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Development of physico-chemical parameters for indigenous drug, Bharangi: *Clerodendrum serratum* and *Premna herbacea*

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Abstract

Objective: To develop physico-chemical parameters for indigenous drugs *Clerodendrum serratum* and *Premna herbacea* commonly known as Bharangi. Materials and Methods: Roots of *C. serratum* and *P. herbacea* were studied for macro and microscopical characters. HPTLC method was developed to generate fingerprint profiles for the two roots and to quantify β- sitosterol (in *P. herbacea* root) using n-hexane: ethyl formate (7:3) as a mobile phase, precoated TLC plates (silica gel 60 F_{254}) as a stationary phase and H_2SO_4 as derivatizing agent. Further, presence of sugar, D-mannitol in *Clerodendrum serratum* was confirmed with the use of paper chromatography. Results: Roots of *C. serratum* are hard, woody, tortuous, earthy brown and bear rootlets. Stratified cork, cortex, phloem and wood, all filled with starch are characteristic features of transverse section of *C. serratum* root. Roots of *P. herbacea* are brown or earthy brown, woody, shows swollen nodes and exfoliated surface exposing the inner orange colored tissue. T.S. of the root shows cork with yellowish orange content, cortex constituted of collenchymatous parenchyma, phloem and wood portion. Presence of starch grains and stone cells in powdered root of *C. serratum* differentiate it from that of *P. herbacea*. β- sitosterol (0.012% w/w) was found to be present in the root *P. herbacea* only. Sugar D-mannitol was present in *Clerodendrum serratum* only. Conclusion: The present study provides the first report regarding comparative study and identification parameters for *C. serratum* and *P. herbacea*. Further, we report presence of β-sitosterol in *P. herbacea* root and HPTLC method to develop distinct chemo-profile for both the roots.

Keywords: Bharangi, *Clerodendrum serratum*, HPTLC, D-mannitol, *Premna herbacea*, β-sitosterol

1. Introduction

There exists a controversy regarding the use of drugs under the common vernacular name, Bharangi. Roots of two different drugs *Clerodendrum serratum* (Linn.) Moon and *Premna herbacea* Roxb. belonging to family Verbenaceae are used as Bharangi [1, 2]. *C. serratum* (Linn.) Moon (synonym: *Volkameria serrrata* Linn. and *C. macrophyllum* Sims.) is found throughout most of India and Srilanka [2, 3, 4]. Root is reported to contain D-mannitol [5], sapogenin mixture, which contained 3 major triterpenoid constituents, oleanolic acid, queretaroic acid (I) and serratagenic acid [6] and sterols visually campesterol and stigmasterol [7].

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P. herbacea (synonym: *Pygmaeopremna herbacea* Moldenke) is distributed throughout the subtropical Himalayas extending southwards, West Bengal, Bihar and Orissa [2,8]. *Premna herbacea* root is used in treatment of rheumatism, asthma, fevers and cough [8, 9, 10]. Previous phytochemical studies include report of presence of sirrutekon and diterpenoid labdanes, clerodens, pimarens, naturally occurring abietans, kaurenes, byrenes, gibrallins and miscellaneous diterpenoid [11,12]. Roots of both the plants are used as Bharangi and are valued in asthma, bronchitis, and other catarrhal affections of the lungs, fever, diseases of the blood, tumors, and inflammations [2, 3, 4, 13, 14]. Root is also valued in the treatment of skin diseases like leucoderma and leprosy [2, 13, 14].

Bharangi is highly valued and important ingredient of many popular Ayurvedic formulations like, Dahmula-arista, Dashmula-kwatha, Chavanprasha-aveleh, Bharngyadi kasayam, Ayaskrti, Haritakiavleh, Mahamanjisthadikwath, Kankasav, Visgarbha tail, Yograjaguggulu vatika [2, 14, 15].

The present study deals with the development of physico-chemical parameters for *C. serratum* and *P. herbacea* roots.

