The Use of Eugenia Caryophllate Extract in Practical Diets as Immunity Promoter for Nile Tilapia, Oreochromis Niloticus (L.) Fingerlinges Challenged with Pathogenic *Aeromonas hydrophila*

Doaa. A. El-Araby

Fish health and Management Dept., Central Laboratory for Aquaculture Research (CLAR). Abbassa Abou Hammad, Sharkia, Egypt.

Abstract

This experiment was conducted to evaluate the use of ethanol-extracted from the medicinal plant, *eugenia Caryophllate* as immunity promoter for Nile tilapia, *Oreochromis niloticus* (L.) fingerlings. Fish (Average 12.27 g) were randomly distributed into four treatments; three replicates each at a rate of 20 fish per 100– L aquarium. Fish were fed one of the tested diets containing similar crude protein (30 %) and gross energy (4.40 kcal / g), in addition to 0.0, 0.5 %, 1.0%, or 1.5 % *eugenia caryophllate* extract. fish of each treatment were challenged by pathogenic *Aeromonas Hydrophila*, which was given by intraperitoneal (I/P) injection and they were kept under observation for 10 days to follow up any abnormal clinical signs and the daily mortality rate. meanwhile total protein and albumin, increased significantly ($P<0.5$) to the highest values at 0.5 % *eugenia caryophllate* extract, as compared to the control. WBCs count and Hb values increased with non- significant differences with increase in *eugenia caryophllate* extract level in fsh diet ($P>0.05$). This present study, showed that 0.5 % *eugenia caryophllate* extract in Nile tilapia diets, increased the fish resistance to *Aeromonas Hydrophila*, indicating the effective role of *eugenia caryophllate* extract in disease prevention in tilapia culture. The reduction in feed cost compared with control diet showed 12.52 % to produce one kg fish gain of treatment containing 0.5 % extracted *Eugenia caryophllate* levels.

Key of words: *eugenia Caryophllate,* Nile tilapia, physiological parameters, *Aeromonas hydrophila*-challenge

Introduction

Tilapia species are widely distributed in many countries of the world. There farming has grown extremely fast in the last decade, where they are cultured worldwide with annual growth rate of about 12.2% **(*El-Sayed, 2006*).** In Egypt, Nile tilapia is a major species in aquaculture system and much

appreciated by consumers. However, the success of intensive tilapia culture depends to a large extent on supplemental feeding.

Some medicinal plants, as natural growth and immunity promaters in fish **(*El Daker, 2004 and Shalaby, 2004*)** Some vegetable herbs edible plants seeds are used as natural tonic and restoratives **(*Boulos, 1983*).** Medicinal and aromatic plants have been used for many years in human nutrition as spices and medical additives for animals to increase dietary energy utilization, imporove the oerformance efficiency and as anew source of protein (***El–Katcha, 1990 and Abdel-Aal and Attia, 1993)*.** Several medicinal herbs are used in the medication of various diseases; like reducing high blood cholesterol, protection from cancer, chronic diseases, as well as stimulating the immune system. Furthermore, these herbs contain also aromatic substances and essential oils used in food industries **(*Evans and Pharm, 1975 and Craig, 1999*)** Antimicrobial substances are now widely used for the treatment of bacterial diseases for fish **(*Sahin et al., 2004 and Jirawan et al., 2005).***

The genus *eugenia caryophllate* occur in the Egyptian as fruticose shrubs or perennial herbs with ovate, entire leaves, and corymbose or paniculateds inflorescnces**. (*Montaser and Hassib, 1956*).** *Eugenia caryophllate* is a native to the Mediterranean, Euro-Siberian and Irano-Siberian regions **(*Aligiannis, et al., 2001)*.** Due to the variability in chemical and aromatic characteristics, *eugenia caryophllate* plants belonging to different species and ecotypes are widely used in agriculture, pharmaceutical and cosmetic industries as a culinary herb, flavoring substance of food products, alcoholic beverages and perfumery for their spicy fragrance. The content of essential oils and extracts of *eugenia caryophllate* species have antimicrobial, antioxidant and other biological activities, which may change based on the differences in cultivation, origin, vegetative stage and growing seasons of the plants **(*Milos et al., 2000; Aligiannis et al., 2001*).**

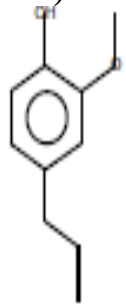
This study was conducted to assess the effects of *Eugenia caryophllate* extract on the immunity promoter, and resistance of Nile tilapia to *aeromonas hydrophila* infection.

