

New Approach to use *Origanium Vulgare* Extract as Immunostimulant to Increase Resistance to *Pseudomonas aeruginosa* and *Pseudomonas flourescence*

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Abstract

The aim of the present study to assess the use of ethanol extracts of *Origanium vulgare* as a growth and 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, 0.0, 0.5%, 1.0%, or 1.5% *Origanium vulgare* extract. Diets were given twice daily at a rate of 3% of live body weight, for six days a week during 10 weeks. After the feeding trial, fish of each treatment were challenged by pathogenic *Pseudomonas aeruginosa* and *Pseudomonas flourescence*, 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. The growth promoting influence of *Origanium vulgare* extract was observed on fish. The maximum growth was observed at 0.5 % *Origanium vulgare* extract as compared to the control. No significant differences in fish survival were reported among the experimental treatments, falling within the range of 93.3-100%. The control fish consumed less diet and gave a higher Feed conversion ratio (FCR), while fish fed diet supplemented with 0.5% *Origanium vulgare* extract demonstrated the highest protein efficiency ratio (PER), apparent protein utilization (APU), and energy utilization (EU). The supplementation of *Origanium vulgare* extract had no significant effect on the fish body composition (dry matter, crude protein, fat, and ash), mean which total protein, albumin, and globulin increased significantly to the highest values at 0.5% *Origanium vulgare* extract, as compared to the control. However, supplementation of *Origanium vulgare* extract did not significantly affect the albumin /globulin ratio (A/G). In conclusion, 0.5% *Origanium vulgare* extract in Nile tilapia diets increased the fish resistance to *Pseudomonas aeruginosa* and *Pseudomonas flourescence*, indicating the effective role of *Origanium vulgare* extract in disease prevention in tilapia culture. Moreover, the reduction in feed cost compared with the control diet showed 12.52% to produce one kg fish gain of treatment containing 0.5% extracted *Origanium vulgare* levels.

Keywords: *Origanium vulgare*; Nile tilapia; Growth performance; Feed utilization; *Pseudomonas aeruginosa*; *Pseudomonas flourescence*

Introduction

Tilapia species are widely distributed in many countries of the world. Their farming has grown extremely fast in the last decade, where they are cultured worldwide with annual growth rate of about 12.2% [1]. In Egypt, Nile tilapia (*Oreochromis niloticus* L) is a major species in aquaculture system and much appreciated by consumers. However, the success of intensive tilapia culture depends to large extent on supplemental feeding.

Medicinal plants as natural growth promoters have significant improvements of body weight, weight gain, and survival rate and feed conversion rate in broilers [2]. Particularly, aromatic plants are considered of massive interest for their flavors and for their medicinal properties, along with human consumption, animal foodstuff and ornamental uses; thus, they are especially appropriate for multifunctional sustainable crop models [3,4]. Several medicinal herbs are used in the medication of various diseases; like reducing high blood cholesterol, protection from cancer, chronic diseases, stimulating the immune system. Furthermore, these herbs contain also aromatic substances and essential oils used in food industries [5]. Antimicrobial substances are now widely used for the treatment of bacterial diseases for fish [6].

The genus *origanum* Bth. of the *Labiatae* occur in the Egyptian as fruticose shrubs or perennial herbs with ovate, entire leaves, and

corymbose or paniculated inflorescences. This genus can be represented by two species, *O. vulgare* Linn and *O. Maru* Linn V. sinaicum Boiss. *Origanum vulgare* is a native to the Mediterranean, Euro-Siberian and Irano-Siberian regions [7]. Due to the variability in chemical and aromatic characteristics, *Origanum* 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 essential oil of *Origanum vulgare* involves more than 20 ingredients, most of which are phenolic antioxidants. Major components are thymol and carvacrol that constitute about 78 to 82% of the total oil. It has been demonstrated that the essential oil derived from oregano possess *in vitro* antimicrobial, antifungal, insecticidal and antioxidant properties. These properties are mainly attributed to thymol and carvacrol. Due

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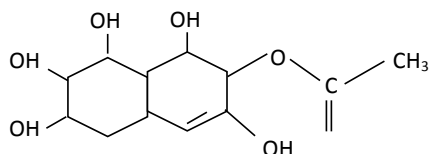
especially to the *in vitro* antimicrobial property, the application of oregano essential oil (OEO) in poultry production would be expected to have both prophylactic and therapeutic results [7,8].

