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INVESTIGATION ON REGIONAL VARIATION IN TOTAL PHENOLIC, ALKALOID CONTENT AND *IN-VITRO* ANTIOXIDANT ACTIVITY OF *LEUCAS ASPERA*

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ABSTRACT

In this study, we investigated and compared the total phenolic, alkaloid content and i*n-vitro* antioxidant activity of *Leucas aspera* collected from four different regions; Tirupathi (Southern zone), Lam (Krishna river region), Jagityala (Northern Telangana) and Hyderabad (Southern Telangana) of Andhra Pradesh, India. Quantitative regional variation was observed in total phenolic content, and alkaloid content in methanolic extracts of *Leucas aspera* from above four regions of Andhra Pradesh. Concentration dependent antioxidant activity was observed for all these extracts and also observed regional variation for scavenging of Superoxide, Hydroxyl and DPPH radicals. Among the four regions, *Leucas aspera* from Jagityala region contains more phenolic content (48.06±0.4µg/100 µg), Tirupathi region contains good alkaloid content (58.6±0.1µg/mg) and Hyderabad zone showed better free radical scavenging activity (IC_{50} value for superoxide radical 156.34µg, Hydroxyl radical 122.34µg and DPPH radical 57.12µg respectively).

INTRODUCTION: *Leucas aspera* (Lamiaceae) is an annual, branched herb, erecting weed to a height of 15-60 cm with stout and hispid acutely quadrangular stem and branches. This weed is distributed throughout India from Himalayas down to Ceylon [1]. This weed is used traditionally as antipyretic and insecticide. Bruised leaves are applied locally in snake bites [2, 3].

The plant contains various phytochemical constituents mainly triterpenoids, Ursolic acid and β-sitosterol, Nicotine, Sterols, glucoside, diterpenes, oleanolic acid, ursolic acid, phenolic compounds [4-(24-hydroxy-1-oxo-5-n-propyltetracosantyl)-phenol] [4-11].

In this investigation, a detailed study was carried out on regional variation in total phenolic and alkaloid contents of the alcoholic extracts of *L. aspera* collected from different regions of Andhra Pradesh like Tirupathi, Lam, Hyderabad and Jagityala, and were screened for *in vitro* free radical scavenging activity of superoxide radical, Hydroxyl radical and DPPH radical.

MATERIAL AND METHODS:

Chemicals: All the chemicals and reagents used were of analytical grade. Folin-Ciocalteu reagent, 1, 1-diphenyl-2-picrylhydrazyl was purchased from Sigma Chemical Company, St. Louis, USA), Riboflavin from Loba Chemie Pvt. Ltd., Bombay, Deoxyribose, Bromo cresol green and Nitroblue tetrozolium were purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai.

Plant Material: The plant material was collected from different climatic zones of Andhra Pradesh i.e.

Tirupathi, Lam, Jagityala and Hyderabad. The Voucher specimens were deposited in the herbarium, College of Pharmaceutical Sciences, Andhra University.

Preparation of Extract: The freshly collected leaves of the plant were shade dried and powdered. The powdered material was then subjected to triple maceration with methanol: water (70:30). The extract thus obtained was concentrated under vacuum at temperature of 43^{o}C by using rotary evaporator (Buchi), dried completely, weighed and stored in desiccators.

Total Phenolic content: Total phenolic content was estimated as GAE (GA equivalents) as described by Singleton, Orthofer and Lamuela-Raventos, 1999 [12]. Briefly, 100µl aliquot of dissolved extract was transferred to 10ml volumetric flask containing 6.0ml ultra pure water, to which was subsequently added 500µl undiluted Folin-Ciocalteu reagent. After 1min, 1.5ml 20g/100ml sodium carbonate was added and the volume was made up to 10ml with ultra pure water. After 30 minutes incubation at 25^{o}C the absorbance was measured at 760nm and compared to GA calibration curve. All experiments were performed thrice; the results were averaged and reported in the form of Mean ± S.E.M (**Table 1**).

Total Alkaloid Content: Total alkaloid content was determined by the Fazel *et al*., 2008 method [13].The plant extract (1mg/ml) was dissolved in 2 N HCl and then filtered. The pH of phosphate buffer solution was adjusted to neutral with 0.1 N NaOH. One ml of this solution was transferred to a separating funnel and then 5 ml of Bromocresol green solution along with 5 ml of phosphate buffer were added.

