Development of Pharmacognostical Parameters and Estimation of β-sitosterol using HPTLC in Roots of *Gmelina arborea* Roxb

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ABSTRACT

This paper deals with the detailed pharmacognostical evaluation of roots of *Gmelina arborea* Roxb. (Verbenaceae), a highly valued plant in Ayurveda as a bitter tonic, stomachic, laxative and useful in fever, indigestion, anasarca and skin problems. It has been used as an ingredient in many preparations like Dashmoola, Panchmoola di kwatha and Chyawanprasha. Chemical constituents of *G. arborea* roots have been studied extensively which include lignans, flavonoids, alkaloids and various phenyl propanoid glycosides but pharmacognosy of roots has not been reported. The main objective of the present work is to develop detailed pharmacognostical and physicochemical parameters recommended by World Health Organization (WHO) and various pharmacopoeias for the roots, as this has not been reported so far for the roots of the plant. Preliminary analysis showed the presence of phenolics and flavonoids, which led us to estimate total phenolics in roots. The present study also focuses on development of high-performance thin-layer chromatographic (HPTLC) method for the quantitative analysis of roots using β-sitosterol as a chemical marker. Major microscopic features of roots are lignified cork, presence of oil globules, scattered stone cell islets in secondary cortex and pitted wood elements. Phytochemical screening reveals the presence of flavonoids, lignans, sterols, tannins, carbohydrate, coumarins and alkaloids. Total phenolics were found 1.89% w/w in roots and β-sitosterol was found 0.120 ± 0.018% in methanolic extract of the roots by the developed HPTLC method. These findings will aid in the standardization of one of the major roots used in a well known Ayurvedic formulation, Dashmoola. Validated HPTLC method developed can be used as a tool for standardization of roots in different formulations using β-sitosterol as a marker.

Key words: β-sitosterol, Dashmoola, *G. arborea*, HPTLC, physicochemical parameters

INTRODUCTION

Many medicinal plants, traditionally used for thousands of years, are present in a group of herbal preparations of the Indian traditional health care system (Ayurveda) and proposed for their interesting multilevel activities. Amongst the medicinal plants used in Ayurvedic preparations for their therapeutic action, some have been thoroughly investigated and some of are still to be explored. One such plant, *Gmelina arborea* Roxb. (Verbenaceae) is well reputed for its multilevel activities in Ayurveda. It is also known as Gambhari, Krishna Vrinlaka, and Shriparni in Sanskrit and other biological names are *Gmelina indica* Burm. F., *Gmelina rheedii* and *Premna arborea*. It is distributed throughout India, Ceylon, Malayan and Philippine Islands. In India, *G. arborea* occurs extensively in the sub-Himalayan tracts, Assam, Northern West Bengal, South Bihar and Orissa and can be planted elsewhere on a large scale.[1,2,3] The roots are used in many well-known Ayurvedic preparations like Dashmoola and Chyawanprasha. It belongs to group of five major roots (Mahat panchmula) of Dashmula di kvatha which is reported to be used in chronic fever, rheumatic affection, haemorrhages, urinary tract infection, anuria and dysuria. The main preparations are Shriparnaadi kwatha and Panchmulaadi kwatha.[4] Further, *Gmelina arborea* and Dashmoola found widely are used in many herbal products like abana, Geriforte, Chyawanprasha, arvindasav, etc.[5] Roots are useful in hallucinations, and for the treatment of piles, abdominal pains, burning sensations and urinary discharges.[6-8] It has been found useful in indigestions, fever,

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and anasarca in the form of infusion or decoction. It is also given as an appetite stimulant, anthelmintic and as galactogogue with liquorice, sugar and honey.[6,8-11] Roots have been reported to possess anti malarial[12] and cardiovascular activity.[13] Chemical constituents of *G. arborea* include lignans[14] flavonoids,[15] alkaloids[16] and various phenyl propanoid glycosides[17] mainly found in the heartwood and bark of the plant. Roots of this plant are highly valued in the traditional system of medicine and the pharmacological and chemical composition of roots has been well explored. However, to our knowledge pharmacognostical and physicochemical parameters have not been developed so far for the roots. Further, heartwood, stem bark and roots have been reported to contain many terpenoids, flavonoids and lignans but a validated analytical method for the estimation of marker compound has not been developed for the roots of this plant. Hence, the present study focuses on the evaluation of physicochemical parameters to develop the monograph detail along with the validated HPTLC method for quantification of β-sitosterol in the roots. This method forms the basis of standardization of the formulation containing roots of *G. arborea* using β-sitosterol as a marker compound.

MATERIALS AND METHODS

Plant Material

Fresh mature roots were collected from fully-grown trees from fields near the outskirts of Mahesana, Gujarat in September, 2006. The authenticity was established by comparing its morphological and microscopical characters with the available literature[3] and by a taxonomist of Gujarat Ayurveda University, Jamnagar. Roots were dried under shade, powdered and then stored in air tight containers for further use. Voucher specimen (LM 16) was deposited at Dept. of Pharmacognosy, L. M. College of Pharmacy, Ahmedabad.

