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Leucas cephalotes (Spreng): Photochemical investigation and antimicrobial activity via cylinder-plate method or cup-plate method

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Abstract
Leucas cephalotes is an annual herb growing widely in India. The Leucas cephalotes (Roth) Spreng whole herb contains new Labdane, Nor Labdane & Abietone-type, Diterpenes named Leucasdins A (1), B (2), C (3), five sterols and eight flavones. The research summaries phytochemical and pharmacological investigations carried out on Leucas Cephalotes. The ethanolic extract of plant was more effective against *V. cholera* than the aqueous extract in terms of zone of inhibition. Ethanolic extract was also more effective against *C. albicans* and *A. niger* than the aqueous extract of same plant with respective strains. Minimum inhibitory concentration was also tested against the selected strains of micro-organisms. Ethanolic extract showed MIC at 250 µg/ml against *S. aureus*, *E. coli* and *V. cholera* while against *C. albicans* and *A. niger* the MIC was found to be 500 µg/ml. Aqueous extract showed MIC at the dose leveling 500 µg/ml for all the test microbial strains. The ethanolic extract of the plant *Leucas cephalotes* Spreng. Was more active against all the test strains than the aqueous extract.

Keywords: Leucas cephalotes Spreng, antimicrobial activity, ethanolic extract

1. Introduction
Medicinal plants are of important therapeutic aid for various ailments because infectious diseases have dramatically increased [1, 2]. Antibiotics have been used to treat infections since 1940's [3] but the development of resistance by a pathogen to many of the commonly used antibiotics provides an impetus for further attempts to search for new antimicrobial agents to combat infections and overcome problems of resistance and side effects of the currently available antimicrobial agents [4, 5]. Scientific experiments on the antimicrobial properties of plant components were first documented in the late 19th century [6]. Naturally occurring antimicrobials can be derived from plants, animal tissues, or microorganisms. The shortcomings of the drugs available today propel the discovery of new pharmacotherapeutic agents in medicinal plants [7]. The antimicrobial activity of some plants was studied against pathogenic yeasts and bacteria. A comparison of the antimicrobial activity of plants to that of different antibiotics has also been carried out which might yield significant information as to whether extracts can be employed as replacement or as an adjuvant to well established chemotherapeutic agents [8]. Hence, this in vitro study was aimed at screening the plants for its composition and antimicrobial activity evaluating its potential use in treating various infections caused by bacteria and/or fungus and determining whether its use in folkloric medicine use justified [9].

2. Materials and Methods
The dried plant stems powder (1 kg) of Leucas cephalotes Spreng. Was taken and subjected to extraction with ethanol and aqueous extraction was done on the marc left after ethanolic extraction. The respective solvents were evaporated and extract were concentrated. The dried weight of the each extract was used to determine the concentration in mg/ml. Extracts were stored in refrigerator and were suspended in DMSO (dimethyl sulfoxide) prior to use.

Strains for antibacterial activity:
- Staphylococcus aureus (MTCC 3160)
- Escherichia coli (NCIM 2065)
- Vibrio cholera (MTCC 3906)
- Strains for antifungal activity:
- Candida albicans (MTCC 227)
- Aspergillus niger (NCIM 501)

Culture Media [10]
The culture media used for antimicrobial assay were procured from HiMedia Bombay, India. Media were prepared using specified quantities of antibiotic assay medium and were thereafter sterilized by autoclaving at 15lb/square pressure at 121°C for 20 minutes.

Media for bacterial growth
a) Nutrient Broth Medium

Beef extract	10 gm
Peptone	10 gm
Sodium chloride	5 gm
Distilled water	1000 ml
pH adjusted	7.0±0.2

b) Nutrient Agar Medium

Beef extract	**10 gm**
Peptone	10 gm
Sodium chloride	5 gm
Agar	20 gm
Distilled water	1000 ml
pH adjusted	7.0±0.2

Media for fungal growth

a) Sabouraud's Dextrose Agar Medium

Dextrose	40 gm
Peptone	10 gm
Agar	15 gm
Distilled water	1000 ml
pH adjusted	5.6±0.2

b) Sabouraud's Dextrose Broth Medium

Dextrose	40 gm
Peptone	10 gm
Distilled water	1000 ml
pH adjusted	5.6±0.2

Experimental Work

Preparation of Test Inoculums [10]

(a) Seeded broth preparation

The various strains of microorganisms were obtained from National Chemical Laboratory, Pune, India. The stock bacterial cultures were maintained in nutrient agar slants at 4°C. Each of the microorganisms was freshly cultured prior to susceptibility testing by transferring them into a separate sterile conical flask containing about 100 ml nutrient broth and incubated overnight at 37°C ± 1°C and termed as seeded broth. A microbial loop was used to remove a colony of each bacterium from pure culture and transferred into nutrient broth.

