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PHYTO-PHARMACOGNOSTICAL EVALUATION AND HPTLC FINGER-PRINTING PROFILE OF LEAVES OF SAPTAPARNA [*ALSTONIA SCHOLARIS* (L.) R. BR.]

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Keywords:

Alstonia scholaris leaves, Pharmacognostical studies, Physicochemical parameters, Phytochemical analysis, HPTLC

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ABSTRACT: Objective: We report the phyto-pharmacognostic evaluation and HPTLC finger-printing profile of the methanolic extract of Saptaparṇa [*Alstonia scholaris* (L.) R. Br.] leaves – unaffected and gall-affected, collected in January 2019. **Materials and Methods:** Physicochemical parameters and phytochemical screening were done by standard procedures. HPTLC finger-printing profile of the methanolic extracts of dried leaves and dried leaves with gall was performed on pre-coated TLC silica gel $60F_{254}$ plates with hexane: EtOAc: MeOH: formic acid (5:4.5:0.5:0.5 v/v) as developing solvent, with observation at 254 and 366 nm. **Results:** Some of the leaves had 15-20 galls on both surfaces. Powder Microscopy of the bitter-tasting green color leaf powder showed a group of epidermal cells, unicellular trichomes, elongated stone cells in the group, prismatic calcium oxalate crystals, single-layered fibers, reticulate vessels, anomocytic stomata, and reddish-brown color tannin matter; epidermis of gall cells, insect body in affected leaves. Phytochemical screening revealed the presence of alkaloids, carbohydrates, terpenoids/steroids, phenolic compounds/ tannins, but an absence of reducing sugars and flavonoids. A large number of bands were observed on HPTLC chromatograms – many showed red fluorescence at 366 nm. Methanolic extracts of leaves, with or without galls, gave similar results as regards the number and position of the bands, an additional band of low R_f was discernible in the extract of leaves with gall. However, there were some instances of variations in peak intensities, a few exhibiting marked differences. **Conclusion:** There is a clear indication that there is a significant difference between concentrations of some phytoconstituents in leaves with and without galls. The phyto-pharmacognostical studies and HPTLC finger-printing profiles can be used for the authentication and identification of the leaves of this medicinally important plant.

INTRODUCTION: There is a global resurgence of interest in traditional Asian schools of medicine, which use plant-based materials and their formulations.

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The authentication of the drugs by phyto-pharmacognostic studies and HPTLC fingerprinting proceed simultaneously with the search for chemical components, which contribute to the biological properties of drugs.

Saptaparṇa, *i.e.*, *Alstonia scholaris* (L.) R. Br. is a reputed medicinal plant in the Indian traditional medicine system of Ayurveda, as well as in traditional medicine systems of South Asian countries. Earlier studies on this plant had been carried out at the University of Calcutta and the

Calcutta unit of the Central Council for Research in Ayurveda and Siddha (CCRAS), which has developed to the present Central Ayurveda Research Institute for Drug Development (CARIDD). At present, a thorough re-investigation of this plant is being carried out at CARIDD in order to gather further information regarding various aspects to supplement the existing literature on this plant. *Alstonia scholaris* (L.) R. Br. (Family Apocynaceae; tribe - Plumeriae; Subtribe - Alstoniinae) is a tall evergreen tree, up to 20 meters high, growing mostly in deciduous forests in sub-Himalayan tracts in India from Jammu eastwards to West Bengal and also in the Western peninsula. It occurs in other countries of the Indian subcontinent, *viz*. Bangladesh, Nepal, Pakistan, Sri Lanka as well as in a number of other Southeast Asian countries. It is called devil tree or dita bark in English, Saptaparṇa (sapta: seven, parna: leaves) in Sanskrit, Saptaporsi in Odiya, Chhatim in Bengali [1-3].

