Pharmacognostical Evaluation and HPTLC Fingerprinting Studies of *Millingtonia Hortensis* L. f. Leaf

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Abstract

Millingtonia hortensis L.f. is commonly known as Cork tree belongs to the family Bignoniaceae. It is an important medicinal plant in Southern Asia such as India, Burma, Thailand and South China. The leaves of the plant were collected freshly and subjected to macro-microscopic, physico-chemical and quality control parameters to fix the authenticity and quality standards of the plant. Microscopical studies showed the presence of wavy epidermal cells with anomocytic stomata; palisade and spongy parenchyma cells; wide pitted vessels with tails at one or both the ends; fibres; glandular trichomes up to 50µ with 16 head cells and unicellular trichomes up to 200µ length. The physico-chemical data showed moisture content as 8.42%, total ash 8.93%, acid insoluble ash 0.098% and alcohol and water soluble extractive values as 5.92% and 24.44% respectively. TLC/HPTLC studies of chloroform and alcohol extracts showed various spots / peaks at 254nm, 366nm and in derivatized plates (Vanillin-sulphuric acid reagent). Quality control parameters such as microbial content and the heavy metals (As, Cd, Pb and Hg) were found to be within the permissible limit. The aflatoxins B_1, B_2, G_1 and G_2 were not detected. The study will be useful for the identification and authentication of the plant in dry form as well as in fresh form. The evaluated phytochemical data will serve as pharmacopoeial standards in the near future for any analytical and biological studies.

Keywords: *Millingtonia hortensis*, pharmacognostical characters, physico-chemical analysis, TLC/HPTLC studies, Quality control parameters.

Introduction

Millingtonia hortensis L.f. the sole species of the genus *Millingtonia* belongs to the family Bignoniaceae (Lindley and Moore, 1866) and originated from South-East Asia and South Asia (Gamble, 1921). It is also found in Central India, Myanmar (Burma) and Thailand. In India it is widely distributed and cultivated in many parts including the semi-arid regions of Rajasthan (Kaushik and Saini, 2008). The plant is commonly known as Indian Cork tree. Some of its other names are Akas Nim, Nim Chameli, Betati Nim, Mini Chameli, Maramall, Tree jasmine, Karkku, Kat Malli and Kavudi (Ramasubramaniaraja, 2010).

It is a very tall deciduous tree which grows up to 25m with straight trunk and a few branches. The leaves are pinnately compound and ornamental. The tree usually blooms from October to December, sheds leaves between January and March and renew during April and May. It flowers at night and shed early in the

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morning. The flowers are long tubular, silvery white with delightful fragrance. The arrangement of flowers is corymbose and bears capsule type of fruits (Mayuranathan, 1981).

It is an important medicinal plant in Southern Asia such as India, Burma, Thailand and Southern China where the leaves are used as tonic in folklore medicine and reported to have many medicinal values like antipyretic, sinusitis and cholagogue. The plants also have reported to possess antibacterial, antifungal, anticonvulsant and larvicidal activities (Ramasubramaniaraja, 2010). Flowers are reported to possess antioxidant, hepatoprotective, antiphlorgistic and antiasthmatic properties (Surendra Kumar *et al.*, 2014). Stem bark is used as antioxidant, antihelmintic drugs and also have other pharmacological activities like induction of apotosis on RKO colon cancer cell lines (Siwapong Tansuwanwong *et al.*, 2006).

Various phyto-constituents have been reported to be identified and isolated from various parts of the plants like lapachol, β-sitosterol and poulownin (roots), β-sitosterol (heart wood and bark), hispidulin, rutinoside, a flavonoid dinatin together with β-carotene (leaves), scutellarein, hispidulin and scutellarein-5-glucuronide (flowers) and acetyl oleanolic acid (fruit) (Aruna Kumari and Sharma, 2013) etc. Studies on the mutagenicity and antimutagenicity of hispidulin and hortensin (flavonoids) of the plant were also reported (Blatter and Millard, 1954).

The present investigation deals with the evaluation of morphological, anatomical, physico-chemical, TLC/HPTLC fingerprints and quality control parameters of leaves of the plant *M. hortensis*. The morphological and anatomical studies will provide the information for correct identification and authentication of the plant material whereas the other phytochemical studies will serve as pharmacopoeial standards for the plant in the near future for any phytochemical and biological related studies.

Material and Methods

Pharmacognostical Studies

The leaves of the plant *M. hortensis* were collected from the herbal garden of Regional Research Institute of Unani Medicine (RRIUM), Chennai, during the month of October 2016 and identified with the help of Flora of Presidency of Madras (Gamble, 1921.). The morphological authenticity of the plant was referred and compared with the herbarium specimen (*M. hortensis* - voucher specimen No. RRIUMCH12525), Department of Survey of Medicinal Plants Unit (SMPU), RRIUM, Chennai.

The fresh leaves were macroscopically examined for shape, size, surface characteristics, texture, color, odour and taste. The macroscopical, microscopical and powder microscopy were carried out using standard methods (Johansen, 1940). Free hand sections of the leaves were taken and its photo micrographs were recorded using MIPS camera attached with the microscope. Quantitative microscopical studies like vein islet number, veinlet termination number, palisade ratio, stomatal number and stomatal index were studied as per the standard procedures (Wallis, 1985; Kokate, 1994; UPI, 2009).

Physico-chemical Parameters

Physico-chemical parameters like foreign matter, total ash, acid insoluble ash and loss on drying at 105°C, alcohol and water soluble extractives were carried out as per the standard method (WHO, 2011).

