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Evaluation of Synthetic Drugs vs. Plant Extracts as Sustainable Antibacterial Agents against Milk-Borne Pathogens: A Greener Approach to Antibacterial Activity

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AI performed all activity of the experimental research. RH contribute in reviewing the manuscript and final data analysis in revision process. AY supervise the research and improved the interpretation of result and the overall quality of the paper.

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***Azadirachta indica, Avicennia marina*, milk-borne pathogens, antibacterial activity, antimicrobial resistance**

ABSTRACT

Milk, rich in proteins, vitamins, and minerals like calcium, is a key nutritional source but prone to contamination by pathogens, especially in regions lacking robust pasteurization. Multidrug-resistant bacteria, driven by antibiotic overuse, heighten health risks. This study evaluates *Azadirachta indica* (Neem) and *Avicennia marina* (Mangrove) extracts against milk-borne pathogens *Staphylococcus aureus*, *Escherichia coli*, and *Enterobacter aerogenes* to address antimicrobial resistance (AMR). Extraction from 50 g dried plant material using methanol, chloroform, and hexane yielded 2.56–8.74%, with methanol extracts most efficient (8.74% for *A. indica*, 4.38% for *A. marina*). Using the disc diffusion method, *A. indica* methanol extract (M1) showed superior antibacterial activity, with zones of inhibition of 38 mm (*S. aureus*) and 35 mm (*E. aerogenes*) at 400 mg/mL, outperforming ciprofloxacin (7 mm) and levofloxacin (14 mm) at 500 mg/ml. Minimum inhibitory concentrations (MICs) confirmed M1's potency (12.5 mg/ml for *S. aureus*, 25.0 mg/mL for *E. aerogenes*), linked to azadirachtin and flavonoids. *A. marina* chloroform extract (C2) was effective against S. aureus (MIC: 25.0 mg/ml) but less so at higher concentrations, possibly due to phytochemical antagonism. Both plants showed minimal activity against MDR *E. coli*, indicating Gram-negative resistance. Methanol's efficacy in extracting bioactive compounds supports its use. These results advocate *A. indica* and *A. marina* extracts as sustainable, multi-target *al*ternatives to combat antimicrobial resistance in dairy safety.

INTRODUCTION

Milk, a staple in human nutrition due to its rich composition of proteins, lipids, vitamins, and minerals, is susceptible to contamination by pathogenic microorganisms during production, handling, and storage, thereby posing substantial risks to public health (Oliver *et al*., 2005). Milk-borne pathogens, such as *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus*, and *Listeria monocytogenes*, are frequently implicated in foodborne outbreaks, with raw or unpasteurized milk serving as a primary reservoir for these bacteria (Claeys *et al*., 2013). The isolation and identification of such microorganisms from milk samples are essential steps in epidemiological surveillance and quality control, typically involving culture-based techniques, biochemical assays, and molecular methods like polymerase chain reaction (PCR) to detect virulence and resistance determinants (Amagliani *et al*., 2012).

The escalating challenge of antimicrobial resistance (AMR) among milk-associated pathogens exacerbates the public health threat, as evidenced by the detection of resistance genes conferring tolerance to beta-lactams, aminoglycosides, and tetracyclines in retail milk products (Verraes *et al*., 2014). This resistance is often linked to the overuse of synthetic antibiotics in dairy farming for mastitis treatment and growth promotion, leading to the selection and dissemination of multidrug-resistant strains through the food chain (Oliver *et al*., 2011). Consequently, conventional synthetic drugs, while historically effective, are increasingly inadequate, necessitating the exploration of alternative antimicrobial agents that minimize ecological impact and resistance development.

