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Evaluation for substitution of stem bark with small branches of *Myrica esculenta* for medicinal use – A comparative phytochemical study

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ABSTRACT

Background: Over exploitation of many traditional medicinal plants like *Myrica esculenta* has become a threat and in the near future, many medicinal plants may be unavailable for use of industry.
Objective: Present study outlines the concept of plant part substitution. Stem bark and small branches of *M. esculenta* are compared on the basis of physicochemical analysis, phytochemical analysis, total phenolic contents, total flavonoid contents and high performance thin layer chromatography (HPTLC) to evaluate the possibilities of using small branches in place of stem bark.
Material and methods: Physicochemical parameters and preliminary phytochemical screening were carried out using standard methods. Total phenolic and total flavonoid contents were estimated spectrophotometrically using Folin-Ciocalteu and aluminum chloride method, respectively. CAMAG HPTLC system equipped with semi-automatic applicator was used for HPTLC profiling. *n*-Hexane, ethyl acetate and ethanol extracts of stem bark and small branches were developed in suitable mobile phase using standard procedures and visualized in UV 254 and 366 nm and in white light after derivatization within anisaldehyde-sulphuric acid reagent.
Results: Phytochemical analysis and HPTLC profile of different extracts showed the presence of almost similar phytochemicals in both stem bark and small branches.
Conclusion: Similarities in phytochemical analysis and HPTLC profile of various extracts suggests that small branches may be used in place of stem bark. The study provides the base for further study to use small branches as a substitute of stem bark of *M. esculenta*.

1. Introduction

The importance of medicinal plants has been realized and well documented by physician and scientists since ancient time. Majority of the population in developing countries depend on traditional system of medicine for their primary health care. Overharvesting of many traditional medicinal plants indirectly poses a risk to the society and has placed many medicinal species at risk of extinction. Commercial exploitation has also sometimes led to traditional medicines becoming unavailable to the indigenous peoples who have relied on them for centuries. In Indian system of medicine the most commonly used medicinal plants are slow-growing trees, with bark and underground parts being the parts mainly utilized. Collection of underground parts, stem or bark of medicinal plants leads to mass scale uprooting from their natural habitat. This is leading to depletion of plant resources in near future. Due to which plant may be difficult to use in traditional system of medicine in near future. Substitution of the plant or plant parts is therefore the need of the hour for preventing medicinal plants from becoming red listed. It will provide greater scope for practitioners of traditional medicine to utilize herbs that are easily available, cost-effective and most appropriate for the clinical condition. There are a number of possible approaches to solve this problem like establishing conservation areas, enforcing laws against collecting underground parts and bark, large-scale cultivation and use of alternative parts of the same plant such as aerial parts in place of underground parts. Substitution with the part of the same plant is likely to be much better accepted by the patients of traditional healer. Possibilities of substitution of aerial parts with

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underground parts as a strategy for conservation of medicinal plants has been studied by researchers in *Eucomis autumnalis*, *Siphonochilus aethiopicus*, *Ocotea bullata*, *Warburgia salutaris* [1] and *Aegle marmelos* [2].

Myrica esculenta (Family: Myricaceae) commonly called Katphala is a medicinal plant widely used in Ayurveda. As per the Ayurvedic literature, stem bark of this plant is used in *gulma* (abdominal tumors), *jvara* (fever), *arsa* (piles), *grahani* (irregular bowel function), *pandu roga* (anemia), *hrillasa* (nausea), *mukha roga* (oral disorders), *kasa* (cough), *svasa* (dyspnea), *agnimandhya* (indigestion), *aruchi* (anorexia) and *kantharoga* (ears, nose, and throat disorders) [3]. Stem bark is also reported for various pharmacological activities likes radical-scavenging [4], antioxidant [4,5], anti-diabetic [6], anxiolytic [7], antibacterial [8], anti-helmintic [9], anti-allergic [10], anti-inflammatory [11,12], antimicrobial [12], mast cell stabilizing [13], anti-asthmatic [14].

