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Development of Analytical Screening Method for Rapid Identification of *Carica Papaya* Seed Adulterants in Black Pepper

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Abstract: Adulteration is a critical issue that undermines the credibility and therapeutic potential of Ayurvedic medicine. Ensuring authenticity through scientific methods and regulatory oversight is essential to preserve the heritage and promote global acceptance of Ayurveda. With regards to above concern HPLC and HPTLC method has been established for identification of adulteration of *Carica papaya* seed in black pepper. The methanolic extracts of *Carica papaya* seeds, along with authenticated samples of *Piper nigrum* and an adulterated mixture, were simultaneously applied onto the TLC plate. After the development, the plates were scanned at a wavelength of 254 nm. The HPLC analysis showing specific Rt at 28.1 min. for the specific marker present in *Carica papaya* seeds and resulting chromatogram of HPTLC revealed a distinct Rf value of 0.69 in both the *Carica papaya* seed extract and the adulterated mixture track, thereby proving the presence of papaya seed in the adulterated sample. This method offers a reliable approach for rapid identification of *Carica papaya* seed adulteration in black pepper

Keywords: HPTLC; adulteration; quality control; standardization; ayurveda; *piperine*

1. Introduction

In recent decades, there has been exponential growth seen in the field of traditional medicine, both in research and public interest [1]. India, with its rich cultural and medicinal heritage, is home to various traditional systems of medicine, including Ayurveda, Siddha, and Unani. These systems have demonstrated remarkable therapeutic potential and have played a vital role in healthcare for centuries [2]. However, one of the major challenges undermining the credibility and effectiveness of traditional medicine is adulteration [3]. The addition of substandard, misidentified, or non-authentic materials in herbal formulations poses a significant risk to safety, efficacy, and public trust [4,5]. As a result, many people are becoming doubtful about using herbal medicines due to concerns about their authenticity and purity [6]. To overcome this issue, there is an urgent need for standardization and quality control in the traditional system of medicine [7]. Ensuring quality, safety, and efficacy is not only essential to protect and preserve the traditional heritage but also crucial to promote the rational and scientific use of natural products in modern healthcare systems [8]. Recognizing this, the Central Council for Research in Ayurveda and Siddha (CCRAS) has laid down preliminary guidelines for testing and ensuring the quality of traditional formulations [9]. These guidelines serve as a foundational step towards building a robust quality assurance framework that will support the standardization, global acceptance, and clinical validation of traditional Indian medicines [10]. Ayurveda, the traditional system of Indian medicine, emphasizes the use of

polyherbal formulations for achieving holistic health and therapeutic efficacy [11]. Among the numerous botanicals utilized, Piper longum L. (Long pepper) and *Piper nigrum* L. (Black pepper) hold significant importance due to their well-documented deepana (appetizer) and pachana (digestive) properties [12]. These two spices are integral components of several classical Ayurvedic formulations, primarily for their roles in enhancing bioavailability and supporting digestive function [13]. The bioactive alkaloid *piperine*, found in both *P. longum* and *P. nigrum*, is responsible for a wide range of pharmacological activities, including: increase the absorption of drug, Anti-inflammatory effects, anticancer potential, antimicrobial action, hepatoprotective effects, antidepressant effects, anti-obesity, antidiabetic action, immunomodulatory effects [14,15]. These multiple therapeutic benefits highlight the importance of ensuring the authenticity and quality of pepper-containing formulations [16]. However, the rising issue of adulteration in polyherbal products poses a significant threat to both efficacy and safety [17]. The *Carica papaya* seeds are particularly known as a popular adulterant in black pepper, it has been practiced and documented due occurrence of their physical resemblance and low cost. This compromises the product quality, integrity and consumer trust. So it is urgent need to develop a method for the identification such type adulteration. To address this concern, the application of modern standardization techniques, such as High-Performance Thin-Layer Chromatography (HPTLC), becomes essential. Such analytical methods aid in confirming the identity, purity, and quality of the herbal ingredients and in detecting potential adulterants [18].

