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RESEARCH ARTICLE

Detection of adulteration of *Decalepis hamiltonii* Wight & Arn. with *Hemidesmus indicus* (L.) R. Br. by pharmacognostic, molecular DNA fingerprinting by RAPD, chemical and HPTLC studies

Sujith T[1], Susikumar S[2], Sunilkumar KN[2], Radha P[3], Shakila R[1*] & Gopinath P[4*]

[1]Department of Chemistry, [2]Department of Pharmacognosy, Siddha Central Research Institute (Central Council for Research in Siddha, Ministry of AYUSH), Anna Hospital Campus, Arumbakkam, Chennai 600 106, Tamil Nadu, India
[3]Siddha Medicinal Plants Garden (Central Council for Research in Siddha, Ministry of AYUSH), Mettur 636 401, Tamil Nadu, India
[4]Department of Chemistry, SRM Institute of Science and Technology, Kattankulathur, Chennai 603 203, Tamil Nadu, India
Email: gopinatp1@srmist.edu.in | r.shakila@gov.in

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ABSTRACT

Hemidesmus indicus (L.) R. Br. (Apocynaceae) root is extensively used in Indian traditional systems due to its biological activities. *Decalepis hemiltonii* Wight & Arn. is another member from the same family resembling *H. indicus* and is adulterated in the herbal market. Aim of the study was to compare and evaluate the distinguishing features based on macroscopy, microscopy, powder microscopy, molecular differences in the genomic DNA by RAPD, physiochemical, phytochemical screening, TLC and HPTLC fingerprint profiling of successive extracts. Microscopically cork, cortex, phloem, xylem, medullary rays and pith; powder microscopically size and shape of the cork cells, fibre, fibre tracheids, vessels, xylem parenchyma cells were different from each other. Polymorphism (75.4 %) was found in eight primers out of 16 primers analyzed. The water soluble extractive and the hexane soluble extractive of *D. hamiltonii* was higher than *H. indicus*. Tannins, flavonoids, steroids and coumarins were present only in *H. indicus* and absent in *D. hamiltonii*. After derivatization, spots at R_f 0.88 (hexane extract), 0.81 (chloroform extract) and 0.55 (ethanol extract) in *H. indicus*; spots at R_f 0.22, 0.45 (chloroform extract), 0.19, 0.35, 0.58, 0.59 (ethanol extract) in *D. hamiltonii* were observed. This study will be helpful to find out adulteration of *D. hemiltonii* in place of *H. indicus* sold in the crude drug market and in herbal formulations.

Introduction

Plants have been the basis of many traditional medicines throughout the world for thousands of years due to their therapeutic efficacy. It is estimated that herbal medicine in developed countries make up to one fourth; while in developing countries it is up to three fourth of all medicines (1). The use of herbal drugs is rapidly increasing worldwide as the herbal drugs were found to be beneficial in treating mild to moderate diseases in all age groups and in averting illnesses thereby promoting health (2). Roots of *H. indicus* is an important plant drug used as a tonic, demulcent, diaphoretic and diuretic as per Siddha literature, it is used to treat a variety of diseases such as leprosy, leucoderma, itching, skin disease, body coolant, asthma, bronchitis, leucorrhoea, dysentery, piles, syphilis, paralysis, urinary disorders and diabetes (3). *Hemidesmus indicus* (L.) R. Br. is commonly known as Indian sarsaparilla, False sarsaparilla in English; Anantamul, Hindisalsa in Hindi; Anantumula, sariva, Dhavalasariva, Krishodari, Nagajihva, Sugandha in Sanskrit; Salsa in Urdu; Upalsan in Marathi; Nannari in Tamil; Narunenti in Malayalam; Sugandhi pala in Telugu; Namdaberu, Sogada beru in Kannada; Onontomulo, Suguddimalo in Oriya; Upalasari, Sariva, Anantvel in Gujarati; Anantamul in Manipuri and Ushba in Persian (4). It is used in in Siddha formulations viz., Carapunka vilvati ilakam, Ilaku cantanatit tailam, Pitta curak kutinir (5), Kumari ilakam, senkathari ennai, Sowbakkiya sundi ilakam, Thippili nei, Pericchangai nei, Maha vilvathi ilakam (6); root bark in Manturati attaik kutinir, Parankip pattai iracayanam (5), Nannari manapagu, Pancha paadana chenduram (7); in Sarivadyasava an Ayurvedic formulation (8). Due to these, *H. indicus* has high demand in traditional medicine system and herbal drug industries. At the same time availability of this plant is decreasing and the cultivation is also less to meet out the commercial demand. This gap is being utilized by crude drug collectors, suppliers and sellers to adulterate similar looking plant species.

Decalepis hamiltonii Wight & Arn. is commonly known as Maredu kommulu, Nannari kommulu, Barre Sugandhi; Swallow root in English; Sariba, Sveta sariva in Sanskrit; Makaliber in Kannada; Magali kizhangu, Peru nannari, Mavilinga kilangu in Tamil; Madina kommulu, Maredu gaddalu in Telugu; Nannari in Malayalam (9) but not found medicinal value like *H. indicus*.

Hemidesmus indicus possesses anti-asthmatic activity (10), diuretic and anti-urolithiatic activity (11), nephroprotective activity (12) and neuroprotective activity (13), *D. hamiltonii* exhibits anti-inflammatory activity (14), neuroprotective activity (15) and hepatoprotective activity (16).

Both plant roots contain 2-hydroxy 4-methoxy benzaldehyde as major compound (17), lupeol, α-amyrin, β-amyrin, lupeol acetate, α-amyrin acetate, β-amyrin acetate (18-20) reported from various extracts of the root. However, in specific, *H. indicus* contains hemidesminine (21), hemidesmin-1, hemidesmin-2 (22), hemidesmol, hemidesterol (23) and *D. hamiltonii* contains bis-2,3,4,6-galloyl-α/β-D-glucopyranoside (decalepin) (24).

D. hamiltonii root is often adulterated with *H. indicus* because of the common availability in South India and bigger in size. Variety of adverse reactions due to adulteration are caused ranging from minor (allergic reactions, tiredness, digestive disorder, temper distraction or muscle weakness, nausea and breathing problems) to medium (misperception, fits, dermatitis, sensory disorders) and severe life threatening effects (cancer, cerebral oedema, unconsciousness, intracerebral haemorrhage, poisoning, metabolic acidosis, multi-organ failure, perinatal stroke, renal or liver failure or death) (25).

There are two reports published in 2017 by World Health Organization (WHO) on the investigation of inferior and fabricated medicines and their influence (26). The ancient Ayurvedic literature, *Charaka Samhita* indicates that medicines (Ayurvedic) have undesirable effects if they are not appropriately prepared or used wrongly (27). However, Indian traditional herbal medicines of Ayurveda, Siddha and Unani (ASU) origin are whispered harmless because of their long time use. According to the Drugs and Cosmetics Act of 1940 (DCA), no safety and efficacy studies are required for marketing approval for ASU drugs (28). At the same time, for trademarked herbal drugs, ethnomedicinal use based drugs and extract based drugs, safety and efficacy studies are mandatory (29). The crucial benchmark for substitution should be the pharmacological activity than the morphology or phytochemicals of the plant drug (30).

