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Pharmaceutical Standardization

Pharmacognostical and high performance thin layer chromatography studies on leaves of *Clerodendrum infortunatum* L.

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Abstract

Background: *Clerodendrum infortunatum* L. commonly known as Bhant plays a significant role in Indian System of Medicine, that is, Ayurveda, due to its medicinal properties. It grows easily in waste places of all areas of India, Bangladesh, and Myanmar. **Aim:** The present study was carried out with a view to lay down its pharmacognostic standards along with high-performance thin layer chromatography (HPTLC) studies in order to document/validate its therapeutic benefits. **Materials and Methods:** In this research, leaves of *C. infortunatum* were subjected to pharmacognostic evaluation parameters such as macroscopy, quantitative microscopy, physicochemical studies, phytochemical screening. HPTLC has been developed for detection and quantification of gallic acid and tyrosine in *C. infortunatum*. Increasing serial dilutions of reference standard gallic acid (20–100 μg/mL) and tyrosine (20–100 μg/mL) were scanned at 254 nm and 280 nm, respectively. **Results:** Microscopy of leaf revealed the presence of anisocytic stomata, sclereids, glandular and covering trichome, and prisms of calcium oxalate crystal. The total ash, water-soluble, and acid insoluble ash values of leaves were 9.95%, 2.15%, and 0.70%, respectively. The maximum extractive value of crude powder was in the water. HPTLC studies revealed that the amount of gallic acid in the crude powder of test sample were high (0.244 mg/g) in comparison to tyrosine (0.081 mg/g). **Conclusion:** The data generated would be of significant use for the authentication of drug and would also serve as a reference for the standardization and quality control of *C. infortunatum*.

Key words: Bhant, *Clerodendrum infortunatum* L., ethnomedicine, pharmacognostic evaluation, standardization

Introduction

The plant *Clerodendron viscosum* Vent. (Synonym: *Clerodendrum infortunatum* Gaetrn.) Family Verbenaceae known as Bhandirah in Sanskrit is an important plant in Indian system of Medicine.[1,2] The species number has been estimated to be 560–580, found along margin of evergreen to semi-evergreen forests up to 1800 m[3] and widely distributed in Asia, Australia, Africa, America,[4] Indo-malaysia and throughout Western Ghats.[3] It grows commonly in waste places and graveyards in all districts in Bangladesh, India, and Burma. It is well known as a medicinal plant because of its wide therapeutic uses.[5] In Ayurveda, the plant is used in postnatal care and to dress fresh wounds, in tumors, cirrhosis, jaundice, in scorpion-sting, and snake-bite.[6] In Indian folk medicine, the plant is used in the treatment of bronchitis, asthma, fever, diseases of the blood, inflammation, burning sensation, and epilepsy.[2] The plant is used in convulsions, cramps, sores, ulcers, and gravel.[6] *C. infortunatum* L. is reported to possess analgesic,[7,8] free radical scavenging,[9,10] anticonvulsant,[11] CNS activity,[12,13] antihyperglycemic,[14] antimicrobial,[15,16] and repellant activities.[17] In spite of the numerous medicinal uses attributed to *C. infortunatum*, there is no pharmacognostic report along with quantitation of biomarkers on the leaf of the plant to determine the anatomical and other physicochemical standards required for quality control of the crude drug. Some of the reasons that have been identified for low usage are lack of stringent

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quality control parameters, not following the traditional purification process and batch to batch variation of active constituents. Taking this into the account it was thought worthwhile to generate data taking gallic acid and tyrosine as a standard marker compound, responsible for the diverse pharmacological activities, that may be useful as a reference in future development of a monograph on *C. infortunatum*, and also to keep a check on intentional/unintentional adulteration.

Therefore, the main aim of the present investigation was to study the macroscopic, microscopic, physicochemical standards, and phytochemical screening along with high-performance thin layer chromatography (HPTLC) studies on leaves of *C. infortunatum* as per WHO guidelines, which could be of use in preparing a monograph of the plant.

Materials and Methods

Collection and authentication

The leaves of *C. infortunatum* were collected from local areas in the month of August 2011 and authenticated also a voucher specimen was submitted for future reference (Ref No. NBRI/CIF/293/2012). The air-dried plant material was first washed with running tap water, then again washed twice with double distilled water and the washed specimen was air-dried. The air dried specimen (leaves) were pulverized and sieved through 80# mesh size and stored in an air-tight container at 25°C for future/further studies.

