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Research article

PHARMACOGNOSTICAL STANDARDIZATION AND HPTLC FINGERPRINT OF *ALSTONIA SCHOLARIS* LINN. BARK

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ABSTRACT: There is increasing global interest in herbal and other forms of traditional medicines. Herbs have long been an important source of numerous effective drugs. As per World Health Organization recommendations, there is a need for investigation of traditional medicinal plants for their potential therapeutic efficacy. The bark of *Alstonia scholaris* (L.) R. Br. (Family: Apocynaceae) locally known as 'Sapthaparni' or 'Satwid', is reported to have anticancer, antihelminthic, antidiarrhoeal, antiasthamatic, antimalarial etc. The present work embodies the study carried out for quality control of herbal drugs which comprises of macroscopy, microscopy, physicochemical properties, phytochemical analysis, fluorescence analysis and HPTLC fingerprint. The anatomical markers present were found to be stone cells, sclereids, cork cells, fibers and prismatic crystals of calcium oxalate. Methanol soluble extractive value was found to be higher than Water, Ethanol and Petroleum ether soluble extractive values. Preliminary phytochemical analysis revealed the presence of tannins, alkaloids, steroids, amino acids, fats, fixed oil, glycosides, proteins, starch and flavonoids. A unique HPTLC fingerprint for *A. scholaris* (L.) R. Br. bark was developed. Results of the present study on pharmacognostical and phytochemical investigation of *A. scholaris* (L.) R. Br. bark will be helpful in developing standards for quality, purity and sample identification of this plant.
Key words: *Alstonia scholaris* L., pharmacognosy, phytochemical analysis, HPTLC fingerprint

INTRODUCTION

Millions of people in the third world use herbal medicines because they believe in them and regard them as their own system of medicine (Chawdhury, 1992). It has been estimated that 80% of people living in developing countries are almost completely dependent on traditional medical practices, for their primary health care needs. Many higher plants are known to be the main source of drug therapy in traditional medicine (Farnsworth *et al.*, 1985 and Martin, 1995). Various tribal sections of India are repositories of rich knowledge on plant genetic resources, which have remained unknown (Khoshoo, 1996). Therefore, there is a need for documentation of research work carried out on traditional medicines (Dahanukar *et al.*, 2000). With this backdrop, it becomes extremely important to make an effort towards standardization of the plant material to be used as medicine. *Alstonia scholaris* (L.) R. Br. (Family: Apocynaceae), also locally known as 'Sapthaparni' or 'Satwid' is a medium to tall tree, about 40 m high with a corky grey to grey-white bark. The outer blaze is cream to yellowish in colour with abundant, milky latex that flows rapidly when cut (Meena *et al.*, 2011). The bark is useful in malarial fevers, abdominal disorders, dyspepsia and skin diseases (Kirtikar and Basu, 1999). The bark is bitter, astringent, digestive, laxative, antihelminthic, antipyretic, stomachic, cardiotonic and tonic (Nadkarni, 1976). The bark extract has been reported to possess antiplasmodial, immunostimulant, anticancer effect and is also hepatoprotective (Lin *et al.*, 1996). In Ayurveda, it is reported that the bark of the plant when soaked in water overnight, can reduce the blood glucose level after oral administration (Bandawane, 2011). Bark is also used as febrifuge, depurative and galactogogue. It is effective in leprosy, skin diseases, prurities, chronic and foul ulcers, asthma, bronchitis and debility (Nadkarni, 1976). In folklore medicine, milky juice is applied on wounds, ulcers and rheumatic pains; mixed with oil and dropped into ear, it relieves ear ache (Arulmozhi, 2010). Some pharmacognostic parameters of *Alstonia scholaris* (L.) R. Br. bark have already been reported in Ayurvedic Pharmacopoeia of India (API).

However, the present study was carried out to re-evaluate pharmacognostic parameters for comparative study and to report some additional important parameters of *Alstonia scholaris* (L.) R. Br. bark; with an aim to enrich the existing pharmacognostic data which may serve as a measure of authentication and quality control for commercial samples of the crude drug. Therefore, the present investigation of *A. scholaris* (L.) R. Br. bark has been undertaken to evaluate and establish quality control as per Indian Pharmacopoeia and World Health Organization (WHO) guidelines, which will help in identification as well as in standardization (Pharmacopoeia of India, 1996 and WHO, 1991).
The WHO accepts fingerprint chromatography as an identification and quality evaluation technique for medicinal herbs since 1991 (WHO, 1991). Fingerprints can be a unique identification utility for herbs and their different species (CHMP, 2005 and Welsh *et al*., 1996). Therefore, HPTLC fingerprint has also been developed for *A. scholaris* (L.) R. Br. bark.

