Simultaneous-HPLC Quantification of Phenolic Acids in Traditionally used Ayurvedic Herb *Diplocyclos palmatus* (L.) Jeffry

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**ABSTRACT**

**Introduction.** The present study deals with the simultaneous HPLC-quantification of phenolic acid(s) in the aerial parts of *Diplocyclos palmatus* (Cucurbitaceae) and evaluation of their bioactivity potential through *in vitro* antioxidant assay’s. **Method.** The HPLC elution was done using C18 column using gradient (binary phases) solvent system at a flow rate of 0.6 ml/min. Total phenolic and, flavonoid contents were determined and the antioxidant potential was estimated by four assay’s viz. DPPH radical scavenging assay, ferric reducing power assay, total antioxidant capacity and 2-deoxy ribose assay. **Results.** The species is rich in three phenolic acids, among which gallic acid (1708 µg/g) is in maximum concentration followed by caffic acid (437 µg/g) and protocateuchic acid (3377 µg/g). Total phenolic content was higher (10.5 mg/g) than flavonoid content (3.78 mg/g) and TAC was found at 0.137 mg/g ASE (ascorbic acid equivalent). IC50 of *D. palmatus* extract for scavenging of hydroxyl radical by 2-deoxy ribose and DPPH was at concentration of 125.61 ± 0.834 (µg/ml) and 353.71 ± 0.663 (µg/ml) respectively. **Conclusion.** The species (aerial part) was rich in phenolic acid with potential bioactivity, identified leads will be useful in further chemical characterization and pharmacological validation.

**Key words:** *Diplocyclos palmatus*, HPLC, Phenolic acid, Anti oxidant, Anti diabetic.

**INTRODUCTION**

*Diplocyclos palmatus* (L.) C. Jeffery (Cucurbitaceae), commonly known as “shiving” is ethnobotanically a highly valuable plant and is distributed widely in India as a climbing weed.1 In traditional systems of medicine, almost every part of the plant has medicinal property and is widely used by traditional healers for inflammation, jaundice and fever conditions.2,3 The *Bhilla* tribe of Maharashtra (India) use seed powder in gynaecological problems,4 whereas tribes of Akole tahsil (Maharashtra) use the decoction of root to cure bronchitis, however literature regarding scientific validation of the same was not available.5 In Ayurvedic system of medicine this species is well known for treating reproductive disorders,6 in male and female infertility and also, to prevent miscarriage.7 The plant is known to have alkaloids, saponins, phenols, steroids and cardiac glycosides etc.8 In *in vitro* studies revealed that *D. palmatus* (aerial part) contain antioxidant and anti-microbial activity.9,10 Seeds have analgesic, anti-inflammatory and antiarthritic activity.11 In *in vivo* anticonvulsant activity of leaves were also studied and chloroform extract was found to be more potential than aqueous and alcoholic extracts in controlling both MES and PTZ convulsions.12 *D. palmatus* seeds have antidiabetic potential, a dose of 150 mg/Kg results in 53.8% decrease in fasting blood glucose level.13 The leaves of the plant are used as an ingredient in special dietary preparation by tribes of Chhattisgarh region, as a tonic and as antidote in snake bite.14 However with the passage of time, importance of this Ayurvedic herb diminishes essentially due to upsurge of various other potential natural dietary sources/ medicinal herb and secondary due to extinction/ concentration of tribals and their associated knowledge.

Hence, through this study an attempt is made to scientifically explore the potential of *D. palmatus* aerial part and bring it to the scientific desk for further promotion of its usage in rural for health benefits. To the best of our knowledge this is first ever report on identification of phenolic acid(s) in targeted species and evaluation of its biological potential.

**MATERIAL AND METHODS**

**Plant materials**

Fresh sample of *D. palmatus* were collected in the month of October to November from the nearby area of Varanasi (Phytogeographical zone: Gangetic plains, 81 m, 25.3176°N, 82.9739°E), Uttar Pradesh, India. The sample was authenticated, and a voucher specimen was deposited in institute’s herbarium (LWG No. 254028).
**Standard solutions and reagents**

Reference standards viz., Gallic acid (≥ 95%), protocatechuic acid (≥ 97%), chlorogenic acid (≥ 97%), Caffeic acid (≥ 97%), ferulic acid (≥ 99%), rutin (≥ 95%), kaempferol (≥ 97%) and quercetin (≥ 97%) and 1-1-diphenyl-2-picrylhydrazyl (≥99%), DPPH were purchased from Sigma-Aldrich. HPLC grade solvents viz., acetonitrile, methanol, water, and all other solvents/chemicals (AR grade) were purchased from Merck, Mumbai, India.

