Hematological Parameters of Red Tilapia (Oreochromis sp.) Fed Essential Oils of Mentha piperita after Challenge with Streptococcus agalactiae

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ABSTRACT

The study focused on investigating the effectiveness of essential oils of peppermint (Mentha piperita) against Streptococcus agalactiae (bacteria causing hemorrhagic disease) in red tilapia with an infective concentration of 10⁶ CFU/mL. The experiment was arranged with 2 control treatments: control 1 - commercial feed + no infection; control 2 - commercial feed + infection of S. agalactiae; and 3 experimental treatments with essential oils concentrations of 0.125%; 0.25% and 0.5%. The study examined the blood cell morphology and physiological blood indices of red tilapia (including hemoglobin; hematocrit; red blood cells count; the total number of white blood cells and thrombocytes, erythrocyte size) at three stages: after 15 days of adding essential oils without infection; five days after infection; and ten days after infection. In this study, fish supplemented with peppermint essential oils stimulated the body to create immunity. However, concentrations of 0.125% and 0.25% showed higher results about this ability in the presence of bacteria while the concentration of 0.5% exhibited a higher antibacterial effect of essential oils.

INTRODUCTION

Tilapia farming is the most popular type of aquaculture globally, with production reported in at least 145 countries and continents (FAO, 2020). Tilapia is an ideal fish for farming because of the relatively short culture period (about six months), good tolerance to a poor stocking environment, high productivity rate and high nutritional values (Miao and Wang, 2020; Mjoun et al., 2010). Although the origin of tilapia is Africa, Asia has dominated its production ever since the fish was introduced to aquaculture. The two most cultured tilapia species in Asia are Nile tilapia (Oreochromis niloticus) and red tilapia (Oreochromis sp.), a hybrid between Oreochromis mossambicus and O. niloticus (Romaña-Eguia et al., 2004). In Vietnam, red tilapia is farmed most at Mekong Delta provinces, with suitable climatic and hydrological conditions.

Streptococcal infection is one of the most critical diseases globally, which heavily reduces marketable products and decreases production and processing efficiency. The two significant bacteria species that affect fish production are Streptococcus iniae and S. agalactiae; however, S. agalactiae was a more prevalent factor in tilapia (Neil Wendover et al., 2011).

The immunoprophylactic methods, including vaccination and immunostimulation, have become famous for activating the immune system and protecting the host from pathogens. Prevention is better than cure. While antibiotics for treatment have faced criticism because of their negative impacts, plant-based immunostimulants provide safe and beneficial effects on the immune response against fish diseases (Cabello, 2006; Citarasu, 2010).

Mentha piperita L. is peppermint, the natural hybrid between M. aquatica L. and M. spicata L. belongs to Lamiaceae. It is an aromatic perennial herb whose oil is one of the most widely used essential oils in the flavoring of...
pharmaceuticals and preparations for cough syrup, chewing gum, oral care or beverages. In addition, its oil has been reported to have antibacterial, antiviral, antiparasitic and antifungal activities (Pushpangadan and Tewari, 2006). The antibiotic activity of peppermint oil was reported by many in vitro studies (McKay and Blumberg, 2006). In in vivo studies, the addition of essential oils to fish diet revealed high growth performance, enhanced hematological parameters and immune response (Adel et al., 2015). Besides, peppermint oils supplemented diet showed a higher survival rate and increased white blood cells in infected fish compared with control (de Souza Silva et al., 2019).

This study aimed to evaluate the antibacterial ability of \textit{S. agalactiae} of red tilapia fed with the dietary peppermint essential oil supplemented before and after infection.

**MATERIALS AND METHODS**

**Biological material and experimental design**

Three hundred healthy red tilapia (about 2.5 months of age) were purchased from the National Breeding Center for Southern Freshwater Aquaculture in Tien Giang province, Vietnam. Fish were stocked in a tank with 200 cm × 100 cm × 80 cm of each. The tanks were aerated continuously 24 h a day. The water used was tap water that has been dechlorinated; changed twice a week, each time changing 2/3 of the water and the temperature was checked continuously 24h a day. The water used was tap water that has been dechlorinated; changed twice a week, each time changing 2/3 of the water and the temperature was checked daily to avoid poor water quality causing stress for the fish.

