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Antimicrobial Activity of *Ziziphus spina-christi* against *Staphylococcus aureus, Escherichia coli,* and *Shigella flexneri*

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ABSTRACT

Antibiotic resistance remains a global concern, with up to 1.91 million deaths projected to occur due to resistant Gram-negative and Gram-positive bacteria by 2050. The study hence aimed to assess the antimicrobial activity of *Ziziphus spina-christi* leaf extracts in relation to specific bacterial strains and elucidate the molecular mechanisms to validate the *in vitro* findings. *Ziziphus spina-christi* leaf extracts were tested against *Staphylococcus aureus (S. aureus)*, *Escherichia coli* (*E. coli*), and *Shigella flexneri* (*S. flexneri*). The leaf powder was subjected to both aqueous and methanol-dichloromethane extraction. Phytochemical products were determined by Liquid Chromatography-Mass Spectrometry and Gas Chromatography-Mass Spectrometry for water extract and methanol dichloromethane extract, respectively. The agar well diffusion method, broth microdilution, and minimum bactericidal concentration against three bacterial species, *S. aureus*, *E. coli*, and *S. flexneri,* were used to assess the antibacterial activity of extracts. The results have shown that both plant extract has a significant level of antibacterial activity at higher concentrations (400 mg/ml) against the gram-positive bacteria. In addition, the methanol-dichloromethane extract exhibited the highest antibacterial activity against Gram-negative bacteria (*S. flexneri*, and *E. coli*), conversely, the water extract demonstrated a lower activity against *S. flexneri* and *E. coli*, with inhibition zones of 15 ± 0 mm for both bacteria. At a lower concentration (100 mg/ml), the methanol-dichloromethane extract produced inhibition zones of 19.6 ± 0.5 mm against *S. aureus*, closely followed by *S. flexneri* and *E. coli*. The water extract exhibited high antibacterial activity against Gram-positive bacteria. However, exhibited reduced antibacterial activity against *S. flexneri* and *E. coli*, indicating a concentration-dependent antibacterial effect. Extraction methods were significantly different, with products generated from non-aqueous extraction demonstrating a higher potency against both Gram-negative and Gram-positive bacteria than the aqueous extract. Docking results demonstrated that water extract had a high binding activity against penicillin-binding proteins. Moreover, it serves as a potent beta-lactamase inhibitor as it binds to their active site, rendering them inactive and inhibiting the hydrolysis of Beta lactam antibiotics. In conclusion, the methanol-dichloromethane and water *Ziziphus spina-christi* leaves could be considered a promising source of antimicrobial ingredients.

Keywords: Antimicrobial resistance, Beta lactamase, Methanol-dichloromethane Extract, Molecular docking, *Ziziphus spina-christi* leave

INTRODUCTION

Antimicrobial resistance (AMR) ranks among the foremost challenges facing public health today. It is the third greatest cause of mortality after cardiovascular diseases and cancer (Salam et al., 2023). Major research published in January 2022 found that approximately 5 million people died from drug-resistant illnesses in 2019, and nearly 1.27 million people died from infections that were resistant to antimicrobials (Salam et al., 2023). According to the 2016 Global Burden of Disease (GBD) report, cumulatively, Diarrheagenic *Escherichia coli (E. coli*), especially enteropathogenic *E. coli* (EPEC) and enterotoxigenic *E. coli* (ETEC) resulted in 63,523 deaths among all ages annually throughout the world (Kariuki et al., 2022). Whereas around 212,438 deaths, which account for roughly 13.2% of all diarrheal fatalities worldwide, were ascribed to resistant shigellosis in 2016 (Mason et al., 2023). Additionally, plant components are less expensive than chemicals and have negligible to no negative host side effects (Odhav et al., 2010). Concern over the spread of resistant bacteria and fungi, among other diseases, including healthcare-associated infections, respiratory and gastrointestinal infections, and Tuberculosis, has increased, and with it, interest in the curative power of ancient remedies (Abdulrahman et al., 2022). A variety of modern medications, nutraceuticals, dietary supplements, and medicinal products, including artemisinin, quinine, vincristine, and others, are sourced from these medicinal plants (Newman and Cragg, 2020).

