Influence of Moringa (*Moringa oleifera*) and Rosemary (*Rosmarinus officinalis*), and Turmeric (*Curcuma longa*) on Immune parameters and Challenge of Nile tilapia to *Aeromonas hydrophila*

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Abstract: The immune potentiating activities of three medicinal plants; Moringa (*Moringa oleifera*), Rosemary (*Rosmarinus officinalis*), and Turmeric (*Curcuma longa*) leaves on Nile tilapia and their challenge with *Aeromonas hydrophila* was assessed. Fish were grouped into 4 treatments; the first one was fed with isonitrogenous and isoenergetic diets supplemented without addition of any supplement (control group) (T1), the other groups were supplemented with 1% dietary *C. longa* (T2), 1% dietary *M. oleifera* (T3) and 1% dietary *R. officinalis* (T4). Fish in all groups were fed for 2 months on the experimental diets. Blood was sampled biweekly. Albumin, globulin, and total protein were measured in serum samples. Additionally, lysozyme and respiratory burst activity were evaluated. After the period of the feeding experiment, treated fish were infected intraperitoneally with *A. hydrophila* and relative percent of survival (RPS) was calculated. It was noticed that serum albumin, globulin, and total protein was considerably improved in treated fish in comparison with control one. As well, serum lysozyme and respiratory burst activity in the fish group fed a diet with *C. longa* (T2) were higher than other groups (T3 and T4) and all the three treated groups were significantly improved in relation to the control group. Results, also, revealed that RPS was 10, 75, 70, and 65% for treated fish groups respectively. It can be concluded that the incorporation of *C. longa*, *M. oleifera*, or *R. officinalis* in diets of Nile tilapia can be helpful for improvement of the immune response towards the emerging diseases.
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1. Introduction

Nile tilapia, *Oreochromis niloticus* is one of great consequence cultured fish worldwide **(Davlin, 1991 & Pullin, 1997)**. *Aeromonas hydrophilia* is among the dangerous pathogenic bacterial pathogens of fish that encountered in great losses of tilapia culture in Egypt **(Karunasagar *et al*., 2003)**. As well, it was found that the use of antimicrobials showed reduced abilities in the prevention or control of fish diseases **(Subasinghe, 1997)**. Also, the continuous application of broad-spectrum antibiotics will lead to the development of resistant strains that are difficult to be controlled, so finding alternatives for antibiotics is an urgent need.

Turmeric (*Curcuma longa*), is one of *Zingiberaceae* family (**Aggarwal *et al*., 2007 & Chan *et al*., 2009**) and is intensely utilized as a food preservative, and beneficial coloring material in several countries especially India. Curcumin is the major active ingredient of *C. longa* (**El-Bahr *et al*., 2007 & Hatcher *et al*., 2008**). Several more values of using turmeric as feed additives. Turmeric can improve the immune response of various fishes in the face of the invading pathogens. **Sahu *et al*. (2008)** have demonstrated that turmeric can improve the immune response and produce prolonged protection of rohu, *Labeo rohita* infected with *A. hydrophila*. Additionally, **Sivagurunathan *et al*. (2011)** have documented that feeding *Zingiber officinale* and *C. longa* together has proven to enhance the immune response of *Cirrhinus mrigala* challenged with *Pseudomonas aeruginosa (Ps. aeruginosa)*. As well, the supplementation of 0.50% turmeric improved the growth parameters and help in the protection of fish against *Ps. fluorescens* (**Mahmoud *et al*., 2014**). In *Common carp*, **Al-Faragi and Hassan (2017)** found that the growth rate was improved and with increased survival rate when challenged with *Flavobacterium columnaris* when turmeric was supplied in fish diets. Furthermore, for *O. niloticus*, Curcumin has proven to have immunomodulatory with antimicrobial properties against *Vibrio alginolyticus* (**Elgendy *et al*., 2016**). For fish health status, **Yusuf *et al*. (2017)** demonstrated that the supplementation of turmeric in

diets of *O. niloticus* help in improvement of growth parameters, intestinal fold length, and lowering the fecal coliform count in relation to total bacterial count.

Rosemary *(Rosmarinus Officinalis* L.) (family Lamiaceae) is frequently used as a spice with a desirable flavor so can be used in food processing. The rosmarinic and carnosic acids are the most important ingredients of *R. officinalis* with antioxidant and antimicrobial activities (**Erkan *et al*., 2008**).

