AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

Dietary administration of *Moringa oleifera* extract enhances growth, fatty acid composition, antioxidant activity and resistance of shrimp *Litopenaeus vannamei* against *Photobacterium damselae*

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Article Info

Article history:
Received 10 January 2021
Revised 24 January 2021
Accepted 27 January 2021
Available online 9 April 2021

Keywords:
Antioxidant activity,
Fatty acid composition,
Immune response,
Litopenaeus vannamei,
Moringa oleifera

Abstract

Adverse effects of antibiotics used for disease control in aquaculture have become a global concern. This has enhanced research on using natural products such as herbs as an alternative for disease control in aquatic animals. This study was conducted to evaluate the effect of *Moringa oleifera* extract (ME) on growth, fatty acid composition, antioxidant activity and resistance of shrimp (*Litopenaeus vannamei*) after challenge with *Photobacterium damselae* bacteria. In total, 600 post-larvae shrimps (initial weight: 0.87 ± 0.1 g) were randomly distributed into 12 fiberglass tanks (n = 50 per tank) and divided into four trial groups. The control group (ME0) was fed with an ME-free basal diet. Other groups were fed with the basal diet supplemented with 0.5 g/kg diet (ME0.5), 1.0 g/kg diet (ME1) and 1.5 (ME1.5) g/kg diet. Shrimps were fed at 10% of body weight for 8 wk. The results showed that *M. oleifera* enhanced shrimp growth (specific growth rate, final weight and weight gain) and DHA compared to the control (ME0), although no significant differences were found for the ME0.5 and ME1 groups. However, both the growth and DHA levels declined in the shrimp fed with ME1.5. The antioxidant activity (SOD, GPx and CAT) and fatty acid composition (PUFA, EPA, DPA, ARA and SFA) of the shrimp fed with ME1 increased while the cumulative mortality of the disease-challenged shrimp significantly ($p < 0.05$) decreased in the ME1 group compared to the other groups. Overall, *M. oleifera* extract at 1g/kg diet seemed to be the most appropriate level for increasing growth, fatty acid composition, antioxidant activity and disease resistance of *L. vannamei*.

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https://doi.org/10.34044/j.anres.2021.55.2.01

Introduction

Sustainable aquaculture development is required to increase aquaculture production to reduce poverty and malnutrition without endangering the environment (Kamble et al., 2014, Akbary and Jahanbakhshi, 2018). Shrimp is an important aquaculture commodity and the white shrimp, *Litopenaeus vannamei* is currently the major cultured species worldwide (Chang et al., 2013; Sudaryono et al., 2015). However, various pathogens are major constraints to the shrimp farming industry. Outbreaks of infectious diseases, such as bacterial infections, have caused severe damage to global shrimp production (Huxley and Lipton, 2009; Traifalgar et al., 2009; Stentiford et al., 2012; Kumaran and Citarasu, 2016).

Antibiotic resistance is a health problem globally (World Health Organization, 2002, 2004; Henning, 2006). This has triggered a growing demand for herbal products as alternative drugs (Ayotunde et al., 2011). Tolerance of plant extracts by the host also suggests that a reliable treatment regimen can be developed from herbal products to treat aquaculture diseases (Dewangan et al., 2010). Plants are accessible in a wide range with reasonable prices, hence herbal protein sources should be considered in aqua feeds (Food and Agriculture Organization of the United Nations, 2007).

There is a demand to recognize protein-rich plant sources for aquatic diets. The drumstick tree, *Moringa oleifera*, a member of the Moringaceae family, is an angiosperm plant with a variety of valuable impacts, depending on the plant part and inception. For example, *M. oleifera* is of great interest because its leaves, flowers and seeds can all be used as food resource (Puycha et al., 2017). Various portions of this species have been used throughout history due to its nutritional and therapeutic importance (Mbikay, 2012; Santos et al., 2012; Leone et al., 2015). The leaves are rich in protein, vitamins and minerals such as calcium, phosphorus, magnesium, ascorbic acid and tocopherol (Egwui et al., 2013), β-carotene and various phenolics and all parts have medicinal properties, such as cardiovascular stimulant, anti-tumor, antipyretic, anti-inflammatory, anti-coagulant, anti-spasmodic, antihypertensive, cholesterol-lowering, antioxidant, anti-diabetic, hepato-protective, antibacterial and antifungal properties (Anwar et al., 2007). Therefore, *M. oleifera* is widely consumed by humans and animals. Information about its impacts on immune response, antioxidant activity and the growth of different aquatic species is available (Shourbela et al., 2020). The antimicrobial influence of *M. oleifera,* has been studied since 1980s and appears to be associated with specific components containing pterygospermin and *Moringa* glycosides, 4-(α-L-rhamnosyloxy)-benzyl isothiocyanate and 4-(α-L-rhamnosyloxy)-phenyl-acetonitrile (Jahn et al., 1986; Viera et al., 2010).

