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PHYTOCHEMICAL ANALYSIS, BIOLOGICAL SCREENING, ANTIMICROBIAL, AND CYTOTOXIC ACTIVITIES OF SOME SELECTED MEDICINAL PLANTS OF GILGIT BALTISTAN

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Abstract

This study addresses the gap by evaluating traditionally used 10 medicinal plants from the Skardu region. The objectives were to quantify key phytochemical components, assess antioxidant, antimicrobial, enzyme inhibitory, and cytotoxic potentials. Moreover, identified species with promising pharmacological relevance. Phytochemical content, antioxidant activity, antimicrobial activity, enzyme inhibition, and cytotoxic activity of methanolic extracts of the selected plants were investigate. *Artemisia siversiana* had the highest Total Phenolic Content (53.8 ± 0.19 µg GAE/mg extract) and free radical scavenging activity 85.7 ± 0.33 with 33.2 IC50 (µg/ml).While, *Capparis spinosa* had the maximum Total Flavonoid Content (41.6 ± 0.41 µg QE/mg extract), and Total Antioxidant Capacity (211.2 ± 0.42 µg AAE/mg extract). The Total Reducing Power was recorded highest in *Thymus leniaris* L**.** 217.7 ± 0.15 µg AAE/mg extract. The antibacterial tests was done against five resistant and two non-resistant bacterial strains respectively. *Artemisia siversiana* and *Thymus leniaris* L. have noteworthy anti-*Pseudomonas aeruginosa* inhibitory activity. *Thymus leniaris* L. exerted a significant 12 mm antifungal zone of inhibition followed by *Artemisia brevifolium* and *Delphinium brunonianum* (10 mm each). *Acantholimon lycopodioides* having highest antidiabetic capacity with an inhibition of alpha- amylase by 76.7% with an IC50 of 9.8 µg/ml. Whereas, *Peganum harmala* was reported with most cytotoxic potential (LD50 = 29.4 µg/ml). Furthermore, highest anti-hemolytic activity was exhibited by *Aconitum heterophyllum* (74.3 ± 0.57 µg/ml). These findings highlight the high phytochemical richness and multifunctional bioactivities of these plant extracts, supporting their potential as antidiabetic, anticancer, and antimicrobial drug application.

Key words: Antidiabetic; Anticancer; Flavonoids; Bioactive Compounds; Pharmaceutical Development

Introduction

The use of medicinal plants for therapeutic purposes is an ancient and enduring human practice, forming the cornerstone of traditional medicine systems worldwide (Ramamurthy *et al*., 2017). These botanical resources have evolved into sophisticated frameworks for developing remedies against a wide spectrum of ailments, a legacy that continues to inform modern pharmacopeia (Lee *et al*., 2022). In many developing nations, plants are deemed a precious natural resource and are formally integrated into healthcare programs, underscoring their socio-economic and cultural importance (Karim *et al*., 2020). A key advantage of plant-derived bioactive compounds is their general biocompatibility, often resulting in better patient tolerance and acceptability compared to synthetic alternatives (Newman & Cragg, 2012). Their paramount value in contemporary drug discovery lies in their vast, unexplored structural diversity, which provides an invaluable reservoir of chemical scaffolds for prospective drug development (Lautié *et al*., 2020). Indeed, plants have served as the foundational basis for conventional therapeutic systems, paving the way for the discovery of numerous modern chemotherapeutic agents (Veeresham *et al*., 2012).

It is estimated that approximately 80% of the global population relies on plant-based therapies for primary healthcare, highlighting their unparalleled reach and significance (Patrício *et al*., 2022). Consequently, the pharmaceutical industry and therapeutic research remain heavily dependent on medicinal and aromatic plants as primary sources of phytochemicals for identifying and developing crude drugs (Mustafa *et al*., 2017; Boukhatem *et al*., 2020). This extensive use is driven by the relative accessibility, cost-effectiveness, and perceived safety of plant-derived medications (Maver *et al*., 2018). Validating this trust, many plants possess well-documented ethnomedicinal properties, making them potential candidates for managing conditions such as rheumatism, gout, and musculoskeletal pain (Yatoo *et al*., 2018).

The medicinal efficacy of plants is intrinsically linked to their phytochemical constituents (Nwozo *et al*., 2023). These encompass both primary metabolites (e.g., proteins, lipids) involved in basic cellular processes and, more critically, secondary metabolites with specialized functions (Alamgir,

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2018; Erb & Kleibenstein, 2020). Secondary metabolites—including phenolic compounds, alkaloids, flavonoids, saponins, and phytosterols—play vital roles in plant defense and confer significant bioactivity (Hussein & El Anssary, 2019; Abbas *et al*., 2023). Their biosynthesis is often upregulated under environmental or induced oxidative stress, leading to their accumulation as part of the plant's adaptive defense mechanism (Ho *et al*., 2020). Plants possess inherent antimicrobial properties as a vital part of their natural defense system, producing a diverse array of secondary metabolites such as alkaloids, flavonoids, tannins, and phenolic compounds. This defense arsenal also includes volatile essential oils, which are renowned for their broad-spectrum bioactivities (Ni *et al*., 2021). These bioactive constituents can inhibit the growth of pathogenic bacteria, fungi, and other microorganisms through mechanisms like disrupting cell membranes, interfering with protein synthesis, and inhibiting enzyme activity. This natural pharmacological activity not only underpins the historical use of many plants in traditional medicine for treating infections but also provides a crucial foundation for modern research seeking new compounds to combat the growing global threat of antimicrobial resistance (Chan *et al*., 2021). A paramount activity exhibited by many of these compounds is antioxidant potential, which is crucial for modulating cellular signaling and countering oxidative damage (Ho *et al*., 2020). Antioxidants are essential for neutralizing reactive oxygen species (ROS)—such as hydroxyl radicals (OH), superoxide (O_2^-), and peroxide radicals (ROO•)—produced during metabolism. Unmitigated ROS generation leads to oxidative stress, a key pathological driver of aging, cancer, and various degenerative diseases (Chikara *et al*., 2018; Hunyadi *et al*., 2019). The cellular response to this stress involves complex signaling pathways, including secondary sulfur metabolism, which plays a crucial role in maintaining redox homeostasis (Chan *et al*., 2019). Bioactive plant compounds that mitigate this oxidative stress and associated inflammation are therefore recognized for promoting health and systemic stability (Kretovich, 2013). Plants possess a diverse array of natural antioxidants with distinct compositions and mechanisms, making them a subject of intense interest for combating life-threatening disorders like neurodegenerative and cardiovascular diseases (Zahra *et al*., 2017; Chan *et al*., 2019; Ni *et al*., 2021).

While historical use involved direct application, modern drug discovery employs advanced technologies to isolate pure phytochemicals. These natural structures serve as direct models or inspiration for synthesizing novel pharmaceuticals, with a plant's pharmacological activity hinging on the identification of these constituents. However, commonly used synthetic antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are under scrutiny for potential links to pancreatic damage and carcinogenesis. This has significantly heightened the global quest for safer, effective natural alternatives from plant sources.

Despite this need, a considerable research gap persists. Many regions with rich biodiversity, such as Gilgit-Baltistan in Pakistan, harbor numerous endemic medicinal plants whose phytochemical profiles and comprehensive pharmacological potentials remain insufficiently characterized and scientifically validated. This study is inherently limited by its *In vitro* nature, the use of a single solvent (methanol) for extraction, and a selected panel of microbial strains. Further *In vivo* studies and compound isolation are required for definitive therapeutic conclusions.

