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Research Article

Antimicrobial Potential of *Lippia Nodiflora* Linn.

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Abstract

Antimicrobial activities of the methanolic extract from *Lippia nodiflora* and isolated compound from the extract were studied by the disc diffusion method. 100 µg/ml of methanolic extract of *Lippia nodiflora* was recorded the minimun inhibitory concentration as compared with standard drug kanamycin and clotrimazole (10 µg). The extracts showed antimicrobial impact on both gram positive and negative bacteria such as *E. coli, P. vulgaris, K. pneumonia, B. cereus, B. subtilis, S. aureus, P. aeruginosa* and *B. clausii* as well as fungi such as *A. niger* and *C. albicans.* The results showed that increasing concentrations of extracts increased the antimicrobial activities against all of the microorganisms. Bacteria were more sensitive than fungi, and gram positive bacteria were more sensitive to *L. nodiflora* extract than gram negative ones. It has been concluded that methanolic extract of *Lippia nodiflora* possess potential antimicrobial activity.

1. INTRODUCTION

Knowledge of medicinal values of plants is recognized by almost every society on earth. The inhabitants of the remote places have good knowledge about the utilization of plants because of the non-availability of synthetic drugs. In addition, for the survival, they use the plant based drugs growing nearby their villages. Based on their right or wrong experiences they discovered the therapeutic agents of these plants in particular diseases. These experiences are transferred from parents to offspring[1]**.** Traditional medicine based on plants has played a key role in the health care system of many countries little- India, China etc[2]. Herbal medicine is still the main stay of about 70- 80% of the world population stays on the herbal medicine.

Lippia nodiflora is the important member of the family verbenaceae showing a variety of medicinal uses. It can be the source of the indigenous medicine. In India, it is found in the warmer parts including Andhra Pradesh, Karnataka, Kerala, and Maharashtra, some parts of Rajasthan, Tamil Nadu, Uttar Pradesh and West Bengal. It is common in wet places along bunds or irrigation canal edges and sliver banks. The plant is rich in many important medicinal useful compounds. The plant contains a variety of constituents such as triterpenoids**,** flavonoids, phenols, steroids, and many others**.** Among these, flavonoids were the most commonly found constituent. Nodifloretin (3), *β*-sitosterol glycoside and stigmasterol glycoside from the leaves of *L. nodiflora*[3]. Nodifloridin A (1) and Nodifloridin B(2) along with lactose, maltose, glucose, fructose, and xylose were isolated from the plant[4].The plant is used as gastroprotective effect[5], anti inflammatory,antineoplastic[6], antioxidant[7] and diuretic[8]. The plant is used for the treatment of diuretic, plant made into a poultice used as maturant for boils, infusion of leaves and tender stalks given to children in indigestion and to women after delivery. *Chutney* made from its leaves and fruits are eaten to relive the irritation of internal piles[10, 11].

Over – usage of antibiotics has resulted in an increase in the resistance of bacteria against these drugs. The use of too many antibiotics can also cause numerous side effects in humans. Since plants with possible antimicrobial activity should be tested against an appropriate microbial model to confirm the activity and to ascertain the parameters associated with it. The effects of plant extracts on bacteria have been studied by a very large number of researchers in different parts of the world[12,13]. It has been suggested that aqueous and ethanolic extracts from plants used in allopathic medicine are potential sources of antiviral, antitumor and antimicrobial agents[14,15]. The selection of crude plant extracts for screening programs has the potential of being more successful in initial steps than the screening of pure compounds isolated from natural products[16].

The present investigation was carried out to find out the antibacterial activity and anti fungal activities of the methanolic extract and isolated compound of *Lippia nodiflora* was estimated.

2. MATERIALS AND METHODS

2.1 Collection of Plant Materials

The plant specimen for the proposed study *Lippia nodiflora* (here after *L. nodiflora*) was collected from the wetland fields and other irrigated fields in and around Madurai District, Tamil Nadu, India. The herbarium of these plants was identified and authenticated by Dr. D. Stephen, Professor, Department of Botany, American college of Arts and Science, Madurai, Tamil Nadu and the specimen was deposited in Department of Pharmaceutical Chemistry, Ultra College of Pharmacy, Madurai, Tamil Nadu, India**.**

2.2 Preparation of *L. nodiflora* Extracts

The fresh whole plant of *L. nodiflora* was washed with distilled water to removed unwanted foreign materials like soil and dusts.