Since 2009, a new bacterial infection has become widespread, which has led to serious economic losses in the shrimp industry (Zhang et al., 2012). *Photobacterium damselae*, is a marine species of the family Vibrionaceae (Farmer and Hickman-Brenner, 2006). This bacterium has been reported as an emerging bacteria in marine aquaculture (Labella et al., 2011). Spontaneous mortalities among tiger shrimps (*Penaeus monodon*) (Song et al., 1993; Vaseeharan et al., 2007) and *L. vannamei* (Viera et al., 2010; Liu et al., 2016) have been reported to be associated with this bacterial species.

Several studies have characterized *M. oleifera* and its beneficial activities in the bodies of aquatic animals (Richter et al., 2003; Alishahi et al., 2010; Yuangsoi and Charoenwattanasak, 2011; Heidarieh et al., 2013; Stadtlander et al., 2013; Abo-State et al., 2014; Lemmens, 2014; Leone et al., 2015; Tekle and Sahu, 2015; Puycha et al., 2017; David-Oku et al., 2018; Shourbela et al., 2020), its antibacterial activity (Viera et al., 2010; Ferreira et al., 2011; Peixoto et al., 2011; Bichi et al., 2012; Oluduro, 2012; Rajamanickam and Sudha, 2013) and antioxidant activity (Chien et al., 2003; Atli and Canli, 2007; Jiang et al., 2009; Luqman et al., 2011; Halliwell and Gutteridge, 2015). Antimicrobial properties of *M. oleifera* have been reported in shrimps including *Penaeus indicus* (Rayes, 2013) and *L. vannamei* (Costa, 2011; Lotaka and Piyatiratitivorakul, 2012).

Previous studies lack information on the growth performance of *Litopenaeus vannamei* as a response to being fed *M. oleifera* meal, which is considered a cheap source of protein in shrimp feed formulations. In addition, despite strong scientific evidence of the bioactivity of *Moringa* against bacteria, investigations of its effects on *Photobacterium damselae* are still under way. Thus, an evaluation of the effects of supplemented diets with *M. oleifera* on growth performance, fatty acid composition and antioxidant activity of *L. vannamei* has been carried out, and additionally, this research aimed to evaluate the bioactive potential from water extract of *M. oleifera* against *P. damselae* in this shrimp species.

Materials and Methods

Preparation of M. oleifera water extract and experimental diets

Seeds of *M. oleifera* plants were collected in mid-November 2015 from the shore area of Tis port, in southeastern Iran, north of the Oman Sea (Chabahar Bay). The seeds were thoroughly washed in distilled water and air-dried at 60°C. Preparation of *M. oleifera* extract (ME) was carried out according to the method of Choi et al. (2015). Briefly, 30 g of dried *M. oleifera* seed was ground, sieved (pore size < 0.5 mm) and added to 750 mL of deionized water and boiled for 4 hr. Then, centrifugation was carried out at 18,500×*g* for 10 min at 10°C. The supernatant was concentrated under reduced pressure at 60ºC. To obtain four experimental diets at inclusion levels of 0 g/kg ME, 0.5 g/kg ME, 1.0 g/kg ME and 1.5 g/kg ME, the ME was mixed with the ingredients of the basal control diet (Table 1), then oil and 30% distilled water were added and further mixed. The resultant wet dough was pelletized into 1 mm particles using a modified handmade grinder (National, Japan). The experimental diets were air-dried and kept at 4°C until use.

Table 1 Ingredients and chemical composition of basal control diet

Ingredient	g/kg diet
Fish meal	300
Soybean meal	80
Wheat meal	70
Squid meal	350
Shrimp meal	100
Yeast	20
Fish oil	20
Lecithin	40
Vitamins and minerals[a]	20
Analyzed chemical composition diet	g/kg diet
Moisture	83
Crude protein	267
Crude lipid	97
Ash	108
Crude fiber	9
Nitrogen free extract	436

[a]Vitamins = vitamin A, 3,000 international units (IU)/g; vitamin D, 2,500 IU/g; vitamin E, 50,000 mg kg^{-1}; Minerals = $MgSO_4$, 20,000 mg kg^{-1};$CuSO_4$; 40,000 mg kg^{-1}; ZnSO4, 15,000

