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Protective Impact of *Moringa oleifera* Plant Extract Against Phorate-Induced Toxicity in the Stomach of Carp Fish, *Cyprinus carpio*

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Abstract: ***This study examined the protective role of Moringa oleifera plant extract (MPE) against stomach tissue damage caused by the pesticide phorate in common carp, Cyprinus carpio (L. 1758). Fish were divided into five groups: a control group and four experimental groups exposed to two sub-lethal concentrations of phorate (0.075 ppm and 0.15 ppm), both individually and in combination with MPE (8 ml/L), for 10 and 20 days. After completion of the exposure period, the stomach tissues were dissected, fixed, dehydrated, cleaned, and paraffin-embedded. Appropriately sized blocks were prepared from the tissues; sections 5-6 µm thick were taken and stained with Hematoxylin and Eosin (H&E). The prepared slides were examined under a light microscope and photographed. After observation, it was found that the fish exposed only to phorate showed severe histopathological alterations, such as tissue degeneration and structural damage, which increased with concentration and exposure duration. In contrast, fish treated with both phorate and MPE exhibited significantly less tissue damage, indicating a protective effect of the extract. These findings demonstrate that phorate induces notable histological harm in fish stomachs, but co-treatment with MPE effectively mitigates these toxic effects. Therefore, Moringa oleifera extract shows strong potential as a natural protective agent against pesticide-induced toxicity, highlighting its value in reducing the harmful impacts of synthetic agrochemicals on aquatic organisms and, by extension, the environment.***

Keywords: *Cyprinus carpio*, phorate, histology, *Moringa olifera*, toxicity, stomach

1.Introduction

In recent years, human activities have brought various pollutants into the aquatic environment that is proven to be toxic to organisms. Indiscriminate use of pesticides is one of them **(Abu Zeid et al. 2021; El Bouhy et al. 2021; Galal et al. 2018; Khalil et al. 2017).** Pesticides are organic compound, frequently used on a large scale to control unwanted and harmful insects and pests to enhance food quality and agricultural production. It is also used to control vector-borne diseases such as malaria, typhoid, etc **(Ross, 2005).** The organophosphate group of pesticides is now becoming popular due to their effectiveness and low toxicity. Phorate is a broad-spectrum organophosphate insecticide, used throughout the world to control the sucking and chewing insects, mites, and soil-dwelling pests in numerous crops such as sugarcane, wheat, beetroot, carrots, potatoes, and paddy fields to enhance crop production. However, their deleterious effects are often noticed in non-target organisms like fish, which humans consume **(Zhu et al., 2017; Hodgson and Lcvi, 1996).**

Indiscriminate use of insecticides on crops causes serious environmental hazards affecting aquatic and land-dwelling animals. Unfortunately, most of the organophosphate insecticides, like phorate, are not biodegradable and tend to persist for years in soil and water**.** Direct and indirect pollution of aquatic environments by pesticides can cause fish mortality, reduce fish production, and gradually accumulate in fish tissues, altering their internal structure and causing tissue damage **(Banerjee & Bhattacharya, 1995; Rodriguez and Fant, 1998).** Histopathological evaluation of the vital organs of fish plays a crucial role in detecting the harmful effects of various pesticides as well as in monitoring aquatic contamination **(Majumdar, 1980).** Histopathological studies show that pesticide exposure causes morphological and physiological changes in fish stomachs, which are vital for food storage and digestion. Pollutant-induced damage from contaminated food and water disrupts digestion, impairs growth and survival, and serves as an indicator of environmental stress in aquatic ecosystems **(Hundet and Prabhat, 2014; Lakshmaiah, G., 2016).**

Being at the top of the aquatic ecosystem, the pesticide-contaminated fish might influence the levels of pesticides in the human body through the food chain and cause several diseases, as fish constitute an important part of animal protein in rural and urban areas (**Jaroli and Sharma, 2005; Murthy, 1986; Pandey et al., 1999).** Considering the nutritional value of fish, maintaining good water quality in the environment is essential for their growth and development.

