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Development of Standardisation parameters and Isolation of Phytomarker Myricetin from stem bark of *Myrica esculenta* Buch. Ham. Ex d. Don

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Abstract
The present study was undertaken to develop standardization parameters and to develop method for isolation of phyto marker myricetin from the stem bark of *Myrica esculenta* Buch. Ham. Ex D. Don, Myricaceae. Different pharmacognostical and phytochemical parameters for quantitative estimation of phytoconstituents and separation of marker myricetin from the methanolic extract by column chromatography were studied. The stem bark of *M. esculenta* revealed 2.47% of total ash, 0.02% of acid insoluble ash, 24% of water soluble extractive, 13% of alcohol soluble extractive, 9.9% of moisture content and foaming index of 125. Preliminary phytochemical screening revealed that the stem bark was credited with phytoconstituents like tannins, phenols, flavonoids and saponins in methanolic and aqueous extract. Stem bark contains 0.53%w/w of total flavonoid while the total phenolic content was found to be 9.18%w/w.43 fractions were isolated by column chromatography. Thin layer chromatographic studies of isolated fractions resulted in separation of yellow colored spot at the R_f value 0.65. The U.V. spectroscopic studies of isolated spots produced peaks at 254 and 375 nm which were similar to the peaks of the reference compound myricetin.

Keywords: Pharmacognosy, phytochemical screening, myricetin, column chromatography, *Myrica esculenta*

1. Introduction
Medicinal values of plants and herbs are immense and they are used for treating various ailments. In recent years, the use of medicinal plants in both crude and prepared form has increased substantially [1]. Herbs are considered to be safe but many unsafe and fatal side effects like toxic effects, allergic reaction, effects of contaminants and interactions with drugs and other herbs have been reported [2-4]. Therefore, a necessary first step towards developing standards for medicinal plants and herbs is must in order to make sure the safe use of these medicines. With reference to the mentioned facts, the present study was undertaken to standardize ethnopharmacological useful stem bark of *Myrica esculenta* and also an attempt was made to isolate the myricetin, which can be used as an analytical marker for determination of its quality, by using a chromatographic and spectroscopic technique.
Myrica esculenta, family Myricaceae commonly known as kaiphal is a small to moderate-sized tree varying from 3 to 15 m from place to place in sub-tropical Himalayas from the Ravi river eastward to the Khasi, Jaintia, Naga, and Lushai hills of India at altitudes of 900 to 2100 m. The plant is known to have varied medicinal properties and the stem bark of this plant has a variety of reported traditional uses for the treatment of asthma, fever, dyspnoea, throat and lung infections, chronic bronchitis, and coughing [5-7]. It has been reported that the stem bark contains a flavonol, myricetin (Hexahydroxy flavone, $C_{13}H_{10}O_8$)which occurs in the form of glycoside myricetrin [8, 9]. It has a widespread occurrence in the nature and has been reported to have varied therapeutic effects. It has been reported to be antioxidant, anti-carcinogenic, anti-inflammatory, anti-artherosclerotic and antithrombotic agent. Preliminary work has also shown that myricetin has hypoglycemic effect in diabetic animals [10].

Material and methods
Chemicals and plant material
Reference standard myricetin (M-6760, ≥ 96% purity), quercetin (Q-4951, ≥95% purity) and natural product reagent (NP) (2- amino ethyl diphenyl borinate) were obtained from Sigma-Aldrich (Bangalore, India).Phloroglucinol, potassium hydroxide, hydrochloric acid, nitric acid, sulphuric acid, acetic acid, picric acid, gallic acid, toluene, chloroform, petroleum ether, methanol, potassium dichromate, acetone, silica gel (60-120 mesh) (analytical grade) were obtained from SD Fine chemicals (Mumbai, India)

Dried stem bark of *Myrica esculenta* was obtained from a commercial supplier of Ahmedabad, Gujarat, India. The plant was identified and authenticated by Dr. Minoo Parabia, Head and Professor, Department of Bioscience, Veer Narmad South Gujarat University, Surat, Gujarat, India. A voucher specimen of the plant was deposited in the herbarium of the department of Pharmacognosy, Anand Pharmacy College, Anand, Gujarat, India (APC/2006/01; PC/2007/01). Dried stem bark of *Myrica esculenta* was milled into powder with the aid of the electrical grinder and stored in air tight bottles for further analysis.