MATERIALS AND METHODS

Bark of *A. scholaris* (L.) R. Br. was collected from Sawantwadi forest area, M. S., India. Herbarium of *A. scholaris* (L.) R. Br. was prepared and authenticated from Blatter Herbarium, St. Xavier's College, Mumbai. Bark was washed under running tap water and blotted dry for further studies. The bark was dried in preset oven at 40 ± 2^0C for about one week, ground into powder and used for further analysis. Organoleptic evaluation can be done by means of sensory organs which includes colour, taste, odour and texture which therefore define some specific characteristic of the material which can be considered as first step towards establishment of identity and purity. The organoleptic characteristics of the bark were carried out based on the method described by Mukherjee (2005). Physicochemical constants such as total ash, acid insoluble ash and water soluble ash; water, ethanol, methanol and petroleum ether soluble extractive values were calculated according to the methods described in Indian Pharmacopoeia (2007). Preliminary phytochemical analysis of powdered bark was performed as described by Khandelwal (2008). Fluorescence analysis was conducted using methods of Kokoski (1958) and Chase and Pratt (1949).
A qualitative densitometric HPTLC analysis was performed with methanolic extract for the development of characteristic fingerprint profile, which may be used for quality evaluation and standardization of the drug. 10 µl of extract was spotted on pre-coated silica gel G60 F_{254} HPTLC plates (Merck) with the help of CAMAG Linomat V applicator. The plate was developed in glass twin trough chamber (20 cm × 10 cm) pre-saturated with mobile phase (Toluene: Ethyl acetate: Methanol: Formic acid, 4:3:1:2). The plate was derivatized using Anisaldehyde-sulphuric acid reagent and scanned using CAMAG TLC Scanner 3.

RESULTS

Pharmacognosy is a simple and reliable tool, by which complete information of the crude drug can be obtained (Gokhale, 2009; Padmavathi *et al.*, 2011; Soni *et al.*, 2011; Bharat and Parabia, 2010). Today with the present surge of interest in the phytotherapeutics, the availability of genuine plant material is becoming scarce. Since crude plant drugs form the basis for the manufacture of numerous medicinal preparations, accurate determination of drug identity forms an essential part of its study. It becomes extremely important to make an effort towards standardization of the plant material as medicine. The process of standardization can be achieved by stepwise pharmacognostic studies (Thomas *et al.*, 2008). These studies help in identification and authentication of the plant material.
Organoleptic evaluation of the drug refers to the evaluation of the material by colour, odour, shape, taste etc. Organoleptic characteristics of bark was done and results are tabulated in Table 1.
The bark is 3-4 mm thick from branches and cut or broken irregularly into curved or flat pieces. It is grayish brown in colour, rough, uneven and fissured. Lenticels are prominent, transversely elongated to rounded in shape and grey to whitish brown coloured. Inner surface of the bark is brownish buff to yellowish brown in colour, somewhat striated and indented, fractured (Fig. 1B).
Transverse section of the bark showed three distinct regions, cork (phellem), cork cambium (phellogen) and secondary cortex (phelloderm). The cork (CR) was multilayered made up of thick cork cells. The cork cambium (CC) forms a narrow zone made up of 2-3 layers of cells. Followed by cork cambium a broad zone of secondary cortex (SC) was present composed of parenchymatous cells. Secondary cortex showed presence of numerous prismatic crystals (PC) of calcium oxalate, sclereids (SCL) and scattered groups of 3-5 stone cells (STC).

