Hepatoprotective effects of dietary Artemisia (Artemisia annua) leaf extract on common carp (Cyprinus carpio) exposed to ambient ammonia

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ABSTRACT

In this study, antioxidant capacity of Artemisia (Artemisia annua) leaf extract (AE) were assessed, in vitro, followed by evaluation of dietary AE administration on hepatic health of common carp (Cyprinus carpio), during ammonia exposure. For this, common carp juveniles were fed diets supplemented with 0, 0.5, 1 and 2 g kg−1 AE for 30 days and then, exposed to 0.5 mg L−1 water unionized ammonia nitrogen for 24 h. Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and total protein, as well as hepatic levels of malondialdehyde (MDA), and activity and gene expression of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were monitored. The results showed that AE had antioxidant properties, in vitro. Moreover, Dietary AE administration had no significant effects on plasma ALT, AST and ALP activities before ammonia exposure, but mitigated the enzymes elevation after the ammonia exposure. Dietary AE administration significantly increased plasma total protein before the ammonia exposure and mitigated/inhibited the ammonia-induced hypoproteinemia. In addition, the fish fed with the AE-supplemented diets showed higher hepatic antioxidant enzyme activity and gene expression and lower hepatic MDA levels, before the ammonia exposure. AE significantly mitigated/inhibited the ammonia-induced increase in hepatic antioxidant enzymes activities and gene expressions as well as hepatic MDA levels. In conclusion, AE is suitable antioxidant agent and can be used as feed additive to prevent hepatotoxicity of common carp during ammonia exposure.

1. Introduction

One of the risks in aquaculture practice is water ammonia elevation and fish toxicity (Randall and Tsui, 2002; Hoseini et al., 2019b). Ammonia is the main nitrogenous waste compound of fish, which is very toxic to aquatic organisms and high stocking density, poor water exchange and overfeeding lead to ammonia accumulation in water (Rajabiesterabadi et al., 2020a). Liver is one of the targets of ammonia toxicity, which faces oxidative stress and hepatocyte damage. A huge body of evidence shows that higher ambient ammonia is hepatotoxic in common carp, Cyprinus carpio (Peyghan and Takamy, 2002; Rama and Manjabhat, 2014), grass carp, Ctenopharyngodon idella (Jin et al., 2017), yellow catfish, Pelteobagrus fulvidraco (Zhang et al., 2018), and Brazilian flounder, Paralichthys orbignyanus (Maltez et al., 2017). These studies have shown that ammonia exposure induced hepatic oxidative stress and change in antioxidant enzymes activity, and elevation in circulating levels of certain hepatic enzymes, including alanine aminotransferase (ALT), aspartate amino transferases (AST), and alkaline phosphatase (ALP).

Considering the important roles of the liver in living organisms, including metabolism, protein synthesize, etc., it is necessary to apply methods for suppressing ammonia toxicity in fish liver. In this regard, strengthening antioxidant system to prevent oxidative damage may be useful method to mitigate hepatotoxicity during ammonia exposure. One of the useful methods to augment fish antioxidant system is dietary supplementation with antioxidant agents, and, among them, herbal compounds have gained a great attention recently (Abdel-Tawwab et al., 2018; Yousefi et al., 2019; Dawood et al., 2020; Yousefi et al., 2020). A huge body of evidence has suggested that dietary herbal additives, such as ginger (Fazelan et al., 2020a), myrcene (Hoseini et al., 2020; Khalili et al., 2020), and eucalyptol (Hoseini et al., 2018a; Fazelan et al., 2020b), are able to augment fish health during toxicant exposure. Moreover, studies have shown that dietary supplementation with various herbal ingredients such as 1,8-cineole (Taheri Mirghaed et al., 2018; Taheri Mirghaed et al., 2019), myrcene (Hoseini et al., 2019a), leaf extract of olive (Rajabiesterabadi et al., 2020a) and

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moringa (Kaleo et al., 2019) mitigated oxidative stress and hepato-toxicity induced by ammonia in fish and crustaceans.

