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ORIGINAL ARTICLE

Antibacterial potencies of selected medicinal plant extracts against shrimp pathogens *Vibrio parahaemolyticus* and *Vibrio harveyi*

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ABSTRACT
Vibriosis is the major problem in shrimp aquaculture across the world and causing huge economic loss to the farmers. In this study, an alternative herbal remedy for the said issue has been undertaken with ten traditional medicinal plants. The selected herbs were extracted with the organic solvents of increasing polarity such as hexane, chloroform, and methanol to extract the maximum bioactive metabolites. Then the extracts were tested for the better antagonistic activity against the shrimp bacterial pathogens V. parahaemolyticus and V. harveyi. Assessment of antibacterial activity using the disc diffusion method revealed that the methanolic extract of all the ten plants exhibited the maximum bioactivity in terms of zone of inhibition ranged from 8.5 to 19.0 mm against V. parahaemolyticus and 9.5 to 18.5mm against V. harveyi respectively. Further assays were conducted only with methanolic extracts due to its better bacterial inhibitory property. The Minimal Inhibitory Concentration (MIC) was ranged from 1.0µg/ml recorded by Solanum trilobatum to 100µg/ml by depicted by Centella asiatica. During Minimum Bactericidal Concentration (MBC) assay, S. trilobatum, C. asiatica,and Leucasa spera have noticed no growth inhibition against the test bacteria in TSA plates and the extracts described both bacteriostatic and bactericidal properties. The above promising results further proposed the dietary supplementation of these herbs for vibriosis management in shrimp aquaculture.
Keywords: Vibriosis; Antibacterial; Herbal extract; MIC; MBC; *Penaues vannamei*

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INTRODUCTION

Aquaculture is a food production sector with the annual growth rate at 6%, preparing itself to be the major source of protein to humankind. The unavoidable development of the sector is attended by adopting some practices which are harmful to live beings [29]. It is a great relief that the use of antibiotics in aquaculture is banned. This is to avoid the increased antibiotic resistance in bacteria, which is pathogenic to aquatic organisms [7]. The use of antibiotics becomes a threat to humans and non-target organisms in the environment in the long run [22].

The vibriosis is a common disease found in shellfish and finfish aquaculture [22]. The disease caused by gram-negative bacteria which falls under Vibrionaceae family. The disease causes huge economic loss to the shrimp culture due to high mortality [2, 9, 20-24]. The various process of shrimp farming such as hatchery operations and grow out shrimp pond was affected by Vibriosis. The favourable environmental factors enhance the multiplication of *Vibrio*sp within the shrimp blood or penetration through stressed host barriers and evolve Vibriosis outbreak [40, 48].

The crustaceans have an exoskeleton acts as a physical barrier effectively control the pathogen penetration from the external environment. The *Vibrio* spare Chitinoclastic in nature responsible for the shell disease in shrimps [10] and may enter through the wounds present on the exoskeleton [18, 2]. The gills are considered the primary entry site for the bacteria as it has thin exoskeleton covering [42]. The exoskeleton is being frequently cleaned by setobranchs [5, 6] but still, the infection may occur. The digestive parts including foregut to midgut trunk [24] do not have the exoskeleton covering and likely the site for pathogen penetration from external factors such as water, feed and sediments [37, 15].

The occurrence of Vibriosis in aquaculture is tackled by administering chemicals for disinfection and antibiotic treatment [41]. The palatability, cost, toxicity and solubility are the infeasible factors which limit the usage of antibiotics in aquaculture. The use of antibiotics in aquaculture is recently banned due to the development of Multiple Antibiotic Resistance Index (MAR) in pathogenic bacterial strains.

The development of multiple antibiotic resistances of the pathogens to antibiotics has increased the search for the alternatives [27]. The usage of medicinal herbs to treat bacterial diseases has administrated in various field like agriculture, medicine for both human and animal [34]. Since ancient times, the medicinal plants have been used as the treatment for bacterial infections are potential, beneficial alternatives in aquaculture also [11]. Though the side-effects of the synthetic antimicrobials can be nullified using medicinal plants to treat the bacterial diseases in aquaculture [3].

