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PHYTOCHEMICAL SCREENING, *IN VITRO* ANTIMICROBIAL AND CYTOTOXIC POTENTIAL OF MEDICINALLY IMPORTANT *PLANTAGO AMPLEXICAULIS*. L

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Abstract

Medicinal plants are considered as the most important source for the development and discovery of natural drugs. The present research work was carried out to investigate the phytochemical constituents, antimicrobial activity and cytotoxic activity of the methanolic and ether extracts of *Plantago amplexicaulis* L. The phytochemical screening revealed that the ether extract was rich in terpenoids, flavonoids, tannins, proteins, and saponins whereas the methanolic extract of *P. amplexicaulis* was rich in terpenoids, flavonoids, phytosterols, tannins, proteins, phenols, and saponins. The antibacterial activity of methanolic extract of P. amplexicaulis had maximum inhibitory zone of 29 mm against Klebsiella pneumonia with 30 mg/mL concentration and the minimum inhibitory zone was recorded as 13 mm at concentration of 10mg/mL against Staphylococcus aureus with maximum MIC value of 8mm against Escherichia coli. The ether extract of P. amplexicaulis had maximum inhibitory zone of 27 mm against K. pneumonia with 30 mg/ml of extract while the minimum zone of inhibition was 12 mm against Shigella flexneri at 10 mg/ml concentration with maximum MIC value of 8mm against *S. flexneri*. 30 mg/ml of methanolic extract in antifungal activity showed maximum zone of inhibition of 25 mm against *Aspergillus fumigatus* and the minimum zone of inhibition was 8 mm against *Fusarium solani* at concentration of 10 mg/ml with maximum MIC value of 8 mm against *F. solani*. 30 mg/ml of ether extract showed maximum zone of inhibition of 24 mm against *F. solani* while the minimum inhibition zone was 7 mm against *A. fumigatus* at concentration of 10 mg/ml with highest MIC value of 9 mm against *A. fumigatus*. 1000 mg/ml of methanol extract showed high mortality rate (293.33%) of brine shrimps while shown lowest (26.66) at 10 mg/ml. Similarly, 10 mg/ml of ether extract revealed high mortality rate (253.33%). The plant was found a potent therapeutic agent against microbial infections and highly cytotoxic.

Key words: Secondry metabolites; Biological activities; *Plantago amplexicaulis*

Introduction

Medicinal plants are the richest source of bioactive compounds which can be classified into different categories such as volatile oils, terpenoids, aromatic compounds, alkaloids, anthocyanin, flavonoids, tannins, sterols and phenols. Due to these chemical compounds, plants are therapeutic in nature and used for treatments of many disorders (Sharifi *et al*., 2022). Aromatic medicinal plants are extensively utilized across diverse sectors including food, perfumery, textiles, and pharmaceuticals. These plants possess medicinal attributes owing to the existence of bioactive elements like saponins, glycosides, quinones, alkaloids, and flavonoids (Ambrin *et al*., 2024). For the detection of these bioactive compounds, phytochemical screening is carried out which is an important tool for the identification of many chemical compounds and secondary metabolites that are present in plants (Adil *et al*., 2024).

As plants contains medicinally important phytochemicals, the use of folk medicines has always instructed the researchers to utilize medicinal plants as herbal remedies against serious health issues such as microbial infections in order to avoid the adverse effects of synthetic drugs. Medicinal herbs are said to be the most important source for the development and discovery of natural drugs. Since the time immemorial, medicinal plants are used as alternative to chemo drugs due to their significant and extraordinary biological potential. However, the potential and mode of action of medicinal plants in several aspects has not yet been investigated, despite the fact that their pharmaceutical formulations behave favorably due to their bioactive compounds (Arafa *et al*., 2022).

Medicinal plants and their secondary metabolites are nowadays being utilized as alternative source for the development of new antibiotics (Masood *et al*., 2023). Antimicrobial medications are commonly used in the treatment of infections but these synthetic medications have a lot of adverse effects on human health. Recently, antibiotics resistance has been increasing which leads to various complicated health problems. Plants are the natural source of several bioactive compounds and antibiotic resistance inhibitors that can be used to reduce antimicrobial drug resistance (Adil *et al*., 2020). Microbial infections are the most alarming health problems which has attracted significant attention of people due to their harmful effects on health and also damages economy of a country. World health organization has reported the serious health problems related to bacterial attack such as permanent rise in multidrug resistance by bacteria, mutation in bacterial strains, deficiency or lack of proper vaccinations in underdeveloped countries, limited antibiotics discovery and prevalence of bacterial infections (Okeke *et al*., 2022).

