Original Article

Phytochemical Markers of *Diospyros paniculata* Dalzell: HPLC Validation for Juglone and Betulin with HPTLC Quantification of Juglone

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ABSTRACT

Introduction: A lesser-known medicinal plant *Diospyros paniculata* has potential therapeutic compounds. Among these, two significant bioactive compounds are Juglone and Betulin. The pharmacological assessment of plant extracts and quality control depend on the precise and effective quantification of these substances. Nevertheless, there aren't many approved analytical techniques for estimating these markers simultaneously in different parts of *D. paniculata.* **Objectives:** This study aimed to develop and validate straightforward, accurate, and economical HPLC and HPTLC procedures for the simultaneous estimation of Juglone and Betulin in the bark and leaves of *Diospyros paniculata.* **Materials and Methods:** To create improved samples, plant materials (bark and leaves) were extracted using methanol as a hot solvent and then further fractionated. A Waters Alliance system with a PDA detector was used for HPLC analysis in isocratic settings that were optimised for efficient separation. Toluene and ethyl acetate in an 8:2 (v/v) ratio was used as the mobile phase for HPTLC, which produced the best resolution. Both the techniques were evaluated for linearity, accuracy, precision and robustness in accordance with the ICH guidelines. **Results:** With an Rf value of 0.88 and a strong correlation coefficient (r^2=0.9917), the HPTLC technique produced clear and repeatable peaks, demonstrating exceptional linearity. The quantification of juglone produced consistent findings using both HPLC and HPTLC techniques. The reliability and reproducibility of the suggested analytical processes were demonstrated by the validation parameters for both methods falling within acceptable ICH limits. **Conclusion:** For the quantification of Juglone and Betulin in *Diospyros paniculata*, the suggested HPLC and HPTLC techniques are accurate, precise, cost-effective and repeatable. These proven methods work well with variety of plant extracts and herbal formulations that contain these phytoconstituents.

Keywords: Juglone, Betulin, *Diospyros paniculata*, Method Development, Method Validation, Quantification.

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INTRODUCTION

Natural products are a vital source of pharmacologically active compounds and have significantly contributed to modern drug discovery and development. A considerable proportion of clinically approved drugs, including those recognized by the US Food and Drug Administration (FDA), are either derived directly from natural products or synthesized from natural precursors (Dong, 2006). Secondary metabolites from plants are particularly valued for their structural diversity and therapeutic properties. According to the World Health Organization (WHO), nearly 80% of the global population relies on plant-based medicines as a primary source of healthcare, especially in resource-limited regions (Hamilton, 1982).

Despite their extensive use, herbal medicines face challenges such as compositional variability, lack of standardization, and concerns regarding safety and efficacy. These limitations highlight the importance of developing reliable, reproducible, and validated analytical methods to ensure the quality, consistency, and authenticity of herbal formulations (Attimarad, 2011 & Ramu, 2018). Chromatographic techniques, particularly High-Performance Liquid Chromatography (HPLC) and High-Performance Thin Layer Chromatography (HPTLC), are widely employed for the qualitative and quantitative evaluation of phytochemicals due to their high sensitivity, precision, and

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robustness (Renger, 2011, Tatke 2013 & Verma 2008). HPLC offers advantages such as high selectivity, reproducibility, and the ability to detect compounds at trace levels, whereas HPTLC, an advanced form of Thin Layer Chromatography, provides rapid separation, minimal sample preparation, and is highly suitable for fingerprint profiling and quality control of herbal drugs (Babula, 2009 & Aithal, 2009). Analytical standardization using biomarkers ensures the authenticity and therapeutic reliability of plant-based medicines (Ahmad, 2019).

