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ASSESSMENT OF ANTIMICROBIAL, ANTIOXIDANT ACTIVITIES AND PHYTOCHEMICAL SCREENING OF ACHYRANTHES ASPERA

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Abstract

Achyranthes aspera L. is a well-known medicinal plant widely used in traditional systems of medicine for the treatment of various infectious and oxidative stress-related disorders. The present study aimed to investigate the phytochemical composition, antioxidant potential, and antimicrobial activity of A. aspera extracts using established in vitro methods. Preliminary phytochemical screening confirmed the presence of major bioactive constituents, including tannins, alkaloids, saponins, steroids, and flavonoids, supporting its therapeutic relevance. Antioxidant activity was evaluated using the DPPH free radical scavenging assay, which demonstrated a concentration-dependent response, with maximum inhibition of approximately 85% at 100 µg/mL. Antibacterial activity assessed by the agar well diffusion method revealed notable inhibitory effects against Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, and Klebsiella aerogenes, with zones of inhibition ranging from 5 to 18 mm, depending on extract concentration. The activity was comparable to the standard antibiotic ampicillin at higher concentrations. Additionally, antifungal evaluation against Candida albicans showed moderate inhibitory activity, with inhibition zones ranging from 6 to 13 mm. All experimental data were statistically analyzed and expressed as mean ± standard deviation. The findings validate the traditional medicinal claims associated with A. aspera and highlight its potential as a natural source of antimicrobial and antioxidant agents. Further studies focusing on bioactive compound isolation and mechanistic pathways are recommended to support its pharmaceutical development.

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1. Introduction

The foundational role of medicinal plants within the healthcare systems of the world has been documented since prehistoric times, and medicinal plants continue to contribute significantly to the prevention and treatment of diseases globally. In many countries, especially developing countries, traditional medicine is still the primary access point to healthcare. Studies estimate that around 80 percent of the global population depends on traditional medicine, and therefore, on plant-based medicines, to satisfy their basic healthcare needs (Ekor, 2014). Interest in the scientific study of plants as medicine has expanded in the past few decades because of the accessibility of plants, the low costs involved, and the fewer side effects in comparison to synthetic drugs.

Antibiotic-resistant microorganisms have emerged as one of the most pressing global health problems because of the misuse and overuse of antibiotics, poor compliance by patients, suboptimal drug selection, and the indiscriminate use of antibiotics in the environment (Wilson et al., 2014). New antimicrobial agents are needed to treat infections caused by antibiotic-resistant pathogens, especially those that are derived from natural products.

The use of traditional medicinal plants for infections from bacteria, fungi, parasites, and viruses has been common for decades. Many rural and indigenous societies use and rely on herbal treatments because they are believed to work and they are culturally accepted. To incorporate herbal medicine into more modern practices and discover new medicinal compounds, the scientific proof of these practices is essential (Muthamizhe et al., 2013) .

Apamarg is the most common name for *Achyranthes aspera L.*, and is part of the Amaranthaceae family, which is composed of a variety of widely spread medicinal plants. It can be found in tropical and subtropical areas as well. It has been used in several ancient practices, including Ayurveda and a variety of folk medicine practices. It has been used to help a wide array of conditions including: asthma, cough, diabetes, inflammation, skin lesions, gastro-intestinal issues, and a variety of chronic infections. The plant has many bioactive materials including, but not limited to, alkaloids, saponins, flavonoids, phenolic compounds, and glycosides. Its bioactive materials are the reason for the variety of pharmacological benefits it has (Chakraborty et al., 2011; Srivastav et al., 2011).

Phytochemicals are bioactive, non-nutrient compounds found in plants that are involved in the plant's defense mechanisms, but also have potential beneficial health effects. These secondary metabolites include alkaloids, flavonoids, tannins, terpenoids, and saponins, and have been found to have antioxidant, antimicrobial, and anti-inflammatory and anti-cancer properties (Mumtaz et al., 2011). Antioxidants in particular are able to reduce free radicals and the oxidative damage that occurs from chronic diseases.

