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Application of anatomy and HPTLC in characterizing species of *Dioscorea* (Dioscoreaceae)

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Abstract

The edible tubers from different species of *Dioscorea* are a major source of food and nutrition for millions of people. Some of the species are medicinally important but others are toxic. The genus consists of about 630 species of almost wholly dioecious plants, many of them poorly characterized. The taxonomy of *Dioscorea* is confusing and identification of the species is generally problematic. There are no adequate anatomical studies available for most of the species. This study is aimed to fill this gap and provides a detailed investigation of the anatomy and micromorphology of the rhizomes and tubers of five different species of *Dioscorea*, namely *D. balcanica*, *D. bulbifera*, *D. polystachya*, *D. rotundata* and *D. villosa*. The primary features that can help in distinguishing the species include the nature of periderm, presence or absence of pericyclic sclereids, lignification in the phloem, types of calcium oxalate crystals and features of starch

grains. The descriptions are supported with images of bright-field and scanning electron microscopy for better understanding of these species. The diagnostic key of anatomical features included in this paper can help distinguish the investigated species unambiguously. Additionally, HPTLC analyses of authentic and commercial samples of the five species are described.

Keywords

Anatomy; Microscopy; HPTLC; *Dioscorea*; Yams

Introduction

Yams—the edible starchy tubers of various species of *Dioscorea* L. (Dioscoreaceae)—are of cultural, medicinal, economic and nutritional importance across their range of distribution and are reported to be good sources of essential nutrients [1]. Several *Dioscorea* species, along with their cultivars, are widely cultivated for their nutritional value. In West Africa, the edible yams of *D. rotundata* Poir. (white yam), *D. cayenensis* Lam. and *D. alata* L. (water yam) are the most commonly cultivated yams that serve as major sources of food for millions of people [2]. Yams are also medicinally important and are reported to be used for treating various conditions including allergy, dermatopathy, cancer, diabetes mellitus, inflammation, lipoperoxidation, renal infections and rheumatoid arthritis [3]. *D. villosa* L., commonly called wild yam, is mainly used for medicinal purposes in the USA, where it has traditionally been used for easing menstrual cramping and for threatened miscarriage. It is also used in the treatment of colic, nausea and morning sickness, inflammation, osteoporosis, menopausal symptoms and various other conditions [4, 5]. Diosgenin, a steroidal sapogenin present in the roots of wild yam, is used as a precursor for the synthesis of steroidal compounds, oral contraceptives and sex hormones [6].

Some species of wild-growing yams are, however, toxic, unpalatable, bitter or acrid in taste and may cause vomiting and diarrhea if eaten raw or under-processed [7]. The presence of high levels of calcium oxalate crystals in wild yams could possibly be the reason for their acrid taste, inflammation and occasional toxicity. Ingestion of high amounts of these sharp needles may cause inflammation of the tongue, mouth and throat [8].

The genus *Dioscorea* consists of about 630 species distributed throughout the tropical and warm temperate regions of the world [9, 10]. The genus is composed almost entirely of dioecious plants which pose difficulties in accurate identification. Male and female plants of a single species were, in the past, described as two distinct taxa. Many of the species of this group show a high degree of polymorphism exhibiting complex patterns of variations across their range of distribution. This has resulted in descriptions of several infra-specific taxa adding to the already long list of synonyms. Thus the taxonomy of *Dioscorea* is confusing and ‘notoriously problematic’ [11]. It is extremely difficult to identify yams in the absence of aerial parts, but in general only the underground part is used commercially. The rootstocks as well as the aerial parts of different species often look alike. *D. villosa* is often confused with *D. polystachya* Turcz. and may easily be mistaken for *D. floridana* Bartlett [12] at the vegetative state. *D. bulbifera* L. may be confused with *D. alata* due to similar

morphology. *D. polystachya* has in the past been mistaken for *D. oppositifolia* L. The common name of the former species ‘Cinnamon vine’ may actually refer to the latter species as it has tri-nerved oblong leaves resembling those of *Cinnamomum* Schaeff. species. The common names of yams are often confusing, because different species are known by the same common name. Several species of *Dioscorea* are called ‘wild yam’ in different regions of the world. The name ‘yam’ is also applied to other different groups of plants. In parts of USA, *Ipomoea batatas* (L.) Lam. (sweet potato), belonging to the morning glory family Convolvulaceae, is referred to as ‘yam’. In New Zealand, the popular vegetable tuber of *Oxalis tuberosa* Molina (Oxalidaceae) is called ‘yam’. The tuberous root of *Pachyrhizus erosus* (L.) Urb. (Fabaceae) is called ‘Mexican yam’, and the cormous root of species of *Amorphophallus* Blume ex Decne. (Araceae) is commonly referred to as ‘elephant foot yam’.

