

Pharmacognostic Screening of *Piper trichostachyon* Fruits and its Comparative Analysis with *Piper nigrum* Using Chromatographic Techniques

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ABSTRACT

Background: *Piper trichostachyon* is a wild, endemic *Piper* species from Western Ghats of India. The folklore healers of Belagavi region use this plant, similar to *Piper nigrum*. **Aims:** The present study investigates the comparison between *P. nigrum* and *P. trichostachyon* using pharmacognostic parameters. **Materials and Methods:** Pharmacognostic evaluation was carried out in terms of morphological, microscopic characters, and phytochemical analysis using standard methods. Comparative physicochemical analysis between *P. trichostachyon* and *P. nigrum* was also carried out through estimation of micro-macro nutrients, high-performance thin layer chromatography (HPTLC) investigation and using piperine as a marker compound for reversed phase-ultra flow liquid chromatographic (RP-UFLC) technique. **Results:** *P. trichostachyon* grows in the forests, and the fruits are morphologically similar to *P. nigrum* fruits, so the name in Kannada "Kaadu Kalu menasu" (wild/forest black pepper). The microscopy revealed the presence of stone cells, starch grains, oil cells and globules, beaker cells, and yellowish brown pigment layer, parenchymatous cells. The presence of alkaloids, oil, and tannins were observed in *P. trichostachyon* fruits. The HPTLC studies visibly indicated differences among two species with 12 peaks and varied banding pattern. RP-UFLC results showed less amount of piperine in *P. trichostachyon* (0.05 ± 0.002 mg/g) than in *P. nigrum* (16.14 ± 0.807 mg/g). **Conclusion:** The study reports on pharmacognostic parameters of *P. trichostachyon* for the 1st time and will be useful for the identification and authentication. The comparative HPTLC and RP-UFLC studies resolve the differentiation impasse among two species. However, further biological efficacy studies are required to establish its use in traditional medicine.

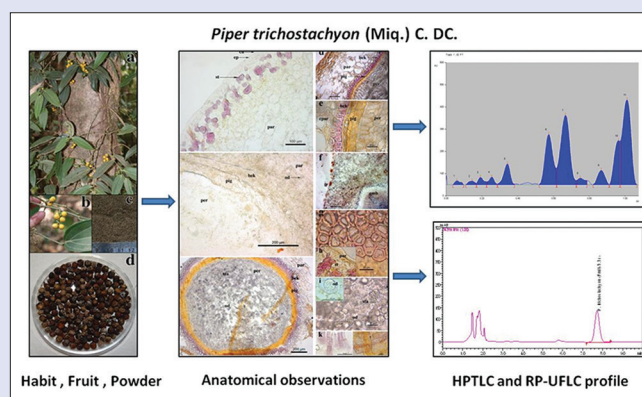
Key words: High-performance thin layer chromatography, pharmacognosy, physicochemistry, *Piper nigrum*, *Piper trichostachyon*, reversed phase-ultra flow liquid chromatography

SUMMARY

- *Piper trichostachyon* grows in the forests, and the fruits are morphologically similar to *Piper nigrum* fruits
- The microscopy of *P. trichostachyon* revealed the presence of stone cells, starch grains, oil cells and globules, beaker cells and yellowish brown pigment

layer, parenchymatous cells

- The high-performance thin layer chromatography studies visibly indicated differences among two species with varied banding pattern
- Reversed phase-ultra flow liquid chromatographic results showed less amount of piperine in *P. trichostachyon* than in *P. nigrum*.



Abbreviation used: HPTLC: High Performance Thin Layer Chromatography, RP-UFLC: Reversed phase-ultra flow liquid chromatographic analysis, DST: Length of line, Maj: Length of large half axis for ellipse RDS - radius for circle, Rf: Retention Factor, TS: Transverse Section, TLC: Thin Layer Chromatography.

