ORIGINAL ARTICLE

Microscopic and HPTLC Fingerprint Analysis a Tool for Authentication and Quality Control of *Nelumbo nucifera*

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Abstract

The demand for herbal raw material has increased and there is a requirement for quality control to look at the integrity of the herbal raw materials. There is a need for an easy and fast technique to check the quality and the authenticity of these raw materials. *Nelumbo nucifera* seeds have been used as traditional medicine for ages for curing various diseases. Its microscopy examination of fruit showed double-layered palisade tissue, rosette scleride, and epidermal pores. The morphology of starch grains were found to be simple, oval, and elliptical to polygonal in shape, which is the unique characteristic of the plant. Phytochemical analysis showed the presence of alkaloids, flavonoids, tannins, phenols, and saponin. A comparative study of cold and hot extraction of seed extract was carried out with respect to the HPTLC fingerprint profile. The HPTLC fingerprint of the cold and hot extract was done using butanol: water: acetic acid (4:1:1v/v/v) it showed the presence of 9 bands (R_F 0.05–0.94) on the plate. The result shows that both cold and hot extract can be used for fingerprinting. Different concentrations of cold extract (100%, 80%, 50%, 20%) was used to standardize the lowest on plate concentration which can give maximum bands with high resolution, the results shows that a lower concentration of 50% can be used for the study. Thus, microscopy and HPTLC fingerprinting data along with morphological information can be used as a quality control tool to check the authenticity of the *Nelumbo nucifera* which has potential therapeutic value.

Keywords HPTLC fingerprinting · Microscopy · Phytochemical · *Nelumbo nucifera*

1 Introduction

The interest in herbal medicine has increased and there is a requirement for quality control to look at the integrity of the herbal raw materials. Nearly about 80% of the population is dependent on the herbs for the treatment, cure, and prevention [1]. Botanical materials can be identified using a variety of techniques, including macroscopic and microscopic descriptions, DNA analysis, and chemical analysis. Chemical identification involves chromatographic or spectroscopic processes to compare a fingerprint to that of a Reference Standard, a monograph description, or a reference chromatogram to establish identification. One of the chromatographic techniques used in USP monographs for botanical materials is thin-layer chromatography (TLC); high-performance thin-layer chromatography (HPTLC) is the most advanced version of TLC [2]. HPTLC is an automated TLC technique that uses sorbents with smaller particle and pore sizes, faster analysis durations, and a development chamber that uses less mobile phase and is more efficient. The UV/Visible/Fluorescent Scanner are a sort of sophisticated densitometer that scans the complete chromatogram qualitatively and quantitatively. HPTLC has gained popularity in pharmaceutical analysis due to advantages such as improved separation efficiency and detection limits, lower cost per analysis, shorter analysis times, no solvent pretreatment (such as filtration and degassing), low mobile phase consumption per sample, and no interference from previous analyses, as each analysis uses a new stationary phase and mobile phase. In addition, the approach allows us to apply numerous sample points (up to 18) in a single run. The full chromatogram may be seen at a glance, allowing for a quick comparison evaluation. When compared to HPTLC, HPLC requires high-quality solvents and columns, resulting in higher analysis expenses [3].

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Plant microscopic and morphological data and HPTLC fingerprint can be utilized as quality control, authentication and validation tool for these medicinal plants. HPTLC has been used for proving the identity of herbal drugs and extracts, an also for the investigation of the presence of adulterations or alterations, which is a key part of establishing purity [4]. The improved HPTLC fingerprints are a tool for authentication, and a method to show the different patterns of phytochemical components present in herbal products [5]. Chemical markers present in plant extracts can be detected, identified, and estimated using high performance thin layer chromatography (HPTLC), which can also be used to standardize herbal formulations [6]. Qualitative Phytochemical analysis will only tell us the broad category of secondary metabolites present in the plant, whereas HPTLC will show the different phytochemical in the form of band on TLC plate, which will be helpful to establish a unique fingerprint pattern for the plant.

Nelumbo nucifera has been placed in the family of Nelumbonaceae, this plant has various other names for example Indian lotus, Chinese water lilly, and holy lotus [7–9]. In India, it is found from north to south, showing immense phenotypic variety with different shapes, sizes and colour shades from pink to white blossoms having 16–160 petals [10]. The seeds are sold as a in Indian market, by the name of 'kamal gatta'. The leaf, rhizome, seed, and flower are generally utilized for the treatment of pharyngoplasty, pectoralgia, spermatorrhoea, hematuria, hepatopathy, hyperlipidemia, hack, hematemesis, epistaxis, hemoptysis, leucoderma, metrorrhagia, loose bowel, fever, cholera, smallpox, and hyperdipsia. This plant is also utilized as a diuretic, anthelmintic and in the treatment of strangury, spewing, and skin infection, as announced in ayurveda. Various pharmacologically dynamic phytoconstituents have been extracted from the leaf, rhizome, seed and flower [11]. Diverse phytochemicals are present in the seed, for example, nuciferine, roemerine, dauricine, armepavine and lotusine [12].

Hence, this plant is of medicinal importance and less data is available to assess the quality of the raw material, the current study was undertaken to build up a simple and easy technique for the inspection of the quality and authenticity of the plant. Two strategies were utilized, microscopic characteristics and HPTLC fingerprinting for the study.

2 Materials and Methods

2.1 Plant Material

N. nucifera dry seeds were collected from the local ayurvedic shop. It was authenticated from Agharkar Research Institute, Pune. All the chemicals utilized were of analytical grade.

