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The Antimicrobial Activity of some of the *Ziziphus spina-christi* Constituents on Multi-drug Resistance Microbial Strains

Ashraf Noor-Aldeen Mohsen [1]*, Samara Mowaffaq Ali [2]

[1,2] Department of Pharmacology, College of Medicine, University of Baghdad, Iraq

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CORRESPONDING AUTHOR:
Ashraf Noor-Aldeen Mohsen e-mail ashraf.noor2306m@comed.uobaghdad.edu.iq

ABSTRACT

Multidrug-resistant (MDR) pathogens such as *MRSA, Escherichia coli, Pseudomonas aeruginosa,* and *Acinetobacter baumannii* remain a major global health challenge, driving the search for alternative antimicrobial agents from natural sources. *Ziziphus spina-christi* (Sidr) is traditionally used for treating infectious diseases and is known to contain bioactive phenolic compounds and cyclopeptide alkaloids with potential antibacterial activity.

This study evaluated the antibacterial properties of phenolic and alkaloid fractions extracted from the leaves and stem bark of *Z. spina-christi* against MDR clinical isolates. A total of 258 isolates were collected from multiple hospitals, of which 50 MDR strains (13 *MRSA*, 13 *E. coli*, 12 *P. aeruginosa*, 12 *A. baumannii*) met the inclusion criteria. Extracts were prepared using Soxhlet extraction followed by acid-base fractionation, and their chemical profiles were confirmed by HPLC. Antibacterial activity was assessed by agar well diffusion, and minimum inhibitory concentrations (MICs) were determined using micro-broth dilution.

Phenolic fractions demonstrated superior antibacterial activity compared with alkaloid fractions. The leaves phenolic fraction showed the strongest effect, with MIC values of 6.25 mg/mL against *MRSA, P. aeruginosa,* and *A. baumannii*, and 3.125 mg/mL against *E.coli*. Stem phenolic showed comparable but slightly lower activity, while stem alkaloids exhibited minimal effects and no inhibition of *MRSA*.

These findings highlight Z. spina-christi, particularly its leaf phenolics, as a promising natural source of antibacterial agents against MDR pathogens.

1. Introduction

1.1. Antimicrobial Resistance and its Causes

The growing resistance of bacteria to antimicrobial agents represents a serious global public health threat, often limiting effective treatment options for infectious diseases[1]. Humans have long battled bacterial infections, and while penicillin initially proved effective, its overuse has driven widespread antibiotic resistance. Today, increasing antibiotic use especially in developing countries has accelerated resistance, leading to higher morbidity, mortality, and clinical complications[2-3]. Antimicrobial resistance causes a major global health burden, with over one million deaths directly attributable to resistant infections annually. The highest impact is linked to pathogens such as *E. coli, Staphylococcus aureus (MRSA), Klebsiella pneumoniae, Streptococcus pneumoniae, Acinetobacter baumannii, and Pseudomonas aeruginosa*, with resistance to β-lactams and fluoroquinolones accounting for most AMR-related deaths [4-5].

Misuse and overuse of antimicrobials are major drivers of resistance, especially in developing countries where over the counter access and weak regulatory systems facilitate inappropriate consumption. Even correct use can contribute to resistance, but unnecessary or excessive use accelerates the problem. Non-compliance often linked to poverty further exacerbates resistance, as some patients stop treatment once symptoms improve or miss doses unintentionally. Additionally, the circulation of poorly manufactured or low-potency antimicrobial products exposes pathogens to sub-therapeutic drug levels, creating ideal conditions for resistance development[6-7].

1.2. Ziziphus Spina Christi (ZSC)

Commonly known as Christ's Thorn Jujube and locally as "Sidr" or "Nabuk," is a deciduous tree native to tropical and subtropical regions and widely distributed across North Africa, Southern Europe, the Mediterranean, Australia, sub-tropical America, and parts of Asia including the Middle East. Christ's Thorn Jujube belongs to the Rhamnaceae family of Rosales order that contains about 60 genus and more than 850 species. this plant has long been used in traditional medicine for its demulcent, depurative, anodyne, and emollient properties. It has been employed to manage various ailments such as stomach and liver disorders, urinary problems, obesity, diarrhea, diabetes, insomnia, and various skin infections[8]. Traditionally, ZSC has been widely employed in Saudi folk medicine to treat various ailments such as palpitations, hypertension, insomnia, irritability, diabetes, wounds, skin disorders, ulcers, and a range of infectious diseases including ringworm, gonorrhea, and other sexually transmitted infections. Phytochemical studies show that *ZSC* leaves are rich in polyphenolic compounds, including phenolics, flavonoids, and tannins, which contribute to their wide range of biological activities. These leaves exhibit multiple pharmacological effects, such as antioxidant, antidiabetic, anticancer, anti-inflammatory, and antimicrobial activities [9-10].

