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Original Article

Bactericidal effect of *Lawsonia inermis* aqueous leaf extract against selective *Vibrio* species of *Penaeus monodon* culture hatchery

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ABSTRACT

The bactericidal effect of aqueous leaf extract of *Lawsonia inermis* against selective *Vibrio* species was evaluated. *Vibrios* are marine bacterium, responsible for disease outbreak affecting commercial production of shrimp species especially *Penaeus monodon*. *Vibrios* were cultivated on Thiosulfate-citrate-bile salts-sucrose (TCBS) agar and seven colonies of *Vibrio* species were isolated based on their colony morphology. The aqueous plant extract was prepared from *Lawsonia inermis* and its antibacterial activity evaluated against selected *Vibrio* species. The antibacterial activity was tested by using the disk diffusion method, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) methods.

Keywords: TCBS, MIC, MBC, *Lawsonia inermis*, *Penaeus monodon*

INTRODUCTION

Aquaculture involves breeding, rearing and harvesting of plants and animals in all types of water including pond, river, lake and ocean. Cultivation of freshwater and saltwater populations under controlled conditions and can be contrasted with commercial fishing, which is the harvesting of wild fish. Broadly speaking, finfish and shellfish fisheries can be conceptualized as akin to hunting and gathering while aquaculture is akin to agriculture [1].

The importance of the fisheries sector in India is demonstrated by the fact that it employs more than five million people [2], contributes to food and nutritional security and employment, supports livelihoods, and raises the socioeconomic status of poor fishing communities. During the past half-century, Indian fish production registered excellent growth, from a meagre 0.75 million tonnes in 1950 to 6.3 million tonnes in 2002 [2,3]. The sector is one of the major contributors to foreign exports. During the past two decades, the inland fisheries in India, which include both capture and culture fisheries, have registered tremendous growth and change. Almost 400 species are reared in the aquatic environment with the aim of harvesting animal or plant protein.

Commercial shrimp farming began in the 1970s, and production grew steeply thereafter. Global production reached more than 1.6 million tonnes in 2003, worth about 9 billion U.S. dollars. About 75% of farmed shrimp is produced in Asia. Infectious diseases pose one of the most significant threats to successful shrimp culture. The Industrial monocultures are very susceptible to disease, which has decimated shrimp populations across entire regions. Cultured shrimps suffer from various diseases due to infectious and non-infectious causes. Infectious diseases are caused by viruses, bacteria, fungi and certa*in* parasites.

Vibriosis, is the bacterial disease caused by *Vibrio* species. It is one of the most prevalent diseases in aquaculture-reared organisms and widely responsible for mortality in shrimp culture worldwide [4,5]. Treatment of bacterial diseases with different herbs has been safely used in organic agriculture, veterinary and human medicine [6], and treatments with medicinal plants having antibacterial activity are a potentially beneficial alternative in aquaculture [7,8].

Therefore, due to increasing the resistance of microorganisms to antibiotics and the cost of modern allopathic medicines, the scientists are now looking for medicinal plants, because most of them

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are safe, cost less and are effective against a wide range of antibiotic resistant microorganisms. In this context, the present study elucidates the antimicrobial activity of aqueous extract of *Lawsonia inermis* against selective *Vibrio* species.

MATERIALS AND METHODS

Plant: The leaves of *Lawsonia inermis* (Family: Lythraceae) were collected during the month of October 2015 from in and around Vellore District, Tamilnadu, India. The plant materials were cleaned with distilled water and shade dried at room temperature. The plant material was authenticated and voucher specimens were kept at the Department of Zoology, Thiruvalluvar University, Vellore Dt., Tamilnadu. The shade dried plant materials were powdered by using electric blender.

Preparation of plant extract: The powdered plant materials were extracted separately to exhaustion in a soxhlet apparatus of aqueous plant extracts (Merk Chemicals, India). The extract was filtered through a cotton plug followed by Whatman filter paper (No.1) and then concentrated by using a rotary evaporator at low temperature (40-50°C) and reduced pressure to get yield. The extract was preserved in airtight containers and kept at 4°C until further use. The aqueous crude extract was taken for *invitro* antibacterial studies.

