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Free radical scavenging and elastase inhibitory activity of different extracts of *Leucas aspera* (Willd.) Link- An *in vitro* study

Asha Gangadharan[1]* and Benny P J[2]

[1]Department of Biochemistry, Mar Athanasius College, Kothamangalam, Eranakulam Dist, Kerala 686691, India
[2]Department of Chemistry, St.Thomas College, Pala, Kottayam Dist. Kerala 686574, India

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Leucas aspera (Willd.) Link has been used for curing various ailments including skin disease in traditional medicine for decades. The aim of the study was to evaluate the *in vitro* antioxidant potential and anti-elastase activity of *L. aspera* (Willd.) Link. The hexane, ethyl acetate, acetone and ethanol extracts were prepared using the Soxhlet extraction method. The phytochemical analysis, *in vitro* free radical scavenging, and anti-elastase activities, were conducted. The total phenol and flavonoids compounds were found to be significantly high for the hexane extract and the ethyl acetate extract respectively. *In vitro* antioxidant assays like percentage reducing power, DPPH radical scavenging activity, the total antioxidant capacity by FRAP assay, revealed that the acetone extract of the plant *L. aspera* possesses significantly the highest activity. The anti elastase assay revealed that all the extracts possess elastase inhibitory activity and the hexane extract possessed significantly highest activity with IC_{50} of 247.42 µg/mL, at a significant level (α) $\leq$0.05. The present result supports the traditional use of *L. aspera*.

Keywords: Anti-elastase activity, Antioxidant activity, *Leucas aspera* (Willd.) Link, Phytochemical analysis.
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Introduction

Elastase is an enzyme capable of degrading extracellular matrix proteins which provides elasticity to connective tissues and has important role in many natural physiological processes. They take part in complete tissue recovery after healing of the wound by degrading the damaged tissue at the wound area. Under normal conditions, the levels of human neutrophil elastase (HNE) are controlled by endogenous factors. However, in chronic non-healing wounds, elevated levels of elastase may degrade growth factors and connective tissue proteins present in the surrounding area. An excessive and uncontrolled elastase activity is also associated with other major inflammatory diseases including rheumatoid arthritis, chronic obstructive pulmonary diseases, cystic fibrosis, ischemic reperfusion, injury, atherosclerosis, psoriasis, and malignant tumors[1]. Elastase inhibitors can be used therapeutically as a measure to restore the normal levels of elastase in the above diseases[2]. It may also be used in the healing of chronic wounds, anti-ageing and ant wrinkle treatments[3]. Due to these various applications, there has been considerable interest in the design of such elastase inhibitors[1].

*Correspondent author
Email: asharaj81@gmail.com

The production of free radicals may lead to the destruction of the extracellular matrix, which became an initiation factor for ageing and various diseases. Antioxidant agents can preserve tissues viable, delay ageing and also facilitate healing of wounds[4]. Reactive oxygen species are deleterious to skin ageing due to their harmful effects on cells and tissues. Topical application of compounds with free radical scavenging properties, significantly protect tissues from oxidative damage[5]. Thus antioxidant compounds with anti elastase property could be used as a cosmetic ingredient to relieve skin ageing[3]. The interest in discovering novel natural cosmetic agents from medicinal plants is increasing among modern researchers as they are safe and easily available without any side effects.

Medicinal plants are immensely rich sources of new pharmaceuticals, cosmetics and other economically important chemicals. Presently numerous pure compounds are isolated from plants for many therapeutic purposes. The plant *L. aspera* (Willd.) Link is a tropical plant mainly used for many diseases in traditional systems of medicine. *L. aspera* belongs to the family Labiatae, commonly known as "Thumbai", which is distributed throughout India from the Himalayas down to Ceylon[6]. It is also known as

Dronapushpi and Gumma Bhaji. It is an annual herb found as a weed in cultivated fields, wastelands and roadsides. The plant has been used traditionally to cure boils, ulcers, bleeding piles, colds, colic, ear-aches, headaches and stomach aches and also for infectious diseases like fever, chronic cough, etc. The juice of the leaves is used to treat psoriasis, chronic skin eruptions and chronic rheumatism[7]. The plant is known to possess several medicinal properties[8,9] such as anticaterogenic[10], hepatoprotective[11], antidiabetic[12], antimicrobial[13], larvicidal[14], insecticidal[15], wound healing[16], anti-inflammatory[17], antidiabetic activity[18] etc. Since *L. aspera* has long been used as a traditional remedy for wound healing and skin diseases, the aim of the present study was to evaluate the antioxidant and anti elastase activity of different extracts of *L. aspera*.

