
Antibacterial activity of medicinal herbs against the fish pathogen *Aeromonas hydrophila*

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Antibiotics play an important role in the disease management of aquaculture practices; however antibiotic resistance develops readily in pathogens following antibiotic treatment. Medicinal herbs play an alternative role to tackle this problem. In the present study, 15 selected plant species belonging to Lamiaceae and Apocynaceae were collected from different localities of Tirunelveli district of Tamilnadu. The antibacterial activity and Minimum Inhibitory Concentration (MIC) of the plant extracts were evaluated against the fish pathogen, *Aeromonas hydrophila*. The antagonistic effect of the methanolic extracts of *Coleus aromaticus* of Lamiaceae and *Tabernaemontana divaricata* of Apocynaceae were found to be most effective against *A. hydrophila*. Regarding Lamiaceae, *C. aromaticus*, *Mentha arvensis* and *Leucas aspera* exhibited lower MIC value of 12.5 mg/ml with inhibition zones of 10.33 mm, 9.67 mm, and 9.33 mm respectively. *T. divaricata* of Apocynaceae showed MIC of 12.5 mg/ml with an inhibition zone of 7.33 mm whereas *Catharanthus roseus* at 25 mg/ml exhibited an inhibition zone of 9.67 mm and *Rauvolfia tetraphylla* at 50 mg/ml showed an inhibition zone of 9.33 mm. Considering overall performance, *C. aromaticus* was found to be the most effective antagonistic agent against *A. hydrophila*.

Key words: Medicinal herbs, Antibacterial activity, Inhibition zone, *Aeromonas hydrophila*.

Introduction

The decline of fish from natural aquatic resources and increasing demands for fish, shrimp and other aquatic organisms by consumers are the two main factors for the expansion of aquaculture nowadays (Dogruoz *et al.*, 2008). Epizootic ulcerative syndrome (EUS) is a dreadful disease of most air breathing fishes caused by the fungus *Aphanomyces invadans* primarily, followed by secondary invasion of the bacterium *Aeromonas hydrophila*. Due to EUS, the most economically important freshwater food fish, the murels, succumb heavily resulting in severe economic loss. Antibiotics play a major role in

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aquaculture in terms of health management. But because of the side effects and the resistance of pathogenic microorganisms, scientists have recently paid attention to extracts and biologically active compounds isolated from medicinal herbs (Essawi and Srour, 2000). Antimicrobial properties of medicinal herbs are being increasingly reported from different parts of the world. Among many herbs used as antimicrobials, the members belonging to Lamiaceae and Apocynaceae are considered as the most effective against pathogens.

Lamiaceae members are used as diuretic, sedative, digestive, antiparasitic, carminative, appetite, anticonvulsant, and anti-inflammatory agents and recommended to treat fever, cough, headache, stomachache, wound healing, heart diseases and dysmenorrhea (Sarac and Ugur, 2007). Apocynaceae species are used in traditional medicine to treat gastrointestinal ailments, fever, malaria, pain and diabetes (Wiert, 2006). The roots, leaves and latex of Apocynaceae were used to treat skin and liver diseases, leprosy, dysentery, ulcers, tumours and ear ache (Rajakaruna *et al.*, 2002). With this background, an attempt was made to study the invitro antimicrobial activity of the methanol extracts of eight species of Lamiaceae and seven species of Apocynaceae against the most common fish pathogen *Aeromonas hydrophila*.

Materials and methods

Mentha arvensis L. (L₁), *Anisomeles malabarica* (L.) R.Br. ex Sims (L₂), *Hyptis suaveolens* (L.) Poit. (L₃), *Ocimum gratissimum* L. (L₄), *Ocimum sanctum* L. (L₅), *Leucas aspera* (Willd.) (L₆), *Coleus aromaticus* Benth. (L₇) and *Ocimum basicilicum* L. (L₈) belonging to Lamiaceae and *Catharanthus roseus* (L.) G.Don (A₁), *Rauwolfia tetraphylla* L. (A₂), *Nerium Oleander* L. (A₃), *Tabernaemontana divaricata* (L.) R. Br. ex Roem. & Schult (A₄), *Allamanda cathartica* L. (A₅), *Thevetia peruviana* Merr. (A₆) and *Plumeria alba* L. (A₇) belonging to Apocynaceae were collected from different locations of Tirunelveli District of Tamilnadu, India (8° 44' N and 77° 44' E). All the species were identified and reconfirmed by herbarium collections (XCH), St. Xavier's College, Palayamkottai, Tamilnadu.

