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Evaluation of Antibacterial Efficacy and Antibiotic Synergy of *Moringa oleifera* Leaves Extracts Against some Clinical Pathogenic Bacterial Strains

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ABSTRACT

The rise of multidrug-resistant bacteria poses a significant threat of "incurable" infections, highlighting the urgent need for new infection-fighting strategies. This study evaluated the antimicrobial activity of aqueous and ethanolic extracts of *Moringa oleifera* leaves against various bacterial pathogens and their potential synergistic effects with commonly used antibiotics. The results showed that the ethanolic extract significantly increased antibiotic sensitivity in several bacterial strains. *Staphylococcus aureus* displayed inhibition zones of 7 mm at a 5% concentration and 15 mm at a 40% concentration Additionally, *Pseudomonas sp*. exhibited inhibition zones ranging from 0 mm at 5% to 8 mm at 40%. In contrast, the aqueous extract demonstrated no antimicrobial activity, with 0 mm inhibition zones across all tested concentrations. Furthermore, the aqueous extract increased the sensitivity of *S. aureus* to antibiotics S10 and C30, indicating a synergistic effect, with p-values < 0.001. However, combinations with Gram-negative bacteria, such as *Escherichia coli* and *Salmonella*, resulted in antagonistic effects, reducing antibiotic effectiveness. While the ethanolic extracts improved efficacy against *S. aureus* and were effective against *Pseudomonas*, they were ineffective against other Gram-negative bacteria, indicating limitations in their antimicrobial spectrum. Further research is needed to enhance the efficacy of Moringa extracts against a broader range of bacterial species. These findings suggest that *Moringa oleifera* has potential as a synergistic agent to boost antibiotic efficacy, but the antagonistic properties observed with certain pathogens highlight the need for careful application, as it may reduce the effectiveness of antibiotics against disease-causing bacteria.

تقييم الفعالية التضادية والتآزرية مع المضادات الحيوية لمستخلصات أوراق نبات المورينجا *Moringa oleifera*) ضد بعض السلالات البكتيرية الممرضة)

آمنة علي الفتحي المجدوب[1]*، أحمد صالح ضو مصباح[1]، مريم بشير فضل[1]، هند محمدخلف الله[2]

يُشكل ارتفاع مقاومة البكتيريا متعددة الأدوية تهديدًا كبيرًا لحدوث "عدوى غير قابلة للعلاج"، مما يبرز الحاجة الملحة لاستراتيجيات جديدة لمكافحة العدوى. وقد هدفت هذه الدراسة لتقييم النشاط المضاد للميكروبات لمستخلص أوراق نبات المورينجا (Moringa oleifera) المائي والإيثانولي ضد مجموعة من مسببات الأمراض البكتيرية، وكذلك تقييم التأثيرات التآزرية المحتملة للمستخلص مع المضادات الحيوية الشائعة الاستخدام. أظهرت النتائج أن المستخلص الإيثانولي له نشاط مضاد للباكتيريا ضد عدة سلالات بكتيرية. حيث أظهرت المكورات العنقوديةالذهبية *Staphylococcus aureus* مناطق تثبيط بمقدار 7 مم عند تركيز 5% و15 مم عند تركيز 40%. بالإضافة إلى ذلك، أظهرت الزائفة الزنجارية *Pseudomonas sp*. مناطق تثبيط تتراوح من 0 مم عند 5% إلى 8 مم عند 40%. في المقابل، لم يظهر المستخلص المائي أي تأثير مضاد للميكروبات مع كل الانواع البكتيرية المختبرة، حيث كانت مناطق التثبيط 0 مم مع جميع التركيزات المستخدمة. أما بالنسبة للفعالية التآزرية لأوراق نبات المورينجا فقد زاد المستخلص

المائي من حساسية S. aureus تجاه المضادات الحيوية S10 وC30، مما يشير إلى وجود تأثير تآزري، ($p < 0.001$). ومع ذلك، أدى خلط المستخلص مع المضادات الحيوية إلى آثار معاكسة "تضادية" عند التعامل مع البكتيريا سالبة الجرام، مثل الإشريكية القولونية Escherichia *coli* والسالمونيلا *Salmonella*، مما قلل من فعالية هذه المضادات الحيوية. كما اظهر المستخلص الإيثانولي فعالية تآزرية مع المضادات الحيوية المستخدمة ضد *S. aureus* و*Pseudomonas*، لكنه لم يظهر فعالية تآزرية ضد البكتيريا السالبة الجرام الأخرى، مما يشير إلى قيود في طيفها المضاد للميكروبات. هناك حاجة لمزيد من الأبحاث لتعزيز فعالية مستخلصات أوراق نبات المورينجا ضد مجموعة أوسع من الأنواع البكتيرية. تشير هذه النتائج إلى أن اوراق نبات المورينجا لديها القدرة على أن تكون عاملًا تآزريا لزيادة فعالية المضادات الحيوية، ولكن الخصائص المعاكسة التي لوحظت مع بعض مسببات الأمراض تسلط الضوء على الحاجة إلى تطبيقها بحذر، حيث قد تقلل من فعالية المضادات الحيوية ضد البكتيريا المسببة للأمراض.

