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Original Article

Botanical and genetic characterization of *Hydrocotyle umbellata* L. cultivated in Egypt

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ABSTRACT

Hydrocotyle umbellata L. is a creeping aquatic herb belonging to family Araliaceae. The plant has been used in folk medicine as an anti-inflammatory, anxiolytic and memory stimulant herb. This study targets the botanical features of the leaf, rhizome and roots, in addition to DNA fingerprinting using rapid amplified (RAPD) and inter simple sequence repeat (ISSR) based polymerase chain reaction (PCR) techniques. Microscopically, leaves and rhizomes are characterized by numerous brown schizogenous ducts containing volatile oils. On the other hand, a total of 34 random amplified polymorphic DNA (RAPD) markers were identified using seven random decamer primers and a total of 15 inter simple sequence repeat (ISSR) markers were identified using five decamer random primers. Both the botanical study and DNA fingerprinting could be useful in identification and authentication of the plant.

1. Introduction

Genus *Hydrocotyle* comprises about 130 species of creeping aquatic plants [1]. Members of the genus are used in Taiwan folk medicine as anti-inflammatory herbs [2]. Moreover, in Brazilian folk medicine the decoction of *Hydrocotyle umbellata* leaves is used orally or as baths for treatment of inflammatory disorders [3].

H. umbellata L. (popularly known as Acaricoba, Water pennywort, Marsh pennywort) (Fig. 1), is a perennial creeping herb, grown widely in the Americas and mainly native to Brazil [4,5]. It is a long petiolated plant characterized by peltate-shaped leaves, simple umbel inflorescence, somewhat exceeding the length of the leaves, bearing numerous small white flowers and flattened capsule-shaped fruits [6,7]. It has a great interest in folk medicine and Ayurvedic medicine because of its potential memory stimulant and anxiolytic effects [8]. The ethanolic extract of the underground parts of *H. umbellata* exhibited significant analgesic, anti-inflammatory and anxiolytic activities [5,7,9]. Literature survey revealed that little information could be traced concerning the botanical features of the organs used in folk medicine; therefore, this study presents the botanical investigation of leaf, rhizome and root systems, as well as the DNA fingerprinting of the plant to characterize the plant botanically and genetically.

2. Materials and methods

2.1. Plant material

Samples of *H. umbellata* L. used in this study; leaves, rhizomes and roots, were collected in May 2014 from El-Orman Botanical Garden, Giza, Egypt. The plant was kindly identified by Eng. Threase Labib, consultant in Orman Garden and National Gene Bank, Ministry of Agriculture. A voucher specimen (No. 17-8-2016) is kept at the Herbarium of Pharmacognosy Department, Faculty of Pharmacy, Cairo University.

2.2. Botanical profiling

Photographs for macroscopical and microscopical studies were taken by Canon power shot A470 digital camera and Leica light microscope equipped with Leica Queen 550IW digital camera (Leica microsystems, Wetzlar, Germany) for botanical examination, respectively. Samples of the leaves, rhizomes and roots were separated and examined either fresh or after keeping in ethanol (70%) containing 5% glycerol, as well as after being dried and reduced to fine powder for botanical examination.

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Fig. 1. Photographs of *H. umbellata* L. (X = 0.45).

Fig. 2. Photographs of *H. umbellata* L. leaf. A. Leaf lamina: upper surface (X = 0.4). B. Leaf lamina: lower surface (X = 0.4).

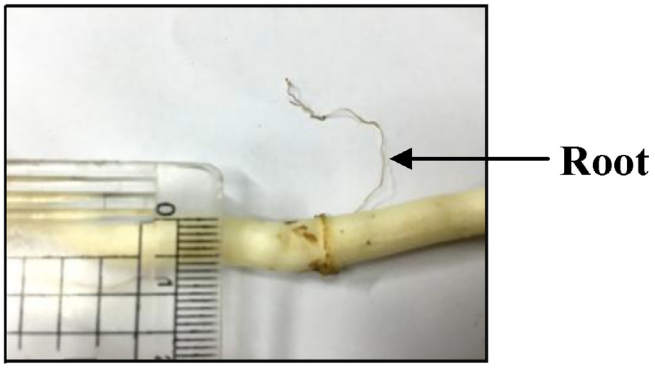

Fig. 3. Photographs of *H. umbellata* L. rhizome. (X = 1.1).

2.3. Genetic profiling

2.3.1. DNA fingerprinting

Freeze-dried whole leaves were ground under liquid nitrogen to a fine powder prior to DNA isolation for RAPD and ISSR analysis. This was carried out in the Central Laboratory, Faculty of Agriculture, Cairo University.

2.3.2. DNA isolation procedure

The bulked DNA extraction was performed according to DNeasy Plant Mini Kit (supplied by QIAGEN).

2.3.3. Oligonucleotide primers for RAPD analysis

Seven primers were obtained from Operon Technologies Inc., Almeda, California, USA, with the following sequences; **A-01:** 5′CAG GCC CTT C 3′, **A-07:** 5′ GAA AGG GGT G 3′, **A-10**: 5′GTA GAC CCG T 3′, **B-01**: 5′ GTT TCG CTC C 3′, **B-07**: 5′ GGT GAC GCA G 3′, **C-12**: 5′ GGC TGT CCG T 3′, **M-01**: 5′ACG GCG TAT G 3′.