1.3. Selected Microbial Strains

1.3.1. ***Methicillin-Resistant Staphylococcus Aureus*** **:** (MRSA) is a Gram-positive coccus forming grape-like clusters and a major cause of hospital- and community-acquired infections, including pneumonia, surgical site, cardiovascular infections, and bacteremia. It shows high mortality worldwide, with widespread resistance to penicillin. The emergence of methicillin-resistant S. aureus (MRSA), mediated by the mecA gene encoding PBP2a, has led to multidrug resistance, making MRSA a serious global health challenge[11-12].

1.3.2. Pseudomonas Aeruginosa : is a motile, aerobic, Gram-negative bacillus with multiple virulence factors, including biofilm formation and

toxin production, enabling severe tissue damage and systemic infection. It is a common nosocomial pathogen causing pneumonia, catheter-associated urinary tract infections, and burn wound infections. The emergence of multidrug-resistant P. aeruginosa, with high mortality rates, poses a serious global health threat due to its strong intrinsic and acquired antibiotic resistance [13-15].

1.3.3. *Escherichia Coli:* (*E. coli*) is a Gram-negative, anaerobic, rod-shaped bacterium commonly found as a commensal in the human intestine, where it contributes to gut homeostasis and vitamin K synthesis. However, pathogenic strains can cause a wide range of intestinal and extra-intestinal infections, including UTIs, gastrointestinal disease, bacteremia, meningitis, and endocarditis. E. coli is also a major food borne pathogen, often linked to contaminated meat, and its increasing antimicrobial resistance-driven by gene transfer, mutations, and efflux pumps-represents a significant public health concern [16-17].

1.3.4. Acinetobacter Baumannii: (*A. baumannii*) is an aerobic, Gram-negative, non-motile coccobacillus and a major nosocomial pathogen causing pneumonia, urinary tract infections, bacteremia, meningitis, and wound infections. It is a key member of the ESKAPE group (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, A.baumannii, Pseudomonas aeruginosa, and Enterobacter spp*.) and is notable for its extreme multidrug resistance, including resistance to carbapenems and last-resort antibiotics. Its ability to form biofilms and persist in hospital environments makes *A. baumannii* a serious global health threat with limited treatment options[18-20].

1.4. Aim of the study

The aim of the present focuses on the following issues:

- Evaluation the antimicrobial activity of phenolic and alkaloid fractions of *Ziziphus spina-christi.*
- Comparing the antimicrobial activity of phenolic and alkaloid fractions between the leaves and bark of *Ziziphus spina-christi*.
- Comparing the antimicrobial activity between phenolic and alkaloid fractions of *Ziziphus spina-christi*
- Determine the minimum inhibitory concentration (MIC) of phenolic and alkaloid fractions of *Ziziphus spina-christi* against selected microbial strains.

2. Materials and Methods

2.1. Study Design

An Experimental / Laboratory-based Study.

In vitro evaluation of antimicrobial activity of plant extracts / fractions.

The Present Study Was Conducted At Tuz General Hospital In Tuz / Saladin And Ghazi Al-Hariri Surgical Specialties Hospital, Specialized Burn Hospital, The National Center For Educational Laboratories In The Medical City Of Baghdad, Iraq. The samples were collected during period November 2024 to October 2025. The duration of the study was 14 months from September 2024 to December 2025.

2.2. Plant material

Identification of Plant

The identification and authentication of *Ziziphus spina-christi* tree which belongs to the Rhamnaceae family, was conducted in (University Of Baghdad / Iraq Natural History Research Center And Museum Plant Environment Department).

Collection of Plant Material

The leaves and stem bark of *Ziziphus spina-christi* were collected from Baghdad, Iraq (University of Baghdad, College of Medicine campus) in November 2024. The geographical coordinates of the collection site were (33°20′46.4″ N and 44°22′40.7″

E). The collected leaves were thoroughly washed with distilled water to remove surface contaminants and debris. Both the leaves and stem bark were then air-dried under shade at room temperature for 10 days to prevent degradation of heat-sensitive compounds. After drying, the plant materials were separately ground into fine powder using an electric grinder. The powdered samples were then weighed, yielding 725 g of leaf powder and 625 g of stem bark powder, and stored in airtight containers until extraction.

2.3. Pilot Study

Powdered material of both the stem bark and leaves was used separately to prepare crude extracts employing different solvents (distilled water, ethanol, and methanol). The extraction was performed using a Soxhlet apparatus. Specifically, 25 g of the powdered leaves were placed in an extraction thimble and extracted with 80% methanol in a round-bottom flask at 50 °C for 8 hours. The solvent was subsequently evaporated under reduced pressure using a rotary evaporator at 45 °C, and the resulting concentrate was dried in an oven at 37 °C for 10 hours to obtain a dry powder, which was stored in a refrigerator until use. The same procedure was repeated for aqueous and ethanolic extractions and also applied to the stem bark to obtain three crude extracts from the leaves and three from the stem bark.

Different concentrations of these extracts (100, 200, 400, and 500 mg/ml) were prepared using dimethyl sulfoxide (DMSO) for alcoholic extracts and distilled water for aqueous extracts. The antibacterial activity of the extracts was evaluated against selected bacterial strains, namely *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*, using the agar well diffusion method on Mueller–Hinton agar (MHA).