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Beside animal's diseases important plants, fruits and vegetables which are crucial parts of a healthy diet are badly affected by plant pathogens. *Aspergillus niger* and *A. flavus* are particularly harmful to various crops such as melons, citrus, pears, apples, strawberries, peaches, corn, tomatoes, grapes, figs, and mangoes as well as other fruits and vegetables because they are typically acidic and thus resistant to invasion by microbes and pathogens (Witasari *et al*., 2022).

In the last few decades, pre-clinical cytotoxicity testing has been based on cell-based assays for drug efficacy testing. *In vitro* cytotoxicity assays (CTAs) evaluate the harmful potential of chemical and natural materials in cell culture models. Plant extracts are examined for their capacity to alter cell viability, cellular growth, and cell damage (Gavanji *et al*., 2023).

P. amplexicaulis L. belongs to the family Plantaginaceae and is commonly known as Spighwol. *P. amplexicaulis* is an annual, mostly acaulescent herb that grows up to two feet and four inches per year. This species is anemophilous, distributed in Darra Adam Khel, KPK Pakistan, and also in, Algeria, Tunisia, Egypt. Arabia, Iran, and Western India (Diaz *et al.,* 2009). *Plantago amplexicaulis* is traditionally used for the treatment of intestinal disorders and as a demulcent in cases of dysentery. Traditionally, the husk of the plant is dissolved in sugar syrup for the treatment of diarrhea. Husk of *P. amplexicaulis* is also used by local people for treating urinary infections, gastric and intestinal inflammation, piles and ulcers (Bencheikh *et al.,* 2024). They are said to be useful in the treatment of intermittent fevers and pulmonary affections.

The present study is aimed to investigate various bioactive compounds, antimicrobial activity and cytotoxic activity of medicinally important *P. amplexicaulis* L. These studies have generally yielded important information for estimating biological potential and developing structure-activity correlations (SAR). These tests are easy to use, affordable, and trustworthy. Clinical trials are costly even though monolayer cultures are the best option for screening and evaluating the efficacy of drugs and for gathering information on the cytotoxicity and biological potential of constituents before conducting *In vivo* investigations.

Materials and Method

Collection of plant: The green plant Plantago amplexicaulis L. was collected from Darra Adam Khel, Pakistan. The plant was identified with the help of the Flora of Pakistan and available literature. The voucher specimen was assigned voucher number Kiran 002 (QUSIT), and deposited in the Herbarium of Qurtuba University of Science and Information Technology, Peshawar, Pakistan.

Extract preparation: The plant sample was shade-dried at normal temperature, ground into a fine powder, and 50 grams of powder were soaked in 200 milliliters of solvent. The powder was stored at room temperature for two weeks and was shaken each day. The impregnated plant material was first filtered through a plain filter and then through Whatman's filter paper 41 (fast filter paper 20 µm). The solvent was completely vaporized by a rotary evaporator under vacuum to obtain the crude extracts. The filtrates acquired were kept in a refrigerator at 4°C (Choudhary & Sekhon, 2011).

Preliminary phytochemical qualitative tests

Test for alkaloids detection: For the detection of alkaloids, 3 mL of ether extract were added to the test sample. After few minutes, red color precipitates were formed indicating the presence of alkaloids while methanolic extract was not involved in the formation of red color precipitates (Joshi *et al*., 2013).

Test for the presence of flavonoids: Flavonoids were tested by the addition methanol and ether extracts to test samples. The formation of Yellow-red precipitates indicated the existence of flavonoids (Stankovic *et al*., 2011).

Test for phenols detection: To test phenols, 3 to 4 drops of extract were added in methanolic extract due to which bluish black color was appeared which indicated the presence of phenols while the same was not formed by the addition of ether extract which reflected the absence of flavonoids (Chlopicka *et al*., 2012).

Test for the presence of proteins: For proteins tests, the methanol and ether extracts were mixed with test samples in a test tube. As a result, violet color was appeared which indicated the presence of proteins (Kalita *et al*., 2013).

Test for saponins detection: The plant extract was mixed with 20mL of distilled water and shaken for 15 minutes. After that, about 1cm of foam was formed in test tube which shown the presence of saponins (Banso & Adeymo, 2006).

