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Pharmacognostical and phytochemical studies on roots of *Smilax perfoliata*– Alternate source for the ayurvedic drug chopachinee

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Abstract
Introduction: *Chopachinee* is one of the important Ayurvedic drug used in the treatment of several diseases. The accepted botanical source of *Chopachini* is *Smilax china*. *Smilax perfoliata* is used as a substitute for the Ayurvedic drug *Chopachini*. *S. perfoliata* is used traditionally in the treatment of epilepsy, psychosis, diseases of nervous system and Parkinsonism. The aim and objectives of the present study is to evaluate the pharmacognostical and phytochemical studies on the roots of *S. perfoliata*
Methods: Pharmacognostical studies comprises of taxonomical, macroscopical, microscopical studies including powder and macerate analysis. The phytochemical study involves determination of physicochemical constants, fluorescence analysis, histochemical tests, preliminary phytochemical screening and HPTLC studies. Quantification of secondary metabolites like total phenolic, flavonoid and alkaloid content was carried out. Quantity of Diosgenin present in the extract was determined by HPLC method.
Results: Transectional view of the roots showed outer periderm and inner ground parenchyma with diffusely distributed vascular strands. The periderm seems to have originated deep in the vascular strands located inner portion of the rhizome. Macerate of root showed group of parenchyma cells, fibre, vessels with scalariform and reticulate thickenings. Powder revealed the presence of cork cells, fibre, starch grains and vessels. Preliminary phytochemical screening of different extracts revealed the presence various phytoconstituents. The phenolic, flavonoid and alkaloid content of alcohol extract was found to be 0.059 mg GAE/g, 0.2639 mg Q/Eg and 0.526 % w/w respectively. The amount of Diosgenin present in root extract was found to be 0.151 %.
Conclusion: The study will help in laying down pharmacopoeial standard for the drug *Smilax perfoliata.*

Keywords: pharmacognosy, phytochemistry, chopachinee, diosgenin, HPLC, *Smilax perfoliata*

Introduction
Chopachini is one of the important drug in Ayurveda. The accepted botanical source of *Chopachini* is *Smilax china* Linn [1]. The plant is indigenous to china. *Smilax china* is widely distributed in parts of Himalayas, Japan, Nepal and most parts of china. It is imported to India particularly to parts of Calcutta and Mumbai. The reference to *Chopachini* was first mentioned in Bhavaprakasha (16th century) and also present in some indigenous medicine text book like *Vaidyaka Hasthasaraya*. Roots are used to treat various diseases like syphilis, mercury poisoning, pains, rheumatism, epilepsy, Parkinsonism, psychosis, diseases of nervous system and hysteria. There are several Ayurvedic formulations like Madhusnuhi churna, Dwipantara Vacha Madhusnuhi Rasaayana, Mahaa Vallaathi Legiyam and Parangi Rasaayanam are available in the market with *Smilax china* as one of the major ingredients [2]. Four species of *Smilax* namely *Smilax aspera* Linn., *S. perfoliata* Lour., *S. wightii* A. DC., and *S. zeylanica* Linn., occur in south India, [3] (Gamble., 1990). It is distributed throughout western Himalayas, kumamon, Nepal, Bengal, Bihar and Deccan peninsula [3]. Different species of the genus including *S. perfoliata*, *S. zeylanica* are used as substitutes for *S. china*. *S. perfoliata* roots are used traditionally in the treatment of epilepsy, psychosis, diseases of nervous system and Parkinsonism diseases [4]. In the present study, roots of *smilax perfoliata* is evaluated for Pharmacognostical and phytochemical studies which help in identification of crude drug and to establish pharmacopoeial standards.

Materials and Methods
Collection and Identification
Roots were collected from Satyavedu region of Chittoor district, Andhra Pradesh in August 2019. The collected plant material was identified and authenticated by Dr. K. Madhava Chetty, Taxonomist, Sri Venkateswara University, Tirupati. Taxonomical identification was carried out using various Floras [3]. The material was washed with water and dried in shade.

Microscopical Studies
Microscopical studies were carried out by taking free hand sections of roots using razor blade. Sections were cleared by warming with a few drops of chloral hydrate, stained with phloroglucinol: conc.HCI (1:1), treated with iodine solution and safranin. Sections were then mounted in glycerin for microscopical observations [5, 6].

Macerate Studies
Small pieces of the roots were taken and immersed in 50 % nitric acid and potassium chlorate. The solution was boiled until brown fumes were observed. The solution was filtered

and washed thoroughly with distilled water until it become free from acid. Material was separated with needle and stained using safranin, and examined under compound microscope [5, 6].

Powder Analysis
Roots were powdered and passed through sieve no.60. A small quantity of powder was treated with different reagents like chloral hydrate, phloroglucinol and conc.HCI (1:1), iodine solution for detection of constituents like lignin, starch and calcium oxalate crystals and observed under a compound microscope [5, 6].

Histochemical Analysis
The roots sections were treated with various reagents such as Ruthenium red for mucilage, Iodine Solution for starch, Millon's Reagent for proteins, Sudan III for fixed oil, 5 % Ferric chloride for tannins, Conc. HCl for crystal, Iodine+60% H_2SO_4 for cellulose and Phloroglucinol + dil. HCl for lignified substances [6].