Several medicinal and herbal plants are used to reduce pollutant toxicity because they are easily available, eco-friendly, and free from side effects. Among these, *Moringa oleifera* (Lam.) *(M. oleifera),* commonly known as the "Miracle Tree," is a traditional medicinal plant from the Moringaceae family, widely distributed in the western and sub-Himalayan regions in India **(Paryab et al., 2017; Pu et al., 2017).** M. oleifera is rich in antioxidants and nutrients that boost growth, immunity, and stress resistance **(Gopal Krishnan et al., 2016; Farzaei et al., 2013).** Its various parts detoxify pesticides and metals, promote health, and offer anticancer, antiulcer, and hepatoprotective benefits. The seeds purify water and

flowers contain quercetin with hepatoprotective effects, while pods combat malnutrition and enhance lactation, and roots treat diarrhea through antispasmodic activity **(Abdel-Latif et al., 2022; Kumar et al., 2021; Aasma Noureen et al., 2018; Ibrahim et al., 2019).**

Therefore, this research primarily focused on studying the protective effects of *M. oleifera* plant extract (MPE) against phorate-induced histopathological changes in the stomach tissue of the freshwater carp fish, *C. carpio*.

2.Material and Methods

2.1 Procurement and preparation of stock solution of phorate:

Phorate, an organophosphate insecticide was selected for this study. A commercial grade of phorate 10% CG (TRIKAL), (C_7 H_{17} O_2 PS_3) (Gyatri Insecticide India PVT LTD, Bahadarpur, Yamuna Nagar, Haryana, batch no. G I - 85, M.W. 260.38), was procured from a local market in Raipur, Chhattisgarh. Commercial formulation of this pesticide is used because only commercial preparation is used in agriculture.

For histological study, 0.075 ppm and 0.15 ppm concentrations of Phorate were taken which were sub-lethal concentrations of 96 hrs LC50 value of phorate. A stock solution was prepared at a concentration of 1mg/ml. Different concentrations were prepared by adding the required amount of distilled water as per the dilution method. In each exposure, fresh stock solutions were used.

2.2 Collection and preparation of *M. olifera* plant extract (MPE):

Different parts of the *M. oleifera* plant, including the root, bark, leaves, and pods, were collected, washed, dried in sunlight and rendered crisp. Equal quantities of these dried parts were finely powdered using an electric blender and stored in an airtight container. Further, 25 g of the powder was placed into a conical flask and added 250 ml of hot (98ºC) distilled water and left to steep for 24h. Subsequently, the solution was first filtered through a muslin cloth and further refined using sterile Whatman filter paper no. - 02 **(Khalil and Korni, 2017).** The resulting extracts were carefully stored in the refrigerator at 4°C and used for one week.

2.3 Experimental animal and Experiment design:

Active and healthy *C. carpio* specimens (measuring 10±2 cm in length and 30±5 g in weight) were collected from the Kurudih fish hatchery in Raipur, treated with 0.1% KMnO4 solution for 2-5 minutes and kept in a glass aquarium filled with dechlorinated water for 15 days to acclimate to the laboratory conditions.

Acclimated fishes were randomly selected and distributed in five groups and each group containing 6 fish. The experiment was continued for 10 and 20 days. The groups are detailed following-

Group I: Fishes served as normal control
Group II: Fishes treated with phorate (0.075 ppm)
Group III: Fishes treated with phorate (0.075 ppm) + MPE (8 ml/L)
Group IV: Fishes treated with phorate (0.15 ppm)
Group V: Fishes treated with phorate (0.15 ppm) + MPE (8 ml/L)

Fish were fed commercial aquarium food pellets twice a day at a rate of 2% of body weight and uneaten food was removed 30 minutes after feeding. The physicochemical quality of the aquarium water, including pH (6.3 ± 0.2), dissolved oxygen (6.4 ± 0.15 mg/L), and temperature (28.5°C ± 1.2°C), was monitored regularly, and the dead fish were removed immediately.