With the need for new sources of antioxidant and antimicrobial agents, this study will assess the phytochemical content, antimicrobial activity, and antioxidant potential of Achyranthes aspera. This study aims to assess and provide scientific evidence for the traditional uses of this species and explore the potential for developing new therapeutic alternatives from this and similar plants.

2. Materials and Methods

2.1 Study Area

The current research was carried out at the Research Laboratory, University of Agriculture, Dera Ismail Khan, Pakistan, for the period of July 2023 to March 2024.

2.2 Collection and Identification of Plant Material

Fresh *Achyranthes aspera L.* leaves were retrieved from the adjacent sugarcane fields in Bhakkar, Pakistan. The retrieved plant materials were cleaned to discard materials such as debris and soil. Taxonomic identification was carried out by Dr. Ilyas Ahmad, a lecturer in Botany at Government Degree College No. 1, Dera Ismail Khan, and based on the detailed morphological characteristics. Identification was also verified by comparison with standard herbarium specimens and appropriate botanical literature.

2.3 Preparation of Plant Extract

The plant leaves were washed with tap water and air-dried under the shade at room temperature for 7 days. The dried leaves were milled into a powder using an electric grinder, and the powder was stored in glass containers that were sealed to avoid air exposure until the powder was needed. For the extraction, 5 grams of the powdered plant material was subjected to Soxhlet extraction, where methanol of 170 ml was used as the extraction solvent. The extraction process was carried out continuously for 24 hours. The resultant extracts were filtered, dried, weighed, and stored at 4°C until future analysis.

2.4 Removal of Solvent

A rotary evaporator facilitated concentrating extracts with a water bath set to 40-45°C. This was to avoid thermal disruption of bioactive components. The extract was evaporated and methanol was removed, resulting in a concentrated crude extract, and was stored in glass containers for use in subsequent experiments.

2.5 Phytochemical Screening

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A. aspera's methanol leaf extracts were subjected to qualitative phytochemical analysis to identify secondary metabolites.

2.5.1 Test for Tannins (Ferric Chloride Test)

200 mg of the leaf extract was put in 10 ml distilled water and boiled. This was filtered, and a few drops of ferric chloride ($FeCl_3$) solution were added to the filtrate. A blue-black or greenish precipitate was indicative of a positive test.

2.5.2 Test for Alkaloids (Dragendorff's Test)

200 mg of the leaf extract was boiled in 10 ml of methanol and filtered. In the resultant solution, 1 % HCl was added, followed by 6 drops of Dragendorff's reagent. If there was a brownish-red precipitate, then the test for alkaloids was positive.

2.5.3 Frothing Test for Saponins

To test for saponins, the filtrate was mixed with 5 ml of distilled water, and then shaken for two minutes. The presence of saponins was indicated by the formation of stable froth.

2.5.4 The Test for Steroids (Liebermann–Burchard Reaction)

200 mg of each of the plant extracts was prepared in a test tube and mixed with 10 ml of chloroform. Then, a 1:1 ratio of acetic anhydride was introduced, and then, with care, concentrated sulfuric acid was added along the sides of the test tube. The formation of a blue-green ring indicated the presence of steroids.

2.5.5 The Test for Flavonoids

To the aqueous filtrate, 5 ml of diluted ammonia solution and concentrated sulfuric acid were added. The formation of yellow color was an indication of the presence of flavonoids.

2.6. Antimicrobial Activity

2.6.1 Microorganisms

Antibacterial activity of *A. aspera* leaf extract was tested on four bacterial strains, namely, *Staphylococcus aureus* (Gram-positive, NCIM 2079), *Bacillus subtilis* (Gram-positive, NCIM 2063), *Pseudomonas aeruginosa* (Gram-negative, NCIM 2036), and *Klebsiella aerogenes* (Gram-negative, NCIM 2098). In addition, for anti-fungal activity, *Candida albicans* (NCIM 3102) was used. All of the mentioned microorganisms were obtained from the institutional lab and were regularly maintained by sub-culturing on Nutrient Agar (for the bacteria) and Sabouraud Dextrose Agar (for the fungi).

