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Studying Protective Effects of Thymol on the Growth Factors of Juvenile Common Carp Following Chronic Mercury (II) Chloride Exposure

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Short running title: Effects of thymol on carp and mercury exposure

ABSTRACT

Background: Heavy metals are non-degradable pollutants, and mercury is one of the most important of them. Mercury can cause harmful effects on the fish, including reducing the growth rate. One of the most effective ways to reduce the side effects of heavy metals is to use herbal compounds or their active ingredients. Thymol is a natural monoterpene which is found in the essential oils of plants such as thyme. The positive effects of this substance have been proven to improve growth indicators in some fish, such as grass carp.

Objectives: This research was conducted to investigate the protective effects of thymol on the growth factors of juvenile carp exposed to mercury metal.

Methods: In the present study, 120 common carp (*Cyprinus carpio*) were randomly divided into 4 groups (control, HgCl2, thymol, and thymol+HgCl2), and each group was replicated three times and included 10 fish. At first, the initial weight of the fish (W0) was recorded, and then the fish were kept for 56 days. The fish in the control group were fed with basic food and kept in mercury (II) chloride-free water. During 56 days, the fish of the thymol and thymol+HgCl2 groups were fed with food containing thymol 100 mg/kg feed. The tank water of the fish of the thymol+HgCl2 and HgCl2 groups contained 0.44 mg/l mercury chloride. After 56 days, the final weight (Wt) of the fish was also measured, and the growth factors (absolute growth, absolute growth rate, SGR, FCR, and FCE) were calculated.

Results: The results of this research showed that thymol has a good protective effect on growth factors following chronic mercury exposure. Thymol in the thymol+HgCl2 group significantly improved absolute growth, absolute growth rate, and FCR compared to the HgCl2 group. Also, thymol led to a significant improvement in SGR and FCE factors, although there was no significant difference with the mercury group.

Conclusions: Therefore, it can be concluded that thymol can effectively reduce the side effects caused by mercury chloride exposure in common carp.

Keywords: common carp, growth indicators, heavy metals, mercury chloride, thymol.

Introduction

The aquaculture industry's most important sectors are food and ornamental fish cultivation. Aquaculture has emerged as a rapidly expanding food-production technology on a global scale. The production of fish for human consumption is one of the most important applications of aquaculture, which is a fast-growing field worldwide (Sasani *et al.,* 2016; Rahmati-Holasoo *et al.,* 2015; Ahmadivand *et al.,* 2020; Rahmati-Holasoo *et al.,* 2020; Malek Ahmadi *et al.,* 2021; Abdulrahman, 2022; Malek Ahmadi *et al.,* 2022; Mirsadeghi et al., 2022; Rahmati-Holasoo *et al.*, 2021; Rahmati-Holasoo *et al.*, 2022a, b; Ziafati Kafi *et al.*, 2022a; Rahmati-Holasoo *et al.*, 2023; Adah *et al.,* 2023, Adah *et al.,* 2024; Mohammed et al., 2024; Rahmati-Holasoo *et al.*, 2024). However, this industry has always faced problems such as the effects of various infectious diseases (Imani & Akhlaghi, 2004) and the effects of environmental pollution (Vutukuru, 2005) on fish health. The *Cyprinidae* are the largest freshwater fish family and rank first in aquaculture production worldwide (about 18 million tonnes) (Action, 2020; Ziafati Kafi *et al.,* 2022b). In Iran, of the 490,000 t of freshwater fish production, 186,000 t are cyprinids, mainly farmed in the three provinces of Khuzestan, Mazandaran, and Gilan (Ziafati Kafi *et al.,* 2022b). One of the most popular farmed cyprinid fish in the world and Iran is Common carp (*Cyprinus carpio*). Its breeding distribution in the world includes almost any region that is warm enough. carp farming can play a decisive role in the prosperity of the country's economy (Abilov *et al.,* 2021).

