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Antibacterial Potential of Moringa oleifera Extracts against MDR Enterobacteriaceae from Hospitalized Patients in Mardan, Pakistan

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Abstract

The escalating resistance of bacterial pathogens to multiple classes of antibiotics poses a major public health threat. This study evaluated the antibacterial potential of *Moringa oleifera* leaf and seed extracts against multidrug-resistant (MDR) strains of *Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella typhi* isolated from hospitalized patients at Mardan Medical Complex, Pakistan. Methanol and ethanol extracts were prepared from leaves and seeds, and their antibacterial activity was assessed using the agar well diffusion method. All extracts demonstrated inhibitory effects against the tested isolates, with methanolic leaf extracts showing the highest antibacterial activity. The crude extracts were further fractionated using high-performance liquid chromatography (HPLC), and the antibacterial activity of individual fractions was evaluated. Several fractions exhibited strong inhibition zones, particularly those derived from methanol leaf extracts. The findings highlight the potential of *M. oleifera*, especially its leaves, as a natural source of bioactive compounds effective against MDR Enterobacteriaceae. Further phytochemical characterization and in vivo studies are recommended to develop novel plant-based antimicrobial therapies.

Keywords: *Moringa oleifera*, multidrug resistance, Enterobacteriaceae, antibacterial activity, HPLC, plant extract

Introduction

The global rise in antimicrobial resistance (AMR) has emerged as a major public health crisis, threatening the effective prevention and treatment of a growing range of infections caused by bacteria, parasites, viruses, and fungi. According to the World Health Organization (WHO), AMR is responsible for nearly 1.27 million deaths annually and is projected to claim up to 10 million lives per year by 2050 if current trends continue unchecked (Paneri & Sevta, 2023). Among the most concerning resistant pathogens are members of the *Enterobacteriaceae* family, including *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter* spp., which are frequently implicated in hospital- and community-acquired infections and exhibit resistance to multiple antibiotic classes(DD Pitout, 2013; Veening & Tamayo, 2018). *Enterobacteriaceae* are common inhabitants of the gastrointestinal tract but are also opportunistic pathogens capable of causing urinary tract infections, bacteremia,

pneumonia, and soft tissue infections(NWOKOLO, 2023; Tamma et al., 2021). The widespread and often indiscriminate use of antibiotics in healthcare and agriculture has accelerated the emergence and dissemination of multidrug-resistant (MDR) strains Of particular concern are extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae*, which confer resistance to a wide range of β-lactam antibiotics, including penicillin and third-generation cephalosporins(Fatima et al., 2023; Rawat & Nair, 2010). The growing prevalence of such strains underscores the urgent need for novel and effective antimicrobial agents. In this context, medicinal plants have gained attention as promising alternatives to synthetic antibiotics(Bashir et al., 2025). Many are rich in bioactive phytochemicals such as alkaloids, tannins, flavonoids, saponins, and phenolic compounds, which exhibit antimicrobial, antioxidant, and anti-inflammatory properties(Cowan, 1999; Doughari et al., 2009). *Moringa oleifera*, commonly known as the drumstick tree, is one such plant with diverse ethnopharmacological uses across Asia and Africa(Klimek-Szczykutowicz et al., 2024). Various parts of the plant particularly the leaves have been used traditionally to treat infections, gastrointestinal disorders, and inflammatory conditions(Fahey, 2005). Recent scientific studies have confirmed the antimicrobial potential of *M. oleifera* leaf extracts against a broad spectrum of bacterial pathogens, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *E. coli*(Anwar et al., 2007; Rahman et al., 2009). However, there is limited research focusing on the efficacy of *M. oleifera* leaf extract against MDR *Enterobacteriaceae* isolates, especially those obtained from clinical settings in low- and middle-income countries. This study, therefore, aims to evaluate the antibacterial activity of *Moringa oleifera* leaf extract against multidrug-resistant *Enterobacteriaceae* isolates. The research seeks to contribute to the global search for alternative antimicrobial therapies and to validate the therapeutic potential of *M. oleifera* as a bioresource for new drug development, especially in settings where antibiotic resistance is widespread, and access to advanced therapeutics is limited.

Methodology

Bacterial Isolates

Multidrug-resistant clinical isolates of *Escherichia coli*, *Salmonella typhi*, and *Klebsiella pneumoniae* were obtained from the culture lab of the Pathology Department at Mardan

Medical Complex (MMC). These isolates were preserved in 50% glycerol stocks at -20°C for further use.

