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A comparative analysis of *in vitro* growth inhibition of waterborne bacteria with bioactive plant *Lippia nodiflora* L. and camphor

Madhu Pandey, Anand Pandey, Shashi Kant Shukla, Rajesh Kumar, Ashutosh Pathak, Rohit Kumar Mishra, Anupam Dikshit*

Biological Product Laboratory, Department of Botany, University of Allahabad, Allahabad 211002, India, Tel. +91 7843987553; email: madhupbpl1989@gmail.com (M. Pandey), Tel. +91 9307014222; email: deep.7890@gmail.com (A. Pandey), Tel. +91 9415603821; email: shashibplau@gmail.com (S.K. Shukla), Tel. +91 9196010036; email: rajeshdubey.au@gmail.com (R. Kumar), Tel. +91 9335329228; email: ashupathaks@rediffmail.com (A. Pathak), Tel. +91 9305231487; email: rohit_ernet@yahoo.co.in (R.K. Mishra), Tel. +91 9335108519; email: anupambplau@rediffmail.com (A. Dikshit)

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ABSTRACT

In the current scenario, due to global deterioration of the environment and climate change, among them water pollution possesses serious threat to most of the populace. Waterborne pathogenic bacteria like *Escherichia coli*, *Vibrio cholerae*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, etc. are responsible for several diseases such as diarrhoea, cholera, salmonellosis, etc. In order to overcome these waterborne bacterial diseases and for purification of water, plants have been of great use for their potential role from very ancient times. The present study deals with the Clinical & Laboratory Standards Institute (CLSI)-recommended broth microdilution antibacterial susceptibility assay of waterborne bacterial pathogens against *Lippia nodiflora* L. petroleum ether (LNPE) and ethanolic extracts (LNEE) prepared from the leaves and flowers together of *Lippia nodiflora* (Verbenaceae), in comparison to camphor. Growth inhibition of tested bacterial pathogens was recorded in from of IC_{50} and MIC values were found to be 0.171, 0.327 (mg/ml) against *E. coli* and *V. cholerae*, respectively, for LNPE extract. Thus, LNPE justifies its potential in inhibiting the growth of tested waterborne bacterial pathogens and creates an interest in further testing its active fraction for *in vivo* trials and organoleptic analysis; making it a good herbal replacement for the conventional water treatment.

Keywords: CLSI; Microorganisms; Antibacterial susceptibility; Microdilution; Diarrhoea; Salmonellosis

1. Introduction

Of all natural resources, water is the most rapidly exhausting resource which is quintessential for mankind. Despite, great efforts by municipal and other associated authorities, potable drinking water is a nightmare especially in urban areas. Prime source of water contamination is due to the mixing of sewage water through various channels to supply water. It was reported that globally 1.1 billion people do not have the proper channel to access safe drinking water and possess greatest risk to the people of developing countries [1,2]. Of the widespread health hazardous

*Corresponding author.

elements present in the different resources of water in the environment, pathogenic microbes being inconspicuous in size and wide in distribution throughout are the most life-threatening causes which can lead to death [3,4]. Common waterborne bacteria such as *Escherichia coli*, *Salmonella typhimurium*, *Vibrio cholerae* and *Klebsiella pneumoniae* cause mortality, malnutrition as well as diminutive growth in children [5]. Contaminated water leads to several waterborne diseases such as diarrhoea, dysentery, typhoid fever, shigellosis and problems of human enteritis are mainly due to the presence of bacterial pathogens [6,7]. Globally around 10 billion episodes of people infected annually by diarrhoeal diseases are known among which 2.2 million deaths occur per annum [8–10].

