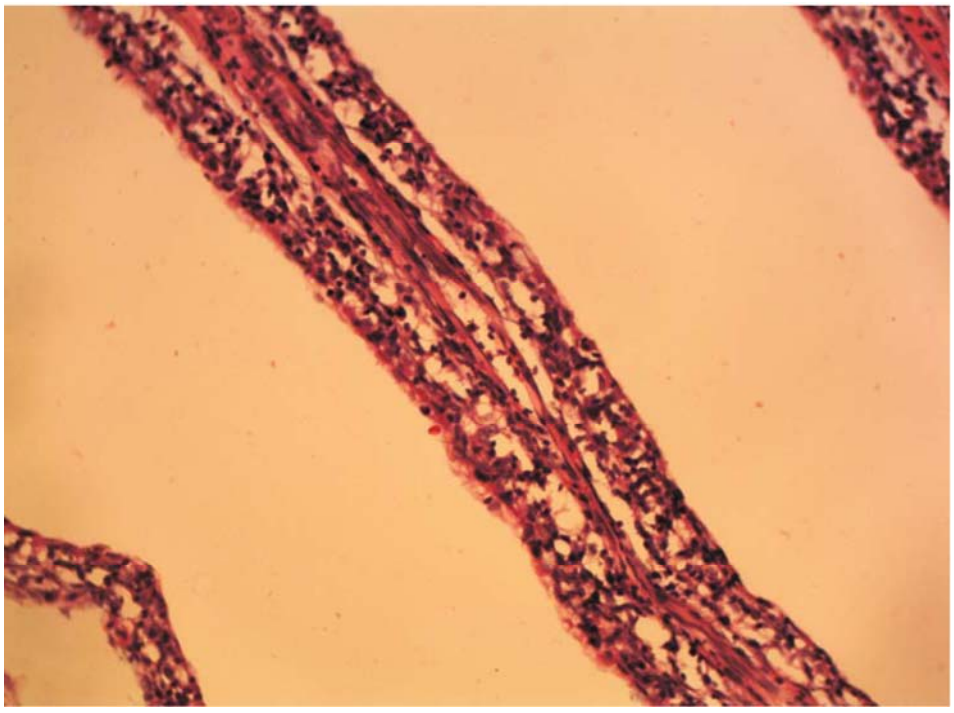

Plate 6.1d Photomicrograph of gills of *O. mossambicus* treated with phenol showing complete necrosis of secondary lamellae (40X).

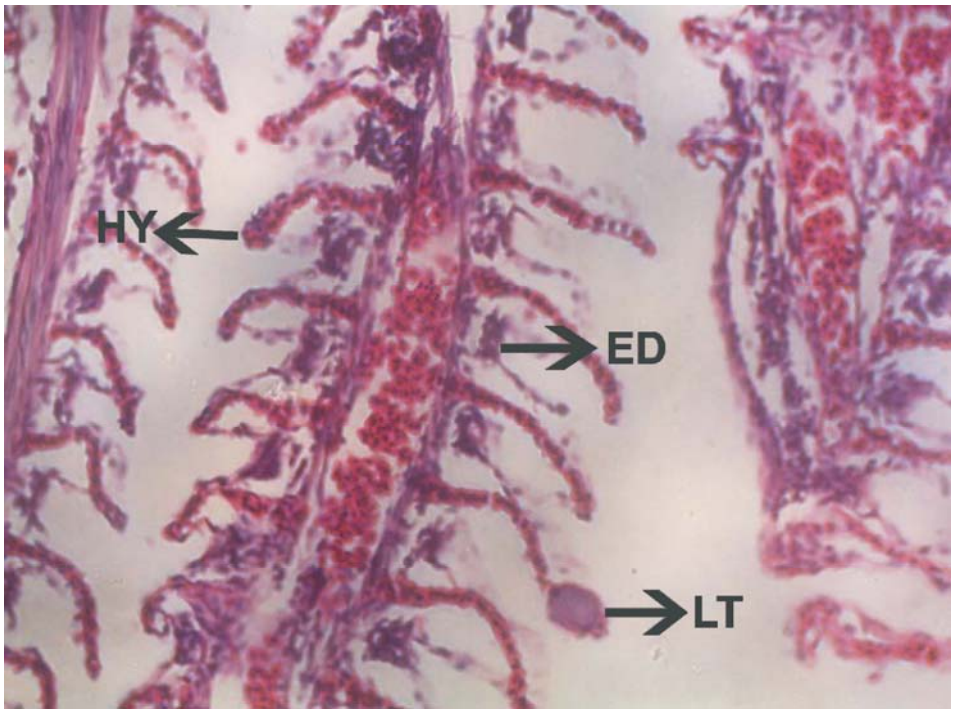

Plate 6.1e Photomicrograph of gills of *O. mossambicus treated* with phenol showing hyperplasia (HY), epithelial desquamation (ED) and lamellar telangiectesis (LT) (40X).

