Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
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ORIGINAL ARTICLE

Reversed Phase High-Performance Liquid Chromatographic Ultra-violet (Photo Diode Array) Quantification of Oleanolic Acid and its Isomer Ursolic Acid for Phytochemical Comparison and Pharmacological Evaluation of Four *Leucas* Species Used in Ayurveda

Pushpendra Kumar Shukla, Ankita Misra, Sharad Srivastava, Ajay K. S. Rawat

Pharmacognosy and Ethnopharmacology Division, CSIR-National Botanical Research Institute, Lucknow, Uttar Pradesh, India

Submitted: 26-10-2015 Revised: 07-12-2015 Published: 11-05-2016

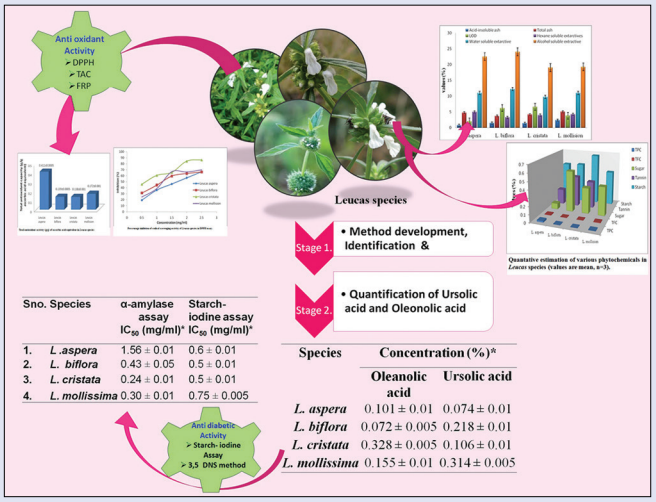
ABSTRACT

Content: Different *Leucas* species are well known as "Dronpushpi," a well-known herb of Ayurveda, used in the treatment of various ailments. **Objective:** Evaluation of four industrially important *Leucas* species for their *in vitro* antidiabetic potential and radical scavenging effect along with high-performance liquid chromatographic quantification of the bioactive triterpenes. **Materials and Methods:** The quantification of triterpenes was carried out on C-18 column with acetonitrile and water (90:10) as the solvent system at a detection wavelength of 210 nm. *In vitro* antidiabetic activity was evaluated by α-amylase inhibition assay based on starch–iodine and 3,5 dinitrosalicylic acid (DNS) method. Antioxidant activity was calculated by five different models, namely total phenolic and total flavonoid content, free radical scavenging activity by 1-1-diphenyl-2-pic-rylhydrazyl (DPPH), ferric-reducing power assay, and the total antioxidant capacity. **Results:** Maximum concentration of oleanolic acid was found in *Leucas cristata*, followed by *Leucas mollissima*, *Leucas Aspera,* and *Leucas biflora*. Ursolic acid was highest in *L. mollissima* and then in *L. biflora, L. cristata,* and *L. aspera,* respectively. In *in vitro* antidiabetic activity, IC_{50} of *L. aspera* (1.56 ± 0.01 mg/ml) and *L. mollissima* (0.75 ± 0.005 mg/ml) were found to be highest in DNS and iodine starch assay. IC_{50} in DPPH assay ranges from 0.6 ± 0.011 to 1.68 ± 0.011 mg/ml. Antioxidant capacity follows the order; *L.* aspera > *L.* mollissima > *L.* biflora > *L. cristata*. **Conclusion:** Promising activities were observed in targeted species, thus *L. mollissima, L. biflora,* and *L. cristata* can be used alternatively as a substitute to *L. aspera*.

Key words: Dronpushpi, *in vitro* antidiabetic activity, oleanolic acid, ursolic acid

SUMMARY

- Physicochemical parameters are within the limit as per the Ayurvedic Pharmacopoeia of India
- Maximum concentration of oleanolic acid was found in *Leucas cristata*; however, ursolic acid was highest in *Leucas mollissima*
- *In vitro* antidiabetic activity of *Leucas aspera* and *L. mollissima* was found to be heighest as compared to other species. However, antioxidant capacity is almost similar in targeted species.
- Promising activities were observed in all the species, thus *L. mollissima, Leucas biflora*, and *L. cristata* can be used alternatively as a substitute to *L. aspera*.



