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Assessment of Invitro Antibacterial Activity of *Moringa oléifera* and *Murraya koenigii* Leaf Extracts Against Clinically Important Bacteria

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ABSTRACT

Resistant gram-negative bacteria are increasing in prevalence, causing health concerns, particularly in hospitals and intensive care units, leading to increased healthcare expenses due to sickness, and death. One frequent bacterial infection that affects many people is Urinary Tract Infection (UTI), both men and women, with women. **Objective:** This study was conducted to assess the anti-microbial activity of leaf extracts from *Murraya Koenigii* (Mk) and *Moringa oleifera* (Mo) against multidrug-resistant Klebsiella pneumoniae (MDR-Kp) in vitro. **Methods:** It was a Preclinical in-vitro study, carried out at Ziauddin University from December 2022 to May 2023. Using a rotary evaporator, MO and MK leaves were extracted. Utilizing the Agar well diffusion assay and the broth dilution assay, the antibacterial activity of both plants were assessed. **Results:** For both extracts, concentrations ranging from 7.812 mg/ml to 500 mg/ml were prepared in 10% Dimethyl Sulfoxide (DMSO). Minimum Inhibitory Concentration (MIC) of *Murraya Koenigii* leaf extract was found to be 15mg/ml against MDR-Kp. *Moringa oleifera* leaf extract did not exhibit any discernible antibacterial action against MDR-Kp at any of the tested concentrations. **Conclusions:** While MOLE did not impede the growth of MDR-Kp strains at the tested doses, MKLE hindered the growth of MDR-Kp strains at 15 mg/ml(MIC).

INTRODUCTION

Resistant gram-negative bacteria are increasing in prevalence, causing health concerns, particularly in hospitals and intensive care units, leading to increased healthcare expenses due to sickness, and death. One frequent bacterial infection that affects many people is Urinary Tract Infection (UTI), both men and women, with women being more likely to develop it due to the easier penetration of microorganisms through the shorter female urethra [1]. The traditional warning signs and symptoms of UTI include discomfort at the cost vertebral angle, dysuria, and frequent or urgent urination [2]. It has been documented that there have been 15 billion cases of UTIs reported worldwide [3]. *Escherichia coli* and *Klebsiella pneumoniae* are the notable pathogens causing resistant UTIs globally, leading to kidney damage, scarring, and failure if untreated [4]. With a percentage of 73.7%, *Escherichia coli* is by far the most dominant organism that causes UTIs, followed by *Klebsiella pneumoniae* (30%), *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* [5]. UTI often occurs due to the reverse movement of bacteria into the bladder, with *Escherichia coli* and *Klebsiella pneumoniae* being more infectious and resistant to complement due to its production of cytotoxin. Unique physical features of the gram negative organisms include type 1 fimbriae, which stick to urothelial cells and travel upstream with the help of

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their flagella [6]. The management of UTI includes treatment with broad-spectrum antibiotics including nitrofurantoin, fosfomycin, trimethoprim-sulfamethoxazole, and quinolones [7, 8]. However, the misuse of antibiotics leads to development of Antibiotic Resistance Worldwide (ABR), causing the strains that are resistant to several medications to arise (MDR strains) [8]. MDR bacteria show resistance to penicillin and cephalosporin as well as to non-β-lactam antibiotics such as fluoroquinolones, trimethoprim-Sulfamethoxazole or aminoglycosides. The frequency of strains of MDR-Ec and MDR-Kp has increased; however, their patterns of their antimicrobial susceptibility vary from country to country [9]. Additionally, research indicates that MDR-Ec and Kp is becoming alarmingly more common worldwide, especially in South Asia [10]. The application of broad spectrum antibiotics has been limited due to resistance mechanisms produced by MDR-Ec and MDR-Kp [11]. Since the organisms have developed resistance to the different antibiotics, it is important to identify new treatment choices that are less expensive, more effective, tolerable, and less likely to cause side effects. Numerous beneficial properties, including antibacterial, antifungal, antipyretic, anti-inflammatory, antiulcer, antispasmodic, diuretic, antihypertensive, cholesterol-lowering, antioxidant, anti-diabetic, hepatoprotective, anticancer, cardiac and circulatory stimulants, have been discovered in *Moringa oleifera* (Mo) leaves [12]. A 2024 study revealed that employing curry leaf extract inhibited *carbapenem-resistant streptococcus pnemoniae*, which provides compelling evidence for further research into the antibacterial activities of curry leaves against MDR-Kp [13]. According to reports, *Murraya koenigii* (Mk) leaves have similar benefits, including the ability to heal wounds, prevent cancer, reduce inflammation, act as an antioxidant, fight infection, fight fungal growth, lower blood pressure, hepatoprotective against hypercholesterolemia, and fight diabetes [14, 15]. This investigation was carried out to assess the antibacterial activity of two consumable leaves over MDR-Kp, taking into consideration their previously reported antibacterial activities against a number of pathogens, in light of their known antibacterial characteristics against a variety of organisms.