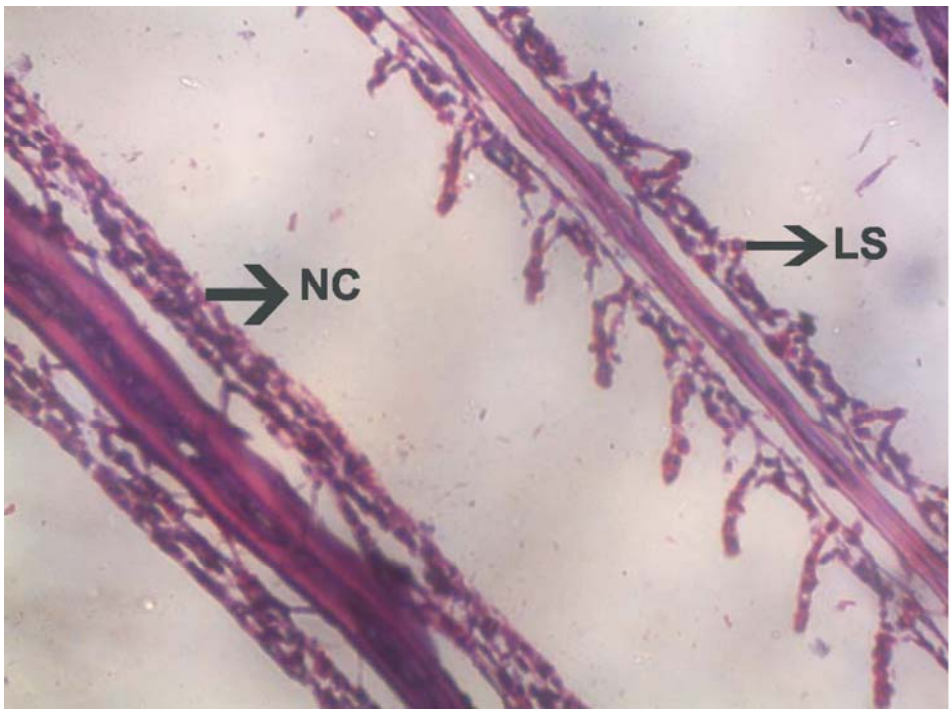

Plate 6.1f Photomicrograph of gills of *O. mossambicus* treated with m-cresol showing lamellar necrosis (NC) and lamellar shortening (LS) (40X).

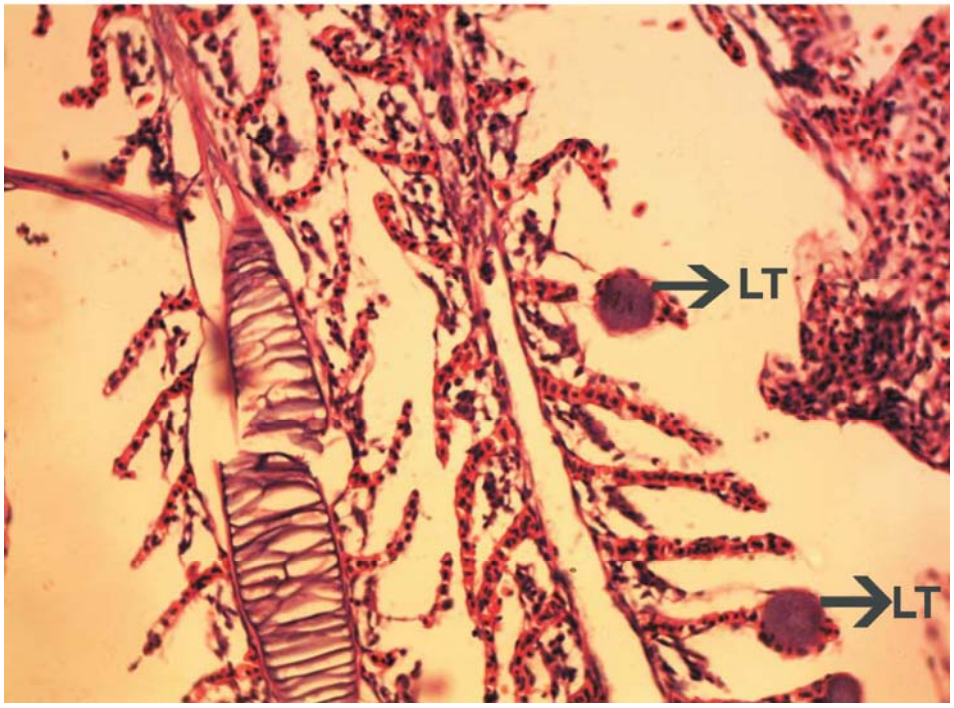

Plate 6.1g Photomicrograph of gills of *O. mossambicus* treated with m-cresol showing lamellar telangiectesis (LT) (40X).

