ORIGINAL ARTICLE

Preliminary phytochemical screening, RP-HPLC, HPTLC and anti-oxidant studies of *Pinus maritimus*

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Abstract

Objective: The aim of the present study is to screen for the phytochemical constituents present in the ethanolic extract of whole plant of *Pinus maritimus* (PM). **Method:** The ethanolic extract of the dried whole plant of PM is subjected to preliminary phytochemical screening which showed the presence of major phytoconstituents such as phenols, flavonoids, and alkaloids. The extract was screened for its antioxidant activity by 2,2'-diphenyl-1-picrylhydrazyl, hydroxyl radical, ferric reducing antioxidant potential, and nitric oxide scavenging assay. Further, the ethanolic extract was subjected to fingerprinting technique high-pressure thin-layer chromatography. Reverse-phase high-performance liquid chromatography (HPLC) was performed to estimate the amount of total phenolics, flavonoids, and alkaloids quantitatively in isocratic mode. **Results:** Phytochemical screening of the ethanolic extract of the plant showed the presence of important active constituents such as alkaloids, flavonoids, phenolics, and terpenoids. The study also revealed the potential antioxidant activity of the extract with IC_{50} value. Reverse-phase HPLC showed 0.119 µg/ml of total phenolics, 0.257 µg/ml of alkaloids, and 0.0016 µg /ml of flavonoids. **Conclusion:** Scientific evaluation of this plant was carried out which is very important for the standardization of the plant-based drug. PM is one which has therapeutic importance as it showed important phytoconstituents.

Key words: *Pinus maritimus*, *In vitro* antioxidant, high-performance liquid chromatography, reverse-phase high-performance liquid chromatography

INTRODUCTION

Pinus maritimus (PM) belongs to Pinaceae family. It is an annual herb and is found extensively grown in the region of Eastern and Southern regions of India and also extended in the region between Southeast Asia. The aerial parts and the roots of the plant are used in the treatment of wide variety of ailments such as inflammation, melasma, and osteoarthritis.[1,2] In spite of its wide usage as an herbal treatment, there is no extensive research on the phytochemical screening which is very important for the standardization of the drug. The literature survey revealed that nine different phytocompounds have been isolated and characterized among which three were proved to be effective antioxidant drug.[3,4] Phenolic derivative from aerial parts of the plant showed presence of pycnogenol.[5] The compound Pycnogenol was reported and the studies also showed its role in oxidative stress in several cell systems by doubling the intracellular synthesis of antioxidative enzymes and by acting as a potent scavenger of free radicals.[2] In this present study, attempt is made in the further screening of phytoconstituents which are of biological importance. The study also deals with the quantitative estimation of total phenolics, flavonoids, and alkaloids by reverse-phase high-pressure chromatography.

MATERIALS AND METHODS

Chemicals and Instrument

Ethanol of analytical grade, 2,2'-diphenyl-1-picrylhydrazyl (DPPH) Sigma grade, naphthyl ethylenediamine

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dihydrochloride gifted, ascorbic acid HiMedia, deoxyribose Sigma grade, dimethyl sulfoxide, EDTA, and other chemicals are of analytical grades are used in the phytochemical screening and antioxidant parameters. High-performance thin-layer chromatography (HPTLC), CAMAG, Switzerland and software WinCATS 4 software are used. Supercon liquid chromatography–mass spectrometer was done by Shimadzu 2010A model. Fourier transform infrared spectra were got from KBr discs and instrument used Thermo Nicole Id5, nuclear magnetic resonance (NMR), that is, C-NMR and H-NMR by Bruker NMR with 500 MHz using TMS as internal standard. For column chromatography, silica mesh size 60–120 Merck and silica gel G, Merck for TLC were used.

Collection of Plant Material

The fresh and healthy whole plant material of PM was collected from Eastern Ghats of India, that is, from Tirupati, Andhra Pradesh. The plant material was authenticated by Dr. K Madhava Chetty and the specimen is preserved in herbarium in the Department of Botany, SV University, Tirupathi, with the herbarium number being 0523.

Preliminary Phytochemical Screening

Extractive value

Ethanolic-soluble extractive value and water-soluble extractive value of air-dried coarse powder of plant materials PM are determined. The percentage of ethanolic-soluble extractive value and water-soluble extractive value with reference to air-dried material was calculated and the values are recorded.

Determination of moisture content

The difference in weight percentage after loss on drying with reference to the air-dried drug was calculated.[7-9]

Ultraviolet (UV) study

A small quantity of dried powder of the plant materials was placed on the grease free microscopic slide and treated with 1–2 drops of chemical reagents such as 1 N HCl, 1 N HNO_3, 1 N H_2SO_4, 1 N NaOH, alcoholic NaOH, alcoholic KOH, and ammonia separately with gentle tilting. The slides were placed in the ultraviolet viewer chamber and viewed in day light, short (254 nm), long (365 nm) UV radiation and the color changes of the powdered drug when treated with different chemical reagents were recorded based on different chemical constituents.[10]

Preliminary phytochemical screening

Five kilograms of the healthy plant material of PM were shade dried and finely ground into coarse powder. This coarse powder was subjected for Soxhlet extraction process using ethanol. The extraction was continued till the solution in the siphon was colorless. Then, the ethanolic extract was subjected to rotary evaporation, where the solvent was recovered leaving behind ethanolic extract. Ethanolic extracts of PM were subjected to preliminary phytochemical screening. The extracts were screened for the presence of alkaloids, carbohydrates, glycosides, saponins, phytosterols, fixed oils and fats, resins, tannins, phenols, flavonoids, proteins and amino acids, terpenes and terpenoids, gums and mucilage, and lignin.[11,12]

In vitro antioxidant activity

Plants play an important role in scavenging the free radicals and thereby proving as a potent antioxidant. In this study, the ethanolic extract of PM was evaluated for their potent antioxidant activity using four important models.

