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HISTOPATHOLOGICAL ALTERATIONS IN THE STOMACH OF FRESHWATER FISH *CHANNA PUNCTATUS* UNDER CHLORPYRIFOS TOXICITY.

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ABSTRACT
The non-judicious use of pesticides sprayed in the agricultural lands ultimately finds their way to aquatic ecosystems. The Aquatic ecosystems face threat of biodiversity loss due to toxicity induced by these agro-chemicals. Other than targeted pests, pesticides affect a wide range of non-target organisms, such as invertebrates and fish inhabiting aquatic environments. Among various physiological changes, histopathological alterations in fish intestines due to organophosphate toxicity is the basic tool for finding the potency of these chemicals in mitigating fish health. Therefore the present study deals with the impact of Chlorpyrifos on histopathology of stomach of *C. punctatus* in which set of twelve fish were exposed to 0, 5, 2.5, 3.5, 4.5, 5.5 and 6.5 µl/L concentration of Chlorpyrifos. The lethal concentration (LC_{50}) value of Chlorpyrifos was calculated as 4.5 µl /L for 96 hours of exposure. The fish were exposed to three sublethal concentrations (0.15, 0.25 and 0.35 µl/L) of Chlorpyrifos for 15 and 30 days respectively. Fishes showed severe histological changes in stomach. The degenerative changes included degeneration of serosa layer, columnar epithelium and goblet cells were analysed and section of stomach also showed loosely arranged of muscle layers, large vacuolation in the mucosa as well as in the sub mucosa region. Presence of scattered blood cells in the submucosa and muscularis layer of the stomach tissue was also observed. This clearly demonstrates that the sublethal concentrations of Chlorpyrifos have a deleterious effect on the stomach of *C. punctatus*. Therefore the pesticide can be considered as a potent toxic pollutant to fishes whose entry to natural water bodies should be checked from all point and non-point sources.

KEYWORDS: Chlorpyrifos, *Channa punctatus*, Histopathology, Stomach.

INTRODUCTION

In the agricultural fields, the use of pesticides to protect the crops from the attack of pests and unwanted plants has been considered as an integral part of the modern agricultural practices worldwide. But the indiscriminate use of it might endanger the aquatic ecosystems and fish farms close to the agricultural fields, as they ultimately reach to these water bodies through different routes runoff and cause harmful effects to the flora and fauna residing in waterbody, which primarily include the non-target organisms such as aquatic insects, molluscs and fish. Chlorpyrifos (O, O-diethyl-O-3,5,6-trichlor-2-pyridyl phosphorothioate; CPF) is a broad spectrum organophosphate insecticide widely used to control insects in agricultural crops (Rusyniak and Nanagas, 2004) and subterranean termites (Rao *et al.,* 2005). It is the second highest selling organophosphate insecticide and is more toxic to fish than any other organochlorine compounds (Tilak *et al*., 2001). The present piece of work includes a detailed account of lethal and sub lethal effects of pesticide on the stomach of fresh water fish, *C. punctatus* (Bloch), On account of its high nutrition and medicinal value, this fish has a commercial importance. The objective of this study was to investigate the histopathological effects of chlorpyrifos on *C. punctatus* exposed to its lethal and sub lethal concentrations.

MATERIALS AND METHODS

For studies, healthy and disease free juveniles of fresh water fish *C. punctatus* were collected from Hanumantal fish market (Jabalpur) M.P ranging from 6 to 10 cm in length and 35 to 48 gms in weight. The collected fishes without least disturbance were transported through polythene bags filled half with water. About 50 fishes were put in each bag and water was well aerated using pressurized air from a cylinder. This mode of transit proved successful since there was no mortality in all consignments throughout the course of this study. The fishes brought to the laboratory were dipped in tubs and were disinfected with 0.01% of $KMnO_4$ solution and washed thoroughly to prevent dermal infection and fishes were maintained in aquariums with dechlorinated water which was continuously aerated. These fishes were acclimatized in the laboratory for two weeks prior to the experimentation. (Joshi *et al*., 2002).

