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Effect of *Rosemarinus officinalis* Leaves Extract on Growth Performance, Innate Immune Response, Antioxidant ctivity, and Disease Resistance of the Nile Tilapia

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ABSTRACT

The current study was carried out to assess the effect of the *Rosemarinus officinalis* extract (RE) as a feed additive on growth performance, immune response, antioxidant status, and disease resistance of the Nile tilapia (*Oreochromis niloticus*). Fish were randomly distributed into four groups. G_1 served as a control, while G_2, G_3, and G_4 were fed the basal diet supplemented with 0.5%, 1%, and 1.5% of RE, respectively. After the experimental period (8 weeks), fish were experimentally infected with *Staphylococcus aureus*. The results displayed enhanced growth performance [final body weight (FBW), weight gain (WG), weight gain% (WG%), and specific growth rate (SGR)] with a significant reduction in feed conversion rate (FCR) in G_3 and G_4. A significant increase of the biometric indices [hebatosomatic index (HIS), splenosomatic index (SSI), and condition factor (K)] was recorded in G_3, followed by G_4 and G_2 compared to G_1. Serum levels of glucose, triglyceride, cholesterol, creatinine and nitric oxide (NO) and activities of alkaline phosphatase (ALP) and alanine aminotransferase (ALT) reduced significantly with increasing the concentration of RE fed. While levels of total protein, albumin, total globulin, γ globulin, superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and resistance against *S.Aureus* were significantly increased in a dose-dependent manner of RE fed. Additionally, the serum activity of lysozyme and level of myeloperoxidase (MPO) increased significantly in treated groups in a descending manner with increasing the concentration of RE in the feed. Thus, in the present work, the addition of ethanolic RE to the Nile tilapia diets proved to enhance growth performance, innate immunity, and physiological status of fish hence, presenting a promising feed additive in aquaculture.

INTRODUCTION

Recommendations have been considered to minimize or replace using the chemical stimulants such as, hormones and antibiotics as feed additives in fish diets with herbs and herbal supplements which are reasonably economical and eco-friendly with fewer side effects **(Toutou *et al.*, 2019)**. This substitution is preferable to avoid the development of microbial resistance to those products and prevent the associated risks to human health

(Baruah *et al.*, 2008). The potential of herbal products is astounding as they can be used to augment growth, minimize stress, improve immunity, and prevent various microbial infections in aquatic animals improving their health for the consumption of the end users **(Shakya, 2017)**. Consequently, aromatic herbs and medicinal plants, along with their extracts, have already been utilized as safe alternative feed supplements to enhance growth performance and economic value **(EL–Dakar *et al.*, 2016)** together with their antimicrobial effects **(Pai & Platt, 1995)**.

The most widely used medicinal herbs worldwide is Rosemary (*R.officinalis*), **(Caillet *et al.*, 2007)**. It is a member of the Family Lamiaceae, known as rosemary or "dew of the sea", and is commonly used for culinary and medicinal purposes, mainly due to its aromatic properties and health benefits **(Holmes, 1999)**. Remarkably, it acts as an antibacterial, antifungal and anti-inflammatory substance **(Ribeiro-Santos *et al.*, 2015)**. Moreover, it works as an antioxidant **(Caillet *et al.*, 2007)** and as an anti-carcinogenic as well **(El-Barbary & Mehrim, 2009)**.

Its active extracts are mainly composed of 1,8-cineole, carnosol, carnosic acid, rosmarinic acid, β- and α-pinene, camphor, and camphene **(Taheri Mirghaed *et al.*, 2018)**. Extracts of rosemary are rich in antioxidants **(Zoral *et al.*, 2017).** It has been determined that rosemary extracts or its active ingredients have anti-inflammatory, hepatoprotective, antithrombotic, diuretic, anti-diabetic, anti-nociceptive, anticancer, and antioxidant activity in humans and experimental animals **(Takayama *et al.*, 2016).** It has also been reported to possess a strong antibacterial activity against gram-positive and gram-negative bacteria **(Jiang *et al.*, 2011).**

Consequently, the interest in the use of rosemary has increased because of its effects to control diseases in human and aquatic animals **(Zoral *et al.*, 2017).** Evidences of rosemary application in fish culture are still scarce, especially with respect to its effects on fish immunity, antioxidant activity and stress responses **(Yousefi *et al.*, 2019)**. Hece, this study was conducted to evaluate the effect of RE as a feed additive on growth performance, innate immunity, antioxidant activity, and tissue architecture of the Nile tilapia.

MATERIALS AND METHODS

Plant Extraction

The dry leaves of *R.officinalis* were purchased from a local herbal market in Zagazig. Leaves were grounded to a fine powder then subjected to exhaustive extraction with ethyl alcohol (95%) using Soxhlet apparatus **(**VELP SCIENTIFICA) till complete extraction. Afterwards, the outcome was evaporated to a thick syrup of dark green color, this thick syrup underwent spectroscopic analysis (The GC- mass techniques) which was determined at the regional center for Mycology and Biotechnology, Al-Azhar University, Egypt. Analysis revealed the richness of RE with monoterpenes [as 1,8-cineol (eucalyptol) and camphor]. Moreover, the elemental analysis indicated the chemical formula to be as follows: $C_{10}H_{18}O$ and $C_{10}H_{16}O$ respectively. While, sesquiterpenes as caryophyllene with the elemental analysis indicated the chemical formula of $C_{15}H_{24}$, and

triterpenes (as betulin and carnosol) with the elemental analysis indicated the chemical formula as $C_{30}H_{50}O_2$ and $C_{20}H_{26}O_4$ respectively (Fig. 1).