Pharmacognostic studies

Macroscopic characteristics

The morphological characteristics of the specimen, that is, leaves were studied and the photographs were taken with the help of Sony Corp. DSC-S980, 12.1 megapixel camera.

Microscopic characteristics

For microscopic studies transverse section (TS) of lamina were used. The fine sections of leaves were cut by free hand. The chlorophyll and the other pigments of the plant were removed by treating the sections with 5% potassium hydroxide (KOH) and 20% chloral hydrate as required. Photographs of different magnifications were taken with Olympus Microscope, Model Olympus (India), attached to YOKO CCD Camera.

Quantitative microscopy

Quantitative microscopy of leaf such as stomatal number, stomatal index, vein-islet, vein termination number, and palisade ratio were determined by using fresh leaves of the plant.[18,19]

Physiochemical parameters

The physical constant evaluation of the drugs is an important parameter in detecting adulteration or improper handling of drugs. It includes ash values (total ash, acid-insoluble ash, and water-soluble ash), extractive values (alcohol soluble, water soluble, and ether extractive values), and moisture content.[19,20]

Phytochemical screening

Preliminary phytochemical investigation of different extracts of leaves of *C. infortunatum* L. shows the presence of sterols, carbohydrates, tannins, terpenoids, flavonoids, and saponin.

High-performance thin layer chromatography fingerprinting

The preliminary phytochemical investigation of the methanolic extract of leaves of *C. infortunatum* showed the presence of tannins and amino acids. Hence, the methanol fraction was used for HPTLC studies to detect and quantify the gallic acid and tyrosine in the respective extract.

Preparation of gallic acid and tyrosine standard solution

A stock solution of standard gallic acid procured from SD Fine Chemicals; Mumbai and tyrosine procured from HiMedia Laboratories Pvt. Ltd. Mumbai, through an authorized institutional supplier M/S Sohan Lal and sons. A stock solutions of concentration 100 µg/mL were prepared by transferring 10 mg of gallic acid and tyrosine in 100 mL volumetric flask and the volume was made up to the mark with methanol and ethanol: Water (70:30), respectively. Further dilutions were made with methanol and ethanol: Water (70:30) to obtain working standards 20, 40, 60, 80, and 100 µg/mL for standard gallic acid and tyrosine.

Preparation of sample solution

A volume of 100 mg of air dried powdered plant material (leaves) was defatted with petroleum ether and then Soxhlet extracted with methanol for 16 h. The methanolic extract was vacuum evaporated and concentrated and 10 mg of the concentrated methanolic extract was re-dissolved in 10 mL methanol to yield a test sample (1000 µg/mL).

Instrumentation and chromatographic conditions

High-performance thin layer chromatography was performed on 10 cm × 10 cm aluminum backed plates coated with Silica gel GF_{254} (Merck, Mumbai, India). Standard solution of gallic acid and sample solution and tyrosine and sample solution was applied to the separate plates maintaining a distance of 10 mm from the edge of plate and 10.0 mm from the bottom edge of the same chromatographic plate by use of a Camag (Muttenz, Switzerland) Linomat V sample applicator equipped with a 2 µL Hamilton (USA) syringe. Ascending development to a distance of 75 mm was performed at room temperature (28 ± 2°C), with toluene: Ethyl acetate: Formic acid, 5:5.5:1 (v/v/v)[21] for gallic acid and n-Butanol: Glacial acetic acid: Water (4:1:1) (v/v/v)[22] for tyrosine, as mobile phase in a Camag glass twin trough chamber previously saturated with mobile phase vapor for 20 min. After development, each plate was dried in an oven and then scanned at 254 nm for gallic acid and 280 nm for tyrosine, with a Camag TLC Scanner with WINCAT software.

Calibration curve of gallic acid and tyrosine

A stock solution of standard gallic acid (100 µg/mL) and tyrosine (100 µg/mL) was prepared in methanol and ethanol: Water (70:30), respectively. From the stock solution, the concentration of 20, 40, 60, 80, and 100 µg/mL was applied to separate HPTLC plates and plate was developed as above and scanned at 254 nm and 280 nm. Calibration graph of gallic acid and tyrosine was constructed by plotting concentration versus spot area (area under the curve [AUC]) of the compounds.

