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PHARMACOGNOSTIC CHARACTERIZATION AND IN VITRO ANTIMICROBIAL EVALUATION OF *IRIS AITCHISONII* (BAKER) BOISS. AGAINST MULTIDRUG-RESISTANT PATHOGENS

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Abstract *In 2019, antimicrobial resistance resulted in approximately 4.95 million deaths worldwide, and South Asia bore the brunt of these deaths. Iris aitchisonii (Baker) Boiss. is utilized by Northern Pakistanis for traditional medical purposes; however, there are no available pharmacognostic standards or data regarding the antimicrobial properties of the species. Stem, flower, and tunic bulb of authenticated I. aitchisonii (voucher 6095) were shade-dried and extracted using distilled water, ethanol, and methanol. In addition to recording organoleptic, microscopic, and micromorphometric characteristics, the constituents were screened qualitatively. The activity of the extracts against six bacterial and three fungal strains was evaluated by using agar-well diffusion methods, comparing the activity to levofloxacin, erythromycin, and terbinafine as controls. Phytotoxicity was tested using Lemna minor with the extracts at 10–1000 µg/mL. All assays were conducted in triplicate. Each of the 135 samples was tested for constituents and found to contain detectable amounts of constituents in 46 of the total number of tests, with the highest quantity found in the stem (17 out of 45 tests). In each of the 18 extract-organism combinations, the zone of inhibition was smaller than or equal to the control; the largest zone of inhibition was seen with flower extract against Proteus mirabilis (26.66 mm ± 3.51 mm; difference = -5.34mm, 95% CI = -22.11 to 11.43; p = 0.371) and the smallest zone of inhibition was seen with bulb extract against Salmonella typhi (-19.00 mm 95% CI -23.16 to -14.84; p = 0.001). All of the nine tested antifungal comparisons exhibited zones of inhibition smaller than the control (p ≤ 0.013). Antifungal phytotoxicity was found to be dependent upon concentration, with a maximum phytotoxicity of 77.77%. Extracts showed measurable but sub-comparator antimicrobial activity and concentration-dependent phytotoxicity. Interpretation is limited by triplicate testing, crude extracts, and diffusion-based endpoints; no therapeutic use is supported. The standards reported enable authentication; fractionation and minimum inhibitory concentration testing are required next.*

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Keywords*:* *Iris aitchisonii*; *Iridaceae*; *phytochemical screening*; *antimicrobial resistance*; *agar-well diffusion*

Introduction

Antimicrobial resistance (AMR) is quickly becoming one of the primary therapeutic challenges of our current century. The latest analysis of global data reports that 4.95 million deaths in 2019 were attributable to bacterial AMR, with 1.27 million being directly attributable to AMR, which translates to 39 million additional deaths due to antimicrobial-resistant infections between 2025 and 2050 (Naghavi et al., 2024). The greatest burden is levied primarily on South Asia and sub-Saharan Africa, where access to diagnostics is limited, and antibiotics are sold without a prescription. An example of this pressing problem can be seen in Pakistan where, since 2016, there have been multiple outbreaks of extensively drug-resistant Salmonella Typhi in the province of Sindh; this left azithromycin as the only reliably effective oral treatment for typhoid in the linked districts (Klemm et al., 2018). The slow progress of antibacterial drug development has compounded the issue further; there are very few new antibacterial agents present in the development pipeline, and the World Health Organization's 2024 Bacterial Priority Pathogens List identifies carbapenem-resistant Acinetobacter baumannii and Enterobacterales as critical priority organisms for which new chemical scaffolds must be developed urgently (Organization, 2024). Although there are limited numbers of chemical scaffolds being explored through drug discovery today, higher plants continue to be a source of chemical scaffolds that may help alleviate the aforementioned crisis. Over the past 40 years, a substantial portion of approved anti-infective agents for human use have come from natural sources or derivatives of natural products (Newman and Cragg, 2020). Of particular interest within the ongoing search for antimicrobial agents, the Iridaceae family is noteworthy, as members of the Iris genus have shown accumulation of a diverse array of isoflavonoids, quinones, terpenoids, and tannins; these classes of compounds have previously been shown to produce

membrane-disrupting and enzyme-inhibitory effects (Khatib et al., 2022). Several species of the genus Iris have been tested for activity against laboratory strains (Gram-positive and Gram-negative bacteria) as well as some types of phytopathogenic fungi (Amin et al., 2021; Hoang et al., 2020). Although the research to date indicates activity against laboratory strains, the evidence documenting this activity has limitations. Most of the published studies only tested either rhizome extract alone or using a single test solvent system or reference laboratory strains rather than a resistant clinical phenotype responsible for mortality. Moreover, different studies reported markedly different responses to plant extracts, potentially attributable to geographical sources, the manner of extraction, and assay methodology, rather than true differences in activity level of the taxa tested. There is also presently very little published comparative data on the responses to plant extracts for different parts of the plant (i.e. stem, flower, bulb) from an authenticated single collection, with all the samples being subjected to the same type of extraction procedure.

The case of Iris aitchisonii (Baker) Boiss. illustrates this lack of information. The species is found throughout the western Himalayan region and is used in traditional medicine in northern Pakistan. While this species has been associated with traditional medicine and used by various communities, very little systematic investigation into its pharmacological properties or potential antimicrobial properties has been conducted (Ajaib et al., 2021). Several deficits have been identified as deficiencies in the research on this plant. First, no validated phytochemical and micro-morphometric standards have been identified for the powdered form of the herbal product. Because these standards do not currently exist, the raw material being sold as I. aitchisonii cannot be authenticated, and the incidence of adulteration cannot be reliably determined, as recognised by the World Health Organisation and national pharmacopoeias, thereby preventing any future assessment of the material. Second, no information is available concerning the relative distribution of secondary metabolites within the stem, flower, and tunic bulb of I. aitchisonii, and how that distribution would be affected by different extraction solvents, thus leaving no scientific basis for selecting plant parts for future research. Lastly, no studies have been conducted on the activities of I. aitchisonii against local gram-negative clinical pathogens or potential non-target toxicity of extracts of this species compared to the known clinical antibiotics.