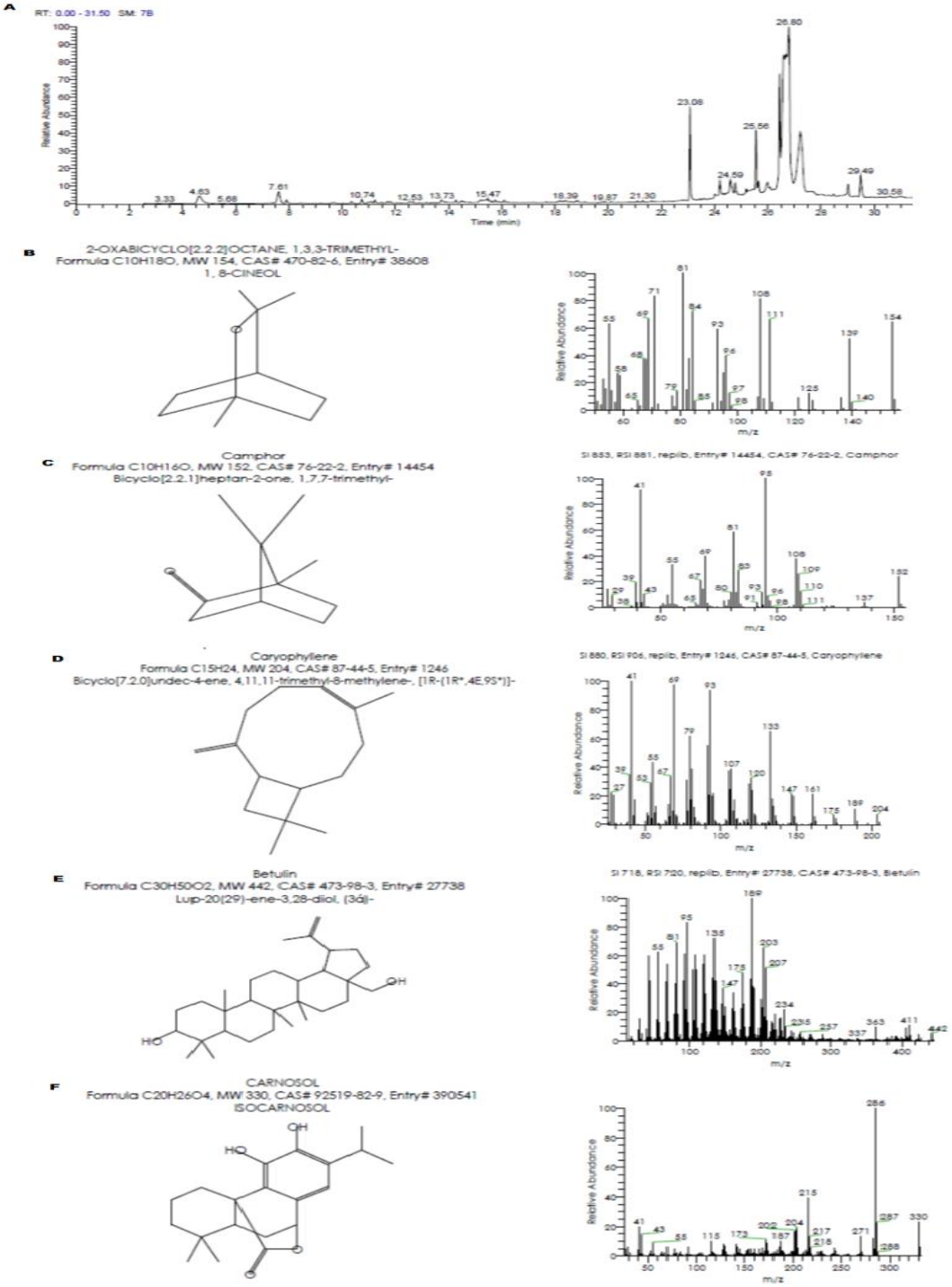

Fig. 1: GC- mass techniques revealing the powerful compounds illustrated .

Fish and cultural conditions

This study was done in the central laboratory for aquaculture research (CLAR), Abbassa, Egypt. The Nile tilapia (N = 144, average body weight 21.38±0.45 g) were checked to be healthy following the guidelines of the Canadian Council for Animal Care **(CCAC, 2005)**. Then, fish specimens were randomly distributed in 100-L glass aquaria supplied with dechlorinated tap water. A continuous aeration was safeguarded using electric air pumping compressors with air stones for 2 weeks, and fish were fed on a basal diet before the onset of the experiment. The aquaria were completely water changed and cleaned day after day. The water quality parameters were kept under the same conditions, including temperature (28.3 ± 1.1 °C), dissolved oxygen (6.18 ± 0.4 mg/L), pH (6.9 ± 0.1), and total ammonia (0.035 ± 0.01 mg/L) with a controlled photoperiod (12 h light: 12 h dark) in the laboratory according to **APHA (1998)**. The study protocol was approved by the Ethics of Animal Use in Research Committee of Zagazig University. Furthermore, experimental procedures were performed in line with the National Institute of Health general guidelines for the Care and Use of Laboratory Animals in scientific investigations.

The experimental design and diets preparation

Fish were equally distributed into four groups with three replicates for each group (12 fish / replicate). The 1st group (G_1) acted as a control group and fed only the basal diet, the 2nd, 3rd, and 4th groups fed the basal diet supplemented with 0.5%, 1%, and 1.5% RE, respectively. The basal and experimental diets were prepared and adjusted to fulfill the recommended nutritional needs of the Nile tilapia according to the National Research Council **(NRC, 2011)**, manufactured in the feed processing laboratory, Nutrition Department, central laboratory for aquaculture research, Abbassa, Egypt (Table 1). The ingredients were mechanically mixed and pelletized using a pellet machine, ultimately producing pellets of 1.5 mm diameter. The prepared diets were air-dried at room temperature for 24 h and stored in a refrigerator at 4°C until further use. The fish were fed by hand till satiety twice daily at 8.00 am and 2.00 pm.

Growth performance

At the start of the experiment, the fish of every group were weighed to attain initial body weight (IBW). At the end of the study, they were weighed to obtain the final body weight (FBW). Fish weight gain (WG) was calculated using the following equation: WG=FBW−IBW (the difference between FBW and IBW). The weight gain% (WG %) was calculated using the following equation: WG % = [(WG / IBW) ×100]. The specific growth rate (SGR) = [(ln final body weight−ln initial body weight) time intervals (days) ×100]. FCR= total dry weight of the diet fed (g) / wet weight gain (g).

Condition factor (K) was calculated using the formula of **Froese (2006):** K =W/ L3 × 100, where W is wet weight (g) and L is length (cm) while Hepato-somatic (HSI). Spleen-somatic (SSI) indexes were determined according to **Schreck and Moyle (1990)** as follows: HIS = 100 × liver weight [g] / total body weight [g], SSI = 100 × spleen weight [g] / total body weight [g].

Sampling

By the end of the feeding trial (8 weeks), three fish/aquarium were randomly selected and anesthetized by 95 mg L-1 clove oil (Oleum, Cairo, Egypt) within 3min. **(Adeshina *et al.*, 2016)**, then blood samples were collected from the caudal blood vessels of fish by clean and sterile syringes without anticoagulant, and serum was separated by centrifuging at 3000 RPM for 10 min. using ordinary centrifuge **(HERMLE, Z 230 A)** according to **Zhu *et al.* (2018)**. The serum obtained was used for the evaluation of some biochemical indicators. Moreover, gills, kidneys, spleen, and posterior intestinal tissues were sampled and fixed at 10% neutral buffer for further histological examination **(Suvarna *et al.*, 2018)**. Additionally, intestinal morphometric analyses were proceeded according to **Yang *et al.* (2018)**.

Table 1: Ingredient of fish meal after adding *Rosmarinus officinalis* extract.

	Control diet	***Rosmarinus officinalis* extract containing diet**		
		0.5%	**1%**	**1.5%**
Ingredients (%)				
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
R. officinalis extract	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

1.Vitamin Premix (Per Kg of Premix); thiamine, 2.5 g; riboflavin, 2.5g; pyridoxine, 2.0 g; inositol, 100.0g; biotin, 0.3g; pantothenic acid, 100.0g; folic acid, 0.75g; para-aminobenzoic acid, 2.5g; choline, 200.0g; nicotinic acetate, 10.0g; menadione, 500.000 IU.

2.Mineral premix (g/Kg of premix) P: CaHPO4.2H2O, 727.2; MgCO4.7H2O, 127.5; KCl, 50.5; NaCl, 60.0; FeC6H5O7.3H2O, 25.0; ZnCO3, 5.5; MnCl2.4H2O, 2.5; CU(OAc)2.H2O, 0.785; CoCL3.6H2O, 0.128; AICI3.6H2O, 0.477; CaLO3.6H2O, 0.295; CrCI3.6H2O, 0.128; AICI3.6H2O, 0.57; 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.

Biochemical parameters measurement

Serum biochemical parameters as glucose, ALT, creatinine, cholesterol, and triglycerides were measured colorimetrically using Biomed, Egy-Chem, Egypt, Diamond Diagnostic Egypt, Spinreact kits (Esteve De Bas, (GI) Spain) according to **Trinder (1969)** and **Bergmeyer *et al.* (1977)** specifically to determine glucose and ALT, respectively. Moreover, the method of **Young (2001)** was used to specify creatinine, cholesterol, and triglycerides. Total protein, total globulin, and albumin were estimated according to **Gornal *et al.* (1949), Doumas and Biggs (1972)** and **Doumas *et al.* (1981)**, respectively.

Immunological assessment

Nitric oxide (NO) (CAT.NO. NO 25 33), differential globulins, and lysozyme were measured according to the method of **Montgomery and Dymock (1961)**, **Ornstein (1964)** and **Schaperclaus *et al.*, 1992**, respectively. To measure myeloperoxidase (MPO) serum levels, an enzyme-linked immunosorbent assay kit was used according to the manufacturer's instructions.

Antioxidant activity

Selective serum oxidative markers; glutathione (GSH) (CAT.NO. GR 25 11), superoxide dismutase (CAT.NO. 8D 25 21), and catalase (CAT.NO. CA 25 17) were estimated colorimetrically using commercial kits from Biodiagnostic, Egypt, according to **Beutler *et al.* (1963)**, **Nishikimi *et al.* (1972)** and **Aebi (1984),** respectively.

Whole-body composition and diet analysis

Samples from each experimental diet and fish group (3 samples / group) were analyzed for moisture by a drying oven (at 85ºC till constant weight), crude protein by macro-Kjeldahl, total lipids by the method of ether extraction, and the muffle furnace for total ash (550ºC / 6 hours) according to the standard methods of **AOAC (1990)**, and for crude fiber (for feed samples only).

