ORIGINAL ARTICLE

Microscopical Evaluation, Phytochemical Screening, and High-Performance Thin-Layer Chromatography Fingerprinting of Leaves of *Diospyros montana* (Roxb.)

Abhijeet V. Puri

Department of Pharmacognosy, St. John Institute of Pharmacy and Research Vevoor, Palghar (E), Maharashtra, India

Abstract

Objective: The study was designed to investigate imperative pharmacognostic insights, phytochemical properties, and high-performance thin-layer chromatography (HPTLC) profile of *Diospyros montana* (Roxb.) (*D. montana*) belonging to the family Ebenaceae. **Methods:** The qualitative microscopy, quantitative microscopy, and other WHO suggested parameters of standardization of dried leaves and powder were carried out as per standard procedures. Leaves were exhaustively extracted with ethanol and fractionated into petroleum ether, chloroform, and ethyl acetate. The established conventional methods were used for phytochemical screening and HPTLC fingerprinting. **Results:** Pharmacognostic assessment of *D. montana* (Roxb.) leaves demonstrates simple petiolated, ovate to oblong-ovate, with serrate margin, and acute apex. Leaf venation indicates brachidodrome, pinnate. The leaf surface demonstrates the unicellular covering trichomes and rananculaceous stomata, veins, vein islet, and vein termination. Transverse section of leaf demonstrates the epidermis layer covered with cuticle and vascular bundles (xylem and phloem) in the midrib region. The mesophyll region is separated into palisade and light parenchyma cells with raphides of calcium oxalate crystals. Collenchyma segments were observed in midrib toward epidermis or aligned to epidermis. Preliminary phytochemical examination demonstrated the presence of steroids, saponins, flavonoids, alkaloids, and tannins. The previously stated phytochemicals were further affirmed by thin-layer chromatography and HPTLC fingerprinting. **Conclusions:** In this present study, we were able to set up some diagnostic parameters for the standardization of leaf of *D. montana* (Roxb.).

Key words: *Diospyros montana* Roxb., Pharmacognostic, Phytochemical analysis, HPTLC

INTRODUCTION

Ebenaceae is a group of blooming plants, which incorporates ebony and persimmon. The family has roughly 500 species of trees and bushes in two genera, *Diospyros* and Euclea which are wide spread mostly in tropics and subtropics.[1] The genus *Diospyros montana* (Roxb.) (Ebenaceae) found in subtropical and tropical territories of the China, India, Indonesia, and the Malay Peninsula. The variety of *Diospyros* comprises 240 species, and 59 of which are dispersed in India. *D. montana* (Roxb.) is a tree with slim stems, smooth bark and youthful shoots which are glabrous or pubescent. Leaves are simple petiolated, alternate, glabrescent or softly pubescent, ovate-oblong with acute apex and decurrent base.[2] Plant image is shown in Figure 1. The plant and parts, particularly the leaves, have been utilized as an anti-inflammatory and antipyretic drug in numerous traditional conventional meds: Chinese, Tibetan drug, and Ayurveda medicine. Literature reveals phytochemical and pharmacological studies on 13 *Diospyros* species.[3] *D. montana* (Roxb.) has been accounted to possess anthelmintic, anticancer, anti-inflammatory, antimalarial, antiviral, prostaglandin synthesis inhibitor, hypolipidemic, antitumor, and as antileukemic agent. In

Address for correspondence:
Abhijeet V. Puri, Department of Pharmacognosy,
St. John Institute of Pharmacy and Research, Palghar (E),
Maharashtra, India. Phone: +91-9960102520.
E-mail: avpuri@rediffmail.com

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Indian ethnomedicine, this plant is locally named as Ebony and its bark is used in jaundice and gum is prescribed in tuberculosis while roots as abortifacient.[4] The present research work, therefore, expects to build up a connection between the phytoconstituents present in the leaves and its different pharmacological activities. Authentication and standardization are essential steps particularly for herbal medications and formulations in traditional systems of medicine.[5] Hence, in this work, endeavor was made to research pharmacognostic parameters such as morphology, proximate values, phytochemical screening, and high-performance thin-layer chromatography (HPTLC) fingerprinting of the leaves of *D. montana* (Roxb.).

Plant taxonomy and Vernacular name[6]

Kingdom: Plantae
Class: Dicotyledons
Sub-class: Gametophyte
Botanical Name: *Diospyros montana* Roxburg.
Family: Ebenaceae
Genus: *Diospryos*
Vernacular Name
Kannada: Balagani
Marathi: Thembhurni
Gujarati: Timbaroa
Sanskrit: Kaakathinduka.

