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USES OF ETHANOLIC *MALVA SYLVESTRIS* LEAVES EXTRACT AS ANTIMICROBIAL ACTIVITY AND IMMUNE STIMULANT IN *OREOCHROMIS NILOTICUS*

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Abstract

This study concern the effect of ethanolic *Malva sylvestris* leaves extract as immune stimulant in *Oreochromis niloticus* and its antibacterial and antifungal effect . 50 specimens of *O. niloticus* were collected alive, randomly subjected to microbial isolation and identification (bacteria and fungi). Ninty apparently healthy *O. niloticus*, were obtained from Central Laboratory for Aquaculture Research, Abbassa, Abo-Hammad,Sharqia, Egypt. The fish were divided into three groups (in three replicate). Fish of the first group act as a control (T_1) in which fed with feed without ethanolic malve leaves extract supplementation during the feeding period and fish of Groups 2and 3 were fed with feed contained 0.5% (T_2) and 1% (T_3) ethanolic malve leaves extract respectively and the fish were fed two times a day for 28 days. Blood samples were taken at zero day, 1 , 2 and 4 weeks of feeding period. At the end of experiment (after 4 weeks of feeding experiment) the challenge infection with pathogenic *Aeromonas sobria* was carried out, mortality rate and relative percent of survival were recorded. The result indicated that Heamatocrite, Lysozome and NBT where enhanced in T_2 and T_3 compared, with control group. Ethanolic Malve leaves extract of concenteration 150 µg /disc had greater effect antibacterial effect against *Aeromonas veronii, A. hydrophilla A. jandaei,* and *A. caviae* except *Aeromonas sobria* and had antifungal effect against *Saprolignia, Asperagillus flavus* and *Pencillium* of concenterationµg 130 /disc. Relative levels of protection were higher in T2 and T3 compared to the control So, ethanolic *Malva sylvestris* enhanced immune response and disease resistance of *O. niloticus*.

INTRODUCTION

Aquaculture is playing an important role in meeting the increasing global demand for animal protein requirements in addition to it is the fastest growing food production sectors (Aklakur *et al.*, 2015). The fish intensification

constitutes one of the most important problems which effect on the development of aquaculture and cause different disease (Rao *et al*., 1992). So good management practices must be ensuring the production of healthy fish by aquaculture. Chemotherapeutics have adverse effects including the increase of antibiotic resistant bacterial strains, suppression of host's immune system, destruction of the microbial population and accumulation of the antibiotic residues in the fish tissue and aquatic environment (Austin, 2012).Recently, natural herbs act as an important part of traditional medicine in different countries in new treatment procedures. In these study has been studied the effect of the ethanolic malve extract as a immune stimulant and anti microbial medicine which is more effective. Moreover, the herb is used in treating of different disease in traditional medicine Cetto (1999) *and* Pellow *et al.* (1985). The *Malva sylvestris* L. plant family is malvaceae and it is a biennial plant and its height about 100 to 120 cm and its flower of plant is 2 or 5 cm, and there is no smell and mucilage test and *Malva sylvestris* found in Europe, northern Africa, and Asia in addition to Mediterranean. All parts of *Malva sylvestris* are used as medicinal compounds especially its leaves and flowers which used from along times as food and drug which used as antioxidant, anticancer, antimicrobial, skin treatment, anti inflammation, and in the treatment of ulcer and its common name of *Malva sylvestris* is common mallow while its local name is Khabazi and it was found that the flowers and leaves rich with mucilage, antocyanines, flavonoids carotenoids, antioxidant , phenolic compounds, ascorbic acid, tannin and tocopherols used for their expectorant properties Yeole *et al.,* 2010. Barros *et al.,* 2010 and Gasparetto *et al.,* 2011.

MATERIALS AND METHODS

This study was carried out in Central Laboratory for Aquaculture Research at Abbassa, Agriculture Research Center, Egypt.

Microbial isolation and identification:

50 naturally infected *O. niloticus* showed skin infection collected randomly from aquaculture fish farm of Central Laboratory for Aquaculture Research (CLAR) Abou Hammad - Sharkia – Egypt.

(I) Bacterial examination:

Sampling and Primary isolation were done according to Noga, 1996 under complete asepticconditions from the kidney, liver, and spleen inoculated on tryptic soy broth, and incubated at 30 C for 24 hours. A loopfull of incubated broth was streaked on tryptic soy agar; the isolated bacteria were identified according to Bergey *et al*., 1994.

(II) Mycological examination:

The isolated fungi were carried out from moribund and diseased *O. niloticus* , the sample were taken from eyes, skin ulcer, fins, gills and mouth and inoculated onto Sabaroud's Dextrose Agar (SDA) medium plates and incubated at 20 ± 2^{o}c for 3 - 4 days and subculture on the Same medium (SDA) for purification, all positive culture were examined for colonial growth, morphological feature and microscopic characteristic. The microscopical examination was done from wet preparation from skin ulcer, eyes, gills, mouth and fins and also from growth on SDA to detect septation of hyphae according to Dvorak and Atanoesk (1969); Mossel (1977); Schipper (1978) and Onions *et al*. (1981).

Malve leaves extract:

The *Malva sylvestris* leaves were collected from area of Zagazig - Elsharqia -Egypt Different components which used in Soxhlet extraction like thimble, water cooling system, and reservoir, by pass tube, siphon tube and condenser. We will take 10 mg of dried of leaves keep in thimble which is loaded into soxhlet vessel having flask containing extractor in 100 ml ethanol for 3.5 hours Vibha *et al.*, 2012 .

Antimicrobial activity:

(I) Antibacterial activity:

Agar-disc-diffusion method was employed for the determination of antibacterial activity of Malve leaves extract against bacteria which isolated

from fish *Aeromonas veronii, A.hydrophilla A. jandaei, ,and A.caviae,* according to (Gonsales *et al.,* 2006).