TLC/ HPTLC Analysis

The TLC/HPTLC analysis was performed for chloroform and alcohol extract of the leaves of *M. hortensis*. The sample (5µl each extract) was applied on pre-coated silica gel 60 F_{254} TLC plate (E Merck) and developed using Toluene: ethyl acetate (1:1) solvent systems as mobile phase for both extracts. The developed plates were scanned densitometrically at 254nm, 366nm and derivatized using spray reagent Vanillin sulphuric acid. The Retention factor (Rf) values, peak area and peak height were determined (Wagner, 1984, Sethi, 1996).

Quality Control Parameters

Quality control parameters like microbial load and aflatoxin were carried out as per the WHO guidelines (WHO, 2007). Heavy metals analysis was done by atomic absorption spectrophotometer (AOAC, 2005). Pesticide residues were analyzed using GC-MS agilent instrument equipped with mass selective detector as per the methods of AOAC (AOAC, 2005).

Results

Macroscopic

Leaves large, imparipinnate and ornamental (Figure-1) Long leaf bears two or three widely spaced pinnae, each with 5-7 smooth leaflets; Leaflets oval, pointed, slightly toothed and 1-3 inches long. Sometimes the lower pinnae again divided and bear one pair of three leaved pinnae, 1-2 pairs of leaflets with one leaflet at the end. The leaves are slightly bitter in taste and odourless.

Microscopic

Petiole - The T. S. of petiole almost circular in outline (Figure-2); measures 3mm in median vertical plane and 5mm in horizontal plane; the upper part somewhat flat and the lower part semicircular; epidermis consists of single layer of thin walled parenchyma cells; cortex consists of 2 - 3 layers of collenchyma, chlorenchyma and parenchyma cells. Pericycle consists of group of sclerenchyma cells in bundles. Closed circular cylindrical vascular system (Figure-3) with xylem towards the centre and phloem outwards with wide pith in the centre. The petiole of the leaflets (Figure-2a, 2b, 2c and 2d) almost circular with two lateral wings on either side.

Leaf - The transverse section of the leaf shows prominent midrib and thick lamina; the lamina slightly raised above the level of the midrib forming shallow concavity on the upper side (Figure-4a).

Midrib - The T. S. of the midrib slight concave on the upper side and semi-circular on the lower side (Figure-4b), measures 500µm in vertical plane; cortex consists of 2 - 3 layers of collenchyma, chlorenchyma and parenchyma cells; the vascular bundle bowl shaped consists of 5 to 7 parallel rows of compactly arranged xylem elements with thin layer of phloem beneath the xylem strand.

Lamina - The T. S. of lamina shows 100µm thick; dorsiventral; upper epidermal cells rectangular (Figure-5a) with thick and smooth cuticle; the lower epidermal cells contain numerous stomata; the mesophyll region differentiated into upper two layer of compactly arranged palisade cells and lower four to five layers of loosely arranged spongy parenchyma cells.

Glandular Trichomes (Figure-5b) - Glandular trichome sessile on the epidermis of the lamina, it has two parts - the basal stalk cell and head. Head cell consists of 16 thin walled cells measuring up to 50µm wide; the glands are embedded on both the surfaces of the epidermal cells at position slightly lower the level of the epidermis.

Leaf Venation

The venation pattern clearly not visible. Veins fairly thick and vein lets form wide, angular islets. The vein-islets wide, distinct, rectangular, squarish or polygonal. The vein terminations arise from the vein islets and are long, slender, either branched or un-branched (Figure-6a).

Covering Trichomes (Figure-6b)

Covering trichomes seen on the epidermis; it is uniseriate and unicellular; the terminal cell of the trichome pointed and narrow whereas the basal cells broad and rectangular.

Epidermal Cells and Stomata

The epidermal cells thin, highly wavy (Figure-7a & 7b) with anomocytic stomata; stomata present only on the lower surface, the guard cells long and elliptical with narrow stomatal pore. The lower surface consists of 67.5 stomata per sq.mm with stomatal index of 24 per sq.mm; the vein islet number 9.25/sq.mm; veinlet termination number 19.25/sq.mm and palisade ratio 6.15 (Table 1).

Powder Microscopy (Figure-8)

Green color powder; evidenced for the presence of epidermal cells with anomocytic stomata (8a), numerous glandular trichomes (8a), covering trichomes (8b); glandular trichome with a single basal stalk cells and 16 head cells up to 50µm wide (8c); spiral vessels up to 30µ (8d); pitted vessels up to 25µ with tails at one or both the ends (8e & 8f) and thin walled fibres with broad lumen up to 30µ (8g).

Physico-chemical Studies

The physico-chemical parameters of the powdered drug were analyzed and the results are shown in Table 2. The loss on drying at 105°C was found to be 7.23% and the content of total ash and acid insoluble ash was found to be 8.92% and 0.099% respectively. The alcohol soluble extractive values 5.94% and water soluble extractive value 24.44 % show the extraction of polar constituents.

TLC/HPTLC Studies

TLC/HPTLC profile of chloroform and alcohol extract of the leaf was developed using Toluene: Ethyl acetate (1:1) as mobile phase. TLC/HPTLC profile at UV-254 nm, UV-366 nm and after derivatized with vanillin - sulphuric acid are shown in Figure-9 and 10. The Rf values of both the extracts are given in Table 3 and 4.