Physicochemical Parameters
Moisture content, ash values (total ash, acid insoluble ash, water soluble ash) and extractive values (water soluble and alcohol soluble extractive value) of *S. perfoliata* root powder was determined following standard procedures [7, 8]. Fluorescence analysis was carried out with various reagents and observed under visible and ultra violet radiations of short (254 nm) and long wavelength (365 nm) [9].

Qualitative Analysis
A known quantity of root powder of *smilax perfoliata* was extracted successively with following solvent such as n-hexane (60-80^0 C), toluene, chloroform, ethyl acetate, ethanol (95 % v/v). Each time before extracting with next solvent the marc was air dried below 50 ^{0}c, weighed, repacked and then extraction was followed by using soxhlet apparatus. Aqueous extract was prepared by macerating with chloroform water for 24 hours. Extracts were filtered and concentrated, extractive values were calculated with reference to air dried drug. Colour and consistency of extracts were noted. For the extract obtained preliminary phytochemical screening is carried out to check the presence various primary and secondary plant metabolites using standard procedure [10, 11].

Quantitative Estimation
Total Alkaloid Content
10 g of coarsely powdered drug was extracted with 4 x 30 mL 0.1 M phosphoric acid under agitation for 30 min, filtered and filtrates combined. Then the acid solution was thrice extracted with 100 ml hexane each time to remove non-polar compound and pH adjusted to 9 with 25% ammonium hydroxide. The pH adjusted solution was extracted with chloroform in a separating funnel until the extract showed negative for Dragendorff's reagent test. Organic phase was dried with anhydrous sodium sulphate and concentrated. The total alkaloid content was determined by gravimetric method [12].

Total Phenolic Content
1 mL of sample and various dilutions of standard were each taken in 10 mL volumetric flask. To each to the volumetric flask, 5mL of distilled water and 0.5 mL of Folin ciocalteu's reagent was added. After 5 minutes 1.5 mL of 20% sodium carbonate solution was added, made up to 10mL with ethanol and incubated for 2 h. The absorbance of the developed intense blue colour was measured at 750 nm in UV-Visible spectrophotometer (Shimadzu UV-1700 spectrophotometer) and the total phenolic present in the extract was calculated and represented as Gallic Acid Equivalent (GAE) [13].

Total Flavonoid Content
1 mL of sample and standards were each taken in 10 mL volumetric flask. 4 mL of distilled water and 0.3 mL of 5 % sodium nitrate was added to each volumetric flask. After 5 minutes 0.3 mL of 10 % aluminium chloride was added followed by 2 mL of 1 M NaOH and made up to 10 mL with ethanol. Solution developed orange yellow colour and absorbance was measured at 510 nm in UV-Visible spectrophotometer. The total flavonoids present in the extract was calculated and represented in Quercetin equivalent (QE) [13].

Estimation of Diosgenin content in alcohol root extract of *S. perfoliata* using HPLC
Diosgenin quantification was performed using HPLC and experimental conditions were maintained isocratic binary system of Acetonitrile / water (90:10), with a flow rate of 1 ml per second and temperature of 35 ^{0} C. 10 mg of diosgenin was weighed and dissolved in 5 ml of methanol by sonicating for 15 minutes. The solution was diluted up to 10 ml with methanol (1mg/ml). Pipette out 1 ml solution from stock solution and diluted up to 10 ml with methanol (100 mg/ml) used as standard. Various concentrations of alcohol extract ranging from 2 to 10 (µg/ml) was measured by UV spectrometer at 291 nm. The corresponding peak areas were plotted against the concentration of diosgenin injected and retention time was noted down.

Results and Discussion
Exomorphology of Plant
Smilax perfoliata is a stout climbing shrub, with woody stem. The leaves are broad having coiled tendrils. Flowers Inflorescence of 10-30 umbels and have berry type of fruits (Fig 1A).

Distribution
India, Malaysia, Nepal, Sri Lanka, Taiwan, Thailand, Vietnam, and south china. In India, particularly it is distributed in West Himalaya and several parts of south India.

Vernacular Names
Sanskrit: Chopachinee; Kannada: Neeru betta balli; Telugu: Pirangi chekka [14].

Pharmacognostical Studies
Morphological Characters
Dried root pieces measures 4 to 12 cm in length and 3 cm in thickness. The dried roots are hard and cylindrical in shape with brownish to blackish externally, yellowish internally in colour. Outer surface is rough and fracture is fibrous. It has a bitter in taste with no characteristic odour. (Fig.1B).

Microscopical Characters

Transectional view of the roots exhibits outer periderm and inner ground parenchyma with diffusely distributed vascular strands. The periderm seems to have originated deep in the vascular strands located inner portion of the rhizome. The periderm cells are darkly stained with red content due to stain used in safranin. The periderm cells also contain black inclusion of tannins. The root has attract type of stele where these are several independent, diffusely distributed monocot type of vascular bundles. The ground tissue of the root consists of wide angular, thin walled compact parenchyma cells. The ground parenchyma cells have no specific cell illusions. The vascular bundles are circular and collateral. The collateral bundles have phloem and xylem one above the other. Most of the vascular bundles are surrounded by thick walled lignified sclerenchyma cells. In some of the vascular bundles the bundles sclerenchyma cell possess dense tannins. The bundle sheath fitness are much enlarged and thicker walled than the xylem elements. The phloem elements are small angular and thick walled the sieve elements has small companion cells attached with xylem element along one corner of the cell. The Meta xylem elements are 40 to 50 um in diameter. The bundle sheath sclerenchyma cells are 70 um in diameter. The vascular bundles are 400um in diameter. In longitudinal section of the root, the xylem elements appear vertically elongated tubes with close spiral lateral wall thickening. Xylem parenchyma cells are vertically elongated wide tubes. Fibres wide, highly thick walled signified cells. (Fig 2)