This study aimed to evaluate the effects of *Origanum vulgare* extract on the growth performance, feed utilization, whole body composition, and resistance of Nile tilapia to *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* infection.

Materials and Methods

Extraction of *origanum vulgare*

A known weight of air dried material of *Origanum vulgare* 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 Di-Methyl Sulf-Oxide (DMSO), the solution was mixed with petroleum ether b.P (40-60°C) and centrifuged at 5000rpm, a deep green powder was obtained, containing Turbinones (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 *Origanum vulgare* extract (Table 1), *Origanum vulgare* 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°C, stored in plastic bags and kept in a refrigerator at -2°C during the experimental period to avoid rancidity [9].

Fish culture and feeding regime

Fingerlings of Nile tilapia, *O. niloticus* L. were obtained from fish hatchery, at the Central Laboratory for Aquaculture Research, Abbassa, Abo-Hammad, Sharkia. Fish were kept in indoor fiberglass tanks for 2 weeks for acclimation to the laboratory condition and they were fed a commercial diet, containing 30% crude protein. Fifteen fish were frozen at -20°C for proximate analysis at initial. Four groups of Nile tilapia ranged from 11 to 13 g were randomly distributed into four groups, each contains 15 fish per 100-L aquarium (triplicate each treatment). Each aquarium was supplied with compressed air via air-stones using aquarium air pumps. Settled fish wastes were cleaned daily by siphonation with a three quarets of aquarium's water, which was replaced by aerated water from the storage tank. Water temperature ranged between 27°C -29°C. The feeding rate was 3% of live body weight, fish fed twice daily at 9.00 and 13.00 hours; six days a week for a period of 10 weeks. Fish in each aquarium were group-weighted every 2 weeks and the amounts of feed given were readjusted accordingly. Dead fish was daily recorded and removed.

Chemical analysis of diets and fish

The tested diets and whole-fish body from each treatment at the

beginning and at the end of experiment were analyzed according to the standard methods for moisture [10], protein, fat and ash. Moisture content was estimated by drying the samples at 85°C in an oven (GCA, model 18 EM, precision scientific group, Chicago, Illinois, USA) to constant weight and calculating weight loss. Nitrogen content was measured using a microkjeldahl apparatus Labconco (Labconco Corporation, Kansas, Missouri, and USA) and crude protein was estimated by multiplying nitrogen content by 6.25. Total lipids content was determined by ether extraction in the multi-unit extraction Soxhlet apparatus (Lab-Line Instruments, Inc., Melrose Park, Illinois, USA) for 16 hours and ash was determined by combusting samples at 550C for 6 hr in a muffle furnace (Thermolyne Corporation, Dubuque, Iowa, USA). Crude fiber was estimated according to method of Goering and Van Soest [11]. Gross energy was calculated according to NRC [9].

Growth parameters

Growth performance was determined and feed utilization was calculated as follows:

$$\text{Weight gain} = W_2 - W_1;$$

Specific growth rate (SGR) = $100 (\ln W_2 - \ln W_1) / T$; where W_1 and W_2 are the initial and final weights, respectively, and T is the number of days of the feeding period;

Ingredients	0.0% Control	At <i>Origanum vulgare</i> extract % in the diets		
		0.50%	1%	1.50%
Fish meal	9.1	9.1	9.1	9.1
Soybean meal	45.5	45.5	45.5	45.5
Ground corn	15.31	15.31	15.31	15.31
Wheat bran	19.21	19.21	19.21	19.21
Starch	4	3.5	3	2.5
<i>Origanum vulgare</i>	0	0.5	1	1.5
Cod fish oil	2.23	2.23	2.23	2.23
Corn oil	1.65	1.65	1.65	1.65
Vitamins premix	1	1	1	1
Minerals Premix	2	2	2	2
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	49.2	48.87	48.51	48.73
GE(Kcal/100 g)	4390.3	4395.7	4403.8	4423
P/E ratio	68.58	68.84	68.99	68.75