(b) Standardization of seeded broth (viable count)

(i) Dilution: In 99 ml of sterile water containing 0.05% Tween 80, 1 ml of seeded broth was added. From this, 1 ml was taken and diluted to 10 ml with sterile water and seeded broth is further diluted upto 10-10 dilution.

(ii) Inoculation into nutrient agar Petri dishes 0.2 ml of seeded broth dilutions were inoculated into solidified nutrient agar medium by spread plate method. Number of colonies of microorganisms formed after inoculation at 37°C ± 1°C. The seeded broth was suitably diluted to contain 106-107 colony forming unit/ml (cfu/ml). It was the working stock and used for microbiological evaluation.

Zone of Inhibition

Cylinder-plate method or Cup-plate method [10]

This method depends upon the diffusion of an antibiotic from vertical cylinder or a cavity through the solidified agar layer of a petridish or plate to an extent such that growth of the added microorganisms is prevented entirely in a circular area of zone around the cylinder or cavity containing the solution of test compound. Diameter of the clear zone produced due to inhibition of microbial growth is measured.

Preparation of stock and standard solutions

The solution of test compounds and standards were prepared at the concentration of 1000 µg/ml by dissolving in dimethyl sulphoxide (DMSO) in small volumetric flasks.

Procedure

The standard and test compounds (ethanolic and aqueous extract of plant) solution were prepared in dimethyl sulphoxide (DMSO) at the concentration of 10 mg/ml. Standard drugs used in the study were streptomycin at the concentration of 1 mg/ml for bacterial assay and fluconazole (1 mg/ml) for the assay of fungi. The petriplates containing 25 ml of sterile nutrient agar were inoculated with standardized innocula (0.1x 108 cell/ml) using sterile Pasteur pipette. Wells of 8
mm diameter were made by steel borer at the centre of each plate. To these wells 0.2 ml of various test and standard compounds solution were dispensed aseptically into each well. The extracts were allowed to diffuse into medium for 1 hour at room temperature. The plates were incubated at 37°C ±1°C for 18 hours for bacteria and 37°C ±1°C for 72 hours for *Candida albicans* and 28°C ± 1°C for *Aspergillus niger* for a period of seven days. Antimicrobial potential of test compound was determined on the basis of mean diameter of zone of inhibition around the wells. The experiment was repeated thrice and the average values were recorded.

As appreciable results in form of significant zone of inhibition was seen so minimum inhibitory concentration of various test compounds was also screened.

Minimum Inhibitory Concentration (MIC) [11]

MIC of extracts was determined using turbidity method in nutrient broth medium for bacterial strains and Sabauraud dextrose broth medium for fungal strains. This method depends upon the growth of a microbial culture in uniform solution of the test solution in a fluid medium that is favorable to its rapid growth in the absence of test compound. Varying concentration of the compounds was added to test organism on liquid culture.

Preparation of stock and standard solutions

The solution of test compounds and standards were prepared at the concentration of 1000 µg/ml by dissolving in dimethyl sulphoxide (DMSO) in small volumetric flasks.

Procedure

The study involved a series of 5 assay tubes containing 1 ml of sterile broth, were serially diluted with 1 ml of the stock solution of the extract, to give concentrations of 500, 250, 125, 62.5 µg/ml respectively. The microbial suspension (0.1 ml) was then added to each test tube aseptically. The racks of assay tubes were incubated at 37°C ± 1°C for 18 hours for bacteria and 37°C ± 1°C for 72 hours for *Candida albicans* was incubated at and 28°C ± 1°C for *Aspergillus niger* for a period of seven days. After incubation the assay tubes were removed, observed for any deposits, shaken to aerate the solution and to suspend microbes which have settled at the bottom of the assay tubes. The lowest concentration of the extracts and the standard drug, which cause apparently a complete inhibition of the growth of microorganisms was taken as the minimum inhibitory concentration of that particular extract. The solvent tubes were also observed for any inhibitory action of DMSO. Two positive controls were maintained without the addition of the extracts or the standard drug. The concentration observed is assumed as minimum inhibitory concentration.