Macroscopic and microscopic examination

The macroscopic examination was carried out with the help of naked eyes and simple hand lens for evaluation of the shape, size, color, and fracture. For microscopic evaluation, the dried bark pieces were boiled in water to soften the tissues and then free hands sections were taken. The resulting sections were boiled in potassium hydroxide for 10 min to clear of interfering pigments in the tissues. The sections were then treated with phloroglucinol and concentrated hydrochloric acid for 10 min and then observed using binocular microscope (Radical PRM-12A) [11, 12].

Quantitative microscopy

2 gm of powdered stem bark was mixed with 50 ml of 10% nitricacid in a casserole. It was boiled and maintained at boilingpointfor 30 seconds diluted with water and strainedthrough a fine cloth held over the mouth of filterfunnel. The was hedresidue was trans ferred to casserole and boiled further with 50 ml of 2.5% sodium hydroxide for 30s. The residue was washed and used for quantitative analysis. The values for 25 fibres were calculated and multi plied by the factor. The average value was calculated and the range for width and length of fibres were calculated [13].

Fluorescence analysis

The fluorescence and general behavior of powdered plants was studied by treating separately with different reagent and was observed immediately in visible light and UV lights (254 and 366 nm) using UV cabinet (Durga Scientific) for fluorescence behavior [14, 15].

Proximate analysis

Different physicochemical constants were assessed which included determination of moisture content, total solids, solvent extractive value (water, alcohol and ether), ash values (total ash, acid insoluble ash and water soluble ash), determination of tannins and foaming index [16-20].

Elemental analysis

The major elements, comprising calcium, potassium, sulphur and trace elements (iron, copper and zinc) were determined according to the method of Shahidi *et al*. The mineral content was determined using inductively coupled plasma atomic emission spectrophotometer (Perkin Elmer, USA, 3300RL) and concentration of each element in the sample was calculated as the percentage of dry matter [21].

Preliminary phytochemical screening

50 gm of powdered stem bark was successively extracted with petroleum ether (60-80 °C b.p.), toluene, chloroform, methanol using soxhlet apparatus. The extraction with each solvent was carried out until the solvent was colorless for 24 h. Finally, the marc left was extracted with water by digesting on a boiling water bath. The extraction was continued till a few drops of the last portion of the extract left no residue on drying. The extracts were taken in a tarred porcelain dish and evaporated to dryness on a water bath and dried at 105 ºC to a constant weight. The percentage extractives were calculated with reference to air dried drug. The presence or absence of the primary (carbohydrates, proteins and amino acids) and secondary phytoconstituents (glycosides, alkaloids, saponins, tannins, steroids, coumarins etc.) was detected by various qualitative chemical tests [22-26].

Estimation of total flavonoid content

The total flavonoid content of stem bark was estimated by colorimetric aluminium chloride method reported by Woisky and Salatino using Shimadzu double beam UV/Visible spectrophotometer 1650 PC [27].

Estimation of total phenolics

The total phenolic content of stem bark was estimated by the modified Folinciocalteu method using Shimadzu double beam UV/Visible spectrophotometer 1650 PC [28].

Chromatographic separation

Preparation of column

Silica gel (60-120 mesh) was weighed and transferred to a clean dry beaker. The beaker was covered with watch glass and kept in oven at 100 °C for 3 h and then cooled in a dessicator. A 90 cm glass column (ID 2.8 cm, Durga make) was cleaned, washed and dried. A piece of glass wool (previously kept overnight dipped in benzene) was introduced with the help of a glass rod to the base of the column. The column was then placed in vertical position with the help of clamps and column stand. The activated silica gel was then poured into the column and was gently tapped by a rubber cork fixed at the end of glass rod.