1-Vitamin premix (per kg of premix): thiamine, 2.5 g; riboflavin, 2.5 g; pyridoxine, 2.0 g; inositol, 100.0 g; biotin, 0.3 g; pantothenic acid, 100.0 g; folic acid, 0.75 g; para-aminobenzoic acid, 2.5 g; choline, 200.0 g; nicotinic acid, 10.0 g; cyanocobalamin, 0.005 g; a-tocopherol acetate, 20.1 g; menadione, 2.0 g; retinol palmitate, 100,000 IU; cholecalciferol, 500,000 IU. 2-Mineral premix (g/kg of premix): CaHPO₄ · 2H₂O, 727.2; MgCO₃ · 7H₂O, 127.5; KCl 50.0; NaCl, 60.0; FeC₂H₆O₇ · 3H₂O, 25.0; ZnCO₃, 5.5; MnCl₂ · 4H₂O, 2.5; Cu(OAc)₂ · H₂O, 0.785; CoCl₂ · 6H₂O, 0.477; CaIO₃ · 6H₂O, 0.295; CrCl₃ · 6H₂O, 0.128; AlCl₃ · 6H₂O, 0.54; Na₂SeO₃, 0.03. 3-Nitrogen-Free Extract (calculated by difference) = 100 - (protein + lipid + ash + fiber). 4-Gross energy (GE) was calculated from (NRC, 1993) as 5.65, 9.45, and 4.1 kcal/g for protein, lipid, and carbohydrates, respectively.

Table 1: Ingredients and chemical analysis of the experimental diets (on dry matter basis) containing different levels of extracted *Origanum vulgare*.

Feed conversion ratio (FCR) = feed intake/weight gain;

Feed efficiency ratio (FER) = weight gain/feed intake;

Protein efficiency ratio (PER) = weight gain/protein intake;

Apparent protein utilization (APU; %) = 100 [protein gain in fish (g) / protein intake in diet (g)].

Energy utilization (EU; %) = 100 [Energy gain in fish (g) / energy intake in diet (g)].

Challenge test

The experimental fish were divided into two groups. The first group comprised fish fed control diet, and challenged I/P with pathogenic *Ps. aeruginosa* and *Ps. fluorescens* (0.2 ml of 4×10^6 CFU). The second group, comprised fish fed diets containing different levels of *Origanum vulgare* extract (0.5, 1.0 and 1.5) and challenged I/P with the same pathogen (0.2 ml of 4×10^6 CFU). Both groups kept under observation for 10 days to record the daily mortality rate. Isolation and identification of pathogens were determined according to method of Schäperclaus et al. [12].

Physiological measurement

At the end of the 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°C and centrifuged at 5000 rpm for 5 minutes at room temperature. The collected serum was stored at -20°C for further assays. Total protein content in plasma was determined colorimetrically according to Henry method [13], Albumin and globulin in plasma according to method of Wotton and Freeman [14].

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 *Origanum* 30.

Statistical analysis

The obtained data were analyzed by one-way ANOVA to evaluate the effect of *Origanum vulgare* extract. Differences between means were tested at the 5% probability level using Duncan Multiple Range test. All the statistical analyses were done using SPSS program version 10 (SPSS, Richmond, USA) as described by Dytham [15].