The use of dried leaves of rosemary increases the protection against *Streptococcus agalactiae* (**Zilberg *et al*., 2010)** and *Streptococcus iniae* in *O. niloticus* (**Abutbul *et al*., 2004**). Furthermore, they can boost the health condition of Seabass (**Yilmaz *et al*., 2012**), improved the growth, disease resistance and immunity of *O. mossambicus* (**Ergün *et al*., 2011**).

Moringa oleifera (family *Moringaceae*), is possessing a wide range of medical advantages with high nutritional value (**Tahany *et al*., 2010**). *M. oleifera* leaves can be incorporated as a protein source in fish diets (**Chiseva, 2006**) with substantial bactericidal activity (**Caceres *et al*., 1991 & Suarez *et al*., 2005**). *M. oleifera* leaves can be administered as protein alternate in the diet of *L. rohita* and *Clarias gariepinus* up to inclusion level of 10% (**Bello and Nzeh, 2013; Arsalan *et al*., 2016; Ezekiel *et al*., 2016 & Mehdi *et al*., 2016**). In addition, seed meal of *M. oleifera* has been successfully used as a protein source in *O. niloticus* diets (**Hashem *et al*., 2017**). As well, **Puycha *et al.* (2017)** documented that the supplementation of leaves of *M. oleifera* helps in growth improvement and efficient utilization of feed of *Pangasius bocourti*.

In this context, the beneficial effects of leaves of three medicinal plants; *Curcuma longa*, *Rosmarinus officinalis*, and *Moringa oleifera* on the non-specific immune response of Nile tilapia and its survival towards *A. hydrophila* were assessed.

2. Materials and Methods

1. Diet preparation: -

Rosemary (*R. officinalis*), Moringa (*M. oleifera*), and Turmeric (*C. longa*) plant leaves were obtained from Kemet Co. (Egypt). Leaves were dried, crushed and minced each alone then mixed with isonitrogenous (25 % protein) and isoenergetic diets with a concentration of 1% of each plant. The mixture was pelleted and let to air dry then stored in a refrigerator at 4°C until use.

2. Fish and Experimental setup: -

A total of 240 obviously healthy *O. niloticus* (with average body weight 35±5 g / fish) were gotten from Central Laboratory for Aquaculture Research (CLAR), Abbassa, Abo-Hammad, Sharkia, Egypt. Fish were transported to Fish health and Diseases Laboratory and were kept 14 days under observation for acclimation in glass aquaria (40×60×50 cm) supplemented with continuous aeration. pH should be maintained at 7±1 and temperature at 25±1°C. One-third of the water column was replaced daily.

Fish were grouped into 4 treatments with 3 replicates (20 fish/aquaria); **group 1 (T1):** Fish fed on basal diet without any additives (control group), **group 2 (T2):** Fish fed diet + 1% *C. longa*, **group 3 (T3)**: Fish fed diet + 1% *M. oleifera*, and **group 4 (T4):** fish fed diet + 1% *R. officinalis*. Fish were fed 3 % of their body weight and the diets were offered twice per day. The experiment continued for 8 weeks.

3. Blood samples: -

Fish were anesthetized with Tricaine Methanesulfonate (MS 222) and blood was biweekly sampled from four fish of each group from the caudal veins **(Lied *et al*., 1975)** into clean dry and the other one without anticoagulant was used for preparation for sera separation. Samples were centrifuged 3000 rpm for 10 minutes for serum separation.

3.1. Determination of serum proteins: -

Total protein (g/dL) **(Lowry *et al*., 1951)** and albumin (g/dL) (**Doumas *et al*., 1971)** were assayed. As well, globulin (g/dL) was calculated by subtraction of albumin from total protein.

3.2. Determination of immune parameters:

Serum lysozyme activity was measured spectrophotometrically **(Schaperclaus *et al*., 1992** & **Gopalakannan and Arul, 2006)**. Respiratory burst activity was measured by the reduction of Nitro Blue Tetrazolium (NBT) **(Graham and Secombes, 1990** & **Stasiak and Baumann, 1996)**.

