Contents lists available at NCBI

The American Journal of Science and Medical Research

Journal homepage: http://ajsmrjournal.com/

Research Article

Effect of the drug Diclofenac on the brain tissue of *Channa punctatus*

Rohini P

Government Degree College, Thorrur, Mahabubabad distrct, Telangana State, India

*Corresponding author:
E-mail: rohinipadma28@gmail.com

ISSN: 2377-6196© 2018 The Authors. Published by Global Science Publishing Group. US.

https://dx.doi.org/10.5281/zenodo.7275472
Received: 28 June 2022;
Accepted: 29 August, 2022;
Published: 10 October, 2022

Keywords:
Diclofenac, Brain, *Channa punctatus*

ABSTRACT

Pharmaceuticals are the biologically active compounds that are designed to exert specific action on the target molecules of human and veterinary animals. The erroneous use of these drugs has resulted in aquatic pollution. Diclofenac is a non-steroidal anti- inflammatory drug that has been usually detected in surface waters worldwide. There are investigations on the toxicity of this drug in aquatic flora and fauna. The acute toxic studies on the histological alterations in fish are very rare. The brain is an important organ which controls all functions of the body. The freshwater fish, *Channa punctatus,* has been exposed to ten different concentrations of diclofenac for 96 hours. The median lethal concentration was found to be 25.28ppm. The fish were exposed to 8.42 ppm and 25.28 ppm concentrations of Diclofenac for 96 hours. The alterations noticed in the exposed fish were degeneration of nerve cells, vacuolization and necrosis of cells, atrophy, swelling of the axon and cellular damage in the interior and posterior regions. The findings of the study reveal that the drug Diclofenac has the toxic potential and can alter the structural integrity of the tissues in the fish. This investigation also signifies that histopathological alterations are the effective biomarkers in the study of pollution.

1. Introduction

Pharmaceuticals are the biologically active compounds that are designed to exert specific action on the target molecules of human and veterinary animals. The erroneous use of pharmaceuticals has resulted in continuous discharge of pharmaceuticals and their metabolites into the environment. Pharmaceuticals enter into the aquatic environment through different pathways and cause untoward effects in biota. The primary source of pharmaceuticals in the environment is human excretion (Williams 2005). The pharmaceuticals administered are incompletely absorbed and most of the parent drug or its metabolites are excreted through urine and feces. These chemicals enter into the wastewater treatment plants and subsequently into surface waters because these chemicals are not completely removed by the waste treatment plants (Zhang *et al.*, 2008).

The improper disposal of unused medication also contributes to aquatic pollution. The unused drugs are directly disposed of into the domestic sewage system (Bound and Voulvoulis, 2005). The application of sewage sludge to land causes pharmaceuticals to leach into surface and ground waters. The veterinary drugs also enter into water through animal excretions. The antibiotics and hormones used in aquaculture practices also cause pharmaceutical pollution. The hospital wastes and effluents from manufacturing industries are released into the waters and contaminate the waters (Larsson *et al.*, 2007).

Most pharmaceuticals are designed to target specific metabolic pathways in humans and animals. As most of the drug targets are evolutionarily conserved across different phyla, they may show impact on non-target organisms like invertebrates and lower vertebrates. Pharmaceutical residues are highly potent and as they are continuously released, even low level exposure may lead to chronic effects on a diverse range of organisms. The adverse effects caused by pharmaceutical compounds include acute toxicity, behavioral abnormalities, physiological changes, development of resistance in pathogenic bacteria, genotoxicity and endocrine disruption.

Pharmaceuticals comprise a wide spectrum of therapeutic classes which are used for the treatment of various disorders in humans. Diclofenac IUPAC name is 2(2,6- dichloroaniline) phenylacetic acid. Its generic name is Diclofenac Sodium, Diclofenac Potassium and Diclofenac Epolamine. Diclofenac is the widely prescribed non- steroidal anti inflammatory drug for treating both acute and chronic pain in various disorders like rheumatoid arthritis, osteoarthritis, spondylitis, ocular

inflammation, gout and dysmenorrhea (Skoutakis *et al*., 1988). It is available in the form of tablets, capsules, suppositories, intravenous solutions and injections. It is usually supplied in the form of either sodium or potassium salt.