This study is based on the hypothesis that the selected medicinal plants from Gilgit-Baltistan contain significant levels of bioactive secondary metabolites, which are responsible for conferring potent *In vitro* antioxidant, antimicrobial, enzyme inhibitory, and cytotoxic activities, thereby substantiating their ethnomedicinal use and highlighting their potential for drug discovery. To test this hypothesis, the study aims to scientifically evaluate the phytochemical composition and biological potential of ten selected plants through the following specific objectives: to prepare methanolic extracts; to quantitatively analyze the phytochemical content, including total phenolic and flavonoid compounds; to evaluate *In vitro* antioxidant capacity through various assays (DPPH, TAC, TRP); to screen for antimicrobial activity against a panel of resistant and non-resistant bacterial and fungal strains; to assess enzyme inhibitory potential against alpha-amylase and protein kinase; and to determine cytotoxic activity using the brine shrimp lethality assay and hemolytic potential on human erythrocytes.

Material and Method

Plant collection and identification: A total 10 medicinal plants (Table 1) were collected from district Skardu during the month of August 2021. The plants were identified by Dr. Sher Wali Khan, Karakoram International University from Flora of Pakistan and online Flora of China. All collected plant specimens were authenticated by comparison with reference materials available in the Herbarium, the Flora of Pakistan, and online databases of the Flora of Pakistan and China. The verified and properly identified specimens were then pressed, dried and deposited in the Herbarium of the University of Baltistan, Skardu, for future reference and documentation.

Extractions and drying: The collected plants were carefully examined and washed thoroughly to remove any unwanted debris, contaminants, or decayed fragments prior to further processing. The material was then air dried for four to six weeks in the shade. The dried plant materials were finely ground using a mechanical grinder to obtain a uniform powder for subsequent extraction procedures (Atta *et al.,* 2023).

Maceration: Plant material was successively extracted using ultrasonic assisted maceration (Fatima *et al*., 2015). The 5g of aerial parts from dried plant materials were first immersed for three days in 25 ml of Methanol (MeOH) with sporadic 25 KHz sonication at 25°C.

Total phenolic content (TPC): The assay was performed in 96-well plate. In each corresponding well of 96-well plate an aliquot of 20 µl was added from the 4 mg/ml stock solution of each extract followed by the addition of 90 µl FC reagent as described by (Atta *et al.,* 2023). The absorbance was measured, and the total phenolic content was calculated and expressed as milligrams of gallic acid equivalent to per milligram of dry plant weight (mg GAE/mg DW).

Table 1. List of the ten medicinal plants investigated from the Gilgit-Baltistan region, including their scientific names, and parts used for extraction.

Plant name	Local name (Balti)	Part used
Allardia glabra Decne.	Tarqan	Aerial part/ Whole plant
Capparis spinosa L.	Traba	Root / Leaves, Flower
Paganum harmala L.	Isman	Aerial part/ Whole plant
Nepeta leucolaena L.	Azumal	Aerial part
Aconitum heterophyllum Wall. ex Royle	Buma	Root part/ Leaves
Acantholimon lycopodioides (Girard) Boiss.	Loxey	Aerial part/ Whole plant
Artemisia brevifolium Wall. ex DC.	Bursay Thurgu	Aerial part/ Whole plant
Delphinium brunonianum Royle	Makhoting	Aerial part/ Whole plant
Thymus linearis Benth.	Tumburuk	Aerial part
Artemisia siversiana Ehrhart	Bursay	Aerial part/ Whole plant

Total flavonoid content (TFC): For performance of assay 96-well plates were used. From the test extracts (4 mg/ml) an aliquot of 20 µl was transferred to each well followed by 10 µl potassium acetate, aluminum chloride 10 µl and 160 µl distilled water was added to the respective wells, following the protocol used by Haq *et al.,* (2012). The results were expressed as milligrams of quercetin equivalents per milligram of dry plant weight (mg QE/mg DW).

Total reducing power (TRP): Potassium ferricyanide colorimetric assay was used to estimate the reducing power of extracts. An aliquot of 100 µl of each test extracts (4 mg/ml DMSO) was added with 200 µl each of phosphate buffer (0.2 M, pH 6.6) and 250 µl potassium ferricyanide (1% w/v in distilled water) followed by incubation at 50°C for 20 minutes in water bath. (Khan *et al.* 2015). The reducing power of each sample was expressed as µg ascorbic acid equivalent per mg of sample (µg AAE/mg).

Total antioxidant capacity (TAC): The phosphomolybdenum-based method served as the evaluation technique for test sample TAC measurement. Test extract at 4 mg/mL DMSO solution received 100 µL of solution volume from 900 µL containing TAC reagent that resulted from mixing 0.6 M sulfuric acid with 28 mM sodium phosphate and 4 mM ammonium molybdate. DMSO served as the detrimental control when the experts conducted this process. The investigation applied the protocol outlined in Khan *et al*., (2015). The antioxidant activity was stated as the number of µg equivalents of ascorbic acid per mg of dry weight (µg AAE/mg of DW).

Free radical scavenging assay: The compounds were screened for antioxidant activity by using 2, 2-diphenyl 1-picrylhydrazyl (DPPH) following Khan *et al*., (2015).

The following formula was computed for determining % free radical scavenging activity:

$$\text{Free radical scavenging activity} = 1 - As/Ac * 100$$

where, As and Ac are the absorbance of sample and negative control respectively.

Antibacterial assay: Microbroth dilution method was used to determine antibacterial potential of test samples against *Staphylococcus aureus* (ATCC-6538), *Bacillus subtilis* (ATCC-6633), *Escherichia coli* (ATCC-25922), *Klebseilla pneumoniae* (ATCC-1705) and *Pseudomonas aeruginosa* (ATCC-15442) and resistant *Escherichia coli and MRSA.* Bacterial inoculum was formed under aseptic conditions keeping the density near to 5×10^4 CFU/ml in pre autoclaved nutrient broth. Test extracts of an aliquot of 5 µl were transferred to the respective wells of 96 well plate followed by the addition of 195 µl of nutrient broth (Fatima *et al.*, 2015).

Antifungal assay: The antifungal activity of the test extracts was evaluated using the agar disc diffusion technique. Spore suspensions of selected fungal strains including *Fusarium solani* (FCBP-0291), *Aspergillus fumigatus* (FCBP-66), *Aspergillus flavus* (FCBP-0064), *Aspergillus niger* (FCBP-0198), and *Mucor* species (FCBP-0300) were prepared in a 0.02% Tween 20 solution, following the methodology of Fatima *et al.*, (2015).

α-amylase inhibition assay: A reaction mixture containing 85 µL of phosphate buffer with pH 6.8, 25 µL of α-amylase enzyme at 0.14 U/mL, test sample at 4 mg/mL in DMSO and 40 µL of starch solution at 2 mg/mL potassium phosphate buffer was used to perform the α-amylase inhibition assay. The test solution measured at 4 mg/mL was combined with α-amylase enzyme (0.14 U/mL) and starch solution (2 mg/mL) in a phosphate buffer solution at 50°C for 30 minutes through a 96-well microplate procedure (Atta *et al.,* 2023). The inhibitory action of the prepared samples were evaluated by determining the percentage of α-amylase inhibition through appropriate calculations:

$$\alpha - \text{amylase inhibition} = (Os - On)/(Ob - On) \times 100$$

where O_n= Absorbance of negative control, O_s = Absorbance of sample and O_b = Absorbance of blank well.

Protein kinase inhibition assay: To evaluate the protein kinase inhibitory activity, test extracts derived from the Streptomyces strain 85E were utilized. The strain was revitalized in tryptone soya broth and incubated at 37°C for 24 hours to ensure optimal growth conditions (Atta *et al.,* 2023).

Brine shrimp lethality assay: A 96 well microplate was used to perform the brine shrimp lethality assay. In order to hatch, the eggs of the test organism *Artemia salina* were

incubated at 30–32°C for 24–48 h in the simulated sea water (38 g/l supplemented with 6 mg/l dried yeast) which was pre-saturated with oxygen while maintaining a temperature range from 30–32°C for 24 to 48 hours to produce hatched larvae. A Pasteur pipette was utilized to gather nauplii after incubation then moved them to a 50 ml beaker. Ten larvae were distributed among different wells in the microplate containing solutions with multiple concentration levels. Each well received artificial seawater solution to maintain the DMSO concentration at or below 1%. The protocol followed by Atta *et al*., (2023) using extracts at the concentrations of 200, 100, 50 and 25 µg/ml.

