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Effect of propolis extract as feed additive on the growth performance and gonadal histology of red tilapia *Oreochromis niloticus* x *O. mossambicus*

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Abstract
The present study was conducted to evaluate the effect of propolis extract as feed additive in the diets at 3, 6 and 12 mL kg^{-1} of feed on the performance, hematological parameters and testes histology of red tilapia, *Oreochromis niloticus* x *O. mossambicus* brood stock. The results performed that there were significant differences ($P<0.05$) among all treatments in terms of final weight, weight gain, specific growth rate and relative growth rate. Fish fed D1 gained the highest final body weight and fish fed D4 gained the lowest value of final body weight. The same trend was observed with weight gain, specific growth rate and relative growth rate. Feeding propolis supplemented diets did affect feed conversion ratio during the feeding trial. Fish fed D4 recorded the lowest FCR compared with the control diet (D1). Propolis as feed additive did not affect the survival rate of the experimental fish. Concerning the hematological parameters, fish fed D3 recorded the highest value of the hemoglobin and red blood cells compared with the control diet (D1). Propolis significantly ($P<0.05$) decreased the serum glucose, urea and increased the serum alanine aminotransferase (ALT). Fish fed D3 and D4 gained the lowest values of glucose and urea. Also, fish fed D4 recorded the higher ALT value compared with the control diet (D1). Also, there were no significant differences in the albumin, total protein and alkaline phosphatase of the serum among all treatments. Sections of testes from fish fed propolis supplemented diets showed seminiferous tubules impacted by spermatozoa compared with the control diet. It can be concluded that the propolis extract can be used as feed additive in the diets for red tilapia, *Oreochromis niloticus* x *O. mossambicus* brood stock up to 6 mL kg^{-1} without any adverse effects.

Keywords: propolis, feed additive, red tilapia, histology

1. Introduction
The intensive culture of tilapia is increasing and it is necessary to face the demand of sufficient quantities of good quality fry. Brood stock management is needed for mass production of fry [1]. It is necessary to find natural products from plant and/or animal products to enhance the performance of fish as high nutritional value useful for human health to develop the fish production without adverse effects on human health [2].
Several studies were performed to study using of natural products from the animal or plant source as feed additives in different types as anti-microbial and anti-oxidants to improve the feed utilization and the performance of animal production such as propolis [3, 4], immunostimulant [5] and hepatoprotective agent [6]. Most of certain natural food ingredients are safer and better than synthetic ones because a lot of these compounds, such as plant phenolic, may be used as an antioxidant and anti-microbial substrate.
Propolis is a resinous mixture that honey bees produce by mixing saliva and bee wax with exudate gathered from botanical sources and is used to build, adapt of their nests, and protects the entrance against intruders [7]. Also, Propolis color varies from green, red to dark brown. It has gained much more attention for its anti-bacterial [8], and anti-carcinogenic properties [9].
It was found that propolis is a mixture contains about 50–70% resins and 10% essential oils, from plant source, mixed with 30–50% wax for appropriate stability and 5–10% pollen, increased from being transported in the bees 'pollen baskets [10]. A chemical analysis of propolis stated the presence of bioflavonoids, some vitamins as B1, B2, B6, C, E, and minerals as manganese, iron, calcium, aluminum and vanadium. Also, the effect of propolis on the cytoplasmic membrane and the inhibition of enzyme activity as well as bacterial motility was indicated [11, 12].

The present study was investigated to examine the effect of propolis as feed additive in the diets for red tilapia *Oreochromis niloticus* x *O. mossambicus* brood stock on the performance, body composition, blood parameters and gonads histology.

2. Materials and Methods

2.1. The extraction of Propolis

Propolis extract was prepared following the method described by Cuesta *et al*. 2005 [13]. The sample of propolis was collected from bee units at Gharbia governorate bee farm. 10 ml of Absolute ether per g crude propolis was used for extraction, kept in air sealed bottles which were continuously shaken in darkness for 24 h at ordinary room temperature. The extract was filtered twice, dried under vacuum and finally stored in pervious bottles at 4°C until use. The extractions were added to the diet during the cooling stage of formulation process to avoid the heating effect.