METHODS

At Ziauddin University, an in-vitro pre-clinical experimental investigation was conducted between December 2022 and May 2023.The urine used in the experiment was taken from the Microbiology Laboratory at Ziauddin Hospital. In order to separate the bacteria MDR-*Kp* from the urine, these samples underwent further processing. A rotary evaporator was used to extract plants. The broth dilution assay and Agar well diffusion methods were employed in estimating the minimum inhibitory concentration. Included were UTI samples that demonstrated MDR-*Kp* growth. Agar plates on which other species were growing were excluded. The Ziauddin University Ethics Review Committee granted Waiver for the research and issued study approval, Reference Code: 5510622HAPHA [16]. Assortment and verification of plant: Fresh MK and MO leaves were purchased from Karachi's retail market. The plants were washed and then left in a shaded spot for two weeks to air dry. Plant authentication was performed by a botanist from the University of Karachi, department of Herbarium. Voucher number 96826 and 96827, were assigned to MK and MO specimens respectively. MO Leaf and MK Leaf Extract Preparation: Both plant leaves, weighing 500 g, were finely pulverized by machine. By immersing the 50g of powder residue in 500 mL of 80% ethanol in a stoppered flask, the residue was extracted. After that, the mixture was passed through Whatman® filter paper, Grade 1, the solvent was removed with a rotary evaporator (at 40°C to 50°C for 5 to 20 minutes duration), and the suspension was kept in airtight bottles. The extraction process was carried out for 48 hours with intermittent pulsating. To create a stock solution, plant extracts (MOLE and MKLE) were dissolved in 10% Dimethyl Sulfoxide (DMSO). Isolation of Bacteria from Clinical Specimens: Gram stain, Microbiological examination, and biochemical tests were used to identify the isolates. Identification of MDR *K-Pneumoniae* (*Kp*) By Modified Kirby-Bauer Disk Diffusion Test: Using antibiotic discs that contained 10 µg of ampicillin, 5 µg of ciprofloxacin, 300 µg of nitrofurantoin, 30 µg of cefuroxime, and 10 µg of gentamicin, isolates were found to be multidrug resistant if they demonstrated resistance to at least one antibiotic in at least three distinct antibiotic groups. Furthermore, Mueller Hinton agar plates were swabbed with the bacterial colonies, and they were cultured at 37ºC for 24 hours [17, 18]. Broth Dilution Assay: The test organisms' MIC values for MOLE and MKLE were determined (2018 CLSI). In order to reconstitute the MOLE and MKLE standard solutions (200 mg/ml), 12.5 ml of distilled water and 2.5 g of leaf extract were mixed [19]. To achieve different concentrations of the stock, the obtained extract (MOLE and MKLE separately) remained serially dilute in MH broth (Mueller Hinton). Using stock solutions in three separate test tubes, three strengths of extracts were prepared, resulting in a 2.5 ml broth with extract concentrations of 500 mg/ml, 250 mg/ml, and 125 mg/ml. The vial held 0.1 mL of the inoculum for each organism used in the operation. The negative control was the MH broth. After being correctly labeled and cultured for 24 hours at 37°C, the bottles were examined for any obvious change in color. The lowest concentration at which the test organisms were unable to grow was determined to be the MIC [20]. The experiment was run in triplicates. The information was

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given as to whether turbidity was present in the falcon tubes or not. Agar Well Diffusion Method: This test was used to check MOLE and MKLE antibacterial activity against MDR-*Kp*. On the surface of MHA plates, the fresh inoculums of MDR bacterial isolates were evenly distributed in comparison with 0.5 McFarland standard. Wells with a diameter of 7 mm were punched into the inoculation plates using a sterile cork borer. A micropipette was used to transfer 50µl of various MOLE and MKLE concentrations (500, 250, 125, 62.5, 31.25, 15.625, 7.81 mg/ml) into the labeled wells. The negative control used in the experiment was 10% DMSO. The plates were incubated at 37°C for 2 days after the extract was allowed to diffuse for 15 minutes. Afterwards, the zone of inhibition, presence or absence was assessed after two days. Using a diameter scale, the zones of inhibition for MKLE and MOLE were determined [21]. Using SPSS version 22.0, data analysis was carried out. ZOI's mean and standard deviation were computed. ZOIs between several groups were compared using the ANOVA test. At the 95% confidence level, a P-value of less than 0.05 was regarded as significant.

