ANTIMICROBIAL AND LIPID PEROXIDE SCAVENGING ACTIVITY OF *LIPPIA NODIFLORA* (VERBENACEAE)

Ashok Kumar Durairaj, Thamil Selvan Vaiyapuri, Upal Kanti Mazumder*, Malaya Gupta

Department of Pharmaceutical Technology, Jadavpur University, Kolkata, 700 032, India.

Summary

The present study deals with the *in vitro* antimicrobial and lipid peroxide scavenging activity of the methanol extract of whole plant of *Lippia nodiflora* (MELN) (Verbenaceae). Lipid peroxide scavenging was monitored by the change in optical density of the prepared concentrations (20-320 µg/ml) and the percentage inhibition was calculated. Ascorbate/FeSO4-induced peroxidation was inhibited by MELN and standard antioxidants such as BHA, BHT. It emphasized that the percentage inhibition of the methanol extract increased with concentration dependent manner. The IC_{50} value of the MELN, BHA and BHT for lipid peroxide scavenging was found to be 226.52 µg/ml, 25.62 µg/ml and 17.13 µg/ml respectively. The antimicrobial activity of MELN was determined by disc diffusion method with three Gram positive, five Gram-negative bacteria and two Fungi. From the present results, it concludes that MELN can be considered as a potential source of natural antioxidant and antimicrobial agent.

Keywords*: Lippia nodiflora, In vitro* lipid peroxidation*,* antimicrobial activity.

Introduction

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 World Health Organization states that infectious and parasitic diseases account for nearly 11 million among the 57 million total deaths in 2003. Although there seems to be a great array of antibacterial and antifungal drugs in clinical use, the appearance of resistant organisms makes these drugs sometimes ineffective or leads to recurrence. Higher plants have been shown to be an important source of new bioactive compounds, including antihypertensive, analgesic, and cytotoxic compounds, among others. Though no plant-derived compound has been found to compete with clinically used antibiotics to date, the great structural variety found in medicinal plants makes them attractive as a source of novel lead compounds. Hence, the higher plants frequently show significant potency against human bacterial and fungal pathogens[1].

For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. The use of plant compounds for pharmaceutical purposes has gradually increased in the world.

According to World Health Organization medicinal plants would be the best source to obtain a variety of drugs. About 80% of individuals from developed countries use traditional medicine, which has compounds derived from medicinal plants. Therefore, such plants should be investigated to better understand their properties, safety and efficiency[2]. The antimicrobial properties of plants have been investigated by a number of researchers worldwide.

The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great advantage in therapeutic treatments. In the recent few years, a number of researches have been

conducted in different countries to prove such efficiency[3, 4,5,6]. Many plants have been used because of their antimicrobial traits, which are due to compounds derived from the secondary metabolism of the plant.

Lipid peroxidation arising from the reaction between free radicals and lipids is considered as an important feature of cellular injury. Several studies suggest that lipid oxidation products ingested with food or produced endogenously represent a health risk. There is increasing involvement in the role of diet and nutrition in human health, pathogenesis and possible prevention of disease. Endogenous antioxidant defense systems, though scavenge and minimize the formation of oxygen free radicals, are not fully active especially in pathological conditions demanding use of exogenous antioxidants. Free radical mediated oxidation of lipids mainly affects the polyunsaturated fatty acids due to their high degree of unsaturation[7].

Lippia nodiflora Mich. (Verbenaceae) is a creeping much-branched herb with small white flowers, a weed of wet ground and grassy pastures[8,9]. The herb is known as Poduthalai in Tamil, Kattuttippali in Malayalam, Bhuiokra in Hindi and Vashira in Sanskrit. The plant is distributed throughout India, Ceylon, Baluchistan and Africa. The plant is used for the treatment of diuretic, aphrodisiac, diseases of heart, ulcers, bronchitis, fever and colds[10]. The 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[11, 12]. The herb possesses cooling and diuretic effect and stop pain in knee joints[13]. Aqueous extract of leaves of the plant was reported for the anti-inflammatory, analgesic and antipyretic activity in rodents[14].

