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

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Key words
Red tilapia, Peppermint, Hematology, Essential oils, *Streptococcus agalactiae*

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 10^6 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* (Romana-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

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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 *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 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, fish were kept stable for 60 days. After that, fish were fed a supplemented diet with essential oils of peppermint (*Mentha piperita*) as arranged in 15 days and the first blood sample was collected (mentioned below as stage 1). Then, fish were challenged with *S. agalactiae* at a density of 10^6 CFU/mL. Five days after infection, the second blood sample was collected (stage 2) and the last one was five days next (stage 3) (Nya and Austin, 2009; Farahi *et al*., 2010; Guo *et al*., 2012); 15 fish of each tank were selected randomly for each blood sample.

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 (*Mentha piperita*) is a commercial one produced by Heber Vietnam Co., Ltd. The essential oil compounds of *M. piperita* include α-Pinene 1.8%, Sabinene 0.2%, β-Pinene 1.4%, β-Myrcene 0.3%, 3-Octanol 0.5%, Limonene 4.3%, Isopulegol 0.9%, Isomenthol 4.8%, Menthone 30.3%, Menthol 32.8%, α-Terpineol 1.9%, Menthyl acetate 5.6%, Piperitone 0.9%, 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 Sahli hemoglobin. 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 = $A \times 5 \times 10 \times 200$ (cells/mm^3), where A is the total number of red blood cells in the five-count zones.

The WBC and thrombocytes count was calculated according to Hrubec *et al*. (2000):

The total WBC and thrombocyte count = (number of WBC of 1500 cells×R)/1500, where R is the number of red blood cells in 1mm^3 of blood.

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

Challenge with 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 10^6 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 $\overline{X} \pm$ 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 Mentha 0.5% (9.20±1.14 g%) was higher when compared to control 1 (7.83±0.82 g%). The hematocrit didn't show a significant difference between the fish supplemented with Mentha 0.5% and control 1. For the RBC count, the total count of WBC thrombocytes, the highest value was obtained in fish supplemented with Mentha 0.5%. The hematological indices of Mentha 0.125% and Mentha 0.25% were lower when compared to control 1 and there was no difference between the two concentrations. The increase of hematological indices in Nile tilapia (*O. niloticus*) with a diet supplemented was also presented in the study of de Souza Silva *et al*. (2019). The results showed that after 60 days, the hematocrit indices, the Hb concentration, the RBC count and the WBC count at Mentha 0.25% were all significantly higher when compared with control (de Souza Silva *et al*., 2019).

Hematological changes in fish (such as increase or decrease in RBC count, WBC count, Hb and Hct value) can help detect diseases affecting the cellular components of blood and in determining the health status of hematopoietic organs (Campbell and Ellis, 2007). In this study, the essential oils of *M. piperita* added to the diet improved the hematological status of fish, which demonstrated the positive effects of the chemical compounds in essential oils on some hematopoietic processes. The improved hematological variables of the herbs were similarly observed in other studies. In the study of Aly *et al*. (2010), the results showed a significant increase in Hct values, the total WBC and lymphocytes when fish diets were supplemented with garlic (*Allium sativum*) and echinacea (*Echinacea purpurea*) (Aly and Mohamed, 2010). Similarly, in the study of Acar *et al*. (2015), citrus (*Citrus sinensis*) essential oils added to the diet improved the hematological parameters of fish.

Table II shows hematological parameters of red tilapia challenged with *S. agalactiae* at a 10^6 CFU/mL density for 5 and 10 days. The hematocrit and the RBC count increased after five days and ten days of infection, but there was no statistically significant difference between the two stages of infection ($p<0.05$). The Hb concentration increased significantly after ten days of infection (8.93±0.78 g%) and there was no significant difference in this index after five days of infection. The values of erythrocyte size after five days and ten days of infection both decreased and were significantly different ($p<0.05$). Bacterial infection in fish can change blood cell physiological parameters as reflected by a decrease in RBC count, which causes a reduction in the hemoglobin concentration and the hematocrit value (Ranzani-Paiva *et al*., 2004; Ndong and Fall, 2011; Omitoyin *et al*., 2019). However, contrasting results were found in the present study, where the investigated hematological parameters increased after each infection stage. This could be explained by the action of bacteria in reducing the amount of normal mature erythrocytes and stimulating the production of immature erythrocytes. According to Groff and Zinkl (1999), stressors such as hypoxia, bacteria, and anemia are responsible for the increased immature erythrocytes (Groff and Zinkl, 1999). The presence of many immature erythrocytes in the peripheral blood of fish after the bacterial infection has also been shown similarly in the studies of Yoshinaga *et al*. (2000) and (Ranzani-Paiva *et al*., 2004; Yoshinaga *et al*., 2000). The significant increase of immature erythrocytes leads to the RBC count increase, which explains the increase in the hematological parameters investigated (Hb concentration and Hct value) and the decrease in erythrocyte size. Mature erythrocytes are oval to elliptical while immature erythrocytes are round and more minor than mature erythrocytes (Campbell and Ellis, 2007).