2.3.4. Oligonucleotide primers for ISSR analysis

Five primers were obtained from Operon Technologies Inc., Almeda, California, USA, with the following sequences; **HB-08**: 5′ GAG AGA GAG AGA GG 3′, **HB-10**: 5′GAG AGA GAG AGA CC 3′, **HB-12**: 5′ CAC CAC CAC GC 3′, **HB-13**: 5′ GAG GAG GAG GC 3′, **HB-14**: 5′ CTC CTC CTC GC 3′.

2.3.5. Statistical analysis

The DNA bands generated by each primer were counted and their molecular sizes were compared with those of the DNA markers (100 bp ladder, Promega Corporation, Madisson, USA). The bands scored from DNA profiles generated by each primer were pooled together. Then, the presence or absence of each DNA band was treated as a binary character in a data matrix (coded 1 and 0, respectively) using computer program SPSS-10.

2.3.6. Polymerase chain reaction (PCR)

PCR was performed in 30-μl volume tubes according to [10]. Each PCR reaction contained 3.00 μl of dNTPs (2.5 mM), 3.00 μl of $MgCl_2$ (25 mM), 3.00 μl of 10 X PCR buffer, 2.00 μl of Operon primer (10 pmol), 0.20 μl of Taq DNA polymerase (5U/μl), 2.00 μl of Template DNA (25 ng) and 16.80 μl of distilled water.

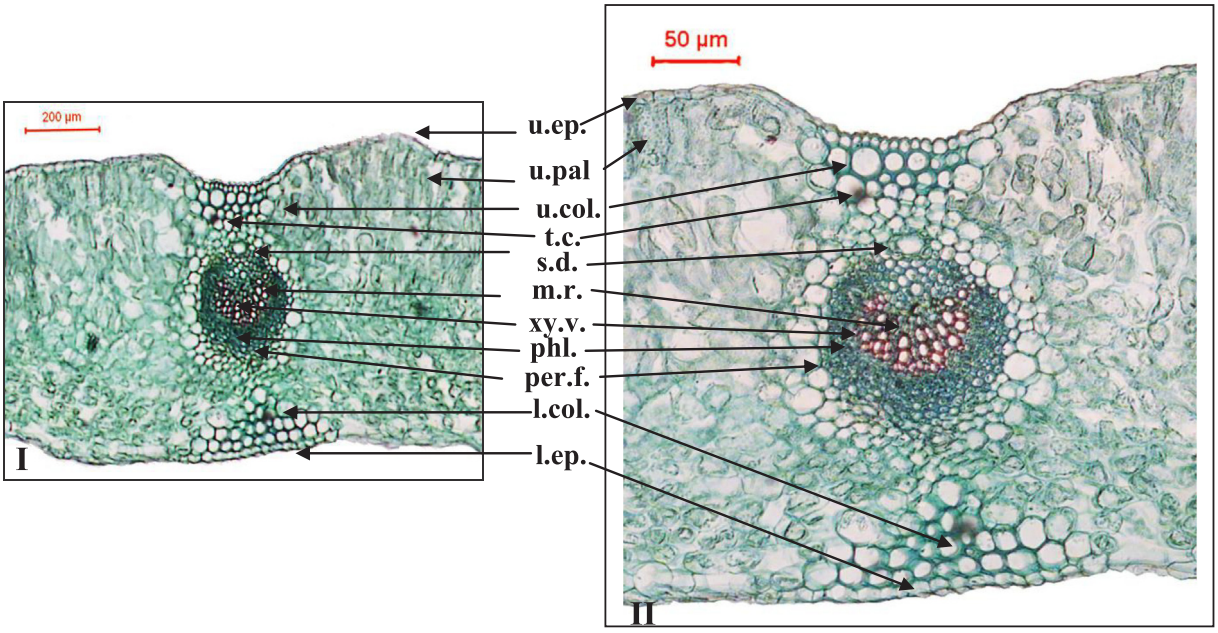

Fig. 4. Photographs of transverse section in leaf lamina of *H. umbellata* L. I: T.S. in leaf lamina (low power) (X = 50), II: T.S. in leaf lamina (high power) (X = 280). l.col., lower collenchyma; l.ep., lower epidermis; m.r., medullary rays; per.f., pericyclic fibre; phl., phloem; s.d., secretory ducts; t.c., tannin cells; u.col., upper collenchyma; u.ep., upper epidermis; u.pal., upper palisade; xy.v., xylem vessel.

