Original Article

Simultaneous RP-HPLC Quantification of Four Phenolics in *Elephantopus scaber* L. and their *in vitro* Pharmacological Validation

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ABSTRACT

Elephantopus scaber (L.) is a widely used traditional medicinal plant, grows in hotter parts of the Indian subcontinent, Tropical Africa and East Asia. The present study was aimed RP-HPLC quantification of phenolics and its pharmacological validation in *Elephantopus scaber* (L.). The chromatographic separation was obtained using RP-C_{18} column, using mobile phase acetic acid: water (1.0: 99.0 V/V) as solvent-A and acetonitrile as solvent-B and 0.6ml/min flow rate was used for proper separation. HPLC method was developed and validated according to ICH guidelines. Four important phenolic compounds i.e chlorogenic acid, ferulic acid, gallic acid and protocateuchine were identified and quantified with the help of developed RP-HPLC method. Result reveals the presence of chlorogenic acid (3.481mg/g), ferulic acid (0.842 mg/g), gallic acid (0.668 mg/g) and protocateuchic acid (0.086 mg/g). Total phenolic and flavonoid content in the methanolic extract was found to be 16.24±1.61 mg/g GAE and 12.87±0.043 mg/g QE. *In vitro* antioxidant and antidiabetic assays were performed as per standard protocols. The IC_{50} value for the *in vitro* DPPH method was found to be (0.167±1.21 mg/ml), in DNS assay (0.522±0.04 mg/ml) and (0.364±0.03 mg/ml) for the starch-Iodine method in methanolic extract of *E. scaber*. This implies that *Elephantopus scaber* which has been generally considered as weeds and exploited for beneficial purposes not only traditionally but also would be used in medicinally based on their biochemical active compounds. Hence, the study aids in authentication of *E. scaber* by identification of marker compounds with validated traditional claims.

Key words: Antidiabetic, Antioxidant, *Elephantopus scaber*, Phytochemical, RP-HPLC.

INTRODUCTION

Elephantopus scaber Linn., belonging to Asteraceae family, commonly known as "Elephant's foot". It is a tropical plant, distributed in the Europe, Asia, Australia and Africa.[1] In India, It is found in Madhya Pradesh, Uttarakhand, Uttar Pradesh, Kerala, Odisha and Maharashtra.[2] Traditionally, this plant is used as folk medicine in tribal communities, in the treatment of dysentery, cardiotonic, stomachic pain, diuretic, antiemetic, gout, dysuria, eczema, spider and snake bite.[3,4] Kani tribals in Tirunelveli hills (Tamil Nadu, India) use leaf powder of *E. scaber* along with the leaves of *Toddalia asiatica* and *Naravelia zeylanica* to cure rheumatism.[5] In Thailand, *E. scaber* has been used as traditional medicine to treat cough (root decoction) as a tonic and whole plant decoction use for the treatment of chapped lips and galactogoue. In Brazil, decotion of the whole plant is used to excite dieresis, reduce fever and eliminate bladder stones, febrifuge and diaphoretic against cough, bronchitis and asthama.[6] Malaysia, tribals community use decoction of root for the contraction of abdominal area and reduce inflammation after childbirth. As well as whole plant parts are also boiled with red bean to remove flatulence.[7] In Taiwan,

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whole plant decoction is used for the treatment of hepatitis, in Nigeria, levees hydro-alcoholic extract use to treat arthritis, in Mauritius to treat diarrhea and urinary problems.[8,9] Several *in vivo* and *in vitro* studies confirmed that *E. scaber* extract has powerful biological activities such as diuretic, emollient, febrifuge, diaphoretic, anthelmintic, antidiarrheal,[10] astringent, antiemetic, analgesic,[11] in cough,[12] antiulcer,[13] chicken pox[14] and toothache.[15]

Deoxyelephantopin (ESD) and isodeoxyelephantopin (ESI) are two sesquiterpene lactones, exhibit antimicrobial, anti-inflammatory, heptoprotoctive and anticancer activity in the plant.[16,17] Other sesquiterpene lactones reported are deoxyelephantopin, isodeoxyelephantopin and scabertopin.[18] It also contains epifriedelinol, lupeol and stigmasterol.[19,20]

Due to the importance of *E. scaber* in traditional as well ayurvedic system of medicine, the present study has been planned to identify the presence of phenolics and validate its bioactivity by targeting antioxidant and antidiabetic potential.