Primary bacterial culture: Water samples from the *P. monodon* hatchery culture tank were taken in a culture flask to identify the presence of *Vibrio* species. Appropriate dilutions were made in sterile PBS, and 100 ml samples were plated on TCBS agar. Seven colonies from different plates, displaying typical morphology on TCBS, were isolated in pure culture and presumptively identified to the species level by the method of Alsina and Blanch.

Maintenance of test bacterial strains: All the microorganisms used in the study were maintained on Nutrient agar (Himedia, Mumbai) slants and kept in refrigerator; sub-cultures were made after every fifteen days.

Antibacterial activity (Agar well diffusion method): The extract obtained from plant material was studied for antimicrobial activity. The water sample from *P.monodon* hatchery was inoculated in 100 ml of nutrient broth in a conical flask and incubated for 24 hrs to activate the strain. 100µl culture inoculums ($1x10^6$cfu/ml) from nutrient broth was inoculated into sterile TCBS agar plates for 48hrs. Different colonies of *Vibrio* species formed in as the TCBS agar plates. The colonies were identified based on the size and colour of the specific species. For pure culture, a loop full of specific colony was inoculated in the Muller Hinton Broth for 24 hours. 100µl of pure culture was inoculated into solidified Muller Hinton agar. Care was taken to ensure proper homogenization.

The experiment was performed under strict aseptic conditions. A well was made in the plates with sterile borer (5mm). The extract compound (50 µl) was introduced into the well and the plates were incubated at 37 ˚C for 24 to 48 hrs. All samples were tested in triplicates. Microbial growth was determined by measuring the diameter of the zone of inhibition. Ciprofloxacin (Himedia, Mumbai, India) is a reference drug used as a control for test organisms.

Determination of Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC): The plant extract which was found to be effective as antimicrobial agent, was later tested to determine the MIC and MBC values for selected strain. MIC was determined using broth dilution method. The plant extract was diluted with 5ml Muller Hinton broth to give the final concentrations of 1000, 500, 250, 125, 62.5, 31.25µg/ml. The different concentration of plant extract was taken in six separate MIC tubes and 100 µl (10^6 CFU/ml) of the pure *Vibrio* species from the TCBS agar was inoculated into the tubes. The tubes were incubated under aseptic condition at 37°C for 24 hrs. Streptomycin which was used as the positive control (15µg) was added into 5 ml of Muller Hinton broth taken in the control tube. The lowest concentration of the extract that produced no visible growth (no turbidity) in the first 24 hrs when compared with the control tubes was considered as initial MIC. The dilutions that showed no turbidity were incubated further for 24 hrs at 37 °C. The lowest concentration that produced no visible turbidity after a total incubation period of 48 hrs was regarded as final MIC. The minimum bactericidal concentration (MBC) was defined as the minimal concentration of the plant extract which completely inhibited the visible growth of the bacteria on solid media in the petriplates that were incubated at 37ºC for 24 hrs.

RESULTS

Agar well diffusion method: In this study the antibacterial activity of *Lawsonia inermis* aqueous leaf extract was evaluated against seven *Vibrio* species (Table:2). The aqueous leaf extract of *Lawsonia inermis* showed more antibacterial activity against all *Vibrio* species like *Vibrio harveyi* (10mm), *Vibrio natriegens* (8mm), *Vibrio alginolyticus* (7.5mm), *Vibrio parahaemolyticus*

(9.5mm), *Vibrio fluvialis* (11mm), *Vibrio anguillarum* (9mm) and *Vibrio vulnificus* (9.5mm). The zone inhibitory activity of plant extract reported in (Table. 2) was compared with standard reference antibiotic Ciprofloxacin (15µg).

Minimum inhibitory concentration (MIC): The MIC value of *Lawsonia inermis* aqueous leaf extract against *Vibrio* species is shown in table 3. Generally, if the extract displayed an MIC in well diluted form (e.g. below 100 µg/ml), then its antimicrobial activity was considered to be the best; if in moderate dilution (e.g., from 100 to 500 µg/ml) the antimicrobial activity was considered to be better; and if in more concentration during dilution (e.g., from 500 to 1000 µg/ ml) the antimicrobial activity was considered as good. If the same was above 1000 µg/ml then the extract was considered inactive. The leaf extract of *Lawsonia inermis* presented best activity against *Vibrio* species like *Vibrio harveyi* (MIC 31.25), *Vibrio natriegens* (MIC 125), *Vibrio alginolyticus* (MIC 125), *Vibrio parahaemolyticus* (MIC 62.5), *Vibrio fluvialis* (MIC 31.25), *Vibrio anguillarum* (MIC 62.5) and *Vibrio vulnificus* (MIC 62.5) (Table : 3).