The Diospyros genus (family Ebenaceae) is known for its rich content of bioactive secondary metabolites, notably naphthoquinones and triterpenoids. Naphthoquinones, characterized by a naphthalene ring fused with a quinone moiety, are naturally pigmented compounds with demonstrated antioxidant, antimicrobial, anticancer, and anti-inflammatory activities (Alakurtti, 2006 & Cos, 2006). Among them, Juglone (5-hydroxy-1,4-naphthoquinone) is prominent for its pharmacological significance. Triterpenoids, comprising six isoprene units with a pentacyclic lupane skeleton, include Betulin, a compound reported for its hepatoprotective, antiviral, anti-inflammatory, and anticancer properties (Amiri, 2020 & Tolstikov, 2005)

Diospyros paniculata Dalzell, commonly known as Indian persimmon or Panicle-flowered ebony, is native to tropical and subtropical regions including India, Sri Lanka, Malaysia, and Thailand (Cosmulescu, 2011; Ghosh, 2012; Gîrzu, 1998; Haj, 2006 & Masfria, 2019). Traditionally, various parts of this plant have been used for treating ailments such as rheumatism, skin infections, ulcers, and poisoning (Nancy, 2011). Preliminary phytochemical studies have identified Juglone and Betulin among its major secondary metabolites (Kale, 2013; Nour, 2013 & Vijayalakshmi, 2012). However, to date, no validated analytical techniques have been reported for the simultaneous quantification of these compounds from different plant parts of *D. paniculata*.

The present study addresses this gap by developing and validating robust HPLC and HPTLC methods for the quantification of Juglone and Betulin in bark and leaf extracts of *D. paniculata*. The dual-method approach facilitates cross-validation of results, enhances the reliability of phytochemical profiling, and provides a foundation for standardization of herbal preparations derived from this species. Furthermore, it contributes to the understanding of the plant's phytoconstituents and supports future pharmacological investigations and quality control efforts.

MATERIALS AND METHODS

Analytical reagent (AR)-grade chemicals and solvents were procured from Hi-Media Laboratories, Sigma-Aldrich, and S.D. Fine Chem. Ltd., while HPLC-grade solvents were obtained from Merck and Hi-Media. Standards of Juglone and Betulin were purchased from Yucca Enterprises (Mumbai, India). Purified water was used from a Millipore Type-1 system.

Instrumentation included a Waters Alliance HPLC system with a PDA detector and Empower software. HPTLC analyses were performed using a CAMAG system equipped with Linomat 5 applicator, Twin Trough chamber, and TLC Scanner 4 (visionCATS software). Additional equipment used included a rotary evaporator (IKA RV 10), analytical balance (Shimadzu Uni Bloc), hot air oven (Innovative DTC 96), and UV chamber (Super Fit).

Plant Material and Extraction

Bark and leaves of *Diospyros paniculata* Dalzell were collected from the Tillari region (Western Ghats, Maharashtra, India), authenticated, and voucher specimens were preserved. The material was shade-dried, coarsely powdered, and stored in airtight containers (Maji, 2013). Methanolic hot solvent extraction was carried out at 20°C, followed by solvent recovery via distillation and concentration under reduced pressure using a rotary evaporator (30°C, 90 rpm) (Chauhan, 2004 & Singh, 2016).

Fractionation of Crude Extracts

Bark extract (20 g) underwent column chromatography on silica gel (60–120 mesh), eluted with increasing polarity solvents from n-hexane to ethyl acetate. Fractions eluted with 100% chloroform were selected for further analysis (Erol, 2023). Leaf extract (5 g) was fractionated following the biphasic method of P. Cos *et al.*, using dichloromethane, citric acid, methanol, and petroleum ether. Methanol-rich fractions were concentrated and stored (Babula, 2005).

Phytochemical Screening and TLC Analysis

Standard qualitative assays were performed to detect alkaloids, tannins, steroids, triterpenoids, flavonoids, phenolics, carbohydrates, quinones, saponins, naphthoquinones, cholesterol, and coumarins. TLC analysis was carried out on silica gel plates (10 × 20 cm), developed using solvent systems (e.g., n-hexane:ethyl acetate, chloroform:methanol), and visualized under UV light, iodine vapours, or 5% sulfuric acid-methanol reagent (Banfi, 2006 & Chauhan, 2010).

HPLC Method Development & Validation

HPLC analysis was performed using a Waters Alliance system with a 2998 PDA detector (Empower software). Method development aimed to achieve optimal separation and quantification of Juglone and Betulin in bark and leaf extracts of *Diospyros paniculata*.

