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ORIGINAL ARTICLE

Quality Analysis of *Manjishta* (*Rubia cordifolia* L.) with Respect to Pharmacognostical and High-Performance Thin-Layer Chromatography Profile of the Genuine Sample: A Cross-Sectional Market Sample Study

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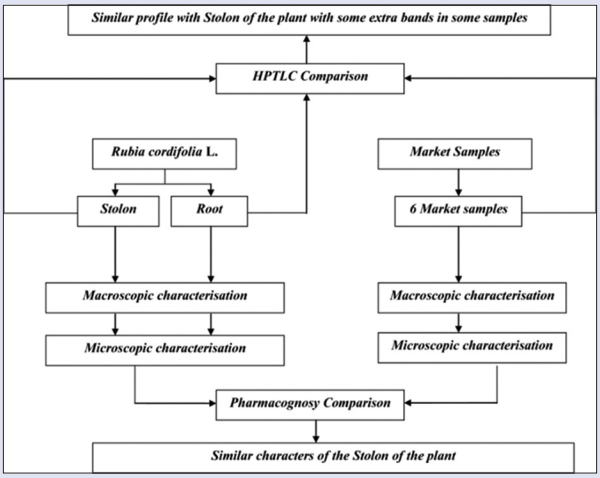
ABSTRACT

Background: The genuineness of Ayurvedic herbs causally determines the effectiveness of Ayurvedic treatment protocols. Research and subsequent evidence-based medicinal practices fundamentally dependant on appropriate identification and standardization of specific herbs are used for health-care purposes. Definite deficits that prevail in this regard in the form of adulteration, substitution, and compromised quality standards are the reasons why many scientific communities and health-related organizations question Ayurvedic sciences. This study aims at raising the very timely subject matter of genuine drug collection based on a model of Pharmacognostical and high-performance thin-layer chromatography (HPTLC) profiling of an Indian medicinal herb "*Manjishta*" (*Rubia cordifolia* L.), a profoundly marketed Ayurvedic drug. **Objectives:** To compare the six market samples of *Manjishta* (Pharmacognostical and with HPTLC profiling) with the genuine root and stolon. **Materials and Methods:** The market samples of *Manjishta* from 6 districts of Kerala were collected, and these samples were compared with the genuine root and stolon (morphology, histology, and with HPTLC profiles). **Results:** Morphological and anatomical evaluation of the market samples was similar with that of the original stolon of *Rubia cordifolia*. HPTLC profiling yielded entirely different peaks in specific samples when compared with that of the genuine stolon. **Conclusion:** The collected market samples of *Manjishta* from different districts of Kerala were almost similar to the stolon of *Rubia cordifolia* L. A clear standard operative procedure should be prepared for medicinal plant part collection with respect to source plant maturity for *Manjishta* or as a matter of fact any other herb and thus the HPTLC profiles should be redefined.

Key words: High-performance thin-layer chromatography, *Manjishta*, market samples, pharmacognosy, *Rubia cordifolia*

SUMMARY

- *Manjishta* is a plant which is used widely in many common Ayurvedic formulations. The accepted source of *Manjishta* is *Rubia cordifolia* Linn. of the *Rubiaceae* family
- In the market, different samples are available as *Manjishta* which are more or less similar in the external appearance
- The present study compared the six market samples of *Manjishta* (Pharmacognostical and with high-performance thin-layer chromatography [HPTLC] profiling) with the genuine root and stolon
- Morphological and anatomical evaluation of the market samples was similar with that of the original stolon of *Rubia cordifolia*
- HPTLC profiling yielded extra peaks in specific samples when compared with that of the genuine stolon.



Abbreviations Used: TLC: Thin-layer chromatography; HPTLC: High-performance thin-layer chromatography; TS: Transverse section; CVA: Cardiovascular accidents; µl: Micro litter; µm: Micro meter; g: Gram; R_f: Retention factor; L.: Linn.

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INTRODUCTION

Ayurveda, the traditional and alternative health-care system of India has been gaining much global attention because of its personalized care strategies and use of natural sources such as herbs for therapeutic purposes. But as far as, the global acceptance of Ayurvedic medicinal products are considered, most of the countries until now have not approved it. The lack of proper standardization and monitoring techniques, adulteration, substitution or improper use, and inappropriate documentation of safety and toxicity drug profiles have significant negative impacts on the global

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approval of Ayurvedic medicinal products. To add to these factors, exploitation of natural sources, deforestation, and hazardous change in the climatic conditions destroy the natural habitat of many important plant species advocated in Ayurvedic Sciences. Increased demands and fewer supplies accelerate the problems of adulteration and substitution, leading to untoward events and toxicities. Hence, proper screening methods of single drugs to be used in drug formulations are crucial for delivering high-quality products in the global market. Simultaneously, Indian medicinal herbs ought to be introduced at various global platforms with their scientific safety and efficacy profiles to initiate global acceptance of Ayurvedic medicaments and therapeutics.

