

Evaluation of Antibacterial Activity of *Achyranthes aspera* Extract against *Vibrio alginolyticus*: An in Vitro Study

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Abstract: Marine organisms are potential sources of unique natural products with pharmacological and biological activities. In mariculture, diseases of microbial origin can cause huge economic losses worldwide. The evolution of microorganism resistance to antibiotics has resulted in a growing need for new antibacterial compounds that are effective in veterinary medicine and characterized by limited undesirable side effects. Increased attention has recently been turned to plants as a promising source for metabolites with antimicrobial activity. *Achyranthes aspera* is one such important plant with various established pharmacological properties. The aim of the present study was to assess the antibacterial activity of the *Achyranthes aspera* extract against *Vibrio alginolyticus* 1374. Aqueous extract of *Achyranthes aspera* was prepared. Different concentrations of the root and stem extracts of *Achyranthes aspera* were transferred to the agar plates, which had been streaked with the bacterium *Vibrio alginolyticus*; 1374. The plates were incubated aerobically at 37°C for 24 h and the zones of inhibition were measured using well diffusion method. *Achyranthes aspera* extract showed significant zones of inhibition. *Achyranthes aspera* showed marked antibacterial activity against *Vibrio alginolyticus* 1374.

Keywords: *Vibrio alginolyticus* 1374, *Achyranthes aspera*, Ciprofloxacin

INTRODUCTION:

In aquaculture, diseases of microbial origin cause high mortality rates and lesions on fish skin, with consequent economic losses worldwide [1]. Bacteria, mainly of the genus *Vibrio* have been identified as the etiological agents responsible for the most common disease outbreaks in fish and shellfish, called *Vibriosis* [1-3]. Moreover, these microorganisms can accumulate in the reared animal's flesh and become a serious threat for human health. For example, *Vibrio vulnificus* is considered as one of the worst foodborne pathogen, mainly causing gastroenteritis. It is often present, together with *V. parahaemolyticus*, in the edible *Mytilus galloprovincialis* from the Italian Apulian farms [4, 5].

Vibrio's have been recognized as human pathogens, and they can be acquired through either ingestion of contaminated seafood or contact of traumatized skin with seawater [6]. Extra intestinal *Vibrio* infections are most commonly cutaneous wounds or otitis externa, where breaks in the skin have become contaminated while swimming or boating in infected marine waters or after handling contaminated raw seafood. Most clinical isolates are recovered from superficial wounds or the external ears [7]. Other *Vibrio cholera*, less common halophilic *Vibrio sp.* include *V.*

vulnificus, *V. alginolyticus*, *V. fluvialis*, *V. hollisae*, *V. damsela*. Molecular methods can be suggested for identification or detection of these species [8].

V. alginolyticus, first identified as a pathogen of humans in 1973, has been predominantly associated with cellulitis and acute otitis media or externa rather than gastroenteritis [6,9]. *V. alginolyticus* occasionally causes life-threatening infections in immunocompromised individuals [10]. Conjunctivitis, acute gastroenteritis, bacteremia and necrotising fasciitis caused by *V. alginolyticus* have also been reported [7]. Bacteremic infections of *V. alginolyticus* are severe and occur only in patients with underlying diseases.

The herb *Achyranthes aspera* Linn. (Amaranthaceae), is a common herb found throughout India and is one of the well-known folk medicine for treating pneumonia and in the treatment of rheumatoid arthritis. The plant parts are widely used for the treatment of upper respiratory tract infections, urinary tract infections and various sexually transmitted diseases caused by bacteria and fungi.

The antimicrobial activity and medicinal qualities of several medicinal plants have been reported [11-15]. The study therefore was undertaken to evaluate the presence of antimicrobial activity of *Achyranthes aspera* Linn against marine *Vibrio alginolyticus*. Aqueous extracts of roots as well as stems were selected for testing the antimicrobial activity.

MATERIALS AND METHODS:

Preparation of medium

Readymade dehydrated medium supplied by Hi Media was used for testing the antimicrobial activity of plant extracts. The dehydrated medium was dissolved in 100 ml of distilled water and heated to boiling to dissolve the medium completely following the instructions given by the manufacturer. The medium was distributed into clean glass tubes and plugged with cotton and sterilized by autoclaving at 15 lbs/sq. inch pressure at 121°C for 20 min.