2.5 Sampling and Histological examination:

At the completion of exposure period of 10 and 20 days, both the control and exposed fish were sacrificed and the stomach tissues were dissected out for histological analysis. The histological sections were prepared by adopting the procedure as described by **Humason (1972).** The tissues were fixed in 10% formalin for 24 hrs, dehydrated with ascending grade of alcohol and embedded in paraffin, blocks were prepared and sections were cut at 5-6 µm thickness. Then the sections were stained with hematoxylin and eosin (H&E) and slides were mounted with DPX mount **(Harris, 1900)**. Prepared slides were viewed under optical microscope Olympus CX41 microscope (X100) and photographs were taken for Histopathological analysis.

Histopathological changes were semi-quantitatively assessed using a four-graded assessment scheme according to **Peebua et al. (2008).** A value of either (-) no structural deterioration, (+) mild structural deterioration, (++) moderate structural deterioration or (+++) severe structural deterioration was assigned to each investigated section. Several sections of each specimen were prepared, and at least three slides, each containing three to four sections, were used for observation and scoring.

3.Observations and Results

3.1. Histology of the stomach of normal/untreated *C. carpio:*

The stomach wall has four distinct layers, namely: 1. Mucosa, 2. Submucosa, 3. Muscularis externa, and 4. Serosa.

The mucosa, the innermost lining, has finger-like folds with gastric epithelium and a narrow tunica propria. The gastric epithelium contains tall, simple columnar cells that secrete mucus and gastric juice **(Fig. 01).** The tunica propria, a thin layer of vascular loose connective tissue, lies between the gastric epithelium and the muscularis mucosa. Beneath it, the muscularis mucosa, the deepest mucosal layer, is made of smooth muscle fibers and separates the mucosa from the submucosa **(Fig. 02).** The second important layer is submucosa, a dense connective tissue layer outside the muscularis mucosa with blood

vessels, lymph vessels, and nerve cells that nourish the mucosa. It is well-developed at the basal portion and extends into the mucosal folds as a thin strip **(Fig. 03).** The third layer is muscularis externa, which covers the submucosa and consists of three muscle layers: outer longitudinal, middle circular and inner oblique muscles, which aid to digestion. The fourth and outermost layer is the serosa, made of areolar tissue, which connects to the mesenteries supporting the stomach **(Fig. 04).**

3.2. - Phorate (0.075 ppm and 0.15 ppm) treated stomach of *C. carpio* (10 and 20 days):

After 10 days of exposure to 0.075 ppm phorate, the stomach showed necrotic, blunt mucosal folds, with some fused. The longitudinal muscle layer detached from the circular muscle layer, and small gaps appeared in the circular muscles. The serosa loosened from the muscles **(Fig. 05).** After 20 days, damage increased, with eroded gastric epithelial cells, mashed gastric glands, and increased mucus cells. Gaps appeared between the tunica propria and submucosa, while the muscularis and serosa were broken, with bulging longitudinal muscles and gaps between the circular muscles and submucosa **(Fig. 06).**

After 10 days of 0.15 ppm phorate exposure, mucosal folds fused and submucosa thinned, due to columnar epithelium proliferation and necrosis. Circular muscles thickened, while longitudinal muscles disorganized **(Fig. 07).** After 20 days, gastric folds became flattened, mucous glands hypertrophied, and mucus scattered in the lumen. Empty spaces appeared in tissues, mucus cells decreased, and the serosa and muscle layers showed further damage and shrinkage **(Fig. 08).**

3.3. – Phorate (0.075 ppm and 0.15 ppm) + MPE (8ml/L) co-treated stomach of *C. carpio* (10 and 20 days):

