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# HPLC analysis of phenolic acids and antioxidant activity of *Dioscorea belophylla* (Prain) voigt haines from Andhra Pradesh, India

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**Abstract**
*Dioscorea belophylla* is of family *Dioscorea*ceae and it is commonly known as yam. The tubers are well known source of steroidal saponin compounds and is of economical and pharmacological interest. In the present study, the rhizome collected from the natural forests of eastern Ghats were investigated for the presence of phytochemicals in the different solvent extracts of tubers. The identification of polyphenolics in the tubers of *Dioscorea belophylla* were carried out by HPLC method. The results of investigation showed the presence of phytoconstituents in different solvent extracts. The total phenoliccontent was found to be 9.231±0.554,23.149±0.600and 14.321±0.21mg/g gallic acid equivalent and the total flavonoid content was found to be 4.335±0.334,15.498±0.461 and8.869±0.658mg/g quercetin equivalent of the dry extract of ethyl acetate,80% methanol and aqueous extracts, respectively. The high percentage radical scavenging activity detected in methanolic exracts(30.52% µg/mL). The HPLC analysis also indicated thepresence of phenols and polyphenolics in various amount in this wild edible plant. Above results indicate that the tubers of *D.belophylla* could be a useful natural antioxidant and antibacterial agent, and may be used for further study on the plant.

**Keywords:** *Dioscorea belophylla*, Phytochemicals, Antioxidant activity, HPLC, terpenoids, saponin

## 1. Introduction

Plants are an important source of medicines and pharmaceutical materials, play a major role in global health, and have been used for thousands of years [1,2]. Nowadays, approximately 80% of antimicrobial drugs, cardiovascular drugs, immunosuppressants and anticancer drugs are of plant origin [3], and natural products and their derivatives represent more than 50% of all the drugs in modern therapeutics, of which higher plants contribute no less than 25% of the total [4,5]. In addition, WHO supports, suggests and encourages traditional/herbal remedies in national healthcare programs as these medicines are easily available, low cost, safe and people rely on them [6]. The WHO also supports and encourages the study of plants and extracting medicines from them, with the initial process usually begins with identifying plant species of interest, especially those with documented traditional use [7], so establishing pharmacognostic standards are very important parameters for evaluation of medicinal plants [6,8]. This is followed by extraction, fractionation, and isolation of the bioactive compound where applicable. In addition, it comprises determination of quantity and quality of bioactive compounds [9,6]. During the last three decades antioxidant-based drugs for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease, and cancer have appeared [10]. Phenolic compounds such as phenolic acid, flavonoid and tannin are commonly found in plants, and they have been reported to have multiple biological effects, including antioxidant activity [11,12]. The antimicrobial properties of plants are due to the presence of phytochemicals such as phenolic and tannin compounds in them [13,14]. Yam is widely grown in many West African countries and manyspecies are eaten in various parts of the world [15]. Yams are edible starchy tubers and are of cultural, economic and nutritional importance in the tropical and subtropical regions of the world [16]. *Dioscorea* species especially from the tropical areas, are a very important source of secondary metabolites, and used in pharmaceutical industry and medicine [17]. The most predominant phytochemical characteristic of yam is the presence of dioscorine alkaloid and diosgenin saponin. Yams have been well respected by the herbalist community for generations due to their potency in enhancing fertility in males. This may be due to the presence of steroidal sapogenins such as diosgenin. It is used as precursor for the synthesis of hormones and are precursors for the

semi synthesis of birth control pills as well as similar hormones and corticosteroids [18]. Its roots contain diosgenin, which is a compound often used in the manufacture of progesterone and other steroid drugs. Tubers of *D.belophylla* (fig 1)used by local people to treat health problems such as anti-rheumatic, dysentery, headache and fever. The leaves are used to malaria. Yam has the high antioxidant activity but low phenolic content. Yam is ranked the seventh of highest antioxidant concentration among the 11 roots and tubers analyzed [19].