Bacterial challenge

At the end of the experiment, 30 fish from each treatment were distributed into three aquaria at a rate of 10 fish / aquarium, with an extra triplicate aquaria group from control fish inoculated 0.2 ml saline solution IP (intraperitoneal) as a negative control group. Fish were challenged with pathogenic *Staphylococcus aureus*, which was previously isolated in the Department of Fish Disease and Management Faculty of Veterinary Medicine, Zagazig University, Egypt. The fish has inoculated IP with 0.2 ml cell suspension containing $4x10^6$cells/ml **(Gaafar *et al.*, 2015)** by using McFarland standard tubes as a dose of 24-h broth from the bacterial pathogen *S.aureus.* The dose was given by IP injection **(Schaperclaus *et al.*, 1992)**. All groups were kept under observation for 15 days to record any abnormal clinical signs and daily mortality. The mortalities were recorded for the calculation of relative percentage survival (RPS) according to **Amend (1981)** as follows:

RPS = [1 – (treatment mortality % ÷ control mortality %)] x 100.

Statistical analysis

Before statistical analyses, the normality of distribution and homogeneity of variances between different treatments were tested using the Kolmogorov–Smirnov test and Bartlett's test, respectively. Then data were analyzed using one-way ANOVA to test the

influence of different concentrations of ROE on the Nile tilapia. The *post-hoc* test was performed using Duncan's Multiple Ranges test to compare means at $P \leq 0.05$. All the statistical analyses were done using SPSS program version 20 (SPSS, Richmond, VA, USA) according to **Dytham (2011)**.

RESULTS

Growth performance and biometric indices

As shown in Table (2) FBW, WG, WG%, and SGR values were significantly augmented in G_3 and G_4 then G_2 compared to the control. Apparently, the FCR was significantly the lowest in G_3, G_4 followed by G_2 compared to the control.

In Table (3), the biometric indices (SSI and K) displayed the highest significant value in G_3 followed by G_2 and G_4 then the control. While, the HIS was the highest in G_3 followed by G_4 then G_2 compared to the control.

Table 2: Effect of *R.officinalis* extract addition to diet on the growth performance of the Nile tilapia fingerlings, (*Oreochromis niloticus*) for 8 weeks.

G	IBW/g	FBW/g	WG /g	WG %	SGR (%/day)	FCR
G1	20.45±0.017	42.03^{c} ±0.564	21.58^{c} ±0.554	105.50^{c}±1.309	1.46^{c} ±0.012	1.194^{a} ±0.013
G2	20.42±0.009	44.82^{b} ±0.528	24.40^{b} ±0.041	119.55^{b}±0.652	1.59^{b} ±0.009	0.986^{b} ±0.006
G3	20.39±0.024	49.09^{a} ±0.697	28.70^{a} ±1.000	140.72^{a}±0.919	1.79^{a} ±0.007	0.818^{c} ±0.039
G4	20.41±0.036	48.80^{a} ±1.179	28.39^{a} ±1.211	139.12^{a}±0.669	1.77^{a} ±0.007	0.878^{c} ±0.041

Means within the same column carrying different superscripts are significantly different at ($P< 0.05$).
G: Groups. IBW: Initial body weight. FBW: Final body weight. Wg: Weight gain. Wg%: Weight gain percent. SGR: Specific growth rate. FCR: Feed conversion ratio. G1:control group fish fed only basal diet. G2: fish fed 0.5% of *R.officinalis* extract added to basal diet. G3: fish fed 1% of *R.officinalis* extract added to basal diet. G4: fish fed 1.5% of *R.officinalis* extract added to basal diet.

Table 3: Effect of *R.officinalis* extract addition to diet on biometric indices of Nile tilapia fingerlings, (*Oreochromis niloticus*) for 8 weeks

Groups	HSI	SSI	K
G1	3.09^{d} ±0.071	0.148^{c} ±0.004	1.64^{c} ±0.033
G2	3.85^{c} ±0.139	0.262^{b} ±0.001	1.81^{b} ±0.050
G3	5.21^{a} ±0.153	0.294^{a} ±0.003	1.98^{a} ±0.024
G4	4.23^{b} ±0.075	0.259^{b} ±0.004	1.77^{b} ±0.015

Means within the same column carrying different superscripts are significantly different at (*P< 0.05*). HIS: Hepatosomatic index, SSI: spleenosomatic index, K: condition factor. G1:control group fish fed only basal diet. G2: fish fed 0.5% of *R.officinalis* extract added to basal diet. G3: fish fed 1% of *R.officinalis* extract added to basal diet. G4: fish fed 1.5% of *R.officinalis* extract added to basal diet.

Serum biochemical parameters of the Nile tilapia

Table (4**)** shows that serum glucose, cholesterol, and triglyceride levels were significantly decreased with increasing concentration of RE in the diet, while serum total protein and albumin increased with increasing concentration of RE in the diet. Total globulin increased significantly in G_4 and significantly decreased in G_2 compared to control and G_3.

Furthermore, ALT and ALP activities revealed a significant reduction with increasing concentration of RE fed, while the serum creatinine level was significantly the lowest in G_3 and G_4 compared to control and G_2(Table 3).

Immunological status of the Nile tilapia

Data presented in Table (5) reveal that, the serum lysozyme activity of *O.niloticus* fed RE increased in the treatment groups compared to the control as it was the highest in G_2 then G_3 and G_4, while the MPO was the highest level in G_2 then G_3 then G_4. Simultaneously, NO was significantly the lowest in G_4 then G_3 then G_2.

In Table (6), γ globulin level was significantly increased in G_4, followed by G_3 then G_2. α_1 globulin decreased significantly in treatment groups compared to control, while α_2 globulin and β globulin decreased significantly in G_2 followed by G_3 compared to G_4 and control.

Table 4: Effect of addition of *R.officinalis* extract to diet on the blood biochemistry of Nile tilapia fingerlings (*Oreochromis niloticus*) for 8 weeks.

G	Glucose (mg dL^{-1})	CH (mg dL^{-1})	TG (mg dL^{-1})	TP (g dL^{-1})	Albumin (g dL^{-1})	Globulin (g dL^{-1})	ALT (UL^{-1})	ALP (UL^{-1})	Creatinine (mg dL^{-1})
G1	68.30^{a} ±1.38	275.00^{a} ±2.89	259.50^{a} ±1.44	6.10^{d} ±0.058	2.00^{c} ±0.058	4.90^{b} ±0.058	27.40^{a} ±0.693	57.50 ^{a}±1.44	0.119^{a} ±0.001
G2	60.10^{b} ±1.15	266.00^{b} ±2.31	187.50^{b} ±0.866	6.65^{c} ±0.029	2.10^{bc} ±0.058	4.55^{c} ±0.029	24.80^{b} ±0.058	51.50 ±0.866	0.111^{a} ±0.005
G3	49.00^{c} ±0.635	168.50^{c} ±0.289	160.50^{c} ±2.60	7.20^{b} ±0.115	2.20^{ab} ±0.058	5.00^{b} ±0.058	23.00^{c} ±0.577	42.50^{c} ±0.28	0.071^{b} ±0.003
G4	36.45^{d} ±0.433	139.50^{d} ±1.44	131.50^{d} ±0.866	8.25^{a} ±0.144	2.35^{a} ±0.029	5.90^{a} ±0.058	21.05^{d} ±0.202	29.00^{d} ±1.155	0.079^{b} ±0.001

Means within the same column carrying different superscripts are significantly different at ($P< 0.05$). G: Groups. CH: cholesterol. TG: triglycerides. TP: Total protein. **ALT**: Alanine aminotransferase. ALP: Alkaline phosphatase. G1:control group fish fed only basal diet. G2: fish fed 0.5% of *R. officinalis* extract added to basal diet. G3: fish fed 1% of *R.officinalis* extract added to basal diet. G4: fish fed 1.5% of *R. officinalis* extract added to basal diet.

Table 5: Effect of *R.officinalis* extract's addition to diet on the Lysozyme, myeloperoxidase, and Nitric oxide of the Nile tilapia fingerlings (*Oreochromis niloticus*) for 8weeks.