The leaves occur in whorls of usually seven, though arrangements of five to ten have been mentioned; the leaves are narrowly obovate to very narrowly spathulate, base cuneate. Lateral nerves are numerous, parallel, and terminate in an intramarginal vein. The tree is fairly widespread in the city of Kolkata, being planted in several places in parks and on street verges. Gall formation is observed on the leaves, particularly during November to January.

Description of the pharmacognosy of the stem-bark is recorded in the Ayurvedic Pharmacopoeia of India [3]. We have recently reported systematic pharmacognostic and phytochemical studies as well as HPTLC profiling on the roots of this plant [4, 5]; no pharmacognostic studies on the roots had been reported earlier to our knowledge.

Different parts of the plant are used as drugs [1-3]: the bark is alterative, anti dysenteric, anthelmintic, astringent, antileishmanial, antimicrobial and antimalarial; decoction of the leaves is used in the treatment of beriberi. The roots [6] show antibacterial and anthelmintic properties, antimalarial activity, and are used in the treatment of leprosy and an enlarged liver with pain. The stem-bark has been used in the antimalarial drug formulation Ayush-64, developed by the CCRAS unit at Calcutta.

Pioneering work on the alkaloidal constituents of *Alstonia scholaris* and *Alstonia macrophylla* were carried out by Professor Asima Chatterjee's research group and her associates at the Chemistry Department, University of Calcutta [7-9]. Other research groups followed suit. A large number of phytoconstituents, particularly indole alkaloids, have been isolated from different parts of plants of *Alstonia* species [7-13]; though more remain to be characterized.

In view of the medicinal importance of *Alstonia* species, research work is still continuing in different countries [10-23]. *A. scholaris* plant formulations are used to treat diarrhea [10], wounds and earache [14], fever [15]. Publications have appeared on phytochemical constituents and antioxidant activity of *A. scholaris* [16], the anti-proliferative activity of triterpenoids and sterols from *A. scholaris* against lung carcinoma cells [17], the antidiabetic effect of *A. scholaris* Linn. Bark [18], ameliorative effect of alkaloidal fraction of *A. scholaris* leaves against acetic acid-induced Colitis [19], the anticarcinogenic and antimutagenic activity of *A. scholaris* [20], the study of the interaction of human serum albumin with *A. scholaris* leaf extract-mediated silver nanoparticles having bactericidal property [21], the ameliorative potential of *Alstonia scholaris* (Linn.) R. Br. against chronic constriction injury-induced neuropathic pain [14], *in-vitro* anti-bacterial and HPTLC study of of *Alstonia scholaris* latex to substantiate its ancient usage [22], astringent, thermogenic, cardiotonic properties of chloroform extracts of *Alstonia scholaris* [23].

A number of recent publications [24-28] have reported the isolation and characterization of new indole alkaloids.

Our present communication reports phyto-pharmacognostic studies and HPTLC studies on the leaves of *A. scholaris*. This follows our earlier reports of systematic pharmacognostic studies, as well as phytochemical studies and HPTLC profiling of extracts of roots of this plant [4, 5].

MATERIALS AND METHODS:

Collection and Authentication of Plant Material:

Alstonia scholaris (L.) R.Br. leaves were collected from CN Block, Sector 5, Salt Lake City, Kolkata in January 2019, **Fig. 1a, b**. The tree was identified

by Professor N. D. Paria, Indira Gandhi National Open University, and formerly of Botany Department, University of Calcutta. A voucher sample of the roots, stem bark, leaves, and flowers of *Alstonia scholaris* have been deposited (Voucher number - CRD/Chem/AS) at CARIDD, Kolkata.