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INTRODUCTION

Piper nigrum L., (family-Piperaceae) known as "King of spices" (vernacular: Kannada - Kari menasu/Kalu menasu, Marathi-Miri, English - black pepper) is one of the highly used medicinal plant by the healers of Belagavi region.^[1] The literature also suggests *P. nigrum* (black pepper) to be a popular spice and a potent medicinal plant used in Ayurveda.^[2] However, folklore healers of Belagavi region use a wild species, *Piper trichostachyon* (Miq.) C. DC., similar to *P. nigrum*.^[1] Genus *Piper* is represented by about 13 species from the Western Ghats and 05 (02 wild; 03 cultivated) species are reported from Belagavi district.^[3,4] *P. trichostachyon* is endemic to Western Ghats of India. It is locally called as "Kadukalu menasu," (Kannada) or "Miri mirch" (Marathi) and in English it is known as "Pouched pepper."^[1,5]

Medicinal plants used in folklore practices are one of the major sources for drug development by the scientific community and industries. The heterogeneity of a less known medicinal plant may be treated as

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substitute or adulterant against a popular well-accepted species. The standardization and comparative analysis of medicinal plants used as herbal drugs is necessary and may also help to differentiate between two. Available literature revealed lack of pharmacognostic studies on the fruits of *P. trichostachyon*. Hence, the present investigation was undertaken with the objective to evaluate various pharmacognostic characters of *P. trichostachyon* fruits. Comparative phytochemical analysis between *P. trichostachyon* and *P. nigrum* was also carried out through the estimation of micro-macro nutrients, high-performance thin layer chromatography (HPTLC) investigation and using piperine as a marker compound for reversed phase-ultra flow liquid chromatographic (RP-UFLC) technique.

MATERIALS AND METHODS

Collection and authentication of plant material

Fruits of *P. trichostachyon* and *P. nigrum* were collected from Belagavi (N 15.63834°, E 074.27841°) and Uttara Kannada (N 14.4721°, E 074.5131°) regions of the Western Ghats in Karnataka state, India, respectively. The plant specimens were scientifically identified and herbaria prepared were deposited at the Regional Medical Research Centre (RMRC), (Indian Council of Medical Research), Belagavi, Karnataka, India, for future reference (voucher numbers - *P. trichostachyon*: RMRC 1072 and *P. nigrum*: RMRC 1205).

Macroscopic and microscopic analysis

Key morphological features of the fruits of *P. trichostachyon* were observed during macroscopic analysis using dissecting microscope (Labomed, India). Sections of the fruits were taken using Leica Germany (Model no - CM 1850) cryostat following the procedure described by Upadhy, *et al.*^[6] The sections were of the thickness of $20 \pm 2 \mu$. Histochemical and powder studies were carried out by using reagents and stains like iodine, concentrated sulphuric acid, concentrated hydrochloric acid (HCL), ferric chloride, Sudan III, ruthenium red, and phloroglucinol with concentrated HCL (1:1).^[7] Similarly, organoleptic characters such as color, odor, and taste were determined for the fruit powder.^[7]

Microphotography

Olympus America Inc., (Model no. BX 41) microscope with inbuilt analog camera (ProgRes C3-Jenoptik) was used for microphotography of the sections and powder. Computer images at $\times 4$, $\times 10$, and $\times 40$ magnifications were captured using inbuilt software (ProgRes® CapturePro 2.1.1-Jenoptik laser optical system). Cell dimensions were represented as DST (length of line), Maj (length of large half axis for ellipse) and RDS (radius for circle) in microscopy as defined in ProgRes® CapturePro 2.1.1-Jenoptik, software.^[6]

Preparation of extracts and preliminary phytochemical analysis

The dried fruit powder was serially extracted by the continuous shaking method given by Ankad *et al.*, using different solvents.^[8] The extracts were stored at 4°C until further use. These extracts were subjected for preliminary phytochemical screening as per standard pharmacognostic methods.^[7]

Fluorescence analysis

Fruit powder (0.5 g) was mixed with 1.5 mL of respective reagent. The mixture was observed under visible light, short ultra-violet (UV) light (254 nm) and long UV light (365 nm) after a minute. Different reagents were used for the analysis of fluorescence activity.^[8,9]

Comparative analysis between *Piper trichostachyon* and *Piper nigrum*

Physicochemical and nutritive content analysis

Powder physicochemical parameters viz., acid-insoluble ash, soluble extractive values, total ash, and water-soluble ash were determined as per standard methods.^[7] The moisture content was estimated by loss on drying method.^[7] Microelements (copper, iron, manganese, sodium, and zinc) and macroelements (calcium, magnesium, phosphorus, potassium, and sulfur) were estimated using atomic absorption techniques.^[10,11] Nitrogen content was determined by Kjeldahl method.^[10,11] Nutritive contents such as percent starch, total carbohydrates, reducing and nonreducing sugars were also estimated.^[10,11] Protein content was calculated using the formula: % proteins = % nitrogen \times 6.125.

