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# EFFECTS OF *ECHINACEA PURPUREA* AND VITAMIN C ON THE HEALTH STATUS, IMMUNE RESPONSE AND RESISTANCE OF *OREOCHROMIS NILOTICUS* TO *AEROMONAS SOBRIA* INFECTION

**Alshimaa A. Khalil** and **Mohamed ElHady**

*Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Zagazig University, Egypt.*

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***Abstract***

This study was conducted to investigate the effect of the dietary supplementation of *Echinacea purpurea* extract (EP) and Vitamin C, either singular or combined on the health status, immune response and resistance of Nile tilapia, *Oreochromis niloticus,* to *Aeromonas sobria* (*A. sobria*) infection. A total number of 120 fish were randomly distributed into four equal groups. The 1st group was fed on a basal diet and was kept as a control. The 2nd and 3rd groups were fed on EP (500 mg $kg^{-1}$) and Vitamin C (400 mg $kg^{-1}$) supplemented diets, respectively. While the diet fed to the 4th group was supplemented by a mixture of EP (500 mg $kg^{-1}$) and Vitamin C (400 mg $kg^{-1}$). After 28 days of feeding trial, some hematological (Rbcs, Wbcs, Hct and Hb) and non-specific immune parameters (serum bactericidal activity, lysozymes, total proteins, albumin and globulins) were evaluated, as well as a challenge test using a pathogenic *A. sobria* strain was performed. The results illustrated that, oral administration of *Echinacea purpurea* (500 mg $kg^{-1}$) or Vitamin C (400 mg $kg^{-1}$) significantly enhanced the serum lysozymes and bactericidal activities, as well as exhibited a relatively similar improving effect on the haematological indices assayed and the protection against *A. sobria* challenge. Whereas, their simultaneous administration showed synergistically enhanced serum lysozymes, bactericidal activities, total proteins and globulins when compared to the use of a single compound, as well as a higher survival was noticed after *A. sobria* challenge.

**Keywords:** *Echinacea purpurea,* Vitamin C, *Aeromonas sobria,* serum bactericidal activity, lysozymes.

## INTRODUCTION

Aquaculture is considered the fastest growing animal food producing sector in the world, and recently it has been revealed to be the main source for increasing fish supply. Rapid development of aquaculture and increasing fish demand lead to intensification of fish culture, amplifying stressors for fish and thus increasing the risk of diseases (Reverter *et al.,* 2014). Infectious diseases represent the main problem facing the fish farming industry, resulting in high economic losses (Bulfon *et al.,* 2015). Motile aeromonads, particularly *Aeromonas hydrophila*, *A. sobria* and *A. caviae* are considered among the most common bacteria associated with diseases in freshwater fishes (Cipriano, 2001). Antibiotics and chemotherapeutics used for prophylaxis and/or treatment of fish diseases have been widely criticized for their side effects such as resistance, toxicity, environmental hazards and residual impacts on public health (Tang *et al.*, 2014). Therefore, seeking for alternative less harmful and more environmentally-friendly approaches for disease management in aquaculture become urgent (Reverter *et al.,* 2014).

The use of immunostimulants in aquaculture as an alternative to the drugs, chemicals and antibiotics is rising and attracting the attention of many researchers (Galina *et al.,* 2009). An immunostimulant is a substance that enhances the non-specific defense mechanism and/or the specific immune response, thus helping the animal to be more resistant to diseases (Anderson, 1992). Although many natural and synthetic substances have been reported to potentiate fish immunity and increase disease resistance, a number of plant extracts (Vaseeharan and Thaya, 2014) and antioxidant vitamins (Ortuño *et al.*, 2001) have recently received particular attention as it is cheap, have a low impact on the environment and easily incorporated into the diet (Guz *et al.*, 2011).

*Echinacea purpurea*, a globally popular herbal medicine, has been known as immunostimulant in human, in other animals and in fish (Tang *et al.*, 2014). In fish, dietary supplementation with *Echinacea purpurea* has been reported to enhance the growth rate, survival rate and promote the immune response and resistance against bacterial infection (Aly *et al.,* 2008; Aly and Mohamed, 2010; Oskoii *et al.,* 2012).The Echinacea immunostimulatory properties appear to involve both non-specific and specific immune function (Rehman *et al.,* 1999).