Despite the prevailing confusions with the identification of different taxa of *Dioscorea*, many species are poorly characterized and there are no adequate comparative studies available. In this situation, comparative anatomical and microscopic studies can play critical roles in distinguishing the species and in authenticating the botanical materials [13, 14]. Plant anatomy studies of fresh materials have played an important historical role in the classification and identification of plants [15]. Nevertheless, for the species of *Dioscorea*, only a very few anatomical studies have been conducted [3, 16–22]. The only comprehensive work on the anatomy of *Dioscorea* was published by Ayensu in 1972 [20]. However, the book only deals with the general anatomy at generic and subgeneric levels and no detailed anatomy of species is provided. Ayensu found ‘a striking uniformity’ in anatomical details between species of *Dioscorea* and did not find any substantial anatomical feature that could clearly define species. The lists of selected diagnostic characteristics provided by Ayensu contain mostly morphological features and characteristics of trichomes and glands rather than anatomical features.

The present study is therefore aimed at providing detailed anatomical descriptions and at identifying the diagnostic features that could help distinguish different species of *Dioscorea*. This study may also serve as a model for comparative studies of other species of the genus in the future. The five species of *Dioscorea* included in the present study are: *D. balcanica* Kosanin; *D. bulbifera* L.; *D. polystachya* Turcz.; *D. rotundata* Poir.; and *D. villosa* L. (Fig. 1).

In addition to microscopic investigations, an HPTLC method was developed for acquisition of fingerprints of the five species of *Dioscorea* and their respective raw materials included in the current research. The rationale for initiating this chromatographic study was the lack of literature on such work on most of the *Dioscorea* species mentioned herein [23–25]. The current work also includes determination of the hexanes/ethyl acetate and the methanol extractive values and their respective percentages of the samples of *Dioscorea* studied based on gravimetric analysis.

Materials and methods

Materials

Authentic samples of rootstocks of *Dioscorea balcanica* Kusanin, *D. bulbifera* L., *D. polystachya* Turcz., *D. rotundata* Poir. and *D. villosa* L., sourced from 2-year old plants grown in the greenhouse at the Maynard W. Quimby Medicinal Plant Garden (MPG) of the University of Mississippi, University, MS, USA were used for anatomical study. Herbarium specimens of all the five taxa (NCNPR # 13121, 13123, 13122, 13124 and 13125) were prepared and housed in the herbarium of MPG. Authentic samples of dried rootstocks (NCNPR # 11620, 11609, 9797, 9167 and 11608) from the same source were deposited in the Repository of Botanicals at the National Center for Natural Products Research (NCNPR), in the School of Pharmacy, University of Mississippi. For HPTLC analysis, 17 samples (Table 1) were used. Commercial samples were purchased online from different suppliers. All the samples were sourced from the USA except # 11609 from India and # 9797 from South Korea.

Methods

Instrumentation and chromatographic conditions for HPTLC—The HPTLC system (Camag, Muttanz, Switzerland) consists of a DigiStore2 digital system along with winCATS software ver.1.4.3 (Camag, Switzerland); automatic developing chamber CAMAG ADC2 and a Linomat 5 sample applicator with 100 µL syringes, twin trough chambers 20 × 10 cm (Analtech, USA) were used for development. Nano-silica HPTLC plates, with UV254, glass-backed, 200 µm, 10 × 10 cm (Sorbent Technologies, Georgia, USA) were used for analysis. Application position from the lower edge of the HPTLC plate was 20 mm at a dosing rate of 90 nL/s. All samples were applied according to the following settings: 10 mm from the bottom of the plate, band width 8 mm; distance between tracks 12.5 mm. A constant application rate of 0.1 µL/s was employed. All remaining measurement parameters were maintained at the default settings. The images were taken using Reprostar 3 along with winCATS 4 software ver.1.3.2 (Camag, Switzerland). The plates were developed in a twin trough chamber 20 × 10 cm (Analtech, USA) using two different solvent systems: (A) hexanes–ethyl acetate: 70–30 and (B) chloroform–methanol–water: 70–30–1. Plates were developed in a Camag automatic developing chamber ADC2 presaturated with 25 mL of mobile phase for 10 min at room temperature (23 ± 2 °C) at a relative humidity of 39 ± 5 % and 10 mL of mobile phase for development of plates at the same temperature and relative humidity. After development, the plates were dried and dipped into a mixture of ethanol, glacial acetic acid, sulfuric acid and *p*-anisaldehyde (186:10:5:0.5) using the Immersion Device (dipping time 2 s, dipping speed 5 cm/s) followed by air drying for 5 min. After spraying the plates were dried at 100 °C for 5–10 min and images were taken under white light transmission mode. A Camag Video Documentation system in conjunction with the Reprostar 3 was used for imaging and archiving the thin-layer chromatograms.

Preparation of samples for light microscopy—Freshly collected samples of yams were fixed in formalin–acetic acid–alcohol (FAA) for 2 days and freehand sections were prepared using razor blades. The sections were treated with chloral hydrate solution and stained with phloroglucinol/HCl. Photomicrographs were prepared using Nikon E600 and

Nikon E600 POL microscopes equipped with Nikon DS-Fiv camera systems and Nikon Elements imaging software (Nikon Inc., Tokyo, Japan).