Leaves were dried, powdered and macerated with 95% methanol (1:5 w/v) and kept at room temperature for 7 days. The filtrates of each species were removed under vacuum at 40°C using a rotary evaporator and the crude methanol extract were stored at 4°C and were used for preliminary screening of phytochemicals viz: alkaloids, steroids, reducing sugars, catechins, anthraquinones, flavanoids, terpenoids, sugars, phenols, saponins, tannins and aminoacids (Brinda *et al.*, 1981). The test pathogen *Aeromonas hydrophila* (MTCC-646) was obtained from Microbial Type Culture Collection (MTCC),

Chandigarh. The bacterial strain was cultivated in nutrient broth at 37°C for 4h equivalent to 0.5 McFarland turbidity standard (3.0×10^8 CFU/ml).

Antibacterial activity of the extracts was determined against *A. hydrophila* by Disc Diffusion Assay (Dulger and Gonuz, 2004). on Muller Hinton Agar (HiMedia). The crude methanolic extracts were dissolved in DiMethyl Sulfoxide (DMSO) and 6 mm sterile discs containing 25 µl of plant extracts were prepared. The inoculated plates were incubated at 37 °C for 16 - 18 h. The antibacterial activity was evaluated by measuring the zone of inhibition surrounding the discs. Standard disc of the antibiotic Ciprofloxacin (5 mcg) served as positive antibacterial control. Paper disc loaded with 25 µl of DMSO served as negative control. All tests were carried out in triplicates to minimize test error (Ramzi *et al.*, 2005).

The Minimal Inhibitory Concentration (MIC) of the plant extracts which showed antibacterial activity was calculated using five concentrations of each extract (3.12, 6.25, 12.5, 25.0, 50 mg/ml) employing the same agar disc diffusion method. The antibacterial activity was evaluated by calculating the percentage of Relative Inhibition Zone Diameter (% RIZD) as follows:

$$\% \text{ RIZD} = \frac{\text{IZD sample} - \text{IZD negative control}}{\text{IZD antibiotic standard}} \times 100$$

where IZD is inhibition zone diameter (mm). All values were expressed as mean \pm SD. The Inhibition Zone Diameter data for all concentrations were subjected to one way analysis of variance (ANOVA) and $P < 0.05$ was considered as significant (Jhon *et al.*, 2006). The software OriginPro 7.5 was employed for the statistical analysis.

Results and discussion

Medicinal plants have been known to synthesize active secondary metabolites with established potent anti-microbial activities, which indeed have formed the basis for their applications in pharmaceuticals, alternative medicines and natural therapies (Hammer *et al.* (1999); Fabricant and Farnsworth, 2001). The antimicrobial activities of the medicinal herbs used in the present study could be due to the presence of various secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, saponins, and steroids against both Gram-positive and Gram-negative organisms (Baser, 1993; Jouad *et al.*, 2001; Anyanwu and Dawet (2005); Koche *et al.*, 2010).

Phytochemical screening of the selected species of Lamiaceae revealed high scoring of alkaloids, reducing sugars, tannins, phenolic compounds and

aminoacids in the extract while triterpenoids and saponins indicated low scores. Steroid, flavonoids and catechin showed moderate scores whereas sugars and anthraquinones were not detected in the extracts. Alkaloids, phenolic compounds, amino acids, reducing sugars and tannins were present in all of the medicinal herbs, while catechin was noticed only in *M. arvensis*, *A. malabarica*, *H. sauveolens*, *O. gratissimum*, *O. sanctum* and *L. aspera*. Similarly flavonoids indicated their presence in five plants viz., *M. arvensis*, *A. malabarica*, *H. sauveolens*, *O. sanctum* and *L. aspera*. Steroids were present in all the seven plants of Lamiaceae studied except *C. aromaticus*. Saponins were recorded in two plants namely *A. malabarica* and *L. aspera* whereas triperpenoids showed their presence only in *C. aromaticus* (Table 1).