Results and Discussion

Morphology

Clerodendrum infortunatum is a terrestrial shrub having square, blackish stem, and hairy leaves with a disagreeable

odor. Macroscopically the leaf was dark green in color, simple, opposite decussate, ovate shaped, margin dentate, hairy on both sides, with reticulate pinnate venation. The average leaf size was 10–20 cm (length) and 9–15 cm (width) [Figure 1].

Flowers: Bluish-purple often white, tinged with pink or red, borne in terminal trichotomous panicles, corolla-tube exceeding the glandular calyx-lobe.[23]

Fruits: Globose drupes seated on the enlarged pinkish or reddish calyx, containing 1–4 pyrenees, black when ripe, seeds oblong.[23]

Microscopic Study

Transverse section of Midrib and Lamina

Epidermis

The TS of the leaf shows three distinct regions such as upper epidermis, lower epidermis, and mesophyll. The epidermis was covered with a thin layer of the cuticle. Below the upper epidermis is a single layer of palisade cells which is followed by 3–5 layers of spongy parenchyma [Figure 2]. Both the epidermis is identical and shows glandular and covering trichomes, but the cells of upper epidermis are larger than the cells of lower epidermis.

Vascular bundles

The central portion of the midrib is occupied by prominent bicollateral vascular bundles with xylem toward the ventral surface and phloem toward the dorsal surface. Vascular bundles were conjoint, collateral, and closed [Figure 3a and b]. The vascular bundles are surrounded by sclerenchymatous fibers with calcium oxalate crystals.

Stomata

Lower surface of the leaf contains more number of anisocytic type stomata as compared to upper surface [Figure 4].

Crystals in lamina

Prism of Calcium oxalate crystals was present in the mesophyll cells [Figure 5].

Figure 1: Photograph of leaf

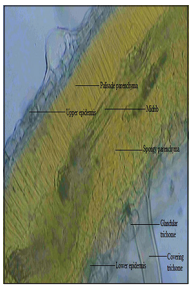

Figure 2: TS of midrib with lamina showing upper and lower epidermis, Palisade cells, and spongy parenchyma

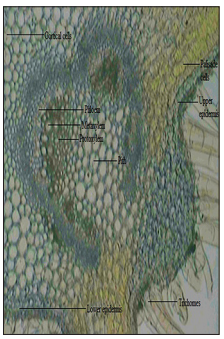

Figure 3: (a) TS of midrib

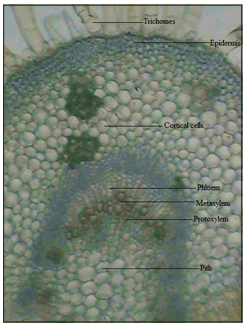

Figure 3: (b) TS of midrib with lamina showing Xylem and phloem

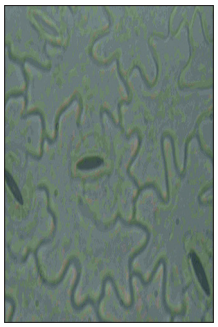

Figure 4: TS of lamina showing anisocytic stomata

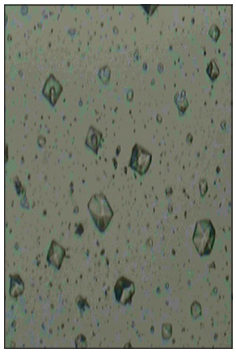

Figure 5: Prism of calcium oxalate crystals

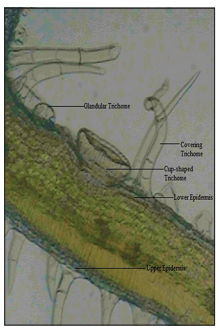

Figure 6: TS of midrib with lamina showing different types of trichome

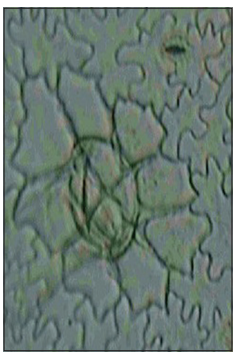

Figure 7: Cicatrix

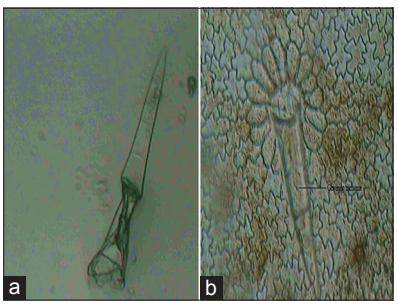

Figure 8: (a) Covering trichome with collapsed cell (b) Multicellular covering trichome

Trichomes

The upper and lower epidermis of the plant contains covering trichome, glandular trichomes, Cup-shaped trichomes [Figure 6], and cicatrix [Figure 7]. Covering trichomes are lignified or nonlignified, uniseriate, multicellular (3–10 cells long) mostly straight with tapering ends, some of the covering trichomes show collapsed cells [Figure 8a]. Covering trichomes with unicellular stalk and unicellular or multicellular head consisting of 4–8 cells [Figure 8b].