HPTLC Fingerprint Profile of Chloroform Extract

HPTLC fingerprint profile of chloroform extract, showed 8 peaks at 254nm and 6 peaks at 366 nm (Figure-11 and 12). Of which one major peak was seen at

254nm (Rf value of 0.37) and two major peaks were seen at 366nm (Rf values 0.73 and 0.79). The others were moderately smaller peaks.

HPTLC Fingerprint Profile of Alcohol Extract

HPTLC fingerprint profile of alcohol extract showed 6 peaks each at 254nm and 366nm (Figure-13 and 14). Of which 1 major peak was noted at both 254nm and 366nm (Rf value of 0.66). The others were moderately smaller peaks.

Quality Control Parameters

The microbial contents were found to be within the permissible limit (Table 5). The other parameters such as heavy metals, aflatoxin and pesticide residue were not detected from the samples (Table 6, 7 and 8) which indicate that the sample is free from toxic substances.

Discussion

The microscopical studies clearly reveal the wavy parenchyma cells of the epidermis in surface view with anomocytic type of stomata on the lower side and no stomata in the upper epidermis. Evidences are seen for the presence of glandular trichome with 16 head cells and a single basal cell immersed in the surface of the epidermis; numerous unicellular covering trichomes are also present on both the surfaces of leaf. The findings of the study are similar to the earlier reports of Metclafe and Chalk, 1957 where the reports stated the presence of external hairs of glandular and non-glandular forms, stomata confirmed to the lower surface surrounded by a fairly number of ordinary epidermal cells.

The usage of fingerprint chromatogram in the study helped in the identification of various phyto-constituents and quality evaluation of the study sample. The preliminary study of microscopy and phytochemistry carried on leaf part of the plant *M. hortensis* will act as a basic tool for correct identification of the plant and serve as phytochemical standards in the coming years, though a long-term study is required to evaluate the therapeutic efficacy and toxicity nature of the plant to establish the plant as a drug in the pharmaceutical industries.

Conclusion

The pharmacognostic studies, physico-chemical properties and TLC/HPTLC fingerprint analysis of the leaf of *M. hortensis* have been carried out for the first time which could serve in the identification and preparation of a monograph of the plant.

Millingtonia hortensis L.f.

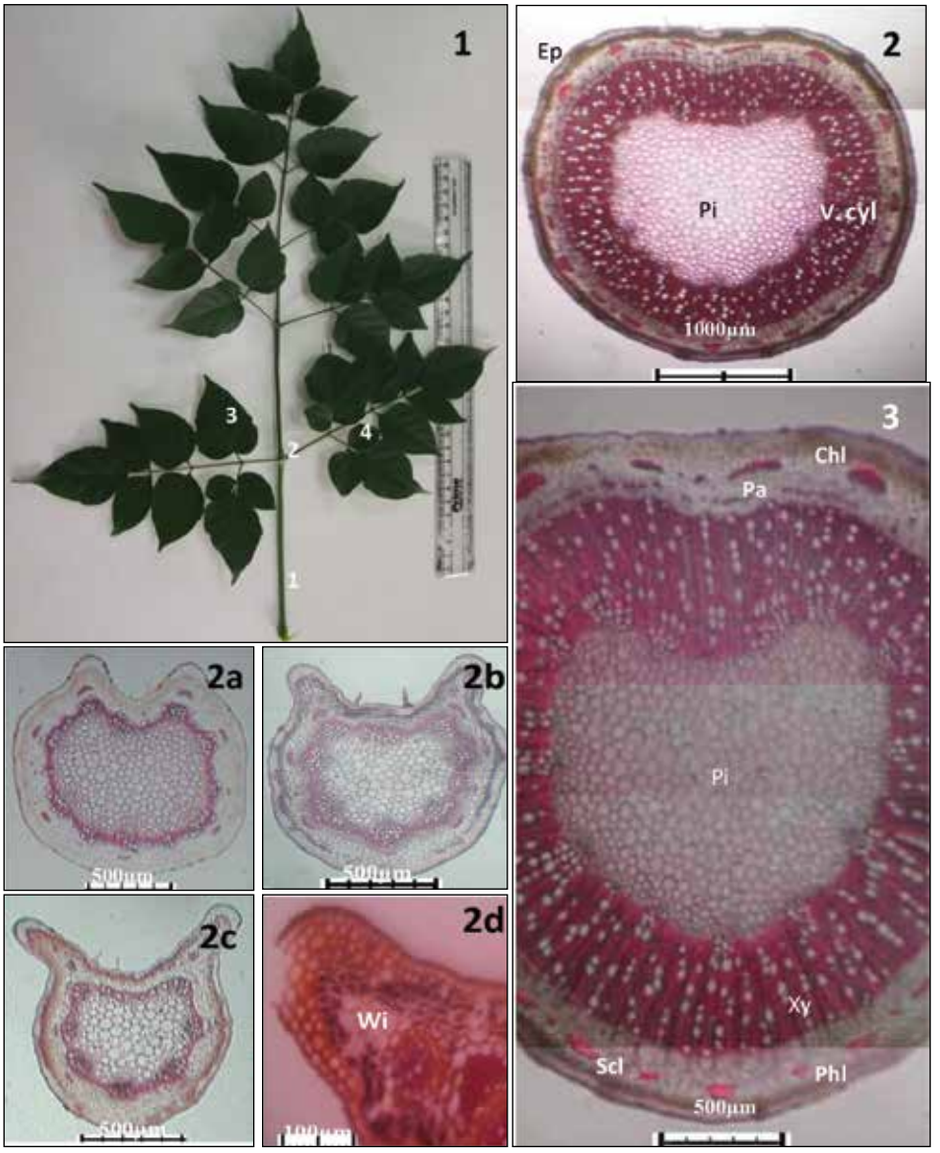


Fig. 1 : Leaf; **Fig. 2 :** T. S. of Petiole; **Fig. 3 :** T. S. of Petiole (Enlarged); **Fig. 2a, 2b, 2c** and **2d :** T. S. of Petiole of the leaflets with lateral wings; Ep – Epidermis, Chl – Chlorenchyma; Pa- Parenchyma, Scl – Sclerenchyma, Xy – Xylem, Ph – Phloem, Pi – Pith, Wi - Wings

