International Journal of Herbal Medicine
Available online at www.florajournal.com

E-ISSN: 2321-2187
P-ISSN: 2394-0514
www.florajournal.com
IJHM 2022; 10(1): 15-21
Received: 07-11-2021
Accepted: 10-12-2021

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Pharmacognostical and HPTLC analysis of *Azadirachta indica* A. Juss: Flower

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Abstract
Traditionally medicinal plants are most valuable for modern medicine that they are used as a source of direct therapeutic agents, the bioactive chemical constituents contributes high medicinal values and used to cure various diseases. and also used as models for discovery of new synthetic compounds and However, the source and quality of raw materials play a pivotal role in guaranteeing the quality and stability of herbal preparations. Hence, we mainly focused on flower part to analyze Pharmacognostical, Physicochemical and HPTLC finger printing studies for the standardization process. Specifically, in Unani system of medicine, the flower part is used to cure diseases like Juzam (Leprosy) Fasade Dam (Putrefaction of blood) and Waja-ul- Mafaasil (Joints pain).

Keywords: Pharmacognostical Studies, HPTLC Finger Print, Physico-chemical Parameters, *Azadirachta indica* A. Juss

1. Introduction
Azadirachta indica A. Juss. (Fam. Meliaceae) is one of the most important medicinal tree, which is used to treat different diseases in Unani System of Medicine as well as traditional system of medicine (Ayurveda, Homeopathic China and European "Materia Medica"). It is typically grown in tropical and sub-tropical region. Neem tree is one of the fast growing tree that can reach a height of 15-20 mts. In neem tree every part contributes high medicinal values like leaf is used to cure leprosy, eye problems, intestinal worms, anorexia, and skin ulcer. Bark is used to cure analgesic, alternative and curative of fever. Fruit is used to cure various diseases like intestinal worms, urinary disorder, eye problems, diabetes, wound and leprosy. Twig is used against cough, asthma, piles, urinary disorder and diabetes. Gum is effective against skin disease like ring worms, scabies wound and ulcers. Seed pulp is used to cure Leprosy and intestinal worms. Moreover, the root, bark, leaf, flower and fruit together is responsible for blood morbidity, itching, skin ulcer, burning sensation and leprosy respectively [1]. Neem flower is considered as one of the best medicinal plant due to its several therapeutic uses *viz*., bile suspension, elimination of intestinal worms and phlegm [2]. The *Azadirachta indica* flower contain chemical constituents like flavanones (flowerine and flowerone), sesquiterpenes, aromatic compounds, fatty acids, fatty acid esters, steroids, few hydrocarbons, azharone, azadirone, isoazadironolide and triterpenoids (Omethylazadironolide and diepoxyazadirol) [3]. The oil extracted from flowers of the tree also possessed cubebene, copaene, humulene, δ-cardinene, and a number of sesquiterpenes, which were responsible for antimicrobial activities [4].

Due to the presence of various bioactive components, it can be used to treat against various diseases like anorexia, nausea belching and intestinal worms [5]. Anthraquinone fraction of dried flower is taken orally for leprosy and hot water extract of flower is taken orally as an anti-hysteric remedy and used externally to treat wound and the extracts from young flower have strong antioxidant potential. An indicator of oxidative stress, malondialdehyde (MDA) was reduced by 46.0% and 50.6% for flower extracts [6]. Furthermore, the flowers of the tree are used as astringent and anthelmintic agents [7].

Herbal formulations have reached extensive acceptability as therapeutic agents for several diseases. The development of authentic analytical methods which can reliably profile the phytochemical composition, including quantitative analyses of marker/bioactive compounds and other major constituents, is a major challenge to scientists. Standardization is an important step for the establishment of a consistent biological activity, a consistent chemical profile, or simply a quality assurance program for production and manufacturing of herbal drugs. WHO specific guidelines for the assessment of the safety, efficacy and quality of herbal medicines as a prerequisite for global harmonization are of utmost importance [8].

Hence, the present study aimed to standardize and assure the quality of *Azadirachta indica* A. Juss. Flower using Pharmocognostic, Physicochemical, HPTLC finger print analysis to assists in validating the flower for usage in herbal formulations in the upcoming era.

2. Materials and methods

2.1 Collection of plant material

The flowers of *Azadirachta indica* A. Juss. were collected from garden of Regional Research Institute in Unani Medicine (RRIUM), Royapuram, Chennai. Lattitude 13.113275° and Longitude 80.25765°. The plant was deposited for future reference. The flowers were shade dried and made it into a coarse powder and kept in an airtight container for further analysis.

2.2 Pharmacognostical Studies

The Pharmacognostical studies such as macroscopical, microscopical and powder microscopy were carried out using Standard method [9]. Fresh flowers were taken for microscopic studies, sections were prepared and stained with safranin and mounted in glycerine. The powder of the drug was treated with various chemical reagents like phloroglucinol with HCl and Jefferey's reagent [10] for clearing the tissues to study the various elements. Photographs were taken in different magnification using (Nikon Eclipse Ci Microscope).

