# Effect of *Oregano Vulgare* essential oil supplement on growth and histopathological changes of gill and spleen of koi fish (*Cyprinus carpio*) under long-term exposure to naproxen

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**Abstract**

Residues of various pharmaceutical substances in surface waters have raised concerns about the possible adverse effects of these pollutants on aquatic organisms in recent years. The study aimed to investigate the effect of feeding with oregano essential oil (OEO) on reducing the damage of gill and spleen tissues in long-term exposure to the non-steroidal anti-inflammatory drug (NSAID) naproxen (NPX). In this study, 180 koi fish were purchased from the ornamental fish breeding center. After the adaptation period, they were divided into four groups with three replications. Accordingly, two groups were fed with a basic diet (C, NP) and two groups with an OEO diet (OE, OE+NP) for 30 days. Then, NP and OE+NP groups were subjected to long-term poisoning with NPX for 14 days. Growth factors were examined on the 30th day of the test and sampling was done to examine the histopathology of the spleen and gills on the 30$^{th}$, 37$^{th}$, and 44$^{th}$ days. The results showed that feeding with OEO increased the growth performance of fish ($P<0.05$) Also, the results of gill pathology examination showed apical hyperplasia, basal membrane hyperplasia, infiltration of blood cells, shortening of the secondary lamellae, protrusion of the epithelium, swelling of squamous cells, fusion of secondary lamellae, and aneurysm, which were reduced in OE+NP treatment. Examination of spleen tissue showed hyperemia, necrosis, vacuolization, hemosiderin, Dilation of sinusoid, and accumulation of melanoma macrophages due to exposure to NPX. Also, the number of side effects decreased in OE+NP treatment. Therefore, the use of this food supplement improves the growth and physiological performance of fish in waters with medicinal pollutants.

**Keywords:** Oregano, Naproxen, Gill, Spleen, Koi fish

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## Introduction

During the past years, water pollution with pharmaceutical compounds has attracted a lot of attention due to the increase in consumption and incomplete removal of drugs during wastewater treatment. The amount of human and veterinary drugs has reached a significant level in surface water, sewage treatment plants, and groundwater (Li, 2014; Comber *et al*., 2018). Nonsteroidal anti-inflammatory drugs (NSAID) are among the drugs that are widely used in medicine and veterinary medicine. These drugs are commonly used due to their relatively low cost, high efficacy, and lack of suitable alternatives (Whitfield-Cargile *et al*., 2016). Naproxen (NPX) is one of the pharmaceutical compounds belonging to NSAID drugs that are frequently detected in aquatic environments around the world (Xu *et al*., 2019). Although pharmaceutical compounds are often detected in small amounts, chronic exposure to these compounds can cause many side effects such as reduced growth and weakened immunity in aquatic animals (Zenker *et al*., 2014; Fabbri and Franzellitti, 2016; Ebele *et al*., 2017).

In recent years, the decrease in availability and increase in the price of fish feed has led to the development of research to find alternative sources to have profitable and sustainable aquaculture (FAO, 2018; Slater *et al*., 2018). This relationship of plant products has been suggested as an alternative to expensive diets in aquatic feed due to their low cost, availability, and high protein (Gatlin *et al*., 2007; Olsen and Hasan, 2012; Kazempoor *et al*., 2022). Also, herbal food supplements improve metabolic and physiological functions and stimulate the activity of the immune system components of aquatic animals (Rao *et al*., 2006; Sahu *et al*., 2007; Ardó *et al*., 2008).

Oregano (Origanum vulgare) belongs to the Lamiaceae family, which is widely distributed throughout the Mediterranean region and Asia (Blahova *et al*., 2020). T The main compounds of this plant include carvacrol (as the main component, 40-70% depending on the origin), flavonoids such as naringin, and phenolic compounds (Petrovici *et al*., 2020). So far, extensive studies have been conducted on the effects of oregano essential oil (OEO) in different species, but few studies have investigated the effects of this plant's extract on aquatic animals (Rashidian *et al*., 2021).