Iron (III) to Iron (II) reducing activity

The ability of the extracts and isolated compounds to reduce iron III was assessed.[13] The reduction potential was measured spectrophometrically at 700 nm. The results were expressed as ascorbic acid equivalents (ASCAEs) in mg of ascorbic acid per g of extract.

Diphenylpicrylhydrazyl radical scavenging assay

The DPPH scavenging assay was carried out to the test extracts and isolated compounds.[14] The scavenging property is read at 517 nm and inhibition of diphenylpicrylhydrazyl radical was calculated.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging was carried out by measuring the competition between deoxyribose and the extracts and isolated compounds for hydroxyl radicals generated from the Fe3+/ascorbate/EDTA/H2O2 system a method carried.[13] Butylated hydroxyl toluene was taken as standard.

Nitric oxide scavenging activity

Nitrite ions generated from sodium nitroprusside react with Griess reagent to form purple azo dye. In the presence of the test components, likely to be scavengers, the amount of nitrites will decrease. The degree of decrease in the formation of purple azo dye will reflect the extent of scavenging.[15] Nitric oxide scavenging assay was assessed for all the test extracts and isolated compounds taking gallic acid as standard.

HPTLC

HPTLC was performed on silica gel 60F254 (10 cm × 10 cm; 0.25 mm layer thickness; Merck). PM extract prepared in 10 ml volumetric flask by taking 250 mg of the extract and diluting with ethanol the concentration was 25 mg/ml and filtered through a 0.45 µ syringe filter from this 4, 6, and 12 mg/ml concentrations which were subjected to HPTLC (CAMAG, Switzerland) analysis. All these dilutions were spotted on a silica gel 60F254 (Merck, Darmstadt, Germany)

TLC plate. The plate was air dried and then developed using the solvent system hexane:chloroform:methanol (3:3.5:3.5 v/v) as mobile phase in a CAMAG twin trough glass chamber previously saturated with mobile phase vapor for 20 min. After developing the plate, it was dried at 65°C for 2 min and then it was scanned using Camag Scanner 3 (CAMAG, Switzerland) at 254 and 366 nm using WinCATS 4 software.[16]

Determination of total phenolics, flavonoids, and alkaloids by reverse-phase HPLC Total phenolics[17]

Mobile phase was prepared by mixing methanol:water (60:40); pH was adjusted to 3.1 with orthophosphoric acid. This solution was filtered using a 0.45 µm filter paper and was sonicated for 10 min. The total volume of the mobile phase prepared was 300 ml.

Standard: The standard concentration of gallic acid taken was 100 µg/ml.

Sample preparation: To 5 mg PM extract, 5 ml of ethanol was added. The solution was vortexed for 5 min. The sample was filtered using 0.45 µm filters. A 20 µl of the sample was injected in the HPLC system.

Wavelength: 254 nm.

Total Flavonoids[18]

With slight modification, mobile phase was prepared by mixing methanol:0.2% acetic acid pH 3.5 (50:50). This solution was filtered using a 0.45 µm filter paper and was sonicated for 10 min. The total volume of the mobile phase prepared was 300 ml.

Standard: The standard concentration of quercetin acid taken was 100 µg/ml.

Sample preparation: To 5 mg PM extract, 5 ml of ethanol was added. The solution was vortexed for 5 min. The sample was filtered using 0.45 µm filters. A 20 µl of the sample was injected in the HPLC system.

Wavelength: 254 nm.

Total Alkaloids[19]

Mobile phase was prepared by mixing methanol:water (90:10). This solution was filtered using a 0.45 µm filter paper and was sonicated for 10 min. The total volume of the mobile phase prepared was 300 ml.

Standard: The standard concentration of berberine acid taken was 100 µg/ml.

Sample preparation: To 5 mg PM extracts, 5 ml of ethanol was added. The solution was vortexed for 5 min. The sample was filtered using 0.45 µm filters. A 20 µl of the sample was injected in the HPLC system.

Wavelength: 220 nm.

Common optimized chromatographic conditions

RESULTS AND DISCUSSION

DISCUSSION

Determination of the physical and chemical parameters in the plant material is very important for the standardization of the drug. Therefore, in this paper, attempt was done to standardize some of the chemical parameters. The coarse plant material of PM was subjected for the determination of moisture content or loss on drying, it was found that the percentage of loss on drying was just 15. If the moisture holding capacity of the drug was high, it would lead to the deterioration of the plant material. As in this case, the loss on drying was negligible, it ensures the stability of the drug. The coarse plant material showed the alcoholic extractive value 12% and water extractive value as 8%, respectively, suggested that most of the active constituents which are of clinical importance are extracted in ethanol compared to water [Table 1].

Many chemical compounds present in the plant materials will behave as chromophores. These chromophores on reacting with the chemical reagent give out characteristic colors when exposed under the UV light. This helps as a major parameter in pharmacognosy for the standardization of the drug. The results are summarized in Tables 2 and 3.