Correspondence:

Dr. Sharad Srivastava,
Pharmacognosy and Ethnopharmacology Division,
CSIR-National Botanical Research Institute,
Lucknow - 226 001, Uttar Pradesh, India.
E-mail: sharad_ks2003@yahoo.com
DOI: 10.4103/0973-1296.182173

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INTRODUCTION

Leucas species (*Lamiaceae*) collectively recognized as "Dronpushpi," a well-known herb of ayurvedic systems of medicines is used for cough, cold, diarrhea, inflammatory skin disorders, and several other ailments. The genus comprises about 80 species and the highest diversity has been found in East Africa.[1] Among them, 43 species are available in India[2] as annual or perennial herbs. *Leucas aspera* (wild) is the most common species with immense medicinal values such as ulcer protective,[3] antifungal, antioxidant, antimicrobial, antinociceptive, and cytotoxic activity.[4] *Leucas biflora* (Vahl.) is a two-flowered perennial herb with nodal roots and many branches arise from a woody root stock. As ethnic medicine in Northern India, the mature leaf decoction is used in conjunctivitis.[5] *Leucas cristata* (Roth.) Spreng, commonly known as gumma, is found abundantly in roadside areas as a weed, especially in rainy season. It possesses stimulant, laxative, diaphoretic, antiseptic,

Cite this article as: Shukla PK, Misra A, Srivastava S, Rawat AK. Reversed phase high-performance liquid chromatographic ultra-violet (photo diode array) quantification of oleanolic acid and its isomer ursolic acid for phytochemical comparison and pharmacological evaluation of four *Leucas* species used in ayurveda. Phcog Mag 2016;12:S159-64.

anthelmintic, insecticidal, germicidal, fungicidal, hypolipidemic, hypoglycemic, antiprotozoal, expectorant, and anti-pyretic properties.[6] Foliage of *Leucas mollissima* Wall. ex Benth. is used in treating headache. The decoction is used orally to treat diabetes mellitus, hepatitis, and is effective in liver diseases.[7]

A variety of chemical markers have been isolated from the *Leucas* species, which include terpenes, fatty acids, steroids, lignans, flavonoids, coumarins, various aliphatic long-chain compounds,[8] phytol,[9] salicylic acid, and caffeic acid.[10] Several recent reports suggested the preventive role of triterpene oleanolic acid and its isomer, ursolic acid in the treatment of type-2 diabetes and other associated complications, namely nonalcoholic fatty liver disease, nephropathy, retinopathy, and atherosclerosis. The antidiabetic potential of these triterpene isomers is mediated due to target signaling of molecules that increase insulin signal transduction; reduce inflammatory and oxidative stress signaling.[11,12] The targeted species of *Leucas* are widely available in Northern and Central part of India and a review of literature suggested that *L. aspera* is the most utilized one. The present study includes comparative phytochemical investigation along with the evaluation of *in vitro* antidiabetic potential. The antioxidant potential was also estimated to explain the radical scavenging effect among them, as this is one of the major underlying causes of diabetes. Oleanolic acid and its isomer ursolic acid were also quantified in targeted species to authenticate their use in the management of diabetes. This also prospects for identification of an alternative/substitute of commercially exploited species, i.e. *L. aspera*.

MATERIALS AND METHODS

Chemicals

Oleanolic acid (≥97% high-performance liquid chromatographic [HPLC]), ursolic acid (≥97% HPLC), ascorbic acid (≥97%), quercetin (≥97%), rutin (≥95%), 1-1-diphenyl-2-pic-rylhydrazyl (≥99%, DPPH), and butylated hydroxytoluene were purchased from Sigma-Aldrich. HPLC grade solvents, namely acetonitrile, methanol, water, and all other solvents/chemicals (AR grade) were purchased from Merck, Mumbai, India.

