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EVALUATION AND IDENTIFICATION OF ANTIBACTERIAL, ANTIFUNGAL, ANTIOXIDANT, AND PHYTOTOXIC ACTIVITIES OF *ASPLENIUM DALHOUSIAE* HOOK. A POTENTIAL MEDICINAL PTERIDOPHYTE

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Abstract

Pteridophytes have been identified as resistant to microbial infections, which may be attributed to the presence of various phytocompounds. The current study focused on the *Asplenium dalhousieae* Hook for detailed morphological, pharmacognostic, and phytochemical evaluation. Here we used the fresh and powdered fronds of *Asplenium dalhousieae* for its macroscopic and microscopic characterization. Phytochemical investigations were carried out by using ethanolic, methanolic, and distilled water extracts. Antimicrobial, phytotoxic, and antioxidant properties were assessed using crude methanolic extracts. The macroscopic characteristic of the plant revealed a dark green color, smooth powdered medicine texture, and an aromatic odor. Different types of cells and their micrometry were detected in microscopic features, confirming that the *Asplenium dalhousieae* has a wide range of cells such as subsidiary cells, scalariform arteries, desmocytic stomata, and polygonal epidermal cells. Phytochemical analysis showed the existence of multiple phytochemical compounds such as flavonoids, phenols, glycosides, alkaloids, coumarin, anthraquinones, quinones, cardiac glycosides, terpenoids, and saponins. Subsequently, plant antimicrobial response against various bacterial and fungal strains (*Streptococcus mitis, Pseudomonas fluorescens, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae Proteus mirabilis,* and *Salmonella typhi* and *Carvularia, Fusarium, Alternaria, Rhizopus, Aspergillus)* determined a significant response when compared with standard drug ciprofloxacin and miconazole respectively. The phytotoxic response against the test plant *Lemna minor* indicated 75% growth of inhibition revealing potent herbicidal aptitude. Further, antioxidant potential revealed 34% inhibition by DPPH and 55.7% inhibition by phosomolybdate complex assay. In conclusion, this study emphasizes the promising therapeutic applications of *Asplenium dalhousieae* in diverse medical contexts, highlighting its substantial potential for future research and development. Further studies on *Asplenium dalhousieae* are needed to identify and isolate cytotoxic chemicals, as well as to use TLC methods to aid the production of natural bioactive compounds.

Key words: Biological activity; Pharmacognostic; DPPH; Micrometry; Phytochemical; Powder drug.

Introduction

For thousands of years, medicinal plants have been used as a significant source of medicine. Many conventional medical systems, for example, Indian Ayurvedic medicine, Traditional Chinese Medicine, and Roman and ancient Greek systems emerged and contributed significantly to the advancement of natural medicine. Medicinal plants provide many advantages, such as availability, affordability, ease of use, and fewer side effects (Xin-Yue *et al*., 2013), whereas poor quality control, inappropriate dosage, perceptible effects, and differences with modern drug systems limit their usage on a large scale. Nevertheless, over 3.3 billion people still rely on medicinal herbs in developing and underdeveloped countries (Singh, 2015).

Asplenium dalhousieae, a member of the Aspleniaceae family, is found mainly in shady places, in moist soil, on field edges, and grows under other grasses. The family, encompassing approximately 700 species distributed worldwide, is further categorized within the class Polypodiales (Tryon & Lugardon, 1991; de Winter & Amoroso, 2003). The habitat of *Asplenium* is easily accessible in the Himalayas widely distributed at 4,000-8,000 feet above sea level throughout Pakistan, Afghanistan, and India, and abundantly found in different parts of Asia (Iwashina & Matsumoto, 2011).

