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Studies on qualitative, quantitative, phytochemical analysis and screening of *in vitro* biological activities of *Leucas indica* (L) *VAR.* Nagalapuramiana

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Abstract
Leucas indica var. nagalapuramiana is a high value medicinal plant of Seshachalam hill range of Eastern Ghats, Andhra Pradesh. The present study was conducted to determine the yield of extract, qualitative, quantitative analysis, antimicrobial activity and antioxidant activity of the crude extracts of *Leucas indica* (L) var. nagalapuramiana. The extracts were prepared by using Hexane, Acetone, Chloroform and Methanol solvents. The yield of extract was calculated for all the four solvents and they are studied for qualitative analysis of phytochemical compounds. The plant extracts were positive for a wide range of bioactive compounds and further they are studied for total phenol and flavonoid contents. The extracts are subjected to test their biological activities by few *in vitro* tests like anti-bacterial activity, anti- fungal activity and anti- oxidant activity. The present study revealed the presence of alkaloids, phenols, flavonoids, steroids, tannins, saponins, and reducing sugars. Quantitative analysis of the total phenols was 105 µgGAE/µg and flavonoids was 62.34 µg Rutin/µg. Antimicrobial activity was found to be moderate and a better antioxidant activity of IC_{50} 365.5 µg was noted in methanol extracts.

Keywords: *Leucas indica* (L) var. nagalapuramiana, Phytochemical Analysis, quantitative analysis, Antimicrobial Properties, antioxidant activity.

1. Introduction
Ethno medicine is the oldest method of curing diseases and Infections. Various plants have been used in different parts of the world to treat human diseases and infections [1, 2, 3]. Plants are used medicinally in different countries and are a source of many potential and powerful drugs [4]. Traditional medicine using plant extracts continues to provide health coverage for over 80% of the world's population, especially in the developing world [5].
India is the largest producer of medicinal herbs and appropriately called as Botanical garden of the world [6]. In India, the herbal remedy is so popular that the Government of India has created a separate department –AYUSH – under the Ministry of Health & Family Welfare. The National Medicinal Plants Board was also established in 2000 by the Govt. of India in order to deal with the herbal medicinal system [7].
Drugs from the plants are easily available, less expensive, safe, and efficient and have less side effects. Natural products of higher plants may possess a new source of antimicrobial agents with possibly novel mechanisms of action [8]. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials [9]. In contrast to synthetic pharmaceuticals based upon single chemicals, many phytomedicines exert their beneficial effects through the additive or synergistic action, eliminating the problematic side effects associated with the predominance of a single xenobiotic compound in the body [10].
The beneficial medicinal effects of plant materials typically result from the combinations of secondary metabolites present in the plant. The therapeutic actions of plants are unique to particular plant species or groups are consistent with this concept as the combinations of secondary products in a particular plant are often taxonomically distinct [11]. Secondary metabolites are synthesized in a specialized cell types and at distinct developmental stages, making their extraction and purification difficult. As a result secondary metabolites that are used commercially as biologically active compounds, are generally high value-low volume products (e.g. -Steroids, Quinines, Alkaloids, Terpenoids and Flavonoids) which are used in drug manufacture by the pharmaceutical industries.

These are generally obtained from plant materials by steam distillation or by extraction with organic or aqueous solvents and the molecular weights are generally less than 2000.

1.1 Plant Characteristics
Leucas indica (L.) R.Br. Ex Vatke. Var. nagalapuramiana.

1.2 Telugu Common Name
Chinna Poola Tummi.

1.3 Description
Erect, herbs, branches - slender, puberulus. Leaves- linear, linear-lanceolate, entire, slightly undulate or serrulate, puberulous on both the sides. Flowers- white, in whorls at the end of the branches. Nutlet- oblong, trigonous, brownish, black.

1.4 Flowering & Fruiting
September –March

1.5 Distribution
Endemic to Chittor district. Occasional on hill slopes among grasses, Srikalahasti, Nagalapuramiana.

1.6 Medicinal Uses
Leaf-Stomachic, dermatitis, sores, swellings, skin diseases, cold, fever, snake bite, scorpion sting.

1.7 Classification

Class	Dicots
SUB-CLASS	Gamopetalae
SERIES	Bicarpellatae
ORDER	Lamiales
FAMILY	Lamiaceae
GENUS	*Leucas*
SPEIES	*Indica*

2. Materials and Methods
2.1 Plant collection
The whole plant was collected from the Seshachalam hills region of Tirupati in Andhra Pradesh, India in the month of June-August 2013.