The industrial development of human life during the last few decades has caused large amounts of pollutants to be produced and introduced into the environment. Finding reliable strategies to eliminate and or modulate the adverse effects is important (Hasankhani *et al.,* 2023). On the other hand, non-compliance with environmental requirements has caused the effects of pollutants on the living environment around us to increase (Vutukuru, 2005). If the concentration of heavy metal pollution in the aquatic environment rises above safe thresholds, it could seriously damage aquatic species (Shahjahan *et al.*, 2022). In general, pollutants can be divided into two types: degradable and non-degradable pollutants, and heavy metal salts are classified as non-degradable pollutants (Güven *et al.*, 1999). Increasing the concentration of these substances has adverse effects on fish, other aquatic organisms, and even aquatic plants (Malik *et al.,* 2010). Heavy metals in the aquatic environment cause pollution of the food cycle and eventually accumulate in the bodies of fish. In this situation, contaminants enter the human body through the consumption of infected fish by people (Farombi *et al.,* 2007, El-Naga *et al*., 2005, Lakshmanan *et al.,* 2009). Nowadays, heavy metal pollution that causes toxicity in fish, is a worldwide problem (Taslima et al., 2022). Among many heavy metals, lead (Pb), mercury (Hg), cadmium (Cd), arsenic (As), chromium (Cr), zinc (Zn), and copper (Cu) are the most worrying (Landis *et al.,* 2003; Agbugui & Abe, 2023). In general, there are two main ways for heavy metals to enter the body of a living organism and then other components of the food chain: one through the digestive tract and the other through permeable membranes such as gills in fish (Yesaki, 1994). Heavy metal accumulation is common in fish species throughout time (Sharma *et al.,* 2024).

Mercury is acknowledged as a contaminant of the environment worldwide. This metal can be found in water, sediments, soil, and the atmosphere. Natural events and industrial processes including gold mine, coal combustion, fuel and chemical waste production, and cement factories are the primary sources of mercury leakage into the aquatic environment. Mercury is also resistant to decompose (Li *et al*., 2009; Lidskog *et al*., 2018; Kimáková *et al*., 2019; Keerthana and Qureshi, 2020; Zulkipli *et al*., 2021). Hg^{2+} is mainly found as an element (Hg^0), in aquatic ecosystems as inorganic compounds (such as HgS, Hg_2Cl_2, and $HgCl_2$), or organic compounds

($(CH_3)_2Hg$ and MeHg). However, the most prevalent type of mercury that companies discharge into aquatic ecosystems is mineral mercury. Compared to the other two forms, this type of mercury has a more toxic effect on fish tissues (Zhang *et al*., 2016a; Zhang *et al*., 2016b; de Almeida Rodrigues *et al*., 2019; Zulkipli *et al*., 2021). Numerous consequences, such as chronic injury to the renal and liver tissues, neurological damage, heart damage, immune system disturbances, reproductive problems, and most notably, growth and development disorders, can be caused by inorganic mercury in aquatic creatures (Chen *et al*., 2021; Huang *et al*., 2010; Zhang *et al*., 2016a; Zhou *et al*., 2020). For example, a study on *Sander vitreus* fish in Canada showed that increasing the amount of mercury accumulated in the muscles of the fish significantly decreased the amount of growth factors (Simoneau *et al.,* 2005).

Although one of the suitable ways to prevent the contamination of fish with heavy metals such as mercury is to prevent the contamination of water and underground resources, another way is to use substances that act as chelators or antioxidants and prevent absorption or effects of heavy metals in the tissues of different animals, including fish, and, as a result, reduce pollution in human and animal societies. The presence of non-degradable toxins such as heavy metals that have already entered the environment and are still present in the environment may be a reason for the importance of this issue. Nowadays, the use of plants in the scientific researches as antioxidants or chelators of various substances has become very common (Arzi *et al*., 2011). Taleghani *et al*., (2019) described that *Rosa damascena* extract reduces side effects of zinc exposure in the liver of common carp (*Cyprinus carpio*). Another study showed that oral consumption of curcumin reduces the effects of lead metal toxicity on the immune and antioxidant responses of common carp (*Cyprinus carpio*) (Giri *et al*., 2021). Oral consumption of *Allium hirtifolium* extract effectively reduced the side effects caused by food poisoning with zinc oxide nanoparticles (Mahboub *et al*., 2022). Also, cinnamaldehyde can also reduce the injuries caused by zinc oxide poisoning in the gill tissue of common carp (*Cyprinus carpio*) (Heidardokht *et al*., 2023).