Shrimp and experimental design

This research was conducted in the Offshore Fisheries Research Center (Chabahar, Iran) in May 2015. In total, 600 post larvae of white leg shrimp *L. vannamei* (mean weight: 0.87 ± 0.01 g) were obtained from a private hatchery (Chabahar, Iran). The shrimp larvae were stocked into two 300 L fiberglass tanks for rearing for 2 wk for acclimatization and were fed with a control feed. One-third of the water in each tank was changed daily. Wastes were removed by siphoning. Thereafter, shrimps were randomly divided into four groups (at three replications for each group) at a stocking density of 50 shrimp for each tank. The control group (ME0) was fed the basal diet without ME. The other shrimp groups were fed with the basal diet supplemented with ME at levels of 0.5 g/kg diet (ME0.5), 1 g/kg diet (ME1) and 1.5 g/kg diet (ME1.5) g/kg diet. During the 2 wk experimental period, shrimps were daily fed with the determined diet at a rate of 10% of body weight on all days of the week. All shrimps were weighed and the amount of food was set and updated according to their actual body weight changes. Feeding was carried out manually three times a day at 0800 hours, 1300 hours and 1700 hours. Monitoring twice a day (0800 hours and 1400 hours) was undertaken of the following water parameters: pH, dissolved oxygen (DO) and water temperature, using a digital pH meter (Model Photoic 20; Labtech International Ltd.; Heathfield, UK), a digital DO meter (Model AVI-660; Labtech International Ltd.; Heathfield, UK) and a mercury-in-glass thermometer, respectively (Akbary et al. 2020). During the experimental trial, average values of the water salinity, pH, dissolved oxygen, temperature and total ammonia nitrogen were maintained at 37 parts per trillion, 8 ± 0.2, 7.5 ± 0.65 mg/L, 28.4°C ± 0.7 and 0.1 ± 0.03 mg/L which were suitable for shrimp culture (Jahanbakhshi et al., 2012; Akbary and Aminikhoei, 2018). Experimental protocols were performed in accordance with the Iranian animal ethics framework under the supervision of the Iranian Society for the Prevention of Cruelty to Animals and the Shiraz University Research Council (IACUC no: 4687/63).

Growth performance analysis

The shrimp growth parameters were evaluated in terms of weight gain percentage (WG), average daily gain (ADG) and specific growth rate (SGR). The growth parameters of protein efficiency ratio (PER) and feed conversion ratio (FCR) were calculated according to Akbary and Jahanbakhshi (2016) and Harikrishnan et al. (2011) as shown in Equations 1–5:

Weight gain = ([Final body weight □ Initial body weight] / Initial body weight) × 100 (1)

Specific growth rate = ([ln Final body weight □ ln Initial body weight] / days) × 100 (2)

Feed conversion ratio = (Wet weight gain / Feed intake) × 100 (3)

Average daily gain = (Final weight - Initial weight) / Time (d) (4)

Protein efficiency ratio = Weight gain / Protein fed (5)

Fatty acid analysis

After the 8 wk experiment, 15 shrimps from each treatment group (five sampled from each tank) were collected, the shell and head of each shrimp were discarded and muscle samples were freeze-dried using a 5–l freeze-drier (DORSA Tech; Tehran, Iran) at -40ºC for 3 d in the dark. The dried muscles were passed through a grinder (M20 Universal; IKA Works Inc.; Wilmington, NC, USA) and stored at -20ºC for fatty acids analysis. Fatty acid esterification was analyzed in a mixture of toluene/methanol based on the method described by Kang and Wang (2005). Fatty acid methyl esters (FAMEs) were extracted with hexane. After the solvent had evaporated, the FAMEs were prepared for injection by dissolving them in iso-octane. Quantitative determination of fatty acids was performed using a gas chromatograph (GC-2010; Shimadzu; Tokyo, Japan). The injector and flame ionization detector temperatures were 260°C and 300°C, respectively. The silica capillary column (Supelco SP-2560; Sigma-Aldrich; St Louis, MO, USA; 100 m, 0.25 mm, film thickness 0.20l m) temperature was programmed in the range 140–200°C at a rate of 15°C/min, then from 200°C to 240°C at a rate of 2°C/min. Nitrogen was used as the carrier gas. Fatty acids were identified by comparing the relative retention time with the reference standards (Supelco, 37 components FAME Mix; Supelco Inc.; Bellefonte, PA, USA). The content of fatty acids was quantified by comparing the area with that of the internal standard and expressed as a percentage of total FAME.

