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Comprehensive phytochemical profiling and bioactivities of *Delphinium vestitum:* An unexplored high-altitude Himalayan herb

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Abstract

High-altitude medicinal plants have been identified as valuable sources of bioactive secondary metabolites because of their ability to thrive in harsh environmental conditions. The main objective of the present study was to scientifically evaluate the ethanobotanically important, unexplored high-altitude plant *Delphinium vestitum.* The phytochemical content, HPLC profile, and antioxidant and antibacterial potential of the methanolic leaf extract of the high-altitude medicinal plant *Delphinium vestitum* were assessed. Phytochemical screening revealed the presence of numerous important phytochemicals, including flavonoids, tannins, phenols, and glycosides. The quantitative analysis revealed significant levels of total phenols (TPC; 191.80 ± 0.02 mg GAE/g), flavonoids (TFC; 90.07 ± 0.03 mg RE/g), and tannins (TTC; 124.36 ± 0.02 mg TA/g) in the leaf extract, establishing the plant's richness in secondary metabolites. High-performance liquid chromatography analysis of the leaf extract of *D. vestitum* revealed the presence of valuable phenolic and flavonoid markers, including gallic acid (41.94 µg/100 mg) and quercetin (0.44 µg/100 mg). The extract exhibited considerable antioxidant activity, as evaluated by the TAC (62.08 µg AAE/mL at 150 µg/mL), DPPH (IC_{50} = 65.60 µg/mL), and ABTS (IC_{50} = 145.63 µg/mL) assays. Furthermore, the antibacterial assay demonstrated significant growth inhibition against *Escherichia coli, Klebsiella pneumoniae, Streptococcus pneumoniae*, and *Staphylococcus aureus,* as assessed by the agar well diffusion method and a resazurin-based assay. These findings suggest that *D. vestitum*, an unexplored high-altitude medicinal plant, is a rich source of bioactive phytochemicals with significant antioxidant and antibacterial potential. The results highlighted the therapeutic importance of the high-altitude traditional medicinal plant *D. vestitum*, the best of the authors' knowledge, the first in-depth scientific report on this plant, supporting its use as a potential source of natural therapeutic agents. Further studies on the isolation and pharmacological evaluation of compounds of this plant are suggested.

Keywords: Traditional medicinal plant, High-altitude, High-performance liquid chromatography, Antioxidant activity, Agar well plate, Resazurin assay

Introduction

The plethora of floral wealth with therapeutic potential, of which 1748 species are reported and well documented from the Indian Himalayas, is integral to indigenous medicinal systems and practices [1-3] . Under extreme environmental conditions, plants usually adapt to grow naturally by producing bioactive phytochemicals such as antioxidants against extreme ultraviolet radiation exposure at relatively high altitudes. These phytochemicals include phenols and their derivatives, flavonoids, terpenes, and tannins [4]. Therefore, the plants that thrive under extremely harsh conditions in the higher Himalayas serve as effective reservoirs for therapeutic agents because of their natural availability and cost-effectiveness, offering alternative therapies either directly or as models for future synthetic molecules [5-7].

The medicinal potential of these plants is related to their complex secondary metabolites with considerable bioactivity that have specific mechanisms in the mitigation of free radical-mediated diseases and may be considered natural substitutes for foodstuffs [8]. The fear of antimicrobial resistance and toxicity induced by currently available synthetic drugs has increased interest in the search for plant alternatives, particularly in biodiversity-rich countries such as India and its Himalayan region. Consequently, the exploration of these plant resources has increased as phytochemicals derived from medicinal plants exhibit structurally different and multitarget antimicrobial effects that can impair the necessary cellular functions of pathogenic microorganisms [9].

The genus *Delphinium*, belonging to the family Ranunculaceae, comprises more than 300 species distributed mainly across temperate regions of the Northern Hemisphere, with the Himalayan region representing a major center of species diversity. Plants of this genus are particularly rich in diterpenoid and norditerpenoid alkaloids, many of which exhibit significant pharmacological activities such as anti-inflammatory, immunosuppressive, analgesic, antitumor, cardiotonic, antihypertensive, and vasodilative effects. Diterpenoid alkaloids exhibit anticancer activity [10]. *Delphinium vestitum* Wall. ex-Royle is a perennial herb, native to the Indian Himalayas, that grows on partly shaded, grassy slopes [11]. Local healers have traditionally utilized different parts of *D. vestitum* for their analgesic and anti-inflammatory properties. Extracts obtained from whole plants of *D. vestitum* have also been traditionally

prescribed for the treatment of dermatological issues, gastric issues, rheumatic pain, snake bites, cuts, wounds, fever, and diarrhea [11,12].

Despite its ethnobotanical importance, *D. vestitum* has not yet been explored for its phytochemical and pharmacological potential. Scientific validation of its traditional uses may not only support the rational development of plant-based therapeutic agents but also contribute to the preservation of indigenous knowledge and sustainable utilization of Himalayan medicinal flora. Hence, in the present study, qualitative phytochemical analysis of various extracts of *D. vestitum* and quantitative phytochemical analysis of the methanolic leaf extract were performed. High-performance liquid chromatography was used to analyze the amount of gallic acid and quercetin in the extract. The *D. vestitum* extract was also explored for its antioxidant and free radical scavenging properties via TAC, DPPH, and ABTS assays. The antimicrobial potential of the extract was analyzed via the agar well plate method and resazurin-based microtiter plate assay.

Results

Qualitative phytochemical analysis of the methanolic leaf extract of *D. vestitum*

Preliminary secondary metabolite screening of the plant extracts prepared using solvents with increasing polarity, i.e., petroleum ether, chloroform, acetone, ethanol, methanol, and water, revealed the presence of a broad spectrum of bioactive secondary metabolites (Table 1). These results clearly demonstrate that solvent polarity plays a crucial role in the extraction efficiency of phytoconstituents.

Table 1: Qualitative phytochemical analysis of various extracts of *D. vestitum* (+ indicates presence, while – indicates absence)

Phytochemical Test	**Petroleum ether**	**Chloroform**	**Acetone**	**Ethanol**	**Methanol**	**Water**	**Observation**
Alkaloids	+	+	+	-	+	-	Presence of brown/reddish precipitate
Flavonoids	+	+	+	+	+	+	Presence of green precipitate
Phenols	+	+	+	+	+	+	Formation of bluish black color
Tannins	+	+	+	+	+	+	Blue- green color formation
Saponins	-	+	+	+	+	+	Presence of foam
Carbohydrates	+	+	+	+	+	+	Formation of a violet ring
Phytosterols	+	+	+	+	+	+	Appearance of red color in the lower layer
Triterpenoids	+	+	+	+	+	-	Presence of a golden yellow layer at the bottom
Cardiac glycosides	+	+	+	+	+	-	Appearance of blue color in the acetic acid layer
Carboxylic acid	+	+	-	-	+	-	Appearance of effervescence
Reducing sugar	+	+	+	+	+	+	Presence of red precipitate

Quantitative phytochemical analysis of *D. vestitum* methanolic extract

Total Phenolic Content (TPC)

The total phenolic content of the *D. vestitum* extract was determined via the Folin–Ciocalteu method and expressed as gallic acid equivalents (GAEs). A calibration curve was prepared using gallic acid in the concentration range of 50–250 µg/mL, which showed good linearity, indicating reliable quantification. The mean absorbance of the sample was recorded as 0.00123, and the corresponding phenolic concentration was calculated using the standard curve equation. The total phenolic content of the extract was 191.80 ± 0.02 mg GAE/g extract.

Total Flavonoid Content (TFC)

The total flavonoid content in *D. vestitum* was estimated via an aluminum chloride colorimetric assay, with rutin used as the reference standard. The rutin calibration curve showed good linearity over the concentration range of 20–100 µg/mL. The regression analysis demonstrated a strong correlation (R^2 = 0.9917), confirming the suitability of the method. The mean absorbance of the sample was 0.010. After accounting for the dilution factor, the total flavonoid content of the extract was calculated as 90.07 ± 0.03 mg RE/g extract.

Total Tannin Content (TTC)

The total tannin content of the *D. vestitum* extract was determined using the Folin–Ciocalteu method and expressed as tannic acid equivalents (TAEs). Tannic acid was used to generate a standard calibration curve in the range of 50–250 µg/mL, which exhibited good linearity, indicating reliable quantification. On the basis of the standard curve and absorbance values, the total tannin content of the extract was calculated as 124.36 ± 0.02 mg TAE/g extract.

High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) analysis was carried out to identify and quantify the major phenolic constituents in the *D. vestitum* extract, with gallic acid (GA) and quercetin (Q) used as reference standards. The chromatogram of the standard mixture exhibited well-resolved, reproducible peaks for gallic acid and quercetin at retention times (RT) of 4.701 min and 22.768 min, respectively. The chromatographic profile of the *D. vestitum* extract presented multiple peaks, indicating the presence of diverse phytoconstituents, among which two major peaks were identified on the basis of retention times matching those of the standards. Gallic acid was detected in the extract at RT 4.620 min, which closely matched the standard GA peak (RT 4.701 min), confirming its presence in the plant sample. Similarly, quercetin was

identified at RT 22.456 min, corresponding closely with the standard quercetin peak at 22.768 min (Figure 1).

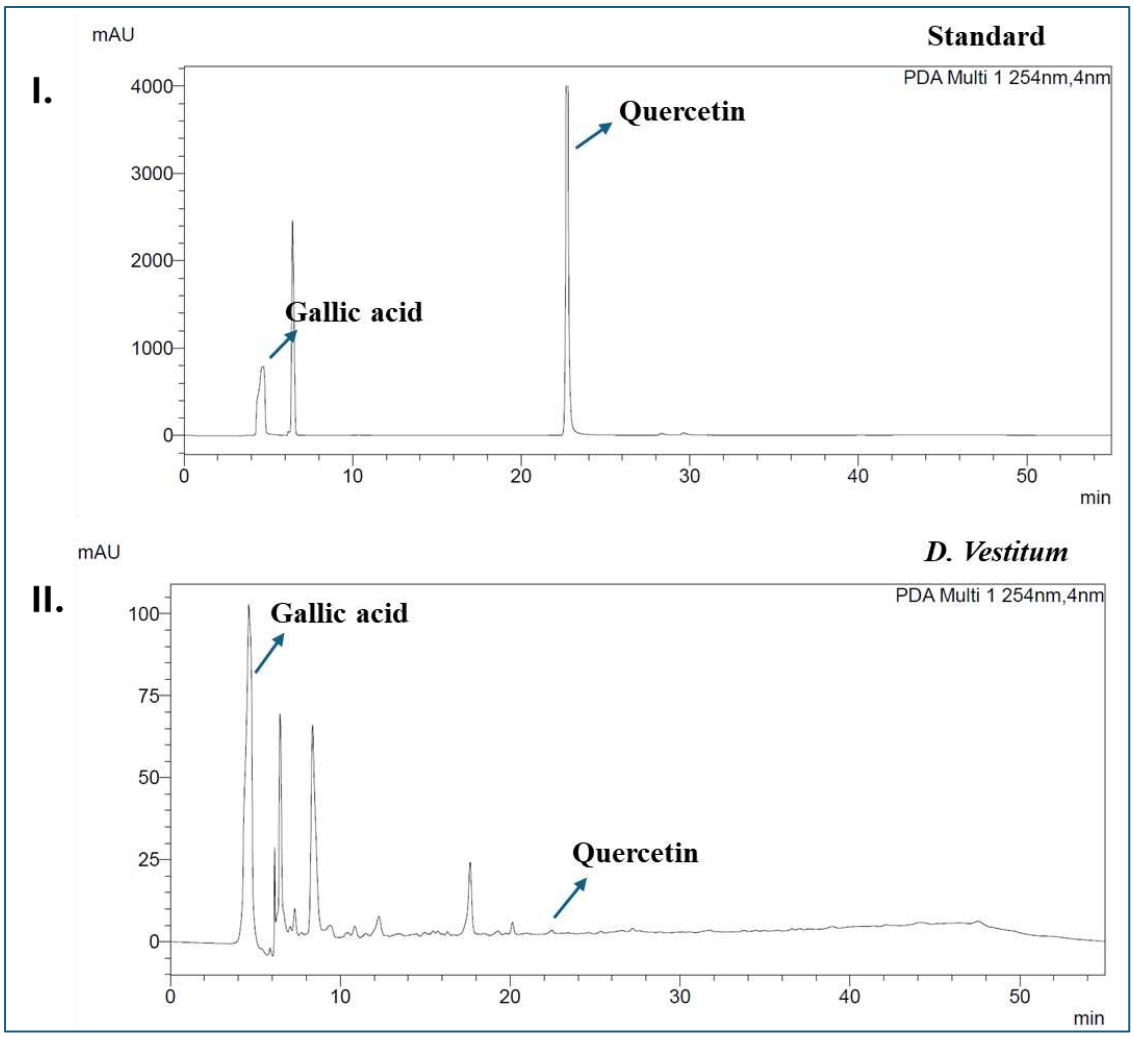


Figure 1: HPLC chromatographs of **I.** Gallic acid and quercetin standard solutions and **II** methanolic leaf extract of *D. vestitum*

Quantitative analysis via HPLC revealed that the gallic acid and quercetin peaks accounted for 39.61% and 0.287 % of the total chromatographic area, respectively. With a calculated average concentration of 41.95 µg/100 mg and 0.44 µg/100 mg extract of gallic acid and quercetin, respectively (Table 2).