High performance thin layer chromatography analysis

A CAMAG made HPTLC system was used during the study. Separation was achieved on a pre-coated HPTLC silica gel G60 F254 plates (MERCK, Germany). Bands of 6 mm length were applied using CAMAG Automated TLC Sampler (ATS-4) equipped with 25- μ L syringe. Application settings given by Upadhy *et al.*^[12] were followed. The plates were developed using 10 mL mobile phase toluene:ethyl acetate (7:3) to a distance of 80 mm in a CAMAG twin trough glass chamber (10 cm \times 10 cm) saturated with the mobile phase at room temperature.^[2] The developed plate was visualized and scanned at 254 nm using CAMAG TLC visualizer.^[12]

Reversed phase-ultra flow liquid chromatographic analysis

Shimadzu chromatographic system (model number LC-20AD) was used to estimate the piperine content in *Piper* species using RP-UFLC technique as described by Upadhy *et al.*^[13] Chromatographic separation of piperine was achieved on RP-18e (LiChrospher 100, 5 μ m, 4.6 \times 250 mm) with a mobile phase consisting of methanol:water (70:30). A chromatographic condition of 1.4 mL/min flow rate at 343 nm with 10 min retention time was set for piperine. The built-in LC-Solution software system was used for data processing.

One mg/mL stock solution of piperine in methanol was prepared. The stock solution was serially diluted with methanol to obtain working concentrations of 0.01, 1, 3, 10, 50, 100, 200, 400, and 1000 μ g/L to obtain calibration curves. All the solutions were stored at 4°C until further use. The system suitability test, limit of detection (LOD), and limit of quantification (LOQ) were assessed followed by Upadhy *et al.*^[13]

Chemicals

Solvents viz., methanol, water, toluene, ethyl acetate used in the analysis of HPTLC and RP-UFLC were of HPLC grade. HPLC grade piperine (97% pure) was procured from Sigma for RP-UFLC analysis. All other chemicals, reagents, and test solutions used in various physicochemical investigations were of analytical grade.

RESULTS

Morphological characters

P. trichostachyon is a large woody climber [Figure 1a] found in moist deciduous forests to evergreen forests of Western Ghats. The leaves are elliptic to lanceolate, apex acute to acuminate and 3 nerved [Figure 1a]. Fruits are globose, 5–9 mm in diameter, yellow when ripe [Figure 1b].



Figure 1: *Piper trichostachyon* - (a) habit; (b) fresh fruits; (c) dried fruits; (d) fruit powder

Medicinal uses and parts used

Fruits of *P. trichostachyon* were reported to be used in the treatment of cough, cold, fever, migraine, sperm count increase, toothache by the folklore practitioners of Belagavi district.^[1]

Macro and microscopic analysis

Organoleptic characters

Fruits turned to grayish brown to black after drying with wrinkled surface [Figure 1c]. Powder showed Khaki gray nature with less pungent, peppery bitter taste compared to *P. nigrum*. The powder was coarse to touch [Figure 1d].

Anatomical description and powder microscopy

Fruit transverse section (TS) was circular in its outline and can be differentiated into pericarp, testa, and inner perisperm with embryo [Figure 2]. Pericarp includes outer epicarp, mesocarp and endocarp. The epicarp in TS showed a single layered epidermis covered by cuticle [Figure 2a and d]. The cells of the epicarp were oval or polygonal in nature followed by 2–4 layers of sub-epidermal layer made of thin walled parenchymatous cells with scattered, single or groups of stone cells [Figure 2a and d]. Stone cells showed heavily thickened elongated walls (thickness DST: 2.863–7.278 μm), with rectangle, round, and/or oval outline.

Mesocarp was largely composed of parenchymatous region [Figure 2b and c] consisting 6–10 layers with thin walled oval and/or polygonal cells [Figure 2b, c and e]. The cells showed accumulation of spheroidal starch grains within. Few parenchyma cells also contain prismatic calcium oxalate crystals. Isolated, elongated, irregular shaped, oleoresin, or oil cells were scattered in the mesocarp region. The inner region of the mesocarp showed compressed parenchymatous layers with scattered, small, 4–5 groups of circularly arranged, fibro-vascular bundles. Innermost layers of mesocarp were made of thick-walled parenchyma cells.