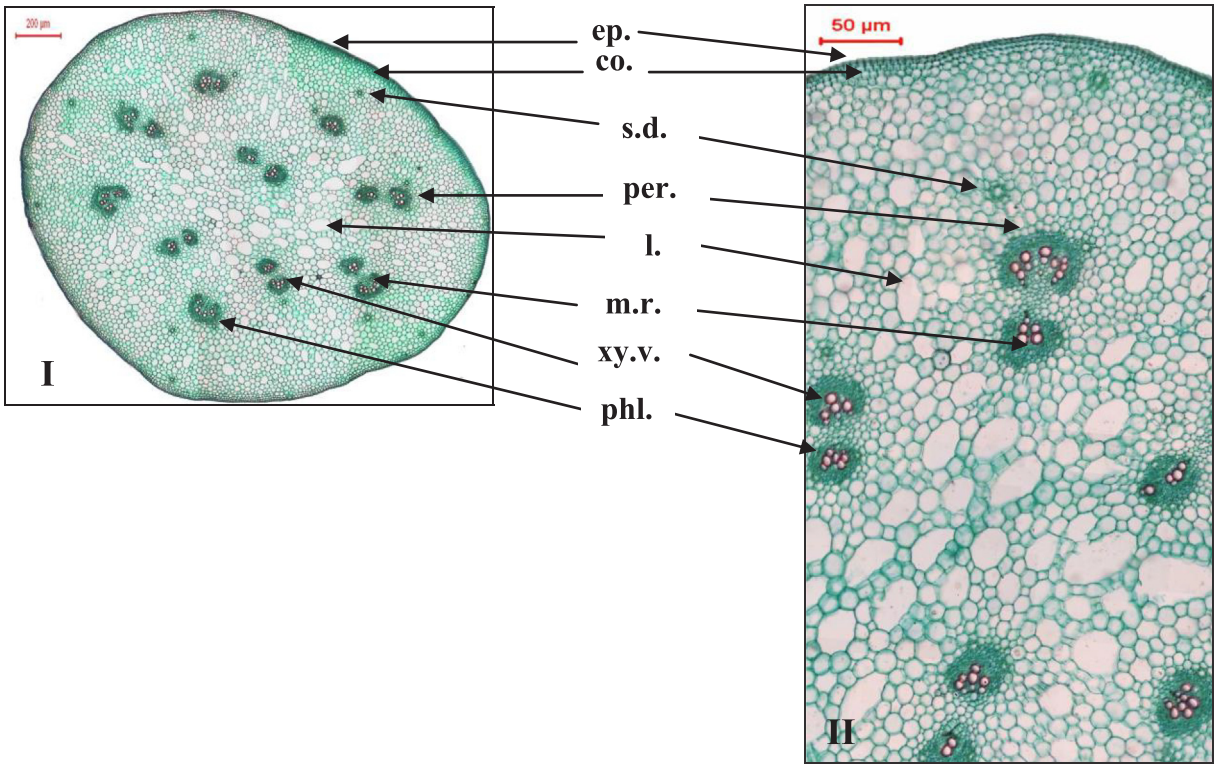

Fig. 5. Photographs of T.S. in leaf petiole of *H. umbellata* L. I. T.S. in leaf petiole (low power) (X = 40). II. T.S. in leaf petiole (high power) (X = 300). **co.**, cortex; **ep.**, epidermis; **l.**, lacuna; **m.r.**, medullary rays; **per.**, pericycle; **phl.**, phloem; **s.d.**, secretory duct; **xy.v.**, xylem vessel.

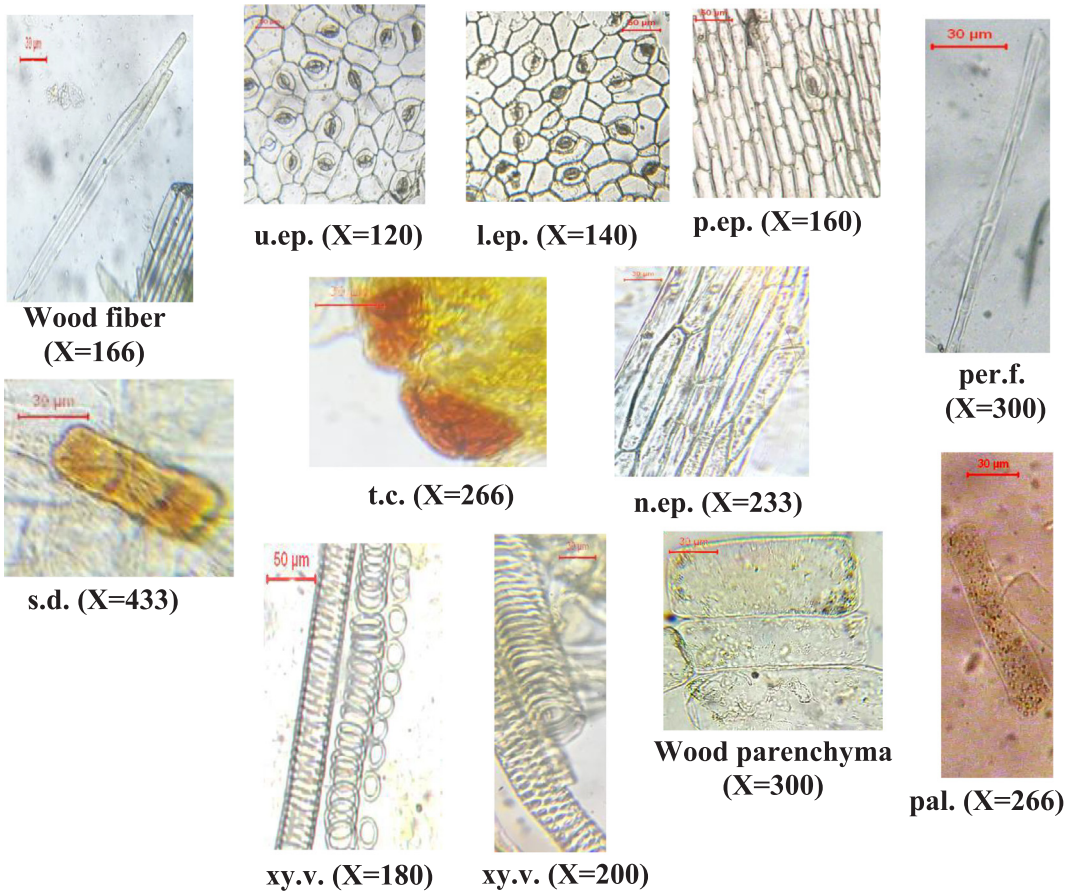

Fig. 6. Powdered leaf.