FIG. 1: (A) AND (B) *ALSTONIA SCHOLARIS* TREE DURING FLOWERING SEASON (JANUARY 2019). SOME OF THE LEAVES BEAR GALLS

Plant Sample Processing: The leaves were dried at ambient temperature in the shade for 7 days. After drying, approximately 170 g of the dried leaves were obtained. Approximately 40g in total was ground with a grinder and sieved to obtain 60 mesh size powder; these were used for organoleptic and microscopical studies. The rest of the leaves, sorted into two groups – those with galls (about 20g) and without galls – were separately ground to coarser powders, which were used for determining Physicochemical Parameters including extractive values and carrying out preliminary phytochemical analysis and HPTLC analyses.

Physicochemical Parameters: These were determined in accordance with standard protocols, as mentioned in Ayurvedic Pharmacopoeia of India/ WHO protocols [29, 30].

Preliminary Phytochemical Analysis: These were done using standard chemical tests.

Solvents and Chemicals: The solvents and chemicals were of GR grade (E. Merck Ltd., Mumbai, India).

Macroscopy of Plant Material: The organoleptic parameters, *viz.* texture, shape, size, color, *etc*. of the plant material were noted by naked eye observation with a simple microscope Olympus OIC DM.

Cytomorphology of Plant Material: For powder microscopy, finely powdered samples (~2 g) were separately treated with different reagents, *viz.* aqueous saturated chloral hydrate (for maceration), 50% glycerin, phloroglucinol in concentrated HCl (for staining lignified tissues) and 0.02 N iodine reagent (for starch grains), mounted on slides with glycerin following a standard protocol and observed under the binocular compound microscope (Olympus OIC- 07964) at 10X and 40X magnifications.

Photomicrographs of the different cellular structures and inclusions were taken using Magcam DC14 camera attached with Olympus CX21i trinocular compound microscope.

RESULTS AND DISCUSSION:

Pharmacognostical Analysis of *Alstonia scholaris* Leaves:

Macroscopy: In the collected samples, the leaves usually occur 7 in the group (Saptaparṇa), occasionally 6-8 in the group; 11-20 cm in length, 2-4 cm in breadth. Few leaves had 15-20 galls on both the surfaces of the leaf. The petiole is short, and the upper surface of the leaf is glossy and dark green compared to the lower surface, which is dull and lighter. Fresh leaves are yielding bitter and milky sap when injured. The leaves are odorless and bitter-tasting.

FIG. 2: MORPHOLOGY OF *ALSTONIA SCHOLARIS* LEAVES – (A) FRESH LEAVES WITH TYPICAL SAPTAPARNA ARRANGEMENT; (B) LEAVES (DRIED) WITHOUT GALLS; (C) LEAVES WITH GALLS

Powder Microscopy: Green color leaf powder shows a group of epidermal cells, unicellular trichomes, elongated stone cells in group, prismatic calcium oxalate crystals, single-layered fibers, epidermis of gall cells, insect body, reticulate vessels, anomocytic stomata and reddish-brown color tannin matter. The powder is odourless and of bitter taste.

