In Vitro Antibacterial Efficacy of *Leucas Cephalotes* (Roth) Spreng. (Lamiaceae) against some Gram Positive and Gram Negative Human Pathogens

Abdul Viqar Khan¹*, Qamar Uddin Ahmed², Athar Ali Khan¹ and Indu Shukla³

¹ Department of Botany, Faculty of Life Sciences, Aligarh Muslim University (A.M.U.), Aligarh-202002, State of Uttar Pradesh (U.P), India
² Department of Pharmaceutical Chemistry, Faculty of Pharmacy, IIUM, 25200-Kuantan, Pahang Darul Makmur, Malaysia
³ Department of Microbiology, Jawaharlal Nehru Medical College, A.M.U., Aligarh-202002, U.P., India

Abstract:

*Leucas cephalotes* (Roth.) Spreng (family: Lamiaceae) is a rainy season weed widely distributed in tropical regions of Asia at elevation up to 1,700 m, medicinally employed for the treatment of skin diseases, fever, hepatic disorders, urinary complaints cough and cold. The traditional uses of this plant strongly suggest its possible antibacterial properties, but its efficacy has not been examined in broad scenes, in present communication, its antibacterial efficacy has been explored. Polar and non-polar extracts (ethyl acetate, methanol, aqueous, benzene, and petroleum ether) at five different concentrations (0.5, 1, 2, 5, and 10 mg/mL) were evaluated for their antibacterial efficacy against seven G+ and eleven G– hospital isolated bacteria. Disc diffusion method was followed to determine antibacterial activity. Phytochemical analysis of plant revealed presence of tannins, glycosides, saponins, steroids, phenolic compounds and flavonoids as secondary metabolites. Maximum antibacterial activity was demonstrated by ethyl acetate and methanol extracts at a minimum concentration (0.5 mg/mL/disc). Extracts were effective on both types of test pathogens. From the present findings, it may be concluded that the plant could be formulated in broad spectrum antibiotic and confirms the traditional uses in pathogenic diseases.

Keywords: *Leucas cephalotes* (Roth) Spreng, Antibacterial efficacy, crude extracts, traditional uses

1. Introduction

Herbal plants are chief sources of potentially useful chemotherapeutic agents to help health care system. The medicinal value of plants lies in a wide variety of secondary metabolites, such as, terpenoids, alkaloids, and flavonoids. These natural compounds often serve as lead compounds whose activities can be increased by structural manipulation through amalgamations with molecular modelling and synthetic chemistry. These metabolites have been found *in vitro* as well as *in vivo* to have antibacterial characteristics. People’s interest in medicinal plants has greatly increased in recent years. This interest has eventually led to the discovery of new biologically-active substances by
the pharmaceutical based industries and the adoption of herbal extracts for self-medication by the general public (Nahrstedt and Butterweck, 2010; Vogel, 1991).

India is one of the World’s top 12 mega biodiversity countries with 10 biogeographic regions and over 40 sites which are known for their high endemism and genetic diversity. India has more than one fourth (8000) of the world’s known medicinal plant species (30,000) of which 90% are found in forests (Khan et al., 2008; 2010; 2011; 2011a; 2012; 2013). Plant species still serve as rich sources of many novel biological active compounds, as very few plants species have been thoroughly investigated for their medicinal properties (Abubakar et al., 2011; Caius,1986; Habbal et al., 2011; Khan et al., 2008; Paul et al., 2011). Even though pharmaceutical industries have produced a number of new antimicrobial drugs in the last years (Jila, 2011; Karuppiah and Rajaram, 2012; Parekh and Chanda, 2010; Ruban and Gajalakshmi, 2012; Srinivasan and Balakrishnan, 2012), resistance to present chemically synthesised drugs by microorganisms has inevitably increased (Ravikumar, 2010; Sati et al., 2011). In general, bacteria have the genetic ability to transmit and acquire resistance to drugs used as therapeutic agents (Goossens et al., 2005; Mathew et al., 2007).