Sample collection:

The seawater was collected from coastal areas of Prakasham district of Andhra Pradesh using new sterile screw capped tubes and subsequently transported to the laboratory for further biological analysis.

Isolation of organism

Thiosulphate Citrate Bile Sucrose (TCBS) agar medium was used for the isolation [16]. TCBS plates were prepared by dissolving 8.9 gm of TCBS agar in 100ml of marine water. It was slightly heated and temperature was reduced to 55°C. Agar medium was poured into 3 petri plates each with 15 – 20 ml, after solidification, 2ml of diluted ($10^{-4} \times 10^{-6}$) samples were transferred to 2 different petri plate by spread plate method. Petri plates were incubated overnight at 37°C for the growth of *V. alginolyticus*.

Identification of the marine bacterial isolate

Sub culturing was done for single colony isolation. A loopful of seed culture from overnight sample was streaked on to new TCBS agar plate and was incubated for 24 hrs. The strain identity was further confirmed by DNA sequencing technique [17].

Inoculum

Culture of test organisms were used as inoculum. Loop full of organism was taken from the slant and transferred to a flask containing sterilized nutrient broth and allowed to grow at 37°C. The 18 hr culture was then plated on a nutrient agar plate to study the morphological character. The cultures from nutrient broth were centrifuged and a suspension of cells were made with the sterile saline (10%). This culture suspension was used for further studies.

Seeding of culture

To a 12 ml of molten sterile nutrient agar medium in tubes (maintained at 45°C), 0.2 ml of the culture suspension was added. The tubes were mixed

thoroughly and poured into sterile plates and care was taken to form a uniform layer.

Reference standard solution

Ciprofloxacin at concentration of 1000 µg/ml was used as positive control for comparison with *Achyranthes aspera* Linn extracts. Selection of these standard was based on its broad spectrum of activity against various bacteria and on its available purity.

Controls for the test

The controls were required to confirm all the necessary nutritional conditions were suitable for the growth of microorganism and for absence of inhibitory substance in the medium. The positive controls were observed by streaking the organism on agar plates for observing morphology of colonies. Any contamination during the assay was ruled out by keeping the negative control. This was checked by adding the sterile saline and observing for growth as a contamination. The results indicated that the medium was free from contamination.

Preparation of plant extracts

Fresh stems and roots of *A. aspera* were collected and dried under shade and then in an oven at a temperature above 50°C. The stems and roots were ground into a fine powder. Then, 100 g of powdered stems and roots were boiled in 2000 ml of distilled water separately in two flasks for 2 h. One thousand and three hundred milliliters of filtrate was obtained from the stems and roots, which was then filtered using a filter paper. It was reduced to obtain a solid residue of 6 g by heating it at 60°C. The solid residue obtained was then stored at a low temperature. Dimethyl sulphoxide was used to dissolve the solid residue made from the stems and roots to make different concentrations. One gram of solid residue was mixed in 10 ml of dimethyl sulphoxide to make 10% concentration and it was kept as a stock solution. From this stock solution, further different concentrations were made.

Determination of zone of inhibition

The antibacterial activity was determined by well diffusion method. 20ml of sterile nutrient agar medium was poured into sterile petri-dishes and allowed to solidify. The petri dishes were incubated at 37°C for 24 hours to check for sterility. The medium was seeded with the organisms by pour plate method using sterile top agar (4 ml) contained 1 ml culture. Bores were made on the medium using sterile borer. Different concentrations of extract were added in to the respective bores.

0.2 % of Ciprofloxacin at concentration of 1000 µg/ml was taken as standard reference. The plates were incubated overnight at 37°C with appropriate positive and negative controls. The petri-dishes were kept in refrigerator at 4°C for ½ hour for diffusion. After diffusion the petri-dishes were incubated at 37°C

for 24 hours and zone of inhibition were observed and measured. Dimethyl sulfoxide was used as the control.

RESULTS & DISCUSSION

Vibrio alginolyticus isolates produced yellow color colonies without pigmentation and showed the faster growth within a day. Then the strain was identified through series of sub culturing and analyzing enzymatic properties. It showed a positive reaction to indole production, Voges-Proskauer, lysine decarboxylase, and ornithine dihydrolase, glucose

fermentation, gelatin hydrolysis, reduction from nitrate to nitrite, alkaline phosphatase and growth in Nutrient broth with 6-10% NaCl. Negative findings included: growth on Simmons' citrate, growth in Nutrient broth with 0% NaCl, H₂S on tri-sugar iron agar (TSI), gas production, urea hydrolysis, and arginine decarboxylase, myo-inositol, lactose fermentation. Characterization of isolates showed gram negative red color, rod shaped bacteria as *Vibrio alginolyticus*. Finally the strain identity was confirmed by sequencing method.