Plant-extracted essential oils, such as thyme (Hoseini and Yousefi 2019) and oregano (Zheng *et al*. 2009), contain thymol (2-isopropyl-5-methylphenol), as a naturally occurring monoterpene. Thymol has been effectively applied as a plant feed addition in fish nutrition to enhance performance, stimulate the digestive tract's structure and function, boost metabolism, and lessen the harm that comes from free radicals (Ran *et al.,* 2016; Ezzat Abd El-Hack *et al*., 2016; Anyu *et al*., 2018). In fish, thymol is an excellent anesthetic with anti-inflammatory activity in several fish species, including the common carp (Yousefi *et al.,* 2018) and the silver catfish (Bianchini *et al.,* 2017). Thymol is antibacterial against *Aeromonas hydrophila* bacteria (da Cunha *et al*.,

2019) and also has growth-stimulating properties (Anyu *et al.*, 2018). In this regard, a study showed that the presence of thymol in the diet, improves the growth performance of Nile tilapia (*Oreochromis niloticus*). In addition, the antioxidant properties of thymol have been proven (Amer *et al*., 2018).

Considering the negative effects of mercury metal poisoning on growth factors in fish and the positive effects of thymol on growth factors and antioxidant responses in fish, this study aims to evaluate the protective effects of thymol on growth factor changes following mercury chloride waterborne toxicity.

Materials and Methods

Diet preparation

To feed the fish, the commercial food for carp fish (Beyza 21 Manufacturing Company, Fars, Iran) was used as basic food. For adding thymol to the basic diet, the food must first be completely and uniformly ground. Basal feeding was supplemented with 100 mg/kg thymol (Merck, Germany) (Morselli *et al*., 2020). After that, every ingredient was well combined, pelletized, let to air dry, and then sieved to produce pellets of the proper size. Fresh feed was made and stored at 4°C every week.

Fish and Experimental Nutrition

Fish were bought from a breeding facility in the province of Gilan. 120 juvenile common carps were transported to the Department of Aquatic Health, Aquatic Research Center, Faculty of Veterinary Medicine, University of Tehran. The fish initially were transferred to the 1000-liter aquariums and 14 days of adaption of fish were completed (Giri *et al*., 2021). Following the adaption period, the fish were harvested and examined for diseases. No external and internal parasites were observed in microscopical examinations. After that, their initial weight (W_0) was determined. The fish's initial weight was recorded at 17.4 ± 1.08 grams on average. The fish were then separated into groups of one to four and placed into twelve 125-liter tanks with a total volume of 100 liters water. Each group consisted of three repetitions, and 10 fish were considered for each repetition. The fish in group 1 (control) were fed the basic diet, and the water had no mercury chloride. In group 2 ($HgCl_2$), the fish were fed the basic food, and their water was supplemented with mercury chloride (0.44 mg/liter) (Gül *et al*., 2004). In group 3 (thymol), fish were fed with food containing thymol (100mg/kg feed) (Morselli *et al*., 2020), with no mercury chloride in their water. In group 4 (thymol+$HgCl_2$), fish were fed with food containing thymol (100 mg/kg feed), and mercury (II) chloride (0.44 mg/liter) was added to fish water. The fish were then held for eight weeks (fifty-six days). The fish were fed daily at the rate of one percent of their body weight twice. The water of the fish was also changed daily by 50%, and to maintain the concentration of mercury in the water, the amount of mercury removed after the water change was calculated and added to the water. Also, fish water was kept constant by using an air pump, and its temperature was kept constant by using an electric heater. The average water temperature during the research period was 24.02±0.8 and pH was 7.7±0.2.