Results and Discussion

In the present study, the experimental diets contained 30% crude protein and 4.4 kcal/g diet (Table 1) are similar to that used by [16]. Changes in body weight (g) of Nile tilapia fed different levels of *Origanum vulgare* extract during experiment are illustrated in Figure 1. Initial body weight at all experimental groups did not differ significantly (Table 2). The fish actively and efficiently grew without any external signs of nutritional deficiency. Growth performance (final weight, weight gain, weight gain % and specific growth rate) increased significantly ($P < 0.05$) with the experienced diets (Table 3). The highest growth was obtained with diet of 0.5% *Origanum vulgare* extract, as compared to the control diet. No significant differences were reported in fish survival rate among different treatments ($P > 0.05$), since it ranged from 93.3 to 100%. This may indicate that *Origanum vulgare* extract enhance fish growth, feed utilization and immunity. The improvement of live body weight, body weight gain, weight gain %, SGR and survival rate may be due to the content of essential oil and extracts of *Origanum species* containing (turbin) antimicrobial, antioxidant and other biological activities [7,8]. Furthermore a better growth performance was observed in Nile tilapia fed on diet contained 0.5% cinnamon and 1% marjoram as a feed additive to tilapia feeds were recommended by Abd El-Maksoud et al., [17].

As compared to control fish fed diet supplemented with 0.5% *Origanum vulgare* extract produced the highest protein efficiency ratio (PER), apparent protein utilization (APU) and energy utilization (EU), (Table 4). Moreover, fish fed diet supplemented with 0.5% *Origanum vulgare* extract improves fish growth and feeding efficiency; this

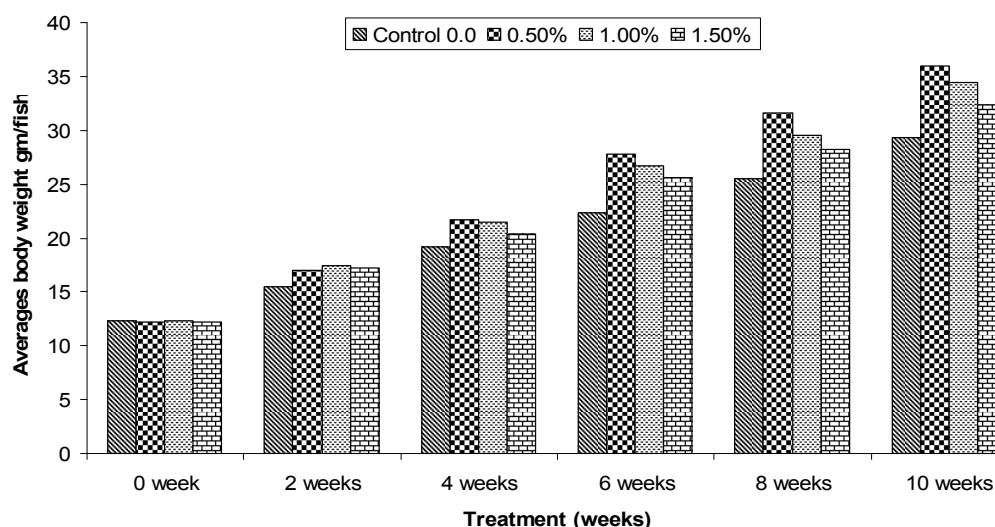


Figure 1: Average of live body weight of Nile tilapia (*O. niloticus*) as affected by diets containing different levels of *Origanum vulgare* extracts.

improvement may be contributed to improve feed intake and nutrient digestibility. In addition, *Origanum vulgare* extract contains several nutrients, especially essential oils, vitamins and minerals that may help in fish growth promotion. These results are in agreement with those of Abdel-Maksoud et al., [17] and Shalaby et al., [18].

The extract of *Origanum vulgare* had no significant ($P>0.05$) effect on the whole body composition (dry matter, crude protein, fat and ash) (Table 2). Similar finding were given by Abdel-Maksoud et al. [17] and Shalaby et al. [18].