Artemisia (Artemisia annua) is a medicinal plant, grown in various regions of the world. The plant leaves contain phenolic compounds and flavonoids, which induce antioxidant effects (Iqbal et al., 2012; Ćavar et al., 2012). In vitro assays have shown that Artemisia leaf extract (AE) had antioxidant capacity, which was higher than that of thymol (Cavar et al., 2012). In vivo studies in broilers have indicated that AE improve antioxidant strength of the animals under normal or stressful conditions (Wan et al., 2016; Wan et al., 2018). However, little is known about the effects of AE on fish. In this regard, feeding rainbow trout, Oncorhynchus mykiss, is a well-known warm-water aquaculture species, which is cultured either extensively in earthen ponds or intensively in tanks (Hoseini et al., 2012). It is one of the main cyprinid species in aquaculture sector, with global production of > 4 million tons in 2017. Therefore, finding methods for mitigating ammonia toxicity is very important in this species.

According to above, it was hypothesized if dietary supplementation with AE may improve hepatic health of common carp in response to ammonia toxicity. For this, plasma ALT, AST, ALP and total protein (as hepatic function tests) and liver MDA levels and SOD, CAT and GPx activity and gene expressions (as hepatic antioxidant status) were determined.

### 2. Materials and methods

#### 2.1. Diets

Four diets were used in this experiment (Table 1): control, control + 0.5 g kg⁻¹ AE (AE0.5), control + 1 g kg⁻¹ AE (AE1), and control + 2 g kg⁻¹ AE (AE2). The feedstuffs were mixed together and transformed to dough by moisturizing. To make feed pellets, the dough was formed to dough by moisturizing. To make feed pellets, the dough was kept at −70 °C for further analysis.

<table>
<thead>
<tr>
<th>Feedstuffs</th>
<th>Control</th>
<th>AE0.5</th>
<th>AE1</th>
<th>AE2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fishmeal</td>
<td>160</td>
<td>160</td>
<td>160</td>
<td>160</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Poultry by-product</td>
<td>130</td>
<td>130</td>
<td>130</td>
<td>130</td>
</tr>
<tr>
<td>Wheat meal</td>
<td>453</td>
<td>452.5</td>
<td>452</td>
<td>451</td>
</tr>
<tr>
<td>Fish oil</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Lysine</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Methionine</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

#### 2.2. Feeding and ammonia challenge

All parts of the study were conducted under a protocol approved by the committee of ethics of the faculty of sciences of the University of Tehran (357; 8 November 2000). Common carp with average weight of 59.6 g were stocked in 12 tanks (100 L) at a density of ~9 g L⁻¹ (15 fish per tank). The tanks were aerated and water flow rate was 0.5 L min⁻¹. After a week of acclimation and feeding with the control diet, the fish were fed either of the above-mentioned diets for 30 days (three tanks per diet). Daily feeding was performed based on 2% of biomass divided into two meals. After two weeks of feeding, the tanks' biomass was recorded to adjust the feed amount. After 30 days of feeding, blood and liver samples were taken from all the tanks; then water flow was ceased and the fish were exposed to 0.5 mg L⁻¹ un-ionized ammonia nitrogen [as described by Taheri Mirghaed et al., 2018] for a period of 24 h. After the ammonia challenge, the second blood and liver samples were taken from all tanks. Water temperature (23.8 ± 1.13 °C), dissolved oxygen (6.39 ± 0.87 mg L⁻¹), pH (7.66 ± 0.33) and un-ionized ammonia nitrogen (0.012 ± 0.003 mg L⁻¹) were measured twice a week as described by Rajabiesterabadi et al. (2020b).

#### 2.3. Sampling and biochemical analyses

At each sampling time, two fish were netted from each tanks and anesthetized by 100 mg L⁻¹ eugenol. Blood samples were taken with heparinized syringes from caudal vein and collected in plastic tubes. Then, the fish were killed by a sharp blow on the head and liver samples were taken and frozen in liquid nitrogen. The liver samples were then kept at −70 °C for further analysis.