Plant extracts, derived from medicinal herbs rich

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in phytochemicals such as alkaloids, flavonoids, and terpenoids, represent a greener paradigm in antibacterial therapy, exhibiting broad-spectrum activity against bacterial pathogens through mechanisms including cell membrane disruption, efflux pump inhibition, and biofilm interference (Cowan, 1999; Savoia, 2012). *Azadirachta indica* (neem), thriving in arid and saline environments, contains potent phytochemicals like azadirachtin, nimbin, and quercetin, which exhibit antibacterial, antifungal, and anti-inflammatory properties, attributed to their ability to disrupt bacterial cell walls and inhibit protein synthesis (Biswas *et al*., 2002; Alzohairy, 2016). Similarly, *Avicennia marina* (grey mangrove), adapted to harsh coastal and saline conditions, produces tannins, flavonoids, and triterpenoids, which confer antimicrobial activity by altering membrane permeability and inhibiting enzymatic functions (Nayak *et al*., 2014). The resilience of both plants to extreme conditions enhances their phytochemical diversity, making them ideal candidates for extracting robust antimicrobial compounds.

Comparative evaluations have demonstrated that these natural compounds can rival or surpass synthetic antibiotics in efficacy, particularly against resistant isolates, while offering advantages in sustainability, reduced toxicity, and lower propensity for inducing resistance (Ncube *et al*., 2008; Gyawali and Ibrahim, 2014). For instance, extracts from plants like *Aloe vera*, *Curcuma longa*, and *Ocimum basilicum* have shown potent inhibitory effects on food spoilage and pathogenic bacteria, positioning them as viable alternatives in food preservation and therapeutic applications (Mostafa *et al*., 2018).

The present study addresses this gap by a comparative assessment of synthetic antibiotics versus selected plant extracts against bacterial strain isolated from milk sample. This approach not only elucidates the microbial burden in milk but also advocates for eco-friendly strategies to combat AMR, aligning with global efforts toward sustainable antimicrobial stewardship.

MATERIALS AND METHODS

Plants extraction preparation

Plant material of two plant species included in this study was collected. *A. indica* (neem) leaves were collected from the premises of Jinnah University for Women, Karachi, Pakistan and *Avicennia marina* (grey mangrove) leaves were collected from sandpit backwaters. Plant samples were washed, sterilized, rinsed with distilled water, and shade-dried. Dried material was ground into fine powder and sieved through a 100-mesh screen for extraction. Three different solvents (methanol, hexane and chloroform) were used for extraction separately for each plant. 50g of fine powder was soaked in 200 ml methanol, hexane and chloroform separately with stirring 24 h, filter through whatman filter paper no. 41 to obtain filtrate. The residues were re-extracted with same solvents separately for 24 h. Combine both the filtrates of same solvents and evaporated to dry under reduced pressure using rotatory evaporator. Total three extracts were prepared from *A. indica* (neem) leaves, including methanol extract (M1), hexane extract (H1) and chloroform extract (C1) while another three extracts were prepared from *Avicennia marina* (grey mangrove) leaves, methanol extract (M2), hexane extract (H2) and chloroform extract (C2). Extract yields were weighed, stored in clean glass vials at 5°C, and percentage yields calculated using the formula: extract yield % (R/S) × 100, where R is the weight of extracted plant residue and S is the weight of the raw plant sample.

Bacterial strains

The antimicrobial efficacy of each plant extract was assessed against three bacterial strains associated with foodborne illnesses. Three bacterial strains were used for antibacterial activity one strain of Gram positive MRSA (*Staphylococcus aureus* JUW-IB2323) and two strains Gram negative (*Enterobacter aerogenes* (JUW-IB2442)) and *Escherichia coli* (JUW-IB2552)) clinical isolates obtained from cow milk at the Department of Zoology, Jinnah University for Women.

Disc diffusion assay

Initial antibacterial activity was screened using the disc diffusion method. Different concentrations (200mg/ml, 300mg/ml and 400mg/ml) of both plant extracts were prepared in 10% DMSO. The bacterial colonies were dissolved in normal saline, and suspensions were standardized to a 0.5 McFarland turbidity, equivalent to ~1.5 × 10^8 CFU/ml (Macfarland standards: 9.95 ml of 10% H_2SO_4 in destilled water +0.05 ml of 1 % $BaCl_2$ in distilled water) and spread on Mueller-Hinton agar plates. Sterile Whatman No. 1 filter paper discs were soaked with 10 µl of varying plant extract concentrations, 10% DMSO (solvent control), and placed on inoculated plates with ciprofloxacin (500 mg/ml) of (10 µl) used as the positive control for the above Gram negative bacteria and levofloxinoxin 500mg/ml of (10 µl) as the positive control for Gram positive MRSA pathogenic bacteria. The plates were incubated at 35°C ± 2°C for 24 h. Inhibition zone diameters were measured with a vernier caliper to assess the antibacterial activity of the extracts.

