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IN VITRO ANTIBACTERIAL ACTIVITY OF MORINGA OLEIFERA LEAF EXTRACTS AGAINST MULTIDRUG RESISTANT UROPATHOGENIC GRAM NEGATIVE BACTERIA

Bushra Rehman[1], Manahil Zubair[2], Aiman Waheed[3], Sajjad Ahmad[4], Dur-E-Najaf Khan[5], Numan Saleh Zada[6]

[1-4]*Department of Microbiology, Institute of Pathology and Diagnostic Medicine, IPDM, Khyber Medical University, Peshawar, Pakistan*
[5]*Department of Pharmacy , Bacha Khan University, Charsadda*
[6]*Pakistan Council of Scientific and Industrial Research, Peshawar*

[1]drbushra.ipdm@kmu.edu.pk, [2]mahahilzee15@gmail.com, [3]draimanwaheed.ibms@kmu.edu.pk, [4]sajjadahmad.ibms@kmu.edu.pk, [5]najafkhan@bkuc.edu.pk, [6]nomansalehzada@yahoo.com

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Corresponding Author:

Abstract

Background: Urinary tract infections (UTIs) are among the most prevalent bacterial infections worldwide and represent a major contributor to antimicrobial consumption in both community and hospital settings. The rapid emergence of multi drug resistant (MDR) uropathogens, particularly among Gram negative bacteria has substantially limited the effectiveness of conventional antibiotics and underscores the urgent need for alternative antimicrobial strategies. Medicinal plants rich in bioactive phytochemicals have gained increasing attention as potential sources of novel antimicrobial agents. Objective: This study aimed to evaluate the in vitro antibacterial efficacy of Moringa oleifera leaf extracts against MDR uropathogenic bacterial isolates obtained from UTIs. Methodology: A cross sectional experimental study was conducted using clinically isolated uropathogens, including Escherichia coli, Klebsiella pneumoniae, Enterobacter and Pseudomonas aeruginosa. Bacterial identification was confirmed through standard microbiological and biochemical techniques including Analytical Profile Index (API 20E) profiling. Antimicrobial susceptibility testing was performed using the Kirby Bauer disc diffusion method to determine Multi drug resistance patterns. Crude leaf extracts of M. oleifera were prepared using methanolic and Dimethyl Sulfoxide (DMSO) based extraction. Antibacterial activity was assessed using the agar well diffusion assay at graded concentrations and compared with ciprofloxacin as a standard control. Results: All tested isolates exhibited resistance to multiple antibiotic classes. M. oleifera leaf extracts demonstrated concentration dependent antibacterial activity against all uropathogens. The highest percentage inhibition was observed against Enterobacter (86%) and K. pneumoniae (85%), followed by P. aeruginosa (76%) and E. coli (72%). Zones of inhibition produced by the plant extract ranged from 17 to 21 mm, indicating substantial antibacterial potential against MDR strains. Conclusion: M. oleifera leaf extracts exhibit significant in vitro antibacterial activity against MDR uropathogenic Gram negative bacteria. These findings support the potential role of M. oleifera as a complementary or alternative antimicrobial agent in the management of drug resistant UTIs. Further studies are warranted to isolate active compounds, elucidate

mechanisms of action and evaluate safety and efficacy in in vivo and clinical settings.

Introduction

UTIs constitute one of the most common bacterial infections affecting individuals across all age groups and remain a significant public health burden globally (1). It is estimated that hundreds of millions of UTI cases occur annually, leading to substantial morbidity, healthcare utilization and economic costs. UTIs range from uncomplicated lower UTIs to severe, complicated infections involving the kidneys, often requiring hospitalization and prolonged antimicrobial therapy (2). Gram negative bacteria, particularly *E. coli*, are the predominant etiological agents, followed by opportunistic pathogens such as *K. pneumoniae*, *Enterobacter* and *P. aeruginosa* (3).