Antioxidant activity assay

At the end of the experimental trial, six shrimp from each tank were randomly collected for antioxidant activity assay. They were frozen immediately in liquid nitrogen and stored at 70ºC. For assay, each shrimp was defrosted and homogenized in 10 × (weight per volume) phosphate buffer solutions (NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.42 g, KH_2PO_4) on ice. The homogenates were centrifuged (3,000 revolutions per minute for 10 min) at 4ºC. The supernatants were kept at -70ºC until required to be analyzed. The SOD activity unit was defined as the amount of specimen which catalyzed decomposition of 1 mol of O_2- into H_2O_2 and O_2 per minute. Absorbance was recorded at 550 nm. glutathione peroxidase (GPx) was conducted using a commercially available chemical colorimetric assay kit (ZellBio GmbH; Ulm, Germany). When H is oxidized to GSSG, GPx decreases to cumene hydro-peroxide. The produced GSSG is reduced to GSH by consuming nicotinamide adenine dinucleotide phosphate (NADPH) through glutathione reductase. The declining NADPH (that is readily measured at 340 nm) will be proportionate to the activities of GPx. Measurement of CAT action was carried out using a calorimetric enzymes assay kit at 405 nm. The CAT performance unit was defined as the amount of specimen catalyzing decomposition of 1 µmole of H_2O_2 to water and O_2 in 1 min. CAT at 0.5 Um/L sensitivity, was determined using this technique. The liquid peroxidation (MDA) level was measured using a commercially available chemical colorimetrical assay kit based on the manufacturer's instructions (MDA assay kit; ZellBio GmbH; Ulm, Germany). It applies MDA-TBA (thiobarbituric acid) adduct generation via reacting MDA and TBA at high temperatures. Measuring the MDA was carried out in an acidic medium at 90–100°C and 535 nm. MDA of 0.1 µM sensitivity was determined using this procedure. Total soluble proteins were measured using the method of Bradford (1976) with bovine serum albumin as a standard. Each enzyme activity was stated as activity (U/mg protein). Each enzymatic assay was performed in triplicate (Bradford, 1976).

To measure the phenoloxidase (PO) activity, four shrimps from each tank were homogenated and centrifuged at 700×*g* and 4°C for 20 min Afterward, the supernatant fluids were eliminated and pellet was washed, resuspended slowly in cacodylate citrate buffer (0.45 M NaCl, 0.01 M CAC, pH: 7.0, 0.10 M $Na_3C_6H_5O_7$) and centrifuged once more. Next, re-suspension of the pellet was done with 200 µL CAC buffer—0.26 M $MgCl_2$, 0.45 M NaCl, 0.01 M $CaCl_2$, pH: 7.0, 0.01 M CAC—and incubation of a 100 µL aliquot was carried out using 50 µL trypsin (1 mg/mL), serving as an activator for 10 min at 25–26°C. Then, 50 µL of L-3,4-dihydroxyphenylalanine (DOPA) was included, followed by 800 µL of CAC buffer 5 min later. The spectrophotometer was used to measure the optical density at 490 nm.

Challenge producer

After 8 wk of the experimental trial, the effect of *M. oleifera* extract included in the feed for the disease resistance (cumulative mortality percentage), was investigated in a shrimp sample (n = 30/group). The Sk7 strain of *P. damselae* was separated from the juvenile shrimps by the Iran Veterinary Organization (IVO), Chabahar province, and then was grown on brain heart infusion broth (BHI, Sigma) at 30°C for 24–48 hr. Bacterial cells were washed twice with sterile phosphate buffered saline solution and then re-suspended in the same solution to obtain a bacterial suspension. The bacterial concentrations were adjusted to lethal dose to kill half the population (LD50) = 7.2×10^4 colony forming units/mL with the suspension optical density (Austin and Austin, 2007). Ultimately, shrimps were immersed into aquarium water which was inoculated with bacteria for 4 hr. The data for cumulative mortality were recorded throughout a 10 d challenge test.

Statistical analysis

Data was presented as mean ± SD. Effects of treatments were analyzed using one-way analysis of variance ANOVA. Duncan's multiple range test was use to compare differences among treatments and results were regarded as significant at $p < 0.05$. The analyses were performed using the SPSS software version 22 (Armonk, NY, USA).

Results

Growth performance and protein efficiency ratio

At the end of the feeding trial the body weight (BW) of the control shrimp was 1.55 ± 0.05 g while the BW of the groups treated with ME ranged between 1.49 ± 0.04 g and 1.71 ± 0.04 g. The growth performance data (Table 2) showed that dietary supplementation with ME at 1 g/kg diet resulted in significant increases in the WG, SGR, ADG and FW compared to the ME 0 and ME 1.5 groups. Noteworthy, the effects of the *M. oleifera* supplement tended to be dosage-dependent whereby the growth performance of shrimps was enhanced as the concentration of ME in feed increased from 0 g/kg to 1 g/kg but the growth declined when the concentration was increased to 1.5 g/kg diet.