The stem bark mainly contains gallic acid, castalagin [15], myricanol, myricanone [16–18], epigallocatechin 3-*O*-gallate, epigallocatechin-(4$\beta\rightarrow$8)-epigallocatechin 3-*O*-gallate, 3-*O*-galloylepigallocatechin-(4$\beta\rightarrow$8)-epigallocatechin-3-*O*-gallate, proanthocyanidin, catechine, delphinidine chloride [19], myriconol [20], quercetin, β-sitosterol, taraxerol and triterpene diol [21]. Removal of stem bark from trunk damages the plant. Due to this, availability of the plant may be difficult in near future for use in Indian system of medicine. Present study is carried out in *M. esculenta* to evaluate the possibilities of using small branches in place of stem bark, which will help sustainable utilization.

2. Material and methods

2.1. Plant material

Stem bark and small branches of *M. esculenta* were collected from Regional Research Institute of Himalayan Folra, Thapala, Ganiadholi, Ranikhet, Distt. Almora (Uttrakhand). Plant material was identified and authenticated by botanist of the Institute, a voucher Specimen (Accession no. 2115) was deposited in Institute.

2.2. Instrumentation

A CAMAG HPTLC system (Muttenz, Switzerland) equipped with a semi automatic TLC applicator Linomat IV, twin trough plate development chamber, Win CATS software version 1.4.2. and Hamilton (Reno, Nevada, USA) Syringe (100 μl).

2.3. Material and reagents

All chemicals, reagents and solvents used during the experimentation were of analytical grade and HPTLC plates were purchased from E. Merck Pvt. Ltd. (Mumbai, India).

2.4. Physicochemical parameters

Stem bark and small branches were studied for various physicochemical standards like foreign matter, loss on drying at 105 °C, total ash, acid-insoluble ash, alcohol soluble extractive, water-soluble extractive and pH of 10% aqueous solution using standard methods [22,23].

2.5. Preliminary phytochemical screening

n-Hexane, ethyl acetate and ethanol extracts of both stem bark and small branches were screened for the presence of phenols, tannins, carbohydrates, saponins, alkaloids, proteins, flavonoids, steroids, furanoids, coumarins, quinone and triterpenoids by the standards methods of Harborne [24] and Kokate et al. [25].

2.6. Estimation of total phenolic and flavonoid content

Five grams of each of shade-dried plant material was pulverized into coarse powder and subjected to ethanolic extraction using Soxhlet apparatus. Extracts were concentrated to dryness. Dried residues were then dissolved in 100 ml of 95% ethanol. Extracts were used for total phenolic and flavonoid assay.

Total phenolics content was determined by using Folin-Ciocalteu assay [26]. An aliquot (1 ml) of extracts or standard solution of gallic acid (20, 40, 60, 80 and 100 μg/ml) was added to a 25 ml volumetric flask, containing 9 ml of distilled water. A reagent blank was prepared using distilled water. One milliliter of Folin-Ciocalteu phenol reagent was added to the mixture and shaken. After 5 min, 10 ml of 7% Na_2CO_3 solution was added to the mixture. Volume was then made up to the mark. After incubation for 90 min at room temperature, absorbance against reagent blank was determined at 550 nm with an UV/Vis spectrophotometer. Total phenolics content was expressed as mg gallic acid equivalents (GAE).

Total flavonoid content was measured by aluminum chloride colorimetric assay [27]. An aliquot (1 ml) of extracts or standard solutions of quercetin (20, 40, 60, 80 and 100 μg/ml) was added to a 10 ml volumetric flask containing 4 ml of distilled water. To the flask, 0.3 ml of 5% $NaNO_2$ was added and after 5 min, 0.3 ml of 10% $AlCl_3$ was added. After 5 min, 2 ml of 1M NaOH was added and volume was made up to 10 ml with distilled water. Solution was mixed and absorbance was measured against blank at 510 nm. Total flavonoid content was expressed as mg quercetin equivalents (QUE).

2.7. HPTLC profiles

HPTLC studies were carried out by following methods of Sethi [28], Stahl [29] and Wagner et al. [30]. Stem bark and small branches were powdered coarsely. Ten gram powdered samples of each of stem bark and small branches were accurately weighed and exhaustively extracted by *n*-hexane, ethyl acetate and ethanol (each 100 ml) separately using Soxhlet apparatus. Extracts were filtered and concentrated under reduced pressure and made up to 10 ml in standard flasks separately.

Mobile phases used for developing the *n*-hexane, ethyl acetate and ethanol extract were toluene: ethyl acetate (5:5 v/v), toluene: ethyl acetate (7:3 v/v) and toluene: ethyl acetate: formic acid (5:5:0.5 v/v), respectively.