Table 2: Quantification of gallic acid and quercetin in *D. vestitum* extract via HPLC (254 nm)

S. No.	Compound	(GA+Q) RT (min)	*Delphinium Vestitum* RT (min)	Standard Peak Area	*Delphinium Vestitum* Peak Area	*Delphinium Vestitum* Area %	Average concentration in *D. vestitum* (µg/100 mg)
1	**Gallic acid (GA)**	4.701	4.620	21,552,897	2,714,415	39.614	41.95
2	**Quercetin (Q)**	22.768	22.456	54,096,749	19,688	0.287	0.44

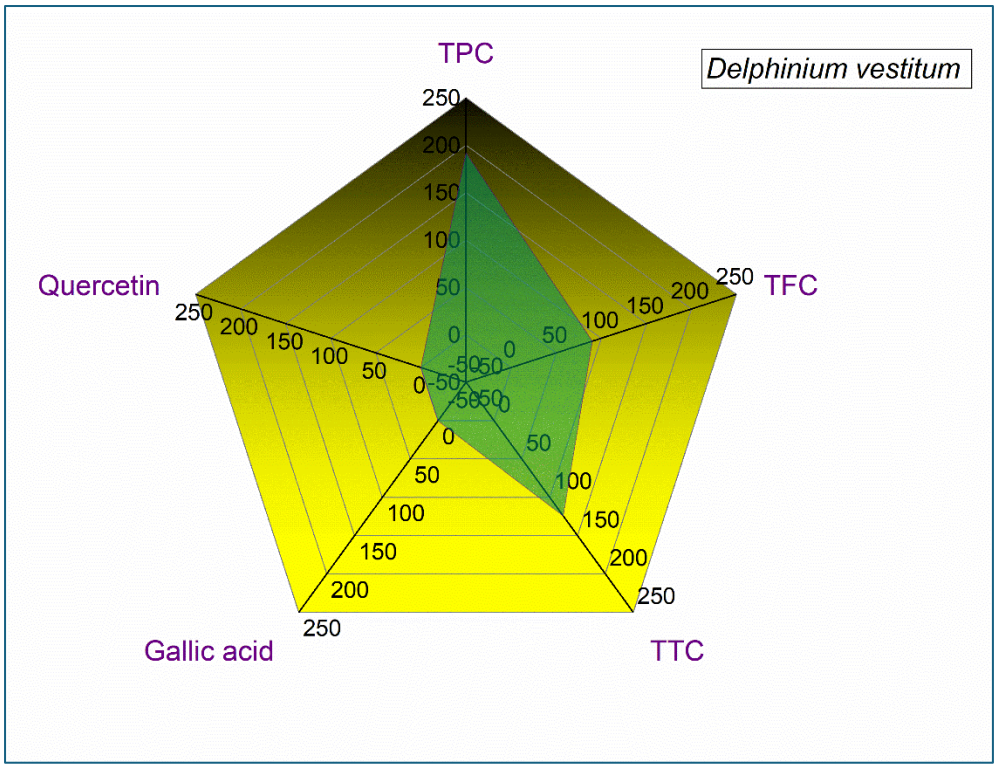


Figure 2: Radar plot showing the overall phytochemical profile of *Delphinium vestitum* (generated in Origin 2018)

Figure 2 shows the relative levels of total phenolic content (TPC) in mg GAE/g, total flavonoid content (TFC) in mg RE/g, total tannin content (TTC) in mg TA/g, quantity detected via HPLC of gallic acid equivalents, and quercetin equivalents in the plant extract (mg/g). The values are plotted on a common radial scale (0–250), allowing visual comparison of the contribution of each phytochemical parameter to the overall phytochemical profile

In vitro assessment of the antioxidant properties of the methanolic leaf extract of *D. vestitum*

Total antioxidant capacity (TAC)

The total antioxidant capacity reflects the overall reducing potential of an extract and is typically measured via the phosphomolybdenum reduction assay, which estimates the cumulative electron-transfer ability by reducing molybdenum (VI) to molybdenum (V). In the present study, the total antioxidant capacity of *D. vestitum* was evaluated via ascorbic acid standard calibration and expressed as ascorbic acid equivalents (AAEs). The extract increased the total reducing potential in a dose-dependent manner. The lowest TAC was recorded at 25 µg/mL (10.82 ± 0.25 µg AAE/mL), and the highest TAC was found at 150 µg/mL (62.08 ± 0.03 µg AAE/mL), confirming greater electron transfer capacity at higher concentrations (Figure 3).

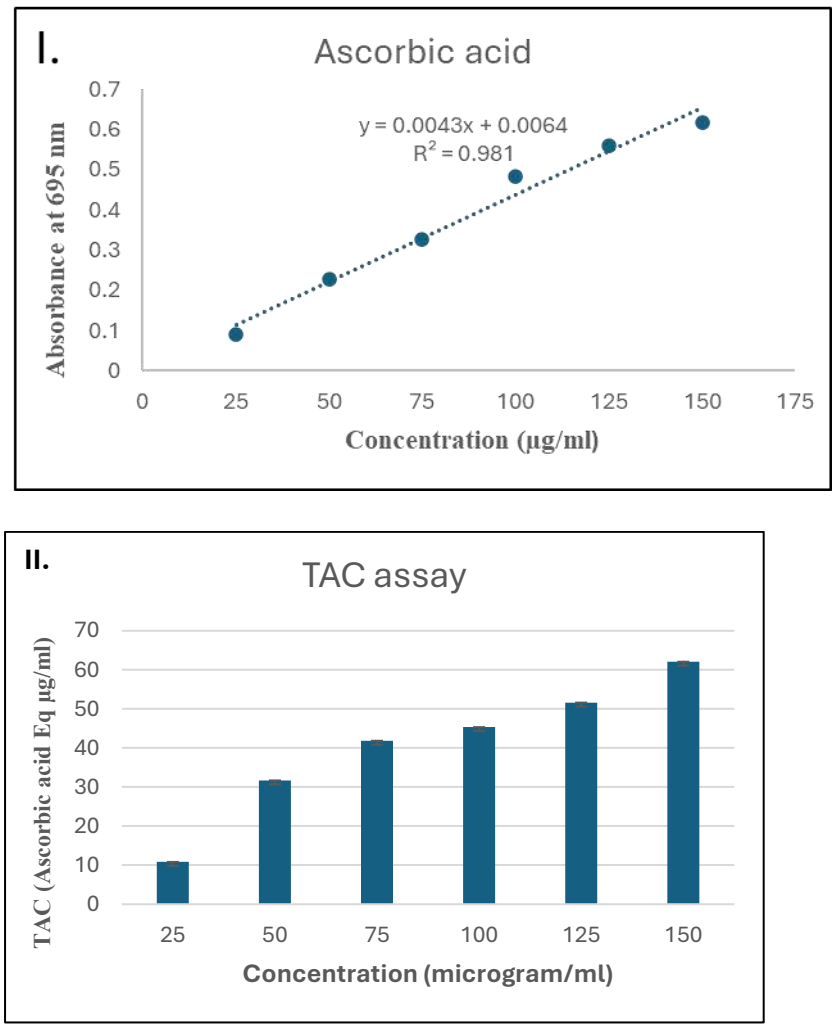
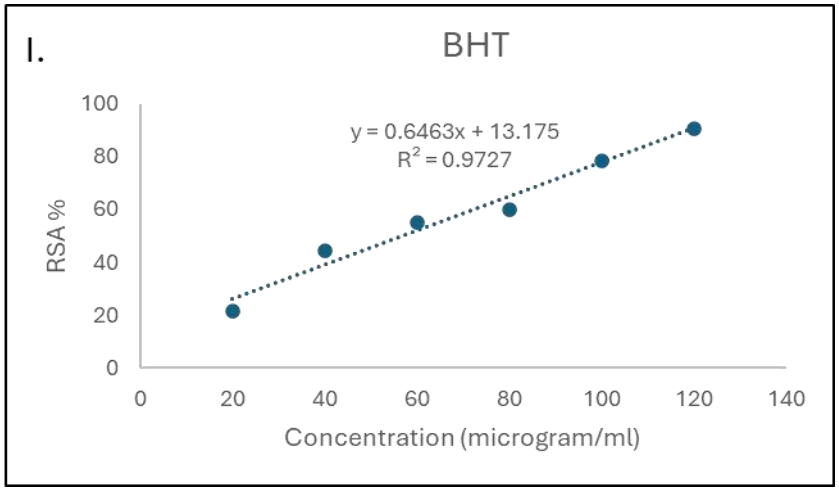


Figure 3: Total antioxidant capacity (TAC) assay standard calibration curve **I.** Standard calibration curve for the total antioxidant capacity (TAC) assay using ascorbic acid as the

reference standard. The absorbance was measured at 695 nm following the phosphomolybdenum method. **II.** The total antioxidant capacity (TAC) of the plant extract was determined by the phosphomolybdenum assay at different concentrations (25–150 µg/mL). TAC was expressed as ascorbic acid equivalents (AAE). Data are presented as mean ± SD (n = 3), showing a concentration-dependent increase in antioxidant activity.

DPPH Free Radical Scavenging Activity

The antioxidant activity of the plant extract was determined via the free radical scavenging activity of DPPH and compared with that of the positive antioxidant standard butylated hydroxytoluene (BHT). Both the standard and the plant extracts increased in a concentration-dependent manner in terms of the ability to scavenge free radicals at concentrations ranging from 20-120 µg/mL. BHT had greater antioxidant activity, with 21.62% inhibition at 20 µg/mL BHT, which increased to 90.79% at 120 µg/mL BHT. On the other hand, the plant extract showed 18.26% at 20 µg/mL concentrations and reached 84.99% at the highest concentrations (Figure 4). The IC_{50} of BHT was determined to be 50.90 µg/mL. The IC_{50} of the plant extract was 65.60 µg/mL (Table 3).



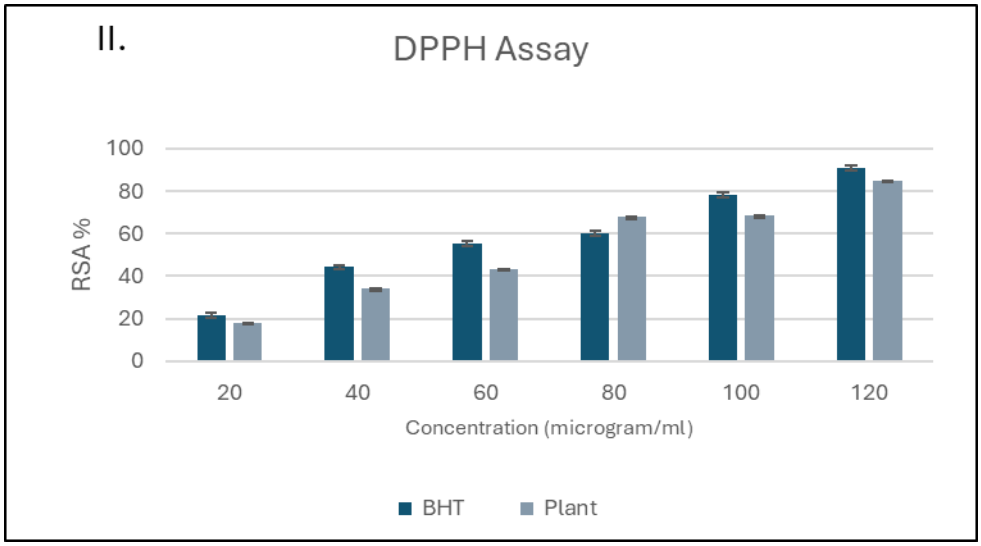


Figure 4: DPPH radical scavenging assay of the *D. vestitum* extract in comparison with the standard antioxidant butylated hydroxytoluene (BHT) at various concentrations (20–120 µg/mL). The radical scavenging activity is expressed as a percentage (RSA%). (**I**) BHT calibration curve showing a linear relationship between concentration and RSA% ($y = 0.6463x + 13.175$; $R^2 = 0.9727$). Data are expressed as mean ± SD (n = 3). (**II**) Concentration-dependent scavenging activity of the plant extract and BHT.

ABTS assay

The ABTS radical cation decolorization assay is a robust method applicable to both hydrophilic and lipophilic antioxidant molecules and is frequently used to assess the antioxidant capacity of plant extracts. In this work, the *D. vestitum* extract displayed concentration-dependent ABTS inhibition, with the lowest inhibition at 100 µg/ml (26.92 %) and the highest inhibition at 150 µg/ml (52.24%), suggesting improved radical cation scavenging as the dose increased (Figure 5).

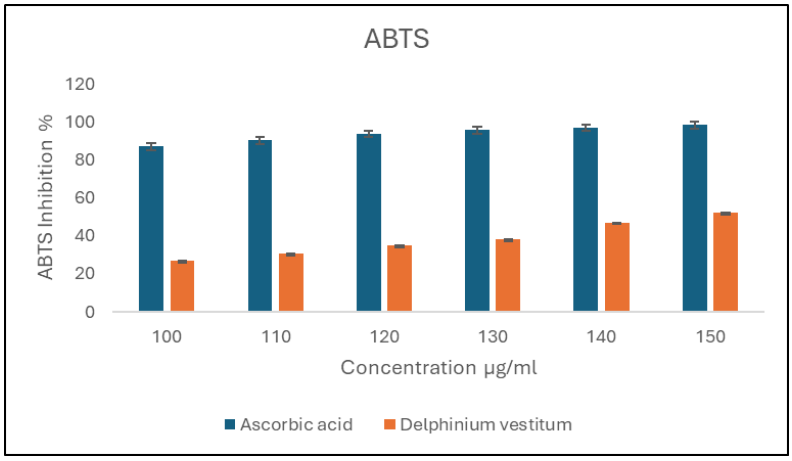


Figure 5: ABTS radical cation scavenging activity of the *D. vestitum* extract compared with the standard antioxidant ascorbic acid at different concentrations (100–150 µg/mL). Antioxidant activity is expressed as percentage ABTS inhibition (%). Results demonstrate a concentration-dependent increase in scavenging activity. Data are presented as mean ± SD (n = 3)

Using ascorbic acid as the reference standard (IC_{50} < 100 µg/mL), *D. vestitum* extract exhibited an IC_{50} value of 145.63 µg/mL, indicating moderate ABTS radical scavenging activity (Table 3).

Table 3: Antioxidant activity of *D. vestitum* assessed using DPPH and ABTS. BHT and ascorbic acid were used as reference standards. Results of DPPH and ABTS assays are expressed as IC_{50} values (µg/mL), defined as the concentration required to inhibit 50% of free radicals, obtained from the standard calibration curve. Lower IC_{50} values indicate higher antioxidant activity.

Assay	Standard used	Standard (IC_{50})	D. vestitum (IC_{50})	Interpretation
DPPH radical scavenging	BHT	IC_{50} = 50.90 µg/mL	IC_{50} = 65.60 µg/mL	*D. vestitum* shows strong DPPH scavenging, slightly lower than BHT
ABTS radical scavenging	Ascorbic acid	IC_{50} < 100 µg/mL	IC_{50} = 145.63 µg/mL	*D. vestitum* shows moderate ABTS scavenging activity

In vitro assessment of the antibacterial properties of *D. vestitum* methanolic leaf extract

Agar well plate method

The antibacterial activity of the *D. vestitum* leaf methanolic extract was evaluated against two gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*) and two gram-positive

bacteria (*Staphylococcus aureus* and *Streptococcus pneumoniae*) via the agar well diffusion method. The plant extract inhibited all the strains tested, confirming its broad-spectrum antimicrobial potential.