(II) Effect of different concentrations of ethanolic malve leaves extract on radial growth of isolated fungi:

Plant extract which could suppress the fungal growth in the disk diffusion method were further tested for their efficiency against the pathogen by using an agar dilution technique Jorgensen *et al.,* 1999. Different concentrations of the extracts; 50, 80, 100, 110 and 130ug of effective plant were amended with Sabouraud' Dextrose Agar Medium (SDAM) (Britania). The amended medium was dispensed into sterile Petri dishes and allowed to solidify and to avoid bacterial contamination 0.5g of antibacterial streptomycin was added to 1L of SDA medium. Two perpendicular lines were drawn at the bottom of each plate to cross each other at the centre of the plate. Each plate was inoculated with tested fungi. A 5-mm diameter mycelia disc of each of the test organisms was inoculated on each amended agar plate. Inoculated plates were incubated at 25±2ºC and growth measured along the perpendicular lines. The radial growth of each test organism of the test extract was recorded after 7 days. Each treatment was replicated four times Then all the culture plates were incubated at 27ºC in dark condition. Calculate the percent inhibition of the mycelia growth over control by using the following formula [Steel and Torry, 1960].

$$\% \text{ of inhibition} = 100x \frac{\text{Diameter of control colony- Diameter of treated colony}}{\text{Diameter of control colony}}$$

(III) Sensitivity test using Antibiograms discs:

Sensitivity of the previous bacterial strains to Antibiograms (Ampicillin, Ciprofloxacin, Norofloxacin, Amikacin, and Amoxicillin) was carried out using disc diffusion method. The culture of *Aeromonas veronii, A. hydrophilla A. jandaei, and A. caviae* was flooded on the surface of tryptic soy agar and the

excess were removed. As well as each of the above antibiotic disks were used and gently pressed on the agar surfaces using sterile forceps. The plates were incubated at 30˚C for 24 hrs. The results were interpreted according to the criteria given by (Finegold and Martin, 1982).

(V) Minimal inhibition concentration determination (MIC):

The minimum inhibitory concentration, of ethanolic malve leaves extract to pathogenic bacteria was determined by broth micro dilution method. 50ml Malve leaf extract was dissolved in 1 ml distilled water. Serial dilutions from (1:10) using tryptic soya broth (TSB) was prepared for each isolate, and inoculated by one loopful of *Aeromonas veronii, A.hydrophilla A. jandaei, ,and A.caviae* broth (24 hrs live). The inoculum's contained 10^2 bacterial cells. The test-tubes were incubated at 30^oC for 18–24 hrs and checked for bacterial growth. A loopful, from each tube, was streaked TSA to check the bacterial growth. The plates were incubated at 30^oC for 24 hrs according to (Ruangpan *et al.*, 1997).

Effect of ethanolic Malve leaves extract as immunostimulant :

Diet preparation:

Commercial basal diet (crude protein 30%) was crushed, and then divided into three parts. The first part was basal diet mixed with sterile saline as a control group. The second and third parts were basal diet mixed with (0.5 and 1 ml of extract / 100gm diet). The diets were reformed into pellets; spread to air dry and stored at 4˚C for the feeding experiment.

Feeding experiments:

Ninty apparently healthy *O. niloticus*, (with average body weight of 50 ± 5 g / fish) were collected from Central Laboratory for Aquaculture Research, Abbassa, Abo-Hammad,Sharqia, Egypt. Healthy fish were kept in an indoor fiberglass tank acclamized to the laboratory conditions. After that, the fish were divided into three groups in three replicates (30 fish per each group). Fish of the first group (T_1) served as a control and fed diet free from ethanolic malve

leaves extract. Fish of Groups 2 and 3 (T_2 and T_3) fed with feed containing 0.5% and 1% ethanol malve leave extract respectively. The fish were fed to satiation two times a day for four weeks. Experiments was conducted in prepared glass aquaria (90 x50 x 35 Cm), supplied with chlorine free tap water .The continuous aeration was maintained in each aquarium using an electric air pumping compressor. Settled fish wastes were cleaned daily by siphoned with three quarters of the aquarium's water, which was replaced by aerated water from the water storage tank. Water temperature was kept at 22 ± 1°C and pH 8.5 throughout whole experiments. .

Blood Sampling:

Twenty fish were randomly collected from each group and were anesthetized via immersing in water containing tricaine methane sulfonate (MS-222) neutralized by sodium bicarbonate. Whole blood (0.5 ml) pooled samples were collected from the caudal vessels of each fish using syringes (1 ml) and 27-gauge needles rinsed with heparin (15 unit/m). A further 0.5 ml blood sample was centrifuged at 2000 rpm for 5 min in order to separate the plasma. The latter was stored at -20°C until uses.

Hematocrit (HCT) Values:

Hematocrit capillary tubes were two-third filled with the whole blood and centrifuged in a hematocrit centrifuge for 5 min and the percentage of the packed cell-volume was determined by the hematocrit tube reader.

Nitroblue Tetrazolium (NBT) Activity:

The NBT (yellow) is reduced to formazan (blue) in the reaction with oxygen radicals from neutrophils and monocytes, the analysis of the production of oxygen radicals by the use of NBT can done by spectroFigmeter. 0.1ml blood was placed into microtiter plate then equal amount of 0.2% NBT solution was added and incubated for 30 min at room temperature, 0.1 ml of NBT blood cell suspension was taken and added to a glass tube contain 1 ml N, N-dimethyl formamide and centrifuged for 5 min at 3000 rpm, the supernatant

fluids was read in spectroFigmeter at 620 nm in 1 ml cuvettes (Siwicki *et al.* 1985).

Lysozyme Activity:

The lysozyme activity was measured using Figelectric colorimeter with attachment for turbidity measurement. A series of dilution was prepared by diluting the standard lysozyme from hen egg-white (Fluka, Switzerland) and mixed with Micrococcus lysodeikticus (ATCC No. 1698 Sigma) suspension for establishing the calibration curve. Ten ml of standard solution or serum were added to 200 ml of Micrococcus suspension (35 mg of Micrococcus dry powder/95 ml of 1/15 M phosphate buffer 5.0 ml of NaCl solution). The changes in the extinction were measured at 546 nm by measuring the extinction immediately after adding the solution which contained the lysozyme (start of the reaction) and after a 20 min incubation of the preparation under investigation at 40˚C (end of the reaction). The lysozyme content is determined on the basis of the calibration curve and the extinction measured (Schaperclaus *et al.,* 1992).

