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Research Article

Antioxidant-Antimicrobial Evaluation of *Clematis Tibetana* Kuntze: A Phytochemical Study from the Cold Desert Biosphere Reserve, Spiti, India

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Abstract

The present study investigates the phytochemical composition, antioxidant capacity, and antimicrobial potential of the methanolic leaf extract of *Clematis tibetana*, a traditionally used medicinal plant from cold desert regions. The extract exhibited substantial levels of phenolics (47.89 µg/mg GAE) and flavonoids (210.68 µg/mg rutin equivalents), surpassing values reported for several other *Clematis* species. Strong antioxidant activity was demonstrated by a DPPH radical-scavenging IC_{50} of approximately 103 µg/mL. Antimicrobial evaluation revealed notable inhibitory effects against *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, and Pseudomonas aeruginosa*. GC–MS profiling identified eight compounds, with major constituents including benzene-propanoic acid derivatives (54.35%), dibutyl phthalate (18.15%), undecane (12.30%), and dodecane (8.88%), many of which are associated with antimicrobial and antioxidant activities. Overall, the findings underscore the phytochemical richness and bioactive potential of *C. tibetana*, supporting its value as a promising source of natural antioxidant and antimicrobial agents and warranting further investigation into its active constituents and therapeutic mechanisms.

Keywords: Antimicrobial, Antioxidant, Cold Desert, GC-MS, Phytochemical

Introduction

Since ancient times, medicinal plants have served as essential resources for disease prevention and treatment across cultures worldwide. Their therapeutic value arises from a remarkable diversity of bioactive metabolites that contribute to broad pharmacological activity [1]. A wide range of naturally occurring compounds-such as alkaloids, terpenoids, polyphenols, and coumarins-along with prominent plant-derived drugs including morphine, artemisinin, and taxol, underscores the longstanding and continuing importance of plant systems in pharmaceutical development [2].

India hosts an extensive network of traditional healers and practitioners who operate alongside formally trained professionals from well-established indigenous medical systems, including Ayurveda, Siddha, Unani, Amchi, and Sowa-Rigpa. The Indian Himalayan region is renowned for its rich biodiversity [3]. The Amchi system-rooted in the Mongolian term *Amrjay* and embedded

in the broader Tibetan medical tradition-is practiced across regions such as Tibet, Mongolia, Bhutan, China, Nepal, Russia, and India. Within India, the Himalayan states of Himachal Pradesh and Sikkim are key centers for the Amchi System of Medicine [4]. The vast reservoir of traditional knowledge accumulated over generations in these regions has contributed significantly to their flourishing botanical diversity, offering a wealth of therapeutic applications. Moreover, medicinal plants continue to form a vital resource for modern drug discovery directed at developing new therapeutic agents [5]. Ethnobotanical studies illuminate the cultural and biological interplay between people and plants, enriching our understanding of human–plant relationships within cultural landscapes [6]. Numerous investigations in indigenous and traditional contexts have documented the biological, medicinal, and pharmacological relevance of diverse plant and fungal species [7].

Within this framework, the genus *Clematis* (Ranunculaceae) stands out as a rich source of bioactive constituents, including glycosides, saponins, alkaloids, and other phytochemicals with recognized therapeutic value [8]. In Pakistan, various *Clematis* species are traditionally utilized as natural food preservatives and as topical treatments for skin disorders. In the Indian Himalayan region, they are used to alleviate pruritic conditions, support wound healing, reduce viral fevers, regulate cardiac disturbances, and manage bacterial infections [9]. Comprising nearly 350 species distributed across temperate and subtropical regions [10], the genus is known for producing an extensive spectrum of secondary metabolites such as triterpenes, steroids, lignans, flavonoids, coumarins, phenolic glycosides, macrocyclic compounds, volatile oils, and fatty acids [11-14]. These chemical constituents underpin a variety of pharmacological activities reported in *Clematis*, including anti-inflammatory, antinociceptive, antitumor, antibacterial, and antioxidant effects [15-17].