In this study, the process of monitoring the peripheral blood of infected red tilapia also observed abnormal erythrocytes with shape variation, such as teardrop cell, helmet cell, bur cell (1, 2, 3 in Fig. 1) or a nuclear variation like binuclear erythrocyte (4 in Fig. 1). The appearance of these erythrocytes also explained the decrease in erythrocyte size. Several studies on fish exposed to bacteria also noted an increase in the number of hemocytoblasts (immature blood cells) associated with the presence of abnormal erythrocytes (Duncan and Lovell, 1994; Miwa and Inouye, 1999). The reason why immature erythrocytes gradually replace mature cells may be due to bacteria affecting the hematopoietic organ leading to a shortened cell cycle. Abnormal erythrocytes can be caused by bacteria affecting the cytoplasm, leading to deformation of erythrocytes, even breakdown of erythrocytes (hemolytic erythrocytes) (Duncan and Lovell, 1994).

Table I. Effect of feeding essential oil of peppermint (*Mentha piperita*) in different concentrations for 15 days on hematological parameters ($\overline{X}$± *SD*) of red tilapia.

Hematological parameters	Control 1	Mentha 0.125%	Mentha 0.25%	Mentha 0.5%
Hct (%)	38.25±5.16^{a}	36.26±4.03^{b}	36.67±6.03^{b}	39.56±4.26^{a}
Hb (g%)	7.83±0.82^{b}	7.24±1.10^{c}	7.51±0.88^{bc}	9.20±1.14^{a}
RBC ($\times 10^6$/mm^3)	1.28±0.27^{b}	1.12±0.31^{c}	1.08±0.18^{c}	1.41±0.29^{a}
WBC and thrombocytes ($\times 10^4$/mm^3)	4.23±1.77^{b}	3.40±1.43^{c}	3.57±1.20^{bc}	5.18±2.47^{a}

a, b, c, The difference is statistically significant ($p<0.05$).

Table II. Hematological parameters and size of erythrocyte (SOE) ($\bar{X}$± *SD*) of red tilapia after five days and ten days of *S. agalactiae* infection.

Hematological parameters		Control 1	Control 2	
			After five days	After ten days
Hct (%)		38.25±5.16^{b}	40.37±5.30^{a}	41.56±3.83^{a}
Hb (g%)		7.83±0.82^{b}	8.06±0.99^{b}	8.93±0.78^{a}
RBC (×10^{6}/mm^{3})		1.28±0.27^{b}	1.69±0.37^{a}	1.81±0.43^{a}
WBC and thrombocytes (×10^{4}/mm^{3})		5.04±2.69^{c}	6.41±2.60^{b}	8.82±2.74^{a}
SOE	Minor axis (µm)	19.00±3.26^{a}	14.49±2.02^{b}	13.80±2.39^{c}
	Major axis (µm)	23.53±2.78^{a}	19.99±2.45^{b}	18.28±2.4^{c}
	Area (µm)	67.48±7.12^{a}	55.03±5.44^{b}	51.13±5.55^{c}
	Perimeter (µm^{2})	352.27±79.78^{a}	227.99±45.05^{b}	198.54±45.34^{c}

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

Table III. Effect of feeding different concentrations of essential oil of peppermint (*M. piperita*) on hematological parameters ($\bar{X}$± *SD*) of red tilapia after challenging it with *S. agalactiae* for 5 days and 10 days.