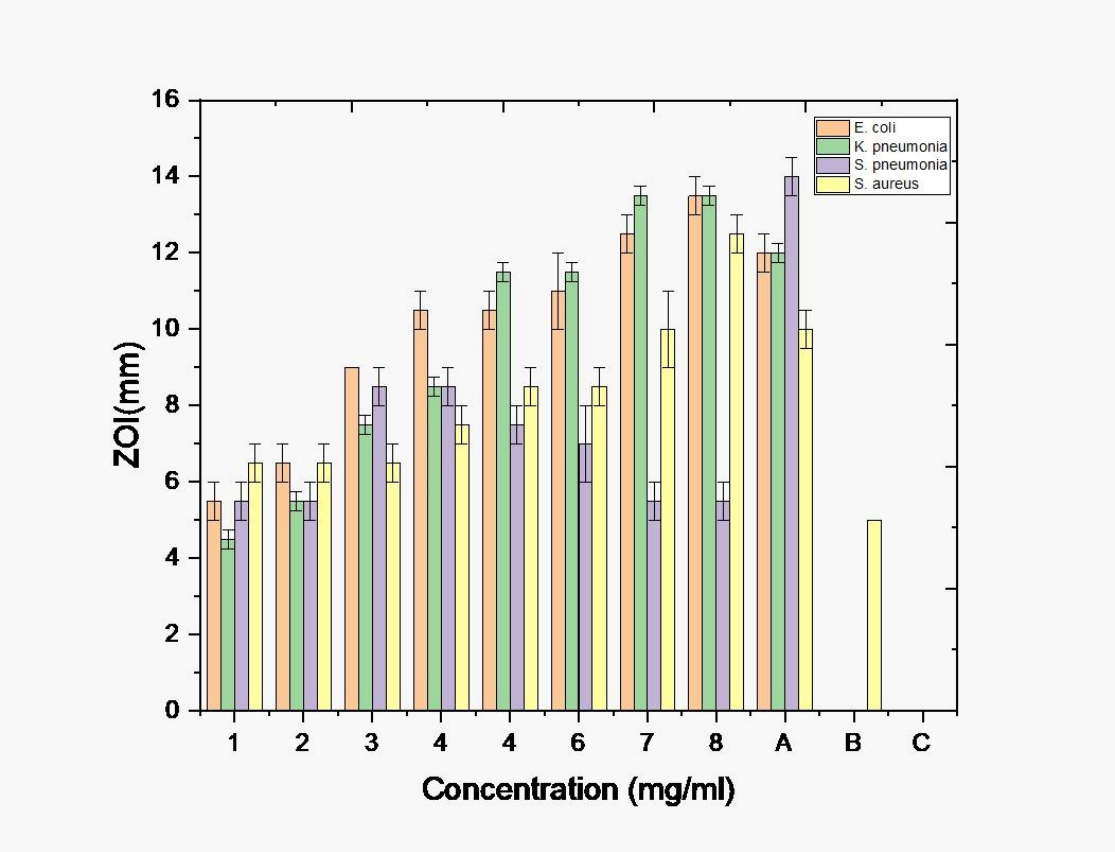


Figure 6. The antibacterial activity of *D. vestitum* was evaluated by the agar well diffusion method against *Escherichia coli, Klebsiella pneumoniae, Streptococcus pneumoniae*, and *Staphylococcus aureus* at different concentrations (1–8 mg/mL). The antibacterial efficacy is expressed as the zone of inhibition (ZOI, mm). A and B represent the positive controls, streptomycin and ampicillin, respectively, whereas C denotes the negative control (solvent control). Data are presented as mean ± SD (n = 3).

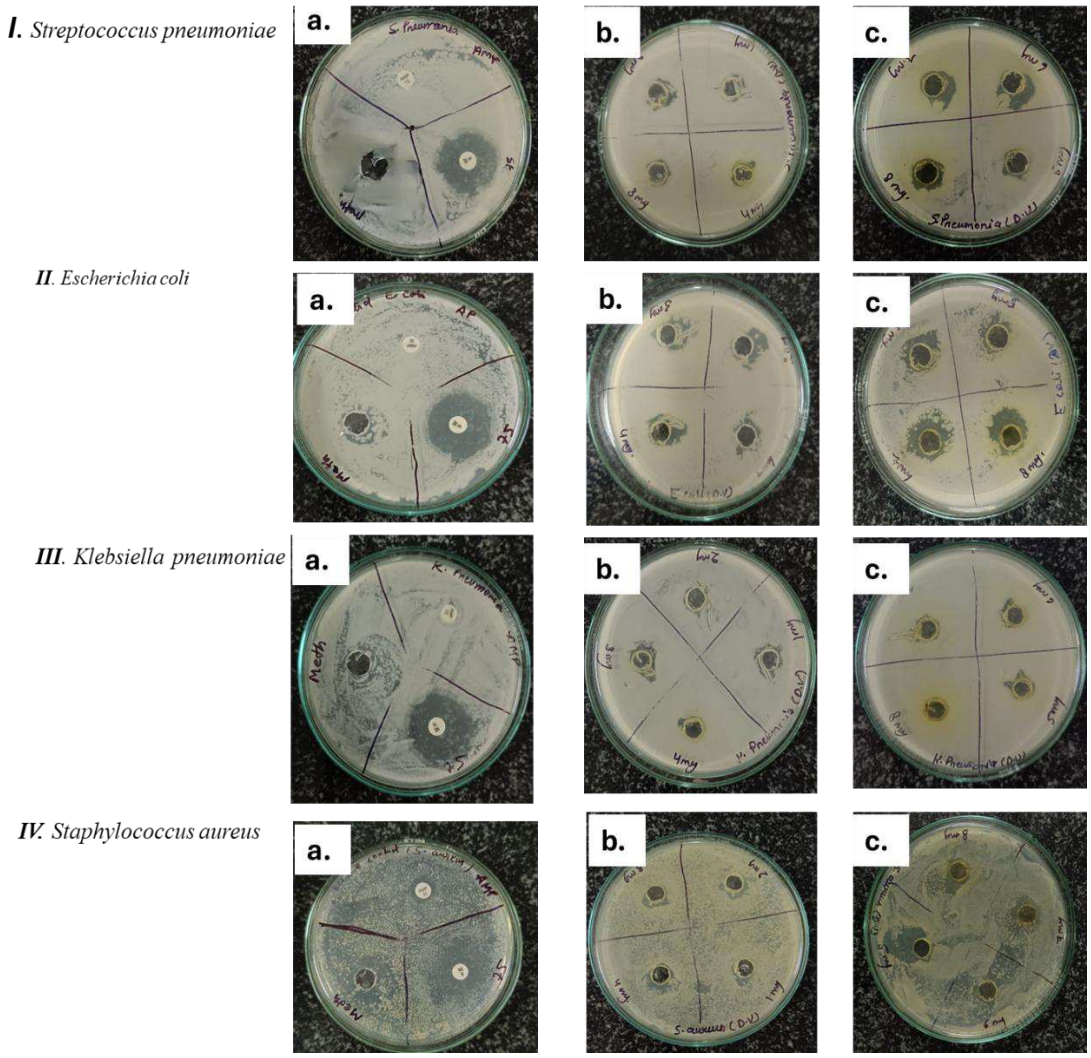


Figure 7: Agar well diffusion assay showing the antibacterial activity of *D. vestitum* methanolic leaf extract. Nutrient agar plates illustrating zones of inhibition produced by the extract against different bacterial strains: **(I)** *Streptococcus pneumoniae*, **(II)** *Escherichia coli*, **(III)** *Klebsiella pneumoniae,* and **(IV)** *Staphylococcus aureus*. Panel **a.** represents positive (streptomycin and ampicillin) and negative controls (methanol), **b.** represents concentrations from 1 to 4 mg/ml, and **c.** represents ZOIs for concentrations from 5-8 mg/ml of plant extract for each of the strains.

Resazurin-based antibacterial assay

The antibacterial activity of *D. vestitum* methanolic leaf extract was further evaluated using the resazurin microtiter plate assay to determine bacterial metabolic activity and growth inhibition. Resazurin acts as an oxidation–reduction indicator, changing from blue/purple (non-viable or metabolically inactive cells) to pink (viable, metabolically active cells). Therefore, the absence of pink coloration indicates effective bacterial growth inhibition. In the current approach,

wells that had a higher percentage of the extract remained blue to purple, showing significant inhibition of the metabolic activity of bacteria. The lower concentrations in the wells showed a more pronounced pink color, characteristic of active bacterial growth. With decreasing extract concentration, the color shifted from purple/blue to pink, indicating that the antibacterial effect was dose dependent.

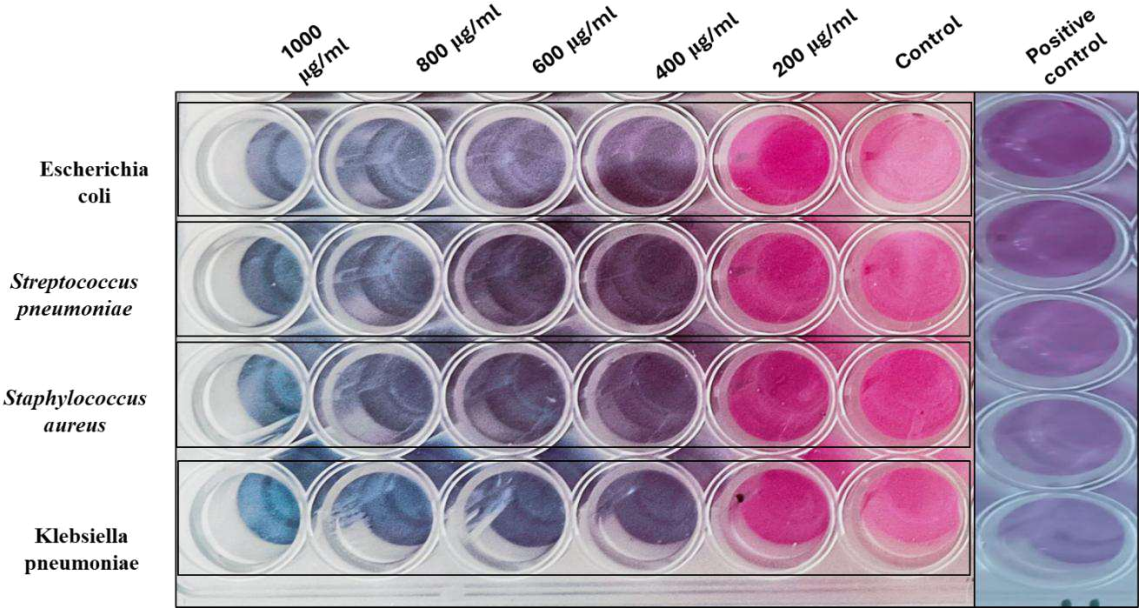


Figure 8. Resazurin-based microtiter plate assay illustrating the antibacterial properties of the plant extract against *Escherichia coli, Klebsiella pneumoniae, Streptococcus pneumoniae,* and *Staphylococcus aureus* (represented in rows). Each bacterial strain was treated with the plant extract at concentrations of 1000, 800, 600, 400, and 200 µg/mL (represented in columns). Gentamycin was used as the positive control, and an untreated bacterial culture served as the negative control. All the assays were performed in triplicate.

Table 4: Growth inhibition of bacterial strains measured by absorbance at 600 nm (OD_{600}). The table shows the optical density values of *Escherichia coli, Streptococcus pneumoniae, Staphylococcus aureus,* and *Klebsiella pneumoniae* after treatment with the *D. vestitum* extract at different concentrations (1000 µg/mL, 800, 600, 400, and 200 µg/mL). The growth control represents untreated bacterial cultures, while the positive control corresponds to gentamycin antibiotics.

Absorbance at 600 nm	1000 µg/ml	800 µg/ml	600 µg/ml	400 µg/ml	200 µg/ml	Control	Positive control
Escherichia coli	0.924 ± 0.003	0.784 ± 0.001	0.544 ± 0.003	0.453 ± 0.002	0.354 ± 0.002	0.343 ± 0.003	1.292 ± 0.002
Streptococcus pneumoniae	0.884 ± 0.002	0.843 ± 0.003	0.573 ± 0.002	0.484 ± 0.003	0.485 ± 0.003	0.443 ± 0.002	1.132 ± 0.001
Staphylococcus aureus	1.164± 0.002	0.993 ± 0.003	0.705 ± 0.003	0.567 ± 0.001	0.425 ± 0.002	0.428 ± 0.027	1.572 ± 0.003
Klebsiella pneumoniae	1.104 ± 0.002	0.945 ± 0.002	0.784 ± 0.002	0.693 ± 0.003	0.447 ± 0.004	0.434 ± 0.003	1.425 ± 0.003

Discussion

In qualitative analysis of *D. vestitum*, alkaloids were detected in petroleum ether, chloroform, acetone, and methanol extracts, as evidenced by the formation of brown to reddish precipitates, whereas ethanol and water extracts tested negative. These findings suggest that the alkaloids in *D. vestitum* are predominantly moderately nonpolar to semipolar. Alkaloids are well known for their pharmacological activities, including antimicrobial, analgesic, and anticancer properties, which may contribute to the biological efficacy of these extracts [13]. Flavonoids were present in all the solvent extracts, as indicated by the formation of a green precipitate. The universal presence of these compounds suggests a broad solubility range, which is consistent with their abundance in *D. vestitum* extracts. Flavonoids are recognized for their antioxidant, anti-inflammatory, and antimicrobial activities, and their consistent detection highlights the therapeutic potential of plants [14]. Phenolic compounds and tannins were also detected in all extracts, as evidenced by bluish-black and blue-green coloration, respectively. The presence of these polyphenolic compounds is significant, as they are strongly associated with antioxidant capacity and free radical scavenging activity [15]. The broad distribution of phenols and tannins indicates their high extractability across a range of solvent polarities. Saponins were absent in the petroleum ether extract but present in the chloroform, acetone, ethanol, methanol, and water extracts, as indicated by stable foam formation. This pattern reflects both the nonpolar and polar nature of saponins. Saponins are reported to possess

antimicrobial, anti-inflammatory, and membrane-permeabilizing properties. Carbohydrates and reducing sugars were detected in all extracts, suggesting that both simple sugars and complex sugars are abundantly distributed in the plant matrix and can be extracted via a wide range of solvents. Phytosterols and triterpenoids were identified in the extracts. These compounds are known for their anti-inflammatory, hepatoprotective, and anticancer potential [16]. Cardiac glycosides detected in organic solvent extracts are clinically significant because of their effects on cardiac muscle contraction, and their anticancer and antiviral potential [17]. Overall, the phytochemical screening results demonstrated that the plant is a rich source of diverse secondary metabolites, with the methanol extract exhibiting the broadest range of phytoconstituents. The presence of multiple bioactive compounds supports the traditional medicinal use of this plant and provides a strong scientific basis for further quantitative analysis, isolation, and pharmacological evaluation.

