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Antibacterial activity of *Moringa oleifera* and *Leucas aspera* flower extract against fish pathogens

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Original Article

Abstract

Today, more attention is focused on the use of medicinal herbal extracts to improve yield in aquaculture as alternatives to chemical agents. The present study evaluates the antioxidant and antibacterial activity of floral extracts of *Leucas aspera* and *Moringa oleifera* against *Aeromonas hydrophila* and *Vibrio parahaemolyticus*, the most common fish pathogens. The presence of bioactive compounds was confirmed using GC-MS/MS analysis. Antioxidant activity was analyzed using the DPPH assay. The IC_{50} value for free radical scavenging by *Moringa oleifera* flower extract and *Leucas aspera* flower in *Oreochromis niloticus* were 36 µg/ml and 73.89 µg/ml respectively. In in-vitro antibacterial analysis, both the extract at 10 mg/ml concentration showed activity against fish pathogen namely, *A. hydrophila* and *V. parahaemolyticus.* The *M. oleifera* flower extract showed maximum zone of inhibition (25mm, 40mm) and the *L. aspera* flower extract (21mm, 35mm). Plant extract based formulated fish feed could, therefore, be used in aquaculture, as a potent substitute against antibiotics for disease management.

Keywords*: Moringa oleifera, Leucas aspera, Aeromonas hydrophila, Vibrio parahaemolyticus, Oreochromis niloticus, antimicrobial*

Introduction

Fish disease outbreaks are one of the important reason for increased mortality rates and decreased productivity in fish farming. *Aeromonas hydrophil*a is one of the common infectious fish pathogen. They are nonspore-forming gram negative rod with monotrichous flagellum (Simmons and Gibson, 2012). *Vibrio parahaemolyticus* is also a gram-negative marine bacterium that inhabits the warm coastal and brackish waters. It is a common causative organism for seafood associated infections in humans (Letchumanan *et al*., 2014). Attack by these pathogens caused significant economic problem in developing countries over the past decades (Assefa and Abuna, 2018). The use of antibiotics is an important treatment procedure applied to control bacterial diseases in fish farming. However, the multiple usage of antibiotics results in antibiotic resistant bacterial emergence which can result in uncontrolled pathogen attack in aquaculture sectors. It is proven that some fish pathogens can cause disease in human, making aquaculture products as a potential risk to the consumers. Hence, there is an urgent need to establish new drugs and alternative therapies to prevent bacterial diseases (Castro *et al*., 2008).

There are large varieties of feed additives available as fish growth promoters or immune stimulators for disease resistance. These are mainly chemical products, hormone or any antibiotic

which can have profound side effects. From the beginning of human history onwards, medicinal plants are used in drug development for various diseases. Traditional folks have always fascinated the researchers by their usage of varieties of a single medicine for multiple diseases. These wild plants can be used for the preparation of natural drugs for a healthy life for humans and animals and can be also considered as a natural source of safer and easily available chemical substitute for growth promoters or immune boosters or can act as antibiotics in aquaculture.

Leucas aspera plant is mainly used as an insecticide and in the treatment of psoriasis. The juice of the flower is used for the treatment of cough and colds and this plant is reported to have anti-inflammatory properties (Das *et al.*, 2012). The flowers of *Moringa oleifera* are rich source of potassium and calcium (Jideani and Diedericks, 2014). Traditional medicine practitioners use the decoction of *M. oleifera* flowers which is used as an excellent herbal tonic for sexual weakness and functional infertility of both males and females (Mehta *et al.*, 2011). In addition to this, these flowers are also used for tumour treatment (Wang *et al.*, 2012). Studies proved that its leaves have immense nutritional value hence, *M. oleifera* is considered as an excellent plant to meet the increasing nutritional demands of the growing body. The aerial parts and root of this plant are useful in treatment of hundreds of diseases. It has been noted that this plant has antimicrobial, immune boosting and detoxifying activity (Sahay *et al.*, 2017). However, no extensive work on antimicrobial activity against fish pathogens has been performed.

Material and methods

Sample Collection

The flowers of *L. aspera* and *M. oleifera* were collected from nearby regions of the Ernakulam district of Kerala State. Each sample was washed separately under running tap water to remove epiphytes, animal castings, attached debris and sand particles and the final washing was done with distilled water. The samples were dried in air and were ground by an electrical mixer until they became fine powder. The powdered samples were stored at -20^{0}C until compound extraction.

