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Physicochemical Analysis and HPTLC Fingerprinting of *Diospyros nigrescens* Leaves

Rupali D. Popeta[1*], **Ganesh A. Thakur** [1]

Department of Chemistry, Mahatma Phule Arts, Science and Commerce College, Panvel, India[1*]

Department of Chemistry, Mahatma Phule Arts, Science and Commerce College, Panvel, India[1]

Abstract: *Standardization of medicinal plants is essential to ensure their identity, purity and quality for safe therapeutic use. The present study aimed to evaluate the physicochemical parameters and establish an HPTLC fingerprint profile of Diospyros nigrescensleaves for quality control and authentication purposes. Physicochemical analysis revealed low foreign organic matter (0.05%), indicating minimal contamination and proper handling of the crude drug. The ethanol-soluble extractive value (16.34%) was slightly higher than the water-soluble extractive value (15.0%), suggesting the presence of significant polar and moderately polar phytoconstituents. The total ash (6.37%), acid-insoluble ash (1.80%) and water-soluble ash (4.10%) values indicated acceptable inorganic content with minimal siliceous impurities, while the sulphated ash value (15.64%) reflected the mineral composition of the plant material. Loss on drying (5.84%) and moisture content (7.16%) were within permissible limits, supporting good stability and storage conditions. HPTLC profiling performed at 254 nm, 366 nmand 540 nm using 5 µL and 10 µL application volumes demonstrated multiple phytoconstituents with reproducible Rf values. A major compound was consistently observed at Rf ≈ 0.91 across all wavelengths, confirming the presence of a predominant constituent. The developed physicochemical standards and chromatographic fingerprint provide reliable reference data for identification, authentication and quality evaluation of Diospyros nigrescensleves, supporting its potential for further pharmacological applications*

Keywords*:* *Diospyros nigrescens*, Physicochemical analysis, HPTLC fingerprinting, Quality control, Herbal authentication

I. INTRODUCTION

Medicinal plants continue to play a vital role in global healthcare systems, acting as valuable sources of bioactive compounds for the prevention and treatment of various diseases [1], [2]. The therapeutic potential of herbal drugs largely depends on their chemical composition, which may vary due to environmental, geographical and processing factors [3]. Therefore, the standardization and quality evaluation of plant materials are essential to ensure their safety, efficacy and reproducibility. Physicochemical analysis and chromatographic fingerprinting have emerged as important tools for the authentication and quality control of herbal drugs, particularly in the context of pharmacognostic and phytochemical research [4].

The genus *Diospyros* (family Ebenaceae) comprises more than 500 species distributed widely in tropical and subtropical regions of the world [5]. Several species within this genus are traditionally used in ethnomedicine for the management of ailments such as inflammation, microbial infections, fever, diabetes and gastrointestinal disorders [6]. These pharmacological activities are primarily attributed to the presence of diverse secondary metabolites, including naphthoquinones, flavonoids, tannins, triterpenoids and phenolic compounds [7]. Among the less explored species, *Diospyros nigrescens* has gained attention due to its potential medicinal properties and regional ethnobotanical significance. However, comprehensive scientific validation and quality assessment of this species remain limited, highlighting the need for systematic investigation.

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Physicochemical parameters, such as ash values, extractive values, moisture content and crude foreign organic matter content, provide fundamental information regarding the purity, identity and quality of plant materials [8]. These parameters help detect adulteration, contamination and improper handling during processing and storage. Establishing physicochemical standards is particularly important for medicinal plants that lack official monographs, as such data serve as reference benchmarks for future research and industrial applications [9].