	Stage	Control 2	Mentha 0.125%	Mentha 0.25%	Mentha 0.5%
Hct (%)	1	38.25±5.16$^{a, B}$	36.26±4.03$^{b, B}$	36.67±6.03$^{b, B}$	39.56±4.26$^{a, A}$
	2	40.37±5.30$^{b, A}$	43.65±4.49^{a,} A	38.49±3.74$^{b, A}$	36.51±6.09$^{c, B}$
	3	41.56±3.83$^{a, A}$	30.95±6.23$^{c, C}$	31.85±5.23$^{c, C}$	35.78±7.84$^{b, B}$
Hb (g%)	1	7.83±0.82$^{b, B}$	7.24±1.10$^{c, B}$	7.51±0.88$^{bc, A}$	9.20±1.14$^{a, A}$
	2	8.06±0.99$^{ab, B}$	8.38±1.21$^{a, A}$	7.65±1.44$^{b, A}$	7.87±0.68$^{ab, B}$
	3	8.93±0.78$^{a, A}$	5.55±1.60$^{c, C}$	6.20±1.22$^{bc, B}$	6.60±1.14$^{b, C}$
RBC (×10^{6}/mm^{3})	1	1.28±0.27$^{b, B}$	1.12±0.31$^{c, B}$	1.08±0.18$^{c, C}$	1.41±0.29$^{a, A}$
	2	1.69±0.37$^{a, A}$	1.81±0.51$^{a, A}$	1.51±0.36$^{b, A}$	1.34±0.30$^{c, A}$
	3	1.81±0.43$^{a, A}$	0,95±0,47$^{c, C}$	1,30±0,40$^{b, B}$	1.34±0.37$^{b, A}$
WBC and thrombocytes (×10^{4}/mm^{3})	1	4.23±1.77$^{b, C}$	3.40±1.43$^{c, B}$	3.57±1.20$^{bc, C}$	5.18±2.47$^{a, A}$
	2	6.41±2.60$^{bc, B}$	8.13±3.77$^{a, A}$	6.87±2.76$^{b, A}$	5.61±2.29$^{c, A}$
	3	8.82±2.74$^{a, A}$	3.95±2.32$^{c, B}$	5.29±2.90$^{b, B}$	5.74±2.39$^{b, A}$

a, b, c, d shows the difference in rows ($p<0.05$). A, B, C shows the difference in columns ($p<0.05$).

WBC 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^{4}/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).

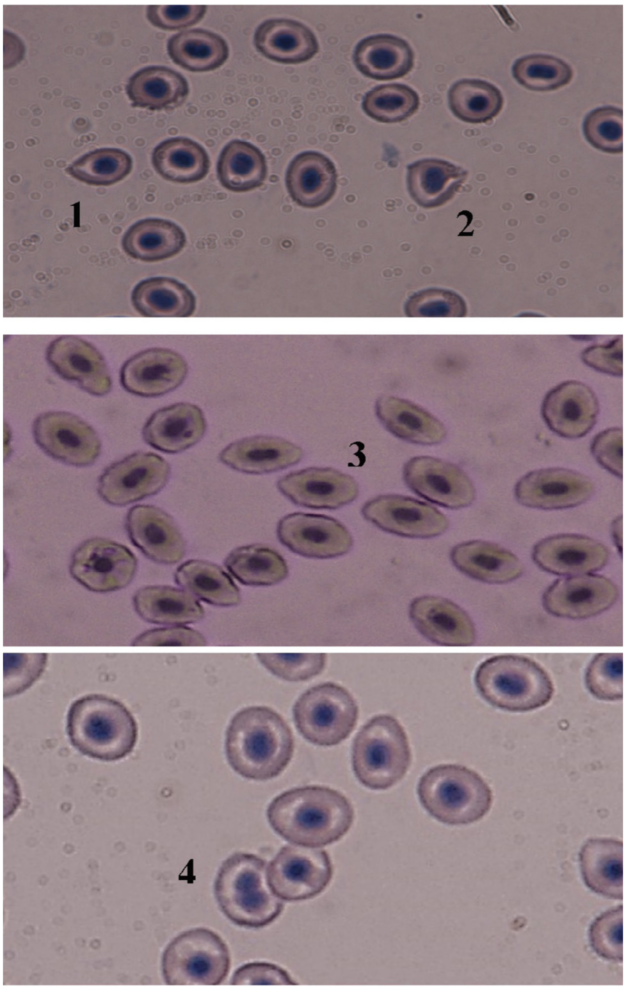

Fig. 1. Peripheral blood smear of infected red tilapia after ten days, showing a teardrop erythrocyte (1), helmet erythrocyte (2), burr erythrocyte (3) and a binuclear erythrocyte (4) (×40 magnification).

In contrast, after ten days of infection, the hematological parameters were lowest at Mentha 0.125%, reflected most clearly in the results of RBC ($0.95\pm0.47\times106/mm^3$) and the total WBC and thrombocyte count ($3.95\pm2.32\times104/mm^3$). 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*., 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 (İşcan *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 10^6 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.

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