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The Study of the Effects of Menthol and Thymol on Kidney and Liver Lesions Caused by Chronic Mercury Poisoning in Zebrafish (*Danio rerio*)

Running Title: Effects of menthol and thymol on kidney and liver lesions caused by chronic mercury poisoning

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ABSTRACT

Heavy metals represent a significant form of environmental pollution, accumulating over time and posing serious risks to human health. Among these, mercury is particularly concerning, as it primarily enters the human body through the consumption of contaminated aquatic animals. The ingestion of mercury-laden fish can lead to severe health issues. Therefore, it is essential to explore strategies to mitigate the adverse effects of this pollution. Natural plant compounds, known for their antioxidant and chelating properties as well as their biodegradability, offer promising solutions for addressing heavy metal contamination. Thymol and menthol are two such compounds that exhibit beneficial effects in this context. Zebrafish (*Danio rerio*) have gained significant attention as a model organism in biological research due to their genetic similarity to humans, ease of breeding, and high reproductive capacity. This study aimed to compare the protective effects of thymol and menthol against chronic mercury poisoning in the liver and kidney tissues of zebrafish. A group of zebrafish was exposed to 0.44 mg/L of mercury to assess the therapeutic effects of thymol and menthol on chronic mercury toxicity. Pathological sampling was performed, and tissue sections were examined microscopically. The findings revealed that the mercury-exposed group exhibited the highest level of tissue damage. While the severity of injuries in the mercury-thymol group was less pronounced than in the mercury-menthol group, neither treatment fully resolved the damage caused by mercury exposure. These results underscore the importance of investigating various substances to mitigate the effects of environmental pollutants. Notably, this study demonstrated that thymol was slightly more effective than menthol in reducing the severity of mercury-induced injuries.
Keywords: *Danio rerio*, Histopathology, Mercuric chloride, Menthol, Thymol

INTRODUCTION

Heavy metals are a major pollutant in environment, causing developmental and health disorder [1,2]. They threaten all types of life including aquatic and terrestrial, by inducing oxidative stress, cytological damage and inflammation [3-5]. Heavy metals may have interaction with other water pollutants, such as microplastics, and worsen their toxicity [6]. Heavy metals, such as mercury, present a serious threat to both aquatic life and seafood consumers, who may face substantial health risks from contamination [7]. Mercury, a particularly toxic heavy metal, adversely affects the human nervous system. Methylmercury compounds, which primarily accumulate in seafood and freshwater fish, contribute to mercury buildup in the human body [8, 9]. The concentration of mercury can magnify through the food chain over time [10].

Zebrafish (*Danio rerio*) is commonly used as an embryonic and larval model in laboratory experiments and developmental toxicology studies due to its genetic and physiological similarities to humans, particularly in the central nervous system, liver, kidneys, and intestines [11]. This makes zebrafish an excellent model for evaluating the toxicity of heavy metals.

The utilization of plant-derived compounds in scientific research has gained traction, particularly for their antioxidant and chelating properties [12]. Researchers are increasingly drawn to these compounds due to their beneficial effects, biodegradability, and potential to mitigate environmental pollution [13,14,5]. Thymol, for example, is used as a plant-based dietary additive in fish nutrition. It enhances digestive performance, boosts metabolism, and reduces damage from free radicals [15-17]). Thymol also exhibits antibacterial properties against *Aeromonas hydrophila* [18] and acts as a growth stimulant in fish [15]. By reducing lipid peroxidation and increasing the activity of antioxidant enzymes, thymol improves the antioxidant status of fish tissues, leading to enhanced performance and reduced adverse effects from environmental stressors [19,16,17].

Similarly, menthol is a relaxant compound [20] recognized for its antioxidant properties and its positive effects on growth performance, immune response, and resistance to ammonia-induced pollution [14,6,21]. The application of menthol as an oral supplement has been shown to enhance growth rates, antioxidant responses, and anti-inflammatory effects [22].

