Extraction, Fractionation, and Characterization of Plumbagin from the Roots of *Plumbago zeylanica*

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KEYWORDS

Plumbago zeylanica, Plumbagin, Soxhlet extraction, TLC, Mass spectrometry.

ABSTRACT

Plumbago zeylanica, a prominent medicinal plant, is renowned for its bioactive constituent, plumbagin, a naphthoquinone with notable pharmacological properties, including anticancer, antimicrobial, and anti-inflammatory effects. The efficient extraction and detailed characterization of plumbagin are essential for its clinical applications. This study aimed to optimize a method for the extraction, fractionation, and characterization of plumbagin from the roots of *P. zeylanica*. A Soxhlet extraction method was utilized with an appropriate solvent system to obtain a crude extract rich in plumbagin. Column chromatography was employed for the fractionation of the crude extract, and thin-layer chromatography (TLC) was used to identify plumbagin-containing fractions by comparing their Rf values with a standard. The isolated compound was subjected to comprehensive characterization using UV-Vis spectroscopy, Fourier-transform infrared (FTIR) spectroscopy, nuclear magnetic resonance (NMR), and mass spectrometry (MS). The optimized extraction and fractionation method yielded 7.2% (w/w) crude extract and 2.81% (w/w) plumbagin. The Rf value of the extract was determined to be 0.86. UV-Vis spectroscopy confirmed the presence of plumbagin by exhibiting characteristic absorption maxima at 265 nm. FTIR analysis supported this identification by revealing peaks corresponding to the naphthoquinone structure. ^{1}H NMR spectroscopy provided further structural insights, confirming the presence of specific protons and carbons within the compound. This study establishes an efficient protocol for extracting and characterizing plumbagin from *P. zeylanica* roots, facilitating future research into its therapeutic potential and enhancing its applicability in the pharmaceutical and medicinal fields.

INTRODUCTION

Medicinal plants have been a cornerstone of traditional medicine for centuries, offering a rich source of bioactive compounds with diverse therapeutic properties.[1] Among these plants, *Plumbago zeylanica* L., commonly known as Ceylon leadwort or doctorbush, stands out for its significant medicinal value.[2] This plant, belonging to the Plumbaginaceae family, is widely distributed across tropical and subtropical regions, including Asia, Africa, and the Americas. *P. zeylanica* has a long history of use in traditional medicine systems, such as Ayurveda, Siddha, and Unani, for treating various ailments, including skin diseases, inflammation, and parasitic infections. [3]

The therapeutic efficacy of *P. zeylanica* is attributed to its diverse array of phytochemicals, including naphthoquinones, flavonoids, terpenoids, and steroids. Among these, plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is a prominent bioactive naphthoquinone compound found in the roots of *P. zeylanica.*[4] Plumbagin has garnered significant attention in recent years due to its remarkable pharmacological activities, including anticancer, antimicrobial, anti-inflammatory, antioxidant, and wound-healing properties.[5]

The anticancer activity of plumbagin has been demonstrated in various in vitro and in vivo studies. It has shown promising effects against a wide range of cancer cell lines, including leukemia, breast cancer, lung cancer, and prostate cancer.[6] The mechanisms underlying plumbagin's anticancer activity involve multiple pathways, such as inducing apoptosis, inhibiting cell proliferation, and suppressing angiogenesis.[7] Furthermore, plumbagin has exhibited synergistic effects with conventional chemotherapeutic agents, suggesting its potential as an adjuvant therapy.[8]

In addition to its anticancer properties, plumbagin possesses potent antimicrobial activity against various bacterial, fungal, and parasitic pathogens. Studies have shown its effectiveness against *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, and *Plasmodium falciparum.*[9] The antimicrobial activity of plumbagin is attributed to its ability to disrupt microbial cell membranes, inhibit DNA synthesis, and interfere with essential metabolic pathways.[10]

Plumbagin's anti-inflammatory activity has also been well-documented. It has been shown to inhibit the production of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, and reduce inflammation in various animal models.[11] This anti-inflammatory effect is mediated through the modulation of signaling pathways, such as NF-κB and MAPK.