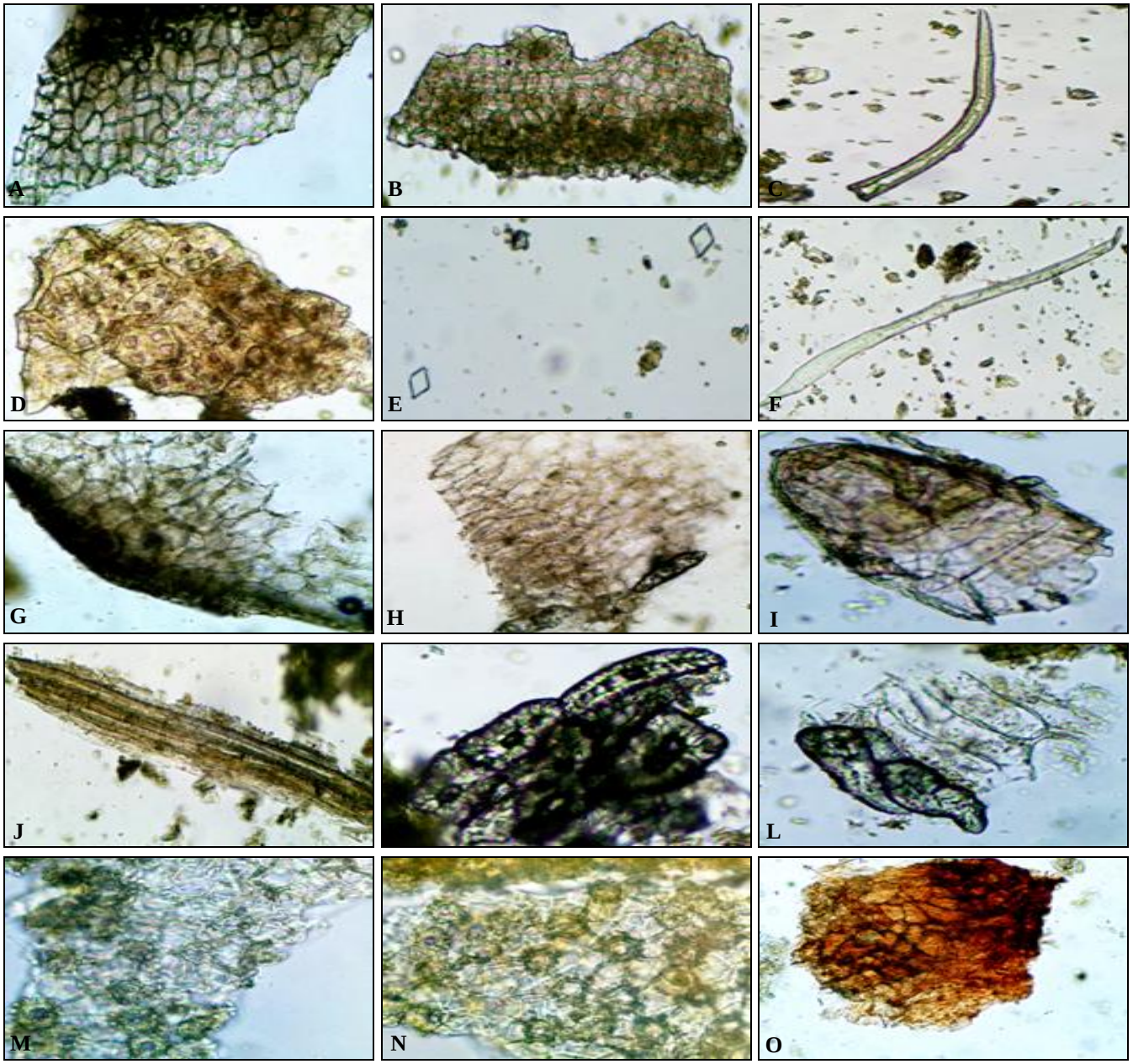

FIG. 3: PHOTOMICROGRAPHS OF *ALSTONIA SCHOLARIS* LEAF POWDER. A, B - Epidermal cells; C - Unicellular trichome; D, E - Prismatic calcium oxalate crystals; F - Fiber; G, H - Epidermal cells of gall cells; I - Insect body; J - Reticulate vessels; K, L - Elongated stone cells; M, N - Anomocytic Stomata; O - Tannin matter.

Physicochemical Parameters: Determination of the following physicochemical parameters were carried out - methanol soluble extractive, loss on drying, total ash, sulphated ash, pH of 10% aqueous suspension **Table 1**. These were determined in accordance with standard protocols as mentioned in Ayurvedic Pharmacopoeia of India/ WHO protocols [29, 30]. 4 g of the dried and powdered plant part (dried leaves) of *Alstonia scholaris* was taken in a 250 ml round bottom flask and refluxed with methanol (100 ml) for 1hr. The whole was kept overnight (for 18 h) at ambient temperature. The extract was filtered through fluted filter paper (Whatman no. 40). 25 ml of this filtrate was taken for determining the methanol extractive – this was heated to constant weight at 106° for 5 h, cooled and weighed. The rest of the filtrate was utilised for preliminary phytochemical testing.

