Effect of chlorpyrifos on the gonadal maturation of freshwater catfish *Heteropneustes fossilis* (Bloch, 1794)

THESIS

SUBMITTED IN FULFILMENT FOR THE AWARD OF THE DEGREE OF

Doctor of Philosophy

IN

APPLIED ANIMAL SCIENCES

Under the Supervision of

Dr. ABHA MISHRA
Associate Professor

Submitted By

ABHISWETA SINGH
M.Sc.

DEPARTMENT OF APPLIED ANIMAL SCIENCES
SCHOOL FOR BIOSCIENCES AND BIOTECHNOLOGY
BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
(A CENTRAL UNIVERSITY)
VIDYA VIHAR, RAEBARELI ROAD, LUCKNOW-226025 (U.P.), INDIA

Enrollment No. 706/15 **Year 2021**

DECLARATION

I hereby declare that this thesis entitled **"Effect of chlorpyrifos on the gonadal maturation of freshwater catfish *Heteropneustes fossilis* (Bloch, 1794)"** submitted by me for the award of the degree of **Doctor of Philosophy** in **Applied Animal Sciences** is an outcome of my original research work and my own efforts carried out under the guidance and supervision of Dr. Abha Mishra, Associate Professor, Department of Applied Animal Sciences, Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow. It has not been submitted for the award of any other degree or diploma, associateship of any university or institution. I also declare that this thesis is essentially free from all kinds of plagiarism.

Place: Lucknow
Date: 21-01-2021

Abhisweta

Signature of candidate
(Abhisweta Singh)

CERTIFICATE

This is to certify that the thesis entitled **"Effect of chlorpyrifos on the gonadal maturation of freshwater catfish *Heteropneustes fossilis* (Bloch, 1794)"** submitted by Abhisweta Singh is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university. The thesis submitted to Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow satisfies all the requirements as stipulated in the Doctor of Philosophy (Ph.D.) regulations-1999 as amended in 2013 and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

Date: 21/1/2021
Place: Lucknow

Abha Mishra

(Dr. Abha Mishra)
Supervisor

22/01/2024

Head of the Department

ACKNOWLEDGEMENTS

Firstly, I would like to thank Almighty God who has bestowed me his immense blessings, given me unbroken health and strength during the span of my research work because of which this thesis was possible. I owe my deep gratitude and indebtedness to Hon'ble Vice Chancellor, Prof. Sanjay Singh and former Vice Chancellor, Prof. R. C. Sobti, Babasaheb Bhimrao Ambedkar University, Lucknow, for giving me an opportunity to carry out my work in an esteemed Central University.

It is a matter of immense pleasure to express my deep veneration and gratitude to ***Prof. Kamal Jaiswal, Dean of School of Life Sciences (Formerly School of Biosciences and Biotechnology) and Head of Department of Zoology (Formerly Applied Animal Sciences), School of Life Sciences,*** *Babasaheb Bhimrao Ambedkar University, Lucknow for supporting and helping me with all sorts of facilities to carry out my research work efficiently throughout my study period.*

I am extremely delighted to express my high regard and indebtedness to my Supervisor ***Dr. Abha Mishra****, Associate Professor, Department of Zoology (School of Life Sciences), Babasaheb Bhimrao Ambedkar University, Lucknow for her constant supervision, invaluable guidance and motivation. Her best suggestions and excellent insight of the objectives made me capable to finish this arduous work efficiently. I am grateful for her perceptive conversations and helpful comments shared during my research work.*

I also express my earnest gratitude towards ***Prof. V. Elangovan (former Head)****,* ***Dr. Venkatesh Kumar R.****,* ***Dr. Suman Mishra****,* ***Dr. Sandhya****,* ***Dr. Neeshima Jaiswal****, Faculty members of Department of Zoology, Babasaheb Bhimrao Ambedkar University, Lucknow for their immense blessings and support whenever required.*

I would also like to thank all the technical and non-teaching staff members, ***Mrs. Kavita, Mr. Abhishek Tiwari, Mr. Deepak, Mr. Ramesh Kumar*** *of Department of Zoology, Babasaheb Bhimrao Ambedkar University Lucknow, for their help during my research work.*

I cannot forget my special thanks to ***Awanish Kumar Singh, Savita, Shani Kumar Bhartiy, Akansha Dwivedi, Umesh, Rituraj, Priyamvada Mishra*** *and* ***Sneha*** *for helping me during my research work whenever needed.*

I am also very thankful to Acube Life Sciences Private Limited, Lucknow for carrying out my gene expression work effecinetly.

This work could never have been possible without the love and support of my family who has been a constant source of strength and support all through these years. I express my love and gratitude towards my Father, ***Mr. Ashok Kumar Singh****, Mother,* ***Sumitra Singh*** *and brother,* ***Abhishek Singh****, for having faith in me, boosting my confidence, endless love and care throughout my life. I also express my heart-felt gratitude to my husband,* ***Mr. Prashant Kumar Singh*** *for his love and support without which I would never have accomplished my thesis. I am thankful to him for believing and encouraging me during my Ph.D. completion.*

I also express my sincere gratitude and thanks to ***Mr. Deepak Kashyap****, the local fish supplier in Lucknow, for providing me healthy fish whenever required and without whom this research could never have been possible.*

I also seek an opportunity to thank ***University Grants Commission*** *for financial assistance and* ***DBT (BT/PR 21245/AAQ/3/830/2016)*** *for financial support to strengthen laboratory for research work.*

The list never ends of mentioning people who have helped me during my research work. I am thankful to the direct and indirect support of people who have helped me completing my thesis on time. Due to lack of space I have to keep my pen down but I do express my gratitude and affection to them.

Abhisweta

Abhisweta Singh

CONTENTS

General Introduction

General Introduction

Agriculture has always been the backbone of the Indian economy since ages. About 70% of the population is dependent on agriculture for livelihood, making agriculture sector essential for the sustenance and growth of the Indian economy (Limbore and Khillare, 2015). Pesticides are one of the most important inputs in maintaining agriculture production as a substantial amount of food production is lost due to insect pests, pathogens, weeds etc. (Bhardwaj and Sharma, 2013). The extensive and inappropriate use of pesticides has caused havoc to non-target living organisms and the environment as they tend to persist in the environment for a long duration because of water solubility, soil adsorption and slower degradation (Bernardes *et al.*, 2015). Water bodies act as a sink for agricultural runoff, become contaminated with pesticide residues and threaten the populations of aquatic organisms. These residues accumulate in aquatic organisms either from the surrounding water or indirectly through food consumption (Thomas and Mansingh, 2002).

Pesticide

The term pesticide covers a wide range of substances or mixture of substances or biological agents that are intentionally released into the environment to deter, destroy or control populations of insects, weeds, rodents, nematode, fungi and other harmful pests (Mahmood *et al.*, 2016). The percentage consumption of pesticides in India is more than the other major countries of the world in general. They are tremendously used to improve crop productivity to feed a large population of individuals, increase yield and

prevent crop losses (Aktar *et al.,* 2009). The extreme use of pesticides has allowed them to persist in the environment for a long duration and is gradually accumulated by the non-target organisms. These pesticides contaminate the water bodies via surface runoff within a few days of application (Sabra and Mehana, 2015). Such pesticides along with their products of biotransformation remain in the environment and interact with the living organisms in multiple ways, according to their chemical nature, dose and targets. They may enter the body of non-target living organisms especially fish in various ways and may lead to deleterious effects on the organism (Lushchak *et al.,* 2018). The continuous impairment of health status of fish and other aquatic organisms has made the study of aquatic toxicology relevant. The most commonly used pesticides are insecticides, herbicides, fungicides and rodenticides.

Classification of pesticides

Pesticides can be classified based on their: (i) mode of entry (ii) pesticide function (iii) chemical composition (Yadav and Devi 2017; **Figure 1**).

Classification on the basis of mode of entry

These include systemic and non-systemic pesticides. The systemic pesticides can be easily absorbed and reach to untreated tissues of the plant whereas non-systemic pesticides also known as contact pesticides act on the target pests only after coming into physical contact. Glyphosate and paraquat are systemic and non-systemic pesticide respectively.

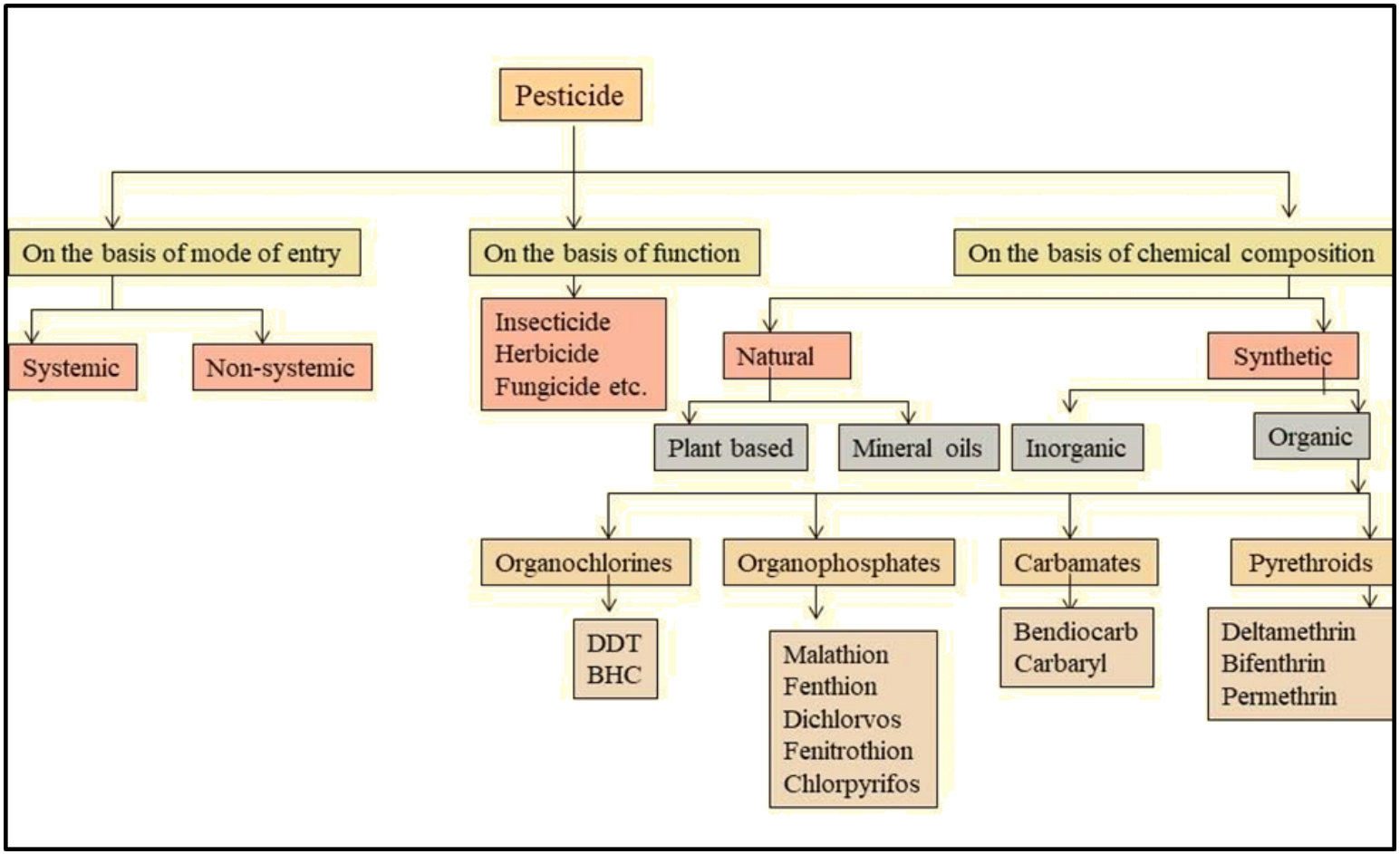


Figure 1: Classification of pesticides (Yadav and Devi, 2017)

Classification on the basis of function

This category includes pesticides classified on the basis of their specificity for their target pests. Some of these pesticides can be used for more than one class. The names of the major ones are mentioned below:

(a) Insecticide: Kills insects and other arthropods. Example, aldicarb.

(b) Fungicide: Kills fungi. Example, azoxystrobin.

(c) Bactericide: Acts against bacteria. Example, copper complexes.

(d) Acaricide: Kills mites. Example, bifenazate.

(e) Herbicide: Kills weeds and unwanted plants. Example, atrazine.

(f) Rodenticides: Controls rodents and mice. Example, warfarin

(g) Termiticides: Kills termites. Example, fipronil.

Classification on the basis of chemical composition

Pesticides can be natural or synthetic (man-made). On the basis of chemical composition, these are broadly divided into four major classes namely; organochlorines, organophosphorus, carbamates and pyrethroids.

(a) Organochlorines

Organochlorines (OC) are chlorinated hydrocarbons widely used as pesticides. These pesticides are one of the major persistent organic pollutants (POPs) with high persistence in the environment (Jayaraj *et al.,* 2016). They tend to accumulate in fat rich tissues such as nerves of insects causing neural dysfunction (Horsak *et al.,* 1964). They

are the first group of pesticides to be synthesized and used in agriculture. The common examples of these pesticides include DDT, endosulfan, aldrin etc.

(b) Organophosphates

Organophosphate pesticides (OP) are primarily used for agricultural practices. They are phosphoric acid ester compounds containing a central phosphorous atom double bonded to either oxygen or sulphur atom, and single bonded to two methoxy (-OCH_3) or ethoxy (-OCH_2, CH3) groups (Horsak *et al.,* 1964). Upon release, acetylcholinesterase enzyme (AChE) at neuromuscular junctions and in chemical synapses rapidly hydrolyzes acetylcholine (ACh) into choline and acetic acid to terminate synaptic transmission (English and Webster, 2012). OPs are toxic because they bind and phosphorylate the active site of AChE leading to irreversible inactivation of AChE. ACh is a neurotransmitter that mediates cholinergic transmission not only in insects but in most of the animal species (Horsak *et al.,* 1964; English and Webster, 2012). This makes OPs toxic to a wide variety of non-target species, including aquatic organisms and human beings. Despite its toxicity, OPs are in demand because they degrade rapidly into other constituents via hydrolysis on exposure to light, soil and air (Jayaraj *et al.,* 2016). Organophosphate pesticides include chlorpyrifos, malathion, parathion, diazinon, fenthion, dichlorvos.

(c) Carbamates

Carbamate pesticides are carbamic acid derivatives that kill insects in a way similar to organophosphate insecticides. They are effective insecticides because of their ability to inhibit esterases including acetylcholinesterase enzyme causing over-stimulation of the

nervous system. Unlike OPs, the inhibitory effect of carbamates on cholinesterase is for a very short duration. They are mainly used for small fleas and ticks (Struger *et al.,* 2016). Carbamates include carbofuran, aldicarb, and carbaryl.

(d) Pyrethroids

They are synthesized from the natural pyrethrins which are extracted from the flowers of *Chrysanthemum cinerariafolium.* They are used widely as insecticides both for domestic and commercial purposes. In medicine, they are used for the topical treatment of scabies and headlice. Pyrethroids mainly target excitable (nerve and muscle) cells affecting sodium and chloride channels. They are more toxic to insects and fish and slightly toxic to mammals and birds (Bradberry *et al.,* 2005; Yadav and Devi, 2017).

Chlorpyrifos

Chlorpyrifos (CPF; O, O-diethyl-O-3, 5, 6-trichlor-2-pyridyl phosphorothioate) is considered as a broad-spectrum systemic organophosphorus insecticide (OP) used widely against pests of wheat, nuts, sweet potatoes, corn, soybeans, citrus fruits and brassica vegetables, as well as for subterranean termites (Yancheva *et al.,* 2019). It has been extensively used both for crop and non-crop purposes since its introduction into the marketplace in 1965 by Dow chemical company (U.S.A.). CPF is the top-selling OP around the globe and has been reported to be highly toxic to aquatic organisms especially freshwater fish (Yancheva *et al.,* 2019; Sunanda *et al.,* 2016). In most of the countries including the United States of America, its usage has been restricted because it was concerned for infants and children (Choi *et al.,* 2006). According to WHO, CPF has been categorized as "Moderately hazardous pesticide, class II pesticide". Like other pesticides,

CPF can easily migrate to the aquatic environment via surface runoff, air drift and tend to accumulate in different groups of non-target aquatic organisms particularly fish (Varò *et al.*, 2002). A notable value of bioconcentration factor (BCF) for CPF was noted in fishes, *Pimephales promelas* (Veith *et al.*, 1979), *Danio rerio* (El-Amrani *et al.*, 2012), *Poecilia reticulate* (Welling and deVries, 1992), and red hybrid *Tilapia* (Thomas and Mansingh, 2002). At pH 7.4, CPF has an average half-life of 53 days at 20^0C and 13 days at 37.5^0C (Freed *et al.*, 1979). It can persist in soil for 60-120 days and degrades by microbial action (Antonious *et al.*, 2016). It can also persist indoors for many months. The chlorine group present on chlorpyrifos is responsible for increasing its lipid solubility and half-life in the body of organisms (Rathod and Garg, 2017; **Figure 2**).

The main reason behind the extreme demand of CPF lies in its biodegradability. It degrades easily via hydrolysis on exposure to light, soil and water. This pesticide can easily enter the body either by ingestion of contaminated food or water, dermal contact or by inhalation of air containing CPF. The activation of CPF can occur by contact, ingestion and vapour action, causing convulsions and paralysis (Deb and Das, 2013). CPF is oxidized to more toxic metabolite chlorpyrifos oxon (CPO) via aerobic metabolism, photolysis and chlorination. The chlorination of water leads to the chemical hydrolysis of CPF forming 3, 5, 6-trichloro-2-pyridinol (TCP) which have been reported in fish tissues. The n-octanol-water partition coefficient (k_{ow}) of CPF is generally 4.8 which indicates its potential to bioaccumulate in terrestrial and aquatic food chains (Koshlukova and Reed, 2014). The physical and chemical properties of CPF have been mentioned in **Table 1.**

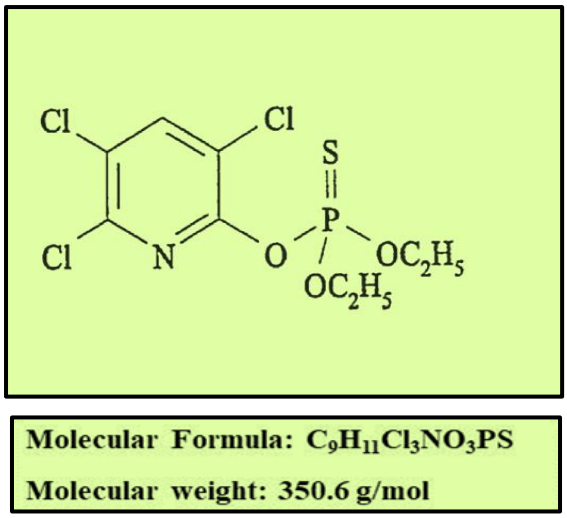


Figure 2: Structure of chlorpyrifos (Rathod and Garg, 2017)

Table 1: Physical and chemical properties of CPF (Koshlukova and Reed, 2014; Gebremariam *et al.*, 2011)

Colour	White/colorless crystalline solid
Odour	Mild mercaptan
Melting point	41-43.5^{0}C
Boiling point	160^{0}C
Vapour Pressure	2.7 mPa at 25^{0}C
Water solubility	2.0 mg/L at 25^0 C
Density	1.398 g/cm^3
Octanol/water partition coefficient (log P or k_{ow})	4.7–5.3
Soil sorption coefficient, K_{oc}	652-30,381 L/kg
Aquatic sediment sorption, K_{oc}	3,000-25,565 L/kg
Stability	Stable in air and non-sensitive to U.V. radiation. Stable in neutral and acidic solution, hydrolyzed by strong bases.
Trade names	Dursban, Lorsban,Empire, Equity,Hilban

Mechanism of Chlorpyrifos (CPF) action

Acetylcholine (ACh) is an important neurotransmitter at the neuromuscular junction, and at synapses in the central nervous system (Horzmann and Freeman, 2016). Acetylcholinesterase (AChE) is a serine hydrolase that rapidly cause hydrolysis of ACh to acetate and choline, terminating impulse transmission at cholinergic synapses or neuromuscular junctions (Čolović *et al.,* 2013). The molecular mechanism of CPF lies in the phosphorylation of the residue serine 203 at the active center of acetylcholinesterase forming an organophosphorous intermediate with acetylcholinesterase. This makes the phosphorylated enzyme more stable with a lower rate of hydrolysis and regeneration of the active enzyme. In the process, the phosphorylated acetylcholinesterase might also become inactive or lose an alkyl group before active enzyme can be regenerated (Kwong, 2002). In most of the cases, the inhibition of AChE by CPF is irreversible (Moretto, 1998).

Once CPF enters the body it gets distributed throughout the body, while some part of CPF may get deposited in fat tissues. Aerobic and anaerobic metabolisms are the major routes of dissipation of CPF. The process of CPF metabolism is same in both human and other animals (Rathod and Garg, 2017). The CPF toxicity is mainly modulated by its metabolic biotransformation which involves cytochrome P450 (CYP) mediated monooxygenase oxidation and/or enzymatic conjugation with glucuronide or sulfate in metabolic pathways. The metabolism of chlorpyrifos to chlorpyrifos-oxon (CPO) by replacing the sulfur group with oxygen is carried out by the CYP2B6 **(Figure 3)**.

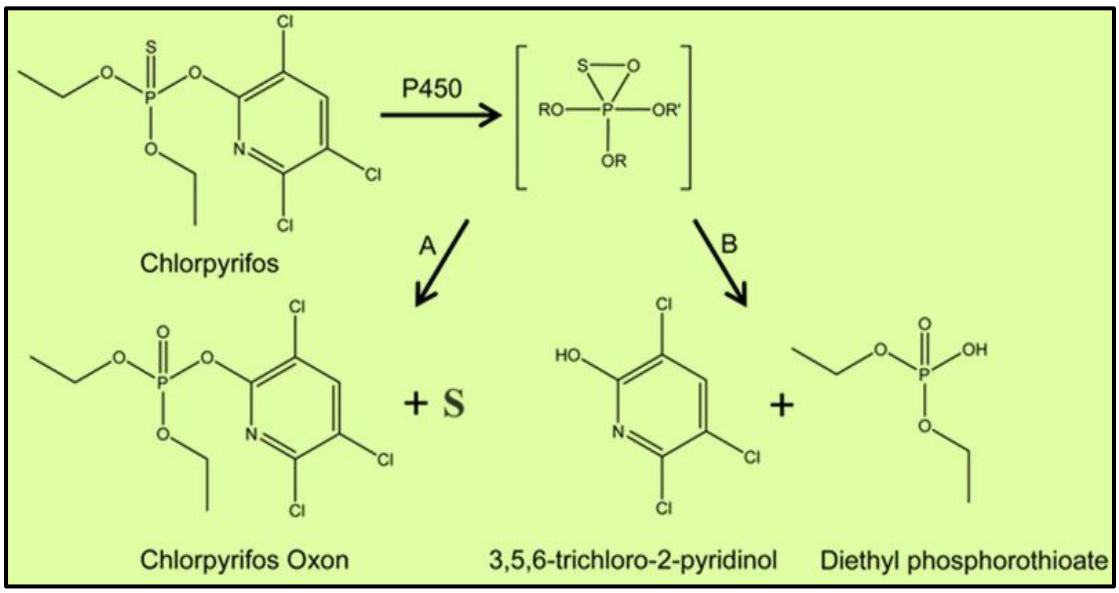


Figure 3: Chlorpyrifos metabolism by Cytochrome P450s (A: Desulfuration pathway; B: Dearylation pathways; S: Sulphur released in desulfuration pathway; D'Agostino*et al.*, 2015)

The chlorpyrifos (CPF) is bioactivated in the liver by cytochrome P450 (CYP) and it is metabolized to chlorpyrifo-oxon (CPO) by oxidative desulfuration. Dearylation of CPF is also performed by P450s forming 3, 5, 6-trichloro-2-pyridinol (TCP). CYP2B6, CYP2C19, and CYP3A4 are the main human P450s isoforms that metabolize CPF. The metabolization of CPF by these isoforms differ in their catalytic efficiency and quantity of metabolite formed (D'Agostino *et al*., 2015). CPO is a highly potent acetylcholine esterase (AChE) inhibitor having almost three times higher affinity toward the active site of AChE than the parent compound CPF, and is considered to be more toxic to organisms including birds, mammals, freshwater fishes and invertebrates (Choi *et al*., 2006; Rathod and Garg, 2017). Both chlorpyrifos and chlorpyrifos-oxon are hydrolyzed to 3, 5, 6-trichloro-2-pyridinol (TCP) which may further hydrolyzes to 3, 5, 6-trichloro-2-methoxy pyridine (TMP) (Sardar and Kole, 2005; **Figure 4**).

In concomitant biodegradation process metabolites such as diethyl phosphate (DEP) and diethyl thiophosphate (DETP) are also produced (Choi *et al.*, 2006; Yadav *et al.*, 2015). TCP is more persistent and mobile than CPF due to its higher water solubility responsible for the widespread contamination of soil and aquatic environments (Yadav *et al.*, 2015). DEP has been found to disrupt thyroid metabolism, blood transport and regulation of thyroid hormones (Yang *et al.*, 2020). The balance of activation and detoxification pathways is a deciding factor for the toxicity of CPF. Bioactivation of chlorpyrifos to chlorpyrifos oxon is entirely dependent on oxidative desulfuration of CPF by various isoforms of CYPs. In contrast, detoxification of the oxon is carried out by various pathways that include various CYPs, carboxylesterases, paraoxonase (Eaton *et al.*, 2008).

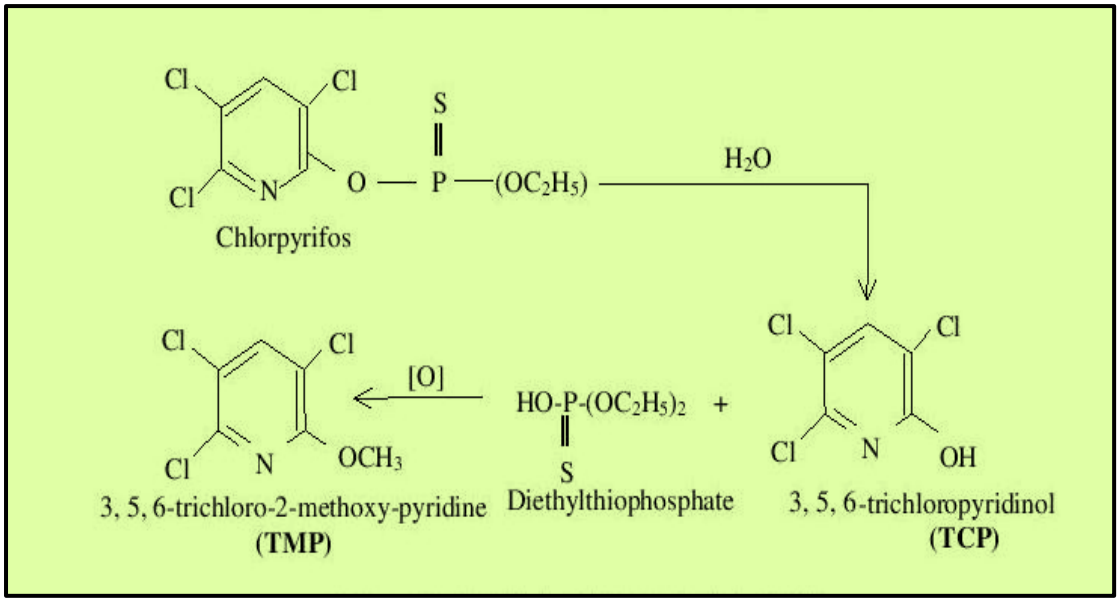


Figure 4: Biodegradation of chlorpyrifos in soil (Sardar and Kole, 2005)

Toxicity of CPF

There are numerous studies to report various mechanisms by which CPF can affect wide range of organisms starting from invertebrates to mammals. However, it is still a matter of concern to investigate toxic levels of CPF in aquatic organisms especially fish to raise awareness about the negative aspect of chlorpyrifos usage. Some of the toxicity levels of CPF are mentioned below:

Acute toxicity

CPF is highly toxic to aquatic living organisms including freshwater fish, aquatic invertebrates, estuarine and marine organisms (Barron and Woodburn, 1995). The 96 hrs LC_{50} values for CPF has been reported to be 3 ppb in rainbow trout, *Oncorhynchus mykiss*, 13.4 ppb in channel catfish, *Ictalurus punctatus*, 203 ppb in fathead minnow, *Pimephales promelas*, 98.67 µg/L and 154.01 µg/L in juvenile and adult *Oreochromis niloticus* respectively (Deb and Das, 2013). The LC_{50} value for 96 hrs was found to be 297 mg/L in mosquito fish, *Gambusia affinis* (Kavitha and Rao, 2008) and 580 µg/L in common carp, *Cyprinus carpio* (Xing *et al*., 2012) which reveals that CPF can be highly toxic to fish. Like acute toxicity, chronic toxicity of CPF also varies between different species, life stages and sex of aquatic organisms (Majumder and Kaviraj, 2018). The seasonal variation in acute toxicity of CPF was studied in freshwater catfish, *Heteropneustes fossilis* which was found to be susceptible to lethal effects of CPF in both duration and concentration dependent manner (Mishra and Verma, 2016).

Behavioural and morphological alterations

The sub-chronic dose of CPF is responsible for inducing specific behavioural and morphological deformities which might also affect physiological functions (Zahan *et*

al., 2019). In the fry fish of teleost *Channa punctatus*, CPF induced behavioural alterations including erratic swimming movements, vertical hanging, loss of balance and irregularities in opercular movement (Devi and Mishra, 2013). In the same study, morphological aberrations induced by CPF included skin lesions, shedding of scales, discoloration of skin, necrosis, scoliosis, damaged skull, eye deformities and excessive mucus secretion. Also, these aberrations were found to be dose and duration dependent. Similar behavioural and morphological alterations were also observed in freshwater catfish, *Heteropneustes fossilis* on exposure to different concentrations of CPF (Mishra and Verma, 2016).

Neurotoxicity

The acute and subchronic exposure of CPF has been reported to induce histopathological alterations in the brain (optic tectum) of teleost *Channa punctatus* which affected motor coordination and visual response of the fish body (Mishra and Devi, 2014). It has been found to induce persistent neurobehavioral alterations (Sledge *et al.,* 2011) and decrease in whole brain activity (Eddins *et al.,* 2010) in zebrafish. CPF has also been reported to produce neurotoxic alterations in mammalian models (Braquenier *et al.,* 2010; Middlemore-Risher *et al.,* 2010) but in fish the data available is very little.

Acetylcholinesterase (AChE) inhibition

CPF is a known AChE inhibitor (Rao *et al*., 2005) and has a tendency to induce various behavioural and physiological abnormalities including hyperactivity, paralysis, loss of

balance, muscle twitching which may lead to the death of fish and other aquatic organisms (Sandahl *et al.,* 2005).

Developmental toxicity

There are numerous studies to support the fact that CPF can induce structural and functional alterations or neurobehavioural teratology which can interfere with the normal development, growth or homeostasis of the organism (Sledge *et al.,* 2011). However, studies in fish supporting the fact are sparse. In zebrafish, CPF exposure during early development induced hatchling's swimming impairment (Levin *et al.,* 2003) and neurobehavioural dysfunction (Levin *et al.,* 2004).

Endocrine disruptor

CPF has been reported to alter normal steroid hormone levels in fish which affects the normal functioning of the reproductive system. CPF has been reported to induce alterations in the estrogen, testosterone and cortisol levels (Oruç, 2010). It has also been reported to act as endocrine disruptor in other organisms such as marine mussels *Mytilus galloprovincialis* (Canesi *et al.,* 2011). However, studies mentioning the role of CPF as an endocrine disruptor in fish are sparse and need to be explored.

Genotoxicity and Mutagenicity

There are various studies to mention about the genotoxic and mutagenic aspects of CPF in mice and other organisms (Ojha *et al.,* 2011; Gupta *et al.,* 2010; Sandal and Yilmaz, 2010; Cui *et al.,* 2011; Amer and Aly, 1992; Sobti *et al.,* 1982; Yin *et al.,* 2009). Since pesticides like CPF have been detected in the aquatic environments, there is a growing

concern to study their genotoxic or mutagenic effects in aquatic organisms. CPF has been reported to cause DNA damage in the tissues of *Channa punctatus (*Ali *et al.,* 2008). In *C. punctatus*, CPF induced single- strand DNA breaks in a concentration dependent manner. However, there is a need to explore role of CPF at genetic level in fish to understand more about its toxicity levels and mechanisms.

Oxidative stress

CPF has been reported to induce oxidative stress by generating reactive oxygen species in various aquatic organisms including *Gambusia affinis* (Kavitha and Rao, 2008), *Oreochromis niloticus* (Oruç, 2010), *Poecila reticulate* (Sharbidre *et al*., 2011). Induction of oxidative stress leads to alterations in the enzymatic and non-enzymatic antioxidant system of fish and other organisms. The altered antioxidant system may disturb physiological functions of organisms especially growth, development and reproduction (Zhou *et al*., 2006).

Histopathological alterations

The exposure of sub-lethal concentration of CPF for 3 (short term exposure) and 7 days (long term exposure) caused severe histopathological alterations in liver and gill tissues of *Channa punctatus* (Devi and Mishra, 2013). These histopathological alterations became more prominent with an increase in the CPF. CPF has been reported to be gonadotoxic (Pandey and Mishra, 2014). Histopathological alterations such as follicle disruption, vacuolization and atresia in oocytes, rupturing of germinal epithelium were also observed in the ovary of *Channa punctatus* when exposed to $1/10^{th}$ and $1/3^{rd}$ of 24 hrs LC_{50} for 3 (short term) and 7 days (long term exposure) (Mishra and Singh, 2018).

In the same study, histochemical analysis showed decrease in calcium and carbohydrate positive reactions in the CPF treated ovary whereas there was an increase in the iron pigments due to the breakdown of heme. This indicates that CPF interferes with the biochemical composition of the ovary.

Research model *(Heteropneustes fossilis)*

Common name

Heteropneustes fossilis (**Figure 5**) is a species of catfish mostly found in India, Nepal, Bangladesh, Pakistan, Bhutan, Myanmar, and Thailand. It is commonly referred as stinging catfish. The common names of *H. fossilis* include 'Singee' and 'Bitchu' in India (Nath and Dey, 1989), 'shingi' or 'shing' in Bangladesh (Ali *et al*., 2014), 'Hunga' in Sri Lanka and 'Lahoord', 'Nullie' and 'Lohar' in Pakistan (Hossain *et al*., 2015).

Synonyms

- *Saccobranchus fossilis (Bloch, 1794)*
- *Silurus fossilis (Bloch, 1794)*
- *Silurus singio (Hamilton, 1822)*
- *Saccobranchus singio (Hamilton, 1822)*
- *Silurus laticeps (Swainson, 1838)*
- *Silurus biserratus (Swainson, 1839)*
- *Saccobranchus microcephalus (Günther, 1864)*

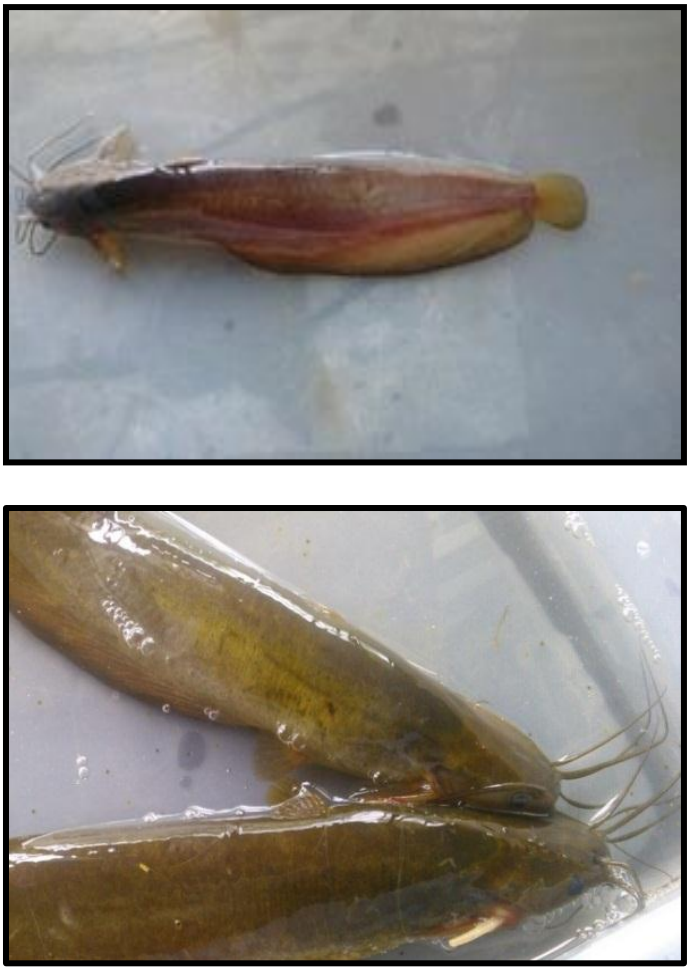


Figure 5: *Heteropneustes fossilis* (Bloch, 1794)

Conservation status (IUCN 3.1)

NE	DD	LC	NT	V	EN	CR	EW	EX

NE: Not evaluated; DD: Data deficient; LC: Least concern; V: Vulnerable; EN: Endangered; CR: Critically endangered; EW: Extinct in the wild; E: Extinct.

Classification

Phylum	Chordata
Subphylum	Vertebrata
Superclass	Gnathostomata
Class	Actinopterygii
Division	Teleostei
Order	Siluriformes
Family	Heteropneustidae
Genus	*Heteropneustes*
Species	*fossilis* (Bloch, 1794)

Habit and Habitat

H. fossilis is mainly found in freshwaters and sometimes in brackish waters. They mainly inhabit ponds, swamps, ditches, marshes and muddy rivers (Jha and Rayamajhi, 2010). It is omnivorous; prefer feeding on insects, plant matter and detritus. The fry are planktivorus whereas fingerlings feed on insects, crustacea and miscellaneous matter (Hossain *et al*., 2015). It can tolerate wide range of temperatures ranging from 16^0C (winter) to 28^0C (summer) (Vasal and Sundararaj, 1978). The catfish is a seasonal breeder. It spawns during the monsoon season (July-August).

Important features

The body of *H. fossilis* is compressed and elongated with head covered with osseous plates at top and sides. The body is black or reddish brown in colour. There are four pairs of barbels in which maxillary and mandibular pairs extend up to pectoral fins and

base of pelvis respectively. Caudal fin is rounded. On either side of vertebral column a pair of accessory respiratory organ (air sacs) extending backwards from the gill chamber is present. A thin ventral line is dominant in male but absent in female. *H. fossilis* is also termed as a stinging catfish because it possesses venom glands lying in the epidermis on the sides of pectoral fin spines (Whitear *et al.,* 1991). These stings act as a defense mechanism against the predators (Dorooshi, 2012) (**Figure 5**).

Heteropneustes fossilis is one of the most demanded and commercially important fish in India and neighboring countries. This fish has high market demand because of its delicious taste, nutritional and medicinal values. It has high protein and low fat content. It is rich in iron (226 mg/100g) and calcium content (221 mg/ 100g) (Hossain *et al*., 2015; Alok *et al*., 1993; Tiwary *et al*., 1998; Paul *et al*., 2015). They are recommended as a diet for anaemic and convalescents (Nushy *et al.,* 2020). Its air breathing adaptation allows it to survive even in swampy areas and water bodies with poor oxygen content. The ability of the fish to survive in any kind of water, make cultivation, storage and transportation in live condition easy, making it an ideal fish species for aquaculture (Puvaneswari *et al.,* 2009).

Despite various reports to elaborate the extensive research performed on *Heteropneustes fossilis* including those mentioned above, there is still paucity of knowledge especially in understanding the interaction of some of the major environmental pollutants especially pesticides with this fish. CPF is one of the most extensively used organophosphate pesticides. Its interaction with *H. fossilis* at physiological levels has not been clearly explained so far. It is known that when such pesticides interfere with the normal biological processes there is a need of some effective measures to reduce or alleviate the

toxic effects of such pollutants. Therefore, the present study is not only an attempt to fill the gap but also, it is for the first time that interaction of CPF with *H. fossilis* has been understood elaborately at biochemical, enzymatic, histological, hormonal and genetic level along with some effective measures to reduce the toxic effects of CPF.

Thesis reports on:

1. Understanding of chlorpyrifos toxicity and its bioremediation in freshwater catfish *Heteropneustes fossilis*.
    A) Effect of chlorpyrifos and antioxidants (curcumin, ascorbic acid and chitosan) on stress regulators and histotexture of body cleaning organs (gill, liver and kidney).
    B) Effect of chlorpyrifos and effective antioxidants (curcumin and ascorbic acid) on biomolecules (protein, carbohydrate and lipid) and histotexture of nutrient absorbing organs (stomach, intestine).
2. Understanding of chlorpyrifos on gonadal maturation of the catfish, *Heteropneustes fossilis.*
    A) Influence of CPF alone and with antioxidants on gamete growth (vitellogenin) and maturation supporting steroid hormone (estradiol, maturation inducing steroid, progesterone) profile.
    B) Chlorpyrifos impact on gene expression linked to gonad development (*NR5A1*) in fry of catfish, *Heteropneustes fossilis.*

Chapter 1

Understanding of chlorpyrifos toxicity and its bioremediation in freshwater catfish Heteropneustes fossilis

[A] Effect of chlorpyrifos and antioxidants (curcumin, ascorbic acid and chitosan) on stress regulators and histotexture of body cleaning organs (gill, liver and kidney).

[B] Effect of chlorpyrifos and effective antioxidants (curcumin and ascorbic acid) on biomolecules (protein, carbohydrate and lipid) and histotexture of nutrient absorbing organs (stomach, intestine).

Chapter 1

Understanding of chlorpyrifos toxicity and its bioremediation in freshwater catfish *Heteropneustes fossilis*

[A] Effect of chlorpyrifos and antioxidants (curcumin, ascorbic acid and chitosan) on stress regulators and histotexture of body cleaning organs (gill, liver and kidney)

Abstract

The present study aims to investigate the toxicity of chlorpyrifos (CPF) and its bioremediation by some powerful antioxidants curcumin, ascorbic acid and chitosan in freshwater fish, *Heteropneustes fossilis* (Bloch, 1794). For the first time effectiveness of different antioxidants were studied against the CPF toxicity in *Heteropneustes fossilis.* In the present study,the live and healthy fish were exposed to sub-lethal dose of CPF (0.174µM/l; $1/10^{th}$ of 96 hrs LC_{50}) and effective concentrations of antioxidants alone and in different combinations to assess their effectiveness against the agro-urban pesticide.The finding of different experimental setup concluded that sub-lethal dose of CPF (0.174µM/l; $1/10^{th}$ of 96 hrs LC_{50}) causes histopathological alterations in major organs of fish (liver, kidney, gill) and increases antioxidative enzyme activities (catalase, peroxidase) and lipid peroxidation significantly. In this study ascorbic acid and curcumin was found to attenuate the CPF toxicity. Chitosan was found to be ineffective in suppressing the CPF toxicity. The decrease in CPF toxicity becomes significant when tested in combinations of antioxidative agents as compared to their alone positive control groups. Therefore, co-incubation of curcumin and ascorbic acid was found to be effective against CPF induced aberrations in antioxidative enzyme levels and anatomical changes. It helps catfish to regain health.

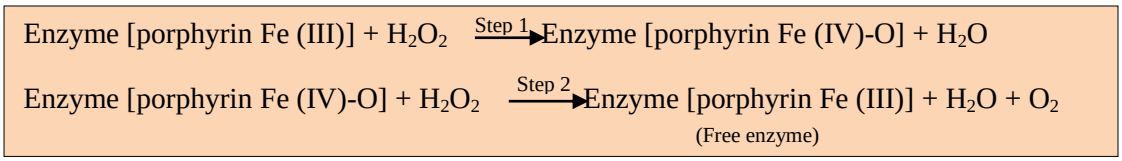
1A.1 Introduction

Chlorpyrifos (CPF) is an organophosphorus (OP) systemic pesticide extensively used in agricultural, industrial, and household applications. The inhibition of acetylcholinesterase (AChE) is the primary acute toxicological effect of CPF (Chiapella *et al.*, 2013). In addition, there are several studies supporting the fact that CPF induces oxidative stress by increasing the formation of highly active moieties called reactive oxygen species (ROS) (Almedia *et al.*,1997; Banerjee *et al.*, 1999; Banerjee 2001; Prakasam *et al.*, 2001), alterations of antioxidant enzyme function and depletion of antioxidant defense system (Ojha *et al.*, , 2011). Reactive oxygen species (ROS) is a term given to various molecules and free radicals derived from molecular oxygen. They may be direct oxidants or oxygen-like electronegative elements produced within the cell either during cellular metabolism or under pathological conditions. The reduction of oxygen produces free radicals such as hydroxyl radical and superoxide anion and non-radical such as hydrogen peroxide (Dennery, 2010; Covarrubias *et al.*, 2008). Free radicals are independent, unstable, and short lived but chemically reactive species consisting of one or more unpaired electrons. They have the potential to oxidize cellular biomolecules (Nandi *et al.*, 2019). ROS act as second messengers and have both beneficial and deleterious roles in influencing normal cell growth and survival. A certain amount of ROS is required for organogenesis, growth and development (Dennery, 2010). They also modify gene expression by the activation of several signaling pathways (Flohe´ and Flohe´, 2011). The condition of oxidative stress is generated when excessive concentration of ROS accumulated in a cell exceeds cellular defense system. A certain amount of ROS is produced by the active cells but cellular antioxidant systems keep the levels low. ROS in excess proves to be detrimental to cells as it induces oxidative damage to lipids, proteins, and DNA. Some of the harmful

oxidized products include are lipid hydroperoxides, carbonylated proteins and DNA with oxidized bases (e.g., 7, 8-dihydro-8-oxoguanine) (Covarrubias *et al.,* 2008). In order to alleviate and restore the damage caused by ROS, cells have developed enzymatic and non-enzymatic antioxidant defense systems. The enzymes such as NADPH oxidases, cyclo-oxygenases, xanthine oxidase and lipoxygenases mainly produce ROS, and enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidases (GPx), and peroxidases (POD) are responsible for degrading or inactivating these ROS. The alterations in the antioxidant enzyme activities serve as a biomarker to assess the exposure of fish or other aquatic organisms to certain toxicants or pollutants (Oost *et al.*, 2003; Sayeed *et al.,* 2003). A balance is maintained between the functions of ROS and antioxidants for normal homeostasis. The disturbance of this balance leads to abnormalities that may affect germ cells; embryogenesis and can have long-term consequences on the mature organism (Dennery, 2010). There are several study reports to support the fact that CPF exposure induced alterations in the antioxidant status in fish (Ural, 2013), earthworm (Wang *et al.,* 2012), rats (Bebe and Panemangalore, 2003), also during in vitro exposure studies (Gultekin *et al.,* 2006; Lee *et al.,* 2012).

Catalase (CAT; E.C. 1.11.1.6) is the most important antioxidant enzyme which is present in almost all aerobic organisms. It is a tetrameric protein with four identical tetrahedrally arranged subunits of 60 kDa, containing four porphyrin haem (iron) groups that allow the enzyme to interact with the H_2O_2 (Scibior and Czeczot, 2006; Ighodaro and Akinloye, 2018; Kaushal *et al.,* 2018). It is also termed as hydrogen peroxide oxidoreductase (Patlolla *et al.,* 2008). In two-step reaction process: catalase breaks down two molecules of hydrogen peroxide into one molecule of oxygen and two molecules of water (Deisseroth and Dounce, 1970). In the first step, the heme is

oxidized by a molecule of hydrogen peroxide to an oxyferryl species. In the second step, second molecule of hydrogen peroxide acts as a reducing agent, regenerate the enzyme and produces a molecule of oxygen and water (Ighodaro and Akinloye, 2018; Chelikani *et al.,* 2004). CAT plays an important role in regulating the concentration of hydrogen peroxide in a cell, protecting the cell from oxidative assault (Nandi *et al.*, 2019; Mueller *et al.*,1998). It also performs an important function in fatty acid metabolism (Oost *et al.*, 2003).



Steps involved in catalase reaction (Nandi *et al.*, 2019)

The excess of accumulation of H_2O_2 is toxic at tissue and cellular level. It is converted to deleterious hydroxyl radical in the presence of Fe^{2+} through Fenton reaction. In order to prevent this free radical induced deleterious effects, catalase primarily located in the peroxisomes breaks down H_2O_2 into water and molecular oxygen, completing the detoxification process. It is one of the first lines of defense mechanisms against the oxidative stress (Abhijith *et al.*, 2016). The enzyme utilizes either iron or manganese as a cofactor to catalyze the degradation reaction. The efficiency of CAT enzyme is quite high; it can easily break down millions of hydrogen peroxide molecules within a second. It can also react with hydrogen donor compounds such as methanol, ethanol, formic acid, or phenols (Ighodaro and Akinloye, 2018).

The membrane phospholipids of aerobic organisms including fish are continually subjected to oxidant challenges from endogenous as well as exogenous sources.

Pesticides and many other chemicals evoke lipid peroxidation (LPO) (Slaninova *et al.*, 2009; Ali *et al.*, 2014). Lipid peroxidation (LPO) has widely been used as a biomarker of oxidative damage in fish leading to impairment of cellular functions under oxidant conditions (Kehrer, 1993; Monteiro *et al.*, 2006; Clasen *et al.*, 2018; Xing *et al.*, 2012). LPO is a set of chain reactions, mostly for polyunsaturated fatty acids which are more prone to oxidative reactions because of their double bonds (Valavanidis *et al.*, 2006). The hydroxyl free radical generated during oxidative stress extracts hydrogen atom from unsaturated fatty acids of phospholopid membrane (Farber, 1994). Increase in LPO mainly occurs due to an increase in MDA levels which are formed due to peroxidation of unsaturated fatty acids.

