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In vitro Antibacterial and Cytotoxic Activities of Various Fractions of *Lippia alba* (Mill.)

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Abstract

Lippia alba (Mill.) (Family: Verbenaceae) is widely known due to both ethnobotanical uses and chemical diversity. The present work was designated to determine the antimicrobial and cytotoxic activities of the aqueous ethanolic extract and various fractions of this plant. The whole plant was made into coarse powder and was extracted with 5% aqueous ethanol. The concentrated ethanolic extract (abbreviated as LAE) was successively partitioned with *n*-hexane (LAH), chloroform (LAC), ethyl acetate (LAA), and the remaining part was designated as water (LAQ). The antimicrobial and cytotoxic activities of extract and all these fractions were evaluated *in vitro* using disc diffusion technique and brine shrimp (*Artemia salina*) lethality assay, respectively. In antimicrobial assay, five Gram-positive and five Gram-negative bacteria and standard antibiotic ciprofloxacin (10 µg/disc) were used. All the samples were tested at four concentrations (200, 400, 600 and 800 µg/disc). Our study showed that the extractive and different fractions of *L. alba* had antibacterial activity against all the tested bacteria except *Pseudomonas aeruginosa.* Among the extractives, LAE and LAH had significant antibacterial activity when compared to standard ciprofloxacin. The zone of inhibition of LAE and LAH were found to be 14.4, 24.4, 13.4, 15.2, 18.5, 24.1, 16.1, 23.5, 23.2 and 14.5, 20.1, 12.5, 17.1, 17.5, 20.1, 12.5, 19.4, and 18.16 mm against *Bacillus cereus, Staphylococcus aureus, Streptococcus-β- haemolyticus, Bacillus subtilis, Sarcina lutea, Escherichia coli, Klebsiella pneumonia, Salmonella typhi* and *Shigella dysenteriae*, respectively, at 800 µg/disc. The median lethal concentration (LC_{50}) in brine shrimp lethality bioassay was determined by extrapolation from graph and the values were found to be 41.9, 23.9, 21.3, 88.7, 6.7 and 7.5 µg/ml for LAQ, LAH, LAC, LAA, LAE and standard vincristine sulfate, respectively. In the study, different mortality rate was observed at different concentration and all the samples showed positive response, indicating the presence of cytotoxic components in *L. alba.* Phytochemical screening revealed that *L. alba* contains saponins, tannins, glycosides, steroids, alkaloids, terpenoids and flavonoids. The results of the study indicate that *L. alba* might be a good source of potent antibiotic and anticancer drugs.

Key words: *Lippia alba*, Verbenaceae, cytotoxic activity, antimicrobial activity.

Introduction

Lippia alba (Mill.) is an aromatic shrub locally known as pichas-lakri, motmotia, bushy matgrass, etc. and is widely distributed in southern United States, northern Argentina, Colombia, and Mexico (Munir *et al.*, 1993; Hennebelle *et al.*, 2008). The plant is naturalized to Indian subcontinent due to its variety of folkloric reputations (Munir *et al.*, 1993) including activity against virus, fungi, bacteria, protozoa, oxidative stress, gastrointestinal disturbance, cancer cell lines, etc. (Abad *et al.*, 1997; Ali *et al.*, 2023; Hennebelle *et al.*, 2008; Oliveira *et al.*, 2006). Traditionally, tea from the leaves of *L. alba* has long been used to treat gastrointestinal disturbance and as a tranquilizer throughout Brazil (Vale et al., 1999). Literature survey reported anticonvulsant activity (Viana *et al.*, 2000), CNS activity (Do Vale *et al.*, 2002), anxiolytic activity (Hatano *et al.*, 2012),

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treatment of migraine (Carmona *et al.*, 2013), analgesic and anti-inflammatory effects (Viana *et al.*, 1998), lipid peroxidation inhibition and antioxidant activity (Saccol *et al.*, 2013), antiulcerogenic activity (Pascual *et al.*, 2001), antimicrobial activity (Oliveira *et al.*, 2006), antifungal activity (Tomazoni *et al.*, 2016), antiviral activity (Abad *et al.*, 1997; Hennebelle *et al.*, 2008) and cytotoxic activity (Santos *et al.*, 2016) of various parts of this plant.

Based on the literature review and folkloric reputation, we selected the plant to evaluate its antioxidant, antimicrobial and cytotoxic activities and in our previous report we presented extensive antioxidant activities, including polyphenolic contents of the whole plant, *L. alba* (Ali *et al.*, 2023). In the present study, we performed phytochemical screening and evaluated the antibacterial and cytotoxic activities of the ethanolic extract (aqueous) and its hexane, chloroform, ethyl acetate and aqueous fractions using *in vitro* assays.