Medicinal plants are used to treat many lethal diseases (Ullah *et al*., 2018; Shinwari *et al*., 2018; Khan *et al*., 2019; Wakeel *et al*., 2019; Jan *et al*., 2021). The *Asplenium dalhousieae* is associated with several synonyms, such as *Asplenium rupium*, *Ceterach alternans*, *Ceterach dalhousiae*, and *Ceterachopsis dalhousiae* Ching (http://www.theplantlist.org). Various taxonomists have conducted studies on the morphology of *A. dalhousiae* (Bir, 1962; Fazalullah & Ali, 2014). Traditionally, it is used in a wide variety of foods. Nonetheless, various species of *Asplenium* species are used in traditional remedies as a single part, such as roots, stems, leaves, or entire plants for the treatment of different ailments. Most people use it as a powder (72%), followed by decoction (17%) and infusion (17%). Herbalists in several regions of Pakistan prescribe the powder of *Asplenium* species for various diseases. The powder and decoction are used to treat skin conditions, colds, jaundice, rickets, diabetes, and cough (Zaman *et al*., 2019). Moreover, the juice of *Asplenium nidus* has been reported to cure oral infections and weakness (Nath *et al*., 2013), and it is used in Tahiti India to relieve many types of body discomfort, particularly chest pain. Crushed leaf extract is used to treat skin disorders on a local level (Yumkham *et al*., 2017). Aerial portions of *Asplenium adiantum-nigrum* L., are used as diuretics, anti-

inflammatory drugs, and treatments for asthma, stomachaches, and kidney stones (Tuzlacı & Aymaz, 2001). On the other hand, the different phytochemicals of *Asplenium dalhousieae* have a wide range of pharmacological effects, which could help with chronic disease prevention (Aulakh *et al*., 2019). *Asplenium* species is rich in flavonoids with antioxidant properties, and the majority of people use it for its nutritional purposes (Cambie & Ferguson, 2003).

Numerous studies investigated the medicinal properties of different *Asplenium* species. However, only a few pharmacognostic research on the *Asplenium dalhousieae* has been conducted in Pakistan, and only limited information exists about the species itself. Therefore, we are aiming to broaden the knowledge of the species and study its pharmacognostic potential. Specifically, we aim to identify the microbiological, phytotoxic, antioxidant, and phytochemical screening properties. The findings of the study can lead to the development of novel plant-based therapies for chronic diseases, providing an alternative and potentially more accessible form of treatment.

Material and Methods

Collection and preservation: *Asplenium dalhousieae*, a medicinal plant, was meticulously gathered from Dadar, from Mansehra district, situated in the Khyber Pakhtunkhwa province of Pakistan. Following collection, the plant was subjected to preservation as a herbarium specimen using proper techniques. Subsequently, it was formally deposited at Hazara University's Herbarium, accompanied by the voucher number Sz/Dr/H-001 (Accession No. 3290). The mature plant's fronds were detached from the rhizome, cleaned with distilled water, and soaked in fixative for 2 to 3 days for cell fixation. The remaining parts of the plants were dried for 20 to 30 days to remove moisture, and the dry components were then ground to create a powder.

Pharmacognostic studies: The plant was macroscopically examined for morphological characteristics, including the color of the fronds, size, aroma, and fracture. For microscopic characterization, a small amount of *Asplenium dalhousieae* powder was immersed in a 70% chloral hydrate solution. It was then heated for 5 minutes using a spirit lamp and allowed to cool to room temperature. Subsequently, a drop of the solution was applied to a microscopic slide and examined for different types of cells and tissue, along with micrometry.

Microscopic analysis: The pre-soaked plant was used for section cutting. A clear section was selected and stained for microscopic investigation, following standard techniques as described by Oruade-Dimaro *et al*., (2010).

Phytochemical analysis: Qualitative phytochemical analysis was carried out using 5g of powdered drug pre-soaked in 50 ml of ethanol, methanol, and distilled water for seven days. The Whatman filter paper was used to filter the extract (Gnana & Estherludia, 2014). The ethanolic, methanolic, and distilled water extracts were tested for different secondary metabolites like carbohydrates, quinones, anthraquinones, alkaloids, phenols, glycosides, cardiac glycosides, flavonoids, coumarins, terpenoids, tannin, phlobatannin, and oils.

