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Isolation and characterization of triterpenes from *Diospyros montana* (Roxb.) leaves

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Abstract

Objective: The objective of this research was to isolate important secondary metabolites of *Diospyros montana* (Roxb.) leaves belonging to the family *Ebenaceae*.

Methods: In this study, we investigated the chemical composition of a pentacyclic triterpenes fraction from the leaves of *D. montana*. Leaves were exhaustively extracted with ethanol and fractionated into petroleum ether, chloroform, and ethyl acetate extracts. The various fractions were further analyzed for phytochemical composition and HPTLC fingerprinting. The fractions were subjected to TLC and spectral analysis by IR, Mass, and ¹HNMR and ¹³CNMR spectra to characterize isolated compounds.

Results: The chemical examination of the leaves of *D. montana* (Roxb.) afforded the isolation of two compounds on column chromatography. These two compounds were confirmed by comparing their chemical and spectral data with that of published works of literature.

Conclusion: The author could isolate a significant amount of Pentacyclic triterpenes: oleanolic acid and β-amyrin from the leaves of *D. montana*. Further preclinical studies may be extended to elucidate their mechanism.

Keywords: *D. montana* (Roxb.), *Ebenaceae*, Pentacyclic triterpenes, Oleanolic acid, and β-amyrin

1. Introduction

A significant study of drug discovery in been dedicated towards testing herbal drugs for treating several diseases and disorders. With very less or negligible side effects, herbal drugs play a huge role in the treatment of several disorders. Presently, conventional medications are not working against some intended diseases due to drug resistance. Additionally, new diseases are emerging which are causing threat full effects on the human race^[1]. Herbal drugs have been playing a noteworthy part in the development of various clinically useful agents^[2]. India has a rich natural heritage source of traditional systems of medicine. These traditional systems of medicines with the points of view of safety, efficacy, and quality will encourage to safeguard the use of natural products in healthcare^[3]. The variety of *Diospyros* comprises 240 species, and 59 of which are dispersed in India. The genus *Diospyros montana* (Roxb.) (*Ebenaceae*) found in subtropical and tropical territories of China, India, Indonesia, and the Malay Peninsula^[4]. *D. montana* (Roxb.) is a tree with slim stems, smooth bark, and youthful shoots that are glabrous or pubescent. *D. montana* (Roxb.) has been accounted to possess anthelmintic, anticancer, anti-inflammatory, antimalarial, antiviral, prostaglandin synthesis inhibitor, hypolipidemic, antitumor, and as antileukemic agent^[5-8]. In Indian ethnomedicine, this plant is locally named Ebony and its bark is utilized in the treatment of jaundice, and gum is prescribed in tuberculosis while roots as an abortifacient^[9]. The genus *Diospyros* possess an enormous amount of juglone based naphthoquinones and pentacyclic triterpenes. *D. montana* (Roxb.) used in indigenous medicine for delirium high fevers and to treat vomiting, Jaundice, diarrhea, and pneumonia. It is antibacterial and effective against organisms like *Bacillus subtilis*. It also shows CNS depressant, spasmolytic, and anticancer agent activity. The literature also revealed that phytochemical screening of this species possesses some imperative phytochemicals like steroids, naphthoquinones, triterpenes, polyphenols, flavonoids, carbohydrates, etc^[10-11]. This plant comprises an extensive variety of structurally varied secondary metabolites and to support this traditional uses with scientific evidences *D. montana* (Roxb.) leaves were selected for this research work. My earlier research work on extraction, phytochemicals screening, and evaluation of free radical scavenging activity has helped me to provide some insightful account of secondary metabolites of the *D. montana* (Roxb.) leaves.^[12] Hence an attempt was made to carry out extensive phytochemicals screening, chromatographic evaluation and isolation of secondary metabolites in form of triterpenic acids from the ethanolic extract of *D. montana* (Roxb.) leaves.

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2. Methods and Material

2.1 General experimental instrument

Ultraviolet-visible (UV-Vis) Spectra was measured using Hitachi Thermo Spectronic Spectrophotometer, Fourier Transform InfraRed (FT-IR) Spectrometer: Thermo Nicolet IR 200 Spectrometer was used to measure the absorbance and spectra. ^1H -(300 MHz) and ^{13}C -(75 MHz) Nuclear magnetic resonance (NMR) spectra were recorded on Bruker Advance II 400 NMR Spectrometer using tetramethylsilane (TMS) as an internal standard and chemical shifts were given in δ (ppm) values. Mass spectra were recorded with Waters Q-TOF Micro mass (ESI-MS). Thin-layer chromatography (TLC) was performed using silica gel pre-coated on aluminum plate 60F₂₅₄ plates with 250 μm thickness procured from E. Merck, Darmstadt, Germany. High-Performance Liquid Chromatography (HPLC) was carried out on the CAMAG instrument.