The plants of *Dioscorea*ceae, have many medicinal properties, due to the presence of phytochemical compounds. Plant parts have high nutritional value and known to exhibit antioxidant activity. Most of the research is focused on the tubers of *Dioscorea* species. The phytochemical screening and antioxidant evaluation as well as HPLC of tuber extracts of *D. belophylla*has not been reported. Therefore, the present study necessitates investigation on the above-mentioned criteria.

**Fig 1:** HPLC Analysis of Phenolic Acids and Antioxidant Activity of *Dioscorea belophylla* (Prain) Voigt Haines from Andhra Pradesh, India

## 2. Materials and Methods

### 2.1. Phytochemical Study

#### 2.1.1. Preparation of the extracts

The tubers of *Dioscoreabelophylla* were collected from east godavari district, eastern Ghats during November 2017. The plant was identified on the basis of flora [20]. And the voucher specimens of the taxa were deposited in Sri Krishnadevaraya University Herbarium, SKU, Anantapuramu, Andhra Pradesh. The collected tubers wereshed dried separately and powdered by mixer. The powder was weighed and preserved in polythene covers and labelled. The tuber powder of 50 g each were weighed and used for soxhlet extraction. Three solvents were used for the extraction based on their polarity viz., hexane, ethylacetate, and methanol.

The dried powder of the aerial parts (50 gm) were defatted with ethyl acetate (boiling point 60-80 °C) in Soxhlet extractor. Theethyl acetateextract was filtered. The marc left after ethyl acetateextract was dried completely in hot air oven below 50 °C and then packed well in Soxhlet apparatus and extracted with 80% methanol (80-90 °C), until the extraction was completed. The 80% methanol extract was filtered., after that the marc was extracted with distill water. The aqueous

extract was filtered. All the obtained extracts were separately evaporated to dryness by rotary evaporator and the percentage yield was calculated for each extract. The dried crude extracts were stored in air tight bottle at 4 °C for farther study [21, 22].

**2.1.2. Qualitative phytochemical analysis**

Various qualitative tests were performed to determine the chemical composition of the extracts according to standard methods [21, 22].

**2.1.3. Determination of total phenolic content (TPC)**

The total phenolic content of the ethyl acetate, 80% methanolic and water extracts was determined by using Folin-Ciocalteu reagent following a slightly modified method of Ainsworth [23]. Gallic acid was used as a reference standard for plotting calibration curve. A volume of 0.5 mL of the plant extract (1 mg/ml) was mixed with 2 ml of the Folin-Ciocalteu reagent (diluted 1:10 with deionized water) and were neutralized with 4 ml of sodium carbonate solution (7.5%, w/v). The reaction mixture was incubated at room temperature for 30 min with intermittent shaking for color development. The absorbance of the resulting blue color was measured at 765 nm using UV-VIS spectrophotometer. The total phenolic contents were determined from the linear equation of a standard curve prepared with gallic acid (20, 40, 60, 80, 120, and 140 µg/ml). The content of total phenolic compounds expressed as mg/g gallic acid equivalent (GAE) of dry extract. All the samples were analyzed in three replications.

**2.1.4. Determination of total flavonoid content (TFC)**

Total flavonoid content ethyl acetate, of 80% methanolic and water tuber extracts was determined in extracts according to colorimetric method described by [24] with some modification. Briefly 0.5 ml extract (1 mg/ml) was added in three bijou bottles and mixed with 2 ml of distilled water. Subsequently add 0.15 ml of sodium nitrite (NaNO2, 5% w/v) into each bottle and the reaction mixture was allowed to stand for 6 min. Then 0.15 ml aluminium trichloride (AlCl3, 10%) was added and allowed to stand for 6 min, followed by addition of 2 ml of sodium hydroxide (NaOH, 4% w/v) to the reaction mixture. Then distilled water was added to the mixture to bring the final volume up to 5 ml. The reaction mixture was mixed thoroughly and allowed to stand for another 15 min. Then absorbance was measured at 513 nm against the same mixture but without extract as a blank using spectrophotometer. Methanol was used as blank. The final absorbance of each sample was compared with a standard curve plotted from quercetin (2.5-80 µg/ml). The total flavonoid content was expressed in mg of quercetin per gram of extract. The whole experiment was conducted in three replicates.

