

ECHINACEA AS IMMUNOSTIMULATORY AGENT IN NILE TILAPIA (*OREOCHROMIS NILOTICUS*) VIA EARTHEN POND EXPERIMENT

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Abstract

Twelve hundred Nile tilapia (*Oreochromis niloticus*), average body weight of 4.5 ± 0.2 g, were used to investigate the effect of Echinacea (*Echinacea purpurea*) on the growth rate and disease resistance of fish reared in earthen ponds. They were equally and randomly distributed in six circular earthen ponds (100 m²) at a stocking rate of 2 fish m⁻². Three ponds were randomly assigned for as treatment replicates and the remaining 3 ponds as controls. The control group (gp.1) was given a balanced diet while the treatment group (gp. 2) was given the same diet supplemented by Echinacea at a rate of 0.25 ppt on a dry weight basis. The fish were fed twice daily at a rate of 3% body weight per day for 6 months. Growth and survival rates were recorded at the end of the experiment. The immunostimulatory effects, of Echinacea, were determined from differences between treatment and control groups in survival rate, total and differential leukocytic counts and nitroblue tetrazolium values. A challenge test was conducted using 90 tilapia from each group (30 fish/replicate) by I/P inoculation with 0.5 ml suspension culture of the pathogen *Pseudomonas fluorescens* (10⁸ bacteria ml⁻¹). The mortality rate was recorded for 7 days post-challenge. Group (2) showed a significant increase in body weight gain, specific growth rate, hematocrit values, lysozyme activities and total leukocytic counts, especially in terms of lymphocytes and eosinophils when compared with the control (gp. 1). The survival rate was significantly increased in gp. (2), with and without challenge, when compared with gp. (1). No significant changes were observed in the monocyte numbers and in the nitroblue tetrazolium test. It may be concluded that, Echinacea can be used as a growth enhancer, immunostimulant and a disease control agent in fish. It is recommended as a means of improving the tilapia aquaculture production under certain conditions.

Keywords: *Oreochromis niloticus*, Immunostimulants, Echinacea, Survival, Growth Performance, NBT, WBCs, Lysozymes, Challenge, *Pseudomonas fluorescens*,

INTRODUCTION

Fish culture is an important industry, production increasing worldwide every year. Some countries have sought to improve productivity and profitability by intensification of fish production methods, which can adversely affect fish health, as it can result in a poor environment for fish thereby increasing susceptibility to infection

(Sakai, 1999). Disease outbreaks are particularly prevalent in rapidly developing aquaculture industries, affecting the economic development of this sector (Yunxia *et al.*, 2001). Various chemotherapeutics have been used to treat bacterial infections in cultured fish for the last 20 years. However, the incidence of drug-resistant bacteria has become a major problem in fish culture (Aoki, 1992). Vaccination is a useful prophylaxis for infectious diseases of fish, but the development of vaccines against intracellular pathogens has not so far been successful. Therefore, the immediate control of all fish diseases using only vaccines is impossible (Sakai, 1999). The most effective method, in our opinion, may be the development of natural disease resistance in fish through the use of immunostimulants which can increase the immunocompetency and disease resistance of fish.

Immunostimulants include a wide range of chemical agents, bacterial components, polysaccharides, animal or plant extracts, nutritional factors and cytokines. The term applies to any compound that modulates the immune system by increasing the host's resistance to disease. Immunostimulants mainly facilitate the function of phagocytic cells and increase their bactericidal activities. Several immunostimulants also stimulate lysosomes and the antibody responses of fish (Sakai, 1999). They attach to specific receptors on the cell surface of the phagocytes and lymphocytes activating these cells to produce enzymes capable of destroying pathogens. Moreover, they can increase the production of chemical messengers (interferon, interleukins and complement proteins) that stimulate other aspects of the immune system and increase the activity of T and B lymphocytes (Raa *et al.*, 1996).