The antioxidant properties of plumbagin contribute to its therapeutic potential. It acts as a free radical scavenger, protecting cells from oxidative damage and reducing oxidative stress. This antioxidant activity is beneficial in various conditions, including cardiovascular diseases, neurodegenerative disorders, and aging.[12]

Moreover, plumbagin has demonstrated wound-healing properties in both in vitro and in vivo studies. It promotes cell migration and proliferation, accelerates wound closure, and enhances collagen synthesis. These wound-healing effects make plumbagin a potential candidate for developing novel wound-healing agents.[13]

Given the diverse pharmacological activities of plumbagin, there is a growing interest in developing efficient methods for its extraction, fractionation, and characterization from *P. zeylanica* roots. Various extraction techniques, including Soxhlet extraction, maceration, and ultrasound-assisted extraction, have been employed. Fractionation techniques, such as column chromatography and preparative thin-layer chromatography, are used to isolate plumbagin from the crude extract. Characterization of the isolated plumbagin is typically performed using spectroscopic techniques, such as UV-Vis, FTIR, NMR, and mass spectrometry.[14]

This study aimed to develop and optimize a method for extracting, fractionating, and characterizing plumbagin from *P. zeylanica* roots. Soxhlet extraction was employed using a suitable solvent system, followed by column chromatography for fractionation. Thin-layer chromatography was used to identify plumbagin-containing fractions, and the isolated plumbagin was characterized using various spectroscopic techniques. The developed method provides a valuable approach for obtaining high-quality plumbagin from *P. zeylanica* roots for further research and potential applications.

MATERIALS AND METHODS

Materials

Plumbago zeylanica roots were collected from Neemuch and its surrounding areas. Botanical authentication was performed by comparing the collected samples with voucher specimens preserved at the Department of Pharmacognosy, BRNCP, Mandsaur, M.P., India. A voucher specimen (BRNCP/P/006/2022) was deposited in the herbarium. All solvents and reagents used were of analytical grade. Methanol, ethyl acetate, hexane, and n-butanol were obtained from Merck. Reagents for phytochemical screening, including ferric chloride, Dragendorff's reagent, and lead acetate, were purchased from Sigma-Aldrich. Chromatographic and spectroscopic analyses utilized reagents, including silica gel, sourced from S D Fine-Chem Ltd, India.

Preparation of Extracts

Powdered *P. zeylanica* root material (100 g) was subjected to Soxhlet extraction with ethanol for 72 h at 55–60°C. The extract was filtered, and the filtrate was concentrated under reduced pressure using a rotary evaporator at less than 50°C[15] The ethanol was evaporated completely, and the resulting concentrated extract was lyophilized and stored at 4°C. The percentage yield of the extracts was calculated using the following formula:

Yield (%) = (Weight of the extract / Weight of the powdered drug) × 100

Fractionation of Extract using Chromatographic Separation and Isolation of Active Constituents

The ethanolic extract of P. zeylanica roots (10g) was fractionated using column chromatography.[16] A glass column (60 cm × 3 cm) was packed with silica gel (60–120 mesh) as the stationary phase. The ethanolic extract was loaded onto the column, and different solvent systems, including n-hexane, n-hexane-ethyl acetate, ethyl acetate 100%, and methanol 100%, were used for elution in a gradient manner.[17] Elution continued until approximately 90% of the loaded extract was eluted. A total of twenty-three fractions were collected, and those exhibiting similar thin layer chromatography (TLC) patterns were pooled together. Ultimately, four distinct fractions (F1–F4) were obtained. The percentage yield of each fraction was calculated relative to the total weight of the ethanolic extract.[18]

Fractions exhibiting a single spot on TLC (n-hexane:ethyl formate, 9:1; Rf 0.37) were pooled and evaporated to dryness, yielding plumbagin.[14] The melting point of the isolated compound was determined. TLC analysis was conducted on precoated silica gel 60 F254 plates. Purity was assessed using UV, FTIR, NMR and Mass spectroscopy.