Ornamental fish trade as pets is an important source of income for many countries (Saxby *et al*., 2010; Ploeg, 2013). Koi fish (*Cyprinus carpio*) belongs to the Cyprinidae family and is one of the ornamental fish with very high commercial value in the world. This fish is used all over the world (especially in China and Japan) due to its high adaptability, easy breeding, and unique beauty for personal entertainment or competitive performances (Luo *et al*., 2021).

The high mortality rate in the ornamental fish trade (between 2-73%)

is due to the high stress of breeding and annual transportation, which causes significant economic losses to breeders (Ploeg, 2007). The statistics of casualties in this industry show the necessity of using efficient strategies to improve physiological performance and increase the survival rate of fish. In this study, the effects of feeding with OEO on the growth performance and histological changes of gill and spleen of koi fish under long-term exposure to NPX were investigated.

## Materials and methods

### *Experimental design*

In this experiment, 180 pieces of koi fish (average weight 5±1 grams and average standard length 6±1 cm) were purchased from the ornamental fish breeding center located in Sari city and transferred to the laboratory. At first, the fish were kept in fiberglass tanks for two weeks for adaptation. During this period, they were fed daily based on 5% of body weight (twice a day) with commercial feed. The hours of darkness: and light were 12:12 during the adaptation period. No fish deaths were reported during the adaptation period. The fish in 12 tanks (containing 15 liters of water) were divided into four groups with three replications (15 fish in each tank) after an adaptation period. The experiment was done in 4 groups with three replications. The fishes were fed two diets of commercial feed (C, NP) and OEO (OE, OE+NP) for 30 days and then exposed to long-term poisoning with NPX drug (OE+NP, NP) for 14 days. Fish were fed twice a day at 9 and 16 hours based on 5% of body weight and additional food was collected from the water surface 2 hours after feeding. 30% of the water in the tanks was replaced with fresh water daily.

### *Food preparation*

The essential oil used was prepared by Pars Ayman Daru Company (Tehran, Iran) in this experiment. The preparation of a diet containing OEO was based on the method of El-Hawarry *et al*. (2018) with a ratio of 2 ml of essential oil per kilogram of commercial feed. This essential oil was sprayed on the commercial feed (basic diet) daily and before each feeding and then it was fed to the fish.

### *Exposure to NPX*

Naproxen powder was obtained from Razak Pharmaceutical Company (Tehran, Iran). Poisoning with NPX was performed according to OECD guideline No. 204 (OECD, 1984). For this purpose, NP and OE+NP groups were exposed to 100 mg/L of NPX (1.5 g per tank) on days 30 to 44 of the experiment. 70% of the water in the tanks was replaced with fresh water daily to add NPX to the tanks. The drug powder was dissolved in 10 mL of distilled water and added to the tank water. Then it was mixed with a plastic rod. During the poisoning period, feeding with both diets continued with the pre-poisoning process.

*Sampling*

Sampling was done to check fish growth on days 0 and 30 (five fish from each treatment). Also, sampling was done to check the pathology of gill and spleen tissues on the 30th, 37th, and 44th days of the experiment. Three fish were selected from each treatment at each sampling time. The gill and spleen of the fish were placed in 10% formalin. Formalin was changed 24 hours after sampling to complete the fixation of the samples.

*Growth performance*

At the end of the feeding period (day 30), weight gain (WG), weight gain percentage (WG %), and specific growth rate (SGR) were calculated based on the following formulas (Abdel-Latif *et al*., 2020).