2.2. The experimental diets

The formulated basal diet containing 30% crude protein and 426.9 kcal/kg dry matter, gross energy. All ingredients were first ground to a small particle size (approximately 250 mm). Four experimental diets were formulated including the control as basal diet (D1) without any supplementation, followed by three diets supplemented with propolis at 3, 6 and 12 mL kg^{-1} (D2, D3 and D4, respectively). The proximate compositions of feed ingredients are given in Table 1. Diets ingredient were passed through a mincer 35 mm diameter like spaghetti strands, air dried and stored in airtight containers at 5º C until fed or analyzed for the chemical composition.

2.3. The experimental design

Red tilapia, *Oreochromis niloticus* x *O. mossambicus* brood were obtained from a local fish hatchery (21 kilo Marriott, Alexandria Governorate, Egypt). Fish were acclimated to laboratory conditions for one week in fiber glass tank 1000 L. At the beginning of the experiment, twelve outdoor concrete tanks 1 cubic meter were each stocked with 20 fish male with an initial average weight 44±0.1 g. The experimental diets were fed to triplicate groups of fish two times at a rate of 2% of body weight for 56 days. Each group of fish was weighed at the beginning and after every 2 week throughout the experimental period. Water temperature and dissolved oxygen were measured every other day using a YSI Model 58 oxygen meter. Parameters averaged (± SD): water temperature, 27.1 ± 0.5 °C: dissolved oxygen, 6.5 ± 0.5 mg $^{-l}$.

2.4. Analytic procedures

The proximate analysis of the diets and fish carcass of crude protein, moisture, and ash were performed by standard procedures [14]. By the end of the feeding trial, all fish were counted and weighed to calculate the final weight using digital scale to the nearest 0.1, total weight gain (TWG; (final body weight – initial body weight), relative growth rate RGR; 100 x (average final weight/ average initial weight), specific growth rate (SGR; [ln final BW – ln initial BW] × 100/days), feed conversion ratio (FCR; dry feed consumed/WG, protein efficiency ratio (PER; WG/protein intake), and survival ([no. of fish at the end of the experiment/no. of fish at the beginning of the experiment] × 100).

2.5. Blood sampling

At the end of the feeding trial blood samples were taken from three fish per tank. Fish were euthanized with Clove oil at 30 mg/l. Blood was sampled from the caudal vein using a 25-gauge needle and a 1-ml syringe (3 fish per tank) pooled into Eppendorf tubes with EDTA. For serum biochemical assays, blood samples of another five fish from a given tank were placed in Eppendorf tubes without anticoagulant. Blood samples in Eppendorf tubes were allowed to clot for 30 min at room temperature (~25 °C). The tubes were then centrifuged at 3500 g for 5 min, and the supernatant serum was collected. The serum was kept frozen at -20 °C until analysis of selected biochemical. Hematocrit levels (expressed as % packed cell volume; PCV), leucocyte levels (expressed per 1000 blood cells).

2.6. Histological analysis

Gonads from the experimental fish (n = 6 per treatment) were dissected after scarification, fixed in 10% neutral buffered formalin for 24 h and processed in different levels of alcohol and embedded in paraffin wax. Sections were prepared as thin as 5 µm with a microtome, mounted on glass slides and stained routinely with hematoxylin and eosin. The slides were examined through the light electric microscope according to Banchfort *et al*.1996 [15].

2.7. Statistical analysis

The data were statistically analyzed by SPSS version 17. Mean ± SD, ANOVA and Duncan ´s multiple range tests [16] were calculated among all treatments.

