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IMMUNOMODULATORY EFFECTS OF THYME AND FENUGREEK IN SHARPTOOTH CATFISH, *CLARIAS GARIEPINUS*

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ABSTRACT

The aim of this study was to investigate the modulation effects of thyme and fenugreek as food additives on sharptooth catfish immunity. Three diet regimes, a basic (control); Thyme diet (1%) and fenugreek diet (1%) were formulated and used to feed fish for 30 successive days. Half of the fish were used to investigate some of the immune parameters as differential leucocytic counts, serum globulins, phagocytic activities, phagocytic index, catalase and glutathione peroxidase. The other half of fish was subjected to challenge infection with *Aeromonas hydrophila* to investigate the disease resistance ability of the fish received the feeding additives. The results showed that, the serum globulins have significantly increased in fish fed with fenugreek, while the total serum protein has significantly increased in fish fed with thyme or fenugreek. Glutathione peroxidase (Gper) and catalase (CAT) increased significantly in the groups fed on fenugreek or thyme diets when compared to the control group. Phagocytic percent and index of fish fed on fenugreek were significantly higher than those of the control and thyme groups. Monocytes were significantly increased in all treated groups, while lymphocytes were significantly decreased in all treated groups. Weight gain of *C. gariepinus* was significantly lower in the group fed on thyme than in the control group, while it was insignificantly higher in fish fed fenugreek. After 30 days of feeding, fish were challenged with *A. hydrophila*, the cumulative mortalities were 40% and 26.67 % in fish fed on diet supplemented with 1% thyme and fenugreek, respectively, compared to 66.7% in the control group. It was concluded that thyme and fenugreek are be able to positively stimulate the immune system of *C. gariepinus* and decrease mortality rate in fish challenged with *A. hydrophila*.

Key words: *Catfish, Thyme, Fenugreek, Immunostimulant effect.*

INTRODUCTION

Fish are exposed to pathogenic microorganisms since they live in an unfavorable environment (Yunxia *et al.,* 2001). Antibiotics and chemotherapeutics have been used to prevent or control bacterial infections in aquaculture for about 20 years (Sakai, 1999). Unfortunately, antibiotics treatment is not successful and sustainable due to increase antibiotic-resistant in bacteria, negative effects on the indigenous microflora of juveniles or adult fish (Balfry and Higgs, 2001), accumulation of antibiotic residues in fish tissue and environment causing human and animal health issues. Vaccination is an effective prophylactic treatment for infectious diseases in fish culture, but it may be very expensive and stressful to fish. A single vaccine is effective against only one specific type of pathogen, but limits the effectiveness for wide range of pathogens due to the complex antigenic structure (Ardo *et al.,* 2008). Therefore using natural food additives has been important to minimize these adverse effects (Abd El-Latif *et al.,* 2004).

In fish culture, there are various active components such as *Allium sativum* (garlic), *Zingiber officinale* (ginger), *Curcuma longa Linn* (turmeric), *Trigonella foenum-graecum* (fenugreek) and others which have been reported to promote various effects like higher growth rates (Shalaby *et al.,* 2006), appetite stimulation, antistress, immune functions (Dügenci *et al.,* 2003; Dorucu *et al.,* 2009; Ergün *et al.,* 2011), skin coloration (Yılmaz and Ergün, 2011), egg hatching rates (Yılmaz and Ergün, 2012a), hematological and biochemical status (Yılmaz and Ergün, 2012b) and also increase disease resistance (Yılmaz *et al.,* 2012; Yılmaz *et al.,* 2013). Several studies have showed that oral administration of fenugreek in *Labeo rohita* and *Oreochromis mossambicus* (Paul *et al.,* 2004; Mostafa *et al.,* 2009), rosemary in *O. niloticus* (Abutbul *et al.,* 2004; Zilberg *et al.,* 2010), thyme, rosemary and fenugreek in *O. mossambicus* (Ergün *et al.,* 2011) improved

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growth performance, disease resistance and increase immunity.

The aim of the present study was to investigate the effects of thyme and fenugreek supplementation diet on some immunological parameters and disease resistance of sharptooth catfish *C. gariepinus* to *Aeromonas hydrophila.*

MATERIALS AND METHODS

Fish

200 live African sharptooth catfish, *Clarias gariepinus* were obtained from a private fish farm at El-Dakahlea Government and transported under hygienic control to the wet laboratory, Faculty of Veterinary Medicine, South Valley University, Qena. Fish were acclimated for 21 days in porcelain aquaria (260×65×70 Cm) according to the protocol of maintaining bioassay fish as was previously described (Ellsaesser and Clem, 1986).