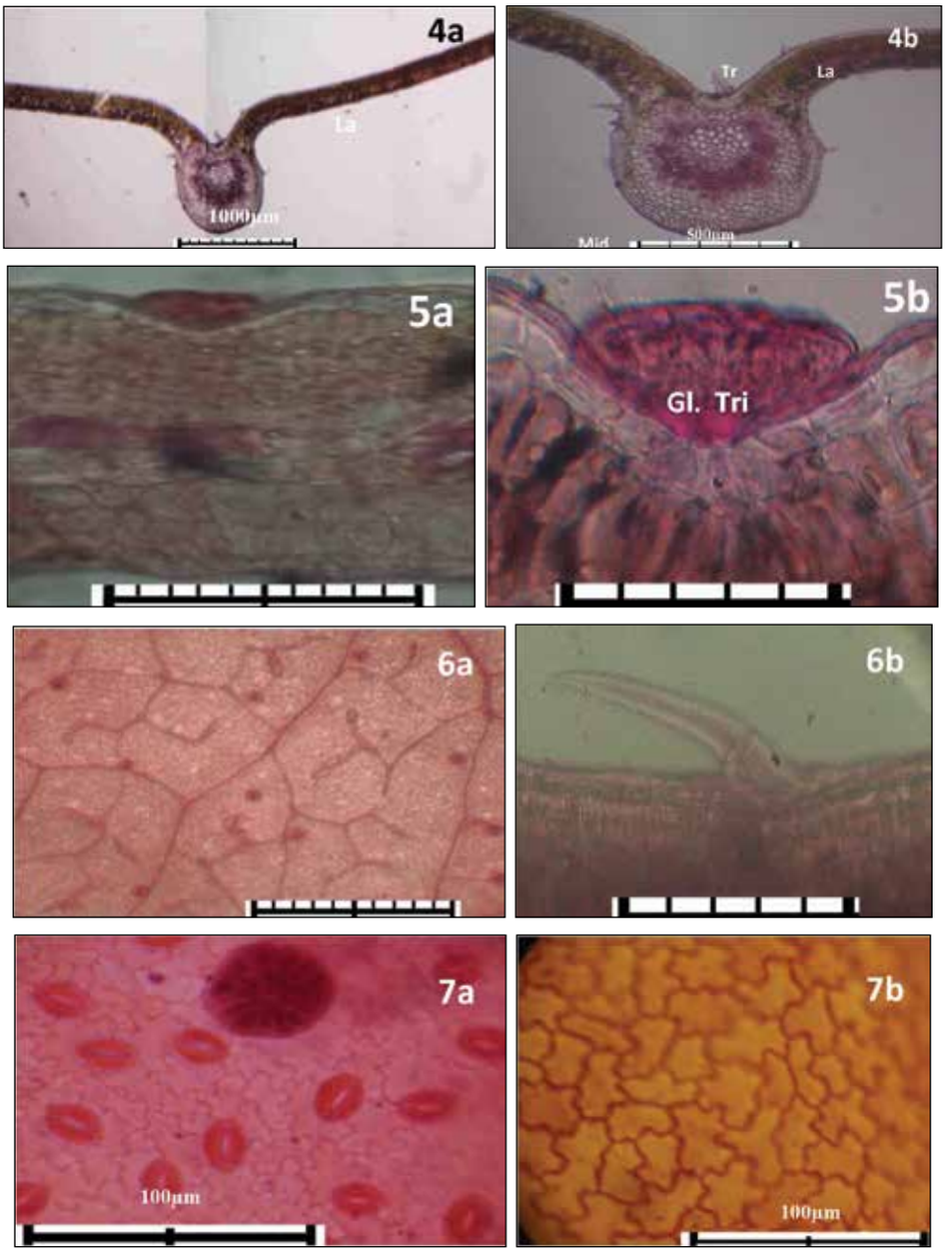
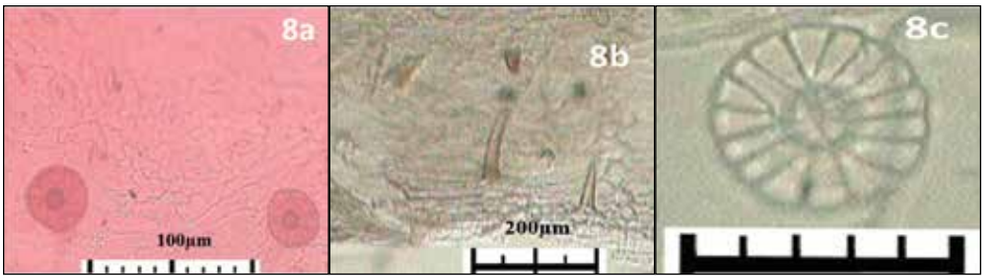