Investigating growth performance.

As mentioned earlier, the initial weight (W_0) of the fish was recorded before the experiment. After 56 days from the beginning of the experiment, the fish were anesthetized using an anesthetic (PI222, Pars Imen Daru, Tehran, Iran), and their final weight (W_t) was recorded. Then, the following factors related to fish growth were measured using formulas (Goddard, 1995):

Absolute growth in weight from Day 0 to Day 56=W_t-W_0

Absolute growth rate (g/day) = (W_t-W_0)/t

$$SGR(\%/day) = [(Ln(W_t)-Ln(W_0))/t] \times 100$$

FCR = food fed/weight gain

FCE (%) = weight gain/food consumed × 100,

In this formula, (Wt) is the final weight, (W0) is the initial weight, and (t) is the duration of feeding (in days).

Statistical Analysis

The study's data were analyzed using GraphPad Prism 9.0 (GraphPad Software Inc., La Jolla, CA, USA) and IBM SPSS statistics for Windows, version 25.0 (IBM Corp., Armonk, NY, USA). The results are presented as mean ± standard deviation. The normality of data and homogeneity of variances were checked using the Kolmogorov-Smirnov test and Levene's test. These were performed with IBM SPSS Statistics for Windows, version 25.0 (IBM Corp., Armonk, NY, USA). Graphs and statistical analyses were performed using GraphPad Software 9.0 (GraphPad Software Inc., La Jolla, CA, USA), and significant differences were determined by one-way analysis of variance (ANOVA), followed by Tukey's test as a post hoc test for comparison. Multiple tests were performed. A p-value of less than 0.05 was considered significant.

Results

The results of the present research showed that the absolute growth in the $HgCl_2$ group decreased compared to the control group, but it was not significantly different. The absolute growth in the thymol group had the highest value compared to the other groups and was significantly different from the control and $HgCl_2$ groups. In the thymol+$HgCl_2$ group, the absolute growth value was significantly higher than the $HgCl_2$ group, and although its value was lower than the thymol group, it was not significantly different.

Also, the performance of thymol on the absolute growth rate was appropriate, so this parameter increased significantly in both the thymol and thymol+$HgCl_2$ groups compared to the $HgCl_2$ group. However, these two groups did not have significant differences with each other.

The specific growth rate (SGR) in the thymol group increased in a way that had a significant difference from the control and $HgCl_2$ groups. This parameter also increased in the Thymol+$Hgcl_2$ group, but none of the groups had a significant difference. Also, the control and $HgCl_2$ groups were not significantly different from each other, although the amount of SGR in the mercury group decreased compared to the control.

Food conversion efficiency (FCE) also increased after the use of thymol, but only the thymol and HgCl2 groups had significant differences with each other, and the other groups did not have significant differences with each other.

Food conversion ratio (FCR) increased in the HgCl2 group compared to other groups; however, this increase was not significantly different from the control group, but it was significantly different from the other two groups. Also, the thymol and thymol+HgCl2 groups had no significant differences from each other. The results of this research can be seen in figure 1A-E. In these graphs, only the groups that have significant differences from each other have been identified.