Standard stock solutions (1000 µg/mL) were prepared in DMSO and diluted with methanol to obtain working solutions (10 µg/mL). Similarly, plant fractions were prepared and diluted for analysis. Juglone was analyzed using methanol:water (50:50, v/v) on a Spherisorb ODS 1 column (4.6 × 250 mm, 5 µm), with a flow

rate of 1.0 mL/min, detection at 224 nm, and 6 min run time. Betulin analysis was optimized with acetonitrile:water (90:10, v/v) on an X-Bridge column (4.6 × 50 mm, 3.5 μm), 0.5 mL/min flow, detection at 206 nm, and 5 min run time.

The developed HPLC method was validated according to ICH Q2(R1) guidelines to ensure reliability and accuracy (ICH, 2005). System suitability was assessed by injecting six replicates of standard solutions of Juglone and Betulin at three concentration levels (low, medium, high), evaluating retention time, USP tailing factor, number of theoretical plates, and %RSD of peak areas, all of which met the acceptance criteria. Linearity was established for Juglone (2–10 μg/mL) and Betulin (20–100 μg/mL) with Correlation Coefficients (R^2) exceeding 0.99. The Limits of Detection (LOD) and Quantification (LOQ) were determined from the standard deviation of the intercept and slope of the calibration curves. Precision was evaluated through intra-day and inter-day studies, with analyses performed in triplicate at three concentration levels, demonstrating %RSD values below 2%. Repeatability was confirmed by analyzing multiple aliquots of each concentration level, showing consistent peak areas. Robustness was tested by introducing deliberate variations in flow rate, mobile phase composition, wavelength, and column temperature, with results indicating negligible impact on method performance. Ruggedness was assessed by different analysts preparing and analyzing samples independently, yielding reproducible results. Specificity and selectivity were verified by confirming the absence of interference from solvents or matrix components in the chromatograms. The validated method was subsequently applied for accurate quantification of Juglone and Betulin in bark and leaf extracts of *Diospyros paniculata*.

HPTLC Method Development & Validation

HPTLC analysis was performed using Silica Gel 60 F254 plates as the stationary phase. Plates were saturated for 20 min in a CAMAG twin-trough chamber, samples were applied with a Linomat 5 applicator, and detection was done using a TLC Scanner 4 at 410 nm. Standard Juglone (1000 μg/mL) and sample stock solutions (1000 μg/mL) were prepared in methanol. Among tested solvent systems, toluene:ethyl acetate (8:2, v/v) provided optimal resolution with an R_f of 0.88 and was selected for validation.

Method validation followed ICH Q2(R1) guidelines (31). System suitability was assessed using triplicate applications at three concentrations, evaluating R_f, %RSD of peak area, and spot symmetry. Linearity was established for 0.2–1.0 μL/spot with $R^2 > 0.99$. LOD and LOQ were calculated from the calibration curve. Specificity confirmed no interference from solvents or matrices. Precision (intra-day and inter-day) showed %RSD < 2%. Ruggedness was tested using different analysts, and robustness by varying the mobile phase (7:3 and 9:1). The validated method was successfully applied to quantify Juglone (2 μL/spot) in bark and leaf extracts of *Diospyros paniculata*.

RESULTS

Methanolic extraction of *Diospyros paniculata* leaves (1.5 kg) and bark (1 kg) yielded 68 g and 45 g of semisolid extracts, respectively. Leaf extract appeared dark green, bark extract brown, both with distinct odor and taste, fully soluble in methanol/ethanol and partially in water, petroleum ether, and chloroform.

Qualitative phytochemical screening revealed alkaloids, steroids, triterpenoids, tannins, flavonoids, phenolics, carbohydrates, naphthoquinones, and saponins in both extracts; coumarins and quinones were unique to leaves, cholesterol to bark. The abundance of secondary metabolites, particularly naphthoquinones and triterpenoids with known pharmacological effects, justified further analysis.

Fractions obtained via column chromatography (bark) and liquid–liquid partitioning (leaf) tested positive for naphthoquinones, steroids, and triterpenoids, confirming their suitability for chromatographic quantification. TLC analysis using various solvent systems showed distinct phytochemical bands under UV and after chemical visualization, indicating effective separation of bioactive constituents as seen in Figure 1.