Materials and Methods

Plant collection

The shoot of *L. aspera* was collected from Aromatic and Medicinal Plant Research Station, Kerala Agricutural University, Odackali, Perumbavoor, Eranakulam, Kerala and was identified by Dr Jomy Augustine, Head, Department of Botany, St.Thomas College, Pala, Kottayam, Kerala. Voucher specimen (Acc. No. 003401) was deposited in the Herbarium of Department of Botany, St.Thomas College, Pala, Kottayam, Kerala, for future reference. Fresh plant materials were first washed under running tap water, then washed with distilled water, and finally were dried in shade at room temperature. Dried leaves were ground into a fine powder and stored in an airtight bottle.

Preparation of plant extracts

The different plant extracts were prepared by soxhlet extraction method using solvents of increasing order of polarity for 72 hours successively until the extract became colourless or till the extraction was completed. The solvents used were hexane, acetone, ethyl acetate, and ethanol. The extracts were concentrated to dryness by vaccum rotary evaporator and the yield of the extracts was determined.

Qualitative phytochemical screening

The phytochemical screening of different extracts of the plant was performed[19,20].

Quatitative phytochemical analysis

Total phenolic compounds

The amounts of phenol content in the extracts were determined by the Folins Ciocalteu method[21]. The absorbance was measured at 765 nm using the Biorad iMark microplate reader. The results were expressed as mg gallic acid equivalents per g of plant extracts (mean±SD) using linear regression analysis.

Total flavonoid content

The flavonoid content of different plant extracts was determined by the aluminium trichloride method[22] using iMark Bio Rad microplate reader. The quantity of flavonoids was expressed as mean±SD of mg in rutin equivalents per g of plant extracts.

In vitro antioxidant activities

Assay of reducing power

Total reducing power was estimated by the method described by the previous reports[23]. Optical density was measured at 700 nm by using Perkin Elmer UV/Vis Double beam Spectrophotometer and was expressed as ascorbic acid equivalents.

$$\text{Percentage reducing power} = \frac{(\text{Absorbance of test solution} - \text{Absorbance of blank})}{\text{Absorbance of blank}} \times 100$$

In vitro DPPH free radial scavenging assay

The *in vitro* free radical scavenging ability of various plant extracts was determined by using α, α-Diphenyl-β-Picryl-Hydrazyl (DPPH) scavenging assay[24] by using iMark BioRad microplate reader. The different plant extracts (20 mg/mL) and standard ascorbic acid, were used. Methanol and DPPH was taken as blank

$$\text{Percentage inhibition} = \frac{(\text{Absorbance of blank} - \text{Absorbance of extract})}{\text{Absorbance of Blank}} \times 100$$

Total antioxidant capacity by FRAP method

The total antioxidant activity of different extracts of *L. aspera* was evaluated by FRAP assay[25]. The absorbance of the coloured product (Ferrous tripyridyltriazine complex) was recorded at 593 nm by iMark Biorad microplate reader. The FRAP values were expressed as mM Fe (II) equivalents per mg.

Total anti-oxidant capacity by ammonium molybdate assay

The total antioxidant capacity was determined by the transformation of Mo (VI) to Mo (V) to form phosphomolybdenum complex using the method of Prieto *et al.* [26] and was expressed in g equivalent of ascorbic acid.

Anti-elastase activity

The effect of hexane, ethyl acetate, acetone, and ethanol extracts of *L. aspera* on porcine pancreatic elastase (PPE, Sigma, Type IV) was assayed by the method described by Siedle *et al.*[27,28] using N-succinyl-Ala-Ala-Ala-nitroanilide (SANA, Sigma) as the

substrate. The release of p-nitro aniline due to proteolysis of N-succinyl-(Ala)-Ala-Ala-Ala-p-Nitroanilide by porcine pancreas elastase in the presence and the absence of inhibitor, was monitored by measuring the absorbance at 410 nm using 96 well microplate assay. The release of p-nitroanilide from the substrate was monitored by measuring the optical density at 410 nm using a microplate reader iMark BioRad. The percentage inhibition of different concentrations of extracts of *L. aspera* on porcine pancreas elastase was calculated in comparison with the control without inhibitor, considering the influence of the buffer, substrate, solvent and test extract. Blanks contained all the components except the enzyme was also taken. Percentage inhibition of elastase was determined using the following equation.

$$\text{Percentage inhibition} = \frac{(\text{control} - \text{control blank}) - (\text{sample} - \text{sample blank})}{(\text{control} - \text{control blank})} \times 100$$

where the control contained enzyme and substrate without extract, the control blank contained only substrate without enzyme and extract, the sample contained enzyme and substrate with extract and the sample blank contained substrate and extract without enzyme. The assay was performed using triplicate parallel samples. IC_{50} values were obtained from dose-effect curves by linear regression analysis.