MATERIALS AND METHODS

Collection of Plant Material and Authentication

Fresh leaves of *D. montana* (Roxb.) were collected from neighborhood cantonment area of Belgaum district (Karnataka) and authenticated by Dr. B. D. Huddar at the Department of Botany, Shri Kadasiddheshwar H.S. Kotambari Science Institute, Vidyanagar, Hubli (Karnataka). A specimen sample (DM-08) is deposited in the Department. of Pharmacognosy at KLES College of Pharmacy, Hubli, Karnataka.

Drying and Size Reduction of Plant

The leaves of *D. montana* (Roxb.) were cleaned to evacuate the adhered foreign material and were washed under tap water, air dried, homogenized to fine powder, and stored in hermetically sealed bottles.

Chemicals and Instruments

Phloroglucinol, glycerine, hydrochloric acid, potassium hydroxide, and all other chemicals used in the study were of analytical grade.

Pharmacognostic Evaluation

Macroscopic characteristics

For morphological observations, fresh leaves of *D. montana* (Roxb.) (approximately 10–13 cm in length) were utilized. Type of leaf base, presence or absence of petiole, and characters of lamina were observed. Lamina comprises of characteristic elements, for composition, incision, shape, venation, margin, apex, base, surface, and texture. The shape, margin, taste, and odor of leaves were determined. The macromorphological features of the leaf were observed under a magnifying lens.[7]

Qualitative microscopy

In this study, transverse sections (T.S.) of leaf *D. montana* (Roxb.) were examined under microscope (10X and 40X). Staining reagents (Phloroglucinol-Hydrochloric) were employed according to standard techniques. The different distinguishing characters were observed with or without staining, and images were recorded.[8,9]

Microscopical Evaluation

Leaf microscopy

The microscopic features of the fresh leaves of *D. montana* (Roxb.) were determined by utilizing standard procedures. Various dermal characters, such as type of trichomes, epidermis cells, and stomata were identified.[10] To observe vascular tissue, T.S. was stained by reagent 0.1% w/v phloroglucinol took after by concentrated hydrochloric acid. The stained and unstained sections were observed under microscope.[11] Diverse layers of cells and recognizing characters were observed.

Quantitative Microscopy[12,13]

Determination of stomatal number and stomatal index

The number of stomata and the number of epidermal cells in each field were counted. The numbers of stomata were counted as stomatal number and the stomatal index utilizing the formula as per standards and were calculated independently for the upper and lower surface.

Determination of vein-islet and vein termination number

Vein islet is the minute area of photosynthetic tissue enclosed by the ultimate division of the conducting patterns. Vein termination number is the number of veinlet terminations per mm of leaf surface. The number of veinlet termination present inside of the square was counted, and the average number of veinlet termination number from the four adjoining squares to get the value for 1^2 mm was calculated as vein termination number.

Determination of Palisade Ratio

The palisade cells under the four epidermal cells (counting cells which are more than half and barring cells which are not as much as half inside of the territory of epidermal cells) were counted. The determination for five groups of four epidermal cells from various part of the leaf was repeated. The average number of cells underneath epidermal cells was calculated known as palisade ratio.

Powder Microscopy[14-19]

Dried leaves of *D. montana* (Roxb.) was sifted through mesh 60, and powder microscopy was carried out to detect specific diagnostic characters. Diverse staining reagents were used for the identification of tissue components. A little amount of dried leaf powder was taken onto a slide; 1–2 drops of 0.1% w/v phloroglucinol and a drop of concentrated hydrochloric acid were included and secured with a coverslip. The slide arrangement was mounted in glycerol and inspected under microscope, and diagnostic characters were recorded.

Physiochemical Analysis

The physicochemical parameters such as percentage of total ash, acid-insoluble ash, water soluble ash, extractives values and moisture content were determined as per standard protocol described by WHO guidelines.[20]

Preliminary Phytochemical Analysis

Fresh shade dried leaves of *D. montana* (Roxb.) were powdered and extracted by ethanol utilizing Soxhlet apparatus. The ethanolic extract was concentrated under reduced pressure utilizing a rotational evaporator and dried in vacuum. A portion of the crude ethanolic extract was suspended in water and fractionated with petroleum ether, chloroform, and ethyl acetate. All the fractions were concentrated under reduced pressure using a rotary evaporator and dried in vacuum and subjected to phytochemical screening.[21-23] The qualitative chemical tests were carried out as described by Harborne.[24] Further, this phytoconstituents were confirmed by thin-layer chromatography (TLC) and HPTLC studies.[25,26]

