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ANALYTICAL INVESTIGATION OF PHYTOCHEMICAL MARKERS IN *PLUCHEA WALLICHIANA* DC THROUGH SPECTROSCOPIC AND CHROMATOGRAPHIC TECHNIQUES

BERA K[1*], CHAUHAN U[2] AND BHAVSAR P

1: Department of Pharmacognosy, Parul Institute of Pharmacy, Parul University, Limda, Vadodara, Gujarat, 391760, India, ORCID ID: 0000-0001-7841-5167

2: Department of Quality Assurance, Parul Institute of Pharmacy, Parul University, Limda, Vadodara, Gujarat, 391760, India

3: Department of Quality Assurance, Parul Institute of Pharmacy, Parul University, Limda, Vadodara, Gujarat, 391760, India, ORCID ID: 0000-0002-9798-782X

***Corresponding Author: Mrs. Madhu M: E Mail: kinjal_savani@yahoo.com**

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ABSTRACT

Background & Objective: *Pluchea wallichiana* DC is commonly used in the management of inflammatory ailments. The primary objective of the study was to identify and quantify its phytoconstituents using spectroscopy and chromatography methods. **Methods:** Preparative thin layer chromatography was utilized to separate gallic acid and lupeol, which were subsequently characterized using infrared, mass, and nuclear magnetic resonance spectroscopy techniques. Additionally, a high-performance thin layer chromatography method was developed and validated for the quantification of gallic acid, β-sitosterol, and lupeol utilizing the Toluene: Ethyl acetate: Formic acid (8:3:0.5 v/v/v) mobile phase. **Result:** Lupeol (Rf 0.75) and β-sitosterol (Rf 0.63) were successfully separated using thin layer chromatography, followed by scanning at 521 nm after derivatization with anisaldehyde-sulphuric acid reagent. Gallic acid (Rf 0.19) was also separated using the same mobile phase, with subsequent scanning at 254 nm. The selected method was found linear at range 200–600 ng/spot with the correlation coefficients 0.998, 0.998, and 0.999, for lupeol,

β-sitosterol, and gallic acid, respectively. Furthermore, the recovery percentages for the phytomarkers were determined to be 99.31% for lupeol, 99.00% for β-sitosterol, and 98.61% for gallic acid. **Conclusion:** The isolation and characterization lupeol and gallic acid were the first ever to be reported from this plant. It is concluded that gallic acid was absent in the leaf, while lupeol, and β-sitosterol are present in root, stem, and leaf parts.

Keywords: β-sitosterol, Gallic acid, High-performance thin layer chromatography, *Pluchea wallichiana*, Lupeol

INTRODUCTION

The asteraceae family involves the *Pluchea* genus, found abundantly across Pakistan, India, Afghanistan, and Iran. *Pluchea* species are characterized by the presence of sesquiterpenes, chlorogenic acid, lactones, orange-yellow pigments, and sitosterol glycosides, known for their cytotoxic, antitumor, and mutagenic properties. Numerous species within this genus exhibit noteworthy antifungal, antibacterial, and anti-inflammatory properties against various microorganisms. *Pluchea wallichiana*, a medicinal plant belonging to this genus, has a history of traditional use in treating bacterial ailments as well as addressing peptic ulcers, burns, and abdominal pain **[1-4]**. The reported chemical constituents of *Pluchea wallichiana* are β-amyrin, β-sitosterol, apigenin, syringaresinol, and pulvitilol **[1]**.

The chromatographic technique facilitates the swift identification of essential chemical constituents present in herbal plants, crucial for their therapeutic properties. Preparative thin layer chromatography (prep TLC) is employed to separate larger quantities of substances compared to analytical TLC, where smaller amounts are typically handled. In prep TLC, the material to be separated is applied as long streaks rather than spots to accommodate larger volumes. After development, specific components are isolated by carefully removing the sorbent layer from the targeted area on the plate and extracting the separated substances with a potent solvent **[5]**. HPTLC presents an effective, quick, and reliable alternative for the quantitative assessment of natural products. One key advantage of HPTLC is its capability for the simultaneous analysis of multiple phytochemical markers. HPTLC proves to be a potent and valuable method for generating a fingerprint profile of plant extracts, enabling a comprehensive analysis of their phytocomposition **[6-12]**.