MATERIALS AND METHODS

Plant material

Fresh aerial part of *E. scaber* was collected in the month of October from the nearby area of Pallode (Phytogeographical zone: Western Ghats, Altitude: 270-meter, latitude: 8° 45' 01.94" N, Longitude: 77° 01' 42.08" E), Kerala, India. The plant material was authenticated by Dr. Sharad Srivastava, Principal Scientist, Pharmacognosy Division, CSIR-NBRI, Lucknow and the voucher specimen (LWG No 254055) was deposited in the Institute's herbarium.

Chemicals

Reference standards i.e. gallic acid, proto-catechuic acid, chlorogenic acid, caffeic acid, ferulic acid, rutin, quercetin, kaempferol, α-amylase, 3, 5-Dinitrosalicylic acid, electronic grade methanol and acetonitrile were purchased from Sigma-Aldrich, USA. HPLC grade solvents viz. acetonitrile, methanol, water and all other solvents/chemicals (AR grade) were purchased from Merck, Mumbai, India.

Preparation of extract

The dried, aerial parts of *E. scaber* was 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) under standard condition through soxhlet extraction, till complete exhaustion of sample (7days; 27 ± 2°C). The pooled extracts were filtered (Whatmann No. 1 filter paper), concentrated in rotary evaporator under controlled conditions (50°C, 40 mbar) and finally lyophilized extracts were quantified.

Anatomical studies

The freshly collected plant material was preserved in 70% ethanolic solution for microscopic studies. The anatomy studies were performed as per standard methods.[21-25] Free hand sectioning was done to obtain thin sections for clear visibility of cellular details. The sections were then serially stained with safranin and fast green to contrast the secondary structures. The stained sections were mounted with glycerin and observed under light microscope. Photomicrographs were taken with digital microscopes (model CX31, Olympus).

Standard and sample stock preparation for HPLC analysis

Primary stock solution of each reference compound was prepared in methanol to obtain a concentration of 1 mg/ml. Consequently, the primary stock solution was diluted to prepare a secondary stock followed by further dilutions to achieve variable concentrations in the range of 0.5–50 mg/ml. The plant sample was prepared by dissolving 1 g of powder into 10 ml of 50% ethanol in water. The solution was centrifuged, supernatant was taken and filtered through 0.45 mm nylon filter (Millipore TM) and is used for HPLC analysis.

HPLC Instrumentation

Qualitative and quantitative analysis of polyphenols was performed by using HPLC-UV (Shimadzu LC-10A, Japan) equipped with dual pump LC-10AT binary system, UV detector SPD-10A at 254 nm, rheodyne injection valve furnished with a 20 ml loop, on phenomenex Luna RP-C 18 column (4.6 X 250 mm, i.d., 5mm pore size) preceded with guard column of same chemistry. Data was integrated by Shimadzu class VP series software and results were obtained by comparison with standards. Results are the mean values of three replicates of the same sample. Elution was carried out at a flow rate of 0.6 ml/min with water: acetic acid (99.0:1.0 v/v) as solvent A and acetonitrile as solvent B using a gradient elution in 0-14 min with 20-35% of solvent B, 14-40 min with 35-50% of solvent B. The buffer and acetonitrile were filtered through 0.45 mm nylon filter and de-aerated in ultrasonic bath before use.

In vitro antioxidant activity

Total flavonoid[26] and phenolic[27] content were expressed in terms of mg/g QE (Quercetin Equivalent) and mg/g GAE (Gallic Acid Equivalent) respectively based on calibration curve of quercetin and gallic acid as standard. The antioxidant potential was analyzed via DPPH radical scavenging[28] and ferric reducing power assay.[29]

In vitro antidiabetic activity (alpha amylase inhibition assay)

Evaluation of anti-diabetic potential was estimated by starch-iodine colour assay[30] and 3, 5-dinitrosalicylic acid (DNS) method.[31] The activity potential of extract was expresses as inhibition (%) of enzyme activity.

Statistical Analysis

Results were expressed as mean ± S.D. Linear regressions analysis was carried out for standards to calculate total phenolic and flavonoid content. IC_{50} values were obtained by graph pad prism 5 software. One-way ANOVA followed by student's *t* test ($p<0.01$) was used to find the significance of *in vitro* anti-diabetic and anti-oxidant assays.

RESULTS AND DISCUSSION

The collected plant was matched with the specimen available at the institute herbarium and found the same Ash values and extractive values were also estimated to ensure the authenticity of sample as per standard protocols. Total ash and acid insoluble ash was found to be 7.17 % and 0.487%. Extractive values viz. hexane, ethanol and water soluble were obtained as 5 %, 10% and 28% respectively. These results are in accordance with reported literature[32] and thus confirming the authenticity of studied material.