2.1.1 Sample Preparation

The outer hard seed coat and plumule were removed from the seed, and the cotyledons were ground into coarse powder using an electric grinder. The powder was sieved to obtain 85 BSS mesh powdered material, which was then stored at room temperature in an airtight container.

2.2 Microscopic Analysis

Transverse section (TS) of whole fruit of *N. nucifera* was studied using Compound microscope BA-210 equipped with 3MPx camera (Motic software). The starch grains were stained with iodine solution and were observed under 100X.

2.3 Phytochemical Analysis

The finely powdered plant material was subjected to rotary shaker overnight using methanol. The methanol extract was used for preliminary screening of various phytochemical present in the seed by using different test specific for each phytochemical [13].

Preliminary screening of phytochemicals.

(a) Test for Terpenoids (Salkowski test): To the 2 mL of chloroform, 5 mL of extract was added, and 3 mL of strong sulphuric acid was added to the mixture. The presence of terpenoids is indicated by the formation of a reddish-brown color at the interface.

(b) Test for Saponin: 1 mL of material was combined properly with 5 mL of distilled water in a test tube and vigorously agitated; if stable foam appeared, it indicated the presence of saponin.

(c) Test for Tannins, phenols (Ferric chloride test): A few drops of 5% ferric chloride solution were added to 1 mL of extract in a test tube. The presence of tannin is indicated by a blue-black color.

Gelatin test: The addition of a 1% gelatin solution containing 10% sodium chloride to the extract resulted in the production of a white precipitate, indicating the presence of tannins.

(d) Test for Alkaloids (Dragendroffs reagent test):

In a test tube few quantities of sample were mixed with 2 mL of Dragendroffs reagent (solution of potassium iodide). If reddish brown colored precipitate is formed indicates the presence of alkaloids.

Mayes's Test: Extract was treated with Mayer's reagent (Potassium mercuric iodide); Formation of a yellow colored precipitate indicates presence of alkaloids.

Hager's Test: Extract was treated with Hager's reagent (saturated picric acid solution), Presence of alkaloids was

confirmed by the yellow colored precipitate indicates the presence of alkaloids.

(e) Test for Flavonoids (Shinoda Test): Magnesium powder and a few drops of strong hydrochloric acid were added to 2 mL of test extract. Flavonoids were identified by the presence of a red or orange coloration.

(f) Test for Steroids: 1 mL of the sample was taken and dissolved in 5 mL chloroform in a test tube, and then an equal volume (5 mL) of strong sulphuric acid was carefully added through the test tube's sides. The presence of steroid is indicated if the upper layer becomes red and the sulphuric acid layer turns yellow with a slight green fluorescence.

(g) Test for detection of Glycosides (Born Trager's): The extracts were incubated in a ferric chloride solution for 5 min before being placed in a boiling water bath. The mixture was cooled before being extracted with benzene in equal parts. The benzene layer was separated and treated ammonia solution. The presence of glycoside is indicated by the formation of a rose pink colour in the ammonia layer.

Legal's test: After treating extracts with sodium nitopruside in pyridine and sodium hydroxide, a pink to crimson colour was formed, indicating the presence of cardiac glycosides.

2.4 HPTLC Fingerprinting

2.4.1 Sample Preparation for HPTLC Analysis

The methanol extract was used for the fingerprinting analysis. Cold extraction was done by taking different concentration 20% (0.2 g), 50% (0.5 g), 80% (0.8 g), and 100% (1 g powder) in 10 mL methanol and sonicated for 15 min and centrifuged at 1000 rpm for 10 min and the supernatant was used as sample.

Hot extraction was done by using Soxhlet apparatus and extracted with methanol for 5 h. The solvent was evaporated by keeping on water bath and the extract was reconstituted in 10 mL methanol.

2.4.2 Chromatography Conditions

HPTLC fingerprint was performed on Merck Silica gel $60F_{254}$ TLC precoated plates. Samples *N. nucifera* seeds cold (20–100% concentration) and hot extraction extracts of 2 μL and 5 μL were applied on the plate, a band of width 8 mm was applied on plate with the help of CAMAG Linomat- V sample applicator.

The mobile phase constituted of butanol: glacial acetic acid: water (4:1:1v/v/v). The plate was developed to a distance 70 mm in a CAMAG twin trough chamber (20×10 cm) previously equilibrated with mobile phase for 20 min.

After development, plate was observed in CAMAG TLC Visualizer and images were taken at 254 nm and white light. The plate was derivatized using anisaldehyde sulphuric acid reagent (ASR), the plate was kept at 110 °C for 3 min till colour bands appeared and were scanned at 366 nm and 540 nm using CAMAG Scanner IV.

