

Phytochemical analysis and invitro assays for antimicrobial activity of *Pluchea lanceolata* extract against multi drug resistant *Vibrio cholerae*

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Abstract: In the present study water samples from different areas of Kanpur were collected and isolated three *Vibrio cholerae* strains. The growth pattern of *V. cholerae* strains were observed on TCBS agar and 3%, 5% and 8% salt concentration. Mostly *V. cholerae* strains were tolerate at 3% to 5% salt concentration but in this study one strain can tolerate at 8% salt concentration. The multidrug resistant activities of these strains were examined by different antibiotics i.e. Kanamycin, Gentamicin, ampicillin, cefixime, streptomycin and oxacilline. Methanolic leaf extract of *Pluchea lanceolata* were analyzed by qualitative phytochemical methods to identified different secondary metabolites. The different concentration (100%, 50%, 25% and 12.5%) of methanolic extract of *Pluchea lanceolata* was used as a biological tool to resolve the antibiotic problem. *P. lanceolata* extract inhibited the growth of isolated strains of *V. cholerae* at all concentrations and zone of diameter increased with the increase of concentrations. This study revealed that the extract of *P. lanceolata* is a potent antibacterial drug in the treatment of *V. Cholerae* infection. It provided to cholera patient if found effective and non toxic through in vivo studies.

Key words: *Vibrio cholerae*, Salt tolerance, *Pluchea lanceolata*, Phytochemical analysis, Multidrug resistance.

I. Introduction

Bacterial contamination in natural water continues to be a major problem in the developing countries and people suffering from many infectious water born diseases. Diarrhoea is one of the most dangerous water born disease by which increases the rate of mortality in every year. The people of India get affected by diarrhoeal infection through various sources and the most important is the lack of hygiene practice that causes severe sickness to them. The worldwide infections of diarrhoeal disease caused by *Vibrio cholerae* and near about 120,000 deaths are estimated each year (Nielsen et. al., 2006). The virulence genes of *V. cholerae* encoded the cholera toxin proteins, during infection they causes profuse, watery diarrhoea (Boyd et.al., 2002). *Vibrio cholerae* infection can quickly lead to a life-threatening situation due to loss of fluid. Such situations are often addressed by infusion of electrolytes to the blood and the simultaneous antibiotic therapy (Hodges et.al.,2010). A recent study from India comprising seven hospitals from New Delhi, Vellore, Bangalore and Ludhiana found that the resistance of enteropathogens to antibiotics was greatest for quinolones, macrolides, aminoglycosides, trimethoprim, β -lactams and sulphonamides of higher generations (Ganguli et.al., 2011). Many plants in India are used as traditional medicine for the treatment of gastrointestinal disorders such as cholera, diarrhoea and dysentery (Chopra et. al.1956, Mukherjee et. al.,1998, Nautiyal et.al.,2000, Maikhuri et al.,2000, Kala 2005, and Muthu et. al.,2006). There impact is particularly large in developing countries due to relative unavailability of medicines and the emergence of widespread drug resistance (Zampini et.al., 2009). The search for natural antimicrobials to use in foods is encouraged by the high prevalence of food-borne diseases and the current popular preference of consuming only natural foods (Rasooli, 2007). Medicinal plant extract were able to disrupt the cell membranes of *V. cholerae* cells, causing increased membrane permeability, a clear decrease in cytoplasmic pH, cell membrane hyperpolarization, and a decrease in cellular ATP concentration (Eduardo et.al., 2010). Several studies have reported the use of herbal preparations for the control of infectious bacteria (Ushimaru et.al.,2007, Oliveira et. al. 2013, and Al Akeel et.al., 2014).The present study was designed to examine the occurrence and emergence of multidrug resistant *Vibrio cholerae* with newer antibiotic-resistance profile in the aquatic environment of Kanpur (U.P.) India. Phytochemicals analyses of *Pluchea lanceolata* leaf extract to find out the chemical constituents that have the antibacterial properties against isolated *Vibrio* spp. These finding have implications for understanding the antibiotic resistance pattern of *Vibrio* species and the feasibility of using *Pluchea lanceolata* leaf extract to prevent spread of pathogenic *Vibrio cholerae* Strains before the initiation of cholera epidemics.

II. Materials And Methods

Study area and sample collection

In the present study water sample were collected from three different areas of district Kanpur (U.P.), India i.e. Kalyanpur, Mandhna and Chaubeypur during May to June, 2014. At each time of sample collection, precaution was taken and samples were aseptically collected in sterile glass bottle according to standard procedure of microbiology and transported to the laboratory.