Quantitative microscopy, physiochemical parameters, and phytochemical screening

These standardization parameters were performed as per the guidelines of Ayurvedic Pharmacopoeia of India. Preliminary phytochemical investigation shows the presence of sterols, carbohydrates, tannins, terpenoids, flavonoids, and saponin. The results are depicted in Tables 1-3, respectively.

High-performance thin layer chromatography fingerprinting

The identity of the gallic acid and tyrosine bands in sample chromatograms was confirmed by the chromatogram obtained from the test sample with that obtained from the reference standard solution and by comparing retention factors of gallic acid and tyrosine from sample and standard solutions. The peak corresponding to gallic acid and tyrosine from the sample solution had same retention factor as that from the gallic acid and tyrosine standard (R_f 0.91 and R_f 0.88, respectively). The chromatograms of standard gallic acid [Figure 9] and tyrosine [Figure 10] and that of gallic acid and tyrosine in leaves of *C. infortunatum* were shown in the Figure 11a and b. The AUC obtained for various tracks of gallic acid and tyrosine were enumerated in Tables 4 and 5. The three-dimension spectra of all tracks of gallic acid and tyrosine scanned at 254 nm and 280 nm, respectively are shown in Figure 12a and b. Spectral comparison of gallic acid and tyrosine (reference standard) with gallic acid and tyrosine present in test samples is shown in Figure 13a and b, respectively. The calibration curve was linear in the range of 20–100 µg/mL, as illustrated in Figures 14 and 15. From the regression equation y = 1261.5× +830.35 and y = 1015.7× +19355, the concentration of gallic acid and tyrosine in test sample was found to be 5.588 µg/mL and 5.396 µg/mL, respectively. The estimated value on per gram basis of the drug was about 0.244 mg/g and 0.081 mg/g of leaves powder respectively.

Conclusion

From the present investigation, it is evident that certain characteristics such as stomata, calcium oxalate crystals, trichomes, and arrangement of vascular bundles can provide useful parameter, which may be of use as a reference. Preliminary phytochemical investigation of different extracts of leaves of *C. infortunatum* shows the presence of sterols, tannins, flavonoids, and saponin, indicating the plant a prominent model for various types of pharmacological activity. The present HPTLC method provided a quick an easy approach

Table 1: Quantitative microscopy of leaf of *Clerodendrum infortunatum* L

Parameter	Values
Vein islet number (1 mm² leaf surface)	16.66
Vein termination number (1 mm² leaf surface)	11.11
Stomatal number (1 mm² leaf surface on lower epidermis)	620.77
Stomatal number (1 mm² leaf surface on upper epidermis)	225.73
Stomatal index (1 mm² leaf surface on lower epidermis)	27.19
Stomatal index (1 mm² leaf surface on upper epidermis)	9.85

Table 2: Physiochemical analysis of leaves of *Clerodendrum infortunatum* L.

Parameter	Value % (w/w)
Ash values	
Total ash	9.95
Water soluble ash	2.15
Acid insoluble ash	0.70
Extractive values	
Water extractive value	13.50
Ethanol extractive value	7.50
Ether extractive value	3.65
Moisture content 3.65	
Foaming index <100	

Table 3: Phytochemical analysis of different extract of *Clerodendrum infortunatum* L.

