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Original Article

Isolation, Characterization and Structural Elucidation of a Bioactive Flavonoid from *Schinus polygama* Leaves Using Chromatographic and Spectroscopic Techniques

Chandra Prakash Bhardwaj[1,*], Piush Sharma[1], Maneesh Napit[2]

[1]Maharishi Arvind College of Pharmacy, Rajasthan University of Health Sciences, Jaipur, Rajasthan, INDIA.
[2]Department of Pharmaceutical Sciences, Apex University, Jaipur, Rajasthan, INDIA.

ABSTRACT

Aim: Isolation, Characterization and Structural Elucidation of a Bioactive Flavonoid from Schinus polygama Leaves Using Chromatographic and Spectroscopic Techniques is the aim of present study. The process of separating and characterizing bioactive substances from natural sources are pivotal in understanding their therapeutic potential. **Materials and Methods:** In the present study, Thin-Layer Chromatography (TLC) with optimized solvent system used. Further this solvent system was employed in column chromatography to isolate active fractions. Active fraction characterized by UV-visible spectroscopy, FT-IR analysis, Nuclear Magnetic Resonance (NMR) and mass spectroscopy. **Results:** *Schinus polygama* (SP) (family Anacardiaceae) extract was subjected to Thin-Layer Chromatography (TLC) using various solvent systems, with the optimized system of Acetic Acid: Ethyl acetate: Toluene (0.4:4:6) yielding a distinct band with 0.64 Rf value, corresponding to a standard flavonoid. This solvent system was employed in column chromatography, leading to the separation of multiple fractions, including the active Fraction (F). UV-visible spectroscopy of Fraction (F) showed absorption peaks at 208, 269 and 365 nm, indicating conjugated π-electron systems. The presence of distinctive functional groups was further verified by FT-IR analysis., including hydroxyl (-OH), alkyl (C-H) and aromatic (C=C) bonds. Nuclear Magnetic Resonance (NMR) spectra revealed specific proton signals consistent with a flavonoid structure, while mass spectrometry identified a molecular ion peak which corresponds to 3,5,7-trihydroxy-2-(4-hydroxyphenyl) chromen-4-one at m/z 286.20 with a molecular formula of $C_{15}H_{10}O_6$. **Conclusion:** These findings suggest that the isolated compound from Schinus polygama leaves ethanolic extract is a flavonoid with potential biological activities. Further studies are recommended to explore its pharmacological properties and potential applications in therapeutic formulations

Keywords: Active fraction, Characterization, Flavonoid, Isolation, Spectroscopy.

Correspondence:
Mr. Chandra Prakash Bhardwaj
Research Scholar, Maharishi Arvind College of Pharmacy, Rajasthan University of Health Sciences, Jaipur, Rajasthan, INDIA.
Email: cpb@rediffmail.com

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INTRODUCTION

Isolation and characterization of bioactive substances derived from natural sources is a fundamental step in drug discovery and development, as these compounds often exhibit therapeutic properties. It is crucial for several reasons. First, natural products have been a rich source of therapeutic agents and isolating individual bioactive compounds allows for the identification of molecules with specific biological activities. This is essential for creating novel medications for a range of illnesses, such as infectious diseases, cancer and cardiovascular conditions. Characterization, on the other hand, is essential for understanding the chemical structure, functional groups and molecular interactions of the isolated compounds. This knowledge provides insight into the mechanisms by which these compounds exert their biological effects, aiding in the design of more potent and selective drugs. Furthermore, proper characterization ensures the reproducibility of bioactive compounds in scientific research and helps avoid issues related to compound purity, which can influence pharmacological outcomes. Researchers can investigate these compounds' potential as lead molecules in drug development, help find new therapeutic agents and support the sustainable use of natural resources in medicine by isolating and fully characterizing them.

In order to obtain a crude extract that is abundant in a variety of chemical elements, process usually starts with extraction of plant or natural material using appropriate solvents. Individual components are then separated according to their physicochemical characteristics using techniques like column

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chromatography, Thin-Layer Chromatography (TLC) or High-Performance Liquid Chromatography (HPLC). Following isolation, by different analytical techniques, including Mass Spectrometry (MS), Nuclear Magnetic Resonance (NMR), Fourier Transform Infrared (FT-IR) spectroscopy and UV-visible spectroscopy are used to characterize the bioactive chemicals. Each technique provides critical information about the molecular structure, functional groups and chemical behaviour of the compound. This thorough characterization is essential to identify compounds with potential medicinal value and to understand their mechanisms of action, paving the way for further biological evaluation and pharmacological studies.

MATERIALS AND METHODS

Preliminary stage: Separation by Thin layer chromatography

One kind of solid-liquid adsorption chromatography is Thin-Layer Chromatography (TLC). It employs particular solvent solution and silica gel 60 F_{254} plates that have been pre-coated having 0.2 mm thick layer as the stationary phase. Through capillary action, the mobile phase ascends through the stationary phase. Spots are manually applied to the TLC plate with a capillary tube and the plates are allowed to air dry before being placed in a TLC chamber at room temperature. The analytes are carried upward by the solvent as it travels up the plate. A pipette with adjustable flow rates is sometimes used to direct sample mixture applied at bottom of plate. The rate of upward movement depends on polarity of analyte, stationary phase and solvent.[1-5]

$$R_f \text{ Value} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

In preliminary TLC analysis of *Schinus polygama* ethanolic extract, the solvent system Acetic Acid: Ethyl Acetate: Toluene (0.4:4:6) produced the most distinct spots with a standard flavonoid. Therefore, this solvent combination (0.4:4:6) was selected for use in column chromatography as mobile phase.

