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Studying Protective Effects of Thymol, Curcumin and Menthol on Gill Histopathological Changes in Mercury-Exposed Juvenile Common Carp (*Cyprinus carpio*)

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ABSTRACT

Heavy metal pollution, particularly from mercury, poses a significant threat to aquaculture sustainability and food safety. This study investigated the protective efficacy of dietary thymol, curcumin, and menthol against mercuric chloride ($HgCl_2$)-induced gill histopathology in common carp (*Cyprinus carpio*). A total of 240 fish (50 ± 2 g) were distributed in a completely randomized design into eight treatments for 8 weeks: Control, Hg (0.44 mg/L $HgCl_2$ in water), Thymol (100 mg/kg feed), Curcumin (15 g/kg feed), Menthol (2.5 g/kg feed), and three co-exposure groups (Hg+Thymol, Hg+Curcumin, Hg+Menthol). Histopathological examination revealed severe lymphoplasmacytic proliferative branchitis, necrosis, and lamellar destruction in the Hg group. Thymol supplementation demonstrated the most potent protective effect, reducing lesions to mild branchitis and edema. Curcumin provided moderate protection, notably mitigating the severity of the injury. Conversely, menthol failed to alleviate mercury-induced pathology and, when administered alone, induced mild branchitis and aneurysms. The results indicate that the protective effects of these phytochemicals are compound-specific. Thymol is highly effective in preventing $HgCl_2$-induced gill damage, curcumin offers significant but moderate protection, while menthol is ineffective and potentially irritating at the tested dose. Thymol is recommended as a promising dietary supplement for enhancing carp resilience in mercury-contaminated aquaculture environments.

Keywords: Common carp, Dietary supplement, Gill histopathology, Mercuric chloride, Thymol.

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INTRODUCTION

Aquaculture has become a vital component of the global food system, currently producing approximately 12% of all aquatic food and serving as an essential source of high-quality animal protein, particularly in tropical and developing regions [1, 2]. However, the sustainable growth of this industry faces serious environmental challenges, with pollution emerging as a primary concern [3]. Among various pollutants, heavy metal contamination presents significant risks to both ecosystem health and food safety [2]. Industrial and agricultural activities continuously release heavy metals into aquatic environments, where these persistent pollutants accumulate in aquatic organisms [4-6]. In this situation, contaminants enter the human body by consuming infected fish [7]. This bioaccumulation process triggers oxidative stress, disrupts cellular functions, and can lead to serious health issues including neurotoxicity, organ damage, cardiovascular disorders, and increased cancer risk, even at relatively low exposure levels [4, 5]. Therefore, developing effective strategies to combat heavy metal pollution is crucial for protecting public health and ensuring sustainable aquaculture [8]. The common carp (*Cyprinus carpio*) holds global significance in aquaculture, ranking as the world's third most farmed finfish [9]. This species is valued not only for its excellent nutritional profile (rich in unsaturated fatty acids and low in saturated fats and cholesterol) but also for its ecological role as an 'environmental engineer' in pond ecosystems [9]. Despite its importance, carp are particularly vulnerable to accumulating waterborne contaminants like mercury, known for its high toxicity and environmental persistence [10]. Plant-based bioactive compounds offer considerable potential for reducing metal toxicity. Thymol, a natural monoterpene phenol from essential oils, demonstrates multiple beneficial properties in fish, including growth promotion, metabolic enhancement, and antioxidant, anti-inflammatory, and antimicrobial activities [11, 12]. It improves digestive efficiency, strengthens immune function, and enhances tissue antioxidant capacity, helping fish cope with environmental stressors [13]. Similarly, curcumin, the active component of turmeric, has shown remarkable hepatoprotective and antioxidant effects in various fish species, including common carp [14, 15]. Curcumin supplementation improves growth performance, enhances protein metabolism, reduces tissue damage, and decreases accumulation of toxins like chromium and ammonia [14, 16, 17]. It also fine-tunes gene expression, reducing inflammatory signals while boosting immune and growth-related genes [17]. Menthol,

commonly used as a fish anesthetic, has also shown protective potential against environmental toxins. Studies indicate it can reduce oxidative stress and strengthen antioxidant defenses in common carp exposed to ammonia [18, 19]. Given mercury's serious threat to aquaculture and the promising protective properties of these natural compounds, our study aimed to evaluate their effectiveness in common carp. We specifically investigated whether dietary thymol, curcumin, and menthol could reduce mercury's damaging effects on gill tissues, contributing to natural strategies for making farmed fish more resilient and safer.