Test for tannin detection: One % of NaCl solution containing gelatin solution was added to the extract, resulting in the formation of white-colored precipitates, which indicated the presence of tannins (Banso & Adeymo, 2006).

Check for terpenoids and phytosterols: Three drops of H_2SO_4 and chloroform were dissolved in the extract. The appearance of red color indicated the existence of terpenoids while yellow color indicated the presence of phytosterols (Ayoola *et al*., 2008).

Biochemical activity

Antifungal activity: For antifungal activity, agar tube reduction method was used to test the antifungal potential of ether, and methanolic extract of *P. amplexicaulis* (Adil *et al*., 2020). Four fungal strains including *F. solanum*, *A. niger, A. fumigatus,* and *P. notatum* were taken from pathology department at the Agriculture University, Peshawar. Sabouraud dextrose agar (SDA) media was used for fungal culture. The stock solution was formed by melting 1 mL of sterile DMSO with about 24 µg of extract per (Dimethlysulphoxide).

Bacterial resistance: The bacteria were stimulated after pouring the nutrient broth into conical flasks and the standard was raised for 24 hours. The agar media was then transmitted in Petri dishes consisting bacteria that had been injected. The media was allowed to solidify under normal conditions. After solidification, cork borer was used to organize the bores in the agar plate.

Cytotoxic activity: Utilizing the brine shrimp assay, it was determined the methanolic and ether extract of *P. amplexicaulis* L. cytotoxic potential by using the following procedure of (Fitri *et al*., 2023).

Artemia salina egg hatching procedures: 38g/L of filtered sea water solution was spread over the 2 parts of 18 to 22 cm long dish. 20 to 25 mg of Artemia salina eggs were dispersed on one part of dish and covered with a black paper while the other part was not covered. At room temperature, the brine shrimp's hatching dish was used to produce eggs. The light lamp was used to illuminate the hatching dish. The movement of brine shrimps nauplii across the perforated barrier from the hidden to the lit portion was examined after 48 hours.

Results

Qualitative phytochemical analysis: The qualitative analysis illustrated that methanol and ether extracts of *Plantago amplexicaulis* were rich in various phytochemicals, including flavonoids, saponins, phytosterols, proteins, tannins, phenols, and terpenoids. It was also observed that the methanolic extract lacked alkaloids, while phenols and phytosterols were not identified in the ether extract of *Plantago amplexicaulis* (Table 1).

Table 1. Qualitative phytochemical screening of *Plantago amplexicaulis*.

Chemical constituents	Methanolic extract	Ether extract
Flavonoids	+	+
Alkaloids	–	+
Phytosterol	+	–
Phenols	+	–
Terpenoids	+	+
Tannins	+	+
Saponins	+	+
Proteins	+	+

Antibacterial activity: The antibacterial activity revealed that 30 mg/ml methanolic extract of *P. amplexicaulis* had maximum inhibitory zone of 29 mm against *K. pneumonia*. Likewise, the minimum inhibitory zone was recorded as 13 mm at concentration of 10 mg/mL against *S. aureus*. In contrast, the ether extract of *P. amplexicaulis* demonstrated the maximum inhibitory zone of 27 mm against *K. pneumonia* with 30 mg/ml of extract while the minimum zone of inhibition was (12mm) against *S. flexneri* at 10 mg/ml concentration of extract (Table 2; Figs. 1 and 2).

Antibacterial activity MIC values: The MIC values recorded for antibacterial activity revealed that methanolic extract of *P. amplexicaulis* had maximum MIC value of 8mm against *E. coli* while the minimum MIC value of 2 mm was observed against *S. aureus* Rosenbach. As well as the ether extract of *P. amplexicaulis* exhibited the highest value of 8mm against *S. flexneri* while the minimum MIC value against *E. coli* was recorded as 2 mm (Table 3).