Fresh bacterial inoculum was prepared by sub-culturing the test organisms from their preservation medium onto blood agar plates (BA) using the streak plate method, followed by incubation at 37 °C for 24 h. Isolated colonies were aseptically picked and suspended in sterile normal saline (0.85%), then adjusted to a turbidity equivalent to the 0.5 McFarland standard using a spectrophotometer. Each standardized suspension was uniformly swabbed onto MHA plates in three directions, rotating the plate by 60° after each streaking, and allowed to dry for 3 min at room temperature.

Five wells (6 mm in diameter) were made in each plate using a sterile cork borer. Each well was filled with one of the prepared concentrations of the extract solution (100, 200, 400, and 500 mg/mL)100 µL, while the fifth well was filled with DMSO as a negative control. Plates were incubated at 37 °C for 24 h, after which the diameters of inhibition zones were measured in millimeters for all extracts. A pilot study was conducted to determine which solvent produced extracts with the highest antibacterial activity.

2.4. Extraction of Plant Material

Extraction of *ZSC* stem bark (600 g) and leaves (700 g) was carried out separately. Initially, the powdered plant materials were defatted via maceration in hexane for 24 hours with occasional stirring to remove lipophilic impurities. The residues were then air-dried at 25 °C to eliminate residual solvent. Subsequently, the defatted materials were subjected to Soxhlet extraction using 80% methanol as the solvent for 8 hours. The obtained extracts were filtered through Whatman No. 1 filter paper and concentrated under reduced pressure using a rotary evaporator at 45 °C to obtain dry methanolic extracts, which were stored in airtight containers at 4 °C until further use.

2.5. Phytochemical Analysis

The qualitative phytochemical analysis of the methanolic crude extracts of *ZSC* leaves and stem bark was performed using standard phytochemical screening procedures to identify the presence

of major bioactive constituents.

Phenols Test (Ferric chloride test)

The addition of two milliliters of distilled water to the crude extract, followed by two to three drops of 10% ferric chloride solution. Formation of a bluish-black coloration indicated the presence of phenolic compounds[21].

Alkaloids Test (Mayer's test)

The crude extract was treated with dilute hydrochloric acid and filtered. A few drops of Mayer's reagent (potassium mercuric iodide) were then added to the filtrate. The appearance of a brownish or reddish precipitate indicated the presence of alkaloids[21].

Saponines Test (Foam test)

Twenty milliliters of distilled water were added to the crude extract in a graduated cylinder and shaken vigorously for 15 minutes. Formation of a stable foam layer indicated the presence of saponins[21].

Glycosids Test (Benedict's test)

Benedict's reagent was added to the filtrate, which was then heated gently in a boiling water bath for 5 minutes. Formation of an orange-red precipitate indicated the presence of reducing sugars[21].

Tannins Test (Lead sub acetate test) and (Braymer's test)

a) Three drops of lead subacetate solution were added to 1 mL of the crude extract; formation of a white gelatinous precipitate indicated the presence of tannins.

b) One milliliter of the extract was boiled with 3 mL of distilled water, filtered, and a few drops of 10% ferric chloride were added. Development of a blue-green color confirmed the presence of tannins[22].

Resins Test (Turbidity test)

One milliliter of the plant extract was dissolved in acetone and heated in a water bath for 2 minutes, followed by filtration. Twenty milliliters of 4% hydrochloric acid were added to the filtrate. The appearance of turbidity indicated the presence of resins[22].

Coumarins (NaOH paper test)

Approximately 0.5 g of crude extract was placed in a test tube, and the mouth of the tube was covered with filter paper moistened with 1 N NaOH. The tube was heated in a water bath for a few minutes. The appearance of yellow fluorescence on the paper under UV light confirmed the presence of coumarins[22].

Flavonoids (Alkaline reagent test)

One milliliter of crude extract was mixed with 2 mL of 2% NaOH solution. The formation of an intense yellow color that became colorless upon addition of a few drops of dilute hydrochloric acid indicated the presence of flavonoids[22].

Terpenes

Two milliliters of crude extract were mixed with 5 mL of chloroform and evaporated on a water bath. A few drops of concentrated sulfuric acid were then added, and the mixture was heated on a water bath. Development of a grey coloration indicated the presence of terpenes[22].

Steroids (Liebermann Burchard test)

The crude extract was dissolved in chloroform and filtered. The filtrate was mixed with a few

drops of acetic anhydride solution, boiled, and cooled. Concentrated sulfuric acid was then carefully added. Formation of a brown ring at the interface indicated the presence of steroids[21].