Fig. 4a : T. S. Leaf; **Fig. 4b :** T. S. of Leaf through midrib;
Fig. 5a : T. S. Leaf through lamina; **Fig. 5b :** Glandular trichomes on the lamina;
Fig. 6a : Vein islet and vein termination of the lamina;
Fig. 6b : Covering trichomes on the lamina;
Fig. 7a : Lower epidermal cells with stomata and glandular trichomes;
Fig. 7b : Upper epidermal cells without stomata



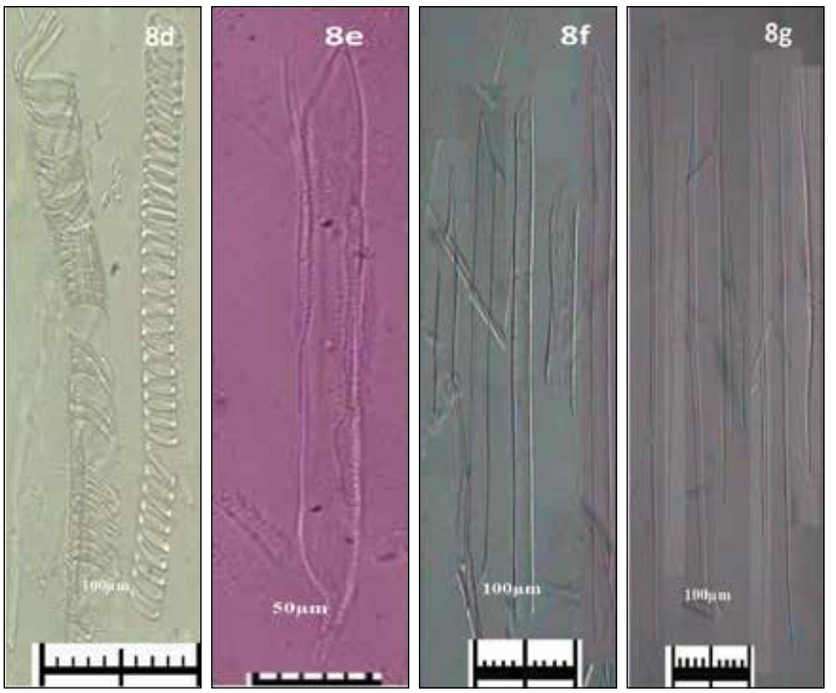


Fig. 8 : Powder Microscopical studies -

a. Epidermal cells with stomata and glandular trichomes;

b. Epidermal cells with covering trichomes;

c. Glandular trichomes

d. Spiral vessels

e. Pitted vessels

f. Pitted vessels

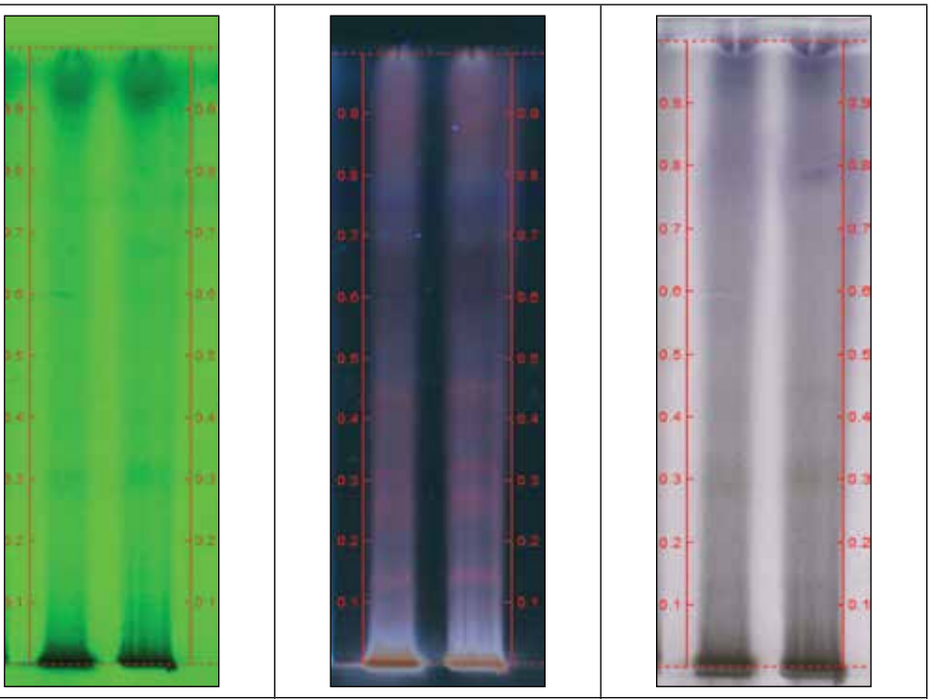
g. Fibres

Table 1 : Quantitative Microscopy

S. No.	Parameters Analysed	Observations	
		Range	Mean
1	Stomatal Number – lower epidermis	63 – 72 / sq. mm.	67.5
2	Stomatal Index – lower epidermis	24.28	24
3	Vein islet number	8.5 – 10.5 / sq. mm.	9.25
4	Veinlet termination number	15.5 – 22.0 / sq. mm.	19.25
5	Palisade ratio	5.5 – 6.8	6.15

Table 2 : Physico-chemical Parameters

S. No	Parameters	Results in % (w/w); n = 3
1.	Foreign matter	Nil
2.	Loss on drying at 105ºC	7.23
3.	Total ash	8.92
4.	Acid insoluble ash	0.099
5.	Alcohol soluble extractive values	5.94
6.	Water soluble extractive values	24.44