Total flavonoid contents (TFC) quantification was determined by the aluminum chloride assay following the protocol of Ayele *et al*., (2022), to quantify flavonoid content, (0.5 ml) of aluminum chloride was added to 1 ml of methanol-made plant extract (triplicate), followed by 0.5 ml of sodium nitrite and 2 ml of sodium hydroxide. The extract was then incubated in the dark for 30 to 40 minutes. The absorbance was measured by spectrophotometer at 510 nm UV. A calibration curve was developed for quercetin as a standard, and the total flavonoid content was determined using an equation derived from the standard calibration curve and expressed as (mg quercetin/g extract). The data were presented as the mean of triplicates with standard deviation (SD).

Total Phenolic Content (TPC) quantification was carried out as Lin *et al*., (2011) described. TPC was evaluated using the Folin-Ciocalteu method. Briefly, the methanolic extract (1 ml) was mixed with Folin-Ciocalteu (1 ml) and incubated for 10 minutes, followed by the addition of sodium carbonate (2 mL) in the dark for 90 minutes. The absorbance was then measured by a spectrophotometer at 765nm. Gallic acid in different dilutions was used to create the standard calibration curve, i.e., 0 ppm, 10 ppm, 20 ppm, 40 ppm, 60 ppm, 80 ppm, and 100 ppm. The data is presented as a mean of triplicates with standard deviation (SD).

Biological activities: To prepare the crude extract, 100 g of fronds powder, was soaked in 300 ml of methanol with steady stirring of the solution and incubated at room temperature for 7 days. The extract was then filtered three times with Whatman filter paper to yield maximum extract. The filtrate was then placed in a rotary evaporator at 50 °C for methanol evaporation to obtain crude extract. The crude extract was then evaluated for antimicrobial, phytotoxic, and antioxidant activity (Seidal, 2006; Handa, 2008).

Antimicrobial activity: The antibacterial and antifungal activities were assessed using the methodology described by Atta-Ur-Rehman *et al*., (2001). The crude extract (20 mg) was dissolved in 20 ml of DMSO, and different bacterial strains, including *Streptococcus mitis*, *Escherichia coli*, *Pseudomonas fluorescens*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Staphylococcus aureus*, and *Salmonella typhi*, were tested against the antibacterial potential of the extract. Ciprofloxacin is considered a positive control, while DMSO is a negative control. To evaluate the antifungal potential, various fungal strains such as *Carvularia*, *Fusarium*, *Alternaria*, *Rhizopus*, and *Aspergillus* were tested against the crude extract, with miconazole used as a positive control and DMSO as a negative control. The zone of inhibition method is used to determine plant extracts' antimicrobial activity. This method involves the diffusion of the extract into an agar medium which is inoculated with bacterial and fungal strains.

Phytotoxic activity: The herbicidal impact of the plant was assessed following the methodology outlined by Atta-Ur-Rehman *et al*., (2001), using *Lemna minor* as the model plant. The e-media was prepared in distilled water (1000 ml) and autoclaved at 121°C for 15 min, then pH was adjusted (5.5 – 5.6) by adding KOH (Potassium Hydroxide) dropwise. The extracts (10 mg) were dissolved in methanol (20 ml) to prepare a stock solution. Ten Petri plates were used, three for each concentration (10, 100, 1000 µl) of the stock solution and one as a control. The organic solvent was allowed to evaporate at room temperature in a laminar flow hood for seven days. Next, e-media (20 ml) and 10 plants of *L. minor* were added to each of the 10 plates, with each plant containing a rosette of three fronds, while the negative control had no extract but simply e-Media with *L. minor*. Subsequently, after that, each plate was kept at 37°C for seven days. After a week, the number of fronds on each plate was counted and noted.

Antioxidant activity: The antioxidant potential of the *Asplenium dalhousieae* was carried out using two standard protocols.

