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The dietary administration of miswak leaf powder promotes performance, antioxidant, immune activity, and resistance against infectious diseases on Nile tilapia (*Oreochromis niloticus*)

Mohammed A.E. Naiel [a,*], Mohamed K. Khames [b], Nashwa Abdel-Razek [c], Amany A. Gharib [d], Khaled A. El-Tarabily [e,f,**]

[a] Department of Animal Production, Faculty of Agriculture, Zagazig University, Zagazig, 44511, Egypt
[b] Fish Nutrition and Feed Technology Department, Central Laboratory for Aquaculture Research, Agriculture Research Center, Abassa, 44661, Abu-Hammad, Sharkia, Egypt
[c] Department of Fish Health and Management, Central Laboratory for Aquaculture Research, Agriculture Research Center, Abbassa, 44661, Abu-Hammed, Sharkia, Egypt
[d] Department of Hatchery and Fish Physiology, Central Laboratory for Aquaculture Research, Agriculture Research Center, Abbassa, 44661, Abu-Hammed, Sharkia, Egypt
[e] Department of Biology, College of Science, United Arab Emirates University, 15551, Al-Ain, United Arab Emirates
[f] Harry Butler Institute, Murdoch University, Murdoch, 6150, Western Australia, Australia

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ABSTRACT

The feeding experiment's main purpose was to estimate the influence of fortification of tilapia diets with miswak leaf powder (MLP) on performance, digestive enzyme activity, chemical body composition, antioxidant status, immune response, and resistance against bacterial and fungal infections. *O. niloticus* fingerlings (15.22 ± 0.06 g) were fed four formulated diets fortified with MLP at 0, 2.5, 5, and 10 g kg^{-1} diet for 56 days. The fish were injected with *Aeromonas hydrophila* for 14 days at the end of the feeding trial, and then the mortality percentage was recorded daily in all treated groups. The obtained results showed that dietary administration of MLP at 2.5 g kg^{-1} recorded significantly higher values in all growth, feed utilization, and feed efficiency parameters. The digestive enzyme activity was significantly ($P < 0.01$) higher in fish fed a low level of MLP (2.5 or 5 g kg^{-1}) when compared with the control group. Furthermore, the higher concentration of MLP (10 g kg^{-1}) significantly reduced the mortality rate induced by *Aeromonas hydrophila* infection, increased crude protein percentage in fish body and represented highly *in vitro* antibacterial and antifungal efficiency. Based on the findings of the challenge tests against *A. hydrophilia*, higher survival was found in infected fish, explaining that MLP at high level of 10 g kg can be used to resist the outbreak of the *A. hydophilia* disease in tilapia. In conclusion, our obtained data showed that applied MLP in tilapia diets could promote performance, feed efficiency, antioxidant status, immune activity, and enhanced resistance of *O. niloticus* against infections.

1. Introduction

Globally, *Oreochromis niloticus* is actually the world's second-largest cultured aquatic fish species after carp (Ismael et al., 2021). According to FAO statistics, 125 countries produced 6,882,202 tonnes of wild and farmed tilapia. Egypt is considered one of the largest producing countries after china and Indonesia, with 17.04 % of the total global production (FAO, 2020). However, the degradation of water bodies and various aquatic diseases caused by the heavy implementation of the industrial revolution have threatened the intensive production of fish (Ali et al., 2020). Due to the highly infectious diseases, enhanced intensive cultivated systems have also been regarded as obstacles to sustainable aquaculture development (Abdelghany et al., 2020). Besides, the high stocking rate of rearing fish induced several infectious diseases, higher mortality rates, reduced growth and led to economic losses (Tan et al., 2018). The conventional treatment protocols applied

* Corresponding author at: Department of Animal Production, Faculty of Agriculture, Zagazig University, Zagazig, 44511, Egypt.
** Corresponding author at: Department of Biology, College of Science, United Arab Emirates University, 15551, Al-Ain, United Arab Emirates
E-mail addresses: mohammednaiel.1984@gmail.com, mnaiel@zu.edu.eg (M.A.E. Naiel), ktarabily@uaeu.ac.ae (K.A. El-Tarabily).

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in fish farms to control disease, such as antibiotics, are costly, hazard the environment, threaten consumer health, and be difficult in a wide-scale application (Lieke et al., 2020). Thus, promoting the antioxidative and immunity status of farmed fish is a vital and prevalent safe and effective strategy for preventing several bacterial and fungal diseases (Abdel--Ghany and Salem, 2020). In the aquaculture industry, medicinal plants and their derivatives are of great potential in promoting the antioxidant and immune status of fish, thereby increasing their capability to prevent diseases and promote growth performance (Awad and Awaad, 2017; Naiel et al., 2020).

Several studies had been investigated numerous valuable properties of some medicinal plants as growth promoters, antibacterial, antifungal, antitoxin, immunostimulant, antioxidant, and anti-stress such as rosemary (*Rosmarinus officinale*) (Naiel et al., 2019); miswak (*Salvadora persica*) (Lebda et al., 2019; Abd El-latif et al., 2021); turmeric (*Curcuma longa*) (Ayoub et al., 2019); *Moringa oleifera* (Abdelhiee et al., 2020); Oregano (*Origanum vulgare*) (Espirito Santo et al., 2020), thyme (*Thymus vulgaries*) (Abd El-Naby et al., 2020b).