3. Results and Discussion

3.1. Growth performance

The effects of propolis extract supplemented diets on the growth performance of Red tilapia brood stock are presented in Table 2. There were significant differences ($P<0.05$) among all treatments in terms of final weight, weight gain, relative growth rate, and specific growth rate. Final body weight was decreased with increasing the inclusion of propolis in the diets and differed significantly among all treatments. The same trend was observed with weight gain, relative growth rate and specific growth rate. Feed conversion ratio was better with fish fed D1 (1.7) and the worst was observed with fish fed D4 (2.2). The lowest value of protein efficiency ratio was observed with fish fed D4 (1.48), and the highest value was observed with fish fed D1 (1.92). Propolis as feed additive did not affect the survival rate of the experimental fish. Propolis has valuable nutritional effect can improve fish culture as natural products for fish diets [17].

In the present study, diet supplemented with 12 mL propolis extract kg^{-1} significantly recorded the lowest final body weight and weight gain. Moreover, propolis additive did not affect the feed intake except fish fed propolis at 3mL kg^{-1}. The supplementation of brown propolis extract at 1.83–2.74 g kg^{-1} with Nile tilapia (*Oreochromis niloticus*) fingerlings had been tested [3] and ethanolic extract of propolis at 10 g kg^{-1} or crude propolis [18] and the results pointed that the supplementation significantly improved the growth indices. These studies suggested that desirable effects of propolis on fish growth performance may due to the propolis extract compounds and its anti-microbial, biological and anti-oxidant activities which resulting in improving digestion and absorption of digestive system. In contrast, few studies [17, 19] explained that, propolis had no beneficial effect on fish weight gain and specific growth rate of rainbow trout (*Oncorhynchus mykiss*) although muscular development increased. This confliction of results

may be due to different doses of propolis and/or its origin especially, propolis analysis may be varied according to some factors such as the suitable exudates, fish species use different climate and other environmental conditions [20, 21].

3.2. Blood parameters

The blood parameters of red tilapia brood stock by the end of feeding trial are presented in Table 3. It was observed that fish fed the control diet (D1) exhibited the higher values of glucose and urea. There were no significant differences (P>0.05) among all treatments in terms of albumin, total protein and alkaline phosphatase in the blood of the experimental fish. Hematological parameters of fish fed tested diets are shown in Table 4. Overall, dietary treatments had a significant effect on hematological indices. RBC showed the lowest value in fish fed D4 when compared with the control group (P<0.05). The present study showed that, fish fed the control diet showed significantly (*P*<0.05) the higher glucose, blood urea nitrogen (BUN) and aspartate aminotransferase (AST) concentrations compared to fish fed diets supplemented with different levels of propolis. These results were in agreement with Deng *et al*. 2011 [6] who explained that the dietary supplementation of 1 g kg^{-1} ethanolic extract of propolis with rainbow trout diets significantly decreased the AST plasma and triglycerides levels. Long-term administration of propolis (56 days) in juvenile rainbow diet especially, with 9 g kg^{-1} diet had no significant alterations in the content of serum TP, albumin, globulin, Low-density lipoprotein cholesterol (LDL), High-density lipoprotein cholesterol (HDL), Triglycerides (TG) and function of liver enzymes expressed as aspartate aminotransferase (AST), alanine aminotransferase (ALT) concentrations [6]. Increasing glucose production in the control group may be happening to meet the increasing demands for energy from fish under stress. So, dietary propolis in this experiment was accompanied with recorded and desirable results of the glucose level in this experiment. Fish fed propolis supplemented diets increased the plasma ALT and Creatine levels significantly (P<0.05). These results in contrast with Abbass *et al*. 2012 [22] who resulted that feeding Nile tilapia with propolis supplemented diets showed significant (P<0.001) lower ALT activity contrary to AST level when compared to the control diet.

In the current study, there were significant effects on hematological indices of the experimental fish. The higher ***RBCs*** count were recorded with fish fed D3 diet supplemented with 6 mL kg^{-1} of propolis extract compared to the control diet (*P*<0.05). Our results are in agreement with Dotta *et al*. 2015 [23] who found that, the using dietary mixtures of propolis and *Aloe barbadensis* extracts improved the hematological parameters of Nile tilapia and caused a significant reduction in the number of gill parasites.

