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MORPHO-ANATOMICAL AND PHYTOCHEMICAL CHARACTERISATION OF TRADITIONALLY USED PLANT *RUELLIA TUBEROSA* L. LEAVES AND ROOTS

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Keywords:

Ruellia tuberosa, Microscopy, Cytomorphology, Physicochemical, Phytochemical, Chromatography

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ABSTRACT: The objective of the present study is to develop the pharmacognostic and phytochemical profiles of *Ruellia tuberosa* L. leaf and root. The present study considers the detail investigation of macroscopy, microscopy, preliminary qualitative phytochemical analysis, physicochemical evaluations, and chromatography profiling. The microscopical investigation was adopted to identify the cellular powder characters of leaves and roots, including diacytic stomata, few sessile glandular trichomes, profuse oil globules, exfoliating cork, few small schizogenous oil cavities, cystolith, *etc*. Physicochemical parameters reveal the data of quality, purity while phytochemical screening reflects the presence of various secondary metabolites. The chromatographic fingerprint data represent the authenticity of plant sample. Data obtained from botanical and chemical screening in combination may be considered as standard for identification and authentication of leaves and roots and may be helpful in developing pharmacopoeial standards.

INTRODUCTION: *Ruellia tuberosa* L., (family: Acanthaceae) plant having traditional medicinal value commonly known as Blue-bell, Spearpod, Minnieroot or Snapgragon root [1]. The other names of the plant, such as cracker plant or popping pod, are for dried pods that pop while rubbed with spit or contact with water [2]. It is a small biennial to perennial plant identified with funnel-shaped striking violet bracteate flowers on dichotomous few-flowered cymes, 12-20 mm long linear hispid calyx lobes, 4-6 cm long corolla tube abruptly expanded above, 12-15 mm wide 2-2.5 mm in diameter purple suborbicular lobes, sessile subcylindrical puberulent capsule (fruit) 2 cm (0.8 in) long having more or less 20 seeds per locule, thick fusiform tuberous roots in cluster [3],

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Erect widely branched stem up to 50 cm tall, mostly basal, finely pubescent ovate to oblong petiolate leaves with alate 1.5 cm long petioles and 4-6 × 1.5-2.5 cm pubescent leaf blades with cuneate base, obtuse apex, undulated margin.

Ruellia tuberosa is found in moist and shady environments. It is native to Central America but presently habitating in many places of tropical south and southeast Asia, preferably in grasslands and roadsides - often as a weed in cultivated fields, and also in xerophile and ruderal habitats [4].

Several studies [5, 6, 7] showed that this plant contains steroids, terpenoids, long-chain aliphatic compounds, and flavonoids. In folk medicine [8] and Ayurvedic medicine [9], it is believed to be diuretic, anti-diabetic, antipyretic, analgesic, antihypertensive, and gastroprotective. In the treatment of gonorrhea, its uses are mentioned [10]. It is also used as a natural dye for textiles [11]. In India, it is used for kidney stone disorders [12]. Antimicrobial activity [13] of the plant leaves is reported. The present study aimed at development of standardization and authentication

parameters of this plant which is not included in API.

MATERIALS AND METHODS:

Material and Reagents: The work has been carried out by using the chemicals, reagents and solvents of Emplura grade of Merck and aluminum supported Thin Layer Chromatography plates were purchased from E. Merck Pvt. Ltd. (Mumbai, India).

Plant Materials Collection and Authentication: The flowering twig, leaves, and roots of *Ruellia tuberosa* were freshly collected from the natural habitat of Salt Lake area, Kolkata (22°31′31.8″ N: 88°21′59.4″ E), West Bengal in the month of May 2019 and authenticated in Department of Pharmacognosy, Central Ayurveda Research Institute for Drug Development, Kolkata, a herbarium was prepared and deposited in the Department, available for reference.

Plant Sample Processing: The plant materials, leaves, and roots were washed thoroughly with aqueous ethanol, each of them dried at ambient temperature (20-23 °C). A small portion of both the fresh and air-dried plant samples was used for macroscopic, organoleptic, and microscopic (Transverse section) studies. The rest of both plant materials were pulverized separately with a grinder (National SM 2000) to obtain fine powder (sieved in 60 #) used for powder microscopy, analysis of physicochemical and phytochemical features. The coarse powder (sieved in 25 #) of both the plant materials was used for chromatographic examinations. The whole and powdered plant samples were stored at room temperature in airtight, light-resistant containers as per standard guidelines [14].

Macroscopy of Plant Material: The morpho-logical and organoleptic parameters *viz.* texture, shape, size, color, odor, *etc*. of the whole plant material were noted mainly by naked eye observation [14, 15] with the help of simple microscope Olympus OIC DM.

Powder Microscopy (Transverse Section): Leaf and root samples were separately taken and transversely cut to obtain sections using hand razor. Few fine transverse sections were selected and treated with different ethanolic gradations (30%, 50%, 70%, 90% and absolute), stained in safranin, light green, etc. and mounted on slides with Canada balsam separately for both the samples followed by observation under the binocular compound microscope (Olympus OIC-07964) at 10× and 40× magnifications [14, 15].

The photomicrographs of different cellular structures and inclusions were taken using Magcam DC14 camera attached to an Olympus CX21i trinocular compound microscope.

Powder Microscopy of Cytomorphological Features: Fine dried powdered samples i.e. leaf and root (~2 g) were separately treated with different solutions *i.e.,* aqueous saturated chloral hydrate (for maceration), 50% glycerin, phloroglucinol in conc. HCl (for staining lignified tissues) and 0.02 N iodine reagent (for starch grains), mounted on slides with 50% glycerin following a standard protocol and observed under the binocular compound microscope (Olympus OIC-07964) at 10× and 40× magnifications [14, 15]. The photomicrographs of different cellular structures and inclusions were taken using Magcam DC14 camera attached to an Olympus CX21i trinocular compound microscope.