The herbs can be used as the immunostimulant, growth enhancer, antimicrobials [4]. The previous reports of Lua *et al* [25], Nguyen *et al* [30] favours the use of herbs such as *Psidium guajava, Piper betle*, *Phyllanthus amarus,* and *Rhodomyrtus tomentosa*in white leg shrimp culture against bacterial pathogens especially AHPND causing vibrios including *V. parahaemolyticus* KC12.020; *V. parahaemolyticus* KC13.14.2 and *V. harveyi* KC13.17.5.The medicinal herbs are also used as feed attractant and stimulating the secretion of gastric juices, thus increasing the feed intake as well as decreasing the FCR (Feed Conversion Ratio) [46].

Besides, the use of disease preventive herbs in white leg shrimp (*P. vannamei*), many other medicinal plants are also studied for preventing and treating diseases in Indian white shrimp *(Fenneropenaeus indicus)*. The methanolic extracts of *Quercusin fectoria Murraya koenigii* and *Psoralea corylifolia*, have strongly suppressed the growth of the pathogenic bacteria isolated from the gut of the infected *Fenneropenaeus indicus*. According to Velmurugan *et al* [44] 9.00 to 14.00mm size of zone of inhibition was obtained against the pathogenic *V. parahaemolyticus* and *V. harveyi* strains. The present work has been undertaken to investigate the antibacterial potencies of ten medicinal plants against the shrimp pathogens such as *V. parahaemolyticus* and *V. harveyi*.

The disease preventive and treatment based medicinal herbs were studied not only in the white leg shrimp culture but also investigated in the Indian white shrimp Besides, not only herbs are studied for disease prevention and treatment for white-leg shrimp *Fenneropenaeus indicus.* The bacterial pathogens from the gut of infected *Fenneropenaeus indicus* were suppressed using three methanolic extracts of *Psoralea corylifolia*, *Murraya koenigii* and *Quercusin fectoria* [43]. The average zone of inhibition ranging between 9.00 to 14.00mm as observed against the pathogens such as *Pseudomanas aeruginosa*, *Staphylococus aureus,* and *V. harveyi* strains. In the above context, the present work has been undertaken to investigate the antibacterial potencies of ten medicinal plants against the shrimp pathogens such as *V. parahaemolyticus* and *V. harveyi*.

MATERIAL AND METHODS

Bacterial Pathogens

The shrimp pathogenic bacterial strains such as *V. parahaemolyticus* and *V. harveyi* were collected from the microbial culture collections of Centre for Marine Sciences and Technology, Manonmaniam Sundaranar University, Rajakkamangalam, Tamilnadu, India.

Medicinal Plants

Ten medicinal plants such as *Solanumtrilobatum, Curcuma longa, Psidium guajava, Ocimum sanctum, Azaclirachta indica, Acalypha fruticosa, Centella asiatica, Bacopa monnieri, Piper betle,*and *Leucasa spera* were collected from different regions of Cuddalore district and washed twice using freshwater to remove epiphytes and other extraneous matter from the leaves of the plants. Then the plant leaves were shade dried, powdered, sieved and stored in airtight containers until further use.

Extraction

A hundred grams of powdered medicinal plants were extracted individually in a Soxhlet apparatus using methanol, chloroform, and hexane as solvents. Extracts were filtered with Whatman No. 1 filter paper, evaporated and concentrated. The crude extracts were used for the antibacterial assay [15].

Antibacterial assay

The individual vibrio strains were seeded in Muller Hinton agar plates. The antibacterial assay was determined using the standard disc diffusion method [6]. Sterile Whatman No. 1 filter paper discs of 5 mm diameter were impregnated with 10 mg/disc of the crude extract, air-dried, and placed on Muller Hintonagar plates seeded with individual organisms and incubated for 24 h at 30^{0}C. The assay was carried out in triplicate. The zone of inhibition was measured in millimeter from the center of the disc and the results were recorded [15].

Minimal Inhibitory Concentration (MIC)

As the methanolic extract having better antibacterial activity in the previous assay, thus MIC was determined only for the methanolic extract of medicinal plants by broth microdilution method. For this, an inoculum of the bacteria was prepared and the suspension was adjusted with an equivalent to 0.5 McFarland standards. The dilutions of crude extracts by 2-fold dilution were prepared using sterile TSB to get various ranges of concentrations (1.0, 10, 25, 50, 75, and 100 µg/ml). 1ml of bacterial suspension was added into each tube. The control tubes contain no extract. After 24 hrs of incubation at 37^{0}C, the test tubes were examined for possible growth, and MIC was determined as the lowest concentration that ended with no growth. The assay was done in triplicate [36].