Materials and Methods

Collection of plant: Fresh *L. alba* (Mill.) whole plant was collected from the University of Rajshahi campus (Rajshahi, Bangladesh) in October-November, 2018 and was identifying by professor A.H.M. Mahbubur Rahman, taxonomist, Department of Botany, University of Rajshahi, Bangladesh. A voucher specimen (PHRU - 211) has been maintained in the Department of Pharmacy, University of Rajshahi.

Preparation of plant material for extraction: The collected whole plant was washed with fresh tap water and then distilled water and shade dried for few days followed by oven drying at 40-45^{o} C for 2 days. The dried materials were ground using a grinder and the powdered materials were kept in air tight container.

Extraction, filtration and fractionation: Powdered sample of *L. alba* (around 1 kg) was extracted in extraction bottle with aqueous ethanol (5% H_2O, 6 litres) for 7 days with occasional shaking. When extraction was finished, the mixture was filtered through cloth and then cotton and was concentrated under reduced pressure at 45° C using a rotary evaporator. The process was repeated twice and the whole filtrates were combined to get concentrated brownish mass (LAE, 59 g, 5.3%, w/w) and stored at 4° C in a refrigerator. A portion of the concentrated ethanolic extract was partitioned using separatory funnel to get four fractions: LAH (*n*-hexane, 6.79 gm), LAC (chloroform, 6.30 gm), LAA (ethyl acetate, 4.78 gm) and highest proportion of LAQ (aqueous, 36.95 gm) fractions.

Antimicrobial screening: Antibacterial screening of LAE, LAH, LAC, LAA, and LAQ was performed using disc diffusion assay method (Bauer *et al.*, 1966) which is based on the ability of sample to diffuse through the nutrient agar gel and create a concentration gradient. The bacteria enlisted in table 1 were used for the assay.

Table 1. List of tested bacteria.

Gram-positive	Gram-negative
Bacillus cereus	*Escherichia coli*
Staphylococcus aureus	*Pseudomonas aeruginosa*
Streptococcus-β-haemolyticus	*Shigella dysenteriae*
Bacillus subtilis	*Salmonella typhi*
Sarcina lutea	*Klebsiella pneumoniae*

All bacterial strains were collected from the Department of Biochemistry and Molecular Biology, University of Rajshahi. The stock cultures of bacteria were incubated for 24 hours at 37°C on nutrient agar medium, following storage at 4°C. Three types of discs were prepared for antibacterial screening, sample discs, standard discs and control/ blank discs. Sterilized filter paper discs having 6 mm in diameter (BBL, Cocksville U.S.A) were prepared in a blank autoclaved petri dish. Sample solution of desired concentration was applied on the discs with the help of a micropipette in an aseptic condition. Test sample LAE, LAH, LAC, LAA and LAQ were dissolved separately in methanol to get a concentration of 200, 400, 600, and 800 µg/disc. Standard discs of ciprofloxacin (10 µg/disc, OXOID Ltd. UK) were used as a reference standard to compare the

antibacterial activity of test materials. Blank discs were used as a negative control to ensure that the residual solvent and the disc itself didn't show any activity.

The sample discs impregnated separately with LAE, LAH, LAC, LAA, and LAQ and standard ciprofloxacin discs were placed on the solidified agar plates, previously seeded with the mentioned organisms with the help of a sterile forceps and the plates were inverted and kept in a refrigerator for 48 hours at 37 ℃. After 48 hours of incubation, the antibacterial activity of the samples was determined by measuring the diameter of the zones of inhibition (in mm) and compared with the standard disc. (Santos *et al.*, 2015).

Cytotoxicity assay (Brine shrimp lethality bioassay): Brine shrimp lethality bioassay is a simple method using zoological organism called brine shrimp nauplii for the primary screening of anticancer, antiviral and cytotoxicity of a test sample. The method was described by Mclaughlin (1991). In this method, shrimp eggs obtained from market were allowed to hatch in artificial sea water (37 g/L non-ionized NaCl salt, pH of the sea water was maintained around 8.5). The nauplii were placed in sea water for 24 h at 37 °C under constant oxygen supply to ensure survival and maturity before use. Five mg of each sample from LAE, LAH, LAC, LAA, and LAQ were accurately weighed and dissolved in 1 ml of dimethyl sulfoxide in different beaker (stock solution concentration: 5 mg/ml). With the help of a micropipette 80, 60, 40, 30, 20, 10, 5, 2.5 and 1.25 µl of the stock solution were transferred in 9 different vials and the final concentration (160, 120, 80, 60, 40, 20, 10, 5 and 2.5µg/ml) were taken in nine different test tubes containing 5 ml sea water and 10 matured nauplii. Tests were carried out in triplicate. Vincristine sulphate was used as the positive control and DMSO was used as negative control. After 24 hours, the vials were observed using a magnifying glass and the number of survived nauplii in each vial was counted, recorded and the percentage of mortality of nauplii was calculated. The LC_{50}, median lethal concentration was obtained by a plot of probit value of % of the shrimps killed vs concentration of the test samples.