Endocarp [Figure 2c] was made up of a row of beaker cells (size WD 11.73–32.87 μm^2) with greatly lignified cell walls on three sides (thickness DST 5.203–20.49 μm) than the outer layer, thus, appear as horseshoe. The beaker cells were followed by a layer of seed coat, formed by testa consisted of 2–3 layers of compressed, elongated cells, followed by a pigment layer, containing a dark – brown tannin substance (thickness DST 15.56–27.12 μm). Endosperm also contains small embryo at the

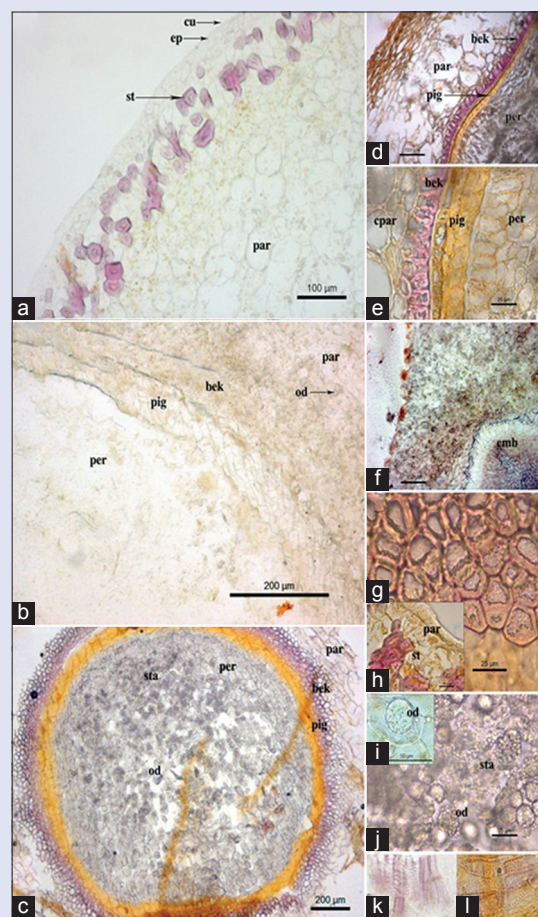


Figure 2: *Piper trichostachyon* - (a) transverse section of fruit showing epicarp region; (b) transverse section of fruit showing mesocarp region; (c) transverse section of fruit with seed region; (d) transverse section of the fruit; (e) transverse section of endocarp region; (f) transverse section showing embryo; (g and h) Stone cells; (i) oil globule; (j) Starch grains and oil globules; (k and l) Xylem vessels; bek: Beaker cells; cpar: Compressed parenchyma cells; cu: Cuticle; emb: Embryo; ep: Epidermis; od: Oil drops; par: Parenchyma cells; per: Perisperm; pig: Pigmented layer; st: Stone cells; sta: Starch grains

central portion [Figure 2f]. Pericarp forms the larger part of the seed made up of thin-walled, rectangular and/or polygonal cells. Some of the cells contain aleurone grains and also calcium oxalate crystals. Inner portions of the pericarp consist oval, round, and/or polygonal shaped cells containing large oil globules [Figure 2b and c].

Powder microscopy showed the presence of varied size and shaped parenchymatous cells [Figure 2h]; stone cells [Figure 2d and g]; starch grains [Figure 2j]; and oil cells [Figure 2i and j]. The presence of horseshoe shaped beaker cells [Figure 2e] and brown polygonal cells of pigment layers were [Figure 2c and e] also observed. Fragments of spiral and pitted xylem vessels [Figure 2k and l]; cells containing oil drops [Figure 2i]; starch grains were seen in powder microscopy.

Histochemical and preliminary phytochemical analysis

Fruit sections of *P. trichostachyon* were treated with different reagents to know various cell components. The tests showed the presence of starch, oil components, lignin, and tannin clearly. The calcium oxalate crystals soluble in concentrated HCL were also occasionally observed.

Table 1: Preliminary phytochemical analysis of *Piper trichostachyon*

Tests	Aqueous	Methanol	Ethyl acetate	Chloroform	Benzene	Pet ether
Carbohydrates	Present	Present	Absent	Absent	Absent	Absent
Proteins	Present	Absent	Absent	Absent	Absent	Absent
Amino acids	Present	Absent	Absent	Absent	Absent	Absent
Steroids	Absent	Absent	Absent	Absent	Absent	Absent
Triterpenoids	Absent	Present	Absent	Absent	Absent	Absent
Glycosides	Absent	Present	Absent	Absent	Absent	Absent
Flavonides	Present	Present	Present	Absent	Absent	Present
Alkaloids	Present	Present	Present	Absent	Absent	Absent
Tannins and phenolic compounds	Present	Present	Present	Present	Present	Absent
Fat and oil	Present	Present	Absent	Absent	Absent	Absent

Preliminary phytochemical investigations against various extracts viz., aqueous, methanol, ethyl acetate, chloroform, benzene, and petroleum ether were carried out, and the results were presented in Table 1. Preliminary phytochemical analysis showed the presence of alkaloids, flavonoids, oil, tannins, and phenolic compounds. Maximum positive results for phytochemical tests were observed in aqueous and alcoholic extracts.