Chemicals and instruments

Charged Coupled Device (CCD, Lawrence and Mayo) camera attached with compound microscope, solvents viz. petroleum ether, benzene, chloroform, acetone, ethanol (95%), n-butanol, ethyl acetate and various reagents like phloroglucinol, glycerine, hydrochloric acid, chloral hydrate were procured from India Scientific, India. Folin Ciocaluteu reagent was procured from Sigma Chemical Co. (St. Louis, MO, USA).

Macroscopic and microscopic observations

Roots were studied for their detailed morphological characters and freehand sections of root were taken and stained with a number of reagents for histochemical examination. All the observations of the microscopical study of roots and powder were made and recorded with the help of a special CCD camera attached with the microscope.

Physicochemical analysis

Physicochemical analysis was performed by the determination of different ash values, extractive values, loss on drying according to the official methods prescribed[18] and the WHO guidelines on quality control methods for medicinal plant materials.[19] The fluorescence properties were studied under ultra violet (UV) light adopting the method described by Kokoshi[20] and Chase & Pratt.[21] A small quantity of root powder was placed on a clean microscopic slide and one to two drops of freshly prepared reagent solution was added, followed by gentle mixing. The behaviour of the sample with different chemical reagents was studied and fluorescence characters were observed on short and long UV light after 1-2 minutes.

Preliminary phytochemical screening

A known quantity of powdered roots was extracted sequentially with petroleum ether, chloroform, ethyl acetate and methanol and then left for the maceration in water for 12 hours. Preliminary phytochemical screening was carried out using standard procedures described by Harborne[22] by subjecting different fractions to tests separately for the presence of various phytoconstituents like alkaloids, flavonoids, sterols, saponins, lignans, coumarins, carbohydrates and tannins.

Estimation of total phenolics[23]

One g of air-dried root powder was extracted with 100 ml methanol by maceration for 24 hours and filtered. The final volume of the filtrate was adjusted to 100 ml using methanol. Five ml of this extract was diluted with an equal volume of methanol and was used for the estimation of phenols. To 10 ml of the methanolic extract, 10 ml of distilled water and 1.5 ml of diluted (1:2) Folin Ciocaulteu reagent were added and the mixture was kept for 5 minutes. After adding 4 ml of 20% Na_2CO_3 solution, the final volume was adjusted to 25 ml using distilled water. The absorbance was measured at 765 nm at an interval of 30 minutes up to 2 hours using distilled water as a blank. The data was compared with similarly prepared set of standard substance gallic acid in a concentration range of 50 µg to 300 µg per 25 ml. The total phenol content C was measured using the following formula:

$$C = A \times 282.6 - 8.451 \quad (A = \text{absorbance})$$

Quantification of β-sitosterol in roots of *G. arborea* by HPTLC

Instruments

Camag Linomat V (Semi automatic spotting device), Hamilton 100µl HPTLC syringe, Camag twin trough

chambers (20 × 10 cm), Camag TLC scanner 3, 120311, Camag CATS 4 integration software, Camag Reprostar – 3

Materials and chemicals

Precoated silica gel 60 F_{254} aluminium plates (Merck, Darmstadt, Germany) with a thickness of 0.1 mm, 10 × 10 cm, ethyl acetate, methanol, toluene, solvent ether, chloroform, distilled water, anisaldehyde sulphuric acid (0.5% anisaldehyde in sulphuric acid in methanol). All the reagents used were of analytical grade.

Preparation of test samples and standard solutions for HPTLC

HPTLC analysis was performed to confirm the presence of β-sitosterol by co chromatography with available reference standard of β-sitosterol procured from Sigma, USA. Five grams of accurately-weighed root powder was extracted with methanol (2 × 50 ml) on a boiling water bath by refluxing for 1 hour. Combined filtrates were evaporated to dryness and used for preparation of stock solution of extract for spotting on plate.

Calibration curve of ß-sitosterol and development of chromatogram

Accurately weighed 10 mg of standard β-sitosterol was dissolved in methanol to prepare a solution of 1 mg/ml strength. Graded concentration of standard solution (1 mg/ml) in 0.5, 1, 2, 5 and 7.5 µl volume was applied on a precoated TLC silica gel 60 F_{254} plate so that the concentration of β-sitosterol was in the range of 500, 1000, 2000, 5000 and 7500 ng/spot. Two spots of methanolic extract (2 µl) were spotted and the plate was developed in a mobile phase, toluene: methanol (9.4:0.6) and dried using a hair dryer on hot mode for 5 minutes. After complete removal of the solvent from the plate, it was derivatized by 0.5% anisaldehyde sulphuric acid reagent followed by heating at 110 °C for 10 minutes and scanned at 523 nm. The calibration curve was obtained by plotting the area versus concentration of each peak corresponding to the respective spot.