HPTLC Fingerprinting[27,28]

The quantitative and qualitative analysis was performed with the assistance of HPTLC instrument. The chromatographic estimation was performed by streaking the extracts in the form of narrow bands of 6 mm length on the precoated silica gel 60 F254 aluminum TLC plate (10 cm × 10 cm), at a consistent application rate of 150 µl/s, and gas flow 10 s/µl was employed with help of Camag 100 µl syringe connected to a nitrogen tank, using a Camag Linomat V (Camag, Muttenz, Switzerland). The space between three bands was kept at 15 mm. 5 µl of 1% concentration solution from each three extracts (ethanol, chloroform, and petroleum ether)

Figure 1: *Diospyros montana* (Roxb.)

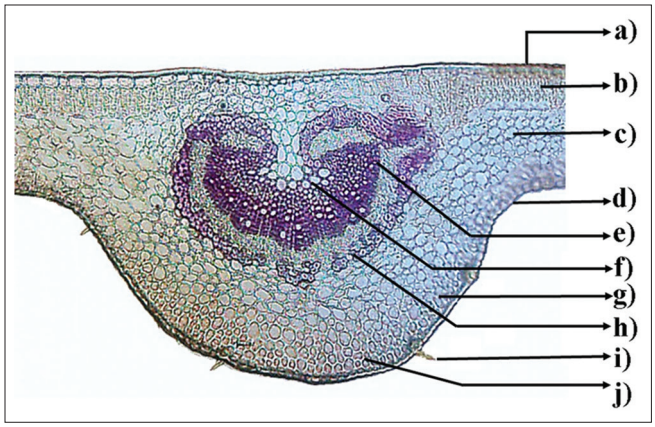

Figure 2: Transverse section of *Diospyros montana* (Roxb.) Leaf. (a) upper epidermis, (b) palisade tissue, (c) spongy tissue; (d) lower epidermis; (e) phloem, (f) xylem, (g) parenchyma, (h) pericycle, (i) trichomes, (j) collenchyma

Table 1: Morphological evaluation of *D. montana* (Roxb.) leaves

Features	Observation
Color	Green
Taste	Acrid
Odor	Odorless
Size	3.8–14×2.5–7.5 cm
Shape	Ovate-oblong
Texture	Hairy
Margin	Rounded toothed
Leaf base	Decurrent
Apex	Acute
Venation	Reticulate
Midrib	Distinct on both surfaces

D. montana: Diospyros montana

was set as a spot. After spotting, the plate was subjected to linear ascending development up to a distance of about 90 mm in a solvent system of cyclohexane: ethyl acetate (8:2 v/v) at Camag Twin Trough glass chamber, which was saturated with the same solvent system at room temperature just 10 min preceding the development. TLC plate was dried in flowing air at room temperature. Densitometric scanning was carried out between wavelength 256 and 366 nm. The chromatograms were integrated, and regression analysis and statistical data were generated using WinCATS evaluation software.

Table 2: Physicochemical parameters of *D. montana* (Roxb.) leaves

Physicochemical parameters	Constant value (%w/w)
Alcohol-soluble extractive value	9.4±1.2
Water-soluble extractive value	7.2±0.8
Chloroform-soluble extractive value	3.8±0.6
Petroleum ether-soluble extractive	1.3±0.3
Moisture content	3.5±0.5
Total ash	6.5±1.5
Water-soluble ash	1.4±0.4
Acid-insoluble ash	2.7±0.7

D. montana: Diospyros montana

Table 3: Quantitative microscopy of *D. montana* (Roxb.) leaves

Characteristics	Values (Average value of three replicates ± SD)
Stomatal number	UE: 144.5LE: 325
Stomatal index	UE: 12.10 ± 0.22LE: 08.36 ± 0.64
Vein islet number	20.00 ± 0.41
Vein terminal number	38.24 ± 0.63
Palisade ratio	5.2 ± 5.4

UE: Upper epidermis LE: Lower epidermis, *D. montana: Diospyros montana*. SD: Standard deviation

RESULTS AND DISCUSSION

Pharmacognostic Characteristics of the Leaf

Macroscopical characteristics

The shape of *D. montana* (Roxb.) leaves is alternate, ovate-oblong or elliptical acute or sub acuminate, base usually rounded, softly pubescent or tomentose when young ultimately glabrescent, rounded toothed margins, leaf base decurrent, venation reticulate, mid rib distinct on both surfaces of the way down into two obtuse lobes, green when fresh and brown when dry, weak odor, and slightly acrid in taste. The observation of organoleptic characteristics is shown in Table 1.