The fixed specimens were also used for the preparation of permanent slides using standard procedures [26, 27]. The specimens were dehydrated using a series of alcohol solutions (10, 20, 30, 50, 70, 95 and 100 %) and passed through graded series of xylene–alcohol solutions up to 100 % xylene. The specimens were then transferred to molten paraffin and specimen blocks were prepared using molds, in Leica Tissue Embedding System (Leica Microsystems, Wetzlar, Germany). Tissues embedded in blocks of paraffin wax were sectioned at a thickness of 10–15 µm using a Leica RM2255 fully automatic rotary microtome and the sections were stained by safranin and counterstained with Fast Green. Diluted iodine solution (1 % w/v) was used to stain the starch grains.

Preparation of samples for scanning electron microscopy—Fresh yam samples were fixed overnight in glutaraldehyde solution (2 % v/v), washed with water and dehydrated by passing through increasing concentrations of acetone in water according to a standard method [28]. The dehydrated specimens were then critical point dried in a critical point drier (Denton Vacuum, Moorestown, NJ, USA) using liquid CO₂ as a cryogenic fluid. The fully dried samples were mounted on aluminum stubs using glued carbon tapes and then coated with gold using a Hummer 6.2 sputter coater (Anatech USA, Union City, CA, USA) supplied with argon gas. Photomicrographs of the specimens were prepared using a model JSM-5600 SEM (JEOL Ltd., Tokyo, Japan).

Preparation of samples for HPTLC—The samples were ground into fine powders, using a Mixer Mill MM 400 (Retsch, Haan, Germany). About 1.0 g of powder of each of the 5 samples of *Dioscorea* species was sonicated in 2.5 mL of hexanes–ethyl acetate for 30 min followed by centrifugation for 5 min at 3000 rpm. This procedure was repeated four times, and followed by pooling of the supernatants. The solvent was evaporated in vacuum to dryness, dissolved in 2 mL methanol and then passed through a 0.45-µm nylon membrane filter. The extracted plant material was left overnight in the centrifuge vials for removal of the solvent. The same procedure was repeated using methanol and 80 % methanol, sequentially, to obtain three extracts of each sample.

Determination of extractive values—The hexanes/ethyl acetate, methanol and 80 % methanol extracts prepared for the HPLTC analysis were used for gravimetric determination of the extractive yields of samples 1–17. However, methanol and 80 % methanol extracts were combined since HPTLC examination revealed similar profiles.

Results

Anatomy and micromorphology

***Dioscorea balcanica* (Fig. 2a–e; Table 2)**—Transection (TS) of the rhizome (Fig. 2a, b) shows the following features: epidermis if present consists of a layer of collapsed cells. Periderm includes 1–2 layers of cork cells, some of which contain tannin, with suberized walls; a continuous band of 1–2 layers of polygonal cells with moderately thickened, lignified and pitted walls; and a 2-layered cork cambium of radially arranged cells with thin,

slightly lignified walls. Cortex consists of 5–6 layers of polygonal parenchyma with slightly thickened, thinly lignified and pitted walls; cells devoid of starch grains. Ground tissue is wide, occupying major portion of the rhizome, made up of several layers of polygonal parenchyma cells with moderately thickened, slightly lignified and pitted walls; cells filled with starch grains. Several vascular bundles (Fig. 2a) traverse the ground tissue, irregularly distributed, collateral, xylem is facing toward the center of the rhizome and phloem is facing the periphery, all the xylem and phloem elements are lignified (Fig. 2c). Idioblasts (Fig. 2b) containing bundles of raphide crystals of calcium oxalate are found in the cortex. Starch grains (Fig. 2d, e) ovoid, spherical, or ellipsoid, both ends obtuse; hilum dot-like, cleft-shaped, or radiating, usually concentric; striations concentric. Occasionally some of the grains show eccentric hilum and eccentric striations; larger grains measuring 14–27 μm long and 11–17 μm in diameter.

Dioscorea bulbifera (Fig. 2f–j; Table 2)—TS of the tuber (Fig. 2f) shows the following features: cork usually absent, sometimes represented by 1–2 layers of collapsed, brownish cells. Sclerenchyma (Fig. 2f, g) band of the periderm is composed of 1–5-layers of tangentially elongated cells, usually rectangular, rarely triangular or oval in shape, measuring 40–200 μm long and 17–46 μm wide, with prominent nucleus; walls striated, 7–11 μm thick, densely lignified, pitted with radially elongated slit-like pits. Cork cambium is indistinguishable or sometimes represented by a narrow layer of tangentially elongated cells with dense cytoplasm and large nuclei. Cortex made up of 10–15 layers of parenchyma cells, devoid of starch except the 1–2 innermost layers of cells containing small starch grains; cells polygonal, slightly tangentially elongated, with intercellular spaces; many of the cells filled with yellow contents. Cells of the ground tissue are polygonal, arranged with small intercellular spaces, filled with starch grains; walls slightly thick, non-lignified. Vascular bundles are few and small, irregularly distributed in the ground tissue, collateral; xylem elements few, lignified, with spiral or scalariform thickening; phloem elements thin-walled, non-lignified. Cells containing yellow substance are commonly found in the cork—especially in lenticels, cortex and ground tissue. Idioblasts containing raphide bundles of calcium oxalate crystals (Fig. 2h) embedded in mucilage are common in outer cortex and occasional in the ground tissue; small, simple, rectangular prismatic crystals of 5–10 μm long and 1.5–2.5 μm wide are abundant in the cells of ground tissue; also present in the cells of ground tissue are solitary styloides of 9–18 μm long, pointed at one end. Starch grains (Fig. 2i, j) simple, usually wedge-shaped, 13–37 μm long and 16–30 μm in diameter, one end narrow, obtuse and slightly curved, the other end truncate, widest near middle, horizontally striated; hilum eccentric, dot-like, towards the narrow end of grain. Starch grains found in the innermost layers of cortex and cells near vascular bundles are smaller, 6–10 $\times$ 5–7 μm , oval or roughly triangular; hilum and striations are indistinguishable.