Hemolytic assay: The analysis of test sample's ability to cause red blood cell damage required its studying with human erythrocytes. 1ml of blood was added in each Eppendorf tube and centrifuged at 10000 rpm for 10 min. The supernatant obtained from centrifugation received proper disposal and the pellet of erythrocytes was rinsed using 1 mL of phosphate-buffered saline (PBS). The washing protocol included a second centrifugation step to remove any remaining plasma contents and other contaminants before preparing the red blood cells for laboratory assessment (Atta *et al.,* 2023). The % age hemolytic activity was calculated by formula:

$$\% \text{ Hemolysis} = \frac{\text{Absorbance of sample} - \text{Absorbance of negative control}}{\text{Absorbance of positive control} - \text{Absorbance of negative control}} * 100$$

Data analysis: MS Excel was used to measure mean and standard deviation of samples.

Results and Discussion

Total phenolic content (TPC): The quantitative analysis of total phenolic content revealed significant variation among the methanolic extracts of the ten selected medicinal plants, as summarized in Table 2. The measured TPC values ranged from a minimum of 0.9 ± 0.23 µg GAE/mg extract in *Acantholimon lycopodioides* to a maximum of 53.8 ± 0.19 µg GAE/mg extract in *Artemisia siversiana*. The majority of the extracts demonstrated substantial phenolic content, with values predominantly falling between 38.8 and 53.8 µg GAE/mg. *Artemisia siversiana* emerged as the most phenol-rich specimen, a finding consistent with the broader literature on the *Artemisia* genus, which is frequently reported to accumulate high levels of diverse polyphenols. For instance, comparable studies have identified significant phenolic constituents in other *Artemisia* species, such as *A. parviflora*, where methanolic extracts were particularly efficacious (Ahameethunisa *et al*., 2012; Hussain *et al*., 2017).

The high phenolic content in these extracts is of considerable pharmacological importance. Phenolic compounds are renowned for their potent antioxidant activity, primarily attributed to the redox properties of their hydroxyl groups, which enable them to act as hydrogen donors, free radical scavengers, and singlet oxygen quenchers (Yildirim *et al*., 2000; Kumar *et al*., 2017). This intrinsic antioxidant capacity allows them to mitigate oxidative stress by neutralizing reactive oxygen species (ROS), thereby protecting cellular components from damage—a fundamental mechanism in preventing a spectrum of chronic and degenerative diseases (Cheynier *et al*., 2012). Consequently, plant extracts with elevated TPC, like that of *A. siversiana*, are strongly implicated in therapeutic applications ranging from wound healing to combating metabolic disorders (Petti and Scully, 2009). The observed variation in TPC across the tested species aligns with established research, such as Katalinic *et al*., (2006), documented wide disparities in phenolic content across 70 medicinal plants, underscoring the chemical diversity within the plant kingdom and the need for species-specific phytochemical profiling.

Total flavonoid content: The analysis of total flavonoid content (TFC) revealed a pronounced disparity among the evaluated plant extracts, as detailed in Table 3. *Capparis spinose* possessed the most substantial flavonoid reservoir, with a quantified value of 41.6 ± 0.41 µg QE/mg extract. This finding aligns with and substantiates prior research on the *Capparis* genus; for example, Tlili *et al*., (2015) documented a broad TFC range of 4.71 to 72.79 mg QE/g in caper materials, positioning our result well within the expected phytochemical profile for this species. In stark contrast, *Delphinium brunonianu* exhibited no detectable flavonoid content, while *Acantholimon lycopodioides* registered a minimal value of 0.2 ± 0.61 µg QE/mg extract. This pattern of distribution underscores the selective biosynthesis of these specialized metabolites across different plant families.

The significant flavonoid concentration in *C. spinose* directly correlates with its documented bioactivity. As supported by Rahnavard & Razavi (2017), the robust *In vitro* antioxidant potential of caper extracts is intrinsically linked to their high levels of phenolic acids and flavonoids. This relationship between phytochemical composition and function is a critical observation. Furthermore, a consistent trend was noted wherein extracts with minimal phenolic content, such as *A. lycopodioides*, correspondingly exhibited low flavonoid levels. This positive correlation underscores the interconnected biosynthetic pathways of these secondary metabolites and reinforces the principle that a plant's overall bioactive potential is often a composite of its major phytochemical classes (Kaurinovic & Vastag, 2019).

The high TFC in several extracts holds considerable pharmacological promise. Flavonoids are renowned for their multi-target therapeutic mechanisms, contributing to anticancer, antioxidant, antibacterial, and antifungal activities (Malik *et al*., 2022). Their efficacy stems from a combination of immunoprotective and detoxifying properties that aid in neutralizing harmful toxins (Atta *et al*., 2023). As potent antioxidants, flavonoids play a dual role: they directly stabilize and scavenge free radicals involved in oxidative cascades, and they indirectly bolster the cellular antioxidant defense system (Jafri *et al*., 2017). Their mechanism extends beyond radical quenching to include the inhibition of key pro-inflammatory and catalytic enzymes, as well as the chelation of metal ions that catalyze ROS formation (Zahra *et al*., 2017; Akhtar & Mirza, 2018). Therefore, the elevated flavonoid content identified in species like *C. spinose* provides a strong biochemical rationale for their ethnomedicinal use and positions them as promising candidates for the development of novel therapeutic agents targeting oxidative stress, microbial infections, and chronic inflammation.

Table 2. Total phenolic content (TPC) of the methanolic plant extracts, expressed as micrograms of gallic acid equivalent per milligram of extract (µg GAE/mg extract) ± S.D.

Serial No.	Sample name	(µg GAE/mg of sample) ± S.D.
1.	*Allardia glabra* Decne.	44.1 ± 0.71
2.	*Capparis spinosa* L.	45.9 ± 0.23
3.	*Paganum harmala* L.	40.7 ± 0.71
4.	*Nepeta leucolaena* L.	42.3 ± 0.19
5.	*Aconitum heterophyllum*Wall. ex Royle	48 ± 0.15
6.	*Acantholimon lycopodioides* (Girard) Boiss.	0.9 ± 0.23
7.	*Artemisia brevifolium* Wall. ex DC.	38.8 ± 0.57
8.	*Delphinium brunonianum* Royle	2.1 ± 0.18
9.	*Thymus linearis* Benth.	42.5 ± 0.35
10.	*Artemisia siversiana* Ehrhart	53.8 ± 0.19

Note: GAE = Gallic acid equivalent, --- = No activity, Sample concentration = 400 µg/ml, Positive control = Gallic acid, Negative control = DMSO and SD = Standard deviation

Table 3. Total flavonoid content (TFC) of the methanolic plant extracts, expressed as micrograms of quercetin equivalent per milligram of extract (µg QE/mg extract) ± S.D.

Serial No.	Sample name	(µg QE/mg of sample) ± S.D.
1.	*Allardia glabra* Decne.	14.5 ± 0.21
2.	*Capparis spinosa* L.	41.6 ± 0.41
3.	*Paganum harmala* L.	34 ± 0.34
4.	*Nepta leucolaena* L.	34.4 ± 0.33
5.	*Aconitum heterophyllum* Wall. ex Royle	8.5 ± 0.73
6.	*Acantholimon lycopodioides* (Girard) Boiss.	0.2 ± 0.61
7.	*Artemisia brevifolium* Wall. ex DC.	31.7 ± 0.26
8.	*Delphinium brunonianum* Royle	---
9.	*Thymus linearis* Benth.	9.1 ± 0.51
10.	*Artemisia siversiana* Ehrhart	3.6 ± 0.13

Note: QE = Quercetin equivalent, --- = No activity, Sample concentration = 400 µg/m, Positive control = Quercetin, Negative control = DMSO and SD = Standard deviation

Table 4. Total reducing power (TRP) of the methanolic plant extracts, expressed as micrograms of ascorbic acid equivalent per milligram of extract (µg AAE/mg extract) ± S.D.