The blood plasma was separated after centrifugation at 4 °C for 10 min (1200 g). Plasma ALT, AST, ALP and total protein were measured using commercial kits (Pars Azmun Co., Tehran Iran) as described before (Ghelichpour et al., 2019). Briefly, for ALT and AST, 100 μL of the samples were mixed with 1 mL of reaction mixture and average of fall in optical density was recorded after 3 min at 340 nm. For ALP, 20 μL of the samples were mixed with 1 mL of reaction mixture and average of fall in optical density was recorded after 3 min at 490 nm. Liver samples were homogenized in Tris-EDTA buffer on ice as
described before (Hoseini and Yousefi, 2019). The homogenized products were centrifuged at 4 °C for 30 min and resultant supernatants were used for antioxidant assays. SOD activity was determined based on the sample ability to prevent reduction in cytochrome c as described by McCord and Fridovich (1969). In a reaction mixture, superoxide radical was created by xantine reaction with oxygen. The rate of cytochrome c reduction inhibition was measured at 550 nm. CAT activity was measured based on decomposition rate of hydrogen peroxide (Goth, 1991). Hydrogen peroxide was diluted in phosphate buffered saline to reach optical density of 0.780 at 405 nm. 200 μL of the samples were mixed with 1 mL of hydrogen peroxide dilution and the reaction was stopped after 1 min by adding 1 mL of ammonium molybdate solution. GPx activity was determined based on Flohé and Günsler (1984) using a commercial kit (Zellbio, Berlin, Germany). MDA content was determined according to Buege and Aust (1978) method. 100 μL of the sample was added to 0.5 mL of reaction mixture [HCl (0.25 N), trichloroacetic acid (15%), thiobarbituric acid (0.375%), BHT (0.01%)] and incubated for 15 min at 95 °C. Optical density was measured at 535 nm.

2.4. Gene expression

RNA of the liver samples was extracted using a commercial kit (RNX-plus kit; Sinagene, Iran); Dnase I (Fermentas, Lithuania) was applied to avoid DNA contamination. After synthesizing complementary DNA (cDNA) by a commercial kit (Fermentas, Lithuania), expression of superoxide dismutase (sod), catalase (cat), and glutathione peroxidase (gpx) gene (Table 2) were determined by measuring optical density using SYBR green dye and normalization based on a housekeeping gene (beta-actin). DDCt method was used to quantify the gene expressions (Hoseinifar et al., 2019).

2.5. Statistical analysis

Data of antioxidant activity of AE were analyzed by t-test. Other data were subjected to two-way ANOVA with sampling time and dietary AE as the factors. Non-normally distributed (plasma AST and ALP) and non-homogeneity variances (hepatic SOD) were log-transformed before ANOVA. Since there were significant interaction effects of the sampling time and dietary AE on all tested parameters, the data were re-analyzed via one-way ANOVA and Duncan tests. Significant differences were judged based on P < .05 and all analyses were performed in SPSS v.22.

3. Results

Antioxidant capacity of AE is presented in Table 3. At all concentrations, BHT had higher antioxidant capacity than AE. There were interaction effects of dietary AE and ammonia exposure on plasma ALT (P = .003), AST (P = .006) and ALP (P = .050) activities (Fig. 1). There were no significant differences in the enzymes plasmatic activities among the dietary treatments, before ammonia exposure. Exposure to water ammonia significantly increased the activity of the enzymes; however, the control group showed highest increases.

Dietary AE levels and ammonia exposure had interaction effects on plasma total protein (P = .005) and MDA (P < .001) levels (Fig. 2). Before ammonia exposure, the AE-treated fish had similar plasma total protein levels, which were significantly higher than the control fish. However, after the ammonia exposure, the lowest and highest plasma total protein levels were observed in the control and AE2 treatment, respectively. Ammonia exposure significantly decreased plasma total protein levels in the control and AE1 treatment, but not AE2. The control group had significantly higher MDA levels compared to the AE1 and AE2 treatments, before ammonia exposure. Moreover, exposure to water ammonia significantly increased plasma MDA levels in the control and AE1, but not AE2 treatments. The highest plasma MDA levels after ammonia exposure was related to the control group.

Dietary AE levels and exposure to water ammonia showed interaction effects on hepatic CAT activity (P = .001) and gene expression (P = .050) (Fig. 3). Before ammonia exposure, the AE1 and AE2 treatments had significantly higher SOD activity and gene expression compared to the control group; the highest gene expression was observed in the AE2 group. After ammonia exposure, hepatic CAT activity and gene expression significantly increased in the control, but not AE1 and AE2 treatments. Moreover, hepatic SOD gene expression significantly increased in the control and AE1, but not AE2 treatment.