INTRODUCTION

A variety of microorganisms can be found in different natural habitats. Some of these microorganisms can function as pathogens or opportunistic pathogens for humans, while others are simply commensal. However, the commensal nature of a microorganism depends on several factors(Van Baarlen *et al*., 2009); (Messerschmidt, 2020). Antibiotics changed medical practice by remarkably reducing the morbidity and mortality associated with bacterial infection. Nevertheless, infectious diseases remain the leading cause of death in the world(Dhingra *et al*., 2020). The overuse and improper use of antimicrobial agents have contributed to the rise of antibiotic-resistant microorganisms (AMR). Additionally, people traveling between countries for tourism and business play a significant role in spreading multidrug-resistant strains(Galindo-Méndez, 2020). Unfortunately, the current development of antibiotics is insufficient, and the future appears grim unless we abandon our current approach of generating synthetic antibiotics that rapidly lose their effectiveness against multidrug-resistant bacteria(Abdallah, Alhatlani, de Paula Menezes, & Martins, 2023). Antimicrobial Resistance has been acknowledged to be one among the top three major public health threats by the World Health Organization (WHO). Antimicrobial-resistant infection has been ranked third as the leading cause of death after cardiovascular diseases(Salam *et al*., 2023). WHO has noted a shortage of new antibiotics to address the problem of antimicrobial resistance. As a result, there has been a growing interest in searching for new antimicrobial compounds from natural sources as an alternative to traditional drugs to tackle the rapidly increasing issue of antimicrobial resistance(Al Saiqali, Kaiser Jamil, & Reddy, 2024). This has given scientists the impetus to search for newer and alternative microbial compounds from medicinal plants Plant-based antimicrobials are a vast and underutilized source of medications, and further investigation of plant antimicrobials is essential(Bakkiyaraj & Pandiyaraj, 2011) (Moyo, Masika, & Muchenje, 2012).Secondary metabolites such as alkaloids, terpenoids, and phenolic compounds can interfere with microbial cell membranes and inhibit essential enzymes necessary for bacterial growth (Basavegowda & Baek, 2022).Medicinal plants have been used by mankind for the treatment of various ailments for thousands of years (Pathak, Budhathoki, Yadav, Niraula, & Kalauni, 2020), WHO evaluated that 80 % of the population of some developing countries relies on herbal medicine for some aspect of primary health care(Kheir, Kafi, & Elbir, 2014).

Moringa (*Moringa oleifera* L.) is a plant of the genus Moringa in the family Moringaceae (Ruttarattanamongkol & Petrasch, 2015). Native to the Indian subcontinent, it has naturalized in tropical and subtropical regions worldwide (Dixit, Tripathi, & Kumar, 2016). It thrives in arid and semi-arid areas and can tolerate extended droughts. The species tolerates soils with a pH range of 4.5 to 8, but neutral or slightly acidic soils are preferable. After only six months, it grows to 4 meters tall, reaching 10 meters in only 20 years (Al-Khalasi, Al-Ghafri, Al-Saqri, & Al-Khatri, 2023). In the traditional Indian medical system, *Moringa oleifera* was used externally in poultices and ointments on wounds to heal infections and abscesses(Abd Rani, Husain, & Kumolosasi, 2018).

This plant is called the miracle tree, the most valuable multipurpose tree in the world. (M. Ahmed *et al*., 2023). It is an incredible source of minerals, polyunsaturated fatty acids, glucosinolates, flavonoids, phenolic acids, carotenoids, tocopherols, and folate (Mursyid, Annisa, Zahran, Langkong, & Kamaruddin, 2019). Moringa leaves enclose more than 14 amino acids, carbohydrates, proteins, vitamins A, C, and E, calcium, magnesium, and other nutrients necessary for the human body, making it a natural energy enhancer As a result of its components, the Moringa plant possesses antioxidant, diuretic, hypotensive, cholesterol-lowering, anti-ulcer, antispasmodic, antipyretic, antiepileptic, anti-inflammatory, antifungal, and antibacterial properties. (M. Ahmed *et al*., 2023). Besides its medicinal and nutritional characteristics, *Moringa oleifera* has a low demand for soil nutrients and can even grow on a stack of granite stones, which creates good opportunities for cultivation in areas where the soil is barren(van den Berg & Kuipers, 2022).The emergence of multidrug-resistant bacteria poses a significant threat to global health, particularly in intensive care units where combination therapy is often the most recommended treatment for microbial infections. This approach is essential, as not all pathogens respond to monotherapy. The strategic use of natural products alongside synthetic antibiotics has gained traction as an effective means to combat resistant microorganisms (Ferreira *et al*., 2018).

