

ORIGINAL RESEARCH ARTICLE

Antibacterial potential, phytochemical constituents, and toxicity assessment of *Azadirachta indica* leaf extracts in combination with antibiotics

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Abstract

The rise of antimicrobial resistance (AMR) has highlighted the need for exploration of alternative therapeutic agents, including medicinal plants. This study investigates the antibacterial potential, phytochemical composition, and toxicity of *Azadirachta indica* leaf extracts, along with their interactions with conventional antibiotics. Extracts prepared in water (AQ), methanol (MeOH), and ethyl acetate (EtOAc) were evaluated for growth-inhibitory effects against a range of bacterial species, including drug-resistant isolates, through both disc diffusion and microdilution methods. Zone of inhibition (ZOI) measurements ranged from 7 to 10 mm, while minimum inhibitory concentration values varied between 2938 and 5875 µg/mL for AQ extracts, and between 1175 and 4700 µg/mL for MeOH extracts. Notably, EtOAc extracts did not inhibit bacterial growth in either assay. Fractional inhibitory concentration (FIC) analysis revealed additive interactions of extracts with β-lactam antibiotics (penicillin G, oxacillin, amoxicillin) and protein synthesis inhibitors (tetracycline, chloramphenicol), particularly against *Bacillus cereus*, *Staphylococcus aureus*, and *Shigella* spp., suggesting a potential role as antibiotic adjuvants. However, antagonistic interactions were also observed in combinations containing gentamicin and polymyxin B. Liquid chromatography–mass spectrometry (LC-MS) analysis confirmed the presence of phytochemicals, including flavonoids, tannins, and phenolics, which are known for their antimicrobial properties. Evaluation of extract safety using brine shrimp (*Artemia franciscana*) assays revealed no toxic responses. To ensure their safety for therapeutic applications, future toxicity evaluations should be conducted on mammalian cell lines to confirm these findings. Future research should focus on isolating bioactive compounds from the extracts, elucidating their mechanisms of action, and optimizing extract-antibiotic formulations to combat resistant bacterial infections effectively.

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1. Introduction

Antimicrobial resistance (AMR) develops as microbes such as bacteria, parasites, fungi, and viruses acquire resistance to drugs, which compromises standard therapies and allows infections to continue unchecked.¹ This issue represents a major challenge to global health by undermining the treatment of routine infections and contributing to extended sickness, long-term complications, and mortality. The impact of AMR on healthcare systems is profound. In the United States alone, more than 2.8 million antimicrobial-resistant infections occur each year, leading to over 35,000 deaths.² Globally, drug-resistant infections were responsible for approximately 5 million deaths in 2019, with nearly 1.3 million directly attributable to AMR. Projections suggest that if effective measures are not implemented, AMR could result in 10 million deaths annually by 2050, surpassing the mortality rates of cancer.² AMR also imposes a substantial economic burden. The World Bank estimates that by 2050, unchecked AMR could reduce global gross domestic product by 3.8% annually and push 28 million people into poverty.² In the healthcare sector, treating AMR threats contributes more than \$4.6 billion to healthcare costs annually in the United States.³ These economic strains are exacerbated by increased hospital stays, the need for more intensive care, and the use of expensive drugs.

In response to the escalating AMR crisis, there is a growing interest in exploring alternative therapeutic options, including the use of medicinal plants. Historically, plants have been a rich source of antimicrobial agents. Indeed, flavonoids, alkaloids, and essential oils extracted from medicinal plants have demonstrated significant antimicrobial properties.⁴ Recent studies have also highlighted the potential of plant-derived compounds to combat multidrug-resistant pathogens.^{4,5} For example, sophoraflavanone G isolated from *Sophora flavescens* Aiton. has potent antibacterial activity against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA), with minimum inhibitory concentrations (MICs) of 0.5–8 µg/mL.⁶ In addition, synergistic interactions of sophoraflavanone G were observed with ampicillin and oxacillin against MRSA.

The integration of medicinal plants into antimicrobial strategies offers a promising avenue to address the challenges posed by AMR.^{7,8} Their diverse chemical structures and mechanisms of action provide a valuable resource for developing new antimicrobial agents that can either serve as alternatives to, or enhance the efficacy of, existing antibiotics.⁹ However, it is crucial to conduct further research to fully understand their potential, ensure their safety, and develop effective formulations for clinical use.

The current research examines the inhibitory effects of *Azadirachta indica* A. Juss. leaf extracts on different bacterial species. Previous studies have demonstrated the antibacterial activity of *A. indica* leaf extracts against *S. aureus*, *Escherichia coli*, *Klebsiella pneumoniae* using standard disc diffusion and agar well diffusion methods.^{10–12} While these assays provide preliminary insights into the inhibitory effects of *A. indica*, they are qualitative and limited in their ability to determine the precise MIC required to inhibit bacterial growth. The absence of microdilution assays, which are the gold standard for quantifying MIC values,¹³ raises concerns regarding the accuracy and reproducibility of these findings. Without MIC determination, it is difficult to assess the true potency of *A. indica* extracts, especially against resistant strains.

Furthermore, many of these studies have not investigated the potential synergistic or antagonistic interactions between *A. indica* plant extracts and antibiotics. The combinatory effects of plant extracts with conventional antibiotics may offer valuable insights into alternative therapeutic approaches to combat AMR. However, the lack of such studies leaves a significant gap in understanding the full potential of these combinations. Addressing these gaps by incorporating rigorous MIC determination and combinational studies would provide more reliable and comprehensive data on the efficacy of plant extracts.

This research aims to bridge existing knowledge gaps by investigating the antibacterial properties of *A. indica* leaf extracts against multiple bacterial strains, including *Bacillus cereus*, *Shigella flexneri*, *Shigella sonnei*, *S. aureus*, MRSA, *E. coli*, *K. pneumoniae*, and extended-spectrum β-lactamase (ESBL)-producing isolates of *E. coli* and *K. pneumoniae*. Liquid chromatography–mass spectrometry (LC–MS) was employed to characterize compounds potentially contributing to the antibacterial effects. We further assessed extract–antibiotic combinations by calculating the fractional inhibitory concentration (FIC) index, which allowed evaluation of synergistic antibacterial effects. Furthermore, an initial toxicity examination was carried out using *Artemia franciscana* nauplii lethality assays (ALA) to evaluate the safety profile of the plant extracts. By integrating these methodologies, this study aims to provide comprehensive and reliable data on the potential of *A. indica* leaf extracts as an alternative to, or complementary strategy against bacterial resistance to, clinical antibiotics.

2. Materials and methods

2.1. Plant supplies

Powdered leaf material of *A. indica* (batch no: NLP1020) was purchased from Sattvic Goods and Services (Melbourne, Australia) and supplied by Aarshaveda. The

plant product was identified on the supplier's website using its Ayurvedic names, *i.e.*, Neem and Indian Lilac. According to the supplier, the material was verified for purity and authenticity, and originated from India, where mature leaves had been dried under ambient conditions before being finely milled. A voucher specimen (NBG-AzI0221GU) has been deposited in the School of Pharmacy and Medical Sciences, Griffith University (Southport, Australia).

2.2. Extract preparation

The extraction of *A. indica* leaf powder followed a previously described method.¹⁴ Briefly, 1 g of powdered leaves was soaked in 50 mL of sterile deionized water (AzI-AQ), methanol (AzI-MeOH), or ethyl acetate (AzI-EtOAc) and kept at room temperature with gentle agitation on a roller for 24 h. Analytical-grade solvents were sourced from Thermo Fisher Scientific Inc. (Melbourne, Australia). After extraction, the mixtures were vacuum-filtered. The aqueous extracts were subsequently lyophilized in a freeze dryer (Martin Christ, Germany) for 72 h, whereas the organic solvent extracts were evaporated in a water bath under a fume hood at 42°C. The dried material was then weighed, redissolved in 10 mL of 1% DMSO (Merck Life Science Pty. Ltd., Bayswater, Australia), filtered through 0.2 µm membranes (Sarstedt Australia Pty. Ltd., Mawson Lakes, Australia), and preserved at -20°C until further testing.

2.3. Antibiotics and bacterial strains

Antibiotics used in this study, either in powdered or disc form, included penicillin G, ciprofloxacin, polymyxin B, oxacillin, amoxicillin, erythromycin, tetracycline, chloramphenicol, gentamicin, vancomycin, Augmentin®, and cefotixin (Merck Life Science Pty. Ltd., Bayswater, Australia). Stock solutions of powdered antibiotics were prepared at 1 mg/mL for microdilution assays and stored at -20°C. Amoxicillin discs were prepared by applying 10 µL of a 10 µg/mL stock solution onto sterile filter paper discs.