Dioscorea polystachya (Fig. 3a–e; Table 2)—TS of the tuber (Fig. 3a, b) exhibits the following characteristics: cork has 2–3 or more layers, with polygonal cells showing thin and suberized walls, often containing tannin; cells of the outermost layers often collapsed. A continuous band of sclerenchyma follows the cork; cells radially arranged, tangentially elongated, walls moderately thickened, lignified and pitted with radially elongated slit-like pits. Cork cambium made up of two layers of tangentially elongated parenchyma cells with

dense cytoplasm and prominent nucleus. Cortex consists of 6–8 layers of compactly arranged, thin-walled, polygonal parenchyma, usually with small starch grains. Ground tissue is wide, made up of polygonal cells arranged with small intercellular spaces, thin-walled, filled with starch grains. Vascular bundles (Fig. 3a, d) few, collateral, irregularly distributed in the ground tissue; xylem elements lignified; phloem elements non-lignified. Idioblasts containing raphide bundles (Fig. 3c) of calcium oxalate embedded in mucilage are very common in the cortex and ground tissue; cluster crystals are rarely observed. Cells filled with yellow substance are commonly found in the cork, cortex and ground tissue, where they usually are associated with vascular tissue. Some idioblasts with brownish granular contents are occasionally found in the cortex and the ground tissue. Starch grains (Fig. 3e) simple, usually oval, semicircular, elliptic, or shell-shaped, 10–39 μm long and 7–29 μm wide; hilum eccentric, radiating, cleft-shaped, or fissured, with eccentric striations.

Dioscorea rotundata (Fig. 3f–j; Table 2)—In TS (Fig. 3f), the tuber shows the following features: wide cork, made up of several layers of compactly arranged polygonal cells, inner layers of cells radially arranged, some of the cells containing tannin in the form of yellow substance. Cork cambium 2-layered, often indistinguishable. Cortex wide, made up of several layers of parenchyma cells, isodiametric or slightly tangentially elongated, thin-walled, with large nuclei and usually with small intercellular spaces; cells devoid of starch grains. Tannin cells are particularly abundant in the cortex. 4–6 innermost layers of the cortex show compact, radially arranged, tangentially elongated, thin-walled parenchyma cells, containing large nuclei. Cortex and the ground tissue are separated by a band of pericyclic sclereids (Fig. 3f, g) which are isodiametric or tangentially elongated, measuring $18\text{--}77 \times 13\text{--}42 \mu\text{m}$, with wide lumen; walls unevenly thickened, densely lignified and pitted with rounded pits. The ground tissue is wide; cells polygonal, thin-walled, nonlignified, arranged with small intercellular spaces, filled with starch grains, most of the cells also contain brown granular content in addition to starch grains. Vascular bundles collateral, irregularly distributed in the ground tissue; xylem consists of vessels and tracheids; phloem elements thin-walled, non-lignified. Tannin cells (Fig. 3f, h) occasionally found in the ground tissue and near vascular bundles. Idioblasts containing bundles of raphide crystals (Fig. 3i) of calcium oxalate are common in outer cortex and occasional in ground tissue, slightly tangentially elongated; raphide bundles measure $55\text{--}120 \mu\text{m}$ long, $25\text{--}30 \mu\text{m}$ wide, embedded in mucilage; individual crystals up to $3 \mu\text{m}$ in diameter; another kind of idioblasts filled with brown granular substance is also abundant in the ground tissue. Starch grains (Fig. 3j) usually shell-shaped, triangular or oval, narrow and obtuse at one end, broad and truncate at the other end, measuring $20\text{--}47 \times 23\text{--}35 \mu\text{m}$; hilum eccentric, slit-shaped, dot-like, or radiating; smaller grains found in the peripheral layers of ground tissue are nearly spherical, $4\text{--}8 \mu\text{m}$ in diameter.