Enrichment of samples

For enrichment of bacterial population, 100ml water samples were filtered through 0.22 μ m pore size Whatmann No.1 filter paper. Dip the filter paper into 50ml autoclaved alkaline peptone water (APW) and incubate at 37^oC for 12 hours.

Isolation of vibrio cholerae Strains

1ml alkaline peptone broth culture were serially diluted and Spread 10⁻⁵-10⁻⁶ dilution on a selective TCBS agar media and incubated at 37^oc for 24 hrs. Observe the isolated colonies and streaked on TCBS agar plate for obtained pure culture and for presumptive identification. The bacterial cultures were confirmed using biochemical test.

Characterization of Vibrio cholerae strains

The shape and type of Gram reaction and microscopically studied using 24 hrs culture from agar plate. The biochemical tests involved catalase, Gelatin hydrolysis, Methyl Red (MR), Voges Proskauer (VP), Indole and Simmon's Citrate. Identification of isolates of obtained in pure culture was based on Gram staining, biochemical characteristics and growth pattern on selective media and according to the procedure recommended in the Bergey's Manual.

Plant material

The plant of *Pluchea lanceolata* were collected from Gangatic zone of Kanpur, (U.P) India, and the taxonomic identity of the plant were identified by Dr. R. K. Pandey Department of Botany K.K.C., Lucknow.

Extraction procedure

The fresh leaves of plant were washed under running tap water, dried at room temperature and then made fine powder using a mixer. 10 g of powder were dissolved in 100 ml of methanol in a conical flask. Plugged the flask with cotton and kept on a rotary shaker at 250 rpm for three days. After three days filtered with the help of Whatmann No.1 filter paper and the solvent was evaporated carefully at low temperature until reach its maximum concentration (Harbone et. al. 1973) supposed final concentration as 100% and it diluted in respected solution to form different concentration as 50%, 25%, and 12.5% then store at 4^oc for phytochemical analysis and testing their antimicrobial properties.

Phytochemical analysis of plant extracts

Plant extracts were screened for the presence of major phytochemicals. The presence of biologically active compounds was determined by qualitative phytochemical analysis of the crude extract of *Pluchea lanceolata* was determined using standard methods. 1ml of extract was taken and a few drops of 5% aqueous ferric chloride were added. If blue black colour appears and disappear on addition of dilute H₂SO₄. It indicates the presence of condensed salts of tannins (Bruneton, 1995). Few ml of extract was taken in 5ml distilled water. Add 2M HCl until acid reaction; add 1 ml of Dragendorff's reagent, red or orange precipitate indicated the presence alkaloids. The method of (Subramanian and Nagarjan, 1969, Ogunyemi, 1979) was used for confirmation of flavonoids. 1ml extract was dried over a water bath and 5-10 drops of concentrated HCl was added followed by Zn powder. A pink, reddish pink or brown colour develops indicates the presence of flavonoids. Small amount of plant extract was dissolved in distilled water and add 1ml of 1% NaOH solution yellow colour indicate the presence of Glycosides. Small amount of extract was dissolved in 2ml distilled water. A few drops of 10% aqueous ferric chloride solution were added. A blue green colour develops show the presence of phenols.

Screening for antimicrobial activity

The antimicrobial activities were screened on MHA media by using disc diffusion method (Bauer et.al.1996, Andrews's et.al.2001) Taken loopful bacterial culture in sterile water and Swabbing on MHA agar plate with the help of sterile ear bud. Marked the swabbed plate with marker as 100%, 50%, 25% and 12.5%. Sterile Whatman No.1 filter paper discs (6 mm in diameter) were impregnated in respective percentage solution

and then placed on the swabbed MHA plates. The multidrug resistant properties were showed by using six antibiotics i.e. Kanamycin, Gentamicin, Amikacin, ampicillin, cefixime and erythromycin. The plates were kept at 4°C for 1 h for diffusion of extract, thereafter were incubated at 37°C for 24 hrs. Antibacterial activities were observed by zone of inhibition in mili meter.

III. Results And Discussions

Isolation and identification of *V.cholerae*

Water samples from different areas of Kanpur were collected and for enrichment of bacterial population, 100ml water samples were filtered through 0.22µm pore size Whatman filter paper. Dip the filter paper into 50ml autoclaved alkaline peptone water (APW) and incubate at 37°C for 12 hours. Spread the 50 micro litre cultured peptone sample on TCBS agar plate and showing the result of isolated bacterial colonies. Streaked selected colonies on TCBS agar plate for obtained pure culture. Identification of isolates obtained from pure culture was based on Gram staining, biochemical characteristics and growth pattern on selective and differential media according to the procedures recommended in the Bergey's Manual of Determinative Bacteriology.

