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Phytotherapeutic Potential of Citrus Peels against Fluoride-Induced Toxicity in *Channa punctatus* Bloch.

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Abstract

This study aims to investigate the protective efficacy of methanolic extracts of *Citrus jambhiri* (lemon) and *Citrus reticulata* (orange) peels against Sodium Fluoride (NaF)-induced toxicity in *Channa punctatus*. Phytochemical screening revealed the presence of alkaloids, phenols, flavonoids, terpenoids, steroids, and saponins in both extracts, with higher concentrations in methanol-based extractions. GC-MS analysis identified potent antioxidant and therapeutic compounds such as linalool, 5-hydroxymethylfurfural, and 4,7-dimethoxycoumarin. *Channa punctatus* was exposed to sublethal NaF concentrations (25 ppm and 50 ppm) for 3 and 7 days. After exposure, the fish were treated with 100 mg/L methanolic peel extracts of orange (OP), lemon (LP), and a combination of both (OP+LP). Fish exposed to 50 ppm NaF showed hyperactivity, erratic swimming, fin necrosis, and depigmentation. Morphological damages like fin rot and haemorrhagic areas were evident in the NaF group but reduced considerably in the combination group, which exhibited nearly normal pigmentation and minimal tissue damage. Cytomorphological examination of RBCs revealed severe abnormalities under fluoride stress, including lobular clumping, nuclear disintegration, and cell fusion. Haematological profile on Day 7 showed significant NaF-induced reductions in RBC count (2.34±0.08 $\times 10^6/mm^3$), haemoglobin (9.26±0.14 g/dL), PCV (24.45±0.24%), and MCHC (4.01±0.21%). Treatment with OP+LP significantly ($p<0.05$) restored these values closer to control levels. Liver biomarker enzymes were elevated due to fluoride toxicity: AST (50.66±2.35 IU/L), ALT (18.53±0.42 IU/L), and ALP (46.88±0.89 IU/L), compared to control values of AST (35.80±0.95 IU/L), ALT (13.80±0.22 IU/L), and ALP (42.44±0.70 IU/L). Co-treatment with OP+LP brought these levels significantly closer to baseline. Histopathological analyses of liver, gills, and kidney tissues from NaF-exposed fish showed severe vacuolation, degeneration, and congestion. These haematological, biochemical and histopathological alterations were significantly reversed to a normal level in the combined OP+LP treated group.

Major Findings: Citrus peel extracts, particularly in combination, demonstrated a strong protective and restorative effect against fluoride-induced haematological, physiological, biochemical, and histopathological alterations. This study substantiates the therapeutic potential of *C. jambhiri* and *C. reticulata* peels as natural detoxifying agents in aquatic toxicology.

Keywords: Bioactive Components, *Channa punctatus*, Fluoride Toxicity, Lemon Peel, Orange Peel, Sodium Fluoride

1. Introduction

Fluorine, the most electronegative and reactive element, is present in abundance in the Earth's crust, accounting for approximately 0.3g/kg. It ranks as the thirteenth most common element, reacting with others to form ionic compounds such as NaF and hydrogen fluoride in water[1]. When these compounds dissociate, they release negatively

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charged fluoride ions, which are known pollutants. Fluoride contamination is widespread, largely affecting groundwater due to the slow dissolution of fluoride-containing rocks such as fluorite, biotite, topaz, basalt, granite (20-3600 ppm), and syenite[2]. Additionally, industrial activities like ceramics, glass production, and agriculture contribute to fluoride pollution, particularly through fertilisers that increase the concentration of anions like fluoride in water bodies[3].

Fluoride, while beneficial in small amounts for dental health, becomes harmful when consumed in excess. The recommended daily intake for fluoride is 2.9 to 3.4 mg, and it can be sourced through food and dental hygiene products[4]. However, long-term exposure to high concentrations of fluoride leads to several health issues. In many regions, particularly in countries like China, India, and parts of Africa, groundwater fluoride levels exceed the WHO's safe limit of 1.5 mg/L. These high concentrations have resulted in widespread fluorosis, affecting teeth, bones, and overall health. India also faces severe fluoride contamination, with more than 70% of the districts in Rajasthan, Gujarat, and Andhra Pradesh affected[5]. In Assam, fluoride concentrations in groundwater ranged from 1.45 to 7.8 mg/L in districts such as Goalpara, Kamrup, Karbi Anglong, and Nagaon[6]. A survey revealed that dental fluorosis affected 9.09% of students in Nagaon, 3.30% in Kamrup, and 7.30% in Karbi Anglong. Community surveys showed similar results, with skeletal fluorosis also prevalent in these regions[7,8].

Fluoride contamination in aquatic ecosystems has emerged as a significant environmental concern, primarily due to industrial effluents and agricultural runoff[9]. Elevated fluoride levels can lead to oxidative stress, disrupting physiological and biochemical processes in aquatic organisms[10]. Studies have demonstrated that fluoride exposure induces oxidative damage and apoptosis in fish species, compromising their health and survival[11]. Although small amounts of fluoride can benefit bone density and dental health, chronic exposure leads to harmful effects such as dental and skeletal fluorosis, brittle bones, arthritis, and even neurological issues like Alzheimer's[12].

In aquatic environments, fluoride pollution poses a significant ecotoxicological threat. Freshwater ecosystems are particularly vulnerable, with fish species being negatively impacted[13]. As fluoride concentrations increase, fish metabolism and enzyme activity are disrupted, affecting vital processes such as the glycolytic pathway and Krebs cycle[14]. Fluoride binds to functional amino acid groups in enzymes, leading to their inhibition. The toxicity also triggers oxidative stress, which damages cellular membranes and reduces mitochondrial fitness[15]. In fish, fluoride exposure leads to physiological changes, including erratic swimming behaviour, scale shedding, and haemorrhaging. Blood composition is also affected, with decreased haemoglobin levels, cell count, and packed cell volume[16,17]. Histopathological studies reveal that fluoride exposure causes structural changes in fish organs such as the gills, liver, and kidneys, and leads to apoptosis, or programmed cell death[18]. As a result, researchers have explored various strategies to mitigate fluoride toxicity, including the use of plant extracts for phytoremediation. This method employs plants or plant-based compounds to absorb and reduce pollutants, offering a cost-effective and environmentally friendly solution to fluoride contamination[19].

Phytoremediation using plant waste products, such as fruit peels, is a promising avenue for fluoride detoxification. Waste products are often discarded but can be repurposed to absorb toxicants from soil and water bodies, reducing the risk of groundwater contamination[20]. Various plants have demonstrated significant fluoride adsorption capacities, and research into their nutraceutical and therapeutic properties continues. *Citrus jambhiri* Lush., commonly known as Kaji Nemu, is a variety of lemon native to Assam. Its peel contains high levels of limonene (84.5%), along with other compounds such as sabinene, beta-myrcene, and linalool[21]. Traditionally, it has been used in Assamese cuisine and for medicinal purposes, particularly for digestive disorders and arthritis. Ethnobotanical studies also report its antimetric properties, indicating its potential use in treating nausea and vomiting[22]. *Citrus reticulata* Blanco., better known as the Nagpur orange, has also gained attention for its medicinal properties. Its peel is a common waste product, yet it contains valuable bioactive compounds such as limonene, hesperidin, naringenin, and linalool. These compounds exhibit antioxidant, anticancer, anti-inflammatory, and radical-scavenging properties[23]. Additionally, *Citrus reticulata* is a rich source of vitamins A, B6, and C, as well as calcium and polyphenols, which are associated with the prevention and management of chronic diseases such as diabetes and Alzheimer's[21].

To mitigate fluoride-induced toxicity, researchers have explored the use of natural antioxidants derived from plant sources. Citrus peels, often considered agro-industrial waste, are rich in bioactive compounds such as flavonoids, phenolic acids, and essential oils. These compounds exhibit potent antioxidant and antimicrobial properties, making

citrus peels a promising candidate for developing natural therapeutics[11]. Recent investigations have highlighted the benefits of citrus peel extracts in enhancing fish health[24]. For instance, dietary inclusion of orange peel fragments improved the antioxidant capacity and hematological profiles of Nile tilapia under stress conditions[25]. Similarly, lemon peel extracts have shown significant antioxidant activity, attributed to their high flavonoid content[26].

While the antioxidant properties of citrus peels are well-documented, their specific role in counteracting fluoride-induced toxicity in freshwater fish remains underexplored. Moreover, comparative studies assessing the efficacy of different citrus species, such as *Citrus jambhiri* (lemon) and *Citrus reticulata* (orange), in mitigating fluoride toxicity are limited. This study uniquely investigates the protective effects of methanolic extracts from two distinct citrus species against fluoride-induced toxicity in *Channa punctatus*. By integrating phytochemical analyses with haematological, biochemical, and histopathological assessments, the current study aims to provide comprehensive insights into the potential of citrus peels as natural detoxifying agents in aquaculture.