2. Materials and Methods

The fresh water fish, *Channa punctatus, were* collected from the waters of Hasanparthy village of Warangal district, Telangana, India.

Analytical grade of Diclofenac sodium (2- [(2-6 Dichlorophenyl) amino] benzene acetic acid sodium salt, 99% pure (CAS 15307- 86-5) was purchased from Sara Exports, Ghaziabad, Uttar Pradesh, India. Diclofenac stock solution was prepared with acetone and ten different concentrations 5 ppm, 10 ppm, 15 ppm, 20 ppm, 25 ppm, 30 ppm, 35 ppm, 40 ppm, 45 ppm and 50 ppm were prepared from the stock solution.

2.1 Experimental procedures for Acclimatization and Test

The experiments were performed according to the standard methods to determine the LC_{50} of *Channa punctatus.* The healthy fish weighing about 100-110g and 20±1.21cm in length were transported to the laboratory in large plastic tanks and filled with water. The fish were washed in 1% potassium permanganate to free from microbial infections. The fishes were acclimatized in 50 liters capacity plastic tubs filled with dechlorinated water prior to experimentation. The fishes were fed *ad libitum* with commercial feed rice bran and oil cake twice a day. Proper aeration was provided with the help of aerators. The fish were maintained in tanks under 12:12 hour light : dark period. The dead fish were removed immediately to keep the water afresh. During acclimatization and test period, water was renewed for every 12 hours followed by the addition of desired concentration of the test compound. The fish were starved one day before experimentation.

2.2 Evaluation of Median Lethal Concentration

The concentration of the toxicant at which 50 percent of the test animals die during a specific period of time is referred to as Median lethal concentration (LC_{50}) or Lethal concentration. A group of 10 healthy fishes were exposed to ten different concentrations of the drug Diclofenac to calculate LC_{50} value. One set of fishes are maintained as control and were kept in tap water. The level of dissolved oxygen, pH, alkalinity, hardness and other parameters were monitored and maintained constantly. The mortality of fish was recorded for every 24 hrs during exposure period in control and ten different concentrations of Diclofenac. The whole experiment was carried out six times with each concentration and control. The median lethal concentration (LC_{50}) value was calculated after 96 hour using probit analysis method (Finney, 1971).

The calculation was done by probit analysis using Microsoft Excel Windows 10. The percent mortality, their logarithm values and probit values were incorporated into excel sheets. Regression analysis was done with the help of Windows 10.

The acute toxicity is usually studied by exposing fish to a chemical for 96 hours as it is the standard duration. The literature also defines acute toxicity of diclofenac at 96 hour period of exposure. Therefore, the fish were exposed for 96 hours to determine acute toxicity of diclofenac.

2.3 Histopathological examination

The histopathological studies were performed by taking the standard methods. (Humason, 1972). The freshwater fish, *Channa punctatus, were* exposed for 96 hours to sublethal and lethal concentrations of Diclofenac. At the end of exposure period, fish were randomly selected for histopathological examination. The live fish was sacrificed and the tissues from the brain were collected. The tissues were stored in Bouin's fixative medium for a period of 24 hours in order to immobilize the structure of the cell while maintaining morphological identity. After fixation the tissues were washed under tap water in order to remove traces of picric acid as it hinders the staining processes. Later the tissues were dehydrated to remove water from the tissues. The gradual removal of water from the tissues is done by using increasing concentrations of alcohol overnight gradually. They were dehydrated with 30%, 50%, 70%, 90% and 100% absolute alcohol for one hour in each concentration as it prevents the putrefaction of tissues. Later the tissues were impregnated with paraffin wax to make it firm for the purpose of section cutting. Prior to this clearing of the tissues is done by clearing agent xylene. Later the tissues were immersed in xylene for ten minutes. Two changes of xylene were given at a ten minutes time interval. Later, the tissues were kept for cold infiltration in a mixture of xylene and wax for two hours. Xylene brings about infiltration of paraffin into the tissues and makes the tissue transparent. For embedding, the tissues were soaked in paraffin wax melted at 57°C. All the tissues were given three to four changes for better impregnation. After the tissues were embedded with wax they were cast into blocks of paraffin. The tissues were fixed as blocks and were prepared, trimmed and kept overnight. The block was coated with lubricating gel to prevent wax block sticking. The blocks were left overnight in cold water to ensure that the wax had completely solidified. The wax block should be trimmed with a razor blade. The sides were cut so as to leave about 2-3 mm of wax around the tissue. The tissue sectioning was done with a rotary microtome, and serial sections were cut with an average 7µof thickness. The sections were deparaffinized in xylene and brought down to water via alcohol grades.