Anatomic study

Transverse section of the stem reveals that the outline is irregularly circular (Figure 1). Epidermis is composed of small barrel shaped cells and contains hairs. Just below the epidermis, 3-4 layered cortex which consist of small, rounded, parenchymatous cells with intercellular spaces. Below the cortex region, pericycle layer is visible which is made up of compactly arranged thick walled sclerenchymatous cells. Vascular bundles are conjoint, collateral and endarch, arranged in a ring. Xylem is surrounded by small, rounded cells of xylem parenchyma. Parenchymatuos medullary ray is present. Pith region is large, central in position; consist of almost uniform parenchymatous cells with intercellular spaces.

RP-HPLC quantification

HPLC quantification of methanolic extract (extractive yield of 11.6 %) of phenolic acid under developed, standardized conditions reveal the presence of four markers (Figure 2) in comparison with reference standard (Figure 3) under same protocols. Among the identified markers chlorogenic acid (3.481 mg/g) was found in highest concentration, followed by ferulic acid,

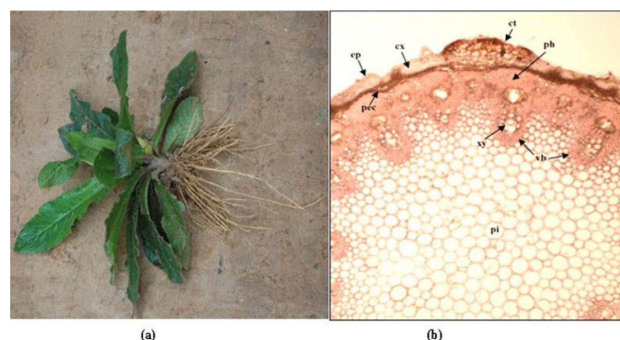

Figure 1: *Elephantopus scaber* Linn herb (a) abd T.S of stem (b). Abbreviation: ep- Epidermis, cx- Cortex, pec- Pericycle, ph- Phloem, xy- Xylem, pi- Pith region.

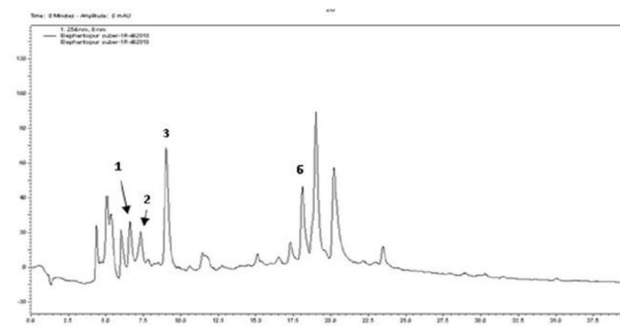

Figure 2: HPLC chromatogram of *E. scaber*. 1- Gallic acid; 2- Protocatechuic acid; 3- Chlogrgenic acid; 4- Ferulic acid.

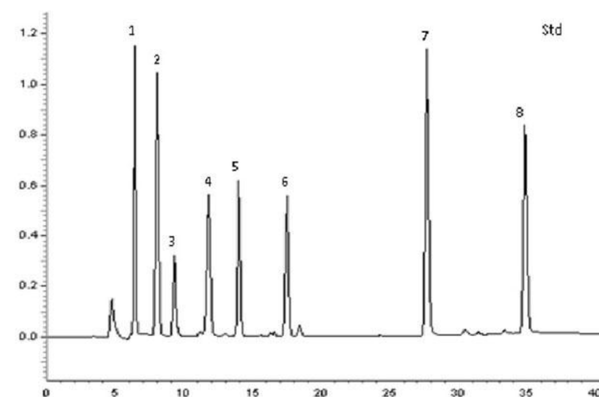

Figure 3: HPLC chromatograms obtained from standard solutions at 254 nm: 1- Gallic acid; 2- Protocatechuic acid; 3- Chlogrgenic acid; 4- Caffeic acid; 5- Rutin; 6- Ferulic acid; 7- Quercetin; 8- Kampferol.

gallic acid and proto catechuic acid as represented in Table 1. Linearity calibration of standards were analysed at 0.5-50 µg/g and stastical parameters viz. regression coefficient, LOD and LOQ are within the acceptable limit of ICH (2005). Method was found to be linear under regression analysis of area Vs concentration of standard(s). In addition, the developed method was also validated for specificity, sensitivity, precision and system suitability.[33] The developed method is very suitable for the identification and quantification of other phenolic compounds, may which play crucial role in the potential of the plant.