Fish were randomly distributed in 5 tanks (60 fish per tank), including two controls (control 1: commercial feed + no infection; control 2: commercial feed + infection) and three experimental lots with supplemented diet at the concentration of Mentha 0.125%, Mentha 0.25% and Mentha 0.5%. After being brought back from the hatchery, the concentration of Mentha 0.125%; Mentha 0.25% and Mentha 0.5% (9.20±1.14 g%) was higher when compared

**Experimental diet preparation and feeding**

Commercial fish feed was mixed well with essential oil at studied concentrations and used a specialized pellet machine to make feed pellets. Fish were fed twice a day at the rate of 5% of body weight (Alsaid et al., 2010).

The used essential oils of peppermint (\textit{Mentha piperita}) is a commercial one produced by Heber Vietnam Co., Ltd. The essential oil compounds of \textit{M. piperita} include \textit{α-Pinene} 1.8%, \textit{Sabinene} 0.2%, \textit{β-Pinene} 1.4%, \textit{β-Myrccene} 0.3%, \textit{3-Octanol} 0.5%, \textit{Limonene} 4.3%, \textit{Isopulegol} 0.9%, \textit{Isomenthol} 4.8%, \textit{Menthone} 30.3%, \textit{Menthol} 32.8%, \textit{α-Terpineol} 1.9%, \textit{Menthyl acetate} 5.6%, \textit{Piperitone} 0.9%, \textit{β-Caryophyllen} 0.5% and not identified compounds 2.3%, 11.6%.

**Hematological analysis and determine RBC size**

For hematological analysis, 15 fish per experimental unit blood was withdrawn from the caudal vein using syringes with a drop of 10% EDTA. The number of red blood cells (RBC) was counted on the Neubauer counting chamber. The total count of white blood cells (WBC) and thrombocytes was performed through the consumption of Giemsa staining. Hemoglobin index is determined using Sahl hematocrit. Hematocrit index was determined by blood centrifugation and measurement of red blood cells/plasma sedimentation ratio.

The RBC count was calculated according to Natt and Herrick (1952) by the equation:

$$RBC = A \times \frac{5 \times 10^{20}}{200} \text{ (cells/mm}^3\text{)},$$

where \(A\) is the total number of red blood cells in the five-count zones.

The WBC and thrombocyte count was calculated according to Herrick et al. (2000):

$$\text{The total WBC and thrombo}y\text{te} = \frac{(\text{number of WBC of } 1500 \text{ cells} \times R)}{1500},$$

where \(R\) is the number of red blood cells in 1 mm\(^3\) of blood.

Fish RBC size was determined from Giemsa staining slides on the microscope and connected with S-EYE software.

**Challenge with \textit{Streptococcus agalactiae}**

Fish were infected by the soaking method (Nguyen et al., 2001). Place 2L of bacterial suspension with a density of 10\(^7\) CFU/mL in a tank containing 18L of dechlorinated water, stir well to obtain 20L of bacteria at a density of 10\(^8\) CFU/mL. Drop a group of 20 fish into the tank, soak for 60 minutes, then take it out and transfer it to the old tank. Do the same with the rest of the treatments.

**Statistical analysis**

The results were submitted to ANOVA one way (p<0.05) using Minitab 18. The significant difference between treatments was determined by the Turkey test (p<0.05). The mean data were presented as \(\bar{X} \pm SD\) (means ± standard deviation).

**RESULTS AND DISCUSSION**

Table I shows the effect of feeding essential oil of peppermint for 15 days on hematological parameters of red tilapia.

The Hb concentration in fish supplemented with \textit{Mentha 0.5%} (9.20±1.14 g%) was higher when compared
Table I. Effect of feeding essential oil of peppermint (Mentha piperita) in different concentrations for 15 days on hematological parameters (X± SD) of red tilapia.