DPPH scavenging activity: To evaluate the antioxidant response, the DPPH free radical scavenging activity was measured by implementing the protocol of Aoshima *et al*., (2004). 1 ml of the methanolic extract and 4 ml of DPPH were gently mixed and incubated at room temperature for 90 minutes. The absorbance was then measured against the blank using a spectrophotometer at 570 nm. A lower value of the absorbance indicates greater DPPH scavenging activity. The antioxidant potential was recorded as the percentage inhibition using the following formula and presented as the mean of three replicates ± SD:

$$\text{DPPH scavenging effect percentage} = 1 - As/Ac \times 100$$

Here, “Ac” represents the absorbance of the solution of DPPH, while “As” is the absorbance of the mixture of the given plant’s extract.

Phosomolybdenium complex assay (PMC): The Phosomolybdenium Complex Assay, as described by Prieto *et al*., (1999), was utilized to determine the overall antioxidant potential. To create the reagent solution, 1.65 ml of H_2SO_4 was dissolved in 50 ml of distilled water, 1.67 g of sodium phosphate was diluted in 50 ml of distilled water, and 0.24 g of ammonium molybdate ($6Mo_7O_{24}$) was dissolved in 50 ml of distilled water. Subsequently, 1 ml of the plant extract was mixed in a sealed flask containing 3.3 ml of the reagent solution and incubated at 95°C for 90 minutes. After cooling, the absorbance of the samples was measured at 695 nm using a spectrophotometer, with a comparison to the blank. Gallic acid served as a reference compound (positive control) at various dilutions (0 ppm, 20 ppm, 40 ppm, 60 ppm, 80 ppm, and 100 ppm) (Fig 4).

Statistical analysis

Each test was conducted in triplicate, and the results are presented as the mean value along with its corresponding standard deviation.

Results and Discussion

Macroscopic and microscopic features: *Asplenium dalhousieae* have brown-scaled fronds, it displays caespitose, alternately arranged fronds, 1.5-3 cm wide and 9-11 cm long. Fronds are yellow green with glabrous upper epidermis, linear sori on the lower epidermis, and obtuse to acute apices. Venation is yellow green each frond pair bearing 10-13 pinnae on a rigid rhizome (Fig. 1A). Micrometric analysis of *Asplenium dalhousieae* reveals precise measurements for its cellular structures. Desmocytic stomata are 44.65 µm length and 35.25 µm width. Polygonal epidermal cells are 63.45 µm in length and 42.3 µm in width, while subsidiary cells are also polygonal, 63 µm in length and 39.95 µm in width.

In contrast, flattened fibers are elongated, with a length of 125 µm and a width of 2 µm. Elongated parenchyma cells are relatively smaller with 32.5 µm length and 20 µm width. Biconvex sporangia are notably larger, 265 µm in length and 18 µm in width. These precise measurements provide valuable insights into the structural characteristics of *Asplenium dalhousieae*. Macroscopic features indicated that the powder drug of the plant was a dark greenish color with an aromatic odor having a bitter taste. The microscopic and micrometry characteristics of the powdered drug revealed multiple types of cells including polygonal epidermal cells, scalariform tracheids, sporangia, branched trichomes, desmocytic stomata, and bean shape guard cells as shown in Fig. 1(B, C, D, E).

Anatomical analysis: The precisely sliced section of pinnae revealed that underneath the outermost single layer of epidermal cells are compactly packed with collenchyma cells and vascular bundles positioned in the central sections of collenchyma cells (Fig. 1F).

Phytochemical analysis: Standard testing protocol for phytochemical analysis revealed the presence of secondary metabolites. Among the 14 phytochemicals screened, the methanolic extract showed the highest abundance of 10 phytochemicals, followed by 8 in the ethanolic extract and distilled water extract respectively (Table 1). The methanolic extract was more effective in extracting the phytochemicals compared to ethanolic and distilled water extract. This could be the fact that methanol has a high polarity and a high tendency to extract polar compounds from plants (Fatiha *et al*., 2012).

All extracts were found to contain various phytochemical constituents, including alkaloids, phenolic content, flavonoids, glycosides, and coumarins (Table 1). The identification of different types of phytochemical constituents in the extracts provides a basis for further research on the potential biological and pharmacological effects of these compounds. For instance, many secondary metabolites are known to have antioxidant, antifungal, antimicrobial, anti-inflammatory, and anticancer properties (Saxena *et al*., 2013; Cör *et al*., 2022).