2.6.2 Agar Well Diffusion Assay

To determine the possible antimicrobial effects of the extract, the Agar Well Diffusion method was used. Microbial suspensions were prepared and spread over agar plates. Using a sterile cork borer, a set of wells were created and different concentrations of the plant extract were placed in each of the wells. The plates were placed in an incubator set to 37 degrees Celsius for a period of 24 hours. The zones of inhibition that formed were measured and then compared to the inhibition zones measured for the standard antibiotic ampicillin.

2.7 Antioxidant Activity (DPPH Assay)

To assess the antioxidant potential of the leaf extract from *A. aspera*, the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was used. Various concentrations of the extract were prepared at 10, 20, 40, 60, 80, and 100 of micrograms per milliliter (µg/mL). First 1 mL of DPPH solution (0.2 mM in methanol) was used, and then added to 2 ml of each of the extract concentrations, and this mixture was incubated in the dark at 20 degrees Celsius for 40 minutes. After this the absorbance was recorded at 517 nm in a UV-Visible spectrophotometer and methanol was used to zero the spectrophotometer. (Guha et al., 2010) All experiments were performed three time.

The following formula was used to calculate the percentage of DPPH radical scavenging activity.

DPPH scavenging activity (%) = $[(A_{(C)} - A_{(t)}) / A_{(C)}] \times 100$

A subscript c refers to the absorbance of the control, and A subscript t refers to the absorbance of the test sample.

2.8 Statistical Analysis

The results of all experiments were given in terms of mean ± standard deviation (SD). The statistical analysis was performed to examine differences in the various treatments and concentrations and to evaluate the dispersion of the mean values.

3. Results

3.1 Phytochemical Screening

The methanolic leaf extract of Achyranthes aspera contains multiple secondary metabolites as revealed through qualitative analysis of phytochemicals. Some of these compounds are known to possess antimicrobial and antioxidant properties.

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Table 1: *Qualitative phytochemical screening of Achyranthes aspera Leaf extract*

Phytochemical Compound	Test Method	Observation	Presence (+) / Absence (—)
Tannins	Ferric chloride ($FeCl_3$) test	Blue-black precipitate	+
Steroids	Liebermann–Burchard reaction	Blue-green ring	+
Saponins	Frothing test	Stable foam formation	+
Flavonoids	H_2SO_4 test	Yellow coloration	+
Alkaloids	Dragendorff's test	Reddish-brown precipitate	+

The medicinal properties of *A. aspera* can be confirmed by the presence of tannins, flavonoids, saponins, and steroids. These primary phytochemicals have antimicrobial, antioxidant, and anti-inflammatory activities, which correlate with the plant's traditional uses.