Phytoconstituents	Petroleum ether extract	Methanol extract	Water extract
Fats and oil	+	-	-
Carbohydrates	-	++	+
Reducing sugar	-	++	+
Proteins	-	-	+
Saponin	+	+	++
Sterols	++	-	-
Terpenoids	++	-	-
Alkaloid	-	-	-
Glycosides	-	-	-
Tannins	-	++	+
Flavonoids	-	++	+

Table 4: R_f range and AUC of standard gallic acid and test sample

Serial number	Samples	Concentration (µg/mL)	Maximum R_F	AUC
Track 1	Standard	20	0.92	2371.6
Track 2	Standard	40	0.91	3156.7
Track 3	Standard	60	0.91	4341.9
Track 4	Standard	80	0.90	5893.0
Track 5	Standard	100	0.90	7310.9
Track 6	Test	1000	0.89	7879.4

AUC: Area under the curve

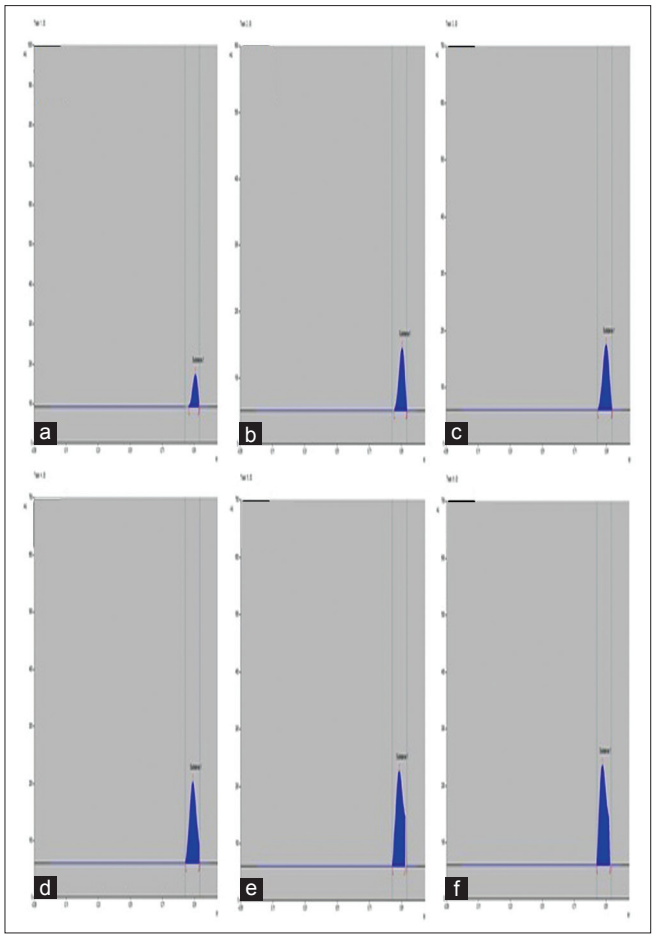

Figure 9: A Typical high-performance thin layer chromatography of gallic acid working standard (a) track 1standard (20 µg/mL), (b) track 2 standard (40 µg/mL), (c) track 3 standard (60 µg/mL), (d) track 4 standard (80 µg/mL), (e) track 5 standard (100 µg/mL), and (f) track 6 test sample: Methanolic extract of leaves of *Clerodendrum infortunatum* (1000 µg/mL)