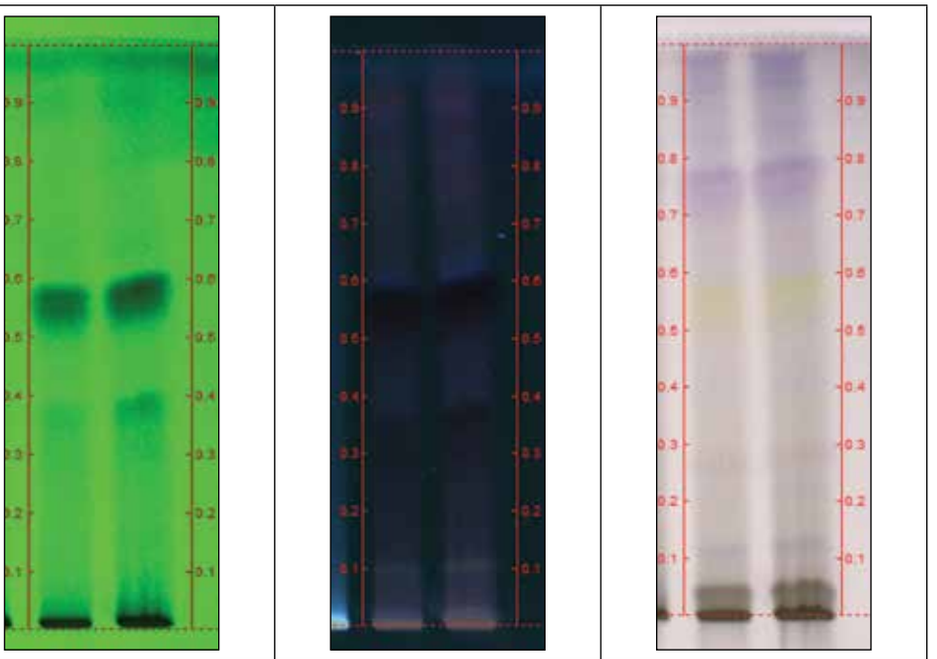


UV-254nm	UV-366nm	Visible light (After derivatized using Vanillin sulphuric acid reagent

Fig. 9 : TLC profile of Chloroform extract

Table 3 : Rf Values of Chloroform Extract (1:1)

Solvent system	Rf Values		
	UV 254nm (9 spots)	**UV 366nm (9 spots)**	**VS reagent (10 spots)**
Toluene : Ethyl acetate (1:1)	0.93 Dark green	0.89 Red	0.93 Dark green
	0.80 Green	0.78 Violet	0.80 Violet
	0.77 Green	0.53 Red	0.76 Light violet
	0.68 Dark green	0.46 Red	0.57 Light violet
	0.61 Green	0.42 Red	0.44 Light blue
	0.52 Green	0.39 Violet	0.32 Light blue
	0.40 Green	0.32 Red	0.29 Dark grey
	0.31 Dark green	0.28 Red	0.26 Light violet
	0.14 Green	0.15 Red	0.18 Light grey
			0.12 Dark grey



UV 254nm	UV 366nm	Visible light (After derivatized using Vanillin sulphuric acid reagent

