RP-HPLC quantification of five phenolic compounds in *Biophytum sensitivum* (L.) DC. (Oxalidaceae) and their biological evaluation

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*Biophytum sensitivum* (L.) DC. (Oxalidaceae) is a widely used medicinal plant in traditional medicinal systems across the globe. The present study deals with the RP-HPLC quantification of five phenolic acids, along with the *in vitro* antioxidant and antidiabetic activity of the aerial parts of the plant as a scientific investigation of traditional claims of its use as an antioxidant and antidiabetic agent. Anatomy of the stem was done to aid in exploring identification parameters for this plant. Among the five identified phenolic markers, caffeic acid exhibits the highest concentration (352.47 µg/gm), followed by ferulic acid (242.28 µg/gm), gallic acid (233.55 µg/gm), chlorogenic acid (192.06 µg/gm), and rutin (64.67 µg/gm). The total phenolic and flavonoid contents in the methanolic extract were found to be 87.0 ± 0.404 mg/gm GAE and 14.268 ± 0.055 mg/gm QE, respectively. The IC\textsubscript{50} value for the *in vitro* DPPH method was 0.164 ± 0.411 mg/ml.

*In vitro* antidiabetic activity was analyzed by the starch-iodine assay and the 3,5-DNS method which displayed IC\textsubscript{50} values of 0.636 ± 0.05 mg/ml and 1.214 ± 0.04 mg/ml, respectively. The *in vitro* study results suggest promising antioxidant and antidiabetic activity of the plant which supports its use in the traditional systems of medicine.

Keywords: *Biophytum sensitivum*, Oxalidaceae, HPLC, Phenolics, Antioxidant, Antidiabetic.

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A61K 36/00, C09K 15/00, A01D 16/02, C07C 39/00

*Biophytum sensitivum* (L.) DC. (Oxalidaceae), commonly known as “little tree plant” due to its miniature, tree-like appearance, is a reputed medicinal plant in traditional systems of medicine, especially Ayurveda. The flowers of this plant are considered as one of the ten sacred plants which are called as “Dasapushpam” in the traditions and culture of Kerala state in India\textsuperscript{1}. *Dashpushpam* is therapeutically active against various common ailments, including headache, dysentery, fever, jaundice and typhoid, etc\textsuperscript{2}. In *Ayurveda*, *B. sensitivum* is utilized for its tonic, stimulant, and expectorant properties. Traditionally, the leaves of this plant are very useful therapeutically and have been utilized in African countries for a long time\textsuperscript{3}. It is used in a variety of pathological complications namely, stomach ache, asthma, insomnia, convulsions, cramps, chest complaints, inflammation, tumors, and remedying chronic skin diseases, and it is also reported to have radioprotection, immunomodulation, antitumor, antioxidant, antibacterial, hypoglycemic, anti-metastatic, anti-angiogenesis, chemoprevention, anti-diabetic and anti-inflammatory activities\textsuperscript{4,5}. Studies have revealed that the leaf extract possesses antibacterial activities comparable to those of standard synthetic antibiotics\textsuperscript{6}. Oral administration of the ethanolic extract of the whole plant at a dose of 200 mg/kg body weight, to alloxan-induced diabetic rats displayed a decrease in the blood glucose level and serum cholesterol level, and an increase in the total protein level\textsuperscript{7}. Studies also reported that the leaf extract of *B. sensitivum* at a dose of 200 mg/kg body weight, stimulates beta cells to increase insulin and shows hypoglycemic effect\textsuperscript{8}. Several workers have attempted to study the quantitative and qualitative phytochemical parameters and have demonstrated presence of good quantities of flavonoids, phenols, and alkaloids in the different extracts of the plant\textsuperscript{9-12}. However, it was observed that there are no reports regarding the major phenolic acids in this plant. Therefore, simultaneous RP-HPLC quantification of the important phenolic acids was undertaken. In the
present study, an attempt has been made to identify and quantify the phenolic compounds in the aerial parts and, through \textit{in vitro} antioxidant and antidiabetic activity studies, an attempt has been made to explore and correlate the scientific basis which supports the use of the plant in traditional systems of medicine.

**Methodology**

**Chemicals**

Reference standards, i.e., gallic acid, protocatechuic 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 water was procured from Merck, India. All the standard compounds (analytical grade) were filtered through nylon 0.45 mm nylon filters (Millipore TM) before use in HPLC.

**Plant materials**

Fresh aerial parts of \textit{B. sensitivum} (L.) DC. were collected in October from an area near Pallode (phyto-geographical zone: Western Ghats, Altitude: 270 m, 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. 254054) was deposited in the Institute’s herbarium.