Results of fish challenging against of *Ps. aeruginosa* and *Ps. fluorescens* for ten days were shown no mortalities in all diets containing different levels of *Origanum vulgare* extract. The highest overall fish mortality rates were observed in the control group (90% and 100%, respectively). This enhanced immune response may be induced by the essential oils content and extracts of *Origanum* species which contains (turbines) antimicrobial, antioxidant and other biological activities [7,8]. Furthermore, study by Sahin et al. [19] prove the antibacterial

Items	0.00%	At <i>Origanum vulgare</i> extract % of the diets		
	(control)	1.50%	1%	0.50%
Moisture	74.64 ± 0.68 ^a	73.77 ± 0.14 ^a	73.75 ± 0.27 ^a	73.84 ± 0.46 ^a
Crude protein	60.00 ± 0.06 ^a	60.79 ± 0.32 ^a	60.59 ± 0.37 ^a	60.68 ± 0.39 ^a
Total lipids	19.72 ± 0.22 ^a	19.79 ± 0.36 ^a	19.67 ± 0.18 ^a	19.97 ± 0.49 ^a
Ash	19.81 ± 0.31 ^a	19.24 ± 0.55 ^a	19.11 ± 0.61 ^a	1.31 ± 0.60 ^a

a-no significant different in the same row from the table at $P<0.05$. Proximate chemical composition of fish at start of this study was 75.22 ± 0.30% moisture; 59.04 ± 0.82% protein; 18.11 ± 0.76% lipid and 21.42 ± 0.57% ash.

Table 2: Proximate chemical analysis on dry matter basis (mean ± SE) of Nile tilapia fed diets containing different levels of *Origanum vulgare* extract.

Items	0.0 %	At <i>Origanum vulgare</i> extract % in the diets		
	(control)	0.50%	1.00%	1.50%
Initial weight (g)	12.27 ± 0.46 ^a	12.17 ± 0.15 ^a	12.37 ± 0.50 ^a	12.23 ± 0.36 ^a
Final weight (g)	29.30 ± 0.67 ^c	36.00 ± 0.85 ^a	34.40 ± 0.55 ^{ab}	32.37 ± 0.74 ^b
Weight Gain (g)	17.03 ± 0.52 ^c	23.83 ± 0.97 ^a	22.03 ± 0.33 ^{ab}	20.14 ± 0.87 ^{ab}
Weight Gain %	138.79 ± 7.03 ^c	195.81 ± 9.99 ^a	178.09 ± 9.13 ^{ab}	164.68 ± 6.02 ^{bc}
SGR (% day)	1.24 ± 0.03 ^c	1.55 ± 0.05 ^a	1.46 ± 0.05 ^{ab}	1.39 ± 0.03 ^b
Survival rate (%)	93.30 ± 3.85 ^a	100 ± 0.0 ^a	100 ± 0.0 ^a	96.03 ± 0.33 ^{ab}

Means the same letter (a/b/c) in the same row is not significantly different at $P<0.05$.

Table 3: Growth performance (means ± SE) of Nile tilapia fed diets containing different levels of *Origanum vulgare* extract.

Items	0.0 % (control)	At <i>Origanum vulgare</i> extract % in the diet		
		0.50%	1.00%	1.50%
Feed intake (g feed/fish)	34.10 ± 0.34 ^c	39.74 ± 0.22 ^a	38.75 ± 0.36 ^a	37.29 ± 0.52 ^b
FCR	2.01 ± 0.05 ^a	1.67 ± 0.07 ^b	1.72 ± 0.06 ^b	1.86 ± 0.04 ^{ab}
PER	1.81 ± 0.05 ^c	2.17 ± 0.08 ^a	2.05 ± 0.05 ^{ab}	1.94 ± 0.04 ^{bc}
APU %	28.37 ± 0.28 ^d	35.73 ± 0.20 ^a	34.08 ± 0.32 ^b	32.40 ± 0.45 ^c
AFU %	41.36 ± 0.40 ^d	50.76 ± 0.28 ^a	46.56 ± 0.42 ^b	44.14 ± 0.60 ^c
EU %	17.32 ± 0.17 ^c	21.84 ± 0.11 ^a	20.63 ± 0.22 ^b	20.40 ± 0.21 ^b

Feed intake, feed conversion ratio (FCR), protein efficiency ratio (PER), apparent protein utilization (APU), apparent fat utilization (AFU) and energy utilization (EU) of Nile tilapia fed diets containing different levels of *Origanum vulgare* extract. Means the same letter (a/b/c) in the same row is not significantly different at $P<0.05$.