In the present investigation the pesticide Chlorpyrifos (O,O-Diethyl 0-3,5,6-trichloropyridin-2 phosphorothioate) with chemical formulla $C_9H_{11}Cl_3NO_3PS$ has been selected . It is a broad spectrum systemic organophosphate pesticide used to kill a number of pests including insects and worms and used on crops, animals, and buildings, It was introduced by

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Dow Chemical Company in United States of America in 1965 (Murray *et al.,* 2001). It is available in granules, wettable powder, dustable powder, liquids, emulsifiable concentrate (Swathi and Singh 2002). Its local name is HILBAN.

Acute toxicity test:
Before the start of acute toxicity test, the range finding test was executed to determine the concentrations to be used for definitive tests. (Table 1) The experiments consisted of a control group and 6 experimental groups. A total number of twelve *C. punctatus* juveniles per experiment were used in this study to determine the zero and 100 % of mortalities, as well as the 96 hours of LC_{50}. The juveniles were divided into 7 groups (each group contained 12 fishes each) and kept in glass aquarium (size 60cm×30cm×40cm) fishes per group were exposed to different concentrations of Chlorpyrifos of concentrations 1.5 µl, 2.5 µl, 3.5 µl, 4.5 µl, 5.5 µl, and 6.5 µl (except control group).

Subacute toxicity experiments
For sub-acute toxicity, the doses for experiments were kept below the LC_{50} value. For these experiments, the various sublethal concentrations of Chlorpyrifos 0.15, 0.25, 0. 35 µl/L for 15 and 30 days respectively were given. Sets of twelve fishes were transferred to four different aquaria. Out of four, one served as control (contained only dechlorinated water) and rest three contained different concentrations of Chlorpyrifos. Six fishes were taken out from each aquarium after 15 days exposure and the rest of the fishes were taken out after 30 days treatment. The fish were sacrificed and the tissues of stomach were excised out. After cutting then into small pieces, the tissue was fixed in freshly prepared fixative (alcoholic bouins solution was used as fixative). The period of fixation was 1-3 days

After the fixation, water molecules from the cells or tissues were removed. The processes are known as dehydration. the tissues were kept along with the progressive Alcohol grades for the following duration: 30 %, and 50 % grades for 5 to 10 hours: in 70 % grade for 10 to 12 hours : 90 % grade for 12 to 14 hours and in 100 % absolute alcohol grade for about 15 hours.

After that the tissues contain alcohol so it has to be removed from the tissue to make it firm for the purpose of section cutting. The tissues were kept in xylene for about 2 to 4 hours, the tissue were soaked in molten wax at a standard temperature, paraffin wax was used in the process of embedding. After embedding the tissue with wax, it was cast into a block of paraffin, this process is known as bock making, the mould of "L" pieces was adjusted in such a way to accommodate the object. The mould was filled with molten paraffin wax. The label carrying all the details of the tissue was fixed on one side of the mould. The mould was gently immersed in cold water so as to cool the wax rapidly. When the block become solid it was removed from water, after that the prepared block was trimmed into correct shape for section cutting. The attached trimmed block was cut into thin sections of desired thickness of the microtome. Sections were cut at 6-8µ thickness and were floated in a water bath between 38-49°C. The sections from water were then mounted on clean glassslides smeared with Mayer's egg albumin. They were then dried on a hot plate at about 50°C for 30 minutes. The sections on the slides were kept for staining after drying.

The slide was kept in xylene for 5 minutes to de paraffinize. Then the slides were passed through the down grade series of 100 %, 90 %, 80 %, 70 %, 50 %, and 30 % ethyl alcohol five minutes each, and the slides were washed in running tap water for 5 minutes. First, the slides were stained in haematoxylin for 10 minutes and then stained slides were washed in running tap water up to 10 dips or the sections turn into blue. After that the slides were dehydrated by passing through ascending order alcohol series up to 70 % alcohol. Secondly, the slides were stained with eosin for quick Dips and the stained slides were dehydrated by passing them through an ascending series (30 %, 50 %, 70 %, 80 %, 90 % and 95 %) of alcohol for 3 minutes each. The slides were allowed in absolute alcohol for complete dehydration. Then the slides were transferred into xylene for clearing and then finally mounted in dpx mountant and then analysed under microscope.