2. Materials and Methods

2.1 Chemicals and Reagents

Ethanol, methanol, chloroform, glacial acetic acid, diethyl ether, and paraffin wax (DK9D692807, melting point: 58-60°C) were obtained from Merck Life Science Pvt. Ltd. Acetone, sodium chloride, sodium hydroxide, and petroleum ether were purchased from Thermo Fisher Scientific India Pvt. Ltd., Mumbai. Wright stain (powder), Giemsa stain (powder), haematoxylin, and eosin were sourced from Sigma-Aldrich Chemicals Pvt. Ltd., USA. Enzyme assay kits were procured from HiMedia Laboratories Pvt. Ltd., Abcam Ltd., India, and Transasia Bio-Medicals Ltd., Mumbai.

2.2 Collection, Identification and Preparation of Plant Extract

Fresh orange and lemon fruits were collected from Satgaon weekly market located in Guwahati, Assam. Authentication and identification of the plants were conducted by the Department of Botany, Cotton University, Guwahati, Assam. The dried and powdered plant materials (50 g) were subjected to extraction with 250 mL of methanol (1:5 w/v) using a Soxhlet extractor for 24 h at a temperature below the solvent's boiling point. Subsequently, the extracts were filtered through Whatman filter paper and concentrated at 40°C using a Rotary evaporator.

2.3 Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

The peels of *Citrus jambhiri* and *Citrus reticulata* were combined with methanol for extraction, followed by GC-MS analysis. GC-MS analysis was conducted using a Perkin Elmer Clarus 680 GC and Clarus 600C MS system with TurboMass Ver. 6.1.2 Software. Helium gas served as the carrier at 1 ml/min. Samples were injected in splitless mode with an injector temperature of 280°C and an ion-source temperature of 180°C. The oven temperature started at 60°C for 1 min, increased at 7°C/min to 200°C (held for 3 min), then at 10°C/min to 300°C (held for 5 min) with a total runtime of 39 min. Mass spectra were acquired in EI+ mode at 70 eV, with a mass range of 50-600 amu. Peaks were identified by comparing mass spectra with the NIST-2014 library 4. Each peak in the chromatogram was subjected to GC-MS library matching to identify the presence of bioactive compounds. Chemdraw software was utilised to illustrate the chemical structures of the identified bioactive compounds.

2.4 Qualitative Phytochemical Screening

Qualitative analysis of different phytochemical constituents of methanol, ethanol, and aqueous extracts of selected citrus peel was carried out by using standard protocols following the methods described by Harborne (1998), Trease and Evans (1989), and Sofowora (1993)[27-29]. The test has been performed to detect the presence of phytochemicals like Alkaloids, Phenols, Tannins, Flavonoids, Glycosides, Terpenoids, Steroids and Saponins in *C. jambhiri* and *C. reticulata*.

2.5 Experimental Design

Channa punctatus (Bloch.), commonly known as snakehead fish or locally as "Goroi", weighing around 50-60 g and measuring 14-17 cm in length, were used for the study. The fish were kept in chlorine-free deep well water, with the water being changed every two days. They underwent a 7-day acclimatisation period during which key water parameters such as temperature, pH, dissolved oxygen, and electrical conductivity were measured following standard APHA (2005) guidelines. The fish were

fed daily with commercial dry pellets (Tokyo pellets). The fish were then randomly divided into six groups of five each. Group A served as the control, while Groups B and C were exposed to 25 ppm and 50 ppm NaF, respectively, in the water. Group D was given 50 ppm NaF and 100 mg/l Orange Peel (OP) extract, Group E received 50 ppm NaF and 100 mg/l Lemon Peel (LP) extract, and Group F was treated with 50 ppm NaF along with 50 mg/l each of orange and lemon peel extracts (OP+LP). Observations were made on the behavioural, morphological, haematological, histological, and biochemical changes in all six groups.

2.6 Study of Behavioural Changes

Behavioural changes were observed to detect any unusual or erratic behaviour in response to NaF exposure. Parameters included rapid and erratic swimming or hyper-mobility, hovering near the water surface, rapid operculum movement, darting, and reduction in overall behavioural activities.

2.7 Study of Morphological Changes

Fish housed in glass aquariums were regularly observed for morphological changes induced by NaF toxicity. Lesions, vacuole formation, loss of body colouration or fins, fin ray abnormalities, and body surface haemorrhages were noted. Fish displaying clinical defects or infections were separated from the group, and any changes resulting from the addition of plant extracts were recorded.

2.8 Haematological Parameters

Blood was collected in EDTA-coated tubes by heart puncture, and parameters such as Haemoglobin (Hb), Red Blood Cells (RBC), White Blood Cells (WBC), and Packed Cell Volume (PCV) were assessed using standard techniques

2.9 Biochemical Estimation

Activity levels of Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), and Alkaline Phosphatase (ALP) were determined using commercially available enzyme kits.

2.10 Histopathological Study

Liver, kidney, and gill tissues were dissected from fishes of all experimental groups on day 7 and processed using the standard Hematoxylin-eosin method (HE). Microphotographs of representative tissue sections were captured using a D-Winter microscope equipped with a microphotograph attachment camera and analysed using Calipro Version 4.6 software for histopathological analysis.

2.11 Statistical Analysis

For the statistical analysis of the data, two-way ANOVA was used using GraphPad Prism software version 8.0.2. Tukey's post hoc test was utilised for multiple comparisons. Each experiment described above was conducted three times to ensure robustness and enhance the reliability of the results and subsequent analysis.

3. Result

3.1 Qualitative Phytochemical Screening

The results of the phytochemical screening of both the citrus fruit peels showed that most all the phytochemicals, *viz.* Alkaloids, phenols, tannins, flavonoids, glycosides, terpenoids, steroids and saponins are present in the extracts of *C. jambhiri* and *C. reticulata* at different levels of concentration, with higher levels observed in the methanol extract of both citrus peels (Table 1).

Table 1. Qualitative phytochemical screening of *Citrus reticulata* and *Citrus jambhiri* using three different solvents

Compounds	*Citrus reticulata*			*Citrus jambhiri*		
	Aqueous	Methanol	Ethanol	Aqueous	Methanol	Ethanol
Glycosides	+	+++	++	+	-	-
Phenols	+	++	+	+	+++	++
Alkaloids	-	+	+	+	+	+
Tannins	-	++	+	+	+	+
Flavonoids	+	+	++	+	++	+
Steroids	+	+	+	+	+	+
Saponin	-	+	+	-	+	+
Terpenoids	-	++	+	+	+	+
+ = low conc., ++ = moderate conc., +++ = high conc., - = absent						

3.2 GC-MS Analysis

GC-MS analysis of lemon peel and orange peel revealed the presence of various phenolic groups, terpenes, and other compounds with antimicrobial, antioxidant, and medicinal properties (Tables 2, 3). *Citrus jambhiri* peel extract revealed the presence of nine important compounds *viz*., 2-methoxy-4-vinylphenol, Linalool, 5-hydroxymethylfurfural, n-hexadecanoic acid, Eicosanoic acid, Ethylbenzene, p-Xylene, Benzene-1,3-dimethyl-., and o-Xylene (Figure 1). *Citrus reticulata* peel extract revealed the presence of seven important bioactive compounds *viz*., 4,7-di(methyloxy)coumarin, 2-methylthiolane, S,S-dioxide, 2-methyl benzaldehyde, 2,3-dihydro benzofuran, 2-methoxy-4-vinylphenol, 3-methyl-2-(2-oxopropyl)furan, and 3-methoxy-5-propylphenol (Figure 2).

3.2.1 *Citrus jambhiri Lush peel*

The following compounds were identified: (Refer Figure 1 and Table 2)

3.2.2 *Citrus reticulata Blanco peel*

The following compounds were identified: (Refer Figure 2 and Table 3)

3.3 Behavioural Changes

No behavioural changes were noted in the control group. However, fish exposed to fluoride-contaminated water showed noticeable behavioural shifts. Initially, they exhibited increased excitability, likely due to a fear response. Their swimming patterns became erratic and jerky, with attempts to jump out of the water and frequent surfacing for air. Irregular operculum movements were also observed. Some fish collided with the container walls, and a few displayed head-down swimming behaviour, occasionally scraping the bottom of the container with their snouts (Table 4).

3.4 Morphological Changes

No mortality or morphological changes were observed in the control group. However, fish exposed to fluoride-treated water displayed several significant alterations. Depigmentation of the body surface was noted, along with excessive mucus secretion, even at low fluoride levels. The fish shed their scales and showed haemorrhaging near the caudal region (Figures 3, 4). Necrosis developed at the base of the pectoral and caudal fins, sometimes resulting in death as the condition spread. Fish adopted an upright swimming posture due to the loss of pectoral, pelvic, and caudal fins, with necrosis particularly evident around the mouth, especially the lips. Additionally, fluoride-treated fish experienced a reduction in body weight, likely due to reduced feeding under stress conditions. Fluoride exposure also disrupted their carbohydrate, protein, and cholesterol metabolism. The livers of affected fish turned mustard yellow, while their gills became pale pink. In contrast, the control group exhibited brownish livers and dark red gills (Figure 5). In fish treated with plant extracts, the liver appeared yellowish-brown, and the gills retained their red colour.