The primary goal of this research study was to establish the macroscopic, microscopic, and micromorphometric standards of I. aitchisonii and to characterise qualitatively the phytochemical profiles of the stem, flower, and bulb extracts of I. aitchisonii in aqueous, ethanolic, and methanolic solvent systems. The secondary goals of this study were to compare the antibacterial activity of the stem, flower, and bulb of I. aitchisonii against six bacterial pathogens (including Salmonella Typhi, Klebsiella pneumoniae, and Acinetobacter baumannii) as well as compare their antifungal activity against three phytopathogenic fungi, and determine the potential phytotoxicity of extracts of this species against Lemna minor as the first measure of non-target biological effects. To our knowledge, this is the first complete pharmacognostic and biological profile of the species I. aitchisonii.

Materials and methods

Plant collection, authentication and herbarium preparation

The complete specimen of Iris aitchisonii (Baker) Boiss. (Iridaceae) was collected from Chamhatti in Abbottabad District of Khyber Pakhtunkhwa, Pakistan. The specimen was verified based on the published characteristics of the Iridaceae family and identified by Prof. Dr. Manzoor Hussain's Department of Botany at Hazara University, Mansehra. A voucher specimen was created by applying the specimen between two sheets of blotting paper, drying it daily until no moisture remained in the paper, and subsequently mounting it on a herbarium mounting board and marking it with a unique number assigned to the herbarium department (receipt no. 01; accession no. 6095) (Figure 1). Using the methods of Balakrishnan et al. (2011), the gross morphological characteristics of the specimen, such as plant habit and sizes of the main parts of the specimen, were documented. Only authenticated materials were used in the experimentation process (Balakrishnan et al., 2011).

Figure-1: Herbarium specimen of I. aitchisonii (Baker) Boiss

Preparation of powdered drug and crude extracts

The three components of the plant, the stem, flower, and tunic bulb, were separated from each other, cut

into small pieces, shade-dried at room temperature for a period of 28 days, and ground into a fine powder. In preparation for testing this plant's pharmacological effects (bioassays), 100g of each component powder was placed in a container with 300mL of 70% methanol, agitated every day for 14 days (intermittently), filtered three times through Whatman filter paper, and concentrated into a semi-solid at 40°C using a rotary evaporator. Both the concentrated extracts and the powdered drugs remained at 40°C until used. For the phytochemical screening tests, individual extracts were produced from the three components by combining 3g of each component powder with 30mL of distilled water, ethanol, or methanol, agitating the mixture every day for six or seven days, and subsequently filtering it.

Pharmacognostic evaluation

Pharmacognostic evaluation was done using the methods (Balakrishnan et al., 2011; Das et al., 1997), where stem powder's organoleptic characteristics were evaluated through direct sensory assessments of colour, odour, flavour, feel (i.e., textural quality), fracture, and surface markings. Microscopic examination of stem powder was accomplished by placing the powder in a solution consisting of 70% chloral hydrate to boil for 30 minutes, mounting the powder with glycerine jelly, staining the prepared specimen with 1% safranin, and sealing the mounted specimen with nail varnish (Khandelwal, 2008). Epidermal anatomy was observed according to Sreelakshmi and his colleagues (Sreelakshmi et al., 2014), with minor modification: The epidermal tissue of completely opened leaves was placed in fixative for 1–2 days, followed by washing with 70% alcohol, followed by boiling pieces of the epidermis in 5% potassium hydroxide for 7–10 minutes. After boiling, the epidermal surfaces were separated from the underlying tissue with a needle and subsequently mounted for viewing. Qualitative characteristics (such as the arrangement and borders of the epidermal cells) and micrometric characteristics (including the number of epidermal cells per unit area, the length/width of each epidermal cell, and the number of filaments and trichomes and their dimensions) were recorded using an ocular micrometer that was calibrated in accordance with a designated procedure. Transverse sections of stems were also prepared using a microtome according to Oruade and his colleagues (Oruade-Dimaro et al., 2010), after the fixation of sections using methyl alcohol, glacial acetic acid and 30–40% (v/v/v, methyl alcohol: glacial acetic acid: formalin) formalin for 48 hours; following fixation, sections were dehydrated by passing them through increasing percentages of ethanol; then, sections were stained with 1% safranin and light green and made permanent by mounting the stained sections in Canada balsam. All stained and mounted specimens were photomicrographed using digital cameras through a light microscope, and micrometry was performed on micrometric data obtained from photomicrographs (Shah et al., 2017).

Preliminary phytochemical screening

Potential phytochemicals were evaluated from sample stem, flower, and bulb extracts using various extraction methods (water, ethanol, methanol) to test for the presence of secondary metabolites. Established methods of qualitative screening were followed (Banu and Cathrine, 2015; Edeoga et al., 2005; Evans, 2009; Parekh and Chanda, 2007), except for minor modifications. Mayer's reagent was used to test for alkaloids; a mixture of chloroform and concentrated sulfuric acid was used to test for phenols, terpenoids, and quinones, whereas dilute NaOH and HCl were used to test for flavonoids; a 10% solution of NaOH was used to test for anthraquinones, coumarins, and glycosides; iodine solution was used to test for carbohydrates and 5% ferric chloride to test for tannins and cardiac glycosides. Phlobatannins were tested using a 2% HCl solution, and saponins were identified based on evidence of frothing; fixed oils were identified using the filter-paper stain test, while mucilage was identified based on the gummy consistency of the extract after standing in water. All results for the chemical constituents were based on tests performed three times per test. For any chemical constituent to be reported as present, a characteristic reaction must have been observed in the three tests.

Test microorganisms and culture conditions

Bacterial strains of Salmonella typhi, Klebsiella pneumoniae, Acinetobacter baumannii, Proteus mirabilis, Staphylococcus epidermidis, and Bacillus subtilis were obtained from the Department of Microbiology, Hazara University, Mansehra, Pakistan. Working cultures were prepared in nutrient broth (4g in 200mL distilled water) with an inoculation volume of 4mL per culture tube using a flame-sterilized inoculation loop/asceptic technique, placed in a biosafety cabinet, and incubated at 37°C for 24 hours. Fungal strains of Alternaria solani, Rhizopus stolonifer, and Fusarium oxysporum were sub-cultured using Sabouraud dextrose agar at an incubation temperature of 28°C for a period of 7 days. To store overnight cultures, they were centrifuged, then resuspended in a 70% glycerol solution and stored at -80 degrees Celsius.

