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Original article

Phytochemical Composition, Antimicrobial Activity, and In Silico Evaluation of *Azadirachta indica* Stem Bark Extracts against Multidrug-Resistant Pathogens

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ABSTRACT

Introduction: Medicinal plants are valuable sources of bioactive compounds with therapeutic potential. This study aimed to profile the bioactive compounds in the stem bark of *Azadirachta indica*, evaluate the antimicrobial activity of its crude and fractionated extracts, and explore molecular interactions with key bacterial enzymes.

Methods: Crude stem bark extracts were obtained via maceration and fractionated using n-hexane and ethanol. Antimicrobial activity was assessed against multidrug-resistant pathogens using standard microbiological assays. Phytochemical composition was analyzed via high-performance liquid chromatography (HPLC), while molecular docking, ADME predictions, drug-likeness, and toxicity assessments were performed in silico.

Results: The crude extract displayed broad antibacterial activity, with inhibition zones of 10–20 mm at 100 mg/mL, showing strongest activity against *Acinetobacter baumannii*. Fractionated extracts exhibited moderate activity (6–12 mm). MIC values ranged from 50–100 mg/mL for the crude extract and ≥100 mg/mL for fractions. HPLC identified key compounds including quercetin, rutin hydrate, kaempferol, gallic acid and sinapic acid. Docking studies showed rutin hydrate, quercetin, nicotiflorin, and astragalin had high binding affinities for *E. coli* DNA gyrase B and *A. baumannii* topoisomerase IV. ADME predictions indicated good gastrointestinal absorption for most compounds, with minimal toxicity and generally favorable drug-likeness profiles, except for rutin hydrate and nicotiflorin.

KEYWORDS: *AZADIRACHTA INDICA*; PHYTOCHEMICAL ANALYSIS; ANTIMICROBIAL ACTIVITY; MULTIDRUG-RESISTANT BACTERIA; MOLECULAR DOCKING; BIOACTIVE COMPOUNDS

INTRODUCTION

Medicinal plants have long served as essential sources of therapeutic agents, forming the foundation of many traditional healing systems around the world (Krishnaprabu, 2020; Bhat, 2021). These plants have been used for centuries across ancient Egyptian, Chinese, Indian, Greek, and Roman medical practices, where knowledge about their healing properties was passed down through generations (Jamshidi-Kia *et al*., 2017; Akbar, 2020). In contemporary research, plant-derived remedies remain highly valued because they often demonstrate good efficacy with fewer adverse effects compared to many synthetic drugs (Shah *et al*., 2019; Qadir and Raja, 2021). With the continuous emergence of infectious diseases and increasing global antimicrobial resistance, the search for new bioactive compounds from natural sources has become even more urgent (Borges *et al*., 2015; Newman and Cragg, 2016). Phytochemical studies therefore play a crucial role in identifying plant constituents with therapeutic potential, supporting drug development and validating long-standing ethnomedicinal claims (Atanasov *et al*., 2021; Rivzi *et al*., 2022).

Azadirachta indica A. Juss. (commonly called neem), a member of the Meliaceae family, is well known for its broad medicinal and agricultural value (Loganathan *et al*., 2021). It is a hardy, drought-tolerant tree widely cultivated across tropical and subtropical regions. The tree bears pinnate leaves, fragrant white flowers, and greenish drupes, while the bark has a long history of use in traditional medicine. Different parts of the plant; including the leaves, flowers, seed oil, roots, and twigs have been utilized for various cultural, therapeutic, and domestic purposes (Maji and Modak, 2021; Wylie and Merrell, 2022). The plant's pharmacological activities stem from its rich phytochemical profile, which includes limonoids such as azadirachtin, nimbin, nimbolide, and salannin, along with flavonoids, phenols, tannins, saponins, steroids, glycosides, alkaloids, and oxalic acid (Biney *et al*., 2021; Moin *et al*., 2021). Neem leaves are commonly used for antimicrobial and antiviral applications, the bark is traditionally employed for antiseptic and digestive purposes, the seed oil is valued for antiparasitic and cosmetic uses, and the oil cake serves as an organic fertilizer and biopesticide (Maji and Modak, 2021; Reedy and Neelimah, 2022).

The therapeutic relevance of *A. indica* has been supported by numerous modern investigations. Neem extracts exhibit strong antimicrobial activity against pathogenic bacteria including *Staphylococcus aureus*, *Bacillus pumilus*, and *Helicobacter pylori*, which explains their increasing use in oral hygiene, wound management, food preservation, and agricultural protection (Borges *et al*., 2015; Wylie and Merrell, 2022). These activities are attributed to the synergistic actions of several bioactive constituents, particularly limonoids and phenolic compounds that contribute to anti-inflammatory, immunomodulatory, and tissue-repair properties. Additionally, neem has demonstrated antiviral, antioxidant, hypoglycemic, hepatoprotective, cardioprotective, anticancer, antiparasitic, and dermatological benefits (Pingali *et al*., 2020; Seriana *et al*., 2021; Oktavia and Infora, 2022).