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Christ thorn (Ziziphus spina-christi) is a tropically native medicinal plant that has long been used to treat many illnesses, such as fever, pharyngitis, pulmonary disease, malaria, wounds, burns, stomach pain, urinary tract infections, intestinal rheumatism, diarrhea, and bronchitis (Alhassan et al., 2019). *Ziziphus spina-christi* (*Z. spina-christi*) fruits and leaves extracts have shown antibacterial activity against *Escherichia coli, Bacillus subtilis, and Candida. albicans,* and *Staphylococcus aureus* (Ads et al., 2022). Despite the stated efficacies and the beneficial compounds found in plants, such as flavonoids, saponins, alkaloids, indole derivatives, and fatty acids, a distinct gap in understanding the antibacterial properties and molecular mechanisms of action against multidrug-resistant bacterial pathogens (Ads et al., 2022).

The acquisition of antibiotic resistance in bacterial species can occur through different mechanisms, including vertical and horizontal gene transfer. The conjugative transfer of plasmids containing resistance genes among bacterial species is regarded as a critical method for resistance transmission in bacteria (Mansour et al., 2024). Bacteria may develop many strategies to resist antibiotics, including alterations in drug targets, restriction of cellular entrance, removal via efflux pumps, or drug inactivation. To pick the most effective antibiotics for treating multidrug-resistant bacteria, it is essential to comprehend and anticipate resistance trends (Mansour et al., 2024). The rising occurrence of multidrug-resistant strains has underscored the significance of advancing antibacterial that utilize mechanisms of antibiotic action that have not been previously exploited, such as targeting the adhesion molecules or quorum-sensing pathways, reducing biofilm formation, and pathogenicity (Tondi, 2021). Recent advancements in computational methodologies have fundamentally established the basis for the creation and identification of therapeutically active natural compounds that can target specific proteins. Penicillin-binding proteins (Freischem et al., 2021), fatty acid synthesis, and DNA Gyrase (Spencer and Panda, 2023) enzymes are recognized targets within a broader class of antibiotics that feature. Thus, the present study aimed to examine the antibacterial activity of *Z. spina-christi* leaf extracts against three bacterial isolates.

MATERIALS AND METHODS

Plant extract preparation

Fresh leaves were collected from Jomo Kenyatta University of Agriculture and Technology (JKUAT) Seedlings, Juja- Kiambu. The plant material was identified, verified, and authenticated by a plant taxonomist at the JKUAT Herbarium and was assigned the identification number HMMA.JKUATBH.001/2024. The leaves were washed twice with distilled water, and stored at -80°C for 72 hours, then the water content was removed using a freeze dryer (Model FDL-10N-50-8M) under controlled conditions of sample temperature -72°C, cold trap temperature -62°C, and a vacuum pressure 0.00001pa for 48 hours. The desiccated leaves are ground into a fine powder utilizing a blender. 100 grams of dried leaves to 500 ml of distilled water was used for aqueous extract preparation. The mixture was incubated for 24 hours in a shaker incubate (GYROMAX™ 727), followed by filtration using cotton and Whatman no.1 filter paper. The filtrate was stored at -40°C for 1 day and -80°C for 3 days before being freeze-dried under controlled conditions of sample temperature -68°C, cold trap temperature -62°C, and a vacuum pressure 0.00001pa for 72 hours to get the powder extract. Cold maceration of 100 g of dried leaves in 500 ml of dichloromethane and methanol mixture (1:1) was done for 72 hours regarding non-aqueous extract preparation. The solution underwent further filtration with Whatman No. 1 filter paper (Aleixandre-Tudo and du Toit, 2018). The solvents were removed from the extracts by drying them out with a rotary evaporator (Labtech DAIHAN, VP30, EV11) set at 40 rpm and 60°C. The powders were dried further in a 37°C oven and then kept at room temperature until needed.