**Preparation of samples**

The plant material was manually screened for any impurities and dried in shade, followed by drying in hot air oven at 45°C and then powdered with an electric grinder. The coarse powder (40 mesh) was subjected to cold maceration with 100% methanol. Extracts were continuously stirred for 6 hrs and kept up to 18 hrs at room temperature. The process was repeated thrice, filtered (whatman No.4) and the pooled filtrate was concentrated in a rotary evaporator (Buchi rotavpour, Switzerland) under controlled condition of temperature (40 ± 2°C) and pressure (40 bar). The extract was finally freeze dried and stored at 4°C for further use. 10 mg/ml of methanol extract was used for *in vitro* methods.

**HPLC Quantification of Phenolics**

**Preparation of stock and working solution**

The standard stock solutions (1 mg/mL) of phenolic acids (PA's) standard viz. gallic acid (GA), protocatechuic acid (PCA), chlorogenic acid (ChA), caffeic acid (CA), rutin (RT), ferulic acid (FA), quercetin (Qu) and kaempferol (Ka) were prepared in HPLC grade methanol and stored at 4°C, until used. Working solutions of lower concentration (0.1 mg/mL) were prepared by appropriate dilution of the stock solutions in methanol.

**Preparation of plant extract.**

The powdered plant material (5 g) was successively extracted thrice in methanol:water (50:50 v/v) overnight on an orbital shaker at room temperature. The combined plant extract (150 mL) was centrifuged (8000 x g) for 10 min to remove debris and concentrated up to half of the volume 75 mL under reduced pressure and temperature on a rotary evaporator (Buchi, USA). The extract was fractionated against ethyl acetate (75 mL) three times with 75 mL 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), filtered with membrane syringe filter (milipore) before injecting into the HPLC.

**HPLC conditions**

Separation followed by 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, rhodyne injection valve furnished with a 20 ml loop, on phenomenex Luna RP-C 18 column (4.6 x 250 mm, i.d., 5 mm pore size) preceded with guard column of same material. 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 (99:1.0 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. Method was also validated for linearity, range, specificity, sensitivity, precision and system suitability.

**In-vitro antioxidant activity**

Total flavonoids and phenolics 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 radical scavenging potential of *D. palmatus* were analysed by four different assay viz. Ferric reducing assay, phos-molybdate assay, 2-deoxyribose assay and DPPH radical scavenging assay.

**RESULTS**

A review of literature on the traditional knowledge about *D. palmatus* reveals that the herb is conventionally used since long ago among tribes and rural people as a potential remedy for many reproductive disorders but due to lack of considerable focus on healthcare system of such communities, this highly valuable herb is losing its charm. However, the therapeutic potential of the species viz. fruit, leaves etc. is sparingly documented. Hence we explored the antioxidant and, anti-diabetic potential of its aerial part to create a preliminary background data source which can be utilised in near future to examine other biological properties. This further aids to the knowledge of locals for its encouraged use due to medicinal action.

**Quantification of identified phenolic acid(s) (PAs)**

The methanolic extraction of the powdered drug yielded an extractive value of 140 mg. HPLC for quantification of the phenolic acids in the extract revealed the presence of three marker compounds out of eight analyzed standard mixture solution (Figure 1). Among the identified markers gallic acid (1708 µg/g), was found in highest concentration followed by caffeic acid (437 µg/g) and protocatechueic acid (337.7 µg/g) as shown Table 1. The identification of PA's was based on a comparison of retention times and UV spectra of reference standard(s) with the corresponding peaks in the extract. It is noteworthy that the identified markers are not common in nature and thus can be used as characteristic marker for quality control of the herb. Beside this, the bioactivity of phenolic acids is well evident as underlying cause of various disorders. Linear calibration for standards were analysed at 0.5-50 µg/g. LOD and LOQ were within the acceptable limit as per ICH guidelines. The method was found to be linear under regression analysis of area Vs. concentration of standard(s). To the best of our knowledge this is first ever report on HPLC quantification of phenolics in *D. palmatus*.