Among the diverse members of this genus, *Clematis tibetana* Kuntze holds prominence due to its ethnomedicinal relevance and phytochemical richness within Himalayan traditional medicine. Traditionally used to treat skin ailments such as "Huangshui disease," its longstanding therapeutic use points toward potential anti-inflammatory properties. Phytochemical investigations have shown that *C. tibetana* is especially enriched with flavonoid glycosides-primarily quercetin, luteolin, kaempferol, and apigenin [18]-and contains triterpenoid saponins such as clematibetosides A–C in its aerial parts [19]. Despite these promising findings, comprehensive biochemical and pharmacological studies on C. tibetana remain limited. The present study addresses this gap by examining leaf extracts of *C. tibetana* with a focus on total phenolic and flavonoid content, antioxidant activity, and antimicrobial potential, aiming to provide a holistic assessment of its bioactive properties in alignment with its ethnomedicinal significance.

Methodology

Collection of Plant and Extraction

Plants were collected in July from the cold desert landscape of Pin Valley in Spiti, India, with their selection and harvesting period guided exclusively by ethnobotanical relevance. After collection, the plant material was thoroughly rinsed under running water and left to air-dry in the shade. Once completely dried, the samples were finely powdered using electric grinders. The powdered material was then subjected to methanolic extraction by soaking in methanol for 48 hours. Following maceration, the mixture was filtered three times using Whatman No. 1 filter paper. The combined filtrate was concentrated through rotary evaporation to remove excess solvent, yielding a crude extract. This extract was subsequently stored at 4°C for later experimental use.

Phytochemical Analysis

Total Phenolic Content: Total Phenolic Content (TPC) was determined using the Folin–Ciocalteu reagent method described by [20], with slight modifications. For the assay, 1 mg/ml of the crude extract was transferred to a test tube, followed by the addition of 2.5 ml of 10% Folin–Ciocalteu reagent. Subsequently, 2 ml of 2% Na_2CO_3 was added, and the mixture was gently shaken to ensure proper mixing. The reaction mixtures were then incubated at room temperature for 15 minutes. Following incubation, absorbance was recorded at 765 nm using a UV spectrophotometer. A calibration curve was prepared using gallic acid standards ranging from 0.025 to 0.125 mg/ml. All measurements were performed in triplicate, and TPC values were calculated from the standard curve and expressed as gallic acid equivalents per milligram of extract.

Total Flavonoid Content: Total Flavonoid Content (TFC) was assessed using the aluminium chloride colorimetric method described by [21]. A rutin calibration curve (0.02–0.1 mg/mL) was prepared for quantification. For the assay, 0.5 mL of each diluted standard solution was mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. The reaction mixtures were incubated at room temperature for 30 minutes, after which absorbance was recorded at 450 nm using a UV spectrophotometer. Flavonoid content was expressed as milligrams of rutin equivalents per microgram of extract.

GC-MS Analysis: GC–MS analysis was performed using a Thermo Scientific TSQ 8000 Gas Chromatograph–Mass Spectrometer coupled with a TRACE 1300 GC system. Data acquisition and processing were carried out with Xcalibur 2.2 SP1 and Foundation 2.0 SP1 software. The separation was achieved on a BP 5MS capillary column (30 m × 0.25 mm, 0.25 µm film thickness) composed of 5% phenyl polysilphenylene-siloxane. A 3.0 µL sample was injected, with the injector temperature maintained at 250°C, the MS transfer line at 240°C, and the ion source at 230°C. The mass spectra were recorded over a range of m/z 30–650. Helium served as the carrier gas at a constant flow rate of 1 mL/min. The oven temperature program began at 50°C (held for 2 minutes), followed by a ramp of 10°C per minute up to 250°C. Compound identification was carried out by comparing the obtained mass spectra with those in the National Institute of Standards and Technology (NIST) library, enabling determination of compound names, molecular weights, and structural information.

Antioxidant Activity: The antioxidant activity of the plant extract was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay following the method of [22], with slight modifications. For the assay, 0.5 mL of the extract at varying concentrations (10–150 µg/mL) was mixed with 3 mL of freshly prepared 0.4% DPPH solution in separate test tubes. After vigorous shaking, the mixtures were incubated in the dark for 30–35 minutes. Absorbance was then measured at 517 nm. Ascorbic acid served as the standard, and methanol was used as the blank. The IC_{50} value-representing the concentration required to inhibit 50% of the DPPH radicals-was calculated using linear regression analysis. Results were expressed as the percentage decrease in DPPH absorbance compared to the control. The scavenging activity of DPPH was determined as:

$$\% \ inhibition = \left[\left(Ac - As \right) / Ac \right] * 100$$

(where, Ac= Absorbance of control; As= Absorbance of test sample)

