Indian Journal of Traditional Knowledge
Vol 20(2), April 2021, pp 351-357

Taxonomy, pharmacognosy and phytochemical characteristics to identify the authentic and substitute botanical sources used as *Agnimantha*

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Received 29 August 2019; revised 02 March 2021

The roots of *Clerodendrum phlomidis* L.f. are known to be used as *Agnimantha* in Ayurvedic system of medicine. Whereas literature emerged from various texts reports other species of *Premna* as *Agnimantha* that leads to controversies in usage of authentic drug source. Hence this study aims to develop pharmacognosy characteristics to identify the authentic and substitute sources of *Agnimantha*. The Ayurvedic verses prescribed for *Agnimantha* are in compliance with *Clerodendrum phlomidis*, *Premna serratifolia* and *Premna mollissima*. Whereas the vertically fissured stem is found prominently in *Clerodendrum phlomidis*. Likewise cork region is composed of homogenous cells with grouped and scattered calcium oxalate crystals, without oils. Whereas heterogeneous cells with oils and scattered calcium oxalate crystals are found in *Premna serratifolia* and *Premna mollissima*. Similarly phytosterols found unique in *Clerodendrum phlomidis* roots. The developed HPTLC method serves as tool to identify and distinguish the authentic from substitute and adulterants. Enthrallingly quantitative HPLC analysis revealed the presence of verbascoside in all sources of *Agnimantha* and comparatively more in substitutes. Hence the study concludes that roots of *Clerodendrum phlomidis* is authentic source of *Agnimantha*, where *Premna serratifolia* and *Premna mollissima* forms the equivalent substitutes.

Keywords: *Agnimantha*, *Clerodendrum phlomidis*, *Dasamula*, *Premna*, HPLC

IPC Code: Int Cl.[21]: A61K 36/00, A61K 36/53, A61K 36/185, A01H 6/50 A01N 65/22

The plant *Clerodendrum phlomidis* L.f. that belongs to family Lamiaceae (earlier Verbenaceae) is known as *Agnimantha* in Ayurveda. *Agnimantha* is one of the major ingredients in Ayurvedic medicines like *Chyavanaprasha*, *Brahmarasayana*, *Gorocanadivati, Narayanataila* and around 25 similar products in which *Dasamula* is included[1]. The roots of *Clerodendrum phlomidis* reported to contain β-sitosterol, γ-sitosterol, ceryl alcohol, clerodin, clerosterol, clerodendrin-A[2]. The dried mature roots of *Agnimantha* are used to treat inflammatory diseases, swellings and rheumatic pain and are also considered to possess analgesic and anti-asthmatic properties[3,4]. The estimated annual trade of *Clerodendrum phlomidis* was 200 to 500 MT which costs Rs. 35-45/kg and *Premna serratifolia* L. was 100 to 200 MT that costs Rs. 10-15/kg[5]. The Ayurvedic Pharmacopoeia of India correlates *Clerodendrum phlomidis* for *Agnimantha*. Similarly part I of first edition in Ayurvedic Formulary of India[6] reports *Clerodendrum phlomidis* is authentic plant source whereas *Premna integrifolia* Willd and *Premna mucronata* Roxb. as substitutes. Additionally, it recommends all *Premna* species can be used as substitutes for *Agnimantha*. Whereas, it is *vice versa* in second edition[7] while in part II of first edition[8], *Clerodendrum phlomidis* is reported as authentic plant source and *Premna obtusifolia* R. Br. and *Premna mucronata* as alternate or substitutes. The comprehensive studies on authentic and substitute sources of *Agnimantha* are poorly documented. Thus, there is lack of clarity regarding utilization of genuine *Agnimantha* that has been referred in the ancient texts. Current practice of Ayurvedic physician and industry is to use one of the species of *Clerodendrum* or *Premna* depending on the availability in a particular area. But for global acceptance as well as for providing safe and effective Ayurvedic products, it is imperative to identify the authentic botanical entity to have regulatory compliance. Hence, the study aimed

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to identify the authentic and substitute species of *Agnimantha* by correlating the Ayurvedic verses and synonyms with botanical characters and differentiate them by taxonomic and pharmacognosy studies.