Validation of developed HPTLC method

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

RESULTS

Macroscopic studies of roots of *G. arborea*

Roots occur as segments 5-15 cm in length and 3-20 mm in diameter, cylindrical to tapering, with secondary and tertiary branches and bearing slender and tapering rootlets. External surface is uneven, dull, rough and longitudinally wrinkled. It occasionally shows rootlet scars on the larger pieces, with some exfoliation of the bark in small areas revealing the paler wood beneath. The freshly fractured surface shows a thin layer of greyish yellow bark, and the pale wood constituted about 80% of the radius (Figure 1). Dried pieces of mature root bark, curved and channelled, thinner ones forming single quills, external surface was rugged due to presence of vertical cracks, ridges, fissures and numerous lenticels. Wood was light to moderately heavy, hard, strong, elastic and lustrous with a smooth feel. Fracture was somewhat tough in bark, brittle and predominant in woody portion.

Organoleptic properties

Externally light brown to greyish yellow and yellowish white wood. Mature root bark when fresh was yellowish in colour. Root odour was indistinct and taste was mucilaginous and sweetish with slight bitterness.

Microscopic studies of roots of *G. arborea*

Microscopic studies were carried out by studying different histological characters of the transverse as well as longitudinal sections of roots and powdered drug. Observations from

Figure 1: Plant photograph and morphology of roots of *G. arborea*

the unstained and stained slides were studied using various reagents viz. phloroglucinol and concentrated hydrochloric acid for visualizing elements with lignin thickening, dilute Iodine solution for starch and ruthenium red for mucilage.

Transverse section of Root

A portion of a transverse section from the middle of a soaked root pieces was found to show the following characteristics (Figure 2).

Tangentially elongated rectangular and lignified cork cells were sometimes broken towards upper layers; there were 15-20 layers in young roots and 30-35 layers in mature roots. Cork was followed by indistinct phellogen. The secondary cortex was composed of several rows of tangentially elongated thin wall parenchymatous cells, densely filled with starch grains and oil globules. Scattered resin ducts and stone cells were either solitary or in groups of two to four cells, occasionally pitted and highly thickened (60-100 μ). Occasional presence of prisms of calcium oxalate crystals in cortical parenchyma was seen. Phloem was relatively narrow, made up of phloem parenchyma (occasionally, with some yellowish brown resin masses in inner cells) interlaid with scattered sieve tubes with companion cells and transverse by uni to biseriate ray cells. The secondary xylem region was separated from phloem by indistinct and narrow cambium represented almost two thirds the bulk of the root. Wood consisted of simple pitted wood parenchyma, numerous xylem vessels and fibres, tracheids and medullary rays. Vessels were radially arranged and found scattered in groups or singly nearer to central region with wide lumen (130-250 μ by 50-100 μ), tracheids 175-300 μ by 30-50 μ. Wood fibres were abundant and with simple pits. Medullary rays were uni to biseriate, oblong rectangular and 60-90 cells deep filled with abundant starch grains and occasionally found pitted.

Powder microscopy

Powdered roots were light yellowish brown in colour with indistinct odour and mucilaginous test. Powder study showed the characteristic features listed below.

Fragments of isolated lignified cork cells were elongated, up to 90 μ in length. Xylem vessels were broadly cylindrical with bordered pitted and rarely with reticulated thickening, 130-250 μ in length and 50-100 μ in diameter. Tracheids pitted, with moderately thick, tapering, beaded walls, with relatively broad lumen and 175-300 μ in length and 30-60 μ in diameter. Xylem fibres were found to have thick heavily lignified walls showing small transverse pits on surface. Xylem parenchymatous cells had moderately thick walls and were found with frequently simple circular pits. Stone cells were scattered or in groups of six to ten cells, irregular in shape and highly thickened with narrow lumen (60-100 μ). Fragments of cortical parenchyma were filled abundantly with starch grains and oil globules. Medullary rays showed presence of starch grains. Starch grains were numerous, simple (4-6 μ) as well as 3 to 5 of them were compound (up to 20 μ), spheroid, ovate or irregular. Occasionally fragments containing mucilage were seen, which turned red when treated with ruthenium. Fragments of cortical and phloem parenchyma were filled with calcium oxalate crystals and resinous mass (Figure 3).

Physicochemical parameters of roots of *G. arborea*

Powdered roots were evaluated for various physicochemical parameters and percentage of total ash, acid insoluble ash, water soluble ash were calculated (Table 1). As the drug is reported to contain various flavolignans, ethyl acetate soluble