Serial No.	Sample name	Total reducing power (µg AAE/mg of sample) ± S.D.
1.	*Allardia glabra* Decne.	123.4 ± 0.54
2.	*Capparis spinosa* L.	108.1 ± 0.32
3.	*Paganum harmala* L.	198 ± 0.15
4.	*Nepeta leucolaena* L.	102.7 ± 0.25
5.	*Aconitum heterophyllum* Wall. ex Royle	163 ± 0.86
6.	*Acantholimon lycopodioides* (Girard) Boiss.	20.3 ± 0.35
7.	*Artemisia brevifolium* Wall. ex DC.	192.5 ± 0.12
8.	*Delphinium brunonianum* Royle	53.1 ± 0.70
9.	*Thymus linearis* Benth	217.7 ± 0.15
10.	*Artemisia siversiana* Ehrhart	108.1 ± 0.02

Note: AAE = Ascorbic acid equivalent, --- = No activity, Sample concentration = 400 µg/ml, Positive control (ascorbic acid) concentration = 100 µg/ml, Negative control DMSO and SD = Standard deviation

Total reducing power (TRP): The evaluation of total reducing power (TRP) provided a critical measure of the electron-donating capacity of the plant extracts, a fundamental mechanism of antioxidant action. The results, presented in Table 4, demonstrated considerable variation among the species. *Thymus linearis* L. exhibited the most potent reducing activity, with a value of 217.7 ± 0.15 µg AAE/mg extract, strongly aligning with previous studies on the *Thymus* genus that report significant reductive capabilities, such as in *Thymus vulgaris* (Amamra *et al*., 2018). This was followed by substantively high values in *Paganum harmala* (198 ± 0.15 µg AAE/mg extract) and *Artemisia brevifolium* (192.5 ± 0.12 µg AAE/mg extract), underscoring their rich content of reductant compounds. In stark contrast, *Acantholimon lycopodioides* recorded the lowest TRP at 20.3 ± 0.35 µg AAE/mg extract, a result consistent with its previously noted minimal Total Phenolic (TPC) and Flavonoid (TFC) content.

This direct correlation between low polyphenolic levels and weak reducing power is not coincidental but causal. Polyphenols, through their hydroxyl groups, are primary agents responsible for reducing free radicals by donating hydrogen atoms or electrons (Chew *et al*., 2009). The TRP assay specifically quantifies this capacity,

wherein bioactive molecules with strong proton (H^+)-donating ability reduce the ferricyanide complex, forming ferrous cyanide (Brewer, 2011). Therefore, a high TRP value is a direct functional indicator of the presence of potent reductones—compounds that terminate free radical chain reactions by stabilizing reactive species, thereby preventing oxidative cascade damage (Ahmed *et al*., 2017).

For drug discovery targeting oxidative stress-related pathologies, this reductive capacity is paramount (Nisar *et al*., 2010). The exceptional TRP of *T. linearis*, *P. harmala*, and *A. brevifolium* provides a robust biochemical rationale for their ethnomedicinal use. It confirms that their extracts are not merely chemically rich but are functionally equipped to act as primary antioxidants. This potent reducing power suggests promising utility in developing therapeutic or nutraceutical agents aimed at mitigating oxidative stress, a key contributor to chronic diseases, thereby validating their further investigation for isolation of specific reducing compounds.

Total Antioxidant Capacity (TAC): The phosphomolybdenum assay provided a comprehensive assessment of the total antioxidant capacity (TAC) by measuring the combined ability of all phytoconstituents in the extracts to reduce Mo(VI) to Mo(V). As presented in Table 5, a wide spectrum of antioxidant potential was observed among the ten medicinal plants. *Capparis spinosa* demonstrated exceptional activity, registering the highest TAC value of 211.2 ± 0.42 µg AAE/mg extract. This outstanding capacity aligns perfectly with its previously noted status as the specimen with the highest total flavonoid content (TFC), underscoring a direct phytochemical basis for its potency. Conversely, *Acantholimon lycopodioides* exhibited the weakest overall antioxidant activity (37.7 ± 0.22 µg AAE/mg extract), a finding consistent with its poor performance across all preceding phytochemical and antioxidant assays.

These results robustly affirm the well-established positive correlation between phenolic compounds and antioxidant strength (Shon *et al*., 2004). Plants like *C. spinosa*, which are rich in both phenolic compounds and flavonoids, inherently possess strong, broad-spectrum antioxidant properties (Asif, 2015). The mechanism is intrinsically linked to the chemical structure of these metabolites. Specifically, flavonoids exert their antioxidant action primarily through the phenolic hydroxyl groups attached to their ring structures; these groups donate hydrogen atoms or electrons to stabilize free radicals, thereby interrupting oxidative chain reactions (Carocho & Ferreira, 2013).

The physiological and therapeutic implication of high TAC is profound. Natural antioxidants from plant sources play a crucial role in resisting oxidative damage at the cellular level, a key factor in the pathogenesis of numerous chronic and degenerative diseases (Moussa & Almagharabi, 2016). Consequently, plants with high concentrations of these phytochemicals are considered premier sources of natural antioxidants, offering a safer and more biocompatible alternative to synthetic options (Jafri *et al*., 2017). The superior and holistic antioxidant profile of *Capparis spinosa*, as evidenced by its leading TAC, TFC, and TRP values, provides a powerful scientific rationale for its ethnomedicinal use. It validates the plant not merely as a repository of chemicals but as a source of integrated, synergistic antioxidant activity with significant potential for development into therapeutic agents aimed at mitigating oxidative stress-related pathologies (Kalaivani & Mathew, 2010).

DPPH free radical scavenging assay (FRSA): The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay is a fundamental and widely validated method for evaluating the primary antioxidant activity of plant extracts by measuring their hydrogen-donating capacity. The results, detailed in Table 6, revealed pronounced differences in this specific activity among the tested species. *Artemisia siversiana* exhibited the most potent radical scavenging activity, with an inhibition of 85.6% and a notably low IC_{50} value of 33.2 µg/ml, indicating a high concentration of fast-acting antioxidant compounds. In stark contrast, *Delphinium brunonianum* showed negligible activity (0.1% inhibition), reflecting minimal direct free radical neutralizing potential under these conditions.

These results are not isolated but are intrinsically interconnected with the quantitative phytochemical profiles established earlier. The superior performance of *A. siversiana* directly correlates with its status as the specimen with the highest total phenolic content (TPC), robustly defending the central hypothesis that bioactive phenolic compounds are primary contributors to measurable antioxidant activity (Hayet *et al*., 2010). Conversely, *D. brunonianum*, which recorded minimal TPC and no detectable flavonoids, demonstrated commensurately weak scavenging capacity. This clear dose-response relationship underscores the principle that the ability of plant extracts to scavenge reactive oxygen species (ROS) is a direct function of their endowment with specific antioxidant phytochemicals (Riaz *et al*., 2012).

The DPPH assay is a critical tool for identifying this property because it provides a stable, rapid model system to gauge a compound's ability to donate a hydrogen atom to stabilize a free radical, mimicking a key mechanism required to counteract oxidative stress in biological systems (Abdel-Hameed, 2009). While free radicals are natural metabolic byproducts, their excessive generation overwhelms endogenous defense systems, leading to oxidative damage to cellular components a pivotal event in aging and disease pathogenesis (Valko *et al*., 2004; Vaghasiya *et al*., 2011). The potent activity of *A. siversiana* validates the pursuit of plant-derived polyphenols as effective and safer alternatives to synthetic antioxidants, which are often burdened by safety concerns (Tzima *et al*., 2018). The significant radical scavenging activity, coupled with a favorable IC_{50}, positions this extract as a particularly promising candidate for further investigation. Furthermore, the variability observed across extracts highlights the necessity of employing a battery of complementary antioxidant assays—such as TAC and TRP—to fully capture the complex and multifaceted nature of antioxidant mechanisms, which can involve single-electron transfer, hydrogen atom transfer, and metal chelation (Sini *et al*., 2011).