2. Materials and methods

2.1 Plant materials

Entire plants of *C. serratum* were procured from hilly regions of Manipur- Imphal in the month of August, and roots of *P. herbacea* were procured from Nepal in the month of September. Herbarium specimens (501 and 502) were deposited in the Department of Pharmacognosy, L. M. College of Pharmacy, Ahmedabad. Roots of both plants were sun dried and powdered separately. The powder materials were sieved using 60 mesh.

2.2 Chemicals

All chemicals and reagents used for the present study were of AR grade.

Acetone, ethyl acetate, ethyl formate, ethanol, hexane, methanol, n-propanol, H_2SO_4, $AgNO_3$, β-sitosterol (Sigma), TLC Aluminum sheets pre-coated with silica gel $60F_{254}$, thickness 0.2mm, (20 x 20cm) (E. Merck).

2.3 Microscopy

Microscopical study was carried out using Leica CTR Mic (Automated research microscope DMLA, Germany).

2.4 Physical parameters

Ash values (total ash, acid insoluble ash, water soluble ash) and extractive values (alcohol soluble, water soluble) were carried out according to the specifications of Ayurvedic Pharmacopoeia [16].

2.5 Fluorescence analysis [17]

0.5 g powdered materials were macerated with 5 ml of methanol, ethanol, 1N methanolic NaOH and 1N HCl separately for 1 h and extract were filtered. Extracts were analysed at different wavelength.

2.6 Paper chromatography of D-mannitol [18]

5 g of dried root powder of *C. serratum* and *P. herbacea* were exhaustively extracted using methanol (2 x 25 ml) separately. Upon concentrating, and keeping in a refrigerator (12° C) for a week, a white crystalline solid was found settled, in methanolic extract of *C. serratum*, which was filtered, recrystallized and weighed.

On the basis of earlier reports, the solid when subjected to the chemical test and paper chromatography was found to be D-mannitol.

Paper chromatography was performed using Whatman No. 1 paper as a stationary phase, n-propanol: ethyl acetate: water (7:1:2) as a mobile phase and visualization was obtained with the use of alkaline $AgNO_3$. The $AgNO_3$ was prepared as a saturated solution in water, which was mixed with acetone in proportions (1:200) and used as a dip. White spots appeared on brown back ground, upon dipping the chromatogram in to a solution of 5% ethanolic NaOH after drying.

2.7 Fingerprinting and Co-Chromatography using β- sitosterol

All solvents were of analytical grade. Chromatogram was developed using precoated TLC plates (silica gel $60F_{254}$, Merck) as stationary phase and n-hexane: ethyl formate (7:3) as a mobile phase. The spots were visualized using 5% aqueous H_2SO_4.

2.7.1 Sample preparation

The concentrated methanolic extract (2.6) was made aqueous by adding 10% water. This 90% aqueous methanolic extract was then extracted with n-hexane (3 x 25 ml). Hexane soluble fraction of both was concentrated to 25 ml and used for the present study.

2.7.2 Estimation of β- sitosterol in P. herbacea by HPTLC method

A calibration curve of β- sitosterol was obtained by plotting the peak area of β- sitosterol against the concentration of β- sitosterol.

Accurately weighed 5 mg of β- sitosterol was dissolved in 5 ml of hexane in a volumetric flask (1.0 mg/ml).

A fixed volume of standard solution (2, 4, 6, 8, 10 µl) and sample solution were spotted as sharp bands on the plate. The plate was then developed in a twin trough chamber containing mobile phase, n-hexane: ethyl formate (7:3). After development, the plate was dipped in to dip chamber containing 5% aqueous H_2SO_4 then heated at 110° C, the bands were scanned at 400 nm. After applying suitable dilution factor and comparing peak height and peak area of standard and sample solution, the amount of β- sitosterol in fresh roots was calculated.

2.7.2.1 Validation of HPTLC method

The method was validated in terms of linearity, precision, repeatability specificity and limit of detection.

Precision

Repeatability

Precision under same condition (same analyte, same apparatus, short interval of time and identical reagent) using the same sample.

Interday and intraday Precision

The intraday precision was determined by analyzing β-sitosterol three times on the same day. The interday precision was determined by analyzing the same daily for 5 days.