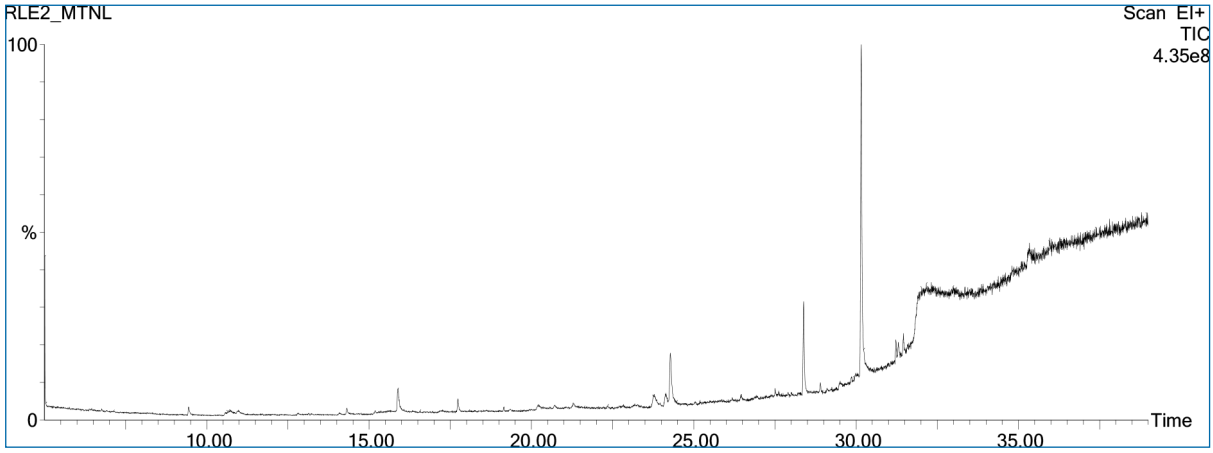
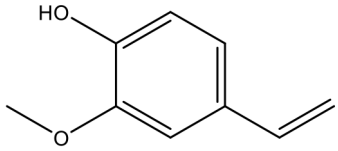
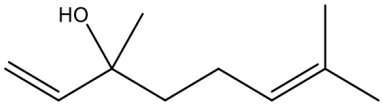
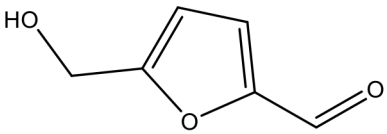
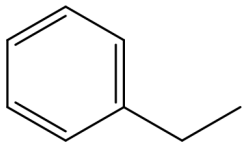
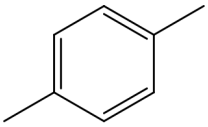
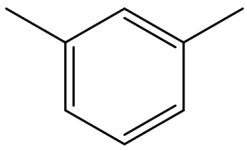
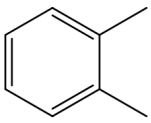


Figure 1. GC-MS chromatogram of methanolic extract of *Citrus jambhiri* Lush peel.

Table 2. Marker bioactive components detected in the methanolic extract of *Citrus jambhiri* peel through GC-MS.

RT value	Molecular formula	Molecular weight (g/mol)	Name of the compound and Chemical structure	Bioactivity
17.719	$C_9H_{10}O_2$	150	 2-METHOXY-4-VINYLPHENOL	It is an aromatic substance used as anti- inflammatory[51], antioxidant[52] and antimicrobial agent[53].
13.137	$C_{10}H_{18}O$	154	 LINALOOL	It is a powerful terpene. It helps in the treatment of conditions such as anxiety[54], depression[55] and helps in the treatment of several cancers[56].
15.958	$C_6H_6O_3$	658	 5-HYDROXYMETHYLFURFURAL	It has an antioxidant property[57], blocks immune-mediated allergic reactions[58]. Downregulates xanthine oxidase and aids in preventing sickle haemoglobin[59].
31.894	$C_{16}H_{32}O_2$	256	 N-HEXADECANOIC ACID	It may help in designing specific inhibitors of phospholipase A(2) as it has anti-inflammatory property[60].
29.513	$C_{20}H_{40}O_2$	340	 EICOSANOIC ACID	It acts as an antibacterial agent[61].
8.140	C_8H_{10}	106	 ETHYLBENZENE	It acts as a good solvent[62].
8.140	C_8H_{10}	106	 P-XYLENE	An important petrochemical building block[63].
8.315	C_8H_{10}	106	 BENZENE, 1,3-DIMETHYL-	It is used in the production of isophthalic acid, which is used as a copolymerizing monomer to alter the properties of polyethylene terephthalate[64].
8.315	C_8H_{10}	106	 O-XYLENE	Used in the laboratory during processing of histology sections and staining[65].

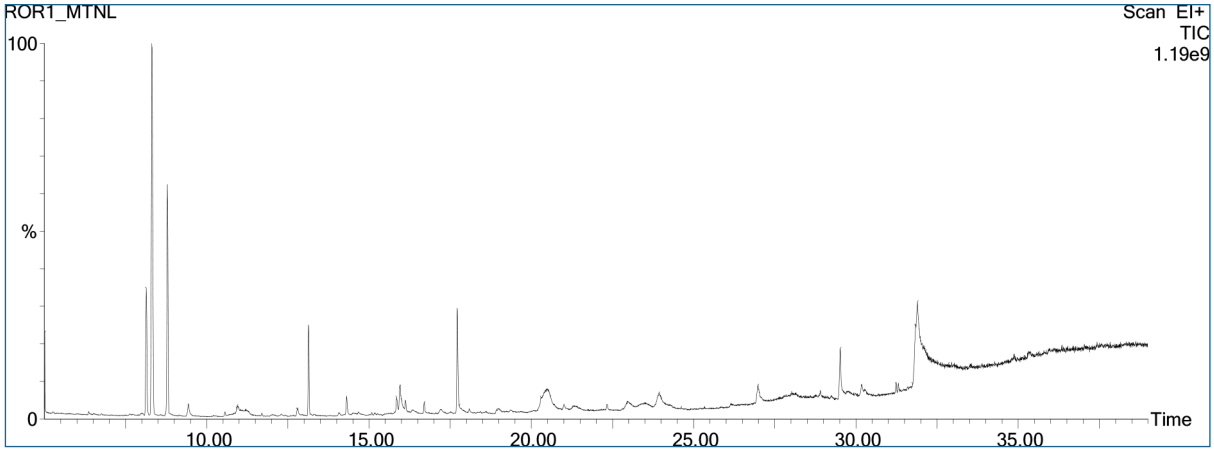


Figure 2. GC-MS chromatogram of methanolic extract of *Citrus reticulata* Blanco peel.

Table 3. Marker bioactive components detected in the methanolic extract of *Citrus reticulata* peel through GC-MS

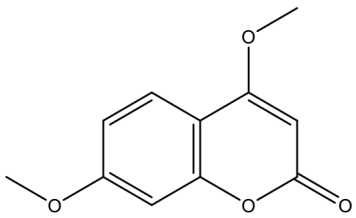
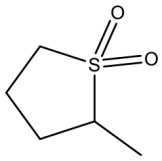
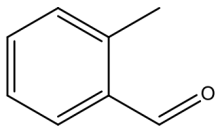
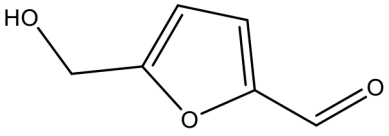
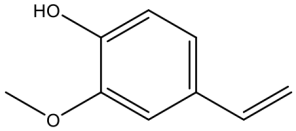
RT value	Molecular formula	Molecular weight	Compounds	Bioactivity
30.159	$C_{11}H_{10}O_4$	206	 4,7-DI(METHYLOXY)COUMARIN	Used in photochemotherapy[66] and therapeutic application in cancer[67].
9.440	$C_5H_{13}O_2S$	134	 2-METHYLTHIOLANE, S,S-DIOXIDE	It has been reported to possess a wide range of therapeutic properties with diverse applications in medicinal chemistry and material science[68].
15.893	C_8H_8O	120	 BENZALDEHYDE, 2-METHYL-	Used in additives like antibacterial and antifungal preservatives[69].
15.893	C_8H_8O	120	 5-HYDROXYMETHYLFURFURAL	It is a flavonoid and has been identified as an antioxidant agent[70].
17.739	$C_9H_{10}O_2$	150	 2-METHOXY-4-VINYLPHENOL	It is an aromatic substance used as anti- inflammatory[51], antioxidant[52] and antimicrobial agent[53].

Table 3. Continued...

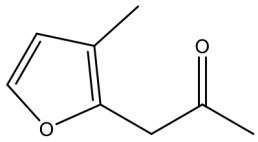
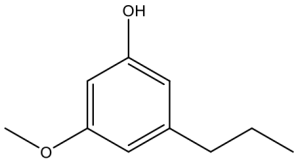
23.781	$C_8H_{10}O_2$	138	 3-METHYL-2-(2-OXOPROPYL)FURAN	It acts as a good antioxidant agent[71].
28.383	$C_{10}H14O_2$	166	 3-METHOXY-5-PROPYLPHENOL	These phenolic component exhibit tremendous antioxidant activity[72].

