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RESEARCH ARTICLE

Botanical and SFE aided need base systemic scientific inaugurate of *Delphinium denudatum* root Wall. an unexplored jeopardise medicinal plant

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Abstract

The endangered medicinal plant *Delphinium denudatum* Wall. is widely recognized by the medical community for its potential in treating neurological disorders. Despite its known health benefits, it has not been thoroughly investigated. Further research is required using scientifically recognized analytical techniques to fully explore its therapeutic potential.

The use of traditional methods for extracting botanicals raises concerns about both environmental damage and potential health risks due to the extensive use of organic solvents. In response to these issues, this research utilizes environmentally-conscious and eco-friendly methods for botanical extraction. Recently, supercritical fluid extraction (SFE) has emerged as a preferred technique for extracting plant material due to its high efficiency and yield. Carbon dioxide (CO_2) is the solvent of choice in SFE because of its effective diffusion into plant material at low temperatures, as well as its environmentally friendly properties.

In this study, the optimal conditions for SFE were determined to be a pressure of 200 bar, a temperature of 80 °C and a CO_2 flow rate of 15 g. min^{-1} to achieve to achieve maximum yield of Delphinium root extract (DRE). The result demonstrated that SFE produced an optimal extract yield with three productivity levels: level-I (0.8 % w/w), level-II (1.2 % w/w) and level-III (0.95 % w/w). Furthermore, a botanical examination verified the integrity of cell structures, while physicochemical evaluations help establish quality standards for the extract.

Phytometabolites mapping of the SFE- derived DRE was performed using HPTLC under various conditions, including daylight, post-derivatization and at 254 nm and 366 nm. Additionally, community reports validated the effectiveness of DDR against harmful microorganisms, with zones of inhibition observed against *Staphylococcus aureus* (ZI: 5.21 ± 0.29); *Pseudomonas aeruginosa* (ZI: 7.48 ± 0.53); *Aspergillus niger* (ZI: 8.15 ± 0.9) at concentrations of 100 µg mL^{-1} and 500 µg mL^{-1} of the DRE respectively.

In conclusion, this research strongly supports the use of *D. denudatum* extracts obtained via SFE as a potential treatment for various pathogenic disease. To the best of our knowledge, there is no previous scientific evidence documenting the successful extraction of *D. denudatum* using SFE. Additionally, the protective properties of SFE-derived DRE against pathogenic microorganisms had not been previously explored. Thus, the present study identifies *D. denudatum* as a promising candidate for the development of drugs to combat pathogenic infections.

Keywords

carbon dioxide (CO_2); Delphinium denudatum; endangered plant; HPTLC; phytometabolites; supercritical fluid extraction (SFE)

Introduction

Botanicals have a broad therapeutic appeal and fulfil many of the world's health need. However, ensuring the quality of botanicals and their products require significant attention. Variability in the quality and quantity of phytoactive constituents is a persistent challenge due to the diverse flora and fauna of plants (1). As a result, the quality of crude herbal drugs is often compromised, raising concerns about the standardization of herbal products (2). Among valuable folk medicines, *Delphinium denudatum* (Ranunculaceae) is a scientifically underexplored medicinal plant (3). It grows in the Himalaya region, where only a few populations (fewer than 7) are found and necessitating immediate efforts to protect this species. A thorough review of the literature confirms that *D. denudatum* root is used in the treatment of neuropsychiatric disorder, nephropathy and leishmaniasis (4). The available literature, also highlights the lack of clear information for identifying similar medicinal plants, as *D. denudatum* has been assigned various vernacular names. Another issue is the use of different plant species that resemble each other or have similar-sounding names, leading to confusion between look-alike and sound-alike drugs (5).

Although *D. denudatum* is an underexplored medicinal plant, initial efforts have been made towards its identification (6). In this study, we aimed to address authentication challenges of *D. denudatum* using advanced methods, specifically supercritical fluid extraction (SFE). In the context of quality control for natural-origin drugs, various analytical techniques are commonly used by the scientific community, including SFE, HPTLC, HPLC and GC-MS (7, 8). While traditional extraction methods such as cold extraction, percolation, Soxhlet extraction and pressurized solvent extraction have contributed to the development of natural product research, these techniques often face quality-related challenges (9). These challenges include high operational temperatures, the use of costly and environmentally hazardous organic solvents, solvent disposal issues, poor compatibility with extractive materials and the low yield of volatile phytoconstituents. Such limitations underscore the need for an eco-friendly extraction technique for botanicals (10). In this context, SFE stands out as the most reliable method. Supercritical fluids, beyond their critical temperature, exhibits dual advantages, behaving like both liquids and gases (11). SFE offers fast recovery, uses an inexpensive, non-combustible solvent (CO_2) and has an easy recycling process, making it a valuable tool for extraction (12). In other studies, water at high temperature (373 °C) has been used for extracting Huadian oil (13), but such high temperature is not suitable for extracting heat-sensitive phytoactive constituents from plants (14). In contrast, CO_2 is an ideal solvent, especially in the extraction of dietary food products and bioactive natural drug candidates, due to its mild supercritical nature (15). Currently, modern extraction techniques like SFE are gaining attention as effective strategies for extracting novel natural compounds from medicinal herbs, as the methods is closely linked to the bioactivity of bioactive compounds.

An intensive literature review confirmed the potential of SFE for extracting phytoconstituents from natural drugs. In lieu of this, the current study primarily focused on extracting DDR using SFE. Various polyphenolic plant constituents have been analysed using green supercritical fluid extraction technique, as documented in earlier studies (16). For example, Supercritical fluid extraction has been used to extract essential medicinal oil from *Psidium guajava* L. leaves and non-volatile constituents were also obtained using green (CO_2) supercritical extraction (17). Another study assessed the antimicrobial activity of extracts from 6 medicinal plants (*Cichorium intybus, Cinnamomum camphora, Commiphora myrrha, Foeniculum vulgare, Nerium oleander* and *Spartium junceum*) obtained via SFE (18).

The present study aimed to analyse the active phytoconstituent's of *D. denudatum*, using state-of-the-art technique supercritical fluid extraction (SFE) technology. The extraction was conducted under specific parameters, followed by an evaluation of the potential of these SFE-based extracts to inhibit the growth of Gram-positive and Gram-negative bacteria, as well as fungal species. Additionally, these SFE extracts were analysed using high-performance thin-layer chromatography (HPTLC) to identify and characterise the bioactive natural constituents. To the best of our knowledge, there is no scientific data demonstrating the successful extraction of *D. denudatum* via SFE. Therefore, this study serves as a scientific introduction of the underutilized *Delphinium denudatum*, which we believe will provide valuable information for future research.

Materials and Methods

Sample collection

The roots of *Delphinium denudatum* (JHFP-5431) were manually collected in autumn from a traditional folk material in New Delhi, India and identified by a qualified taxonomist.

Experimental chemicals and reagents

All experimental chemicals and reagents used in the study were of high analytical grade and were sourced from Merck (Darmstadt, Germany).

Super critical fluid extraction (SFE) aided pytochemical mapping

The extraction of *D. denudatum* root was performed using an advanced supercritical fluid extraction unit (SFE-500M1 -2-C50; Waters Corp., Milford, MA, USA). The powder root material (10.05 ± 0.5 g) was evenly packed into the extraction vessel to prevent unwanted exposure to external air. To enhance the extraction efficiency, 1.5 g w/w of laboratory-grade glass wool was packed into the vessel filters to

fill the hollow area of the vessel. The extraction was conducted at three different pre-adjusted pressures (150, 200, 250 bar) and temperatures (60° C, 70 °C, 80 °C) to obtain an optimal yield. The final optimized extract was stored in a refrigerator at -4 °C for further use in chromatographic analysis and microbial studies, including the mapping of secondary phytoactive constituents.

Non cellular morphological investigation of D. denudatum roots

A detailed morphological study was conducted to examine the external features of *D. denudatum* roots, including their dimension, size, contour, tint, aroma, taste and fracture characteristics (19).

Computer aided cellular and powder examination

An authenticated root specimen was subjected to transverse section (TS), analysis to examine its cellular architecture using a modified method (20). Fresh root pieces were placed in a 100 mL volumetric glass beaker containing 70 % lactic acid and heated in an electronic boiling water bath (Hi media-12 hole) for about 30 min. The lactic acid softened the root tissues, facilitating cellular analysis. The specimen, preserved in a solution of formalin-glacial acetic acid and methanol (30 % formalin, 15 mL; 70 % ethanol, 80 mL, glacial acetic acid, 10 mL), was used for T.S analysis of the plant.

Root tissues staining was performed using a mixture of Toluidine Blue O (TBO) 0.25 % in an aqueous buffer medium (0.5 g benzoic acid in 100 mL distilled water, pH 5.5), diluted with ion-free water. The stained section were examined under a laboratory microscope (Olympus BX 41, Tokyo, Japan). Root powder of optimum size (40 mesh size) was selected for individual cellular analysis and cellular histology was corroborated using advance imagining equipment (Motic microscopic system) connected to a computer.

