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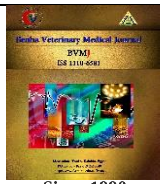

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Original Paper

Control of motile Aeromonas septicemia in Nile tilapia (*Oreochromis niloticus*) using some herbal additives

Doaa A. Yassen, Eman A. Abd El-Gawad*, Amel M. El Asely, Hiam S. Elabd, Adel A. Shaheen, Amany A. Abbass**

Department of Aquatic Animal Medicine, Faculty of Veterinary Medicine, Benha University

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ABSTRACT

This work aimed to investigate the effect of dietary thyme (*Thymus vulgaris*) and rosemary (*Rosmarinus officinalis*) on controlling *Aeromonas hydrophila* (*A. hydrophila*) infection in Nile tilapia (*Oreochromis niloticus*). Fish were divided into three groups in duplicate and were fed on experimental diets (0% control, 1.5% thyme, and 0.5% rosemary) for 6 weeks before the challenge trial with *A. hydrophila.* Three sampling points were carried out after 2-, 4-, and 6-weeks post-feeding. Results revealed that serum lysozyme activity significantly increased after 2 weeks in both treated groups compared to controls. Serum nitric oxide recorded a significant decrease in thyme supplemented group during the experiment. Nile tilapia-fed thyme or rosemary exhibited a significant ($P< 0.05$) increase in total leukocyte count, neutrophils, and monocytes throughout the experimental period. Meanwhile, lymphocytes recorded a significant increase in both treated groups after 4 and 6 weeks of feeding compared to the control. In addition, thyme treated group showed a significant increase in superoxide dismutase (SOD) and glutathione reductase (GR) enzymes activities after 4 weeks of feeding. Malondialdehyde (MDA) decreased significantly at 6 weeks of feeding compared to the control. The challenged fish fed 1.5 % thyme and 0.5 % rosemary for 4 and 6 weeks showed high survivability, 90 %, compared to only 10 % of the control. In conclusion, dietary supplementation with thyme and rosemary in Nile tilapia could increase fish's resistance to *A. hydrophila* by improving immune-hematological parameters.

1. INTRODUCTION

The aquaculture sector has developed rapidly. This impressive industrial development has been accompanied by the expansion of semi-intensive culture to the intensive system that adversely affecting fish due to the stressful aquatic environment (Wang et al., 2015).
Bacterial diseases can cause significant economic losses through mortality/morbidity, poor growth rate, low quality of flesh, or reduced trade resulting in reduced profit margins (Smith et al., 2003).
Aeromonas hydrophila is the most pathogenic bacteria causing a high mortality rate among cultured freshwater fishes (Janda and Abbott, 2010; Youssuf, et al., 2020).
Immunostimulants dietary supplementation is recommended in aquaculture as an alternative method to antibiotic treatment due to their impacts on human health and aquatic ecosystem (Reverter et al., 2017). Currently, a wide variety of medicinal plants and their bioactive compounds possess antioxidant effects, improving fish's general physiological and immunological status (Abd El-Gawad et al., 2020; Abdel-Tawwab et al., 2021; Soror et al., 2021).
The health status of fish is evaluated by measuring biochemical and oxidative indices, which are essential markers for the effectiveness of medicinal plants' dietary supplementation (Zahran et al., 2018; El-Houseiny et al., 2019; Abd El-Naby et al., 2020).
Thyme has a potent antimicrobial and antioxidant activities and enhances resistance against fish diseases (Yassen et al., 2017; Abd El-Naby et al., 2020; Tasa et al., 2020) due to its very high contents of thymol, p-cymene, carvacrol, eugenol, and 4-allylphenol (Rota et al., 2008; Dauqan and Abdullah, 2017).
Rosemary is well known for its antioxidant and immunological properties in aquaculture applications (Nail et al. 2020; Yousefi et al. 2020). The primary chemical constituents of rosemary are carnosic acid and rosmarinic acid, which have exceptionally high antioxidant activity (Erkan et al., 2008; Charles, 2012).
This study aimed to evaluate the efficiency of thyme and rosemary as dietary herbal plants to control the infection with *Aeromonas hydrophila* in in Nile tilapia (*Oreochromis niloticus, O. niloticus*).