2.3 Physico-chemical analysis

The coarsely powdered flower of Neem were taken to proceed for the qualitative physicochemical parameters like foreign matter, loss on drying at 105 °C, total ash and acid - insoluble ash at 600 °C, pH and extractive values for different solvents like hexane, alcohol and aqueous were carried out as per the standard methods [11].

2.4 TLC/ HPTLC analysis

Preparation of samples

The coarse powder (5 g) of dried flower of the Neem was extracted with chloroform and ethanol using hot extraction method. The extract was filtered and made up to 10 ml in a standard flask with respective solvents separately.

Development of TLC/HPTLC finger print Profile

HPTLC technique is widely employed in pharmaceutical industry in process development, identification and detection of adulterants in herbal product and helps in identification of pesticide content, mycotoxins and in quality control of herbs and health foods [12]. HPTLC finger printing profile is very important parameter of herbal drug standardization for the proper identification of medicinal plants. High performance thin layer chromatography (HPTLC) has become an effective and powerful tool for the estimation of chemical and biochemical markers [13]. HPTLC profile of plant extracts was generated in solvent systems of different polarities in order to ascertain the total number of chemical moieties which will also help in designing the method of isolation and characterization of bioactive compounds [14]. Chromatographic fingerprint is a rational option to meet the need for more effective and powerful quality assessment to the traditional system of medicine throughout the world [15]. Hence, it is powerful tool for the analysis of traditional system of herbal medicine.

TLC/HPTLC Instrument conditions

CAMAG HPTLC instrument, sample applicator-CAMAG Linomat-IV applicator with N2 gas flow, photodocumentation system-Digi store-2 documentation system with Win Cats & video scan software, scanner-CAMAG HPTLC scanner-3 (030618), Win Cats-IV, development chamber-CAMAG TLC 10x10, 10x20 twin trough linear development chamber, stationary phase-Aluminium plate pre-coated with silica gel 60 F254 (E. Merck), plate thickness-0.2 mm, scanning wavelength-254 nm and 366 nm, laboratory condition-26 ± 5 °C and 53% relative humidity.

The TLC chromatogram was carried out using spray techniques in ATS4, CAMAG HPTLC system. Apply the each extract (5 µl and 10 µl each) separately on TLC plate.

Develop the plate using Chloroform: Methanol (9.8:0.2) for chloroform extract and Toluene: Ethyl acetate: Formic acid (8.8:1.2:0.01) for alcohol extract as mobile phase.

The plates were dried at room temperature and observed the spots at UV-254 nm, UV-366 nm and the plates were scanned at 254 nm to record the finger print spectrum. Finally the plate were dipped in vanillin-sulphuric acid and heated at 105° till coloured spots appeared.

3. Results and Discussion

3.1 Pharmacognostical studies

Macroscopic features: Dried flowers brown to deep brown; individual flowers 5 to 6 mm long and 6 to 11 mm wide, pentamerous, bisexual, regular and hypogynous; calyx 5, short, united at base; corolla 5, free, spathulate, spreading, 4.5 to 5.5 mm long 2 mm wide; stamens 10, monoadelphous, staminal tube inserted at base of corolla; gynoecium tricarpellary, syncarpous, superior, trilocular, two ovules in each locule, style 1, stigma 3-lobed; taste mildly bitter:odour indistinct.