Table 1: Extractive values

Parameter	*Pinus maritimus*
Moisture	15%
Alcohol-soluble extractive	12%
Water-soluble extractive	8%

Detector	Shimadzu SPD-10A UV–VIS, Japan
Pump	Shimadzu LC-10ATVP, Japan
Software	Baseline chromatography Data System N2000
Injection valve	7725i Rheodyne 20 µl, USA
Syringe	50 µl Hamilton, Switzerland
Column	Phenomenex Gemini-NX-5 µm C18(2) 110 Å, LC Column 250×4.6 mm, Ea
Part No	00G-4041-EO
Dimensions	250×4.6 mm ID
Flow rate	1 ml/min

The plant material was subjected to Soxhlet extraction using ethanol. The ethanolic extract was subjected for the qualitative determination of phytoconstituents. The study revealed the presence of alkaloids, glycosides, phenols, tannins, terpenoids, and flavonoids which are of pharmacologically important [Table 4].

Reactive oxygen species which are produced in a cell by various metabolic processes are responsible for the damage of DNA and to bring abnormality in the cell. It is reported that the plant produces some important phytoconstituents which neutralizes the effect of these reactive oxygen species. Therefore, in the present study, the ethanolic extract of PM was subjected to various antioxidant models which showed IC_{50} values and is summarized in Table 5, Graphs 1-4, this indicates that the plant material is good antioxidant.

The HPTLC profile developed for ethanolic extract will help in proper identification and quantification of compounds. The extract fingerprinting through HPTLC revealed the presence of various phytoconstituents at near UV and far UV radiations [Figures 1-5].

Table 2: Fluorescent study of crude extract of *Pinus maritimus*

Extract	Daylight	UV light	
		Short 254 nm	Long 365 nm
Ethanol	Mustard yellow	Green	Light blue

Table 3: Fluorescent analysis of *Pinus maritimus* extract under various treatments

Treatment	Daylight	UV light	
		254 nm (Short λ)	365 nm (Long λ)
Powder	Olive green	Dark brown	Burgundy
Powder+H_2O	Dark green	Dark brown	Light green
Powder+1 N HCl	Brownish-yellow	Dark brown	Dark green
Powder+1 N HNO_3	Brownish-yellow	Dark brown	Dark green
Powder+1 N H_2SO_4	Brownish-yellow	Dark brown	Dark green
Powder+1 N NaOH	Dark green	Dark brown	Mustard yellow
Powder+Alc. NaOH	Dark green	Dark brown	Orange
Powder+1 N KOH	Dark green	Dark brown	Dark green
Powder+Alc. KOH	Dark green	Dark brown	Mustard yellow
Powder+ Ammonia	Dark green	Dark brown	Neon green

The literature reveals that the presence of phenols and flavonoids in the plant extract is responsible for the plant to become a potent medicinal plant. Here, the amount of

Table 4: Phytochemical analysis of plant materials

Test	*Pinus maritimus*
Detection of alkaloids	
Hager's test	+++
Dragendorff's test	+
Detection of carbohydrates	
Molisch's test	-
Benedict's test	-
Barfoed's test	-
Detection of glycosides	
Modified Borntrager's test	++
Legal's test	++
Detection of phytosterols	
Salkowski's test	+++
Liebermann–Burchard's test	+++
Detection of phenols	
Ferric chloride test	+++
Detection of tannins	
Gelatin test	+++
Lead acetate test	++
Detection of flavonoids	
Alkaline reagent test	+++
Lead acetate test	+++
Zinc hydrochloric acid test	+++
Shinoda test	+++
Detection of proteins and amino acids	
Ninhydrin test	-
Detection of terpenes and terpenoids	
Copper acetate test	+++
Detection of saponins	
Froth test	+++
Detection of oil and fat	
Stain test	-
Detection of resins	
Acetone water test	++
Gums and mucilage	-
Detection of lignin	++

Table 5: IC_{50} in µg/ml

IC_{50} in µg/ml			
Sample	DPPH	Hydroxyl radical	Nitric oxide
Standard	82.88±0.52	39.63±0.36	145.66±0.58
Pinus maritimus	383.5417	81.92857	538.6486

total phenolic and flavonoids was estimated quantitatively by reverse-phase high-pressure liquid chromatography using column Phenomenex Gemini-NX-5 µm C18 (2) 110 Å, LC column 250 × 4.6 mm. The elution being isocratic system. For alkaloids, mobile phase was methanol:water (90:10), flow rate 1 ml/min and absorption maxima 220 nm were fixed [Figures 6-8]. Flavonoids were estimated where mobile phase is methanol:0.2% acetic acid pH 3.5 (50:50), flow rate 1 ml/min and absorption maxima 254 nm were adjusted [Figures 9-11]. For the estimation of total phenolics parameters such as mobile phase methanol:water (60:40), flow rate 1 ml/min and absorption maxima 254 nm were adjusted [Figures 12-14]. The Rt values of the standards were compared with its same Rt values in the sample chromatogram, area of both standard and sample was noted. The concentration of total phenolics, flavonoids, and alkaloids was calculated and is summarized in Table 6.