Diets and feed additives

Three different diets with or without feed additives in the form of dry pellets, representing 3 diet variants, were formulated to be used in feeding of fish. The experimental diet was prepared using a hand palletizer. The basic diet, (control) was formulated of grounded yellow corn 34.9%, vegetable bean meal 28.6%, fish meal 17.0%, wheat bran 9.3%, mineral mixture 1.7% and vitamin mixture 1.0%. The two other experimental diets were formulated as a Fenugreek diet (basic diet with 1% of *Trigonella foenum-graecum* fenugreek) and thyme diet (basic diet with 1% *Thymus vulgaris,* thyme). Fenugreek and thyme were obtained from the local market in the form of powder.

Experimental design

One hundred and twenty acclimated apparently healthy African sharptooth catfish, *C. gariepinus* were randomly selected with a body weight range of 40 – 90 g to be used for the experiment groups. The experiment was done in 3 replicates (40 fish each) where fish in each replicate were divided into 2 sets, one set was used for evaluating the immune blood parameters of fish (blood set) while the other set was used for challenge experiment (challenge set). The blood set has three equal groups that represent the three nutritional treatments (corresponding to the three diets variants) with 15 fish in each. Control group received the basic diet, the second group received the thyme diet and the third group received the fenugreek diet. The challenge set has the same three groups as the first set, but with 2 additional non-challenge control groups. These two groups received the basic diet, where one group of which was injected with distilled water, while the other one remained un-injected. Each group was fed twice daily for 30 successive days. At the end of the 30 days feeding; blood samples were taken from the caudal vein from each group to measure the immune and hematological parameters.

Hematological studies

By the end of the feeding experiment, fish of all groups of the blood sets were anasthetized using tricaine methanesulfonate (MS-222, Argent Chemical Labs, Redmond, Washington, USA), and blood samples were collected from the caudal vein and the samples were divided into 2 portions. Sodium citrate (3%) was added to one part for differential leukocytic count and phagocytic assay. The second part of blood sample was allowed to clot, centrifuged for serum separation. Total proteins and albumin were determined by "Protein" and "Albumin" kits (Spectrum, Egyptian Company for Biotechnology, Obour City, Cairo, Egypt) according to manufacture's recommedation. Globulin levels were calculated mathematically by subtracting albumin value from total protein value and expressed in g/dl according to Khalil, (2000). Catalase assay was calculated according to Aebi (1984) using catalase kits (Biodiagnostic test kits). Glutathione peroxidase were measured according to Paglia and Valentine (1967) using Biodiagnostic test kits.

Phagocytosis assay

Phagocytic assay was done according to Kwahara *et al.* (1991). Fifty µg *Candida albicans* culture was added to 1 ml of citrated blood collected from fish and shaken in water bath at 25°C for 5 hours. Smears of the blood were then stained with Giemsa stain. Phagocytosis was estimated by determining the proportion of phagocytic cells which contained intracellular yeast cells in a random count of 200 phagocytes (Abu-Elala *et al.,* 2013) and expressed as percentage of phagocytic activity (PA). The number of phagocytized yeast cells was counted in the phagocytic cells to estimate the phagocytic index (PI).

Experimental challenge test

Bacterial strain: An *Aeromonas hydrophila* strain isolated from clinical cases of infected *C. gariepinus* showing signs of septicemia, were used in the experimental infection. The strain was identified by Gram stain, motility test, and various biochemical characters according to Austin and Austin (2007) and preserved in glycerol at -80°C. The *Aeromonas hydrophila* strain was passed three times in healthy sharptooth catfish through intraperitoneal injection before using for experimental challenge.

Bacterial challenge suspension and counts

Colony forming units (cfu) counts in bacterial suspensions were determined using spectrophometery optical density values at wavelength of 600 nm and standard-plate-count method with ten-fold serial dilution (Elkamel and Thune, 2003).

Experimental challenge

The challenge groups of each challenge set were challenged through I/P injection with 0.5 ml of sterile saline containing 0.3×10^6cfu/ml pathogenic strain of *Aeromonas hydrophila*. One group of the challenged set was injected with sterile saline (sham control) and another group was left un- injected as a control. The clinical signs, post mortem lesions and mortalities were recorded daily for up to two weeks. Re-isolation and identification of bacteria was done from freshly dead fish as mentioned above.