A similar trend was also observed for the protein efficiency (PER) ratio, as the PER significantly increased as the dosage of ME increased (7.93 ± 0.74) to 1 g/kg diet and then significantly reduced in the group fed ME at 1.5 g/kg diet.

Fatty acid composition

The fatty acid composition of shrimps fed different experimental diets is presented in Table 3. Diet supplemented with ME at 1 g/kg diet produced significant increases in SFA, C12:0, C14:0, C15:0, C16:0, C17:0 C18:0, PUFA, DPA and EPA compared with the other ME groups and the control group. The dosage-dependent trend was also evident, as the level of the fatty acids increased as the concentration of ME increased but declined as the concentration of ME reached 1.5 g/kg diet. However, the MUFA level was not significantly different among all groups. A significant increase in DHA was observed in shrimp diets with ME 0.5 and ME 1 compared with those of shrimp fed the control diet (ME0).

Table 2 Growth performance of *Litopenaeus vannamei* fed experimental diets containing different levels of *Moringa oleifera* extract (ME) for 8 wk

Parameters	Experimental diets			
	ME (g/kg)			
	0	0.5	1	1.5
Initial body weight (g)	0.88 ± 0.01[a]	0.87 ± 0.01[a]	0.86 ± 0.01[a]	0.87 ± 0.00[a]
Feed conversion ratio (%)	1.01 ± 0.07[a]	1.03 ± 0.02[a]	1.00 ± 0.01[a]	1.05 ± 0.04[a]
Weight gain (%)	76.68 ± 5.57[c]	92. 66 ± 7.25[ab]	98.81 ± 6.21[a]	70.63 ± 5.59[bc]
Specific growth rate (%)	0.93 ± 0.21[bc]	1.07 ± 0.05[ab]	1.13 ± 0.05[a]	0.87 ± 0.06[c]
Average daily gain (g/d)	1.26 ± 0.57 [c]	1. 54 ± 0.37 [b]	1.64 ± 0.21[a]	1.19 ± 0.19[d]
Protein efficiency ratio	1.86 ± 0.12[c]	5.38 ± 0.70[b]	7.93 ± 0.74[a]	1.42 ± 0.12[c]

Mean ± SD (n =3) with different lowercase superscripts in the same row are significantly ($p < 0.05$) different.

Table 3 Fatty acid composition (% total fatty acids) of whole body of *Litopenaeus vannamei* fed experimental diets containing different levels of *Moringa oleifera* extract (ME) for 8 wk

Fatty acid	Experimental diet			
	ME (g/kg diet)			
	0	0.5	1	1.5
C12:0	0.27 ± 0.02 [d]	0.54 ± 0.01[c]	0.87 ± 0.07[a]	0.66 ± 0.02[b]
C14:0	1.18 ± 0.20[b]	0.60 ± 0.03[d]	1.44 ± 0.01[a]	0.76 ± 0.03[c]
C15:0	1.91 ± 0.02 [b]	1.58 ± 0.08 [d]	2.57 ± 0.03 [a]	1.65 ± 0.01 [c]
C16:0	21.76 ± 0.14 [b]	21.47 ± 0.02 [b]	24.53 ± 0.06 [a]	20.79 ± 0.10 [c]
C17:0	0.91 ± 0.03 [b]	0.84 ± 0.01 [c]	1.14 ± 0.01 [a]	0.80 ± 0.03 [c]
C18:0	8.79 ± 0.45 [d]	10.34 ± 0.12 [b]	11.53 ± 0.04 [a]	10.09 ± 0.04 [c]
C20:0	0.29 ± 0.00 [b]	0.34 ± 0.01 [a]	0.21 ± 0.03 [c]	0.35 ± 0.05 [a]
C22:0	0.25 ± 0.01 [c]	0.30 ± 0.01 [b]	0.23 ± 0.02 [c]	0.38 ± 0.01 [a]
SFA	35.38 ± 1.17 [c]	36.05 ± 2.05[b]	48.32 ± 1.43 [a]	35.60 ± 1.15[c]
C16:1n	1.74 ± 0.01 [a]	1.79 ± 0.03[a]	1.70 ± 0.01[a]	1.77 ± 0.01[a]
C18:1n-9	20.94 ± 0.18 [a]	21. 00 ± 0.13[a]	20.99 ± 0.11[a]	20.90 ± 0.10[a]
C20:1n-9	3.00 ± 0.09[a]	2.93 ± 0.02[a]	2.94 ± 0.01[a]	2.96 ± 0.02[a]
MUFA	26.02 ± 0.18 [a]	25.97 ± 0.16[a]	25.90 ± 0.12[a]	26.03 ± 0.11[a]
C18:2n-6	16.93 ± 0.48 [a]	17.02 ± 0.17[a]	17.01 ± 0.18[a]	16.95 ± 0.27[a]
C18:3n-3	0.87 ± 0.02 [b]	0.91 ± 0.03 [ab]	0.90 ± 0.03 [ab]	0.90 ± 0.01 [a]
C20:3n-6	0.76 ± 0.03 [a]	0.75 ± 0.01[a]	0.71 ± 0.03 [a]	0.75 ± 0.01[a]
C20:4n-6	3.01 ± 0.01 [c]	3.49 ± 0.02 [b]	3.90 ± 0.07 [a]	3.80 ± 0.05 [a]
C20:5n-3	5.75 ± 0.24 [d]	7.36 ± 0.06 [b]	7.94 ± 0.03 [a]	6.57 ± 0.01[c]
C22:4n-6	0.37 ± 0.02 [a]	0.36 ± 0.01[a]	0.35 ± 0.01[a]	0.34 ± 0.06 [a]
C22:5n-3	0.18 ± 0.01 [c]	0.26 ± 0.01 [b]	0.36 ± 0.03 [a]	0.26 ± 0.04 [b]
C22:6n-3	8.89 ± 0.01 [c]	12.08 ± 0.05 [a]	10.77 ± 0.08 [ab]	9.46 ± 0.35 [bc]
PUFA	37.88 ± 0.18 [c]	42.80 ± 0.60 [b]	45.71 ± 0.43 [a]	40.68 ± 0.37 [cb]