1. Initiation of lipid peroxidation

   $LH + R^{\bullet}$ or $HO^{\bullet} \longrightarrow L^{\bullet} + RH$, or HOH

   LH = Polyunsaturated lipid

2. Propagation of lipid peroxidation

   $L^{\bullet} + O_2 \longrightarrow LOO^{\bullet}$

   $L^{\bullet}$: Carbon centered lipid radical

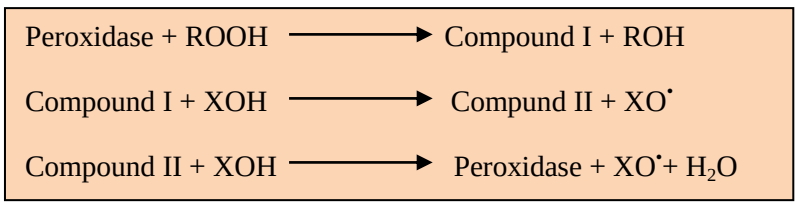
   $LOO^{\bullet}$: Lipid peroxyl radical

3. $LH + LOO^{\bullet} \longrightarrow L^{\bullet} + LOOH$ (Lipid hydroperoxide)

Steps involved in Lipid Peroxidation (Valavanidis *et al.*, 2006)

The lipid hydroperoxide (LOOH) formed as a result of termination of lipid peroxidation decomposes into several reactive species including aldehydes (malondialdehyde), lipid alkoxyl radicals ($LO^{\bullet}$), alkanes, alcohols and lipid epoxides. These reactive species are highly toxic and active mutagens (Esterbauer *et al.*, 1990; Valavanidis *et al.*,2006). These products have the potential to form DNA adducts which may further lead to mutations and alterations in pattern of gene expression (Marnett, 1999). The membranes which are peroxidized lose their permeability and integrity.

Peroxidases are a large family of isoenzymes present almost in all living organisms ranging from invertebrates to humans (Cheng *et al.*, 2008). They are present in cytosolic, mitochondrial and peroxisomal compartments (Lubos *et al.*, 2011). Their molecular weight ranges from 35-100 Kd. The enzyme contains feriprotoporphyrin IX, with four pyrrole nitrogens bound to the Fe (III) (O'Brien, 2000). In animals, peroxidases have organ, tissue, cellular and sub-cellular specific functions. These peroxidases include glutathione peroxidase, myeloperoxidase, eosinophil peroxidase, lactoperoxidase and thyroid peroxidase (Klebanoff, 2005). Such peroxidases participate in various metabolic activities including oxidative stress in which they enzymatically reduce hydrogen peroxide to water. They also play an important role in protective mechanisms including xenobiotic detoxification, immunity, hormone biosynthesis and pathogenesis of inflammatory diseases (Lubos *et al.*, 2011). They are oxido-reductases and undergo a series of redox reactions after binding with the substrate. The oxidation of xenobiotics by POD involves reaction cycle during which intermediate forms of peroxidases (Compound I and II) are formed. The reaction cycle initiates by reaction of the POD (native enzyme; Fe (III) form) with hydrogen peroxide forming compound I, which contains two oxidizing equivalents more than the resting enzyme (Khan *et al.*, 2014).



Steps involved in the reaction cycle of Peroxidase(POD) (O'Brien, 2000)

In fish, POD plays a protective role by preventing auto-oxidation of fish lipids. This function is important throughout the biological cycle of fish, right from the egg

development to the gonad maturation, since these processes involve lipid transport and oxidative breakdown of fatty acids for source of energy (Mazeaud *et al.,* 1979). It also protects membrane damage induced by the lipid peroxidation (Ullah *et al.,* 2015). There are numerous studies in the support of high activity of POD in various fish species (Matkovics *et al.*,1977, Mazeaud *et al.*,1979; Wdzieczak *et al.,* 1982). They are considered to be one of the major defense mechanisms to combat oxidative stress (Carmel-Harel and Storz, 2000). Unlike CAT, that can only reduce H_2O_2, peroxidases can reduce both H_2O_2 and various lipid peroxides (Oost *et al.,* 2003).

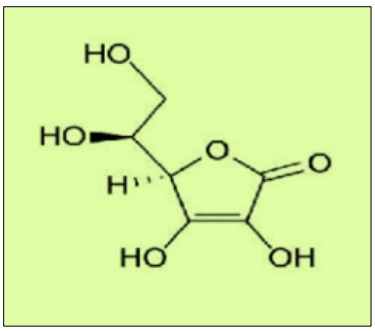
In case of depletion of antioxidant enzymes, need of natural antioxidants supplementation arises to overcome the oxidative stress. Ascorbic acid, curcumin and chitosan are some of the potent antioxidants whose effectiveness against the CPF induced stress in *H. fossilis* have been evaluated in the present study.

L-ascorbic acid (vitamin C; Asc) is an essential micronutrient necessary for various biological functions. It is water soluble dibasic acid having 5 membered lactone ring structure (Pehlivan, 2017; **Figure 6**). Asc is vital for growth and development, metabolism, iron absorption, xenobiotic detoxification and oxidative protection. It acts as a cofactor for various enzymes involved in tyrosine metabolism, collagen synthesis, and neurotransmitter dopamine conversion to norepinephrine (Pehlivan, 2017; Beyer, 1994). It is a powerful antioxidant, reducing agent, scavenger of free radicals and harmful oxygen derived species including hydrogen peroxide, hydroxyl radical and singlet oxygen (Arrigoni and Tullio, 2002; Hacişevki, 2009). Among all the properties, antioxidant property of ascorbic acid has been widely studied (Bindhumol *et al.,* 2003;

Duarte and Lunec, 2005; Padayatty *et al.,* 2003). Beyer (1994) has also mentioned its role in the inhibition of mutagenesis and carcinogenesis.

Curcumin (diferuloylmethane; [1, 7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione]; Cur; **Figure 7**) is a phenolic compound derived from rhizomes of *Curcuma longa* belonging to ginger family, *Zingaberaceae* (Sökmen and Khan, 2016). Like ascorbic acid, curcumin is another powerful antioxidant which has the potential to scavenge reactive oxygen species and inhibit lipid peroxidation (Reddy and Lokesh, 1994). The unique antioxidant property of curcumin owes to its structure which contains b-diketo group, phenyl rings, carbon–carbon double bonds, methoxy, carbonyl and hydroxyl groups (Menon and Sudheer, 2007; Wright 2002). In addition to this, curcumin also possess anti-inflammatory, anti-viral, anti-fungal properties and anti-bacterial properties (He *et al.,* 2015; Ghosh *et al.,* 2015; Moghadamtousi *et al.,* 2014).

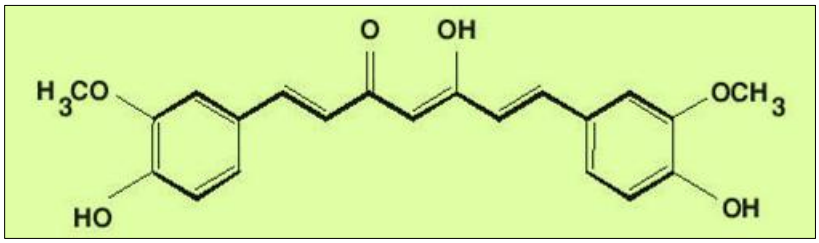
Chitosan (Chi; **Figure 8**) is a nontoxic natural straight-chain biopolymer of glucosamine and N-acetyl glucosamine. It is derived from chitin present in fungal cell walls and crustacean exoskeleton by alkaline deacetylation (Ngo and Kim, 2014; Avelelas *et al.,* 2019). It also shows antioxidant (Trung and Bao, 2015), anti-microbial property (Sousa *et al.,* 2009), anti-tumor, hypocholesterolemic and immunity enhancing effects (Xia *et al.,* 2011; Nishimura *et al.,* 1984). Chi has gained importance in various sectors including agrochemicals industry, wastewater treatment and cell culture because of its drug delivery, biocompatibility and biodegradability properties (Trung and Bao, 2015; Avelelas *et al.,* 2019). Although, Chi has been found to enhance the immune system in *Cyprinus carpio* (Gopalakannan and Arul, 2006) and antioxidant levels in *Oreochromis mossambicus* (Gobinath and Ravichandran, 2020), there are not much studies to illustrate its potential in fish and other aquatic organisms.



Molecular formula: $C_6H_8O_6$

Molecular weight: 176.12 g/mol

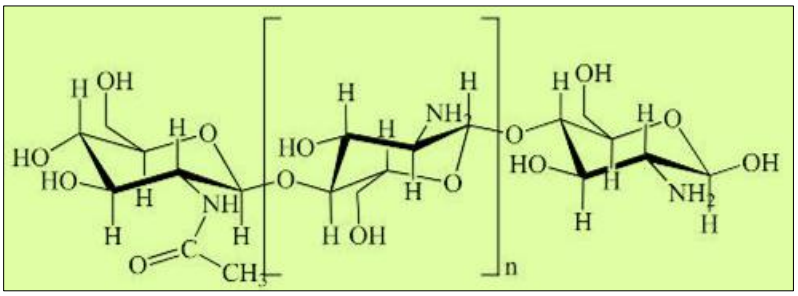
Figure 6: Structure of L-ascorbic acid (Pehlivan, 2017)



Molecular formula: $C_{21}H_{20}O_6$

Molecular weight: 368.38 g/ mol

Figure 7: Structure of curcumin



Molecular formula: $C_{56}H_{103}N_9O_{39}$

Molecular weight: 1526.5 g/mol

Figure 8: Structure of chitosan (Islam *et al.*, 2016)

Histopathological changes are widely used as biomarkers to evaluate the health of fish exposed to different contaminants (Camargo and Martinez, 2007). This category of biomarker allows examining of important organs responsible for vital functions in fish, such as respiration, excretion and bioaccumulation and biotransformation of xenobiotics (Gernhöfer *et al.*, 2001). The histopathological alterations observed in these organs provide signs of damage to animal health (Teh *et al.*,1997).

1A.2 Materials and Methods

1A.2.1 Chemicals

The organophosphorus pesticide, Chlorpyrifos was purchased from the local agricultural farm under the trade name Hilban® (20% EC CPF, $C_9H_{11}Cl_3NO_3PS$, CAS No. 2921-88-2). All chemicals including L-ascorbic acid (Asc, $C_6H_8O_6$, CAS No. 50-81-7; Molychem), curcumin (Cur, $C_{21}H_{20}O_6$, CAS No. 458-37-7; Himedia), and chitosan (Chi, $C_{56}H_{103}N_9O_{39}$, CAS No. 9012-76-4; Biochemika) were of analytical grade and obtained from local scientific suppliers.

1A.2.2 Animal collection and their acclimatization

The live, healthy and adult *Heteropneustes fossilis* (Bloch, 1794) of relatively uniform size (15±3 cm) and weight (50±10gm) were collected from local fish suppliers in Lucknow, Uttar Pradesh, India in the last week of January (Resting phase). Fish were brought to the laboratory carefully in large plastic containers having freshwater to avoid any stress and injuries as much as possible. They were given potassium permanganate treatment (1%) to avoid any dermal infection (**Figure 9**). The fish were acclimatised in laboratory conditions for two weeks under normal photoperiod and temperature (12h: 12h; light: dark and 23 ± 2^0 C). Fish was fed goat liver daily *ad libitum*. The water was changed daily in order to remove the faecal matter and excess feed.

Figure 9: Fish acclimatization set up

To avoid any cruelty to the animals, we followed national guidelines of the ethical committee to exhibit experiments.

1A.2.3 Experimental setup

In this study, minimum but effective concentration of CPF (0.174µM/l; $1/10^{th}$ of 96 hrs $LC_{50;}$ Mishra and Verma, 2016) and antioxidants viz., Asc (5 mg/l; Kumar *et al.*, 2009), Cur (10 mg/l; Cao *et al.*, 2015), and Chi (150 mg/ l; Dautremepuits *et al.*, 2004) were selected. Fish were divided into fourteen different experimental groups as mentioned in the **Table 2.** In each group five fish were introduced in duplicates. The experimental setup was maintained for three days (72 hrs) by replacing with the fresh dose of chemicals every other day to avoid accumulation of metabolic wastes, keeping the same chemical constituents in each water tank.

After the completion of experimental duration, fish from each group was sacrificed by decapitation. The liver, kidney, gill from each fish belonging to each group were isolated carefully and weighed. A small piece of each tissue was fixed in Bouin's fixative for 24 hrs for histological study, and the remaining was stored at -20^0 C for biochemical analysis.

Table 2: Different experimental groups to study bioremedials [ascorbic acid (Asc), curcumin (Cur), chitosan (Chi)] effects on chlorpyrifos (CPF) induced stress in *Heteropneustes fossilis*. In each group five fish were introduced in duplicates.The setup was maintained for three days (72 hrs)

Group No	Description	References
I	Negative control (with freshwater)	
Positive control		
II	CPF (0.174µM/l; 1/10th of 96 hrs LC_{50})	Mishra and Verma , 2016
III	Asc (5 mg/l)	Kumar *et al.,* 2009
IV	Cur (10 mg/l)	Cao *et al.,* 2015
V	Chi (150 mg/l)	Dautremepuits *et al.*, 2004
VI	Asc + Cur	
VII	Asc + Chi	
VIII	Cur + Chi	
Experiment cluster 1		
IX	CPF + Asc	
X	CPF + Cur	
XI	CPF + Chi	
Experiment cluster 2		
XII	CPF + Asc + Cur	
XIII	CPF + Asc + Chi	
XIV	CPF + Cur + Chi	

1A.2.4 Antioxidant enzyme activity and lipid peroxidation analysis

The tissues (liver, kidney and gill) were homogenized in 10% phosphate buffer (pH-7.4). The homogenised solution was centrifuged at 10,000 rpm for 25 minutes at 4^0 C. The supernatant was used for the analysis of antioxidant enzymes.

Catalase (CAT) assay

The activity of catalase enzyme or catalase assay was carried out by measuring the decomposition rate of H_2O_2 (Hydrogen peroxide) at 240nm (Aebi, 1984). The assay system contained 2.9 ml of freshly prepared 30Mm H_2O_2 in phosphate buffer (50 Mm, pH 7.0) and 0.1 ml of enzyme source (100 µl of supernatant). At 240nm, changes in the absorbance were measured for 1 min (at the interval of 30s) against the blank containing distilled water. The unit for the enzyme activity was Units /min/gFW. The catalase activity was calculated as follows:

Enzyme calculated mg protein/ml = A_{240nm} X 0.0667

Enzyme activity = ΔA_{240nm} /min X V_A X $1/P_L$ X $1000/\varepsilon$ X mg protein/ml reaction mixture

Where,

ΔA_{240nm} /min: change in absorbance per minute

V_A: Volume of assay mixture (ml)

P_L : Pathlength (1 cm)

ε: Molar extinction coefficient (43.6 $mM^{-1}cm^{-1}$)

Lipid Peroxidation (LPO) assay

The lipid peroxidation was determined by the method of Rotruck *et al.*,1973. For the LPO assay, 1.0 ml of the supernatant was added to 2 ml of thiobarbituric acid-

hydrochloric acid-trichloroacetic acid (TBA-HCl-TCA) mixture. The TBA-HCl-TCA mixture was prepared by mixing 0.37% TBA, 0.24N HCl and 15% TCA in the ratio 1:1:1. After adding the mixture, it was boiled for 15 minutes, allowed to cool followed by the centrifugation at 3000 rpm for 10 minutes. The supernatant was taken and observed at 532nm. The unit for lipid peroxidation was units/gm tissue. Lipid peroxidation was calculated as follows:

$$\text{Enzyme activity (Unit/gm tissue)} = V_A / V_S \text{ X } 1/ P_L \text{ X } A_{532nm} / \varepsilon \text{ X } 10^9$$

Where,

V_A: Volume of assay mixture (ml)

V_S: Volume of sample (ml)

P_L: Pathlength (cm)

A_{532nm} = Absorbance at 532nm.

ε: Molar extinction coefficient ($1.5 \text{ X } 10^5$ cm ml^{-1})

Malondialdehyde (MDA) is a secondary end product of the oxidation of polyunsaturated fatty acids, which on reacting with thiobarbituric acid (TBA) forms a coloured complex whose absorbance can be recorded at 532nm. This assay is also known as thiobarbituric acid-reactive substances (TBARS) assay.

Peroxidase (POD) assay

The activity of POD activity was determined by measuring the coloured product formed due to oxidation of pyragallol (Ohkawa *et al.*, 1979). To 3.0 ml of pyrogallol solution, 0.1 ml of supernatant and 0.5 ml of H_2O_2 was added and mixed. The absorbance was

taken at 430nm and recorded at every 30 sec. for upto 3 minutes. The unit for the enzyme activity was Units /min/gFW. The peroxidase activity was calculated as follows:

$$\text{Enzyme activity (units/ min/ gFW)} = \Delta A_{430nm} \text{ /min X } V_A / V_S \text{ X } 1/ \varepsilon \text{ X } 1/P_L$$

Where,

ΔA_{430nm} /min: change in absorbance per minute

V_A: Total assay volume (ml)

V_S: Sample volume (ml)

ε : Molar extinction coefficient (25 $mM^{-1}cm^{-1}$)

P_L: Pathlength (cm)

All the absorbance were obtained by using UV-visible spectrophotometer (EVOLUTION 201, Thermoscientific).

1A.2.5 Statistical analysis

Data of samples were represented as means ± SEM. The significance of values obtained from different groups was tested by using one-way analysis of variance (ANOVA). The intergroup comparisons were done by Newman-keuls' test ($P<0.05$).

1A.2.6 Histopathology

The histopathological studies were carried out by the steps as shown in **Table 3**.

Table 3: Steps of the histopathological studies

S.N.	Steps	Descriptions
1	Tissue fixation	Dissected tissues were fixed in Bouin's fixative for 24 hrs and trimmed.
2	Cleaning	Fixed tissues were washed in distilled water (15 min; two times).
3	Dehydration process	Tissues were passed through increasing order of graded alcohols as 30%, 50%, 70%, 90%, 100% (30 min each).
4	Tissue clearing	In xylene:100% alcohol (1:1) followed by xylene (15 min each).
5	Embedding of tissues	Tissue was passed twice through xylene:wax (1:1)(15 min), followed by Wax_1, Wax_2 and Wax_3 (30 min each).
6	Block preparation	Tissue was placed in L piece (metal angle) filled with wax and the air bubbles were removed by using hot needle. The block was allowed to cool.
7	Block Trimming	The block was carefully trimmed removing extra wax till the tissue was slightly visible and placed on the block holder for sectioning.
8	Tissue sectioning	Sectioning of tissues with 5 µm thickness was done on rotatry microtome (Weswox optic, ARMT-1090A).
9	Spreading of tissues	Slides were made ready with Mayer's egg albumin. Tissues were spread on slides kept on hot plate (30^0-35^0 C).
10	De-paraffinization	Slide with sections were dipped in xylene and then xylene:100% alcohol (1:1) (15 min each).
11	Hydration	Sections were passed through graded alcohols in descending order (100%, 90%, 70%, 50% and 30%) (10 min each).
12	Washing	In distilled water (10 min).
13	I^{st} staining	The slides were passed through Haematoxylin, tap water and distilled water (5-10 min each).
14	Dehydration	The slides were dehydrated by passing through graded alcohols in ascending order (30%, 50%, 70% alcohol) (10 min each).
15	II^{nd} staining	The slides were passed through Eosin, 90% alcohol, 100% alcohol, xylene:100% alcohol (1:1) (2 min each).
16	Mounting	Tissues were mounted in DPX and covered with cover slip.
17	Examination	The tissue sections were observed under Bright field microscope (Olympus CX41) at 20X magnification using micropublisher 3.3 RTV camera (Qimaging, BC, Canada)

1A.3 Results

The present study elaborates the toxic effects of chlorpyrifos (CPF) on antioxidant enzyme activity (CAT, POD), lipid peroxidation and histology of tissues and its bioremedial by natural antioxidants (Cur, Asc and Chi). This approach explains the toxicity levels of CPF in the major tissues of *Heteropneustes fossilis* and the role of antioxidants in attenuating the toxicity.

1A.3.1 Effect of antioxidants (Cur, Asc and Chi) on CPF induced antioxidant enzyme activity and lipid peroxidation

Effect on Catalase (CAT) activity

There was a significant overall change in the CAT activity in the tested tissues of the experimental groups (I to XIV) ($p < 0.001$; $F = 14.0$ for the liver; $F = 13.2$ for the kidney and $F = 9.9$ for gill; **Figure 10**). The CAT activity (units/min/gFW) in the liver, kidney and gills of the negative control group was recorded to be 46.15, 34.79, and 22.72 respectively. The CPF treated group (II) was found to have increased CAT activity in the order 64.39 units/min/gFW, 54.22 units/min/gFW and 39.50 units/min/gFW for liver, kidney and gill respectively. This increased CAT activity was significantly different ($p < 0.05$) from the negative and other positive control groups. The increase was recorded to be more in the liver than the kidney followed by the gill. The effect of the antioxidants alone or in mix groups (III-VIII) was similar to the negative control group (I) for liver, kidney and gill tissues. The groups having CPF in addition to one or two antioxidants (group IX-XIV) recorded variations in the CAT activity in comparison to the control group. In group IX (Asc+CPF) the CAT activity was found to be 58.7 units/min/gFW for liver, 50.86

units/min/gFW for kidney, 35.71 units/min/gFW for gills. In group X (Cur+CPF) the CAT activity was recorded to be 62.12 units/min/gFW for liver, 50.10 units/min/gFW for kidney, and 37.93 units/min/gFW for gill. In XI (Chi+CPF) the CAT activity was found to be 66.66 units/min/gFW for liver, 55.03 units/min/gFW for kidney, 41.61 units/min/gFW for gill. It was noticed that in these three groups (IX, X, XI) only Asc and Cur was slightly decreasing the CAT activity in comparison to the CPF treated group (II). Chi was found to be ineffective against the increased CAT activity. Despite the decrease noticed in the CAT activity from the combination of CPF with single antioxidant (Asc or Cur), they were not found significantly different from the CPF treated group (group II). In group XII (Asc+Cur+CPF), the CAT activity was found to be 49.89 units/min/gFW for liver, 42.64 units/min/gFW for kidney, 27.38 units/min/gFW for gill. In group XIII (Asc+Chi+CPF) the CAT activity was recorded as 55.30 units/min/gFW for liver, 42.42 units/min/gFW kidney and 29.87 units/min/gFW for gill. In group XIV (Cur+Chi+CPF) the CAT activity was recorded as 59.79 units/min/gFW for liver, 50.59 units/min/gFW kidney and 32.14 units/min/gFW for gill. The interesting part of the result to be noticed was that, group XII (Asc+Cur+CPF) attenuated the CPF induced increase in the CAT activity significantly ($p < 0.05$) when compared to the CPF treated group (group II) in all the three tested tissues. In the Group XIII (Asc + Chit + CPF) there was a significant decrease in the CAT activity to the level of negative control in kidney and gill tissues but in the liver, there was no such significant decrease. In this group, Asc alone decreased the CPF toxicity and produced an additive effect in combination with Cur and Chi as Chi alone (group XI) or in combination with Cur (group XIV) was found to be ineffective in influencing the CPF induced increase in the CAT activity in the tested tissues.

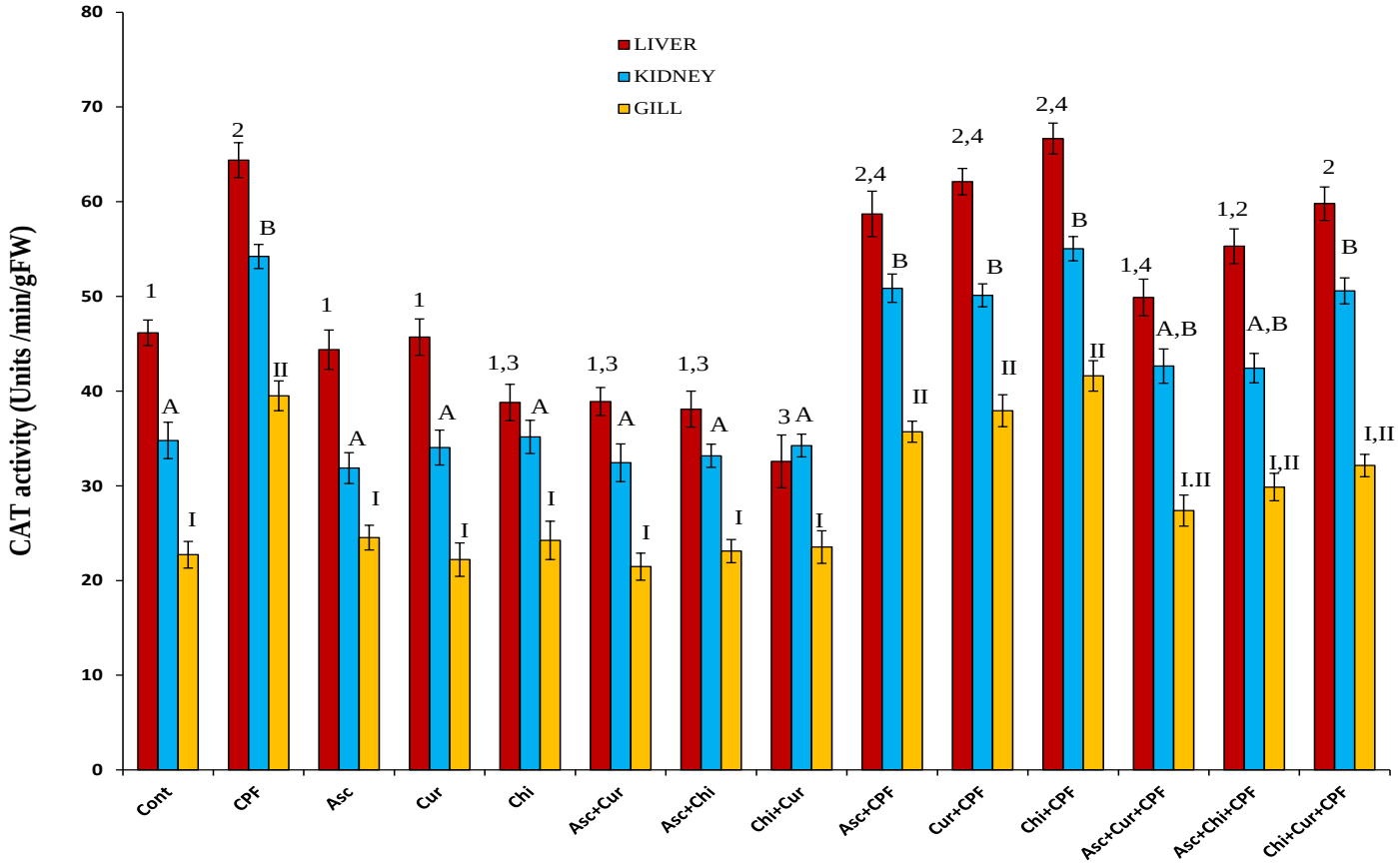


Figure 10: Effect of antioxidants (ascorbic acid: Asc, 5 mg/l; curcumin: Cur, 10 mg/l and chitosan: Chi, 150 mg/l) alone and in combination on chlorpyrifos (CPF; 0.174µM/l, 1/10th of 96 hrs LC_{50}) induced catalase (CAT) activity in different tissues (liver: red bar; kidney: blue bar; gill: yellow bar) of catfish, *Heteropneustes fossilis*. Each group had five fish in duplicates for three days (72 hrs). Values expressed as mean ± SEM. Data were analyzed by one way ANOVA ($p < 0.001$) and Newman- Kuels' test ($p < 0.05$). Numerical 1, 2, 3 and 4 show intergroup comparison between liver groups. Alphabets A and B show an intergroup comparison between kidney groups. Roman I and II show an intergroup comparison between gill groups. Groups superscripted with same symbols are insignificant, and those with different letters or numbers are significantly different in intergroup comparison.

Effect on Lipid Peroxidation (LPO)

The result showed significant changes in the LPO level in all the groups (I-XIV) for all the three tested tissues ($p < 0.001$; $F = 12.4$ for the liver; $F = 10.5$ for kidney and $F = 3.3$ for gill; **Figure 11**). The LPO level (units/gm tissue) noticed in liver, kidney and gill tissues from the negative control group (I) was 0.55, 0.29 and 0.19 respectively. In comparison to the negative control group, the CPF treated group (II) was found to increase the LPO significantly ($p < 0.05$) in liver, kidney and gill. The LPO noticed in the CPF treated group (II) was 2.65 units/gm tissues for liver, 1.85 units/gm tissue kidney and 1.21 units/gm tissue for gill. However, like CAT activity, there was no significant alteration recorded in the LPO level among groups having single antioxidant alone or two antioxidants in combination (III, IV, Vor VI, VII, VIII respectively). The groups having CPF (IX-XIV) recorded an increase in the LPO in comparison to the control group. In group IX (Asc+CPF) the LPO level in liver, kidney and gill was found to be 2.35 units/gm tissue, 1.54 units/gm tissue and 1.18 units/gm tissue respectively. In group X (Cur+CPF) the LPO was noted as 2.48 units/gm tissue for liver, 1.56 units/gm tissue for kidney and 1.19 units/gm tissue for gill respectively. In group XI (Chi+CPF) the LPO level noted in liver, kidney and gill was 2.5 units/gm tissue, 1.81units/gm tissue and 1.20 units/gm tissue respectively. Among these three groups, it was noted that only Asc (group IX) and Cur (group X) not Chi (group XI) had showed a small decrease in the LPO level. However, in comparison to the CPF treated group (II) they were not significantly different. The inclusion of single antioxidant (experiment cluster 1; groups IX-XI) was found unable to suppress the increase in the LPO in comparison to the groups having two antioxidants (experiment cluster 2; groups XII-XIV).

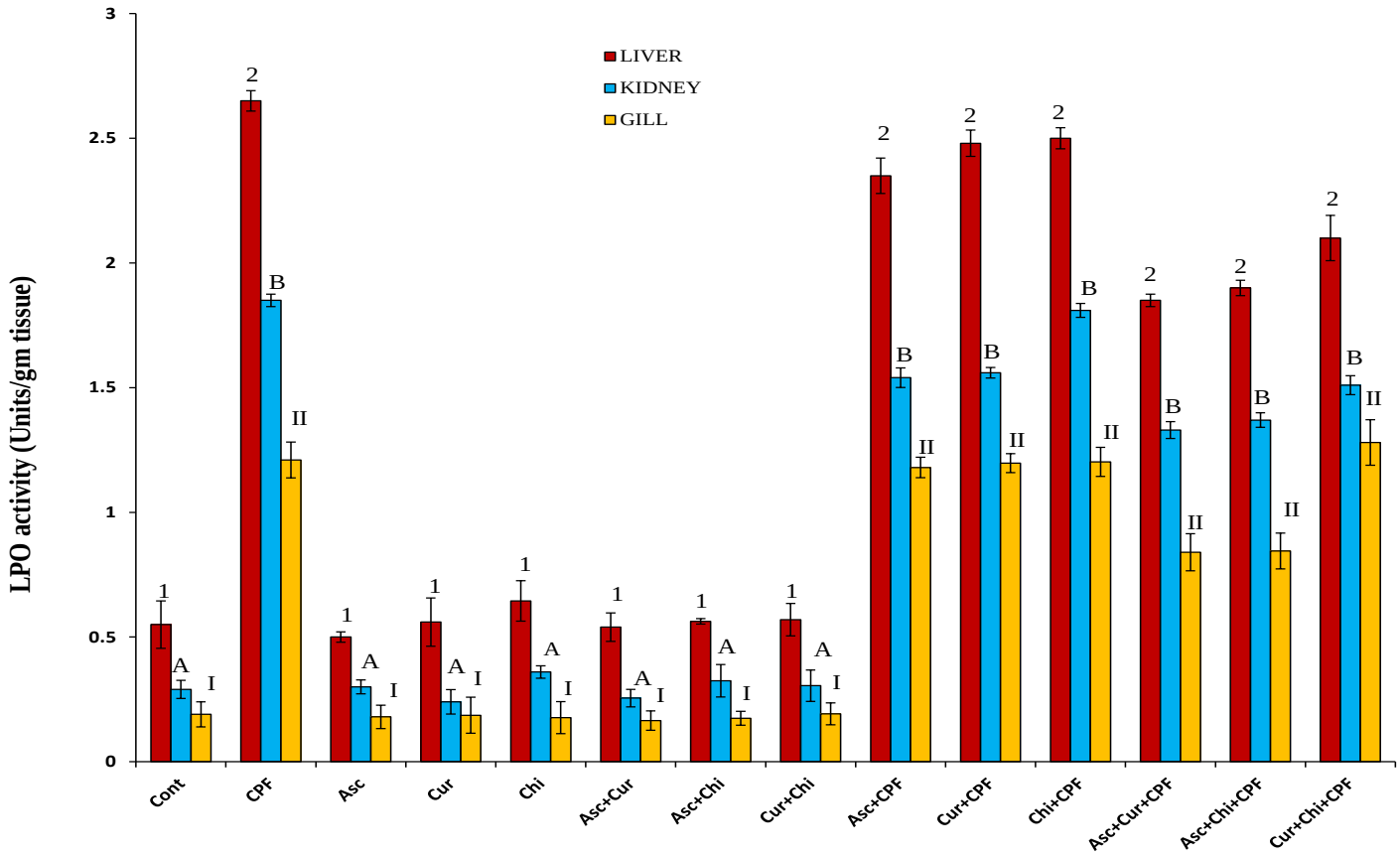


Figure 11: Effect of antioxidants (ascorbic acid: Asc, 5 mg/l; curcumin: Cur, 10 mg/l and chitosan: Chi, 150 mg/l) alone and in combination on chlorpyrifos (CPF; 0.174µM/l, 1/10th of 96 hrs LC_{50}) induced lipid peroxidation (LPO) in different tissues (liver: red bar; kidney: blue bar; gill: yellow bar) of catfish, *Heteropneustes fossilis*. Each group had five fish in duplicates for three days (72 hrs). Values expressed as mean ± SEM. Data were analyzed by one way ANOVA ($p < 0.001$) and Newman- Kuels' test ($p < 0.05$). Numerical 1 and 2 show intergroup comparison between liver groups. Alphabet A and B show an intergroup comparison between kidney groups. Roman I and II show an intergroup comparison between gill groups. Groups superscripted with same symbols are insignificant, and those with different letters or numbers are significantly different in intergroup comparison.

Among the experiment cluster 2, the group having Asc and Cur (group XII) was found to have LPO as 1.85 units/gm tissue for liver, 1.33 units/gm tissue for kidney, 0.84 units/gm tissue for gill. In group XIII (Asc+Chi+CPF) the LPO in liver, kidney and gill was 1.9 units/gm tissue,1.37 units/gm tissue and 0.84 units/gm tissue respectively. The LPO in XIV (Cur+Chi+CPF) group was 2.1units/gm tissue, 1.51 units/gm tissue and 1.28 units/gm tissue for liver, kidney and gill respectively. The combination of Asc and Cur was found to be more effective than the group having Asc and Chi (group XIII) in attenuating the CPF induced increase in LPO. The group XIV was ineffective in surpassing the increased LPO.

Effect on Peroxidase (POD) activity

There was significant changes in the POD activity in all the tested tissues ($p<0.001$; $F= 115.7$ for the liver; $F= 85.0$ for kidney and $F= 69.3$ for gill; **Figure 12**). The POD activity (units/min/gFW) in liver, kidney and gill of the fish from negative control group was 226.10, 175.10and 133.59. The POD activity in CPF treated group (II) for liver, kidney and gill were noted to be 313.18 units/min/gFW, 248.91 units/min/gFW,189.81 units/min/gFW respectively. In comparison to the negative control the POD activity in the liver, kidney and gill increased significantly ($p < 0.05$) in CPF exposed fish (group II). The POD activity was more in liver than kidney followed by the gill. The antioxidants in single or in dual combination mode (group III-VIII) did not bring any significant alterations in the POD activity in all the three tested tissues. The groups having CPF in addition to one or two antioxidants (groups IX-XIV) recorded variations in the POD activity as compared to the control group. In group IX

(Asc+CPF) the POD activity in liver, kidney and gill were found to be 290.30 units/min/gFW, 236.99 units/min/gFW, 178.76 units/min/gFW. In group X (Cur+CPF) the POD activity was found to be 292.38 units/min/gFW, 246.08 units/min/gFW, 180.68 units/min/gFW in liver, kidney and gill respectively. In group XI (Chi+CPF) the POD activity was recorded to be 295.86 units/min/gFW in liver, 251.00 units/min/gFW in kidney and 189.71 units/min/gFW in gill. Among the three groups (IX, X, XI) a small decrease in the POD activity was noticed only in groups having Asc and Cur but not Chi. In group XI (Chi+CPF) a small decrease was noticed only in liver. This indicates that Chi was ineffective against the CPF induced POD activity. The decrease in the POD activity by single antioxidant (groups IX and X) was not found significantly different from the CPF treated group (II). In group XII (Asc+Cur+CPF) the POD activity in liver, kidney and gill was recorded as 280.40 units/min/gFW, 209.87 units/min/gFW, 160.96 units/min/gFW respectively. The POD activity in liver, kidney and gill tissues of group XIII (Asc+Chi+CPF) was recorded as 284.60 units/min/gFW, 230.39 units/min/gFW, 170.58 units/min/gFW respectively. In group XIV (Cur+Chi+CPF) the POD activity in liver, kidney and gill was 287.18 units/min/gFW, 235.29 units/min/gFW, 180.21 units/min/gFW respectively. Among these three groups, the one having the combination of Asc and Cur (group XII) was recorded to be the most effective group in reducing the enzyme activity significantly as compared to the CPF alone. The group XIII was found to be less effective in comparison to the group XII and group XIV did not score a significant decrease in POD activity in all the three tested tissue.

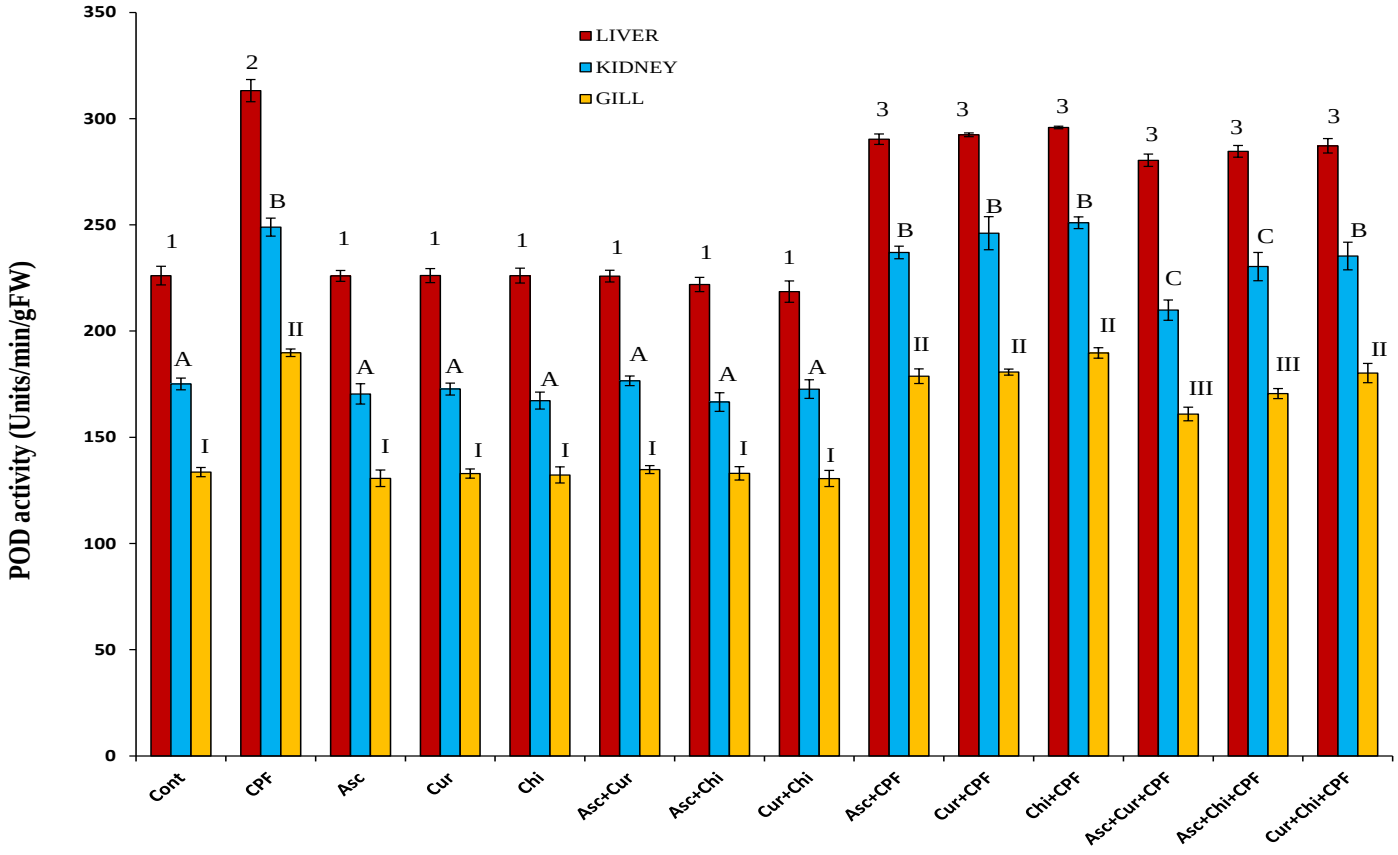


Figure 12: Effect of antioxidants (ascorbic acid: Asc, 5 mg/l; curcumin: Cur, 10 mg/l and chitosan: Chi, 150 mg/l) alone and in combination on chlorpyrifos (CPF; 0.174µM/l, 1/10th of 96 hrs LC_{50}) induced peroxidase (POD) activity in different tissues (liver: red bar; kidney: blue bar; gill: yellow bar) of catfish, *Heteropneustes fossilis*. Each group had five fish in duplicates for three days (72 hrs). Values expressed as mean ± SEM. Data were analyzed by one way ANOVA ($p < 0.001$) and Newman- Kuels' test ($p < 0.05$). Numerical 1, 2 and 3 show intergroup comparison between liver groups. Alphabet A, B and C show an intergroup comparison between kidney groups. Roman I, II and III show an intergroup comparison between gill groups. Groups superscripted with same symbols are insignificant, and those with different letters or numbers are significantly different in intergroup comparison.

1A.3.2 Effect of antioxidants against CPF induced histopathological alterations

The antioxidants (Cur, Asc,) alone or in combinations did not show any alteration in tissue texture (quality wise) as compared to the tissues of negative control group (photomicrographs not shown). The group having combination of Cur and Asc (group VIII) was found to suppress the CPF induced histotoxicity when compared to CPF alone (group II).

Effect on liver

The histological section of the liver from the negative control group showed no abnormalities (**Figure 13A**). It was parenchymatous in appearance containing polygonal hepatic cells with distinct spherical nucleus amongst the sinusoids. The central vein was evident around which the hepatocytes were radially arranged. The exposure of CPF induced severe histopathological alterations in the fish liver including degeneration of hepatocytes, sinusoids dilation, melanomacrophage appearance, granular cytoplasm, nuclear hypertrophy and blood cells among the hepatocytes (**Figure 13B**). However, fish exposed to CPF with combination of antioxidants (Cur and Asc) recorded attenuated histological abnormalities (**Figure 13C**). Such liver sections were found to be intact as compared to the CPF treated group (positive control).

Effect on gill

The histological sections of gills from the negative control group showed normal structure without any aberrations. The structure consisted of long thin filaments called primary lamellae whose surface area was increased by the formation of regular semilunar folds known as secondary lamellae which lied on a basement membrane

supported by the pillar cells. The spaces between the pillar cells, called lacunae were distinct. The filaments were found to be covered with epithelial covering (**Figure 13D**). The anomalies in the CPF treated group included hypertrophy and hyperplasia of epithelial cells that resulted in shortening, curling and clubbing of secondary lamellae with an increased number of mucous cells, degeneration of pillar cells. Vacuolization was also observed (**Figure 13E**). These histopathological abnormalities were reduced to a greater extent in the groups containing CPF with both the antioxidants (Cur and Asc, **Figure 13F**).

Effect on kidney

The histological sections of the kidney of the negative control group recorded no lesions. It showed numerous renal corpuscles, well-developed glomeruli and a system of tubules (**Figure 13G**). In CPF treated groups irregular renal tubules, shrinkage of glomeruli, damaged renal corpuscle, disruption of tubules, haemorrhage and cytoplasm degeneration causing vacuolization were observed (**Figure 13H**). However, such abnormalities reduced to a greater extent in group containing combination of antioxidants (Cur and Asc) with CPF (**Figure 13I**). Such tissues were found to be intact with negligible alterations similar to the control group.

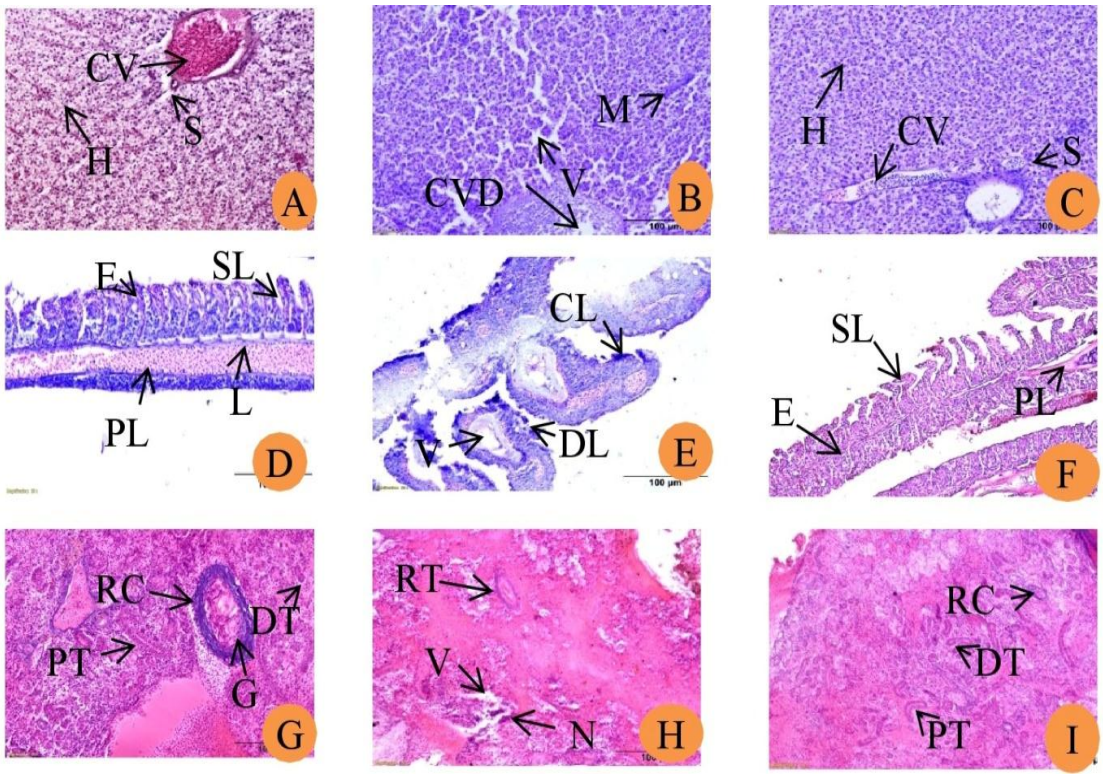


Figure 13:.The combined bioremediation by ascorbic acid (Asc; 5 mg/l) and curcumin (Cur; 10 mg/l) on histopathological alterations induced by chlorpyrifos (CPF; 0.174 µM/l, $1/10^{th}$ of 96 hrs LC_{50}) on liver, gill, kidney of catfish, *Heteropneustes fossilis*, for three days (72 hrs) using haematoxylin-eosin double staining. **Liver**: (A) control (B) CPF (C) Asc + Cur + CPF, **Gill**: (D) control (E) CPF (F) Asc + Cur + CPF **Kidney**: (G) control (H) CPF (I) Asc + Cur + CPF, Where; CV:central vein, H: hepatocyte, S: sinusoids, CVD: central vein degenerated,V: vacuolization, M:Melamomacrphage aggregation, PL: primary lamellae, L: lacunae, SL: secondary lamellae, E: epithelial cell, CL: curled lamellae, DL: damaged lamellae,RC: renal corpuscle, DT: distal tubule, G: glomerulus, PT: proximal tubule, RT: ruptured tubule, N: necrosis, [Image captured with 200X].

1A.4 Discussion

The present study was aimed to investigate the toxicity levels of the organophosphorus pesticide, chlorpyrifos in the freshwater catfish, *Heteropneustes fossilis*. This was important because studies about the interaction of the pesticide with the aquatic organism especially fish at the physiological level is meager. The initial understanding of the toxicity of chlorpyrifos on the health status of *H.fossilis* was necessary before exploring its role in other important parameters such as steroidogenesis and development. The present study also aimed to explore the role of three powerful antioxidants as a bioremediation against the chlorpyrifos toxicity.