Material and Methods

Extraction of *Eugenia caryophllate*:

A known weight of air-dried material of *eugenia caryophllate* was subjected to exhaustive extraction with ethyl alcohol (95%) using soxhlet apparatus. The obtained extract was then cooled, filtered and evaporated under vacuum to a thick syrup, which was dissolved in DMSO, the solution

was mixed with petroleum ether b.P (40-60˚C) and centrifuged at 5000rpm, a deep brown powder was obtained **(Doaa, 2016),** containing Eugenol (an active principal which protecting Fresh water fishes from bacterial diseases).



Diet Preparation:
Four experimental diets were prepared containing 30% crude protein and 7% lipid in addition to different levels 0.0, 0.5, 1.0, 1.5 % of *Eugenia caryophllate* extract (Table 1), *eugenia caryophllate* were obtained from local market and extracted in Lab. The dry ingredients of each diet were thoroughly mixed, 100 mL of water per kg diet was added and all contents were blended using kitchen blender to make a paste. Pelleting of diets was carried out by passing the blended mixture through laboratory pellet machine with 1 mm diameter matrix. The pellets were dried in a drying oven model (Fisher oven 13 – 261 – 28A) for 24 hours at 85 ^{o}C, stored in plastic bags and kept in a refrigerator at -2 ^{o}C during the experimental period to avoid rancidity **(*NRC, 1994).***

Challenge Test:
The experminted fish were divided into two groups. The first group comprised fish fed control diet, and challenged I/P with pathogenic *Aeromonas hydrophila*(0.2ml of 4 x 10^6 CFU). The second group, comprised fish fed diets containing different levels of *eugenia caryophllate* extract (0.5, 1.0 and 1.5) and challenged I/P with the same pathogen (0.2ml of 4 x 10^6 CFU). Both groups kept under observation for 10 days to record the daily mortality rate. Isolation and identification of pathogens were carried out according to ***Schäperclaus et al. (1992).***

Preparation of blood samples:
Fish were not fed in the 24 our immediately prior to samling . Fish were anaesthetized with buffered MS222 (30 mg/L) and blood was collected with a hypodermic syringe from the caudal vein. The blood collection lasted less than 3 min in order to avoid cortisol rise induced by the manipulation during sampling. The extracted blood was divided in two sets of Eppendorf tubes One set contained heparin, used as an anticoagulant, for hematology (hemoglobin, and red blood cell counting). The second set, without anticoagulant, was left to colt at 4°C and centrifuged at 5000 rpm for 5 min at room temperature. The collected serum

was stored at 20° C for further assays.

Physiological Measurement:

At the end of experiment feeding trial, fish were fasted for the 24 hour immediately prior to sampling and five fish per aquaria were randomly chosen and anesthetized with tricaine methane sulfate (20 mg/L). The blood samples were put in micro centrifuge tubes, without anticoagulant, left to clot at 4^{o}C and centrifuged at 5000 rpm for 5 minutes at room temperature. The collected serum was stored at 20^{o}C for further assays. Total protein content in plasma was determined colorimetrically according to ***Henry (1964),*** while plasma Albumin was estimated according to ***Wotton and Freeman (1982).***

Blood parameter: -

Blood parameter including Hb %, WBCs count and differential leucocytic count were measured according to method described by **(*Franco, 1984*) and (*Davis and Lewis, 1999)*.**

Economical evaluation:

The cost of feed required to produce a unit of fish biomass was estimated using a simple economic analysis. The estimation was based on the local retail sale market price of all the dietary ingredients during the time of this study. These prices (in LE/kg) were as follows: herring fish meal, 10; soybean meal, 2.0; corn meal, 1.50; starch, 3.0; Wheat bran, 1.25; fish oil, 7.0; corn oil, 5.0; vitamin premix, 7.0; mineral mixture, 3.0; extracted *eugenia caryophllate* 30.