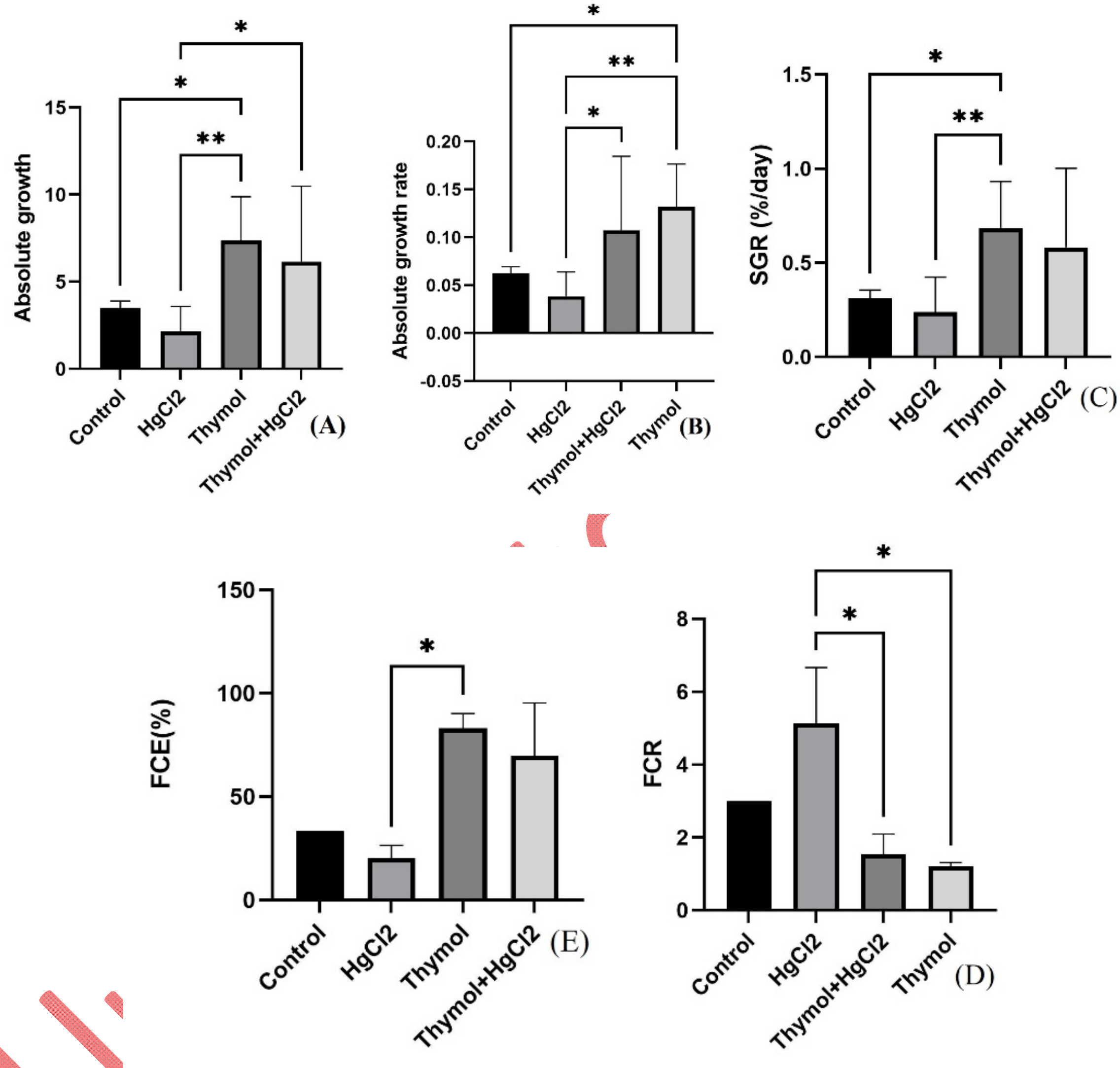
Absolute growth
15
10
5
0
Control
HgCl2
Thymol
Thymol+HgCl2
*
**
*
(A)
Absolute growth rate
0.20
0.15
0.10
0.05
0.00
-0.05
Control
HgCl2
Thymol+HgCl2
Thymol
*
**
*
(B)
SGR (%/day)
1.5
1.0
0.5
0.0
Control
HgCl2
Thymol
Thymol+HgCl2
*
**
(C)
FCE(%)
150
100
50
0
Control
HgCl2
Thymol
Thymol+HgCl2
*
(E)
FCR
8
6
4
2
0
Control
HgCl2
Thymol+HgCl2
Thymol
*
*
(D)

Figure 1. Protective effect of thymol on growth factors of common carp after chronic mercury exposure. A) Absolute growth; B) Absolute growth rate; C) Specific growth rate (SGR); D) Food conversion efficiency (FCE); E) Food conversion ratio (FCR). * ($p < 0.05$), ** ($p < 0.01$).