High-Performance Thin-Layer Chromatography (HPTLC) is a widely accepted, rapid and cost-effective analytical technique for the qualitative and semi-quantitative evaluation of phytochemicals [10]. HPTLC fingerprinting enables the visualization of complex phytochemical profiles and provides characteristic chromatographic patterns that can be used for authentication, detection of adulterants and batch-to-batch consistency assessment [11]. Compared with conventional chromatographic methods, HPTLC offers advantages such as minimal sample preparation, simultaneous analysis of multiple samples, high resolution and reproducibility. The technique is increasingly recommended by regulatory authorities for herbal drug standardization because it generates reliable chemical fingerprints that reflect the presence of multiple constituents rather than a single marker compound [12].

Despite the pharmacological importance of the *Diospyros* genus, there is a lack of scientific literature focusing on the physicochemical standardization and chromatographic profiling of *Diospyros nigrescens* leaves. Establishing a detailed HPTLC fingerprint along with physicochemical parameters can contribute significantly to the identification and quality control of this species. Such studies are also essential for supporting the development of herbal formulations, ensuring raw material authenticity and facilitating regulatory acceptance in the herbal and nutraceutical industries.

Therefore, the present study aims to evaluate the physicochemical characteristics and develop an HPTLC fingerprint profile of *Diospyros nigrescens* leaves. The findings of this research are expected to provide baseline data for the authentication, standardization and quality assurance of this medicinal plant, thereby promoting its safe utilization in traditional medicine and potential pharmaceutical applications. Furthermore, the generated chromatographic fingerprints may serve as valuable reference standards for future phytochemical and pharmacological investigations involving this species.

II. MATERIALS AND METHODS

Chemicals and Reagents

Analytical grade solvents were used for HPTLC fingerprinting. Toluene (99%, MCR-11407), methanol (99%, SJ7SA71233) and ammonia solution (25% LR, CAS No. 1336-21-6) were procured from Molychem, India. Ethyl acetate (99%, SJ7SA71233) was obtained from Merck, India, while diethylamine (99%, CAS No. 109-89-7), ethanolwas purchased from Lobachemie, India. Pre-coated silica gel 60 F254 aluminium plates (Merck, Germany) were used as the stationary phase for chromatographic analysis.

Plant Material Collection

Fresh leaves of *Diospyros nigrescens* were obtained from Amboli Ghat, Maharashtra, India and authenticated at St. Xavier’s College, Mumbai. The plant material was washed thoroughly with running tap water to remove surface impurities and allowed to dry under shade for nearly two weeks. After complete drying, the leaves were finely powdered using an electric blender to obtain material suitable for further studies [13].

Physicochemical Analysis

Determination of Foreign Organic Matter

Fresh leaves were washed thoroughly with water to remove adhering dust and impurities and then air-dried. Approximately 500 g of the sample was spread on a clean white muslin cloth and foreign matter was separated by visual inspection using a 6× magnifying lens. The collected impurities were weighed and the percentage of foreign organic matter was calculated [14].

Determination oftotal ash

The amount of organic impurities present in the sample was evaluated by determining the total ash value. For this purpose, 2 g of the sample was accurately weighed into a pre-weighed silica crucible. The sample was then incinerated by gradually increasing the temperature up to 550 ± 20 °C for 4 hoursor until it became free from carbon. After incineration, the crucible was allowed to cool and weighed repeatedly until a constant weight was achieved. The percentage of total ash was calculated by comparing the weight difference with the initial weight of the sample taken[14], [15].

Determination of Acid insoluble ash

The total ash moistened with concentrated hydrochloric acid, evaporated to dryness and heated in an oven at 135 °C ± 2 °C for three hours. After cooling, 25 mL of dilute HCl was added, covered and heated on a water bath for 10 minutes. The acid-insoluble residue was filtered through ashless Whatman No. 1 filter paper and washed thoroughly with distilled water. The filter paper containing the residue was subsequently ignited in a crucible at a temperature not exceeding 550 °C ± 20 °C for1 hours. After ignition, the crucible was placed in a desiccator, allowed to cool and then weighed. The percentage of acid-insoluble ash was calculated with reference to the initial weight of the leaf powder[14], [15].