<table>
<thead>
<tr>
<th>Hematological parameters</th>
<th>Control 1</th>
<th>Mentha 0.125%</th>
<th>Mentha 0.25%</th>
<th>Mentha 0.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hct (%)</td>
<td>38.25±5.16a</td>
<td>36.26±4.03b</td>
<td>36.67±6.03b</td>
<td>39.56±4.26b</td>
</tr>
<tr>
<td>Hb (g%)</td>
<td>7.83±0.82a</td>
<td>7.24±1.10b</td>
<td>7.51±0.88b</td>
<td>9.20±1.14b</td>
</tr>
<tr>
<td>RBC (×10^6/mm³)</td>
<td>1.28±0.27a</td>
<td>1.12±0.31b</td>
<td>1.08±0.18b</td>
<td>1.41±0.29b</td>
</tr>
<tr>
<td>WBC and thrombocytes (×10^6/mm³)</td>
<td>4.23±1.77a</td>
<td>3.40±1.43b</td>
<td>3.57±1.20b</td>
<td>5.18±2.47b</td>
</tr>
</tbody>
</table>

a, b, c, The difference is statistically significant (p<0.05).
Table II. Hematological parameters and size of erythrocyte (SOE) ($X \pm SD$) of red tilapia after five days and ten days of *S. agalactiae* infection.

<table>
<thead>
<tr>
<th>Hematological parameters</th>
<th>Control 1</th>
<th>Control 2</th>
<th>After five days</th>
<th>After ten days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hct (%)</td>
<td>38.25±5.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40.37±5.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36.67±6.03&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>39.56±4.26&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hb (g%)</td>
<td>7.83±0.82&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.06±0.99&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38.49±3.74&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36.51±6.09&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>RBC ($\times10^6$/mm$^3$)</td>
<td>1.28±0.27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.69±0.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.95±6.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>31.85±5.23&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>WBC and thrombocytes ($\times10^5$/mm$^3$)</td>
<td>5.04±2.69&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.41±2.60&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.51±0.88&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>9.20±1.14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SOE Minor axis (µm)</td>
<td>19.00±3.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.49±2.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.65±1.44&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>7.87±0.68&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Major axis (µm)</td>
<td>23.53±2.78&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.99±2.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.72±1.81&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.01±1.14&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Area (µm)</td>
<td>67.48±7.12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>55.03±5.44&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.32±1.20&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.29±2.90&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Perimeter (µm$^2$)</td>
<td>352.27±79.78&lt;sup&gt;a&lt;/sup&gt;</td>
<td>227.99±45.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.32±2.58&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.74±2.39&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a, b, c</sup>, the difference is statistically significant ($p<0.05$).

BWC had a marked change between three stages (before infection, five days and ten days after infection). Specifically, the total WBC and thrombocytes count reached 6.41±2.60×10$^5$/mm$^3$, showing a slight increase after five days of infection (increased 27.18%), and the growth rate was faster (increased 37.6%) in the next five days. The presence of bacteria inside the body causes the number of WBC to change with a tendency of gradually increasing over time of infection. The WBCs play a role in protecting the body against foreign agents by phagocytosis and antibody production. When bacteria are present, the body produces more WBC to strengthen immunity (Martins et al., 2008).

The hematological parameters of red tilapia five days and ten days after challenging with *S. agalactiae* and feeding with essential oil of peppermint (*M. piperita*) in different concentrations are presented in Table III. After five days of infection, the improvement of the hematological index was more pronounced at Mentha 0.125%. The hematocrit and the total WBC and thrombocyte count reached the highest value when compared with control 2 and with other concentrations of essential oils; Hb was higher when compared with Mentha 0.25% but not statistically significant when compared with control 2 and Mentha 0.5%; RBC had no difference with control 2 but higher than Mentha 0.25% and Mentha 0.5%. Other studies also showed that fish infected with bacteria significantly increased the hematocrit if supplemented with natural essential oils. Specifically, the study of (Nafiqoh et al., 2020) tested guava essential oils to stimulate the immunity.
of catfish when infected with *A. hydrophila* (Nafiqoh *et al.*, 2020), the results recorded a significant increase in Hct. Thus, at five days after infection, fish were fed with a diet supplemented at a low concentration (0.125%), the hematological indexes increased. In addition, the increase in RBC count explained the rise in Hct and Hb values. Several studies have shown that bioactive substances from plants cause an increase in the number of blood cells, activate immunity and enhance the natural defenses of some fish species. Research by Harikrishnan *et al.* (2003) using some herbs to treat fish infected with *A. hydrophila*, the results showed an increase in WBC count along with an increase in Hct and Hb value (Harikrishnan *et al.*, 2003).