Effect of CPF on the antioxidant enzyme activity and lipid peroxidation

An optimal quantity of hydrogen peroxide is essential for regulating eukaryotic signal transduction. It is produced in response to many stimuli such as cytokines and growth factors and is involved in regulating biological processes including cell proliferation, differentiation and apoptosis (Veal *et al.,* 2007). The catalase enzyme is responsible for decomposition of hydrogen peroxide and maintaining an optimum level of the molecule in the cell which is also prerequisite for cellular signaling processes (Nandi *et al.,* 2019). This makes obvious to record CAT activity in the liver, kidney and gill of the fish from the negative control group (I). In group II, the sublethal concentration of CPF increased the CAT activity significantly in comparison to the negative control group. CPF induces the formation of reactive oxygen species (ROS) which further leads to oxidative stress (Xing *et al.,* 2012, Verma *et al.,* 2007) The increased antioxidant activity is a part of the defense mechanism to combat the reactive oxygen species (ROS) generated during the oxidative stress (Kaur and Jindal, 2017; John *et al.,* 2001). The increased CAT activity, a sign of increased peroxide concentrations, is an

important strategy of all aquatic organisms to remove H_2O_2 from cells and fight against the oxidative stress (Karadag *et al.*, 2014). Similar result was also observed in *Danio rerio* (Nunes *et al.*, 2018; Jin *et al.*, 2015), in *Cyprinus carpio* (Karadag *et al.*, 2014), in *Capoeta trutta* (Yildirim *et al.*, 2011), *Labeo rohita* (Kumari *et al.*, 2014), *Catla catla* (Abhijith *et al.*, 2016), *Brycon cephalus* (Monteiro *et al.*, 2006) and *Geophagus brasiliensis* (Filho *et al.*, 2001). The increased CAT activity also indicates increase in the superoxide dismutase (SOD) activity that converts the parental ROS superoxide, into H_2O_2 (Ullah *et al.*, 2015). In response to chlorpyrifos exposure for 15 days, the CAT activity was also found to be increased in the liver and kidney of *Ctenopharyngodon idella* (Kaur and Jindal, 2017). Topal *et al.* (2017) reported an increase in the catalase activity in brain tissues of rainbow trout exposed to imidacloprid pesticide. The increase in the CAT activity leads to the elimination of ROS (Oruc, 2012). Also, Wang *et al.* (2014) reported that the CAT activity is not only induced by the chlorpyrifos but, its activity also increases with time in zebrafish, *Danio rerio*. This might be because of the fact the chlorpyrifos induces ROS generation, and CAT are one of the major ROS-scavenging enzyme (Anderson *et al.*, 1995). In order to keep the ROS production at steady level, the activities of CAT was increased to eliminate the redundant ROS (Mittler, 2002). The increase in the duration of toxicant exposure has been found to decrease the CAT activity (Kaur and Jindal, 2017). This result corroborate the statement of Alves *et al.*, (2002) that pesticides exposure may elicit pro-oxidant conditions that induce adaptive responses such as increase in the antioxidant enzyme activities.

Lipid peroxidation (LPO) was increased in all the tested tissues of fish exposed to sub-lethal concentration of CPF. CPF has also been found to induce LPO in mosquito fish,

Gambusia affinis (Kavitha and Rao, 2008) and milkfish, *Chanos chanos* (Palanikumar *et al.,* 2013). Similarly, organophosphate pesticides have been found to increase LPO in catfish, *Ictalurus nebulosus* and carp, *Cyprinus carpio* (Hai *et al.,* 1997). Another organophosphate pesticide, Folisuper 600 BR® was also reported to induce LPO levels in freshwater fish, *Brycon cephalus* (Monteiro *et al.,* 2009). LPO act as a biomarker of oxidative damage of cell membranes induced by pesticides and xenobiotics. The increased LPO is a result of increased malondialdehyde formation as a result of excessive ROS production due to pesticide exposure. The increased LPO also alters the enzymatic and non-enzymatic antioxidant system of the organism (Yonar and Sakin, 2011). Xing *et al*. (2012) mentioned lipid peroxidation as one of the mechanisms of CPF induced toxicity. The high level of LPO levels is also associated with reduction in reduced glutathione (GSH) levels as a result of increased ROS. The low level of GSH makes the cellular membranes of fish more prone to harmful and toxic electrophilic compounds including organophosphates (Monteiro *et al.,* 2009).

In the present study, CPF also increased the POD activity significantly in liver, kidney and gill. The increased POD activities indicates oxidative stress (Ullah *et al*., 2015) and pollutants such as CPF induces oxidative stress and hence, POD activity (Oost *et al.,* 2003). An optimal POD activity is necessary for maintaining homeostasis and redox balance as observed in the tested tissues of the negative control group (I) (Mulgund *et al.* 2015). Induction in the POD activity depicts a possible defense strategy in response to the elevated level of LPO due to CPF exposure. In this way; it actually performs detoxifying function by terminating radical chain propogation (Oost *et al.,* 2003). It plays an important role against lipid peroxidation, elimination of H_2O_2

and harmful organic compounds. Similar result was also reported in *Channa punctatus* (Sayeed *et al.,* 2003), *Oreochromis niloticus* (Almeida *et al.,* 2002) and *Carassius auratus* (Zhang *et al.,* 2004). The metabolites of pollutants induce oxidative damage by the uncoupling of mitochondrial oxidative phosphorylation generating reactive oxygen species. These reactive species are further detoxified by the oxidation steps carried out by the peroxidases (Zhang *et al.,* 2004). Hsieh and Hsu (2013) also mentioned that increase in the level of ROS increases the level of POD activity. The peroxidase activity was higher in liver than kidney followed by the gill. This is because liver is the major detoxifying organ and is constantly exposed to many and exogenous and endogenous free radicals (Wu *et al.,* 2017). Initially, superoxide dismutase (SOD) removes superoxide by generating H_2O_2 which is further scavenged by CAT and POD system. POD removes H_2O_2 by reducing it to water. In common carp, *Cyprinus carpio* POD activity was initially decreased but with the increasing concentration of pesticide exposure the POD activity was found to be increased. The increased concentration of TBARS (Thiobarbituric acid reactive substances) level during lipid peroxidation induces the activity of POD to maintain the level of LPO (Song *et al.,* 2006).

Among all the tested tissues, liver possessed high antioxidant activity and lipid peroxidation, as compared to kidney and gills because liver being the major organ for purification faces more oxidative reactions and maximum ROS generation. Additionally, exposure of fish to pollutants trigger biochemical processes related to detoxification, making liver as an indicator of water contamination (Sherry, 2003; López *et al.,* 2011).

Effect of CPF on the histology of tissues

In the present study, CPF was found to induce alterations in the tissue architecture of liver, kidney and gills. The histology of liver tissue from control group showed numerous hepatocytes with homogenous cytoplasm and distinct nucleus. The exposure of sub-lethal concentration of CPF for 72 hrs induced vacuolation, hepatocyte degeneration, necrotic hepatocytes, and granular cytoplasm. Similar observations were also mentioned by Stalin *et al*., (2019) in *Channa punctatus* where histological lesions were found to be increased with number of days of CPF exposure. The CPF revealed degenerative changes and hyperaemia at 48, 72 and 96 hrs of exposure in liver of rainbow trout (Topal *et al.*, 2014), vacuolization and pyknotic nuclei in *Cyprinus carpio* (Xing *et al.*, 2012), necrosis and hypertrophy of hepatocytes in *Channa punctatus* (Devi and Mishra, 2013). The structural changes in the liver are the result of the oxidative stress due to the toxicant exposure since liver is the primary organ for detoxification of xenobiotics (Ganeshwade, 2011).

In case of gills, the secondary lamellae were shortened, curled and clubbed with an increase in the mucous cells in CPF treated group. It also induced degeneration of pillar cells and vacuolization. Similar observations were also recorded in *Channa punctatus* by Devi and Mishra (2013) where sublethal concentration of CPF resulted into fusion of secondary lamellae, edema, hypertrophy and lifting of epithelial cells. Topal *et al*. (2014) also recorded similar aberrations in *Oncorhynchus mykiss* when exposed to CPF for 24, 48, 72 and 96 hrs. Similarly, aberrations including thinness and elongation of secondary lamellae were also found in *Cyprinus carpio* (Jaffer *et al.*,2017), destruction of gill lamellae and increased vacuolization in *Poecilia reticulate* (De Silva and Samayawardhena, 2002), hyperplasia of branchial epithelial cells in *Carassius auratus*

gibelio (Cristina *et al.,* 2008), haemorrhage in primary lamellae and desquamation of secondary lamellae in *Gambusia affinis* (Cengiz and Unlu, 2003) and epithelial necrosis and aneurysm *Cirrhinus mrigala* (Velmurugan *et al* ., 2009).

The kidney sections from CPF treated group showed disruption of renal corpuscle and tubules, shrinkage of glomeruli, haemorrhage and vacuolization. Similar pathological lesions were also noted in *Cyprinus carpio* (Pal *et al.,* 2012; Xing *et al.,* 2012), *Heteropneustes fossilis* (Srivastava *et al.,* 1990; Deka and Mahanta, 2012), *Barbonymus gonionotus* (Mostakim *et al.,* 2015), *Clarias gariepinus* (Al-Otaibi *et al*., 2018). Since kidney receives maximum portion of postbranchial blood and maintains a stable internal environment, renal aberrations act as a good indicator of environmental pollution (Ortiz *et al.,* 2003).

Effect of antioxidants (Asc, Cur, and Chi) against CPF induced toxicity

The groups having single antioxidants (Asc, Cur, and Chi) or combination of two antioxidants did not show any significant alterations in the CAT activity, LPO and POD activity of the tested tissues. It might be because ascorbic acid (Fetoui *et al.,* 2008), curcumin (Manju *et al.,* 2012) and chitosan (Ngo and Kim, 2014) are powerful antioxidants and have protective roles against the oxidative stress. In the present study, single antioxidant was found to be less effective in attenuating the toxic effects of CPF than the combination of two antioxidants. Amongst all the different combinations, it was only the combination of Asc + Cur which showed ameliorative effects against CPF induced toxicity whereas combination of Chi + Cur was found to be least effective. The CAT activity, LPO and POD activity which was increased in the CPF treated groups was found to be reverted to the normal state effectively by the combination of Asc +

Cur in comparison to the other two groups Asc + Chi and Chi + Cur. Alterations in histology of tissues were found to be attenuated by the combination of Asc + Cur. Ascorbic acid has been found to normalize increment in the CAT activity and LPO level in *Oreochromis niloticus* when exposed to sub-lethal concentration of CPF for 96 hrs (Ozkan *et al.,* 2012). In the same study, Asc (Vitamin C) improved the TBARS level in brain tissue of the fish preventing the oxidative damage. Datta and Kaviraj (2003) and Korkmaz *et al.* (2009) mentioned about the recuperative effect of ascorbic acid against stress induced by the pesticides (deltamethrin and cypermethrin) in tissues of *Clarias gariepinus* and *Oreochromis niloticus* respectively. Both ascorbic acid and curcumin have potential to lower LPO by radical scavenging activity (Sharma *et al.,* 2010). In non-enzymatic reactions Asc acts as an electron donor preventing other compounds from being oxidized and reducing the formation of ROS. Also, it has also been found to ameliorate histopathological aberrations induced by CPF (Ozkan *et al.,* 2012). Scavenging of ROS by Asc helps in regaining the peroxidase (POD) activity (Yadav *et al*., 2015). The present result was also similar to Tripathi and Shasmal (2011) where Asc increased the CAT activity in various organs of *Heteropneustes fossilis* after exposure to CPF which might be because of Asc induced increase in hormone profile. Like Asc, the protective effect of Cur against oxidative stress has been found parallel to many authors (Madkour, 2012; Samuhasaneeto *et al.,* 2009). Cur has been found to alleviate the increased lipid peroxidation and histological lesions in *Anabas testudineus* ensuring its protective action. The different pathway by which Cur exerts its action includes scavenging of free radicals, stimulation of antioxidant enzyme pathways and increasing the antioxidant contents in cell (Manju *et al.,* 2012). The present result is in agreement with Yonar (2018) where administration of Cur was found to reverse the

CPF induced toxicity. Jiang *et al*. (2016) also mentioned in his study that Cur improved growth performance and antioxidant status of crucian carp *Carassius auratus*. Induction of antioxidant enzyme transcription by activating nuclear factor erythroid 2 (Nrf2) signaling pathway is another mechanism by which Cur exerts its action (Jiang *et al.,* 2016). Cur has been reported to regulate Nrf2 signaling which actively participates in free radical scavenging (Kwak *et al.,* 2004). In the present study the combination of Asc + Cur normalized CPF induced alterations in CAT, LPO and POD levels, biomolecular contents and histology of tissues (Elballat, 2016). This might either be due to synergistic effect when the combined action of the two antioxidants is greater than the individual ones or additive effect where the outcome is the sum of the two individual antioxidants (Tomczyk, 2019; Uri, 1961; Elballat 2016). Also, it was also found that antioxidant effect of curcumin enhanced in the presence of ascorbic acid (Khalil *et al.,* 2012). The effectiveness of the Asc + Cur in reverting oxidative stress and histopathological alterations was also mentioned by Tarasub *et al.* (2012); Jeon *et al.* (2008). However, amongst the three combinations of antioxidants, Chi (Chitosan) was found to be less effective in alleviating the CPF induced oxidative stress which might be either due to its poor solubility in water at neutral pH or its high molecular weight (Sun *et al.,* 2007). The weak antioxidant property of Chi also owes to its chemical inertness and poor H-atom donating ability (Ivanova and Yaneva, 2020).

[B] Effect of chlorpyrifos and effective antioxidants (curcumin and ascorbic acid) on biomolecules (protein, carbohydrate and lipid) and histotexture of nutrient absorbing organs (stomach, intestine)

Abstract

The present study aims to investigate the effects of chlorpyrifos (CPF) on the biomolecular content and its bioremediation by curcumin and ascorbic acid considering them effective from the previous antioxidant enzyme activity study in freshwater fish, *Heteropneustes fossilis* (Bloch, 1794). The biomolecules such as glycogen, protein and lipid are essential for normal growth, metabolism and reproductive events. In the present study, CPF toxicity was studied on the biomolecular changes of important energy source compounds, glycogen, protein and lipid in tissues viz. muscles, liver, kidney, gill of fish. The live and healthy fish were exposed to sub-lethal dose of CPF (0.174µM/l; 1/10th of 96 hrs LC_{50}) and effective concentration of antioxidants, curcumin and ascorbic acid alone and in combination for 72 hrs to assess their effectiveness against the CPF toxicity. The present experimental setup concluded that sub-lethal dose of CPF (0.174µM/l; 1/10th of 96 hrs LC_{50}) causes histopathological alterations in stomach and intestine. The understanding of different experimental setups concluded that sub-lethal dose of CPF decreases the glycogen and protein content and increases the lipid content significantly. However, co-incubation of curcumin and ascorbic acid was found to be effective against CPF induced alterations in biomolecular content and tissue architecture and restored the aberrations.

1B.1 Introduction

Organophosphate pesticides are used to enhance crop production. These pesticides via agricultural runoff, air drift, overspray and improper disposal enter into different aquatic habitats and disturbed normal physiology of aquatic animals including fish. Fish bioaccumulate these pesticides and transmit to other organism via food chain (McConnell *et al.,* 1998). Chlorpyrifos (CPF) is one of the most extensively used organophosphate pesticides. It has been reported to cause behavioural and developmental defects (Levin *et al.,* 2003; Devi and Mishra, 2013; Mishra and Verma, 2017), neurotoxic (Sledge *et al.*, 2011; Mishra and Devi, 2014) and genotoxic effects (Ali *et al.,* 2008; Mishra *et al.,* 2014), oxidative stress and histopathological aberrations (Sabra and Mehana, 2015; Mishra and Singh, 2018) in non-target animals including fish. Fish serve as useful bio-indicator to assess level of water contamination (Oost *et al.*, 2003).

The histopathological changes in fish tissues and biomolecular tests are necessary to monitor the acute or chronic toxicity effects of pesticides in ecosystem (Fredianelli *et al.*, 2019). Carbohydrate, protein and lipid are essential biochemical components of all animal tissues. They are important source of energy required for various metabolic functions. The monosaccharides not only stimulate oocyte maturation but have also been found to play an important role in maintaining the quality of gametes (Mishra and Joy, 2004). Proteins are the building block of all tissues. They serve as major source of energy and repair in various physiological functions including reproduction (Jagtap *et al.*, 2011). Apart from being important structural constituent of tissues (Begum, 2005), they are essential for vitellogenesis and gonad development. The exposure of excessive organophosphorus pesticides has been reported to induce alterations in these

biochemical profiles in a variety of fishes (Barbieri and Ferreira, 2011). A sufficient amount of energy is required during stress conditions which are supplied from glycogen, protein and lipid (Kadam and Patil, 2016).

The generation of ROS can be combated by the antioxidants such as curcumin and ascorbic acid (Tvrdá *et al.*, 2016, Padayatty *et al.*, 2003). Curcumin, a polyphenol, present in turmeric (*Curcuma longa*) has been used as a spice and herbal medicine in India since ages. It has antioxidant, anti-inflammatory and anticancer properties (Tsuda, 2018). It has been found to improve the antioxidant and protein status in fish *Anabas testudineus* (Manju *et al.*,2012). Ascorbic acid, commonly known as Vitamin C, is a known ROS scavenger (Aly *et al.*, 2010). It is a water soluble compound essential in reducing oxidative damage and maintaining normal metabolic functions (Padayatty *et al.*, 2003).

The present study was performed to understand the attenuating effect of curcumin and ascorbic acid on CPF induced manipulation in glycogen, protein and total lipid content and histopathology of tissues, stomach and intestine of freshwater catfish, *Heteropneustes fossilis*.

1B.2 Materials and Methods

1B.2.1 Chemicals

A commercial grade of pesticide, Hilban® (CPF, 20% EC, $C_9H_{11}Cl_3NO_3PS$, CAS No. 2921-88-2) was purchased from the local agricultural farm. All other chemicals including L-ascorbic acid (Asc; $C_6H_8O_6$, CAS No. 50-81-7, Molychem; **Figure 6**) and curcumin (Cur;$C_{21}H_{20}O_6$, CAS No. 458-37-7 Himedia; **Figure 7**) were of analytical grade and obtained from local scientific suppliers, Lucknow.

1B.2.2 Animal collection and their acclimatization

The live and healthy adult *Heteropneustes fossilis* (Bloch, 1794) of relatively same size (15±3 cm) and weight (50±10 gm) were collected from the local fish market in the last week of January (resting phase). Fish were brought to the laboratory carefully, avoiding any injuries as much as possible. After giving, potassium permanganate treatment (1%) to prevent any dermal infection, the fish were kept in aquaria to acclimatize for one week under normal photoperiod and temperature (12h: 12h; light: dark and 23 ± 2^0 C). Fish were fed with goat liver daily *ad libitum*.

The experiments were conducted as per national guidelines of the ethical committee to avoid any kind of cruelty to the fish.

1B.2.3 Experimental setup

In the present study, acclimatized fish were incubated with fixed concentration of CPF 0.174µM/l; $1/10^{th}$ of 96 hrs LC_{50}; Mishra and Verma, 2016), Cur (Group10 mg/l; Cao *et al.*, 2015), Asc (5 mg/l; Kumar *et al.*, 2009) and co-incubated with Cur and Asc. Fish were maintained with individual antioxidants and their combination. In order to evaluate combined antioxidant effect against CPF toxicity, fish were maintained with Cur, Asc and CPF. Control fish were set side by side with only freshwater. The experimental setup with five fish in each group was maintained for 72 hrs and the media was changed with fresh dose of chemicals every 24 hrs to maintain same chemical constituents and to avoid any accumulation of metabolic wastes. At the end of experimental period, fish from each group was sacrificed and tissues (muscles, liver, kidney and gill) were dissected and stored at -20^0C for glycogen, protein and lipid analysis. A small part of tissue (stomach, alimentary canal) was fixed in Bouin's fixative for 24 hrs for histotexture study.

1B.2.4 Glycogen, protein and total lipid analysis

Glycogen estimation

The glycogen content was determined by the method described by Seifter and Dayton (1950) using anthrone reagent. For this estimation, 100 mg of tissues (muscles, liver, kidney, gill) were taken and homogenized in 2 ml of 0.6% saline. This mixture was incubated for 30 minutes in a boiling water bath. To the homogenate 4 ml of 60% ethanol was added and kept in a refrigerator (4^0C) for 24 hrs for precipitation of glycogen followed by centrifugation at 3000 rpm for 20 minutes. The precipitate was dried and resuspended in 1 ml of distilled water. To 0.25 ml of this solution, 1.75 ml of 0.2% anthrone reagent (0.2g of anthrone in 100 ml of 95% sulphuric acid solution) was added carefully and mixed. This solution was mixed and kept in boiling water for 15 minutes and cooled immediately. The colour developed was read at 620nm spectrophotometrically (Evolution 201, Thermoscientific) against a reagent (anthrone) blank. The amount of glycogen was calculated as per the following equation and expressed as µg/100 mg of tissue weight.

$$\text{Glycogen content } (\mu g) = \frac{100 \times U}{1.11 \times S}$$

Where,

U: Optical density of the test solution

S: Optical density of 100 µg glucose standard

1.11: Factor for the conversion of glucose to glycogen

Protein estimation

The total protein estimation was performed by the method of Lowry *et al.* (1951). The method is based on the principle that Folin - Ciocalteau reagent reacts with proteins

containing different amounts of aromatic residues giving a blue colour whose absorbance can be recorded at 750nm. In this estimation, 100 mg of each tissue (muscles, liver, kidney, gill) was taken and homogenized in 2 ml of 0.6% saline. 0.5 ml of the homogenate was precipitated with 0.5ml of 10% TCA and then centrifuged at 10,000 rpm for 15 min. 0.1ml of the supernatant was added to 1ml of hot 1N NaOH solution and kept for 5 minutes, followed by the addition of 5 ml of alkaline copper reagent and incubation for 10 minutes at room temperature. This was followed by the addition of folin-ciocalteau reagent (0.5 ml). The volume was made upto 10 ml by adding 2.5 ml of distilled water and kept for 30 minutes. Finally, the coloured complex was measured using a spectrophotometer (Evolution 201, Thermoscientific) at 750nm against a reagent blank solution. The protein content was calculated as per the standard solution curve of BSA. The standard solution of BSA was prepared by dissolving 25 mg of BSA in 50 ml of distilled water. The protein content was calculated as per the following equation and expressed as µg/100 mg of tissue weight.

$$\text{Protein content } (\mu g) = \frac{\text{O.D. of test sample}}{\text{O.D. of standard sample}} \times \text{ concentration of standard sample}$$

Total lipid estimation

Total lipid content was measured by Folch *et al.* (1957).The tissues (muscles, liver, kidney, gill) were washed with saline and dried with filter paper and kept in oven at 60^0C. A weighed amount of each tissue (500 mg) was homogenized with 7.0 ml of methanol and filtered through a Whatman No 1 filter paper into a weighed tube. After filtration, the residue was scraped and homogenized in 14 ml chloroform. This step was repeated with 10 ml of chloroform-methanol (2:1, v/v) mixture and the resulting filterate was evaporated to dryness. The dried lipid residue was dissolved in 5 ml of chloroform - methanol mixture and the dissolved lipid extract was mixed with 1.0 ml of

0.1 N KCI and the contents were shaken well and allowed to stand for some time. The upper aqueous phase was removed with a Pasteur pippette and the lower chloroform phase was again washed 3 times with 2.0 ml of chloroform-methanol (2:1, v/v mixture). The washed lower layer of chloroform-methanol was aspirated. The lower layer was utilized to estimate the total lipid concentration gravimetrically as per the following equation and was expressed as µg/ 100 mg tissue weight.

Lipid content in given sample = Container with dried filtrate – Weight of test tube

1B.2.5 Statistical analysis

Data of samples were represented as means ± SEM. The significance of values obtained from different groups was tested by using one-way analysis of variance (ANOVA). The intergroup comparisons were done by Newman-keuls' test ($P<0.05$).

1B.2.6 Histopathology

The histopathological studies were carried out as summarized in **Table 3**.

1B.3 Results

The present study elaborates the toxic effects of chlorpyrifos (CPF) on biochemical content and histology of tissues and its bioremedial by natural antioxidants (Cur, Asc). This approach explains the various toxicity levels of CPF in the major tissues of *Heteropneustes fossilis* and the role of antioxidants in attenuating the toxicity.

1B.3.1 Effect of antioxidants against CPF induced toxicity on biomolecular content

Effect on the glycogen content

The glycogen content in the tissues (muscles, liver, kidney and gill) exposed to sub-lethal concentration of CPF (group II) was found to be depleted significantly as compared to the control ($P< 0.001$; $F= 32.35$ for the muscle; $F= 30.85$ for the liver; $F= 54.78$ for kidney and $F= 173.43$ for gill; **Figure 14**).

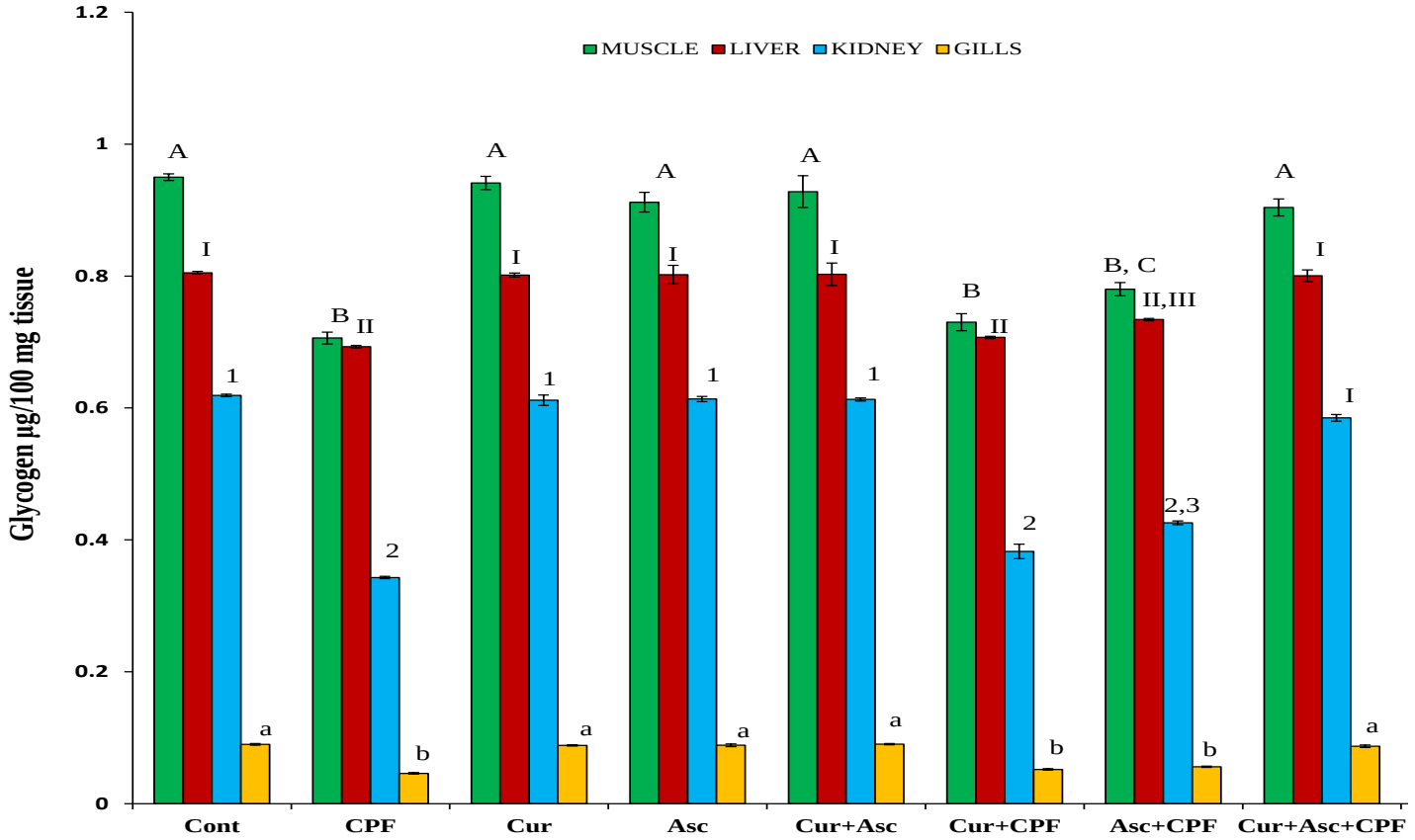


Figure 14: Effect of chlorpyrifos (CPF; 0.174µM/l, 1/10th of 96 hrs LC_{50}) and different combinations of antioxidants (ascorbic acid: Asc, 5 mg/l; curcumin: Cur, 10 mg/l) on glycogen content in different tissues (muscle: green bar; liver: red bar; kidney: blue bar; gills: yellow bar) of *Heteropneustes fossilis* for three days (72 hrs). Values are mean ± SEM of 5 fish in duplicates. Data were analyzed by one way ANOVA ($p< 0.001$) and Newman- Kuels' test ($p < 0.05$). Alphabets, A, B,… shows intergroup comparison between muscles. Roman I, II,..shows intergroup comparison between liver.Numericals,1,2,…shows intergroup comparison between kidney. Small letters, a,b….shows intergroup comparison between gill. Groups marked with same symbols are not significant and those with different letters are significantly different in intergroup comparison.

The negative control group (I) recorded glycogen content (µg/100 mg tissue) in the tissues as: 0.95 for muscle, 0.80 for liver, 0.61 for kidney and 0.09 for gill whereas values for the glycogen content (µg/100 mg tissue) in CPF treated group (II) was found as: 0.70 for muscles, 0.69 for liver, 0.34 for kidney and 0.04 for gill. The percentage decrease in the glycogen content was in the order: gill (48.8%)> kidney (44.5%)> muscle (25.6%)> liver (13.9%). The group having Cur (group III), Asc (group IV) and their combination (group V) showed no significant alteration in the carbohydrate content in all the tissues with respect to the control group. However, these groups (III, IV and V) showed varied effect on glycogen content compared to the CPF toxicity alone (II). In group VI (Cur + CPF), Cur was unable to suppress the CPF effect over the glycogen content in the tissues. Similarly, in group VII (Asc + CPF), Asc was also found to be ineffective in restoring the decrease in the glycogen content due to the CPF exposure. In group VIII (Asc + Cur + CPF), the combination of Cur and Asc with CPF was found to restore the glycogen content significantly in all the tissues in comparison to the CPF treated group (II). In the same group, the increment percentage in comparison to the CPF treated group (II) was 89.13% for gill >70.5% for kidney > 28.04% for muscle > 15.49% for liver.

Effect on the protein content

The protein content (µg/100 mg tissue) in the negative control group (I) was noted as 115.68 for muscle, 131.9 liver, 97.16 kidney and 77.32 for gill respectively. The protein content in the tissues exposed to sub-lethal concentration of CPF (group II) was found to be depleted significantly as compared to the control ($P< 0.001$; $F= 4.09$ for the muscle; $F= 15.58$ for the liver; $F= 6.10$ kidney and $F= 4.88$ for the gill; **Figure 15**). In CPF treated group (II) the protein content (µg/100 mg tissue) decreased to a level of 84.62 in muscle, 91.88 in liver, 58.12 in kidney and 35.4 in gill.

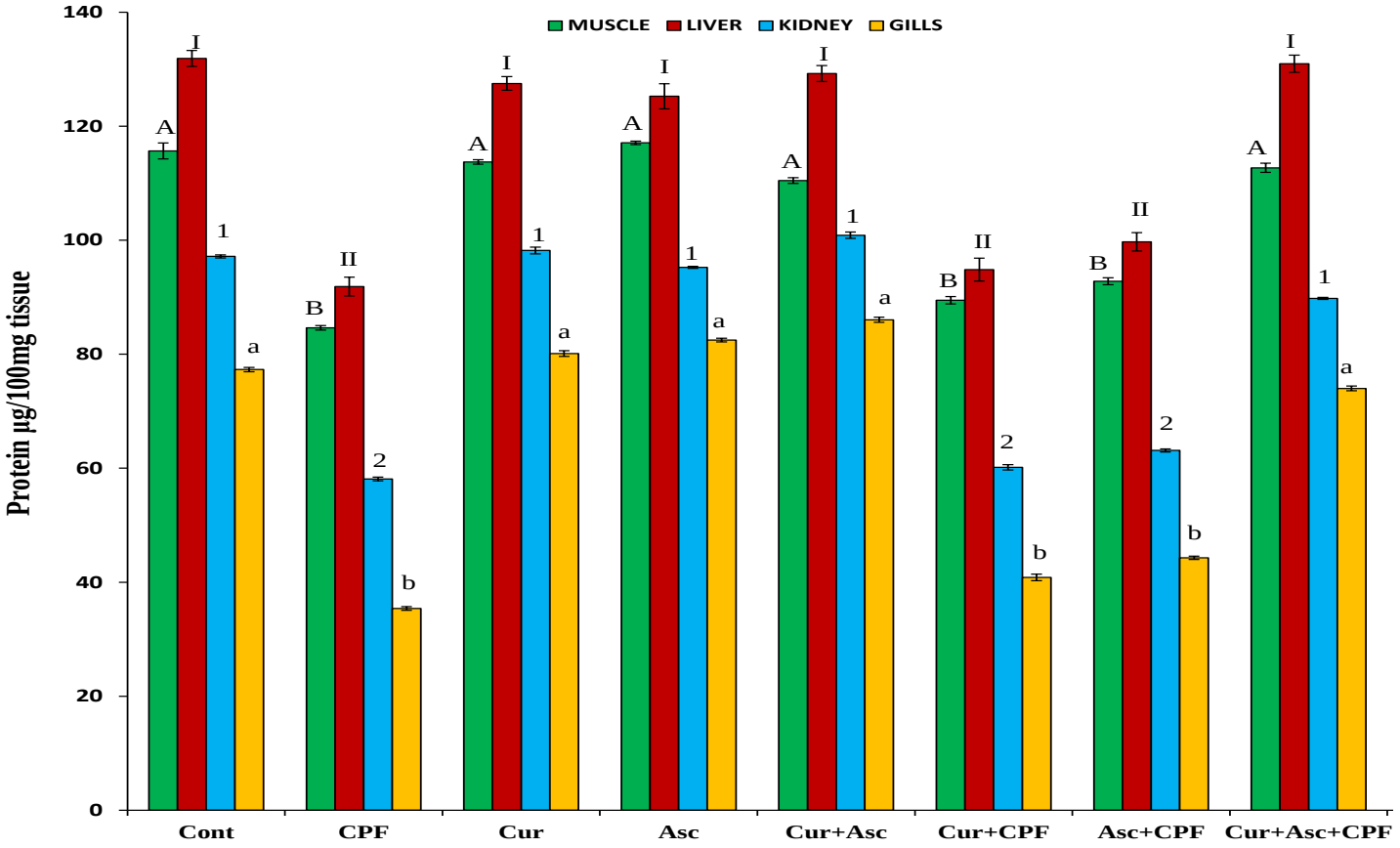


Figure 15: Effect of chlorpyrifos (CPF; 0.174µM/l, 1/10th of 96 hrs LC_{50}) and different combinations of antioxidants (ascorbic acid: Asc, 5 mg/l; curcumin: Cur, 10 mg/l) on protein content in different tissues (muscle: green bar; liver: red bar; kidney: blue bar; gils: yellow bar) of *Heteropneustes fossilis* for three days (72 hrs).Values are mean ± SEM of 5 fish in duplicates. Data were analyzed by one way ANOVA (p< 0.001) and Newman- Kuels' test (p < 0.05). Alphabets, A,B,… shows intergroup comparison between muscles. Roman I,II,.. shows intergroup comparison between liver.Numericals,1,2,…shows intergroup comparison between kidney. Small letters, a,b….shows intergroup comparison between gill. Groups marked with same symbols are not significant and those with different letters are significantly different in intergroup comparison.

The group having only Cur (III), only Asc (IV) and their combination (group V) did not bring any alteration in the protein content with respect to the control group. In groups having CPF with single antioxidant (groups VI and VII), protein content was found to be depleted in comparison to the control group. The combination of Cur and Asc (group VIII) with CPF had restored the normal protein level significantly in all the tissues, in comparison to the groups having only CPF (II) and CPF with single antioxidant (VI and VII).

Effect on the total lipid content

The total lipid content (μg/100 mg tissue) in the tissues of the negative control group (I) was found to be 27.69 for muscle, 39.29 for liver, 2.29 for kidney, 0.77 for gill. The total lipid content was found to be increased in all the tissues exposed to the sub-lethal concentration of CPF (II) ($P< 0.001$; $F= 7.16$ for the muscle; $F= 13.07$ for the liver; $F= 4.93$ kidney and $F= 43.49$ for the gill; **Figure 16**).

The total lipid content (μg/100 mg tissue) increased to a level of 34.01 in muscle, 47.05 in liver, 4.13 in kidney and 3.11 in gill. The increased lipid content values were found to be significantly different from the negative control group. The group having only curcumin (III), ascorbic acid (IV) or their combination (V) showed no significant alteration in the total lipid content in all the tissues with respect to the control group. The group having only curcumin, only ascorbic acid and their combination showed varied effect on the total lipid content as compared to the CPF toxicity alone. As in the case of glycogen and protein, curcumin (VI) and ascorbic acid (VII) were unable to suppress CPF effect over the total lipid content in liver, kidney and gill. However, the combination of curcumin and ascorbic acid (group VIII) was found to be effective in suppressing the CPF toxicity in the tissues. In group VIII, the total lipid content (μg/100 mg tissue) restored to a level of 28.94 in muscle, 38.68 in liver, 2.37 in kidney and 1.20 in gill.

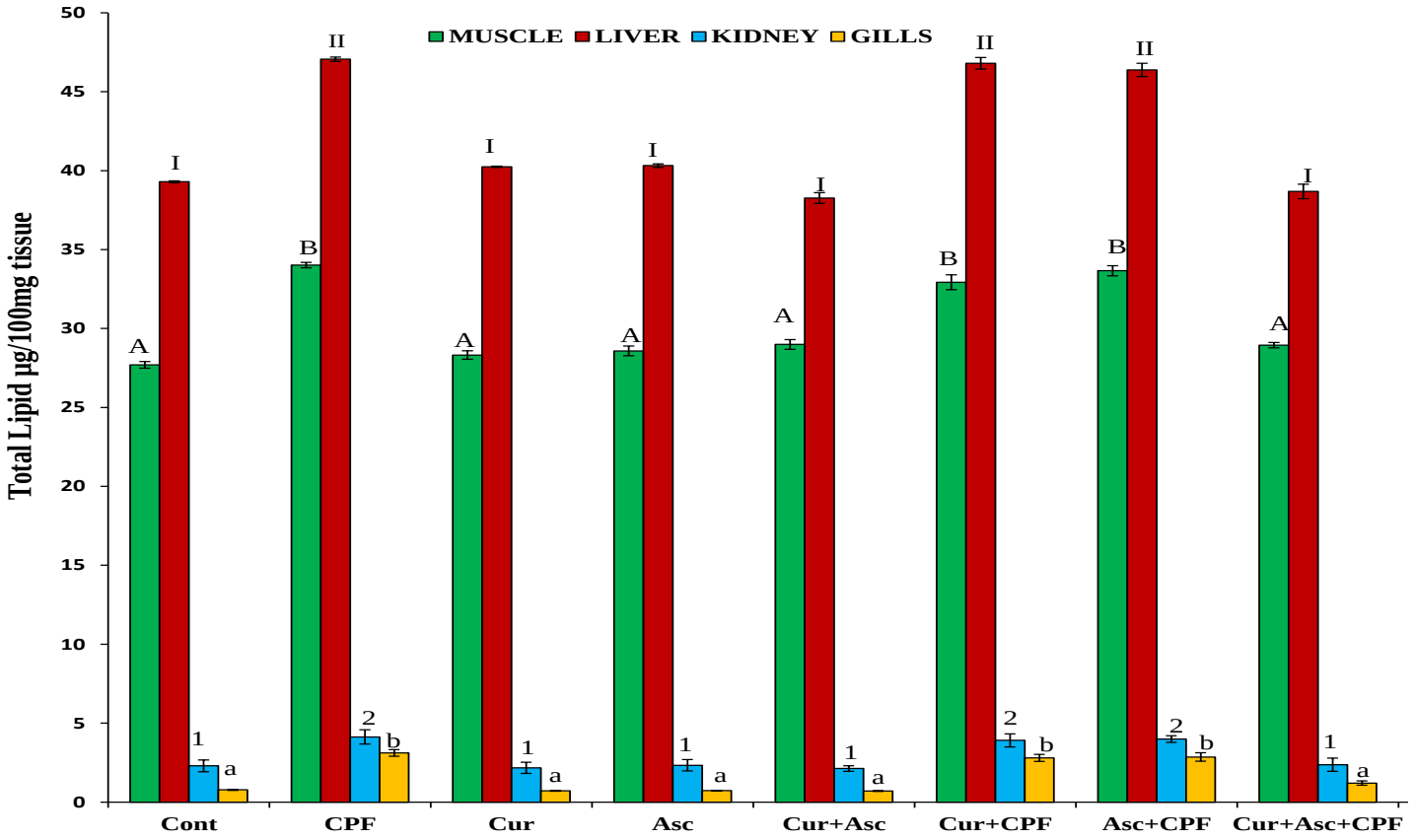


Figure 16: Effect of chlorpyrifos (CPF; 0.174µM/l, 1/10th of 96 hrs LC_{50}) and different combinations of antioxidants (ascorbic acid: Asc, 5 mg/l; curcumin: Cur, 10 mg/l) on lipid content in different tissues (muscle: green bar; liver: red bar; kidney: blue bar; gills: yellow bar) of *Heteropneustes fossilis* for three days (72 hrs).Values are mean ± SEM of 5 fish in duplicates. Data were analyzed by one way ANOVA (p< 0.001) and Newman- Kuels' test (p < 0.05). Alphabets, A,B,… shows intergroup comparison between muscles. Roman I,II,..shows intergroup comparison between liver.Numericals,1,2,…shows intergroup comparison between kidney. Small letters, a,b….shows intergroup comparison between gill. Groups marked with same symbols are not significant and those with different letters are significantly different in intergroup comparison.

1B.3.2 Effect of antioxidants against CPF induced histopathological alterations

Effect on stomach

The histological sections of the stomach from the negative control group fishes were normal depicting different layers of the stomach that are mucosa, submucosa, muscularis and serosa layers. The mucosa was the innermost layer folded into variable depths, composed of superficial epithelium and lamina propria. The submucosa layer was vascularized with a layer of connective tissue extending into the lamina propria. The muscularis layer and serosa layer was distinct (**Figure 17J**). In CPF treated group histopathological alterations were observed. These were vacuolization in the muscularis layer, thinning of submucosa layer, rupture and disintegration of the mucosa layer (**Figure 17K**).These histopathological alterations were found to be suppressed in the group having both CPF and antioxidants (Cur and Asc). The different layers of the stomach was intact and distinct similar to the control group (**Figure 17L**).

Effect on the intestine

The histological sections of the intestine from the negative control group fishes showed four normal histological layers that are: mucosa, submucosa, muscularis and serosa. The mucosal layer was produced into villi lined by epithelial cells. The submucosa layer was formed of connective tissue fibers while the muscularis layer was distinct divided into inner and outer muscle muscle fibers. The serosa layer was distinct (**Figure 17M**). In the CPF treated fishes, there was an alteration in the histoarchitecture of the intestine. There was atrophy and rupture of submucosa layer, vacuolization in the mucosa layer; disintegration and thinning of muscularis layer (**Figure 17N**).Such histological abnormalities induced by the CPF were found to be suppressed or attenuated on treatment with two antioxidants (Cur and Asc). The histological sections of intestine from this group were found to have normal architecture similar to the control group (**Figure 17O**).

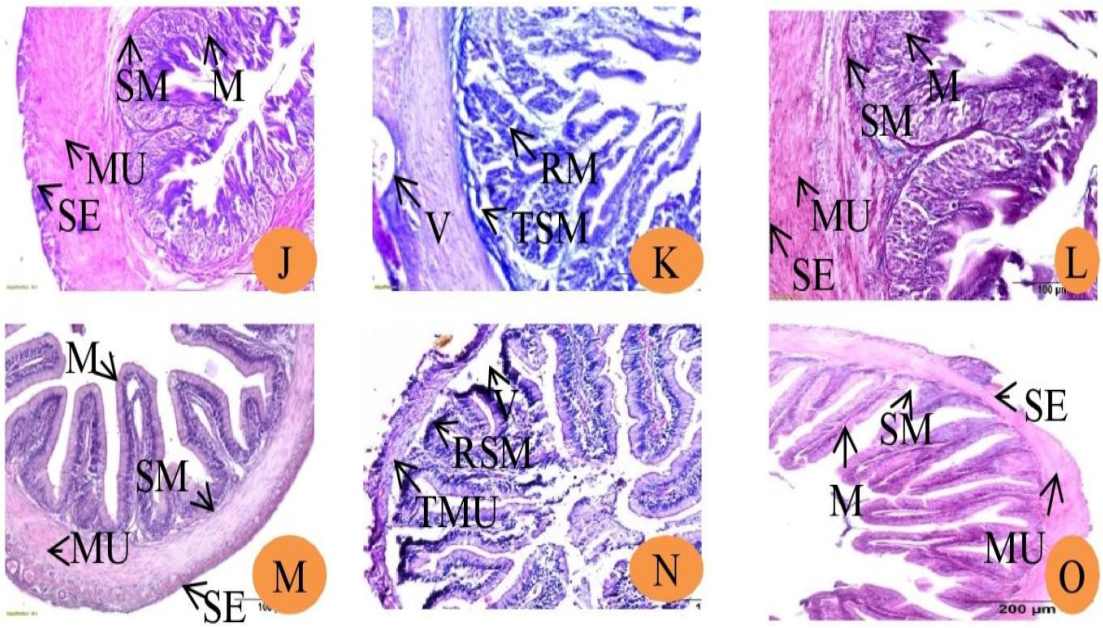


Figure 17: The combined bioremediation by ascorbic acid (Asc; 5 mg/l) and curcumin (Cur; 10 mg/l) on histopathological alterations induced by chlorpyrifos (CPF; 0.174 µM/l, $1/10^{th}$ of 96 hrs LC_{50}) onstomach and intestine of catfish, *Heteropneustesfossilis*, for three days (72 hr) using haematoxylin-eosin double staining. **Stomach**: (J) control (K) CPF (L) Asc + Cur + CPF, **Intestine**: (M) control (N) CPF (O) Asc + Cur + CPF.SE: serosa,MU:muscularis, SM: submucosa M:mucosa, RM: ruptured mucosa,TSM: thinning of submucosa, RSM: ruptured submucosa, TMU:thinning of muscularis.[Image captured with 200X].

1B.4 Discussion

Effect of CPF on glycogen, protein and total lipid content

In the present study, CPF decreased the glycogen and protein content but increased the total lipid content in all the tested tissues of fish when compared to the control group. The glycogen and protein content was found to be decreased in time dependent manner in freshwater fish, *Channa gachua* exposed to different concentrations of CPF (Kadam and Patil, 2016). Saradhamani and Kumari (2011) also revealed depletion in glycogen and protein content in gills, liver, kidney and muscle of *Oreochromis mossambicus* exposed to 0.045 ppm of CPF. The CPF induced depletion in the glycogen and protein content over control was also noted in freshwater fish *Catla catla, Labeo rohita, Cirrhinus mrigala* (Tilak *et al.*, 2005), guppy fish (Khan, 2017), *Channa striatus* (Revathi and Krishnamurthy, 2018). Similar to the present result; exposure of organophosphate pesticide Dimethoate decreased the glycogen and protein content but increased the cholesterol level in freshwater fish, *Puntius ticto* (Ganeshwade, 2011). Malathion, an organophosphate was reported to decrease the glycogen and protein content in important tissues such as gill, liver, kidney, muscle and brain over the control tissues in freshwater fish, *Ctenopharyngodon idella* (Satyavardhan, 2013). Alterations in the protein content and amino acids were noted by Magar and Shaikh (2012) exposed to malathion ($1/5^{th}$ of LC_{50} 96 hrs) in gill and liver of *Channa punctatus.*The depletion in the glycogen content might be due to the fact it is the major source of energy required whenever there is lack of food, hypoxic or stress conditions (Kadam and Patil 2016). The reduction in the protein content might be either due to the disruption in protein synthesis or increased protein degradation to amino acids. These amino acids are utilized in TCA (Tricarboxylic acid cycle) as keto acids in order to cope with high energy demands during oxidative stress

conditions. The decrease in the protein content might also be due to its involvement in cell repair and formation of important constituents of cell membranes including lipoproteins (Ramalingam and Ramalingam, 1982; Ganeshwade, 2011). Ramalingam and Ramalingam (1982) mentioned about quantitative and qualitative alterations in tissue amino acids of fishes exposed to toxicants including pesticides.

In contrast to glycogen and protein, the total lipid content was found to be increased in all the tested tissues (muscle, liver, kidney and gill) of fish exposed to CPF over the control group. Similar to the present result, sub-lethal concentration of phosphamidon, an organophosphate insecticide was found to elevate the total lipids significantly in liver and ovary of freshwater fish, *Puntius conchonius* (Gill *et al.*, 1990). Malathion, an organophosphate was found to increase hepatic total lipid level in males of *Clarias batrachus.* In the same study, Malathion also increased the cholesterol levels in all the tissues of *C. batrachus* (Lal and Singh, 1987). Since total lipid is a combination of cholesterol, triglycerides and phospholipids, an increment in these components can lead to increment in total lipid (Javed *et al*., 2017). The increased total lipid level might either be due to stimulated lipogenic activity in liver resulting to increased lipid synthesis than consumed by other tissues or increased rate of hydrolysis of circulating triglycerides (Vadhva and Hasan,1986; Lal and Singh, 1987).Vadhva and Hasan (1986) mentioned about dose dependent increase in the levels of total lipid, esterified fatty acids and cholesterol in different parts of CNS (Central Nervous System) of fish *H. fossilis* exposed to organophosphate Dichlorvos. Qayoom *et al.* (2014) supported the fact that organophosphorus compounds can bring alterations in the lipid metabolism of fishes. The other reason for the significant increase in the total lipid content could be the estrogenic nature of CPF eliciting disruption in the hormone levels (Ventura *et al*., 2016).

Effect of CPF on the histology of tissues

The sub-lethal concentration of CPF induced histopathological alterations in both stomach as well as intestine. These included disintegration and atrophy of the mucosa layer, thinning and rupture of submucosa layer and vacuolization in the muscularis layer. Similar histological alterations were also noted in stomach of *Channa punctatus* including degeneration of serosa and mucosa layer, vacuolation in mucosa and sub-mucosa region, and degeneration of columnar epithelium when exposed to three different sublethal concentration of CPF (Jain *et al.,* 2018). Das and Gupta (2013) mentioned about histological aberrations in intestine of *Esomus danricus* when exposed to sublethal concentration of organophosphate pesticide, Malathion for 28 days. The pathological lesions in the intestine of the studied fish are in agreement with different investigators who studied the effects of different toxicants on fish intestine (Sakr *et al.,* 1991; Cengiz and Unlu, 2006; Maurya *et al.,* 2019).

