한국어병학회지 제36권 제1호 (2023)
J. Fish Pathol., 36(1) : 075~093

www.ksfp.org
pISSN 1226-0819, eISSN 2233-5412
http://dx.doi.org/10.7847/jfp.2023.36.1.075

Efficacy of dietary propolis and its nanoparticles on immune-response, stress indicators, and prevention of *Pseudomonas aeruginosa* infection in *Oreochromis niloticus*

Fatma M. M. Korni[*†], Asmaa N. Mohammed[**], Usama K. Moawad[***], Nour El-Houda y. Hassan[****], Ahmed Farghali[*****] and Rehab K. Mahmoud[******]

[*]*Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt.*
[**]*Department of Hygiene, Zoonoses and Epidemiology, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt*
[***]*Department of Histology and Cytology, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt*
[****]*Department of Forensic medicine and toxicology, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt*
[*****]*Departement of Materials Science and Nanotechnology, Faculty of Postgraduate Studies for Advanced Sciences, Beni-Suef University*
[******]*Department of Chemistry, Faculty of Science, Beni-Suef University, Beni-Suef, Egypt*

Nanotechnology seeks to improve material effectiveness to have a greater impact on medicine. The current study was performed to determine how effective dietary propolis (PR) and propolis nanoparticles (PR-NPs) were at improving immune and anti-oxidant status, lowering cortisol levels as stress indicators, and preventing *Pseudomonas aeruginosa* infection in *O. niloticus*. Histopathology was carried out. Fish ($N = 516$, 25 ± 2g) were fed basal diets supplemented with 1 gm PR/kg, 2 gm PR/kg, 1 gm PR-NPs/kg, 2 gm PR-NPs/kg feed and control diet for 28 days. When compared to the control, interlukin-1, IgM, complement 5, and levels of lysozyme were dramatically higher in PR and PR-NPs fed-groups. Antioxidant enzymes and glutathione levels were higher in fish fed PR and PR-NPs, but malondialdehyde levels were lower. Cortisol levels decreased in feeding groups compared to the control. When compared to *O. niloticus* group fed 1 gm PR/kg and the positive control, propolis nanoparticles successfully prevented *P. aeruginosa* infection and the mortality rate was zero. When compared to the negative control, the positive control group's histopathological findings revealed severe histopathological changes. In low and high groups fed PR-NPS, normal structures were observed as well as high concentrations of PR after being injected with *P. aeruginosa*. The group that was fed low concentrations of PR after being injected with *P. aeruginosa* showed only minor histopathological changes. Conclusion: Dietary supplementation of PR or PR-NPs may have a beneficial effect on aquaculture, with PR-NPs having a superior effect.

Key words: Propolis, Propolis nanoparticles, *O. niloticus*, Immune status, Stress indicators, Prevention of *P. aeruginosa,* Histopathological examination.

[†]Corresponding author: Fatma M. M. Korni
Tel: +0201100921799; Fax: 0822327982
E-mail: naglaamostafa1@yahoo.com

Introduction

Aquaculture is a rapidly expanding industry that accounts for third of the world's fisheries production (Lowther, 2005). Aquaculture accounts for about 70% of total fish production in Egypt (GAFRD, 2012).

Oreochromis niloticus, (*O. niloticus*), Nile tilapia can resist adverse environmental and stress conditions, so it is a commercially valuable fish that is recommended for aquaculture in tropical and subtropical countries (El-Sayed, 2006; Nguyen 2007). After carp and salmonids, tilapia is third most valuable fish in aquaculture, and the first one in Egypt (FAO 2009).

Nanotechnologies used in aquaculture with wide range (Huang et al., 2015). One of their central aims is to improve the effectiveness of substances by altering their size, as at the nanoscale materials may have a greater effect on both of biology and medicine (Sahlan et al 2017).

Propolis (PR) is a viscous waxy resin substance produced by bee saliva from an enzyme, pollen, and wax mixture. It has antibacterial activity against numerous Gram-positive and negative bacteria (Seven et al. 2011) as well as anti-inflammatory (Funakoshi-Tago et al. 2015), and anti-oxidant activities (Yonar et al., 2014, Gul Baykalir et al. 2016). Furthermore, it is known to enhance the immunologic response and resistance to disease of various farmed fish species. Natural products like PR may have many aquaculture applications without risk and side effects when compared with the synthetic products that are used for the same purpose, which have negative impacts on environmental, animal and human health due to their eco-toxicity (Jesús et al 2018).

Propolis nanoparticles (PR-NPs) are easy to be absorbed by the body because of their smaller size, and they may be more effective at increasing the dissolvability of PR (Seven et. al. 2020). Some researchers have suggested that PR-NPs could be used as antibacterial agents (Hasan et al., 2014). Propolis nanoparticles were previously used successfully to prevent *Microcystis aeruginosa* and as an anti-oxidant agent in *O. niloticus* (Abdel mageid et al 2020). Furthermore, they have the potential to be useful in veterinary science in regards to health, performance, and consistent food production.

The hypothalamus pituitary adrenal axis (HPA-axis) is stimulated by stress, increasing blood cortisol and locomotor activity (Carrasco and Van de Kar, 2003). Cortisol is the most abundant corticosteroid in teleosts, and plasma levels increase significantly during stress, suppressing immunity and making the fish vulnerable to bacterial infections (Mommsen et al., 1999).

The most serious issue in in Egypt's aquaculture practices is bacterial diseases, causing high mortality and resulting economic losses (Noor El-Deen et al. 2010). *Pseudomonas aeruginosa (P. aeruginosa)* causes significant economic losses in tilapia farming (Thomas et al 2014). It is one of the world's most dangerous malignant strains, causing severe health problems in both humans and animals (Rossolini et al 2005).

Propolis and its nanoparticles have been studied as supplementary feed in a variety of teleosts. Their supplementation improves innate immune responses in tilapia by increasing antibody levels, complements, and lysozyme activity (Abd-El-Rhman, 2009; Abbass et al 2012; Ümit et al 2018; Mohamed et al 2019; Abdelmagid et al 2022). Also, the consumption of PR and its extract significantly reduces cortisol levels in fish subjected to low-temperature stress (Segvic-Bubic et al., 2013). Furthermore, they reduced malondialdehyde levels and increase the activity of glutathione s-transferase in fish tissues (Ferreira et al., 2013; Aldemir et al., 2014). Moreover, the dietary supplementation of PR and its nanoparticles were used as as an antibacterial agent against several Gram-positive and negative bacteria (Jess et al 2018). Therefore, the purpose of this study was to determine the impacts of dietary PR and PR-NPs on improving

immune response, mitigating stress and preventing *P. aeruginosa* infection in *O. niloticus*. Also, histopathological examinations were carried out to assess the results.

Material and methods

Ethical committee

The Beni-Suef Institutional Animal Care and Use Committee (BSU-IACUC, number 022-332) of the Faculty of Veterinary Medicine, Beni-Suef University, Egypt, approved all experiments.

Source of PR

Propolis brown powdered color was purchased from Imtenan Company Cairo, Egypt.

Table 1. Chemical composition (g/kg on dry weight basis) of diets of experiments

Ingredients	g/kg
Soybean meal (42.7% CP)	300
Fish meal (65.0% CP)	400
Corn meal	100
Wheat gluten	50
Corn oil	20
Rice bran	60
Fish oil	10
Mineral Premix*	30
Vitamin Premix**	30
Total	1000
Proximate chemical composition	
Crude protein	462
Dry matter	93.2
Crude fibers	49
Crude lipids	135
Ash	103

*Mineral premix: Zn 4 g, Mn 1.4 mg, Fe 14 g, Mg 10 g, Co 30 mg, Cu 350 mg, Se 35 mg, I 40 mg.
**Vitamin premix include (/kg in premix): vitamin A 67 IU, vitamin E 7.4 g, vitamin D 16.2 IU, vitamin K3 340 mg, vitamin B1 670 mg, vitamin B2 1000 mg, vitamin B6 800 mg, vitamin B12 1.4 mg, vitamin C 10 g, D-pantothenic acid 2.65 g, folic acid 330 mg, nicotinamide 5.35 g, choline chloride 35 g, biotin 34 mg, inositol 8 g.

Synthesis of PR–NPs

The commercial PR was placed in ball milling of a photon vessel for 24 hours at a speed of 200 rpm under the conditions described in Table 2.

Characterizations of PR and PR–NPs

X-ray differentiation analysis (XRD) was used to determine the phase formation and crystallinity of the nanoparticles. Using the Fourier Transform Infrared, the chemical bonds variation was investigated (FT-IR, Bruker Vertex 70). The microstructures of all the nanocomposites were characterised using a high-resolution transmission electron microscope (HRTEM, JEOL-JEM 2100). A field emission scanning electron microscope was used to characterise the morphology of the PR-NPs (FESEM, Quanta FEG 250). The hydrodynamic size and zeta potential were studied (experimentally optimized) by a Malvern (Malvern Instruments Ltd) and previously described methodology, (Moaty, Farghali et al. 2017).

Preparation of diets

Using a mortar and pestle, the commercial feed diet was finely ground. To create five fish diets, the PR and its nanoparticles were immediately combined with the previously made fine powder. Diet 1 contained no additives (control), diet 2 contained 1 g PR/kg of feed, diet 3 contained 2 g PR/kg of feed, diet 4 contained 1 g PR-NPs/kg of feed, and diet 5 contained 2 g PR-NPs/kg of feed. To create a homogeneous mixture, the fish diet contents were mixed with distilled water. The mix has been injected through an extruder to create extruded strings, which were then dried at 40°C for 24 hours before being broken down to a length of about 2 mm (Rattanachaikunsopon and Phumkhachorn 2010).

Experimental fish collection

For experiments, 372 *O. niloticus* with body weight average of 25 ± 2 g were taken alive from the fish hatchery of Abo-Saleh in Beni-Suef, Egypt. The col-

lected samples were brought to the wet lab of Fish Diseases and Management Department at Beni-Suef University's Faculty of Veterinary Medicine in Egypt.