MATERIALS AND METHODS

Fish and Experimental Nutrition

Fish were bought from a breeding facility in the province of Giulan. A total of 260 common carp (*Cyprinus carpio*) were transported to the Department of Aquatic Health, Aquatic Research Center, Faculty of Veterinary Medicine, University of Tehran. The fish's initial weight was recorded at 17.4±1.08 g on average. The fish were initially transferred to the 1000-L aquariums until 14 days of adaption of fish were completed [15]. Following the adaption period, the fish were harvested and examined for diseases. No external and internal parasites were observed in microscopical examinations. After acclimatization, 240 fish were randomly distributed into a completely randomized design with 8 treatments and 3 replicates each (10 fish per replicate). The experiment was conducted in 24 aquaria (125 L, 100 L water volume) for 8 weeks (56 days). The fish were fed a commercial diet (21 Beiza Co., Iran; 32-38% crude protein, 7-10% crude fat) twice daily at 1% of their body weight. Water quality parameters were maintained as follows: temperature 26.2 °C, pH 7.5, TDS 473 ppm, with continuous aeration and a 12h:12h light-dark cycle. A 50% water exchange was performed daily using dechlorinated tap water.

Experimental Design and Treatments

The experimental groups were designed as follows:

1. **Control:** Basic diet without $HgCl_2$ exposure
2. **Hg:** Basic diet + 0.44 mg/L $HgCl_2$ in water
3. **Thymol:** Basic diet + 100 mg thymol/kg feed
4. **Curcumin:** Basic diet + 15 g curcumin/kg feed
5. **Menthol:** Basic diet + 2.5 g menthol/kg feed
6. **Hg+Thymol:** 0.44 mg/L $HgCl_2$ + 100 mg thymol/kg feed
7. **Hg+Curcumin:** 0.44 mg/L $HgCl_2$ + 15 g curcumin/kg feed
8. **Hg+Menthol:** 0.44 mg/L $HgCl_2$ + 2.5 g menthol/kg feed

The $HgCl_2$ concentration was maintained at 0.44 mg/L by replenishing after daily water changes. Experimental diets were prepared by thoroughly mixing the compounds with the basal diet, adding distilled water (6 ml/10 g feed), extruding through a mincer, air-drying for 24 h, and refrigerating in sealed bags. Diets were prepared weekly to ensure freshness.

Sample Collection and Histopathological Analysis

At the end of the experimental period, fish were humanely euthanized followed by euthanasia via spinal cord transection. For histopathological examination, gill tissues were carefully dissected and immediately fixed in 10% neutral buffered formalin (NBF), routinely processed, dehydrated, embedded in paraffin wax, sectioned at 5 µm in thickness (Rotary Microtome RM2 145; Leica, Wetzlar, Germany) and stained with Haematoxylin and Eosin. Sections were examined using a light microscope (Motic, BA310 Epi-LED FL), and micrographs were taken using a camera (Tucsen, MIchrome 20).

Microscopic Examination and Scoring

Histopathological lesions in gill tissues were evaluated and scored semi-quantitatively based on severity: (-) normal, (+) mild, (++) moderate, (+++) severe. The analysis focused on histopathological indicators, including lymphoplasmacytic branchitis, edema, necrosis, and aneurysms.