Table (1)

Ingredients	% Control	At *eugenia caryophllate* extract % in the diets		
		0.5%	1%	1.5%
Fish meal	9.10	9.10	9.10	9.10
Soybean meal	45.50	45.50	45.50	45.50
Ground corn	15.31	15.31	15.31	15.31
Wheat bran	19.21	19.21	19.21	19.21
Starch	4.00	3.50	3.00	2.50
Eugenia caryophllate	0.00	0.50	1.00	1.50
Cod fish oil	2.23	2.23	2.23	2.23
Corn oil	1.65	1.65	1.65	1.65
Vitamins premix	1.00	1.00	1.00	1.00
Minerals Premix	2.00	2.00	2.00	2.00
Total	100	100	100	100
Chemical analysis %				
Dry matter	91.68	91.46	91.53	91.69
Crude protein	30.11	30.26	30.38	30.41
Crude fat	7.11	7.22	7.39	7.48
Ash	8.13	8.33	8.17	8.06
Fiber	5.45	5.32	5.55	5.32
NFE	49.20	48.87	48.51	48.73
GE(Kcal/100 g)	4390.3	4395.7	4403.8	4423.0
P/E ratio	68.58	68.84	68.99	68.75

Results and Discussion

In the results study, the experimental diets contained 30% crude protein and 4.4 kcallg diet (Table 1) are similar to that hsed by ***Ablel – wahab et al. (2007)*** *and* ***Ahmad (2008).***

The present results agree with those of *Shalaby (2004)* who found that feeding fenugreek seed meal to Nile tilapia improved growth rate and immunity may be due to the content of essential oil and extracts of *eugenia caryophllate species* containing (Eugenol) antimicrobial, antioxidant and other biological *activities* ***(Milos, et al., 2000 and Aligiannis et al., 2001).*** Furthermore, a better growth performance was observed in Nile tilapia fed on diet contained 0.5 % cinnamon***, (Abdel Wahab et al., 2007*)** and 1% marjoram ***(Abd El–Maksoud et al., 1999)*** as a feed additive to tilapia.

Results of fish challenging against of *Aeromonas hydrophila* for ten days are shown in Tables (2). No mortalities were observed in all diets containing different levels of *Eugenia caryophllate* extract. The highest overall fish mortality rates were observed in the control group (100 %). This enhanced immune response may be induced by the essential oils content and extracts of eugenia caryophllate species which contains (Eugenol) antimicrobial, antioxidant and other biological activities ***(Aligiannis et al., 2001 and Milos et al., 2000). Sahin et al. (2004)*** proved the antibacterial

effect of oils and extracts of *Eugenia caryophllate* species on many bacterial species like *Escherichia coli, Enterobacter sp., Bacillus sp., Salmonella sp., Staphylococcus aureus, Klebsiella pneumoniae, Listeria monocytogenes and Campylobacter jejuni.* ***Abdel – Wahab et al. (2007)*** found that 0.5 % cinnamon level in the diet is enough to eliminate the harmful microbes in the gut, improve absorption and control blood sugar to a certain extent giving reasonable growth enhancement.

Total protein and albumin, values increased significantly ($P \leq 0.5$) with *Eugenia caryophllate* extract, reaching the highest value at 0.5 % of the diet, as compared to the control (Table 3 & fig 1). The results indicate the improvement of fish health when fed diet with *eugenia caryophllate* extract. Moreover, the measurement of total protein, and albumin in serum or plasma are of considerable diagnostic value in fish, as it affects the general nutritional status as well as the integrity of the vascular system and liver function **(*Schäperclaus et al., 1992*).**

Blood Parameter: -

Results of hemoglobin concentration, as shown as (table 4 & fig 2) and leucocytic count in case of *eugenia caryophllate* diet fed groups are summarized in (table 5& fig 3) It shows that diets containing 0.5,1 and 1.5 % of *eugenia caryophllate* significantly increased some of the examined blood parameters. Hemoglobin concentration and leucocytic count increased significantly in fish fed on diets containing 0.5, 1 and 1.5 % *eugenia caryophllate* in comparison with the control group. Lymphocyte and Monocyte count increased significantly in fish fed on diets containing all *eugenia caryophllate* doses compared with the control group. *eugenia caryophllate* has some constituents that may play a role in the immune system stimulation and in the function of organs related to blood cell formation such as thymus, spleen, and bone marrow **(*Jeorg and Lee, 1998*) Alsi , (*Faisal, 2003*)** reported significantly increased values of haemoglobin concentration in *O. niloticus* and leukocyte count. The employment of hematological techniques, including enaluation of hemoglobin concentration and leukocyte count has provided valuable knowledge for fishery biologists in the assessment of fish health **(*Blaxhall, 1972*)** Addition of *eugenia caryophllate* to fish diets increased hemoglobin concentration and leukocyte count **(*Martins et al ,2002*).** *eugenia caryophllate* contains allicin, which promotes biogenic performance due to its positive effect on the ontestinal flora, thereby improving digestion

availability of natural feed, supply of nutrients and utilization of energy which influences the growth of fish **(*Khalil et al., 2001*).** The *eugenia caryophllate* show immune enhancing activates that include promotion of lymphocyte synthesis, cytokine release, phagocytosis and natural killer cell activity **(*Kyo et al., 1998*)**