Bacterial Culturing and Identification

Isolates were revived by inoculating into Luria-Bertani (LB) broth and incubated at 37°C for 24 hours. Subculturing was performed on nutrient agar plates for colony isolation. Bacteria were identified based on colony morphology and confirmed by Gram staining.

Plant Material Collection and Preparation

Moringa oleifera leaves and seeds were collected from the Mardan region and authenticated by the Department of Botany, Abdul Wali Khan University. The plant parts were shade-dried at room temperature for four weeks and ground into fine powder using a mechanical grinder.

Extraction of Plant Compounds

Eighty grams of dried leaf and seed powder were separately macerated in 400 mL of methanol and ethanol. Samples were incubated at 30°C for seven days in a shaking incubator. Extracts were filtered through Whatman No.1 filter paper and concentrated using a rotary evaporator at 40°C. The semi-solid crude extracts were stored at 4°C.

Antibiotic Susceptibility Testing

Antibiotic resistance profiling was performed using the Kirby-Bauer disc diffusion method on Mueller-Hinton Agar, following CLSI guidelines. Antibiotics tested included amoxicillin-clavulanic acid, ceftazidime, imipenem, fosfomycin, cefoperazone-sulbactam, and polymyxin B. Zones of inhibition were measured and interpreted as per CLSI standards.

Antibacterial Activity of Moringa Extracts

Antibacterial activity of the crude extracts was evaluated using the agar well diffusion method. Bacterial lawns were prepared on nutrient agar plates, and 100 µL of each extract (dissolved in DMSO) was introduced into wells. Plates were incubated at 37°C for 24 hours, and inhibition zones were measured in millimeters.

HPLC Fractionation of Plant Extracts

High-performance liquid chromatography (HPLC) was employed to fractionate the methanol and ethanol extracts. One gram of crude extract was dissolved in 10 mL of 60% methanol, centrifuged, and filtered. The filtrate was injected into a C-18 column, and peaks were recorded. Fractions were collected and tested individually for antibacterial activity using the well diffusion method.

Results

Antibiotics susceptibility of isolates of bacteria

Enterobacteriaceae were clinically isolated from different culture samples for confirming the multidrug resistant bacteria. 9 different classes of antibiotics were tested against various isolates of *Enterobacteriaceae* maximum no of antibiotics show resistant to collected culture and minimum of them show sensitive to isolates show in following table 1.

Table 1: Antibiotics Susceptibility of culturing bacteria

S. No	Antibiotics	Symbol of Antibiotics	*Escherichia coli*	*Klebsiella pneumonia*	*Salmonella typhi*
1.	Nitrofurantoin	NFT	Sensitive	Resistant	**Resistance**
2.	Sulbactam-Cefoperazone	SCF	Resistance	Sensitive	**Sensitive**
3.	Ceftazidime	CAZ	Resistance	Resistance	**Sensitive**
4.	Amoxicillin-Clavulanic acid	AMC	Resistance	Resistance	**Resistance**
5.	Piperacillin-Tazobactam	TZP	Resistance	Resistant	**Sensitive**
6.	Piperacillin	PIP	Resistance	Resistant	**Resistance**
7.	Meropenem	MEM	Sensitive	Sensitive	**Sensitive**
8.	Fosfomycin	FOS	Sensitive	Sensitive	**Resistance**
9.	**Amikacin**	**AK**	**Sensitive**	**Sensitive**	**Resistance**

Moringa oleifera plant extracts activity against *Enterobacteriaceae*

Moringa oleifera plant (leaves and seeds) were collected from the local area known for being a medicinal property with the help of herbal medicine experts. Plant parts (leaves and seeds) were placed to dry at room temperature, (25 ^{0}C). Plant parts were crushed manually to make powder. Plant crude extract was obtained by soaking plant powder in two solvents i-e., ethanol, and methanol. Different concentrations of plant parts extract were dissolved in DMSO to prepare the stock solution for antimicrobial activity by using a well diffusion