Preparation of extract and chromatographic development

The stem bark was defatted using petroleum ether (40-60°C) to remove the oily and fatty material. After defatting with petroleum ether, the drug was kept at room temperature for the removal of solvent. After drying, the powder was extracted with methanol by cold maceration. The extract thus obtained was dried under reduced pressure. The alcoholic extract (1 g) was dissolved in a minimum quantity of chloroform and mixed with silica gel equal to the weight of extract, stirred with a glass rod and dried in air. Completely dried mixture was introduced into the column with the help of a glass rod to prevent the sticking of the mixture to the wall of the column. The extract was then chromatographed using chloroform: methanol (gradient) as an eluent. The elution was maintained at 30-35 drops/min. 43 fractions (each of 10 ml) were collected and analyzed by thin layer chromatography on Silica gel 60 F254TLC plate (10×10 cm, 0.2 mm thick, Merck Ltd., Mumbai, India) in chloroform: acetic acid: methanol: water (60:32:12:8) as mobile phase. Identical fractions were pooled together. The spots were visualized by spraying the chromatograms with NP-PEG reagent followed by observation at 366 nm which gave orange fluorescence. The spot without spray was scrapped and dissolved in minimum quantity of methanol for obtaining spectroscopic data. It was scanned in U.V. mode between 200 to 400 nm.

Results

Morphological evaluation

The outer surface of the bark was rough, with deep vertical

wrinkles, grey to brownish grey in color, 1.1-2.3 cm thick, slightly quilled, fissured longitudinally and transversely. The inner surface was dark brown and smooth; its fracture was hard and taste bitter. The macroscopic characters are as shown in Figure 1.

Fig 1: Stem Bark of *Myricaesculenta*Buch. Ham. Ex D. Don, Myricaceae

Microscopic evaluation

Transverse section of mature bark showed multilayered cork, composed of rectangular, tangentially elongated, thin walled cells and some were filled with reddish brown matter. A wide zone called secondary cortex composed of thin walled rectangular to polygonal, parenchymatous cells filled with reddish brown matter was found. A number of stone cells were found in singles or in groups, polygonal or oval, thick walled, lignified with simple pits and radiating canals, found scattered throughout secondary cortex. Secondary phloem consisted of phloem fibres, crystal fibres, stone cells and phloem parenchyma traversed by phloem rays and all these microscopic characters are as shown in Figure 2(A, B). Powder study revealed the presence of parenchyma, stone cells, pericyclic fibre and crystal fibre as shown in Figure 3 (A-D).