Statistical analysis

Results were expressed as Mean±SD and compared using univariate analysis of variance followed by Duncan test with a significant level (α) 0.05. The $P \leq 0.05$ was considered statistically significant. Linear Regression analysis was performed to derive the regression equation, and IC_{50} values were calculated. All the calculations were performed using IBM-SPSS Statistics-22.

Results

Preparation of plant extracts

The different extracts of *L. aspera* were prepared using hexane, ethyl acetate, acetone, and ethanol by using sequential soxhlet extraction method and evaporated to dryness by rotary evaporator. The yields of different extracts of *L. aspera* were found to be 46.1, 69.1, 9.2, and 4.9 mg/g dry weight respectively.

Qualitative phytochemical analysis

Phytochemical analysis of different extracts of *L. aspera* revealed the presence of alkaloids, flavonoids, phenolic compounds and tannins, carbohydrates, proteins, oils, steroids and terpenoids in the different extract. Preliminary phytochemical analysis of different extracts of *L. aspera* is shown in Table 1.

Quantitative phytochemical analysis

Total phenolic compounds

The phenol content of the plant extracts was determined and the results are given in Table 2 as mean±standard deviation of triplicates and expressed as mg/g plant extracts in gallic acid equivalence. In the present study, the total phenol compounds were found to be 5.85±1.3, 52.26±4.19, 36.57±3.23, and 2.92±0.05 mg/g extract in gallic acid equivalent for hexane, ethyl acetate, acetone and ethanol extract respectively. It was found that the phenol content was high for ethyl acetate extract among the different extracts of the plant (Table 2).

Table 1 — Qualitative phytochemical analysis

Phytochemical tests	Hexane extract	Ethyl acetate extract	Acetone extract	Ethanol extract
Carbohydrate				
Molisch's test	-	-	+	+
Barfoed's test	-	-	+	+
Benedict's test	-	-	+	+
Fehling's test	-	-	+	+
Alkaloid test				
Wagner's test	-	-	+	+
Mayer's test	-	-	+	+
Flavanoids				
Alkaline reagent test	-	+	-	+
Protein	-	-	-	+
Biuret test				
Phenols				
Lead acetate test	+	+	+	+
Ferric chloride test		+	+	+
Oils				
Spot test	+	+	-	-
Steroids/terpenoids				
Salkowski's test	+	-	-	+

Table 2 — Quantitative analysis of phytochemicals in *Leucas aspera* (Willd.) Link extracts

Leucas aspera extract	Total phenol (Gallic acid equivalents)*	Flavanoids (Rutin equivalents)*
Hexane extract	5.85 ± 1.3	-
Ethyl acetate extract	52.26 ± 4.19	6.19 ± 1.68
Acetone extract	36.57 ± 3.23	2.60 ± 1.21
Ethanol extract	2.92 ± 0.05	-

*Values expressed as mean±SD

Estimation of flavonoid content

The amount of flavonoid content in the ethyl acetate and acetone extract of *L. aspera* was found to be 6.19±1.68 and 2.60±.21 mg/g plant extract in rutin equivalent respectively (Table 2). It can be concluded that the ethyl acetate extracts of *L. aspera* contain much more flavonoid content than acetone extract.

***In vitro* antioxidant activity**

Determination of reducing power

The present study showed the reductive capabilities of the different extracts of *L. aspera* compared to that of vitamin C. The percentage reducing the power of extracts was measured and expressed as Mean±SD as 33.13±3.23, 18.97±4.5, 52.74±5.33, and 29±1.73% for hexane, ethyl acetate, acetone, and ethanol extracts respectively and were compared statistically. From the results, it can be concluded that all the tested extracts possess the reductive capability and the percentage reducing capability of the acetone extract was significantly higher when compared with other extracts. Hence, the antioxidant property is more for acetone extract. The results are shown in Table 3.