2.3.7. PCR program and temperature profile of randomly amplified polymorphic-PCR

The DNA amplifications were performed in an automated thermal cycle (model Techni 512) programmed for one cycle at 94 °C for 4 min, followed by 45 cycles of 1 min at 94 °C, 1 min at 37 °C, and 2 min at 72 °C. The reaction was finally stored at 72 °C for 10 min.

2.3.8. PCR program and temperature profile inter simple sequence repeat-PCR

The DNA amplifications were performed in an automated thermal cycle (model Techni 512) programmed for one cycle at 94 °C for 4 min followed by 45 cycles of 1 min at 94 °C, 1 min at 57 °C, and 2 min at 72 °C. The reaction was finally stored at 72 °C for 10 min.

2.3.9. Gel electrophoresis and staining

Amplified products were analysed by electrophoresis on 1.5% agarose gel and finally stained with ethidium bromide. The run was performed for about 30 min at 80 V in mini submarine gel BioRad.

3. Results and discussion

3.1. Morphological investigation of Hydrocotyle umbellata L.

3.1.1. The leaf (Fig. 2)

The leaves are green in colour, simple, alternate, petiolate with characteristic odour and taste. The lamina is peltate in shape, rounded (2–6 cm wide), glabrous, with crenate margins, showing palmate venation, with upper surface being darker in colour than the lower one and attached from its center to long slender petiole. The petiole is long, cylindrical, glabrous, measuring 16 to 20 cm in length and 0.5 to 0.7 cm in width, green to greenish purple in colour.

3.1.2. The rhizome (Fig. 3)

The rhizomes are cylindrical in shape (0.5–1 cm in diameter), creamy white in colour, grow horizontally below the soil surface, with characteristic odour and taste, thick, fleshy, solid, showing swollen nodes from which it gives roots downwards and aerial parts upwards.

3.2. Anatomical investigation of Hydrocotyle umbellata L.

3.2.1. The leaf (Figs. 4, 5 and 6)

Hydrocotyle umbellata L. leaf was subjected to a detailed anatomical study including the lamina, the petiole and the powdered form.

3.2.2. The lamina (Fig. 4)

A transverse section in the leaf lamina (Fig. 4) shows upper and lower epidermises, enclosing a dorsiventral mesophyll and several rows of irregularly shaped parenchyma cells, with wide intercellular spaces, showing few tannin cells (stained dark blue with ferric chloride) of its spongy tissue. The palisade tissue consists of two to three rows of radially elongated columnar cells with thin straight anticlinal wall, loosely packed and containing green plastids interrupted in the midrib region by collenchymatous cells. The upper epidermis consists of polygonal cells having straight anticlinal wall, covered with smooth cuticle and showing paracytic stomata. The lower epidermis is similar to the upper one, but smaller in size.

The neural epidermis is polygonal, axially elongated, with straight slightly thickened walls, covered with smooth cuticle and devoid of stomata.

The midrib is slightly depressed on the upper side, showing one crecent-shaped collateral vascular bundle with xylem directed toward the upper and phloem toward the lower sides, the vascular bundle is surrounded by nearly continuous patches of non-lignified pericyclic

Table 1
Microscopical measurements of the elements of different organs of *H. umbellata* L. (in microns).

Elements	Length	Width	Height	Diameter
Leaf				
Upper epidermis	41–58-75	33–41-50	3.5–7-10	N.A
Stomata	25–33-41	16–20-25	N.A	N.A
Lower epidermis	35–42-60	14–21-28	N.A	N.A
Stomata	7–10-13	3–5-9	N.A	N.A
Neural epidermis	98–102-105	12–17-19	N.A	N.A
Petiole epidermis	50–68-112	7–9-12	6–7-9	N.A
Palisade cells	112–123-131	22–26-30	N.A	N.A
Pericyclic fibres (Lamina)	166–176-183	5–6-7	N.A	N.A
Wood fibres	294–306-324	6–12-18	N.A	N.A
Xylem vessels	N.A	N.A	N.A	19–22-17
Pitted xylem vessels	N.A	N.A	N.A	25–28-35
Wood parenchyma	111–113-116	30–43-50	N.A	N.A
Secretory ducts	48–57-67	13–16-20	N.A	N.A
Tannin cells	37–56-63	18–24-33	N.A	N.A
Rhizome				
Epidermis	42–90-120	30–31-33	6–7-9	N.A
Wood fibres	300–313-333	8–3-16	N.A	N.A
Spiral xylem vessels	N.A	N.A	N.A	22–37-56
Wood parenchyma	100–113-145-	33–38-56	N.A	N.A
Secretory ducts	67–75-86	26–30-33	N.A	N.A
Tannin cells	33–40-50	16–25-26	N.A	N.A
Starch garanules	N.A	N.A	N.A	5–16-21
Xylem vessels	N.A	N.A	N.A	30–45-52
Root				
Piliferous layer	40–55-80	20–25-35	5–7-9	N.A
Wood parenchyma	100–175-200	30–40-55	N.A	N.A
Wood fibre	257–300-321	5–7-8	N.A	N.A
Xylem vessels	N.A	N.A	N.A	12–17-24
Starch granules	N.A	N.A	N.A	4–15-21

*N.A; Not Applicable.