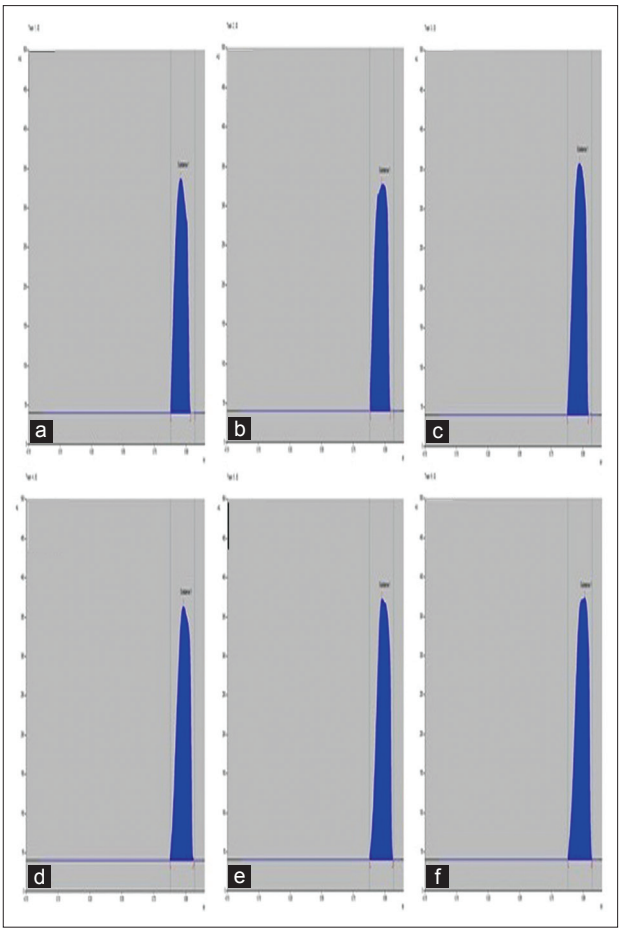

Figure 10: A typical high-performance thin layer chromatography of tyrosine working standard (a) track 1standard (20 µg/mL), (b) track 2 standard (40 µg/mL), (c) track 3 standard (60 µg/mL), (d) track 4 standard (80 µg/mL), (e) track 5 standard (100 µg/mL), and (f) track 6 test sample: Methanolic extract of leaves of *Clerodendrum infortunatum* (1000 µg/mL)

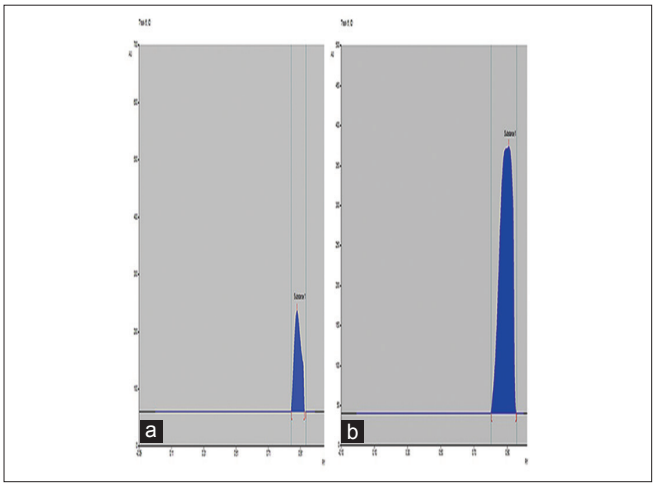

Figure 11: (a) A typical high-performance thin layer chromatography of gallic acid in leaves of *Clerodendrum infortunatum* (b) A typical high-performance thin layer chromatography of tyrosine in leaves of *Clerodendrum infortunatum*

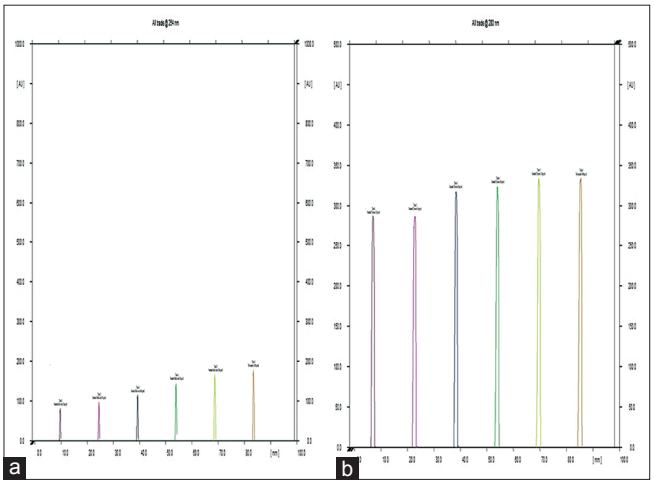

Figure 12: (a) Three-dimension-spectra of standard gallic acid with gallic acid present in test sample scanned at λ 254 nm (b) Three-dimension-spectra of standard tyrosine with tyrosine present in test sample scanned at λ 280 nm

for detection and quantitation of biomarker gallic acid and tyrosine in *C. infortunatum* leaves. The proposed HPTLC method was found to be simple, precise, and accurate which can be used for the quality control of the raw materials as well