Through a comprehensive literature review, it has been observed that there is limited information available on the *Pluchea wallichiana* plant. This article aims to fill this knowledge gap by presenting a detailed

analysis of both qualitative and quantitative phytochemicals present in the plant.

MATERIAL AND METHODS

Plant material

The *Pluchea wallichiana* was procured from the Parul University medicinal garden. Herbarium was submitted to the Department of Botany, Maharaja Sayajirao University of Vadodara (Gujarat), India, for authentication.

Phytomarkers, Chemicals and Solvents

Lupeol, β-sitosterol, and gallic acid were procured from Yucca Enterprises in Mumbai. All solvents (Toluene, ethyl acetate, formic acid, and methanol) utilized in the study were of chromatography grade, and all chemicals employed met the standards of analytical reagent grade.

Instrument

A CAMAG HPTLC system, featuring an applicator (LINOMAT 5) with a syringe (100 µl), scanner, and software (WinCATS), was employed for the analysis.

Methanol extract preparation

The dried powdered root, stem, and leaf of *Pluchea wallichiana* material was separately extracted with methanol using a soxhlet apparatus. The methanol extract was concentrated by evaporation on a water bath at 40°C and stored in airtight containers.

Separation of phytomarkers through Preparative Thin-Layer Chromatography

Methanolic extracts from the leaf, stem, and root were applied onto the glass plates (10 cm x 20 cm) coated with 0.25 mm thick silica gel 60, and then placed in a development chamber saturated with a mobile phase [Toluene, ethyl acetate, and formic acid (8:3:0.5 v/v/v)]. After confirming the Rf values of the phytoconstituents and standards, the silica gel region containing the desired phytoconstituents was carefully scratched off. The collected silica material was dissolved in methanol, filtered, and the filtrate was evaporated to yield crystals of the phytoconstituents.

Spectral analysis and structure elucidation

The unexplored phytoconstituents from purified scratched samples were identified using mass spectroscopy, nuclear magnetic resonance (NMR), and infrared spectroscopy (IR).

CHROMATOGRAPHIC EVALUATION

Preparation of standard solutions

1mg/ml solution for each marker (Lupeol, β-sitosterol, and gallic acid) were prepared, which were used as stock solutions. Subsequently, 1 ml from each stock solution was diluted with methanol to get a final volume of 10 ml, yielding 100 µg/ml solutions.

Preparation of test sample solution

Accurately weighed individually 20 mg of methanol extracts (root, stem, leaf) of *Pluchea wallichiana* dissolved in methanol (5 ml).

Instrumentation and chromatographic conditions

The TLC aluminum precoated plate (Silica gel $60F_{254}$, 20×10 cm) was procured from E-Merck, Mumbai, India, and used for the separation of selected markers. A TLC sample applicator (CAMAG Linomat 5) with a syringe (100 µl) was used for sample application. 2 µl of each standard (Lupeol, β-sitosterol, and Gallic acid) and 13 µl of the extract (root, stem, and leaf) were spotted on the plate as 80 mm band. Plates developed after application in a glass twin-trough chamber that was already pre-saturated for 20 minutes at room temperature. Toluene, ethyl acetate, and formic acid (8:3:0.5 v/v/v) were used as the mobile phase. After 80 mm chromatographic run, the plates were scanned using a Camag TLC scanner at 254 nm to determine gallic acid. Anisaldehyde sulfuric acid reagent used for derivatization to detect lupeol, and β-sitosterol. After being sprayed with the reagent, the plate was heated for 5 minutes at 100°C. The derivatized plate was scanned at 521 nm to determine lupeol and β-sitosterol.

HPTLC method validation

The HPTLC procedures were validated using the guidelines established by the International Conference on Harmonization **[13]**.

Linearity

A standard linearity with a predetermined range (200–600 ng/spot) was established for each. On the TLC plate, varying quantities of the standard solution were spotted in fivefold (2 to 6 µl). Peak areas were plotted against the corresponding amount per spot to create the calibration curve.

Precision

The system's repeatability was assessed by spotting the standard solution (400 ng/spot) seven times under identical chromatographic conditions, calculating the mean retention time. Intraday precision was evaluated by analyzing three different concentration levels three times within the same day, while interday precision was determined by repeating the analysis on three different days. Standard solutions containing concentrations of 300, 400, and 500 ng/spot were used, and the % RSD was calculated based on peak area measurements from three replicates of each concentration.