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After, washed plant material was dried under shade at room temperature without direct exposure of sunrays. It was then coarsely grounded by using mechanical device. The powdered plant material was passed through sieve no 40 and stored in an airtight container for further use.

The coarsely powdered plant materials of *L. nodiflora* (2000 g) were extracted separately to exhaustion in a soxhlet apparatus for 72 hours by using Petroleum ether (60-80˚C) and Methanol (95 %) solvent (Merk & Spectrum Chemicals, India) systems. All the extracts were filtered through a cotton plug followed by what mann filter paper (No.1) and then concentrated by using a rotary evaporator at low temperature (40-50˚C) and reduced pressure to get 20.6 g and 100.2 g respectively. The extracts were preserved in airtight containers and kept at 4˚C until further use.

2.3 Isolation of Phytoconstituents from *L. nodiflora*

The isolation of phytoconstituent is done by column chromatographic method. The constituents of methanol extract (MELN) were isolated by column chromatography. Identification and purity determination were done by thin layer chromatography techniques. The fractions collected were further chromatographed to know the number of constituents present. Silica gel was used as stationary phase. The column was first eluted with 100 % petroleum ether. The polarity of mobile phase was gradually increased with chloroform, ethyl acetate, acetone, and methanol. The fractions collected and were concentrated. The dried fraction was kept on vials with suitable label and kept for further use.

2.4 Microbial strains used

Antibacterial effect of *L. nodiflora* was determined against 4 gram negative bacteria *viz., Escherichia coli* MTCC 118*, Proteus vulgaris* MTCC 426*, Klebseilla pneumonia* M 4020 and *Pseudomonas aueroginosa* (Clinical isolate obtained from Vijay Clinical Laboratory, Madurai) and 4 gram positive bacteria *viz., Bacillus cereus* MTCC 1305, *Bacillus subtilis* MTCC 619*, Bacillus clausii (Probiotic spores obtained from medical store)* and *Staphylococcus aureus* MTCC 96. Antifungal effect of *L. nodiflora* was determined against 2 different fungal strains *viz., Aspergillus niger* MTCC 872 and *Candida albicans* MTCC 183.

2.5 Antibacterial assay

The spectrum of antibacterial activity was studied using as test agent a range of 8 different strains of human pathogenic bacteria of which there were one standard drug (Kanamycin). *In vitro* antibacterial assay was carried out by disc diffusion technique[17] in whatman No.1 filter paper discs with 4 mm diameter were impregnated with known amount test samples of the *L. nodiflora.* The discs were immersed in different test concentrations (*L. nodiflora* methanolic extract - 250 and 500 µg, compound isolated from *L. nodiflora* - LNC_1 25 and 50 µg) allowed to evaporate. The positive control contained a standard drug disc. Sterile discs used as negative control. The impregnated discs along with control were kept at the center of agar plates, seeded with test bacterial cultures. The discs were then placed individually using a sterile forceps in appropriate grids which were marked on the undersurface of the plated Petri plates and kept for incubation at room temperature (27°C±2) for 24 hrs. After incubation, plates were observed for zones of inhibition and recorded in millimeters.

2.6 Antifungal assay

Stock cultures were maintained in Sabouraud dextrose agar and 2 different species of fungal pathogen were maintained in Sabouraud Dextrose broth for 24 hours until used for antifungal activity. *In vitro* antifungal activity was determined by using the disc diffusion technique[17]. Two different species of fungal pathogens inoculated by spread plate method using 0.1 ml of 24 hours old culture, maintained in Sabouraud Dextrose broth. Whatman No.1 filter paper (4 mm) discs impregnated with test samples of the *L. nodiflora.* The discs were immersed in different test concentrations (*L. nodiflora* methanolic extract - 250 and 500 µg, compound isolated from *L. nodiflora* - LNC_1 25 and 50 µg) allowed to evaporate. Clotrimazole used as positive control. Incubating the fungal petriplates for 32 hrs at 30°C, then plates were observed for zones of inhibition and recorded in millimeters.