Antibacterial and antifungal assays

The agar-well diffusion method was used to measure the antibacterial (V) activity of the samples (Perez and Anesini, 1994). Clay agar was prepared at 20g / L, pH 7, and autoclaved at 120 degrees Celsius for 15 minutes. 10-15 mL was poured into flat-bottom sterile plates (10-15). The inoculated plates were then distributed evenly across a flat-bottom plate using sterile cotton swabs. Each inoculated plate was then bored with a sterile cork borer to make 4 uniform 6 mm to 7 mm holes. Each well received either 20µL of the stem, flower or bulb extract, or Levofloxacin and Erythromycin, respectively (positive control), or Extraction Solvent (Negative Control). Measurements of zones of inhibition were made after 24 hours of

incubation at 37 degrees Celsius.

Fungal activity was measured as per Valsaraj et al. (1997) but with slight modifications. Potato Dextrose Agar (PDA) was prepared at 15g / L, autoclaved at 120 degrees Celsius for 10 minutes (Valsaraj et al., 1997). Plates were swabbed with a cotton swab, and holes of approximately 10 mm were bored into the plates with a sterile cork borer and then charged with extracts. Stock solutions were 1g extract/10mL dimethyl sulfoxide and Terbinafine at 12 mg/mL in dimethyl sulfoxide (positive control) and solvent only (negative control). The plates were incubated overnight, and the zones of inhibition were measured. All diffusion assays were completed in triplicate.

Phytotoxic assay

Phytotoxicity was evaluated according to the following protocols by Atta-ur-Rahman et al. (2001) (Choudhary and Thomsen, 2001). 10, 100, 1000 µg/mL (10, 100, 1000 µg/mL) extracts were tested with three replicate plates at each concentration. Each replicate plate received 20 mL of E-Medium plus test extract or E-Medium alone (negative control). Healthy Lemna minor L. with intact roots were transferred to each plate and counted by fronds. After 7 days of growing in ambient laboratory conditions, frond counts were repeated, and the growth inhibition was calculated as: % Inhibition = 100 - [(fronds in test sample)/(fronds in negative control)] x 100.

Data analysis

Data analysis was performed using Microsoft Excel. All results have been expressed as mean ± standard deviation. All zones of Inhibition and Frond Counts have been expressed as Mean ± Standard Deviation, and percentage growth inhibition was calculated relative to the negative control using Microsoft Excel to perform descriptive statistics.

Results

Flow of Analytical and Plant Materials

The amount of plant material collected exceeded that which was originally required for the intended testing. After inspecting and removing any diseased, damaged, or otherwise unrepresentative plant materials, sufficient amounts of stems, flowers, and tunic bulbs were collected and processed. All 9 of the micro-organisms (Table 1) that are part of the testing protocol (6 bacterial and 3 fungal) were successfully subcultured and were used in the bioassays; none of the cultures were lost due to contamination during the subculturing process. Each of the individual assays was conducted three times (3), resulting in triplicates (n=3) for each assay. There were no recorded observations that were not accounted for in the pharmacognosy, phytochemistry, antimicrobial, or phyto-toxicity areas; therefore, the analysis becomes a complete case with zero (0) missing data.

Table 1. Bacterial and fungal strains used for the in vitro antimicrobial evaluation of *Iris aitchisonii* (Baker) Boiss

No.	Test microorganism	Type / Gram reaction
Bacterial strains		
1	*Salmonella typhi*	Gram-negative bacterium
2	*Klebsiella pneumoniae*	Gram-negative bacterium
3	*Acinetobacter baumannii*	Gram-negative bacterium
4	*Proteus mirabilis*	Gram-negative bacterium
5	*Staphylococcus epidermidis*	Gram-positive bacterium
6	*Bacillus subtilis*	Gram-positive bacterium
Fungal strains		
7	*Alternaria solani*	Filamentous fungus
8	*Rhizopus stolonifer*	Filamentous fungus
9	*Fusarium oxysporum*	Filamentous fungus
Strain identity was confirmed by standard morphological and biochemical procedures.		

Pharmacognostic Analysis

Pharmacognostic, organoleptic and qualitative microscopic characteristics of the stem powder are described in Table 2. Stem powder appears brownish grey, is fine textured, somewhat bitter and somewhat fragrant. Under a microscope, the above data show that the single-cell epidermis has an entire cell configuration with a smooth cell margin; three types of trichomes were identified (i.e., stellate, unicellular and cylindrical). The powder contains no macro- or micro-hairs and no oil droplets; however, the powder does contain several filament structures.

Table 2. Organoleptic and qualitative microscopic characters of the stem powder of *Iris aitchisonii* (Baker) Boiss

No.	Parameter	Observation
Organoleptic (macroscopic) characters		
1	Taste	Slightly bitter
2	Odour	Slightly aromatic
3	Texture	Fine
4	Colour	Light brown
Qualitative microscopic characters		
5	Epidermal cell type	Entire
6	Epidermal layers	Single-layered
7	Epidermal cell margin	Smooth
8	Macro-/micro-hairs	Absent
9	Trichomes	Present
10	Trichome types	Stellate, unicellular and cylindrical
11	Oil droplets	Absent
12	Filaments	Present
Observations were made on shade-dried, coarsely powdered stem material examined under a compound light microscope after clearing in chloral hydrate. Each character was recorded independently by two observers; only concordant observations are reported.		

Quantitative Micromorphometric Characteristics

Details of the quantitative micromorphometric characteristics are listed in Table 3. Reticulate tracheids were the longest of the three types of tracheary elements at 20.00 µm, while spiral tracheids were the shortest at 6.50 µm. Among the structures of trichomes and filaments, stellate trichomes were the

largest at 40.50 × 6.00 µm, while filaments were the smallest at 5.11 × 0.65 µm. The dimensions of the epidermal cells were 12.40 × 7.00 µm, with the average number of epidermal cells being 30–31 per microscopically viewed area at 400 × while the total number of trichomes and filaments per area was 3 each.