Challenge test:

After 30 days of feeding ethanol guava leaf extract supplementation diets, the fish of each group were divided into two subgroups; the first subgroup was challenged I/P with pathogenic *A. sobria* (0.2 ml of 5 x 10^5 CFU). The second subgroup was injected I/P with 0.2 ml of saline solution as a control. Both subgroups kept under observation for 10 days to record the daily mortality rate.

Statistical Analysis:

The obtained data of fish were subjected to one-way ANOVA. Differences between means were tested at the 5% probability level using Duncan test. All the statistical analyses were done using SPSS program version 10 (SPSS, Richmond, USA).

RESULT AND DISCUSSION

Fish may display many behavioral and physical changes, some of which give valuable clues as to the nature of the disease, that many signs are common to a multitude of bacterial diseases (Miyazaki and Jo, 1985). The isolates bacteria were identified according to the morphological and biochemical characters to *Aeromonas veronii, A. hydrophilla A. jandaei, A. caviae* and *A. sobria* present in (Table 1).This was carried out basically according to the key of Bergy's Manual of Systemic Bacteriology Bergey *et al.,* (1994). *Aeromonas* species facultative anaerobic, Gram-negative bacteria and short rod shape (Daskalov, 2016) and belong to the family Aeromonadaceae (Colwell *et al.*, 1986). These bacteria have found in both cold and warm-blooded animals, including humans, and known as psychrophilic and mesophilic (Nerland, 1996). These bacteria cause hemorrhagic septicemia, fin rot and soft tissue rot in addation to it was recently reported that epizootic ulcerative syndrome caused by *Aeromonas sobria* resulted in great damage to fish farms (Rahman *et al*., 2002). Aeromonas species are important pathogens of various fish species, causing motile aeromonad septicaemia (MAS) and often occurring serious damage in the aquaculture (Min and Ying, 2005 and Wahli *et al*., 2005).

Table 1. Biochemical characters of *Aeromonas jandiae*, *A. veronii*, *A. hydrophila*, *A. caviae* and *A. sobria* isolated from cultured *O. niloticus.*

Items	*A.jandiae*	*A.veronii*	*. A. hydrophila*	*A. sobria.*	*A.*caviae
Gram-stain	**–ve**	**–ve**	**–ve**	**–ve**	**–ve**
Shape	**Short rod**	**Short rod**	**Short rod**	**Short rod**	**Short rod**
Motility	+	+	+	+	+
Cytochrom oxidase	+	+	+	+	+
O/F	F	F	F	F	F
Growth on 5°C	-	-	+	+	-
Growth on 0%NaCl	+	+	+	+	+
3.5%NaCl	+	+	-	+	+
6% NaCl	-	-	-	-	-
Catalase	+	+	+	+	+
H_2S (TSI)	-	-	+	+	-
Indol	+	+	+	+	+
Starch hydrolysis	-	-	-	+	-
Methyl red	+	-	+	+	-
Vogaus proskauer	+	+	+	+	-
Citrate	+	+	+	+	+
Gelatin liquefaction	-	-	+	+	+
Heamolysis	γ	-	β	β	-
Acid	production	From:			
Arabinose	-	-	+	-	+
Salicin	-	+	-	-	+
Sucrose	-	+	-	+	+
Inositol	-	-	-	-	-
Maltose	+	+	+	+	+
Glucose	+	+	+	+	-
Mannitol	+	+	+	+	+
Tween 80	+	+	+	+	
Ornithen decarboxylase	+	+	+	±	-
Lysin decarboxylase	+	+	+	±	-
Arginie dehydrolase	-	-	-	-	-

The suspected isolates of fungi were identified as *Saprolignia*, *Asperagillus flavus* and *Pencillium* according to microscopic character and structure of growth on sabroud dextrose agar. The morphological characteristics of the *Saprolegnia* sp are cottony shape and microscopically was branched and non septated hyphae and isolated from the skin lesions. *Asperagillus flavus*

isolated from intestine and its color is yellow at first and turned to dark yellow green with branched septated hyphae with condia. *Pencillium* isolated from gills is fast growing, in green and microscopically, chains form finger like shape (phialide) carried by conidiophore. Neish, 1997 reported that every freshwater fish is affected at by least one species of fungus during its lifetime and attack all the life stages of fish life from egg to adult fish. Meyer, 1991 decided that fungal diseases are the second most serious cause the death between fish in aquaculture. *Aspergillus flavus, Aspergillus terreus* and *Aspergillus japonicas* caused aspergillomycosis disease of African fish tilapia (Oreochromis sp) and its infectious through contamination of fish feed Saleem *et al.,* 2012. Firouzbakhsh *et al*., 2005 isolated *Aspergillus niger* from common carp from its eggs and from the environment and said that *Aspergillus niger* caused the internal and external infection in fishes. *Fusarium sp., Saprolegnia sp., Penicillium sp.* and *Mucor sp*., were reported in the eggs of *Aspencer percicus* Jalilpoor *et al*., 2006. Refai *et al*., 2010 isolated eight different genera, *Aspergillus, Rhizopus, Mucor, Saprolegnia, Fusarium* and *Pencillium* from *Oreochromis sp*. and *Clarias gariepinas* and said that *Penicillium sp., Aspergillus sp.,* and *Rhizopus sp.,* are present normally on fish in addition to some fungal genera cause fish diseases under favorable predisposing environment.