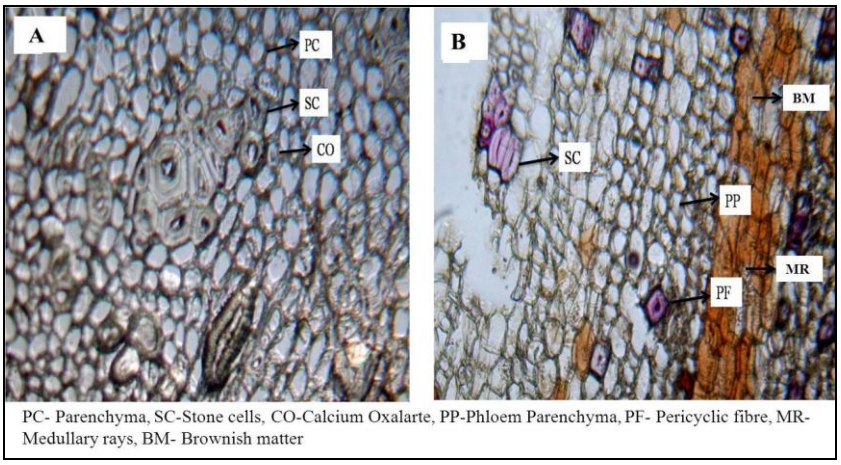

Fig 2(A, B): Transverse section of stem bark of *M. esculenta*

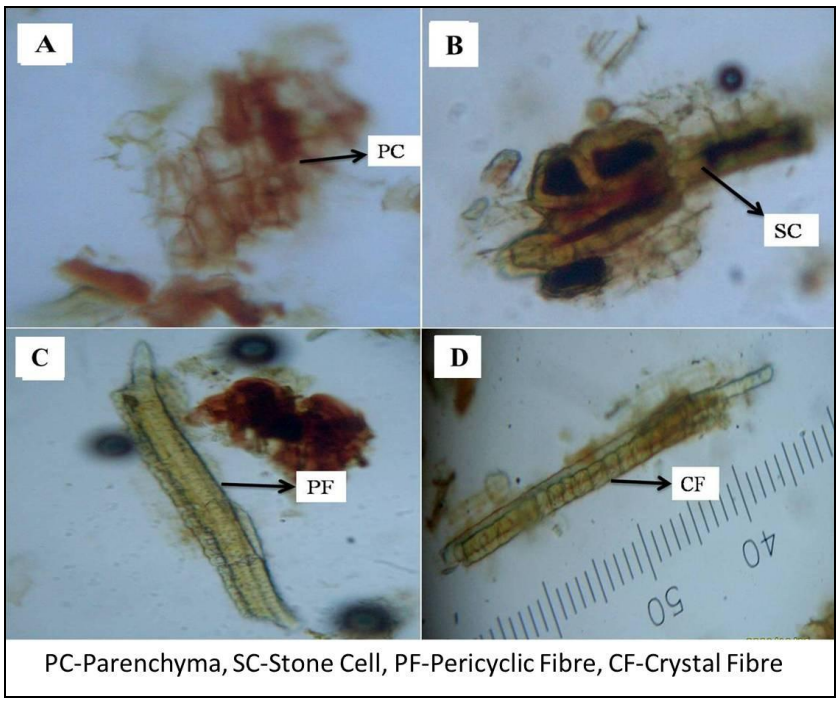

Fig 3(A-D): Powder characteristics of stem bark of *M. esculenta*

Quantitative microscopy

The width and length of fibre present in the stem bark was found to be 25.8-51.6-77.4 µm and 251.55-461.18-709.5 µm respectively.

Fluorescence analysis

The results of the fluorescent properties of the powdered stem bark treated with several reagents are as presented in Table 1.

Table 1: Fluorescent properties of stem bark of *M. Esculenta*

Reagent	Daylight	U.V. (254 nm)	U.V. (365 nm)
Methanol	Brown	-	Brown
1 M NaOH in methanol	Brownish black	Green	Brownish black
1 M NaOH in water	Brown	-	Brown
1 M HCl in methanol	Brown	-	Green
1 M HCl in water	Brown	Blue	Dark brown
50% HNO_3	Brown	-	Green
50% H_2SO_4	Brown	-	Green
HNO_3	Orange	-	Green
CH_3COOH	Brown	-	Greenish brown
1% picric acid	Brown	-	Green
10 % $K_2Cr_2O_7$	Brownish green	Purple	Green
5% I_2	Brown	Purple	Green
Dilute NH_3	Brown	-	Brownish green
5% $FeCl_3$	Green	-	Green

Proximate analysis

Results for standardization parameters of stem bark are as presented in Table 2.

Table 2: Standardization parameters of stem bark of *M. esculenta*

Sr. No.	Standardization parameters	(%)
1	Moisture content	9.93±0.38
2	Total solids	90.07±0.38
3	Solvent extractive value	
	Water soluble extractive	24.0±0.60
	Ethanol soluble extractive	13.0±0.20
	Methanol soluble extractive	21.0±0.50
	Ether soluble extractive	2.70±0.10
4	Ash values	
	Total ash	2.47±0.12
	Acid-insoluble ash	0.02±0.001
	Water-soluble ash	0.60±0.015
5	Foaming index	125
6	Tannin content	22.00±1.58

Elemental analysis

The elemental content like calcium, potassium, sulphur and trace elements were analyzed in the powdered plants and the results are as tabulated in the Table 3.

Table 3: Elemental analysis of stem bark of *M. esculenta*

Minerals	(%)
Calcium	3.155±0.18
Iron	0.123±0.16
Potassium	2.939±0.23
Magnesium	1.061±0.4
Sodium	0.060±0.03
Phosphorus	0.030±0.01
Sulphur	0.277±0.34
Zinc	0.006±0.001

Preliminary Phytochemical screening

The results of color, consistence and extractive value of extract were as presented in Table 4.