Determination of minimum inhibitory concentration (MICs) of the effective plant extract

The MIC of *A. indica* methanol (M1) and chloroform

(C1) extracts, effective against *Staphylococcus aureus* and *Enterobacter aerogenes*, was evaluated using the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) protocols (CLSI, 2018). Overnight cultures in Mueller-Hinton broth (MHB) were adjusted to a 0.5 McFarland standard (~1.5 × 10^8 CFU/ml) at 630 nm and diluted to ~1.5 × 10^6 CFU/ml. Stock solutions of M1 and C1 (400 mg/ml in 10% DMSO) were serially diluted two-fold in MHB within 96-well microtiter plates (400–0.781 mg/ml). Each well contained 100 µl extract and 100 µl inoculum, yielding final concentrations of 200–0.391 mg/ml. Positive (bacteria in MHB), negative (MHB alone), and solvent (10% DMSO) controls were included, with ciprofloxacin (500 mg/ml) as the reference. Plates were incubated at 37°C for 24 h. MIC was the lowest concentration preventing visible turbidity. Non-turbid wells were subcultured (10 µl) on Mueller-Hinton agar to determine the minimum bactericidal concentration (MBC), defined as no colony growth after 24 hours at 37°C. Experiments were conducted in triplicate, with MIC values reported as the mean.

RESULTS AND DISCUSSION

Plant extract yield

The ethnobotanical data of *Azadirachta indica* (neem) and *A. marina* (mangrove) using three solvents (methanol, chloroform, and hexane) are presented in Table I. Extraction was performed using 50g of dried plant material for each plant, with yields calculated as the percentage dried extract residue. The extract yields ranged from 2.56 to 8.74% across the solvents. For *A. indica*, methanol extraction yielded the highest residue (8.74%), followed by chloroform and hexane. For *A. marina*, methanol extraction also produced the highest yield (4.38%), followed by chloroform and hexane extract, respectively. Yields are reported as mean ± standard deviation from triplicate extractions. Methanol consistently provided the highest extraction efficiency for both plants, likely due to its polarity, which effectively solubilizes bioactive compounds such as flavonoids and alkaloids (Sultana *et al*., 2007).

Antibacterial assays

The antibacterial efficacy of organic extracts from *A. indica* (neem) and *Avicennia marina* (grey mangrove) was evaluated using the disc diffusion method, a standard technique for assessing zone of inhibition (ZOI) as an indicator of antimicrobial potency. Extracts were prepared in methanol (M1 for *A. indica*, M2 for *A. marina*), chloroform (C1 and C2), and hexane (H1 and H2) at concentrations of 200, 300, and 400 mg/mL, and tested against *Enterobacter aerogenes*, *Escherichia coli*, and *Staphylococcus aureus*. Standard antibiotics (ciprofloxacin [S] and levofloxacin [L] at 500 mg/mL) and DMSO (control) were included for comparison. Data are presented in Tables II, III, IV, with ZOI values reported as mean ± standard deviation (mm).

Table I. Extract yields (%) leaves of selected plant species.

Plant species	Extraction solvent	Extract yield (%)
Azadirachta indica	Methanol (M1)	8.74
	Hexane (H1)	3.54
	Chloroform (C1)	4.26
Avicennia marina	Methanol (M2)	4.38
	Hexane (H2)	2.56
	Chloroform (C2)	3.75

Table II. Antibacterial activity of *A. indica* and *A. marina* against *Enterobacter aerogenes*.