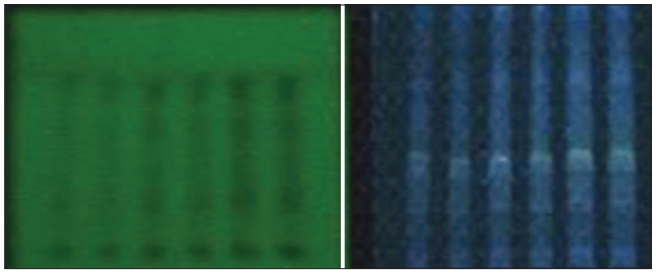
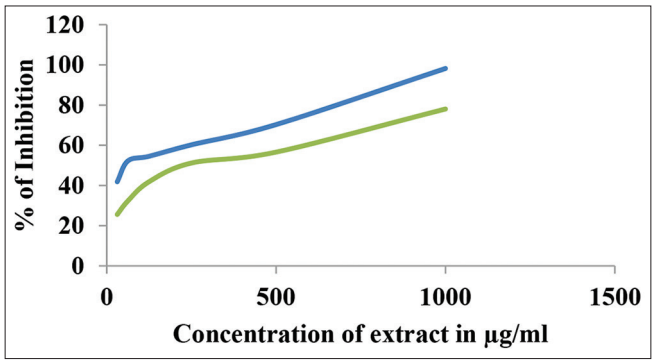
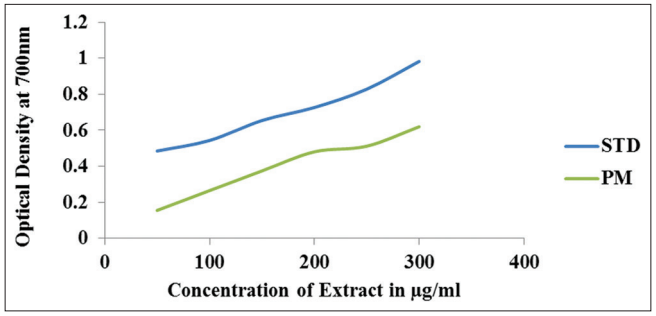


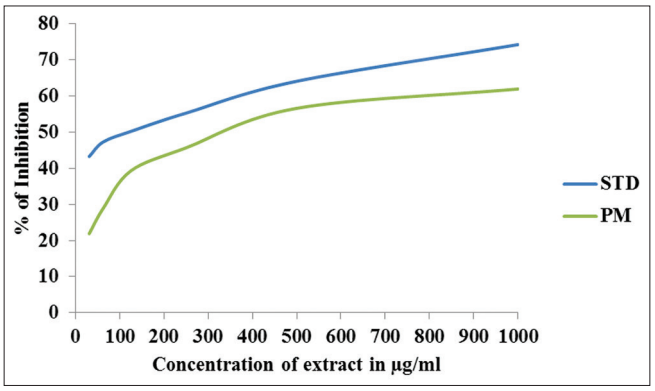
Figure 1: (a) High-performance thin-layer chromatography (HPTLC) chromatogram plate of *Pinus maritimus* (PM) extracts (256 nm). (b) HPTLC chromatogram plate of *Pinus maritimus* extract (366 nm)



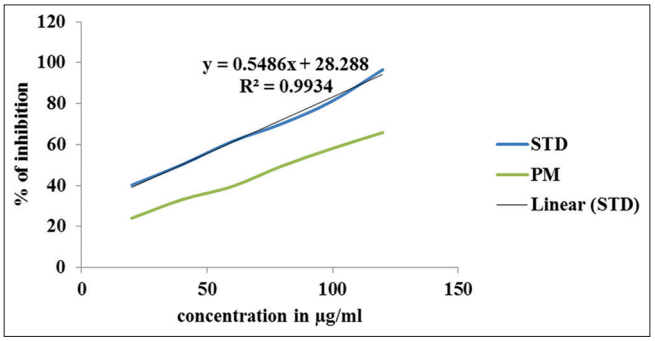
Graph 1: Antioxidant activity by 2,2'-diphenyl-1-picrylhydrazyl method



Graph 2: Antioxidant activity by ferric reducing antioxidant potential method



Graph 3: Antioxidant activity by nitric oxide method



Graph 4: Antioxidant activity by hydroxyl radical method

Table 6: Content of phytoconstituents in the ethanolic extract of *Pinus maritimus* in µg/ml

Phytochemical constituent	Phenolics	Alkaloids	Flavonoids
Purity	98	98	98
Sample area	2,172,707.25	735,218.5	85,855.945
Standard area	1,787,689.125	279,502.344	5,049,533
Sample weight in mg	1	1	1
Standard weight in mg	0.1	0.1	0.1
Sample dilution ml	1	1	1
Standard dilution ml	100	100	100
Content of phytoconstituents in the ethanolic extract of PM in mg/ml	0.119106453	0.257784647	0.001666269