Table 4. Behavioural changes observed in different experimental groups

Nature of behaviour	Control	25ppm NaF	50ppm NaF	50ppm NaF+OP	50ppm NaF+LP	50ppm NaF+OP+LP
Hyper movement	-	+++	++++	+++	+++	++
Operculum movement	+	++	+++	++	++	+
Imbalance of swimming	-	+	+++	++	++	+
Fin movement	+	++	+++	+	++	+
Jumping	-	+	++	+	+	+
Opening the mouth on the water surface	+	++	+++	+	++	+

+= low conc., ++= moderate conc., +++= high conc., - = absent



Figure 3. Morphological changes in *Channa punctatus* Bloch. **(A)** Control; no morphological changes observed; **(B)** 25ppm NaF exposed and **(C)** 50ppm NaF exposed; skin colour changes, damaged fins and rotten operculum; **(D-F)** Combined peel formulation; little pigmentation and no fin damage.

3.5 Haematological Study

3.5.1 Cytomorphological Changes

In the untreated control fish, the RBCs were normal, with fully intact cell membranes. However, in fluoride-treated fish, the outer cell membrane showed signs of disintegration, and significant clumping of RBCs was observed. At higher fluoride concentrations, lobular clumping became apparent, along with the disintegration of the nuclear membrane, pallore formation, tear drop cells, granulation and cell fusion. On treatment with citrus fruit peel extracts, these changes of RBCs were greatly

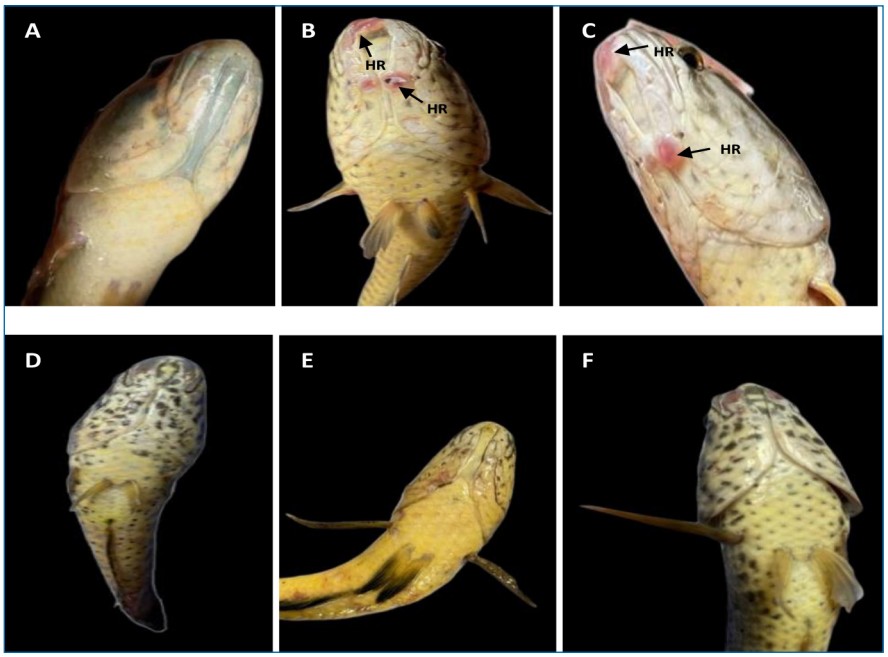


Figure 4. Morphological changes in the ventral side of *Channa punctatus* Bloch. **(A)** Control; no changes observed; **(B)** 25ppm NaF exposed and **(C)** 50ppm NaF exposed; ulcer formation and haemorrhages (HR); **(D)** 50ppm NaF + OP**, (E)** 50ppm NaF + LP**,** and **(F)** 50ppm + OP + LP; little pigmentation and no fin damage.



Figure 5. Colour changes in the liver and gills of *Channa punctatus* Bloch**. (A)** Control; red gills and brown liver **(B)** 25ppm NaF exposed and **(C)** 50ppm NaF exposed; yellow liver and faded red gills; **(D)** 50ppm NaF + OP**, (E)** 50ppm NaF + LP**,** and **(F)** 50ppm + OP + LP; progressive change from yellow to brownish colour liver and pinkish red gills.

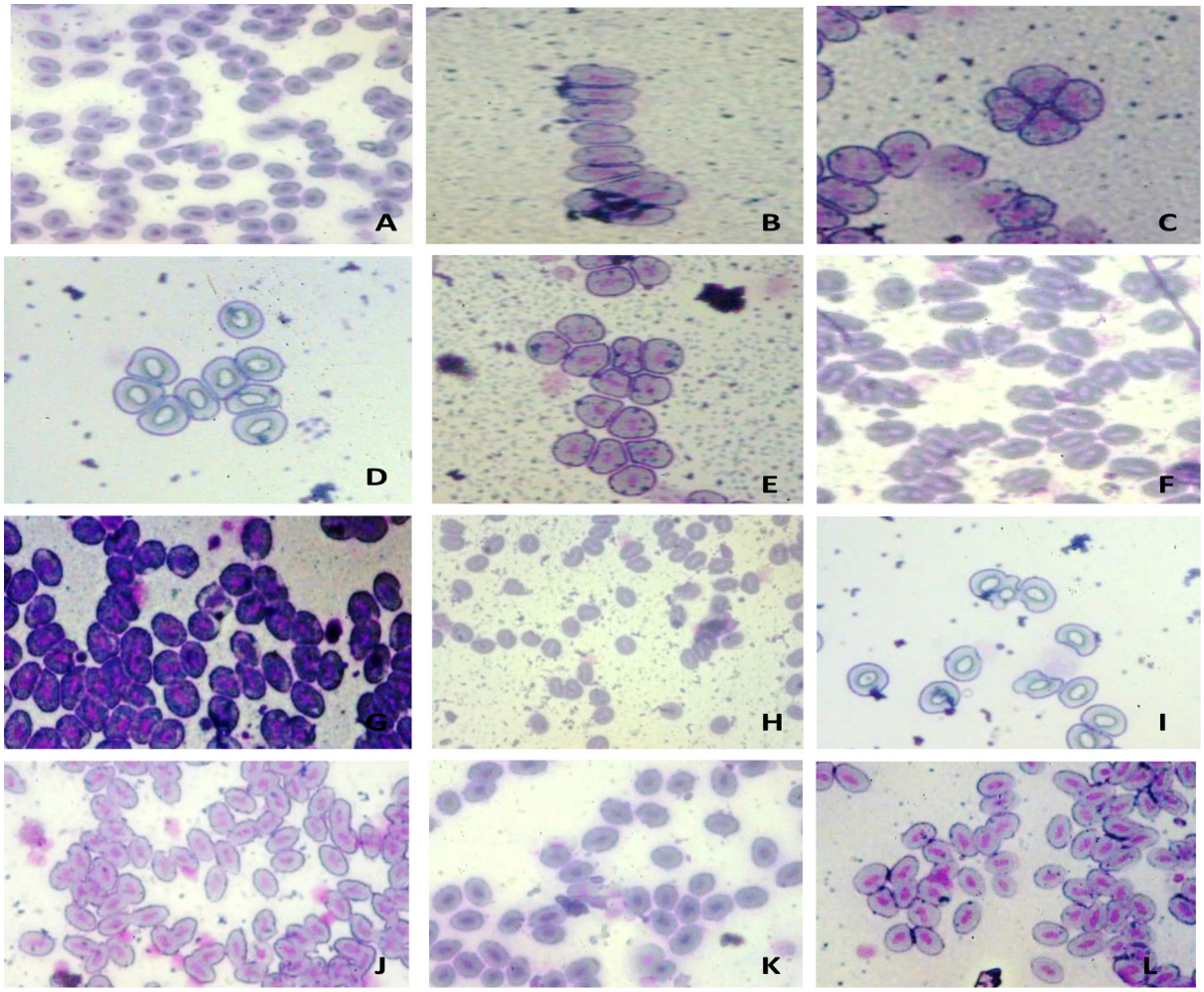


Figure 6. Cytomorphological changes in RBC's of *Channa punctatus* Bloch. **(A)** Control; no changes observed, RBC's with fully intact cell membrane; **(B–D)** 25ppm NaF exposed; RBC cell shape change, cell stacking, ring shaped cells, formation of rosette structure, lobular clumping of cells; **(E-I)** 50ppm NaF exposed; prominent clumping of cells, tear drop formation, pallore formation, granulation, disintegration of nucleus, cell membrane disintegration, cell fusion, cell shape changed to ring shape; **(J)** 50ppm NaF + OP; recovery of cell shape**,** less clumping of cells **(K)** 50ppm NaF + LP; cell membrane intact, oval shaped cells and **(L)** 50ppm NaF + OP + LP; highest recovery with intact cell membrane and oval shaped cells.

reduced with oval-shaped cells and intact cell membranes (Figure 6).

3.5.2 Numerical Changes

Exposure to different fluoride concentrations in water led to significant changes in several blood parameters. These included alterations in Total Erythrocyte Count (TEC), hemoglobin levels, Packed Cell Volume (PCV), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), and Total Leukocyte Count (TLC) at both 25 ppm and 50 ppm fluoride levels. Blood samples were collected from the fish hearts on days 3 and 7 to assess these parameters.