Samples were spotted in the form of bands of width 10 mm with a 100 μl Hamilton syringe on aluminum TLC plates pre-coated with Silica gel 60 F_{254} of 0.2 mm thickness with the help of TLC semi-automatic applicator Linomat IV attached to CAMAG HPTLC system, which was programmed through Win CATS software version 1.4.2. 10 μl of each extracts of stem bark and small branches were applied in two tracks as 10 mm bands at a spraying rate of 10 s/μl. Track 1 was stem bark and track 2 was small branches for each of extracts applied.

Development of plate upto a migration distance of 80 mm was performed at 27 ± 2 °C with mobile phase for each extracts in a CAMAG HPTLC chamber previously saturated for 30 min. After development the plate was dried at 60 °C in an oven for 5 min and visualized under wavelength 254 nm and 366 nm for ultraviolet detection. Developed plate was then dipped in anisaldehyde sulphuric acid reagent for derivatization and dried at 105 °C in hot air oven till color of band appears and visualized under white light. Images were captured by keeping plates in photodocumentation chamber and R_f values were recorded by Win CATS software.

Table 1
Physicochemical parameters of stem bark and small branches of *M. esculenta*.

S. no.	Parameters	Results	
		Stem bark	Small branches
1.	Foreign matter (% w/w)	Nil	Nil
2.	Loss on drying (% w/w)	6.47	6.81
3.	Total ash (% w/w)	1.010	1.856
4.	Acid insoluble ash (% w/w)	0.187	0.320
5.	Alcohol soluble extractive value (% w/w)	23.57	5.03
6.	Water soluble extractive value (% w/w)	18.36	3.52
7.	pH of 10% aqueous solution	4.64	4.88

3. Results

Physicochemical parameters like foreign matter, loss on drying at 105 °C, ash values, acid insoluble ash, extractive values and pH are given in Table 1.

Phytochemical analysis of different extracts of stem bark and small branches are shown in Table 2. Results reveal the presence of similar phytochemicals in stem bark and small branches except for quinones which were found present in ethyl acetate extract of stem bark and absent in ethyl acetate extract of small branches.

Total amount of phenolics and flavonoids content of ethanolic extract of stem bark and small branches of *M. esculenta* are summarized in Table 3. Results indicate that in comparison to small branches, stem bark had high total phenolic and flavonoid content.

HPTLC profile of *n*-hexane extract of stem bark and small branches (Table 4 and Fig. 1) showed three and two bands, respectively when visualized under UV at 254 nm out of which one band at R_f 0.49 was found similar. At UV 366 nm stem bark and small branches showed six and five bands, respectively out of which four bands at R_f 0.42 (blue), 0.51 (blue), 0.74 (florescent blue), 0.91 (florescent red) were found similar. Band at R_f 0.83 was also found common to both parts but with different color. Visualization under white light after derivatization with anisaldehyde sulphuric acid reagent, stem bark and small branches showed seven and five bands, respectively out of which five bands at R_f 0.41 (gray), 0.62 (blue), 0.73 (purple), 0.82 (purple), 0.97 (purple) were found similar.

HPTLC profile of ethyl acetate extract of stem bark and small branches (Table 5 and Fig. 2) showed seven and two bands, respectively at UV at 254 nm out of which two bands at R_f 0.47 (black), 0.67 (black) were found similar. At UV 366 nm stem bark and small branches showed eleven and seven bands, respectively out of which six bands at R_f 0.18 (red), 0.49 (florescent blue), 0.65 (florescent blue), 0.75 (florescent blue), 0.85 (purple), 0.90 (red) were found similar. Visualization under white light after derivatization with anisaldehyde sulphuric acid reagent stem bark and small branches showed ten and seven bands, respectively out of which seven bands at R_f 0.07 (blue), 0.09 (purple), 0.48 (blue), 0.55 (blue), 0.61 (dark blue), 0.75 (purple), 0.99 (dark blue) were found similar.

Table 3
Total phenolic and total flavonoid content of ethanol extracts of stem bark and small branches of *M. esculenta*.

S. no.	Plant parts	Total phenolics mg of GAE/g dry weight[a]	Total flavonoids mg of QUE/g dry weight[a]
1.	Stem bark	276.78 ± 5.36	121.68 ± 6.81
2.	Small branches	31.24 ± 2.57	12.94 ± 1.12

[a] Values are expressed as Mean ± SD.