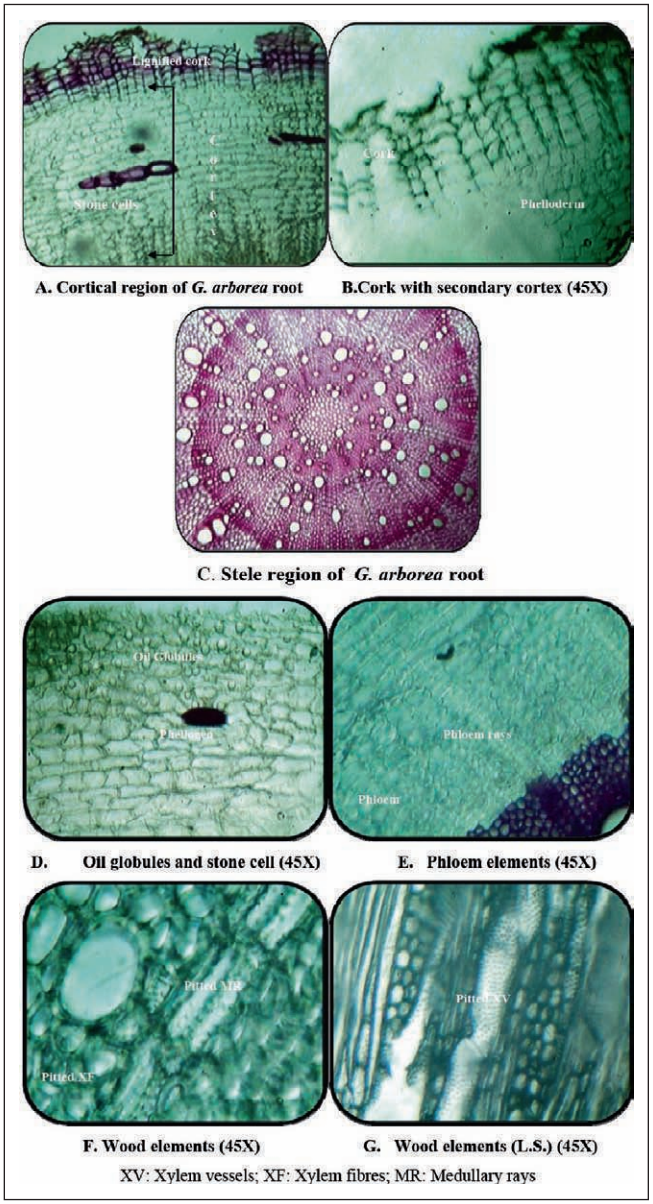

Figure 2: Microscopical characters of roots of *G. arborea*

extractive value was also determined for the roots along with the water soluble, alcohol soluble and ether soluble extractive values. The results of fluorescence analysis of the drug powder are presented in (Table 2).

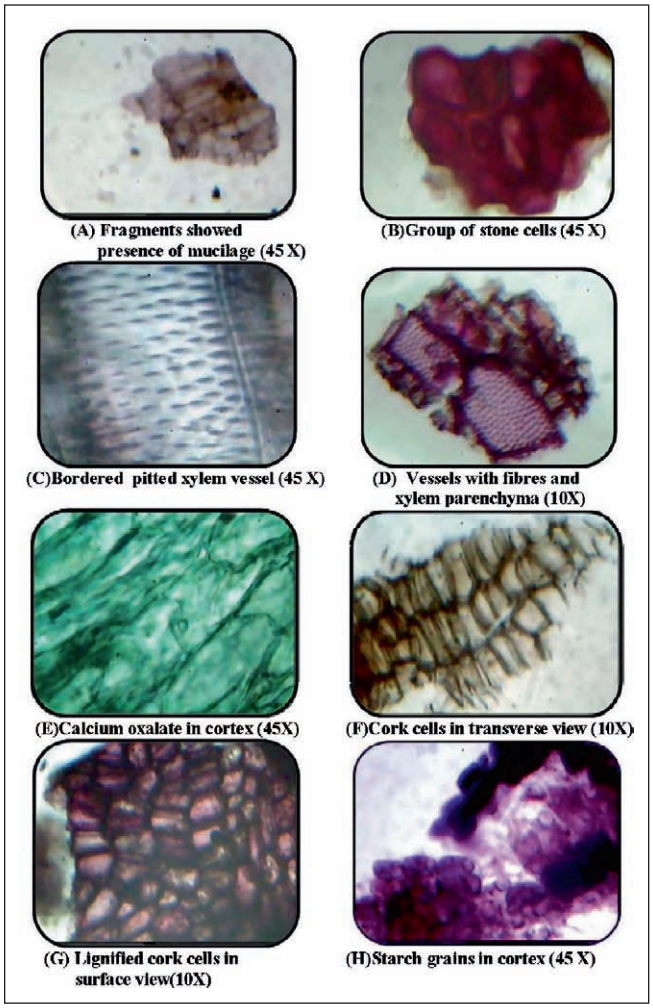

Figure 3: Powder study of roots of *G. arborea*

Phytochemical screening

Preliminary phytochemical analysis showed the presence of various phytoconstituents through positive tests for flavonoids, lignans, sterols, tannins, carbohydrate, coumarins and alkaloids. It showed negative tests for saponins. Preliminary phytochemical screening revealed the presence of phenolics and tannins in methanolic and aqueous fraction and flavonoids and lignans in ethyl acetate fraction (Table 3). Presence of abundant phenolics and tannins in the preliminary screening led us to estimate the total phenolics content in the sample. The root powder was estimated for and found to contain 1.89% w/w phenolics.