*Leucas cephalotes* (Roth.) Spreng. is a rainy season weed and commonly found ascending up to 600-1,800 m particularly in the hilly regions of Nepal, India, Pakistan, Bangladesh and Myanmar. In the East Asia this plant is found in Afghanistan and Western China in open areas at elevation of 1,700 m (Khan, 2002). Traditionally, its decoction is used in the treatment of malarial fever and for snake bite. The dried inflorescences are smoked and the smoke exhaled through the nose to treat nose bleeds. Dried leaves along with tobacco (1:3) are smoked to treat bleeding as well as itching piles and fresh leaves eaten as a potent herb. The juice of leaves is used topically in psoriasis, skin eruptions, scabies and internally for the treatment of urinary complaints. The flowers are administered in the form of syrup or with honey for cough and colds (Baburao et al., 2010; Manimekalai et al., 2011). Pharmacologically, *L. cephalotes* has been reported to exert hepatoprotective action in CCl₄ induced hepatotoxicity in animals, its juice has been reported to act as an antibilious in herbal therapy for jaundice, and helps cure induced filariasis. The whole plant’s powder has 70% herbal composition and is patented to cure epileptic convulsions and cerebral function disorders, it acts as antipyretic, stimulant, expectant, aperient, diaphoretic, insecticidal, emmenagogue, antioxidant, anti-inflammatory and anti-diabetic (Mishra, 2002; Qamaruddin et al., 2002). Various classes of phytochemicals have been isolated from different parts of *L. cephalotes* i.e. labdane, norlabdane, flavones, laballenic acid (octadeca-5, 6-dienoic acid), luuric acid, glutaric acid, adipic acid, tridecanoic acid, β- sitosterol and its glucoside. The volatile compounds of inflorescence and seeds were found to contain caryophyllene oxide 26.56%; δ-fenchene 12.02%; α-cardinal 2.13%; 1-hepten-3-ol 6.53%; menthol 6.30%; deca hydronaphthalene 5.15% and trans-caryophyllene 4.05% (Singh et al., 1978; Kamat and Singh, 1994; Miyaichi et al., 2006).

*L. cephalotes* is traditionally employed for the treatment of skin diseases, urinary complaints, fever, liver disorders, cough and cold which are suggestive of its possible antimicrobial properties, however, its antibiotic efficacy has not been examined in broad scenes. Hence, in present communication, its antibacterial analysis has been carried out.

![Leucas cephalotes (Inflorescence)](image)

### 2. Materials and Methods

#### 2.1 Plant Material

Fresh plants’ material was collected after the rainy season in the month of September 2010 from the wild localities of its natural distribution from rural areas of Aligarh and was authenticated by Dr. Athar Ali Khan (Taxonomist) Department of Botany, Faculty of Life Sciences, Aligarh Muslim University.
University, Aligarh, 202002, India and was deposited at the same department (Voucher Specimen No. AVKLC: 013).

2.2 Preparation of Extracts and Fractions

Plant extracts were prepared according to the following protocol: Pulverized plant material (500 g) of whole plant was refluxed with 95% methanol (MeOH) on hot water bath for 1 hr. Mother liquor (crude MeOH extract) was filtered out and residual plant material was again refluxed with 95% methanol for 1 hr, the whole process was repeated four times to obtain maximum yield of methanol soluble compounds from the plant material. The extract was evaporated to dryness at 35°C under reduced pressure (Harborne, 1988). Dried methanolic extract (67.5 g) was further refluxed with petroleum ether (60-80°C) for 1 hr and filtered. Residual methanolic extract was again refluxed with petroleum ether for 1 hr and the entire process was repeated five times until there was no coloration. Petroleum ether extract was evaporated under reduced pressure to obtain petroleum ether soluble extract (13.58 g). Petroleum ether insoluble fraction of methanolic extract was refluxed with benzene for 1 hr, filtered and refluxed again with benzene for 1 hr and filtered. The process was repeated five times until there was no coloration. Benzene was evaporated under reduced pressure to obtain benzene soluble extract (13.83 g). Benzene insoluble fraction of the methanol extract was further refluxed with ethyl acetate for 1 hr, filtered and refluxed again with ethyl acetate for 1 hr and filtered, the whole process was repeated 5 times to ensure maximum yield. Ethyl acetate was evaporated under reduced pressure to obtain ethyl acetate soluble extract (17.05 g). Ethyl acetate insoluble fraction of the methanolic extract was again refluxed with methanol (95%) for 1 hr, filtered and was then repeatedly refluxed five times with methanol. The methanol soluble fraction was evaporated under reduced pressure to obtain methanolic extract (11.09 g), while methanol insoluble residue was discarded. (Figure 1)

![Figure 1](diagram)

**Figure 1:** Strategic preparation of non-polar and polar extracts of whole plant *L. cephalotes* based on different polarities of organic solvents.
2.3 Preparation of Aqueous Extract
Shade dried 500 g pulverized plant material was poured with double distilled water, and left for 72 hrs at room temperature. The flask was then refluxed over hot water bath for about 2 hrs and the mother liquor was filtered, the entire process was repeated 4 times to obtain maximum yield of water soluble components (43.12 g). The filtrate, thus obtained, was evaporated to complete dryness under reduced pressure by using rotary evaporator and eventually freeze dried. The plant extracts thus obtained were kept carefully in closed bottle at -20°C until further use.