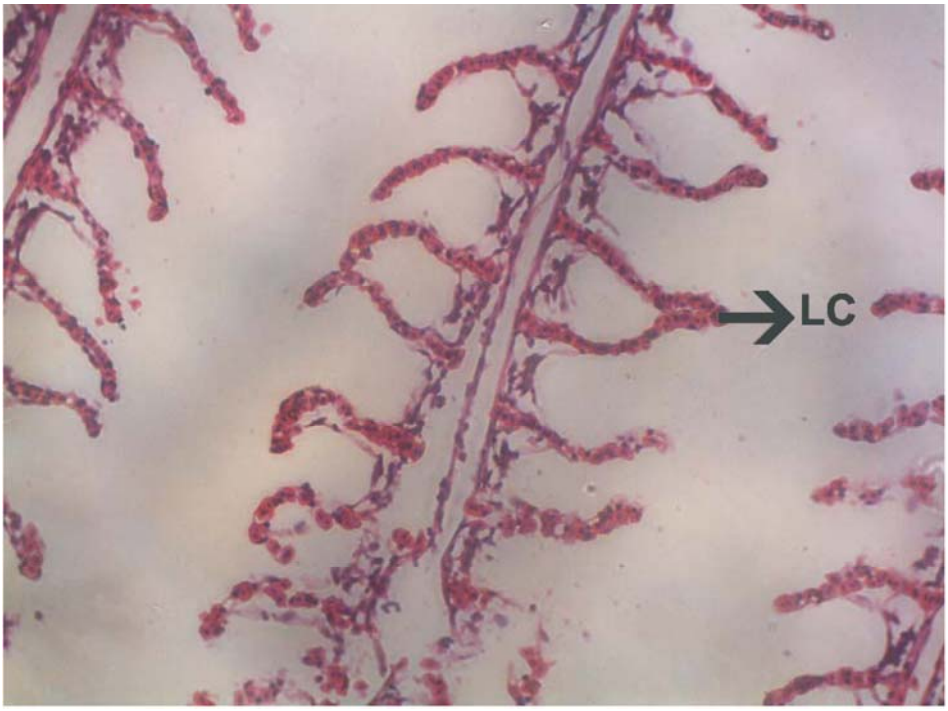

Plate 6.1h Photomicrograph of gills of *O. mossambicus* treated with m-cresol showing lamellar clubbing (LC) (40X).

Plate 6.2 Histopathological changes observed in the liver tissue of *O. mossambicus* on exposure to different phenolic compounds.

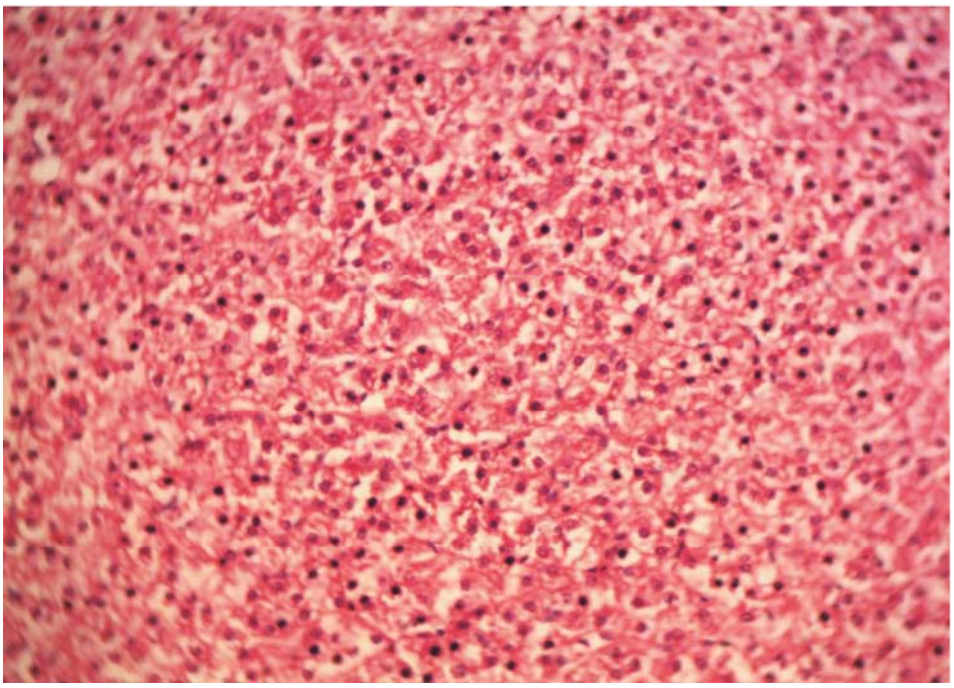

Plate 6.2a Photomicrograph of control liver of *O. mossambicus* showing normal architecture (40X).