Effect of antioxidants (Asc and Cur) against CPF induced toxicity

The alterations in glycogen, protein and total lipid content and histology of tissues were found to be attenuated by the combination of Asc + Cur. The total glycogen and protein content in different tissues of *Tilapia mossambica* (Joshi and Desai, 1988), *Cirrhinus mrigala, Labeo rohita* and *Catla catla* were found to be decreased on exposure to CPF (Tilak *et al.,* 2005). However, ascorbic acid administration to CPF treated fishes was found to increase the protein content (Tripathi and Shasmal, 2011). In addition, Cur facilitates growth by increasing the protein concentration suggesting that Cur in very low doses can be used in aquaculture to increase the quality and quantity of fish. In the present study the combination of Asc + Cur normalized CPF induced alterations in biomolecular contents and histology of tissues (Elballat, 2016). This might either be due

to synergistic effect when the combined action of the two antioxidants is greater than the individual ones or additive effect where the outcome is the sum of the two individual antioxidants (Tomczyk, 2019; Uri, 1961; Elballat, 2016). Also, it was also found that antioxidant effect of curcumin enhanced in the presence of ascorbic acid (Khalil *et al.*, 2012).

Chapter 2

Understanding of chlorpyrifos on the gonadal maturation of freshwater catfish Heteropneustes fossilis

[A] Influence of chlorpyrifos alone and with antioxidants on gamete growth (vitellogenin) and maturation supporting steroid hormones (estradiol, maturation inducing steroid, progesterone) profile

[B] Chlorpyrifos impact on gene expression linked to gonad development (*NR5A1*) in fry of catfish, *Heteropneustes fossilis*

Chapter 2

Understanding of chlorpyrifos on the gonadal maturation of freshwater catfish *Heteropneustes fossilis*

[A] Influence of chlorpyrifos alone and with antioxidants on gamete growth (vitellogenin) and maturation supporting steroid hormone (estradiol, maturation inducing steroid, progesterone) profile

Abstract

Chlorpyrifos (CPF) is a broad-spectrum organophosphate insecticide extensively used for outdoor and indoor uses. It is an acetylcholinesterase inhibitor, neurotoxic as well as endocrine disruptor. The present study elaborates interference of the CPF in gonad function at the steroidal level during prespawning phase of the freshwater catfish, *Heteropneustes fossilis*. The matured fish of early pre-spawning stage were exposed to different combinations of CPF, gonadotropin (hCG) and natural antioxidants (curcumin and ascorbic acid) for 24 hrs to measure vitellogenin (Vtg) and ovarian steroid. Results showed that the CPF increased vitellogenin level in liver, serum and ovary. The gonadotropin further induced this increase. Among steroidal cascade, CPF induced E_2 (17β-estradiol) many fold along with significant increase in P_4 (progesterone) and 17α, 20β-DHP (17α, 20β-dihydroxy-4-pregnen-3-one). In the ovarian steroidal level also gonadotropin registered an additive effect with CPF. Antioxidants co-incubation did not produce any significant change on either vitellogenin or in steroids content. The results clearly signals major influence of CPF in gonadal physiology of freshwater catfish, *Heteropneustes fossilis*.

2A.1 Introduction

Chlorpyrifos (CPF) is the most extensively used broad-spectrum chlorinated organophosphate insecticide. The extensive use of pesticide has allowed it to reach the water bodies via surface runoff and bioaccumulate in aquatic organisms including fish (Thomas and Mansingh, 2002). Despite being an acetylcholinesterase inhibitor (Topal *et al.*, 2014), CPF is an endocrine disruptor (Ventura *et al.*, 2012, Andersen *et al.*, 2002, Kojima *et al.*,2004, Grünefeld and Bonefeld-Jorgensen, 2004), teratogenic (Farag *et al.*, 2003), developmental neurotoxic (Slotkin, 2004). However, study reporting the role of chlorpyrifos in catfish steroidogenesis is very less.

Human chorionic gonadotropin (hCG) is a placenta derived, structurally related variant of pituitary gonadotropin. It has been reported to induce oocyte maturation both *in vivo* and *in vitro* in various species of fish including catfish (Sorbera *et al.*, 1999; Canario and Scott, 1990; Mishra and Joy, 2006). Besides gamete maturation, hCG has a role in ovulation. This is due to the fact that hCG is composed of follicle stimulating hormone (FSH) which has an important role in the gonadal maturation and luteinizing hormone (LH) involved in bringing ovulation process to spawning (Putra and Mulah, 2019).

Curcumin (Cur), derived from the rhizome *Curcuma longa*, is a powerful antioxidant, anti-inflammatory and hepatoprotective agent and has been reported to improve the growth and quality of fish in aquaculture (Manju *et al*., 2012, Mishra and Singh, 2020a). So far, there is no study to assert the role of curcumin on fish at the steroidal level. Ascorbic acid (Asc) is another antioxidant to contribute an important role in fish health (Blom and Dabrowski, 1995; Mishra and Singh, 2020b). Both these antioxidants are promising suppressor of CPF induced stress on fish that influenced most of the tissue physiology (Mishra and Singh, 2020a, b).

Vitellogenins (Vtgs) are glycolipophosphoproteins prominent in various fishes including both viviparous and oviparous teleosts (Bradley and Grizzle, 1989). They are synthesized in the liver of all sexually mature oviparous females in response to the circulating estrogens. They are transported to the ovary via the blood, sequestered by reverse micropinocytosis into the oocytes where they are proteolytically cleaved into the yolk proteins lipovitellin and phosvitin which are essential source of nutrients for the successful development of oocytes and embryos of oviparous vertebrates, including fish (Nath and Sundararaj, 1981b; Mewes *et al.*, 2002). At the end of vitellogenesis the ovary is packed with fully yolked oocytes ready to undergo maturation and ovulation (Reading *et al.,* 2017). Like other teleosts, *Heteropneustes fossilis,* is a seasonal breeder whose reproductive event depends on gonadotrophins (Goswami *et al.*, 1985). In response to various endogenous and environmental factors such as innate biorhythms, nutritional status, water temperature and photoperiod (DeVlaming, 1972), the brain (hypothalamus) produces gonadotropin-releasing hormone (GnRH) which stimulates the pituitary gonadotrophs to secrete two gonadotrophins, GTH I and GTH II which are homologous to follicle stimulating hormone (FSH) and Luteinizing hormone (LH) respectively (Nagahama and Yamashita, 2008; Minniti *et al.*, 2009). The FSH stimulates the theca and granulose cells of the ovarian follicle to produce 17β-Estradiol (E_2), which commands the liver to synthesize Vtgs and secrete them, into the bloodstream (Nath and Sundaraj, 1981a). In aquaculture, circulations of Vtgs are the sign of puberty onset and progression of gonad maturation in female fishes (Reading *et al.*, 2017). Also, they are used as an effective biomarker to assess exposure of aquatic animals to endocrine-disrupting

chemicals (EDCs) including those EDCs that can mimic the action of estrogens (Schwaiger and Negele, 1998, Schwaiger *et al*., 2002; Park *et al.,* 2010a).

17β-estradiol (E_2) concentration is higher in females than males and is a major steroid, regulating the ovarian development by inducing hepatic synthesis of vitellogenin during oocyte growth period (Devlin and Nagahama, 2002; Nagahama and Yamashita, 2008). The GTH (FSH) stimulates the ovarian granulosa layer to enhance the activity of cytochrome P450 aromatase, the key enzyme involved in the conversion of testosterone produced by the thecal layer to E_2 (Nagahama and Yamashita, 2008). In fish, E_2 regulates important reproductive function by binding and activating the intracellular estrogen receptors (ERs). They also play an important role in regulation of oogenesis and gonadotropin, testicular development, non-gonadal tissue gene expression, sex change or sex differentiation through regulation of hormonal synthesis (Nelson and Habibi, 2013; Park *et al.,* 2010a).

The steroidogenic pathway initiates with the side-chain cleavage of cholesterol by cytochrome P450 (P450scc) forming pregnenolone which further gets converted to progesterone (P_4) via 3β-hydroxysteroid dehydrogenase (3β-HSD) or to 17α-hydroxypregnenolone via cytochrome 17α-hydroxylase (P450c17) (Nagahama and Yamashita, 2008). P_4 is an important progestin that mediates not only the oocyte growth and maturation but also spermatogenesis and sperm maturation in teleosts (Zeilinger *et al*., 2009). Progesterone and 17α-hydroxypregnenolone are converted to 17α-hydroxyprogesteone followed by the production of 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DP) by 20β-hydroxysteroid dehydrogenase (20β-HSD). The 17α, 20β-DP is a

maturation inducing hormone (MIH). It is an important steroid regulating oocyte maturation occuring prior to ovulation, an essential step for successful fertilization (Nagahama and Yamashita, 2008; Mishra and Joy, 2006). Chlorpyrifos has been reported as ovatoxic and embryotoxic in most of the ruminants (Nandi *et al.*, 2011).

H. fossilis is a freshwater catfish. It is commonly used as laboratory fish due to its table size and sturdy nature. It is a favorite model to understand reproductive limitation of the catfish and their alternate mechanism.

The present study is an attempt to understand the interference of the CPF with the steroidogenesis of *H. fossilis*. This is for the first time where interplay of chlorpyrifos, hCG and antioxidants have been studied in *H. fossilis* with reference to gonadal physiology.

2A.2 Materials and Methods

2A.2.1 Chemicals

The steroid standards were purchased as 17β-estradiol (E_2, CAS Number: 221093-45-4; Sigma Chemicals, USA), 4-pregnene-3,20-dione (P_4, CAS Number*:* 57-83-0; Sigma Chemicals, USA) and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP, CAS Number: 1662-06-2; Cayman Chemicals, USA). The solvent, methanol and acetonitrile were of HPLC grade (Merck, India). The synthetic hormone, human chorionic gonadotropin (hCG: Ovidac®, Bayer Zydus Pharma Private Limited) was purchased from local medical stores. A commercial grade of pesticide, Hilban® (20% EC CPF) purchased from retail agricultural shop (Hindustan Insecticide Limited, India). All

other chemicals and reagents used during the experimental process were of analytical grade and procured from HiMedia Laboratory Private Limited, India.

2A.2.2 Animal collection and their acclimatization

The adult catfish, *Heteropneustes fossilis* (Bloch, 1794) of early prespawning (1-5th May) season of relatively same size (16 ± 5 cm) and weight (140 ± 15 gm) was purchased from local fish market in Lucknow, Uttar Pradesh, India. The fish were brought to the laboratory carefully in large containers containing fresh water avoiding any sort of stress and injuries as much as possible. The fish were treated with 0.5% potassium permanganate to prevent dermal infection and were allowed to acclimatize for one week in plastic tanks containing freshwater (pH ~ 7.5) under normal photoperiod and temperature (12h : 12h; light : dark and 25 ± 2^0 C). The water was renewed daily to remove faecal matter and metabolic wastes accumulated during acclimatization. They were fed with minced goat liver *ad libitum*.

National guidelines of the ethical committee were followed to exhibit experiments and to avoid any cruelty to the animals.

2A.2.3 Quantitative estimation of vitellogenin

Experimental design

To study the quantitative estimation of vitellogenin, mature adult females of *Heteropneustes fossilis* were divided into eight groups of three fish in duplicates for 24 hrs.The group I was a control having only freshwater and group II received fish treated with synthetic hormone (hCG hormone : 100 IU/fish; Mishra and Joy, 2006). The group III and IV received hCG treated fish in curcumin (Cur : 10 mg/l; Cao *et al.,* 2015) water and

ascorbic acid (Asc : 5 mg/l; Kumar *et al.*, 2009) water respectively. The group V received hCG treated fish in freshwater containing combination of Cur and Asc. The group VI and VII received untreated fish and hCG treated fish respectively into chlorpyrifos (CPF : 0.174 µM/l, $1/10^{th}$ of 96 hrs LC_{50}; Mishra and Verma, 2016) mixed water. The last group VIII received hCG treated fish in freshwater containing CPF in addition to Cur and Asc.

Sampling

At the end of the experimental period, blood from fish of each group was collected by caudal severance and allowed to clot by leaving it undisturbed at room temperature for at least 30 minutes. The clotted blood was centrifuged 1,000 - 2,000 g for 10 minutes in a refrigerated centrifuge and the resulting supernatant called serum was decanted into clean tubes, sealed, and frozen at -20 °C until assayed. The same fish from each group was sacrificed and tissues such as liver and ovary were dissected out and stored at -20 °C until assayed.

Procedure

Vitellogenin in serum samples and tissues (liver and ovary) were determined by the alkali-labile protein phosphorus (ALPP) method according to the Nath and Sundararaj (1981a) and Gagné (2014). The tissues were homogenized in ice-cold 50 mM Tris-acetate buffer (homogenization buffer, pH 8) containing 100 mM NaCl, protease inhibitor (apoprotinin, 1 µg/ml), reductive agent (dithiothreitol, 0.1 mM) and calcium/magnesium chelator (EDTA, 0.1 mM). The homogenate was centrifuged at 12,000 g for 20 minutes at 2^0 C and the supernatant was collected for the assay. The protein was precipitated by adding acetone to 200 µL of freshly thawed sample

(supernatant as well as serum) to make a final concentration of 35 % (v/v). It was mixed and placed on ice for 15 minutes followed by centrifugation at 10,000 g for 5 minutes at 4^0 C. The supernatant was discarded and the pellet was washed successively with hot ethanol, chloroform : ether : ethanol (1:2:2), acetone, and finally ether to remove any traces of lipid and free phosphates. The washed pellet was dried (air) and dissolved in alkaline solution (100 µL of 1M NaOH) and incubated for 60 minutes at 37^0 C. In the spectrophotometric assay, phosphomolybdic acid complex reagent was used to determine the released inorganic phosphates in each sample. The standard phosphate solution was prepared by 1.74 g of monobasic potassium phosphate (KH_2PO_4) in 100 ml of 1M NaOH. In a clean microplate, 20 µL each of the blank (NaOH alone), phosphate standard and sample (pellet dissolved in NaOH) was added in duplicate to 125 µL of water. To this 5 µL of 6.1 N trichloroacetic acid (TCA), 25 µL of molybdate reagent (1 g of ammonium molybdate dissolved in 100 ml of 0.9 M sulphuric acid, H_2SO_4) and 25 µL of ascorbate reagent (1 g of L-ascorbic acid dissolved in 100 ml distilled water) was added and mixed thoroughly by repeated pipetting for atleast 5 minutes to develop colour. The absorbance of the phosphomolybdate complex was determined at 815nm using SpectraMax® ABS plus microplate reader. For turbidity correction if present, the absorbance at 444nm was also measured. The alkali-labile phosphorus content in individual sample was calculated with a reference to a phosphorus standard curve. The standard curve was prepared by plotting different concentrations (20, 40, 80, 120, 160, 200, 240, 280, 300, 320 µg/ml) of the standard stock solution against their respective absorbance values. A brief outline of vitellogenin determination procedure has been illustrated in **Figure 18.**

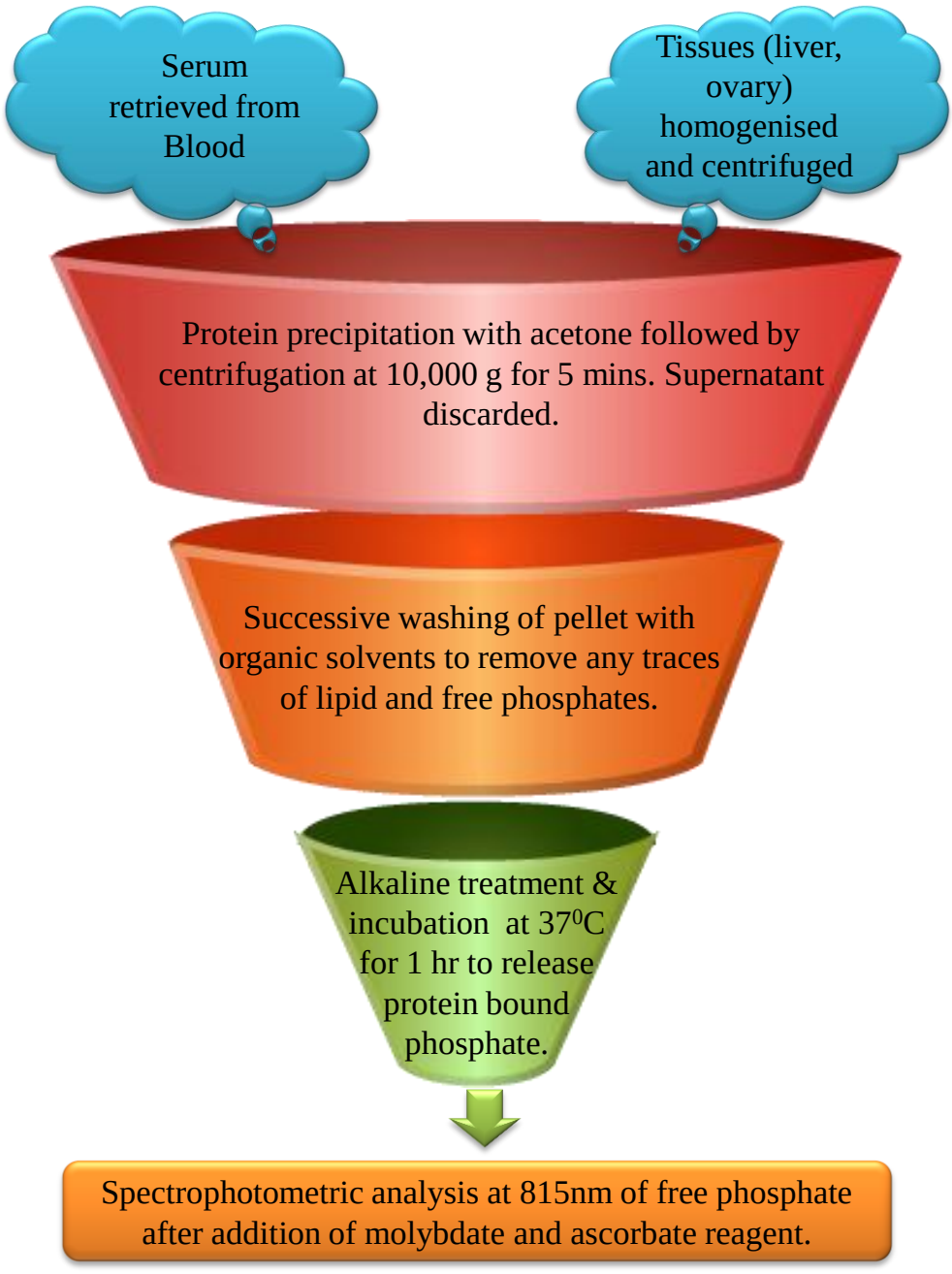


Figure 18: A brief illustration of steps for vitellogenin estimation

Data calculation

The quantity of vitellogenin was estimated indirectly by determining the levels of alkali-labile phosphates in sample by the method of Gagné (2014).

Turbidity correction for absorbance at 815nm by the following equation:

$$A_{815nm}\,\text{Corrected} = (1.045 \times A_{815}\,\text{nm}) - (0.043 \times A_{444}\,\text{nm})$$

Equation to determine phosphate (vitellogenin) concentration:

$$[A_{815nm}\,S - A_{815nm}\,B] \times St\ P_{conc.} / [A_{815nm}\,St - A_{815nm}\,B] \times (S_A/T_A) \times (1\ ml \times S_V)$$

$$= \text{Phosphate concentration (µg/ml) or (µg/ml/mg tissue weight)}$$

Where,

$A_{815nm}S$ = Absorbance of sample at 815nm.

A_{815nm} B = Absorbance of blank at 815nm.

A_{815nm} St = Absorbance of standard solution at 815nm.

St $P_{conc.}$ = Standard phosphate concentration.

S_A = Sample volume (µL)

S_V = Supernatant volume (µL)

T_A= Total assay volume (µL)

2A.2.4 Steroid analysis

Extraction

The steroid extraction was followed as per the Mishra and Joy (2006). The ovary of each fish from each group was homogenized in 4 volumes of cold 0.2 N perchloric acid with an ultrasonic homogenizer at 0°C for 10 – 15 seconds. The homogenate obtained was centrifuged at 21,000*g* for 20 min at 0°C. The supernatant collected was extracted

with hexane twice followed by diethylether three times. The ether phase obtained was stored at -20°C till chromatography after completing pooling, evaporating and drying process under liquid N_2.

HPLC analysis

The HPLC analysis of standards and samples was performed as per the Bhattacharyya *et al.*, (2002). Standard steroids, 17β-estradiol, 4-pregnene-3,20-dione and 17α,20β-dihydroxy-4-pregnen-3-one were used to compare fish ovarian samples from different groups. Each standard compound and sample (1 mg) was separately dissolved in mobile phase (1 ml) forming a solution of 1 mg/ml. The sample was filtered (0.2 µm) and 10 µl of each sample was subjected to reversed-phase HPLC (Waters 2998) on an X-Bridge C-18 column (250X4.6 mm) packed with 5 µm particles size. The mobile phase used was acetonitrile : water (40 : 60, v/v) at a constant flow rate of 1 ml/min for 30 minutes. The absorbance of the effluent was measured at 230nm with Photodiode Array Detector. The activation of column was done with 100% methanol followed by the mobile phase before injecting the standard or sample. Firstly, the retention time (RT) and chromatogram of individual standard was obtained followed by the standard mix in order to verify their retention times. After standard run, each sample was injected separately into the HPLC column. A chromatogram showing retention time, peak height and peak area for each standard/sample was obtained using Empower 2.0 software (**Figure 22, 23** and **24**).

Qualitative analysis includes identification of different steroids present in each sample by matching the RT and peak profile of different standard steroids to each sample. After the qualitative analysis in HPLC analysis, the next step is quantification. In the

quantification step of HPLC analysis, the concentration of standard steroid present in the injected volume (10 µl) is compared to its peak area (or peak height) which is established as 'known' concentration. The concentration of different steroids present in the sample (unknown) can be determined by comparing its peak areas (or peak heights) to the 'known' concentration of standard steroids. The unmatched peaks, if occurs in samples requires more identification and screening.

In HPLC, quantitative analysis based on percentage area gives a proper approximation of relative quantity of component present in the sample if all the components are detected equally in the detector and eluted. The calculation is based on area of each peak expressed as a percentage of total area covered by all peaks. The height percentage of each peak is expressed as a percentage of all peaks and can also be used for quantitative analysis.

2A.2.5 Statistical analysis

Data of samples were represented as means ± SEM. The significance of values obtained from different groups was tested by using one-way analysis of variance (ANOVA). The intergroup comparisons were done by Newman-keuls' test ($P < 0.05$).

2A.3 Results

Vitellogenin estimation in liver, serum and ovary

The hCG (group II) increased the Vtg content significantly in liver and serum but not in the ovary of early prespawning phase of *H. fossilis* (**Figure 19**: liver, $F = 34.32$, $P < 0.001$; **Figure 20**: serum, $F = 37.23$, $P < 0.001$; **Figure 21**: Ovary, $F = 5.49$, $P < 0.05$) with respect to the control group (group I). The percentage increase in the Vtg content after hCG treatment was: liver (88.88 %), serum (85.27 %) and ovary (3.33 %).

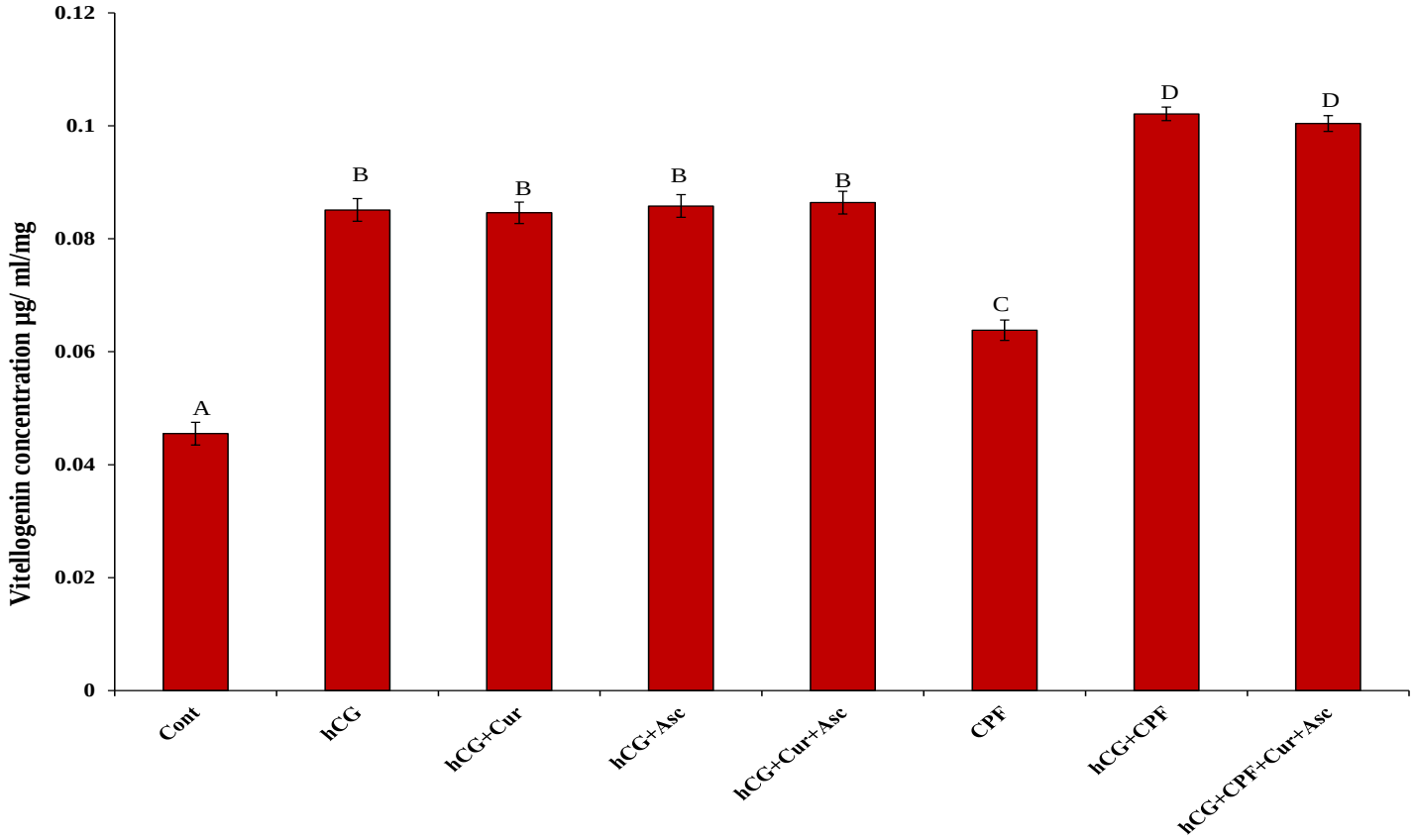


Figure 19: Effect of human chorionic gonadotropin (hCG) and chlorpyrifos (CPF) with different combination of antioxidants (curcumin, Cur and ascorbic acid, Asc) for 24 hrs on vitellogenin content in liver of freshwater catfish, *Heteropneustes fossilis* during early prespawning period (May).Values are mean ± SEM of 3 fish in duplicates. Data were analyzed by one way ANOVA ($P<0.001$) and Newman- Kuels' test ($P<0.05$). Groups marked with same symbols are not significant and those with different symbols are significantly different in intergroup comparison.

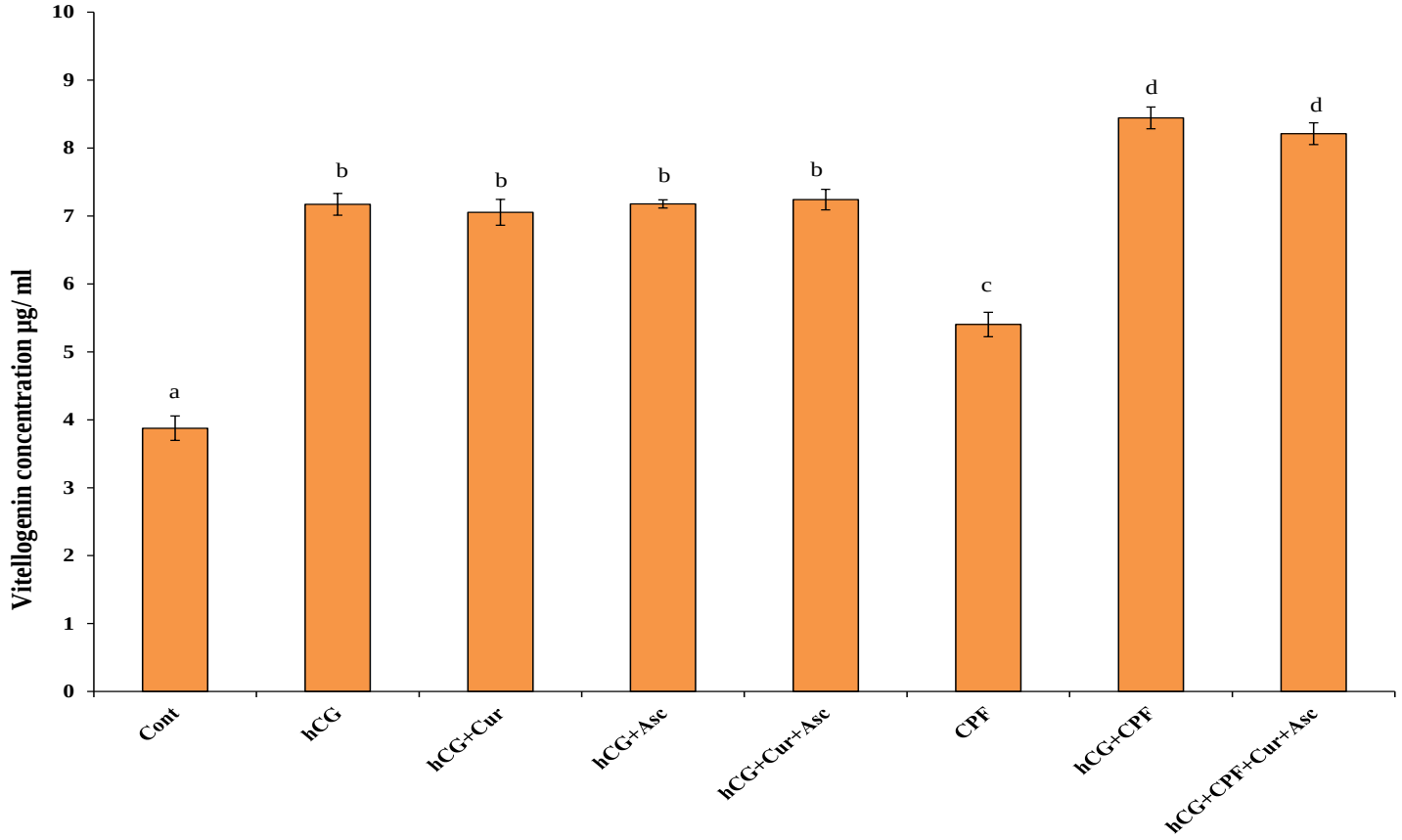


Figure 20: Effect of human chorionic gonadotropin (hCG) and chlorpyrifos (CPF) with different combination of antioxidants (curcumin, Cur and ascorbic acid, Asc) for 24 hrs on vitellogenin content in serum of freshwater catfish, *Heteropneustes fossilis* during prespawning period (May).Values are mean ± SEM of 3 fish in duplicates. Data were analyzed by one way ANOVA ($P<0.001$) and Newman- Kuels' test ($P<0.05$). Groups marked with same symbols are not significant and those with different symbols are significantly different in intergroup comparison.

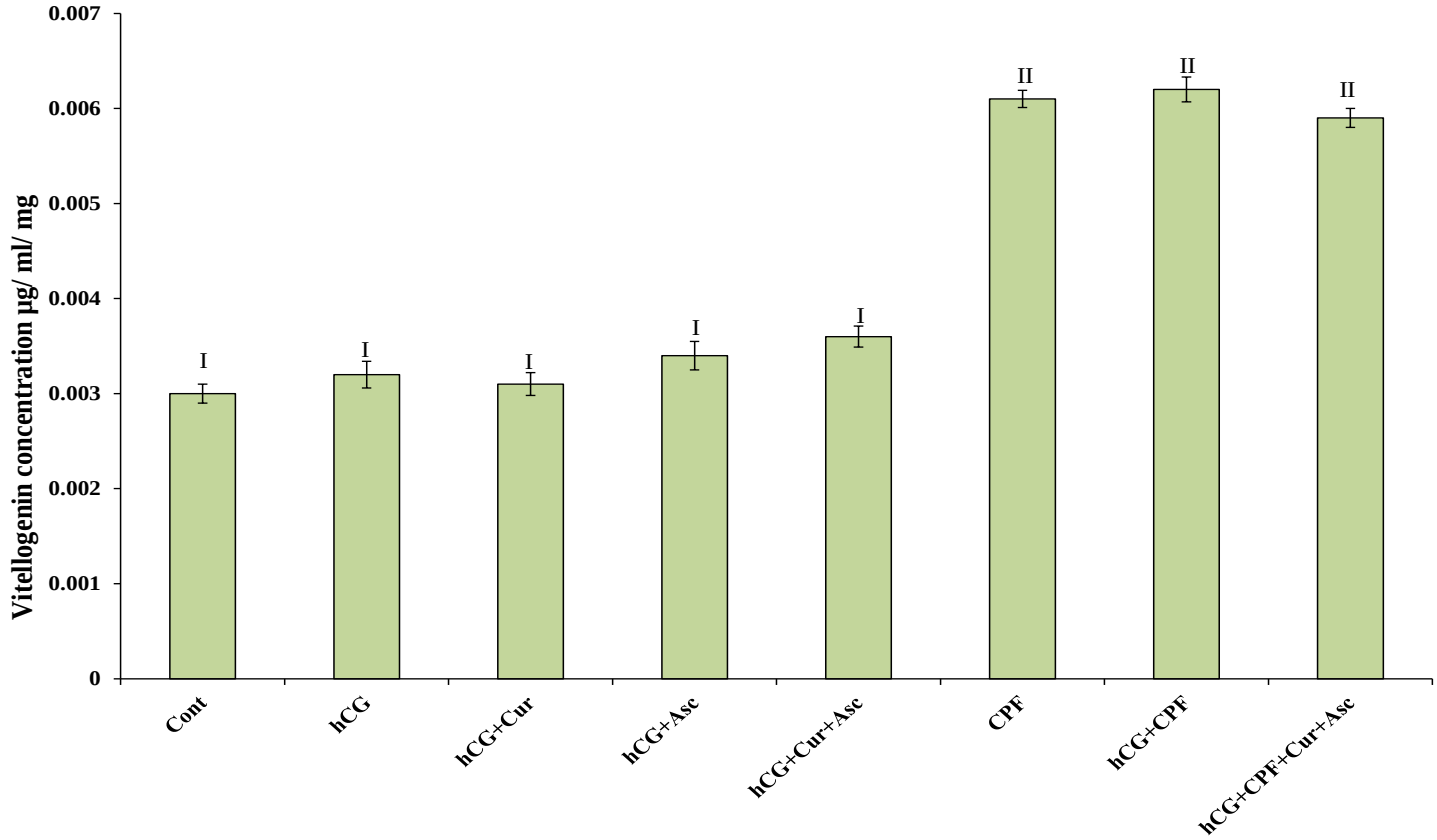


Figure 21: Effect of human chorionic gonadotropin (hCG) and chlorpyrifos (CPF) with different combination of antioxidants (curcumin, Cur and ascorbic acid, Asc) for 24 hrs on vitellogenin content in ovary of freshwater catfish, *Heteropneustes fossilis* during prespawning period (May).Values are mean ± SEM of 3 fish in duplicates. Data were analyzed by one way ANOVA ($P<0.001$) and Newman- Kuels' test ($P<0.05$). Groups marked with same symbols are not significant and those with different symbols are significantly different in intergroup comparison.

In group III, IV and V respectively, hCG in combination with curcumin (Cur), ascorbic acid (Asc) and both, increased the Vtg in liver and serum in comparison to the control group whereas in ovary the increase was found to be insignificant. There was no influence of singular or dual combination of antioxidants on gonadotropin induced Vtg content as compared to hCG injected group. Also, there was no such significant difference observed among these three groups. The administration of CPF (group VI) promoted a significant increase in the Vtg content both in the tissues (liver and ovary) and the serum, in reference to the group I (control). The CPF induced increase was by 1.4-fold in liver, 1.39-fold in serum and 2-fold in ovary with reference to the control group. The gonadotropin injected fish kept in CPF treated water (group VII) recorded significant increase in Vtg content in liver (1.2-fold), serum (1.1-fold) and ovary (1.9-fold) in comparison to hCG treated group (II). In contrast to liver and serum, the increase in the ovary was not found to be significant with reference to CPF treated group (VI). An inappreciable decrease was noticed in the Vtg content in the group VIII for both the tissues and the serum indicating no significant role of antioxidants on Vtg content. The Vtg content was highest in the liver and least in the ovary.

Steroid analysis

High-performance liquid chromatography analysis of standard steroids and the steroids extracted from the ovary of fish from different groups have been depicted in **Figure 22**, **23** and **24**. The standard steroid compounds showed distinct peak and height at a specific time interval whereas the chromatogram of different samples showed alterations in peak areas and heights. The retention time and individual peak

of standard steroids in the HPLC chromatogram were marked as reference. The HPLC profile of standard steroid namely, 17β-estradiol (E_2) has covered an area of 9025966 and a height of 911782 at 15.112 minutes (**Figure 22 A**), 4-pregnene-3,20-dione (P_4) has covered an area of 20967877 and a height of 1563699 at 21.112 minutes (**Figure 22 B**) and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) covered an area of 247222854 and a height of 2395023 at 15.497 minutes (**Figure 22 C**). A mixture of standard steroids was also injected into the system in order to verify their retention times **(Figure 22 D)**.

The HPLC profiles of ovary samples of fish from different groups were compared to the profiles of the standard steroids for qualitative and quantitative analysis (**Figure 23** and **Figure 24**). The presence of steroids (qualitative analysis) in the samples were identified by comparing the retention time (RT) with that of the standard steroids. The quantity of the steroids (quantitative analysis) was determined by comparing unknown peak areas to the known peak areas of the standards.

The gonadotrophin and CPF treatment alone and in combination with antioxidants produced significant changes in the concentrations of E_2, P_4 and 17α,20β-DP in early prespawning phased *H. fossilis* (**Figure 25:** $F = 192.14, 586.71, 435.99$ respectively; $P < 0.001$). In the control group a definite concentration of P_4 was noticed with very low concentrations of E_2 and 17α,20β-DP. The administration of hCG stimulated the progestin pathway involving P_4 and 17α,20β-DP with a simultaneous increase in the concentration of E_2. The administration of curcumin in hCG injected fish brought no significant change in the concentrations of E_2 and 17α,20β-DP with an increase in the

P_4 concentration as compared to the hCG injected group. However, the administration of Asc in gonadotropin injected fish induced a significant increase in P_4 and 17α, 20β-DP with a simultaneous decrease in E_2 concentration as compared hCG alone group. When gonadotrophin injected fish kept in co-incubation with the antioxidants (Cur and Asc) a significant increase (2-fold) noticed in the concentration of P_4 and 17α,20β-DP with a decreased (1-fold) E_2 concentration in comparison to the other groups of hCG. In CPF treated group, E_2 was found to be increased with a decrease in P_4 as compared to the control group. Also, in the same group a small increase was noted in the 17α,20β-DP concentration.The administration of CPF to hCG injected fish showed similar E_2 levels with a slight but significant increase in P_4 and 17α, 20β-DP in comparison to the CPF alone group.The combination of CPF, antioxidants and hCG marked a significant decrease in the E_2 but no significant change in 17α,20β-DP level as compared to the CPF and hCG group.

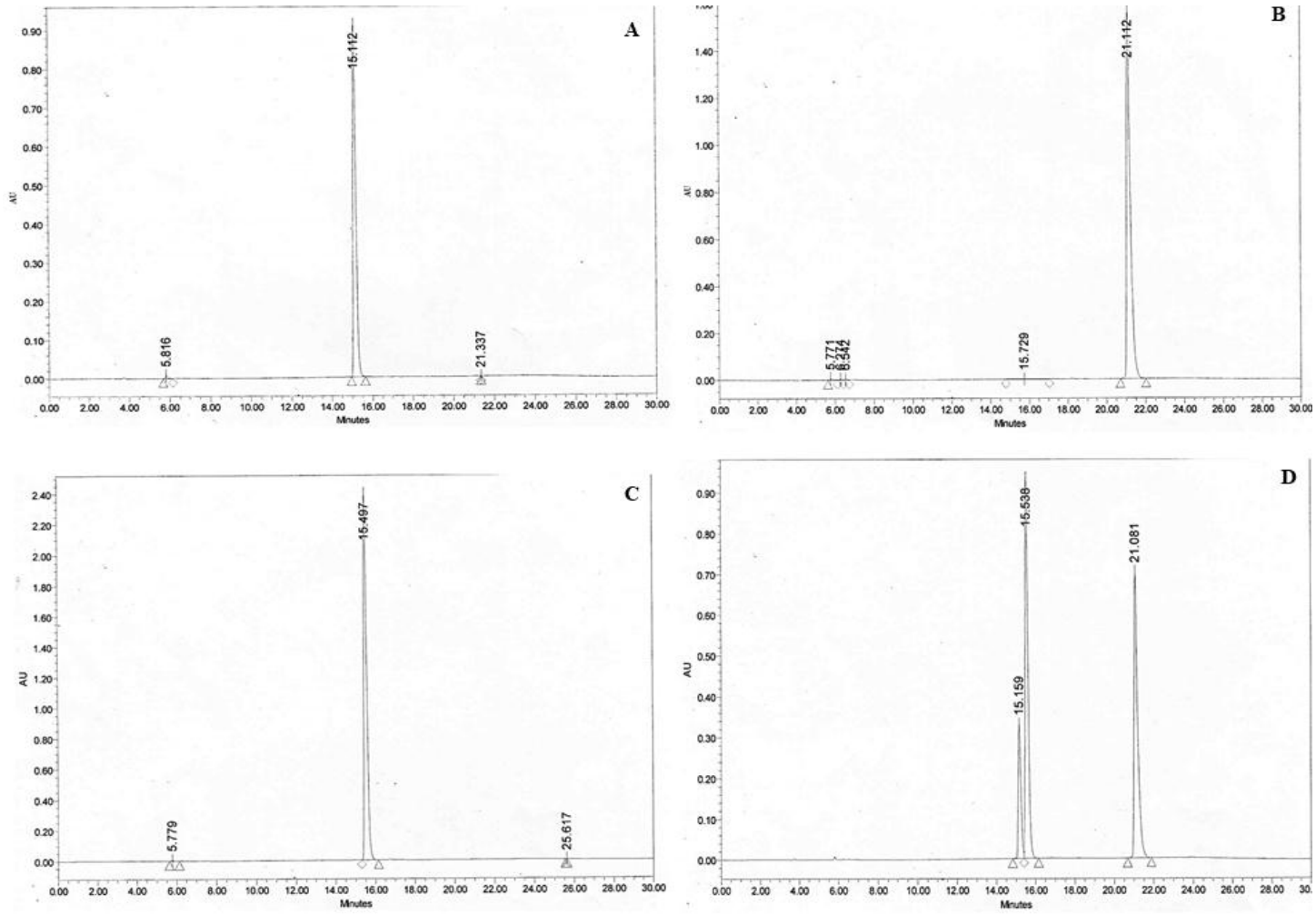


Figure 22: The typical HPLC chromatograms of standard steroids: 17β-estradiol, E_2 (**A**) 4-Pregnene-3, 20-Dione, P_4 (**B**) 17α, 20β-Dihydroxy-4-pregnen-3-one, 17α,20β-DP (**C**) Standard mix (**D**) showing their respective peaks when applied on reversed-phase HPLC (Waters 2998) on an X-Bridge C-18 column (250X4.6 mm) packed with 5µm particles size. The mobile phase used was acetonitrile-water (40:60, v/v).

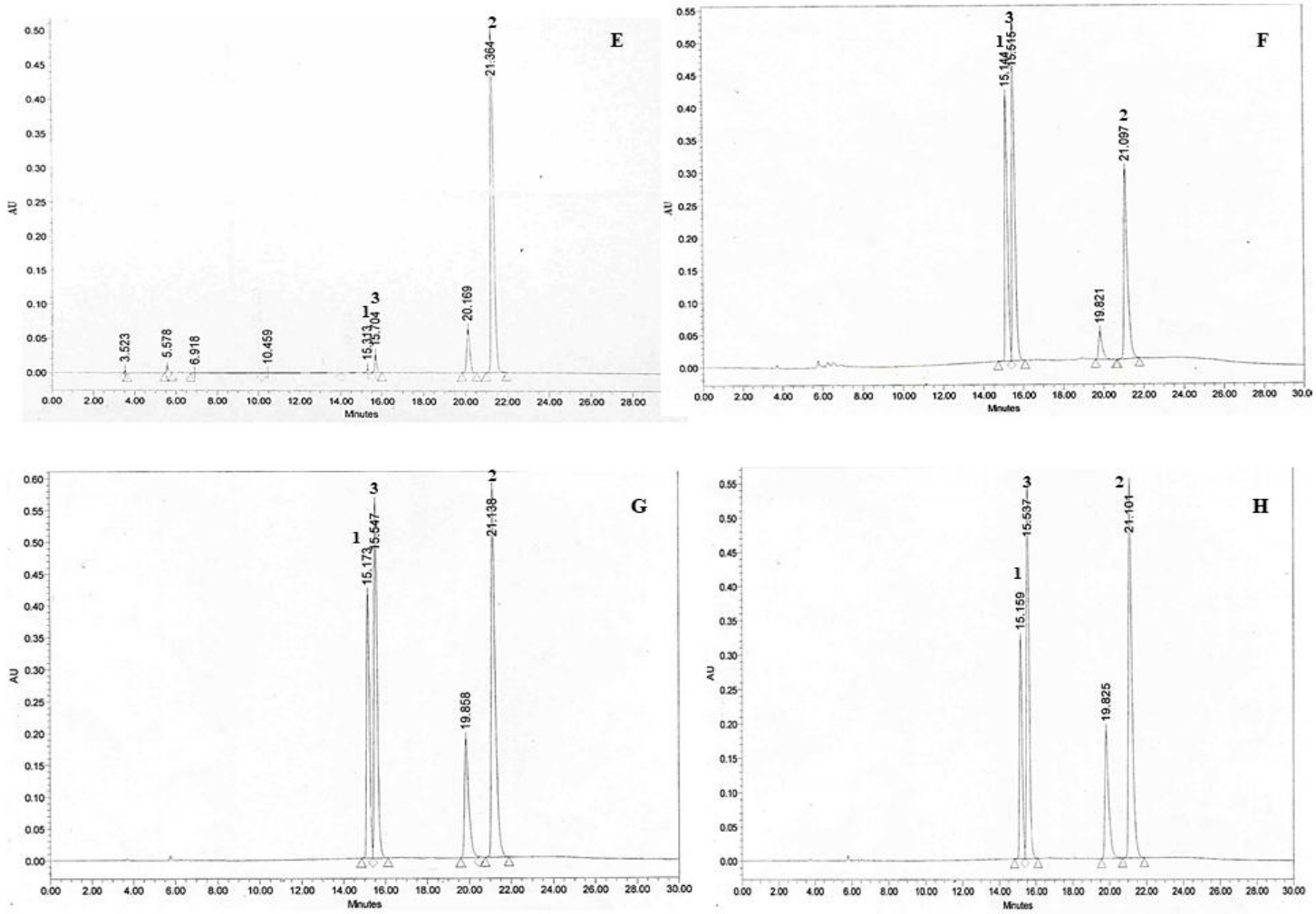


Figure 23: The typical HPLC chromatograms of control (**E**) hCG (**F**) hCG+ Cur (**G**) hCG+Asc (**H**) showing respective peaks as : **1**: 17β-estradiol (E_2); **2**: 4-Pregnene-3, 20-Dione (P_4); **3**: 17α, 20β-Dihydroxy-4-pregnen-3-one (17α 20β-DP) when applied on reversed-phase HPLC (Waters 2998) on an X-Bridge C-18 column (250X4.6 mm) packed with 5µm particles size. The mobile phase used was acetonitrile-water (40:60, v/v).