Material and Methods

Plant materials

The mature roots of *Clerodendrum phlomidis*, *Premna serratifolia* and *Premna mollissima* of minimum four to five accessions of each were collected from different bio geographic regions in southern India. Collected plant samples were authenticated and each sample was assigned with voucher numbers and voucher specimens were deposited in FRLH Herbarium, Bengaluru, India.

Etymology and botanical correlations

The botanical correlations were carried out using *Namarupavignanam*[9].

Macroscopy and organoleptic analysis

The macroscopic and organoleptic characters are analyzed by following the pharmacognostic parameters by Wallis[10].

Microscopy

Anatomy

Collected roots were cut into small pieces and fixed in FAA (Formalin, Acetic acid and 70% Ethyl alcohol - 5 mL: 5 mL: 90 mL) immediately after collection[11]. Transverse sections were taken using sharp razor blades and the sections were stained with Toluidine blue as per method published[12]. The stained sections were washed with distilled water and mounted on clean slide. Descriptive terms of anatomical features are followed as per standard anatomical nomenclature[13]. Photomicrographs of different magnifications were taken with Nikon Lab Photo 2 Microscopic unit.

Histochemistry

The fresh root specimens from each collection were allowed to saturate in water and transverse sections were taken using sharp razor blades. The sections were stained using specific reagents for localizing starch, lignin, calcium oxalate, tannins and total lipids[14].

Powder microscopy

The roots are powdered and sieved using Mesh no 16 to a pore size of 1 mm as per the Bureau of Indian Standards, the powder is macerated with Jeffery's maceration fluid (1:1 of 10% nitric acid and 10% chromic acid) mixture and heated in water bath until a bleaching effect was observed[15]. The enduring acid in bleached powder is subjected to serial water wash and neutralized by adding few drops of ammonium hydroxide. The macerated powder was then stained with Toluidine Blue-'O' and observed for powder characters.

Physicochemical analysis

Physicochemical parameters like moisture content, total ash, acid insoluble ash, alcohol solubility and water solubility were carried out as per quality standards of Ayurvedic Pharmacopeia of India[6].

Phytochemical screening

The phytochemical screening was performed by using standard procedures[16,17] in order to establish a chemical profile. The powdered roots were successively extracted using different solvents of increasing polarity viz., hexane, ethyl acetate, chloroform, methanol and water for eight cycles at 60-70^{o}C and concentrated to 25 mL at 40-45^{o}C using rotary evaporator and screened for phytoconstituents.

HPTLC finger printing

The powder of roots of *Agnimantha* sources of 1 g each was refluxed with 50 mL methanol in water bath and filtered using Whatman No 1 filter paper. The residues were extracted until it became colourless. The extracts were filtered and concentrated to 10 mL at 40-45^{o}C using rotary evaporator. The qualitative HPTLC analysis was performed using 20 µL of methanol root extracts of *Agnimantha* sources using solvent system (Methanol: Chloroform 19: 1 v/v). The plates were dried and viewed under TLC visualize before and after derivatization with anisaldehyde sulphuric acid.

HPLC

The quantitative HPLC analysis was performed with methanol root extracts of *Agnimantha* sources in Isocratic elution at a flow rate of 1.2 mL /min employed on Phenomenex Luna, C_{18}, 2.5 µ (250 x 4.6 mm) reverse phase at ambient temperature. The mobile phase consisted of Orthophosphoric acid: Acetonitrile 70:30 % (V/V). The PDA Detector at 330 nm and 20 µL sample was injected.

Results

Taxonomy

The *Agnimantha* comprises of two genera namely *Clerodendrum* L. and *Premna* L. that belong to the family Lamiaceae. Earlier classification systems placed these genera under the family Verbenaceae. However, the APG IV system, the current system of classification in vogue globally, based on morphology and molecular phylogeny has transferred several

genera that were traditionally treated under Verbenaceae such as *Tectona* L. f., *Clerodendrum* L., *Premna* L., *Vitex* L., *Gmelina* L., *Callicarpa* L. and *Citharexylum* L. to Lamiaceae.

Key 1: Taxonomical keys to identify plant sources of *Agnimantha*

The following key is helpful in identifying the different plants used as drug sources collectively called *Agnimantha*.