Table 5. Total antioxidant capacity (TAC) of the methanolic plant extracts, expressed as micrograms of ascorbic acid equivalent per milligram of extract (µg AAE/mg extract) ± S.D.

Serial No.	Sample name	Total antioxidant capacity ± S.D. (µg AAE/mg of sample)
1.	*Allardia glabra* Decne.	96.6 ± 0.17
2.	*Capparis spinosa* L.	1.2 ± 0.42
3.	*Paganum harmala* L.	174.9 ± 0.31
4.	*Nepta leucolaena* L.	98.9 ± 0.41
5.	*Aconitum heterophyllum* Wall. ex Royle	97.5 ± 0.64
6.	*Acantholimon lycopodioides* (Girard) Boiss.	37.4 ± 0.67
7.	*Artemisia brevifolium* Wall. ex DC.	101.8 ± 0.55
8.	*Delphinium brunonianum* Royle	87.8 ± 0.37
9.	*Thymus linearis* Benth.	116.5 ± 0.23
10.	*Artemisia siversiana* Ehrhart	193.4 ± 0.17

Note: AAE = Ascorbic acid equivalent, --- = No activity, Sample concentration = 400 µg/ml, Positive control (ascorbic acid) concentration = 50 µg/ml, Negative control = DMSO and SD = Standard deviation

Table 6. Free radical scavenging activity (%) of the methanolic plant extracts (200 µg/ml) as determined by the DPPH assay, along with the calculated half-maximal inhibitory concentration (IC50) values (µg/ml). Data are presented as mean ± S.D.

Serial No.	Sample name	% Scavenging (200 µg/ml) ± S.D.	IC50 (µg/ml)
1.	*Allardia glabra* Decne.	34.6 ± 0.11	NA
2.	*Capparis spinosa* L.	10.4 ± 0.26	NA
3.	*Paganum harmala* L.	38.4 ± 0.65	NA
4.	*Nepta leucolaena* L.	1.2 ± 0.43	NA
5.	*Aconitum heterophyllum* Wall. ex Royle	55.8 ± 0.63	99.5
6.	*Acantholimon lycopodioides* (Girard) Boiss.	10.4 ± 0.86	NA
7.	*Artemisia brevifolium* Wall. ex DC.	37.7 ± 0.22	NA
8.	*Delphinium brunonianum* Royle	0.1 ± 0.71	NA
9.	*Thymus linearis* Benth.	48.2 ± 0.53	NA
10.	*Artemisia siversiana* Ehrhart	85.7 ± 0.33	33.2

Note: Sample concentration = 200 µg/ml, Positive control (Ascorbic Acid) concentration = 50 µg/ml, Negative control =DMSO, Not Applicable = NA and SD = Standard deviation

Table 7. Antibacterial screening results of the methanolic plant extracts against non-resistant and resistant bacterial strains, indicating activity and the corresponding minimum inhibitory concentration (MIC in µg/ml).

Serial No	Sample	Non-resistant bacterial strains										Resistant bacterial *Strains*			
		S. aureus		*Bacillus subtilis*		*Klebseilla pneumoniae*		*E. coli*		*Pseudomonas aeruginosa*		MRSA		*Escherichia coli*	
		Activity	MIC	Activity	MIC	Activity	MIC	Activity	MIC	Activity	MIC	Activity	MIC	Activity	MIC
1.	*Allardia glabra*	---	NA	---	NA	---	NA	---	NA	---	NA	---	NA	---	NA
2.	*Capparis spinosa*	---	NA	Active	100	---	NA	Active	100	---	NA	---	NA	---	NA
3.	*Paganum harmala*	---	NA	---	NA	---	NA	Active	50	---	NA	---	NA	---	NA
4.	*Nepeta leucolaena*	---	NA	---	NA	---	NA	Active	50	---	NA	---	NA	---	NA
5.	*Aconitum heterophyllum*	---	NA	---	NA	---	NA	Active	50	---	NA	---	NA	---	NA
6.	*Acantholimon lycopodioides*	---	NA	---	NA	---	NA	Active	50	---	NA	---	NA	---	NA
7.	*Artemisia brevifolium*	---	NA	---	NA	---	NA	Active	200	---	NA	---	NA	---	NA
8.	*Delphinium brunonianum*	---	NA	---	NA	---	NA	Active	50	---	NA	---	NA	---	NA
9.	*Thymus linearis*	---	NA	---	NA	---	NA	---	NA	Active	200	---	NA	---	NA
10.	*Artemisia siversiana*	---	NA	---	NA	---	NA	Active	50	Active	100	---	NA	---	NA
+ive	**Ciprofloxacin**	Active	10	Active	5	Active	5	Active	1.25	Active	10	Active	1.25	Active	10
-ive	**DMSO**	---	NA	---	NA	---	NA	---	NA	---	NA	---	NA	---	NA

For non-resistant bacterial strains: Positive control = Ciprofloxacin, Negative control = DMSO, Positive = Bacterial activity, --- = No activity, NA = not applicable

For resistant bacterial strains: --- = no anti-bacterial activity, NA= not applicable, Positive control = Ciprofloxacin (10 µg/ml), Negative control = DMSO; MRSA = Methicillin resistant *Staphylococcus aureus*

Antimicrobial assays: This section details the evaluation of the plant extracts for their ability to inhibit microbial growth, encompassing both antibacterial and antifungal activities. The investigation is driven by the critical global challenge of antimicrobial resistance (AMR), largely accelerated by the rampant use of conventional antibiotics, which compromises treatment efficacy and poses a severe threat to public health (Ali *et al*., 2022). In this context, screening medicinal plants for their inherent antimicrobial properties represents a vital strategy for discovering novel pharmacophores that could lead to the development of new, effective therapeutic agents (Malik *et al*., 2022).

Antibacterial assay: The evaluation of antibacterial activity revealed a distinct spectrum of action for the methanolic plant extracts against a panel of clinically relevant bacteria, including antibiotic-resistant strains. As summarized in Table 7, the extracts demonstrated variable

but promising activity, primarily against non-resistant Gram-negative pathogens.

A key finding was the broad susceptibility of *Escherichia coli* to most extracts, with only *Allardia glabra* and *Thymus linearis* L. showing no inhibition. In contrast, antibacterial effects were more selective against other strains. *Capparis spinosa* exhibited mild, singular activity against *Bacillus subtilis*. Most notably, *Artemisia siversiana* and *Thymus linearis* L. exhibited potent and specific activity against *Pseudomonas aeruginosa*, a notorious opportunistic pathogen known for its intrinsic and acquired resistance mechanisms. For all active extracts, the Minimum Inhibitory Concentration (MIC) values ranged from 50 to 200 µg/ml, indicating a tangible bactericidal or bacteriostatic effect. However, it is significant to mention that none of the extracts showed inhibitory activity against the methicillin-resistant *Staphylococcus aureus* (MRSA) or resistant *E. coli* strains under the tested conditions, highlighting both a limitation of this screening and the formidable nature of these resistant phenotypes.

The observed antibacterial effects can be rationally linked to the phytochemical profiles established earlier. Extracts with high phenolic and flavonoid content, such as *A. siversiana* and *C. spinosa*, displayed significant activity, supporting the established mechanism where these compounds can complex with and disrupt bacterial cell walls and membranes (Mehmood *et al*., 2015). The specific activity of most extracts against *E. coli* aligns with findings from related ethnopharmacological studies (Atta *et al*., 2023).