Weight gain (WG) (g) =Final weight-Initial weight

Weight gain % (WG%) = 100 (Final weight-Initial weight)/ Initial weight

Specific Growth Rate (SGR) = 100 [Ln Final weight (g) –Ln Initial weight (g)] /time in days

*Histopathological examination*

Histological examinations were performed based on the method of Alavinezhad *et al*. (2021). Briefly, after dehydrating by alcoholic solution and cleaning with xylene, the samples were placed in paraffin. Then tissue sections with a diameter of 5 μm were prepared using a microtome. Staining was done with the hematoxylin-eosin (HandE) method. Finally, the prepared histological slides were examined by optical microscope (Olympus BX51; Olympus, Tokyo, Japan) to evaluate pathological features.

*Data analysis*

The normality of the data was determined by the Kolmogorov-Smirnov test and the paired t-test at 5 % was then employed to compare means and the relation between the measured factors. Significant differences between treatments were considered by one-way analysis of variance (One-way ANOVA). Duncan's test was used at a significant level of 0.05 to compare means. Statistical analyzes were performed by SPSS 21 and Excel 2013 software.

## Results

*Growth performance*

The results of growth parameters are shown in Table 1. Based on the obtained results, there was no significant difference in the initial weight of the fish ($p$>0.05). After 30 days of feeding, final weight, WG, WG%, and SGR showed a significant increase in OE and OE+NP groups compared to C and NP groups ($p$<0.05), while there was no significant difference between group C and NP and also between group OE and OE+NP ($p$>0.05).

**Table 1: Comparison (Mean±SE) of koi fish (*Cyprinus carpio*) growth parameters (WG: Weight Gain; SGR: Specific Growth Rate) in four experimental groups**

| Growth Parameters | experimental group | | | |
|---|---|---|---|---|
| | C | OE | OE+NP | NP |
| Initial weight (mg) | 5.06±0.11 a | 5.15±0.1 a | 5.05±0.23 a | 5.14±0.09 a |
| Final weight (mg) | 8.89±0.16 b | 11.79±0.21 a | 11.91±0.22 a | 8.84±0.2 b |
| WG | 3.83±0.2 b | 6.65±0.17 a | 6.86±0.19 a | 3.7±0.28 b |
| SGR% | 75.97±5.03 b | 129.18±3.68 a | 136.52±9.15 a | 72.25±6.84 b |
| WG% | 1.88±0.1 b | 2.76±0.05 a | 2.86±0.13 a | 1.77±0.53 b |

Different lowercase letters in each row indicate significant differences between treatments ($p<0.05$).

*Gill histopathology*

The results of gill histopathology in koi fish are shown in Figure 1. Based on obtained results, the fish in group C had normal and healthy gills.