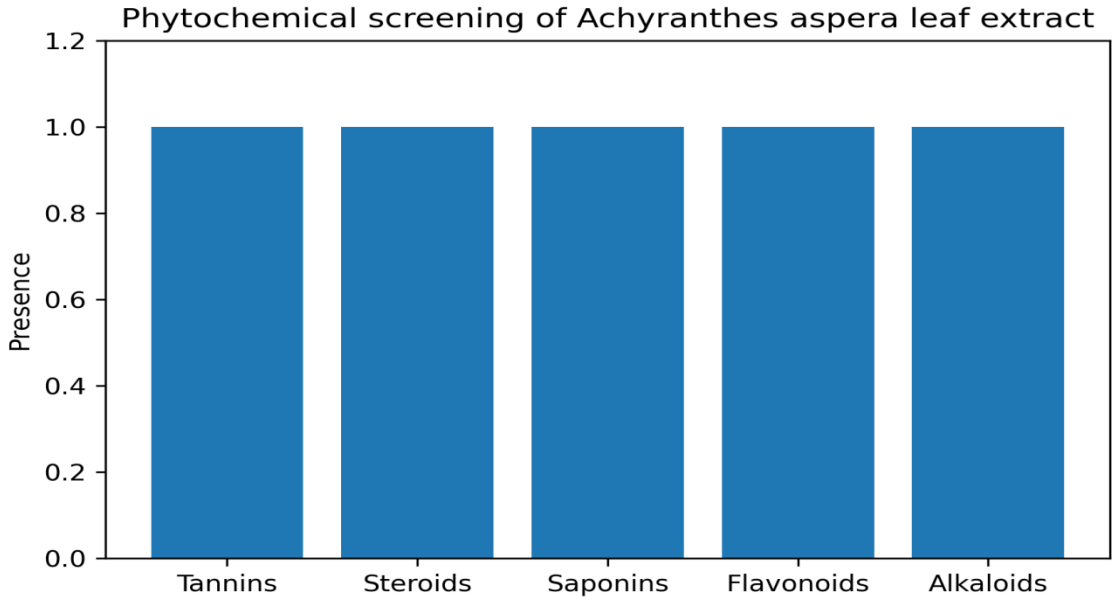

Figure.1 Phytochemical profile of Achyranthes aspera Leaf extract

3.2 Antibacterial Activity

Using the agar well diffusion method, antibacterial properties of the leaf extract of *A. aspera* were tested against four pathogenic bacterial strains. The extract showed an increase in antibacterial activity which was concentration dependent.

Table 2: *Antibacterial activity of Achyranthes aspera Leaf extract (Zone of inhibition in mm, Mean ± SD)*

Bacterial Strain	50 µL Extract	100 µL Extract	200 µL Extract	Ampicillin Control
Staphylococcus aureus	10.0 ± 0.5	15.0 ± 0.4	18.0 ± 0.3	20.0 ± 0.4
Bacillus subtilis	8.0 ± 0.5	12.0 ± 0.3	16.0 ± 0.4	19.0 ± 0.5
Pseudomonas aeruginosa	5.0 ± 0.4	8.0 ± 0.7	10.0 ± 0.5	18.0 ± 0.5
Klebsiella aerogenes	6.0 ± 0.5	9.0 ± 0.6	12.0 ± 1.0	17.0 ± 0.7

The methanolic extract of *A. aspera* showed considerable antibacterial activity against both Gram-negative and Gram-positive bacteria. The greatest inhibition was against *Staphylococcus aureus* and was also significant against *Bacillus subtilis*. Gram-negative bacteria showed much less sensitivity, possibly due to their outer protective membrane. The findings suggest antibacterial activity of the extract is dose-dependent, as higher concentrations produced inhibition zones approaching those of the standard antibiotic, ampicillin.

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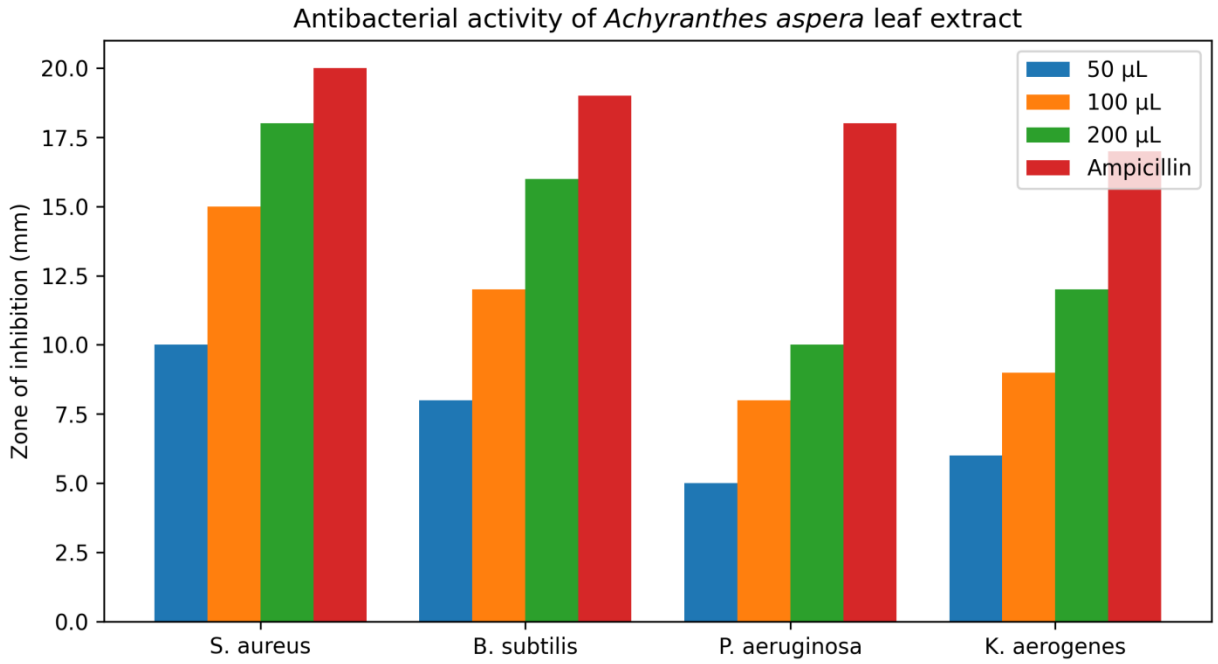