HPTLC profile of ethanol extract of both stem bark and small branches (Table 6 and Fig. 3) showed two bands with similar R_f when visualized under UV at 254 nm. At UV 366 nm stem bark and small branches showed three and five bands, respectively and all the three bands at R_f 0.54 (purple), 0.73 (florescent blue), 0.84 (red) in HPTLC profile of stem bark were appeared in small branches also. Visualization under white light after derivatization with anisaldehyde sulphuric acid reagent, stem bark and small branches showed two and three bands respectively. Here also both the two bands at R_f 0.24 (purple), 0.66 (purple) in HPTLC profile of stem bark were appeared in small branches.

4. Discussion

Stem bark of *M. esculenta* is mentioned as ingredient in some Ayurvedic formulations. Removal of stem bark from the trunk of this tree may make this plant weak and susceptible to damage by insects and natural elements. The availability of this plant may be difficult in near future for use in Indian system of medicine. Therefore conservation and protection of this plant from extinction has become a matter of urgency. The present study carried out in *M. esculenta* to evaluate the possibilities of using small branches in place of stem bark outlines the concept of plant substitution and will help sustainable utilization. Data of physicochemical parameters (Table 1) may be useful to pharmaceutical industries for the authentication and batch to batch consistency of the commercial samples. Both the parts of *M. esculenta* were found to possess little moisture and hence can be stored at room temperature without fear of spoilage. Almost similar results for qualitative phytochemical analysis of various extracts of stem bark and small branches of *M. esculenta* (Table 2) indicates the presence of analogous compounds in both the parts of this plant and is obviously of great

Table 2
Phytochemical analysis of extracts of stem bark and small branches of *M. esculenta*.

Phytochemicals	Stem bark			Small branches		
	n-Hexane	Ethyl acetate	Ethanol	*n*-Hexane	Ethyl acetate	Ethanol
Alkaloids	−ve	+ve	+ve	−ve	+ve	+ve
Carbohydrates	−ve	+ve	+ve	−ve	+ve	+ve
Coumarins	−ve	−ve	−ve	−ve	−ve	−ve
Flavonoids	−ve	+ve	+ve	−ve	+ve	+ve
Furanoids	−ve	+ve	+ve	−ve	+ve	+ve
Phenols	−ve	+ve	+ve	−ve	+ve	+ve
Proteins	−ve	−ve	−ve	−ve	−ve	−ve
Quinone	−ve	+ve	+ve	−ve	−ve	+ve
Saponins	−ve	−ve	−ve	−ve	−ve	−ve
Steroids	−ve	+ve	+ve	−ve	+ve	+ve
Tannins	−ve	+ve	+ve	−ve	+ve	+ve
Triterpenoids	−ve	−ve	+ve	−ve	−ve	+ve

Table 4
R_f value of *n*-hexane extract of *M. esculenta*.

S. no.	Wavelength	R_f value	
		Stem bark	Small branches
1.	254 nm	0.49, 0.69, 0.88	0.49, 0.78
2.	366 nm	0.42, 0.51, 0.59, 0.74, 0.83,0.91	0.42, 0.51, 0.74,0.83,0.91
3.	Visible light after derivatization	0.41, 0.62, 0.65, 0.73, 0.82, 0.92, 0.97	0.41, 0.62, 0.73, 0.82, 0.97