For the detection of adulterants, different techniques, viz., thin layer chromatography (TLC), high performance thin layer chromatography (HPTLC), high-performance liquid chromatography (HPLC), high-resolution melting (HRM), liquid chromatography–mass spectrometry (LCMS), nuclear magnetic resonance (NMR), polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP), random amplified polymorphic DNA (RAPD), sequence characterised amplified region (SCAR), single nucleotide polymorphism (SNP) etc. are available for different plants (31). Near-infrared (NIR), infrared (IR), Raman, liquid chromatography-circular dichroism (LC-CD), liquid chromatography-mass spectrometry (LC-MS), thin layer chromatography-surface enhanced Raman spectroscopy (TLC-SERS) and thin layer chromatography-mass spectrometry (TLC-MS) (32). But depending on the facilities, comparatively cheaper and easier technique is adapted for detection of adulteration. Plants from different genera, families, species, cultivars (cultivated variety) and sibling plants can be distinguished by DNA fingerprinting method (33). Sequence characteristic amplified region (SCAR) marker which is an advanced technique, RAPD finger prints and MALDI-TOF were developed for *H. indicus* by earlier researchers (34). In the present study, authors have selected different primers for RAPD analysis (35). Pharmacopoeial parameters such as macroscopy, microscopy, powder microscopy, physiochemical, TLC identification along with HPTLC finger printing have been carried out for both samples.

Materials and Methods

Collection of Samples

Root samples of *H. indicus* were collected from Palayamkottai, Tirunelveli district, Tamil Nadu during the month of January 2019 and *D. hemiltonii* were collected from Salem district, Tamil Nadu, India authenticated by the Pharmacognosist of this Institute.

Macroscopy, Microscopy & *Powder Microscopy*

The macro-morphological study was carried out by following the standard methods (36-38). The anatomical studies were carried by following standard procedures (38-40). For powder microscopic study the plant material after cleaning, dried properly, powdered and passed through sieve No. 80. The mounting and staining was carried out by standard methods (40-41). Photograph of sections and powder were made under different magnifications with the help of Olympus BX51 microscope fitted with Olympus camera.

Genomic DNA Isolation

The genomic DNA was extracted by modified cetyl trimethyl ammonium bromide (CTAB) method (42).

Purification of DNA

The silica membrane based column (Qiagen) was placed in collection tube, 400 µl of equilibrium buffer was added to the column and centrifuged at 10000 rpm for 1 min. Collected buffer was discarded. 400 µl of equilibrium buffer was added to the DNA samples, mixed and loaded into the column (This step was repeated till the DNA sample was completed). Break through was collected. 500 µl of vanadium salt in alcohol high concentration (wash buffer 1) was added, centrifuged at 10000 rpm for 1 min and buffer was collected. 500 µl of vanadium salt in alcohol low concentration (wash buffer 2) was added, centrifuged at 10000 rpm for 1 min and

buffer was collected. The column was centrifuged with empty collection tube to completely remove the wash buffer for 2 min. 50 µl of tris-EDTA buffer (elution buffer) was added to the column placed in new collection tube. Incubated at room temperature for two min and centrifuged at 10000 rpm for one min and eluted sample was saved (elution 1). Previous step was repeated (DNA may elute in this fraction also) (eluted sample was saved as elution 2) Quantization of eluted DNA samples was done by loading into the Agarose gel.

RAPD Analysis

Genomic DNA polymorphism was determined by Random Amplified Polymorphic DNA (RAPD) method (43). Amplification reactions were carried out in a total volume of 40 µl PCR reaction containing 200 ng genomic DNA, 4 µl 1X reaction buffer, 20 µl of 2X PCR master mix, 1 µl of standard arbitrary decamer oligonucleotides (Operon Technologies Inc. USA) and 17 µl of distilled water. Total of 16 primer sets were used. Amplification products were separated on 1.5 or 2% agarose gel in tris-borate-EDTA buffer (TBE buffer) and stained with ethidium bromide and visualized in the UV light.

DNA amplification was performed in the thermal cycler (Eppendroff, Hamberg, Germany) programed for 42 cycles as follows: the first step consisted of holding the sample at 94 ˚C for 5 min for complete denaturation of template DNA. The second step comprise of 40 cycles and each cycle comprise of three temperature steps i.e. 30 s at 94 ˚C, for denaturation of template, one min at 45 ˚C primer annealing followed by 1 min and 30 s at 72 ˚C for primer extension. The third step comprise of only one cycle i.e. 5 min at 72 ˚C for complete polymerization followed by holding at 4 ˚C. After completion of PCR, amplified products were stored at -20 ˚C for further use.

Data Analysis

The RAPD-PCR bands were scored as '1' for the presence and '0' for absence (44). From the Genetic similarity data among accession between the two samples were determined with respect to the similarity (dissimilarity) index method calculated using the Jaccard's similarity coefficient. Distances between individuals were calculated by clustering analysis (nearest neighbour method) with the help of the StatistiXL program (version 2) (45).

Chemicals, Solvents and Reagents

All the chemicals and solvents used were AR grade (Merck). For visualizing the developed spots in TLC, reagent containing vanillin (1 gm) sulphuric acid (5%) in ethanol (VSA) was used.

Instrument for HPTLC

For HPTLC, aluminium plate (Merck) pre coated with Silica gel $60F_{254}$ of 0.2 mm thickness was used. Automatic sampler ATS4 for application on TLC plate, twin trough chamber (10 × 10 cm) for plate development, visualizer for photo documentation under UV-visible conditions, Scanner 4 with winCATS software for finger prints, TLC plate heater for derivatization (all from CAMAG, Switzerland).

Physico-Chemical Parameters

All the physiochemical parameters for *D. hamiltonii* Wight & Arn. and *H. indicus* (L.) R. Br. were carried out as per standard methods (46).

Phytochemical Screening

The phytochemical tests for phenol, tannin, flavonoids, triterpenoids, proteins, glycosides, reducing sugar, anthraquinones, quinones, alkaloids, saponins, cardiac glycoside, steroids, coumarins and acids were done by using the standard methods (47).

Preparation of Extracts

Powdered root samples of *D. hamiltonii* and *H. indicus* (25 gm) were extracted successively with n-hexane, chloroform and ethanol using Soxhlet apparatus for 6 hrs. Concentrated and dried and the corresponding weights were recorded for calculating the yield as successive extractive values. The extract residue were re-dissolved in corresponding solvents and sonicated for ten minutes then filtered and made up to 2 ml and transferred into sample vials for TLC application.