Experimental fish management

Fish sample (372 *O. niloticus* with body weight average of 25 ± 2 g) was added to three fiber glass tanks of 500 L each in size and supplied with tape water without chlorine. The fish received a commercial fish ration consisting of 3% body weight and they were acclimated in these tanks for 14 days (Bresikl factory, Egypt). After acclimation, fish were moved into 90 × 25 × 40 cm glass aquariums. Through an air pump, regular artificial aeration was applied to each tank. The water rate of exchange in the aquaria was 10% daily. Fish received food equal to 3% of their weight (Table 1). Throughout the trial, water quality was checked two times a week. Water thermometer from Yellow Spring Instrument Co., USA, were able to determine the water's temperature (25 ± 1°C), dissolved oxygen content (D.O.; 7±2 mg/L), and pH (7-8) using a D.O meter (Yellow Spring Instrument Co., USA) and pH indicator paper (Fisher Scientific, Denver, CO, USA). Also, total ammonia nitrogen (TAN) was measured using a Multiparameter Photometer (bench, HI83200, HANNA, Romania). The measurement of the concentration was determined using the Nessler method with reagent kits HI93700A-0 and HI93700B-0. In addition, the TAN level was measured in parts per million (ppm). The mean values for TAN levels in fish aquaria after feeding on PR and PR-NPs at 1 gm/kg and 2 gm/kg each were 0.5 ± 0.02, 0.45 ± 0.0 mg/L, 0.07 ± 0.0 mg/L, and 0.06 ± 0.0, respectively, while for the control group it was 0.9 ± 0.13 mg/L

Table 2. Ball milling process conditions for preparing PR-NPS

Description	Process Condition
Ranged from 1.5~1.8 cm	Balls diameters
7.5 cm	Vessel diameter
Stain steel	Materials of vessel
Porcelain	Materials of used balls
10:1	Ball/Natural-zeolite mass ratio
200 rpm	Speed
10 hrs	Time

Design of experiments and regime of feeding

Six groups of 14 fish each with three replicates were formed from the 252 *O. niloticus*. The first and second groups of fish (the negative and positive controls, respectively) received diet 1, while the third group of fish received diet 2. The fish in the fourth group were also fed diet 3. Diets 4 and 5 were administered to the fifth and sixth groups, respectively. All groups received 3% of their body weight of their respective diets twice daily for the duration of the four-week experiment (28 days). Three fish from each group with their replicates were used for blood and gill sampling after the feeding period (28 days). Except for the negative control group, 11 remaining fish in each group were injected with *P. aeruginosa* along with their replicates while the negative group with its replicate was injected with physiological saline. Three fish from every group with their replicates were collected 96 hours after injection for histopathological examination. *P. aeruginosa* prevention mortality was calculated using the remaining eight fish in each group and their replicates (Table 3).

1) Estimation of immune parameters and stress indicators in serum, gills and plasma of experimental feeding groups

The 3 fish from every group were rapidly netted and anaesthetized with tricaine methane sulfonate (Sigma-Aldrich Chemical Co., Germany, 4 mg/l) just at finish of the feeding (28 days), and the blood and gills were collected for serum separation, blood was drawn from caudal veins without the use of an anticoagulant. The serum was collected and stored at -20°C. Test kits were used to measure interleukin 1 β (IL-1) (CUSABIO, Fish Interleukin 1 ELISA Kit Catalog Number. CSB-E13259Fh, China). IgM levels were de-

Table 3. Numbers of apparently healthy *C. gariepinus* for each experiment

Numbers of apparently healthy *O. niloticus*	The experiment
A total of 252 *O.* niloticus feeding for 28 days (6 groups × 14 fish in each group × 3 replicates)	3 fish from each group with 3 replicates were used for blood and gill sampling 8 fish from each group with 3 replicates were used for prevention of *P. aeruginosa* 3 fish from each group with 3 replicates were used for histopathology
A total of 120	Experimental infection and determination of the median lethal dose (LD50) of *P. aeruginosa*
Total: 372 *O. niloticus* with body weight average of 25 ± 2 g	

termined using the commercial test kits' manufacturer's instructions (CUSABIO, Fish immunoglobulin M ELISA Kit Catalog Number. CSB-E12045Fh, China). Complement 5 (CUSABIO, Fish Complement 5 (C5 ELISA Kit Catalog Number. CSB-F13502F, China) and lysozyme were also measured using commercial test kits (CUSABIO, Fish lysozyme (LZM, ELISA Kit Catalog Number. CSB-E17296Fh, China). Malondialdehyde (MDA) was measured according to the manufacturer's instructions for the commercial test kits (Bio-diagnostic Company, Egypt).

Gills were carefully removed. On ice, 1tissue gram was weighed and homogenized in 5 mL PBS using a motorized mini handheld homogenizer. The homogenates were then centrifuged for 20 minutes at 3000 rpm to obtain the supernatant. The supernatant was collected, placed on ice, and used immediately for biochemical assays. Using colorimetric kits (cat no: MAK187D, GR 2511), the tissue supernatant was used to measure total anti-oxidant capacity and glutathione peroxidase, GSH (stress indicators).

Furthermore, cortisol levels in plasma samples were determined using the Cortisol ELISA kit® (Calbiotech, catalogue No. CO103S, Canada) according to the manufacturer's instructions. The results were calculated using an automatic ELISA reader (SUNRISE®; Tecan, Austria) as described by Schlaghecke et al., (1992).

2) *P. aeruginosa* prevention in *O. niloticus*

(1) Bacterial strain

The *P. aeruginosa* strain was obtained from a previous study, and it was obtained from diseased *O. niloticus* (Elgohary et al 2020).

(2) Determination of median lethal dose, LD_{50} of *P. aeruginosa* in healthy *O. niloticus*

Five groups of eight *O. niloticus* each had three replicates, totaling 120 *O. niloticus*. *P. aeruginosa* cultures were adjusted to densities of 3 × 108, 3 × 107, 3 × 106, and 3 × 105 CFU/mL after growing them overnight. The dose of each dilution was 300 µL/fish intraperitoneally injected, and the fish in the fifth group served as the control and received 300 µL of physiological saline injections. For two weeks, all fish groups were closely observed, and daily mortalities were recorded.

(3) *P. aeruginosa* prevention in *O. niloticus*

At the end of feeding period, the first (negative control) group and its replicates were challenged intraperitoneally with 300 µL of physiological saline.. *P. aeruginosa* was administered intraperitoneally to fish in the second group (positive control), third, fourth, fifth, and sixth groups along with their replicates at doses of 300 µL of 3 × 106 CFU/mL each. For two weeks, the injected groups were kept in a different glass aquarium. According to Amend (1981),

the mortality was noted, and relative percent survival (RPS) was computed using the formula: RPS = 1 (% of mortality in treated groups /% of mortality in control group) 100.

3) Histopathological examination

Three fish from every group and the replicates were euthanized by an overdose of MS222 (150 mg/l) 96 hours after infection. All feeding groups had their gills, liver, spleen, kidney, and intestine tissue samples taken. The obtained specimens were embedded in paraplast®, cleared in xylene, fixed in 10% neutral buffered formalin, dehydrated in various grades of ethyl alcohols (50% ~ 100%), and sectioned at 4-5 m using a rotary microtome. Haematoxylin and Eosin (H&E stain) was used to colour the prepared sections, and light microscopy was used to examine the results (Suvarna et al., 2019).

Statistical Analyses

All data were subjected to statistical analysis using the Advanced Models 16.0 software's one-way ANOVA (post hoc test; Dunnet's test) (SPSS, Tokyo, Japan). Statistical significance was defined as a P-value 0.05.

Results

Characterizations of PR and PR–NPs

onoids, phenolics, and aromatic compounds make up the majority of the chemical classes found in propolis. Additionally, terpenes, volatile oils, and bee wax are found in propolis. The FTIR spectra of proplis are shown in Fig. 1. The peaks appear in the region 1019.75 cm^{-1}. The band at 1633.21 cm^{-1} related to the stretching of O-H groups. C=O groups at 1446.12 cm^{-1}. Evidence of polyphenol present in the propolis is clearly visible. There were bands accordingly O-H at 3355.20 cm^{-1} and C-H band at 2923.37 cm^{-1}. In addition, a band at 877 cm^{-1} was present, which was associated with the angular deformation of C-H outside the aromatic plane. At 1376.99-1261, the N-O, C-O, and C-N groups all have symmetric effects. 86 cm^{-1}. XRD patterns of the PR-NPs do not show any characteristic reflection peaks (Fig. 2) and has an amorphous structure, and a broad peak can be observed on the pattern at 2θ -21.72°. The morphology of the PR-NPs was determined by HRTEM as shown in Fig. 3 It is evident that the nanoparticles are small, with an average size of 50 nm in the nanometer range (Fig. 4). In addition, the HRTEM view reveals that the PR-NPs have layered, spherical, and hexagonal structures. The PR-NPs had a zeta potential of -34. The highly negative zeta potential cationic particles are nontoxic and safe (Fig. 5).

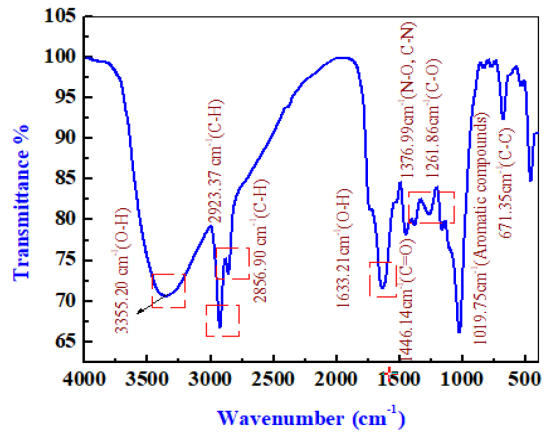

Fig. 1. FTIR spectra of PR-NPs.