The miswak (*Salvadora persica* L.) tree is commonly originated in several Asian countries. In traditional medicine, various parts of this tree were widely used for dental hygiene (Haque and Alsareii, 2015). In addition, miswak root has several pharmacological properties such as antioxidant (Kumari et al., 2017), immunostimulants (Syawal et al., 2008), antibacterial (Ahmadi et al., 2019), antitoxic (Abd El-Naby et al., 2020a) and antifungal (Alili et al., 2014). Also, fortified common carp diets with miswak roots promoted the immune status and survival rate (Syawal et al., 2008). Besides, Lebda et al. (2019) found that supplemented tilapia diets with miswak roots powder (1 %) enhanced antioxidant status and non-specific immune activity. The mainly bioactive molecules that have been linked with the leaves, roots and bark of the miswak tree are alkaloids such as salvadorine and trimethylamine; chlorides; fluoride; tannins; resins; flavonoids; sodium bicarbonate; calcium, benzyl isothiocyanate and other derivatives including silica, sulfur, ascorbic acid, sterols, trimethylamine and saponins (Halawany, 2012).

Several pharmacologic studies investigated the potential of the roots, stems, and leaves of *S. persica* against bacterial infection due to the presence of essential volatile oil (Akhtar et al., 2011; Al Bratty et al., 2020). Also, the miswak leaves are medically used for treating some respiratory and digestive diseases (Haque and Alsareii, 2015). In addition, there is rare literature about the benefits of using miswak leaf on the performance and health of Nile tilapia.

Thus, our main purpose was to estimate the desired supplemented level of miswak leaf powder (MLP) that could promote the immunity of farmed tilapia against the microbial infection incorporated with enhanced performance. To investigate this hypothesis, 56 days feeding trial was accomplished with three MLP levels to determine the performance, feed efficiency, antioxidant and immune response, relative survival percentage, chemical analysis of the fish body, and *in vitro* antibacterial and antifungal tests were also inspected.

2. Materials and methods

2.1. Ethical approval statement

The existing trial followed the general protocol guidelines for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee of Zagazig University (NO: ZU-IACUC/1/F/57/2019).

2.2. Fish rearing conditions and feeding experiment

Nile tilapia juveniles (*Oreochromis niloticus,* 15.22 ± 0.06 g; average weight ± SE) were purchased from a governate fish hatchery belonging to the Central Laboratory for Aquaculture Research, Abassa, Abu Hammad, Sharkia, Egypt. The experimental fish were transferred using polyethylene bags filled with $\frac{1}{3}$ dechlorinated water and supplied with $\frac{2}{3}$ oxygen then allocated into the fiberglass ($1 \times 1x1m^3$) for the acclimation period (14 days). All experimental fish were apparently healthy and had no observed lesions after the initial examination.

A total of healthy two hundred fish were randomly allocated into 20 experimental glass aquariums (10 fish per aquarium; $40 \times 65 \times 35$ cm). Each of the four experimental groups was allotted five replicate aquariums, siphoned daily to remove solid waste and provide clean, dechlorinated tap water. Photoperiod was applied as 12L:12D. The feeding trial was extended for 56 days. The miswak (*S. persica*) leaf powder examined levels (0, 2.5, 5.0, and 10 g MLP Kg^{-1} diets were selected followed by Toutou et al. (2019) study. The fish were fed daily *ad-libtium*.

All examined water quality parameters were measured biweekly before water exchange. All measured water quality parameters were found to be within the acceptable ranges, according to Boyd and Tucker (2012). The temperature, pH, dissolved oxygen, and total NH_4-N values were 26 ± 1 °C, 8.1 ± 0.1, 8.7 ± 0.2 mg L^{-1}, and 0.12 ± 0.02 mg L^{-1}, respectively.

2.3. Preparation of experimental diet

Four experimental diets were formulated to contain 0.0, 2.5, 5.0, and 10 g MLP kg^{-1} diet. All ingredients were mingled with oil, and then water was added until the apparent solid dough was obtained. Each formulated diet was extruded using a mincer. The obtained pelleted diets were air-dried and stored at 4 °C in plastic bags till use. All ingredients and analyzed composition of the basal-diet are illustrated in Table 1.

Table 1
Ingredients (air-dry basis, g Kg^{-1} diet) and analyzed basal diet chemical composition (%).

Ingredients	MLP levels (g Kg^{-1} diet)			
	0.0	2.5	5	10
Fish meal	100	100	100	100
Yellow corn	120	120	120	120
Soybean meal 44 %	360	360	360	360
Corn gluten meal 60 %	50	50	50	50
Rice bran	100	100	100	100
Wheat bran	120	120	120	120
Wheat flour	99.2	96.7	94.2	89.2
Vegetable oil	15	15	15	15
Fish oil	15	15	15	15
[1]Vitamin premix	10	10	10	10
[2]Mineral premix	10	10	10	10
Ascorbic acid	0.8	0.8	0.8	0.8
Miswak leaf powder (MLP)	0.0	2.5	5	10
Total	1000	1000	1000	1000
Analyzed composition (%)				
Moisture	9.45	10.18	10.02	10.10
Dry matter	90.55	89.82	89.98	89.9
Crude protein	30.25	30.22	30.20	30.22
Crude lipids	8.12	8.22	8.60	8.40
Crude fiber	6.21	6.11	6.13	6.14
Ash	10.45	10.52	10.60	10.61
Nitrogen free extract (NFE)[3]	44.97	44.93	44.47	44.63
Gross energy (Kcal g^{-1})[4]	4070.37	4039.39	4061.52	4048.24

[1] Composition of mineral premix kg^{-1}: manganese,53 g; zinc,40 g; iron, 20 g; copper, 2.7 g; iodine, 0.34 g; selenium, 70 mg; cobalt, 70 mg and calcium carbonate as carrier up to 1 kg. [2]Composition of vitamin premix kg^{-1}: vitamin A, 8000000 IU; vitamin D_3, 2000000 IU; vitamin E, 7000 mg; vitamin K_3, 1500 mg; vitamin B_1, 700 mg; vitamin B_2, 3500 mg; vitamin B_6, 1000 mg; vitamin B_{12}, 7 mg; biotin, 50 mg; folic acid, 700 mg; nicotinic, 20000 mg; pantothenic acid,7000 mg. [3] NFE = 100 – (crude protein + crude lipids + ash + crude fiber). [4] Calculated based on the values for protein, lipid and carbohydrate as 23.6, 39.5 and 17.2 Kcal/g, respectively.