Statistical analysis:

Statistical analysis was carried out using of S.A.S. (2001) program, version 8.2. The significance differences between treatments means were tested by Duncan Multiple Range test (Steel and Torrie, 1982). Data was presented in means± standard error of means (S.E.M). Level of significance was set at <0.05.

RESULTS

Serum total protein and globulin values were significantly higher in fish supplement with thyme and fenugreek when compared to the control group. Higher value of globulins was observed in fish supplemented with fenugreek. Phagocytic percent and phagocytic index of catfish received fenugreek diet were significantly higher than those of the other groups. Serum of fish fed on diet with thyme and fenugreek showed significant increase in the glutathione peroxidase and catalase activities compared to fish fed on basic diet. There was insignificant increase in total weight gain in fish fed on fenugreek diet compared to the thyme and control groups, but there were significant decrease in total weight gain in fish fed on thyme diet compared to other groups. Cumulative mortalities of catfish challenged with *A. hydrophila* were significantly less in fish fed on fenugreek diets than that of fish fed on thyme or basic diets (table 1).

Table 1: Effect of thyme and fenugreek diets on some haematological and immunological parameters of sharptooth catfish.

Parameter / groups	Basic(control) n=15	Thyme diet n=15	Fenugreek diet n=15
Lymphocyte %	40.75±3.84 [a]	36.25 ±1.015 [b]	35.417 ±0.949 [b]
Monocyte %	34.833±3.214 [a]	37.75±1.045 [b]	38.333±1.548 [b]
Neutrophil %	13.917±2.778 [a]	14.166±0.833 [a]	14.666±0.837 [a]
Basophile %	8.666±1.669 [a]	8.833±0.660 [a]	8.583±0.701 [a]
Eosinophil %	1.833±0.717 [a]	3±0.246 [b]	3±0.213 [b]
Phagocytic %	26.416±3.528 [a]	27.978±1.019 [a b]	29.291±0.568 [b]
Phagocytic index	1.075±0.033 [a]	1.122±0.027 [a b]	1.190±0.042 [b]
Total protein mg/dl	3.17±0.202 [a]	3.80±0.196 [b]	4.29±0.157 [c]
Albumin	1.17±0.07 [a]	1.48±0.13 [b]	1.71±0.08 [b]
Globulin	2±0.174 [a]	2.32±0.130 [a b]	2.58±0.172 [b]
Glutathione peroxidase mU/ l	0.31±0.037 [a]	0.48±0.051 [b]	0.52±0.060 [b]
Catalase U/ l	319.28±11.413 [a]	360.78±15.130 [b]	377.62±13.144 [b]
total weight gain gm	2.833±0.548 [a]	1.583±0.259 [b]	3.166±0.423 [a]
Cumulative Mortality percent	66.7%	40%	26.67%

Means in the same raw with different letters are significantly different ($p<0.05$).

DISCUSSION

Strengthening the defense mechanism of fish through prophylactic administration of natural plant products is one of the most promising methods of controlling diseases in aquaculture (Agarwal and Singh 1999; Devasagayam and Sainis 2002) and is considered a promising alternative to chemotherapy and vaccines (Secombes, 1994). Using natural plants as immunostimulants in fish is more useful than using of antibacterial drugs that cause adverses side effects for fish, environment and consumers. Therefore, we investigated the effects of thyme (*Thymus vulgaris*) and fenugreek (*Trigonella foenum-graecum*), as herbal medicine plants on some immunological, hematological parameters and defense mechanisms of sharptooth catfish, *Claries gariepinus* after 30 days of feeding.

Results indicated that fenugreek improved body weight gain of *C. gariepinus*. These results could be attributed to the action of fenugreek as appetizer (Coutteau *et al.,* 2011). In addition (Frankic *et al.,* 2009) reported herbs stimulate the secretion of pancreatic enzymes, important factors in nutrient digestion and assimilation. This can explain the higher, though not significant, weight gain obtained

with fenugreek supplementation. Several studies have reported that oral administration of fenugreek in *Labeo rohita* and *Oreochromis mossambicus* (Paul *et al.*, 2004; Mostafa *et al.*, 2009 and Ergün *et al.*, 2011) improved growth performance, disease resistance and increased immunity.