According to above facts, we aimed to explore if dietary supplementation with thymol and menthol is beneficial to reduce histopathological lesions in the liver and kidney of zebrafish exposed to mercury.

MATERIAL AND METHODS

Feed Preparation and Experimental Diets

A commercial fish feed was first ground into a fine powder using an electric grinder and subsequently divided into four experimental batches. The control group received a basal diet without any additives or mercury exposure. The positive control group was fed the basal diet while being exposed to 0.44 mg/L of $HgCl_2$ in water [23]. Two treatment groups received either a thymol-supplemented diet [100 mg/kg thymol, [24]] or a menthol-supplemented diet [2.5 g/kg menthol, [25]], both with concurrent exposure to 0.44 mg/L of $HgCl_2$. Thymol and menthol were purchased from Sigma-Aldrich Co. (Mo. USA) with ≥99% purity.

For the supplemented diets, thymol or menthol was thoroughly mixed with the ground feed using an electric mixer. Distilled water (6 mL per 10 g of feed) was then added to create a uniform paste, which was extruded through a meat grinder to form pellets. These pellets were air-dried for 24 hours at 25°C. The prepared feed was sealed in zip-lock bags and stored at 4°C, with fresh batches prepared weekly to maintain feed quality and stability.

Fish Acclimation and Husbandry

A total of 170 zebrafish, averaging 4 ± 0.3 cm in length and weighing 1 ± 0.1 g, were obtained from a commercial supplier for this study. Prior to experimentation, all fish underwent a 2% NaCl bath (20 g/L for 45 minutes) for pathogen control, followed by a 14-day quarantine period in 50-L aquaria under controlled environmental conditions. During the quarantine, the water temperature was maintained at 26 ± 1°C under a 12:12 hour light:dark photoperiod. Adequate aeration was provided through air stones to keep dissolved oxygen levels above 6 mg/L. Water quality was managed through daily 30% water changes, and waste was removed via siphoning several hours post-feeding. After the acclimation period, 120 fish of uniform size were selected for the main experiment.

Experimental Design

The zebrafish were randomly assigned to four treatment groups, with 30 fish per group (10 fish per replicate), housed in 25-L glass aquaria, each containing 20 L of water. The control group received the basal diet and kept in clean water, while the positive control group received the basal diet and kept in water containing 0.44 mg/L of $HgCl_2$. The two treatment groups were fed either the thymol-supplemented diet (100 mg/kg) or the menthol-supplemented diet (2.5 g/kg), both with concurrent exposure to 0.44 mg/L of $HgCl_2$.

Each aquarium (30 fishes) was divided into three mesh-separated compartments (10 fishes) to maintain consistent water chemistry while preventing fish movement between sections. Fish were fed twice daily at a rate of 1.5% of their body weight per feeding throughout the 8-week experimental duration. Mercury chloride ($HgCl_2$, Merck, analytical grade) was dissolved in deionized water and added daily to achieve the target concentration of 0.44 mg/L, ensuring consistent exposure levels.

Tissue Sampling and Histopathological Analysis

At the end of the experimental period, fish were humanely euthanized followed by euthanasia via spinal cord transection. For histopathological examination, kidney and liver tissues were carefully dissected and immediately fixed in 10% neutral buffered formalin (NBF) for 24 hours before being transferred to fresh NBF for long-term preservation.

The fixed tissues underwent standard histological processing, beginning with dehydration through a graded ethanol series (50-100%), clearing in xylene, and embedding in paraffin using a Leica EG1150H embedding station. Tissue sections of 4 µm thickness were cut with a Leica RM2235 rotary microtome and mounted on glass slides for further analysis.