2.4. Performance and feed efficiency indices

At the end of the feeding trial (56 days), the fish were collected, counted, and weighed to determine the final live body weight. The performance and feed utilization parameters were calculated according to the following formulas:

Weight gain (WG; g) = FW – IW,

Specific growth rate (SGR; % day^{-1})^2 = 100 × [(Ln FW) - (Ln IW) /trial period in days],

Feed conversion ratio (FCR; g/g) = FI (g) /WG (g);

Protein efficiency ratio (PER) = WG (g)/Protein intake (PI; g);

Fish survival rate (%) = 100 × [total final number of living fish at the end of the experiment /initial number of fish];

Where FW, final fish weight (g); IW, initial fish weight (g) and FI, the total amount of feed intake (g).

2.5. The pathogenic bacterium challenge test

The Gram-negative bacterial strain (*Aeromonas hydrophila*) used in the challenge experiment was identified and acquired from the Department of Fish Biology and Ecology, Central laboratory for Aquaculture Research, Abassa, Abu Hammad, Sharkia, Egypt. The bacterial strain was cultivated using Tryptic Soy Broth (Himedia, Mumbai, India) and then incubated for 24 h at 25 °C. The culture broth solution was centrifuged at 3000 *g* for 10 min. The supernatant was discarded, and the obtained pellets were washed twice by phosphate-buffered saline as described by Naiel et al. (2020). The optical density (OD) was then measured using the prepared solution at 456 nm, which parallels 1×10^7 cells ml^{-1} (Bailone et al., 2010). At the end of the feeding trial, the selected fish from each group (20 fish from each group) were injected intraperitoneally with 0.1 mL of bacterial suspension as described by Zahran et al. (2018). Fish were prolonged with the same feeding regime during this post-challenge period (14 days), and mortality was verified and accounted for all treated groups daily.

The recorded mortality data have been used to calculated Relative Live Percentage (RLP) following Amend (1981) equation:

RLP = 1 - [(recorded mortality percentage in treated groups (%))/ (recorded mortality percentage in non-treated group (%))] ×100.

2.6. Chemical analysis of diets and fish

The chemical composition from diet and fish samples have been measured and the moisture, dry matter, crude protein, crude fat, and ash content were calculated using Thiex et al. (2012) standard methods. The nitrogen-free extract (NFE) was determined statically using the following formula:

NFE ($g\ kg^{-1}$) = 1000 – (crude protein + crude lipids + ash + crude fiber).

In addition, the gross diet energy was estimated based on the values for protein, lipid and carbohydrate as 23.6, 39.5 and 17.2 Kcal/g, respectively, as illustrated in Table 1. All analyses were performed in five replicates.

2.7. Blood samples collection

At the end of the bacterial challenge test, the blood samples were collected after starvation for 24 h. Six fish from each treated group were anesthetized (120 mg L^{-1} aminobenzoic acid, Sigma-Aldrich Chemie GmbH, Germany). The samples were then collected from the caudal vein using an aspiration syringe. A small portion was blended with EDTA dipotassium salt as an anticoagulant (0.5 mg ml^{-1} blood) into Eppendorf tubes for respiratory burst. The other portion was left to clot at room temperature to obtain serum into a plain centrifuge tube. The collected serum was centrifuged at 4 °C for 10 min at 3000 rpm and then stored at −20 °C until use and the digestive enzymes, antioxidant activity and other immune parameters were then determined

2.8. Blood digestive enzymes and antioxidant activity analysis

The serum digestive enzymes (amylase and lipase) levels were quantified following the commercial kits manufacturer instructions (Biodiagnostic, Bio. Co., Egypt). In addition, serum glutathione reductase (GSH), catalase (CAT), malonaldehyde (MDA) and superoxide dismutase (SOD) levels were assessed calorimetrically using commercial diagnostic kits (Diamond Diagnostic Co. for Modern Laboratory Chemicals, Egypt) following the recommended instructions.

2.9. Immunity assay

The Sunyer and Tort (1995) method was used to determined complement C3 activity, while Overkamp et al. (1988) procedure was applicable for determining the immunoglobulin M (IgM) levels. The serum lysozyme levels were estimated based on the lysis method of *Micrococcus lysodeikticus* (Sigma–Aldrich) according to Lee et al. (2002) procedure with a small modification. Briefly, 0.25 mL from the sample was blended with 0.75 mL from *M. lysodeikticus* suspension (0.2 mg/mL in 0.05 M PBS, pH 6.2). After incubating the mixture for 5 min at 25 °C, the OD of the obtained solution was measured using a spectrophotometer (BM Co. Germany) at 540 nm. Each specified unit induced an absorbance reduction of 0.001. Using a stander curve created with lyophilized hen egg-white lysozyme (Sigma-Aldrich), the lysozyme unit was defined and represented in (μg/mL). The respiratory burst activity of the whole blood sample was estimated according to microtiter plate wells method as described by Abreu et al. (2009). The prepared supernatant was estimated at 620 nm using nitroblue tetrazolium (NBT) blood assay and the NBT levels were expressed in (μg/mL).