Fluorescence Analysis: The coarsely powdered samples *i.e.* leaf and root (~ 0.5 g each) were treated separately with different (18 in number) reagents (5 ml each) such as, acids and alkaline solutions along with other solvents (including distilled water) inside clean test tubes, which were shaken well and allowed to stand for about 24 h. The individual solutions were observed under normal daylight and UV (254 nm and 365 nm) light for their characteristic colors and compared with the standard color chart [16].

Physicochemical Evaluation: The physico-chemical constant like ash values, loss on drying, extractive values and pH value of the plant material were determined by using coarse powder as per standard guidelines [5].

Extractability was studied with different solvents like hexane, acetone, chloroform, ethyl acetate, methanol, ethanol, water, and aqueous ethanol. Extractions were performed by conventional cold and hot extraction method [14, 15].

Phytochemical: The finely powdered plant materials both leaves and roots were subjected to Soxhlet extraction for 1 h, with methanol and extracts were evaporated to dryness and used for screening the presence of secondary metabolites [17].

Fingerprint Analysis by High-Performance Thin Layer Chromatography (HPTLC): The methanol extract of the plant materials gave the maximum extractive value; the same was used for the fingerprinting analysis. For this, the coarsely powdered plant material (1 g) was extracted with methanol (25 ml) using a Soxhlet apparatus. The extract was filtered, and the final volume made up to 20 ml using methanol and used for the fingerprinting analysis by High-Performance Thin Layer Chromatography (HPTLC).

The extract (2 µL) was applied in the form of 8 mm band, 15 mm from the bottom of a 5 × 10 cm preactivated aluminum supported precoated silica gel $60F_{254}$ plate, with the help of ATS-4 applicator attached to a CAMAG HPTLC system. The plate was developed in a pre-saturated twin trough chamber using the mobile phase as hexane: ethyl acetate: acetone: 1,4-dioxan: formic acid (4:3:2:1:0.5, v/v) to a distance of 8 cm, dried for 5 min in ambient air. Images of the developed plate were captured under 254 nm and 366 nm UV light. Densitometric scanning [18] of the developed plate at 254 nm was performed. An image was also captured using visible light after derivatizing the plate with an aqueous 20% sulphuric acid [19].

Fingerprint Analysis by High-Performance Liquid Chromatography (HPLC): This was carried out with an HPLC equipment (Agilent model Infinity 1260), equipped with quaternary LC-2010 AHT VP pumps, a variable wavelength programmable UV/VIS detector, SPD-10AVP column oven, and Class-VP software for analysis. The chromatographic separation was performed using a Phenomenex C_{18} (250 mm × 4.6 mm, 5 µm particle sizes) column at 25 ºC. The optimized mobile phase was found to be methanol: water (0.1% aqueous orthophosphoric acid) 80:20 (v/v) at a flow rate of 0.5 ml/min. An autosampler with injection volume 20 µl was used for sample loading and the peaks were detected at 254 nm UV.

RESULTS AND DISCUSSION:

Morphological Characters:

Leaf: The fresh Leaves are opposite, petiolate, petiole with up to 2 cm long petiole, lamina elliptic-oblong or oblong-obovate to oblanceolate, 4-7 cm long, 1.2-2.5 cm wide, nearly glabrous, dark-green, sub-acute or obtuse or rounded at apex, cuneate to attenuate at base, minutely hairy on both the sides margin nearly is smooth-edged or entire to slightly undulated. Leaves are arranged oppositely along the stem **Fig. 1a, b**.

Root: Fresh Roots are elongated, slender, fusiform, tuberous finger-like, thick and cylindrical, 2.5 cm. to 8 cm. long, 0.2 to 0.4 cm. thick, off white to rusty brown in color, present in clusters.

FLOWERING TWIG **FRESH LEAVES** **FRESH TUBEROUS ROOTS**

FIG. 1: MORPHOLOGY OF FRESH *R. TUBEROSA* WHOLE PLANT, LEAVES AND ROOTS

Microscopy (Transverse Section):

Leaf: Dorsoventral T.S through lamina shows single-layered upper and lower epidermis with thin cuticle, diacytic stomata on lower epidermis, glandular sessile trichomes and few covering trichomes, collenchymatous hypodermis, ground cortical parenchyma with prominent double-layered compact palisade and loosely arranged spongy

mesophyll tissue zone with chlorophyll and oil globules; spiral xylem vessels associated with sclerenchymatous fibers present discontinuing the mesophyll tissue. In midrib, a crescent-shaped collateral vascular zone consisting of xylem strands, phloem, and sclerenchymatous fibers, *etc*. embedded in ground tissue **Fig. 2a**.