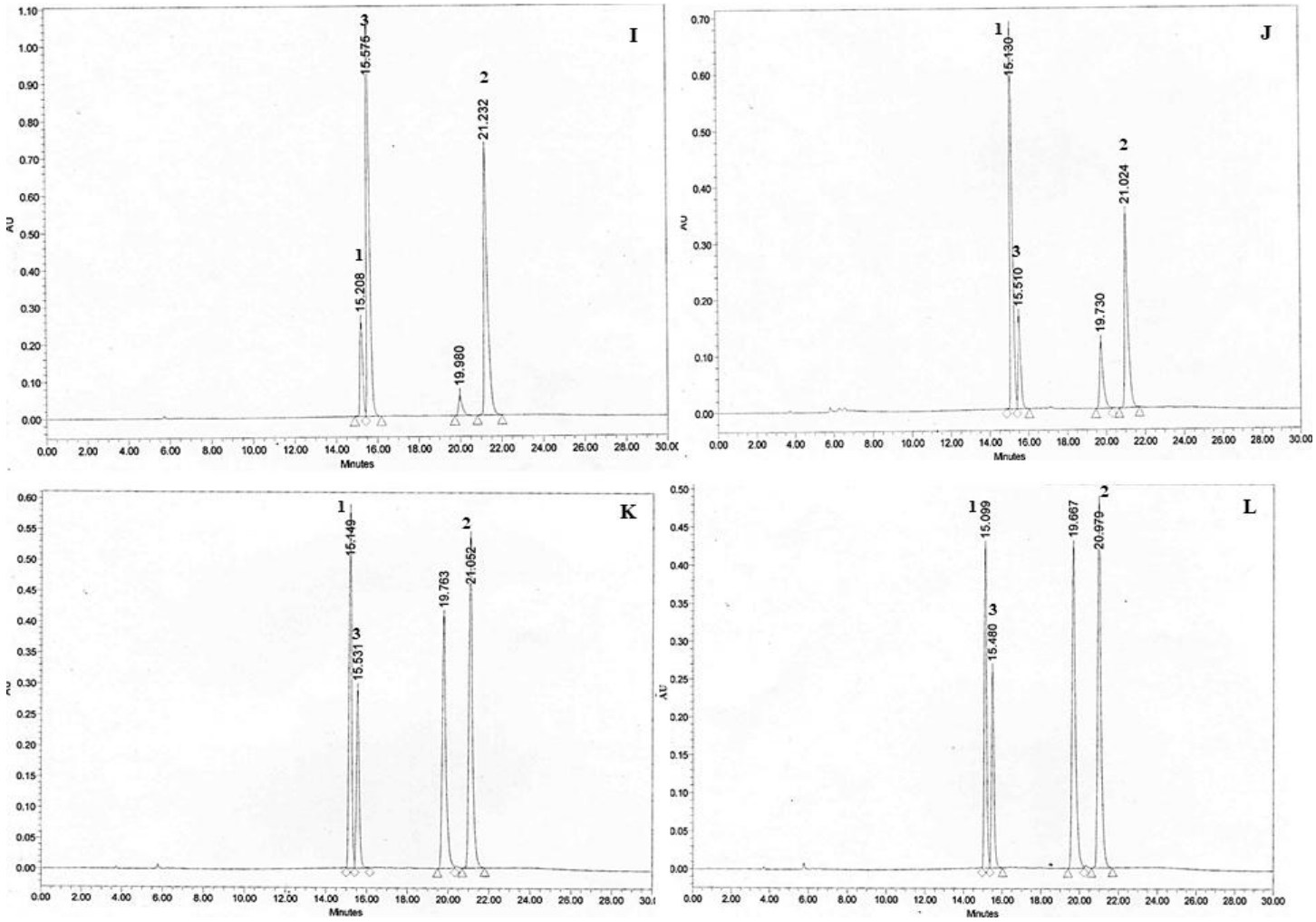


Figure 24: The typical HPLC chromatograms of hCG+Cur+Asc (**I**) CPF (**J**) CPF+hCG (**K**) CPF+hCG+Cur+Asc (**L**) showing respective peaks as : **1**: 17β-estradiol (E_2); **2**: 4-Pregnene-3, 20-Dione (P_4); **3**: 17α, 20β-Dihydroxy-4-pregnen-3-one (17α 20β-DP) when applied on reversed-phase HPLC (Waters 2998) on an X-Bridge C-18 column (250X4.6 mm) packed with 5µm particles size. The mobile phase used was acetonitrile-water (40:60, v/v).

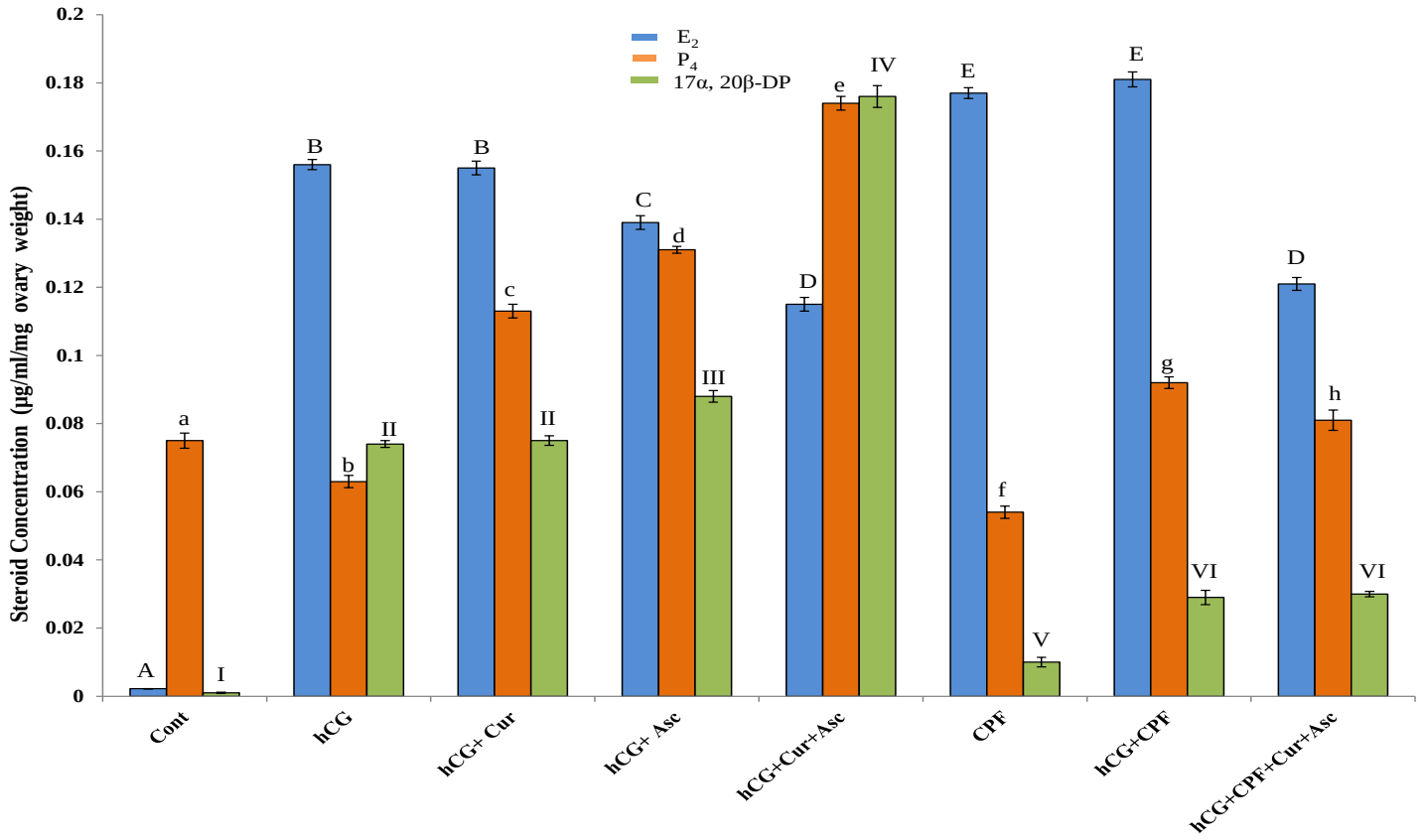


Figure 25: Effect of human chorionic gonadotropin (hCG) and chlorpyrifos (CPF) with different combination of antioxidants (curcumin, Cur and ascorbic acid, Asc) for 24 hrs on steroid concentrations (E_2: 17β-estradiol; P_4: 4-Pregnene-3,20-dione; 17α,20β-DP: 17α,20β-dihydroxy-4-pregnen-3-one) in ovary of freshwater catfish, *Heteropneustes fossilis* during prespawning period (May).Values are mean ± SEM of 3 fish in duplicates. Data were analyzed by one way ANOVA ($P<0.001$) and Newman- Kuels' test ($P<0.05$). Different symbols used to denote different steroid for intergroup comparison viz., capital letters denoted comparison for E_2, small letter for P_4 and roman number for 17α,20β-DP. Groups marked with same symbols are not significant and those with different symbols are significantly different.

2A.4 Discussion

In the present study, control group *H. fossilis* showed the presence of vitellogenin in the liver, serum and ovary owing to its prespawning period as reported earlier (Nath and Sundaraj, 1981a; Sundararaj and Vasal 1976; Lamba *et al*., 1983). The level of E_2 starts increasing during the late preparatory period, reaches maximum during the prespawning period and drop steeply during the spawning period (Sundararaj *et al.*, 1982). In catfish and many other teleosts the level of E_2 is important as it regulates the hepatic synthesis of vitellogenin. During prespawning period, increase in the vitellogenin concentration and ovarian weight (due to deposition of yolk proteins) follows the pattern similar to the E_2 (Sundararaj *et al*., 1982). During this period the pituitary of the fish actively participates in gonadotropin secretion stimulating the ovarian follicles to produce estrogen. The estrogen released promotes the hepatic synthesis of vitellogenin which are further sequestered and incorporated into the ooplasm (Nath and Sundararaj, 1981a). Also, the granulosa cells of the ovary hypertrophy and show 3β-hydroxysteroid dehydrogenase activity initiating steroidogenesis (Sundararaj and Anand, 1972). In gonadotropin injected *H. fossilis* group (II) the level of Vtg was increased significantly in the liver, serum but not in the ovary with respect to the control group I. This is because the administration of hCG is effective enough to induce Vtg synthesis by promoting estradiol secretion but do not promote the uptake of Vtg and formation of yolky oocytes as that was already being done by the intrinsic gonadotropin. Also, hCG is important in maintaining the yolky oocytes (Nath and Sundararaj, 1981a, 1981b). Pattern of change in Vtg was similar in serum as in liver that reflect change in overall Vtg synthesis and will influence yolk accumulation in growing oocytes. The hCG injected fish in presence of individual antioxidants, curcumin (group III), ascorbic acid

(group IV) and combination of antioxidants (group V) had steady level of Vtg in 24 hrs treatment. The curcumin has been reported to improve both the hepatic vitellogenin synthesis and nutrient deposition in the ovulating eggs of teleost fish improving the quality of the embryo and the larvae (Saraswati *et al*., 2013, Dewi *et al*., 2017) whereas the ascorbic acid may act as a stabilizer, protector, enhancer or inhibitor in relation to the steroid concentration in endocrine functioning (Waagbø *et al*., 1989). CPF (group VI) emerged as follicle maturation and ovulation enhancer in this, 24 hr *in vivo* study. It has been found to promote Vtg synthesis in tissues (liver and ovary) and serum. As per results, the ovary recorded increase as compared to the liver and serum due to CPF, whereas external gonadotropin increased Vtg in liver and serum, but not in ovary. Both hCG and CPF increased Vtg in liver followed by serum as compared to control group. Though increase of Vtg was less in CPF incubation as compared to the hCG injected fish. This increase was further enhanced when hCG injected fish kept in CPF water (group VII) or with Cur and Asc water (group VIII). The results interpreted that both hCG and CPF have stimulatory role in Vtg synthesis in liver that promote yolk accumulation *in vivo* condition within 24 hr. Results suggest CPF did its estrogenic function in ovary without getting influenced from the presence of gonadotropin or antioxidants alone or in combination (Yu *et al*., 2015, Grünfeld and Bonefeld-Jorgensen, 2004). This might be because chlorpyrifos is estrogenic (Ventura *et al*., 2016) and like estradiol, it initiates estrogenic effects by binding with estrogen receptors (ERα and ERβ) (Yu *et al*., 2015, Grünfeld and Bonefeld-Jorgensen, 2004).

In the present study, the teleost ovary was detected with low E_2 and 17α, 20β-DP but good detectable amount of P_4 as per their early prespawning phase (Nagahama and Yamashita, 2008). The gonadotropin injection increased the E_2 to enhance

vitellogenesis process to bring follicle toward maturation by liquidation of yolk (Sundararaj and Anand, 1972). The gonadotropin injected fish also registered increase in P_4 and 17α, 20β-DP providing maturational competence that may culminate into ovulation. This result is consistent with Degani and Boker (1992) and Kim *et al*., 2007. The hCG induced *in vitro* oocyte maturation of ovarian follicles of freshwater cyprinid, *Barilius vagra* with a significant increase in the E_2 (Zuberi *et al.,* 2011) and catfish *H. fossilis* (Mishra and Joy, 2006). The administration of hCG induces the expression of 20β-HSD (hydroxysteroid dehydrogenase) required for the production of 17α, 20β-DP, a reported MIS (Maturation inducing steroid) (Nagahama and Yamashita, 2008). The hCG injected fish in curcumin water maintains the 17α, 20β-DP production with a significant increase in the P_4. Curcumin with gonadotropin treatment has been found to improve the liver performance, increase E_2 concentration and accelerate gonadal maturation three times than the control treatments in female striped catfish *Pangasionodon hypophthalmus* (Arfah *et al*., 2018). In the same catfish, Dewi *et al*., 2017 reported curcumin to improve the transfer of hormones and nutrients to the developing oocytes. So far, there has been no thorough study eliciting the role of Cur in the gonadal maturation in fish except few to show it as assistant. Besides fish, curcumin has been found to increase the FSH, LH and estradiol levels, improving laying performance and egg quality in heat stressed hens (Liu *et al*., 2020). The hCG injected fish in Asc water, has significantly increased the P_4 and 17α, 20β-DP levels followed by a decrease in the E_2 level. The findings of Dabrowski and Ciereszko (2001) also reported stimulation of progesterone synthesis by ascorbic acid by avian and bovine ovarian cells. The shift towards MIS indicates an impressive role of ascorbic acid in gonad maturation in fish (Jiying *et al*., 2014). The combination of antioxidants with

hCG has significantly elaborated the MIS production as compared to the other groups which indicates the synergistic approach of the antioxidants (Loftsson, 2014). Dabrowski and Ciereszko (2001) reported combination of ascorbic acid with other antioxidants is necessary for the enhancement of quality of gametes. CPF has been found to stimulate the estradiol synthesis reflecting its estrogenic nature (Yu *et al*., 2015, Ventura *et al*., 2016). It also triggered P_4 and 17α,20β-DP level which might be due to the inhibition of 17α-hydroxylase or C17,20-lyase (P450c17) which leads to an increased amount of substrate available for 20β-HSD, the enzyme needed for the synthesis of 17α, 20β-DP (Thibaut and Porte, 2004). Similarly as another estrogenic compound, Bisphenol A, induces ovarian steroidogenic genes (Zhang, 2014). CPF inhibited hCG induced 17α, 20β-DP production which might be due to the suppression of C_{21} steroids within the follicle (Mishra and Verma, 2017). Similarly, the administration of CPF to the combination of hCG and antioxidants decreased the E_2 level when compared to the CPF alone. It seems, the synergistic approach of the two antioxidants was observed to maintain the hormone balance, that is, the tonic level of hormones was maintained. With hCG injected fish in CPF water alone or with antioxidants significant increase in progesterone and MIS, facilitates oocyte maturation and ovulation *in vivo* as compared to control group. CPF significantly reduced gonad major biochemicals (carbohydrate, protein and lipid) that are essential for healthy gametes (Mishra and Devi, 2020) but present study suggested it as inducer of gonad steroid that are necessary for maturation and ovulation. Because of this CPF will be resulted in the production of week ova, which can mature early *in vivo* condition in 24 hr study.

[B] Chlorpyrifos impact on gene expression linked to gonad development (*NR5A1*) in fry of catfish, *Heteropneustes fossilis*

Abstract

Chlorpyrifos (CPF), an organophosphate insecticide has been reported to induce not only neurotoxic effects but also genotoxic and mutagenic effects. However, reports claiming the role of CPF at the gene level especially in fish are very less in number. The present study identifies the effect of CPF on the *NR5A1* during the fry stage of the freshwater catfish, *Heteropneustes fossilis*. The protein encoded by the *NR5A1* gene is a transcriptional activator that plays an important role in sex determination. It regulates various steroidogenic enzymes and several genes involved in steroidogenesis, reproduction and sexual differentiation.In this study, twenty days post hatch, fish fry were exposed to CPF for 96 hrs to understand its influence on *NR5A1* gene expression pattern. CPF has been found to trigger *NR5A1* gene expression. The results clearly signals major influence of CPF in gonadal physiology in fry stage of fish.

2B.1 Introduction

Chlorpyrifos (O,O'-diethyl-O-3,5,6-trichloro-2-pyridyl phosphorothionate, CPF) is the most immensely used organophosphate insecticide against major pests in agriculture. CPF inhibits acetylcholinesterase irreversibly in the central and peripheral nervous systems of insects leading to their death. The immeasurable use of this pesticide has allowed it to reach aquatic bodies via surface runoff and tend to bioaccumulate inside the bodies of aquatic organisms including fish (Thomas and Mansingh, 2002). Despite being neurotoxic, endocrine disruptor and teratogenic, CPF has been reported to be genotoxic (Ojha *et al.*, 2011) and mutagenic (Amer and Aly, 1992). In recent studies, it has been found that CPF can bind to DNA forming DNA adducts and increase chromosomal aberrations. Such agrochemicals affect chromosomal DNA directly or indirectly inducing chronic genotoxicity and reproductive toxicity (Li *et al*., 2015). Such pesticides which are also endocrine disrupting chemicals (EDCs) could disrupt hormonal signals, producing certain products that could interfere with sex determination and differentiation processes in various aquatic organisms (Mizoguchi and Valenzuela, 2015).

In teleosts, a number of genes have been linked to sex determination or differentiation process. Sex determination is a process where the sex of a developing embryo is determined. Although, it is genetically known, it is still a delicate process to be influenced by several factors such as environmental factors including climate and temperature change or endocrine disruptors especially in sensitive organisms including fish **(Figure 26).** Despite the fact that gonadal plasticity is more common in fish than mammals, there is a need of more studies regarding sex determination mechanisms and factors controlling it (Hofsten and Olsson, 2005).

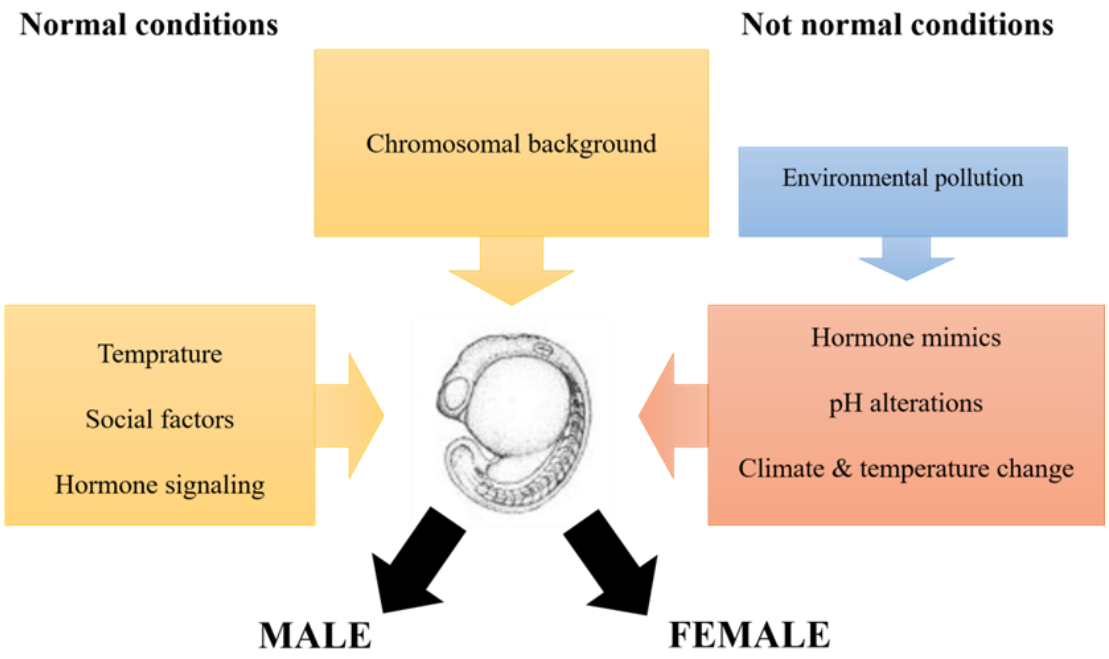


Figure 26: Factors influencing sex determination and differentiation in teleosts. Chromosomal sex determination is a default pathway, but other conditions may also influence this (Hofsten, 2004).

The *NR5A1* (nuclear receptor subfamily 5, group A, member 1) is one of the two FTZ-F1 homologues (Fushi Tarazu Factor-1) present in the mammalian genome that regulates ftz (fushi tarazu) expression. Other homologue is *NR5A2* (nuclear receptor subfamily 5, group A, member 2) which participates in cholesterol metabolism and steroid synthesis. Such genes have been isolated from several teleost species (Liu *et al.,* 1997; Watanabe *et al.,* 1999; Higa *et al*., 2000; Chai and Chan, 2000; Hofsten *et al.,* 2002). However, there are four different FTZ-F1 genes in teleosts unlike higher vertebrates which have only two. These genes are majorly involved in steroidogenesis or reproductive functions (Hofsten and Olsson, 2005). *NR5A1* is also known as steroidogenic factor-1 (SF-1), embryonal long terminal repeat-binding protein (ELP), adrenal-4- binding protein (Ad4BP) (Hofsten, 2004; Hofsten and Olsson, 2005). It regulates the transcription of genes that controls the expression of the steroidogenic cytochrome P450 enzymes in the endocrine tissues, such as the adrenal cortex, testis, and ovary (Sakai *et al.*, 2008) and metabolism (Chai and Chan, 2000). Ijiri *et al.* (2008) reported about the important role of *NR5A1* in the transcriptional regulation of *cyp19a1a* in the vitellogenic follicle of teleost fish, *Oreochromis niloticus*. Cyp19a1a converts androgens into estrogens, a rate limited step in estrogen synthesis (Zhang *et al.*, 2014). Besides this, it is also involved in the sex determination and differentiation in vertebrates including mammals and fish (Hofsten and Olsson, 2005, Cheshenko *et al.*, 2008). *NR5A1* plays a crucial role in regulating the balance between androgen and estrogen production during steroid biosynthesis which is essential for proper development of sex characteristics (Hofsten and Olsson, 2005). Critical period for

gonad differentiation usually starts in catfish 30-40 days post hatch. In this duration sex specific gene expression starts to multiply (Raghuveer *et al*., 2011). So far, there has been no report on analyzing the impact of pesticide on gonadal differentiation.

The larval stages of fish (fry or fingerlings) act as sensitive biomarkers of environmental contamination. Since, the Indian catfish, *H. fossilis* is the most demanded freshwater catfish because of its nutritive qualities and culture potential and previous studies reflected the toxic effects of CPF on *H. fossilis,* the fry of this catfish was chosen for gene expression study. The present study is an attempt to understand the interference of the CPF with the *NR5A1* gene expression in *H. fossilis*.

2B.2 Materials and Methods

2B.2.1 Animal collection and their acclimatization

For *NR5A1* expression study, live and healthy fry of *H. fossilis* (20 days post hatch, dph; 6±1 cm) was procured from the local hatchery in Lucknow. The fry after bringing to the laboratory carefully, were treated with 0.5% potassium permanganate to prevent dermal infection and allowed to acclimatize for three days in laboratory under normal photoperiod and temperature (12h: 12h; light: dark and 25 ± 2^0 C). The water was renewed at every two hours in order to maintain oxygen levels and to remove faecal matter and metabolic wastes accumulated during acclimatization. They were fed with minced boiled egg *ad libitum*. After acclimatization, the fish fry were divided into three groups of three each in duplicates. The group I was a control containing only fresh water and in group II and III, the fry were exposed to CPF (0.174µM/l, $1/10^{th}$ of 96 hrs LC_{50}; Mishra and Verma, 2016) for 24 and 96 hrs respectively. At the end of the

experimental duration, the fry were snap-freezed in liquid N_2 and given to Acube LifeSciences Private, Lucknow for expression study of *NR5A1* gene.

National guidelines of the ethical committee were followed to exhibit experiments and to avoid any cruelty to the animals.

2B.2.2 Procedure for gene expression study

Total RNA was isolated from each sample by Trizol method and 1000 ng was used for cDNA synthesis using Reverse transcriptase (Thermo scientific) following the manufacturer's protocol. The RNA and Oligo dT/ Random hexamer primers were used for the first strand of cDNA synthesis. Primer sequences are mentioned in **Table 4.** The Real Time PCR reaction mixture of 20 µl containing 10 µl of SYBR Green Supermix (Bio Rad, USA) and 2 µl of cDNA was run for 35 cycles followed by Denaturation at 95ºC for 30sec, Annealing 55ºC (Gradient) for 30sec in Bio-Rad CFX96 system. The mRNA expression levels were normalized to that of Housekeeping gene (GAPDH) and the results were analyzed against the control group.

2B.2.3 Statistical analysis

Data of samples were represented as means ± SEM. The significance of values obtained from different groups was tested by using one-way analysis of variance (ANOVA). The intergroup comparisons were done by Newman-keuls' test ($P<0.05$).

Table 4: Primer sequences used in the *NR5A1* expression study

Experiment	**Gene**	**Direction**	**Sequence 5'-3'**
Gene specific primer for qPCR study	*NR5A1*	Forward primer	ATGCTGGAAGCGCAGAGC
		Reverse primer	AATGGTGGTCGCGCGTTT
Housekeeping gene	*GAPDH*	Forward primer	ACCCACTCCTCCACCTTTGA
		Reverse primer	CTGTTGCTGTAGCCAAATTCGT

2B.3 Results

The 20 dph fry of *H. fossilis* exposed to sublethal dose of CPF for 96 hrs showed 5-fold increase in the *NR5A1* expression as compared to the control group but there was no change in gene expression level at 24 hrs duration study **(Figure 27** ; F= 5.39, P< 0.05).

2B.4 Discussion

The sublethal dose of CPF increased *NR5A1* gene expression multi folds in a duration dependent manner among young fry. The gene expression was significantly increased in 96 hrs duration study as compared to the 24 hrs duration study. The *NR5A1* is an activator of important steroidogenic enzymes including cytochrome P-450 aromatase (P-450arom) (Ijiri *et al.,* 2008). P-450arom catalyzes testosterone to 17β-estradiol, playing an important role in ovarian development. In ovarian follicles of *Oryzias latipes*, *NR5A1* has been found to regulate P-450arom at the transcriptional level (Watanabe *et al.,* 1999). This is similar to the findings of Fan *et al*. (2007) in human cancer cell lines where atrazine, a common pesticide and also a potent endocrine disruptor was found to increase the aromatase expression by inhibiting phosphodiesterase. This results in increased cAMP levels which further leads to elevated transcription of aromatase *CYP19* gene (cytochrome P450, family 19, subfamily A, polypeptide 1 (CYP19A1) and hence, increased estrogen production. Increase in cAMP further increases the gene expression. Since *NR5A1* is a member of orphan receptor group that regulates transcription constitutively but its activity is increased after binding to ligands (Fan *et al.,* 2007). CPF like bisphenol A, another endocrine disrupting chemical, has a potential to disrupt endocrine functions by altering normal endogeneous hormone levels (Ventura *et al.,* 2012, Andersen *et al.,* 2002, Kojima *et al.,*2004, Grünefeld and Bonefeld-Jorgensen,

2004). In *Gobiocypris rarus*, bisphenol A has been found to mediate its actions on steroidogenesis by involving androgen and estrogen receptor signaling, *NR5A1* pathway and epigenetic regulation (Zhang *et al.*, 2014). In the present study, CPF like bisphenol A, seems to be altering the mRNA expression level of *NR5A1* and epigenetic regulation (Zhang *et al.*, 2014, Cheshenko *et al.*, 2008). In granulosa cells of mice, *NR5A1* has reported to stimulate *cyp 17a1* expression by binding to its promoter regions. Cyp 17a1 (17α-hydroxylase or 17,20-lyase) is an important enzyme for androgen formation (Park *et al.*, 2010b). *NR5A1* is a member of orphan receptor family involved in the regulation of oocyte development and ovulation. It gets activated by pesticides and other xenobiotic substances. Since *NR5A1* regulates various ovarian genes including P450scc (CYP11A1), 3β HSD (3β hydroxysteroid dehydrogenase), P450c17 (17α hydroxylase or 17, 20 lyase and P450 aromatase (estrogen synthase), any alteration in its expression might affect ovarian cycle (Mlynarczuk and Rekawiecki, 2010). In the same study they also reported about the stimulation of *NR5A1* by estradiol in mice. This also elicits about the role of estrogen mimics which can influence *NR5A1* expression and hence reproductive processes. The stimulation of *NR5A1* expression by such mimics might trigger unwanted alterations in the regulation of reproductive processes in humans and animals. Also, the intereference in the normal *NR5A1* expresssion by environmental contaminants might have profound effects on gonad development and differentiation not only in fish but across various range of animal species. In developing gonads of American bullfrog, *R. catesbeiana* exposure to estrogenic chemicals has been found to alter *NR5A1* levels and accelerate gonadal differentiation. The *NR5A1* levels was found to be increased which suggests that exposure to endocrine disrupting chemicals disrupts estrogen dependent gonadal differentiation in amphibians (Wolff *et al.*, 2015).

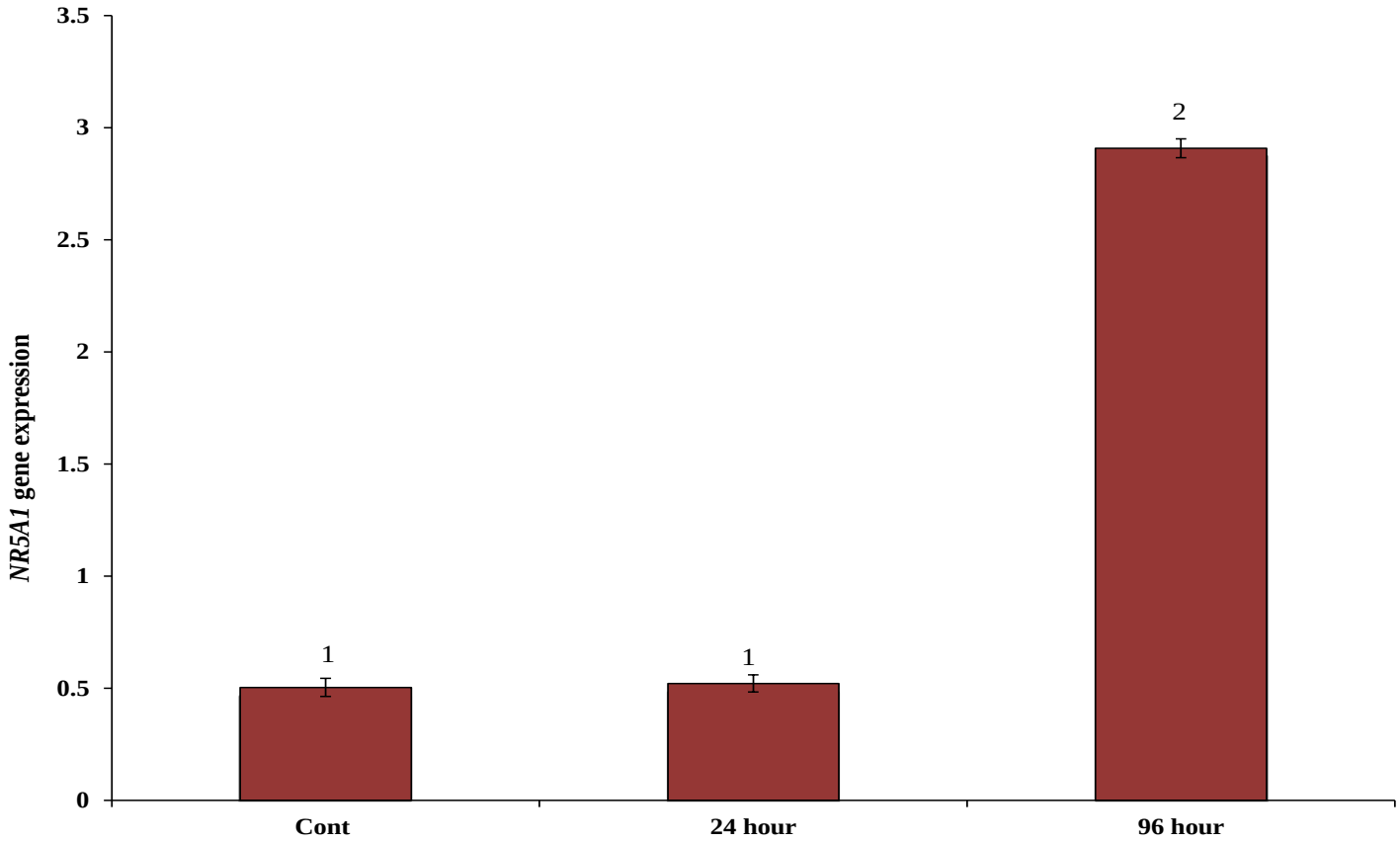


Figure 27: Effect of chlorpyrifos (0.174 µM/l) on the *NR5A1* expression in 20 day fry of freshwater catfish, *Heteropneustes fossilis*. Values are mean ± SEM of 3 fish in duplicates. Data were analyzed by one way ANOVA ($P < 0.001$) and Newman - Kuels' test ($P < 0.05$). Groups marked with same number are not significant and that with different number is significantly different in intergroup comparison.

The present study suggests that CPF support early onset of gonad differentiation which may leads to early sexual maturity in fish. This might result in poor fecundity power and week gamete development as compared to the healthy environment fish. Long term duration study is required further to set a trend for CPF impact on catfish reproductive physiology.

Consolidated Summary and Conclusion

Consolidated Summary and Conclusion

In agricultural countries like India, a major percentage of crops are eaten away by various pests. In order to prevent huge economic loss, usage of pesticides during farming practices has become a necessity. The increased production of food has also led to increased demand of various kinds of pesticides. One such pesticide is chlorpyrifos (CPF) which comes under the category of organophosphates (OPs). Although CPF is best used for controlling pests of various fruits, vegetables and nuts; its extensive usage has led to the contamination of soil, air, surface water and ground water. The increasing use of CPF especially in India is of major concern as it is not only responsible for death of several aquatic organisms including fish but have also gained the potential to enter into human body via food chain. To understand the extent of toxicity of such pesticide on tissue architecture and normal physiological processes, researchers around the globe picked up several model organisms to understand the toxicity of CPF or similar pesticide. Among all the research model organisms, fish is considered to be a best model to understand pesticide exposure effect on humans. The more CPF is sprayed over the crops; more it tends to bioaccumulate in aquatic organisms via surface runoff. Fish, an ideal marker for water pollution, eaten by majority of communities, therefore, it becomes essential to explore and study the toxicity impact of CPF by taking fish as a research model. The exploration of various toxicity levels of CPF on fish gives an important indication of its impact on humans and other non-target organisms.

In the present study, *Heteropneustes fossilis* has been chosen as an ideal research model to study the toxicity of CPF by seeing its high demand especially in Indian markets. People prefer eating this fish because of its taste, nutritional values and medical reasons.

There have been various reports to mention about the bioaccumulation of CPF in *H. fossilis* but none has described what extent CPF can hamper the normal physiology of fish. In vertebrates important physiological processes are interconnected which means abnormalities in the functioning of one system might affect the functioning of other systems. This work is an effort to analyze important toxicity levels of CPF on *H. fossilis* which includes enzymatic, biochemical, histological, hormonal and genetic levels. Each of these levels plays an important role in providing a suitable environment for proper gonad development and maturation in fish. In addition, the research work also focuses on the different combinations of bioremedials to attenuate CPF toxicity.

In the present study, the first chapter dealt with different parameters to evaluate toxicity of sub-lethal concentration of CPF on antioxidant enzymes (catalase, peroxidase), lipid peroxidation, biochemical parameters (glycogen, protein, lipid) and histology of major tissues such as liver, kidney, gill, stomach and intestine. Antioxidant enzymes are important defense mechanisms to fight stress whereas glycogen, protein and lipid are not only source of energy but are also vital for vitellogenesis and gonadal development. The sub-lethal dose of CPF induced a significant increase in antioxidant enzymes (catalase, peroxidase) and lipid peroxidation level in liver, kidney and gill indicating oxidative stress. This induced stress was alleviated by the introduction of natural antioxidants [Ascorbic acid (Asc); Curcumin (Cur); Chitosan (Chi)] as a bio-remedial when combined with CPF. However, the single antioxidant impact was found to be less as compared to the dual combinations of antioxidants. Among all the groups, the combination of Cur and Asc was found to be most effective in reducing oxidative stress as it brings down the antioxidant enzyme level. CPF also decreased the glycogen and protein content and increased the total lipid content in major tissues of fish. The

decrease in the glycogen and protein content explains immediate energy requirement to avoid unhealthy and toxic effects of CPF on normal metabolism of fish whereas increase in the lipid content asserts the estrogenic role of CPF. This effect was also supported by the tissue histological study. The combination of Asc and Cur reduced the CPF induced damage to liver, kidney, gill, stomach and intestine. These results strongly suggests that the dual combination of Cur and Asc act in a synergistic manner to attenuate CPF induced stressed/toxic effect on antioxidant enzymes, biomolecular content and internal anatomy of the vital tissues.

In the second chapter, CPF impact on vitellogenin, hormonal levels and gene expression of *NR5A1* was studied. Vitellogenins (Vtgs) are glycolipophosphoproteins essential for the successful development of oocytes and embryos of oviparous vertebrates, including fish. Steroids such as 17β-Estradiol (E_2), 4-Pregnene-3, 20-dione (P_4), 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DP) play an important role in the regulation of oocyte growth and maturation whereas *NR5A1* gene not only controls the expression of the steroidogenic cytochrome P450 enzymes in the endocrine tissues but is also involved in the sex determination and differentiation in vertebrates including mammals and fish. These three (vitellogenin, steroid levels and *NR5A1* gene) are important key points in deciding proper development and reproductive fitness in fish. The involvement of antioxidants (Cur, Asc) and their impact on the Vtg and hormone levels is explained in the present research work. This study is for the first time where an interplay of CPF, human chorionic gonadotropin (hCG) and antioxidants (Cur and Asc) were studied on the steroidogenesis. The *NR5A1* gene expression was studied with and without CPF in fry of freshwater catfish, *Heteropneustes fossilis*. The study concludes that CPF is an endocrine disruptor with a potential to perturb sensitive steroidal and

genetic pathway that regulate reproductive functions. It induces increased vitellogenesis in fish which may lead to decreased fertility and egg production in females, or lead to reduced gonad size or feminization of genetic male fish. The antioxidants act as a balancer to chlorpyrifos induced toxicity, maintains the tonicity of hormones. Thus, the excess use of the pesticide has deterimental effect both at the steroidal and genetic level in aquaculture.

Thus, the present work is an effort to draw the attention towards the hazardous effect of pesticide, chlorpyrifos on catfish, *Heteropneustes fossilis*. There is a need of awareness among the people who perform agriculture practices to optimize the usage of CPF in order to protect environment and the biota who could come in contact with toxicant.

References

References

Abhijith, B. D., Ramesh, M., R.K. Poopal, R. K. (2016). Responses of metabolic and antioxidant enzymatic activities in gill, liver and plasma of *Catla catla* during methyl parathion exposure. *JOBAZ.*, **77**: 31-40.

Aebi, H. (1984). Catalase in vitro. *Methods Enzymol*. **105**: 121-126.

Al-Otaibi, A. M., Al-Balawia, H. F. A., Ahmada, Z and Sulimana, E. M. (2018). Toxicity bioassay and sub-lethal effects of diazinon on blood profile and histology of liver, gills and kidney of catfish, *Clarias gariepinus*. *Braz. J. Biol*., **79(2)**: 326-336.

Ali, D., Nagpure, N.S., Kumar, S., Kumar, R., Kushwaha, B. (2008). Genotoxicity assessment of acute exposure of chlorpyrifos to freshwater fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell electrophoresis. *Chemosphere,* **7**: 1823-31.

Ali, M. F., Rahman, M. M., Bashar, M. K., Rahmatullah, R., Hadiuzzaman, M. and Amin, M. R. (2014). Comparative study on induced breeding of shing, *Heteropneustes fossilis* (Bloch) between HCG and PG with different combination. *Int. J. Fish Aquat. Stud*., **2(2)**: 104-108.

Almedia, M. G., Fanini, F., Davino, S., Aznar, A. E., Koch, O. R., and Barros, S. B. M. (1997). Pro and antioxidant parameters in rat liver after short-term exposure to hexacholorobenzene. *Hum. Exp. Toxicol.,* **16**: 257–261.

Almeida, J. A., Diniz, Y. S., Marques, S. F. G., Faine, L. A., Ribas, B. O., Burneiko, R. C., Novelli, E. L. B. (2002). The use of the oxidative stress responses as biomarkers in Nile tilapia (*Oreochromis niloticus*) exposed to in vivo cadmium contamination. *Environ. Int*., **27**: 673–679.

Alok, D., Krishnan, T., Talwar, G. P., and Garg, L. C. (1993). Induced spawning of catfish, *Heteropneustes fossilis* (Bloch), using D-Lys^{6} salmon gonadotropin-releasing hormone analog. *Aquaculture*, **115**: 159-167.

Alves, S. R. C., Severino, P. C., Ibbotson, D. P., da Silva, A. Z., Lopes, F. R. A. S., Sa´enz, L. A., Bainy, A. C. D. (2002). Effects of furadan in the brown mussel *Perna perna* and in the mangrove oyster *Crassostrea rhizophorae. Mar. Environmen. Res*., **54**: 241–245.

Aly, N., Gendy, K. E. L.,Mahmoud, F. and Sebae, A. K. (2010). Protective effect of vitamin C against chlorpyrifos oxidative stress in male mice. *Pestic. Biochem. Phys*. **97(1)**: 7-12.

Amer, S.M. and Aly, F.A.E. (1992). Cytogenic effects of pesticides. IV. Cytogenic effects of the insecticides Gardona and Dursban. *Mutat. Res.,* **279**: 165-70.

Andersen, H.R.,Vinggaard, A.M., Rasmussen, T.H., Gjermandsen, I.M., Bonefeld-Jorgensen, E.C. (2002). Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity in vitro. *Toxicol. Appld. Pharmacol.*, **179**: 1-12.

Anderson, D., Prasad, K., Stewart, R. (1995). Changes in isozyme profiles of catalase, peroxidase and glutathione reductase during acclimation to chilling in mesocotyls of maize seedlings. *Plant Physiol*., **109**: 1247–1257.

Antonious, F. G., Turley, T.E., Abubakari, M., Snyder, C.J. (2016). Dissipation, half-lives, and mass spectrometric identification of chlorpyrifos and its two metabolites on field-grown collard and kale. *J. Environ. Sci. Heal. B.,* 1–5.

Aktar, M.W., Sengupta, D., Chowdhury, A. (2009). Impact of pesticides use in agriculture: their benefits and hazards. *Interdisc. Toxicol.*, **2(1)**: 1–12.

Amer, S. M. and Aly, F. A. E. (1992). Cytogenetic effects of pesticides. IV. Cytogenetic effects of the insecticides Gardona and Dursban. *Mutat. Res*., **279**: 165-170.

Arfah, H., Sudrajat, A.O., Supriyadi, M.A., Zairin, M. (2018). Gonad maturation of female striped catfish *Pangasionodon hypophthalmus* (Sauvage, 1878) using combination of pregnant mare serum Gonadotropin+Antidopamine, Vitamin E, and curcumin extract mixed feed outside its spawning season. *Int. J. Fish Aquat. Stud*., **6(5)**: 52-57.

Arrigoni, O. and Tullio, M. C. D. (2002). Ascorbic acid: much more than just an antioxidant. *Biochim. Biophys. Acta,* **1569***:* 1-9.

Avelelas, F., Horta, A., Pinto, L. F.V., Marques, S. C., Nunes, P. M., Pedrosa, R., Leandro, S. M. (2019). Antifungal and antioxidant properties of chitosan polymers obtained from nontraditional *Polybius henslowii* sources. *Mar. Drugs*, **17**: 239.

Banerjee, B. D., Seth, V., Bhattacharya, A., Pasha, S. T., and Chakraborty, A. K. (1999). Biochemical effects of pesticides on lipid peroxidation and free radicals scavengers. *Toxicol. Lett.* **107**: 33–77.

Banerjee, B. D. (2001). Pesticide-induced oxidative stress: perspectives and trends. *Rev. Environ. Health.* **16**:1–40.

Barbieri, E. and Ferreira, L. A. A. (2011). Effects of the organophosphate pesticide Folidol 600® on the freshwater fish, Nile tilapia *(Oreochromis niloticus). Pestic. Biochem. Phys.* **99(3):** 209-214.

Barron, M. G. and Woodburn, K. B. (1995). Ecotoxicology of Chlorpyrifos. In *Reviews of Environmental Contamination and Toxicology,* **144**: 1-93.

Bebe, F. N. and Panemangalore, M. (2003). Exposure to low doses of endosulfan and chlorpyrifos modifies endogenous antioxidants in tissues of rats. *J. Environ. Sci. Health B*, **38(3)**: 349-363.

Begum, G. (2005). Toxicity of cypermethrin on total lipids and free fatty acids in liver, muscle, kidney and ovary of *Clarias batrachus* (L) and recovery response. *Toxicol. Environ. Chem.* **87(2):** 253-260.

Bernardes, F.F. M., Pazin, M., Pereira, C.L., Dorta, D.J. (2015). Impact of Pesticides on Environmental and Human Health. In: Toxicology Studies - Cells, Drugs and Environment (Ana Cristina Andreazza and Gustavo Scola eds). IntechOpen Ltd., London, U.K., 195-233.

Beyer, R. E. (1994). The role of ascorbate in antioxidant protection of biomembranes: Interaction with vitamin E and coenzyme Q. *J. Bioenerg. Biomemb*., **26 (4)**: 349-358.

Bhardwaj, T. and Sharma, J.P. (2013). Impact of pesticides application in agricultural industry: An Indian scenario. *Int. J. Res. Agric. Food Sci.,* **4 (8)**: 817-822.

Bhattacharyya, S., Sen, U., Bhattacharyya, S.P., Mukherjee, D. (2002). High performance liquid chromatographic separation of steroids from ovarian follicles of fresh water perch *Anabas testudineus:* Identification and Characterization of the Maturation-Inducing Hormone. *J. Exp. Zool*., **292**: 565–572.

Bindhumol, V., Chitra, K. C., Mathur, P. P. (2003). Bisphenol A induces reactive oxygen species generation in the liver of male rats. *Toxicology*, **188**: 117-124.

Blom, J. H. and Dabrowski, K. (1995). Reproductive success of female Rainbow trout (*Oncorhynchus mykiss*) in response to graded dietary ascorbyl monophosphate levels. *Biol. Reprod*., **52**: 1073-1080.

Bradberry, M. S., Cage, A.S., Proudfoot, A.T., Vale, A. J. (2005). Poisoning due to pyrethroids. *Toxicol.Rev.*, **24 (2)**: 93-106.

Bradley, J.T. and Grizzle, A.M. (1989). Vitellogenin induction by estradiol in Channel catfish, *Ictalurus punctatus. Gen. Comp. Endocrinol*., **73**: 28-39.

Braquenier, J.B., Quertemont, E., Tirelli, E., Plumier, J.C. (2010). Anxiety in adult female mice following perinatal exposure to chlorpyrifos. *Neurotoxicol. Teratol*., **32**: 234-239.

Camargo, M. M. P. and Martinez, C.B.R. (2007). Histopathology of gills, kidney and liver of a neotropical fish caged in an urban stream. *Neotrop. Ichthyol*., **5(3)**: 327-336.

Canario, A.V.M., Scott, A.P. (1990). Effects of steroids and human Chorionic gonadotrophin on in vitro oocyte final maturation in two marine flatfish: The Dab, Limanda limanda, and the Plaice, *Pleuronectes platessa. Gen. Comp. Endocrinol*., **77**: 161-176.

Canesi, L., Negri, A., Barmo, C., Banni, M., Gallo, G., Viarengo, A. and Dondero, F. (2011). The organophosphate chlorpyrifos interferes with the responses to 17b-estradiol in the digestive gland of the Marine mussel *Mytilus galloprovincialis. PLoS One.*, **6(5)**: e19803.

Cao, L., Ding, W., Du, J., Jia, R., Liu, Y., Zhao, C., Shen, Y., Yin, G. (2015). Effects of curcumin on antioxidative activities and cytokine production in Jian carp (*Cyprinus carpio* var. Jian) with CCl4-induced liver damage. *Fish Shellfish Immun.* **43**: 150-157.

Carmel-Harel, O., and Storz, G. (2000). Roles of the glutathione- and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces Cerevisiae* responses to oxidative stress. *Annu. Rev. Microbiol.*, **54**: 439–61.

Cengiz, E.I. and Unlu, E. (2003). Histopathology of gills in mosquitofish, *Gambusia affinis* after long-term exposure to sublethal concentrations of malathion. *J. Environ. Sci. Health. B*. **38 (5)**: 581–589.

Cengiz, E. I. and Unlu, E. (2006). Sublethal effects of commercial deltamethrin on the structure of the gill, liver and gut tissues of mosquito fish, *Gambusia affinis*: A microscopic study. *Environ. Toxicol. Pharmacol*., **21**: 246–253.

Chai, C. and Chan, W.K. (2000). Developmental expression of a novel Ftz-F1 homologue, ff1b (NR5A4), in the zebrafish *Danio rerio*. *Mech. Dev*., **91**: 421-426.

Chelikani, P., Fita, I., and Loewen, P.C. (2004). Diversity of structures and properties among catalases. *Cell. Mol. Life Sci.,* **61**: 192–208.

Cheng, G., Salerno, J.C., Zehong Cao, Z., Pagano, P. J., Lambeth, J. D. (2008). Identification and characterization of VPO1, a new animal heme-containing peroxidase. *Free Radic. Biol. Med*., **45**: 1682–1694.

Cheshenko, K.,Pakdel, F., Segner, H., Kah, O., Eggen, R.I.L. (2008). Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. *Gen. Comp. Endocrinol*., **155**: 31–62.

Choi, K., Joo, H., L., Rose, Hodgson, E. (2006). Metabolism of chlorpyrifos and chlorpyrifos oxon by human hepatocytes. *J. Biochem. Mol. Toxicol*, **20(6)**.

Chiapella, G., Flores-Martín, J., Ridano, M. E., Reyna, L., De Potas, G.M., Panzetta-Dutari, G.M., Genti-Raimondi, S. (2013). The organophosphate chlorpyrifos disturbs redox balance and triggers antioxidant defense mechanisms in JEG-3 cells. *Placenta*, **34**: 792-798.

Clasen, B., Loro, V. L., Murussi, C. R., Tiecher, T. L., Moraes, B., Zanella, R. (2018). Bioaccumulation and oxidative stress caused by pesticides in *Cyprinus carpio* reared in a rice-fish system. *Sci. Total Environ.*, **626**: 737–743.

Čolović, M.B., Krstić, D. Z., Lazarević-Pašti, Bondžić, A.M. and Vasić, V. M. (2013). Acetylcholinesterase Inhibitors: Pharmacology and toxicology. *Curr. Neuropharmacol.*, **11**: 315-335.

Covarrubias, L., García, D.H., Schnabel, D., Vidal, E. S., Obregón, S. C. (2008). Function of reactive oxygen species during animal development: Passive or active? *Dev. Biol.*, **320**: 1-11.

