EFFICIENT USE OF CURCUMIN ON THE IMMUNE RESPONSE OF NILE TILAPIA AGAINST MOTILE AEROMONAS SEPTICEMIA

Hala Ayoub and Mohamed El Tantawy

Fish Health and Management Department, Central Laboratory For Aquaculture Research, Agricultural Research Center, Egypt.

Received 4/ 10/ 2015 *Accepted 10/ 11/ 2015*

Abstract

Turmeric (*Curcuma longa* Linn.) is a native plant of southern Asia and is cultivated extensively throughout the tropical parts of the world. The aim of this study was to evaluate the dietary dosages of turmeric that enhance immune response and disease resistance against the opportunistic pathogen *Aeromonas hydrophila* in *Oreochromis niloticus*. Three different dosages of turmeric at 1, 3 and 5% kg^{-1} feed were given to the fish for 60 days at 4% body weight. At every 14-day interval, blood was collected and serum was separated. At the end of experiment fish was challenged by I/P with 0.2 ml *A. hydrophila* /fish. The mortality (%) was recorded for 10 days post challenge. Some of immune parameters including lysozyme activity and Nitroblue tetrazolium (NBT) were significantly ($P<0.05$) higher on 42 days of feeding of 3.0 g of turmeric per kg of feed. Challenge study indicated 0, 75, 90, and 80% survivability in the group of fish fed with 0, 1, 3 and 5% of turmeric per kg of feed respectively. Feeding of turmeric might have maintained long-term protection in fish by elevating the nonspecific immune system such as Nitroblue tetrazolium (NBT) and lysozyme activity. The result showed that turmeric at a dose of 3% kg^{-1} feed for 60 days provided the greatest protection against challenge by *A. hydrophila*.

Key words: Curcumin – Immnostimulant – Aeromonas hydrophila

INTRODUCTION

The aquaculture industry in Egypt has grown considerably in recent years and is now recognized as a significant supplier of food products for Egyptian consumers. Nile tilapia is one of the most important cultured fish species in many parts of the world including Egypt (Davlin, 1991 and Pullin, 1997). Since tilapia is a top priority fish in the tropics. Motile Aeromonas Septicemia (MAS), caused by *Aeromonas hydrophila* is among the dangerous diseases encountered in freshwater fish culture (Karunasagar *et al.,* 2003). Various

chemotherapeutants have been used for the treatment or prevention of diseases. However, the use of antimicrobial agents in aquaculture has resulted in more resistant bacterial strains. These resistant bacterial strains could have a negative impact on the therapy of fish diseases, human diseases and the environment of the fish farms (Smith *et al.*, 1994). An alternative method of protection is the use of medicinal herbs that can inhibit colonization and exert inhibitory effects against undesired microorganisms. Nowadays herbs or herbal products play a significant role in aquaculture. Many kinds of herbal medicines have been used allover the world to control fish diseases and have produced satisfactory results (Rajendran 1990). Turmeric (*Curcuma longa* Linn.) is a native plant of southern Asia and is cultivated extensively throughout the tropical parts of the world, (Gupta and Balasubrahmanyam, 1998). Many biological activities have been attributed to the extracts of *C. longa* and to its active compound (Curcumin). Milobedzka *et al.* (1910) identified the chemical structure of Curcumin as diferuloylmethane or 1,6-heptadiene-3,5-dione-1,7-bis (4-hydroxy-3-methoxyphenyl)-(1E, 6E). Turmeric is a well known indigenous herbal medicine having many significant biological activities. It is an antiviral, antibacterial, antifungal and an excellent anti-inflammatory herbal product (Baum and Ng, 2004 and Gul *et al.*, 2004). *C. longa* is extensively used as a spice, food preservative and coloring material in India, China and South East Asia. For the last few decades, extensive work has been done to establish the biological activities and pharmacological actions of turmeric and its extracts, (Chattopadhyay *et al.,* 2004). Turmeric extract and Curcumin have also been used widely as a hepatoprotective agent, (Mesa *et al.*, 2000). Little of the research on herbs has investigated matters of immuno-modulation and disease resistance in aquatic animals.

Antibiotics have been used in fish culture as a treatment for bacterial disease, which can cause residue problems if good practice has not been implemented as part of the drug regime. In addition, measures that are more stringent have been exercised by trade counter parts in regard to contamination

with antibiotics. Our objective to evaluate Curcumin as feed additives to enhance immunity in *oreochromis niloticus*.