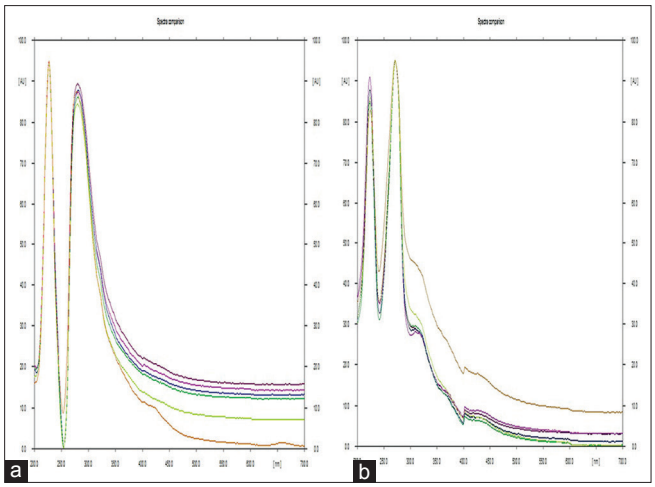

Figure 13: (a) Spectral comparison of standard gallic acid with gallic acid present in test sample at λ 227 nm, (b) Spectral comparison of standard tyrosine with tyrosine present in test sample at λ 271 nm

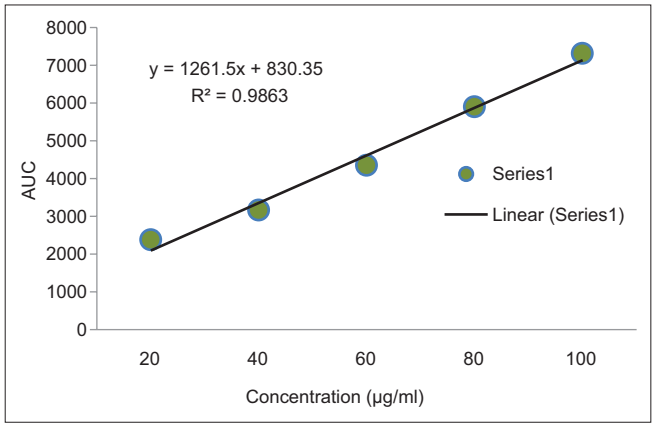

Figure 14: Calibration curve for standard gallic acid

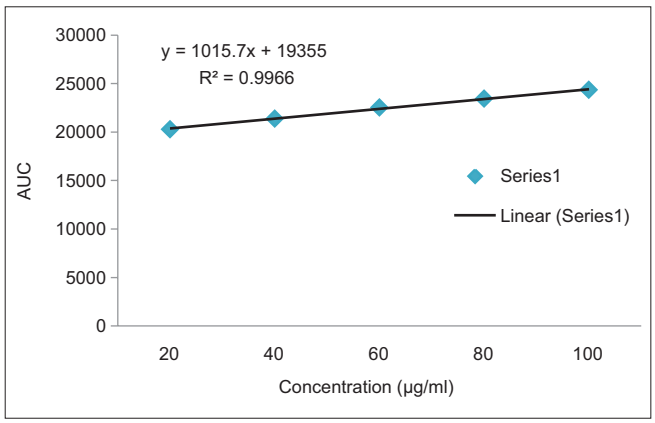

Figure 15: Calibration curve for standard tyrosine

as formulations. Hence, the present study attempts to outline basic requirements necessary to develop scientific/technical standards to justify the medicinal plant worth exploring for further research work and also to keep a check on intentional/unintentional adulteration.

Table 5: R_f range and AUC of standard tyrosine and test sample

Serial number	Samples	Concentration (µg/mL)	Maximum R_f	AUC
Track 1	Standard	20	0.87	20292.0
Track 2	Standard	40	0.88	21388.8
Track 3	Standard	60	0.88	22540.2
Track 4	Standard	80	0.89	23449.0
Track 5	Standard	100	0.88	24340.5
Track 6	Test	1000	0.89	24835.8

AUC: Area under the curve

Acknowledgment

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हिन्दी सारांश

भांट के पत्तों का भेषजज्ञानीय मानकीकरण

सुमन वर्मा, राजीव गुप्ता

क्लिरोडेन्ट्रम इन्फारचूनेट्म साधारण तया भांट के नाम से जाना जाता है, जो अपनी औषधीय गुणों के कारण आयुर्वेद में एक महत्वपूर्ण भूमिका निभाता है। यह भारत, बांग्लादेश और म्यांनमार के सभी क्षेत्रों के अपषिष्ट स्थानों में पाया जाता है। वर्तमान अध्ययन का हेतु औषधि के भेषज ज्ञान के सभी मानकों को एक साथ स्थापित कर इस औषधि के चिकित्सा पद्धति में उपयोग बढ़ाना है। स्थापित किये गये मानकों से औषधि का मूल्यांकन और पहचान करना सुलभ होगा।