**Preparation of the plant extract**

The dried, aerial parts were ground with a laboratory grinder and sieved through 40 mesh sieve (up to 500 mm) to obtain a fine powder. Dried and powdered plant material (5 gm) was extracted three times successively with methanol:water (50 mL, 50:50 v/v) overnight on an orbital shaker at room temperature. The combined plant extract (150 mL) was centrifuged (8000 x gm) for 10 min to remove debris and concentrated up to half of the volume 75mL under reduced pressure on a rotary evaporator (Büchi, USA). The extract was fractionated against ethyl acetate (75 mL) three times with 75mL each and then concentrated on a rotary evaporator. The obtained residue was freeze-dried (Freezone 4.5, Labconco, USA) under high vacuum (133 x 104 mBar). A sample (5 mg) of the extract was dissolved in HPLC grade methanol (10 mL) before injecting into the HPLC.

**Microscopy**

The freshly collected plant material was preserved in 70 % ethanolic solution for anatomical studies. The anatomical study was performed according to standard and well-established methods. Free hand sectioning was done to obtain thin sections so that cellular details were clearly visible. Multiple transverse sections were cut and a fine section was chosen and washed with 30% alcohol, followed by 50 % alcohol. Then the section was double stained, first with safranin for 5 min, and then with fast green. The stained section was then treated with different dilutions of xylene, namely, 30 %, 50 %, 70 % and 90 % sequentially. Finally, the stained section was mounted with glycerin on a clean glass slide and observed under light microscope. The photomicrograph was taken with an Olympus, model CX31, digital microscope at magnification of ocular 10X and objective 10X.

**Standard and sample stock preparation for HPLC**

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

**HPLC conditions**

Separation, followed by qualitative and quantitative analysis of polyphenols was performed using an HPLC-UV instrument (Shimadzu LC-10A, Japan) equipped with a 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-C18 column (4.6 x 250 mm, i.d., 5 mm pore size) preceded with a guard column of the same material. Data were 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 and 14-40 min with 35-50 % of solvent B. The buffer and acetonitrile
were filtered through a 0.45 mm nylon filter and de-aerated in an ultrasonic bath before use. The method was validated for linearity, range, specificity, sensitivity, precision, and system suitability.\(^{14}\)

**In vitro antioxidant and antidiabetic activities**

Total flavonoid\(^{15}\) and phenolic\(^{16}\) contents were expressed in terms of mg/gm of QE (Quercetin Equivalent) and mg/gm GAE (Gallic Acid Equivalent) respectively based on the calibration curves of quercetin and gallic acid as standard. The antioxidant potential was analyzed using the DPPH radical scavenging assay.\(^ {17}\) The antidiabetic assay was performed based on the alpha amylase inhibition assay using two methods. The first method was carried out with slight modification based on the starch-iodine test.\(^ {18}\) Inhibition of enzyme activity was calculated as:

\[
\text{Inhibition of enzyme activity (\%)} = \left( \frac{C - S}{C} \right) \times 100
\]

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

The second assay was performed using the 3,5-dinitrosalicylic acid (DNS) method.\(^ {19}\) The results were expressed as % inhibition, calculated using the formula:

\[
\text{Inhibition activity (\%)} = \frac{\text{Abs (control)} - \text{Abs (extract)}}{\text{Abs (control)}} \times 100
\]

**Results**

**Microscopy**

The outline of the transverse section is smoothly circular and possesses the typical distinguishing characters of the stem. The epidermis is single layered, and made up of compactly arranged, rectangular, parenchymatous cells without or with very little intercellular space. The entire epidermis/epidermal layer is covered by a homogenously thick layer of cuticle followed by cortex, which is 5-6 celled thick. The hypodermis region is composed mainly of collenchymatous cells, oval in shape, thick-walled due to deposition of cellulose or pectin with little intercellular spaces. The pericycle is single layered and present in the form of patches. In the vascular system, secondary tissues are more abundant, xylem is present in the form of vertically radiated cylinders, particularly made of vessels and tracheids. The phloem parenchyma is somewhat fissured, probably due to the pressure exerted by the xylem. Pith is in the center, cells are round, thin walled with intercellular spaces (Fig. 1).

**RP-HPLC quantification**

The methanol extract for the analytical work represents an extractive yield of 11.6 %. HPLC for quantification of the phenolics in the extract revealed the presence of five marker compounds out of eight analyzed (Fig. 2). Among the identified markers, caffeic acid (352.47 µg/gm) exhibits the highest...
concentration followed by ferulic acid (242.28 µg/gm), gallic acid (233.55 µg/gm), chlorogenic acid (192.06 µg/gm), and rutin (64.67 µg/gm) (Table 1). Linearity calibration for standards were analysed at 0.5-50 µg/gm. LOD and LOQ were within the acceptable specified range. The method was found to be linear under regression analysis of area and concentration of standard(s).