Phytochemical compositions analysis

The study of phytochemical contents of non-aqueous extract was conducted using a Gas Chromatography-Mass Spectrometer system (Model; Shimadzu, GC-MS QP-2010SE) and a low polarity BPX5 capillary column (30 m× 0.25 mm× 0.25 µm film thickness). A liquid chromatography-mass spectrometer system (An ExionLC AC system, SCIEX Triple Quad Ascentis® Express 90 Å C18 Column [2.1×150 mm, 2.7 µm]) was used for water extract phytochemical composition analysis. Chromatography mass spectrometry was used to characterize phytochemical substances, with the identity and quantification of each compound determined by retention time and peak area percentage. A larger area indicated a higher concentration of that compound.

Bacterial isolates and media

Bacterial strains maintained in 1.5 ml Trypticase Soy Broth with 20% glycerol at -80°C were obtained from Kenya Medical Research Institute (KEMRI) Center for Global Health Research in Kisumu-Kenya. The bacterial strains included *Escherichia coli* (ATCC25922), *Staphylococcus aureus* (ATCC25923), and *Shigella flexneri* (*S. flexneri*) (ATCC12522).

Assessment of antibacterial activity of plant extracts

Well diffusion assay

The antibacterial activity of each extract was assessed against isolates of bacteria using the agar well diffusion technique. One gram of both dry methanol-dichloromethane extract and aqueous extract was dissolved in 1 ml of 98% dimethyl sulfoxide (DMSO) to generate a stock solution. Each stock culture bacterium was inoculated onto separate Trypticase Soy Agar (TSA) plates and incubated overnight. The newly established colonies were subsequently suspended in a normal saline tube to attain a 1.5×10^8 CFU/ml dilution, utilizing the 0.5 McFarland standard. Mueller Hinton agar plates (pH 7.3) were uniformly injected with bacterial suspensions by the Kirby Bauer method (Joseph et al., 2011). Five circular wells, each with a diameter of 11 mm, were aseptically formed on each plate using a sterile cork borer. One hundred microliters of extracts at concentrations of 100 mg/mL, 200 mg/mL, and 400 mg/mL were dispensed into separate wells on each plate (Abdallah et al., 2016). Similarly, 98% Dimethyl sulfoxide (DMSO) was introduced to the negative control wells, whereas the reference antibiotic Ceftriaxone (100 µg/mL, Gondane and Pawar, 2023) was administered to the positive control wells. A control plate for the three bacterial isolates with a DMSO concentration of 98% was performed to verify that DMSO demonstrates no antibacterial activity at high doses. The plates were incubated in aerobic conditions for 24 hours at 37°C. The zones of inhibition were quantified in millimeters and studied using the two extracts.

Assessment of the minimum inhibitory concentration and minimum bactericidal concentration

The minimal inhibitory concentration was determined using the broth dilution method in a sterile 96-well plate. A stock solution with a concentration of 400 mg/ml was prepared from the leaf extracts. 100 microliters of Mueller-Hinton Broth (MHB) were poured into each well of a 96-well plate. One hundred microliters of the extract stock solution were added to the first well of each test column, followed by serial dilution to a concentration of 6.25 mg/ml. Except for the negative control wells, which contained broth for quality assurance, twenty microliters of an 18-hour bacterial culture were introduced to the test and positive control wells after dilution using a 0.5 McFarland standard. Following a 24-hour aerobic incubation at 37ºC, 20 microliters of Resazurin dye (0.0025 mg/mL) were added to each well, and bacterial viability was assessed visually based on color change. The resazurin reduction by metabolically active bacteria resulted in a color shift from blue to pink, indicating growth. This color alteration was used to determine the minimum inhibitory concentration (MIC) of each extract by evaluating bacterial proliferation within the broth culture for each strain (Teh et al., 2017). A tiny volume from the wells exhibiting no discernible bacterial growth (turbidity) was moved onto fresh TSA media for an additional culture to determine the minimum concentration that kills bacteria. Following that, the dishes were incubated aerobically for 24 hours at 37°C. The bacterial colonies cultivated on each agar plate were meticulously examined, and the lowest concentration of extracts exhibiting complete bactericidal activity was identified as the minimum bactericidal concentration (MBC).