3.3. Carcass composition

Data on the carcass composition of red tilapia are performed in Table 5. There were no significant differences of lipid and ash contents of the whole body of red tilapia. Slight increase of protein content was observed with fish fed D1. There were no significant differences (*P* > 0.05) in the carcass parameters in terms of protein, lipid and ash contents. Abdel-Hakim *et al*. 2014 [24] found that, the whole body, dry matter, crude protein and ash percentages of monosex Nile tilapia (*Oreochromis niloticus*) fingerlings were significantly (*P*<0.05) influenced by propolis (Bee Glue) dietary treatment. Similarly, Bae *et al*. 2012 [25] found that propolis level supplementation of 0.5% increased the whole-body protein and lipid contents of juvenile eel, *Anguilla japonica*. Also, Wafaa *et al*. 2014 [26] obtained that the higher DM content of Nile tilapia fed by diet supplemented with black cumin seed while the whole body CP content was significantly (P<0.05) enhanced by green tea, black seed and propolis extract groups. So, the higher values of whole protein of fish groups supplemented with propolis supplementation may be due to that flavonoids compounds in propolis improve nutrient metabolism, feed ingestion and absorption.

3.4. Histological analysis

The histological examination of red tilapia testes fed propolis extract supplemented diets for 56 days at levels of 0, 3, 6 and 12 mL kg^{-1} of feed are shown in Fig.1. (A) Control group without supplementation showed normal structure of the male testes with degeneration in spermatogonia in some individual seminiferous tubules with lowering in spermatozoa (d). Also, the same results were observed with fish fed propolis supplemented diet at 3 mL kg^{-1} (B). With increasing the level of propolis extract in the diets at 6 and 12 mL kg^{-1} (C, D) showed seminiferous tubules impacted by spermatozoa (s). These results are similar to Didik *et al*. 2012 [27] who used propolis as natural material for sex reversal of tilapia and found that the best results of Propolis for sex reversal in NIFI red tilapia showed with the highest percentage of male was 5 mL kg^{-1}.

Table 1: The basal diet composition and proximate analysis.

%	D1,Control diet
Ingredients	
Fish meal (72%)	10
Soybean meal (44%)	40
Yellow corn	15
Wheat bran	18
Wheat flour	14
Soybean oil	2
Vitamin	0.5
Minerals	0.5
Chemical composition(% DM)	
Dry Matter	89.96
Crude protein	30.75
Ether extract	5.05
Crude fiber	5.71
Nitrogen Free Extract[1]	42.85
Crude ash	5.6

[1] Nitrogen Free Extract was calculated by the difference: =100 - (moisture + protein + lipid + ash + Crude fiber).

Table 2: Growth performance and feed utilization of red tilapia, *Oreochromis niloticus* x *O. mossambicus* brood stock (initial wt 44±0.1 g) fed Propolis as feed additive in the diets for 56 days. Values are mean ± SD of triplicate groups.

Experimental diets (mL propolis kg^{-1})				
Parameters	**D1 (0)**	**D2 (3)**	**D3 (6)**	**D4 (12)**
IBW (g fish^{-1})	44.2±1.1	44.4±0.2	44.1±0.1	44.3±1.1
FBW (g fish^{-1})	97.4±0.1^{d}	86.8±0.1^{c}	86.4±0.1^{b}	85.4±0.1^{a}
TWG (g fish^{-1})	53.2±0.1^{d}	42.8±0.1^{c}	42.3±0.1^{b}	41.1±0.1^{a}
RG R (%)*	220.7±0.6^{d}	197.3±0.6^{c}	196±0.0^{b}	193±0.0^{a}
SGR (% day^{-1})	1.41±0.03^{d}	1.21±0.03^{c}	1.20±0.03^{b}	1.17±0.03^{a}
FI (g fish^{-1})	90.1±0.0^{b}	87.5±2.3^{a}	90.02±0.0^{b}	90.6±0.0^{b}
FCR	1.7±0.1^{a}	2.04±0.6^{b}	2.1±0.02^{c}	2.2±0.01^{d}
PER	1.92±0.0^{d}	1.6±0.04^{c}	1.53±0.1^{b}	1.48±0.01^{a}
Survival %	95±0.1^{a}	96±0.0 ^{a}	95±0.0 ^{a}	96±0.0 ^{a}