The mixture was shaken and the complex formed was extracted with chloroform by vigorous shaking. The extracts were collected in a 10 ml volumetric flask and diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm. All experiments were performed thrice. The results were averaged and reported in the form of Mean ± S.E.M. (Table 1).

***In-vitro* Antioxidant Activity**: The alcoholic extracts of *Leucas aspera* from four regions were screened for free radical scavenging activity against Superoxide radical, Hydroxyl and DPPH radicals at different concentrations. The Percentage Inhibition and 50% Inhibition Concentration's (IC_{50}) were calculated. All experiments were performed thrice and the results were averaged.

Superoxide Radical Scavenging Activity: Superoxide radical scavenging activity of the extract was measured according to McCord and Fridovich method, 1969 [14]. It depends on light induced superoxide generation by riboflavin and the corresponding reduction of nitroblue tetrazolium. All the solutions were prepared in phosphate buffer (pH 7.8). The optical density was measured at 560nm.

Hydroxyl Radical Scavenging Activity: Hydroxyl radical scavenging activity was measured according to the method of Elizabeth and Rao, 1990 [15], by studying the competition between deoxyribose and test extract for hydroxyl radicals generated by Fenton's reaction. The damage imposed on deoxyribose due to the free radicals was determined calorimetrically by measuring the thiobarbituric acid reactive substances (TBARS) at 532 nm.

DPPH Radical Scavenging Activity: DPPH radical scavenging activity was measured according to the method of Braca *et al*., 2003 [16]. An aliquot of 3ml of 0.004% DPPH solution in ethanol and 0.1ml of plant extract at various concentrations were mixed and incubated at 37˚C for 30 min. and absorbance of the test mixture was read at 517nm.

Calculation of Percentage Inhibition: The percentage inhibition of superoxide production by the extract was calculated using the formula:

$$\text{Inhibitory ratio} = \frac{(Ao - A_1)}{A_0} \times 100$$

Where, A_0 is the absorbance of control; A_1 is the absorbance with addition of plant extract/ ascorbic acid.

Calculation of 50% Inhibition Concentration: The optical density obtained with each concentration of the extract/ascorbic acid was plotted taking concentration on X-axis and percentage inhibition on Y-axis. The graph was extrapolated to find the 50% inhibition concentration of extract/ ascorbic acid.

Statistical Analysis: Values were expressed as means ± standard deviation. Analysis of variance was conducted and differences between variables were tested for significance by one-way ANOVA and linear regression analysis was used to calculate IC_{50} values. All determinations were done at least in triplicate and all were averaged.

RESULTS AND DISCUSSION:

Total Phenolic content: The alcoholic extracts of *Leucas aspera* collected from different climatic regions of Andhra Pradesh i.e., Tirupathi, Lam, Hyderabad and Jagityala showed variation in Phenolic content (Tirupathi- 3.6±0.1, Lam- 44.5±0.2, Hyderabad-38.2±0.2 and Jagityala-48.06±0.4 mg/gm). Hyderabad region contains more phenolic content compared to other regions (**Table 1**).

Total Alkaloid Content: Regional variation in total alkaloid content also observed in four regions of *leucas aspera* extracts i.e Tirupathi- 58.6±0.1, Lam-24.1±0.3, Hyderabad-28.5±0.2 and Jagityala- 22.4±0.3 mg/gm respectively. Tirupathi region contains more alkaloid content compared to other regions (Table 1).

TABLE 1: REGIONAL VARIATION IN TOTAL PHENOLIC AND ALKALOID CONTENT OF *LEUCAS ASPERA* FROM DIFFERENT REGIONS

Region	Total phenolic content (µg/100µg)	Total alkaloid content (µg/mg)
Tirupathi	3.6±0.1	58.6±0.1
Lam	44.5±0.2	24.1±0.3
Hyderabad	38.2±0.25	28.5±0.2
Jagityala	48.06±0.4	22.4±0.3

Superoxide Radical Scavenging Activity: Superoxide anion plays an important role in the formation of more reactive species such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induce oxidative damage in lipids, proteins, and DNA. Therefore, studying the scavenging activity of plant extracts/compounds on superoxide radical is one of the most important ways of clarifying the mechanism of antioxidant activity. The extracts of *Leucas aspera* produced concentration dependent inhibition of superoxide anion i.e. IC_{50} value of *L. aspera* from Tirupathi region-670.25, Lam-540.12, Hyderabad-156.34 and Jagityala- 380.24µg/0.1ml respectively (**Table 2, Table 5, Fig. 1 and Fig. 4**). *Leucas aspera* from Hyderabad region showed better inhibition of superoxide radicals.