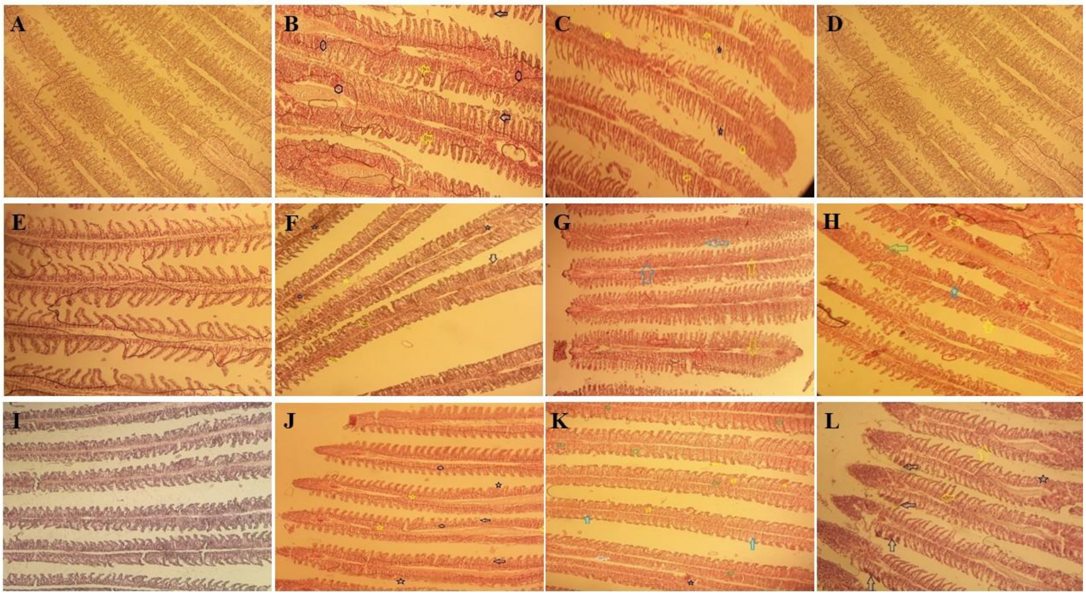

**Figure 1: The effect of OE feeding and NPX poisoning on koi fish gill tissue on different days (400x). Pictures A to D are related to the 30th day, pictures E to H are related to the 37th day, and pictures I to L are related to the 44th day. A: C treatment day 30 (normal tissue). B: OE treatment on day 30 (black arrow: apical hyperplasia, yellow arrow: basal membrane hyperplasia, hexagon: blood cell infiltration). C: OE+NP treatment on day 30 (yellow arrow: primary hyperplasia, black star: Squamous cells, hexagon: fusion of secondary lamellae). D: NP treatment on day 30 (normal tissue). E: C treatment on day 37 (normal tissue). F: OE treatment on day 37 (black arrow: apical hyperplasia, yellow arrow: basal membrane hyperplasia, black star: blood cell infiltration, yellow star: edema). G: OE+NP treatment on day 37 (blue arrow: fusion of secondary lamellae, yellow arrow: blood cell infiltration, red arrow: primary hyperplasia). H: NP treatment on day 37 (yellow arrow: epithelial protrusion, green arrow: apical hyperplasia, blue arrow: basal membrane hyperplasia, yellow circle: aneurysm, red star: fusion of secondary lamellae, hexagon: shortening of the secondary lamellae). I: treatment C day 44 (normal tissue). J: OE treatment on day 44 (arrow: edema, black star: fusion of the secondary lamellae, yellow star: blood cell infiltration, hexagon: basal membrane hyperplasia). K: OE+NP treatment on day 44 (white arrow: shortening of the secondary lamellae, blue arrow: basal membrane hyperplasia, star: attachment of secondary lamellae, hexagon: blood cells infiltration). L: NP treatment on day 44 (black arrow: apical hyperplasia, yellow arrow: basal membrane hyperplasia, star: shortening of the secondary lamellae).**

The most observed complications were in OE+NP and NP, which included apical hyperplasia, basal membrane hyperplasia, infiltration of blood cells, shortening of the secondary lamellae, protrusion of the epithelium, swelling of squamous cells, fusion of secondary lamellae, and aneurysm.The most destructive effect was observed in NP treatment on days 37 and 44, which included the protrusion of the epithelium, basal membrane hyperplasia, infiltration of blood cells, and fusion of secondary lamellae. These complications were less destructive in OE+NP treatment.

*Spleen histopathology*

The results of spleen histopathology in koi fish are shown in Figure 2. According to the obtained results, the spleen tissue in all groups was normal on day 30 and no changes were observed. The fish in the control treatment had normal and healthy spleen tissue on the 37th and 44th days of the experiment. The most observed complications in OE+NP and NP included hyperemia, necrosis, vacuolation, hemosiderin, Dilation of sinusoid, and accumulation of melano-macrophages. The most destructive effect in the NP group on days 37 and 44 included complications such as Dilation of sinusoid, hemosiderin, accumulation of melano-macrophages, hyperemia, necrosis, and vacuolation. These complications were observed to a lesser extent in OE+NP treatment.

