Bulletin of Pure and Applied Sciences
Zoology (Animal Science), Vol.42A, No.1,
January-June 2023: P.87-105

Print version ISSN 0970 0765
Online version ISSN 2320 3188
DOI: 10.48165/bpas.2023.42A.1.8

Original Research Article *Content Available online at: https://www.bpasjournals.com/zoology*

Preventive Role of Methanolic Extract of Ocimum sanctum L., Camellia sinensis (L.) Kuntze and Their Combined Formulation against Sublethal Concentration of Sodium Fluoride Exposed to Channa punctatus (Bloch.)

[1]Uma Dutta*, [2]Trishna Kalita and [3]Shahdukht Parsha Hazarika

Author's Affiliation:
[1]Head of the Department and Associate Professor, Department of Zoology, Cotton University, Guwahati, Assam 781001, India
E-mail: uma.dutta@cottonuniversity.ac.in
2Research Scholar, Department of Zoology, Cotton University, Guwahati, Assam 781001, India
E-mail: trishnakalita91@gmail.com
[3]Post Graduate student, Department of Zoology, Cotton University, Guwahati, Assam 781001, India
E-mail: Hazarika.parsha@gmail.com

***Corresponding author:**
Uma Dutta,
Head of the Department and Associate Professor, Department of Zoology, Cotton University, Guwahati, Assam 781001, India
E-mail: uma.dutta@cottonuniversity.ac.in

Article Info:
Received on 23.12.2022
Revised on 07.02.2023
Approved on 09.03.2023
Accepted on 16.03.2023
Published on 16.06.2023

ABSTRACT:
In the recent years, Fluoride has emerged as one of the most harmful pollutants in the world and its presence has been shown to negatively impact the quality of groundwater in many countries. According to World Health Organization (WHO), when its concentration exceeds 1.5 mg/L limit, it may cause threats to the health. Exposure to high concentrations of fluoride has been known to have deleterious effects on the biological system of human as well as other animals. Based on this background, a study has been conducted to show the effects of sodium fluoride induced toxicity in the freshwater fish *Channa punctatus* (Bloch.). The fishes were exposed to sublethal doses of 25 ppm and 50 ppm of sodium fluoride for a period of 3 and 7 days. The damaging effects of sodium fluoride were manifested in the morphological, behavioural, haematological, biochemical and histological parameters of the fishes. The fishes were then further exposed to 100 mg/L of methanolic leaf extracts of tulsi, tea and a combined formulation of the two extracts to investigate the ameliorative properties in the revival of fluoride toxicity in the fishes. However, the results showed that the impact of toxicity caused by exposure to 25 ppm was much less pronounced as compared to 50 ppm of sodium fluoride. These effects were revealed to be effectively reduced by the addition of the plant extracts. Both the selected plant extracts showed positive results. However, their combined formula revealed the highest level of effectiveness in the remedial activity of fluoride induced toxicity.

Keywords: Fluoride, Sublethal, Toxicity, Channa punctatus, Leaf Extract, Remediation

How to cite this article: Dutta U., Kalita T. and Hazarika S.P. (2023). Preventive Role of Methanolic Extract of Ocimum sanctum L., Camellia sinensis (L.) Kuntze and Their Combined Formulation against Sublethal Concentration of Sodium Fluoride Exposed to Channa punctatus (Bloch.). *Bulletin of Pure and Applied Sciences-Zoology*, 42A (1), 87-105.

INTRODUCTION

Fluoride has emerged as one of the most toxicity inducing hazards in the world. Its high impact has been seen across the Indian subcontinent, particularly in Rajasthan, Gujarat, Haryana, Delhi and Assam (Kisku and sahu, 2019). They enter the groundwater due to weathering of fluoride containing minerals and sediments, volcanic ash, as well as anthropogenic activities including industrial effluents, agricultural fertilizers and combustion of hydrocarbons (Jhaet al., 2013; Brindha & Elango, 2011). According to World Health Organization (WHO), the permissible limit of fluoride concentration in drinking water is 1.5mg/L. Fluoride ingestion above the level of 1.5 mg/L causes fluorosis, a crippling disease affecting almost every organs and tissues in the body. Severe health concerns including tooth decay and skeletal damage of the body have been documented in recent studies worldwide. Ingested fluorides are rapidly absorbed in the gastrointestinal system; 35-48 percent of the fluoride is retained by the body and stored in calcified tissues and skeletal tissue. The remaining fluoride is primarily eliminated in the urine. The development of fluorosis in animals is caused by the prolonged use of fluoride-rich foodstuff and water in endemic regions. Symptoms of fluorosis include tooth discoloration, difficulties in mastication, bone lesions, lameness, debility, and death (Patra et al., 2000).The majority of studies indicated that an excessive intake of fluoride leads to an increased generation of reactive oxygen species, which in turn causes multiple organ toxicity. On the other hand, an excessive intake of fluoride remarkably weakens the body's self-defense system. For instance, it decreases the level of Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GSH), total and protein thiols, and other antioxidants. Increased reactive oxygen species (ROS) activity and oxidative stress are actually the primary causes of fluoride-induced organ toxicity (Sharma et al., 2017). The constant exposure to fluoride contaminated water increase the severity of effects in the aquatic organisms by accumulating in the bones and flesh of the fish (Camargo, 2003). A major contributor of fluorosis in human is the dietry consumption of fluoride contaminated fish (Ganta et al., 2015). As a result, this poses an alarming threat at various levels of the entire food chain, providing indication of its detrimental effects for humans.

Although there are various cost-effective techniques available which are often employed for de-fluoridation of water, however, the benefits of these techniques have not yet reached the rural areas due to certain limitations (Jha et al., 2013). There also have been several studies on the use of plant-based approaches to remediate fluoride from the environment in the recent years. Phytoremediation of fluoride using Tulsi (*Ocimum sanctum*) leaves (Bayu et al., 2021), and Tea Ash (Deshmukh et al., 2018) have been reported to show promising therapeutic effects in the removal of fluoride from contaminated water. Along with phytoremediation abilities, Tulsiand Tea leaves also possess other ameliorative properties including antiseptic, antimicrobial, antioxidant and anti-inflammatory properties (Borah et al., 2018; Prasanth et al., 2019).Thus, *Ocimum sanctum* (tulsi) and *Camellia sinensis* (tea) were selected and their leaves were chosen to investigate if they possess suitable remedial properties to reduce the harmful effects of sodium fluoride toxicity in *Channa punctatus* Bloch.

MATERIALS AND METHODS

Chemicals and reagents:

Ethanol, methanol, chloroform, glacial acetic acid, diethyl ether and paraffin wax (DK9D692807, melting point: 58-60^{o} C) were purchased from Merck Life Science Private Limited. Acetone, sodium chloride, sodium hydroxide, petroleum ether fromThermo Fisher Scientific India Private Limited, Mumbai; Wright stain (powder), giemsa stain (powder), haematoxylin, eosin were brought from Sigma-Aldrich Chemicals Private Limited, USA; kits for Enzyme assay from HiMedia Laboratories Private Limited; Abcam Limited, India; Transasia Bio-Medicals Limited, Mumbai.

Collection of plants:

The two plants *Ocimum sanctum* L. (Tulsi) and *Camellia sinensis* (L.) Kuntze (Tea) were selected

for the experiment. Leaves of Tulsi plant collected from the kitchen garden. (Location: Guwahati, Bagharbari. Latitude 26.139866, Longitude 91.824767). The tea leaves were collected from Wild Mahseer, Balipara Division Addabarie Tea Estate, Sonitpur District, Balipara, Assam 784101 (Latitude: 26.838695660874674, Longitude: 92.80979766809682). Plants were identified and authenticated by the Department of Botany, Cotton University, Guwahati, Assam.