The historical and traditional use of echinacea species were noted among Native Americans, among whom the compound was used to treat sore mouths, toothache, colds, tonsillitis, septic diseases, snakebite, coughs and general inflammatory conditions (Hobbs 1989). During the European colonization, echinacea began to be known among the settlers and over the past 50 years has become one of the most popular remedies.

Echinacea seems to activate the macrophages and other immunological functions in lab animals and humans and there is considerable evidence of the role played by the polysaccharidic fraction in the immunostimulatory effect of echinacea preparations. The polysaccharides (heteroglycans), isolated from *E. purpurea*, have been particularly investigated in their capacity to activate macrophages and other components of the immune system in mice, rats and humans (Bauer, 1999). Less consistent data are available on the lipophilic components present in echinacea preparations. However, some, such as the alkylamides and in particular isobutylamides, produce a strong stimulatory effect on the phagocyte-function and on the lipoxigenase-inhibiting activity (Bauer, 1998; Emmendorffer *et al.*, 1999).

The present study aimed to investigate the effect of Echinacea on survival and growth rates of Nile tilapia (*Oreochromis niloticus*), its immunostimulatory and disease control effects via changes in various hematological and immunological measures, and its impacts on resistance to challenge infection tests.

MATERIALS AND METHODS

1-Experimental fish

Twelve hundred Nile tilapia (*Oreochromis niloticus*), with average body weight of 4.5 ± 0.2 g, were obtained from the hatchery of the WorldFish Center, Abbassa, Egypt. They were equally and randomly distributed among 6 circular ponds (100 m²) at a stocking density of 2 fish/m². Three ponds, chosen at random, served as a control (gp. 1) and were given a balanced diet, while the remaining three ponds, used to evaluate the effect of Echinacea, were given a balanced diet mixed with echinacea (0.25 ppt feed) as feed additive (gp. 2). Fish were fed 3 % of their body weight three times/day using clean plastic feeders (1 feeder/pond).

Echinacea (*Echinacea purpurea*) extract (1.5% chicoric acid grade) was procured from SEKEM Company, Egypt. The experiment was extended to six month (November, 2005 – April, 2006), at the end of which survival and growth measure were determined, physiological samples taken and challenge infection tests performed.

2- Feeding and formulation

A balanced dietary ration was prepared (Table 1), dietary ingredients being obtained from several specialized suppliers and prepared locally in our Center in the form of pellets. Basal diets were prepared by grinding the corn to granules using 0.5 mm mesh (Thomes-Willey Laboratory Mill Model 4). Ingredients were mixed mechanically by horizontal mixture (Hobarts model D300T) at a low speed for 30 minutes. Oil (vegetable & cod liver) was added gradually to assure the homogeneity of the ingredients, the mixing speed increased for 5 min during the addition of water (600 ml water) until the mixture began to clump. Pellets were then prepared using a pellet machine (CPM California pellet mill Co.) with 0.5 cm diameter, and pellets were left for 24 hrs to air dry.

Table 1. Analysis and ingredient of the used basal diet

Ingredients		Protein (%)		Metabolic energy (Joule)	
Name	(%)	ingredients	feed	ingredients	feed
Fish meal	8.00	0.72	5.76	4000	32000
Soybean meal	52.9	0.48	25.39	2870	151823
Ground corn	29.1	0.11	3.17	1240	36084
Wheat flour	5.00	0.13	0.67	2700	13500
Vegetable oil	2.00	0.00	0.00	9100	18200
Cod liver oil	2.00	0.00	0.00	9100	18200
Dicalcium phosphate	1.00	0.00	0.00	0.00	0000
Mineral mix.	0.07	0.00	0.00	0.00	0000
Vitamin mix.	0.05	0.00	0.00	0.00	0000
Vitamin C	0.03	0.00	0.00	0.00	0000
Total	100.15	0.00	34.99	0.00	269807