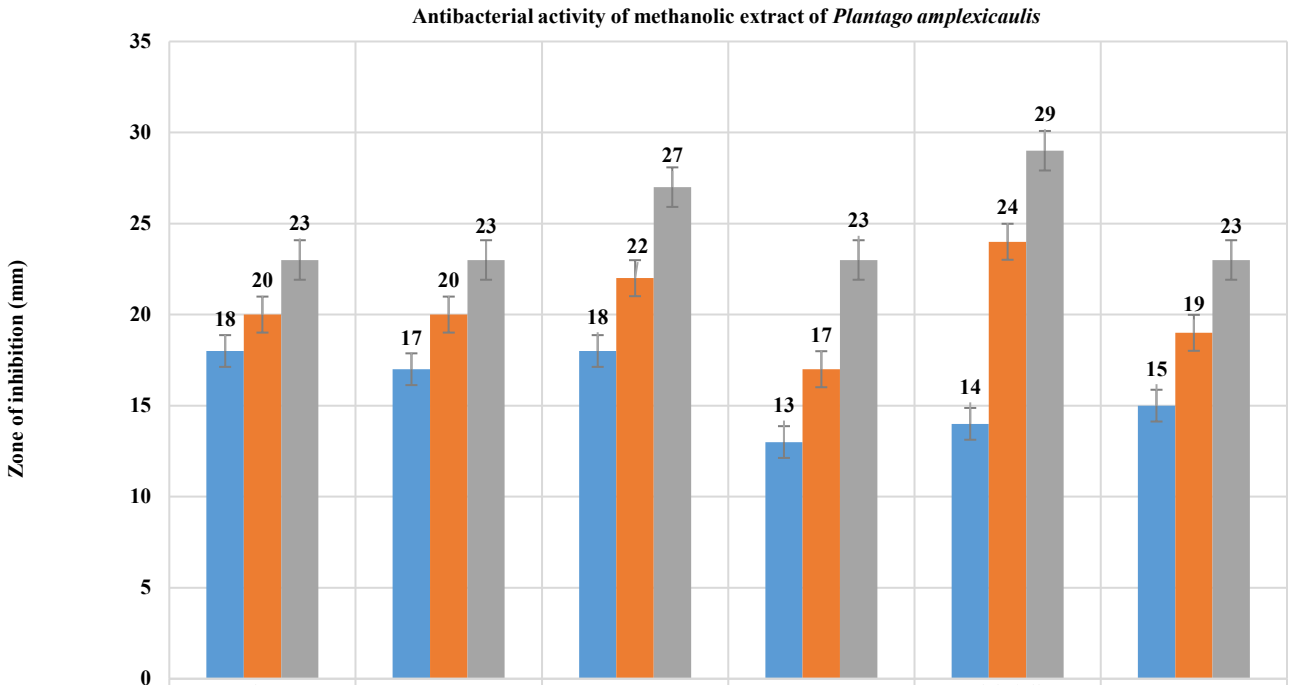
Antifungal activity: The methanolic extract of *P. amplexicaulis* L. in antifungal activity showed maximum zone of inhibition of 25 mm against *A. fumigatus* with 30mg/ml concentration of extract. In the same way, the minimum zone of inhibition was (8mm) against *F. solani* at concentration of 10 mg/ml. The highest zone of inhibition of 24 mm was shown by 30 mg/ml of ether extract of *P. amplexicaulis* against *F. solani*. Similarly, the lowest inhibition zone was 7 mm against *A. fumigatus* at concentration of 10 mg/ml, (Table 4; Figs. 3 and 4).

Antifungal activity MIC values: For antifungal activity, the MIC values of methanolic extract of *P. amplexicaulis* had significant MIC value of 8 mm against *F. solani* and the lowest MIC value against *P. notatum* was reported as 2 mm. Similarly, the highest MIC value of 9 mm against *A. fumigatus* was shown in ether extract of *P. amplexicaulis* and the lowest MIC value against *F. solani* was observed as 2 mm., respectively (Table 5).

Cytotoxic activity: The two different extracts of *Plantago amplexicaulis* L. were investigated for their cytotoxic potential to kill brine shrimp napulii. 1000 mg/ml of dose of methanolic extract showed the highest (293.33%) death rate of brine shrimps. 10 mg/ml of methanolic extract validated the lowest mortality rate (26.66%). Similarly, ether extract of *P. amplexicaulis* revealed strong cytotoxic potential with high mortality rate of 253.33 at concentration of 10 mg/ml. Thus, this plant was reported as a potent therapeutic agent having strong antimicrobial and cytotoxic potential, respectively (Table 6).