Preparation of plant extract:
The dried and powdered plant materials (50gm) were extracted with 250 ml of methanol (1: 5 w / v) by using Soxhlet extractor for 24 hoursat a temperature below the solvent's boiling point. The extracts were concentrated at 40°C using a Rotary evaporator after being filtered using Whatman filter paper.

Experimental design:
Freshwater fish *Channa punctatus* (Bloch.) commonly known as snakehead fish and indigenously known as "Goroi", was used for the present study. They weighed approximately 50-60 gm each, having a length of 14-17 cm. The fish were kept in chlorine free deep well water. The water in which the fish were kept was changed every alternate day. The fish were acclimatized for 7 days. The physicochemical characteristics water like temperature, pH, dissolved oxygen and electrical conductivity were monitored throughout the acclimatization period and the trial periods according to the standard methods of APHA, 2005. They were fed daily with commercial dry feed pellets (Tokyo pellets). Fishes are randomly divided into 6 groups (each group containing 5 fishes). Group A is considered as control group. Group B and Group C treated with 25ppm and 50 ppm sodium fluoride respectively added in the water medium. Group D fishes were treated with 50 ppm sodium fluoride and 100 mg/l tulsi extract. Group E fishes were treated with 50 ppm sodium fluoride and 100 mg/l tea extract. The group F was treated with 50 ppm sodium fluoride along with 50 mg/l of tulsi extract and 50 mg/l of tea extract. The 6 groups of the selected animal model were observed for behavioural, morphological, haematological, histological and biochemical parameters.

Study of behavioral changes:
The fishes were checked for any unusual or erratic behavior upon the addition of sodium fluroide to the medium. The parameters considered under observation were rapid and erratic swimming or hyper-mobility, hovering near the water surface, rapid operculum movement and darting. In addition, it was noted if the fishes showed a reduction in the behavioral activities.

Study of morphological changes:
The fishes kept in the 6 glass aquarium were frequently and minutely observed throughout the whole duration of the experiment in order to record the morphological changes due to toxicity induced by sodium fluoride (NaF). It was noted whether the fishes developed any lesions or vacuole formation, and/or if they showed any loss of body coloration or fins and fin rays. The fishes were also checked for any hemorrhage on their body surface. Any fishes with any observable clinical defects or infections were separated from the rest of the group. Furthermore, it was also recorded whether the addition of the plant extracts caused any visible changes in the effects of toxicity.

Haematological parameters:
Haematological parameters were assessed by the standard technique. The current investigation shows that blood parameters including haemoglobin (Hb), red blood cells (RBC), white blood cells (WBC), and packed cell volume (PCV) correlate with one another. Blood was collected in EDTA-coated tubes by puncturing the heart of the fish.

Biochemical estimation:
Aspartate Aminotransferase (AST) activity, Alanine Aminotransferase (ALT) activity and the activity of Alkaline Phosphatase (ALP) determined by commercially available enzyme kits.

Histopathological study:
To carry out histopathological studies, the liver, kidney and gills were dissected out from the fishes of all the experimental groups on day 7 of the experiment and are processed following the standard Hematoxylin-eosin method (HE). Microphotograph of the different selected areas of representative section of target tissues taken with the help of microphotograph attachment camera in microscope D-Winter (German Company) using the software Calipro Version 4.6 for histopathological analysis.

Data analysis:
The data obtained from each experiment was expressed as mean ± standard error (SEM) in average of 5 observations each. The difference in the results between the control and treated group was analyzed using one-way ANOVA analysis. The difference was considered the levels of significant at $p < 0.05$.

RESULTS

Behavioral changes:
Fish behavior is one of the most important and ideal parameters for studying the impact of sub-lethal impacts of fluoride toxicity in fishes. In the present study, the fishes when exposed to sub-lethal doses of NaF for 7 days exhibited unusual patterns of behavior while the fishes in the control group were observed to show normal behavior activities. In the control group, there were no visible changes observed in the behavioral patterns of the fish. They swim actively at a regular rate with relatively normal movements of body and operculum. However, upon exposure to sub-lethal concentrations of sodium fluoride, the most pronounced response observed in the fish was hyperaxcitability. The fishes showed erratic swimming with abrupt stops in their trajectory. Some of the fishes would exhibit fright response while others would show an aggressive stance of dominance over the others. It was noted that the fishes kept in 50 ppm NaF started showing the effects within 36 to 48 hours of exposure, whereas those exposed to 25 ppm showed relatively inconspicuous alterations in their behavior. The experimental animals subjected to 50 ppm NaF treatment showed marked patterns of hyperexcitability, imbalanced and jerky swimming with unusual fin movement. The fishes also showed abrupt jumping and darting behavior. They also manifested abnormal and arrhythmic operculum movement and frequent hovering at the water surface. The fishes were seen vertically darting towards the water surface and standing there maintaining the vertical posture of the body with respect to the base, gasping for air. The above-recorded responses were much heightened in case of the 50 ppm group in comparison with the 25 ppm group. It was, however, observed in later groups that there was decreased in activity (Table 1).

Table 1: Impact of sodium fluoride in behavior of fish observed within 72 hour of exposure

Nature of behavior	Control	25ppm NaF	50ppm NaF	Tulsi extract	Tea extract	Combined formulation of extracts
Hyper excitability	-	+++	++++	+++	++	+
Operculum movement	+	++	+++	++	+++	++
Imbalance of swimming	-	+	+++	++	+	+
Fin movement	+	++	+++	++	+	++
Jumping and darting	-	+	++	+	-	-
Vertical posture of opening mouth at water surface	+	++	+++	++	+	+

- = Normal effect; + = mild effect; ++ = Moderate effect; +++ = Strong effect

Morphological Changes:
Morphological changes are essential parameter for the study of sub-lethal toxicity of sodium fluoride in fishes showed in figure 1. In control group, there were no significant changes observed in morphology of the body appearance. Nevertheless, the fishes that were kept in 25 and 50 ppm of NaF showed distinct changes in body morphology. There was lesion formation seen in the ventral side of the body,

mostly around the head region and underside of fins. There was loss of coloration more strikingly observed with vacuolation in fishes treated with 50 ppm NaF. The black colored coat on the skin that extends along the whole length of the body of *Channa punctatus* was seen to disappear over time during the experimental period. The fishes treated with 25 ppm of NaF were observed to show faint manifestations of the same responses but not so much to the extent of causing much damage. The lateral-ventral side of the fish body showed hemorrhage in the batch of 50 PPM NaF.

However, upon treatment with the leaf extracts, it was observed that there was not much change seen in the body morphology of the fish as compared to those batches that were only treated with sodium fluoride.