Plant species	Extracts	Zone of inhibition (mm) at concentration		
		200 mg/ml	300 mg/ml	400 mg/ml
Azadirachta indica (Neem tree)	M1	16± 0.1	25± 0.04	35± 0.2
	C1	07± 0.2	2± 0.5	25± 0.2
	H1	1± 0.3	14± 0.03	21± 0.2
Avicenia marina (Mangrove plant)	M2	03± 0.01	04± 0.2	05± 0.2
	C2	04± 0.04	02± 0.3	04± 0.2
	H2	02± 0.3	03± 0.3	05± 0.1
Standard	S 500mg/ml	16± 0.5		
	L 500mg/ml	06± 0.1		
DMSO	Control	0		

S, Ciprofloxacin; L, Levofloxacin.

The methanol extract of *A. indica* (M1) exhibited significant concentration dependent inhibitory activity against *E. aerogenes* and *S. aureus*, with ZOI increasing concentration-dependently (16–35 mm for *E. aerogenes*; 26–38 mm for *S. aureus*), surpassing the standard drugs at 500mg/ml concentrations. *A. indica* methanol extract (M1) exhibited the lowest MIC (12.5 mg/ml) and MBC (25.0 mg/ml) against *S. aureus*, indicating superior potency, likely due to bioactive compounds like azadirachtin and flavonoids (Biswas *et al*., 2002). For *E. aerogenes*, M1 showed an MIC of 25.0 mg/mL and MBC of 50.0 mg/ml (Table V). This aligns with studies demonstrating that *A. indica* contains bioactive compounds such as azadirachtin, nimbin, and flavonoids, which disrupt bacterial cell

membranes and inhibit enzymatic activity (Biswas *et al*., 2002; Alzohairy, 2016).

Table III. Antibacterial activity of *A. indica* and *A. marina* against *Escherichia coli.*

Plant species	Extracts	Zone of inhibition (mm) at concentration		
		200 mg/ml	300 mg/ml	400 mg/ml
Azadirachta indica (neem tree)	M1	01± 0.2	01± 0.02	02± 0.1
	C1	00± 0.3	00± 0.02	01± 0.1
	H1	00± 0.1	00± 0.2	01± 0.1
Avicenia marina (mangrove plant)	M2	00± 0.00	01± 0.1	00± 0.0
	C2	01± 0.2	01± 0.1	01± 0.1
	H2	00± 0.02	01± 0.2	01± 0.2
Standard	S 500mg/ml	05± 0.4		
	L 500mg/ml	17± 0.1		
DMSO	Control	0		

S, Ciprofloxacin; L, Levofloxacin.

Table IV. Antibacterial activity of *A. indica* and *A. marina* against *Staphylococcus aureus.*

Plant species	Extracts	Zone of inhibition (mm) at concentration		
		200 mg/ml	300 mg/ml	400 mg/ml
Azadirachta indica (neem tree)	M1	26± 0.1	30± 0.1	38± 0.2
	C1	23± 0.2	23± 0.04	29± 0.2
	H1	16± 0.3	19± 0.4	25± 0.2
Avicenia marina (mangrove)	M2	18± 0.3	15± 0.1	17± 0.2
	C2	18± 0.4	22± 0.2	07± 0.2
	H2	05± 0.3	05± 0.4	15± 0.3
Standard	S 500mg/ml	07± 0.1		
	L 500mg/ml	14± 0.1		
DMSO	Control	0		

S, Ciprofloxacin; L, Levofloxacin.

The chloroform extract (C1) demonstrated high efficacy against *S. aureus* (23–29 mm) particullarly at 200 mg/ml, consistent with reports that chloroform enhances extraction of lipophilic antimicrobials (Sultana *et al*., 2007). The chloroform extract (C1) required higher concentrations (MIC: 25.0–50.0 mg/ml; MBC: 50.0–100.0 mg/ml) (Table V), aligning with studies noting methanol's efficacy in extracting polar antimicrobials (Alzohairy, 2016).

Hexane extract (H1) was notably active against *S. aureus* (16–25 mm) at lower concentrations and *E. aerogenes* (1–21 mm) at higher ones (Table II), corroborating findings that non-polar solvents extract terpenoids with antibacterial properties (Cowan, 1999). However, all *A. indica* extracts displayed minimal activity against *E. coli* (0–2 mm), likely due to the outer membrane barrier and efflux pumps in Gram-negative bacteria, which limit penetration of phytochemicals (Nikaido, 2003). Organic solvents, particularly methanol and chloroform, enhanced extraction efficiency for bioactive compounds compared to aqueous methods, likely due to better solubility of lipophilic antimicrobials (Zhang *et al*., 2018).