Determination ofwater-soluble ash

Water-soluble ash was calculated by boiling the total ash with distilled water, filtering and subtracting the weight of the insoluble residue from the totalash[14], [15].

Determination of sulphated ash

One gram of the powdered plant material was accurately weighed into a silica dish and ignited until the material was completely charred. The remaining residue was moistened with 1 mL of concentrated sulphuric acid, gently heated until the white fumes subsided and then incinerated at 800 °C ± 25 °C until the black residue disappeared [14], [15].

Extractive Values

Water-soluble and ethanol-soluble extractive values were determined by macerating 4 g of powdered leaves with 100 mL of respective solvents in stoppered flasks for 24 h with intermittent shaking. The filtrates were evaporated to dryness, dried at 105 °C, cooled in a desiccator and weighed to calculate extractive values [14].

Loss on Drying

Approximately 5 g of powdered leaf material was placed in a weighing bottle and dried in a hot air oven at 100 ± 2 °C until a constant weight was obtained. The loss in weight was recorded as loss on drying [14].

Moisture Content

Moisture content was determined using the Karl Fischer titration method. A known quantity of powdered sample was titrated with Karl Fischer reagent using a Karl Fischer titrator after standardization with distilled waterand moisture percentage was calculated [16].

HPTLC Fingerprint

Sample Preparation

One gram of powdered *Diospyros nigrescens* leaves was extracted with 20 mL methanol in a conical flask on a rotary shaker overnight. The extract was filtered through Whatman No. 41 filter paper and the filtrate was collected for subsequent analysis [17].

Mobile Phase

Several solvent combinations were tested for the extract and optimal separation was achieved using Toluene: Ethyl Acetate: Diethylamine: Methanol: Ammonia (1:5:3:1:2 v/v/v/v/v) [17].

Sample Application

Bands of sample (8 mm length, 5 µL and 10 µL volume) were applied using a spray application technique. The samples were spotted on pre-coated silica gel 60 F254 aluminum plates (5 × 10 cm) using a CAMAG Linomat 5 applicator connected to an HPTLC system and operated through WINCATS software [13], [17].

Chromatogram Development and Spot Detection

After sample application, the chromatogram was developed in a twin-trough glass chamber (10 × 10 cm) pre-saturated with the mobile phase consisting of Toluene: Ethyl Acetate: Diethylamine: Methanol: Ammonia (1:5:3:1:2 v/v/v/v/v)for 15 minutes. The plates were then air-dried and observed under ultraviolet radiation as well as in daylight. The chromatograms were subsequently scanned using a densitometer at 254 nm, 366 nmand 540 nm with a Densitometry TLC Scanner 3. The Rf values and fingerprint data were recorded using WINCATS software [13], [17].

III. RESULT AND DISCUSSION

Proximate analysis

The physicochemical evaluation of the crude drug revealed important quality control parameters indicating the purity, identity and stability of the plant material. The percentage of foreign organic matter was found to be 0.05%, which is within acceptable limits, indicating minimal contamination and proper handling during collection and processing. Low foreign matter content reflects adherence to good agricultural and collection practices and ensures the authenticity of the crude drug [18], [22].

The ethanol-soluble extractive value (16.34%) was slightly higher than the water-soluble extractive value (15.0%), suggesting that the plant contains a considerable proportion of moderately polar to polar phytoconstituents such as phenolics, flavonoids, glycosides and other secondary metabolites soluble in hydroalcoholic solvents. Extractive values are considered important indicators of the approximate chemical composition of plant materials and provide preliminary evidence regarding the presence of bioactive constituents responsible for therapeutic activity [18], [19], [23]. The relatively high extractive values observed in this study may indicate the presence of significant phytochemical diversity, which supports the pharmacological potential of the plant.