2. MATERIAL AND METHODS

2.1. Experimental fish

One hundred and fifty *O. niloticus* fingerlings, of an average body weight 18±1.5 g, were obtained from a private fish farm at El Sharkia Governorate, Egypt. Fish were transported to the wet lab of Aquatic Animals

* Correspondence to: * eman.mahmoud@fvtm.bu.edu.eg. **amanyabbass3@fvtm.bu.edu.eg.

Diseases and Management, Faculty of Veterinary Medicine, Benha University, Egypt. Fish were maintained for 14 days under adjustable water parameters as previously adopted by Yassen et al. (2017). Random fish samples were taken to examine fish health status as described by Austin and Austin (1989).

2.2. Experimental diets and design

Dried Thyme and Rosemary were purchased as the whole plant from the traditional market in Egypt. The experimental diets were prepared according to Yassen et al. (2017)**.** After the acclimation period, the fish was divided into six circular fiberglass tanks (250 L capacity) at 25 fish per tank. The tank presents three groups in duplicate. The first group was kept as a control group and fed a basal diet. The second group was fed on 1.5 % thyme-treated diet, and the third group was fed 0.5 % rosemary-supplemented diet. Fish was hand-fed twice daily at a rate of 3% of body weight for six weeks. During the experiment, the water temperature was adjusted at 25±2 °C and the dissolved oxygen level at 5.4±0.2 mg/l.

This study and all experimental procedures involving animal care and use were performed according to the protocol approved by Benha University Institutional Animal Care and Use Committee.

2.3. Sampling

At the end of 2-, 4-, and 6-weeks of feeding, blood samples were taken from the caudal blood vessels of six anesthetized fish (MS-222, 100 µg/ml). Serum samples were prepared without anticoagulant, left in a slant position for one hour, and centrifuged at 3000 rpm. for 15 minutes. The serum was collected, pooled (n=3), and stored at 20 °C for immunological assay according to Yassen et al. (2017)**.** In addition, another blood sample with EDTA was collected for hematological parameters analysis. Liver samples were taken immediately from euthanized fish in a clean Eppendorf tube containing phosphate buffer saline (PBS, pH 7.4) and kept at -80 °C until use for antioxidant enzymes assay.

2.4. Immune-hematological parameters assay

Serum lysozyme activity was measured according to Schlitz (1987), and serum nitric oxid**e** was assessed following the method described by Rajaraman et al. ***(***1998)**.** Blood elements (RBCs and WBCs) were counted according to Kanaev **(**1985) using a Neubauer-improved hemo-cytometer (Germany). Hemoglobin concentration using the cyanomethoglobin method and differential leukocyte count (DLC) were carried out according to Stoskopf (1993). The Packed cell volume (PCV %) and blood indices (MCV, MCH, and MCHC) were estimated after the method described by Dacie and Lewis (1991).

2.5. Lipid peroxidation and antioxidant enzymes assay

The weighted liver tissues were rinsed firstly with PBS to remove clotted blood, then homogenized using cold PBS (pH 7.4), and centrifuged at 4°C at 4000 ×g for 15 min at 4°C. The supernatants were taken for measuring Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Reductase (GR), and Malondialdehyde (MDA) levels following the manufacture protocol of Bio-diagnostic commercial kits (Egypt)**.**

2.6. Efficacy of herbal plants in controlling A. hydrophila

At the end of each sampling point 2-, 4-, and 6-weeks post-feeding, fish (n= 10/treatment) from each group in duplicate were intra-peritoneal (I.P.) injected with 0.2 ml *A. hydrophila* suspension (1×10^8 cells/ml). The bacterial suspension was prepared, and the required concentration was adjusted after Yassen et al. (2017)**.** Mortalities, clinical signs, and developed lesions were recorded daily over 2 weeks.

2.7. Statistical analysis

The data was analyzed by One-Way Analysis of Variance (ANOVA) and Duncan's multiple range tests to determine significant differences between groups using the statistical package for the social sciences (SPSS) software (Version 17.0). Antioxidant enzyme activity was analyzed by independent–sample *t*-Test. A value of $P < 0.05$ was considered significant.

3. RESULTS

3.1. Immune-hematological parameters

Serum lysozyme activity increased significantly ($P< 0.05$) in both treated groups for two weeks compared to the control. Nitric oxide significantly decreased in the group fed 1.5 % thyme during the experimental periods. However, fish fed 0.5 % rosemary showed a significant decrease in nitric oxide levels after 6 weeks of feeding compared to controls (Table 1).