RESULTS AND DISCUSSION

LC_{50} values:
During the 96h acute toxicity experiment, water in each aquarium was aerated and had the same conditions as the acclimation period. After every 24 hours of the experiment the dead fishes were removed and the number of survivals was recorded, the lethal concentration values were obtained and analyzed by Finney's probit analysis method (Elia *et al.,* 2002). Regression line was drawn on the basis of two variables. Log dose and empirical probit and was used to

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determine the expected probit necessary for LC determination. (Fig 1) In the present study, the calculated 96 h LC_{50} value of Chlorpyrifos is 4.5 mg/L, (Table 2).

Table 1: Rate of mortality of juvenile *C. Punctatus* exposure to Chlorpyrifos for 96 hrs

concentration	Log dose	24	48	72	96	total
control	0	0	0	0	0	0
1.5	0.176091	0	0	0	1	1
2.5	0.39794	0	0	1	1	2
3.5	0.544068	1	1	1	2	5
4.5	0.653213	1	1	2	2	6
5.5	0.740363	1	2	3	3	9
6.5	0.812913	2	2	4	4	12

Table 2: Mortality and empirical probit values for the concentration of Chlorpyrifos after 96 h for calculation of LC_{50}.

Conc.	*Log dose*	mortality	% age	probit
1.5	0.176091	1	8.34	3.59
2.5	0.39794	2	16.67	4.01
3.5	0.544068	5	41.67	4.77
4.5	0.653213	6	50	5
5.5	0.740363	9	75	5.07
6.5	0.812913	12	100	7.33

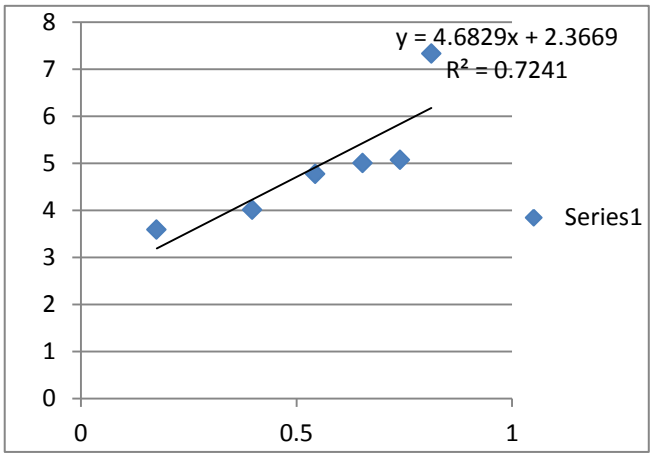

Fig 1: The logarithm of Chlorpyrifos concentration against the empirical probit values of the mortality of *C. punctatus* individuals for calculation of LC_{50}.

Histopathology:
Stomach is one of the prime organ of fish alimentary canal which has role in digestion of food eaten.

Histology of untreated stomach of *C. punctatus*:
FIG 2. The control fishes showed normal histology of stomach tissue when stained with Hematoxylene Eosin (HE) stain when visualised under 400x magnification,

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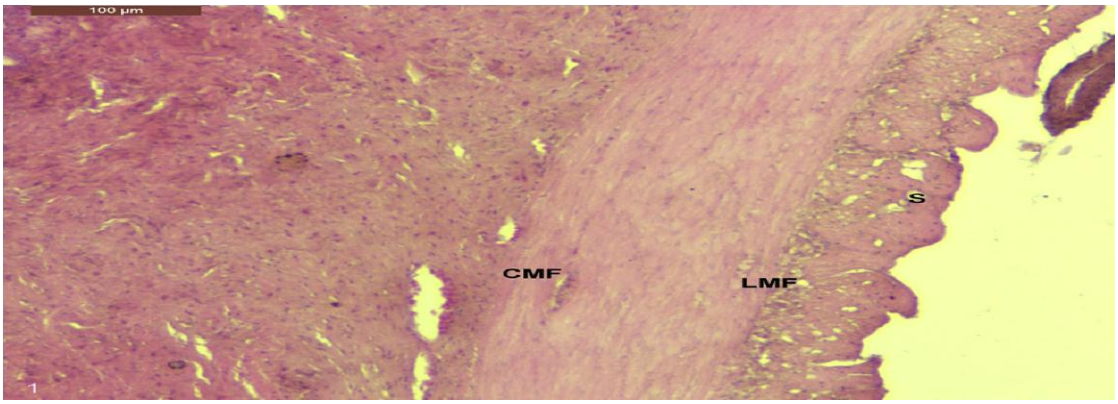

FIG .2. Photomicrograph of transverse section of the Stomach of untreated *Channa punctatus* (haematoxylene eosine stain) X 400. S- Serosa, LML-Longitudinal muscle layer, CML-Circular muslce layer, SM-Sub mucosa, GC-Goblet cells, CE- Columnar epithelium, LP- Lamina propria, BM- Basement membrane.