Despite the plant's extensive study, most research has focused on the leaves and seed kernels, whereas the stem bark, though traditionally used has received comparatively less scientific attention. This gap is scientifically relevant because the bark contains significant amounts of terpenoids and other compounds that may possess therapeutic relevance. With the persistent rise of multidrug-resistant (MDR) bacteria worldwide, identifying new antimicrobial agents from understudied plant parts has become a priority. In addition to laboratory screening, modern computational approaches such as molecular docking enhance this process by predicting how phytochemicals may interact with microbial targets, supporting the interpretation of in vitro findings and guiding compound prioritization.

Based on these considerations, the present study was designed to (i) identify the phytochemical constituents of the ethanolic stem bark extract of *Azadirachta indica*, (ii) evaluate its antimicrobial activity against selected MDR bacterial isolates, and (iii) assess the binding potential of the detected compounds to relevant microbial protein targets using molecular docking analysis. These combined approaches provide insight into the therapeutic value of neem stem bark and contribute to the ongoing search for plant-based antimicrobial candidates.

MATERIALS AND METHODS

MATERIALS

Test Organisms and Standardization of Inocula

The multidrug-resistant bacterial species used in this study were obtained from the Microbiology Laboratory of the institution. The organisms included *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella aerogenes*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. All isolates were reconfirmed through standard biochemical and microbiological tests to ensure purity. Prior to antimicrobial assays, bacterial inocula were standardized to 0.5 McFarland turbidity by adjusting the turbidity of overnight cultures in sterile saline using a spectrophotometer (Oluyele *et al*., 2023).

Plant Sample Collection and Authentication

Air-dried stem bark of the dicotyledonous plant *Azadirachta indica* (Neem/Margosa tree; family Meliaceae) was used for this study. Plant materials were collected from the local environment. The stem bark and the whole plant were identified and authenticated at the university's Herbarium and Taxonomic Unit. Both the stem bark and whole plant were assigned the voucher number PSBHT-270 and deposited at the herbarium for future reference.

Preparation of Extract and fractionation

Fresh *Azadirachta indica* (neem) bark was collected, cleaned, and air-dried at room temperature. The dried bark was pulverized, and 2200g of the powdered material was macerated in 5 L of 70% ethanol in an airtight container for 96 hours, with occasional shaking to enhance extraction (Oluyele and Oladunmoye, 2017) with slight modifications). The mixture was then filtered through Muslin cloth to remove solid residues. The resulting filtrate was concentrated by air-drying at room temperature to facilitate solvent evaporation, yielding the crude ethanol extract.

To obtain fractions, exactly 65 g of the crude extract was dissolved in 150 mL of distilled water and transferred into a 500 mL separatory funnel using a previous method with some modifications (Oluyele, 2025). Liquid–liquid partitioning was first carried out using n-hexane. An equivalent volume (100 mL) of n-hexane was added, and the mixture was shaken vigorously and allowed to stand for 15 min to allow complete separation of the organic and aqueous layers. The n-hexane layer was collected, and the extraction was repeated three times until the organic layer became colourless. All n-hexane fractions were pooled and concentrated to dryness in vacuo using a rotary evaporator. The remaining aqueous phase was subsequently extracted with ethanol by adding an equal volume of ethanol (100 mL) to the aqueous mixture. The mixture was stirred and filtered to obtain the ethanol-soluble fraction, as ethanol is completely miscible with water and does not form a separate layer. The ethanol extract was concentrated under reduced pressure and stored appropriately for further analysis.

Assay of Antibacterial Activity of *Azadirachta indica* Extract

Antibacterial activity was evaluated using the agar well diffusion method (Oluyele *et al*., 2025). Standardized bacterial suspensions (1 mL) were spread on Mueller-Hinton agar, and 6 mm wells were filled with 100 µL of extract (100 mg/mL in 5% DMSO). Amoxicillin and Ofloxacin served as positive controls. Plates were pre-diffused for 15 min at room temperature and incubated at 37 °C for 24 h, after which zones of inhibition were measured. MICs were determined by broth dilution (100–3.125 mg/mL) with subculturing (Oluyele *et al*., 2025). Broth only and broth plus inoculum without extract served as negative and growth controls, respectively. The MIC was defined as the lowest concentration showing no visible turbidity.