Reference bacterial strains included *S. sonnei* (American Type Culture Collection [ATCC] 25931), *Salmonella enterica* serovar *typhimurium* (ATCC 14028), *S. flexneri* (ATCC 12022), *B. cereus* (ATCC 14579), *K. pneumoniae* (ATCC 13883), ESBL-producing *K. pneumoniae* (ATCC 700603), *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923), and MRSA (ATCC 43300), sourced from the ATCC through In Vitro Technologies (Noble Park North, Australia). In addition, an ESBL-producing *E. coli* clinical isolate was provided by Gold Coast University Hospital (Southport, Australia). Cultures were maintained on Mueller–Hinton

(MH) agar and broth, with MRSA grown at 35°C and all other strains at 37°C for 18–24 h.¹⁴

2.4. Antibacterial susceptibility test

The antibacterial activity of the plant extracts was examined using a modified Kirby–Bauer disc diffusion approach.¹⁴ Overnight bacterial cultures were prepared in MH broth and standardized to a 0.5 McFarland turbidity. For the assays, 100 µL of each suspension was spread evenly across MH agar plates (37°C incubation, or 35°C in the case of MRSA). Sterile 6.0 mm Whatman filter paper discs were loaded with 10 µL of extract dissolved in 1% DMSO, while discs containing reference antibiotics were included as comparators. Each assay was carried out in triplicate, and all plates were subsequently incubated under the same temperature conditions for 18–24 h.

2.5. Calculation of MIC

MICs of the extracts and reference drugs were assessed through a 96-well plate microdilution assay.¹⁴ In each well, 100 µL of MH broth was dispensed, and the first well of each row received 100 µL of extract or antibiotic solution. Serial twofold dilutions were then prepared across the row. Each well was subsequently inoculated with 100 µL of a 1:100 dilution of a 0.5 McFarland bacterial suspension, except for sterile controls. Plates were incubated at 37°C for 20–24 h. Following incubation, 40 µL of p-iodonitrotetrazolium violet (INT) (0.4 mg/mL, Sigma Aldrich, Australia) was added, and plates were further incubated at room temperature for 2–4 h. MIC values were identified as the lowest concentration of the extract or antibiotic that completely suppressed bacterial growth, confirmed by the absence of a red or pink color change. All assays were conducted in duplicate. The antibacterial activity of extracts was interpreted according to the classification proposed by Eloff,¹⁵ where MIC values are categorized as outstanding (<20 µg/mL), excellent (21–40 µg/mL), very good (41–80 µg/mL), good (81–160 µg/mL), average (161–320 µg/mL), and weak (>320 µg/mL).

2.6. Fractional inhibitory concentration analysis

Extracts and antibiotics showing antibacterial effects (MIC ≤6000 µg/mL for extracts; ≤2.5 µg/mL for antibiotics) were selected for subsequent combination testing. Extract–antibiotic mixtures were prepared in equal volumes (1:1 ratio), and their effects against the bacterial panel were assessed using the sum of fractional inhibitory concentration (ΣFIC) approach.

The ΣFIC values for each combination were calculated using the following formulas:

$$FIC(A) = \frac{\text{MIC of A in combination}}{\text{MIC of A alone}}$$

$$FIC(B) = \frac{\text{MIC of B in combination}}{\text{MIC of B alone}}$$

$$\Sigma FIC = FIC(A) + FIC(B)$$

Outcomes were interpreted as follows: synergistic when $\Sigma FIC \leq 0.5$, additive when $0.5 < \Sigma FIC \leq 1.0$, indifferent when $1.0 < \Sigma FIC \leq 4.0$, and antagonistic when $\Sigma FIC > 4.0$.¹⁶

2.7. Toxicity assays

The safety of the extracts was evaluated through the ALA.¹⁴ Briefly, 400 μL of each extract (2 mg/mL in artificial seawater) was combined with 400 μL of artificial seawater containing newly hatched *A. franciscana* nauplii (≤ 1 day old) in 48-well plates. Negative controls consisted of seawater alone (32 g/L, Red Sea), while sodium azide (1 mg/mL) served as the positive control. All assays were carried out in parallel and incubated at $25 \pm 1^\circ\text{C}$ for 24 h. Survival of the nauplii was then recorded, and lethal concentration 50 (LC_{50}) values, representing the concentration required to kill 50% of the test organisms, were determined using probit analysis. The LC_{50} values represent averages obtained from three separate experiments.

2.8. LC-MS-based metabolomic profiling

Untargeted metabolic profiling of the extracts was conducted using a Vanquish UHPLC system (Thermo Fisher Scientific) coupled to an Orbitrap Exploris 120 mass spectrometer. Separation was achieved on an Accucore™ RP-MS column with a flow rate of 0.6 mL/min, employing a 24-min gradient elution.¹⁴ The mobile phases consisted of ultrapure water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). Mass spectrometric analysis was performed in negative ionization mode using electrospray ionization, with predefined parameters for spray voltage, temperature, and gas flow. Data were processed in Compound Discoverer™ 3.3, employing the natural product unknown ID workflow. Detected metabolites were filtered against blanks and annotated through databases such as MzCloud, ChemSpider, and MassList. Identified metabolites were exported into Excel spreadsheets, and their relative levels were reported as percentages of the overall detected signal.

2.9. Statistical analysis

For disc diffusion assays, inhibition zone diameters (zone of inhibition [ZOI]) were measured in millimeters, with 6 mm taken as the baseline for non-active extracts. Data are expressed as mean $\pm$ standard error of the

mean and visualized using bar graphs based on at least three independent replicates. Statistical comparisons were carried out using one-way ANOVA, where $p < 0.01$ (* significant) and $p < 0.001$ (** highly significant) indicated statistical significance. MIC data from broth microdilution tests were analyzed in triplicate, and outcomes for each extract and antibiotic were reported separately. All MIC and FIC values were calculated following two independent experiments on separate days to ensure the reproducibility of the findings.

3. Results

3.1. Antimicrobial susceptibility analysis

The antimicrobial activity of aqueous (AZI-AQ), methanol (AZI-MeOH), and ethyl acetate (AZI-EtOAc) extracts was evaluated against ten bacterial strains using disc diffusion (ZOI) and broth microdilution (MIC) assays. The results varied significantly among the bacterial strains, with the MeOH extract demonstrating the most promising antibacterial potential (Figures 1 and 2, Table 1). In contrast, AQ and EtOAc extracts were largely inactive, exhibiting high MIC values and minimal ZOI.

B. cereus exhibited moderate antibacterial susceptibility, particularly to the MeOH extract, which produced a ZOI of 9.0 mm and the lowest MIC value (1175 $\mu\text{g/mL}$). The AQ extract produced a ZOI of 7.0 mm, but its high MIC value (5875 $\mu\text{g/mL}$) suggested weak inhibition. The EtOAc extract was entirely inactive, as indicated by a ZOI of 6.0 mm and an MIC exceeding 10,000 $\mu\text{g/mL}$.

S. flexneri was substantially more susceptible compared to *S. sonnei*. *S. flexneri* was moderately susceptible to the MeOH extract (ZOI = 10.0 mm, MIC = 4700 $\mu\text{g/mL}$), whilst the AQ extract exhibited only weak inhibitory activity (ZOI = 7.0 mm, MIC = 5875 $\mu\text{g/mL}$). The EtOAc extract was completely inactive. In contrast, *S. sonnei* was far less susceptible, showing no significant inhibition across all extracts, as evidenced by ZOI values of 6.0 mm (equivalent to the filter paper control). However, the AQ extract had an MIC of 2938 $\mu\text{g/mL}$, and MeOH exhibited an MIC of 4700 $\mu\text{g/mL}$, suggesting some minor inhibition at high concentrations. The difference in response between these two strains of the same species suggests strain-dependent variation in susceptibility to plant extracts. *Salmonella typhimurium* followed a trend similar to *B. cereus* and *S. flexneri*, with moderate activity in the MeOH extract (ZOI = 8.7 mm, MIC = 4700 $\mu\text{g/mL}$), but weak activity for the AQ extract (ZOI = 7.0 mm, MIC = 5875 $\mu\text{g/mL}$). The EtOAc extract was inactive against both bacteria.