One of the many grave concerns regarding Ayurvedic single drugs is obviously adulteration. Some amount of published works are available on the credibility and quality standards of market samples of single drugs such as Ativisha *(Aconitum heterophyllum),* Rasna *(Pluchia lanceolata),* Bala *(Sida cordifolia),* Dasamoola (a combination of 10 drugs), Guggulu *(Commiphora mukul),* and Pippalimoola (root of *Piper longum*).[1] However, compared to the huge turnover of specific drugs which are very common in multiple Ayurvedic formulations, very minimal studies have been carried out which identify the genuine nature of market samples, thus creating a prominent deficit in this arena. *Manjishta* is such a plant which is used widely in many common formulations such as *Ashwagandharishta, Aravindasava, Chandanasava, Ushirasava, Dasamoolarishta, Devadavyarishta, Manjistadi kwatha, Mahamanjistadi Kwatha, Jathyadi taila, Jatyadi ghrita, Kalyanaka Ghrita, Phalaghrita, Manjishthadi taila, Arimedadi taila, Pinda taila, Kumkumadi taila,* and *Bala taila*.[2] It is a medicinal plant species among the 70 recognized plants with high trade sourced from tropical forest. The accepted source of *Manjishta* is *Rubia cordifolia* Linn. of the *Rubiaceae* family, which is a perennial herbaceous climber naturally distributed throughout hilly districts of India. The roots of the plant are very long, cylindrical, flexuose with a thin red bark. Stem is also very long with a white bark. Young stem is green, quadrangular, and sometimes prickly on angles, glabrous, and shiny. Leaves are simple, in whorls of 4 from each node, ovate, cordate – ovate to ovate lanceolate, acute apex, scabrous above and the margins are with minute white prickles. Petioles are triangular and with many sharp recurved prickles. Inflorescence is terminal panicles of cymes. Fruits are globose or slightly 2 – lobed, dark purplish or black colored, fleshy with two small seeds.[3,4] It is used effectively in conditions such as *Kushta* (skin ailments), *Vataraktha* (inflammatory joint disorders), *Pakshaghata* (Hemiplegia and other cardiovascular accidents), *Vyanga* (Leukoderma), etc., by Ayurvedic physicians in India. Majority of Ayurvedic texts prescribe the root of *Manjishta* for medicinal uses. However, as per Ayurvedic Pharmacopeia of India, the *kaanda* (stem) of *Rubia cordifolia* Linn. is the pharmacologically useful part to be used as *Manjishta*.[5]

In the market, different samples are available as *Manjishta* which are more or less similar in external appearance. Studies are available about the sources and controversies of the market samples of the drug. In 1993, there was a report which compared the stem and root of *Rubia cordifolia* and *Rubia tinctorium* using pharmacognostic and phytochemical tools. They had also compared the various Indian market samples of *Manjishta* and concluded the presence of *Rubia tinctorium* (*Irani Madder*) in the Indian Markets.[6] In CCRAS, database on medicinal plants reported the presence of root of *Rubia tinctorium* L., root of *Oldenlandia umbellata* L. and even stem pieces of *Schleichera oleosa* Lour and some other subspecies of *Rubia* in the market in the name of *Manjishta*.[7,8] Another study reported that there was no *Rubia tinctorium* L. in the South Indian market and *Rubia cordifolia* alone was readily available. However, the presence of other subspecies of *Rubia* and other plants were not excluded in the study.[9] Later, some researchers had reported the presence of *Rubia tinctorium* in the market as there was no sufficient supply of genuine *R. cordifolia* against the demand. They had also reported the quality parameters to identify both these samples.[10] A study conducted in 2013 reported the problems of adulteration of *Manjishta* with *Iranian Manjishta* and recommended to avoid borrowing the powder form of *Manjishta* as it was difficult to identify the adulteration.[11] As per the latest report available, there was no adulteration of *Rubia cordifolia* with *Rubia tinctorium,* but they were unable to establish whether the samples were of root or of stem.[12] Another important finding is that even though high-performance thin-layer chromatography (HPTLC) profiling of the stem of *Rubia* was conducted in some studies, the market sample HPTLC profiling with the genuine root and stem (stolon) was not done.

Kerala is a leading state of Ayurvedic drug manufacturing and nationwide distribution. All the aforementioned studies were carried out by taking samples from 3 to 5 different areas of the country. Only one sample from Thiruvananthapuram, Kerala was included, that too in one of the study. The present study failed to establish the correct identity. Moreover, in Kerala, >700 small scales to large scale Ayurvedic drug manufacturers are using this drug in many highly demanding formulations. However, no studies have been carried out in establishing the quality standards of the drug in the Kerala market. Hence, this study aimed at ascertaining the identity of six different market samples of *Manjishta* from Kerala through macroscopic and microscopic evaluation and comparing them with the genuine root and stem (stolon). The study also aimed at HPTLC profiling of these samples. Although histological and morphological characteristics of different market samples may appear similar, only a qualitative assessment study shall identify the different active principles in the sample. If these identified active principles in the sample under consideration differed from the standards, then the source of such samples should be ascertained and further phytochemical and pharmacological studies with these samples should be carried out to develop a detailed drug profile. Furthermore, measures are to be adopted to promote the cultivation of the qualitatively identified sources for the medicinal uses.

MATERIALS AND METHODS

Genuine samples of the root and stem (stolon) with a maturity of 1 year were collected from Herbal Garden of Vaidyaratnam P. S. Varrier (VPSV) Ayurveda College, Kottakkal. The samples were authenticated and specimens were deposited at the Herbarium of Center for Medicinal Plant Research (CMPR), Arya Vaidya Sala, Kottakkal, Kerala (Herbarium Voucher No: 9054). Market samples of *Manjishta* were collected from the local markets of Ernakulam, Kottakkal, Kannur, Kozhikode, Thrissur, and Thiruvananthapuram.

Samples were named as follows:

- Sample 1: Roots of *Rubia cordifolia*
- Sample 2: Stolon/Stem of *Rubia cordifolia*
- Sample 3: Sample collected from Ernakulam Market
- Sample 4: Sample collected from Kottakkal Market
- Sample 5: Sample collected from Kannur Market
- Sample 6: Sample collected from Kozhikode Market
- Sample 7: Sample collected from Thrissur Market
- Sample 8: Sample collected from Thiruvananthapuram Market.

Macroscopic study

External morphological characters were studied and documented as per the standard protocol given in Ayurveda Pharmacopoeia of India. Characters such as size, color, odor, taste, external markings, and fracture were noted and compared with that of original root and stem.[12]

Microscopic study

Samples were processed as per the standard protocol.[13] The compound microscope used for microscopic study was Leica DM 1000 LED. Trinocular "Leica" microscope attached with "Leica DFC 295" digital camera connected to the computer and Leica Application Software LAS Version 3.6.1 were used for the viewing and transferring microscopic images. NK System Plant Microtome, Automatic MT3 is used for taking fine sections of thick materials. Thin transverse sections (TSs) of the roots were taken by hand and also using microtome. Sections were stained with diluted aqueous saffranin, washed thoroughly and mounted in 40% glycerin and observed under the microscope. Specimens were double stained with saffranin and fast green as per the procedure given by Johansen, 1940.[14] The microscopic evaluation of sections was carried out to confirm the structural details of the drugs. Microscopic images were transferred using the computer controlled microscopic system and camera.