Groups	Lysozyme (μg mL^{-1})	Myeloperoxidase (U L^{-1})	Nitric oxide (μmol L^{-1})
G1	4.12 ^{c} ±0.110	18.50 ^{d} ±0.520	79.50^{a} ±0.289
G2	6.05 ^{a} ±0.119	40.05 ^{a} ±0.664	71.33^{b} ±0.667
G3	4.86 ^{b} ±0.064	32.70 ^{b} ±0.462	22.00^{c} ±0.577
G4	4.61 ^{b} ±0.042	23.65 ^{c} ±0.606	12.00^{d} ±0.289

Means within the same column carrying different superscripts are significantly different at ($P< 0.05$). G1:control group fish fed only basal diet. G2: fish fed 0.5% of *R. officinalis* extract added to basal diet. G3: fish fed 1% of *R. officinalis* extract added to basal diet. G4: fish fed 1.5% of *R.officinalis* extract added to basal diet.

Table 6: Effect of *R. officinalis* extract's addition to diet on globulin differentiation of the Nile tilapia fingerlings (*Oreochromis niloticus*) for 8 weeks.

Groups	α1globulin (g dL^{-1})	α2globulin (g dL^{-1})	β globulin (g dL^{-1})	γ globulin (g dL^{-1})
G1	1.35 ^{a} ±0.015	1.30 ^{a} ±0.029	1.30 ^{a} ±0.058	0.950 ^{d} ±0.029
G2	1.13 ^{b} ±0.011	1.19 ^{b} ±0.003	0.925^{b} ±0.072	1.30 ^{c} ±0.058
G3	1.15 ^{b} ±0.029	1.07 ^{c} ±0.014	1.00 ^{b} ±0.058	1.77 ^{b} ±0.014
G4	1.055 ^{c} ±0.003	1.26 ^{a} ±0.020	1.09 ^{ab} ±0.087	2.50 ^{a} ±0.115

Means within the same column carrying different superscripts are significantly different at ($P< 0.05$). G1:control group fish fed only basal diet. G2: fish fed 0.5% of *R. officinalis* extract added to basal diet. G3: fish fed 1% of *R.officinalis* extract added to basal diet. G4: fish fed 1.5% of *R.officinalis* extract added to basal diet.

Antioxidant status of the Nile tilapia

Data in Table (7) show increased levels of SOD, CAT, and GSH in G_4, G_3 then G_2 compared to control (G_1).

Table 7: Effect of *R. officinalis* extract's addition to diet on antioxidant enzymes of the Nile tilapia fingerlings (*Oreochromis niloticus*) for 8weeks.

Groups	Superoxidedismutase (U L^{-1})	Catalase (U L^{-1})	Glutathione (mmol L^{-1})
G1	3.77 ^{d} ±0.014	124.00 ^{d} ±2.31	2.67 ^{d} ±0.017
G2	4.84 ^{c} ±0.035	138.00 ^{c} ±0.577	3.03 ^{c} ±0.020
G3	5.80 ^{b} ±0.231	147.33 ^{b} ±2.03	3.91 ^{b} ±0.026
G4	7.15 ^{a} ±0.087	235.50 ^{a} ±0.289	4.30 ^{a} ±0.130

Means within the same column carrying different superscripts are significantly different at ($P< 0.05$). G1:control group fish fed only basal diet. G2: fish fed 0.5% of *R. officinalis* extract added to basal diet. G3: fish fed 1% of *R. officinalis* extract added to basal diet. G4: fish fed 1.5% of *R. officinalis* extract added to basal diet.

Whole-body composition

Table (8) shows decreasing moisture and lipid content in G_4, G_3 then G_2, while protein and ash content was significantly increased in G_4, G_3 then G_2 compared to G_1 (control).

Table 8: Effect of *R.officinalis* extract's addition to diet on body chemical composition of the Nile tilapia fingerlings (*Oreochromis niloticus*) for 8 weeks.

Groups	Moisture %	Protein %	Lipid %	Ash %
G1	78.18 ^{a} ±0.691	45.58 ^{d} ±0.014	39.06 ^{a} ±0.364	15.35 ^{c} ±0.378
G2	75.16 ^{b} ±0.098	52.06 ^{c} ±0.596	21.35 ^{b} ±0.777	19.39 ^{b} ±0.261
G3	75.87 ^{b} ±0.398	61.52 ^{b} ±0.330	19.09 ^{c} ±0.523	18.84 ^{b} ±0.410
G4	72.68 ^{c} ±0.651	65.03 ^{a} ±1.04	16.12 ^{d} ±0.264	26.59 ^{a} ±0.564

Means within the same column carrying different superscripts are significantly different at ($P< 0.05$). G1:control group fish fed only basal diet. G2: fish fed 0.5% of *R.officinalis* extract added to basal diet. G3: fish fed 1% of *R.officinalis* extract added to basal diet. G4: fish fed 1.5% of *R.officinalis* extract added to basal diet.

Histological examination

Though the histomorphological examination of the posterior intestine of the control group was similar to the anterior part, however, the villi were broad and short, the crypts were deeper, the mucosal epithelial lining was free of cilia, and contains more goblet cells. The mucosa appeared to be formed from a single epithelial layer of columnar cells, followed by the lamina propria and ill-distinct muscularis mucosa. The submucosa showed loose C.T, reticular and elastic fibers beside some adipocytes. It enclosed blood vessels and lymphatics. The muscular coat was formed from an inner circular and outer longitudinal smooth muscle fiber. A thin mesothelial layer encoded the outermost muscle layer (serosa) (Fig. 2A).

The posterior part of the intestine of all the treated groups was nearly similar to the control however, a low or moderate number of lymphocytes were seen infiltrating the lamina epithelialis, which was most prominent in the G_3, G_4 (Fig. 2C, D) respectively. It reflects the enhanced immune mechanisms provoked by the administered RE.

Morphometric indices in Table (9) display increasing villi length, width, muscle thickness, and intraepithelial lymphocytes (IEL) with increasing concentration, while decreasing goblet cell count with increasing concentration.

Means within the same column carrying different superscripts are significantly different at ($P< 0.05$). MT: muscular thickness. GCC: Goblet cell count. IELI: Intra epithelial lymphocytic infiltration. G1:control group fish fed only basal diet. G2: fish fed 0.5% of *R. officinalis* extract added to basal diet. G3: fish fed 1% of *R. officinalis* extract added to basal diet. G4: fish fed 1.5% of *R. officinalis* extract added to basal diet.

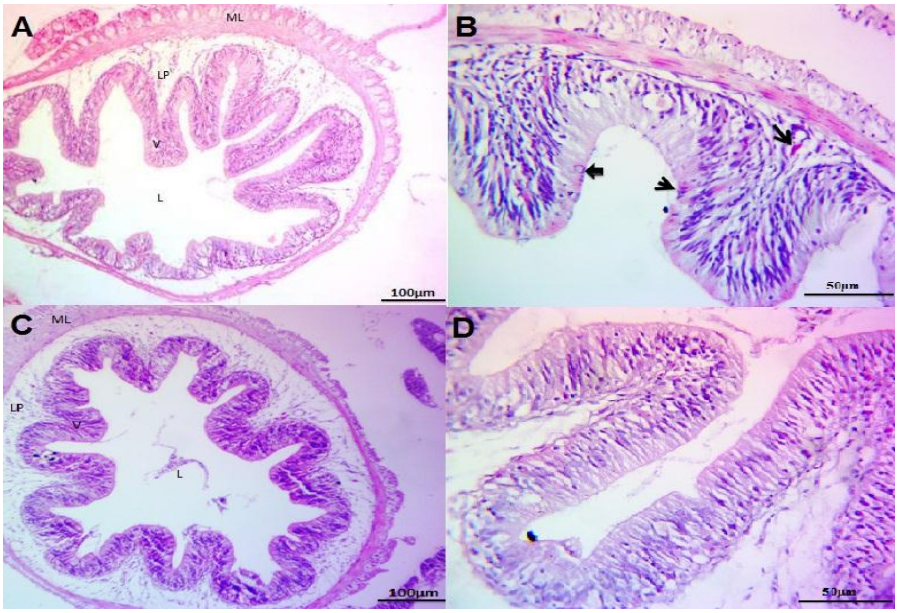

Fig. 2: Photomicrograph from the posterior intestine of control

(A) showing the villi are broad and short, the crypts are deeper, the mucosal epithelial lining is free of cilia and contains more goblet cells The mucosa shows a single epithelial layer of columnar cells, followed by the lamina propria and ill-distinct muscularis mucosa. The submucosa shows loose C.T, reticular and elastic fibers beside some adipocytes, it encloses blood vessels and lymphatics. A thin mesothelial layer encoded the outermost muscle layer (serosa), scale bars = 100 um. (B) Representative photomicrograph of the fish posterior intestine G_2 showing single layer of the columnar epithelium which contain a few goblet cells (thick arrow) and infiltrated by a few lymphocytes (arrow head) beside numerous granulocytes in the lamina propria (small arrow), scale bars = 50 um. (C) Representative photomicrograph of the fish posterior intestine G_3 showing apparently free lumen (L), short thick villi (V) and lamina propria (LP), scale bars = 100 um. (D) Representative photomicrograph of the fish posterior intestine G_4 showing nearly normal columnar epithelium with limited goblet cells and intraepithelial lymphocytic infiltrations, scale bars = 50 um.