Column chromatography

To isolate flavonoids from ethanolic extract of *Schinus polygama*, silica gel column chromatography was applied. A vertical borosilicate glass column was thoroughly dried, cleaned with acetone and prepared with a wet packing method using silica gel (60-120) as the adsorbent. Toluene was used to create a slurry of silica gel, which was then poured into the column. 1 g of the extract was placed on top of the packed column and a gradient elution method was followed. The column was eluted with toluene, followed by various eluents in the ratio of Acetic Acid: Ethyl Acetate: Toluene (0.4:4:6). The fractions collected were concentrated and TLC was used to confirm the presence of individual compounds in each fraction (Figure 1).[6]

Spectroscopic characterization

UV-visible Spectroscopy

The isolated fraction (F) of *Schinus polygama* ethanolic extract was analyzed using a Shimadzu UV-1700 UV-visible Spectrophotometer, scanning across wavelengths from 200 to 800 nm. The characteristic peaks were observed and recorded.[7-10]

Figure 1: Isolation of components by column chromatography.

FT-IR

FT-IR spectroscopy was performed on the separated fraction (F) of the *Schinus polygama* ethanolic extract to identify its functional groups. The dried sample was ground with KBr to create pellets, then analyzed using a Perkin Spectrum BX spectrophotometer and a Thermo Nicolet 6700 spectrum analyzer. A 400 mg KBr disc containing 2% of the finely powdered sample was prepared and IR spectra recorded within range of 400 to 4,000 cm^{-1}.[11-14]

NMR Spectroscopy

NMR spectroscopy was employed to determine structure of compound present in isolated Fraction (F) of the *Schinus polygama* ethanolic extract. This analysis was conducted using Jeol Resonance Fourier Transform Nuclear Magnetic Resonance spectroscopy.[15-19]

Mass Spectroscopy

Mass spectrometry was used to ionize molecules, allowing them to be sorted and segregated based on the ratio of their mass to charge. The molecular weight of the isolated fraction (F) from the ethanolic extract of *Schinus polygama* leaves was determined using a micrOTOF-Q 228888.10348 mass spectrometer.[20-26]

RESULTS

Initial TLC preparation for the estimate of active ingredients

TLC from an ethanolic extract of *Schinus polygama*.

For Flavonoid

Mobile Phase-Acetic Acid: Ethyl Acetate: Toluene (0.4:4:6).

Thin-Layer Chromatography (TLC) was conducted on the ethanolic extract of *Schinus polygama* using various solvent systems, selected based on a review of relevant literature. TLC

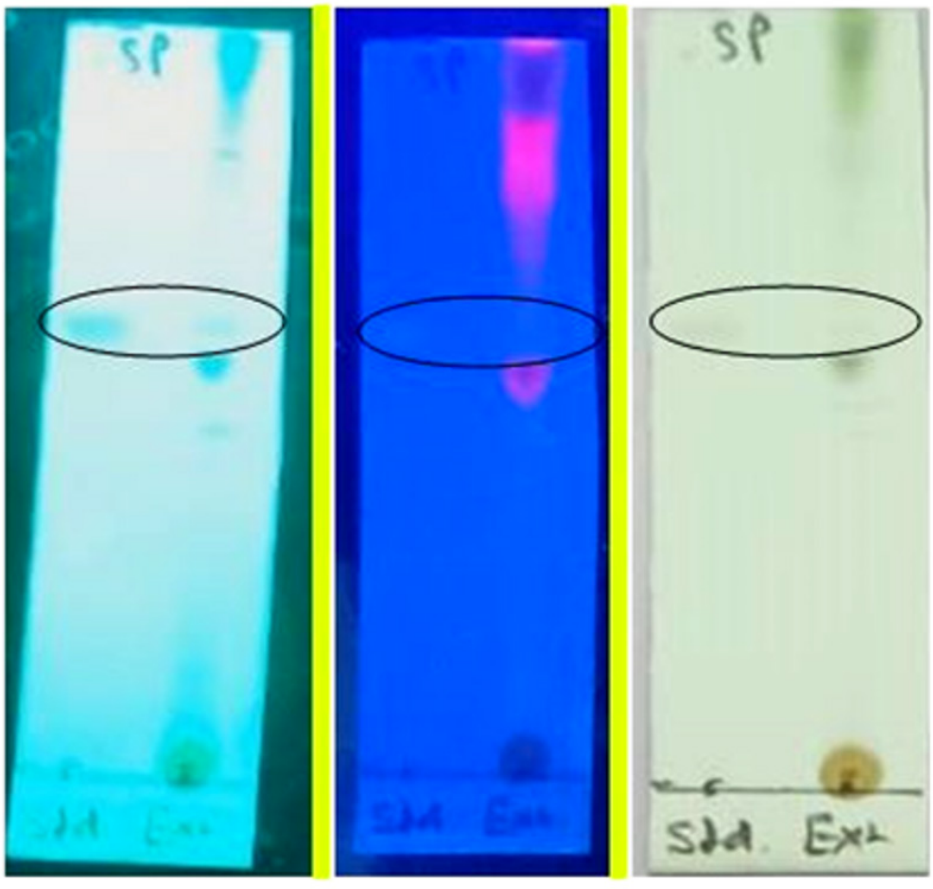


Figure 2: TLC estimation through UV lamp for ESP with Kaempferol (Std. Flavonoid). (Std.=Standard, ESP=Ethanolic extract of *Schinus polygama* leaves)

performed in Acetic Acid: Ethyl Acetate: Toluene (0.4:4:6) that were clearly visible bands of *Schinus polygama* ethanolic extract with Std. Flavonoid. The Rf values of *Schinus polygama* ethanolic extract with Std. Flavonoid was found to be 0.64 and 0.64 (Table 1) (Figure 2).