method. Then the 7-day-old cultured *Enterobacteriaceae* spread on agar plate through spreader, incubating the culture for 24 hours at 37 ^{0}C. Zones of inhibition were noted which caused by different plant part extract. Leaves of *Moringa* showed maximum antibacterial activity with 18mm Zone of Inhibition against *E. coli*, followed by *K. pneumonia* 15mm and *S. typhi* was 14mm Zone of Inhibition at 50µl, with ethanol-based extract, while methanol-based extracts show 16mm against *E. coli* followed by *K. pneumonia* with 14mm and *S. typhi* with 18mm. Seed of *Moringa oleifera* displayed the maximum zone of inhibition in ethanol-based extract against *E. coli* strain with 10mm followed by *K. pneumonia* with 10mm and *S. typhi* 16mm zone of inhibition. In methanol extract the maximum sensitivity was recorded against *E. coli* with 13mm, followed by *k. pneumonia* 16mm and *S. typhi* 18mm zone of inhibition at 50µL of concentration show in table 2 and figure 1. With the concentration of 100µL, leaves part of *Moringa* at ethanol extract revealed the maximum sensitivity which was against E. *coli, k. pneumonia* and *S. typhi* with19mm, 16mm, and 16mm zone of inhibition. While 17mm, 16mm and 19mm zones of inhibition were recorded against *E. coli, k. pneumonia* and *S. typhi* with 100µL concentration of *Moringa* leaves in methanol-based extract, showed in table 3 and figure 1 respectively.

Table 2: Antimicrobial profile of *Moringa oleifera* plant against *Enterobacteriaceae (50µL)*

Plant	Parts used	Extracts	Zone of inhibitions (mm)		
			E. coli	***K. pneumonia***	***S. typhi***
Moringa oleifera	Leaves	Methanol	16mm	14mm	**18mm**
		Ethanol	18mm	15mm	**14mm**
	Seeds	Methanol	13mm	16mm	**18mm**
		Ethanol	**10mm**	**10mm**	**16mm**

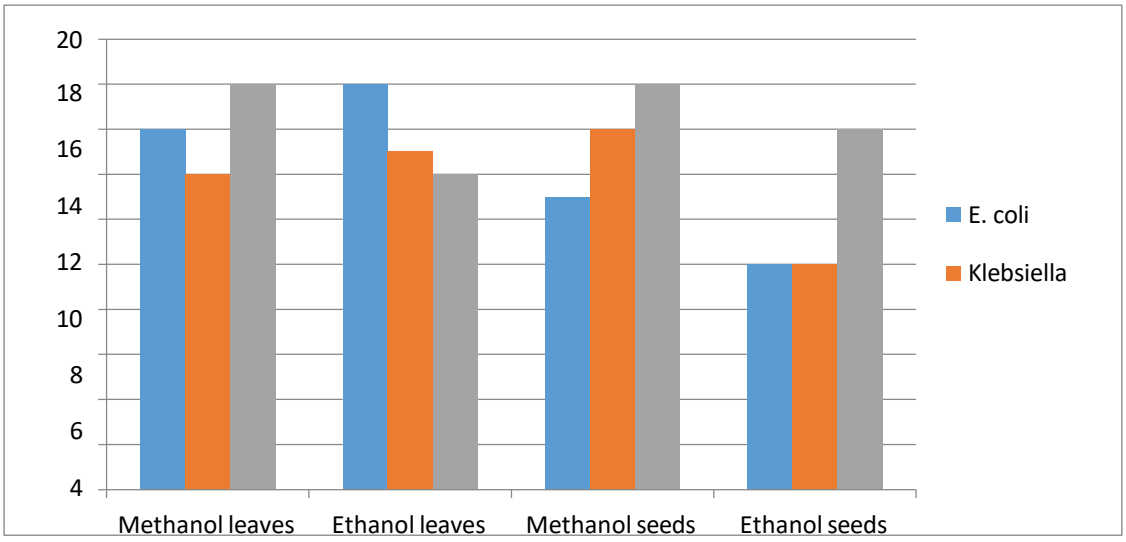

Figure 1: Antibacterial profile of Moringa oleifera plant against Enterobacteriaceae (50µL)

Table 3: Antimicrobial profile of *Moringa oleifera* plant against *Enterobacteriaceae (100µL)*

Plant	Parts used	Extracts	Zone of inhibitions (mm)		
			E. coli	***k. pneumonia***	***S. typhi***
Moringa oleifera	Leaves	Methanol	17mm	16mm	**19mm**
		Ethanol	19mm	16mm	**16mm**
	Seeds	Methanol	14mm	17mm	**18mm**
		Ethanol	**12mm**	**14mm**	**17mm**