In contrast, after ten days of infection, the hematological parameters were lowest at Mentha 0.125%, reflected most clearly in the results of RBC (0.95±0.47×10⁶/mm³) and the total WBC and thrombocyte count (3.95±2.32×10⁴/mm³). The Hct and Hb values were lower than those of control 2 and Mentha 0.5%, but there was no significant difference compared with Mentha 0.25% (p>0.05).

In this study, the physiological indices of RBC at Mentha 0.5% after five days of infection decreased as low as those observed in normal fish – control 1 (Hb and RBC count have no significant difference, the difference in Hb values is 4.7%). After ten days of infection, no difference in Hb and RBC count; the difference in Hct was 6.9%. Regarding the total WBC and thrombocyte count, the changeover the time of infection was not significant and the difference compared with control 1 was shallow (the highest difference value was 0.36 times at ten days of infection).

In general, the effect of peppermint essential oils in infected fish is reflected in the correlation between hematological parameters (such as the increase or decrease in the total WBC and thrombocyte count and physiological indices of RBC). This is similar to other studies on Nile tilapia and red tilapia when applying plant extracts as a supplemented diet to treat fish diseases caused by bacteria (Harikrishnan *et al.*, 2011; Van Hai, 2015; Vallejos-Vidal *et al.*, 2016). At Mentha 0.125% and 0.25%, the fish created more WBC (the higher result at Mentha 0.125% after five days of infection), showing that essential oils’ antibacterial ability was still low. Therefore, the body itself must increase WBC to combat the pathogen *S. agalactiae*. This result proved that at concentrations 0.125% and 0.25%, essential oils could stimulate the body’s immunity. After ten days of infection, the total WBC and thrombocyte count at the two concentrations simultaneously decreased (Mentha 0.25% was higher 33.92% than Mentha 0.5%). This could be explained by the rapid increase in WBC that previously significantly reduced the number of bacteria. In addition, the accumulation of compounds believed to have antibacterial properties of peppermint oils reduces the virulence of *S. agalactiae*. It can be said that the antibacterial ability of peppermint essential oils at low concentrations requires a longer time. As mentioned above, the Mentha 0.5% showed no significant change (p>0.05) in physiological indices of RBC compared with normal fish; total WBC and thrombocyte count also indicated slight variation by each stage of blood collection. This result demonstrated the antibacterial ability of essential oil at the higher concentration was more effective. The presence of chemical compounds in peppermint oils with solid antibacterial activity such as menthol, menthone, pulegone has been demonstrated through many studies (Sivropoulou *et al.*, 1995; Ushimaru *et al.*, 2007; de Souza Silva *et al.*, 2005).
2019; Nafiqoh et al., 2020). In this study, the results of chemical compounds testing of essential oils used for the experiment showed that menthol accounted for the highest percentage (32.8%), followed by menthone (30.3%). For these two compounds, the antibacterial role of menthol was assessed to be more predominant (Işcan et al., 2002).

CONCLUSION

Adding essential oils of peppermint to fish diets can improve hematological parameters. In addition, fish were fed with supplemented diet before challenging with *S. agalactiae* with a density of 10⁶ CFU/mL, which may stimulate the body to create immunity against bacteria (at concentrations of 0.125% and 0.25%); and can help the body resist them (at a concentration of 0.5%).

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Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES


Hematological Parameters of Red Tilapia