The stone cells are of various sizes, shape and thickness. Simple and spherical to oval starch grains are also present in parenchymatous cells. Inner to the secondary cortex, secondary phloem cells consisting of phloem parenchyma and many sieve tubes are present. Sclereids (SCL) can be seen in the form of radially oblique dark lines (Fig 1C – G). Powder microscopy of bark showed presence of medullary rays (MR), stone cells (STC), prismatic crystals (PC) of calcium oxalate, sclereid (SCL), beaded phloem parenchyma (PP), cork cells showing pitted and lignified walls (CCPW), starch grains (SG) and fibers (FB) (Fig: 1H – O). Loss on drying is the loss of mass expressed as percent w/w (Mukherjee, 2005). Percentage of the total ash, acid insoluble ash and water soluble ash were determined. Extractive value using different solvents was determined. Results are tabulated in Table 2. The change in the colour of the bark powder and various extracts under UV radiation with reference to day light was observed. Results of fluorescence analysis of extracts and powder are tabulated in Table 3 and 4 respectively. The preliminary phytochemical screening of bark powder was carried out using various solvents *viz.* Petroleum ether, ethanol, methanol and water. These extracts were subjected to various qualitative chemical analysis which showed presence of tannins, alkaloids, steroids, amino acids, fats and fixed oils, glycosides, proteins, starch, flavonoids and resins and the results are tabulated in Table 5. HPTLC, now a days is applied to obtain "Fingerprint" patterns of herbal formulations, quantification of active ingredients and also detection of adulteration (Sanja *et al.*, 2009). HPTLC fingerprint profile of methanolic extract of bark showed distinct band pattern before and after spraying with derivatizing reagent anisaldehyde sulphuric acid. *Rf* values under different wavelengths before and after derivatization are tabulated in Table 6 (Fig. 2).

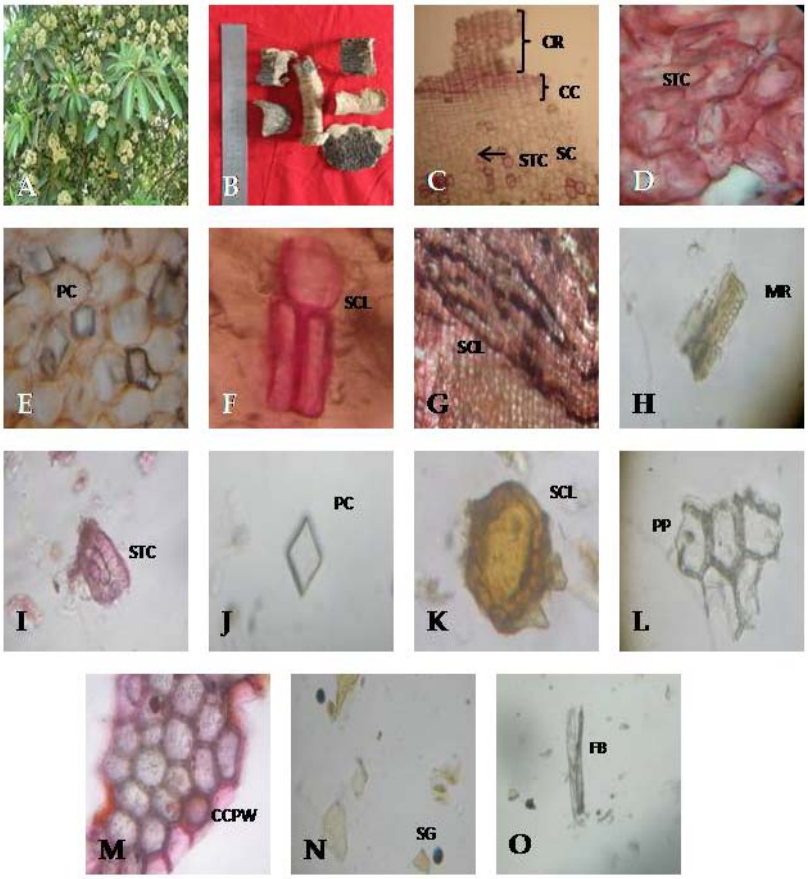

Fig. 1 – Macroscopic, microscopic and powder characteristic of *Alstonia scholaris* (L.) R. Br. Bark. A: Tree, B : Bark, C: T. S. of bark showing cortex (CR),cork cambium (CC) and secondary cortex (SC), D: T. S. of bark showing sclereids (SCL), E: T. S. of bark showing prismatic crystal (PC), F: T. S. of bark showing sclereid (SCL), G: Collapsed phloem elements (CP); H: Powder microscopy showing medullary rays (MR), I: Powder microscopy showing stone cell (STC), J: Powder microscopy showing prismatic crystal (PC), K: Powder microscopy showing sclereid (SCL), L: Powder microscopy showing beaded phloem parenchyma (PP), M: Powder microscopy showing cork cells with pitted and lignified walls (CCPW), N: Powder microscopy showing starch grain (SG), O: Powder microscopy showing fibres (FB).