Standardize physicochemical profile

The physicochemical profile of the plant sample was analysed by determining the ash values, extractive values and loss on drying, following scientifically approved guidelines and methods (21). The Karl Fischer method was employed to determine the water (moisture) content, ensuring the quality of the product. The determination of ash values provides an estimate of inorganic compounds, sandy material and other unwanted contaminants present in the *Delphinium* root.

Phytoextraction by analytical grade (AR) solvent

The sample extraction was performed using a standard laboratory method. Shed-dried and cleaned plant material (20 g) was powdered and extracted using methanol (100 mL) as the organic solvent through Soxhlet extraction. The powdered plant material was packed into a thimble and extracted with methanol for 18-24 h. Statistically, three extraction replicates (n=3) were completed within this time frame.

After extraction, the solvent was removed by placing the beakers in a water bath set at 50 °C to obtain a solvent-free extract. The mixture was filtered through cleaned filter paper and the remaining solvent was evaporated to dryness using a rotary evaporator (Buchi, Switzerland). The dehydrated extract was stored in a refrigerator for further phytochemical analysis (22).

Fluorescence examination of powdered drug

The fluorescence study of *Delphinium* root powder was conducted using a previously established method (23). The powdered drug was treated with a variety of analytical grade (AR) chemicals and examined under short-wave (254 nm) and long-wave (366 nm) ultraviolet (UV) light in a controlled UV chamber (Camag, Switzerland). The results were then documented.

Chromatographic biography for bioactive phytoconstituents of D. denudatum root

Thin layer chromatography (TLC) was performed on an aluminum HPTLC plate uniformly coated with silica gel 60 (Camag, Switzerland). The sample-loaded HPTLC plate was placed in a twin-trough TLC chamber saturated with the mobile phase (toluene: ethyl acetate: formic acid; 6:4:1, v/v/v) for 10 min. The developed HPTLC plate was scanned at wavelengths of 254 nm and 366 nm using WinCats software (CAMAG, Switzerland). Additionally, the HPTLC plate was sprayed with anisaldehyde sulfuric acid (visualizing reagent) to detect various phytoconstituents of *D. denudatum*.

Antimicrobial study of D. denudatum root

The antimicrobial potential was assessed using a slightly modified agar well diffusion technique. Briefly, wells measuring 6 mm in diameter were created in the agar. A microbial suspension (100 µL) was prepared and each well was filled with 80 µL of extract (125 mg/mL in DMSO). The incubation period was set at 37 °C for 24 h for bacterial strains and at 30 °C for 4-7 h for fungal strains, as previously described (24). A unit volume of uncontaminated microbial culture (50 µL) was obtained using a volumetric pipette and poured onto a sterile glass Petri plate (4 cm wide). Autoclaved molten and aseptically cooled Mueller Hinton agar was used for bacterial studies, while potato dextrose agar (PDA) was utilized for fungal studies. The 2 types of agar media were dispensed into pre-sterilized Petri plates and blended with the inoculum. After allowing the agar media to solidify (to a depth of 4 ± 0.5 mm), identical wells (5.0 mm in diameter) were created on the agar surface using a sterile metallic borer.

Subsequently, 100 µL and 500 µL of each extract (30 % w/v) were filled into the designated wells for bacterial and fungal studies respectively. The chosen concentration of phytoextract was determined through an extensive literature review. The experimental plates were sealed with analytical grade paraffin and set aside under laboratory conditions (25 °C for 15 min) to allow proper diffusion of the phytoextract into the wells, followed by incubation at 37 °C for 72 h. In our study, we used the following microbial strains: *Staphylococcus aureus* (Gram-positive bacteria), *Pseudomonas aeruginosa* (Gram-negative bacteria) and *Aspergillus niger* (fungus). Dimethyl sulfoxide (DMSO; 5.0 % v/v) was selected as a negative control, while cefixime and fluconazole served as positive controls

against the microbes.

Statistical Analysis

Statistical analysis was performed, and the data were expressed as relative concentrations of normalized values. Results are presented as means ± standard deviations (SD) based on 3 consecutive repetitions (n=3). Statistical differences were analysed using one-way ANOVA with Graph Pad Prism 9.0, with differences considered statistically significant at $p < 0.05$. The values reported are the means ± SD of the quantified data.

Results

Supercritical fluid extraction (SFE)

The extract yield was assessed by altering pressure and temperature to optimize the extraction conditions via supercritical fluid extraction (SFE). The observed % yield was directly influenced by these variables. The combination of different extraction parameters, such as pressures and temperatures, accounted for the varying % yields of the SFE extracts (level-1, level-2 and level-3). The effects of altered pressures and temperatures on the extraction yields of *Delphinium* extracts are illustrated in Fig. 1. It was observed that extraction yields increased with a combination of elevated SFE pressure and temperature. Specifically, an extraction pressure of 150 bar and a temperature of 60 °C resulted in a lower SFE yield (0.8 % w/w, SFE level-1 extract). In contrast, a pressure of 200 bar and a temperature of 80 °C produced a higher extraction yield (1.2 % w/w, SFE level-2 extract). The mid-range SFE extraction yield (0.95 % w/w, SFE level-3 extract) was achieved at an extraction pressure of 250 bar and a temperature of 70 °C, as discussed in relation to the SFE level-3 extract. All experimentally collected extracts of *D. denudatum* are collectively referred to as "SFE-assisted phytoextracts."

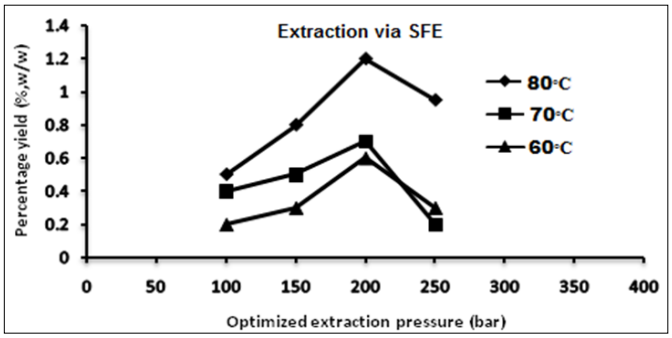

Fig. 1. Results of SFE assisted extraction yield (0.8 % w/w, 1.2 % w/w and 0.95 % w/w) at altered pressure (bar) and temperature (°C) of *D. denudatum* root extracts. Amalgamation of selected pressures (100, 150, 200 and 250 bar) and selected temperatures (60, 70 and 80 °C) were employed to optimized extraction protocol for the proposed study.

Non cellular morphological investigation of *D. denudatum* root

The outer surface of the *D. denudatum* root was rough and hard, with a reddish-brown hue. It was tough to break and exhibited various noticeable longitudinal wrinkles. The dimensions were mathematically calculated to be 4-7 cm in length and 5-12 cm in width, with an elliptical contour. The root had a bitter taste and was devoid of any significant aroma (Fig. 2 A).

Computer added cellular and powder examination

The transverse section (T.S.) of the *D. denudatum* root revealed a thin-walled periderm consisting of a single to double layer of phelloderm and phellogen. The cortex region contained thin-walled parenchyma cells (Fig. 2B). The phloem parenchyma comprised numerous slender-shaped cells, arranged in clusters and tightly packed together (Fig. 2C). The endodermis was cylindrical, composed of 2 or 3 layers of irregularly organized parenchymatous cells (Fig. 2D). Starch granules were well distributed, while thick -walled stone cells were abundant in the parenchymatous region. Calcium oxalate crystals were identified as prismatic in shape and were plentiful in the xylem cells of the root (Fig. 2E). The results from the powder study confirmed the individual cellular structures as shown in Fig. 2F where xylem cells (X: not continuously arranged) are illustrated; Fig. 2G displays parenchyma (Pr: hexagonal in shape); and Fig. 2H shows stone cells (St: globular in shape), which are intermittently distributed throughout the powder.

Standardize physicochemical profile

The results of the physico-chemical analysis confirmed that the extraction yields reflect the quantity of phytoconstituents present in the *Delphinium* extracts. The highest yield was recorded for the methanol extract (7.15 ± 0.24 % w/w), while the lowest yield from Soxhlet extraction was found in petroleum ether (0.13 ± 0.12 % w/w) when cold extraction was employed, followed by chloroform, which yielded 2.96 ± 0.25 % w/w (Table 1). Additionally, the physico-chemical studies established the loss on drying at 3.77 % and the moisture content at 2.56 %. The data on ash values for the *Delphinium* root suggest the presence of various naturally accumulated organic and inorganic materials as well as other unwanted contaminants. The total ash content in the *D. denudatum* sample was found to be 4.97 % w/w, while the acid-insoluble ash was 2.19 % w/w and the water-soluble ash was 2.78 % w/w. Our results ranked the ash values in the following order: total ash > water-soluble ash > acid-insoluble ash (Table 2). Preliminary phytochemical screening indicated the presence of secondary metabolites, including polyphenolics, terpenoids, alkaloids, amino acids, glycosides and tannins. Furthermore, the fluorescence study of the powdered drug was conducted after treatment with analytical-grade chemicals and reagents (Table 3).