Macerate Studies

The macerate study of root revealed the presence of groups of parenchyma cells, pitted parenchyma cells, fibre, vessels with scalariform thickening and reticulate thickening. (Fig 3)

Powder Analysis

Powder studies showed parenchyma cell with starch grain, cork cells, fibre, Vessel with reticulate thickenings and starch grain. (Fig 4)

Histochemical Tests

The sections of roots were treated with different reagents to determine the presence of cell contents (Table 1).

Physico-Chemical Parameters

The results obtained from physicochemical constants, fluorescence analysis and successive solvent extraction helps to lay down pharmacopoeial standards for the crude drug. The moisture content was found to be 8.14 % w/w, total ash 1.64 % w/w, acid insoluble ash 1.45 % w/w, water soluble ash 1.45 % w/w, alcohol soluble extractive value 12.45 % w/w and water soluble extractive value 22.44 % w/w (Table 2).

Fluorescence Analysis

The drug exhibited characteristics colors under visible and UV light (Table 3).

Preliminary Phytochemical Analysis

On successive solvent extraction of dried powder of *smilax perfoliata* with solvents using n-hexane, toluene, chloroform, ethyl acetate, ethanol (95%) and Water, the percentage of yield was found to be very less in non-polar solvents such as n-hexane, toluene and Chloroform, while in ethyl acetate, aqueous extract and ethanol extract showed relatively high yield. The percentage of yield, color were noted down (Table 4). The extracts of successive solvent extraction were subjected to phytochemical screening which revealed the presence of alkaloids, carbohydrates and glycosides, Phytosterols, saponins, phenolic compounds, proteins and amino acids and flavonoids (Table 5). The total phenolic, flavonoid and alkaloid content of alcohol extract was found to be 0.059 mg GAE/g, 0.2639 mg QE/g and 0.526 % w/w respectively (Table 6) (Fig 5-6). The amount of Diosgenin present in root extract was found to be 0.151 % (Fig 7-8).