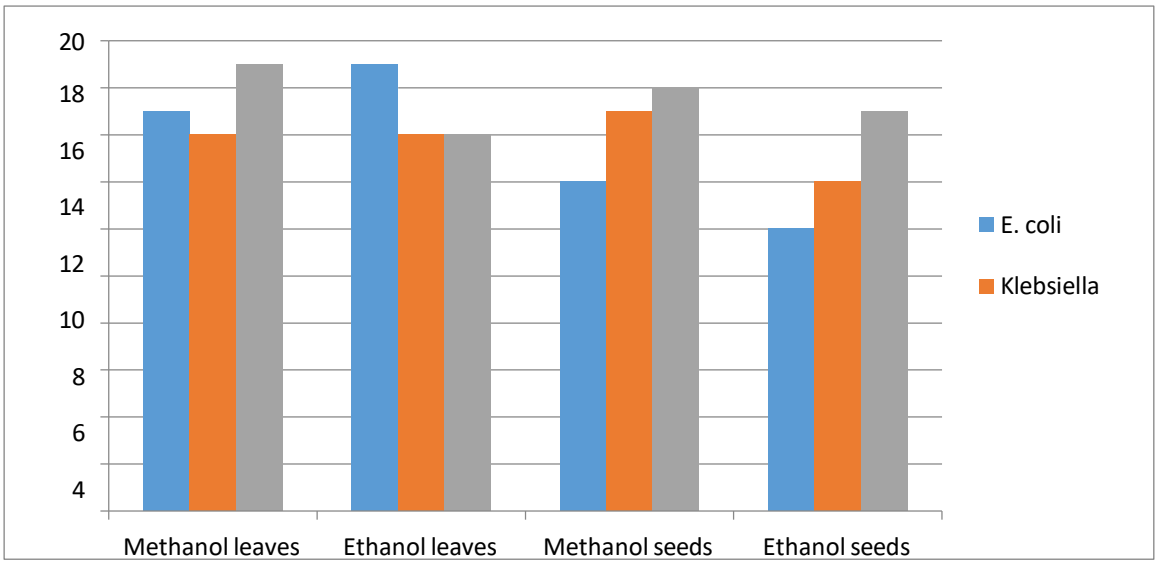

Figure 2: Antibacterial profile of *Moringa oleifera* plant against *Enterobacteriaceae (100µL)*

HPLC fractionation

From gradient HPLC fractionation different numbers of fraction were collected from extracts of different parts of *Moringa oleifera* plant. The following table shows all fractions which were conserved in test tubes for further experimentation.

Table 4: List of selected solvent extracts and its HPLC fractions

Parts of *Moringa oleifera*	Solvent extracts	HPLC fractions
Leaves	Methanol Ethanol	**A1 A2 A3 A1 A2 A3**
Seeds	**Methanol** **Ethanol**	**A1 A2 A3 A4 A5 A6 A1 A2 A3 A4 A5 A6**

Antibacterial activity of *Moringa oleifera* HPLC fractions

***Moringa oleifera* (leaves) and Methanol leaves extract**

Methanol extract of leaves of *M. oleifera* was subjected to fractionation by HPLC, from which three fractions is obtained and proceed to check the antibacterial activity as shown in figure 3**.**

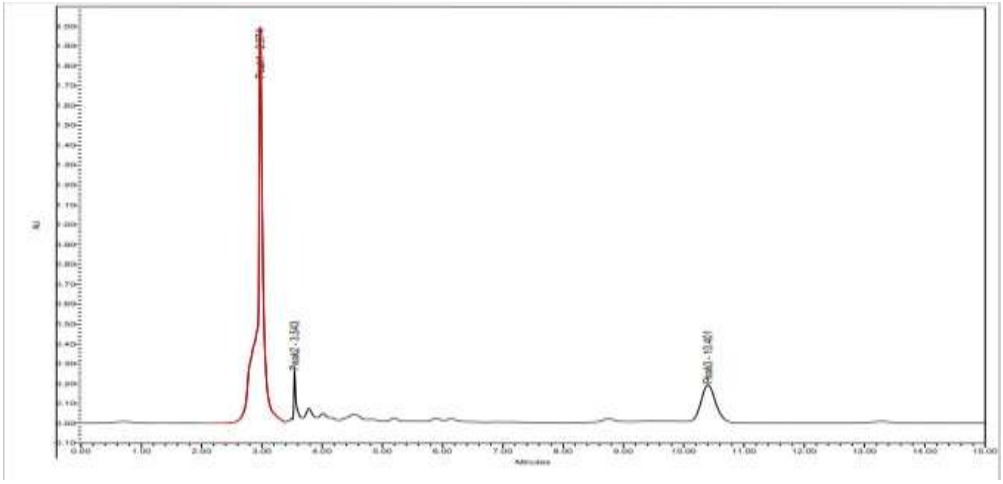

Figure 3: chromatograph showing HPLC fractions of methanol extracts of ***Moringa oleifera*** **leaves**