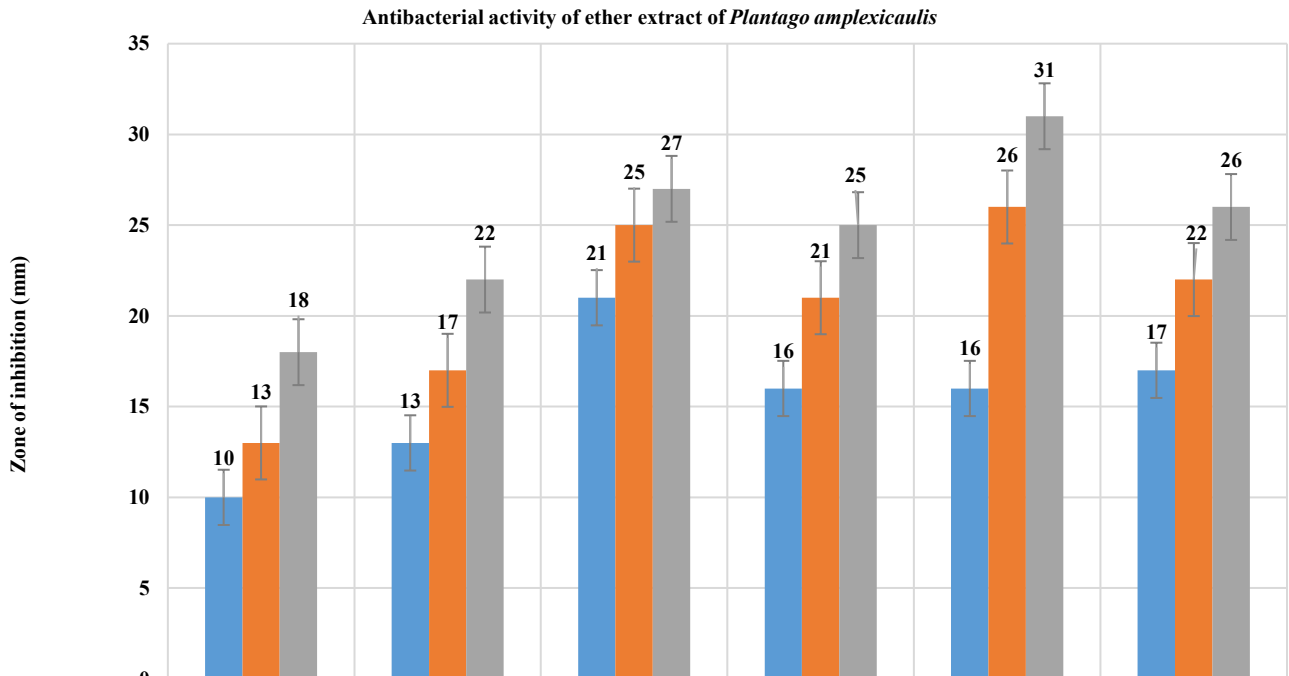
Table 2. Antibacterial activity of methanolic and ether extracts of *Plantago amplexicaulis* L.

Bacteria	Zone of inhibition (mm)							
	+ve Control	_ve Control	Methanolic extract Mg/ml			Ether extract Mg/ml		
			10	20	30	10	20	30
Pseudomonas aeruginosa	30	0	18	20	23	10	13	18
Escherichia coli	26	0	17	20	23	13	17	22
staphylococcus epidermidis	31	0	18	22	27	21	25	27
Staphylococcus aureus	28	0	13	17	23	16	21	25
Klebsiella pneumonias	33	0	14	24	29	16	26	31
Salmonella typhi	27	0	15	19	23	17	22	26
Shigella flexneri	30	0	19	26	28	12	21	27