Mobile Phases

The mobile phase for n-hexane extract, hexane:ethyl acetate (8:0.5, v/v); for successive chloroform, toluene:ethyl acetate:formic acid (10:2:0.2, v/v/v); for successive ethanol, toluene:ethyl acetate: methanol (8:2:0.7, v/v/v) were finalized.

TLC/HPTLC Procedure

Hexane (10 µl), chloroform (20 µl) and ethanol (10 µl) extracts in 3 different plates (8x10 cm) as 8 mm bands was applied on silica gel $60F_{254}$ coated aluminium plate using ATS4 applicator from 10 mm from left side and 10 mm from bottom of the plate The plates were developed in the respective mobile phases in presaturated twin trough chamber (10×10 cm). The plates were developed up to 90 mm from the bottom. The developed plates were air dried, viewed under UV 254 nm, 366 nm and the images were documented using Visualizer followed by dual wavelength scanning using Scanner 4 at λ 254 nm (D_2 lamp/absorption mode) and λ 366 nm (Hg lamp/fluorescence mode) with a slit dimension of 6×0.45 mm and scanning speed of 20 mm/s. Then, the TLC plates were dipped in a dip tank containing VSA reagent and heated at 100 °C or till the appearance of coloured spots. Immediately the derivatized TLC plates were photo documented at white light followed by scanning at λ 520 (W lamp/absorption mode) for finger prints.

Results and Discussion

Macroscopy, Microscopy and Powder Microscopy

The detailed macroscopic (Supplementary Table 1; Supplementary Fig 1 & 2), microscopic (Table 1) and powder microscopic characters (Table 2) are reported. The problem arises literally from the market samples in the name of Nannari, Actually, in Siddha, Nannari botanically equated *H. indicus* (L.) R. Br. ex Schult. and Malai nannari is referred as *D. hamiltonii* Wight & Arn. Macroscopically size,

Table 1. Microscopic characters of *D. hamiltonii and H. indicus*

Characters	***Decalepis hamiltonii*** (48)	***Hemidesmus indicus*** (48-51)
Diagrammatic TS	Brown colored narrow cork followed by off-white wide zone of cortex and phloem having centrally located porous xylem (Fig 3A).	Narrow cork, cortex, phloem and wide zone of central core xylem (Fig 3B); Root stock shows narrow cork, cortex, phloem and central parenchymatous pith encircled by wide zone of xylem (Fig 3C).
Cork	Different layers of cells, occurring one after the other, such as; wavy, thick walled, compressed, suberized, rectangular, tangentially elongated, 5 to 10 exfoliating rows of cell layers filled with reddish brown content; 4 to 8 rows of thin walled non-suberized cells rows; 5 to 10 rows of compressed, rectangular and a few polygonal, suberized cork filled with brownish content (Fig. 4A).	Different layers of tissues such as compressed, thick walled, suberized, rectangular, tangentially elongated, 5 to 25 cells rows of exfoliating, narrow band of cells filled with reddish or purplish brown color content (Fig. 4B & 4C).
Phellogen	Distinct colorless 1 or 2 row cells.	Distinct colorless1 or 2 row cells.
Phelloderm	Narrow zone of compressed, thin and thick walled cells.	Compressed 4 to 16 rows of cells containing brownish content (tannin) and prismatic crystals of calcium oxalate.
Cortex	Wide zone of thin walled parenchymatous cells showing presence of a few sclereids, prismatic crystals, latexcanalsand starch grains.	Wide zone of thin walled, polygonal, parenchymatous cells with abundant round to oval, helmet shaped, simple and multi component starch grains with hillum; non-articulated laticiferous canals, cavities, brownish content, oil globules and prismatic crystals; rarely a few sclereids between phelloderm and cortex regions in roots above 1 cm thickness.
Phloem	Tangentially elongated to rectangular, thin walled, compressed cells consists of sieve elements, non-articulated, branched laticiferous cells, ceratenchyma, prismatic crystals and abundant simple and compound starch grains.	Narrow zone of phloem cells consisting of sieve elements, thin walled tangentially elongated, larger towards periphery and become smaller compressed rectangular cells towards inner side; ceratenchyma, laticiferous cells, a few starch grains and prismatic crystals are found distributed in the region; in root stock pith region contain groups of primary inner phloem consisting of compressed collapsed cells, without any cell content and fibres.
Xylem	Various sized, round to oval, mostly single or 2 or 3 grouped diffused porous vessels,a few showingtylosis; thick walled wide lumen fibre and fibretracheids; thin walled uni-seriate xylem ray cells and thick-walled axial parenchyma cells embedded with a few starch grains, oil globule and prismatic crystals.	Very wide zone of xylem consist variously sized, round to oval, mostly single or 2 or 3 grouped, diffused porous vessels showing a few tylosis and resin like brownish content; thick walled xylem fibres with wide lumen; thick walled xylem axial parenchyma parenchyma containing a few starch grains, oil globules and prismatic crystals of calcium oxalate.
Medullary ray		Phloem rays uni-seriate, cells being larger in size than that of other phloem cells; mostly uni-seriate xylem rays, rarely bi and tri-seriate with a few starch grains, oil globules and prismatic crystals of calcium oxalate
Pith	Centrally located protoxylem.	Centrally located protoxylem in root; thin walled parenchymatous cells embedded with abundant starch grains, a few laticiferous cells, prismatic crystals in root stock.

external morphology, color, odour, taste are different and microscopically (Fig. 1-2) cork, cortex, phloem, xylem, medullary rays and pith characters are different from each other. Depending upon thickness of the root and root stock, place of stone cells, sclereids and fibres may be present or absent. Powder microscopically size and shape of the cork cells, fibre, fibre tracheids, vessels, xylem parenchyma cells are different (Fig. 3-4).

Molecular DNA fingerprinting by RAPD

DNA isolation was done by modified CTAB-based protocol and the isolated DNA is shown in Fig. 5. Total DNA extracted is 3 µl in which 2 µl of sample was used for PCR. Sixteen primers were selected for polymorphism. Out of sixteen primers used, eight primers produced the most polymorphic bands. The list of eight primers are presented (Supplementary Table 2) and the banding pattern of genomic DNA is shown in Fig. 6. The percentage of polymorphism is found to be 75.4 %.

The number of different bands developed in molecular DNA fingerprinting by RAPD, play an important role in differentiating the plants (45), especially Primers that successfully amplify DNA showed different patterns. Information on polymorphism of these both plants can be used as a reference for detection of authentic herbal drug. Out of sixteen primers, eight primers showed polymorphic bands and totally sixty one bands appeared in which forty six were polymorphic. In OPH-05 all six bands are polymorphic and this may be suitable one for distinguishing both plants.