Table 9: Effect of *R. officinalis* extract's addition to diet on the morphometric of posterior intestine in the Nile tilapia fingerlings (*Oreochromis niloticus*) for 8 weeks.

Group	Villi Tall (µm)	Villi Width (µm)	MT (µm)	GCC	IELI
G1	583.95 ^{a} ±15.81	208.25 ^{c} ±0.72	143.28 ^{c} ±6.42	2.33 ^{a} ±0.333	34.00 ^{a} ±1.00
G2	372.48 ^{c} ±11.07	215.53 ^{c} ±3.10	143.93 ^{c} ±6.88	2.33 ^{a} ±0.333	13.33 ^{c} ±0.333
G3	359.83 ^{c} ±6.75	310.44 ^{b} ±1.64	187.53 ^{b} ±8.61	2.00 ^{ab} ±0.577	18.67 ^{b} ±0.882
G4	510.96 ^{b} ±7.39	335.88 ^{a} ±9.52	297.08 ^{a} ±4.63	1.0000^{b}±0.0001	5.67 ^{d} ±0.333

Means within the same column carrying different superscripts are significantly different at ($P< 0.05$). MT: muscular thickness. GCC: Goblet cell count. IELI: Intra epithelial lymphocytic infiltration. G1:control group fish fed only basal diet. G2: fish fed 0.5% of *R. officinalis* extract added to basal diet. G3: fish fed 1% of *R. officinalis* extract added to basal diet. G4: fish fed 1.5% of *R. officinalis* extract added to basal diet.

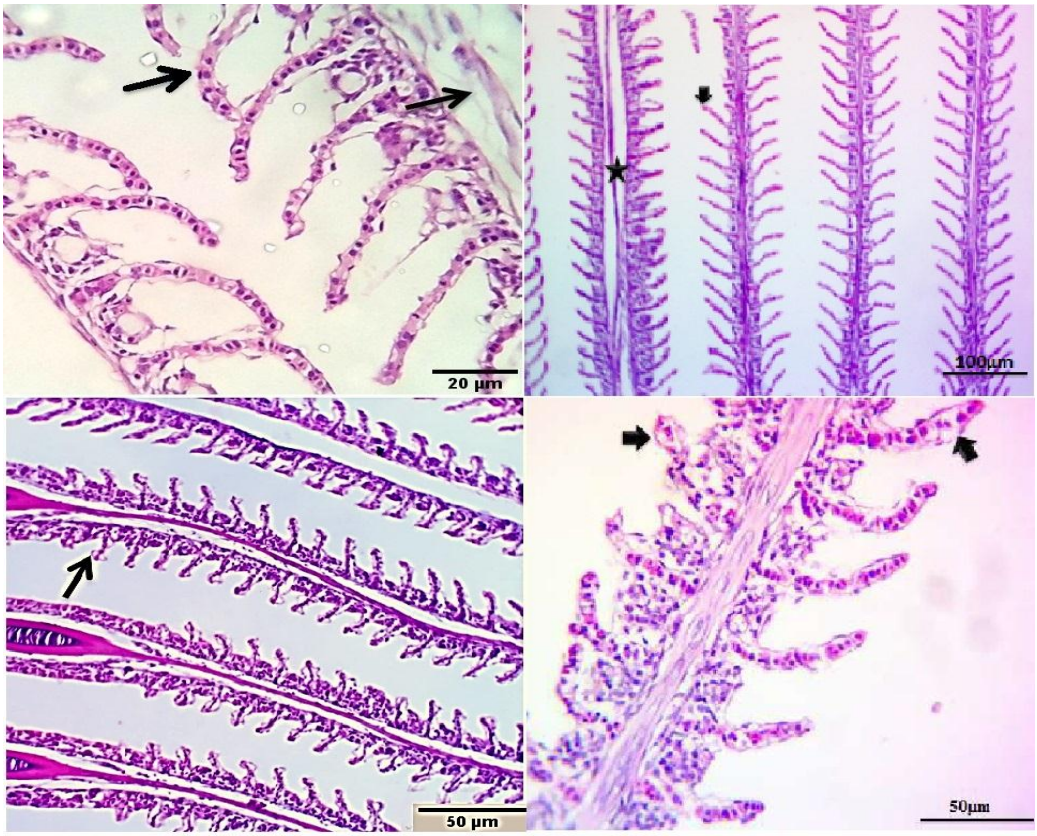

Fig. 3: Photomicrograph from gills control group

(A) showing normal primary and secondary gill filaments (arrows) with normal pavement cells, scale bars = 20 um. (B) Photomicrographs from the gills of G_2 showing normal gill filament (star) and secondary lamella (arrow). (C) Photomicrograph from the gills of G_3 showing epithelial lifting of some secondary gill filaments (arrow) and focal epithelial denudation from the tip of other filaments, scale bars = 50 um. (D) Photomicrographs from the gills of G_4 showing nearly normal lamella with slight congested capillary (arrows).

Examined serial sections from gills of the control fish revealed ordinary histological structures; the primary and secondary gill filaments were normal with normal pavement cells, lamellar epithelial cells, pillar cells, mucus-secreting cells (goblet cells), chloride cells, capillary channels (afferent and efferent venules), and a few mononuclear cells (Fig. 3A). Gills of G_2 revealed comparatively normal gill filament and secondary lamella (Fig.3B). Gills of G_3 denoted minor changes represented by the epithelial lifting of some secondary gill filaments and focal epithelial denudation from the tip of other filaments (Fig.3C). Gills of G_4 pointed out nearly normal gill filament and slightly destructed a few secondary lamellas (Fig.3D).

Kidneys of the control group pointed out normal renal glomerular, tubular, and interstitial structures with preserved bowman's capsular histomorphology, glomerular capillary morphology, and tubular epithelial length and widths with a centrally located nucleus. Minimal degenerative changes in a few numbers of renal tubules are seen in Fig. (4A). Kidneys of the treatment groups showed comparatively healthy, normal counter parts of the nephron units with a standard morphological appearance which was more

standardized and homologous to the control one in G_3 and less standardized in G_2, G_4, where more numbers of renal tubules suffered degenerative changes, mostly of hydropic type. A few glomeruli were lobulated in G_4 (Figs.4 B, C & D).