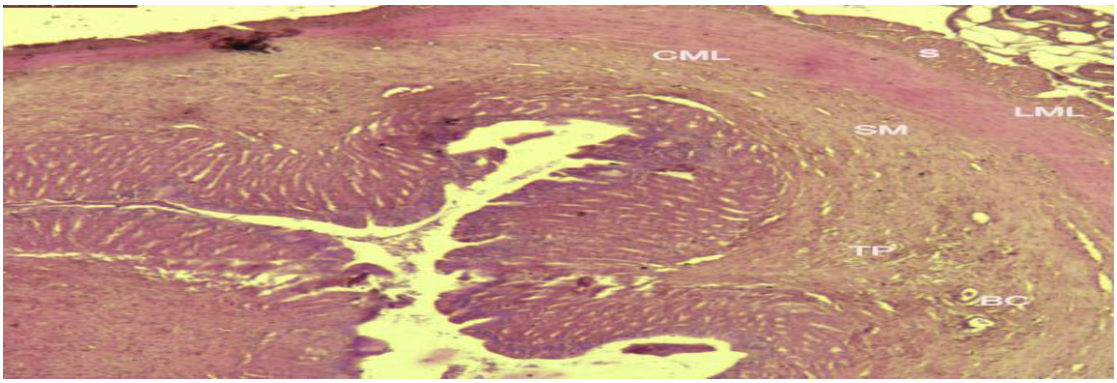

FIG .3. Photomicrograph of transverse section of the stomach of *Channa punctatus* after 15 days exposure to 0.15 µl/L Chlorpyrifos (haematoxylene eosine stain) X 400.

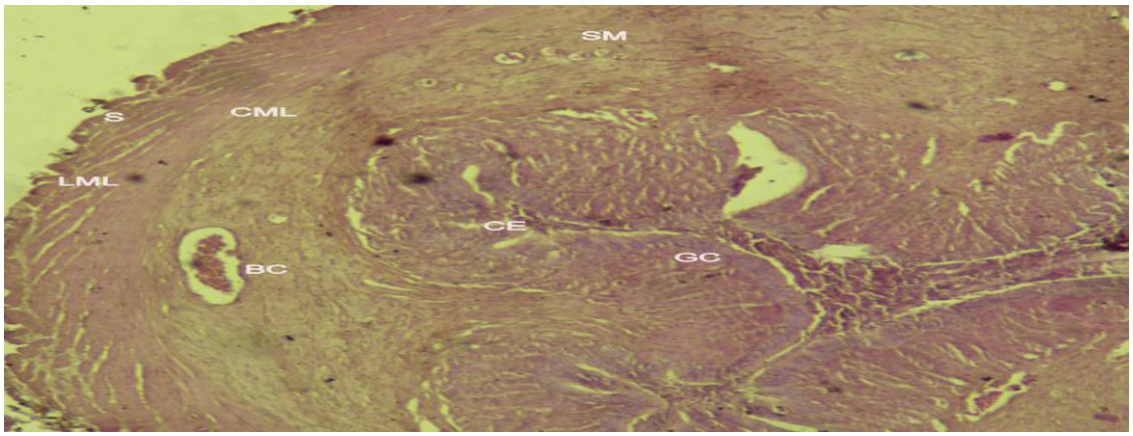

FIG .4. Photomicrograph of transverse section of the stomach of *Channa punctatus* after 15 days exposure to 0.25 µl/L Chlorpyrifos (haematoxylene eosine stain) X 400.