High Performance Liquid Chromatography (HPLC) Analysis of *Azadirachta indica* Extract

For HPLC analysis, 2.0 g of the extract was dissolved in 20 mL of a 1:1 (v/v) mixture of acetonitrile and methanol and stirred for 30 minutes. The aqueous fraction was removed, and the organic layer was transferred to a 25 mL volumetric flask and adjusted to volume with the same solvent mixture. Chromatographic separation was conducted on an Agilent 1200 reversed-phase HPLC system equipped

with a Hypersil BDS C18 column (250 mm × 4.0 mm i.d.). The mobile phase consisted of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B), delivered at a flow rate of 0.6 mL/min, with a 20 µL injection volume. Detection was carried out at 280 nm. Reference standards were analyzed under identical conditions to generate comparative chromatograms, and constituents in the extract were identified by matching retention times and UV spectra with those of the standards (Oluyele, 2025).

Protein Preparation

The X-ray crystallographic structures of *A. baumannii* topoisomerase IV (PDB ID: 2XKJ) and *E. coli* DNA gyrase B (PDB ID: 7C7O) were obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) (www.rcsb.org). Missing residues, loop regions, and side-chain anomalies were resolved, followed by energy optimization using the OPLS4 force field in the Protein Preparation Wizard of the Schrödinger suite (2021). The receptor grid was generated using the Glide Grid Generation tool, targeting the co-ligand binding site.

Structure-based virtual screening

The prepared compounds from *C. albidum* and the standard ligand were screened against *A. baumannii* topoisomerase IV (PDB ID: 2XKJ) and *E. coli* DNA gyrase B (PDB ID: 7C7O) using the extra precision (XP) GLIDE docking protocol in the Maestro Schrödinger suite (v2021). This scoring function is widely recognized for its reliability and ability to distinguish binding affinities effectively, though it requires a longer computational runtime (Oluyele and Akinyeke, 2025).

Pharmacokinetic, Drug-Likeness, and Toxicity Predictions

Pharmacokinetic properties, medicinal chemistry parameters, and drug-likeness profiles of the selected phytochemicals were assessed using the SwissADME web platform (http://www.swissadme.ch), while toxicity predictions were performed using the ProTox-II online server (https://tox-new.charite.de/protox_II).

Ethical approval for research methods

Not applicable

Statistical Analysis

All in-vitro antimicrobial experiments were conducted in triplicate, and data were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) to compare the antibacterial activities of the crude extract and solvent fractions of *Azadirachta indica* at different concentrations. Differences among means were considered statistically significant at $P < 0.05$, and Duncan's Multiple Range Test (DMRT) was applied as a post-hoc test to separate statistically significant means. Statistical analysis was not applied to molecular docking, MMGBSA binding energy calculations, QSAR predictions, or pharmacokinetic evaluations, as these analyses are computational and based on deterministic scoring functions rather than experimental replicates.

RESULTS AND DISCUSSION

As shown in Table 1**,** the crude extract of *A. indica* demonstrated clear antibacterial activity against all multidrug-resistant test isolates. At 100 mg/ml, zones of inhibition ranged from 10 mm to 20 mm, with *A. baumannii* exhibiting the highest susceptibility (20 mm), and *S. aureus* alongside *K. pneumoniae* (14 mm) exhibiting the least. At 50 mg/ml, zones of inhibition ranged between 8mm-15mm, while at 25mg/ml, zones of inhibition ranged from 5mm-10mm and no inhibition was observed at 12.5 mg/mL except for *Escherichia coli* (8mm)**.**

Table 1: Zone of Inhibition of *A. indica* crude extract against selected MDR pathogens

TEST ISOLATE	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml
Escherichia coli	15mm	13mm	10mm	8mm
Enterococcus faecalis	10mm	8mm	5mm	0.0mm
Staphylococcus aureus	14mm	10mm	8mm	0.0mm
Acinetobacter baumannii	20mm	15mm	9mm	0.0mm
Klebsiella pneumoniae	14mm	11mm	9mm	0.0 mm

As depicted in Table 2, the ethanolic fraction exerted moderate activity, producing inhibition zones between 6–10 mm at 100 mg/ml, with stronger effects against *A. baumannii*, *E. coli*, and *K. pneumoniae*. At 50mg/ml, zones of inhibition ranged between 4mm-8mm and at 25mg/ml, no inhibition was observed except for *Staphylococcus aureus* and *Acinetobacter baumannii* (5mm). No inhibition was observed at 12.5mg/ml.

Table 2: Zone of Inhibition of *A. indica* Ethanolic Fraction against Selected MDR Pathogens

TEST ISOLATE	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml
E. coli	10mm	7mm	0.0 mm	0.0 mm
E. faecalis	6mm	4mm	0.0 mm	0.0 mm
S. aureus	8mm	5mm	5mm	0.0 mm
A. baumannii	10mm	8mm	5mm	0.0 mm
K. pneumoniae	10mm	7mm	0.0 mm	0.0 mm

As presented in Table 3, the n-hexane fraction demonstrated antibacterial activity, with inhibition zones ranging from 9–12 mm at 100 mg/ml. The highest effect was observed against *K. pneumoniae* (12 mm). The n-hexane fraction showed minimal residual activity at lower concentrations (25–50 mg/ml). At 12.5mg/ml, no inhibition was observed except for *Enterococcus faecalis* (4mm).