High performance thin layer chromatography (HPTLC) profiling of *D. denudatum*

The chromatographic profiling revealed that the methanolic, chloroform and petroleum ether extracts were well distinguished using the following mobile phases: (Hexane: Ethyl acetate: Formic acid; 60:20:20, v/v/v), (Hexane: Methanol; 90:10, v/v) and (Chloroform: Ethyl acetate: Formic acid; 60:35:05, v/v/v) respectively. The methanol extract yielded clear spots at a wavelength of 254 nm, while satisfactory and compact spots for the chloroform extract were observed at a wavelength of 366 nm. The spraying agent, anisaldehyde sulfuric acid, proved effective for the petroleum ether extracts. After air-drying, the developed plate was placed in an oven at 110 °C for 10 min to achieve com-

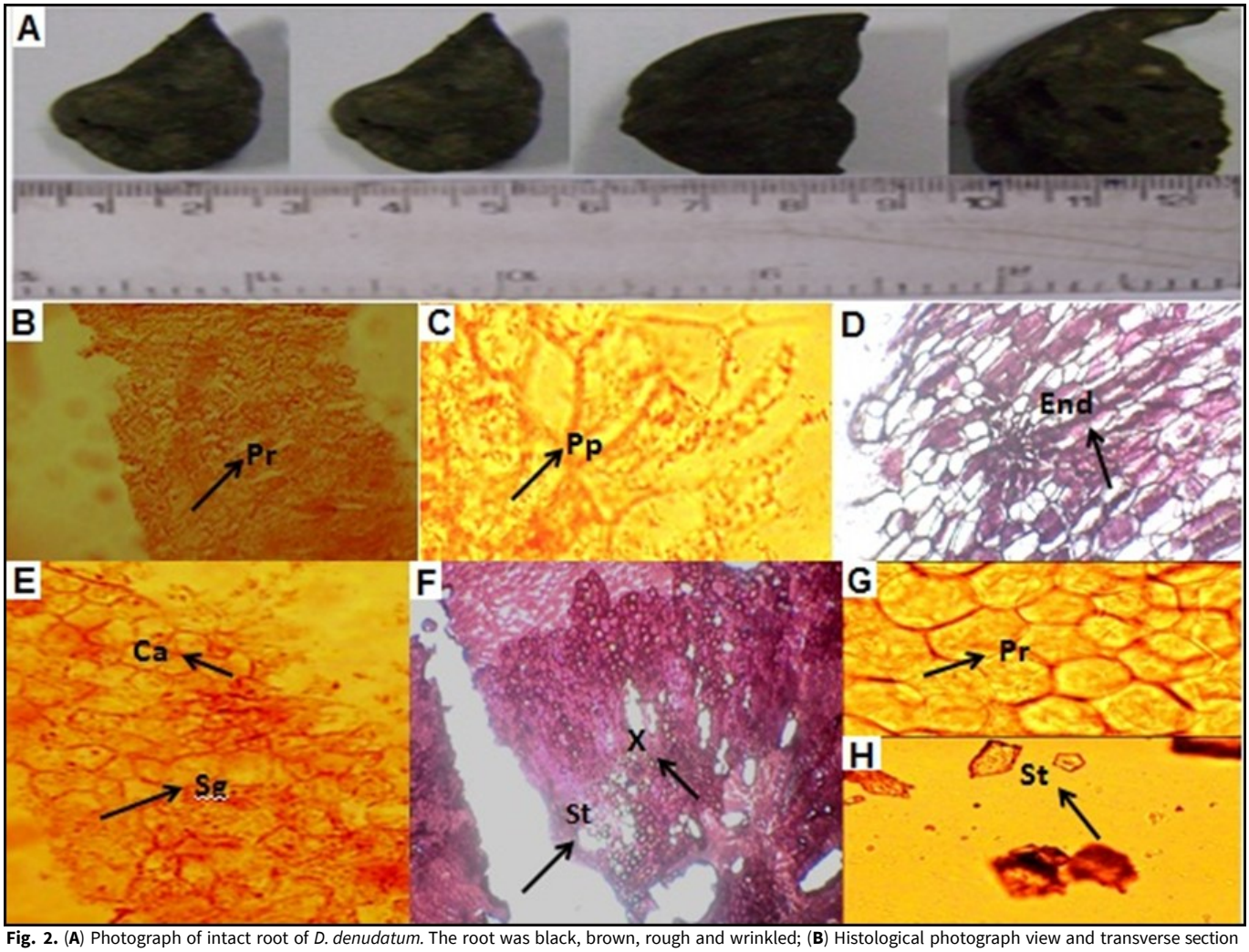

Fig. 2. (**A**) Photograph of intact root of *D. denudatum*. The root was black, brown, rough and wrinkled; (**B**) Histological photograph view and transverse section (T.S) by Motic microscope (dual slide holder with rack less stage; 100X) of *D. denudatum* root. **Pr**: parenchyma (rectangular and irregular arrangement); (**C**) **Pp**: Phloem parenchyma (continuous distribution); (**D**) **End**: Endodermis (closely packed); (**E**) **Sg**: Starch grain (discontinuous distribution); **Ca**: Calcium oxalate (oval cluster and arrange in a groups); (**F**) **X**: xylem cells (not continuous arrangement); (**G**) **Pr**: parenchyma (hexagonal); (**H**) **St**: stone cells (globular intermittently spread).

Table 1. Percentage extractive yield of *D. dendatum* with different solvent by traditional techniques

Extraction Technique	Solvent use for extraction (mL)	% yield (% w/w)^{n*}
Cold extraction	Petroleum ether	0.13 ± 0.12^{a}
	Chloroform	2.96 ± 0.25^{b}
	Methanol	1.47 ± 0.273^{c}
	Aqueous	1.31 ± 0.41^{d}
Hot extraction	Petroleum ether	0.43 ± 0.41^{e}
	Chloroform	3.64 ± 0.45^{f}
	Methanol	4.83 ± 0.43^{g}
	Aqueous	1.75 ± 0.31^{d}
Soxhlet extraction	Petroleum ether	0.88 ± 0.35^{h}
	Chloroform	3.76 ± 0.51^{f}
	Methanol	7.15 ± 0.24^{d}

Traditional extraction results expressed in term of % yield (% w/w) as mean of triplicate *(n=3) ± standard deviations. Values in lower artistic letters indicate significant differences (P < 0.05) between different extracts.

pact spots. A total of 7, 5 and 7 phytoconstituents were identified in the HPTLC fingerprints of the methanolic, chloroform and petroleum ether extracts of *D. denudatum* (Table 4).

Based on the HPTLC study conducted on extracts

Table 2. Results of physicochemical parameters of *D. denudatum* root powder

Physicochemical parameters	Value ± SD*
Loss on drying	3.77 ± 0.13
Moisture content	2.56 ± 0.25
Total ash	4.97 ± 0.05
Acid insoluble ash	2.19 ± 0.05
Water soluble ash	2.78 ± 0.06

*Physicochemical results expressed as mean of three values (n=3) ± standard deviations at p< 0.05.

obtained through traditional extraction techniques, we established the chromatographic parameters for subsequent studies. The optimal mobile phase (Hexane: Ethyl acetate: Formic acid; 60:20:20, v/v/v) was selected for the SFE-assisted *D. denudatum* extracts, based on the high retention factors (Rf) of the phytoconstituents. The HPTLC fingerprint profiles of the SFE-assisted *D. denudatum* extracts were then executed at three different levels: level I (150 bar at 60 °C), level II (200 bar at 70 °C) and level III (250 bar at 80 °C), using different chromatographic conditions (daylight, after derivatization, short wavelength 254 nm and long wavelength 366 nm).

The results under various chromatographic conditions were documented: in daylight, the SFE level-1 extract

Table 3. Results of *D. denudatum* root powder analysis with the treatment of different laboratory reagents

Treatment with laboratory reagents	Color of extract under laboratory light	Color of extract (λ=254 nm)*	Color of Extract (λ=366 nm)**	Phytoconstituents specific to each reagents
Plant powder + Ammonia solution	Reddish brown	Greenish brown	Brown	Alkaloids
Plant powder + Double distilled water	Earthy brown	Dark brown	Brown	Tannins
Plant powder + 5 % $FeCl_3$ reagent	Black	Yellowish Black	Reddish black	Phenols
Plant powder + Acetic acid	Light brown	Light brown	Brown	Triterpenoids
Plant powder + concentrated HNO_3	Green	Greenish black	Green	Proteins
Plant powder + Lugol Solution	Black	Bluish black	Blue	Starch
Plant powder + concentrated HCl	Brown	Reddish brown	Dark brown	Phlobatannins
Plant powder + concentrated H_2SO_4	Dark yellow	Dark brown	Black	Carbohydrates
Plant powder + 5 % Aqueous NaOH	Light orange	Orange	Orange	Anthocyanins
Plant powder + 5 % Aqueous KOH	Light orange	Orange	Orange	Flavonoids

*Short distance wavelength-254 nm; **Long distance wavelength-366 nm.