Physico-chemical Analysis

The physico-chemical parameters were carried out in duplicates and the mean values are presented (Supplementary Fig. 3). In the physicochemical point of view, the total ash of *D. hamiltonii* is 10.77 % which is approximately three time more than the total ash value of *H. indicus* (3.69 %). Similarly the acid insoluble ash value of *D. hamiltonii* is 4.07 % and that of *H. indicus* is 0.93 % which means that the siliceous matter adhered on the root of *D. hamiltonii* is high. The water soluble extractive value of *D. hamiltonii* is 20.88 % which is higher than *H. indicus* even in the presence of higher content of siliceous matter. This indicates that *D. hamiltonii* contains more polar compounds than *H. indicus* which are soluble in water.

Table 2- Powder microscopic characters of *D. hamiltonii* and *H. indicus*

Characters	***Decalepis hamiltonii* (**48)	***Hemidesmus indicus* (**48-51)
Cork	Thin and thick walled suberized cells filled with brownish content, up to 150µ in length and up to 100µ width in surface view (Fig. 5).	Thin walled, suberized cells filled with reddish or purplish brown color content up to 100µ in length and up to 60µ width in surface view (Fig. 6).
Parenchymatous cells	Thin and thick walled cells	Thin walled cells
Sclereids	Varying in shape, up to 220µ in length and up to 75µ in width	Varying in shape, up to 200µ in length and up to 70µ in width
Fibres	Thick walled, wide lumen, sharp ends with a few fork and pegged, size up to 750µ in length and up to 60µ in width	Thick walled, wide lumen, sharp and blend ends with a few pegged out growth, size up to 750µ in length and up to 40µ in width
Vessels	Simple perforated, mostly pitted and a few bordered pitted. Pitted vessels up to 650µ in length and up to 160µ in width	Tailed, simple perforated, pitted vessels up to 400µ in length and up to 80µ in width
Fibre tracheids	Varying in shape with numerous simple pits up to 900µ in length and up to 60µ in width	Thick walled wide lumen, sharp and blend ends with a few pegged out growth, size up to 750µ in length and up to 40µ in width
Xylem parenchyma	Non lignified, ray parenchymatous up to 150µ in length and up to 60µ in width; axial parenchyma up to 50µ in length and up to 60µ in width	Lignified, Ray and axial cells up to 100µ in length and up to 40µ in width
Latex canals	non-articulated, branched, embedded with prismatic crystals,	non-articulated
Starch grains	abundant round to oval, simple and compound starch grains 10-30 µ in size	abundant round to oval, helmet shaped, simple and multi component with fissure hillum, 10-40 µ in size
Crystals	Prismatic crystals of calcium oxalate	Prismatic crystals of calcium oxalate

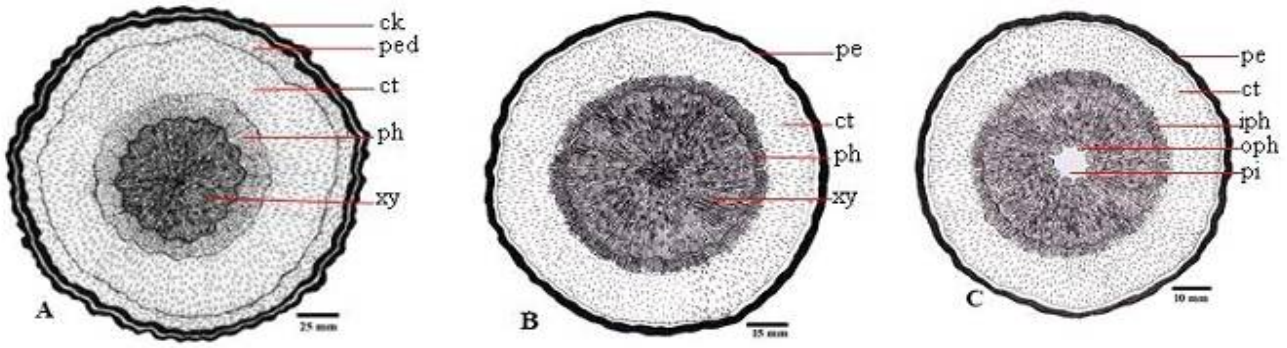

Fig. 1. Microscopy diagrammatic TS. **A**. *D. hamiltonii* root; **B.** *H. indicus* root; **C**. *H. indicus* root stock.

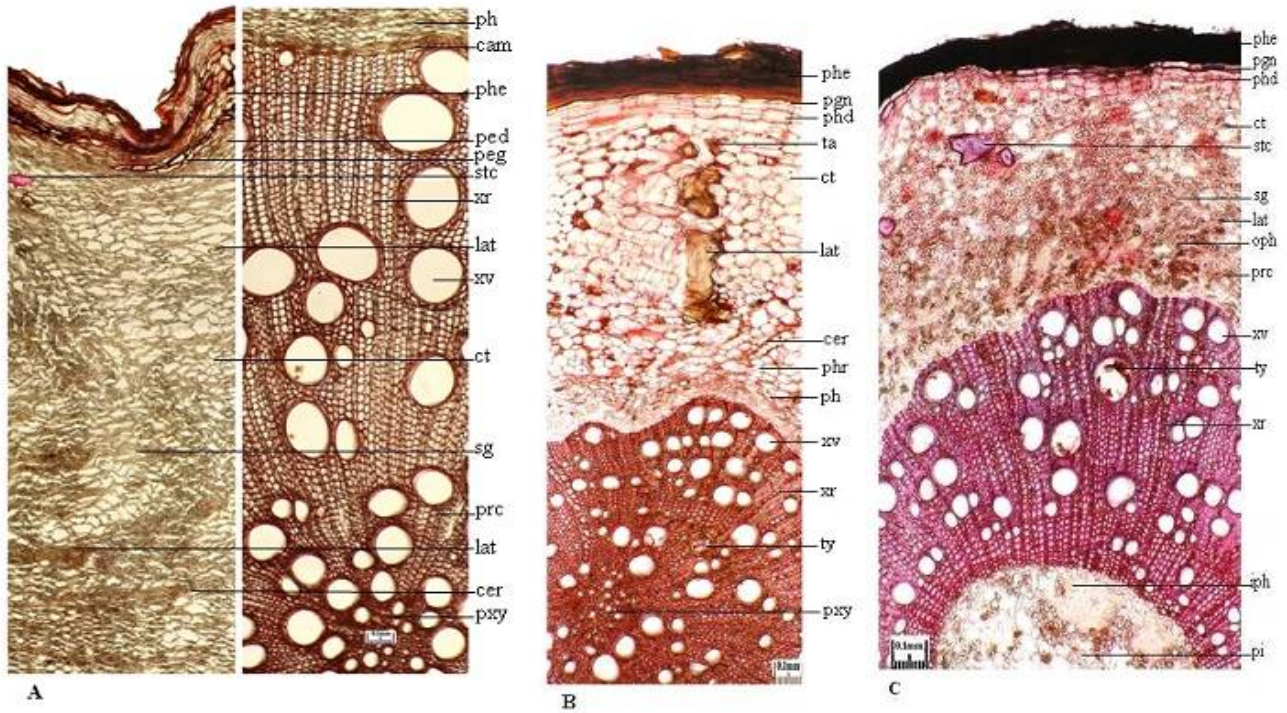

Fig. 2. Microscopy detailed TS- **A**. *D. hamiltonii* root; **B.** *H. indicus* root; **C**. *H. indicus* root stock.