Screening of Flowers for Phytochemical Analysis

Qualitative Analysis: *L. aspera* and *M. oleifera* flowers were extracted successively with water and methanol and were subjected to preliminary phytochemical screening for the presence of different phyto constituents. The extracts were tested for the presence of carbohydrates, proteins, phytosterol, steroids, flavonoids, glycosides, saponins, tannins, phenols, alkaloids and terpenoids according to the standard protocols using Molisch's reagent (alpha naphthol), Biuret reagent (sodium hydroxide (NaOH) and hydrated copper (II) sulfate, together with potassium sodium tartrate), liebermann- burchar test (acetic anhydride and concentrated sulphuric acid added to chloroform solution of test), sulphuric acid-acetic acid test, Aluminium chloride tests (1%), Keller kiliani test (glacial acetic acid and 5% Ferric chloride along with Sulphuic acid), foam test with distilled water, Ferric chloride test, Ellagic acid tests (5% glacial acetic acid along with 5% Sodium nitrite), Wagner's reagent (aqueous solution of iodine and potassium iodide) and Salkowski reagent (concentrated Sulfuric acid added to chloroform solution of test) respectively (Pandith, 2012) (Table 1).

Quantitative analysis

Determination of total phenolic content: The amount of total phenolic content in the sample was calculated by the Folin-Ciocalteu method. The absorbance of test samples, blank, control, Gallic acid standard was measured at 725 nm using a UV- Visible spectrophotometer (Alhakmani *et al.*, 2013).

Determination of total flavonoid content: The amount of total flavonoid content in the sample is calculated by the Aluminium chloride method. The absorbance of test samples, blank, control, Quercetin standard is measured at 510 nm using a UV- Visible spectrophotometer (Sankhalkar and Vernekar, 2016)

Table 1. Preliminary qualitative phytochemical analysis of various ethanol and aqueous extracts of *M. oleifera* and *L. aspera* flower.

Chemical Constituent	Ethanol extract		Water extract	
	Leucas aspera flowers	*Moringa oleifer*a flowers	*Leucas aspera* flowers	*Moringa oleifera* flowers
Carbohydrates	++	++	+	+
Proteins	-	+	-	-
Phytosterols	++	++	-	-
Phenolic Compounds	+	++	-	-
Cardiac glycosides	++	++	+	+
Alkaloids	++	++	+	+
Flavonoids	++	++	-	-
Saponins	-	-	-	-
Tannins	-	-	-	-
Terpenoids	+	+	-	-
Steroids	+	+	+	+

Antioxidant activity of ethanol and water extracts of L. aspera flowers and M. oleifera flowers

In-vitro analysis of free radical scavenging activity (DPPH• assay): Antioxidant activity was determined by the DPPH

assay. The absorbance of test samples, blank, control, Ascorbic acid standard was measured at 517 nm using a UV-Visible spectrophotometer. Ascorbic acid was used as a standard compound and IC_{50} values were calculated. The percentage of free radical scavenging activity of the test was calculated using the equation, DPPH scavenging effect (% inhibition) = $(A_0 - A_1)/A_0$ x 100 and IC_{50} values for the tests was calculated and compared with that of standard (Blois, 1958).

Antibacterial activity of ethanol and water extracts of L. aspera flowers and M. oleifera flowers

Well diffusion assay (Wikler, 2006): The modified well diffusion method was conducted to evaluate the antimicrobial activity of the ethanolic extract of *L. aspera* and *M. oleifera* flowers. Muller – Hinton agar medium with wells containing extracts were incubated with fish pathogens, *A. hydrophila* and *V. parahaemolyticus* for 24 hours, separately. After incubation, the diameter of the zone of inhibition was measured in millimetres.

GC-MS analysis: The gas chromatograph interfaced to a mass spectrometer (GC – MS) analysis was carried out using an Agilent, model no: 7890 A Gas Chromatograph coupled to a mass detector of Agilent make -5975c, (GC-MS) equipped with a DB 5ms capillary column (30 m× 0.25 mm × 0.25 μm). Injection temperature was maintained at 250°C and ion source temperature at 230°C. Helium gas (99.99%) was used as the carrier gas at a constant flow rate 1ml/min and an injection volume of 1μl was employed at a split ratio of 50:1. The instrument was set to an initial temperature of 40°C, and maintained at this temperature for 5 min. At the end of this period, the oven temperature was raised up to 280°C and maintained for 10 min. The mass spectra of compounds in samples were obtained by electron ionization (EI) at 70 eV. Total GC running time was 60 min. Relative percentage amount of each component was calculated by comparing its average peak area to the total area. Software adopted to handle mass spectra and chromatograms was a Turbo mass.