Quantitative analysis of phenol, flavonoids, and tannins of *D. vestitum* was performed. The total phenolic content of the extract was 191.80 ± 0.02 mg GAE/g extract. Similar levels of phenolic content have been reported in several plants, indicating their potential therapeutic applications. One study reported that the total phenolic content of the fruits and bark of wild *Zanthoxylum armatum* was 185.02 ± 2.15 mg GAE/g and 171.13 mg GAE/g, respectively [18]. *Delphinium denudatum* root methanolic extract has been shown to yield 90.43 ± 0.07 mg GAE/g [19]. Several medicinal plants have been reported with remarkably high phenolic contents, highlighting their high therapeutic potential. *Ocimum tenuiflorum* (Black tulsi) exhibited exceptionally high phenolic content of nearly 900 mg GAE/g in acetone extract and about 800 mg GAE/g in methanolic extract [20]. Similarly, *Quercus robur* and *Cinnamomum zeylanicum* showed significant phenolic content of 397.03 ± 0.05 mg GAE/g and 309.23 ± 0.05 mg GAE/g extract [21]. The moderate phenolic content observed in the present study suggests that the plant is a rich source of phenolic compounds, which are known for their strong antioxidant and free radical scavenging activities. Phenolic compounds play a significant role in preventing oxidative stress by donating hydrogen atoms or electrons to neutralize reactive oxygen species. The elevated TPC value obtained in this study may contribute to the plant's biological activities, including antioxidant, anti-inflammatory, and antimicrobial properties [22].

The total flavonoid content of *D. vestitum* extract was determined to be 90.07 ± 0.03 mg RE/g extract. Similar levels of flavonoids have been reported in a previous study**,** which reported that the total flavonoid contents of the fruits and bark of wild *Zanthoxylum armatum* were 103.7 ± 1.39 mg QE/g and 91.27 ± 3.13 mg QE/g, respectively [18]. The TFC of *D.*

denudatum root methanolic extract was reported to be 5.72 ± 0.37 mg RE/g [19]. Plants possessing high flavonoid content are often regarded as valuable medicinal resources. In previous studies, the acetone extract of *Ficus semicordata* showed very high flavonoid content of 500.35 ± 0.04 mg QE/g, while the extracts of *Crassocephalum crepidioides* and *Aesculus indica* exhibited flavonoid content of 348.78 ± 0.002 mg QE/g and 717.70 ± 0.60 mg QE/g, respectively [23,24]. Flavonoids are important secondary metabolites with well-documented antioxidant, anticancer, and cardioprotective activities. The low to moderate flavonoid content observed in this study indicates that the plant possesses significant bioactive potential. Flavonoids contribute to antioxidant activity mainly through metal chelation and free radical scavenging mechanisms. The presence of flavonoids in the extract may enhance its pharmacological efficacy and support its traditional medicinal use.

The total tannin content of the *D. vestitum* extract was calculated as 124.36 ± 0.02 mg TAE/g extract. This indicates a significant tannin content in the studied plant. Similarly, in accordance with the present study, previous studies reported 468.35 ± 21.44 mg TAE/g of tannin in the n-hexane extract of *Helichrysum italicum* and 38.86 mg TAE/g in *Byttneria herbacea* Roxb, two traditional medicinal herbs [25,26]. Plants rich in tannins are often used in traditional medicine for the treatment of infections and inflammatory conditions. A significant level of tannin, 659 mg TAE/g and 616 mg TAE/g, is reported in *Ocimum gratissimum* (green tulsi) and *Ocimum tenuiflorum* (black tulsi), respectively [20]. Similarly, *Aesculus indica* showed tannin content of 342.47 ± 8.55 mg TAE/g [24]. Tannins are polyphenolic compounds known for their astringent, antimicrobial, and antioxidant properties. They play important roles in plant defense mechanisms and contribute to various biological activities. The relatively low to moderate tannin content observed in this study suggests that the extract may possess strong antimicrobial and protein-binding properties, which can be beneficial in pharmaceutical and nutraceutical applications. Moreover, tannins have been reported to inhibit lipid peroxidation and reduce oxidative damage, further supporting the medicinal value of the plant.

Quantitative analysis via HPLC revealed the presence of gallic acid and quercetin in *D. vestitum* extract. The GA peak accounted for 39.61% of the total chromatographic area, with a calculated average concentration of 41.95 μg/100 mg extract, indicating the accumulation of this phenolic acid (Table 2). Gallic acid is known as a powerful antioxidant, anti-inflammatory, and antimicrobial agent, suggesting that its high content may be responsible for most of the biological properties observed in the extract [27]. In contrast, quercetin was detected at relatively low concentration, contributing to only 0.29% of the total peak area, with an average

concentration of 0.44 µg/100 mg extract (Table 2). Although present at trace levels, quercetin is a potent flavonoid with well-documented antioxidant and radical-scavenging properties, and even low concentrations can exert notable biological effects through synergistic interactions with other phenolic compounds [28].

In the present study, the total antioxidant capacity of *D. vestitum* was evaluated via ascorbic acid standard calibration and expressed as ascorbic acid equivalents (AAEs). The extract increased the total reducing potential in a dose-dependent manner. The lowest TAC was recorded at 25 µg/mL (10.82 ± 0.25 µg AAE/mL), and the highest TAC was found at 150 µg/mL (62.08 ± 0.03 µg AAE/mL), confirming greater electron transfer capacity at higher concentrations (Figure 3). A similar dose-dependent increase in TAC values has been widely observed in plant extracts, where higher concentrations result in a greater reduction in the phosphomolybdenum complex due to greater availability of reducing phytochemicals [29]. These findings support the strong cumulative antioxidant and reducing capacity of the plant extract. This supports the view that plant-based antioxidants often act through multiple mechanisms, including electron transfer and radical quenching, leading to a measurable increase in TAC in a concentration-dependent manner.

The antioxidant activity of the *D. vestitum* extract was also determined via the free radical scavenging activity of DPPH and compared with that of the positive antioxidant standard butylated hydroxytoluene (BHT). Both the standard and the plant extracts increased in a concentration-dependent manner in terms of the ability to scavenge free radicals at concentrations ranging from 20-120 µg/mL. BHT had greater antioxidant activity, with 21.62% inhibition at 20 µg/mL BHT, which increased to 90.79% at 120 µg/mL BHT. On the other hand, the plant extract showed 18.26% at 20 µg/mL concentrations and reached 84.99% at the highest concentrations (Figure 4). The IC_{50} of BHT was determined to be 50.90 µg/mL. The IC_{50} of the plant extract was 65.60 µg/mL (Table 3). Similarly, other studies reported similar results, such as the ethanolic extracts of *Punica granatum* and *Astragalus alopecurus* exhibited IC_{50} values of 16.10 µg/mL and 99.02 µg/mL, respectively [30]. One previous study reported IC_{50} of BHT and hemp seed oil equal to 49.50 µg/mL and 9.76 µg/mL, respectively [31]. The smaller IC_{50} of BHT indicates that it is more efficient at scavenging free radicals than the plant extract is . However, the plant extract displayed good antioxidant activity, indicating the presence of bioactive phytoconstituents of the plants capable of donating hydrogen atoms or electrons in the process of neutralization of free radicals.

The ABTS radical cation decolorization assay is a robust method applicable to both hydrophilic and lipophilic antioxidant molecules and is frequently used to assess the antioxidant capacity of plant extracts [32]. In this study, the *D. vestitum* extract displayed concentration-dependent ABTS inhibition, with the lowest inhibition at 100 µg/ml (26.92 %) and the highest inhibition at 150 µg/ml (52.24%), suggesting improved radical cation scavenging as the dose increased (Figure 5). This pattern follows earlier studies reporting enhanced ABTS scavenging capacity in medical plants with increasing concentrations, indicating a direct relationship between extract dose and the antioxidant response. Using ascorbic acid as the reference standard (IC_{50} < 100 µg/mL), *D. vestitum* extract exhibited an IC_{50} value of 145.63 µg/mL, indicating moderate ABTS radical scavenging activity (Table 3). Similarly, previous studies have reported IC_{50} values for ascorbic acid and the ethanolic extract of *Vernonia amygdalina* of 127.74 µg/mL and 256.89 µg/mL, respectively. IC_{50} values of leaf ethanol extract of *Nelumbo nucifera* were shown to be 22.8995 µg/mL [33,34]. The higher IC_{50} value suggests that the antioxidant potential of the *D. vestitum* extract is lower than that of the standard but still biologically significant.

The zone of inhibition against *E. coli* increased from 5.5 ± 0.5 mm at 1 mg to the maximum of 13.5 ± 0.5 mm at 8 mg, indicating strong dose-dependent antibacterial action. Similarly, *S. aureus* showed enhanced sensitivity with increasing concentration, where the zone of inhibition increased from 4.5 ± 0.25 mm (1 mg) to 13.5 ± 0.25 mm at 8 mg. In the case of *S. pneumoniae*, moderate inhibition was observed at lower concentrations (6.5 ± 0.5 mm at 3 mg), which increased notably at higher concentrations, reaching 12.5 ± 0.5 mm at 8 mg of *D. vestitum* extract. In contrast, *K. pneumoniae* exhibited a different pattern, where the maximum inhibition was recorded at intermediate concentrations (8.5 ± 0.5 mm at 3 mg), followed by reduced inhibition at higher concentrations (5.5 ± 0.5 mm at 8 mg). The positive control (streptomycin) demonstrated strong activity against all the test organisms (*E. coli* 12.0 ± 0.5 mm*; S. aureus* 10.0 ± 0.25 mm; *S. pneumoniae* 12.0 ± 0.5 mm*; K. pneumoniae* 14.0 ± 0.5 mm). Ampicillin showed inhibition only agains*t S. aureus* (5.0 ± 0.0 mm), whereas the negative control (methanol) produced no inhibition zones, confirming that the antimicrobial activity was due to the methanolic leaf extract of *D. vestitum,* as shown in Figures 6 and 7.

The present findings confirm that the *D. vestitum* methanolic extract exhibits promising antibacterial activity against both gram-positive and gram-negative bacteria, as evidenced by a measurable zone of inhibition. These antimicrobial effects of plant-derived bioactive compounds are frequently associated with phenolic metabolites. Plant phenolics and related

metabolites are reported to act through multiple mechanisms, including membrane disruption, interference with metabolic enzymes, inhibition of bacterial DNA/ protein synthesis, and oxidative stress induction in microbial cells. These mechanisms explain the broad-spectrum nature of the inhibition observed in the well diffusion assay [35,36].

Differences in susceptibility among bacterial strains can be explained by variations in cell wall architecture. Gram-negative bacteria possess an outer membrane that functions as a major permeability barrier and works synergistically with efflux systems, limiting the entry and accumulation of antimicrobial agents. In contrast, gram-positive bacteria lack this outer membrane, and their cell envelope structure often allows greater penetration of antimicrobial agents; therefore, they show relatively greater sensitivity to plant extracts. Interestingly, despite this barrier, the sample exhibited strong inhibition against gram-negative *E. coli*, indicating that the active constituents are capable of destabilizing the outer membrane or bypassing permeability restrictions [35,37]. The reduced inhibition against *K. pneumoniae* at higher concentrations may be due to reduced diffusion or dispersion of active compounds at higher doses because of the aggregation of the plant extract in the well. In addition, *K. pneumoniae* is a capsulated organism, and its capsular polysaccharides form a physical protective barrier that can reduce antimicrobial penetration, enhancing its resistance against *D. vestitum* extract [38,39].

Compared with the plant sample, the standard antibiotic ampicillin showed significantly lower inhibitory activity against certain bacterial strains. Similarly, previous studies have shown that many bacterial strains are gradually developing resistance to ampicillin, reducing its effectiveness against infections. These studies have reported that prolonged exposure to ampicillin enabled bacterial strains to adapt through changes in membrane permeability, biofilm formation, and resistance-related gene expression, and that gram-negative bacteria commonly resist ampicillin by producing beta-lactamase enzymes that inactivate the antibiotic [40,41]. The reduced effectiveness of standard antibiotics may indicate the emergence of antibiotic resistance, which has become a major global health concern. The declining efficiency of conventional antibiotics highlights the urgent need to discover and develop new antimicrobial agents that are safer, effective, and capable of overcoming resistant pathogens.

In the resazurin assay, resazurin absorbs more at approximately 600 nm, and its reduced form is resorufin, which is formed when bacteria are metabolically active. As shown in Table 4, the absorbance of the resazurin plate at 600 nm shows the higher OD_{600} values for reduced bacterial growth and higher antibacterial activity of the extract, and a lower OD_{600} for high bacterial

growth and less antibacterial potential. As shown in Figure 8, blue to purple coloration indicates inhibition of bacterial metabolic activity, whereas pink coloration signifies bacterial viability due to the reduction of resazurin to resorufin. The column with 400 µg/mL plant extract showed the minimum inhibitory concentration for all four bacterial strains.

In previous studies involving resazurin-based antibacterial assays, the petroleum ether extract of *Picrorhiza kurroa* exhibited a minimum inhibitory concentration of 5000 µg/mL against *Bacillus subtilis* and 2500 µg/mL against *Pseudomonas aeruginosa*. The water extract of *Saraca asoca* showed a minimum inhibitory concentration of 150 µg/mL against *Pseudomonas aeruginosa.* Aqueous extract of medicinal plants like *Ocimum sanctum*, *Adhatoda vasica*, *Leptadenia reticulata*, and *Cocculus hirsutus* exhibited minimum inhibitory concentration of 500 µg/mL, 500 µg/mL, 250 µg/mL, and 250 µg/mL, respectively, against *Mycobacterium tuberculosis* [42-43].