TABLE 2: ***IN-VITRO*** **CONCENTRATION DEPENDENT PERCENTAGE INHIBITION OF SUPEROXIDE RADICAL BY ALCOHOLIC EXTRACTS OF *LEUCAS ASPERA* AND ASCORBIC ACID**

Leucas aspera extract Region Wise	Percentage inhibition of Superoxide radical						
	Quantity of extracts/ascorbic acid (µg/0.1 ml)						
	50	100	250	500	750	1000	2000
Tirupathi region	12.42±2.1	20.42±1.5	32.19±2.2	41.18±2.3	54.25±2.6	76.63±2.4	78.27±3.4
Lam region	11.44±1.3	20.59±2.3	32.19±2.5	48.20±2.4	62.42±3.2	71.24±2.3	73.20±2.3
Hyderabad region	28.10±1.6	46.90±1.7	54.25±2.1	65.20±2.4	73.20±3.2	82.35±1.5	85.62±3.2
Jagityala region	16.34±2.1	29.90±2.2	43.30±1.5	56.37±2.3	68.46±2.6	71.24±2.1	75.65±3.2
Ascorbic acid	43.17±0.7	52.41±0.2	61.10±0.2	75.31±1.3	81.52±1.6	86.48±0.5	87.17±1.4

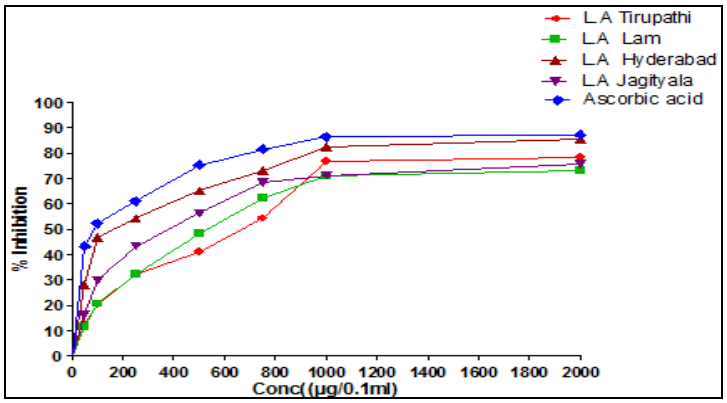

FIG. 1: *IN-VITRO* CONCENTRATION DEPENDENT PERCENTAGE INHIBITION OF SUPEROXIDE RADICAL BY ALCOHOLIC EXTRACTS OF *LEUCAS ASPERA* (LA) AND ASCORBIC ACID

Hydroxyl Radical Scavenging Activity: A single hydroxyl radical can result in formation of many molecules of lipid hydroperoxides in the cell membrane, which may severely, disrupts its function, and lead to cell death. The extracts of *Leucas aspera* from four regions produced concentration dependent inhibition of hydroxyl radical i.e. IC_{50} value of *L. aspera* from Tirupathi region-304.25, Lam-210.26, Hyderabad-122.34 and Jagityala-252.12 µg/ml respectively (**Table 3, Table 5, Fig. 2 and Fig. 4**). *Leucas aspera* from Hyderabad region showed better inhibition of hydroxyl radicals.