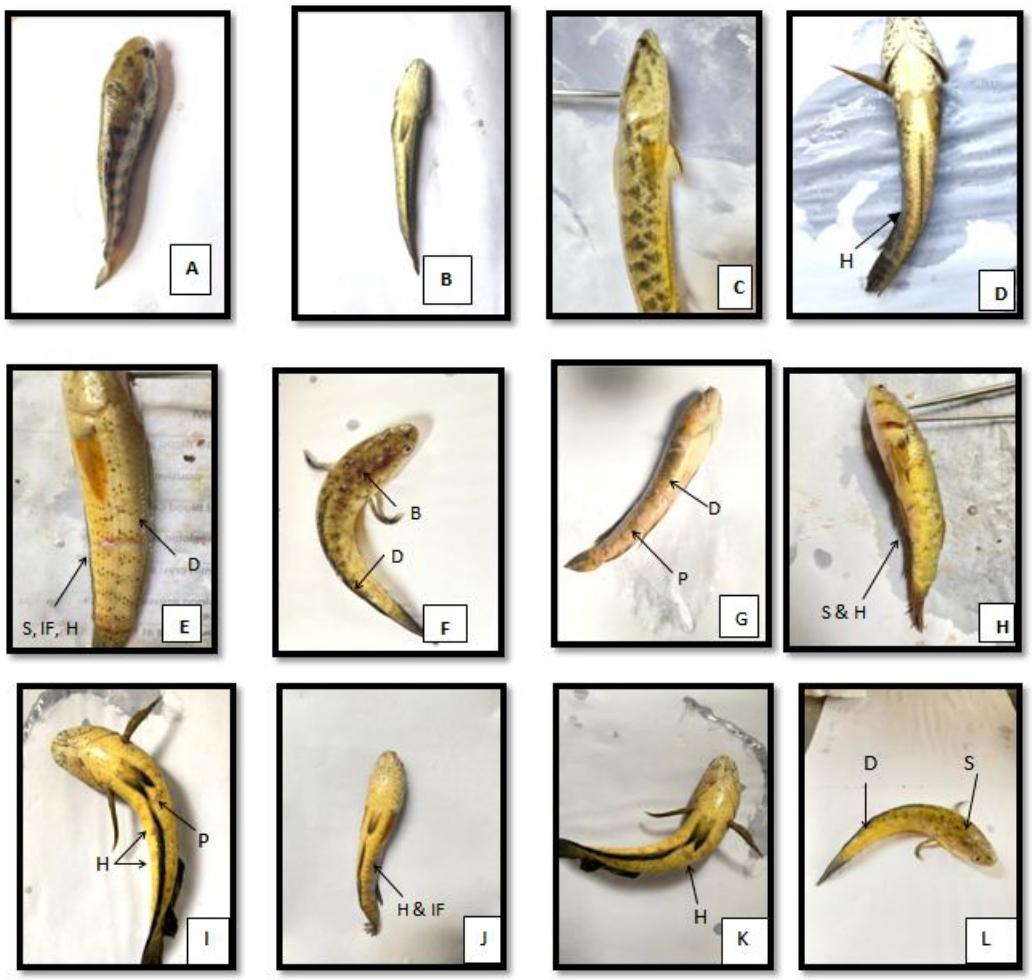

Figure 1: (A-C) control group with normal appearance, A-Dorsal morphology, B-Ventral profile, C-Lateral profile. (D-F) 25ppm NaF exposed group. (G-I) 50ppm NaF group. (J-L) NaF treated groups exposed to plant extracts, J- Ventral morphology of fish treated with tulsi leaf extract showing mild hemorrhage. K-Ventral view of fish treated with tea leaf extract showing some light hemorrhagic areas. L- Dorsal view of fish exposed to extract combination showing slight discoloration and scale shedding in the head region. S: Scale shedding, H: Hemorrhage, D: Discoloration, B: Blister, P: Pigmentation, IF: Inflammation, SF: Fading of skin color.

Haematological Study

A Study of Numerical Changes in the Erythrocytes

The experimental fishes when exposed to 25 ppm and 50 ppm of sodium fluoride (NaF), the values of some of the blood parameters changed significantly as compared to the untreated group of fishes (Table 2).

Table 2: The numerical changes in some blood parameters of the haematological study of *Channa punctatus* Bloch

Sl. No.	Parameters	Days	Control	Treated-25PPM	Treated- 50 PPM	Tulsi leaf Extract	Tea leaf Extract	Extract Combination
1	Haemoglobin content (g / dL)	7	10.18±0.17	9.7±0.16 ***	9.38±0.08 ****	9.56±0.07	9.68±0.18	9.74±0.06
2	PCV (%)	7	28.22±0.87	25.93±0.79*	23.54±0.53*	27.29±0.62	27.47±0.76	27.65±0.56
3	Total RBC count (TEC) (10^6 / MM^3)	7	3.16±0.16	2.84±0.08	2.27±0.04	3.02±0.06	3.07±0.08	3.11±0.04
4	Total WBC count (TLC) (10^3 / MM^3)	7	8.48±0.36	8.89±0.24	9.24±0.28**	8.14±0.14	8.28±0.16	8.26±0.38
5	MCV (mm^3)	7	102.22±2.42	95.13±2.32*	92.36±2.40*	99.64±2.30	99.87±1.88	101.56±2.42

Data represented as mean ± SE of 3 observations (p>0.05)
(*indicates p<0.05, ** indicates p<0.01 *** indicates p<0.001 **** indicates p<0.0001

The haemoglobin showed a gradual decline with increase of concentration of sodium fluoride. The haemoglobin decreased to 9.7±0.10 at 25 ppm and 9.38±0.08 at 50 ppmNaFtreated groups as compared to control group. The addition of tulsi leaf extract resulted in a elevating the haemoglobin percentage value to 9.98±0.10, while the extract made of tea leaves showed an elevation to 10.07±0.06. A combined formulation of both the extract showed a raise in the haemoglobin percentage to 10.13±0.12. The packed cell volume (PCV) showed a gradual decrease with increase of concentration. The PCV decreased from 28.22±0.87% to 25.93±0.79% at 25 ppm and to 23.54±0.53% at 50 ppm concentration of NaF. The addition of tulsi leaf extract resulted in a elevating the PCV percentage value to 27.29±0.62%, while the extract made of tea leaves showed an elevation to27.47±0.76%. A combined formulation of both the extract showed a raise in the PCV percentage to 27.65±0.56%. The total erythrocyte count (TEC) showed a steady decrease with increase in concentration. The value of TEC decreased from 3.16±0.16 to 2.84±0.08 and 2.27±0.04 at 25 ppm and 50 ppm NaF treated groups respectively. The addition of tulsi leaf extract and tea leaf extract raised the TEC to 3.02±0.06 and 3.07±0.08, respectively. But a combination of the two extracts showed better increase of TEC value of 3.11±0.04 which was almost similar to the TEC value recorded in control groups. The total leucocyte count (TLC) first showed anincrease at 25 ppm and then a sharp increase at 50 ppm NaF treated group. The values recorded at the two different concentrations were 8.89±0.24 and 9.24±0.28 respectively. The addition of leaf extracts of tulsi, tea and combination of tea and tulsi showed a decrease in the TLC recorded as 8.14±0.14, 8.28±0.16 and 8.26±0.38, respectively. The Mean Corpuscular Volume **(**MCV) values showed a gradual decrease with increase in the concentrations of fluoride. The MCVsrecorded at 25 ppm and 50 ppmwas 95.13±2.32 and 92.36±2.40. However, the MCV values showed an increase with the addition of tulsi leaf extract, tea leaf extract and combined leaf extract which were recorded as 99.64±2.30, 99.87±1.88 and 101.56±2.42 respectively.

Microscopic Analysis of Erythrocytes

Light microscopic studies of erythrocytes of control group (Group A) animals showed flattened, bilaterally concave disc shaped appearance (Figure 2-A). Additionally, in peripheral blood film of control fish, negligible numbers of stomatocytes, tear drop cells as well as ring shaped cells were observed which were statistically not significant. Erythrocytes of treated (25 ppm) group of fish showed some distortions in the membrane and irregular appearance in shape (Figure 2 (B-C)). Besides, the erythrocytes showed a relatively greater number of stomatocytes and tear drop cells with hyperchromatic nuclei, which were statistically significant. Erythrocytes of 50ppm NaF treated group (Group B) of fish showed some distortions in the membrane and asymmetry in its oval shape (Figure-2 (D-E)).