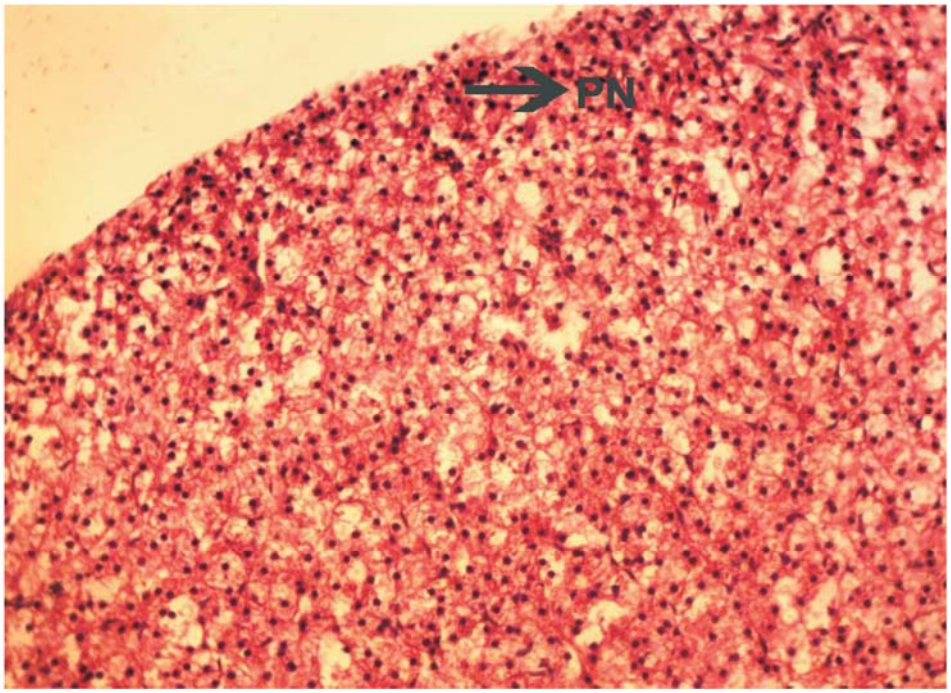

Plate 6.2b Photomicrograph of liver of *O. mossambicus* treated with phenol showing pyknotic nuclei (PN) (40X).

