Research Article

ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITY OF *LEUCAS ASPERA* L

Archana Borah[1*], Raj Narayan Singh Yadav[1], Bala Gopalan Unni[2]
1. Department of Life sciences, Dibrugarh University. Dibrugarh-786004. Assam, India.
2. Biotechnology Department (Biochemistry and Molecular Biology Laboratory), North East Institute of Science &Technology, Jorhat, Assam, India.
*Corresponding author's E-mail: archana_jorhat@yahoo.com

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ABSTRACT

During the course of metabolism several reactive oxygen species are formed which causes oxidative stress. Plants are the natural source of phytochemicals which can prevent the oxidative stress. Antioxidant property of *Leucas aspera* was assayed by phosphomolybdate method and was found to be high in ethanolic extract (19.588mM of ascorbic acid/g of sample) followed by methanol and ethanol extract. Free radical scavenging activity was measured by several *in vitro* standard methods. Total phenolic and flavonoid content was found to be 80.249 mg/g dry wt. and 0.927 mg/g dry wt. respectively. Glutathione content was estimated to be 6022.972 µM/g fresh wt. Antioxidant vitamins were also estimated and contained vitamin C content (0.084 mg/g fresh wt.) and vitamin E (645.69 mg/g fresh wt.). The study shows that the *L.aspera* is a promising source of antioxidant.

Keywords: Antioxidant activity, Glutathione, Phenolics, Flavonoids.

INTRODUCTION

Oxidative stress is the state resulting from the imbalance in the level of pro oxidant and antioxidant. Oxidative stress is initiated by free radicals, which seek stability through electron pairing with biological macromolecules such as protein and DNA in healthy human cells and cause protein and DNA damage along with lipid peroxidation. These changes contribute to cancer, atherosclerosis, cardiovascular diseases, ageing and inflammatory diseases[1,2]. Epidemiological studies have strongly suggested that consumption of certain plant materials may reduce the risk of chronic diseases related to oxidative stress on account of their antioxidant activity and promote general health benefits[3]. Leafty vegetables apart from being a good source of minerals also contain antioxidant vitamins and pigments. They are also known for their therapeutic value[4]. Recently much attention has been made on the use of plants as a source medicine with strong antioxidant properties.

Leucas aspera is a small herbaceous plant, erect plant belonging to the family Lamiaceae. It grows as a weed on wastelands and road side all over India. The plant is used as an insecticide and indicated in traditional medicine for coughs, colds, painful swellings and chronic skin eruptions[5].

In the present study an attempt has been made to evaluate the antioxidant and radical scavenging activity of *L.aspera* by different *in vitro* model.

MATERIALS AND METHODS

Plant material: Fresh mature leaves and stem of *Leucas aspera* were collected during the month of March and April from Dibrugarh, Assam.

Preparation of plant extract: The leaves and stem of *Leucas aspera* was dried at room temperature, powdered and used for extraction. 1 gm of powder extracted with 10 ml of 90% methanol, 70% acetone and 80% ethanol for 12 hours with occasional shaking. The supernatant was collected and concentrated under reduced pressure in a rotary evaporator. All extracts were kept in a refrigerator until use.

Total antioxidant activity by phosphomolybdenum method

The antioxidant activity is based on the reduction of Mo (VI) to Mo (V) by the test sample and the subsequent formation of green phosphate/Mo (V) complex at acidic P^{H}.[6] An aliquot of 0.1 ml sample was mixed with 1 ml of reagent solution (0.6 M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes were incubated at 95ºC for 90 min and cooled at room temperature. The absorbance was measured at 695 nm against blank. The antioxidant activities of samples were expressed as mM of ascorbic acid/g of sample.

DPPH radical scavenging assay

The radical scavenging activity of Leucas extracts and TROLOX was measured by DPPH method[7]. 1 ml of 0.135mM DPPH solution in methanol was mixed with 1 ml of extract. The reaction mixture was vortexed and left in the dark at room temperature for 30 min. The absorbance was measured at 517 nm. A reaction mixture without test sample was served as control. The ability to scavenge DPPH radical was calculated by the following equation:

% scavenging activity = Control Abs – Test Abs / Control Abs* 100

Ferrous ion chelating activity

Ferrous ion chelating activity was assayed by the standard method[8]. The assay is based on the principle of the Fe^{2+} chelating ability of the test samples by measuring the ferrous iron-ferrozine complex formed at 562 nm. To

different concentration of sample extracts were added 0.1 ml of 2mM ferrous chloride, 0.2 ml of 5mM ferrozine and 3.7 ml of methanol. The solution was allowed to react for 10 min. The absorbance at 562 nm was measured against blank. The percentage of ferrous ion chelating activity was calculated as follows:

% ferrous ion chelating activity = Control Abs – Test Abs / Control Abs * 100

Superoxide scavenging activity

Superoxide scavenging activity was based on the inhibitory action of superoxide dismutase on the rate of base catalyzed auto-oxidation of pyrogallol[9]. The assay medium contained 1 ml of different concentration of test sample, 2ml of water, 3ml of 0.05M Tris buffer, P^H 8.2, and the reaction was started by addition of 0.02 ml pyrogallol (60mM) and recorded during 1 min at 420 nm. The percentage of superoxide scavenging activity was calculated as follows:

% scavenging activity = Control Abs – Test Abs / Control Abs * 100

Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity was determined by spectrophotometric method[10]. Test samples of different concentrations were dissolved in DMSO. To the 1 ml of test solution 1 ml of sodium nitroprusside (5mM) in phosphate buffer saline was mixed and incubated for 30 min at 25ºC. Assay medium without test solution served as control. After 30 min 1 ml of incubated solution was taken out and equal amount of Griess reagent was added. The absorbance of the chromophore formed during the diazotization of the nitrile with sulphanilamide and the subsequent coupling with naphthyethylene diamine dihydrochloride was measured at 546 nm. The percentage scavenging activity was calculated as follows:

% scavenging activity = Control Abs – Test Abs / Control Abs * 100

Determination of total phenolic compounds

Preparation of crude phenolic extracts. The soluble crude polyphenols were isolated by standard method[11]. The air dried grounded sample of *L.aspera* (1 gm) was extracted six times with a 20 ml mixture of acetone/methanol/water (7:7:6 by vol.) at room temperature. After each centrifugation, the supernatant were collected, combined and evaporated to near dryness. This residue was dissolved in 25 ml methanol. The methanolic extract was referred to as Extract A.

Isolation of phenolic acids: 10 ml of extract A was evaporated to dryness and the residue was suspended in double distilled water and treated with 30 ml of 4 M NaOH. The resulting hydrolyzed solution was acidified to pH 2 with 6 M HCl and extracted six times with diethyl ether (1:1, vol/vol). The diethyl ether extracts were combined and evaporate to dryness. The residue containing phenolic acids, both liberated form esters was dissolved in methanol. The methanolic extract was referred to as Extract B.

Quantification of phenolics: Total content of phenolics in extract A and extract B was estimated by Folin-Ciocalteu's method[12]. 0.5 ml of sample extract was taken and final volume was adjusted to 3 ml by addition of distilled water. 0.5 ml of Folin-Ciocalteu's (50% v/v) was added to the reaction mixture. After 5 min incubation at room temperature 2 ml of 20% sodium carbonate (w/v) was added. After 3 min incubation absorbance at 760 nm was taken along with blank. Results were expressed as gallic acid equivalent per gram of dried sample.

Determination of Flavonoids

The amount of flavonoids was determined by spectrophotometric method[13]. 1 ml of plant extract was mixed with 1 ml of 2% aluminum trichloride in ethanol. The mixture was diluted with ethanol to 25 ml and allowed to stand for 40 min at 20ºC and the absorbance was measured at 415nm against the sample blank. The results were expressed as rutin equivalent per gram of dried sample.

Determination of glutathione

Glutathione was extracted by grinding 0.5g of plant tissues in 1% picric acid (w/v) under cold condition. After centrifugation at 10,000g for 10 min, the supernatant were collected immediately for assay. Glutathione was estimated using Ellman's reagent[14]. Briefly, to 0.1 ml sample was added 2ml of 0.1M sodium phosphate buffer (pH 8.0) followed by 0.2ml of freshly prepared 5,5-Dithiobis,2-nitrobenzoic acid (DTNB) solution (0.6 mM in 0.1M phosphate buffer, pH 8.0). After 10 min absorbance was measured at 412nm spectrophotometrically.

Estimation of vitamin C

The total vitamin content of *L.aspera* was estimated using Folin phenol reagent[15]. 0.5g of plant tissues was ground in oxalis acid. It was then centrifuged at 5000 rpm for 5min. 0.2-0.5ml supernatant was diluted to 2ml with distilled water and after that 0.2ml of diluted Folin reagent was added to the extract. The tubes were vigorously shaken. After 10 min absorbance was measured at 760nm.

Estimation of vitamin E

The vitamin E was estimated by phosphomolybdate method[6]. 0.5g of plant was extracted in hexane. It was then centrifuged. 0.2ml supernatant was mixed with 2ml of reagent solution (0.6M sulphuric acid, 28mM sodium phosphate, and 4mM ammonium molybdate) and incubated at 37ºC for 90 min with vigorous shaking. Absorbance of the aqueous phase at 695 nm was measured against appropriate blank.

Statistical analysis

The analyses were performed in triplicate. The experimental results obtained were expressed as mean ± S.D. Statistical analysis was performed using minitab 15. Data were analyzed by ANOVA ($p > 0.05$) followed by

Tukey's test. The IC_{50} values were calculated by linear regression and were compared by t-test, $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

The results of the total antioxidant activity of *L.aspera* in different solvent extract by phosphomolybdate method are presented in Table 1. The phosphomolybdate method is routinely applied to evaluate the total antioxidant capacity of plant extracts[16]. The antioxidant activity was found to be high in ethanol extract (19.588 mM of ascorbic acid/g of sample). ANOVA shows the significant differences in the antioxidant activity of *L.aspera* in methanol and acetone extract; acetone and ethanol extract but there is no significance difference in ethanol and methanol extract.