MATERIALS AND METHODS

Preparation of feeding:

Commercial fish feed containing 25% protein was crouched and divided into four groups. Crude Curcumin was obtained from (kimet Company) minced and were added by 0%, 1%, 3% and 5% to fish feed then pelted and stored for use.

Fish:

A total number of 160 apparently healthy *Oreochromis niloticus* with an average body weight of 30±2g were collected randomly from Central laboratory For Aquaculture Research. Acclimation was done for two weeks, and the fish were divided into four groups (each group in 4 replicates where 10 of the fish were kept in an aquarium (70×40×50 cm). group 1(G1) a fish fed diet free from Curcmin (Control group), group 2 (G2) fish fed diet contained Curcumin 1%, group 3 (G3) fish fed diet contained Curcumin 3% and group 4 (G4) fish fed diet contained Curcmin 5%. In addition to 20 fish used for application treatment, kept as group 5 (G5).

Antibacterial susceptibility test:

The disc diffusion assay (Kirby-Bauer Method) was used to screen the herbal extracts for antibiotic activity (Prescott *et al*., 1990) using the Curcumin ethyl extract.

Challenge test:

A well identified *Aeromonas hydrophila* strain was obtained from Fish Disease Department of Central Laboratory for Aquaculture Research. The strain was activated by cultivation on TSA at 28 ^{o}C for 18 h for 3 cultivates then standards at (1.5 x 10^{8} CFU/ml) and 0.2 ml / fish were injected intra-peritoneal, 20 fish from each group while the control was injected 0.2 ml sterile saline

/fish. Mortality was recorded for 10 days and relative percent of survival were calculated.

Application of Curcumin in treating ulcers:

Twenty *Oreochromis niloticus* fish experimentally infected by *A. hydrophila* through scraping and swapping methods showed skin lesions, tail and fin rot were subjected to feeding by 3% Curcumin containing 25% protein at a level of 4% feed daily for 60 day. The relative percent of survival, skin lesions and healing were recorded.

Blood collection and serum preparation:

Four fish from each treatment were anaesthetized and blood was collected into 3 ml test tube without anticoagulant and allowed to clot for 2 h at room temperature in a slanting position. The tubes were centrifuged at 4000 × g for 20 min by using under cooling centrifuge and the supernatant serum was separated and collected in Eppendroff and stored at -20 °C. Another blood samples with Heparin anticoagulant were collected at the same time.

Lysozyme activity:

A turbidometric assay using lyophilized *Micrococcus lysodeikticus* (Sigma–Aldrich) was followed to determine the lysozyme activity in serum as described by Ellis (1990). Thus, 2 ml of *M. lysodeikticus* at a concentration of 0.2 mg ml $^{-1}$)1 (w ⁄ v) in 0.05 mol l $^{-1}$ sodium phosphate buffer, pH 6.2 (SPB; Sigma–Aldrich) was added to 100 µl of serum sample. As a negative control, SPB replaced serum. The decrease in OD at 450 nm was recorded after 0 and 20 min at 25 °C. A unit of lysozyme activity was defined as the amount of serum causing a reduction in absorbance of 0.001 unit min $^{-1}$.

Nitroblue tetrazolium assay (NBT):

Leukocytes activities were assayed by the reduction of Nitro Blue Tetrazolium, (Andrson and siwicki 1994). 100 µl of heparinised blood from fish of each group was mixed with 100 ml of 0.2 % NBT Solution (Sigma, USA) and incubated for 30 minute at 25 ^{O}C.· After incubation, 50 µl from the above

mixture was added with 1 ml of N'N diethyl methyl formamide (Sigma) and then centrifuged at 6000 x g for 5 minutes. The O.D of the supernatant was measured at 450 nm.

Statistical analysis:

The obtained data were subjected to two-way ANOVA to test the effect of curcumin concentration and days for experiment as the two factors simultaneously tested. The Duncan's multiple range tests to use as a post hoc test to compare between means at P 0.05. The software SPSS version 11.0 (SPSS, Richmond, Virginia, USA) was used as described by Dytham (1999).

RESULTS

Antibacterial susceptibility test:

Ethyl extract of Curcumin showed inhibition zone of 8 mm in comparison with control 5 mm and Ciprofloxacin 17 mm**,** Table (1).