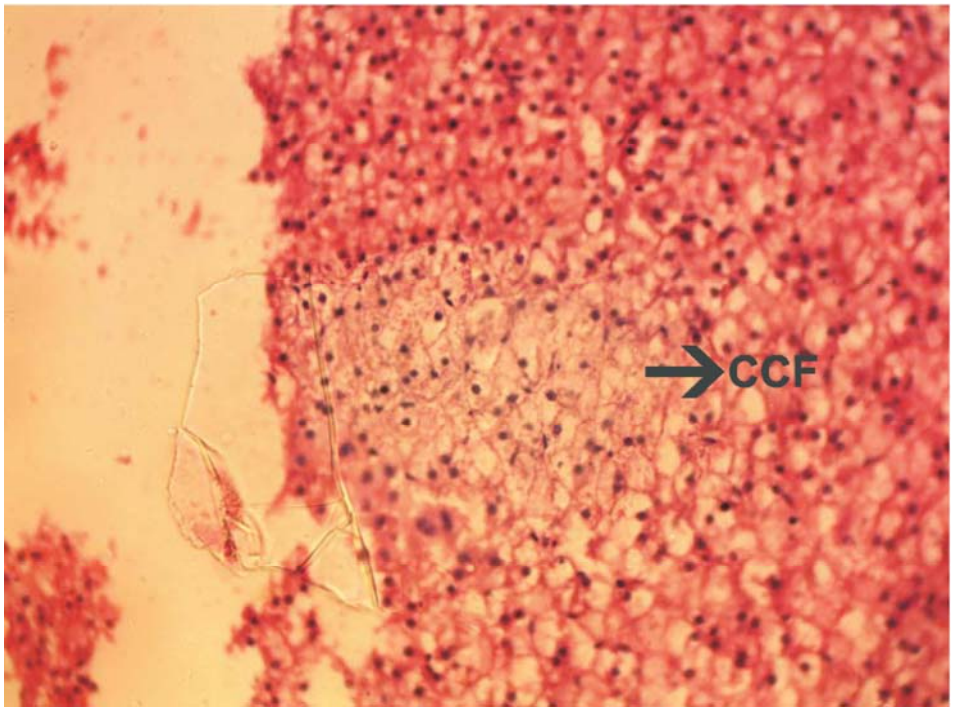

Plate 6.2c Photomicrograph of liver of *O. mossambicus* treated with phenol showing clear cell foci (CCF) (40X).

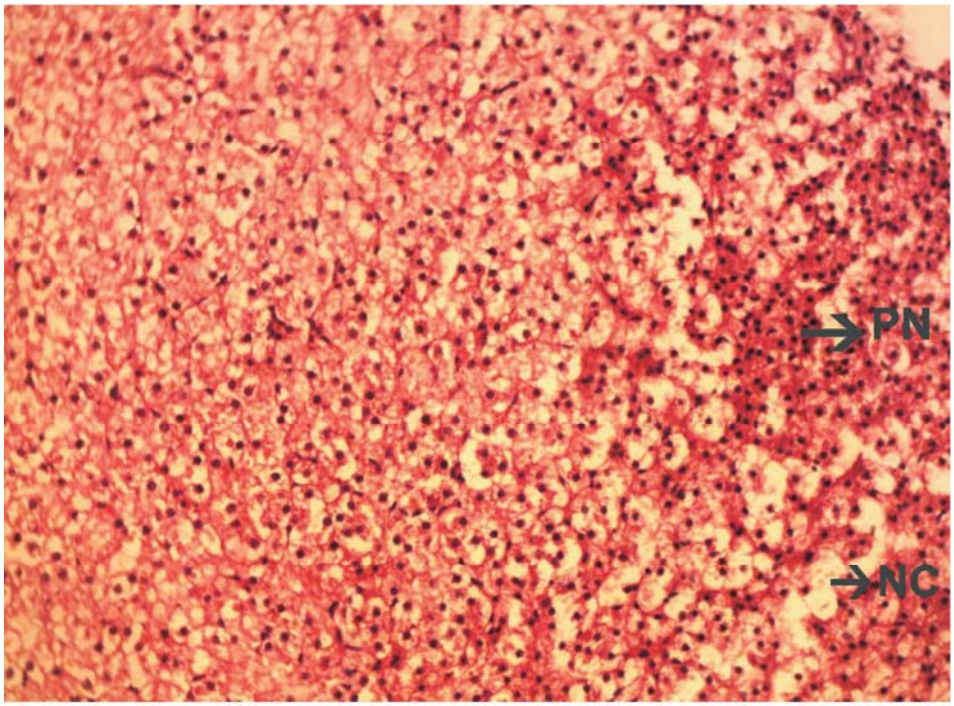

Plate 6.2d Photomicrograph of liver of *O. mossambicus* treated with m-cresol showing pyknotic nuclei (PN) and necrosis (NC) (40X).

Plate 6.3 Histopathological changes observed in the renal tissue of *O. mossambicus* on exposure to different phenolic compounds.