The staining was done with Hematoxylin Eosin. They were washed under running tap water for fifteen minutes. They were dehydrated via graded alcohol to 90%. They were counterstained in 0.49% eosin in 90% alcohol. The slides were rinsed in two changes of absolute alcohol. They were cleared in two changes of xylene of half an hour each and were mounted in DPX.

3. Results and Discussion

The results of the mortality of *Channa punctatus* on exposure to ten different concentrations of Diclofenac are presented in the Table-1 and graphically represented in Figure-1.

There was no mortality in 5ppm concentration of Diclofenac. There was 10 % mortality in both 10 ppm and 15ppm concentrations of diclofenac. 20% mortality was observed in 20ppm and 40% mortality in 25 ppm concentration. Fifty percent mortality was noticed in 30 ppm and 60% mortality in 35 ppm. There was 80% mortality in 40ppm, 90% mortality in 45ppm and 100 % in 50ppm of Diclofenac. The 96 hour LC_{50}

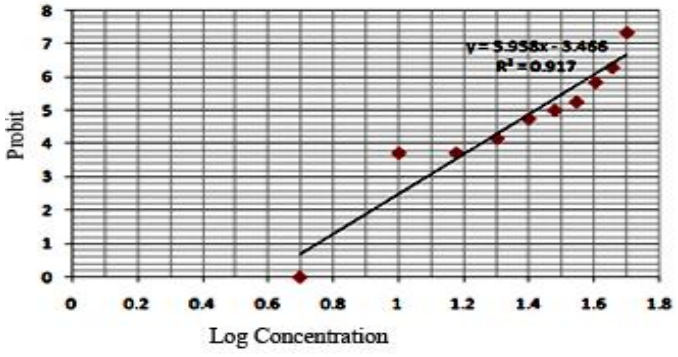
Table-1. Mortality of *Channa punctatus* on exposure to different concentrations of Diclofenac for 96 hours

S.No	Concentration of Diclofenac	Log Concentration	No. of fishes exposed	No. of fishes died at 96 hr	Probit Kill	Percent Kill
1	5 ppm	0.698	10	0	0	0
2	10 ppm	1	10	1	3.72	10
3	15 ppm	1.176	10	1	3.72	10
4	20ppm	1.301	10	2	4.16	20
5	25ppm	1.397	10	4	4.75	40
6	30ppm	1.477	10	5	5	50
7	35ppm	1.544	10	6	5.25	60
8	40ppm	1.602	10	8	5.84	80
9	45ppm	1.653	10	9	6.28	90
10	50ppm	1.698	10	10	7.33	100

value of diclofenac in *Channa punctatus* was found to be 25.28mg/L or 25.28ppm. One third of the median lethal concentration, 8.42 mg/L or 8.42ppm was taken as sublethal concentration for further evaluation.

The present study has shown a positive relationship between the mortality and level of concentration, as the concentration has increased, the rate of mortality also has increased. The same has been evidenced from various studies.