2.4 Phytochemical Analysis
2.4.1 Test for Alkaloids
a) Dragendorff’s Test: Dragendorff’s reagent (potassium bismuth iodide) did not give reddish brown precipitate indicative of alkaloids presence.
b) Mayer’s Test: Mayer’s reagent (potassium mercuric iodide) did not give cream precipitate indicative of alkaloids presence.

2.4.2 Test for Glycosides
a) Keller-kiliiani Test (for deoxy sugar): 1 mL of glacial acetic acid containing traces of FeCl₃ and 1 mL of conc. H₂SO₄ were added. A reddish-brown colour formed at the junction of two layers and the upper layer turned bluish green.

2.4.3 Test for Saponins
a) Foam Test: Powdered drug residue was taken in a test tube and shaken vigorously with a small amount of NaHCO₃ and water. Characteristic honeycomb like froth was obtained.

2.4.4 Test for Steroids
a) Salkowaski reaction: Residue of extract was taken in 2 mL of CHCl₃ and 2 mL of conc. H₂SO₄ added from the side of the test-tube and then shaken for a few minutes. Red colour developed in the CHCl₃ layer.

2.4.5 Test for Tannins and Phenolic Compounds
a) Ferric chloride reagent: FeCl₃ sol. was added to test sol. Dark green or deep blue colour was obtained.
b) Lead acetate Test: A 10% w/v solution of basic lead acetate in distilled water was added to test sol. As a result precipitation occurred.

2.4.6 Test for Flavonoids
a) Shinoda Test: Test residue was dissolved in 5 mL ethanol (95% v/v) and reacted with few drops of conc. HCl and 0.5 g of Mg metal. Pink, crimson or magenta colour was developed.
b) Ammonia Test: Filter paper strips were dipped in the alcoholic extract and ammoniated Strips turned yellow.

2.4.7 Test for Amino Acids and Proteins
a) Ninhydrin Test: 0.1% w/v solution of Ninhydrine was dissolved in n-butanol and then added to test sol. A violet or purple colour was developed.
b) Cysteine Test: 2 ml of 40% w/v NaOH and 2 drops of 10% w/v lead acetate solution were added to 5ml of test sol, resulting solution is boiled for few min to obtain black precipitate.
c) Biuret Test: Residue is taken in water, 4% NaOH solution is added then few drops of 1% CuSO₄ sol were added, violet or pink colour was obtained.

2.4.8 Test for Sugars
a) Molisch Test: A few drops of α-naphthol and then conc. H₂SO₄ were added to test solution. Violet colour ring was formed at the junction of two layers.
b) Fehling’s Test: Fehling A and Fehling B solution were mixed in equal amount and boiled then added with test solution and again heated. Yellow to brick red colour was obtained.
c) Benedict’s Test: Test solution and Benedict’s reagent were mixed and heated in water bath for 5-10 minutes. Yellow to red or green colour were obtained.

2.4.9 Test for Total Flavonoids Content
Twenty grams of plant powder was extracted repeatedly with 250 mL of 80% aqueous methanol (MeOH) at room temperature (29°C). The whole solution was filtered through Whatman filter paper No: 42 (125 mm). The filtrate was evaporated to
dryness using rotary evaporator at standard temperature. Quantitative Result: Flavonoids ≤8.09%.

2.4.10 Test for Total Tannins Content

One gram of plant powdered drug was extracted with 100 mL of distilled water by shaking, using shaker for an hour and left overnight, solid material was allowed to settle and the liquid was filtered through a Whatman no. 1 filter paper, the first 20 mL of filtrate was discarded and then 10 mL of the filtrate was put into a conical flask. To it 750 mL of water and 25 mL of Indigo sulphuric acid sol. was added and titrated with 0.1 N KMnO₄ solutions. The flask was shaken vigorously till a golden yellow end point was reached. A blank determination was also performed and percentage calculated. Each mL of KMnO₄ solution is equivalent to 0.004157 gm of total tannins. Quantitative Result: Total Tannins ≤0.1% (Harborne, 1988).

2.5 Microorganisms

The whole plant extracts of *L. cephalotes* were tested for possible antibacterial activity against eighteen (18) human pathogenic bacteria listed in table 1. The authenticated microorganisms were procured from stock culture, Department of Microbiology, Jawaharlal Nehru Medical College, Aligarh-202002, U.P., India. The bacterial strains were sub-cultured at 37°C for 24 hrs and maintained on nutrient agar media.