Cui, Y., Guo, J., Bujin Xu, B. and Chen, Z. (2011). Genotoxicity of chlorpyrifos and cypermethrin to ICR mouse hepatocytes. *Toxicol. Mech. Methods*., **21(1)**: 70-74.

Cristina, S. A., Cristina, M. M., Anca, D. (2008). Malathion induced histological modifications in gills and kidney of *Carassius auratus gibelio*. *Lucrări stiinŃifice Zootehnie si Biotehnologii*, **41(1)**: 448-453.

D'Agostino, J., Zhang, H., Kenaan, C. and Hollenberg, P. F. (2015). Mechanism-based inactivation of human cytochrome P450 2B6 by chlorpyrifos. *Chem. Res. Toxicol*., **28(7)**: 1484-1495.

Dabrowski, K. and Ciereszko, A. (2001). Ascorbic acid and reproduction in fish: endocrine regulation and gamete quality. *Aquac. Res*., **32**: 623-638.

Das, S. and Gupta, A. (2013). Histopathological changes in the intestine of Indian flying barb (*Esomus danricus*) exposed to organophosphate pesticide, Malathion (EC 50). *G.J B.A.H.S*.,**2 (2)**: 90-93.

Datta, M. and Kaviraj, A. (2003). Ascorbic acid supplementation of diet for reduction of deltamethrin induced stress in freshwater catfish *Clarias gariepinus*. *Chemosphere*, **53**: 883–888.

Dautremepuits, C., Paris-Palacios, S., Betoulle, S., Vernet, G. (2004). Modulation in hepatic and head kidney parameters of carp (*Cyprinus carpio L.*) induced by copper and chitosan. *Comp. Biochem. Physiol.C. Toxicol. Pharmacol.*, **137(4):** 325-333.

De Silva, P. M. C. S. and L. A. Samayawardhena, L.A. (2002). Low concentrations of lorsban in water result in far reaching behavioral and histological effects in early life stages in Guppy. *Ecotoxicol. Environ. Safety*, **53**: 248-254.

De Vlaming, L. V. (1972). Environmental control of teleost reproductive cycles: a brief review. *J. Fish Biol.* **4**: 131-140.

Degani, G. and Boker, R. (1992). Sensitivity to maturation-inducing steroids and gonadotropin in the oocytes of Blue gourami *Trichogaster trichopterus* (Anabantidae, Pallas, 1770). *Gen. Comp. Endocrinol.,* **85**: 430-439.

Deisseroth, A. and Dounce, A.I. (1970). Catalase: Physical and chemical properties, mechanism of catalysis, and physiological role. *Physiol. Rev*., **50 (3)**: 319-375.

Dennery, P.A. (2010). Oxidative stress in development: Nature or nurture? *Free Radical Bio. Med.,* **49**:1147-1151.

Deb, N. and Das, S. (2013). Chlorpyrifos toxicity in fish: A Review. *Curr. World Environ.,* **8(1)**: 77-84.

Deka, S. and Mahanta, R. (2012). A study on the effect of organophosphorus pesticide malathion on hepato-renal and reproductive organs of *Heteropneustes fossilis* (Bloch). *The Sci. Probe*, **1(1)**: 1-13.

Devi, Y. and Mishra, A. (2013). Study of behavioural and morphological anomalies of fry fish of fresh water teleost, *Channa punctatus* under chlorpyrifos intoxication. *Int. J. Pharm. Bio. Sci*., **4(1)**: 865-874.

Devlin, R. H. and Nagahama, Y. (2002). Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture*, **208**: 191–364.

Dewi, C.D., Ekastuti, D.R., Sudrajat, A.O., Manalu, W. (2017). Improved vitellogenesis, gonad development and egg diameter in catfish (*Pangasianodon hypophthalmus*) supplemented with turmeric (*Curcuma longa*) powder. *Aquac. Res*., **49(2)**: 651-658.

Dorooshi, G. (2012). Catfish stings: A report of two cases. *J. Res. Med. Sci*., **17(6)**: 578-581.

Duarte, T. and Lunec, J. (2005). Review: When is an antioxidant not an antioxidant? A review of novel actions and reactions of vitamin C. *Free Radic. Res*., **39(7)**: 671–686.

Eaton, D. L., Daroff, R.B., Autrup, H., Bridges, J., Buffler, P., Costa, L. G., Coyle, J., McKhann, G., Mobley, W. C., Nadel, L., Neubert, D., Rolf Schulte-Hermann, R., Spencer, P. S. (2008). Review of the toxicology of chlorpyrifos with an emphasis on human exposure and neurodevelopment. *Crit. Rev. Toxicol*., **S2**:1-125.

Eddins, D., Cerutti, D., Williams, P., Linney, E., Levin, E. D. (2010). Zebrafish provide a sensitive model of persisting neurobehavioral effects of developmental chlorpyrifos exposure: Comparison with nicotine and pilocarpine effects and relationship to dopamine deficits. *Neurotoxicol. Toxicol.*, **32**: 99-108.

El-Amrani, S., Pena-Abaurrea, M., Sanz-Landaluze, J., Ramos, L., Guinea, J., Camara, C. (2012). Bioconcentration of pesticides in zebrafish eleuthero embryos (*Danio rerio). Sci. Total Environ.*, **425**: 184-90.

Elballat, S. E. (2016). Protective effect of curcumin and vitamin C each alone and in combination on cisplatin-induced sperm abnormalities in male albino rats. *JOBAZ*, **76**: 52-59.

English, A. B. and Webster, A.A. (2012). Acetylcholinesterase and its Inhibitors. In: Primer on the Autonomic Nervous System, 3rd edition (David Robertson, Italo Biaggioni, Julian F.R.Paton, Phillip A. Low eds.). Academic Press, 631-633.

Esterbauer, H., Eckl, P., Ortner, A. (1990). Possible mutagens derived from lipids and lipid precursors. *Mutat. Res*., **238**: 223-233.

Fan, W. Q., Yanase, T., Morinaga, H., Gondo, S., Okabe, T., Nomura, M., Komatsu, T., Morohashi, K. I., Hayes, T. B., Takayanagi, R., Nawata, H. (2007). Atrazine-induced aromatase expression is SF-1 dependent: Implications for endocrine disruption in wildlife and reproductive cancers in humans. *Environ. Health Perspect.*, **115(5)**: 720–727.

Farag, A.T., El Okazy, A.M., El-Aswed, A.F. (2003). Developmental toxicity study of chlorpyrifos in rats. *Repro. Toxicol.,* **17**: 203-208.

Farber, J. L. (1994). Mechanisms of cell injury by activated oxygen species. *Environ. Health Perspect*., **102(10)**: 17-24.

Fetoui, H., Garouia, E. M., Fatma Makni-ayadi, F. M., Zeghal, N. (2008). Oxidative stress induced by lambda-cyhalothrin (LTC) in rat erythrocytes and brain: Attenuation by vitamin C. *Environ. Toxicol. Pharmacol*., **26**: 225–231.

Filho, D. W., Torres, M. A., Tribess, T. B., Pedrosa, R. C., Soares, C. H. L. (2001). Influence of season and pollution on the antioxidant defenses of the cichlid fish acará (*Geophagus brasiliensis*). *Braz. J. Med. Biol. Res*., **34**: 719-726.

Flohe´, R. B. and Flohe´, L. (2011). Basic principles and emerging concepts in the redox control of transcription factors. *Antioxid. Redox Signal.,* **15(8)**: 2335-2381.

Folch, J., Lees, M., Sloane, S. G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226 (1)**: 497-509.

Fredianelli, A. C., Pierin, V. H., Uhlig, S. C., Galeb, L. A. G. (2019). Hematologic, biochemical, genetic, and histological biomarkers for the evaluation of the toxic effects of fipronil for Rhamdia quelen. *Turk. J. Vet. Anim. Sci.*, **43(1)**: 54-59.

Freed, V.H., Chiou, C.T., Schmedding, D. W. (1979). Degradation of selected organophosphate pesticides in water and soil. *J. Agric. Food Chem.,* **27(4)**: 706-708.

Gagné, F. (2014). Neuroendocrine disruption. In Biochemical Ecotoxicology: Principles and methods (Andre, C., Auclair, J., Lacaze, E., Quinn, B. eds.). Academic Press, pp.145-170.

Ganeshwade, R. M. (2011). Biochemical changes induced by dimethoate in the liver of fresh water fish *Puntius ticto* (HAM). (2011). *Biol Forum.*, **3(2)**: 65-68.

Gebremariam, S. Y., Beutel, M. W.,Yonge, D. R., Flury, M., Harsh, J. B. (2011) Adsorption and desorption of chlorpyrifos to soils and sediments. *Rev. Environ. Contam. Toxicol.*, **215**:123-75.

Gernhöfer, M., Pawert, M., Schramm, M., Müller, E. and Triebskorn, R. (2001). Ultrastructural biomarkers as tools to characterize the health status of fish in contaminated streams. *J. Aquat. Ecosyst. Stress and Recovery*, **8**: 241–260.

Ghosh, S., Banerjee, S., Sil, P.C. (2015). The beneficial role of curcumin on inflammation, diabetes and neurodegenerative disease: A recent update. *Food Chem. Toxicol*., **83**:111–24.

Gill, T.S., Pande, J., Tewar, H. (1990). Sublethal effects of an organophosphorus insecticide on certain metabolite levels in a freshwater fish, *Puntius conchonius* Hamilton. *Pestic. Biochem. Phys.*, **36(3)**: 290-299.

Gobinath, T. and Ravichandran, S. (2020). Chitosan from crustacean shell waste and its protective role against lead toxicity in *Oreochromis mossambicus. Toxicol. Rep*., **7**:296-303.

Gopalakannan, A. and Arul, V. (2006). Immunomodulatory effects of dietary intake of chitin, chitosan and levamisole on the immune system of *Cyprinus carpio* and control of *Aeromonas hydrophila* infection in ponds. *Aquaculture*, **255**: 179–187.

Goswami, S.V., Lamba, V.J., Sundararaj, B.I. (1985). Gonadotrophin-induced oocyte maturation in the catfish, *Heteropneustes fossilis* (Bloch), requires steroidogenesis in both interrenal and ovary. *Gen. Comp. Endocrinol.*, **57**: 53-63.

Grünefeld, H.T. and Bonefeld-Jorgensen, E.C. (2004). Effect of in vitro estrogenic pesticides on human oestrogen receptor alpha and ß mRNA levels. *Toxicol. Letts.,* **151**: 467-80.

Gultekin, F., Patat, S., Akca, H., Akdogan, M., Altuntas, I. (2006). Melatonin can suppress the cytotoxic effects of chlorpyrifos on human HepG2 cell lines. *Hum. Exp. Toxicol*., **25**: 47 – 55.

Gupta, S. C., Mishra, M., Sharma, A., Balaji, T.G.R.D., Kumar, R., Ranjit K.Mishra, R. K., Chowdhuri, D.K. (2010). Chlorpyrifos induces apoptosis and DNA damage in Drosophila through generation of reactive oxygen species. *Ecotoxicol. Environ. Saf.,* **73**: 1415-1423.

Hacışevki, A. (2009). An overview of ascorbic acid biochemistry. *J. Fac. Pharm.*, **38(3)**: 233-255.

Hai, D. Q., Varga, S. I., Matkovics, B. (1997). Organophosphate effects on antioxidant system of carp (*Cyprinus carpio*) and catfish (*Ictalurus nebulosus*). *Comp. Biochem. Physiol*., **117C(1)**: 83–88.

He, Y., Yue, Y., Zheng, X., Zhang, K., Chen, S., Du, Z. (2015). Curcumin, inflammation, and chronic diseases: How are they linked? *Molecules*, **20**: 9183–213.

Higa, M., Kanda, H., Kitahashi, T., Ito, M., Shiba, T., Ando, H. (2000). Quantitative analysis of fushi tarazu factor 1 homolog messenger ribonucleic acids in the pituitary of salmon at different prespawning stages. *Biol. Reprod*., **63**: 1756-1763.

Hofsten, J. V., Karlsson, J., Jones, I., Olsson, P-E. (2002). Expression and regulation of fushi tarazu factor-1 and steroidogenic genes during reproduction in Arctic char (*Salvelinus alpinus*). *Biol. Reprod*., **67**: 1297-1304.

Hofsten, J. V. (2004). Developmental and reproductive regulation of NR5A genes in teleosts. Molecular biology, Umeå University.

Hofsten, J. V. and Olsson, P.E. (2005). Zebrafish sex determination and differentiation: Involvement of FTZ-F1 genes. *Repro. Biol. Endocrinol*., **3(1)**: 63.

Horsak, R.D., Bedient, P.B., Hamilton, M.C., Thomas, B. F. (1964). Pesticides. In: Environmental Forensics (Robert D. Morrison, Brian L. Murphy eds.). Academic Press, pp.143-165.

Hossain, M. Y., Islam, R., Zoarder Faruque Ahmed, Z. F., Rahman, M. M., Md. Alomgir Hossen, M. A., S. M. Abu Naser, S. M.A., Rasel, R. I. (2015). Threatened fishes of the world: *Heteropneustes fossilis* (Bloch, 1794) (Siluriformes: Heteropneustidae). *Ribarstvo*, **73**: 77-79.

Horzmann, K.A. and Freeman, J. L. (2016). Zebrafish Get Connected: Investigating neurotransmission targets and alterations in chemical toxicity. *Toxics*, **4**:19.

Hsieh, Y. S. and Hsu, C. Y. (2013). Oxidative stress and anti-oxidant enzyme activities in the trophocytes and fat cells of queen honeybees (*Apis mellifera*). *Rejuvenation Res*., **16(4)**: 295-303.

Ighodaro, O. M. and Akinloye, O. A. (2018). First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria J. Med.*, **54(4)**: 287-293.

Ijiri, S., Kaneko, H., Kobayashi, T., Wang, D. S., Sakai,F., Prasanth, B.P., Nakamura, M., Nagahama, Y. (2008). Sexual dimorphic expression of genes in gonads during early differentiation of a teleost fish, the nile tilapia, *Oreochromis niloticus. Biol. Reprod*., **78**: 333–341.

Islam, S., Bhuiyan, M. A. R., Islam, M. N. (2016). Chitin and chitosan: Structure, properties and applications in biomedical engineering. *J. Polym. Environ*., **25**: 854-866.

Ivanova, D. G. and Yaneva, Z. L. (2020). Antioxidant properties and redox-modulating activity of chitosan and its derivatives: biomaterials with application in cancer therapy. *BioRes.*, **9**: 64-72.

Jaffer, N. S., Rabee, A. M., Al-Chalabi, S. M.M. (2017). Biochemical, hematological parameters and histological alterations in Fish *Cyprinus carpio* L. as biomarkers for water pollution with chlorpyrifos. *Hum. Ecol. Risk Assess.*, **23**: 605-616.

Jagtap, J. T., Shejule, K. B., Ubarhande, S. B. (2011). Acute effect of TBTCL on protein alteration in freshwater bivalve, *Lamellidens marginalis. Int. Multidiscip. Res. J.* **1(8)**: 13-16.

Jain, S., Lone, M. M., Rukhsar, S. (2018). Histopathological alterations in the stomach of freshwater fish *Channa punctatus* under chlorpyrifos toxicity. *TFR.*, **7(3)**: 96-102.

Javed, M., Md. Irshad Ahmad, M. I., Usmani, N., Ahmad, M. (2017). Multiple biomarker responses (serum biochemistry, oxidative stress, genotoxicity and histopathology) in *Channa punctatus* exposed to heavy metal loaded waste water. *Sci. Rep*.,**7(1)**: 1-11.

Jayaraj, R., Megha, P., Sreedev, P. (2016). Organochlorine pesticides, their toxic effects on living organisms and their fate in the environment. *Interdiscip Toxicol.*, **9(3–4)**: 90–100.

Jeon, H. Y., Kim, J. K., Lee, J. E., Shin, H. J. (2008). A mixture of curcumin, vitamin C, and E prolongs the antioxidant effect to beyond that of each component alone in vivo. *Food Sci. Biotechnol*., **17**: 1151-1155.

Jha, B.R. and Rayamajhi, A. (2010). *Heteropneustes fossilis*. *The IUCN Red List of Threatened Species* 2010: e.T166452A135875733.

Jiang, J., Wu, X. Y., Zhou, X. Q., Feng, L., Liu, Y., Jiang, W. D., Wu, P., Zhao, Y. (2016). Effects of dietary curcumin supplementation on growth performance, intestinal digestive enzyme activities and antioxidant capacity of crucian carp *Carassius auratus*. *Aquaculture*, **463**: 174–180.

Jin, Y., Liu, Z., Peng, T., Fu, Z., (2015). The toxicity of chlorpyrifos on the early life stage of zebrafish: A survey on the endpoints at development, locomotor behavior, oxidative stress and immunotoxicity. *Fish Shellfish Immunol*., **43(2)**: 405-414.

Jiying,W., Baoshan, L, Xudong, L, Jingjing, M., Shixin, W., Limin, Z. (2014). Dietary vitamin A, ascorbic acid and α-tocopherol affect the gonad development and reproductive performance of starry flounder *Platichthys stellatus* broodstock. *Chin. J. Oceanol. Limn*., **32(2)**: 326-333.

John, S., Kale, M., Rathore, N., Bhatnagar, D. (2001). Protective effect of vitamin E in dimethoate and malathion induced oxidative stress in rat erythrocytes. *J. Nutr. Biochem*., **12**: 500–504.

Joshi, U. M. and Desai, A. K. (1988). Biochemical changes in the liver of fish, *Tilapia mossambica* (Peters), during continuous exposure to monocrotophos. *Ecotoxicol. Environ. Saf*., **15**: 272-276.

Kadam, P. and Patil, R. (2016). Effect of chlorpyrifos on some biochemical constituents in liver and kidney of fresh water fish, *Channa gachua* (F.Hamilton). IJSR, **5(4)**: 1975-1979.

Karadag, H., Firat, O., Firat, O. (2014). Use of oxidative stress biomarkers in *Cyprinus carpio* L. for the evaluation of water pollution in ataturk dam lake (Adiyaman, Turkey). *Bull. Environ. Contam. Toxicol.*, **92**: 289–293.

Kavitha, P. and Rao, J. V. (2008). Toxic effects of chlorpyrifos on antioxidant enzymes and target enzyme acetylcholinesterase interaction in mosquito fish, *Gambusia affinis*. *Environ. Toxicol. Pharmacol*., **26**: 192–198.

Kaushal, J., Seema, Gursharan Singh, G., Arya, S. K. (2018). Immobilization of catalase onto chitosan and chitosan–bentonite complex: A comparative study. *Biotechnol. Rep*., **18**: e00258.

Kaur, M., and Jindal, R. (2017). Oxidative stress response in liver, kidney and gills of *Ctenopharyngodon idellus* (cuvier & valenciennes) exposed to chlorpyrifos. *MOJ Biol. Med*., **1(4)**: 103-112.

Kehrer, J. P. (1993). Free radicals as mediators of tissue injury and disease. *Crit. Rev. Toxicol.*, **23(1)**: 21-48.

Khalil, O. A. K., Oliveira, O. M. M. F., Vellosa, J. C. R., Quadros, A. U. Q., Dalposso, L. M., Karam, T. K., Mainardes, M. R., Najeh Maissar Khalil, N. M. (2012). Curcumin antifungal and antioxidant activities are increased in the presence of ascorbic acid. *Food Chem*., **133**: 1001–1005.

Khan, A. A., Rahmani, A. H., Aldebasi, Y. H., Aly, S. M. (2014). Biochemical and pathological studies on peroxidases–An updated review. *Glob. J. of Health Sci*., **6(5)**: 87-98.

Khan, S. (2017). Sublethal effect of chlorpyrifos on some biochemical constituents of guppy fish. *World J. Pharm. Pharm. Sci.*, **6(7)**: 2167-2172.

Kim, D. J., Bae, J. Y., Kim, E. O. (2007). Changes in sex steroid hormones and ovarian development during artificial maturation of female eel, *Anguilla japonica. Integr. Biosci*., **11**: 117-124.

Klebanoff, S. J. (2005). Myeloperoxidase: friend and foe. *J. Leukoc. Biol.*,**77(5)**: 598-625.

Kojima, H., Katsura, E., Takeuchi, S., Niyama, K., Kobayashi, K. (2004). Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using Chinese hamster ovary cells. *Environ. Health Perspect.*, **112**: 524-31.

Korkmaz, N., Cengiz, E. I., Unlu, E., E. Uysal, E., Yanard, M. (2009). Cypermethrin-induced histopathological and biochemical changes in Nile tilapia (*Oreochromis niloticus*), and the protective and recuperative effect of ascorbic acid. *Environ. Toxicol. Pharmacol*., **28**: 198–205.

Koshlukova, S. E. and Reed, N. R. (2014). Chlorpyrifos. Encyclopedia of toxicology, **1**: 583-585.

Kumar, P., Prasad, Y., Patra, A. K., Ranjan, R., Swarup, D., Patra, R. C., Pal, S. (2009). Ascorbic acid, garlic extract and taurine alleviate cadmium-induced oxidative stress in freshwater catfish (*Clarias batrachus*). *Sci. Total Environ.* **407:** 5024-5030.

Kumari, K., Khare, A., Dange, S. (2014). The applicability of oxidative stress biomarkers in assessing chromium induced toxicity in the fish *Labeo rohita. BioMed Res. Int.,* **2014**: 782493.

Kwak, M. K., Wakabayashi, N., Kensler, T. W. (2004). Chemoprevention through the Keap1–Nrf2 signaling pathway by phase 2 enzyme inducers. *Mutat. Res*., **555**: 133–148.

Kwong, T. C. (2002) Organophosphate Pesticides: Biochemistry and clinical toxicology. *Ther Drug Monit,* **24**:144–149.

Lal, B. and Singh, T. P. (1987). Impact of pesticides on lipid metabolism in the freshwater catfish, *Clarias batrachus*, during the vitellogenic phase of its annual reproductive cycle. *Ecotox. Environ. Safe*., **13**: 13-23.

Lamba, V.J., Goswami, S.V., Sundararaj, B.I. (1983). Circannual and circadian variations in plasma levels of steroids (cortisol, estradiol-17p, estrone, and testosterone) correlated with the annual gonadal cycle in the catfish, *Heteropneustes fossilis* (Bloch). *Gen. Comp. Endocrinol*., **50**: 205-225.

Lee, J. E., Park, J. H., Shin, I. C., Koh, H. C. (2012). Reactive oxygen species regulated mitochondria-mediated apoptosis in PC12 cells exposed to chlorpyrifos. *Toxicol. Appl. Pharmacol*., **263**:148–162.

Levin, E. D., Chrysanthis, E., Yacisin, K., Linney, E. (2003). Chlorpyrifos exposure of developing zebrafish: effects on survival and long-term effects on response latency and spatial discrimination. *Neurotoxicol. Teratol.,* **25**: 51-57.

Levin, E. D., Holly A. Swain, H. A., Donerly, S., Linney, E. (2004). Developmental chlorpyrifos effects on hatchling zebrafish swimming behavior. *Neurotoxicol. Teratol.,* **26**: 719-723.

Li, D., Huang, Q., Lu, M., Zhang, L., Yang, Z., Zong, M., Tao, L. (2015). The organophosphate insecticide chlorpyrifos confers its genotoxic effects by inducing DNA damage and cell apoptosis. *Chemosphere*, **135**: 387-393.

Limbore, N.V. and Khillare, S. K. (2015). An analytical study of Indian agriculture crop production and export with reference to Wheat. *Rev. Res.,* **4(6)**: 1-8.

Liu, D., Le Drean, Y., Ekker, M., Xiong, F., Hew, C.L. (1997). Teleost FTZ-F1 homolog and its splicing variant determine the expression of the salmon gonadotropin IIß subunit gene. *Mol Endocrinol.,* **11**: 877-890.

Liu, M.,Lu, Y., Gao, P., Xie, X., Li, D., Yu, D., Yu, M. (2020). Effect of curcumin on laying performance, egg quality, endocrine hormones, and immune activity in heat-stressed hens. *Poult. sci.*, **99(4)**: 2196-2202.

Loftsson, T. (2014). Degradation Pathways. In Drug stability and Pharmaceutical Scientists. Academic Press, UK, 63-104.

Lowry, O. H., Rosebrough, N. J., Farr, A. I., Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193(1)**: 265-275.

Lubos, E., Loscalzo, J., Diane E. Handy, D. E. (2011). Glutathione Peroxidase-1 in health and disease: From molecular mechanisms to therapeutic opportunities. *Antioxid. Redox Signal*., **15(7)**: 1957-1997.

López, E. L., Díaz, J. E. S., Soto, C., Favari, L. (2011). Responses of antioxidant enzymes, lipid peroxidation, and Na+/K+-ATPase in liver of the fish *Goodea atripinnis* exposed to Lake Yuriria water. *Fish Physiol. Biochem*., **37**: 511–522.

Lushchak, V.I., Matviishyn, M.T., Husak, V.K., Janet M. Storey, Storey, K. B. (2018). Pesticide Toxicity:A Mechanistic Approach. *EXCLI J.*, **17**: 1101-1136.

Mahmood, I., Imadi, S.R., Shazadi, K., Gul, A., Hakeem, R. K. (2016). Effects of Pesticides on Environment. In: Plant, Soil and Microbes (Hakeem K., Akhtar M., Abdullah S. eds). Springer, Cham, Switzerland , 253-269.

Madkour, N. K. (2012). Protective effect of curcumin on oxidative stress and DNA fragmentation against lambda cyhalothrin-induced liver damage in rats. *J. Appl. Pharm. Sci.*, **2 (12)**: 76-81.

Magar, R. S and Shaikh, A. (2012). Biochemical changes in proteins and amino acids in *Channa punctatus* in responses to sublethal treatment with the insecticide malathion. *Trends Life Sci*., **1(3)**: 19-23.

Manju, M., Akbarsha, M. A., Oommen, O. V. (2012). In vivo protective effect of dietary curcumin in fish *Anabas testudineus* (Bloch). *Fish Physiol. Biochem.*, **38(2)**: 309-318.

Majumder, R. and Kaviraj, A. (2018). Acute and sublethal effects of organophosphate insecticide chlorpyrifos on freshwater fish *Oreochromis niloticus. Drug Chem. Toxicol.*, **42(5)**: 487-495.

Marnett, L. J. (1999). Lipid peroxidation—DNA damage by malondialdehyde. *Mutat. Res*., **424**: 83–95.

Matkovics, B., Novak, R., Hanh, H. D., Szabo, L., Varga, S. I., Zalesna, G. (1977). A comparative study of some more important experimental animal peroxide metabolism enzymes. *Comp. Biochem. Physiol*., **56B**: 31-34.

Maurya, P. K., Malik, D. S., Yadav, K. K., Gupta, N., Kumar, S. (2019). Haematological and histological changes in fish *Heteropneustes fossilis* exposed to pesticides from industrial waste water. *Hum. Ecol. Risk Assess.*, **25**: 1251-1278.

Mazeaud, F., Maral, J., Michelson, A. M. (1979). Distribution of superoxide dismutase and glutathione peroxidase in the carp: erythrocyte manganese SOD. *Biochem. Biophys. Res. Commun*., **86(4)**: 1161-1168.

McConnell, L. L., Lenoir, J. S., Datta, S., Seiber, J. N. (1998). Wet deposition of current-use pesticides In the Sierra Nevada Mountain Range, California, USA. *Environ. Toxicol. Chem*., **17(10)**: 1908–1916.

Menon, V. P., Sudheer, A. R. (2007). Antioxidant and anti-inflammatory properties of curcumin. *Adv. Exp. Med. Biol*., **595**: 105-25.

Mewes, K. R., Latz M., Golla, H., Fischer, A. (2002). Vitellogenin from female and estradiol-stimulated male river Lampreys (*Lampetra fluviatilis* L.*). J. Exp. Zool.*, **292**: 52-72.

Middlemore-Risher, M. L., Buccafusco, J. J., A.V. Terry, A. V. (2010). Repeated exposures to low-level chlorpyrifos results in impairments in sustained attention and increased impulsivity in rats. *Neurotoxicol. Teratol.*, **32**: 415-424.

Minniti, F., Maisano, M., , Giannetto, A., , Mauceri , A., Fasulo, S. (2009). GTH I and GTH II in the pituitary gland of swordfish (*Xiphiasgladius L.). Ital. J. Zool*., **76(3)**: 269-278.

Mishra, A and Devi, Y. (2014). Histopathological alterations in the brain (optic tectum) of the fresh water teleost *Channa punctatus* in response to acute and subchronic exposure to the pesticide chlorpyrifos. *Acta Histochem*., **116(1)**: 176-81.

Mishra, A. and Devi, Y. (2020). Gonad biochemical profile alteration by chlorpyrifos in freshwater fish *Channa punctatus. Int. J. Pharm. Bio. Sci*., **11(2)**: 84-94.

Mishra, A. and Joy, K. P. (2004). Ovarian monosaccharides (glucose and fructose): hormonal effects and their role in final oocyte maturation and egg quality in catfish *Heteropnuestes fossilis,* Bloch. *Indian J. Exp. Biol.* **42:** 1084-1090.

Mishra, A., and Joy, K.P. (2006). HPLC-electrochemical detection of ovarian estradiol-17 and catecholestrogens in the catfish *Heteropneustes fossilis:* Seasonal and periovulatory changes. *Gen. Comp. Endocrinol*., **145**: 84–91.

Mishra, A., Mishra, N., Rawat, A., Verma, S. (2014). Effect of an organophosphate insecticide, chlorpyriphos on mortality and haematology of freshwater butter catfish *Ompok bimaculatus* (Bloch, 1794). *Pensee*., **76(5)**: 452-461.

Mishra, A. and Singh, P. (2018). Impact of chlorpyrifos on the ovary of the freshwater fish, *Channa punctatus*. *Int. J. Pharm. Bio. Sci*., **9(3)**: 219-225.

Mishra, A. and Singh, A. (2020a). Effect of curcumin, ascorbic acid against chlorpyrifos toxicity on histotexture and bimolecular content of fresh water catfish, *Heteropneustes fossilis*. *J. Exp. Zool. India*, **23(2)**: 1043-1049.

Mishra, A. and Singh, A. (2020b). Attenuation of chlorpyrifos induced toxicity in freshwater catfish, *Heteropneustes fossilis. Biochem. Cell Arch*., 20(2): 5065-5072.

Mishra, A. and Verma, S. (2016). Acute toxicity bioassay of organophosphorus pesticide, chlorpyrifos on freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794). *Int. J. Fish Aquat. Stud.* **4(6)**: 388-393.

Mishra, A. and Verma, S. (2017). In vitro effects of chlorpyrifos on gonadotropin-induced oocyte maturation and steroidal interplay of freshwater catfish, *Heteropneustes fossilis. Int. J. Pharma Bio. Sci.*, **8(3)**: 912-920.

Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci*., **7**: 405–410.

Mizoguchi, B. A. and Valenzuela, N. (2015). Ecotoxicological Perspectives of Sex Determination. *Sex. Dev.*, **10(1)**: 45-57.

Mlynarczuk, J. and Rekawiecki, R. (2010). The role of the orphan receptor SF-1 in the development and function of the ovary. *Reprod. Biol*., **10(3)**: 177-193.

Moghadamtousi, S.Z., Kadir, H.A., Hassandarvish, P., Tajik, H., Abubakar, S., Zandi, K. (2014). A review on antibacterial, antiviral, and antifungal activity of curcumin. *Biomed Res. Int.*, **2014**: 1-12.

Monteiro, D. A., De Almeida, J. A. D., Rantin, F. T., Kalinin, A. L. (2006). Oxidative stress biomarkers in the freshwater characid fish, *Brycon cephalus*, exposed to organophosphorus insecticide Folisuper 600 (methyl parathion). *Comp. Biochem Physiol C*., **143**:141–149.

Monteiro, D. A., Rantin, F. T., Kalinin, A. L. (2009). The effects of selenium on oxidative stress biomarkers in the freshwater characid fish matrinxã, *Brycon cephalus* (Günther, 1869) exposed to organophosphate insecticide Folisuper 600 BR® (methyl parathion). *Comp. Biochem. Physiol*. *C*., **149**: 40–49.

Moretto, A. (1998). Experimental and clinical toxicology of anticholinesterase agents. *Toxicol. Lett.*, **102-103**: 509-513.

Mostakim, G. M., Zahangir, M. M. Z., Mishu, M. M., Rahman, M. K., Islam, M. S. (2015). Alteration of blood parameters and histoarchitecture of liver and kidney of silver barb after chronic exposure to quinalphos. *J. Toxicol*., **3**: 1-8.

Mueller, S., Riedel, H. D., Stremmel, W. (1998). Direct evidence for catalase as the predominant H_2O_2 -removing enzyme in human erythrocytes. *Blood.*, **90(12)**: 4973-4978.

Mulgund, A., Doshi, S., Agarwal, A. (2015). The role of oxidative stress in endometriosis. In Handbook of Fertility: Nutrition, Diet, Lifestyle and Reproductive Health, 273-281.

Nagahama, Y.,Yamashita, M. (2008). Regulation of oocyte maturation in fish. *Develop. Growth Differ*., **50**: 195–219.

Nandi, S., Gupta, P.S.P., Roy, S. C., Sellappan, S., Ravindra, J.P. (2011). Chlorpyrifos and endosulfan affect buffalo oocyte maturation, fertilization, and embryo development in vitro directly and through cumulus cells. *Environ. Toxicol.*, **26**: 57-67.

Nandi, A., Yan, L. J., Jana, C. K., Das, N. (2019). Role of catalase in oxidative stress- and age-associated degenerative diseases. *Oxid. Med. Cell Longev.,* **2019**: 1-19.

Nath, P. and Dey, S. C. (1989). Fish and fisheries of North East India. Arunachal Pradesh. Narendra Publishing House, Delhi, 1-143.

Nath, P. and Sundararaj, B. I. (1981a). Isolation and identification of female-specific serum lipophosphoprotein (vitellogenin) in the catfish, *Heteropneustes fossilis* (Bloch). *Gen. Comp. Endocrinol*., **43**: 184- 190.

Nath, P. and Sundararaj, B. I. (1981b). Induction of Vitellogenesis in the hypophysectomized catfish, *Heteropneustes fossilis* (Bloch): Effects of Piscine and Mammalian Hormones. *Gen. Comp. Endocrinol*., **43**: 191-200.

Nelson, E.R. and Habibi, H.R. (2013). Estrogen receptor function and regulation in fish and other vertebrates. *Gen.Comp. Endocrinol*., **192**: 15-24.

Ngo, D. H. and Kim, S. K. (2014). Antioxidant effects of chitin, chitosan, and their derivatives. *Adv. Food Nutr. Res*., **73**: 15-31.

Nishimura, I.C., Nishimura, S., Nishi, N., Saiki, I., Tokura, S., Azuma, I. (1984). Immunological activity of chitin and its derivatives. *Vaccine,* **2(1)**: 93-99.

Nunes, M. E. M., Müller, T. E., Murussi, C., do Amaral, A. M. B., Gomes, J. L. C., Marins, A. T., Leitemperger, J., Rodrigues, C. C. R., Fiuzaa, T. L., Costa, M. D., Eduardo S. Severo, E. S., Rosemberg, D. B., Loro, V. L. (2018). Oxidative effects of the acute exposure to a pesticide mixture of cypermethrin and chlorpyrifos on carp and zebrafish – A comparative study. *Comp. Biochem. Physiol.,C.,* **206–207**: 48–53.

Nushy, N. H., Zafar, A., Khatun, M., Rohani, F., Rana, M. (2020). Comparative growth performance assessment of Shing (*Heteropneustes fossilis*) feeding with prepared and commercial diet. *J. Aquac. Mar. Biol.*, **9(1)**: 10–13.

O'Brien, P. J. (2000). Peroxidases. *Chem. Biol. Interact*., **129**: 113–139.

Ohkawa, H., Ohishi, N., Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.*, **95(2)**: 351-358.

Ojha, A., Yaduvanshi, S. K., Pant, S. C., Lomash, V., Srivastava, N. (2011). Evaluation of DNA damage and cytotoxicity induced by three commonly used organophosphate pesticides individually and in mixture, in rat tissues. *Environ. Toxicol.*, **28(10)**: 543-552.

Oost, V. D., R., Beyer, J. and Vermeulen, N. P. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ. Toxicol Pharmacol.*, **13**: 57-149.

Ortiz, J. B., Canales, M. L. G. D., Sarasquete, C. (2003). Histopathological changes induced by lindane (γ-HCH) in various organs of fishes. *Sci. Mar*., **67 (1)**: 53-61.

Oruç, E. Ö. (2010). Oxidative stress, steroid hormone concentrations and acetylcholinesterase activity in *Oreochromis niloticus* exposed to chlorpyrifos. *Pestic Biochem. Phy*., **96**: 160-166.

Oruc, E. (2012). Oxidative Stress Responses and Recovery Patterns in the Liver of *Oreochromis niloticus* exposed to Chlorpyrifos-Ethyl. *Bull. Environ. Contam. Toxicol*., **88**: 678–684.

Ozkan, F, Gündüz, S. G., Berköz, M., Hunt, A. O., Yalın, S. (2012). The protective role of ascorbic acid (vitamin C) against chlorpyrifos-induced oxidative stress in *Oreochromis niloticus*. *Fish Physiol. Biochem.* **38(3)**: 635-643.

Padayatty, S. J., Katz, A.,Wang, Y., Eck, P., Kwon, O., Lee, J.H., Chen,S., Corpe,C., Anand Dutta, A., Sudhir K Dutta, S. K., Mark Levine, M. (2003). Vitamin C as an antioxidant: Evaluation of its role in disease prevention. *J. Am. Coll.Nutr*., **22(1)**: 8-35.

Pal, S., Kokushi, E., Koyama, J., Uno, S., Ghosh, A. R. (2012): Histopathological alterations in gill, liver and kidney of common carp exposed to chlorpyrifos. *J.Environ. Sci. Health B*: Pesticides, Food Contaminants, and Agricultural Wastes, **47(3)**: 180-195.

Palanikumar, L., Kumaraguru, A. K., Ramakritinan, C. M., Anand, M. (2013). Toxicity, biochemical and clastogenic response of chlorpyrifos and carbendazim in milkfish *Chanos chanos*. *Int. J. Environ. Sci. Technol.,* **11**: 765-774.

Pandey, H. and Mishra, A. (2014). Histopathological effects of chlorpyrifos, an organophosphate insecticide, on ovary of fresh water teleost *Channa punctatus* during pre-spawning season. In Biodiversity and Environment (Serajuddin, M., Tripathi, M., Kanaujia, A., Arshad, M. eds.). Excellent Publishing House, pp.86-88.

Park, C.B., Jun-ya Aokia, J.Y., Leec, J.S., Nagaed,M., Leee,Y.D., Sakakuraf, Y., Hagiwarab, A., Soyanoa, K. (2010a). The effects of 17β-estradiol on various reproductive parameters in the hermaphrodite fish *Kryptolebias marmoratus*. *Aquat. Toxicol*., **96**: 273–279.

Park, M., Shin, E., Won, M., Kim, J.H., Go, H., Kim, H.L., Ko, J.J., Lee, K., Bae, J., (2010b). FOXL2 interacts with steroidogenic factor-1 (SF-1) and represses SF-1-induced CYP17 transcription in granulosa cells. *Mol. Endocrinol*., **24**: 1024–1036.

Patlolla, A. K., Barnes, C., Yedjou, C., Velma, V. R., Tchounwou, P. B. (2008). Oxidative stress, DNA damage, and antioxidant enzyme activity induced by hexavalent chromium in sprague-dawley rats. *Environ. Toxicol.,* **24(1)**: 66-73.

Pehlivan, F. E. (2017). Vitamin C: An Antioxidant Agent. In Vitamin C (Hamza, A.H. ed). IntechOpen.

Paul, B. N., Sridhar, N., Chanda, S., Saha, G. S., Giri, S. S. (2015). Nutrition facts *Heteropneustes fossilis* (Singhi).

Prakasam, A., Sethupathy, S., Lalitha, S. (2001). Plasma and RBCs antioxidant status in occupational male pesticide sprayers. *Clin. Chim. Acta.,* **310**: 107–112.

Putra, W. K. A. and Mulah, A. (2019). Combination effects human chorionic gonadotropin hormon and ovaprim distribution on the time latency, percentage of fertilization, hatching and survival of silver pompano (*Trachinotus blochii*) larve fish. *IOP Conf. Ser.: Earth Environ. Sci.* **278**: 012063.

Puvaneswari, S., , Marimuthu, K., Ramasamy Karuppasamy, R., Haniffa, M. A. (2009). Early embryonic and larval development of Indian catfish, *Heteropneustes fossilis. Eur. Asia J. BioSci.*, **3(12)**: 84-96.

Qayoom, I., Balkhi, M. H., Mukhtar, M., Farooz A. B., Feroz A. S. (2014). Biochemical toxicity of organophosphate compounds in fishes. *SKUAST J. Res.* **16(1)**: 1-13.

Raghuveer, K., Senthilkumaran, B., Sudhakumari , C. C., Sridevi, P., Rajakumar , A., Singh, R., Kumar, R. M., Majumdar, K. C. (2011). Dimorphic expression of various transcription factor and steroidogenic enzyme genes during gonadal ontogeny in the air-breathing catfish, *Clarias gariepinus*. *Sex Dev*., **5**: 213–223.

Ramalingam, K. and Ramalingam, K. (1982). Effects of sublethal levels of DDT, malathion and mercury on tissue proteins of *Sarotherodon mossambicus* (Peters). *Proc. Indian Acad. Sci. (Anim Sci.*), **91(6)**: 501-505.

Rathod, A. L. and Garg, R. K. (2017). Chlorpyrifos poisoning and its implications in human fatal cases: A forensic perspective with reference to Indian scenario. *J. Forensic Leg. Med.*, **47**: 29-34.

Rao, J. V., Begum, G., Pallela, R., P. K. Usman, P. K. and Rao, R. N. (2005). Changes in behavior and brain acetylcholinesterase activity in mosquito fish, *Gambusia affinis* in response to the sub-lethal exposure to chlorpyrifos. *Int. J. Environ. Res. Public Health*, **2(3)***:* 478-483.

Reading, J. B., Sullivan, C. V., Schilling, J. (2017). Vitellogenesis in fishes. In Reference Module in Life Sciences; Elsevier: Amsterdam, The Netherlands, pp.1-12.

Reddy, A. C.P., Lokesh, B. R. (1994). Effect of dietary turmeric (curcuma longa) on iron induced lipid peroxidation in the rat liver. *Fd. Chem. Toxic*. Vol., **32 (3)**: 279-283.

Revathi., T. and Krishnamurthy, R. (2018). Impact of pesticide chlorpyrifos on protein alterations in a freshwater fish, *Channa striatus. Int. J. Pharm. Biol. Sci.,* **8(3)**: 926-931.

Rotruck, J. T., Pope, A. L., Ganther, H. E., Swanson, A. B., Hafeman, D. G., Hoekstra, W. G. (1973). Selenium: biochemical role as a component of glutathione peroxidase. *Science,* **179(4073)**: 588-590.

Sabra, F. S. and Mehana, E.S. E. D. (2015). Pesticides toxicity in fish with particular reference to insecticides. *Asian J. Agric. Food Sci., **3**(1)*: 40-60.

Sakai, N., Terami, H., Suzuki, S., Haga, M., Nomoto, K.,Tsuchida, N., Morohashi, K., Saito, N.,Asada, M., Hashimoto, M., Harada, D., Asahara, H., Ishikawa, T., Shimada, F , Sakurada, K. (2008). Identification of NR5A1 (SF-1/AD4BP) gene expression modulators by large-scale gain and loss of function studies. *J. Endocrinol*., **198**: 489–497.

Sakr, S. A., Gabr, S. A., El-Saadany, M. M. (1991). Effect of diazinon on freeze-fracture images of microvilli of intestinal epithelial cells of *Tilapia nilotica*. *Z Ernährungswiss*., **30**: 268-275.

Samuhasaneeto, S., Thong-Ngam, D., Kulaputana, O., Suyasunanont, D., Klaikeaw, N. (2009). Curcumin decreased oxidative stress, inhibited NF-κB activation, and improved liver pathology in ethanol-induced liver injury in rats. *J. Biomed. Biotechnol*., 1-8.

Sandahl, J. F., Baldwin, D.H., Jenkins, J. J., Scholz, N. L. (2005). Comparative thresholds for acetylcholinesterase inhibition and behavioral impairment in coho salmon exposed to chlorpyrifos. *Environ. Toxicol. Chem*., **24**: 136-145.

Sandal, S. and Yilmaz, B. (2010). Genotoxic effects of chlorpyrifos, cypermethrin, endosulfan and 2, 4-D on human peripheral lymphocytes cultured from smokers and nonsmokers. *Environ. Toxicol.*, **26(5)**: 433-442.

Saradhamani, N. and Kumari, S. B. (2011). Impact of insecticide (chloropyrifos) on the biochemical components of the fish, *Oreochromis mossambicus* (Peters, 1852). *Curr. Biotica.,* **5(1)**: 79-84.

Saraswati, R., Manalu, W., Ekastuti, D.R. , Kusumorini, N. (2013). The role of turmeric powder in lipid metabolism and its effect on quality of the first quail's egg. *J. Indonesian Trop. Anim. Agric.*, **38(2)**: 123-130.

Sardar, D. and Kole, R. K. (2005). Metabolism of chlorpyrifos in relation to its effect on the availability of some plant nutrients in soil. *Chemosphere*., **61**: 1273–1280.

Satyavardhan, K. (2013). Effect of fenvalarate and malathion on biochemical constituents of freshwater fish, *Ctenopharyngodon idella* (valenciennes). *World Appl. Sci. J*., **27(5)**: 649-655.

Sayeed, I., Parvez, S., Pandey, S., Hafeez, B.B., Haque, R., Raisuddin, S. (2003). Oxidative stress biomarkers of exposure to deltamethrin in freshwater fish, *Channa punctatus* Bloch. *Ecotoxicol. Environ. Saf*., **56**: 295–301.

Schwaiger, J. and Negele, R.D. (1998). Plasma vitellogenin - a blood parameter to evaluate exposure of fish to xenoestrogens. *Acta. Vet. Brno*., **67**: 257-264.

Schwaiger, J., Mallow, U., Ferling, H., Knoerr, S., Braunbeck, Th., Kalbfus, W., Negele, R.D. (2002). How estrogenic is nonylphenol? A transgenerational study using rainbow trout (*Oncorhynchus mykiss*) as a test organism. *Aquatic Toxicol*., **59**: 177–189.

Scibior, D. and Czeczot, H. (2006). Catalase: Structure, Properties, Functions. *Postepy Hig. Med. Dosw*., **60**: 170-180.

Seifter, S. and Dayton, S. (1950). The estimation of glucose with the anthrone reagent. *Arch. Biochem. Biophy.*, **25(1)**: 191-200.

Sharbidre, A. A., Metkari, V. and Patode, P. (2011). Effect of methyl parathion and chlorpyrifos on certain biomarkers in various tissues of guppy fish, *Poecilia reticulata*. *Pestic. Biochem. Phys*., **101**: 132-141.

Sharma, P., Shankar, S., Asha Agarwal, A., Singh, R. (2010). Variation in serum lipids & liver function markers in lindane exposed female wistar rats: Attenuating effect of curcumin, vitamin C & vitamin E. *Asian J. Exp. Biol. Sci*., **1 (2)**: 440-444.

Sherry, J. P. (2003). The Role of Biomarkers in the Health Assessment of Aquatic Ecosystems, Aquatic Ecosystem Health & Management, **6(4)**: 423-440.

Slaninova, A., Smutna, M., Modra, H., Svobodova, Z. (2009). A review: Oxidative stress in fish induced by pesticides. *Neuroendocrinol. Lett.,* **30(1)**: 2-12.

Sledge, D., Yen, J., Morton, T., Dishaw, L., Petro, A., Donerly, S., Elwood Linney, E., Levin, E. D. (2011). Critical duration of exposure for developmental chlorpyrifos-induced neurobehavioral toxicity. *Neurotoxicol. Teratol.,* **33**: 742-751.

Slotkin, T. A. (2004). Guidelines for developmental neurotoxicity and their impact on organophosphate pesticides: a personal view from an academic perspective. *Neurotoxicology*., **25**: 631-40.

Sobti, R.C., Krishan, A., Pfaffenberger, C.D. (1982). Cytokinetic and cytogenetic effects of some agricultural chemicals on human lymphoid cells in vitro: organophosphates. *Mutat. Res.,* **102**: 89-102.

Sökmen, M. and Khan, M. A. (2016). The antioxidant activity of some curcuminoids and chalcones. *Inflammopharmacol*., **24**: 81–86.

Song, S. B., Xu, Y., Zhou, B. S. (2006). Effects of hexachlorobenzene on antioxidant status of liver and brain of common carp (*Cyprinus carpio*). *Chemosphere*., **65**: 699–706.

Sorbera, L.A., Asturiano, J.F., Carrillo, M., Cerda, J., Kime, D.E., Zanuy, S. (1999). In vitro oocyte maturation in the sea bass: effects of hCG, pituitary extract and steroids. *J. Fish Biol*., **55**: 9–25.

Sousa, F., Guebitz, G. M., Kokol, V. (2009). Antimicrobial and antioxidant properties of chitosan enzymatically functionalized with flavonoids. *Process Biochem*., **44**: 749–756.

Srivastava, S. K., Tiwari, P. R., and Srivastav, A. K. (1990). Effects of Chlorpyrifos on the Kidney of Freshwater Catfish, *Heteropneustes fossilis*. *Bull. Environ. Contam. Toxicol.,* **45**: 748-751.

Struger, J., Grabuski, J., Cagampan, S., Sverko, E., Marvin, C. (2016). Occurrence and distribution of carbamate pesticides and metalaxyl in southern ontario surface waters 2007–2010. *Bull. Environ. Contam.Toxicol*., **96**: 423–431.

Stalin, A., Suganthi, P., Mathivani, S., Paray, B. A., Sadoon, M. K.A., Gokula, V., Musthafa, M. S. (2019). Impact of chlorpyrifos on behavior and histopathological indices in different tissues of freshwater fish *Channa punctatus* (Bloch). *Environ. Sci. Pollut. Res*., **26**: 17623–17631.

Sun, T., Zhou, D., Mao, F., Zhu, Y. (2007). Preparation of low-molecular-weight carboxymethyl chitosan and their superoxide anion scavenging activity. *Eur. Polym. J.*, **43**: 652–656.