3 Result and Discussion

3.1 Morphological and Microscopic Characters

3.1.1 Morphology of Fruit

Individual fruit falls under a category of nut type. Each fruit encloses only one seed (Fig. 1a). Size of fruit ranges from 0.5 to 2 cm. Fruit shape is oval to globular. Color of the fruit is blackish brown and fruit surface is smooth and shining possibly because of the presence of waxy coating. Fruit has anterior and posterior end. At the anterior end, there is a
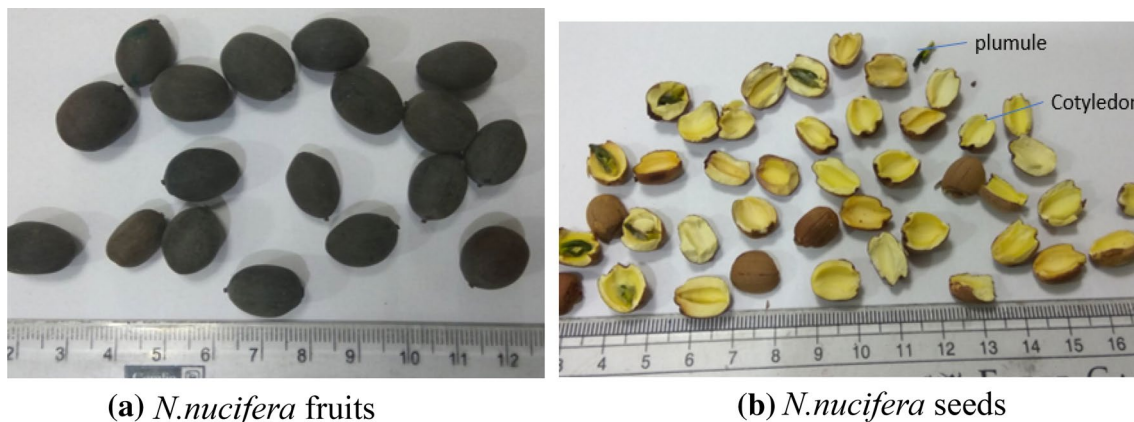

(a) *N.nucifera* fruits

(b) *N.nucifera* seeds

Fig. 1 Morphology **a** *N. nucifera* fruits, **b** *N. nucifera* seed

scar which represents fallen style or stigma. Posterior end has a small stipe.

3.1.2 Microscopic Characters of Fruit

3.1.2.1 Transvers Section (T.S) of Fruit Fruit Pericarp is very hard and unbreakable. On the surface of the fruit wall there are minute openings/pores are present. These pores are lined with hexagonal cells for gaseous exchange (Fig. 2d).

3.1.3 TS of the Fruit Wall have Following Layers

3.1.3.1 Epidermis Outermost layer is epidermis; (Fig. 2c) it is composed of compactly arranged cells. These cells are interrupted by pores. Epidermal cells are followed by two layered elongated palisade cells. Palisade cells are compactly arranged and made up of parenchyma cells.
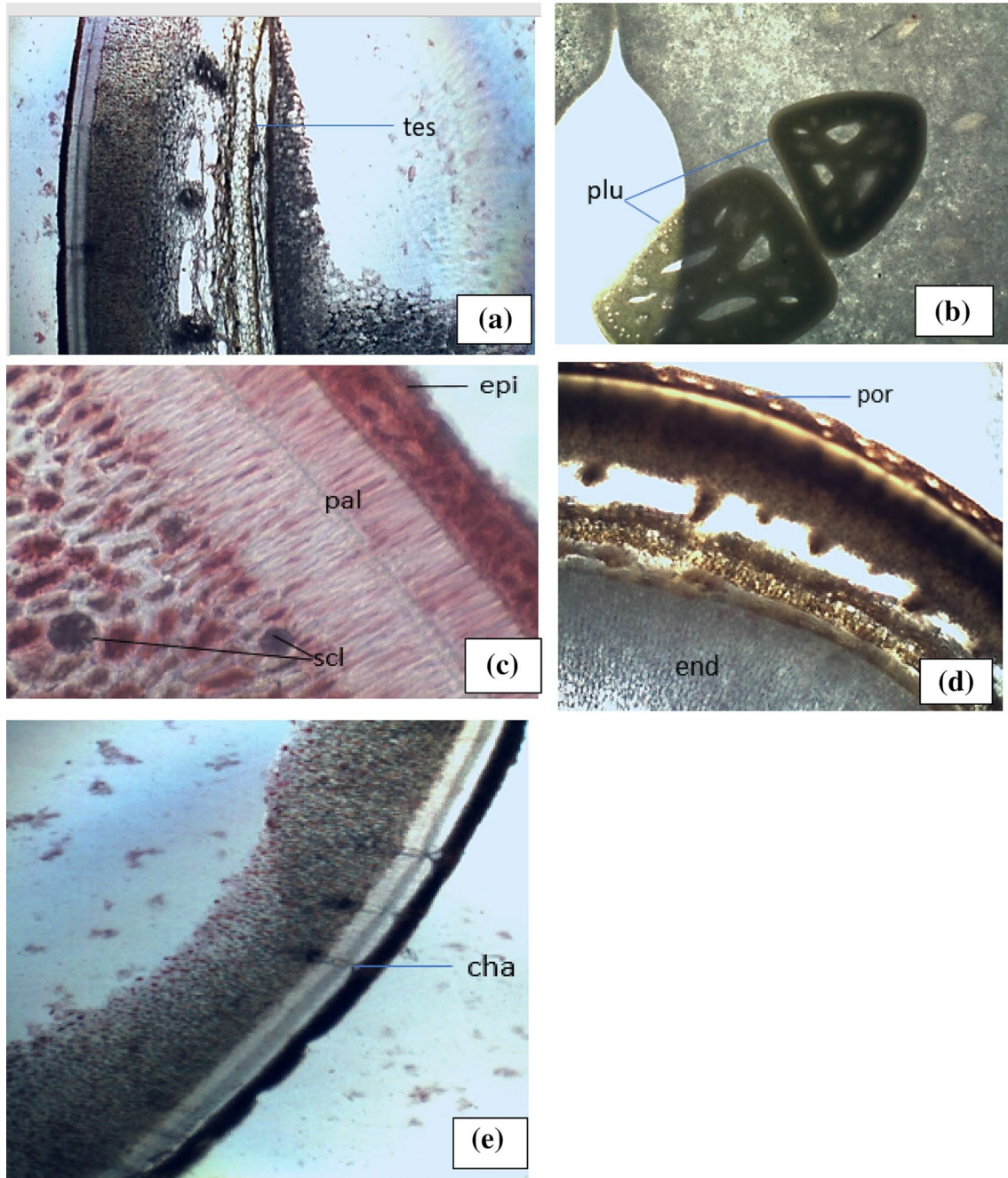