High-performance thin-layer chromatography

For HPTLC profiling, ethanolic extracts of the samples were prepared by taking 3 g of each samples in a round bottomed flask and refluxed with 100 ml of ethanol continuously for 3 h. The extracts were filtered, concentrated and made up to 10 ml in a standard flask. 5 µl of test sample solutions were applied on the plate using Camag automatic thin layer chromatography (TLC) sampler 4 attached to Camag HPTLC system on an aluminum backed precoated silica gel 60 F_{254} TLC plate (E. Merck) of uniform thickness of 0.2 mm plate in the form of bands with width 8 mm using Hamilton syringe (100 µl). The plate was developed in the solvent system of toluene: ethyl acetate (8.5:1.5) in a twin trough chamber to a distance of 9 cm. Then, they were observed under ultraviolet light at 254, 366 nm after derivatization with Anisaldehyde sulfuric acid reagent, and the retention factor R_f value and color of the resolved bands were recorded. Densitometric scanning of the plates was done by using Camag TLC scanner 3 at 254, 366, and 550 nm.

RESULTS

Macroscopy of samples 01–08

Organoleptic characters of different samples were recorded [Table 1 and Figure 1]. Samples 2–8 were almost identical in characters such as color, external markings, fractures, and cut surfaces. The main differences between these samples were in dimension and odor. Sample 01, the original root was entirely different from others in most of the features. All the samples were having bitter to astringent tastes. Crushed pieces of samples in water showed purplish color except for sample 03, which showed a light orange color.

Microscopy of Samples 01 and 02

Root - Sample 1 - [Figure 2a-f] - TS of the root almost circular in outline with outermost cork layers followed by secondary cortex, phloem and xylem regions. The cork well developed and composed of 4–8 layers of tangentially elongated cells of almost equal sizes; walls with wavy outline; often filled with reddish brown depositions; outer portions ruptured at places. Cortex made up of up to 12 layers of parenchyma cells which were broader in size in outer region and smaller in size in the inner region; some of the cells filled with red colored depositions; acicular crystals of calcium oxalate present in some cells of outer cortex. Phloem 8–10 cells wide composed of irregularly arranged thin-walled parenchyma cells of comparatively smaller size. Xylem consisted of vessels, fibers, and parenchyma; round to oval vessels of varying sizes (diameter ranging from 30 µm to 130 µm) scattered throughout wood region. Big vessels arranged at the peripheral region. Medullary rays were uni-multi seriate; made up of radially elongated cells. Pith absent and center portion occupied by lignified xylem elements.

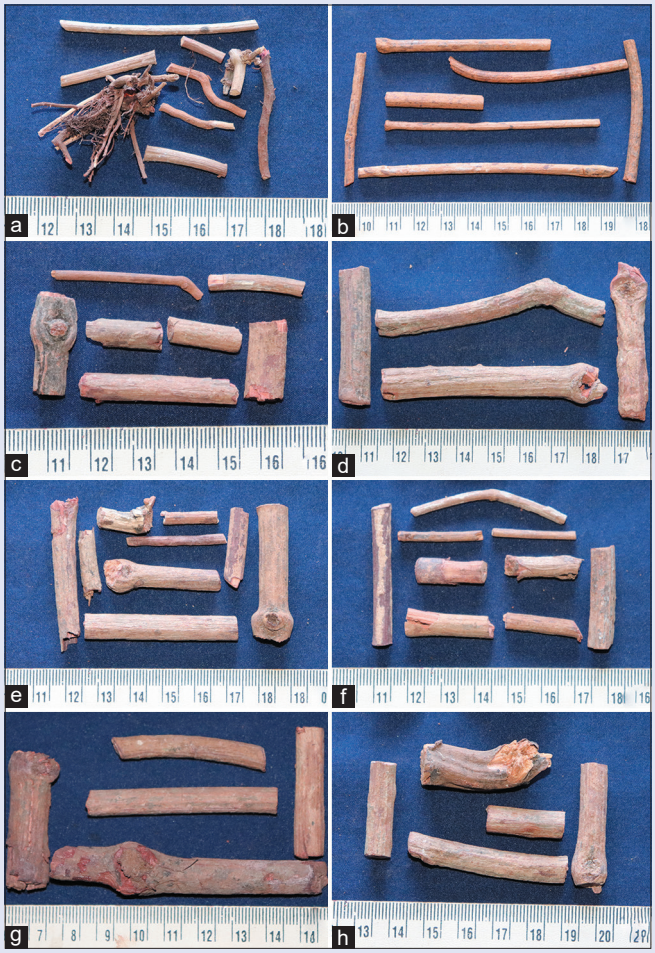

Figure 1: Macroscopy of the samples of *Manjishta*. (a and b). Genuine sample; (a) *Rubia cordifolia* root; (b) *Rubia cordifolia* stolon; (c-h). Market samples; (c) Ernakulam; (d) Kottakkal; (e) Kannur; (f) Kozhikode; (g) Thrissur; (h) Thiruvananthapuram

Stolon – Sample 2 – [Figure 2g-l] – Stem TS round to oval in outline with well-defined cork, cortex, phloem, xylem, and pith regions. Detailed TS showed an outer cork region comprising of 5–12 layers of tangentially elongated, thin-walled polygonal cells of various sizes, often ruptured at places. Cortex consisted of 5–10 layers of thin-walled parenchyma cells with intercellular spaces, filled with black granular masses of sandy crystals and acicular crystals at places. Phloem consisted of up to 20 layers of parenchyma cells with irregular margins. Some of the cells filled with black granular masses of sandy crystals. Xylem region showed the presence of vessels, fibers, and parenchyma. Vessels of varying size (diameter ranging from 20 to 160 µm) distributed uniformly throughout the xylem region. Protoxylem elements seen at the center covering the pith region. Center portion occupied by round to oval shaped parenchymatous cells containing sandy crystals and acicular crystals. In mature stems, the pith cells get crushed giving a hollow appearance in the pith region.