1a. Flower foetid, under 1 cm long; drupes with 1-pyrene:

1b. Flowers fragrant; over 3 cm long; drupes with 4-pyrenes *Clerodendrum phlomidis*

2a. Leaves entire at margins, pubescent beneath; calyx copular *Premna mollissima*

2b. Leaves crenate-serrate at margins, glabrous or glabrescent; calyx shortly campanulate *Premna serratifolia*

Etymology and botanical correlation

Based on the analysis, *Clerodendrum phlomidis* shows a high degree of correlation than *Premna serratifolia* and *Premna mollissima*. This implies that *Clerodendrum phlomidis* is the most authentic drug source of *Agnimantha*. However, both *Premna mollissima* and *Premna serratifolia* show close correlation with *Clerodendrum phlomidis* as shown by their scores which only differ marginally and thus could be considered as possible substitute species of *Agnimantha*.

Macroscopy and organoleptic characters

The roots of all three *Agnimantha* species are woody in nature. The root bark of *Clerodendrum phlomidis* is pale brown with vertical fissures. Whereas *Premna serratifolia* comprise peeling and reddish brown when young and turns brown at maturity. *Premna mollissima* has a grey coloured smooth bark. Similarly colour of root powder is yellow in *Clerodendrum phlomidis*, dark to light brown in *Premna serratifolia* and grey coloured in *Premna mollissima*. The powder of all three roots does not show any detectable taste however, they emit a mild odour and are distinctively gritty.

Key 2: Based on macroscopic and organoleptic characters to identify crude or powdered roots of *Agnimantha*

1. Root powder mildly pungent: *Clerodendrum phlomidis*

2. Root powder strongly pungent:

2. a. Root bark longitudinally cracked, reddish brown, initially sweet and bitter in taste later *Premna serratifolia*

2. b. Root bark longitudinally wrinkled, greyish brown, astringent in taste *Premna mollissima*

Microscopy

The transverse sections of roots showed distinct differentiating characters among three species. The cork region composed of 10-15 rows of homogenous thin walled cells with absence of oils and fibres arranged in radial lines found unique in *Clerodendrum phlomidis* (Fig. 1a). Whereas, in *Premna serratifolia* (Fig. 1b) and *Premna mollissima* (Fig. 1c) cork is composed of distinct heterogeneous thin and thick walled cells with oils. Likewise the secondary cortex is composed of parenchymatous cells in *Premna serratifolia* and collenchymatous parenchyma cells in *Premna mollissima*. Microscopic characters to identify and differentiate authentic and substitute sources of *Agnimantha* are given in key 3. The comparative root anatomical characters of all three *Agnimantha* sources are summarized in Table 1.

1. Cork composed of 10-15 layers; cells homogeneous, thin walled; parenchymatous cells without oil *Clerodendrum phlomidis*

2. Cork composed of 4-8 layers; cells heterogeneous, thick walled; parenchymatous cells with oil:

2. a. Secondary cortex consists of parenchymatous cells *Premna serratifolia*

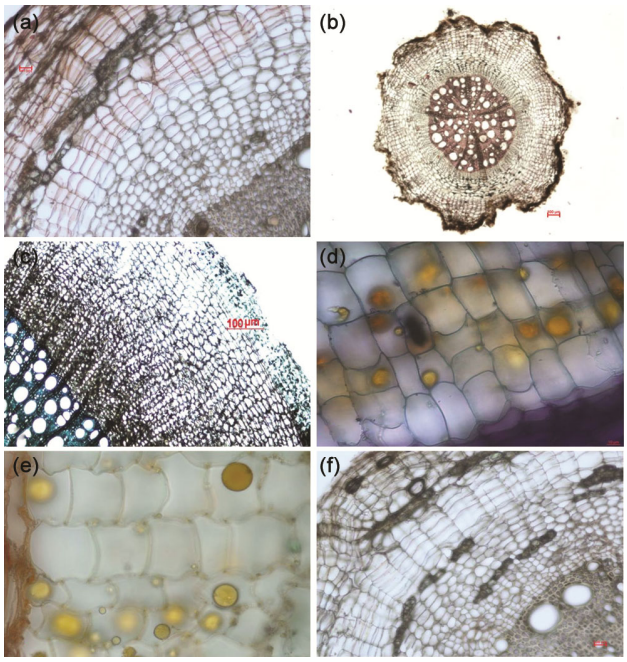

Fig. 1 (a-f) — Comparative root anatomy and histochemistry of *Agnimantha* sources Cross section of roots showing cortex, secondary phloem and oils in a and f *Clerodendrum phlomidis*; b-c. *Premna serratifolia*; d-e. *Premna mollissima*