In recent years, the clinical management of UTIs has been increasingly complicated by the alarming rise in antimicrobial resistance (AMR) (4). Uropathogens exhibiting resistance to multiple antibiotic classes, including β lactams, fluoroquinolones, aminoglycosides and carbapenems are now frequently reported, especially in recurrent, catheter associated and hospital acquired infections (5). The emergence of MDR strains not only limits therapeutic options but also contributes to increased treatment failure, prolonged illness and higher healthcare costs (6). In low and middle income countries including Pakistan, the burden of MDR uropathogens is particularly pronounced due to widespread antibiotic misuse, limited diagnostic stewardship and high rates of empirical therapy (7).

The escalating threat of AMR has intensified global efforts to identify alternative and adjunct antimicrobial strategies. Among these, medicinal plants represent a promising and underexplored reservoir of bioactive compounds with antimicrobial properties (8). Plant derived secondary metabolites such as flavonoids, phenolic acids, tannins, saponins and alkaloids have been shown to exert antibacterial effects through diverse mechanisms, including disruption of bacterial cell membranes, inhibition of essential enzymes and interference with nucleic acid synthesis (9). Importantly, the multi targeted nature of these compounds may reduce the likelihood of rapid resistance development compared with conventional antibiotics (10).

M.oleifera, a nutrient rich plant belonging to the family Moringaceae, has been widely utilized in traditional medicine and nutrition across tropical and subtropical regions (11). Its leaves are particularly rich in bioactive phytochemicals and have demonstrated antioxidant, anti-inflammatory and antimicrobial activities in previous studies (12, 13). Despite growing evidence supporting its broad spectrum antibacterial potential, limited data are available regarding its efficacy against MDR uropathogenic bacteria, especially secondary isolates associated with complicated and recurrent UTIs (14).

Given the increasing prevalence of MDR uropathogens and the urgent need for novel antimicrobial alternatives, the present study was designed to evaluate the *in vitro* antibacterial

activity of *M. oleifera* leaf extracts against clinically relevant MDR Gram negative bacteria isolated from urinary tract infections. By focusing on uropathogenic isolates and employing standardized microbiological assays, this study aims to provide robust experimental evidence supporting the potential role of *M. oleifera* as a natural antimicrobial agent and to contribute to the growing body of research exploring plant based solutions to combat AMR.

Materials and Methods

Study Design

This study was conducted as a cross sectional *in vitro* experimental investigation to evaluate the antibacterial activity of *M. oleifera* leaf extracts against MDR uropathogenic Gram negative bacteria. The experimental workflow comprised bacterial isolation and confirmation, antimicrobial susceptibility profiling, preparation of plant extracts and assessment of antibacterial activity using standardized diffusion assays.

Bacterial Isolates

Clinically identified uropathogenic bacterial isolates were obtained from the Public Health Reference Laboratory, Khyber Medical University, Peshawar. The isolates included *E. coli*, *K. pneumoniae*, *Enterobacter* and *P. aeruginosa* which are frequently implicated in complicated and recurrent UTIs. All isolates were subcultured on selective and differential media and preserved under appropriate laboratory conditions until further analysis.

Culture and Identification of Uropathogens

Bacterial isolates were cultured on MacConkey agar and Cystine Lactose Electrolyte Deficient (CLED) agar and incubated at 37 °C for 18 to 24 h. Preliminary identification was performed based on colony morphology, lactose fermentation characteristics and Gram staining. Definitive identification was achieved using standard biochemical tests, including oxidase, indole, and citrate utilization assays followed by confirmation using the API 20E system in accordance with the manufacturer's instructions. Only confirmed Gram negative isolates were included in subsequent analyses.

Antibiotic Susceptibility Testing and MDR Classification

Antimicrobial susceptibility testing was performed using the Kirby Bauer disc diffusion method on Mueller Hinton agar (MHA). Bacterial suspensions were standardized to 0.5 McFarland turbidity and uniformly inoculated onto MHA plates. Commercial antibiotic discs representing multiple antimicrobial classes commonly used for UTIs were applied aseptically. Plates were incubated at 37 °C for 18 to 24 h, after which zones of inhibition were measured in millimeters and interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines.