Fig. 2 TS of *N. nucifera* fruit. **a**, **b**, **d**, **e** under 4X and **c** under 40X

3.1.3.2 Multilayered Scleride In the two zones i.e., palisade and scleride zone exists a channel, which opens to the exterior through a pore and ends in a chamber. In scleride zone, rosette shaped calcium oxalate crystals are observed. This is one of the important taxonomic characteristics which will help in identification of *N. nucifera* fruit (Fig. 2c).

3.1.3.3 Testa It is thin, papery, brownish in colour and easily separable. Testa encloses endosperm enveloping embryo (Fig. 2a).

Inside the thick pericarp, two thin light brown papery seed coats i.e., the outer testa and inner tegmen are present. These enclose two cream coloured cotyledons having first two leaves and plumule. Radical is insignificant, and its axis surrounds the embryo. (Figs. 1b, 2b) Endosperm consists of cells containing starch grains.

3.1.3.4 Starch Grains The starch grains are simple, oval, and elliptical in shape. No striation was observed on the starch grains (Fig. 3).

The small oval shape of starch granules of seeds has also been reported by J. Man et al. [14]. Similar results were observed by Y. Zhang et al. [15], Zebin Guo et al. [16] Oval, elliptical shaped starch granules with smooth surface [17].

These microscopic characters are unique for *Nelumbo nucifera* and it can be used as a quality control tool. The present work is the first report on microscopic characterization of *N. nucifera* fruit (Table 1).

3.2 Phytochemical Analysis

The results of the phytochemical screening (Table 2) revealed the presence of flavonoids, tannins, phenols, terpenoids, alkaloids and saponin. However, glycoside was
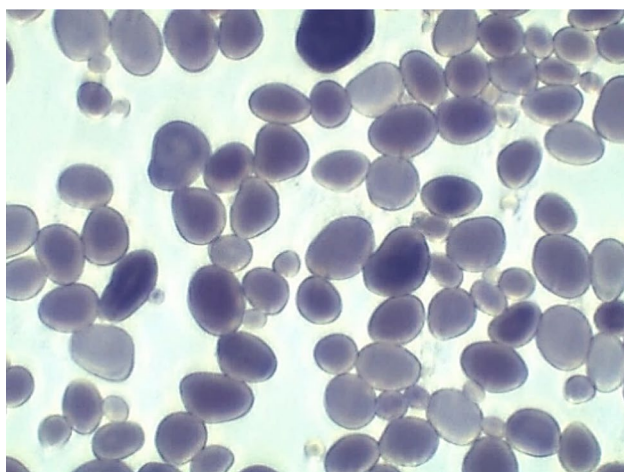

Fig. 3 Starch granules of *N. nucifera* seed stained with iodine (under 100X)

Table 1 Details of abbreviations

Label	Description
pal	Double layered palisade
scl	Rosette scleride
epi	Epidermis
cha	Channels
por	Epidermal pore
end	Endosperm
tes	Testa
pul	Plumule

absent in the methanol seed extract. Similar results were reported by Saratha et al. [18] except for tannins and saponin, as they were found to be absent in the ethanol extract, this may be due to the different solvent used for extraction, another research done by Yamini et al. [19] showed presence of all the phytochemicals in the ethanol extract.

3.3 HPTLC Analysis

For derivatisation of TLC plates, general reagent 'Anisaldehyde Sulphuric acid' was used. Anisaldehyde sulfuric acid is a universal reagent for herbal products, which makes color differentiation possible based on different classes of secondary metabolites present in the plant. The use of anisaldehyde sulfuric acid as a staining reagent is more informative, as it exposes monoterpenes, triterpenes, phenolic, saponin, essential oils and steroid with specific colours [20] as mentioned in Table 5. On a colourless background, different coloured zones appear, which are fluorescent under UV light (365 nm) [20].

Table 2 Phytochemical analysis of *Nelumbo nucifera* seed

Phytochemical	Method	Result
Terpenoids	Salkowski test	+
Phenols, Tannins	Ferric chloride test	+
	Lead acetate test	+
	Gelatin test	+
Flavonoid	Shinoda test	+
	Sulphuric acid test	+
	NaOH test	+
Steroids	Libermann Burchard test	+
Alkaloids	Mayers test	+
	Wagners test	+
	Dragendroffs test	+
Glycoside	Born Trager's test	−
	Legal's test	−
Saponin	Foam test	+

Key: "+" present, "−" absent

As presence of various phytoconstituents was detected in qualitative phytochemical analysis performed earlier (Table 2), the HPTLC profile (Fig. 4) of *Nelumbo nucifera* extract is in good agreement with the results of phytochemical tests.