Understanding these mechanisms highlights the importance of exploring synergistic interactions that can enhance the efficacy of antibiotics (Li, Plésiat, & Nikaido, 2015). Plant extracts can enhance antibiotic effectiveness by modifying membrane permeability, increasing drug absorption, and inhibiting resistance enzymes, thereby restoring the efficacy of antibiotics against resistant strains (Sinaga, Hanafi, & Yantih, 2021).

Many in vitro studies have demonstrated the inhibitory activity of the variant extracts from different parts of M.*oleifera* on Gram-positive bacteria(Kheir *et al*., 2014) *(Enterococcus faecalis*(Al Saiqali *et al*., 2024)*Streptococcus mutans*(Shafiq, Mahdee, & Mohammed Hasan, 2024), *Staphylococcus aureus* (Al-Khalasi *et al*., 2023; Al Saiqali *et al*., 2024), and *Staphylococcus epidermidis*(Mursyid *et al*., 2019)) and Gram-negative bacteria (, *Proteus vulgaris*(Moyo *et al*., 2012), *Klebsiella pneumoniae*(Wahyuningsih, Sumaryono, & Chaidir, 2021), *Escherichia coli*(Al Saiqali *et al*., 2024; Moyo *et al*., 2012; Wahyuningsih *et al*., 2021), and *Pseudomonas aeruginosa*(Wahyuningsih *et al*., 2021)).

The aim of this study was to evaluate the antimicrobial activity of aqueous and alcoholic extracts of *Moringa oleifera* leaves against various bacterial pathogens, as well as to investigate the potential synergistic effects of the Moringa extracts with commonly used antibiotics.

MATERIALS AND METHODS

This study utilized an experimental research design with a static-group comparison approach, conducted at the microbiology laboratory of the Department of Medical Laboratories, College of Medical Technology, Wadi Al-Shati University.

Plant material

Fresh Moringa leaves were collected from trees cultivated on a farm in the Ashkida-Al-Shati area. The leaves were carefully harvested and placed in clean, dry, and sealed plastic bags. They were then washed and rinsed with distilled water to eliminate dust and impurities. After washing, the leaves were air-dried at room temperature. Once fully dried, they were ground into a fine powder using an electric grinder and stored in airtight containers for subsequent analysis.

Extraction process

To obtain the aqueous extract,100 grams of ground M. *oleifera* leaves powder was added to 500 ml of sterile distilled water, mixed thoroughly, and allowed to infuse for 24 hours at room temperature. The mixture was then filtered using Whatman No.1 filter paper. The filtrate was collected and placed in sterile glass Petri dishes and dried in an oven at 40°C for 24 to 48 hours until completely dry. The dried extract was then scraped off with a sterile scalpel and stored in the refrigerator until use (Khan *et al*., 2013) .

To obtain the alcoholic extracts,100 grams of ground M. *oleifera* leaves powder was dissolved in 500 ml of 75% ethyl alcohol by stirring continuously. The mixture was soaked in a 1000 ml opaque glass beaker for 24 hours at room temperature. The solution was then filtered using Whatman No.1 filter paper. The supernatant filtrate was collected and placed in sterile glass Petri dishes and dried in an oven at 40°C for 24 to 48 hours until completely dry. The dried extract was then scraped off with a sterile scalpel and is stored in the refrigerator until use(Khan *et al*., 2013).

Preparation of Culture Media

Nutrient broth, nutrient agar, and Mueller-Hinton Agar (MHA) media were prepared according to the methods described in. Kirby1996

Bacterial Strains

Clinical bacterial isolates included: *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella species*, *Pseudomonas species*, and *Salmonella species*. The bacterial isolates were activated by culturing in nutrient broth and subsequently subculturing on nutrient agar. It was obtained from the Microbiology Laboratory at Wadi Al-Shati University**.**

Antibiotics

The study employed several antibiotics: Ampicillin (AMP) at 10 µg/ml, Amoxicillin (AML) at 10 µg/ml, Ceftazidime (CAZ) at 30 µg/ml, Ceftriaxone (CRO) at 30 µg/ml, and Imipenem (IPM) at 35 µg/ml; Streptomycin (S) at 10 µg/ml and Amikacin (AK) at 30 µg/ml; Tetracycline (TE) at 10 µg/ml; Erythromycin (E) at 15 µg/ml; Chloramphenicol (C) at 30 µg/ml; Nitrofurantoin (F) at 300 µg/ml; and Aztreonam (ATM) at 30 µg/ml. These antibiotics were essential for evaluating the antibacterial activity of the extracts and determining potential synergistic effects against various bacterial strains