Table 2
Molecular size in base pairs of amplified DNA fragments produced by seven decamer primers in *Hydrocotyle umbellata* L.

Band No.	M.W bp	Primer Name						
		A01	A07	A10	B01	B07	C12	M01
1	985	0	1	0	0	0	0	0
2	560	0	1	0	0	0	0	0
3	435	0	1	1	0	0	0	0
4	360	0	1	0	1	0	0	1
5	330	0	1	1	1	0	0	1
6	290	1	1	1	1	1	1	1
7	250	1	1	1	1	1	1	1
8	210	0	1	1	1	0	1	1
9	165	0	0	0	1	0	1	0
10	130	0	0	0	1	0	0	0
11	110	0	0	0	0	0	1	0
Total		2	8	5	7	2	5	5

(1) = presence of bands; (0) = absence of bands.

fibres and traversed by uni- to biseriate medullary rays consisting of polygonal slightly elongated cells.

The upper and lower cortical tissue of the midrib consist of 2–3 rows of collenchymatous cells, followed by 3–4 rows of parenchymatous cells showing secretory ducts secreting volatile oil (stained red with Sudan III).

3.2.3. The petiole (Fig. 5)

A transverse section in the petiole (Fig. 5) is nearly oval in shape. It is formed of outer polygonal axially elongated epidermal cells with straight anticlinal walls, covered with smooth cuticle and showing rare paracytic stomata, followed by parenchymatous cortex. The ground tissue is formed of several rows of rounded parenchymatous cells with

Table 3
Molecular size in base pairs of amplified DNA fragments produced by five decamer primers in *Hydrocotyle umbellata* L.

Band No.	M.W bp	Primer Name				
		HB-8	HB-10	HB-12	HB-13	HB-14
1	625	0	0	1	1	0
2	400	0	0	0	1	0
3	375	1	0	0	0	0
4	330	0	0	1	1	0
5	270	1	1	1	1	1
6	205	1	0	0	1	1
7	170	0	1	0	0	0
Total		3	2	3	5	2

narrow intercellular spaces containing numerous schizogenous secretory ducts lined with epithelial cells secreting volatile oil (stained red with sudan III) placed opposite to vascular bundles. The pericycle is composed of 1–2 rows of rounded parenchyma cells.

The vascular system is complex, showing irregularly distributed, sometimes inversely oriented crescent-shaped collateral vascular bundles, separated by uni- to biseriate polygonal slightly elongated parenchymatous medullary rays. Numerous lacunae are present in the center of the ground tissues [11,12].

3.2.4. Powdered leaf (Fig. 6)

The powdered leaf is green in colour, with characteristic odour and taste. Microscopically, it is characterized by presence of the upper and lower leaf epidermises, neural epidermis, petiole epidermis, palisade cells, tannin cells, non-lignified fusiform septate pericyclic fibres, non-lignified wood fibres, lignified spiral, annular, reticulate and pitted xylem vessels, elongated thick-walled wood parenchyma and schizogenous secretory ducts. The measurements of different elements detected are summarized in Table 1.

3.2.5. The rhizome (Figs. 7 and 8)

A transverse section in the rhizome (Fig. 7) is more or less circular in outline, formed of one row of thin-walled elongated parenchyma cells, covered with smooth cuticle and devoid of stomata, followed by several rows of parenchymatous cortex containing starch granules (stained blue with iodine), tannin cells (stained dark blue with ferric chloride) and few scattered schizogenous secreting ducts. The endodermis is indistinct. The pericycle is formed of 2–3 rows of parenchyma cells. The vascular tissue is formed of collateral vascular bundles traversed by trito multi-seriate medullary rays. The central pith is wide, constituting about two third of the diameter and formed of parenchyma cells containing starch granules and few tannin cells.

3.2.6. Powdered rhizome (Fig. 8)

The powder is yellowish-brown in colour, with characteristic odour and taste. It is characterized microscopically by the presence of epidermal cells, parenchymatous cells containing simple, oval or rounded starch granules stained blue with iodine, rounded tannin cells, brown schizogenous ducts, lined with thin-walled epithelial cells, non-lignified slightly thickened wood fibres, with undulating walls showing moderately wide to narrow lumen, having acute tapering apices and some are septated, lignified pitted and spiral xylem vessels, thin-wall elongated medullary rays, wood parenchyma. The measurements of different elements detected are summarized in Table 1.

3.2.7. The root (Figs. 9 and 10)

A transverse section in the root (Fig. 9) is circular in outline, formed of piliferous layer followed by the cortex, which is composed of 5–6 rows of thin-walled parenchymatous cells, packed with starch granules stained blue with iodine. The piliferous layer consists of straight thin-walled polygonal slightly elongated parenchyma cells covered with

