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In vitro Antibacterial Effect of Bushy Matgrass (*Lippia alba* Mill.) Extracts

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ABSTRACT

Extracts in organic solvents (namely petroleum sprit, dichloromethane, ethyl acetate and methanol) of one of the important medicinal plants- *Lippia alba* were evaluated for their antibacterial activities against five pathogenic bacteria such as *Bacillus subtilis*, *Sarcina lutea*, *Xanthomonas campestris*, *Escherichia coli* and *Klebsiella pneumoniae* by estimating zones of inhibition as produced by disc-diffusion method on Nutrient agar medium. All the bacteria showed susceptibility against the petroleum sprit and ethyl acetate extracts, whereas, the dichloromethane and methanol extract showed least activities. It was found that antibacterial activity of the petroleum sprit extract showed maximum zone of inhibition against *S. lutea* (22 mm) followed by *X. campestris* (14 mm) *B. subtilis* (12 mm) and *E. coli* (12 mm). Ethyl acetate extracts showed highest activity 12 mm zone of inhibition against both *S. lutea* and *X. campestris*. The minimum inhibitory concentration of petroleum sprit and ethyl acetate extracts were ranged between 32-256 μg mL^{-1} for tested bacteria. This study demonstrates the potential of *Lippia alba* as a source of antibacterial agent that could be effectively used for future health care purposes.

Key words: *Lippia alba*, medicinal plants, antibacterial activity, disc diffusion assay, MIC value

INTRODUCTION

Medicinal plants have been subjected to detailed study since time immemorial and practically there is no much difference in the value of medicinal herbs and method of treatment used in the ayurvedic, the allopathic and the homeopathic literature (Pavia *et al*., 1995). Different extracts from traditional medicinal plants have been tested to identify the sources of the therapeutics effect (Parekh and Chanda, 2007). Over the past 20 years, there has been an increased interest in the investigation of natural materials as sources of new antibacterial agents. In the recent years, the development of resistance of pathogens against antibiotics has become a difficult issue caused by the indiscriminate use of modern antibiotics (WHO, 1983; Kunin, 1993; Okemo *et al*., 2003; Arya *et al*., 2010; Dash *et al*., 2011). As a result some natural products have been approved as new antibacterial drugs but there is still an urgent need to identify novel substances that are active towards pathogens with high resistance (Barbour *et al*., 2004). Considering the high cost of the synthetic drugs and their side effects, wide varieties of natural plants can be considered as a vital source for anti-microbial agents (Geyid *et al*., 2005). Therefore, the demand for new and effective anti-microbial agents with broad-spectrum of activity from natural sources is increasing day by day (Rahman *et al*., 2008).

Lippia alba is a shrub belongs to the Verbenaceae family, is abundantly present between the south of the United States of America (Florida), the north of Argentina and also in Bangladesh (Yasmin *et al*., 2009). The versatility of this plant makes it a species of high potential economic interest (De Albuquerque *et al*., 2007). Various chemical constituents are reported in *Lippia alba* like citral (major compound), linalool (<5%), iridoids (geniposide, theveside and shanzhizide methyl ester) along with geniposidic acid, caryoptoside, 8-epiloganin and mussaenoside etc. (Hennebelle *et al*., 2006; Barbosa *et al*., 2006).

Lippia alba leaves are claimed to possess a wide range of pharmacological effects used as a sedative and also against states of excitement, respiratory ailments, hypertension, headaches, digestive troubles, flatulence pain, diarrhea, anemia, skin diseases, sore throat, nausea and vomiting, flu, cold, to heal wounds and as syrup against cough and bronchitis. An infusion of the roots is also used against bad colds and coughs (Di Stasi *et al*., 2002; Gazzaneo *et al*., 2005; Oliveira *et al*., 2006). Hence, the purpose of the present investigation was to evaluate the antibacterial activity of *Lippia alba* for the discovery of potential antibacterial agents that might be used for the management of bacterial infectious diseases.

MATERIALS AND METHODS

Plant material: Healthy, disease free and mature *Lippia alba* plant was collected directly from Islamic university campus, Kushtia, Bangladesh on July, 2010. This plant was then botanically identified and the name of the plant, time, place and date of collection were recorded.

Preparation of the extracts: Collected *Lippia alba* leaves were cleaned with deionized water and dried in shade and pulverized into fine powdered substances by a grinding machine. Each 10 g of powder was weighted with the electric balance and transferred into four separate 100 mL conical flasks. Then each 40 mL of petroleum spirit, dichloromethane, ethyl acetate and methanol was added in the flasks, respectively. The conical flasks were closed by foil paper and placed on a shaker at 37°C temperature for 24 h. The crude extracts were then filtered by passing the extracts through Whatman No. 1 filter paper and then concentrated under vacuum at 40°C by using a rotary evaporator. The residual extracts were stored in refrigerator at 4°C in small and sterile plastic bottles.