Estimation of β-sitosterol in roots of *G. arborea*

β-sitosterol along with other phytosterol have been reported in the roots and heartwood of this plant which was further confirmed in the present study by the phytochemical screening of the roots. HPTLC method was developed to quantify β-sitosterol in methanolic extract of the roots of *G. arborea*. β-sitosterol was estimated with very good resolution and the minimum interference with other constituents in mobile system toluene: methanol (9.4:0.6) and detected as a violet colored band with 0.5% anisaldehyde sulphuric acid reagent followed by heating at 110°C for

Table 1: Physicochemical parameters of the roots of *G. arborea*

Sr. No.	Parameters	% w/w
1.	Total ash value	1.712
2.	Acid insoluble ash	0.29
3.	Water soluble ash	0.03
4.	Moisture content	67.8
5.	Water soluble extractive	20.76
6.	Alcohol soluble extractive	10.89
7.	Ethyl acetate soluble extractive	1.86
8.	Petrol ether soluble extractive	0.15

Table 2: Fluorescence characteristics of the powdered roots of *G. arborea* with different reagents

Treatment	Ordinary light	UV light	
		254 nm	365 nm
Powder as such	Yellowish brown	No color	No color
In 1N NaOH	Yellowish	Dark brown	Light brown
In 1N HCl	Yellow brown	Dark brown	Brown black
In 1N HNO_3	Slightly red	Brown	Brown
In Conc. H_2SO_4	Dark reddish brown	Black	Black
In 5% $FeCl_3$	Brown yellow	Dark brown	Brown
In Iodine	Brown black	Black	Black
In Ammonia	Creamiest yellow	Greenish brown	Brown
In Acetic acid	Pale brown	Black	Black
In Picric acid	Yellow	Black	Black
In Conc. HCl	Dark brown	Black	Black
In Ammonia + HNO_3	Dark yellow	Dark brown	Pale brown
In Methanol	Cream yellow	Dark green	Green black
In Ethanol	Yellow brown	Green	Green
In Distilled water	Yellow	No color	Yellow

10 minutes at R_f 0.31 ± 0.03 (Figures 4 and 5). It was then scanned at its absorption maxima 523 nm after derivatization. Four other bands resolved at different R_f 0.12, 0.22, 0.43 and 0.7 were found to be resolved in methanolic extract in the developed mobile system. The identity was confirmed by co-chromatography and overlain absorption spectra with reference standard, when scanned at 523 nm (Figure 6). The method was precise and found to be linear in the range of 500-7500 ng/spot with the correlation coefficient of 0.992 (Figure 7). Limit of detection and limit of quantification

Table 3: Preliminary phytochemical screening of the roots of *G. arborea*

Sr. No.	Tests for phytoconstituents	Petroleum ether	Chloroform	Ethyl acetate	Methanol	Water
1.	Alkaloids	--	+	--	+	--
2.	Flavonoids	--	--	++	++	+
3.	Lignans	--	--	++	++	--
4.	Saponins	--	--	--	--	--
5.	Sterols	++	++	+	--	--
6.	Carbohydrates	--	--	+	++	++
7.	Phenolics and tannins	--	--	++	+++	+++
8.	Coumarins	+	+	--	--	--
9.	Fats and fixed oils	+	+	+	--	--