Table 1. Phytochemicals of different species of Lamiaceae and Apocynaceae

S. No.	Phytochemicals	Lamiaceae							Apocynaceae							
		L ₁	L ₂	L ₃	L ₄	L ₅	L ₆	L ₇	L ₈	A ₁	A ₂	A ₃	A ₄	A ₅	A ₆	A ₇
1.	Steroids	+	+	+	+	+	-	+	+	+	+	-	-	-	+	+
2.	Triterpenoids	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
3.	Reducing sugar	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4.	Sugars	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5.	Alkaloids	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+
6.	Phenolic compounds	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7.	Flavonoids	+	+	+	-	+	+	-	-	+	-	+	-	+	+	+
8.	Catechin	+	+	+	+	+	+	-	-	+	-	+	-	+	+	+
9.	Saponins	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-
10.	Tannins	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
11.	Anthroquinones	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12.	Amino acids	+	+	+	+	+	+	+	+	-	+	-	+	-	+	-

L₁ - *M. arvensis*, L₂ - *A. malabarica*, L₃ - *H. sauveolens*, L₄ - *O. gratissimum*, L₅ - *O. sanctum*, L₆ - *L. aspera*, L₇ - *C. aromaticus*, L₈ - *O. basillicum*, A₁ - *C. roseus*, A₂ - *R. tetraphylla*, A₃ - *N. oleander*, A₄ - *T. divaricata*, A₅ - *A. cathartica*, A₆ - *T. peruviana*, A₇ - *P. alba* (+) indicates presence and (-) indicates absence

In Apocynaceae, reducing sugars, phenolic compounds and tannins showed high scoring in the extracts while catechin and steroids indicated moderate scores. Alkaloids, flavonoids, sugars and aminoacids showed low scores. Triterpenoids, saponins and anthraquinones were not detected in the extracts. Tannins were present in all the six plants except *T. peruviana* and alkaloids indicated their presence in *C. roseus*, *R. tetraphylla*, *N. oleander* and *P. alba*. The catechin was present in five plants except *C. roseus* and *N. oleander* (Table 1). The methanolic extracts of most of the members of Lamiaceae showed positive antibacterial activity whereas only a few members of the family Apocynaceae showed moderate activity against the tested pathogen, *A. hydrophila*. It is also found that when the concentration of the extracts decreased from 50 mg/ml to 3.12 mg/ml, decrease in inhibition zones was observed with almost no inhibition at least concentrations.

Among Lamiaceae species, the most pronounced activity was noticed in the methanolic extract of *C. aromaticus* (L₇) which recorded a zone of 20.67 mm at 50 mg/ml with 81.6 % RIZD, whereas a zone of 12.33 mm was observed at 25 mg/ml, 10.33 mm at 12.5 mg/ml and no inhibition at lower concentrations. *M. arvensis* (L₁), *A. malabarica* (L₂), *L. aspera* (L₆), and *O. basclicum* (L₈) also showed remarkable activity against *A. hydrophila*. *M. arvensis* (L₁), showed maximum inhibitory zone of 13.67 mm (53.97 % RIZD) at 50 mg/ml, 12.66 mm (50.02 % RIZD) at 25 mg/ml, and 9.33 mm (40.78 % RIZD) at 12.5 mg/ml. *A. malabarica* (L₂) and *L. aspera* (L₆) exhibited a zone of 9.67mm (38.18% RIZD) and 8.33 mm (32.89 %RIZD) at MIC of 12.5 mg/ml, whereas *O. basclicum* (L₈) showed its activity by a zone of 9.33 mm (36.83 % RIZD) at 25 mg/ml. The methanolic extracts of other plants like *H. sauveolens* (L₃), *O. gratissimum* (L₄) and *O. sanctum* (L₅) showed very little activity i.e., less than 10 mm against the pathogen. *C. aromaticus* (L₇), *A. malabarica* (L₂), *M. arvensis* (L₁) and *L. aspera* (L₆) were found to exhibit lower MIC value of 12.5 mg/ml with the inhibition zones of 10.33 mm, 9.67mm, 9.33 mm and 8.33 mm respectively. Among the Lamiaceae members examined, *C. aromaticus* was found to be the most effective antagonistic agent against *A. hydrophila* (Fig. 1 and 2).