Plant materials

Aerial part of four *Leucas* species, namely *L. aspera* (LWG No. 254029), *L. biflora* (LWG No. 254030), *L. cristata* (LWG No. 254031), and *L. mollissima* (LWG No. 254033) were collected from Tendukheda forest, Tamiya, Madhya Pradesh, India, during September–October. Samples were authenticated by Dr. Sharad Srivastava, Scientist, Pharmacognosy Division, CSIR-NBRI, and voucher specimens were deposited in institute's herbarium. Collected samples were washed, shade dried, and powdered for further studies.

Preparation of extract

The dried, aerial parts of *Leucas* spp. were grinded (lab grinder) and sieved through 40 mesh (up to 500 mm) to obtain fine powder. About 100 g of powdered sample was defatted with petroleum ether and then treated with methanol (ethanol stabilized) through Soxhlet extraction, until complete exhaustion of sample (7 days; 27 ± 2°C). The pooled extracts were filtered (Whatman No. 1 filter paper), concentrated in rotary evaporator under controlled conditions (50°C, 40 mbar), and finally lyophilized extracts were quantified.

Physicochemical and phytochemical characterization

Physicochemical parameters, namely moisture content, total ash, acid insoluble ash, and extractive values (hexane, alcohol, and water-soluble extractives), were evaluated and quantified.[13] Phytochemical estimation of various primary and secondary metabolites, i.e. sugar, starch, phenolic,[14] flavonoid,[15] and tannin[16] was also done as per the standard protocols.

High-performance liquid chromatographic quantification of oleanolic acid and its isomer ursolic acid in *Leucas* species

Reversed phase-HPLC ultra-violet-2996 (Cyber Lab, LC 100, USA) was used for the qualitative and quantitative analysis of analytes. Chromatographic separation was performed on phenomenex C_{18} column (250 mm × 4.6 mm, 5.0 µm,) with C_{18} guard column in isocratic mode via binary mobile phase consisting of acetonitrile: Methanol (90:10) at 0.8 ml/min. Mobile phases were duly filtered through 0.22 µm millipore filter and degassed ultrasonically for 15 min before delivered to column for separation and run time was 30 min. Data acquisitions were performed using ChromeStation software. The injection volume was 25 µl for standard and sample. Detection was done at a wavelength is 210 nm.

In vitro (alpha amylase inhibition assay) antidiabetic activity

Starch–iodine color assay

Assay was carried out with slight modification based on the starch–iodine test.[17] Methanol extract (500 µL) of varied concentrations were added to 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 containing 6 mM sodium chloride) containing 0.04 units of α-amylase solution and were incubated at 37°C for 10 min, then 500 µL soluble starch (1% w/v) was added to each reaction well and again incubated at 37°C for 15 min. 1 M HCl (20 µL) was added to stop the enzymatic reaction, followed by the addition of 100 µL of iodine reagent (5 mM I_2 and 5 mM KI). The color change was noted and the absorbance was read at 620 nm on a micro plate reader. The control reaction representing 100% enzyme activity did not contain any plant extract to eliminate the absorbance produced by plant extract; appropriate extract controls without the enzyme were also included. Acarbose is used as a positive control and the inhibition of enzyme activity was calculated as:

Inhibition of enzyme activity (%) = (C − S)/C × 100

Where S is the absorbance of the sample and C is the absorbance of blank (no extract).

3,5-dinitrosalicylic acid method

The inhibition assay was performed using 3,5-dinitrosalicylic acid (DNS) method.[18] Methanol extract (500 µL) of varied concentrations were added to 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 containing 6 mM sodium chloride) containing 0.04 units of α-amylase solution and were incubated at 37°C for 10 min, followed by addition of 500 µL of a 1% starch solution (0.02 M sodium phosphate buffer, pH 6.9) in all the test tubes. The reaction was stopped with 1.0 ml of DNS reagent. The test tubes were then incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted after adding 10 ml of distilled water and absorbance was measured at 540 nm. The control samples were also prepared accordingly without any plant extracts and were compared with the test samples containing various concentrations of the plant extracts. Acarbose is used as a positive control and results were expressed as % inhibition calculated using the formula.