Values followed by the same superscript letters in the same row are not significantly different ($P > 0.05$).
* Relative growth rate %= 100 x (average final weight/ average initial weight)

Table 3: Biochemical parameters of red tilapia brood stock, *Oreochromis niloticus* x *O. mossambicus* fed Propolis supplemented diets for 56 days.

Parameters	**Experimental diets (mL Propolis kg^{-1}diet)**			
	D1 (0)	**D2 (3)**	**D3 (6)**	**D4 (12)**
Glucose (mg dl^{-1})	174.0±1.0 ^{d}	132.2±1.1^{c}	102.3±1.3^{a}	112.9±1.5^{b}
BUN (mg dl^{-1})	4.45±0.70 ^{a}	2.54±0.52^{c}	1.52±0.21 ^{b}	2.04±0.02 ^{bc}
Creatinine (mg dl^{-1})	1. 6±0.8^{a}	1.3±0.7^{b}	1.52±0.1^{a}	2.02±0.1^{c}
AST (µ ml^{-1})	150.0±1.0^{b}	125.1±1.2^{a}	126.1±1.2^{a}	150.3±1.9^{b}
ALT (µ ml^{-1})	25.0±0.7^{a}	34.0±1.3^{b}	35.0±0.8^{c}	43.0±1.1 ^{d}
Albumin (g dl^{-1})	0.7±0.01	0.7±0.02	0.6±0.01	0.9±0.01
TP (g dl^{-1})	2.6±0.1	2.6±0.1	2.7±0.2	2.6±0.1
Alkaline phosphatase (IU L^{-1})	22.4±1.8	23.9±0.9	24.6±2.0	22.9±2.6

Means within the same row having different superscript letter were significantly differ at 0.05 levels.
BUN: blood urea nitrogen, AST: aspartate aminotransferase, ALT: alanine aminotransferase, TP: total protein.

Table 4: Hematological parameters of red tilapia brood stock, *Oreochromis niloticus* x *O. mossambicus* fed Propolis supplemented diets for 56 days.

Parameters	**Experimental diets (mL Propolis kg^{-1}diet)**			
	D1 (0)	**D2 (3)**	**D3 (6)**	**D4 (12)**
Hb	7.8±0.2^{b}	9.8±0.1^{c}	10.4±0.1^{d}	7.3±0.2^{a}
RBC (10^{6}/mm^{3})	5.2±0.8^{ab}	5.9±1.0^{bc}	6.4±0.1^{c}	4.8±0.1^{a}
Total Leukocyte (10^{6}/mm^{3})	21.2±1.0^{b}	28.2±2.2^{d}	17.1±1.4^{a}	24.1±1.2^{c}
Lymphocytes (µl)	30.0±1.0^{c}	21.0±1.1^{a}	28.0±1.0^{b}	21.0±1.0^{a}
Monocytes (µl)	4.0±1.0^{a}	7.0±1.1^{b}	6.0±1.3^{b}	3.0±1.2^{a}
PCV (%)	24.3±1.0^{b}	32.2±2.2^{c}	21.5±1.8^{d}	24.9±1.1^{a}
MCV (fl)	46.1±1.2^{a}	54.2±1.6^{b}	46.7±2. 1^{b}	46.2±1.8^{a}
MCH (pg)	14.2±1^{a}	16.6±1^{b}	17.0±1.4^{b}	14.3±1.1^{a}
MCHC	30.8±1.5	30.3±1.3	31.3±1.0	30.8±1.9

Means within the same row having different superscript letter were significantly differ at (p<0.05) levels.
RBC, red blood cells; Hb, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin.