3- Growth performance

The growth parameters of experimented fish were calculated at the end of the 6th month, when all fish were weighed individually, and their body gain was calculated (*Innes, 1982*). Specific growth rates (SGR) and condition factor (CF) were calculated as described by *Laird and Needham (1988)*:

$$\text{SGR} = \frac{\ln[\text{final mean body weight (g)}] - \ln[\text{initial mean body weight (g)}]}{\text{time interval (days)}} \times 100.$$

$$\text{CF} = \frac{\text{weight (g)}}{[\text{length (cm)}]^3}$$

4- Hematological analysis and immunological tests

Thirty tilapia from each of the echinacea treatment and control groups (10 fish/replicate) were anaesthetized with MS-222. Blood samples were collected from the caudal vein to be used as whole blood and for serum separation. The whole blood was used for the estimation of total and differential leukocytic count according to the methodology of *Stoskoph (1993)*, as well as for determination of hematocrit values (*Wintrob 1967*) and nitroblue tetrazolium reduction assay (*Anderson et al., 1992*). Blood serum was used to quantify lysozyme activity, as described by *Sankaran and Gurnani (1972)*. Serum lysozyme was determined via turbidometric assay according to *Sankaran and Gurnani (1972)*. *Micrococcus lysodeikticus* lyophilized cells (Sigma Chemical Co.) were suspended in phosphate buffer (pH 7.2) at a concentration of 0.25 mg/ml and used as a substrate solution. Two hundred microliters of serum, diluted with an equal volume of phosphate buffered saline (PBS), were added to 1.3 ml of the substrate solution at 25 °C and measured immediately at an optical density of 450 nm. After 30 minutes incubation in a humidified environment at 25°C, the optical density was again measured. A lyophilized hen egg-white lysozyme (Sigma Chemical .Co.) was used to develop the standard curve. Serum lysozyme values were expressed as µg/ml equivalent of hen egg-white activity.

5- Survival and mortality after the challenge infection

The survival rate of the experimental fish was calculated at the end of the sixth month. The challenge test was done in 3 replicates where 30 fish from each pond of both control and echinacea treatment groups were transferred to glass aquaria and then inoculated with pathogenic *Pseudomonas fluorescens*. The inoculation was done via i/p route using 0.5 ml culture suspension of pathogenic *Pseudomonas fluorescens* containing 10⁸ bacteria ml⁻¹ that had been previously isolated from moribund fish and studied for their pathogenicity. The challenged fish from each pond were observed for 7 days in order to record the mortality.

6- Statistical analysis

Statistical analysis was performed by one-way analysis of variance (*Duncan 1955*). Multiple Range Test was carried out to determine differences between treatment means (significance level $P < 0.05$). Standard errors were also estimated. All analyses were run on the computer using the SAS program (*SAS, 2005*).

RESULTS

Body weight gain and specific growth rates in group 2 were significantly higher than those of the control group (gp. 1), although there was no significant change in condition factor between experimental and control groups. The blood and sera of group 2 showed a significant increase in hematocrit and lysozyme activity and a non-significant increase in the nitroblue tetrazolium values when compared with gp. (1). Total leukocytic counts were significantly higher in gp. (2) than in gp. (1) due to the significantly more elevated values of the lymphocytes and eosinophils. By contrast, there were no statistically significant differences in neutrophils, monocytes and basophils counts between treatment and control fish. Survival rates during the experimental period did not differ significantly between control and treatment groups. Mortality rates following the challenge infection, was non-significantly lower in gp. (2) than in gp. (1) (Table 2, Figs. 1 and 2).

Table 2. Survival, growth performance, some immunological and hematological parameters in gps. (1 & 2) as well as the mortality after challenge infection