Antimicrobial Assay: The antimicrobial activity of the plant extract was evaluated using a modified broth microdilution assay following Clinical and Laboratory Standards Institute [23] guidelines. Bacterial strains-including *Staphylococcus aureus* (Gram-positive) and *Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *Escherichia coli* (Gram-negative)-were cultured to mid-log phase, diluted, and inoculated into 96-well plates at a density of 4×10^5 cells per well. The extract, prepared in Luria Broth, was added at concentrations ranging from 25 to 800 µg/mL, and plates were incubated at 37°C for 24 hours. Following incubation, bacterial growth was assessed by measuring optical density at 600 nm, providing an estimate of cell density and viability. The percentage survival relative to untreated controls was calculated to determine the extract's inhibitory effect. IC_{50} values-defined as the concentration that inhibits 50% of bacterial growth-were derived by fitting dose–response data to a nonlinear regression model with a variable slope (GraphPad Prism, Version 8.0), incorporating minimum and maximum inhibition parameters as well as a HillSlope factor. All assays were conducted in triplicate across three independent experiments, and IC_{50} values were reported in µg/mL as mean ± Standard Error (SE).

Growth Curve Assay: The antimicrobial activity of the plant extracts was further verified through growth curve analysis, following NCCLS guidelines with minor modifications [24]. For this assay, 5 mL of bacterial culture with an initial optical density of OD_{600} = 0.02 was transferred into sterile test tubes. The respective IC_{50} concentration of the plant extract, as determined from the microdilution assay, was added and mixed thoroughly. The tubes were sealed with sterile cotton plugs and incubated at 37 ± 2°C in a shaking incubator set to 180 rpm for 24 hours. During incubation, bacterial growth was monitored by recording OD_{600} at 3-hour intervals until the completion of the 24-hour growth period.

Statistical Analysis: For the Antioxidant (DPPH) assay, data were analyzed using two-way ANOVA followed by Sidak's multiple comparison test to assess differences between *Clematis tibetana* leaf extract and ascorbic acid across varying concentrations. Results are presented as mean ± SE from three replicates. Statistical significance was defined as ns ($p > 0.05$), * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$). For the growth curve assay, values are expressed as mean ± SE of three replicates, and significance between Untreated (UT) and treated groups at 24 hours was determined using an unpaired two-tailed t-test, applying the same significance thresholds. All statistical analyses were performed using GraphPad Prism version 8.0.

Results

Total Phenolic Content

The total phenolic content of the methanolic leaf extract of *C. tibetana*, determined using the Folin–Ciocalteu method, was 47.89 ± 0.18 µg GAE/mg (Figure. 1A–B).

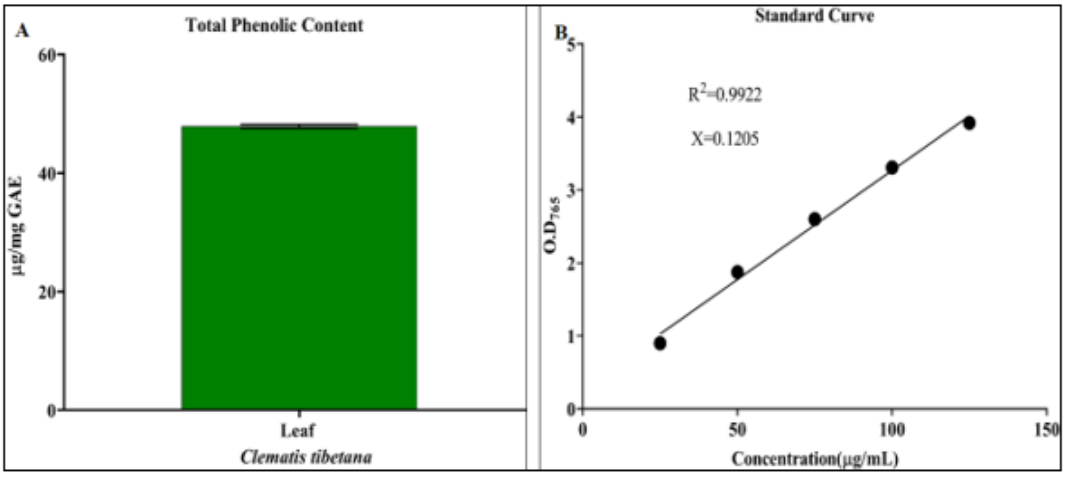


Figure 1: TPC (A) Total phenolic content of the leaf extract of *C. tibetana* (B) The standard curve of gallic acid used for measuring TPC. The finding of three distinct studies reported as mean ± SE.