Table 1. Antimicrobial susceptibility test of curcumin and Ciprofloxacin against *Aeromonas hydrophila*.

Antimicrobial substance	Inhibition zoon mm.
Curcumin Ethyl extract	8
Ethanol (Ethyl alcohol) control	5
Ciproloxacin	17

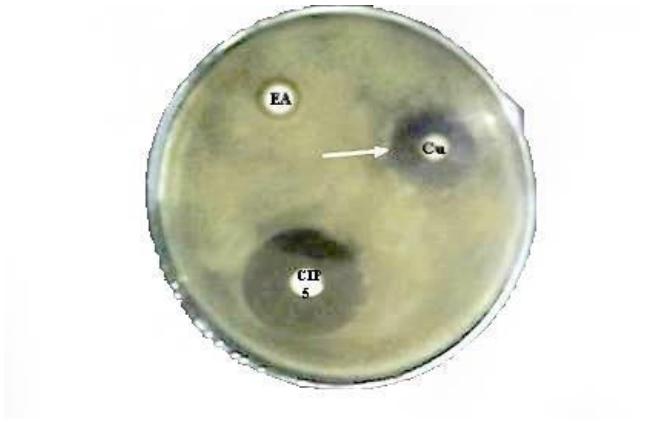

Photo 1. Antibacterial susceptibility test showing 8 mm inhibition zone of Curcumin against *Aeromonas hydrophila*, (arrow).

Challenge test:

It's obvious that group 3 gives more protection to fish against *Aeromonas hydrophila*, Table (2) and (3).

Table 2. Daily death.

Group	Days										Deaths	Survival	RPS.
	1	2	3	4	5	6	7	8	9	10			
G1	0	0	0	0	2	5	7	6	0	0	20	0	0
G2	0	0	0	0	2	2	1	0	0	0	5	15	75
G3	0	0	0	0	0	1	1	0	0	0	2	18	90
G4	0	0	0	0	1	2	1	0	0	0	4	16	80

Table 3. Cumulative death.

Group	Days									
	1	2	3	4	5	6	7	8	9	10
G1	0	0	0	0	2	7	14	20	20	20
G2	0	0	0	0	0	2	4	5	5	5
G3	0	0	0	0	0	1	2	2	2	2
G4	0	0	0	0	1	3	4	4	4	4

Application test:

Mortality percent was 20% and relative percent of survival was 80%, the survivals showed gradual healing of the skin at 10^{th} day post feeding, the fin and tail rot did not progress. Complete healing of the skin at 37 day post feeding, Table (4).

Table 4. Results of application of Curcumin on experimentally infected *Oreochromis niloticus* by *Aeromonas hydrophila* feeding on Curcumin 3% for 42 day.

Application group													
	Dead fish per day										**Deaths**	**Survival**	**RPS.**
Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**			
No of fish	0	0	0	0	0	1	2	1	0	0	4	16	80

Lysozyme activity:

At 14 day G3 gives the highest value of lysozyme activity (2.10±0.28) then G4 (1.98±0.04**)** in compared to control. The highest result in lysozyme was G3 **(**3.09±0.05) at 42 day of feeding experiment as shown in Table (5).

Table 5. Lysozyme activity of *Oreochromis niloticus* sera of groups Curcumin 1, 3, 5% and control (means ±SE) at 14, 28 and 42 days.

Day	**Treatment**			
	Control group	**Test groups**		
	G1	**G2**	**G3**	**G4**
14	1.04±0.02 ^{g}	1.56±0.03 ^{f}	2.10±0.28 ^{d}	1.98±0.04 ^{de}
28	1.72±0.12 ^{ef}	2.61±0.04 ^{c}	2.94±0.04 ^{ab}	1.71±0.07 ^{ef}
42	2.58±0.18 ^{c}	2.70±0.03 ^{bc}	3.09±0.05 ^{a}	1.74±0.05 ^{ef}
Two way ANOVA		*P* value		
Treatment		0.0001		
Day		0.0001		
Treatment X day		0.0001		

Means having the same letters in the same column are not significantly different at ($P < 0.05$).

Nitro Blue Tetrazolium activity:

The highest result in NBT activity was G3 **(**0.7601±0.003) at 42 day of feeding experiment in compared to control and other groups as shown in **Table (6)**.