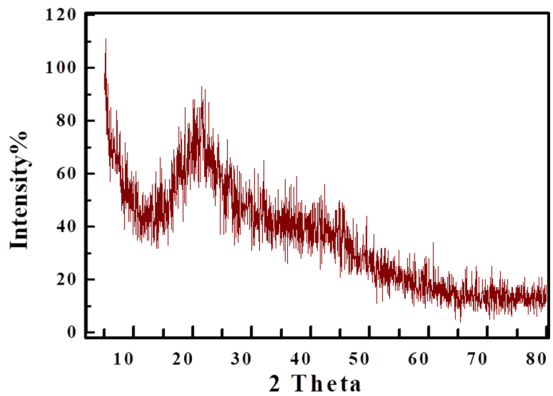

Fig. 2. XRD patern of PR-NPs.

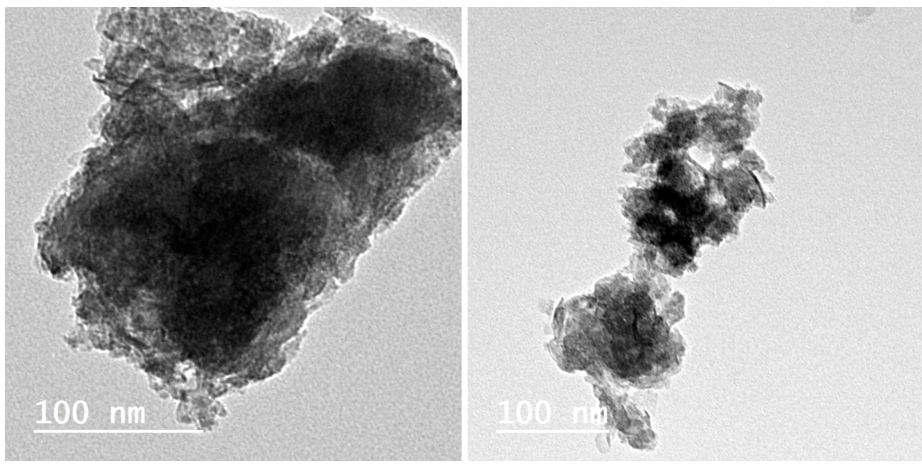

Fig. 3. HRTEM images of PR-NPs.

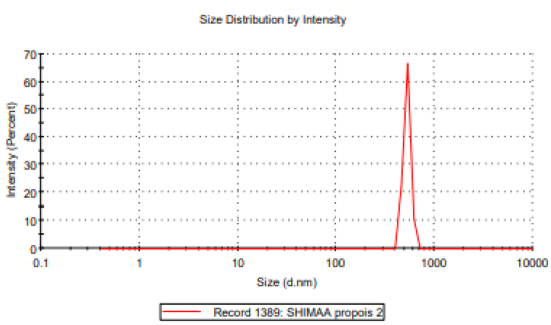

Fig. 4. Particle size of the prepared PR-NPs.

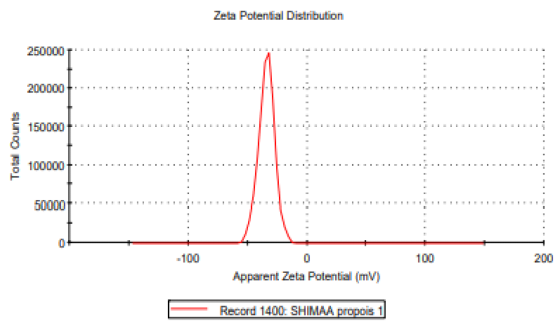

Fig. 5. Zeta potential prepared PR-NPs.

Immunological parameters of feeding *O. niloticus* at different concentration of PR and PR-NPs

Significant changes were seen in the IL-1, IgM, complement 5, and lysozyme of groups fed various PR and PR-NPs concentration throughout the study. After 28 days of feeding, they were elevated significantly ($P<0.05$) in groups fed various concentrations of PR and PR-NPs compared to the control (Table 4).

Stress indicators in the groups of *O. niloticus* fed different concentration of PR and PR-NPs

Table 4. Immunological parameters of *O. niloticus* fed different concentration of PR and PR-NPs

Tested compounds	Serum parameters	IL-1β (pg/ml)	IgM (ug/ml)	Complement	Lysozyme (ng/ml)
Propolis	1 gm PR/kg	4.95±0.005	1.8±0.05	0.84±0.04	5.87±0.06
	2 gm PR/kg	5.36±0.005	2±0.05	1.1±0.03	5.99±0.003
Propolis nanoparticles	1 gm PR-NPs/kg	5.77±0.008	2.3±0.08	1.34±0.05	6.32±0.12
	2 gm PR-NPs/kg	5.97±0.005	2.7±0.05	1.69±0.08	6.46±0.05
Control negative		4.52±0.005	1.4±0.05	0.55±0.04	5.15±0.1

The mean values of immunological parameters (mean± SE) of *O. niloticus* fed different concentration of PR and PR-NPs

Table 5. Effects of dietary PR and PR-NPs on the serum malondialdehyde (MDA) level of the experimental feeding groups

Tested compounds	Tested conc. (gm/kg feed)	MDA
Propolis crude	1.0	5.4920±0.48[a]
	2.0	4.2886±0.8[ab]
Propolis nanoparticles	1.0	5.1640±0.38[bc]
	2.0	2.7460±0.52[ab]
Control fish	NA	6.6180±0.47[c]

Data are expressed as mean ± SE (n = 3) with different superscript letters [(a,b,&c)] in the same column are significantly different at $P \leq 0.05$.

The serum obtained from groups fed 2 gm PR-NPs/kg feed had the highest anti-oxidant activity and was significantly different from the others. Furthermore, in comparison to the control, anti-oxidant activity was increased throughout all treated groups. The data, however, revealed significant differences between both the control group and the groups with PR and PR-NPs.

The GSH levels increased in all treated groups compared to the control, demonstrating a significant distinction between the experimental eating groups and the control. The greatest GSH levels were detected in the groups given 2 gm PR-NPs/kg feed. MDA levels in the treated groups' gills were significantly lower than in the control group. When compared to the control, the treated groups fed 2 gm of both the PR and PR-NPs recovered significantly. Cortisol levels decreased in all feeding groups when compared to control (Table 5 & 6)

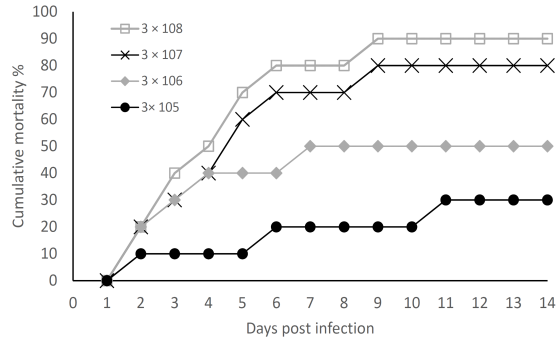

Fig. 6. Median lethal dose (LD_{50}) of *P. aeruginosa* in apparently healthy *O. niloticus*.

Prevention of *P. aeruginosa* infection in *O. niloticus* using PR and PR-NPs

1) The LD_{50} of *P. aeruginosa* in apparently healthy *O. niloticus*

In the initial week of the experiment, fish death occurred and the LD_{50} was 3×10^6 CFU/mL (Fig. 6).

2) Prevention of *P. aeruginosa* infection in *O. niloticus* using PR and PR-NPs

There was no mortality but 100% RPS was recorded in the groups fed the high concentrations of PR and in the group fed high and low concentrations of PR-NPs then injected with *P. aeruginosa* strain and

Table 6. Effects of dietary PR and PR-NPs on gills tissue homogenate total anti-oxidant and glutathione level of the experimental feeding groups of *O. niloticus*

Tested compounds	Tested conc. (gm/kg feed)	total anti-oxidant	Glutathione	Cortisol
Propolis	1.0	0.1909±0.002[c]	105.6571±5.4[c]	0.0256±0.002*
	2.0	0.2232±0.005[c]	113.4900±3.2[b]	0.017±0.0005*
Propolis nanoparticles	1.0	0.2353±0.001[b]	114.5344±4.1[b]	0.013±0.0005*
	2.0	0.2632±0.003[b]	136.5901±4.1[b]	0.009±0.0005*
Control group	NA	0.1827±0.006[a]	89.9417±3.2[a]	0.062±0.0008

Data are expressed as mean ± SE (n = 3) with different superscript letters [(a,b,&c)] in the same column are significantly different at $P \leq 0.05$.

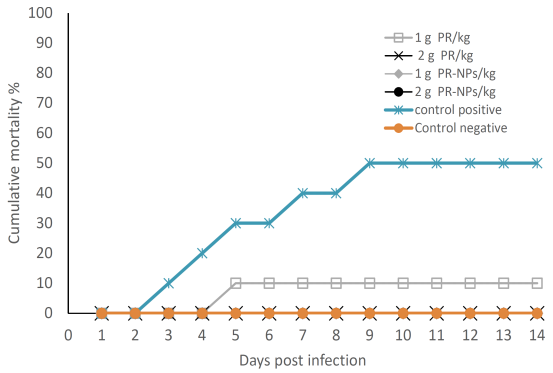

Fig. 7. Preventive effect of *O. niloticus* against *P. aeruginosa* infection using PR and PR-NP.

in the group fed a normal diet then injected with physiologic saline (control negative), respectively. However, a 10% mortality rate and 87.5 RPS were reported in the group of low concentrations of PR then injected with *P. aeruginosa* strain. On the other hand, a 50% mortality rate was found in the control positive group.