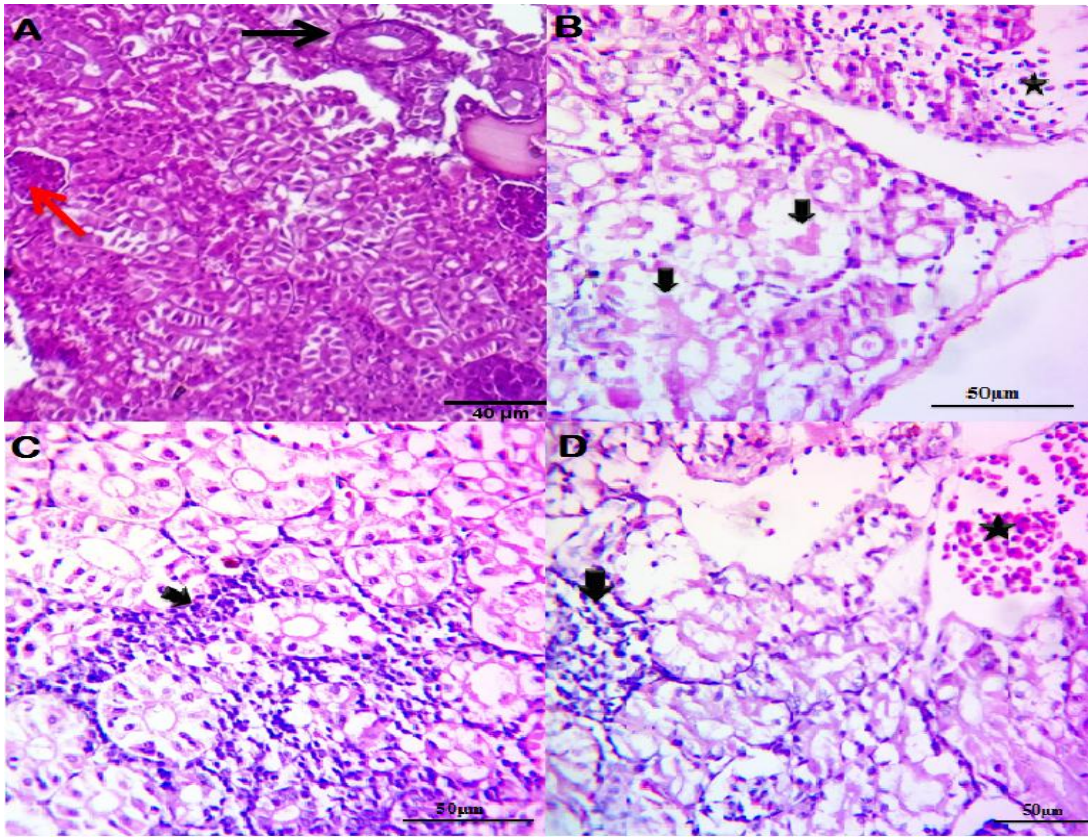

Fig. 4: Photomicrographs from the kidney of control group.

(A) showing normal renal glomerular, tubular and interstitial structures with preserved Bowman's capsular histo-morphology, glomerular capillary morphology (red arrows) and tubular epithelial length and widths, minimal degenerative changes in a few numbers of renal tubules were seen, scale bar = 40 um. (B) Representative photomicrograph of the fish kidney G_2 showing edema infiltrated with inflammatory cells (star) and necrotic tubular epithelium (arrows), scale bar = 50 um. (C) Representative photomicrograph of the fish kidney G_3 showing intense interstitial lymphocytic aggregations (arrow), scale bar = 50 um. (D) Representative photomicrograph of the fish kidney G_4 showing congested blood vessels (star) besides lymphocytic infiltrations (arrow), scale bar = 50 um.

The spleen of control fish showed characteristic proliferative aggregations of melano-macrophages, both perivascular and interstitial. The blood vessels and the splenic sinusoids were mild to moderately congested, and the latter occupied large areas of the tissue constituting the red pulp of the spleen. Splenic cords are a mesh of fibroblast-like cells with foci of various blood cells. White pulp, consisting mainly of lymphoid cells, typically surrounds arterial vessels, sometimes assuming a nodular pattern. Melano macrophage centers (MMC) form small clusters in the parenchyma. The melano macrophage (MM) is a characteristic immune cell type prevalent in the spleen (Fig.5A).The spleen of the treatment groups revealed, histological structures comparable to the control group, but the

lymphoid elements were more prevalent, particularly around the small size blood vessels forming aggregations or ill distinct nodular arrangement, and the splenic cords were outstanding, especially in G_3. Melano-macrophage centers (MMC) were mild to moderately activated in all groups, but they were markedly activated in G_2 (Figs.5B, C & D).

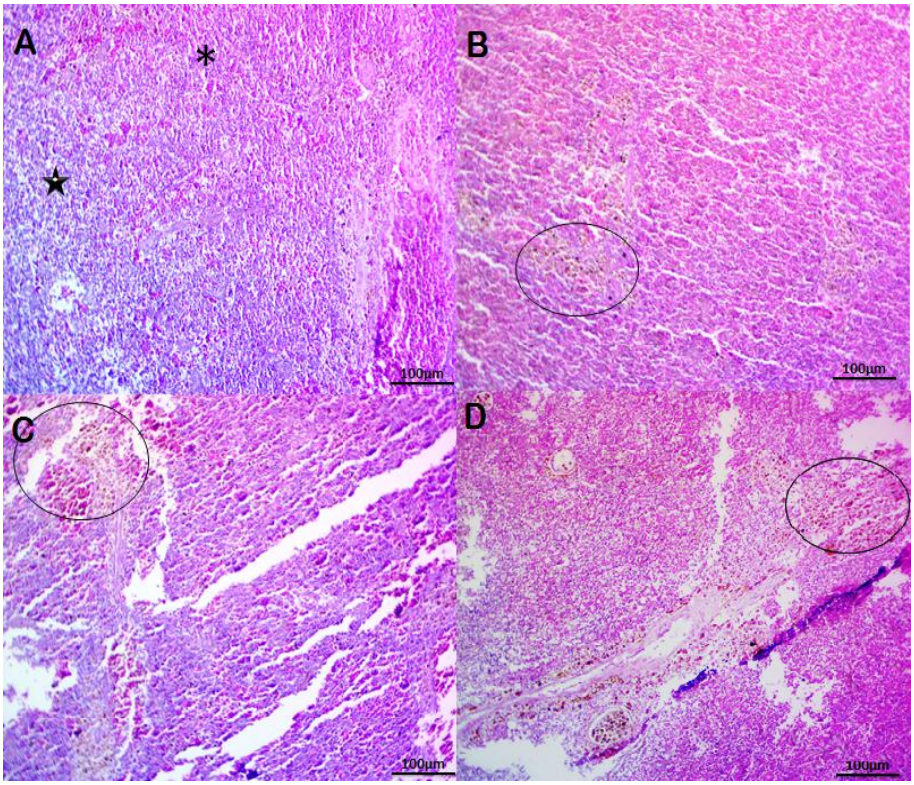

Fig. 5: Photomicrographs from the spleen of G_1 (control group)
A) showing normal white (close star) and red pulps (open star), scale bar = 100 um. (B) Representative photomicrograph of the fish spleen of G_2 showing apparently normal splenic structures with limited melanomacropahges (circle), scale bar = 100 um. (C) Representative photomicrograph of the fish spleen of G_3 showing intense melanomacropahges (circle), scale bar = 100 um. (D) Representative photomicrograph of the fish spleen of G_4 showing moderate to severe melanomacropahges centers (circle), scale bar = 100 um.

Bacterial challenge test

Figure (6) shows the capability of RE to compete for the *S.aureus* infection for the groups G_3 and G_4 followed by G_2 by 100% and 55.71% RPS, respectively.

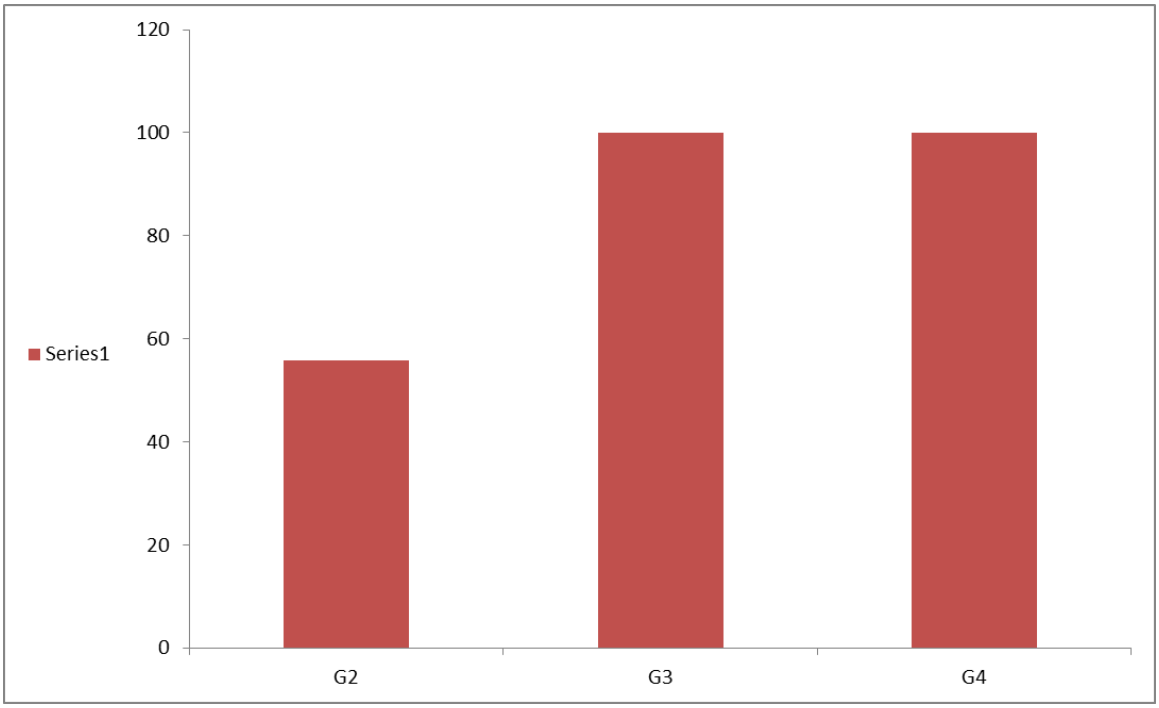

Fig.6: The RPS of the Nile tilapia fed RE against *s.aureus* infection.