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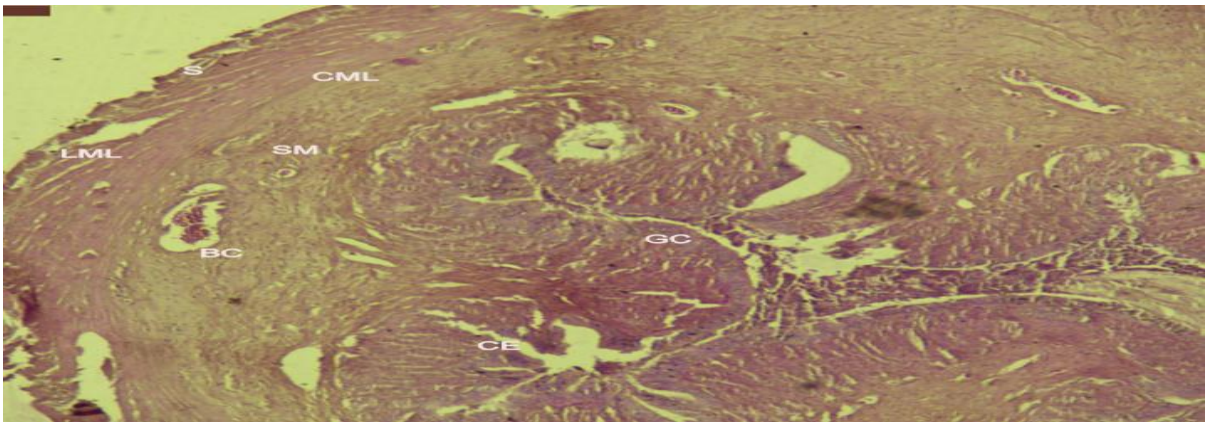

FIG .5. Photomicrograph of transverse section of the stomach of *Channa punctatus* after 15 days exposure to 0.35 µl/L Chlorpyrifos (haematoxylene eosine stain) X 400.

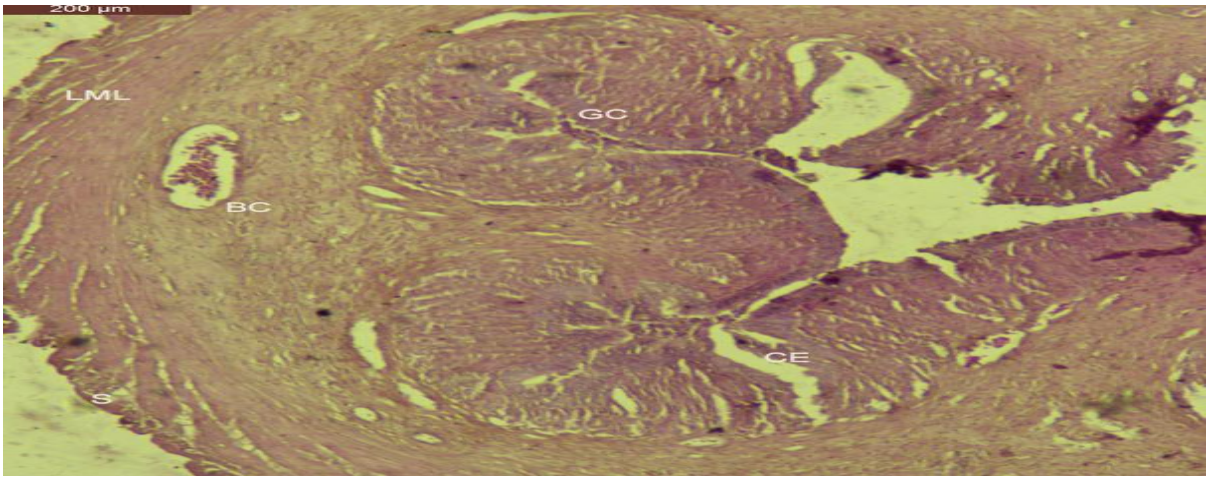

FIG .6. Photomicrograph of transverse section of the stomach of *Channa punctatus* after 30 days exposure to 0.15 µl/L Chlorpyrifos (haematoxylene eosine stain) X 400.