Multidrug resistance was defined as resistance to at least one antimicrobial agent in three or more distinct antibiotic classes. Only MDR confirmed isolates were subjected to plant extract testing.

Plant Material Collection and Authentication

Fresh, mature leaves of *M. oleifera* were collected from the Pakistan Forest Institute, Peshawar. Botanical authentication was performed by a qualified plant taxonomist at the Department of Botany, University of Peshawar. Collected leaves were thoroughly washed to remove debris and contaminants prior to processing (15).

Preparation of *Moringa oleifera* Leaf Extract

The cleaned leaves were shade dried at ambient temperature until constant weight was achieved and subsequently pulverized into fine powder using an electric grinder. The powdered plant material was subjected to maceration using methanol at a plant to solvent ratio of approximately 1:5–1:10 (w/v) (16, 17). The mixture was sealed and maintained at room temperature for four weeks with intermittent agitation (18).

Following maceration, the extract was filtered using Whatman filter paper and the solvent was evaporated under reduced pressure at 45°C using a rotary evaporator (19, 20). The resulting crude extract was further air dried to obtain a solid residue. Stock solutions were prepared by dissolving the dried extract in DMSO to achieve concentrations of 25, 50 and 75 mg/mL. All solutions were sterilized using a 0.22 µm syringe filter and stored at 4 °C until use.

Evaluation of Antibacterial Activity

The antibacterial activity of M. *oleifera* leaf extract was evaluated using the agar well diffusion method. MDR bacterial isolates were cultured overnight and adjusted to 0.5 McFarland turbidity before being uniformly spread onto MHA plates to form a bacterial lawn. Wells of 6 mm diameter were aseptically punched into the agar using a sterile cork borer.

Each well was loaded with 100 µL of the prepared plant extract at specified concentrations. Ciprofloxacin was used as a positive control while DMSO served as a negative control. Plates were incubated at 37 °C for 18 to 24 h. Zones of inhibition were measured in millimeters and antibacterial efficacy was expressed as both absolute zone diameters and percentage inhibition relative to the standard antibiotic.

Statistical Analysis

All experiments were performed in triplicate to ensure reproducibility. Results were recorded as mean values of inhibition zones. Data were tabulated and graphically represented to compare antibacterial activity across bacterial species and extract concentrations. Due to the exploratory *in vitro* nature of the study, descriptive analysis was primarily employed.

Results

Identification and Characterization of Uropathogenic Isolates

A total of four Gram negative uropathogenic bacterial species were included in the study: *E. coli*, *K. pneumoniae*, *Enterobacter* and *P. aeruginosa*. Following incubation on selective and differential media, all isolates demonstrated characteristic colony morphology consistent with their respective species. Lactose fermenting organisms (*E. coli*, *K. pneumoniae*, and *Enterobacter*) produced pink to mucoid colonies on MacConkey agar and yellow colonies on CLED agar, whereas *P. aeruginosa* exhibited non lactose fermenting, colorless colonies on both media.

Microscopic examination following Gram staining confirmed that all isolates were Gram negative bacilli. Species level identification was further validated using biochemical assays, including oxidase, indole and citrate utilization tests and confirmed by API 20E profiling. The combined cultural, microscopic and biochemical characteristics supported accurate identification of all isolates (Table 1). Representative growth patterns on selective media and Gramstained micrographs are shown in Figure 1 and Figure 2, respectively.