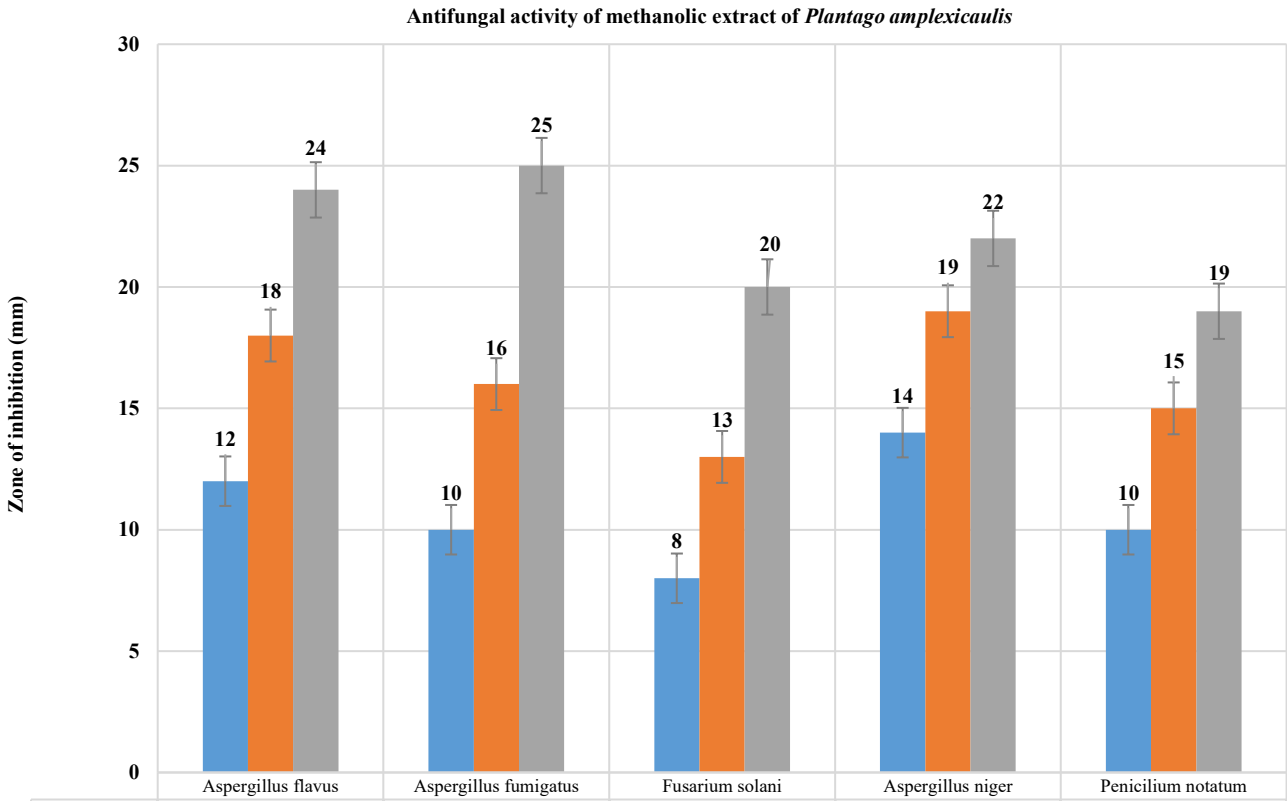
	Pseudomonas aeraginose (Schroeter) Migula	Escherichia coli	Staphylococcu epidermidis Rosenbach	Staphylococcus aureus Rosenbach	Klebsiella pneumonia (Schroeter) Trevisan	Salmonella typhi (schroeter) warren et scott
10mg/ml 10	18	17	18	13	14	15
20mg/ ml 13	20	20	22	17	24	19
30mg/ ml 13	23	23	27	23	29	23

Fig. 1. Antibacterial activity of methanolic extract of *Plantago amplexicaulis*.