Total Flavonoid Content

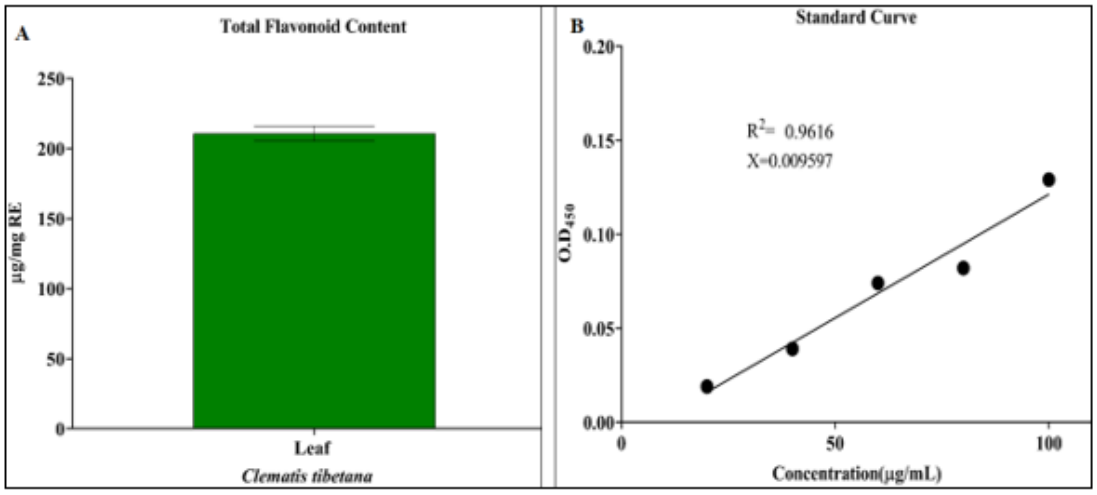


Figure 2: TFC (A) Total flavonoid content of the leaf extract of *C. tibetana* (B) The standard curve of Rutin used for measuring total flavonoid content. The finding of three distinct studies reported as mean ± SE.

The total flavonoid content of *C. tibetana* leaf extract was measured as 210.68 ± 3.11 µg rutin equivalents per mg (Figure. 2A–B).

GC-MS Analysis

GC–MS analysis of the *C. tibetana* leaf extract revealed a total of eight distinct peaks in the chromatogram. Among these, four compounds were present in notable abundance. The most predominant was Benzene-propanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, with a Retention Time (RT) of 32.694 minutes and a peak area of 54.3%. This was followed by dibutyl phthalate (RT 33.343; 18.15%), undecane (RT 10.811; 12.30%), and dodecane (RT 14.140; 8.88%). The remaining four compounds were detected in trace amounts, including heptadecane (RT 20.081; 3.80%), 11,14-eicosadienoic acid methyl ester (RT 30.749; 1.37%), adenosine, N,6-didehydro-1,9-dihydro-1-methyl- (RT 33.483; 0.24%), and cyclononasiloxane, octadecamethyl- (RT 44.776; 0.90%). Overall, the GC–MS profile of *C. tibetana* leaf extract indicates a complex mixture comprising alkanes, alkylbenzenes, benzoic acid esters, and fatty acid methyl esters (Table 1).

Table 1

No.	Compound	Rt	P.A.%	Nature	Biological Properties	References
1	Undecane	10.811	12.30	Alkane	Not reported	-
2	Dodecane	14.140	8.88	Alkane	Antioxidant, Antimicrobial	[25]
3	Heptadecane	20.081	3.80	Alkane	Antimicrobial	[26]
4	11,14-Eicosadienoic acid, methyl ester	30.749	1.37	Fatty acid methyl ester	Not reported	-
5	Benzene-propanoic acid 3,5-bis(1,1-dimethylethyl)-4-hydroxy-,	32.694	54.35	Alkyl benzene	Antioxidant	[27]
6	Dibutyl phthalate	33.343	18.15	Benzoic acid ester	Antimicrobial	[28]
7	Adenosine, N,6-didehydro-1,9-dihydro-1-methyl-	33.483	0.24	Purine ribonucleoside	Not reported	-
8	Cyclononasiloxane, octadecamethyl-	44.776	0.90	Cyclic siloxanes	Not reported	-