**2.2. Antioxidant Study**

**2.2.1. DPPH Radical Scavenging Activity**

The free radical - scavenging activity of each extracts was determined as described by Chan *et al*. with slight modification [25]. Different dilutions of the extract (10, 20, 40, 60 and 80 µg/ml) were prepared. DPPH solution was also prepared by dissolving 6.0 mg of DPPH in 100 ml methanol. Then, 1 ml of extract from each dilution was added into the test tube containing 2 ml of DPPH solution. Control was prepared by adding 1 ml of methanol to 2 ml of DPPH solution. The mixture was shaken vigorously and was left to stand in the dark for 30 min. Quercetin was taken as reference compound. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm. The scavenging activity of extract on DPPH radical was calculated using the following equation: Inhibition% = [(A0 -A1) /A0] x 100; A0 is the absorbance of control and A1 is absorbance of test.

**2.2.2. Calculate $IC_{50}$ value**

The 50% inhibition ($IC_{50}$) of antioxidant activity was calculated as the concentrations of samples that inhibited 50% of scavenging activity of DPPH radical's activity under these conditions [26]. The effective concentration of sample required to scavenge DPPH radical by 50% ($IC_{50}$ value) was obtained by linear regression analysis of dose-response curve plotting between% inhibition and concentrations. The data were presented as mean values ± standard deviation (n = 3).

**2.2.3. High Performance Liquid Chromatography (HPLC) Analysis -HPLC Analysis**

The chromatographic analysis was carried out following the method as described by Marco Ciulua [27] with minor modification. The mobile phase contains acetonitrile (Solvent A) and aqueous trifluoro acetic acid (TFA, 0.01% v/v) (Solvent B), the column was thermostatically controlled at 220 C and the injection volume was kept at 20 µl. A gradient elution was performed by varying the proportion of solvent A to solvent B. The gradient elusion was 1% A and 99% B with flow rate 0.5 ml/min in 5 min, from 1% to 25% A with flow rate 0.5 ml/min for 16 min, 45% A, with flow rate 0.5 ml/min for 8 min. from 45 to 1% A with flow rate 0.5 ml/min in 5 min. The mobile phase composition back to initial condition (solvent A: solvent B: 1: 99) in 34 min and allowed to run for another 1 min, before the injection of another sample. Total analysis time per sample was 35 min. The various concentrations of (20, 40. 60, 80 and 100 µg/ml) phenols working standards were injected into the HPLC column separately and the retention times were noted and used to identify the vitamins in the sample. HPLC Chromatograms of all phytocompounds were detected using a photo diode array UV detector at four different wavelengths (210, 245, 275 and 290 nm) according to absorption maxima of analysed compounds. Each compound in the plant extract were identified by its retention time and by spiking with standards under the same conditions.

**3. Results and Discussion**

**3.1. Qualitative phytochemical studies**

The phytochemical screening methods are employed for identification of the species-based specific compounds in herbal medicines [28, 29]. The existence of secondary plant metabolites including alkaloids, flavonoids, glycosides, tannins, steroids, etc. might be attributed to the various pharmacological effects such as anti-inflammatory, antibacterial, antiviral, antioxidant and anticancer [30, 31]. Phytochemical screening revealed the presence of alkaloids, terpenoids, phenols in all extracts. Saponins were present in ethyl acetate and methanolic tuber extracts. (Table 1). Similarly, phytochemical screening of *D.pentaphylla* showed the saponins.