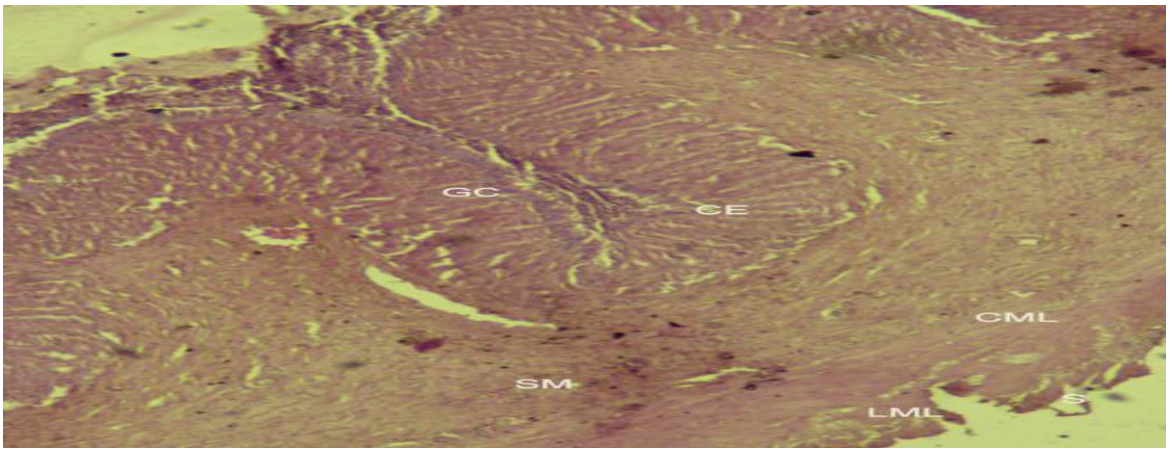

FIG .7. Photomicrograph of transverse section of the stomach of *Channa punctatus* after 30 days exposure to 0.25 µl/L Chlorpyrifos (haematoxylene eosine stain) X 400.

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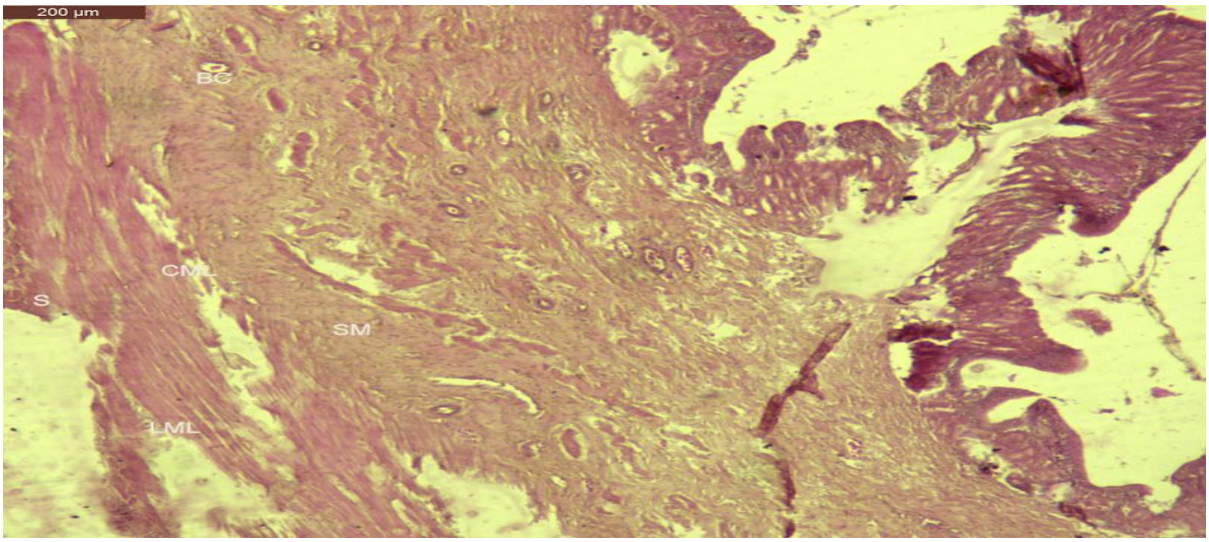

FIG .8. Photomicrograph of transverse section of the stomach of *Channa punctatus* after 30 days exposure to 0.35 µl/L Chlorpyrifos (haematoxylene eosine stain) X 400.