Sundararaj, B.I. and Anand, T.C. (1972). Effects of Piscine and Mammalian Gonadotropins on Gametogenesis in the Catfish, *Heteropneustes fossilis* (Bloch). *Gen. Comp. Endocrinol*., **3**: 683-702.

Sundararaj, B. I. and Vasal, S. (1976). Photoperiod and temperature control in the regulation of reproduction in the female catfish *Heteropneustes fossilis*. *J. Fish. Res. Board Can*., **33**: 959-973.

Sundararaj, B.I., Goswami, S.V., Lamba, V.J. (1982). Role of testosterone, estradiol-17β, and cortisol during vitellogenin synthesis in the catfish, *Heteropneustes fossilis* (Bloch). *Gen. Comp. Endocrinol*., **48**: 390-397.

Sunanda, M., Rao, S.C.J., Neelima, P., Rao, G.K., Simhachalam, G. (2016). Effects of chlorpyrifos (an organophosphate pesticide) in fish. *Int. J. Pharm. Sci. Rev. Res.*, **59**: 299-305.

Tarasub, N., Junseecha, T., Tarasub, C., Ayutthaya, W.D.N. (2012). Protective effects of curcumin, vitamin C, or their combination on cadmium-induced hepatotoxicity. *J. Basic Clin. Pharm.* **3(2)**: 273-81.

Teh, S. J., S.M. Adams, S. M., Hinton, D. E. (1997). Histopathologic biomarkers in feral freshwater fish populations exposed to different types of contaminant stress. *Aquat. Toxicol*., **37**: 51-70.

Thibaut, R. and Porte, C. (2004). Effects of endocrine disrupters on sex steroid synthesis and metabolism pathways in fish. *J. Steroid Biochem. Mol. Biol*., **92**: 485-494.

Thomas, C. N. and Mansingh, A. (2002). Bioaccumulation, elimination, and tissue distribution of chlorpyrifos by red hybrid tilapia in fresh and brackish waters, *Environ. Technol*., **23(11)**: 1313-1323.

Tilak, K. S.S., Veeraiah, K. and Rao, D. K. (2005). Biochemical changes induced by chlorpyrifos, an organophosphate compound in sublethal concentrations to the freshwater fish *Catla catla, Labeo rohita* and *Cirrhinus mrigala*. *J. Environmen. Biol*., **26(2)**: 341-347.

Tiwary, B. K., Kirubagaran, R., Ray, A. K. (1998). Testosterone-mediated complete feminization with precocious ovarian maturation in catfish, *Heteropneustes fossilis* (Bloch). *Aquac. Res*., **29**: 333-336.

Tomczyk, M. O. (2019). Synergistic, antagonistic and additive antioxidant effects in the binary mixtures. *Phytochem. Rev.*, **63**: 103.

Topal, A., Atamanalp, M., Oruç, E., Demir, Y., Beydemir, S., Işık, A. (2014). In vivo changes in carbonic anhydrase activity and histopathology of gill and liver tissues after acute exposure to chlorpyrifos in rainbow trout. *Arh. Hig. Rada. Toksikol*., **65**: 377-385.

Topal, A., Alak, G., Ozkaraca, M., Yeltekin, A. C., Comakl, S., Acıl, G., Kokturk, M., Atamanalp, M. (2017). Neurotoxic responses in brain tissues of rainbow trout exposed to imidacloprid pesticide: Assessment of 8-hydroxy-2-deoxyguanosine activity, oxidative stress and acetylcholinesterase activity. *Chemosphere*., **175**: 186-191.

Tripathi, G. and Shasmal, J. (2011). Concentration related responses of chlorpyriphos in antioxidant, anaerobic and protein synthesizing machinery of the freshwater fish, *Heteropneustes fossilis*. *Pestic. Biochem. Phys*., **99(3)**: 215-220.

Trung, T. S., and Huynh N. D. B. (2015). Physicochemical properties and antioxidant activity of chitin and chitosan prepared from pacific white shrimp waste. *Int. J. Carbohydr. Chem.*, **2015**: 1-6.

Tsuda, T. (2018).Curcumin as a functional food-derived factor: degradation products, metabolites, bioactivity, and future perspectives. *Food Funct*., **9(2)**: 705-714.

Tvrdá, E., Tušimová, E., Kováèik, A., Paál, D., Greifová, H., Abdramanov, A., Lukáè, N. (2016). Curcumin has protective and antioxidant properties on bull spermatozoa subjected to induced oxidative stress. *Anim. Reprod. Sci.*, **172**: 10-20.

Ullah, S., Hasan, Z., Dhama, K. (2015). Toxic effects of endosulfan on behaviour, protein contents and antioxidant enzyme system in gills, brain, liver and muscle tissues of rohu, *Labeo rohita* . *Int. J. Pharmacol*., **12**: 1-10.

Ural, M.S. (2013). Chlorpyrifos-induced changes in oxidant/antioxidant status and haematological parameters of *Cyprinus carpio carpio*: Ameliorative effect of lycopene. *Chemosphere* ,**90**: 2059–2064.

Uri, N. (1961). Mechanism of antioxidation. In: Autoxidation and Antioxidants (Lundberg W.O., ed.) Interscience Wiley, New York (1), pp.133–169.

Vadhva, P. and Hasan, M. (1986): Organophosphate dichlorvos induced dose- related differential alterations in lipid levels and lipid peroxidation in various regions of the fish brain and spinal cord. *J. Environ. Sci. Health B*., **21(5)**: 413-424.

Valavanidis, A., Vlahogianni, T., Dassenaki, M., Michael Scoullos, M. (2006). Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicol. Environ. Safety*, **64**: 178–189.

Varo´, I., Serrano, R., Pitarch, E., Amat, F., Lo´pez, F.J., Navarro, J.C. (2002). Bioaccumulation of chlorpyrifos through an experimental food chain: Study of protein HSP70 as biomarker of sublethal stress in fish. *Arch. Environ. Contam.Toxicol*. **42**: 229–235.

Vasal, S. and Sundararaj, B. I. (1978). Thermal tolerance and preference of the Indian catfish *Heteropneustes fossilis*. *Environ. Biol. Fishes*., **3(3)**: 309-315.

Veal, E. A., Day, A. M., Morgan, B. A. (2007). Hydrogen Peroxide Sensing and Signaling. *Mol. Cell.,* **26(1)**: 1-14.

Veith, G.D., DeFoe, D.L., Bergstedt, B.V. (1979). Measuring and estimating the bioconcentration factor of chemicals in fish. *J. Fish Res. Board Can.,* **36(9)**: 1040-8.

Velmurugan, B., Selvanayagam, M., Cengiz, E. I., Unlu, E. (2009). Histopathological changes in the gill and liver tissues of freshwater fish, *Cirrhinus mrigala* exposed to dichlorvos. *Braz. Arch. Biol. Technol.,* **52 (5)**: 1291-1296.

Ventura, C., Núñez, M., Miret, N., Lamas, D.M., Randi, A., Venturino, A., Rivera, E., Cocca, C. (2012). Differential mechanisms of action are involved in chlorpyrifos effects in estrogen-dependent or -independent breast cancer cells exposed to low or high concentrations of the pesticide. *Toxicol. Letts*., **213**: 184-93.

Ventura, C., Nieto, M.R.R., Bourguignon, N., Lux-Lantos, V., Rodriguez, H., Caod, G., Randie, A., Cocca, C., Núñez., M. (2016). Pesticide chlorpyrifos acts as an endocrine disruptor in adult rats causing changes in mammary gland and hormonal balance. *J. Steroid Biochem. Mol. Biol.,* **156**: 1–9.

Verma, R. S., Mehta, A., Nalini Srivastava, N. (2007). In vivo chlorpyrifos induced oxidative stress: Attenuation by antioxidant vitamins. *Pest. Biochem. Physiol*., **88**: 191–196.

Waagbø, R., Thorsen, T., Sandnes, K. (1989). Role of dietary ascorbic acid in vitellogenesis in rainbow trout (*Salmo gairdneri*). *Aquaculture*, **80**: 301-314.

Wang, J. H., Zhu, L.S., Liu, W., Wang, J., Xie, H. (2012). Biochemical responses of earthworm (*Eisenia foetida*) to the pesticides chlorpyrifos and fenvalerate. *Toxicol. Mech. Methods*, **22(3)**: 236–241.

Wang, J., Wang, J., Zhu, L., Xie, H., Shao, B., Hou, X. (2014). The enzyme toxicity and genotoxicity of chlorpyrifos and its toxic metabolite TCP to zebrafish *Danio rerio*. *Ecotoxicology*, **23(10)**: 1858-69.

Watanabe, M., Tanaka, M., Kobayashi, D., Yoshiura, Y., Oba, Y., Nagahama, Y. (1999). Medaka (*Oryzias lapites*) FTZ-F1 potentially regulates the transcription of P-450 aromatase in ovarian follicles: cDNA cloning and functional characterization. *Mol. Cell Endocrinol.*, **149**: 221-228.

Wdzieczak, J., Zalesna, G., Wujec, E., Peres, G. (1982). Comparative studies on superoxide dismutase, catalase and peroxidase levels in erythrocytes and livers of different freshwater and marine fish species. *Comp. Biochem. Physiol.*, **73B**: 62–69.

Welling, W. and de Vries, J.W. (1992). Bioconcentration kinetics of the organophosphorus insecticide chlorpyrifos in guppies (*Poecilia reticulata*). *Ecotoxicol. Environ. Saf.*, **23**: 64-75.

Whitear, M., Zaccone, G., Ricca, M. B., Cascio, P.L. (1991). Fine structure and histochemistry of the venom gland in the Indian stinging catfish (*Heteropneustes fossilis*). *J. Zool*., **224(3)**: 479-489.

Wolff, S. E., Veldhoen, N., Helbing, C. C., Ramirez, C.A., Malpas, J.A., Propper, C.A. (2015). Estrogenic environmental contaminants alter the mRNA abundance profiles of genes involved in gonadal differentiation of the American bullfrog. *Sci. Total Environ*., **521-522**: 380-387.

Wright, J. S. (2002). Predicting the antioxidant activity of curcumin and curcuminoids. *J. Mol. Struct*., **591**: 207–217.

WHO. (2009). WHO Specifications and Evaluations for Public Health Pesticides. Chlorpyrifos *O, O*-diethyl *O*-3, 5, 6-trichloro-2-pyridyl phosphorothioate. World Health Organization, Geneva.

Wu, P., Liu, Y., Jiang, W. D., Jiang, J., Zhao, J., Yong-An Zhang, Y. A., Zhou, X. Q., Feng, L. (2017). A comparative study on antioxidant system in fish hepatopancreas and intestine affected by choline deficiency: different change patterns of varied antioxidant enzyme genes and Nrf2 signaling factors. *Plos One*., **12(1)**: e0169888.

Xia, W., Liu, P., Zhang, J., Jie Chen, J. (2011). Biological activities of chitosan and chitooligosaccharides. *Food Hydrocolloids*, **25**: 170-179.

Xing, H., Xu Wang, X., Sun, G., Xuejiao Gao, X., Xu, S., Wang, X. (2012). Effects of atrazine and chlorpyrifos on activity and transcription of glutathione S-transferase in common carp (*Cyprinus carpio* L.). *Environ. Toxicol. Pharmacol*., **33**: 233-244.

Yadav, M., Shukla, A. K., Srivastva, N., Upadhyay, S. N., Dubey, S. K. (2015). Utilization of microbial community potential for removal of chlorpyrifos: A review. *Crit. Rev. Biotechnol.*,36(4): 727-742.

Yadav, I. C. and Devi, N.L. (2017). Pesticides Classification and Its Impact on Human and Environment. In Environmental science and engineering (Kumar, A., Singhal J.C., Techato, K., Molina, L.T., Singh, N., Kumar, P., Kumar, P., Chandra, R., Caprio, S., Upadhye, S., Yonemura, S., Rao, S. Y., Zhang, T.C., Sharma, U.C., Abrol, Y.P. eds.). Studium Press LLC, USA, pp.140-158.

Yancheva, V., Velcheva, I., Georgieva, E., Mollov, I., Stoyanova, S. (2019). Chlorpyrifos induced changes on the physiology of common carp (*Cyprinus carpio* Linnaeus, 1785): A laboratory exposure study. *Appl. Ecol. Environ. Res*., **17(2)**: 5139-5157.

Yonar, M. E. and Sakin, F. (2011). Ameliorative effect of lycopene on antioxidant status in *Cyprinus carpio* during pyrethroid deltamethrin exposure. *Pestic. Biochem. Physiol*., **99**: 226–231.

Yildirim, N. C., Benzer, F., Danabas, D. (2011). Evaluation of environmental pollution at Munzur river of Tunceli applying oxidative stress biomarkers in Capoeta trutta (Heckel, 1843). *J. Anim. Plant Sci.*, **21(1)**: 66-71.

Yonar, M. E. (2018). Chlorpyrifos-induced biochemical changes in *Cyprinus carpio*: Ameliorative effect of curcumin. *Ecotoxicol. Environmen. Saf*., **151**: 49–54.

Yang, F. W., Zhao, G. P., Ren, F. Z., Pang, G. F., Lia, Y. X. (2020). Assessment of the endocrine-disrupting effects of diethyl phosphate, a nonspecific metabolite of organophosphorus pesticides, by in vivo and in silico approaches. *Environ. Int.*, **135**: 105383.

Yin, X.H., Zhu, G.N., Li, X.B., Liu, S.Y. (2009). Genotoxicity evaluation of chlorpyrifos to amphibian Chinese toad (Amphibian: Anura) by comet assay and micronucleus test. *Mutat Res.*, **680(1-2)**: 2-6.

Yu, K., Li, G., Feng, W., Liu, L., Zhang, J., Wu, W., Xu, L., Yan, Y. (2015). Chlorpyrifos is estrogenic and alters embryonic hatching, cell proliferation and apoptosis in zebrafish. *Chem. Biol. Interact*., **239**: 26–33.

Zahan, M. N., Islam, M. J., Mahajebin, T., Rahman, M. S., A.K.M.M Hossain, A. K.M.M. (2019). Toxicity bioassay of chlorpyrifos on some local fish species of northern Bangladesh. *Int. J. Agril. Res. Innov. Tech.*, **9(1)**: 42-47.

Zeilinger, J., Hartmann, T. S., Maser, E., Goller, S., Vonk, R. and Lange, R. (2009). Effects of synthetic gestagens on fish reproduction. *Environ. Toxicol. Chem*., **28 (12)**: 2663–2670.

Zhang, J., Shen, H., Wang, X., Wu, J., Xue, Y. (2004). Effects of chronic exposure of 2,4-dichlorophenol on the antioxidant system in liver of freshwater fish *Carassius auratus*. *Chemosphere,* **55**: 167–174.

Zhang, Y., Gao, J., Xu, P., Yuan, C., Qin, F., Liu, S., Zheng, Y.,Yang, Y.,Wang, Z. (2014). Low-dose bisphenol A disrupts gonad development and steroidogenic genes expression in adult female rare minnow *Gobiocypris rarus. Chemosphere*, **112**: 435–442.

Zhou, B., Liu, W., Siu, W. H. L., Desmond O'Toole, D., Lam, P.K.S., Wu, R.S.S. (2006). Exposure of spermatozoa to duroquinone may impair reproduction of the common carp (*Cyprinus carpio*) through oxidative stress. *Aquat Toxicol*., **77**: 136-142.

Zuberi, A., Naeem, M., Jalali, S. (2011). Effect of human chorionic gonadotropin (hCG) on in vitro oocyte maturation in freshwater cyprinid, *Barilius vagra*. *Afr. J. Biotechnol*, **10(74)**: 16986-16993.

List of Publications

J. Exp. Zool. India Vol. 23, No. 2, pp. 1043-1049, 2020 | www.connectjournals.com/jez | *ISSN 0972-0030*

EFFECT OF CURCUMIN, ASCORBIC ACID AGAINST CHLORPYRIFOS TOXICITY ON HISTOTEXTURE AND BIMOLECULAR CONTENT OF FRESH WATER CATFISH, *HETEROPNEUSTES FOSSILIS*

Abha Mishra* and Abhisweta Singh

Department of Zoology, Babasaheb Bhimrao Ambedkar University, Lucknow - 226 025, India.
*e-mail : drabhamishra@gmail.com

(Received 20 February 2020, Revised 12 May 2020, Accepted 18 May 2020)

ABSTRACT : Chlorpyrifos (CPF) is one of the most extensively used organophosphorus pesticides. The contamination of water bodies due to surface runoff or overspray is of major concern to animal health. The present study implicates the ameliorative effect of curcumin and ascorbic acid on CPF toxicity in fresh water catfish, *Heteropneustes fossilis*. Study focus on the biomolecular changes of important energy source compounds, glucose, protein and lipid in tissue *viz*. muscles, liver, kidney, gill and histotexture of stomach, alimentary canal, liver, kidney and gill. These biomolecules are essential for normal growth, metabolism and reproductive events. The understanding of different experimental setups concluded that sub-lethal dose of CPF decreases the glucose and protein content and increases the lipid content significantly. It also damaged histotexture. However, co-incubation of curcumin and ascorbic acid was found to be effective against CPF induced aberrations in biomolecules and anatomical changes. It helps catfish to regain health.

Key words : Anti-oxidants, ascorbic acid, curcumin, chlorpyrifos, *Heteropneustes fossilis.*

INTRODUCTION

Organophosphate pesticides used to enhance crop production. These pesticides via agricultural runoff, air drift, overspray and improper disposal enter into different aquatic habitats and disturbed normal physiology of aquatic animals including fish. Fish bioaccumulate these pesticides and transmit to other organism via food chain (McConnell *et al*, 1998). Chlorpyrifos (CPF) is one of the organophosphate pesticides. CPF caused behavioural and developmental defects (Levin *et al*, 2003; Devi and Mishra, 2013; Mishra and Verma, 2017), neurotoxic (Sledge *et al*, 2011; Mishra and Devi, 2014) and genotoxic effects (Ali *et al*, 2009; Mishra *et al*, 2014), oxidative stress and histopathological aberrations (Sabra and Mehana, 2015; Mishra and Singh, 2018) in non target animals including fish. Fish serve as useful bioindicator to assess level of water contamination (Van der Oost *et al*, 2003).

The histopathological changes in fish tissues and biochemical tests are necessary to monitor the acute or chronic toxicity effects of pesticides in ecosystem (Fredianelli *et al*, 2019). Carbohydrate is an important biochemical component of all animal tissues. It serves as a reservoir of energy in the form of glycogen. The monosacharides stimulate oocyte maturation and play important role in gamete quality maintenance (Mishra and Joy, 2004). Protein, building block of all tissues, is another major biochemical component serving as a major source of repair and energy for most of physiological functions including reproduction (Jagtap *et al*, 2011). Lipids are one of the most important structural components (Begum, 2005) and major source of stored chemical energy in fish body. They are vital for vitellogenesis and gonadal development. The exposure of organophosphorous pesticides has been found to induce alteration in biochemical profile in a variety of fishes (Barbieri and Ferreira, 2011). CPF released reactive oxygen species (ROS) by oxidative stress, and initiate a cascade of harmful reaction that caused severe hazards in the body (Miladinovic *et al*, 2018).

The generation of ROS can be combated by the anti-oxidants such as curcumin and ascorbic acid (Tvrdá *et al*, 2016; Padayatty *et al*, 2003). Curcumin, a polyphenol, present in turmeric (*Curcuma longa*) has been used as a spice and herbal medicine in India since ages. It has anti-oxidant, anti-inflammatory and anticancer properties

(Tsuda, 2018). It has been found to improve the anti-oxidant and protein status in fish *Anabas testudineus* (Manju *et al*, 2012). Ascorbic acid, commonly known as Vitamin C is a known ROS scavenger (Aly *et al*, 2010). It is a water soluble compound essential in reducing oxidative damage and maintaining normal metabolic functions (Padayatty *et al*, 2003).

The present study was performed to understand the attenuating effect of curcumin and ascorbic acid on CPF caused manipulation in glucose, protein and total lipid content and histopathology in different tissue of freshwater catfish, *Heteropneustes fossilis*.

MATERIALS AND METHODS

A commercial grade of pesticide, Hilban® (CPF; 20% EC) was purchased from the local agricultural farm. All other chemicals including L-ascorbic acid (Asc; Molychem) and curcumin (Cur; Himedia) were of analytical grade and obtained from local scientific suppliers, Lucknow.

Animal collection and acclimatization

Live and adult *Heteropneustes fossilis* (40 – 45 g) of resting phase (24 January, 2019) were collected from the local fish market. Fish were brought to laboratory, BBAU, Lucknow and kept in aquaria to acclimatize for one week under normal photoperiod and temperature. Fish were fed with goat liver daily *ad libitum*.

The experiments were conducted as per national guidelines of the ethical committee to avoid any kind of cruelty to the fish.

Experimental design

Acclimatized fish were incubated with fixed concentration of CPF (0.174µM/l; 1/10th of 96 hrs LC_{50}; Mishra and Verma, 2016), Cur (10 mg/l; Cao *et al*, 2015), Asc (5 mg/l; Kumar *et al*, 2009) and co-incubation with Cur and Asc. CPF toxicity was studied in presence of antioxidant separately as Cur and CPF, Asc and CPF. To evaluate combined antioxidant effect on CPF toxicity, fish

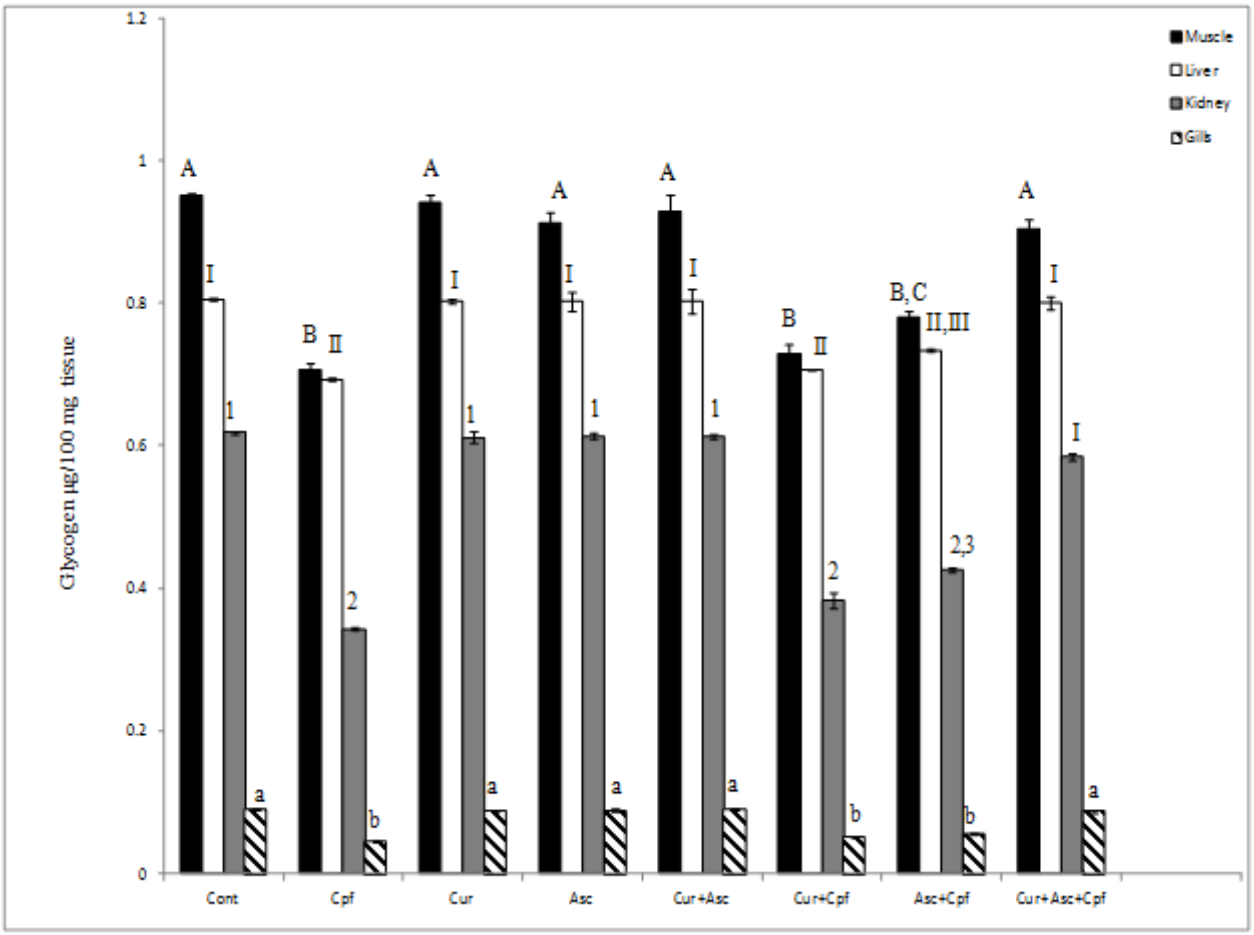


Fig. 1 : Effect of CPF and different combinations of anti-oxidants for 72h on glycogen content in muscle, liver, kidney and gill of *Heteropneustes fossilis*. Values are mean ± SEM of 5 fish in duplicates. Data were analyzed by one way ANOVA ($p < 0.001$) and Newman- Kuels' test ($p < 0.05$). Alphabets, A, B,… shows intergroup comparison between muscles. Roman I, II,..shows intergroup comparison between liver.Numericals,1,2,…shows intergroup comparison between kidney. Small letters, a,b….shows intergroup comparison between gill. Groups marked with same symbols are not significant and those with different letters are significantly different in intergroup comparison.

were maintained with Cur, Asc and CPF. Control fish were set side by side with only freshwater. The experimental setup with five fish in each group was maintained for 72hrs and the media was changed with fresh dose of chemicals every 24hrs to maintain same chemical constituents and to avoid any accumulation of metabolic wastes. At the end of experimental period, fish from each group was sacrificed and tissues (muscles, liver, kidney and gill) were dissected and stored at -20^{0}C for energy molecule analysis. A small part of tissue (stomach, alimentary canal, liver, kidney and gill) was fixed in Bouin's fixative for 24hrs for histotexture study.

Biochemical analysis

100mg of tissue (muscles, liver, kidney, gill) was taken and homogenized in 2ml of 0.6% saline and then centrifuged at 10,000rpm for 15min. 0.1ml of supernatant was taken for further estimation. Protein content was estimated by folin phenol reagent method (Lowry *et al*, 1951), tissue glycogen content was estimated by Seifter and Dayton (1950), and total lipid content was measured by Folch *et al* (1957) with UV-visible spectrophotometer (EVOLUTION 201, Thermoscientific).

Histotexture study

After 24hrs of fixation of tissues in Bouin's fluid, tissues were processed for paraffin blocks preparation and 5 microns thick sectioning (rotator microtome, Weswox, India). Microsections were double stained with haematoxylin-eosin for microscopic examinations (Olympus India).

Statistical analysis

Data of biomolecules analysis were represented as means ± SEM. The significance of values obtained from different groups tested by using one-way analysis of

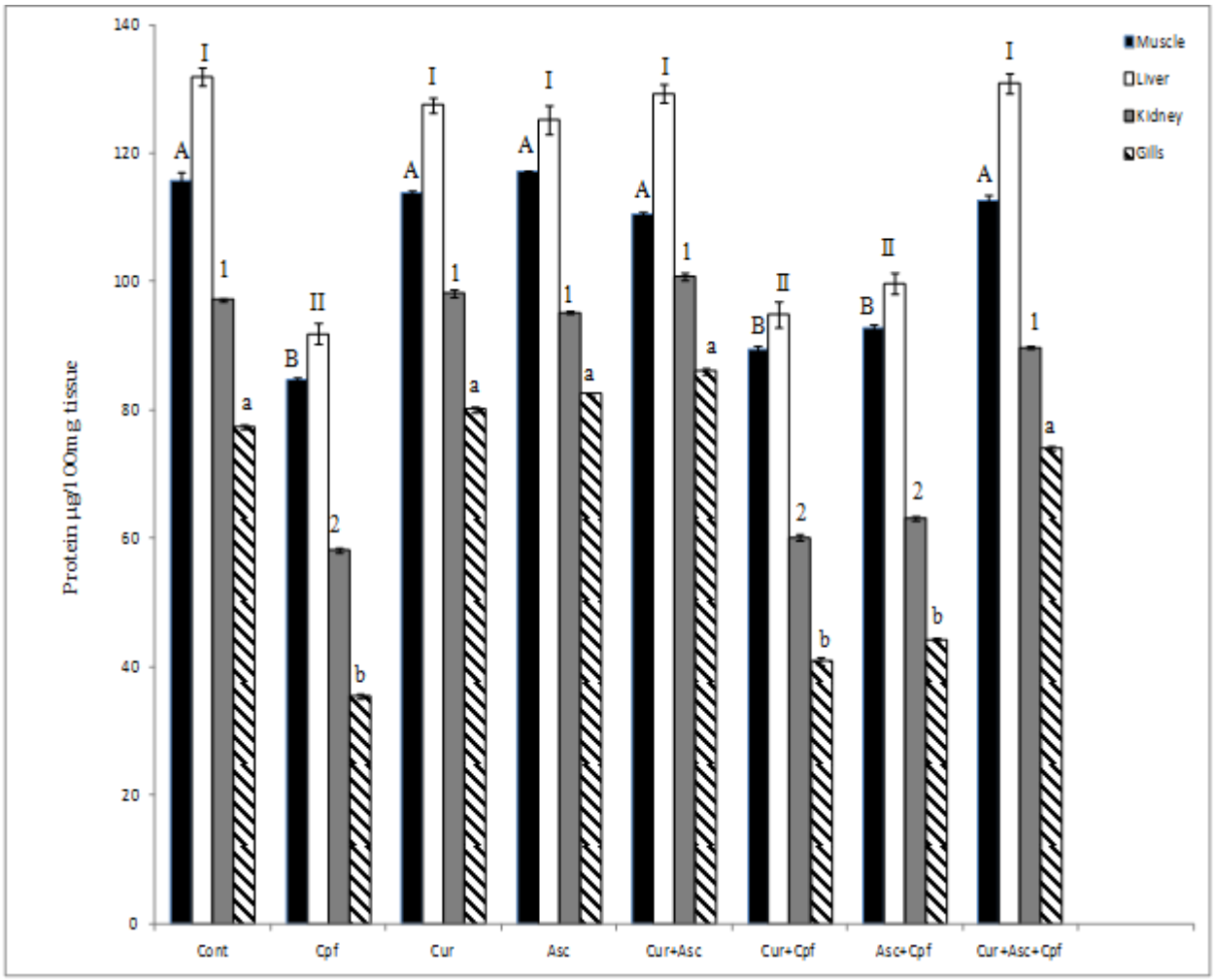


Fig. 2 : Effect of CPF and different combinations of anti-oxidants for 72h on protein content in muscle, liver, kidney and gill of *Heteropneustes fossilis*. Values are mean ± SEM of 5 fish in duplicates. Data were analyzed by one way ANOVA ($p < 0.001$) and Newman-Kuels' test ($p < 0.05$). Alphabets, A, B,… shows intergroup comparison between muscles. Roman I, II,.. shows intergroup comparison between liver. Numericals,1,2,…shows intergroup comparison between kidney. Small letters, a,b….shows intergroup comparison between gill. Groups marked with same symbols are not significant and those with different letters are significantly different in intergroup comparison.

variance (ANOVA). Intergroup comparisons were done by Newman-keuls' test (P<0.05).

RESULTS AND DISCUSSION

Effect of chlorpyrifos on biomolecules and histotexture

The glycogen and protein content in the tissues (muscles, liver, kidney and gill) exposed to sub-lethal concentration of CPF was found to be depleted significantly as compared to control (Figs. 1 and 2, P< 0.001). The decrease of glycogen was more in gill followed by kidney, muscle and liver. Higher glycogen depletion in gill reflected direct exposure and high energy consumptive processes to cope-up contaminated environment (Barron and Woodburn, 1989). Whereas, pattern of decrease in protein, was maximum in liver followed by muscle, kidney and gill. However, CPF was found to increase the total lipid content in all the tissues significantly in the order liver>muscles>kidneys>gills (Fig. 3, P< 0.001). The observed decline in protein and glycogen content indicates intense energy requirements to cope up stressed state (Barron and Woodburn, 1989; Tamizhazhagan *et al*, 2017). The total lipid content was found to be increased in all the tissues exposed to CPF. CPF act as an endocrine disruptor acting through estrogen receptor alpha (α) pathway (Ventura *et al*, 2016). The lipogenic activity increased by fatty acid synthesis or by inhibition of fatty acids β-oxidation and their utilization. It may result in increase in lipid content as done by malathion (Lal and Singh, 1987).

The normal structure of fish liver is mainly composed of large hexagonal cells. The hepatocytes are large and granulated, with distinct nucleus. After 72hrs of CPF exposure, necrotic changes with vacuolated hepatocytes

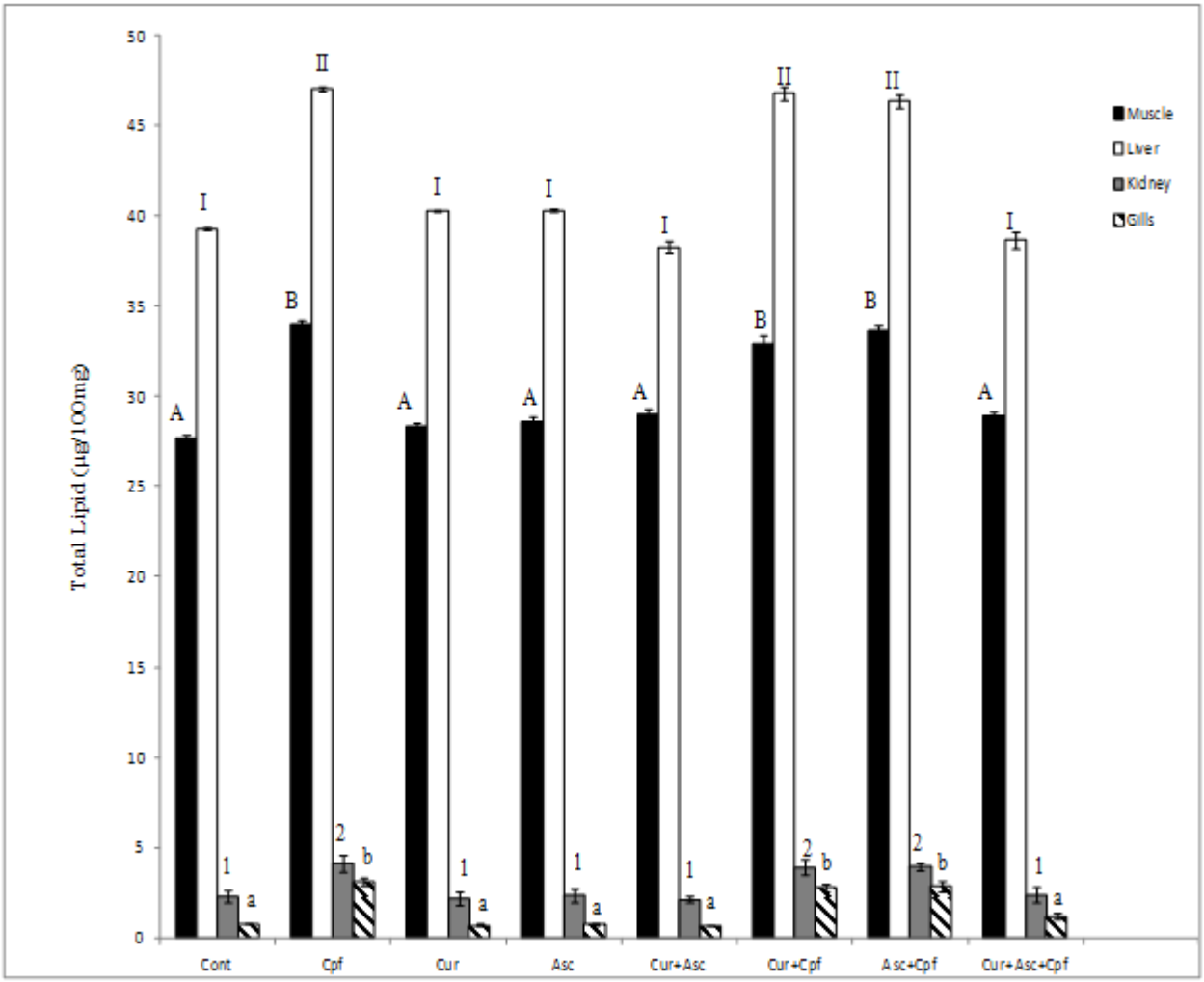


Fig. 3 : Effect of CPF and different combinations of anti-oxidants for 72h on lipid content in muscle, liver, kidney and gill of *Heteropneustes fossilis*. Values are mean ± SEM of 5 fish in duplicates. Data were analyzed by one way ANOVA (p< 0.001) and Newman- Kuels' test (p < 0.05). Alphabets, A,B,… shows intergroup comparison between muscles. Roman I, II,.. shows intergroup comparison between liver.Numericals,1,2,…shows intergroup comparison between kidney. Small letters, a,b….shows intergroup comparison between gill. Groups marked with same symbols are not significant and those with different letters are significantly different in intergroup comparison.

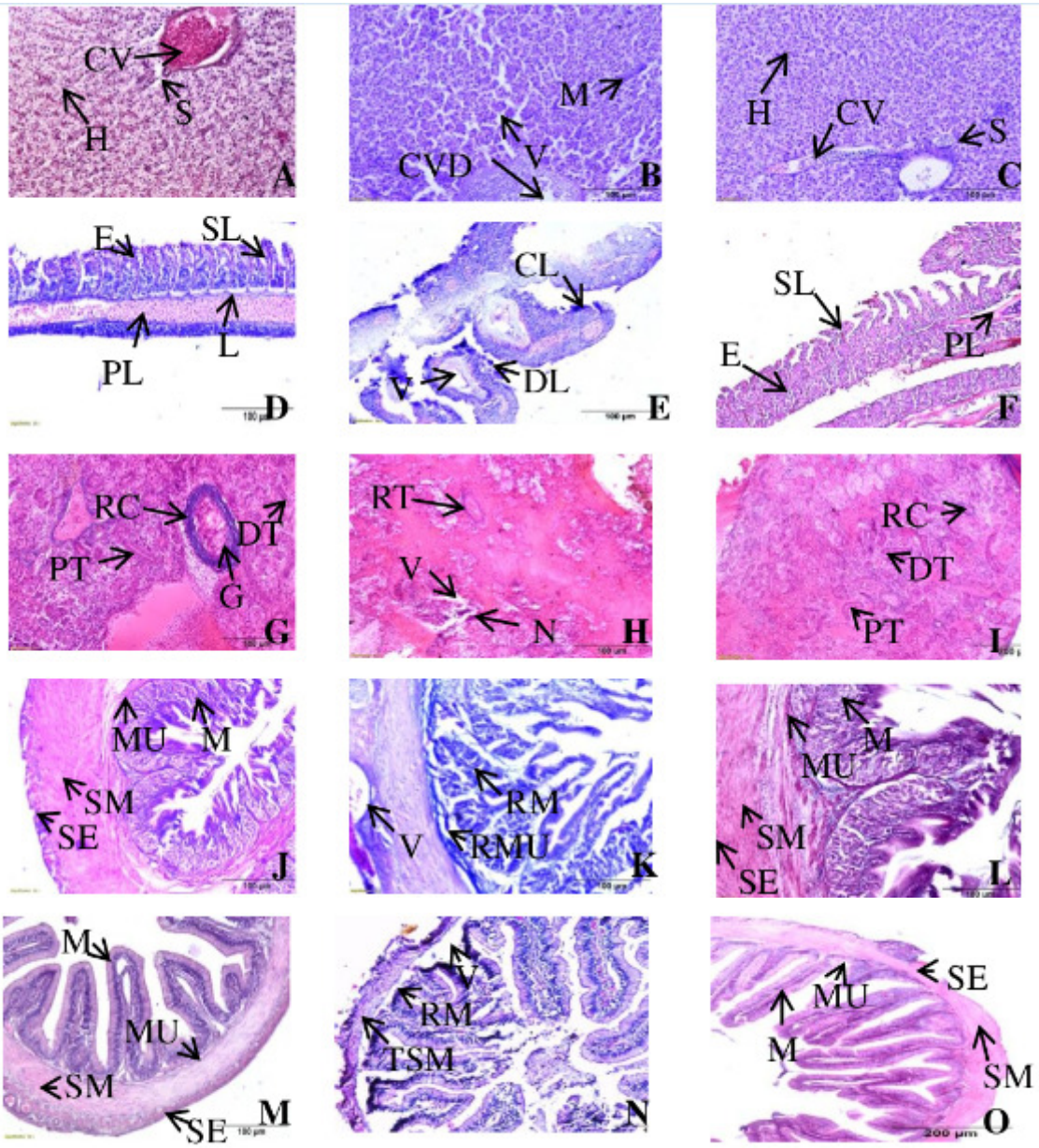


Fig. 4 : The combined bioremediation by ascorbic acid (Asc : 5 mg/l) and curcumin (Cur; 10 mg/l) on histopathological alterations induced by chlorpyrifos (CPF: 0.174 µM/lm 1/10th of 96 hrs LC_{50}) in catfish, *Heteropneustes fossils*, for three days (72 days) using haematoxylin-eosin double staining. Liver: (A) control (B) CPF (C) Asc + Cur + CPF, Gill: (D) control (E) CPF (F) Asc + Cur + CPF Kidney: (G) control (H) CPF (I) Asc + Cur + CPF, Stomach: (J) control (K) CPF (L) Asc + Cur + CPF, Intestine: (M) control (N) CPF (O) Asc + Cur + CPF. Where, CV : control vein, M : Melamomacrphage aggregation, PL: primary lamellae, L: lacunae, SL: secondary lamellae, E : epithelial cell, CL: curled lamellae, DL: damaged lamellae, RC : renal corpuscle, DT: distal tubule, G: glomerulus, PT: proximal tuble, RT: ruptured tubule, N: necrosis, SE : serosa, SB: submucosa, MU: muscularis, M: mucosa, RM: Ruptured Muscularis, TSM: thinning of submucosa [Image captured with 200X].

and indistinct nuclei were witnessed. Similarly, degenerative changes were also observed in gills, kidney, stomach and intestine. The primary and the secondary lamellae of gill were either fragmented or fused in CPF treated group. Hypertrophy and hyperplasia of epithelial cells, curling of secondary lamellae (CL) and degeneration of filaments (DL) was also noticed. CPF affected kidney by causing shrinkage of glomeruli, disruption of tubules, cytoplasm degeneration causing vacuolization (V). The stomach and intestine of *H. fossilis* is composed of mucosa (M), submucosa (SM), muscularis (MU) and serosa (S) layers, among which submucosa and mucosa was found to be vacuolated, serosa layer to be degenerated, and microvilli to be disrupted in CPF treated group. CPF caused atrophy of villi, necrosis and degeneration of mucosal epithetlium of intestine (Fig. 4).

Effect of curcumin, ascorbic acid and their combination treatment on CPF induced alteration in biomolecules and histotexture

The group having curcumin, ascorbic acid and their combination showed no significant alteration in the carbohydrate (Fig. 1), protein (Fig. 2) and total lipid content (Fig. 3) in all tissues with respect to the control group. The mild fall in carbohydrate level, suggested curcumin and ascorbic acid role in metabolism (Masuda *et al*, 2001; Iqbal *et al*, 2003; Aggarwal, 2010).

The group having curcumin, ascorbic acid and their combination with CPF showed varied effect on biomolecules as compared to CPF toxicity alone. Alone curcumin and ascorbic acid were unable to suppress CPF effect over carbohydrate, protein and lipid level in tissues. These two groups witnessed tissue aberrations, however, these aberrations were found to be less severe when compared to CPF alone (pictures not shown).

The coincubation of curcumin and ascorbic acid with CPF significantly revert biomolecular depletion as compared to CPF alone (Figs. 1, 2, 3). It may be due to synergistic response of antioxidants (Uri, 1961; Elballat, 2016) against CPF toxicity. The coincubation was found to be effective enough to attenuate the histopathological abnormalities caused by the CPF. The liver exhibited close to normal architecture with intact hepatocytes (Tarasub *et al*, 2012). The lamellae, arches and rays of gills were intact with no vacuolation. Similary, kidney cells also retained normal structure. The different layers of stomach and intestine were also found to be undisturbed (Fig. 4).

CONCLUSION

CPF decreases the glycogen and protein content and increases the total lipid in major tissues of fish. This indicates immediate energy requirement to avoid unhealthy and toxic effects of CPF on normal metabolism of fish. The individual combination of anti-oxidants, curcumin and ascorbic acid with CPF was found to lighten the stress effect as indicated in content of three major biomolecules. Results strongly suggested that the dual combination of curcumin and ascorbic acid act synergistic in attenuating the CPF induced stressed/toxic effect on glycogen, protein, lipid content and internal anatomy of the vital tissues.

ACKNOWLEDGEMENT

Authors thankfully acknowledge financial assistance from DBT (BT/PR 21245/AAQ/3/830/2016).

Conflict of interest : The authors declare that they have no conflict of interest.

REFERENCES

Aggarwal B B (2010) Targeting Inflammation-Induced Obesity and Metabolic Diseases by Curcumin and Other Nutraceuticals. *Ann. Rev. Nutr.* **30**, 173–199. doi: 10.1146/annurev.nutr. 012809. 104755.

Ali D, Nagpure N S, Kumar S, Kumar R, Kushwaha B and Lakra W S (2009) Assessment of genotoxic and mutagenic effects of chlorpyrifos in freshwater fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. *Food Chem. Toxicol.* **47**(3), 650-656. doi: 10.1016/ j.fct.2008.12.021.

Aly N, Gendy K E L, Mahmoud F and Sebae A K (2010) Protective effect of vitamin C against chlorpyrifos oxidative stress in male mice. *Pestic. Biochem. Phys.* **97**(1), 7-12. doi:10.1016/j.pestbp. 2009.11.007.

Barbieri E and Ferreira L A A (2011) Effects of the organophosphate pesticide Folidol 600® on the freshwater fish, Nile Tilapia *(Oreochromis niloticus). Pestic. Biochem. Phys.* **99**(3), 209-214. doi: 10.1016/j.pestbp.2010.09.002.

Begum G (2005) Toxicity of cypermethrin on total lipids and free fatty acids in liver, muscle, kidney and ovary of *Clarias batrachus* (L) and recovery response. *Toxicol. Environ. Chem.* **87**(2), 253-260. doi.org/10.1080/02772240500043223.

Cao L, Ding W, Du J, Jia R, Liu Y, Zhao C, Shen Y and Yin G (2015) Effects of curcumin on antioxidative activities and cytokine production in Jian carp (*Cyprinus carpio* var. Jian) with CCl4-induced liver damage. *Fish Shellfish Immunol.* **43**, 150-157. doi: 10.1016/j.fsi.2014.12.025.

Devi Y and Mishra A (2013) Study of behavioural and morphological anomalies of fry fish of fresh water teleost, *Channa punctatus* under chlorpyrifos intoxication. *Int. J. Pharm. Biol. Sci.* **4**(1), 865 - 874.

Elballat S E (2016) Protective effect of curcumin and vitamin C each alone and in combination on cisplatin-induced sperm abnormalities in male albino rats. *J. Basic Appl. Zool.* **76**, 52-59.doi: 10.1016/j.jobaz.2016.11.001.

Folch J, Lees M and Sloane S G H (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J. bio. Chem.* **226**(1), 497-509.

Fredianelli A C, Pierin V H, Uhlig S C and Galeb L A G (2019) Hematologic, biochemical, genetic, and histological biomarkers for the evaluation of the toxic effects of fipronil for *Rhamdia quelen*. *Turk. J. Vet. Anim. Sci.* **43**(1), 54-59.doi: 10.3906/vet-1806-71.

Barron M G and Woodburn K B (1995) Ecotoxicology of chlorpyrifos. In: *Reviews of Environmental Contamination and Toxicology* (ed: George W Ware) **144**, Springer science, New York. doi:10.1007/978-1-4612-2550-8_1.

Iqbal M, Sharma S D, Okazaki Y, Fujisawa M and Okada S (2003) Dietary supplementation of curcumin enhances antioxidant and phase II metabolizing enzymes in ddY male mice: possible role in protection against chemical carcinogenesis and toxicity. *Pharmacol. Toxicol.* **92**, 33-38. doi:10.1034/j.1600-0773. 2003.920106.x.

Jagtap J T, Shejule K B and Ubarhande S B (2011) Acute effect of TBTCL on protein alteration in freshwater bivalve, *Lamellidens marginalis. Int. Multidiscip. Res. J.* **1**(8), 13-16.

Kumar P, Prasad Y, Patra A K, Ranjan R, Swarup D, Patra R C and Pal

S (2009) Ascorbic acid, garlic extract and taurine alleviate cadmium-induced oxidative stress in freshwater catfish (*Clarias batrachus*). *Sci. Total Environ.* **407**, 5024-5030.doi: 10.1016/j.scitotenv.2009.05.030.

Lal B and Singh T P (1987) Impact of pesticides on lipid metabolism in the freshwater catfish, *Clarias batrachus*, during the vitellogenic phase of its annual reproductive cycle. *Ecotox. Environ. Safe.* **13**, 13-23.doi: 10.1016/0147-6513(87)90038-8.

Levin E D, Chrysanthis E, Yacisin K and Linney E (2003) Chlorpyrifos exposure of developing zebrafish: effects on survival and long-term effects on response latency and spatial discrimination. *Neurotoxicol. Teratol.* **25**(1), 51-57. doi: 10.1016/s0892-0362(02)00322-7.

Lowry O H, Rosebrough N J, Farr A I and Randall R J (1951) Protein measurement with the Folin phenol reagent. *J. bio. Chem.* **193**(1), 265-275.

Manju M, Akbarsha M A and Oommen O V (2012) *In vivo* protective effect of dietary curcumin in fish *Anabas testudineus* (Bloch). *Fish Physiol. Biochem.* **38**(2), 309-318. doi: 10.1007/s10695-011-9508-x.