Figure 2. Antibacterial activity of Achyranthes aspera Leaf extract against selected bacterial strains.

3.3 Antifungal Activity

The extract was also tested against *Candida albicans* to assess its antifungal activity. The extract showed moderate antifungal activity, which was nonetheless significant.

Table 3: ***Antifungal activity of Achyranthes aspera Leaf extract against Candida albicans (Zone of inhibition in mm, Mean ± SD)***

Fungal Strain	50 µL Extract	100 µL Extract	200 µL Extract	Fluconazole Control
Candida albicans	7.0 ± 0.41	10.0 ± 0.82	13.0 ± 1.23	18.0 ± 0.75

The extract showed concentration-dependent activity against *Candida albicans*. The inhibitory zones in the extract were smaller than the standard antifungal drug fluconazole. The results still showed potential antifungal activity of *A. aspera* which may be due to the presence of saponins and flavonoids.

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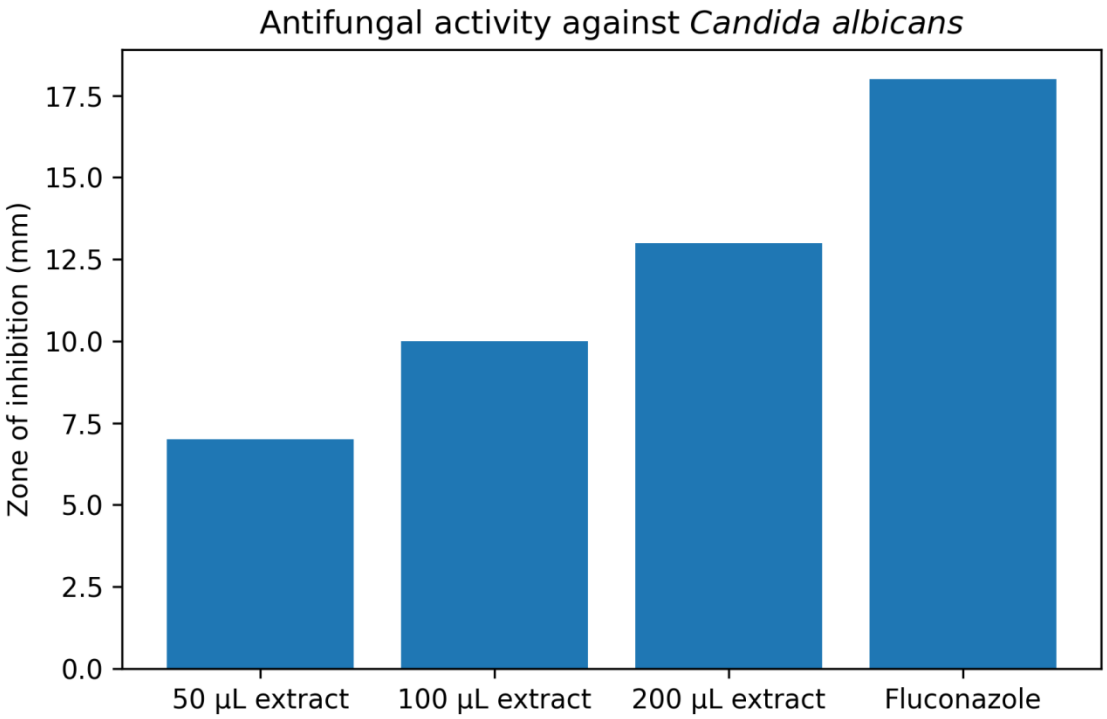

Figure 3. Antifungal activity of Achyranthes aspera Leaf extract against Candida albicans.