Table 4: Successive solvent extraction of stem bark of *M. esculenta*

Solvent	Color of extract	Consistency of extract	Extractive value (%w/w)
Petroleum ether	Light yellow	Semisolid	0.50
Toluene	Light brown	Solid	5.08
Chloroform	Brownish pink	Semisolid	0.78
Acetone	Dark brown	Solid	1.56
Methanol	Dark brown	Solid	1.88

The results of preliminary qualitative chemical tests of various extracts of stem bark obtained by successive solvent extraction are as tabulated in Table 5.

Table 5: Qualitative chemical test of different extracts of stem bark of *M. esculenta*

Phytoconstituents	Pet. ether	Toluene	Chloroform	Acetone	Methanol	Water
Steroids	+	-	-	-	-	-
Triterpenoids	-	-	-	-	+	+
Reducing sugars	-	-	-	-	-	-
Carbohydrates	-	-	-	-	-	-
Alkaloids	-	-	-	-	-	-
Phenolic compounds	-	-	-	-	+	+
Anthraquinones	-	-	-	-	-	-
Saponins	-	-	-	-	+	+
Tannins	-	-	-	-	+	+
Flavonoids	-	-	-	-	+	+
Mucilage	-	-	-	-	-	-

+ Present, - Absent

Total flavonoid content

The total flavonoid content was calculated as quercetin equivalent with reference to standard curve, Y=0.0961X+0.0114, R^2 = 0.9948 (Figure 4). Total flavonoid content was found to be 0.53% w/w.

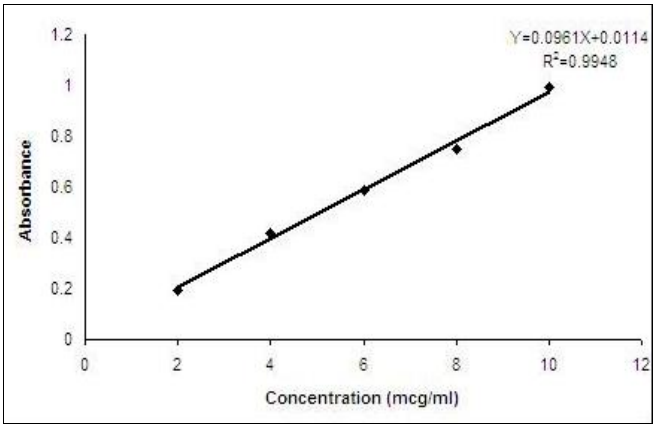

Fig 4: Calibration curve of standard quercetin

Total phenolic content

Total phenolic content had been reported as gallic acid equivalent with reference to standard curve, Y=0.0915X-0.0072, R^2=0.9996 (Figure 5). Total phenolic content *was* found to be 9.18% w/w calculated as gallic acid equivalent.

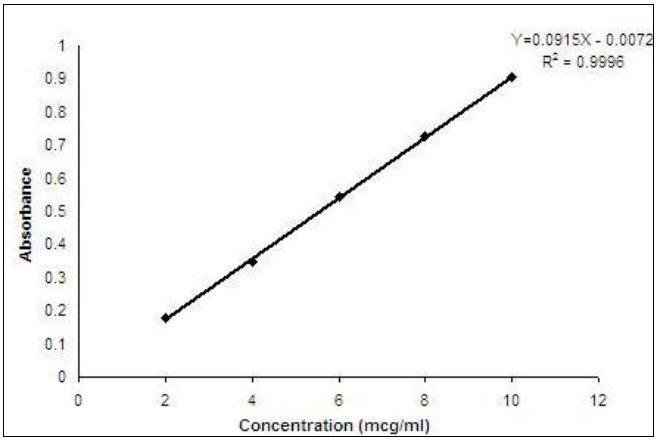

Fig 5: Calibration curve of standard gallic acid

Chromatographic separation

Fraction No. 27-40 showed the presence of yellow colored spot at R_f 0.65 in visible light typical of flavonol glycoside. The plate on spraying with NP-PEG reagent resulted in development of orange spot in visible mode and on observation in UV 254 nm gave bright orange fluorescence which confirmed the presence of flavonol glycosides as shown in Figure 6 (A, B). The separated spot without derivatization was scrapped and dissolved in methanol. On scanning it in Shimadzu double beam UV/visible spectrophotometer 1650 PC in U.V. mode between 200 to 400 nm produced two peaks at 254 and 375 nm which is a characteristic of flavonolaglycone,myricetin. Further, U.V. spectra of reference standard produced two peaks at same wavelength, which confirmed the presence of myricetin.