2.2 Collection and authentication of plant material

Fresh leaves of *D. montana* (Roxb.) were collected from the 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.

2.3 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.

2.4 Extraction, Fractionation

Fresh leaves of *D. montana* (Roxb.) were shade dried at room temperature, pulverized, and 100g of coarse powder was further subjected to continuous hot percolation (Soxhlation) with Ethanol (95%). After the exhaustive extraction, the solvent was removed under reduced pressure (Buchi) using a rotary flash evaporator then finally dried in desiccators over sodium sulfite. This procedure was repeated for 6-7 times to receive quantity sufficient amount of ethanolic extract for further processing some part of ethanolic extract was reserved for preliminary phytochemical investigations. Fractionation was carried out with Petroleum ether (40-60 °C), Chloroform, and Ethyl acetate in a succession of increasing order of their polarity. Each fraction was dried over anhydrous sodium sulfate and then evaporated to dryness^[13-15].

2.5 Preliminary phytochemical analysis

The ethanolic extract and its fractions of petroleum ether, chloroform, and ethyl acetate were subjected to phytochemical screening for detections of phytoconstituents as carbohydrates, glycosides, phytosterol steroids, triterpenoids, tannins, phenolics, alkaloids, and flavonoids present in them using standard protocols^[16-17].

2.6 Thin-layer chromatography (TLC)

Based on the preliminary phytochemical analysis, ethanolic extract and fractions were subjected to TLC. The semi-solid extract was subjected to chromatography by taking a small amount of the extract diluted with a solvent. In this method, pre-coated silica gel plate (Silica Gel 60F₂₄₅) was used. Glass capillaries were used to apply the spot on the TLC plate,

approximately 1 μL of sample volume applied using capillary at a distance of 1cm and the 3 tracks and developed in the airtight chamber already saturated with the same solvent system. In the twin trough chamber with different solvent systems, chloroform: ethyl acetate (80:20) solvent system-I. Insolvent system-II, petroleum ether: benzene (40:10) used. After pre-saturation with the mobile phase, it allowed developing for 20min. After chromatograph was developed, plates were dried in a hot air oven and subjected to visualization in the UV chamber (254, 365nm). Here, freshly prepared anisaldehyde sulfuric acid was used as a visualizing agent. The distance traveled by phytoconstituents was noted by calculating its retention factor (Rf) value, and the chromatograms were photographed^[18].

2.7 HPTLC fingerprinting

The quantitative and qualitative analysis was performed with the assistance of the HPTLC instrument. The chromatographic estimation was performed by streaking the extracts in the form of narrow bands of 6 mm length on the pre-coated silica gel 60 F₂₅₄ aluminum TLC plate (10cm \times 10cm), at a consistent application rate of 150 $\mu\text{L/s}$, 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 the three bands was kept at 15mm. 5 μL of 1% concentration solution from every three extracts (ethanol, chloroform, and petroleum ether) was set as a spot. Subsequently spotting, the plate was subjected to linear ascending development in a Camag Twin Trough glass chamber comprising the mobile phase, which was saturated with the same mobile phase at room temperature before the development. The TLC plate was dried in flowing air at room temperature. Densitometric scanning was carried out between wavelength 256 and 366 nm. The chromatograms were interpreted, and regression analysis and statistical data were obtained employing WinCATS software^[19-20].

2.8 Isolation of phytoconstituents

Column chromatography methods were used for the separation and purification of compounds. Isolation of desired compounds from a mixture was carried out by this technique.

Details of column chromatography

Adsorbent: Silica gel for column chromatography.

Activation 110 °C for 1 hour.

Length of the column: 45cm.

Diameter: Outer- 4.2cm, Inner- 3.8cm

Length of the adsorbent: 30cm.

Rate of elution: 30-40 drops/min.

The volume of elute collected: 5mL each.

Eluent: Cyclohexane: Ethyl acetate (8:2).

2gm of ethanolic extract was dissolved in 10 mL of ethanol and mixed with 2gm of silica gel (60-120 mesh size) and dried in a vacuum oven at 45 °C. Then the sample was charged to the column and was eluted with the mobile phase. About 180 fractions of 5mL each were collected in the test tubes and each fraction was subjected to TLC to analyze the eluting number of the compound by single spotting. These fractions were grouped according to their Rf values of phytoconstituents judged from the TLC analysis. Eluents of the same Rf values were pooled together. Isolated & separated compounds DM1 and DM2 were dissolved in pure methanol and evaporated on the water bath^[21-22].