Fig (1) shows the concenteration of the ethanolic extract of *Malva sylvestris* leaves extract which were tested against *A. veronii, A. hydrophilla A. jandaei, A. caviae* and *A. sobria* .Ethanol extract of *Malva sylvestris* leaf displayed antibacterial activity against *A hydrophila, A. veronii, A.sobria, A. jandaei*, and *A. caviae*. The maximum inhibition zone obtained with 150µl/disc against *A. caviae* and *A. hydrophila* 55 and 54 mm recpectively. The study conducted by Pirbalouti *et al*., 2009 who mention that *M. sylvestris* was good for skin disorders, as well as having good antimicrobial and anti-inflammatory activity and detected that *Malva sylvestris* exhibited maximum antibacterial activity against *S. aureus* (3.1 mm) at 15mg/ml. The MIC of ethanolic malve leaves extract were 50 µgm/ml against *A. hydrophila, A. veronii, A. jandaei* and

A. caviae. (Robert, 2000) recorded that The MIC is the highest dilution in which there is no macroscopic growth. The MIC is also used as a comparative index for other antimicrobial agents.

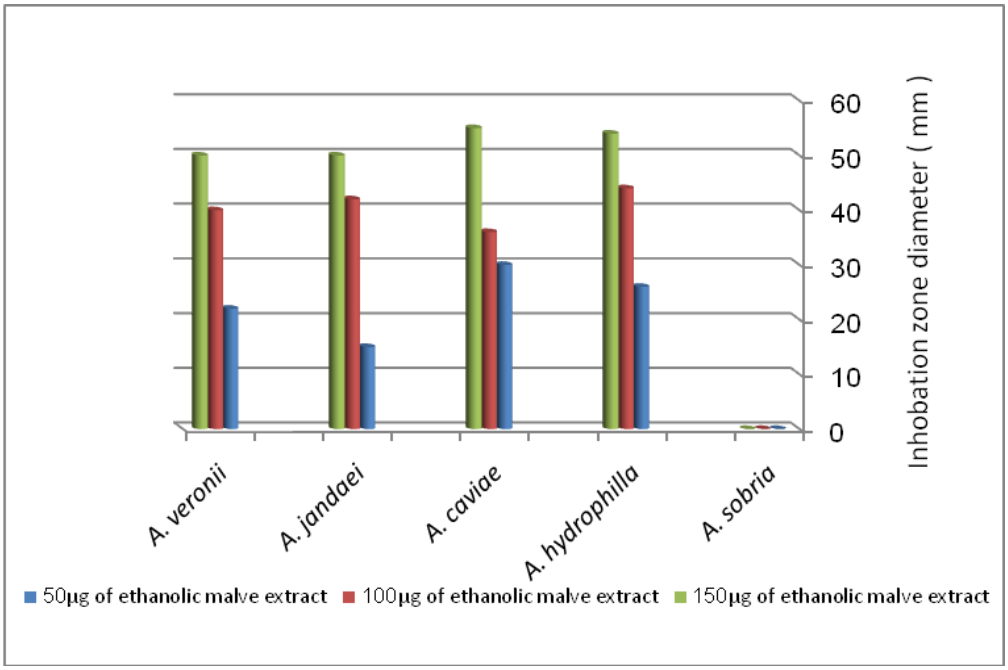

Fig.1. Show the antibacterial assay of ethanolic malve extract against some pathogenic bacteria.

The growth of *Saprolignia*, *Asperagillus flavus* and *Pencillium* were decreaed by increasing concentrations of extract of the malve leaves in which the percentage of inhibition zone increased to 61.1, 66.6 and 83.3% for Saprolignia, *Asperagillus* and *Pencillium* respectively with the same concentration (130 ug / ml) of the malve leaves extract (Table. 3) and this result was agree with Magro *et al*., 2006 who reported that the most effective aqueous extracts of leaves had the concentration 0.60 g/ml, which inhibited the growth of all the tested fungi: *Aspergillus candidus, Aspergillus niger, Penicillium sp.*, and *Fusarium culmorum* , that indicates the antifungal activity depends on the solvent used for extraction and the parts of plant in the same time some references about antifungal activity of seed were not found. Amelio, 1999 reported that *Malva sylvestris* L. componant (malv one A: 2-methyl-3-methoxy-

5,6-dihydroxy-1,4-naphthoquinone may be responsible for antimicrobial activity. Also, *Malva sylvestris* L pharmaceutical compounds including phenols, tannin, terpenes have antifungal activity Barros *et al*., 2010 and Castillo *et al.,* 2012.

Table 3. Percentage of inhibition of malve extract on the fungi (%).

Concenteration(ug)	*saprolignia*		*asperagillus flavus*		*pencillium*	
	Mean(cm)	**%inhibation**	**Mean(cm)**	**%inhibation**	**Mean(cm)**	**%inhibation**
50	6.7	27.7	6	33.3	2.7	70
80	6.8	25.5	5.4	40	1.9	78.8
100	6.6	26.6	5	44.4	1.8	80
110	6.2	31.1	3.2	64.4	1.7	81.1
130	3.5	61.1	3	66.6	1.5	83.3

The results in (Table. 2) demonstrated that *A. hydrophila, A. jandaei, A. sobria* and *A. caviae* were resistance to Ampicllin and Amoxicillin. Lewin *et al.,* 1992 decided that bacterial resistance to antimicrobial drugs has become wide spread in aquaculture. The Gram-negative bacteria were sensitive for Ciprofloxacin, Norfloxacin,Amikacin and Trimethoprim –Sulfamethoxazoleand it resisted to Ampicillin and Amoxicillin.These results agreed with Attia, 2004 and Abou El-Atta and El-Tantawy, 2008.

Table 2. Antimicrobial sensitivity of various bacterial isolated from *O. niloticus* to the antibiograme with the inhibition zone diameters (mm) measurements.