SFA = saturated fatty acid; MUFA = mono-unsaturated fatty acid; PUFA = polyunsaturated fatty acid.
Mean ± SD (n =3) with different lowercase superscripts in the same row are significantly ($p < 0.05$) different.

Table 4 Total antioxidant capacity and antioxidant enzyme activities of *Litopenaeus vannamei* fed experimental diets containing different levels of *Moringa oleifera* extract (ME) for 8 wk

Antioxidant enzyme (U mg protein)	Experimental diet			
	ME (g/kg diet)			
	0	0.5	1	1.5
PO	18.96 ± 1.05 [b]	20.00 ± 0.11 [a]	20.26 ± 0.37 [a]	20.30 ± 0.35 [a]
SOD	11.42 ± 0.73 [d]	33.32 ± 0.16 [b]	39.73 ± 0.43 [a]	23.48 ± 0.77 [c]
GPX	172.39 ± 3.82 [d]	257.37 ± 1.72 [b]	286.29 ± 0.35 [a]	207.09 ± 0.55 [c]
CAT	0.45 ± 0.01 [d]	1.01 ± 0.01[b]	1.29 ± 0.02 [a]	0.94 ± 0.01 [c]
MDA	2.52 ± 0.13 [b]	2.29 ± 0.00 [c]	1.82 ± 0.24 [d]	2.67 ± 0.11 [a]

PO = phenoloxidase, SOD = superoxide dismutase, GPX = glutathione peroxidase; CAT = catalase; MDA = malondialdehyde.
Mean ± SD (n =3) with different lowercase superscripts in the same row are significantly different ($p < 0.05$).

Antioxidant activities

Data of antioxidant activities are shown in Table 4. Dietary supplementation with ME at 1 g/kg diet resulted in a significant decrease in malondialdehyde (MDA) values. It should be noted that MDA declined with the dosage of ME (2.29 ± 0 U mg protein and 1.82 ± 0.24 U mg protein for ME0.5 and ME1, respectively) but increased (2.67 ± 0.11 U mg) again when the dosage of ME was 1.5 g/kg diet. On the contrary, superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) activity significantly increased in all the ME-treated groups compared to the control. However, differences among the ME-treated groups were significant in the following order ME1 > ME0.5 > ME1.5. The activity of phenoloxidase (PO) for ME1 (20.26 ± 0.37) did not significantly differ with the other ME-treated groups, but significantly increased compared to the control group.

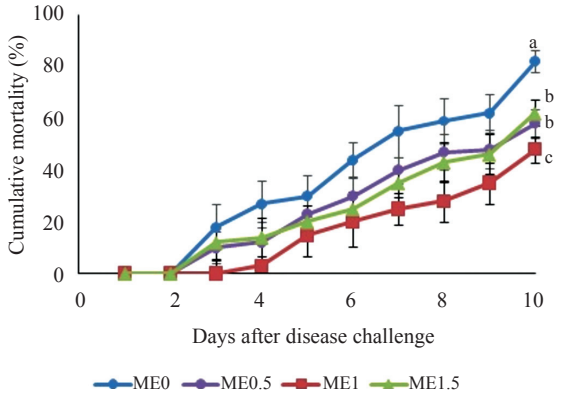

Fig. 1 Cumulative mortality (%) of *Litopenaeus vannamei* fed experimental diets containing different levels of *Moringa oleifera* extract (ME) for 8 wk and subsequently challenged with *Photobacterium damselae*, where different lowercase letters at 10 d after challenge indicate significant ($p < 0.05$) differences, error bars = ± SE and ME0 = 0 g/kg diet, ME0.5 = 0.5 g/kg diet, ME1 = 1.0 g/kg diet and ME1.5 = 1.5 g/kg diet