2.7 Minimum Inhibitory Concentration (MIC)

The methanolic extract of *L. nodiflora* antimicrobial activity were further tested against all the organisms for the evaluation of its antibacterial and antifungal efficiency at different concentrations (50µg/ml, 100µg/ml, 250 µg/ml, 500 µg/ml, 1 mg/ml and 10mg/ml) by using the filter paper disc diffusion method. The zone of inhibition was calculated in mm.

Activity index was calculated by comparing the zone of inhibition by plant extract with that of standard drug.

$$\text{Activity Index} = \frac{\text{Inhibition zone of test sample (extract)}}{\text{Inhibition zone of standard antibiotic}}$$

2.8 Statistical Analysis

Tests were carried out in triplicates. The mean values were calculated from the triplicate values. Values are expressed as the Mean ± SD and differences between groups were considered to be significant if $p < 0.05$.

3. RESULTS AND DISCUSSION

Disc diffusion methods are extensively used to evaluate the antimicrobial activity of natural substances and plant extracts[27]. In this antimicrobial activity four gram negative, four gram positive and two fungal strains were used to evaluate the possible antimicrobial activities of methanolic extract and isolated compound of *L. nodiflora.* The present study showed that the methanolic extract and isolated compound of *L. nodiflora* were very effective against all micro organisms used in this research (Table 1). Minimum inhibitory concentration of the methanolic extract of *L. nodiflora* at the concentration of 100µg/ml as compared with standard drug kanamycin and clotrimazole (10 µg). The results indicated that by increasing the concentration of extracts, the antimicrobial activities also increase. Gram positive bacteria were more sensitive than gram negative bacteria. In both gram negative and gram positive bacteria tested, *K. pneumonia, P. aueroginosa* and *B. subtilis* showed more antibacterial activities than the other tested species.

Methanolic extract was the most effective against all bacterial pathogens. Thirteen mm zone of inhibition observed against *Klebsiella pneumonia, Bacillus cereus* and *Bacillus subtilis*, 12 mm against *Escherichia coli* and *Proteus vulgaris* and 11 and 10 mm against *Pseudomonas aueroginosa* and *Staphylococcus aureus* respectively in 250 µg/disc methanolic extract of *L. nodiflora.* Methanolic extract of *L. nodiflora* in 500 µg/disc showed the highest inhibition zone against *P. vulgaris, K. pneumonia, B. cereus* and *B. subtilis* and lowest inhibition zone against *B. clausii.* Results of fungus exhibited same zone of inhibition (11 at 250 µg per disc) for both *Aspergillus niger* and *Candida albicans* and in 500 µg per disc, highest zone of inhibition (14 mm) occurred against *A. niger* and lowest zone of inhibition (11 mm) occurred against *C. albicans* (Table 1 and Figure 1).

Among the gram negative bacteria tested, *K. pneumonia* and *P. aueroginosa* showed highest zone of inhibition (15 mm) in the isolated compound at the dose of 25µg per disc whereas *P. vulgaris* showed the lowest zone of inhibition (11 mm) at the dose of 25 µg per disc. Seventeen mm zone of inhibition observed against *K. pneumonia,* 16 mm zone of inhibition observed against *P. aueroginosa* and 14 mm zone of inhibition observed against *E. coli* and *P. vulgaris* at the dose of 50µg per disc. Among the 4 tested gram positive bacteria, *B. subtilis* exhibited the highest zone of inhibition (15 and 16 mm) which was followed by *S. aureus* (12 and 13 mm) and *Bacillus cereus* (11 and 12 mm) and *B. clausii* showed the lowest zone of inhibition (7 and 9 mm) in the dose of 25 and 50µg of isolated compound per disc respectively (Figure 2).