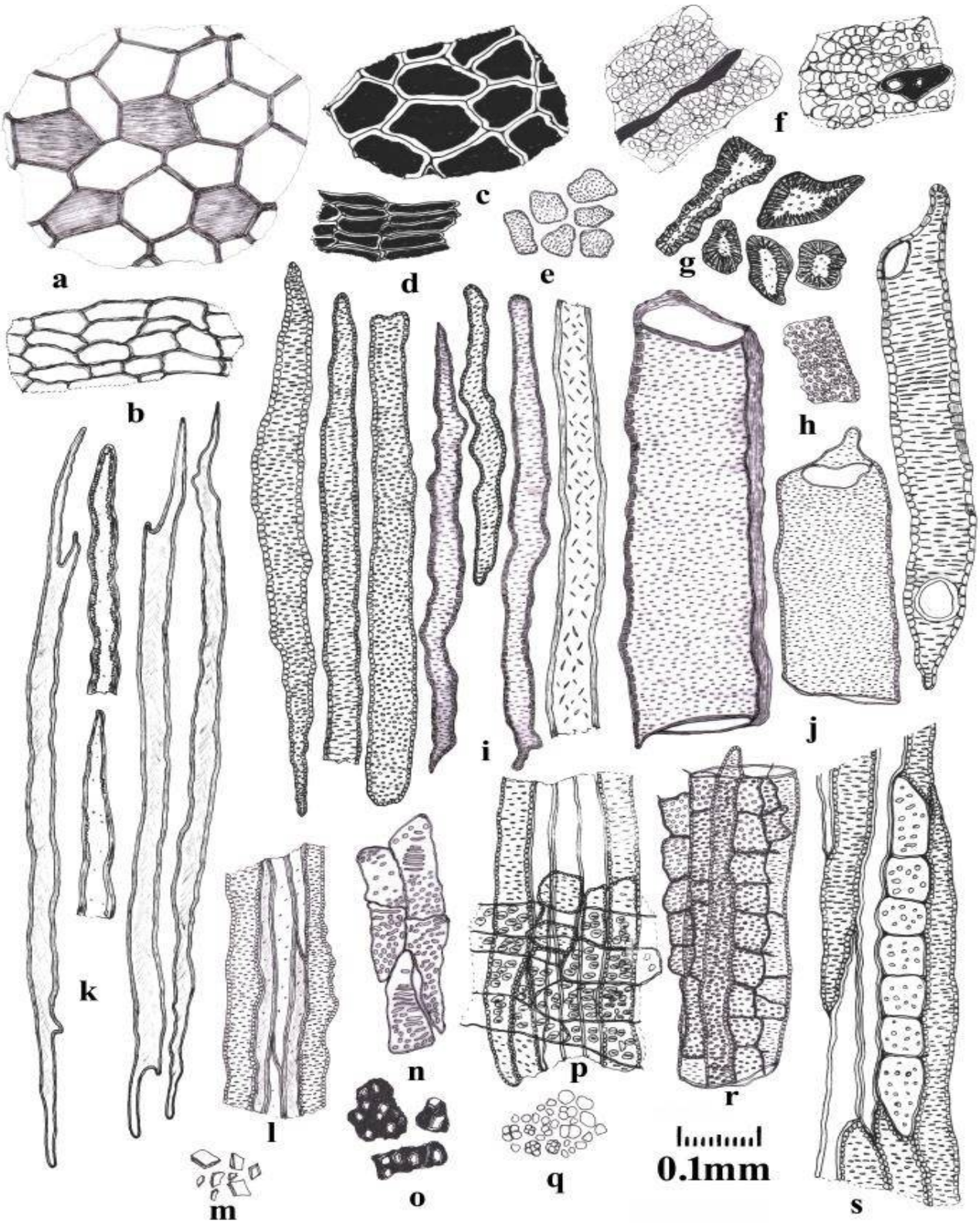

Fig. 3. Powder microscopy of *D. hamiltonii* . **a**, cork cells in surface view with brownish content; **b**, cork cells in sectional view; **c**, suberized cork cells in surface view; **d**, suberized cork cells in sectional view; **e**, xylem axial parenchyma cells; **f**, parenchymatous cells embedded with starch grains and latex canals; **g**, stone cells; **h**, bordered pitted vessels; **i**, fibretracheids; **j**, pitted vessels; **k**, fibres; **l**, fragment of fibre associated with fibretracheids; **m**, prismatic crystals of calcium oxalate; **n**, xylem ray parenchymatous cells; **o**, latex embedded with prismatic crystals; **p**, radially cut medullary rays crossing with fibre and fibretracheids; **q**, starch grains; **r**, tangentially cut vessels and fibre tracheid associated with xylem axial parenchymacells; **s**, tangential longitudinally cut medullary rays associated with fibre and fibretracheids.

The water and alcohol soluble extractive of *H. indicus* are almost equal. The alcohol soluble extractive value of both species are more or less equal which means that the presence of similar quantity of polar compound which are soluble in organic solvents. The *n*-hexane soluble extractive value of *D. hamiltonii* is 4.37 % and *H. indicus* 2.54 % which is an indication of presence of higher volume of low polar compounds in *D. hamiltonii* than *H. indicus*. With the difference in physicochemical values would facilitate the authentication of drugs (52). In a previous study on *D. hamiltonii* and *H.*