Conclusion

Phytochemical screening revealed that *D. vestitum* is a rich source of diverse secondary metabolites, including phenols, flavonoids, alkaloids, saponins, tannins, and terpenoids, with the methanol extract exhibiting the broadest range of phytoconstituents. The presence of multiple bioactive compounds supports the traditional medicinal use of this plant. Quantitative analysis of the extract revealed high concentrations of phenols, flavonoids, and tannins in the leaves. The HPLC results demonstrate that *D. vestitum* is a rich source of phenolic compounds, with gallic acid as the major contributor and quercetin as a minor yet biologically relevant flavonoid. The qualitative and quantitative profiles obtained by HPLC strongly support the antioxidant findings and highlight the therapeutic potential of *D. vestitum* as a natural source of bioactive phenols and flavonoids. TAC, DPPH, and ABTS assays, as well as antimicrobial assays, validated the plant's antioxidant and antimicrobial potential. Overall, the findings of this study highlight that the investigated high altitude medicinal plant is a valuable source of bioactive phytochemicals, scientifically support its traditional medicinal use, suggest its potential application in the development of natural antioxidant and antimicrobial formulations, and provide a strong scientific basis for further quantitative analysis, isolation, and pharmacological evaluation.

Materials and methods

Sample collection and authentication

Fresh leaves of *D. vestitum* Wall. ex Royle was collected in July from the Tungnath region (30°28′N lat; 79°13′E long, 3680 m a.s.l) in the Rudraprayag district, Uttarakhand, India (Figures 9 and 10) from our institutional nursery of the High Altitude Plant Physiology Research Center (HAPPRC), H.N.B. Garhwal University. The recorded temperature during sampling was 17 °C. The identification and authentication of *D. vestitum* Wall. ex Royle fresh plant was done by the Botanical Survey of India (BSI) and provided an accession number of 1893. A voucher specimen was stored in the herbarium of BSI. The plant name has been checked in the online database World Flora Online, a global plant species database available at http://www.worldfloraonline.org/.

Figure 9: Picture of *D. vestitum* Wall. ex-Royle at the sampling site

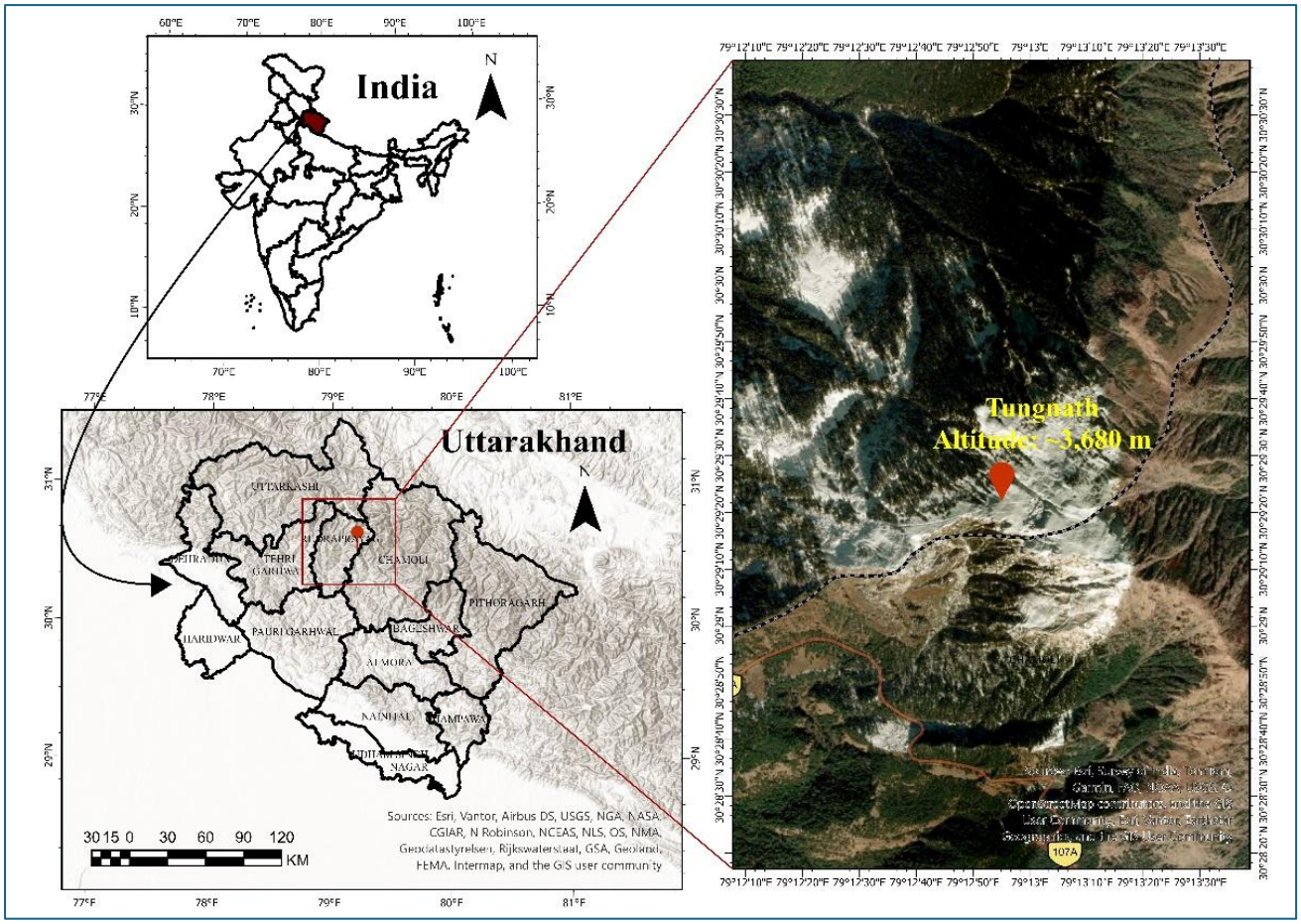


Figure 10: Location map of the study area, Tungnath (3680 m a.s.l)

Preparation of *D. vestitum* extracts

Fresh leaves from *D. vestitum* were rinsed well with tap water, washed with double-distilled water, and air-dried in the shade for 15 days. The dried leaves were ground to powder and extracts were prepared with the solvents in increasing order of polarity viz. petroleum ether, chloroform, acetone, ethanol, methanol, and water, following the maceration method of extraction at a 1:10 ratio by keeping the mixture in an orbital shaker at 50 rpm for 48 hrs at room temperature. The extract obtained was filtered through Whatman filter paper No. 41 in a preweighed empty flask. The flask was kept open in a water bath until the solvent completely disappeared. The weight of the flask containing the remaining compounds was taken, which provided the yield of the extract. Once the yield was measured, respective solvents were added to the extract to obtain a final concentration of 1 mg/mL plant extract (Figure 11), which was stored at 4 °C.

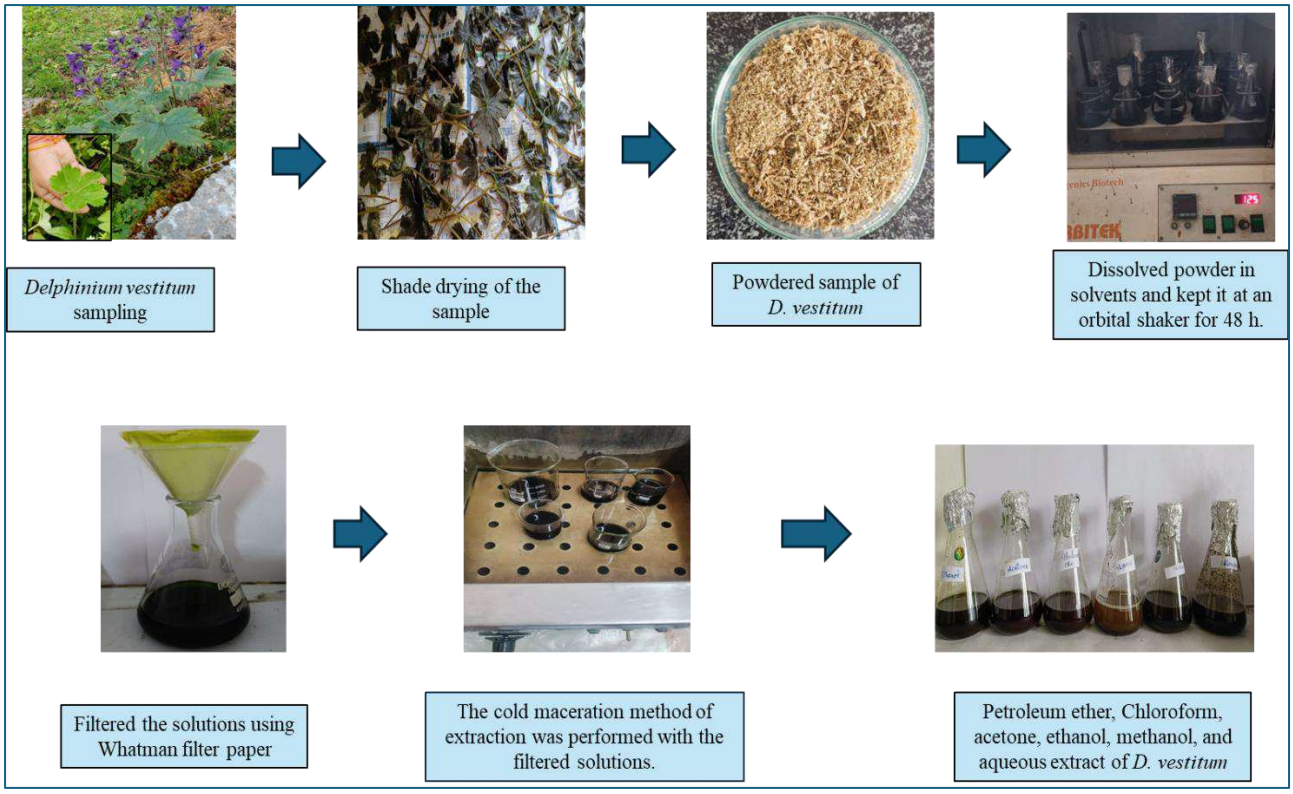


Figure 11: Preparation of various extracts (petroleum ether, chloroform, acetone, ethanol, methanol, and water) of *D. vestitum* leaves via the maceration method.

Qualitative analysis of phytochemicals

Preliminary screening of phytochemicals

The phytochemical analysis of the *D. vestitum* leaf extracts was performed at a concentration of 1 mg/mL to determine the presence or absence of key phytochemicals in the extract [44].

Detection of Alkaloids

Wagner's test: The extract was tested via Wagner's test, which involved treating the methanolic extract with 1.5% v/v HCL and Wagner's reagent, to detect the presence of alkaloids.

Detection of Flavonoids

Ammonia test: Plant extract (0.5 ml) was mixed with 4 ml of a 1% NH_3 (ammonia) solution, and to this mixture, 1 ml of concentrated sulfuric acid was added. The occurrence of strong yellow coloration was observed as an indication of the presence of flavonoids.

Detection of Phenols

Ferric chloride test: In the phenol test, 1 ml of the methanolic extract was mixed with 2 ml of distilled water. A few drops of 5% ferric chloride solution were subsequently added to the mixture. Upon the addition of 5% ferric chloride solution, the mixture underwent a color change and developed a dark green color. The dark green color indicates the presence of phenols in the tested extract.

Detection of Tannins

Braymer's test: In the tannin test, 1 ml of the methanolic extract was mixed with 3 ml of distilled water, followed by the addition of a few drops of 10% aqueous iron chloride solution. The addition of the iron chloride solution results in the development of an intense color, which can appear as green, purple, or blue-black. This intense color change indicates the presence of tannin in the tested extract.

Detection of Saponins

Foam test: The procedure involved taking 0.5 ml of the methanolic extract and adding 10 ml of distilled water. The mixture was then vigorously agitated or shaken to facilitate the extraction of saponins. After agitation, the formation of froth or foam on the surface was observed. Saponins, known for their ability to produce stable foams, are present when a significant amount of froth is formed. The absence of frothing suggests the absence or low concentration of saponins in the sample.

Detection of Carbohydrates

Molish's test: Alcoholic α-naphthol (2 drops) was added to 2 ml of filtrate. Then 1 ml of concentrated sulphuric acid was added along the side of the test tube. The formation of a violet ring confirmed the presence of a carbohydrate.

Detection of Phytosterol

Hesse response: Chloroform (2 ml) was added to 5 ml of extract. Then, 2 ml of concentrated sulfuric acid was added along the sides of the test tube. The pink/red color in the chloroform layer indicated the presence of phytosterols.

Detection of Triterpenoids

For Salkowski's test: 1 ml of plant extract was taken, and a few drops of concentrated sulphuric acid were added to it. After vigorous shaking, the mixture was allowed to stand. The presence of triterpenoids was confirmed by observing a golden yellow layer at the bottom of the test tube.

Detection of Cardiac Glycosides

Keller-Killani test: The extract (2 mL) was mixed with 1.5 mL of glacial acetic acid, and 1 drop of 5% ferric chloride was added. Then, concentrated sulfuric acid was added along the side of the test tube. The blue color of the acetic acid layer confirms the presence of cardiac glycosides.

Detection of Carboxylic Acids

Effervescence test : In the carboxylic acid test, 1 ml of the methanolic extract was mixed with a few milliliters of 10% sodium bicarbonate solution. Upon mixing, there is very little effervescence or bubbling seen in the mixture. This slight effervescence suggests the presence of carboxylic acids in the tested extract.

Detection of Reducing sugars

Fehling test: In the reducing sugar test, 1 ml of each Fehling solution A and Fehling solution B was added to 1 ml of the plant extract. The solution was boiled in a water bath. The presence of red precipitate confirmed the presence of reducing sugars in the extract.

Quantitative phytochemical analysis of the methanolic leaf extract of *D. vestitum*

Estimation of Total Phenolic Content

In preparation for the stock solution, 20 mg of gallic acid was dissolved in 20 mL of methanol. The aliquots (50-250 µg/mL) in methanol were prepared using the stock solution. Take the 200 µg/mL of methanolic leaf extract as test samples. To the 200 µl test sample and to all standard samples, one milliliter of Folin Ciocalteu reagent (1:1, v/v) was added. This was followed by the addition of 1 mL of 7.5% sodium carbonate, and the mixture was allowed to stand at room temperature for 2 hours. The absorbance was read with the UV-visible Spectrophotometer at 726 nm [45,46].