The spectrum of activity observed here particularly the potent but narrow targeting of *P. aeruginosa* by specific plants—holds valuable therapeutic implications. Monotherapy with such narrow-spectrum agents offers a targeted approach, which is strategically advantageous in reducing the non-selective pressure that drives the emergence of resistance commonly associated with broad-spectrum antibiotics (Rios & Recio, 2005). This principle is supported by studies demonstrating that narrow-spectrum antimicrobials can effectively target pathogens like *Clostridium difficile* while better preserving the protective diversity of the native microbial community, compared to broad-spectrum agents (Rea *et al*., 2011). This targeted efficacy, especially against a resilient pathogen like *P. aeruginosa*, underscores the potential of these plant extracts as valuable starting points for developing novel antibacterial agents to complement the existing, but diminishing, antimicrobial arsenal.

Antifungal assay: The antifungal screening against a panel of five pathogenic fungal strains revealed a varied but promising spectrum of inhibitory activity for the plant extracts (Table 8). The results demonstrate that the efficacy was both species-specific and fungus-dependent.

Notably, *Thymus linearis* L. exhibited the most potent single activity, producing a substantial zone of inhibition (ZOI) of 12 mm against *Fusarium solani*, alongside a mild effect (6 mm ZOI) against *Mucor* species. Broad activity against *Aspergillus fumigatus* was observed, with several extracts including *Artemisia brevifolium* and *Delphinium brunonianum* showing strong inhibition (ZOI of 10 mm). *Allardia glabra*, *Capparis spinosa*, and *Acantholimon lycopodioides* also displayed moderate activity against various strains. However, no extract inhibited *Aspergillus flavus*, and certain species like *Nepta leucolaena* showed no activity across the fungal panel. This pattern of selective inhibition aligns with findings from comparable phytochemical studies (Atta *et al*., 2023).

The observed antifungal properties can be directly attributed to the secondary metabolites identified in the phytochemical analysis. The presence of flavonoids and phenolic compounds, in particular, correlates with antifungal activity, as these classes of metabolites are well-documented for their mycostatic and mycocidal effects (Kalboush & Hassan, 2019). Their mechanisms of action are multifaceted: they can disrupt critical fungal processes such as cell division, damage the integrity of the cell membrane, and inhibit the synthesis of essential cell wall components like chitin and glucan (Castillo-Reyes *et al*., 2015; Rashid *et al*., 2017). The structural diversity of phenolic compounds further enhances their antifungal potential by enabling interactions with multiple cellular targets (Ansari *et al*., 2013).

The significant activity against resilient pathogens like *A. fumigatus* and *F. solani* is of particular therapeutic relevance. It underscores the validity of ethnobotanical knowledge and positions these plants, especially *T. linearis*, as valuable sources of lead compounds for developing new antifungal agents. This is crucial in the context of rising fungal resistance and the limited arsenal of existing antifungal drugs, reinforcing the strategic importance of investigating traditional medicinal plants for novel antimicrobial therapeutics (Sukanya *et al*., 2009).

Table 8. Antifungal activity of the methanolic plant extracts, measured as the zone of inhibition (mm) against selected fungal strains using the agar disc diffusion method. Sample concentration: 100 µg/disc. Positive control: Amphotericin B (20 µg/disc). '---' indicates no activity.

Serial No.	Sample name	Zone of Inhibition (mm)				
		***Mucor* sp.**	***Aspergillus niger***	***Aspergillus flavus***	***Aspergillus fumigatus***	***Fusarium solani***
1.	*Allardia glabra* Decne.	---	8	---	8	---
2.	*Capparis spinose*	---	8	---	---	7
3.	*Paganum harmala*	---	---	---	8	---
4.	*Nepeta leucolaena*	---	---	---	---	---
5.	*Aconitum heterphyllum*	---	---	---	---	---
6.	*Acantholimon lycopodioides*	---	7	---	8	---
7.	*Artemisia brevifolum*	---	---	---	10	---
8.	*Delphinium brunonianum*	---	---	---	10	---
9.	*Thymus linearis* L.	6	---	---	8	12
10.	*Artemisia siversiana*	---	---	---	9	---
13.	Amphotericin B	12	12.5	9	10	12
14.	DMSO	---	---	---	---	---

Note: sample concentration = 100 µg/disc, positive control concentration = 20 µg/disc, Positive control = Amphotericin B, Negative control = DMSO

Table 9. Alpha-amylase inhibitory activity of the methanolic plant extracts, showing percentage inhibition at 200 µg/ml and the half-maximal inhibitory concentration (IC50 in µg/ml).

Serial No.	Sample name	% Inhibition (200 µg/ml)	IC_{50} (µg/ml)
1.	*Allardia glabra* Decne.	29.4	NA
2.	*Capparis spinose*	57	130.4
3.	*Paganum harmala*	48.4	NA
5.	*Nepeta leucolaena*	49.1	NA
6.	*Aconitum heterphyllum*	64.1	140.3
8.	*Acantholimon lycopodioides*	76.7	9.8
9.	*Artemisia brevifolum*	49.9	NA
10.	*Delphinium brunonianum*	33.0	NA
11.	*Thymus linearis*	49.1	NA
12.	*Artemisia siversiana*	17.9	NA
13.	Acarbose	81.1	33.7
14.	DMSO	---	---

Note: --- = No α-amylase inhibition activity, Sample concentration = 200µg/ml, Positive control = Acarbose (50 µg/ml); Negative control = DMSO

Table 10. Protein kinase inhibition (as bald or clear zones of inhibition in mm) and alpha-amylase inhibitory activity (showing percentage inhibition at 200 µg/ml and IC50 in µg/ml) of the methanolic plant extracts

Serial No.	Sample	Protein kinase inhibition assay		α-amylase inhibition assay	
		Bald zone	Clear zone	% Inhibition (200 µg/ml)	IC_{50} (µg/ml)
1.	*Allardia glabra* Decne.	7 mm	---	29.4	NA
2.	*Capparis spinose*	---	---	57	130.4
3.	*Paganum harmala*	---	6.5 mm	48.4	NA
4.	*Nepeta leucolaena*	---	---	49.1	NA
5.	*Aconitum heterophyllum*	---	---	64.1	140.3
6.	*Acantholimo lycopodioides*	---	7 mm	76.7	9.8
7.	*Artemisia brevifolium*	7.5 mm	---	49.9	NA
8.	*Delphinium brunonianum*	7 mm	---	33.0	NA
9.	*Thymus linearis*	---	---	49.1	NA
10.	*Artemisia siversiana*	---	8 mm	17.9	NA

α-amylase inhibition assay: The evaluation of alpha-amylase inhibitory activity revealed significant potential in several plant extracts for managing postprandial hyperglycemia, a key therapeutic target in type 2 diabetes mellitus. As summarized in Table 9, the methanolic extracts exhibited a broad range of inhibition. *Acantholimon lycopodioides* demonstrated outstanding activity, inhibiting 76.7% of the enzyme with a remarkably low IC_{50} value of 9.8 µg/ml, indicating a high potency that surpasses many other tested species. This finding is consistent with reports on other members of the *Acantholimon* genus, such as *A. acerosum* and *A. caryophyllaceum*, which have also shown promising amylase inhibitory effects (Kiziltas *et al*., 2022). Substantial activity was also noted for *Aconitum heterophyllum* (64.1% inhibition, IC_{50} 140.3 µg/ml) and *Capparis spinosa* (57% inhibition, IC_{50} 130.4 µg/ml), while the remaining extracts showed moderate to low inhibition (17.9% to 49.1%).