RESULTS

A) Broth dilution test: Findings for the broth dilution test for MOLE and MKLE for MDR-Kp were shown in Figs. 1 and 2. The experiment was run in triplicate. For every plant extract, three different concentrations (500 mg/ml, 250 mg/ml, and 125 mg/ml) were assessed. MOLE: The concentration of MOLE in the falcon tubes H1, H2, and H3 was 500 mg/ml; the concentration in tubes I1 I2, and I3 was 250 mg/ml; and the concentration in tubes J1, J2, and J3 was 125 mg/ml. MKLE: The concentrations of MKLE in Falcon tubes C1, C2, and C3 were 500 mg/ml, D1, D2, and D3 were 250 mg/ml, and E1, E2, and E3 were 125 mg/ml. The three tubes for MOLE (H, I, and J) and MKLE (C, D, and E) have been shown to have a murky color, demonstrating unfavorable outcomes. Moreover, the negative control group displayed no effect. B) Agar Well Diffusion Method: The results of the Agar well diffusion assay were shown in Fig. 3 and 4. The MDR-Kp strains were tested at seven different concentrations: 7.81, 15.62, 31.25, 62.5, 125, 250, and 500 mg/ml for both extracts in seven labeled wells; DMSO was used as a negative control in the eighth well. MKLE inhibit the growth of MDR-Kp at almost all tested concentrations. The MIC was found to be 15.625mg/ml. Antibacterial activity was absent in the negative control. The Experiment was run in triplicates. In figure 4 it was depicted that MOLE did not inhibit the growth of MDR-Kp at any tested concentration. Label Tubes H, I and J: All Tubes Were Turbid Showing No Inhibitory Activity.

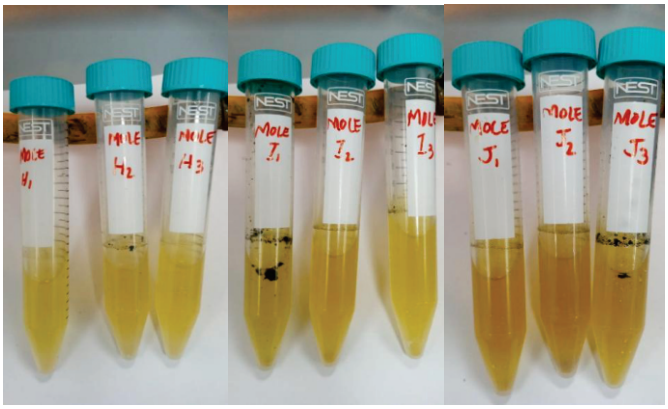

Figure 1: Result of Antibacterial Activity of Mole against MDR-Kp

Label Tubes C, D and E: All Tubes Were Turbid Showing No Inhibitory Activity

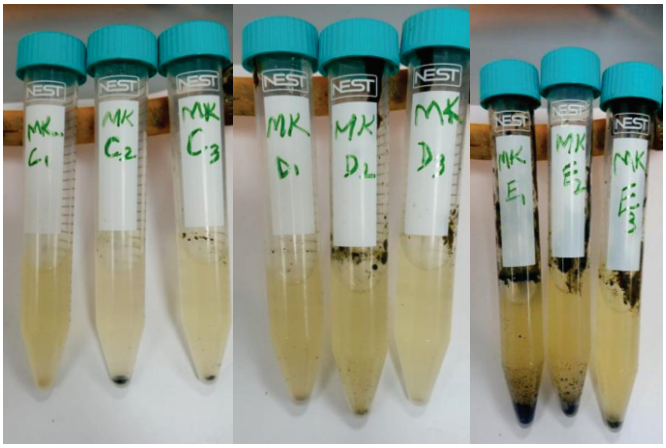

Figure 2: Result of Antibacterial Activity of Mkle against MDR-Kp

Various ZOIs were calculated using a diameter scale as per CLSI standards as follows:

Label 1 (500 mg/ml): Zone of inhibition of 18.3mm
Label 2 (250 mg/ml): Zone of inhibition of 17.3mm.
Label 3 (125 mg/ml): Zone of inhibition of 17.6mm.
Label 4 (62.5 mg/ml): Zone of inhibition of 15.3mm.
Label 5 (15.625 mg/ml): Zone of inhibition of 11.6mm.
Label 6 (lower concentrations): No inhibitory effects.
Label C (control group): No inhibitory effects.