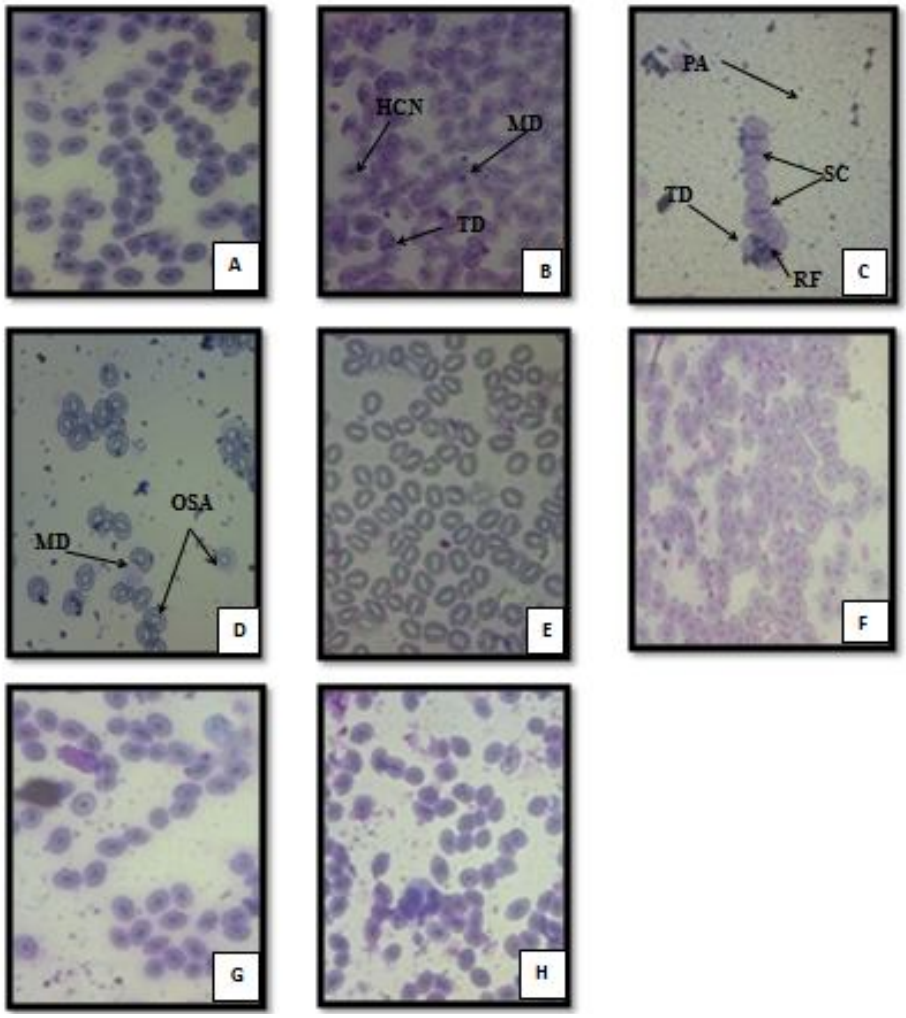

Figure 2: A-Cytomorphological status in RBCs of control group, (B-C) RBCs of fishes of group B exposed to 25 PPM sodium fluoride, (D-E) RBCs of fishes of group C exposed to 50 PPM Sodium Fluoride, (F-H) RBCs of Group D, E and F respectively showing greatly reduced distortions and resembling the erythrocytes of control group.PA: Platelet Aggregation, CD: Cell disappearance, HCN: Hyperchromatic Nucleus, MD: Membrane disintegration, SC: Stomatocytes, TD: Tear drop cells, OSA: Oval Shape Asymmetry. (All images Leishman stain, 40x)

Besides, the erythrocytes showed far greater number of stomatocytes and tear drop cells with hyperchromatic nuclei as well as vacuolated cytoplasm and roleaux formation, which were statistically significant. Erythrocytes of tulsi extract treated group (Group C) of fish showed lesser visible deformation in the membrane and irregularity in its shape as compared to the NaF treated groups (Figure 2-F).

Besides, the erythrocytes only a few number of stomatocytes, which were statistically not very significant. Erythrocytes of tea extract treated group (Group D) of fish showed lesser visible deformation in the membrane and irregularity in its shape (Figure 2-G) as compared to the NaF treated groups. Besides, there was very negligible number of toxic granules observed in the blood film, which were statistically not very significant. Erythrocytes of group F of fish showed lesser visible deformation in the membrane and irregularity in its shape (Figure 2-H) as compared to the NaF treated groups. Besides, a few numbers of membrane disintegration and platelet aggregation were observed, which were statistically not very significant.

Histopathological Analysis

Histopathological studies in fishes have been widely used as key indicators of the various effects of a toxicant when they are exposed to it. These analyses also help in identifying any major damage to the vital organs such as kidney, liver and gills.

Histological Analysis of Liver

The liver histology in control group revealed the presence of systematically arranged round hepatocytes with centrally located spherical nucleus surrounded by homogenous cytoplasm. Nucleus showed a prominent nucleolus in the nucleoplasm (Figure 3-A). In the histology of liver of group B and group C fishes, the shape of the hepatocytes was seen to have undergone distortion with considerable swelling of the cells and blood streaks seen among hepatocytes with congested blood vessels. Broken cellular membranes were identified with irregular structure of the hepatocytes. There was formation of vacuoles observed in the hepatic cells with distinct necrotic progress (Figure 3 (B-C)). Fluoride exposed tissue upon coming in contact leaf extract of tulsi (Group D) some alleviation in the severity of the histological changes in liver caused by sodium fluoride. There were fewer necrotic cells and distorted nucleus with some sinusoidal dilation and blood streaks observed (Figure 3-D) In the group E, the tissues revealed some recovery of the damage observed earlier although there were still signs of sinusoidal dilation and pyknosis in the nucleus distinctly observed (Figure 3-E). In the combined formulation of plant extracts, tissues exhibited extensive degree of recovery with much fewer necrotic cells and less sinusoidal congestion. But the visible damages still observed in the hepatocyte and their membranes (Figure 3-F).