**Antioxidant activity**

Quantification of phenolic acid(s) lead us towards identification/detection of their bioactivity, in lieu of which polyphenolic content was spectroscopically analysed in reference to standard compound (0.1 mg/mL). Total phenolics were found to be higher than total flavonoids exhibiting straight line regression equation and statistically accepted regression coefficient (Table 2).

The reducing power of extract was estimated by reducing the ferric ion in presence of folin’s phenol reagent. The reducing power of *D. palmatus* increases linearly (eq: y = 0.0013x - 0.0746, r²= 0.988) with increase in concentration as shown in Figure 2, similar to reference standard(s) viz. ascorbic acid, quercetin, rutin and BHT. In order to analyse the antioxidant potential of non polar part of *D. palamtus* extract, total antioxidant capacity (TAC) was evaluated by phosphomolydneum method. IC₅₀ of extract was found to be 0.137 mg/g ASE (ascorbic acid equivalent) based on calibration curves of standard ascorbic acid (0.1 mg/mL). Linear curve fitting was observed by five dilutions (20-100 µl) of standard exhibiting straight line regression equation and statistically accepted regression coefficient.

IC₅₀ of *D. palmatus* extract in 2-deoxy ribose was found at a concentration of 125.61 ± 0.834 (µg/ml). The scavenging effect of DPPH radical was concentration dependant and potentially varied for ascorbic acid, quercetin, rutin, butylated hydroxy toluene (BHT) and plant extract.
Ascorbic acid exhibits maximum inhibition of 77.57% which is followed by quercetin, rutin and BHT having inhibition of 72.43, 71.48, and 62.10% respectively.

Inhibition (%) of extract varies from 31.25 – 66.9% at a concentration range of 0.2 mg/ml to 1 mg/ml. IC$_{50}$ of extract was observed to be 353.71 ± 0.663 (µg/ml), significantly different from standard and decreased in order of $D.\text{palmatus}$ extract $>$ rutin $>$ quercetin $>$ ascorbic acid $>$ BHT and hence indicating that BHT is potent inhibitor of free radical in all.

In addition to this, the traditional claim of $D.\text{palmatus}$ as antidiabetic was well reported in seed, however an attempt was made to evaluate the same in aerial part(s). Antidiabetic potential of $D.\text{palmatus}$ aerial part are evaluated by in vitro models via inhibition of alpha amylase enzyme. Inhibitory activity of extract on a-amylase was observed at a concentration range of 0.2 mg/ml to 1 mg/ml. IC$_{50}$ of extract was observed to be 353.71 ± 0.663 (µg/ml), significantly different from standard and decreased in order of $D.\text{palmatus}$ extract $>$ rutin $>$ quercetin $>$ ascorbic acid $>$ BHT and hence indicating that BHT is potent inhibitor of free radical in all.

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CONCLUSION

The available literature on $D.\text{palmatus}$ reveals its traditional medicinal usage, although scientific literature scarcely available on its pharmacological properties. Fruit and seeds are the most commonly used parts and thus we made an attempt to explore the medicinal potential of its aerial part, which can be utilized for various medicinal purposes in near future. It is interesting to note that the extract contains high content of phenolic acid(s) and is characterized by presence of therapeutically valuable phenolics viz. chlorogenic acid, gallic acid, caffic acid and protocatechuic acid. This is further confirmed by high phenolic content accompanied with high antioxidant activity. The traditional claim of species as antidiabetic drug was also revalidated through in vitro assays. Hence, the study was conducted to explore and highlight the therapeutic properties of this underutilized Ayurvedic herb. The study needs be further elaborated for more detailed chemical characterization and pharmacological inspection of $D.\text{palmatus}$.

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CONFLICT OF INTEREST
None

REFERENCES

GRAPHICAL ABSTRACT

SUMMARY

- The investigation on aerial part of D. palmatus, reveals the presence of three phenolic markers.
- Gallic acid was found in highest concentration followed by caffeic acid and proto catechuic acid.
- IC₅₀ of test extract was obtained at concentration of 125.61 and 353.71 ug/ml for hydroxyl radical scavenging activity by 2-deoxy ribose and DPPH stable radical respectively.
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