Hence, more studies pertaining to the use of plants, as therapeutic agents should be emphasized. The objective of this research was to evaluate the lipid peroxidation and antimicrobial Activity (against Gram positive, Gram negative bacteria and Fungi) of *Lippia nodiflora* methanol extract by *in vitro* model.

Materials and Methods

Plant material

The whole plant of *Lippia nodiflora* Mich. (Verbenaceae) was collected in the month of January 2005 from wet places of Mallasamudram, Namakkal District, Tamilnadu, India. The plant material was identified and authenticated by Prof. Revenna, H.O.D, Department of Botany, Kuvempu First Grade College, Channapatna, Karnataka, India and the voucher specimen (DAKJU-04/2005) was maintained in our laboratory for future reference. The collected plant material was washed with tap water to remove the adhering material and shade dried. The dried plant material was coarsely powdered, sieved #40 and defatted with petroleum ether (60-80^0 C), further extracted with methanol in a soxhlet apparatus. The percentage yield of petroleum ether (2.91% w/w) and methanol (21.42% w/w) were obtained.

The methanol extract was then distilled, evaporated and dried in vacuum. Phytochemical screening of the petroleum ether extract revealed the presence of tannins, steroids, saponins, gums and terpenoids and alkaloids, glycosides, flavonoids, saponins, terpenes, tannins and steroids in methanol extract. The methanol extract thus obtained was directly used in the assay of lipid peroxidation and antimicrobial activity.

Chemicals and Drugs

Thiobarbituric acid (TBA), Butylated hydroxytoluene (BHT), Butylated hydroxyanisole (BHA) from Loba Chemie Pvt Ltd, Mumbai, India. The antibiotics used in this study were tetracycline (Hindustan Antibiotics, Pimpri, India), amphotericin B (Criticare, India) obtained from the respective manufacturer.

Microorganisms used

Bacterial Strains includes Gram- positive strains such as *Staphylococcus aureus* (MTCC 96), *Staphylococcus epidermidis* (MTCC 435), *Proteus vulgaris* (MTCC 1429), Gram-negative strains like *Escherichia coli* (MTCC 433), *Salmonella paratyphi A* (MTCC 735*), Salmonella paratyphi* B (Clinical isolate), *Klebsiella pneumonia*

(MTCC 432), *Salmonella typhimurium* (MTCC 98) and Fungal Strains of *Candida albicans* (MTCC 183), *Cryptococcus neoformans* (Clinical isolate) were collected from stock culture of Indian Institute of Chemical Biology, Kolkata, Central Drug Laboratory, Kolkata and Plant Pathology Laboratory, Calcutta University, Kolkata, India. The microbial isolates were maintained on agar slant at 4° C. The strains were subcultured on a fresh appropriate agar plate 24 h prior to antimicrobial test. For this antimicrobial evaluation, Nutrient Agar and Sabouraud Dextrose Agar were used for the activation of bacteria and fungi, respectively. Muller Hinton Agar (MHA) was used for the diffusion assays. The nutrient agar media and standard discs of 6 mm diameter were procured from M/S Hi media, Mumbai, India.

Lipid Peroxide scavenging activity

Lipid peroxidation induced by Fe^{2+}-ascorbate system in rat liver homogenate was estimated as thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa *et al* (1979) [15]. The reaction mixture contained rat liver homogenate 0.1 ml (25% w/v) in Tris-HCl buffer (40 mM, pH 7.0), 0.1ml of KCl (30 mM), 0.1 ml of ferrous iron (0.16 mM), 0.1 ml of ascorbic acid (0.06 mM); and various concentrations of MELN (20-320 µg/ml) in a final volume of 0.5ml. The reaction mixture was incubated at 37° C for 1 h. From this, after the incubation period, 0.4 ml was removed and treated with 0.2 ml sodium dodecyl sulphate (SDS-8.1%), 1.5 ml thiobarbituric acid (TBA-0.8%) and 1.5 ml acetic acid (20%, pH 3.5). The total volume was made up to 4.0 ml by adding distilled water and then kept in a water bath at 100° C for 1 h. After cooling, 1.0 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The butanol-pyridine layer was removed and its absorbance at 532 nm was measured to quantify TBARS. The percentage inhibition of lipid peroxidation was determined by comparing the results of the test compounds with that of the control not treated with the extract concentration needed for 50% inhibition was calculated. BHA and BHT were used as standard.