The total ash value (6.37%) represents the total inorganic content present in the plant material, including physiological and non-physiological ash. The acid-insoluble ash (1.80%) indicates the presence of siliceous matter such as soil or sand contamination, which was found to be relatively low, confirming acceptable purity. The water-soluble ash (4.10%) reflects the amount of inorganic components soluble in water, which may include naturally occurring mineral salts. Ash values are widely used as reliable parameters to evaluate the cleanliness and quality of crude drugs and to detect adulteration or substitution with inorganic materials [18], [20], [24].

The sulphated ash value (15.64%) was comparatively higher than the total ash value, which may be attributed to the conversion of organic matter into stable sulphated inorganic residues during sulphation, indicating the presence of mineral constituents in the plant material. Sulphated ash determination is considered more precise because sulphuric acid treatment ensures complete oxidation of organic matter and conversion into measurable inorganic salts [20], [24].

The loss on drying (5.84%) and moisture content (7.16%) were within permissible limits, suggesting low moisture levels that reduce the risk of microbial growth, enzymatic degradation and deterioration during storage. Adequate moisture control also contributes to improved shelf life, physicochemical stability and prevention of fungal contamination, which is critical for maintaining the therapeutic efficacy of herbal drugs [18], [21], [25].

Overall, the physicochemical parameters generated in this study (Table 1.) establish robust baseline reference standards for the plant material, demonstrating its acceptable purity, compositional consistency and physicochemical integrity.

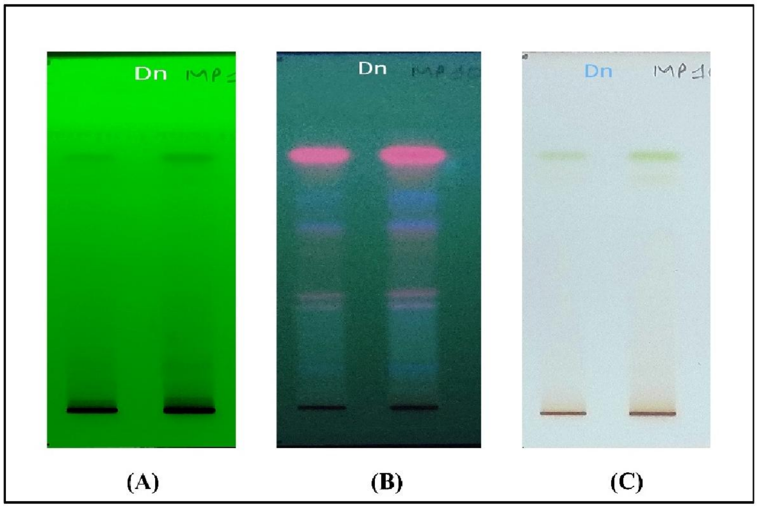
These findings provide critical quality control benchmarks that can support raw material authentication, detection of adulteration and batch-to-batch uniformity in herbal preparations. The defined parameters further enhance the reliability, safety and regulatory compliance of the plant for phytopharmaceutical development, thereby strengthening its suitability for advanced phytochemical characterization and pharmacological evaluation.

Table 1. Physicochemical analysis of *Diospyros nigrescens* leaves.

Sr. No.	Parameters	% Content in Diospyros nigrescens
1.	Foreign organic matter	0.05
2.	Ethanol soluble extractive	16.34
3.	Water soluble extractive	15.0
4.	Total ash	6.37
5.	Acid-insoluble ash	1.80
6.	Water soluble ash	4.10
7.	Sulphated ash	15.64
8.	Loss on drying	5.84
9.	Moisture content	7.16

HPTLC Fingerprinting

HPTLC fingerprinting of the sample was performed at 254 nm, 366 nm and 540 nm using two application volumes (5 µL and 10 µL) to evaluate the effect of concentration on peak resolution and phytochemical distribution. HPTLC analysis at multiple wavelengths is commonly employed to detect diverse classes of phytoconstituents, as different compounds exhibit characteristic absorption or fluorescence under specific wavelengths [26], [27]. The HPTLC fingerprint profile of *Diospyros nigrescens* leaves is shown in figure 1.