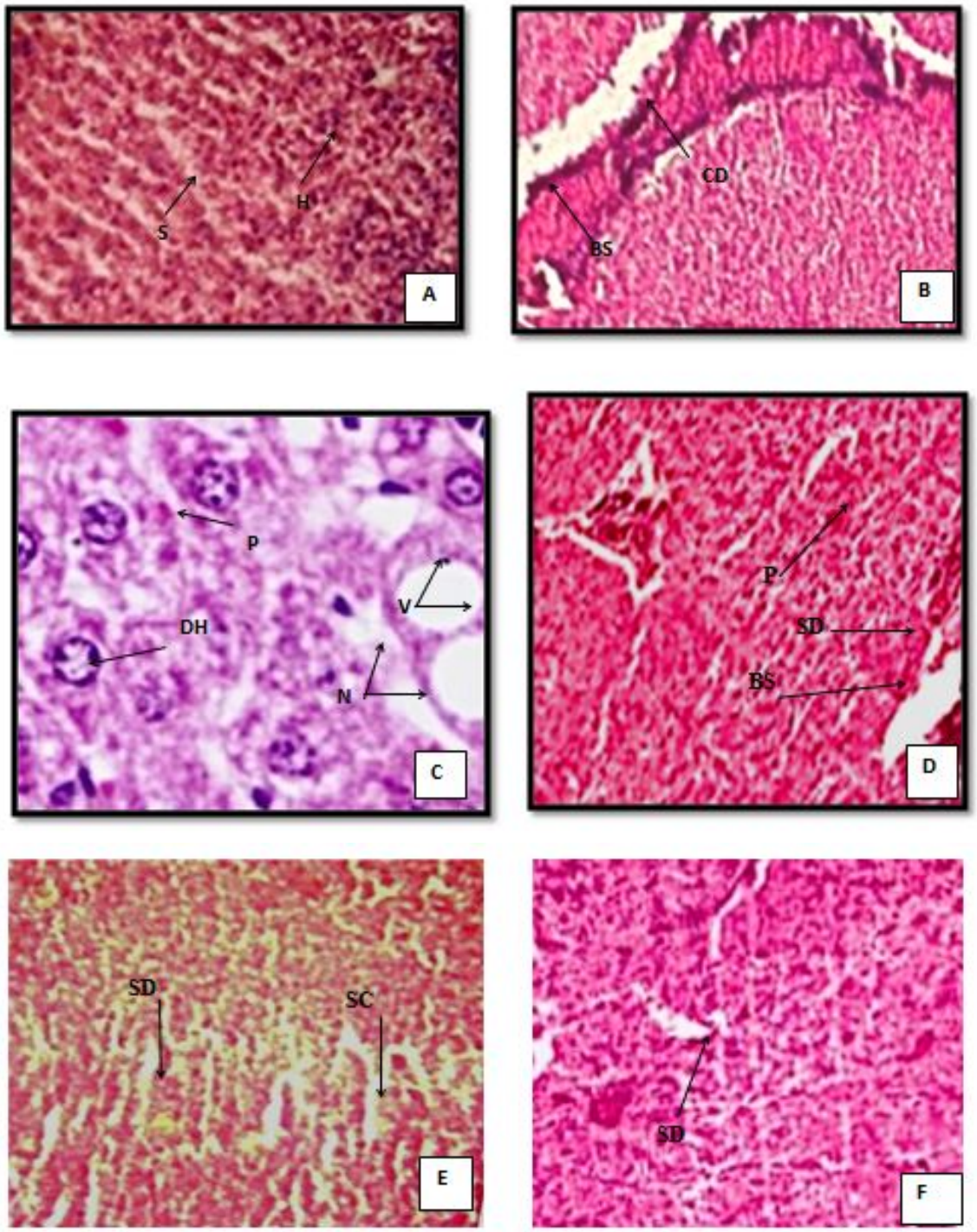

Figure 3:A- Control: Shows normal histology with hepatocytes and sinusoids, (B-C) Histological changes in liver of 50 PPM NaF treated fish, D- Histological changes in liver of fishes treated with tulsi leaf extract- Histological changes in liver of fluoride exposed fish treated with tea leaf extract, F- Histological changes in liver of fluoride exposed fish treated with combined formulation of extracts: Hepatocytes, S: Sinusoids, BS: Blood Streaks, CV: Congestion of vessels, P:Pyknotic nucleus, V: Vacuoles, DH: Distorted Hepatocytes, N: Necrosis, SD: Sinusoidal Dilution. (H&E, 40x)

Histological analysis of kidney

The kidney histology of control model revealed normal nephrons with lymphoid tissues present interstitially. Blood vessels were identified and glomerulus was systematically arranged. There were some hematopoietic cells detected in the interstitial tissues (Figure 4-A). The Kidney of 50ppm fluoride treated group showed marked degeneration with enlargement of nuclei. Cellular hypertrophy was identified. Epithelium showed vacuolation and relatively less number of distal convoluted tubules. Necrosis of glomerulus along with glomerular shrinkage was observed (Figure 4-B). Exposure to tulsi leaf extract in methanol base showed some extent of recovery in the damaging effects of sodium fluoride. There was reversal in the vacuolation of epithelium recorded earlier. Cellular hypertrophy was seen to have decreased in severity (Figure 4-C). In the tea leaf extract treated group, the distortions observed earlier due to fluoride exposure did not show much of a strikingly curative effect other than reducing the severity of necrotic formations earlier observed in the glomerulus (Figure 4-D). In the combined plant extract treated group, tissue showed similar characteristics as observed in tissue structure exposed to Tulsi leaf extract. There were fewer vacuolations identified as compared to the histological structure of kidney in fishes exposed to sodium fluoride (Figure 4-E)

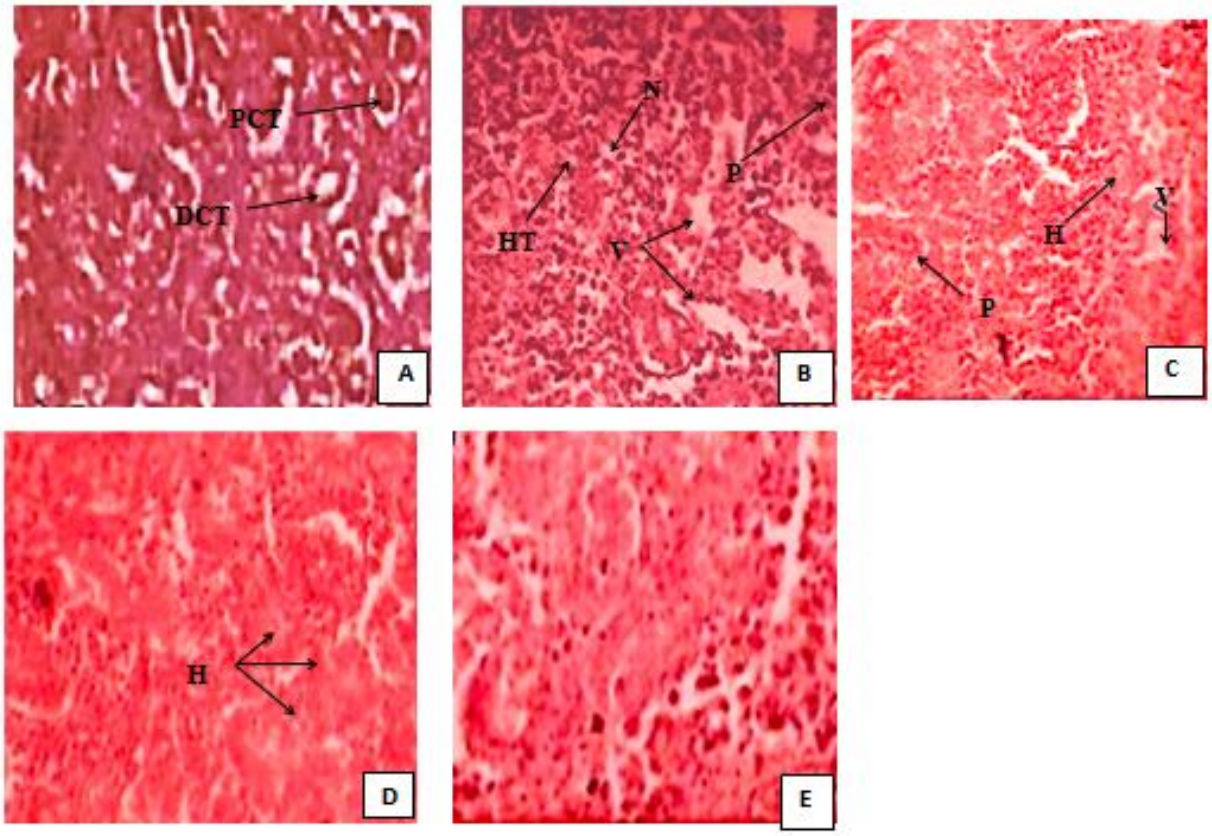

Figure 4: A-Shows normal kidney histology with glomerulus and Proximal Convolute Tubules (PCT) and Distal Convoluted Tubules (DCT), B- Histological changes in kidney of NaF treated (50 PPM) experimental fish, C- Histological changes in kidney of fishes treated with Tulsi leaf extract, D- Histological changes in kidney of fishes treated with Tea leaf extract, E- Histological changes in kidney of fishes treated with combined formulation of extracts. HT: Heterotrophy of epithelium of renal tubules, V: Vacuoles P: Pyknosis, N: Necrosis. (H&E, 40x)