2. b. Secondary cortex consists of collenchymatous parenchyma *Premna mollissima*

Histochemistry

The histochemical evaluation of *Agnimantha* root samples revealed the presence of starch, lignin and absence of total lipids, tannins and alkaloids in all three species. When compared, all the three profiles of *Agnimantha* sources almost look alike with the exception of *Premna serratifolia* (Fig. 1d) and *Premna mollissima* (Fig. 1e) which showed the presence of oils which is absent in *Clerodendrum phlomidis* (Fig. 1f).

Powder microscopy

The root powders of all three *Agnimantha* sources are characterized with presence of lignified cork cells, pitted vessels, fibres with sharp ends. Apart, stone cells found to be present in *Clerodendrum phlomidis* and *Premna serratifolia* and are absent in *Premna mollissima*. Likewise calcium oxalate crystals are observed in *Clerodendrum phlomidis* and *Premna mollissima* whereas absent in *Premna serratifolia*. The comparative account of powder microscopic characters of *Agnimantha* sources are detailed in Table 2.

Physicochemical analysis

The moisture content of all the powdered roots of *Agnimantha* sources of all accessions collected from different geographic regions found to be in acceptable range. Similarly ash and extractive values are within prescribed Ayurvedic Pharmacopeia of India limits for *Agnimantha*[16]. Interestingly *Clerodendrum phlomidis* showed slight divergence in total ash (6.2-11%) that exemplifies the presence of silica matter. As compared with alcohol, water solubility extractive values are more is found to be more that implies water is the best solvent for formulations. The comparative physicochemical results obtained are summarized in Table 3.

Phytochemical screening

The preliminary phytochemical screening with root extracts showed inconsistency in phytoconstituents. carbohydrates, glycosides, flavonoids and saponins were found to be similar in all three species referred as *Agnimantha*. Phytosterols were found unique in

Table 1 — Comparative microscopic characters of *Agnimantha*

Characters	*Clerodendrum phlomidis*	*Premna serratifolia*	*Premna mollissima*
Cork	Composed of 10-15 rows of thin-walled cells	Composed of 4-8 rows of two distinct types of cells (thick and thin walled) arranged alternatively	Composed of 8-10 layers of thin walled suberised cells.
Cortex	Parenchymatous cells	Parenchymatous cells	Collenchymatous parenchyma cells
Secondary phloem	Includes rectangular sieve elements with distinct companion cells	Includes tubular sieve elements of thick walled	Includes tubular sieve elements of thick walled
Secondary xylem	Vessels solitary	Vessels solitary	Vessels solitary
Wood	Diffuse porous and scattered	Diffuse porous and dense	Diffuse porous and scattered
Axial parenchyma	Paratracheal	Initial paratracheal	Paratracheal
Ray parenchyma	Bi-triseriate; cell walls thick and lignified	Biseriate; cell walls thick and lignified	Bi-triseriate; cell walls thin and non-lignified
Fibers	Occurs in radial lines	Occurs in groups	Occurs in bands
Medullary rays	2-3 seriate; cells pitted	1-seriate; occasionally 2-3 seriate, cells pitted	1-4 seriate, cells pitted
Xylem fibers	Thick walled, lignified and are straight and radiate expanding	Thick walled and lignified with narrow lumen and straight, arranged in compact vertical lines	Thick walled, lignified and arranged in compact vertical lines

Key 3: Based on anatomical characters to differentiate roots of Agnimantha

Table 2 — Comparative powder microscopic characters of *Agnimantha*

Sl. No.	Characters	*Clerodendrum phlomidis*	*Premna serratifolia*	*Premna mollissima*
1	Starch	Abundant, simple, spherical to ovoid in shape	Simple, spherical have concentric hilum	Simple, angular have concentric hilum
2	Stone cells	Present	Present	Absent
3	Vessels	Simple, circular-elliptical and bordered pitted	Simple, circular pitted	Simple, circular-elliptical pitted
4	Fibers	Libriform, tips blunted	Libriform, tips sharp	Libriform, tips sharp
5	Calcium oxalate crystals	3-8 prisms in groups and scattered	Absent	Scattered