Minimal Bactericidal Concentration (MBC)

Tubes without bacterial growth in the MIC test were streaked onto triplicate TSA plates to estimate MBC against tested bacteria. Bacterial growth was observed after 12hrs incubation. The minimum concentration of crude extracts that prevent bacterial growth is reported as the MBC value [36].

RESULTS AND DISCUSSION

The vibriosis mainly caused by *Vibrio harveyi*, *Vibrio parahaemolyticus,* and *Vibrio alginolyticus* is the most prevalent bacterial disease in global shrimp farming [47, 35]. *Vibriosis* was effectively controlled by the usage of antibiotics in shrimp aquaculture. However, the Marine Product Export Development Authority has banned the use of Chloramphenicol, Furazolidone, Neomycin, Nalidixicacid; Sulphamet, Nitrofurantoin, Oxytetracycline, Cotrimoxazole and Chloramphenicol were banned in Indian shrimp aquaculture [28]. The development of MAR pathogenic strains and the presence of residual antibiotics in processed shrimp tissue has led to the search of a needful replacement for antibiotics in various process in aquaculture [19]. The synthetic antibiotics have to be replaced by antibacterial herbal molecules and probiotics. Though the studies are available using probiotic strains to enhance the competitive inhibition in the gut of the crustaceans, killing and eliminating of pathogenic bacterial strains are the immediate need in disease control. The antibacterial herbals are being used to secure the health of the human and farmed animals for many decades-long in Asia–Pacific countries like India, China, Korea and Japan. The interest of avoiding internal microbial stress by using medicinal herbs as a kind of dietary supplement in the aquaculture is developing idea in recent years as it has many positive effects on growth and the immune response of the cultured species [17].

The antibacterial activity of the crude extracts of the selected medicinal plants was screenedagainst the shrimp bacterial pathogens such as *V. parahaemolyticus* and *V. harveyi*. Amongst the solvents used, the maximum antagonistic activity was registered by methanolic extract which was followed by moderate range activity recorded by chloroform extract. Whereas, the hexane extracts of medicinal plants showed the least inhibitory activity against the test of bacterial strains.

Out of 3 tested organic solvents of increasing polarity from hexane (non-polar), chloroform (mid polar), and methanol (polar), methanol extracts alone showed a very good range of bioactivity against the test bacteria. Among the 10 methanolic extracts of medicinal plants, three (*S. trilobatum, C. Longa* and *P. guajava*) showed a better bacterial growth inhibitory effect. Among these, the methanolic extract of *S. Trilobatum* exhibited the highest inhibitory activity by forming a zone of inhibition of 19.0 ± 0.71mmagainst *V. parahaemolyticus*. Next to this, *C. longa* recorded a zone of inhibition of 16.0 ± 0.77mm, followed by *P. Guajava*showed a zone of inhibition of 14.5±0.63 mm. The least active plant *C. asiatica*also exerted an inhibitory effect of 8.0±0.41mm against *V. parahaemolyticus* (Table 1).

Similarly, the methanolic extract of *S. trilobatum, C. longa,* and *P. Guajava* exhibited a better antagonistic activity against the shrimp bacterial pathogen *V. harveyi* and was ranged from 14.0 to 18.5 mm. The moderate level of inhibitory activity (11.5 to 13.0 mm) was registered by the methanolic extracts of *A. fruticosa, A.indica, O. sanctum,* and *P. betle*. Further, the least antibacterial activity in terms of zone of inhibition from 8.0 to 9.5mm was inferred by the methanolic extracts of *B. monnieri, C. asiatica* and *L. aspera* against the tested bacterial strain (Table 2). There have been few numbers of studies reported antibacterial effects of medicinal plants on *V. parahaemolyticus* and *V. harveyi* [4, 14. 44, 45, 31, 32, 38, 39]. Similarly, the crude extract of *S. trilobatum* recorded better antibacterial activity against *V. cholerae* and *E. coli* [33]. *S. trilobatum* leaf extract found to possess maximum antibacterial activity than fruit and root extracts [24].