Diet formulation: Rice bran (15 g), *L. aspera flower* (Thumba) /*M. oleifera* were taken as principal ingredients. Paste was prepared separately at different concentrations (10, 20, 30 and 40%). Other ingredients like milk powder (10 g), cornflour (5 g) and eggs (5 g) were added and mixed well. Agar powder (5 g) was added as a binding agent. The constituents were boiled, cooled at room temperature. After cooling, cod liver oil (the volume was determined based on diet percentage), Glycine (5g), as well as vitamin mixture of vitamin B complex (5 g) and vitamin E (5 g) were added. It was kept under refrigeration for 12 hours. Then it was squeezed over polythene sheet and dried at room temperature for 48 hours. The dried nodules were crushed into small pellets and they were sun dried (to avoid fungal infection), weighed and stored in the bottle (Pandey, 2013). A control feed is also formulated by avoiding the plant extracts during feed preparation.

Experimental design: Active and healthy fish were chosen from acclimatization tank. Fishes of different age groups namely, 3, 6 and 12 months were used for each treatment. Fish belonging to 1st group were fed on a diet with *M. oleifera* and the 2nd were fed with *L. aspera*. The third group were fed with control feed (feed prepared without plant extract) and the final group of fishes were fed with synthetic feed (commercial feed) for 30 days.

Growth parameters: At the beginning of the experiment, the total weight of the fishes in each group was determined by weighing them on an electronic weighing balance. All fishes were weighed at the end of the experiment. Growth parameters such as body weight gain (BWG), and specific growth rate (SGR) were calculated as per the protocol of Ali *et al.* (2008) and Afuang *et al.* (2003). The results were analysed using one way ANNOVA and significance $p<0.05$.

BWG (in g) = (Final weight –Initial weight/Final weight)

SGR = (Ln Final weight –Ln Initial weight / Time interval) X 100

Results

A high quantity of secondary metabolites and proteins were present in ethanol extract of both *M. oleifera* and *L. aspera* flowers, when compared to the water extract of these flowers. The ethanol extract of *M. oleifera* contained greater presence of carbohydrates, proteins, phytosterols, phenolic compounds, cardiac glycosides, alkaloids, flavonoids, terpenoids and steroids. In the case of *L. aspera* flower, except for proteins, saponins and tannins all other metabolites were detected in ethanol extract (Table 1 & 2). But, some secondary metabolites like amino acids, phytosterols, phenolic compounds, flavonoids, saponins, tannins and terpenoids were absent in water extract for *M. oleifera* and *L. aspera* flowers. Hence, the free radical scavenging activity was tested only for ethanol extract and was determined by DPPH assay showing the capability of scavenging the free radicals. The IC_{50} value for free radical scavenging by *M. oleifera* flower extract was 36 μg/mL and for *L. aspera* flower it was 73.89 μg/mL.

The agar diffusion assay was performed according to the guidelines "Susceptibility testing of bacteria isolated from aquatic animals" of the Clinical and Laboratory Standard Institute (CLSI, 2006). The results of the antimicrobial screening by agar diffusion are given in Table 3.

Table 2. Preliminary quantitative phytochemical analysis of ethanol and aqueous extracts of *M. oleifera* and *L. aspera* flower.

TESTS	*M. oleifera* flower (ethanol)	*L. aspera* flower (ethanol)	*M. oleifera* flower (water)	*L. aspera* flower (water)
Total Phenol	19.40 ± 0.418	16.08 ± 0.852	15.63 ± 0.098	12.29 ± 0.082
Total flavonoids	13.27 ± 1.01	7.44 ± 1.96	7.28 ± 0.00	5.66 ± 0.28

In GC-MS/MS analysis of plant extracts, twenty five compounds were identified in the methanolic extract of *M. oleifera* flowers. In *M. oleifera,* the compounds like urea, beta- D-glucopyranose, 3-ethylthiolane,nonanoic acid, dibutyl phthalate, hexadecanoic acid, 9,12 octadecadienoic acid, ethyl oleate, octadecanoic acid,