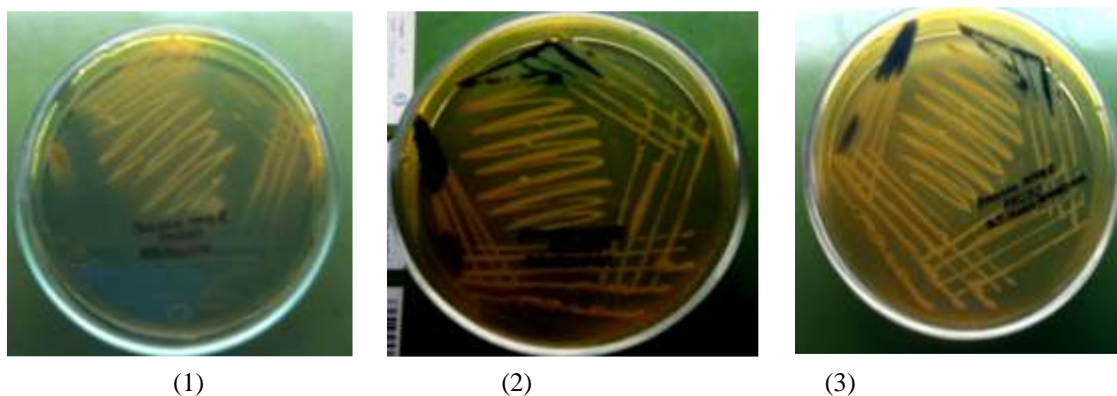


Fig.1. Streaking of isolated colonies on TCBS agar plate to obtain pure culture.

Biochemical test for strains identification

Catalase Test: After addition of 3% H₂O₂ bubbles were arises. This observation indicated that bacterial colony was showing positive catalase test.

Gelatin Hydrolysis Test: Inoculated gelatin tube after keeping 4°C observed as liquid. This liquification of gelatin was showing positive gelatin hydrolysis test.

Indole Production Test: Chery red or dark brown colour ring was observed in inoculated tryptone broth hence shows positive indole production test.

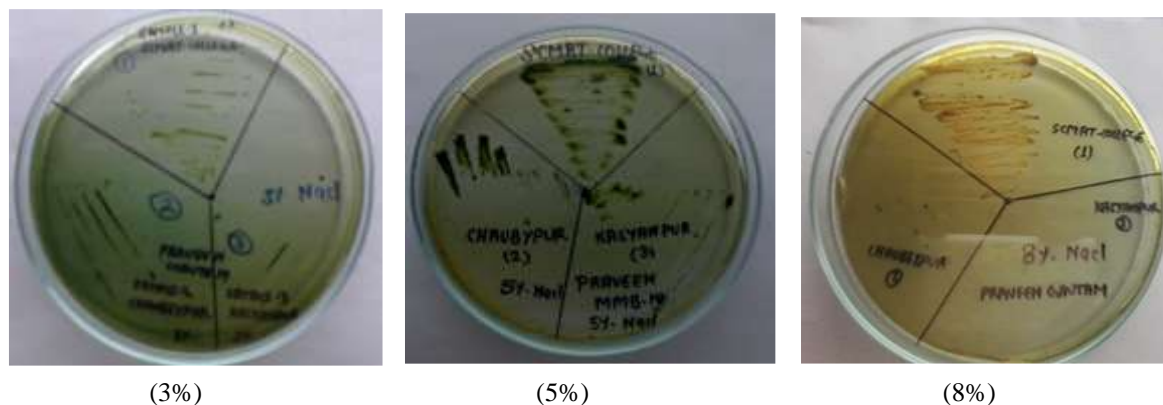
MR Test: After the addition of methyl red, at pH 4 the colour of broth become Pink or red shows MR +ve test.

VP Test: No colour change observed in all inoculated VP broth after addition of VP reagents. This observation showed VP -ve.

Simmon's Citrate Test: In all Bacterial slants growth were observed and the medium colour was turned green to blue. This observation shown Citrate +ve test.

Salt tolerance

Various species of *Vibrio cholerae* have different characteristics in salt tolerances that could be used for identification. According to salt tolerance of *Vibrio* spp, *Vibrio cholerae* can grow at 3% and 5% salt concentration and cannot grow at 8% salt concentration. But in this experiment I found that the isolated *Vibrio cholerae* sample-1 can grow at 8% NaCl whereas sample-2 and sample-3 cannot grow at 8% NaCl which indicates them to be obligate halophiles. From the above observation it is clear that the three suspected culture was identified as *Vibrio cholera*.