Table 3 shows the MIC values of methanolic extracts of chosen medicinal plants. MIC was determined as the lowest concentration that ended with no growth in the broth microdilution method. The highest MIC of 1µg/ml was exhibited by the methanolic extract of *S. trilobatum* against both *V. parahaemolyticus* and *V. harveyi*. The moderate level of MIC was ranged from 10 to 50µg/ml which was recorded by the methanolic extracts of *C. longa, P. guajava, O. sanctum, A.indica,* and *P.betle*. However, the least level of MIC (75 to 100µg/ml) was notified by the methanolic extracts of *A. fruiticosa, B. monnieri, L. aspera* and *C. asiatica*. The herb of *C. longa* with 86.5% curcumin value against 24 pathogenic bacteria isolated from the chicken and shrimp showed the highest antimicrobial activity for ethanol extract with the MIC value of 3.91 to 125 ppt [22]. The hexane and methanol extracts of *C. longa* demonstrated antibacterial effect against 13 bacteria, namely, *Vibrio harveyi, V. alginolyticus, V. vulnificus, V. parahaemolyticus, V. cholerae, Bacillus subtilis, B. cereus, Aeromonas hydrophila, Streptococcus agalactiae, Staph. aureus, Staph. intermedius, Staph epidermidis,* and *Edwardsiella tarda*. Correspondingly, in this study methanolic extract of *C. longa* registered comparatively better antibacterial activity with the zone of inhibition ranged from 15.0 to 16.0mm against *V. harveyi* and *V. parahaemolyticus* with the MIC1.0 and 10µg/ml respectively. Also, *C. longa* showed a better MBC value of 100µg/ml. Further, crude aqueous mixture and water-soluble methanol extract from leaf and bark of *Psidium guajava*, showed the strong antibacterial activity against multidrug-resistant *V. cholerae* O1. The *in vitro* minimum inhibitory concentration of the crude aqueous mixture and water-soluble methanol extract, which was bactericidal against 10^7 CFU/mL of *V. cholerae* was determined to be 1,250 mg/mL and 850 mg/mL, respectively [26]. Accordingly, the present results evidenced the antibacterial potencies of *P. guajava* with good MIC and MBC values against the shrimp bacterial pathogens. The rest of the tested extracts entered moderate to least level of antibacterial efficacies against *V. parahaemolyticus* and *V. harveyi*.

The minimum concentration of crude extracts that prevent bacterial growth is reported as the MBC value.Methanolic extracts of *S. trilobatum, C. asiatica,* and *L. aspera* registered no growth inhibition but in the above mentioned three extract acts *S. Trilobatum* alone recorded the highest MIC value of 1µg/ml. Next to that, the methanolic extract of *C. longa, P. guajava* and *B. Monnierin* exhibited a better MBC value of 100µg/ml. The medium level of 75µg/ml was inferred by *O. sanctum* and *A. indica*. However, the least MBC value of 50µg/ml was showed by the methanolic extract of *A. fruiticosa*a nd *P. betle* (Table 4).

In this study, the methanolic extract of five medicinal plants such as *S. trilobatum, C. longa, P. guajava, C. asiatica* and *L. aspera* exhibited bacteriostatic activity as their MBC/MIC ratio was ranged from 4 to 100. Whereas, the methanolic extract of the other five medicinal plants like *O. sanctum, A. indica, A. fruiticosa, B. monnieri,* and *P. betle* showed the bactericidal activity with the MBC/MIC ratio ranged from 0.6 to 3 (Table 5).

According to the report of Canillac and Mourey (2001), if the ratio of MBC/MIC is lower than or equal to 4, the extract is considered bactericidal; in contrast, if this ratio is higher than 4, the extract is bacteriostatic. Likewise, in this investigation the methanolic extract of five medicinal plants such as *S. trilobatum, C. longa, P. guajava, C. asiatica,* and *L. aspera* exhibited bacteriostatic activity as their MBC/MIC ratio was ranged from 4 to 100. Whereas, the methanolic extract of the other five medicinal plants like *O. sanctum, A. indica, A. fruiticosa, B. Monnieri,* and *P. betle* showed the bactericidal activity with the MBC/MIC ratio ranged from 0.6 to 3.