Antibiotic againt	**Symbol**	**Concentration (mcg)**	**Susceptible zoon (mm)**	**Inhibition zoon (mm) and sensitivity reaction of *bacterial isolates* to antibiograme**				
				A. veronii	*A. hydrophilla*	*A. jandaei*	*A. Sorbia*	*A.caviae*
Ampicillin	AM	10	≥17	20 (S)	2(R)	0.00(R)	0.0(R)	0.0 (R)
Ciprofloxacin	CIP	5	≥21	40.0 (S)	40.0 (S)	35.0 (S)	35.0 (S)	41.0 (S)
Norofloxacin	Nor	10	≥17	35.0(S)	35.0(S)	25.0(S)	30(S)	35.0(S)
Amikacin	AK	30	≥17	22.0 (S)	22.0 (S)	25.0(S)	25.0 (S)	0.00 (R)
Amoxicillin	AMX	25	≥14	10.0(R)	1(R)	0.00(R)	15.0(S)	0.00(R)

The results in Fig (2) indicated that the value of heamatocrite at zerotime was 40±0.57 which increased significantly at the second week of feeding and reached to, 53±1.85 and 56±0.88 for T2 and T3 respectively in comparison with T1 was 39±0.33. At the fourth week of feeding experiment, hematocrite value showed significant increase with T_2 and T_3 58±1.66 and 59±0.33 in contrast to T1 (control) but there was no significant between T_2 and T_3 so The elevated hematocrite-value show the effect of ethanolic malve leaves extraction on the health of the fish status (Abd El-Rhman, 2009) that due to *Malva sylvestris* L. component such as mucilage, flavonoids, tannin, phenolic compounds, ascorbic acid, carotenoids tocopherols, and antocyanines Barros *et al*., 2010; Gasparetto *et al*., 2011 and Cui-lin CH and Zhen-ya, 2006.

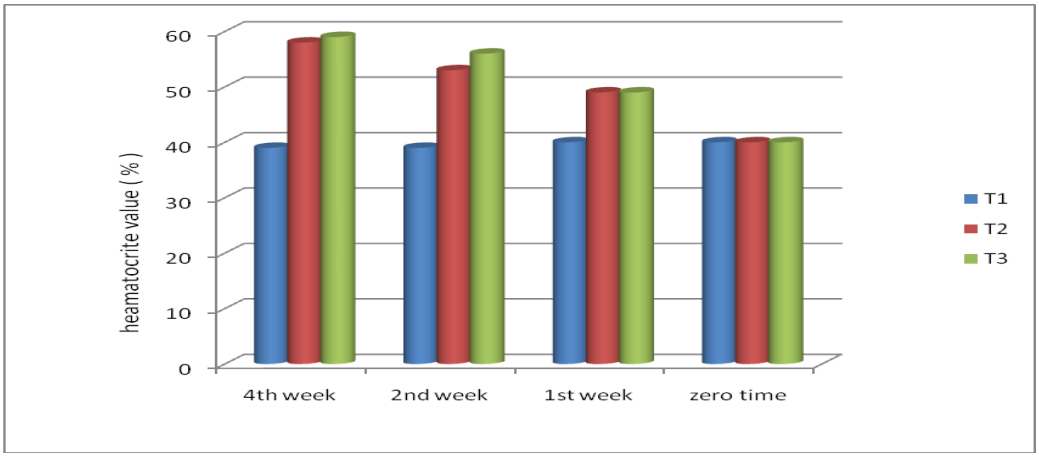

Fig. 2. Effect of Malve ethanolic extraction supplemented diet on heamatocrite value in *O. niloticus* at first, second and fourth week of feeding experiment.

T1= basal diet free from *Malva sylvestris* extract. T2= Fish fed basal diet supplemented with 5 ml/kg. T3= Fish fed basal diet supplemented with 10 ml /kg

The results in Fig (3) indicated that the initial lysozome was ١.3±0.205 at the beginning of experiment and increased significantly at the second week of feeding experiment and reached to, 2.49±٠.109 and 2.75±٠.095 for T2 and T3 respectively in comparison with T1 was ١.٥±0.250. At the fourth week of feeding experiment, lysozome value showed significant increased with T2 and T3 3.2±٠.١4٧ and 3.5±٠.073 in contrast to T1 = 1±0.042 but there was no

significant between T2 andT3. Lysozyme is a fish defence element, which causes lysis of bacteria and activation of the complement system and phagocytes by acting as an opsonic (Magnadottir, 2006). The increase of serum lysozome value may attributed to antioxidant of *Malva sylvestris* such as polyphenols vitamin C, vitamin E, B-carotene and other important phytochemicals, which are based on their ability to scavenge different free radical leading to the protection of biological molecules against oxidation (Rackova *et al*., 2009).

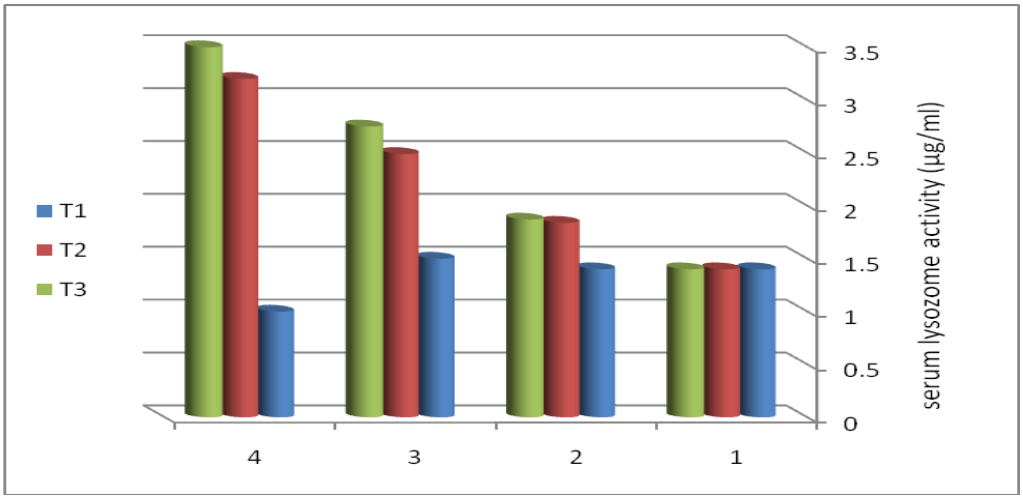

Fig. 3. Effect of Malve ethanolic extraction supplemented diet on lysozome value (µg/ml) in *O. niloticus* at first, second and fourth week of feeding experiment.

T1= basal diet free from *Malva sylvestris* extract. T2= Fish fed basal diet supplemented with 5 ml/kg.
T3= Fish fed basal diet supplemented with 10 ml /kg.