Evaluation of Antimicrobial Studies

Preparation of Sample Solution

Stock solution of the MELN at the concentration of 1000µg/ml was prepared using DMSO for the samples. DMSO was sterilized by filtration using G-5 filter. Stock solutions were diluted by the sterile DMSO at the concentration of 100, 200, 300, 400 and 500 µg/ml. The solvent DMSO was used as control.

Antimicrobial Assay

Antibacterial and antifungal sensitivity tests were performed by the disc diffusion methods[16]. Agar cultures of the test organisms were prepared. Three to five similar colonies were selected and transferred with loop in to 5 ml of Tryptone soya broth, a highly nutritious versatile medium, which is recommended for general laboratory purpose and used for the cultivation of aerobes and facultative anaerobes, including some fungi. The broth cultures were incubated for 24 h at 37° C. The inoculum for each organism was prepared from broth cultures. The concentration of cultures was to 10^{8} colony-forming units (1×10^{8} cfu/ml). For the antimicrobial evaluation, sterile impregnated discs with MELN, at concentrations of 100-500 µg/ml was aseptically placed on the sensitivity agar plates with the help of a sterile fine pointed forceps at a suitable distance apart so that the respective disc can produce clear zones of inhibition around them. All the plates were then incubated either at 16-18 h at 37° C for bacteria and at 30° C for 24 h in case of fungi. The zone of all the doses of MELN for different organism was measured at the end of incubation period. The degree of sensitivity was determined and recorded by measuring the easily visible clear zone of growth inhibition produced by the diffusion of extract from the respective discs into the surrounding medium on agar surface around the discs. The zones showing complete inhibition was compared with reference standard drugs, tetracycline (4µg/ml) and amphotericin B (10µg/ml) [17,18].

Statistical Analysis

All treatments were performed in triplicate and each data point in the results is the mean of three readings in each case. The statistical significance of the values was expressed as mean ± SEM.

Results and Discussion

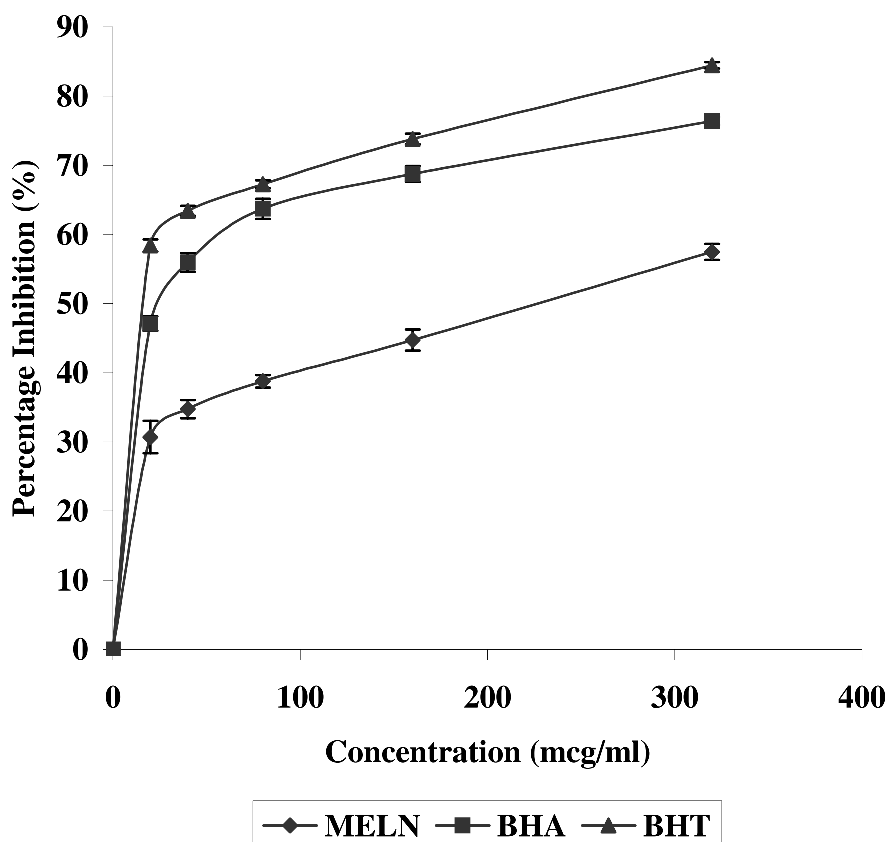
New sources of antimicrobial compounds from plants are of great interest in academia and the food and pharmaceutical industry due to a growing tendency to replace synthetic compounds with natural ones. The present study illustrated the *in vitro* lipid peroxidation and antimicrobial activities of *Lippia nodiflora.*