The MeOH extract demonstrated activity against *S. aureus*, exhibiting a ZOI of 9.0 mm and an MIC of 2350 $\mu\text{g/mL}$. In contrast, neither the AQ nor EtOAc

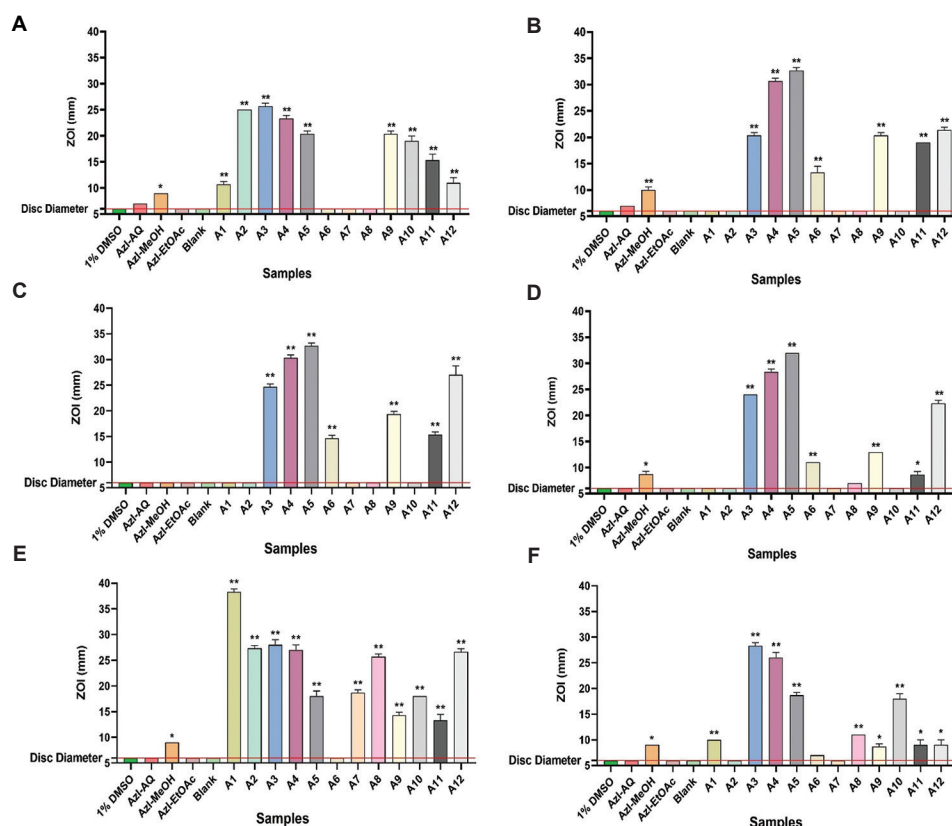


Figure 1. Antibacterial effects of *Azadirachta indica* leaf extracts were assessed using the disc diffusion method against bacterial species: (A) *Bacillus cereus*, (B) *Shigella flexneri*, (C) *S. sonnei*, (D) *Salmonella typhimurium*, (E) *Staphylococcus aureus*, and (F) methicillin-resistant *Staphylococcus aureus* (MRSA). Extracts tested included aqueous (AzI-AQ), methanolic (AzI-MeOH), and ethyl acetate (AzI-EtOAc). Negative controls consisted of 1% DMSO and sterile water (Blank). A panel of reference antibiotics was included for comparison: A1 (penicillin G), A2 (erythromycin), A3 (tetracycline), A4 (chloramphenicol), A5 (ciprofloxacin), A6 (polymyxin B), A7 (oxacillin), A8 (amoxicillin), A9 (gentamicin), A10 (vancomycin), A11 (Augmentin®), and A12 (cefoxitin). The y-axis line at 6 mm denotes the disc diameter employed in the study. Results are expressed as mean inhibition zones ($\pm$ standard error of the mean) based on three independent experiments. * $p < 0.01$, ** $p < 0.001$.

extracts exhibited any activity. Interestingly, MRSA showed a similar susceptibility pattern, with MeOH (ZOI = 9.0 mm, MIC = 2350 μ g/mL) being active whilst AQ and EtOAc extracts were ineffective. The MeOH extract was not significantly affected by the resistance mechanism of MRSA, indicating that its active compounds may act through a different pathway.

E. coli was also moderately susceptible to the MeOH extract, with a ZOI of 9.3 mm and an MIC of 4700 μ g/mL. The AQ and EtOAc extracts were inactive in both assays. The MeOH extract exhibited consistent activity against ESBL *E. coli*, with a ZOI of 9.3 mm and an MIC of 4700 μ g/mL. This suggests that the resistance mechanism in this strain does not interfere with the extract's mode of action.

None of the extracts showed activity against *K. pneumoniae* in the disc diffusion assay, with all producing ZOI values of only 6.0 mm in the disc diffusion

assay for all extracts. However, the MeOH extract produced an MIC of 4700 μ g/mL, suggesting weak antibacterial activity. AQ and EtOAc extracts were completely inactive against this bacterium. ESBL-producing *K. pneumoniae* was completely resistant to all extracts, with no measurable antibacterial effects in either assay. Thus, the ESBL-producing *K. pneumoniae* strain was the most resistant bacterium screened in this study.

3.2. Combinational analysis: Fractional inhibitory concentration determination

The Σ FIC analysis revealed varying degrees of interaction between *A. indica* extracts and reference antibiotics against the tested bacterial strains (Table 2). Additive interactions were observed in 20 instances, indicating cases where the combination moderately enhanced antibacterial activity. Indifferent interactions were the most common class of interaction, with 32 occurrences, suggesting that the extract-antibiotic combinations did not significantly

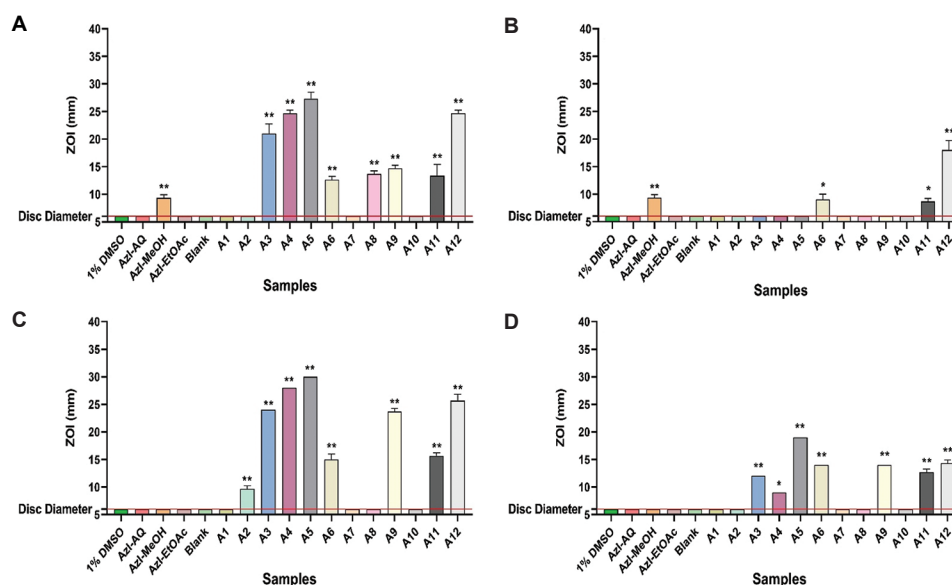


Figure 2. Antibacterial effects of *Azadirachta indica* leaf extracts were assessed using the disc diffusion method against bacterial species: (A) *Escherichia coli*, (B) ESBL-producing *Escherichia coli*, (C) *Klebsiella pneumoniae*, (D) ESBL-producing *Klebsiella pneumoniae*. Extracts tested included aqueous (AzI-AQ), methanolic (AzI-MeOH), and ethyl acetate (AzI-EtOAc). Negative controls consisted of 1% DMSO and sterile water (Blank). A panel of reference antibiotics was included for comparison: A1 (penicillin G), A2 (erythromycin), A3 (tetracycline), A4 (chloramphenicol), A5 (ciprofloxacin), A6 (polymyxin B), A7 (oxacillin), A8 (amoxicillin), A9 (gentamicin), A10 (vancomycin), A11 (Augmentin®), and A12 (cefoxitin). The y-axis line at 6 mm denotes the disc diameter employed in the study. Results are expressed as mean inhibition zones ($\pm$ standard error of the mean) based on three independent experiments. * $p < 0.01$, ** $p < 0.001$.