In this context, a systematic attempt was made to develop an HPTLC and HPLC method specifically aimed to identifying adulterants in Black pepper. The analytical findings of this study not only contribute to the establishment of pharmacopoeial standards for crude drugs but also provide the path for the development of adulterant detection markers, thereby enhancing the global credibility and scientific validation of Ayurvedic medicine.

2. Materials and Methods

2.1. Chemicals, Equipments and Drugs:

All chemicals and reagents used were of analytical grade. Standard of *piperine* was purchased from Merck, Begaluru, India. Methanol, Ethyl acetate, Formic acid and Methanol were used.

2.2. Equipment

A Camag HPTLC system comprising Linomate 5 automatic sample, 100 µL Hamilton Syringe with Camag TLC Scanner 4 and Camag Vision CAT software (version 3.2 SP2), Camag Twin trough chamber, pre-coated silica gel plates 60F254 (20 cm × 10 cm) was used for the present study.

2.3. Drugs

Kalimirch (*P. nigrum*) were procured from the local market of Raipur, Chhattisgarh and *Carica papaya* seeds was collected by self. It was authenticated and evaluated in Drugs Testing laboratory AvamAnusandhan Kendra, Raipur, Chhattishgah (Authenticated by Dr. N.S. Chauhan with Specimen Voucher No.-DTLAK/2024/Drug Auth./19, Date-25/06/24).

2.4. Preparation of Sample

The Kalimirch (*Piper nigrum*) (coded as PN) and Papaya (*Carica papaya*) seeds (coded as CP) raw sample powdered. The individual drugs were powdered separately and sieved through 85 meshes and prepared an adulterated sample by mixing equal amount of Kalimirch and *Carica papaya* seeds powder (coded as PNCP). [19,20].

2.5. Physico-chemical Analysis

Physico-chemical evaluations of the (PN, CP and PNCP) were conducted in accordance with the standards specified in the Ayurvedic Pharmacopoeia of India (API) [21]. These tests were performed to assess the quality, authenticity, and purity of the formulation and its constituent raw materials [22] (Tables 1 and 2).

Table 1. Organoleptic characteristics of *Piper nigrum* and *Carica papaya.*

S. No.	Test Parameter	*Piper nigrum*	*Carica papaya*
1.	Shape	Round shape	Oval shape
2.	Size	4–5 mm	6 mm
3.	Colour	Grayish Black	Black
4.	Odour	Pungent Aromatic	Aromatic
5.	Taste	Pungent taste	Taste less
6.	Skin Characteristic	Smooth Skin	Hairy Skin

Table 2. Physico-chemical test.

S. No.	Test Parameter	*P. nigrum*	*C. papaya*	*P. nigrum* + *C. papaya* (50:50)	API Limit for *P. nigrum*
1.	Loss on Drying	4%	7%	5%	-
2.	Total Ash	4%	1%	4.5%	NMT-5
3.	Acid Insoluble Ash	0.5%	1%	0.7%	NMT-0.5
4.	Water Soluble Extractive	8%	16%	12%	NLT-6
5.	Alcohol Soluble Extractive	10%	14%	11%	NLT-6

2.6. Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) is a widely adopted, rapid, and cost-effective analytical technique for the separation and qualitative identification of chemical constituents, particularly in herbal and pharmaceutical preparations [23]. The TLC study was conducted for detection of *Carica papaya* seed adulteration in black pepper sample by using mobile phase; Toluene: Ethyl acetate: Glacial acetic acid (6:4:0.1 *v*/*v*/*v*). The colour intensity of spot and Rf value PN sample is complying with marker compound *piperine* but PNCP extract showing less intense colour spot and CP extract did not showing any spot in separation (Figures 1 and 2).

Solvent System	Toluene: Ethyl acetate: Glacial acetic acid (6:4:0.1)
Spot A	*Piper nigrum*
Spot B	*Carica papaya*
Spot C	*Piper nigrum* + *Carica papaya mixture*
Spot D	*Piperine Standard*

Figure 1. Solvent system used for TLC development.