Inhibition of Lipid peroxidation

The effect of MELN and standard antioxidants namely BHA and BHT on the *in vitro* inhibition of lipid peroxidation is illustrated in Fig 1. The generation of lipid peroxidase by Fe^{2+}-ascorbate in rat liver homogenate seems to be inhibited by MELN with IC_{50} value of 226.52 µg/ml. The IC_{50} value of BHA and BHT were 25.62 µg/ml and 17.13 µg/ml, respectively.

Lipid peroxidation is the oxidative deterioration of polyunsaturated lipids. Peroxidative reactions for nonbiological olefinic substances are known. The peroxidative process leads to the formation of free radical intermediates, which can lead to autocatalysis[19]. Unsaturated lipids in liver tissue were very susceptible to peroxidation when they were exposed to reactive oxygen species (ROS). In the present study, the liver tissue was incubated in presence of a ROS generating system, ascorbate/FeSO4. Lipid peroxides generated by the induction of Fe^{2+}/ascorbate on rat liver homogenate was found to be inhibited by the addition of the extract. MELN exhibited significant effect at quantities of 20-320µg/ml inhibited the formation of lipid peroxides in a dose dependent manner. The results indicate that MELN had potent lipid peroxidation.

Figure 1. Effect of methanol extract of *Lippia nodiflora* (MELN) on lipid peroxidation



Screening of Antimicrobial Activity

In this antimicrobial activity three Gram-positive organism, five Gram-negative organisms and two fungal strains were used to evaluate the possible antimicrobial activities of methanol extract of *Lippia nodiflora.* All the three Gram-positive bacteria of *Staphylococcus epidermidis, Staphylococcus aureus* and *Proteus vulgaris* were exhibited significant at 200-500 µg/ml, 300-500 µg/ml and 400-500 µg/ml, respectively. Gram-negative bacteria, *Salmonella paratyphi A* exhibited significant effect from 300–500 µg/ml. *Escherichia coli* and *Salmonella paratyphi B* were showed the inhibition at 400-500 µg/ml in concentration dependent manner.

Klebsiella pneumonia and *Salmonella typhimurium* did not show any positive result. Fungal strains of *Candida albicans* and *Cryptococcus neoformans* observed the significant activity at 400 and 500µg/ml.

DMSO used as control and was not showed antimicrobial activity. The antimicrobial activity was compared with standard drug tetracycline at 4µg/ml for bacterial strains and amphotericin B at 10µg/ml used for the fungal organisms.

In vitro antimicrobial activity of MELN revealed the presence or absence of inhibition by measuring the zone diameters from different bacterial and fungal organisms. Disc diffusion methods are extensively used to evaluate the antimicrobial activity of natural substances and plant extracts[20]. The inhibition of antimicrobial activity of the bacterial and fungi species as it is shown in Table 1.