Accuracy (% recovery)

The usual addition method was utilized to calculate the recovery percentage of lupeol, β-sitosterol, and gallic acid in order to assess the accuracy of the procedure. A predetermined

volume of reference solutions (gallic acid, β-sitosterol, and lupeol) was introduced at 50, 100, and 150% of the phytomarker concentration detected in the extract samples and further calculated.

Robustness

The suggested method's robustness was tested by making minor modifications to the process, such as altering the migration distance and wavelength. The robustness of the approach was determined as %RSD, which gave an indication of its ability to withstand changes.

Limit of Detection and Quantification

The Limit of Detection (LOD) was calculated using the formula LOD = 3.3σ/S. Similarly, the Limit of Quantification (LOQ), was determined by LOQ = 10σ/S, where σ is the standard deviation and S is the slope.

RESULT

Authentication of plant

The plant *Pluchea wallichiana* was authenticated by Dr. Padamnabhi S. Nagar, associate professor of the department of botany, faculty of science, Maharaja Sayajirao University of Vadodara (Gujarat), India.

Preliminary identification using thin layer chromatography (TLC)

The selected mobile phase [Toluene: Ethyl acetate: Formic acid (8:3:0.5)] used to separate lupeol, β-sitosterol, and gallic acid. The resolution was mention in **Figure 1**.

Spectroscopic identification of isolated phytocompound

FTIR Spectroscopy

The identification of functional groups in the active compounds within the extract was accomplished through the utilization of FTIR spectrum analysis. As the phytocompound underwent FTIR analysis, the distinct peaks in the infrared radiation region facilitated the separation of functional groups in the individual components. The peaks' values served as key indicators, allowing for the precise characterization of the various functional groups present in the phytocompound **[14]**. **Figure 2** revealed the IR spectra of phytocompound-1. The IR spectra of phytocompound-1 indicated the presence of different functional groups like C-C, C-CH_3, and CH-OH at 1094 cm^{-1}, 1375 cm^{-1}, and 3276 cm^{-1}.The C-H vibrations of the unsaturated segment were detected at 878 cm^{-1}. Simultaneously, the C=C vibration, found at approximately 1639 cm^{-1}, exhibited a relatively weak intensity. The stretching and bending vibrations associated with the methyl group were identified by prominent bands at 2934 cm^{-1} and 1455 cm^{-1}, respectively **[15]**. The FTIR spectrum of phytocompound-2, represented in **Figure 3**, reveals distinctive features. The pronounced broad band peak at 3492 cm^{-1} signifies the stretching vibration of

hydroxyl groups. Additionally, a strong and narrow peak at 1702 $cm^{-1}$is attributed to stretching vibration of carbonyl group. The presence of a peak at 2953 cm-1 confirms the existence of C-H bonds. Notably, the bands at 1452 and 1420 cm^{-1} are indicative of typical stretching vibrations of C-C bonds within the aromatic ring **[16, 17]**. FTIR evaluation indicated the presence of functional groups of lupeol in phytocompound-1 and gallic acid in phytocompound-2.

Mass spectroscopy of isolated phytocompounds

Both isolated phytocompounds underwent mass spectroscopy to determine their molecular masses. The mass spectra for phytocompound-1 and phytocompound-2 are presented in **Figure 4 and Figure 5**, respectively. Phytocompound-1 exhibited a molecular ion at m/z 427.3 $[M-H]^+$, with a formula identified as $C_{30}H_{50}O$ in the positive ion mode, indicating the presence of lupeol **[18]**. In the case of phytocompound-2, the molecular ion was detected at m/z 169.12 $[M-H]^-$, and its formula was determined as $C_7H_6O_5$, identifying it as gallic acid in the negative ion mode. Fragment ions at m/z 169.0166 were observed, corresponding to deprotonated ions of gallic acid **[19]**.