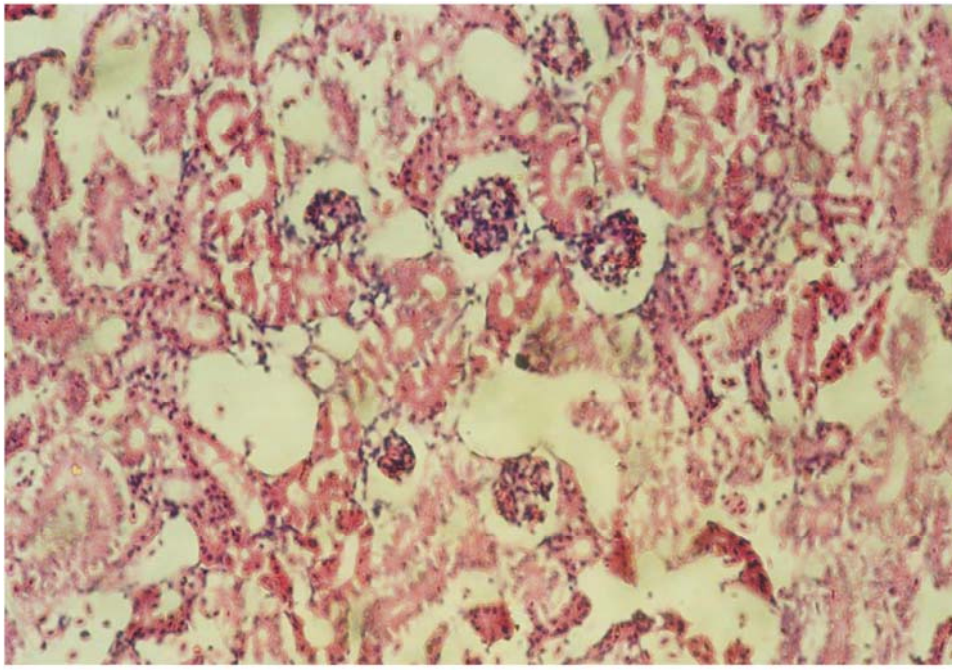

Plate 6.3a Photomicrograph of control kidney of *O. mossambicus* showing normal architecture (40X).

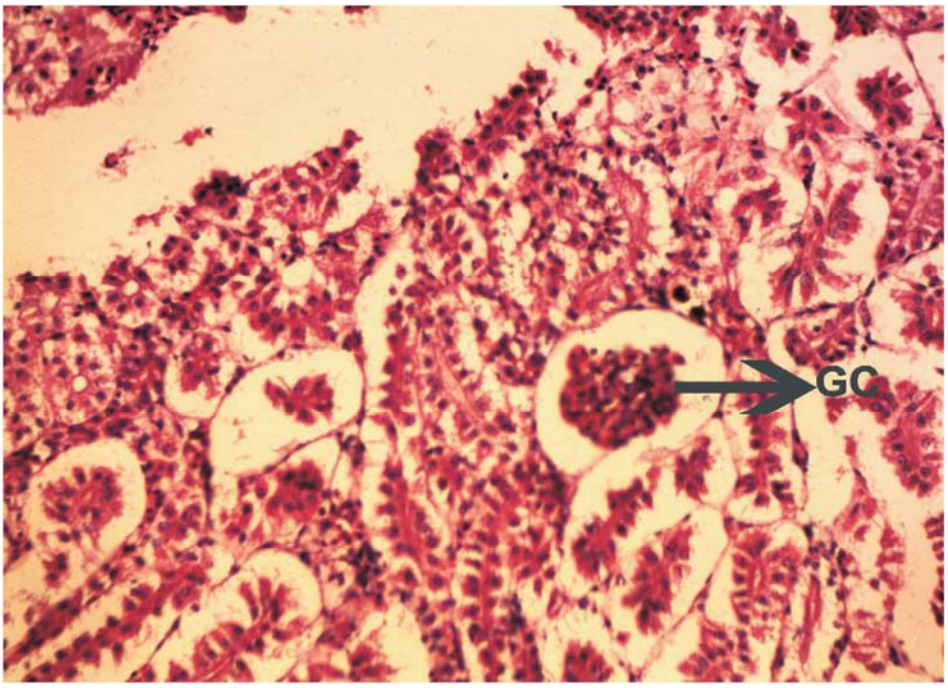

Plate 6.3b Photomicrograph of kidney of *O. mossambicus* treated with phenol showing glomerular congestion (GC) (40X).