Table 3. Quantitative micromorphometric characters of the stem powder of *Iris aitchisonii* (Baker) Boiss

Cell / structure	Length (µm)	Width (µm)	Frequency (n per field)
Epidermal cells	12.40	7.00	30–31
Spiral tracheids	6.50	3.10	—
Pitted tracheids	14.50	6.00	—
Reticulate tracheids	20.00	5.00	—
Unicellular trichomes	13.60	4.00	3
Stellate trichomes	40.50	6.00	3
Cylindrical trichomes	11.50	7.00	3
Filaments	5.11	0.65	3

Values are arithmetic means of measurements taken on 10 randomly selected structures per slide across three slides, recorded with a calibrated ocular micrometer at 400× magnification. Frequency denotes the mean number of structures counted per microscopic field at 400×. Em dash (—) indicates a structure for which frequency was not counted.

Phytochemical Screening

A total of 135 qualitative phytochemical tests on three parts of three different plants using three solvents were performed (Table 4), of which 46 (34.1%) produced a positive reaction. Stem extracts produced 17 of the 45 (37.8%), followed by flower extracts with 16 out of 45 (35.6%), and bulb extracts had 13 of the 45 (28.9%). When grouped by solvent, distilled water produced the most positives at 19 of the 45 (42.2%), followed by ethanol with 14 of 45 (31.1%), and methanol produced 13 of the 45 (28.9%).

Table 4. Preliminary phytochemical screening of methanol, ethanol and aqueous extracts of the stem, flower and bulb of *Iris aitchisonii* (Baker) Boiss

Phytochemical constituent	Stem			Flower			Bulb		
	MeOH	EtOH	Aq.	MeOH	EtOH	Aq.	MeOH	EtOH	Aq.
Alkaloids	+	+	+	−	−	−	−	−	−
Phenols	−	−	+	+	+	+	−	−	−
Flavonoids	−	−	+	+	+	+	+	+	−
Saponins	−	+	−	−	−	−	−	−	+
Anthraquinones	−	−	−	−	−	−	−	−	+
Carbohydrates	−	−	−	−	−	−	−	−	−
Quinones	−	+	+	−	−	−	−	−	+
Tannins	+	+	+	−	−	−	+	+	+
Terpenoids	−	−	−	+	+	−	−	−	−
Glycosides	+	+	+	−	−	−	−	−	+
Cardiac glycosides	+	−	+	+	+	−	−	−	−
Coumarins	−	−	+	−	−	−	−	−	+
Phlobatannins	−	−	−	−	−	−	−	−	−
Fixed oils	−	−	−	+	+	+	−	−	−
Mucilages	−	−	−	+	+	+	+	+	+

+, constituent detected; −, constituent not detected. MeOH, methanol; EtOH, ethanol; Aq., distilled water. Screening was performed in triplicate using standard qualitative phytochemical tests; a constituent was recorded as present only when the characteristic reaction was observed in all three replicates. Carbohydrates and phlobatannins were not detected in any part or solvent.

The percentage distribution of phytochemicals among plant organs varied considerably. The stem extracts contained positive results for alkaloids, glycosides, and tannins, whereas neither flower nor bulb extracts contained any alkaloids. All flower extracts contained positive results for phenols, flavonoids, mucilages, and fixed oils, while neither stem nor bulb extracts contained any fixed oils. In contrast, tannin and mucilage were the only phytochemicals found in all three of the bulb extracts. Carbohydrates and phlobatannins were absent from all parts of the plant, regardless of the solvent used.

Antibacterial Activity

Table 5 displays the zones of inhibition for the three extracts and the antibiotic control tested on six different strains of bacteria. Of the 18 different

extract-organism combinations, the extracts had less activity than the control. The largest zone of inhibition caused by an extract was produced by the flower extract against the bacterium Proteus mirabilis at 26.66 ± 3.51 mm, representing 83.3% of the control zone (32.00 ± 7.93 mm) with a mean difference between the two of −5.34 mm (95% CI=−22.11 to 11.43; p=0.371). All bulb extracts appeared active against *Salmonella typhi* (11.00±1.00mm), which represented 36.7% of the control zone and had a mean rank difference of −19.00mm (95% CI, −23.16 to −14.84; p=0.001). Antibacterial activity of stem extracts against six bacterial pathogens, showing inhibition zones compared with standard antibiotic controls shown in Figure 2.

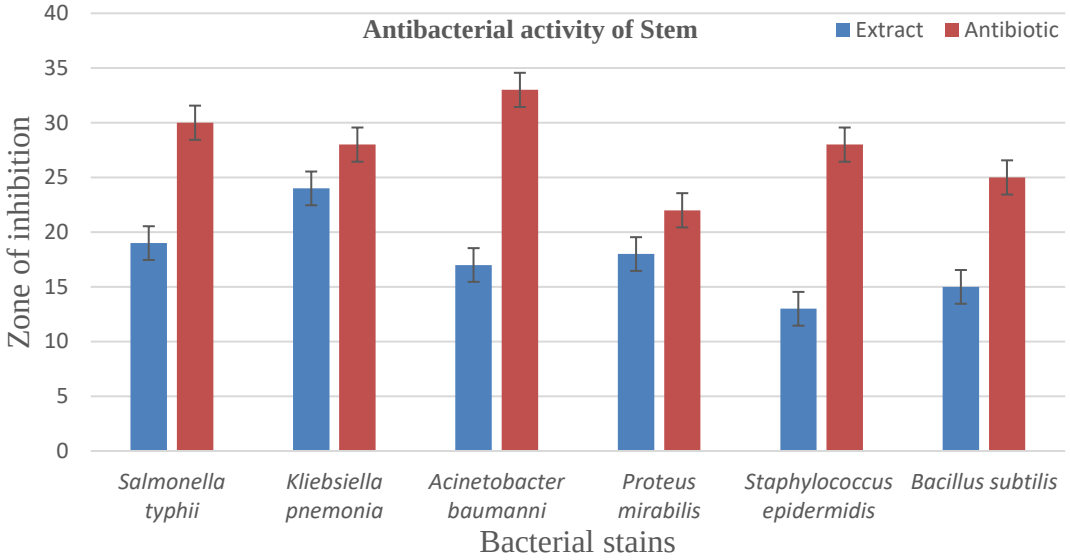

Figure 2: Antibacterial activity of stem of *Iris aitchisonii* (Baker) Boiss.