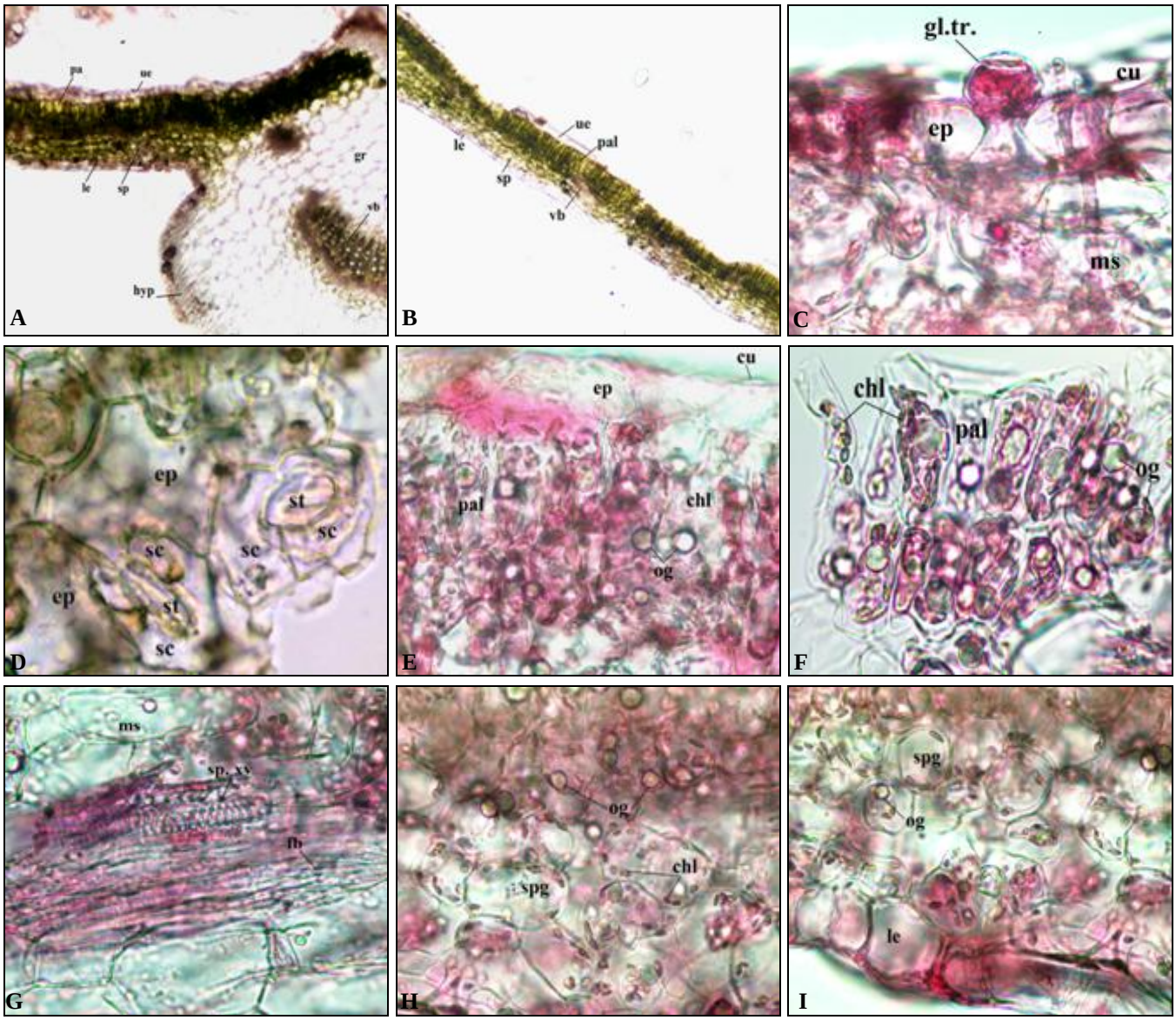


FIG. 2: MICROSCOPY (T.S.) OF *R. TUBEROSA* LEAF

a, b: T.S. through midrib and lamina showing upper (ue) and lower epidermis (le), hypodermis (hyp) , palisade (pal) and spongy (sp) parenchyma, vascular bundle (vb) and ground tissue (gr); c: the epidermis (ep) with thin cuticle (cu) showing sessile glandular trichome (gl.tr.) and mesophyll tissue (ms); d: epidermis (ep) showing diacytic stomata (st) with subsidiary cells (sc); e, f: palisade parenchymatous (pal) zone showing profuse oil globules (og) and chlorophyll (chl); g: vascular zone showing spiral xylem vessels (sp.xv.) with a tuft of fibers (fb); h, i: Spongy tissue (spg) having oil globules (og) and chlorophyll (chl) attached with lower epidermis (le).

Root: T.S shows outermost exfoliating cork cell layers arranged in parallel rows containing brownish cell content, single phelloderm layer, collapsed epidermis, collenchymatous hypodermis and ground cortical parenchyma followed by a ring shaped wavy vascular tissue zone consisting of phloem, exarch xylem stands alternating with sclerenchymatous fibers, bi to triseriate medullary ray cells encircling the wide parenchymatous pith region. Prismatic crystals of calcium oxalate, cystoliths, and few small schizogenous cavities (sz) containing oil globules are scattered in the cortex and central pith region **Fig. 2b**.