Linearity

The range of concentration of the standard compound was determined for linearity. The obtained test results must be in direct proportion to the concentration of analyte in the sample calibration curve for the analyte. The results were expressed in terms of correlation coefficient of the linear regression analysis.

Accuracy

The accuracy of an analysis was determined by calculating systemic error involved. Accuracy of the above method was ascertained by adding known concentration of β-sitosterol to the prequantified sample solution and estimating the quantity of β-sitosterol using the proposed method.

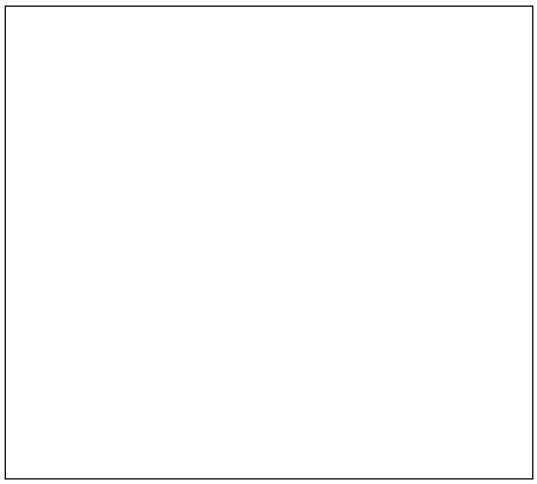

Fig. 1a, Morphology of *C. serratum* root

3. Results and Discussion

Roots of *C. serratum* are hard, woody and tortuous, 8-10 cm in length and 2-3 cm in diameter. Externally roots are earthy brown and show ridges, longitudinal striations, wrinkles, circular warts, rootlets and rootlet scars. Rootlets are thin, wiry, 8-15 cm in length and 0.5 cm in diameter. Fracture is hard and fractured surface is pale yellowish brown. Roots possess characteristic odour and a slight bitter taste.

Transverse section of the root shows stratified cork (Ck) and a narrow cortex (Ct) containing reddish brown content in the initial layers and plenty of simple starch grains (Sg) through out. It also shows groups of lignified stone cells (St) and mesocortical fibres (Fb). Stone cells are isolated or in groups of 3-4, and are 42-112 µ in length and 28-55 µ in width. They are of two types, a) oval and thick walled with striations and, b) somewhat polygonal and comparatively thin walled. Fibers are in a group of 3-5, lignified and striated. Phloem (Ph) is parenchymatous and filled with starch grains. Few stone cells are also observed in this region.

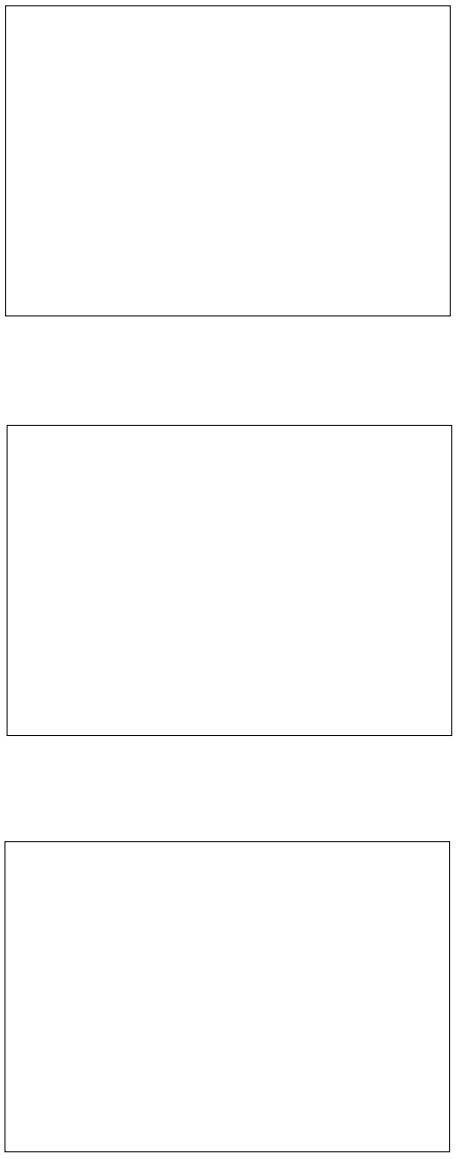

Fig. 1b, Transverse Section of *C. serratum* root

Xylem vessels (Xv) are of varying size (56.2-140 µ), scattered, lignified and pitted in nature. Medullary rays (Mr) are uni to tetra seriate and lignified. Abundant starch grains, (12-36 µ), are contained in xylem parenchyma and medullary ray cells. Calcium oxalate crystals are totally absent.