Market Samples

Ernakulam sample – Sample 3 – [Figure 3a-d] – TS of the sample was almost circular with wavy outline. Cork was composed of 10–15 layers of tangentially elongated cells. Cortex was thin-walled parenchymatous,

Table 1: Macroscopic characters of *Rubia cordifolia* samples

Observation	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
Physical state	Solid, fresh as well as dried sample	Solid, fresh as well as dried sample	Solid, dried sample	Solid, dried sample	Solid, dried sample	Solid, dried sample	Solid, dried sample	Solid, dried sample
Source	VPSV Ayurveda college Kottakkal	VPSV Ayurveda college Kottakkal	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
External characters	Twisted, slender or hairy, round roots	Slender, more or less cylindrical, flattened, wiry	Slender, some are thick 5 mm - 10 mm in diameter	Thick, 10 mm - 15 mm diameter. Skin easily peel off	Slender, some are thick 5 mm - 10 mm in diameter. nodes and internodes present	Slender, some chopped pieces	Thick pieces, 10 mm - 15 mm diameter, skin easily peel off. Prominent nodes and inter nodes present	Thick, 10 mm - 15 mm diameter. Nodes are not much prominent
Size	Long, wiry, twisted, cut surface round	Cylindrical pieces, small pieces	Pieces of 1 cm - 5 cm long	Pieces of 1 cm - 5 cm long	Pieces of 1 cm - 3 cm long	Pieces of 1 cm - 7 cm long	Pieces of 1 cm - 5 cm long	Pieces of 1 cm - 5 cm long
Colour	Purple brown	Bright purple brown externally and internally	Pale purple brown externally, purple brown internally	Pale purple brown externally, purple brown internally	Pale brown externally, purple brown internally	Pale brown externally, purple brown internally	Pale purple brown externally, dark brown to black patches, purple brown internally	Pale brown externally, purple brown internally
Surface characters and texture	Twisted, smooth	Longitudinally grooved, scabrous	Longitudinally grooved, scabrous	Longitudinally grooved with some longitudinal cracks, scabrous	Longitudinally grooved, scabrous	Longitudinally grooved, slightly scabrous	Longitudinally grooved with some longitudinal cracks, scabrous	Longitudinally grooved with some longitudinal cracks, scabrous
Fracture	Fibrous fracture	Short fracture	Short to slight splintery fracture	Short to slight splintery fracture	Slight splintery fracture	Short splintery fracture	Splintery fracture	Splintery fracture
Taste	Bitter and astringent	Bitter and astringent	Bitter and astringent	Bitter and astringent	Bitter and astringent	Bitter and astringent	Bitter and astringent	Bitter and astringent
Solubility in water	Produce dark purplish color	Produce purplish color	Light orange color	Produce purplish color	Produce purplish color	Produce purplish color	Produce purplish color	Produce purplish color

VPSV: Vaidyaratnam P. S. Varrier

up to 10 cells wide, with intercellular spaces, some cells with reddish depositions and some with black powdery mass of sandy crystals and acicular crystals. Phloem was composed of 10–12 layers of irregularly shaped parenchymatous cells. Xylem was comparatively wider with vessels of varying sizes (diameter ranging from 20 µm to 200 µm) scattered throughout. Center was hollow due to the disintegration of parenchymatous cells. Protoxylem elements covered the pith region. The presence of acicular crystals was observed in the remnants of pith cells.

Kottakkal sample – Sample 4 [Figure 3e-h] – TS of the sample was circular. Cork was composed of 10–12 layers of tangentially elongated cells. Cortex was thin-walled parenchymatous, comparatively wide, with intercellular spaces. Phloem was narrow and was composed of irregularly shaped parenchymatous cells. Granular masses of sandy crystals, reddish depositions were limited in number. Xylem was comparatively wider with vessels of varying dimensions (diameter ranging from 30 µm to 250 µm) scattered throughout. Center was hollow due to the disintegration of parenchymatous cells. Protoxylem elements covered the pith region.

Kannur sample – Sample 5 [Figure 3i-l] – TS of the sample was circular with wavy outline. Outer layers were ruptured at places. Cork was composed of 10–12 layers of tangentially elongated cells. Cortex was thin-walled parenchymatous, up to 10 cells wide, with intercellular spaces. Phloem was composed of 12–15 layers of irregularly shaped parenchymatous cells. Granular masses of sandy crystals, reddish depositions were limited in number. Xylem vessels of varying sizes (diameter ranging from 25 µm to 160 µm) were scattered throughout. Center was hollow due to the disintegration of parenchymatous cells and it was comparatively wider. Protoxylem elements covered the pith region.

Kozhikkode sample – Sample 6 [Figure 3m-p] – TS of the sample was circular with wavy outline. Cork was composed of 6–10 layers of tangentially elongated cells. Cortex was thin walled parenchymatous, up to 10 cells wide, with intercellular spaces and majority of cells with reddish depositions. Sandy crystals were present in the majority of the cells of cortex. Phloem was composed of 10–12 layers of irregularly shaped parenchymatous cells. Xylem was comparatively wider with vessels of varying dimensions (diameter ranging from 25 µm to 200 µm) scattered throughout. Center was hollow due to the disintegration of parenchymatous cells. Protoxylem elements covered the pith region. The presence of acicular crystals and sandy crystals was observed in pith cells.