After 10 days of phorate (0.075 ppm) exposure combined with MPE, tissue damage was slightly reduced compared to phorate alone, showing mild damage in stomach tissue, including mild submucosal connective tissue damage and gastric epithelial peeling **(Fig. 09).** After 20 days, the phorate and MPE-treated group showed mild pathological changes, including no epithelial separation but increased mucus cells. The stomach structure appeared better organized, though small empty spaces were noted in the longitudinal muscle region **(Fig. 10).**

After 10 days of phorate (0.15 ppm) and MPE exposure, mild stomach tissue deterioration was observed compared to phorate alone showing usual mucosal fold arrangements, still some were blunt due to necrosis and fusion due to proliferation. Mild hypertrophy and hyperemia were noted **(Fig. 11).** After 20 days, damage increased slightly, with abnormal cellular arrangements, thickened mucosal folds, peeling epithelium and damage to connective tissue, though less severe than the phorate-only exposure group **(Fig. 12).**

Tables 1: Summarized Histopathological alterations noticed in the stomach of *C. carpio* of untreated, Phorate-treated, and Phorate + MPE co-treated group

Exposure group	Histopathological alterations											
	Mucosal degeneration		Vacuolation		Focal necrosis		Muscularis detachment		Loosened serosa		Complete degeneration	
	10 d	20 d	10 d	20 d	10 d	20 d	10 d	20 d	10 d	20 d	10 d	20 d
G1	-	-	-	-	-	-	-	-	-	-	-	-
G2	+	++	+	+	-	+	+	++	-	+	+	+
G3	++	+++	++	+++	+	++	+	+++	++	++	++	+++
G4	+	+	_	+	_	-	-	+	-	-	_	-
G5	+	++	+	+	+	+	-	++	-	+	+	+

(-) Normal Histological structure; (+) mild histological deteriorations; (++) moderate histological deteriorations; (+++) Severe histological deteriorations;

G1- Control, **G2-** Phorate (0.075 ppm), **G3-** Phorate (0.15 ppm), **G4-** Phorate + MPE (0.075 ppm+8ml), **G5-** Phorate + MPE (0.15 ppm+8ml)

Plate – 1: Photomicrograph of T.S. of untreated/normal stomach of *C. carpio* showing normal gastric architecture: (Formalin, H&E, 10X and 40X)

Fig. 01–04: untreated/normal stomach (10 and 20 days)

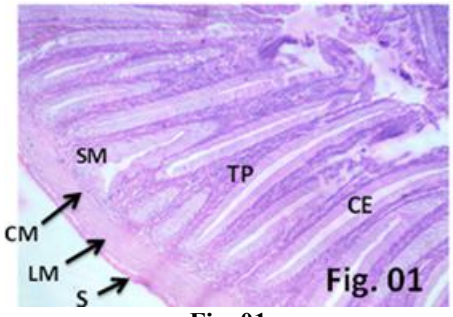

Fig. 01

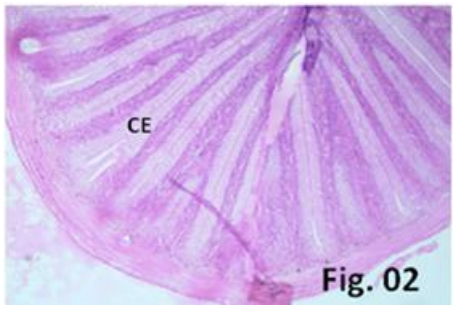

Fig. 02

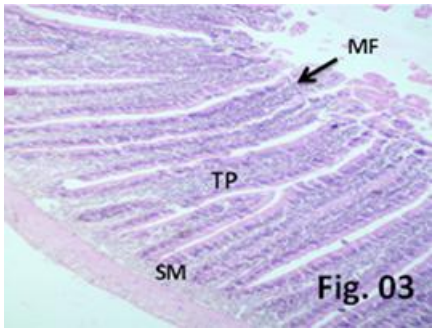

Fig. 03

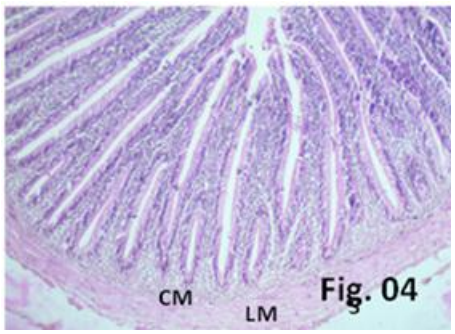

Fig. 04

3.1. Histology of untreated/normal stomach of *C. carpio* showing normal gastric architecture: (Formalin, H&E, 10X and 40X).