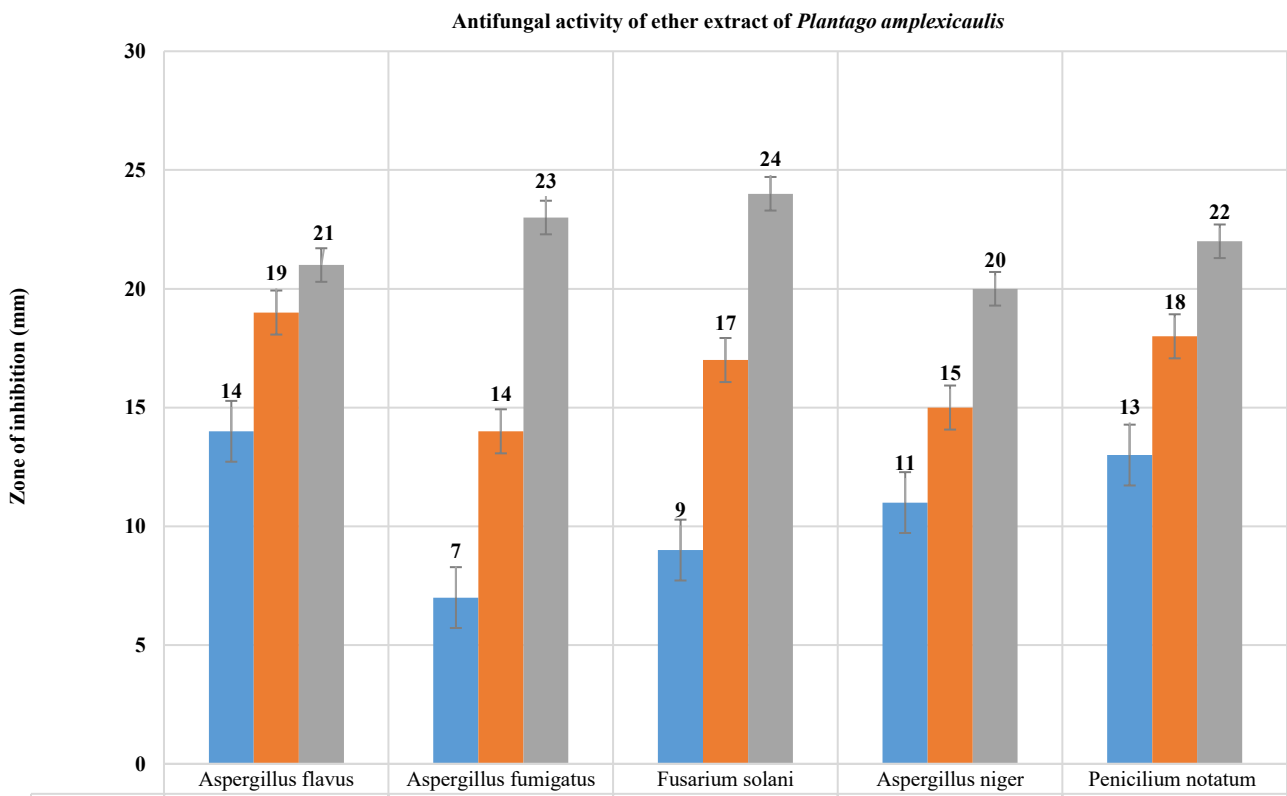
	pseudomonas aeraginosa (Schroeter) Migula	Escherichia coli	Staphylococcu epidermidis Rosenbach	Staphylococcu aureus Rosenbach	Klebsiella pneumonia (Schtroeter) Trevisan	Salmonella typhi (Schroeter) Warren et Scott
10mg/ml 10	10	13	21	16	16	17
20mg/ml 13	13	17	25	21	26	22
30mg/ml 13	18	22	27	25	31	26

Fig. 2. Antibacterial activity of ether extract of *Plantago amplexicaulis*.



	Aspergillus flavus	Aspergillus fumigatus	Fusarium solani	Aspergillus niger	Penicilium notatum
10 mg/ml 10	12	10	8	14	10
20mg/ml 13	18	16	13	19	15
30mg/ml 13	24	25	20	22	19

Fig. 3. Antifungal activity of methanolic extract of *Plantago amplexicaulis*.



	Aspergillus flavus	Aspergillus fumigatus	Fusarium solani	Aspergillus niger	Penicilium notatum
10mg/ml 10	14	7	9	11	13
20mg/ml 13	19	14	17	15	18
30mg/ml 18	21	23	24	20	22

Fig. 4. Antifungal activity of ether extract of *Plantago amplexicaulis*.

Table 4. Antifungal activity of methanolic and ether extract of *Plantago amplexicaulis* L.

Fungi	Zone of inhibition (mm)							
	+ve Control	_ve Control	Methanolic extract (Mg/ml)			Ether extract (Mg/ml)		
			10	20	30	10	20	30
Penicilium notatum	26	0	10	15	19	13	18	22
Aspergillus niger	31	0	14	19	22	11	15	20
Fusarium solani	28	0	8	13	20	9	17	24
Aspergillus fumigatus	30	0	10	16	25	7	14	23
Aspergillus flavus	34	0	12	18	24	14	19	21

Table 3. Antibacterial activity MIC values.

***Plantago amplexicaulis* L.**		
MIC (mg/ml) value		
Test for organisms	**Methanolic extract (mg/ml)**	**Ether extract (Mg/ml)**
Escherichia coli	8	2
Pseudomonas aeruginosa	6	4
Staphylococcus epidermidis	4	6
Staphylococcus aureus	2	4
Klebsilla pneumonia	3	6
Salmonella typhi	4	3
Shigella flexneri	3	8

Table 5. Antifungal activity MIC values.