The plate was scanned at 366 nm and 540 nm according to US pharmacopeia [2, 3] at 366 nm bands showed fluorescence and at 540 nm (white light) coloured bands were observed after derivatisation with ASR, shown in Fig. 4a, b. *N. nucifera* seed when subjected to soxhlet extraction gave nine bands on the TLC plate from R_F 0.05–0.94 (Table 3). Similarly, *N. nucifera* seed (cold extraction) showed nine bands from R_F 0.05–0.92. There was no difference observed in the number of bands and the R_F values between cold and hot extraction, which shows that phytochemical components were not affected by heat treatment, hence both the extraction methods can be used for fingerprinting analysis. The number of bands observed in the present study were more as compared to the study reported by [21] S. Rai et al. which showed six bands at RF 0.19, 0.36, 0.40, 0.48, 0.61 and

0.74, using chloroform: methanol (7:1 v/v) as solvent system and hexane: ethyl acetate solvent system (7:3 v/v), showed nine bands at R_F 0.10, 0.15, 0.27, 0.39, 0.42, 0.51, 0.61, 0.77 and 0.85 on the plate. Another study reported three bands on tlc plate with R_F 0.25–0.99, using mobile phase Toluene: Ethyl acetate: Formic acid [18]. Attarde et.al has worked on *N. nucifera* seed oil and it shows 10 spots on the tlc plate, using mobile phase Chloroform: Methanol (9:1 v/v) [22]. These differences would be due to the difference in the mobile phase used, as different solvent systems will give different pattern on TLC plate, depending on the eluent strength of mobile phase which is related to the polarity of the solvents. The elution power of the mobile phase depends on the nature of the compound, more nonpolar the compound the less time it will remain on the stationary phase and the more polar the compound it will stay more time on the stationary phase [23]. Many researchers have also worked on different parts of the plant such as leaves, flower and rhizome. Recent studies are reported on *N. nucifera* leaf, to detect Rutin an important phytochemical present in
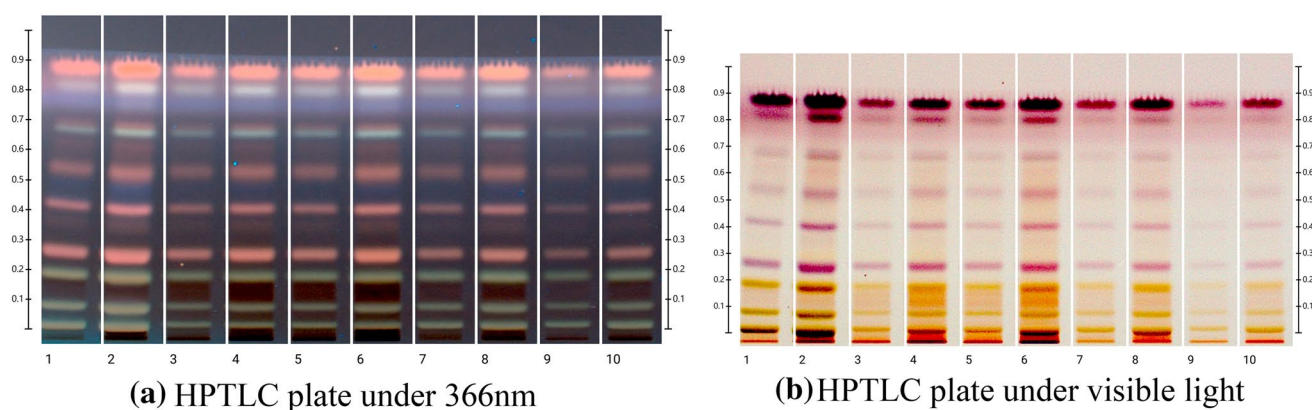

(a) HPTLC plate under 366nm

(b) HPTLC plate under visible light

Fig. 4 HPTLC fingerprint of *N. nucifera* seed after derivatisation with ASR. Track 1 & 2 Hot extract (2, 5 µL), Track 3—10 Cold extract, Track 3 & 4—100%, Track 5 & 6—80%, Track 7 & 8—50% and Track 9 & 10—20%

Table 3 Peak no, R_F value and % Peak area of *N. nucifera* seeds scanned at 366 nm and 540 nm derivatisation with ASR

Peak No	Track 2 (Soxhlet extract)				Track 4 (cold extract-100%)			
	540 nm		366 nm		540 nm		366 nm	
	Max R_F	Peak Area %	Max R_F	Peak Area %	Max R_F	Peak Area %	Max R_F	Peak Area %
1	0.027	15.80	0.053	7.40	0.043	15.07	0.056	5.38
2	0.111	10.50	0.118	7.60	0.117	4.40	0.121	5.67
3	0.220	14.86	0.230	6.47	0.217	11.94	0.229	4.46
4	0.303	14.02	0.300	17.64	0.305	13.21	0.303	15.37
5	0.467	9.86	0.467	13.72	0.464	7.55	0.462	9.13
6	0.588	5.89	0.585	7.31	0.585	5.28	0.583	5.74
7	0.735	3.95	0.735	7.05	0.727	4.62	0.725	8.99
8	0.880	7.57	0.881	14.36	0.869	13.07	0.867	19.02
9	0.941	17.57	0.942	18.45	0.927	24.88	0.926	26.24

the plant, using mobile phase Butanol: Glacial acetic acid: Water: Formic acid (7:1:1:0.25v/v/v/v) [24]. Another study was done on *N. nucifera* rhizome to develop an analytical method for Betulinic acid, using mobile phase n-hexane: ethyl acetate: acetic acid (7:3:0.3v/v/v) [25]. Darapaneni et al. worked on the leaves to estimate Quercetin by HPTLC using n-Butanol: Glacial Acetic Acid:Water:0.1% Formic Acid (7:1:1:0.25v/v/v/v) as mobile phase [26]. Sranujit et al. has worked on *N. nucifera* flower extract and found various phytochemicals using HPTLC method, different mobile phases and derivatizing reagent were used to confirm the presence of phenols, alkaloids and steroids [27].