Table 4. Results of HPTLC fingerprint profile of *D. denudatum* extracts

D. denudatum extract*	Solvents use for mobile phase (mL)	Detection wavelength (nm) and visualizing agent	No of spot and Rf values
Methanol extract	Hexan: Ethyl acetate: Formic acid (60:20:20, v/v/v)	254	**(7)** 0.19, 0.33, 0.41,0.61, 0.73, 0.74, 0.67
Chloroform extract	Hexane: Methanol (90:10, v/v)	366	**(5)** 0.31, 0.55, 0.35,0.54, 0.42
Petroleum ether extract	Chloroform: Ethyl acetate: Formic acid (60:35:05, v/v/v)	Anisaldehyde sulphuric acid	**(7)** 0.21, 0.43, 0.25, 0.36, 0.45, 0.33, 0.53

*Extracts use for HPTLC fingerprint profile obtained by tredetinal techniques (Soxhlet extraction) through organic sovents. Extract selection based on perecntage yield (w/w).

(tracks 1-3) exhibited 10 compounds; the SFE level-2 extract (tracks 4-6) showed 9 compounds and the SFE level-3 extract (tracks 7-9) contained 5 compounds (Fig. 3A). Similar results were obtained after derivatization of the developed HPTLC plate (Fig. 3B). The HPTLC chromatogram showed that the SFE level-1 extract (tracks 1-3) contained 12 compounds, the SFE level-2 extract (tracks 4-6) had 11 compounds and the SFE level-3 extract (tracks 7-9) revealed 4 compounds in daylight (Fig. 3C) and after derivatization (Fig. 3D).

Encouraging results were also observed when scanning the developed HPTLC plate of SFE-assisted *D. denudatum* root extract at different wavelengths: 254 nm (Fig. 4A) and 366 nm (Fig. 4B). The chromatographic HPTLC chromatogram for different level extracts showed that the SFE level-1 extract (tracks 1-3) contained 10 compounds, the SFE level-2 extract (tracks 4-6) had 11 compounds and the SFE level-3 extract (tracks 7-9) contained 2 compounds at 254 nm (Fig. 4C). In contrast, the HPTLC plate for different level extracts scanned at 366 nm exhibited the following compounds: SFE level-1 extract (tracks 1-3) had 11 compounds, SFE level-2 extract (tracks 4-6) had 9 compounds, and SFE level-3 extract (tracks 7-9) had 10 compounds as discussed in Fig. 4D and Table 5.

Antimicrobial study

The results of the antimicrobial study were confirmed by measuring the zone of inhibition (ZI) and minimum inhibitory concentration (MIC) of standard drugs (cefixime and fluconazole) and *D. denudatum* SFE level-2 extracts, along with DMSO as a negative control. The inhibitory effect of cefixime was recorded at a low concentration (MIC; 25 µg/mL) against Gram-positive (*S. aureus*: ZI; 13.43 ± 0.15 mm) and Gram-negative (*P. aeruginosa*: ZI; 19.27 ± 0.11 mm) bacteria. In comparison, the antibacterial effects of *D. denudatum* SFE level-2 extracts were observed at a dose of MIC; 100 µg/mL, resulting in ZIs of 5.21 ± 0.29 mm for *S. aureus* and 7.48 ± 0.53 mm for *P. aeruginosa*. The standard antibacterial dose (MIC; 25 µg/mL) was found to be ineffective against fungi, as no zone of inhibition was observed with standard fluconazole. However, fluconazole demonstrated a significant antifungal effect (ZI; 21.37 ± 0.9 mm) at a dose of MIC; 250 µg/mL. Based on the phytochemical investigation results, *D. denudatum* root (DDR) appears to contain several potent phytoactive compounds, as shown in Table 6. The antibacterial action of DDR is attributed to the presence of phytoactive compounds such as alkaloids, carbohydrates, terpenoids and tannins. The zones of inhibition (ZIs) indicate that the antibacterial potential of *D. denudatum* extracts varied, with values ranging from 5.21 ± 0.29 mm for *S. aureus* to 7.48 ± 0.53 mm for *P. aeruginosa*. The antifungal effect was notably strong, with a ZI of 21.37 ± 0.9 mm observed at a dose of MIC; 250 µg/mL. Among the results, *P. aeruginosa* exhibited the largest zone of inhibition (ZI; 7.48 ± 0.53 mm), while *S. aureus* showed the lowest (ZI; 5.21 ± 0.29 mm). The antifungal effect (ZI; 21.37 ± 0.9 mm) was also considerable. These results significantly validate the antibacterial properties of *D. denudatum* SFE level-2 extracts against pathogenic bacteria. Additionally, the *D. denudatum* SFE level-2 extracts exhibited antifungal potential (ZI; 8.15 ± 0.9 mm) against *A. niger* at a MIC of 500 µg/mL (Table 6).

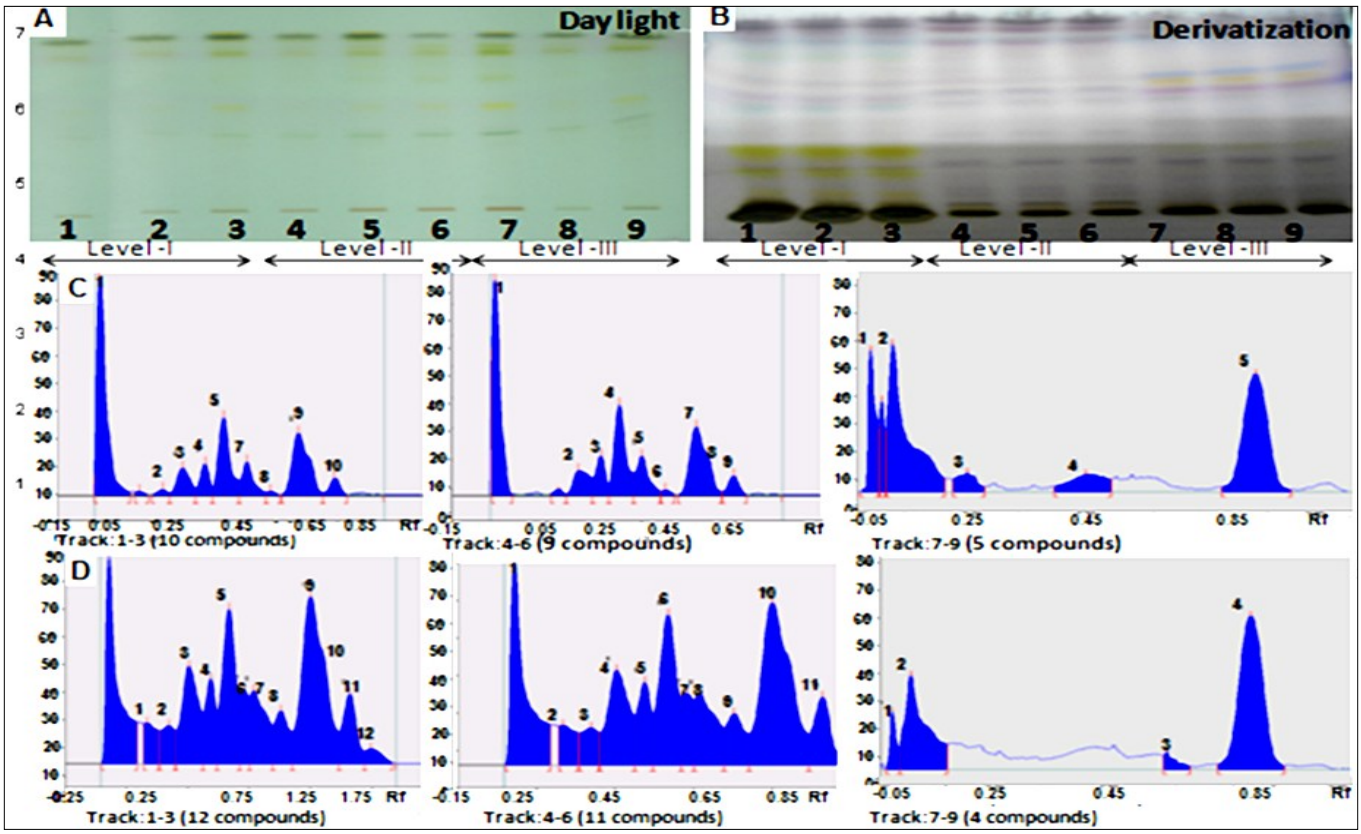

Fig. 3. High performance thin layer chromatography fingerprint profile of *D. denudatum* root extract via SFE at 3 different levels of pressure and temperature (level I:150 bar at 60 °C; 200 bar at 70 °C and 250 bar at 80 °C); **A**: Photograph of developed HPTLC plate in day light, **B**: Photograph of developed HPTLC plate after derivatization; (**C**) Chromatogram of day light developed HPTLC plate of different level extracts; level-1 extract: track 1-3 (10 compounds); level-2 extract: track 4-6 (9 compounds); level-3 extract: track 7-9 (5 compounds); (**D**) Chromatogram after derivatization of developed HPTLC plate of different level extracts; level-l extract: track 1-3 (12 compounds); track 4-6 (11 compounds); track 7-9 (4 compounds); Each level (I-III) shown different *Delphinium* extract, obtained by SFE extracts.