Nuclear magnetic resonance spectroscopy (NMR)

Nuclear Magnetic Resonance (NMR) analysis was conducted to elucidate the structure and identify unknown phytocompounds. The proton spectrum also showed signals at δ 4.69ppm, 4.58 (H-29, d, d, 2H), 2.414 (H-19, m, 1H),1.421 (H-18, t, H), 1.188 (H-15, d. 1H), 3.183 (H-3, t, 1H), 0.921 (H-23, s. 3H), 0.774 (H-5, t, 1H), and 1.695 (H-30, s, 3H). Additionally, the proton NMR spectrum exhibited seven singlet methyl protons at δ 0.707, 0.774, 0.89, 0.981, 0.957, 1.042, and 1.669 ppm. The proton of H-3 manifested as a triplet at δ 3.224 ppm. Furthermore, two olefinic protons at 4.7 and 4.581 were observed, indicative of the exocyclic double bond in the compound **[20]**. **Figure 6** displays the 1H NMR spectrum of phytocompound-1 with the molecular formula $C_{30}H_{50}O$. In the spectrum of phytocompound-2, peaks emerge at δ values of 12.24, 9.19, 8.84, 6.91, 3.37, 2.25, and 0.002 ppm **(Figure 7)**. It also exhibits an identifiable acid group signal at 12.15 ppm and characteristic features evident hydroxyl proton signal at 8.84 and 9.19 ppm **[21, 22]**. The hydroxyl and carboxylic protons have broad signals in the upper spectra, which are indicative of the interaction with other -OH groups that are possibly water remnants that are found between 9 and 11 ppm **[23]**. The assessment confirmed the presence of lupeol

as phytocompound-1 and gallic acid as phytocompound-2."

Chromatographic Evaluation

Quantification of phytomarkers (lupeol, β-sitosterol, and gallic acid) in *Pluchea wallichiana* leaves, roots, and stems was conducted using HPTLC. The solvent system comprising toluene, ethyl acetate, and formic acid (8:3:0.5) demonstrated higher resolution. The Rf values and quantities for each phytomarker are detailed in **Table 1**. In **Figure 8**, gallic acid was revealed at 254 nm, while lupeol and β-sitosterol were visualized using anisaldehyde sulfuric acid reagent. A chromatogram of selected phytomarkers is presented for further verification in **Figure 9**. HPTLC analysis revealed a higher concentration of lupeol and β-sitosterol in leaves, while gallic acid was predominantly present in root while absent in leaves.

Linearity of phytomarkers

The developed method was found linear over the range of 200-600 ng/spot. The linear equation and other observations mentioned in **Table 2**.

Repeatability

%RSD for repeatability of method for lupeol, β-sitosterol and gallic acid, was found less than 2 and under the acceptance criteria **(Table 2)**.

Intraday and Interday precision

Three replicated of three concentration of phytomarker mixture (Lupeol, β-sitosterol, and Gallic acid) at concentration level of 300, 400, 500 ng/spot were used to determine intraday and interday precision, the %RSD of the method was found to be less than two **(Table 3)**, which is under the acceptance criteria.

Accuracy:

The accuracy of the method was performed by recovery method, here a triplicate was prepared and accuracy calculated by standard addition at 50, 100, and 150%. The % recovery for found to be 99.31,99.00 and 98.61for lupeol, β-sitosterol and gallic acid **(Table 4)** which was under the acceptance criteria between 98 to 102%. So, it can be said that the given method is accurate.

Robustness

The robustness analysis was carried out under the change in condition like duration of saturation of chamber and change in wavelength that did not show any major deviation as mention in **Table 3**.

LOD and LOQ:

Table 2 represents the value of LOD and LOQ of lupeol, β-sitosterol and gallic acid.

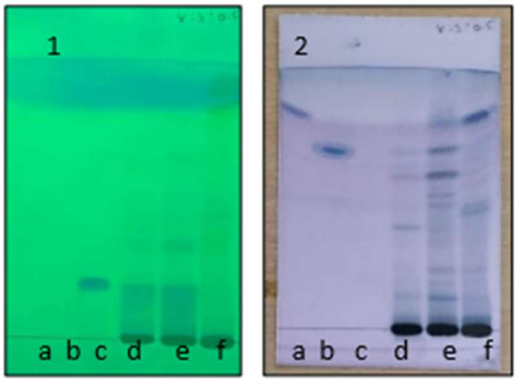

Figure 1: 1) At 254 2) After derivetization with anisaldehyde sulphuric acid reagent where; a) Lupeol b) β-sitosterol c) Gallic acid d) Root extract e) Stem extract f) leaf extract