Table 3. Antibacterial activity of ethanol extracts of of *M.oleifera* and *Leucas aspera* flower

Sample	Zone of inhibition(mm)	
	A. hydrophila	*V. parahaemolyticus*
Moringa flower extract	25 mm	40 mm
Thumba flower extract	21 mm	35 mm

oleic acid, methyl 17-methyl-octadecanoate, bis (2-ethylhexyl) phthalate, 3-piperidinol, eicosane, dodecane, 8-hexadecenal, ethyl tetracosanoate, 5-cyclopropyl carbonyl oxy pentadecan, hexahydropyridine, 13-tetradecenoic acid, gamma.-sitosterol and pyridine-3-carboxamide were confirmed. Among the identified compounds ethyl oleate (31.19%), hexadecanoic acid (13.26%) and 9, 12-octadecadienoic acid (10.79%) were the most prevailing ones, which possesses antifungal, antioxidant and antimicrobial activity respectively.

Whereas twenty six compounds namely, methyl-βD-galactopyranoside, tetradecenoic acid, n-hexadecanoic acid, ethanone derivatives, oleic acid, eicosane, alloaromadendrene oxide, 1H-benzoimidazole, 1-isobutyl-2-phenoxymethyl-,2,6,10-dodecatrien-1-ol, 3,7,11-trimethyl-, (Z,E)-, oxirane, 3,7,11-tridecatrienoic acid, 1H-Indole, 5-methyl-2-phenyl-, 13-octadecenal, Benzo[qr]naphtha [2,1,8,7-fghi] pentacene, 1-nonadecene octadecane, pyridine-3-carboxamide, cholestan-7-one, cyclic 1,2-ethanediyl acetal, (5.alpha.)-, 2-methyl-pentanoic acid [4-(2-methyl-pentanoylsulfamoyl) -phenyl]-amide and .gamma.-sitosterol were the phytocompounds present in *L. aspera* flower. Hexadecanoic acid (18.25%, 17. 40%), Benzo [qr] naphtha (2, 1, 8, 7-fghi) pentacene (13.34%) and methyl-βD-galactopyranoside (12.21%) were the major compounds detected in *L. aspera* flower extract and these bioactive compounds exhibits antimicrobial activity, antioxidant and antitumor activity respectively. The presence of these bioactive compounds justifies the use of the whole plant for phytopharmaceutical importance. Chromatogram with the peaks of the test compounds with respect to retention time is shown in Fig. 1 and 2.

All diets were willingly accepted by fishes, showing no issues regarding palatability of the prepared diet. No mortality was observed during the experimental period and the data indicates that the moringa flower added fish feed, significantly improved fish growth when compared to Thumba flower and the control feed fed groups. The results indicate that the body weight gain of the fishes fed with moringa diet for 3, 6, and 1year old fishes were 0.76, 0.34 and 0.02% respectively. But Thumba flower diet showed 0.34, 0.11 and 0.006% body weight gain