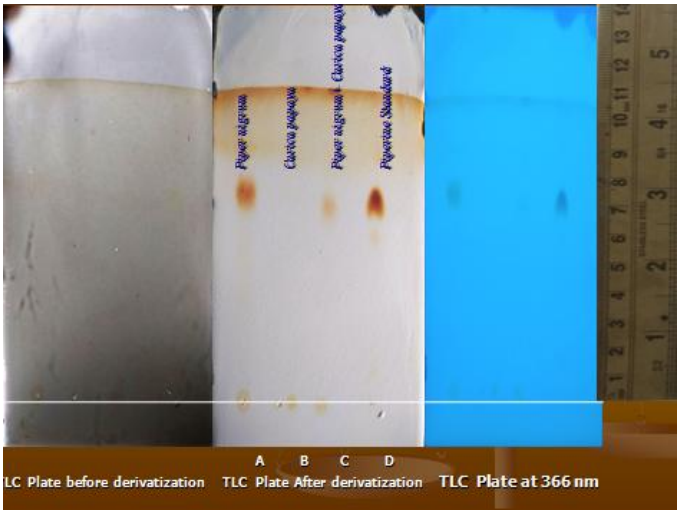

Figure 2. Image of TLC plate at visible light, and after derivatization with iodine. Vapour and at wavelength 254 nm.

2.7. High performance Liquid Chromatography

2.7.1. Preparation of Sample Solutions and Standard Solution

Accurately weighted 100 mg amount of powdered drug of PN, CP, PNCP (1:1) and 10 mg of *Piperine* standard, each was dissolved in 10 mL methanol in a 10 mL of volumetric flask, gives 10 mg/mL concentration sample solution for all samples and 1000 µg/mL, for standard *piperine* solution. The solution was filtered through 0.45 µm Millipore filter and degassed by sonication for 30 min [24,25].

2.7.2. Chromatographic Condition and Analysis

HPLC (YL, 9100, Younglin), Hamilton syringe- 100 µL, UV & PDA-detector was used for the analysis. The data was acquired on the YL Clarity Software (South Korea) and YL C18-4E Column was used. Injections were carried out using a 25 µL loop at room temperature and the flow rate was 1 mL/min. The 25 µL of each, PN, CP, PNCP and *Piperine* standard was injected through the sample loop at room temperature, and the flow rate was 1 mL/min. Detection was performed at 342 nm with 40 min run time and mobile phase (Acetonitrile:Water (50:50)) was used (Figure 3).

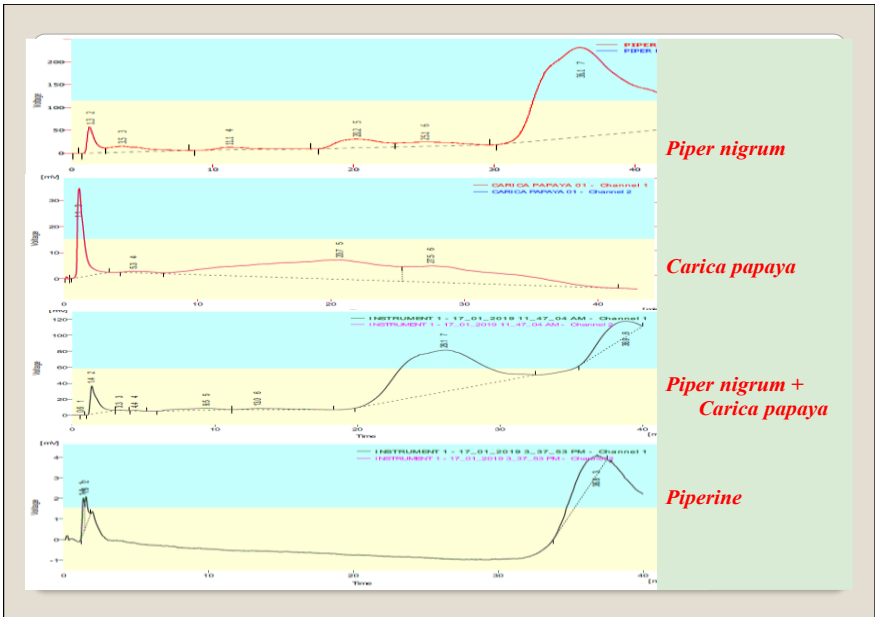

Figure 3. HPLC Chromatograms of *P.nirum*, *C.papaya*, Mixture of *P.nirum*, *C.papaya* and *piperine* standard.