Table 6. Nitro Blue Tetrazolium activity of *Oreochromis niloticus* heparinised blood of groups Curcumin 1, 3, 5% and control (means ±SE) at 14, 28 and 42 days.

Treatment	**Mean**			
	Control group	**Test groups**		
Day	**G1**	**G2**	**G3**	**G4**
14	0.5808±0.001 ^{g}	0.5417±0.002 ^{h}	0.7435±0.003 ^{b}	0.5261±0.002 ^{i}
28	0.5923±0.003 ^{f}	0.6535±0.003 ^{d}	0.7598±0.004 ^{a}	0.6400±0.007 ^{e}
42	0.5967±0.002 ^{f}	0.6727±0.002 ^{c}	0.7601±0.003 ^{a}	0.6741±0.007 ^{c}
Two way ANOVA	*P* value			
Treatment	0.0001			
Day	0.0001			
Treatment X day	0.0001			

Means having the same letters in the same column are not significantly different at ($P < 0.05$).

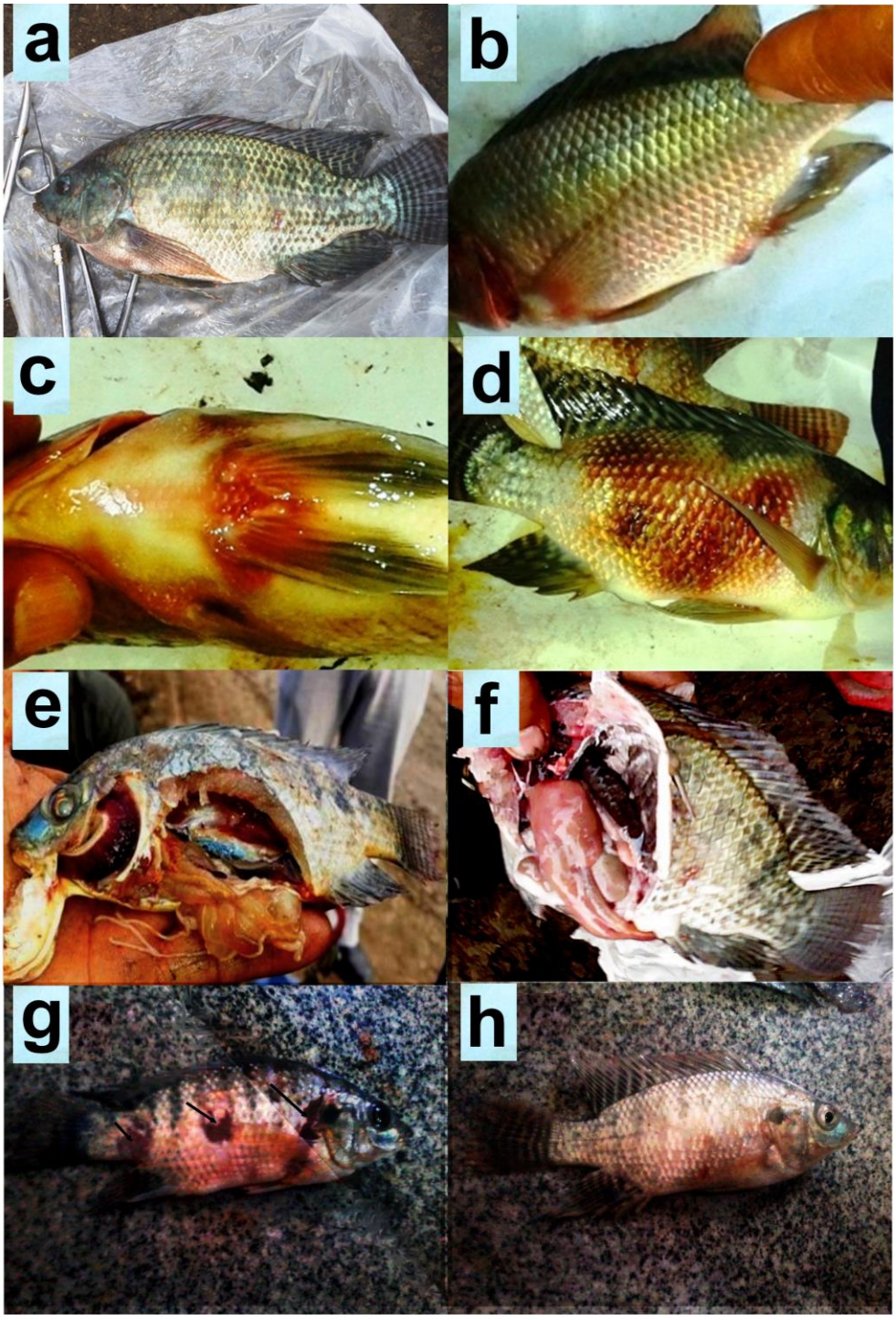

Plate (1):