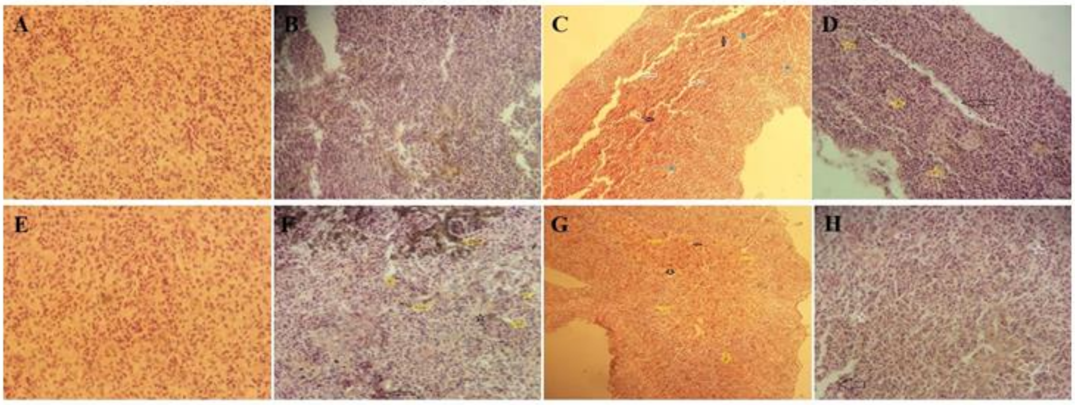


**Figure 2: The effect of OE feeding and NPX poisoning on koi fish spleen tissue on different days (400x). Pictures A to D are related to the 37th day and pictures E to H are related to the 44th day. A: C treatment on day 37 (normal tissue). B: OE treatment on day 37 (normal tissue). C: OE + NP treatment on day 37 (white arrow: Dilation of sinusoid, black arrow: hemosiderin, blue star: necrosis). D: NP treatment on day 37 (black arrow: Dilation of sinusoid, yellow star: necrosis). E: C treatment on day 44 (normal tissue). F: OE treatment on day 44 (black arrow: hyperemia, yellow arrow: macrophage, black star: necrosis, yellow star: vacuolation). G: OE + NP treatment on day 44 (black arrow: vacuolation, yellow arrow: necrosis). H: NP treatment on day 44 (black arrow: Dilation of sinusoid, white arrow: necrosis, white star: vacuolation).**

## Discussion

Nowadays, the use of phytobiotics as a functional diet in fish has become important due to the improvement of growth, health status, immune responses, and protection against diseases (Kazempoor *et al*., 2022). The results showed that feeding with OEO improved the growth performance of fish. In similar studies, Rashidian *et al*. (2021) and Abdel-Latif *et al*. (2020) reported improved growth performance in fish fed with OEO. However, more research is needed to identify the action mechanisms of OEO on fish growth. Rashidian *et al*. (2021) suggested that the high levels of polyphenols and flavonoids in OEO probably cause positive effects. The effects of growth stimulation in groups fed with OEO can be caused by the improvement of taste, which leads to a subsequent increase in the level of feed consumption (Abdel-Latif and Khalil, 2014). Synergistic effects between carvacrol and thymol are effective on fish health, secretion of digestive enzymes (such as protease, lipase, and amylase), and improvement of digestive activity and intestinal microbial population (Zheng *et al*., 2009; Puvača *et al*., 2013; Radhakrishnan *et al*., 2015; Ran *et al*., 2016; Zhang *et al*., 2020).

The results of gill tissue in fish fed with OEO showed a mild level of hyperplasia, blood cell infiltration, and fusion of secondary lamellae. Soares *et al*. (2017) reported changes in hyperplasia, the fusion of lamellae epithelium, and dilation of blood vessels caused by the use of the essential oil of Vervain (*Lippia sidoides*) in black pacu (*Colossoma macropomum*), which is consistent with the results of this study. Changes such as hyperplasia and hypertrophy of epithelial cells and fusion of some secondary lamellae are defense mechanisms that generally lead to an increase in the distance between the external environment and the blood. These mechanisms act as a barrier to preventing the entry of pollutants (Mallatt, 1985; Poleksic and Mitrovic-Tutundizc, 1994; Fernandes, 2003; Laurén and Wails., 2018). The observation of hyperplasia and integration of secondary lamellae shows the positive performance of feeding with OEO in improving the defense mechanism of fish gills.