Table 1 Lysozyme activity and nitric oxide level of *O. niloticus* fed diets supplemented with 0 % (control), 1.5 % thyme and 0.5 % rosemary for 2-, 4-, and 6-weeks.

Groups	Lysozyme activity	Nitric oxide
================ 2 weeks ================		
Control	207.51 ± 7.29^b	24.91 ± 0.01^a
Thyme 1.5%	229.43 ± 7.33^a	19.73 ± 0.03^b
Rosemary 0.5%	222.10 ± 0.001^a	23.17 ± 0.03^a
================ 4 weeks ================		
Control	207.39 ± 7.35	34.07 ± 1.67^b
Thyme 1.5%	200.02 ± 0.01	10.60 ± 0.01^c
Rosemary 0.5%	200.04 ± 0.01	38.60 ± 0.90^a
================ 6 weeks ================		
Control	200.04 ± 0.001	52.60 ± 0.01^a
Thyme 1.5%	200.04 ± 0.002	42.07 ± 0.01^b
Rosemary 0.5%	214.75 ± 7.35	41.26 ± 0.17^c

Values (means± S.E; n=3) with different letters in the same column are significantly different ($P < 0.05$).

Total RBCs count and PCV significantly increased after 4 and 6 weeks in thyme treated group. Total WBC exhibited a significant increase after 2 and 4 weeks of feeding, accompanied by a significant increase in monocytes and neutrophils during the experimental period. Meanwhile, lymphocytes revealed only a significant increase after 4 and 6 weeks of feeding compared to controls. MCV and MCH exhibited a significant decrease after 2 and 4 weeks of feeding, while MCHC significantly increased after 2 weeks and then decreased (Table 2).

Rosemary treated group revealed a significant increase in total RBCs count after 2 and 4 weeks of feeding with an increase in PCV in all sampling points. Hemoglobin concentration significantly increased after 2 weeks of feeding then decreased after 6-week feeding. Total WBCs recorded a significant increase after 2 and 4 weeks of feeding, with monocytes increasing throughout the feeding experiment. MCH and MCHC showed a significant decrease after 4 weeks of feeding; MCV revealed a significant increase after 6 weeks compared to the control (Table 2).

3.2. Antioxidant enzymes activity

Malondialdehyde (MDA) level showed a significant increase after 4 weeks, then decreased at 6 weeks post-feeding compared to the control. Superoxide dismutase (SOD) enzyme activity significantly increased after 2 and 4 weeks of feeding. Meanwhile, catalase (CAT) enzyme activity decreased at 2 and 6 weeks compared to the control-fed basal diet. Hepatic GR enzyme in thyme treated group showed a significant increase in activity after 4 and 6 weeks (Table 3).

3.3. Protective efficacy of thyme and rosemary against A. hydrophila *infection*

Oreochromis niloticus fed with 1.5% thyme and 0.5 % rosemary for 4 and 6 weeks and challenged with *A. hydrophila* showed high survivability, 90 % compared to 10 % of the control. Meanwhile, groups fed either thyme or rosemary for 2 weeks recorded 40 % and 80 % survivability, respectively (Fig 1). Fish fed on a control diet and experimentally infected with *A. hydrophila* showed redness and congestion of the skin and anal opening with ulcer formation. In addition, internal organs showed congestion with enlarged gall bladder compared to treated groups which revealed no clinical signs or postmortem lesions.

Table 2 Hematological parameters of *O. niloticus* fed diets supplemented with 0 % (control), 1.5 % thyme and 0.5 % rosemary for 2, 4, and 6 weeks