Some traditional ways for the purification of drinking water which are in common practice are the use of camphor, chlorine tablets [11,12] bleaching powder, iodine [13], potassium permanganate [14], etc. which are in constant use by concerned government authorities. The use of synthetic disinfectants is itself a source of contamination to the ground water table and thus possesses serious harm to the environment; causing irreparable damage. The rich source of medicinal plants in the Indian subcontinent provides excellent opportunity for the use of herbs as a novel method of water purification with the aid of their antimicrobial activity. Several plants are categorised from the primeval era for the purification of water as well as in the treatment of waterborne diseases, such as *Moringa oleifera*, *Mentha arvensis*, *Citrus reticulata*, *Strychnos potatorosum* and many more [15,16]. Studies reflect that plants have always played a tremendous role in water purification in the form of disinfectants, coagulants, flocculants and antimicrobials [17].

Both plants and water are equally important in the ecological balance and an play important role in the natural purification of water resources. The present study puts forward yet another benchmark proving the potential of herbs as a source of antibacterial. The selected plant i.e. *Lippia nodiflora* is a perennial herb with creeping nature, with club-shaped arrangement of white-coloured flowers. It grows in mesic habitats generally near the riverside in sandy and stony soil [18,19] and is an excellent source of herbal antimicrobial.

2. Materials and methods

2.1. Collection of plant material and preparation of extracts

The plant of *L. nodiflora* (syn. *Phyla nodiflora* L.) belonging to family Verbenaceae was collected from Shantipuram, Phaphamau, Allahabad [17,20] and belongs to the family Verbenaceae. Collected plant materials were washed thoroughly by tap water and were shade dried before secondary metabolite extraction. 10 g of dried plant material was chopped into small pieces and soaked in 50 ml ethanol and petroleum ether. Further, after an overnight incubation at room temperature, they were filtered by Whatman No. 1 filter paper and condensed in a rotary evaporator to obtain the crude ethanolic extract (LNEE) and petroleum ether extract (LNPE).

2.2. Procurement and maintenance of cultures

All bacterial cultures used in the study were procured from MTCC, Chandigarh, India. The procured cultures were *E. coli* (MTCC No. 8936), *V. cholerae* (MTCC No. 3906), *S. typhimurium* (MTCC No. 3231) and *K. pneumoniae* (MTCC No. 4032). The slant cultures were maintained in nutrient agar medium with regular sub-culturing at an interval of 15 d. Seven days prior to antibacterial testing, fresh reculture was done (Fig. 1).

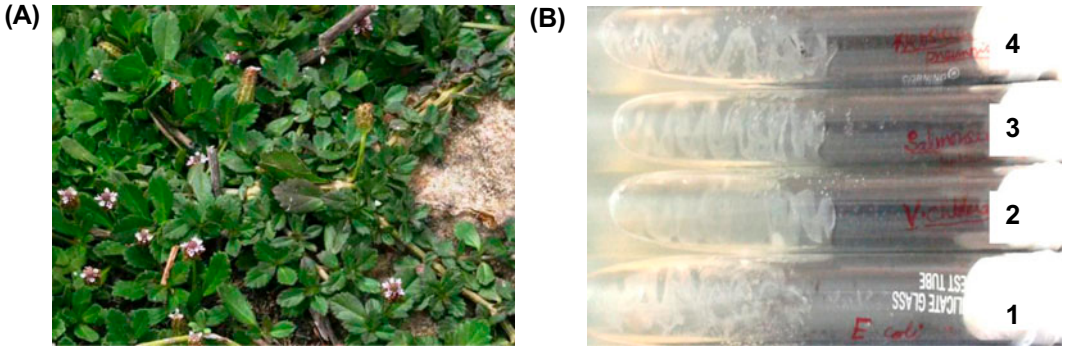

Fig. 1. (A) *Lippia nodiflora* L. in natural habitat and (B) cultures of (1) *E. coli*, (2) *V. cholerae*, (3) *S. typhimurium* and (4) *K. pneumoniae*.