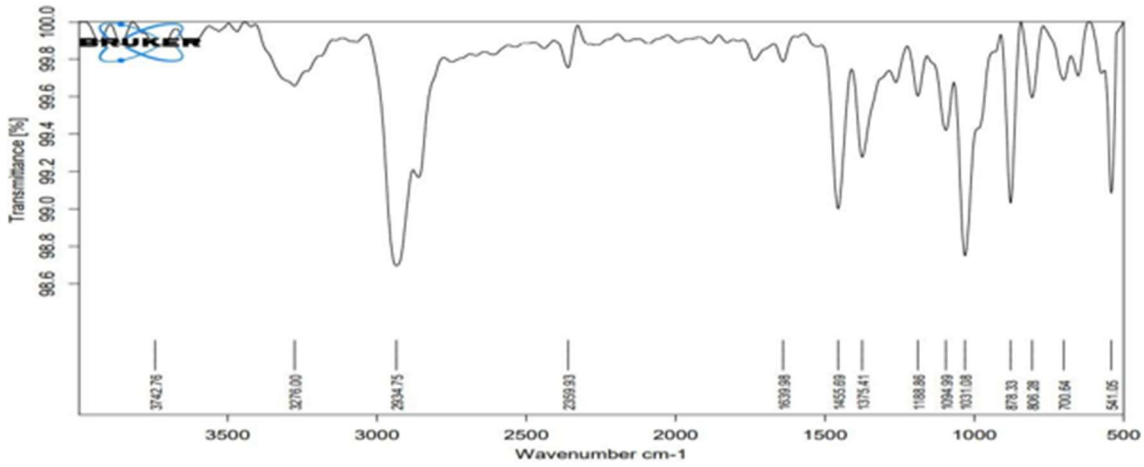

Figure: 2 IR spectra of phytocompound-1

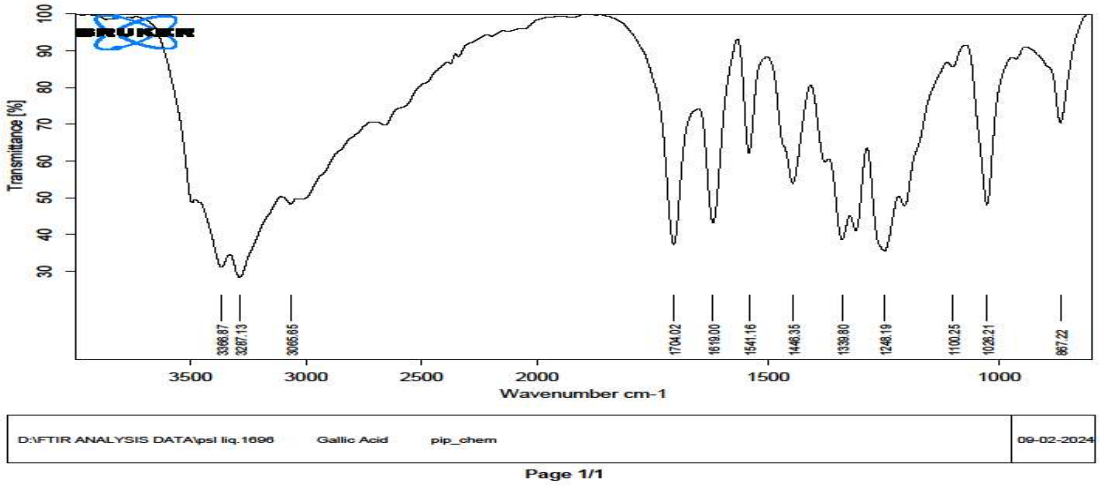

Figure 3: IR spectra of phytocompound-2

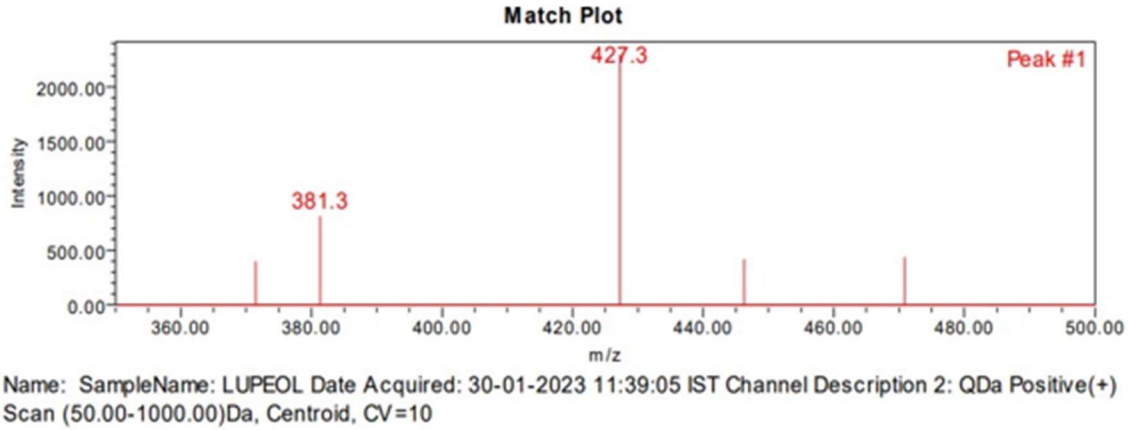