(A) (B) (C)

Figure 1. HPTLC fingerprint profile of *Diospyros nigrescens* leaves at wavelengths (A) 254 nm, (B) 366 nm, and (C) 540 nm.

Table 2. Rf values of the methanol leaf extract of *Diospyros nigrescens* at 5 µL application volume.

Peak no.	**At 254nm**		**At 366 nm**		**At 540 nm**	
	Max. Rf	**Peak Area %**	**Max. Rf**	**Peak Area %**	**Max. Rf**	**Peak Area %**
1	0.007	10.26	0.006	2.95	0.004	26.48
2	0.033	3.70	0.033	2.56	0.916	73.52
3	0.096	4.84	0.096	3.34	-	-
4	0.166	11.10	0.164	5.69	-	-
5	0.914	70.10	0.363	2.52	-	-
6	-	-	0.404	3.13	-	-
7	-	-	0.647	9.71	-	-
8	-	-	0.759	5.61	-	-
9	-	-	0.829	13.89	-	-
10	-	-	0.914	50.60	-	-

Table 3. Rf values of the methanol leaf extract of *Diospyros nigrescens* at 10 µL application volume.

Peak no.	**At 254nm**		**At 366 nm**		**At 540 nm**	
	Max. Rf	**Peak Area %**	**Max. Rf**	**Peak Area %**	**Max. Rf**	**Peak Area %**
1	0.006	3.54	0.004	0.92	0.004	8.95
2	0.039	3.51	0.039	2.37	0.913	91.05
3	0.093	2.53	0.093	1.79	-	-
4	0.160	11.89	0.160	5.23	-	-
5	0.831	15.87	0.254	2.08	-	-
6	0.911	62.66	0.366	2.84	-	-
7	-	-	0.410	3.26	-	-
8	-	-	0.649	11.72	-	-
9	-	-	0.760	8.11	-	-
10	-	-	0.829	9.83	-	-
11	-	-	0.913	51.86	-	-

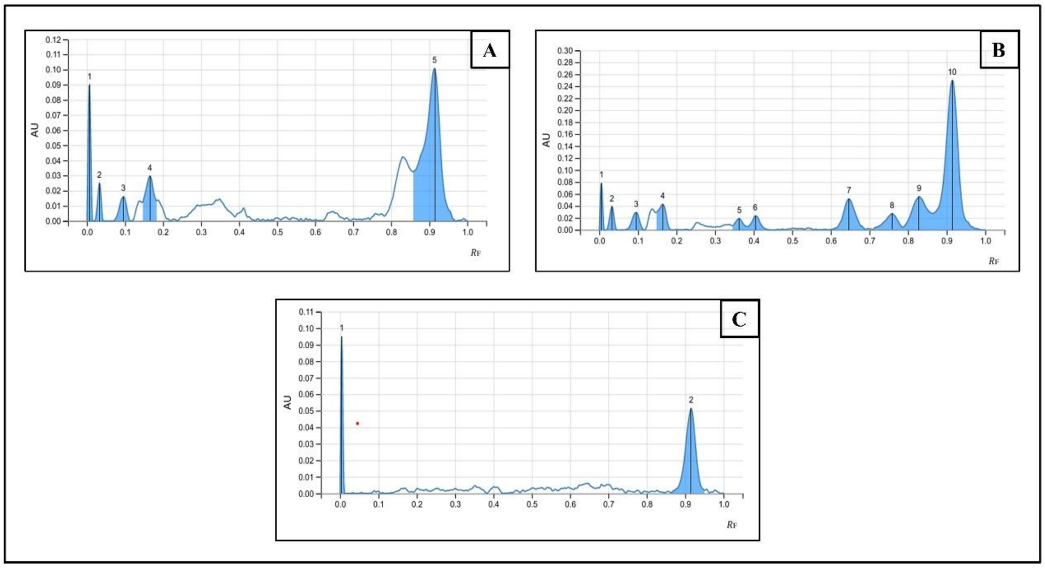


Figure 2. HPTLC chromatogram of the methanol leaf extract of *Diospyros nigrescens* at 5 µL application volume at wavelengths (A) 254 nm, (B) 366 nm, and (C) 540 nm.