Nilofer *et al*. (2013) [32] reported the phytochemical screening of root tubers of *D. pentaphylla, D. alata*, *D. oppositifolia*, *D. bulbifera*, *D. glabra* and *D. pubera*. Powdered tubers were treated with methanol and ethyl acetate. Phytochemical screening revealed that, in the ethyl acetate extracts, terpenoids and saponins were present in *D. pentaphylla*,*D. alata*, *D. bulbifera*. Terpenoids were present in all methanolic

extracts, except *D. glabra*. Saponins were present in all methanolic extracts except *D. alata*. The *D. belophylla* tuber extracts were dissolved in respective solvents and subjected to preliminary qualitative phytochemical screening for tannins, saponins, flavonoids, terpenoids, steroids, anthraquinones, phlobatannins, glycosides, reducing sugars and alkaloids using standard procedures. The genus *Dioscorea* is known to contain a large number of biologically active compounds, including polyphenols, carotenoids, and terpenes [33].

**Table 1:** Results of phytochemical screenings of successive extracts of *D.belophylla*

| Phytochemicals | Ethyl acetate | Methanol | Aqueous |
|---|---|---|---|
| Saponins | + | +++ | ++ |
| Flavonoids | + | +++ | ++ |
| Triterpenoids | ++ | ++ | +++ |
| Alkaloids | + | ++ | + |
| Glycosides | + | + | ++ |
| Phenolic compounds | ++ | +++ | + |
| Steroids | - | ++ | ++ |
| carbohydrates | - | + | + |
| coumarins | - | - | - |
| Tannins | + | + | + |

### 3.2. Determination of the total phenolic and flavonoid content

Estimation of total phenols, flavonoids and saponins were performed in this study. *D. belophylla* tuber aqueous (14.321±0.21μgGAE/g), ethanolic (9.231±0.554μgGAE/g) and methanolic extracts (23.149±0.600 μgGAE/g) showed high total phenolic contents compared to other solvent extracts *D. belophylla* tuber ethanolic (8.869±0.658μgGAE/g), ethanolic (4.335±0.334μgGAE/g) and aqueousextracts (15.498±0.461μgGAE/g) showed high total flavonoid contents compared to other solvent extracts. Terpenoids have antihepatotoxic properties, thus helping to prevent liver damage (cirrhosis). They equally have antimicrobial or antiseptic properties. Total saponins in tuber extracts of methanolic (13.521±0.051) aqueous extracts (24.735±1.456) (mg/g DW) (table 2). Saponins are expectorants, cough suppressants and administered for hemolytic activities [34]. *D. villosa* have five steroidal saponins; Dioscin, Protodioscin, Meprotodioscin, Perrisaponin and Progenin II. It also has a spirostanolglycoside. Other constituents include phytosterols (beta-sitosterol), alkaloids and tannins that make this plant useful as an antiinflammatory, diuretic, antispasmodic, cholagogue, diaphoretic and vasodilator. A decoction of the root is used to alleviate many of the symptoms of menopause. It is also used to treat irritable bowel syndrome, gastritis, painful menstruation, and in small doses is especially helpful in treating the nausea of pregnant women [35]. *Dioscorea* attributes antimicrobial activities to the presence of secondary metabolites, Diosgenyl saponins the most abundant steroid saponins, reported to exert a large variety of biological functions [36].

The methanolic extract has been shown to be the most potent, rich in phenols and flavonoids, known for its antioxidant and therapeutic properties.