Dioscorea villosa (Fig. 4a–g; Table 2)—TS of rhizome (Fig. 4a, b) shows the following features: cork, when present, consists of few to many layers of collapsed cells; some of the cells contain tannin. Sclerenchyma is not observed. Cork cambium prominent, made up of 3–5 layers of thin-walled, radially arranged, tangentially elongated cells, non-lignified or thinly lignified. Cortex narrow, parenchymatous; cells of outer layers are tangentially elongated and the inner ones polygonal, filled with small starch grains; walls

slightly thick, non-lignified. Ground tissue is wide, made up of large polygonal parenchyma cells, filled with starch grains; cell walls moderately thickened and slightly lignified. Vascular bundles (Fig. 4d) collateral, irregularly distributed in the ground tissue; xylem elements lignified; vessels and tracheids irregularly arranged, anastomosing; tracheidal fibers (Fig. 4e) with thickened, pitted and lignified walls are occasionally present at the branching of vascular bundles, they are not observed in the regular bundles; phloem elements thin-walled, lignified. Idioblasts containing bundles of raphide crystals (Fig. 4b, c) of calcium oxalate are common in the cortex as well as the ground tissue; raphides 33–68 μm long, 1.2–1.8 μm in diameter, found in bundles of about 32–38 μm in diameter, embedded in mucilage. Prismatic crystals (Fig. 4f) of calcium oxalate, measuring about $12 \times 10 \mu\text{m}$, are occasional in cork cells. Some of the cork cells also contain several very small prismatic crystals. Tannin cells commonly observed in the cork, occasional in cortex and rarely found in the ground tissue. Starch grains (Fig. 4g) usually nearly circular, oval, or ellipsoid in shape, rounded at both ends, up to $40 \times 26 \mu\text{m}$; cortex usually contains only small grains, whereas the cells of ground tissue contain both small and large starch grains; hilum nearly concentric, dot-like; striations faint.

Diagnostic key to five species of *Dioscorea* using micromorphological features

1. Phloem elements and cells of ground tissue are lignified; hilum and striations of starch grains are usually concentric:
 2. Cortical sclerenchyma present, 1–2-layered; cortical parenchyma cells slightly lignified, devoid of starch grains; vascular bundles regular..... *D. balcanica*
 2. Cortical sclerenchyma absent; cortical parenchyma cells non-lignified, containing small grains of starch; vascular bundles often irregularly anastomosing..... *D. villosa*
 1. Phloem elements and cells of ground tissue are non-lignified; hilum and striations of starch grains are usually eccentric:
 3. Pericycle well-developed, 4–6-layered; pericyclic sclereids present; cells of ground tissue usually contain brown granular contents *D. rotundata*
 3. Pericycle undifferentiated; pericyclic sclereids absent; cells of ground tissue devoid of such brown granular contents:
 4. Starch grains usually oval, semi-circular or elliptic, rounded at both ends; hilum radiating, cleft-shaped, or fissured; cluster crystals of calcium oxalate are rarely observed in the ground tissue..... *D. polystachya*
 4. Starch grains usually wedge-shaped, rounded at one end and straight at the other end; hilum dot-like; cluster crystals not observed *D. bulbifera*
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HPTLC analysis

Examination of the HPTLC profiles of the hexanes–ethyl acetate extract (Fig. 5a, b) revealed that tracks 1, 7, 8, 10, 11, 13, 16 and 17 showed a band corresponding to diosgenin (R_f 0.32). In contrast to others, samples in tracks 9 and 17 (samples # 9161 and # 13064, respectively) showed a peculiar band at R_f 0.12. This band has strong intensity in track 9, whereas it is faint in track 17. Inspection of the HPTLC profiles of the methanol extracts (Fig. 5c) also showed peculiar bands for track 9. The sample # 9161, although labeled and sold as *Dioscorea villosa*, thus did not fully match that or any other species of *Dioscorea* studied. Diosgenin was not observed in methanol extract perhaps due to its high non-polarity.

Discussion

Anatomy and micromorphology

A detailed study of the anatomy of five species of *Dioscorea*, namely *D. balcanica*, *D. bulbifera*, *D. polystachya*, *D. rotundata* and *D. villosa*, was carried out. The study showed that all five species have some similarities in their basic arrangement of tissues as seen in TS. The rhizomes/tubers have a thin layer of cork, usually in collapsed state, and some cells contain tannin as yellow substance. Cork cambium is narrow, producing secondary tissues externally. The outer cortex is parenchymatous, with few to many layers. The central ground tissue is wide, occupying the major portion of the rhizome/tuber, parenchymatous; the cells are filled with starch grains. Vascular bundles are collateral, irregularly distributed in the ground tissue; xylem, consisting of vessels and/or tracheids and parenchyma, is arranged facing the center with phloem facing the periphery of the rhizome/tuber. Idioblasts containing calcium oxalate crystals embedded in mucilage are found in all five species (Figs. 2, 3, 4; Table 2).