Column Chromatography

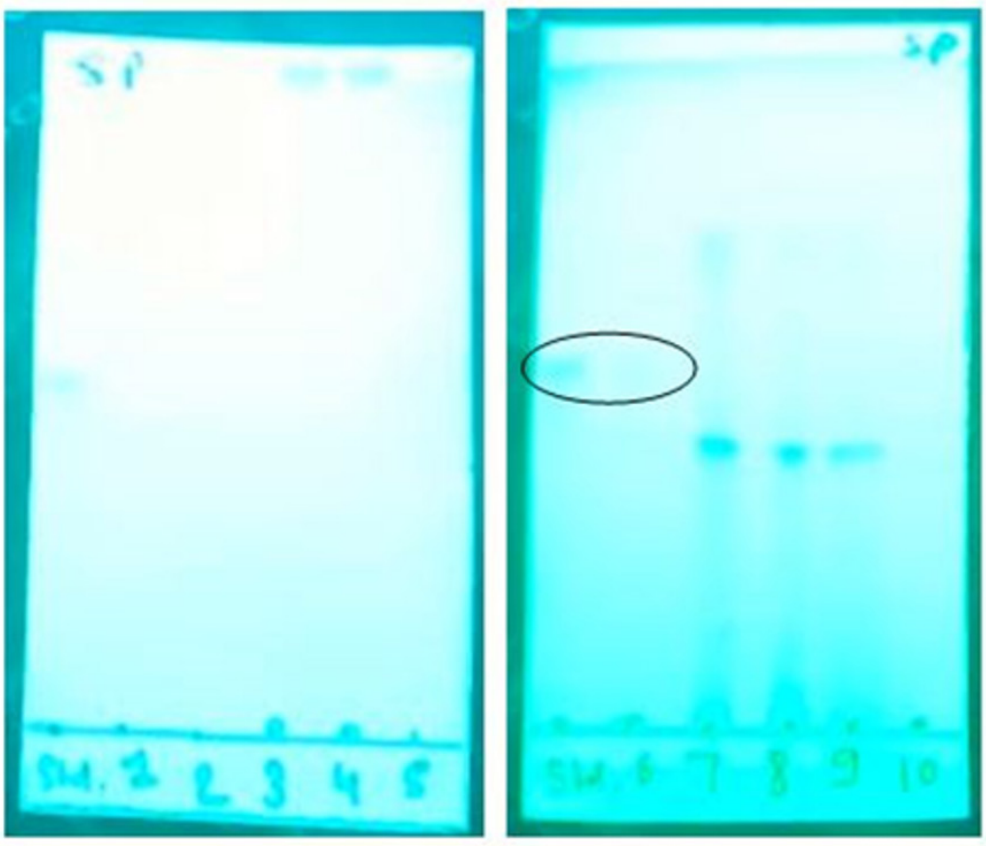
Fractions obtained from silica gel column chromatography of the *Schinus polygama* ethanolic extract were analyzed by TLC to identify different phytocompounds. The collected fractions were then properly prepared and analyzed using UV spectroscopy.

Table 1: TLC of *Schinus polygama* leaves ethanolic extract.

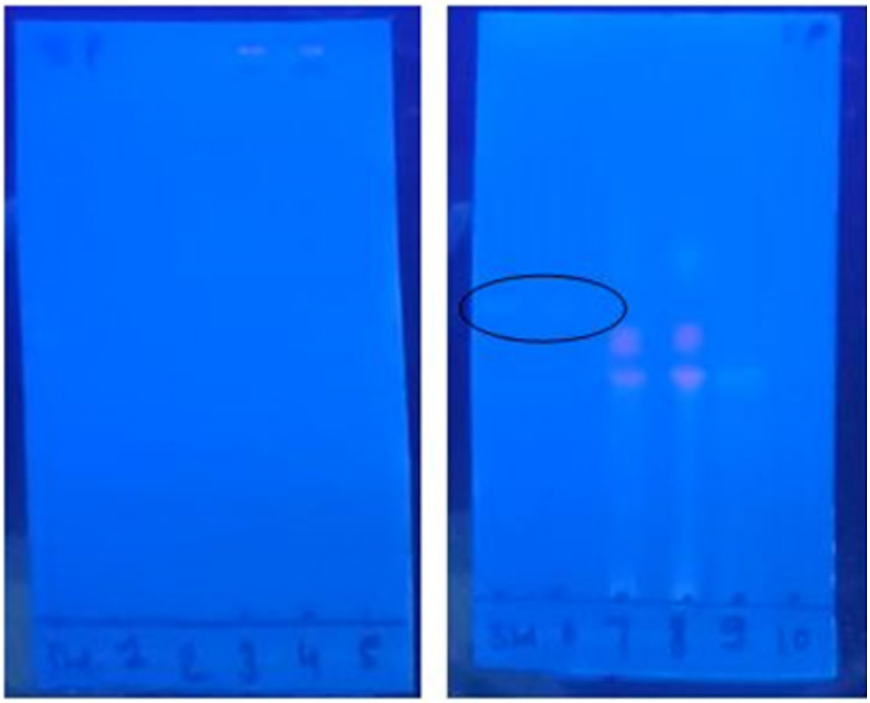
Sl. No.	Solvent system	No. of spots	Colour of spots at Wavelength (365nm)	Colour of spots at Wavelength (254)	R_f value (Extract)	R_f value Kaempferol (Std. Flavonoid)
1.	Acetic Acid: Ethyl Acetate: Toluene (0.4:4:6)	09	Florescence (Std.) Florescence (SP) Florescence Pink Florescence Pink - Dark Purple Pink	Green (Std.) Light Green (SP) Light Green Dark Green Light Green Light Green Green Dark Green Dark Green	- 1.4/5=0.28 2.4/5=0.48 2.9/5=0.58 3.2/5=0.64 3.9/5=0.78 4.0/5=0.80 4.3/5=0.86 4.9/5=0.98	3.2/5= 0.64