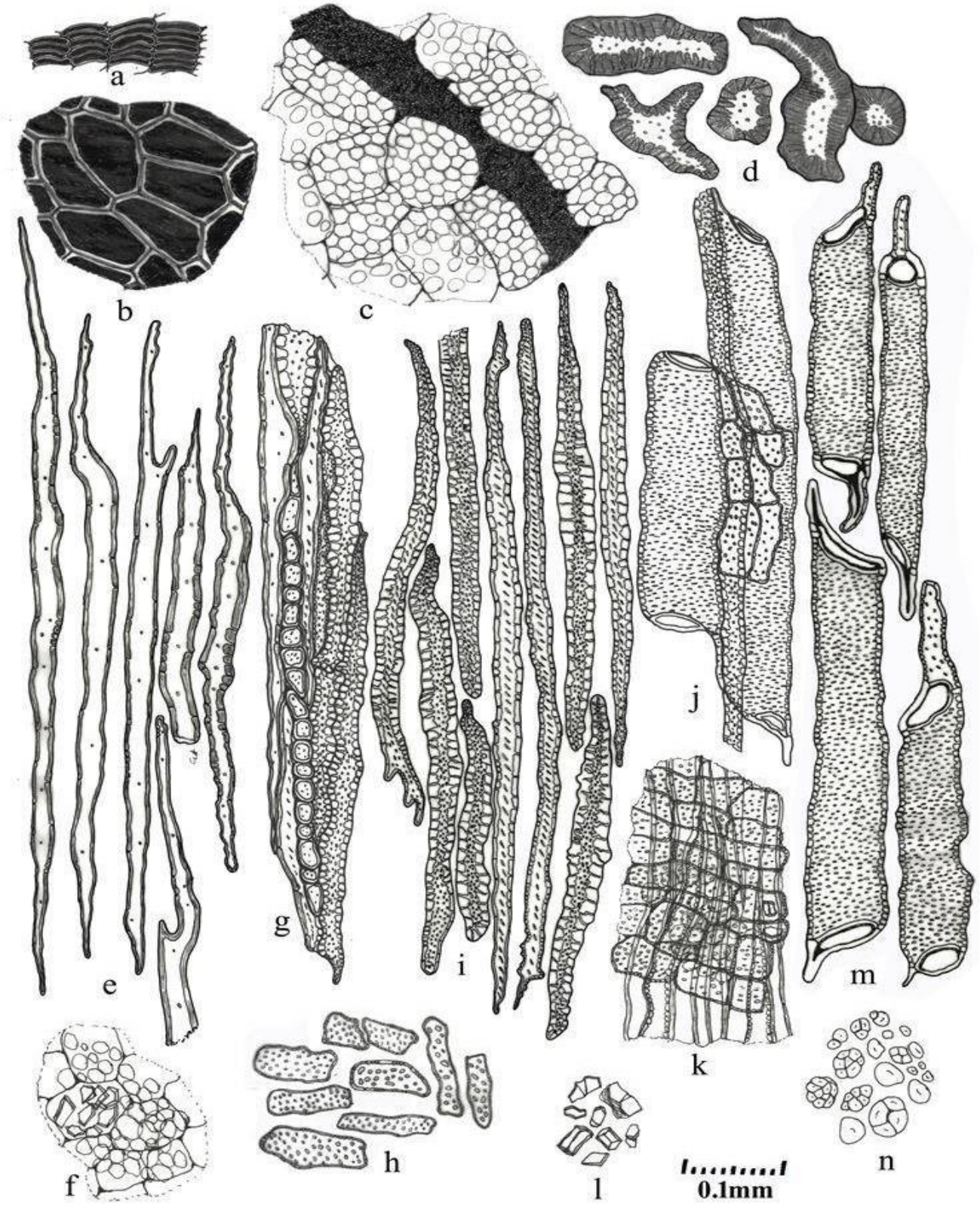

Fig. 4. Powder microscopy of *H. indicus* root and root stock. **a**. cork cells in sectional view; **b**. cork cells in surface view; **c**. fragment of parenchyma cells associated with laticiferous canal; **d**. stone cells and sclereids; **e**. thick walled, wide lumen, sharp end fibres; **f**. parenchymatous cells embedded with starch grains and prismatic crystals of calcium oxalate; **g**. tangential longitudinally cut xylem ray associated with fibre and fibre tracheids; **h**. lignified xylem parenchyma; **i**. thick walled fibre tracheids; **j**. longitudinally cut fragment of fibre tracheid associated with xylem parenchyma and pitted vessels; **k**. radial longitudinally cut xylem ray crossing with fibre, fibre tracheids and ray cells embedded with starch grains and prismatic crystals of calcium oxalate; **l**. prismatic crystals of calcium oxalate; **m**. tailed, simple perforated, pitted vessels; **n**. starch grains.

indicus collected from Maharashtra, physicochemical parameters have been compared (53) and the microscopic and chromatographic studies (hand TLC) has been reported (54).

Phytochemical Screening

D. hamiltonii and *H. indicus* root powders were subjected to phytochemical screening and their

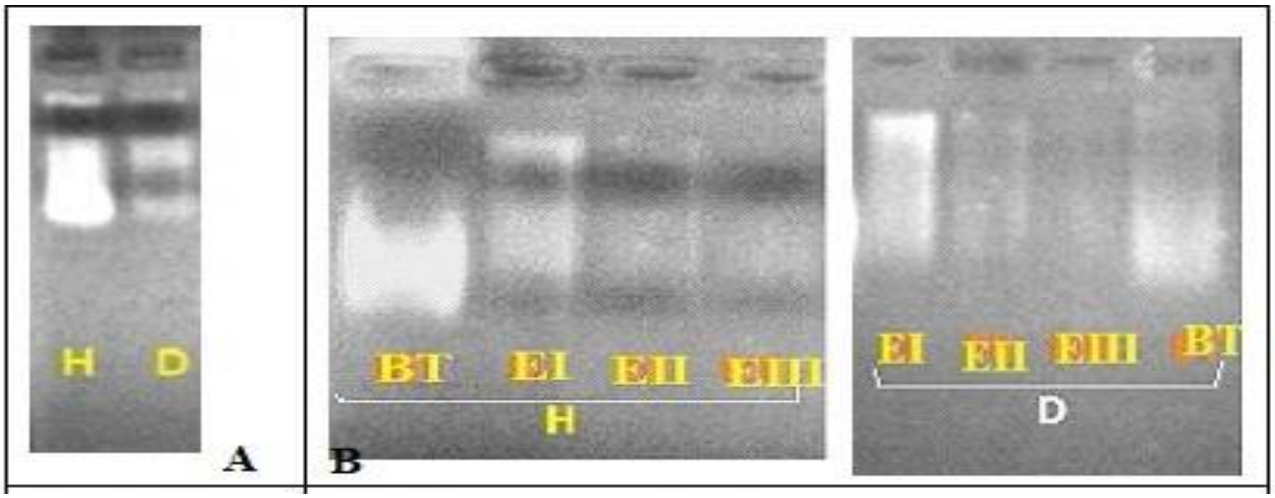

Fig. 5. A. Isolated DNA; **B**.Column purified DNA. H: *H. indicus*, D: *D. hamiltonii*, BT: Break through, EI: Elution I, EII: Elution II, EIII: Elution III.