Histopathological findings

1) Control negative group

The histopathological examination revealed normal organization of the gill lamellae and filaments (Fig. 8A). The hepatopancreas showed a normal hepatic tissue architecture; normal central vein and surrounding hepatocytes, as well as pancreatic tissue (Fig. 8B). The spleen showed a normal histological picture of both red and white pulps as well as melanomacrophage centers (Fig. 8C). The intestine showed normal arrangement of all intestinal layers; mucosa, submucosa, muscularis and serosa (Fig. 8D). The kidney showed the normal histological structure of renal glomeruli and tubules (Fig. 8E).

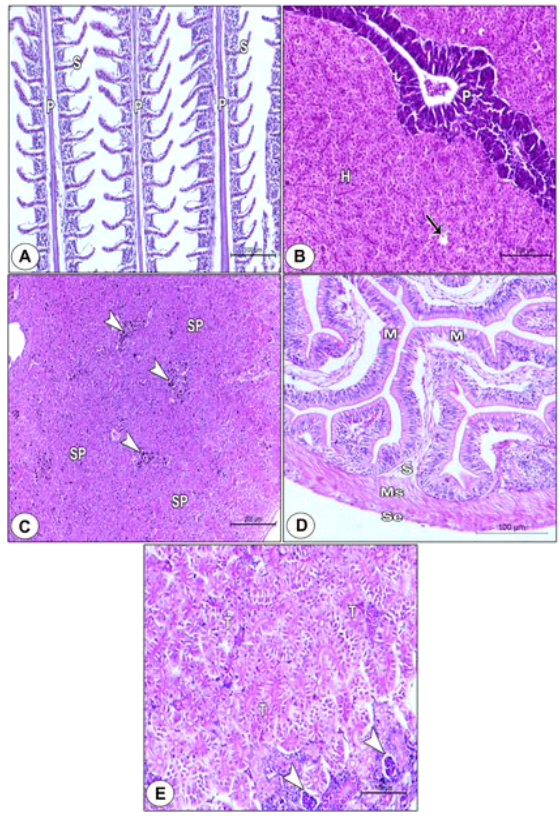

Fig. 8. Photomicrograph of control negative group of *O. niloticus* showing: A) Normal histological picture and arrangement of primary (P) and secondary (S) lamellae of gills. B) Normal histological structure of central vein (arrow), hepatocytes (H), and pancreatic acini (P) of hepatopancreas. C) Normal histological architecture of splenic pulps (SP) and melanomacrophage centers (arrowheads) of the spleen. D) Normal histological arrangement and picture of intestinal layers; mucosa (M), submucosa (S), muscularis (Ms), and serosa (Se). E) Normal histological structure of renal glomeruli (arrowheads) and tubules (T). H&E stain: scale bar; 100, 100, 200, 100 and 100, respectively.

2) Control positive group

The histopathological examination of the control positive group showed marked alternation in all examined organs (gills, liver, spleen, intestine, and kidney) in the form of degenerative and necrotic changes of most internal organs, as well as inflammatory cells infiltration. The gills showed severe gill damage; some gills' primary lamellae showed severe degenerative changes, the epithelial cells of some secondary lamellae showed hypertrophy and hyperplasia leads to fusion of the lamelle. (Fig. 9A) marked sloughing off and losses of some secondary lamellae were detected. The hepatopancreas showed degenerative

changes in the form of vacuolar degeneration (cytoplasmic vacuolization) and marked nuclear condensation as well as infiltration of mononuclear cells in the hepatic parenchyma (Fig. 9B). The splenic parenchyma showed an increase in the number and extension of melanomacrophage centers with lymphocytic depletion (Fig. 9C). The intestinal epithelium showed marked hyperplasia and massive mononuclear cells infiltration in the intestinal propria-submucosa (Fig. 9D). The kidneys showed marked degenerative

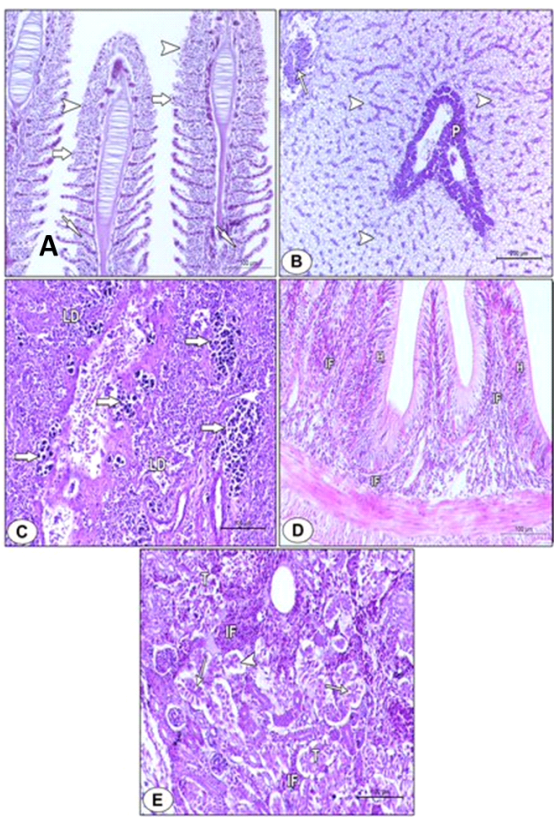

Fig. 9. Photomicrograph of control positive group of *O. niloticus* showing: A) Degenerative changes of the epithelial cells lining the primary lamellae (thin arrows), hypertrophy and hyperplasia of secondary lamellae (thick arrows), and sloughed secondary lamellae (arrowheads). B) Vacuolar degeneration of hepatocytes (arrowheads), mononuclear cells infiltration (arrow) and normal pancreatic acini (P). C) A large number of melanomacrophage centers (thick arrows) with lymphocytic depletion (LD) of splenic parenchyma. D) Marked hyperplasia (H) of the intestinal epithelium and mononuclear cells infiltration (IF) in the propria-submucosa. E) Degenerative changes (T) and sloughed off (arrows) of tubular epithelial cells as well as shrinkage of a renal glomerulus (arrowhead) and mononuclear cells infiltration (IF). H&E stain: scale bar; 100, 200, 100,100 and 100, respectively.

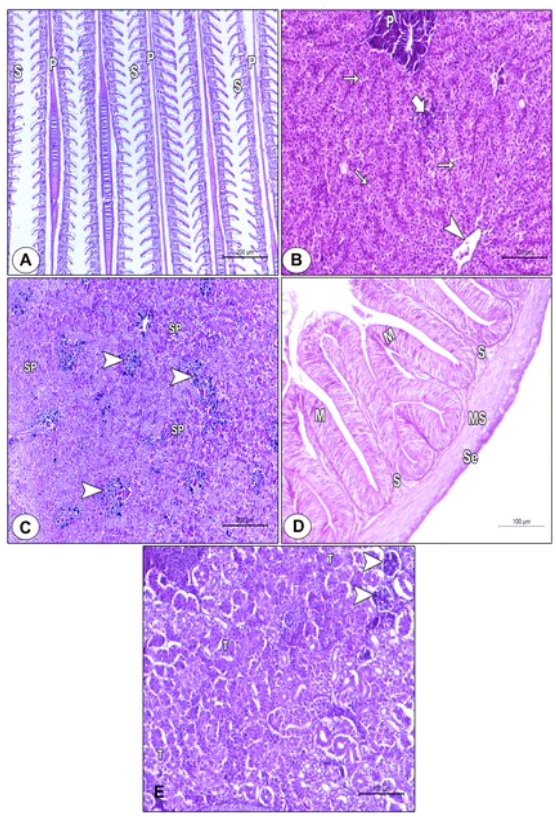

Fig. 10. Photomicrograph of a fish group of *O. niloticus* fed diet 2 (1 g PR/kg of feed) showing: A) Normal histological architecture of primary (P) and secondary (S) lamellae. B) Degeneration of hepatocytes (thin arrows), area of necrosis (thick arrow), dilated central vein (arrowhead), and normal pancreatic tissue (P) of the hepatopancreas. C) Normal histological picture of splenic parenchyma; splenic pulps (SP) and melanomacrophage centers (arrowheads). D) Normal histological architecture of all intestinal layers; mucosa (M), submucosa (S), muscularis (Ms), and serosa (Se). E) Normal histological structure of renal glomeruli (arrowheads) and tubules (T). H&E stain: scale bar; 200, 100, 200,100 and 100, respectively.

changes of the tubular epithelial cells in the form of vacuolization and necrosis. Some tubular epithelium was noticed sloughed off and lost. Degenerative changes of the endothelium lining the glomerular tuft, slight congestion of most blood vessels, and mononuclear cells infiltration were noticed (Fig. 9E).

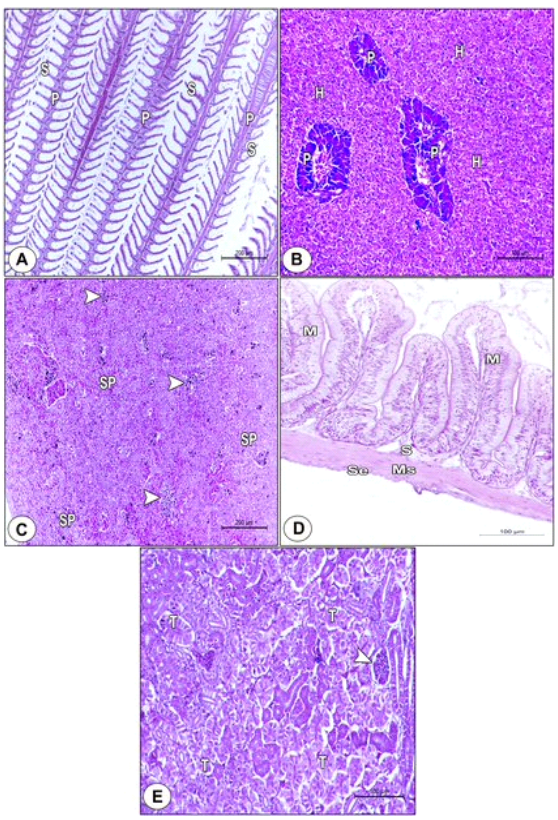

Fig. 11. Photomicrograph of a fish group of *O. niloticus* fed diet 3 (2 g PR/kg of feed) showing: A) Normal histological arrangement of primary filaments (P) and secondary lamellae (S). B) Normal histological picture of hepatocytes (H) and pancreatic acini (P) of hepatopancreas. C) Normal histological architecture of splenic parenchyma; pulps (SP) and melanomacrophage centers (arrowheads). D) Normal histological arrangement and picture of intestinal layers; mucosa (M), submucosa (S), muscularis (Ms), and serosa (Se). E) Normal histological structure of renal glomerulus (arrowhead) and tubules (T). H&E stain: scale bar; 200, 100, 200,100 and 100, respectively.