TLC of all collected fractions

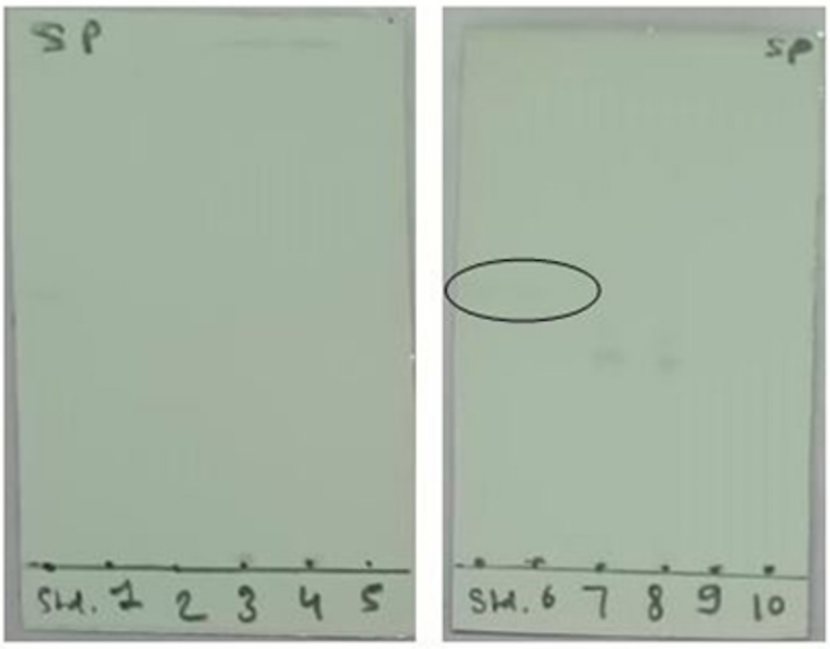
A) TLC of all collected fractions of Schinus polygama ethanolic extract



(a) Short Ultra-violet (254 nm)



(b) Long Ultra-violet (365 nm)



(c) Visible Light

Figure 3: TLC estimation by UV lamp for ESP fractions after column chromatography with Kaempferol (Std. Flavonoid) a) Short-Ultra violet (254 nm), b) Long-Ultra violet (365 nm), c) visible light. (Std.= Standard, ESP = Ethanolic extract of *Schinus polygama*). TLC of all fractions (A, B, C, D, E, F, G, H and I) of *Schinus polygama* ethanolic extract-

Table 2: Collected Fraction from Column Chromatography of *Schinus polygama* ethanolic extract.

Sl. No.	Eluent composition	Fraction collected	Remarks
1	Acetic Acid: Ethyl Acetate: Toluene (0.4:4:6)	01 (A)	White colour mixture of compound
2		02 (B)	Mixture of Light yellowish coloured compound
3		03 (C)	Dark Yellowish coloured mixture of compound
4		04 (D)	Mixture of Yellowish colour compound
5		05 (E)	Mixture of white colour compound
6		06 (F)	Mixture of Light yellowish coloured compound
7		07-08 (G) (G1 & G2)	Dark Greenish coloured mixture of compound
8		09 (H)	Mixture of Light Greenish coloured compound
9		10 (I)	Mixture of White coloured compound

Table 3: R_f values of all obtained fractions of ESP after column chromatography.

Sl. No.	Fraction	Solvent system	No. of spots	Colour of spots at Wavelength (365 nm)	Colour of spots at Wavelength (254 nm)	R_f value (Extract)	R_f value (Std. Flavonoid)
1.	A	Acetic Acid: Ethyl Acetate: Toluene (0.4:4:6)	-	-	-	-	3.2/5=0.64
2.	B		-	-	-	-	
3.	C		03	Dark Purple Fluorescence Pink	Green Green Light Green	4.6/5=0.92 4.8/5=0.96 4.9/5=0.98	
4.	D		03	Dark Purple Fluorescence Pink	Green Green Light Green	4.6/5=0.92 4.8/5=0.96 4.9/5=0.98	
5.	E		01	-	Light Green	4.4/5=0.88	
6.	F		01	Fluorescence	Light Green	3.1/5=0.63	
7.	G1		06	Fluorescence Fluorescence Pink Pink Fluorescence Fluorescence	Green Light Green Dark Green Light Green Light Green Green	1.4/5=0.28 2.4/5=0.48 2.9/5=0.58 3.0/5=0.60 3.6/5=0.72 4.0/5=0.80	
8	G2		06	Fluorescence Fluorescence Pink Pink Fluorescence Fluorescence	Green Light Green Dark Green Light Green Light Green Green	1.4/5=0.28 2.4/5=0.48 2.9/5=0.58 3.0/5=0.60 3.6/5=0.72 4.0/5=0.80	
9	H		02	Fluorescence Fluorescence	Light Green Dark Green	2.9/5=0.58 1.4/5=0.28	
10	I		-	-	-	-	

R_f value Following the completion of the TLC estimation, the active ingredient in fraction (F) of the *Schinus polygama* ethanolic extract was confirmed comparing the standard flavonoid and using mobile phase Acetic Acid: Ethyl Acetate: Toluene (0.4:4:6).