2.6. Isolation of Phenolic Fraction From Crude Methanolic Extract

Approximately 40 g of the crude methanolic extract of *ZSC* leaves was placed in a 1000 mL round-bottom flask, to which 800 mL of 2% acetic acid solution was added. The mixture was subjected to extraction using a reflux condenser in a water bath at 100 °C for 1 hour and then was allowed to cool to room temperature. The cooled mixture was first filtered through muslin cloth and then through Whatman No. 1 filter paper. An equal volume of n-propanol was subsequently added to the filtrate, followed by the gradual addition of sodium chloride until saturation was achieved, resulting in the formation of two distinct layers. The upper organic layer, containing the phenolic compounds, was carefully separated using a separatory funnel. This layer was then concentrated under reduced pressure using a rotary evaporator and the resulting residue was dried. The dried phenolic fraction was stored in sealed glass tubes at 4 °C until further use.

The same procedure was repeated to isolate of phenolic compounds from 40 g of the crude methanolic extract of the stem bark[23-24].

2.7. Isolation of Alkaloid Fraction From Crude Methanolic Extract

Approximately 20 g of the crude methanolic extract of *ZSC* leaves was acidified with 2% sulfuric acid (H_2SO_4) dropwise until the pH reached 1-2. The acidified solution was then basified by the gradual addition of 10% ammonium hydroxide (NH_4OH) until the pH reached 9. The resulting basic solution was transferred to a separatory funnel, and 30 mL of chloroform was added. The mixture was shaken several times and allowed to stand until two distinct layers formed. The lower chloroform layer, containing the alkaloid fraction, was collected. This extraction step was repeated three times, and the chloroform layers were combined. The pooled chloroform extract was then concentrated and stored in tightly sealed glass tubes at 4 °C until further use.

The same procedure was followed to isolate the alkaloid fraction from 20 g of the crude methanolic extract of the stem bark[25-26].

2.8. Analysis of Phenolic And Alkaloid Fractions Obtained From Leaves And Steam Bark of *Ziziphus Spina Christi* Methanolic Extract Using HPLC

The phytochemical constituents of phenolic and alkaloid fractions were identified and quantified by using high-performance liquid chromatography (HPLC)[27].

The separation occurred on Shimadzu 10AV-LC liquid chromatography equipped with binary delivery pump model LC-10A Shimadzu, the eluted peaks were monitored using UV –Vis 10 A-SPD spectrophotometer.

Determination of Alkaloids In *Ziziphus* Leaves And Stem Bark Extract

The alkaloid fractions that were isolated from leaves and stem bark were dissolved in 1 ml HPLC grade CH_3OH for further analysis by HPLC according to the optimum separation of the authentic standard, then the concentrations were determined by comparison the area of standard with that of sample under the same separation conditions.

The extract of alkaloids according to the enclosed procedure was separated on FLC (Fast Liquid Chromatography) column,

Column : 3μm particle size, Phenomenex C-18 (50 x 4.6 mm I.D) column,

Mobile phase : were 0.01M phosphate buffer

pH 6.2: acetonitrile (75:25, V/V)

Injection volume : 20 µl

Detection : UV set at 310 nm

Flow rate : 1.4 ml/min.

Determination of Phenols And Flavonoids

The phenolic fractions that were isolated from leaves and stem bark were resuspended in 1.0 ml HPLC grade methanol by vortexing, the mixture was passed through 2.5 µm disposable filter, and stored at 4°C for further analysis, then 20 µl of the sample injected into HPLC system according to the optimum conditions. The concentration of each phenolic compound was calculated by comparing the peak area of the standard with that of the sample under the same separation conditions.

The main phenolic and flavonoid compounds were separated on FLC (Fast Liquid Chromatography) column under the optimum conditions:

Column : Phenomenex C-18 ,3µm particle size (50 x 2.0 mm I.D) column,

Mobile phase : linear gradient of, solvent A 0.1% phosphoric acid: solvent B was of acetonitrile added 0.1% phosphoric acid, linear gradient program from 0%B to 100%B for 15 minutes

Injection volume : 20 µl

Flow rate : 1.0 ml/min.

Detection : UV at 280 nm.

2.9. Determination of Alkaloids

Different swabs including (urine, sputum, wound, foley catheter, burn and ear swab) were taken (using sterile cotton swab) from 258 patients of both sexes aged between 16-80 years old in Tuz General Hospital in Tuz / Saladin, Ghazi Al-Hariri Hospital For Specialized Surgery, Specialized Burn Hospital and The National Center For Educational Laboratories In The Medical City Of Baghdad, Iraq, during the period November 2024 to October 2025. These swabs were cultured on MacConkey Agar and blood agar then incubated for 24 hour at 37°C.

2.9.1. Identification of Bacteria

Morphological and Cultural Characteristics

Gram staining was used to identify the bacterial isolates, which allowed for the initial distinction between Gram-positive and Gram-negative organisms. In order to examine each isolate's cultural characteristics, it was also cultivated on adequate selective and differential media. Reliable confirmation of the bacterial species under investigation was made possible by the combination of Gram stain results and cultural characteristics.

Identification Using (Identification And Susceptibility Testing, IST Kits)

Confirmation of bacterial isolates identity was performed using IST card (DL biochemical identification system), which utilizes multiple colorimetric and turbidimetric biochemical reactions to determine species identity.