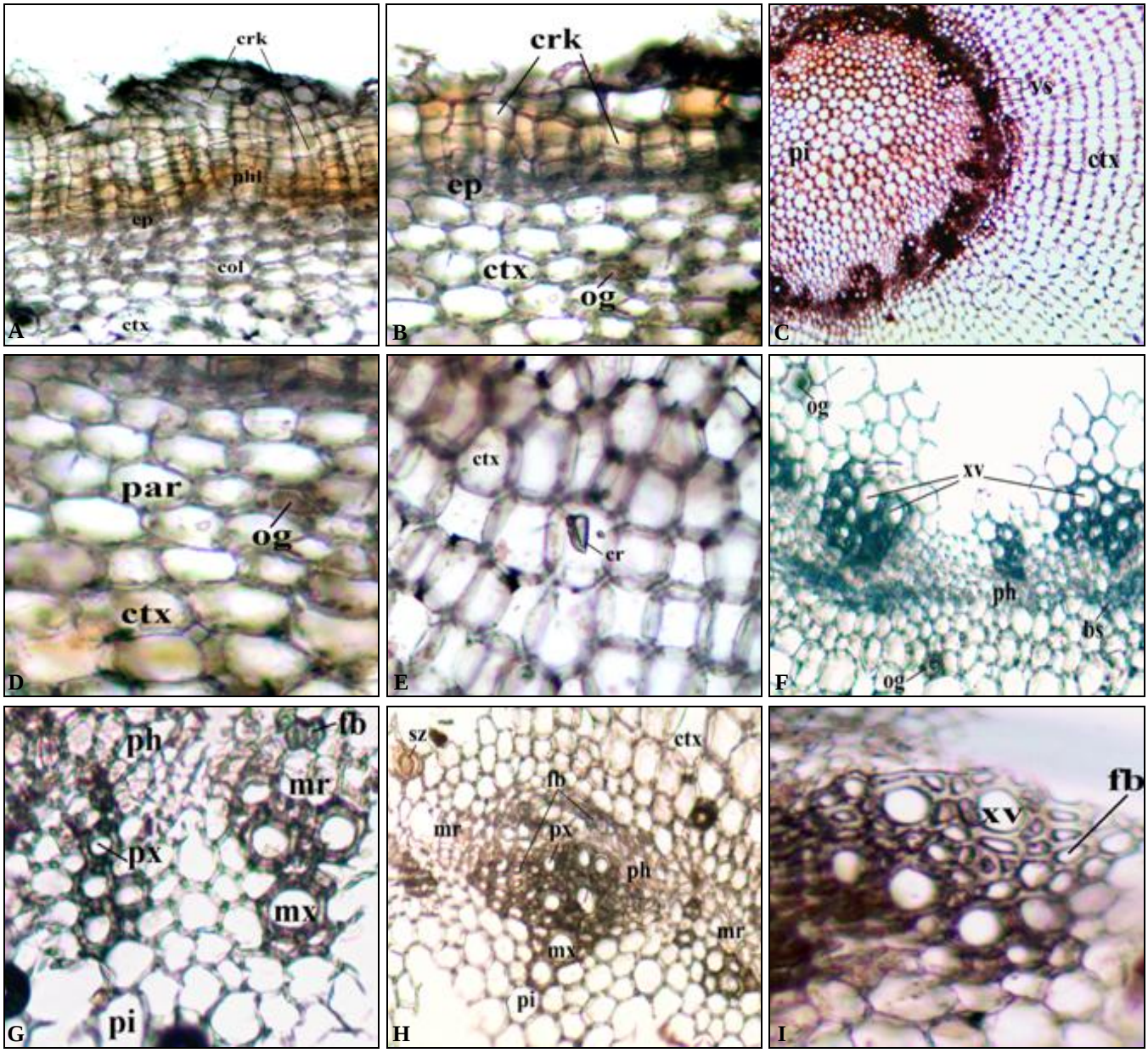


FIG. 3: MICROSCOPY (T.S.) OF *R. TUBEROSA* ROOT

a, b: Outermost exfoliating cork layers (crk) composed of cork cells arranged in parallel rows containing brownish cell content followed by single cell layered phelloderm (phl), collapsed epidermis(ep), collenchymatous (col) hypodermis and cortex (ctx) as ground tissue containing few oil containing cavity or oil glands (og); c: Cortex (ctx) and vascular zone (vs) encircling the wide pith region (pi); d, e: Cortex (ctx) with parenchymatous cells (par) containg few prismatic crystals (cr) and few oil glands (og); f, g,h,i: Vascular region showing xylem vessels (xv) of two types *i.e.* metaxylem (mx) and protoxylem (px), fibre (fb), medullary rays (mr), phloem (ph), very few small schizogenous cavity (sz) containing oil and pith (pi).

Powder Microscopy (Cytomorphological Features): Leaf: Fine powder dark green in colour with no salient taste and odour, shows the presence of groups of an epidermal cell with diacytic stomata, uniseriate nonglandular trichome, sessile and stalked glandular trichome, aseptate fibres with reticulate striations on thick fiber wall, parenchyma with dense cell contents and chlorophyll, few fragments of dark reddish-brown crystalline mass and prismatic crystals of Ca-oxalate **Fig. 3a**.

Root: Fine powder grayish brown to rusty brown in color with minute creamish flakes having no salient taste and odour, shows the presence of fragmented thick-walled irregular brown cork cells, aseptate fibers, spiral vessels in groups, number of Cystolith (cys) deposition inside specialized lithocyst cells associated with parenchyma, fragments of vascular tissue, prismatic crystals of Ca-oxalate, cystoliths and very few fragmented squarish to polygonal medullary ray cells **Fig. 3b**.