Table 3: Zone of Inhibition of *A. indica* n-Hexane Fraction against selected MDR Pathogens

TEST ISOLATE	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml
E. coli	10mm	8mm	0.0 mm	0.0 mm
E. faecalis	9mm	6mm	5mm	4mm
S. aureus	9mm	7mm	5mm	0.0 mm
A. baumannii	10mm	8mm	7mm	0.0 mm
K. pneumoniae	12mm	0.0 mm	0.0 mm	0.0 mm

As shown in Table 4, the crude extract exhibited MICs ranging from 50–100 mg/mL, while both the ethanolic and n-hexane fractions recorded MICs ≥100 mg/mL for most organisms. Notably, *E. faecalis* remained the least susceptible isolate, with MIC values exceeding 100 mg/ml for all fractions.

Table 4: Minimum Inhibitory Concentration of *A. indica* crude extract and fractions

Test Organism	Crude Extract	n-hexane Fraction	Ethanolic Fraction
E. coli	50 mg/ml	100 mg/ml	100 mg/ml
E. faecalis	100 mg/ml	>100 mg/ml	>100 mg/ml
S. aureus	50 mg/ml	>100 mg/ml	>100 mg/ml
A. baumannii	50 mg/ml	100 mg/ml	100 mg/ml
K. pneumoniae	50 mg/ml	100 mg/ml	100 mg/ml

As presented in Table 5 and illustrated in Figure 1, chromatographic profiling of the *Azadirachta indica* crude extract revealed the presence of multiple phenolic acids and flavonoid derivatives. Nine major compounds were identified based on their retention times and spectral characteristics, including gallic acid (1.116 min), sinapic acid (1.480 min), caffeic acid (2.543 min), rutin hydrate (4.321 min), quercetin (5.321 min), kaempferol (6.654 min), astragalin (8.762 min), and two distinct peaks corresponding to nicotiflorin at 7.432 and 10.234 min. As shown in Figure 1, the chromatogram displays clear and well-resolved peaks, with quercetin exhibiting the highest peak area among the identified compounds, followed by rutin hydrate and caffeic acid.

Table 5: Compounds Identified in *Azadirachta indica* Extract

Component	Retention	Area	Height	External Units
Gallic acid	1.116	241.4631	22.134	24.8591 ppm
Sinapic Acid	1.480	78.2412	4.712	8.1462 ppm
Caffeic Acid	2.543	149.8122	8.842	15.3292 ppm
Rutin Hydrate	4.321	152.8142	5.824	15.7612 ppm
Quercetin	5.321	361.4280	6.847	0.0000
Kaempferol	6.654	87.1165	3.921	8.9141 ppm
Nicotiflorin	7.432	139.4610	5.742	13.6212 ppm
Astragalin	8.762	113.9621	6.892	15.1983 ppm
Nicotiflorin	10.234	123.7460	8.564	16.8720 ppm