For identification of Gram-negative bacilli, including *P. aeruginosa* and *A. baumannii*, non-ferment. IST card (DL-120NE) was used.

For identification of *Enterobacteriaceae*, including *E. coli*, Enterobact. IST card (DL-120E) card was used.

For identification of Gram-positive cocci, including MRSA, Staph. IST card (DL-120STAPH) was used.

The susceptibility test was conducted also by IST card from DL biotech to determine whether the bacterial isolates were MDR or not.

Molecular Identification by Real Time-Polymerase Chain Reaction (RT-PCR)

Due to their high precision and specificity, molecular methods are increasingly used[28]. After DNA extraction by using QIAamp DNA Mini Kit (Cat. No. 51304), RT-PCR was performed as follows

1. Materials Required for RT-PCR :

- One-step RT-PCR kit, Species-specific primers and TaqMan Master Mix.
- Internal control (RNase).
- Thermal cycler (Quant Studio™ 5).

2. Prepare the reaction mix (20 µL total volume):

- 10 µL TaqMan Master Mix.
- 0.5 µL Forward Primer (10 µM).
- 0.5 µL Reverse Primer (10 µM).
- 0.5 µL of mecA-specific probe-VIC.
- 2 µL Template DNA.
- 6.5 µL Nuclease-free water to complete the final volume (20µL).

Note: The mecA-specific probe-VIC was applied to detect the mecA gene in *MRSA* isolates. Each bacterial isolate was identified using a specific target gene: oprL gene for *P. aeruginosa*, blaOXA-51-like gene for *A. baumannii*, and blaSHV gene for *E. coli*.

3. RT-PCR : The RT-PCR tubes containing the reaction mixtures were transferred to a thermal cycle and the process was started as shown in Table 1.

2.10. Antibacterial Activity Of *Ziziphus Spina-Christi*

Phenolic And Alkaloid Fractions of Methanolic Extract Of Leaves

A different concentrations of the phenolic fraction from the leaves were prepared (10, 40, 70, and 100 mg/mL) using DMSO to evaluate their antibacterial activity. For all bacterial strains, suspensions equivalent to a 0.5 McFarland standard were prepared and inoculated onto Mueller-Hinton agar (MHA) plates.

Five wells (6 mm in diameter) were made using a sterile cork borer; each of the first four wells was filled with one of the prepared concentrations (10, 40, 70 and 100 mg/mL), while the fifth well contained DMSO as a negative control. The plates were then incubated at 37°C for 24 hours. After incubation, the diameters of the inhibition zones were measured.

The same procedure was repeated for the alkaloid fraction of the leaves, and the inhibition zones were similarly recorded for comparison.

Phenolic And Alkaloid Fractions of Methanolic Extract Of Stem Bark

A different concentrations of the phenolic fraction from the leaves were prepared (10, 40, 70, and 100 mg/mL) using DMSO to evaluate their antibacterial activity. For all bacterial strains, suspensions equivalent to a 0.5 McFarland standard were prepared and inoculated onto Mueller-Hinton agar (MHA) plates.

Five wells (6 mm in diameter) were made using a sterile cork borer; each of the first four wells was filled with one of the prepared concentrations (10, 40, 70, and 100 mg/mL), while the fifth well contained DMSO as a negative control. The plates were then incubated at 37°C for 24 hours. After incubation, the diameters of the inhibition zones were measured. The same procedure was repeated for the alkaloid fraction of the leaves, and the inhibition zones were similarly recorded for comparison.

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) defined as the lowest concentration of an antimicrobial agent that inhibits the growth of a microorganism. the MIC values for phenolic and alkaloid fractions were measured using the micro-broth dilution method in a 96-well sterile microplate, This method involves adding bacterial broth to a 96-well plate, where they are exposed to various concentrations of fractions [29].

2.11. Statistical Analysis

Data were analyzed by the Statistical Package for Social Sciences (SPSS) ver.23. and ANOVA test was applied. The means were compared using Dun-

Table 1. Thermal Cycling Conditions (Standard protocol was used)

RT-PCR steps	Temperature	Time	Cycle
Initial Denaturation	95°C	3 minutes	1
Denaturation	95°C	15 seconds	45
Annealing / Extension	60°C	30 seconds	

can's multiple range test under the level of significantly 0.05.

3. Results and Discussion

The source of the samples collected and their percentages are listed in Table 2.

The pilot study results showed that the methanolic extract exhibited the highest activity compared to the other extracts; therefore, methanol was used as the extraction solvent.

3.1. Methanolic Extract of *Ziziphus Spina Christi*

Extraction of 600 grams of stem bark using 80% methanol as a solvent using soxhlet, yielded approximately 68 grams of methanolic extract and Extraction of 700 grams of leaves, produced approximately 63 grams of methanolic extract.

Results of preliminary qualitative phytochemical analysis of methanolic extracts of leaves and stem bark of Ziziphus spina Christi are listed in Table 3.

Identification of the constituents of phenolic and alkaloid compounds was conducted by comparing the retention times of the analyzed samples with reference standards under identical chromatographic conditions.