3. Results and Discussion

The antimicrobial activity in terms of zone of inhibition and minimum inhibitory concentration of different extracts of *Leucas cephalotes* Spreng. was tested against three bacterial strains *viz. Staphylococcus aureus* (MTCC 3160), *Escherichia coli* (NCIM 2065), *Vibrio cholera* (MTCC 3906) and two fungal strains *viz*. *Candida albicans* (MTCC 227), *Aspergillus niger* (NCIM 501) according to the method mention under section 8.2.1.3 and 8.2.1.4. The aqueous and ethanolic extracts

of the plant respectively exhibited antimicrobial activity against the test strains (Table: 1-2, Plate 25-29, Fig. 1).The ethanolic extract showed more activity against *S. aureus* than the aqueous extract. The aqueous extracts of plant also showed activity against all the test strains but zone of inhibition was better for *E. coli* than the other test strains. The ethanolic extract of plant was more effective against *V.cholera* than the aqueous extract in terms of zone of inhibition. Ethanolic extract was also more effective against *C. albicans* and *A. niger* than the aqueous extract of same plant with respective strains. Minimum inhibitory concentration was also tested against the selected strains of micro-organisms. Ethanolic extract showed MIC at 250 µg/ml against *S. aureus*, *E. coli* and *V.cholera* while against *C. albicans* and *A. niger* the MIC was found to be 500 µg/ml. Aqueous extract showed MIC at the dose leveling 500 µg/ml for all the test microbial strains. Therefore it was concluded that the ethanolic extract of the plant *Leucas cephalotes* Spreng. Was more active against all the test strains than the aqueous extract. All the results were compared with standard drugs. Streptomycin was used as standard for antibacterial activity and Fluconazole for antifungal activity and DMSO used as a control. No activity was observed with DMSO.

Table 1: Data showing the diameter of the zone of inhibition for the ethanolic and aqueous extracts of *Leucas cephalotes* Spreng.

S. No.	Name of Organism	Diameter of Zone of Inhibition (mm)				
		Ethanol Extract (1mg/ml)	Aquous Extract (1mg/ml)	Streptomycin (1mg/ml)	Fluconazole (1mg/ml)	DMSO
1	*S. aureus* (MTCC 3160)	20.07	13.64	33	n.p	-
2	*E. coli* (NCIM 20650	25.12	15.23	30	n.p.	-
3	*V. cholera* (MTCC 3906)	19.63	12.54	32	n.p.	-
4	*C. albicans* (MTCC 227)	20.26	11.35	n.p	26	-
5	*A. niger* (NCIM 501)	18.25	9.46	n.p.	23	-

***S. aureus*:** *Staphylococcus aureus*; ***E. coli*:** *Escherichia coli*; ***V. cholera*:** *Vibrio cholera*; ***C. albicans*:** *Candida albicans*; ***A. niger*:** *Aspergillus niger*, **n.p** = Not Performed, **(-)** = no inhibition

Antimicrobial activity of *Leucas cephalotes* Spreng. On different strains of Microorganisms