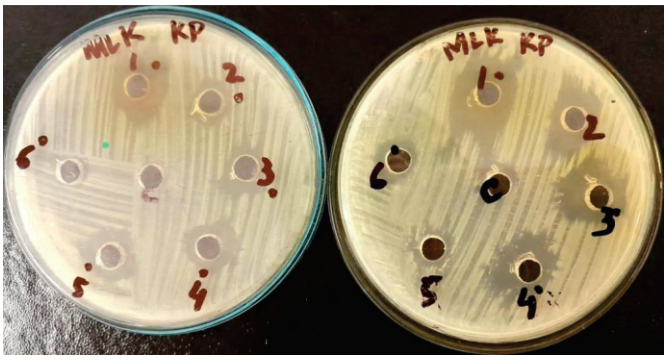

Figure 3: ZOI of Murraya Koenigii Leaf Extract (Mkle) For Mdr K.Pneumoniae –Agar Well Diffusion Using Mh Agar Plate

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ZOIs were calculated using a diameter scale as per CLSI standards as follows:
Label 1(500 mg/ml): No inhibitory effects.
Label 2(250 mg/ml): No inhibitory effects.
Label 3(125 mg/ml): No inhibitory effects..
Label 4(62.5 mg/ml): No inhibitory effects.
Label 5(15.625 mg/ml): No inhibitory effects.
Label 6(lower concentrations): No inhibitory effects.
Label C(control group): No inhibitory effects.

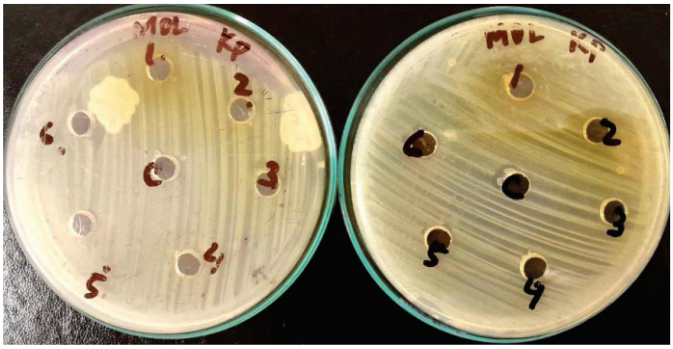

Figure 4: ZOI of Moringa Oleifera Leaf Extract (Mole) For MDR K.Pneumoniae –Agar Well Diffusion Using Mh Agar Plate

Table 1 illustrated the dose-dependent bactericidal activity of MKLE against MDR-Kp. This study's maximum dose, 500 mg/ml, resulted in an incredibly outstanding mean zone of inhibition of 18.3 mm. The mean ZOI dropped to 17.3 mm at 250 mg/ml. This drop further suggests that MKLE's potency may be reduced when the concentration was lowered from 125 to 15 mg/ml, even though it still shows antibacterial action. At doses below 15 mg/ml, no inhibitory potential was seen, indicating the absence of ZOIs. Because MKLE's antibacterial efficacy was concentration-dependent, employing larger doses has remained crucial for achieving the optimum outcomes.

Table 1: ZOI of MKLE for MDR-Klebsiella pneumoniae –Agar Well Diffusion

S. No.	Concentration of Herb (mg/mL)	ZOI (mm) (Mean ± SD)	p-value*
1	500	18.3 ± 0.57	*0.001
2	250	17.3 ± 0.57	
3	125	17.6 ± 0.57	
4	62.5	15.3 ± 0.57	
5	31.25	12.6 ± 2.08	
6	15.625 (MIC)	11.6 ± 0.57	
7	7.8125	-	
8	Control	-	

*Comparison of ZOIs of MKLE against MDR-*Klebsiella pneumoniae* at various concentrations by ANOVA