### 3.3. Antioxidant Study

#### 3.3.1. DPPH radical scavenging activity assay

The antioxidant activity of ethyl acetate, 80% methanolic and aqueous extracts of the tuber of. *D.belophylla*was determined by using In Vitro DPPH method. The free radical DPPH in solution is purple in color with its odd electron and gives a strong absorption maximum at 517 nm and the color of DPPH turns from purple to yellow. Result of antioxidant activity test can be seen in Table 2. The antioxidant activity was increased by increasing the extract concentration in a dose-dependent manner, the greater the color change, the greater the antioxidant capacity, which is represented by the lower IC50 value. The IC50 values of quercetin, 80% methanolic and water extracts were found to be 30.521±0.051, and 24.735±1.456 µg/ml respectively. The result indicates that the methanolic extract has the strongest antioxidant activity among the extracts, and this result is consistent with its high phenolic and flavonoid content. The water extract demonstrated moderate activity, reflecting its lower phenolic and flavonoid content, while the ethyl acetate extract showed weak antioxidant activity. Murugan and Mohan [37] reported the DPPH radical scavenging activity of *D. esculenta* tuber methanolic extract which showed 79.33% scavenging activity at 1000 µg/mL. The reducing power of methanolic extract was very potent in *D.alata* tubers [37] and methanolic extract of *D. esculenta* tubers showed high reducing power[38]. Phenolic compounds are the principal antioxidant constituents of natural plant products are composed of phenolic acid and flavonoids. These compounds are potent radical terminators by donating a hydrogen atom to the radical and preventing lipid oxidation at initial step. These compounds have multiple biological effects like anticancer, anti-proliferative, antimicrobial, wound healing and antibacterial activities including antioxidant activity [14]. The findings align with earlier studies that reported the antioxidants activity in the genus *Dioscorea* [22],

**Table 2:** Phenolic acid, flavonoid and saponins content in *D.belophylla*. Result of DPPH free radical scavenging by the studied extracts and quercetin

| Solvent | TPC (GAEmg/g DW) | TFC (QEmg/g DW) | TAC (ASE mg/g DW) | TSC (mg/g DW) | $IC_{50}$µg/ml |
|---|---|---|---|---|---|
| Ethyl acetate | 9.231±0.554 | 4.335±0.334 | 1.784±0.075 | - | - |
| Methanol | 23.149±0.600 | 8.869±0.658 | 22.981±0.980 | 13.521±0.051 | 30.521± 0.051 |
| aqueous | 14.321±0.21 | 15.498±0.461 | 11.428±0.405 | 24.735±1.456 | 24.735±1.456 |

#### 3.3.2. High Performance Liquid Chromatography (HPLC) Analysis -HPLC Analysis

The presence of multiple phytocompounds in methanolic tuber extracts of *D.belophylla* tuber extracts were further confirmed by HPLC analysis. The individual phytocompounds, present in *Dioscorea* tuber extracts compared with reference standards like Diosgenin, Gallic acid, Quercetin and Rutin respectively. The approximate retention time of the compounds gallic acids, rutin, quercetin in the tuber extract were more or less tallied and very adjacent to the standard phenolic compounds in the HPLC chromatograms of diosgenin, curcumin, gallic acids, rutin, and quercetin (Fig 2). Three peaks were observed with a retention time of 3.5, 4.3, and 7.4 min respectively. These results clearly indicate that saponin compounds are the major

constituents among the methanolic tuber extracts of the *D.belophylla* followed by diosgenin, flavonoids and phenols.