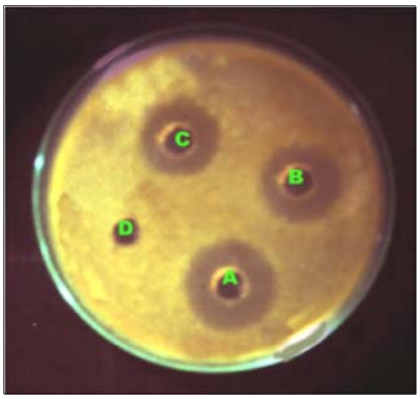


Plate No. 25: Zone of inhibition *Staphylococcus aureus* MTCC 3160

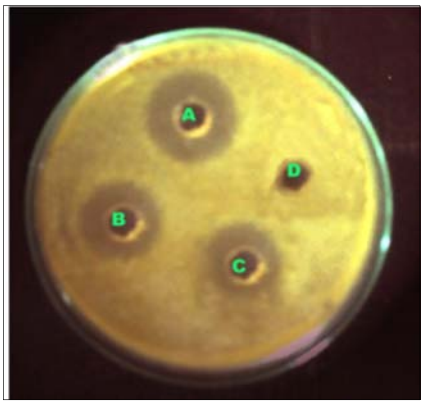


Plate No. 26: Zone of inhibition *Escherichia coli* NCIM 2065

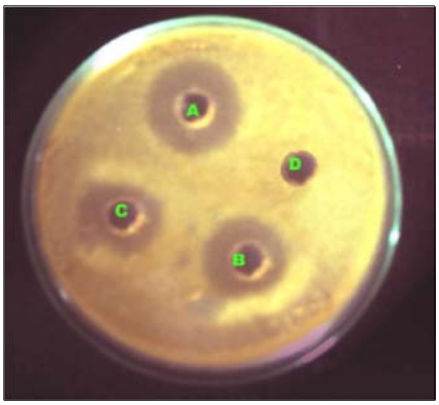
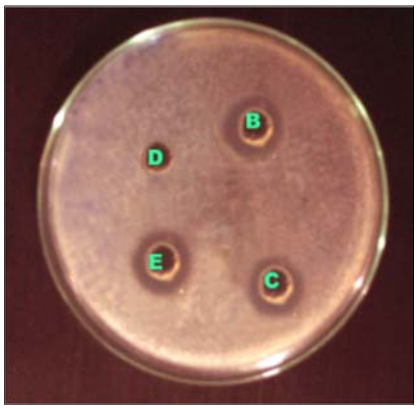
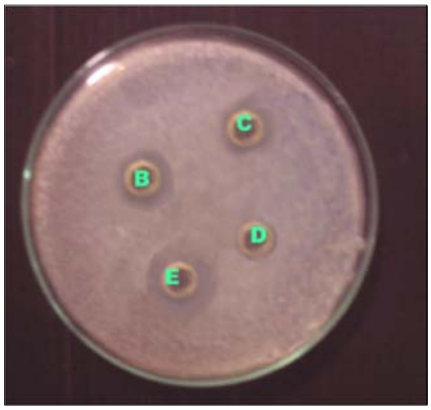


Plate No. 27: Zone of inhibition Vibrio cholera MTCC 3906





A: Streptomycin; B: Ethanolic extract; C: Aqueous extract; D: DMSO; E: Fluconazole