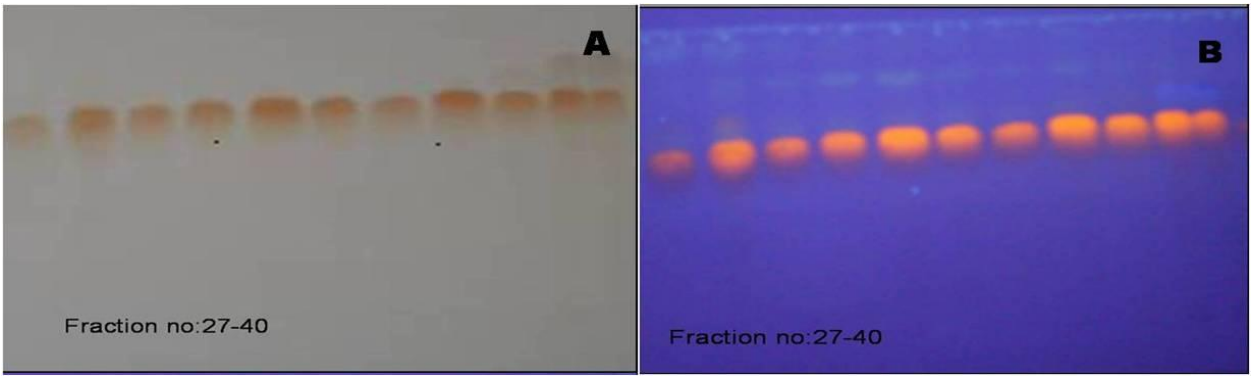

Fig 6(A, B): Photograph of TLC profile of fractions 27 to 40 (A) visible mode, (B) fluorescence mode

Discussion

In the present work macroscopic and microscopic evaluations was carried. This can be used for identifying the genuine drug and distinguishing it from its substitutes and adulterants. The physicochemical studies and the proximate analysis helped in identifying the quality and purity of the plant.

Fluorescence analysis of the plants revealed the presence of starch and phenolic compound. Reaction with acid and alkali showed fluorescence indicating that phenolic compounds like flavonoids, flavones and coumarins may be present.

The total ash value indicative of inorganic salts and acid insoluble ash value indicating the presence of silicaceous matter was found to be within the standard limits. Among the various solvent extractive values, water and alcohol soluble extractive was found to be highest indicating the presence of polar and non-polar constituents. Foaming index was found to be 125 indicating the presence of saponin. Stem bark showed the presence of 22% of tannins.

On elemental analysis, calcium was the most abundant macro element present in the plants. This was followed closely by potassium, magnesium, sulphur and iron. However, sodium, phosphorus and zinc content were found to be present in lower amounts.

Stem bark yielded solid extracts on successive solvent extraction. The extractive value was found to be higher (5.08%) for toluene extract, showing the presence of both polar and non-polar constituents. Preliminary qualitative chemical tests of various extracts obtained by successive solvent extraction revealed phytoconstituents like tannins, phenolics, flavonoids and saponins in methanolic and aqueous extract. Moreover, steroidal compound was also found to be present in the petroleum ether extract of stem bark.

A marker compound myricetin was isolated using chromatographic technique and was further confirmed by U.V. spectroscopic method by comparing the spectral data of isolated compound with that obtained with the reference standard myricetin.

The results obtained from pharmacognostical and phytochemical investigation can serve as standards for identification of genuine crude drug and also for differentiating it from the other species of genus *Myrica*. The developed chromatographic method can be used for isolation of marker myricetin and can be utilized for further scientific investigation.

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