3. Results and Discussion

The preliminary qualitative analysis of various extracts showed the presence of triterpenoid, steroids, saponins, flavonoids, and tannins shown in (Table 1).

(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 $\leq 1.77\%$).

Table 1: Qualitative phytochemical analysis of *D. montana* (Roxb.) leaves

Phytochemicals	Ethanol extract	Pet-ether extract	Chloroform extract	Ethyl Acetate extract
Glycosides	-	-	-	-
Phytosterol & Steroids	+	+	+	-
Triterpenoids	+	-	+	+
Tannins & Phenolics	+	-	-	+
Alkaloids	-	-	-	-
Flavonoids	+	-	-	+

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

The TLC analysis of ethanolic extract which was eluted using the mobile phase (cyclohexane: ethyl acetate) (80:20) showed after spraying with anisaldehyde sulfuric acid. Five spots with different Rf values were observed that can be useful for further isolation (Table 2, Fig. 2).

Table 2: TLC profile of ethanolic extract of *D. montana* (Roxb) leaves

Sr No	Extract	Colour of Spots	Spots	Rf-value
Solvent system Cyclohexane: Ethyl acetate (80:20)				
1	Ethanolic	Green, Blue, Purple, Violet Brown, Pale yellow	5	0.29,0.42,0.48,0.62,0.85

Spray reagent: Anisaldehyde Sulphuric acid, Rf: Retention factor, *D. montana*: *Diospyros montana*

3.1 HPTLC

Ethanolic extract showed 10 spots at the max Rf 0.02,0.07,0.11,0.2,0.29,0.39,0.44,0.66,0.83,0.93. Depicted in (Table 3, Fig. 1) which proposes the occurrence of at least 10 different components in the ethanolic extract. It proved from (Table 3) and the chromatogram as shown (Fig. 1) that out of 10 components, the components with Rf values 0.29, 0.39, 0.44, 0.88, 0.93 at 366nm were found to be more predominant. Thus the developed chromatogram will be specific with selected solvent system Cyclohexane: Ethyl acetate (80:20), Rf value serves the better tool for standardization of the drug. Characteristic TLC/HPTLC fingerprinting provided the base for the isolation, purification, characterization, and identification of compounds from *D. montana*.

Table 3: HPTLC fingerprinting of ethanolic extract of *D. montana* (Roxb.) leaves

Track	Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	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	10.94	0.32	29.8	2620.1	11.46
1	6	0.34	6.20	0.39	256.5	32.03	0.41	88.60	5676.9	24.82
1	7	0.42	89.90	0.44	121.5	15.17	0.47	10.0	3240	14.17
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	3858.8	16.87
1	10	0.89	51.10	0.93	92.60	11.56	1.00	2.60	5115.9	22.37