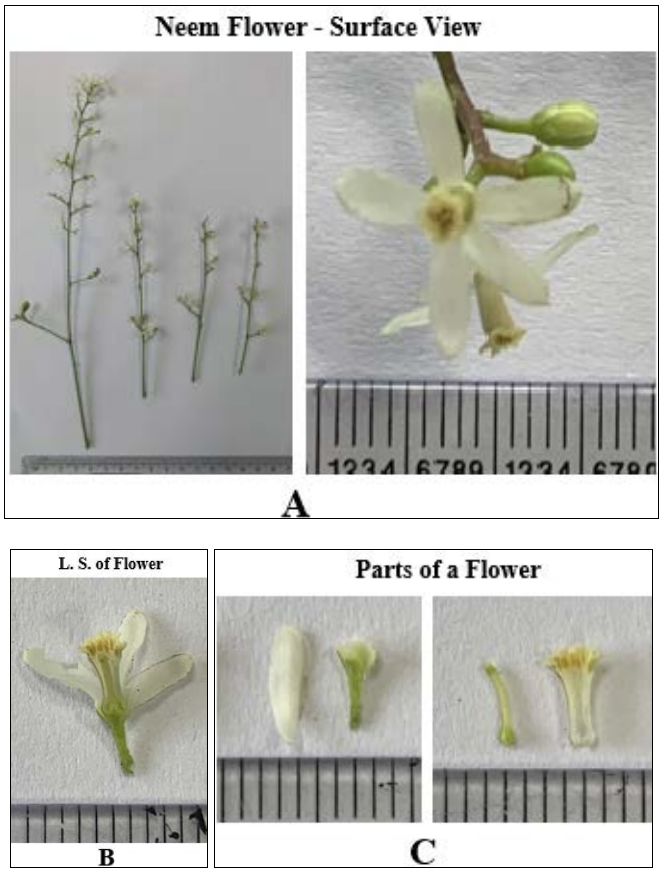


Fig 1: *Azadirachta indica* A. Juss. Flower A. Neem Flower - Surface view; B. L.S. of Flower; C. Parts of flower (Calyx, Corolla, Androecium, Gynoecium)

Microscopic features

T.S. of Pedicel: T. S. of Pedicel almost circular in outline and slightly angled: epidermis single layered with thick cuticle; trichomes thin walled elongated unicellular conical of varying length; cortex consists of 2 to 3 layers of collenchyma cells followed by 5 to 10 layers of thin walled, round to oval polygonal parenchyma cells with intercellular spaces; vascular bundles arranged in the form of ring with pith in the centre; vascular bundle consisting of xylem below and phloem above; pericycle consisting of group of thick wall sclerenchyma cells; numerous calcium oxalate crystals or druses present in the parenchyma cell of the cortex and pith.

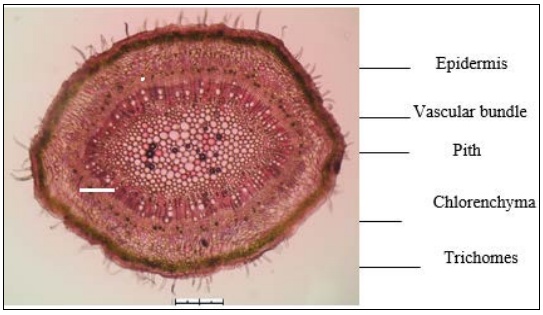


Fig 2: T. S. of Pedicel

Calyx: T. S. of calyx shows epidermis consisting of thin walled parenchyma cells; numerous trichomes are thin walled elongated unicellular of varying lengths upto 300 µ; mesophyll region consisting of calcium oxalate crystals 15 to 20 layers of thin walled polygonal parenchyma cells with intercellular spaces; 5 to 6 vascular strands present in the centre.

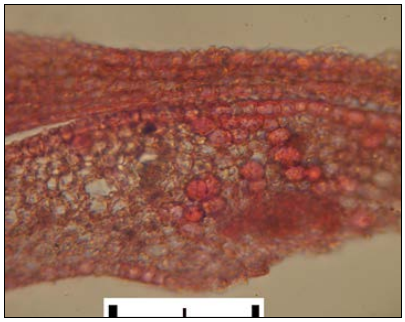


Fig 3: T. S. of Flower – Calyx & Corolla

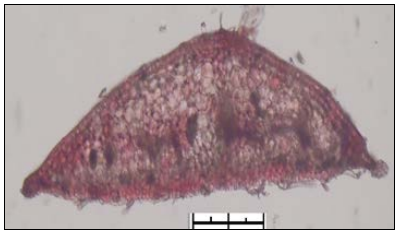


Fig 4: A-T. S. of Calyx

Fig 5: B- T. S. of Calyx – A Portion Enlarged

Corolla: T. S. of corolla shows epidermis consisting of rectangular parenchyma cells; non glandular thin walled unicellular conical trichomes upto 300 µ long; glandular trichomes of about 15 µ; mesophyll region consisting of numerous crystals thin walled parenchyma cells; 2 to 3 vascular strands present in the centre.

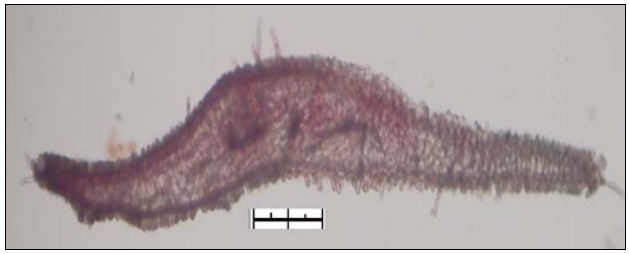


Fig 6: A-T. S. of Corolla

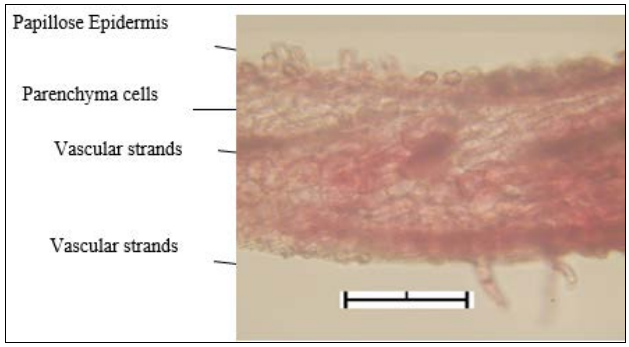


Fig 7: B- T. S. of Corolla – A Portion Enlarged

Androecium: T. S. of anther shows epidermis of staminal tube composed of thick walled rectangular parenchyma cells and the endothecium of the anther walls