Respiratory burst is one of bactericidal mechanisms in fish (Ellis, 1999 and 2001) and it considered the best indicator of their health status (Anderson, 1992). The results in Fig (4) indicated that the initial NBT was ١.٤±0.٢91 at the beginning of experiment and increased significantly at the second week of feeding and reached to, ٢.9±٠.072 and 3.45±٠.215 for T2 and T3 respectively in comparison with T1 was ١.٥±0.5. At the fourth week of feeding experiment, NBT value showed significant increase with T2 and T3 3.3±٠.١3٧ and 4.3±٠.441 in contrast to T1 = 1.35±0.17 but there was no significant between

T2 andT. The significant increase in NBT values may be attributed to its anti-oxidant effect of the componant *Malva sylvestris* such as mucilage, flavonoids, tannin, phenolic compound, ascorbic acid, cartenoid, tocopherols and atocyanine (Barros *et al.*, 2010 and Gasparetto *et al.*, 2011).

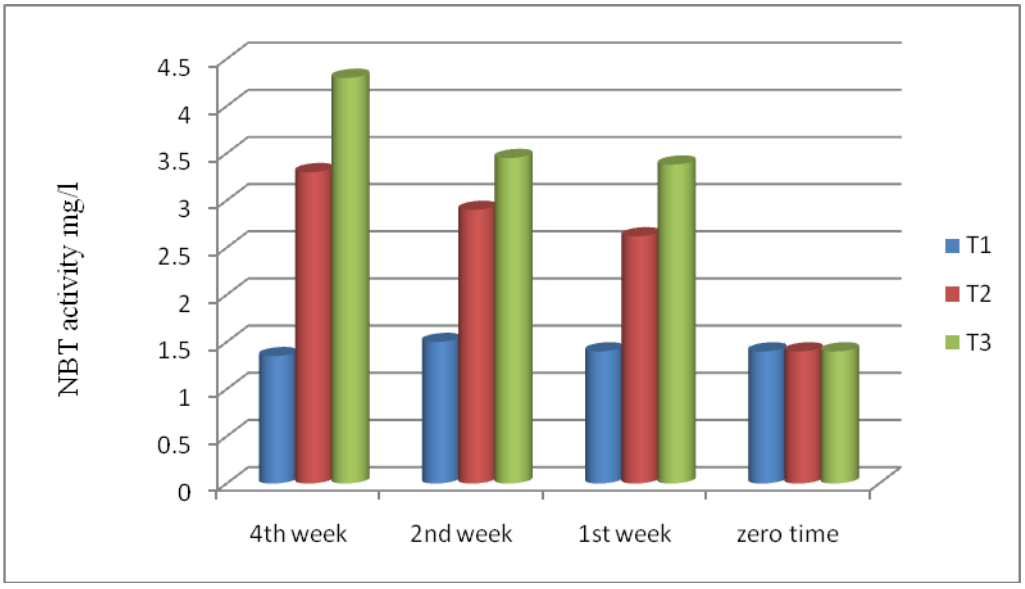

Fig. 4. Effect of Malve ethanolic extraction supplemented diet on NBT value in *O. niloticus* at first, second and fourth week of feeding experiment.

T1= basal diet free from *Malva sylvestris* extract. T2= Fish fed basal diet supplemented with 5 ml/kg. T3= Fish fed basal diet supplemented with 10 ml /kg

Table (4) show the relative level of protection in the treated fish to determine the effect of Malve ethanolic extraction to increase fish resistance to *A. sobria* . Malve ethanolic extraction supplemented diet groups reduced mortality which induced by *A. sobria* when compared with the control group table (4). Fish mortality was 0 % in fish fed Malve extracts supplemented diets and 50% in fish fed the control diet. The clinical signs of dead fish with tail and fin rot scale loss and external hemorrhage while the postmortem finding was septicemic lesions of the internal organs that agree with Noga, 2010. *A. sobria* was re-isolated from liver, kidneys and spleen of the moribund and recently dead fish and these results indicate that the Malve ethanolic extraction enhance

the immune system of the Nile tilapia and showed their capacity to reduce mortalities associated with infection by *A. sobria*.

Table 4. Mortality rate and Relative level of protection in treated *O.niloticus* due to challenge with *Aermonus sobria.*

Treatment	*A. sobria*	
	Mortality%	**RLp%**
T1	50	0
T2	0	100
T3	0	100

T1= basal diet free from *Malva sylvestris* extract. T2= Fish fed basal diet supplemented with 5 ml/kg. T3= Fish fed basal diet supplemented with 10 ml /kg

REFERENCE

Abd-El-Rhman; M.M.Azza, 2009. Antagonism of *Aeromonas hydrophila* by propolis and its effect on the performance of Nile tilapia, *Oreochromis niloticus.* Fish and Shellfish immune., 27: 454–459.

Abou El-atta M. and M.M. El. Tantawy (2008). Bacterial causes of skin ulcers affection in Tilapia nilotica (*Orechromis niloticus*) with special references to its control.8th international symposium on tilapia in aquaculture. P 1419-1436.

Aklakur, M.; M.A. Rather, and N. Kumar, 2015. Nano Delivery: An Emerging Avenue for Nutraceuticals and Drug Delivery. Critical Review in Food Science and Nutrition. DOI**:** 10.1080/10408398.2013.839543.

Amelio, F.S., 1999. Botanicals, a phyto-cosmetic desk reference. CRC Press LLC, Boca Raton, USA.

Anderson, D.P., 1992. Immunostimulants, adjuvants, and vaccine carriers in fish: applications to aquaculture. Ann. Rev. J. Fish. Dis., 2: 281–307.

Attia, Y.M.A., 2004. Studies on problems affecting gills of cultured fishes. Ph.D. thesis Vet. Sci. (Fish Disease) Fact.of Vet. Med. Zagazig University.

Austin, B., 2012. Developments of vaccination against fish bacterial disease In: Infectious diseases in aquaculture prevention and control.1st ed by Austin, B. Woodhead publishing limited, UK. pp 218.