Phytochemical Screening of Extracts and Fractions

Preliminary phytochemical screening of the ethanolic extract and the isolated fractions was carried out to identify the presence of various secondary metabolites, such as alkaloids, flavonoids, tannins, saponins, glycosides, and terpenoids, using standard methods.[19] Test solutions of the extract and fractions were prepared in various solvents (aqueous, alcoholic, petroleum ether, diethyl ether, chloroform, etc.) depending on the solubility of the target compounds. Clear, transparent solutions were used whenever possible to ensure accurate results and facilitate observation.[20]

Thin Layer Chromatography of Isolated compound

Silica gel was used as the stationary phase for adsorption TLC. To minimize band broadening, fine, uniform silica gel particles were employed. A slurry of silica gel and water was evenly spread on a glass plate, air-dried, and activated in an oven at 100–110°C for 30 min to ensure linear solute migration. A chloroform:ethyl acetate:hexane:acetic acid (10:5:5:0.3) mobile phase was used. The TLC chamber was saturated with the mobile phase by lining it with filter paper wetted with the

solvent system. Following activation, the sample (µg–mg range) was applied to the TLC plate using a capillary tube. The plate was developed in the chamber with a solvent depth of ~0.5 cm.[21] After development, the plate was removed, the solvent front was marked, and the solvent was evaporated in an oven. Plumbagin appeared as a yellow spot on the silica gel plate. The Rf value was calculated as:

$$Rf = \frac{\text{Distance traveled by solute}}{\text{Distance traveled by solvent}}$$

Characterization of Isolated Plumbagin

The isolated plumbagin from the fractions was subjected to various spectroscopic techniques for characterization. The melting point of the isolated fraction was determined using a Tempo melting point apparatus. A capillary tube, sealed at one end, was filled with a small amount of the dried, finely powdered fraction and packed by gentle tapping. The capillary tube was inserted into the heating block of the apparatus, and the temperature was gradually increased until complete melting was observed.[22]

UV-Vis spectra of the isolated fraction were recorded on a Shimadzu UV-1601 double-beam spectrophotometer over a wavelength range of 200–800 nm using matched quartz cuvettes with a 10 mm path length.[23] FTIR spectra of the isolated fraction were obtained using a Shimadzu 8400S FTIR spectrophotometer in the 4000-400 cm^{-1} range. Samples were prepared as KBr pellets using a Shimadzu hydraulic press.[24] ^1H NMR spectra of the isolated fraction were recorded in D_2O on a Bruker Avance-II 300 MHz FT-NMR spectrometer at the SAIF, Punjab University, Chandigarh.[25] Mass spectroscopy was carried out by MALDI-TOF (Micromass Tof-Spec 2E instrument, USA) at SAIF, Punjab University, Chandigarh to determine molecular weight of different dendrimers formulations.[26-27]

RESULTS

Extraction Yield and Fractionation

The Soxhlet extraction process was utilized to isolate bioactive compounds from the roots of *Plumbago zeylanica*, a widely recognized medicinal plant. Ethanol was chosen as the solvent due to its excellent ability to dissolve a broad spectrum of phytochemicals, including plumbagin. The extraction was performed over 72 hours at a temperature range of 55-60°C, ensuring maximum yield of the desired compounds. Following solvent evaporation under reduced pressure, the crude extract yield was determined to be 7.2% w/w.

Subsequent fractionation of the crude extract via column chromatography led to the separation of various bioactive fractions. A gradient elution system was used, starting with non-polar solvents (n-hexane) and gradually increasing polarity with ethyl acetate and methanol. This technique allowed for the differential separation of compounds based on their adsorption to silica gel. The process yielded four major fractions, with Fraction F3 (2.81% w/w) being identified as the one containing plumbagin. The percentage yield of each fraction is summarized in Table 1.