Table 5: The body composition of red tilapia brood stock, *Oreochromis niloticus* x *O. mossambicus* fed Propolis supplemented diets for 56 days.

Experimental diets (mL Propolis kg^{-1}diet)					
Items	Initial	D1 (0)	D2 (3)	D3 (6)	D4 (12)
Dry matter	23.4	18.5±2.7^{a}	21.3±2.7^{ab}	22.5±1.6^{ab}	22.8±0.6^{b}
protein	61.2	62.4±0.9^{b}	61.7±0.4^{b}	59.1±0.7^{a}	60.1±0.6^{a}
Lipid	9.2	8.5±0.8^{a}	8.4±1.5^{a}	8.2±1.3^{a}	8.1±1.1^{a}
Ash	9.7	10.5±1.1^{a}	8.5±1.0^{a}	10.1±1.1^{a}	9.2±1.1^{a}

Values followed by the same superscript letters in the same row are not significantly different (P > 0.0

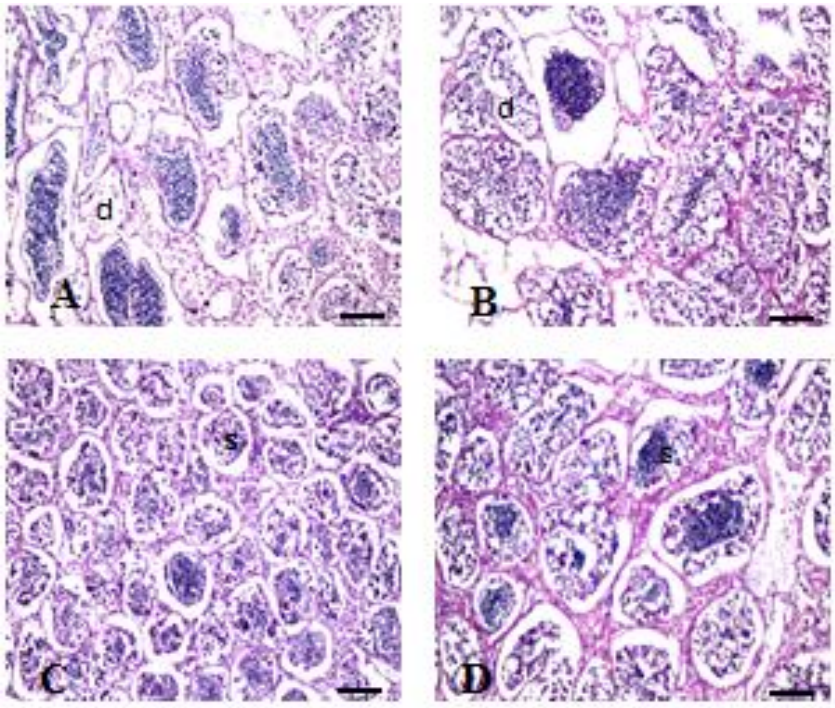

Fig 1: Histopathological section in testes of Red tilapia fed different levels of propolis extract as feed additive (A, Control) without any supplementation, followed by three diets with propolis at 3, 6, 12 mL kg^{-1} of feed (B, C, D, respectively). (A) Showing normal histological structure of testes with degeneration in spermatogonia in some individual seminiferous tubules (d). (B) Showing degeneration in spermatogonia (s) in some individual seminiferous tubules. (C, D) Showing seminiferous tubules impacted by spermatozoa. (H&E staining); scale bars = 40 µm.

4. Conclusion

It can be concluded that, the effectiveness of propolis extract as a natural product in the diets for red tilapia brood stock up to 6 mL kg^{-1} improve the performance of fish, blood parameters and gonads statement without any adverse effects. Further research is required to study the fertilizing capacity in males and some function indices of liver and kidney.

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