The tissue sections were stained using the hematoxylin and eosin (HE) method according to a standardized protocol. This process involved several key steps: first, the sections were deparaffinized in xylene with three changes of 5 minutes each. Next, they were rehydrated through a descending series of ethanol concentrations (from 100% to 70%). The sections were then stained with hematoxylin for 5 minutes, followed by differentiation in acid alcohol. Afterward, counterstaining was performed with eosin for 2 minutes. Finally, the sections were dehydrated through an ascending series of ethanol concentrations, cleared in xylene, and mounted with DPX mountant.

Prepared slides were examined under a light microscope (Nikon, Eclipse E600) (40-400× magnification) equipped with a Tucsen microscope digital camera (GT12, China). The analysis focused on histopathological indicators, including necrosis, inflammation, and other tissue alterations in both gill and liver specimens.

RESULTS

After eight weeks of exposure, significant differences were observed between the treatment groups. Microscopic examination of liver tissue samples revealed normal architecture in the control group (Fig. 1a). In contrast, the mercury-treated group showed pronounced fatty changes, characterized by the presence of intracytoplasmic vacuoles in all hepatocytes (Figs. 1b and c). Additionally, multifocal lymphoplasmacytic hepatitis was observed, marked by mild and localized infiltration of inflammatory cells, predominantly mononuclear cells such as lymphocytes and plasma cells (Fig. 1d). The mercury-menthol group exhibited moderate fatty changes, while the mercury-thymol group showed only mild fatty changes, often absent in several sections (Figs. 1e and f). The mercury-thymol group displayed a reduced severity of these lesions compared to the mercury-menthol group, although neither treatment completely reversed the damage. The mercury-menthol group also showed improvement compared to the mercury-exposed group, but the protective effect was less pronounced than with thymol.

