Original Article

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

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ABSTRACT

Background: Heavy metals are non-degradable pollutants, and mercury is one of them. It can harm fish and reduce its 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 in plants' essential oils, 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.

Methods: In the present study, 120 common carp (*Cyprinus carpio*) were randomly divided into 4 groups (control, $HgCl_2$, thymol, and thymol+$HgCl_2$). Each group was replicated three times and included 10 fish. At first, the initial weight of the fish (W_0) was recorded, and then the fish were kept for 56 days. The control group fish were fed basic food and kept in mercury (II) chloride-free water. During 56 days, the fish of the thymol and thymol+$HgCl_2$ groups were fed with food containing thymol 100 mg/kg feed. The tank water of the fish of the thymol+$HgCl_2$ and $HgCl_2$ groups contained 0.44 mg/L mercury chloride. After 56 days, the final weight (W_t) of the fish was also measured, and the growth factors (absolute growth, absolute growth rate, specific growth rate [SGR], food conversion ratio [FCR], and food conversion efficiency [FCE]) were calculated.

Results: This research showed that thymol has a protective effect on growth factors following chronic mercury exposure. Thymol in the thymol+$HgCl_2$ group significantly improved absolute growth, absolute growth rate, and FCR compared to the $HgCl_2$ group. Also, thymol significantly improved SGR and FCE factors, although there was no significant difference with the mercury group.

Conclusion: 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

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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, Rahmati-Holasoo et al., 2022b; 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 various infectious diseases (Imani & Akhlaghi, 2004) and environmental pollution (Vutukuru, 2005). The Cyprinidae are the largest freshwater fish family and rank first in aquaculture production worldwide (about 18 million tons) (Action, 2020; Ziafati Kafi et al., 2022b). In Iran, of 490000 tons of freshwater fish production, 186000 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 produced large amounts of pollutants introduced into the environment. Finding reliable strategies to eliminate or decrease the adverse effects is important (Hasankhani et al., 2023). On the other hand, non-compliance with environmental requirements has increased the effects of pollutants on the living environment (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 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 pollute the food cycle and eventually accumulate in the bodies of fish. In this situation, contaminants enter the human body by consuming infected fish (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, mercury (Hg), cadmium, arsenic, chromium, zinc, and copper 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 (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 mines, coal combustion, fuel and chemical waste production, and cement factories, are the primary sources of mercury seepage into the aquatic environment. Mercury is also resistant to decomposition (Li et al., 2009; Lidskog et al., 2018; Kimáková et al., 2019; Keerthana & Qureshi, 2020; Zulkipli et al., 2021). In aquatic ecosystems, Hg^{2+} is mainly found as an element (Hg^0) inorganic compounds, such as HgS, Hg_2Cl_2, and $HgCl_2$, or organic compounds, like $(CH_3)_2Hg$ and MeHg. However, mineral mercury is the most prevalent type of mercury companies discharge into aquatic ecosystems. 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 shows that increasing the amount of mercury accumulated in the fish muscles significantly decreases the number 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 solution. Using plants in scientific research 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 the side effects of zinc exposure in the liver of common carp. Another study showed that oral consumption of curcumin reduces the effects of lead metal toxicity on the immune and antioxidant responses of common carp (Giri et al., 2021). Oral consumption of *Allium hirtifolium* extract effectively reduces the side effects caused by food poisoning with zinc oxide nanoparticles (Mahboub et al., 2022). Also, cinnamaldehyde can reduce the injuries caused by zinc oxide poisoning in the gill tissue of common carp (Heidardokht et al., 2023).

Plant-extracted essential oils, such as thyme (Hoseini & 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 from free radicals (Ran et al., 2016; Ezzat Abd El-Hack et al., 2016; Aanyu 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* (da Cunha et al., 2019) and has growth-stimulating properties (Aanyu 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

Commercial food for carp fish (Beyza 21 Manufacturing Company, Fars, Iran) was used to feed the fish. The food must first be entirely and uniformly ground to add thymol to the essential diet. 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 Giulan. One hundred and twenty juvenile common carp were transported to the Department of Aquatic Health, Aquatic Research Center, Faculty of Veterinary Medicine, University of Tehran. The fish were initially transferred to the 1000-L aquariums until 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. Afterward, their initial weight (W_0) was determined. The fish's initial weight was recorded at 17.4±1.08 g on average. They were then separated into groups of one to four and placed into twelve 125-L tanks with a total volume of 100 liters of 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/L) (Gül et al., 2004). In group 3 (thymol), fish were fed food containing thymol (100 mg/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/L) was added to fish water. The fish were then held for 8 weeks (56 days). The fish were fed daily at the rate of one percent of their body weight twice. The fish's water was also changed daily by 50%, and to maintain the mercury concentration 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 using an air pump, and its temperature was kept constant using an electric heater. The average water temperature during the research period was 24.02±0.8 °C and pH was 7.7±0.2.