Spectroscopic characterizations

Active constitutes estimation by UV-spectroscopy

Schinus polygama ethanolic extract's separated fraction (F) has its UV spectrum captured using a Shimadzu 1700 twin beam-UV-vis spectrophotometer. The isolated fraction's UV spectra were recorded in a solution containing Acetic Acid: Ethyl Acetate: Toluene (0.4:4:6) spanning a 200-800 nm scanning range. The isolated compound's λ_{max} was then calculated. Acetic Acid: Ethyl Acetate: Toluene (0.4:4:6) served as the blank. Three peaks at 208, 269 and 365 nm were identified as the wavelength of the isolated Fraction (F) of the ethanolic extract of *Schinus polygama*.

Active constitutes estimation by FTIR-Spectroscopy

(A) Infrared spectra of isolated fraction (F) of Schinus polygama ethanolic extract

The IR spectrum of the isolated (F) fraction from the *Schinus polygama* ethanolic extract revealed several key functional groups. A strong, broad peak for the -OH group appeared at 3449.65 cm^{-1}, while C-H stretching peaks for alkane were observed at 2981.10 and 2901.53 cm^{-1}. The C=C stretching of conjugated alkene was identified at 1640.20 cm^{-1}. Other notable peaks included the O-H bending at 1417.17 cm^{-1}, C-O stretching for alkyl aryl ether at 1259.99 and 1230.32 cm^{-1}, C-O stretching for vinyl ether at 1206.76 cm^{-1} and C-O stretching for alcohol at 1077.18 and 1045.84 cm^{-1}. Additionally, C=C bending appeared at 974.85 cm^{-1} and C-H bending at 878.83 cm^{-1}.

^{1}H NMR-Spectroscopy

^{1}H NMR spectrum of the isolated (F) fraction from *Schinus polygama* ethanolic extract was obtained using an NMR spectrophotometer, with Tetramethylsilane (TMS) as internal standard. The signals were identified and labelled as Singlet (s), Doublet (d), Triplet (t) and Multiplet (m) accordingly.

(A) ^{1}H NMR spectra of isolated Fraction (F) of ESP

The ^{1}H-NMR spectrum of isolated fraction (F) of ESP revealed several proton signals. The H-1 proton appeared at 3.30 (d) ppm, 4.83 (d) ppm, 5.02 (d) ppm, 6.14 (d) ppm, 6.16 (d) ppm and 6.39 (d) ppm. Additionally, the H-2 protons were observed at 6.87 (ddd) ppm and 8.00 (ddd) ppm.

Mass-Spectroscopy

A mass spectrum of isolated Fraction (F) of ESP was recorded on Bruker micrOTOF-Q mass spectrometer.

Mass spectra of isolated Fraction (F) of ESP showed molecular ion [M^+] peaks at mlz 286.20. The carbon (15), Hydrogen (10) and Oxygen (6) present in isolated Fraction (F) of ESP which corresponds to the molecular formula $C_{15}H_{10}O_6$ of 3,5,7-trihydroxy-2-(4 hydroxyphenyl) chromen-4-one according to their fragments (69, 133, 147, 165, 213, 241, 257, 286 and 487 m/z).

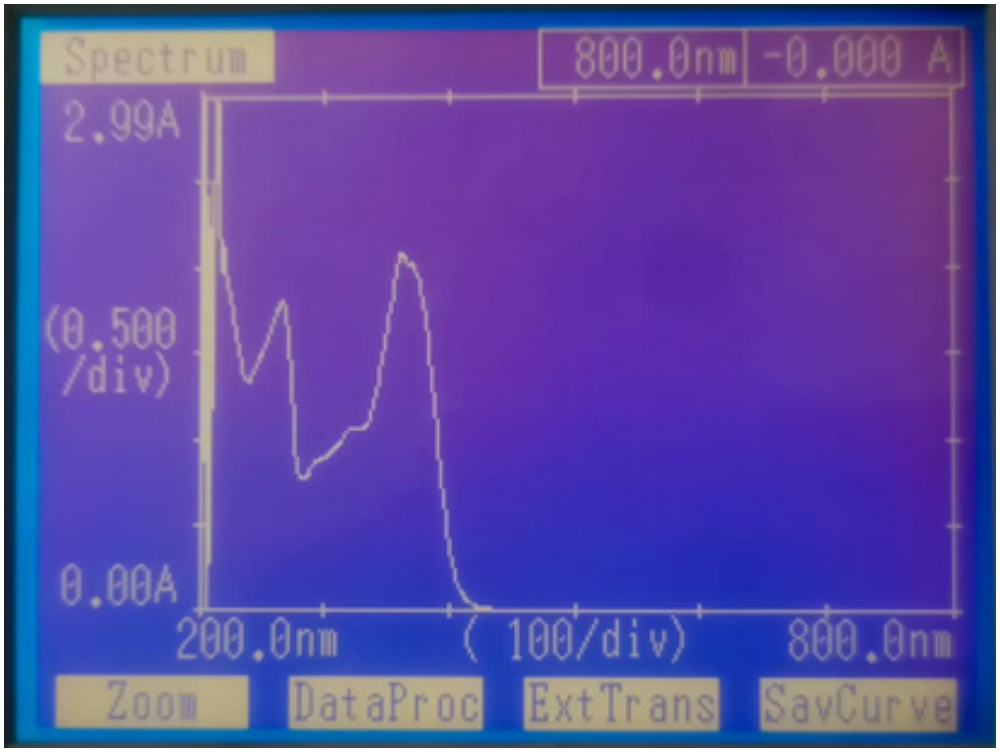


Figure 4: The estimation of active constituents in the (F) fraction of *Schinus polygama* ethanolic extract was performed using UV spectroscopy after column chromatography.