The antimicrobial activity of the diterpenoids and flavone is probably due to the membrane disruption by terpenes [21,22] and their activity might be due to their ability to form complex with extracellular, soluble proteins and to complex with bacterial cell walls[22] and disrupt microbial membranes[23]. Plant extracts have great potential as antimicrobial compounds against microorganisms. Thus, they can be used in the treatment of infectious diseases caused by resistant microbes. The antifungal activity might be due to binding with the sterol of fungi and form a pore or channel, which leads to increase the permeation of cell membrane and increase the leakage of variety of small molecules. Otherwise it may induce the oxidative damage in the fungi cell[24]. MELN showed significant inhibition against *Staphylococcus epidermidis, Staphylococcus aureus* and *Proteus vulgaris* (Gram positive), *Salmonella paratyphi A*, *Escherichia coli* and *Salmonella paratyphi B* (Gram negative) and *Cryptococcus neoformans* and *Candida albicans* (Fungi).

The possible way of antimicrobial activity of plant extract may be by inhibiting the cell wall synthesis, metabolism, protein synthesis and DNA synthesis in microorganism[24]. The preliminary phytochemical analysis revealed that the presence of flavonoids, saponins and triterpenoids in the methanol extract of the *Lippia nodiflora*. This

Table 2. Antimicrobial activity of methanol extract of *Lippia nodiflora* Mich. (as mm inhibition zone)

S. No	Name of the Organism	Zone of Inhibition in mm						
		100 µg/ml	200 µg/ml	300 µg/ml	400 µg/ml	500 µg/ml	Tetracycline 4µg/ml	Amphotericin B 10µg/ml
1.	*Proteus Vulgaris* (MTCC 1429)	-	-	-	7	10	15	-
2.	*Staphylococcus epidermidis* (MTCC 435)	-	10	14	18	20	16	-
3.	*Staphylococcus aureus* (MTCC 96)	-	-	-	7	9	10	-
4.	*Salmonella paratyphi A* (MTCC 735)	-	-	-	8	10	14	-
5.	*Klebsiella pneumonae* (MTCC 432)	-	-	-	-	-	10	-
6.	*Escherichia coli* (MTCC 433)	-	-	-	9	10	11	-
7.	*Salmonella paratyphi B* (Clinical isolate)	-	-	-	7	9	12	-
8.	*Salmonella typhimurium* (MTCC 98)	-	-	-	-	-	10	-
9.	*Cryptococcus neoformans* (Clinical isolate)	-	-	8	10	12	-	15
10.	*Candida albicans* (MTCC 183)	-	-	8	9	11	-	13

apparent antimicrobial activity may be due to the presence of flavonoids and triterpenoids.

From the present study, it can be concluded that the methanol extract of *Lippia nodiflora* had significant inhibition of *in vitro* lipid peroxidation and antimicrobial activity. The components responsible for the activity of MELN are currently unclear. Further research work is needed to isolate the active components, responsible for the study.

Acknowledgement

One of the author ASHOKKUMAR.D, Senior Research Fellow, grateful to AICTE, New Delhi, India for providing financial support to this work.

References

1. Cerdeiras MP, Albores S, Etcheverry S, et al. Antimicrobial activity of *Xanthium cavanillesii* extracts. Pharm Biol 2007; 45 (3): 251–254.
2. Gislene GF Nascimento, Juliana Locatelli1, Paulo C Freitas, et al. Antibacterial activity of plant extracts and phytochemicals on antibiotic resistant bacteria. Braz J Microbiol 2000; 31: 247-256.
3. Suresh Babu K, Srinivas PV, Praveen B, et al. Antimicrobial constituents from the rhizomes of *Rheum emodi*. Phytochemistry 2003; 62: 203–207.
4. Gezer K, Duru ME, Kivrak I, et al. Free-radical scavenging capacity and antimicrobial activity of wild edible mushroom from Turkey. Afr J Biotechnol 2006; 5 (20): 1924-1928.
5. Rasadah MA, Houghton PJ. Antimicrobial activity of some species of Bignoniaceae. ASEAN Review of Biodiversity and Environmental Conservation (ARBEC). 1998; 3: 1-3.
6. Ocksook Yi, Eduardo M Jovel, Neil Towers GH, et al. Antioxidant and antimicrobial activities of native Rosa, sp. from British Columbia, Canada. Int J Food Sci Nutr 2007; 58 (3): 178-189.
7. Hilda Priya D'Souza, Ramachandra Prabhu H. In vitro inhibition of lipid peroxidation in fish by turmeric (*Curcuma longa*). Indian J Clin Biochem 2006; 21(2): 138-141.