Antibacterial Activity Assay

The antibacterial activity of aqueous and ethanolic Moringa oleifera extracts was evaluated using the disc diffusion method(Kirby, 1996). An extract was prepared by dissolving the dry plant extract powder in sterile deionized distilled water to obtain various concentrations of the aqueous and ethanolic extracts. The concentrations were prepared as follows: 5% (w/v), 10% (w/v), 20% (w/v), and 40% (w/v). Each concentration was thoroughly mixed and allowed to stand for a specific period to ensure complete dissolution before further use in the experiments. The density of suspension inoculated onto the media for susceptibility test was determined by comparison with 0.5 McFarland standard of Barium sulphate solution, ensuring a consistent bacterial

concentration across all test plates. This standardized suspension was then evenly spread onto agar plates. Sterile Whatman No.1 filter paper discs (5 mm diameter) saturated with 20 microliters (µL) of each extract at concentrations of 5, 10, 20, and 40 mg/mL were placed on the agar surface. All treatments were repeated three times. The plates were incubated at 37°C for 24 hours. After incubation, the zones of inhibition around each disc were measured and recorded in millimetres (mm)(Harley, 2008).

drying oven at 40°C for 24 to 48 hours until dry. The residue was then scraped off with a sterile scalpel, and the product was stored in the refrigerator until use.

Testing the Synergistic Effect of Moringa oleifera Extracts with Antibiotics

A uniform layer of bacteria was spread onto the surface of Muller Hinton Agar plates using a sterile cotton swab. Prepared antibiotic discs were then saturated with 20 µL of Moringa *oleifera* extract using semi-automatic pipettes. These extract-soaked discs were placed onto the agar plates teeming with bacteria. The plates were then incubated for 18-24 hours at 37°C. After incubation, the diameter of the zone of inhibition (the area with no bacterial growth) around each disc was measured. This zone represents the area where the Moringa *oleifera* extract inhibited bacterial growth. Finally, the size of the Moringa oleifera extract zone of inhibition was compared to the zone produced by the unsaturated antibiotic discs (Standard antibiotic discs were used as a control)، The disc was also treated with distilled water for use as a negative control sample in the dish. Three replicas of each treatment were used to compute the means of the results of the investigates(H. A. E. Ahmed, Kheiralla, & Ali, 2020).

Statistical Analysis

A one-way ANOVA test was conducted using the Statistical Package for the Social Sciences (SPSS, version 27) to assess the antibacterial activity of different concentrations of *Moringa oleifera* extracts and specific antimicrobials. The significance level for the differences was set at $p < 0.05$.

RESULTS AND DISCUSSION

The antibacterial efficacy of varying concentrations (5%, 10%, 20%, and 40%) of a Moringa plant-derived aqueous and alcoholic extract was evaluated against a panel of bacterial species, as presented in Table 1. The diameter of the inhibition zones (in millimetres) was measured, and the statistical significance of the observed changes was assessed using p-values. Additionally, the minimum inhibitory concentration (MIC) was determined. determined (N/D) for any of the organisms. The p-values reported are greater than 0.05, indicating that the results are not statistically significant.

The data presented in Table 1 shows that the aqueous extract did not exhibit any antimicrobial activity against the tested organisms. This is evident from the fact that the zone of inhibition diameter was 0 mm for all the test organisms across the different extract concentrations of 5%, 10%, 20%, and 40%. Additionally, MIC was not

Table (1): Antimicrobial Activity of Aqueous Extract of Moringa *oleifera* against test organism.

Organisms	Zone of inhibition (mm)				MIC	p- value
	5 %	10 %	20 %	40 %		
S. aureus	0	0	0	0	N/D	p>0.05 NS
Salmonella sp.	0	0	0	0	N/D	p>0.05 NS
E. coli	0	0	0	0	N/D	p>0.05 NS
Pseudomonas sp.	0	0	0	0	N/D	p>0.05 NS
Klebsiella sp.	0	0	0	0	N/D	p>0.05 NS

N/D = Not Determined, NS = Not Significant

The data presented in Table 2 shows that the ethanolic extract exhibited varying levels of antimicrobial activity. For the Gram-positive bacterium S. *aureus*, a statistically significant ($p<0.001$) dose-dependent antibacterial effect was observed. The diameter of the inhibition zone increased from 7 mm at the 5% concentration to 15 mm at the 40% concentration. The MIC for S. aureus was determined to be 5%. However, the extract did not exhibit significant antibacterial activity against *Salmonella sp*., *E. coli*, and *Klebsiella*

sp., all Gram-negative bacteria. No inhibition zones were observed at any concentration, resulting in an undetermined MIC, except s, aureus and Pseudomonas sp.