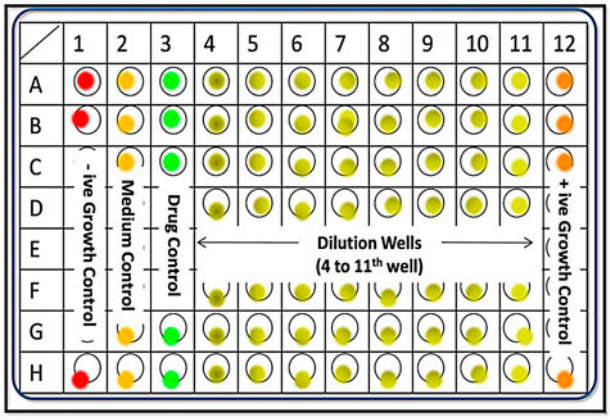

Fig. 2. Pictorial representation of CLSI-recommended broth micro-dilution protocol.

2.3. Antibacterial test

In vitro antibacterial assay performed in a 96-well micro-titre plates in duplicate and minimum inhibitory concentration (MIC) of LNPE and LNEE was determined by CLSI-recommended broth micro-dilution method [21]. Stock solution (50 mg/ml) of extracts was prepared in DMSO. In brief, the initial bacterial inoculum suspension was prepared as per 0.5 McFarland standards (corresponding to a CFU of 1.5×10^7 cell/ml). The MIC and IC_{50} were obtained spectrophotometrically by SpectraMax Plus384 (Molecular Devices Corporation, USA) at 480 nm, after an incubation of 24 h at 35 ± 2°C (Fig. 2).

2.4. Phylogenetic study

To analyse the variability in the MICs of the waterborne bacterial pathogenic strains of *E. coli*, *V. cholerae*, *S. typhimurium* and *K. pneumoniae* against the LNEE and LNPE, phylogenetic treatment of 16S ribosomal RNA was performed. The gene sequence was procured from the gene Bank NCBI database, USA in their FASTA format for further alignment and phylogenetic study. The sequences were aligned in MEGA 4 (version 4.0.1) software and gene alignment was obtained for both the DNA sequence and the amino acid sequence by ClustalW program and 1,000 bootstrapped N-J plotting method in MEGA 4. Further, the gene sequences were blasted for procurement of more genetic homologues which were aligned in ClustalW and phylogeny was obtained by N-J plotting [22–25].

3. Results and discussions

3.1. Antibacterial test

Effect of LNEE, LNPE and commercially available camphor in the form of IC_{50} and MICs on some waterborne pathogens has been summarised in Table 1. In the present study, commercially available camphor was found effective against all the pathogens with IC_{50} values i.e. 0.810, 0.078, 1.403 and 1.101 (mg/ml) and MICs values 2.750, 1.724, 8.638, 2.023 (mg/ml), respectively, for *E. coli*, *V. cholerae*, *S. typhimurium* and *K. pneumoniae*. Comparatively, on the other hand, LNPE was found to be more potent with IC_{50} values 0.069, 0.086, 0.740, 0.485 for the above said pathogens, respectively, whereas MIC values of 0.171, 0.327 (mg/ml) were recorded only for *E. coli* and *V. cholerae*, respectively. *In vitro* antibacterial activity of essential oil of *M. biflora* was assayed against waterborne bacterial pathogens i.e. *E. coli* and *S. typhimurium* in which IC_{50} and MIC values recorded were 0.164 and 0.104 mg/ml and 0.172 and 0.202 mg/ml, respectively [15] *M. arvensis* was also found to be an effective growth inhibitor of studied waterborne bacteria viz. *E. coli*, *V. cholerae*, *S. typhimurium*, *K. pneumoniae* with IC_{50} and MICs as 0.97; 2.46, 0.28,0.72, 0.85, 2.22; 0.81, 2.06 respectively [26]. In another study; it has been found that lichens such as *Hypotrachyna cirrhata* and *Flavoparmelia caperata* are also good inhibitors of some enterobacterial members i.e. *K. pneumoniae*,

Table 1
Antibacterial assay of *L. nodiflora* L. and commercial camphor against waterborne bacterial pathogens