Fig-1: Colonies on NA plate

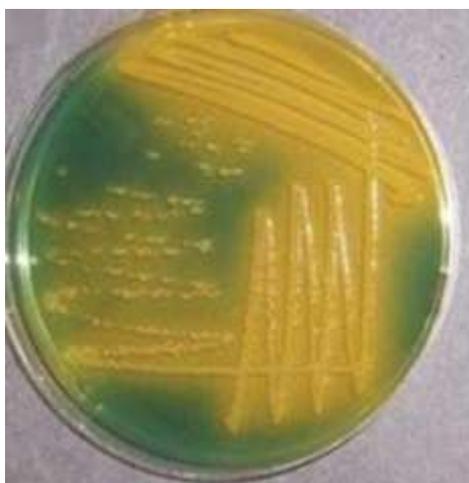


Fig-2: Colonies on TCBS agar medium

The ability of test substance (plant extract) to inhibit bacterial growth was confirmed by the appearance of zone of inhibition around the well containing the test solution after a specified incubation period. The negative control was checked for the absence of growth, thereby indicating the sterility of the medium. The remaining plates were examined for the presence or absence of growth. The positive control without *Achyranthes aspera* Linn. Extract was checked to ensure that the test strain was capable of showing

adequate growth in the medium. In reading the end points, a faint haze of growth of a single colony was evident for antimicrobial activity. A dense film of growth or more than one colony was considered as evidence that the plant extract failed to inhibit the growth. Both root as well as stem extracts were found to exhibit wide range of activity against *Vibrio alginolyticus*. The results are summarized in Table 1 & 2.

Table-1: Zone of inhibition of stem and root extracts

Concentrations	100		150		200		250 μ l	
2.5	12	14	12	16	13	16	14	17
5	13	15	13	16	14	17	14	18
7.5	13	16	14	17	15	17	15	18
10	14	16	15	16	16	20	16	21

Table-2: Mean values of stem and root extracts at different volumes and concentrations

	Extracts	N	Mean \pm S.D	T	P
100 μ l	Root	4	15.25 \pm 0.96	3.576	0.012*
	Stem	4	13 \pm 0.82		
150 μ l	Root	4	16.25 \pm 0.5	3.973	0.007**
	Stem	4	13.25 \pm 1.29		
200 μ l	Root	4	17.5 \pm 1.73	2.777	0.032*
	Stem	4	14.5 \pm 1.29		
250 μ l	Root	4	18.5 \pm 1.73	3.79	0.009**
	Stem	4	14.75 \pm 0.96		

* Significant , ** Highly significant , S.D. Standard deviation.

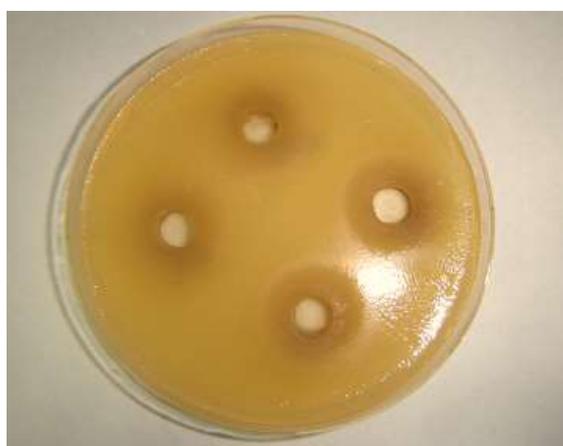


Fig-3: Zone of inhibition formed by *Achyranthes aspera* root extracts at different concentrations and volumes