Investigating growth performance

As mentioned, the fish's initial weight (W_0) 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

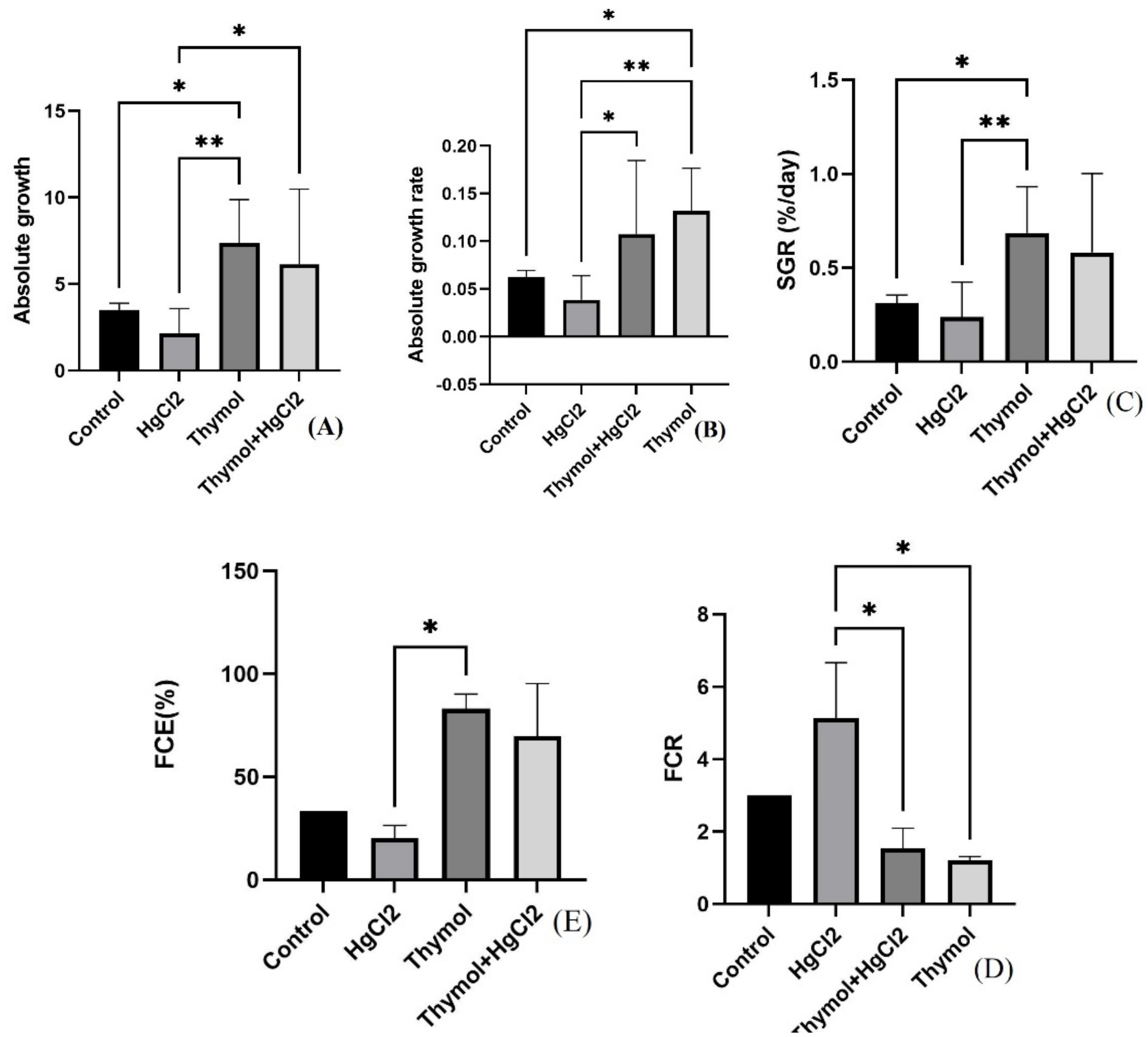

Figure 1. Protective effect of thymol on growth factors of common carp after chronic mercury exposure

A) Absolute growth, B) Absolute growth rate, C) SGR, D) FCE, E) FCR

$^{*}P<0.05$; $^{**}P<0.01$.

following factors related to fish growth were measured using Equations 1, 2, 3, 4 and 5 (Goddard, 1995):

1. Absolute growth in weight from day 0 to day 56=W_t-W_0

2. Absolute growth rate (g/d)=(W_t-W_0)/t

3. Specific growth rate (SGR) (%/d)=[(Ln(W_t)-Ln(W_0))/t]×100

4. Food conversion ratio (FCR)=Food fed/weight gain

5. Food conversion efficiency (FCE) (%)=Weight gain/Food consumed×100

In this formula, (W_t) is the final weight, (W_0) is the initial weight, and (t) is the duration of feeding (in days).