Fig 1: A. Habitat of *Smilax perfoliata*, B. Crude Drug

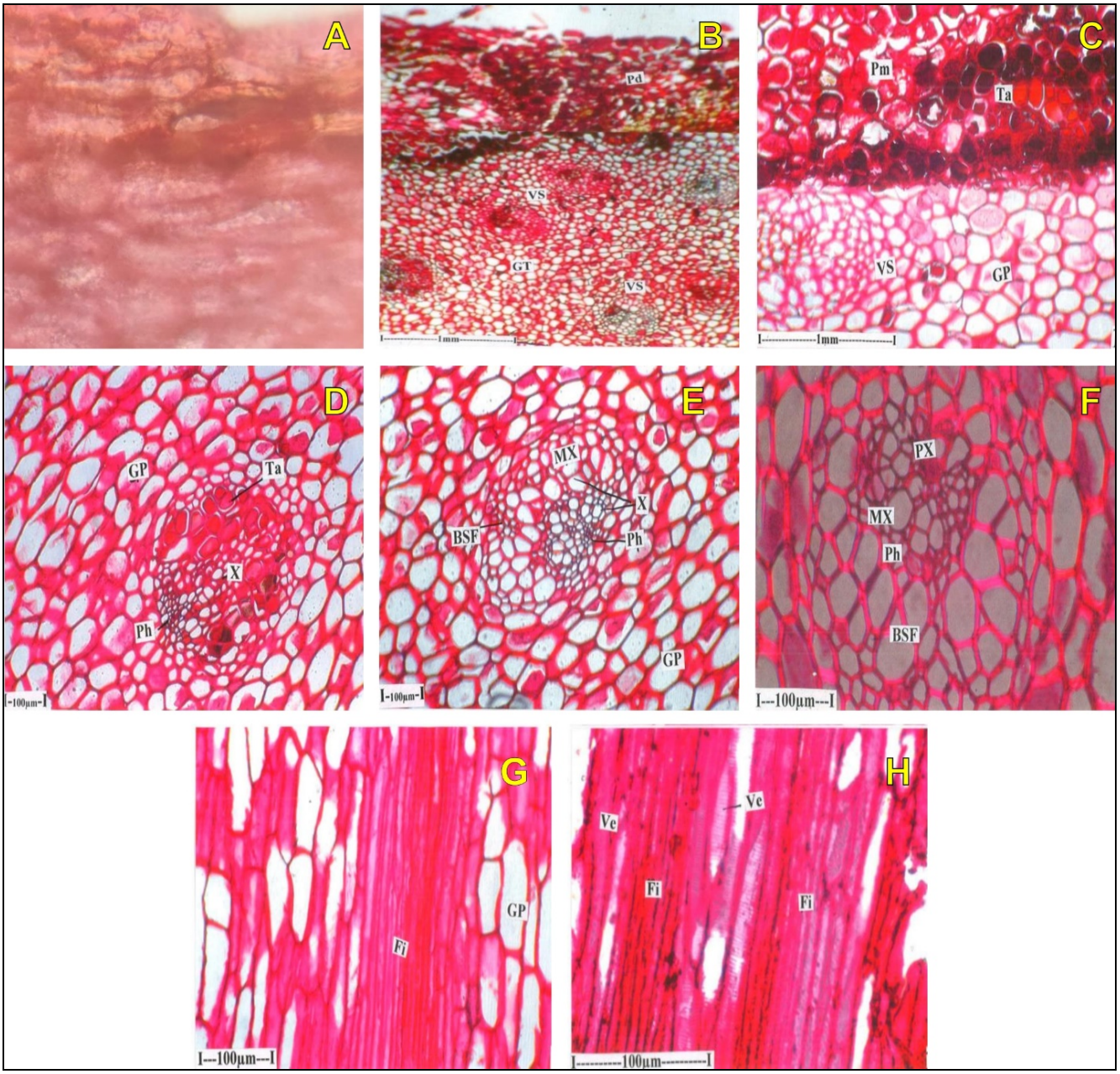

Fig 2: Microscopy of *Smilax perfoliata* root

A. Cork and cortex region, **B.** outer periderm and inner vascular bundles, **C.** outer Phelleus of Periderm and inner vascular zone, **D.** Vascular bundle enlarged, **E.** Two Vascular bundles, **F.** Vascular bundles enlarged, **G.** Xylem elements, **H.** vessels and fibre

PM: Phellem, Ta: Tannins, VS: Vascular Strand, Ph: Phloem; GT: Ground tissue, X: Xylem, BSF: Bundle sheath Fibre, Px: Proto xylem, MX: Meta Xylem, GP: Ground tissue, P: Phloem, X: Xylem, Fi: Fibre, GP: Ground tissue, Ve: Vessels

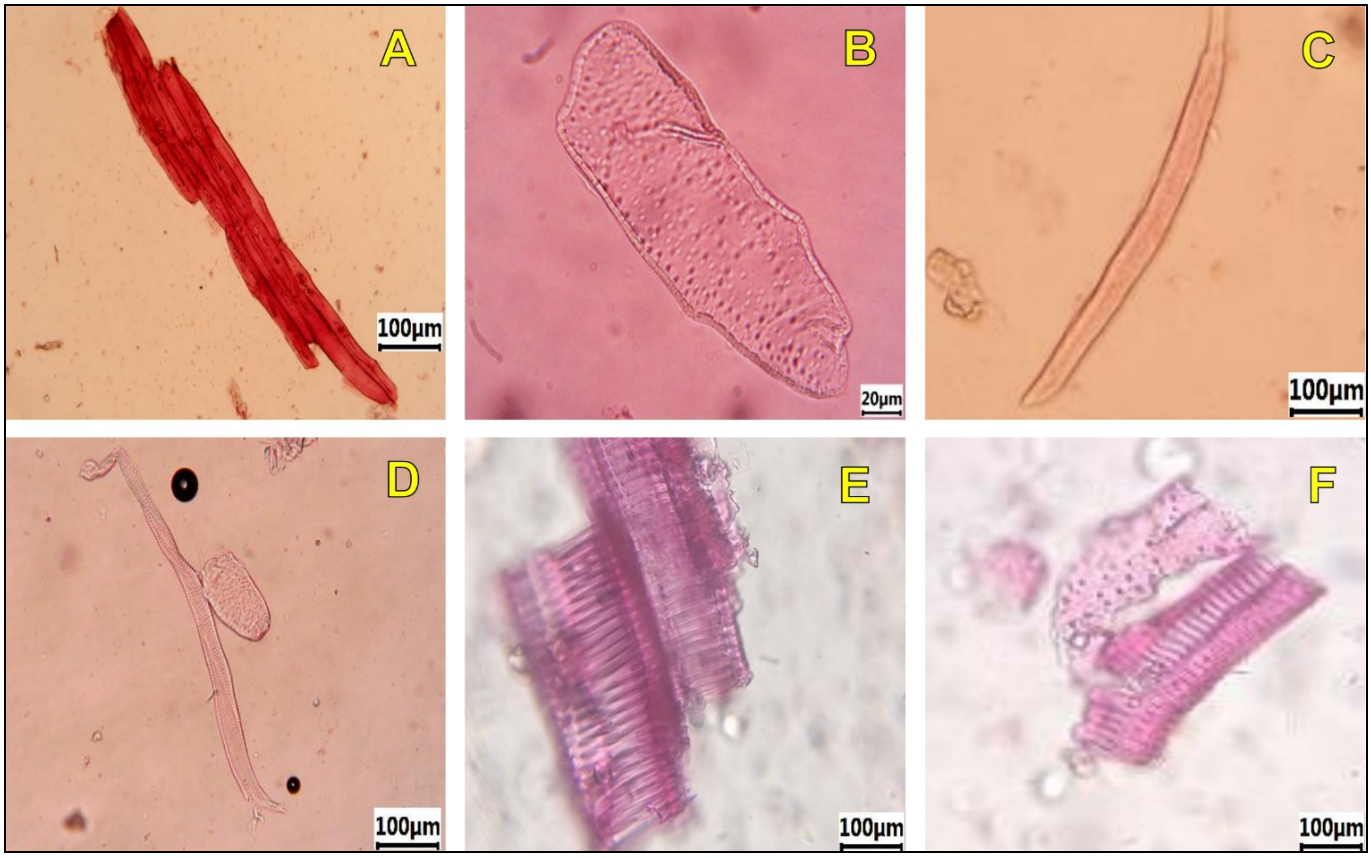

Fig 3: Macerate studies of roots of *S. perfoliata*
A. Group of parenchyma Cells, **B.** Parenchyma Cell, **C.** Fibre, **D.** Vessel with Scalariform thickening, **E.** Vessel with Scalariform thickening, **F.** Vessel with Reticulate thickening