MIC values (mg/ml)		
Fungi	**Methanolic extract**	**Ether extract**
Penicilium notatum	2	6
Aspergillus niger	6	7
Fusarium solani	8	2
Aspergillus fumigatus	3	8
Aspergillus flavus	5	9

Table 6. Cytotoxic activity of methanolic and ether extract of *Plantago amplexicaulis*.

Extracts	Extracts (mg/ml)	Total no. of larvae	No. of survival larvae	No. of death of larvae	% Mortality
Control		30	30	0	0
	10	30	22	08	26.66
Methanolic extract	100	30	18	12	273.33
	1000	30	12	18	293.33
	10	30	24	06	253.33
Ether extract	100	30	16	14	46.66
	1000	30	10	20	66.66

Discussion

Many plant extracts contain bioactive compounds such as saponins, alkaloids, and essential oils that can disrupt bacterial cell membranes. This disruption leads to leakage of cellular contents and ultimately cell death (Zengin *et al*., 2020). For instance, essential oils like tea tree oil disrupt bacterial membranes, leading to antimicrobial effects (Arif *et al*., 2023). Some plant extracts can induce the production of reactive oxygen species within bacterial cells, leading to oxidative damage and cell death. Polyphenolic compounds like tannins can exhibit this mechanism (Adil *et al*., 2024; Naseer *et al*., 2025). Because of their unique characteristics, they have found various applications such as combating bacteria, being utilized in industrial, household, and healthcare items, incorporated into consumer products, medical device coatings, and optical sensors, as well as being used in cosmetics, pharmaceuticals, and the food industry (Rehman *et al*., 2023). Additionally, they have been employed in diagnostics, orthopedic treatments, drug delivery systems, and exhibit promising potential as anticancer agents, augmenting the efficacy of cancer-fighting drugs (Salam *et al*., 2023). *P. amplexicaulis* L. was found to have antibacterial potential and showed high zone of inhibition, against *K. pneumonia* (Schroeter) Trevisan. Munshi *et al*., (2022), studied *Boerhaavia diffusa* and reported similar antibacterial activity against *S. typhi*, *K. pneumonia* which was in line with the findings of *P. amplexicaulis*. Ether extract demonstrated the maximum inhibitory zone against *K. pneumonia* which showed similarity with the findings (effectivity of *Conyza bonariensis* against *S. aureus*) of Philip *et al*., (2022). The ether extract of *P. amplexicaulis* was more effective against *K. pneumonia*, however the plant's methanolic extract showed high zone of inhibition against *K. pneumonia*. These observations are in agreement with the findings of Taufik *et al*., (2022) who reported maximum zone of inhibition against *K. Pneumonia*. and *E. coli*. According to the antifungal activity, a 30 mm concentration of *P. amplexicaulis* L. methanolic extract demonstrates the highest zone of inhibition against *A. fumigatus*. These results were in accordance with the findings of Barimani *et al*., (2020) who reported that *Raphanus sativus* was highly effective against *A. fumigations*. Similarly, ether extract of *P. amplexicaulis* showed that *F. solani* exhibited the highest zone of inhibition at a concentration of 30 mm. It coincided with Adil *et al*., (2020). The ether extract obtained from

Murraya koenigii exhibited the highest level of growth inhibition against *P. notatum*. According to the antifungal activity, 30 mm concentration of *P. amplexicaulis* methanolic extract showed the highest zone of inhibition against *A. fumigatus*. Barimani *et al*., (2020) reported of strong antifungal efficacy of *Raphanus sativus* against *A. fumigations*. Similar to this, an ether extract of *P. amplexicaulis* showed that *F. solani* exhibited the highest zone of inhibition at a concentration of 30 mm. According to Rahman *et al*., (2022), *P. amplexicaulis* exhibited cytotoxic activity, indicating that both its methanolic and ether extracts were effective against brine shrimp. Additionally, the ether extract of *Murraya koenigii* showed maximal inhibition of *P. notatum* growth. These results were in line with those of (Jakovljevic *et al*., 2020), who tested brine shrimp and noted comparable results.

Conclusion

The current study revealed that *Plantago amplexicaulis* L., has medicinally important bioactive constituents including flavonoids, alkaloids, and tannins, among others, that can be used to cure infectious disorders and shield the host from microbial illnesses. The study also indicates the significant results of the crude extract against multidrug resistant (MDR) bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*. In this regard, the plant is highly medicinal and can be used for future drug development.

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