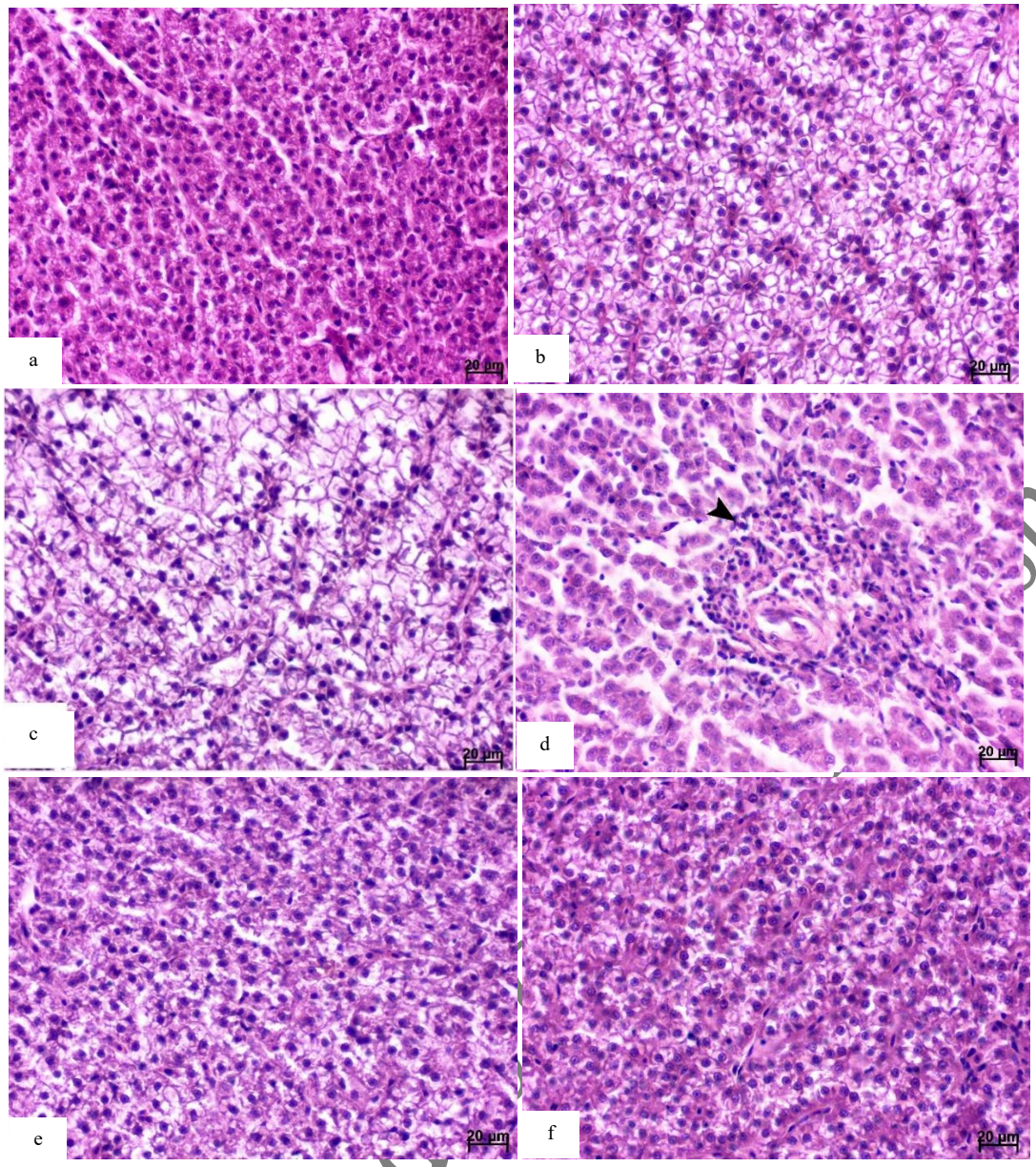
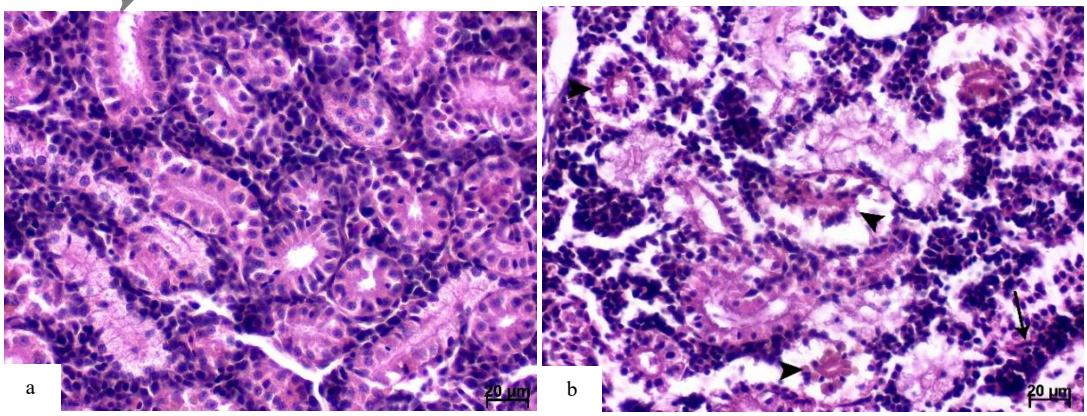


Fig. 1 Microscopic findings in liver tissue in different groups. (a): Normal liver tissue in the control group. (b-c) Severe fatty changes and (d) Multifocal hepatitis (arrowhead) in the mercury group. (e) Moderate fatty changes in the mercury-menthol group. (f) Mild fatty changes in the mercury-thymol group (H&E).

Microscopic evaluation of kidney tissue sections from the control group revealed a normal appearance (Fig. 2a). Conversely, the mercury group demonstrated moderate to severe necrosis of the epithelial cells lining the urinary tubules, accompanied by mild multifocal lymphoplasmacytic nephritis and moderate proteinuria (Fig. 2b). The mercury-menthol group also exhibited moderate necrosis and degeneration of epithelial cells, along with moderate proteinuria (Fig. 2c). In contrast, most sections from the mercury-thymol group appeared normal, with no significant pathological alterations, aside from mild degeneration of the epithelial cells lining the urinary tubules (Fig. 2d).