2.2 Collection of microorganisms
The microorganisms were collected from the Department of Biotechnology, Acharya Nagarjuna University, Guntur and they were reconfirmed by gram staining and sub culturing in appropriate selective media.

2.3 Extraction Procedure
The aerial parts of *Leucas indica* were carefully separated, cleaned, shade dried, mechanically grinded and coarsely powdered. The powder was subjected to solvent extraction with Hexane, Acetone, Methanol, and Water. The Extracts were concentrated by using the Rotary Evaporator and the yield of the Extract was noted with respect to the dried plant material.

2.4 Qualitative Phytochemical Analysis [12, 13, 14, 15]
The Bioactive compounds were analysed by the qualitative tests for the solvent extracts. It was screened for alkaloids, steroidal compounds, flavonoids, saponins, phenolic compounds, tannins, steroids, coumarins and cardiac glycosides by using standard procedures.

2.5 Determination of total phenols
The total phenol content was determined by the Folin Ciocalteu procedure by Skerget *et al*. 2005. Briefly, different concentrations of the extracts were taken to that 0.1 ml of Folin Ciocalteu reagent and 2.5 ml of 0.2 N Na_2CO_3 were added and incubated for 30 min at room temperature. Distilled water was used as blank. Absorbance was measured at 760 nm using Thermo Fisher double beam spectrophotometer. Gallic acid was used as standard and the results were expressed as µg of gallic acid equivalents per gram dry mass of extract (µg GAE/gDM).

2.6 Estimation of total flavonoid content
The total flavonoid content was determined by the aluminium chloride calorimetric assay. In a test tube, 0.3 ml of extracts, 3.4 ml of 30% methanol, 0.15 ml of $NaNO_2$ (0.5 M) and 0.15 ml of $AlCl_3.6H_2O$(0.3M) were mixed. 1 ml of NaOH was added after 5 min. The absorbance was taken at 506 nm against the blank. The standard curve with the reference of rutin standard solution was made. The total flavonoid content was expressed with the rutin equivalents per g of dried fraction.

2.7 Antimicrobial activity
Antimicrobial assay was performed by agar well diffusion method [17]. 50 µl of the plant extracts at a concentration of 100 µg/ml were added to the each well by using the sterile micro pipette and allowed to diffuse at room temperature for 2 Hrs. The respective extracts were maintained and the experiment was repeated thrice, and average values of zone of inhibition were recorded in mm for antimicrobial activity. The 50 µl of antibiotic compound Streptomycin (100 µg/ml) was used as a Standard for the antibacterial study.

2.8 Antioxidant Properties [18]
1, 1- Diphenyl-2-Picrylhydrazyl radical (DPPH) scavenging activity

The free radical scavenging activity of the extract was measured by using 1, 1- Diphenyl-2-Picrylhydrazyl radical (DPPH) as described by Brand- Williams with some modifications. The extracts were prepared at 1 mg/ml concentration with DMSO solution. The mixture was made uniform and 100-500 µg/ml concentrations were made as working solutions. Further 0.004% (W/V) solution of DPPH in methanol was added to the solution. The mixture was shaken and incubated for 60 min in the dark at room temperature. The absorbance was measured at 517 nm against blank. The DPPH scavenging activity (I %) was calculated as follows:

$$I\% = \left[\frac{(Ao - As)}{Ao}\right] \times 100$$

Where Ao is the absorbance of the DPPH solution without sample extract and As is the absorbance of sample with DPPH solution.

3. Results
3.1 Yield of Extract
The extracts (Hexane, Acetone, Methanol and Aqueous extracts) of *Leuca indica* (L) var. nagalapuramiana were prepared by the solvent extraction method. In the present study the yield of crude extract is obtained by measuring its dry

weight. Yield was found to be low in hexane extract due to its low polarity and the yield was found to be more in methanol (28.896%) and aqueous extracts (22.758%). The color of the extract was found to be dark brown in hexane extract, dark green in acetone and methanol extracts and dark red in aqueous extract. The yield of extracts have shown in the table 1 & Plate 1.