Regarding the Gram-negative bacterium *Pseudomonas sp.*, a statistically significant ($p<0.001$) concentration-dependent antibacterial effect was observed. The diameter of the inhibition zone increased from 0 mm at the 5% concentration to 8 mm at the 40% concentration. The MIC for Pseudomonas sp. was determined to be 10%.

Table 2: Antimicrobial Activity of Ethanolic Extract of Moringa *oleifera* against test organism.

Organisms	Zone of inhibition (mm)				MIC	p- value
	5%	10%	20%	40%		
S. aureus	7	8	11	15	5%	p<0.001 *
Salmonella sp.	0	0	0	0	N/D	p>0.05 NS
E. coli	0	0	0	0	N/D	p>0.05 NS
Pseudomonas sp.	0	7	7	8	10%	p<0.005*

Klebsiella sp.	0	0	0	0	N/D	p>0.05 NS

*N/D = Not Determined, NS = Not Significant, *= Statistically significant*

Data from Table 3demonstrates a significant enhancement (p-value < 0.001) in the sensitivity of *S. aureus* to antibiotics S10***Streptomycin*** and C30***Chloramphenicol*** when saturated with a *Moringa* aqueous extract. This effect was consistent across all tested concentrations (5%, 10%, 20%, and 40%) compared to unsaturated controls. Interestingly, the effectiveness of antibiotics E15erythromycin and TETetracycline noticeably decreased. Conversely, *Salmonella* exhibited increased sensitivity to antibiotics, particularly the AMB antibiotic, at 10% and 40% saturation with the *Moringa* extract. Furthermore, antibiotics CRO (10% saturation) and IPM Imipenem (10%, 20%, and 40% saturation) proved to be significantly more effective (p-value < 0.05) when combined with the *Moringa* extract compared to unsaturated controls. *Escherichia coli* displayed a similar pattern, with increased sensitivity (p-value < 0.001) to IPM antibiotic saturated with the *Moringa* extract across all concentrations tested. Additionally, the bacteria demonstrated heightened sensitivity (p-value < 0.001) to TE Tetracycline (10% and 20%), C30***Chloramphenicol*** (10%, 20%, and 40% saturation), and F300 Nitrifuraintion (10% saturation) compared to unsaturated controls. In contrast, *Pseudomonas* exhibited a distinct response, showing increased sensitivity (p-value < 0.001) to the IMP antibiotic when saturated with the *Moringa* extract at all tested concentrations. Additionally, the bacteria showed increased susceptibility to antibiotics ATM (at 20% and 40% saturation levels) and AK (at 40% saturation) when combined with the *Moringa* extract compared to unsaturated controls (p-value < 0.001). Similarly, *Klebsiella* mirrored the effect observed in other bacteria, demonstrating increased sensitivity (p-value < 0.001) to TE and C30***Chloramphenicol*** antibiotics saturated with the *Moringa* extract across all studied concentrations.

Table 3: Synergistic Effects of Aqueous Moringa oleifera Extract on Inhibition Zone Diameters of Various Bacteria in Combinat ion with Antibiotics

No.	Organisms			Inhibition Zone Diameter (mm)			
1	Staphylococcus aureus	Antibiotic type (control)		TE	S10	E15	C30
				38	7	7	35
		Extracts conc.	5%	35*	13*	35*	29*
			10 %	35*	15*	35*	31
			20 %	39	15*	35*	31
			40 %	35*	21*	25*	35
2	Salmon	Antibiotic type		AMP	IPM	CAZ	CRO
		(control)		0	0	35	0
		Extracts conc.	5%	0	33	0	15
			10 %	0	37*	0	15
			20 %	0	37*	0	17*
			40 %	0	37*	0	16
3	E. coli	Antibiotic type (control)		TE	IPM	F300	C30
				19	19	30	17
		Extracts conc.	5%	23*	31	25*	25*
			10 %	23*	33*	25*	25*
			20 %	23*	37*	27*	25*
			40 %	23*	35*	25*	25*
4	Pseudomonas sp.	Antibiotic type (control)		TE	AML	ATM	AK
				0	0	0	0
		Extracts conc.	5%	12*	0	0	0
			10 %	14*	0	7*	0
			20 %	14*	0	7*	0
			40 %	12*	0	9*	9*
5	Klebsiella sp.	Antibiotic type (control)		TE	IPM	CRO	C30
				16	16	25	11
		Extracts conc.	5%	19*	25	17*	25*
			10 %	19*	27*	17 *	25*
			20 %	19*	29*	17*	25*
			40 %	19*	31*	17*	25*

(*) indicate a statistically significant difference (p-value < 0.05) compared to the non-infused antibiotics (controls).