In vitro DPPH radical scavenging assay

The percentage of DPPH radical scavenging activity of hexane, ethyl acetate, acetone extract and ethanol extract was found to be 74.64±4.91, 44.57±1.11, 77.54±2.33, and 15.44±0.41% respectively. The results are shown in Table 3. In the present study percentage of radical scavenging activity was found to be increased in dose-dependent manner. Also, the acetone extract had a high percentage of DPPH Radical scavenging activity when compared to the other extracts.

FRAP (Ferric reducing antioxidant power) assay

Benzie and Strain modified method[25] was adopted for the FRAP assay. This assay measures the ability of the compound to reduce the intense blue ferric tripyridyltriazine complex to its ferrous form, thereby changing its absorbance. Results are expressed in mM (Fe (II)/mg dry mass and compared with that of ascorbic acid. The results were 29.47±0.80, 1.6±9.184, 33.87±6.25, and 22.86±1 mM (Fe (II)/mg dry mass respectively for hexane, ethyl acetate, acetone and ethanol extract of *L. aspera* respectively and that of ascorbic acid was 78.67±0.67 (Table 3). The acetone extract possessed the highest FRAP when compared with other extracts was found to be lower when compared with standard ascorbic acid (Table 3).

Ammonium molybdate assay

The antioxidant activity of the extracts was evaluated by analyzing the transformation of Mo (VI) to Mo (V) to form phosphomolybdenum complex[26]. The total antioxidant capacity of hexane, ethyl acetate, acetone, and ethanol extract of *L. aspera* were found to be 23.8±7.87, 31.74±5.36, 44.24±1.0, and 18.44±3.7 in ascorbic acid equivalent respectively (Table 3).

Anti-elastase assay of *L. aspera*

The different concentrations of the extracts were prepared (10, 100, and 1000 µg/mL) in DMSO and were tested for their anti-elastase activity. The effects of the extracts on porcine pancreas elastase were evaluated and percentage inhibition was expressed as mean±SD. The results are shown in Table 4 and Fig. 1. The results revealed that all the extracts possessed inhibitory activity towards porcine pancreatic elastase and percentage inhibition was increased in a dose-dependent manner. The concentration of extract exerting 50 percentage inhibition (IC_{50}) towards porcine pancreas elastase was calculated for each extract using linear regression analysis. The IC_{50} were found to be 247.42, 744.28, 483, and 409.69 (µg/mL) for hexane, ethyl acetate, acetone and ethanol extract respectively. It was observed that the hexane extract possessed significantly the highest anti-elastase activity as it had a lower IC_{50} value when compared with other extracts at a significant level (α) $\leq$ 0.05.

Table 3 — Antioxidant activity of different extracts of *Leucas aspera* (Willd.) Link

Sample	Extract	Total antioxidant activity (Vitamin C equivalents)*	DPPH Scavenging activity (%)*	FRAP [mM Fe Equivalents/mg]*	Percentage reducing power (%)*
Leucas aspera	Hexane	23.8±7.87	74.64±4.91	29.47±0.808	33.13±3.23
	Ethyl acetate	31.74±5.36	44.57±1.11	1.6±9.184	18.97±4.5
	Acetone	44.24±1.0[a,b,d,e]	77.54±2.33[b,d]	33.87±6.25[a,b,d]	52.74±5.33[a,b,d]
	Ethanol	18.44±3.7	15.44±0.41	22.86±1	29±1.73
	Ascorbic acid	85.54±0.98	94.97±.05	78.67±0.67	87.28±0 .58

P <0.05:- a: significantly differ from hexane extract; b: significantly differ from ethyl acetate extract; c: significantly differ from acetone extract; d: significantly differ from ethanol extract; e:significantly differ from standard ascorbic acid

*Values expressed as mean±SD

Table 4 — Anti-elastase activity and IC_{50} of different extracts of *Leucas aspera* (Willd.) Link

Extract	Concentration of extract (µg/mL)*	Elastase inhibition (%)*	Regression equation	IC_{50} (µg/mL)
Hexane extract	10	33.60±2.03	Y=0.031X+42.330	247.42[b,c,d]
	100	57.75±1.00		
	1000	72.84±2.77		
Ethyl acetate extract	10	15.57± 0.55	Y=0.047X+ 15.01	744.28[a,c,d]
	100	20.76±1.76		
	1000	61.98±3.43		
Acetone extract	10	22.95±1.52	Y=0.045X+28.26	483[a,b,d]
	100	42.20±1.52		
	1000	72.28±2.12		
Ethanol extract	10	9.11±1.00	Y=0.0330X+ 36.48	409.69[a,b,c]
	100	33.66±1.37		
	1000	68.35±4.68		