+ Less, ++ Moderate, +++ High, -- Negative

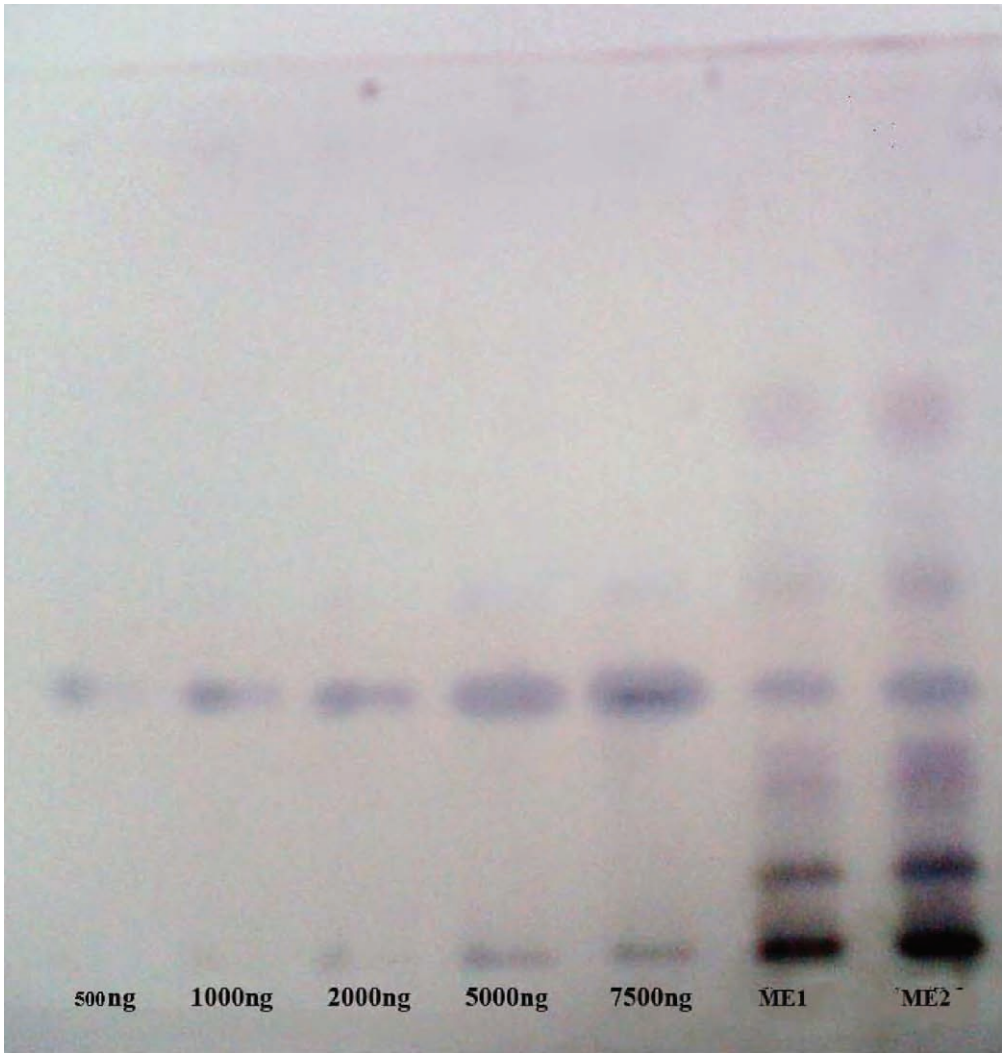

Figure 4: Densitometric chromatogram of β-sitosterol with methanolic extract of roots of *G. arborea* after derivatization with 0.5% anisaldehyde sulphuric acid reagent followed by heating at 110 °C for 10 minutes

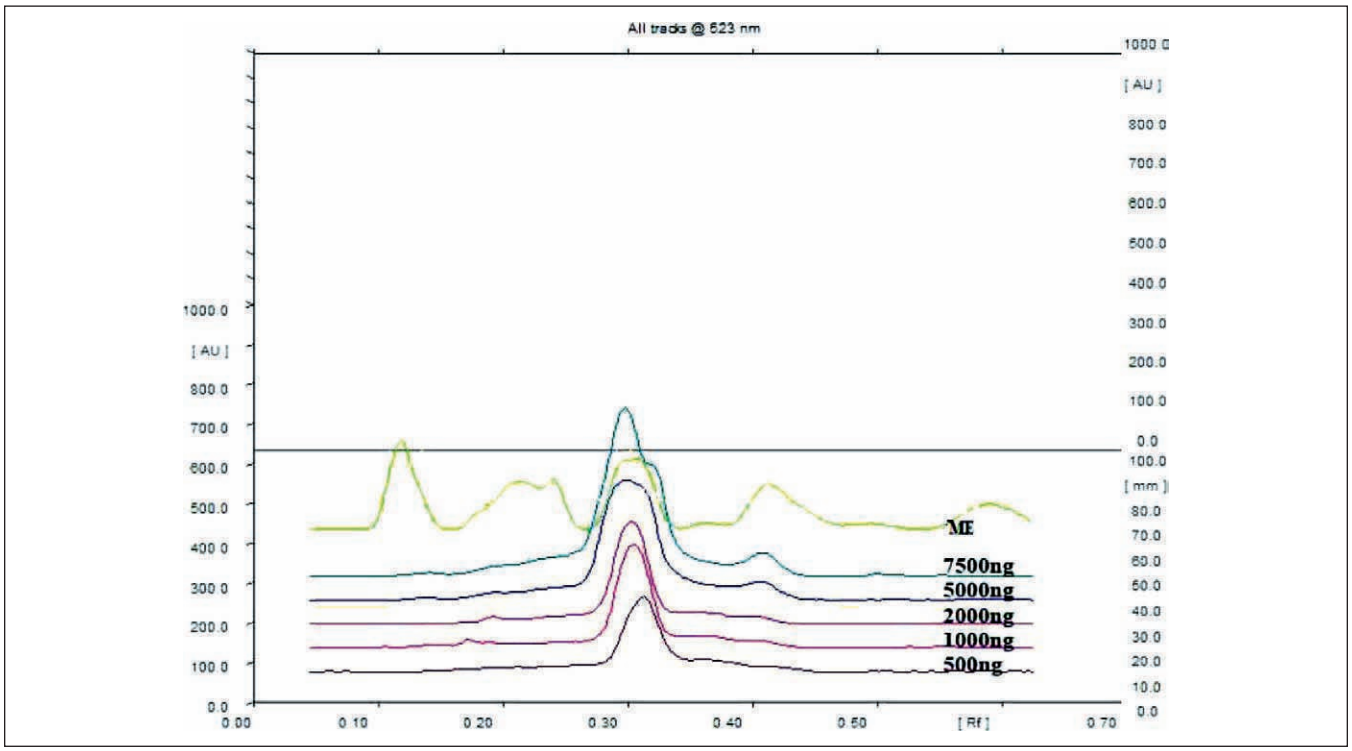

Figure 5: Densitometric chromatogram of β-sitosterol with methanolic extract of roots of *G. arborea* scanned at 523 nm