Discussion

The industry currently uses over 40 different metals and metal alloys. Heavy metal pollution is now at risk to human health. Mercury is one of the most important environmental pollutants whose presence in the environment can be worrying (Landis *et al*., 2003). Fish are the main source of mercury contamination for humans because, like other heavy metals, mercury may enter fish bodies through the food chain or via water. Mercury can then have an impact on both fish and human culture (Farombi *et al*., 2007; El-Naga *et al*., 2005; Lakshmanan *et al*., 2009). Growth reduction is one of the many negative impacts of mercury exposure in fish. Fish health and the surrounding environment are just two of the many variables that affect fish growth. Therefore, investigating the growth factors in fish is one of the ways to evaluate the health level of fish. Furthermore, the economic significance of the fish growth rate implies that the decline in growth resulting from contaminants like mercury may potentially have economic implications (Simoneau *et al*., 2005, Zhou *et al*., 2024).

Although preventing the entry of heavy metals into the environment is one of the most effective ways to reduce their negative effects, considering their cumulative and long-lasting effects on the environment, one should think of another way, and the use of plant active ingredients is one of the best (Arzi *et al*., 2011). Considering the importance of this type of pollution, this study aimed to find a way to reduce the negative effects of this type of pollution.

In a study by Gül *et al*., (2004) conducted on the Chub (*Squalius cephalus* previously named *Leuciscus cephalus*), which belongs to the Cyprinidae family, it was found that the 96-h LC50 of mercury chloride in this fish is 0.55 mg/L, and the dose of 0.44 mg/L did not cause specific behavioral or appearance effects. So, 0.44 mg/L was selected in this study.

A 2020 study found that 100 mg thymol/kg of feed increased the growth performance of herbivorous carp (Morselli *et al*., 2020). This was consistent with the results of the present study. The present research also showed that the dose of 100 mg thymol/kg of fish food significantly improved the growth factors compared to the control group. Also, the thymol group had a significant difference from the $HgCl_2$ group in terms of all factors related to fish growth. A study conducted by Simoneau *et al*. (2005) on walleye (*Sander vitreus*) in Canadian lakes showed that increasing the amount of mercury can cause a decrease in growth indicators in fish. The result of the present research also showed that the amount of growth factors in the group whose water contained HgCl2 decreased compared to the control group in such a way that the average absolute growth, absolute growth rate, specific growth rate (SGR), and food conversion efficiency (FCE) in the HgCl2 group decreased, and the food conversion ratio (FCR) increased compared to the control group, although the amount of this change was not significantly different from the control group. However, the reduction of the growth indicators shows the same direction as the results of these two studies. Heidardokht *et al.* (2023) showed that oral administration of cinnamaldehyde had a favorable protective effect on injuries caused by zinc oxide water poisoning in the gill tissue of common carp. Also, Mahboub *et al.* (2022) showed that *Allium hirtifolium* extract has a good protective effect against common carp poisoning with zinc oxide metal nanoparticles. Taleghani *et al.* (2019) described the favorable protective effect of oral consumption of *Rosa damascena* extract on zinc oxide poisoning in common carp. The present study described that thymol has a reasonable ability to improve the growth factors of common carp when exposed to mercury chloride. The $HgCl_2$+thymol group had a significant difference from the $HgCl_2$ group in terms of absolute growth, absolute growth rate, and FCR. Also, the amount of SGR and FCE improved in the $HgCl_2$+thymol group compared to the $HgCl_2$ group, although there was no significant difference. A study conducted by Giri *et al.* (2021) showed that curcumin can improve growth factors in common carp exposed to lead metal.