8. Chopra RN, Chopra IC, Handa KL, Kapur LD. Indigenous Drugs of India, 2nd ed. Calcutta, India: UN Dhur & Sons Private Limited. 1958.
9. Chopra RN, Nayar SL, Chopra IC. Glossary of Indian medicinal plants, New Delhi, India: Council of Scientific and Industrial Research. 1956.
10. Gamble JS. Flora of the Presidency of Madras, Vol 2, Calcutta, India: Botanical survey of India. 1957.
11. Kirthikar KR, Basu BD. Indian Medicinal Plants, Vol 3, 2nd ed. Delhi, India: M/S. Bishen Singh Mahendra Pal Singh. 1975.
12. Nadkarni KM. Indian Materia Medica, Vol 1, Bombay, India: Popular Prakashan. 1954.
13. Anonymous, The Useful Plants of India, 3rd Edn, New Delhi, Publication and Information Directorate, Council of Scientific and Industrial Research. 1986
14. Forestieri AM, Monforte MT, Ragusa S, et al. Anti-inflammatory, analgesic and antipyretic activity in rodents of plant extracts used in African medicine. Phytother Res 1996; 10:100-106.
15. Ohkawa H, Onishi Yagi K. Assay for lipid peroxidation in animal tissue by Thiobarbituric acid reaction. Anal Bio Chem. 1979; 95: 351-358.
16. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk susceptibility test. 6th ed. Wagne. Approved standard. M2-A6. PA: NCCLS. 1997.
17. Ananrthanarayanan R, Jayaram Paniker CK. Textbook of Microbiology, Madras, Orient Longman Limited. 1987.
18. Pelczar MJ, Chan JR, Krieg NR. Microbiology, 5th Edn, New Delhi, Tata McGraw-Hill Publishing Company Limited. 1997.
19. Plaa GL, Witschi. Chemicals, Drugs, and Lipid peroxidation, Annu Rev Pharmacol Toxicol 1976; 16: 125-142.
20. Bartner A, Pfeiffer KP, Batner H. Applicability of disc diffusion methods required by the pharmacopoeias for testing antibacterial activity of natural compounds. Pharmazie 1994; 49: 512-516.
21. Urzua A, Caroli M, Vazquez L, et al. Antimicrobial study of the resinous exudate and diterpenoids isolated from *Eupatorium salvia* (Asteraceae). J Ethnopharmacol 1998; 62: 251–254.
22. Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev 1999; 12: 564–582.
23. Tsuchiya H, Sato M, Miyasaki T, et al. Comparative study on the antibacterial activity of phytochemical flavanones against methicilin-resistant *Staphylococcus aureus*. J Ethnopharmacol 1996; 50: 27–34.

24. Goodman & Gillman. The Pharmacological Basis of Therapeutics. In: Antimicrobial agents, and Antifungal agents. William A Petri Jr, John E Bennett, Joel G Hardman, Lee E Limbard. eds. 10th edn. Newyork: McGraw-Hill, Medical Publishing Division, 2001: 1298-99.

[*]Corresponding Author
Address for Correspondence:
Prof. (Dr). Upal Kanti Mazumder,
Department of Pharmaceutical technology,
Jadavpur University,
Kolkata,
West Bengal,
India-700 032.
Phone: +91-9433435247
E-mail: ashokkumardurairaj@yahoo.co.in