Estimation of Total Flavonoid Content

To prepare the standard stock solution, 10 mg of rutin was dissolved in 10 ml of methanol. Different concentrations of the standard solution, ranging from 20 to 100 micrograms per

milliliter, were made with methanol. Take two hundred micrograms per milliliter of the methanolic extract from the plant sample. In both the test sample and the standard solution, 2 mL was taken, and 0.1 mL of 10% aluminium chloride hexahydrate was added. Then, 0.1 mL of potassium acetate and 2.8 mL of deionized water were added, and the mixture was left to sit at room temperature for 40 minutes. The absorbance was measured at 415 nm using a UV-visible spectrometer. The total flavonoid content was expressed as milligrams of rutin equivalent (RE) per gram of plant sample [45].

Estimation of Total Tannin Content

Tannin-like components in a high-concentration alkaline solution reduce the phosphotungstomolybdic acid, producing a bright blue solution, the intensity of which depends on the amount of tannins. The Folin-Ciocalteu method was employed to determine the total tannin content (TTC). A total of 0.1 mL of methanolic plant extract (1 mg/mL) was dissolved in 7.5 mL of distilled water in a 10 mL volumetric flask. A total of 0.5 mL of Folin-Ciocalteu phenol reagent and another 1 mL of 35% Na_2CO_3 solution were added, and the resulting mixture was subsequently diluted with 10 mL of distilled water. After proper shaking, the mixture was incubated at room temperature for 30 min. A standard tannic acid solution (20 to 100 µg/mL) was prepared. The absorbance was measured at 725 nm after comparison with that of a standard blank solution. The total tannin content was expressed as milligrams of tannic acid equivalents per gram of dry extract (mg TA/g). All analyses were carried out in triplicate [46].

High-Performance Liquid Chromatography

Chemicals and reagents

A mixed standard solution of gallic acid (GA) and quercetin (Q) from HiMedia was used as a reference for peak identification and chromatographic comparison. HPLC-grade solvents from HiMedia were used for the mobile phase and sample preparation.

Preparation of the standard and the sample

A mixed standard solution (GA+Q) was injected as a reference under identical chromatographic conditions as the plant extract sample. The methanolic extract was filtered prior to injection to remove particulate matter.

Instrumentation and conditions

The methanolic extract was analyzed via HPLC (Shimadzu, Japan) with a PDA SPD-M20 detector. A 20 µL sample loop was included with the LC-20AD dual pump system and SIL-20AC autoinjector (with cooler). The compounds were separated on a Shimadzu RP-C18 column (250 × 4.6 mm, 5 µm), protected by a guard column with the same packing material. Chromatographic separation was performed via an isocratic mobile phase composed of water:acetonitrile (80: 20 % v/v). The methanolic extract of the leaf was injected in triplicate (20 µL), and the run time was maintained for 50 min. Shimadzu LabSolutions software was used for data acquisition and peak integration, with detection at 254 nm (PDA channel 1; bandwidth 4 nm). Peak identification was performed by comparing the retention times with those of available standards. The plain mobile phase was injected as a blank control for the detection of background peaks [47].

In vitro assessment of the antioxidant properties of *D. vestitum*

Determination of Radical Scavenging Activity via the DPPH Assay

The free radical scavenging ability of *D. vestitum* extract was tested via the DPPH assay. A fresh 0.1 mM DPPH solution was prepared by dissolving DPPH in methanol and storing it in the dark to prevent its photodegradation. The concentrated methanolic plant extract was taken, and then different concentrations (20 to 120 micrograms per milliliter) were made by gradually diluting the solution. Butylated hydroxytoluene (BHT) was used as the standard for comparison.

For the test, 1 mL of DPPH solution was combined with 1 mL of each sample or standard in separate test tubes. The mixtures were gently shaken and left in the dark at room temperature for 30 minutes. A control sample was prepared by mixing 1 mL of DPPH with 1 mL of methanol. After the reaction time, the amount of light absorbed was measured at 517 nm using a UV-visible spectrophotometer, with methanol used as the reference. All experiments were carried out in triplicate.

The percentage of DPPH radical scavenging activity was calculated using the following equation: $\%\ \text{inhibition} = \frac{\text{Ao}-\text{As}}{\text{Ao}} \times 100$

where Ao is the absorbance of the control and As is the absorbance of the sample or standard. The IC50 value, defined as the concentration required to inhibit 50% of DPPH radicals, was determined by plotting the percentage inhibition against the concentration and was calculated via linear regression [48].

Determination of Total Antioxidant Capacity (TAC)

The total antioxidant capacity (TAC) of *D. vestitum* extract was measured via the phosphomolybdenum method. To prepare the reagent solution, 28 mM sodium phosphate, 4 mM ammonium molybdate, and 0.6 M sulfuric acid were mixed together. Fresh reagent was added before each test. The plant extract was made in methanol at different concentrations, ranging from 25 to 150 micrograms per milliliter. Ascorbic acid prepared in methanol was used as the standard for comparison.

For the test, 0.3 mL of each sample or standard was added to 3.0 mL of phosphomolybdenum reagent in sealed test tubes. The tubes were placed in a water bath at 95°C for 90 minutes. After that, the mixtures were cooled down to room temperature. The absorbance was measured at 695 nm using a UV-visible spectrophotometer and compared to that of a blank reagent solution. All measurements were performed three times each.

A calibration curve was generated using ascorbic acid, and the TAC was expressed as micrograms of ascorbic acid equivalent per milliliter (µg AAE/mL) of the extract [49].

Determination of ABTS Radical Cation Scavenging Activity

The scavenging activity of the ABTS radical cation by the *D. vestitum* extract was tested. To make the ABTS radical solution, 7 mM ABTS was mixed with 2.45 mM potassium persulfate in equal parts. This mixture was left in the dark at room temperature for 16 hours so that the ABTS radical cation fully formed. Before testing, the ABTS radical solution was diluted with methanol so that it had an absorbance of 0.70 ± 0.02 at 734 nm. The plant extract was made in methanol and diluted to obtain concentrations between 100 and 150 micrograms per milliliter. Ascorbic acid was used as the reference.

For the test, 2 mL of each sample or standard was combined with 2 mL of the diluted ABTS radical solution.

The mixture was kept in the dark at 27°C for 6 minutes. The absorbance was measured at 734 nm using a UV-Visible spectrophotometer. A control sample was made using methanol instead of the plant extract. All the measurements were done three times. The percentage inhibition of ABTS radicals was calculated via the following equation: % Inhibition = $\frac{\mathrm{Ao}-\mathrm{As}}{\mathrm{Ao}} \times 100$

where A0 represents the control absorbance and As represents the absorbance of the test sample.

The IC_{50} value was determined from the dose–response curve by plotting percentage inhibition against concentration [50].

In vitro assessment of the antibacterial properties of *D. vestitum* methanolic leaf extract

Chemicals and reagents

Nutrient agar, nutrient broth, and resazurin sodium salt were used for antibacterial analysis. Streptomycin, ampicillin, and gentamycin were used as standard antibiotics (positive controls). Methanol was used as the solvent and the negative control. All the chemicals and media used were of analytical grade.

Bacterial strains

The antibacterial activity of the test sample was evaluated against four bacterial strains, namely, *Escherichia coli* (clinical strain), *Staphylococcus aureus* (ATCC-25293*)*, *Klebsiella pneumoniae (*MTCC-4030), and *Streptococcus pneumoniae* (ATCC-49169), which were procured from Hi-Media. The *Escherichia coli* strain was obtained from V.C.S.G. Government Medical College. The antibacterial discs streptomycin and ampicillin were also procured from Hi-Media Laboratories Pvt. Ltd., Mumbai, India. The strains were maintained on nutrient agar slants at 4 °C and subcultured prior to experimentation.

Preparation of sample solutions

The leaf powder of *D. vestitum* was dissolved in methanol to prepare a 10 mg/mL stock solution. Working concentrations corresponding to 1, 2, 3, 4, 5, 6, 7, and 8 mg/mL were prepared by serial dilution for the agar well diffusion assay, and 200, 400, 600, 800, and 1000 µg/mL were used for the resazurin-based microdilution assay. Methanol alone served as the negative control.

Standardization of inoculum

Fresh bacterial cultures were grown in nutrient broth and adjusted to a 0.5 McFarland standard (1×10^8 CFU/mL). The standardized suspension was used for both the agar diffusion and microdilution assays.

Agar well diffusion assay

Preliminary antibacterial screening was performed via the agar well diffusion method on nutrient agar plates. The sterile nutrient agar plates were uniformly inoculated with standardized bacterial suspensions via a sterile cotton swab to obtain a confluent lawn culture. Wells of approximately 6 mm in diameter were aseptically punched using a sterile cork borer. Each well was loaded with the methanolic leaf extract of *D. vestitum* at various concentrations (1 to 8 mg). Streptomycin and ampicillin were used as positive controls, whereas methanol served as a negative control. The plates were allowed to stand at room temperature for 30 min to facilitate diffusion and were then incubated at 37 °C for 24 h. After incubation, the zones of inhibition (mm) were measured [51].

Resazurin-based microplate assay for MIC determination

The minimum inhibitory concentration (MIC) of the extract was determined using the resazurin-based microtiter plate method. Briefly, 96-well microplates were filled with 100 µL of nutrient broth. An equal volume of methanolic leaf extract of *D. vestitum* at different concentrations (200 µg/mL to 1 mg/mL), methanol, and a positive control (40 mg/mL gentamycin) were added to the wells, followed by the addition of 10 µL of a standardized bacterial inoculum. The plates were incubated at 37 °C for 24 h. After incubation, 10 µL of resazurin solution (0.01% w/v) was added to each well, and the mixture was further incubated for 1 h. Color changes were visually monitored. Wells retaining blue/purple color were considered indicative of bacterial growth inhibition, whereas those with a pink color indicated bacterial viability. The MIC was defined as the lowest concentration showing no color change [52].

Statistical analysis

All the quantitative analysis experiments were performed in triplicate, and the results are expressed as the mean and standard deviation. The linear regression coefficient (R^2) for the quantification of phytochemical content was analyzed via Microsoft Office Professional Plus 16. A radar plot of phytochemical analysis and antimicrobial potential graphs is prepared using Origin 2018 software.

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Author contributions

P.S. conceived the concept of the article and supervised. N.B. wrote the manuscript, performed the experiments, and analyzed the results; S.A. reviewed the manuscript and analyzed the results; S.K. reviewed the manuscript and analyzed the results; M.R. reviewed the manuscript. P.S.R. proofread the manuscript. All the authors reviewed and approved the manuscript.

Competing interests

The authors declare no competing interests.

Ethical issues: None

Data availability

The datasets generated or analyzed during the current study are available from the corresponding author on reasonable request.

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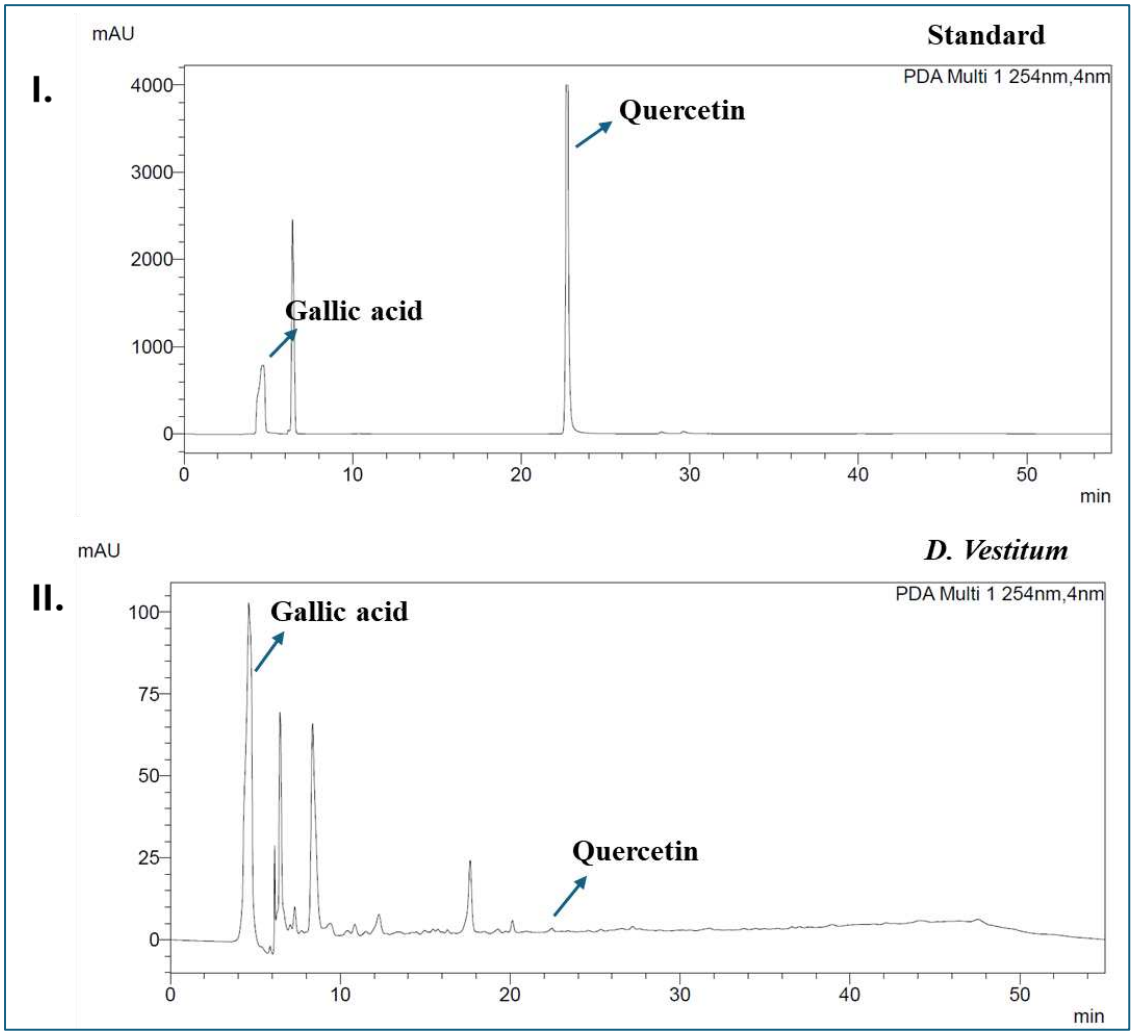