Groups	**RBCs**	**PCV%**	**Hb g/dl**	**MCV**	**MCH**	**MCHC**	**TLC**	**Monocytes**	**Lymphocytes**	**Neutrophil**
================ 2 weeks ================										
Control	2.48^{b} ± 0.08	32.50^{b} ± 0.29	8.35^{b} ± 0.55	131.60^{a} ± 3.2	33.71^{a} ± 1.88	25.70^{c} ± 1.70	14.40^{c} ± 0.40	22.20^{b} ± 0.58	40.20 ± 0.58	24.60^{b} ± 0.75
Thyme 1.5%	2.66^{b} ± 0.02	22.00^{c} ± 0.001	8.86^{b} ± 0.15	83.36^{b} ± 0.77	33.31^{a} ± 0.59	40.27^{a} ± 0.67	18.60^{b} ± 0.40	26.20^{a} ± 0.66	42.00 ± 0.71	29.00^{a} ± 0.71
Rosemary 0.5%	4.48^{a} ± 0.07	36.60^{a} ± 0.24	11.5^{a} ± 0.07	81.78^{b} ± 1.38	25.74^{b} ± 0.48	31.48^{b} ± 0.32	26.20^{a} ± 1.74	25.60^{a} ± 0.93	41.20 ± 0.86	25.60^{b} ± 0.24
================ 4 weeks ================										
Control	1.55^{b} ± 0.05	17.00^{b} ± 0.00	6.20 ± 0.00	110.00^{a} ± 3.3	40.12^{a} ± 1.22	36.47^{a} ± 0.00	13.40^{b} ± 0.60	23.20^{b} ± 0.37	39.40^{c} ± 0.51	24.40^{b} ± 0.51
Thyme 1.5%	2.62^{a} ± 0.04	22.60^{a} ± 0.24	6.64 ± 0.32	86.29^{b} ± 0.80	25.34^{b} ± 1.14	29.35^{b} ± 1.24	20.60^{a} ± 1.08	25.00^{a} ± 0.71	44.00^{a} ± 0.32	27.80^{a} ± 0.66
Rosemary 0.5%	2.60^{a} ± 0.08	22.40^{a} ± 0.0	6.80 ± 0.36	86.19^{b} ± 0.66	26.18^{b} ± 1.22	30.35^{b} ± 1.29	19.20^{a} ± 1.20	26.60^{a} ± 0.51	42.00^{b} ± 0.71	25.20^{b} ± 0.37
================ 6 weeks ================										
Control	3.10^{b} ± 0.00	26.75^{c} ± 0.4	7.60^{a} ± 0.37	86.29^{b} ± 1.54	24.52^{a} ± 1.19	28.38^{a} ± 0.98	17.00 ± 1.38	24.00^{b} ± 0.71	40.80^{b} ± 0.86	24.20^{b} ± 0.58
Thyme 1.5%	5.02^{a} ± 0.22	44.00^{a} ± 0.63	6.78^{ab} ± 0.26	88.17^{b} ± 3.17	13.58^{b} ± 0.67	15.40^{c} ± 0.49	17.80 ± 0.20	26.40^{a} ± 0.51	43.60^{a} ± 0.68	28.40^{a} ± 0.51
Rosemary 0.5%	2.82^{b} ± 0.05	33.00^{b} ± 0.01	6.26 ^{b} ± 0.40	117.16^{a} ± 2.06	22.19^{a} ± 1.31	18.97^{b} ± 1.22	18.80 ± 1.36	26.00^{a} ± 0.55	41.60^{b} ± 0.65	25.00^{b} ± 0.32

Values (means ± S.E) (n= 3) with different letters in the same column are significantly different ($P < 0.05$).

Table 3 Lipid peroxidation and anti-oxidant enzymes activity of *O. niloticus* fed diets supplemented with 0 % (control), 1.5 % thyme for 2, 4, 6 weeks.

Groups	MDA	SOD	CAT	GR
================ 2 weeks ================				
Control	274.76 ± 0.01	810.31^{b} ± 0.01	3.02^{a} ± 0.001	160.22^{a} ± 0.02
Thyme 1.5%	317.86 ± 0.02	879.37^{a} ± 0.06	2.16^{b} ± 0.12	89.02^{b} ± 0.02
================ 4 weeks ================				
Control	172.40^{a} ± 0.001	1086.20^{b} ± 0.001	3.37 ± 0.11	81.06^{b} ± 0.00
Thyme 1.5%	581.40^{b} ± 0.20	1568.92^{a} ± 0.02	3.15 ± 0.11	97.31^{a} ± 0.01
================ 6 weeks ================				
Control	394.22^{b} ± 0.16	1293.10 ± 0.001	1.97^{a} ± 0.01	61.59^{b} ± 0.001
Thyme 1.5%	344.29 ^{a} ± 0.23	1432.34 ± 0.66	1.26^{b} ± 0.07	90.92^{a} ± 0.001

Values (means± S.E) (n= 3) with different letters in the same column are significantly different ($P < 0.05$).