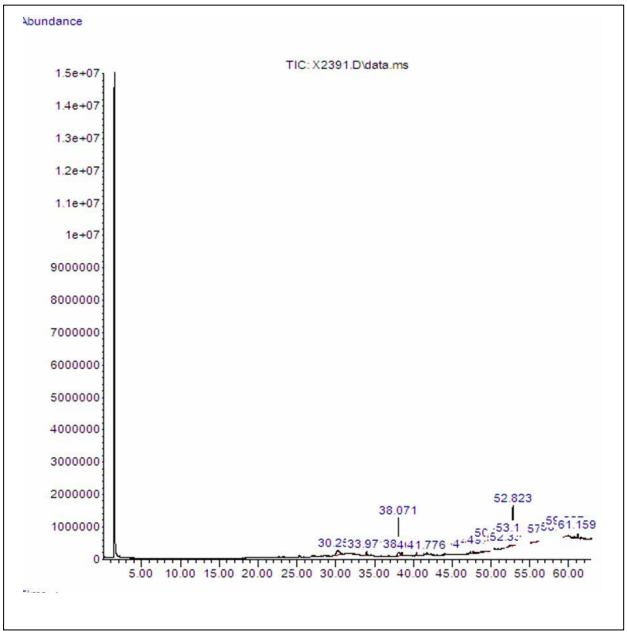

Fig.1. Chromatogram of methanol extract of *M. oleifera* flowers by GC-MS

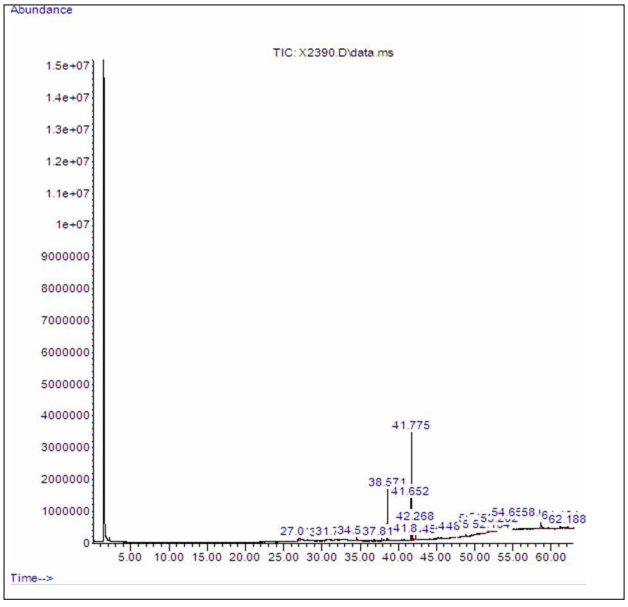

Fig. 2. Chromatogram of methanol extract of *L. aspera* flowers by GC-MS

(Fig.3 and 4) and synthetic feed fed groups showed 0.28, 0.097 and 0.002% whereas control feed given group showed 0.23, 0.084 and 0.0015% weight gain.

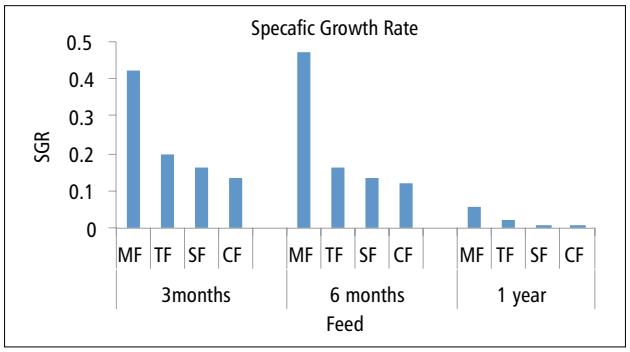

Fig. 3. Showing results of body weight gain

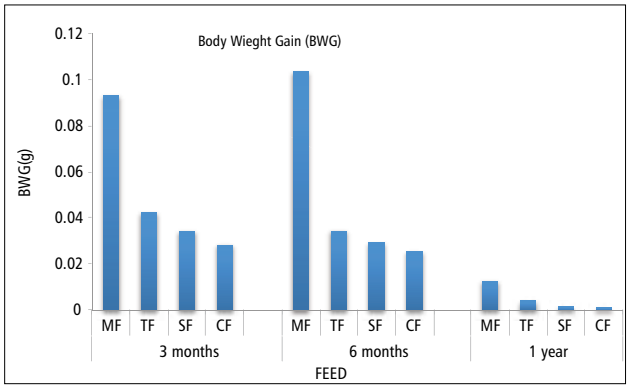

Fig. 4. Specific growth rate

Discussion

To overcome economic losses because of infectious diseases in aquaculture, it is necessary to act upon every health constraint based on scientifically proven and recommended as well as locally applicable methods. To overcome this, huge amounts of antibiotics are used by farmers. In this study, the antimicrobial activity of plant extracts against the fish pathogen were examined. For the preliminary screening, ethanol and water extract were collected and compared for their phytochemical screening and we could observe that ethanol extract contained most of the antimicrobial compounds.

Widespread use of antibiotics can result in development of disease resistance. It was evident from the previous work that *A. hydrophila* shows resistance against antibiotics like amoxicillin, ampicillin etc. (Belem-Costa and Cyrino, 2006) due to the multiple usage of antibiotics. Antimicrobial activity of *M. oleifera* has been reported earlier (Oluduro, 2012; Onyuka *et al*., 2013). In this study, we could observe that *M. oleifera* flower extract is efficient against selected fish pathogens (zone of inhibition: 25 mm, 40 mm) when compared to *L. aspera* flower extract (Table 3). But this study shows divergence from the results of Ekenam *et al.* (2011) who reported that *M. oleifera* extract was inactive against *A. hydrophila.* However, Vanden and Vlietinck (1991) reported strong antibacterial activity of *M. oleifera* against *P. aeruginosa*. Mangathayaru *et al.* (2005) studied the antibacterial activity of *L. aspera* flowers and showed good antibacterial activity for methanol extract and methanol fraction with maximum activity for the alkaloidal residue. But, the analysed data are not available against fish pathogens. The activity of the flower extract against both pathogens, in the present study, may be indicative of the presence of a broad spectrum of antibiotic compounds in the ethanolic extract of *M. oleifera* and *L. aspera* flowers (Srinivasan *et al*., 2001).