TABLE 1: PHYSICOCHEMICAL EVALUATION OF *ALSTONIA SCHOLARIS* LEAVES

S. no.	Parameters	Result (in w/w %)
1	Loss on Drying	10.9%
2	Total Ash	6.85%
3	Sulphated ash	12.1%
4	Methanol soluble extractive	12.7%
5	pH of 10% aqueous suspension	7.0

Preliminary Phytochemical Analysis: The methanolic extract, obtained as described in the previous section was utilised for preliminary phyto-chemical testing – 2 ml of the extract being taken for each test. The results are given in **Table 2**.

TABLE 2: QUALITATIVE PHYTOCHEMICAL TESTS OF METHANOLIC EXTRACTS OF *ALSTONIA SCHOLARIS* LEAVES WITHOUT GALLS

S. no.	Test/Reagent used	Observation
1a	Alkaloids - Mayer's test (in dil. HCl)	+
1b	Alkaloids - Dragendorff's test (in dil. HCl)	+
2a	Carbohydrates – Molisch's test	++
2b	Reducing sugars - Fehling's test	-
3	Flavonoids - Shinoda test	-
4	Terpenoids/ Steroids – (Liebermann-Burchardt Test)	++ (deep green changing to light violet and finally to deep violet)
5	$FeCl_3$ test Phenolic compounds/ tannins	++ (transient deep blue-green coloration changing to greenish-brown – greenish brown ppt. after 5 min)
6	Amino Acids - Ninhydrin test	-
7	20% NaOH test	+ (deep yellow coloration, on addition of conc. HCl – fine white ppt.)
8	Lead acetate test	+

Note:- (+) Trace amount, (++) Higher amount, (-) Absent.

HPTLC Finger-Printing Profile of *Alstonia scholaris* Roots:

Chromatography Experiment: Dried and powdered *Alstonia scholaris* leaves (AlScL) and leaves with galls (AlScLG) were extracted by refluxing separately with methanol; the extracts were subjected to HPTLC analysis.

Sample Preparation: General Procedure - 1g of the dried and powdered plant part (dried leaves and dried leaves with gall separately) of *Alstonia scholaris* was taken in a 100ml round bottom flask and refluxed with methanol (25ml) for 1hr. The whole was kept overnight (for 18 hr.) at ambient temperature. The extract was filtered through fluted filter paper (Whatman no. 40). The filtrate was taken for HPTLC profiling. The extracts were designated AlScLM and AlScLgM, respectively.

Stationary Phase: Precoated (support on Aluminum Sheets) Silica Gel Plate. Specification: TLC Silica Gel $60F_{254}$, Merck.

Mobile Phase – Hexane: EtOAc: MeOH: Formic Acid (5:4.5:0.5:0.5) v/v.

Sample Application: Applied volume - 5µL as 8mm band and applied at 12 mm from the base of the plate, with a CAMAG ATS4. The plate size was 10 × 5 cm.

Development: Developed up to 80mm in CAMAG Twin trough chamber, Plate preconditioning – temperature 27 °C; relative average humidity was 48%.

Observation: The chromatograms were visualized in CAMAG TLC visualiser, and scanned using a CAMAG TLC Scanner 4.