Plate No. 28: Zone of inhibition against Candida albicans MTCC 227

Plate No. 29: Zone of inhibition *Aspergillus niger* NCIM 501

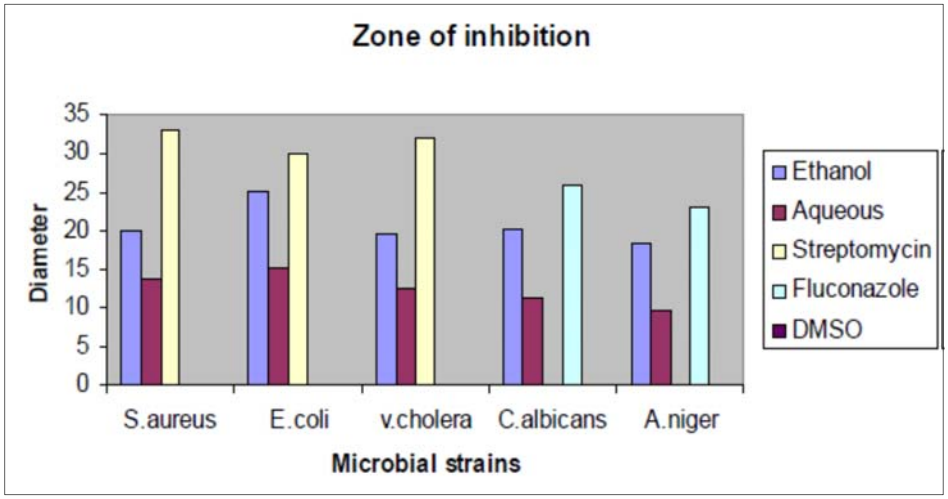
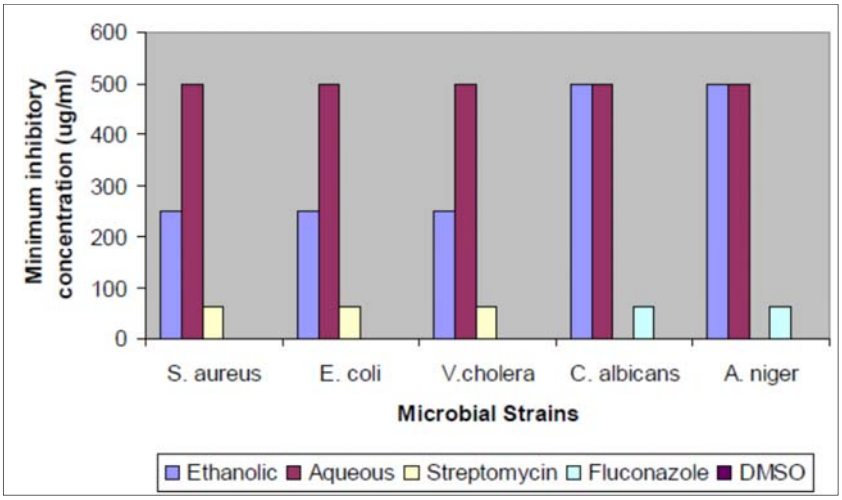


Fig 1: Diagram showing the diameter of the zone of inhibition for the ethanolic and aqueous extracts of *Leucas cephalotes* Spreng

Table 2: Data showing the minimum inhibition concentration for the ethanolic and aqueous extracts of *Leucas cephalotes* Spreng.

S. No.	Name of Organism	Minimum inhibitory concentration (MIC) (µg/ml)				
		Ethanol Extract (1mg/ml)	Aquous Extract (1mg/ml)	Streptomycin (1mg/ml)	Fluconazole (1mg/ml)	DMSO
1	*S. aureus* (MTCC 3160)	250	500	62.5	n.p	-
2	*E. coli* (NCIM 2065	250	500	62.5	n.p.	-
3	*V. cholera* (MTCC 3906)	250	500	62.5	n.p.	-
4	*C. albicans* (MTCC 227)	500	500	n.p	62.5	-
5	*A. niger* (NCIM 501)	500	500	n.p.	62.5	-

***S. aureus*:** *Staphylococcus aureus*; ***E. coli*:** *Escherichia coli*; ***V. cholera*:** *Vibrio cholera*; ***C. albicans*:** *Candida albicans*; ***A. niger*:** *Aspergillus niger*, **n.p** = Not Performed, **(-)** = no inhibition



***S. aureus*:** *Staphylococcus aureus*; ***E. coli*:** *Escherichia coli*; ***V. cholera*:** *Vibrio cholera*; ***C. albicans*:** *Candida albicans*; ***A. niger*:** *Aspergillus niger*

Fig 2: Diagram showing the diameter of the minimum inhibition concentration for the ethanolic and aqueous extracts of *Leucas cephalotes* Spreng.

4. Conclusion

The ethanolic extract showed more activity against *S. aureus* than the aqueous extract. The aqueous extracts of plant also showed activity against all the test strains but zone of inhibition was better for *E. coli* than the other test strains. The ethanolic extract of plant was more effective against *V.cholera* than the aqueous extract in terms of zone of inhibition. Ethanolic extract was also more effective against *C. albicans* and *A. niger* than the aqueous extract of same plant with respective strains. Minimum inhibitory concentration was also tested against the selected strains of micro-organisms. Ethanolic extract showed MIC at 250 µg/ml against *S. aureus*, *E. coli* and *V.cholera* while against *C. albicans* and *A. niger* the MIC was found to be 500 µg/ml. Aqueous extract showed MIC at the dose leveling 500 µg/ml for all the test microbial strains. Therefore it was concluded that the ethanolic extract of the plant *Leucas cephalotes* Spreng. Was more active against all the test strains than the aqueous extract.

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