Different concentrations of cold extract were tried to optimize on plate concentration, to check for the lowest concentration which will give good resolution and maximum number of bands. As seen in Fig. 4b and Table 4 it can be observed that the peak at R_F 0.212 is missing at 540 nm, as the band intensity has reduced at a lower concentration of 20%. Whereas at 366 nm (Table 4) all the bands are present at 20%, this could be due to the fluorescence capacity of the band at longer wavelength. Other concentrations from 100 to 50% (Table 3 and 4) are showing all the bands with good resolution at 540 and 366 nm. Hence, for the fingerprint analysis a minimum concentration of 50% (5 µL) can be applied on to the plate for maximum number of bands.

Pictorial representation of the bands on the TLC plate and chromatogram at different wavelengths are represented in Figs. 4, and 5, Tables 3, and 4.

These results show that both cold and hot extract can be used for the study of fingerprinting profile, to identify various phytochemical present in the seed, for checking the quality and authenticity of *N. nucifera* seeds (Table 5).

4 Conclusion

The present study showed the microscopic characteristics, phytochemical analysis, and HPTLC fingerprinting profile of *N. nucifera* seeds. Both the techniques are easy and fast to perform, and the findings of this study can be used as a tool for the establishment of quality standards, authentication and also in identification of the medicinally important phytochemicals present in the *N. nucifera* seed.

Table 4 Peak no, RF and % Peak area of *N. nucifera* seeds of different concentration (cold extract) scanned at 540 nm and 366 nm after derivatisation with ASR

| Peak No | Track 6 (80%) | | | | Track 8 (50%) | | | | Track 10 (20%) | | | |
| | 540 nm | | 366 nm | | 540 nm | | 366 nm | | 540 nm | | 366 nm | |
	Max R_F	Peak Area %	Max R_F	Peak Area %	Max R_F	Peak Area %	Max R_F	Peak Area %	Max R_F	Peak Area %	Max R_F	Peak Area %
1	0.034	16.51	0.051	5.19	0.038	15.93	0.049	6.23	0.050	12.26	0.050	8.01
2	0.111	5.04	0.115	5.38	0.115	4.32	0.114	5.79	0.124	5.66	0.120	5.91
3	0.213	11.26	0.222	4.86	0.212	7.86	0.22	5.27	0.310	8.96	0.228	6.39
4	0.303	10.29	0.300	12.03	0.303	9.52	0.298	10.47	0.460	5.18	0.306	8.65
5	0.459	7.07	0.455	9.33	0.455	6.14	0.452	8.14	0.577	4.54	0.456	5.91
6	0.574	5.58	0.571	6.74	0.572	4.69	0.566	5.68	0.727	5.21	0.567	3.73
7	0.721	5.26	0.717	10.89	0.721	5.19	0.715	9.40	0.868	18.17	0.721	6.94
8	0.863	12.48	0.858	19.45	0.863	13.20	0.858	18.44	0.925	40.02	0.862	19.00
9	0.921	26.51	0.920	26.12	0.921	33.17	0.919	30.5			0.920	35.47