DISCUSSION

Despite the availability of antioxidants in RE **(Zoral *et al.*, 2017)** and its powerful anti-inflammatory, hepato-protective, and antioxidant activity in experimental animals and humans **(Takayama *et al.*, 2016)**, in addition to its strong antibacterial activity against Gram-positive and Gram-negative bacteria **(Jiang *et al.*, 2011)**, the researches in aquaculture are still limited, especially on the effects of RE on fish blood biochemistry, immunity, and antioxidant activity **(Yousefi *et al.*, 2019)**. Therefore, to fill the gap in literature, the present study was performed to illustrate the potential role of RE as a feed additive for the Nile tilapia on the general health and immune response.

Regarding the results of growth performance, the priority was noticed of G_3 and G_4 in FBW, WG, WG%, and SGR with no significant difference in between, followed by G_2 then G_1 (control) The same manner was noted in the FCR result; recording the lowest in G_3 and G_4 with no significant difference in between, then came G_2 in order, and finally G_1 (control). While the biometric indices showed the priority to G_3. In addition, **Hassan *et al.* (2018)** stated that, the 1% rosemary supplementation increased weight gain, SGR, and protein efficiency ratio (PER) in a significant degree of the Nile tilapia. The current results disagree with those mentioned by **Hernandez *et al.* (2015)** who reported that, the growth and feed intake were not affected by RE. This enhancement may be due to the stimulation of the digestion through increasing the bile production, or by stimulation of the pancreas and increased secretion of digestive enzymes as reported by **Yılmaz *et al.* (2013).** The previous authors noted that pancreatic enzymes are important factors in nutrient digestion and assimilation **(Frankic *et al.*, 2009)**. Moreover, they stimulate the liver function **(Dzubak *et al.*, 2006)** as observed in the current study. Additionally, the intestinal morphometry result indicates that, the increased absorptive intestinal surface

with increasing RE fed influencing the absorption, augment the function of the gastrointestinal tract **(Raja *et al.*, 2011)**, subsequently increases the feed utilization coordinating with the beneficial effects of rosemary on fish health. The aforementioned resut is in the same line with the other obtained result in this study. In addition, **Hoseini *et al.* (2018)** found that, the 1,8-cineole administration significantly improved *Oncorhynchus mykiss* growth performance and serum biochemical parameters.

Fish health and physiology could be estimated via assessment of serum biochemical and innate immune parameters **(Fazio, 2019)**. Hence these parameters are used for determining the effect of feed additives on fish **(Yılmaz *et al.*, 2018)**. It is worthy to mention that, ALP, ALT, glucose, total protein and albumin are typically used to evaluate liver function and nutrient metabolism **(Liu *et al.*, 2020)**.

Studies have demonstrated the connection between lipid metabolism and growth performance **(Zhang *et al.*, 2019).** Thus**,** the levels of triglycerides and cholesterol (energy metabolites) are important health parameters for fish **(Coz-Rakovac *et al.*, 2005)**. They are generally used as indicators of liver function **(Wagner and Congleton, 2004)**, and are the main energy reserve in fish **(Abarra *et al.*, 2017)**.

Regarding the present results, a significant reduction was detected in serum glucose, cholesterol, and triglyceride levels with increasing concentration of RE supplemented to the diet. This indicates the hepatoprotective effect of RE which may be due to the action of betulin (a component of RE which is a triterpenes) that increases insulin sensitivity and inhibits maturation of sterol regulatory element-binding protein, and subsequently reduces the synthesis of cholesterol and fatty acids **(Tang *et al.*, 2011)**. The obtained result agrees with that of **Hernandez *et al.* (2015)** who reported a reduction in plasma level of glucose after 12 weeks of administrating rosemary extract to gilthead seabream (*Sparus aurata*). Additionally, **Yousefi *et al.* (2019)** suggested that, the oral administration of rosemary leaf powder has the potential to prevent cortisol and glucose elevation. **Hernandez *et al.* (2015)** cleared that rosemary extract reduced the plasma levels of glucose and triglycerides after four weeks, and glucose, HDL/LDL cholesterol ratio, and plasma alanine aminotransferase after 12 weeks administration to gilthead seabream (*Sparus aurata*).

Serum ALT and ALP activities decreased significantly with the increasing concentration of RE. Similarly, **Takayama *et al.* (2016)** discussed the hepatoprotective effect of RE. Moreover, **Hernandez *et al.* (2015)** reported a reduction in ALT level in gilthead seabream (*Sparus aurata*) fed rosemary extract supplementation after 12 weeks of administration.

Notably, creatinine is a traditional screening indicator of kidney function and renal structural integrity **(Abdel-Tawwab *et al.*, 2014)**. Results of serum creatinine level were significantly reduced with increasing the RE concentration, supported by the present histological finding of renal tissue of the control group. The present finding disagrees with that of **Yılmaz *et al.* (2012)** who found that, rosemary did not change serum creatinine of sea bass (*Dicentrarchus labrax*) after a 45-day feeding trial.

it is noteworthy mentioning that**,** the non-specific immune response is the first line of defense system to protect fish against invading pathogens **(Van Doan *et al.*, 2017)**.

Considering the present results, serum total protein, total globulin, and γ globulin increased significantly with increasing concentration of RE, suggesting immunomodulatory effects of RE on the Nile tilapia. Thus, dietary supplementation of

RE affects positively the welfare of the cultured tilapia, as increased total protein and globulin may indicate an improved function of the protein synthesized organ as the liver and strong fish immunity **(Asadi *et al.*, 2012);** a result which coordinates with the rest of the current results.

To illustrate, **Yousefi *et al.* (2019)** stated that, the beneficial effects of dietary rosemary leaves powder on fish health by is determined by affecting its blood parameters, especially at the highest levels (2 and 3% rosemary leaves powder). Those results are in the same line with those of **Hoseini *et al.* (2018)** who found that the 1,8-cineole administration improved *Oncorhynchus mykiss* serum biochemical parameters significantly.

For RE, it enhances the production of lysozyme compared to control indicating an enhancement in immunological function **(Kurian *et al.*, 2020)**. However, reducing lysozyme and NO level with increasing RE level may be contributed to increasing the antimicrobial activity of RE with increasing its concentration. RE antimicrobial properties contributed to their different polyphenol compositions **(Moreno *et al.*, 2006);** mainly rosmarinic acid, carnosol and carnosic acid (**Govaris *et al.*, 2007**). Those compositions interact with the cell membrane, causing changes in the genetic material and nutrients, altering the transport of electrons, leakage of cellular components and production changes in fatty acid. In addition, it also produced an interaction with the membrane of proteins producing the loss of membrane functionality and its structure **(Fung *et al.*, 1977)**. **Ivanovic *et al.* (2012)** demonstrated that the effectiveness of rosemary is related to a possible synergy between the rosmarinic phenolic acid and the carnosic acid diterpene. So that rosemary and its derivatives used as a food preservative to inhibit the growth of common food bacteria contributing to food spoilage as stated by (**Camo *et al.*, 2008)**.

This ability may compensates the need for activation of fish lysozyme and NO.