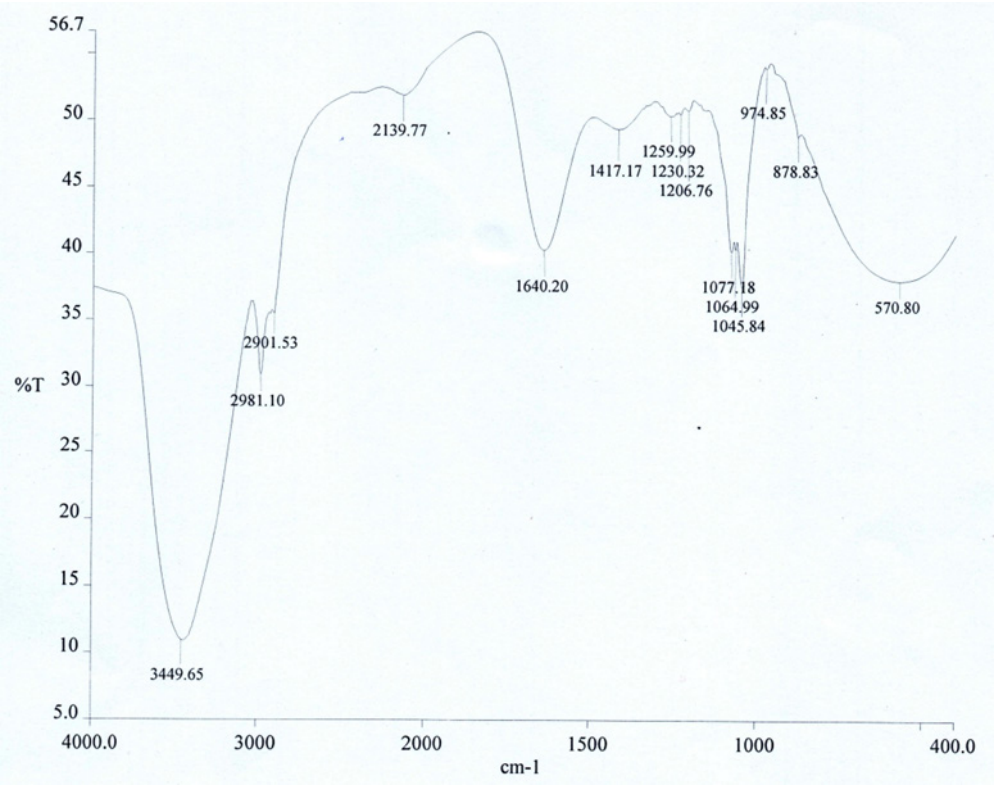


Figure 5: Infrared spectra of isolated fraction (F) of *Schinus polygama* ethanolic extract.

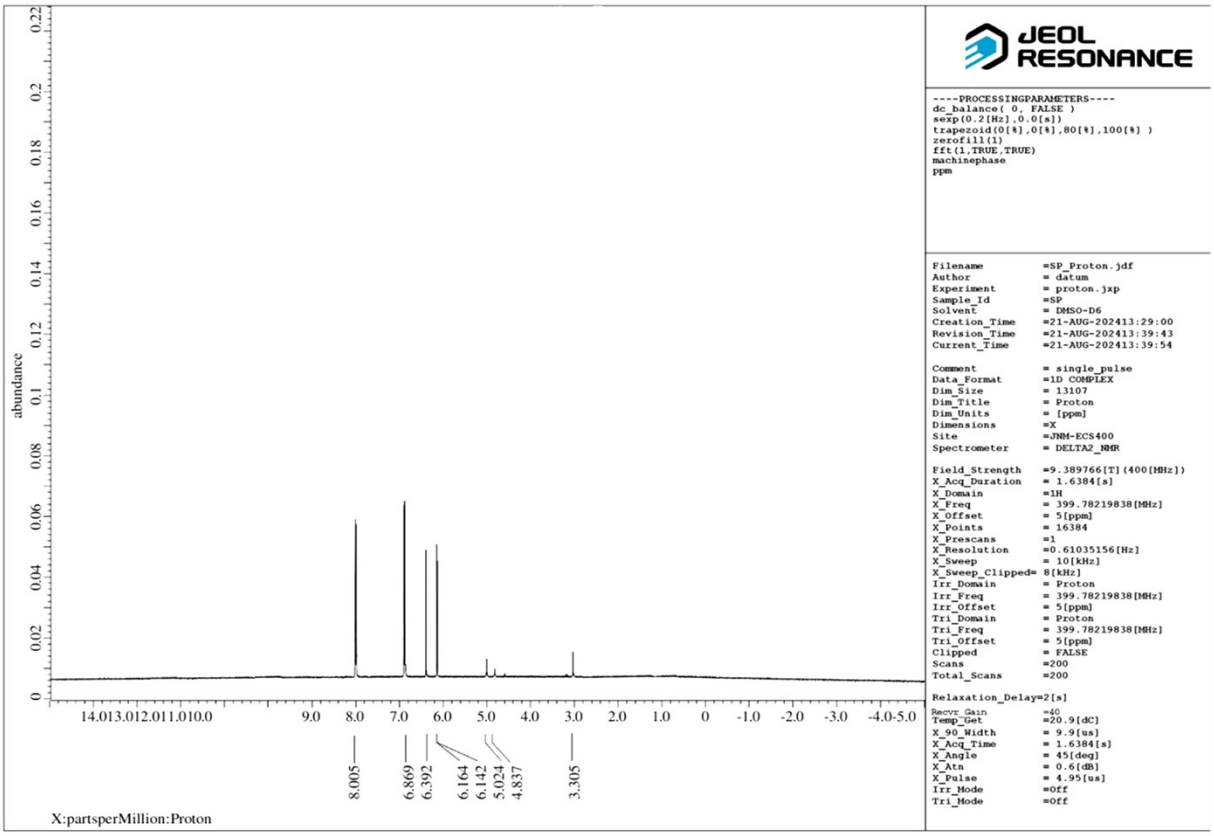


Figure 6: ^{1}H-NMR spectra of the isolated Fraction (F) of SP.