T.S. of Leaf

T.S. of *D. montana* (Roxb.) leaf [Figure 2] lamina shows well-developed upper and lower epidermis covered by thin cuticle and made up of thin-walled rectangular cells. Leaf is isobilateral as palisade tissue is two layered with columnar cells and sponge tissues that are loosely arranged. The midrib of the leaf shows well-developed vascular bundles with xylem and phloem. In the mesophyll tissue, lignified vascular bundle of the veins is vertically transparent and vascular bundle is usually accompanied by sclerenchyma. Midrib also shows upper and lower epidermis with well-developed thin

Table 4: Qualitative phytochemical analysis of *D. montana* (Roxb.) leaves

Phytochemicals	Ethanolic extract	Petroleum ether extract	Chloroform extract	Ethyl acetate extract
Carbohydrates	+	+	-	+
Glycosides	-	-	+	-
Phytosterol steroids	+	+	+	-
Triterpenoids	+	-	+	+
Tannins and phenolics	+	-	-	+
Alkaloids	-	-	-	-
Flavonoids	+	-	-	+

+: Present, -: Absent, *D. montana: Diospyros montana*

Table 5: TLC profile of the ethanolic extract of *D. montana* (Roxb.) leaves

Extract	Solvent system	Spray reagent	Number of spots	Rf value
Ethanolic extract	Cyclohexane: ethyl acetate (80:20)	Anisaldehyde Sulfuric acid	5	0.29,0.42,0.48,0.62,0.85

D. montana: Diospyros montana. TLC: Thin-layer chromatography

Table 6: HPTLC of the ethanolic extract of *D. montana* leaves

Track	Peak	Start Rf	Start Height	Maximum Rf	Maximum Height	Maximum %	End Rf	End Height	Area	Area %
1	1	0.01	31.3	0.02	67.10	8.37	0.05	0.20	833.9	3.24
1	2	0.06	0.00	0.07	18.10	2.26	0.08	5.00	89.8	0.39
1	3	0.1	6.70	0.11	22.40	2.8	0.12	5.40	192.7	0.84
1	4	0.18	4.80	0.20	42.90	5.36	0.22	11.0	838.9	3.67
1	5	0.26	13.80	0.29	87.70	10.9	0.32	29.8	2620	11.4
1	6	0.34	6.20	0.39	256.5	32.0	0.41	88.60	5676	24.8
1	7	0.42	89.90	0.44	121.5	15.1	0.47	10.0	3240	14.1
1	8	0.65	24.00	0.66	36.00	4.49	0.67	27.30	404.6	1.77
1	9	0.74	39.30	0.83	56.10	7.01	0.83	55.90	3858	16.8
1	10	0.89	51.10	0.93	92.60	11.5	1.00	2.60	5115	22.3

HPTLC: Highperformance thin-layer chromatography, *D. montana: Diospyros montana*