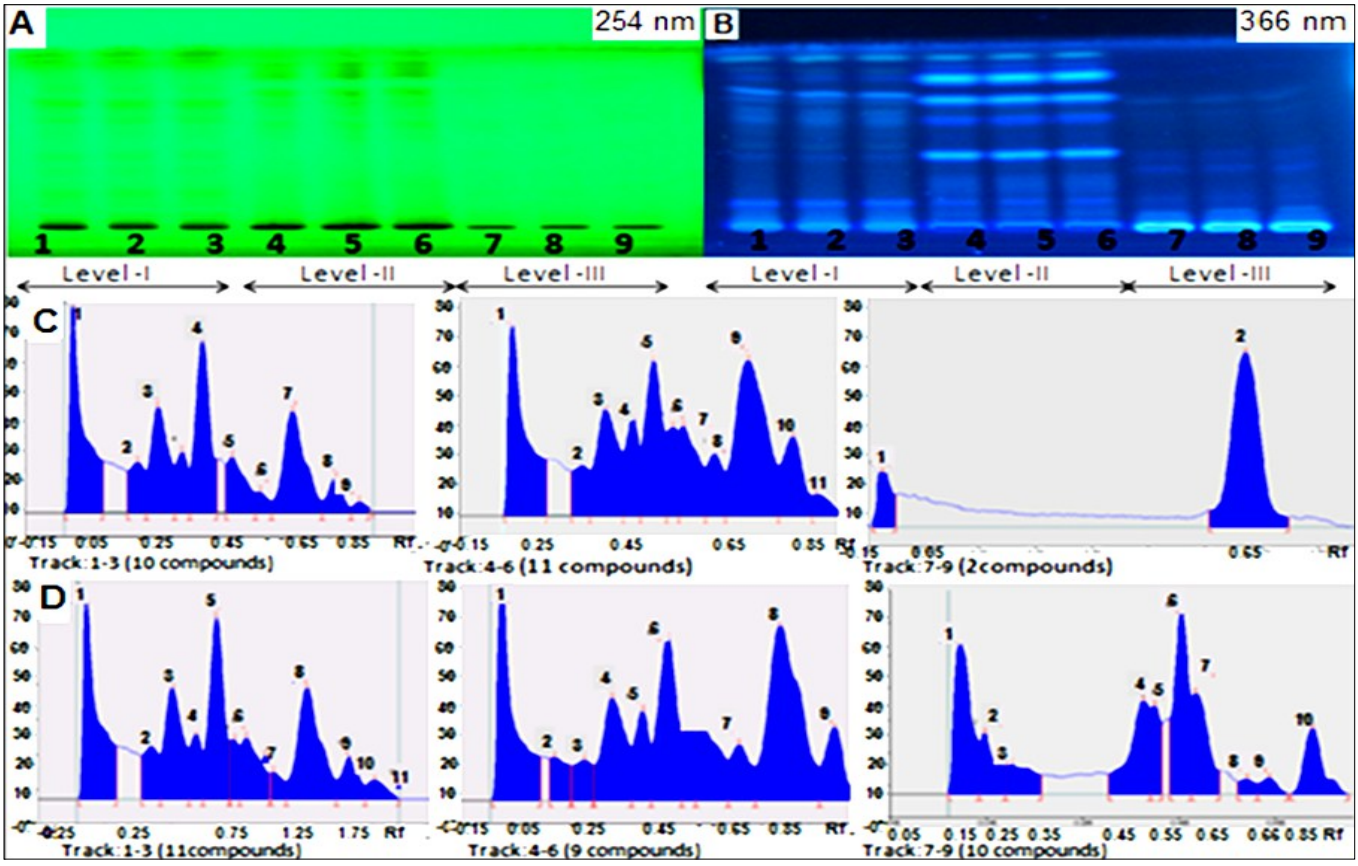

Fig. 4. High performance thin layer chromatography fingerprint profile of *D. denudatum* root extract via SFE at 3 different levels of pressure and temperature (level I:150 bar at 60 °C; 200 bar at 70 °C and 250 bar at 80 °C); **A**: Photograph of developed HPTLC plate at 254 nm, **B**: Photograph of developed HPTLC plate at 366 nm; (**C**) Chromatogram of developed HPTLC plate of different level extracts scan at 254 nm; level-1 extract: track 1-3 (10 compounds); level-2 extract: track 4-6 (11 compounds); level-3 extract: track 7-9 (2 compounds); (**D**) Chromatogram of developed HPTLC plate of different level extracts scan at 366 nm; level-l extract: track 1-3 (11 compounds); track 4-6 (9 compounds); track 7-9 (10 compounds); Each level (I-III) shown different *Delphinium* extract, obtained by SFE extracts.

Table 5. Results of HPTLC fingerprint profile of SFE assisted *D. denudatum* extracts at different levels under controlled pressures (bar) and temperatures (°C)

Detection conditions and visualizing agents	SFE assisted *D. denudatum* extract	HPTLC track numbers	Number of compounds and Rf values*
Day light	Level-1	1-3	**(10)** 0.12, 0.13, 0.34, 0.51, 0.33, 0.64, 0.27, 0.41, 0.29, 0.81
	Level-2	4-6	**(9)** 0.71, 0.25, 0.15, 0.64, 0.22, 0.81, 0.49, 0.85, 0.72
	Level-3	7-9	**(5)** 0.51,0.73,0.54, 0.16, 0.35
Anisaldehyde sulphuric acid	Level-1	1-3	**(12)** 0.49, 0.53, 0.31,0.41, 0.38, 0.95, 0.57, 0.93, 0.43, 0.82, 0.76, 0.41
	Level-2	4-6	**(11)** 0.91, 0.48, 0.81, 0.38, 0.27, 0.53, 0.61, 0.31, 0.29, 0.54, 0.22
	Level-3	7-9	**(4)** 0.85, 0.54, 0.05, 0.91
Short wavelength (254 nm)	Level-1	1-3	**(10)** 0.37, 0.52, 0.43, 0.57, 0.23, 0.43, 0.61, 0.28, 0.45, 0.66
	Level-2	4-6	**(11)** 0.71, 0.94, 0.62, 0.55, 0.49, 0.72, 0.59, 0.77, 0.18, 0.11, 0.88
	Level-3	7-9	**(2)** 0.73, 0.13
Long wavelength (366 nm)	Level-1	1-3	**(11)** 0.98, 0.51, 0.32, 0.11, 0.64, 0.88, 0.45, 0.52, 0.40, 0.22, 0.70
	Level-2	4-6	**(9)** 0.12, 0.43, 0.39, 0.22, 0.56, 0.07, 0.75, 0.27, 0.69
	Level-3	7-9	**(10)** 0.01, 0.83, 0.72, 0.05, 0.19, 0.13, 0.40, 0.85, 0.91, 0.75

SFE assisted *D. denudatum* root extraction carried out at selected pressures (100, 150, 200 and 250 bar) and selected temperatures (60, 70 and 80 °C) employed to optimized extraction protocol for the proposed study. *Mobile phase: Hexane: Ethyl acetate: Formic acid (60:20:20, v/v/v).

Table 6. Antimicrobial effect of *D. denudatum* super critical (SFE-level-2) extract and standard (Cefixime and Fluconazole) drugs

Antimicrobial Activity	Name of *D. denudatum* extract and antimicrobial agents	Pathogenic strains	Zone of inhibition (ZI mm)^{n*}	Minimum inhibitory concentration (MIC µg mL^{-1})
Anti bacterial	*D. denudatum* SFE-level-2 extract	*S. aureus*	5.21 ± 0.29^{a}	100
		P. aeruginosa	7.48 ± 0.53^{a}	100
	Cefixime	*S. aureus*	13.43 ± 0.15^{b}	25
		P. aeruginosa	19.27 ± 0.11^{c}	25
Anti fungal	*D. denudatum* SFE-level-2 extract	*A. niger*	8.15 ± 0.9^{d}	500
	Fluconazole	*A. niger*	21.37 ± 0.9^{e}	250

Antimicrobial results expressed in term of zones of inhibition (ZI mm) as mean of triplicate (n=3) ± standard deviations. Data in column of minimum inhibitory concentration (MIC) of *D. denudatum* SFE-level-2 extract (100 µg mL^{-1}), Cefixime (25 µg mL^{-1}) and Fluconazole (250 µg mL^{-1}) are expressed in µg mL^{-1}. Values in lower artistic letters indicate significant differences ($P < 0.05$) between different microbial strains using the same extract.

Discussion

Plants hold significant medicinal importance in traditional medicine systems and are increasingly sought after in herbal and nutraceutical markets for their potential benefits to human health (25). *D. denudatum* is primarily considered an endangered plant due to the limited availability of its flora and fauna (26). Consequently, the roots of this plant are of high medicinal value, especially considering their benefits for the health of local communities (27). *Delphinium* is a unique botanical species that thrives at high altitudes in the Himalayan region, where it requires specific environmental conditions to grow for a limited period (28). As a result, this rare and interesting plant has not been extensively studied for its biological properties. While traditional healers and herbalists recognize the significance of this species, there is still a considerable lack of scientific research focusing on analytical techniques, bioactive potentials and the medicinal applications of this plant (29).