Gynoecium: T. S. of gynoecium shows stigma sticky, parenchymatous epidermal cells, elongated into extensive papillae; style thin walled, rectangular; ovary superior, trilocular.

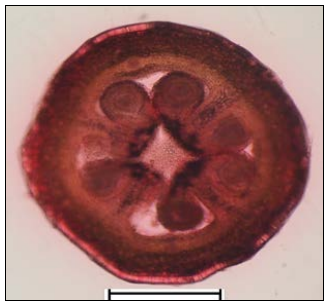


Fig 8: T. S. of Gynoecium

Pollen Grain: Porous, 4-colporate, spherical upto 25 µ, with a smooth exine.

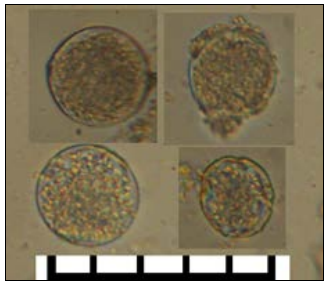


Fig 9: Pollen grains

Powder features: Yellowish-brown; numerous unicellular trichomes as well as glandular trichome base; fragments of parenchymatous papillose epidermal cells; fragments of anther wall; spiral vessels upto 20 µ; rosette of calcium oxalate crystals upto 10 µ; unicellular trichomes upto 300 µ; glandular trichomes upto 15 µ and yellowish-brown pollen grains upto 25 µ with distinct exine and intine.