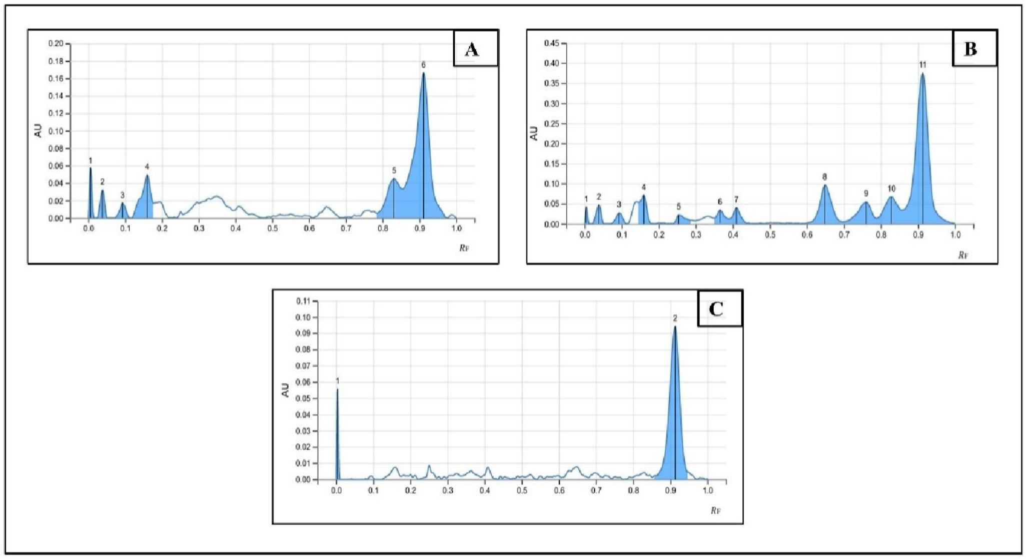


Figure 3. HPTLC chromatogram of the methanol leaf extract of *Diospyros nigrescens* at 10 µL application volume at wavelengths (A) 254 nm, (B) 366 nm, and (C) 540 nm.

The Rf values of the methanolic leaf extract of *Diospyros nigrescens* at application volumes of 5 µL and 10 µL are presented in Tables 2 and 3, and Figures 3 and 4, respectively. At 254 nm, the chromatogram obtained with 5 µL application showed five peaks, with a major peak at Rf ≈ 0.914 contributing 70.10% of the total peak area, indicating the presence of a dominant UV-absorbing constituent. Similarly, the 10 µL application also exhibited a prominent peak at Rf ≈ 0.911 with a peak area of 62.66%, confirming consistency in the phytochemical profile. However, the 10 µL volume showed slightly improved detection of intermediate peaks, suggesting better sensitivity due to higher sample loading. Detection at 254 nm primarily corresponds to conjugated systems and aromatic compounds, which are commonly present in plant secondary metabolites [26], [28].

At 366 nm, a greater number of peaks were detected for both volumes, indicating the presence of fluorescent phytoconstituents such as phenolics and flavonoids. The 5 µL sample showed ten peaks, whereas the 10 µL sample showed eleven peaks, demonstrating that increased application volume enhanced compound detectability. In both cases, a major peak near Rf ≈ 0.914 was observed with high peak area percentages (50.60% for 5 µL and 51.86% for 10 µL), indicating a predominant fluorescent component. Fluorescence detection at 366 nm is widely used for identifying polyphenolic compounds, coumarinsand flavonoids after chromatographic separation [27], [29].