Table V. MIC's of the most effective plant extract against *S. aureus* and *E. aerogenes.*

Plant species	Ex-tracts	Bacterial Strain	MIC (mg/mL)	MBC (mg/mL)
Azadirachta indica	M1	*S. aureus*	12.5 ± 0.2	25.0 ± 0.3
		E. aerogenes	25.0 ± 0.3	50.0 ± 0.4
	C1	*S. aureus*	25.0 ± 0.3	50.0 ± 0.4
		E. aerogenes	50.0 ± 0.4	100.0 ± 0.5
Avicennia marina	M2	*S. aureus*	50.0 ± 0.4	100.0 ± 0.5
		E. aerogenes	200.0 ± 0.6	400.0 ± 0.7
	C2	*S. aureus*	25.0 ± 0.3	50.0 ± 0.4
		E. aerogenes	100.0 ± 0.5	200.0 ± 0.6

For *A. marina* extracts, chloroform (C2) and methanol (M2) showed selective inhibition against *S. aureus*. Methanol (M2) and chloroform (C2) extracts showed ZOI of 15–18 mm and 7–22 mm, respectively, at lower concentrations (Table IV). Chloroform extract (C2) showed notable MIC activity against *S. aureus* (MIC: 25.0 mg/ml; MBC: 50.0 mg/ml), while methanol (M2) less potent (MIC: 50.0–200.0 mg/ml; MBC: 100.0–400.0 mg/ml) (Table V). For *E. aerogenes*, all *A. marina* extracts had higher MICs (100.0–200.0 mg/ml), reflecting Gram-negative resistance due to outer membrane barriers (Nikaido, 2003). However, the efficacy declined at high concentration 400 mg/ml against *S. aureus*. (e.g., C2: 7 mm). The reduction may result from antagonistic interactions among bioactive compounds, precipitation of active compounds, or microbial defense mechanisms such as efflux pumps and biofilm formation as reported in high-concentration scenarios (Savoia, 2012; Gyawali and Ibrahim, 2014). High concentrations may lead to aggregation of phenolics or flavonoids, reducing bioavailability and promoting oxidative degradation. Both extracts were inactive against MDR *E. coli* and *E. aerogenes* (Fig. 1A, B). Hexane extract (H2) was less effective (5–15 mm against *S. aureus*) with negligible

activity against *E. aerogenes* and *E. coli*. These findings are consistent with studies indicating that *A. marina* contains tannins and terpenoids with selective efficacy against Gram-positive bacteria due to their simpler cell wall structure (Vadlapudi *et al*., 2010). The lack of activity against *E. coli* and *E. aerogenes* aligns with literature highlighting Gram-negative resistance to plant extracts due to lipopolysaccharides and efflux systems (Tegos *et al*., 2002) (Fig. 1C).