		Lippia nodiflora L.				Commercially available camphor	
		Ethanolic extract		Petroleum ether extract			
S. no.	Selected pathogens	IC_{50} (mg/ml)	MIC (mg/ml)	IC_{50} (mg/ml)	MIC (mg/ml)	IC_{50} (mg/ml)	MIC (mg/ml)
1	*E. coli*	2.480	2.530	0.069	0.171	0.810	2.750
2	*V. cholerae*	2.490	2.526	0.086	0.327	0.078	1.724
3	*S. typhimurium*	No activity	No activity	0.740	No activity	1.403	8.638
4	*K. pneumoniae*	No activity	No activity	0.485	No activity	1.101	2.023

S. typhimurium and *V. cholerae* with IC_{50} values 1.46, 1.52, 1.71 mg/ml and 2.95, 3.40, 2.51 and MIC values 2.24, 2.28, 2.48 mg/ml and 4.40, 4.96, 3.29 ,respectively [27]. The studied bacterial pathogens were also found non-resistant for *Trachyspermum ammi* L. oil with MIC values of 0.128, 0.107 and 0.109 for *E. coli*, *V. cholerae*, *S. typhimurium*, respectively [28].

LNEE was found to be effective only on *E. coli* and *V. cholerae* with IC_{50} (2.480, 2.490 mg/ml) and MIC values of 2.530 and 2.526 (mg/ml), respectively. It is clear from the above results that *L. nodiflora* L. may be a potential source of herbal antimicrobial. In developing countries, free chlorine bleaching, solar disinfections, coagulants, biosand/ceramic filtration and camphor are frequently used as cost-effective water potability agents [20]. Though commercially available, camphor can be a good source of antibacterial for water treatment but it is more conceivable that plant can be a better source of water potability machinery therefore, enhancing the drinking water quality and making it microbe-free by inhibiting their growth at low doses and making the water fit for drinking.

On the basis of previous studies, it can be thus proved that herbal medicinal plants are found to be effective in inhibiting the growth of pathogenic bacterial forms. The present study dealing with *L. nodiflora* proved to be much better for treating and inhibiting the growth of waterborne bacterial pathogens and since the volatile compounds are very meagre in green plants; therefore, it does not have a characteristic aroma and it is abundantly available which makes it a suitable replacement for conventional treatment protocols for obtaining potable water (Figs. 3–5).

3.2. Phylogenetic analysis of waterborne pathogens

For the reduction of the experimentation cost and determination of the variability in the MICs against different extracts for their susceptibility against the

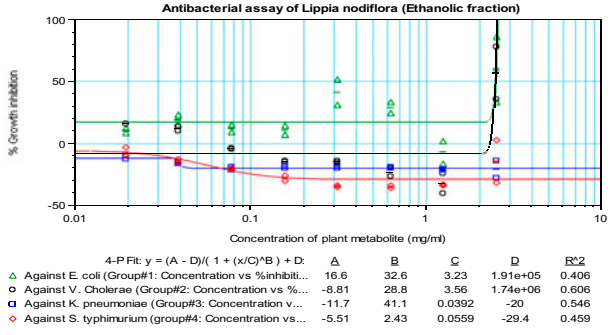

Fig. 3. Percent growth inhibition curve of *L. nodiflora* L. ethanolic fraction against *E. coli*, *V. cholerae*, *S. typhimurium* and *K. pneumoniae*.

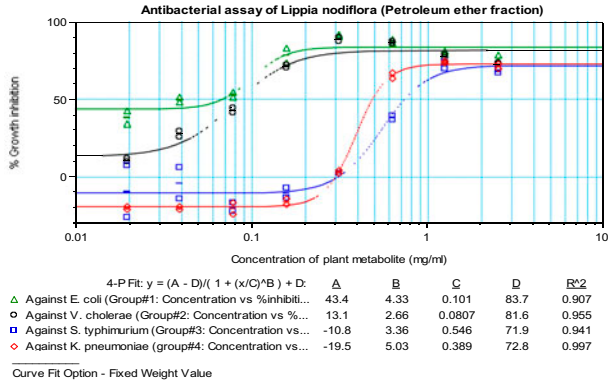

Fig. 4. Percent growth inhibition curve of *L. nodiflora* petroleum ether fraction against *E. coli*, *V.cholerae*, *S. typhimurium* and *K. pneumoniae*.