(MF) = Mucosal folds, (CM) = Circular muscles,
(CE) = Columnar Epithelium, (LM) = Longitudinal muscles,
(SM) = Submucosa, (S) = Serosa,
(TP) = Tunica propria

Plate – 2: Photomicrograph of T.S. of phorate-treated stomach of *C. carpio* showing severe damage in the gastric architecture: (Formalin, H&E, 10X and 40X)

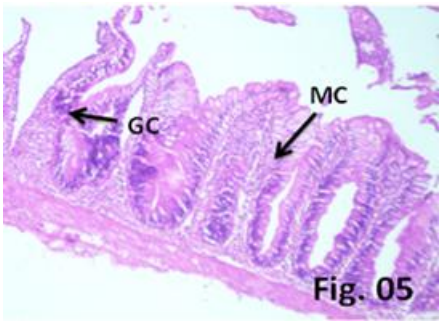

Fig. 05 - Phorate (0.075 ppm, 10 days)

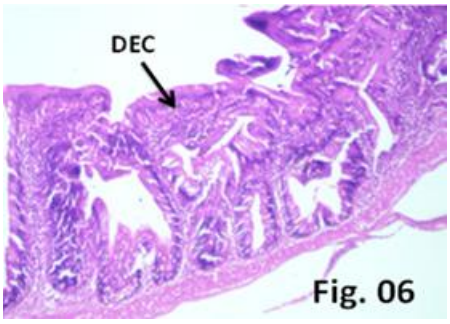

Fig. 06 - Phorate (0.075 ppm, 20 days)

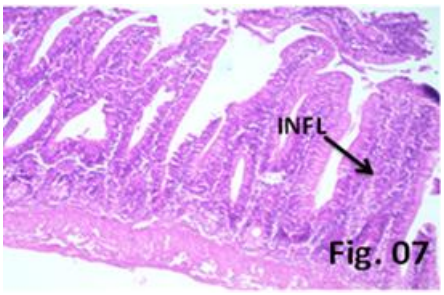

Fig. 07 - Phorate (0.15 ppm, 10 days)

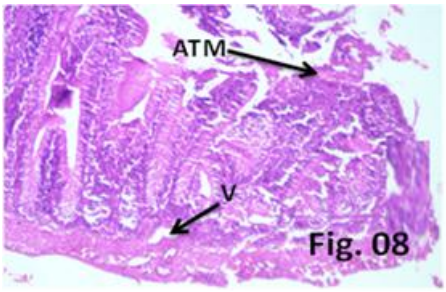

Fig. 08 – Phorate (0.15 ppm, 20 days)

3.2. Histopathology of phorate treated stomach of *C. carpio* showing severe deteriorations in the gastric architecture: (Formalin, H&E, 10X and 40X).

(GC) = Goblet cells, (INFL) = Inflammation of cells,
(MC) = Mucous cells, (ATM) = Autolysis of mucosa,
(DEC) = Degeneration of epithelial cells, (V) = Vacuolization,

Plate – 3: Photomicrograph of T.S. of phorate + MPE co-treated stomach of *C.carpio* showing mild damage in the gastric architecture: (Formalin, H&E, 10X and 40X)