Table 1. Minimum inhibitory concentrations (MIC) of *Azadirachta indica* leaf extracts (AzI-AQ, AzI-MeOH, AzI-EtOAc) and control antibiotics evaluated against a panel of ten bacterial species

Extract type or antibiotic	Bacterial species and MIC ($\mu\text{g/mL}$)									
	<i>Bacillus cereus</i>	<i>Shigella flexneri</i>	<i>Shigella sonnei</i>	<i>Shigella typhimurium</i>	<i>Shigella aureus</i>	MRSA	<i>Escherichia coli</i>	ESBL-producing <i>E. coli</i>	<i>Klebsiella pneumoniae</i>	ESBL-producing <i>Klebsiella pneumoniae</i>
AzI-AQ	5875	5875	2938	5875	-	-	-	-	-	-
AzI-MeOH	1175 ^a	4700	4700	4700	2350	2350	4700	4700	4700	-
AzI-EtOAc	-	-	-	-	-	-	-	-	-	-
A1	-	-	-	0.625	1.25	-	-	-	-	-
A2	-	-	-	0.08	0.31	-	-	-	-	-
A3	0.625	0.31	0.625	0.08	0.16	0.04	0.31	-	0.625	-
A4	-	2.5	1.25	1.25	-	-	2.5	-	2.5	-
A5	0.02	0.02	0.02	0.08	0.16	0.625	0.02	-	0.02	0.16
A6	0.31	0.31	0.31	-	-	-	0.02	0.02	0.02	0.04
A7	-	-	-	1.25	0.16	-	-	-	-	-
A8	-	-	-	0.625	0.625	-	-	-	-	-
A9	0.625	2.5	0.625	0.16	-	-	0.625	-	0.625	-
A10	-	-	-	0.625	1.25	1.25	-	-	-	-

Notes: MIC values of the extracts are categorized by activity: $>10,000 \mu\text{g/mL}$ are considered inactive (-); low activity ranges from 2000 to 6000 $\mu\text{g/mL}$; moderate activity ranges from 1000 to 2000 $\mu\text{g/mL}$, denoted with ^a. Active antibiotics are shown in bold, whilst those with MIC $>2.5 \mu\text{g/mL}$ are marked as inactive (-). Reference antibiotics: A1 (penicillin G), A2 (erythromycin), A3 (tetracycline), A4 (chloramphenicol), A5 (ciprofloxacin), A6 (polymyxin B), A7 (oxacillin), A8 (amoxicillin), A9 (gentamicin), A10 (vancomycin).

impact each other's effectiveness. Antagonistic interactions were noted in 17 cases, demonstrating instances where the

combination reduced antibacterial efficacy. These results not only highlight the potential of *A. indica* extracts

Table 2. Fractional inhibitory concentration (Σ FIC) indices from interactions between *Azadirachta indica* leaf extracts and reference antibiotics

Bacteria	Extracts	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
<i>Bacillus cereus</i>	AzI-AQ	0.62 ^a	2.06	2.06	1.50	1.03	-	1.00a	2.50	8.30b	1.25
	AzI-MeOH	0.51 ^a	2.25	2.25	1.50	2.25	-	0.51 ^a	0.51 ^a	8.81 ^b	2.00
<i>Shigella flexneri</i>	AzI-AQ	-	-	0.63 ^a	0.75 ^a	1.03	31.50 ^b	-	-	1.25	-
	AzI-MeOH	-	-	0.63 ^a	0.75 ^a	2.06	31.50 ^b	-	-	>4 ^b	-
<i>Shigella sonnei</i>	AzI-AQ	-	-	1.25	0.75 ^a	1.06	31.75 ^b	-	-	0.75 ^a	-
	AzI-MeOH	-	-	4.50 ^b	1.00 ^a	2.06	31.50 ^b	-	-	1.00 ^a	-
<i>Shigella typhimurium</i>	AzI-AQ	-	-	1.25	1.00 ^a	2.06	4.53 ^b	-	-	>4 ^b	-
	AzI-MeOH	-	-	0.63 ^a	1.00 ^a	1.06	4.53 ^b	-	-	>4 ^b	-
<i>Shigella aureus</i>	AzI-MeOH	1.00 ^a	1.25	1.13	-	2.19	-	2.25	0.75 ^a	-	1.00 ^a
MRSA	AzI-MeOH	-	-	1.03	-	1.50	-	-	-	-	2.00
<i>E. coli</i>	AzI-MeOH	-	-	1.06	1.00 ^a	1.06	>4 ^b	-	-	1.06	-
ESBL-producing <i>E. coli</i>	AzI-MeOH	-	-	-	-	-	63.00 ^b	-	-	-	-
<i>Klebsiella pneumoniae</i>	AzI-MeOH	-	-	1.25	>4 ^b	2.13	63.00 ^b	-	-	>4 ^b	-

Notes: The Σ FIC values of *A. indica* extracts—aqueous (AzI-AQ), methanolic (AzI-MeOH), and ethyl acetate (AzI-EtOAc) were assessed in combination with standard antibiotics against *Bacillus cereus*, *Shigella flexneri*, *Shigella sonnei*, *Shigella typhimurium*, *Shigella aureus*, MRSA, *E. coli*, ESBL-producing *E. coli*, and *Klebsiella pneumoniae*. Additive effects (Σ FIC>0.5–1.0) are indicated with ^a; indifferent effects (>1.0–4.0) are shown with the exact values; and antagonistic effects (>4.0) are indicated with ^b. A dash (–) indicates inactivity of either the extract or antibiotic. Reference antibiotics: A1 (penicillin G), A2 (erythromycin), A3 (tetracycline), A4 (chloramphenicol), A5 (ciprofloxacin), A6 (polymyxin B), A7 (oxacillin), A8 (amoxicillin), A9 (gentamicin), A10 (vancomycin).

Abbreviation: *E. coli*: *Escherichia coli*.

in combination therapy but also emphasize the need for careful selection, as antagonistic interactions may compromise treatment effectiveness. Further investigation is necessary to determine the mechanisms behind these interactions and optimize extract-antibiotic pairings for enhanced therapeutic outcomes.

3.3. LC-MS-based metabolomic profiling and compound identification

LC-MS was employed for metabolomic profiling of the extracts, and compounds were identified by comparison across several databases. The profiling aimed to capture a broad range of metabolites, with particular focus on flavonoids, phenolic acids, tannins, and terpenoids. The majority of detected compounds showed moderate to low polarity, aligning with their elution in the 30–90% acetonitrile gradient region of the chromatogram (Figure S1).¹⁷ Polar compounds such as organic acids and amines appeared at the beginning of the chromatogram, whereas less polar metabolites eluted later at higher acetonitrile levels because of their stronger interaction with the non-polar stationary phase. To maintain reliability, only compounds confirmed by at least one database were retained in the final list (Table S1). The study emphasized

phenolic acids, tannins, flavonoids, and terpenoids (Table 3) due to their documented biological importance.¹⁸

3.4. Toxicity examination

The plant extracts were evaluated for toxicity through the *Artemia franciscana* nauplii lethality bioassay.¹⁴ Toxicity was assigned to extracts that produced LC₅₀ values at or below 1000 μ g/mL after a 24-h treatment period.¹⁹ Results showed no significant difference between extracts and the negative control (artificial seawater) ($p>0.05$), as none of the extracts caused $\geq 50\%$ mortality at 1000 μ g/mL. Therefore, the tested extracts were considered non-toxic.

4. Discussion

This investigation assessed *A. indica* leaf extracts against clinically important bacterial pathogens, including drug-resistant strains. Both aqueous (AQ) and methanolic (MeOH) extracts demonstrated inhibitory activity, suppressing the growth of four and nine out of ten tested organisms, respectively, highlighting their potential as candidates for antibacterial drug development. Methanol-derived extracts exhibited the highest levels of inhibition in disc diffusion and broth microdilution assays, in contrast to the weaker activity observed with

Table 3. LC-MS–based tentative identification and percentage relative abundance of phytochemicals detected in *Azadirachta indica* extracts