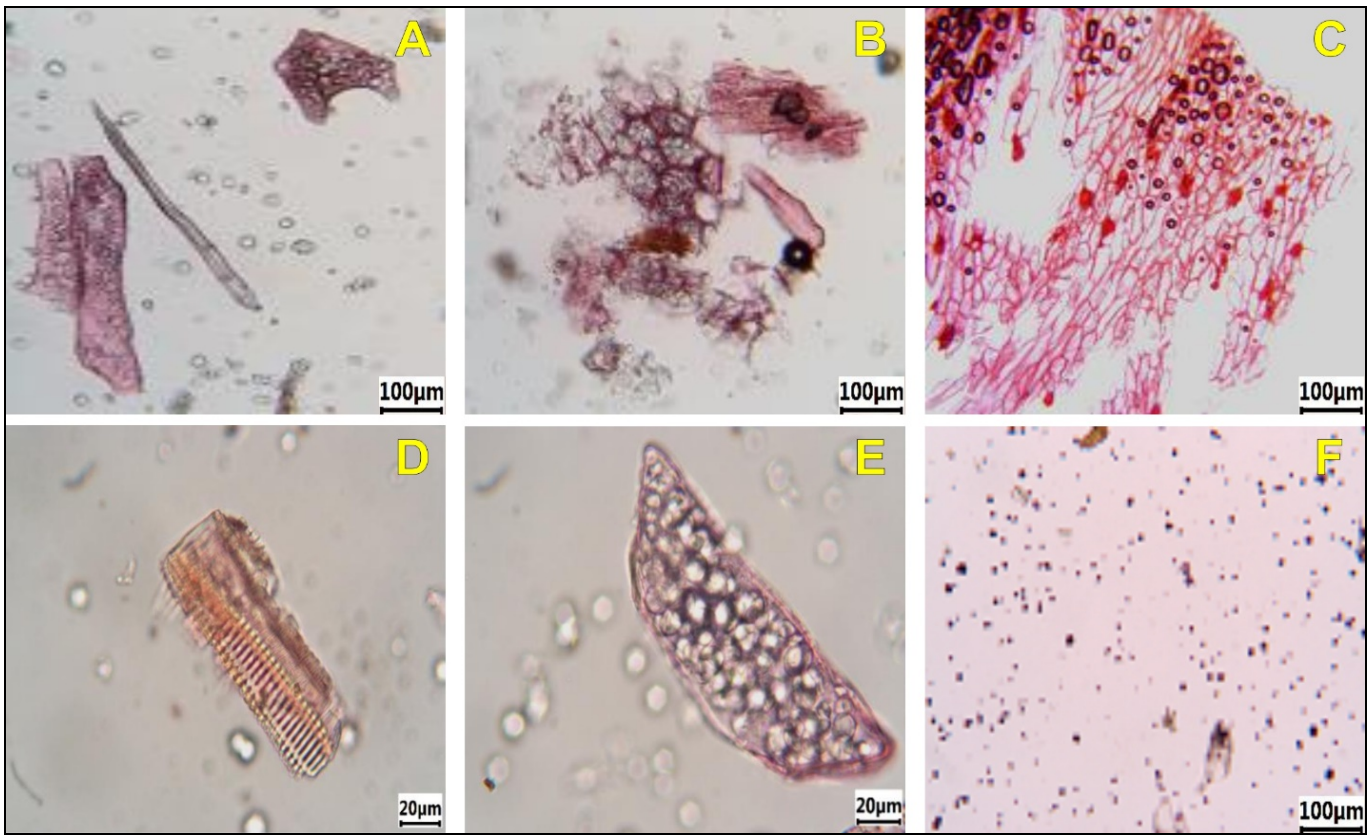

Fig 4: Powder analysis of roots of *S. perfoliata*
A. Fibre, **B.** Cork Cells, **C.** Cork Cells, **D.** Vessel with Reticulate thickening, **E.** Parenchyma with Starch grain, **F.** Starch grains

Table 1: Histochemical tests for roots section of *S. perfoliata*

S. No	Reagent	Test for	Reaction	Result
1	Phloroglucinol + dil. HCL	Lignin	Pink	+
2	Ruthenium Red	Mucilage	Red	+
3	Iodine Solution	Starch	Blue	+
4	Millon's Reagent	Protein	No Brick red	+
5	Sudan red III	Fixed oil	Red	_
6	5% Ferric chloride	Tannins	Bluish Black	+
7	Conc. HCL	Crystals	Effervescence	_
8	Chlor zinc iodine	Cellulose	Blue	+
9	Iodine +60% H_2SO_4	Cellulose	Blue	+