2.10. In vitro antibacterial and antifungal activity of MLP extract

The three levels of MLP (2.5, 5 and 10 g) were tested by the disc diffusion method for their antibacterial and antifungal potential in five selected species. Each selected level was weighed and extracted using 100 mL 80 % ethanol by distillation method then stored in dark Durham's bottle. The bottles were incubated under 24 °C for 24 h within 130 rpm shaking water bath as described by Hashemi Karouei and Azizi (2012). The obtained solution was then centrifuged at 5000 *g* for 15 min and filtered by Whatman filter paper (ashless, 150 mm). The prepared solvent was evaporated at 40 °C by a water bath to make the final volume at one-fifth of the original volume. The prepared extract was stored within airtight bottles at 4 °C for further *in vitro* antibacterial and antifungal studies.

The examined bacterial and fungal strains were obtained from the Fish Biology and Ecology Department, Central laboratory for Aquaculture Research, Abassa, Abu Hammad, Sharkia, Egypt. Each selected bacterial strain was identified followed Clark (1984) method by using selective media and conventional biochemical procedures. The examination and identification of used fungal strains were performed as described by Abdel-Razek (2019) and Abd El-Tawab et al. (2020) methods. The tested bacterial strains were *Aeromonas hydrophilia*, *Aeromonas jandaei*, *Pseudomonas fluorescens*, *Staphylococcus aureus*, and *Streptococcus agalactiae*. While, the examined fungal strains were *Aspergillus niger, Aspergillus fumigatus*, *Rhizopus* spp., *Fusarium* spp., and *Candida* spp. The *in vitro* antibacterial and antifungal tests for MLP levels were accomplished by the agar disc diffusion method as described by Parekh et al. (2006). Prior to the antimicrobial examination, each

bacterial strain was incubated for 18 up to 24 h at 37 °C on the Muller Hinton medium. Three sterile 6 mm diameter filter disks were saturated with each MLP extract and the fourth was saturated with ethanol (control). The saturated disks were added on the Sabouraud's Dextrose agar petri dishes seeded with each fungal strain, while TSA medium (Himedia, Mumbai, IN) was used for bacterial stains. Then, the petri dishes were incubated at 28−30 °C for two days. The antibacterial or antifungal activity was evaluated by estimating the inhibition zone surrounding the discs. The antibacterial or antifungal activity of the induced inhibition zone was defined as the mean produced diameter. All dimensions have been estimated in five replicates.

2.11. Data analysis

All calculated data were examined for normality and homogeneity distribution by Levene's test. The percentage of data was arscinc transformed before analysis. The one-way analysis of variance (ANOVA) was applied to verify the differences between MLP levels in 95 % confidence value ($P < 0.05$) using the SPSS software (v.22.0). Tukey's range test was used to compare means and identified significance letters. All measurement data were represented as mean ± SE. While, antibacterial and antifungal data were presented as mean ± SD.

3. Results

3.1. Growth performance and feed utilization parameters

The influence of several levels of MLP in the tilapia diets on growth, efficiency, and utilization of feed for 56 days is illustrated in Table 2. The growth and feed efficiency parameters like FW, WG, SGR, FI, and PER showed higher values in the tilapia fish fed low levels of the MLP. Compared with the fish group fed the basal diet, the fish group fed an enriched diet with MLP at 2.5 g kg^{-1} verified the high values of FW, WG, SGR, FI, and PER. Fortified tilapia diets with MLP improved the FCR values. The lowest FCR values were recorded in fish group fed 2.5 and 5 g kg^{-1} MLP level compared to other treated groups. The survival rate showed no significant variance between all treated groups.

3.2. Serum digestive enzymes, antioxidants and immunity parameters

The influence of supplemented diets with several levels of MLP on Nile tilapia fish serum digestive enzymes, antioxidant indices and immune responses are represented in Table 3. The digestive enzymes (lipase and amylase) recorded the high levels in the fish group fed diets supplemented with 2.5 or 5 MLP g kg^{-1} compared to those in another group. However, the fish group fed basal diet and 10 g MLP-supplemented diet groups displayed low digestive enzyme levels. Fortified tilapia diets with a high level of MLP (5 or 10 g kg^{-1}) significantly promoted antioxidant activity through increasing GSH, CAT and SOD levels. The lowest MDA values were exhibited in the tilapia group fed diets enriched with 5 g MLP kg^{-1}. The immune response showed a highly significant improvement in all MLP supplemented groups. The lysozyme, complement C3 and NBT serum levels showed high significant ($P < 0.001$) enhancement with increasing MLP levels. The highest lysozyme, complement C3 and NBT values were detected in fish groups fed 5 g MLP kg^{-1}. Moreover, the IgM concentration showed no significant differences among all experimental groups.

3.3. Body chemical composition analysis

The results of body chemical analysis showed that crude protein percentage in fish meat was significantly higher in groups fed diets incorporated with MLP at 5 and 10 g kg level over those fed lower level of MLP incorporation and the control groups without MLP treatment. In contrast, the crude fat in the fish body showed no significant variations among experimental treatment groups (Table 4). The moisture and ash levels revealed a significant decrease with increasing MLP concentration in feed diets (Table 4). The lowest moisture and ash levels were recorded at the 10 g MLP kg^{-1} supplemented group.

3.4. Resistance against A. hydrophila infection

Data illustrated in Table 5 showed that all MLP treated groups, the survival rate after the pathogenic bacterium challenge was higher than the fish group fed the basal diet. Fish fed supplemented diets with 10 g MLP kg^{-1} verified the best RLP (100 %) and lowest MR values compared with those in the fish group fed the basal diet.