Figure 4: Mass Spectrum of phytocompound-1

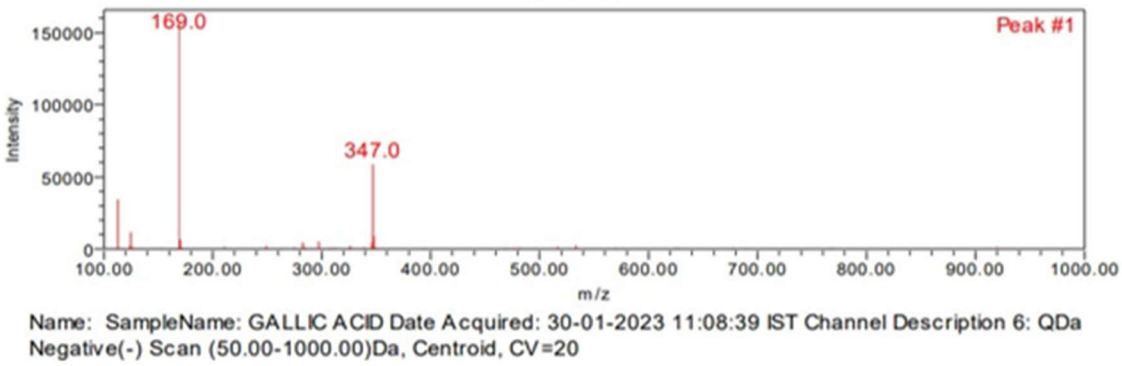

Figure 5: Mass Spectrum of phytocompound-2

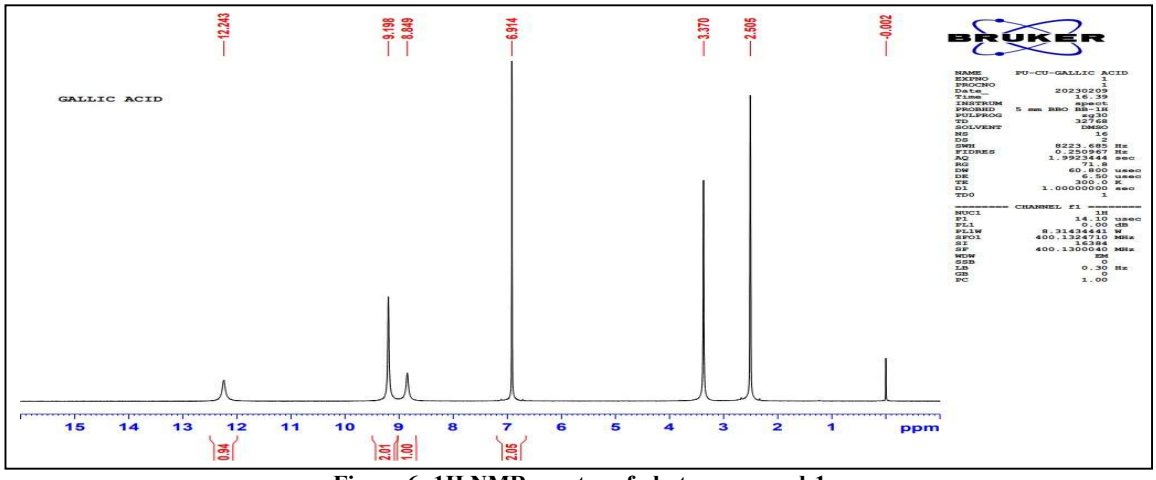

Figure 6: 1H NMR spectra of phytocompound-1