HPLC Method Development & Validation

Optimal chromatographic conditions were established after several trials. Juglone showed a sharp, symmetrical peak using methanol:water (50:50) with a retention time (RT) of 3.38 ± 0.1 min, 7784 plates, and a tailing factor of 1.4. Betulin was best resolved using acetonitrile:water (90:10), with an RT of 3.07 ± 0.1 min, 4578 plates, and a tailing factor of 1.1. System suitability tests confirmed stable retention times (~3.38 min for Juglone, ~3.07 min for Betulin) as shown in Figure 2, acceptable tailing (1.09–1.61), and theoretical plates (Juglone: 7784–10985; Betulin: 4160–4578). Repeatability was validated with %RSD <2% across concentrations. Linearity was excellent with $R^2 = 0.9961$ (Juglone) and 0.9993 (Betulin). LOD/LOQ were 0.625/1.89 μg/mL (Juglone) and 2.58/7.81 μg/mL (Betulin) as shown in Figure 3. Precision studies (intra- and interday) showed %RSD <2% (Table 1), confirming reproducibility. Robustness tests with minor changes in flow rate, mobile phase composition, wavelength, and temperature demonstrated stable performance (%RSD <2%). Ruggedness (analyst-to-analyst variability) also met acceptance criteria (<2% RSD). Specificity was confirmed by the absence of interfering peaks in blanks, and selectivity by clear separation of analytes from complex plant matrices. Quantification revealed higher accumulation in bark: Juglone 1.739 μg/10 mg (bark) vs. 0.507 μg/10 mg (leaf); Betulin 52.96 μg/10 mg (bark) vs. 29.61 μg/10 mg (leaf).

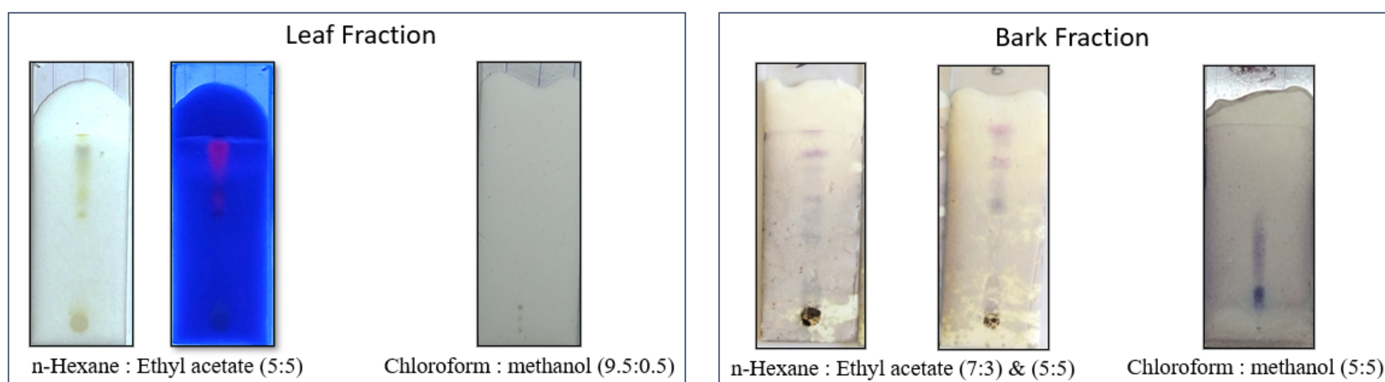

Figure 1: TLC showing the presence of various phytochemical spots of leaf and bark fractions.

Table 1: %RSD of Juglone and Betulin for Intraday and Interday parameter in HPLC.