The antibacterial activity of standard kanamycin showed the maximum zone of inhibition against *Proteus vulgaris* and minimum zone of inhibition was observed against *Pseudomonas aueroginosa, Bacillus cereus* and *Bacillus clausii.* Table 2 shows the activity index of methanolic extract and isolated compound of *L. nodiflora*. From the activity index results, the methanolic extract showed good activity against all the bacteria except *Proteus vulgaris.*

Phytochemical investigations on *L. nodiflora* have resulted in the isolation of several flavones glycoside, including lippiflorin A & B, nodiflorin A & B, nodifloritin A & B, alkaloids, essential oils, resin, stigmasterol, sugars, mono and diflavonesulphates of neotin, jaceosidin, hispidulin and 6-hydroxyluteolin[18]. In the latest study a

new triterpenoid (lippiacin) anda benzofuranonerengyolone (halleridone) was isolated for the first time from the methanolic extract of the *L. nodiflora* aerial parts[19]. The antibacterial activity may be due to several agents, such as presence of alkaloids, Flavanoids, tannin and oil[20]. The antifungal activity of extracts in this research is in accordance with studies on the leaf extract of *L. rugosa* against *Aspergillus flavus*[21]. *Lippia multiflora* and *Lippia chevaliori* extracts showed good antifungal activity against *A. flavus* due to terpenoids, particularly citral, geraniol and citronelol[22]. Since there are some kinds of terpenoids in the *L. nodiflora* composition, we can say that there is antifungal activity in *L. nodiflora.*

L. rehmannii showed high antifungal activity due to the presence of the oil contents β-caryophyllene and β-caryophyllene Oxide, which were the major compounds that are in *L. nodiflora* as sesquiterpenes[23].There are numerous studies about the antibacterial activity in species of *Lippia. L. origanoides* have high antibacterial activity due to the presence of monoterpenoids. The disc diffusion method showed highly significant inhibition zones for all microorganisms tested like gram positive bacteria (*S. aureus*) and fungus (*C. albicans*)[24]. Thin layer chromatography examination of the methanolic extracts of *L. nodiflora,* and reported that this plant containing a group of flavanoids[5]. According to some researchers, flavanoides have antibacterial activity[26].

Table 1: Antimicrobial activity of methanolic extract and isolated compound of *L. nodiflora* Linn.

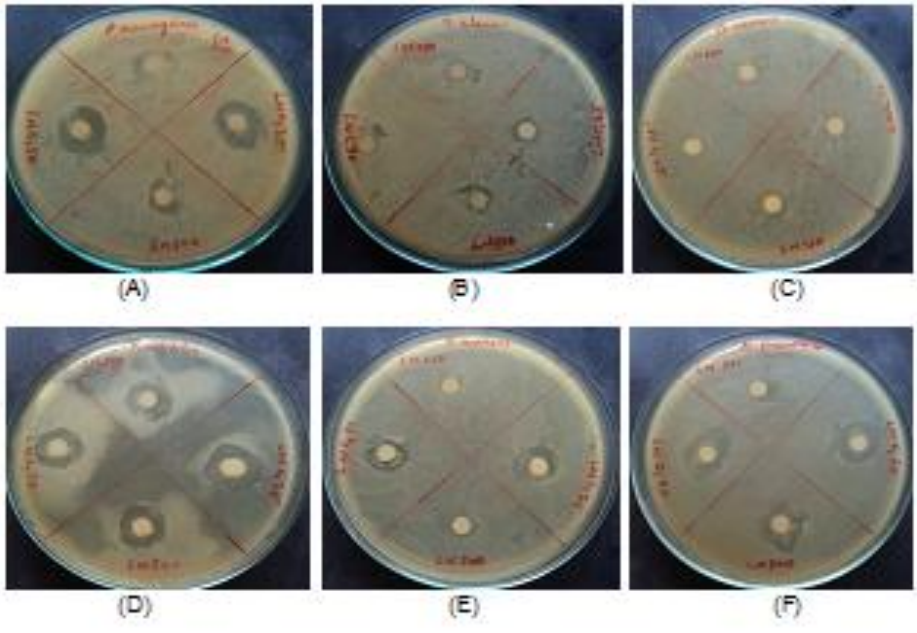
Microorganism	Mean ± SEM of diameter of zone of inhibition (in mm)				
	Methanolic extract of *L. nodiflora*		Isolated compound of *L. nodiflora*		Standard*
	LN250µg/disc	LN500µg/disc	$LNC_1$25µg/ disc	$LNC_1$50µg/disc	
Escherichia coli	12 ± 0.79	13 ± 0.34	14 ± 1.16	14 ± 0.29	20 ± 2.19
Proteus vulgaris	12 ± 0.33	14 ± 0.67	11 ± 1.12	14 ± 0.48	23 ± 1.52
Klebseilla pneumonia	13 ± 0.87	14 ± 0.72	15 ± 0.54	17 ± 1.16	18 ± 1.10
Pseudomonas aueroginosa	11 ± 1.12	13 ± 0.19	15 ± 0.29	16 ± 0.52	16 ± 0.96
Bacillus cereus	13 ± 0.94	14 ± 0.33	11 ± 0.48	12 ± 0.91	14 ± 0.92
Bacillus subtilis	13 ± 0.77	14 ± 0.56	15 ± 0.93	16 ± 0.38	21 ± 1.10
Bacillus clausii	8 ± 0.45	10 ± 0.39	7 ± 0.58	9 ± 1.71	13 ± 0.56
Staphylococcus aureus	10 ± 0.55	11 ± 0.54	12 ± 1.11	13 ± 0.41	13 ± 0.15
Aspergillus niger	11 ± 0.23	14 ± 0.98	14 ± 0.87	14 ± 1.00	15 ± 0.54
Candida albicans	11 ± 0.76	11 ± 0.59	12 ± 0.48	13 ± 0.59	14 ± 0.36