3.5. In vitro antibacterial and antifungal activity of ethanolic MLP extract

The obtained results from the *in vitro* antibacterial examination of MLP extract against some selected bacterial strains were illustrated in Fig. 1. The ethanolic extract of 10 g MLP displayed strong antibacterial activities against all gram-negative bacterial strains (*Aeromonas hydrophilia*, *Aeromonas jandaei*, and *Pseudomonas fluorescens*). The 10 g MLP extract showed mild antibacterial ability against gram-positive bacterium (*Staphylococcus aureus*, and *Streptococcus agalactiae*).

The antifungal activity of MLP ethanolic extract against *Aspergillus niger*, *Aspergillus fumigatus*, *Rhizopus* spp., *Fusarium* spp., and *Candida* spp. are presented in Fig. 2. The high level of MLP (10 g kg^{-1}) exhibited strong antifungal activity against *Rhizopus* spp. and *Candida* spp. All tested MLP levels significantly reduced the development of *Aspergillus niger*, *Aspergillus fumigatus*, and *Fusarium* spp. Generally, the high concentration of MLP extract revealed higher antifungal activity against all tested fungal strains.

Table 2
Performance, feed efficiency and survival rate of Nile tilapia fed diets containing 0.0, 2.5, 5.0 and 10 g MLP kg^{-1} diet.

Parameters	Experimental Diets (g Kg^{-1} diet)				*P value*
	CNT	$MLP_{2.5}$	MLP_5	MLP_{10}	
IW(g)	15.22 ± 0.06	15.26 ± 0.03	15.16 ± 0.04	15.33 ± 0.05	0.524
FW (g)	39.61 ± 0.12^{d}	56.60 ± 0.28^{a}	55.08 ± 0.09^{b}	45.03 ± 0.15^{c}	<0.001
WG (g)	24.39 ± 0.08^{d}	41.34 ± 0.37^{a}	39.92 ± 0.22^{b}	29.70 ± 0.03^{c}	<0.001
FI (g)	41.18 ± 0.13^{c}	51.47 ± 0.74^{a}	49.37 ± 0.32^{b}	43.02 ± 0.21^{c}	0.021
FCR (g/g)	1.69 ± 0.23^{a}	1.24 ± 0.15^{c}	1.23 ± 0.31^{c}	1.45 ± 0.12^{b}	<0.001
SGR (%)	1.37 ± 0.18^{d}	1.89 ± 0.02^{a}	1.85 ± 0.21^{b}	1.54 ± 0.24^{c}	<0.001
PER (g/g)	1.97 ± 0.87^{c}	2.69 ± 0.29^{a}	2.70 ± 0.38^{a}	2.30 ± 0.17^{b}	<0.001
SR (%)	100 ± 0.15	100 ± 0.03	100 ± 0.29	100 ± 0.36	0.895

Values within the same row having different superscripts are significantly different ($P < 0.05$).
Data were presented as the mean ± standard error.
MPL, miswak leaf powder; IW, initial weight (g); FW, final weight (g); WG, weight gain (g); FI, feed intake (g); FCR, feed conversion ratio (g/g); SGR, specific growth rate (%/d)^{2}; PER, protein efficiency ratio (g/g); SR, survival rate (%); CNT, control.

Table 3
Digestive enzymes, antioxidant and immunity activities of Nile tilapia fed diets containing 0.0, 2.5, 5.0 and 10 g MLP kg^{-1} diet.

Parameters	Experimental Diets (g Kg^{-1} diet)				*P value*
	CNT	$MLP_{2.5}$	MLP_5	MLP_{10}	
Digestive Enzymes					
Amylase (U/L)	138.11 ± 1.15^{c}	159.67 ± 0.88^{a}	157.67 ± 1.41^{a}	146.67 ± 0.61^{b}	0.002
Lipase (U/L)	23.67 ± 1.45^{c}	66.33 ± 1.05^{a}	63.02 ± 1.62^{a}	38.33 ± 1.85^{b}	<0.001
Oxidative stress assay					
GSH (mmol/L)	0.48 ± 0.24^{c}	0.70 ± 0.34^{b}	0.91 ± 0.44^{a}	1.03 ± 0.40^{a}	0.011
Catalase (U/mL)	33.67 ± 0.45^{b}	38.33 ± 0.54^{b}	48.01 ± 0.53^{a}	50.10 ± 0.58^{a}	<0.001
MDA (mmol/L)	14.60 ± 0.16^{a}	13.53 ± 0.19^{b}	11.67 ± 0.19^{c}	12.23 ± 0.18^{c}	<0.001
SOD (U/mL)	3.04 ± 0.09^{c}	3.70 ± 0.12^{bc}	4.47 ± 0.22^{ab}	4.97 ± 0.23^{a}	<0.001
Immunity parameters					
IgM (ug/mL)	18.56 ± 0.54	17.87 ± 0.23	20.23 ± 0.18	19.06 ± 0.38	0.667
Complement C3 (ug/mL)	40.33 ± 0.85^{c}	55.01 ± 0.12^{b}	66.33 ± 0.57^{a}	65.12 ± 0.68^{a}	<0.001
Lysozyme(μg/mL)	1.27 ± 0.08^{c}	1.89 ± 0.09^{b}	2.75 ± 0.24^{a}	2.84 ± 0.19^{a}	<0.001
NBT (μg/mL)	4.31 ± 0.42^{c}	7.01 ± 0.06^{ab}	8.20 ± 0.14^{a}	6.55 ± 0.53^{b}	0.014

Values within the same row having different superscripts are significantly different ($P < 0.05$). Data were presented as the mean ± standard error. MPL, miswak leaf powder; CNT, control, GSH, glutathione reductase; MDA, malondialdehyde; SOD, super oxide dismutase; IgM, immunoglobulin M; NBT, Nitroblue tetrazolium blood test.