Methanol extract fractionation shows that fraction 2 (A2) is more effective against *E. coli* having 17±0.23mm inhibition zone. Fraction 3 (A3) showed maximum inhibition zone 16±0.13mm against *K. pneumonia* and fraction 2 (A2) were active against *S. typhi* with inhibition zone 17±0.23mm as shown in table 5.

Figure 4: Antibacterial activity of selected plant extracts; *moringa oleifera* (leaves) methanol extract HPLC fraction against; (a) *E. coli* (b) *K. pneumonia* (c) *S. typhi*.

Table 5 Antibacterial activity of HPLC fractions of *Moringa oleifera* leaves dissolve in methanol solvent.

Plant	Solvent	Fractions of HPLC	Zone of inhibition (mm) against bacterial growth		
***Moringa oleifera* (leaves)**	Methanol		***E. coli***	***K. pneumonia***	***S. typhi***
		A1	16±0.23	10±6.33	**13±0.26**

A2	17±0.32	14±0.13	**17±0.44**
A3	15±0.44	16±0.13	**16±0.23**

±=standard error of given value

Ethanol leaves extract

From an ethanol extract *of M. oleifera* (leaves), only three HPLC fractions were obtained, and they were then tested for antibacterial activity. as shown in figure 5.

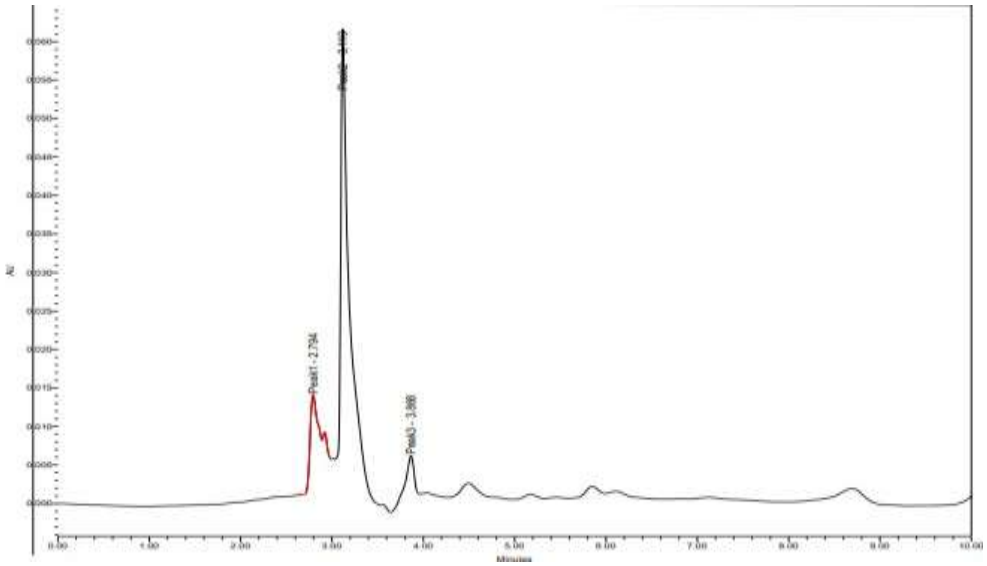

Figure 5: HPLC fractions of ethanol extract of *Moringa oleifera* (leaves)

HPLC fractionations of *M. oleifera* leaves ethanol extract were showing activity against MDR. A1 and A3 have 18±0.32mm zone of inhibition against *E. coli*, A2 has more active potential against *K. pneumonia* have zone of inhibition is 16±0.33mm, A3 was effective against *S. typhi* with 21±0.33 inhibition zone as shown in the following figure 6 and table 6.

Figure 6: Antibacterial activity of selected plant extracts; *Moringa oleifera* (leaves) ethanol

extract HPLC fraction against; (A) *E. coli* (B) *K. pneumonia* (C) *S. typhi*

Table 6: Antibacterial activity of HPLC fractions of *Moringa oleifera* leaves dissolve in ethanol solvent.