Parameters evaluated	Control group (gp. 1)	Echinacea group (gp. 2)
Body Gain (gm)	161.83 ^B ± 3.96	183.16 ^A ± 3.8
Specific Growth Rate	1.49 ^B ± 0.01	1.54 ^A ± 0.01
Condition Factor	0.017 ^A ± 0.001	0.017 ^A ± 0.001
Nitroblue tetrazolium (mg/ml)	0.71 ^A ± 0.022	0.75 ^A ± 0.02
Lysozymes (µg/ml)	8.89 ^B ± 0.08	9.47 ^A ± 0.15
Hematocrit (%)	29.35 ^B ± 0.71	31.48 ^A ± 0.77
Total leukocytic count (10 ³ /µl)	40.57 ^B ± 0.48	42.47 ^A ± 0.56
Neutrophils (10 ³ /µl)	6.96 ^A ± 0.16	7.07 ^A ± 0.11
Lymphocytes (10 ³ /µl)	31.49 ^B ± 0.42	33.1 ^A ± 0.58
Eosinophils (10 ³ /µl)	0.60 ^B ± 0.04	0.79 ^A ± 0.06
Basophils (10 ³ /µl)	0.24 ^A ± 0.04	0.21 ^A ± 0.04
Monocytes (10 ³ /µl)	1.27 ^A ± 0.08	1.27 ^A ± 0.09
Survival (%)	69.83 ^A ± 7.16	76.67 ^A ± 4.69
Mortality after challenge (%)	33.33 ^A ± 5.27	28.33 ^A ± 4.4

Fig. 1. Body gain, Survival and the mortality after challenge infection in both echinacea treated and control groups

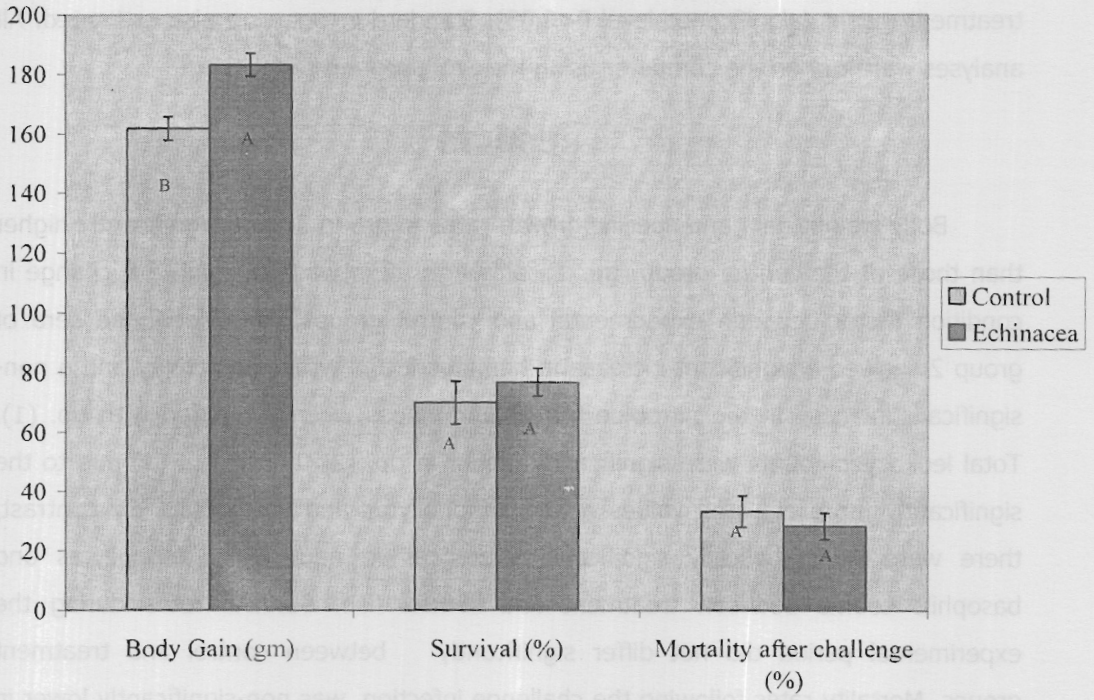
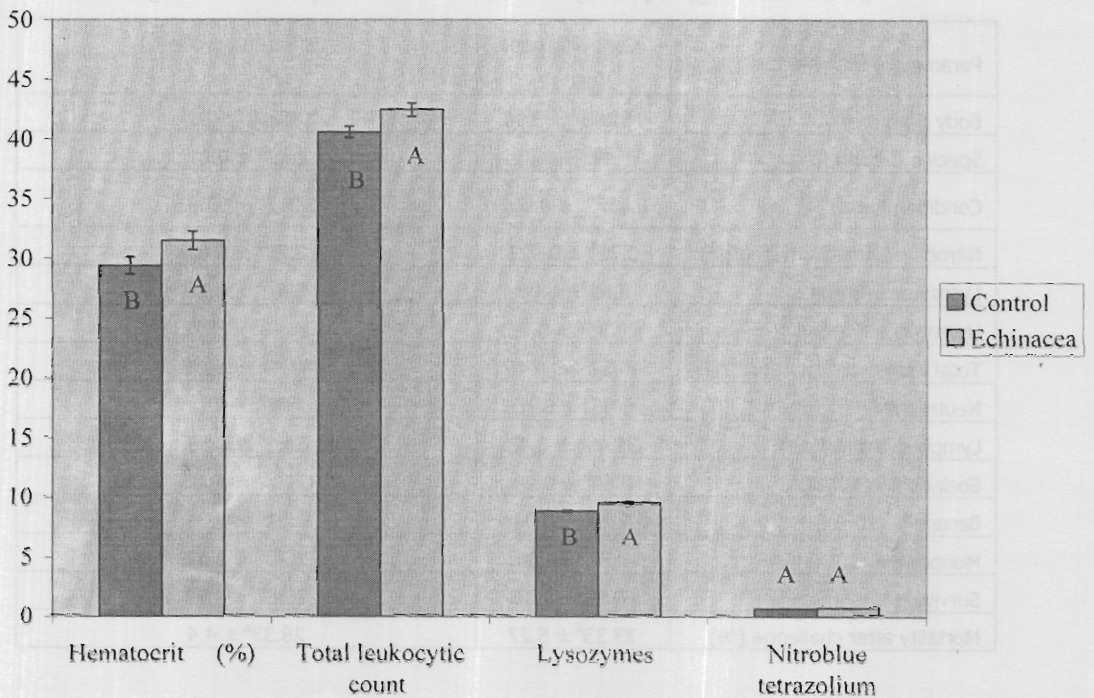