Table 4
Whole body composition (% wet weight basis) of Nile tilapia fed diets containing 0.0, 2.5, 5.0 and 10 g MLP kg^{-1} diet.

Parameters	Experimental Diets				*P value*
	CNT	$MLP_{2.5}$	MLP_5	MLP_{10}	
Moisture	76.69 ± 0.19^{a}	73.68 ± 0.39^{b}	73.50 ± 0.82^{b}	73.80 ± 0.56^{b}	0.001
DM	23.31 ± 0.22^{b}	26.32 ± 0.30^{a}	26.50 ± 0.33^{a}	26.20 ± 0.26^{a}	0.001
CP	58.69 ± 0.44^{d}	61.40 ± 0.14^{c}	63.22 ± 0.24^{b}	65.01 ± 0.35^{a}	<0.001
CF	18.17 ± 0.12	18.54 ± 0.42	20.89 ± 0.13	18.95 ± 0.48	0.106
Ash	20.71 ± 0.04^{a}	17.11 ± 0.44^{b}	13.22 ± 0.63^{c}	12.74 ± 0.16^{c}	<0.001

Values within the same row having different superscripts are significantly different ($P < 0.05$). Data were presented as the mean ± standard error. MPL, miswak leaf powder; CNT, control, DM, dry matter; CP, crude protein; CF, crude fat.

Table 5
Relative level of protection (RLP) of Nile Tilapia fish fed MLP supplemented diet against *Aeromonas hydrophilia* challenge test.

MLP (g Kg^{-1} diet)	Total number	Dead fish	SR (%)	MR (%)	RLP (%)
CNT (0.0)	20	7	65	35	–
2.5	20	5	75	25	28.57
5	20	3	85	15	95.71
10	20	0	100	0	100

MLP, miswak leaf powder; CNT, control; SR, survival rate (%); MR, mortality rate (%); RLP, relative level of protection (%).

4. Discussion

Despite the wide spectrum usage of medicinal plants, until now, there is still a lack of information about the pharmacological role and safe levels of MLP in farmed Tilapia fish against pathogenic infectious diseases. Several parts of the miswak (roots, branches, leaves and flowers) were used to promote the growth and health status of fish when used as feed additives (Lebda et al., 2019; Abd El-Naby et al., 2020a).

The existing trial illustrated that fortified tilapia diets with a low level of MLP (2.5 or 5 g Kg^{-1}) significantly enhanced production, feed efficiency, and chemical composition of the fish body (increased crude protein percentage and reduced ash content). The obtained results were similar to Toutou et al. (2019) and Lebda et al. (2019) in tilapia fish. El Mostehy et al. (1983) investigated that the miswak had high quantities of several compounds (fluoride, sulfur, silica, ascorbic acid, saponins, tannins, flavonoids, and other sterols). Also, fortified Nile tilapia diets with ginseng herb containing some bioactive compounds (saponin triterpenoid glycosides) promoted the performance, feed efficiency and utilization as well as enhanced some hematological indices (Goda, 2008). Moreover, supplemented Nile tilapia diets with ginseng saponins or some related triterpenoid compounds at levels 50, 100, 150, 200 or 250 mg kg^{-1} exhibited significantly higher performance than fish fed a basal diet (Shibata, 2001).

In addition, the improvement of fish growth and feed utilization process may be attributed to the influence of some potent bioactive molecules existing in MLP (alkaloid) (Deswal et al., 2016) on promoting nutrient digestibility process throughout enhancing digestive enzyme activity thereby resulting in an increment of fish growth (Chakraborty et al., 2014). Moreover, the fish production improvement following the dietary administration of MLP could be associated with several factors like species of fish, treatment period, miswak-supplemented form and levels (Yousefi et al., 2019), the feed palatability, improved appetite, and, subsequently, increased feed consumption (Kubiriza et al., 2019). The higher growth performance incorporated with low level of miswak may be due to the presence of saponin. Rezaei (2020) investigated that high levels of saponin in the diet can reduce feed consumption and weight, as well as prevent active nutrients, such as vitamins and minerals, from being absorbed in the intestine, decreasing fertility and protein digestibility.

The utilization and efficiency of feed are linked to the intestinal microbiome activity and stimulation of digestive enzymes, with high feed utilization and digestibility enriches fish performance and general health status (Dawood et al., 2019). The digestive enzymes such as protease, lipase, and amylase play an important role in the digestion and absorption of feed ingredients. The overall body metabolism is enhanced in cooperation with the high activity of the digestive enzymes (Dawood and Koshio, 2016). Herein, our study reveals that the inclusion of a 2.5 or 5 g MLP in the feed diet significantly promoted the activity of both amylase and lipase enzymes in the gastrointestinal tract. The effects of MLP in low levels may be due to the role of saponins on enhancing the