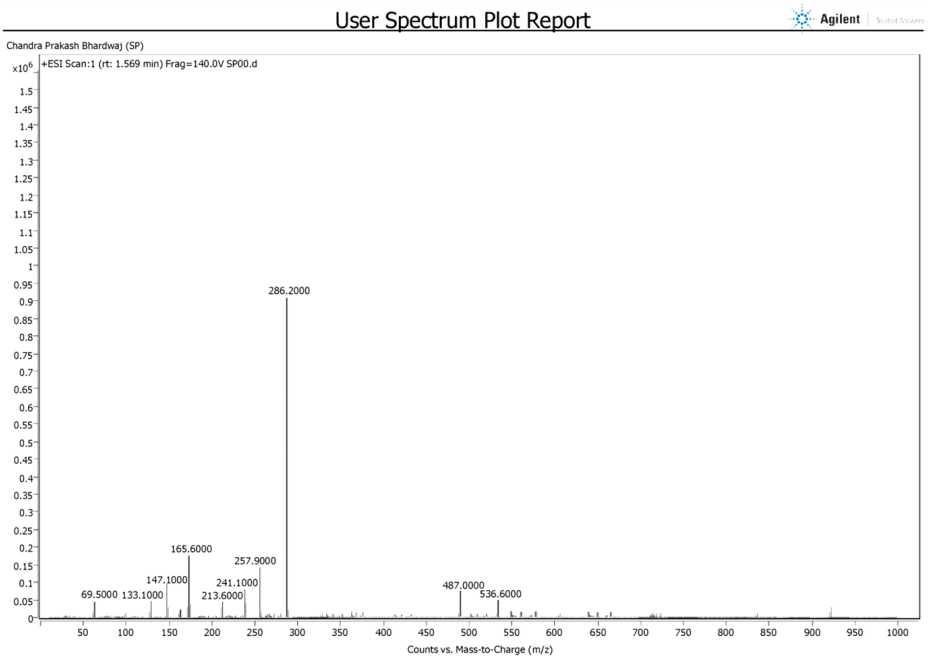
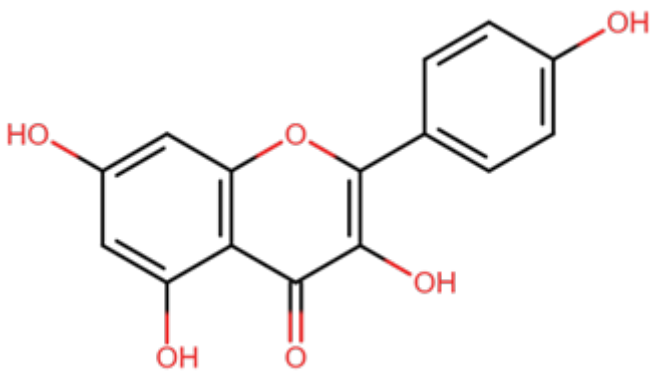


Figure 7: Mass spectrum of the isolated Fraction (F) of SP.



3,5,7-trihydroxy-2-(4 hydroxyphenyl) chromen-4-one.

DISCUSSION

Different solvent systems were used in the preliminary TLC of *Schinus polygama* ethanolic extract (the solvent system was chosen based on a review of the literature). Acetic Acid: Ethyl Acetate: Toluene (0.4:4:6) TLC was performed using standard flavonoid, which showed up as distinct bands in SP. The ESP and Std. Flavonoid R*f* values were determined to be 0.64 and 0.64, respectively. Thus, mobile phase for column chromatography was Acetic Acid: Ethyl Acetate: Toluene (0.4:4:6). Using mobile phase of Acetic Acid: Ethyl Acetate: Toluene (0.4:4:6) for ESP, active constituents are separated from column chromatography to get Fractions 01 (A), 02 (B), 03 (C), 04 (D), 05 (E), 06 (F), 07-08 (G1 & G2), 09 (H) and 10 (I) (Table 2).

R_f value Resulting from the TLC estimation process, the active components in fractions (F) of ESP with mobile phase are also confirmed. In comparison to the standard flavonoid (Figure 3, Table 3), Acetic Acid: Ethyl Acetate: Toluene (0.4:4:6). The fractions were appropriately sampled and analysed using the UV spectrum. After recording the isolated fraction (F) of ESP's UV spectra over a 200-800 nm scanning range, the compound's λ_{max} was calculated and the wavelength of the (F) fraction of ESP was discovered to have three peaks at 208, 269 and 365 nm (Figure 4).