Phytochemical screening of LAE: The ethanolic extracts, LAE was tested for the presence of a variety of phytochemicals (alkaloids, saponins, tannins, glycosides, steroids, terpenoids, and flavonoids) and the findings were expressed as (+) for the presence and (-) for the absence of phytochemicals. Detail methods have been represented in our previous papers (Khatun *et al.*, 2017; Ahmed *et al.*, 2020).

Results and Discussion

Antimicrobial screening: The crude ethanolic extract of *L. alba* LAE and its four fractions LAH, LAC, LAA and LAQ were tested at four concentrations (200, 400, 600 and 800 µg/disc) for antibacterial activity against five Gram-positive and five Gram-negative bacteria (Table 1). Standard antibiotic, ciprofloxacin (10 µg/ml) discs were used for comparison. The results of the antibacterial activity are shown in tables 2 & 3 and figure 1.

All the extracts showed significant antibacterial activity against the tested microorganisms, except *Pseudomonas aeruginosa*. Among the extractives LAE and LAA displayed highest activity compared to other. Activity of aqueous extract was found to be zero. There are many literature reports about the antimicrobial effects of *L. alba* (Klein *et al.*, 2013). Our study is in line with these reports that could be helpful to find active principle responsible for activity as an antibacterial medicine.

Brine shrimp lethality bioassay: The result of cytotoxicity using brine shrimp lethality bioassay is shown in figure 2. The median lethal concentration (LC_{50}) was determined by extrapolation from the graph and the values were found to be 41.9, 88.7, 23.9, 21.3, 6.7 and 7.5 µg/ml for LAQ, LAA, LAH, LAC, LAE and standard vincristine, respectively. In this study, mortality rate at different concentrations was found to be increased with increasing concentration of the sample (Figure 2). Among the tested samples, crude ethanolic extract, LAE showed significant cytotoxic activity, similar to standard

vincristine sulphate indicating that the fraction is cytotoxic as well as biologically active.

Phytochemical screening: Phytochemical screening demonstrated the presence of alkaloids, flavonoids, glycosides, saponins, steroids, tannins, and terpenoids in CME. However, alkaloid, saponin and tannin are absent in LAH and flavonoids and

Table 2. Antibacterial activity of different fractions of *L. alba* and standard against Gram positive pathogenic bacteria.

Name of sample	Conc (µg/disc)	Zone of inhibition (mm)				
		Name of the Gram (+) bacteria*				
		SA	*BC*	*SB*	*BS*	*SL*
LAE	200	18.3	8.0	7.7	8.7	9.8
	400	19.0	10.5	9.4	10.5	13.2
	600	23.5	11.7	11.4	11.4	15.9
	800	24.4	14.4	13.4	15.2	18.5
LAH	200	11.4	8.8	8.40	10.8	8.1
	400	16.3	10.5	11.4	11.8	11.9
	600	17.4	12.1	11.8	13.7	15.4
	800	20.1	14.5	12.5	17.1	17.5
LAC	200	8.5	8.3	8.6	9.1	7.2
	400	13.1	10.2	10.0	10.0	11.0
	600	15.2	10.8	10.3	13.1	12.9
	800	17.9	13.2	11.9	14.0	17.6
LAA	200	9.7	8.4	7.7	9.4	7.1
	400	13.7	10.0	10.4	10.5	11.1
	600	16.4	11.1	11.1	13.8	13.7
	800	18.4	14.1	12.1	14.4	17.7
Ciprofloxacin	10	24.8	15.9	15.8	16.2	19.4

**SA = Staphylococcus aureus, BC = Bacillus cereus, SB = Streptococcus-β- haemolyticus, BS= Bacillus subtilis, SL= Sarcina lutea*. LAQ didn't show any remarkable activity, hence data is omitted.

Table 3. Antibacterial activity of different fractions of *L. alba* and standard against Gram negative pathogenic bacteria.