Today, supercritical fluid extraction (SFE) is recognized as an emerging green technology for the efficient extraction of high-quality herbal drug extracts. SFE extracts are free from hazardous chemicals, as the extraction process utilizes environmentally friendly solvents such as water (H_2O) or carbon dioxide (CO_2) (29). While water is the preferred extraction solvent and is abundantly available in nature, CO_2 is gaining popularity due to its favorable operational requirements (30). Moreover, SFE minimizes the risk of extract degradation compared to traditional extraction methods, which often involve direct heat and higher temperatures (31). The plant extracts obtained through SFE are of high quality and free from solvents, even when ethanol is used as a co-solvent. The high penetration and diffusivity of CO_2 within the inner matrix of plant cells make it an ideal choice for extracting bioactive compounds. However, the low polarity of CO_2 poses challenges for extracting polar compounds. To address this issue, previous studies have suggested the addition of a co-solvent (either an organic solvent or water) to enhance extraction efficiency (32). In our study, we conducted state-of-the-art SFE-assisted extraction using CO_2 as the solvent at different pressures (150 bar, 200 bar and 250 bar) and temperatures (60 °C, 70 °C and 80 °C) to optimize the standardized extraction procedure and calculate the % yield.

In this context, the combination of supercritical carbon dioxide (CO_2) and ethanol as a co-solvent was preferred for extracting Delphinium denudatum root (DDR). Previous research has shown that increasing the extraction pressure from low to high enhances the solvent density, resulting in improved solvating capacity and higher extrac-

tion yields (12 However, the relationship between temperature and extraction yield in SFE is not always linear. Consequently, an increase in operational temperature can lead to either a low or high extraction yield from plant material or it may have no effect at all. This variation depends on the effectiveness of solvent density and the vapor pressure of the solute.

The results obtained were consistent with the previously mentioned explanation, showing that high % yields were achieved under optimized SFE operational conditions of 200 bar and 70 °C. The primary objective of this study was to identify the optimal extraction conditions for Delphinium denudatum using the sophisticated SFE technique. Additionally, we conducted extractions using traditional methods to compare and select the best extraction approach. The traditional techniques employed in our study included cold extraction, hot extraction and Soxhlet extraction. While these methods have been utilized by various authors for extracting plant constituents, they also have some drawbacks. Notably, the yields from these traditional techniques were lower than those obtained through SFE (33). Cold extraction, for instance, requires prolonged solvent and solute contact time for effective extraction (34). On the other hand, heat-assisted extraction methods (hot extraction and Soxhlet extraction) operate at higher temperatures, which have been reported to degrade bioactive compounds. Furthermore, the use of organic solvents, such as benzene, poses health risks and environmental concerns, whereas SFE extracts are free of hazardous chemicals (35, 36).

The present study provides a comprehensive botanical analysis of *Delphinium* root to facilitate the identification and understanding of adulterations associated with inferior quality plant material. Consequently, we conducted detailed assessments of morphological, cellular, physicochemical and quality parameters, which are exceptional and can be used to identify *Delphinium* species and their herbal products. Additionally, the phytometabolites were evaluated using supercritical fluid extraction (SFE).

The morphological and cellular examination of various plant tissues is crucial for the botanical and pharmacognostical assessment of herbal drugs (37). Root studies, in particular, hold significant importance in establishing the genetic and taxonomical profiles of closely related species. These studies are also essential for the initial identification of herbal drugs and for botanical verification in both the herbal industry and plant-based research (38).

This report presents the macroscopic and microscopic characteristics of the root of *D. denudatum*. It highlights key morphological features, including tissue arrangement, root size, shape, texture, color and scar, providing valuable information for future research, standardization and differentiation between adulterated and high-quality herbal products. Based on this analysis, it was observed that *D. denudatum* possesses compound trifoliate leaves that are alternately attached to the stems. The roots are highly wrinkled, conical in shape and have a rough surface.

Our study aligns with previously published reports on the root anatomy of plants in the *Rutaceae* family (39). Experiments have been conducted to explore the various characteristics and features of the roots belonging to this family. Notably, the root structure of *D. denudatum* shares similarities with *Aconitum* in several aspects, such as root shape, outer shell texture, surface color and rough root scars. These observed root features are common within the family *Delphinieae*. Additionally, the root morphology of *Delphinium* is quite similar to that of *Aconitum*, including the presence of a parenchyma layer, stone cells and pitted starch granules. Variations in morphological and microscopic features have been examined in the roots of *Delphinium* (40). Additionally, the root morphology of *Delphinium* is quite similar to that of *Aconitum*, including the presence of a parenchyma layer, stone cells and pitted starch granules (41). Variations in morphological and microscopic features have been examined in the roots of *Delphinium*. The diversity in root characteristics among different *Delphinium* species underscores the significant role of these anatomical features in distinguishing and authenticating plants at the botanical level.

Delphinium roots are known for their medicinal value due to the presence of C19 and C20 alkaloids, which exhibit various pharmacological actions (4, 25, 42). The SFE-assisted HPTLC analysis was conducted to identify bioactive constituents that can serve as a markers for quality assessment and standardization of botanical and herbal products. The bioactive constituents found in the roots of *Delphinium*, including C19 alkaloid, C20 alkaloids, resins, saponin glycosides, highlights the significance of this plant in biological processes. These constituents indicate the potential of root extracts for nephroprotective, psychoactive and antimicrobial effects against various health issues (3). Previously, several derivatives of the norditerpenoid lycoctonine alkaloid have been isolated from the roots of *D. denudatum*, exhibiting significant antimicrobial activity against human pathogens and antifeedant activity against insects such as *Spodoptera littoralis* and *Lepidoptera decemlineata* (43, 44). An earlier report confirmed the de-addiction properties of *D. denudatum* root extracts in morphine addiction (45). The cellular study elucidates the characteristics of the cellular arrangement in the structural components of *D. denudatum* roots. The fluorescence properties of the powdered material at altered wavelengths are associated with the authenticity and standardization of the plant material (46). The powdered drug was treated with a range of organic and inorganic reagents for fluorescence analysis at short (254 nm) and long (366 nm) wavelengths to identify the phytoconstituents.

Since the plant is obtained from wild sources, there is a significant risk of contamination, which can indicate potential adulteration. Consequently, the ash values were calculated mathematically to assess quality (47). The ash value establishes a direct link to the quality parameters of *D. denudatum*. Total ash can indicate the presence of inorganic substances, such as carbonates, silicates, oxalates and phosphates. High total ash values raise concerns about adulteration and question the quality and purity of

the botanicals and their products (48). Previous literature suggests that acid-insoluble ash is indicative of associated silica; therefore, elevated levels of acid-insoluble ash may result from contamination with sandy materials (49). Inorganic elements can also be quantified through water-soluble ash analysis. In our study, the total ash content in the *D. denudatum* sample was found to be 4.97 % w/w, while the acid-insoluble ash was 2.19 % w/w and the water -soluble ash was 2.78 % w/w. Our results establish the following order for ash values: total ash > water-soluble ash > acid-insoluble ash (50).

Introductory phytochemical screening explores the presence of secondary metabolites, including polyphenolics, terpenoids, alkaloids, amino acids, glycosides and tannins, which have been discussed in earlier reports and are responsible for the bio-efficacy of *Delphinium*. These metabolites also exhibit effective pharmacological activity (19). *Delphinium denudatum* is recognized as a source of C19 and C20 diterpene alkaloids, such as delbrunine, delcarpum, delbruline and delbrusine (14). Earlier studies have indicated that the structure-activity relationship (SAR) is crucial for biological activity, depending on the character and position of different substituents on the compounds (33). Generally, alkaloids with substituents such as OH, OCH_3, CH_3, CH_2OH, CH_2CH_3 and others are more favorable for drug formulation than those with 1 or 2 esters; these substituents are predominantly found in diterpene alkaloids. Structurally, *Delphinium* alkaloids are claimed to have antimicrobial activities, a mechanism that has been confirmed by various researchers (37).

Based on the utilization of supercritical fluid extraction (SFE) for extracting *Delphinium* roots, retention factors (Rf) were determined using high-performance thin-layer chromatography (HPTLC) at different SFE extract levels (level-1, level-2 and level-3). The Rf values for various constituents of *Delphinium* were assessed under four different conditions: daylight, after derivatization, at 254 nm and at 366 nm. Furthermore, this study represents the first attempt to map various phytometabolites in root samples via SFE-assisted HPTLC fingerprinting. Several studies have reported that a range of solvents is used to detect phytoconstituents from root samples, employing a mobile phase of (Ethanol: Formic acid: Chloroform; 60:20:20 v/v/v) for *Delphinium* extract. In contrast, this study selected (Toluene: Ethyl acetate: Formic acid; 60:40:10 v/v/v), which provided clearer separation and visualization of the phytoconstituents (22, 23).