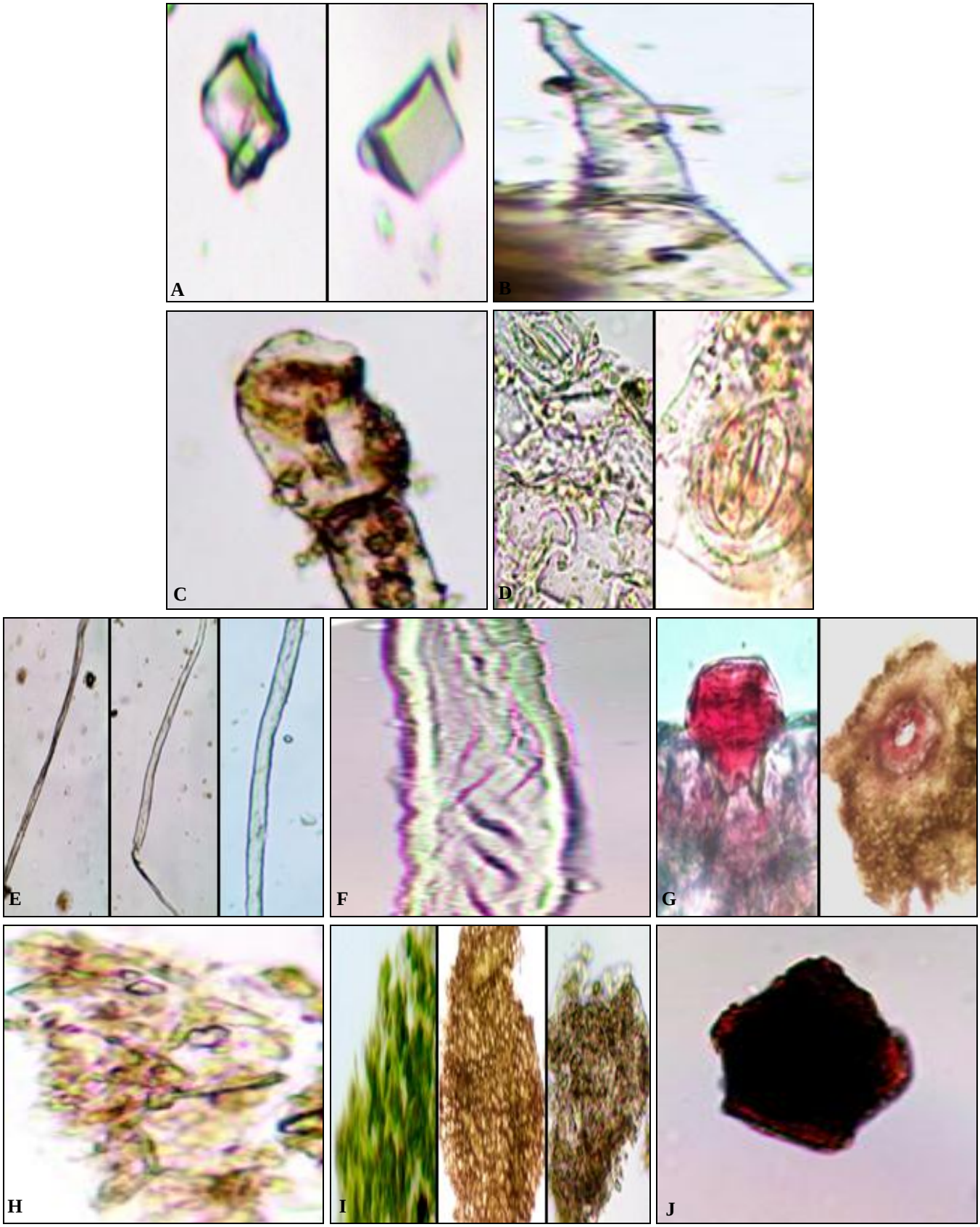


FIG. 4: POWDER MICROSCOPY OF *R. TUBEROSA* LEAF

a: Prismatic crystals of Ca-oxalate ; b: Uniseriate nonglandular trichome; c: Glandular trichome with oval gland and stalk; d: Groups of epidermal cell with diacytic stomata; e: Aseptate fibers; f: Reticulate striations on thick fibre wall; g: Sessile glandular trichome on an epidermal layer (longitudinal & dorsal view); h, i: Parenchymatous cells with cell contents and chlorophyll; i: Reddish-brown crystalline mass.

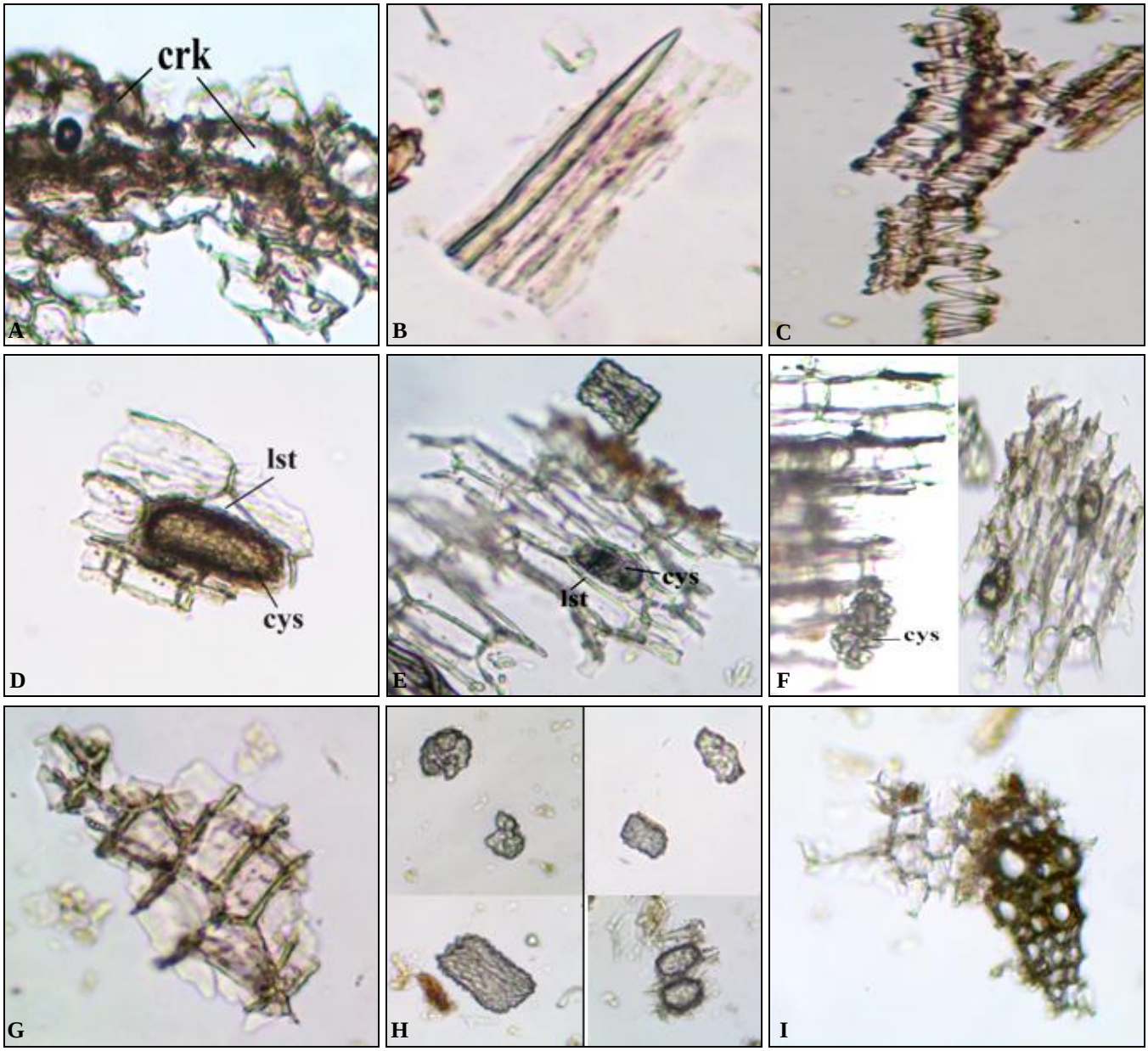


FIG. 5: POWDER MICROSCOPY OF *RUELLIA TUBEROSA* ROOT

a: Fragmented cork cells in the group; b: Aseptate fibre with tapering end; c: Spiral vessels in groups; d, e, f: Cystolith (cys) deposition inside lithocyst cells (lst); g: Squarish to polygonal medullary ray cells; h: Broken aggregated cystoliths or calcium carbonate crystals; i: Fragmented portion of vascular tissue with vessels and fibers.