DISCUSSION

The field of medicine has confirmed that plants could serve as the basis for drugs that prevent and cure illnesses in people. Antimicrobial resistance has been identified by the WHO as a global fitness safety problem for which politicians and the general public must take comprehensive action. Surprisingly, because of their numerous issues, especially in developing nations like Pakistan, MDR pathogens were currently regarded as a general warning signal [22]. Considering the information above, it was necessary to investigate novel antimicrobial compounds obtained from healing flora in order to combat the growing threat posed by these harmful bacteria. In this study, two edible plants, MO and MK, were used to test it against MDR-Kp [23]. Ethanolic plant extracts were tested in vitro for their antibacterial efficacy against MDR Kp, which was known to cause UTI and other infections in humans. Out of the two extracts examined for this experiment, only MKLE exhibited antimicrobial action against MDR-Kp, while MOLE failed to demonstrate antibacterial activity against MDR-Kp. In line with this research, another investigation discovered that Moringa has negligible or nonexistent antimicrobial properties for MDR-Kp [24]. In this study MOLE was unresponsive to sensitivity for Kp, which was in accordance with the other studies who also reported no sensitivity of MOLE for Kp using broth dilution assay [24]. A study carried out in Saudi Arabia reported positive ZOIs ranging between 2 and 3 mm by MOLE using concentrations between (45 to 200 mg/ml) for MDR-Kp, which was in contrasts with this results. This study highlights that MDR-Kp strains that it tested may have developed resistance for the MOLE [25]. Another study Soulaimani B *et al.*, in 2020 negates this finding and supports the exceptional antibacterial activity of MOLE against MDR-Kp. According to literature many reasons may have been responsible for the inactivity of the MOLE, including the nature of solvent used and bacterial resistance. It has been discovered that bacteria that were resistant to many drugs do so by means of various changes and modifications in their structure [22, 26]. Since well-evolved resistance genes were present, it becomes more challenging to eradicate the expanding bacterial population using herbal extracts [27, 28]. Gram-negative bacteria have a double lipid layer, which increases resistance. Changes to this membrane, such as modifications to its hydrophobic characteristics or changes in its porins, can also increase resistance [25]. Furthermore, the majority of resistant infections, including UTIs, were linked to the formation of biofilm. By acting as a barrier, biofilm shields microorganisms from the effects of antimicrobials. Target genes linked to virulence in gram-negative bacteria another possible explanation for the plant extract potential lack of effectiveness against MDR-Kp could be related to the biofilm-forming Kp (fimH gene and papC gene) found in Kp isolates suffering from urosepsis [26]. Therefore, it was essential to look for possible biofilm impeders with a unique or multimodal mode of action. The establishment of a robust resistant bacteria biofilm may be a contributing factor to the inefficiency of the MOLE against MDR-Kps, in addition to the resistance mechanisms previously highlighted,

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particularly the efflux pumps. Furthermore, microbes have the ability to alter the chemical properties of the active substances in plant extracts, which further lessens their antimicrobial efficacy [23]. A significant amount of ethno medical research would be necessary to compare plant extracts with antimicrobial qualities since regional variables like environment influence the genomic and bodily alterations that occur in plants around the globe. Consequently, as this study also showed, a herb that works for a microbe in a particular area of the biosphere might be in-effective in different area. It was strongly advised to combine these plant extracts with additional plants and antibiotics to create a new ecological medication in order to combat such a resistant bacterium. MKLE was shown to be efficacious against MDR-Kp in this research at all tested concentrations (15 to 500 mg/ml), demonstrating the wide ZOIs between 11mm to 18mm. This investigation yielded a MIC of 15.625 mg/ml for MKLE against MDR Kp, which was consistent with previous investigations on the MDR-Kp employing other plant extracts. The ethyl acetate extract of MK leaves gave MIC value between 15.63-1000 µg/mL against *S. aureus, E. coli* and other bacteria [25]. MK Leaves contain a pharmacologically significant amount of alkaloids (such as mahanine, girinimbine, and koenimbine), flavonoids (such as quercetin, rutin, catechin, and myricetin), triterpenoid (mahanimbine), and phenolic compounds (such as caffeic acid, ferulic acid, and pcoumaric acid). The most common mechanism of antimicrobial action reported by these phytochemicals was the disruption of the plasma membranes of the tested bacteria (*Escherichia coli, Staphylococcus aureus*) that it assumes might have been responsible for the activity of MKLE against MDR-Kp. Since MDR pathogenic microorganisms because UTIs, this research has demonstrated that novel MKLE was effective against MDR-Kp and has provided a preview of upcoming future therapeutic improvements. Therefore, further studies investigating the mechanism of action behind its activity should be carried out to endorse its antibacterial potential.

CONCLUSIONS

MKLE showed antibacterial potential against MDR-Kp and inhibited its growth at 15 mg/ml concentration however, at all tested concentrations, MOLE failed to demonstrate the ability to impede the growth of MDR-Kp strains.

Authors Contribution

Conceptualization: HA, ZI, MOI

Methodology: HA, ZI, AA, MOI

Formal analysis: HUR, SM

Writing, review and editing: HA, ZI, AA, MOI, HUR, SM

All authors have read and agreed to the published version of the manuscript

Conflicts of Interest

All the authors declare no conflict of interest.

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