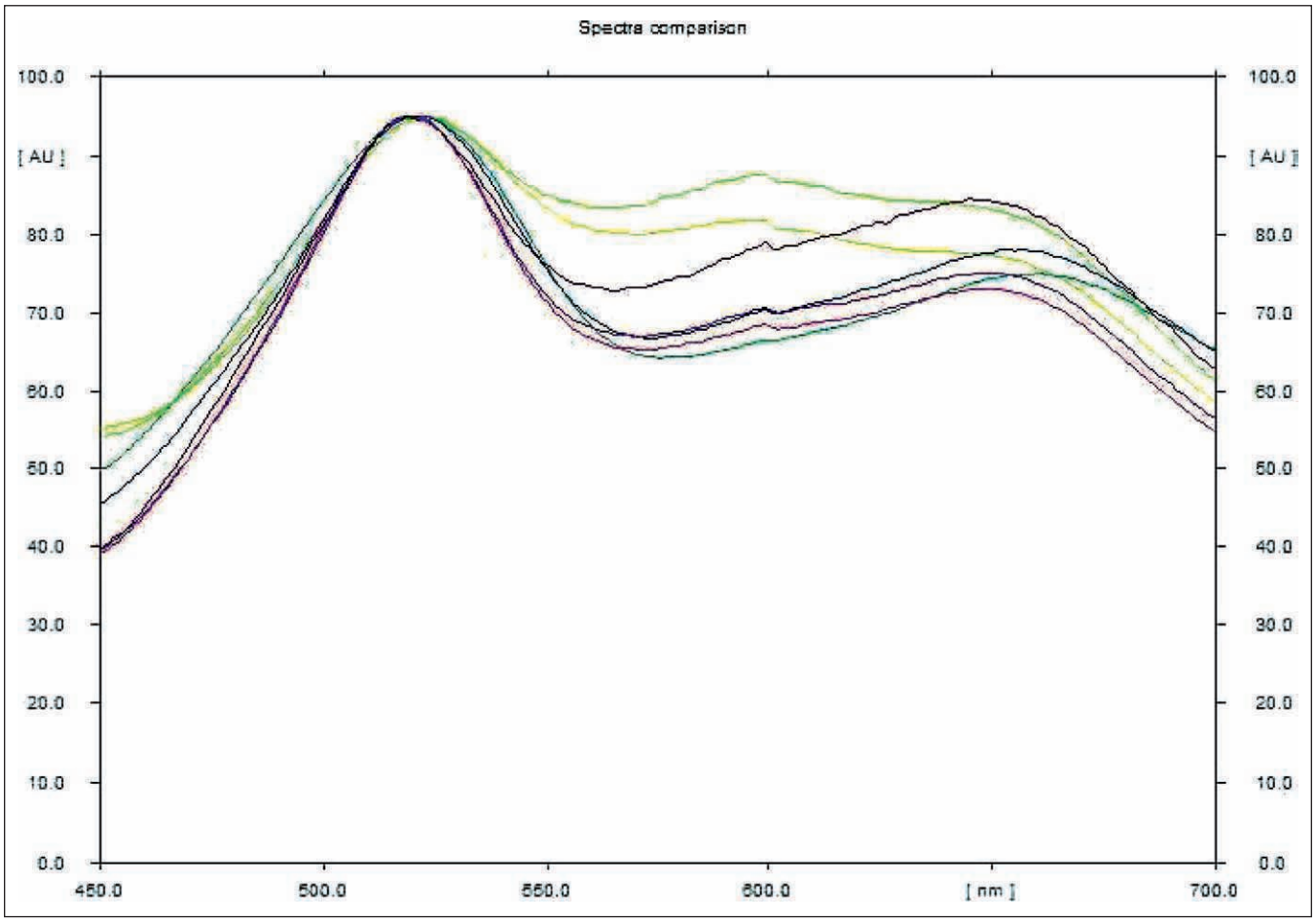

Figure 6: Overlain UV Spectrum of β-sitosterol with methanolic extract of roots of *G. arborea*