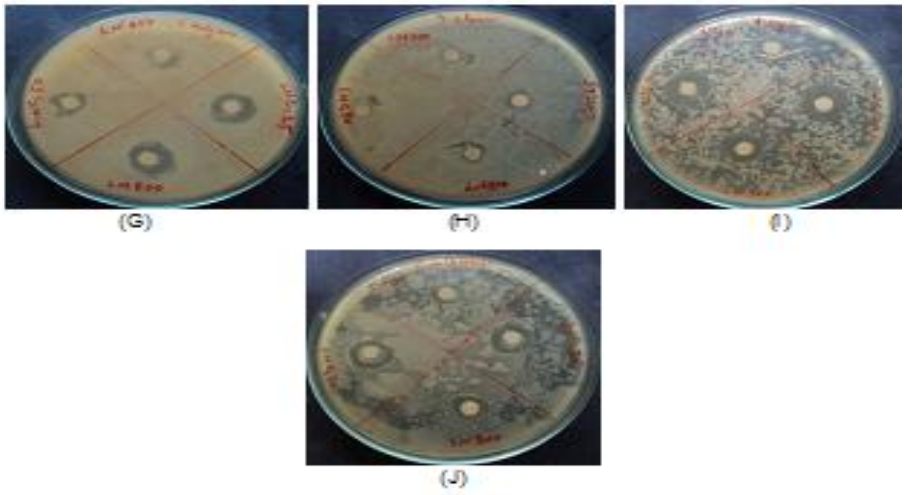
SEM – Standard Error of the Mean
Standard - Kannamycin for bacteria and Clotrimazole for fungi*

Table 2: Activity index of methanolic extract and isolated compound of *L. nodiflora* Linn.