Table 1. Antibacterial activity of medicinal plant extracts against *V. parahaemolyticus*

Name of the Medicinal Plant	Organic solvent extracts		
	Hexane	**Chloroform**	**Methanol**
Solanum trilobatum	8.5 ± 0.42	13.0 ± 0.62	19.0 ± 0.71
Curcuma longa	6.0 ± 0.23	9.5 ± 0.44	16.0 ± 0.77
Psidium guajava	6.0 ± 0.21	8.0 ± 0.42	14.5 ± 0.63
Ocimum sanctum	5.5 ± 0.23	7.0 ± 0.23	13.0 ± 0.44
Azadirachta indica	NA	6.0 ± 0.21	11.5 ± 0.62
Acalyphafruticosa	NA	6.5 ± 0.23	10.0 ± 0.44
Centella asiatica	NA	5.5 ± 0.21	8.0 ± 0.41
Bacopa monnieri	NA	NA	8.5 ± 0.33
Piper betle	6.5 ± 0.42	7.0 ± 0.62	13.0 ± 0.62
Leucas aspera	NA	NA	8.5 ± 0.53

NA: No Activity

Table 2. Antibacterial activity of medicinal plant extracts against *V. harveyi*

Name of the Medicinal Plant	Organic solvent extracts		
	Hexane	Chloroform	Methanol
Solanumtrilobatum	8.0 ± 0.66	12.5 ± 0.77	18.5 ± 0.62
Curcuma longa	6.5 ± 0.44	10.5 ± 0.41	15.0 ± 0.71
Psidiumguajava	6.0 ± 0.23	9.0 ± 0.44	14.0 ± 0.63
Ocimum sanctum	5.0 ± 0.23	7.5 ± 0.62	12.5 ± 0.62
Azadirachtaindica	NA	7.0 ± 0.42	12.0 ± 0.44
Acalyphafruticosa	NA	6.0 ± 0.23	11.5 ± 0.61
Centellaasiatica	NA	5.5 ± 0.41	9.0 ± 0.41
Bacopamonnieri	NA	NA	8.0 ± 0.41
Piper betle	7.0 ± 0.71	6.5 ± 0.53	13.0 ± 0.62
Leucas aspera	5.0 ± 0.23	5.0 ± 0.23	9.5 ± 0.71

NA: No Activity

Table 3. MIC of medicinal plant extracts against *V. parahaemolyticus* and *V. harveyi*

Name of the Medicinal Plant	Minimal Inhibitory Concentration (µg/ml)	
	V. parahaemolyticus	***V. harveyi***
Solanumtrilobatum	1	1
Curcuma longa	10	1
Psidiumguajava	25	10
Ocimum sanctum	25	25
Azadirachtaindica	75	50
Acalyphafruticosa	75	75
Centellaasiatica	100	100
Bacopamonnieri	75	75
Piper betle	25	50
Leucas aspera	75	100

NA: No Activity

Table 4. MBC of medicinal plant extracts against *V. parahaemolyticus* and *V. harveyi*

Name of the Medicinal Plant	Minimal Bactericidal Concentration (µg/ml)	
	V. parahaemolyticus	***V. harveyi***
Solanum trilobatum	NGI	NGI
Curcuma longa	100	100
Psidium guajava	100	50
Ocimum sanctum	75	75
Azadirachta indica	75	50
Acalypha fruticosa	50	50
Centella asiatica	NGI	NGI
Bacopa monnieri	75	100
Piper betle	50	75
Leucas aspera	NGI	NGI

NGI: No Growth Inhibition

Table 5. MBC/MIC ratio of medicinal plant extracts against *V. parahaemolyticus* and *V. harveyi*

Name of the Medicinal Plant	MBC/MIC ratio	
	V. parahaemolyticus	***V. harveyi***
Solanum trilobatum	>100	>100
Curcuma longa	10	100
Psidium guajava	4	5
Ocimum sanctum	3	3
Azadirachta indica	1	1
Acalypha fruticosa	0.6	0.6
Centella asiatica	>100	>100
Bacopa monnieri	1	1.3
Piper betle	2	1.5
Leucas aspera	>100	>100

CONCLUSION

Out of the organic solvents used, methanol was found to exert the highest antibacterial potencies. Further, all the ten medicinal plants were reported to have a different range of growth inhibitory effects in the disc diffusion method and as well in MIC by broth microdilution method. The present results revealed that among the 10 medicinal plants, five plants are having a bacteriostatic effect and the rest of the five plants displaying bactericidal activity against the shrimp bacterial pathogens such as *V. parahaemolyticus* and *V. harveyi*. Moreover, these results suggested the dietary supplementation of the screened medicinal plants in polyherbal combination for shrimp aquaculture which is an environmentally friendly alternative to various antimicrobial drugs in combating *Vibriosis*.

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