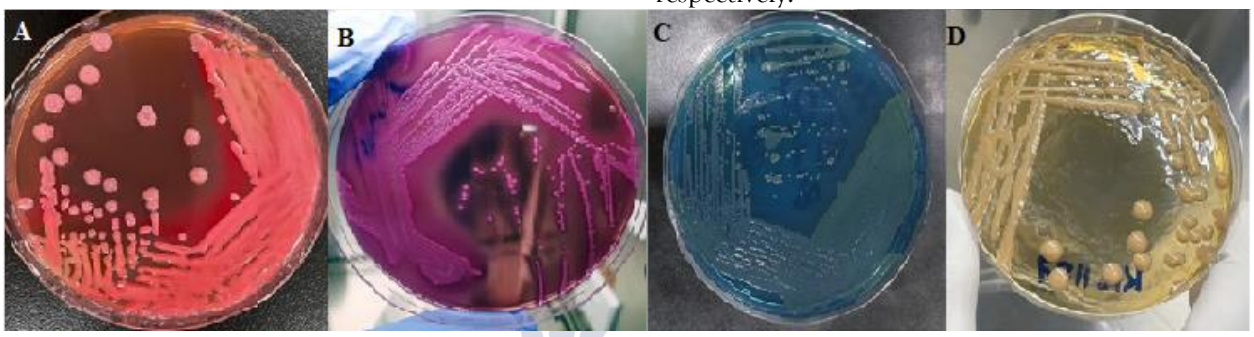


Figure 1: Growth characteristics of uropathogenic isolates on MacConkey (A,B) and CLED agar (C,D)

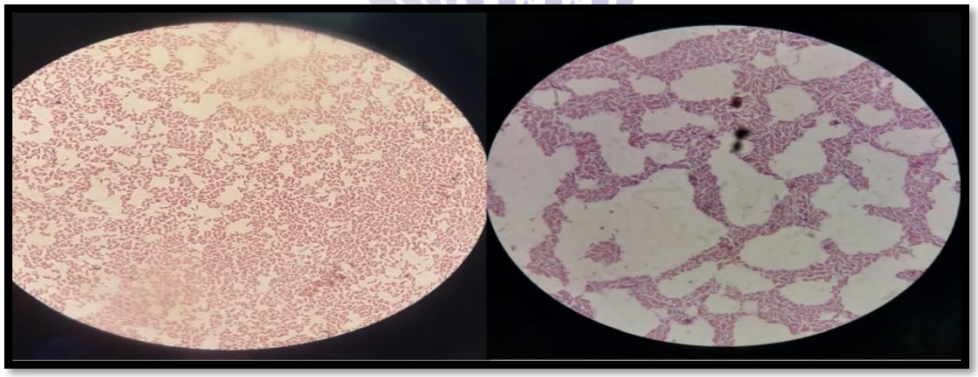


Figure 2: Gram stained micrographs of uropathogenic isolates showing Gram negative bacilli

Table 1: ***Morphological And Biochemical Identification Of Uropathogenic Isolates***

Bacterial isolate	Growth on MacConkey agar	Growth on CLED agar	Gram reaction	Key biochemical reactions	API system confirmation
Escherichia coli	Pink to red colonies (lactose fermenter)	Yellow colonies	Gram negative rods	Indole positive, Citrate negative, Oxidase negative	API 20E – *E. coli*
Klebsiella pneumoniae	Large, mucoid pink colonies (lactose fermenter)	Large, mucoid yellow colonies	Gram negative rods	Citrate positive, Indole negative, Oxidase negative	API 20E – *K. pneumoniae*
Enterobacter	Large, mucoid pink	Large,	Gram	Citrate positive,	API 20E –

spp.	colonies (lactose fermenter)	mucoid yellow colonies	negative rods	Indole negative, Oxidase negative	*Enterobacter* spp.
Pseudomonas aeruginosa	Colorless colonies (non-lactose fermenter)	Blue-green colonies	Gram negative rods	Oxidase positive, Indole negative, Citrate negative	Biochemically confirmed