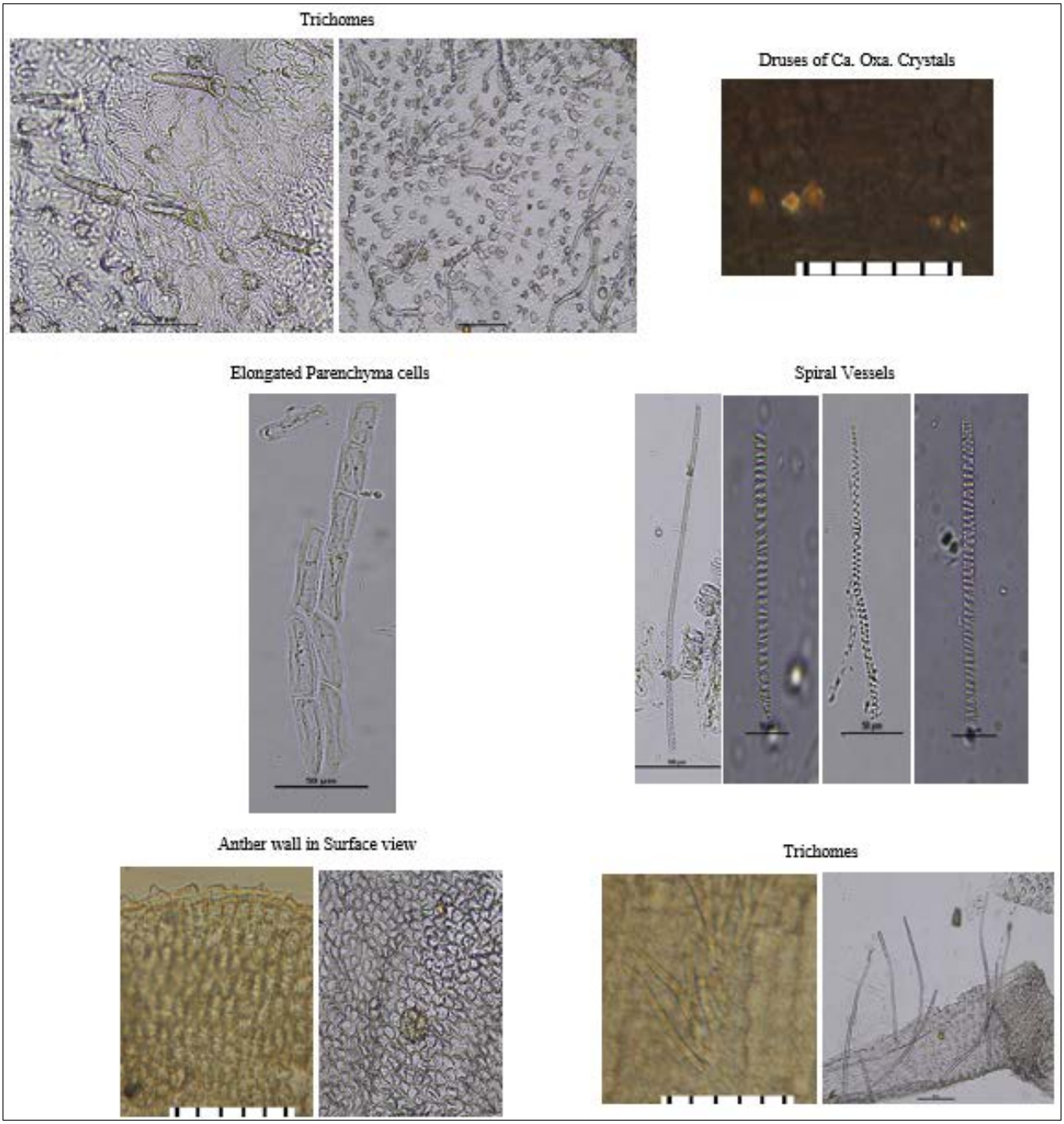


Fig 10: Powder features of *Azadirachta indica* A. Juss floral parts

3.2 Physicochemical Parameters

Physicochemical parameters of flower powder of Neem were estimated based on the methods recommended by World Health Organization (WHO) and API. The result shown in Table 1. Percent weight loss on drying value was found to be 9.60% presence of moisture content. The less value of moisture content of drugs could prevent content bacterial, fungal or yeast growth through storage. The total ash and acid insoluble ash values were found to be 7.81% and 1.02% respectively. It indicates the less amount of siliceous matter in acid insoluble ash. The pH of 5% w/v solution was found to be 6.8% respectively. The hexane soluble extractive values (1.92%) showed presence of low polar compounds, alcohol soluble extractive value (4.62%) indicated the extraction of polar constituents and water soluble extractive value (19.92%) due to presence of polar constituents and inorganic constituents.

Table 1: Physicochemical parameters of flower of *A. indica*.

Parameter	Value in percentage (%)
Foreign matter	Nil
Loss on drying at 105 °C	9.60%
Total ash	7.81%
Acid-insoluble ash	1.02%
Hexane extractive	1.94%
Alcohol extractive	4.62%
Water extractive	19.91%
pH	6.8
Volatile oil content	0.66%

3.3 TLC/HPTLC finger print studies of chloroform extract

The TLC studies of chloroform extract are tabulated in Table -2. In UV–254, 366 nm and visible light (Vanillin-sulphuric acid) it shows 5, 9 and 15 spots respectively with different Rf values (Fig. 8). The finger print of the chloroform extract was scanned at 254 nm shows 15 peaks of which peaks at R_f 0.09, 0.87, 0.93 and 0.97 were the major whereas peaks at Rf 0.03, 0.06, 0.16, 0.19, 0.31, 0.35, 0.44, 0.50, 0.59, 0.69 and 0.83 were moderately smaller peaks (Fig. 9).

The finger print of the chloroform extract was scanned at 366 nm shows 10 peaks of which peaks at R_f 0.06, 0.09 and 0.82 were the major whereas peaks at Rf 0.02, 0.18, 0.26, 0.29, 0.34, 0.59 and 0.91 were moderately smaller peaks (Fig.10).

Table 2: R_f values of the chloroform extract

Solvent System	Rf Values		
	UV- 254 nm	**UV – 366 nm**	**After derivatisation with vanillin–sulphuric acid reagent**
Chloroform: Methanol (9.8: 0.2)	0.88 (Dark green)	0.84 (Fluorescent red)	0.98 (Dark blue)
	0.18 (Light green)	0.79 (Dark blue)	0.91 (Light grey)
	0.10 (Dark green)	0.71 (Light red)	0.88 (Light pink)
	0.07 (Light green)	0.60 (Red)	0.78 (Light grey)
	0.04 (Light green)	0.57 (Light red)	0.71 (Light grey)
		0.50 (Light red)	0.70 (Grey)
		0.39 (Light red)	0.61 (Grey)
		0.29 (Light red)	0.57 (Light grey)
		0.26 (Fluorescent blue)	0.43 (Light violet)
			0.37 (Light grey)
			0.30 (Light grey)
			0.23 (Grey)
			0.20 (Dark violet)
			0.10 (Pink)
			0.05 (Dark grey)