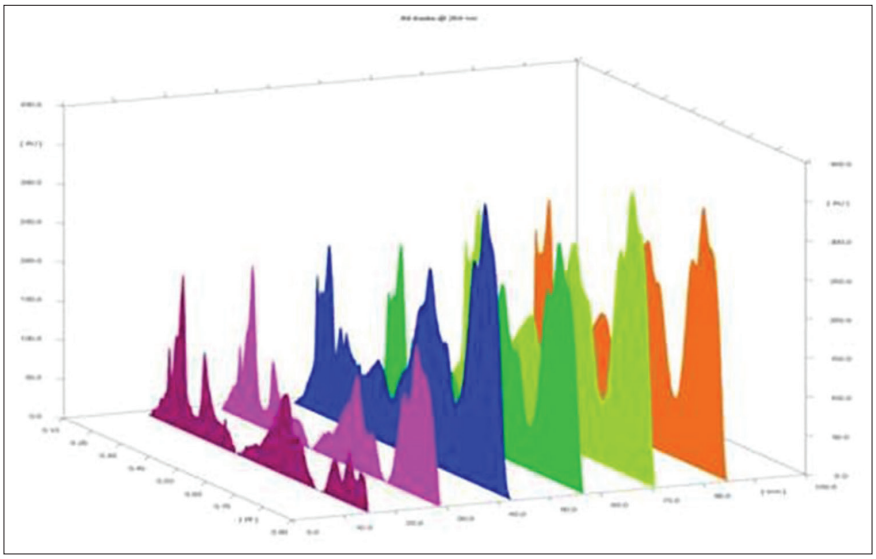


Figure 2: Overlay spectra of *Pinus maritimus* extract at 254 nm

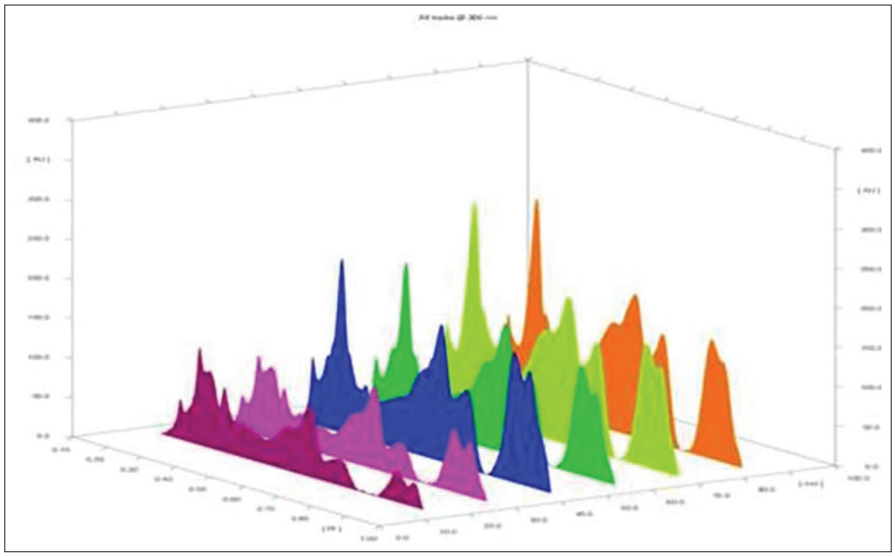


Figure 3: Overlay spectra of *Pinus maritimus* extract at 366 nm

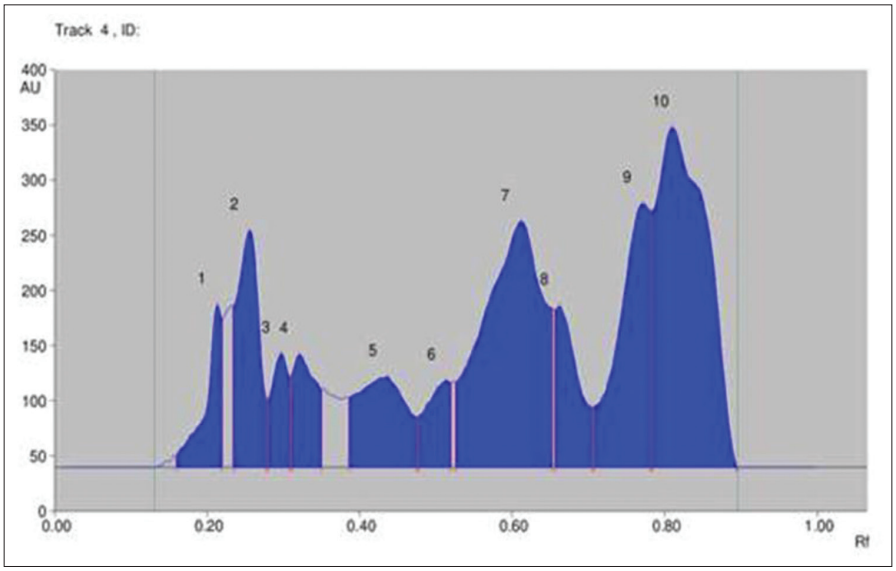


Figure 4: High-performance thin-layer chromatography densitometric chromatogram of *Pinus maritimus* extract at 254 nm

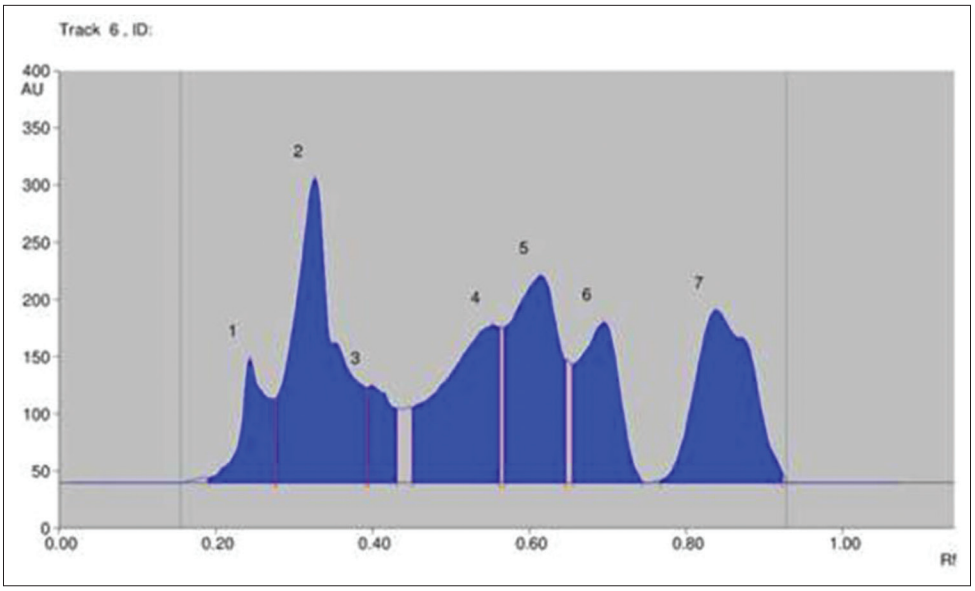


Figure 5: High-performance thin-layer chromatography densitometric chromatogram of *Pinus maritimus* extract at 366 nm