General Comments: The HPTLC chromatograms are given in **Fig. 4** (visualized at 254 nm), and **Fig. 5** (visualized at 366 nm). Corresponding Densitometric fingerprint profiles are given in **Fig. 6** and **Fig. 7.**

TABLE 3A: R_f VALUES AND RELATIVE AREAS OF THE HPTLC PEAKS OF *ALSTONIA SCHOLARIS* NORMAL LEAVES WITHOUT GALLS – METHANOL EXTRACT (AlScLM) VISUALISED AT 254 nm

S. no.	R_f	Height of the Peak (AU)	Relative Area (%)
1	0.06	26.6	7.25
2	0.17	10.7	2.42
3	0.28	68.8	20.29
4	0.38	95.3	36.58
5	0.43	30.9	7.10
6	0.47	17.1	4.60
7	0.53	23.8	5.44
8	0.60	19.4	5.79
9	0.66	14.3	4.02
10	0.71	21.7	6.52

The more intense and sharper bands have been listed, along with their R_f values, absolute intensities, and relative areas – **Table 3a, b** (visualization at 254 nm); **Table 4a, b** (visualization at 366 nm). In the solvent system studied, the methanolic extracts of both sets of leaves – with or without galls – gave very similar results as regards the number and position of the bands, which were nearly identical. However, there were some instances of variations in peak intensities, some exhibiting marked differences.

This is a clear indication that there is a significant difference between concentrations of some phytoconstituents in leaves with and without galls. An additional band of low R_f was discernible in the extract of leaves with gall.

TABLE 3B: R_f VALUES AND RELATIVE AREAS OF THE HPTLC PEAKS OF *ALSTONIA SCHOLARIS* LEAVES WITH GALLS – METHANOL EXTRACT (AlScLgM) VISUALISED AT 254 nm

S. no.	R_f	Height of the Peak (AU)	Relative Area (%)
1	0.03	25.7	2.82
2	0.06	17.6	2.43
3	0.28	36.0	10.02
4	0.38	128.2	44.74
5	0.42	31.6	5.38
6	0.46	30.9	7.36
7	0.52	34.6	8.66
8	0.65	31.8	7.23
9	0.70	45.0	11.37

FIG. 4: PHOTOGRAPHY OF HPTLC PLATE – VISUALISATION AT 254 nm - Plate 1. Track -1 (T-1) - *Alstonia scholaris* leaves without galls (AlScLM); Track-2 (T-2) - *Alstonia scholaris* leaves with gall (AlScLgM).

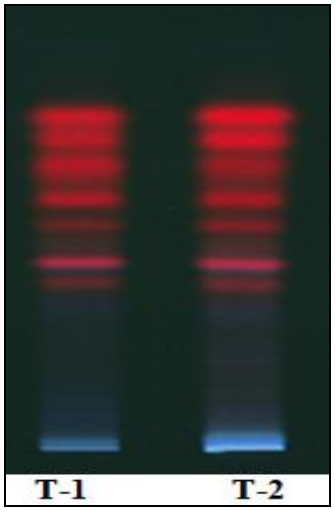

FIG. 5: PHOTOGRAPHY OF HPTLC PLATE – VISUALIZATION AT 366 nm - PLATE 2. Track -1 (T-1) - *Alstonia scholaris* leaves without galls (AlScLM); Track-2 (T-2) - *Alstonia scholaris* leaves with gall (AlScLgM)

TABLE 4A: R_f VALUES AND RELATIVE AREAS OF THE HPTLC PEAKS OF *ALSTONIA SCHOLARIS* NORMAL LEAVES WITHOUT GALLS – METHANOL EXTRACT (AlScLM) VISUALISED AT 366 nm

S. no.	R_f	Height of the Peak (AU)	Relative Area (%)	Colour of Fluorescence
1	0.07	31.7	5.41	Light blue
2	0.16	14.9	2.04	Bluish black
3	0.28	16.8	2.26	Bluish black
4	0.34	30.2	3.76	Red
5	0.38	125.2	17.81	Light red
6	0.43	49.4	4.98	Brownish red
7	0.46	39.1	5.07	Red
8	0.52	87.4	12.41	Red
9	0.60	109.7	22.19	Red
10	0.66	73.8	11.71	Red
11	0.70	84.9	12.36	Red