3) Fish group fed 1 gm PR/kg of feed

The gills showed normal gill lamellae and filaments (Fig. 10A). The hepatopancreas showed mild degenerative and necrotic changes of hepatocytes with dilatation of central veins and normal pancreatic tissues (Fig. 10B). The kidney, spleen, and intestine showed normal histological structure (Fig. 10C, D, and E).

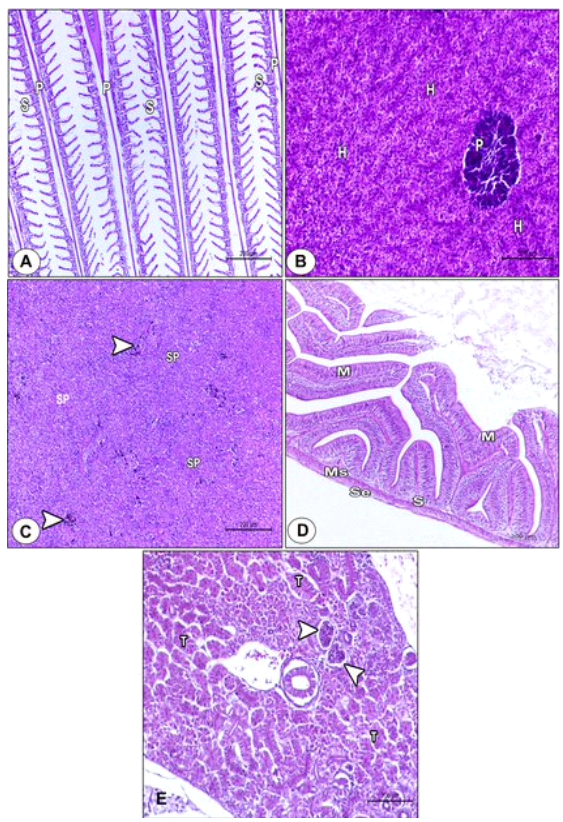

Fig. 12. Photomicrograph of a fish group of *O. niloticus* fed diet 4 (1 g of PR-NPs/kg of feed) showing: A) Normal histological picture of primary filaments (P) and secondary lamellae (S). B) Normal histological structure of hepatocytes (H) and pancreatic acini (P) of hepatopancreas. C) Normal histological architecture of melanomacrophage centers (arrowheads) and splenic pulps (SP). D) Normal histological arrangement of intestinal layers; mucosa (M), submucosa (S), muscularis (Ms), and serosa (Se). E) Normal histological structure of renal glomeruli (arrowheads) and tubules (T). H&E stain: scale bar; 200, 100, 200, 200 and 100, respectively.

4) Fish group fed 2 gm PR/kg of feed

The gills showed the normal histological structure of primary filaments and secondary lamelle that were situated perpendicular to the primary filaments (Fig. 11A). The hepatopancreas showed a normal picture of hepatic tissues and pancreatic acini (Fig. 11B). The spleen showed normal histological architecture of splenic pulps and melanomacrophage centers (Fig. 11C). The intestine showed a normal histological picture of all intestinal layers (Fig. 11D). The kidney showed normal renal tubules and glomeruli (Fig. 11D). Normal histological structure of renal glomerulus (Fig. 11E)

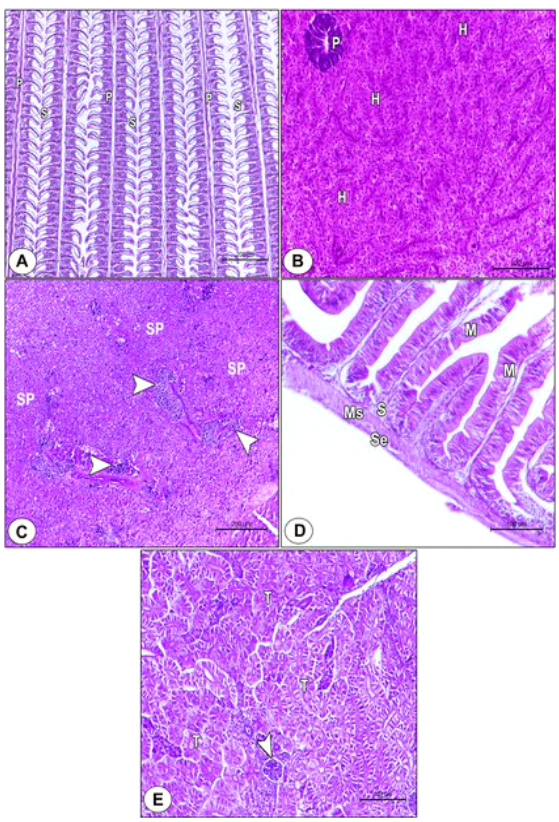

Fig. 13. Photomicrograph of fish group of *O. niloticus* fed diet 5 (2 g of PR-NPs/kg of feed) showing: A) Normal histological picture of primary (P) and secondary (S) lamellae. B) Normal histological structure of hepatocytes (H), and pancreatic acini (P) of hepatopancreas. C) Normal histological architecture of splenic pulps (SP) and melanomacrophage centers (arrowheads). D) Normal histological structure of intestinal layers; mucosa (M), submucosa (S), muscularis (Ms), and serosa (Se). E) Normal histological structure of renal glomerulus (arrowhead) and tubules (T). H&E stain: scale bar; 200, 100, 200, 100 and 100, respectively.

5) Fish group fed 1 g of PR-NPs/kg of feed

The gills, hepatopancreas, spleen, intestine, and kidney showed normal histological structures (Fig. 12A, B, C, D, and E).

6) Fish group fed 2 g of PR-NPs/ kg of feed

The gills, hepatopancreas, spleen, intestine, and kidney showed normal histological pictures (Fig. 13A, B, C, D, and E).

Discussion

The improvement of aquaculture feed is a significant step towards the establishment of successful and economic aquaculture production as feed represents the highest operational expense in the aquaculture industry (Ghanawi et al., 2011). In the current study, different concentrations of propolis and its nanoparticles were used as immune-stimulants, ant-stress and prevent *P. aeruginosa* infection in *O. niloticus*. Histopathological examination was used to assess the effects of these compounds on the fish organs.

In the current study, the chemical structures and characters of PR and its nanoparticles were studied. Propolis has more than 300 chemical constituents which have been identified in PR from different regions (Bankova et al 200; Xu et al 2009; Schmidt 1997). The Flavonoids, phenolics, and aromatic compounds are the main chemical substances present in PR which may be responsible for the bactericidal effects of PR against *P. aeruginosa* (Fatoni 2008; Sabir 2005). Although PR contains volatile oils, terpenes, and bee wax, these components are thought to play a minor role in the chemical properties and effects of the substances.

Regarding the FTIR spectra of PR, the peaks appear in the region 1019.75 cm^{-1} related to aromatic moieties of the flavonoids and Lipids. The band at 1633.21 cm^{-1} related to the stretching of O-H groups. Evidence of polyphenol present in the propolis is clearly visible. Thus, the presence of bands accordingly O-H at 3355.20 cm^{-1} and C-H band at 2923.37 cm^{-1} accurately confirm the polyphenol and ester moieties. The presence of terpenes is suggested from the weak absorbance of C=C at 1725 cm^{-1} (Olegário et al 2009).

Furthermore, the HRTEM view revealed that the PR-NPs had a small size (50 um) as a result of the milling procedure, which resulted in a greater antibacterial impact than PR. The electrostatic attraction or repulsion between particles in the liquid suspension of the PR-NPs was evaluated using the zeta potential, which is considered the most important parameter for describing the stability of PR-NPs in aqueous environments. Particles were considered stable in colloidal dispersions with the absence of steric stabilization when the zeta potentials of the particles are greater than +30 mV or lower than −30 mV (Kumar et al 2013). The zeta potential showed that the PR-NPs are most stable in an aqueous medium. The biomolecules presented in the PR-NPs surfaces consisted of negatively charged groups and are responsible for the more prominent stability. Previous studies had reported that cationic particles are toxic, whereas those with high negative zeta potential are safe and nontoxic (Mahmoud et al 2018).

Particle size distributions were determined for the prepared nanoparticles using DLS and were found to be 2119 nm. The hydrodynamic size reflects how the particles move in a liquefied form. The falling plane indicates how the material particles interact with each other, so the measured hydrodynamic (DLS) size of the particles was expected to be bigger than the partial size that was estimated using SEM or TEM. The particle translational diffusion coefficients will be contingent not only on the size of the particle "core," but also on any surface construction that will be effect the diffusion rapidity, as well as the type of ions in the intermediate and concentration.

Regarding the effect of PR and PR-NPs on the immune response of *O. niloticus*, the levels of IL-1β, IgM, complement 5, and lysozyme were significantly higher in the PR and PR-NPs when compared to the control group. These results were matched with the findings of Ümit et al (2018) who found that the non-specific immune response of Mozambique tilapia could be enhanced by dietary supplementation with PR and that the optimal level would be 2 $g\,kg^{-1}$. Abbass et al (2012) and Mohamed et al (2019) reported that the PR-extract showed an enhanced immune response for tilapia under cold stress. These effects of propolis are associated with its structure including its rich flavonoid, terpenoid, and phenolic acid content (Prytzyk et al. 2003; Tatli Seven et al. 2009, 2012).