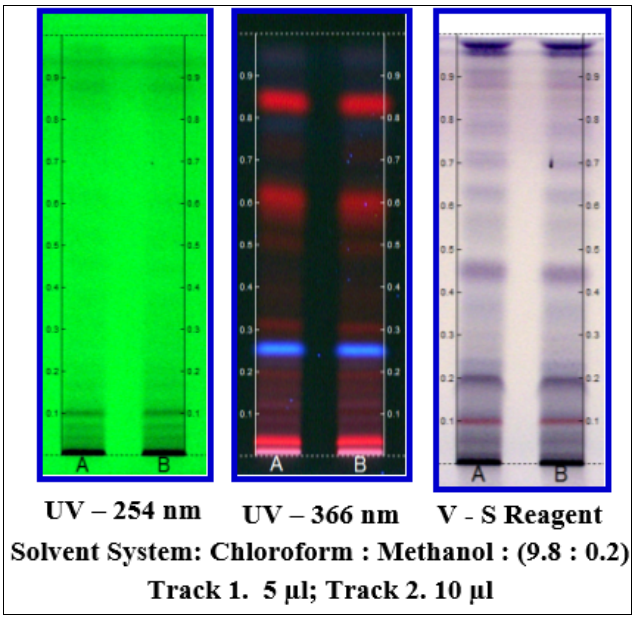
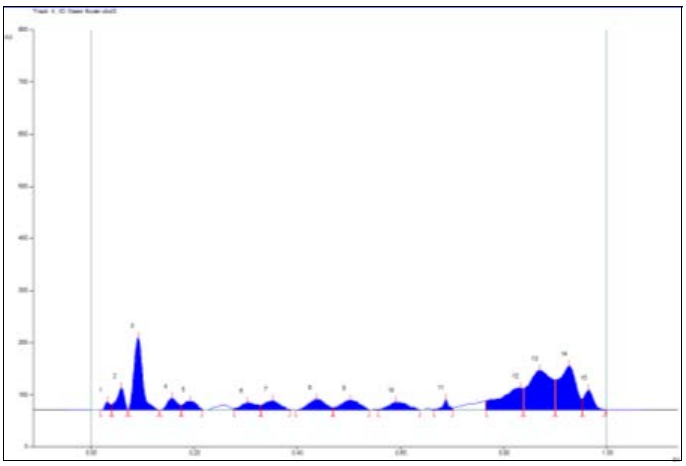
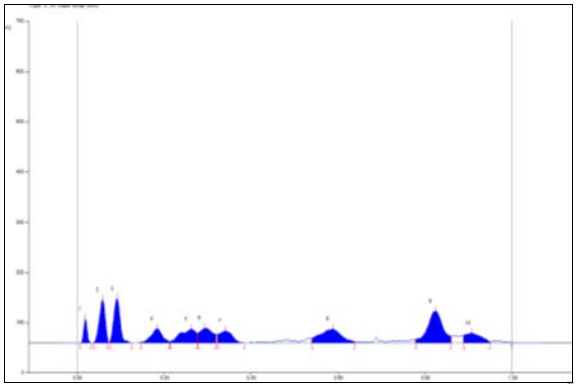


Fig 11: Photo Documentation of TLC of *A. indica* (Flower) in Chloroform extract



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.02 Rf	0.0 AU	0.03 Rf	15.4 AU	2.66 %	0.04 Rf	10.0 AU	127.7 AU	0.99 %
2	0.04 Rf	10.3 AU	0.06 Rf	42.0 AU	7.27 %	0.07 Rf	0.0 AU	511.9 AU	3.98 %
3	0.08 Rf	2.1 AU	0.09 Rf	138.5 AU	23.96 %	0.13 Rf	0.2 AU	1907.6 AU	14.82 %
4	0.14 Rf	0.0 AU	0.16 Rf	22.0 AU	3.81 %	0.17 Rf	9.0 AU	358.7 AU	2.79 %
5	0.18 Rf	9.2 AU	0.19 Rf	16.4 AU	2.84 %	0.22 Rf	1.8 AU	348.7 AU	2.71 %
6	0.28 Rf	2.2 AU	0.31 Rf	14.0 AU	2.42 %	0.33 Rf	8.4 AU	351.5 AU	2.73 %
7	0.33 Rf	8.6 AU	0.35 Rf	17.1 AU	2.97 %	0.39 Rf	0.8 AU	442.5 AU	3.44 %
8	0.40 Rf	0.2 AU	0.44 Rf	20.2 AU	3.50 %	0.47 Rf	3.8 AU	543.4 AU	4.22 %
9	0.47 Rf	4.0 AU	0.50 Rf	19.0 AU	3.28 %	0.54 Rf	0.1 AU	561.8 AU	4.37 %
10	0.56 Rf	1.3 AU	0.59 Rf	14.5 AU	2.50 %	0.64 Rf	0.2 AU	460.3 AU	3.58 %
11	0.67 Rf	1.4 AU	0.69 Rf	19.3 AU	3.34 %	0.70 Rf	4.0 AU	183.6 AU	1.43 %
12	0.77 Rf	17.0 AU	0.83 Rf	41.9 AU	7.24 %	0.84 Rf	40.8 AU	1522.3 AU	11.83 %
13	0.84 Rf	41.0 AU	0.87 Rf	76.1 AU	13.17 %	0.90 Rf	58.0 AU	2738.0 AU	21.28 %
14	0.90 Rf	57.5 AU	0.93 Rf	83.8 AU	14.50 %	0.95 Rf	20.6 AU	2238.7 AU	17.40 %
15	0.95 Rf	21.1 AU	0.97 Rf	37.8 AU	6.54 %	1.00 Rf	0.7 AU	571.3 AU	4.44 %