Parameter	Intervals	Juglone (%RSD)			Betulin (%RSD)		
		2 ppm	6 ppm	10 ppm	20 ppm	60 ppm	100 ppm
Intra	Day 1	0.06465	0.421963	0.32424	0.56622	0.55845	0.36036
day	Day 2	0.1255	0.034819	0.02313	0.937076	0.14103	0.20885
	Day 3	0.35839	0.052551	0.08162	1.145364	0.76193	0.51628
Inter	1st hr	0.77777	0.580086	0.33594	1.877007	0.44822	0.11567
day	4th hr	0.35839	0.064072	0.08416	0.797184	0.86037	0.17977
	8th hr	0.09848	0.089866	0.18191	0.555102	0.84737	0.17453

HPTLC Method development and validation for Juglone

Optimal chromatographic conditions were established after several trials. Toluene:ethyl acetate (8:2, v/v) provided the best resolution (R_f = 0.88 ± 0.01) with a clean baseline, selected for validation. System suitability confirmed sharp, consistent peaks across 0.2–1.0 µL/spot concentrations (Figure 4), with %RSD < 2% for peak areas and no tailing or fronting. Linearity was excellent (R^2 = 0.9917, Figure 5), with LOD and LOQ of 0.091 and 0.276 µg/spot, respectively. Specificity was demonstrated by the absence of interference peaks in blanks and plant matrices (Figure 4). Precision and repeatability were confirmed with %RSD < 2% in intra- and interday studies. Ruggedness testing showed analyst-to-analyst reproducibility (%RSD < 2%). Robustness was maintained under minor mobile phase variations (7:3, 9:1). Quantification revealed higher Juglone content in bark extract (1.462 µg/mL) than leaf extract (0.546 µg/mL), with clear, distinct peaks.

DISCUSSION

For the quantitative assessment of two important phytoconstituents - Juglone, a naphthoquinone, and Betulin, a triterpenoid in the bark and leaf extracts of *Diospyros paniculata* Dalzell, the current work effectively developed, refined, and validated reliable chromatographic techniques. High-Performance Thin-Layer Chromatography (HPTLC) and High-Performance Liquid Chromatography (HPLC) were both used in the method development process, and ICH Q2(R1) guidelines were followed for validation.

For both Juglone and Betulin, the HPLC approach offered superior resolution, selectivity, and measurement accuracy. Betulin was well resolved at 3.07 ± 0.1 min using an acetonitrile: water (90:10, v/v) mobile phase, but Juglone demonstrated excellent retention at 3.38 ± 0.1 minutes using a methanol: water (50:50, v/v) mobile phase. In linearity investigations, both substances showed low limits of detection and quantification, high correlation coefficients (r2 > 0.996), and acceptable values for system appropriateness characteristics including tailing factor and theoretical plate count. Assessments of precision, repeatability, robustness, and ruggedness revealed %RSD values less than 2%, demonstrating the methodologies' dependability.

According to quantitative study, the bark has a higher concentration of both betulin and juglone than the leaves, suggesting that the bark is a more phytochemically rich portion of the plant. In particular, it was discovered that the amount of Juglone in the bark was 1.739 µg/10 mg and in the leaves, 0.507 µg/10 mg, whilst the amount of Betulin in the bark was 52.96 µg/10 mg and in the leaves, 29.61 µg/10 mg. Thus, it is shown that the established HPLC procedures are easy to use, inexpensive, reproducible, and appropriate for regular quality monitoring and standardization of *Diospyros paniculata* and its phytopharmaceutical applications.

Juglone's HPTLC method was also created and verified with great selectivity and accuracy. The best separation among the tested mobile phases was found to be achieved with toluene:

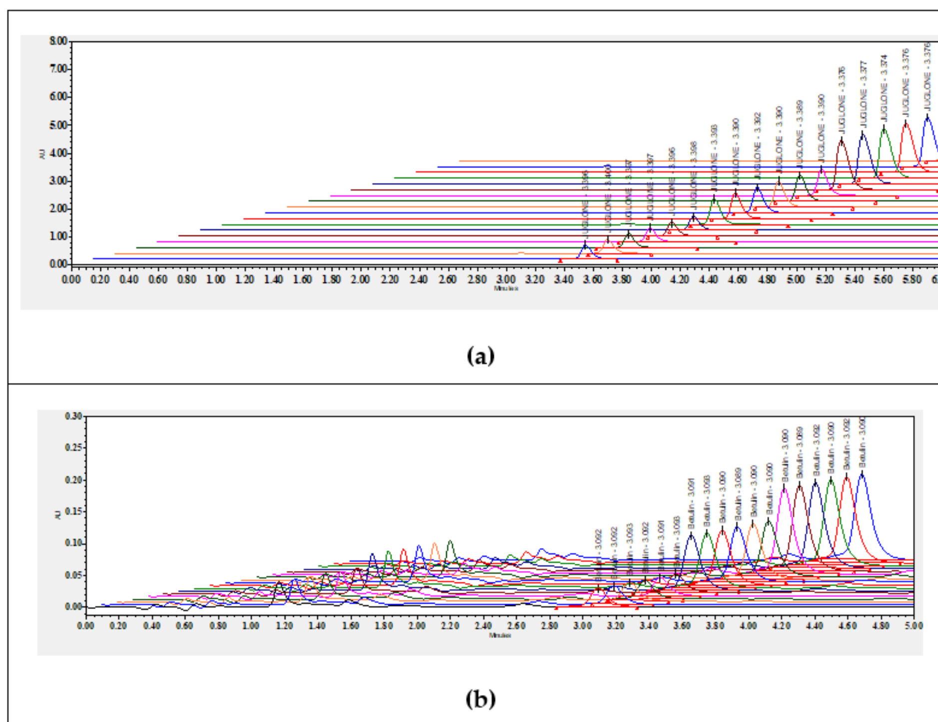

Figure 2: System suitability/ Repeatability Chromatograms of a) Juglone replicates (2, 6, 10 ppm) and b) Betulin replicates (20, 60, 100 ppm).

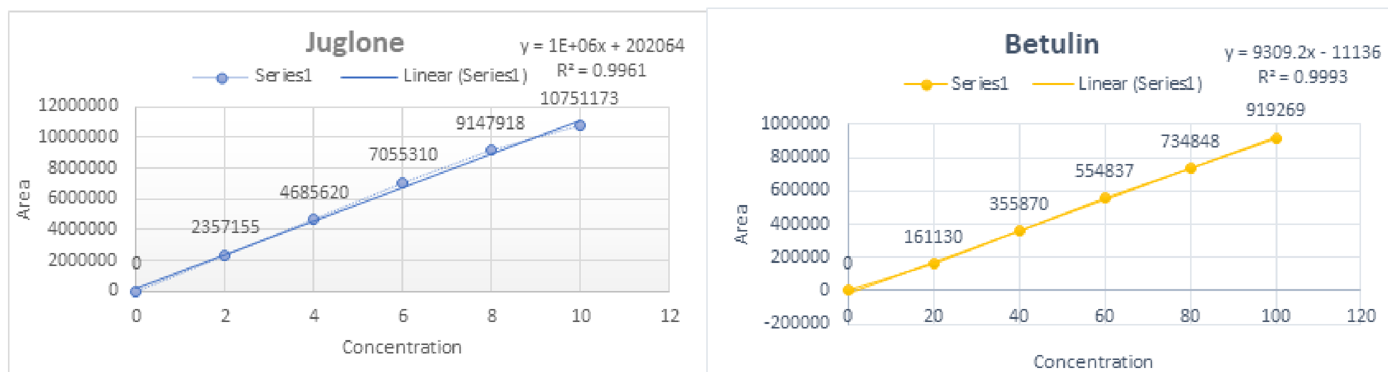

Figure 3: Linearity curves of (a) Juglone and (b) Betulin in HPLC.

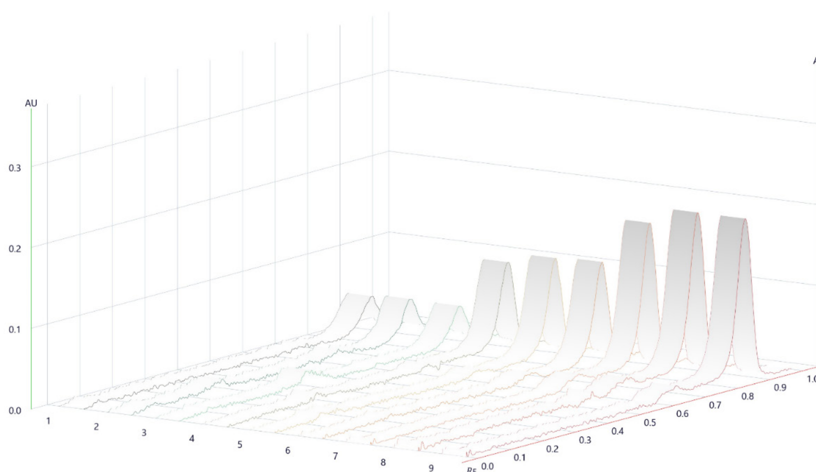

Figure 4: HPTLC System suitability Chromatograms of Juglone (0.2, 0.6, 1 µL/spot).