Fluorescence Analysis: Dried powders of leaves and roots were separately treated with different reagents reveals the presence of chromophoric compounds in them. Lesser fluorescence was observed under normal daylight and short UV (254 nm) light for root powder, indicating a very small amount of chromophores in the sample. The fluorescence characters are noted in **Table 1** and **Table 2**.

TABLE 1: FLUORESCENCE ANALYSIS OF *R. TUBEROSA* LEAF POWDER

S. no.	Fluorescence Analysis Reagents	Visible/Day Light	Short UV (254 nm)	Long UV (366 nm)
1	1N HCl	Pink	Brownish with a greenish tinge	Light pinkish tinge '+ ve'
2	1N NaOH	Greenish brown	Light pinkish tinge	No color
3	1N NaOH + Methanol	Leafy greens	Brownish tinge	Light pinkish tinge '+ ve'
4	50% KOH	Yellowish-brown	Brownish black	Blackish grey
5	50% H_2SO_4	Black	No color	Fade bluish tinge
6	Conc. H_2SO_4	Black	Fade pinkish tinge	No color

7	Conc. HNO_3	Yellow straw	Fade pinkish tinge	Pale bluish tinge '+ ve'
8	Acetic acid	Light green	Pinkish tinge	Bluish tinge
9	50% HNO_3	Light pink	Fade bluish tinge	No color
10	Iodine solution	Reddish violet	No color	No color
11	Distilled water	Opaque solution	Light pale brownish tinge	Bluish tinge
12	Chloroform	Rusty green	No color	No color
13	Acetone	Green	Pale brownish tinge	Bluish tinge
14	Ammonia	Reddish-brown	Brownish tinge	Light blue
15	Ethanol	Light green	Fade blue	No color
16	Toluene	Yellowish green	No color	No color
17	$K_2Cr_2O_7$	Rusty brown	Yellowish green	Black
18	$FeCl_3$	Blackish brown	Brownish with greenish tinge	Pale blackish-grey

TABLE 2: FLUORESCENCE ANALYSIS OF *R. TUBEROSA* ROOT POWDER

S. no.	Fluorescence Analysis Reagents	Visible/Day Light	Short UV (254 nm)	Long UV (366 nm)
1	1N HCl	No color	Pale creamish with a pink tinge	White
2	1N NaOH	No color	Pale bluish gray	Gray '-ve'
3	1N NaOH + Methanol	No color	Greyish	No color
4	50% KOH	Pink	Brownish black	Blackish grey
5	50% H_2SO_4	Greyish	Bluish gray	Pale creamish with a gray tinge
6	Conc. H_2SO_4	Black	Fade pinkish tinge	No color
7	Conc. HNO_3	Black	Fade pinkish tinge	Pale bluish tinge
8	Acetic acid	Blue	Pinkish tinge	Bluish tinge
9	50% HNO_3	No color	Pale creamish with a yellow tinge	Bright cream
10	Iodine solution	Yellow	No color	No color
11	Distilled water	No color	White	White
12	Chloroform	No color	Light pinkish yellow	White
13	Acetone	Greyish	Bluish gray	Faint grayish
14	Ammonia	No color	Pale bluish gray	Creamy white
15	Ethanol	No color	White	Creamy white
16	Toluene	No color	No color	Pearl white
17	$K_2Cr_2O_7$	Orange	Yellowish	Dark grey
18	$FeCl_3$	Rust brown	Brownish	Pale grey

Physicochemical: As demonstrated in **Table 3**, it was revealed that total ash, water-soluble ash, acid insoluble ash values were comparatively higher in *R. tuberosa* roots. The extractive values of different solvents for the plant samples revealed maximum and least extraction by hexane and methanol, respectively. Based on the best extractive yield in methanol, the same was used for the subsequent fingerprinting analyses.

TABLE 3: PHYSICO-CHEMICAL EVALUATION OF *R. TUBEROSA* LEAF AND ROOT[a]

Physicochemical Parameters	Results in Percentage			
	Leaf		Root	
Loss on drying (LOD)	13.41 ± 0.13		12.34 ± 0.17	
Total ash value	11.67 ± 0.12		12.33 ± 0.15	
Water soluble ash value	7.15 ± 0.19		3.53 ± 0.13	
Acid insoluble ash value	2.43 ± 0.11		2.34 ± 0.28	
Sulphated Ash	2.29 ± 0.09		2.65 ± 0.22	
pH value (10% aq. suspension)	6.09 ± 0.03		6.12 ± 0.09	
Extractive values	**Cold extraction**	**Hot extraction**	**Cold extraction**	**Hot extraction**
Hexane	3.12 ± 0.13	3.24 ± 0.04	3.79 ± 0.04	6.23 ± 0.34
Acetone	5.38 ± 0.11	6.13 ± 0.08	8.95 ± 0.04	13.65 ± 0.23
Chloroform	9.35 ± 0.16	9.12 ± 0.12	15.67 ± 0.05	16.92 ± 0.14
Ethyl acetate	9.27 ± 0.09	8.76 ± 0.03	17.32 ± 0.32	17.46 ± 0.21
Methanol	8.31 ± 0.31	11.76 ± 0.05	17.71 ± 0.07	21.07 ± 0.13
Alcohol	7.19 ± 0.33	6.45 ± 0.13	12.61 ± 0.05	10.98 ± 0.21
Water	5.23 ± 0.21	5.94 ± 0.07	12.34 ± 0.03	6.54 ± 0.19
Hydroalcoholic (1:1)	6.37 ± 0.43	7.12 ± 0.04	11.23 ± 0.06	8.87 ± 0.43