(3%) (5%) (8%)
Fig. 2. Showing salt tolerance at 3%,5% and 8% TCBS agar plate.

Morphological & Biochemical Test	Sample-1	Sample-2	Sample-3
Gram reaction	-	-	-
Shape	Comma	Comma	Comma
Pigments	+	+	+
Catalase	+	+	+
Gelatin hydrolysis	+	+	+
Indole	+	+	+
MR test	+	+	+
VP test	-	-	-
Citrate test	+	+	+
Salt tolerance	3%, 5% & 8%	3%, 5%	3%, 5%

Table:1. Morphological and Biochemical characteristics.

Phytochemical analysis

Phytochemical results shows that the methanolic extract of *pluchea lanceolata* is rich in tannins, phenols, alkaloids,flavonoids and glycosides.

Susceptibility against *Pluchea lanceolata* extract at different cocentration

The extract of *Pluchea lanceolata* was used as a biological tool to resolve the antibiotic resistant *V. cholerae* problem. The extract showed promising effect against the isolated *Vibrio cholerae* at different concentrations (100%, 50%, 25% and 12.5%). The zone diameter at different concentration of extract is shown below. *Pluchea lanceolata* extract inhibits the growth of *V. cholerae* at all concentrations and zone diameter increases with the increase of concentrations.



(1) (2) (3)
Fig.3. Methanolic extract showing zone of inhibition at different concentration.

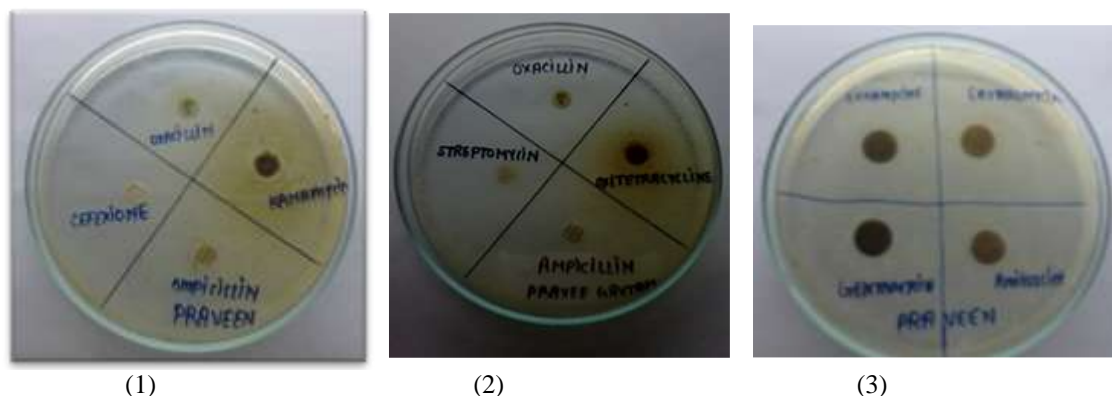


Fig.4. Bacterial strains showing multidrug resistant with different antibiotics.

Pathogen	Zone of inhibition in mm with different antibiotics					
	Cefixime	Streptomycine	Oxacilline	Gentamicin	Kanamycin	Ampicilline
Sample-1	0	0	2	1	3	0
Sample-2	0	0	2	1	1	0
Sample-3	0	0	2	1	1	0

Table:3. Zone of inhibition in mm with different antibiotics

Pathogens	Zone of inhibition in mm with different concentration			
	100%	50%	25%	12.5%
Sample-1	10	8	6	2
Sample-2	9	5	4	2
Sample-3	10	5	3	2

Table:4. Zone of inhibition in mm with different percentage of *Pluchea lanceolata* extracts

IV. Conclusions

From the literature study and experimental results analysis that *Pluchea lanceolata* is a traditional medicine containing important bioactive compounds having antibacterial properties against isolated new multidrug resistant strains of *Vibrio cholerae*. This additional antibiotic resistance might have contributed to the initial selection of the new strain because this antibiotic is used to treat cholera patients. Phytochemical study would help to establishment of some compounds that could be used to formulate new and more potent antimicrobial drugs of natural origin against *Vibrio cholerae*. This is the first report on *Pluchea lanceolata* for its antibacterial activity against the strains of *Vibrio cholerae*, especially against multiple antibiotic resistant isolates.

Pluchea lanceolata is also used to treat various human diseases like anti-inflammatory, and analgesic activity. The alkaloids and flavonoids of the plant possess various applications in the field of medicine and greatly used in rheumatoid arthritis and neurological diseases. The plant is still used in their native places due to its medicinal properties. Hence, this article would be further useful to the researchers and other clinical persons to understand its basic mechanism of action.

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