Histological analysis of gills

Gill histology of control group showed normal arrangement of gill arches with long primary lamellae on which secondary gill lamellae were evenly distributed with bilateral arrangement (Figure 5-A). In the fluoride treated group (50ppm), there was occurrence of epithelial hyperplasia in the epithelium present between the secondary lamellae leading to fusion of lamellae. There was also hypertrophy of secondary lamellae observed in certain areas. This caused changes in the normal shape and arrangement of the gill structure due to degeneration of lamellae (Figure 5 (B-D). In the tulsi leaf extract treated group, gill structure of the fish showed abnormalities observed before. However, there were fewer distorted secondary lamellae and epithelial hyperplasia (Figure 5-E). Subjugation to tea leaf extract showed retention of the damages generated earlier, but there were slightly lower levels of lamellar fusion and degeneration as compared to that observed in the fluoride exposed group (Figure 5-F). In the combined plant extract treated group, gill histology showed a higher degree of reversal of the effects produced by sodium fluoride. There was less primary and secondary degeneration. Destruction to the epithelial cells was seen to have undergone a reduction in severity (Figure 5-G).

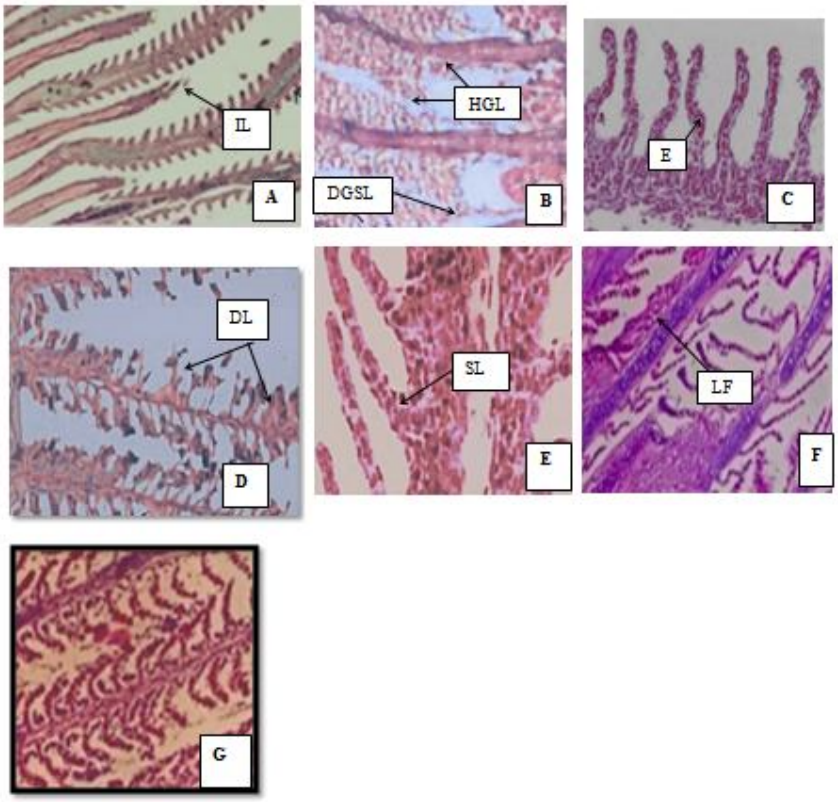

Figure 5: A- Normal gill structure in control group, (B-D) Distortions in the arrangement of gill observed in 50 PPM NaF treated experimental fish, E- Gills showing gradual recovery of structure in fishes treated with Tea leaf extract, F- Gills of fishes treated tulsi leaf extract showing some lamellar fusion and gradual recovery of the gills, G- Histological changes in the gills of fish treated with combined formulation of extracts. HGL: Hyperplasia in gill lamella, DGSL: Degenerating secondary lamella, EL: Epithelial Lifting, DL: Degeneration of lamella.SL: Secondary lamella, PL: Primary Lamella, LF: Lamellar Fusion, IL: Interfilamental lamellar space. (H&E, 40x)

Biochemical analysis

The Aspartate transaminase (AST) activity in the liver of control group of fish was found in 4.11±0.15 IU/L to 4.15±0.13 IU/L. Exposure to sodium fluoride showed a trend of increasing AST activity as compared to the untreated group. The AST activity in the animal group exposed to 50 ppm NaF showed an increase 5.89±0.15 IU/L to 6.12±0.21 IU/L with increase in exposure time (Table 3), which was statistically significant. AST activity showed decreasing trend when the fishes were exposed to the different plant extracts. The values recorded for different extracts exposure have been given in Figure 6.

Table 3: AST (IU/L) activity in *Channa punctatus* exposed to sodium fluoride and different plant extracts

Exposure Time	Control	NaF (25 PPM)	NaF (50 PPM)	Tulsi Leaf Exposure	Tea Leaf Exposure	Combined Extract Exposure
Day 3	4.11±0.15	4.85±0.19 ****	5.89±0.15 ****	5.21±0.16 ****	5.16±0.14****	5.18±0.23
Day 7	4.15±0.13	5.10±0.17 ***	6.12±0.21 ****	4.87±0.21 **	4.63±0.18	4.60±0.16

Data represented as mean ± SE of 5 fishes
(*indicates p<0.05, ** indicates p<0.01 *** indicates p<0.001 **** indicates p<0.0001

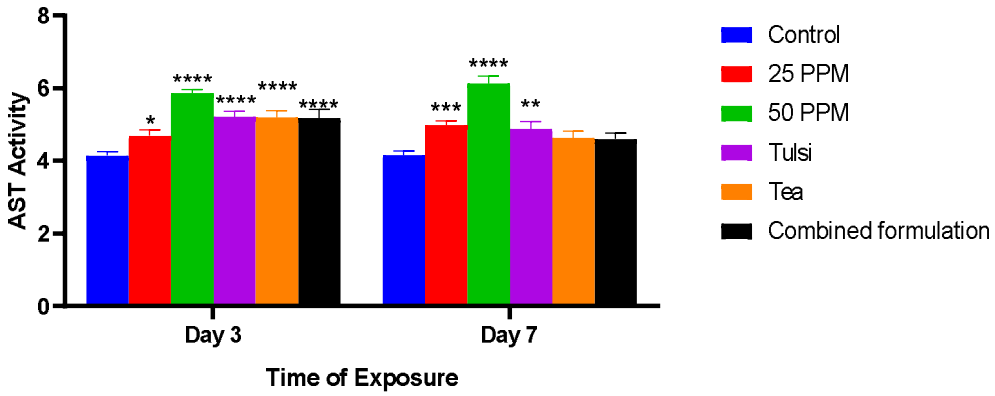

Figure 6: **AST Activity in different group of experimental fish**

The Alanine transaminase (ALT) activity in the liver of control group of fishes was found to range from 10.57±0.09 IU/L to 10.62±0.13IU/L. The fishes when exposed to 50 ppm of sodium fluoride showed a slight increase in trend of ALT activity as compared to the ALT activity observed in the control group which ranged from 13.78±0.08 IU/L to 14.34±0.12 IU/L (Table 4), which is statistically significant. However, ALT activity showed decreasing trend of ALT activity when exposed to the different plant extracts with sodium fluoride. The values recorded for different extract exposure have been given in Figure 7.