Figure 5: HPTLC Calibration curve of Juglone in the concentration range of 0.2-1 µL/spot.

ethyl acetate (8:2, v/v), which produced a distinct and crisp peak with an R_f value of 0.88 ± 0.01. The method's LOD and LOQ values were 0.091 µg/spot and 0.276 µg/spot, respectively, demonstrating excellent linearity (r^2 = 0.9917). Excellent technique consistency was indicated by system appropriateness based on spot repeatability and area accuracy, which demonstrated %RSD values <2%. Additionally, the technique remained stable under slight variations in the composition of the mobile phase and demonstrated good specificity without matrix influence. According to the quantification results, the content of juglone in bark was higher (1.462 µg/mL) than in leaves (0.546 µg/mL), which was in line with the HPLC data.

For high-throughput screening and comparative analysis of juglone in herbal extracts and formulations, the validated HPTLC method is easy to use, quick, and perfect.

CONCULSION

For the measurement of Juglone and Betulin in *Diospyros paniculata*, the validated HPLC and HPTLC procedures provide sensitive, repeatable, and dependable analytical instruments. The use of these techniques to the standardization, verification, and quality control of herbal extracts, raw materials, and derived phytopharmaceuticals can be expanded. In order to promote its selection in next pharmacognostic and pharmacological research, the paper further emphasizes the phytochemical richness of the bark above the leaf.

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ABBREVIATIONS

HPLC: High-Performance Liquid Chromatography; **HPTLC:** High-Performance Thin-Layer Chromatography; **PDA:** Photodiode Array Detector; **ICH:** International Council for Harmonisation; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **Rf:** Retention Factor; **RSD:** Relative Standard Deviation; **RT:** Retention Time; **UV:** Ultraviolet; **DMSO:** Dimethyl Sulfoxide; **AR:** Analytical Reagent; **TLC:** Thin Layer Chromatography; **WHO:** World Health Organization; **FDA:** Food and Drug Administration; **HPLC-grade:** High-Performance Liquid Chromatography grade; **µg/mL:** Microgram per millilitre; **µg/spot:** Microgram per spot; **µg/10 mg:** Microgram per ten milligram; **v/v:** Volume per volume.

CONFLICT OF INTEREST

The authors state that they have no known Conflict of interest or personal interests that might have influenced the work presented in this study.

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CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Sneha Wali: Conceptualization, Methodology, Investigation, Data Curation, Formal Analysis, Writing Original Draft. Parixit Bhandurge: Supervision, Project Administration, Method Validation, Review & Editing. Nikhil Gawas: Experimental Support, Sample Preparation, Data Collection, Documentation, Visualization. Priya Shetti: Instrumental Analysis.

SUMMARY

This study developed and validated HPLC and HPTLC methods for simultaneous estimation of Juglone and Betulin in *Diospyros paniculata* Dalzell. Methanolic extracts of bark and leaves were analyzed following ICH Q2(R1) guidelines. In HPLC, Juglone and Betulin were separated using methanol (50:50) and acetonitrile (90:10) mobile phases with retention times of 3.38 and 3.07 min, respectively, showing good linearity ($R^2 > 0.996$) and precision. HPTLC analysis using toluene acetate (8:2) gave Juglone an Rf of 0.88 ± 0.01 with $R^2 = 0.9917$. Both methods were accurate, robust, and reproducible. Quantitative results revealed higher concentrations of Juglone (1.739 µg/10 mg) and Betulin (52.96 µg/10 mg) in bark compared to leaves, establishing the bark as a richer phytochemical source suitable for quality control of *D. paniculata* extracts.

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