Table 1: Physico-Chemical Evaluations

Solvent	Initial Weight of the Powder (gm)	Final Weight of the Powder (gm)	Weight of the Crude Extract (gm)	Crude Extract %	Colour of the Extract
Hexane	50	44.563	5.437	10.874	Dark Brown
Acetone	50	40.415	9.585	19.17	Dark Green
Methanol	50	35.552	14.448	28.896	Dark Green
Water	50	38.621	11.379	22.758	Dark Red

Plate 1: Yield of Extract

3.2 Phytochemical Analysis

The qualitative analysis of bioactive compounds for the four extracts have been analyzed in this study and there is wide range of phytochemical compounds present in the four extracts shown in table 2. The hexane being highly nonpolar in nature was able to extract very less compound characterized like steroids, saponins and reducing sugars. Methanol extract was found to have a wide range of bioactive compounds like alkaloids, phenols, flavonoids, steroids, tannins, saponins and reducing sugars. The Acetone extract was positive for reducing sugars, saponins, tannins, steroids, flavonoids and alkaloids. The aqueous extract was found to have alkaloids, phenolics, flavonoids, steroids, saponins and reducing sugars. The presence of bioactive constituents indicates that the *L. indica* can be used in a multitude of ways for the beneficiary of population.

Table 2: Phytochemical Analysis of Whole Arial Part Extracts of *Leucas indica* (L) var. nagalapuramiana

***Leucas indica* (L) var. nagalapuramiana**					
S. NO	**Tests**	**Hexane Extract**	**Acetone Extract**	**Methanol extract**	**Water extract**
01.	**Alkaloids**				
	Mayers	Negative	Positive	Positive	Positive
	Dragon	Negative	Positive	Negative	Positive
	Wagners	Negative	Positive	Positive	Positive
	Hagers	Negative	Positive	Negative	Positive
02.	**Phenolics**				
	$Fecl_2$ Test	Negative	Positive	Positive	Positive
03.	**Flavanoids**				
	Lead Acetate Test	Negative	Positive	Positive	Positive
	NaOH Test	Negative	Positive	Positive	Negative
	Ethyl acetate Test	Negative	Negative	Negative	Negative
04.	**Anthraquinone Test**				
	Borntrager's Test	Negative	Negative	Negative	Negative
05.	**Steroids**				
	Salkowski's Test	Positive	Positive	Positive	Positive
06.	**Tannins**				
	$Fecl_2$ Test	Negative	Positive	Positive	Negative

	Lead acetate Test	Negative	Positive	Positive	Negative
	Pot. dichromate Test	Negative	Positive	Positive	Negative
07.	**Saponins**				
	Vigorous Shaking Test	Positive	Positive	Positive	Positive
08.	**Anthocyanins**				
	Ammonia-HCl Test	Negative	Negative	Negative	Negative
09.	**Leuco- Anthocyanin**				
	Iso Amyl Alcohol Test	Negative	Negative	Negative	Negative
10.	**Coumarins**				
	NaOH Test	Negative	Negative	Negative	Negative
11.	**Reducing Sugars**				
	Keller-Kiliani Test	Positive	Positive	Positive	Positive

3.3 Phenol & Flavonoid Quantitative Determination

The total phenol content was high in the methanol extract i.e. of 105.68 µgGAE/µg followed by water, acetone and hexane extracts shown in table 3 & plate 2. There is a gradual increase in the phenol content with the increase of concentration of extract. The total flavonoid content of the four extracts at different concentrations was measured and was found that methanol extract was showing good result i.e. 62.34 µg Rutin/µg shown in table 4 & plant 3.

Table 3: Total Phenol content

% of Phenol content µg GAE/µg				
Concentration of extracts (µg/ml)	**Hexane Extract**	**Acetone Extract**	**Methanol Extract**	**Water Extract**
100	15.69	18.5	25.6	22.8
200	22.54	26.9	45.86	41.8
300	30.41	38.9	72.8	68.4
400	42.89	54.78	88.96	76.5
500	54.58	65.8	105.68	95.8