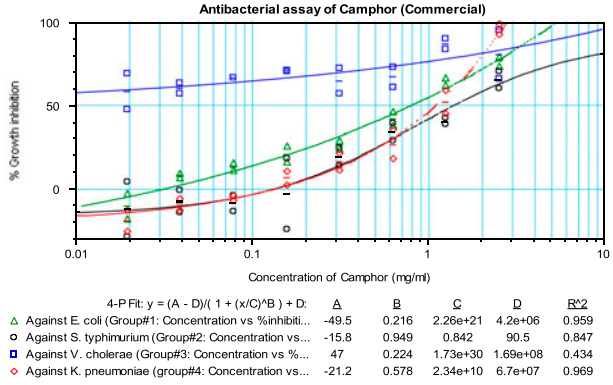

Fig. 5. Percent Growth inhibition curve of commercially available camphor against *E. coli*, *V. cholerae*, *S. enterica* subsp., *K. pneumoniae*.

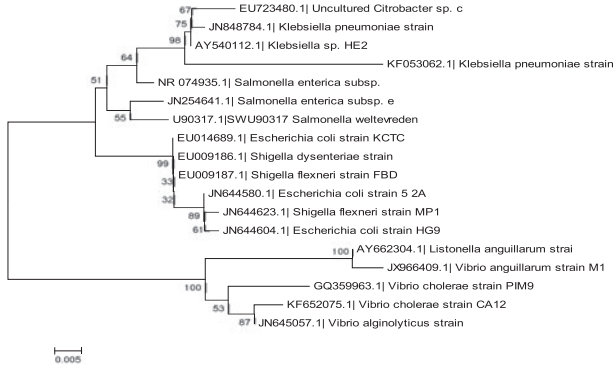

Fig. 6. Molecular phylogenetic tree constructed using the 16S rRNA gene sequence of various waterborne bacteria found homologous in the blastx search of the studied bacterial pathogenic strains.

plant extract, phylogenetic analysis of the bacterial strains was done. The bacterial 16S rRNA gene sequence of *E. coli*, *V. cholerae*, *S. typhimurium* and *K. pneumoniae* was selected for the phylogenetic analysis. For the selected strains, the gene sequences were procured from GenBank NCBI database in their FASTA format. The gene sequences were aligned in the MEGA 4 (version 4.0.1) Software [24,29] in the ClustalW format (Fig. 6).

Phylogenetic studies were also done previously in the case of waterborne bacteria by using ClustalW computer program and GENETYX MAC 10.1 software. Phylogeny was constructed by the maximum likelihood of the DNA [15,26]. Similar studies were also done with reference to different species belonging to *Malassezia* species [30] and plant growth promoting rhizobacteria for obtaining the homologous strains [31]. The ClustalW alignment of 16S rRNA gene was subjected for the 1,000 bootstrapped N-J plotting (Neighbour-joining) for deciphering the phylogeny of the bacteria studied [22,25]. The phylogenetic placing of the studied bacterial pathogens was in strict accordance with their susceptibility to various LNEE and LNPE. *E. coli and V. cholerae* though distant in the phylogeny has similar susceptibility to the *L. nodiflora* L. extracts. The strong homogeneity in the 16S rRNA gene sequence is reflected by their nearness in the tree and also reflected by susceptibility (MICs) against LNPE and LNEE as well as commercially available camphor (Fig. 7).