Max: Maximum, Rf: Retention factor, *D. montana*: *Diospyros Montana*

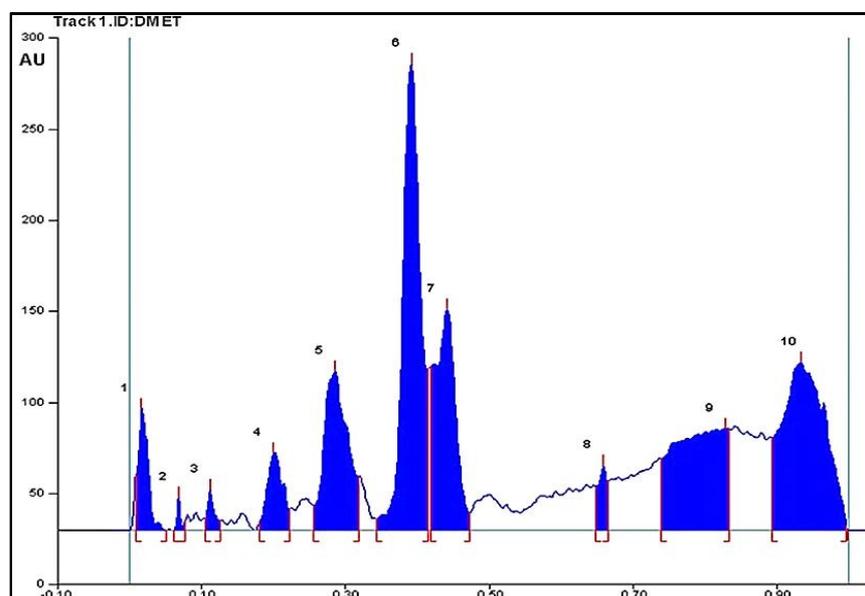


Fig 1: HPTLC Chromatogram of Ethanolic extract of *D. montana* (Roxb.) leaves

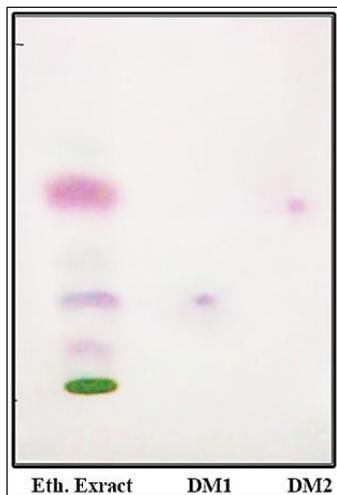
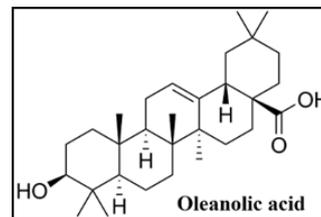


Fig 2: TLC of ethanolic extract and isolated compounds



Spectral characteristics Spectra of DM1

IR (KBr, cm⁻¹): Peaks with wave number shown in Fig.3. 3420.91 cm⁻¹(OH), 1692.04 cm⁻¹(COOH), 1458.35cm⁻¹ & 1381.40cm⁻¹ (C-H str.) 1280.18cm⁻¹, 1180.27 cm⁻¹

¹H-NMR: Peaks at following δ values are shown in Fig.4. ¹H (DMSO-d₆, δ, PPM) 5.12 (s,1H), 2.9-3.0 (s, 1H, 3H), 2.0-2.1 (t, 1H, 3H) 1.80-1.85 (q, 1H, 4H) 0.87-0.93 (m, 1H, 13H) 0.75 (s, 1H, 3H) 0.68 (s, 1H, 5H).

¹³CNMR (DMSO): Peaks at following values shown in Fig.5. ¹³C NMR (DMSO-d₆, δ, PPM) 15.56d, 16.50s, 21.54s, 23.31s, 27.45d, 28.69d, 30.68s, 33.18s, 38.84d, 39.5t, 40.09s, 42.09s, 47.29d, 47.56s, 55.24s, 77.29s, 125.08s, 138.66s

Mass spectra: Peaks observed are shown in Fig.6. Molecular formula - C₃₀H₄₈O₃

Molecular weight - 451; (ESI-MS) (m/z) - 451.5 ([M⁺], C₃), other peaks appeared a, 407.4, 235.5, 219.7, 181.5, and 130.8. From the melting point, IR, 1HNMR, 13CNMR, and Mass spectra, compound DM1 was designated as Oleanolic acid.

In the present study, we have isolated two different compounds DM1 and DM2 which were obtained by subjecting ethanolic extract to column chromatography.

3.2 Analysis of compound DM1

Physical state- White amorphous powder;
Rf value- 0.32 Cyclohexane: Ethyl acetate (80:20)
Melting point- 289-296 °C
Soluble in Ethyl acetate, Ethanol, and Methanol.
The compound DM1 gave a positive response for Salkowski and Liebermann-Burchard test for triterpenoids.