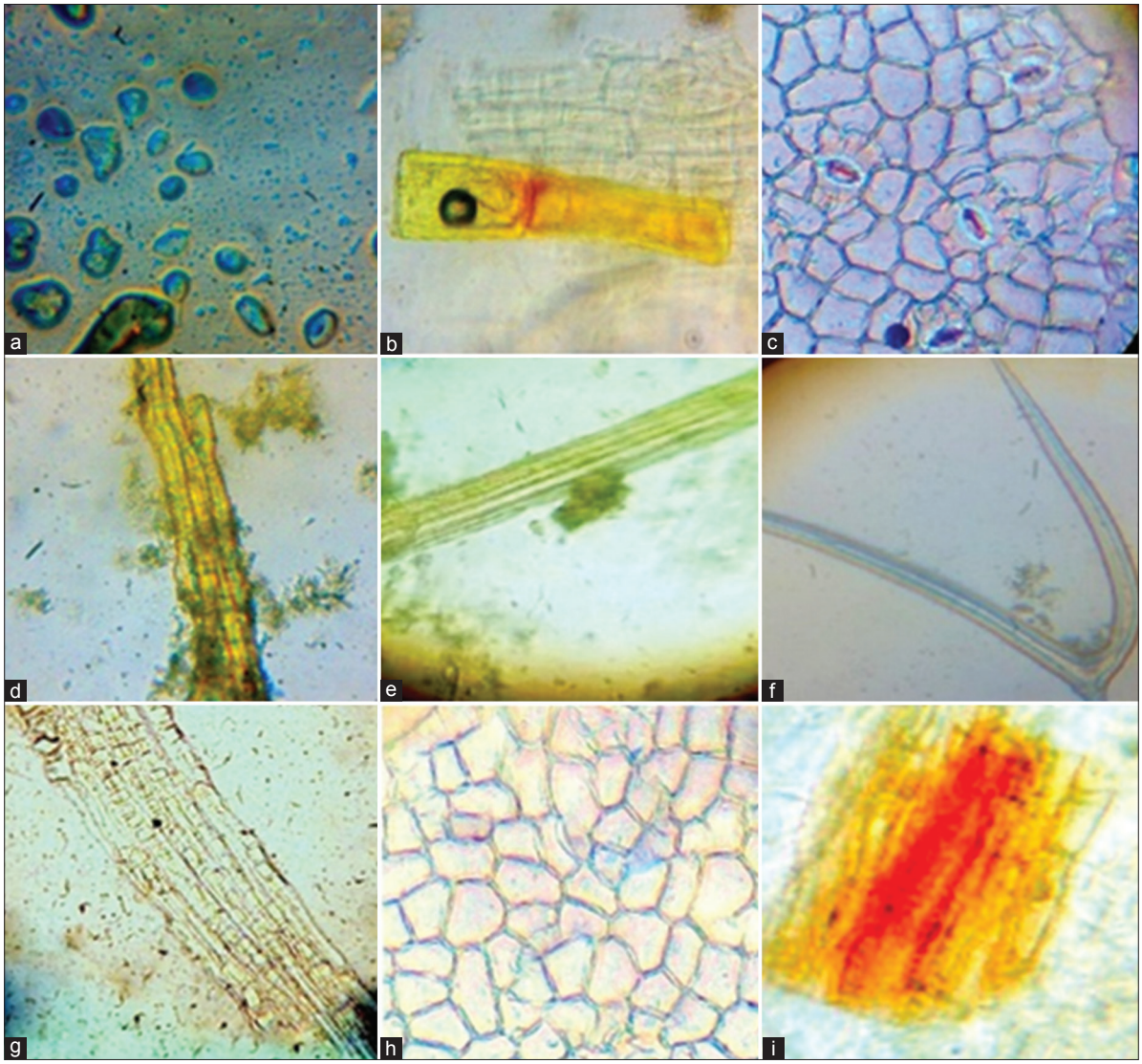

Figure 3: Powder microscopy of *Diospyros montana* (Roxb.) Leaf. (a) Starach grains, (b) xylem vessel, (c) anomocyticstomata, (d) phloem fiber, (e) non-lignifies fibers, (f) unicellular biserate covering trichome, (g) calcium oxylate crystal, (h) epidermis cells, (i) lignified xylem fiber