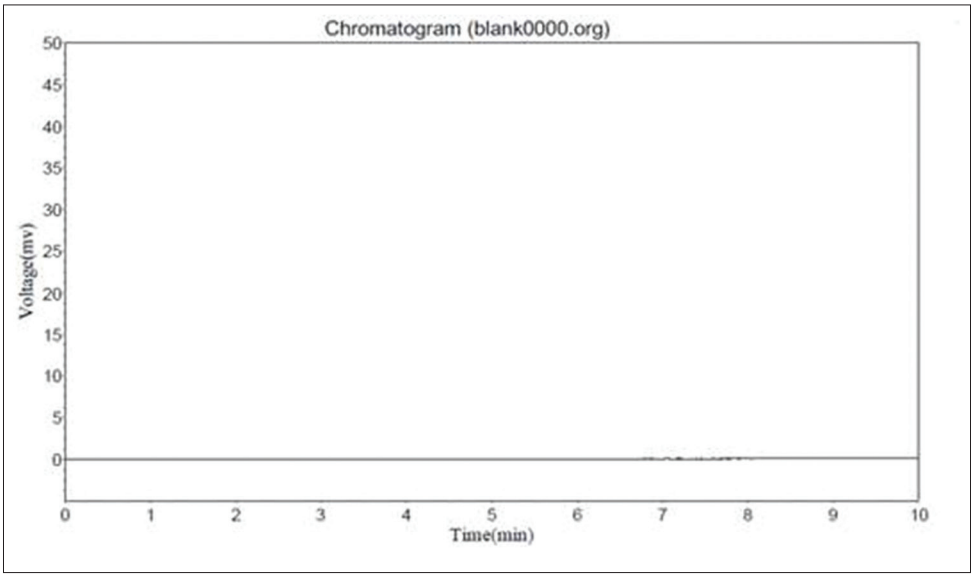


Figure 6: Chromatogram for blank for alkaloid estimation

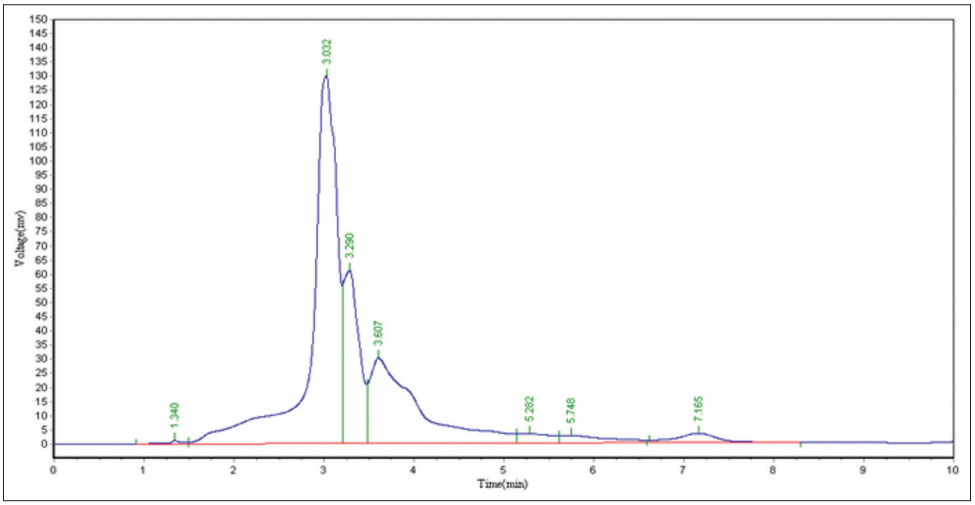


Figure 7: Chromatogram of ethanolic extract of *Pinus maritimus* for alkaloid estimation