Table 5. Antibacterial activity of stem, flower and bulb extracts of *Iris aitchisonii* (Baker) Boiss. against six bacterial pathogens

Test organism	Plant part extract			Standard antibiotic
	Stem	Flower	Bulb	
Salmonella typhi	19.66 ± 3.21	14.33 ± 1.52	11.00 ± 1.00	30.00 ± 2.00
Klebsiella pneumoniae	24.00 ± 2.00	18.66 ± 4.04	16.33 ± 4.04	28.66 ± 6.02
Acinetobacter baumannii	17.00 ± 2.00	21.00 ± 1.00	16.00 ± 4.58	33.00 ± 3.00
Proteus mirabilis	18.66 ± 3.51	26.66 ± 3.51	21.00 ± 6.55	32.00 ± 7.93
Staphylococcus epidermidis	13.00 ± 2.00	21.00 ± 3.60	14.66 ± 2.51	28.00 ± 2.00
Bacillus subtilis	15.00 ± 5.29	22.66 ± 3.05	13.00 ± 2.00	25.66 ± 4.04
Values are zones of inhibition (mm, mean ± standard deviation) from three independent experiments, measured by the agar-well diffusion method and inclusive of the well diameter.				

Rank order of plant parts varied among the organisms tested.

Stem extracts had the largest zones of inhibition against *Klebsiella pneumoniae* (24.00±2.00mm; 83.7% of control), with a mean difference from the control of −4.66mm (95% CI, −18.00 to +8.68; p=0.311). A flower extract had the largest zones against both Gram-positive bacteria and against P. mirabilis and *Acinetobacter baumannii*. Extracts from bulbs produced the smallest zones for four of the six organisms tested. In general, differences between the control antibiotic and the stem extracts against *K. pneumoniae, P. mirabilis and Bacillus subtilis*, and the flower extracts against *K. pneumoniae, P. mirabilis, Staphylococcus* epidermidis and *B. subtilis* did not reach the conventional 5% significance level. Antibacterial activity of flower extracts against six bacterial pathogens, with comparatively strong inhibition against Proteus mirabilis and Gram-positive bacteria shown in Figure 3, and antibacterial activity of bulb extracts against six bacterial pathogens, demonstrating generally smaller inhibition zones than stem and flower extracts shown in Figure 4.