Antibiotic Susceptibility Patterns and Multidrug Resistance Profiles

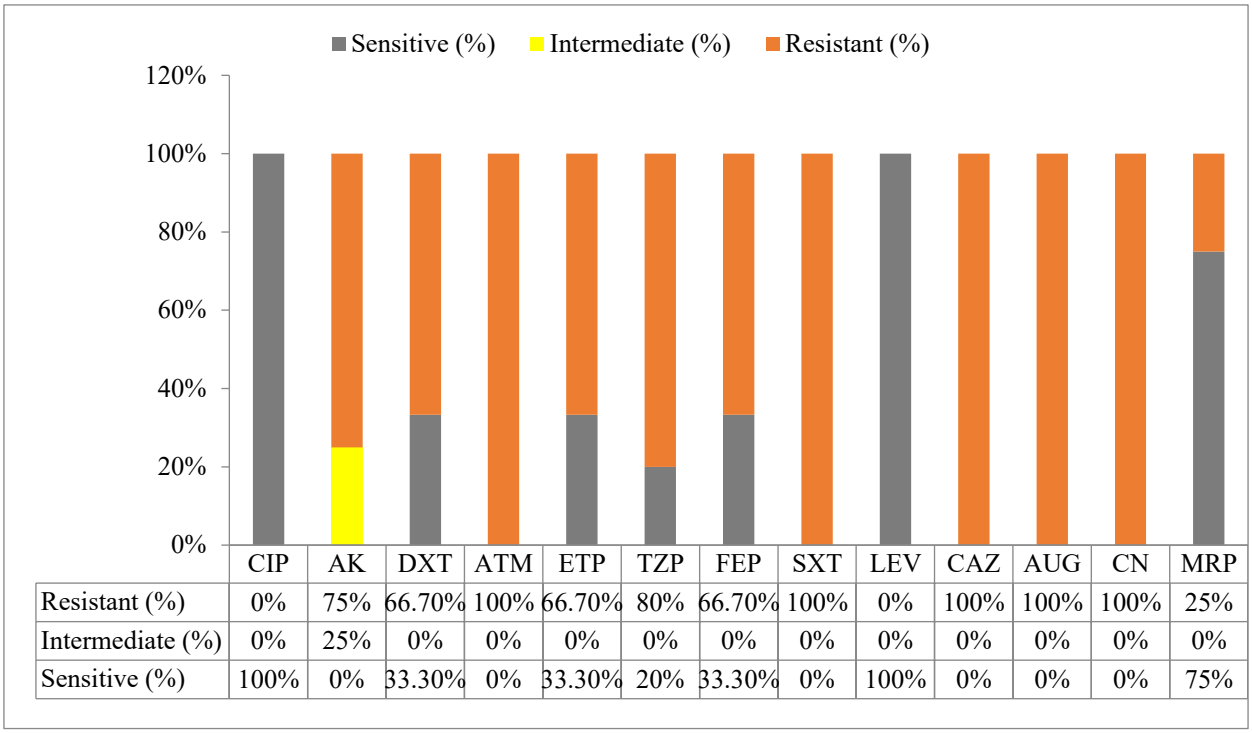
Antimicrobial susceptibility testing revealed extensive resistance among all tested uropathogenic isolates. Resistance was observed across multiple antibiotic classes, including β lactams, fluoroquinolones, aminoglycosides, carbapenems and sulfonamides. *K. pneumoniae* and *Enterobacter* exhibited resistance to the highest number of antimicrobial classes, while *E. coli* and *P. aeruginosa* also demonstrated MDR phenotypes.

Based on established criteria, all isolates fulfilled the definition of multidrug resistance showing resistance to at least one agent in three or more antibiotic classes. Detailed antibiotic resistance profiles and MDR classification are summarized in Table 2. Comparative susceptibility trends across isolates are illustrated in Figure 3.

Table 2: ***Antibiotic Resistance Profiles And Multi Drug Resistance Classification Of Uropathogenic Isolates***

Bacterial Isolate	Resistant Antibiotics*	Number of Antibiotic Classes	MDR Status
Escherichia coli	ATM,CAZ, CN	3	MDR
Pseudomonas aeruginosa	AK, ATM, TZP, CAZ, CN, FEP	4	MDR
Klebsiella pneumoniae	AK, DXT, TZP, FEP, SXT, AUG	5	MDR
Enterobacter spp.	DXT, ETP, TZP, FEP, SXT, AUG, CN	6	MDR

*Abbreviations:
AK: Amikacin; ATM: Aztreonam; AUG: Amoxicillin:clavulanate; CAZ: Ceftazidime; CN: Gentamicin; DXT: Doxycycline; ETP: Ertapenem; FEP: Cefepime; SXT: Trimethoprim:sulfamethoxazole; TZP: Piperacillin:tazobactam.
MDR definition: Resistance to ≥1 antibiotic in ≥3 different antimicrobial classes.