Figure-1. Mortality of *Channa punctatus* on exposure to different concentrations of Diclofenac expressed through Probit kill and Log concentration



The acute toxicity values of diclofenac for different fishes were reported by earlier workers. Ajima *et al*., (2015) have studied the acute toxicity of Diclofenac in *Clarius gariepinus* and 96 hrs. LC_{50} value was found to be 25.12 mg/L. Praskova *et al*., (2011) have studied acute toxicity in both juvenile and embryonic stages of *Danio rerio* and the LC_{50} mean values of diclofenac were found to be 166.6 ± 9.8 mg/L and 6.11 ± 2.48 mg/L respectively.

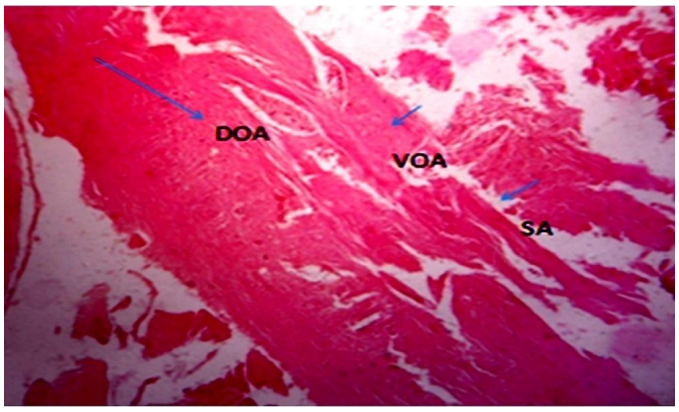
The acute toxicity study has clearly indicated that the rate of mortality for the fixed time increases with increase in concentration and for a particular concentration with increase in exposure time (Nilkhant and Sawant, 1993). The death of fish in higher concentrations of diclofenac may be due to hypoxemia or due to impaired oxygen uptake by the gills. While in lower concentrations, the slow intrusion of the drug might have primarily induced alterations in physiology and ultimately leading to death. Das and Sahu (2005) have reported that the major cause of mortality might be due to the damage of the respiratory epithelium by oxygen consummation during the formation of a mucus covering over the gills of fish.

3.1 Histological alterations in Brain

The brain of fish has five distinct regions namely telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon. Telencephalon consists of olfactory bulbs, olfactory lobes and cerebral hemispheres. The roof of the telencephalon is covered with membranous tissue and lateral ventricles are absent. The diencephalon contains the third ventricle and is composed of epithalamus, thalamus and hypothalamus. The mesencephalon is the center of sense and locomotion and consists of optic tectum and ventral tegmentum. The metencephalon occupies the interior portion of the dorsal wall of the fourth ventricle and is composed of cortex and medulla. The metencephalon includes cerebellum and pans varoli and is the integration center between auditory sense and sense of lateral line organs. The main part of the myelencephalon is medulla oblongata.

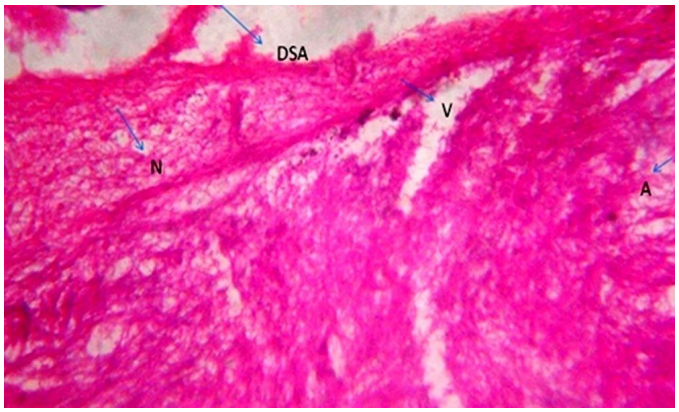
The brain of control fish has (Figure-2) shown the presence of neuronal cells, pyramidal cells and nissl substances. The fish exposed to sublethal concentrations of Diclofenac have shown degeneration of neuronal cells, swelling of pyramidal cells, loss of nissl substances, vacuolization and dystrophic changes (Figure-3). The fish exposed to lethal concentrations of Diclofenac have shown degeneration of nerve cells, vacuolisation and necrosis of cells in the brain, atrophy, swelling of axon and cellular damage in the interior and posterior regions (Figure-4). The damage was more intense in lethal concentration than sublethal concentration. The damage in the brain tissue may lead to abnormalities in the

physiological function which further may result in behavioral changes.