On the other hand, one of the micronutrients that affect the immune system is vitamin C (Ascorbic acid), which is required for normal growth and is involved in various physiological functions in fishes (Zhou *et al.,* 2012). Many fish species cannot synthesize vitamin C. The inability to synthesize vitamin C is owing to a lack of L-gulonolactone oxidase enzyme that is responsible for synthesis of vitamin C de novo (Roy and Guha, 1958). Therefore, an exogenous source of vitamin C is required in fish diets, with a recommended value ranged from 20 to 50 mg $kg^{-1}$ (Lovell, 1991). However, the use of vitamin C at dietary levels higher than standard doses has been found to improve the growth rate, survival rate and innate immunity of fishes (Ibrahem *et al.*, 2010 and Zhou *et al.,* 2012).

There is no available information in the literature addressed the effect of simultaneous administration of the two immunostimulants; *Echinacea purpurea* and vitamin C on fish. The aim of the current study was to investigate the potential interaction between *Echinacea purpurea* extract (500 mg $kg^{-1}$) and vitamin C (400 mg $kg^{-1}$) on the health status, immune response and resistance of Nile tilapia (*Oreochromis niloticus*) against *Aeromonas sobria* challenge.

## MATERIAL AND METHODS

### Fish:

Nile tilapia, *Oreochromis niloticus* (*O. niloticus*) with average body weight of 78 ± 13g $fish^{-1}$ were obtained from a private fish hatchery in Abbassa - Sharkia governorate – Egypt. They were transferred to the Fish Diseases and Management wet lab. at Faculty of Veterinary Medicine, Zagazig University, Egypt. Fish were kept in well prepared glass aquaria for 2 weeks under observation to be acclimated to the new environment and they were fed a basal diet.

**Diets used for fish:**

A balanced basal diet ($D_1$) contained 30% crude protein was prepared in the Fish Research Unit at Faculty of Veterinary Medicine, Zagazig University, Egypt. Three test diets were also prepared; $D_2$ was a basal diet in which *Echinacea purpurea* extract (EP) (500 mg $kg^{-1}$) was incorporated, $D_3$ was a basal diet in which Vitamin C (400 mg $kg^{-1}$) was incorporated, while $D_4$ was a basal diet in which a mixture of EP (500 mg $kg^{-1}$) and Vitamin C (400 mg $kg^{-1}$) were incorporated. The prepared diets were air-dried at room temperature for 8 hours and then kept in dark plastic bags at 4°C.

**Experimental Design:**

A total number of 120 apparently healthy *O. niloticus* were randomly distributed into four equal groups ($G_1$, $G_2$, $G_3$ and $G_4$). Each group consisted of 3 replicates with 10 fish per replicate. Fish of $1^{st}$ group ($G_1$) were fed on the balanced basal diet ($D_1$) and was kept as a control group, while fish of $2^{nd}$, $3^{rd}$ and $4^{th}$ groups ($G_2$, $G_3$ and $G_4$) were fed on the experimental diets ($D_2$, $D_3$ and $D_4$), respectively. Fish of all groups were fed twice daily at a rate of 3% of biomass for 28 days. Water was partially exchanged daily, and the water quality was monitored and kept within the normal range; dissolved oxygen was not less than 5.0 mg $L^{-1}$, and ammonia-N and nitrite-N concentrations were less than 0.1 mg $L^{-1}$, and water temperature was 26 ± 2° C along the experiment. At the end of feeding period, survival rates were recorded, and some hematological and immunological parameters were evaluated.

**Hematological and immunological aspects:**

At the end of the feeding period, blood samples were collected from the caudal vessels of fish of each group using sterile heparinized syringes. These blood samples were used for estimation of total erythrocytic (Rbcs) and leukocytic (Wbcs) counts and the hemoglobin (Hb) and haematocrit (Hct) values (Blaxhall and Daisley, 1973). While, another blood samples were withdrawn into Eppendorf tubes without anticoagulant and centrifuged at 3000 rpm for 15 minutes for serum separation which used for determination of lysozymes (Parry *et al.*, 1965) and serum bactericidal activities (SBA) against *A. sobria* (Kajita *et al.*, 1990). The total serum proteins and albumin content were measured by biuret and bromocresol green binding method (Savory *et al.*, 1976), while globulins was calculated by distracting albumin from total proteins.

**Challenge test:**

At the end of the feeding period, ten fish from each group were I/P challenged with 0.5 ml fresh culture suspension of *A. sobria* containing $10^7$ bacterial cells $ml^{-1}$, while another 10 fish were I/P injected with 0.5 ml sterile physiological saline (0.9% NaCl). The challenged fish were kept under daily observation for up to 14 days. The daily mortalities were recorded and the relative level of protection (RLP) among the challenged fish was calculated (Ruangroupan *et al.*, 1986) using the following equation:

**RLP** = 1- (percentage of treated mortality ÷ percentage of control mortality) X 100

**Statistical analysis:**

Data were analyzed using SPSS Statistics program, where the analysis of variance (ANOVA) and Duncan`s Multiple Range Test (Duncan, 1955) were used to detect the differences between treatments (levels of significance are expressed as $P \leq 0.05$).