The decrease in fish weight gain observed with thyme supplemented diets could be attributed to the antimicrobial activity of thyme which may be masked by diet composition and/or environment, in that no effect of thyme on growth performance (Lee *et al.*, 2003b). These results are supported by those of Saleh *et al.* (2014) who showed that addition of thyme at the selected levels had no beneficial value on live body weights or body weight gain of broiler chicken at any time along the experimental period.

Several types of leukocytes participate in the cellular immune response, including lymphocytes, monocytes, granulocytes (neutrophils, eosinophils and basophils), and cytotoxic cells (Nakanishi, 1999). Fernández *et al.* (2002) recorded that macrophages can be used as indicators to evaluate the health of fish. These cells play an important role in killing pathogens as in immune response. In this study, monocyte have significantly increase in catfish fed diet contains 1% thyme or fenugreek when compared to basic diet which is in line with the results of Gültepe *et al.* (2014) who showed that monocyte was elevated in *Oreochromis mossambicus* fed with diets supplemented with thyme, rosemary and fenugreek.

Either increase or decrease in different leucocytes was pronounced in fish because all leucocytes were calculated as a percentage of the whole leucocytic count which constitutes 100 %. The decrease of the percentage of lymphocytes in fish groups may be attributed to the significant increase of other leucocytic cells.

The present study indicated that both phagocytic % and phagocytic index of macrophages significantly increased in sharptooth catfish fed on fenugreek at concentration 1% in comparison with fish fed on thyme or basic diets. The enhancement of phagocyte function is one of the most immediate and key effects produced plant extracts on the host immune system of fish. This result supported by Gültepe *et al.* (2014) who reported, *Oreochromis mossambicus* fed with a diet supplemented with fenugreek significantly increased phagocytic activity compared to control group. Fenugreek is rich in flavonoids (such as apigenin, kaempferol and quercetin) and saponins (such as diosgenin and yamogenin) that protect cells from oxidative damage (Kaviarasan *et al.*, 2004) and have immunostimulatory properties (Bin-Hafeez *et al.*, 2003). On the other hand, thyme has no significant effect on phagocytosis. These results are in line with Pérez-Rosés *et al.* (2015) who investigated the in vitro activity of thyme on phagocytosis by human neutrophils. They showed inhibition in phagocytosis.

Blood serum protein is a fairly labile biochemical system, precisely reflecting the condition of the organism and the changes happening to it under influence of internal and external factors. The serum protein level is an important indicator of humoral defense system of fish and increases especially in the fish fed with plant extracts (Misra *et al.*, 2006). In the current study total protein in serum was significantly higher in fish fed on diet containing fenugreek or thyme when compared to basic diet. An investigation done by Toghyani *et al*. (2011) proved that serum total protein and globulin concentrations increased significantly in birds fed diet supplemented with thyme powder. They attributed this increase to the components of thyme oils, thymol and carvacrols which possess potent antioxidant properties and consequently, elevate immune responses of chicks. Over and above, Tollba *et al.* (2010) found that mixture of volatile oils including thyme, oregano, *C.verum* and capsicum added to two groups of chicks diets at 1 or 2 g /kg feed in the experimental period which lasted at 12 wks of age, increased the total protein significantly as well as albumin and globulin comparing to un-supplemented control group. Elevated serum total protien level may be due to high level of protein and other nutrients in *T. foenum-graecum L* seeds. This could be supported by the idea of Hoffman (1966) who cited that, serum protein levels are sensitive to nutritional influences.

Globulins concentration is significantly higher in fish fed on *T. foenum-graecum*, supplement compared to the other groups. The increase in globulin levels may be due to the immune stimulant effect of *T. foenum-graecum* supplementation in his work, that is in line with the findings of Abdel Zaher *et al.* (2009) who showed that, fish fed diet containing different levels as 0.5, 1 and 1.5 % of *T. foenum-graecum* seeds meal, increased the serum globulin significantly. The increase in the serum globulin levels is thought to be associated with a stronger innate response in fish (Wiegertjes *et al.*, 1996). The increase in globulin was suggested previously to indicate that, fish are immunologically strong (Nayak *et al.*, 2004).