Retention time (minutes)	Molecular weight	Empirical formula	Putative compounds	Relative abundance (% of total area)		
				AQ	MeOH	EtOAc
1.622	192.06319	C ₇ H ₁₂ O ₆	D-(-)-Quinic acid	0.12	0.17	-
1.648	356.0376	C ₁₄ H ₁₂ O ₁₁	(+)-Chebulic acid	0.24	-	-
1.671	134.02139	C ₄ H ₆ O ₅	D-(+)-Malic acid	-	5.76	-
1.881	322.03203	C ₁₄ H ₁₀ O ₉	Digallic acid	0.06	-	-
1.893	332.07405	C ₁₃ H ₁₆ O ₁₀	6-Galloylglucose	0.11	0.08	-
1.973	126.03156	C ₆ H ₆ O ₃	Pyrogallol	-	0.52	-
2.215	244.05813	C ₁₀ H ₁₂ O ₇	1-O-Galloylglycerol	-	0.11	-
2.928	484.08511	C ₂₀ H ₂₀ O ₁₄	Hamamelitannin	-	S	-
3.238	324.1782	C ₁₄ H ₂₈ O ₈	Caryophyllan	0.04	-	-
3.504	306.07383	C ₁₅ H ₁₄ O ₇	(+) -Gallocatechin	-	0.02	-
3.385	212.06823	C ₁₀ H ₁₂ O ₅	Propyl gallate	-	-	0.87
4.292	484.08512	C ₂₀ H ₂₀ O ₁₄	1,6-Bis-O-(3,4,5-trihydroxybenzoyl) hexopyranose	-	0.14	-
4.679	388.13699	C ₁₇ H ₂₄ O ₁₀	Geniposide	0.02	0.04	-
5.428	178.02645	C ₉ H ₆ O ₄	Aesculetin	-	0.03	-
6.89	192.02685	C ₆ H ₈ O ₇	Citric acid	0.24	0.15	-
7.114	196.07336	C ₁₀ H ₁₂ O ₄	Cantharidin	0.08	0.08	-
8.728	288.08412	C ₁₂ H ₁₆ O ₈	Phlorin	0.02	-	-
9.117	294.09484	C ₁₁ H ₁₈ O ₉	Tuliposide B	S	-	-
9.559	596.17414	C ₂₇ H ₃₂ O ₁₅	Hovetrichoside D	0.03	-	-
9.677	478.07486	C ₂₁ H ₁₈ O ₁₃	8-Hydroxyluteolin 8-glucuronide	-	0.03	-
9.758	342.13117	C ₁₆ H ₂₂ O ₈	Coniferin	-	S	-
9.996	610.15321	C ₂₇ H ₃₀ O ₁₆	Rutin	S	10.23	1.27
10.216	304.05805	C ₁₅ H ₁₂ O ₇	Nigrescin	S	0.10	-
9.758	342.13117	C ₁₆ H ₂₂ O ₈	Coniferin	-	S	-
10.252	636.0961	C ₂₇ H ₂₄ O ₁₈	1,2,6-Trigalloyl-β-D-glucopyranose	-	0.58	-
10.468	490.205	C ₂₂ H ₃₄ O ₁₂	Cymorcin diglucoside	0.02	-	-
10.494	450.11711	C ₂₁ H ₂₂ O ₁₁	Hovetrichoside C	0.02	-	-
10.58	610.15312	C ₂₇ H ₃₀ O ₁₆	Quercetin 3-O-rhamnoside-7-O-glucoside	0.02	-	-
10.66	434.04844	C ₁₉ H ₁₄ O ₁₂	Ellagic acid arabinoside	S	-	-
10.679	222.0526	C ₁₁ H ₁₀ O ₅	Isofraxidin	S	-	-
10.706	480.09005	C ₂₁ H ₂₀ O ₁₃	Myricetin 3-O-β-D-galactopyranoside	-	0.95	-
10.869	192.04216	C ₁₀ H ₈ O ₄	7-Hydroxy-6-methoxy-2H-chromen-2-one	1.32	0.55	9.26
10.885	302.00606	C ₁₄ H ₆ O ₈	Ellagic acid	1.93	4.17	-
10.893	318.00087	C ₁₄ H ₆ O ₉	flavellagic acid	0.02	-	-
10.931	254.07899	C ₁₂ H ₁₄ O ₆	Balticol C	0.02	-	-
10.955	568.21535	C ₂₇ H ₃₆ O ₁₃	Citrusin B	0.04	-	-
10.965	192.04212	C ₁₀ H ₈ O ₄	Scopoletin	-	1.06	-
10.971	190.13559	C ₁₃ H ₁₈ O	Heptanophenone	0.07	-	1.67
10.987	286.04744	C ₁₅ H ₁₀ O ₆	Maritimetin	0.02	-	-
11.026	418.16258	C ₂₂ H ₂₆ O ₈	Lirioresinol A	S	-	-

(Cont'd...)

Table 3. (Continued)

Retention time (minutes)	Molecular weight	Empirical formula	Putative compounds	Relative abundance (% of total area)		
				AQ	MeOH	EtOAc
11.026	176.04729	C ₁₀ H ₈ O ₃	4-Methylumbelliferone hydrate	0.28	-	-
11.066	262.04771	C ₁₃ H ₁₀ O ₆	Maclurin	S	S	-
11.103	524.22562	C ₂₆ H ₃₆ O ₁₁	Mascaroside	0.09	-	-
11.135	304.05816	C ₁₅ H ₁₂ O ₇	(2R,3R) -Taxifolin	0.03	-	-
11.233	464.09493	C ₂₁ H ₂₀ O ₁₂	Hyperoside	-	1.41	-
11.287	196.10975	C ₁₁ H ₁₆ O ₃	Angupyrone E	0.15	-	-
11.287	464.09536	C ₂₁ H ₂₀ O ₁₂	Myricitrin	0.61	6.71	-
11.348	312.12097	C ₁₅ H ₂₀ O ₇	Nivalenol	-	S	-
11.42	126.03156	C ₆ H ₆ O ₃	Phloroglucinol	0.16	0.16	-
11.445	522.2101	C ₂₆ H ₃₄ O ₁₁	Lariciresinol 4-O-glucoside	0.05	0.13	-
11.485	304.058	C ₁₅ H ₁₂ O ₇	Taxifolin	0.03	0.08	-
11.487	302.04207	C ₁₅ H ₁₀ O ₇	Bracteatin	S	-	-
11.487	450.11609	C ₂₁ H ₂₂ O ₁₁	Astilbin	0.06	0.25	0.40
11.493	594.15831	C ₂₇ H ₃₀ O ₁₅	Nictoflorin	-	0.36	-
11.502	598.18966	C ₂₇ H ₃₄ O ₁₅	Phloretin 3',5'-Di-C-glucoside	-	0.16	-
11.549	506.10615	C ₂₃ H ₂₂ O ₁₃	Quercetin 3- (6"-acetylgalactoside)	0.03	-	-
11.669	378.16759	C ₂₀ H ₂₆ O ₇	Carinol	S	-	-
11.684	594.158	C ₂₇ H ₃₀ O ₁₅	5,7-Dihydroxy-2-(4-hydroxyphenyl)-4-oxo-4H-chromen-3-yl 6-O-(6-deoxyhexopyranosyl) hexopyranoside	-	3.85	3.61
11.685	362.13776	C ₁₉ H ₂₂ O ₇	Machaerol B	S	-	-
11.759	448.10062	C ₂₁ H ₂₀ O ₁₁	Trifolin	0.13	0.50	1.92
11.83	302.04253	C ₁₅ H ₁₀ O ₇	Quercetin	1.08	6.59	1.43
11.845	436.13653	C ₂₁ H ₂₄ O ₁₀	Phloridzin	-	0.02	-
11.899	374.15775	C ₁₇ H ₂₆ O ₉	Deoxyloganin	0.02	-	-
11.999	264.09963	C ₁₄ H ₁₆ O ₅	1'-Acetoxyeugenol acetate	0.04	-	-
12.002	316.05803	C ₁₆ H ₁₂ O ₇	Isorhamnetin	-	0.13	-
12.018	680.37682	C ₃₆ H ₅₆ O ₁₂	Tenuifolin	-	S	-
12.076	404.11076	C ₂₀ H ₂₀ O ₉	Chalconaringenin 2'-xyloside	S	-	-
12.079	464.09535	C ₂₁ H ₂₀ O ₁₂	Quercetin-3β-D-glucoside	-	0.06	0.36
12.183	286.04753	C ₁₅ H ₁₀ O ₆	Fisetin	-	0.05	0.69
12.192	534.1007	C ₂₄ H ₂₂ O ₁₄	Kaempferol 3-O-(6-malonyl-glucoside)	-	0.10	-
12.194	338.09994	C ₁₆ H ₁₈ O ₈	4-Methylumbelliferyl-α-D-glucopyranoside	0.04	0.09	-
12.214	462.17169	C ₂₀ H ₃₀ O ₁₂	Bioside	0.04	-	-
12.258	288.06315	C ₁₅ H ₁₂ O ₆	Maesopsin	-	0.07	-
12.331	188.10475	C ₉ H ₁₆ O ₄	Azelaic acid	0.24	0.25	3.23
12.356	170.02143	C ₇ H ₆ O ₅	Gallic acid	3.32	0.15	-
12.359	286.04733	C ₁₅ H ₁₀ O ₆	Kaempferol	0.04	0.16	0.37
12.371	582.2314	C ₂₈ H ₃₈ O ₁₃	(7'R)-(+)-Lyoniresinol 9'-glucoside	0.04	-	-
12.433	348.142	C ₁₅ H ₂₄ O ₉	Ajugol	0.02	0.03	-
12.477	432.1056	C ₂₁ H ₂₀ O ₁₀	Afzelin	-	0.27	-
12.49	346.10525	C ₁₈ H ₁₈ O ₇	Hamilcone	S	-	-

(Cont'd...)