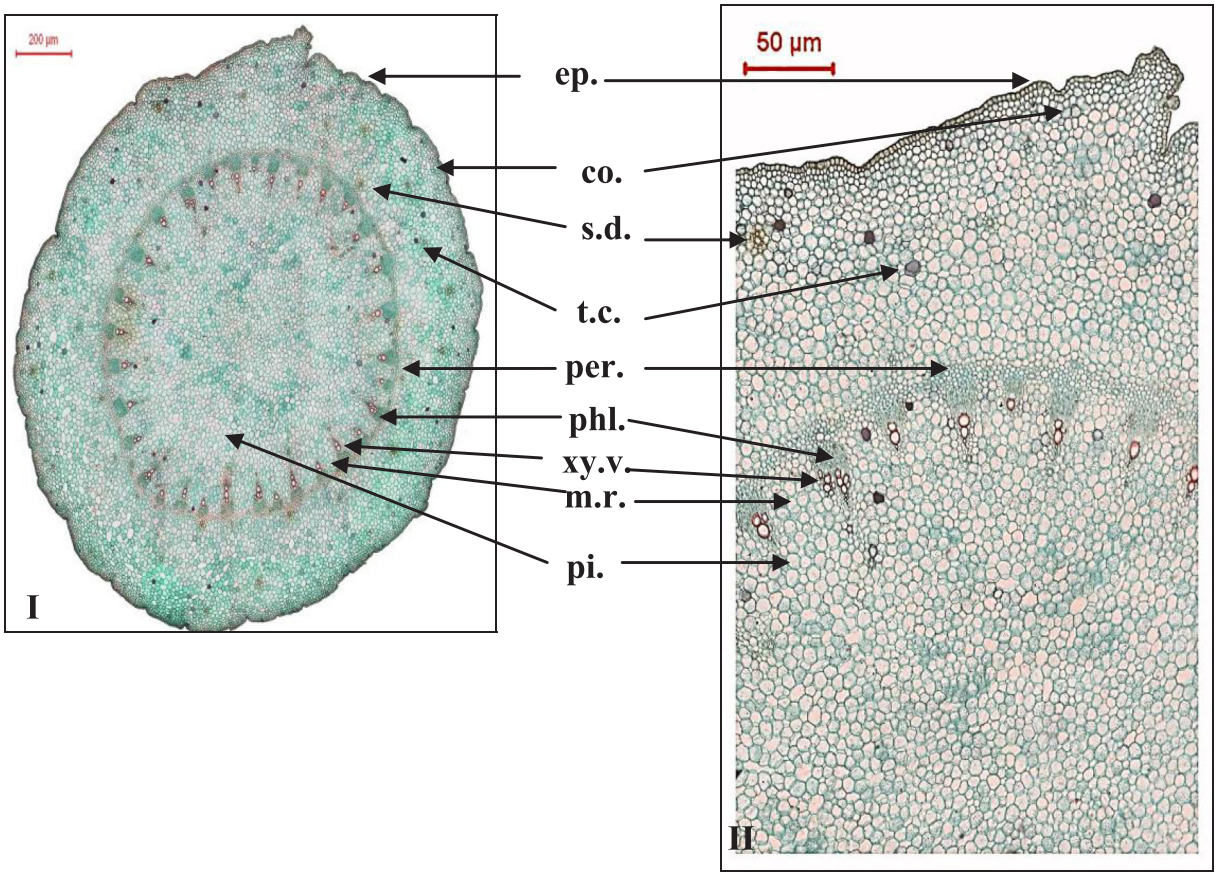

Fig. 7. Photographs of T.S. in rhizome of *H. umbellata* L. I. T.S. in rhizome (low power) (X = 50). II. T.S. in rhizome (high power) (X = 320). **co.**, cortex; **ep.**, epidermis; **m.r.**, medullary rays; **per.**, pericycle; **phl.**, phloem; **pi.**, pith; **s.d.**, secretory duct; **t.c.**, tannin cells; **xy.v.**, xylem vessel.

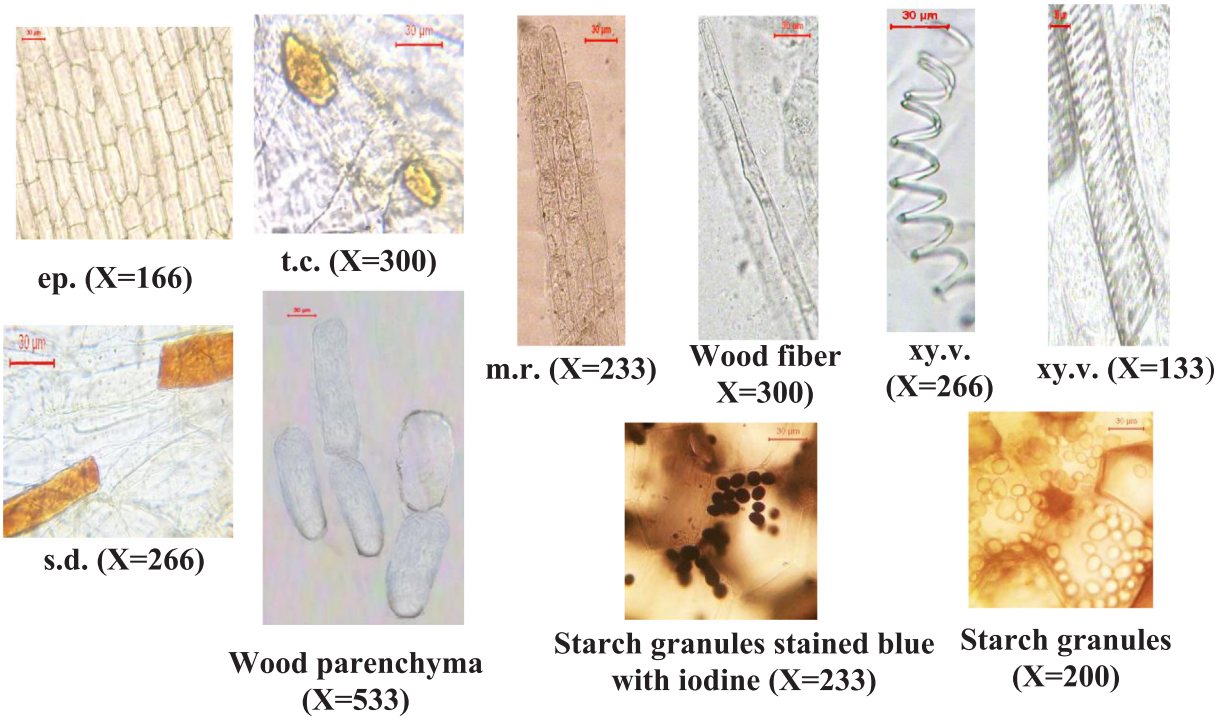

Fig. 8. Powdered rhizome.