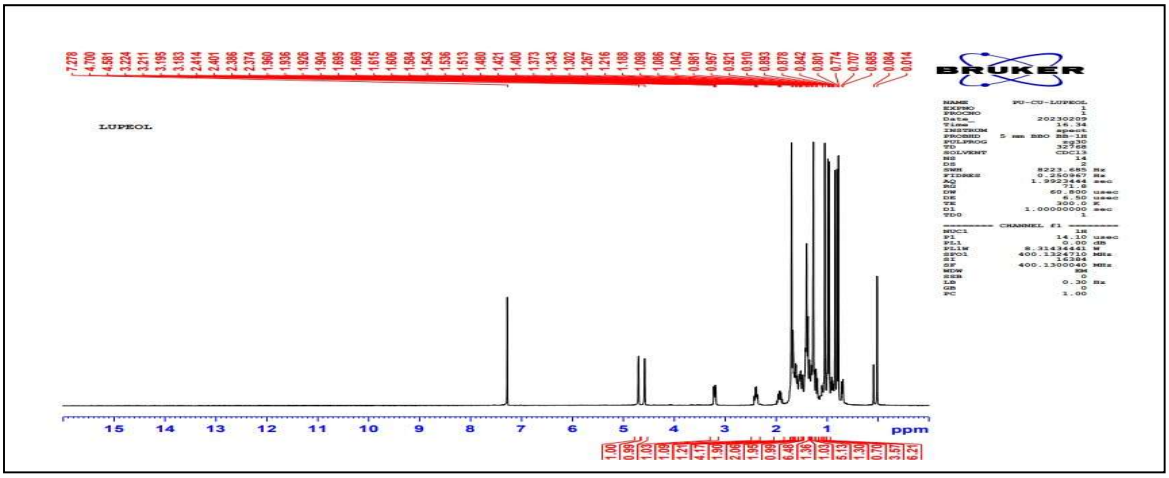

Figure 7: 1H NMR spectra of phytocompound-2

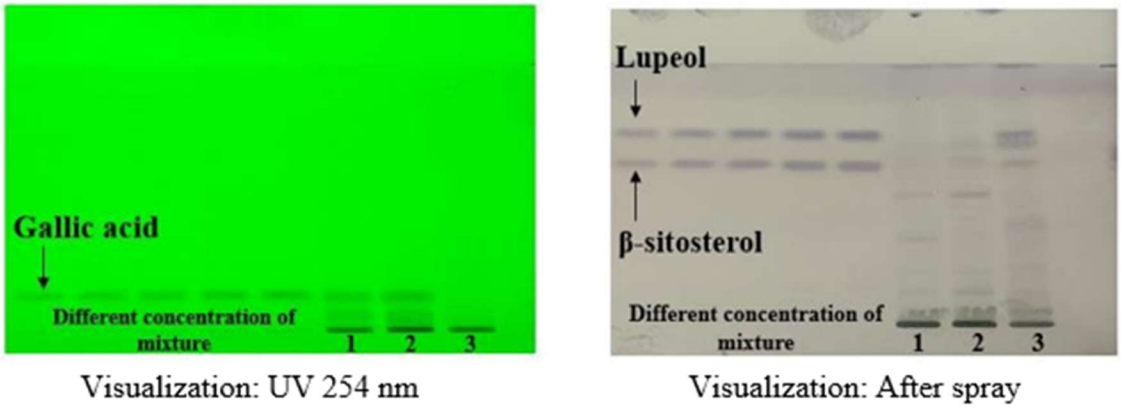

Figure 8: Developed HPTLC plate 1) Root extract. 2) Stem extract. 3) Leaf extract