Histologically, the stomach of *C. punctatus* was composed of mucosa (M), submucosa (SM), muscularis and serosa (S) layers. The innermost layer is mucosa (M) consists of columnar epithelium (CE) with epithelial (EC) and goblet (GC) or secreting cells, it also consists of tubular gastric glands with oxyntic and peptic cells, supported on the lamina propria (LP). The submucosal (SM) layer consists of blood vessels and dense connective tissue rich in collagen fibres. The muscular layer (ML) consists of two sub-layers: an internal one, with smooth muscle fibres oriented in a circular way, circular muscle layer (CML) and an external one, with longitudinal smooth muscle fibres, Longitudinal muscle layer (LML) both interspersed with loose connective tissue Functions in mixing of food with digestive enzymes. Thickening of the inner circular muscular layer occurred in the pyloric region. The serosa (S) is the outermost layer and consists of dense connective tissue, blood vessels and nerves, also consists of flattened cells covering the outer boundary.

Histopathology of stomach tissue under Chlorpyrifos toxicity:
Fig. (3,4,5) stomach of *C. punctatus* exposed for 15 days to various sub lethal concentration 0.15 µl/L, 0.25 µl/L and 0.35 µl/L of Chlorpyrifos, the following changes were observed in the stomach of fish.

At lower concentrations 0.15 µl/L for 15 days slight changes viz degeneration of Serosa layer and formation of vacuoles in the sub mucosa and microvilli region were noticed. However 0.25 µl/L and 0.35 µl/L concentration showed many changes viz large vaculation were seen in the mucosa as well as in the sub mucosa region, Degeneration of columnar epithelium and basement membrane, severe damage of micro villi occurs, goblet or secretory cells are fully distorted.

Fig. (6,7,8). Stomach of *C. punctatus* exposed for 30 days to sub lethal concentration 0.15 µl/L, 0.25 µl/L and 0.35 µl/L of Chlorpyrifos.

Very little changes in stomach were observed after exposure to 0.15 µl/L for 30 days. Section of stomach showing loosely arranged of muscle layers and degeneration of serosa layer were also noticed. however After exposure to 0.25 µl/L, the stomach of *C. punctatus* showed many changes viz, high degeneration of columnar epithelium and goblet cells, Damage of the mucosal epithelial layer which was detached at places from the basal layer was also seen, At 0.35 µl/L concentrations, large vaculation in serosa and muscularis layers were observed , hypermic blood vessel, and presence of scattered blood cells in the submucosa & muscularis layer or all over the entire stomach tissue were observed. These results are more or less similar to the results of Mohanta *et al.* (2010) who reported the disintegration of glomerulus, shrinkage of Bowman's capsule, vacuolation formed around the capsule and the enlargement of tubular lumen, distal and proximal tubules were so high that they almost lost their characteristics in fish *C. punctatus* after

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Tannery Effluents intoxication. Samanta *et al.* (2016) noted the degenerative changes in columnar epithelial cells, vacuolated basal region, brush border disappearance, distortion in gastric glands and fusion of submucosa with mucosal folds were frequently observed in fish *A. Testudineus* stomach after Almix Exposure. Damage in gastric glands observed under present study was also reported by Crespo *et al.*, (1986). Ghanbahadur and Ghanbahadur (2012) also noted the same result and reported that vacuolization occurs in sub mucosa and shrinkage of mucosal folds were observed in stomach of *Rasbora daniconius* when exposed to endosulfan.

CONCLUSION

Fresh water ecosystems are faced with spatially alarming high levels of pesticide concentration. Although, the *C. punctatus* is considered as robust and tolerant fish species however, the present study conducted was able to show the severety of toxic effects on histopathological changes in stomach of the fish. The observed abnormal behavior and altered histopathology of stomach demonstrate the severe adverse effects due to acute exposure of tested toxicant Chlorpyrifos in *C. punctatus.* In the present study the toxicity tests showed that Chlorpyrifos are toxic to fresh water fish species which constitute the non- target organism. The sensitivity of *C. punctatus* to the toxicants is evaluated for short and long term duration. The sub-lethal concentrations caused considerable deterioration to fish health effecting the structural functioning of stomach tissue. The findings clearly indicated that the Chlorpyrifos are significantly toxic to *C. punctatus* at sub-lethal levels. Thus the present study demonstrates that the toxicity of Chlorpyrifos may be a threat to fauna, flora as well as human beings.

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