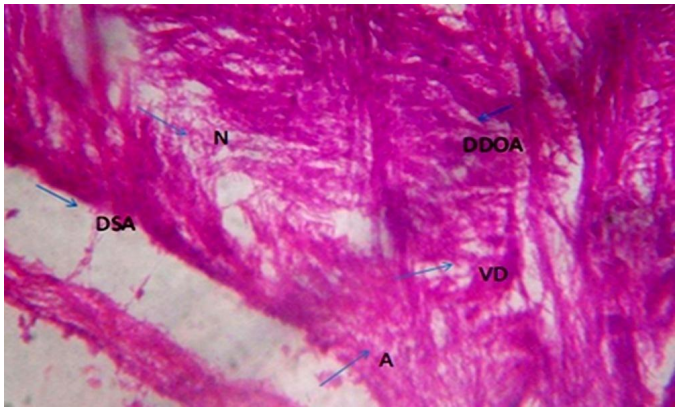
DOA- Dorsal olfactory area, VOA- Ventral olfactory area, SA- Septal area

Figure-2. Photomicrograph showing the brain tissue in control



DSA- Degenerated septal area, N-Necrosis, V-Vacuolation, A-Atrophy

Figure-3. Photomicrograph showing the brain tissue after exposure to sublethal concentration of Diclofenac



DSA- Degenerated septal area, N- Necrosis, DDOA- Degenerated dorsal olfactory area, A- Atrophy, VD-Vacuolar degeneration

Figure-4. Photomicrograph showing brain tissue after exposure to lethal concentration of Diclofenac

Meyer (1958) has noticed that lead acts directly on the cerebral vasculatures including blood-brain barrier and causes cerebral edema. Das and Mukherjee (2000) have reported the neurotoxic nature of hexachlorocyclohexane which induced vacuolation of brain parenchyma and moderate swelling of pyramidal cells of the cerebrum, loss of Nissl substances and glial cell reaction with evidence of glial nodule formation.

Santhakumar *et al.,* (2000) have exposed *Anabas testudineus* to sublethal concentrations of monocrotophos 1.9 mg/L, 4.75 mg/L and 9.5 mg/L for 21 days and reported that the pesticide has caused rupture of cortex, atrophy of molecular and granular layer, necrosis of neuro fibrillar region, vascular dilation, nuclear pyknosis, fibrosis, vacuolisation, cerebral edema and interzonal detachment.

Karuppasamy (2000) has observed vacuolation, dilation of blood capillary, fibrosis, agglutination of neurons and loss of definite demarcation between layers on the optic tectum of *Channa punctatus*. Loganathan *et al*., (2006) have observed similar results on exposure to 10 ppm of zinc in *Labeo rohita*. Altinok and Capkin (2007) have reported telangiectasis and necrosis between the molecular and granular layers of the cerebellum where Purkinje cells are located in *Oncorhynchus mykiss* after exposure to methiocarb. Patnaik (2011) have seen neuronal cell degeneration, swelling of pyramidal cells, vacuolization and dystrophic changes in *Cyprinus carpio communis* L. exposed to sublethal concentration of lead and cadmium.

Bose *et al*., (2013) have revealed generalized congestion and dilation of meningeal vessels along with infiltration of mononuclear cells in brain, atrophy, degeneration of granular and molecular layer, necrosis of brain cells, degeneration of nerve cells, dissolution of nissl bodies, swelling of the axon and vacuolization of myelin sheath of the nerve fibers. Chamarthi *et al*., (2014) have reported mild degenerative changes in neural cells after 24 hours sublethal exposure of quinalphos in *Cyprinus carpio.* There was structural damage, necrotic changes in neural cells and intracellular edema after 7 days of exposure. The exposure for 14 days has caused more degenerative changes, increased necrotic condition of neural cells and cytoplasmic vacuolization. Slight degenerative changes and vacuolization were seen from day 21 to day 30.