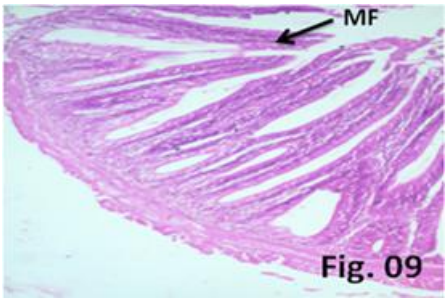

Fig. 9 - Phorate + MPE (0.075 ppm+8 ml/L, 10 days)

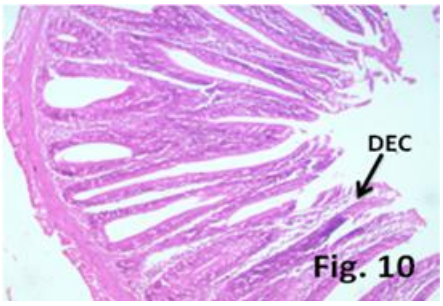

Fig. 10 - Phorate + MPE (0.075 ppm + 8 ml/L, 20 days)

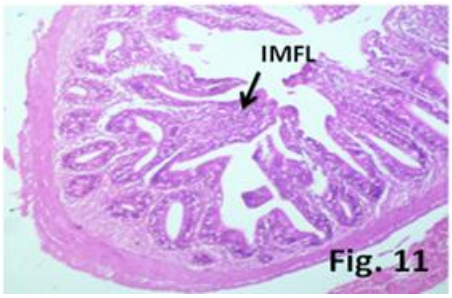

Fig. 11 - Phorate + MPE (0.15 ppm + 8 ml/L, 10 days)

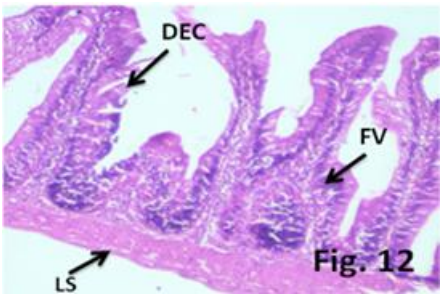

Fig. 12 - Phorate + MPE (0.15 ppm + 8 ml/L, 20 days)

3.3. Histopathology of phorate + MPE co-treated stomach of *C. carpio* showing mild deteriorations in the gastric structure: (Formalin, H&E, 10X and 40X).

(MF) = Mucosal folds, (LS) = Loosened serosa
(DEC) = Degenerated epithelial cell, (FV) = Formation of vacuoles,
(IMFL) = Inflammation of mucosal folds,

4.Discussion

Fish histopathology is an important tool for toxicological studies and monitoring water pollution **(Sreelatha et al., 2011).** It reveals damage to organs like the gills, liver, and gastrointestinal tract, which affects survival and growth, and increases susceptibility to diseases due to pesticides and heavy metals **(Joseph and Raj, 2011).**

In the present investigation, stomach tissues damage in fish increased with higher concentrations and longer exposure of phorate. These organs could not detoxify the harmful substances, leading to degeneration. However, MPE administration reduced the toxic effects of phorate, resulting in mild tissue damage compared to fish exposed only phorate.

The stomach plays a key role in food storage and digestion. Toxic pollutants from contaminated food and water can cause morphological and physiological changes in gastrointestinal tissues, affecting digestion and can negatively impacting fish growth and survival **(Banerjee & Bhattacharya, 1995; Rodrigues & Fanta, 1998; Yadav et al., 2019).** Thus, pathological changes in stomach tissue can indicate exposure to environmental stress.

This study found severe damage to the stomach of *C. carpio* exposed to sub-lethal concentrations of phorate for 10 and 20 days, with hypertrophy of the mucosa and an increased number and size of mucous and gastric glands has occurred. Hyperactive mucous glands released excess mucus into the gastric lumen. This finding is similar to the report by **Ghosh (1990)** on cadmium- and arsenic-induced mucous cell activities in *H. fossilis* and *N. notopterus*. The hypertrophied submucosa showed increased vascularization, with large blood vessels engorged with red blood cells. Similar changes were observed in the stomach and intestines of *Labeo rohita* exposed to CuSO4 and O. niloticus exposed to cadmium **(Pandey & Saxena, 1992; Kaoud et al., 2011).**