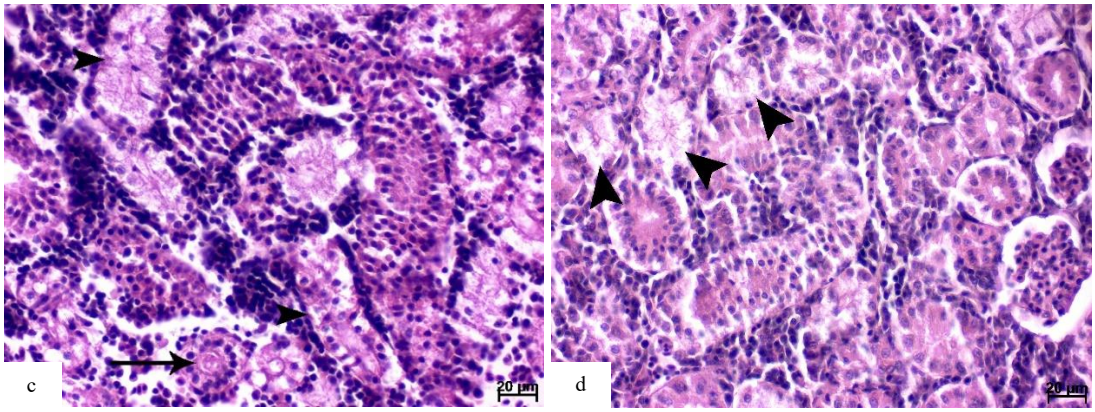


Fig. 2 Microscopical findings in kidney tissue in different groups. (a) Normal kidney tissue in the control group. (b) Severe epithelial cells necrosis in the urinary tubules (arrowheads) accompanied by mild multifocal nephritis (arrow) in the mercury group. (c) Degeneration of the epithelial cells of the urinary tubules (arrowheads) and proteinuria (arrow) in the mercury-menthol group. (d) Epithelial cells degeneration of the urinary tubules (arrowheads) in the mercury-thymol group, H&E.

DISCUSSION

Mercury is a pervasive and alarming environmental contaminant that significantly affects aquatic ecosystems around the globe. As a persistent bio accumulative toxin, mercury poses serious risks to fish populations, primarily due to their direct physiological interactions with contaminated water [26]. The results of the current study, in conjunction with existing literature, reveal consistent patterns of mercury-induced pathology across various fish species, while also underscoring critical interspecies differences in toxicological responses.

The liver plays a crucial role in fish physiology, characterized by its high metabolic rate and its functions in antioxidation and detoxification [5]. In contrast, the kidney is vital for osmoregulation [27]. As a result, the health of these organs is often assessed in toxicological studies. Research indicates that the liver and kidney tend to accumulate the highest concentrations of mercury in fish that are naturally exposed to ambient mercury levels [28]. This accumulation underscores the importance of these organs for histopathological examinations in the context of mercury exposure.

Previous research has demonstrated that exposure to mercury typically induces vacuolation in the liver tissue of zebrafish [29]. Hepatocyte lesions characterized by vacuolation can be categorized into two types: diffuse and focal vacuolation [30]. The presence of vacuoles resulting from heavy metal contamination is indicated by numerous small vacuoles appearing in the cytoplasm, which eventually merge to form a larger vacuole that displaces both the cytoplasm and nucleus toward the cell's periphery. Additionally, changes in the nucleus, such as chromatin condensation and increased nuclear optical density, have been observed [30]. In the kidneys, mercury exposure can lead to inflammation [31], interstitial inflammation, and necrosis of kidney tissue [32]. Furthermore, a study by Kaewamatawong *et al*. [33] found that tilapia exposed to 2 mg of mercury per kilogram for three days developed severe tubulonephrosis and exhibited hyaline casts in their kidney tissue.in the present study, we also obtained similar findings.