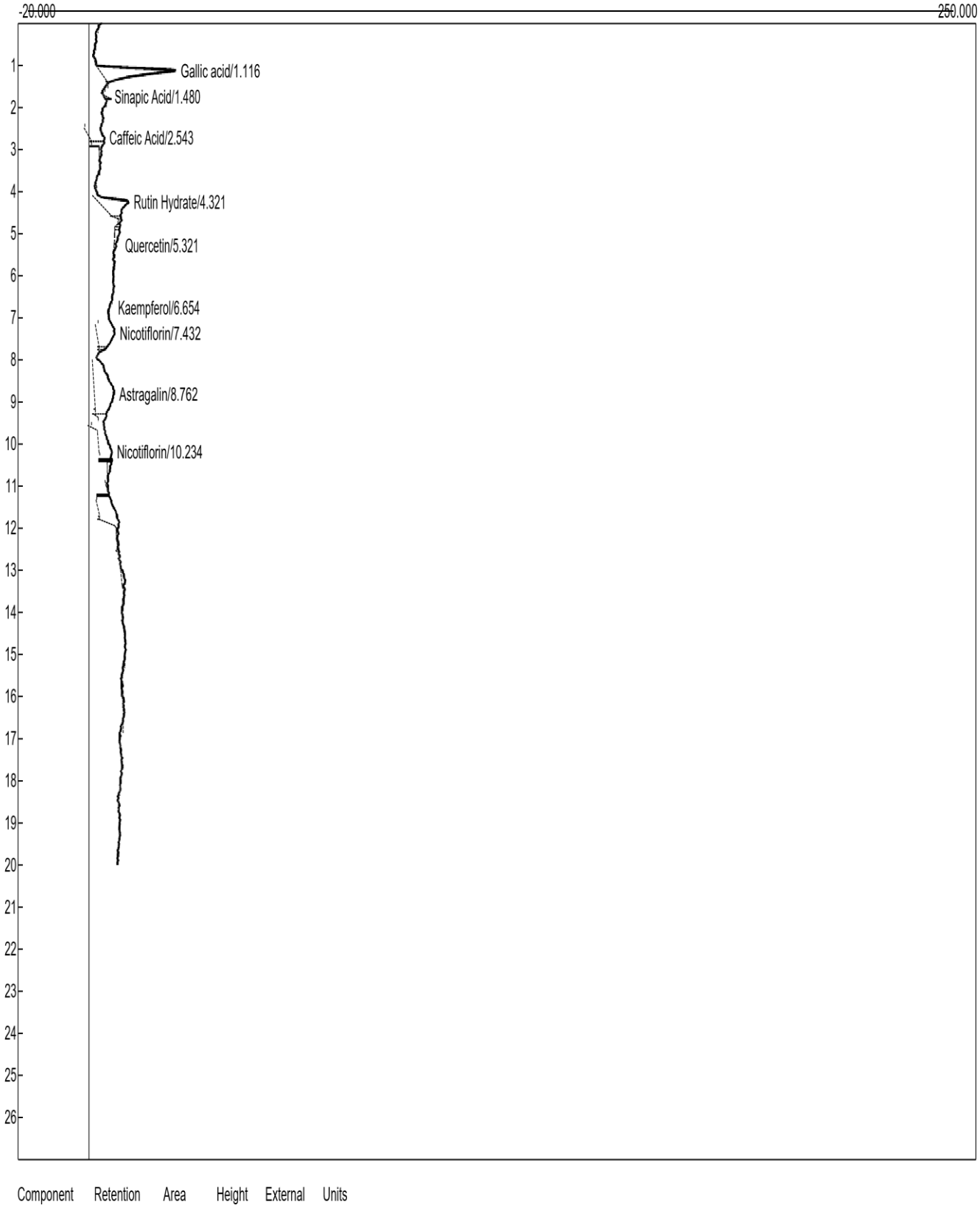

Figure 1: Chromatography of *Azadirachta indica* crude extract

Molecular Docking

Docking against *E. coli* -7C7O

The docking scores, binding free energies, and residue interactions of the phytochemicals with *E. coli* DNA gyrase B are summarized in Table 6. Among all ligands, rutin hydrate showed the strongest binding affinity with an XP-GScore of –9.327 kcal/mol and MMGBSA ΔG_bind of **–**52.6 kcal/mol. Rutin formed multiple hydrogen bonds with GLU50, ASN46, and ASP73. Other flavonoids including quercetin (XP-GScore –6.829 kcal/mol; ΔG_bind –45.4 kcal/mol), nicotiflorin (–7.415 kcal/mol; –39.5 kcal/mol), and astragalin (–6.499 kcal/mol; –43.9 kcal/mol) also displayed strong affinities. Phenolic acids such as gallic acid (–5.059 kcal/mol; 25.3 kcal/mol), caffeic acid (–4.817 kcal/mol; –24.2 kcal/mol), and sinapic acid (–4.565 kcal/mol; 13.4 kcal/mol) recorded weaker scores and fewer stabilizing interactions. Residues commonly involved in ligand stabilization included ASP73, GLY77, ASN46, and GLU50.

Docking against *A. baumannii* -2XKJ

The docking results for *A. baumannii* Topoisomerase IV are presented in Table 7. **Rutin hydrate** again showed the highest affinity (XP-GScore –8.085 kcal/mol), forming interactions with ALA468, GLU1016, ARG1012, ARG561, and ASP518. Astragalin (–7.079 kcal/mol) and nicotiflorin (–6.787 kcal/mol) also demonstrated strong binding.

Quercetin (–5.920 kcal/mol; ΔG_bind –19.18 kcal/mol) and kaempferol (–5.352 kcal/mol; –17.73 kcal/mol) showed favourable binding free energies despite moderate docking scores. In contrast, phenolic acids showed weaker scores, ranging from –4.209 to –4.773 kcal/mol.

ADME Properties of compounds from *A. indica*

Pharmacokinetic predictions for the compounds are shown in Table 8. Most compounds exhibited high gastrointestinal absorption, except rutin hydrate and nicotiflorin, which showed low GI absorption. Only astragalin was predicted to cross the blood–brain barrier. Quercetin and kaempferol were identified as substrates or inhibitors of key CYP enzymes, including CYP3A4 and CYP2D6. Rutin and nicotiflorin were predicted as P-glycoprotein substrates. All compounds showed poor skin permeability, with Log Kp values between –6.58 and –10.35 cm/s.