Antioxidant activity

Bioactive phenolic acids are the class of compounds that effectively inhibits free radicals because of their scavenging activity and therapeutically beneficial as they are common underlying cause of several disorders viz., cardiovascular, diabetes, aging, arthritis, cancer and inflammatory disorders.[28] Thus, in order to estimate the radical scavenging effect of the extract DPPH radical was used. Reducing power assay is selected to determine the total capacity (antioxidant) potential of extract as it is easy and precisely determines the reducing power by converting ferric ion in ferric chloride to ferrous. Total phenolic and flavonoid content in the methanolic extract was found to be 16.24 ± 1.61 mg/g GAE and 12.87 ± 0.043 mg/g QE. The DPPH radical scavenging activity of *E. scaber*, varies from 36.03% to 93.64 % at a concentration range of 0.1- 0.5 mg/ml and the IC_{50} was observed at 0.167±1.21 mg/ml. Ascorbic acid, quercetin and rutin are used as reference standard in DPPH radical scavenging activity (Table 2). While, Sheeba *et al.* 2012 analysis the *in vitro* antioxidant potential in methanolic extract of *E. scaber* by using DPPH model and curcumin as standard. Thy reported the IC_{50} value was 48±5 µg/ml.[34] Our study validates and confirms the previously obtained data.

Table 1. HPLC quantification of phenolics in aerial part of *E. scaber*.

Compound	Value (mg/g)*
1. Gallic acid	0.668 ± 0.05
2. Proto catechuic acid	0.086 ± 0.001
3. Chlorogenic acid	3.481± 0.015
4. Ferulic acid	0.842± 0.012

*n=3, values are mean ± S.D; §Rt are the mean values from ten replicates ± S.D.

Table 2: IC_{50} value of *Elephantopus scaber* Linn. in DPPH radical scavenging assay

Sl. no.	Species	IC_{50}*
1.	*Elephantopus scaber* (mg/ml)	0.167±1.21
2.	Quercetin (ug/ml)	5.93± 0.115
3.	Rutin (ug/ml)	6.8 ± 0.173
4.	Ascorbic acid (ug/ml)	3.86 ± 0.057

*n=3, values are mean ± S.D

In ferric reducing power assay, the sample (2-10 mg/ml) responds in par with the reference standard viz. ascorbic acid, quercetin, BHT (butylated hydroxy anisole) and, rutin. The absorbance was found to be linearly increasing with concentration at a regression coefficient of 0.983 (regression equation: 70.65x + 0.191) as shown in Figure 4. This reflect that the reducing capacity of extract increase with concentration. Natural phenolic acids (phenolic and flavonoid) in the plants serve as an indicator of their free radical scavenging activity and quantification of the same can be useful in accessing the antioxidant potential of species. Total flavonoid content (TFC) and Total Phenolic Content (TPC) were significantly ($P < 0.01$) rich in of *Elephentopus.*

Antidiabetic activity

The antidiabetic activity of *E. scaber* was determined by inhibition of biochemical activity of alpha amylase enzyme. Two models selected were iodine starch assay and 3, 5 di-nitro salicylic acid assay (Figure 5). Inhibitory activity of extract on α-amylase was observed in range of 0.1-0.5 mg/ml. In starch iodine assay, IC_{50} of extract was found to be 0.522 ± 0.05 mg/ml while it was 0.364 ± 0.04 mg/ml in DNS method. However, IC_{50} of the reference compound acarbose was found to be less than 0.025 mg/ml and 0.032 mg/ml in starch iodine assay and DNS method respectively (Figure 5). Potential activity was observed in crude extract of *E. scaber* in both the models, however as the standard acarbose is pure molecule, analysis of variance among these two (extract and standard) are statistically non-significant.

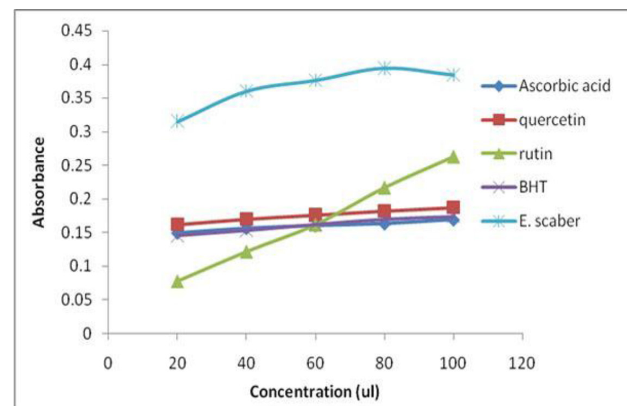

Figure 4: Ferric reducing power of *E. scaber* and reference standard was shown by increase in absorbance with the concentration.