Fig. 2a, Powder study of *C. serratum* root

Fig. 2b, Powder study of *P. herbacea* root

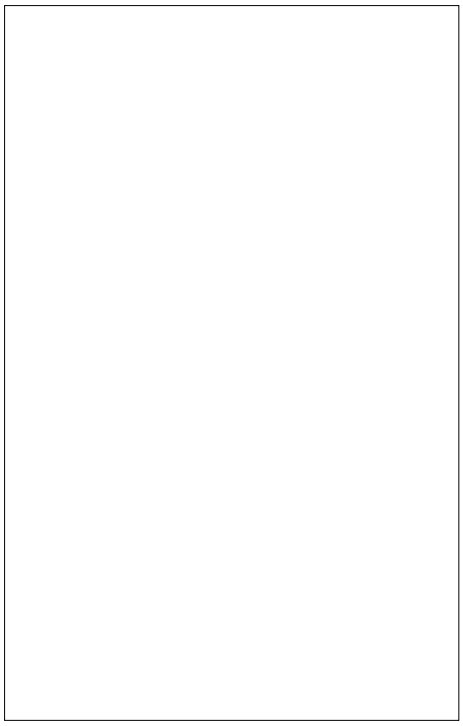

Fig. 3a, Morphology of *P. herbacea* root

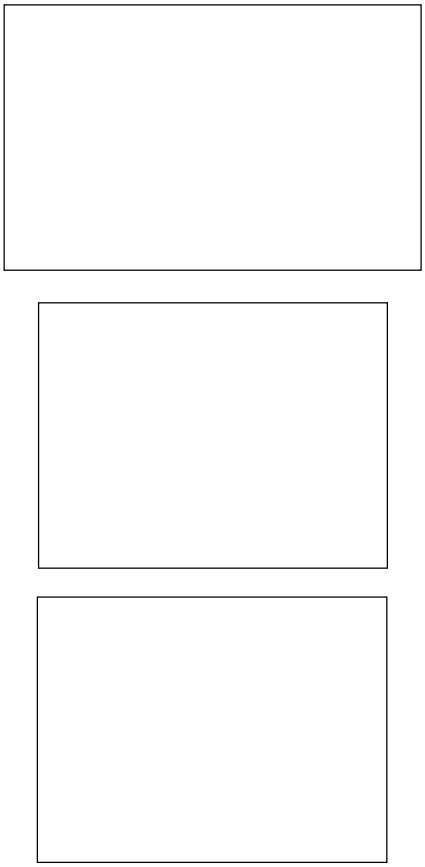

Fig. 3b, Transverse Section of *P. herbacea* root

Fig. 4a, Paper chromatogram of D-mannitol in *C. serratum* root

Fig. 4b, Co-Chromatography of β-sitosterol and n-hexane fraction of *C. serratum* and *P. herbacea* of root

Powder of *C. serratum* is buff in color having characteristic odour and slight bitter taste. The diagnostic characters include plenty of simple starch grains, cork fragments in surface view, composed of multilayered polygonal light brown cells, and lignified, thick walled, pitted stone cells. Xylem vessels are usually isolated, lignified and are bordered pitted.

Roots of *P. herbacea* are woody, somewhat tortuous, 15-18 cm in length and 2-3.5 cm in diameter. Surface shows swollen nodules, corky warts, longitudinal wrinkles, rootlets or rootlet scars and silvery patches of lichen. Externally roots are brown or earthy brown in color. Outer surface gets exfoliated exposing the inner orange colored tissue. Fracture hard and fractured surface is yellowish brown in color. Roots are aromatic and tasteless.