RESULTS

Histopathological examination of gill tissues revealed distinct morphological alterations across the experimental groups, demonstrating the toxic impact of mercuric chloride ($HgCl_2$) and the varying protective efficacies of the dietary supplements. In histopathological examination, exposure to $HgCl_2$ alone induced the most severe gill injury. There was severe lymphoplasmacytic proliferative bronchitis and severe multifocal necrosis of the lamellar epithelium (Fig. 1).

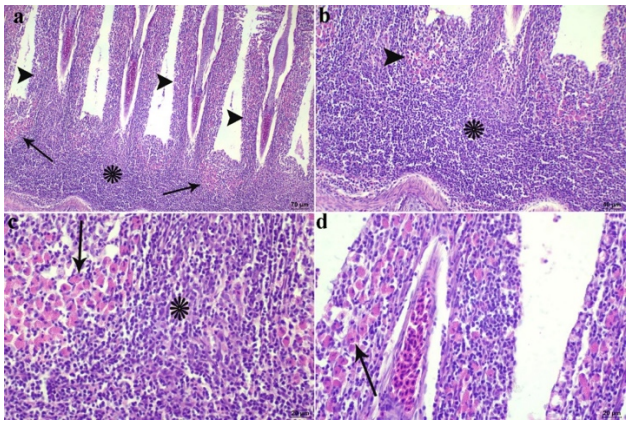

Fig. 1 (a-d) Histopathological findings of gills in the mercuric chloride-treated group. Note Severe lymphoplasmacytic branchitis (arrowheads) accompanied by focal necrosis (arrows) and diffuse infiltration of inflammatory cells in the base of lamellae, H&E.

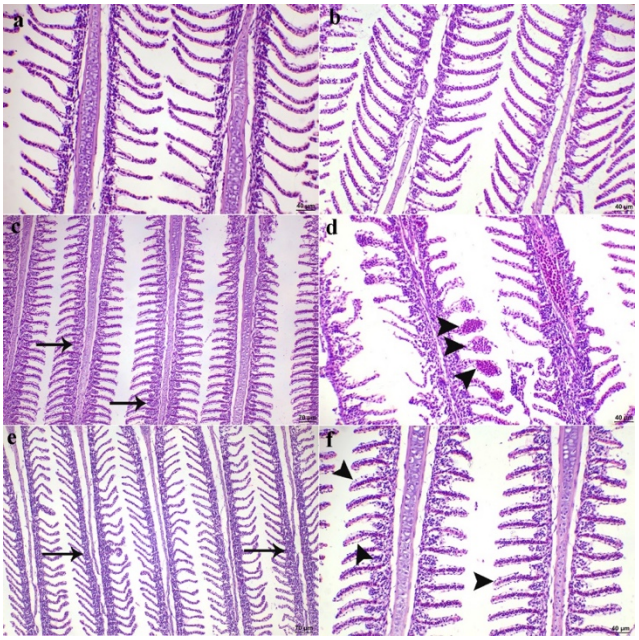

Fig. 2 Histopathological findings of gills in the Thymol, Curcumin, Menthol and Mercury-Thymol groups. (a-b): Normal gill histology in Thymol (a) or Curcumin (b) group. (c-d): Mild lymphoplasmacytic bronchitis (arrows) and aneurysm (arrowheads) in the Menthol-treated group. (e-f): Mild lymphoplasmacytic branchitis (arrows) and edema (arrowheads) in the Mercury-Thymol group, H&E.