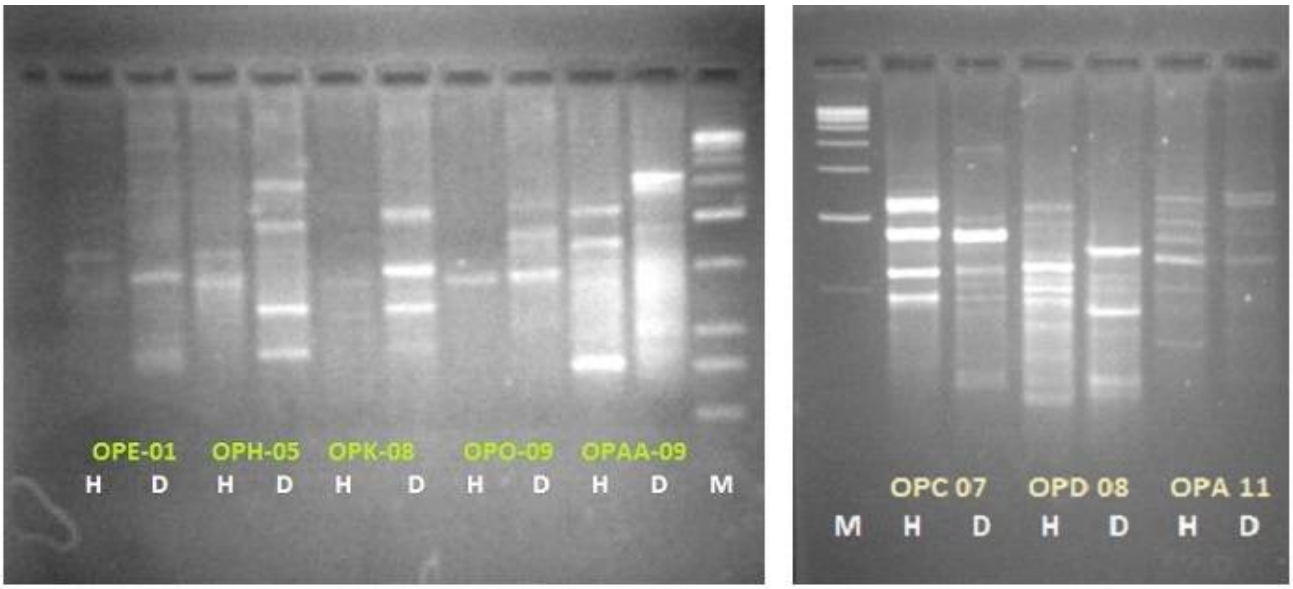

Fig. 6. Banding pattern of genomic DNA with random primers. H: *H. indicus*, D: *D. hamiltonii*, M: Marker.

presence, absence were documented (Supplementary Table 3). Tannins, flavonoids, steroids and coumarins were present only in *H. indicus* and absent in *D. hamiltonii.*

TLC Photo Documentation/HPTLC Chromatographic Studies

The TLC of all the extracts are shown in Fig. 7 and Supplementary Table 4. In the TLC of hexane extract, under 254 nm, a spot at R_f 0.78 appears in *D. hamiltonii* and absent in *H. indicus*; but under 366 nm no distinct spot appears; after derivatization a spot at R_f 0.88 (brown) appears only in *H. indicus* and not in *D. hamiltonii.*

In the TLC of chloroform extract, under 254 nm, two spots at R_f 0.19 and 0.33 appears only in *D. hamiltonii* whereas two spots at R_f 0.28 and 0.81 (all green) appear only in *H. indicus*; under 366 nm six additional spots at R_f 0.28 (blue),0.33 (ash), 0.36 (brown), 0.39 (ash), 0.52 (blue) and 0.61 (light green) appear in *D. hamiltonii* which are missing in *H. indicus;* after dipping in VSR two spots at R_f 0.20 (pink) and 0.45 (blue) appear in *D. hamiltonii* which are absent in *H. indicus* but one extra brown spot at R_f 0.81 appear.

In the ethanol extract, under UV 254 nm, three spots at R_f 0.33, 0.43 and 0.82 (all green) appear in *D. hamiltonii* which are missing in *H. indicus*; under UV 366 nm, seven spots at R_f 0.07 (ash), 0.16 (blue), 0.18 (blue), 0.47, 0.52, 0.56 (all ash) and 0.70 (sky blue) appears only in *D. hamiltonii* but not in *H. indicus*; in the derivatized TLC of ethanol extract of *D. hamiltonii* spots at R_f 0.19, 0.35 (both violet) and 0.59 (yellow) appear whereas in *H. indicus*, a light pink spot at R_f 0.55 appear. In the comparative TLC of both species, appearance of the above mentioned additional spots may be considered as distinguishing spots to identify the authentic *H. indicus* from the adulterated *D. hamiltonii.*

Under UV 254 nm, the HPTLC of hexane extract of *D. hamiltonii* (Supplementary Fig. 4) showed major peaks (5, 7 and 8) at R_f 0.32 (area 22.47%), 0.75 (27.05%) and 0.85 (19.35%); *H. indicus* showed major peaks (5, 8 and 9) at R_f 0.33 (13.38%), 0.76 (13.15%) and 0.88 (53.83%); under 366 nm *D. hamiltonii*

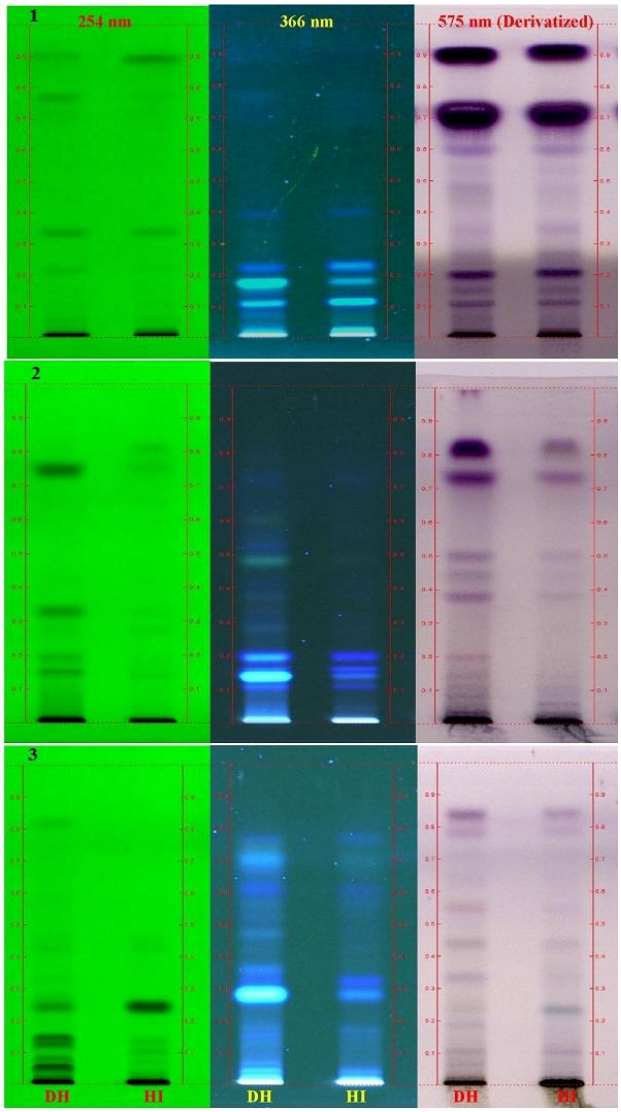

Fig. 7. TLC profile of *D. hamiltonii* and *H. indicus* root. 1. Hexane extract; 2. Chloroform extract; 3. Ethanol extract.