Barros, L.M.; I.C. Carvalho and F.R. Ferreira, 2010. Food and Chemical Toxicology, 48: 1466–1472.

Bergey's, D.H.; J.G. Holt and P.H.A. Sneath, 1994. Bergey's Manual of Determinative Bacteriology, R. E. Buchaman and N.E. Gibbons. 9th Ed, Bohimore, Williams and Wiikins.

Castillo, F.; D. Hernandez; G. Gallegos; R. Rodriguez, C.N., Aguilar, 2012. Fungicides for Plant and Animal Diseases 81-106.

Cetto, A. 1999. Ethno pharmacological study of *Equisetum myriochaetum* and *Cecropia obrusifolia* Doctoral Thesis, Science School, National University of Mexico, 97.

Colwell, R.R.; M. MacDonell; T.O.M. Brien and J. De Ley, 1986. Proposal to recognize the family Aeromonadaceae fam. nov. Int J Syst Bacteriol, 36: 473–477.

Cui-lin, C.H. and w. Zhen-ya, 2006. Bacteriostatic activity of anthocyanin of *Malve sylvestris*.Journal of Forestry Research, 17 (1): 83-85.

Daskalov, H., 2006 "The importance of Aeromonas hydrophila in food safety," Food Control, 17 (6): 474–483.

Dvarak, J. and M. Atanoesk, 1969. Mycologial diagnosis of animal dermatophytosis. Academic Pp. 213.

Ellis, A.E., 1999. Immunity to bacteria in fish. FSFI., 9: 291–308.

Finegold, S.M. and W.J Martin, 1982. Diagonistic microbiology. 6th edition. The C.V. Mosby company. London.

Firouzbakhsh, F.; E.H.A. Ebranhim; Z. Morusavi and A.R. Khosravi, 2005. Isolation and Identification of Pathogenic and saprophytic fungi from gill lesion in cultivated cyprinids (common carp, silver carp and grass carp). J. Fac. Vet. Med. Univ. Tehran., 60 (1): 15-19.

Gasparetto, J.C.; C.A. FerreiraMartins; S.S. Hayashi; M.F. Otuky; R. Pontarolo, 2011. Ethnobotanical and scientific aspects of Malva sylvestris L.: a millennial herbal medicine. Journal of Pharmacy and Pharmacology, 64: 172–18.

Gonsales, G.Z.; R.O. Oris; J.R.A. Fernandes; P. Rodrigues and S.R.C. Funari, 2006. Antibacterial activity of propolis collected in different regions of Brazil. Venomous Animals and Toxins Including Tropical Diseases, 12: 276–84.

Jorgensen, J.H.; J.D. Turnidge and J.A. Washington, 1999. Antibacterial susceptibility tests: dilutionand disk diffusion methods. In: Murray PR, Pfaller MA, Tenover FC, Baron EJ, Yolken RH, ed. Manual of Clinical Microbiology, 7th ed. Washington, DC: ASM Press: 1526-1543.

Jalilpoor, J.; S.A. Mosouleh and M. Masoumzadeh, 2006. Fungal flora in Acipenserpersicus eggs with particular emphasis on Saprolegniasp. (Oomycetes) and mortality during mass incubation at the ShahidBehesti hatchery. J. App. Icthyol., 22 (suppl.1): 265-268

Lewin, C.S.; C. Michel and D.J. Alderman, 1992. Chemotherapy in Aquaculture: From Theory to Reality, O.I.E., Paris., 288–301.

Magnadóttir, B., 2006. Innate immunity of fish (overview). Fish Shellfish Immunol., 20: 137–151.

Magro; A Carolino; M Bastos M; Mexia. A Dev Iberoam Micol. , (2006), Efficacy of plant extracts against stored products fungi. Review, 23: 176-178.

Meyer, F.P., 1991. Aquaculture Diseases and Health Management. Anim. Sci., 69: 4201-4208.

Min, L.I.U. and H.A.N.Ying, 2005. Isolation and identification of *Aeromonas sobria* from infected carps and its sensitivity to drugs" Journal of Northeast Aquacultural University, 4: 486-489.

Miyazaki, T. and Y. Jo., 1985. A histopathological study of motile aeromonad disease in ayu. Fish pathology, 20: 55 – 59.

Mossel, A.A.D., 1977. Microbiology of foods. Univ. of Utricht.Faculty of Vet. Med. Netherland.

Neish, G.A., 1997. Observations on Saprolegniasis of adult sockeye salmon, Oncorhynchus nerka (Walbaum). J. Fish. Biol., 10: 513-522.

Nerland, A.H.,1996. The nucleotide sequence of the gene encoding GCAT from *Aermonas salmonicida* Sap. *Sabmonicida*. J. Fish Dis., 19: 145 – 150.

Noga, E.J., 1996. Fish disease, diagnosis and treatement. Mosby International, 376.

Noga, E.J.; R. Smolowitz and L.H. Khoo, 2000. "Pathology of shell disease in the blue crab, CallinectessapidusRathbun, (Decapoda: Portunidae) ". J of Fish Dise., 23 (6): 389-399.

Noga, E.J., 2010. Fish disease. 2nd Edn., US, WileyBlackwell. PP: 185-186.

Onions, A.H.S.; D.Allsopp, and H.O.W. Eggins, (1981). Smith's introduction to industrial mycology. 7th Ed. Edward Annold.

Pellow, S.; P. Chopin; S.E. File; M. Briley, 1985. Validation of open-closed arm entries in an elevated plus-maze as a measure of anxiety in the rat, J Neurosci Methods, 14 (3): 149-67.

Pirbalouti, A.G.; M. Yousefi; H. Nazari; I. Karimi and A. Koohpayeh, 2009. Evaluation of burn healing properties of *Arnebia euchorma* and *Malva sylvestris*. Electronic Journal of Biology, 5(3): 62-66.

Plump, J.A., 1994. Health maintenance of cultured Fishes principal microbial diseases CRC press Boca Roton, Vol 1, USA.