Thrissur sample – Sample 7 [Figure 3q-t] – TS of the sample was circular with wavy outline. Cork was composed of 10–15 layers of tangentially elongated cells. Cortex was thin-walled parenchymatous, up to 10 cells wide, with intercellular spaces, some cells with reddish depositions. Sandy crystals were present in the majority of the cells of cortex. Phloem was composed of up to 15 layers of irregularly shaped parenchymatous cells. Xylem was comparatively wider with vessels of varying dimensions (diameter ranging from 20 µm to 180 µm) scattered throughout. Center was hollow due to the disintegration of parenchymatous cells. Protoxylem elements covered the pith region. The presence of acicular crystals and sandy crystals was observed in pith cells

Thiruvananthapuram sample – Sample 8 [Figure 3u-x] – TS of the sample was almost circular in outline. Cork was composed of 8–12 layers of tangentially elongated cells with reddish depositions at places. Cortex was thin walled and parenchymatous, up to 10 cells wide, with intercellular spaces, some cells with reddish depositions and some with black powdery mass of sandy crystals. Phloem was composed of up to 15 layers of irregularly shaped parenchymatous cells. Xylem was

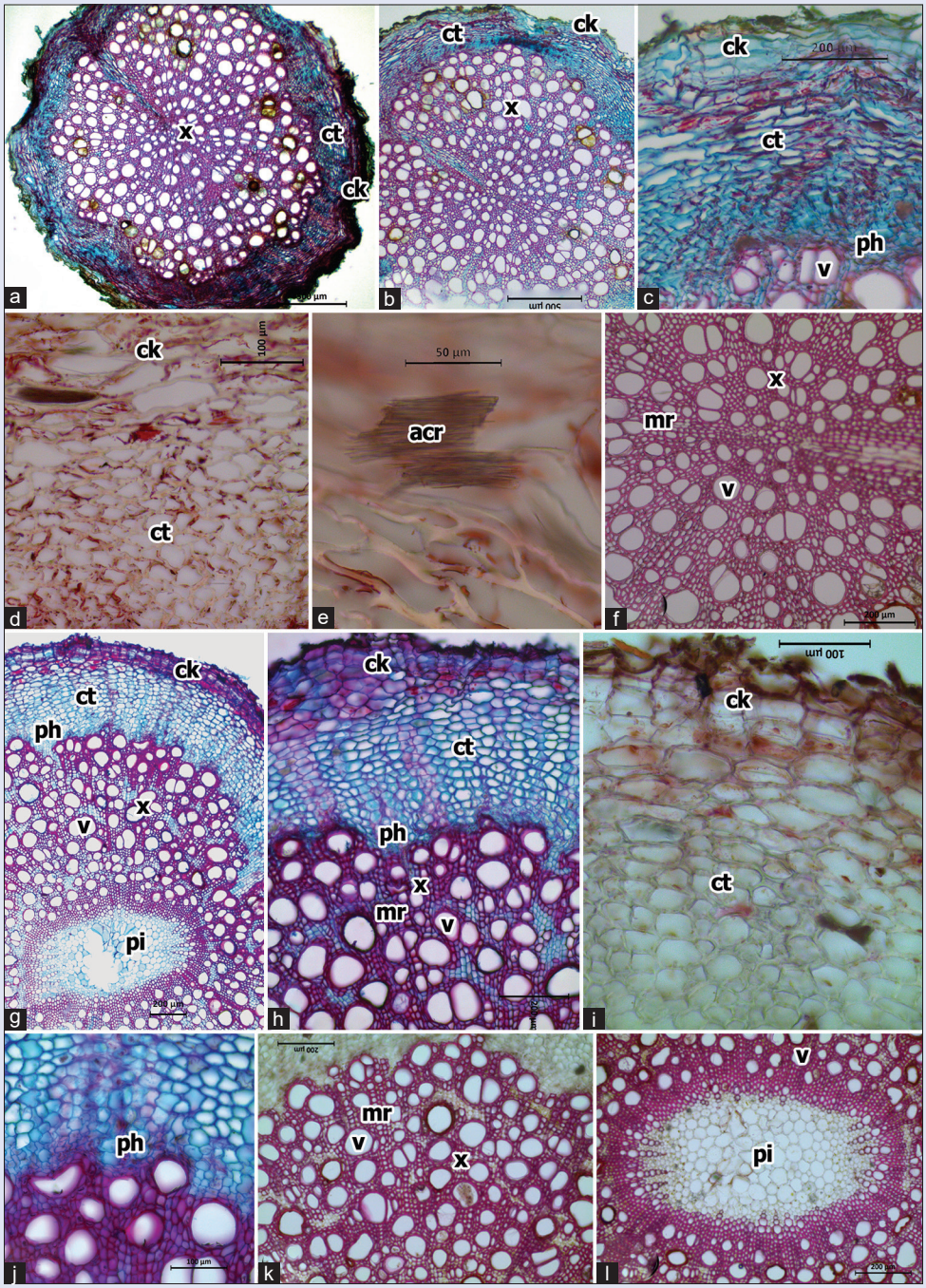

Figure 2: Microscopy of *Rubia cordifolia* L. (a-f) Root TS. (a) TS entire; (b) TS portion enlarged; (c) TS outer portion enlarged; (d) TS cortical portion enlarged; (e) cortical cells containing acicular crystals; (f) stelar portion enlarged. (g-l) Stem TS. (g) TS detailed; (h) TS portion enlarged; (i) TS outer portion enlarged; (j) TS showing phloem region; (k) TS showing xylem region; (l) TS pith region. acr, acicular crystals; ck, cork; ct, cortex; mr, medullary rays; ph, phloem; pi, pith; v, vessels; x, xylem

comparatively wider with vessels of varying dimensions (diameter ranging from 40 µm to 150 µm) scattered throughout. Center was hollow due to the disintegration of parenchymatous cells. Protoxylem elements covered the pith region.

High-performance thin-layer chromatography profile

HPTLC chromatograms and densitometric profiles of eight samples at 254 nm, 366 nm, and 550 nm were recorded [Figure 4]. The Rf values of the separated compounds of each samples were noted [Tables 2-4]. At 254 nm, sample 04 having the maximum number of peaks (14 peaks), sample 05 and 08 with 13 peaks each, followed by sample 07 (11 peaks), Rf values of many peaks of these samples were similar indicating the presence of same chemical constituents. *Rubia cordofolia* root sample (sample 01) had only seven peaks, and its stem/stolon (sample 02) had nine peaks. Peak intensities were different for each sample, but the presence of peaks was almost similar in all the market samples except for sample 5. Sample 05 showed some unique peaks that were not observed in the genuine