Fluorescence analysis

Fluorescence analysis of fruit powder was carried out after treating with several chemicals viz., water, methanol, acids, and sodium hydroxide. The observations were presented in Table 2.

Comparative physicochemical analysis

The quantitative parameters such as ash values, extractive values, estimation of micro- and macro-elements, and nutritive contents were studied for *P. trichostachyon* and *P. nigrum*. The results were represented in Table 3.

Ash values, moisture content, and extractive values

The estimated ash values, moisture content, and extractive values were showed in Table 3 given as percent values (w/w). The estimated total ash values for both species were almost equal. The water-soluble ash and acid-insoluble ash values were higher in *P. nigrum* ($2.36 \pm 0.12\%$ and $0.47 \pm 0.02\%$) than in *P. trichostachyon* ($1.23 \pm 0.06\%$ and $0.44 \pm 0.02\%$). The moisture contents in the fruits of both the species were within the range of standard deviation [Table 3]. The water-soluble extractive was higher in *P. nigrum* ($16.7 \pm 0.84\%$) whereas ethanol soluble extractive was higher in *P. trichostachyon* ($12.87 \pm 0.66\%$).

Estimation of micro, macro elements and nutritive contents

Estimation of macro elements (viz., nitrogen, potassium Phosphorus sulfur, calcium, magnesium, sodium), microelements (zinc, iron, copper, manganese) and nutritive contents were presented in Table 3. Nitrogen, Phosphorus, sulfur, calcium, magnesium, and sodium contents were higher in *P. trichostachyon* than in *P. nigrum*. Fruits of *P. nigrum* estimated to contain 2-fold more amount of iron content (250.20 ± 12.51 ppm) than that in *P. trichostachyon* (108.40 ± 5.42 ppm). Amount of copper was almost equal in both the fruits. The percent nutritive content was higher in the fruits of *P. trichostachyon* in terms of total carbohydrates and protein ($31.32 \pm 1.57\%$ and $16.16 \pm 0.81\%$) than that in *P. nigrum* ($29.13 \pm 1.46\%$ and $10.72 \pm 0.54\%$).

Comparative high-performance thin layer chromatography analysis

Methanol extracts were used for the HPTLC analysis. A better separation with toluene:ethyl acetate (7:1 v/v) was observed for band separation during the plate development. *P. nigrum* methanol

Table 2: Fluorescence analysis of *Piper trichostachyon*

Treatment	Visible light	UV light (254 nm)	UV light (365 nm)
Powder	Khaki gray	Gray	Smoky gray
Powder + water	Light brown	Dark green	Green
Powder + methanol	Vanilla green	Green	Florescent cream
Powder + concentrated H ₂ SO ₄	Brown	Door country green	Green
Powder + concentrated HCL	Dark brown	Door country green	Green
Powder + 1N NaOH	Brown	Door country green	Florescent cream
Powder + acetic acid	Light brown	Dark green	Green
Powder + nitric acid	Brown	Green	Green
Powder + formic acid	Brown	Green	Green

UV: Ultraviolet

Table 3: Estimation of ash values, moisture content, extractive values, micro, macro and nutritive content

Estimation of	Fruit	
	<i>Piper trichostachyon</i>	<i>Piper nigrum</i>
Ash values % (w/w)		
Total ash	5.21±0.26	4.92±0.25
Water-soluble ash	1.23±0.06	2.36±0.12
Acid-insoluble ash	0.44±0.02	0.47±0.02
Moisture % (w/w)	4.31±0.21	4.55±0.23
Extractive values % (w/w)		
Water-soluble extractive	9.71±0.49	16.7±0.84
Ethanol soluble extractive	12.87±0.66	11.97±0.56
Macro elements (%)		
Nitrogen	2.64±0.13	1.72±0.09
Potassium	0.17±0.01	0.20±0.01
Phosphorus	1.56±0.09	1.44±0.07
Sulphur	0.34±0.02	0.27±0.01
Calcium	0.86±0.04	0.52±0.03
Magnesium	0.68±0.03	0.46±0.02
Sodium	0.12±0.01	0.11±0.01
Micro elements (ppm)		
Zinc	19.00±0.95	11.60±0.58
Iron	108.40±5.42	250.20±12.51
Manganese	75.40±3.77	51.80±2.59
Copper	53.00±2.65	51.60±2.58
Nutritive contents (%)		
Carbohydrates	31.32±1.57	29.13±1.46
Reducing sugars	5.61±0.28	8.34±0.42
Nonreducing sugars	25.71±1.29	20.79±1.04
Starch	10.27±0.51	10.7±0.56
Protein	16.16±0.81	10.72±0.57