Phenols and flavonoids protect plants from oxidative damage. They have also been studied extensively as antioxidant protectants for human beings and play a beneficial role in reducing the risk of coronary heart disease, diabetes, hypertension and some types of cancer (Hossain and Rahman, 2011). Diets rich in phytonutrients may supply a variety of phytoestrogens such as isoflavones, resveratrol, lignans etc. capable of producing a range of pharmacological effects. But the solvent for extraction is also considered as an important factor in this effect. The selection of solvents for plant secondary metabolite extraction reliant on low toxicity, ease of evaporation, rapid physiologic absorption of the extract, preservative action etc. Water is a universal solvent and traditional medicine practitioners use water extract for the treatment. Organic solvents are found to be more efficient in antimicrobial action. There are also reports which have supported the fact that some water soluble flavonoids had reduced antimicrobial activity; higher activity of ethanolic extract can be due to the presence of a higher amount of polyphenols (Altemimi *et al*., 2017). The possible reason for the decreased activity of aqueous extract could be ascribed to the presence of polyphenol oxidase enzyme, which degrades polyphenols in water extracts, whereas in methanol and ethanol they are present as inactive form. Free radical scavenging is a significant mechanism for the inhibitory activity towards lipid peroxidation and an excellent marker for antioxidant activity. In the current study the IC_{50} value for free radical scavenging by *M. oleifera* flower extract was 36 μg/mL whereas for *L. aspera* flower it was 73.89 μg/mL. The free-radical scavengers (antioxidants) have potential to prevent, delay or restructure the process of ageing.

The results about GC-MS analysis has led to the identification of a number of compounds from the fractions of both ethanolic flower extracts. GC-MS is a key technological tool for secondary metabolites profiling in plant species. The analysis of both the floral extracts showed the presence of complex mixture of numerous compounds. Most of these

bioactive compounds exhibit antimicrobial, antioxidant and antitumor activity. The presence of these bioactive compounds justifies the use of the whole plant for phyto pharmaceutical importance. Further investigation may lead to isolation of bio-active compounds and their structural elucidation and screening of pharmacological activity will be helpful for further targeted product development.

Bioactive compounds present in these plants can stimulate feed intake, improve secretion of digestive enzyme, increased growth rate and activate immune responses. In the present study, no mortality was observed during the feeding experiments. Moreover, the results indicate that there is an increased body weight gain, which is 2.71 fold and 1.21fold in the three month old fishes that respectively fed on Moringa and Thumba flower diet when compared to that of the fishes that fed on synthetic feed. For six months old fishes there were 3.5 and 1.13 fold increase in body weight gain respectively for *M. oleifera* and *L. aspera* flower feed. Eventhough the weight gain in 1 year old fishes were slow, on comparison with the synthetic feed it was found that there was an increase of 10 and 3 fold respectively for *M. oleifera* and *L. aspera* flower feed. The results clearly depict the herbal feed acceptance by the fishes of different age groups. Moreover, the increased body weight gain evidently indicates the nutritional quality of the supplemented feed.

Nutrition can be considered to be the heart of aquaculture. Nutrients in the feed sway growth, reproduction as well as the health of the fishes. It can also alleviate to the stressors which are both physiological and environmental and pathogens. The present study provides evidence that medicinal plant extracts could be used as a potentanti-pathogenic agent in the aquaculture sector. The natural components with potential bioactivity could be used as an alternative therapy for bacterial fish disease. *M. oleifera* contains active phytochemical components and this plant can be used for fish immune boosters or protector from bacterial diseases. The same was also found to have potent antioxidant property. However, it is important to note that research in antimicrobial at *in vitro* level may not always communicate the same effect in *in vivo* condition. So it is important to study their mode of action in *in vivo* which is yet to be studied to elucidate the complete action of any drug for field application.

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