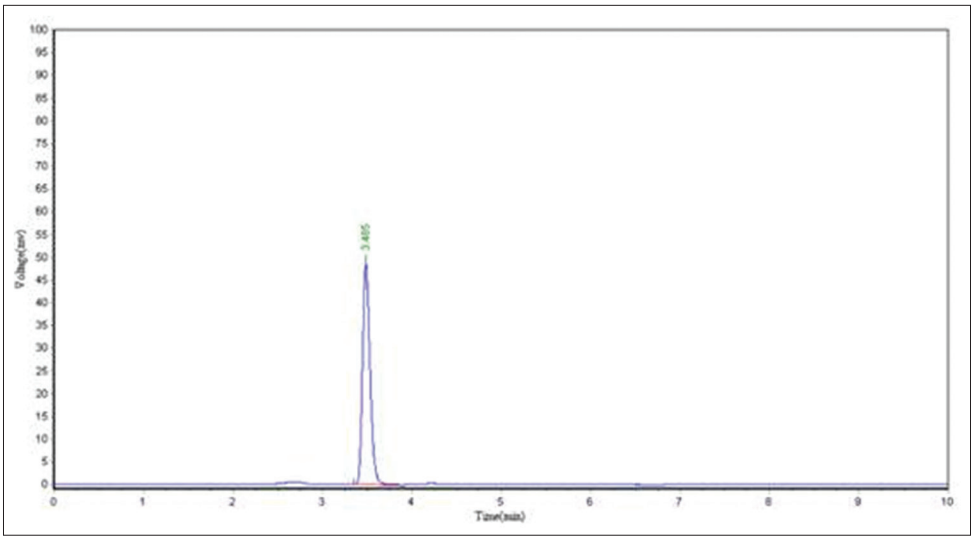


Figure 8: Chromatogram of berberine standard for alkaloid estimation

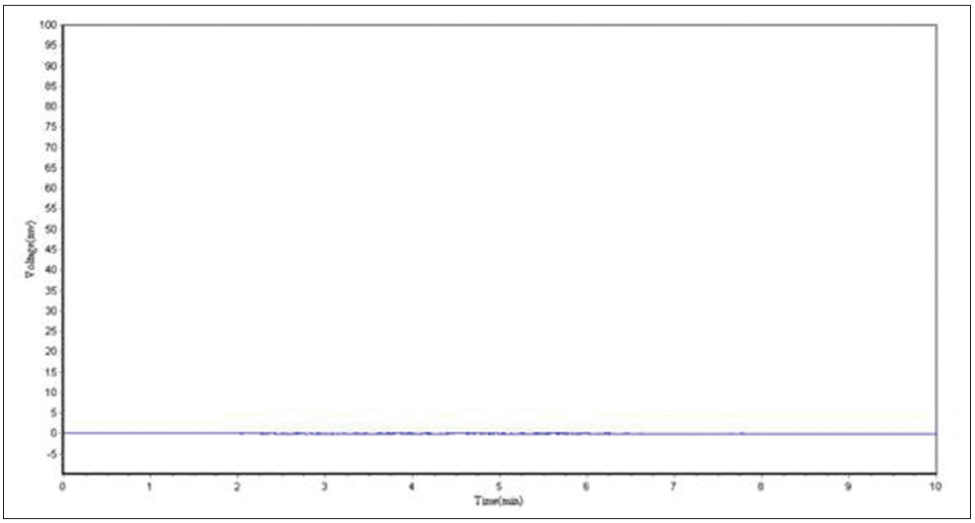


Figure 9: Chromatogram for blank for flavonoids estimation

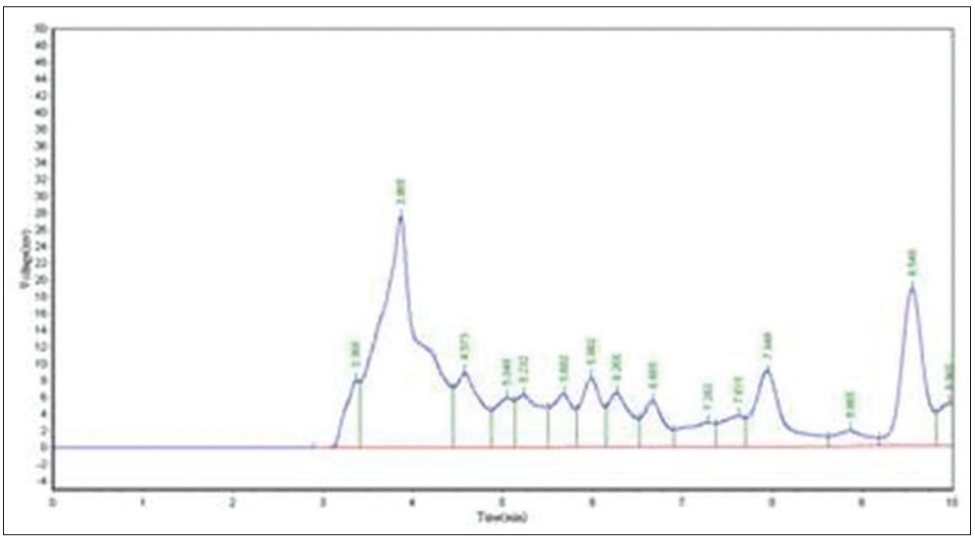


Figure 10: Chromatogram for ethanolic extract of *Pinus maritimus* for flavonoids estimation