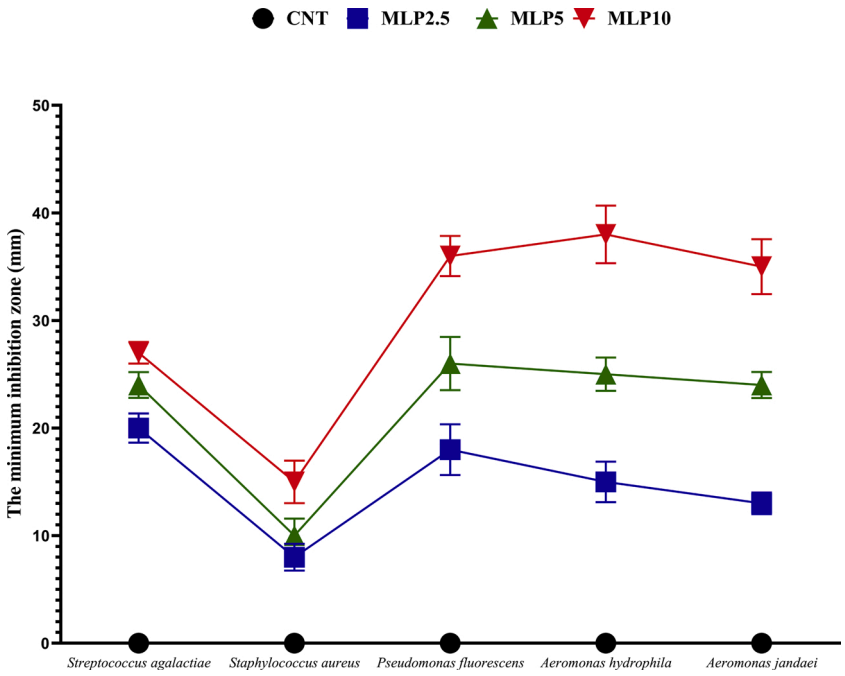

Fig. 1. The *in vitro* antibacterial activity (safe zone, mm) of ethanolic MLP extract against five selected bacterial strains. Data were presented as mean ± SD. While, MLP, miswak leaf powder and CNT, control.

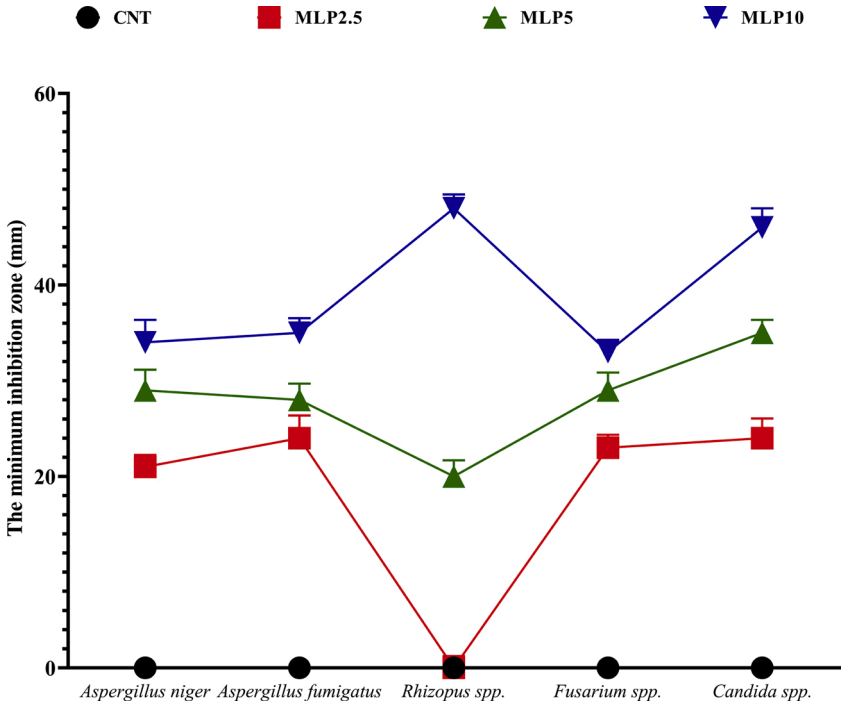

Fig. 2. The *in vitro* antifungal activity (safe zone, mm) of ethanolic MLP extract against five selected fungal strains. Data were presented as mean ± SD. While, MLP, miswak leaf powder and CNT, control.

intestinal membrane permeability to digested dietary nutrients and activated the digestive enzyme secretions (Francis et al., 2005). These obtained results were in line with Serrano Jr et al. (2000) and Francis et al. (2001) findings in carp and tilapia, respectively.

Nowadays, the antioxidant parameters showed important indices used in the intensive culture system to exhibited the fish general health status (Fazio et al., 2013a; Fazio, 2019). Thus, blood stress markers have been determined, such as GSH, catalase, MDA, and SOD. They effectively indicated the fish health status in several recent studies (Fazio et al., 2013b; Abd El-Rahman et al., 2019). In the present study, fortified tilapia diets with a high level of MLP significantly stimulated the GSH, catalase and SOD activity as well as reduced the MDA levels. Likewise, the obtained results were in similarity to Lebda et al. (2019) finding that the miswak dietary administration enhanced the gene expression of CAT and GPx, which implies the highly antioxidant efficiency of miswak on fish hepatocytes. The main antioxidant activity of

MLP may be due to the presence of several antioxidant molecules in miswak such as benzyl isothiocyanate, aniline, carvacrol, benzyl nitrile, naphthalene, and benzaldehyde (Noumi et al., 2011; Kumari et al., 2017). In addtion, Al-Dabbagh et al. (2018) proved the highest antioxidant activity of miswak because it is enriched with high amounts of the total polyphenolic and flavonoid compounds that display an antioxidant role. Moreover, Ibrahim et al. (2015) exhibited that high miswak content from total phenolic and flavonoid molecules is responsible for the effective reported antioxidant role of miswak through modulating the hydrogen peroxide scavenging ability and 1,1-diphenyl-2-picrylhydrazyl (DPPH) mechanisms. These findings were incorporated with Chakraborty et al. (2014) who investigated that alkaloids, flavonoids, pigments, phenolics, terpenoids, and steroids have been stated to promote several biological activities such as performance, consumption of feed, immunostimulant, anti-stress and increased antimicrobial properties of fish. In the same context, several studies provide support to our obtained results (Citarasu, 2010; Chakraborty and Hancz, 2011).