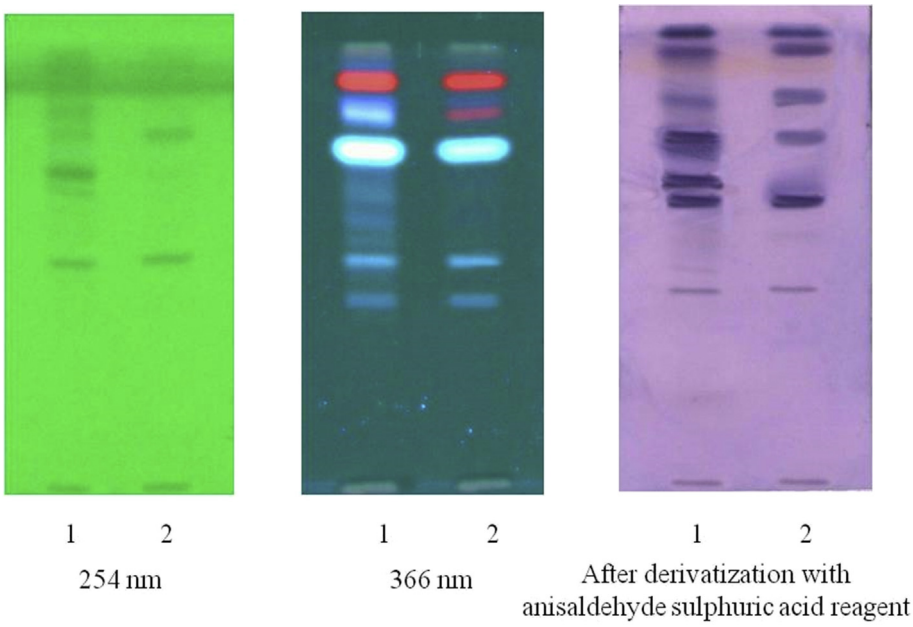

Fig. 1. HPTLC profile of *n*-hexane extracts of stem bark and small branches of *M. esculenta*. (Track 1: stem bark, track 2: small branches).

Table 5
R_f value of ethyl acetate extract of *M. esculenta*.

S. no.	Wavelength	R_f value	
		Stem bark	Small branches
1.	254 nm	0.07, 0.12, 0.36, 0.47, 0.61, 0.67, 0.84	0.47, 0.67
2.	366 nm	0.11, 0.15, 0.18, 0.33, 0.38, 0.44, 0.49, 0.65, 0.75, 0.85, 0.90	0.18, 0.30, 0.49, 0.65, 0.75, 0.85, 0.90
3.	Visible light after derivatization	0.07, 0.09, 0.30, 0.36, 0.48, 0.55, 0.61, 0.75, 0.93, 0.99	0.07, 0.09, 0.48, 0.55, 0.61, 0.75, 0.99

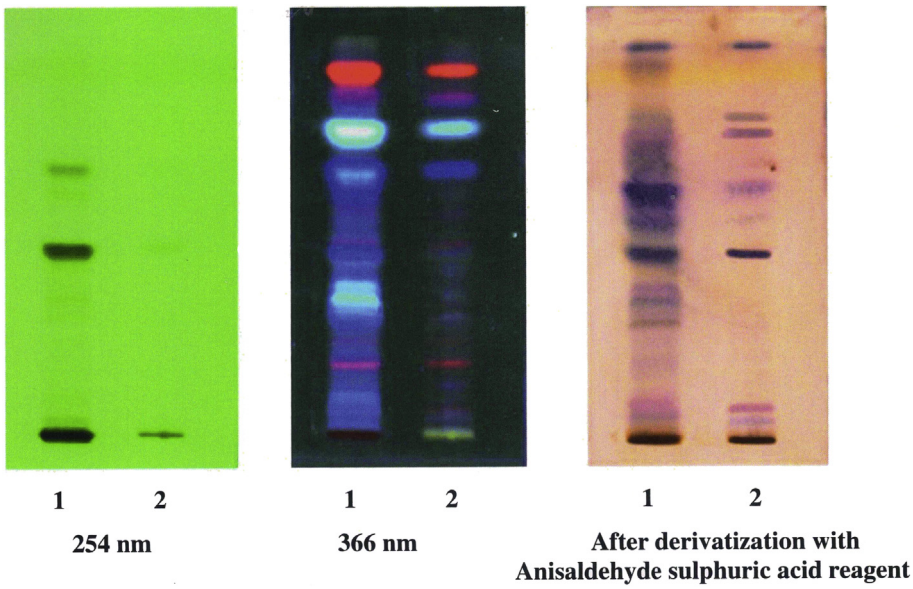

Fig. 2. HPTLC profile of ethyl acetate extracts of stem bark and small branches of *M. esculenta*. (Track 1: stem bark, track 2: small branches).

Table 6
R_f value of ethanol extract of *M. esculenta*.