Table 3. (Continued)

Retention time (minutes)	Molecular weight	Empirical formula	Putative compounds	Relative abundance (% of total area)		
				AQ	MeOH	EtOAc
13.039	274.08413	C ₁₅ H ₁₄ O ₅	Phloretin	0.07	-	-
13.697	314.18749	C ₂₀ H ₂₆ O ₃	Kahweol	-	-	0.50
13.777	428.18319	C ₂₄ H ₂₈ O ₇	Heteroflavanone B	S	-	-
13.851	226.12029	C ₁₂ H ₁₈ O ₄	Allixin	0.06	-	-
13.888	472.20995	C ₂₆ H ₃₂ O ₈	Kushenol H	0.02	-	-
13.949	208.11007	C ₁₂ H ₁₆ O ₃	β-Asarone	0.02	0.02	-
14.102	722.31464	C ₃₆ H ₅₀ O ₁₅	Physagulin G	0.11	-	-
14.137	272.0684	C ₁₅ H ₁₂ O ₅	Naringenin	0.03	0.02	-
14.499	470.19435	C ₂₆ H ₃₀ O ₈	Zapoterin	-	0.07	-
14.552	406.12623	C ₂₀ H ₂₂ O ₉	Anadanthoside	S	-	-
14.559	558.28306	C ₃₁ H ₄₂ O ₉	Glycinoeclepin B	0.02	-	-
15.587	530.21479	C ₂₈ H ₃₄ O ₁₀	7-Acetoxy-6-hydroxylimonin	-	0.39	-
16.063	324.20863	C ₂₂ H ₂₈ O ₂	Cannabinol monomethyl ether	0.04	-	-
16.337	328.22487	C ₁₈ H ₃₂ O ₅	Corchorifatty acid F	0.09	0.48	3.05
16.382	312.20833	C ₂₁ H ₂₈ O ₂	Tetrahydrogestrinone	-	-	1.27
16.687	216.18777	C ₁₆ H ₂₄	Cyperotundone	S	-	-
16.72	408.23001	C ₂₆ H ₃₂ O ₄	Methylinderatin	S	-	-
16.786	504.34497	C ₃₀ H ₄₈ O ₆	Arjungenin	0.53	0.08	-
16.977	822.40365	C ₄₂ H ₆₂ O ₁₆	Glycyrrhizin	0.04	-	-
17.081	468.2149	C ₂₇ H ₃₂ O ₇	Exiguaflavanone E	-	0.02	-
17.127	646.37157	C ₃₆ H ₅₄ O ₁₀	Gypsogenin 3-O-β-D-glucuronide	-	S	-
17.201	574.3505	C ₃₃ H ₅₀ O ₈	Favolon B	S	-	-
17.366	676.23644	C ₃₃ H ₄₀ O ₁₅	Icariin	0.03	-	-
17.552	256.07338	C ₁₅ H ₁₂ O ₄	Isoliquiritigenin	0.02	-	-
17.56	254.11522	C ₁₃ H ₁₈ O ₅	Aplojaveediin C	0.02	-	-
17.574	452.18324	C ₂₆ H ₂₈ O ₇	Derrichalcone	S	-	-
17.673	650.32998	C ₃₄ H ₅₀ O ₁₂	Thapsigargin	-	0.11	-
17.728	294.1828	C ₁₇ H ₂₆ O ₄	(±)-Gingerol	-	0.05	-
18.457	466.19849	C ₂₇ H ₃₀ O ₇	Nimbolide	-	0.07	-
19.166	354.14656	C ₂₁ H ₂₂ O ₅	Xanthohumol	-	-	3.13
22.132	412.37029	C ₂₉ H ₄₈ O	Stigmasterol	-	S	-

Notes: Relative percentage abundance (%) indicates the proportion of each compound detected in the extract, based on its peak area in chromatographic analysis. It reflects how much of each compound is present relative to the total composition. Compounds detected in very small amounts ≤0.01% are considered minor and marked with 'S'. Phytochemicals that do not present in either of the extracts are denoted by dash (-). Abbreviation: LC-MS: Liquid chromatography-mass spectrometry.

ethyl acetate (EtOAc) extracts. These differences are likely explained by variations in phytochemical composition, since methanol and water extract a greater range of mid-polar to polar compounds, while EtOAc yields fewer non-polar constituents.²⁰ Variability in antibacterial activity may also be influenced by differences in cell penetration and solubility. The comparatively lower activity observed

for EtOAc extracts may stem from the limited diffusion of their compounds through agar media, reducing their effectiveness.²¹ In addition, differences in broth solubility can affect MIC values.

The antibacterial activity of *A. indica* extracts varied based on the solvent used for extraction, with MeOH and AQ extracts displaying different levels of inhibition

across bacterial strains. The AQ extracts showed MIC values ranging from 2938 to 5875 µg/mL against four gastrointestinal pathogens: *B. cereus*, *S. flexneri*, *S. sonnei*, and *S. typhimurium*. This outcome points to limited inhibition, which may be attributed to water-soluble constituents such as flavonoids and tannins. However, the relatively high MIC values indicate that these extracts may not be highly potent as standalone antimicrobial agents. The MICs obtained in our study (1175–5875 µg/mL) fall into the weak activity range as classification mentioned in section 2.5. The MeOH fraction demonstrated stronger activity across a broader spectrum of bacteria, with MICs spanning 1175–4700 µg/mL for nine strains. The lowest MIC of 1175 µg/mL was observed against *B. cereus*, indicating a moderate inhibitory effect. In contrast, *S. flexneri*, *S. sonnei*, and *S. typhimurium* had higher MIC values of 4700 µg/mL, suggesting low activity against these Gram-negative enteric pathogens.

The MeOH extract also exhibited MIC values of 2350 µg/mL against *S. aureus* and MRSA, demonstrating equal effectiveness against both strains, which could indicate potential activity against antibiotic resistance mechanisms. However, MIC values of 4700 µg/mL against *E. coli*, ESBL-producing *E. coli*, and *K. pneumoniae* suggest weaker inhibition against these Gram-negative bacteria. Interestingly, the extract was not active against ESBL-producing *K. pneumoniae*, highlighting possible resistance mechanisms that limit its efficacy. However, differences in bacterial susceptibility may be influenced by factors such as cell wall composition, endogenous enzymes, and efflux pump activity.^{22,23} The relatively strong activity against *B. cereus* suggests that the extract contains specific compounds effective against spore-forming Gram-positive bacteria. Conversely, the higher MIC values against *Shigella* and *Salmonella* species imply that these pathogens may have structural or biochemical resistance mechanisms that reduce the extract's effectiveness.^{24,25} Overall, the *A. indica* methanolic extracts' antibacterial activity is encouraging, whilst their efficacy varies across bacterial strains. However, the relatively high MIC values indicate that their standalone use may be limited.

A previous study reported low antibacterial activity of *A. indica* leaf and seed extracts using aqueous and 80% ethanol solvents. Leaf extracts showed MICs of 128–256 mg/mL against *S. aureus*, while seed extracts were more active with MICs of 8–64 mg/mL.²⁶ Notably, these MIC values are very high and generally would not be considered to be active by most standards. In contrast, the methanol extract tested in our study had an MIC of 2350 µg/mL against *S. aureus* and MRSA, which is nearly 100 times more potent than the 80% ethanol leaf extract examined in

the earlier study (based on MIC values), and approximately 3 times as potent as the corresponding seed extract tested in that study. While the differences in extraction solvents (80% ethanol in the earlier study, versus 100% methanol in our study) are likely to contribute to these differences, the scale of the differences indicates that other factors may also contribute, including the plant source as well as its storage and handling. The authors in the earlier study state that they used the same plant material that they used in an earlier study. Depending on the time that the plant material had been stored and the storage conditions, there may have been substantial losses of volatile compounds. In addition, specific antibacterial compounds may have become inactivated (e.g., through oxidation) during the prolonged storage period. Furthermore, methodological differences in the microdilution assays used in the two studies may also account for these differences. Our study used iodinitrotetrazolium salt (INT) as a colorimetric indicator, which allows rapid and sensitive determination of the MIC values. In contrast, Arsene *et al.*'s study²⁶ relied on visual examination of the treated cultures to evaluate the point at which growth was not visually apparent. This method lacks sensitivity and is prone to experimenter error. These differences may likely contribute to the differences in MIC between the two studies.

In addition, only the 80% ethanol seed extract was tested in combination with antibiotics in the earlier study,²⁶ showing additive effects with kanamycin and synergistic interactions with ampicillin and nitrofurantoin against *S. aureus*. Notably, it also exhibited synergy with ampicillin, kanamycin, and nitrofurantoin against *E. coli*.²⁶ Notably, the FIC assay used in that study followed similar protocols (including the visual growth detection method) and would be subject to the same inaccuracies as already discussed for the MIC results obtained in that study. A separate study demonstrated limited antibacterial efficacy of neem seed oil against *E. coli* and *K. pneumoniae*, with an MIC ranging from 3750 to 7500 mg/mL.²⁷ However, the combination of the extract with standard antibiotics was not evaluated in that study. Another investigation reported that aqueous and methanolic leaf extracts exhibited antibacterial effects, respectively, with MICs of 500 and 60 µg/mL against *S. aureus* and 500 and 125 µg/mL against MRSA.²⁸ That study used Soxhlet extraction (rather than maceration used in our study), and therefore, the phytochemical composition may vary substantially between the extracts tested in the two studies. This may account for the differences in MIC values between the studies. However, that study demonstrated no antibacterial activity against Gram-negative strains, including *K. pneumoniae*, *E. coli*, ESBL-producing *E. coli*, and *Salmonella* species, and failed to assess the combinatorial interactions of extracts

with antibiotics. Furthermore, that study did not report extraction yield or the volumes of extracts added in broth assays,²⁸ making dose normalization impossible. Dilution procedures were vaguely described (highest to lowest concentration) without specifying the dilution factor, starting concentration, or final solvent levels. In some cases, 10% DMSO was used at stock concentrations as high as 80 mg/mL for diffusion assays,²⁸ but it was unclear whether the same preparations were applied to broth microdilution, raising concerns about solvent-related artifacts. Furthermore, ATCC codes or strain provenance were not provided, which reduces reproducibility and introduces variability in susceptibility profiles. The absence of appropriate reference antibiotics for benchmarking was another major limitation; that study used cefiderocol as the only comparator in broth assays,²⁸ a Gram-negative-specific agent unsuitable for Gram-positive pathogens such as *S. aureus*.