DPPH Free Radical Scavenging Activity

The DPPH free radical scavenging activity of *C. tibetana* leaf extracts was evaluated, and IC_{50} values were calculated using a regression equation generated from extract concentration versus percentage inhibition [29]. A higher percentage of inhibition reflects stronger antioxidant activity, whereas a higher IC_{50} value indicates weaker radical-scavenging potential. The methanolic leaf extract of *C. tibetana* demonstrated an IC_{50} value of 103.14 ± 1.45 µg/mL (Figure 3).

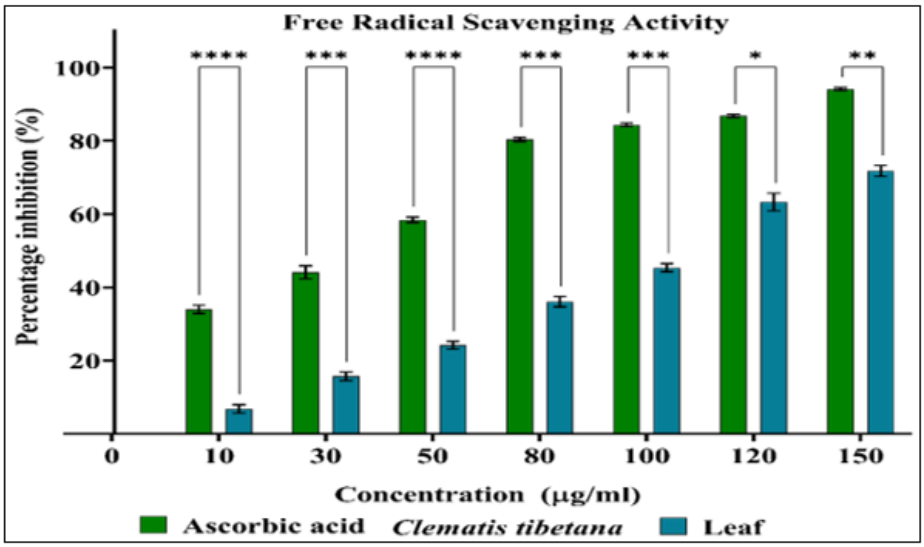
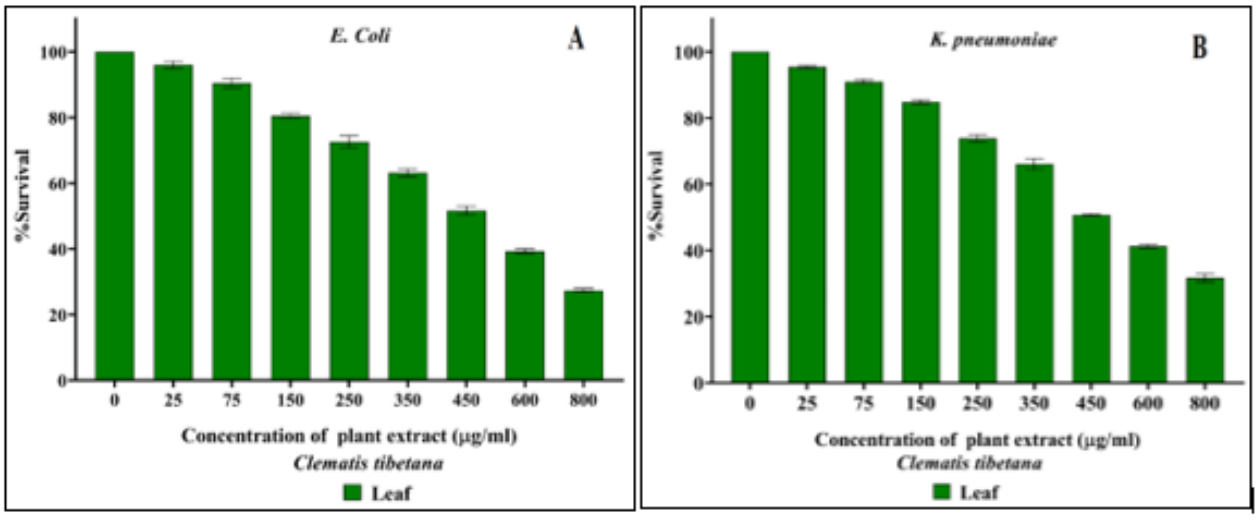


Figure 3: DPPH free radical scavenging activity of C. tibetana. The results of three different studies were reported as mean ± SE where *means p<0.5, ** means p < 0.01 *** means p < 0.001, and **** means p < 0.0001.