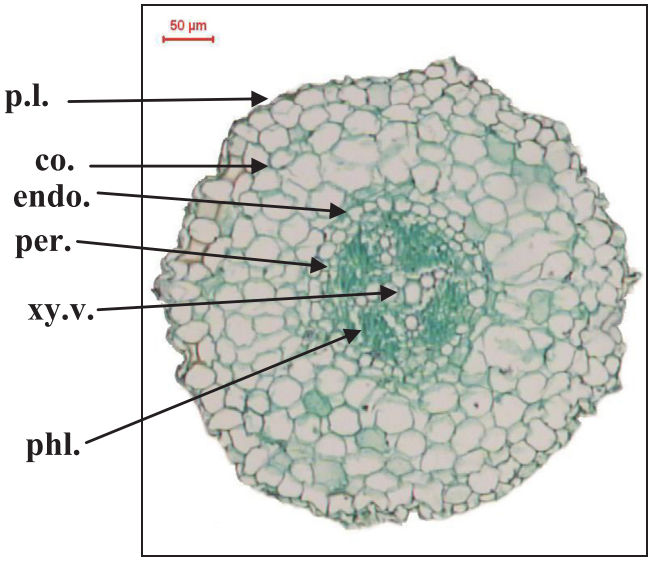

Fig. 9. T.S. in root (X = 160). **co.**, cortex; **endo.**, endodermis; **p.l.**, piliferous layer; **per.** pericycle; **phl.**, phloem; **xy.v.**, xylem vessel.

smooth cuticle and devoid of stomata. The endodermis is distinct, formed of one row of tangentially elongated thin-walled parenchymatous cells devoid of content, followed by one row of parenchymatous pericycle. The vascular tissue consists of alternating xylem and phloem arranged in a circle. The **xylem** is alternating with phloem and present in four radial groups (tetrarch) with the protoxylem toward the prephery and metaxylem toward the center of the section (exarch).

3.2.8. Powdered root (Fig. 10)

The powder is yellowish-brown in colour, odourless with characteristic taste. Microscopically, it is characterized by the presence of epidermal cells, non-lignified wood fibres, parenchyma cells containing starch granules similar to those present in rhizome, wood parenchyma, annular and pitted lignified xylem vessels. The measurements of the elements are summarized in Table 1.

3.3. DNA fingerprinting of Hydrocotyle umbellata L.

3.3.1. Analysis of RAPD data

The banding profile produced by seven decamer primers used in RAPD analysis of *H. umbellata* L. is illustrated in Fig. 11. The RAPD electrophoretic profile of the DNA sample showed distinguishable bands and generated 34 fragment patterns. The distribution of these bands is represented in Table 2. A total of **34** different fragments have been recorded showing 8 bands by primer OPA-07, 7 bands by primer OPB-01, 5 bands by primer OPA-10, OPC-12 and OPM-01, 2 bands by OPA-01 and OPB-07 primers.

3.3.2. Analysis of ISSR data

The obtained banding profiles produced by five primers used in ISSR analysis are represented in Fig. 12. The ISSR electrophoretic profile of the DNA sample showed distinguishable bands and generated 15 fragment patterns. The produced banding profiles by the five primers used in ISSR analysis were represented in Table 3. A total of **15** different fragments showing five bands generated by HB-13 primer, 3 bands produced by HB-8 and HB-12 primers, followed by two bands generated by HB-10 and HB-14 primers.

4. Conclusion

This study aimed to characterize the plant on both botanical and genetic levels. Microscopically *H. umbellata* L. leaves and rhizomes are characterized by the presence of numerous schizogenous secreting ducts. Seven and five primers were used to reveal RAPD and ISSR fragments, respectively. 34 fragments were generated by RAPD analysis, while 15 fragments were produced by ISSR analysis. The results suggest the use of OPA-07 and OPB-01 primers in RAPD analysis and HB-13 primer in ISSR analysis for selective discrimination and identification of the plant.

Author contributions

HE, SM and EA participated in study concept and design, analysis and interpretation of data, and critical revision of the manuscript for important intellectual content. SA drafted the manuscript, carried out the transverse sections, and participated in analysis and interpretation of the obtained data.