Plant	Solvent	Fractions of HPLC	Zone of inhibition (mm) against bacterial growth		
***Moringa oleifera* (leaves)**	Ethanol		***E. coli***	***K. pneumonia***	***S. typhi***
		A1	18±0.32	15±6.33	**18±0.26**
		A2	19±0,33	16±0.28	**17±0,29**
		A3	18±0.32	14±0.33	**21±0.33**
		±=standard error of given value			

***Moringa oleifera* (seeds)**

Methanol seeds extract

Total three out of six HPLC fractions were collected from the methanol extracts of *Moringa oleifera* (seeds). These fractions were analyzed to check against MDR bacteria.

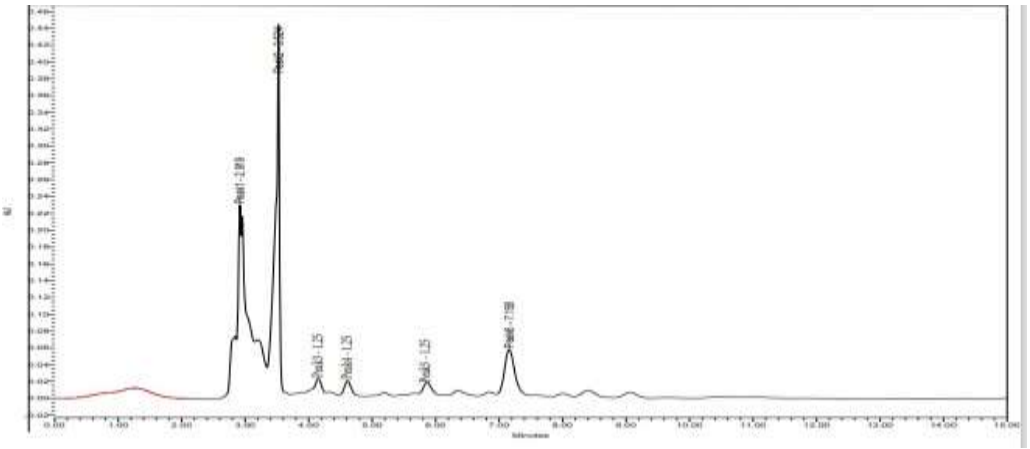

Figure 7: HPLC fractions of methanol extract of *Moringa oleifera* (seeds)

HPLC fractionations of *M. oleifera* seeds methanol extract was effective against MDR. A1 has a zone of inhibition 12±0.32m against *E. coli* while A1 was more effective against *K. pneumonia* with zone of inhibition 17±0.28mm, A3 were effective against *S. typhi* with 16±0.33 inhibition zones as shown in figure 8 and table 7.

Figure: 8 antibacterial activity of selected plant extracts; *moringa oleifera* (seeds) methanol extract HPLC fraction against; (A) *E. coli* (B) *K. pneumonia* (C) *S. typhi*

Table 7: Antibacterial activity of HPLC fractions of *Moringa oleifera seeds* dissolving in methanol solvent

Plant	Solvent	Fractions of HPLC	Zone of inhibition (mm) against bacterial growth		
***Moringa oleifera* (seeds)**	Methanol		***E. coli***	***K. pneumonia***	***S. typhi***
		A1	12±0.32	17±6.33	**14±0.26**
		A2	14±0.32	16±0.28	**08±0,29**
		A3	13±0.32	15±3.11	**16±0.33**
		±=standard error of given value			

Ethanol seeds extract

Four HPLC fractions total were isolated from an ethanol seeds extract of *Moringa oleifera*, and these fractions had strong antibacterial activity as shown in figure 9.