However, all five species are different in certain characteristics (Table 2). Morphologically, the rootstocks of *Dioscorea balcanica* and *D. villosa* are similar in their rhizomatous appearance. They are narrowly cylindrical, highly branched with short branches, moderately woody and horizontally elongated. On the other hand, the rootstocks of the other three species are tuberous, fleshy, broadly ovoid or ellipsoid (Fig. 1). The major differences in the microanatomy of the five species are given below.

Periderm containing a sclerenchymatous ring occurs as 1–5 layers of lignified cells in *D. balcanica*, *D. bulbifera* and *D. polystachya*. The arrangement, shape and size of the cells, however, vary between these species as mentioned in Table 2. Walls of these cells are densely lignified and striated in *D. bulbifera* but they are thinly or moderately lignified in the other two species. Cell walls are pitted with reticulate pits in *D. balcanica* while *D. bulbifera* and *D. polystachya* show slit-like pits. Sclerenchyma in the periderm is not observed in *D. rotundata* and *D. villosa*. The cell walls of the cortical cells are lignified in *D. balcanica*, but non-lignified in other species. Cortical cells in *D. balcanica* and *D. rotundata* are devoid of starch. Small grains of starch are observed in the cortex of *D. polystachya* and *D. villosa*. In *D. bulbifera*, only the 1–2 innermost layers of the cortex contain such small starch grains, while the outer layers of cells do not have them. Of the five species studied, only *D. rotundata* shows a well-developed pericycle, consisting of 1–2 layers of sclereids. The pericycle is not differentiated in the other four species.

In the genus *Dioscorea*, raphides and rarely styloid crystals have been reported [20, 29, 30]. During the present study, cluster crystals and other unusual forms of crystals were also observed. Raphides embedded in mucilage in idioblasts are found in all five species. Two new or unusual types of raphide crystals were observed in *D. polystachya* (Raman et al., in prep.). Cluster crystals of 22–28 µm in diameter are only observed in *D. polystachya*. Rectangular prisms of calcium oxalate are only occasionally seen in the cork cells in *D. villosa*. Simple styloid crystals of calcium oxalate measuring 9–18 µm long are commonly found in the cells of ground tissue in *D. bulbifera*. Short rectangular prismatic crystals are also commonly found in most of the cells of ground tissue in this species. These crystals are

also occasionally found in the cork cells of *D. villosa*. The cell walls of the ground tissue are slightly lignified in *D. balcanica* and *D. villosa*. This lignified ground tissue perhaps contributes to the hardness of the rhizomes in these two species. The cell walls of the ground tissue in the tubers of the other three species are non-lignified. Tracheidal fibers are only rarely observed in *D. villosa*.

According to Esau [31], there is no previous study confirming lignification in the walls of sieve elements. However, during the present study lignification in the walls of phloem elements was observed in two species of *Dioscorea*, namely *D. balcanica* and *D. villosa*. Starch grains in the cells of ground tissue are variously shaped. A comparison of the morphology of starch grains is provided in Table 2.

HPLTC fingerprints (Fig. 5) were obtained from two extracts (hexanes–ethyl acetate and methanol) prepared from 11 voucher samples and 6 commercial samples of *Dioscorea* species (Table 1). Diosgenin, which was detected in eight samples in the hexanes–ethyl acetate extract, is one of the medicinally useful constituents and is utilized as a marker for quality control of *Dioscorea* species sold as herbal products or food supplements. The presence of multiple bands near the baseline in the methanol extract is ascribed to the presence of abundant quantities of the polar steroidal glycosides (saponins). It was also observed that sample # 9161 (track 9) showed an anomalous profile compared to other samples of *D. villosa*, which might indicate that the sample is possibly misidentified. The results of the current investigation demonstrate the possible utility of the HPTLC fingerprints of the hexanes–ethyl acetate extract and the methanol extract in discriminating between the studied samples of *Dioscorea* species.

In case of the polar fraction (combined methanol and 80 % methanol extracts), it is observed that there is a wide range in the extractive values of the voucher samples of *D. villosa* of 95.8–160 mg/g dry weight of raw material or 9.6–16 % w/w. In contrast, the commercial samples showed a narrow range of 134.0–152.0 mg/g dry weight of raw material. In the medium polarity portion (hexanes–ethyl acetate), the range of the extractive values for the voucher samples was 10.9–29.2 mg/g dry weight of raw material or 1.1–2.9 % w/w, whereas it was 13.3–22.6 mg/g dry weight of raw material or 2.7–13.3 % w/w for the commercial samples. The two voucher samples of *D. bulbifera* showed widely different values. Samples of *D. rotundata* also exhibited widely different results. *D. balcanica* and *D. polystachya* showed results within the above-mentioned ranges.

Conclusion

The present study of the tubers and rhizomes of five species of *Dioscorea* clearly indicates the differences in their micromorphology and anatomy that can help in characterization of these species. This paper provides a detailed description of each of five species with light microscopy and SEM images. A diagnostic key using anatomical features has been provided for easy identification of the species. The primary features that can help in distinguishing the species include the presence or absence of various sclerenchymatous structures, lignification in phloem, types of calcium oxalate crystals and features of starch grains. Lignification in phloem, a rare phenomenon, is observed in two species of *Dioscorea*. Similar studies of

different species of yams at different growth levels may help to better understand the species complex. The results of the current work demonstrate the possible utility of HPTLC fingerprint analysis in discriminating between the species studied. However, the gravimetric analysis is found to be unsuitable for discriminating the species studied owing to the wide variation in the extractive values of the samples studied.