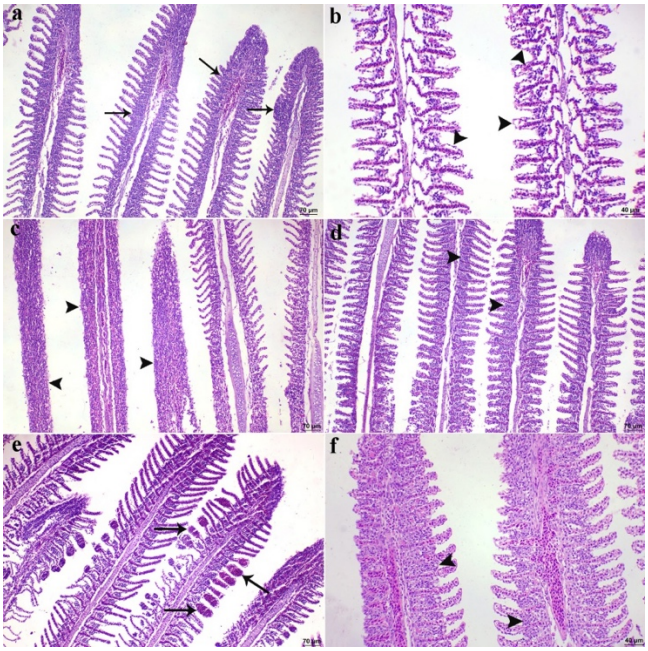

Fig. 3 Histopathological findings of gills in the Mercury-Curcumin and Mercury-Menthol groups. (a-b): Moderate lymphoplasmacytic bronchitis (arrows) and edema (arrowheads) in the Mercury-Curcumin group. (c-e): severe lymphoplasmacytic bronchitis (arrowheads) and aneurysm (arrows) in the Mercury-Menthol group, H&E.

Microscopically, the gill structure in the Control group appeared normal. Similarly, fish fed diets supplemented with Thymol or Curcumin exhibited normal gill histology, indicating no adverse effects from these compounds alone (Fig. 2a&b). In contrast, the Menthol-treated group showed mild lymphoplasmacytic bronchitis, aneurysm in several lamellar capillaries, and mild edema (Fig. 2c-d). The co-administration of bioactive compounds with mercury yielded differential protective outcomes. The Mercury-Thymol group showed a remarkable reduction in pathology, presenting only mild lymphoplasmacytic branchitis and mild lamellar edema (Fig. 2e-f). In the Mercury-Curcumin group, a moderate protective effect was observed, with lesions presenting as moderate lymphoplasmacytic branchitis and moderate edema (Fig. 3 a-b). Conversely, the Mercury-Menthol group exhibited moderate to severe lymphoplasmacytic proliferative branchitis, moderate necrosis, and aneurysms (Fig. 3c-e).

A semi-quantitative summary of the histopathological findings is presented in Table 4-1.

Table 4-1 Histopathological alterations in the gill tissue across different experimental groups. Lesion severity is scored as: Severe (+++), Moderate (++), Mild (+), Normal (-).

Group / Lesion	Branchitis	Edema	Necrosis	Aneurysm
Control	-	-	-	-
Mercury	+++	++	+++	++
Thymol	-	-	-	-
Curcumin	-	-	-	-
Menthol	+	+	+	+
Mercury+Thymol	+	+	-	-
Mercury+Curcumin	++	++	+	+
Mercury+Menthol	+++	++	++	+

The severity of gill histopathological alterations across experimental groups is presented in Figure 4. The mercury-exposed group showed the highest scores for all lesions, particularly branchitis and necrosis. Notably, co-treatment with thymol substantially mitigated mercury-induced damage, reducing all pathological scores to mild levels, whereas menthol provided minimal protection.

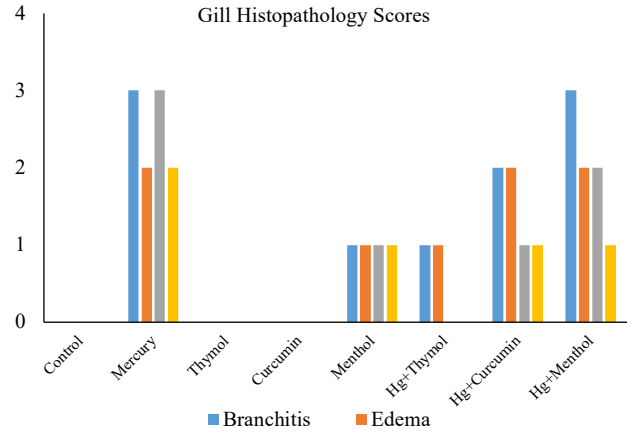

Fig. 4 Semi-quantitative scoring of gill histopathological alterations. Mercury chloride exposure caused severe injury, while thymol supplementation provided the most effective protection, reducing lesion severity to mild levels.