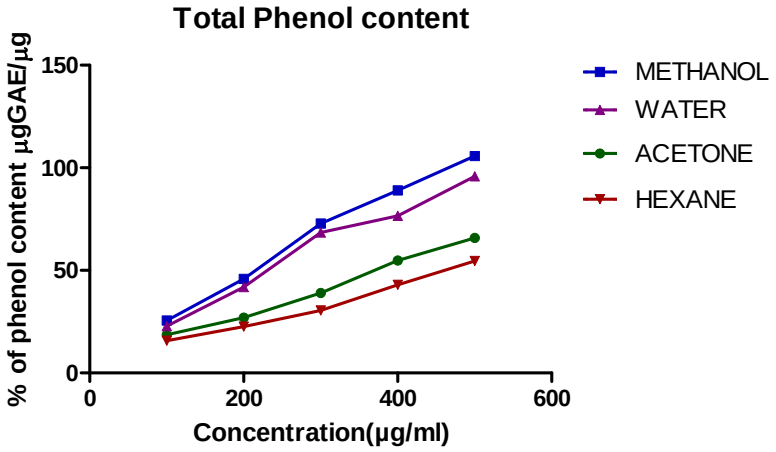

Plate 2: Total Phenol Content

Table 4: Total Flavonoid content

	% of Flavonoid content µg Rutin/µg			
concentration of extracts (µg/ml)	**Hexane Extract**	**Acetone Extract**	**Methanol Extract**	**Water Extract**
100	00	10.28	17.25	00
200	00	15.56	26.35	00
300	00	32.47	29.12	13.20
400	00	41.58	43.52	18.25
500	00	50.24	62.34	24.69

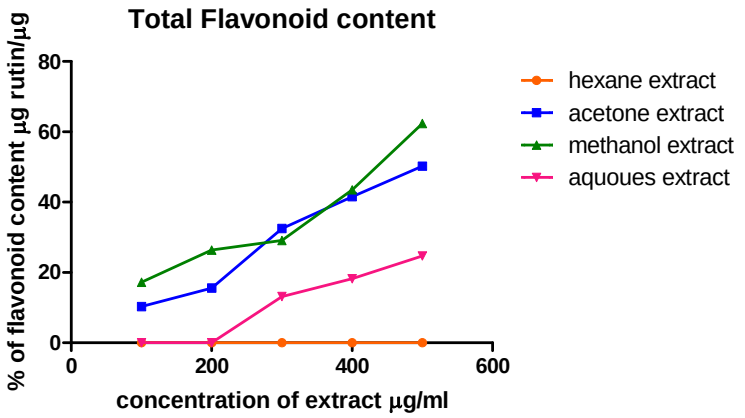

Plate 3: Total Flavonoid Content

3.4 Antimicrobial screening

The antimicrobial activities have been tested against nine bacterial and four fungal strains. Of all the four extracts methanol extract was found to show better antimicrobial activity and the activity was found to be same for both gram positive and gram negative bacteria shown in Plate 4 & 5.

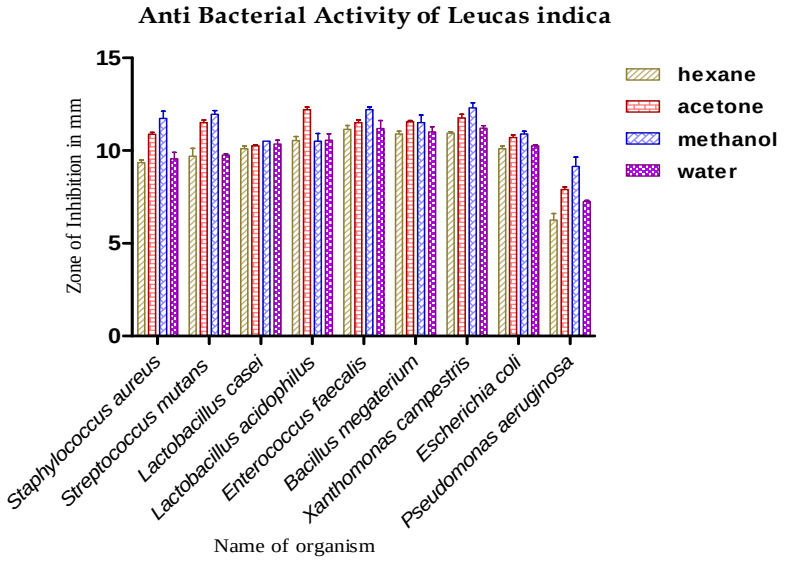

Plate 4: Antibacterial Activity of Whole Arial Part Extracts of *Leucas indica* (L.) var. nagalapuramiana