The study data revealed that RBC counts decreased to 2.9±0.06 and 2.34±0.08 $\times 10^6$ mm^3 when exposed to 25 ppm and 50 ppm NaF, respectively. However, treatment with fruit peel extracts brought the RBC counts closer to normal levels. Haemoglobin levels also significantly dropped to 9.62±0.12 and 9.26±0.14 g/dL at 25 ppm and 50 ppm NaF, but returned to near normal with fruit peel extract treatment (Table 5). Similarly, PCV, MCV, MCH, MCHC, and TLC all showed a marked decline with higher NaF concentrations, but improved with fruit peel extract,

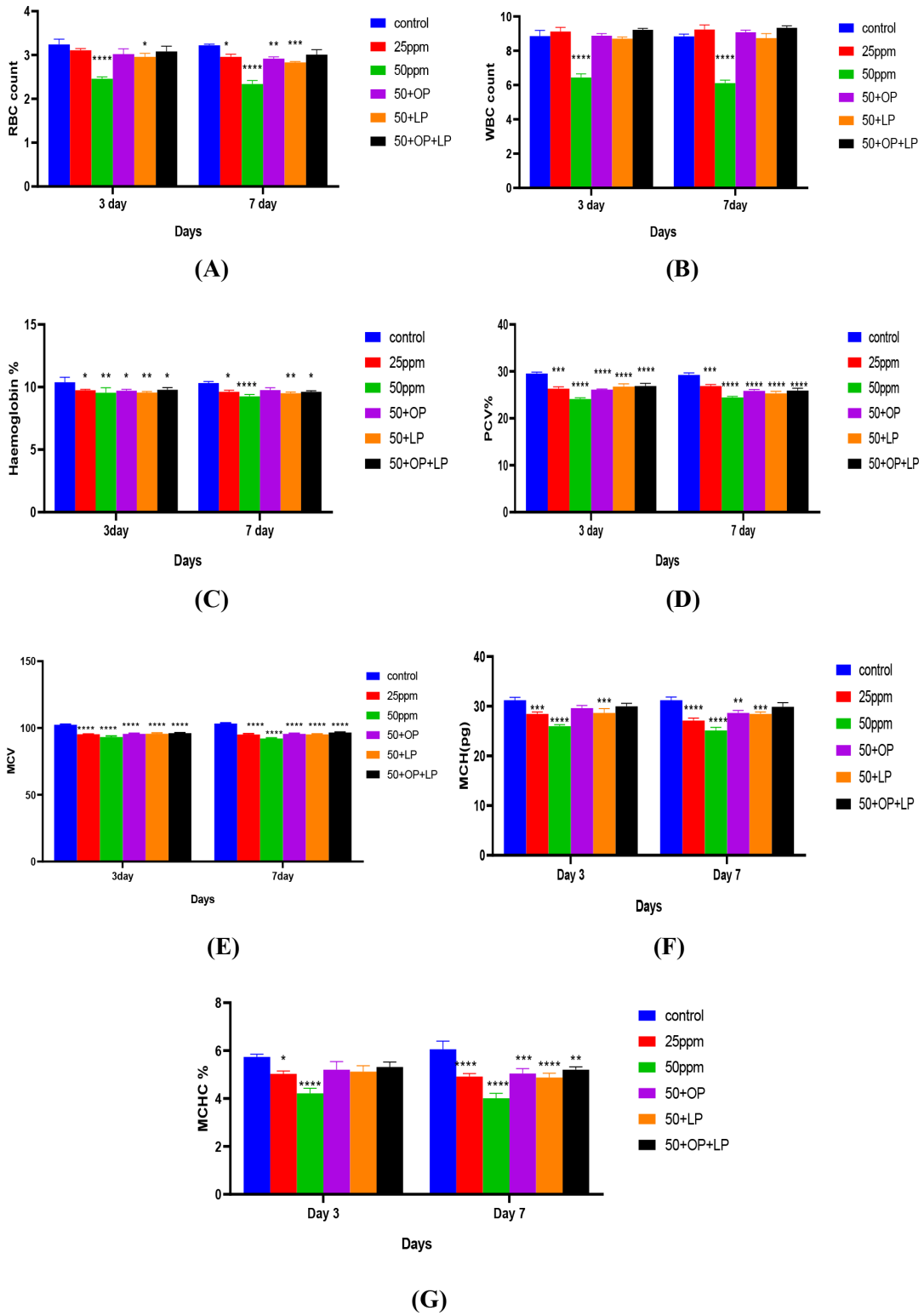
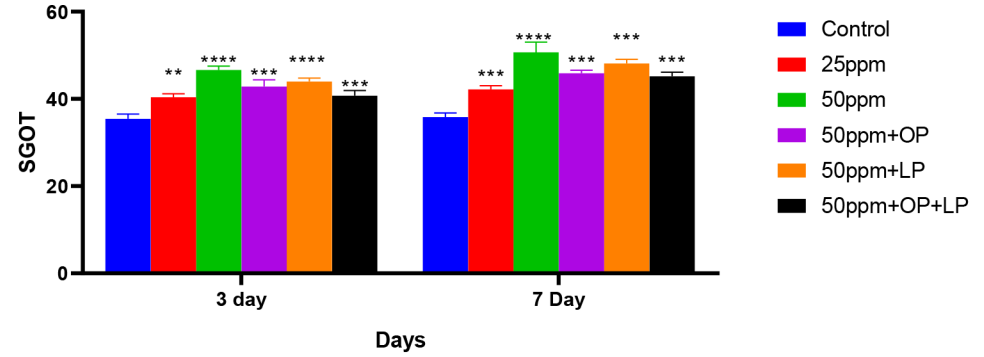
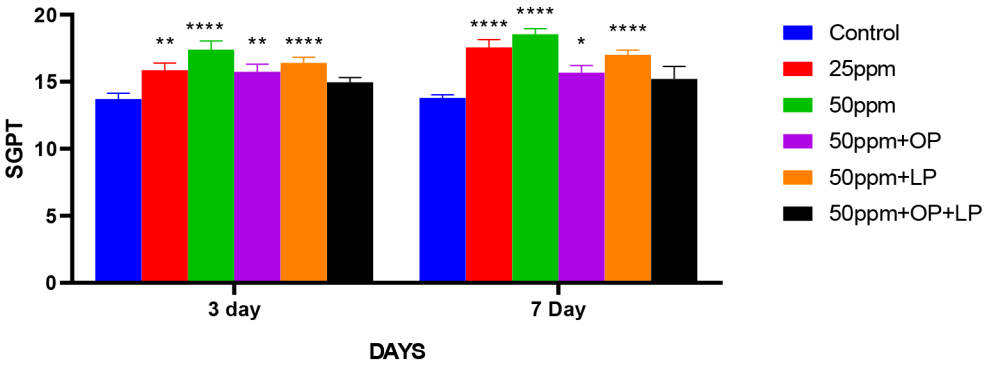


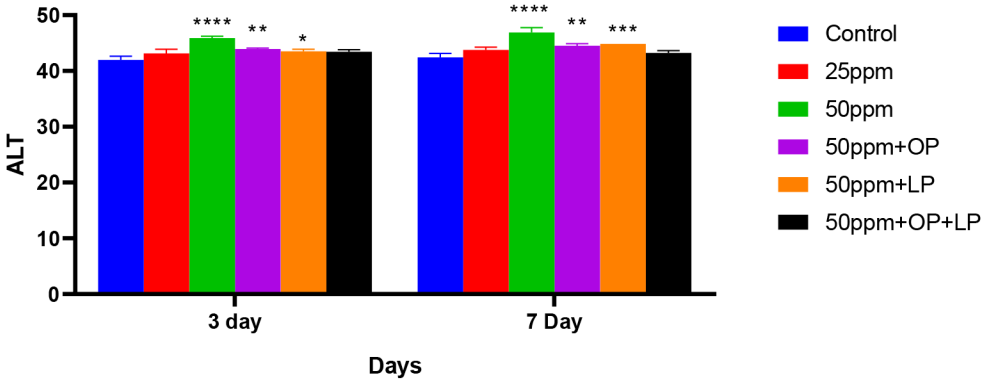
Figure 7. Graphical representation of blood parameter changes in *Channa punctatus* Bloch. on exposure to NaF and Citrus peel extracts on 3rd and 7th days **(A)** RBC count, **(B)** WBC count, **(C)** Haemoglobin%, **(D)** PCV%, **(E)** MCV, **(F)** MCH, **(G)** MCHC%.



(A)



(B).



(C)

Figure 8. Enzyme Activity (IU/L) in different groups of experimental fish **(A)** AST, **(B)** ALT, **(C)** ALP.

approaching control values. WBC counts increased at low fluoride concentrations but decreased at higher levels. On day 7, the control WBC count was 8.84±0.13 $\times 10^3$. With 25 ppm NaF, it rose to 9.24±0.26 $\times 10^3$, but dropped to 6.11±0.18 $\times 10^3$ at 50 ppm. Treatment with fruit peel extract reduced these variations, moving values closer to the control (Figure 7). Cell size also significantly decreased with increased NaF concentrations, measuring 9.58±0.26 µm at 25 ppm and 9.06±0.33 µm at 50 ppm, compared to 10.22±0.32 µm in the control group. Fruit peel extract treatment helped restore cell size closer to normal values.