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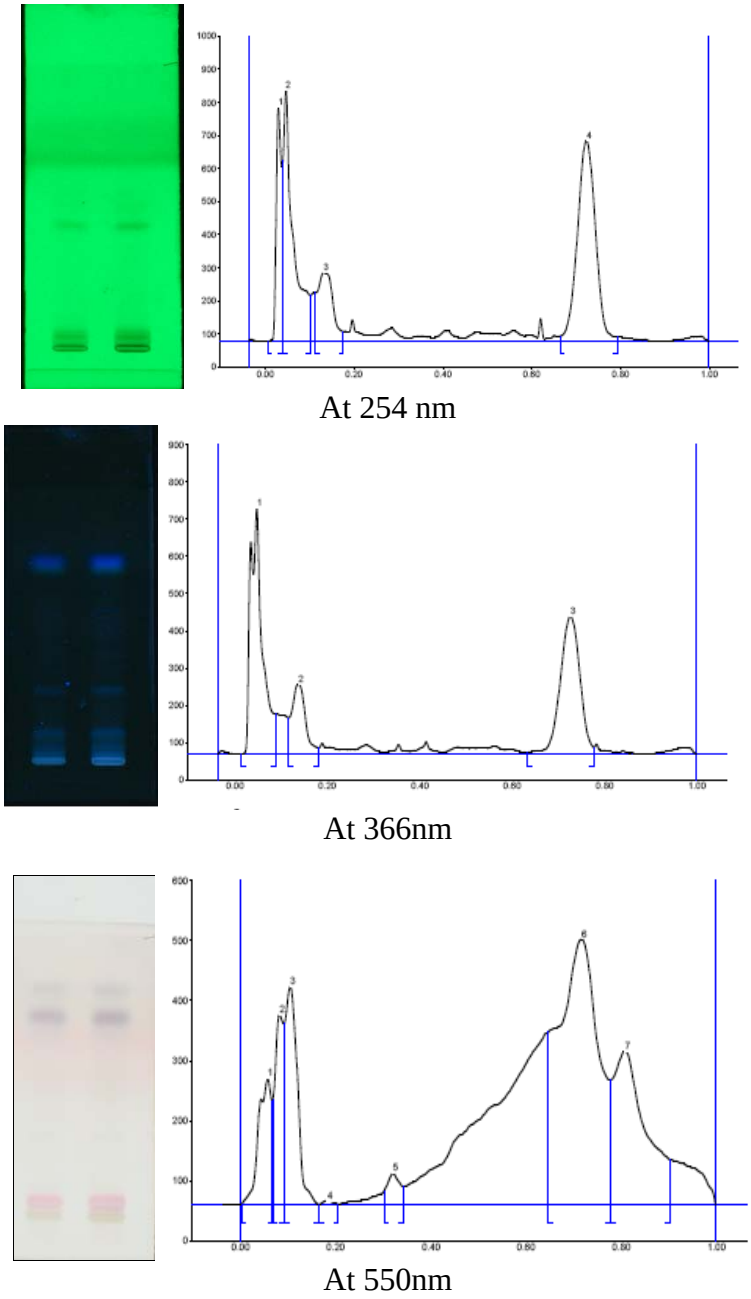

Fig. 2 – HPTLC fingerprint of methanolic extract of *Alstonia scholaris* (L.) R. Br. Bark

Table 1: Organoleptic evaluation of *Alstonia scholaris* (L.) R. Br. bark

Features	Observation
Colour	Outer surface is grayish brown, inner surface is brownish buff to yellowish brown
Odour	No odour
Taste	Bitter
Texture	Rough, spongy, uneven fissured and exuding milky sap when cut

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Table 2: Physico-chemical analysis of *Alstonia scholaris* (L.) R. Br. bark

No.	Parameters	Results (%)
1.	Ash values	
	a. Total ash content	10.47
	b. Acid insoluble ash	2.2
	c. Water soluble ash	4.3
2.	Extractive values	
	a. Aqueous	15.25
	b. Ethanolic	15.15
	c. Methanolic	16.21
	d. Petroleum ether	5.81
3.	Loss on drying	3.58

Table 3: Fluorescence analysis of *Alstonia scholaris* (L.) R. Br. bark extracts

No.	Extract	Observation		
		Day light	UV light	
			254 nm	366nm
1.	Aqueous	Yellow	Purplish pink	Bluish white
2.	Ethanolic	Yellow	Purplish pink	White
3.	Methanolic	Pale Yellow	Purplish pink	White
4.	Petroleum ether	Colourless	Purplish pink	White