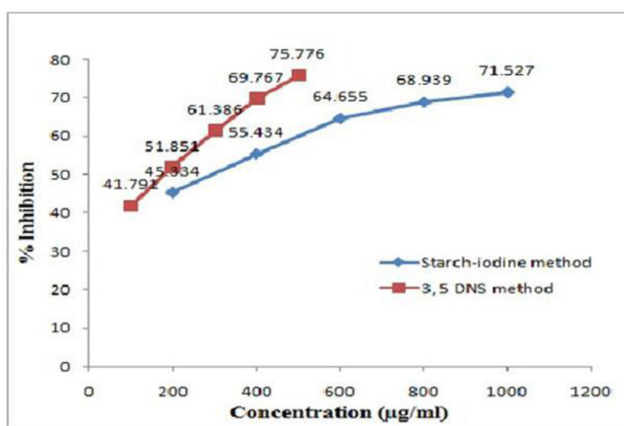

Figure 5: ***In vitro*** **anti diabetic activity of** ***E. scaber*** **extract by α- amylase inhibition methods. Graph point on the line represents the inhibition exhibited by sample (Y axis) with corresponding concentration (X axis) and standard deviation (S.D; trend lines represent S.D on x axis and y axis).**

This is preliminary study, however the result authenticate the traditional claim of *Elephantopus*, which need to be worked further on chemical and bioactivity guided fractionation to identify potential phytomolecules responsible for the same.

In addition, phenolic compounds are able to show antioxidant potential in many ways because they are the good hydrogen donating compounds which can react with reactive oxygen and nitrogen.[35] Identified phyto-compounds are efficient in quenching of oxygen radical and the rate of quenching of singlet oxygen by Gallic acid, proto catechuic acid, Chlorogenic acid and Ferulic acid acid is typically depends on the potential of species.[36] Therefore, this study may be helpful in the future for the selection of *Elephantopus* species and establish the importance of medicinal properties of plants by correlating with the traditional uses and scientific knowledge to discover the active potential medicine which will be utilized by the pharmaceutical industries.

CONCLUSION

In conclusion, it is evident from the present study that aerial parts of *E. scaber* contain considerable amount of different phenolics acids which in turn reflects in rich antioxidant activity. Further, the identified marker compound can be used to evaluate the batch to batch consistency of the herbal products made out of this plant.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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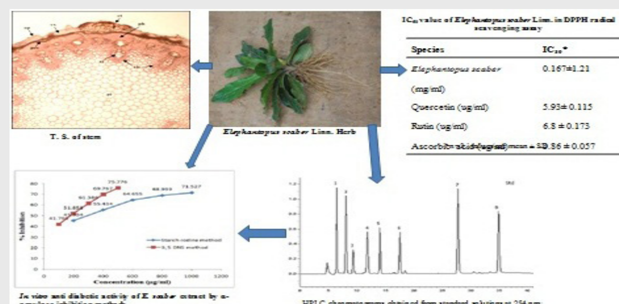
PICTORIAL ABSTRACT

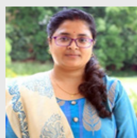


SUMMARY

- Physicochemical parameters are found within the limit as per Ayurvedic Pharmacopoea of India.
- Identification of phenolic bioactive compounds of *E. scaber* collected from Pallode, Kerala.
- Develoment of HPLC-UV method for the quantification of phenolic compounds.
- Promising *in-vitro* antioxidant and anti-diabetic potential were observed in the *E. scaber*.

About Authors

Dr. Pushpendra Kumar Shukla: Young Scientist in Pharmacognosy division, CSIR- National Botanical Research Institute, Lucknow. He is working in the area of analytical chemistry and chemotaxonomy, isolation of active metabolites and handling of sophisticated instruments like HPTLC, HPLC, OPLC, AAS, for identification and quantification of active metabolites in plant extract. He has 21 publications in peer reviewed journals.



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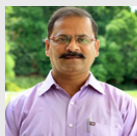
Dr. Bhanu Kumar: Senior Research Fellow in Pharmacognosy Division at CSIR-National Botanical Research Institute; Lucknow, INDIA. She is working in the area of anatomical description of medicinal plants, chemotaxonomy, product development, and quality control of herbal drugs. He has 12 publications in peer reviewed journals. He is awarded for the best research story writing under the scheme of AWASAR by DST, Govt. of India in the year 2019-20.



Dr. Sharad Srivastava: Senior 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- rospection 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 145 publications in peer reviewed journals, 17 patents and developed some technologies/ formulations, few has already been transferred to industry.

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