Resistance of shrimp L. vannamei challenged with P. damselae

After disease challenge, the first mortem was observed at 1 d and mortality stopped at 14 d after challenge. Supplementing the diet with any amount of ME significantly reduced mortality compared to the control group (Fig. 1). The lowest cumulative mortality was observed in the ME1-supplemented diet (45±8.5%) at 10 d after challenge with an LD50 of *P. damselae* (Fig.1). After 10 d of inoculation, the cumulative mortality percentages in diets supplemented with ME 0.5 and ME 1.5 were 58.3±8.90% and 58.5±9.09%, respectively. The cumulative mortality percentage for ME0 group increased from 20±1.5% on the 3rd day to 80±7.5% on the 10th day (Fig. 1).

Discussion

The positive impacts of *M. oleifera* water extract supplemented in feed were evident for *L. vannamei*, with supplementation of *M. oleifera* seed extract at 1g/kg (ME1) diet being the most appropriate level compared to the ME0 and ME1.5 groups without affecting the FCR. These results were in agreement with a report by Lotaka and Piyatiratitivorakul (2012) using the same species of shrimp with dry leaf powder of *M. oleifera*. Similar results were also reported for *Macrobrachium rosenbergii* fed with 0.25–0.5% *M. oleifera* leaf extract (Kaleo et al., 2019). Likewise, the positive effect was reported of water extract of *M. oleifera* on growth performance parameters such as the FCR, WG and SGR in Nile tilapia (*Oreochromis niloticus*) for 250–500 mg/kg (Stadtlander et al., 2013; Tekle and Sahu, 2015; Shourbela et al., 2020) as well as in African catfish (*Clarias gariepinus*) fed with 10% *M. oleifera* leaves (David-Oku et al., 2018) and Bocourti's catfish (*Pangasius bocourti*; Puycha et al., 2017). Increased growth rates and diet utilization can be linked to immune system properties and high digestibility, absorption and assimilation ability, through enhanced gastrointestinal enzymes and enhanced intestinal microflora due to *M. oleifera* prebiotic activity (Richter et al., 2003; Yuangsoi and Charoenwattanasak, 2011; Abo-State et al., 2014).

The current study also showed an adverse effect on growth when the shrimps were overfed with ME (at 1.5 g/kg diet). This was consistent with studies involving other aquatic species. Puycha et al. (2017) indicated that dietary *Moringa* sp. leaf should be considered in the diet of *P. bocourti* in such a way that the amount was not over 100 g/kg fish. The adverse growth of fish overfed with *M. oleifera* related to the presence of toxic materials or anti-nutritional factors (Kaleo et al., 2019). Phytates, tannins, glucosinolates, non-starch polysaccharides (NSPs), protease inhibitors, saponins, lectins and gossypols are ingredients from these plants which have a bitter taste that may cause low acceptability of feed to the experimental fish (Egwui et al., 2013). However, the toxic concentration of *M. oleifera* in the diet is dependent on the size, species and the plant part of *M. oleifera* utilized in the feed formulation (Kaleo et al., 2019).

Based on current knowledge, this was the first study on the effects of *M. oleifera* extract on the fatty acid composition of shrimps. The results showed there was a significant increase in DHA observed in shrimps fed diets containing ME0.5 and ME1. ME1 also produced significant increases in SFA, C12:0, C14:0, C15:0, C16:0, C17:0 C18:0, PUFA, DPA and EPA while the MUFA level was not significantly different among all groups. The increment of unsaturated fatty acids in the ME-treated shrimp may have been a direct effect from the high levels of the fatty acids in *M. oleifera* seed (such as 76% PUFA; Lalas and Tsaknis, 2002). In addition, foods high in protein and carbohydrates are likely to cause excessive satisfaction of energy within the aquatic species, which is converted to fatty acids and stored as lipids (Rønnestad and Hamre, 2001). Wood and Enser (1997) indicated that diets containing antioxidants can enhance the

fatty acid profile of meat. This may be an additional reason for the enhancement of the fatty acids content recorded in the current study because one of the important ingredients of *M. oleifera* leaves is phenolic compounds which are responsible for antioxidant activity (Makkar and Becker, 1997; Wei and Shibamoto, 2007). In accordance with the current study, Zhang et al. (2019) demonstrated that the fatty acid profile in the *longissimus dorsi* muscle of pigs was significantly modified when the animals were fed *Moringa* leaf meal diets; they also showed that the relative percentage of total unsaturated fatty acid and monounsaturated fatty acid was higher in the meat of pigs fed a 6% *M. oleifera* leaf meal diet than in the meat of those receiving the control diet.