Fig 12: HPTLC finger print profile of chloroform extract of *A. indica* (Flower) at 254 nm



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.01 Rf	0.0 AU	0.02 Rf	47.4 AU	10.78 %	0.03 Rf	0.2 AU	312.1 AU	3.59 %
2	0.04 Rf	0.1 AU	0.06 Rf	85.8 AU	19.52 %	0.07 Rf	2.8 AU	973.6 AU	11.21 %
3	0.08 Rf	2.2 AU	0.09 Rf	87.8 AU	19.97 %	0.13 Rf	0.1 AU	1078.6 AU	12.42 %
4	0.15 Rf	0.8 AU	0.18 Rf	28.5 AU	6.49 %	0.21 Rf	2.5 AU	606.5 AU	6.98 %
5	0.22 Rf	2.6 AU	0.26 Rf	26.9 AU	6.13 %	0.28 Rf	19.1 AU	822.0 AU	9.46 %
6	0.28 Rf	19.5 AU	0.29 Rf	28.8 AU	6.56 %	0.32 Rf	16.9 AU	730.9 AU	8.41 %
7	0.32 Rf	16.8 AU	0.34 Rf	23.7 AU	5.39 %	0.39 Rf	0.0 AU	589.0 AU	6.78 %
8	0.54 Rf	9.0 AU	0.59 Rf	27.8 AU	6.32 %	0.64 Rf	2.2 AU	1131.2 AU	13.02 %
9	0.78 Rf	6.7 AU	0.82 Rf	63.2 AU	14.38 %	0.86 Rf	13.2 AU	1843.0 AU	21.22 %
10	0.89 Rf	15.0 AU	0.91 Rf	19.6 AU	4.46 %	0.95 Rf	2.3 AU	599.5 AU	6.90 %

Fig 13: HPTLC finger print profile of chloroform extract of *A. indica* (Flower) at 366 nm

3.4 TLC/HPTLC finger print studies of alcohol extract

The TLC studies of alcohol extract are tabulated in Table - 3. In UV–254, 366 nm and visible light it shows 3, 11 and 8 spots respectively with different Rf values (Fig. 5). The finger print of the alcohol extract was scanned at 254 nm shows 9 peaks of which peaks at R_f 0.01 and 0.07 were the major whereas peaks at Rf 0.03, 0.12, 0.20, 0.23, 0.40, 0.57 and 0.97 were moderately smaller peaks (Fig. 6). The finger print of the alcohol extract was scanned at 366 nm shows 6 peaks of which peaks at R_f 0.01 was major peak whereas peaks at Rf 0.03, 0.07, 0.12, 0.20 and 0.23 were moderately smaller peaks (Fig. 13).

Table 3: R_f values of the alcohol extract

Solvent System	Rf Values		
	UV- 254 nm	UV – 366 nm	After derivatisation with vanillin – sulphuric acid reagent
Chloroform: Methanol (9.8: 0.2)	0.20 (Light green)	0.94 (light blue)	0.99 (Dark grey)
	0.06 (Dark green)	0.40 (Dark blue)	0.69 (Light grey)
	0.02 (Light green)	0.30 (Light blue)	0.44 (Light grey)
		0.21 (Grey)	0.31 (Dark grey)
		0.20 (Light red)	0.23 (Grey)
		0.16 (Light violet)	0.14 (Light grey)
		0.14 (Light red)	0.07 (Light yellow)
		0.10 (Light violet)	0.02 (Light grey)
		0.08 (Light red)	
		0.04 (Violet)	
		0.02 (Light red)	