Antibacterial Assay

The antimicrobial activity of the methanolic *C. tibetana* leaf extract was evaluated using the broth microdilution method across a concentration range of 0–800 µg/mL. The survivability of various Gram-positive and Gram-negative bacterial strains was assessed at each concentration, and the results were plotted as a bar graph of concentration versus survivability (Figure 4). Based on the percentage survivability data, IC_{50} values (half-maximal inhibitory concentrations) were calculated using GraphPad Prism 8.0, applying the log inhibitor versus normalized response model (Figure 4).



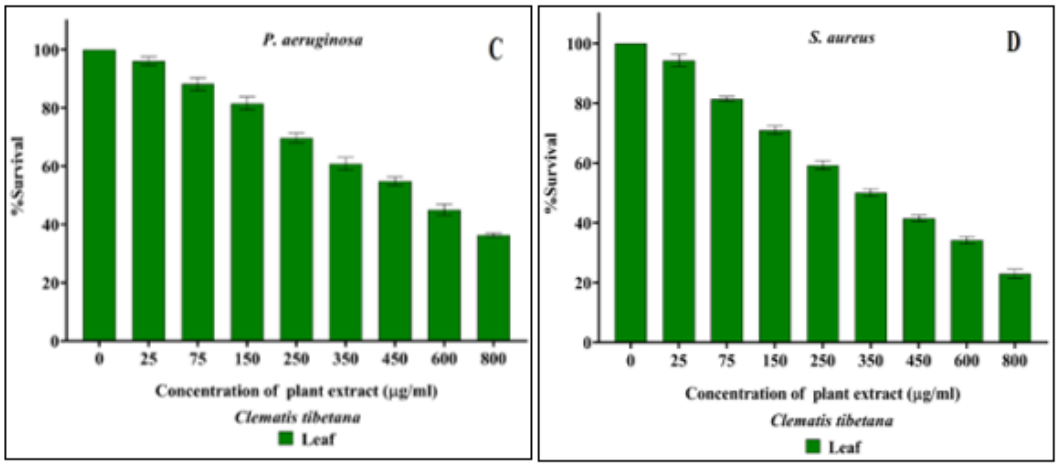


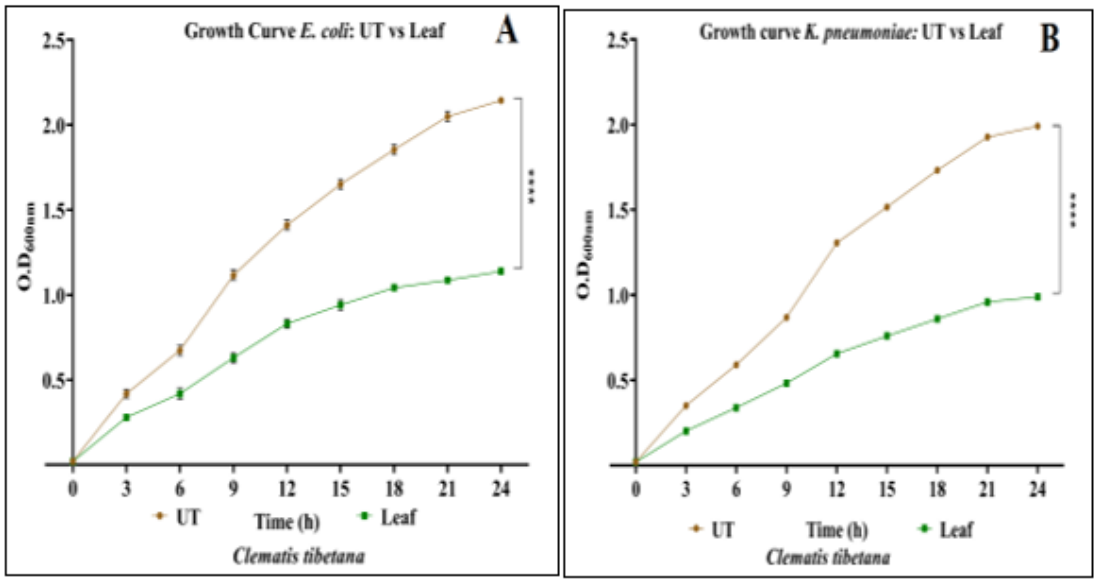
Figure 4: (A-D): Antimicrobial activity of leaf ME of *C. tibetana* shown as survival percentage graph (A) *E. coli* (B) *K. pneumoniae* (C) *P. aeruginosa* (D) *S. aureus.* (All values in triplicate given as mean ± SE). ME= Methanolic Extract.