2.6 Antibacterial Activity

National Committee for Clinical Laboratory Standards (NCCLS, 2003; 2004) were strictly followed to perform antimicrobial disc susceptibility testing using disc diffusion method to test plant extracts against test strains. Standardized inoculums agar plates were inoculated using a sterile swab dipped into culture inoculums adjusted to 1.5 x 10⁸ bacterial/mL using 0.5 Farland turbidity standard, the agar was streaked in three directions turning the plates by 60° by each streak. All the extracts were sterilized by filtration through 0.045 μm membrane filter and then autoclaved at 122°C. The paper disc (Whatman filter paper no 1) with 0.5, 1, 2, 5, and 10 mg/mL plant extracts were dried and placed on the agar surface with the help of sterile forceps. Finally, the sensitivity discs were placed with forceps carefully to make complete contact with the surface of the medium on the plate. Plates were kept at room temperature for 30 minutes (Pre-diffusion time). Inoculated petri dishes were incubated at 37°C overnight and at the end of the period, inhibition zones formed on the medium were evaluated in mm. The experiments were repeated thrice. The mean of the triplicate of the results has been summarized in table 1.

2.7 Statistical Analysis

All values are expressed as the mean ± the standard error of the mean (SEM); linear regression analyses and correlation coefficients to determine the relationship between two variables, they were calculated using MS-DOS software (GraphPad InStat statistical program).

3. Results

Results of *in vitro* antibacterial study are presented in table 1 and figure 2. It was observed that non polar extracts (Petroleum ether (PE) and benzene extracts (BZ)) had lower range of antimicrobial inhibition against test pathogens as compared to polar extracts (Ethyl acetate (EtOAc), methanol (MeOH) and aqueous (AQ) extracts). BZ extract was able to inhibit the growth of two Gram-positive and four Gram-negative pathogens, while PE extract revealed antibacterial activity against five Gram-positive and three Gram-negative pathogens. On the other hand EtOAc, MeOH and AQ extracts showed higher antibacterial activity as they were able to restrict the growth of eleven bacterial strains (five Gram-positive and six Gram-negative pathogens). EtOAc fraction showed the highest antibacterial activity against *S. aureus*, *S. albus* and *P. shigelloides*. MeOH extract also demonstrated good antibacterial activity; however, the zone of inhibition was lower than the EtOAc extract, while moderate sensitivity was observed for AQ extract. As seen in the Table 1; EtOAc, MeOH and AQ plant fractions showed greater inhibition effects then PE and BZ. This showed the presence of some moderate or strong polar components (secondary metabolites).
Table 1
Antibacterial activity of *Leucas cephalexos* (whole plant) inhibition zone (mm)

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<th>Gram-positive bacteria</th>
<th>Gram-negative bacteria</th>
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4. Discussion

Present communication brought to light an interesting finding for antibacterial approach of the traditionally important plant *L. cephalotes*. It was opted that both traditionally practiced solvent that is, Water and Lab practiced EtOAc and MeOH (polar) and PE and BZ should be used as non-polar solvents. Aqueous plant extract also inhibited the growth of five Gram-positive and six Gram-negative pathogenic bacterial strains as demonstrated by EtOAc and MeOH extracts. The most appropriate method would be that in which the extracts were the same as that used in folk medicine or phytotherapy, although in the lab the use of methanol or ethanol extract is much more common. But the zone of inhibition at the same concentration was more pronounced in the later extracts (EtOAc and MeOH) which might be due to two reasons, firstly, the nature of biological active components like tannins, flavonoids, saponins which could be enhanced in presence of the above polar solvents, it is documented that above secondary metabolites are well known for antimicrobial activity. Secondly, the stronger extraction capacity of EtOAc and MeOH could have produced greater number of active constituents. The inference can be drawn that the antibacterial activity observed with the EtOAc, MeOH and AQ extracts suggest the presence of bioactive compounds which can serve as antimicrobial agents or it can be further studied to formulate compounds which are less toxic and cheaper to help the ailing community. Findings also justify traditional uses of plant in curing fever, urinary, chest and GIT infections.

Medicinal plants constitute an effective source of traditional and modern medicine in many countries like China, India, Africa as well as other Asian nations. Herbal medicine has shown to have genuine utility and about 80% of population depends on it as a primary health care as WHO advocates. During the last thirty years, the development of resistance and various undesirable side effects of present chemically synthesised drugs and unavailability of antibiotic to the rural populace has prompted scientific community to search for new antimicrobials, mainly of plants’ origin to overcome the afore mentioned disadvantages and needs.

Conflict of Interest

We declare that we have no conflict of interest.

Acknowledgements

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References