Dietary AE levels and exposure to water ammonia showed interaction effects on hepatic GPx activity (P = .001) and gene expression (P = .030) (Fig. 4). Before ammonia exposure, the AE1 and AE2 treatments had significantly higher CAT activity and gene expression compared to the control group. After ammonia exposure, hepatic CAT activity and gene expression significantly increased in the control, but not AE1 and AE2 treatments. Dietary AE levels and exposure to water ammonia showed interaction effects on hepatic GPx activity (P = .002) and gene expression (P < .001) (Fig. 5). Before ammonia exposure, the AE1 and AE2 treatments had significantly higher GPx activity compared to the control group. Ammonia exposure significantly increased hepatic GPx activity in the control, but not AE1 and AE2 treatments. The AE2 treatment had significantly higher GPx gene expression compared to the control and AE1 treatments, before ammonia exposure. Ammonia exposure significantly increased GPx gene expression in the control and AE1, but not AE2 treatment.

4. Discussion

Ammonia toxicity is a major threat to aquaculture practice, which occurs when ambient ammonia levels increases; under this situation, internal ammonia excretion is impaired that lead hyperammoniema (Ip et al., 2004). The fish liver is a target of internal ammonia accumulation as it is the main site for ammonia detoxification (Peyghan and Takamy, 2002).

ALT, AST and ALP are enzymes with high concentration in hepatocytes that are leaked into circulation during hepatocyte damage. The present results were similar to previous studies on common carp during ammonia exposure, for example, Peyghan and Takamy (2002) found

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequences</th>
<th>Accession number</th>
<th>Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>beta-actin</td>
<td>F: CCTGATGCCAACACCGTGGCTG</td>
<td>JQ619774.1</td>
<td>98%</td>
</tr>
<tr>
<td>sod</td>
<td>F: TGGAGTCTGGGAGGAGGAGCAGA</td>
<td>XM_019111527.1</td>
<td>97%</td>
</tr>
<tr>
<td>cat</td>
<td>F: TTGTTGCCACATGACGACGAC</td>
<td>GQ376154.1</td>
<td>99%</td>
</tr>
<tr>
<td>gpx</td>
<td>F: CTCAACAGGGAAGTGGCAGAAGTG</td>
<td>XM_019093635.1</td>
<td>99%</td>
</tr>
</tbody>
</table>
hepatic injuries and elevation in circulating levels of ALP in common carp exposed to ammonia. Moreover, Rama and Manjabhat (2014) reported significant elevation in plasma ALT and AST activities of common carp following 96 h exposure to ammonia. Moreover, higher plasma ALP and AST activities in fish under stressful conditions might be due to hemolysis and release of the enzymes from erythrocyte intro circulation (Taheri Mirghaed et al., 2017); in this regard, previous studies have shown ammonia exposure decreased erythrocyte number and hemoglobin levels, which might be indications of hemolysis (Yang et al., 2010; Hoseini et al., 2019a). The present results showed that dietary AE supplementation, although had no significant effects on plasma ALT, AST and ALP activities, significantly mitigated the enzymes activity after ammonia exposure. These results suggest AE had hepatoprotective effects in common carp exposed to ammonia. Similar to the present findings, dietary supplementation with myrcene or 1,8-cineole significantly mitigated ammonia-induced elevation in ALT, AST and ALP activities in plasma of common carp (Taheri Mirghaed et al., 2018; Hoseini et al., 2019a).

Plasma total protein level indicates fish health; moreover, as most of plasma proteins are synthetized in the liver, it is indicator of liver function, too. AE administration significantly increased plasma total protein levels, which indicates higher health of the fish. The results are in line with previous studies on common carp that showed dietary administration of herbal ingredients, such as 1,8-cineole (Hoseini et al., 2018b), Ocimum basilicum extract (Amirkhani and Firouzbakhsh, 2015) and lavender extract (Yousefi et al., 2020), significantly increased plasma total protein levels. Decrease in plasma total protein indicates liver dysfunction and altered protein synthesis probably due to hepatocyte damage. Similar to the present study, exposure of crucian carp, Carassius auratus (Ren et al., 2016) and yellow catfish (Zhang et al., 2018) led to significant decrease in plasma total protein levels. Moreover, the results of plasma total protein were in line with the ALT, AST and ALP activity, as dietary AE administration significantly mitigated/prevented hypoproteinemia after ammonia exposure.