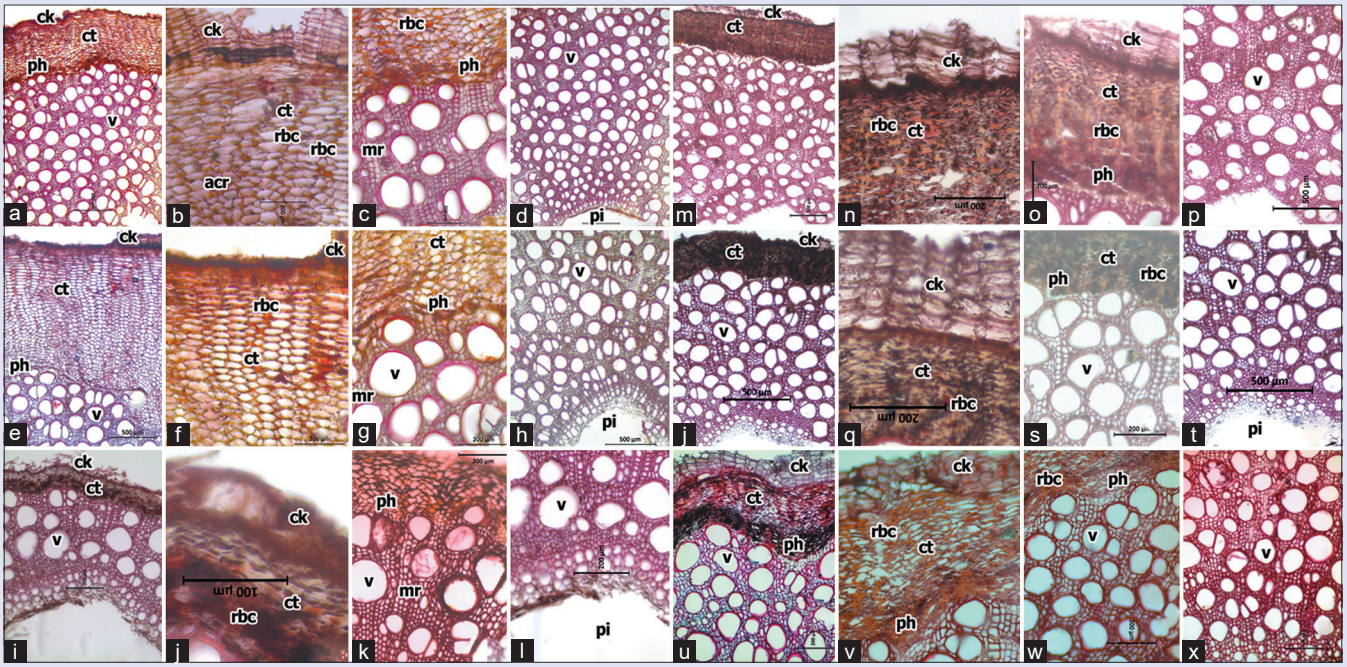

Figure 3: Microscopy of the market samples of *Manjishta*. (a-d) Ernakulam market; (e-h) Kottakkal market; (i-l) Kannur market; (m-p) Kozhikode market; (q-t) Thrissur market; (u-x) Thiruvananthapuram market. acr, acicular crystals of calcium oxalate; ck, cork; ct, cortex; mr, medullary rays; ph, phloem; pi, pith; rbc, reddish brown content; v, vessels

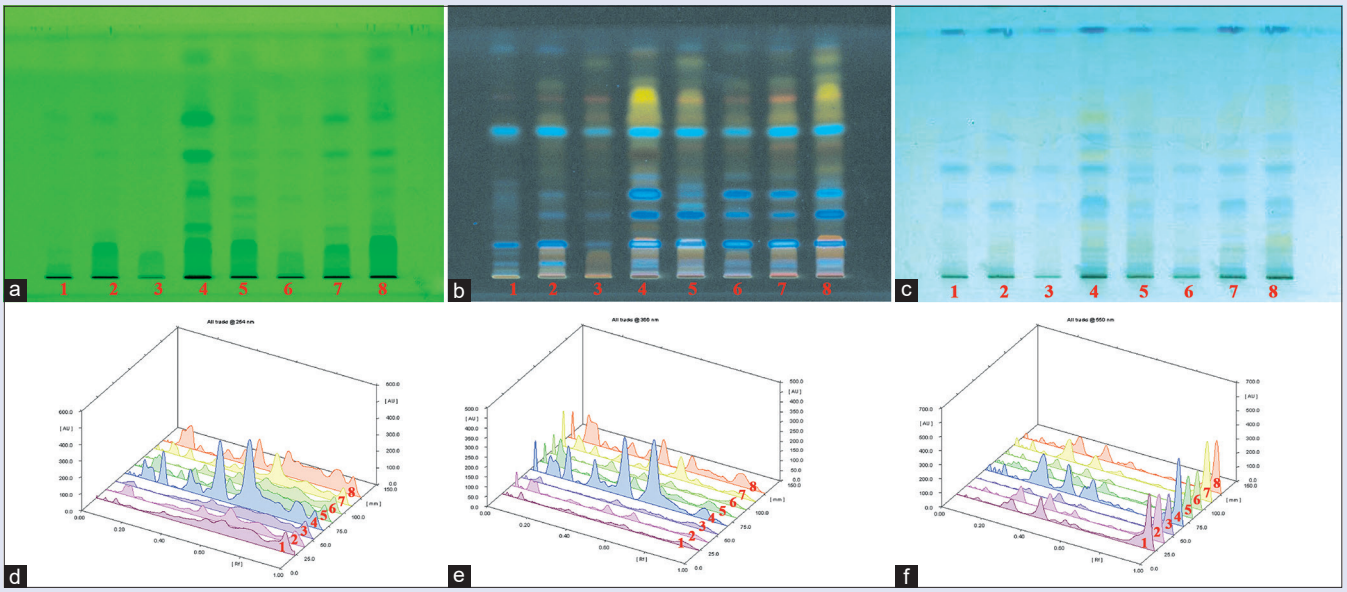

Figure 4: High-performance thin-layer chromatography. Comparison of the genuine and market samples of *Manjishta*. (a-c) TLC profile of samples at 254 nm, 366 nm, and after derivatistion; (d-f) Densitometric profile at 254 nm, 366 nm, and after derivatistion. 1, *Rubia cordifolia* root; 2, *Rubia cordifolia* stolon; 3, Market sample procured from Ernakulam; 4, Kottakkal; 5, Kannur; 6, Kozhikkode; 7, Thrissur; 8, Thiruvanathapuram

sample (sample 02) as well as other market samples. Similar R_f values uniformly present in all the samples studied indicate the presence of similar phytoconstituents.