In comparison, our work adhered to standardized microdilution protocols: We reported extraction yield, controlled DMSO at $\leq 1\%$, employed a defined dilution scheme (4-fold pre-step followed by two-fold dilutions), and tested well-characterized ATCC strains alongside a panel of reference antibiotics covering both Gram-positive and Gram-negative pathogens. This methodological rigor likely explains the lower MICs observed in our study compared with inflated values reported in previous literature. We also would like to acknowledge that lower MIC values, while indicative of antibacterial activities of the complex extract mixtures, should be studied cautiously with regard to their pharmacological relevance when compared to extracts that possess far greater potency.

Antimicrobial activity of plant extracts against bacterial pathogens is attributed to multiple mechanisms of action, depending on the bioactive compounds present. One of the primary mechanisms is cell wall and membrane disruption, where phenolics, flavonoids, and tannins destabilize bacterial cell walls, increasing permeability and leading to lysis.^{4,8} Inhibition of protein synthesis is another key mechanism, where alkaloids and flavonoids interfere with ribosomal function, preventing bacterial translation and growth.^{18,29} Furthermore, certain phytochemicals act as efflux pump inhibitors, reducing bacterial resistance by preventing the expulsion of antimicrobial agents.¹⁸ In addition, polyphenols and flavonoids induce oxidative stress by generating reactive oxygen species, leading to cellular damage and bacterial death. These diverse mechanisms not only contribute to the antibacterial potential of plant extracts but also offer potential synergy with conventional antibiotics, enhancing their efficacy against resistant bacterial strains.

Combination FIC assays further highlighted distinct patterns. We observed additive interactions between

both AQ and MeOH extracts and β -lactam antibiotics (penicillin G, oxacillin, amoxicillin) against *B. cereus* and *S. aureus*, suggesting enhanced cell wall-targeting effects. Additive interactions were also seen with tetracycline and chloramphenicol against *S. flexneri*, *S. sonnei*, and *S. typhimurium*, as well as with chloramphenicol against *E. coli*. These results support the hypothesis that neem phytochemicals may interfere with bacterial protein synthesis pathways in a complementary manner to ribosome-targeting antibiotics. By contrast, both AQ and MeOH extracts displayed strong antagonism with polymyxin B against multiple Gram-negative strains, implying that extract constituents may interfere with the antibiotic's membrane-disrupting activity. The observed antagonism between the AQ and MeOH extracts and polymyxin B against multiple Gram-negative bacteria may be attributed to interactions between bioactive phytochemicals and the antibiotic. Compounds within the extracts may interact with polymyxin B, limiting its uptake and diminishing its ability to disrupt bacterial membranes.³⁰ In addition, the pH-dependent activity of polymyxin B may have been influenced by the introduction of plant extracts into the broth medium. Changes in the local pH could alter the charge properties of polymyxin B, leading to a diminished binding affinity to bacterial membranes and a subsequent reduction in its antimicrobial potency.³¹ This suggests that specific phytochemical properties, such as pH-modulating effects, may contribute to the antagonistic interactions observed. Similarly, antagonistic interactions were noted, particularly with gentamicin, where both extracts reduced its efficacy against *B. cereus*, *S. flexneri*, *S. typhimurium*, and *K. pneumoniae*. In addition, the MeOH extract antagonized tetracycline against *S. sonnei* and *K. pneumoniae*, indicating possible interference with its mechanism of action. While these postulations remain speculative, they provide some insight into the safety of using such combinations in therapy, as antagonistic combinations abolish the effectiveness of antibacterial treatments, putting patients at risk of exacerbated infections.

A previous study of neem extracts reported synergy only in selective contexts, such as additive or synergistic effects of 80% ethanol seed extracts with kanamycin, nitrofurantoin, or ampicillin against *S. aureus* and *E. coli*.²⁶ Indeed, that study represents methodological clarity in those reports, including a detailed combination design (checkerboard) and defined solvent controls and confidence in their conclusions. In contrast, our study applied standardized FIC analysis, allowing a more rigorous and quantitative assessment of interactions. However, we did not test the combination of extracts with kanamycin, nitrofurantoin, or ampicillin.

Extracts' MICs should not be directly compared with purified antibiotics, as they represent complex mixtures in which active constituents are present at relatively low abundance. The high MIC values observed, therefore, emphasize the importance of bioassay-guided fractionation and compound isolation, which may yield individual phytochemicals with substantially lower MICs that approach pharmacologically relevant benchmarks. Overall, our findings reinforce that while crude neem extracts are not highly potent as standalone agents, they can enhance the efficacy of selected antibiotics through additive effects, particularly against Gram-positive bacteria. At the same time, the observed antagonism with polymyxin B emphasizes the importance of evaluating potential negative interactions before clinical consideration. By addressing methodological gaps in the literature, our study provides a more reliable assessment of neem's antibacterial potential and its role in combination therapy.

The absence of synergistic interactions between extracts and antibiotics suggests that while the extracts exhibited some antibacterial potential, they did not significantly enhance antibiotic efficacy beyond an additive effect. This could be due to the extracts lacking strong potentiating compounds or the failure of the potentiating compounds to target bacterial resistance mechanisms effectively. In addition, potential compound-antibiotic interactions may have resulted in competitive binding, preventing synergy. It is also important to note that the concentrations of some of the extracts may have been suboptimal and thus unable to elicit synergistic interactions. While no synergy was observed, the additive effects indicate some potential for combination use. Future studies should place emphasis on isolating specific bioactive compounds and assessing their ability to improve antibiotic activity against resistant bacteria.

LC-MS studies highlighted several phytochemicals in the plant extracts, particularly flavonoids, phenolic acids, tannins, terpenoids, as well as several other compounds. The flavonoids included astilbin (Figure 3A), (+)-gallocatechin (Figure 3B), kaempferol (Figure 3C), myricitrin (Figure 3D), naringenin (Figure 3E), hyperoside (Figure 3F), nictoflorin (Figure 3G), quercetin (Figure 3H), quercetin 3-O-rhamnoside-7-O-glucoside (Figure 3I), rutin (Figure 3J), and pyrogallol (Figure 3K). Tannins detected in the extracts comprise gallic acid (Figure 3L), digallic acid (Figure 3M), 6-galloylglucose (Figure 3N), 1-O-galloylglycerol (Figure 3O), (+)-chebulic acid (Figure 3P), 1,6-bis-O-(3,4,5-trihydroxybenzoyl) hexopyranose (Figure 3Q), 1,2,6-trigalloyl- β -D-glucopyranose (Figure 3R), ellagic acid arabinoside (Figure 3S), and ellagic acid (Figure 3T). Earlier

investigations have also reported additional phytochemicals in *A. indica* leaf extracts, such as genistein 7-O-glucoside, (–)-epicatechin, nimonol, zafaral, meliacinanthridine, nimocinol, isomeldenin, rutin, β -sitosterol, ellagic acid, ferulic acid, lupeol, quercetin, stigmasterol, terpinen-4-ol, sugiol, 4-cymene, nimbiol, α -terpinene, and vitamin E.^{32–35}

Quercetin has previously demonstrated antibacterial activity against resistant strains of *E. coli*, and *K. pneumoniae*, with MICs ranging from 16 to 256 μ g/mL depending on the strain.³⁶ It disrupts bacterial membranes and inhibits the expression of efflux pumps and carbapenemase genes. In the same study, the combination of quercetin and meropenem showed synergistic effects against carbapenem-resistant *E. coli* and *K. pneumoniae*, reducing meropenem MICs from 1024 μ g/mL to 8–512 μ g/mL.