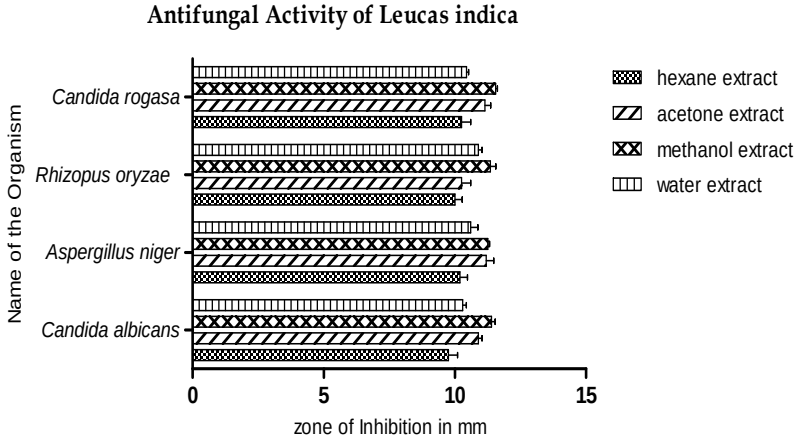

Plate 5: Anti-fungal efficiency of *Leucas indica* (L.) nagalapuramiana

3. 5 Antioxidant Activity

The DPPH antioxidant activity for the four extracts was determined and IC50 values were calculated. The percentage inhibition of scavenging activity was shown in table 5 & plate 6. Different concentrations of the four extracts were analyzed and aqueous extract showed best IC50 value than the other extracts. Hexane and Acetone extracts were found to have least scavenging activity. Highest activity of free radical scavenging was observed at 500 µg/ml concentration.

Table 5: Antioxidant activity of *Leucas indica* (L.) nagalapuramiana DPPH

Conc (µg/ml)	Hexane extract	IC $_{50}$	Acetone extract	IC $_{50}$	Methanol extract	IC $_{50}$	Water extract	IC $_{50}$	Standard % of inhibition (Ascorbic acid)
100	28.2		29.5		37.8		34.5		50.67
200	33.4		35.7		46.3		40.2		53.25
300	41.8	448.25	44.5	396.95	52.3	365.9	46.6	269.8	79.08
400	47.4		50.6		58.8		51.3		83.51
500	52.8		56.7		65.8		58.7		91.26

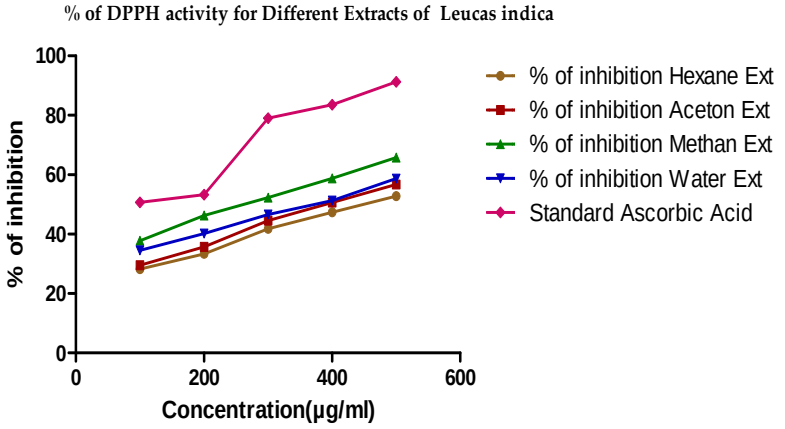

Plate 6: % of DPPH activity for different extracts of *Leucas indica*

4. Discussion and Conclusion

Genus *Leucas* sparsely distributed in Bangladesh and India has a significant role in the Ayurveda medicine. The present study was conducted on qualitative and quantitative phytochemical analysis, and *in vitro* biological activities such as anti-bacterial, anti- fungal and antioxidant activities of *Leucas indica* var. nagalapuramiana.The water and methanol extracts of *L. indica* evaluated in this work has different varieties of phytochemicals that could be considered as responsible for antimicrobial & anti-oxidant activities. Alkaloids are the most significant compounds play a metabolic role in the living systems and are involved in the protective function in animals. Steroidal alkaloids are medicinally evolved. Flavonoids have been used against the cancer causing tumors and it inhibits the promotion of growth and progression of tumors [19]. Phenols when mixed with the flavonoid compounds in plants are reported to show multiple activities like antioxidant, anti-carcinogenic, anti-inflammatory etc [20]. Tannins inhibit the pathogenic fungi and antimicrobial activity of extracts showed better activity by the presence of tannins. Saponins cause the leakage of proteins and degradation of cell wall enzymes from the cell [21].

The plant based compounds have the effective dosage response and minimal side effects when compared to the synthetic compounds. The studies conducted on*Leucas indica* var. nagalapuramiana for *in vitro* biological activities are validated. The presence of most general phytochemicals might be responsible for their therapeutic effects. It further reflects a hope for the development of many more novel chemotherapeutic agents or templates from such plants which in future may serve for the production of synthetically improved therapeutic agents.

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