3.6 Biomarker Enzyme Profile

Significant alterations in the activities of AST, ALT, and ALP were observed in Channa punctatus following NaF exposure and subsequent treatment with OP and

Table 5. Numerical changes in some blood parameters of the haematological study of different experimental groups of *Channa punctatus* Bloch. when exposed to different concentrations of NaF and Orange and Lemon peel extract on the 3rd and 7th days

S. No.	Parameters	Day	Control	25ppm NaF	50ppm NaF	50ppm NaF + OP	50ppm NaF + LP	50ppm NaF + OP + LP
1.	RBC (TEC×10^6/mm^3)	3	3.24±0.12	3.11±0.04	2.46±0.04 ****	3.02±0.12	2.96±0.08 *	3.08±0.12
		7	3.22±0.03	2.96±0.06*	2.34±0.08 ****	2.92±0.04 **	2.83±0.02 ***	3.01±0.11
2.	WBC (TLC×10^3/mm^3)	3	8.85±0.34	9.12±0.24	6.44±0.21 ****	8.88±0.12	8.64±0.18	9.22±0.08
		7	8.84±0.13	9.24±0.26	6.11±0.18 ****	9.08±0.12	8.74±0.26	9.34±0.11
3.	Haemoglobin (g/dL)	3	10.38±0.4	9.74±0.18*	9.54±0.24 **	9.70±0.12 *	9.56±0.18 **	9.78±0.18 *
		7	10.32±0.12	9.62±0.12*	9.26±0.14 ***	9.76±0.18	9.50±0.11 **	9.62±0.09 *
4.	PCV (%)	3	29.21±0.44	26.32±0.38 ***	24.11±0.26 ****	26.11±0.14 ****	26.74±0.62 ****	26.88±0.58 ****
		7	29.24±0.0.43	26.88±0.32 ***	24.45±0.24 ****	25.84±0.31 ****	25.33±0.41 ****	25.92±0.50 ****
5.	MCV (mm^3)	3	102.45±0.45	95.28±0.25 ****	93.22±0.84 ****	95.56±0.52 ****	95.66±0.66 ****	96.11±0.38 ****
		7	103.32±0.52	95.11±0.62 ****	92.11±0.44 ****	95.64±0.46 ****	95.2±0.41 ****	96.44±0.59 ****
6.	MCH (pg)	3	31.22±0.56	28.44±0.42 ***	25.66±0.44 ****	29.62±0.52	28.64±0.88 ***	29.98±0.61
		7	31.20±0.65	27.11±0.52 ****	25.12±0.64 ****	28.64±0.52 **	28.44±0.41 ***	29.88±0.84
7.	MCHC (%)	3	5.74±0.11	5.03±0.12 *	4.22±0.21 ****	5.2±0.34	5.10±0.28	5.32±0.21
		7	5.72±0.32	4.92±0.12 ****	4.01±0.21 ****	5.04±0.21 ***	4.88±0.18 ****	5.20±0.12 **
8.	RBC size (µm)	3	10.29±0.14	9.62±0.08	9.14±0.12 **	9.72±0.45	9.64±0.32	9.80±0.13
			10.22±0.32	9.58±0.26	9.06±0.33 **	9.82±0.12	9.44±0.12	9.85±0.52

LP extracts (Figure 8). On Day 7, AST levels increased significantly from 35.80±0.95 IU/L (control) to 50.66±2.35 IU/L after exposure to 50 ppm NaF ($p<0.0001$). Treatment with OP+LP reduced AST to 45.18±0.94 IU/L ($p<0.001$), showing the most notable recovery. ALT levels rose significantly from 13.80±0.22 IU/L (control) to 18.53±0.42 IU/L after 50 ppm NaF exposure ($p<0.0001$). Treatment with OP+LP brought ALT down to 15.20±0.93 IU/L, close to control values. ALP activity increased from 42.44±0.70 IU/L (control) to 46.88±0.89 IU/L in the 50 ppm NaF group ($p<0.0001$). Treatment with OP+LP significantly lowered ALP levels to 43.21±0.43 IU/L ($p<0.001$).

3.7 Histological Study

3.7.1 Liver

The liver tissue of Channa punctatus on Day 7 displayed significant alterations under different treatments

Table 6. Liver enzyme activity of *C. punctatus* when exposed to NaF and Orange and Lemon peel extract on the 3rd and 7th days

S. No.	Biomarker enzyme	Day	Control	25ppm NaF	50ppm NaF	50ppm NaF +OP	50ppm NaF +LP	50ppm NaF +OP+LP
1.	AST (IU/L)	3	35.41±1.10	40.38±0.79 **	46.60±0.91 ***	42.84±1.52 ***	43.99±0.79 ***	40.72±1.21 ***
		7	35.80±0.95	42.19±0.81 ***	50.66±2.35 ****	45.88±0.67 ***	48.11±0.95 ***	45.18±0.94 ***
2.	ALT (IU/L)	3	13.70±0.43	15.85±0.55 **	17.39±0.65 ****	15.74±0.58 **	16.40±0.41 ****	14.96±0.36
		7	13.80±0.22	17.55±0.59 ****	18.53±0.42 ****	15.67±0.53 *	17.00±0.36 ****	15.20±0.93
3.	ALP (IU/L)	3	41.97±0.69	43.15±0.75	45.88±0.33 ****	43.95±0.14 **	43.51±0.39 *	43.42±0.37
		7	42.44±0.70	43.78±0.49	46.88±0.89 ****	44.54±0.38 **	44.85±0.001 ***	43.21±0.43

Values are represented as mean±mean standard error, N=3, values are significant at p<0.05 (* indicate p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 compared to the values of the control group using two-way ANOVA)

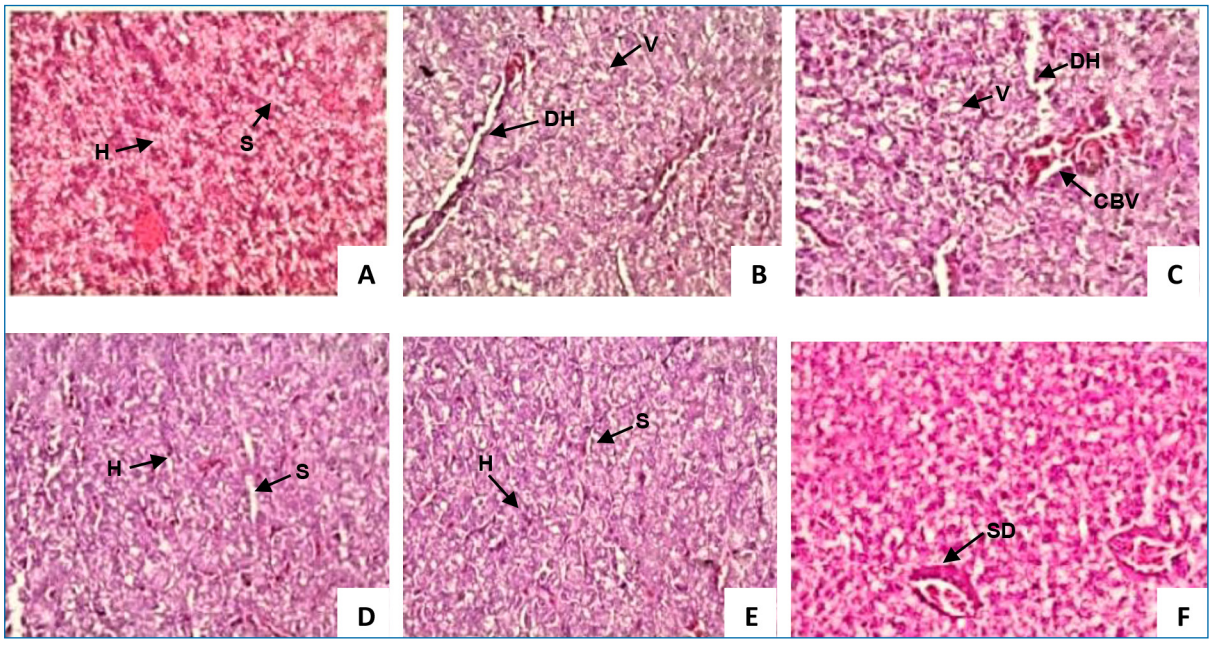


Figure 9. Histology of liver tissue of *Channa punctatus* on Day 7 (A - Control, B - 25ppm NaF, C - 50ppm NaF, D - 50ppm NaF+LP, E - 50ppm NaF+OP and F - 50ppm NaF+OP+LP; H - Hepatocyte, S - Sinusoid, DH- Disintegration of hepatocytes, V - Vacuolation, CBV - Congestion in blood vessels). Magnification- 40X.