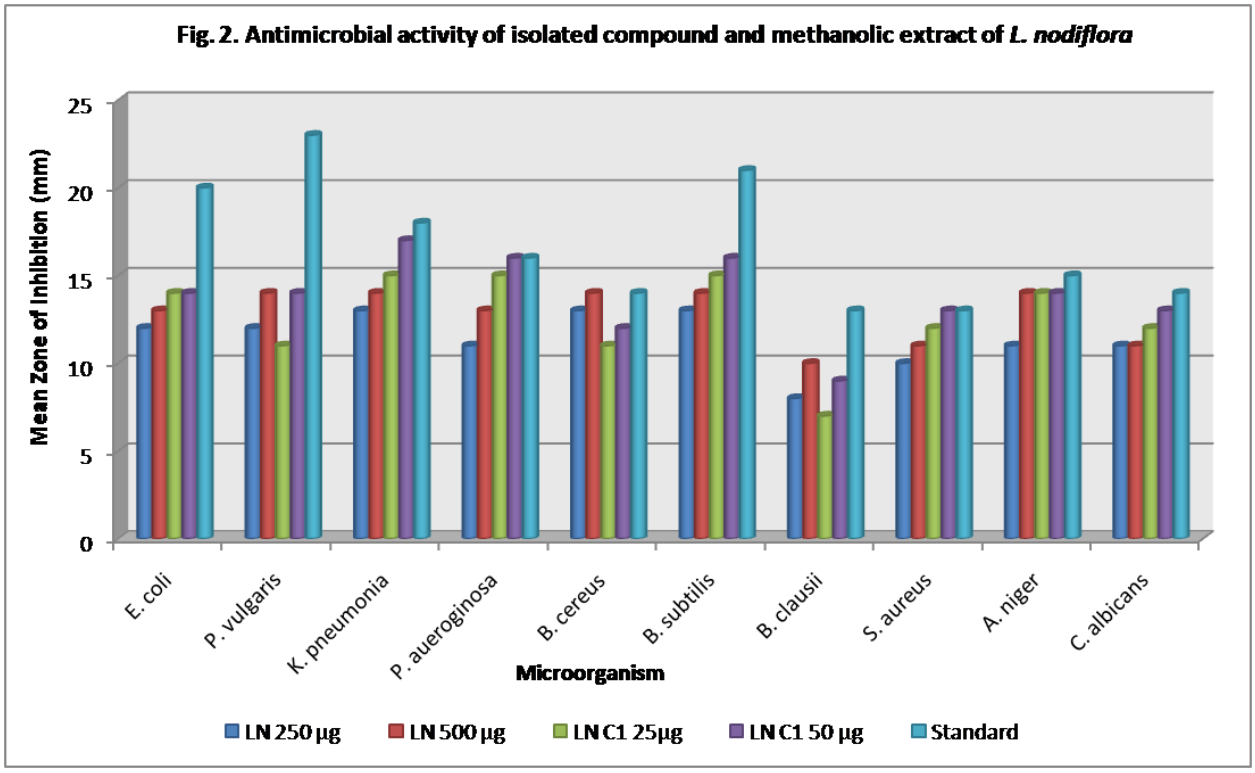
Microorganism	Activity index			
	Methanolic extract of *L. nodiflora*		Isolated compound of *L. nodiflora*	
	LN 250µg/disc	LN 500µg/disc	$LNC_1$25µg/ disc	$LNC_1$50µg/disc
Escherichia coli	0.60	0.65	0.70	0.70
Proteus vulgaris	0.52	0.61	0.48	0.61
Klebseilla pneumonia	0.72	0.78	0.83	0.94
Pseudomonas aueroginosa	0.69	0.81	0.94	1.00
Bacillus cereus	0.93	1.00	0.79	0.86
Bacillus subtilis	0.62	0.67	0.71	0.76
Bacillus clausii	0.62	0.77	0.54	0.69
Staphylococcus aureus	0.77	0.85	0.92	1.00
Aspergillusniger	0.73	0.93	0.93	0.93
Candida albicans	0.79	0.79	0.86	0.93

Fig. 1: Antimicrobial activity of methanolic extract and isolated compound of *L. nodiflora*





A – P. aeuriginosa; B – B. clausii; C – B. cereus; D – B. subtilis; E – S. aureus; F- K. pneumonia; G – P. vulgaris; H – E. coli; I – A. niger; J – C. albicans



4. CONCLUSION

Development of new antimicrobial compounds against different microorganisms is becoming critically important, as infectious diseases are still one of the leading causes of death in the world. The pharmaceutical industry is searching for new lead compounds with novel chemical structures to overcome the increasing resistance to known antibiotics. Plants can be a useful source of these lead compounds. The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great advantage in therapeutic treatments. In that way, methanolic extract and isolated compound of *L. nodiflora* showed antimicrobial activity against 4 gram negative, 4 gram positive bacteria and 2 fungi. The observed activity may be due to the presence of flavanoides, terpenoides, sesquiterpenoids, phenolic acid, alkaloids and other components.

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