Fish antioxidant system is responsible for protecting fish against oxidative conditions such as ammonia exposure. SOD detoxifies superoxide ion by decomposing it into hydrogen peroxide, the product that is decomposed to water and oxygen by CAT and GPx. The present

<table>
<thead>
<tr>
<th>Concentrations (μg mL⁻¹)</th>
<th>12.5</th>
<th>25</th>
<th>50</th>
<th>75</th>
<th>100</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>AE</td>
<td>13.9 ± 0.61</td>
<td>18.1 ± 1.30</td>
<td>24.3 ± 1.23</td>
<td>35.1 ± 1.85</td>
<td>55.3 ± 3.77</td>
<td>79.0 ± 3.67</td>
</tr>
<tr>
<td>BHT</td>
<td>21.9 ± 0.91***</td>
<td>49.9 ± 1.45***</td>
<td>50.2 ± 2.59***</td>
<td>85.0 ± 3.18***</td>
<td>90.2 ± 1.77***</td>
<td>92.8 ± 1.15**</td>
</tr>
</tbody>
</table>

Fig. 1. Effects of dietary AE levels and ammonia exposure on plasma ALT, AST and ALP activity (mean ± SE) in common carp. Different letters above the bars show significant differences among the treatment.
study showed AE had antioxidant activity, although lower than BHT. Similarly, (Ćavar et al., 2012) found antioxidant activity of AE collected from Bosnia. AE stimulated antioxidant system of common carp and lowers oxidative stress in the present study. These results are partially in line with the previous studies on common carp, showing dietary AE supplementation significantly increased blood CAT and GPx activity and decreased blood MDA levels, with no significant effects on SOD activity (Sarhadi et al., 2020). Moreover, other dietary herbal ingredients such as eucalyptol (Fazelan et al., 2020b), myrcene (Hoseini et al., 2020; Khalili et al., 2020), ginger (Fazelan et al., 2020a), palm
fruit extract (Hoseinifar et al., 2017) and turmeric (Giri et al., 2019) significantly stimulated antioxidant system of common carp. It is well-known that ammonia toxicity induces oxidative stress in fish. For example, Hegazi et al. (2010) showed that ammonia toxicity significantly increase hepatic SOD, CAT, GPx activity and MDA levels in Nile tilapia, Oreochromis niloticus. Similarly, in yellow catfish, Zhang et al. (2018) reported that ammonia toxicity significantly increased plasma antioxidant enzymes activity and MDA levels along with up-regulation in hepatic antioxidant enzymes gene expression. Similar results were obtained in the present study; however, dietary AE supplementation mitigated (AE1) or inhibited (AE2) oxidative stress and changes in antioxidant enzymes activity. This effect might be due to pre-activation of antioxidant system, as AE-treated fish had higher antioxidant enzymes activity before ammonia exposure or due to antioxidant compounds presented in AE. Similarly, dietary supplementation with 1,8-cineole for two weeks mitigated oxidative stress caused by a 24-h ammonia exposure period in common carp (Tahteri Mirghaed et al., 2019). Likewise, 30 days feeding with a diet supplemented with myrcene significantly suppressed oxidative stress caused by a 24-h ammonia exposure period in common carp (Hoseini et al., 2019a). Long-term feeding of common carp with a diet supplemented with olive leaf extract significantly mitigated plasma MDA elevation after a 3-h ammonia exposure period (Rajabiesterabadi et al., 2020a).

In conclusion, dietary AE is a potential antioxidant agent and dietary supplementation at 2 g kg⁻¹ is hepatoprotective in common carp during ammonia exposure. It seems that antioxidant system stimulation by AE prevented oxidative stress in carp during ammonia exposure, which in turn, protected hepatocytes against free radicals. This leads to lower plasma levels of hepatic enzymes and higher protein levels during ammonia exposure. Therefore, this extract can be used as feed additive during the periods with high risks of ammonia toxicity (i.e. high stocking density and transportation).

**References**