Table 1: Percentage Yields of Various Fractions from Ethanolic Extract of *P. zeylanica*

Fraction	Colour	% Yield (W/W)
F1	Yellowish	28.13
F2	Yellowish brown	42.55

F3	Orange	2.81
F4	Yellowish brown	22.37

Phytochemical Screening

Phytochemical analysis of the ethanolic extract and its fractions confirmed the presence of multiple secondary metabolites, reinforcing the pharmacological potential of *P. zeylanica*. The screening revealed the presence of flavonoids, tannins, naphthoquinones, carbohydrates, glycosides, and saponins (Table 2). Fraction F3, which contained plumbagin, demonstrated a strong presence of naphthoquinones, validating its identity. Other fractions also exhibited bioactive constituents, suggesting potential synergistic effects among various phytochemicals.

Table 2: Phytochemical Screening of Ethanolic Extract of *P. zeylanica* Roots

S. No.	Chemical Constituent	Result
1	Flavonoids	Positive
2	Steroids	Negative
3	Tannins & Phenolics	Positive
4	Protiens	Negative
5	Carbohydrates	Positive
6	Reducing sugar	Positive
7	Napthaquinone	Positive
8	Amino acid	Negative
9	Glycoside	Positive
10	Cardiac glycoside	Negative
11	Anthraquine glycoside	Negative
12	Saponin	Positive
13	Coumarin	Negative
14	Alkaloids	Negative

Thin-Layer Chromatography (TLC) Analysis

TLC was employed to confirm the presence of plumbagin in Fraction F3. Using a solvent system consisting of $CHCl_3$: Ethyl Acetate: Hexane: Acetic Acid, TLC revealed a distinctive yellow spot under UV-visible light. The Rf value of 0.86 closely matched the standard plumbagin Rf value (0.90), indicating the presence of the desired compound (Figure 1).

Figure 1. TLC analyzing plumbagin in extract

Spectroscopic Characterization

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis was conducted on the isolated plumbagin (F3) to determine its functional groups. The IR spectrum displayed characteristic absorption bands at 3456 cm^{-1}, corresponding to the O-H stretch of a hydroxyl group. Additional peaks were observed at 2921 cm^{-1} and 2866 cm^{-1} (C-H stretching vibrations), 1508 cm^{-1} (C=C stretching), and 1643 cm^{-1} (aromatic ketone stretching). These results strongly confirmed the presence of plumbagin within the fraction (Figure 2).