Table 4: ALT (IU/L) activity in *Channa punctatus* when exposed to sodium fluoride and different plant extracts

Exposure Time	Control	NaF (25 PPM)	NaF (50 PPM)	Tulsi Leaf Exposure	Tea Leaf Exposure	Combined Extract Exposure
Day 3	10.57±0.09	11.68±0.08***	13.78±0.08****	13.54 ± 0.18****	13.06±0.17****	13.04±0.16****
Day 7	10.62±0.13	12.23±0.11***	14.34±0.12****	12.86±0.23***	12.27±0.21***	11.94±0.22**

Data represented as mean ± SE of 3 observations
*indicates p<0.05, ** indicates p<0.01 *** indicates p<0.001 **** indicates p<0.0001

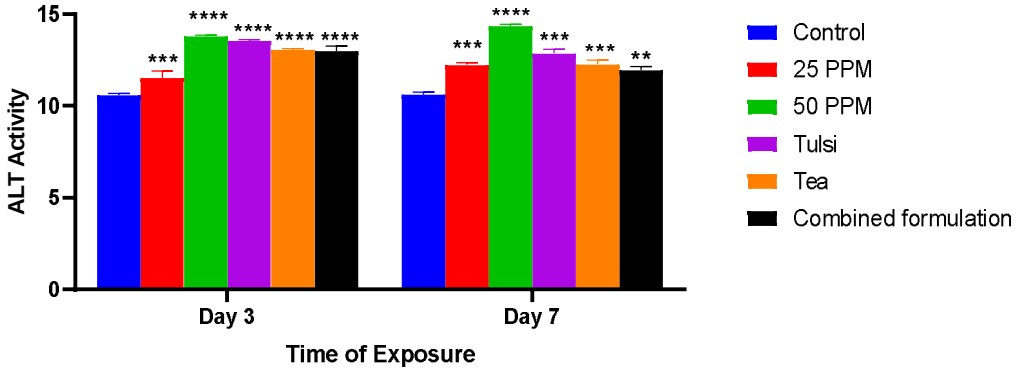

Figure 7: ALT Activity in different group of experimental fish

The Alkaline phosphatase (ALP) activity in control fishes was found to be in the range of 0.76±0.05 IU/L to 0.89±0.0.02 IU/L. However, when treated with 50 ppm of sodium fluoride, there was an increase in trend of alkaline phosphatase observed, as compared to the treated group of fish, (Table 5), which was statistically significant. When the fishes were exposed to tulsi leaf extract, tea leaf extract and a combination of both tulsi and tea leaf extracts, there was a decreasing trend observed in the ALP activity. The values of the ALP activity are shown in figure 8.

Table 5: ALP (IU/L) activity in *Channa punctatus* when exposed to sodium fluoride and different plant extracts

Exposure Time	Control	NaF (25 PPM) Exposed	NaF (50 PPM) Exposed	Tulsi Leaf Exposure	Tea Leaf Exposure	Combined Extract Exposure
Day 3	0.76±0.05	1.37±0.07***	1.42±0.07***	1.36±0.07**	1.32±0.18**	1.28±0.14**
Day 7	0.89±0.02	1.58±0.09****	1.73±0.14****	1.07±0.11	1.15±0.21	0.96±0.23

Data represented as mean ± SE of 3 observations
*indicates p<0.05, ** indicates p<0.01 *** indicates p<0.001 **** indicates p<0.0001

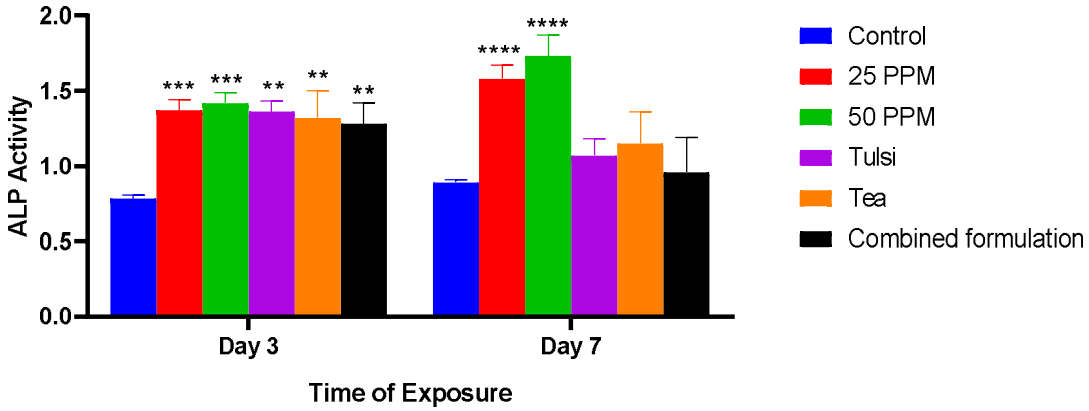

Figure 8: **ALP Activity in different groups of experimental fish**

Thus, the results obtained from the above biochemical indicates that the natural leaf extracts of tulsi, tea and a combinatorial extract are beneficial in resisting the deleterious effects of fluoride on *Channa punctatus*, which indirectly affects human health as a consequence of the dietary consumption of these fishes.

DISCUSSION

Behavior has been one of the principle tools in studying toxicological effects in a particular organism. It helps in determining the physiological and physical well-being of an organism. In the present study, the alterations observed in the behavioral parameters in the fishes may be due to difficulty in maintaining equilibrium due to exposure to high concentrations of fluoride. The fishes exhibited unusual behavior in swimming and other behavioral aspects. Initially, after coming into contact with such a relatively high concentration (50 ppm) of sodium fluoride showed highly erratic swimming with rapid movements which may be attributed to disorientation in their maneuvering ability. The fishes' avoidance reactions to the toxicant may have manifested as irregular swimming, restlessness, fin flickering, hyperexcitability, springing out, and movement of the fish as seen in the current study. Changes in the sensitivity of the chemoreceptors may be the source of the avoidance response.. Similar observations were reported by researchers like Svecevieus (2001) and Agarwal (1991). The rapid movement of gills may be due to the fluoride exposure and its uptake by the gills which may affect respiration causing respiratory distress. This finding agrees to the observations made by Pandey et al. (2005). This explains the frequent vertical posture of fish at the column and surface of water with open mouth. This behavior in *Channa punctatus* occurs as there is comparatively a higher level of dissolved oxygen present in the top layers around the surface of water (Kramer et al. 1987), as oxygen from the atmosphere diffuses into these top layers and replenishes it with higher oxygen level.

Changes in body coloration were noted in the present study which may be attributed to pigmental changes. Similar reports of pigmental changes were observed by Santha et al. (2000); Karuppasamy (2001); Subathra and Karuppasamy (2003); Sivakumar et al. (2006) due to various metal toxicity. When the fishes were kept in water treated with sodium fluoride and leaf extracts of tulsi and tea separately and then in mixtures of them both, they showed gradual reduction and reversal of the adverse effects of fluoride exposure. Both tulsi and tea contains an array of phytochemicals which have been long known to have antioxidant and anti-inflammatory properties. These bioactive components present in both these plants can protect the animal body from the damaging effects of the sub-lethal concentrations of fluoride.

Thehaemoglobin, TEC and MCV revealed a significant decrease upon fluoride exposure to the experiment fishes upon 7 days experiment.