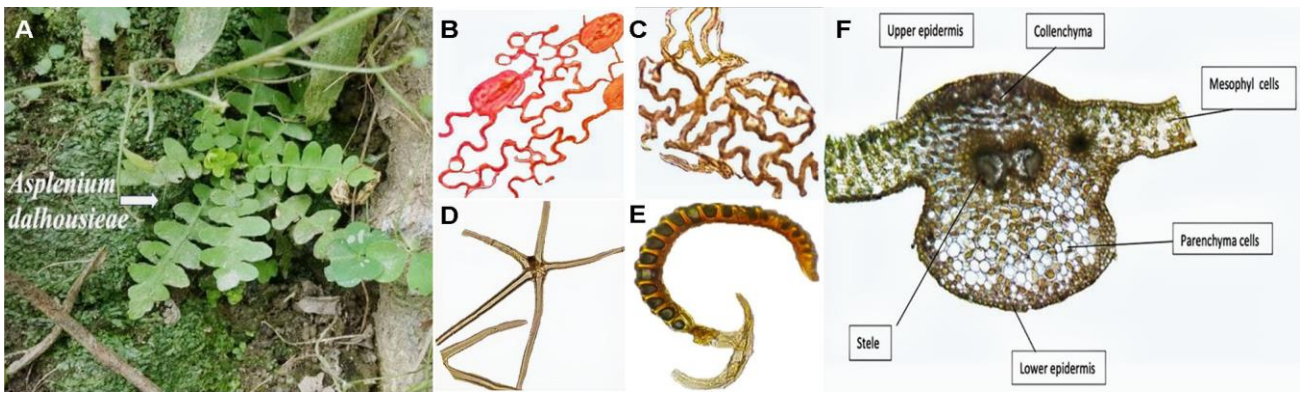

Fig. 1. A) Whole plant in a natural habitat B) subsidiary cells along with stomata C) subsidiary cells D) trichomes E) sporangia F) transverse section of stipe.

Table 1. Qualitative analysis of phytochemical compounds of *Asplenium dalhousieae.*

S. No.	Phytochemicals	Tests	Extracts		
			Ethanolic	Methanolic	Distilled water
1.	Carbohydrate	Molish's test	+	+	+
2.	Phenolic content	Sodium hydroxide test	+	+	+
3.	Alkaloid	Mayer's reagent test	+	+	+
4.	Flavonoids	Shinoda test	+	+	+
5.	Coumarin	Coumarin test	+	+	+
6.	Glycosides	Keller-Killani test	+	+	+
7.	Cardiac glycosides	Ferric chloride test	-	+	-
8.	Tannin	Ferric chloride test	-	-	-
9.	Phlobatanins	Phlobatanins test	-	-	-
10.	Quinones	Quinones test	-	+	-
11.	Anthraquinones	Anthraquinones test	+	+	+
12.	Terpenoids	Salkowaski test	-	+	-
13.	Saponins	Foam test	+	-	+
14.	Oils	Spot test	-	-	-

Key: + = Present; - = Absent

Table 2. Quantitative analysis of two secondary metabolites in *Asplenium dalhousieae.*

TPLC mgGAE/100gDw	TFC mgQE/100gDw
7.923 ± 0.141	22.83 ± 1.52

Quantification of flavonoids and phenolic content: The quantification of flavonoid content (TFC) from the frond utilized the aluminum chloride colorimetric method, while the determination of phenolic content (TPLC) was carried out using the Folin-Ciocalteu reagent method. A 20mg sample of the methanolic extract was employed for both analyses. The total flavonoid and phenolic contents were identified as moderate (Table 2). This moderate presence of flavonoids and phenolic compounds in the plant extract suggests the potential existence of bioactive compounds with implications for treating conditions associated with oxidative stress and inflammation, as discussed by Amaral *et al*., (2009).