**Antioxidant activity**

Ascorbic acid, quercetin, and rutin were used as the reference standards to compare their radical scavenging activity against that of the plant extract. Total phenolic content in the methanolic extract was found to be 87.0 ± 0.404 mg/gm GAE and the total flavonoid content was observed to be 14.26 ± 0.055 mg/gm QE. The radical scavenging activity was analyzed within the range 0.1 - 0.5 mg/mL and the IC₅₀ was determined as 0.164±0.411 mg/mL (Table 2).

**Antidiabetic activity**

The antidiabetic activity of *B. sensitivum* (L.) DC. was determined by inhibition of biochemical activity of the alpha amylase enzyme. Two model systems involved are the iodine starch assay and the 3, 5-dinitrosalicylic acid assay. Inhibitory activity of the extract on α-amylase was observed in the range 0.1-0.5 mg/mL. In the starch iodine assay, the IC₅₀ of the extract was found to be 0.636 ± 0.05 mg/mL, while it was calculated to be 1.214 ± 0.04 mg/mL using the DNS method. However, the IC₅₀ of the reference compound, acarbose, was found to be less than 0.025 mg/mL and 0.032 mg/ml in the starch iodine color assay and the DNS method, respectively (Fig. 3). Analysis of variance between the plant extract and standard acarbose are statistically significant, the potential biological activity was obtained in the plant extract by both model systems.

**Discussion**

In nature, there is great diversity among the living beings, especially among the plants. Plants of different families vary in their morphology, anatomy, and physiology, and as a result they also differ in their metabolite composition. Each and every plant family produces a number of secondary metabolites in response to the prevailing environmental conditions to maintain their proper growth and survival. In this process, there develops a group of plants which accumulate one or other metabolite and emerges as a plant of significant therapeutic value. Our ancestors were very rich in the ethnopharmacological knowledge but it is not well documented. The need for identifying and exploring the therapeutic potential of such useful medicinal plants is of very much significance to conserve our indigenous traditional knowledge. A number of studies have shown that the phytochemical screening and chromatographic analysis have played a crucial role in the Pharmacognosy and quality control of herbal drugs. In this connection, this study was conducted to strengthen the previous reports of this plant having antioxidant and antidiabetic activity, and to report on some of the major phenolic acids as marker compounds in this plant. The botanical study of the

<table>
<thead>
<tr>
<th>Phenolic compounds</th>
<th>Value (µg/gm)*</th>
<th>Rₜ§</th>
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<tbody>
<tr>
<td>Gallic acid</td>
<td>233.55 ± 0.05</td>
<td>6.38 ± 0.030</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>192.06 ± 0.01</td>
<td>9.19 ± 0.060</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>352.47 ± 0.05</td>
<td>12.03 ± 0.113</td>
</tr>
<tr>
<td>Rutin</td>
<td>64.67 ± 0.02</td>
<td>14.82 ± 0.419</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>242.28 ± 0.01</td>
<td>18.20 ± 0.427</td>
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* n=3, values are mean ± S.D; §Rₜ are the mean values from ten replicates ± S.D.

<table>
<thead>
<tr>
<th>Plant extract and references</th>
<th>IC₅₀*</th>
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<tbody>
<tr>
<td><em>B. sensitivum</em></td>
<td>0.164±0.411 (mg/mL)</td>
</tr>
<tr>
<td>Quercetin</td>
<td>5.931±0.115 (µg/mL)</td>
</tr>
<tr>
<td>Rutin</td>
<td>6.801±0.173 (µg/mL)</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>3.861±0.057 (µg/mL)</td>
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*mean ± S.D, n=3

Fig. 3—In vitro antidiabetic activity of *B. sensitivum* 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).
stem revealed the presence of distinct anatomical features which may add to the identification and authentication parameters of this plant. The phenolic compounds and flavonoids are considered to be involved directly in the radical scavenging activity of the natural products. The above study suggests that the targeted plant is a rich source of phenolics and flavonoids as compared with a known antioxidant activity having plant. The RP-HPLC study clearly indicates the presence of 5 major phenolic acids (out of eight analyzed) in relatively higher quantity, which might synergistically with other phytomolecules impart antioxidant activity, as compared with a few other plants reported to have antioxidant activity. The in vitro study results exhibited promising antioxidant and antidiabetic activity of the plant which supports its use in the traditional systems of medicine.

Conclusion

The study indicates that *B. sensitivum* (L.) DC. is rich in many phenolic acids and flavonoids. Since, among the phenolic acids, caffeic acid is present in good quantity, it can be used as a marker compound of this plant to monitor the batch to batch variations in the single drugs made by using this plant. It also has good antioxidant and antidiabetic properties as evident from the in vitro assays. It is a commonly available plant in India having several therapeutic values, it can become a plant of interest for the commercial production of herbal formulations having antioxidant or antidiabetic properties.

Acknowledgement

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References