The physiological relevance of this inhibition is profound. Alpha-amylase is a pivotal enzyme in carbohydrate metabolism, responsible for hydrolyzing dietary starch into absorbable simple sugars, thereby directly influencing postprandial blood glucose levels (Zinjarde *et al*., 2011). Unchecked activity of this enzyme contributes to the hyperglycemia that characterizes diabetes mellitus and exacerbates its complications (Patel *et al*., 2012; Sangeetha & Vedasree, 2012). Current pharmaceutical strategies often employ synthetic enzyme inhibitors, but these can be associated with adverse gastrointestinal effects, driving the search for safer, natural alternatives (Liyanagamage *et al*., 2020). The potent inhibition exhibited by extracts like that of *A. lycopodioides* validates their traditional use and positions them as promising candidates for developing functional foods or nutraceuticals for glycemic control (Gondi & Prasada, 2015).

The inhibitory activity is intrinsically linked to the phytochemical profile of the plants. Specific phenolic compounds and flavonoids, such as quercetin, ferulic acid, and catechin, are well-documented for their ability to bind to the active site of alpha-amylase, obstructing its function (Etxeberria *et al*., 2012). Furthermore, the antidiabetic potential of these extracts is likely amplified by their concomitant antioxidant capacity, as demonstrated in earlier assays. Oxidative stress is both a consequence and a driver of diabetic pathology; therefore, plant extracts that offer dual action—inhibiting carbohydrate-hydrolyzing enzymes and mitigating oxidative damage—provide a comprehensive therapeutic approach (Ranilla *et al*., 2010; Ali *et al*., 2022). This multifaceted mechanism, where flavonoids reduce oxidative stress while other compounds enhance insulin sensitivity or directly inhibit enzymes, is recognized as a hallmark of effective plant-based antidiabetic agents (Hsieh *et al*., 2014). The exceptional activity of *A. lycopodioides* warrants its prioritization for further phytochemical isolation to identify the specific compound(s) responsible for its potent enzyme inhibition.

Protein Kinase: The protein kinase inhibition assay, utilizing *Streptomyces* 85E as a model organism, served as a preliminary screen for detecting compounds that interfere with cell signaling pathways critical for proliferation and

differentiation. The results, summarized in Table 10, revealed distinct inhibitory patterns among the extracts. A bald zone of inhibition—indicating a bacteriostatic effect that prevents aerial hyphae formation without killing the mycelia was observed for *Allardia glabra* (7 mm), *Artemisia brevifolium* (7.5 mm), and *Delphinium brunonianum* (7 mm). Conversely, a clear zone of inhibition suggesting a bactericidal or lytic effect was exhibited by *Paganum harmala* (6.5 mm), *Acantholimon lycopodioides* (7 mm), and most notably *Artemisia siversiana* (8 mm). This activity aligns with previous reports on the cytotoxic and anticancer potential of the *Artemisia* genus (Maqsood *et al*., 2015).

The significance of this assay lies in its connection to oncogenesis. Protein kinases are fundamental regulators of the cell cycle, differentiation, and apoptosis. Their dysregulation, often through genetic mutations leading to hyperactivation, is a hallmark of numerous cancers, driving uncontrolled cellular proliferation (Silva-Pavez & Tapia, 2020; Fatima *et al*., 2022). Consequently, protein kinase inhibitors represent a major frontier in targeted cancer therapy (Evan & Vousden, 2001). The observed inhibition of microbial kinase activity suggests the presence of phytochemicals capable of interfering with analogous signaling pathways in eukaryotic cells.

The inhibitory activity is likely mediated by the rich polyphenolic and flavonoid content identified in these plants. Natural polyphenols are recognized for their ability to modulate various oncogenic kinase pathways, thereby offering a chemopreventive strategy (Fabbro *et al*., 2012; Bano *et al*., 2022). The presence of both bald and clear zones indicates diverse mechanisms of action, ranging from signal disruption to direct cellular damage. While the current assay shows mild to moderate inhibition, it provides a crucial positive signal. The results strongly suggest that dose-dependent studies and evaluation against specific human kinase targets are warranted to fully appraise the antitumor potential of these extracts. This foundational evidence supports their further investigation as sources of novel leads for the development of antitumor and anti-infective agents (Gautam *et al*., 2017).

For Protein kinase inhibition assay

Note: Surfactin B (20µg/disc) = Positive control, --- = No activity, DMSO = Negative control, Sample concentration = 100µg/disc

The values are:

Control	Standard	Bald zone	Clear zone
Positive Control	Surfactin B	22.3 mm	---
Negative Control	DMSO	---	---

For α-amylase inhibition assay

Note: --- = no α-amylase inhibition activity, Sample concentration = 200µg/ml, Positive control = Acarbose (50 µg/ml); Negative control = DMS

The values are:

Control	Standard	%Inhibition (200 µg/ml)	IC_{50} (µg/ml)
Positive control	Acarbose	81.1	33.7
Negative control	DMSO	---	---

Brine shrimp lethal assay: The brine shrimp (*Artemia salina*) lethality assay is a well-established, rapid, and cost-effective preliminary screen for evaluating the general cytotoxic potential of plant extracts. This bioassay is particularly valuable as a first-tier assessment because the biological systems of the shrimp nauplii show a meaningful correlation with cytotoxic effects in mammalian cell lines, providing indicative data for antitumor, insecticidal, and antimicrobial properties (Ramamurthy *et al*., 2013; Rojo-Arreola *et al*., 2020). In the current study, the median lethal concentration (LD_{50}) was determined for each extract across a concentration gradient (200, 100, 50, and 25 µg/mL).

The results, presented in Table 11, revealed a substantial range of cytotoxic potency. *Paganum harmala* extract demonstrated remarkable toxicity, with a notably low LD_{50} value of 29.4 µg/mL, classifying it as highly active in this model. The remaining extracts exhibited moderate to low cytotoxicity, with LD_{50} values spanning from 57.8 to 200 µg/mL. The pronounced activity of *P. harmala* is consistent with a substantial body of literature affirming its potent pharmacological profile, which includes documented anticancer effects against colon and breast cancer cell lines, alongside antimicrobial, antioxidant, and antifungal activities (Patel *et al*., 2012; Khalid *et al*., 2024). The cytotoxic activity observed in our study for other species, such as *Thymus linearis* (LD_{50} = 122.7 µg/mL), finds support in related research. For instance, essential oil from *Thymus vulgaris* demonstrated significant cytotoxicity in both brine shrimp nauplii and cancer cell lines, underscoring the cytotoxic potential inherent in the Thymus genus and validating the use of this bioassay as a preliminary indicator (Niksic *et al*., 2021). This concordance validates the assay's utility in identifying biologically active species.

The observed cytotoxicity is mechanistically attributed to the complex mixture of defensive secondary metabolites within the extracts, such as alkaloids (notably in *P. harmala*), flavonoids, phenolics, and saponins. These compounds can induce cell death through various pathways, including membrane disruption, enzyme inhibition, and interference with cell division (Aziz *et al*., 2013). The significant lethality of *P. harmala* at low concentrations suggests the presence of particularly potent bioactive agents, warranting its prioritization for further investigation.

From a therapeutic discovery perspective, a low LD_{50} in this assay signals promising bioactivity that merits more sophisticated evaluation. It suggests that effective *In vivo* or *In vitro* (against specific cancer cell lines) effects could be achievable at relatively low, potentially less toxic doses (Atta *et al*., 2023). However, this assay is a preliminary indicator; it does not elucidate specific mechanisms or selective toxicity toward cancer cells versus normal cells. Therefore, while the potent cytotoxicity of *P. harmala* and other active extracts highlights their potential as sources of anticancer or antimicrobial leads, it necessitates follow-up studies—such as selective cytotoxicity assays on normal and cancerous mammalian cell lines to accurately appraise their therapeutic index and safety profile before any clinical relevance can be ascertained (Mushtaq *et al*., 2018).

Table 11. Cytotoxic effect of the methanolic plant extracts assessed by the brine shrimp (*Artemia salina*) lethality assay, showing percentage mortality at different concentrations and the calculated median lethal dose (LD50 in µg/ml). Positive control: Doxorubicin (40 µg/ml).