Antimicrobial activity: The investigation of plant extracts for their antimicrobial potential is a pivotal area of research, given the diverse array of secondary metabolites produced by plants, known for their antimicrobial properties (Othman *et al*., 2019). In our current study, we assessed the antimicrobial activity of the methanolic extract obtained from *A. dalhousieae* against both bacterial and fungal strains. Notably, the *A. dalhousieae* methanolic extract demonstrated inhibitory effects on several bacterial strains, including *Pseudomonas fluorescens*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. The maximum inhibitory zones were observed for *Staphylococcus aureus* (31 mm) and *Pseudomonas fluorescens* (29 mm), while the smallest zone was noted for *Klebsiella pneumoniae* (12 mm). Fig. 2 (A) illustrates the varying zones of inhibitory effects against other bacterial strains tested (Table 3).

In terms of antifungal activity, the extract displayed its most pronounced inhibitory effect against *Alternaria* species, resulting in a 27 mm zone of inhibition. In contrast, the least inhibitory effect was observed against *Rhizopus*, with a 12 mm zone of inhibition (Table 4; Fig. 2B). Fungal infections pose significant challenges to treatment, particularly in immunocompromised individuals, discovering plant extracts with antifungal properties of paramount importance in the quest for novel antifungal agents (Peyclit *et al*., 2021).

Table 3. Antibacterial activity of crude extract of *Asplenium dalhousieae* against selected bacterial strains.

Organisms	Inhibitory zone in diameter DMSO (mm) (mean ±standard deviation)		
	Frond	Antibiotic	Control
Streptococcus mitis	18 ± 2.0	18 ± 0.5	19 ± 3.2
Pseudomonas fluorescens	29 ± 4.04	30 ± 2.5	12 ± 2.5
Pseudomonas aeruginosa	27 ± 1.5	30 ± 2.5	12 ± 2.5
Klebsiella pneumoniae	12 ± 2.0	35 ± 2.5	15 ± 3.0
Proteus mirabilis	19 ± 1.0	32 ±2 .5	22 ± 2.5
Escherichia coli	27 ±3.0	28 ± 1.5	11 ± 3.0
Staphylococcus aureus	31 ± 1.1	30 ± 2.0	12 ± 6.5
Staphylococcus aureus	20 ± 2.0	30 ± 2.5	11 ± 1.5

Table 4. Antifungal activity of crude extract of *Asplenium dalhousieae* against selected fungal strains.

Organisms	Inhibition zone DMSO (mm) (mean ±standard deviation)		
	Frond	Antibiotic	Control
Carvularia	18 ± 1.0	16 ± 2.6	19 ± 1.0
Fusarium	20 ± 1.7	20 ± 5.8	17 ± 1.5
Alternaria	27 ± 2.5	30 ± 1.5	18 ± 3.6
Rhizopus	12 ± 3.6	20 ± 2.6	14 ± 4.1
Aspergillus	22 ± 2,5	28 ± 2.5	20 ± 5.0