Name of Sample	Conc (µg/disc)	Zone of inhibition (mm)				
		Name of the Gram (-) bacteria*				
		EC	*KP*	*PA*	*ST*	*SD*
LAE	200	16.8	9.8	-	16.2	11.4
	400	18.3	11.4	-	18.4	13.5
	600	22.8	14.2	-	22.74	16.7
	800	24.1	16.1	-	23.5	23.2
LAH	200	11.1	-	-	10.8	10.1
	400	15.7	10.2	-	15.4	12.1
	600	17.1	10.6	-	16.7	15.8
	800	20.1	12.5	-	19.4	18.1
LAC	200	10.1	9.4	-	10.1	8.7
	400	14.0	11.0	-	13.1	11.2
	600	16.1	12.1	-	15.0	14. 9
	800	17.7	14.0	-	17.2	15.2
LAA	200	10.7	9.0	-	10.1	9.78
	400	14.1	11.1	-	13.4	12.4
	600	17.1	12.7	-	15.7	15.4
	800	18.7	14.3	-	17.4	16.2
Std	100	25.0	17.8	16.7	24.9	25.2

**EC = Escherichia coli, KP= Klebsiella pneumoniae, PA= Pseudomonas aeruginosa, ST = Salmonella typhi, SD= Shigella dysenteriae*, (-) indicates no activity, LAQ didn't show any remarkable activity, hence data is omitted.

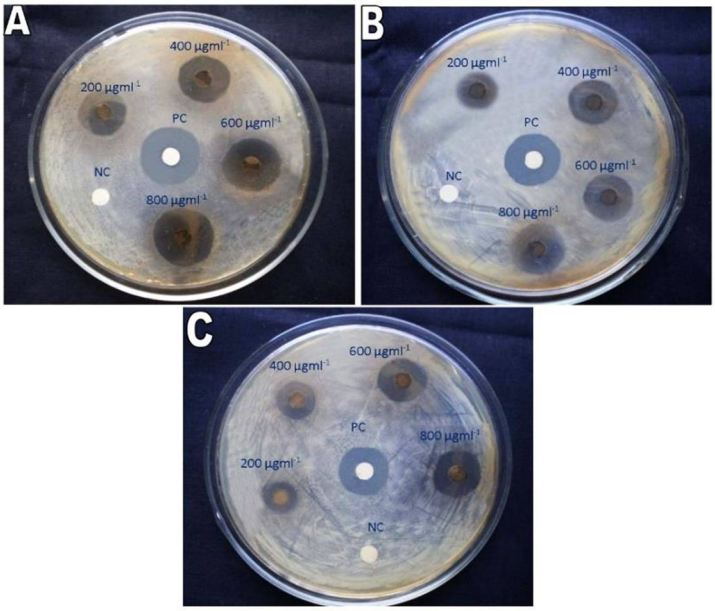

Figure 1. Antibacterial activity of (A) LAE against *Staphylococcus aureus* (B) LAH against *S. aureus* and (C) LAA against *Escherichia coli.*

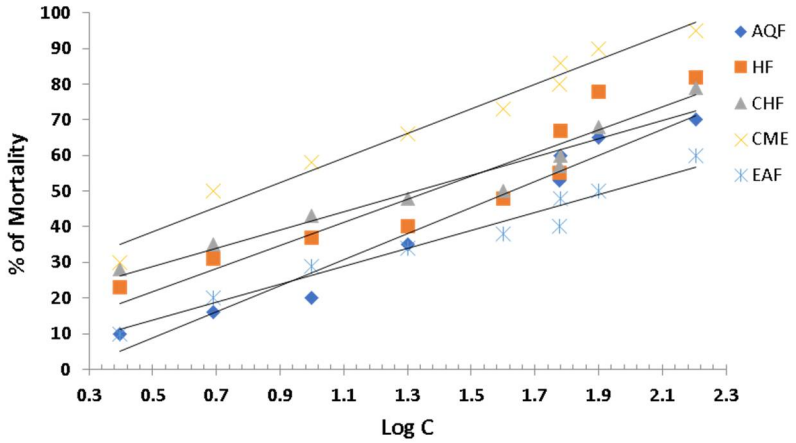

Figure 2. Determination of cytotoxic activity of LAE and four fractions using brine shrimp lethality bioassay.

Table 4. Phytochemical test results of different extractives of *L. alba.*

Tests	CME	LAH	LAC	LAE	LAQ
Saponins	+	-	++	++	+++
Tannins	+	-	+++	+	++
Glycosides	+	++	++	+	+
Steroids	+++	++	++	+	-
Alkaloids	+	-	+++	++	+++
Terpenoids	++	++	+++	+++	++
Flavonoids	+	+	+++	+++	-

NB: + = presence in mild amount, ++ = presence in moderate amount, +++ = present in large amount, (-) not detected.

steroids are absent in LAQ fraction (Table 4). It has been reported that terpenes and flavonoids in extract might be responsible for antimicrobial activity (Oliveira *et al.*, 2014), hence our findings from phytochemical screening validate the significant antibacterial activity of the extracts and fractions.

Conclusions

In our present study, we found the crude aqueous ethanolic extract of *L. alba* possesses remarkable cytotoxic activity against brine shrimp nauplii and moderate antibacterial activity against gram-positive and gram-negative microorganisms, except *Pseudomonas aeruginosa*. Hence, the whole plant can be a potent source of antibacterial and cytotoxic agent.

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