4. Challenge test:

4.a. Preparation of the bacterial strain: -

A. hydrophila was previously isolated from the liver of diseased *O. niloticus* from Abbassa fish farm, and the identified strain was kindly provided by Fish health Laboratory, Central Laboratory for Aquaculture Research, (CLAR), Abbassa, which was used for the challenge test.

The bacterial isolate was then sub-cultured in brain heart infusion (BHI) broth at 28 C for 24 hrs. Bacterial pellets were then captured after centrifugation of the broth solution. Bacterial pellets were then suspended in sterile physiological buffer saline (PBS) solution and adjusted at McFarland No. 0.5 (1.5×10^8 CFU/ml).

4.b. Experimental challenge: -

After the end of the experiment, fish in all groups were (IP) intraperitoneally challenged with the prepared *A. hydrophila* strain (0.2 ml of per fish) (**Wakabayashi *et al*., 1981 & Badran, 1987)**.

Daily mortalities were recorded for 10 consecutive days and the relative percent survival

(RPS) was estimated **(Amend, 1981)** with this formula;

RPS = 1 - (% mortality in challenged fish / % mortality in control fish) x 100.

Statistical analysis:

Serum measurements were analyzed (means ± SD) using one-way analysis of variance (ANOVA) using (software SPSS version 17) (SPSS Inc., Chicago, IL, USA). Differences between means were determined and compared using Duncan's test and considered significant at $p < 0.05$.

3. Results

1. Blood parameters:

It was found that serum proteins (total protein, albumin, and globulin) values in all treatments were significantly elevated than control one (**Table 1** and **Fig. 1, 2 & 3**).

By the end of the 8th week, there was a substantial elevation ($P < 0.05$) in serum total protein values (g/dL) in the group fed on diet with *C. longa*, *M. oleifera*, and *R. officinalis* when compared with control group (3.85±0.03, 3.76±0.02, 3.57±0.02, and 2.74±0.04 respectively) and the albumin values (g/dL) were (1.77±0.15, 1.55±0.08, 1.50±0.01 and, 1.27±0.03 respectively). Meanwhile, the globulin values (g/dL) were (2.48±0.04, 2.26±0.03, 2.23±0.02 and 1.43±0.03 respectively).

Table 1: Effect of different treatments on serum total protein, albumin & globulin of *O. niloticus* after the end of the feeding experiment.

Treatment	Serum total protein (g/dL)	Serum albumin (g/dL)	Serum globulin (g/dL)
T1	2.74±0.04c	1.27±0.03c	1.43±0.03c
T2	3.85±0.03a	1.77±0.15a	2.48±0.04a
T3	3.76±0.02a	1.55±0.08b	2.26±0.03b
T4	3.57±0.02b	1.50±0.01b	2.23±0.02b

Means with the same letters within the same column are not significantly different at $P < 0.05$.

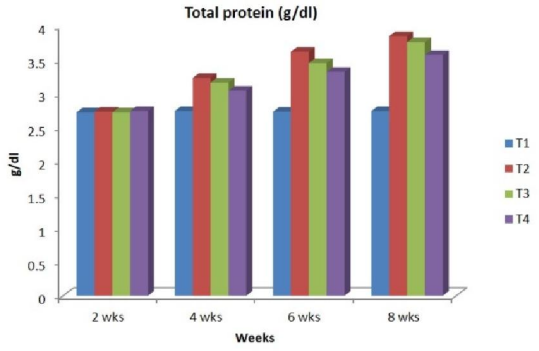

Fig. 1: - Total protein values (g/dL) in sera of different experimental treatment groups of Nile tilapia.

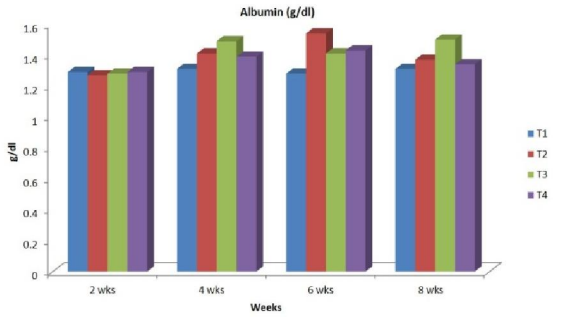

Fig. 2: - Albumin values (g/dL) in sera of different experimental treatment groups for Nile tilapia.