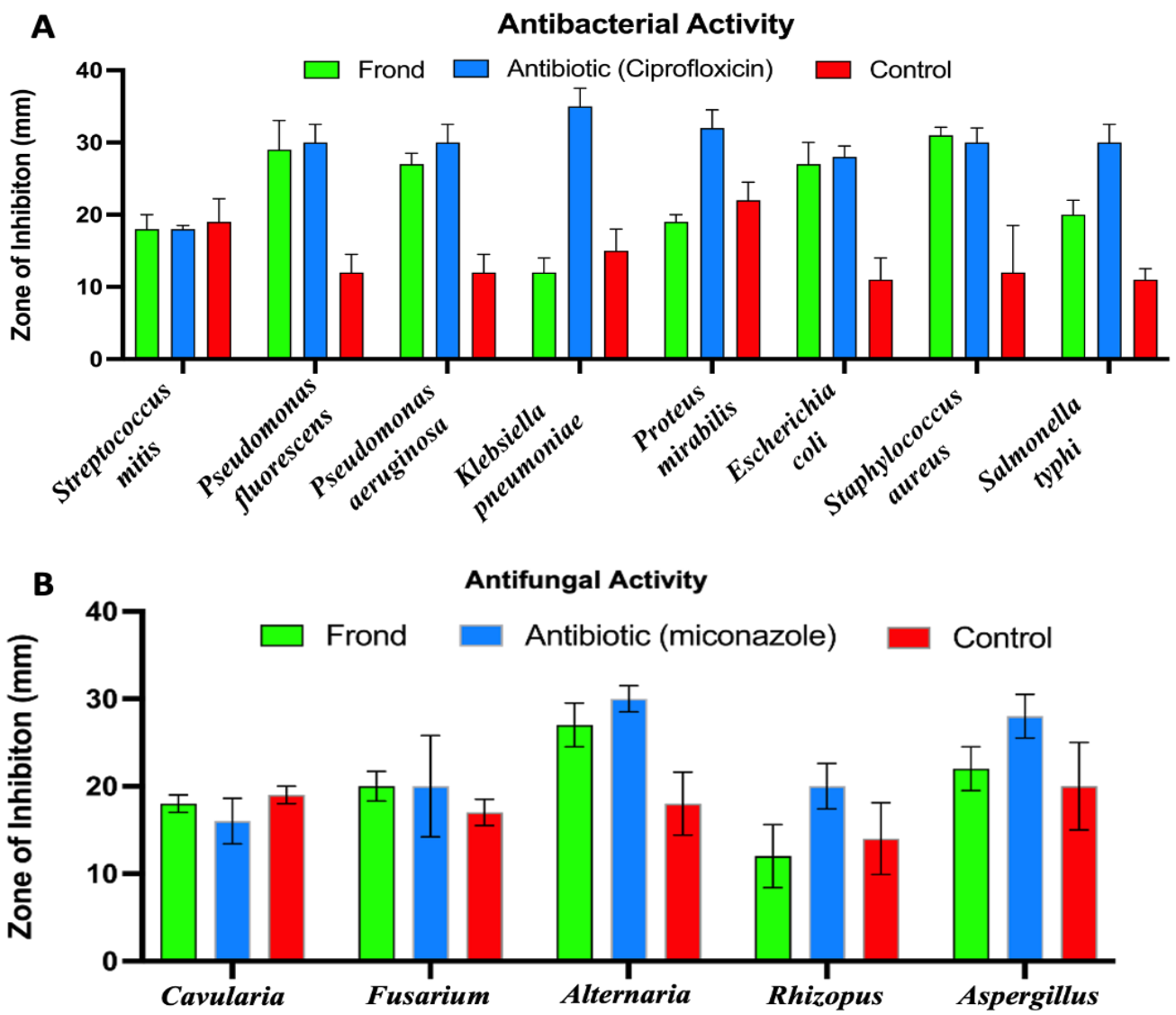

Fig. 2. Antimicrobial activities of methanolic extract of *Asplenium dalhousieae* A) Antibacterial activity B) Antifungal activity.

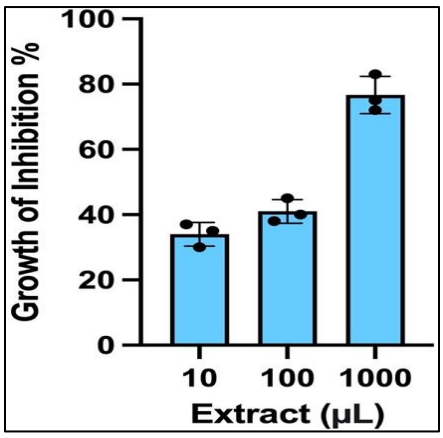

Fig. 3. Phytotoxic activity of crude extract of *Asplenium dalhousieae*.