Inhibition activity (%) = Abs (control) − Abs (extract) × 100/Abs (control)

In vitro antioxidant activity

Total flavonoid[15] and phenolic[14] content were expressed in terms of mg/g of quercetin equivalent (QE) and mg/g gallic acid equivalent (GAE)

based on calibration curve of quercetin and gallic acid as standard. The anti-oxidant potential was analyzed via DPPH radical scavenging assay,[19] ferric reducing power assay,[20] and total antioxidant capacity.[21]

Statistical analysis

Results were expressed as mean ± standard deviation. Linear regression analysis was carried out for standards to calculate total phenolic and flavonoid content. IC_{50} values were obtained by GraphPad Prism 5 software (San Diego, CA, USA). One-way ANOVA followed by Student's *t*-test ($P < 0.01$) was used to find the significance of *in vitro* antidiabetic and antioxidant assays.

RESULTS AND DISCUSSION

Physicochemical and phytochemical studies

Physicochemical parameters of *L. aspera*, *L. biflora*, *L. cristata*, and *L. mollissima* were studied and compared quantitatively [Figure 1]. The study reveals that alcohol soluble extractive was highest in all the four species as compared to other extractive values; *L. biflora* shows the maximum, i.e. 24%. However, hexane and water-soluble extractive varies from 3.25–4.25% to 9.75–12.25%, respectively; however, moisture content was maximum in *L. cristata* (6.6%). Total ash and acid insoluble ash ranges between 3.27–5.05% and 0.75–2.4%, respectively. The values are observed within the specified limit of API.[13]

Analysis of phytochemicals [Figure 2] reveals that starch was found abundantly in the four species than other metabolites, and concentration ranges from 0.448% to 0.664%. Sugar, tannin, phenolic, and flavonoid content vary considerably among the species, i.e., 0.076–0.053%, 0.2–0.4%, 0.0015–0.018%, and 0.0014–0.0079%, respectively. It was observed that the species were not significantly different in the content of phytochemicals.

High-performance liquid chromatographic quantification of oleanolic acid and its isomer

Quantification of targeted chemical marker, namely oleanolic and ursolic acid in different *Leucas* species was carried out on the basis of calibration curve of standards. Five dilutions of standard were used in a concentration range of 0.125–1 mg/ml and various calibration values were obtained [Table 1]. Maximum concentration of oleanolic acid was found in *L. cristata*, followed to *L. mollissima*, *L. aspera*, and *L. biflora*. Ursolic acid was highest in *L. mollissima*, and then in *L. biflora*, *L. cristata*, and *L. aspera*, respectively [Figures 3-6 and Table 2].

In vitro biological assays

In vitro antidiabetic activity

In vitro antidiabetic activity was evaluated by α-amylase inhibition assay based on starch–iodine and DNS method. Data of starch–iodine method reveal that activity increases linearly with concentration, i.e., 0.1–0.5 mg/ml of tested plant extract [Figure 7a]. IC_{50} follows the order, *L. mollissima* (0.75 ± 0.005) > *L. aspera* (0.6 ± 0.01) > *L. biflora* = *L. cristata* (0.5 ± 0.01), whereas acarbose exhibit IC_{50} at <25 µg/ml. Results of α-amylase inhibition by DNS show that with increase in the concentration of inhibitors, degradation of starch reduces and thus indicating the inhibition of enzyme activity. The inhibition (%) by *Leucas*

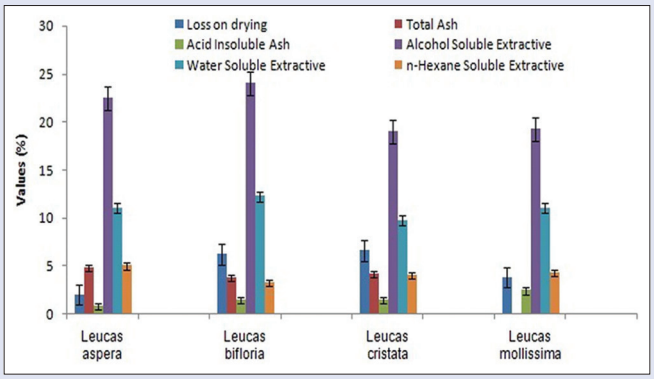

Figure 1: Physico-chemical parameter of *Leucas* species (values are mean, *n* = 3)