Recently, using medicinal plants as an immunostimulant to promote immune activity is rapidly increments to protect fish from infectious pathogens (Abd El-Naby et al., 2019; Abd El-Hakim et al., 2020). The main indicators of the adaptive immune responses are complement C3, lysozyme, and NBT activities (Magnadóttir, 2006). Lysozyme showed high effectiveness against a wide range of gram-negative and gram-positive bacteria (Watts et al., 2001). The complement activity also represented an indication of an innate immune activity, enhanced resistance against the pathogen (Chiu et al., 2008; Biller-Takahashi et al., 2012).

The current results are in agreement with obtained data from previous experiments that showed the high efficiency of some herbs and their derivatives to stimulate the immune activity in fish infected with bacterial diseases. Reyes-Cerpa et al. (2018) showed that supplemented fish diets with several plant extracts (*Rosmarinus officinalis*, *Melissa officinalis* and *Hypericum perforatum*) promoted the upregulation of the immune relative genes. At the same trend, fortified beluga fish (*Huso huso*) diets with *Carthamus tinctorius*, *Rosa canina* seeds, and *Allium cepa*, revealed high lysozyme, complement, and IgM levels (Akrami et al., 2015; Dadras et al., 2016). Besides, Mirghaed et al. (2018) investigated that an increment in cineole dietary concentrations significantly promoted total complement and lysozyme serum activities in rainbow trout. Also, dietary administration of *Cynodon dactylon* leaf for 60 days indicated high enhancement in immune responses (lysozyme and complement) in Indian carp (*Catla catla*) (Kaleeswaran et al., 2011). The miswak contents from ascorbic acid presented highly effective in boosting the immune status and diminishing the adverse effects of pathogens (El Mostehy et al., 1983). Thus, the significant enhancement of NBT, lysozyme, and complement activities proposes the immunomodulatory properties of MLP in Nile tilapia.

Previous studies investigated the high antibacterial and antifungal properties of several herbal ethanolic extracts such as *Origanum vulgare*, *Ocimum tenuiflorum*, *Cymbopogon*, *Rosmarinus officinalis*, thyme, and *Aloe vera* (Aqil et al., 2005; Oskay et al., 2009; Mehrotra et al., 2010; Abd El-latif et al., 2021). Regarding the results obtained from the *in vitro* antibacterial and antifungal experiments, the MLP extract showed strong antibacterial and antifungal effects against all examined strains. In line with our *in vitro* antibacterial trial, the high level of MLP-exhibited the highest RLP and survival rate and lowest mortality rate compared with the control group.

Sofrata et al. (2008) illustrated that implanted miswak in agar plate had strong antimicrobial activity against all tested bacterial strains. Also, Abhary and Al-Hazmi (2016) described similar antimicrobial results for miswak ethanolic extract against *Escherichia coli*, *Staphylococcus aureus*, *Lactobacillus acidophilus*, *Streptococcus mutans* and *Pseudomonas aeruginosa*. The miswak antimicrobial activity may be due to the presence of volatile active antibacterial molecules. Hence, extracted miswak by ethanol showed high antimicrobial activity against gram positive bacterium, which proposes that there are selective semi-polar antimicrobial agents in miswak.

Similar to our antifungal results, Saddiq and Alkinani (2019) investigated that ethanolic extract of a high concentration of miswak (100 mg/mL) revealed a prohibiting effect on the development of the tested fungal strains (*A. niger*, *A. flavus*, and *A. fumigatus*). Also, Naseem et al. (2014) exhibited that miswak has high potential antifungal properties against *Candida albicans*. The powerful antifungal efficiency of miswak may be attributed to high amounts of salvadorine and sulfur compounds (Al-Bayati and Sulaiman, 2008).

In conclusion, fortified tilapia diets with low levels of MLP promoted growth and enhanced feed utilization. While, the high level of MLP may be ascribed to activate antioxidant and immune status, as verified by promoted complement activity, respiratory bursts and lysozyme levels. Also, MLP showed high potential antibacterial and antifungal properties against common infected pathogens by diminishing mortality rate. Hence, MLP at 5 up to 10 g kg^{-1} diet might be a safe and valuable immunomodulatory agent that could be applied in the farm management against infectious diseases, promoting general fish health status for sustainable aquaculture.

CRediT authorship contribution statement

Mohammed A.E. Naiel: Methodology (support); Formal analysis (lead); Data Curation (lead); Writing - Original Draft (lead); Writing - Review & Editing (lead). **Mohamed K. Khames:** Conceptualization (lead); Methodology (lead); Formal analysis (support); Data Curation (support); Writing - Review & Editing (support). **Nashwa Abdel-Razek:** Methodology (support); Formal analysis (support); Data Curation (support); Writing - Review & Editing (support). **Amany A. Gharib:** Validation (lead); Visualization (lead); Methodology (support); Writing - Review & Editing (support). **Khaled A. El-Tarabily:** Investigation (lead); Supervision (lead); Conceptualization (support); Methodology (support); Validation (support); Visualization (support); Writing - Original Draft (support); Writing - Review & Editing (support).

All Authors had been equally revised and approved the final revised manuscript version.

Declaration of Competing Interest

The authors report no declarations of interest.

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