DISCUSSION

Heavy metal pollution now threatens human health. Mercury is one of the most important environmental pollutants, the presence of which in the environment can be of concern to aquatic organisms and humans [20]. Exposure to mercuric chloride (HgCl) causes immunosuppressive and hematological changes in fish, resulting in significant biological damage even at low concentrations, highlighting its threat to aquaculture and the need for effective monitoring [6]. The results of the present study clearly demonstrate that thymol and curcumin, unlike menthol, effectively protect common carp gills from mercury-induced damage. These findings reveal important differences in how plant-derived compounds interact with toxic metals in aquatic organisms. In this study, severe lymphoplasmacytic branchitis and necrosis were observed in mercury-exposed fish. This aligns with established research showing gills as primary targets for waterborne metals [21, 22]. The gill's direct contact with contaminated water makes it particularly vulnerable to toxic insults [23].

Thymol emerged as the most effective protective agent in our study. Fish receiving thymol supplementation alongside mercury showed only mild lymphoplasmacytic branchitis, representing remarkable protection against mercury's severe effects. The strong protective effect of thymol observed in present study aligns with its known antioxidant and anti-inflammatory properties [24, 25], which appear to effectively counteract mercury-induced damage, as evidenced by the remarkable reduction in gill histopathology.

Other studies have also shown thymol's beneficial effects in mercury-exposed fish, including improved antioxidant status in brain tissue [26] and enhanced growth performance [13]. As the present study showed, using a dose of 100 mg/kg seems optimal, as higher concentrations of thymol can cause side effects.

Curcumin provided significant but moderate protection against mercury damage. While it reduced lesion severity from severe to moderate, it couldn't prevent damage completely. This partial protection may be linked to curcumin's well-documented antioxidant properties, which are often associated with the activation of pathways like Nrf2 in other species [14,17]. Curcumin's metal-binding ability might also reduce mercury's biological activity [15]. However, curcumin's effects are tissue-dependent, which could explain why it offered only partial gill protection despite its known antioxidant capacity [27].

Contrary to expectation, menthol failed to protect gill tissue and even caused mild injury when administered alone. The 2.5 g/kg dose appears problematic for gill, potentially acting as an irritant to this tissue. Although menthol shows benefits in other contexts-reducing ammonia toxicity [18], enhancing immunity [28], and serving as an anesthetic [29, 30], its interaction with gill tissue seems unfavorable. Menthol's high tissue penetration may result in a failure to reduce mercury-induced inflammation in gills [31]. While menthol has shown protective effects in other organs [11]. Our results reveal that compound efficacy varies significantly between tissues.

In summary, present study demonstrates that natural compounds vary greatly in their ability to protect fish from environmental toxins. Thymol shows excellent potential as a dietary supplement for aquaculture operations facing mercury contamination challenges. Curcumin offers moderate benefits, while menthol appears unsuitable for gill protection at tested dose. These results highlight the need for compound-specific and tissue-specific safety evaluations in aquaculture applications. Future research should explore the molecular mechanisms behind thymol's superior performance and curcumin's partial protection to optimize their use in sustainable aquaculture practices.

CONCLUSION

This study demonstrates that the protective effects of dietary phytochemicals against mercury-induced gill toxicity in common carp are compound-specific. Histopathological analysis revealed that thymol provided superior protection, nearly preventing tissue damage, likely due to its potent antioxidant and anti-inflammatory properties. Curcumin offered moderate protection, while menthol was ineffective and exhibited intrinsic irritant properties at the tested dose. These findings recommend thymol as a practical dietary supplement for aquaculture in mercury-impacted environments and underscore the necessity for a compound-specific assessment of phytochemical safety and efficacy.

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.

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Conflicts of Interest

The authors have not declared any conflict of interests.

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