Erhunmwunse *et al*., (2014) have noticed severe degeneration of dark stained purkinje neurons, odema, vacuolar changes with empty spaces which appeared as moth eaten area and proliferation of glial cells in the brain tissues on exposure to various concentrations (18 mg/L, 32 mg/L and 75 mg/L) of glyphosate for a period of 7 – 28 days and revealed that glyphosate may be neurotoxic to post juvenile African catfish, *Clarius gariepinus.*

The histological changes which occurred on diclofenac exposure in the present study might be a part of defense mechanism. Vacuolization in brain tissue may be the result of glycolysis leading to microsomal and mitochondrial dysfunction. Severe necrosis of neuronal cells in the cerebrum has indicated loss of nissl substances (Das and Mukherjee, 2000). These changes could be related to possible inhibition or decreased cholinergic activity on exposure to toxicants.

4. Conclusion

The results of the present study have clearly depicted that the drug Diclofenac alters the histological integrity of the brain tissue. This results in the deterioration of the health status of fish and may lead to imbalance in the ecosystem. The drug is potentially harmful to non-target organisms like fish. Measures have to be taken to reduce pharmaceutical residues

at different stages like manufacturing, consumption and waste management.

Conflicting Interests

The authors have declared that no conflicting interests exist.

References

[1] **Williams RT.** 2005. Human health pharmaceuticals in the environment-An introduction. In Williams RT, ed, *Human Pharmaceuticals: Assessing the Impact on Aquatic Ecosystems*. Society of Environmental Toxicology and Chemistry, Pensacola, FL, USA; 1–46.

[2] **Zhang Y, Geiben S and Gal C.** 2008. Carbamazepine and diclofenac: Removal in wastewater treatment plants and occurrence in water bodies. Chemosphere; 73 (8) : 1151–1161.

[3] **Bound JP and Voulvoulis N.** 2005. Household Disposal of Pharmaceuticals as a Pathway for Aquatic Contamination in the United Kingdom. Environmental Health Perspectives; 113(12) : 1705-1711.

[4] **Larsson DGJ, de Pedro C and Paxeus N.** 2007. Effluent from drug manufactures contains extremely high levels of pharmaceuticals. J Hazard Mater.; 148: 751–755.

[5] **Skoutakis VA, Carter CA, Mickle TR, Smith VH, Arkin CR, Alissandratos J and Petty DE.** 1988. Review of diclofenac and evaluation of its place in therapy as a nonsteroidal anti-inflammatory agent. Drug. Intell. Clin. Pharm.; 22(11) : 850-859.

[6] **Farre M, Ferrer I, Ginebreda A, Figueras M, Olivella L, Tirapu L, Vilanova M and Barcelo D.** 2001. Determination of drugs in surface water and wastewater samples by liquid chromatography–mass spectrometry: methods and preliminary results including toxicity studies with *Vibrio fischeri*. J. Chromatogr. A; 938 : 187–197.

[7] **Paje M, Kuhlicke U, Winkler M and Neu T.** 2002. Inhibition of lotic biofilms by Diclofenac.Applied Microbiology and Biotechnology,; 59 (4-5) : 488-492.

[8] **Carlsson G, Orn S and Larsson DGJ.** 2009. Effluent from bulk drug production is toxic to aquatic vertebrates. Environmental Toxicology and Chemistry; 28 (12) : 2656–2662.

[9] **Chovanec A, Hofer R, Schiemer F.** 2003. Chapter 18. Fish as bioindicators. In: Markert BA, Breure AM and Zechmeister HG. eds.Trace Metals and other Contaminants in the Environment Volume 6, Bioindicators & Biomonitors — Principles, Concepts and Applications. 639-676.