Statistical analysis

The study data were analyzed using GraphPad Prism software, version 9.0 (GraphPad Software Inc., La Jolla, CA, USA) and IBM SPSS statistics for Windows, version 25 (IBM Corp., Armonk, NY, USA). The results are presented as Mean±SD. The normality of data and homogeneity of variances were checked using the Kolmogorov-Smirnov and Levene's tests. These tests were performed with IBM SPSS Statistics for Windows, version 25.0. Graphs and statistical analyses were performed using GraphPad Software, and significant differences were determined by one-way analysis of variance

(ANOVA), followed by the Tukey post hoc test for comparison. Multiple tests were performed. A $P<0.05$ was considered significant.

Results

The present research results 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.

The 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.

FCE also increased after the use of thymol, but only the thymol and $HgCl_2$ groups had significant differences with each other, and the other groups did not have significant differences with each other.

FCR increased in the $HgCl_2$ group compared to other groups; however, this increase was not significantly different from the control group but significantly different from the other two groups. Also, the thymol and thymol+$HgCl_2$ groups had no significant differences. The results of this research can be seen in Figure 1. Only the groups with significant differences have been identified in these graphs.

Discussion

The industry currently uses over 40 different metals and metal alloys. Heavy metal pollution now threatens 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 primary source of mercury contamination for humans because, like other heavy metals, mercury may enter fish bodies through the food chain or water. Mercury can impact 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 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 adverse 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 adverse 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 LC_{50} 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 result was consistent with the results of the present study. The present research also shows that the dose of 100 mg thymol/kg of fish food significantly improves the growth factors compared to the control group. Also, the thymol group significantly differs from the $HgCl_2$ group regarding all factors related to fish growth. A study conducted by Simoneau et al. (2005) on walleye (*S. vitreus*) in Canadian lakes shows that a higher amount of mercury can decrease growth indicators in fish. The results of the present research also show that the number of growth factors in the group whose water contained $HgCl_2$ decreased compared to the control group in such a way that the average absolute growth, absolute growth rate, SGR, and FCE in the $HgCl_2$ group decrease, and FCR increases compared to the control group. However, 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) reported 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 *A. 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 *R. damascena* extract on zinc oxide poisoning in common carp. The present study shows that thymol can reasonably improve the growth factors of common carp when exposed to mercury chloride. The $HgCl_2$+thymol group differed significantly 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 the difference was not significant. A study by Giri et al. (2021) showed that curcumin can improve growth factors in common carp exposed to lead metal.

Conclusion

Essential oils from plants like oregano and thyme include thymol, a dietary supplement. It raises fish production and reproduction and enhances animal performance. The findings of this study demonstrate that oral thymol in common carp can positively decrease the adverse effects of chronic mercury metal poisoning.

Ethical Considerations

In the current study, all experimental protocols were approved by the University of Tehran Veterinary Ethical Review Committee, Tehran, Iran (Code: 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.

Compliance with ethical guidelines

The authors declared no conflict of interest.

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Authors' contributions

Conceptualization and validation: Hooman Rahmati-Holasoo and Alireza Nassiri; Methodology: Alireza Nassiri, Mehdi Soltani and Sara Shokrpoor; Investigation: Hooman Rahmati-Holasoo and Alireza Nassiri; Visualization: Hooman Rahmati-Holasoo, Alireza Nassiri and Sara Shokrpoor; Project administration and supervision: Hooman Rahmati-Holasoo; Writing the original draft: Alireza Nassiri and Sara Shokrpoor; Review and editing: All authors.

Conflict of interest

The authors declared no conflict of interest.

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مقاله پژوهشی

مطالعه اثرات محافظتی تیمول بر فاکتورهای رشد ماهی کپور معمولی در مواجهه مزمن با کلرید جیوه

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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.

روش کار: در مطالعه حاضر ۱۲۰ ماهی کپور معمولی به طور تصادفی به ۴ گروه (شاهد، $HgCl_2$، تیمول و تیمول+$HgCl_2$) تقسیم شدند و هر گروه شامل ۱۰ ماهی بود و ۳ تکرار داشت. ابتدا وزن اولیه ماهی ها(W0) ثبت شد و سپس ماهی ها به مدت ۵۶ روز نگهداری شدند. ماهیان گروه کنترل با غذای اصلی تغذیه و در آب بدون کلرید جیوه (II) نگهداری شدند. طی ۵۶ روز، ماهیان گروه تیمول و تیمول+$HgCl_2$ با غذای حاوی تیمول ۱۰۰ میلی گرم بر کیلوگرم خوراک تغذیه شدند. آب مخزن ماهی های گروه تیمول+$HgCl_2$ و $HgCl_2$ حاوی ۰،۴۴ میلی گرم در لیتر کلرید جیوه بود. پس از ۵۶ روز، وزن نهایی ماهی نیز اندازه گیری شد و فاکتورهای رشد، رشد مطلق، نرخ رشد مطلق، FCR، SGR و 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 + HgCl2 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.

نتیجه‌گیری 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.

کلیدواژه‌ها: تیمول، فلزات سنگین، فاکتورهای رشد، کلرید جیوه، کپور معمولی

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