Drug-Likeness and Physicochemical Characteristics

Drug-likeness assessment (Table 9) showed that most compounds complied with Lipinski's rule of five. Exceptions were rutin hydrate and nicotiflorin, which had three rule violations each due to high molecular weight and extensive hydrogen bonding features.

Bioavailability scores ranged from **0.17** for rutin, nicotiflorin, and astragalin to **0.56** for gallic acid, sinapic acid, and caffeic acid. Synthetic accessibility values varied between 1.22 and 6.84, with phenolic acids being the easiest to synthesize.

Toxicity Predictions

Toxicity results (Table 10) showed that most compounds fall within toxicity classes 4 or 5, indicating low acute toxicity. Rutin had the lowest predicted LD_{50} (100 mg/kg), while astragalin and nicotiflorin had the highest (5000 mg/kg). Some compounds exhibited specific toxicity alerts: rutin and sinapic acid were predicted immunotoxic, quercetin was predicted mutagenic, and caffeic acid showed hepatotoxicity.

TABLE 6: **Docking score, MMGBSA, and *E. coli* -7C7O residues-ligand interaction.**

Ligands	XP GScore	MMGBSA dG Bind	H-bond and Pi-Pi stacking with 7C7O
Gallic Acid	-5.059	25.3	ASP 73
Sinapic Acid	-4.565	13.4	GLY 77, THR 165
Caffeic Acid	-4.817	-24.2	ARG 36, ARG76, ASP 73, GLY 77
Rutin Hydrate	-9.327	-52.6	GLU 50, ASN 46, ASP 73
Quercetin	-6.829	-45.4	ASN 46, ASP 73, GLY 77
Kaempferol	-5.636	-38.4	VAL 43
Nicotiflorin	-7.415	-39.5	ASN 46, GLU 50
Astragalin	-6.499	-43.9	ASP 49, ASN 46

TABLE 7: Docking score, MMGBSA, and *A. baumannii* -2XKJ residues-ligand interaction.

Ligands	**XP GScore**	**MMGBSA dG Bind**	**H-bond and Pi-Pi stacking with 7C7O**
Gallic Acid	-4.773	-6.22	ASN 1024, ARG 1012
Sinapic Acid	-4.209	-10.36	ALA 466,
Caffeic Acid	-4.617	-10.11	ARG 1012, PRO502
Rutin Hydrate	-8.085	3.76	ALA 468, GLU 1016, ARG 1012, ARG 561, ASP 518
Quercetin	-5.920	-19.18	ARG 561, PRO 501, GLU 1016, ARG 1012
Kaempferol	-5.352	-17.73	ARG 561, PRO 501, GLU 1016, ARG 1012
Nicotiflorin	-6.787	45.26	GLN 1020, GLU 1016, ARG 1012, ARG 561
Astragalin	-7.079	28.35	GLU 1016, HIS 1006, THR 1002, GLU 1010, ARG 1012

TABLE 8: PHARMACOKINETIC PROPERTIES OF COMPOUNDS FROM *A. INDICA*

Ligand	GI Abs	BBB Perm	P-gp Sub	CYP1A2 Inhibitor	CYP2C19 Inhibitor	CYP2C9 Inhibitor	CYP2D6 Inhibitor	CYP3A4 Inhibitor	Log Kp (cm/s)
Gallic Acid	High	-	-	-	-	-	-	+	-6.86
Sinapic Acid	High	-	-	-	-	-	-	-	-6.63
Caffeic Acid	High	-	-	-	-	-	-	-	-6.58
Rutin Hydrate	Low	-	+	-	-	-	-	-	-10.35
Quercetin	High	-	-	+	-	-	+	+	-7.05
Kaempferol	High	-	-	+	-	-	+	+	-6.70
Nicotiflorin	Low	-	+	-	-	-	-	-	-9.91
Astragalin	High	+	-	-	-	-	-	-	-8.52

Legend : BBB, Blood-Brain Barrier; GI, gastrointestinal, - = non-inhibitor, + = inhibitor

Table 9: Drug-Likeness and Physicochemical Profile of Selected *A. indica* Compounds

Ligand	Lipinski's Violations	BS	PA Alert	SA	MW (g/mol)	HA	AHA	RB	HBA	HBD	TPSA (Å²)
Gallic Acid	0	0.56	1	1.22	170.12	12	6	1	5	4	97.99
Sinapic Acid	0	0.56	0	2.17	224.21	16	6	4	5	2	75.99
Caffeic Acid	0	0.56	1	1.81	180.16	13	6	2	4	3	77.76
Rutin Hydrate	3	0.17	1	6.84	644.58	45	16	6	17	11	278.66
Quercetin	0	0.55	1	3.23	302.24	22	16	1	7	5	131.36
Kaempferol	0	0.55	0	3.14	286.24	21	16	1	6	4	111.13
Nicotiflorin	3	0.17	0	6.48	594.52	42	16	6	15	9	249.20
Astragalin	2	0.17	0	5.29	448.38	32	11	4	10	7	190.28

Legend: MW, Molecular weight; *HA, Num. heavy atoms; *AHA, Num. arom. Heavy atoms; *RB, Num. rotatable bonds; *HBA, Num. H-bond acceptors; *HBD, Num. H-bond donors; *BS, Bioavailability, SA, synthetic accessibility.