In the current study, glutathione peroxidase and total anti-oxidant activities increased with the increasing levels of dietary PR and PR-NPs. The highest activity levels were observed in the group fed 2 gm PR-NPs/kg feed. For the MDA, a significant recovery was observed in the groups fed 2 gm of both PR and PR-NPs/kg feed when compared with the control. These results were supported by Enis et al. (2011) who demonstrated that pre-treatment, post-treatment, and simultaneous treatments with PR attenuated the oxytetracycline induced oxidative stress by significantly decreasing the levels of MDA in the tissues. In addition, the PR significantly increased the levels of reduced GSH, and the catalase, glutathione peroxidase, and superoxide dismutase activities as was previously reported by Shapour et al. (2013).

Propolis has anti-oxidant properties, and its effects are related to its structure, which includes rich flavonoids, terpenoids, and phenolic acids (Prytzyk et al. 2003; Tatli Seven et al. 2009, 2012). In the current study, the superior effect of PR-NPs may be related to its smaller size (50 nm), so they may be more easily

absorbed by the body (Sahlan et al. 2017). These results were supported by Abdel Mageid et al (2020), who reported that nano forms of propolis were more effective than PR as an anti-oxidant agent. Blood levels of cortisol are used mainly as stress indicator (Wendelaar Bonga 1997) because of the extreme sensitivity of the hypothalamo–pituitary–interrenal (HPI) axis. The increase in serum cortisol level can be seen as the sensitive signal of fish stress (Mommsen et al. 1999). In this study, plasma cortisol levels of all groups feeding PR and its nanoparticles were decreased than control negative. These results were supported by the findings of Šegvi´c-Bubi´ (2013). Mohamed et al (2019) and Abdelmagid et al (2022) The protective role of PR and its nanoparticles might be related to its antioxidant effect and the ability to control the peroxidation of unsaturated fatty acids, preventing the production of cholesterol, and subsequent formation of cortisol (Kitabchi 1967).

The dietary incorporation of high concentrations of PR and PR-NPs succeeded in better preventing *P. aeruginosa* infection with zero mortality rate and 100% RPS. However, a 10% mortality rate and 87.5 RPS were reported in the group fed low concentrations of PR then injected with *P. aeruginosa*. On the other hand, the positive control group showed 50% mortality rates. These results were augmented by the study of (Sforcin et al., 2000) who investigated the antimicrobial activity of PR against *P. aeruginosa.* Sabir (2005) reported that some constituents in PR could limit the bacterial enzyme RNA polymerase ability to attach to the DNA. Consequently, bacterial DNA replication does not occur. At the same time, important compounds of PR have the ability to prevent the action of the enzyme restrictive endonucleases that do not occur in RNA transcription ending with disrupted cell division. The bactericidal effects of PR may be related to flavonoids and its hydroxyl group. These results were confirmed by the findings of Fatoni (2008) and Sabir (2005) who confirmed that the hydroxyl group of flavonoids may be able to decrease toxic effects of bacteria. In the current study, the PR-NPS showed better effect for preventing of *P. aeruginosa* infection than low concentration of PR. The superior antibacterial activity of PR-NPs might be attributed to their smaller size (50 nm) which was obtained by milling procedure, as well as their stability as described by zeta potential in this study. As a result, PR-NPs may be more effective by enhancing PR dissolvability, allowing PR-NPs to be absorbed more easily by the body (Seven et al 2018). Seven et al (2018) added to these findings by indicating that PR-NPs may more easily penetrate bacteria's outer membrane, allowing active antibacterial chemicals to damage bacterial cell walls. Gonsales et al. (2006) and Afrouzan et al. (2012) found that PR-NPs were more effective against *S. aureus* than tetracycline antibiotics. Also, Mageid et al (2020) demonstrated that PR-NPs had higher significant effect on competing toxicity of *Microcystis aeruginosa* in *O. niloticus*. Hasan et al. (2014) reported that PR-NPs had more antibacterial effect than PR against *Bacilus subtilis, S. aureus, E. coli and Salmonella sp*.

The histopathological alterations of the control positive group may be attributed to the extracellular toxins produced by *P. aeruginosa* "enterotoxin, phytotoxic factor, proteolytic enzymes, exotoxins, hydrocyanic acid, phospholipase, and slime" (Magdy et al., 2014). Exotoxins elaborated by *P. aeruginosa* are considered the most important factor in its pathogenicity as they induced hepatic necrosis, hemorrhages, and necrosis of renal tubules (Pinghui, 1974 and Iglewski et al., 1977). Additionally, *O. niloticus* fish group fed diet 2 (1 g PR/kg of feed) then challenged intraperitoneally with the *P. aeruginosa* showed a normal histological picture of most internal organs and a marked decrease of hepatic degenerative changes. Whereas the fish groups fed on dietary PR-NPs (1 g and 2 g of PR-NPs/kg of feed), then challenged intraperitoneally with the *P. aeruginosa* showed a normal histological picture of all examined organs. The obtaining results showed that although

the natural form of propolis in low dose (1 g PR/kg of feed) gives an acceptable histopathological picture of most examined organs and minimizing the hepatopancreatic damage, the nanoform of propolis in both concentrations used (1 g and 2 g of PR-NPs/kg of feed) were more effective as hepatoprotective substance. The hepatoprotective activities of PR may be due to its adverse pharmacological properties (Olczyk et al. 2013) and its phenolic compounds that have a hepatoprotective function which is attributed to their antioxidant activities (Banskota et al., 2001). Whereas the more effectiveness of PR-NPs may be attributed to their small-sized particles which induced a good absorbance (Abdelmagid et al., 2019) and has more effective antibacterial activity than propolis (Afrouzan et al. 2012). Moreover, propolis have the tendency to return the plasma ammonia concentration to its normal level due to its contents of natural antioxidants, flavonoids, and phenolic compounds that have the efficiency to remove the excess ammonia (Essa et al., 2006 and Radwan et al., 2008). Also, Banskota et al., 2001 reported that administration of propolis reduces the hyperammonemia to its normal level and attributed the anti-hyperammonemia activity of propolis to the antioxidant capacity of its polyphenolic and flavoniod compounds results in the prevention of oxidative stress (Lemarié et al., 2004), and so reduce the opportunity of *P. aeruginosa* infection results in normal histopathological pictures of examined organs understudy especially when PR-NPs used which have a smaller size and more easily absorbed (Sahlan et al. 2017).

Conclusion

Supplementation of fish diet with either PR or PR-NPs may promise a beneficial effect for aquaculture due to its potential improving effect on immune-response, reducing stress indicators and succeeded in preventing of *P. aeruginosa* infection in *O. niloticus*. The highest effect was found in groups fed PR-NPs which succeeded in better prevention of *P. aeruginosa* infection with no mortality and histopathological alterations compared to a 10% mortality rate and mild histopathological alterations in the traditional PR-fed group and 50% mortality rate with severe histopathological changes in the positive control group.

Declarations

Funding

No funding was received.

Conflict of Interest

The authors declare that they have no conflict of interest.

Availability of data and material:- all the data are involved in the main manuscript and are available from the corresponding author, upon reasonable request.

Code availability (software application or custom code):- not applicable

References

Abdel mageid A. D.; Adel A. Shaheen; Nahed Sh. Gad, Rania M. Zahem, 2020. Ameliorative effect of propolis and nanopropolis supplementation against oxidative stress induced by *Microcystis aeruginosa* in *Oreochromis niloticus*. Egyptian Journal of Aquatic Biology & Fisheries. 24(1): 573-582.

Abdelmagid AD, Said AM, Gawad EAA, Shalaby SA, Dawood MAO, 2022. Propolis nanoparticles relieved the impacts of glyphosate-induced oxidative stress and immunosuppression in Nile tilapia. Environ Sci Pollut Res Int. 2022 Mar; 29(13): 19778-19789. doi: 10.1007/s11356-021-17201-2. Epub 2021 Oct 31. PMID: 34718976.

Abdelmagid, A. Shaheen, N. Gad andR. Zahem. 2019. Biochemical and histopathological effect of propolis and nanopropolis supplementation on alleviating dietary *Microcystis aeruginosa* toxicity on Nile tilapia, *Oreochromis niloticus*. Benha Veterinary Medical Journal, 36(2): 150-160.

Abd-El-Rhman, A. M. M. Antagonism of Aeromonas hydrophila by propolis and its effect on the perform-

ance of Nile tilapia, Oreochromis niloticus. Fish Shellfish Immunol., 27(3): 454-459 (2009). doi:10. 1016/j.fsi.2009.06.015.

Acar Ü., (2018). Effects of diet supplemented with ethanolic extract of propolis on growth performance, hematological and serum biochemical parameters and disease resistance of Mozambique tilapia (*Oreochromis mossambicus*) against Streptococcus iniae. Aquaculture, 495, 339-344.

Afrouzan H, Amirinia C, Mirhadi SA, Ebadollahi A, Vaseji N, Tahmasbi G., 2012. Evaluation of antimicrobial activity of propolis and nanopropolis against *Staphylococcus aureus* and *Candida albicans*. Afr J Microbiol Res. 6: 421-425.

Aldemir, O. S., Z. Selamoglu, M. F. Gulhan, O. Cakir, I. Ozdemir, S. D. Dastan, and H. Dogan. Role of propolis on oxidative stress in various tissues of fish. Fresenius Envir. Bull., 23(12C): 3547-3551 (2014).

Amend, D. F., 1981. Potency testing of fish vaccines. International symposium on fish Biologics: Serodiagnostics and Vaccines. Developmental biology. 49, 447-454. Aquaculture, 310, 395-400.