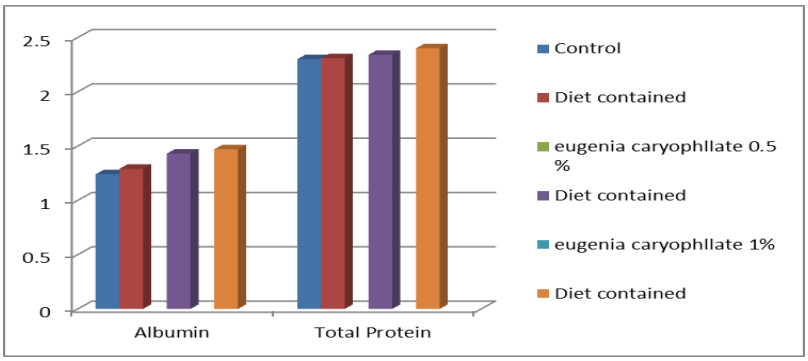
The economical evaluation of the experimental diets contained different *extracted Eugenia caryophllate* levels 0.0, 0.5 %, 1 % and 1.5 % are shown in Table (9). The highest reduction in feed cost compared with control diet showed to produce one kg fish gain of treatment containing 0.5 % extracted *Eugenia caryophllate.* The reduction in feed cost compared with control diet showed 12.52 % to produce one kg fish gain of treatment containing 0.5 % extracted *Eugenia caryophllate* levels. Previous studies showed that the use of spices in small amounts gave lower incidence cost and higher profit index of fish species ***(Abd-Elmonem et al., 2002; Sakr, 2003; Shalaby et al., 2003 and El-Dakar et al., 2004)***.

Table (2): *Mortality rate (%) of fingerlings nile tilapia o. niloticus fed diets containing different levels of eugenia caryophllate extract for 70 days and challenged by Aeromonas Hydrophila for 10 days.*

items		**no. of injection fish**	**bacteria dose (4 x 10^6 cfu)**	**injection route**	**mortality rate (%) after 10 days of injection**
control 0.0		10	0.2 ml	I / p	100 %
***eugenia caryophllate* levels % in the diet**	**0.5%**	10	0.2 ml	I / p	No
	1 %	10	0.2 ml	I / P	No
	1.5 %	10	0.2 ml	I / P	No

Table (3): *The effect of Eugenia caryophllate extract (0.5, 1, 1.5 %) on serum protein (g/dl), S.albumin (g/dl) in comparing with control of O.niloticus in aquarium during the Laboratory experiment. (Fig. 2)*

Treatment	Albumin	Total Protein
Control	1.24	2.30
Diet contained *eugenia caryophllate* 0.5 %	1.29	2.31
Diet contained *eugenia caryophllate* 1%	1.43	2.34
Diet contained *eugenia caryophllate* 1.5 %	1.47	2.4



(**Fig. (1):** The effect of *eugenia caryophllate* extract (0.5 , 1, 1.5 %) on serum protein (g / dl), S. albumin (g / dl) in comparing with control of *O. niloticus* in aquarium during the Laboratory experiment

Table (4) *Effect of eugenia caryophllate diet on blood parmeter of O. niloticus*

Treatment	Hb%
C	8.2
Diet contained *eugenia caryophllate* 0.5 %	9
Diet contained *eugenia caryophllate* 1%	9.6
Diet contained *eugenia caryophllate* 1.5 %	10.9

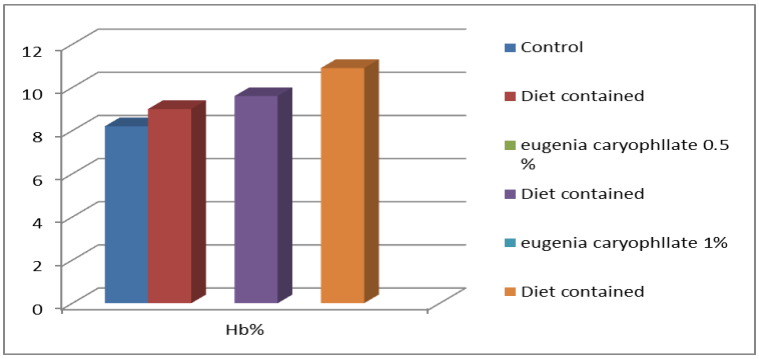


Fig (2)