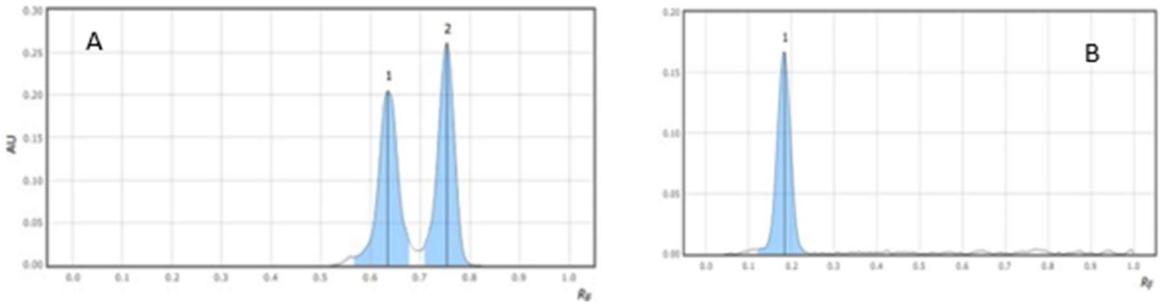

Figure 9. Chromatogram of A) 1. β-sitosteroland 2. Lupeol B) Gallic acid

Table 1: Rf values and amount of phytomarkers present in extracts

Sr no	Standard	Rf value	Amount of Phytomarkers (%)		
			Root	Stem	leaf
1	Lupeol	0.75	1.35%	0.55%	0.75%
2	β-sitosterol	0.63	0.79%	0.78%	0.33%
3	Gallic acid	0.19	4.56%	6.67%	Absent

Table 2: Data of linearity, repeatability, LOD and LOQ of phytomarkers

Parameters		Lupeol	β-sitosterol	Gallic acid
Linearity	Equation	y = 1.292x + 128	y = 1.307x +132.86	y = 1.195x +88.64
	Regression co-efficient	0.998	0.9983	0.9995
Repeatability	% RSD	1.05	1.48	1.37
LOD		57.56	58.12	53.15
LOQ		174.44	176.14	161.06

Table 3: Data for intraday interday and robustness precision

Drug	Concentration (ng/spot)	Precision		Robustness	
		Intraday	Interday	Saturation time	Wavelength
		%RSD	%RSD	%RSD	%RSD
Lupeol	300	1.4481	1.5366	1.01	1.37
	400	1.7918	1.8310	1.42	1.18
	500	1.6404	1.5691	1.19	1.53
β-sitosterol	300	1.5266	1.5570	1.14	1.36
	400	1.8294	1.4921	1.47	1.82
	500	1.8306	1.6759	1.01	1.19
Gallic acid	300	1.0311	1.6866	1.14	1.38
	400	1.7158	1.5648	1.06	1.78
	500	1.3188	1.7033	1.58	1.80

Table 4: Accuracy

Drug	Standard spiked %	Amount of sample (ng/band)	Amount of standard (ng/band)	Total amt. (ng/band)	Amount recovered (ng/band)	% Recovery
Lupeol	50	200	100	300	297	99
	100	200	200	400	399.89	99.2
	150	200	300	500	498.7	99.74
β-sitosterol	50	200	100	300	298.05	99.35
	100	200	200	400	397.2	99.3
	150	200	300	500	491.77	98.354
Gallic acid	50	200	100	300	292	98.4
	100	200	200	400	392	98
	150	200	300	500	497.2	99.44

SUMMARY

The present study attempts to identify the unknown phytoconstituents (lupeol and gallic acid) present in plant as well as quantification of phytochemical constituents like lupeol, β-sitosterol and gallic acid from methanol extracts of *Pluchea wallichiana* (root, stem, leaf). The preparative TLC utilized to isolate unknown phytoconstituents which was further confirmed and identified by IR, Mass, and NMR, as lupeol and gallic acid. A sensitive and reliable HPTLC method had been developed for qualitative and quantitative determination of lupeol, β-sitosterol and gallic acid in the *Pluchea wallichiana*. The methanolic extract of plant parts was chromatographed on silica gel 60 F_{254} plates with toluene: ethyl acetate: formic acid (8:3:0.5 v/v/v), as mobile phase. Detection and quantitation were performed by densitrometric scanning at 254nm for gallic acid while lupeol β-sitosterol scanned at 521nm for after spraying with anisaldehyde sulphuric acid reagent. The selected linearity range was 200-

600 ng/spot. Correlation coefficients for lupeol, β-sitosterol and gallic acid were found to be 0.998, 0.998 and 0.999, respectively. Percentage recovery was found to be in range 99.31% for lupeol, 99 % for β-sitosterol and 98.61% for gallic acid. The method was found precise, repeatable, robust and accurate as the percentage RSD was less than 2.

CONCLUSION

Through the use of IR, NMR, Mass, lupeol and gallic acid were isolated and identified for the first time in the current work. HPTLC, verifying the amount of phytoactive is crucial step to determine the quality of herbal plant. Thus, HPTLC was used to quantify lupeol, β-sitosterol, and gallic acid in *Pluchea wallichiana* DC. The developed HPTLC technique might be used as an analytical and evaluative parameter in the assessment of quality of plant. It is concluded that gallic acid was absent in leaf while lupeol and β-sitosterol is present in root, stem and leaf parts.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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