On the other hand the quantity of each compound was determined using equation 1.

$$conc\left(\frac{\mu g}{ml}\right) = \frac{area\ of\ the\ sample}{area\ of\ the\ standard}\ x\ conc.of\ standard\ x\ dilution\ factor$$

Analysis of the Phenolic Fraction in Leaves and Stem Bark By HPLC

The results of the HPLC analysis of standards and phenolic fraction in the leaves and stem bark are presented in Tables 4-6 and Figure S1 in supplementary material.

The results of the HPLC analysis of the standards and the alkaloid fraction of the leaves and stem bark are presented in Tables 7-9, and Figure S2 in supplementary material.

Table 2. Source of samples

No	Source of sample	Count	Percentage %
1.	Sputum	89	34.5 %
2.	Urine	67	26 %
3.	Blood	36	14 %
4.	Wound	18	7 %
5.	Burn	15	5.8 %
6.	Ascites fluid	18	7 %
7.	Stool	5	1.9 %
8.	Endotracheal tube aspiration	6	2.3 %
9.	Ear wound swab	4	1.6 %
Total		258	100 %

3.2. Identification of Bacteria

3.2.1. Morphological And Cultural Characteristics

Methicillin-Resistant Staphylococcus Aureus: From the total cultured samples, only[41] isolates showed feature resemble to *S. aureus*. In Mannitol Salt Agar, where *S. aureus* fermented mannitol and rendered the medium yellow, the isolates displayed a color change. The colonies looked yellow, round, and smooth. Under a 100X objective, Gram-stained cells appeared spherical and grouped in clusters resembling grapes.

Pseudomonas Aeruginosa: Out of the total cultured samples, 34 isolates exhibited characteristics consistent with *Pseudomonas aeruginosa*.. The isolates produced diffusible pigments on Mueller-Hinton agar that gave the medium a sweet, grape-like smell and a blue-green or yellow-green color typical of pyocyanin and pyoverdine. The colonies were huge, flat, smooth, metallic, and occasionally had a mucoid appearance. Gram staining revealed rod-shaped, Gram-negative bacteria.

Escherichia Coli: Out of the total cultured samples, 57 isolates exhibited features consistent with *E. coli* The isolates produced smooth, spherical, pink-red lactose-fermenting colonies on MacConkey agar, occasionally accompanied by a bile salt precipitate. Gram staining revealed rod-shaped, Gram-negative bacteria grouped either alone or in pairs.

Acinetobacter Baumannii: Out of the total cultured samples, 28) isolates exhibited features consistent with *Acinetobacter baumannii*. The isolates produced smooth, convex, opaque colonies on MacConkey agar that were pale pink in color and consistent with non-lactose fermentation. Gram-negative coccobacilli appeared as short, plump rods organized singly, in pairs, or sometimes in tiny clusters when stained with Gram.

3.2.2. Results of IST Card

The DL IST system demonstrated accurate biochemical identification of all four bacterial species studied. The card system also provided reliable MIC profiles essential for determining antimicrobial susceptibility and categorizing isolates as sensitive, intermediate or resistant, as showed in Figure S3, Figure S4, Figure S5 and Figure S6 in supplementary material.

Out of the 41 isolates that exhibited phenotypic characteristics consistent with *Staphylococcus aureus*, only 13 were confirmed as *MRSA* and MDR.

Among the 34 isolates resembling *Pseudomonas aeruginosa*, only 25 were confirmed as *P. aeruginosa*, of which 12 were identified as MDR.

From 57 isolates that appeared similar to *Escherichia coli*, 29 were confirmed as *E. coli*, and 13 of these demonstrated multidrug resistance MDR.

Finally, of the 28 isolates that showed morphological similarity to *Acinetobacter baumannii*, 12 were confirmed as *A. baumannii* and were also classified as MDR.

3.3. Molecular Identification

The real-time polymerase chain reaction (RT-PCR) assay was performed to detect specific target genes in each bacterial isolate: OprL gene in *P.aeruginosa*, blaOXA-51-like gene in *A.baumannii*, blaSHV gene in *E.coli* and mecA gene in *MRSA*. Figure S7 in supplementary material illustrates the positive and negative control reactions.

All the 13 *E. coli* isolates yielded positive results for the blaSHV gene. (Figure S8)

All the 13 *P. aeruginosa* isolates yielded positive results for the OprL gene. (Figure S9)

All the 13 *MRSA* isolates yielded positive results for the mecA gene. (Figure S10)

All the *A. baumannii* isolates yielded positive results for the blaOXA-51 like gene. (Figure S11 in supplementary material).

3.4. Antibacterial Activity of *Ziziphus Spina-Christi* Constituents

Inhibition zones for all fractions against *MRSA,*

Table 3. Results of phytochemical analysis

N.O	Secondary metabolites	Leaves extract	Stem bark extract
1.	Glycosides	--	+ +
2.	Alkaloids	+ + +	+ +
3.	Tannins	+ + +	+ + +
4.	Resins	+	+
5.	Saponin	+	+ +
6.	Coumarin	+ + +	+ + +
7.	Phenols	+ + +	+ + +
8.	Flavonoid	+ + +	--
9.	Terpenes	--	+
10.	Steroid	--	--

O.aeruginosa, E.coli and A. Baumannii are listed in the Tables 10-13 and depicted Figures S12-S15 in supplementary material.