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Fig. 1.
Morphology of *Dioscorea* species (2-year-old plants) showing leaves and entire tubers/
rhizomes

Fig. 2.

Anatomy of *Dioscorea balcanica* rhizome (a–e) and *D. bulbifera* tuber (f–j) [a–d, f–i light microscopy (LM); d-2, i-2, i-3 polarized light (PL); e, j SEM. a, c, f, g stained in phloroglucinol/HCl; b stained in safranin/fast green; d-1, i-1 stained in 1 % iodine]. a Transection (TS) of rhizome showing distribution of vascular bundles (starch cleared using KOH). b TS of portion of rhizome showing lignified layer of secondary cortex and a crystal idioblast. c Vascular bundle showing lignified phloem and xylem elements. d–e, i, j starch grains. f TS of a portion of tuber. g Enlarged view of sclerenchymatous secondary cortex. h

Raphide crystal bundle. *Bars* **a** = 1 mm; **b–d, g, h, i-2, i-3** = 50 μm ; **e** = 10 μm ; **f** = 500 μm ; **i-1, j** = 20 μm (*Ck* cork, *Cr* raphide crystal, *Cx* cortex, *Gt* ground tissue, *Id* idioblast, *Lc* lenticel, *Ph* phloem, *Pn* phellogen, *Sc* secondary cortex, *Sl* sclerenchyma, *Vb* vascular bundle, *Xy* xylem)

Fig. 3.

Anatomy of tubers of *Dioscorea polystachya* (a–e) and *D. rotundata* (f–j) (a, d, e-2, f-i, j-2 LM; b fluorescence light, UV Ex = 330–380 nm; c, e-1, j-1 SEM; e-3, j-3 PL; a-1, e-2, j-2 stained in iodine; a-2, d, f, g stained in phloroglucinol/HCl). a-1, a-2 TS of portion of tuber showing distribution of starch grains and vascular bundles (starch cleared using KOH), respectively. b TS of tuber showing portions of cork, phellogen and cortex (two crystal idioblasts are seen in cortex). c A raphide crystal in ground tissue. d TS of vascular bundle showing lignified xylem elements and non-lignified phloem. e, j Starch grains. f Partial view

of TS of tuber. **g** A portion of tuber showing sclerenchymatous ring between cortex and ground tissue. A tannin cell (**h**) and an idioblast containing a raphide bundle (**i**) in cortex (*Ck* cork, *Cr* raphide crystals, *Cx* cortex, *Gt* ground tissue, *Id* idioblast, *Pe* pericyclic sclereids, *Ph* phloem, *Pn* phellogen, *St* starch, *Tc* tannin cells, *Vb* vascular bundle, *Xy* xylem). Bars **a-1**, **a-2** = 1 mm; **b** = 200 μ m; **e-1**, **e-2**, **j-1** = 20 μ m; **c**, **d**, **e-3**, **g-j**, **j-2**, **j-3** = 50 μ m; **f** = 500 μ m

Fig. 4.

Anatomy of *Dioscorea villosa* rhizome (**a, b, d, e** LM; **c, g-1** SEM; **f, g-2, g-3** PL; **a, b, d, e** stained in phloroglucinol/HCl). TS of rhizome showing lignified parenchymatous ground tissue (**a**), raphide bundles in cortex (**b, c**) and vascular bundle with lignified phloem and xylem (**d**). **e** Longitudinal section through a branching vascular bundle showing presence of tracheidal fibers among tracheids and vessels. **f** A prism crystal in the cork. **g** Starch grains (Ck cork, Cx cortex, Gt ground tissue, Ph phloem, Tf tracheidal fiber, Vb vascular bundle, Xy xylem). Bars **a** = 500 μ m, **c, f** = 20 μ m; **d, g** = 50 μ m; **b, e** = 100

Fig. 5.

HPTLC profiles of *Dioscorea* samples (**a, b** hexanes–ethyl acetate extracts, **c** methanol extracts, **a** UV 366 nm, **b, c** white light). Track 1 *D. balcanica* (1 NCNPR # 11620); 2, 3 *D. bulbifera* (2# 7862, 3# 11609); 4 *D. polystachya* (# 9797); 5, 6 *D. rotundata* (5# 9167, 6# 9463); 7–17 *D. villosa* (7# 5365, 8# 12152, 9# 9161, 10# 9412, 11# 9413, 12# 9800, 13# 10219, 14# 10227, 15# 11608, 16# 12166, 17# 13064); 18 Diosgenin

Table 1

Extractive values of samples (1–17) of *Dioscorea* spp.