Among the Apocynaceae family, *T. divaricata* (A₄) showed better activity of 11.67 mm (46.07 % RIZD) at 50 mg/ml followed by *C. roseus* (A₁) for a zone of 10.33 mm (40.78 % RIZD) at 50 mg/ml con.) and *R. tetraphylla* (A₂) exhibited a zone of 9.33 mm (36.83 % RIZD) at 50 mg/ml. Other plants showed no activity against the pathogen. *T. divaricata* (A₄) was found to have the MIC of 12.5 mg/ml, with an inhibition zone of 7.33 mm whereas *C. roseus* (A₁) at 25 mg/ml showed an inhibition zone of 9.67 mm and *R. tetraphylla* at 50 mg/ml with an inhibition zone of 9.33 mm. Among the Apocynaceae members examined, *T. divaricata* was found to be more effective by exhibiting a zone of 11.67 mm at 50mg/ml con. (Fig. 1 and 2)

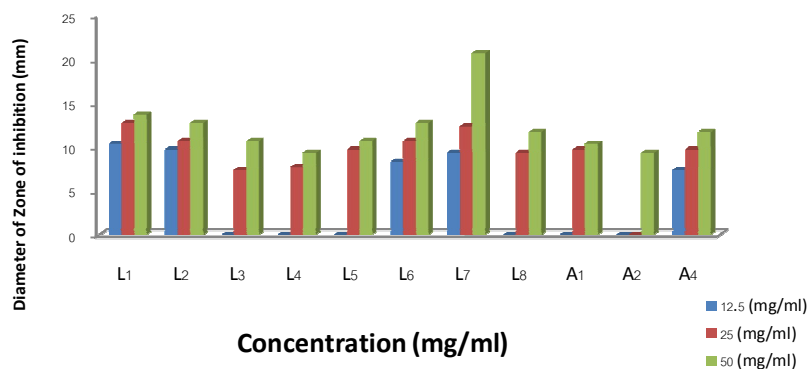


Fig. 1. Zone of Inhibition of different species of Lamiaceae and Apocynaceae against *Aeromonas hydrophila*

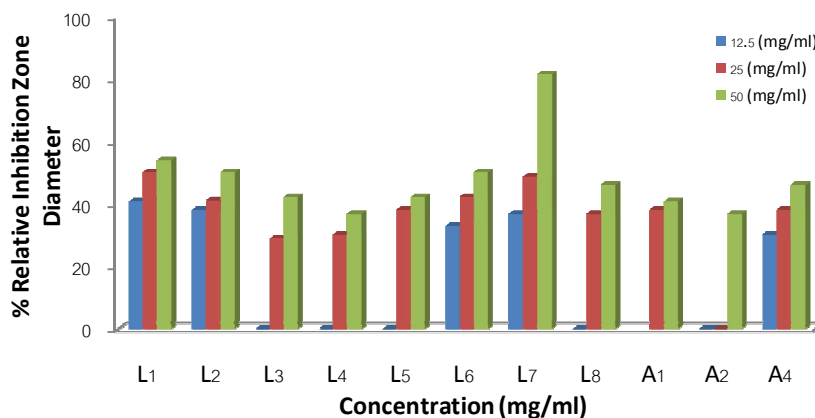


Fig. 2. % RIZD of different species of Lamiaceae and Apocynaceae against *Aeromonas hydrophila*

The antimicrobial activity of Lamiaceae and Apocynaceae members has been tested against many Gram positive and Gram negative bacteria by different research groups world wide highlighting the importance of herbs as alternative therapeutic agents. In our study, *C. aromaticus* leaf extracts possessed potent antimicrobial properties against *A. hydrophila*. In particular, methanol extract with higher concentration (50 mg/ml) was active against *A. hydrophila*, suggesting that the active principles may be useful in the topical treatment of superficial skin infections of *A. hydrophila* (Sunitha *et al.*, 2010). Herbal immunostimulants as an alternative to drugs, chemicals and antibiotics have been recommended to control fish diseases in fish culture. The application of *Mentha piperita* and *Ocimum basclicum* against *A. hydrophila* was investigated and found to have enhanced immunity in common carp (Abasali and Mohamad, 2010).

Antagonistic activity of the medicinal herbs *Curcuma longa*, *Ocimum sanctum*, and *Azadirachta indica* against *A. hydrophila* was found to be effective in Goldfish following disease challenge (Harikrishnan and Balasundaram, 2008). The antibacterial activity of the ethanol extracts of *T. peruviana* against six bacterial and five fungal pathogens. The extracts showed maximum activity was tested against *Escherichia coli*, *Enterobacter aerogenes* and *Alcaligenes faecalis* (Patil *et al.*, 2007). In the present study, enhancement of inhibition zone with an increase in the concentrations suggested concentration dependent activity. Further studies on phyto constituents and purification of individual bioactive components can reflect the exact antimicrobial potential of the medicinal herbs.

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