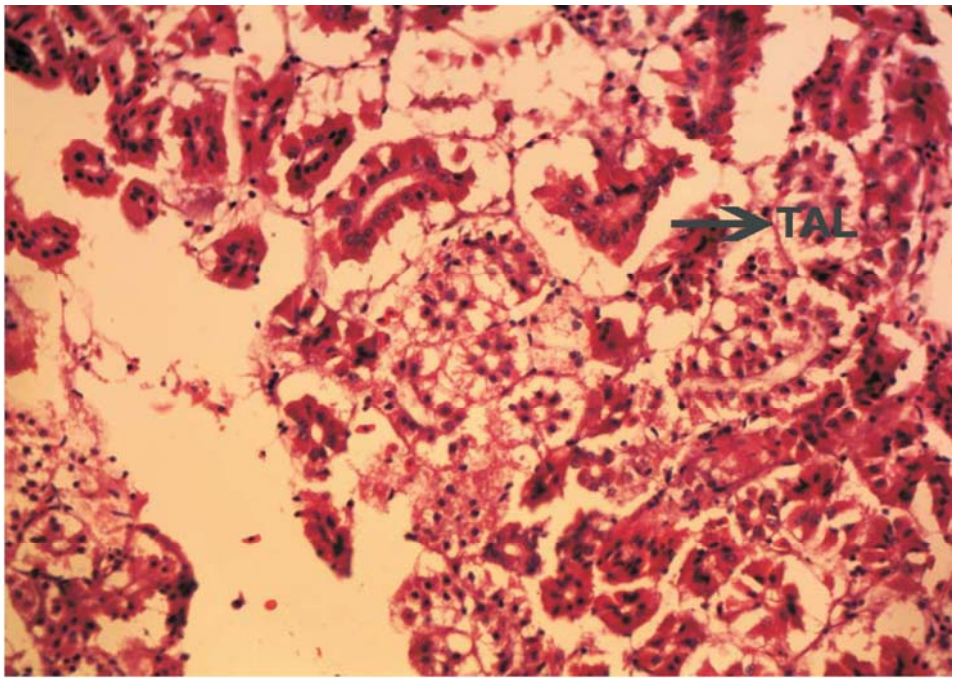

Plate 6.3c Photomicrograph of kidney of *O. mossambicus* treated with phenol showing tubular architectural loss (TAL) (40X).

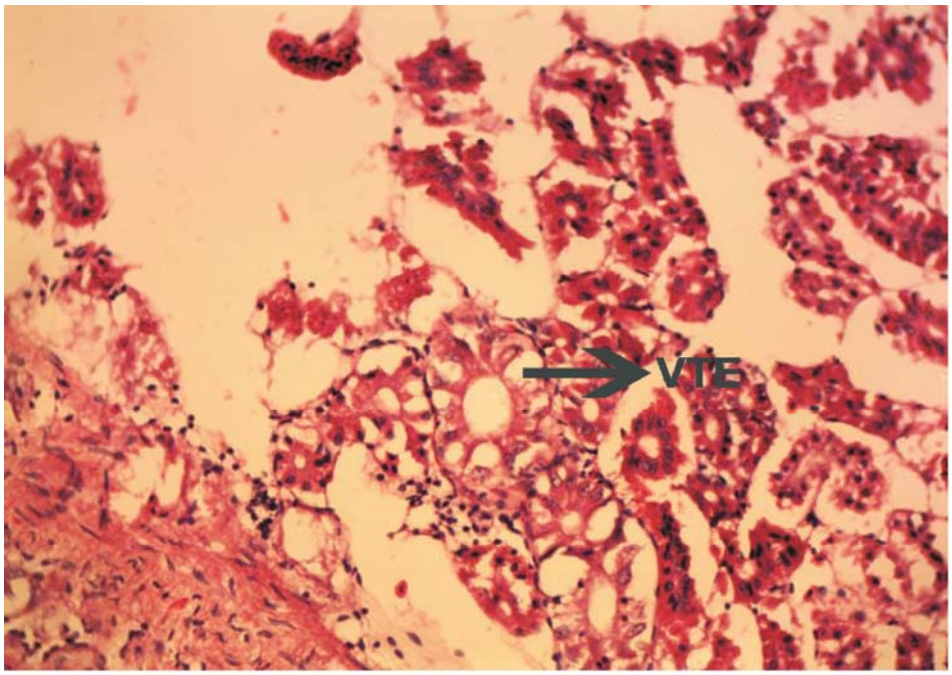

Plate 6.3d Photomicrograph of kidney of *O. mossambicus* treated with m-cresol showing vacuolation of tubular epithelium (VTE) (40X).

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Chapter 7

SUMMARY & CONCLUSIONS

Summary and conclusions

Many pollutants exist in the aquatic environment, for short or long periods, at sub-lethal levels. These levels are not noticed because they do not cause immediate fish mortality. However, the consequences of such effects are morphological and physiological, causing illness and reducing fitness for life. Therefore, the simple fact that a sub-lethal concentration is considered safe because it does not kill any fish does not means that it can be used indiscriminately because contaminant effects can weaken fish, rendering them more susceptible to mortality from other causes. In many cases, alterations at the cellular or sub cellular level are not by themselves diagnostic of a particular type of pollutant. Hence in the present investigation a combination of biochemical and histopathological studies has been adopted for determining a specific response to a particular pollutant. In the present study freshwater-adapted euryhaline teleost *O. mossambicus* was exposed to different phenolic compounds at both *in vitro* and *in vivo* conditions.

Important findings of the study can be summarized as;

- 96 h LC_{50} values of phenol and m-cresol in *O. mossambicus* were found to be 31.5 $mg.l^{-1}$ and 22 $mg.l^{-1}$ respectively.
- Behavioural changes observed on exposure to both the phenolic compounds included intense and frequent avoidance reactions, consisting chiefly of agitated, erratic and violent swimming bouts.
- The lack of cortisol response suggests that, similar to other xenobiotics phenol and m-cresol can act as an endocrine disruptor and as such impair steroidogenesis. Fish exhibiting an impaired cortisol stress response may be at a disadvantage in coping with other environmental stressors.