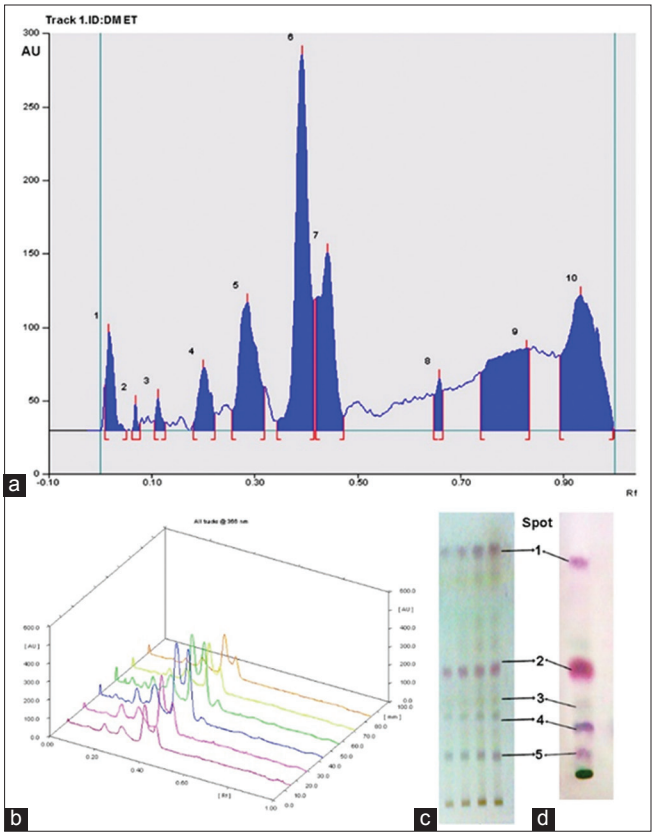

Figure 4: Thin-layer chromatography (TLC) and high-performance thin-layer chromatography (HPTLC) profile of the ethanolic extract of *Diospyros montana* (Roxb.) leaves. (a) HPTLC fingerprinting of the ethanolic extract of *D. montana* leaves. (b) HPTLC three-dimensional evaluation. (c) HPTLC photo documentation of ethanolic extract of *D. montana* leaves.(d)TLC of ethanolic extract of *D. montana* leaves.

cuticle. Both the epidermal cells are rectangular. Epidermis is followed by 2–3 layers of collenchymatous tissue and 2–4 layers of thin-walled parenchymatous cells. Vascular bundle is surrounded by parenchyma tissue and pericycle layer. Vascular bundle is shield shaped and well developed.

Powder Microscopy

The powder microscopy of *D. montana* (Roxb.) leaves [Figure 3] indicates the presence of solitary starch grains, spiral xylem vessels, polygonal epidermal cells, anomocytic (rananculaceous) stomata, non-lignified phloem fibers, lignified xylem fiber, biserate covering trichomes and spherophides with calcium oxalate crystals.

Physicochemical Parameters

Physicochemical parameters include extractive value, ash value, and loss on drying which are determined and mentioned in Table 2.

Quantitative Microscopy

Quantitative microscopy showed anomocytic (ranunculaceous) kind of stomata exist on both the surface of leaves. The stomatal number, stomatal indexes of the upper surface and lower surface, vein islet number, and vein termination and palisade ratio were observed. The results are depicted in Table 3.

Preliminary Phytochemical Screening

The preliminary qualitative analysis of various extracts showed the presence of triterpenoid, steroids, saponins, flavonoids, and tannins in which the results are expressed in Table 4. The TLC analysis of ethanolic extract which was eluted using mobile phase (cyclohexane:ethyl acetate) (80:20) showed the presence of five spots [Table 5 and Figure 4] after spraying with anisaldehyde sulfuric acid.

HPTLC

HPTLC of ethanolic extract was carried out for isolation and identification of the different components. In long UV-366 nm, maximum 10 spots were observed [Table 6 and Figure 4] at the maximum Rf = 0.02, 0.07, 0.11, 0.2, 0.29, 0.39, 0.44, 0.66, 0.83, and 0.93. This suggests the presence of at least 10 different components in ethanolic extract. Hence, it is also clear [Table 6] from the chromatogram [Figure 4] that, out of 10 components, the component with Rf values 0.29, 0.39, 0.44, 0.88, and 0.93 at 366 nm was found to be more predominant (percentage area is more with 24.82%, 22.37%, 16.87%, 14.17%, 11.46%, and 8.36%, respectively) and remaining components were found to be very less in quantity (percentage area for all the spots was ≤1.77%). Thus, the developed chromatogram will be specific with selected solvent system cyclohexane:ethyl acetate (80:20) and will serve the better tool for standardization of the drug.

CONCLUSION

From above study, it can be concluded that pharmacognostic and phytochemical evaluation possibly will help as a valuable resource for the identification, authentication, and building the official monograph of *D. montana* (Roxb.). The present work was embraced with a perspective to set down benchmarks which could be valuable to recognize the authenticity of this medicinally useful plant. Microscopical studies have demonstrated the presence of unicellular biserate covering trichomes; ranunculaceous type stomata, collenchyma, epidermal cells, and vascular bundle (lignified xylem, phloem fibers) phytochemical investigation showed the presence of carbohydrates, flavonoids, triterpenoid, and steroids as phytoconstituents. The microscopic characters, the physiochemical studies, and phytochemical analysis can be utilized for the quality control of the crude drug. Such a

pharmacognostic study will be helpful for standardizing crude drugs and can be used to differentiate closely related species. Results obtained from the qualitative evaluation of HPTLC analysis of leaves can provide standard fingerprints and can be used as a reference for the identification and quality control of the drug. This could also serve in establishing data for preparation of monograph of this plant. Various physicochemical parameters were established which can be important in detecting adulteration and mishandling of the crude drug. Further studies are materialized for the isolation and identification of individual phenolic compounds and also *in vivo* studies are needed for better understanding their mechanism of action.

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