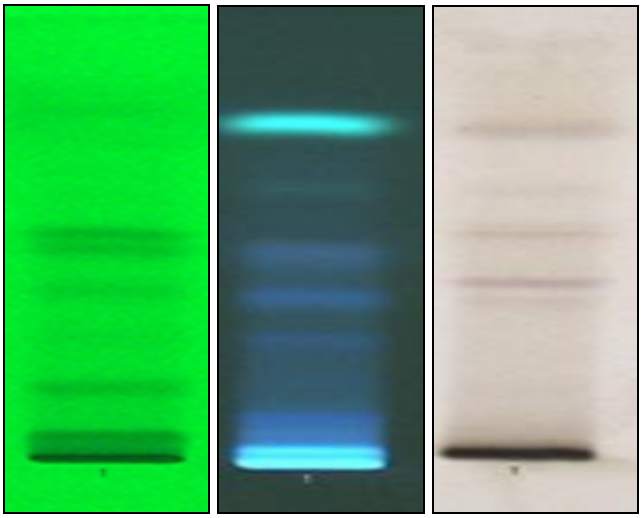
[a]Values are expressed as Mean ± S.D.

Phytochemical: The results of the phytochemical screening are noted in **Table 4,** which reveals the presence of alkaloids, steroids, phenolics, glycosides, tannins, *etc*.

TABLE 4: PHYTOCHEMICAL SCREENING OF *R. TUBEROSA* LEAVES AND ROOTS

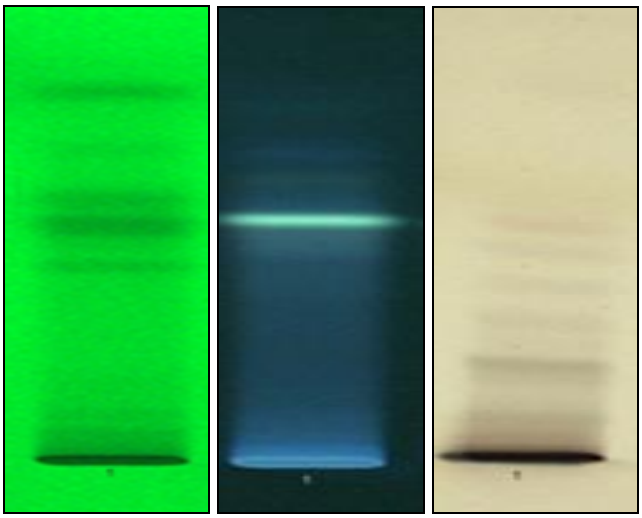
Phytochemical class	Leaves	Roots
Alkaloids	+	+
Steroids	+	+
Triterpenoids	-	+
Flavonoids	+	+
Tannins	+	+
Glycoside	+	+
Phenolic	+	+
Saponins	+	+

High-Performance Thin Layer Chromatography (HPTLC): The HPTLC experimental condition was optimized by using pre-activated and precoated TLC silica gel 60 F_{254} plates and different combinations of polar and apolar solvents as the mobile phases (data not shown). Best result was obtained with Hexane: Ethyl acetate: Methanol (5:3:2, v/v) as the mobile phase, which showed nine bands at R_f values of 0.02, 0.13, 0.24, 0.35, 0.45, 0.48, 0.57, 0.69 and 0.76 for leaves and ten bands at R_f values of 0.03, 0.07, 0.13, 0.27, 0.39, 0.49, 0.54, 0.65, 0.73 and 0.79 for roots when visualized under UV at 254 nm. Densitometric scanning at 254 nm of the developed plates gives the relative ratios of the peaks, which are noted in **Table 5**. Their densitometric scanned pictures as fingerprint profiles are represented in **Fig. 8**. The pictorial representation of the developed plate of leaves and root methanolic extracts are given in **Fig. 6** and **7**.