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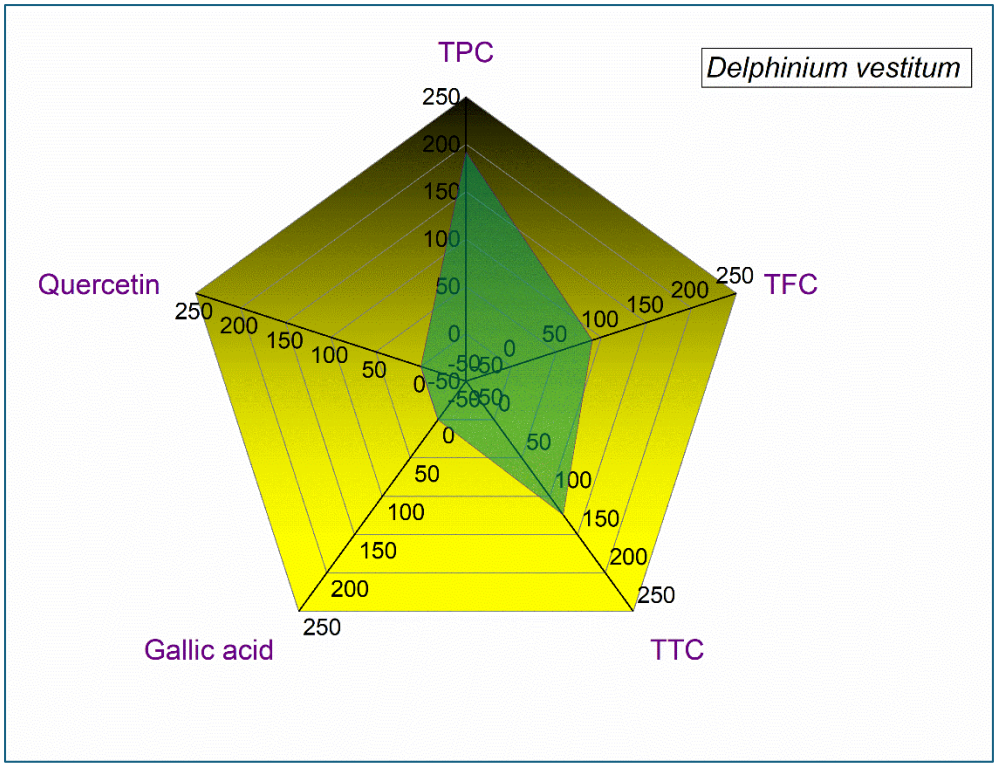

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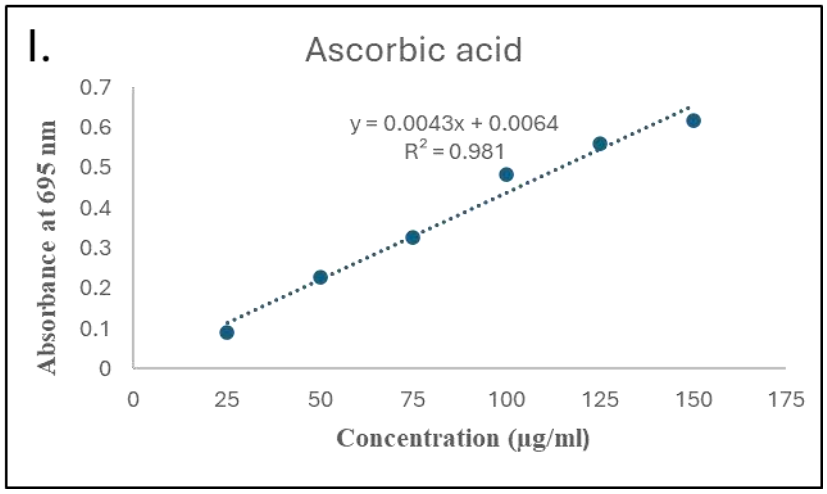

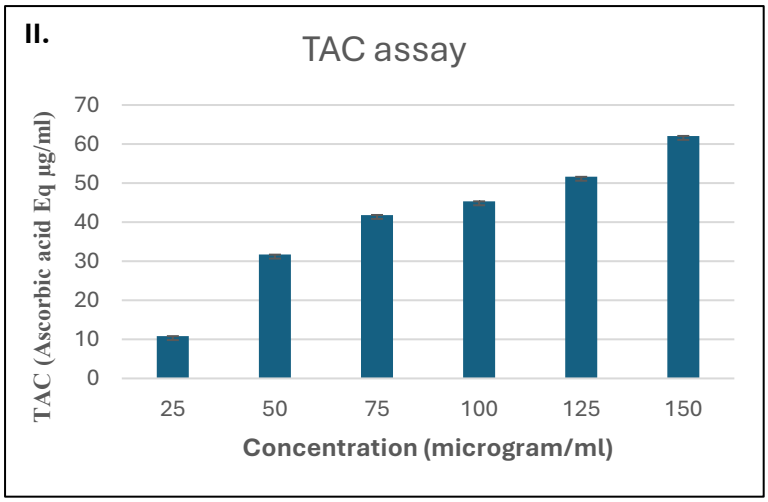

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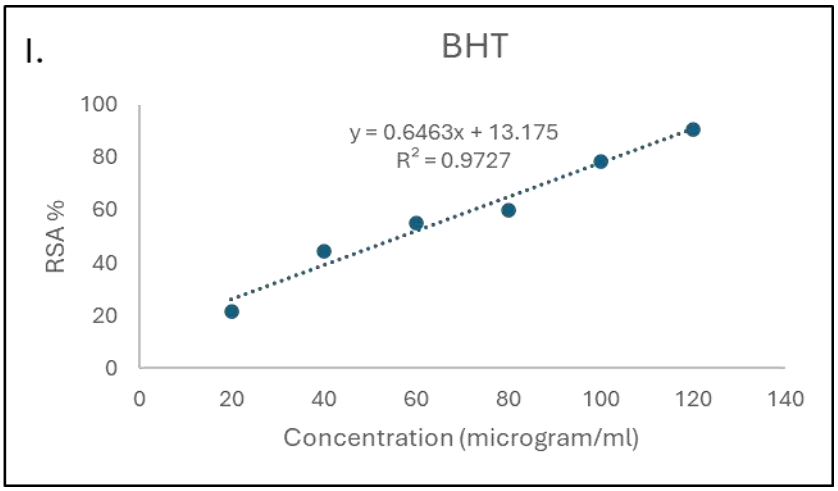

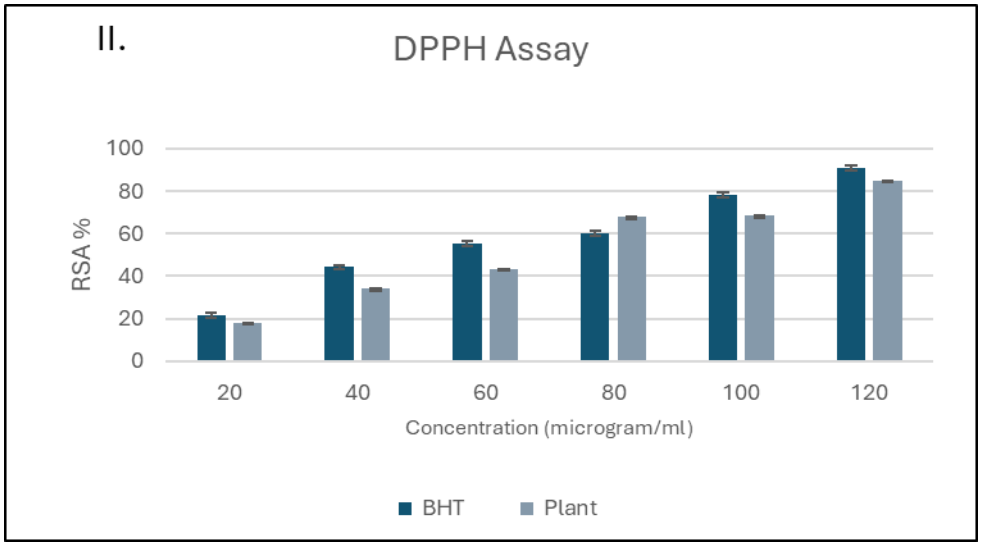

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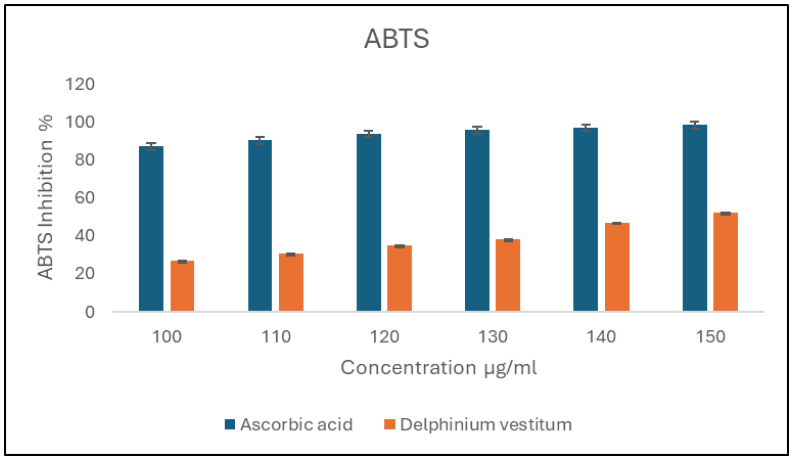

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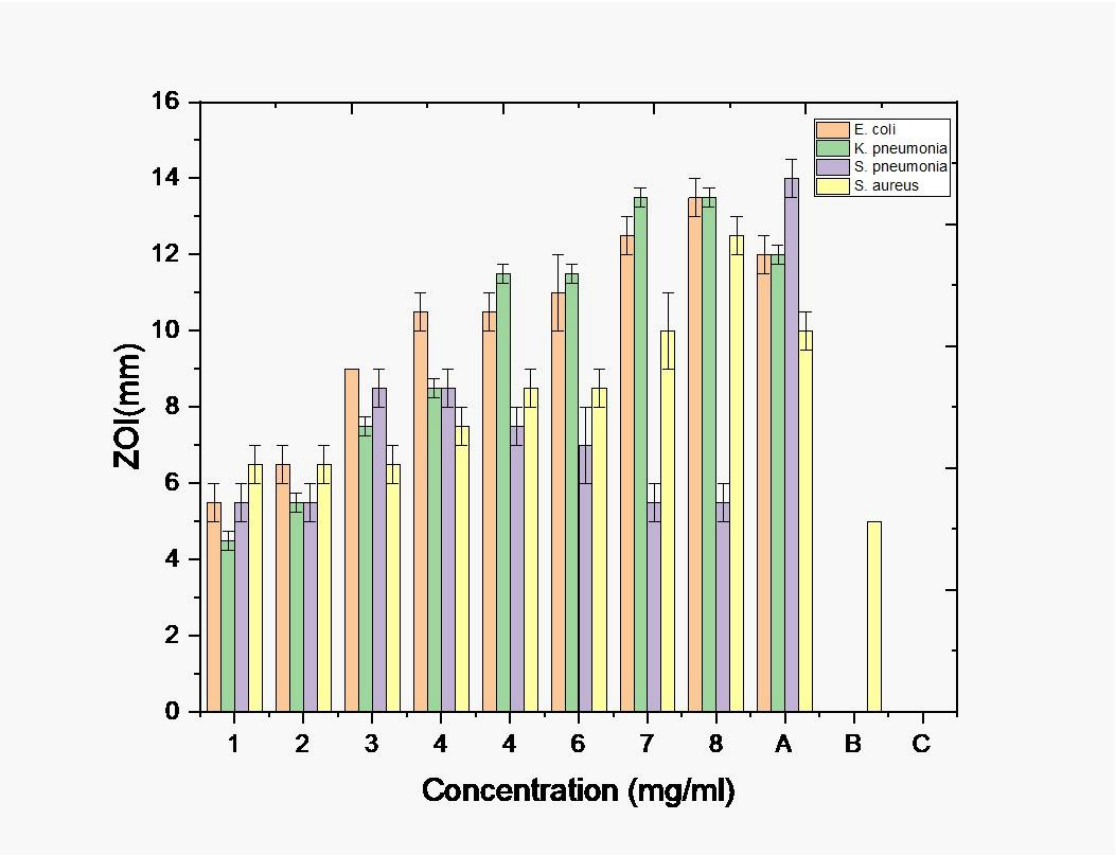

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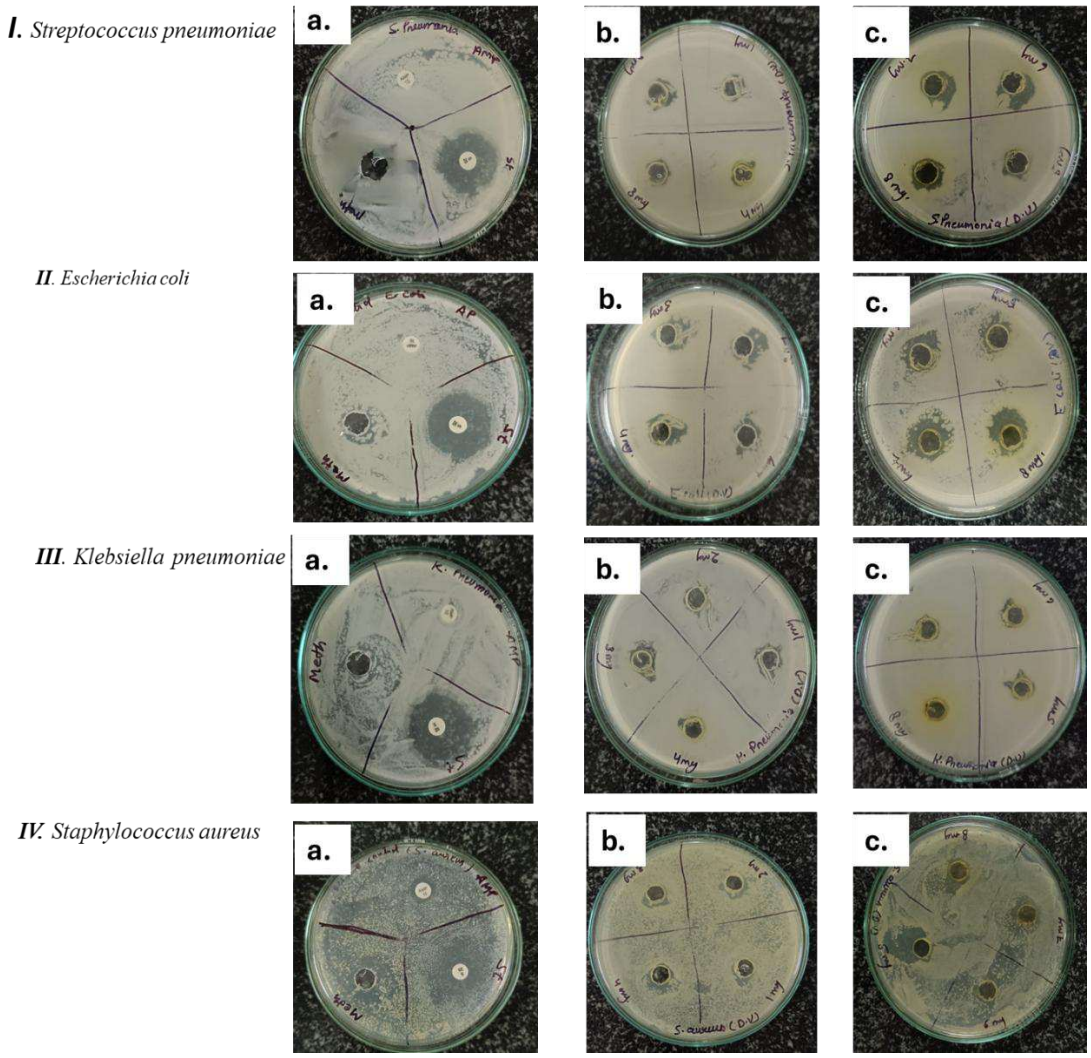