Tested bacteria: Antibacterial activity of *Lippia alba* leaf extracts were investigated against two gram positive (*Bacillus subtilis* and *Sarcina lutea*) and three gram-negative (*Xanthomonas campestris, Escherichia coli* and *Klebsiella pneumoniae*) bacterial isolates, obtained from the Microbial Type Culture Collection (MTCC) of Microbiology Laboratory of the Biotechnology and Genetic Engineering Department, Islamic University, Kushtia, Bangladesh. The tested bacteria were cultured on Nutrient agar (HiMedia, Mumbai, India) at 37°C for 24 h. The cultures were sub cultured regularly (every 30 days) and stored at 4°C.

Inoculum preparation: Ten milliliter of distilled water was taken into the screw cap tube and pure colony of freshly cultured bacteria was added into the tube and vortex was done. The OD (optical density) was measured with the colorimeter and bacterial population were confirmed to be within in 10^7-10^8 mL^{-1} and then plated out as inoculums (Gur *et al*., 2006).

Antibacterial bioassay: The *in vitro* antibacterial activities of the test samples were carried out by disc diffusion method (Bauer *et al*., 1996; NCCLS Document M2-A7, 2000). Dried and sterilized filter paper discs (6 mm diameter) were impregnated with known amount of the test substances (extracts) dissolved in solvents (400 μg $disc^{-1}$) using micropipette(s) and the residual solvents were completely evaporated. Discs containing the test materials (400 μg $disc^{-1}$) were placed on nutrient agar medium uniformly seeded with the test bacteria. Standard disc of nalidixic acid (30 μg $disc^{-1}$) and blank discs (impregnated with solvents followed by evaporation) were used as positive and negative control, respectively. These plates were then kept at low temperature (4°C) for 24 h to allow maximum diffusion of test samples and then incubated at 37°C for 24 h to allow maximum growth of the microorganisms. The test materials having antibacterial activity inhibited the growth of the bacteria and a clear, distinct zone of inhibition was visualized surrounding the disc. The antibacterial activity of the test agents was determined by measuring the diameter of zone of inhibition in millimeter. Minimum Inhibitory Concentration (MIC) values of the extracts showing significant results (petroleum sprit and ethyl acetate extracts) were determined in the present study following the serial dilution technique (Reiner, 1982). The experiment was carried out in triplicate and the average zone of inhibition was calculated.

Statistical evolution: The antibacterial activity was determined by measuring the diameter of zone of inhibition in millimeter (mm) scale that is the Mean±SEM (standard error mean) of triplicates. The MIC endpoint was considered as the lowest drug concentration of antibacterial agent inhibiting the total growth of bacteria.

RESULTS

Antibacterial activities: Among all tested extracts, petroleum spirit and ethyl acetate extracts were found to be most active and significant than corresponding organic extracts (Table 1). Petroleum sprit extract was found to have maximum zone of inhibition against *S. lutea* (22 mm) while *X. campestris* (14 mm), both *B. subtilis* and *E. coli* (12 mm) and *K. pneumoniae* (9 mm) were found to be relatively less active. Ethyl acetate extract was most effective against both *S. lutea* and *X. campestris* (12 mm) (Table 1) and was also significantly active against both *E. coli* and *K. pneumoniae* (11 mm) and *B. subtilis* (10 mm). Both of the dichloromethane and methanol extracts showed no sensitivity against *E. coli*. Dichloromethane extract was also inactive against *X. campestris*, whereas, methanol extract against *S. lutea* and *K. pneumoniae*. The positive control (nalidixic acid) produced higher zone of inhibition against the tested bacteria whereas, negative controls (disc containing only solvent) were found to be inactive (Table 1).