	CIP	AK	DXT	ATM	ETP	TZP	FEP	SXT	LEV	CAZ	AUG	CN	MRP
Resistant (%)	0%	75%	66.70%	100%	66.70%	80%	66.70%	100%	0%	100%	100%	100%	25%
Intermediate (%)	0%	25%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Sensitive (%)	100%	0%	33.30%	0%	33.30%	20%	33.30%	0%	100%	0%	0%	0%	75%

Figure 3: Antibiotic susceptibility patterns of uropathogenic isolates.

Antibacterial Activity of *Moringa oleifera* Leaf Extract

The antibacterial activity of M. *oleifera* leaf extract was evaluated against all MDR uropathogenic isolates using the agar well diffusion assay. The plant extract demonstrated clear zones of inhibition against all tested bacteria, whereas no inhibitory effect was observed with the negative control (DMSO), confirming that antibacterial activity was attributable solely to the extract.

The inhibitory effect of the extract was concentration dependent. Zones of inhibition ranged from 17 to 21 mm across bacterial species at the tested concentrations. The highest percentage inhibition was observed against *Enterobacter* (86%) and *K. pneumoniae* (85%), followed by *P. aeruginosa* (76%) and *E. coli* (72%). Although the standard antibiotic ciprofloxacin produced larger inhibition zones overall (20 to 29 mm), the plant extract exhibited substantial antibacterial activity even against highly resistant isolates.

Comparative inhibition zones between the plant extract, standard antibiotic and negative control are presented in Table 3. The relative antibacterial performance of *M. oleifera* extract across bacterial species is graphically represented in Figure 4, while representative agar well diffusion plates are shown in Figure 5.

Table 3: ***Comparative zones of inhibition and percentage inhibition of Moringa oleifera leaf extract against MDR uropathogens***

Bacterial Isolate	Zone of Inhibition (Standard Drug, mm)	Zone of Inhibition (Plant Extract, mm)	Zone of Inhibition (Negative Control, mm)	Percentage Inhibition (%)
Escherichia coli	29	21	0	72
Enterobacter spp.	22	19	0	86
Klebsiella pneumoniae	20	17	0	85
Pseudomonas	26	20	0	76

aeruginosa

Standard drug: Ciprofloxacin Negative Control: Dimethyl sulfoxide (DMSO)