extracts showed thick bands which were not differentiable from one another. So, the extracts were diluted 3 times with methanol to have

the separated bands. The peaks were identified after the development at 254 nm without derivatization. The HPTLC densitogram and profile image of *P. trichostachyon* and *P. nigrum* were represented in Figure 3a-d.

An autogenerated Table 4 shows the number of peaks, retention factor (Rf) values, area, and % area [Table 4]. Both the plant species showed 12 peaks. Three peaks at Rf 0.06, 0.75, and 0.87 were similar in *P. trichostachyon* and *P. nigrum*. The peaks at Rf 0.75 and 0.87 showed 2 times greater the percent area in *P. nigrum* compared to the peaks of *P. trichostachyon*. Other peaks showed different Rf values and percent height and area indicating the varied chemical nature among two species.

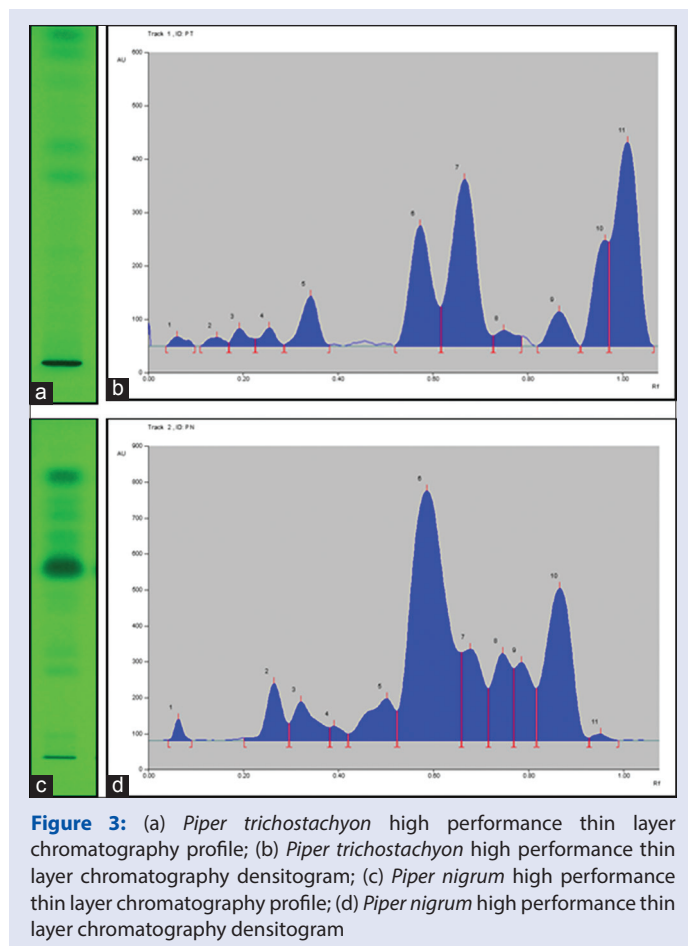


Figure 3: (a) *Piper trichostachyon* high performance thin layer chromatography profile; (b) *Piper trichostachyon* high performance thin layer chromatography densitogram; (c) *Piper nigrum* high performance thin layer chromatography profile; (d) *Piper nigrum* high performance thin layer chromatography densitogram

Comparative quantification of piperine using reversed phase-ultra flow liquid chromatography

Methanol fruit extracts were used for quantitative determination of piperine, and results were expressed as mg/g on dry weight basis. Calibration curve was constructed from nine different concentrations of standard piperine against its area under the curve with a coefficient of determination (R^2) 0.989 [Figure 4a]. The regression equation ($y = 65,960.285x + 1,648,534.102$) showed significant relationship between peak areas and concentrations [Figure 4a]. This was used to estimate piperine content from sample extracts of both the species. LOD and LOQ of piperine were determined as 0.13 and 0.40 $\mu\text{g/mL}$, respectively. The lowest calibrator used during the study was 0.01 $\mu\text{g/mL}$. The relative standard deviation values were <2% indicating the method to be precise and reproducible. Validation of the method was carried out by spiking 50 μL (100 μg) of standard piperine to an equal volume of *P. trichostachyon* fruit extracts to obtain recovery within the range of 94–100%.