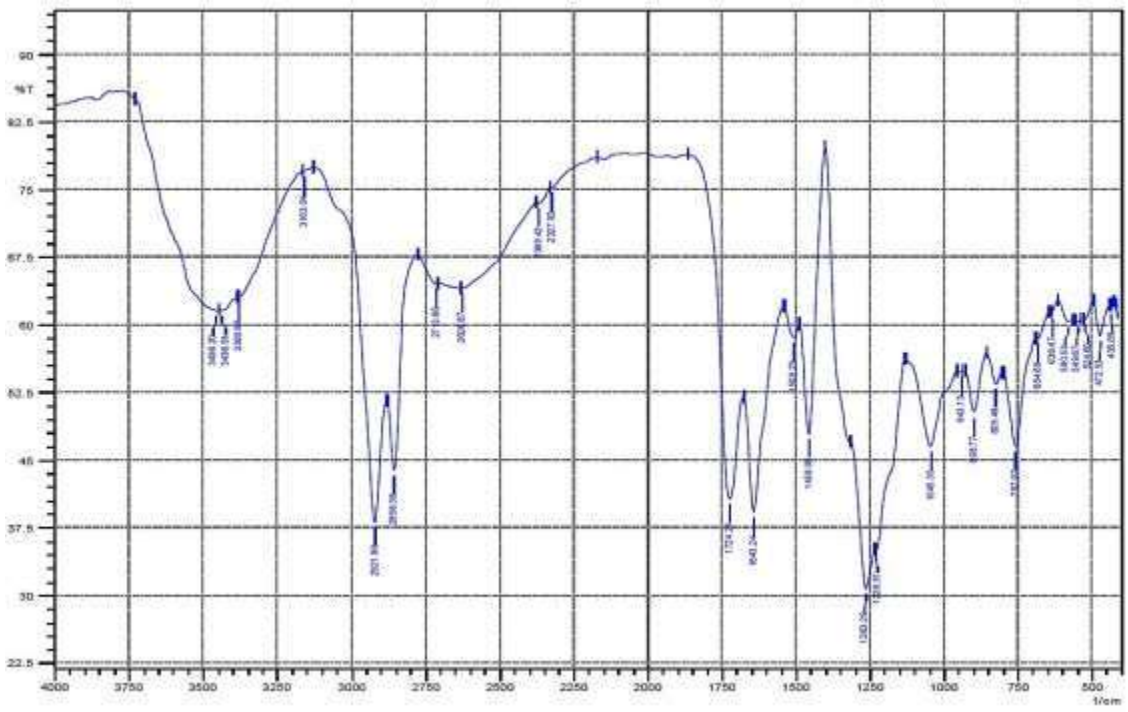

Figure 2: FTIR spectrum of selected fraction (F3)

Nuclear Magnetic Resonance (NMR) Analysis

^{1}H NMR Spectrum: The ^{1}H NMR spectrum of Fraction F3 revealed two distinct proton regions: one between δ 0 to 2.5 and another between δ 6.5 to 7.5. The single peak at δ 2.59 suggested the presence of a methyl group (Me-2), while the peaks at δ 5.64 and δ 7.74 corresponded to hydrogen-bonded protons, likely in a vinyl environment. The multiplet observed at δ 7.58 was attributed to aromatic protons, confirming the structure of plumbagin.

Mass Spectrometry (MS)

Mass spectrometry of the isolated compound displayed major peaks at m/z 187.9 (M+1), 186.5, and 178.1, matching the reported fragmentation pattern of plumbagin. The standard molecular weight of plumbagin is 188.1794 g/mol, aligning well with the observed spectral data.

UV-Visible Spectroscopy

The UV spectrum of the isolated plumbagin in methanol displayed a prominent absorption peak at λmax 265 nm (Figure 3), indicative of a conjugated π-electron system. This result further supports the presence of aromatic and conjugated ketone structures within the isolated compound.

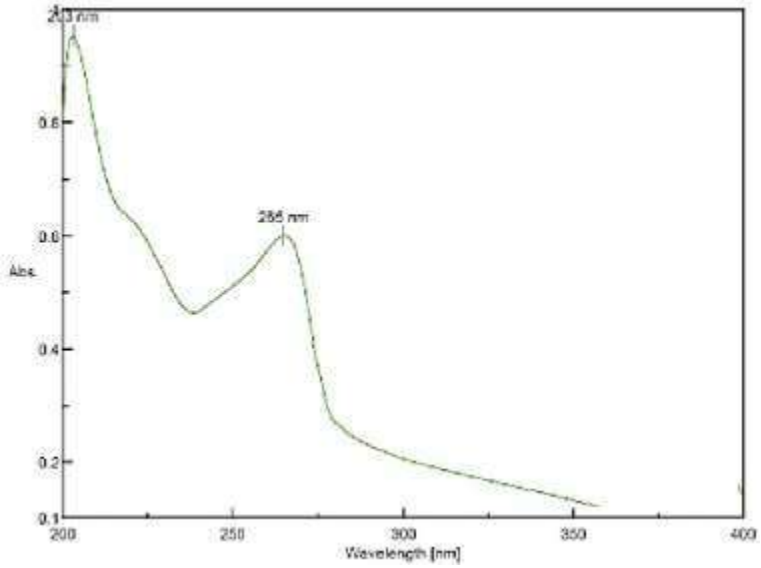

Figure 3: UV-visible spectrum of selected fraction (F3)