The total erythrocyte count (TEC) showed a steady decline as the concentration of fluoride increased. This decrease in TEC is may be due to acute erythroblastic anemia associated with erythropenia (Wintrobe et al., 1934).Many Researchers (Goel et al., 1985; Goel and Sharma 1987; Pamila et al., 1991) previously reported similar results with a considerable decrease in RBC and Hb% concentration in fish exposed to various heavy metals. Christensen et al., (1972) stated that the variations in the haematological parameter may be explained in terms of the reduction of oxygen consumption in fish resulting in death due to heavy metal pollution. This may explain the decrease in the haemoglobin percentage observed in the fluoride exposed experimental models in the present study. The total leucocyte count (TLC) showed a steep increase in 25 ppm and 50 ppm of sodium fluoride concentration. White blood cells are involved in the regulation of immunological processes and increase in their number under the exposure of any toxicant is a defence mechanism of the fish against stress conditions (Mishra and Niyogi, 2011). Similar findings were also observed by other researchers (Nath and Banerjee, 1995; Mazon et al., 2002) in the fishes exposed to different heavy metals.

When the experimental fishes treated with fluoride were exposed to plant extract of tulsi and tea leaves, there was significant reduction of toxic effects noted in the blood parameters of *Channa punctatus*. Tulsi and tea both contain a number of important phytochemicals with several medicinal properties such as antioxidant, anticancerous, anti-inflammatory, etc. Thus, some of the bioactive components leaf extract of tulsi might have been effective in the reduction of some of the alterations seen in the blood parameters in the animal model exposed to fluoride.

The biochemical markers selected for assessment in the present study were alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). Aziz *et. al.* (2014) reported that fluoride increased the alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in freshwater fish *Oreochromis mossambicus.* Likewise, from the analysis of the results derived from our experiment it was found that sublethal concentrations of fluoride exposure to *Channa punctatus* increased the ALP, ALT and AST activity in their liver. AST is being used as an enzymatic biomarker, alteration of which signifies damages in tissue and organ damages of fish due to xenobiotic exposure (Philip and Rajarsee, 1996). In the present investigation, serum AST level was found to be increased in 25 ppm and 50 ppm sodium fluoride exposed fishes as compared to control indicating increased stress and tissue damage as well as efficient use of amino acids for metabolic activities to meet the fluctuating energy demand for maintaining a balanced glycolytic pathway and TCA cycle. According to Campbell (1984) and Tietz (1987) increase in AST level in plasma is mainly due to the damage of parenchyma cells of liver. Similar finding of increased AST level has also been observed by Mathan (2006) and attributed it to cell damage under toxicity stress. The hepatic derived enzyme alanine aminotransferase (ALT) plays a key role in the synthesis and deamination of amino acids which enables inter conversion of carbohydrate and protein metabolism for meeting high energy demand under stress conditions (Waarde and Henegaurajen, 1982). In the present investigation, increased ALT level in the plasma of sodium fluoride exposed fishes indicated hepatocellular damage which further released to the blood stream leading to its elevation in plasma, as supported by (Shenoy et. al., 2001).When experimental fishes treated with fluoride were subjected to tulsi and tea leaf extracts, the fishes seemed to had a fall in the activities of ALP, AST and ALT.

Liver is one of the vital organs in our body since it is known to be the chief organ of detoxification. Fluoride treated experimental fishes showed extensive damage to the liver histology. Marked changes in the hepatocytes were observed with swelling, degeneration and distortion of the cells. Necrosis was also identified in some of the cells. Shastry and

Agarwala (1975) reported necrosis in liver of *Heteropneustes fossilis* after being injected with carbon tetrachloride. The liver cord disarray, cell vacuolation and cell necrosis were observed in this study which was also reported by Shastry and Sharma (1979) in endrin treated liver of Indian fish. Some histopathological studies evidences such as, liver tissue lesions were reported in freshwater fish *Cirrhinus mrigala* by Velmurugan et al.,(2007).

Kidney is another vital organ present in our body which is imperative to the removal of metabolic wastes from our body and also in the maintenance of the electrolytic, acid-base equilibrium inside our body. Exposure to sublethal concentrations of fluoride manifested certain degrees of degenerative progressions and necrotic growths in the nephrons, damage to the epithelium and hypertrophy and structural distortion of the glomerulus. Gupta and Dalela(1987) reported similar histopathological changes in kidney of *Notopterus notopterus*. There were vacuolation in the epithelial cells identified in the histology of kidney. Similar findings were reported by Bhatnagar et al., (2007).Similar histological alterations in the kidney of *Channa punctatus* (Bloch) due to the effects of sub-lethal concentrations of zinc were reported in previous study (Gupta and Srivastava, 2006). The degenerative damages develop due to the presence of high concentration of heavy metals in the renal tissue (Kumari and Ramkumar, 1997; Venkataramana and Radhakrishnaiah, 1987).

In the present study, treatment of *Channa punctatus* with sublethal concentrations of sodium fluoride revealed that it manifested several damaging impacts on the histological structure of gills. There was disruption of the normal structure of the gills observed in the fluoride treated fish. Occurrence of epithelial hyperplasia in the epithelium present between the secondary lamellae leading to fusion of lamellae was also recorded. Dhanapakiam et al., (2004) reported similar findings in the gill histology of *Labeorohita.* Hyperplasia in gill and lamellar fusion was also reported by Abdel-Hameid (2008) in fishes treated with arsenic. Alterations in the gill structure including epithelial lifting were also observed in fish after exposure to copper (Figueiredo-Fernandes et al., 2007; Peebuaet al., 2008). Lead metal was also seen to exhibit similar damaging effects on gills. It caused hyperplasia of epithelial cells between secondary lamellae led to fusion and got separated from pillar system, vacuolation and necrosis of lamellar epithelial cells and resulted in hyperplasia of lamellar epithelial cells (Chavan and Muley, 2014).

The histopathological analysis on different tissues of *Channa punctatus* presents a unique evidence of fluoride toxicity in fishes and how its sub lethal concentration causes several damages on these tissues.

CONCLUSION

From the present study, it is arrived at the conclusion that exposure of high concentrations can induce several toxicological effects in freshwater fish Channa punctatus, which can severely harm the body morphology of fishes causing various abnormal appearances to occur on their body surface such as lesion, inflammations, pigmentations, etc. in addition to causing organ level toxicity. The morphology of the organ also showed several peculiar deformities and destruction as result to high concentrations of NaF. Various marked alterations have been recorded in the behavioural, histological and biochemical alterations of *Channa punctatus* Bloch.as a result of sublethal concentrations of fluoride exposure.Thus, such toxic effects can be ameliorated by the use of leaf extract of *Ocimum sanctum* L. and *Camellia sinensis* (L.) Kuntze as dose and time dependent variables of plant extracts showed notable remediation of the deleterious effects of the fluoride induced toxicity. Although both methanolic leaf extracts of tulsi and tea showed promising results in the reduction of the toxic stress, Tea leaf extract exhibited higher ameliorative potential than that shown by tulsi leaf extract used. It was also revealed that combined formulation of the two plant extracts showed even higher efficacy in the mitigation of the toxic manifestations in the fish. Thus, the analyses of the results obtained from the present study have revealed that the use of plant extracts is an extremely effective way of

reduction of toxic stress induced by sublethal concentrations of fluoride exposure.

Acknowledgements

The authors acknowledge the Department of Zoology, Cotton University, Panbazar, Guwahati, Assam, India for allowing them to carry out the present work.

Conflict of Interest

The authors declare no conflict of interest.

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