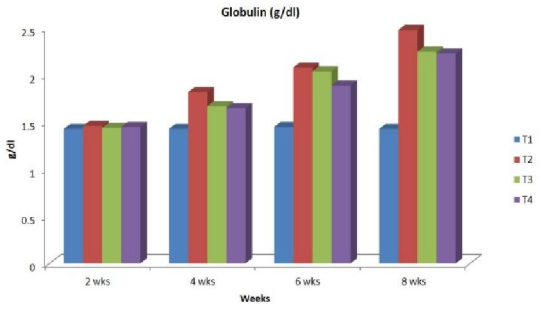

Fig. 3: - Globulin values (g/dL) in sera of different experimental treatment groups for Nile tilapia in comparison to control one.

2. Immune parameters: -

2.a. Serum lysozyme activity: -

Our results revealed that T2, T3, and T4 groups enhanced the lysozyme activity from 2nd week until the end of the experiment in relation to the control group (**Table 2** and **Fig. 4**). Additionally, by the end of 8th week of feeding, curcumin group (T2) showed elevated serum lysozyme activity over the other treatments (T3, and T4) and control group (T1) (3.46±0.03, 3.27±0.02, 3.16±0.02 and 1.30±0.01 respectively).

2.b. Respiratory burst activity by measuring Nitro blue tetrazolium activity (NBT):

The results of NBT in this study revealed that the T2 group was appreciably elevated than other groups (T3 and T4). By the end of 8th week, the results were (0.678±0.01, 0.655 ±0.01 and 0.632±0.01) ($P < 0.05$) (**Table 2** and **Fig. 5**).

However, the best results obtained from moringa group by the end of the 8th week in relation to the control one (0.655±0.01 and 0.510±0.01 respectively).

Table 2: Effect of treatment groups on NBT and lysozyme values at the end of the experiment for *O. niloticus*.

Treatment	NBT (mg/ml)	Lysozyme (µg/ml)
T1	0.510±0.01 d	1.30±0.01d
T2	0.678±0.01a	3.46±0.03a
T3	0.655±0.01 b	3.27±0.02a
T4	0.632±0.01 c	3.16±0.02b

Means with the same letters within the same column are not significantly different at $P < 0.05$.

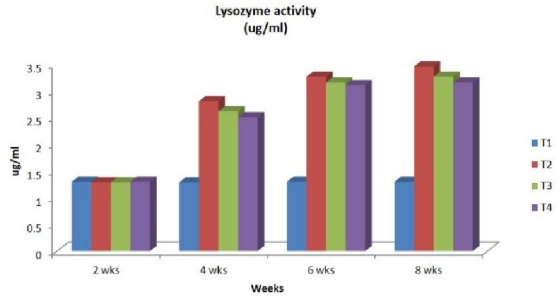

Fig. 4: - Lysozyme activity (µg/ml) of different treatment groups for Nile tilapia sera.

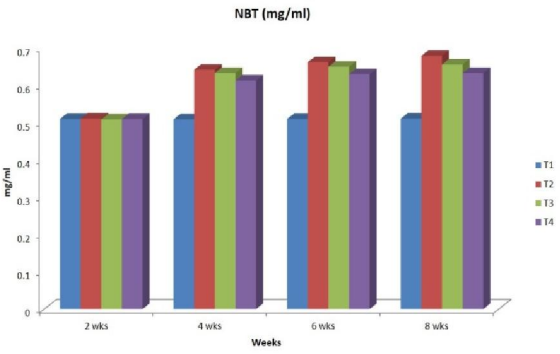

Fig. 5: - NBT values (mg/ml) of different treatment groups in sera of Nile tilapia**.**

***3.* Resistance to challenge test: -**

The results of RPS gave good protection to *O. niloticus* which were 10 % in T1 (control), 75% in group T2, 70% in T3 and 65% in T4 after challenge with the virulent *A. hydrophila* strain (**Table 3**). The best protection was obtained by Curcumin group followed by Moringa then Rosemary.

Table 3: -Relative percent survival (RPS%) after a challenge by *A. hydrophila*.

Treatment	Mortality rate %	RPS%
T1	90%	10%
T2	25%	75%
T3	30%	70%
T4	35%	65%

Discussion

Immunostimulants not only enhances the immune system but also help in preventing infectious diseases **(Watanuki *et al.*, 2006)**. Phytobiotics, medicinal herbs possess immune-stimulating abilities with proven bactericidal activities against numbers of pathogenic bacteria affecting man, animal, and fishes and are possible alternatives to antibiotics (**Khan *et al.*, 2009**).