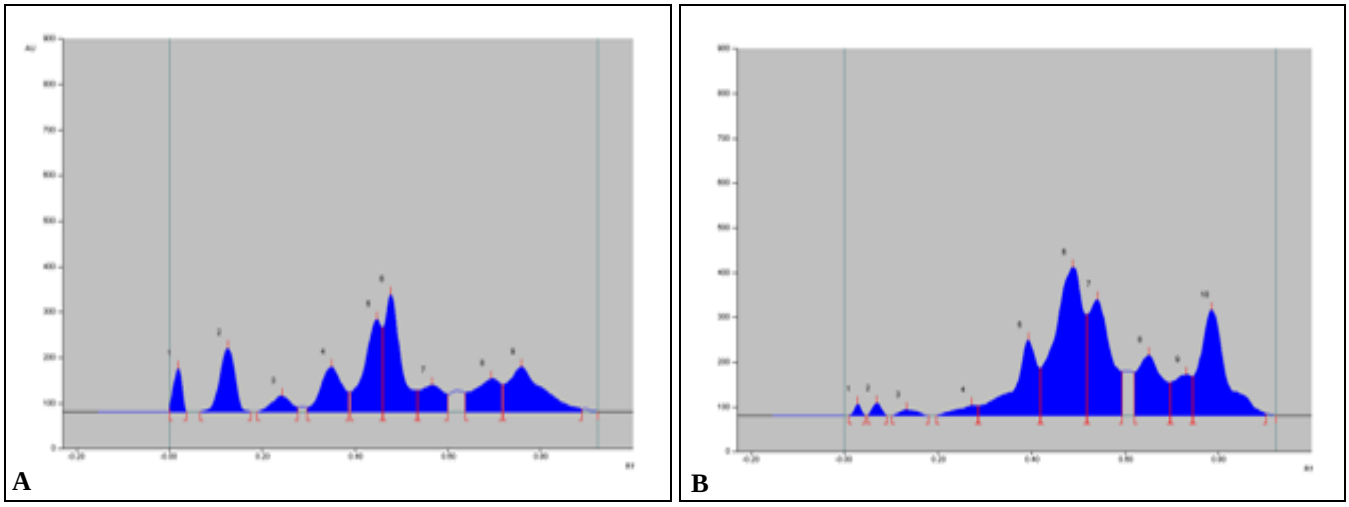
254 nm **366 nm** **WL**

FIG. 6: HPTLC PROFILES OF *R. TUBEROSA* LEAVES METHANOL EXTRACT



254 nm **366 nm** **WL**

FIG. 7: HPTLC PROFILES OF *R. TUBEROSA* ROOTS METHANOL EXTRACT



A **B**

FIG. 8: DENSITOGRAM DISPLAY OF HPTLC PROFILES OF *R. TUBEROSA* METHANOL EXTRACT OF (A) LEAVES AND (B) ROOTS AT 254 nm

TABLE 5: RELATIVE RATIOS OF THE HPTLC PEAKS[a] OF *R. TUBEROSA* LEAVES AND ROOTS

Leaves		Roots	
R_f values	Relative ratio (%)	R_f values	Relative ratio (%)
0.02	3.99	0.03	0.54
0.13	0.52	0.07	0.77
0.24	3.36	0.13	0.70
0.35	0.55	0.27	1.48
0.45	7.76	0.39	12.76
0.48	0.51	0.49	30.02
0.57	6.76	0.54	18.29
0.69	9.51	0.65	10.76
0.76	7.05	0.73	5.30
-	-	0.79	19.37

[a]The peaks were recorded by visualizing the chromatogram spots at 254 nm

High-Performance Liquid Chromatography (HPLC): HPLC method was developed for the best separation of the chemical constituents of the *R. tuberosa* leaves and roots methanol extracts. Separated peaks were detected under UV (254 nm). The HPLC fingerprint analysis in **Fig. 9.**

It shows ten peaks for leaves and eight peaks for roots, and their corresponding retention times with areas under the curves are noted in **Table 6** and **Table 7**.

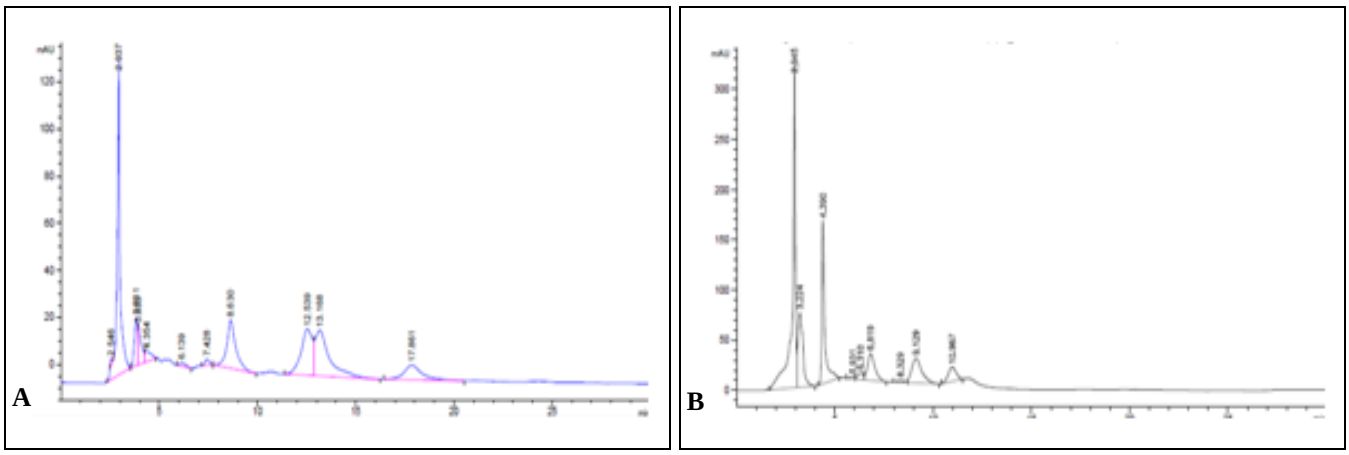


FIG. 9: HPLC CHROMATOGRAM OF (A) LEAVES AND (B) ROOTS METHANOLIC EXTRACT

TABLE 6: RELATIVE RATIOS OF THE HPLC PEAKS[a] *R. TUBEROSA* LEAVES

Peak Retention time (Minute)	Relative ratio (%)
2.546	0.46
3.831	6.3
3.933	5.52
4.354	2.80
6.139	0.86
7.428	1.12
8.630	19.07
12.539	22.77
13.168	28.91
17.861	12.01

TABLE 7: RELATIVE RATIOS OF THE HPLC PEAKS[a] *R. TUBEROSA* ROOTS

Peak Retention time (Minute)	Relative ratio (%)
3.224	25.40
4.390	32.70
5.931	0.71
6.310	2.47
6.819	13.13
8.329	1.35
9.29	16.94
10.967	7.57

[a]The peaks were recorded by detecting the chromatogram at 254 nm

CONCLUSION: The present investigations furnished a set of qualitative and quantitative phytopharmacognostic characters along with the HPLC fingerprinting profile of *R. tuberosa* leaf and roots. These data can serve as diagnostic tools for the establishment of quality standards, authentication, and identification of the medicinally important plant and help in compiling a suitable monograph of this.

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CONFLICTS OF INTEREST: Nil

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