## RESULTS AND DISCUSSION

Recently, there has been increased interest in the possibility of using certain natural products as immunostimulants, capable of improving fish health status, immune response and disease resistance against infections (Bulfon *et al.*, 2015). The fish health status in response to dietary supplementation, environmental alteration and/or stress could be monitored through the haematological analysis (Shalaby *et al.*, 2006). In the current study, improvement of the hematological indices (Rbcs count, Wbcs count, Hct and haemoglobin %) of *O. niloticus* was noticed after feeding on *Echinacea purpurea* (500 mg $kg^{-1}$) and/or vitamin C (400 mg $kg^{-1}$) supplemented diets (Table 1), that is indicating a positive effect on the health status of fish. Similarly, improvement in fish health status and different hematological indices have been previously recorded after dietary supplementation with *Echinacea purpurea* (Aly *et al.,* 2008 and Oskoii *et al.*, 2012) and vitamin C (Ibrahem *et al.,* 2010 and Zhou *et al.,* 2012). The increased hematological parameters might be a result of a stimulatory effect on hematopoiesis process of fish by active ingredients in *Echinacea purpurea* as suggested by Aly and Mohamed (2010) and Oskoii *et al.* (2012). Whereas, vitamin C is a powerful antioxidant vitamin that help in protecting fish against oxidative damage of various tissues including blood cells as proved by Sahoo and Mukherjee (2003).

The total leukocytic count of all treated groups was significantly increased in comparison with the control group as demonstrated in table (1). These findings correspond with the results of Aly *et al.* (2008) who stated that Wbcs counts were significantly increased in EP-fed group; and this may explain the efficacy of EP in terms of the health status and non-specific immune response. These results can be also explained by Sun *et al.* ( 2001) who discussed the effect of EP in which it make a modulation of the non-specific cellular immune system by polysaccharides, glycoproteins, caffeic acid derivatives and alkylamides. In addition, the various immune cells (macrophages, monocytes and natural killer cells) were stimulated in vitro by Echinacea extract. Whereas, feeding fish with much higher dietary vitamin C concentrations are necessary for fish growth and improvement of its immune responses (Ortuño *et al.,* 2003) including white blood cells.

The dietary supplementation of *Echinacea purpurea* (500 mg $kg^{-1}$) and/or vitamin C (400 mg $kg^{-1}$) caused significant increases in serum lysozymes and serum bactericidal activity (SBA) as illustrated in table 2, that is suggesting a stimulation of the fish immune system and improvement in health status. These results agree with the findings of Aly *et al.* (2008) and Ibrahem *et al.*

(2010) who reported the immunostimulatory effects of *Echinacea purpurea* and vitamin C supplemented diets on *O. niloticus*, respectively. The serum lysozymes play an important role in the defense of fish as a bactericidal enzyme inducing bacteriolysis and preventing the growth of bacteria (Bulfon *et al.*, 2015). Therefore, the increased SBA activities could be related to the higher concentration of serum lysozymes (Misra *et al.*, 2007) and other various humoral factors which involved in innate and/or adaptive immunity and are increased in the serum to protect the fish from infection (Maqsood *et al.*, 2010). Furthermore, it was found that the diet contained a mixture of EP and vitamin C resulted in a significantly higher serum total protein and globulins when compared to other groups (table 3). The increase in serum/plasma total proteins and globulins is also considered a strong innate-immune response in fishes (Reverter *et al.*, 2014). Moreover, Serum globulins, mainly gamma globulins, are the origin of immunoglobulins, so their level in blood can be used as a indicator to the concentration of antibodies and consequently the immune activity of fish (Goda, 2008). These results are in accordance with that of Andrade *et al.* (2007) who mentioned that high Vitamin C in fish feed could enhance protein and globulin synthesis. Also, Oskoii *et al.* (2012) reported that serum total protein, albumin, and globulin levels showed a significant increase in EP-fed fish.

The enhancement that observed in the immune functions could be attributed to the polysaccharides, glycoproteins, caffeic acid derivatives, alkamides and isobutylamides that present in Echinacea and were found to be responsible for its immunomodulatory effects (Bauer, 1998).Whereas, the antioxidant activity of vitamin C could, in part at least, enhance the immunity by maintaining the structural and functional integrity of immune cells as stated by Chew (1995).