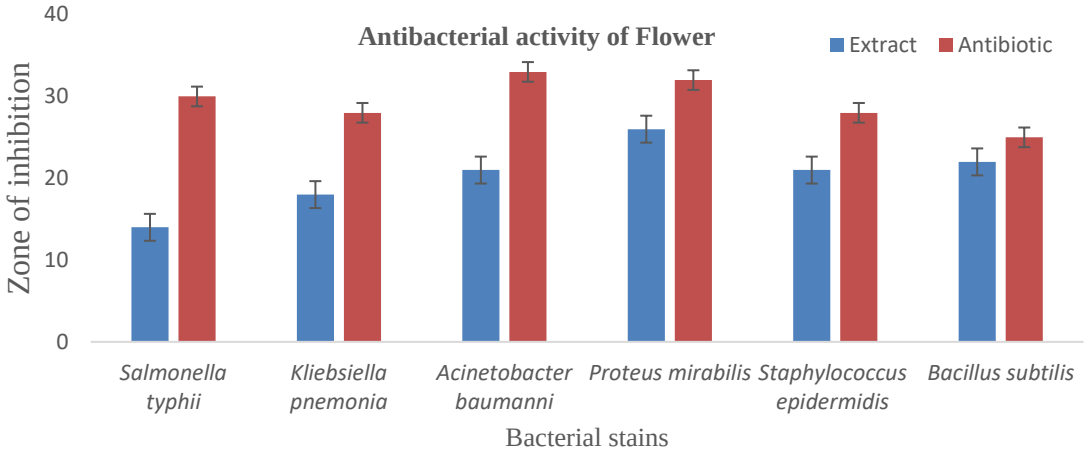

Figure 3: Antibacterial activity of flower of Iris aitchisonii (Baker) Boiss

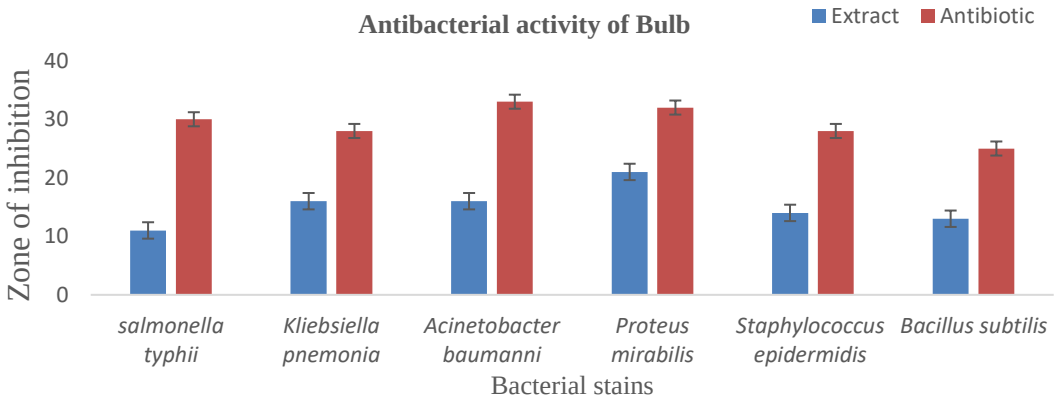

Figure 4: Antibacterial activity of bulb of Iris aitchisonii (Baker) Boiss

Antifungal activity

Antifungal activity was measured using the same method as for antibacterial activity; the results are presented in Table 6. All nine combinations of extracts (bulb, stem, flower) produced zones significantly smaller than those produced by the antifungal control (all $p \leq 0.013$). The stem extract had the highest activity against Fusarium oxysporum (20.00±1.00mm; 53.1% of control), the flower extract had the highest activity against Alternaria solani (21.33±1.10mm; 61.5% of control), and the bulb extract had the highest activity against Rhizopus stolonifer (19.66±1.52mm; 52.7% of control). The flower extract against R. stolonifer had the lowest level of activity (12.00±2.mm; 32.1% of control; mean difference of −25.33mm, 95% CI, −29.96 to −20.70; $p<0.001$). Antifungal activity of stem extracts against Alternaria solani, Rhizopus stolonifer, and Fusarium oxysporum compared with terbinafine shown in figure 5 and antifungal activity of flower extracts against three phytopathogenic fungi, showing the highest activity against Alternaria solani shown in figure 6 also antifungal activity of bulb extracts against three phytopathogenic fungi, with the greatest inhibition observed against Rhizopus stolonifer shown in figure 7.

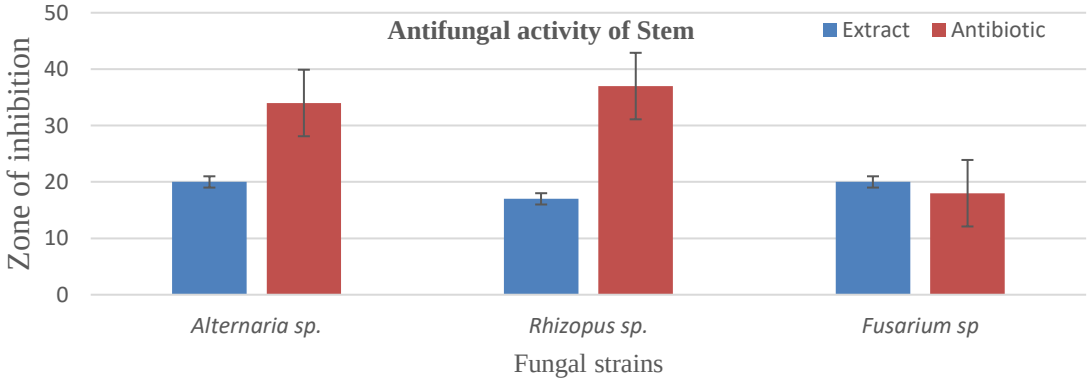

Figure 5: Antifungal activity of stem of Iris aitchisonii (Baker) Boiss

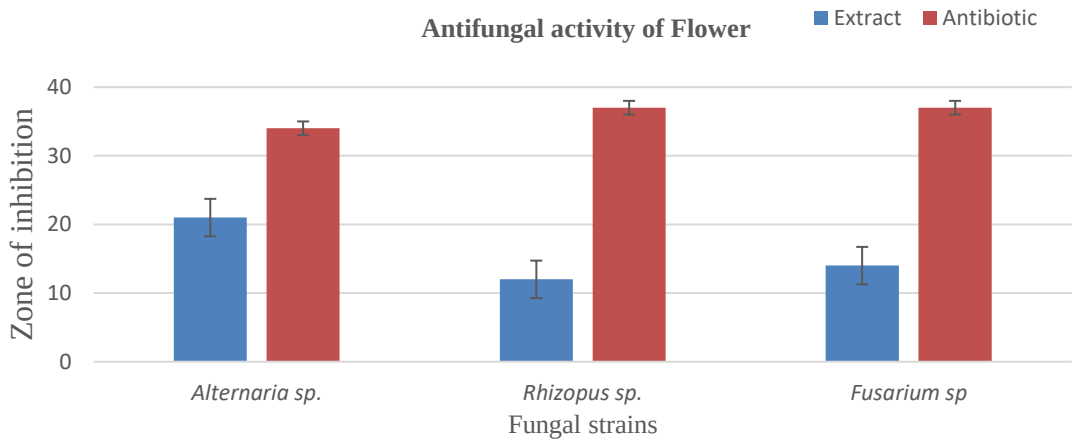

Figure 6: Antifungal activity of flower of *Iris aitchisonii* (Baker) Boiss

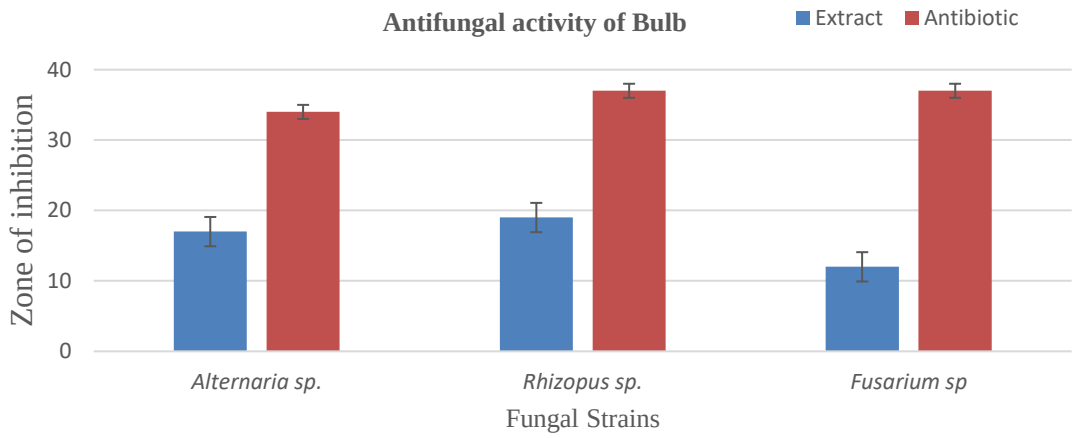

Figure 7: Antifungal activity of Bulb of *Iris aitchisonii* (Baker) Boiss

.Table 6. Antifungal activity of stem, flower and bulb extracts of *Iris aitchisonii* (Baker) Boiss. against three phytopathogenic fungi

Test organism	Plant part extract			Standard antifungal
	Stem	Flower	Bulb	
Alternaria solani	20.66 ± 1.52	21.33 ± 1.10	17.00 ± 3.00	34.66 ± 3.05
Rhizopus stolonifer	17.00 ± 1.00	12.00 ± 2.00	19.66 ± 1.52	37.33 ± 2.08
Fusarium oxysporum	20.00 ± 1.00	14.33 ± 2.51	12.66 ± 2.08	37.66 ± 4.04
Values are zones of inhibition (mm, mean ± standard deviation) from three independent experiments.				