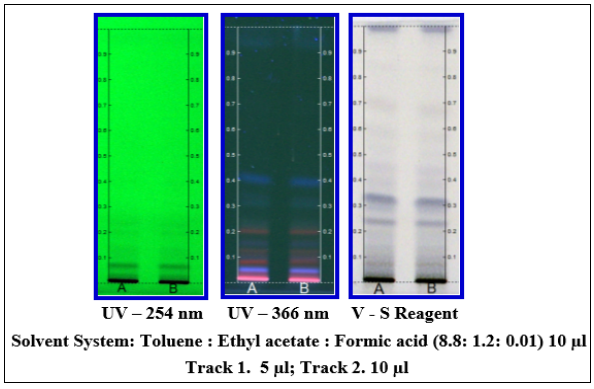
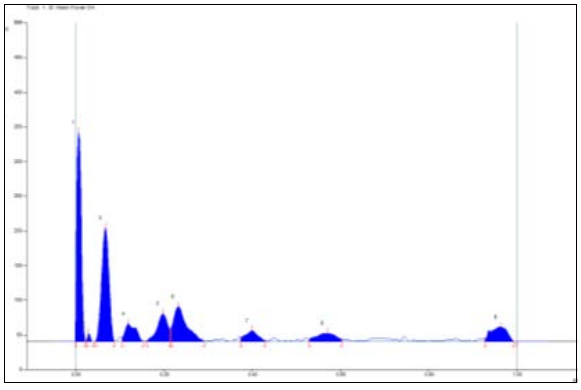
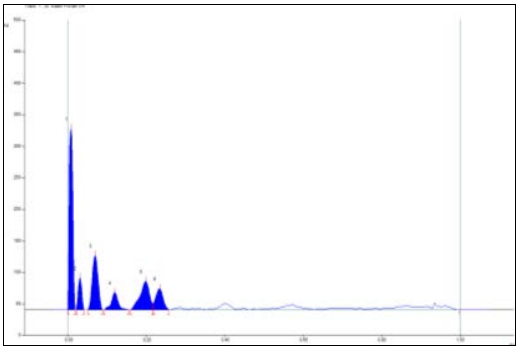


Fig 14: Photo Documentation of TLC of *A. indica* (Flower) in alcohol Extract



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.00 Rf	0.0 AU	0.01 Rf	301.5 AU	46.99 %	0.02 Rf	1.7 AU	2591.8 AU	28.76 %
2	0.03 Rf	3.9 AU	0.03 Rf	11.7 AU	1.82 %	0.04 Rf	0.0 AU	60.0 AU	0.67 %
3	0.05 Rf	0.6 AU	0.07 Rf	163.8 AU	25.52 %	0.09 Rf	0.3 AU	2272.7 AU	25.22 %
4	0.11 Rf	5.6 AU	0.12 Rf	25.5 AU	3.98 %	0.15 Rf	0.1 AU	528.3 AU	5.86 %
5	0.16 Rf	1.7 AU	0.20 Rf	40.3 AU	6.27 %	0.22 Rf	18.5 AU	798.2 AU	8.86 %
6	0.22 Rf	19.3 AU	0.23 Rf	50.2 AU	7.82 %	0.29 Rf	0.2 AU	1260.9 AU	13.99 %
7	0.38 Rf	6.1 AU	0.40 Rf	15.5 AU	2.41 %	0.43 Rf	1.5 AU	373.3 AU	4.14 %
8	0.53 Rf	3.3 AU	0.57 Rf	12.0 AU	1.87 %	0.60 Rf	3.5 AU	449.8 AU	4.99 %
9	0.93 Rf	4.3 AU	0.97 Rf	21.2 AU	3.31 %	0.99 Rf	0.2 AU	676.7 AU	7.51 %

Fig 15: HPTLC finger print profile of alcohol extract of *A. indica* (Flower) at 254 nm



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.00 Rf	0.0 AU	0.01 Rf	287.6 AU	54.09 %	0.02 Rf	1.9 AU	2299.3 AU	41.00 %
2	0.02 Rf	0.2 AU	0.03 Rf	50.9 AU	9.57 %	0.04 Rf	2.2 AU	394.3 AU	7.03 %
3	0.05 Rf	0.5 AU	0.07 Rf	86.5 AU	16.27 %	0.09 Rf	0.7 AU	1082.7 AU	19.31 %
4	0.09 Rf	0.0 AU	0.12 Rf	27.6 AU	5.19 %	0.16 Rf	0.0 AU	396.1 AU	7.06 %
5	0.16 Rf	0.2 AU	0.20 Rf	45.4 AU	8.53 %	0.22 Rf	10.2 AU	907.2 AU	16.18 %
6	0.22 Rf	10.6 AU	0.23 Rf	33.7 AU	6.34 %	0.26 Rf	0.1 AU	528.5 AU	9.42 %

Fig 16: HPTLC finger print profile of alcohol extract of *A. indica* (Flower) at 366 nm

4. Conclusion

Morphology as well as various microscopical parameters of the *A. indica* were studied and described along physico-chemical parameters and TLC/ HPTLC Finger Print studies for authentification of crude drug which will help in quality control and detection of adulteration of the raw drug. It exhibits a set of diagnostic characters, which will help to identify the drug in fragmentary condition as well as in whole form. This study will contribute to the existing knowledge over the standardization aspects of the raw drug *A. indica*.

5. Acknowledgment

Authors are thankful to the Director, CCRUM, New Delhi for financial support.

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