Table 1: Antibacterial activity of different extracts of *L. alba* (400 μg $disc^{-1}$)

	Diameter of zone of inhibition (mm)				
Tested bacteria	Petroleum spirit extract	Dichloromethane extract	Ethyl acetate extract	Methanol extract	Nalidixic acid (30 μg)
B. subtilis	12±0.23	7±0.62	10±0.25	7±0.34	27±0.73
S. lutea	22±0.50	8±0.34	12±0.23	-	30±0.64
X. campestris	14±0.25	-	12±0.31	8±0.50	27±0.43
E. coli	12±0.20	-	11±0.67	-	28±0.37
K. pneumoniae	9±0.71	7±0.71	11±0.40	-	29±0.23

Data are given as Mean±SEM (standard error mean), -: No zone formation

Table 2: MIC values of Petroleum spirit extract and of ethyl acetate extract of *L. alba*

	Petroleum spirit extract of *L. alba* (μg mL^{-1})										Ethyl acetate extract of *L. alba* (μg mL^{-1})									
Tested bacteria	512	256	128	64	32	16	8	4	2	0	512	256	128	64	32	16	8	4	2	0
B. subtilis	-	-	-	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+
S. lutea	-	-	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+
X. campestris	-	-	-	-	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+
E. coli	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+
K. pneumonia	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+

+: No zone formation; -: Formation of inhibition zone

MIC values: Only the MIC values of petroleum spirit and ethyl acetate extracts were investigated and found between 32-128 μg mL^{-1} (Table 2). The lowest MIC value was 32 μg mL^{-1} against *E. coli* and *K. pneumoniae* showed by ethyl acetate extract of *L. alba* (Table 2).

DISCUSSION

Herbal remedies used in folk medicine provide an interesting and still largely unexplored source for the creation and development of potentially new drugs for chemotherapy which might help overcome the growing problem of resistance and also the toxicity of the currently available commercial antibiotics (Alam *et al*., 2009). There are a lot of antimicrobial drugs of which some are discovered or established and over 250,000 undiscovered flowering plants with medicinal properties exist worldwide (Madureira, 2008). Hence, the last decade witnessed an increase in the investigations on plants as a source of human disease management (Aiyelaagbe, 2001; Prashanth *et al*., 2001; Mounissamy *et al*., 2002; Woldemichael *et al*., 2003) and more natural antimicrobials have driven scientists to investigate the effectiveness of inhibitory compounds such as extracts from plants (Nasar-Abbas and Halkman, 2004). The World Health Organization (WHO, 2000) has estimated that between the years 2000 and 2020 nearly one billion people will be infected and more than 200 million will develop the disease. Therefore, it is of great interest to carry out a screening of these plants in order to validate their use in folk medicine and to reveal the active principle by isolation and characterization of their constituents.

The research of Alviano and Alviano (2009) showed that, the essential oils from two chemotypes i.e., *Lippia alba* and *Lippia alba* f. *intermedia*, were active against all microorganisms assayed showing large inhibition zones, superior or similar to those obtained for the positive controls (vancomycin and methicillin). Oliveira *et al*. (2006) carried out an ethnopharmacological study that revealed good agreement of the major use (MUA) of *Lippia alba* (MUA= 92.0%) and to a lesser extent, for *Lippia alba* f. *intermedia* (MUA= 66.7%), as sedatives. But Holetz *et al*. (2002) observed that, *Lippia alba* extracts were considered inactive against some common tested bacterial and fungal strains whereas, Nogueira *et al*. (2007) reported that, the essential oils extracted from *Lippia alba* displayed significant inhibitory activity against *Bacillus subtilis, Escherichia coli, Lactobacillus casei* and *Staphylococcus intermedis* showing inhibition zone higher than positive control.

The findings demonstrated promising antibacterial activity of petroleum sprit and ethyl acetate soluble fractions of *Lippia alba* extracts. The petroleum sprit soluble fraction of *L. alba* showed moderate to potent antibacterial activity against *K. pneumoniae* (9 mm), *E. coli* (12 mm), *B. subtilis* (12 mm), *X. campestris* (14 mm) and *S. lutea* (22 mm), whereas, the dichloromethane soluble

partitionate demonstrated mild antibacterial activity against *S. lutea* (8 mm). In case of ethyl acetate extract, the result revealed that it exhibited almost same antibacterial activity against the tested bacteria ranging from 10-12 mm zone of inhibition, whereas, methanol fraction exhibited very poor antibacterial activities. The zone of inhibition and MIC value suggest that the petroleum spirit and ethyl acetate extracts can significantly inhibit bacterial growth in a lower dose dictates its potential as a source of active chemicals that might be used for the discovery of new antibacterial agent.

CONCLUSIONS

The results of present study supports the traditional usage of the *L. alba* plants and suggests that some of the plant extracts possess compounds with high antibacterial properties that can be used as antibacterial agents in developing new drugs for the therapy of infectious diseases caused by pathogenic bacteria. Further purification and characterization of the active principles from the effective extracts will provide a better understanding of the antimicrobial mechanism. This objective will be achieved in parallel with the investigation on more pathogenic agents currently going on.

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