Atle Foss, Albert K. Imsland, Bjørn Roth, Edward Schram, Sigurd O. Stefansson, 2009. Effects of chronic and periodic exposure to ammonia on growth and blood physiology in juvenile turbot (*Scophthalmus maximus*). Aquaculture, 296 (1-2) 45-50,ISSN 0044 8486,https://doi.org/10.1016/j. Aquaculture. 2009.07.013.

Bankova, V. S., Castro, S. L., Marcucci, M. C., 2000. "Propolis: recent advances in chemistry and plant origin," Apidologie, 31(1): 3-15.

Banskota, A.H., Tezuka, Y., Adnyana, I.K., Ishii, E., Midorikawa, K., Matsushige, K., Kadota, S. 2001. Hepatoprotective and anti-Helicobacter pyloriactivities of constituents from Brazilian propolis. Phytomedicine 8(1): 16-23.

Beutler E., Duron O., Kelly M. B., 1963. Effects of ammonia stress, dietary linseed oil and Edwardsiella ictaluri challenge on juvenile darkbarbel catfish *Pelteobagrus vachelli*. J lab Clin. Med., 61,882.

Carrasco, G.A., & Van de Kar, L.D. (2003). Neuroendocrine pharmacology of stress. European Journal of Pharmacology, 3: 272-364.

De Aguiar, S.C., Zeoula, L.M., Franco, S.L. 2013. Antimicrobial activity of Brazilian propolis extracts against rumen bacteria in vitro. World J Microbiol Biotechnol 29: 1951-1959. https://doi.org/10.1007/s11274-013-1361

El-Gendy, M., Gouda, A., and Shehab El-Din, M. 2015. Effect of zeolite on feeding rates and growth performance for Nile tilapia (Oreochromis niloticus). International Journal of Scientific Essa, M.M., Subramanian, P., 2006. Pongamia pinnata modulates oxidant antioxidant imbalance during hyperammonemic rats. Fund. Clin. Pharm., 3: 299-303. search in Agricultural Sciences, 2: 18-24.

Elgohary, J., Abd Elatief, N. G. F., Alaa E. E., Mahmoud A. M., 2020. Pathological, bacteriological and seasonal prevalence of Aeromonas hydrophila, vibrio vulnificus, Proteus vulgaris and Pseudomonas aeruginosa; infecting Oreochromis niloticus in some Egyptian fish farms Islam. Egyptian Journal of Aquatic Biology & Fisheries 24(5): 467-482.

El-Sayed, A.F.M., 2006. Tilapia culture. CABI.

Engler, P., Pierre Caillis, Samue Voller, Laura Labrie, 2018. Dietary Supplementation of a Mixture of Saponin-Rich Plants to Reduce Ammonia-Nitrogen Excretion in Juvenile Nile Tilapia (Oreochromis niloticus). Sustainable Food Production. 2: 6-12.

Enis M. Yonar , Serpil Mişe Yonar, Sibel Silici., 2011. Protective effect of propolis against oxidative stress and immunosuppression induced by oxytetracycline in rainbow trout (Oncorhynchus mykiss, W.). Fish Shellfish Immunol. Aug;31(2):318-25. doi: 10.1016/j.fsi.2011.05.019. Epub 2011 May 30.

FAO 2009. The State of World Fisheries and Aquaculture 2008. FAO, Rome.

Fatoni A., 2008. Effect of propolis Trigona spp. Bukittinggi against several bacterial the small intestine of cattle and searches active component. Bogor (Indonesia): Bogor Agricultural University.

Ferreira, D., H. C. Rocha, L. C. Kreutz, V. L. Loro, A. Marqueze, G. Koakoski, J. G. S. da Rosa, D. Gusso, T. A. Oliveira, M. S. de Abreu, and L. J. G. Barcellos. Bee products prevent agrichemical-induced oxidative damage in fish. PLoS One, 8(10): 11 (2013). doi:10.1371/journal. pone.0074499.

Funakoshi-Tago M, Okamoto K, Izumi R, Tago K, Yanagisawa K, Narukawa Y, Kiuchi F, Kasahara T, Tamura H. 2015. Antiinflammatory activity of flavonoids in Nepalese propolis is attributed to inhibition of the IL-33 signaling pathway. Int Immunopharmacol. 25: 189-198.

GAFRD (General Authority for Fish Resources Development) 2012. Fish statistics year book. Egyptian Ministry of Agriculture. 89 pp.

Ghanawi, J., Roy, L., Davis, D.A., Saoud I.P., 2011. Effects of dietary lipid levels on growth performance of marbled spinefoot rabbitfish Siganus rivulatus

Gul Baykalir B, Tatli Seven P, Gur S, Seven I., 2016. The effects of propolis on sperm quality, reproductive organs and testicular antioxidant status of male rats treated with cyclosporine-A. Anim Reprod. 13: 105-111.

Guo Honghui, Lin Wang, Hou Jie, Wang Lingkai, Zhang Dandan, Wu Xueyang, Li Li, Li Dapeng , 2018.The Protective Roles of Dietary Selenium Yeast and Tea Polyphenols on Growth Performance and Ammonia Tolerance of Juvenile Wuchang Bream (Megalobrama amblycephala). Frontiers in Physiology. 9: 1371.

Kitabchi, A.E. (1967) Ascorbic acid in steroidogenesis. Nature, 215: 1385-1386.

Hasan, A.E.Z.; Artika, I.M.; Fahri, V.R.; Sari, N. 2012. Application of nanoparticle technology for medicine preparate (Antibiotic base on natural product, Propolis Trigona spp.). Chem. Prog. 5: 1-7.

Huang S., Ling Wang , Lianmeng Liu, Yuxuan Hou, Lu Li., 2015. Nanotechnology in agriculture, livestock, and aquaculture in China. A review. Agron. Sustain. Dev. 35: 369-400 DOI 10.1007/s13593-014-0274-x.

Iglewski, B.H.; Liu, P.V. and Kabat, D. (1977). Mechanism of action of Pseudomonas aeruginosa exotoxin Aiadenosine diphosphate-ribosylation of mammalian elongation factor 2 in vitro and in vivo. Infection and immunity. 15(1): 138-144.

Jesús Alberto de la Cruz-Cervantes, Flaviano Benavides-González, Jesús Genaro Sánchez-Martínez, María de la Luz Vázquez-Sauceda, Antonio Joel Ruiz-Uribe, 2018. Propolis in Aquaculture: A Review of Its Potential, Reviews in Fisheries Science & Aquaculture, 26:3, 337-349, DOI: 10.1080/23308249.2018. 1424798.

Kanbur, M., Eraslan, G. & Silici, S., 2009. Antioxidant effect of propolis against exposure to propetamphosin rats. Ecotox Environ Safe, 72: 909-915.

Koracevic, D., Koracevic, G., Djordjevic, V., Andrejevic, S., Cosic, V., 2001. Method for the measurement of antioxidant activity in human fluids, J. Clin. Pathol. 54(5): 356-361.

Kumar, K. M.; Mandal, B. K.; Kumar, K. S.; Reddy, P. S.; Sreedhar, B., 2013. Biobased green method to synthesise palladium and iron nanoparticles using Terminalia chebula aqueous extract. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 102: 128-133.

Lemarié G, Dosdat A, Covès D, Dutto G, Gasset G, Person-Le Ruyet J., 2004. Effect of chronic ammonia exposure on growth of European seabass (Dicentrarchus labrax) juveniles. Aquaculture; 229: 479e91.

Lia,M. , Na Yua, Jian G. Qinc, Erchao Lia, Zhenyu Dua, Liqiao Chena, 2014.

Li M, Yu N, Qin JG, Li E, Du Z, Chen L. Effects of ammonia stress, dietary linseed oil and Edwardsiella ictaluri challenge on juvenile darkbarbel catfish Pelteobagrus vachelli. Fish Shellfish Immunol. 2014 May; 38(1): 158-65. doi: 10.1016/j.fsi.2014.03.015. Epub 2014 Mar 19. PMID: 24657724.

Liu, L., Gao, Z.Y., Su, X.P., Chen, X., Jiang, L., Yao, J.M. 2015. ACS Sustainable Chemistry & Engineering 3, 432.

Lowther, A., 2005. Highlights from the FAO database on aquaculture production statistics. FAO Aquaculture Newsletter, 33: 22-24.

Magdy, I.; El-Hady, M.; Ahmed, H.; Elmeadawy, S. & Kenwy, A. (2014). A contribution on *Pseudomonas aeruginosa* infection in African catfish (*Clarias gariepinus*). Res. J. Phar. Biol. Chem. Sci., 5(5): 575-588.

Mahmoud, R., Kotp, A. A., El-Ela, F. I. A., Farghali, A. A., Moaty, S. A., Zahran, H. Y., & Amin, R. (2021). Green synthesis of Iron Nanoparticles of Clove and Green Coffee origin with an in vivo Hepatoprotective Investigation. Journal of Environmental Chemical Engineering, 106320.

Marcaida G, Felipo V, Hermenegildo C, Minana MD, Grisolia S., 1992. Acute ammonia toxicity is mediated by NMDA type of glutamate receptors. FEBS Lett. 296:67e8.

Ming Li a,b , Na Yu a, Jian G. Qin c , Erchao Li a, Zhenyu Du a , Liqiao Chen a 2014. Fish& shell fish immunology 38;158-165.

Moaty SA, Farghali A, Moussa M, Khaled R, 2017. Remediation of waste water by Co–Fe layered double hydroxide and its catalytic activity. J Taiwan Inst Chem Eng 71: 441-453.

Mohamed S. Hassaan, Ayman G. EL Nagar, Heba S. Salim, Kevin Fitzsimmons, Ehab R. El-Haroun, 2019. Nutritional mitigation of winter thermal stress in Nile tilapia by propolis-extract: Associated indicators of nutritional status, physiological responses and transcriptional response of delta-9-desaturase gene, Aquaculture,Volume 511,2019,734256,ISSN 0044-8486, https://doi.org/10.1016/j.aquaculture.2019.734256.