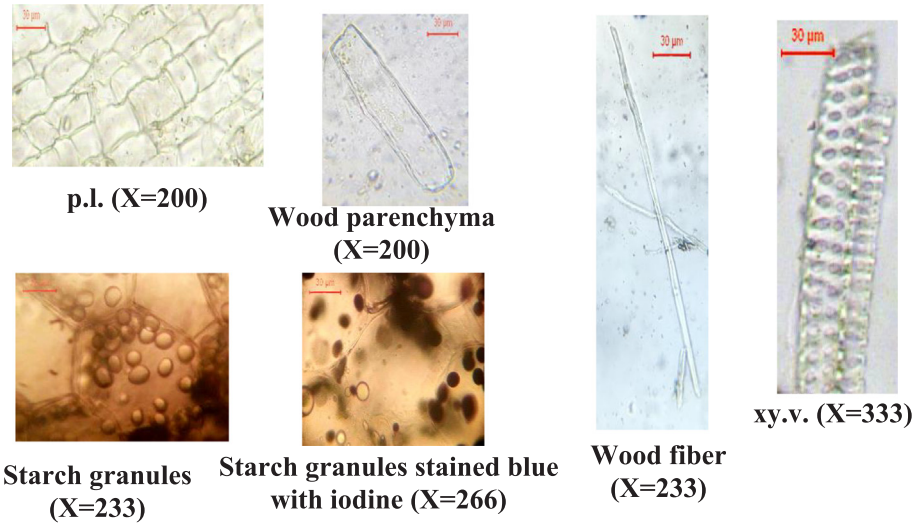

Fig. 10. Powdered root.

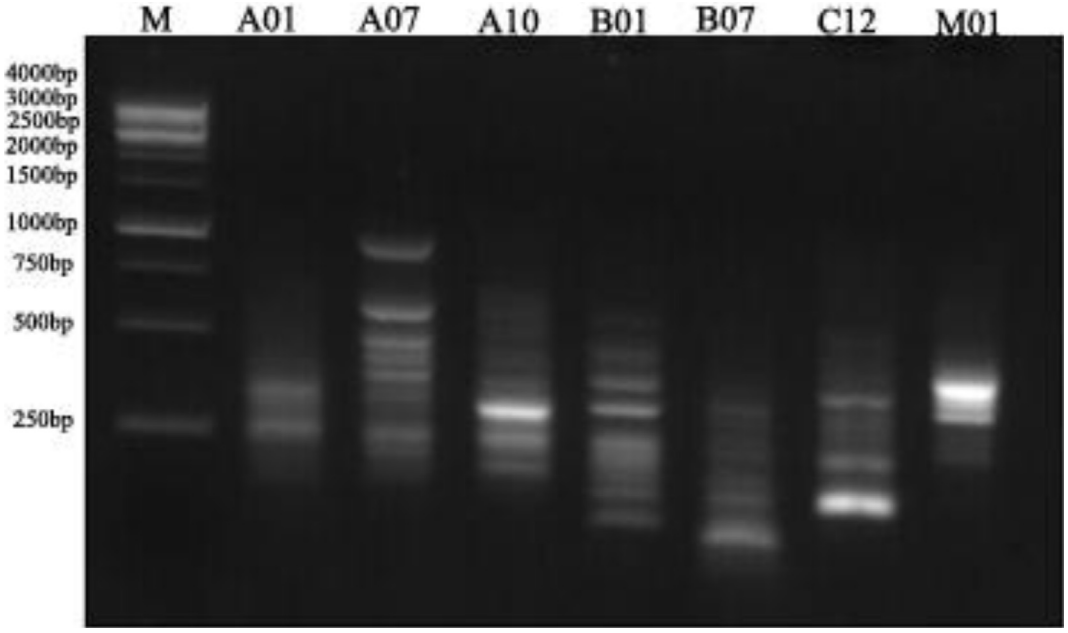

Fig. 11. The RAPD electrophoretic profile of the DNA sample amplified with the seven decamer primers.

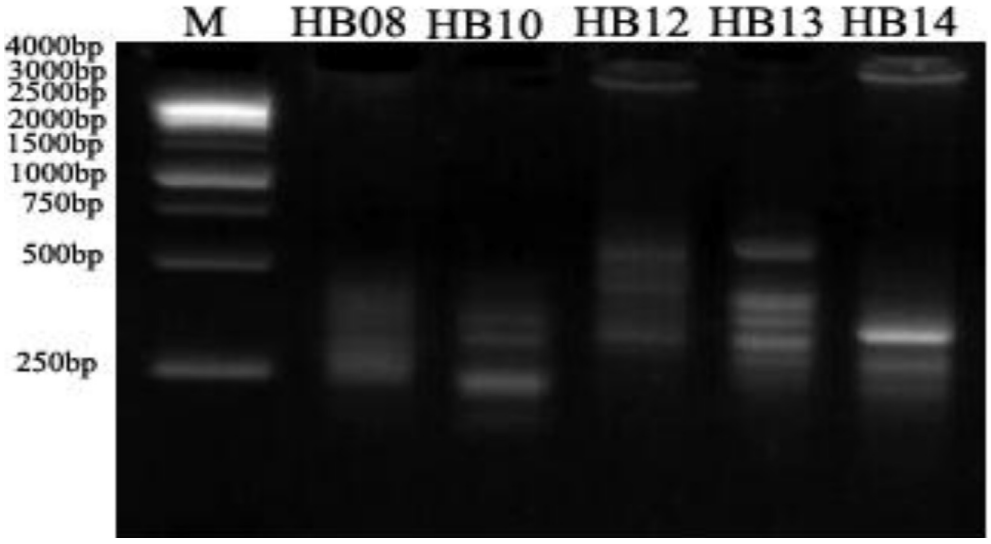

Fig. 12. The ISSR electrophoretic profile of the DNA sample amplified with the five.

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