Table 4: Feed intake, feed conversion ratio (FCR), protein efficiency ratio (PER), apparent protein utilization (APU), apparent fat utilization (AFU) and energy utilization (EU) of Nile tilapia fed diets containing different levels of *Origanum vulgare* extract.

Treat.	T. protein	S. albumin g/dl	Globulin g/dl	A/G ratio %	GOT U / l	GPT U / l
Control	4.32 ± 0.17 ^c	1.33 ± 0.06 ^c	2.99 ± 0.17 ^c	0.44 ± 0.02 ^a	89 ± 3.78 ^a	95 ± 3.58 ^a
Diet contained <i>origanum vulgare</i> 0.5%	5.64 ± 0.22 ^a	1.74 ± 0.05 ^a	3.90 ± 0.20 ^a	0.45 ± 0.02 ^a	70 ± 7.52 ^b	82 ± 3.56 ^b

Means the same letter (a/b/c) in the same row is not significantly different at $P<0.05$.

Table 5: The effect of *Origanum vulgare* extract (0.5%) on serum protein (g/dl), S. albumin (g/dl), A/G ratio (%) and transaminases enzymes in comparing with the control of *O. niloticus* in aquarium during the Laboratory experiment.

Items	<i>Origanum vulgare</i> Levels in experimental rations			
	Control-0.0	0.50%	1%	1.50%
Price/kg feed P.T	2.78	2.93	3.08	3.23
FCR (kg feed/kg gain)	2.01	1.67	1.72	1.86
Feed cost/kg gain P.T	5.59	4.89	5.3	6.01
Reduction cost in kg gain	100	12.52	5.19	-7.51

Table 6: Economic efficiency for production of one Kg gain of fingerlings Nile tilapia *O. niloticus* fed diets containing different levels of extracted *Origanum vulgare*.

effect of oils and extracts of *Origanum* species on many bacterial species like *Escherichia coli*, *Enterobacter sp.*, *Bacillus sp.*, *Salmonella sp.*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Listeria monocytogenes* and *Campylobacter jejuni*. Total protein, albumin, and globulin values increased significantly ($P \leq 0.5$) with *Origanum vulgare* extract, reaching the highest value at 0.5% of the diet, as compared to the control (Table 5). On the other hand, *Origanum vulgare* Extract showed negligible insignificantly effect on the albumin/globulin (A/G) ratio (Table 5). Glutamic Oxaloacetic Transaminase (GOT) and Glutamic Pyruvate Transaminase (GPT) values decreased with increasing *origanum vulgare* extract and the highest values was obtained at the control diet (Table 5). The results indicate that the improvement of fish health was took place when fed diet with *Origanum vulgare* extract. Moreover, the measurement of total protein, albumin and globulin 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 [12].

The economical evaluation of the experimental diets contained different extracted *Origanum vulgare* levels 0.0, 0.5%, 1% and 1.5% are shown in (Table 6). The highest reduction in feed cost compared with the control diet showed to produce one kg fish gain of treatment supplemented with 0.5% extracted *Origanum vulgare*. The reduction in feed cost compared with the control diet showed 12.52% to produce one kg fish gain of treatment supplemented with 0.5% extracted *Origanum vulgare* levels. Previous studies showed that the use of spices in small amounts gave lower incidence cost and higher profit index of fish species [18].

Conclusion

It could be concluded that *Origanum vulgare* extract improved growth performance and feed efficiency of Nile tilapia and strengthen its resistance to *Ps. auroginsia* and *Ps. fluorescens* infection. Thus, the feeding of *Origanum vulgare* extract at level of 0.5% in diets for Nile tilapia is recommended as an immunostimulant.

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