Profiles with a retention time of 7.774 ± 0.09 min for standards and samples were obtained [Figure 4b-d]. Clear, sharp standard peaks ensured purity (98%) and also reduced compatibility issues between extraction solvent and mobile phase in the analysis. The autoscaled chromatograms were generated for standard piperine [Figure 4b] and fruit extracts of *P. trichostachyon* [Figure 4c] and *P. nigrum* [Figure 4d]. The higher content of piperine in *P. nigrum* fruit extract produced broad and flat-topped peak, which was resolved by further diluting the extract to 1.0% [Figure 4d]. Fruits of both the species showed the content of piperine. However, piperine content in fruits of *P. nigrum* (16.14 ± 0.81 mg/g) was 99 times higher than that in *P. trichostachyon* fruits (0.05 ± 0.01 mg/g).

DISCUSSION

According to Hegde *et al.*, every plant shows unique nature in terms of its botany, chemistry, and therapeutic potency. Thus, it is essential to study pharmacognostic characters of a medicinal plant, not only for quality control standardization but also to understand its structure and biology.^[14] *P. trichostachyon* is wild, endemic species, seen in semievergreen forests; whereas *P. nigrum* is widely cultivated in Southern states of India.^[3,4] However, wealth of India recorded grown *P. trichostachyon* along with the cultivated *P. nigrum* in Southern parts of Western Ghats during the early 20th century.^[5] *P. trichostachyon* grows in the forests, and the fruits are morphologically very similar to the *P. nigrum*, so the name in Kannada “Kaadu Kalu menasu” (wild/forest black pepper).

The morphological and biosystematic studies suggested that *P. trichostachyon* is one of the putative parents of *P. nigrum*^[15] with 75–90% similarity.^[16,17] This may be the reason that dried fruits of both

Table 4: High performance thin layer chromatography peak table of *Piper trichostachyon* and *Piper nigrum*

Peak	Retention factor	<i>Piper trichostachyon</i>				<i>Piper nigrum</i>				
		Height AUC	Height (%)	Area AUC	Area (%)	Retention factor	Height AUC	Height (%)	Area AUC	Area (%)
1	0.06	18.6	1.33	327.1	0.74	0.06	64.5	2.36	814.2	0.79
2	0.09	11.1	0.79	98.4	0.22	0.26	174.8	6.39	4192.8	4.07
3	0.14	16.4	1.18	397.3	0.89	0.32	128.4	4.70	4474.7	4.35
4	0.19	25.2	1.80	482.1	1.09	0.39	47.1	1.72	925.0	0.90
5	0.26	32.3	2.31	722.1	1.63	0.47	100.9	3.69	2456.2	2.39
6	0.34	86.6	6.19	2248.9	5.07	0.50	114.7	4.19	3001.2	2.91
7	0.58	250.0	17.89	8212.9	18.50	0.59	735.8	26.91	41,404.3	40.21
8	0.67	301.4	21.57	11,591.4	26.11	0.68	294.2	10.76	9169.0	8.91
9	0.75	35.5	2.54	1322.8	2.98	0.75	289.5	10.59	8856.8	8.60
10	0.87	85.9	6.14	2532.2	5.70	0.79	254.6	9.31	6779.2	6.58
11	0.96	179.8	12.86	4118.1	9.28	0.87	507.8	18.57	20,423.5	19.84
12	1.01	354.9	25.40	12,343.2	27.80	0.95	21.6	0.79	467.6	0.45