Fig. 10 : TLC profile of Alcohol extract

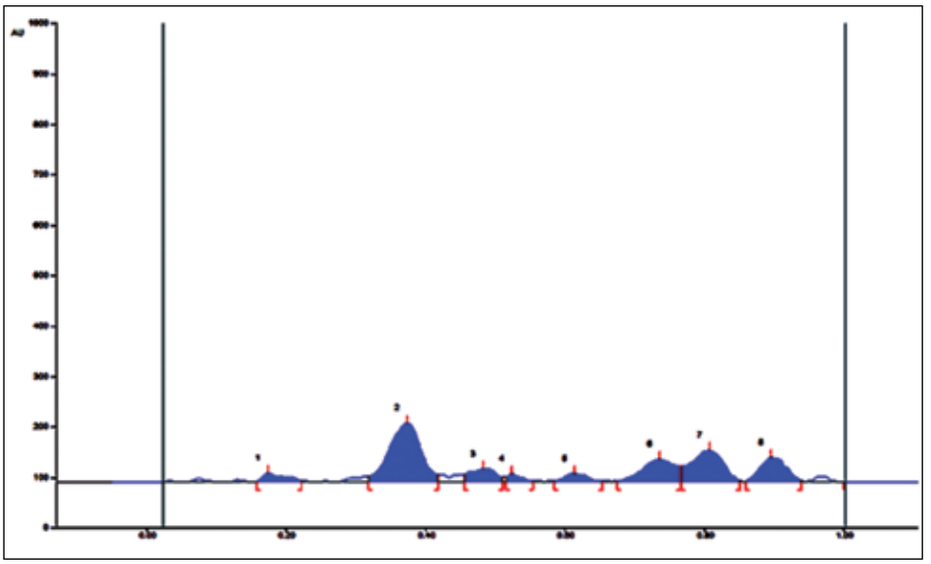


Fig. 11 : HPTLC fingerprint profile of Chloroform extract at 254nm

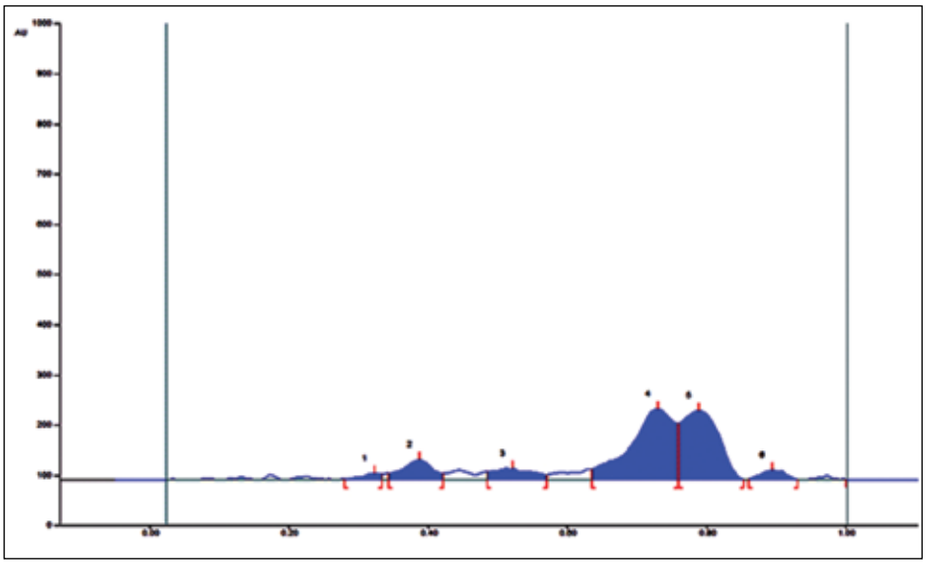


Fig. 12 : HPTLC fingerprint profile of Chloroform extract at 366nm

Table 4 : Rf Values of Alcohol Extract

Solvent system	Rf Values		
	UV 254nm (7 spots)	UV 366nm (7 spots)	VS reagent (10 spots)
Toluene : Ethyl acetate (1:1)	0.90 Green	0.91 Red	0.91 Violet
	0.80 Green	0.88 Light violet	0.83 Violet
	0.64 Green	0.85 Red	0.77 Dark violet
	0.57 Dark green	0.80 Light violet	0.57 Yellow
	0.38 Green	0.78 Red	0.39 Light violet
	0.29 Green	0.59 Violet	0.32 Yellow
	0.15 Green	0.57 Dark red	0.28 Light violet
		0.33 Dark red	0.20 Light violet
		0.30 Red	0.16 Yellow
		0.11 Light yellow	0.11 Blue