Fig. 2. Some immunological and hematological parameters in the echinacea treated and control groups



DISCUSSION

The echinacea supplemented diet improved the feed conversion which agreed with the results of *Maass (2005)* who reported that the echinacea botanicals (herbs and/or spices), administered as feed additive, and improved feed conversion. The mode of action of the herb mixtures is through the enhancement of the digestive functions (*Przybilla and Weib, 1998*).

The fish blood packed cell volume is an indicator of the health status and can be helpful in detecting any abnormal changes including improvement through the use of immunostimulants. Anemic fish may have hematocrit values as low as 10%. The reduced hematocrit values may indicate that the fish are not eating properly or were suffering from infections (*Blaxhall, 1972*). The blood and sera of fish of gp. 2 showed a significant increase in mean hematocrit and lysozyme activity that indicated improved health and immune status of the echinacea treated group.

The nitroblue tetrazolium (NBT) assay was used to determine the activity of the phagocytes, especially neutrophils and monocytes (*Jabs et al., 1980*). A non significant increase in the NBT values was recorded in gp. 2., possible as a result of the apparent none statistically significant increase in neutrophils seen during the hematological examination, which also indicated the role of echinacea in increasing the number and activities of blood cellular elements in the experimental fish.

The significantly increased total leukocytic count in gp. 2 was associated with the increase in lymphocytes and eosinophils. This may explain the efficacy of echinacea in terms of the health status and non-specific immune response that lowered the mortality rate in gp. 2 during the post-challenge test period. However, the significant increase in lymphocytes might also indicate the specific and non-specific immunostimulant role of echinacea. *Bauer (1996)* found in vitro and in vivo pharmacological effects associated with extracts from the aerial parts of *E. purpurea* and the alcoholic extracts of the roots of *E. angustifolia*, *E. purpurea* and *E. pallida*. The effects were mainly linked to a modulation of the non-specific cellular immune system by polysaccharides, glycoproteins, caffeic acid derivatives and alkylamides. Moreover, the various immune cells (macrophages, monocytes and natural killer cells) were stimulated in vitro by echinacea extract (*Bauer, 1998, 1999; Rininger et al., 2000; Sun LZ-Y et al., 2001*).