P <0.05: a = significantly differ from hexane extract; b = significantly differ from ethyl acetate extract; c = significantly differ from acetone extract; and d = significantly differ from ethanol extract

*Values expressed as mean±SD

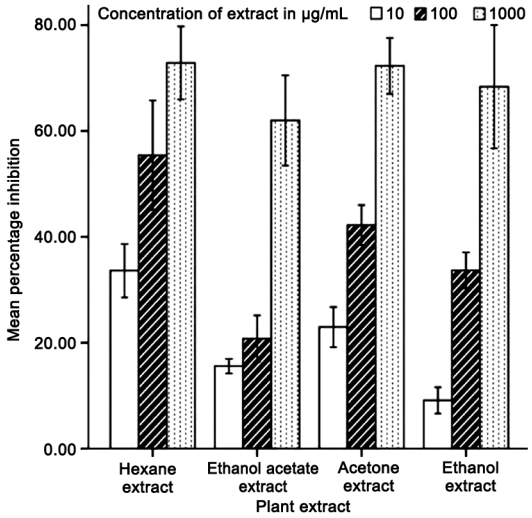

Fig. 1 — Anti-elastase activity of different extracts of *Leucas aspera* (Willd.) Link.

Discussion

The plant *L. aspera* is a tropical plant mainly used for many diseases in traditional systems of medicine[9]. The preliminary phytochemical screening revealed the presence of carbohydrates, proteins, phenolic compounds, alkaloids, flavonoids, terpenoids, steroids, and oils in different extracts of *L. aspera*. The quantitative analysis showed that the amount of total phenol content was observed to be more for ethyl acetate extract of *L. aspera* followed by acetone extract. The concentration of total phenolic compounds was found to be significantly low for hexane and alcohol extracts compared to the ethyl acetate and acetone extract. In plants of the genus *Leucas*, phenolics are found in abundance[8]. From the amount of flavonoid content in mg/g plant extract in rutin equivalents, it can be concluded that the ethyl acetate extracts of *L. aspera* contain much more flavonoid content than acetone extracts. This result supports the detailed review of *L. aspera* by Makhija *et al.*[8].

Free radicals are generated in our body due to oxidative stress and depletion of the dietary antioxidants, which may lead to an increase in pro-oxidant conditions for initiating diseases[29]. The present study showed that different extracts of *L. aspera* possessed significant antioxidant potential and reductive capability. The acetone extract possessed the highest antioxidant activity followed by ethyl acetate and alcohol extracts when compared statistically at a significance level of 0.05. The present results support the previous reports of antioxidant potential of *L.* aspera[30-33]. The identification of free radicals as the promoters of the ageing implies that the interventions inhibiting them should be able to reduce the ageing rate and disease pathogenesis[34]. Thus it can be concluded that the plant *L. aspera* with high antioxidant properties can be used for free radical-induced tissue damage.

Skin-care substances of natural origin have the potential to inhibit matrix metalloproteases like elastase[35]. As an anti-ageing agent, elastase inhibitors are important in preventing skin sagging[36]. It was observed that the hexane extract possesses significantly higher anti-elastase activity due to its lower IC_{50} value compared with other extracts

(significance level (α) ≤0.05). It can be coordinated with its high DPPH Scavenging effect. The anti-elastase effect may be due to the effect of phytochemical compounds present in the extract having free radical scavenging effect.

Uncontrolled elastase activity may function as a nonspecific indicator of inflammation, infection, cardiovascular complications, and obesity. The elastase inhibitors of plant origin can be used to treat these complications without any side effects[37]. They are also useful as anti-ageing, anti-wrinkling, and wound healing agent. The present study supports the medicinal value of *L. aspera* considering its antioxidant potential and anti-elastase activity. *L. aspera* was reported to contain various secondary metabolites[7,9,38-41]. The present study supports previous studies on pharmacological effects of *L. aspera*[42,43].

Conclusion

Natural products are extremely popular agents among the human population. From this study, it can be concluded that *L. aspera* extracts exhibited remarkable antioxidant and elastase inhibitory activity. Thus, the use of the plant for skin diseases is promising. The result supports the traditional use of *L. aspera* in skincare practices. A systematic screening, bioactivity guided purification, and characterization are essential to finding out the specific active constituent responsible for the profound therapeutic activity. The compound identified would be of great medicinal importance as it can be used to develop an effective therapeutic agent. Traditional knowledge coupled with scientific validation can provide effective remedies to eradicate diseases and to give betterment of mankind.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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