TABLE 3: *IN-VITRO* CONCENTRATION DEPENDENT PERCENTAGE INHIBITION OF HYDROXYL RADICAL BY ALCOHOLIC EXTRACTS OF *LEUCAS ASPERA* AND ASCORBIC ACID

Leucas aspera extract Region Wise	Percentage inhibition of Hydroxyl radical Quantity of extracts/ ascorbic acid (µg/0.1 ml)						
	50	100	250	500	750	1000	2000
Tirupathi region	25.46±1.2	31.19±2.2	47.25±2.1	60.09±2.2	68.35±3.2	80.28±2.2	82.34±2.5
Lam region	21.10±0.2	39.68±2.3	53.21±2.5	65.37±2.3	75.69±3.2	82.34±2.4	85.32±3.2
Hyderabad region	27.52±2.1	47.02±2.3	61.24±2.1	69.27±1.5	83.03±1.6	86.47±2.6	87.16±2.4
Jagityala region	19.72±1.6	30.28±2.2	49.77±2.5	64.22±2.4	73.17±1.5	84.63±2.4	85.09±2.7
Ascorbic acid	31.67±1.2	40.30±1.2	55.61±1.1	72.27±2.1	81.52±1.6	84.70±1.6	84.85±3.2

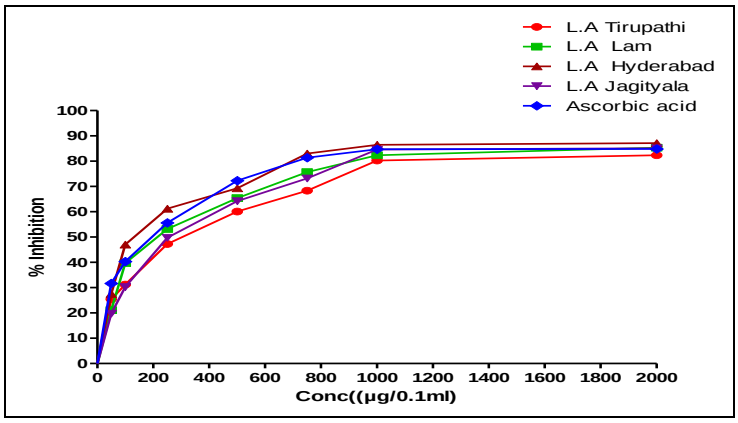

FIG. 2: *IN-VITRO* CONCENTRATION DEPENDENT PERCENTAGE INHIBITION OF HYDROXYL RADICAL BY ALCOHOLIC EXTRACTS OF *LEUCAS ASPERA* (LA) AND ASCORBIC ACID

DPPH Radical Scavenging Activity: All extracts from four regions showed better inhibition of DPPH radicals (IC_{50} value of *L. aspera* from Tirupathi region-280.34, Lam-55.16, Hyderabad-57.12 and Jagityala-240.32 µg/ml respectively) and this is concentration dependent (**Table 4, Table 5, Fig. 3 and Fig. 4**). *Leucas aspera* from Lam, Hyderbad regions showed better inhibition of DPPH radicals. Preliminary phytochemical screening of the alcoholic extracts of *L. aspera* showed the presence of alkaloids, saponins, carbohydrates, phytosterols, terpenoids and flavonoids. Natural antioxidants such as plantphenols, flavonoids and tannins possess potent antioxidant activity [17]. Sterols like β-sitosterol [18], terpenoids [19], oleanolic acid and ursolic acid [20] were reported to possess antioxidant activity. However, these active constituents alone or in combination may be responsible for the observed antioxidant activity.

TABLE 4: *IN-VITRO* CONCENTRATION DEPENDENT PERCENTAGE INHIBITION OF DPPH RADICAL BY ALCOHOLIC EXTRACTS OF *LEUCAS ASPERA* AND ASCORBIC ACID

Leucas aspera extract Region Wise	Percentage inhibition of DPPH radical Quantity of extracts/ ascorbic acid (µg/0.1 ml)						
	50	100	250	500	750	1000	2000
Tirupathi region	17.81 ±0.2	24.43±2.1	48.31±1.2	61.27±1.4	68.42±2.1	76.38±2.2	78.41±2.1
Lam region	48.58±0.1	58.57±1.2	65.18±2.1	72.20±2.2	76.38±2.1	81.24±2.4	84.62±2.4
Hyderabad region	48.31±1.2	56.28 ±1.2	65.45±2.2	70.45±2.4	76.38±2.5	85.70±3.1	86.37±2.4
Jagityala region	20.65±1.3	41.57±1.5	51.15±2.2	65.72±2.2	72.33±2.1	83.67±3.2	84.21±2.2
Ascorbic acid	45.30±2.1	75.61±2.1	81.82±2.4	86.52±2.2	88.18±2.1	90.15±2.2	90.45±3.4