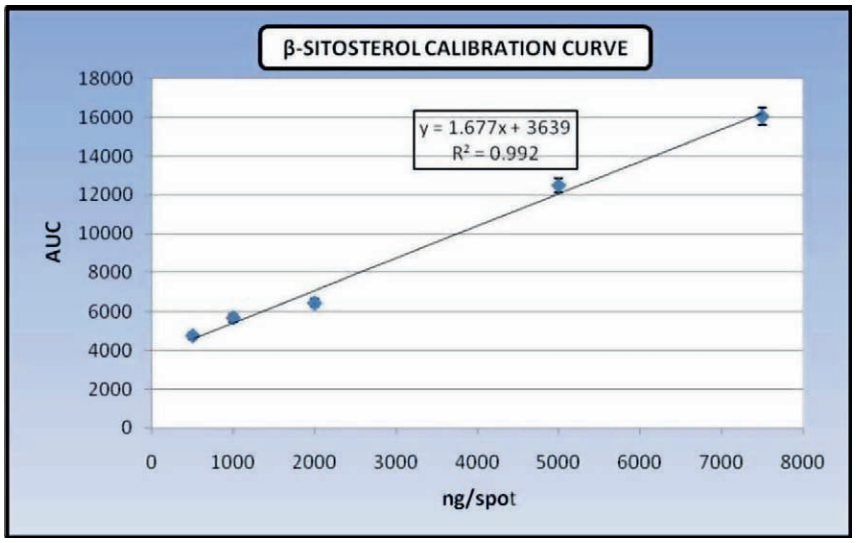

Figure 7: Calibration curve of standard β-sitosterol

Table 4: Summary of validation parameters of β-sitosterol for the developed HPTLC method

Sr. No.	Parameter	Results
1.	Linearity	0.992
2.	Precision	
	• Repeatability of measurement	3.76%
	• Repeatability of application	3.65%
	• Intraday	2.47-4.21%
	• Interday	2.83-3.91%
3.	Range of linearity	500-7500 ng/spot
4.	Limit of quantification	500 ng
5.	Limit of detection	100 ng
6.	Accuracy	94.85-98.66%
7.	Specificity	Specific

was found to be 100 ng/spot and 500 ng/spot, respectively. The percentage content of β-sitosterol was found to be 0.120 ± 0.018 in the methanolic extract of the roots of this plant.

Validation of HPTLC method for estimation of β-sitosterol in roots of G. arborea

The correlation coefficient was found to be 0.992 with RSD, 2.83-3.91% in the developed method. The intraday and interday coefficient of variation for β-sitosterol varied from 2.83-3.91%, and 2.47-4.21%, respectively. RSD for repeatability of measurement of peak area based on 7 times measurement of 250 ng/spot of standard β-sitosterol was found to be 3.76%. RSD for repeatability of measurement of peak area based on a 7 time measurement of 1 µl/spot of sample extract was found to be 3.65%.

Percentage recovery of β-sitosterol was found to be in the range of 94.85-98.66, which indicated that the developed method was accurate and satisfactory, even though it was a derivatization method. The minimum detectable limit was found to be 100 ng/spot. Summary of all validation parameters for the developed HPTLC method for β-sitosterol are listed in Table 4.

DISCUSSION

The improvement in the quality control and standardization of herbal drugs has led to the development of effective quality medicines from plants. In the present study, roots were evaluated qualitatively as well as quantitatively by studying various physicochemical parameters, phytochemical screening and by estimating for the presence of secondary metabolites. Detailed pharmacognostical studies for bark and heartwood has been reported for this drug but roots have not been studied for major microscopic and physicochemical properties. Further, root forms the major ingredient in Dashmoola and other preparations, so the present study is useful in establishing a monograph detail for the roots of *G. arborea*. Root powder was evaluated for its ash values, extractive values and loss on drying. In the present study, detailed physicochemical parameters and method for estimation of phenolics were developed and determined for the first time for the roots of *G. arborea* and to our knowledge this has not been reported earlier. Hence this analysis aids to set up certain standards and contribute towards the development of quality parameters for this drug.

The new HPTLC method developed for β-sitosterol was validated for specificity, linearity, accuracy, and precision. Accuracy was validated by analysis of spiked blank and standard addition samples and precision by performing

replicate analyses on a single day and on different days. It is apparent from the results that validation data for a developed quantitative HPTLC method for analysis of β-sitosterol meet the acceptance criteria for accuracy, precision, linearity and for detection and quantification limits. Thus, the developed HPTLC method can be used for routine analysis of β-sitosterol as a chemical marker compound in crude drugs as well as in herbal formulations containing *G. arborea*.

CONCLUSION

Standardization of crude drugs has become very important for identification and authentication of a drug of natural origin. The present study was developed to establish the pharmacognosy, preliminary phytochemical analysis with other various physicochemical standards for the development of a monograph detail of highly valuable drug ingredient of Dashmoola preparation of Ayurveda. Development of detailed pharmacognostical parameters will be helpful in the correct identification of the plant specimen and authentification of the roots. Further, this is the first report for the estimation of total phenolics in roots and development of a validated HPTLC method for the characterization of β-sitosterol in the roots of *G. arborea*. Roots are widely used in many herbal and Ayurvedic formulations where the developed method can be used for the standardization of a chemo marker to ascertain the quality of the formulation.

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