The gill is the organ that has the most contact with polluted water in fish, and toxic substances in the environment cause changes in its vital functions and morphological structure (Poleksić and Mitrovic-Tutundzic, 1994). In this study, exposure to NPX caused significant damage to the gill tissue. Hoeger *et al*. (2005) and Mehinto *et al*. (2010) have investigated the adverse effects of exposure to NSAID drugs on gill tissue in fish. Gröner *et al*. (2017) reported that diclofenac poisoning in rainbow trout led to epithelial lift and hyperplasia in the gill tissue, which is consistent with the results of this study. A relatively severe occurrence of epithelial lift due to intoxication with the NSAID diclofenac has been reported in other studies (Schwaiger *et al*., 2004; Triebskorn *et al*., 2004). Mohebi Derakhsh *et al*. (2020) reported that diclofenac caused epithelial hyperplasia, clubbing, the fusion of lamellae, and increased mucous cells in the gill tissue of carp. Based on the results of Mohebi Derakhsh *et al*.

(2020), diclofenac caused vasodilation and the accumulation of blood cells in the gill tissue of carp fish. These histopathological changes were also observed in koi fish as a result of NPX poisoning. Epithelial lift indicates a disturbance in osmotic regulation due to damage to the epithelium (Wood, 2017). Fusion and hyperplasia occur due to the increase of mitotic divisions in the gill epithelial layers (Karlsson *et al*., 1985), which is a common event in chronic exposure of fish to lethal external compounds (Mallatt, 1985; Wood, 2017). The proliferation of epithelial cells in combination with the proliferation of chloride cells and mucous cells is an adaptive mechanism to reduce absorption by increasing the blood and water diffusion distance (Wood, 2017). Also, the accumulation of red blood cells in the secondary lamellae is due to the destruction of capillary pillar cells (Hadi and Alwan, 2012).

Based on the obtained results, exposure to NPX caused hyperemia, necrosis, vacuolation, hemosiderin, Dilation of sinusoid, and accumulation of melano-macrophages in the fish spleen. Other studies have not reported tissue damage in the fish spleen due to NSAID poisoning. Tan *et al*. (2013) stated that they reported that the simultaneous use of sunitinib and diclofenac leads to congestion, hyperplasia, and signs of extramedullary hematopoiesis in the spleen tissue. Liu *et al*. (2021) reported that tilapia poisoning with NPX does not cause the accumulation of this drug in the spleen tissue. Also, Schwaiger *et al*. (2004) stated that long-term diclofenac poisoning did not cause any tissue damage in the spleen organ of rainbow trout. The difference in results can be caused by several factors such as fish species, environmental conditions, duration of poisoning, amount, and type of drug used. In addition, spleen damage is attributed to liver portal vein dysfunction (O'Brien *et al*., 2004). Therefore, the liver is the main organ in detoxification, and poisoning with NPX drugs can cause malfunction and serious damage to the spleen tissue of fish.

## Conclusion

Generally, the obtained results showed that feeding with OEO led to the improvement of growth and physiological performance of fish exposed to NPX by reducing the damage to gill and spleen tissues. Therefore, it is recommended to use this food supplement in waters contaminated with pharmaceutical pollutants to prevent damage to farmed fish. It is also recommended to investigate factors involved in improving growth performance due to feeding with OEO, such as the activity of digestive enzymes and intestinal microbial population, and the effect of NPX poisoning on tissue and liver enzymes during future studies.

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