The data presented in Table 4 showed that several bacteria displayed increased antibiotic sensitivity when treated with an ethanolic extract of *M. oleifera* leaves. Notably, *S. aureus* exhibited a significant increase in sensitivity to antibiotics C30***Chloramphenicol*** and S10***Streptomycin*** at all tested concentrations (p-value < 0.05) compared to the control (non-infused antibiotics). Furthermore, antibiotics combined with a 20% ethanolic extract of *M. oleifera* leaves displayed significantly improved effects against *S. aureus* compared to the control, with statistically significant differences (p-value < 0.05). Similarly, *Pseudomonas* bacteria displayed heightened sensitivity to IPM Imipenem antibiotics at concentrations of 10%, 20%, and 40% (p-value < 0.05). Additionally, they showed increased sensitivity to CROCeftriaxone antibiotics impregnated with a 20% ethanolic extract of *M. oleifera* leaves (p-value < 0.05).

Escherichia coli also exhibited statistically significant heightened sensitivity to antibiotics C30*Chloramphenicol*, F300 Nitrifuraintion, and TE, impregnated with the extract across all concentrations compared to the control. Furthermore, there was increased sensitivity to IPM antibiotics at concentrations of 10%, 20%, and 40% (p-value < 0.05). *Salmonella* showed a significant increase in sensitivity to the TE antibiotic impregnated with the ethanolic extract of *M. oleifera* leaves at all studied concentrations compared to the control. Additionally, it demonstrated significantly heightened sensitivity to other antibiotics (ITM and AK amikacin) saturated with the extract (p-value < 0.05). *Klebsiella* bacteria exhibited significantly increased sensitivity to antibiotics TE, CROCeftriaxone, and C30 saturated with the extract at all concentrations (p-value < 0.05). Moreover, there was heightened sensitivity to IPM antibiotics saturated with 10% and 40% concentrations of the extract (p-value < 0.05).

Table4: Synergistic Effects of Ethanolic Moringa Oleifera Extract on Inhibition Zone Diameters of Various Bacteria in Combination with Antibiotics

No.	Organisms			Inhibition Zone Diameter (mm)			
1	Staphylococcus aureus	Antibiotic type (control)		TE	S10	E15	C30
				38	7	35	15
		Extracts conc.	5%	35*	11*	33*	27*
			10 %	35*	21*	31*	31*
			20 %	35*	21*	31*	31*
			40 %	35*	21*	29*	31*
2	Salmonella sp.	Antibiotic type (control)		AMP	IPM	CAZ	CRO
				0	35	0	15
		Extracts conc.	5%	25*	33*	0	27*
			10 %	0	37*	0	15
			20 %	0	37*	0	13*
			40 %	19*	37*	0	15
3	E. coli	Antibiotic type (control)		TE	IPM	F300	C30
				19	30	17	23
		Extracts conc.	5%	20	31*	19*	27*
			10 %	25*	33*	17	25*
			20 %	29*	37*	13*	25*
			40 %	13*	35*	7*	21
4	Pseudomonas sp.	Antibiotic type (control)		TE	IMP	ATM	AK
				0	0	0	0
		E	5%	0	25*	0	0
			10 %	0	29*	0	0
			20 %	0	19*	13*	0
			40 %	0	19*	13*	9*
5	Klebsiella sp.	Antibiotic type (control)		TE	IPM	CRO	C30
				16	25	11	19
		Extracts conc.	5%	17*	25	11	25*
			10 %	19*	29*	15*	23*
			20 %	19*	29*	12*	23*
			40 %	18*	29*	0	23*

(*) indicate a statistically significant difference (p-value < 0.05) compared to the non-infused antibiotics (controls).

Discussion

The rise of multidrug-resistant bacteria and strains with low antibiotic sensitivity poses a significant threat of "incurable" infections, highlighting the urgent need for new infection-fighting strategies. Plants have historically been vital sources of natural health products, and their antimicrobial properties, derived from secondary metabolites like alkaloids and phenolic compounds, make them effective therapeutic alternatives(Sanusi, Mainasara, Maishanu, Saidu, & Dirisu, 2017).The antimicrobial mechanisms of plant extracts include disrupting cell walls and membranes,

inhibiting microbial enzyme activity and biofilm formation, modulating gene expression, and inducing cell death (Adelakun *et al*., 2024).