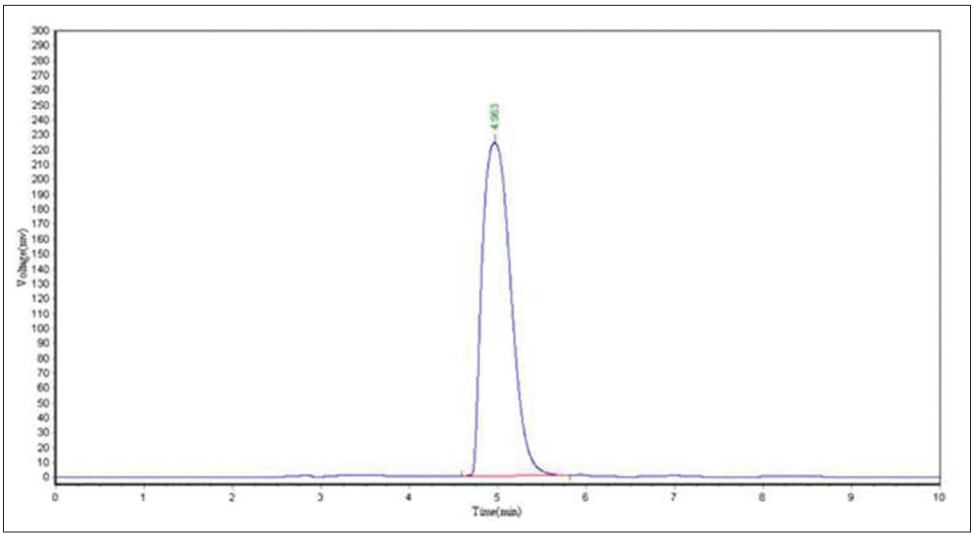


Figure 11: Chromatogram of quercetin standard for flavonoid estimation

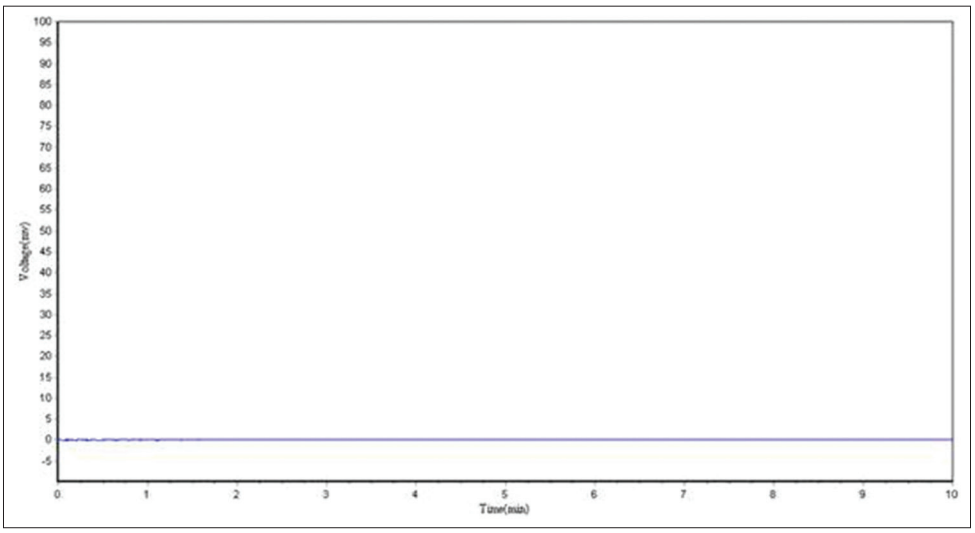


Figure 12: Chromatogram for blank for phenolic estimation

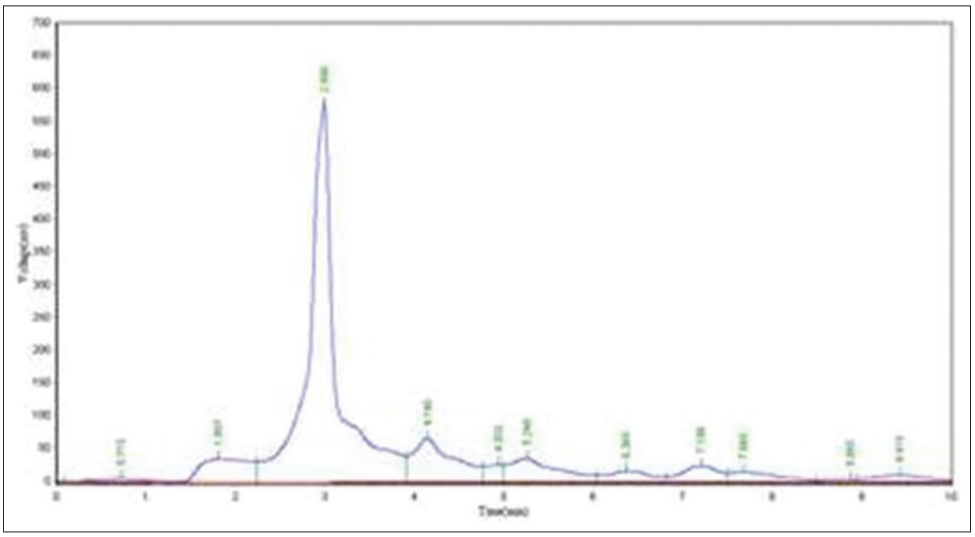


Figure 13: Chromatogram for ethanolic extract of *Pinus maritimus* for phenolic estimation

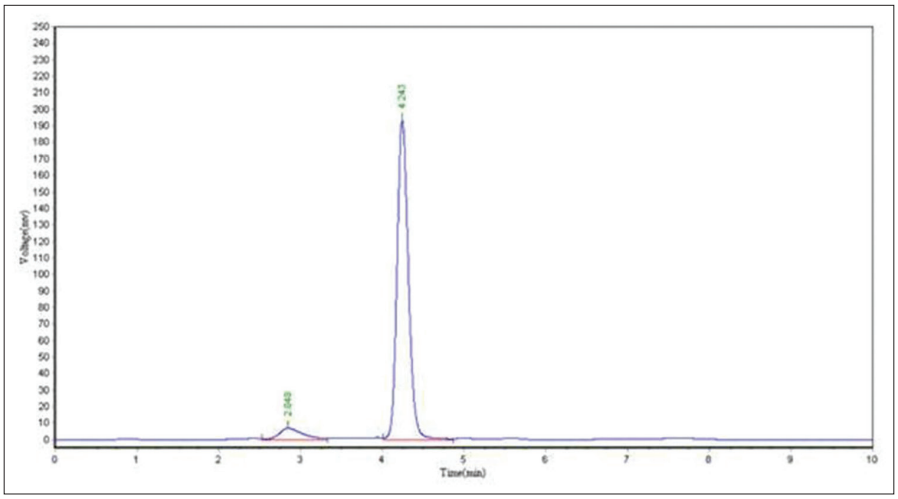


Figure 14: Chromatogram for gallic acid standard for phenolic estimation

The presence of these constituents reveals that the plant is of pharmacologically important.

CONCLUSION

PM is a potent medicinal plant and is rich in secondary phytoconstituents which plays a key role in various pharmacological parameters. Further investigation is being under progress for the isolation and characterization of novel phytoconstituent.

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AUTHORS' CONTRIBUTIONS

All the authors have equal contribution.

CONFLICTS OF INTEREST

There are no conflicts of interest.

SOURCE OF FUNDING

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