showed major peaks (2, 3 and 4) at R_f 0.09 (30.72%), 0.15 (56.94%) and 0.20 (10.86%); *H. indicus* showed major peaks (2, 4 and 6) at R_f 0.09 (46.27%), 0.21 (19.86%) and 0.90 (20.97%); under after derivatization with VSR, at 520 nm, for *D. hamiltonii* showed major peaks (10 and 12) at R_f 0.68 (31.67%) and 0.89 (28.75%); *H. indicus* showed major peaks (3, 8 and 9) at R_f 0.18 (13.65%), 0.69 (42.05%) and 0.89 (28.14%). The HPTLC finger print profile of *n*-hexane extract of *D. hamiltonii* at 254 nm shows three additional peaks at R_f 0.13 (area 2.86%), R_f 0.65 (19.35%), R_f 0.94 (4.65%) which are not present in *H. indicus*; at the same time, *H. indicus* shows a peak at R_f 0.67 (7.53%) which is not visible in *D. hamiltonii*; at 366 nm, *D. hamiltonii* shows a peak at R_f 0.05 (48%) but it is absent in *H. indicus; H. indicus* shows additional peaks at R_f 0.43 (1.98%) and 0.90 (20.97%) than *D. hamiltonii*; at 520 nm *D. hamiltonii* shows three extra peaks at R_f 0.04, 0.21 and 0.72 which are not found in *H. indicus.*

Under UV 254 nm, the HPTLC of chloroform extract of *D. hamiltonii* (Supplementary Fig. 5) showed major peaks (5 and 10) appeared at R_f 0.31 (area 13.51%) and 0.73 (43.35%); for *H. indicus* showed major peaks (3, 9 and 10) at R_f 0.25 (11.30%), 0.74 (26.41%) and 0.79 (30.90%); under 366 nm, *D. hamiltonii* showed major peaks (1 and 2) at R_f 0.11 (64.97%) and 0.17 (25.05%); *H. indicus* showed major peaks (1, 2 and 3) at R_f 0.11 (18.52%), 0.14 (32.66%) and 0.17 (45.88%); after derivatization with VSR, at 520 nm, *D. hamiltonii* showed major peaks (11 and 12) at R_f 0.71 (34.76%) and 0.79 (35.39%); *H. indicus* showed major peaks (9 and 10) at R_f 0.71 (42.50%) and 0.80 (37.84%). The HPTLC finger print profile of chloroform extract of *D. hamiltonii,* at 254 nm, shows peaks at R_f 0.06 (1.05%) and 0.53 (6.06%) but they are not shown in *H. indicus*; and *H. indicus* shows additional peaks at R_f 0.44 (4.26%) and 0.79 (30.90%); at 366 nm, *D. hamiltonii* shows four extra peaks at R_f 0.26 (1.12%), 0.46 (4.47%), 0.50 (2.27%) and 0.70 (2.10%) but are not noted in *H. indicus*; and *H. indicus* shows a peak at R_f 0.14 (area 32.66%) but it is not visible in *D. hamiltonii*; at 520 nm, *D. hamiltonii* shows three peaks at R_f 0.17 (2.35%), 0.25 (0.46 %), 0.29 (1.47 %) which are missing in *H. indicus*; while *H. indicus* shows two peaks at R_f 0.04 (0.85 %) and 0.89 (3.73%) but are not visible in *D. hamiltonii.*

The HPTLC of successive ethanol extract of *D. hamiltonii*, under UV 254 nm (Supplementary Fig. 6), showed major peaks (1, 3, 4, 5 and 10) appeared at R_f 0.04 (area 13.78%), 0.11 (12.58%), 0.13 (14.70%), 0.22 (24.56%) and 0.79 (11.67%); *H. indicus* showed major peaks (5, 7 and 9) at R_f 0.24 (46.88%), 0.43 (10.79%) and 0.75 (28.95%); under 366 nm, *D. hamiltonii* showed major peaks (2 and 7) at R_f 0.25 (75.96%) and 0.67 (14.29%); *H. indicus* showed major peaks (2, 3, 5 and 6) at R_f 0.28 (30.79%), 0.33 (17.11%), 0.76 (14.71%) and 0.90 (21.02%); after derivatization with VSR, at 520 nm, *D. hamiltonii* showed major peaks (8, 9, 10, 11 and 12) at R_f 0.54 (15.82%), 0.63 (10.26%), 0.72 (10.41%), 0.79 (16.58%) and 0.84 (24.75%); *H. indicus* showed major peaks (6, 7 and 8) at R_f 0.72 (21.62%), 0.80 (17.31%) and 0.85 (25.08%). The HPTLC finger print profile of ethanol extract of *D. hamiltonii,* at 254 nm, shows a peak at R_f 0.69 (4.69%) but it is not seen in *H. indicus*; at 366 nm, *D. hamiltonii* shows four peaks at R_f 0.41 (0.60%), 0.44 (1.49%), 0.51 (0.78%), 0.67 (14.29%) %) which are not seen in *H. indicus*; whereas *H. indicus* shows a peak at R_f 0.90 (21.02%) but it is not visible in *D. hamiltonii*; at 520 nm, *D. hamiltonii* shows four additional peaks at R_f 0.04 (0.85%), 0.06 (1.01%), 0.16 (1.57%), 0.63 (10.26%) which are not visible in *H. indicus;* but *H. indicus* showed a peak at R_f 0.95 (1.43%) but it is missing in *D. hamiltonii.* The presence or absence of distinguishing peaks may be useful for the authentication of warranted drug (55-56).

Conclusion

The present study that both roots shows differences size and shape of the cork cells, fibre, fibre tracheids, vessels, xylem parenchyma cells, polymorphism in eight primers, water and hexane soluble extractives, tannins, flavonoids, steroids, coumarins, difference in TLC spot in the ethanol

extract which can be used for the identification of plant of interest and differentiate the authentic plant root from the adulterant available in crude drug market.

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Authors' contributions

ST performed the analytical studies and literature study, SS and KNS carried out the macroscopic, microscopical, powder microscopical studies, RP helped in data acquisition in RAPD, RS and GP designed the topic, edited and revised the manuscript with valuable suggestions.

Conflict of interests

Authors do not have any conflict of interests to declare.

Supplementary files

Table 1. Macroscopic characters of *D. hamiltonii and H. indicus*

Table 2. Details of polymorphism found in *H. indicus* and *D. hamiltonii*

Table 3. Phytochemical screening of *D. hamiltonii* and *H. indicus* root

Table 4. R_f values and color spots of *D. hamiltonii* and *H. indicus* root

Fig. 1. Photograph of *D. hamiltonii* Wight & Arn. **A**. Dried roots, **B.** Powder

Fig. 2. Photograph of *H. indicus* (L.) R. Br. ex Schult. **A**. Dried roots, **B**. Powder

Fig. 3. Physicochemical constants of *D. hamiltonii* and *H. indicus* root

Fig. 4. HPTLC 3D chromatogram and peak table of hexane extracts

Fig. 5. HPTLC 3D chromatogram and peak table of chloroform extracts

Fig. 6. HPTLC 3D chromatogram and peak table of ethanol extracts

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