(Figure 9). The control group showed normal hepatocytes and well-defined sinusoids. Exposure to 25 ppm NaF led to mild disintegration of hepatocytes and vacuolation. Severe vacuolation, hepatocyte disintegration, and congestion in blood vessels were observed at 50 ppm NaF. However, in groups treated with 50 ppm NaF and lemon peel extract, there was a notable reduction in hepatocyte disintegration, with only mild vacuolation. Similarly, treatment with orange peel extract resulted in moderate improvement, though vacuolation and congestion in blood vessels persisted. The combination of LP and OP showed the

most substantial protective effect, with almost normal hepatocyte structure and minimal signs of vacuolation or congestion.

3.7.2 Gills

The gill structure also exhibited significant histopathological changes across treatments (Figure 10). The control group demonstrated intact primary and secondary gill lamellae. Exposure to 25 ppm NaF caused mild degeneration of secondary lamellae, while 50 ppm NaF led to pronounced degeneration, epithelial lifting, and lamellar fusion. The group treated with 50 ppm NaF and LP showed partial recovery, with reduced lamellar fusion and epithelial lifting. Similarly, the group treated with OP exhibited moderate improvement, though some degeneration of the secondary lamellae was still evident. The combination of LP and OP resulted in the greatest restoration of gill architecture, with minimal epithelial lifting and lamellar fusion, closely resembling the control group.

3.7.3 Kidney

Kidney tissue analysis (Figure 11) revealed marked differences between the control and treated groups. The control group exhibited normal renal tubules, glomeruli, and blood vessels. In contrast, exposure to 25 ppm NaF caused mild vacuolisation and haemorrhages. Severe vacuolisation, haemorrhages, and degeneration of glomeruli were observed at 50 ppm NaF. Treatment with 50 ppm NaF and LP showed a notable decrease in vacuolisation and glomerular degeneration. Similar results were observed in the OP-treated group, although haemorrhages persisted. The combination of lemon and orange peel extract provided the most significant recovery, with normal renal architecture and reduced signs of vacuolisation and glomerular degeneration.

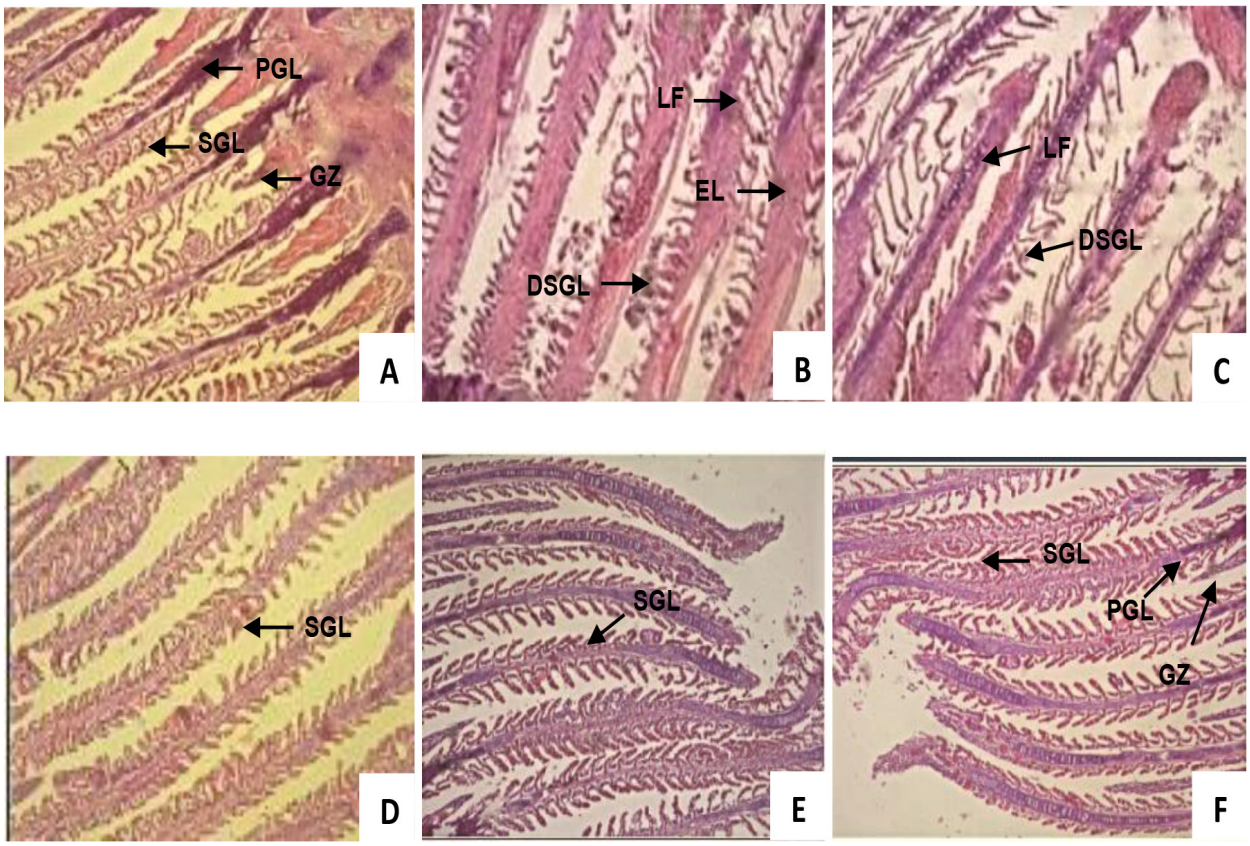


Figure 10. Histology of gills of *C. punctatus* on Day 7 (A - Control, B - 25ppm NaF, C - 50ppm NaF, D - 50ppm NaF+LP, E -50ppm NaF + OP and F - 50ppm NaF+OP+LP; PGL – Primary gill lamella, SGL - Secondary gill lamella, GZ - Growth Zone, DGSL - degeneration of secondary lamella, EL- epithelial lifting, LF- lamellar fusion). Magnification - 40X.

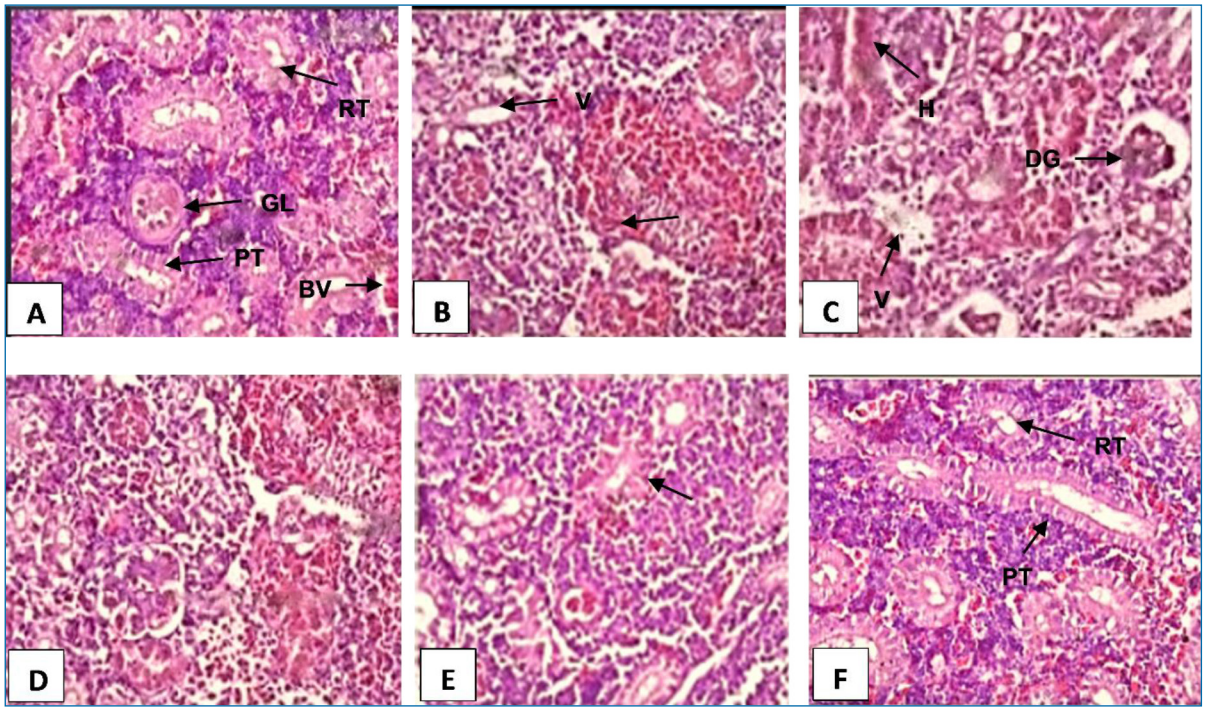


Figure 11. Histology of the kidney of *C. punctatus* on Day 7 (A - Control, B - 25ppm NaF, C - 50ppm NaF, D -50ppm NaF + LP, E - 50ppm NaF + OP and F - 50ppm NaF + OP + LP, RT – Renal tubule, GL – Glomerulus, PT – Proximal tubule, BV – Blood vessel, H – Haemorrhages, V – Vacuolisation, DG – Degeneration of glomerulus). Magnification – 40X.