Against resistant *E. coli* strains, naringenin showed MIC values between 1 and 8 mg/mL, whereas rutin exhibited stronger activity with MICs of 125–1000 mg/mL.^{37,38} Both compounds, when combined with amikacin, showed a synergistic antibacterial effect against multidrug-resistant *E. coli*. The combination disrupted the bacterial cell wall and inner membrane, as evidenced by increased leakage of alkaline phosphatase, K^+ and proteins.^{37,38} This suggests enhanced membrane permeability as a key mechanism.

Gallic acid has additive antibacterial effects when combined with β -lactams (ampicillin, amoxicillin, penicillin G) against *S. aureus* and MRSA, by reducing the MICs of these antibiotics from 62.5 to 8–16 μ g/mL.³⁹ Gallic acid had synergistic effects in combination with gentamicin and norfloxacin against *S. aureus*. When combined with norfloxacin, it reduced the MIC from 156 to 49 μ g/mL, and with gentamicin, the MIC dropped from 49 to 3 μ g/mL.⁴⁰ A separate investigation examined gallic acid for both antibacterial and antibiofilm effects against *S. flexneri*, reporting an MIC of 2000 μ g/mL and minimum bactericidal concentration (MBC) of 8000 μ g/mL. The authors of that study also reported that gallic acid inhibited bacterial growth by disrupting cell membranes and altering morphology.⁴¹ It also significantly reduced biofilm formation by penetrating the extracellular polymeric matrix and downregulating *mdoH* gene and OpgH protein expression, leading to decreased polysaccharide content. However, no studies in combination with antibiotics were conducted in that work.

Pyrogallol enhanced the activity of gentamicin and norfloxacin against *S. aureus*, inducing synergy by lowering the MIC of gentamicin from 49.21 to 2.44 μ g/mL and norfloxacin from 156 to 78 μ g/mL.³⁹ Pyrogallol demonstrates strong antibacterial activity against MRSA, with both MIC and MBC at 16 μ g/mL. Its mechanism involves disrupting the bacterial cell

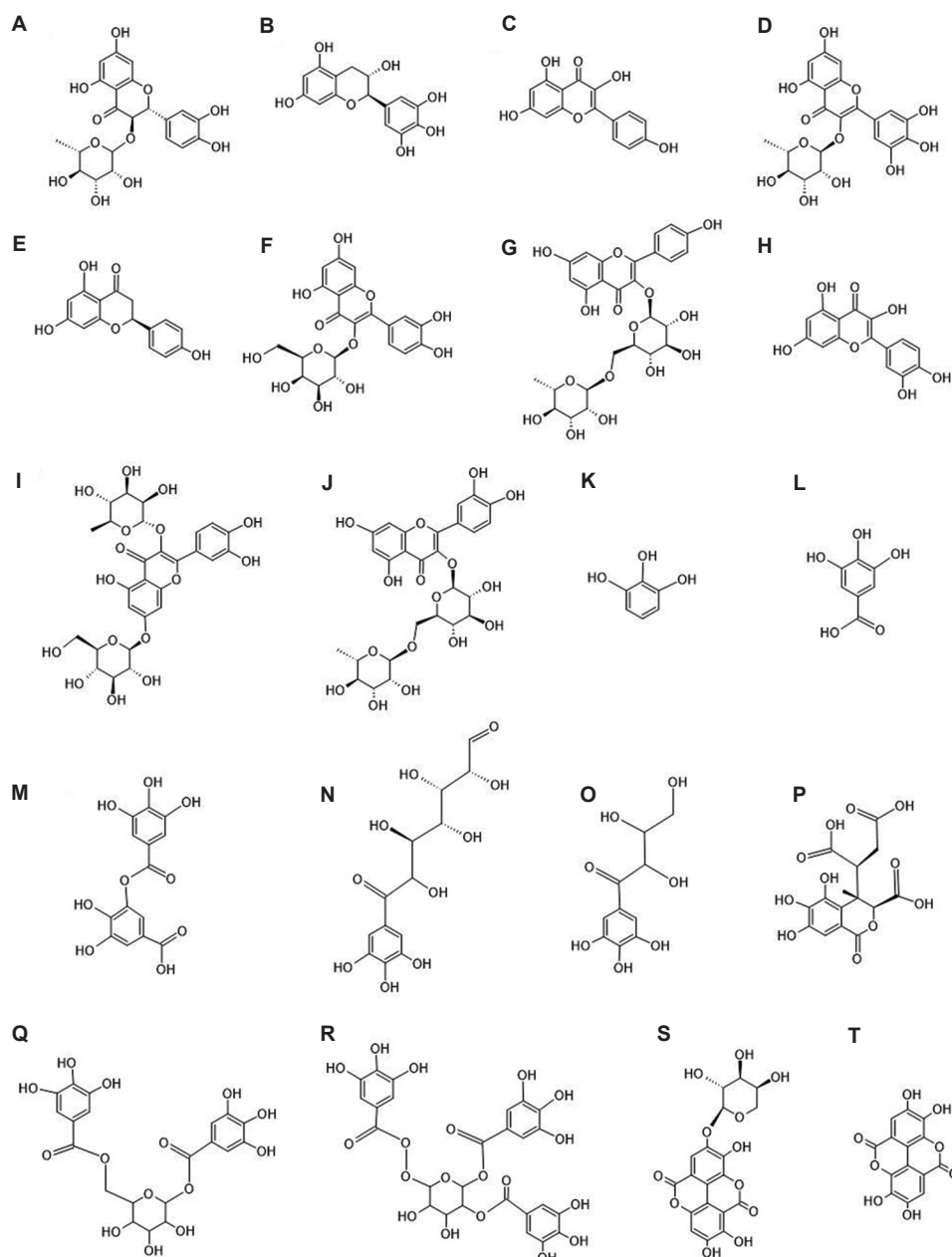


Figure 3. Structures of key phytochemicals detected in *Azadirachta indica* leaf extracts. (A) Astilbin, (B) (+)-gallocatechin, (C) kaempferol, (D) myricitrin, (E) naringenin, (F) hyperoside, (G) nictoflorin, (H) quercetin, (I) quercetin 3-O-rhamnoside-7-O-glucoside, (J) rutin, (K) pyrogallol, (L) gallic acid, (M) digallic acid, (N) 6-galloylglucose, (O) 1-O-galloylglycerol, (P) (+)-chebulic acid, (Q) 1,6-bis-O-(3,4,5-trihydroxybenzoyl) hexopyranose (R) 1,2,6-trigalloyl-β-D-glucopyranose, (S) ellagic acid arabinoside, and (T) ellagic acid. Structures were prepared using ChemSketch (Freeware) 2023.2.4.

membrane by modifying components like fatty acids and peptidoglycans.⁴²

Among the compounds detected by LC-MS, quercetin, naringenin, rutin, gallic acid, and pyrogallol are of particular interest due to their well-documented antibacterial activity in the literature. These phytochemicals have been reported to inhibit *S. aureus*, MRSA, *E. coli*, resistant *E. coli*,

K. pneumoniae, and *Shigella* strains, which overlap with the pathogens tested in this study. While our current data reflect their relative abundance in the extracts, we suggest that these compounds may contribute to the antibacterial activity observed. Future studies are underway to evaluate these molecules as antibacterial agents, both individually and in combination with conventional antibiotics, against the same panel of bacterial strains.

Overall, this study demonstrates the antibacterial potential of methanolic and aqueous *A. indica* leaf extracts, which showed notable activity against various bacterial strains. Additive interactions with penicillin G, oxacillin, tetracycline, and chloramphenicol suggest potential for use as antibiotic adjuvants. Whilst no synergistic interactions were observed, the ability of the extracts to inhibit several bacterial strains and selectively enhance antibiotic activity is encouraging. The absence of toxicity in *Artemia franciscana* assays reinforces the potential of *A. indica* as a safe and viable option for future antimicrobial applications. Future work should focus on fractionation, bioactive compound isolation, mechanism elucidation, and expanded toxicity assessments using mammalian cell lines to better define its therapeutic potential.

5. Conclusion

This study demonstrates the antibacterial potential of *A. indica* leaf extracts against various pathogens, including resistant strains. FIC analysis showed additive effects with some antibiotics, particularly β -lactams and protein synthesis inhibitors, suggesting enhanced efficacy. The antagonism noted in the combination of polymyxin B and gentamicin highlights the need for caution with the use of combination therapies. Phytochemical analysis revealed flavonoids, tannins, and phenolics as key antimicrobial constituents, with extract efficacy influenced by solvent polarity. All extracts were non-toxic in *Artemia franciscana* assays, supporting their safety, although this invertebrate model is only a preliminary assessment of toxicity. Further studies will focus on isolating active compounds, evaluating cytotoxicity on mammalian cells, and understanding mechanisms behind both synergistic and antagonistic interactions.

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Conflict of interest

The authors declare that they have no competing interests.

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Investigation: All authors

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Writing—review & editing: All authors

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Availability of data

Data supporting the findings and conclusions are available on reasonable request from the corresponding author.

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