The order of Rf values observed in our study for the different conditions was daylight > derivatization > 254 nm > 366 nm. Ultimately, the results indicated that HPTLC scanning at 366 nm of the SFE-assisted level-2 extract exhibited the greatest number of phytoconstituents, represented by numerous resultant peaks and Rf values. This may be attributed to the higher compatibility of the active constituents of *D. denudatum* with the CO_2 solvent used in SFE extraction. A similar outcome was previously reported, indicating that the % compatibility of phytoconstituents is higher in CO_2 compared to other solvents (32). Previous studies have noted that SFE is a fast and environmentally friendly solvent-free extraction technique that minimizes harm to phytoconstituents (22). This suggests that the active constituents of *D. denudatum* remained intact even after SFE extraction. Due to the presence of these bioactive natural constituents, subsequent antimicrobial activity was assessed using the agar well diffusion assay method. The presence of secondary phytometabolites may be responsible for the observed antimicrobial activity, which aligns with findings from previous studies. The antibacterial action of *D. denudatum* SFE level-2 extracts can be attributed to these secondary phytometabolites and variations in the photoactive compounds significantly influence the potential for antibacterial activity (41).

As no previous antimicrobial studies have reported on the SFE-assisted extract of *D. denudatum*, the proposed study advocates for the use of *D. denudatum* root extract (DDR) in addressing various life-threatening diseases caused by Gram-positive bacteria, Gram-negative bacteria and fungal infections. Our results are directly linked to the bioactive phytoconstituents present in DDR, which have been cited in earlier reports (29). Therefore, given this information, *D. denudatum* could be a promising candidate for antimicrobial preparations, signalling potential for further therapeutic discoveries.

In the present study, the antimicrobial properties of SFE-assisted *D. denudatum* root extract (DDR) at various concentrations were evaluated and correlated with the zone of inhibition. The microbial strains tested included Gram-positive *Staphylococcus aureus*, Gram-negative *Pseudomonas aeruginosa* and the fungus *Aspergillus niger*. The SFE-assisted level-2 *Delphinium* extract demonstrated strong antibacterial and antifungal activity against the selected pathogens.

This study further confirms the significance of *Delphinium* extract with respect to minimum inhibitory concentration (MIC) determination, as even the lowest concentration exhibited potent antimicrobial action. The antimicrobial activity is attributed to the presence of various phytoconstituents, as it is well established that plant secondary metabolites act as widespread antimicrobial agents (29). These secondary metabolites and plant-derived compounds are considered toxic to microbes, demonstrating a strong bactericidal and fungicidal effect against several microbial strains (18). The biocidal effect of SFE level-2 DDR on harmful microbes can be partially explained by the observation that microbes lose their DNA replication capacity, while the cellular proteins become inactivated during treatment with SFE level-2 DDR (13). Additionally, it is believed that *Delphinium* phytoconstituents bind to the active sites of proteins, leading to denaturation of microbial proteins. In contrast, extracts obtained through traditional techniques have limitations as antimicrobial candidates due to various degradation factors, including the degradation of plant secondary metabolites at high temperatures, the use of harmful solvents, and prolonged exposure of solute to solvent (15). However, these limitations can be overcome by utilizing SFE as an extraction method, given its high extraction yield for DDR due to the rapid diffusivity of carbon dioxide (CO_2) and the

extensive surface exposure of plant material (31). Overall, the green extract from *Delphinium* exhibited superior antimicrobial activity against all experimental organisms. Based on these findings, *D. denudatum* root extract could be considered a promising candidate for antibiotic drug delivery against life-threatening diseases caused by pathogens.

Conclusion

In conclusion, the present study explored the diverse botanical properties of the less-publicized medicinal plant *Delphinium denudatum Wall.* Compared to traditional extraction techniques, supercritical fluid extraction (SFE) can be considered a nature-friendly method, utilizing carbon dioxide (CO_2) as a safe solvent for extracting natural products. The highly structured chromatographic phytoprofiling using SFE and high-performance thin-layer chromatography (HPTLC) highlighted the rich botanical composition of *D. denudatum*. The application of phytobiography through chromatographic analysis established a clear relationship between the SFE extracts, particularly the level-2 extracts of *Delphinium*. Overall, the results of this study strongly suggest that SFE-based *D. denudatum* phytoextracts can be developed as a drug regimen against various pathogenic diseases. To the best of our knowledge, there is no scientific data indicating that *D. denudatum* has been sufficiently studied using SFE. This study also explores the computer-aided anatomical standards of *Delphinium*. Furthermore, the botanical identification of *D. denudatum* is essential to maintain product efficacy and to promote its medicinal traits, providing valuable information for species recognition in future commercial applications.

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Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

References

1. Sookying S, Duangjai A, Saokaew S, Phisalprapa P. Botanical aspects, phytochemicals and toxicity of *Tamarindus indica* leaf and a systematic review of antioxidant capacities of *T. indica* leaf extracts. Front Nutr. 2022;20(9):977015. https://doi.org/10.3389/fnut.2022.977015
2. Mahendran G, Rahman LU. Ethnomedicinal, phytochemical and pharmacological updates on peppermint (*Mentha piperita*)- a review. Phytother Res. 2020;34:2088-139. https://doi.org/10.1002/ptr.6664
3. Yan Y, Jiang H, Yang X, Ding Z, Yin T. Grandiflolines A-F, new anti -inflammatory diterpenoid alkaloids isolated from *Delphinium grandiflorum*. Front Chem. 2022; 19(10):1012874. https://doi.org/10.3389/fchem.2022.1012874
4. Wang WJ, Zha B, Zhao JY. Three new diterpenoid alkaloids from *Delphinium naviculare* var. *lasiocarpum.* Phytochem Lett. 2019;33:12-16. https://doi.org/10.1016/j.phytol.2019.06.009
5. Arunachalam K, Parimelazhagan T. Antioxidant and antimicrobial potential of methanolic extract of Indian sacred grove *Gymnostachyum febrifugum* Benth. root. IJPBR. 2011;2(3):67-71.
6. Gupta AK, K Mahfoozurrahman, Danish K. Physicochemical study of *Delphinium denudatum* Wall. (Ranunculales: Ranunculaceae) and their antioxidant activity. Braz J Biol Sci. 2019;6 (12):161-69. https://doi.org/10.21472/bjbs.061215
7. Gopalakrishnan K, Udayakumar R. GC-MS analysis of phytocompounds of leaf and stem of *Marsilea quadrifolia* (L). Int J Biochem Res Rev. 2014;4(6):517-26. https://doi.org/10.9734/IJBCRR/2014/11350
8. Hassim N, Markom M, Rosli MI, Harun S. Scale-up approach for supercritical fluid extraction with ethanol-water modified carbon dioxide on *Phyllanthus niruri* for safe enriched herbal extracts. Sci Rep. 2021;4:11(1):15818. https://doi.org/10.1038/s41598-021-95222-0
9. Zhang QW, Lin LG, Ye WC. Techniques for extraction and isolation of natural products: a comprehensive review. Chin Med J. 2018;13:20. https://doi.org/10.1186/s13020-018-0177-x
10. Osorio-Tobon JF. Recent advances and comparisons of conventional and alternative extraction techniques of phenolic compounds. J Food Technol. 2020;57:4299-315. https://doi.org/10.1007/s13197-020-04433-2
11. Pronyk C, Giuseppe M. Design and scale-up of pressurized fluid extractors for food and bioproducts. J Food Eng. 2009;95:215-26. https://doi.org/10.1016/j.jfoodeng.2009.06.002
12. Montesantos N, Maschietti M. Supercritical carbon dioxide extraction of lignocellulosic bio-oils: The potential of fuel upgrading and chemical recovery. Energies. 2020;13:1600. https://doi.org/10.3390/en13071600
13. Hu H, Zhang J, Chen G. Extraction of Huadian oil shale with water in sub-and supercritical states. Fuel. 1999;78(6):645-51. https://doi.org/10.1016/S0016-2361(98)00199-9
14. Norodin NSM, Salleh LM, Mustafa NM. Supercritical carbon dioxide (SC-CO_2) extraction of essential oil from *Swietenia mahagoni* seeds. Mater Sci Eng. 2016;162:12-30. https://doi.org/10.1088/1757-899X/162/1/012030
15. Krakowska-Sieprawska A, Rafińska K, Walczak-Skierska J, Kiełbasa A, Buszewski B. Promising green technology in obtaining functional plant preparations: Combined enzyme-assisted supercritical fluid extraction of flavonoids isolation from *Medicago sativa* leaves. Materials (Basel). 2021;14 (11):2724. https://doi.org/10.3390/ma14112724
16. Tyskiewicz K, Konkol M, Roj E. The application of supercritical fluid extraction in phenolic compounds isolation from natural plant materials. Molecules. 2018;23(10):2625. https://doi.org/10.3390/molecules23102625
17. da Silva CG, Lucas AM, Santo ATDE, Almeida RN, Cassel E, Vargas RM. Sequential processing of *Psidium guajava* L. leaves: steam distillation and supercritical fluid extraction. Braz J Chem Eng. 2019;36(1):487-96. https://doi.org/10.1590/0104 6632.20190361s20170215
18. Suleiman WB. *In-vitro* estimation of supercritical fluid extracts of some plants for their antimicrobial potential, phytochemistry and GC-MS analyses. Ann Clin Microbiol Antimicrob. 2020;19:29. https://doi.org/10.1186/s12941-020-00371-1
19. Chen Y, Zhang X, Guo Q. Plant morphology, physiological characteristics, accumulation of secondary metabolites and antioxidant activities of *Prunella vulgaris* under UV solar exclusion. J Biol Res. 2019;52:17. https://doi.org/10.1186/s40659-019-0225-8