3.4 The Antioxidant Activity (DPPH Radical Scavenging Assay)

The DPPH free radical scavenging assay was used to determine the antioxidant potential of *A. aspera* leaf extract. A steady increase in scavenging activity was noted as extract concentration increased.

Table 4: *DPPH radical scavenging activity of Achyranthes aspera Leaf extract (Mean ± SD)*

Concentration (µg/mL)	% DPPH Scavenging Activity
10	21.0 ± 0.9
20	33.0 ± 1.1
40	47.0 ± 1.3
60	62.0 ± 1.5
80	73.0 ± 1.2
100	86.0 ± 1.0

The extract from *A. aspera* methanol shows considerable positive and proportional antioxidant activity. At 100 µg/mL, the maximum inhibition was reached at 86%, demonstrating considerable capacity to eliminate the neutral radicals. The presence of the redox active constituents such as flavonoids and tannins, as well as some other phenolic compounds, could be the reason for this activity.

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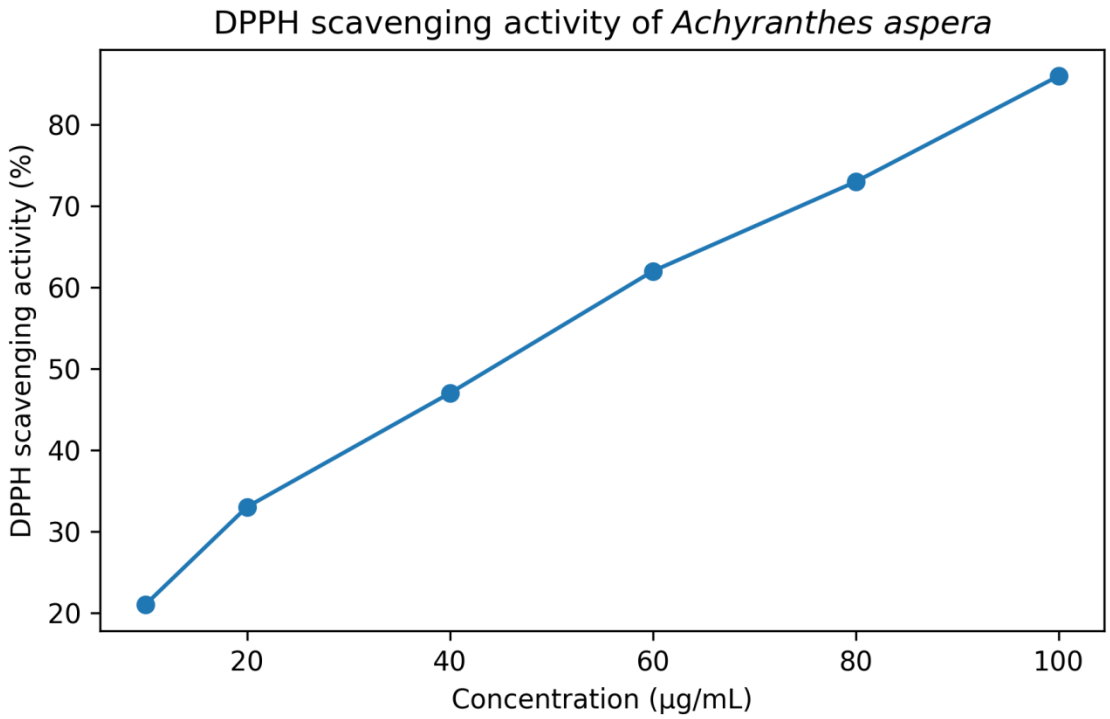

Figure 4. DPPH radical scavenging activity of Achyranthes aspera Leaf extract at different concentrations.

4. Discussion

The current research confirmed that bioactive substances such as tannins, flavonoids, saponins, and steroids, which in the case of the methanol leaf extract of Achyranthes aspera, are known to have antioxidant and antimicrobial properties. The presence of these secondary metabolites validates the findings of other studies with comparable phytochemical profiles and biological activity (Abhang et al., 2024).