- Depletion of total carbohydrates in liver points to the increased utilization of stored energy for detoxification mechanisms since phenolics are often excreted as glucuronyl derivatives by conjugating with UDP-glucuronic acid.
- Depression of glucose-6-phosphatase activity may be a reflection of damage to the microsomal membrane as the enzyme is localized exclusively in the membranes of the endoplasmic reticulum.
- Blood glucose homeostasis was not maintained on exposure to phenolics which may be due to the lack of cortisol response and decreased glucose-6-phosphatase activity.
- On exposure to phenolic compounds gills, liver and kidney showed an elevated pyruvate level compared to control. This might be due to high rate of glycolysis taking place which is the only energy-producing pathway for the animal when it is under stress conditions.
- Stimulation of LDH in muscle of *O. mossambicus* on exposure to phenolic compounds suggests that the final product of glycolysis - pyruvate was preferentially used to produce lactate. Lactate is an important gluconeogenic substrate which helps to cope with the high and rapid energy demand under toxic stress.
- A significant decrease in LDH activity was observed in liver and kidney of m-cresol treated fishes and in gills of fishes treated with phenol was observed which may be due to increased tissue damage.
- Elevation in the levels of AST and ALT in different tissues of *O. mossambicus* can be considered as a response to the stress induced by phenolic compounds to generate keto acids like α-ketoglutarate and oxaloacetate for contributing to gluconeogenesis and/or energy production necessary to meet the excess energy demand.

- An elevation in ACP activity suggests an increase in lysosomal mobilization and cell necrosis due to the toxicity of phenolics. This increase also suggests the supply of phosphate group for energy metabolism.
- Elevated GDH activity was found in almost all tissues treated with phenol; this increased activity may have helped in funneling more α-ketoglutarate into TCA cycle for energy generation.
- Liver and muscle of both the treated groups showed decreased protein content which may be related to impaired food intake, the increased energy cost of homeostasis, tissue repair and the detoxification mechanism during stress.
- The responses of antioxidant enzymes such as catalase, super oxide dismutase, glutathione peroxidase, glutathione-S-transferase and non-enzymatic antioxidant glutathione shows that the organism is experiencing severe oxidative stress which confirms that phenolic compounds can act as potent free radical generators.
- Indicators of lipid peroxidation such as malondialdehyde, conjugated diene and hydroperoxide levels show that extensive lipid peroxidation occurs on exposure to different phenolic compounds.
- Branchial functioning was impaired and hence the ionic homeostasis in *O. mossambicus* was affected on exposure to sub-lethal concentrations of both phenol and m-cresol.
- The blood responses indicate adaptation to hypoxic conditions arising from pro-oxidants, damage to gills and perhaps oxygen-level fluctuations.
- *In vitro* studies show that phenol, m-cresol and 4-nonylphenol have a strong RBC membrane-destabilizing effect, eventually leading to haemolysis.

- Both *in vivo* and *in vitro* studies showed that the phenolic compounds taken for the study affected the lysosomal membrane stability and resulted in the disruption of cellular homeostasis to the point where membrane integrity was compromised.
- Lesions observed in gills, liver and kidney show intracellular degenerative process suggestive of underlying metabolic disorders. The observed abnormal behaviour and altered histopathology of vital organs demonstrate the severe adverse effects on exposure to phenolic compounds in *O. mossambicus*.

Since most environmental contamination occurs at low concentrations, but is of chronic nature, there is a particular need for methods and more subtle techniques to register effects of low concentrations of chemicals. The results obtained show that a combination of biochemical, haematological, *in vivo* and *in vitro* investigations on biological membranes, studies of oxidative stress responses, ion regulatory enzyme studies and histopathological investigation gave hints of the underlying mechanism of toxic injury. Phenolic compounds taken for the present investigation were phenol, m-cresol and 4-nonylphenol which have *pKa* values 9.89, 10.99 and 10.28 respectively. Phenols with *pKa* values > 8.0 exhibit polar narcosis. The mode of action of polar narcosis is not well characterized, but it is assumed to be a non-specific disruption of the functions of the biological membranes causing progressive lethargy, unconsciousness and death. From the results obtained, it is possible to consider that the phenolic compounds can cause a potential risk to the health of aquatic organisms even at very low concentrations ($1/10^{th}$ of LC_{50} value) for a short duration of three weeks, which can in turn affect the growth and vital functions. Prolonged exposure to phenolic compounds may cause serious physiological problems ultimately leading to the death of fish.

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