DISCUSSION

Adulteration, substitution, and problems of controversial identity are the main threats to the quality assurance of Ayurvedic drugs. According to a study published in 2016, 60% of the herbal drugs were adulterated.[15] The main reasons for this may be attributed to the scarcity of genuine drugs, high cost, and the controversies about the identity of the genuine drug. Even though a few studies on quality standards of some market samples were conducted and published, they never ascertained a clear conclusion about the identity of the samples available in the market.

Manjishta is an extensively using Ayurvedic drug in conditions such as skin ailments, rheumatic conditions, as a blood purifier as well as a rejuvenator.[5] There are no direct references about the exact part of

Table 2: High-performance thin-layer chromatography - retention factor values of the samples at 254 nm

	Sample							
	1	2	3	4	5	6	7	8
Total number of peaks	7	9	8	14	13	8	11	13
R_f	0.04	0.10	0.09	0.03	0.04	0.09	0.02	0.03
	0.10	0.13	0.30	0.05	0.13	0.31	0.10	0.11
	0.34	0.17	0.41	0.10	0.17	0.41	0.19	0.14
	0.49	0.31	0.49	0.14	0.24	0.48	0.32	0.19
	0.56	0.41	0.63	0.20	0.31	0.62	0.34	0.33
	0.64	0.49	0.76	0.33	0.34	0.76	0.42	0.42
	0.77	0.63	0.88	0.37	0.41	0.93	0.48	0.49
		0.77	0.96	0.42	0.48	0.96	0.62	0.55
		0.89		0.48	0.62		0.77	0.64
				0.63	0.72		0.89	0.68
				0.72	0.77		0.96	0.79
				0.77	0.86			0.89
				0.87	0.96			0.96
				0.96				

R_f: Retention factor

Table 3: High-performance thin-layer chromatography - retention factor values of the samples at 366 nm

	Sample							
	1	2	3	4	5	6	7	8
Total number of peaks	4	7	7	12	10	9	11	12
R_f	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
	0.10	0.10	0.10	0.10	0.11	0.10	0.10	0.11
	0.61	0.12	0.49	0.13	0.19	0.13	0.19	0.13
	0.92	0.30	0.62	0.19	0.30	0.24	0.32	0.19
		0.49	0.70	0.24	0.41	0.32	0.41	0.24
		0.62	0.85	0.33	0.48	0.48	0.48	0.33
		0.94	0.94	0.37	0.62	0.58	0.62	0.42
				0.41	0.70	0.62	0.70	0.49
				0.48	0.72	0.93	0.84	0.63
				0.63	0.86		0.89	0.71
				0.77			0.94	0.79
				0.89				0.89

R_f: Retention factor

this plant which is to be used in Ayurvedic classical text books. As per Ayurvedic principles, if the part of a drug is not mentioned specifically which is to be used in therapeutics, then the root should be selected as the medicinal plant part.[16] The meaning of the term *Manjishta* indicates the one which can augment complexion and purify blood. Synonyms such as *Aruna, Rakthangi, Kala,* and *Kalameshika* indicate the color of the therapeutically intended part of the plant.[17] The accepted botanical source of *Manjishta* is *Rubia cordifolia* L. of *Rubiaceae* family. Morphologically, it is having fibrous and hairy roots ranging from dark red to dark brown in color.

Another peculiarity of this plant is that it is having two types of stem. A sub-arial stem modification – the stolon is circular in cross section and red in color. The second type is the arial stem which is green in color and is quadrangular in cross section. Hence, as per the morphological description as told in the classics, these two parts with red color, one being the root and the other being the stolon may be intended as the useful plant part. During the *Samhitha* (500 BC to 600 AD), there was no systematic categorical description of the morphological features of a plant. Most of the plants were recognized either by their names, synonyms, or uses. Hence, most probably, the parts seen below the soil were considered as roots. In case of *Manjishta*, the roots and some parts of stolon too are underground portions. One of the vernacular names (Malayalam) for this plant is "*Chovvallikkodi,*" meaning a plant with red-colored weak stem (can be climber or creeper).[18] This indicates that there were chances that the part used in Kerala was its red-colored stem portion, i.e., the stolon and not the root. As per Ayurvedic Pharmacopoeia of India, the official plant part of *Manjishta* is the dried stem,[13] whereas ICMR Quality standards of Indian Medicinal plants state the official plant part of *Rubia cordifolia* Linn. is both dried root and stem.[19]

Hence, as per the above references, both root and stem, preferably the red-colored subarial stem part, i.e., the stolon of *Rubia cordifolia* Linn. may be used as the source for *Manjista*. Studies published on the different market samples of *Manjishta* sold in various Indian markets concluded that the samples were of another species of *Rubia,* namely *Rubia tinctorum*.[7,8,10] However, a study strongly objected these findings stating that there was no *Rubia tinctorum* in the Indian markets, instead they may be subspecies of *Rubia cordifolia* itself.[12] In all these studies, only one sample was included from Kerala, i.e., from Thiruvanathapuram. This study thus aimed to conclude the exact identity of the market sample of *Manjishta* with special regards to Kerala markets, which sold raw drugs to around 700 Ayurvedic pharmacies nationwide.

Morphologically, most of the samples were similar to sample number 2, i.e., the original stolon collected from V.P.S.V Ayurveda College, Kottakkal. None of the samples were matching with sample 01, i.e., the genuine root. Hence, the chances of *Manjishta* roots coming in the market are comparatively less based on morphological observations. The main difference in the market samples was in their dimensions and thickness. The market samples collected from Kottakkal, Kannur, Thiruvananthapuram, and Thrissur were very thick even up to 15 mm diameter with prominent nodes, whereas those from Ernakulam and Kozhikkode were a mixture of thin and thick pieces, and most of them were chopped. The original root collected (Sample 1) was very thin, hairy, twisted, smooth, and bark red to brown color. All other samples were pale purple brown externally and purple brown internally.