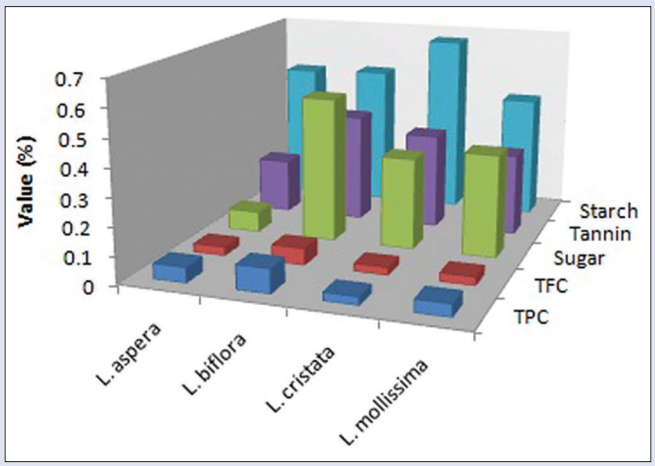

Figure 2: Quantitative estimation of various phytochemicals in *Leucas* species (values are mean, *n* = 3)

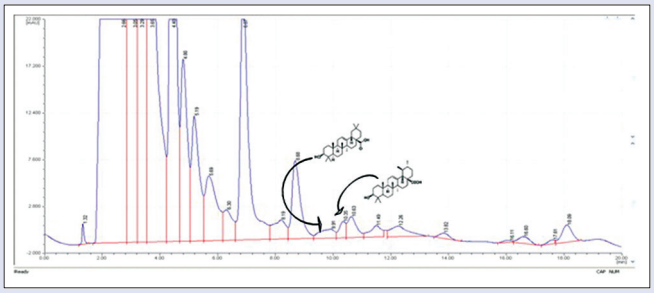

Figure 3: High-performance liquid chromatographic ultra-violet chromatogram of *Leucas aspera*

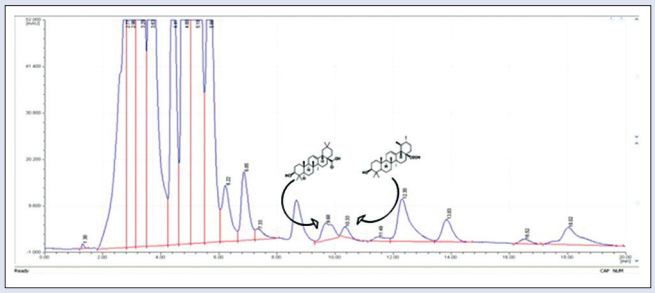

Figure 4: High-performance liquid chromatographic ultra-violet chromatogram of *Leucas cristata*

species varies from 9.09% to 87.95% [Figure 7b] and acarbose exhibits 36.67–95.74% inhibition at a concentration of 10–200 µg/ml, respectively. IC_{50} of acarbose was observed at 32 ± 0.01 µg/ml, whereas within the *Leucas* species, IC_{50} was maximum in *L. aspera* (1.56 ± 0.01 mg/ml), followed by *L. biflora* (0.43 ± 0.05), *L. mollissima* (0.30 ± 0.01), and then *L. cristata* (0.24 ± 0.01) [Table 3]. Thus indicating that *L. cristata* exhibits the most promising activity. However, the inhibitors were significantly indifferent ($P < 0.01$) from each other. The bioactive potential of *Leucas* species is suspected due to the presence of identified markers in synergy with other phytomolecules.