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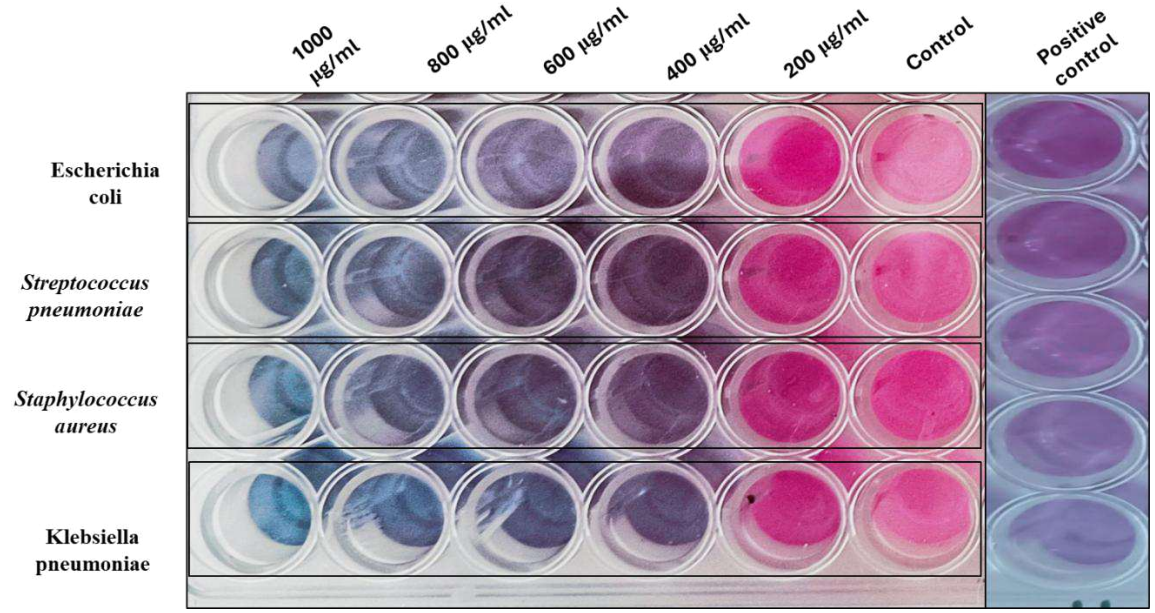

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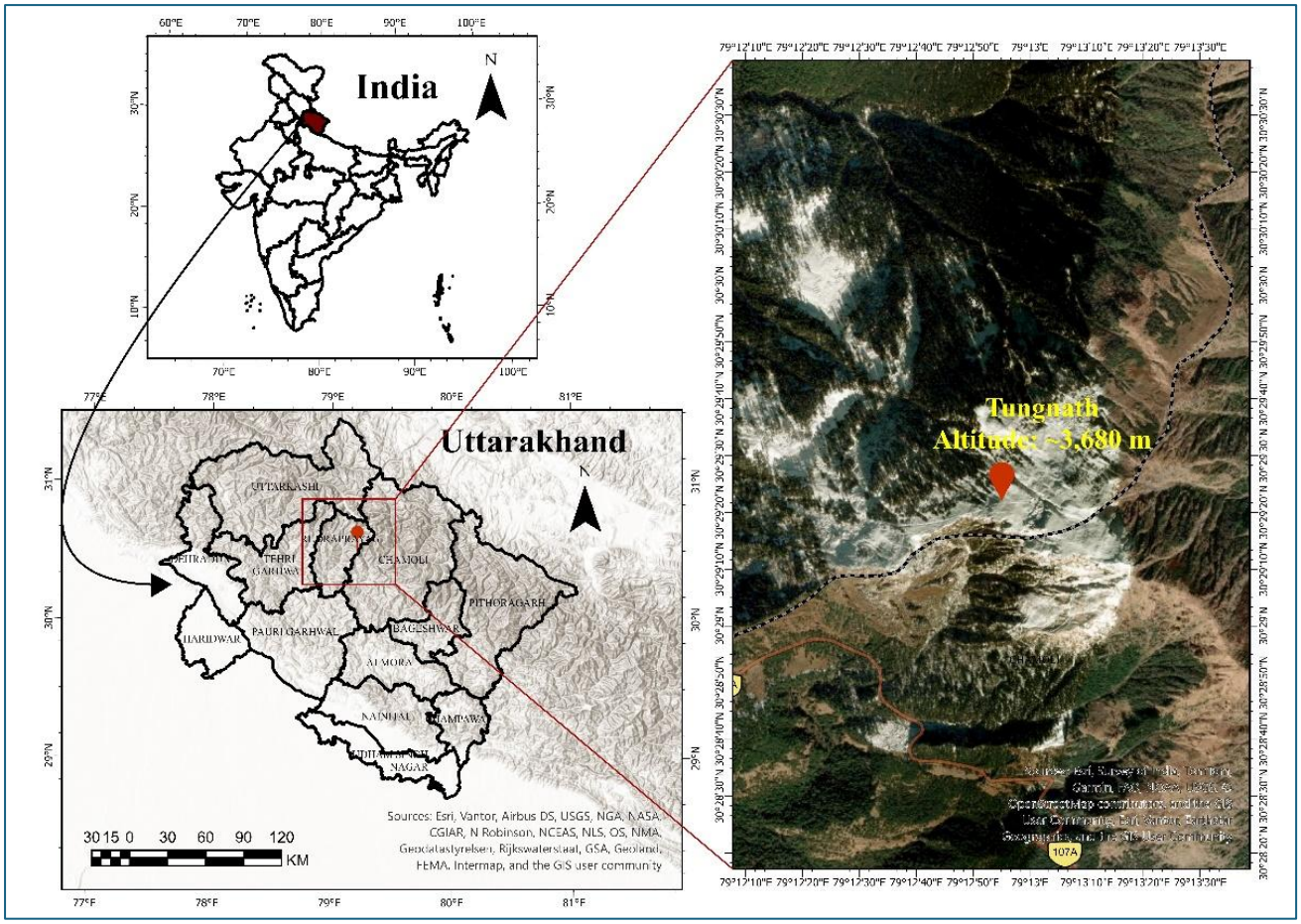

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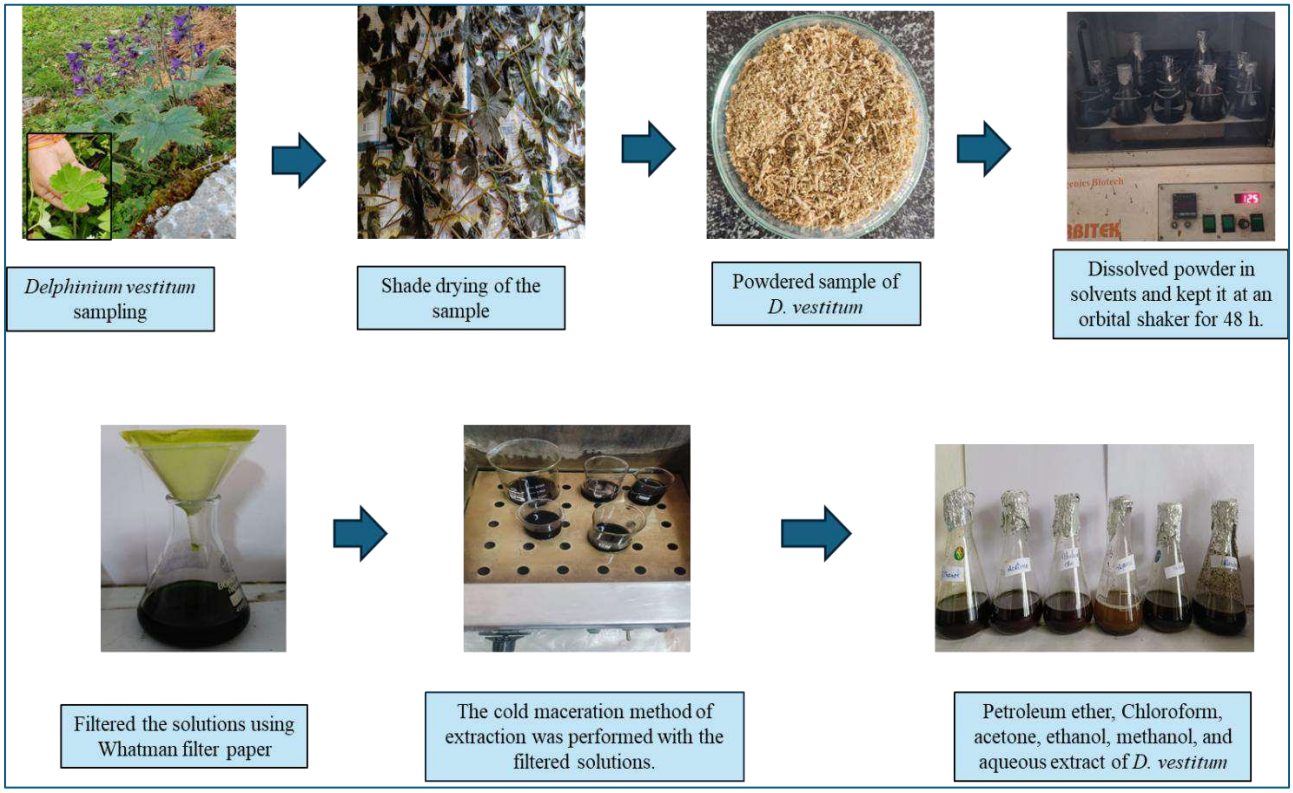

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Table 1: Qualitative phytochemical analysis of various extracts of *D. vestitum* (+ indicates presence, while – indicates absence)

Phytochemical Test	**Petroleum ether**	**Chloroform**	**Acetone**	**Ethanol**	**Methanol**	**Water**	**Observation**
Alkaloids	+	+	+	-	+	-	Presence of brown/reddish precipitate
Flavonoids	+	+	+	+	+	+	Presence of green precipitate

Phenols	+	+	+	+	+	+	Formation of bluish black color
Tannins	+	+	+	+	+	+	Blue- green color formation
Saponins	-	+	+	+	+	+	Presence of foam
Carbohydrates	+	+	+	+	+	+	Formation of a violet ring
Phytosterols	+	+	+	+	+	+	Appearance of red color in the lower layer
Triterpenoids	+	+	+	+	+	-	Presence of a golden yellow layer at the bottom
Cardiac glycosides	+	+	+	+	+	-	Appearance of blue color in the acetic acid layer
Carboxylic acid	+	+	-	-	+	-	Appearance of effervescence
Reducing sugar	+	+	+	+	+	+	Presence of red precipitate

Table 2: Quantification of gallic acid and quercetin in *D. vestitum* extract via HPLC (254 nm)

S. No.	**Compound**	**(GA+Q) RT (min)**	***Delphinium Vestitum* RT (min)**	**Standard Peak Area**	***Delphinium Vestitum* Peak Area**	***Delphinium Vestitum* Area %**	**Average concentration in *D. vestitum* (µg/100 mg)**

1	**Gallic acid (GA)**	4.701	4.620	21,552,897	2,714,415	39.614	41.95
2	**Quercetin (Q)**	22.768	22.456	54,096,749	19,688	0.287	0.44

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Assay	Standard used	Standard (IC_{50})	D. vestitum (IC_{50})	Interpretation
DPPH radical scavenging	BHT	IC_{50} = 50.90 µg/mL	IC_{50} = 65.60 µg/mL	*D. vestitum* shows strong DPPH scavenging, slightly lower than BHT
ABTS radical scavenging	Ascorbic acid	IC_{50} < 100 µg/mL	IC_{50} = 145.63 µg/mL	*D. vestitum* shows moderate ABTS scavenging activity

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Absorbance at 600 nm	1000 µg/ml	800 µg/ml	600 µg/ml	400 µg/ml	200 µg/ml	Control	Positive control
Escherichia coli	0.924 ± 0.003	0.784 ± 0.001	0.544 ± 0.003	0.453 ± 0.002	0.354 ± 0.002	0.343 ± 0.003	1.292 ± 0.002
Streptococcus pneumoniae	0.884 ± 0.002	0.843 ± 0.003	0.573 ± 0.002	0.484 ± 0.003	0.485 ± 0.003	0.443 ± 0.002	1.132 ± 0.001
Staphylococcus aureus	1.164± 0.002	0.993 ± 0.003	0.705 ± 0.003	0.567 ± 0.001	0.425 ± 0.002	0.428 ± 0.027	1.572 ± 0.003

Klebsiella pneumoniae	1.104 ± 0.002	0.945 ± 0.002	0.784 ± 0.002	0.693 ± 0.003	0.447 ± 0.004	0.434 ± 0.003	1.425 ± 0.003