TABLE 4B: R_F VALUES AND RELATIVE AREAS OF THE HPTLC PEAKS OF *ALSTONIA SCHOLARIS* LEAVES WITH GALLS – METHANOL EXTRACT (AlScLgM) VISUALISED AT 366 nm

S. no.	R_f	Height of the Peak (AU)	Relative Area (%)	Colour of Fluorescence
1	0.03	64.3	3.40	Light blue
2	0.06	10.9	0.72	Light blue
3	0.27	13.2	1.35	Bluish black
4	0.33	46.1	5.08	Red
5	0.38	158.4	20.53	Light red
6	0.42	54.6	4.40	Brownish red
7	0.46	62.6	5.49	Red
8	0.52	107.6	13.20	Red
9	0.59	70.1	9.78	Red
10	0.65	131.0	16.30	Red
11	0.70	168.7	19.76	Red

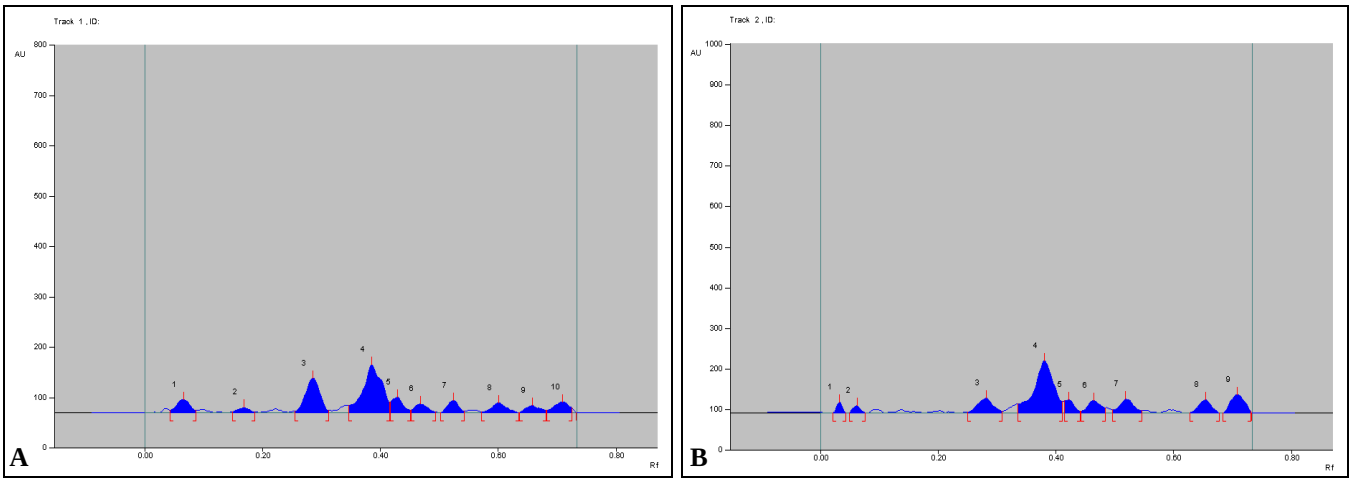

FIG. 6: DENSITOMETRIC FINGERPRINT PROFILES AT 254 nm OF *ALSTONIA SCHOLARIS*.
A: *ALSTONIA SCHOLARIS* NORMAL LEAVES WITHOUT GALLS – METHANOL EXTRACT (AlScLM)
B: *ALSTONIA SCHOLARIS* LEAVES WITH GALLS – METHANOL EXTRACT (AlScLgM)