Glutathione peroxidase and catalase activities in blood serum showed significant increase in fish groups fed on diets contained fenugreek and thyme compared to the control group. Fenugreek and thyme been reported to possess strong antioxidant properties (Yadav and Sehga l1997, Komes *et al.*, 2011). Thyme has a strong antimicrobial and antioxidant activity due to its very high contents of thymol, p-cymene, carvacrol, eugenol and 4-allylphenol (Lee *et al.*, 2005; Rota *et al.*, 2008). Biphenyl compounds antioxidants in fenugreek and thyme are also strong

antioxidants and are involved in preventing lipid peroxidation (Chatterjee *et al.,* 2009). Fenugreek supplementation in diet resulted in lowered lipid peroxidation and increased level of antioxidants in alloxan diabetic rats (Ravikumar and Anuradha, 1999).

In this study, stimulation of the immune system of catfish as a result of feeding of thyme and fenugreek have positively impacted the resistance of fish to *A. hydrophila* infection as was indicated by the significantly lower mortality rates of fish challenged with virulent *A. hydrophila*. It was reported that fenugreek also increase disease resistance against *Streptococcus agalactiae, S. iniae* and *Aeromonas hydrophila* in *Oreochromis sp.* (Zilberg *et al.,* 2010) and *O. niloticus* (Mostafa *et al.* 2009), respectively. Lee *et al.* (2005); Rota *et al.* (2008) reported that thyme has a strong antimicrobial (assessed as inhibitors of microbial growth). The antifungal and antibacterial activity exhibited by the Thymus genus essential oil has been demonstrated by several researchers (Karaman *et al.,* 2001; Rasooli and Mirmostafa 2003).

In conculsion, Results of the current study proved that dietary supplementation of thyme and fenugreek enhanced the immune response and antioxidant effect of catfish. This modulation of the fish immunity has greatly enhanced the resistance of challenged fish to *A. hydrophila.* So, these herbs and spice can be used as immunostimulants to enhance the immune responses and increase the resistance of *C. gariepinus* against *A. hydrophila* and moreover they have economical benefits.

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المحفزات المناعية للزعتر والحلبة فى الاسماك القطية (القراميط)

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ان الهدف من هذه الدراسة هو التحقق من حدوث تنشيط فى الجهاز المناعى لاسماك القراميط عند تغذيتها على الزعتر والحلبة كمكملات غذائية. وقد تم وضع ثلاثة أنظمة غذائية، العليقة الضابطة والعليقة المحتوية على الحلبة والزعتر. وتم تغذية الاسماك عليها لمدة ٣٠ يوما متتابعة. وقد تم استخدام نصف عدد الأسماك لفحص التأثير على بعض المعايير المناعية مثل نشاط الخلايا الاكولة والجلوبيولين ، الكتاليز والجلوتاثيون. بينما تعرض النصف الأخر من الأسماك الى العدوى الصناعية بميكروب الايروموناس هيدروفيلا للتاكد من زيادة قدرة الأسماك التى تم تغذيتها على المكملات الغذائية على مقاومة الامراض. أوضحت النتائج وجود زيادة معنوية فى الجلوبيولين فى الأسماك التى تغذت على الحلبة، بينما زيادة نسبة البروتين الكلى فى الأسماك التى تغذيت على عليقة بها حلبة أوالزعتر. هناك زيادة معنوية فى الكتاليز و الجلوتاثيون فى المجموعات التى تغذيت على عليقة تحتوى على الزعتر أو الحلبة مقارنة بالمجموعة الضابطة. وقد ظهرت زيادة معنوية فى نشاط الخلايا الأكولة فى مجموعة الأسماك التى تغذيت على الحلبة مقارنة بكل من المجموعة الضابطة والمجموعة التى تغذيت على الزعتر. وهناك زيادة معنوية فى خلايا وحيدة النواة ونقص فى الخلايا الليمفاويه فى كل مجموعات الأسماك عند مقارنتها بالمجموعة الضابطة. لوحظ نقص معنوى فى الوزن فى المجموعة التى تغذيت على الزعتر بينما زيادة غير معنوية فى المجموعة التى تغذيت على الحلبة مقارنة بالمجموعة الضابطة. وقد أوضحت العدوى الصناعية بميكروب الأيروموناس هيدروفيلا ان معدل النفوق كان ٤٠، ٢٦.٦٧ % فى مجموعة الأسماك التى تلقت نظام غذائى يحتوى على الزعتر والحلبة على التوالى مقارنة ٦٦.٧% فى المجموعة الضابطة. وأستنادا الى النتائج وقلة سعر المكملات الغذائية يوصى الباحثون باستخدام الزعتر والحلبة كمكمل غذائي فى عليقة الأسماك للحد من نسبة النفوق الناجمة من بعض مسببات الأمراض.