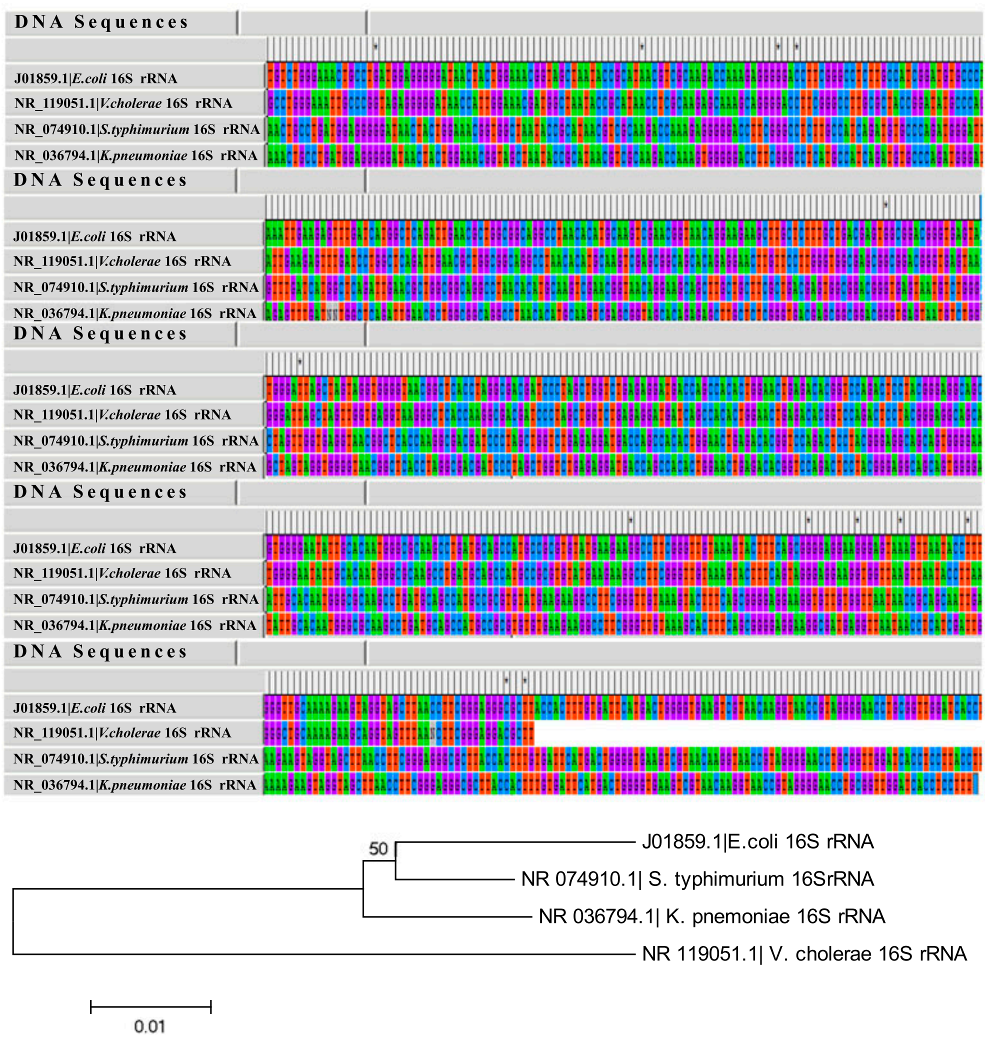

Fig. 7. Alignment of the 16S rRNA ribosomal gene sequence of selected waterborne pathogens by bootstrapped 1,000 ClustalW program in MEGA 4 (Version 4.0.1) and its phylogenetic tree constructed by NJ-plotting method.

4. Conclusion

The present study undertakes the comparative analysis of ethanolic and petroleum ether extract of *L. nodiflora* as well as commercially available camphor against four most prevalent waterborne bacterial pathogens responsible for some of the fatal diseases in the light of phylogenetic analysis. The LNPE was found to be potentially a good growth inhibitor and hence creates an interest in further testing of the active fraction for *in vivo* trials and organoleptic analysis. Moreover, being abundantly available, its commercial usage will not be causing any harm to the flora. Therefore, the use of LNPE can be a justified herbal replacement over the conventional water treatment.

Conflict of interest

Authors have no conflict of interest.

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