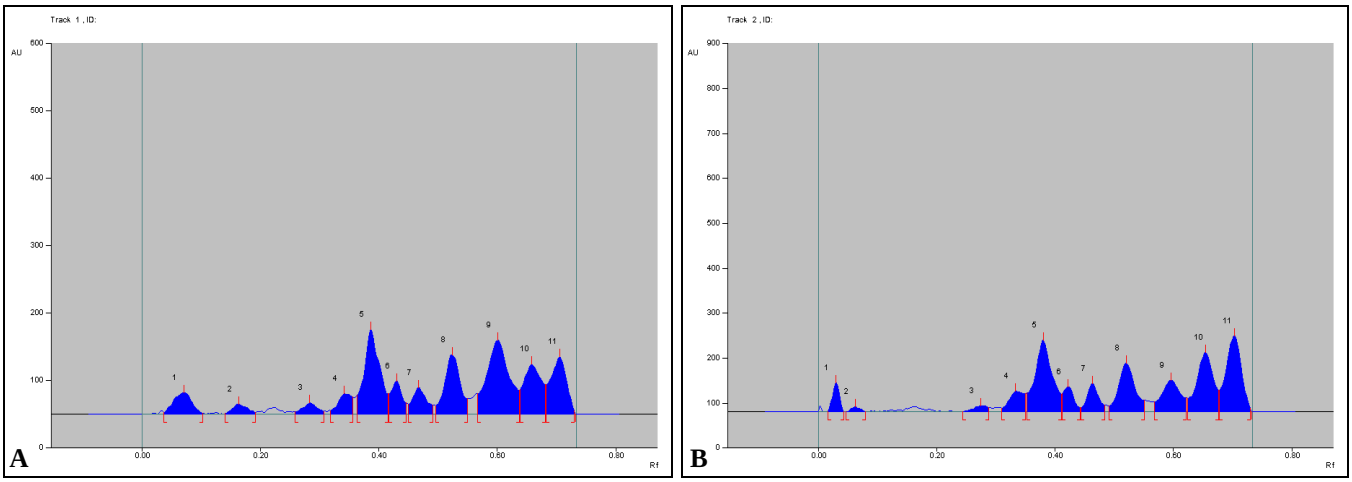

FIG. 7: DENSITOMETRIC FINGERPRINT PROFILES AT 366 NM OF *ALSTONIA SCHOLARIS*
A: *ALSTONIA SCHOLARIS* NORMAL LEAVES WITHOUT GALLS – METHANOL EXTRACT (AlScLM).
B: *ALSTONIA SCHOLARIS* LEAVES WITH GALLS – METHANOL EXTRACT (AlScLgM).

CONCLUSION: This communication furnishes an account of phyto-pharmacognostic studies of leaves of Saptaparṇa [*Alstonia scholaris* (L.) R.Br.]. Anomocytic Stomata, unicellular trichomes, elongated stone cells, prismatic calcium oxalate crystals act as cellular diagnostic characters in Saptaparṇa leaves. Preliminary chemical analysis of classes of secondary metabolites showed the presence of alkaloids, carbohydrates (positive Molisch's test), terpenoids/steroids (positive

Liebermann-Burchardt test), phenolic compounds/ tannins (positive $FeCl_3$ test); but the absence of reducing sugars and flavonoids. HPTLC finger-printing profiles of the methanolic extracts of both dried leaves and dried leaves with gall were performed on precoated (support on Aluminum sheets) TLC Silica Gel $60F_{254}$ plates with hexane: EtOAc: MeOH: formic acid (5:4.5:0.5:0.5 v/v) as developing solvent, with observation at 254 and 366 nm. A large number of bands were obtained - many of which showed red fluorescence at 366 nm. In the HPTLC chromatograms, the methanolic extracts of both sets of the leaves - with or without galls - gave very similar results as regards the number and position of the bands, which were nearly identical. However, there were some instances of variations in peak intensities, a few exhibiting marked differences. This is a clear indication that there is a significant difference between concentrations of some phytoconstituents in leaves with and without galls. An additional band of low R_f was discernible in the leaves with gall extract. The phyto-pharmacognostic studies and HPTLC finger-printing profiles can be used for the authentication and identification of the leaves of this medicinally important plant.

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CONFLICTS OF INTEREST: The authors declare no conflict of interest.

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