20. Cabi E, Dogan M, Mavi O, Karabacak E, Baser B. *Elymus sosnowskyi* (Hackel) Melderis (Poaceae), a rare endemic species in Turkey. Turk J Bot. 2010;34(2):105-14. https://doi.org/10.3906/bot-0902-4

21. Harborne JB. Phytochemical methods. Chapman and Hall Ltd., London. 1973. p. 49-188.

22. Mushtaq A, Akbar S, Zargar MA, Wali AF, Malik AH, Dar MY, et al. Phytochemical screening, physicochemical properties, acute toxicity testing and screening of hypoglycaemic activity of extracts of *Eremurus himalaicus* baker in normoglycaemic Wistar strain albino rats. Biomed Res Int. 2014;867547. https://doi.org/10.1155/2014/867547

23. Mukherjee PK. Quality control of herbal drugs. 1st Edition, Business Horizon, Pharmaceutical Publishers, New Delhi. 2002; p. 380-422.

24. Daoud A, Malika D, Bakari S, Hfaiedh N, Mnafgui K, Kadri A, Gharsallah N. Assessment of polyphenol composition, antioxidant and antimicrobial properties of various extracts of date palm pollen (DPP) from two Tunisian cultivars. Arab J Chem. 2019;12 (8):3075-86. https://doi.org/10.1016/j.arabjc.2015.07.014

25. Zhao ZG, Yan HF, Zheng R, Saeed K, Fu M, Tao X, Zhang ZY. Anthocyanins characterization and antioxidant activities of sugarcane (*Saccharum officinarum* L.) rind extracts. Ind Crops Prod. 2018;113:38-45. https://doi.org/10.1016/J.INDCROP.2018.01.015

26. Mantena VRKR, Soni D, Rao GVB. Anti asthmatic activity of methanolic extract of *Delphinium denudatum* Wall. Int J Pharm Sci. 2011;3(4):167-70.

27. Werff HVD, Consiglio T. Distribution and conservation significance of endemic species of flowering plants in Peru. Int J Biodivers. 2004;13(9):1699-713. https://doi.org/10.1023/B:BIOC.0000029334.69717.f0

28. El Aziz MMA, Ashour AS, Melad ASG. A review on saponins from medicinal plants; chemistry, isolation and determination. J Nanomed Res. 2019;7(4):282-88. https://doi.org/10.1 5406/jnmr.2019.07.00199

29. Passos CP, Silva RM, Silva FAD, Coimbra MA, Silva CM. Enhancement of the supercritical fluid extraction of grape seed oil by using enzymatically pre-treated seed. J Supercrit Fluids. 2009;48:225-29. https://doi.org/10.1016/j.supflu.2008.11.001

30. Nagavekar N, Singhal RS. Enhanced extraction of oleoresin from *Piper nigrum* by supercritical carbon dioxide using ethanol as a co-solvent and its bioactivity profile. J Food Process Eng. 2018;41:12670. https://doi.org/10.1111/jfpe.12670

31. Karami Z, Emam-Djomeh Z, Mirzaee HA, Khomeiri M, Mahoonak AS, Aydani E. Optimization of microwave assisted extraction (MAE) and Soxhlet extraction of phenolic compound from licorice root. J Food Technol. 2015;52:3242-53. https://doi.org/10.1007/s13197-014-1384-9

32. Vatai T, Skerget M, Knez Z. Extraction of phenolic compounds from elder berry and different grape marc varieties using organic solvents and/or supercritical carbon dioxide. J Food Eng. 2009;90:246-54. https://doi.org/10.1016/j.jfoodeng.2008.06.028

33. Sun Q, Shi J, Scanlon M, Xue SJ, Lu J. Optimization of supercritical CO_2 process for extraction of tocopherol rich oil from Canola seeds. LWT Food Science and Technology. 2021;145:111435. https://doi.org/10.1016/j.lwt.2021.111435

34. Dumont MJ, Narine SS. Soap stock and deodorizer distillates from North American vegetable oils: Review on their characterization, extraction and utilization. Int Food Res J. 2007;40(8):957-74. https://doi.org/10.1016/j.foodres. 2007.06.006

35. Fikselova M, Silhar S, Marecek J, Francakova H. Extraction of carrot (*Daucus carota* L.) carotenes under different conditions. Czech J Food Sci. 2008;26:268-74. https://doi.org/10.17221/9/2008-CJFS

36. Mustafa A, Turner C. Pressurized liquid extraction as a green approach in food and herbal plants extraction: A review. Anal Chim Acta. 2011;703:8-18. https://doi.org/10.1016/j.aca.2011.07.018

37. Sattler R. Kaplan's principles of plant morphology: A critical review. Bot Rev. 2022;88:257-70. https://doi.org/10.1007/s12229-022-09280-8

38. Martinez-Gomez J, Atluri TAM, Rose IJ, Holliday AJ, Strock CF, Lync JP, et al, Developmental morphology and anatomy shed light on both parallel and convergent evolution of the umbellate inflorescence in monocots, underlain by a new variant of metatopy. Front Plant Sci. 2022;13:873505. https://doi.org/10.5281/zenodo.6012529

39. Turabekova MA, Rasulev BF, Dzhakhangirov FN, Toropov AA, Leszczynska D, Leszczynski J. *Aconitum* and *Delphinium* diterpenoid alkaloids of local anaesthetic activity: Comparative QSAR analysis based on GA-MLRA/PLS and optimal descriptors approach. Environ Carcinog Ecotoxicol Rev. 2014;32:213-38. https://doi.org/10.1080/10590501.2014.938886

40. Orellana MR, Lopez-Pujol J, Blanche C. Genetic diversity in *Delphinium staphisagria* (Ranunculaceae), a rare Mediterranean dysploid larkspur with medicinal uses. Genetica. 2009; 135:221-32. https://doi.org/10.1007/s10709-008-9271-9

41. Utelli AB, Roy BA, Baltisberger M. Molecular and morphological analyses of *European aconitum* species (Ranunculaceae). Pl Syst Evol. 2000;224(3):195212. https://doi.org/10.3390/molecules23102625

42. Diaz JG, Herz W. Acylated flavonol tetraglycosides from *Delphinium gracile*. Phytochemistry. 2000;71(4):463-68. https://doi.org/10.1016/j.phytochem.2009.11.011

43. Rahman A, Nasreen A, Akhtar F, Shekhani MS, Clardy J, Parvez M, Choudhary MI. Antifungal diterpenoid alkaloids from *Delphinium denudatum* Wall. J Nat Prod. 1997;60(5):472-74. https://doi.org/10.1021/np960663n

44. Bhat BA, Mir WR, Sheikh BA, Rather MA, Dar TH, Mir MA. *In-vitro* and *in-silico* evaluation of antimicrobial properties of *Delphinium cashmerianum* L., a medicinal herb growing in Kashmir, India. J Ethnopharmacol. 2022;291:115046. https://doi.org/10.1016/j.jep.2022.115046

45. Zafar S, Ahmad MA, Siddiqui TA. Protective role of *Delphinium denudatum* (Jadwar) against morphine induced tolerance and dependence in Mice. J Ethnopharmacol. 2001; 78:95-98. https://doi.org/10.1016/s0378-8741(01)00317-8

46. Amina M, Musayeib Al, Alarfaj NM, El-Tohamy NA, Al-Hamoud MF, Alqenaei GA. The fluorescence detection of phenolic compounds in *Plicosepalu scurviflorus* extracts using biosynthesized ZnO nanoparticles and their biomedical potential. Plant. 2022;11:361. https://doi.org/10.3390/plants11030361

47. Prakash A, Janmeda P, Pathak P. Development and standardization of quality control parameters of different parts of *Trianthema portulacastrum* L.. SN Appl Sci. 2019;1:1108. https://doi.org/10.1007/42452-019-1074-3

48. Dave R, Nagani K, Chanda S. Pharmacognostic studies and physicochemical properties of the *Polyalthia longifolia* var. *pendula* leaf. Pharmacogn Mag. 2010;2:572-76. https://doi.org/10.1016/S0975-3575 (10)80062-8

49. Mercy LS, Gnanamani A, Ilavarasan R. Physicochemical, phytochemical and high performance thin layer chromatography analysis of the whole plant of *Orthosiphon thymiflorus* (Roth.) Sleesen. Asian J Pharm Clin Res. 2015;8(1):181-84.

50. Yin T, Cai L, Ding Z. An overview of the chemical constituents from the genus *Delphinium* reported in the last four decades. R Soc Chem. 2020;10:13669-86. https://doi.org/10.1039/D0RA00813C