The -OH group was strong, a broad peak occurred at 3449.65 cm^{-1}, alkane C-H stretching peaks appeared at 2981.10 and 2901.53 cm^{-1} and conjugated alkene C=C stretching peaks appeared at 1640.20 cm^{-1}, according to the infrared spectra of the isolated fraction (F) of *Schinus polygama* ethanolic extract. At 1417.17 cm^{-1}, the O-H bending peak 1259.99 and 1230.32 cm^{-1} for the C-O stretching peak of alkyl aryl ether, 1206.76 cm^{-1} for vinyl ether, 1077.18 and 1045.84 cm^{-1} for alcohol, 974.85 cm^{-1} for C=C bending and 878.83 cm^{-1} for C-H bending Table 4 and Figure 5.

Table 4: FTIR Spectrum Frequency Range of isolated fraction (F) of *Schinus polygama* ethanolic extract.

Sl. No.	Fraction	Frequency Range	Group Absorption (cm^{-1})	Appearance	Group	Compounds Pass
6	F	3550-3200 (cm^{-1})	3449.65	Strong, Broad	O-H stretching	Hydroxyl Group
		3000-2840 (cm^{-1})	2981.10	Medium	C-H stretching	Alkane
		3000-2840 (cm^{-1})	2901.53	Medium	C-H stretching	Alkane
		1650-1600 (cm^{-1})	1640.20	Medium	C=C stretching	Conjugated alkene
		1420-1330 (cm^{-1})	1417.17	Medium	O-H bending	Alcohol
		1275-1200 (cm^{-1})	1259.99	Strong	C-O stretching	Alkyl aryl ether
		1275-1200 (cm^{-1})	1230.32	Strong	C-O stretching	Alkyl aryl ether
		1225-1200 (cm^{-1})	1206.76	Strong	C-O stretching	Vinyl ether
		1085-1040 (cm^{-1})	1077.18	Strong	C-O stretching	Alcohol
		1085-1040 (cm^{-1})	1045.84	Strong	C-O stretching	Alcohol
		980-960 (cm^{-1})	974.85	Strong	C=C bending	Substitute
		900-700 (cm^{-1})	878.83	Strong	C-H bending	Substitute

The isolated fraction (F) of ESP in the ^{1}H-NMR spectra revealed that the ^{1}H-1 proton was present at 3.30 (d) ppm, 4.83 (d) ppm, 5.02 (d) ppm, 6.14 (d) ppm, 6.16 (d) ppm, 6.39 (d) ppm, H-2 protons were present at 6.87 (ddd) ppm and H-2 protons were present at 8.00 (ddd) ppm (Figure 6).

Molecular ion [M+] peaks were seen at m/z 286.20 in the mass spectra of the isolated fraction (F) of ESP. According to its fragments (69, 133, 147, 165, 213, 241, 257, 286 and 487 m/z), the carbon (15), hydrogen (10) and oxygen (6) found in the isolated fraction (F) of ESP correspond to chemical formula $C_{15}H_{10}O_6$ of 3,5,7-trihydroxy-2-(4 hydroxyphenyl) chromen-4-one (Figure 7).

CONCLUSION

Present study focused on the isolation and characterization of a bioactive flavonoid compound from *Schinus polygama* (SP), a plant belonging to the family Anacardiaceae. Initially, TLC was employed to analyze the *Schinus polygama* ethanolic extract using various solvent systems. The optimal system of Acetic Acid: Ethyl Acetate: Toluene (0.4:4:6) yielded a distinct R_f value of 0.64 for ESP and a standard flavonoid, which provided clear bands for the target compound. This solvent system was subsequently used for column chromatography, successfully isolating multiple fractions, including the bioactive Fraction (F).

Spectroscopic techniques played a key role in characterizing the isolated compound. UV-visible spectroscopy of Fraction (F) revealed three absorption peaks at 208, 269 and 365 nm, suggesting the presence of conjugated π-electron systems typical of flavonoids. FT-IR (Fourier-transform infrared) spectroscopy further confirmed existence of characteristic functional groups such as Hydroxyl (-OH), alkyl (C-H) and Aromatic (C=C) groups. Nuclear Magnetic Resonance (NMR) spectra provided detailed proton signals that were consistent with the structure of a flavonoid, while mass spectrometry confirmed molecular ion peak at m/z 286.20, corresponding to a compound with molecular formula $C_{15}H_{10}O_6$. This was identified as 3,5,7-trihydroxy-2-(4-hydroxyphenyl) chromen-4-one, flavonoid known for its potential bioactive properties.

The comprehensive analysis conducted in present study provided valuable insights into chemical composition of *Schinus polygama*. The combination of TLC, column chromatography and various

spectroscopic methods (MS, UV-vis, NMR & FT-IR) enabled the precise isolation and structural elucidation of a key flavonoid compound.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

FTIR: Fourier: transform infrared spectroscopy, **UV:** Ultra violet, **MS:** Mass spectroscopy, **NMR:** Nuclear magnetic resonance, **TLC:** Thin layer chromatography, **SP:** *Schinus polygama*, **ESP:** Ethanolic extract of *Schinus polygama* leaves.

SUMMARY

A bioactive flavonoid compound was isolated and characterized from *Schinus polygama* using various chromatographic and spectroscopic techniques. The optimal solvent system, Acetic Acid: Ethyl Acetate: Toluene (0.4:4:6), was used for TLC and column chromatography, yielding multiple fractions, including the bioactive fraction (F). Spectroscopic analysis, including UV-vis, FT-IR, NMR and MS, revealed the isolated compound to be 3,5,7-trihydroxy-2-(4-hydroxyphenyl) chromen-4-one, a flavonoid with molecular formula $C_{15}H_{10}O_6$, which has potential bioactive properties. The comprehensive analysis provided valuable insights into the chemical composition of *Schinus polygama*, enabling the precise isolation and structural elucidation of the key flavonoid compound.

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