In our study, the aqueous extract of *Moringa oleifera* leaves exhibited no antibacterial effect against any of the bacteria examined. Our results align with previous studies indicating that aqueous extracts of plants generally display little to no antimicrobial activity(Abdalla, Alwasilah, Mahjoub, Mohammed, & Yagoub, 2016; Adetitun, Araoye, Akinyanju, & Anibijuwon, 2013).This reinforces the notion that aqueous extracts may not possess the antibacterial potency against Gram-negative strains. Nevertheless, our findings contrast with those of Sanusi *et al.* (2017), who reported that water extracts of *Moringa oleifera* exhibited antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* (Sanusi *et al*., 2017).Additionally, Abdallah and Ali (2019) found that similar water extracts demonstrated antimicrobial effects against Micrococcus spp., *Shigella spp.*, *Salmonella typhi*, and *Enterococcus faecalis* (Abadallah & Ali, 2019). This discrepancy may be attributed to environmental factors, such as the season of harvest and the physiological stage of the plant at the time of leaf collection, which can significantly affect the chemical composition and concentration of bioactive compounds in the extracts, leading to variations in their antibacterial activity(Moyo *et al*., 2012) .Bubonja-Šonje *et al.*

(2020)noted that the low activity recorded in the disc diffusion test could be due to several factors, including a low amount of bioactive secondary metabolites in the extract embedded in the disc or due to the well-known limitations of the disc test, such as poor diffusion of secondary metabolites in the agar medium among others(Bubonja-Šonje, Knežević, & Abram, 2020).

Our study demonstrated that the ethanolic extract of *Moringa oleifera* leaves was strongly inhibitory against *Staphylococcus aureus*, with the diameter of the inhibition zone reaching up to 15 mm at the 40% concentration. This finding is particularly significant when compared to the previous study by Adetitun, Araoye *et al.* (2013), which reported minimal inhibitory

effects, with zones of inhibition around 1 mm across various concentrations(Adetitun *et al*., 2013).This observation aligns with those of other researchers, such as Eladl, Attia *et al*. (2024), who also reported that ethanolic extracts of Moringa oleifera exhibited activity against *S. aureus*(Eladl, Attia, Abdullatif, & El-Ganiny, 2024). The ethanolic extract is rich in bioactive compounds, including flavonoids and phenolic acids, known for their antimicrobial properties. The observed efficacy of this extract can be attributed to the enhanced solubility of its active constituents in organic solvents(NGEMENYA, ITOE, AWAH, ASONGANA, & NDIP, 2024).This could explain the strong inhibitory effect observed against *Staphylococcus* aureus (Enerijiofi, Akapo, & Erhabor, 2021; Sinaga *et al*., 2021). Similarly, Anzano, de Falco *et al.* (2022) indicated that apolar extracts demonstrated antimicrobial activity against Gram-positive pathogens, showing a dose-dependent reduction in microbial viability. Specifically, Azano reported around 50% effectiveness at a concentration of 4 mg/mL against *Staphylococcus species*, paralleling our results of significant inhibition(Anzano *et al*., 2022).Additionally, our extract demonstrated moderate activity against *Pseudomonas sp*. Similar to (Suhartono, Ismail, & Muhayya, 2019) .While Adetitun, Araoye *et al*. (2013) noted limited activity against this bacterium(Anzano *et al*., 2022), our findings revealed a more pronounced antibacterial response at higher concentrations. However, the extract showed no activity against the other Gram-negative bacteria examined. These results are consistent with previous studies indicating that plant extracts typically exhibit greater efficacy against Gram-positive bacteria (Bagheri, Martorell, Ramírez-Alarcón, Salehi, & Sharifi-Rad, 2020; Bobis, Dezmirean, Tomos, Chirila, & Al. Marghitas, 2015). The difference in bacterial response was possible due to the nature of the bacterial species (Isitua, Ibeh, & Olayinka, 2016).Moreover, the study conducted by Malhotra and Mandal (2018) found that the ethanol extract of *Moringa oleifera* exhibited significant antibacterial activity against various bacterial strains, with the largest inhibition zones recorded at 25 mm against *S. aureus* and 22 mm against *Escherichia coli.* These findings further suggest that the antibacterial efficacy of the ethanol extract is greater against Gram-positive bacteria compared to Gram-negative strains(Malhotra & Mandal, 2018).Gram-negative bacteria demonstrate higher resistance to antibiotics compared to Gram-positive bacteria because of their thin peptidoglycan layer and an outer membrane rich in lipopolysaccharides. This structure obstructs antibiotic penetration and promotes resistance mechanisms, including efflux pumps (Masoumian & Zandi, 2017).

Synergism refers to the interaction of two or more drugs that produces a greater effect than that of each drug individually. Pharmacodynamic synergy occurs when multiple pathways are targeted, enhancing antimicrobial effects through complementary actions (Vaou *et al*., 2022). The combined use of plant extracts can significantly improve pharmacological action by targeting these pathways simultaneously, thereby reducing the required doses and minimizing side effects (Jeong, Jung, Yum, & Hwang, 2023).The enhanced antibacterial activity of combinations of plant extracts has been well-documented in the literature (Archana & Bose, 2022) However, some interactions may diminish efficacy by neutralizing each other or forming inactive complexes (Masoumian & Zandi, 2017). Therefore, employing checkerboard synergy assays is crucial for evaluating the synergistic effects of selected medicinal plants (Jeong *et al*., 2023).The exploration of synergistic mechanisms of herbal ingredients from plant extract will not only help researchers to discover new phytomedicines or drug combinations but also help to avoid the possible negative synergy(Khodaie, Patel, Deore, Surana, & Byahatti, 2024).