Clerodendrum phlomidis that serves to differentiate the authentic and substitutes. Likewise proteins and resins were present only in *Premna mollissima.*

HPTLC fingerprint

The methanol root extracts of *Clerodendrum phlomidis*, *Premna serratifolia* and *Premna mollissima* from different geographic regions showed 18 phytochemicals with disparity in chemical fingerprints that facilitate to distinguish the authentic and substitute species recommended for *Agnimantha*. The compounds with Rf values 0.06, 0.72, 0.32 and 0.63 were found similar in all three species of five accessions. The *Clerodendrum phlomidis* found unique with additional compounds with Rf 0.82, 0.11, 0.42, 0.50 and 0.95 to differentiate among substitute species likewise the bands with Rf 0.44, 0.07 in *Premna mollissima* and 0.24, 0.43 in *Premna serratifolia*.

HPLC analysis

The compound verbascoside is a phenylpropanoid glycoside well-known for its antioxidant, anti-inflammatory and photoprotective activity[18]. Based on the similarity in pharmacological activity, the methanol root extracts of one accession of all *Agnimantha* sources and the selected active principle marker compound verbascoside are subjected to quantitative HPLC analysis to compare the similarity among authentic and recommend substitute species. Interestingly Verbascoside is found to be present in all the recommended species of *Agnimantha* with a sharp peak of verbascoside at retention time of 19.62/min (Fig. 2a). Similarly the amount of verbascoside present in the samples was estimated by using calibration curve. The quantitative studies revealed that, verbascoside content varied from authentic and substitute species. Based on obtained chromatograms, *Premna mollissima* (Fig. 2b) has got more content that is of 2.03% while *Premna serratifolia* (Fig. 2c) 0.23% and *Clerodendrum phlomidis* (Fig. 2d) with 0.086%. The results obtained from HPLC quantitative studies revealed that substitute species are rich

Table 3 — Comparative physicochemical analysis of species recommended as *Agnimantha*

Standards	*Clerodendrum phlomidis*	*Premna serratifolia*	*Premna mollissima*	*API Limits for *Clerodendrum phlomidis*
Moisture content	3.0-6.6	4.2-6.2	3.8-6.0	Not more than8%
Total ash	6.2-11	2.2-4.7	1.8-4.5	Not more than 6%
Acid insoluble ash	0.9-3.1	0.8-1.9	0.1-0.8	Not more than 1%
Alcohol solubility	1.6-7.4	4.8-6.7	9.3-12.6	Not less than 2%
Water solubility	6.9-15.2	6.9-11.4	17.6-22.5	Not less than 5%