Serial No.	Sample name	% Mortality				LD_{50} (µg/ml)
		(200 µg/ml)	(100 µg/ml)	(50 µg/ml)	(25 µg/ml)	
1.	*Allardia glabra* Decne.	57	47	27	17	121.6
2.	*Capparis spinose*	67	57	47	47	57.8
3.	*Paganum harmala*	67	67	57	47	29.4
4.	*Nepeta leucolaena*	67	67	57	47	29.4
5.	*Aconitum heterophyllum*	57	47	47	37	100
6.	*Acantholimon lycopodioides*	67	47	27	17	113.4
7.	*Artemisia brevifolium*	57	47	47	37	100
8.	*Delphinium brunonianum*	67	57	47	47	57.8
9.	*Thymus linearis*	67	47	37	37	122.7
10.	*Artemisia siversiana*	67	57	47	27	62.8
13.	Doxorubicin	97	87	67	47	5.7
14.	DMSO	20	NA	NA	NA	---

Note: Sample concentration = 200 µg/ml; DMSO = Negative control; Doxorubicin (40 µg/ml) = Positive control

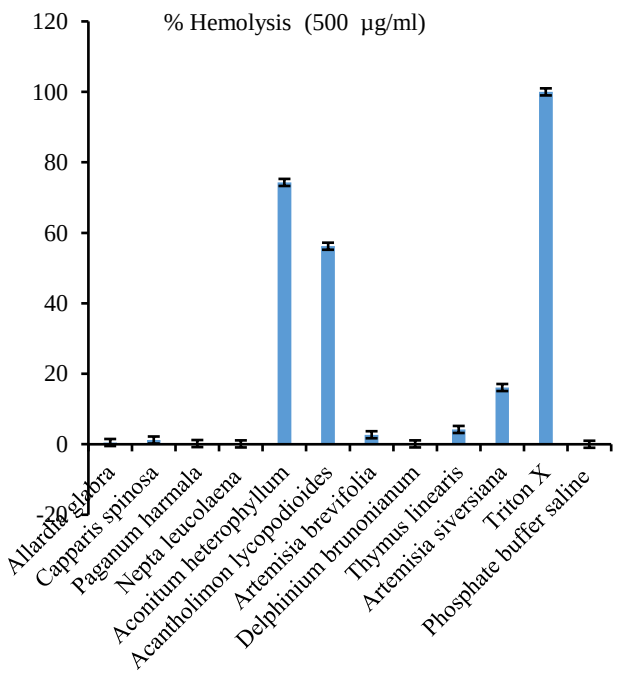

Fig. 1. Hemolytic activity of selected medicinal plants.
Note: Triton x = Positive control, Phosphate buffer saline = Negative control

Hemolytic assay: The hemolytic assay on human erythrocytes serves as a crucial preliminary toxicity screen to evaluate the membrane-damaging potential of plant extracts, which is particularly relevant for assessing the safety profile of compounds intended for therapeutic use, especially via injectable routes. This assay measures the propensity of phytochemicals to disrupt the phospholipid bilayer of red blood cells, causing hemoglobin release—a significant concern for *In vivo* applications (Amin & Dannenfelser, 2006; Vinjamuri *et al*., 2015).

The results revealed (Fig. 1) a broad spectrum of hemolytic activity among the tested extracts, providing critical insight into their biocompatibility. *Aconitum heterophyllum* exhibited the most pronounced hemolytic effect, with 74.3 ± 0.57% hemolysis, indicating a strong interaction with and destabilization of the erythrocyte membrane. This aligns with the well-documented, potent bioactive profile of *Aconitum* species, which are known for their cytotoxic, anticancer, and anti-inflammatory properties mediated by diterpenoid alkaloids (Li *et al*., 2022). The significant bioactivity of this genus underscores its medicinal value and has spurred research into biotechnological methods to improve the yield of its key chemical constituents (Tiwari *et al*., 2023). In stark contrast, *Nepta leucolaena* and *Delphinium brunonianum* showed negligible hemolysis (0.1%), suggesting a markedly safer profile with minimal membrane-disruptive effects at the tested concentration.

The observed hemolysis is directly attributable to the presence of secondary metabolites, such as saponins and certain alkaloids, which can integrate into lipid membranes, alter their fluidity and integrity, and lead to osmotic lysis (Iwasa *et al*., 2001; Santos-Filho, 2016). The strong activity in *A. heterophyllum* serves as a clear indicator of its potent, non-selective cytotoxic capacity. However, it is vital to interpret these results within a broader mechanistic context. As noted by Costa-Lotufo *et al*., (2005), significant cytotoxicity observed in other assays (like brine shrimp lethality) may not always correlate directly with hemolytic activity, as cell death can be induced through other targeted pathways—such as enzyme inhibition or apoptosis without primary membrane lysis.

From a drug discovery perspective, this assay provides essential dual insights. First, high hemolytic activity, as seen in *A. heterophyllum*, raises a critical safety flag, indicating a potential risk of hemolytic anemia and necessitating extensive further toxicity studies before therapeutic development. Second, extracts with potent bioactivity in other assays (e.g., antimicrobial, enzyme inhibition) but low hemolysis, such as *N. leucolaena*, are particularly promising. They suggest the presence of bioactive compounds with more specific mechanisms of action and a potentially higher therapeutic index. Therefore, while hemolysis identifies extracts requiring caution, it also helps prioritize those with a safer pharmacological profile for subsequent isolation of specific, target-selective therapeutic leads.

The results of this study conclusively validate the proposed hypothesis. The significant levels of bioactive secondary metabolites particularly phenolic compounds and flavonoids quantified in the plant extracts were directly and consistently correlated with their potent *In vitro* biological activities. The high phenolic content in *Artemisia siversiana* corresponded with its superior antioxidant and antibacterial effects, while the elevated flavonoids in *Capparis spinosa* were linked to its strong total antioxidant capacity and enzyme inhibition. The remarkable alpha-amylase inhibition by *Acantholimon lycopodioides* and the pronounced cytotoxicity of *Peganum harmala* further demonstrate that the observed pharmacological properties are a direct function of the plants' distinct phytochemical profiles. This clear cause-and-effect relationship substantiates the ethnomedicinal use of these species and robustly confirms their potential as viable sources for future drug discovery.

Conclusion

This comprehensive phytochemical and biological investigation provides robust scientific validation for the ethnomedicinal use of ten selected plants from Gilgit-Baltistan. The findings collectively demonstrate that these species are rich repositories of diverse bioactive secondary metabolites, notably phenolic compound flavonoids included within phenolic compounds, which directly underpin their significant pharmacological potentials. Key highlights include the exceptional antioxidant and free radical scavenging capacity of *Artemisia siversiana*, linked to its high phenolic content; the strong flavonoid-associated total antioxidant and enzyme inhibitory activity of *Capparis spinosa*; and the potent, specific alpha-amylase inhibition by *Acantholimon lycopodioides*, highlighting its promising antidiabetic application. Furthermore, the study identifies *Peganum harmala* as a source of potent cytotoxic compounds and underscores the significant and targeted antimicrobial, particularly antifungal, efficacy of *Thymus linearis* and *Artemisia brevifolium*. The results confirm a strong correlation between the plants' phytochemical profiles and their observed biological activities, thereby substantiating their traditional uses and cementing their role as valuable candidates for modern drug discovery pipelines aimed at addressing oxidative stress, microbial resistance, diabetes, and cancer.

Future research should focus on the bioactivity-guided isolation and structural elucidation of the specific compounds responsible for the observed potent activities, followed by in-depth mechanistic studies and *In vivo* validation in suitable disease models. Furthermore, investigating synergistic interactions, conducting comprehensive toxicity profiling, and developing sustainable cultivation methods for these high-potential species are essential steps toward translating these findings into clinically relevant therapeutic agents or standardized phytomedicines.

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