2.8. High performance Thin Layer Chromatography

2.8.1. Preparation of Methanolic Extract

Accurately weighted 0.1 g each of powdered PN, CP, PNCP (1:1) and each of them were extracted with 10 mL of methanol into three different volumetric flask of 10 mL capacity. The entire sample was sonicated in an ultrasonic bath for 10 min. and strained it with membrane filter 0.45 µm to get clear solution. Aliquot of all methanolic extracts was used for HPTLC study [26–28].

2.8.2. Preparation of Standard Solution

Standard stock solution was prepared by dissolving 10 mg of *piperine* in 10 mL of methanol and sonicated, which yields a solution of concentration 1000 µg/mL and the working standard was prepared from this stock solution.

2.8.3. Pre-Conditioning

To avoid possible interference (irregular base lines in scanning densitometry) from non-volatile impurities in quantitative analysis, the plate was prewashed with methanol, dried, and activated for 30 min at an optimum temperature 70 °C.

2.8.4. Optimization of the Mobile Phases

The development of mobile phases was carried out on hit and trial basis with different combinations of solvents system in specified ratios. Some mobile phases; Toluene:Ethyl acetate:Glacial acetic acid (6:4:0.1) *v*/*v*/*v* had been optimized for the estimation of marker and the investigation of foreign adulterants.

2.8.5. Chromatographic Condition and Analysis

A Camag HPTLC system comprising Linomate 5 sample applicator with Camag TLC Scanner 4 and Camag Vision CAT software 4.0 (4.0.24047.1) were used.

The standard solutions and test samples were spotted in the form of bands (8 mm bandwidth) with 100 µL Hamilton syringe on pre-coated silica gel plates (Merck, 60F254, 20 cm × 10 cm) using Camag Linomate V applicator. The plates developed upto 80 mm with a solvent system; Toluene: Ethyl acetate: Glacial acetic acid ((6:4:0.1) *v*/*v*/*v*) in Camag glass twin-trough chamber previously saturated mobile phase for 30 min at 250 °C. The densitometric scanning was performed on Camag TLC Scanner 4 at absorbance 254 nm (deuterium lamp, slit dimension 6.0 × 0.45 mm) and operated by multilevel VisionCATS planar chromatography manager software (Figures 4–7, Table 3).

Sample Solvent	Methanol
Tank	: TTC 10 × 10
Mobile Phase	: Toluene: Ethyl acetate: Glacial acetic acid (6:4:0.1) *v*/*v*/*v*
Saturation time	: 20 min.
Use saturation pad	: True
Use smart Alert	: False
Volume front through	: 5 mL
Volume rear through	: 5 mL
Drying time	: 5 min.
Drying temperature	: Room temperature

Figure 4. HPTLC Method description.