At 540 nm after derivatization, only two peaks were observed in both volumes, with a major peak at Rf ≈ 0.916 (5 µL: 73.52%; 10 µL: 91.05%). The higher peak area in the 10 µL application suggests improved visualization of derivatized constituents due to increased concentration. Post-derivatization detection enhances the visualization of compounds that are otherwise weakly absorbing in UV regions, enabling improved qualitative profiling [26], [28]. Figure 5 shows densitogram display of the methanolic leaf extract of *Diospyros nigrescens* at application volumes of 5 µL and 10 µL.

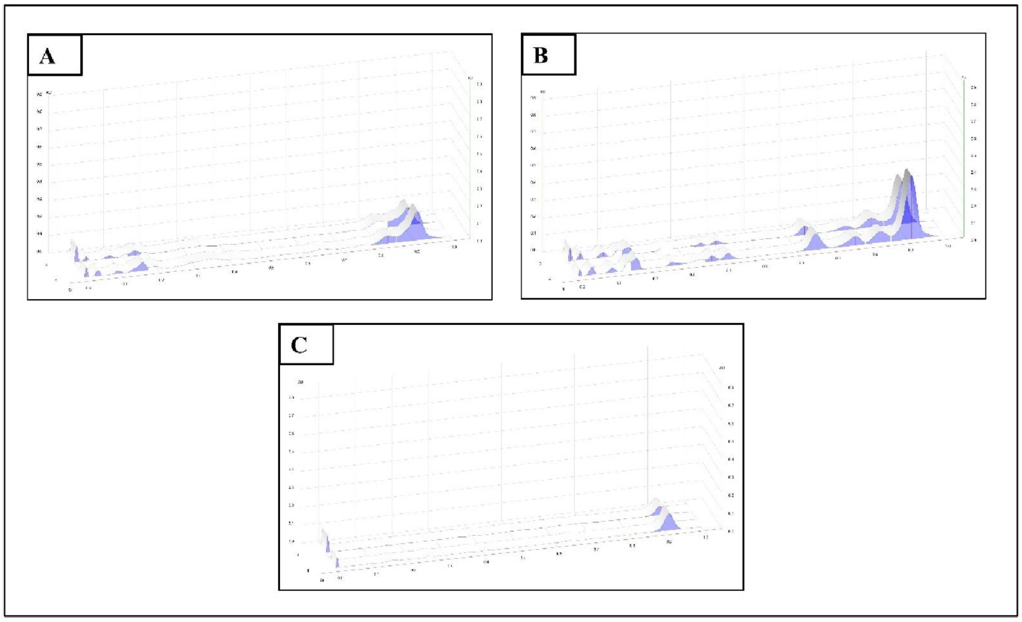


Figure 4. HPTLC densitogram display of the methanol leaf extract of *Diospyros nigrescens* at 5 µL and 10 µL application volume at wavelengths (A) 254 nm, (B) 366 nm, and (C) 540 nm.

Overall, both application volumes exhibited similar Rf values, confirming reproducibility of the chromatographic method. However, the 10 µL volume provided better peak intensity and detection of additional minor components, while the 5 µL volume showed adequate resolution with lower sample consumption. The results indicate that the developed HPTLC method is reliable for phytochemical profiling, with 10 µL being more suitable for comprehensive fingerprint analysis. The reproducible chromatographic pattern obtained in this study can serve as a characteristic chemical fingerprint for authentication, quality control and batch-to-batch consistency evaluation of the plant material [26], [27], [30].

IV. CONCLUSION

The present study provides essential physicochemical parameters and HPTLC fingerprint data for the standardization and quality assessment of the investigated plant material. The physicochemical results confirmed the purity and suitability of the sample, while the HPTLC profiling revealed distinct and reproducible chromatographic patterns, indicating the presence of diverse phytoconstituents. The obtained Rf values and densitometric profiles can serve as reliable reference standards for authentication, detection of adulteration and quality control. Overall, the study supports the scientific validation of the plant and offers a useful foundation for further pharmacological and phytochemical investigations.

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