The echinacea treated group in the current work, exhibited significantly higher survival throughout the experimental period and a non-statistically significant lower mortality rate post challenge infection when compared with the control group. Polinacea (*Echinacea angustifolia* root extract) demonstrated immunostimulatory activity by reducing the *Candida albicans* mortality both in normal and cyclosporin A-treated mice *Morazzonia et al., (2005)* that showed the efficient immunostimulatory and bacteriocidal effect of echinacea. Moreover, *Roesler et al., (1991)* noticed that the

Echinacea purpurea increased the proliferation of phagocytes and migration of granulocytes in the peripheral blood resulting in excellent protection against prevailing pathogens.

It may be concluded that, echinacea acts as both an immunostimulant and a disease control agent in fish. It may be recommended as a dietary supplement in order to improve aquaculture production, after further studies are running to evaluate cost-benefits.

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الاكينيسيا كمنشط مناعي في أسماك البلطي من خلال تجربة في أحواض ترابية

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أجريت هذه الدراسة على ١٢٠٠ سمكة من أسماك البلطي يتراوح وزنها ما بين $4,5 \pm 0,2$ جم لاستبيان تأثير الاكينيسيا على معدل نمو ومقاومة الأسماك للأمراض من خلال تربيتها في أحواض ترابية. تم توزيع هذه الأسماك بالتساوي وبطريقة عشوائية على ستة أحواض ترابية دائرية (٢م^{١٠٠}) بمعدل كثافة ٢ سمكة/م^٢ ، وبطريقة عشوائية تم اختبار ثلاثة أحواض كمجموعة معاملة حيث تم تغذيتها على غذاء صناعي متوازن ومزود بالاكينيسيا بمعدل ٠,٢٥ جزء في الألف من وزن العليقة الجافة ، أما الثلاثة أحواض الأخرى فقد تغذت على الغذاء الصناعي فقط كمجموعة ضابطة ، وقد تم تغذية جميع الأسماك بنسبة ٣% من وزن الجسم يومياً لمدة ٦ أشهر. وقد تم تسجيل معدل النمو ومستوى الإعاشة في نهاية التجربة ، وقد تم تسجيل معدل النمو ومستوى الإعاشة في نهاية التجربة ، كما تحديد الفرق بين المجموعتان في معدلات النمو والعدد الكلي والنوعي لكرات الدم البيضاء واختبار التترازوليوم المناعي. وقد تم عمل اختبار تحدى على ٩٠ من أسماك البلطي في كل مجموعة من خلال الحقن البروتوني لميكروب السيدوموس فلوريسس الممرض (٠,٥ مللي/سمكة كل ١ مللي يحتوي على 10^8 خلية بكتيرية) ، ثم تم تسجيل معدل النفوق بعد العدوى لمدة ٧ أيام . وأوضحت المجموعة المعاملة بالاكينيسيا تزايد معنوي في زيادة الوزن ومعدل النمو ونسبة الهيموجلوبين ونشاط أنزيم الليسوزيم والعدد الكلي لكرات الدم البيضاء وخاصة الليمفوسيت والايذبنوفيل وذلك بالمقارنة بالمجموعة الضابطة ، كما زادت نسبة الإعاشة في المجموعة المعاملة بالاكينيسيا في نهاية التجربة وكذلك بعد العدوى التجريبية ، كما لم يلاحظ تغيير معنوي في خلايا الموفوسيت والتترازوليم المناعي. وقد خلص العمل إلى إمكانية استخدام الاكينيسيا كمحفز للنمو ومنشط مناعي وللسيطرة على الأمراض في الأسماك ، ويوصى باستخدامه كوسيلة لتحسين إنتاج أسماك البلطي تحت ظروف خاصة .