S. no.	Wavelength	R_f value	
		Stem bark	Small branches
1.	254 nm	0.23, 0.54	0.23, 0.54
2.	366 nm	0.54, 0.73, 0.84	0.25, 0.45, 0.54, 0.73, 0.84
3.	Visible light after derivatization	0.24, 0.66	0.24, 0.66, 0.90

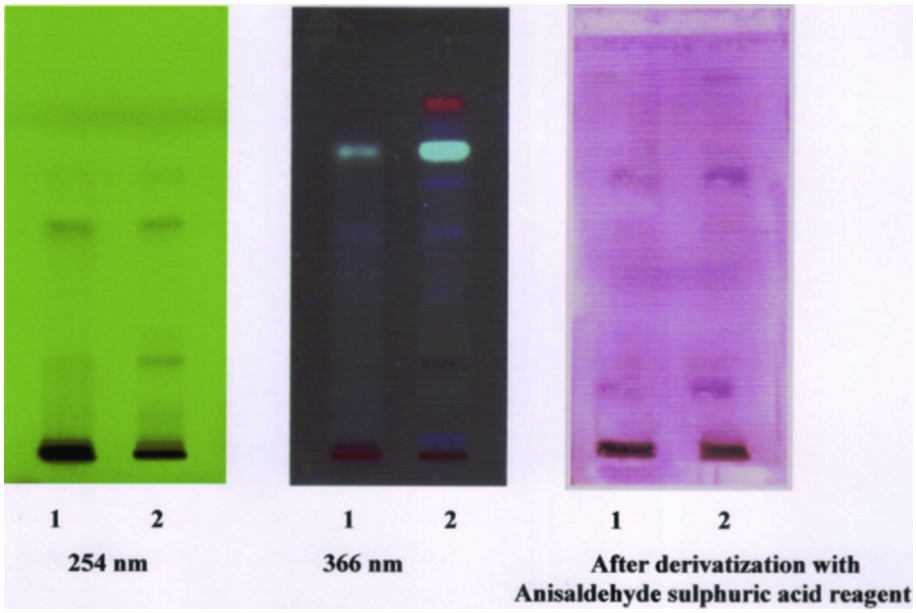

Fig. 3. HPTLC profile of ethanol extracts of stem bark and small branches of *M. esculenta*. (Track 1: stem bark, track 2: small branches).

importance for a scientist's suggestion because healers may substitute the plant parts of the same plant they traditionally use. Less quantity of active phytochemicals like total phenolics and total flavonoids in small branches (Table 3) may be compensated by using more quantity of small branches in formulations because this approach may satisfy the requirements of sustainable harvesting, yet simultaneously provide for primary health care needs.

In spite of the availability of a many sophisticated analytical techniques, thin layer chromatography is still one of the fastest, cheapest and most effective methods to obtain a characteristic analytical fingerprint of a plant extract [30]. Comparative evaluation of HPTLC profiles *n*-hexane, ethyl acetate and ethanol extracts of stem bark and small branches carried out to reveal the chemical pattern showed many similar bands which again indicate the presence of many similar compounds in stem bark and small branches of *M. esculenta*. Similarities in both phytochemical analysis as well as HPTLC profiles of stem bark and small branches suggest that small branches may have nearly similar active constituents like stem bark and may be investigated in detail for use as a substitute of stem bark. With the help of our preliminary results we demonstrate the possibilities of plant part substitution in this plant but phytochemical investigations can never substitute pharmacological investigations in determining the therapeutic value of the plant material. Further investigation on comparison and confirmation of same for pharmacological activities on these aspects is needed to support the findings and coming to the conclusion, so a detailed research covering aspect of pharmacology and toxicity, a major concern with respect to plant part substitution, will be following soon. Plant part substitution is the need of the hour for conservation of medicinal plants as many medicinal plants are becoming red listed. It can provide greater scope for the physicians to utilize raw drugs that are easily available, cost effective and most appropriate for the clinical conditions. We therefore suggest that every investigation on underground parts or stem bark of medicinal plant may include an investigation on aerial parts of plant also, even though those might not be the parts traditionally used. This study is an initiative to add solution inputs to global concern for the management of traditional medicinal plant resources which now-a-days has become a matter of urgency. Investigations like this, may protect more species from extinction, and allow the recovery of threatened medicinal plants. Results of qualitative evaluation of HPTLC profiles will also be helpful in the identification and quality control of the drug and can provide standard HPTLC profiles with selected solvent system. The HPTLC profiles can also be used as a reference for the proper identification/authentication of the drug.

5. Conclusion

Similarities in HPTLC profiles and phytochemical analysis of various extracts of stem bark and small branches suggests that small branches may have almost similar active constituents like stem bark. Hence, the study provides the base for further study to recommend small branches in place of stem bark which can save the plant from destruction. Investigations like this, may protect many plant species from extinction, and allow the recovery of threatened medicinal plants.

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