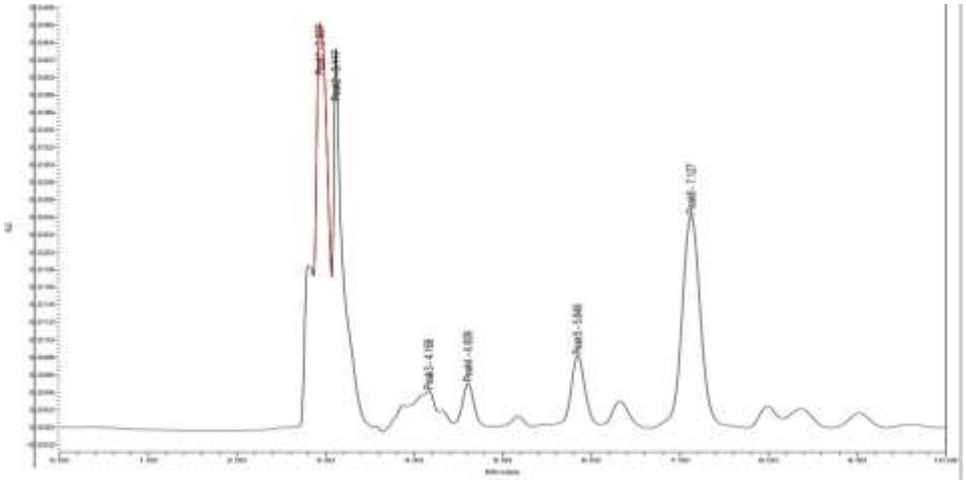

Figure 9: HPLC fractions of ethanol seeds extract of *Moringa oleifera*

Fraction 4 (A4) prevents the growth of *E. coli* have zone of inhibition 08±0.44mm. Fraction 2 (A2) have zone of inhibition 10±0.39mm were active against *K. pneumonia* while *S. typhi* having maximum zone 19±0.16mm as compared to other bacteria as shown in figure 10 and table 8.

Figure: 10 antibacterial activity of selected plant extracts; *moringa oleifera* (seeds) ethanol extract HPLC fraction against**;** (A) *E. coli* (B) *K. pneumonia* (C) *S. typhi*

Table 8 Antibacterial activity of HPLC fractions of *Moringa oleifera* seeds dissolved in ethanol solvent.

Plant	Solvent	Fractions of HPLC	Zone of inhibition (mm) against bacterial growth		
***Moringa oleifera* (seeds)**	Ethanol		***E. coli***	***K. pneumonia***	***S. typhi***
		A1	10±0.32	14±6.33	**19±0.26**
		A2	09±0,28	10±0.39	**18±0,16**
		A3	12±0.29	08±3.11	**16±0.33**
		A4	**08±0.44**	**09±0.27**	**14±0.26**

±=standard error of given value

Discussion

The rapid emergence of multidrug-resistant (MDR) bacterial pathogens poses a serious global public health threat, significantly undermining the effectiveness of conventional antibiotics. This alarming situation has led to an urgent need for the exploration of alternative and complementary therapies that are both effective and sustainable. One such alternative is the

utilization of medicinal plants, which have been historically employed in traditional systems of medicine for the treatment of infectious diseases(Anand et al., 2019). Moringa oleifera, commonly known as the drumstick tree or “miracle tree,” has received increasing scientific attention due to its wide array of pharmacologically active compounds with potential antimicrobial properties(Pachava et al., 2018; Tshabalala et al., 2019). In the present study, the antibacterial potential of Moringa oleifera extracts (leaves and seeds) against MDR strains of Escherichia coli, Klebsiella pneumoniae, and Salmonella typhi members of the Enterobacteriaceae were investigated. These strains were isolated from the Mardan region and confirmed to exhibit resistance to multiple antibiotics. The extracts were prepared using two different solvents methanol and ethanol to assess the influence of extraction medium on antibacterial efficacy. The study also aimed to fractionate these extracts using high-performance liquid chromatography (HPLC) to identify specific bioactive components responsible for antimicrobial activity. Our findings revealed that both methanolic and ethanolic extracts of M. oleifera exhibited significant antibacterial activity against the tested MDR isolates, with variations in efficacy depending on the plant part and solvent used. The methanolic leaf extract demonstrated the highest antibacterial activity, producing zones of inhibition measuring 22 mm against S. typhi, 20 mm against K. pneumoniae, and 18 mm against E. coli. These results are consistent with previous studies, such as those by Rahman et al. (2010) and Fadia et al. (2021), who reported that M. oleifera leaves are rich in flavonoids, phenolic acids, and other secondary metabolites with strong antimicrobial properties. In contrast, ethanolic leaf extracts showed relatively moderate antibacterial activity, indicating that methanol may be more efficient at extracting bioactive compounds from M. oleifera leaves(FadiaTaufik et al., 2021; Rahman et al., 2010). The seed extracts of M. oleifera also demonstrated antibacterial properties, albeit with slightly lower efficacy compared to leaf extracts. Methanolic seed extracts exhibited zones of inhibition of 21 mm against S. typhi, 17 mm against K. pneumoniae, and 14 mm against E. coli. On the other hand, ethanolic seed extracts produced smaller inhibition zones, with 17 mm for S. typhi, 14 mm for K. pneumoniae, and 12 mm for E. coli. These results suggest that while both leaves and seeds possess antibacterial properties, the leaves, particularly when extracted with methanol, offer superior antimicrobial potential. This difference in activity may be attributed to the higher

concentration of flavonoids, saponins, alkaloids, and phenolic compounds found in the leaves compared to the seeds.