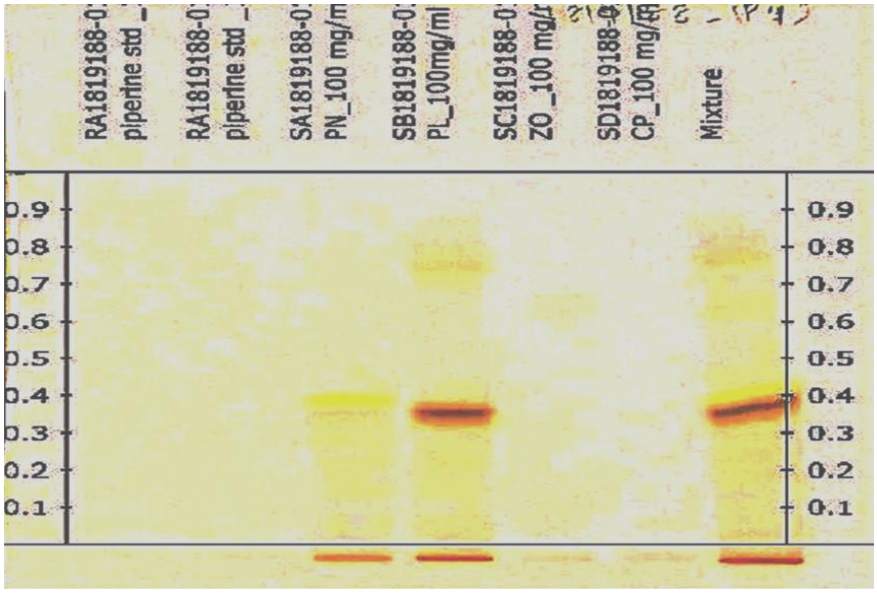

Figure 5. HPTLC fingerprint at visible light after derivatization by iodine vapour.

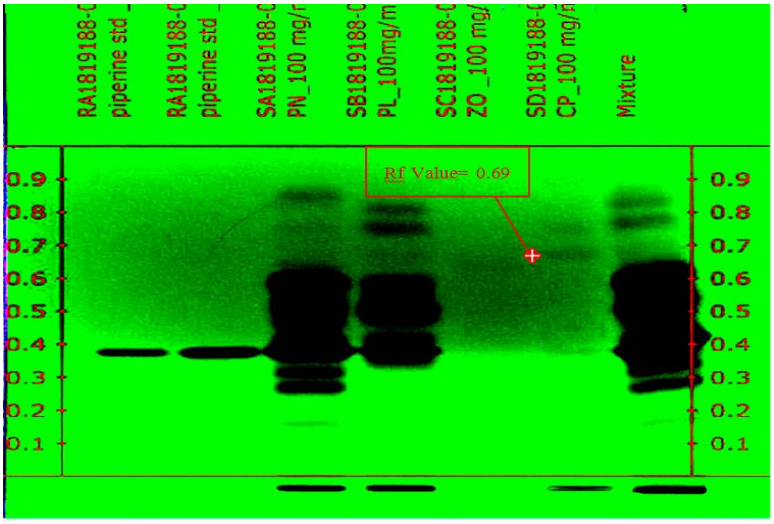

Figure 6. HPTLC fingerprint at wavelength 254 nm.

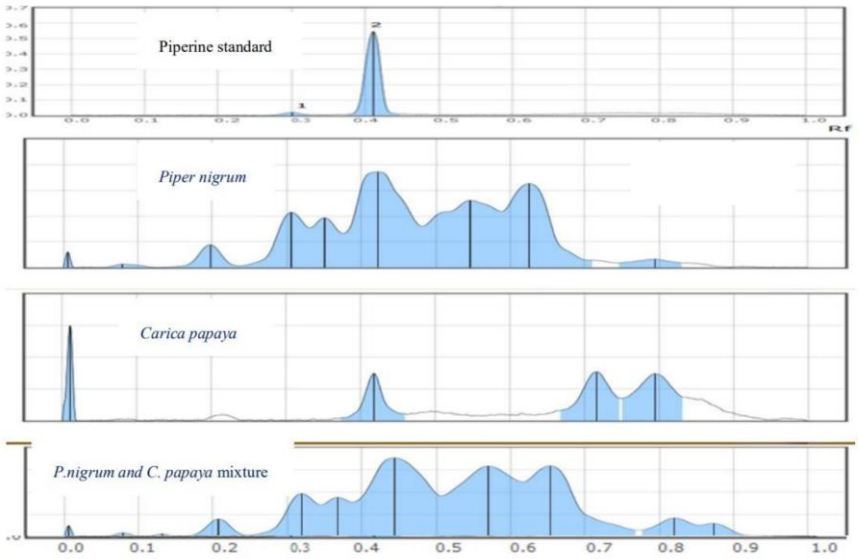

Figure 7. HPTLC Chromatograms of *P. nirum*, *C. papaya*, Mixture of *P. nirum*, *C. papaya* and *piperine* standard.