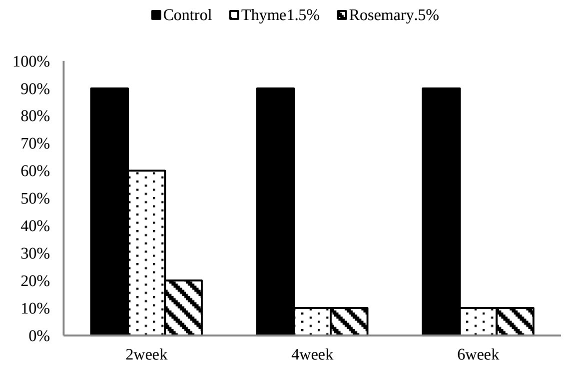

Figure 1 Mortality rate (%) of *O. niloticus* fed diets supplemented with 0 % (control), 1.5 % thyme and 0.5 % rosemary and challenged with *A. hydrophila.*

4. DISCUSSION

Bacterial disease outbreaks in aquaculture represent a significant risk to the economy worldwide. This problem is often addressed with vaccines and antibiotics application. However, some obstacles exist, such as vaccines being bacterial specific, antibiotic residues in body tissues, and increased multi-resistant bacteria (Cabello 2006; Syahidah et al., 2015; Youssuf, et al., 2020). The enhancement of fish's immune system is considered the most promising method of preventing fish diseases in aquaculture (Abd El-Latif et al., 2021). Recently use of natural plants and their extracts as a source of immunomodulatory agents in aquaculture becoming popular. Many research studies study the effect of herbal plants on growth performance, antioxidant activity, immunological response, and disease resistance in different fishes (Ibrahim et al., 2019; Soror et al., 2021; Habiba et al., 2021)

Blood parameters are considered crucial indicators of fish's nutritional and physiological status; hence infection, stress, and nutritional deficiency are always accompanied by a low level of them (Rehulka, 2002). Leukocytes play an essential role in innate immunity and their count is considered an indicator of a fish's health condition (Harikrishnan et al., 2003). The current study revealed enhancement of RBCs, PCV, and Hb in group-fed thyme. Also, dietary supplementation of thyme or rosemary in *O. niloticus* diet significantly increased leukocyte counts and DLC compared to the control. These results were supported by Zaki et al., (2012), Gültepe et al. (2014), and Yassen et al. (2017), who recorded significant improvement in hematological parameters of Nile tilapia-fed thyme-supplemented diets. Similarly, a significant increase in RBC, Hb, and PCV was recorded in Nile tilapia-fed garlic and fenugreek-supplemented diets (Shalaby et al., 2006, Basha et al., 2018).

In our study, MCV and MCH in thyme treated group exhibited a significant decrease after 2 and 4 weeks, while MCHC only increased after 2 weeks of feeding. Meanwhile, fish-fed rosemary 0.5% recorded a significant decrease in MCH and MCH after 4 weeks of feeding and a significant increase in MCV value after 6 weeks. These results follow Kanani et al. (2014), who reported a significant decrease in MCH and MCV in juvenile *Huso huso* fed with garlic. It is also assumed that the decrease or increase in blood indices may be attributed to a defense reaction against thyme and rosemary, which occurs by stimulating erythropoiesis (Fazlolahzadeh et al. 2011).

Serum lysozyme activity plays a role in the lysis of bacterial pathogens, activation of phagocytosis, and hemolytic complement activity. The bactericidal action of this enzyme involves the hydrolyzation of the peptidoglycan of bacterial cell walls resulting in cell lysis, preventing adhesion and colonization of bacterial pathogens and hence prevention of infections and disease (Nya and Austin, 2011). In this study, fish fed with thyme and rosemary showed a significant increase in lysozyme activity in the first 2 weeks post-feeding. The serum lysozyme activity was reported to be enhanced in Nile tilapia fed on thyme seed meal (Zaki et al., 2012), and also a significant increase of lysozyme activity was recorded in rainbow trout, *Oncorhynchus mykiss* fed on carvacrol, and thymol treated diets (Giannenas et al., 2012). There was a strong relationship between hematopoietic and immune cells and the ability to guard against foreign pathogens and stressors. Additionally, in a previous study by Yassen et al. (2017), the immune response of Nile tilapia was significantly enhanced with thyme and rosemary dietary supplementation. The increase in lysozyme activity could be attributed to an increase in the neutrophils and lymphocytes mainly produced by them (Fischer et al., 2006). The immunostimulatory effect of thyme may be due to the stimulatory effect of the primary essential oil of thymol and carvacrol derived from thyme (Azad et al., 2014).