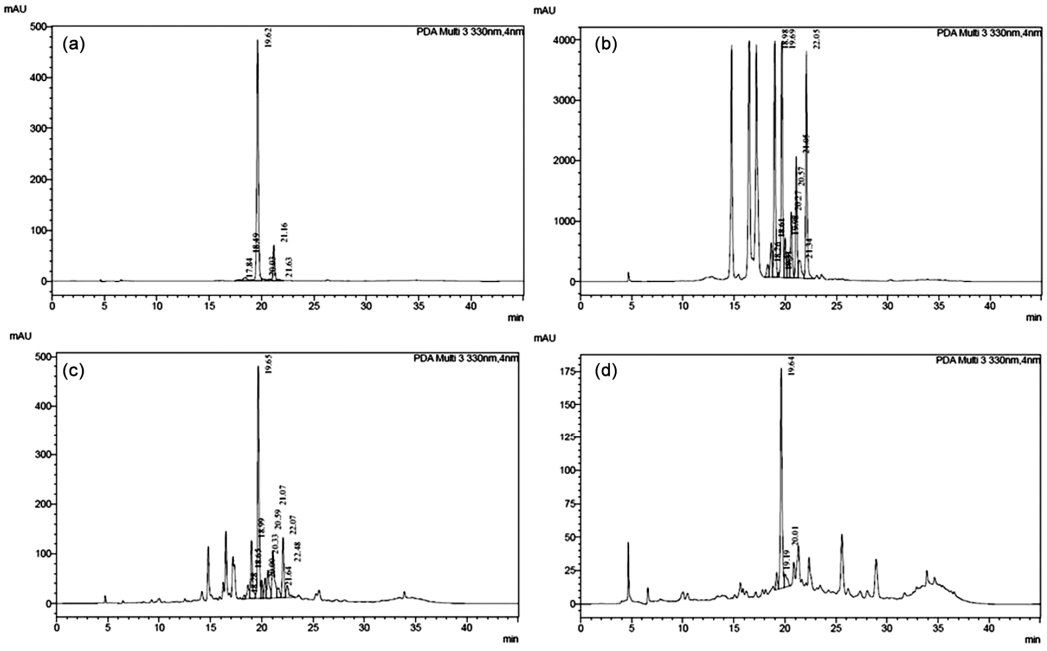

Fig. 2 — (a-d) HPLC Chromatogram of Verbascoside in *Agnimantha* samples (a. Verbascoside; b. *Premna mollissima*; c. *Premna serratifolia*; d. *Clerodendrum phlomidis*)

in active principle compounds as compared to authentic species.

Discussion

In the present scenario of increasing demand for herbal drug sources leads to drug substitution and adulteration with respect to its distribution and availability. On the other hand, plant resources are exploited exponentially to fulfil the needs and demands of herbal industries, which lead to rarity of plant resources. Hence, it is obligatory to document and validate the plant sources in trade to maintain quality of herbal products and to understand market dynamism and for planning conservation strategies for the over exploited botanicals. *Agnimantha* is known to be the plant with controversies in identification and usage of authentic species and plant parts. Moreover, the existing literature reports different species of *Clerodendrum* and *Premna* as *Agnimantha* but there are no concrete conclusions drawn for the correct identification and differentiation of authentic and substitute sources of *Agnimantha*. In the present study, botanical correlation of Sanskrit synonyms and taxonomic characterization revealed that roots of *Clerodendrum phlomidis* is the authentic source of *Agnimantha*, whereas *Premna serratifolia* and *Premna mollissima* are the possible substitute sources that possess equalling similarities with respect to morphology, microscopy and phytochemicals of authentic species. In addition, the quantification studies revealed that all three botanical sources of *Agnimantha* possess the marker compound verbascoside in variable quantities. However, the study finds the other substitute sources *Premna serratifolia* and *Premna mollissima* found to be equivalent to *Clerodendrum phlomidis* in terms of all parameters studied. Interestingly the principle active constituent found to be more in substitute sources such as *Premna mollissima* and *Premna serratifolia* than the authentic source *Clerodendrum phlomidis*, that leads this study to recommend both *Premna serratifolia* and *Premna mollissima* could be the potential sources of *Agnimantha*.

Conclusion

The present pharmacognostic study on *Agnimantha* resolves the existing controversies with respect to species to be used as *Agnimantha* and provides taxonomic and pharmacognostic standards to identify and differentiate the authentic source from substitute and adulterants. The distinct characters like vertically fissured stem; cork with homogenous type of cells; presence of calcium oxalate crystals, sterols and absence of oil found unique in roots of *Clerodendrum phlomidis* to differentiate from other roots. Many of these characters match with the descriptions of Ayurvedic texts. The study concludes that *Clerodendrum phlomidis* is the authentic source and *Premna serratifolia* and *Premna mollissima* forms the equivalent substitute sources for *Agnimantha*.

Acknowledgements

Thanks are due to the National Medicinal Plant Board, Government of India for financial support. Thanks are also due to Mr. Esakkimuthu and Mr. Joshuva David, Department of Plant Science, Manonmaniam Sundaranar University for help in anatomical studies and microphotography.

Funding

This work was supported by the National Medicinal Plant Board, Government of India [Grant No.:Z.18017/187/CSS/R&D/KR-02/2009-10-NMPB].

Conflict of Interest

All authors have none to declare.

Author Contributions

SMV: Collection of raw drugs; conducted experimental part of the research study, writing, editing the manuscript; RK: Collection of raw drugs for study, authentication, editing and review of the manuscript; RC: Evaluated and interpreted the microscopic results, captured microscopic images, editing and review of the manuscript

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