TABLE 10: TOXICITY PREDICTION OF COMPOUNDS FROM *A. INDICA*

Ligand	HT	CG	IT	MG	CT	Toxicity Class	PA (%)	LD50 (mg/kg)
Gallic Acid	-	+	-	-	-	4	70.97	2000
Sinapic Acid	-	-	+	-	-	4	70.97	1772
Caffeic Acid	-	+	-	-	-	5	70.97	2980
Rutin Hydrate	-	-	+	-	-	5	100	100

Quercetin	-	+	-	+	-	3	100	159
Kaempferol	-	-	-	-	-	5	70.97	3919
Nicotiflorin	-	-	-	-	-	5	72.9	5000
Astragalin	-	-	-	-	-	5	72.9	5000

Legend : + denotes active, - denotes inactive. HT-Hepatotoxicity, CG-Carcinogenicity, IT-Immunotoxicity, MG-Mutagenicity, CT-Cytotoxicity, PA-Prediction Accuracy. *If swallowed, Class 1 is fatal, Class 2 is fatal, Class 3 is toxic, Class 4 is harmful, Class 5 may be harmful, and Class 6 is not harmful. *

DISCUSSION

The present study reaffirms *Azadirachta indica* as a valuable reservoir of antimicrobial phytochemicals, particularly against multidrug-resistant bacterial pathogens. The crude ethanolic extract demonstrated markedly greater antibacterial activity than both the n-hexane and ethanol fractions, a pattern commonly reported in medicinal plant research where unfractionated extracts outperform isolated fractions due to the retention of chemical diversity and synergistic interactions. In this study, the crude extract produced substantial inhibition zones across all tested organisms, with *A. baumannii* showing the highest susceptibility (20 mm). This heightened sensitivity aligns with earlier reports that *Acinetobacter* species exhibit membrane fragility when exposed to phytochemical-rich extracts (Dhiman *et al*., 2021). The concentration-dependent decline in inhibition zones further confirms the potency of the crude extract. *E. coli* also remained susceptible at the lowest concentration tested (8 mm at 12.5 mg/mL), consistent with findings that phenolic-rich plant extracts readily disrupt Gram-negative outer membranes (Cushnie and Lamb, 2021).

Fractionation markedly reduced antibacterial potency. The ethanolic fraction showed inhibition zones of 6–10 mm at the highest concentration, while the n-hexane fraction showed only 9–12 mm zones. This loss of activity can be attributed to disruption of synergistic interactions among phytochemical classes, including flavonoids, phenolic acids, terpenoids, and glycosides. Such synergy is crucial for broad-spectrum efficacy and is often diminished when phytochemicals partition into separate solvent phases. Similar trends have been documented in neem and other medicinal plants, where crude extracts outperform fractions or isolated compounds (Süntar, 2020; Biney *et al*., 2021; Chatterjee *et al*., 2023). The antibacterial performance observed in this study is further corroborated by the work of Singaravelu *et al*. (2019), who evaluated the concentration-dependent antibacterial activity of *Azadirachta indica* crude bark extracts against both Gram-positive and Gram-negative bacterial pathogens. Using agar well diffusion and broth microdilution assays, the authors demonstrated that crude neem bark extracts exhibited broad antibacterial activity against *Enterococcus faecalis*, *Pseudomonas aeruginosa,* and *Proteus mirabilis* across all tested concentrations, while *Staphylococcus aureus* showed measurable susceptibility only at higher extract concentrations (>500 µg/mL). This reduced sensitivity of *S. aureus* was attributed to the protective nature of its thick peptidoglycan layer, necessitating higher phytochemical concentrations to achieve effective inhibition. These findings closely mirror the present study, where *S. aureus* similarly exhibited lower susceptibility compared to Gram-negative isolates, and where antibacterial efficacy increased with extract concentration. Importantly, Singaravelu *et al*. emphasized the superior efficacy of crude bark extracts, highlighting the role of multiple phytochemical constituents acting synergistically rather than isolated compounds. This reinforces the current observation that preservation of phytochemical complexity in the crude stem bark extract of A. indica underpins its enhanced antibacterial potency against multidrug-resistant pathogens.