Rahman M.; P. Colque-Navarro; I. Kuhn; G. Huys; J.Swings and R. Mollby, 2002. Identification and characterization of pathogenic *Aeromonas veroniibiov A. sobria* associated with epizootic ulcerative syndrome in fish in Bangladesh. Applied and Environmental Microbiology, 68: 650–655.

Rackova, L.; K. Drabikova; V.Jancinova; T. Perecko; J. Smidrka; J. Harmatha; J. Toth; D. Kostalova; S. Bezek; M. Stefek; R. Nosal, 2009. Structural aspects of antioxidant action of selected natural. Free Rad. Res., 43: S27-97.

Rao, B.R.R; P.N. Kaul; K.V. Syamasundar and S**.** Ramesh, 2005. Chemical profiles of primary and secondary essential oils of palmarosa (*Cymbopogon martini* (Roxb.) Wats. Var *motia* Burk.). Industrial Crops and Products, 21: 121-127.

Refai, M.; K. Laila.; A. Mohamed; M. Kenawy and S.M.S. Shimaa, 2010. The assessment of Mycotic settlement of freshwater fishes in Egypt. J. Amer. Sci., 6 (11): 595602.

Ruangpan, L., S.Prapadson, , and K.Sangrungruang, 1997. Minimal concenteration of five chemotherapeutants against Vibrio Bacteria and their Transfer of R-plasmid. In T.W.Flegel and I.H.MacRae (eds.), Diseases in Asian Aquaculture. Fish Health Section, Asian Fisheries Society, Manila, 221-226.

Saleem, M.J.; A.A.U. Hanan; Nisa and T.A. Qasir, 2012. Occurrence of Aflotoxinin Maize seed under different condition. Int. J. Agri. Biol., 14: 473-476

Schaperclaus, W.; H.Kulow and K. Schreckenbach, 1992. Fish disease. Rotterdam, the Netherlands: A.A. Balkema., 101–105.

Schipper, M.A.A., 1978. A studies on certain spp., of mucor with a key to all accepted spp. Studies in mucology, No.17.

Siwicki, A.K., M. Studnicka and B. Ryka, 1985. Phagocytic ability of neutrophils in carp. Bamidgeh., 37: 123-128.

Steel, R.G.D. and J.H. Torrie, 1960. Principle & procedures of statics, McGraw-Hall Book company ,Inc. ,New York.

Vibha P.; S. Pallavi; G. Devendra, 2012. A Comprehensive Study On Different Methods Of Extraction From Guajava Leaves For Curing Various Health Problem . International Journal of Engineering Research and 2012, pp.490-496

Yeole, N.B.; P. Sandhya; P.S. Chaudhari and P.S. Bhujbal, 2010. Evaluation of *Malva sylvestris* and *Pedalium murexmucilage* as suspending agent. International Journal of PharmTech Research, 2 (1): 385-389.

Wahli, T.; S.E. Burr; D. Pugovkin; O. Mueller and J.Frey, 2005. "*Aeromonas sobria*, a causative agent of disease in farmed perch, Percafluviatilis L.Journal of Fish Diseases, 28 (3): 141-150.

استخدام مستخلص أوراق الخبيزة الكحولي كمضاد للميكروبات ومحفز لمناعة اسماك البلطي النيلي

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الملخص العربـي

تهدف هذه الدراسة لاختبار مستخلص أوراق الخبيزة الكحولي علي الاستجابة المناعية للأسماك البلطي النيلي و دراسة تأثيرها كمضادة للبكتريا و الفطريات. تم تجميع حوالي ٥٠ عينة من اسماك البلطي النيلي لعزل و التعرف علي كل من البكتريا . تم جمع ٩٠ سمكة من المعمل المركزي لبحوث الثروة السمكية العباسة ابوحماد – شرقية وتم عمل أقلمة للأسماك لمدة أسبوعين ثم تم تقسمها إلي ثلاث مجموعات كل مجموعه بها ثلاث مكررات بواقع ٣٠ سمكة لكل مجموعة. تم تغذية المجموعة الأولي بعلف خالي من المستخلص كمجموعة ضابطة و المجموعة الثانية تم تغذيتها علي علف تحتوي علي ٠.٥ % من مستخلص أوراق الخبيرة و المجموعة الثالثة تم تغذيتها علي ١ % من مستخلص أوراق الخبيرة و قد تمت هذه التغذية يدويا مرتين يوميا لمدة أربع أسابيع (٢٨ يوما). تم أخذ عينات الدم و البلازما لقياس اختبارات المناعة في بداية التجربة وعند أسبوع وأسبوعين وأربع أسابيع و تم عمل عدوة اصطناعية بالحقن البروتوني للأسماك ببكتريا الأيرمونس سوبريا مع إبقائها تحت الملاحظة لمدة ١٠ يوما. وأسفرت النتائج علي انه بوجود تأثير محفز للمناعة حيث زادت الهيماتوكريت و الليزوزوم و ذلك بالمقارنة مع المجموعة الضابطة. وجد ان مستخلص الخبيزة الكحولي بتركيز ١٥٠ ملي له تأثير مضاد لكل من الأريمونس فيروني و الأريمونس جاندي و الاريمونس كافي و الاريمونس هيدروفيلا فيما عدا الأريمونس سوبريا و كان لتركيز ١٣٠ ملي تأثير مثبط علي أنواع الفطريات المعزولة المتمثله في الأسبراجلس فلافس و سبرولجنيا و البنسيليوم. اوضحت النتائج انه بالعدوى الصناعيه بإيروموناس فيروني لوحظ ان المجموعهT_2 ، T_3 التي تغذت على ٠,٥% و١% من المستخلص فى العليقه سجلت نسبة نفوق (صفر %) منخفضه بالمقارنه بالمجموعه الضابطه التى سجلت نسبه نفوق ٥٠% .أثبتت الدراسة ان أضافه مستخلص الخبيزة له تاثير ايجابى على رفع المناعه و تحسين الصحه وزيادة مقاومة

الاسماك للامراض لذلك نوصى باضافة مستخلص نبات الخبيزة بنسبه ٠,٥% في علف الاسماك لما له من تاثير رافع للمناعة ومقاوم للامراض فى اسماك البلطي النيلي.