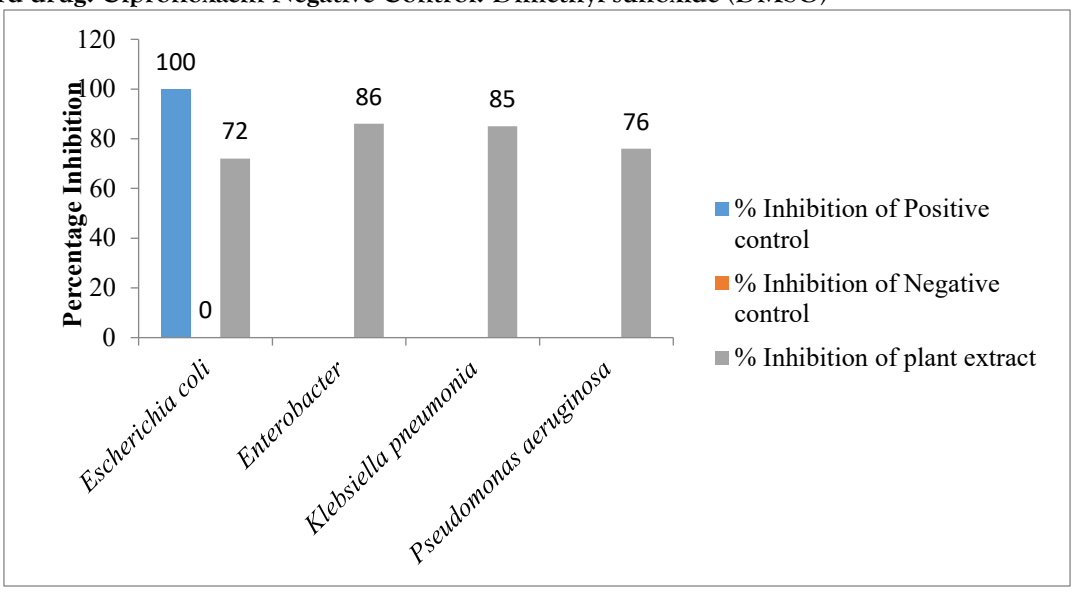


Figure 4: Comparative antibacterial activity of M. oleifera leaf extract and ciprofloxacin

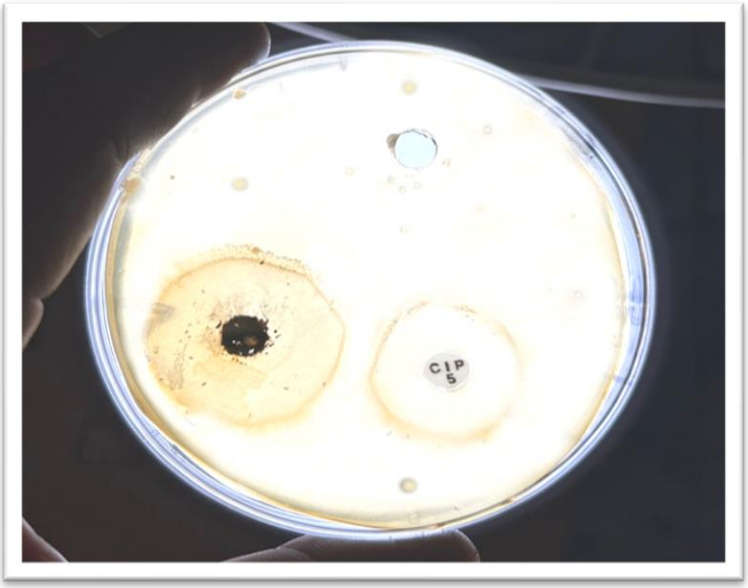


Figure 5: Agar well diffusion assay showing zones of inhibition produced by M. oleifera extract

Discussion

The present study evaluated the *in vitro* antibacterial activity of *M. oleifera* leaf extracts against MDR Gram negative uropathogenic bacteria isolated from UTIs. The findings demonstrate that *M. oleifera* exhibits substantial antibacterial efficacy against clinically relevant MDR pathogens, including *E. coli*, *K. pneumoniae*, *Enterobacter* and *P. aeruginosa*. These results are particularly significant in the context of the escalating global burden of AMR, which has markedly compromised the effectiveness of standard antibiotic therapies for UTIs (21).

A notable strength of this study lies in the use of secondary and clinically relevant MDR uropathogenic isolates**,** rather than laboratory reference strains. Many previous investigations assessing the antimicrobial potential of medicinal plants have relied primarily on nonresistant or reference bacterial strains, limiting their

translational relevance. In contrast, the present work directly addresses the growing clinical challenge posed by MDR uropathogens commonly implicated in complicated, recurrent and hospital acquired UTIs. The high resistance rates observed across multiple antibiotic classes in this study are consistent with contemporary regional and global surveillance data, underscoring the urgent need for alternative antimicrobial strategies (22).

The *M. oleifera* leaf extract demonstrated concentration dependent antibacterial activity against all tested MDR isolates. Particularly strong inhibitory effects were observed against *Enterobacter* and *K. pneumoniae*, organisms that exhibited the most extensive resistance profiles during antibiotic susceptibility testing. This finding is noteworthy, as these pathogens are frequently associated with treatment failure due to ESBL production, carbapenem resistance, and limited therapeutic options. The ability of *M. oleifera* extract to inhibit these highly resistant organisms suggests that its antibacterial mechanisms may differ fundamentally from those targeted by conventional antibiotics (23).