Phytotoxicity

Phytotoxicity was evaluated against Lemna minor (Table 7). For all three plant parts, the amount of growth inhibition increased with concentration. At 10µg/mL growth inhibition ranged from 11.11% (flower) to 18.51% (stem), and at 100µg/mL ranged from 25.92% (stem and flower) to 44.44% (bulb). At 1000µg/mL the stem and flower plants inhibited growth by 40.74% and 77.77%, respectively. The bulb extracts were found to produce the greatest growth inhibition at the two highest levels, and the flower extracts had the lowest levels of inhibition at the highest concentration of 1000µg/mL. Concentration-dependent phytotoxicity of stem, flower, and bulb extracts against Lemna minor, with maximum inhibition produced by bulb extract shown in Figure 8.

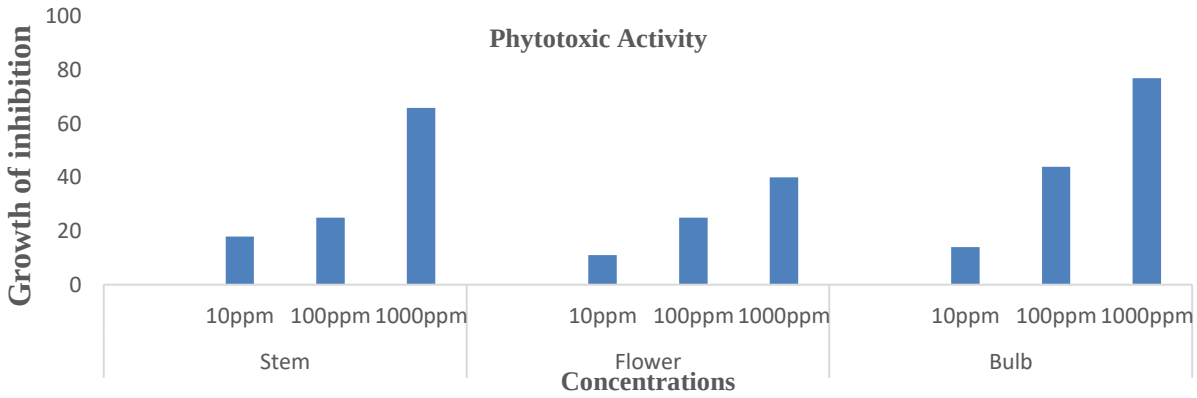

Figure 8: Phytotoxic activities of stem, flower and bulb of Iris aitchisonii (Baker) Boiss

Table 7. Phytotoxic activity of stem, flower and bulb extracts of *Iris aitchisonii* (Baker) Boiss. Against *Lemna minor* L.

Plant part	Concentration	Fronds before treatment (n)	Fronds after treatment (mean ± SD)	Negative control (n)	Growth inhibition (%)
Stem	10 µg/mL	26	22 ± 2	27	18.51
	100 µg/mL	25	20 ± 5	27	25.92
	1000 µg/mL	26	9 ± 5	27	66.66
Flower	10 µg/mL	25	24 ± 3	27	11.11
	100 µg/mL	26	20 ± 3	27	25.92
	1000 µg/mL	28	16 ± 4	27	40.74
Bulb	10 µg/mL	25	23 ± 3	27	14.81
	100 µg/mL	26	15 ± 1	27	44.44
	1000 µg/mL	29	6 ± 2	27	77.77

Growth inhibition (%) was calculated as [(fronds in negative control − fronds after treatment) / fronds in negative control] × 100. Values for fronds after treatment are means ± standard deviation of three replicates counted seven days after treatment.

Discussion

Main findings

This research represents the first comprehensive biological and pharmacognostic assessment of Iris aitchisonii (Baker) Boiss. The stem powder was evaluated for organoleptic, qualitative, and micromorphometric characteristics, resulting in standards of comparison for the raw material when authenticated (Tables 2 and 3). The phytochemical analysis (Table 4) demonstrated that 46 of 135 tested chemical compounds were found in this plant material, with the majority of positive results (17/45) coming from the stem and the most productive solvent (19/45) being distilled water. Antibacterial effectiveness was present but at lower levels than the antibiotic control (Table 5) in all 18 combinations of extracts and bacteria tested, with the flower extract being closest to the control zone against Proteus mirabilis (83.3% of control zone mean difference: -5.34 mm, 95% CI-22.11 to 11.43; p = 0.371). All nine of the antifungal comparisons were significantly lower than the control (Table 6). The phytotoxicity exhibited against Lemna minor was shown to increase with increasing concentrations of the extracts from all organs tested, with a maximum of 77.77% observed for the bulb extract at 1000µg/mL (Table 7). The modest and null findings reveal a great deal of information given the lack of positive results. None of the extracts produced sufficient therapeutic-grade activity under the conditions tested.

Interpretation and potential mechanisms

The organ-specific distribution of the tested plant extracts provides a potential explanation for the activity observed in Tables 5 and 6, although this is still just a theory at this point. All three of the stem extracts contained tannins, and all three of the bulb extracts contained tannins, while flowers were dominated by flavonoids and phenols. Both classes of compounds have established mechanisms of action, where flavonoids may impact nucleic acid synthesis; impair energy metabolism via interaction with the phospholipid bilayer and porins (Górniak et al., 2019; Kováč et al., 2022); and tannins may act via iron chelation, membrane disruption, and inhibition of fatty-acid biosynthesis (Kováč et al., 2022). Iris, a genus of flowering plants, contains a greater variety of isoflavonoids than any other genus or family. Likewise, other members of this family produce some of the same phenolic compounds and, therefore, may show similar biological activities. Extracts of the flowers, in particular the extracts with the highest concentrations of flavonoids and phenolic compounds, were able to inhibit the growth of three of the most common Gram-positive bacteria at the

greatest extent. However, other than the flower extracts, it is possible that the activity is due solely to herbaceous interactions (i.e., it may be an additive effect) with other non-flavonoid compounds that are present in the extracts. In addition, because of the large size of high molecular weight tannins, the limited movement of these tannins through agar may obscure their true potential inhibitory strength against the bacteria (Kováč et al., 2022). There is not enough evidence to support an assertion that any of the individual chemical classes were responsible for the antimicrobial properties because the work was not completed to identify the individual classes using minimum inhibitory concentration testing and fractionation.