Nitric oxide is an intracellular mediator produced by macrophages in various live cells and can be used as an inflammatory marker for disease status and progression (Murphy, 1999). *O. niloticus* fed 1.5 % dietary thyme showed a reduction of nitric oxide production during the whole experimental period. Similar findings were reported in Nile tilapia-fed curcumin (Elgendy et al., 2016) and rainbow trout-fed thymol (Giannenas et al., 2012). This observation could be attributed to the scavenger action of antioxidant compounds of thyme against any free radical or due to the increase of macrophage and neutrophils cells count with humeral acidic secretions and their peroxidase pathways as inhibitory precursors that prevent the synthesis of any harmful and unstable compounds in live tissues (Cuesta et al., 2005). Nile tilapia fed 0.5 % rosemary treated diet revealed a significant increase after 4 weeks, then decreased after 6 weeks of feeding. In the same manner, Yoshida et al. (1995) recorded that the African catfish, *Clarias gariepinus*, fed on a glucan-treated diet, showed an increase in respiratory burst activity over 30 days but not over 45 days. Thus, the effective administration period should be investigated for each immunostimulant.

Malondialdehyde (MDA) level is a marker of cellular membrane damage caused by free radicals (Gaweł et al., 2004). To keep a balance between antioxidants and free radicals, SOD, CAT, and G.R. represent the first line of defense against oxidative stress (Lushchak, 2011). The present results exhibited a significant increase in GSH-RX and SOD activities with a significant decrease in MDA level after 6 weeks of feeding the group fed thyme. In the same manner, previous studies have reported decreased MDA levels and a significant increase of antioxidant enzymes in rainbow trout-fed thyme oils (Sönmez *et al.*, 2015) and Nile tilapia-fed thyme (Yassen et al., 2017). The increase in antioxidant enzyme activities could be attributed to increasing the neutrophils as secretory cells of these compounds. The increased SOD enzyme activity with dietary thyme supplementation could be attributed to the enhanced production of highly reactive superoxide radicals (O2_) and increased the production of H2O2 that can be metabolized to molecular oxygen and water by CAT and G.R. (Yilmaz et al., 2006). In our study, hepatic G.R. activity was significantly increased along experimental periods, suggesting that thyme may have increased GSH due to increased biosynthesis or other antioxidants.

Herbal immunostimulants became an attractive alternative way of conferring protection against diseases by enhancing nonspecific defense mechanisms' activity (Raa, 1996). Therefore, it is essential to test immunostimulants' efficacy in treating fish against diseases (Sakai et al., 2001). Dietary supplementation of thyme at a concentration of 1.5% or rosemary at a dose of 0.5 % for 6 weeks improved the survival rate of *O. niloticus* against challenge with the pathogen *A. hydrophila* compared to the control fed the basal diet. The enhancement of immune-hematological parameters in our study by 1.5 % thyme or 0.5 % rosemary incorporated diet could explain reducing in the percentage of mortality in *O. niloticus*. The present findings are supported by the results of Abutbul et al. (2004), Zilberg et al. (2010), and Yilmaz et al., (2013) in tilapia-fed rosemary and *O. mossambicus* fed thyme (Gültepe et al., 2014). Fish resistance against diseases was enhanced with the oral administration of herbal plants (Sharma et al., 2010; Alishahi and Abdy, 2013; Kazeem et al., 2017). The potent antimicrobial activity of essential oil thymol and carvacrol derived from thyme has been widely tested in vitro against a wide range of pathogenic bacteria (Al-Yaqout and Azad, 2010; Azad et al., 2014). Antimicrobial activities in essential oils from spices and culinary herbs appear to be associated with phenolic compounds (Davidson and Naidu, 2000), which are beneficial in enhancing neutrophil production that mainly remove bacteria by the production of reactive oxygen species during respiratory burst or secretion of hydrolytic lysozymes enzyme (Fischer et al., 2006).

5. CONCLUSION

In conclusion, dietary supplementation of 1.5 % thyme for 6 weeks or 0.5 % rosemary for 4 weeks of feeding could improve the cellular immunity of *O. niloticus* and increase protection against *A. hydrophila* infection. In addition, oral administration of thyme enhances antioxidant enzyme activity in Nile tilapia, increasing its resistance against environmental oxidative stress.

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