Table (5): *Effect of eugenia caryophllate diet on Leucocytic and differential leucocytic of O. niloticus*

Treatment	Monocyte	Granulocyte	Lymphocyte	WBCs
Control	9.3	8.7	9.3	53.9
Diet contained *eugenia caryophllate* 0.5 %	11.5	9.4	11.5	61.1
Diet contained eu*genia caryophllate* 1%	17.5	16.8	17.5	87.3
Diet contained *eugenia caryophllate* 1.5 %	35	34.1	35	90.7

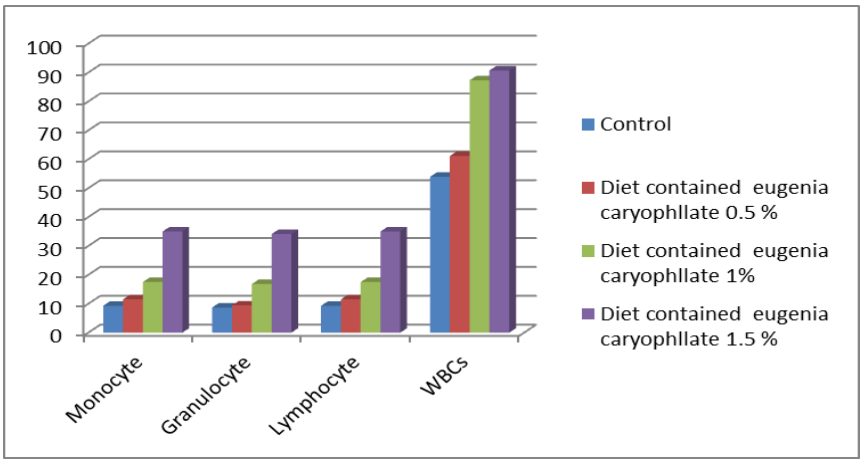


Fig (3)

Conclusion:

It could be concluded that *Eugenia caryophllate* extract improved growth performance and feed efficiency of Nile tilapia and strengthen its resistance to *Aeromonas Hydrophila* infection. Thus, the use of *Eugenia caryophllate* extract at level of 0.5 % in Nile tilapia diets is recommended as an immunostimulant.

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الملخص العربى

استخدام مستخلص نبات الفرنقل فى العلائق كمحفز لمناعة أصبعيات البلطى النيلى ومميت للبكتريا الممرضة (ايروموناس هيدروفيلا)

دعاء احمد العربي محمد زيد

قسم صحة الأسماك ورعايتها - المعمل المركزى لبحوث الثروة السمكية – العباسة أبو حماد – شرقية

تمت لتقييم استخدام نبات القرنفل المستخلص بواسطة الكحول الايثيلى كمحفز لمناعة لاصبعيات البلطى النيلى، بمتوسط وزن (12027 جرام) والتى تم توزيعها بعشوائية إلى أربع معاملات، منها ثلاث معاملات كل واحدة تضم 20 سمكة لكل 100 لتر ماء فى الحوض الزجاجى ، السمك تم تغذيته بواسطة العلف المختبر الذى يحتوى على 30% بروتين خام وطاقت النمو 4.40 كيلو كالورى على الجرام، كما تحتوى على صفر، 0.5، 1، 1.5% من مستخلص نبات القرنفل. العلف تم تغذيته مرتين يومياً بمعدل 3% من وزن الجسم فى اليوم ، لمدة 6 أيام فى الأسبوع على مدى 10 أسابيع. بعد تغذية السمك بالمعاملات المختلفة يتم حقنه ببكتريا الممرضة من نوع ايروموناس هيدروفيلا ، وذلك داخل الغشاء البروتينى. السمك حيث يتم مراقبته لمدة عشرة أيام لتتبع أى أعراض غير طبيعية ومعدل الوفيات يومياً. واستخدام عليقة 0.5 و 1 و 1.5 % من مستخلص نبات القرنفل يزيد من مناعة السمكة. فى هذه الدراسة نشير إلى أن العليقة المحتوية على 0.5 و 1 و 1.5 % من مستخلص القرنفل يزيد من مقاومة الأسماك للبكتريا الممرضة ايروموناس هيدروفيلا . ولذلك يوصى باستخدام العليقة المحتوية على مستخلص القرنفل فى مقاومة الأمراض فى مزارع البلطى النيلى.