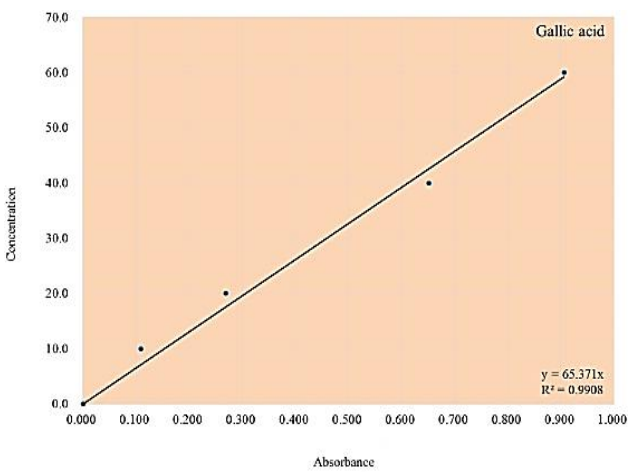

Fig. 4. Standard calibration curve of ascorbic acid for total antioxidant determination.

Phytotoxic activity: Phytotoxicity refers to the toxicity of a substance towards plant growth and development (Huang *et al*., 2021). The phytotoxicity assay of *Asplenium dalhousieae* was conducted to determine the effect of the plant extract on the growth of *Lemna minor*, a standard plant used in phytotoxicity studies. The plant extract was tested at 10 ppm, 100 ppm, and 1000 ppm concentrations. The results of the phytotoxicity assay showed that the plant extract completely inhibited the growth of *Lemna minor* at all tested concentrations, indicating 100% growth inhibition (Fig. 3). This suggests that the plant extract is highly phytotoxic to *Lemna minor*. The phytotoxicity assay is an important tool in the evaluation of the potential environmental impact of plant extracts. However, it is important to note that phytotoxicity assays may vary depending on the test organism, concentration, and exposure duration (Carvalho *et al*., 2014).

Antioxidant activity: The methanol extract of *Asplenium dalhousieae* demonstrated a moderate level of antioxidant potential, as evidenced by IC50 values of 34.4% in the DPPH assay and 55.73% in the Phosphomomybdenum assay (Table 6). In comparison, *A. trichomanes* exhibited a lower IC50 value of 2.27 mg/ml, while *A. scolopendrium* had a significantly higher IC50 value of 113 mg/ml when evaluated against ascorbic acid (vitamin C) with an IC50 of 0.018 mg/ml (Ismail *et al*., 2019). These variations highlight the diversity in antioxidant activity among different fern species and emphasize the potential of *Asplenium alternan* as a source of moderate antioxidant activity.

Table 5. Phytotoxic activity of crude methanolic extract of *Asplenium dalhousieae*.

Test plant	Plant sample (µg/ml)	Replicates	No. of frond (before)	No of frond (after)	No. of frond in control	Mean + (standard deviation)	% Growth regulation
Lemna minor	10	R1	34	28	36	25+(±2.5)	30
		R2	30	25	36		
		R3	31	23	36		
	100	R1	35	25	36	22+(±2.51)	38
		R2	32	20	36		
		R3	30	22	36		
	1000	R1	36	12	36	09+(±2.0)	75
		R2	35	8	36		
		R3	32	9	36		

Table 6. The antioxidant activity of *Asplenium dalhousieae* of methanol extract is presented as the means of three independent values.

DPPH scavenging			Phosphomomybdenum complex	
Absorbance of control	Absorbance of sample	Percent inhibition	Absorbance of sample	Percent inhibition
0.807nm	0.530nm		2.326	
0.805nm	0.553nm	34.40%	2.152	55.73%
0.809	0.509nm		2.504	

Conclusion

In conclusion, *Asplenium dalhousieae* emerges as a valuable resource rich in bioactive compounds, notably flavonoids and phenolics, with demonstrated antibacterial, antioxidant, phytotoxic, and phytochemical properties. These findings underscore its potential significance in the pharmaceutical sector. The anti-bacterial activity displayed promising avenue for the development of novel agents against harmful pathogens, possibly serving as a foundation for the synthesis of active semi-synthetic analogs. Further exploration, including research into fern toxicity, *In vivo* potential, and application against multidrug-resistant pathogens, holds promise for unlocking the full potential of these compounds in various therapeutic contexts.

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