[10] **Finney DT**. Probit Analysis, 3rd *edit*.Cambridge University London, 1971.

[11] **Humason GL.** 1972. Animal tissue technique, III Edn., W.H. Freeman and Company, San Francisco.

[12] **Ajima MNO, Bala OA, Audu S and Ugwoegbu KC.** 2015. Chronic diclofenac (DCF) exposure alters both enzymatic and hematological profile of African catfish, *Clarias gariepinus*. Drug and Chemical Toxicology; 38(3) : 383-90.

[13] **Praskova E, Voslarova E, Siroka Z, Plihalova L, Macova S, Marsalek P, Pistekova V, and Svobodova Z.** 2011. Assessment of diclofenac LC_{50} reference values in juvenile and embryonic stages of the zebrafish (*Danio rerio*). Polish Journal of Veterinary Sciences; 14 (4) : 545–549.

[14] **Nilkant GV and Sawant KB.** 1993. Studies on accumulation and histopathology of gills after exposure to sublethal concentration of hexavalent chromium and effect on the oxygen consumption in *Scylla serrata* (Forskal). Poll. Res; 12(1) :11-18.

[15] **Das S and Sahu B**. 2005. Interaction of pH with mercuric chloride toxicity to penaeid prawns from a tropical estuary, East Coast of India: enhanced toxicity at low pH. Chemosphere; 58(9) : 1241-1248

[16] **Meyer M. 1958.** Neuropathologie ed.Greenfield, J.G.Edward Arnold. Pentschew A. Handbuch der Speziellen Pathologischen.

[17] **Santhakumar M, Balaji M, Saravanan KR, Soumady D and Ramudu K.** 2000. Effect of monocrotophos on the optomotor behavior of an air-breathing fish, *Anabas testudineus* (Bloch). Journal of Environmental Biology; 21(1) : 65-68.

[18] **Karuppasamy R.** 2000. Tissue Histopathology of *Channa punctatus* (Bloch) under Phenyl Mercuric Acetate Toxicity. Bulletin of Pure and Applied Science; 19 : 109-116.

[19] **Loganathan K, Velmurugan B, Howrelia JH, Selvanayagam M and Patnaik BB.** 2006. Zinc induced histological changes in the brain and liver of *Labeo rohita* (Ham). J Environ Biol.; 27(1) : 107-110.

[20] **Altinok I and Capkin E**. 2007. Histopathology of Rainbow Trout exposed to sublethal concentrations of Methiocarb or Endosulfan. Toxicologic Pathology; 35(3) : 405-410.

[21] **Patnaik BB, Howrelia HJ, Mathews T and Selvanayagam M.** 2011. Histopathology of gill, liver, muscle and brain of *Cyprinus carpio communis* L. exposed to sublethal concentration of lead and cadmium. African Journal of Biotechnology; 10 : 12218-12223.

[22] **Bose MTJ, Ilava zhahan M, Tamilselvi IR, Vishwanathan M.** 2013. Effect of heavy metals on the histopathology of gills and brain of freshwater fish, *Catla catla*. Biomed. Pharmacol. J; 6(1)

[23] **Chamarthi RR, Bangeppagari M, Gooty JM, Mandala S, Tirado JO and Marigoudar SR.** 2014. Histopathological Alterations in the Gill, Liver and Brain of *Cyprinus carpio* on Exposure to Quinalphos. American Journal of Life Sciences; 2 (4) : 211-216.

[24] **Erhunmwunse NO, Ekaye SA, Ainerua MO and Ewere EEJ.** 2014. Histopathological changes in the brain tissue of Africa Catfish exposure to Glyphosate Herbicide. Appl. Sci. Environ. Manage.; 18 (2) : 275-280.

[25] **Das BK and Mukerjee SC**. 2000. A histopathological study of carp (*Labeo rohita*) exposed to hexachlorocyclohexane. Vet. Arhiv.; 70 : 169-180.