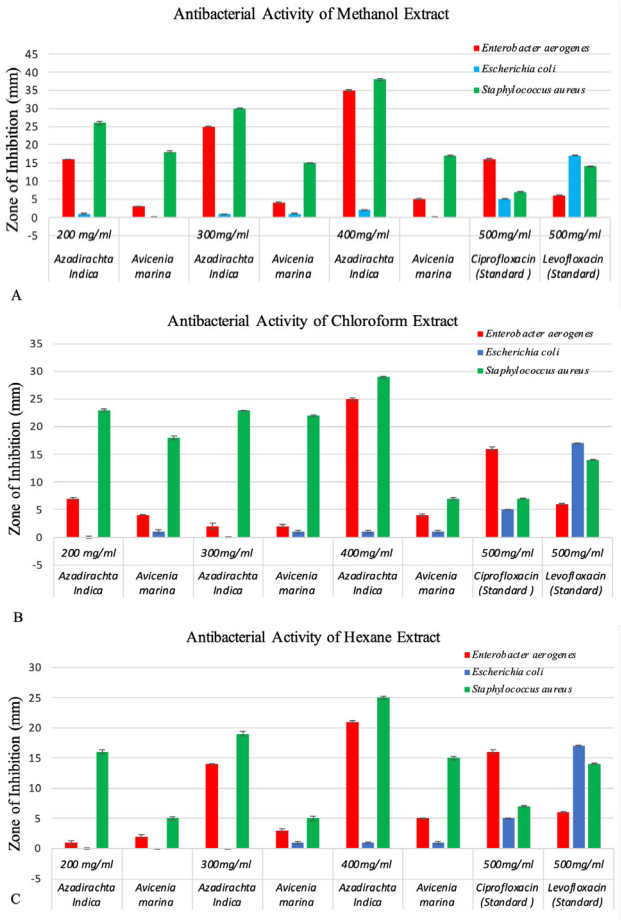

Fig. 1. Comparative antibacterial activity of *A. indica* and *A. marina* methanol (A), chloroform (B), and hexane (C) extracts.

The superior performance of *A. indica* extracts, particularly M1, against *S. aureus* (38 mm at 400 mg/mL vs. ciprofloxacin 7 mm and levofloxacin 14mm) and *E. aerogenes* (35 mm vs. ciprofloxacin 16 mm and levofloxacin 6mm) (Fig. 1A) underscores the potential of plant-based efficacy at low concentrations suggests potential as eco-friendly alternatives (Savoia, 2012). DMSO controls showed no activity, confirming extract-specific effects. Bactericidal activity supports therapeutic potential (Pankey and Sabath, 2004). These results corroborate studies showing that plant extracts target multiple bacterial pathways (e.g., membrane disruption, enzyme inhibition), reducing the likelihood of resistance compared to single-target antibiotics (Cowan, 1999; Mostafa *et al*., 2018). The limited efficacy against MDR *E. coli* reflects the challenge of overcoming Gram-negative resistance, as noted in prior research (Nikaido, 2003; Li *et al*., 2015). The observed decline in *A. marina* activity at higher concentrations supports reports of concentration-dependent antagonism or compound precipitation, which may reduce bioavailability (Savoia, 2012).

Organic solvents (methanol, chloroform) outperformed hexane in extracting bioactive compounds, consistent with findings that polar solvents efficiently extract phenolics and flavonoids with antimicrobial properties (Tuney *et al*., 2006). The synergistic potential of combining *A. indica* extracts with antibiotics, as suggested by enhanced efficacy at lower doses, aligns with studies demonstrating reduced antibiotic doses and resistance when paired with phytochemicals (Hemaiswarya *et al*., 2008). Environmentally, plant-based drugs provide sustainable options with lower ecological footprints through renewable cultivation, contrasting chemical-intensive antibiotic synthesis (Ncube *et al*., 2008). However, challenges such as variability in extract potency due to cultivation or extraction methods necessitate standardization, as emphasized in prior studies. These findings advocate plant extracts as greener adjuncts or alternatives in antimicrobial stewardship (Ncube *et al*., 2008).

CONCLUSION

The study revealed that A. indica and A. marina exibit significant antibacterial activity, especially in methanol and chloroform extracts. The methanol extract of *A. indica* showed the strongest inhibition against *S. aureus* and *E. aerogenes*, attributed to its efficient extraction of bioactive compounds such as flavonoids and alkaloids. *A. marina* extracts were selectively active against *S. aureus* but less effective against Gram-negative bacteria due to membrane barriers. Overall, methanol proved the most effective solvent. These findings highlight A. indica as a promising ecofriendly antimicrobial source and support further studies on bioactive compounds isolation and synergistic application with antibiotics.

DECLARATIONS

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Ethical statement

This study was conducted in accordance with Ethical research standards, ensuring integrity, transparency, and responsible data handling.

Generative AI and AI-assisted technology statement

The data presented in this article are original and research work performs solely by the authors, without the use of AI or automated data generation tools.

Statemenet of conflict of interest

The authors have declared no conflict of interest.

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