In vitro antioxidant activity

Antioxidant activity of *Leucas* species were calculated by five different models having variable mechanism of action. Polyphenolic content, namely total phenolic and flavonoid content were estimated [Table 4], total phenolic content was highest in *L. aspera* (5.55 ± 0.05 mg/g GAE), followed by *L. mollissima* (0.97 ± 0.005 mg/g GAE), *L. biflora* (0.87 ± 0.005 mg/g GAE), and then *L. cristata* (0.30 ± 0.057 mg/g GAE). However, flavonoid content varies from 0.28 ± 0.005 to 1.59 ± 0.005 mg/g QE. The reducing power assay of extract served as a significant indicator of its potentiality as a reducing agent, which in turn signifies its anti-oxidant activity. Result showed that the reducing power of four species (methanolic extract) increases linearly with increase in concentration, i.e. *L. aspera*, *L. biflora*, *L. cristata*, and *L. mollissima* having regression coefficient (r^2) of 0.983, 0.980, 0.888, and 0.990, respectively. This is found similar to standards, i.e. ascorbic acid (0.998), quercetin (0.997), and rutin (0.998).

Free radical scavenging activity of DPPH is most widely used for screening of medicinal plants having anti-oxidant activity. The scavenging effect of DPPH radical on four *Leucas* species was concentration-dependent and varied among samples as well as standards (ascorbic acid, quercetin, and rutin) [Figure 8]. Ascorbic acid exhibits the maximum inhibition of 77.57% (IC_{50} at 3.86 ± 0.057 µg/ml), which is followed by quercetin and rutin having inhibition of

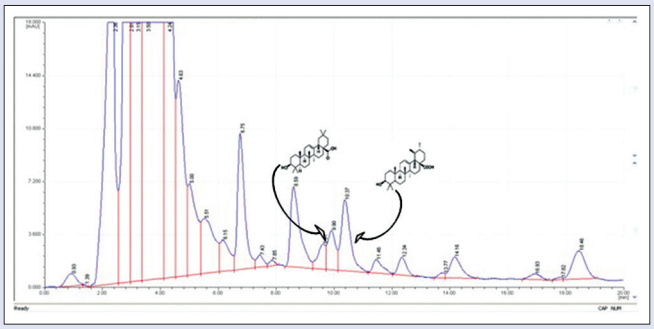

Figure 5: High-performance liquid chromatographic ultra-violet chromatogram of *Leucas mollissima*

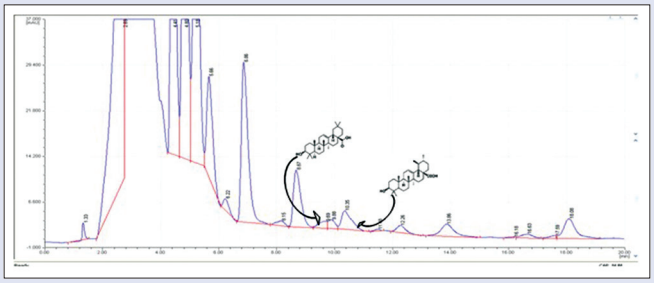

Figure 6: High-performance liquid chromatographic ultra-violet chromatogram of *Leucas biflora*

Table 1: Statistical analysis of calibration curves in high-performance liquid chromatography determination of triterpenes chemical markers

Calibration parameters*	Oleanolic acid	Ursolic acid
R_t value	9.99	10.38
Slope	84,086	83,394
Intercept	1708	28,268
Correlation coefficient (*r*)	0.992	0.999
Linearity range (mg/ml)	0.125-1.0	0.125-1.0
LOD	11.8	11.8
LOQ	35.92	35.8
SD	302,077.2	298,609.7
SE	135,097.1	133,546.3
Average	443,163.4	466,087.4
P (95%)	0.0059	0.00023

*n=5. SD: Standard deviation; SE: Standard error; LOD: Limit of detection; LOQ: Limit of quantification

Table 2: Quantification (%) of oleanolic acid and its isomer ursolic acid in four species of *Leucas*