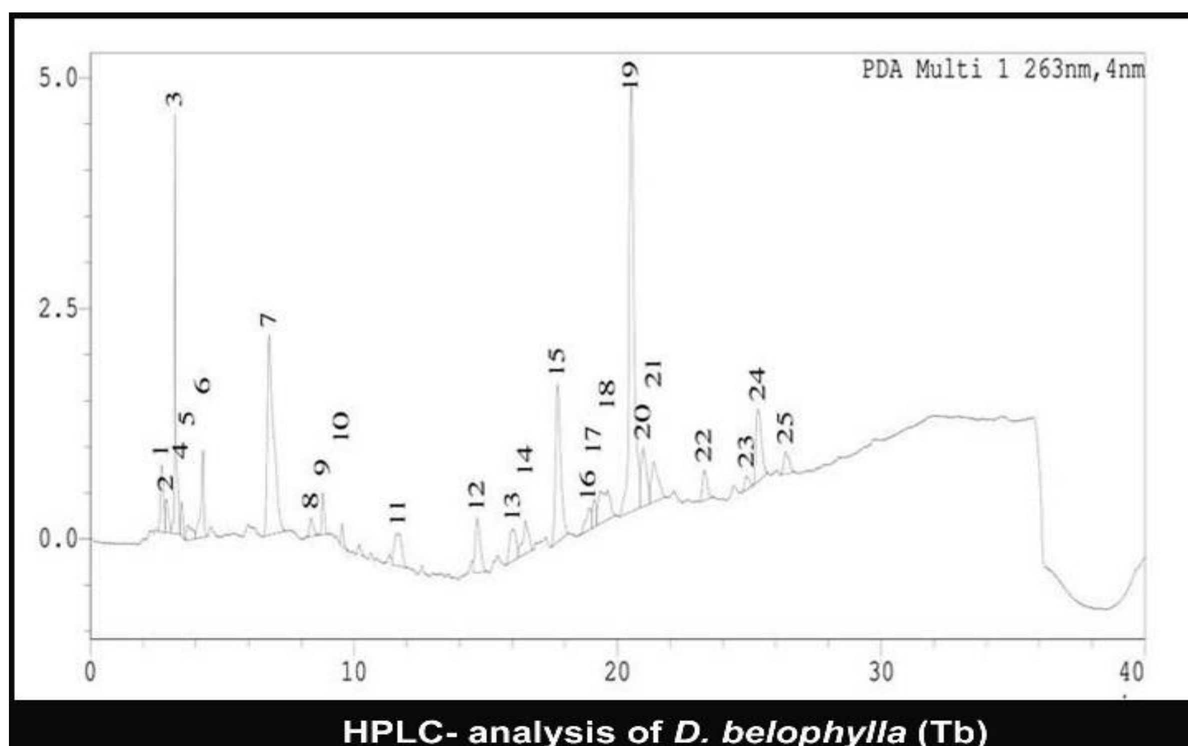


**Fig 2:** HPLC chromatogram of *D.belophylla* tuber extract

*Dioscorea* species have been identified as a possible source of phenols as well as phenolic acids. Zhao *et al*. [41] evaluated the total phenolic acids of two yam species (*D. oppositifolia* and *D. hamiltonii*) using an HPLC system. The presence of flavonoids has been reported in wide varieties of yams. A recent study by Padhan *et al*. [42] investigated the flavonoid content of nine *Dioscorea* species including *D. alata*, *D. oppositifolia*, *D. hamiltonii*, *D. bulbifera*, *D. pubera*, *D.pentaphylla*, *D. wallichii*, *D. glabra* and *D. hispida*. Their findings revealed flavonoid content ranging from 0.62 to 0.85 mg/g dry weight, of which levels detected in *D. alata* and *D. hispida* were significantly lower compared to other *Dioscorea* species. In addition, the authors reported potential antioxidant activities of the yam tuber extracts to range from 1.63 to 5.59%. *D. bulbifera* and *D. pubera* with significantly higher amount of bioactive compounds such as flavonoids exhibited higher radical scavenging activity compared to other *Dioscorea* species irrespective of the screening method (DPPH, ABTS, nitric oxide and superoxide radical scavenging assay) used [42].Alkaloids have been reported in several species of yams (*D. alata*, *D. oppositifolia*, *D. hamiltonii*, *D. bulbifera*, *D. pubera*, *D. pentaphylla*, *D. wallichii*, *D. glabra* and *D. hispida*) at values between 7.2 and 16 mg per 100 g dry weight [43] alkaloid content in different *Dioscorea* species varied from 7.2 to 16 mg/100 g [42]. Alkaloid derivatives have analgesic, antitussive, and antibacterial pharmacological activities [42]. *D. belophylla*is a medicinal plant of immense importance. The phytochemical screening indeed has revealed the presence of saponins, phenols and terpenoids in all solvent extracts. The extracts of tuber have indicated radical scavenging potentials. The overall results of the present study have provided evidence *D.belophylla* containalkaloids, phenols, terpenoids and saponin compounds by HPLC. tuber extracts revealed the presence of high antioxidant activity. Thus, the results provided a strong scientific evidence for considering the wild *D.belophylla*as natural antioxidants and might be used for treatment in oxidative stress-induced disease conditions. Therefore, *D.belophylla*extracts are good sources of phytochemicals.

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