Table 3. Rf value of Samples complying with *piperine* marker.

S.No.	Test Parameter	*Piperine*	*P. nigrum*	*C. papaya*	*P. nigrum* + *C. papaya* (50:50)
1.	Rf Value	0.38	0.38	0.69	0.38, 0.69

3. Results and Discussions

The study employed a combination of physicochemical analysis, HPLC chromatographic profiling, and HPTLC fingerprinting to investigate the adulteration of *Piper nigrum* with *Carica papaya* seeds. These analytical methods collectively provided conclusive evidence indicating the presence of papaya seed as an adulterant.

Physicochemical tests revealed that the inclusion of papaya seed significantly altered the characteristic test values of the formulation suggesting a deviation from the standard profile. The Loss on Drying, Total Ash, Acid insoluble ash, Water soluble extractive, Alcohol soluble extractive value has been changed by the addition of carica seeds in black pepper sample (PNCP).

In the HPLC chromatographic analysis, the chromatogram of the authentic sample was well resolved and exhibited a distinct peak corresponding to standard *piperine*. However, in the adulterated sample (PNCP), an additional peak appeared at a retention time (Rt) of 28.1 min. The CP extract showing less intense peaks at Rt at 27.5 and PNCP is showing Rt at 28.1 min, which clearly shows the presence of adulterant peak which is not

resembling with standard marker. This unique peak is indicative of Carica and was absent in the standard there by confirming adulteration.

Furthermore, HPTLC fingerprinting was performed for additional verification. Extracts of CP, along with authenticated samples of PN, and an adulterated mixture (PNCP), were simultaneously applied onto the TLC plate. Following with development, the plates were scanned at a wavelength of 254 nm. The resulting chromatogram revealed a distinct Rf value of 0.69 in both the CP extract and PNCP, which is absent in the PN extract and standard marker track, thereby it is proving that the presence of papaya seed adulterant in the PNCP sample.

In contrast, PN, and PNCP, all exhibited bands corresponding to the standard *piperine* with an Rf value of 0.38. The presence of both Rf values (0.38 and 0.69) in the adulterated sample (PNCP) further confirms the addition of papaya seed adulterant, as it introduces a specific marker absent in the pure ingredients.

4. Conclusions

Adulteration remains a critical concern in the realm of Ayurvedic medicine, threatening its therapeutic efficacy, safety, and global credibility. Ensuring the authenticity and quality of raw materials is fundamental to safeguarding the integrity of traditional formulations. In this study, HPLC and HPTLC techniques were successfully established as effective tools for the identification of *Carica papaya* seed adulteration in *Piper nigrum*. The HPTLC fingerprinting revealed a distinct Rf value of 0.69, specific to C. papaya, in both its extract and the adulterated sample, confirming the presence of the adulterant. This method provides a simple approach for routine quality control and can serve as a valuable tool for standardization of pepper-containing Ayurvedic formulations and it can be adopted by various laboratories and researcher for the quick detection of *C.papaya* seed adulteration Implementing such scientific validation techniques is vital not only to detect adulteration but also to reinforce the global trust and acceptance of Ayurveda as a credible and evidence-based traditional healthcare system.

Author Contributions

K.S.K.: conceptualization, methodology, A.K.S.P.: data curation, software; U.S.: Analysis, investigation; writing—original draft preparation; N.S.C.: supervision, visualization; M.K.K.: software, validation; S.R.I.: writing—reviewing and editing. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement

Not applicable.

Data Availability Statement

All data will be available as per demand.

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Conflict of Interest Disclosure

The authors declare no conflict of interest.

Use of AI and AI-assisted Technologies

No AI tools were utilized for this paper.

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