a: Normal fish.

b: tilapia fish feed on curcumin and challenged by *A. hydophila* showed signs but less degree than that not fed.

c, d: showed hemorrhagic spots on the skin**,** corneal opacity in**.**

e, f: Postmortem of tilapia fish challenged with *A. hydrophila* intra-peritoneal showing septicemia, enlarged liver, spleen and kidney with congestion, intestine flaccid and filled with muco-serous fluid.

g: Tilapia fish showed septicemia, arrows showed skin abrasions and ulcers.

h: Tilapia fish showed healing of skin abrasions and ulcers after treatment by Curcumin 3% feeding.

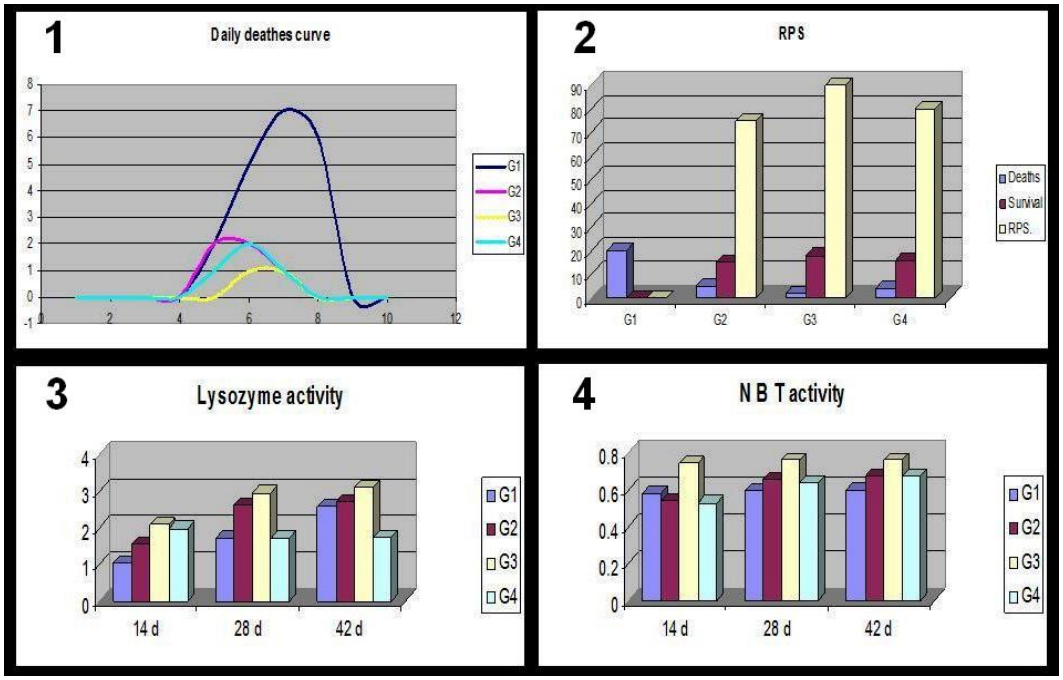

Plate (2):

Diagram (1) showing daily death curve in groups of challenge test.

Diagram (2) showing relative percent of survivability in challenge test.

Diagram (3) showing relation of curcumin percent and mean Lysozyme concentration.

Diagram (4) showing relation of curcumin percent and mean X4 NBT.

DISCUSSION

Bacterial infections are among the important infectious diseases. Hence, over 50 years of extensive researches have been launched for achieving new antimicrobial medicines isolated from different sources. Despite progress in development of antibacterial agents, there are still special needs to find new antibacterial agents due to development of multi-drug resistant bacteria Wise *et al.* (1998).