The IC_{50} values indicated that Gram-positive bacteria were more susceptible to the extract than Gram-negative bacteria. *S. aureus* exhibited the lowest IC_{50} (328.1 ± 6.24 µg/mL), followed by *E. coli* (450.7 ± 4.79 µg/mL) and *K. pneumoniae* (486.3 ± 0.41 µg/mL). *P. aeruginosa* showed the highest resistance, with an IC_{50} of 510.86 ± 11.57 µg/mL (Table 2).

Table 2

No.	Bacterial Strain	Leaf/IC_{50} µg/mL
1	*Klebsiella pneumonia*	486.3 ± 0.41
2	*Staphylococcus aureus*	328.1± 6.24
3	*Escherichia coli*	450.73± 11.57
4	*Pseudomonas aeruginosa*	510.86 ± 6.24

Growth Curve Assay



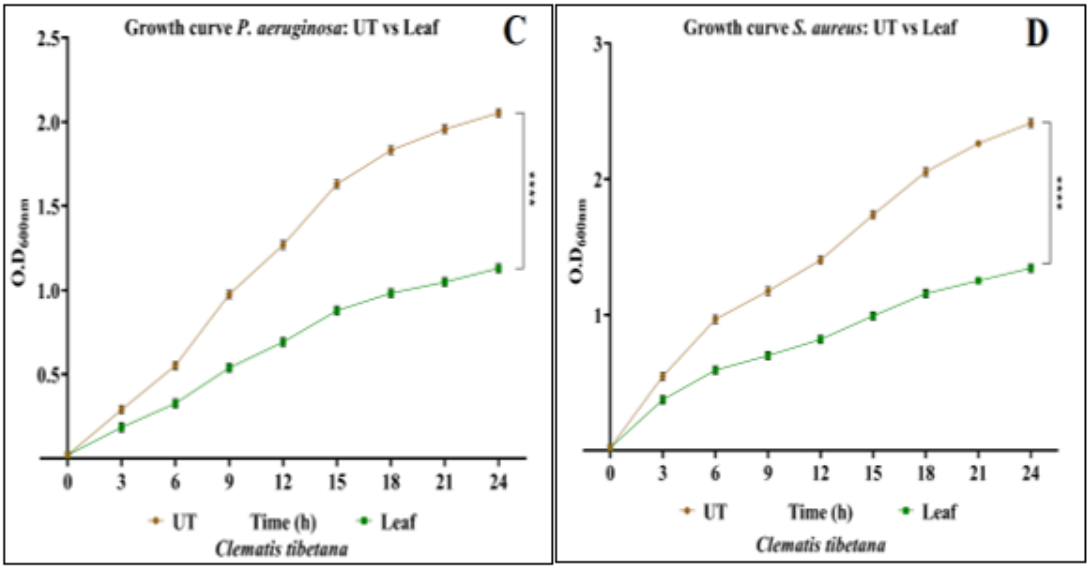


Figure 5: (A-D): Growth curves of (A) E. coli, (B) K. pneumoniae, (C) P. aeruginosa, and (D) S. aureus, values represent mean ± SE of three replicates. Statistical significance between UT and treated samples at 24 h evaluated using an unpaired two-tailed t-test (ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

The growth curve assay was performed to validate the IC_{50} values of the *C. tibetana* leaf extract obtained from the antimicrobial assay. The results demonstrated that the corresponding IC_{50} concentrations effectively inhibited the growth of all tested bacterial strains, as shown in the plotted 24-hour growth profiles comparing Untreated (UT) and extract-treated cultures, with measurements recorded every 3 hours. While the untreated groups exhibited a typical sigmoidal growth pattern, the treated groups consistently displayed suppressed growth, confirming the inhibitory effect of the extract across all bacterial strains (Figure 5).