4. Discussion

The present study investigated the ameliorative effects of methanolic extracts from *Citrus jambhiri* (lemon) and *Citrus reticulata* (orange) peels on NaF-induced toxicity in *Channa punctatus*. The findings demonstrate that citrus peel extracts can mitigate the adverse effects of fluoride exposure, as observed by improvements in behavioural, haematological, biochemical, and histopathological parameters.

Exposure to NaF resulted in noticeable behavioural changes in *C. punctatus*, including hyperactivity, erratic swimming, and excessive mucus secretion, initially. With prolonged fluoride exposure, the fish displayed gulping behaviour, often adopting a vertically erect position, and occasionally leaping out of the water. These reactions might be due to oxygen depletion caused by the fluoride-water mixture, with more severe effects observed at higher fluoride concentrations[30]. These observations are consistent with previous studies indicating that fluoride toxicity disrupts normal fish behaviour due to neurotoxic effects and oxidative stress. A study reported that prolonged exposure to NaF at 30 mg/L significantly disrupts neurotransmission and oxidative balance in the zebrafish brain, as evidenced by a 29.8% reduction in acetylcholinesterase activity, a 39.3% decrease in glutamate uptake, a 41.4% increase in reactive oxygen species, and diminished antioxidant defenses, including a 55.3% reduction in superoxide dismutase and a 26.1% decrease in catalase activity, collectively indicating fluoride's potential role in promoting neurodegenerative changes[31]. Additionally, accelerated operculum movement and frequent operculum opening were also observed. Similar findings were reported in previous studies on metal toxicity in fish when exposed to fluoride and arsenic[32]. These hyperactive behaviours likely stem from internal stimuli disruption or toxicant accumulation, leading to synaptic transmission issues[33]. Behavioural changes are sensitive indicators of water pollution, and increased operculum movement suggests oxygen depletion, consistent with previous studies that reported that toxicants reduce RBCs' oxygen-carrying capacity by damaging them[34]. Pigment disturbances and colour changes in the fish, observed in this study, were also reported in previous studies involving mercury and copper exposure. The goldfish, *Carassius auratus,*

gradually lost their colour, becoming pale, and exhibited deformities, particularly in the caudal peduncle area, at a Hg concentration of 0.06 ppm[35]. These changes are early signs of toxicity due to liver or brain damage. The results can be corroborated with a study that reported that fluoride exposure leads to tissue degeneration and impaired physiological functions in *Channa punctatus*[36]. Haemorrhages, necrosis, and lesions near fins were also observed, which could be attributed to impaired blood supply and nutrient deficiencies. Excessive mucus secretion, which serves as a protective barrier, was also observed, aligning with earlier findings. When exposed to a sublethal concentration (0.10 mg/L) of copper sulphate, *Carassius auratus* exhibited hypertrophy in mucus cells, while blood capillaries shrank due to reduced blood supply[37]. The proliferation of mucous cells leads to increased mucus production, which helps prevent the absorption of toxic compounds through the gills. However, these alterations in the gills interfere with gas exchange and reduce the uptake needed for metabolic and detoxification processes[38].

NaF exposure led to significant reductions ($p<0.05$) in RBC count, haemoglobin concentration, and packed cell volume (PCV), indicating anaemia and compromised oxygen transport. These haematological disruptions are corroborated by studies showing that fluoride toxicity impairs hematopoietic functions in freshwater larvicidal fish, *Channa striatus*[39]. The observed decrease in Mean Corpuscular Volume (MCV) and Mean Corpuscular Haemoglobin Concentration (MCHC) further supports the occurrence of microcytic hypochromic anaemia, a condition previously reported in fluoride-exposed fish, *Oryctolagus cunniculus*[40]. The declining trend in the haematological profile of *C. punctatus* with increasing fluoride concentration suggests a toxic impact on hematopoietic tissue and fish metabolism. This decrease likely indicates hemolysis and nutrient absorption failure, leading to anaemia and respiratory distress. The decline in RBC count and hematocrit in our study aligns with findings by Vosylienė and Mikalajūnė[41], in rainbow trout exposed to metal mixtures, and Huseen and Mohammed[42], who reported similar decreases in fish exposed to metal toxicity. Conversely, white blood cell counts initially increased at lower fluoride concentrations but decreased at higher levels, a pattern reported in other toxicant studies, likely as an adaptive response to tissue damage.

Biochemical assessments revealed significantly ($p<0.05$) elevated levels of hepatic enzymes, including Aspartate Transaminase (AST), Alanine Transaminase (ALT), and alkaline phosphatase (ALP), in NaF-exposed fish, suggesting hepatic stress and damage. These enzymes are markers of cellular breakdown and membrane damage. Similar results have been reported in fish exposed to other toxicants. The study on freshwater fish *Heteropneustes fossilis* (Bloch.) exposed to sub-lethal fluoride concentrations (35 mg/L and 70 mg/L) for 45 and 90 days showed significant increases in AST and ALT levels ($p<0.01$) at both time points for both low and high doses[43]. These findings align with prior research indicating that fluoride exposure induces hepatotoxicity through a misbalance in oxidative biochemistry[44]. The increase in these enzymes reflects compromised liver function, as they are released into the bloodstream upon hepatocellular injury.

Histopathological examinations confirmed the detrimental effects of fluoride, revealing vacuolation, cellular degeneration, hypertrophy and congestion in the liver, gills, and kidneys of exposed fish. Such tissue alterations have been documented in earlier studies, highlighting the systemic toxicity of fluoride in aquatic organisms. The gill damage observed may impair respiratory efficiency, leading to hypoxic conditions and further physiological stress, as was observed in *Labeo rohita*[45]. A study by Wang *et al.*[46] revealed that Fluoride (F) and Lead (Pb) exposure significantly reduced growth performance, with the combined F + Pb treatment having a more pronounced inhibitory effect on male zebrafish compared to females. Histopathological analysis also showed extensive liver damage, including focal necrosis, karyolysis, and pycnotic nuclei in zebrafish exposed to F and Pb[46].

Treatment with methanolic extracts of *C. jambhiri* and *C. reticulata* peels ameliorated the adverse effects induced by fluoride exposure. Phytochemical analyses confirmed the presence of bioactive compounds such as flavonoids, phenolic acids, and essential oils in the citrus peels, which are known for their antioxidant properties. The antioxidant activity of these compounds likely contributed to the restoration of haematological parameters, normalisation of hepatic enzyme levels, and preservation of tissue integrity observed in treated fish. The combined treatment with both citrus extracts demonstrated a synergistic effect, offering enhanced protection against fluoride-induced toxicity. This synergism may be accredited to the complementary actions of different phytochemicals present in the two citrus species, which together provide

a more robust antioxidant defence. Such synergistic effects of plant-based antioxidants have been reported in previous studies.

Recent studies have highlighted the efficacy of various fruit peels, such as those from citrus and pomegranate, in reducing heavy metal toxicity in aquatic organisms, showcasing their potential as eco-friendly solutions for improving aquatic health[47]. The use of fruit peels not only aids in mitigating the adverse effects of fluoride but also aligns with sustainable practices aimed at enhancing fish health and promoting human nutrition through healthier fish consumption[48]. The protective effects of citrus peel extracts can be attributed to their high content of flavonoids and phenolic compounds, which exhibit strong free radical scavenging activities. These antioxidants neutralise Reactive Oxygen Species (ROS) generated during fluoride-induced oxidative stress, thereby preventing cellular and tissue damage[49]. Additionally, the anti-inflammatory properties of citrus phytochemicals may contribute to the observed amelioration of tissue inflammation and degeneration[50].

5. Conclusion

The present study highlights the significant phytochemical and therapeutic potential of *Citrus jambhiri* and *Citrus reticulata* peel extracts, particularly in mitigating NaF-induced toxicity in Channa punctatus. Qualitative phytochemical screening confirmed the presence of vital bioactive compounds such as alkaloids, flavonoids, terpenoids, and phenols, with the methanolic extracts showing the highest concentration levels. GC-MS analysis further identified several compounds with known antioxidant, anti-inflammatory, and antimicrobial properties in both citrus peels. Exposure to NaF resulted in distinct behavioural, morphological, haematological, enzymatic, and histopathological abnormalities in fish, indicating severe systemic stress and toxicity. These included erratic behaviour, fin necrosis, discoloured organs, disrupted RBC morphology, altered liver enzyme levels, and extensive tissue degeneration in the liver, gill, and kidney. However, treatment with citrus peel extracts, especially the combined application of lemon and orange peels, significantly ameliorated these effects. Improvements were evident in the restoration of normal behaviour, organ morphology, blood parameters, enzymatic balance, and tissue architecture.

The findings strongly suggest that *Citrus jambhiri* and *Citrus reticulata* peels possess potent protective properties against fluoride-induced toxicity, likely due to their rich phytochemical content and synergistic bioactive compounds. These citrus peels, often considered agro-waste, may serve as promising, eco-friendly, and cost-effective therapeutic agents in aquaculture and environmental toxicology.

6. Acknowledgement

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