High levels of protection against *A. sobria* challenge were noticed in *O. niloticus* fed on either *Echinacea purpurea* (500 mg $kg^{-1}$) or vitamin C (400 mg $kg^{-1}$) (Table 4). These results are in accordance with the results of Aly *et al.* (2008) and Guz *et al.* (2011) who reported the efficient effect of *Echinacea* *purpurea* in improving the fish resistance against bacterial challenge. Also, it was previously noted that the dietary supplementation with vitamin C could enhance the fish resistance against challenge infection as recorded by Ibrahem *et al.* (2010) and Zhou *et al.* (2012). The improvement of disease resistance and effective protection against pathogen challenge or infection could be attributed to the enhanced immune activity as explained by Ai *et al.* (2006) and Guz *et al.* (2011).

On the other hand, feeding *O. niloticus* on a mixture of *Echinacea purpurea* extract and vitamin C induced a significantly higher serum lysozymes, SBA, total proteins and globulins when compared to the use of a single compound (Table 2 & 3), and also a lower mortality was noticed after *A. sobria* challenge (Table 4). These findings might be attributed to a potential synergistic action between *Echinacea purpurea* and vitamin C on the fish immune response. The inclusion of a mixture of herbal extracts in fish diets could bring a synergistic action on various physiological functions including growth performance, immune response and diseases resistance as documented by Ji *et al.* (2007). As well, it is likely that certain Echinacea preparations can display synergism (Hudson, 2012). In addition, Ortuño *et al.* (2001) found that the simultaneous dietary administration of vitamins C and E synergistically enhanced the seabream immune response especially the respiratory burst activity.

In conclusion: the current results not only demonstrate the beneficial effect of *Echinacea purpurea* (500 mg $kg^{-1}$) and/or vitamin C (400 mg $kg^{-1}$) in improving the fish health status and immune resistance against *A. sobria* infection, but also suggest a potential synergistic action between the two immunostimulants. Therefore, it is recommended to be simultaneously incorporated in fish diets, but after further investigations including; assessing of their cost effectiveness, understanding the mechanism of interaction, and investigating the degree and duration of the resistance induced.

**Table 1.** Effect of *Echinacea purpurea* and Vitamin C supplemented diets on some hematological indices of *O. niloticus*.

| Group | Diet | Supplemented Material | Rbcs count ($10^6/mm^3$) | Wbcs count ($10^3/mm^3$) | Haemoglobin (g/dl) | Haematocrit (%) |
|---|---|---|---|---|---|---|
| **$G_1$(control)** | $D_1$(Basal) | -- | 1.05±0.07$^{b}$ | 18.00±1.15$^{b}$ | 3.90±0.49$^{b}$ | 16.57±2.06$^{b}$ |
| **$G_2$** | $D_2$ | EP | 1.57±0.22$^{a}$ | 28.66±3.71$^{a}$ | 6.51±0.11$^{a}$ | 29.79±2.81$^{a}$ |
| **$G_3$** | $D_3$ | Vitamin C | 1.21±0.00$^{ba}$ | 30.33±1.45$^{a}$ | 5.52±0.29$^{a}$ | 20.53±2.82$^{b}$ |
| **$G_4$** | $D_4$ | EP + Vitamin C | 1.33±0.15$^{ab}$ | 26.00±1.15$^{a}$ | 6.17±0.08$^{a}$ | 23.53±1.76$^{ab}$ |

Means carrying different superscript in the same column are significantly different (P≤0.05).

**Table 2.** Effect of dietary supplementation of *Echinacea purpurea* and Vitamin C on serum total protein, albumin and globulin of *O. niloticus*.

| Group | Diet | Supplemented Material | Total protein (g/dl) | Albumin (g/dl) | Globulin (g/dl) |
|---|---|---|---|---|---|
| **$G_1$ (control)** | $D_1$ (Basal) | -- | 3.50±0.20b$^{c}$ | 1.34±0.04$^{b}$ | 2.20±0.18$^{bc}$ |
| **$G_2$** | $D_2$ | EP | 3.35±0.15$^{c}$ | 1.59±0.03$^{a}$ | 1.75±0.17$^{c}$ |
| **$G_3$** | $D_3$ | Vitamin C | 4.00±0.10$^{b}$ | 1.30±0.02$^{b}$ | 2.66±0.06$^{b}$ |
| **$G_4$** | $D_4$ | EP + Vitamin C | 4.58±0.08$^{a}$ | 1.55±0.01$^{a}$ | 3.03±0.07$^{a}$ |

Means carrying different superscript in the same column are significantly different (P≤0.05).