Discussion

Since ancient times, humans have relied on plant-based remedies to treat various ailments, particularly in regions where modern medical facilities were limited or costly [30]. Cold desert regions, such as the Spiti Valley, are especially known for their longstanding dependence on medicinal plants for treating health conditions [31]. Members of the Ranunculaceae family have traditionally been used to manage wounds, bacterial infections, inflammation, and numerous other diseases [32-34]. In this context, the present study explored the phytochemical profile, GC–MS composition, antioxidant potential, and antimicrobial activity of *Clematis tibetana*, a lesser-studied species belonging to this family.

The Total Phenolic Content (TPC) of *C. tibetana* leaf extract was 47.89 ± 0.18 µg/mg GAE, which is comparatively higher than values reported for other *Clematis species*. While *Singh et al.* [35] reported TPC values ranging from 4.44 to 181.91 µg GAE/mg across 12 medicinal plants from Mizoram, the TPC of C. tibetana falls well within this range, confirming that it is a phenolic-rich species. Similarly, the Total Flavonoid Content (TFC) of *C. tibetana* (210.68 ± 3.11 µg/mg RE) is comparable to the flavonoid content reported for Taxus baccata methanolic extracts (204.26 ± 6.02 mg/g RE) [36], underscoring the flavonoid abundance in this plant.

The antioxidant potential, evaluated using the DPPH assay, revealed an IC_{50} value of 103.14 ± 1.45 µg/mL. This value aligns with previously reported IC_{50} ranges for other Ranunculaceae members, including *Delphinium elbursense, Aconitum moldavicum,* and *Aconitum toxicum* [37,38], further emphasizing the strong radical-scavenging capacity of C. tibetana.

GC–MS analysis identified eight compounds in the leaf extract, four of which are known for significant biological activities. Dodecane, an alkane, is reported to possess antimicrobial and free-radical-fighting properties (*Nandhini et al.,* 2015). Heptadecane has been associated with antimicrobial activity in *Paracoccus pantotrophus* extracts (*Faridha Begum et al.,* 2016). The alkyl benzene derivative benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester is recognized for its antioxidant potential (*Li et al.,* 2014). Additionally, dibutyl phthalate-an ester of benzoic acid-has been detected in *Ipomoea carnea* and linked to antimicrobial activity (*Khatiwora et al.,* 2012). Collectively, these bioactive constituents support the antioxidant and antimicrobial capabilities observed in *C. tibetana* leaf extract.

The antimicrobial evaluation revealed a clear distinction between the susceptibility of gram-positive and gram-negative bacteria. The extract displayed stronger activity against the gram-positive *Staphylococcus aureus* (IC_{50} = 328.1 ± 6.24 µg/mL), whereas gram-negative strains, including *E. coli, K. pneumoniae*, and *P.*

aeruginosa, required higher concentrations for inhibition (450.73–510.86 µg/mL). A similar trend has been reported for *Ranunculus muricatus* (*Nazir et al.,* 2013), suggesting that the antibacterial pattern observed in C. tibetana is consistent with other members of the Ranunculaceae family. Overall, these findings highlight the promising antimicrobial potential of *C. tibetana* leaf extract.

Conclusion

The present study demonstrates that *Clematis tibetana* leaves possess substantial levels of phenolic and flavonoid compounds, which correlate strongly with their notable antioxidant activity. In addition, the leaf extract exhibited significant antibacterial effects against both gram-positive and gram-negative bacteria, underscoring its potential as a promising therapeutic agent. The GC–MS profile revealed a diverse array of bioactive constituents, providing scientific validation for the plant's traditional medicinal applications.

Overall, the findings position *C. tibetana* as a valuable natural source of antioxidant and antimicrobial compounds with potential applications in pharmaceutical and nutraceutical formulations. However, further investigations are required to isolate the key bioactive molecules, elucidate their mechanisms of action, and evaluate their safety and toxicity profiles through in-depth preclinical studies.

This work also emphasizes the importance of examining underexplored plant species from extreme environments, as they may harbour unique bioactive compounds with significant health benefits and therapeutic potential.

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Declarations

None.

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Conflict of Interest

The authors declare no conflict of interest.

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