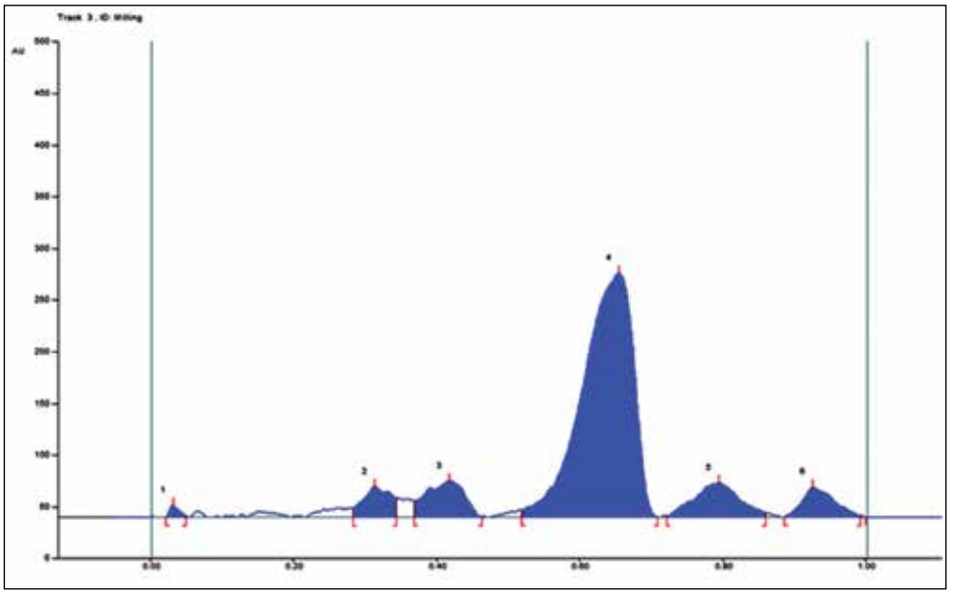


Fig. 13 : HPTLC fingerprint profile of Alcohol extract at 254nm

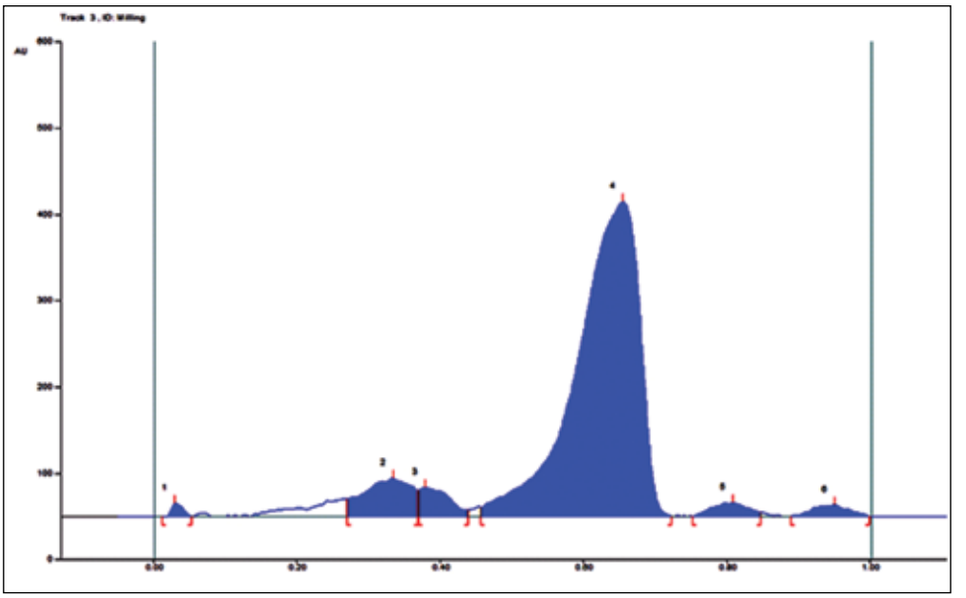


Fig. 14 : HPTLC fingerprint profile of Alcohol extract at 366nm

Table 5 : Microbial Load

S. No.	Parameter Analyzed	Results	Limits
1	Total Bacterial Count	2,600 CFU / gm	105 CFU / gm
2	Total Fungal Count	Absent	103 CFU / gm
3	Enterobacteriaceae	Absent	103 CFU / gm
4	*Salmonella* Spp.	Absent	Nil
5	*Staphylococcus aureus*	Absent	Nil

Table 6 : Heavy Metals

S. No.	Parameter Analyzed	Results	Limits
1	Lead	0.0142 ppm	10 ppm
2	Arsenic	Nil	3 ppm
3	Cadmium	Nil	0.3 ppm
4	Mercury	Nil	1 ppm

Table 7 : Estimation of Aflatoxins

S. No.	Aflatoxins	Results
1	B_1	Not detected
2	B_2	Not detected
3	G_1	Not detected
4	G_2	Not detected

Table 8 : Analysis of Pesticide Residues

S. No.	Pesticide Residues	Results
1	Organo Chlorine Group	ND
2	Organo Phosphorus Group	ND
3	Acephate	ND
4	Chlordane	ND
5	Dimethoate	ND
6	Endosulphan	ND
7	Endosulfan	ND
8	Endosulfon	ND
9	Ethion	ND
10	Endosufon sulphate	ND
11	Fenthion	ND
12	Lindane	ND
13	Methoxychlor	ND
14	Phorate sulfoxide	ND
15	Phorate sulfone	ND
	ND – Not detected	

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सारांश

मिलिंगटोनिया हार्टेंसिस एल.एफ लिफ का भेषजज्ञान मूल्यांकन एवं एच.पी.टी.एल.सी अगुलांक अध्ययन

[1]मागेश्वरी एस, [1]पवन कुमार सागर, [1]मीरा देवी श्री पी, [1]मुरूगेश्वरन आर., [2]रामप्रताप मीणा
[3]शमसूल आरीफीन और [1]आसिया खानम

मिलिंगटोनिया हॉर्टेंसिस एल.एफ. आमतौर पर कार्क पेड़ के रूप में जाना जाता है जोकि बिगनोनिएसी परिवार के अन्तर्गत आता है। यह दक्षिणी एशिया देशों जैसे भारत, बर्मा, थाईलैंड तथा दक्षिण चीन में पाया जाने वाला एक महत्वपूर्ण औषधीय पौधा है। पौधों की प्रमाणिकता और गुणवत्ता मानकों के लिए पौधे की ताजा पत्तियों को एकत्रित किया गया और मैक्रो–माइक्रोस्कोपिक, भौतिक रसायनिक और गुणवत्ता नियंत्रण मापदंडों का अध्ययन किया गया। माइक्रोस्कोपिक अध्ययन में पाया गया कि इसमें अंदर एनोमोसाइटिक स्टोमेटा के साथ वेवी एपिडर्मल सेल, पेलिसेड और स्पोंजी पैरेन्काइमा सेल्स, एक या दोनों किनारों पर टेल्स के साथ वाइडपिटेड वेसिल्स, फाइबर्स, 16 हेड सेल्स के साथ 50µ तक गलेनड्यूलर ट्राईकोम्स और 200µ लम्बाई के एक कोशिए ट्राईकोम्स विद्यमान है। भौतिक–रसायनिक आकड़ों से पता चलता है कि इसमें नमी 8.42%, कुल ऐश 8.93%, एसिड इन्सोल्यूबिल ऐश 0.098% और एल्कोहोल और वाटर सोल्यूबिल एक्स्ट्रैक्टिव मात्रा क्रमशः 5.92% और 24.44% थी। क्लोरोफॉर्म एवं एल्कोहोल एक्स्ट्रेक्ट के टी.एल.सी./एच.पी.टी.एल.सी. अध्ययन ने 254 एन.एम, 366 एन.एम और डेरीवेटाइज़्ड प्लेट्स (बेनीलिन–सल्फ्यूरिक एसिड रिएजेन्ट) के विभिन्न स्पॉट्स/पीक्स दर्शाए। औषधि की गुणवत्ता नियंत्रण मानदंड जैसे कि सूक्ष्म दर्शीय जीवाणु, भारी धातुऐं (जैसे, एएस, सीडी, पीबी, और एचजी) भी स्वीकार्य सीमा के भीतर पाए गए। एफलैटॉक्सिन जैसे, बी1, बी2, जी1, एवं जी2 भी अनुपस्थित पाए गए। यह अध्ययन दवा के सूखे एवं ताजा रूप में पहचान और प्रमाणीकरण करने के लिए बहुत ही उपयोगी साबित होगी। किसी भी विश्लेषणात्मक और जैविक अध्ययन के लिए मूल्यांकित किए गए फाइटोकेमिकल डेटा निकट भविष्य में भेषजकोशकीय मानकों के रूप में काम करेंगे।