Our study reveals that both ethanolic and aqueous extracts of *Moringa oleifera* significantly enhance the sensitivity of *Staphylococcus aureus* to antibiotics chloramphenicol and streptomycin. This finding aligns with the work of Das *et al*. (2015), who demonstrated that combining ethanolic *Moringa* extracts with antibiotics like amoxicillin and ciprofloxacin resulted in significant increases in inhibition zones for various bacterial strains. For instance, the inhibition zone for *S. aureus* increased from 22 mm to 24 mm when combined with amoxicillin, illustrating a consistent synergistic effect(Das, Juyal, & Ali, 2015).Similarly, we found that *Escherichia coli* exhibited a significant increase in sensitivity to antibiotics chloramphenicol, nitrofurantoin, and tetracycline after treatment with *Moringa* extracts. This observation is supported by El-Wafa and Abd El-All (2016), who reported that the methanol extract of *Moringa* enhanced the efficacy of imipenem against *E. coli*, raising the inhibition zone from 30 mm to 37 mm (El-Wafa & Abd El-All, 2016).This suggests that *Moringa* extracts may inhibit crucial microbial enzymes, enhancing the effectiveness of conventional antibiotics. Furthermore, our results demonstrated a significant increase in the sensitivity of *Pseudomonas* to Imipenem antibiotics when treated with ethanolic extracts of *Moringa oleifera*. This finding is particularly relevant in light of Dzotam *et al*. (2015), who investigated the synergistic effects of *Moringa* extracts with various antibiotics against multidrug-resistant bacteria. Their study highlighted that while some combinations showed indifference, the combination of *Moringa* extracts with

tetracycline resulted in significant synergy, underscoring the potential for enhancing antibiotic efficacy (Dzotam, Touani, & Kuete, 2015).In addition, the study by Tahany *et al.* (2010) supports our findings by demonstrating that *Moringa* extracts significantly increased inhibition zones when combined with antibiotics such as amoxicillin and tetracyclin against strains like *Staphylococcus aureus* and *Escherichia coli*. This suggests that the active compounds in *Moringa* may not only enhance antibiotic effectiveness but also potentially disrupt bacterial cell walls or inhibit essential enzymes, further supporting the notion of *Moringa* as a valuable adjunct in antibiotic therapy (Tahany *et al*., 2010).Moreover, the work of Ilanko *et al.* (2019) highlights the complexity of interactions between *Moringa* extracts and antibiotics. Their findings revealed that while some combinations, particularly with ampicillin and chloramphenicol, resulted in synergistic effects, other combinations showed antagonistic interactions with certain Gram-positive bacteria. This emphasizes the need for careful consideration of these interactions to optimize treatment strategies(Ilanko, McDonnell, van Vuuren, & Cock, 2019) .In the case of *Salmonella*, our study found a notable increase in sensitivity to the tetracycline antibiotic when treated with both ethanolic and aqueous extracts of *Moringa oleifera*. This supports the use of *Moringa* as an adjunct therapy for infections caused by this pathogen, consistent with the broader literature advocating for the incorporation of natural products into antibiotic regimens(NGEMENYA *et al*., 2024) . Lastly, the sensitivity of *Klebsiella* to antibiotics tetracycline, ceftriaxone, and chloramphenicol improved significantly with the application of *Moringa* extracts, echoing findings from Ahmed *et al*. (2020). Their study indicated that the combination of *Moringa* extracts with ampicillin significantly increased the inhibition zone against *Bacillus subtilis*, reinforcing the idea that the active compounds in *Moringa* can enhance the efficacy of antibiotics against resistant infections (H. A. E. Ahmed *et al*., 2020).

Conclusion

The study demonstrated that *Moringa oleifera* leaves aqueous extracts exhibited no antibacterial activity against the tested bacteria. In contrast, the alcoholic extract showed significant efficacy against S. aureus and *Pseudomonas.* However, it was ineffective against other Gram-negative bacteria, indicating limitations in its antimicrobial spectrum. Further research is needed to enhance its efficacy against a broader range of bacterial species. Additionally, the M. *oleifera* leaves extracts significantly enhanced antibiotic sensitivity in all examined bacteria. These findings indicate Moringa's potential as a synergistic agent to boost antibiotic efficacy, emphasizing the need for further exploration of its mechanisms and application. However, the antagonistic properties observed in certain combinations against specific Gram-negative and Gram-positive bacteria pathogens indicate that the use of M. *oleifera* leaves as a dietary supplement should be approached with caution, as it may reduce the effectiveness of antibiotics against disease-causing bacteria.

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