AUC: Area under curve

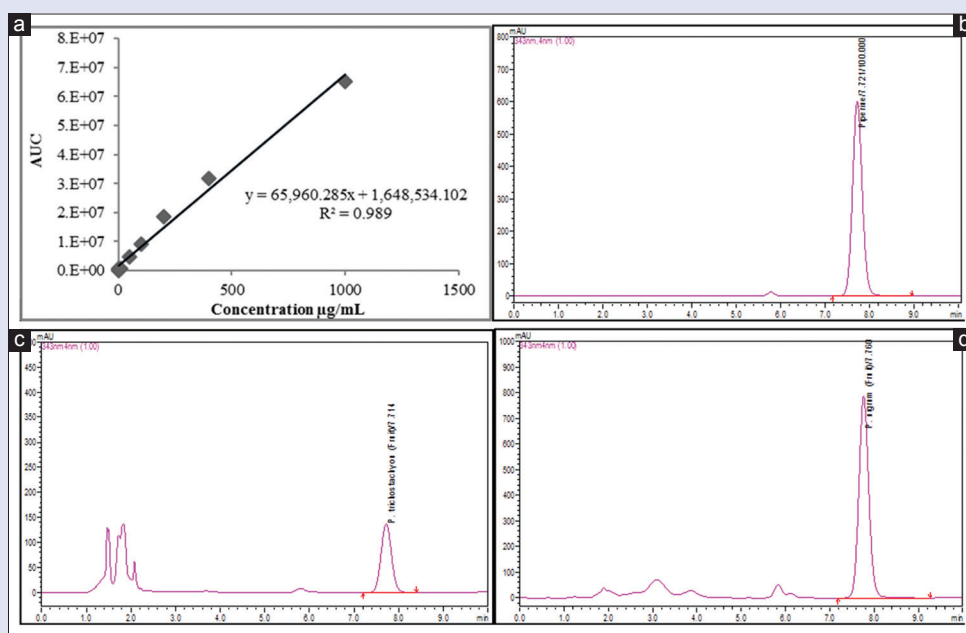


Figure 4: (a) Standard piperine calibration curve; (b) Piperine reversed phase-ultra flow liquid chromatography chromatogram; (c) *Piper trichostachyon* reversed phase-ultra flow liquid chromatography chromatogram; (d) *Piper nigrum* reversed phase-ultra flow liquid chromatography chromatogram

species looks similar and difficult to differentiate. However, the taste of *P. trichostachyon* fruits was not as pungent and spicy as that of *P. nigrum*. The fruits were bitter in nature than the fruits of *P. nigrum*.

The TS and powder microscopy revealed that *P. trichostachyon* had many similarities such as the presence of stone cells, starch grains, oil cells and globules, beaker cells, and yellowish brown pigment layer and parenchymatous cells compared to TS of *P. nigrum* fruits.^[2] Preliminary phytochemical investigation showed the presence of alkaloids. Fluorescence study showed various colors indicating the presence of various inorganic and organic phytoconstituents.

Total ash, acid-insoluble ash, and water-soluble ash estimations indicate the presence of inorganic and silica components in the sample studied.^[14] The comparative analysis of ash contents and extractive values in *P. trichostachyon* and *P. nigrum* lies within the range, similar to that of *P. nigrum*, as reported in Ayurvedic pharmacopeia.^[2] Extractive values using water and ethanol indirectly indicated the presence of secondary metabolites in the powder sample.^[14] Comparable amounts of nutritive contents were observed in both the species, whereas iron content was higher in *P. nigrum* than that of *P. trichostachyon*. *P. trichostachyon* was rich in total macronutrients compared to *P. nigrum* whereas it was *vice versa* for total micronutrient.

The analytical separation techniques such as HPTLC and HPLC were among most popular methods of choice used for quality control of raw material and finished herbal products.^[18] These techniques were used because of their simplicity and reliability.^[18] Results of HPTLC analysis from the fruit extracts of both species revealed variations in their densitograms. The HPTLC studies visibly indicate the differences among two species. Dried fruits of the species can be differentiated using this technique.

Results showed the presence of piperine in both *P. trichostachyon* and *P. nigrum* fruits. Present study results correlate with earlier reports by Upadhyaya et al.,^[13] Parmar et al.,^[19] and Jayashree et al.,^[20] for the amount of piperine in *P. nigrum*. Piperine is the major chemical constituent responsible for the aromatic odor and spicy nature of *P. nigrum*.

Thus, less amount of piperine in *P. trichostachyon* was indicative for its nonspiciness. However, earlier reports suggest *P. trichostachyon* to contain cyclopiperstachine, cyclostachine, I-piperettylpyrrolidine, trichoplein, trichonine, trichostachine, and cubebinin.^[19] Further studies in *P. trichostachyon* are required to assess its biological efficacy to establish its use in traditional medicine.

CONCLUSION

The study reports macroscopic, microscopic, and physicochemical parameters for *P. trichostachyon* in comparison with *P. nigrum*. These parameters may help in differentiating among these species. Comparative HPTLC fingerprint analysis and estimation of marker compounds using RP-UFLC technique showed clear differentiation among *P. trichostachyon* and *P. nigrum*. Thus, both the techniques, combined with pharmacognostic parameters can serve as a tool for identification, authentication, and quality control between species of *Piper*.

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Conflicts of interest

There are no conflicts of interest.

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