The transverse section shows cork made up of 5-6 layers of tangentially elongated and radially arranged cells with yellowish orange content. Cortex is wide, constituted of collenchymatous parenchyma containing blue coloring matter. Phloem is wide and parenchymatous. Wood portion is big and occupies 2/3rd area of the total section. It is composed of radial strips of pitted vessels, xylem fibers and xylem parenchyma. Medullary rays are uni to biseriate run up to the cortex, and are radially elongated, lignified and pitted.

P. herbacea root powder is grayish green in color with aromatic odour and bland taste. It characteristically shows cork, composed of multilayer, radially elongated, thin walled cells in transverse view. Orange colored pigment is observed in these cells (Pc). Starch grains and stone cells are absent.

Data of total ash values, extractive values and fluorescence analysis are mentioned in Table 1 and 2. Presence of D-mannitol was confirmed by performing co-chromatography with standard D-mannitol on paper.

Fingerprinting and quantification of β-sitosterol in the root samples were done by HPTLC method. Under the conditions provided both roots showed almost similar chemo-profile. In both the roots two major spots resolve at R_f 0.26 and 0.89 along with other minor spots. Of these two major compounds, the one at R_f 0.89 was found to be similar in both roots, showing identical spectral characteristic (275 and 375 nm).

Table 1, Ash and extractive values of *C. serratum* and *P. herbacea* roots

Sample	Ash values % w/w			Extractive values % w/w	
	Total Ash	Acid insoluble	Water soluble	Alcohol soluble	Water soluble
C. serratum	2.6	2.25	0.48	11.35	18.61
P. herbacea	3.85	3.31	1.67	14.41	8.2

Table 2, Fluorescence analysis of root powders of *C. serratum* and *P. herbacea*

Treatment	Wavelength	*C. serratum*	*P. herbacea*
As such	Visible	Pale brown	Grayish green
	Short wave	Greenish yellow	Greenish yellow
	Long wave	White	---
Methanol	Visible	Greenish yellow	Yellowish brown
	Shortwave	Yellowish green	Yellowish green
	Long wave	Light bluish	Greenish blue
1N NaOH	Visible	Light brown	Light yellow
Methanolic	Short wave	Dark green	Yellowish green
	Long wave	Absent	Bluish green
Ethanol	Visible	Light yellow	Brownish yellow
	Short wave	Yellowish green	Greenish yellow
	Long wave	Pale blue	Pale blue
1N HCl	Visible	Light yellow	---
	Short wave	Yellowish green	Yellowish green
	Long wave	Greenish brown	Dark green

Table 3, Summary of validation parameters

Parameters	Results
Precision (% C.V.)	
• Repeatability of Measurement	0.063
• Repeatability of Application	0.053
• Interday	0.0173-0.0321
• Intraday	0.0141-0.0353
Linearity	0.9964
Range	2-10 µg/spot
Limit of Detection	0.5 µg/spot
Limit of Quantification	1 µg/spot
Accuracy	98.94-99.75 %
Specificity	Specific

Further, compounds resolving at R_f 0.26 in both the roots were found to be of different chemical nature, exhibiting pinkish purple color in *P. herbacea*, whilst in *C. serratum* it showed light brown color. In case of *P. herbacea* compound at R_f 0.26 was found to be β-sitosterol upon comparing it with the standard. The chromatogram patterns of the test sample *P. herbacea* and standard revealed that the peak corresponding to R_f 0.26 were super imposable. The spectrum characteristics corresponding to this peak were also found exactly matching (correlation co-efficient, 0.9964), indicating the standard and test are identical. Linearity range of standard β-sitosterol was observed between 2-10 µg/spot concentration. The method of chromatography using n-hexane: ethylformate (7:3) as a mobile phase and 5% aqueous H_2SO_4 as detecting agent gave good resolution of β-sitosterol without any interference of the other compounds present in root sample, *P. herbacea*. The amount of β-sitosterol in *P. herbacea* was found to be 0.012 % w/w. The method was validated in terms of linearity, precision accuracy, specificity, and limit of detection (Table 1, 2, 3). This is the first report of presence of β-sitosterol in roots of *P. herbacea.*

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