Species	Concentration (%)*	
	Oleanolic acid	Ursolic acid
Leucas aspera	0.101±0.01	0.074±0.01
Leucas biflora	0.072±0.005	0.218±0.01
Leucas cristata	0.328±0.005	0.106±0.01
Leucas mollissima	0.155±0.01	0.314±0.005

*Values are mean±SD. SD: Standard deviation

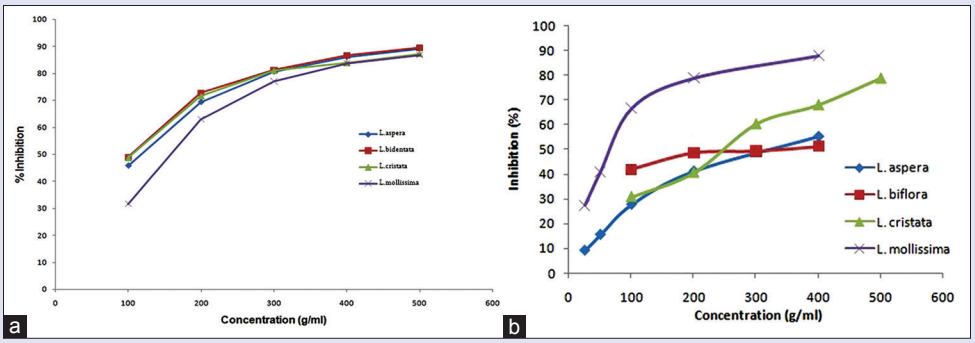

Figure 7: (a) Anti-diabetic assay by starch–iodine color reagent. (b) Anti-diabetic assay by 3,5 dinitrosalicylic acid method color reagent

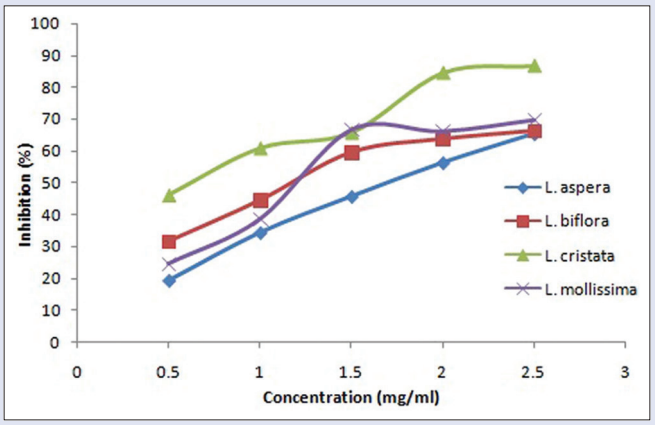

Figure 8: Percentage inhibition of radical scavenging activity of *Leucas* species in 1-1-diphenyl-2-pic-rylhydrazyl assay

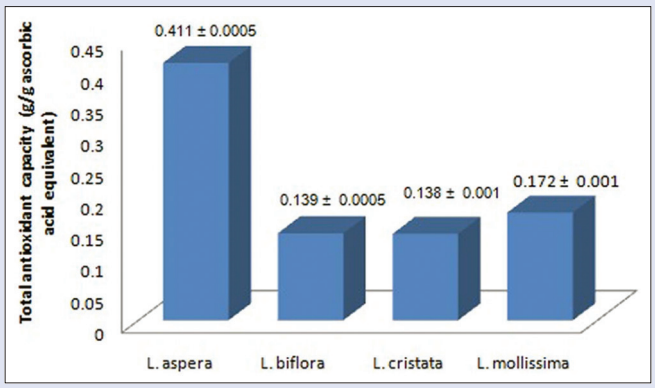

Figure 9: Total antioxidant activity (g/g) of ascorbic acid equivalent in *Leucas* species

Table 3: IC_{50} of *Leucas* species obtained from *in-vitro* antidiabetic assay

Species	α-amylase assay IC (mg/ml)*	Starch–iodine assay IC_{50} (mg/ml)*
Leucas aspera	1.56±0.01	0.6±0.01
Leucas biflora	0.43±0.05	0.5±0.01
Leucas cristata	0.24±0.01	0.5±0.01
Leucas mollissima	0.30±0.01	0.75±0.005