Objectives in the context of existing literature

The primary goal of the current study was to address the aforementioned gap in the literature. All of the available literature (and reviews) relating to the genus Iris recommends that proper identification of Iris species is best accomplished through the use of morphologically distinct characteristics unique to an individual species; however, these species-specific characteristics are absent for the majority of species which do not belong to the commercially produced or accepted group of orris extract (Khatib et al., 2022). The tables that constitute this study provide adequate information to fulfil the purpose of the first objective of the study regarding I. aitchisonii, one of the more commonly used ethnomedicinal plants in Pakistan; however, no previously published laboratory studies exist for this plant species (Ajaib et al., 2021). The objectives of the second portion of the study also complement the literature in that the majority of previous studies relating to the antimicrobial activity of Iris have focused on the bioactivity of Iris in their rhizome or root only, using a single solvent on standard laboratory reference strains (Khatib et al., 2022). Comparing the antimicrobial activities of the three plant components of Iris, using three different solvents, and determining the potential role that solvent may have in developing methods of preparation for medicinal or anti-microbial activities demonstrates that the distribution of antimicrobial activity and active compounds varies according to the plant organ, solvent used for extraction, and whether they originate from the same plant collection. Furthermore, comparisons of the results from one commercial laboratory to another will not give comparable results; therefore, both inter-laboratory and intra-laboratory comparisons cannot be relied upon.

Comparison with other studies

For purposes of comparison, our findings with respect to the effects of methanol extracts of fifteen Iris species on oral bacterial biofilms are similar to but of less magnitude than previous studies by other researchers that showed dominance of flavonoid and other phenolic constituents (Hoang et al., 2020) as active components against bacteria and fungi using the underground portions of the plants as the starting materials and different endpoints. Crude extracts are expected to demonstrate less apparent potency than isolated compounds, and this is consistent with our findings. In agreement with the general tendency of flower extract activity against Gram-positive versus Gram-negative bacteria observed in the literature, we found that flower extracts were more active against Gram-positive bacteria due to the barriers afforded by the outer membrane of the Gram-negative bacteria (Górniak et al., 2019).

Limitations and Strengths

Several limitations restrict the interpretation of our data. The assays were performed in triplicate, and because of the wide ranges of confidence intervals, very few of the antibacterial comparisons reached significance, which suggests imprecision in the antibacterial comparisons rather than equivalence with the standard antibiotic control. The method of agar-well diffusion measures apparent activity rather than intrinsic activity. Because of the lack of determination of minimum inhibitory and minimum bactericidal concentration values, it is not possible to make a direct connection between the inhibition observed in agar well diffusion against organisms and the actual levels of antibacterial activity. All extracts were crude extracts, and although methanol and/or methanol mixed with another solvent was used, the constituents linked to activity remain unknown. The screening was a qualitative screen, and chromatographic confirmation of active constituents could not be conducted. Micromorphometric data did not contain dispersion measures, and all samples were collected from one location and during one season, which likely precludes an assessment of seasonal and/or geographical variations. All of the fungi tested were phytopathogenic fungi, not human pathogenic fungi. The strengths of the study are: All three plant materials and solvents were processed uniformly by one researcher from an authenticated, voucher-deposited collection, and all data have been completely recorded with no missing observations. In addition, null results are reported along with positive results.

Implications

In combination, these data do not support the recommendation of I. aitchisonii for the use of clinical or therapeutic purposes, nor can any inference be drawn based on in vitro inhibition (Naghavi et al., 2024; Newman and Cragg, 2020). These data may have immediate applications in the area of quality control since the data listed in Tables 2 and 3 provide a means by which powdered material in a supply chain may be authenticated in cases where adulteration is otherwise undetectable. Future studies will use bioassay-guided fractionation of the aqueous stem and flower extracts from this study as a logical next step for further examination. The primary focus of future research efforts should be the determination of minimum inhibitory concentrations against

molecularly characterised resistant clinical isolates, the establishment of chromatographic quantities of the active constituents described in Table 4, cytotoxicity testing on mammalian cells relative to the data reported for the Lemna bioassay (Table 7), and the study of seasonal and geographical variation in activity. In light of the current trend of increasing antimicrobial resistance globally and in Pakistan (Klemm et al., 2018; Naghavi et al., 2024; Newman and Cragg, 2020; WHO, 2024), it is still beneficial to have an early characterisation of previously under-researched local plant materials, provided that the limitations and/or status of any data are clearly stated.

Conclusions

This study establishes the first pharmacognostic standards for *Iris aitchisonii* and documents organ- and solvent-dependent phytochemical distribution. All extracts showed antimicrobial activity below the reference controls, alongside concentration-dependent phytotoxicity. These findings support authentication of the crude drug but not therapeutic use; bioassay-guided fractionation and minimum inhibitory concentration testing are needed before any application is considered.

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Statements and Declarations

Ethical approval and consent to participant

Not applicable.

Consent for publication

All the authors agree to publish the manuscript.

Author's Contributions

MS and IS designed the research study. MS performed the research. MS and IS provided resources for data analysis and interpretation of results., MS and IS performed software. MS, and IS investigated and performed analysis. MA and IS proofread the manuscript. All authors contributed to editorial changes in the manuscript. All authors have read and agreed to the published version of the manuscript.

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Competing Interests

The authors declare that they have no financial or none, financial competing interests related to this work...

Consent to Participate

Not applicable.

Data Availability

All data generated or analyzed during this study are included in this published article.

Declaration of Generative AI and AI-assisted Technologies in the Writing Process

During the preparation of this work, the author(s) have not used any ChatGPT (OpenAI) tool.