To further dissect the antibacterial properties of the plant, the study employed HPLC fractionation of both leaf and seed extracts. The methanolic leaf extract was separated into three fractions A1, A2, and A3 using a C-18 column on a PerkinElmer HPLC system. All three fractions showed varying degrees of antibacterial activity, with fraction A2 exhibiting the strongest inhibition, particularly against E. coli and K. pneumoniae. This suggests that fraction A2 contains a potent phytochemical or combination of compounds responsible for the observed antimicrobial effect. This is in line with the findings of Karthik and Sudha (2013), who reported that ethanol and methanol-based extracts of M. oleifera leaves contained phytochemicals that exhibited significant antibacterial activity against Enterobacteriaceae. Similarly, the HPLC fractions of the methanolic and ethanolic seed extracts were assessed for their antibacterial activity. For methanolic seed extracts, fraction A1 showed maximum inhibition against K. pneumoniae, while fraction A3 was most effective against S. typhi. Ethanol seed extract yielded four fractions, of which fraction A1 demonstrated the highest antibacterial activity, and fraction A4 the least. These findings highlight that the effectiveness of HPLC fractions is both solvent- and fraction-dependent. The presence of bioactive constituents such as steroids, terpenoids, and phenolic acids previously reported in M. oleifera seeds(Das et al., 2022) could be responsible for the observed antibacterial effects. The agar well diffusion assay used to determine the antibacterial efficacy of the extracts provided a clear and reproducible method for evaluating antimicrobial potential. The development of distinct zones of inhibition around the wells indicates that the plant extracts possess compounds capable of diffusing through the agar and inhibiting bacterial growth. These results collectively suggest that M. oleifera, particularly its methanolic leaf extract and its HPLC-derived fractions, contains highly potent antibacterial agents that can serve as alternatives or adjuncts to existing antibiotic therapies(Mesas Hernández et al., 2022). Furthermore, the study supports the hypothesis that phytochemicals in medicinal plants can exert synergistic effects when used in combination with conventional antibiotics, although this study focused on extracts alone. The ability of M. oleifera extracts to inhibit MDR pathogens without the use of synthetic drugs points to their therapeutic

potential. Moreover, their low toxicity as suggested in preliminary toxicity assessments in other studies makes them ideal candidates for future drug development. While the findings are promising, some limitations should be acknowledged. The study did not investigate the exact chemical composition of each HPLC fraction through spectroscopic or mass spectrometric techniques, which would be necessary to isolate and characterize the individual bioactive compounds. Additionally, in vivo studies and cytotoxicity assays are essential to establish the safety profile and pharmacokinetics of these plant-derived compounds.

Conclusion

The present study concludes that the extracts derived from different parts of the Moringa oleifera plants specifically the leaves and seeds contain bioactive compounds with significant antimicrobial activity. These compounds demonstrated inhibitory effects against multidrug-resistant (MDR) bacterial strains, underscoring the medicinal potential of M. oleifera in combating infections caused by resistant pathogens. The findings confirm the traditional use of this plant in herbal medicine and support its role as a natural source of therapeutic agents effective against a broad spectrum of bacterial pathogens. Thus, M. Oleifera holds promise as a complementary or alternative treatment option in the ongoing fight against antimicrobial resistance.

Recommendations

Based on the findings of this study, it is recommended that further in-depth research be conducted on the HPLC fractions of M. Oleifera extracts. Future studies should focus on the isolation, identification, and characterization of the specific bioactive compounds responsible for antibacterial activity. In addition, in vivo studies and toxicity profiling are essential to evaluate the safety and therapeutic efficacy of these compounds. Such research may contribute to the development of novel, plant-based antibacterial agents that could serve as effective alternatives to conventional antibiotics in the treatment of MDR bacterial infections.

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