An increment in fatty acids in the ME-fed *L. vannamei* observed in the current study was highly beneficial to shrimp health. Rønnestad and Hamre (2001) indicated that if the balance of fatty acids in an aquatic species is not maintained, then individuals will experience poor growth, enlargement of the liver due to the accumulation of neutral lipids and undeveloped membranes that require PUFA (such as the chloride cells in the gills). Therefore, the positive impact of *M. oleifera* on PUFA and other fatty acids in *L. vannamei* in the current research revealed its efficiency as a nutritional supplement.

The current results indicated that the antioxidant activity (SOD, GPx, CAT) of shrimp significantly increased in the ME1 group compared to the other groups, but it led to a significant decrease in MDA values. The SOD and GPX are sensitive oxidative stress biomarkers (Shourbela et al., 2020). Consistent with the current study, Kaleo et al. (2019) showed that *M. rosenbergii* fed with 0.5% *M. oleifera* leaf extract improved their CAT, SOD and GPx activities. SOD is an effective enzyme for scavenging ROS and protection of cells from free radicals (Chien et al., 2003). The antioxidant potential of *M. oleifera* by various mechanisms, such as reducing peroxide radicals and converting them into oxygen and water, has been confirmed in previous studies (Atli and Canli, 2007; Luqman et al., 2011; Halliwell and Gutteridge, 2015). *M. oleifera* contains active biological compounds (polyphenols, glycosides, anthocyanin, tannins and thiocarbamates). These active compounds expel free radicals and active antioxidant enzymes and inhibit oxidases (Luqman et al., 2011) which provides further clarification for the antioxidant activity associated with *M. oleifera*. Likewise, in the current study, the phenoloxidase (PO) activity of the shrimps fed with different levels of ME significantly increased compared to the control group.

Based on the results from the current research, supplementing the diet with any concentration of ME, significantly decreased mortality caused by *P. damselae* compared to the control group, with the lowest cumulative mortality percentage being observed in the ME1-supplemented diet. The bactericidal activity of *M. oleifera* plant extract has been well documented (Viera et al., 2010). Previous studies described its antibacterial activities against *Escherichia coli*, *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Salmonella enteritidis* (IH) and *Aeromonas caviae* (Vieira et al., 2010; Costa, 2011; Peixoto et al., 2011). However, the influence of extracts against *Photobacterium* has not been widely studied. Ethanolic extract of *M. oleifera* produced suitable antimicrobial activity against many bacterial species (Rajamanickam and Sudha, 2013). In addition, several studies confirmed the antimicrobial action of methanolic, ethanolic and aqueous *M. oleifera* leaves (Pal et al., 1995; Bichi et al., 2012; Oluduro, 2012; Onsare et al., 2013; Rayes, 2013; Abd El-Gawad et al., 2020). Peixoto et al. (2011) showed that the antibacterial ability of *M. oleifera* was related to compounds existing in aqueous and ethanolic *Moringa* leaf extracts. Furthermore, some studies have proved its antibacterial activities in cultured shrimps (Viera et al., 2010; Ferreira et al., 2011; Peixoto et al., 2011; Bichi et al., 2012; Oluduro, 2012; Rajamanickam and Sudha, 2013). Nevertheless, no specific research has been identified to date on its influence on the *Photobacterium* genus. Shrimps have an innate system of defense against infectious diseases, without recognition or long-term memory (Rendón and *Balcázar,* 2003). Therefore, it is vital to investigate diets which improve its immune system against bacteria.

The current study showed the capability of *M. oleifera* seed extract as a growth promoter and as an antibacterial and antioxidant for *L. vannamei* post larvae. Thus, it may be used to replace artificial antimicrobials and growth promoters but only at a concentration not exceeding 1g/kg (ME1) diet. However, further research is required on the safety issues of this plant extract, including its potential toxicity and environmental effects. Detailed studies on its impacts on *L. vannamei* in the grow-out period would be of interest and could make an important contribution in shrimp culture industries worldwide.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This study was a product of a project financially supported by the Iran National Science Foundation (INSF) and also funded by a research grant from Shiraz University. Valuable assistance was provided by personnel in the Iranian Fisheries Science Research Institute (IFSRI), and Offshore Fisheries Research Center, Chabahar, Iran.

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