Excess levels of free radicals induce oxidative stress and trigger inflammation, which is harmful to living cells [34]. This imbalance can lead to cellular damage, contributing to various health issues in both aquatic and terrestrial organisms. As mercury has been found to induce severe oxidative stress and inflammation in fish [35], the observed pathological changes in the liver and kidney are likely related to the formation of free radicals and subsequent inflammatory responses. The detrimental effects of mercury on these vital organs underscore the importance of understanding how environmental toxins impact aquatic life.

On the other hand, thymol and menthol are phenolic compounds known for their antioxidant and anti-inflammatory functions [14, 17]. These compounds have garnered attention for their potential therapeutic benefits in mitigating the adverse effects of oxidative stress in fish. Dietary administration of thymol and menthol has been shown to effectively improve antioxidant capacity and suppress inflammation in different fish species [16, 17, 14, 6]. Although the antioxidant and anti-inflammatory effects of thymol and menthol have been studied in fish, their influence on tissue structure and histology remains underexplored. Recent studies have demonstrated that dietary thymol administration can significantly mitigate the pathological effects of zinc toxicity on liver and gill tissues in Nile tilapia (*Oreochromis niloticus*) [36]. Similarly, the dietary inclusion of essential oil from *Thymus vulgaris*, which is a rich source of thymol, has been shown to alleviate pathological damage to gill, kidney, liver, and spleen tissues in African catfish (*Clarias gariepinus*) exposed to thiamethoxam, a commonly used pesticide [37]. Furthermore, dietary menthol administration has been effective in reducing lesions in the gill, intestine, and liver of Nile tilapia exposed to chlorpyrifos, another hazardous chemical [22]. In all cases, the healthier tissues were accompanied by improved antioxidant capacity and reduced inflammation, emphasizing the notion that the benefits derived from thymol and menthol are closely linked to their antioxidant properties. This suggests that these compounds may play a critical role in maintaining cellular integrity and function in the face of environmental stressors. Thymol exposure disrupts zebrafish embryonic development in a dose-dependent manner [38]. Overall, it is concluded that menthol might be a good analgesic for this species, qualifying it as a substance of interest for prospective studies [39]. The present study investigated the protective effects of thymol and menthol against mercury-induced liver and kidney damage in zebrafish. Our results demonstrate that chronic exposure to mercury leads to significant histopathological alterations, consistent with previous reports. The observed lesions, such as necrosis and inflammation, are indicative of oxidative stress and cellular damage. The administration of thymol and menthol attenuated these changes, with thymol showing a more pronounced protective effect. These findings are consistent with existing literature on the antioxidant and anti-inflammatory properties of plant-derived compounds. However, the incomplete reversal of damage suggests that additional mechanisms or higher doses may be required for complete protection.

Overall, our study highlights the potential of thymol and menthol as natural agents for mitigating heavy metal toxicity in aquatic organisms, although further research is needed to optimize their protective effects.

In conclusion, our study demonstrates that thymol and menthol can partially protect zebrafish liver and kidney tissues from mercury-induced damage. Thymol exhibited a stronger protective effect than menthol. According to the aforementioned facts, it is speculated that the reduced pathological changes observed in the kidney and liver of zebrafish in the present study can be attributed to the antioxidant properties of thymol and menthol. By enhancing the body's natural defense mechanisms against oxidative stress, these compounds may help preserve tissue architecture and function, ultimately contributing to better overall health in fish exposed to toxic substances. These findings suggest that plant-derived compounds may offer a promising strategy for reducing the adverse effects of environmental pollutants in aquatic ecosystems. Further research is warranted to explore higher doses or combinations of these compounds for enhanced efficacy. More research is needed to understand exactly how thymol and menthol protect fish tissues. Additionally, their potential uses in fish farming and pollution control should be explored to help reduce the harmful effects of pollutants on aquatic ecosystems.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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