Table 2: Physiochemical constants of the root of *S. perfoliata*

S. No	Moisture Content (% w/w)	Ash Value (% w/w)			Extractive Value (%w/w)	
		Total Ash	Water soluble ash	Acid insoluble ash	Water soluble	Alcohol Soluble
1.	8.143	1.64	1.47	1.24	22.44	12.45

Table 3: Fluorescence analysis of the roots of *S. perfoliata*

S. No	Reagents	Visible Light	Short wave (254 nm)	Long wave (365 nm)
1.	Powder as such	Ivory	Pastel green	No fluorescence
2.	50% H_2SO_4	Moody Manoor	Manoor	No fluorescence
3.	50% HNO_3	TerracottaN	Green Gold	No fluorescence
4.	5% KOH	TerracottaN	Light green	No fluorescence
5.	Methanol	Mid Cream	Autumn Gold	No fluorescence
6.	Ethanol	Mid Cream	Diced olive	No fluorescence
7.	Acetone	Sandalwood	Light green	No fluorescence
8.	1N HCL	Mid cream	Diced olive	No fluorescence
9.	IN Methanolic NaoH	Copper leaf	African Dessert	No fluorescence
10.	IN ethanolic NaoH	Brick red	Mehendi – N	No fluorescence
11.	Dilute ammonia Solution	Milan red	Mehendi – N	No fluorescence

Note: The color mentioned in Table are based on the "Asian paints" Ace Exterior Emulsion, Asian paints Limited, Mumbai

Table 4: Successive solvent extraction values and nature of extracts of root of *S. perfoliata*

S. No	Solvent	Colour	Consistency	Extractive Value (% w/w)
1.	n- hexane	Brown	Semi-solid	1.66
2.	Toluene	Brown	Semi-solid	0.11
3.	Chloroform	Pale Brown	Semi-solid	0.63
4.	Ethyl acetate	Dark Brown	Semi-solid	1.34
5.	Ethanol (95 % v/v)	Dark Brown	Semi-solid	3.94
6.	Water	Pale Brown	Semi-solid	2.21

Table 5: Preliminary Phytochemical Screening of successive solvent extracts of *S. perfoliata*

Test For	n-hexane extract	Toluene Extract	Chloroform extract	Ethyl acetate	Ethanol extract	Water extract
Alkaloids			+	+	+	-
Carbohydrates and Glycosides					+	+
Phytosterols	+	+			+	
Fixed oil and Fats	+	+				
Phenolic Compounds and Tannins					+	–
Saponins					+	+
Flavonoids				+	+	+
Proteins and Amino Acid					+	+
Volatile Oils	-					
Gum and Mucilage						-