Mommsen, T.P., Vijayan, M.M., Moon, T.W. (1999). Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Reviews in Fish Biology and Fisheries, 9(3): 862-112.

Nguyen, M., N.H., Thodesen, J. and Gjerde, B., 2007. Genetic improvement of farmed tilapias: composition and genetic parameters of a synthetic base population of *Oreochromis niloticus* for selective breeding. Aquaculture, 273(1), pp.1-14.

Noor El-Deen A.E., Sohad M.D., Azza H.M. H. and Hakim A.S. 2010. Studies on Aeromonas hydrophila in Cultured Oreochromis niloticus at Kafr El Sheikh Governorate, Egypt with Reference to Histopathological Alterations in Some Vital Organs. World Journal of Fish and Marine Sciences 6(3): 233-240.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, Anal. Biochem. J. 95(2): 351-358.

Olegário LS, Andrade JKS, Andrade GRS, Denadai M, Cavalcanti RL, da Silva MAAP, Narain N., 2019. Chemical characterization of four Brazilian brown propolis: An insight in tracking of its geographical location of production and quality control. Food Res Int. Sep;123,481-502. doi: 10.1016/j.foodres.2019.04.004. Epub 2019 May 13. PMID: 31284998.

Omnia M. A, Nabila M.A., Nadia R.R., 2014. Biochemical effects of propolis and bee pollen in experimentally- induced hyperammonemia in rats. BENHA veterinary medical journal. 27(1): 8-24.

Olczyk, P.; Wisowski, G.; KomosinskaVassev, K.; Stojko J.; Klimek K.; Olczyk, M. and Kozma, E.M. 2013: Propolis modifies collagen types I and III accumulation in the matrix of burnt tissue. Evid. Based Comp. Alternat. Med 2013: 423809.

OZTURK , H., Mert PEKCAN, Meltem SIRELI, Ulvi Reha FIDANCI, 2014. Effects of propolis on in vitro rumen microbial fermentation. Ankara Üniv Vet Fak Derg, 57: 217-221.

Pinghui V. Liu (1974): extracellular Toxins of Pseudomonas aeruginosa, Journal of Infectious Diseases, Volume 130,Issue Supplement Pp. S94-S99.

Prytzyk E, Dantas AP, Salom~ao K, Pereira AS, Bankova VS, De Castro SL, Aquino Neto FR., 2003. Flavonoids and trypanocidal activity of Bulgarian propolis. J Ethnopharmacol. 88: 189-193.

Quaiyum MA, Jahan R, Jahan N, Akhter T, Sadiqul IM 2014. Effects of Bamboo Charcoal Added Feed on Reduction of Ammonia and Growth of Pangasius hypophthalmus. J Aquac Res Development 5, 269. doi:10.4172/2155-9546.1000269

Quaiyum MA, Jahan R, Jahan N, Akhter T, Sadiqul IM, 2014. Effects of Bamboo Charcoal Added Feed on Reduction of Ammonia and Growth of Pangasius hypophthalmus. J Aquac Res Development 5, 269. doi:10.4172/21559546.1000269.

Radwan, R.R., Shaban, E.A., Kenawy, S.A. 2008. Hepatoprotective Efficiency of Combined Administration of Natural Antioxidants (Rutin and Vitamin E) and Cysteine in Hyperthermic Irradiated Rats. Egyptian J. Hospital Medicine, 32: 441-454.

Rattanachaikunsopon, P., Phumkhachorn, P., 2010. Assessment of synergistic efficacy of carvacrol and cymene against Edwardsiella tarda in vitro and in Tilapia (Oreochromis niloticus). African Journal of Microbiology Research. 4: 420-425.

Rossolini G.M., Mantengoli E., 2005. Treatment and control of severe infections caused by multiresistant Pseudomonas aeruginosa, Clinical Microbiology and Infection, 11(4): 17-32, ISSN 1198-743X, https://doi.org/10.1111/j.1469-0691.2005.01161.x.

Sabir A., 2005. The inflammatory response on rat dental pulp following ethanolic extract of propolis (EEP) application. Maj Ked Gigi (Dent J). 38: 77-83.

Sahlan M, Dienayati D, Hamdi D, Zahra S, Hermansyah H, Chulasiri M., 2017. Encapsulation process of propolis extract by casein micelle improves sunscreen activity. Makara J Technol. 21: 1-6.

Santacruz-Reyes AR, Chien Y-H. 2012. The potential of Yucca schidigera extract to reduce the ammonia pollution from shrimp farming. Biores Technol. 113, 311-4. doi: 10.1016/j.biortech.2012.02.132

Segvic-Bubic, T., J. Boban, L. Grubisi c, Z. Trumbic, M. Radman, M. Percic, and R. Coz Rakovac. Effects of propolis enriched diet on growth performance and plasma biochemical parameters of juvenile European sea bass (Dicentrarchus labrax L.) under acute low temperature stress. Aquacult. Nutr., 19(6): 877-885 (2013). doi:10.1111/ anu.12032.

Schmidt, J. O., 1997 "Chemical composition and application," in Bee Products－Properties, Applications, and Apitherapy, A. Mizrahi and Y. Lensky, Eds., pp. 15–26, New York, New York, NY, USA.

Schnitzler P, Neuner A, Nolkemper S, Zundel C, Nowack H, Sensch KH., 2010. Antiviral activity and mode of action of propolis extracts and selected compounds. Phytother Res. 2 Šegvi´c-Bubi´c, T.; Boban, J.; Grubiši´c, L.; Trumbi´c, Z.; Radman, M.; Per˘ci´c, M.; Což-Rakovac, R. Effects of propolis enriched diet on ˘ growth performance and plasma bio-chemical parameters of juvenile European sea bass (Dicentrarchus labrax L.) under acute low temperature stress. Aquac. Nutr. 2013, 19, 877-885. 4: 20-28

Seven I, Tatli Seven P, Silici S., 2011. Effects of dietary

Turkish propolis as alternative to antibiotic on growth and laying performances, nutrient digestibility and egg quality in laying hens under heat stress. Rev Med. 162: 186-191.

Seven P. T., Ismail Seven , Selcan Karakus, Seda Iflazoglu Mutlu , Gozde Arkali , Yesim Muge Sahin, Ayben Kilislioglu 2020. Turkish Propolis and Its Nano Form Can Ameliorate the Side Effects of Cisplatin, Which Is a Widely Used Drug in the Treatment of Cancer. Plants, 9: 1075.

Seven, P. T., 2008. The effects of dietary Turkish propolis and vitamin C on performance, digestibility, egg production and egg quality in laying hens under different environmental temperatures. Asian-Australas. J. Anim. Sci., 21(8): 1164-1170. doi:10.5713/ajas. 2008.70605.

Sforcin, J.M., Fernandes, A., Jr., Lopes, C.A., Bankova, V., Funari, S.R., 2000. Seasonal effect on Brazilian propolis antibacterial activity. J. Ethnopharmacol. 73: 243-249.

Shalaby, M. A., Mohamed k. Khames2 , Adel Fathy1, Amany A. Gharieb1 , Eman A. Abdel-Hamid 2021. The Impact of Zeolite on Ammonia Toxicity, Growth Performance and Physiological Status of the Nile Tilapia (Oreochromius niloticus). Egyptian Journal of Aquatic Biology & Fisheries, 25(1): 643-663.

Shapour Kakoolaki, Zeliha Selamoglu Talas, Oguz Cakir, Osman Ciftci , Ilknur Ozdemir, 2013. Role of Propolis on Oxidative Stress in Fish Brain. Basic and Clinical neuroscience. 4(2): 153-158.

Shin, K.W., Kim, SH., Kim, JH., 2016. Toxic effects of ammonia exposure on growth performance, hematological parameters, and plasma components in rockfish, Sebastes schlegelii, during thermal stress. Fish Aquatic Sci 19, 44. https://doi.org/10.1186/s41240-016-0044-6

Shokri H, Katiraee F, Fatahinia M, Minooeianhaghighi MH. 2017. Chemical composition and antifungal potential of Iranian propolis against Candida krusei strains. J Apicult Res. 56: 581-587.

Siddik, M.A.B., Nahar, A., Ahamed, F. and Hossain, M., 2014. Over-wintering growth performance of mixed-sex and mono-sex Nile tilapia Oreochromis niloticus in the northeastern Bangladesh. Croatian Journal of Fisheries, 72(2): pp.70-76.

Talbot C. and Hole R. 1994. Fish diets and the control of eutrophication resulting from aquaculture. J Applied Ichth 10: 258-270.

Tatli Seven P, Yilmaz S, Seven I, Cerci IH, Azman MA, Yilmaz M., 2009. Effects of propolis on selected blood indicators and antioxidant enzyme activities in broilers under heat stress. Acta Vet Brno. 78: 75-83.

Tatli Seven P, Yilmaz S, Seven I, Tuna Kelestemur G., 2012. The effects of propolis in animals exposed oxidative stress. In: Volodymyr L, editor. Oxidative stress: environmental induction and dietary antioxidants. Croatia: InTECH; p. 267-288.

Thomas J, Thanigaivel S, Vijayakumar S, Acharya K, Shinge D, Seelan TSJ, Mukherjee A, Chandrasekaran N., 2014. Pathogenicity of Pseudomonas aeruginosa in Oreochromis mossambicus and treatment using lime oil nanoemulsion. Colloids Surf B: Biointerfaces 116: 372-377.

Trenzado CE, Morales AE, Palma JM, Higuer M., 2009. Blood antioxidant defenses and hematological adjustments in crowded/uncrowded rainbow trout (Oncorhynchus mykiss) fed on diets with different levels of antioxidant vitamins and HUFA. Aquaculture. 149: 440e7.

Xu, Y., Luo, L., Chen, B. and Fu, Y.,2009. “Recent development of chemical components in propolis,” Frontiers of Biology in China, 4(4): 385-391.

Manuscript Received : Jan 15, 2023
Revised : Mar 04, 2023
Accepted : Apr 08, 2023