MIC results reinforced the superiority of the crude extract, which produced MICs of 50–100 mg/mL, in contrast to MIC values ≥100 mg/mL observed for both fractions. *Enterococcus faecalis* was the least susceptible isolate, consistent with reports of its intrinsic resilience against oxidative and phenolic stress (Borges *et al*., 2015). The strong activity of the crude extract correlates with the HPLC findings, which revealed abundant phenolic acids and flavonoids including quercetin, gallic acid, caffeic acid, rutin, kaempferol, astragalin, and nicotiflorin. Quercetin—present in the highest concentration—has broad-spectrum antimicrobial actions such as cell wall disruption, interference with nucleic acid synthesis, and

membrane permeabilization (Cushnie and Lamb, 2021; Said *et al*., 2022). Gallic acid and caffeic acid are known to induce oxidative stress and destabilize bacterial membranes (Jayanthi and Lalitha, 2021), while rutin and astragalin have reported antibacterial and anti-inflammatory activities, often mediated through enzyme inhibition and ROS generation (Tungmunnithum *et al*., 2022).

The synergistic interactions among these compounds likely underpin the superior efficacy of the crude extract. Flavonoids frequently enhance membrane permeability, enabling deeper penetration of phenolic acids and other bioactives (Chakraborty *et al*., 2022). Fractionation disrupts this interplay by segregating compounds into different solvent layers; for instance, flavonoids concentrate in ethanol while terpenoids migrate to n-hexane. The moderate activity of the n-hexane fraction suggests that non-polar constituents such as limonoids—including azadirachtin and nimbolide—may also contribute to antibacterial effects (Rahal *et al*., 2019; Moin *et al*., 2021), though not sufficiently to match the potency of the crude mixture. Similar observations have been reported where crude neem extracts show stronger antimicrobial and antifungal activity than isolated flavonoids or triterpenoids (Wylie and Merrell, 2022).

The observed pattern in bacterial susceptibility—where Gram-negative organisms showed greater sensitivity than Gram-positive—may relate to differences in cell wall architecture. Although Gram-negative bacteria possess an outer membrane, many phenolic acids disrupt its lipid components, while the thick peptidoglycan layer and teichoic acids of Gram-positive bacteria can impede diffusion of bulky flavonoids (Borges *et al*., 2015). Collectively, these findings support the established view that *A. indica* stem bark is a potent antimicrobial agent whose efficacy is greatly enhanced when its full complement of secondary metabolites is preserved.

Based on the strong inhibitory activity observed, *E. coli* and *A. baumannii*—the most responsive organisms—were selected for in-silico evaluation targeting DNA gyrase B and Topoisomerase IV. Molecular docking revealed that rutin hydrate, quercetin, astragalin, and nicotiflorin exhibited high binding affinities for both enzymes, suggesting potential dual-target inhibition, a desirable mechanism for combating multidrug-resistant Gram-negative pathogens. Key interactions with catalytic residues such as ASP73, GLY77, ARG1012, and GLU1016 align with established roles of flavonoids in stabilizing or obstructing the topoisomerase catalytic pocket. These findings are in agreement with Oluyele and Akinyeke (2025), who reported that seed-derived compounds from *Persea americana* displayed strong binding affinity toward DNA gyrase B in *Salmonella typhi*. Likewise, Oyedemi *et al*. (2020) observed that phytochemicals from *Ligustrum lucidum* and *Lobelia inflata* exhibited comparable enzyme-targeting interactions, reinforcing the relevance of plant-derived flavonoids as potential antimicrobial agents.

Although rutin hydrate had the highest affinity, its poor pharmacokinetic characteristics—including low gastrointestinal absorption and multiple Lipinski violations—limit its direct therapeutic potential. Conversely, quercetin and kaempferol demonstrated favourable binding energies and more acceptable drug-likeness profiles. Phenolic acids showed modest docking scores but superior pharmacokinetic and safety characteristics, making them promising candidates for combination or supportive therapy. Toxicity predictions indicated low acute toxicity for most compounds, though potential mutagenicity (quercetin) or immunotoxicity (rutin) highlights the need for further biological validation.

CONCLUSIONS

In conclusion, *Azadirachta indica* stem bark exhibited strong antibacterial activity, with the crude extract outperforming its fractions due to synergistic interactions among multiple phenolic and flavonoid constituents identified by HPLC. In-silico analyses further revealed that several of these compounds, particularly rutin hydrate, quercetin, astragalin, and nicotiflorin, possess strong affinity for DNA gyrase B and Topoisomerase IV in *E. coli* and *A. baumannii*, indicating potential dual-target inhibition relevant for addressing multidrug resistance. While certain flavonoids showed pharmacokinetic limitations, phenolic acids displayed favourable drug-likeness and safety profiles. Future work should prioritize isolation and characterization of individual compounds, synergy testing with antibiotics or combined phytochemicals, in vivo efficacy and toxicity studies, and formulation or structural optimization to enhance bioavailability,

thereby supporting the development of neem-derived antimicrobial agents for combating resistant pathogens.

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Supplementary Materials

None

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