Free radical scavenging activity of *L.aspera* was measured by DPPH method. The results are presented in Table 2. The highest activity was found in ethanol extract (IC_{50} 35.335µg/ml) which is followed by acetone and methanol (IC_{50} 119.237µg/ml, and 157.765µg/ml respectively). The t-test analyses showed that there is significant difference among the DPPH radical scavenging activity of different extracts and standard trolox.

The superoxide radical scavenging activities of different solvent extracts of *L.aspera* were determined using a base catalyzed pyrogallol auto- oxidation. Table 2 shows that the methanol extract has high superoxide radical scavenging activity (IC_{50} 1887.132µg/ml). The IC_{50} value for acetone and ethanol was 2232.228µg/ml and 4897.645µg/ml respectively. The activities were significantly less than the standard rutin.

Ntiric oxide is a diffusible free radical which plays many roles as an effectors molecule in diverse biological systems including neuronal messenger, vasodilatation, antimicrobial and anti tumor activities[17]. Although nitric oxide and superoxide radicals are involved in host defense, overproduction of these two radicals contributes to the pathogenesis of some inflammatory diseases [18]. Ethanol extract exhibited higher nitric oxide scavenging activity (IC_{50} 3460.854µg/ml) (Table 2). This is followed by acetone and methanol (IC_{50} 3660.755µg/ml and 5456.497µg/ml respectively). The t-test revealed that there is significant difference between the mean of rutin and methanol extract

Iron is capable of generating free radicals from peroxides by Fenton reactions, and minimization of the Fe^{2+} concentration in the Fenton reaction affords protection against oxidative damage[19]. The addition of different plant extracts interferes with the ferrous ferrozine complex and the formation of the red coloured complex decreased with the increasing concentration. Table 2. Shows that the acetone extract has the highest iron chelating activity (IC_{50} 146.425µg/ml). All the extract showed significantly higher activity than the standard rutin.

Table 3 shows the various antioxidant components of *L.aspera*. The total crude phenolics and phenolic acid was found to be 80.249mg/g dry wt. and 2.394mg/g dry wt. respectively. Total Flavonoids content was 0.927 mg/g dry wt. Glutathione content was found to be 6022.972µM/g fresh wt. Glutathione constitute an important source of non protein thiols both in animal and plant cells and it has the crucial function of cell defense and antioxidizing protection. This tri peptide is part of the ascorbate-gluthathione cycle that helps to prevent or minimize damage caused by reactive oxygen species[20].

The antioxidant vitamin content of *L.aspera* is presented in Table 4. The vitamin C and E content was found to be 0.084mg/g fresh wt. and 645.69mg/g dry wt. These two vitamins are the natural source of antioxidant which protects the body from free radical damage.

Table 1: Antioxidant activity of different solvent extracts of *L.aspera* by Phosphomolybdate method (values are mean ± S.D)

Solvent extracts	Methanol	Ethanol	Acetone
Total antioxidant activity (mM of ascorbic acid/g of sample)	19.446 ± 0.049	19.588 ± 0.835	7.14 ± 0.279

Table 2: Radical scavenging and iron chelating activity (IC_{50} values) of different solvent extract of *L.aspera* and reference compounds (Each value represents mean ± S.D. *p < 0.05.)

Activity	Extracts/Reference	IC_{50} (µg/ml)
DPPH scavenging activity	Methanol	157.23 ± 0.768*
	Acetone	119.237 ± 2.671*
	Ethanol	35.335 ± 11.100*
	Trolox	2.42 ± 0.005
Iron chelating activity	Methanol	684.400 ± 73.410*
	Acetone	146.425 ± 23.39*
	Ethanol	1257.237 ± 53.369*
	Rutin	25275.95 ± 175.61
Superoxide scavenging activity	Methanol	1887.132 ± 263.287*
	Acetone	2232.228 ± 171.891*
	Ethanol	4897.645 ± 149.707*
	Rutin	510.85 ± 22.47
Nitric oxide scavenging activity	Methanol	5456.497 ± 298.749*
	Acetone	3660.755 ± 479.925
	Ethanol	3460.854 ± 301.678
	Rutin	3592.31 ± 319.65

Table 3: Antioxidant components of *L.aspera* Values are expressed as mean ± S.D for five determinations.

Sample	Total crude phenolics (mg/g dry wt.)	Total phenolic acid (mg/g dry wt.)	Total Flavonoids (mg/g dry wt.)	Glutathione (µM/g fresh wt.)
L.aspera	80.249 ± 10.77	2.394 ± 0.36	0.927 ± 0.02	6022.972± 849.01

Table 4: Antioxidant vitamin content of *L.aspera* Values are expressed as mean ± S.D for three determinations

Sample	Vitamin C (mg/g fresh wt.)	Vitamin E (mg/g fresh wt.)
L.aspera	0.084 ± 0.001	645.69 ± 107.554

CONCLUSION

The above results show that the *L.aspera* exhibits antioxidant activity. The various *in vitro* model shows that this plant is a significant source of natural antioxidant to combats oxidative stresses. However, the active principle responsible for antioxidative activity is yet to be investigated.

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