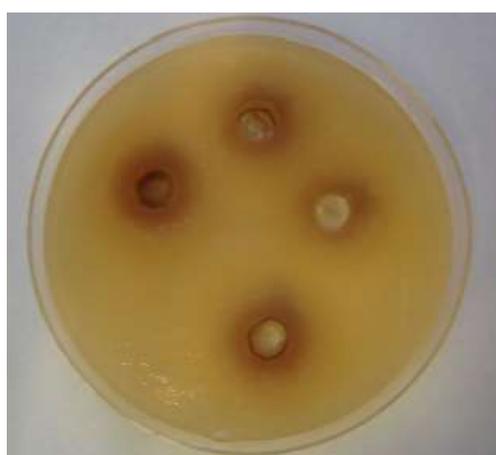


Fig-4: Zone of inhibition formed by *Achyranthes aspera* stem extracts at different concentrations and volumes

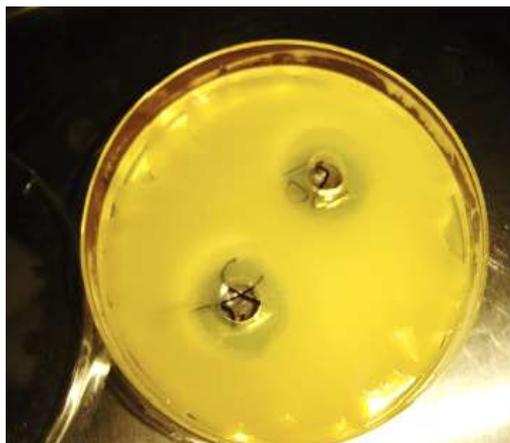


Fig-5: Zone of inhibition formed by 0.2% Ciprofloxacin.

The lowest concentration of the extract, which inhibited the growth of the tested microorganism, was found to be 2.5% for both the stem and root extracts. The minimum zone of inhibition of 14 mm was seen at 2.5% concentration and 100 μ l volume of root extract. While the maximum zone of inhibition of 21 mm was seen at 10% concentration and 250 μ l volume. Similarly, the minimum zone of inhibition of 12 mm was seen at 2.5% concentration and 100 μ l volume of stem extract while the maximum zone of inhibition of 16 mm was seen at 10% concentration and 250 μ l volumes [Table 1]. The mean value of zone of inhibition of the stem and root extracts was 13 and 15.2 at the lowest volume and 14.7 and 18.5, respectively, at the highest volume [Table 2]. While for Ciprofloxacin at 0.2% concentration, the zone of inhibition obtained was 19 mm at 10 μ l volume.

Herbal medications have been well known for ages. Our ancestors had been known to use plant life to treat and alleviate illnesses that affect the human body. Medicinal plants are today being once again preferred because of the various reasons such as their easy availability, negligible side effects, low cost of treatment, and their effectiveness. The present study proved that *A. aspera* possesses a significant antibacterial activity against *Vibrio alginolyticus*, which is the causative organism playing a major role in the pathogenesis of several infections.

The antibacterial activity of *A. aspera* can be attributed to the alkaloids and tannins. Tannin is a phenolic compound which is soluble in water and it could be one of the components responsible for the antibacterial activity [18]. Extracts of the leaves and callus of this plant in various solvents have been reported to show antimicrobial activity [19]. Prabhat *et al* [20] reported that methanolic extracts possess antimicrobial activity while Khan *et al* [21] reported that the ethanol and chloroform extracts of the seeds of *A. aspera* show mild-to-moderate antibiotic activity against *Bacillus subtilis*, *E. coli*, and *Pseudomonas aeruginosa*. Jebashree *et al* [22] demonstrated the

anticariogenic activity of *A. aspera* by using ethyl acetate extracts of *A. aspera*, which showed high antibacterial activity against *S. mutans* than other solvent extracts. However, in the present study, aqueous extract of *A. aspera* was used, which is most easy and safe to obtain and showed a marked antibacterial activity.

Any antimicrobial agent is considered effective, given the size of inhibition zone produced by it measures 2 mm or more. In the present study, the minimum zone of inhibitions obtained were 12 mm and 14 mm for stem and root extracts of *A. aspera*, respectively. It has proved to have potent antibacterial property.

CONCLUSION

This observation may be of practical importance especially in the treatment of secondary infections where bacteria act as an opportunistic organism. The search for antimicrobial principles from plants and microbial life share a common philosophy. The microbial source has been extensively studied by researchers. It can therefore be concluded that natural products of plant origin can be exploited for the search of natural pesticides as well as for medicinal products. Further studies can be conducted in future to assess the safety levels of such herbs.

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