(a) Track 2 soxhlet extract at 366nm
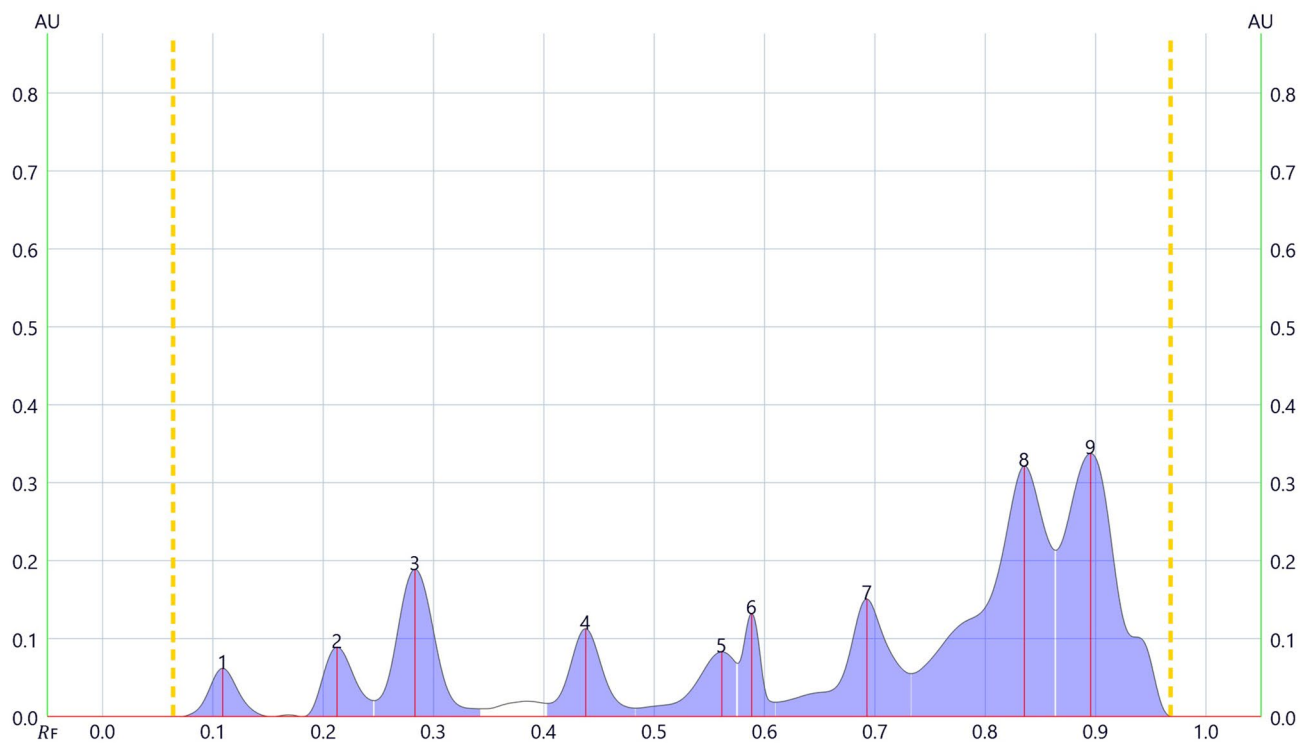

(b) Track 4 cold extract at 366 nm
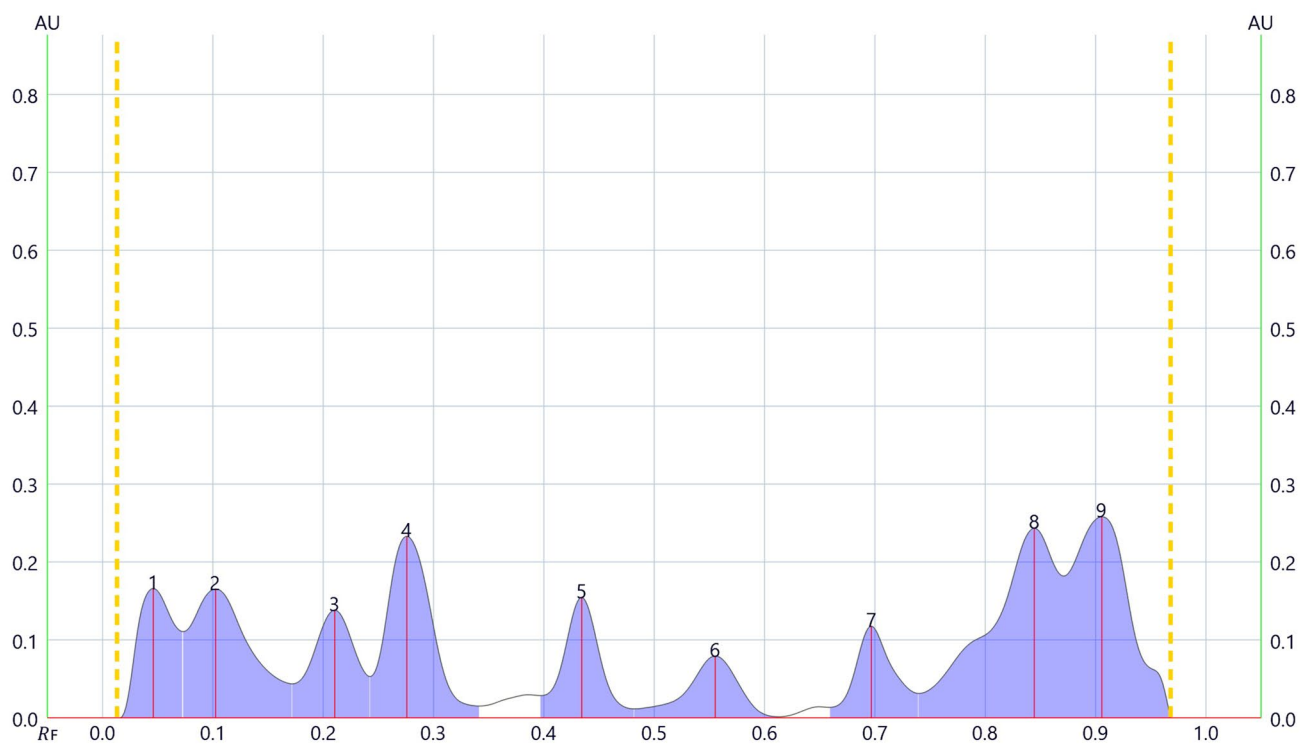
Fig. 5 Chromatogram of HPTLC fingerprint of *N. nucifera* seed after derivatisation with ASR