Conclusion

Essential oils produced from plants like oregano and thyme include thymol, a plant dietary supplement. It is employed to raise fish production and reproduction performance as well as enhance animal performance (Morselli *et al*., 2020; Hoseini and Yousefi, 2019; Zheng *et al*., 2009). The findings of this study demonstrated that oral thymol in common carp can positively decrease the adverse effects of chronic mercury metal poisoning.

Authors' contributions

The study conception and design were prepared by HRH, AN and SS. Investigation, methodology and formal analysis were performed by HRH, AN and MS. The first draft of the manuscript was written by AN. The manuscript edited by HRH and AN. All authors commented on previous versions of the manuscript, read and approved the final version of manuscript.

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Ethical considerations

In the current study, all experimental protocols were approved by the University of Tehran Veterinary Ethical Review Committee (Approval ID: IR.UT.VETMED.REC.1402.066). All methods were carried out in accordance with relevant guidelines and regulations of the University of Tehran Veterinary Ethical Review Committee.

Declarations

Competing interests

The authors declare that they have no conflict of interests.

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چکیده

زمینه مطالعه: فلزات سنگین آلاینده های تجزیه ناپذیر هستند و جیوه یکی از مهم ترین آنهاست. جیوه می تواند اثرات مضری بر روی ماهی از جمله کاهش سرعت رشد داشته باشد. یکی از موثرترین راه ها برای کاهش عوارض فلزات سنگین، استفاده از ترکیبات گیاهی یا مواد موثره آنهاست. تیمول یک مونوترپن طبیعی است که در اسانس گیاهانی مانند آویشن یافت می شود. اثرات مثبت این ماده برای بهبود شاخص های رشد در برخی از ماهی ها مانند کپور علف خوار ثابت شده است.

هدف: این تحقیق به منظور بررسی اثرات محافظتی تیمول بر روی فاکتورهای رشد ماهیان کپور جوان در مواجهه با فلز جیوه انجام شد.

مواد و روش کار: در مطالعه حاضر 120 ماهی کپور معمولی به طور تصادفی به 4 گروه) شاهد، $HgCl_2$، تیمول و تیمول ($HgCl_2$+تقسیم شدند و هر گروه شامل 10 ماهی بود و 3 تکرار داشت. ابتدا وزن اولیه ماهی ها (W_0)ثبت شد و سپس ماهی ها به مدت 56 روز نگهداری شدند. ماهیان گروه کنترل با غذای اصلی تغذیه و در آب بدون کلرید جیوه (II) نگهداری شدند. طی 56 روز، ماهیان گروه تیمول و تیمول $HgCl_2$+با غذای حاوی تیمول 100 میلی گرم بر کیلوگرم خوراک تغذیه شدند. آب مخزن ماهی های گروه تیمول $HgCl_2$+و $HgCl_2$ حاوی 0.44 میلی گرم در لیتر کلرید جیوه بود. پس از 56 روز، وزن نهایی ماهی نیز اندازه گیری شد و فاکتورهای رشد) رشد مطلق، نرخ رشد مطلق، SGR، FCRو (FCE محاسبه شد.

نتایج: نتایج این تحقیق نشان داد تیمول اثر محافظتی خوبی بر عوامل رشد بدنبال مواجهه مزمن با جیوه دارد. تیمول در گروه تیمول + $HgCl_2$ به طور قابل توجهی رشد مطلق، نرخ رشد مطلق و FCR را در مقایسه با گروه $HgCl_2$ بهبود بخشید. همچنین تیمول منجر به بهبود قابل توجهی در فاکتورهای SGR و FCE شد، اگرچه تفاوت معنی‌داری با گروه جیوه وجود نداشت.

نتیجه گیری نهایی: بنابراین می توان نتیجه گرفت که تیمول می تواند به طور موثری عوارض ناشی از مواجهه با کلرید جیوه را در ماهی کپور معمولی کاهش دهد.

کلمات کلیدی: تیمول، فلزات سنگین، فاکتورهای رشد، کلرید جیوه، کپور معمولی