In the present study, changes in the shape of gastric folds, ulceration of the surface epithelium and lamina propria, pronounced desquamation, and damage to the submucosal layers were observed in the stomach of *C. carpio* exposed to sub-lethal phorate concentrations. Similar alterations were noted in the stomach of *Mystus armatus* when exposed to sub-lethal concentrations of paper mill effluent **(Isaiarasu and Haniffa, 1987).** Prolonged exposure to phorate damages the plasma membranes in the stomach cell, causing cell detachment and debris accumulation in the lumen. A similar effect was reported by **Murugesan (1988)** in the stomach of *Heteropneustes fossilis* exposed to textile mill effluent.

In this study, the most significant damage occurred in the mucosal folds after intoxication. Similar damage was reported by **Konar (1983)** and **Jha and Pandey (1989).** Increased mucous secretion, particularly at higher concentrations of the tested chemicals, suggests a protective response against gastric acidity and toxic chemicals. Oedema between the mucosa and submucosa may result from toxicant absorption. Additionally, epithelial degeneration and inflammatory cell infiltration were observed in the gastrointestinal tract of tilapia exposed to carbofuran **(Soufy et al., 2007).**

At higher concentrations, musculature became loosely arranged due to necrosis, disrupting peristalsis and allowing toxicants to remain longer in the digestive tract, leading to further damage. Vacuolation in various layers has been reported by other researchers, and it was also observed in the present study. Reports on different fish species also show similar tissue damage to visceral organs when exposed to pesticides and heavy metals **(Sastry and Malik, 1979; Banerjee and Bhattacharya, 1995).**

Meanwhile, the MPE combination with phorate moderated the disruptive effects of phorate on stomach function, leading to reduced pathological changes compared to phorate alone. *M. oleifera* promoting cell regeneration in

stomach tissue through its amino acids such as, methionine and cysteine, trace elements and various phenolics and mitigates tissue damage, as evidenced by several authors **(Pari and Kumar, 2002; Afuang et al., 2003; Ekam et al., 2012).** Furthermore, MPE also contains caffeine and cinnamic acids, protecting cellular mitochondria from apoptosis and lipid peroxidation as shown in previous studies **(Zhao et al 2021; Espíndola et al., 2019; Li et al. (2017).**

Christian et al., (2016) and **Jyotsna and Swarnalatha, (2016)** have also explored the remedial effects of different parts of *M. oleifera* against toxins in their studies. **Yadav et al. (2020)** found that *Moringa* leaf extract (MLE, 12 ml/L) significantly reduced Imidacloprid-induced toxicity and tissue damage in zebra fish, *Danio rerio*, without harming the fish, supporting the findings of current study. **El-Bakry et al. (2016)** also identified that the polyphenols present in *M. oleifera* act as free radical scavengers, contributing to the leaf extract's antioxidative activity.

5.Conclusion

The overall evaluation showed that both low and high sub-lethal concentrations of phorate initially caused mild deterioration in the stomach tissues of exposed fish, which became severe with prolonged exposure in a concentration- and duration-dependent manner. Co-administration of *M. oleifera* plant extract (MPE) significantly mitigated these harmful effects, particularly at lower concentrations of the toxicant. Although some improvement occurred at higher concentrations, it was less pronounced, likely due to reduced *Moringa* content over time and with increasing toxicity. Overall, MPE demonstrates potential as a natural tool for aquaculture pond management, helping assess and enhance the biosafety of aquatic organisms exposed to toxicants.

6.Future Scopes

In this study, administering MPE (8 ml/L) reduced phorate-induced toxicity in the stomach tissues of C. carpio, indicating its protective potential. Previous studies also show that Moringa oleifera supplementation in fish feed enhances growth, digestion, immunity, antioxidant capacity, and resistance to toxicity and physiological stress in various fish species.

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