Whereas RE enhances the production of MPO compared to control however it reduced with increasing concentration of RE fed, which may be due to increasing the power of anti-inflammatory effect of RE with increasing its concentration, this may be due to the anti-inflammatory action of compounds detected in RE as camphor, caryophyllene, carnosole, and 1,8 cineol, as supported by **(Ribeiro-Santos *et al.*, 2015)**.

Those results could be indicated by the intestinal morphometry in the current study that revealed an increase in IELI with increasing RE fed to compensate for the need for activation of fish lysozyme, NO, and MPO. Thus, RE is considered an appropriate immunomodulator, which is expected to provide activation of immune responses only when necessary, to fight pathogenic organisms **(Brum *et al.*, 2018)**. Markedly, the absence of disease is related to the lack of alteration in the immunological parameters after the supplementation which is considered a beneficial result **(Dotta *et al.*, 2014)**.The immune-modulatory substances mustn't cause unnecessary activation of the immune responses, since this represents an additional energy cost to the organism, which may impair growth and development. Besides, many of the defense substances produced by immune response are extremely toxic and lytic which may cause damage to the animal's tissues. Those results are symmetrical with the histological finding of spleen tissue in this study showing that melano-macrophage centers (MMC) were mild to moderately activated in all groups, but they were markedly activated in G_2.

On the other hand**,** stress can increase the production of reactive oxygen species in the body, whereas excessive reactive oxygen species can cause lipid peroxidation increasing the amount of lipid peroxides in the body, **(Dawood *et al.*, 2019)**. This high level of reactive oxygen species can cause damage and disfunction in body tissues **(Ameur *et al.*, 2012)**.

The significant increase of SOD, CAT, and GSH in treatment groups compared to control with the highest value in G_4 may be explained as reported in the study of **Brake (1997)**. He stated that, the high nutritive value of rosemary from vitamins and minerals may be responsible for oxidative status enhancement via scavenging of free radicals; and prevention of the autointoxication of immunological cells by maximization of macrophage production. The previous author added that, the rise in SOD, CAT and GSH may be due to the flavonoid and phenolic compounds in herbals being responsible for their high antioxidant capacity **(Reyes-Cerpa *et al.*, 2018)**. This explanation coordinates with the recorded GC-mass results revealing the richness of RE with powerful antioxidant carnosol ($C_{20}H_{26}O_4$). Thus, the present finding suggests that RE supplementation could stimulate antioxidant protection by carnosol (phenolic compound) to convert reactive oxygen species into safe compounds to defend biological materials from oxidation. The aforementioned suggestion is similar to that reported by **Hoseini and Yousefi (2018)**. In addition, the present result agrees with that of **Yilmaz (2019)** who found a positive correlation between the antioxidant enzyme activity and innate immune response in finfish or shellfish fed diets supplemented with various feed additives. **Mukherjee *et al.* (2019)** reported that**,** the plant extract act as a potent free radical scavenger in fish tissues, which in turn reduced the production of reactive oxygen species in cells and stimulated fish health.

Concerning chemical body composition, it may contribute to the abilities of rosemary to precipitate proteins and affect nutrient digestibility **(Manafi *et al.*, 2014)**. This result disagrees with that of **Hassan *et al.* (2018)** who found that, the 1% rosemary supplementation had an insignificant effect on the carcass composition of the Nile tilapia. Additionally, **Hoseini *et al.* (2018)** found that the 1,8-cineole administration did not affect the body composition of *Oncorhynchus mykiss*.

The results obtained in the present study reflected the challenge test against *S.aureus*, the RE supplementation to the diets G_3 and G_4 prevent the mortality against *S.aureus*, and at G_2 it enhances RPS by 55.71%. This reduced rate of mortality is undoubtedly attributed to enhanced non-specific immune responses and the antioxidant effects of ROE and its constituents which have antibacterial activity **(Pietta, 2000)**, especially monoterpenes as 1,8-cineol and camphor (detected in this work's extract), which increase fluidity and permeability of the microbial cytoplasmic membrane, disturb the order of membrane implanted proteins, inhibit cell respiration, and alter ion transport pathways **(Jrah *et al.*, 2011)**. Similarly, **Jiang *et al.* (2011)** found that rosemary extract exhibited strong antibacterial activity against gram-positive and gram-negative bacteria. Additionally, numerous studies have reported that oral administration of rosemary in the Nile tilapia (*O. niloticus*) **(Zilberg *et al.*, 2010)** and Mozambique tilapia **(Ergün *et al.*, 2011).** Other researchers added that rosemary supplementation improved disease resistance and immunity as reported in the study of **Gowda and Ledoux (2008)** which agrees with the results of the current study.

CONCLUSION

R.officinalis extract in the diet of the Nile tilapia interferes positively in the metabolism of glucose and lipids, reducing the serum concentration of glucose, cholesterol, and triglycerides. Additionally, immunological indicators (serum protein, immune globulins) were enhanced significantly with increasing RE fed, while lysozyme activity, NO, and melano-macrophages centers were significantly enhanced compared to control but in a descending manner with increasing RE fed. This is recorded a benefit, since there is no excessive stimulation of defense mechanisms, which consume energy and should be activated only against pathogens. In conclusion, RE improves the health and well being of the Nile tilapia by enhancing its immunological and physiological parameters.

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الملخص العربي

تأثير مستخلص نبات إكليل الجبل على أداء النمو والمناعه غير المتخصصه ونشاط مضادات الأكسده ومقاومة الأمراض للبلطي النيلي.

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أجريت هذه الدراسه لتقييم تأثير مستخلص أوراق نبات إكليل الجبل كإضافه لعليقه أسماك البلطي على أداء النمو والمناعه الغير متخصصه ونشاط مضادات الأكسده ومقاومة الأمراض ضد العدوى التجريبيه باستخدام *Staphylococcus aureus* . قسمت الاسماك الصحيه (ن=144 بمتوسط وزن أولي=21.38 ±0.45 جم) بشكل عشوائي إلى أربع مجموعات فى ثلاث تكرارات. تم تغذية المجموعه الأولى (G_1) على نظام غذائي أساسي كمجموعه ضابطه و بينما تم تغذية المجموعه الثانية والثالثة والرابعة (G_2، G_3، G_4) على نظام أساسي مكمل ب 0.5 % و1 % و 1.5 % من مستخلص نبات إكليل الجبل على التوالي لمدة 8 أسابيع. أظهرت النتائج زياده معنويه قى معدلات النمو بما فى ذلك وزن الجسم النهائي FBW وإكتساب الجسم WG ونسبة إكتساب الجسم WG% ومعدل النمو النوعي SGR ، مع إنخفاض معنوي في التحويل الغذائي FCR في المجموعات الثالثة (G_3) و الرابعة (G_4) مع زياده معنويه في القياسات البيومتريه بما قي ذلك HIS و SSI و K وذلك في المجموعه الثالثة (G_3) متبوعه بالمجموعتين الثانية (G_2) والرابعة (G_4) مقارنه مع المجموعه الضابطة (G_1).كما أظهرت مستويات الجلوكوز والدهون الثلاثيه والكوليستيرول والإنزيمات الناقله لمجموعة الأمين و الكرياتينين و النيتريك اوكسيد (NO) انخفاضا معنويا فى مصل الدم مع زيادة تركيز المستخلص فى العليقه. في حين أظهرت مستويات البروتين الكلى و الالبيومين و الجلوبيولين الكلى و الجاما جلوبيولين و انزيمات مضادات الاكسده (SOD, CAT, GSH) و المقاومه ضد عدوى *S.aureus* زياده معنويه فى علاقه طرديه مع زياده تركيز المستخلص فى العليقه. كما أظهرت مستويات الليزوزيم (lysozyme) و الميلوبيروكسيداز(MPO) زياده معنويه فى المجموعات الثانية والثالثة والرابعة (G_2، G_3، G_4) مقارنة بالمجموعة الضابطة (G_1) على الرغم من أنها فى علاقة عكسيه مع زيادة تركيز المستخلص فى العليقه حيث انها تقل مع زياده تركيز المستخلص فى العليقة. لذلك فإن النتائج تؤكد أن إضافة مستخلص نبات إكليل الجبل بإمكانه أن يعزز صحة البلطي النيلي.