+ = present, - = absent

Estimation of Total Phenol and Flavonoid Content

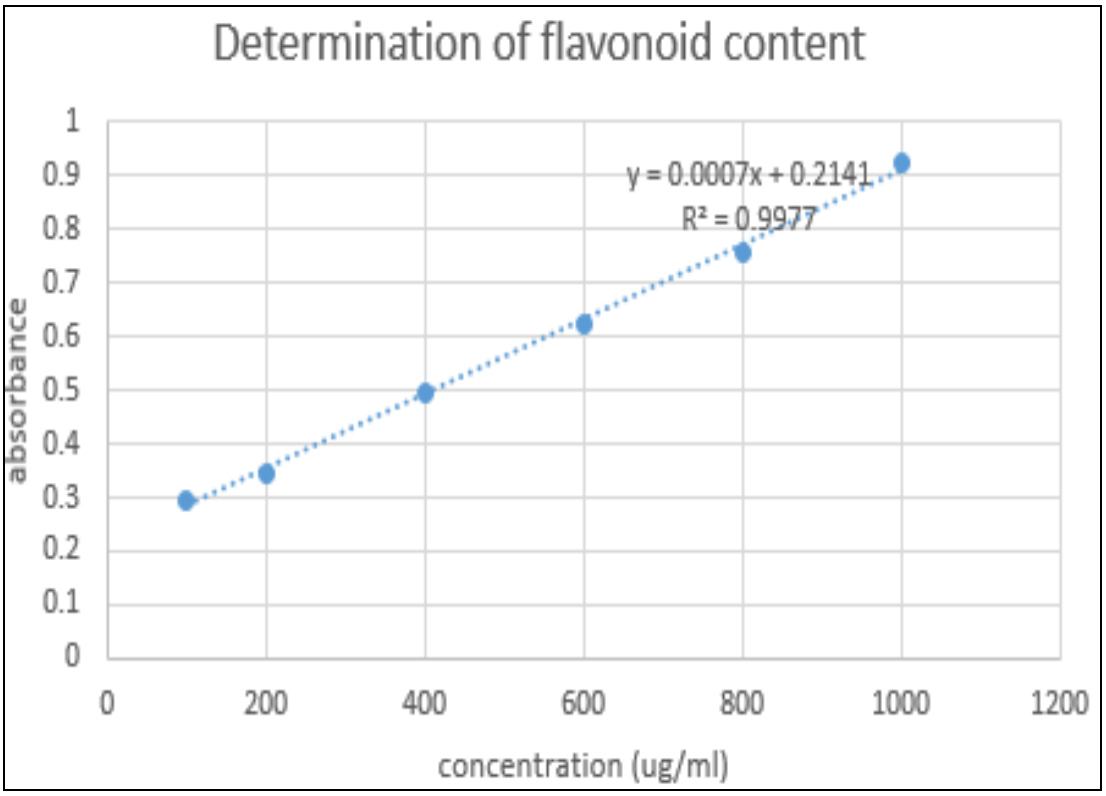

Fig 5: Standard Calibration curve of Quercetin

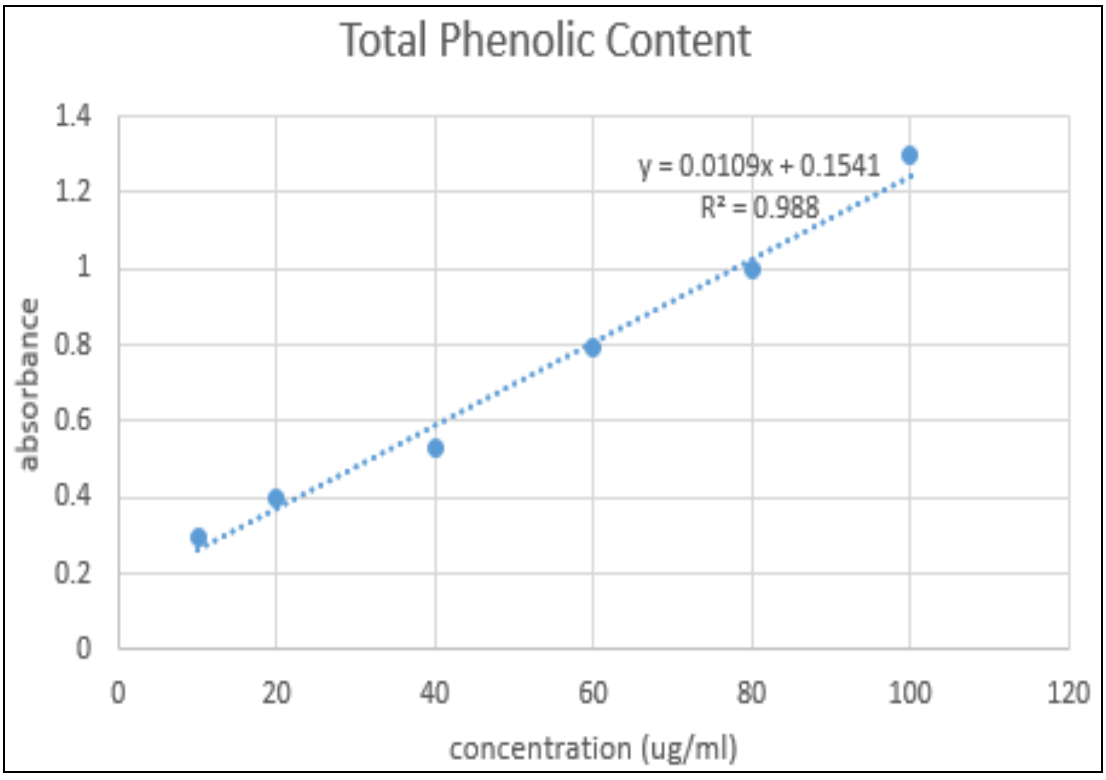

Fig 6: Standard Calibration curve of Gallic Acid

Table 6: Total Phenol and Flavonoid content of alcohol root extract of *S. perfoliata*

S. No	Parameters	Alcohol Extract
1	Phenolic Content	0.059 mg GAE/g
2	Flavonoid Content	0.2639 mg QE/g
3	Alkaloid content	0.526 %w/w

Estimation of Diosgenin content in *S. perfoliata* by HPLC analysis

The HPLC method is a precise and accurate for determination of diosgenin. Amount of Diosgenin alcohol extract was found to be 0.151 %. Retention time of Standard diosgenin and total alcohol extract was found to be 2.96 and 2.93 minutes.