*Mean±SD; *n*=3. SD: Standard deviation

Table 4: Total phenolic and flavonoid content in *Leucas* species

Species	Total phenolic content (mg/g)* GAE	Total Flavonoid content (mg/g)* QE
	y=83.96x+0.170 r^2=0.970	y=101.8x+0.043 r^2=0.932
Leucas aspera	5.55±0.057	1.59±0.0057
Leucas biflora	0.87±0.005	0.31±0.0057
Leucas cristata	0.30±0.057	0.28±0.0057
Leucas mollissima	0.97±0.005	1.56±0.0057

*Mean±SD; *n*=3. SD: Standard deviation; GAE: Gallic acid equivalent; QE: Quercetin equivalent

Table 5: IC_{50} value of *Leucas* spp. in 1-1-diphenyl-2-pic-rylhydrazyl radical scavenging assay

Species	IC_{50} (mg/ml)*
Leucas aspera	1.68±0.011
Leucas biflora	0.66±0.05
Leucas cristata	1.25±0.005
Leucas mollissima	0.6±0.011

*Mean±SD; *n*=3. SD: Standard deviation

72.43 (IC_{50} at 5.93 ± 0.115 µg/ml) and 71.48 (IC_{50} at 6.8 ± 0.173 µg/ml), respectively. *Leucas* species, namely *L. aspera*, *L. biflora*, *L. cristata*, and *L. mollissima* have IC_{50} at 1.68 ± 0.011, 0.66 ± 0.05, 1.25 ± 0.005, and 0.6 ± 0.011 mg/ml, respectively [Table 5]. However, the IC_{50} of samples was statistically different ($P < 0.01$) than standard.

The total antioxidant capacity of *Leucas* (methanolic extract) was measured by phosphor-molybdenum method based on regression analysis of ascorbic acid, having regression equation (y = 4.804x + 0.136) and coefficient; r^2 = 0.987. Data shows that maximum total anti oxidant capacity was observed in *L. aspera* (0.411 ± 0.0005 mg/g ASE), followed by *L. mollissima* (0.172 ± 0.001 mg/g ASE) and *L. cristata* having 0.131 ± 0.005 mg/g of ascorbic acid equivalent [Figure 9]. It is observed that all the four species exhibit promising *in vitro* antioxidant activity and there is insignificant difference between them.

CONCLUSION

From the above study, it is inferred that bioactive triterpenes, namely oleanolic acid and ursolic acid are present in significant amount in all the evaluated species and thus exhibiting promising antidiabetic and antioxidant potential. Maximum concentration of oleanolic acid was found in *L. cristata*; however, ursolic acid was highest in *L. mollissima*. *In vitro* anti-diabetic studies showed that *L. aspera* (1.56 ± 0.01 mg/ml) and *L. mollissima* (0.75 ± 0.005 mg/ml) have significant potential. It is also evident that the potentiality of all the species as natural antioxidants and anti-diabetic were also comparable among each and hence the less explored species, i.e. *L. biflora*, *L. cristata*, and *L. mollissima* can be used as a substitute by herbal industries for *L. aspera*, which is widely recognized and exploited in various ayurvedic formulations by the name of "Dronpushpi."

Acknowledgment

The authors are thankful to the Director of CSIR-NBRI for providing facilities and encouragement throughout the work.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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Sharad Srivastava

ABOUT AUTHOR

Dr. Sharad Srivastava, is Principal Scientist in Pharmacognosy Division at CSIR-National Botanical Research Institute; Lucknow, India. He has made significant contributions to quality control of crude drugs/products, chemotaxonomy, bio-prospection, and natural product development, and have developed quality parameters of single crude drugs (more than 70 medicinal plants) and also identified biomarkers for their quality control. He has contributed 30 monographs of single herbal drugs in Ayurvedic Pharmacopoeia of India. He has 115 publications in peer-reviewed journals, 17 patents, and developed some technologies/formulations, few has already been transferred to industry.