The observed antibacterial activity can be plausibly attributed to the rich phytochemical composition of *M. oleifera* leaves, including flavonoids, phenolic acids, tannins, and saponins. These bioactive compounds are known to exert antimicrobial effects through multiple mechanisms, such as disruption of bacterial cell membranes, interference with enzymatic pathways, inhibition of nucleic acid synthesis and impairment of quorum sensing. The multi target nature of these phytochemicals may explain the effectiveness of the extract against MDR organisms and suggests a reduced likelihood of rapid resistance development compared with single target antibiotics (24).

Interestingly, while *P. aeruginosa* is recognized for its intrinsic resistance mechanisms such as low outer membrane permeability and active efflux pumps, the *M. oleifera* extract still produced measurable inhibition (25). This observation further supports the hypothesis that plant derived compounds may bypass conventional resistance pathways. The comparatively lower inhibition observed against *E. coli* may reflect strain specific resistance mechanisms or differences in membrane composition, however, the extract nonetheless demonstrated meaningful antibacterial activity even against this globally dominant uropathogens (26).

This study directly addresses a critical gap in existing literature by focusing on the *in vitro* evaluation of *M. oleifera* against MDR uropathogenic isolates, rather than susceptible strains or non urinary pathogens. Moreover, while many prior studies have reported antimicrobial activity of *M. oleifera* in general terms, few have systematically linked its efficacy to pathogens of high clinical relevance in UTIs or contextualized findings within the framework of antimicrobial resistance. By integrating resistance profiling with plant extract efficacy, the present work provides a more clinically meaningful assessment and establishes a stronger rationale for further pharmacological development (27).

Conclusion

In conclusion, this study demonstrates that *M. oleifera* leaf extract possesses significant *in vitro* antibacterial activity against MDR Gram negative uropathogens, including *Enterobacter*, *K, pneumoniae*, *P. aeruginosa*and *E. coli*. The extract exhibited concentration dependent inhibition and retained efficacy against pathogens showing extensive resistance to multiple antibiotic classes. These findings highlight the potential of *M. oleifera* as a promising source of alternative or adjunct antimicrobial agents for the management of drug resistant UTIs.

Strengths and Limitations

A key strength of this study is the use of clinically isolated MDR uropathogens, enhancing the translational relevance of the findings. Additionally, standardized microbiological and susceptibility testing methods were employed to ensure reproducibility and reliability.

However, the study is limited by its reliance on *in vitro* assays which do not fully capture pharmacokinetic, pharmacodynamic and host pathogen interactions occurring *in vivo*. Furthermore, crude extracts were used rather than

purified bioactive compounds which may result in variability in antibacterial potency.

Future Perspectives

Future investigations should focus on the isolation and characterization of specific antibacterial compounds within M. *oleifera* leaves, elucidation of their molecular mechanisms of action, and evaluation of potential synergistic effects with conventional antibiotics. *In vivo* studies and toxicity profiling are essential to assess safety, bioavailability, and therapeutic potential. Ultimately, well designed clinical trials will be required to validate the applicability of M. *oleifera* based interventions in the clinical management of MDR UTIs.

Author Contributions

M.Z. conceptualized the study, conducted laboratory experiments, collected and analyzed data, and drafted the original manuscript. B.R contributed to study design, data interpretation, and critical revision of the manuscript. A.W and S.A contributed to writing and reviewing the original draft and data analysis.

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Ethics Statement

Ethical approval was not required for this study as it involved *in vitro* experiments using previously identified bacterial isolates and did not involve human participants or animal subjects.

Data Availability Statement

All data generated or analyzed during this study are included in this published article. Additional details are available from the corresponding author upon reasonable request.

Conflict of Interest

The authors declare no conflict of interest.

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