**Table 3.** Effect of dietary supplementation of *Echinacea purpurea* and Vitamin C on some immunological parameters of *O. niloticus*.

| Group | Diet | Supplemented Material | Lysozyme activity (U/ml) | Serum bactericidal activity ($10^5$ cfu/ml) |
|---|---|---|---|---|
| **$G_1$ (control)** | $D_1$ (Basal) | -- | 0.174±0.0023[c] | 27.7±0.37[a] |
| **$G_2$** | $D_2$ | EP | 0.209±0.0002[b] | 14.6±1.64[b] |
| **$G_3$** | $D_3$ | Vitamin C | 0.214±0.0000[b] | 17.1±1.41[b] |
| **$G_4$** | $D_4$ | EP + Vitamin C | 0.305±0.0028[a] | 10.1±0.40[c] |

Means carrying different superscript in the same column are significantly different (P≤0.05).

**Table 4.** Effect of dietary supplementation of *Echinacea purpurea* and Vitamin C on *Aeromonas sobria* challenged *O. niloticus*.

| Group | Diet | Supplemented Material | Mortality (%) | RLP (%) |
|---|---|---|---|---|
| **$G_1$ (control)** | $D_1$ (Basal) | -- | 40.00 | 0.00 |
| **$G_2$** | $D_2$ | EP | 20.00 | 50.00 |
| **$G_3$** | $D_3$ | Vitamin C | 20.00 | 50.00 |
| **$G_4$** | $D_4$ | EP + Vitamin C | 10.00 | 75.00 |

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# تأثير القنفذية الأرجوانية وفيتامين ج على الحالة الصحية و الاستجابة المناعية ومقاومة البلطي النيلي للإصابة بالإيروموناس سوبريا

**الشيماء عبد العزيز خليل، محمد الهادي أحمد**

*قسم أمراض ورعاية الأسماك - كلية الطب البيطري - جامعة الزقازيق- مصر.*

## الملخـص العربــي

لقد أجريت هذه الدراسة لمعرفة تأثير كلاً من القنفذية الأرجوانية وفيتامين ج المضافين الي العليقة سواء معاً أو كل علي حدى على الحالة الصحية والاستجابة المناعية ومقاومة البلطي النيلي لعدوى الإيروموناس سوبريا. لقد تم توزيع عدد ١٢٠ سمكة عشوائيا إلى أربع مجموعات متساوية. و تمت تغذية الأسماك في المجموعة الأولي على عليقة ذات مكونات أساسية وبدون أي اضافات وتم الاحتفاظ بها كمجموعة ضابطة. و تم تغذية الأسماك في المجموعة الثانية والثالثة على عليقة مزودة بالقنفذية الأرجوانية (٥٠٠ ملجم/ كجم) واخرى مزودة بفيتامين ج (٤٠٠ ملجم/ كجم) على التوالي. في حين أن المجموعة الرابعة تمت تغذيتها بعليقة مزودة بخليط من القنفذية الأرجوانية (٥٠٠ ملجم/ كجم) وفيتامين ج (٤٠٠ ملجم/ كجم). بعد ٢٨ يوما من التغذية، تم تقييم بعض مكونات الدم (عدد كرات الدم الحمراء، الكريات البيضاء، هيماتوكريت، والهيموجلوبن) وكذلك بعض الانشطة المناعية الغير مخصصة (قدرة مصل الدم على قتل البكتريا، نشاط الليزوزيم ، كمية البروتين الكلي، الألبيومين والجلوبيولين)، فضلا عن اجراء اختبار العدوى اللاصطناعية باستخدام سلالة ممرضة من الإيروموناس سوبريا. وقد كشفت النتائج أن تناول القنفذية الأرجوانية (٥٠٠ ملجم/ كجم) أو فيتامين ج (٤٠٠ ملجم/ كجم) في العليقة أديا الى زيادة معنوية في نشاط الليزوزيم وقدرة مصل الدم على قتل البكتريا ، وكذلك أظهرا تحسن مماثل نسبياً في قياسات الدم وفي تحفيز الحماية ضد العدوي بالأيرموناس سوبريا. في حين أن تناولهما معاً في عليقة واحدة أدى الى تعزيز الزيادة في نشاط كلاً من الليزوزيم وقدرة مصل الدم على قتل البكتريا ، وكذلك كمية البروتين الكلي والجلوبيولين بالمقارنة مع استخدام احداهما فقط في العليقة فضلاً عن ملاحظة نسبة إعاشة أعلى في الاسماك بعد العدوي بالأيرموناس سوبريا.