Table 4: Fluorescence analysis of *Alstonia scholaris* (L.) R. Br. bark powder

No.	Treatment	Observation		
		Day Light	UV light	
			254 nm	366 nm
1.	Powder as such	Buff	Grey	Buff
2.	Powder + 1N NaOH in methanol	Brown	Black	Brown
3.	Powder + 1N NaOH in methanol, dried, mounted in Nitrocellulose in amyl acetate	Brown	Black	Buff
4.	Powder + 1N HCl	Buff	Grey	Buff
5.	Powder + 1N HCl, dried, mounted in Nitrocellulose in amyl acetate	Buff	Buff	Buff
6.	Powder + 1N NaOH in water	Brown	Buff	Buff
7.	Powder + 1N NaOH in water, dried, mounted in Nitrocellulose in amyl acetate	Brown	Black	Black
8.	Powder + HNO_3 (1:1)	Buff	Buff	Buff
9.	Powder + H_2SO_4 (1:1)	Dark brown	Black	Black
10.	Powder + 1% Picric acid	Buff	Black	Black
11.	Powder + Acetic acid	Brown	Grey	Grey
12.	Powder + 5% Iodine	Orange	Black	Black
13.	Powder + 5% $FeCl_3$	Brownish black	Black	Black
14.	Powder + 25% NH_3 in HNO_3	Brown	Grey	Buff
15.	Powder + Methanol	Buff	Buff	Yellow
16.	Powder + conc. HNO_3	Brown	Black	Black
17.	Powder + 10% $K_2Cr_2O_7$	Buff	Black	Black
18.	Powder + 50% KOH	Brown	Black	Black

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Table 5: Preliminary Phytochemical Analysis of *Alstonia scholaris* (L.) R. Br. Bark

No.	Tests	ME	EE	PE	AE
1.	Tannins	+	+	+	+
2.	Alkaloids	+	+	+	+
3.	Steroids	+	+	+	ND
4.	Aleurone grains	ND	ND	ND	ND
5.	Saponins	ND	ND	ND	ND
6.	Acid compounds	ND	ND	ND	ND
7.	Amino acid	+	+	+	ND
8.	Carbohydrates	ND	ND	ND	ND
9.	Fats & fixed oils	ND	ND	+	ND
10.	Glycosides	+	+	+	+
12.	Protein	ND	ND	+	+
12.	Starch	ND	ND	+	+
13.	Flavonoids	+	+	+	+
14.	Resins	+	+	+	+
15.	Essential oil	ND	ND	ND	ND
16.	Anthraquinones	ND	ND	ND	ND

Key: ME: Methanol extract; EE: Ethanol Extract; PE: Petroleum ether extract; AE: Aqueous extract; +: Present; - : Absent

Table 6: HPTLC fingerprint of methanolic extract of *Alstonia scholaris* (L.) R. Br. Bark

Sr. No.	Before derivatisation		After derivatisation
	254 nm	366 nm	550 nm
1.	0.03	0.05	0.06
2.	0.05	0.14	0.08
3.	0.13	0.73	0.10
4.	0.72	-	0.18
5.	-	-	0.32
6.	-	-	0.72
7.	-	-	0.81

DISCUSSION

Alstonia scholaris (L.) R. Br. is currently being used in the treatment of various disease conditions without standardization. The standardization of a crude drug is an integral part of establishing its correct identity. For inclusion of a crude drug in Pharmacopoeia, pharmacognostic parameters and standards must be established. The results of these investigations could, therefore, serve as a basis for proper identification, collection and further research work of the plant bark. Organoleptic evaluation of drug refers to the evaluation of drug by colour, odour, size, shape, taste and special features including touch, texture etc. Since the majority of information on the identity, purity and quality of the material can be drawn from these observations, they are of importance before any further testing can be carried out. The transverse section of bark showed presence of cortex, secondary cortex, cork cambium, stone cells, prismatic crystals of calcium oxalate and sclereids. The powder microscopy of bark showed presence of medullary rays, stone cells, prismatic crystals of calcium oxalate, sclereids, beaded phloem parenchyma, cork cells showing pitted and lignified walls, starch grains and fibers. Moisture is an inevitable component of crude drugs, which must be eliminated as far as possible. Insufficient drying favours spoilage by molds and bacteria and makes possible the enzymatic destruction of active principles. Loss on drying was performed according to the method described by Mukherjee (2005) and was found to be 3.58%. Equally important in the evaluation of the crude drugs, is the ash value, water soluble ash value and acid insoluble ash value determination. Ash of any organic material is composed of their non-volatile inorganic components.