Minimum bactericidal concentration (MBC): The results for Minimum Bactericidal Concentration (MBC) were similar to Minimum Inhibitory Concentration (MIC) results, but in MBC confirmation was made by absence of growth in culture plates. It is expressed in table 3.

DISCUSSION

The medicinal plants constitute an effective source of both traditional and modern medicines. In this study was conducted to investigate the *invitro* antibacterial activity of aqueous leaf extract of *Lawsonia inermis* against *Vibrio* species of *P.monodon* hatchery. The plant extract showed high antibacterial activity against *Vibrio vulnificus, Vibrio alginolyticus* and *Vibrio natriegens* (Table:2,3). The strong antibacterial activity of plant extract might be due to presence of various phytochemical constituents, such as alkaloids, tannins, saponins, cardenolides, flavonoids and polyphenols. Phenolic compounds are generally noted for their antimicrobial activities [9]. These phyto-chemical constituents may be present in the *Lawsonia inermis* extract which is responsible for its antibacterial effect.

The *Lawsonia inermis* extract can be used as an alternative therapeutic agent to prevent and control the outbreak of diseases, mainly in hatcheries. Since the plant substance is natural, their hazardous potential is lower when compared with other products. The results proved that the *Lawsonia inermis* plant has a high potential for use as an alternative therapy to control bacterial of crustacean diseases [10]. This is to be expected because the outer membrane of the bacteria is known to be a barrier for the penetration of numerous antibiotic molecules, and the periplasmic space contains enzymes, which are capable of breaking down foreign molecules introduced from outside [11]. It was interesting to note that the extract as able to control *Vibrio* species. It has been concluded form this study that *Lawsonia inermis*, can be used as a potent antibacterial drug in the crustacean aquaculture instead of synthetic antibiotics.

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Table: 1: Colony morphologies on TCBS agar

Species	Colony morphology
Vibrio harveyi	Small (diameter, 2 to 5 mm) colonies; light green with dark
Vibrio natriegens	Small (diameter, 2 to 5 mm) colonies; light green
Vibrio alginolyticus	Large (diameter, 5 mm) colonies, spreading; light blue
Vibrio parahaemolyticus	Large (diameter, 5 mm) colonies, spreading; light blue
Vibrio fluvialis	Small (diameter, 2 to 5 mm); dark blue-green
Vibrio anguillarum	Very small (diameter, 2 mm); light blue
Vibrio vulnificus	Small (Diameter 2 to 5mm) colonies; light green

Table: 2: Antibacterial activity of aqueous leaf extract of *Lawsonia inermis* against *Vibrio* species of *P.monodon* hatchery

***Vibrio* Spp.**	**Zone of Inhibition (in mm)**	**Ref Drug Ciprofloxacin (15µg)**
Vibrio harveyi	10 ± 0.30	14.5 ± 0.15
Vibrio natriegens	8 ± 0.10	14.5 ± 0.15
Vibrio alginolyticus	7.5 ±0.32	14.5 ± 0.15
Vibrio parahaemolyticus	9.5± 0.15	14.5 ± 0.15
Vibrio fluvialis	11 ± 0.32	14.5 ± 0.15
Vibrio anguillarum	9 ± 0.12	14.5 ± 0.15
Vibrio vulnificus	9.5 ± 0.26	14.5 ± 0.15

Table 3: Determination of MIC and MBC of aqueous leaf extract of *Lawsonia inermis* against *Vibrio* species of *P.monodon* hatchery

Vibrio Spp.	**MIC (µg/ml)**	**MBC (µg/ml)**	**Ref Drug** Ciprofloxacin (15µg)
Vibrio harveyi	31.25	31.25	15
Vibrio natriegens	125	125	15
Vibrio alginolyticus	125	125	15
Vibrio parahaemolyticus	62.5	62.5	15
Vibrio fluvialis	31.25	31.25	15
Vibrio anguillarum	62.5	62.5	15
Vibrio vulnificus	62.5	62.5	15

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