In this study the ethyl extract of curcumin was 8mm inhibition zone in comparison with ciprofloxacin, 17 mm and ethyl alcohol disc 5 mm and this agree with Abdul Kader and Haniffa (2011). The antibacterial activity of curcumin was discussed by Schraufstatter and Bernt (1949). In this study Curcumin extract resulted in inhibition zone of 8mm against *Aeromonas hydrophila* and this was agreed with that obtained by Lawhavinit *et al*. (2010) that demonstrated the hexane and methanol extracts of *C. longa* had antibacterial effect against 13 bacteria, as *A. hydrophila* and other 12 bacterial strain. Abdul Kader and Haniffa (2011), evaluated the antimicrobial potency of aqueous extract of *Curcuma longa* (Turmeric, rhizome) against pathogenic *Aeromonas hydrophila* isolated from infected fresh-water fish, *Channa striatus* was assessed by disc diffusion assay and resulted in (8 mm) inhibition zone.

In this study The relative percent of survival protection against *A. hydrophila* ranged from 75 – 90 in fish groups fed on curcumin, this indicate that curcumin had antibacterial effect and played role in inhibition of the growth of the bacteria in addition to augmentation of the non specific immune system to overcome the destroying effect of the bacteria and preventing septicemia. The antimicrobial property of turmeric has been attributed to the presence of essential oil, alkaloid, curcumins and other curcuminoids as turmeric oil, turmerol and veleric acid, Cikricki *et al.*, 2008. Saad *et al*., 2013 showed the improving of immune status of sea bass fed ration containing turmeric and was attributed to a significant increase in serum total protein, albumin and globulins (ß-globuline protein fraction).

In this study, application of curcumin showed mortality percent 20% and relative percent of survival was 80%. The survivals showed gradual healing of the skin at 10^{th} day post feeding 3% curcumin, the fin and tail rot don't progress, and complete healing of the skin at 37 day post feeding. The signs were noticed externally on the 5^{th} day after injection as hemorrhagic spots appeared at the site of injection and the lesions progressed subsequently. The infected *L. rohita* individuals were treated with the application of turmeric powder suspension (2g/L^{-1} water) using the dipping method. The infected individuals were allowed to swim in the turmeric water for 10 min daily until the lesions healed completely (21 days), Dhanaraj *et al*. (2009).

Neutrophil and macrophages contain lysozyme and other hydrolytic enzymes in their lysosomes. Fish macrophages can also produce nitric oxide (NO), which form potent bactericidal agents like peroxynitrites and the hydroxyl free radical (Secombes, 1996).

In this study the results of lysozyme activity of sera in all groups increase gradually in relation of period of feeding and the best results obtained from group (3) feeding 3% curcumin kg^{-1} feed post 42 day while in group (4) decrease of lysozyme concentration and this results need to more search to know the cause, that curcumin in large dose may have adverse on WBCs, liver, and kidney, Table (5). These results agree with Jiraporn, *et al.* (2012), El-Bahr and Saad (2008) and Manal *et al.* (2014). Jiraporn, *et al*. (2012), concluded that turmeric extract adding feed from 0.3 to 5.0% to sand goby can significantly induce digestive enzyme activities, consumption behavior and growth rate enhanced too, while El-Bahr and Saad (2008) attributed the desired effect of turmeric to its potent antioxidant and its hepatoprotective properties. On other focus Manal *et al.* (2014) described the improvement of immunity and performances attributed to hyperplasia of lymphoid follicles and melanomacrophage centers of spleen. Survivability in the fingerlings of *Labeo rohita* after challenged with *Aeromonas hydrophila* fed with 1.0 g of turmeric per kg of feed for 60 days at 4% body weight and feeding of turmeric might have maintained long-term protection in fish by elevating the nonspecific

immune system such as Nitroblue tetrazolium (NBT), lysozyme and serum bactericidal activity.

Phagocytosis is the first step of macrophage to kill invading microorganisms, Phagocytic and neutrophil activities can act as indicators of the non-specific immune response, Weeks and Warinner (1986), their roles in defense mechanisms have been reviewed by Secombes (1996), these cells can engulf bacteria and kill them principally by production of reactive oxygen species (ROS) during the so-called respiratory burst. These products include the superoxide anion (O2 "), H2O2 and the hydroxyl free radical (OHv) which has potent bactericidal activity and can be measured by NBT activities.