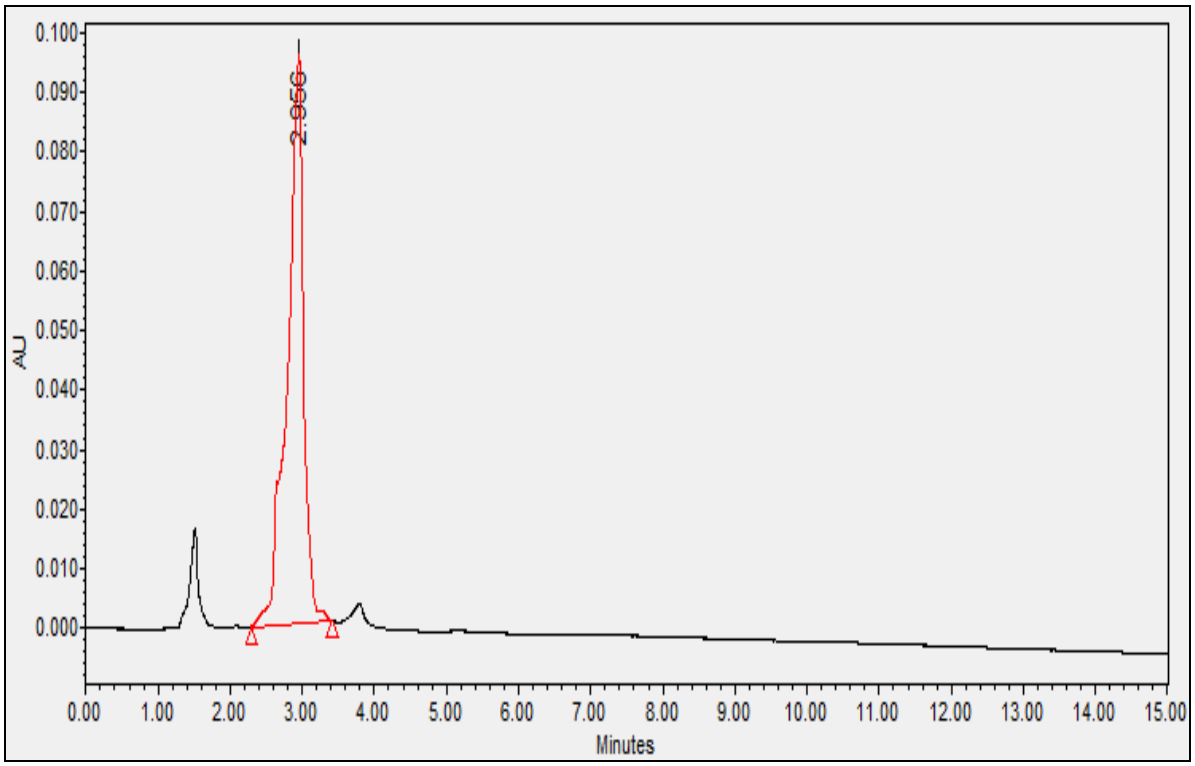

Fig 7: Chromatogram of Standard Diosgenin

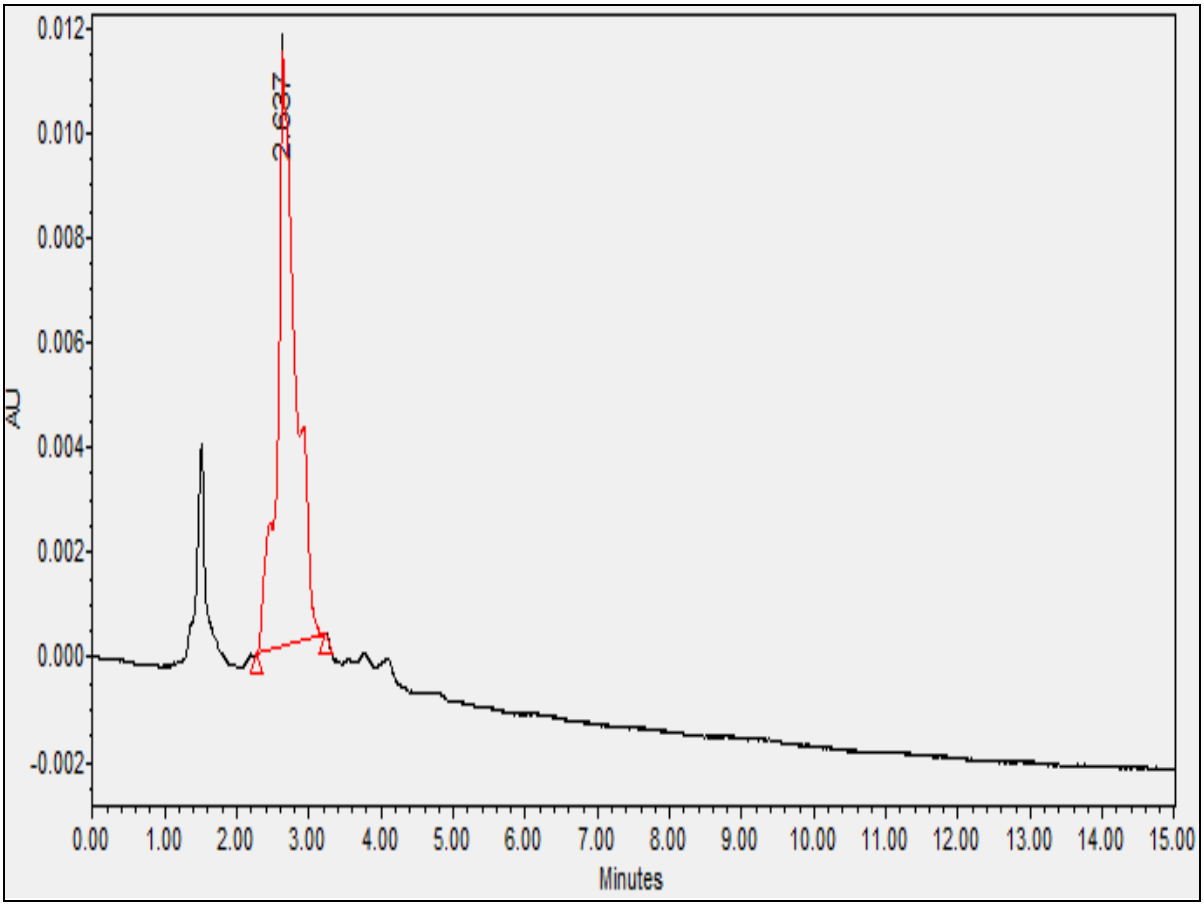

Fig 8: Chromatogram of Total alcohol extract of *S. perfoliata*

Conclusion

The present study provides detailed pharmacognostical, phytochemical studies of *Smilax perfoliata* root which helps in the identification and to differentiate from closely related species. Further research should focus on the pharmacological evaluation and isolation of active principles from the plant source.

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