Serum proteins (albumin, and globulin) have a substantial part in fish immunity **(Kumar *et al.*, 2007)** and the rise of their values are an important indicator that fish have excellent humoral immunity of fish (**Wiegertjes *et al.*, 1996**), and they were increased especially when plant extracts were incorporated in fish diets (**Misra *et al.*, 2006**).

Our findings indicate an improvement of the fish health status and these results were in concordance with that of **Bairwa *et al.* (2012), Basha *et al.* (2013)** and **Sahu *et al.* (2007)** in *L. rohita*. As well, a significant increase in these values was documented in *Cyprinus carpio* (*C. carpio*) fed on diets containing Chinese herbs (**Yuan *et al.*, 2007**). Nevertheless, the best values of serum total protein were obtained from curcumin group which agrees with **El-Bahr and Saad (2008)**, **Elgendy *et al.* (2016)**, and **Hassan *et al.***

(2018) who attributed the preferred effect of turmeric to its potent antioxidant and hepatoprotective properties. Additionally, **Abdel Zaher *et al*. (2009)** illustrated that diet incorporated with turmeric seed meal considerably improved the globulin value in Nile tilapia serum. The elevation of serum globulin is strongly associated with a potent innate immunity of fish (**Nayak *et al*., 2004**).

The lysozyme, one of the most important elements of fish defense mechanisms and it possesses its action by the activation of the complement system and phagocytosis (**Magnadóttir, 2006)**. Additionally, lysozyme possesses bactericidal activity (**Saurabh and Sahoo, 2008**). Our findings were parallel to that obtained by **Ardo *et al*. (2008)** who documented an increase the lysozyme activity in Nile tilapia fed on *Astragalus membranaceus* and *Lonicera japonica* herbs each alone or together after one week of feeding.

NBT assay is predominantly measurement of the production of free oxidative radicals by leukocytes in the defense in the face of fish pathogens (**Cook *et al*., 2003 & Sahoo *et al*., 2005)**. The results were in parallel to that of **Antony *et al*. (1999)** who reported that Curcuminoids have good inhibitory effects on reactive oxygen species (ROS) production. Furthermore, the augmentation of the fish immune system fed on Curcumin diet was attributed to through activation of secretion of the digestive enzymes **(Rojtinnakorn *et al*., 2012).** A similar finding was defined by **Richter *et al*. (2003)** who documented that *M. oleifera* leaf can replace about 30% of fish meal in the diet of *O. niloticus*.

It was clear that immune response of fish was good and that appear from survivability and protection that may be attributed to polysaccharide in *C. longa*, which had been described to display the phagocytic activity **(Gonda *et al*., 1992).** In the field of aquaculture, Curcumin showed antibacterial activity against fish pathogenic bacteria in *O. niloticus* **(Muniruzzanian and Chowdhury, 2004 & Rattanachaikunsopon and Phumkhachorn, 2010)** and increase serum bactericidal activity and activate phagocytosis **(Sahu *et al*., 2008)**. However, Moringa groups gave good protection and survival rate which agree with the findings of **Hussein (2016)**. As well, Curcumin mechanism based on suppression of bacterial cell proliferation (**Rai *et al*., 2008**) and disruption of prokaryotic cell division (**Kaur *et al*., 2010**).

Conclusions

Phytobiotics are not only served as a natural substitute of antibiotics overuse but also, they can boost the fish immunity in the face of emerging diseases. From this study, it can be concluded that the leaves of *C. longa*, *R. officinalis*, and *M. oleifera* can enhance the immunity and elevate the protection of *O. niloticus* to *A. hydrophila*. As well, they should be recommended as additives in diets of *O. niloticus,* especially *C. longa* leaves which gave the best results in enhancement of non-specific immunity without the hazardous effects of antimicrobial overuse.

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Conflict of Interest

The authors declare no conflicts of interest.

List of Abbreviations

NBT: Nitro Blue Tetrazolium**, RLP:** Relative level of protection**, MS 222:** Tricaine Methanesulfonate 222**, RBT:** Respiratory burst activity, **BHIA:** Brain heart infusion agar, **PBS:** Physiological buffer saline.

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