Minimum inhibitory concentration (MIC)

Methicillin-Resistant Staphylococcus Aureus

The results of the (MIC) assay revealed that the phenolic fraction of the leaves, phenolic fraction of the stem, and alkaloid fraction of the leaves exhibited inhibitory activity at a concentration of 6.25 mg/mL. In contrast, the alkaloid fraction of the stem showed no inhibitory effect at any tested concentration, as illustrated in Figure S17 in supplementary material.

Escherichia Coli

The results of the (MIC) determination revealed that the phenolic fraction of the stem exhibited an MIC value of 6.25 mg/mL, whereas the phenolic fraction of the leaves, alkaloid fraction of the leaves and alkaloid fraction of the stem showed an MIC values of 3.125 mg/mL, Figure S18 in supplementary material.

Pseudomonas Aeruginosa

The results of the (MIC) assay showed that the phenolic fraction of the stem exhibited an MIC value of 3.125 mg/mL, whereas the phenolic fraction of the leaves, alkaloid fraction of the leaves, and alkaloid fraction of the stem demonstrated MIC values of 6.25 mg/mL, as shown in Figure S19 in supplementary material.

Acinetobacter Baumannii

The results of the (MIC) determination indicated that the phenolic fraction of the stem had an MIC value of 3.125 mg/mL, whereas the phenolic fraction of the leaves, alkaloid fraction of the leaves, and alkaloid fraction of the stem exhibited MIC values of 6.25 mg/mL, as shown in Figure S20 in supplementary material.

4. Conclusions

According to the present study HPLC analysis showed that the phenolic fraction of the leaves was rich in bioactive compounds such as rutin, tannins, and quinic acid, while the stem fraction also exhibited high levels of rutin along with notable amounts of quinic acid and luteolin. These polyphenolic constituents are well known for their strong antimicrobial activities. Likewise,

Table 4. Analysis of standard by HPLC

Seq.	Compounds	Retention time	Area
1.	Tannins	2.135	378836
2.	Quinic acid	2.93	345382
3.	Gallic acid	3.87	346347
4.	Chlorogenic acid	5.192	361632
5.	Coumaric acid	6.27	342886
6.	Rutin	7.352	331712
7.	Quercetin	8.035	319075
8.	Naringin	8.87	312825
9.	Luteolin	9.933	318218
10.	Ellagic acid	10.862	308424

Table 5. Analysis of the phenolic fraction of leaves by HPLC

Seq.	Compounds	Retention time	Area	Conc. (μg/ml)	%
1.	Tannins	2.165	319500	843.37	16.75
2.	Quinic acid	2.915	245878	711.9	14.14
3.	Gallic acid	3.848	102896	297.08	5.9
4.	Chlorogenic acid	5.163	148861	411.63	8.18
5.	Coumaric acid	6.24	77867	227.09	4.51
6.	Rutin	7.327	295340	890.35	17.7
7.	Quercetin	8.002	169629	531.62	10.56
8.	Naringin	8.838	129117	412.74	8.2
9.	Luteolin	9.917	124833	392.28	7.8
10.	Ellagic acid	10.852	96994	314.48	6.25

Table 6. Analysis of the phenolic fraction of steam bark by HPLC

Seq	Compounds	Retention time	Area	Conc. (μg/ml)	%
1.	Tannins	2.117	140969	372.1	12.43
2.	Quinic acid	2.923	132563	383.8	12.82
3.	Gallic acid	3.86	109620	316.5	10.58
4.	Chlorogenic acid	5.185	71323	197.2	6.6
5.	Coumaric acid	Not detected	----	----	----
6.	Rutin	7.35	228979	690.3	23.06
7.	Quercetin	8.012	88131	276.2	9.2
8.	Naringin	9.517	26164	83.63	2.8
9.	Luteolin	9.93	119537	375.64	12.54
10.	Ellagic acid	10.865	91900	297.96	9.95

Table 7. Analysis of standard by HPLC, conc: 100. (µg/ml)

Seq.	Compounds	Retention time	Area
1.	Ziziphine	2.688	309131
2.	Mauritine	4.173	332763
3.	Jujubine	5.183	328753
4.	Nummularine	6.125	332670

Table 8. Analysis of the alkaloid fraction of leaves by HPLC

Seq	Compounds	Retention time	Area	Conc. (µg/ml)	%
1.	Ziziphine	2.645	299419	968.6	41.52
2.	Mauritine	4.185	202246	607,8	26.06
3.	Jujubine	5.115	120481	366.45	15.71
4.	Nummularine	6.048	126209	379.4	16.26