4.1 Phytochemical Relevance

The presence of tannins, flavonoids and saponins in the leaf extract of *A. aspera*, confirms previous studies that these are the primary constituents of the methanolic extract of this plant. These groups of compounds are speculated to be responsible for free radicals scavenging and antimicrobial activity, as they are able to donate hydrogen and interact with the membranes of microbes (Jain et al., 2024). Such metabolites are recognized for the considerable antimicrobial properties of various perennial herbs, and justify the antioxidant use of *A. aspera* in the preparation of herbal medicines.

4.2 Antibacterial Activity

The extract of *A. aspera* showed good antibacterial activity with a relatively high zone of inhibition on *Staphylococcus aureus* and *Bacillus subtilis*. This finding reports agree with earlier reports in which methanolic extracts of *A. aspera* exhibited significant antibacterial activity against the same Gram positive bacterial strains using well diffusion and MIC methods. Gram-negative strains are particularly hard to inhibit due to the complexity of their outer membrane. Thus, our extract demonstrating activity gives credence to the possible presence of a compound in the extract that can either permeate or alter the structural integrity of the outer membrane. The increase in the inhibition zones with respect to concentration is consistent with the *A. aspera* antibacterial activity literature, and supports the concentration effect.

4.3 Antifungal Activity

The antifungal activity results are in accordance with other researchers and *A. aspera* as well as other strains reporting that antifungal activity, particularly against *Candida albicans*, is less than the antibacterial activity exhibited against the same strain. Some reports state that ethyl acetate and methanol fractions have cellular activity against fungi, particularly dermatophytes and yeasts. The activity paralleled the polarity of the extracts suggesting that in future studies, refinement of the extraction methods can further improve the antifungal activity.

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4.4 Antioxidant Properties

At the maximum concentration tested, *A. aspera* extract showed the highest DPPH inhibition and increased free radical scavenging activity. Similar strong antioxidant activity for methanolic *A. aspera* leaf extract has also been reported in peer-reviewed research. A phenolic antioxidant activity also corresponds to the antioxidant radical scavenging activity; therefore, it can be concluded that the radical scavenging activity of *A. aspera* is due to the phenolic constituents, as *A. aspera* leaf extract is justified to be an antioxidant and a likely protector against diseases resulting from oxidative stress and hyper reactive oxygen species (ROS) due to the DPPH radical scavenging activities.

5. Conclusion

This study shows the methanolic leaf extract of Achyranthes Aspera contains important secondary metabolites like tannins, saponins, flavonoids, and steroids which are linked to various biological functions. The extract showed a concentration-dependent pattern of antibacterial activity against both Gram-positive and Gram-negative bacteria. The extract showed the most activity against *Staphylococcus aureus* and *Bacillus subtilis*. The extract also showed moderate antifungal activity against *Candida albicans*. The extract showed increased DPPH radical scavenging activity with increased concentration signifying the extract contains potent antioxidants which could be due to phenolic and flavonoid constituents. The findings support the traditional use of this plant and show that the plant can be a potential natural source for antimicrobial and antioxidants. Further studies need to be done in regards to the isolation of the compounds, evaluation of toxicity and mechanism studies to evaluate the possible therapeutic uses of the extract.

6. Recommendations

The findings of this study have led to the following recommendations:

1. Upon separating and characterizing the individual bioactive compounds responsible for the biological activity, further studies may be undertaken.
2. Quantitative Phytochemical Analysis

The measurement of primary bioactive constituents and the correlation of their levels to the biological activity can be achieved through the use of sophisticated analytical tools such as High-Performance Liquid Chromatography (HPLC), Gas Chromatography-Mass Spectroscopy (GC-MS), or Liquid Chromatography-Mass Spectroscopy (LC-MS).

3. Mechanistic Studies

Other researchers may wish to study the specific antimicrobial and antioxidant mechanisms of the extracts of *A. aspera* at the molecular and cellular levels.

4. Toxicological Evaluation

Pharmaceutical and nutraceutical uses of *A. aspera* extracts may be investigated at the molecular level following studies to determine the extracts' acute and chronic toxicity.

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