The results of Nitroblue tetrazolium also in this study revealed that group (3) was higher than other groups, Table (6) and this agree with Antony *et al.*, (1999); Hilda *et al.* (2006) and Manju *et al.* (2012). According to Antony *et al.* (1999), curcuminoids have dose dependent inhibitory effects on reactive oxygen species production and myeloperoxidase release by activated neutrophils, however, our results showed that, at higher doses, the activities of curcumin remained unaltered. Hilda *et al.* (2006), turmeric with the active curcuminoids and water soluble turmerin, has antioxidant properties and hence effectively inhibits the free radical damage to biomolecules. Manju *et al.* (2012) applied two doses of curcumin 0.5 and 1% were supplemented in the 40% protein feed and fed to Anabas testudineus (Bloch) for the periods, 2 and 8 weeks and the lipid peroxidation product, thiobarbituric acid reactive substances content either decreased or unaffected. The glutathione content increased while the antioxidant enzyme activity pattern varied with time and dose. The histological analysis also confirmed the safety of curcumin retaining the normal arrangement of hepatocytes, hepatopancreas, and macrophage-melanocyte centers. The immunostimulant status of fish fed curcumin attributed to enhancement of the immune system through activation of secretion of the digestive enzymes and growth rate. Rojtinnakorn *et al*. (2012) showed that all turmeric extract fed fish had significant higher specific activities of digestive enzymes and indicated that growth rate enhanced in follow up.

CONCLUSION

Curcumin is safe for feeding *Oriochromis niloticus* as it increases survivability against *Aeromonas hydrophila* disease, due to its antibacterial activity, antioxidant activity, hepatoprotective effect, enhance phagocytosis, lysozyme production and digestive enzyme, palatable for fish, increase and enhance non specific immunity in fish, so, we advised to add Curcumin to fish food at a rate of 1-3%.

ACKNOWLEDGEMENTS

Grateful thanks to Prof. Dr. Azza Mohamed Abd El-Rhman, Prof of Fish Health and Management, Central lab. For Aquaculture Research for her advices and help, and we wish to express our deepest appreciation to Prof. Dr. Safwat Abd El ghany Abd El mageed Gobran professor of fish production and aquaculture system.

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كفاءة استخدام نبات الكركم على الاستجابه المناعيه لاسماك البلطى النيلى ضد التسمم الدموى الايروموناسى

هاله فؤاد محمد احمد ايوب ، محمد مصطفى سيد احمد الطنطاوى

قسم بحوث صحة الاسماك و رعايتها- المعمل المركزى لبحوث الثروة السمكية بالعباسة – مركز البحوث الزراعية.

الملخص العربى

نبات الكركم من جنس كركوما لونجا نبات محلى بشرق اسيا يزرع بكثافة بالمناطق الاستوائية فى العالم. الهدف من هذا البحث تقييم الجرعات الغذائية المختلفة من الكركم لتحفيز المناعة و المقاومه للجراثيم الانتهازيه خاصة ايروموناس هيدروفيلا فى البلطى النيلى. استخدم الكركم بجرعات ١، ٣، ٥ % لكل كجم عليقه و تم التغذية عليه لمده ٦٠يوم بمعدل ٤% من الوزن الحى يوميا. تم تجميع عينات دم كل ١٤ يوم وتم فصل السيرم وفى نهاية التجربة تم عمل اختبار التحدى باستخدام ميكروب الايروموناس هيدروفيلا بجرعة ٠.٢ مل بالحقن البريتونى وتم حساب معدل النفوق و معدل الحماية بعد ١٠ ايام. وتم اجراء بعض الاختبارات المناعيه ومنها الليزوزيم والينتروبلوتترازوليم وكانت عند ($P<0.05$) اعلى فى اليوم ٤٢ من التغذية على ٣% لكل كجم عليقة. كانت نتيجة اختبار التحدى ٠، ٧٥، ٩٠، ٨٠% حماية للمجموعات على التوالى. و بالتالى من تلك النتائج يتبين ان التغذية على الكركم ادت الى زيادة كفاءة الجهاز المناعى ووضحت فى نتائج الليزوزيم والينتروبلو تترازوليم و يمكن استخدام تركيز ٣% للحماية الاعلى ضد التسمم الدموى الايروموناسى فى البلطى النيلى.