Table 9. Analysis of the alkaloid fraction of steam bark by HPLC

Seq.	Compounds	Retention time	Area	Conc. (µg/ml)	%
1.	Ziziphine	2.697	212950	688.86	45,43
2.	Mauritine	3.913	28169	84.65	5,58
3.	Jujubine	4.172	169200	514,67	33.94
4.	Nummularine	6.113	75821	227,9	15.03

Table 10. Inhibition zone of four fraction in (mm) against *MRSA*

Extract	**Conc. (mg/ml)**				**Mean of extract**	**P.value**
	10	**40**	**70**	**100**		
PS	16.31	23.15	20.54	21.77	20.440 b	**0.0008**
AS	6.15	7.76	4.62	1.62	5.040 c	
PL	25.00	25.53	26.00	27.23	25.940 a	
AL	18.77	19.54	20.23	22.38	20.230 b	
Mean Conc.	16.55 a	18.99 a	17.850 a	18.250 a		
p.value	0.980					

AL alkaloid of leave, AS alkaloid of stem, PL phenol of leaves, PS phenol of stem

Table 11. Inhibition zone of four fraction in (mm) against *P. aeruginosa*

Extract	Conc. (mg/ml)				Mean of extract	p.value
	10	40	70	100		
PS	20.50	21.83	20.00	21.42	20.938 a	**0.172**
AS	21.00	21.00	20.00	18.50	20.125 a	
PL	20.00	21.25	18.08	20.25	19.895 a	
AL	19.50	19.50	19.00	19.00	19.250 a	
Mean of Conc.	20.250 a	20.895 a	19.270 a	19.793 a		
p.value	0.181					

AL alkaloid of leave, AS alkaloid of stem, PL phenol of leaves, PS phenol of stem
Letters indicate the results of post hoc multiple comparison tests performed after one-way ANOVA analysis . Values sharing the same letter within the same column or row are not significantly different from each other at the chosen significance level ($p \geq 0.05$), whereas values with different letters indicate statistically significant differences ($p < 0.05$).

Table 12. Inhibition zone of four fraction in (mm) against *E. coli*

Extract	Conc. (mg/ml)				Mean of extract	p.value
	10	40	70	100		
PS	15.00	16.54	12.77	17.38	15.420 a	**0.308**
AS	14.76	16.00	14.00	10.77	13.880 a	
PL	20.23	14.31	12.54	17.31	16.100 a	
AL	14.77	13.31	12.54	12.00	13.160 a	
Mean Conc.	16.190 a	15.040 a	12.963 a	14.360 a		
p.value	0.314					

AL alkaloid of leave, AS alkaloid of stem, PL phenol of leaves, PS phenol of stem
Letters indicate the results of post hoc multiple comparison tests performed after one-way ANOVA analysis . Values sharing the same letter within the same column or row are not significantly different from each other at the chosen significance level ($p \geq 0.05$), whereas values with different letters indicate statistically significant differences ($p < 0.05$).

HPLC profiling of the alkaloid fractions revealed the presence of four major cyclopeptide alkaloids: ziziphine, mauritine, jujubine, and nummularine, with ziziphine emerging as the predominant compound in both plant parts. Together, these findings highlight the potential role of these chemical constituents in driving the biological activity of the extracts.

Both phenolic and alkaloid fractions isolated from *Ziziphus spina-christi* leaves and stem bark exhibit significant antibacterial activity against multidrug-resistant (MDR) bacterial strains, including *MRSA, P. aeruginosa, E. coli and A. baumannii*. Among tested extracts, The phenolic extracts exhibited the strongest and most consistent antibacterial effects than the alkaloid fractions, as ev-

Table 13. inhibition zone of four fraction in (mm) against *A. Baumannii*

Extract	Conc. (mg/ml)				Mean of extract	p.value
	10	40	70	100		
PS	21.08	22.08	20.00	21.00	21.040 a	**0.050**
AS	20.00	21.00	20.25	17.17	19.605 b	
PL	21.08	20.58	17.83	20.00	19.872 b	
AL	19.00	18.58	17.00	18.50	18.270 b	
Mean Conc.	20.290 a	20.560 a	18.770 a	19.168 a		
p.value	0.292					

AL alkaloid of leave, AS alkaloid of stem, PL phenol of leaves, PS phenol of stem

Letters indicate the results of post hoc multiple comparison tests performed after one-way ANOVA analysis . Values sharing the same letter within the same column or row are not significantly different from each other at the chosen significance level ($p \geq 0.05$), whereas values with different letters indicate statistically significant differences ($p < 0.05$).

idenced by larger inhibition zones. The superior performance of the fraction is likely due to its rich composition of bioactive compounds.

The antibacterial assessment of *Ziziphus spina-christi* extracts revealed that the leaves and stem bark fractions had largely comparable antibacterial activity, with only slight differences observed between them. Although their inhibition zones, MIC values, and activity against multidrug-resistant (MDR) pathogens were closely aligned, the leaves regularly demonstrated a slight advantage. The minor variations may be attributed to small difference in the concentration and substantial compositional of bioactive compounds.

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