(c) Track 2 soxhlet extract at 540nm
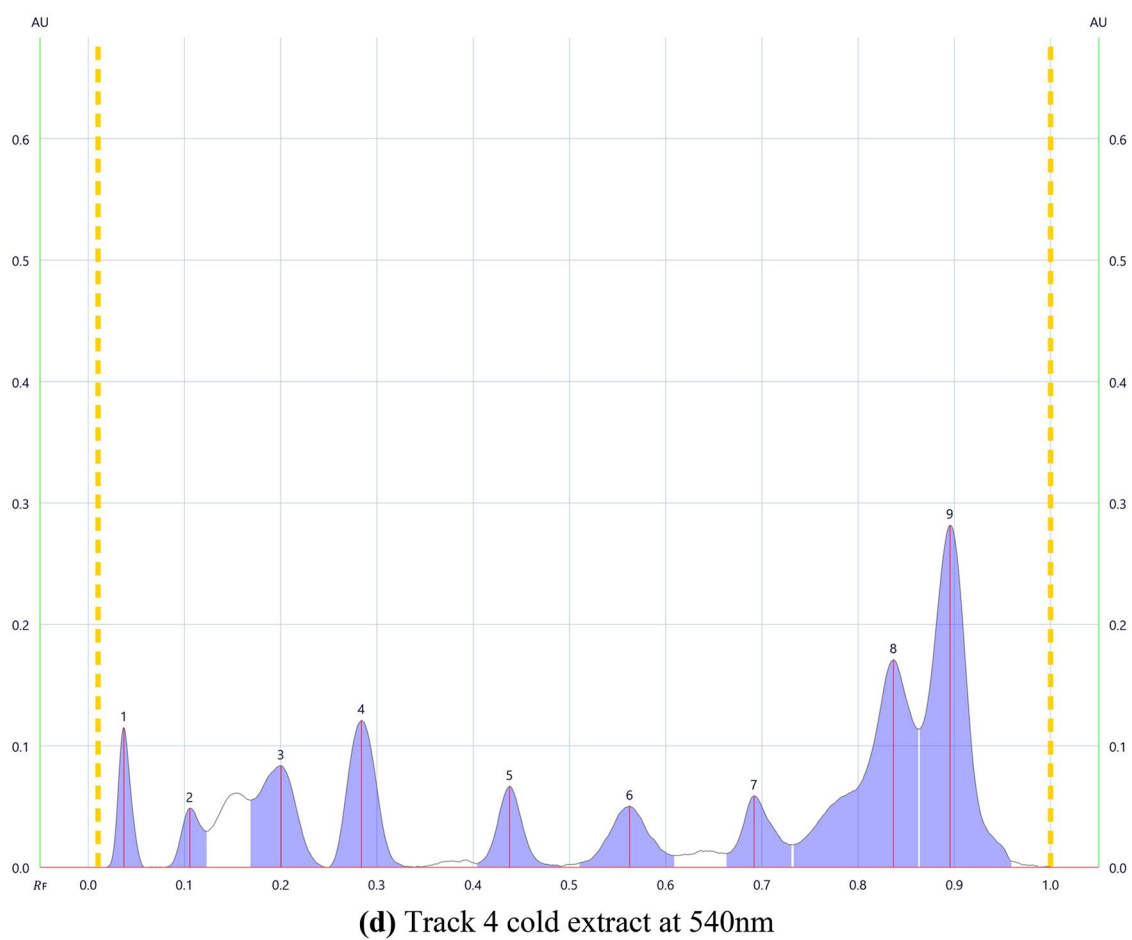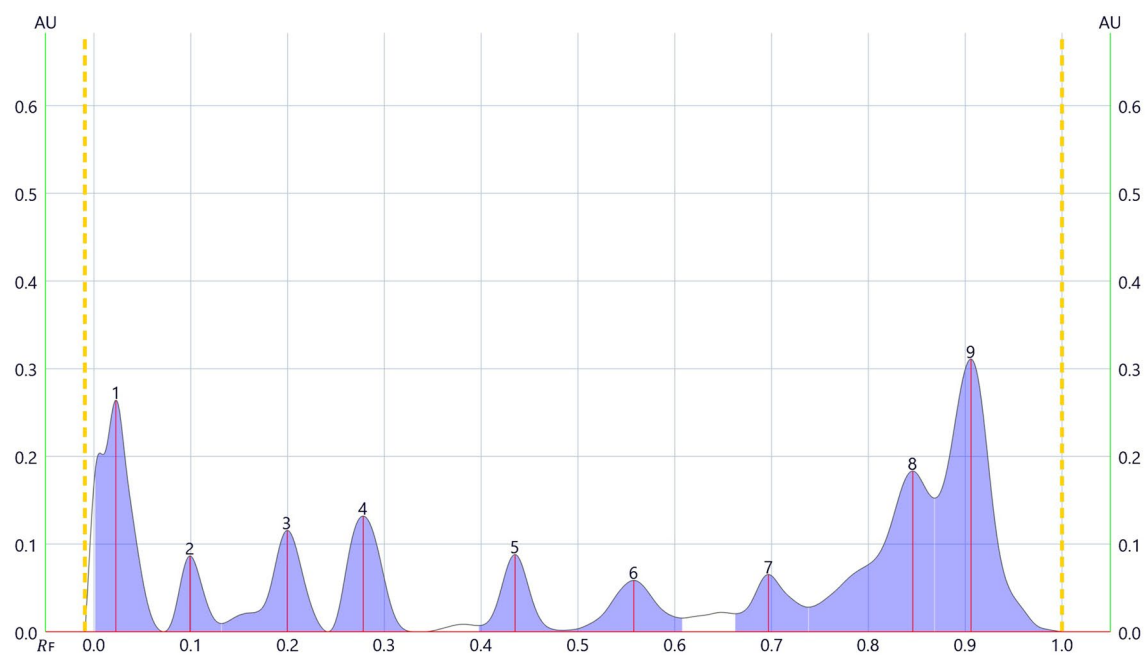

(d) Track 4 cold extract at 540nm

Fig. 5 (continued)

Table 5 Color band and the corresponding compounds seen on TLC plate after derivatisation with ASR at 540 nm (White light) [28, 29]

Track no	Color	RF	Name of the compound
1–10	Reddish brown	0.04	Steroid
	Yellow	0.11, 0.22	Saponin, phenols
	Pinkish violet	0.30, 0.46, 0.58, 0.72, 0.87, 0.93	Terpens, Glycoside

Declarations

Conflict of interest The authors have no conflicts of interest regarding this investigation.

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