Track no.	Sample ID	Accession date	Species	Type	50 % hexanes-EtOAc extract		Combined MeOH and 80 % MeOH (polar fraction)	
					Yield (mg/g raw material)	Yield (% w/w)	Yield (mg/g raw material)	Yield (% w/w)
1	11620	6/14/2012	<i>D. balcanica</i>	V	16.3	1.6	129.6	13.0
2	7862	12/3/2009	<i>D. bulbifera</i>	V	23.7	2.4	64.0	6.4
3	11609	6/14/2012	<i>D. bulbifera</i>	V	17.3	1.7	93.4	9.3
4	9797	9/16/2011	<i>D. polystachya</i>	V	18.3	1.8	91.0	9.1
5	9167	8/16/2010	<i>D. rotundata</i>	V	12.3	1.2	55.7	5.6
6	9463	6/24/2011	<i>D. rotundata</i>	C	19.4	1.9	60.6	6.1
7	5365	6/3/2009	<i>D. villosa</i>	V	29.2	2.9	101.0	10.1
8	12152	6/27/2012	<i>D. villosa</i>	V	21.7	2.2	141.0	14.1
9	9161	5/2/2011	<i>D. villosa</i>	C	18.6	1.9	151.1	15.1
10	9412	4/15/2011	<i>D. villosa</i>	C	22.6	2.7	134.0	13.4
11	9413	4/15/2011	<i>D. villosa</i>	C	15.8	1.6	150.6	15.1
12	9800	9/19/2011	<i>D. villosa</i>	V	20.1	2.0	160.0	16.0
13	10219	5/2/2012	<i>D. villosa</i>	C	13.3	1.3	147.6	14.8
14	10227	5/4/2012	<i>D. villosa</i>	V	11.1	1.1	95.8	9.6
15	11608	6/14/2012	<i>D. villosa</i>	V	10.9	1.1	112.9	11.3
16	12166	6/27/2012	<i>D. villosa</i>	V	22.5	2.3	122.0	12.2
17	13064	10/15/2012	<i>D. villosa</i>	C	21.6	2.2	152.0	15.2

V voucher sample, C commercial sample

Comparison of anatomy and micromorphology of tuber/rhizome of five *Dioscorea* species

Feature	<i>D. balcanica</i>	<i>D. bulbifera</i>	<i>D. polystachya</i>	<i>D. rotundata</i>	<i>D. villosa</i>
Periderm					
Cork layers	1–3	1–2	2–3 to few	Many	Few-to many
Sclerenchyma	1–2 layered; cells 11–33 × 8–20 µm	1–5-layered; cells 40–200 × 17–46 µm	2–3-layered; cells 70–175 × 25–55 µm	Undifferentiated	Undifferentiated
Phellogen	2-layered	1-layered	2-layered	2-layered	3–5 layered
Cortex	5–6-layered; cell walls lignified; devoid of starch	10–15-layered; cell walls non-lignified; small grains of starch only found in cells of 1–2 innermost layers	6–8-layered; cell walls non-lignified; containing small grains of starch	Many layered; cell walls non-lignified; devoid of starch	Few layered; cell walls non-lignified; containing small grains of starch
Pericycle	Undifferentiated	Undifferentiated	Undifferentiated	Sclereids 1–2-layered	Undifferentiated
Calcium oxalate crystals					
Raphides	40–55 µm long	70–80 µm long	180–190 µm long	55–120 µm long	33–68 µm long
Druces	Not observed	Not observed	Present	Not observed	Not observed
Prisms	Not observed	Present	Not observed	Not observed	Present
Styloides	Not observed	Present	Not observed	Not observed	Not observed
Ground tissue	Cells 18–68 µm in diameter; walls slightly lignified	Cells 18–128 µm in diameter; walls non-lignified	Cells 60–175 µm in diameter; walls non-lignified	Cells 35–137 µm in diameter; walls non-lignified	Cells 20–85 µm in diameter; walls slightly lignified
Vascular bundles	Many	Few	Few	Few	Many
Vessel diameter	Up to 53 µm	Up to 35 µm	Up to 105 µm	Up to 48 µm	Up to 30 µm
Phloem	Lignified	Non-lignified	Non-lignified	Non-lignified	Lignified
Starch grains					
Shape	Oval, circular or elliptic in outline; obuse at both the ends	Wedge-shaped, one end narrow obuse and slightly curved, other end truncate	Oval, semi-circular or elliptic	Shell-shaped, triangular or oval, obtuse at one end, broad and truncate at the other end	Usually nearly circular, oval or elliptic, rounded at both the ends
Size	14–27 × 11–17 µm	13–37 × 16–30 µm	10–39 × 7–29 µm	20–47 × 23–35 µm	11–40 × 8–26 µm
Hilum	Dot-like, cleft-shaped or radiating; usually concentric, rarely eccentric	Dot-like; eccentric	Radiating, cleft-shaped, or fissured; eccentric	Slit, dot-like or radiating; eccentric	Dot-like; nearly concentric
Striations	Usually concentric, rarely eccentric	Eccentric, nearly horizontal	Eccentric	Eccentric	Striations faint