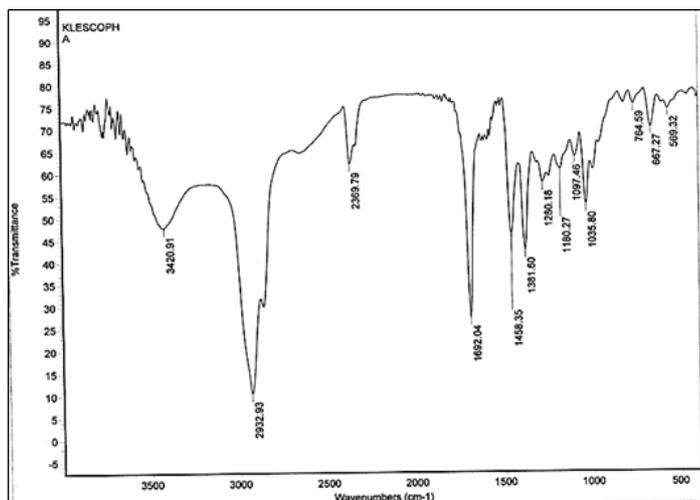


Fig 3: IR spectra of the isolated compound DM1

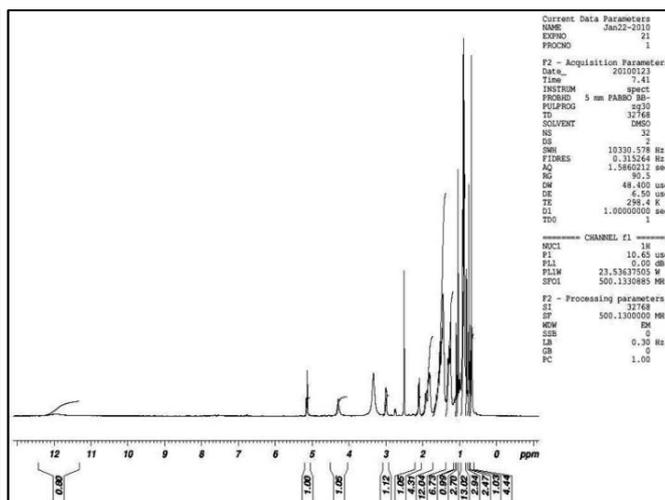


Fig 4: ¹H-NMR spectra of the isolated compound DM1

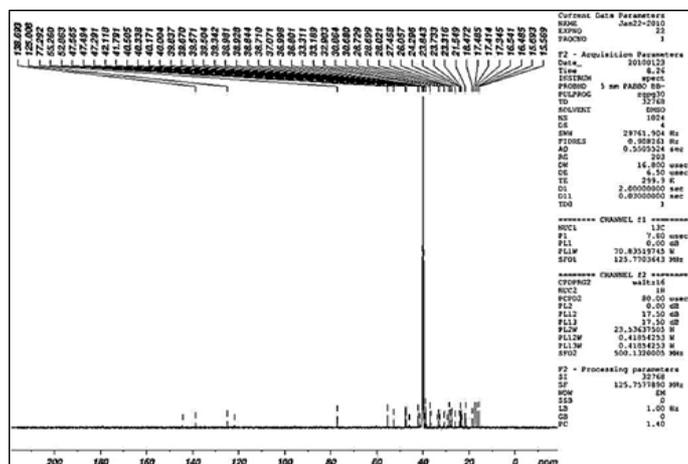


Fig 5: ¹³C-NMR spectra of the isolated compound DM1

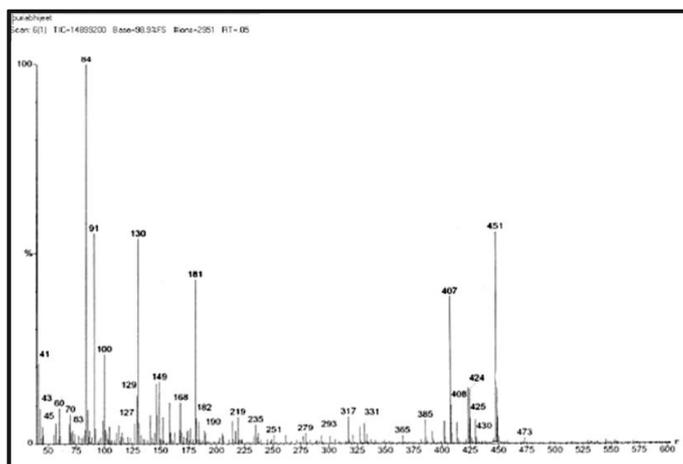


Fig 6: Mass spectra of the isolated compound DM1

4. Conclusion

Phytochemical investigation showed the presence of imperative phytochemicals such as flavonoids, triterpenoid, and steroids. HPTLC and TLC analysis of leaves provided standard fingerprints for the identification and isolation of triterpenes. This phytochemical evaluation afforded two compounds on column chromatography and repeated crystallizations. I was able to isolate two known triterpenes compounds from an ethanolic fraction of *D. montana* (Roxb.) leaves and were confirmed as pentacyclic triterpenes compound DM1 was confirmed as oleanolic acid and DM2 as β -amyrin. These two compounds were identified by comparing their chemical and spectral data with that of published works of literature [25]. The secondary metabolite isolated from this extract must account for the pharmacological activities exhibited by the crude ethanolic extract of the plant. The current pharmacological data of oleanolic acid and β -amyrin together with those of other researchers could be a basis for further investigations for the development of new drugs of natural origin to fight against several diseases. Further preclinical studies may be extended to elucidate their mechanism.

5. Acknowledgment

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6. Conflict of Interest

The authors declare no conflict of interest.

7. Abbreviations

TLC- Thin-layer chromatography
 HPLC -High-Performance Liquid Chromatography
 FT-IR -Fourier Transform InfraRed Spectrometer
 NMR -Nuclear magnetic resonance
 Rf -Retention Factor
 UV - Ultraviolet chamber
 TMS-Tetramethylsilane
 DMSO- Dimethyl sulfoxide

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