TS of the genuine root and stolon of *Rubia cordifolia* L. (Samples 1 and 2, respectively) were compared with that of market samples. Most of the characters of the market samples were having similarities with that of genuine stolon. All market samples were having a cork of thickness ranging from 8 to 12 layers which was comparable with that of original stolon. Comparatively wide cortical region observed in Kottakkal sample. Reddish deposits were seen in the cortical region of all the market samples except from those of Kottakkal and Kannur where the deposits are in minimum quantities. However, in original stolon, reddish deposits were less or not seen, instead black granular masses of sandy and acicular crystals were seen. The limited number of sandy crystals and acicular crystals was there in Eranakulam, Kottakkal, and Kannur samples. In Thiruvananthapuram, Thrissur, and Kozhikkode samples, only sandy crystals were seen in the cortical region. The phloem of the original sample was about 20 layered with granular masses of sandy crystals. The phloem in Ernakulam, Kottakkal, and Kozhikkode samples was comparatively narrower than this ranging from 10 to 15 layers. Major portion occupied by xylem elements in most of the samples, but some sample pieces from Kannur market showed narrow xylem region. Xylem vessels were varying in size from 20 µm to 250 µm in different samples, and the vessels with lesser diameter were observed in Kannur sample. The center portion occupied by narrow hollow pith in all the

Table 4: High-performance thin-layer chromatography - retention factor values of the samples at 550 nm

	Sample							
	1	2	3	4	5	6	7	8
Total number of peaks	7	9	5	15	16	7	10	12
R_f	0.05	0.10	0.24	0.03	0.02	0.02	0.02	0.02
	0.11	0.15	0.27	0.05	0.04	0.10	0.04	0.10
	0.28	0.28	0.42	0.07	0.07	0.27	0.07	0.15
	0.42	0.42	0.53	0.10	0.10	0.42	0.10	0.20
	0.50	0.50	0.92	0.16	0.12	0.50	0.15	0.25
	0.55	0.55		0.25	0.16	0.53	0.28	0.29
	0.60	0.59		0.29	0.23	0.91	0.42	0.42
		0.73		0.42	0.28		0.54	0.48
		0.91		0.50	0.38		0.73	0.54
				0.52	0.42		0.91	0.60
				0.55	0.51			0.74
				0.63	0.58			0.92
				0.67	0.60			
				0.72	0.67			
				0.91	0.77			
					0.91			

R_f: Retention factor

samples, but in Kannur sample showed the presence of stem pieces with large pith occupying almost half of the section. All these descriptions are almost similar to the description of standard drug, i.e., the stolon. However, some dissimilarity such as width of cortical region, presence of crystals and reddish brown depositions in cortical cells, area occupied by xylem and pith cells, and xylem vessel size are observed which may be due to the difference in the maturity level of samples or the mixing of similar plants or sub species of *Rubia cordifolia*. Detailed study is required for confirming the causes of dissimilarities and effect on the therapeutic activities of the drug.

The HPTLC comparison of the alcoholic extracts of different samples were spotted and compared. The genuine root and stolon were having seven spots and nine spots, respectively. However, in other samples, the number of spots was very high, especially in those collected from Kottakal, Kannur, and Thiruvananthapuram, indicating the presence of more number of chemical constituents than the original one. Some R_f values of these samples were similar, but the samples from Kannur show some unique peaks which was different from others and even from the genuine one. There were no previously published comparable works for making a conclusion about these differences in the number of peaks of different samples. TLC of ethanol extract of the stolon in n-butanol: acetic acid: water showed a maximum of 6 spots, whereas in an another reference, methanol extract had a maximum of four spots.[5,19] However, these references were not comparable with this study as the systems used for analysis were different.

Minute observations of HPTLC graphs revealed the presence of some more very small peaks in the genuine stolon. However, correct R_f values of these fine observations were not obtained as those peak intensities were very less indicating lower concentrations of corresponding chemical constituents. This in turn may be due to the differences in age-related maturity states of the collected samples. The genuine sample collected was from a 1 year matured plant. In Ayurvedic texts, there are clear guidelines for the collection of drugs as per season.[20]Furthermore, there are methods for the collection of fruits, seeds, flowers, and leaves whereby their maturity can be identified.[21] However, for the parts such as roots and bark, there are no clear cut guidelines for detecting their maturity. A standard operating procedure for the collection of medicinal plant parts as per season, maturity, and locality is currently not published. But fact remains that maturity of a plant part is causally associated with its phytochemical profile.[22,23] Very limited studies have reported the differences in phytochemical constituents based on plant maturity. One such study was that of *Ocimum sanctum*.[24] In the case of samples from Kannur market, there were many peculiar bands on HPTLC graph while comparing with that of the genuine drug. As aforementioned in a study, this sample could be a subspecies of *Rubia cordifolia*.

Quality assurance and standardization procedures are not only aimed at identifying a plant, but also it has to ensure the quality standards and therapeutic values of the source. Quality assurance may be done using the phytochemical parameters such as HPTLC. The phytochemical parameters may vary as per many factors such as soil, climate, and maturity. The ultimate standardization results should be based on their therapeutic indices because more number of chemical constituents many not always is an indicator of good therapeutic activity. Hence, the standards for each plant should be redefined on the basis of maturity of the source, quality standards, and therapeutic actions. To ensure these facts, a standard operating procedure for collection and quality assurance should be defined for plants which rule the local markets. This shall be an elementary step toward evidence-based Ayurvedic practices and research.

CONCLUSION

The collected six market samples of *Manjishta* from different districts of Kerala were pharmacognostically similar to the stolon of *Rubia cordifolia* L. Morphological and anatomical evaluation of the market samples was similar with that of the original stolon of *Rubia cordifolia*. HPTLC profiling yielded entirely different peaks in specific samples when compared with that of the genuine stolon. A clear standard operative procedure should be prepared for medicinal plant part collection with respect to source plant maturity for *Manjishta* or as a matter of fact any other herb, and thus, the HPTLC profiles should be redefined.

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Conflicts of interest

There are no conflicts of interest.

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