Controlled incineration of crude drug results in an ash residue consisting of an inorganic material (metallic salts and silica). This value varies within fairly wide limits and is therefore an important parameter for the purpose of evaluation of crude drugs (Indian Pharmacopoeia, 2007). The total ash is particularly important in the evaluation of purity of drugs, i.e. the presence or absence of foreign/indigenous inorganic matter such as metallic salts and/or silica (Kalidas *et al.*, 2009). The total ash value, water soluble ash value and acid insoluble ash value of *A. scholaris* (L.) R. Br. bark were found to be 10.47%, 2.20% and 4.30% respectively. Since the ash value is constant for the given drug, this value is one of the diagnostic parameter of the drug. Extractive values are primarily useful for the determination of exhausted or adulterated drugs. The extraction of any crude drug with a particular solvent yields an extract containing different phyto-constituents. It is also useful for evaluation of crude drug, which gives an idea about the nature of the chemical constituents present in a crude drug and is useful for the estimation of specific constituents, soluble in that particular solvent used for extraction (Khandelwal, 2008). Water, ethanol, methanol and petroleum ether soluble extractive values were calculated and were found to be 15.25%, 15.15%, 16.21% and 5.81% respectively. The methanolic extractive value was found to be higher (16.21%) than the other solvents used viz. water, ethanol and petroleum ether, revealing presence of large amount of methanol soluble constituents in the bark. The fluorescence method is adequately sensitive and enables the precise and accurate determination of the analyte over a satisfactory concentration range without several, time consuming dilution steps prior to analysis of pharmaceutical samples (Brinda *et al.*, 1981). Kalidas *et al.* (2009) suggested that a non-fluorescent compound may fluoresce if mixed with impurities that are fluorescent. Therefore, the results obtained from the present fluorescent studies will also help to check any impurities present in bark powder of *A. scholaris* (L.) R. Br. Presence or absence of certain important compounds in an extract is determined by colour reaction of the compound with specific chemicals. This procedure is a simple preliminary prerequisite before going for detailed phytochemical investigation. Various tests have been conducted qualitatively to find out the presence or absence of bioactive compounds (Lalitharani, 2013). Different chemical compounds such as tannins, alkaloids, steroids, amino acids, fats and fixed oils, glycosides, protein, starch, flavonoids and resins are detected in *A. scholaris* (L.) R. Br. bark extracts which could make the plant useful for treating different ailments as having a potential of providing useful drugs for human use.

Currently HPTLC is often used as an alternative to HPLC for the quantification of plant products because of its simplicity, accuracy, cost-effectiveness and rapidity (Aktar *et al.*, 2008). HPTLC fingerprint has better resolution and estimation of active constituents can be done with reasonable accuracy in a shorter time (Pawar *et al.*, 2011). Chromatographic fingerprint is a rational option to meet the need for more effective and powerful quality assessment to ITM (Indian Traditional Medicine) and TCHM (Traditional Chinese Herbal Medicine). The optimized chromatographic finger print is not only an alternative analytical tool for authentication, but also an approach to express the various patterns of chemical ingredients present in the herbal drugs. HPTLC finger print analysis has become the most potent tool for quality control of herbal medicines because of its simplicity and reliability. It can serve as a tool for identification, authentication and quality control of herbal drugs. HPTLC fingerprint profile along with their *Rf* values have been recorded, which would serve as a reference standard for the scientist engaged in research on the medicinal properties of plant (Mauji *et al.*, 2011).

The various morphological, microscopical, physicochemical and phytochemical standards developed in this study will help for botanical identification and standardization of the bark in crude form.

CONCLUSION

Plant materials are used throughout developed and developing countries as home remedies, over-the-counter drug products and raw materials for the pharmaceutical industry and represent a substantial proportion of the global herbal drug market. Therefore it is essential to ensure reproducible quality of herbal products. Thus in recent years there has been an emphasis on standardization of medicinal plants of therapeutic potential. Identification and evaluation of plant drugs by pharmacognostical studies is reliable, accurate and inexpensive method. Since *A. scholaris* (L.) R. Br. bark is known for its various medicinal properties, the present study could be useful to supplement information with respect to its identification, authentication and standardization. The information generated can also be useful for preparation of monograph of the plant, which could be incorporated in the preparation of Indian Herbal Pharmacopoeia.

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