Masuda T, Maekawa T, Hidaka K, Bando H, Takeda Y and Yamaguchi H (2001) Chemical studies on antioxidant mechanisms of curcumin: analysis of oxidative coupling products from curcumin and linoleate. *J. Agr. Food Chem.* **49**, 2539–2547. doi: 10.1021/jf001442x.

McConnell L L, Lenoir J S, Datta S and Seiber J N (1998) Wet deposition of current-use pesticides In the Sierra Nevada Mountain Range, California, USA. *Environ. Toxicol. Chem.* **17**(10), 1908–1916. doi:10.1002/etc.5620171003.

Miladinovic, C D, Borozan S and Ivanovic S (2018) Involvement of cholinesterases in oxidative stress induced by chlorpyrifos in the brain of Japanese quail. *Poultry Sci.* **97**(5), 1564-1571. doi: 10.3382/ps/pey018.

Mishra A and Joy K P (2004) Ovarian monosaccharides (glucose and fructose): hormonal effects and their role in final oocyte maturation and egg quality in catfish *Heteropnuestes fossilis*, Bloch. *Indian J. Exp. Biol.* **42**, 1084-1090.

Mishra A and Devi Y (2014) Histopathological alterations in the brain (optic tectum) of the fresh water teleost *Channa punctatus* in response to acute and subchronic exposure to the pesticide chlorpyrifos. *Acta Histochem.* **116**, 176-181. doi: 10.1016/j.acthis.2013.07.001.

Mishra A and Verma S (2017) In vitro effects of chlorpyrifos on gonadotropin-induced oocyte maturation and steroidal interplay of freshwater catfish, *Heteropneustes fossilis*. *Int. J. Pharma. Bio. Sci.* **8**(3), 912-920. doi: 10.22376/ijpbs.2017.8.3.b912-920.

Mishra A and Singh P (2018) Impact of chlorpyrifos on the ovary of the freshwater fish, *Channa punctatus*. *Int. J. Pharma. Bio. Sci.* **9**(3), 213-218. doi: 10.22376/ijpbs.2018.9.3.b219-225.

Mishra A and Verma S (2016) Acute toxicity bioassay of organophosphorus pesticide, chlorpyrifos on freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794). *Int. J. Fish Aquat. Stud.* **4**(6), 388-393.

Mishra A, Mishra N, Rawat A and Verma S (2014) Effect of an organophosphate insecticide, chlorpyriphos on mortality and haematology of freshwater butter catfish *Ompok bimaculatus* (Bloch, 1794). *Pensee.* **76**(5), 452-461.

Padayatty S J, Katz A, Wang Y, Eck P, Kwon O, Lee J H, Chen S, Corpe C, Dutta A, Dutta S K and Levine M (2003) Vitamin C as an antioxidant: evaluation of its role in disease prevention. *J. Am. Coll. Nutr.* **22**(1), 18-35. doi: 10.1080/07315724.2003.10719272.

Sabra S F and Mehana E E (2015) Pesticides Toxicity in Fish with Particular Reference to Insecticides. *Asian J. Agr. Food Sci*. **3**(1), 40-60.

Seifter S and Dayton S (1950) The estimation of glucose with the anthrone reagent. *Arch. Biochem. Biophy.* **25**(1), 191-200.

Sledge D, Yen J, Morton T, Dishaw L, Petro N, Donerly S, Linney E and Levin E D (2011) Critical duration of exposure for developmental chlorpyrifos-induced neurobehavioral toxicity. *Neurotoxicol. Teratol.* **33**(6), 742-51. doi: 10.1016/j.ntt.2011.06.005.

Tamizhazhagan V, Pugazhendy K, Sakthidasan V, Jayanthi C, Kim K H, Swawicka B, Pandiyan V, Kasinathan M, Baranitharan M and Ramarajan K (2017) The Toxicity Effect of Monocrotophos 36% E.C. on the Biochemical Change in *Catla catla* (Hamilton, 1882). *Glob. J. Pharmaceu. Sci.* **2**(2). doi: 10.19080/GJPPS.2017.02.555585.

Tarasub N, Junseecha T, Tarasub C and Na Ayutthaya W D (2012). Protective Effects of Curcumin, Vitamin C, or their Combination on Cadmium-Induced Hepatotoxicity. *J. Basic Clin. Pharm.* **3**(2), 273-81. doi: 10.4103/0976-0105.103821.

Tsuda T (2018) Curcumin as a functional food-derived factor: degradation products, metabolites, bioactivity, and future perspectives. *Food Funct*. **9**(2), 705-714. doi: 10.1039/c7fo01242j.

Tvrdá E, Tušimová E, Kováèik A, Paál D, Greifová H, Abdramanov A and Lukáè N (2016) Curcumin has protective and antioxidant properties on bull spermatozoa subjected to induced oxidative stress. *Anim. Reprod. Sci.* **172**, 10-20. doi: 10.1016/j.anireprosci.2016.06.008.

Uri N (1961) Mechanism of antioxidation. In: *Autoxidation and Antioxidants* (Lundberg W O eds.) Interscience Wiley, New York **1**, 133–169.

Van der Oost R, Beyer J and Vermeulen N P (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ. Toxicol. Phar.* **13**(2), 57-149. doi: 10.1016/s1382-6689(02)00126-6.

Ventura C, Nieto M R R, Bourguignon N, Lantos V L, Rodriguez H, Cao G, Randi A, Cocca C and Nunez M (2016) Pesticide chlorpyrifos acts as an endocrine disruptor in adult rats causing changes in mammary gland and hormonal balance. *J. Steroid Biochem. Mol. Biol*. **156**, 1-9. doi: 10.1016/j.jsbmb.2015.10.010.

DocID: https://connectjournals.com/03896.2020.20.5065

Biochem. Cell. Arch. Vol. 20, No. 2, pp. 5065-5072, 2020 | www.connectjournals.com/bca | *ISSN 0972-5075*

ATTENUATION OF CHLORPYRIFOS INDUCED TOXICITY IN FRESHWATER CATFISH, *HETEROPNEUSTES FOSSILIS*

Abha Mishra* and Abhisweta Singh

Department of Zoology, School of Life Sciences, Babasaheb Bhimrao Ambedkar University , Lucknow-226 025, India.
*Corresponding author e-mail : drabhamishra@gmail.com; abhishweta02@gmail.com

(Received 8 May 2020, Revised 12 September 2020, Accepted 19 September 2020)

ABSTRACT : Curcumin, ascorbic acid and chitosan (antioxidants) alone or in different combinations were used for the first time to access efficacy against agro-urban pesticide, chlorpyrifos (CPF) toxicity in the freshwater fish, *Heteropneustes fossilis*. The finding of different experimental setup concluded that sublethal dose of CPF (0.174µM/l; 1/10th of 96 hrs LC_{50}) causes histopathological alterations in main clearing/detoxifying organs (liver, kidney, gill), increases antioxidative enzymes (catalase, peroxidase) and lipid peroxidation significantly. Fish in CPF medium when supplemented with antioxidative agents (ascorbic acid, curcumin and chitosan) found with suppressed toxic effects. This decrease in CPF toxicity becomes significant when tested in combinations of antioxidative agents as compared to their alone positive (with CPF) control groups. Ascorbic acid and curcumin were most effective combination against CPF toxicity as compared to other conbinations.

Key words : Ascorbic acid, chlorpyrifos, chitosan, curcumin, *Heteropneustes fossilis*.

INTRODUCTION

Chlorpyrifos, an organophosphorus pesticide, used in the agricultural area to get a good crop. CPF inhibits acetylcholinesterase in the nervous system that causes acute and chronic effects and dragged toward death (Fulton and Key, 2001). Pesticide caused oxidative stress in non-target organisms including fish. Oxidative stress begins with an imbalance between the reactive oxygen species (ROS) production and the defence system to prevent the cellular damage (Bagchi *et al*, 1995; Chambers *et al*, 2001; Sánchez-Bayo, 2012). The ROS production mechanisms included inactivation of antioxidant enzymes and depletion of free radicals scavengers (Winston and Giulio, 1991). The ROS may lead to lipid peroxidation, protein oxidations, alteration of gene expression and redox status of the cell. These reactions initiated by the accumulation of free radicals and cholinergic hyperactivity after AChE inhibition (Slaninova *et al*, 2009).

In recent years, to assess the aquatic environment quality and various physiological changes in organism, more emphasis is made on the study of biochemical biomarkers. Among them, the antioxidant defence system is essential, that include enzymes induced by oxidative stress (Ahmad *et al*, 2000; Vlahogianni *et al*, 2007). Catalase (CAT) is an essential antioxidant enzyme in scavenging H_2O_2, which is the primary precursor of hydroxyl radical, a highly toxic form of ROS (Vlahogianni *et al*, 2007). Thus, increase in the CAT activity signifies its fight against H_2O_2 production. Reactive oxygen species leads to lipid peroxidation (LPO), which occurs naturally in small amounts in the body. The end products of lipid peroxidation, reactive aldehydes as malondialdehyde, 4-hydroxy-nonenal and acrolein are highly toxic to cellular viability. It is a self-propagating chain reaction leading to significant tissue damage (Mylonas and Kouretas, 1999). Peroxidase (POD) common in animals, plants and microorganisms, act as a free radical scavenging enzyme (Zhang *et al*, 2017).

In aquatic toxicology, histopathological alterations are indicators to assess the fish adaptations in both acute and chronic situations of polluted aquatic ecosystems (Javed *et al*, 2017). CPF caused significant histopathological changes like necrosis, inflammation, degeneration and haemorrhage in substantial tissues of fish (Devi and Mishra, 2013).

There are specific enzymatic and nonenzymatic antioxidants existing in the intracellular and extracellular environment to terminate the scavenging activities of ROS. Ascorbic acid (vitamin C) is a monosaccharide antioxidant. It acts as a reducing agent against ROS (Padayatty *et al*, 2003). Curcumin (diferuoyl methane), a natural phenol in turmeric (*Curcuma longa*) has been a part of traditional African and Asian medicine since 4000 years, to treat various diseases. It has many biological targets and cellular effects (Epstein *et al*, 2010). It is considered an

antioxidant, anti-inflammatory and ROS scavenger (Tvrdá *et al*, 2016). Chitosan is an N-acetylated derivative of the polysaccharide chitin, with antimicrobial and hepatoprotective properties. It has extensive application in agriculture, biotechnology, food industry and medicine because of its biocompatible, bioactive, biodegradable and adsorbent qualities (Mehrpak *et al*, 2015; Romanazzi *et al*, 2009).

The present study evaluated natural antioxidants (ascorbic acid, curcumin and chitosan) efficacy in attenuating the toxicity of sub-lethal concentration of chlorpyrifos in oxidative enzymes and histopathological alterations in liver, kidney and gills of *Heteropneustes fossilis*.

MATERIALS AND METHODS

Chemicals

Chlorpyrifos (20% EC CPF, $C_9H_{11}Cl_3NO_3PS$, Cas No. 2921-88-2) purchased from the local agricultural farm as Hilban®. All chemicals including L-ascorbic acid (Asc, $C_6H_8O_6$, Cas No. 50-81-7; Molychem), curcumin (Cur, $C_{21}H_{20}O_6$, Cas No. 458-37-7; Himedia), and chitosan (Chi, $C_{56}H_{103}N_9O_{39}$, Cas No. 9012-76-4; Biochemika) were of analytical grade and obtained from local scientific suppliers.

Animal collection and maintenance

Live adult *H. fossilis* (30–45 g) collected from local fish suppliers in the last week of January. Fish were acclimatised in laboratory conditions for two weeks under normal photoperiod and temperature (12h: 12h; light: dark and 23 ± 2^0 C). Fish fed goat liver daily *ad libitum*.

To avoid any cruelty to the animals, we followed national guidelines of the ethical committee to exhibit experiments.

Experimental setup

In this study, we selected established effective minimum concentrations of CPF and biomolecules viz., Asc, Cur, and Chi. Fish divided into fourteen experiment groups (Table 1). The experimental setup maintained for three days (72 hrs) by replacing with the fresh dose of chemicals every other day to avoid accumulation of metabolic wastes and keeping the same chemical constituents in each water tank.

After the completion of experimental duration, fish from each group sacrificed by decapitation. The liver, kidney and gill from each fish belonging to each group were isolated carefully and weighed. A small piece of tissues were fixed in Bouin's fixative for histological study, and the remaining stored at -20^0C for enzyme analysis.

Antioxidant enzyme activity and lipid peroxidation analysis

The tissues homogenized in 10% phosphate buffer (pH-7.4). The homogenised solution was centrifuged at 12,000 rpm for 20 minutes at 4^0 C. The supernatant used for the analysis of antioxidant enzymes.

Catalase (CAT) assay

For CAT activity (Aebi 1984), 0.1 ml of supernatant was added to 2.9 ml of freshly prepared 30Mm H_2O_2 in phosphate buffer (pH 7.0) and measured at 240 nm for 1 min.

Lipid Peroxidation (LPO) assay

For LPO (Rotruck *et al*, 1973), 1.0 ml of supernatant was added to 2 ml of thiobarbituric acid-hydrochloric acid-trichloroacetic acid (1:1:1) mixture. It was boiled for 15 minutes, allowed to cool and centrifuged at 3000 rpm for 10 minutes. The supernatant was taken and observed at 532 nm.

Peroxidase (POD) assay

In POD activity (Ohkawa *et al*, 1979), 3.0 ml of pyrogallol solution, 0.1ml of supernatant and 0.5 ml of H_2O_2 was added and mixed. The absorbance was taken at 430 nm.

All the absorbance were obtained by using UV-visible spectrophotometer (EVOLUTION 201, Thermoscientific).

Histological study

After the fixation of tissues in Bouin's fixative for 24 hrs, tissues were dehydrated and embedded in filtered paraffin. The paraffin tissue blocks were sectioned using rotator microtome (Weswox, India) instrument of 7 microns thickness. The sections after passing through series of graded alcohol (100%, 90%, 70%, 50% and 30%) and water were double stained in haematoxylin and eosin, cleared in xylene and mounted in DPX (dibutyl phthalate polystyrene xylene) for microscopic examinations.

Statistical analysis

Data for enzyme analysis were represented as means ± SEM. The significance of values obtained from different groups tested by using one-way analysis of variance (ANOVA; $p < 0.001$). Intergroup comparisons made by Newman-keuls' test ($p < 0.05$).

RESULTS AND DISCUSSION

In the current study, a sub-lethal dose of CPF treated group showed a significant increase in antioxidant enzymes (CAT, POD) and LPO level in gill, kidney and liver symbolize stress condition. Among the bio-remedial

groups, when the low concentration of different antioxidant agents (Asc, Cur and Chi) used with CPF, the antioxidant activities were reduced. However, the mono-molecule impact was less as compare to the remedy given in dual combinations of antioxidant agents. Among double combinations, the Cur and Asc were more influential in reducing oxidative stress as it brings down the antioxidant enzyme level. The effect supported by individual tissue anatomical texture. The combo of Asc and Cur reduced the CPF induced damage to gill, liver and kidney. This is the first report in experimenting with the potential additive ability of antioxidant agents against CPF. Synergism might arise either due to the regeneration of one antioxidant by the sacrificial oxidation of another antioxidant or due to a combination of two or more antioxidants with different mechanisms (Choe and Min, 2009). Antioxidants can delay or inhibit oxidation and its hazards (Gultekin *et al*, 2001).

Effect of biomolecules (Cur, Asc and Chi) on CPF induced antioxidant enzyme activity and lipid peroxidation

The oxidative stress is a part of the action mechanism of CPF (Gultekin *et al*, 2001). Oxidation can produce free radicals, leading to chain reactions that may damage cells. In the presence of the antioxidants, cells are protected against oxidative stress by an interacting network of antioxidant enzymes (Bagchi *et al*, 1995). They can decompose hydrogen peroxide and minimize the hazards due to oxidation. However, with the worse of the disease condition, antioxidant enzymes activity decreased as a result of an increase in oxidation reactions (Williams, 1928). The enzymes activity presented fluctuations in unhealthy or stress state (Williams, 1928; Weil-Malherbe *et al*, 1948). In our study liver, antioxidant enzymes activity were influenced more as compared to kidney and least in the gill. The liver immerges as the principal target organ in oxidative stress. A similar pattern was noticed by others too (Weil-Malherbe *et al*, 1948).

Effect on Catalase (CAT) activity

The CAT activity showed a significant overall change in experimental groups (I to XIV) and within the tested tissue (liver, kidney and gill) ($p < 0.001$; $F= 14.0$ for the liver; $F= 13.2$ for the kidney and $F= 9.9$ for gill; Fig. 1). The enzyme activity significantly increased ($p < 0.05$) in the CPF treated group as compared to negative and other positive control groups. However, the increase was more in the liver than the kidney followed by gill. Effect of positive controls with biomolecules alone or in mix groups

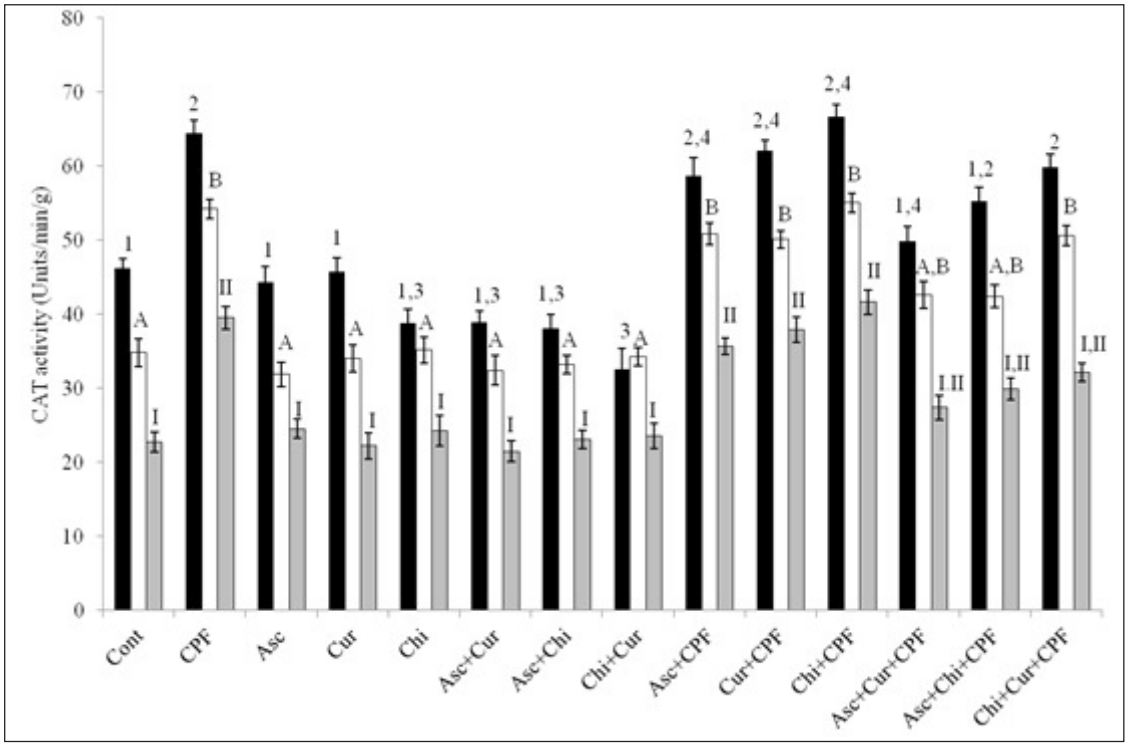


Fig. 1 : Effect of antioxidants (ascorbic acid: Asc, 5 mg/l; curcumin: Cur, 10 mg/l and chitosan: Chi, 150 mg/l) alone and combination on chlorpyrifos (CPF; 0.174µM/l, 1/10th of 96 hrs LC_{50}) induced catalase (CAT) activity in different tissue (liver: solid black bar; kidney: hollow bar; gill: solid gray bar) of catfish, *Heteropneustes fossilis*. Each group had five fish in duplicates for three days (72 hrs). Values expressed as mean ± SEM. Data were analyzed by one way ANOVA ($p < 0.001$) and Newman- Kuels' test ($p < 0.05$). Numerical 1, 2, 3 and 4 show intergroup comparison between liver groups. Alphabets A and B show an intergroup comparison between kidney groups. Roman I and II show an intergroup comparison between gill groups. Groups superscripted with same symbols are insignificant, and those with different letters or numbers are significantly different in intergroup comparison.

(III-VIII) was just like negative control group (I) for liver, kidney and gill tissue. The increase in the CPF induced CAT activity was attenuated significantly ($p < 0.05$) in the group XII (Asc + Cur + CPF) when compared to positive control (group II) in the tested tissue. Group XIII (Asc + Chit + CPF) had a significant decrease in CAT activity to the level of negative control in kidney and gill tissue, but in the liver, there was no significant decrease. In this Asc alone decreased CPF toxicity and produced an additive effect in combination with Cur and Chi. The Chi was unable to influence significantly CPF toxic effect alone (XI) or in combination with Cur (XIV) except in case of gill.

Effect on Lipid Peroxidation (LPO)

The LPO level showed significant changes (groups I to XIV) in all the three tested tissue ($p < 0.001$; F= 12.4 for the liver; F= 10.5 for kidney and F= 3.3 for gill; Fig. 2). The LPO found to increased significantly ($p < 0.05$) in CPF treated group in comparison to the negative control in the liver, kidney and gill. However, like CAT activity, there was no significant increase in LPO level among groups with biomolecule alone or dual combination (positive control; III, IV, V or VI, VII, VIII respectively). Experimental cluster 1 (CPF with mono biomolecule) and experimental cluster 2 (CPF with dual biomolecule combinations) both slightly reduced the toxic effect of CPF alone. The pattern of suppression was same in liver, kidney and gill.

Effect on Peroxidase (POD) activity

The POD activity shown significant changes in all tested tissue ($p < 0.001$; F= 115.7 for the liver; F= 85.0 for kidney and F= 69.3 for gill; Fig. 3). The POD activity in liver, kidney and gill increased significantly ($p < 0.05$) in CPF exposed fish (group II) when compared to negative control. In single or in dual combination mode biomolecules were able to truncate CPF increase in antioxidant enzyme activity. However, their level of significance varies with a combination and with tissue. In all three tissue dual combination of biomolecules with CPF (experiment cluster 2; group XII, XIII) were significantly reducing enzyme activity as compared to CPF alone. However, group XIV had not to score a significant decrease in POD activity in liver, kidney and gill tissue.

The sublethal concentration of CPF leads to a significant increase in CAT, LPO, POD in liver, kidney and gill. It suggests the considerable role of the antioxidant enzymes during oxidative stress as a defence mechanism. Therefore, antioxidant enzymes activity used as an

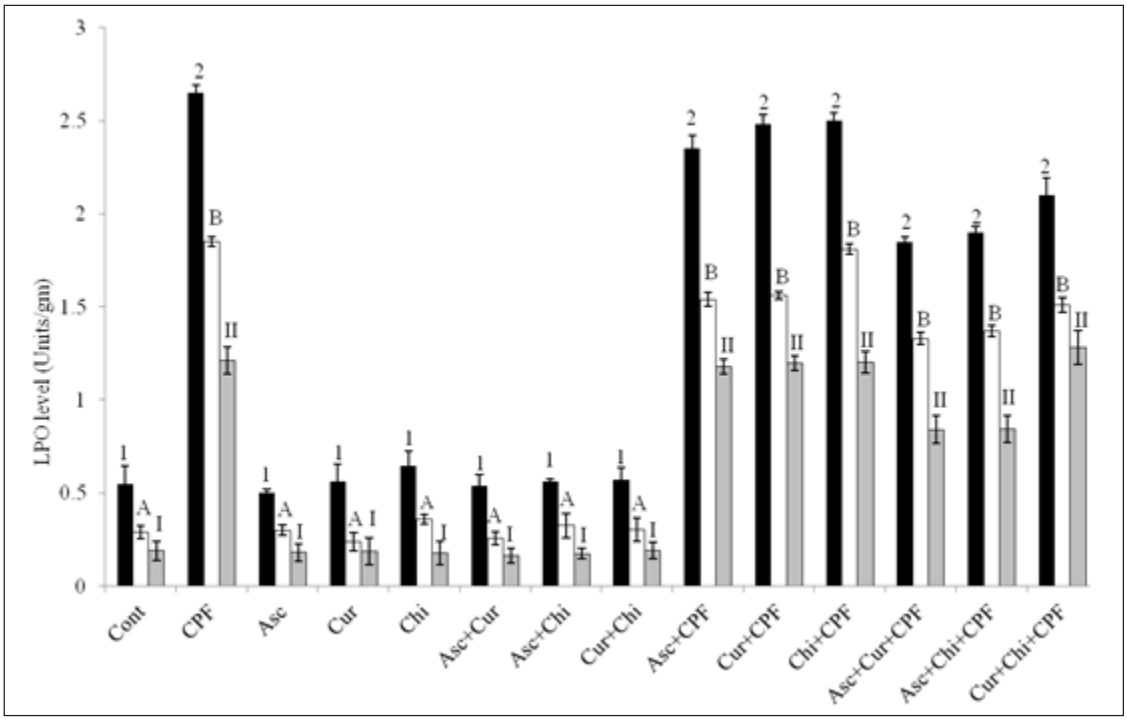


Fig. 2 : Effect of antioxidants (ascorbic acid: Asc, 5 mg/l; curcumin: Cur, 10 mg/l and chitosan: Chi, 150 mg/l) alone and combination on chlorpyrifos (CPF; 0.174µM/l, 1/10th of 96 hrs LC_{50}) induced lipid peroxidation (LPO) in different tissue (liver: solid black bar; kidney: hollow bar; gill: solid gray bar) of catfish, *Heteropneustes fossilis*. Each group had five fish in duplicates for three days (72 hrs). Values expressed as mean ± SEM. Data were analyzed by one way ANOVA ($p < 0.001$) and Newman- Kuels' test ($p < 0.05$). Numerical 1 and 2 show intergroup comparison between liver groups. Alphabet A and B show an intergroup comparison between kidney groups. Roman I and II show an intergroup comparison between gill groups. Groups superscripted with same symbols are insignificant, and those with different letters or numbers are significantly different in intergroup comparison.

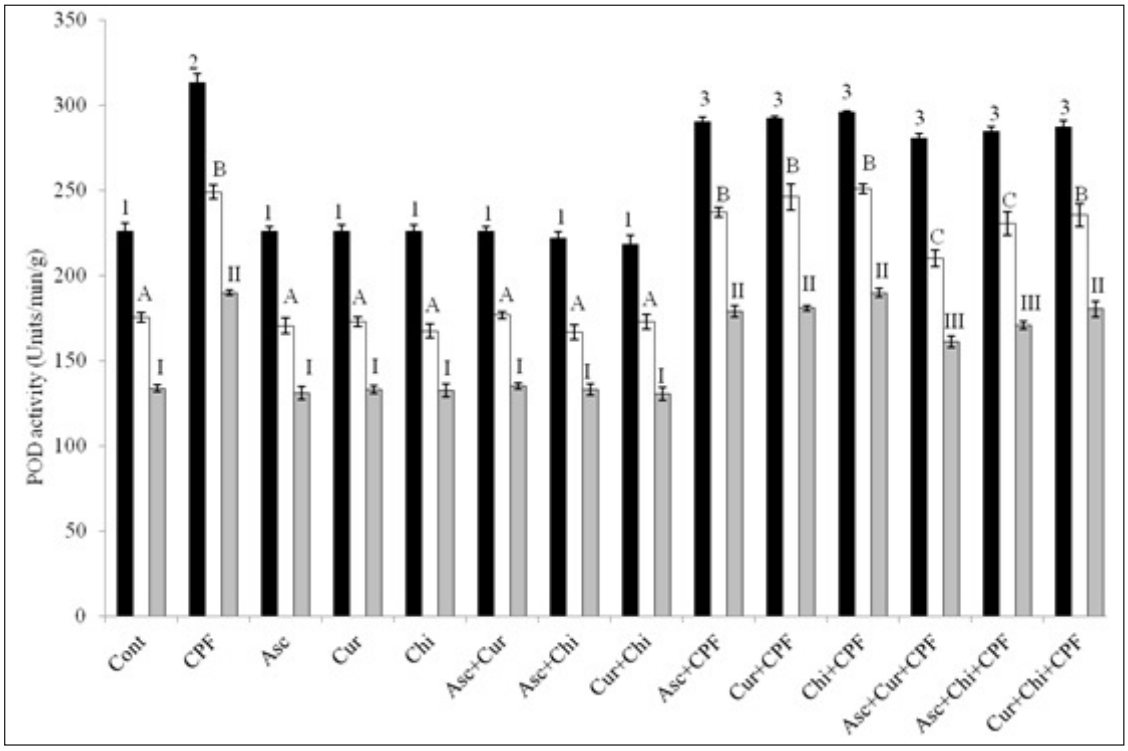


Fig. 3 : Effect of antioxidants (ascorbic acid: Asc, 5 mg/l; curcumin: Cur, 10 mg/l and chitosan: Chi, 150 mg/l) alone and combination on chlorpyrifos (CPF; 0.174µM/l, 1/10th of 96 hrs LC_{50}) induced peroxidase (POD) activity in different tissue (liver: solid black bar; kidney: hollow bar; gill: solid gray bar) of catfish, *Heteropneustes fossilis*. Each group had five fish in duplicates for three days (72 hrs). Values expressed as mean ± SEM. Data were analyzed by one way ANOVA ($p < 0.001$) and Newman- Kuels' test ($p < 0.05$). Numerical 1, 2 and 3 show intergroup comparison between liver groups. Alphabet A, B and C show an intergroup comparison between kidney groups. Roman I, II and III show an intergroup comparison between gill groups. Groups superscripted with same symbols are insignificant, and those with different letters or numbers are significantly different in intergroup comparison.

indicator of oxidative stress (like pesticide contamination) in the aquatic body (Kaur and Jindal, 2017). The administration of antioxidants; Asc, Cur and Chi, reduced CPF mounted free radicals and made mild to a significant decrease in the enzyme activity. Similarly, grape seed extract suppresses an increase in catalase during oxidative stress (Singh *et al*, 2013). Some other studies noticed a sharp decrease in antioxidant enzymes level during oxidative stress condition as a mechanism of interference in the enzyme cascade (Kaur and Jindal, 2017). The antioxidative enzymes perform differently in different fish species and tissue; this may be due to the difference in victim species, habitat and the causative agent's synergistic approach (Bagchi *et al*, 1995). Ascorbate, as well as antioxidants belonging to phenolics (Cur and Chi), can quench singlet oxygen reacts with lipids at a much higher rate than triplet oxygen (Singh *et al*, 2013). They are relieving results into deactivation of singlet oxygen by energy or charge transfer to ground state triplet oxygen (Decker, 2002). Asc reportedly found as an antioxidative agent and is considered as a protector against CPF toxicity like oxidative stress, neurological defects, haematological damages (Ambali and Ayo, 2012; Ozkan *et al*, 2012). The current study presents strong evidence of its oxidative damage reversal image caused by CPF. Cur alone effects were additive in the presence of Vitamin E against CPF toxicity for oxidative stress and histopathological changes in rat similar to current findings in fish (Hassani *et al*, 2015). Chi has anti-inflammatory and wound healing property (Azuma *et al*, 2015). Chi values show the additive result with Cur in the current study.

Effect of biomolecules (Cur, Asc and Chi) on CPF induced histopathological changes

Biomolecules (Cur, Asc, Chi) alone or in combinations not shown any damage in tissue texture (quality wise) as compared to negative control group animals (photomicrographs not shown). Among their combo trials with CPF, only Cur along with Asc was able to suppress CPF induced histotoxicity when compared to CPF alone.

Effect on liver

The histological section of liver from untreated group fish was parenchymatous in appearance containing polygonal hepatic cells with spherical nucleus amongst the sinusoids. The central vein was evident, and there was no lesion observed (Fig. 4A). All the positive control groups (with biomolecule alone or in dual combinations) except CPF have the same histological texture as in

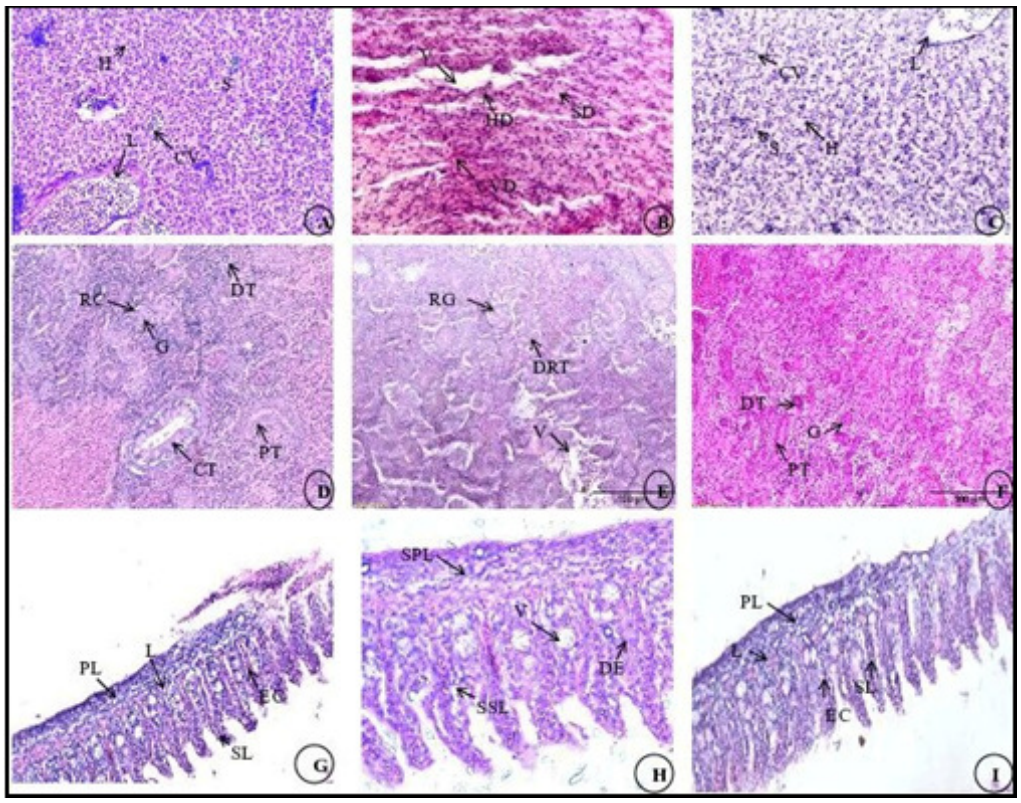


Fig. 4 : Bioremediation by ascorbic acid (Asc; 5 mg/l) and curcumin (Cur; 10 mg/l) (combo effect) on chlorpyrifos (CPF; 0.174 µM/l, 1/10th of 96 hrs LC_{50}) induced histopathology in catfish, *Heteropneustes fossilis*, after three days (72 hr) using haematoxylin-eosin double staining. Liver: (A) control (B) CPF (C) Asc + Cur + CPF, Kidney: (D) control (E) CPF (F) Asc + Cur + CPF, Gill: (G) control (H) CPF (I) Asc + Cur + CPF. Where, L: lobule, H: hepatocyte, S: sinusoids, CV: central vacuole, SD: sinusoid dilation, HD: hepatocyte degeneration, V: Vacuolization, RC: renal corpuscle, DT: distal tubule, G: glomerulus, PT: proximal tubule, CT: collecting tubule, DRT: disrupted renal tubule, RG: ruptured glomerulus,PL: primary lamellae, L: lacunae, SL: secondary lamellae, EC: epithelial cell, DE: damaged epithelium, SPL: shrinkage of primary lamellae, SSL: shrinkage of secondary lamellae, [Image captured with 200X].

negative control. The exposure of chlorpyrifos induced severe histopathological changes in the fish liver including hepatocyte degeneration, sinusoids dilation, granular cytoplasm, Vacuolization (Fig. 4B). These histological abnormalities were attenuated more in the fish co-incubated with CPF and the dual combination of biomolecules (Cur and Asc; Fig. 4C). In this photomicrograph, liver was intact as compared to CPF treated group (positive control).

Effect on kidney

The histological sections of the kidney in the control group showed numerous renal corpuscles, well-developed glomeruli and a system of tubules (Fig. 4D). In CPF treated groups irregular renal tubules, glomerular vacuolization, damaged renal corpuscle, severe degeneration in the tubules cells, haemorrhage and diffused erythrocytes in the interstitial fluid seen (Fig. 4E). However, such abnormalities reduced to a greater extent in groups containing a dual combination of antioxidants (Cur and Asc) with CPF (Fig. 4F).

Effect on gill

In control, the histological sections of gills showed primary and secondary lamellae, mucous cells. Secondary lamellae comprise pillar cells with a protective covering of mucous cells (Fig. 4G). In CPF treated groups the anomalies included hypertrophy and hyperplasia of epithelial cells that resulted in shortening, curling and clubbing of secondary lamellae with an increased number of mucous cells, degeneration of pillar cells, damaged epithelium was also observed (Fig. 4H). Gills of fish treated with the antioxidant in mono and the dual combination did not show toxic effect on gill anatomy. CPF induced anomalies reduced to a greater extent in the groups containing CPF and antioxidants (in dual mode; Cur and Asc, Fig. 4I).

CPF treated groups presented histopathological changes in liver, kidney and gill sections as compared to control. Oxidative stress released free radicals which disturb cellular protein, lipid and a nucleic acid that maintain the integral structure of the organ (Ortiz-Ordoñez *et al*, 2011). In liver damage, we found mainly hepatocyte degeneration and sinusoids dilation. That can be minimized

Table 1 : Experimental design to study bioremedials [ascorbic acid (Asc), curcumin (Cur), chitosan (Chi)] effects on chlorpyrifos (CPF) mounted stress in *Heteropneustes fossilis*. Setup maintained for three days (72 hrs). In each group five fish used in duplicate.

Group No.	Description	Reference
I	Negative control (with freshwater)	
Positive control		
II	CPF (0.174µM/l; of 1/10th 96 hrs LC_{50})	Mishra and Verma (2016)
III	Asc (5 mg/l)	Kumar *et al* (2009)
IV	Cur (10 mg/l)	Cao *et al* (2015)
V	Chi (150 mg/l)	Dautremepuits *et al* (2004)
VI	Asc + Cur	
VII	Asc + Chi	
VIII	Cur + Chi	
Experiment cluster 1		
IX	CPF + Asc	
X	CPF + Cur	
XI	CPF + Chi	
Experiment cluster 2		
XII	CPF + Asc + Cur	
XIII	CPF + Asc + Chi	
XIV	CPF + Cur + Chi	

in the presence of antioxidant biomolecules (Asc, Cur, Chi) in single or in additive mode (Adikwu and Deo, 2013). In the kidney, CPF treated groups shown irregular renal tubules, glomerular vacuolization, damaged renal corpuscle, severe degeneration and haemorrhage (Al-Amoudi, 2013). In gill, CPF treated groups the anomalies included hypertrophy and hyperplasia of epithelial cells. Degeneration of pillar cells, necrosis in primary lamellae also observed (Fontaínhas-Fernandes *et al*, 2008). The results of coincubation of CPF with antioxidising biomolecules reflect the microscopic examination of the respective tissue. Histopathological damage caused by CPF was more in the liver and so its reversal in the presence of antioxidants. Like antioxidizing enzymes, the histological parameters had more intact texture in dual biomolecule combination as compared to single biomolecule in the presence of CPF.

CONCLUSION

The present study evaluated the toxicity of chlorpyrifos to various tissues (liver, kidney, gills) in catfish, *H. fossilis* and investigated the potential mitigation of these harmful effects by antioxidant compounds (bioremedials; Asc, Cur and Chi). The experimental design used CAT, LPO and POD assays and histological samples to assess the efficacy of each bioremedial in isolation and combination with others. It was determined that individual compound did not significantly reduce chlorpyrifos toxicity; however, combinations of the bioremedials (Asc and Cur) decreased oxidative stress significantly in *H. fossilis*.

ACKNOWLEDGEMENTS

Authors thankfully acknowledge financial assistance from DBT (BT/PR 21245/AAQ/3/830/2016) and UGC (706/15) for Research Fellowship.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

REFERENCES

Adikwu E and Deo O (2013) Hepatoprotective effect of vitamin C (ascorbic acid). *Pharmacol. Pharma.* **4**, 84–92.

Aebi H (1984) Catalase *in vitro*. *Methods in Enzymol.* **105**, 121-126.

Ahmad I, Hamid T, Fatima M, Chand H S, Jain S K, Athar M and Raisuddin S (2000) Induction of hepatic antioxidants in freshwater catfish (*Channa punctatus*) is a biomarker of paper mill effluent exposure. *Biochim. Biophys. Acta* **1523**, 37-48.

Al-Amoudi W M (2013) Curcumin ameliorates nephrotoxicity and histopathological alterations induced by chlorpyrifos in albino rats. *J. Pharmacol. Res*. **3**(1), 64-69.

Ambali S F and Ayo J O (2012) Vitamin C attenuates chronic chlorpyrifos-induced alteration of neurobehavioral parameters in Wistar Rats. *Toxicol. Int.* **19**(2), 144-152.

Azuma K, Osaki T, Minami S and Okamoto Y (2015)Anticancer and anti-Inflammatory Properties of chitin and chitosan oligosaccharides. *J. Funct. Biomater.* **6**(1), 33–49.

Bagchi D, Bagchi M, Hassoun E A and Stohs S J (1995) *In vitro* and *in vivo* generation of reactive oxygen species, DNA damage and lactate dehydrogenase leakage by selected pesticides. *Toxicol.* **104**, 129-140.

Cao L, Ding W, Du J, Jia R, Liu Y, Zhao C, Shen Y and Yin G (2015) Effects of curcumin on antioxidative activities and cytokine production in Jian carp (*Cyprinus carpio* var. Jian) with CCl4-induced liver damage. *Fish Shellfish Immunol.* **43**, 150-157.

Chambers J E, Carr R L, Boone S and Chambers H W (2001) The metabolism of organophosphorus insecticides. In: *Handbook of Pesticide Toxicology*, Academic Press USA, pp. 919–927.

Choe E and Min D B (2009) Mechanisms of Antioxidants in the Oxidation of Foods. *Compr. Rev. Food Sci. F.* **8**(4), 345-358.

Dautremepuits C, Paris-Palacios S, Betoulle S and Vernet G (2004) Modulation in hepatic and head kidney parameters of carp (*Cyprinus carpio* L.) induced by copper and chitosan. *Comp Biochem. Physiol.C. Toxicol. Pharmacol.* **137**(4), 325-333.

Decker E A (2002) Antioxidant mechanisms. In: Akoh C C and Min D B (eds) *Food lipids*, 2nd ed. Marcel Dekker Inc New York, pp. 517–542.

Devi Y and Mishra A (2013) Study of behavioural and morphological

anomalies of fry fish of fresh water teleost, *Channa punctatus* under chlorpyrifos intoxication. *Int. J. Pharm. Biol. Sci.* **4**(1), 865 - 874.

Epstein J, Sanderson I R and Macdonald T T (2010) Curcumin as a therapeutic agent: the evidence from *in vitro*, animal and human studies. *Br. J. Nutr.* **103**(11), 1545-1557.

Fontaínhas-Fernandes A, Luzio A, Garcia-Santos S, Carrola J and Monteiro S (2008) Gill histopathological alterations in Nile Tilapia, *Oreochromis niloticus* exposed to treated sewage water. *Braz. Arch. Biol. Techn.* **51**(5), 1057-1063.

Fulton M H and Key P B (2001) Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. *Environ. Toxicol. Chem*. **20**(1), 37–45.

Gultekin F, Delibas N, Yasar S and Kilinc I (2001) *In vivo* changes in antioxidant systems and protective role of melatonin and a combination of vitamin C and vitamin E on oxidative damage in erythrocytes induced by chlorpyrifos-ethyl in rats. *Arch. Toxicol.* **75**(2), 88-96.

Hassani S, Sepand M R, Jafari A, Jaafari J, Rezaee R, Zeinali M, Tavakoli F and Razavi-Azarkhiavi K (2015) Protective effects of curcumin and vitamin E against chlorpyrifos-induced lung oxidative damage. *Hum. Exp. Toxicol.* **34**(6), 668-676.

Javed M, Ahmad M I, Usmani N and Ahmad M (2017) Multiple biomarker responses (serum biochemistry, oxidative stress, genotoxicity and histopathology) in *Channa punctatus* exposed to heavy metal loaded waste water. *Sci. Rep.* **7**(1675), 1-11.

Kaur M and Jindal R (2017) Oxidative stress response in liver, kidney and gills of *Ctenopharyngodon idellus* (Cuvier and Valenciennes) exposed to chlorpyrifos. *MOJ Biol. Med.* **1**(4), 103-112.

Kumar P, Prasad Y, Patra A K, Ranjan R, Swarup D, Patra R C and Pal S (2009) Ascorbic acid, garlic extract and taurine alleviate cadmium-induced oxidative stress in freshwater catûsh (*Clarias batrachus*). *Sci. Total Environ.* **407**, 5024-5030.

Mehrpak M, Banaee M, Haghi B N and Noori A (2015) Protective effects of vitamin C and chitosan against cadmium-induced oxidative stress in the liver of common carp (*Cyprinus carpio*). *Iran J. Toxicol.* **9**(30), 1360-1367.

Mishra A and Verma S (2016) Acute toxicity bioassay of organophosphorus pesticide, chlorpyrifos on freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794). *Int. J. Fish Aquat.* **4**(6), 388-393.

Mylonas C and Kouretas D (1999) Lipid peroxidation and tissue damage. *In Vivo* **13**(3), 295-309.

Ozkan F, Gündüz S G, Berköz M, Hunt A O and Yalýn S (2012) The protective role of ascorbic acid (vitamin C) against chlorpyrifos-induced oxidative stress in *Oreochromis niloticus*. *Fish Physiol. Biochem.* **38**(3), 635-643.

Ohkawa H, Ohishi N and Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* **95**(2), 351-358.

Ortiz-Ordoñez E, Uría-Galicia E, Ruiz-Picos R A, Duran A G, Trejo Y H, Sedeño-Díaz J E and López-López E (2011) Effect of yerbimat herbicide on lipid peroxidation, catalase activity, and histological damage in gills and liver of the freshwater fish *Goodea atripinnis*. *Arch. Environ. Contam. Toxicol.* **61**(3), 443-452.

Padayatty S J, Katz A, Wang Y, Eck P, Kwon O, Lee J H, Chen S, Corpe C, Dutta A, Dutta S K and Levine M (2003) Vitamin C as an antioxidant: evaluation of its role in disease prevention. *J. Am. Coll. Nutr.* **22**(1), 18-35.

Romanazzi G, Gabler F M, Margosan D, Mackey B E, Joseph L and Smilanick J L (2009) Effect of chitosan dissolved in different acids on its ability to control postharvest gray mold of table grape. *Phytopathology* **99**(9), 1028-1036.

Rotruck J T, Pope A L, Ganther H E, Swanson A B, Hafeman D G and Hoekstra W G (1973) Selenium: biochemical role as a component of glutathione peroxidase. *Science* **179**(4073), 588-590

Sánchez-Bayo F (2012) Insecticides mode of action in relation to their toxicity to non-target organisms. *J. Environment Analytic. Toxicol.* **S4**, 1-9.

Singh S, Kaur S and Budhiraja R D (2013) Chlorpyrifos-induced oxidative stress in rat's brain and protective effect of grape seed extract. *J. Phytopharmacol.* **2**(3), 26-33.

Slaninova A, Smutna M, Modra H and Svobodova Z (2009) A review: oxidative stress in fish induced by pesticides. *Neuro. Endocrinol. Lett.* **30**(1), 2-12.

Tvrdá E, Tušimová E, Kováèik A, Paál D, Greifová H, Abdramanov A and Lukáè N (2016) Curcumin has protective and antioxidant properties on bull spermatozoa subjected to induced oxidative stress. *Anim. Reprod. Sci.* **172**, 10-20.

Vlahogianni T, Dassenakis M, Scoullos M J and Valavanidis A (2007) Integrated use of biomarkers (superoxide dismutase, catalase and lipid peroxidation) in mussels *Mytilus galloprovincialis* for assessing heavy metals' pollution in coastal areas from the Saronikos Gulf of Greece. *Mar. Pollut. Bull.* **54**(9), 1361-137.

Weil-Malherbe H and Schade R (1948) Studies on the liver catalase of normal and cancerous rats. *Biochem. J.* **43**(1), 118–125.

Williams J (1928) The decomposition of hydrogen peroxide by liver catalase. *J. gen. Physiol.* **11**(4), 309-337.

Winston G W and Di G R T (1991) Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquat. Toxicol.* **19**, 137–161.

Zhang Y, Zhang L, Feng L, Mao L and Jiang H (2017).Oxidative stress of imidaclothiz on earthworm *Eisenia fetida. Comp. Biochem. Physiol.* **191**, 1–6.

Document Information

Analyzed document	Abhisweta Singh Final Thesis.docx (D90339209)
Submitted	12/21/2020 7:52:00 AM
Submitted by	O. P. Saini
Submitter email	gbl.bbau@gmail.com
Similarity	1%
Analysis address	gbl.bbau.bbau@analysis.urkund.com

Sources included in the report

SA	**Babasaheb Bhimrao Ambedkar University, Lucknow / Sneha Verma.pdf** Document Sneha Verma.pdf (D30081254) Submitted by: shodhganga.bbau@gmail.com Receiver: gbl.bbau.bbau@analysis.urkund.com	8
W	URL: https://oer.unn.edu.ng/read/oxidative-stress-biomarkers-haematological-parameters- ... Fetched: 8/31/2020 5:50:17 PM	1
W	URL: https://www.researchgate.net/publication/221956127_Sub-lethal_effects_of_profenofo ... Fetched: 11/18/2019 12:35:09 PM	1
W	URL: https://dyuthi.cusat.ac.in/jspui/bitstream/purl/2823/1/Dyuthi-T0843.pdf Fetched: 12/17/2020 7:43:31 PM	1
J	**Toxicity induced changes of CU and PB on the humoral and cellular factors on Anabas Testudineus Bloch1792** URL: 7b817414-c36f-4e31-9f0b-f3cf2e51355f Fetched: 3/16/2019 8:25:45 AM	1
W	URL: https://www.science.gov/topicpages/p/pesticide%252Bchlorpyrifos%252Bcpf Fetched: 12/21/2020 7:53:00 AM	1