TABLE 5: *IN VITRO* 50% INHIBITION CONCENTRATION (IC_{50}) OF ALCOHOLIC EXTRACTS OF *LEUCAS ASPERA* AND ASCORBIC ACID ON FREE RADICALS SCAVENGING ACTIVITY

Leucas aspera extract Region Wise	Quantity of various extracts (µg) Free radicals, reactive oxygen species		
	Superoxide radical	Hydroxyl radical	DPPH radical
Tirupathi region	670.25	304.25	280.34
Lam region	540.12	210.26	55.16
Hyderabad region	156.34	122.34	57.12
Jagityala region	380.24	252.12	240.32
Ascorbic acid	80.24	190.20	60.24

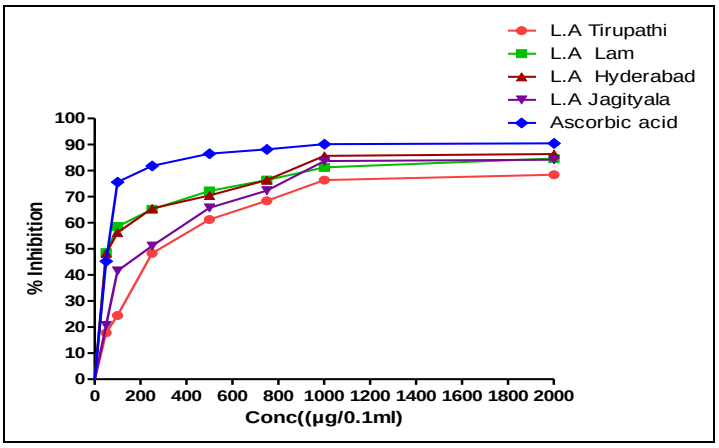

FIG 3: *IN-VITRO* CONCENTRATION DEPENDENT PERCENTAGE INHIBITION OF DPPH RADICAL BY ALCOHOLIC EXTRACTS OF *LEUCAS ASPERA* (LA) AND ASCORBIC ACID

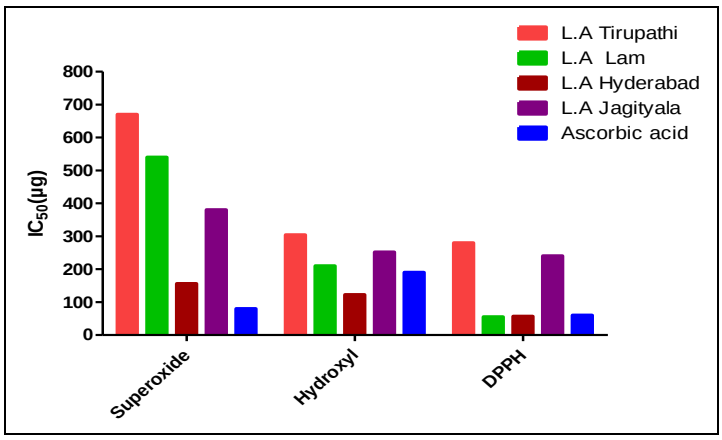

FIG 4: *IN VITRO* 50% INHIBITION CONCENTRATIONS (IC_{50}) FOR SUPEROXIDE, HYDROXYL AND DPPH RADICAL BY ALCOHOLIC EXTRACTS OF *LEUCAS ASPERA* (LA) AND ASCORBIC ACID

CONCLUSION: In present study, we found clear regional variability in total phenolic and alkaloid content. Among the four regions i.e. Tirupathi, Lam, Hyderabad and Jagityala, *Leucas aspera* from Jagityala region contains more phenolic content; Tirupathi region contains good alkaloid content.

The results of *in-vitro* antioxidant activity of alcoholic extracts of *L. aspera* collected from different regions clearly showed regional variation in free radical scavenging activity and also produced dose dependent inhibition of free radical generation of superoxide anion, hydroxyl radical and DPPH radicals which were compared to standard antioxidant drug, ascorbic acid**.** Hyderabad region showed better superoxide and hydroxyl radical scavenging activity and Lam and Hyderabad region showed better inhibition of DPPH radicals. Investigation on regional variation of biological activities for these extracts is in progress.

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