DISCUSSION

The successful isolation of plumbagin from *P. zeylanica* roots validates the effectiveness of Soxhlet extraction and column chromatography as reliable methods for extracting bioactive naphthoquinones. The crude ethanolic extract (7.2% w/w) contained a diverse range of phytochemicals, as confirmed by phytochemical screening. These results highlight the rich medicinal potential of *P. zeylanica*, particularly due to the presence of flavonoids, tannins, and glycosides, in addition to plumbagin.

The use of gradient elution in column chromatography proved effective for fractionating the extract into its individual components. The identification of plumbagin in Fraction F3 (2.81% w/w) aligns with previous literature, reinforcing its consistent yield across extraction studies. The phytochemical diversity among fractions suggests that *P. zeylanica* contains other pharmacologically active constituents that could work synergistically with plumbagin.

TLC provided a rapid and reliable confirmation of plumbagin presence, with an Rf value of 0.86, closely resembling standard plumbagin (0.90). Minor deviations may be attributed to variations in solvent polarity, adsorption properties of the silica gel, or experimental conditions.

Spectroscopic characterization methods (FTIR, NMR, MS, and UV-Vis) provided conclusive evidence for the structural identity of the isolated compound. The FTIR results confirmed the presence of key functional groups, including hydroxyl and ketone groups, characteristic of plumbagin. ^{1}H NMR data provided insights into the proton environment, revealing distinct aliphatic and aromatic regions consistent with standard plumbagin spectra. Mass spectrometry confirmed the molecular weight, and UV-Vis spectroscopy highlighted the conjugated nature of the compound, reinforcing its naphthoquinone framework.

The slight discrepancy between the observed and reported Rf values and spectral peaks can be attributed to solvent purity, environmental conditions, and instrument calibration variations. However, the overall spectral alignment with reference data strongly supports the successful isolation and characterization of plumbagin. This study contributes to the growing body of research on *P. zeylanica*, presenting a highly efficient method for extracting and purifying plumbagin. The results reinforce its pharmacological relevance in antimicrobial, anticancer, and anti-inflammatory applications.

CONCLUSION

This study successfully developed and implemented a method for extracting, isolating, and characterizing plumbagin from the roots of *Plumbago zeylanica*. The process began with Soxhlet extraction using ethanol, chosen for its broad solubility of plant metabolites. This method yielded a 7.2% w/w extract, demonstrating the efficiency of ethanol in extracting plumbagin from the root material. Following extraction, the crude extract underwent fractionation using column chromatography, yielded a 2.81% w/w plumbagin fraction, which was structurally confirmed using TLC, FTIR, NMR, MS, and UV-Vis spectroscopy. Thin-layer chromatography played a key role in identifying the fractions containing plumbagin by comparing their Rf values to a standard plumbagin sample.

The isolated plumbagin was then subjected to rigorous characterization using various spectroscopic techniques. UV-Vis spectroscopy confirmed the presence of plumbagin with characteristic absorption maxima at 265nm. FTIR spectroscopy further validated the compound's identity by revealing characteristic peaks associated with the naphthoquinone moiety, a key structural feature of plumbagin. ^{1}H NMR spectroscopy provided detailed structural information, confirming the presence of specific protons and carbons in the molecule. The spectra obtained were consistent with reported values for plumbagin, solidifying the compound's identification. High-resolution mass spectrometry analysis determined the molecular formula of the isolated compound as $C_{11}H_8O_3$, further confirming its identity.

This study's findings contribute significantly to the existing knowledge on *P. zeylanica* and its bioactive constituents. The developed method provides a reliable and efficient approach for obtaining high-quality plumbagin from the plant's roots. This research provides a valuable platform for future studies aimed at harnessing the therapeutic potential of *P. zeylanica* for various health applications.

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