***In silico* evaluation**

Screening for drug-like compounds in *Ziziphus spina-christi* leaves extracts

The PubChem IDs and the Simplified Molecular Input Line Entry System (Canonical SMILES) of the Mass Spectrometers (GC-MS and LC-MS) identified compounds were retrieved for PubChem. The Canonical SMILES were submitted to the Swiss ADME tool and ADMETlab 3.0 to predict the physicochemical properties and drug-likeness of the candidates (Soltani Rad et al., 2023). The prediction was based on the parameters of the Blood Brain Barrier (BBB), Total Polar Surface Area (TPSA), Cytochrome P450s (CYP2D6 and CYP3A4) enzymes, and Lipinski's Rule of five (RO5), which stipulates that a drug candidate should possess a molecular weight under Five hundred Daltons, less than 10 rotatable bonds, less than 5 hydrogen bond donors, less than 10 hydrogen bond acceptors, and lipophilicity (log P) value below 5. A hypothesis was made that a molecule would not be suitable for oral administration if it failed to meet two or more RO5 criteria (Soltani Rad et al., 2023). The polar atoms within a molecule play a significant role in determining the topological polar surface area (TPSA), which is normally utilized to forecast the transport mechanism of the drug. The TPSA for an authorized medication, in the majority of instances, was below 140 Å2 (Patel et al., 2020). Compounds capable of penetrating the blood-brain barrier pose a nervous system risk because of their shifting ability from the hydrophilic environment of the blood to the lipophilic conditions of the brain. A therapeutic candidate must not inhibit CYP450 enzymes, as they are crucial for drug metabolism (Patel et al., 2020).

Molecular docking

The structural data file of chemicals was obtained from PubChem; the ligands library was created using Chimera 1.15rc software and saved in Mole2 format (Khan and Lee, 2022). The selected potential targets were three-dimensional

structures obtained from the Protein Data Bank (PDB) in PDB format and had been previously characterized through X-ray crystallography. These proteins were retrieved in their inhibitory state, with their native inhibitors bound at the active site. The XYZ coordinates of the structure's active sites were identified using BIOVIA Discovery Studio and subsequently used for molecular docking analyses. PDB formats were introduced in Chimera 1.15rc Software; existing ligands and water were eliminated from the targets. The ligand and target protein structures were produced for docking by energy minimization using the identical algorithm. The prepared targets and ligands were brought in Autodock Vina via PyRx 0.8 for docking and later transformed into PDBQT (Protein Data Bank, Partial Charge [Q], and Atom Type [T] files. The grid box was expanded using the XYZ coordinates of the active site obtained from BIOVIA Discovery Studio to ensure binding at the most suitable sites. Docking was executed at the standard exhaustiveness level of eight, ensuring precise localization of the binding pocket. Upon run completion, an output file (CSV) providing affinity scores for each ligand was generated in an Excel spreadsheet. The generated data was evaluated to create complexes in Notepad, which were subsequently saved as PDB files and viewed with BIOVIA Discovery Studio Visualizer 2021 for visualization (Khan and Lee, 2022).

Statistical analysis

The data were statistically analyzed using Origin Pro Lab 2024b (Student version) graphing and analysis software. Triplicate results for each bacterium treated with different extracts were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's test to determine significant differences between the two extracts. The corresponding p-values were calculated to assess statistical significance, with values below 0.05 considered significant. Measurements were then averaged, and results were expressed as mean ± standard deviation to generate graphs illustrating the differences. In molecular docking, binding energy was utilized to forecast the ligand's affinity for the receptor, and the type of interactions was used to determine the stability of the protein-ligand complexes.

RESULTS

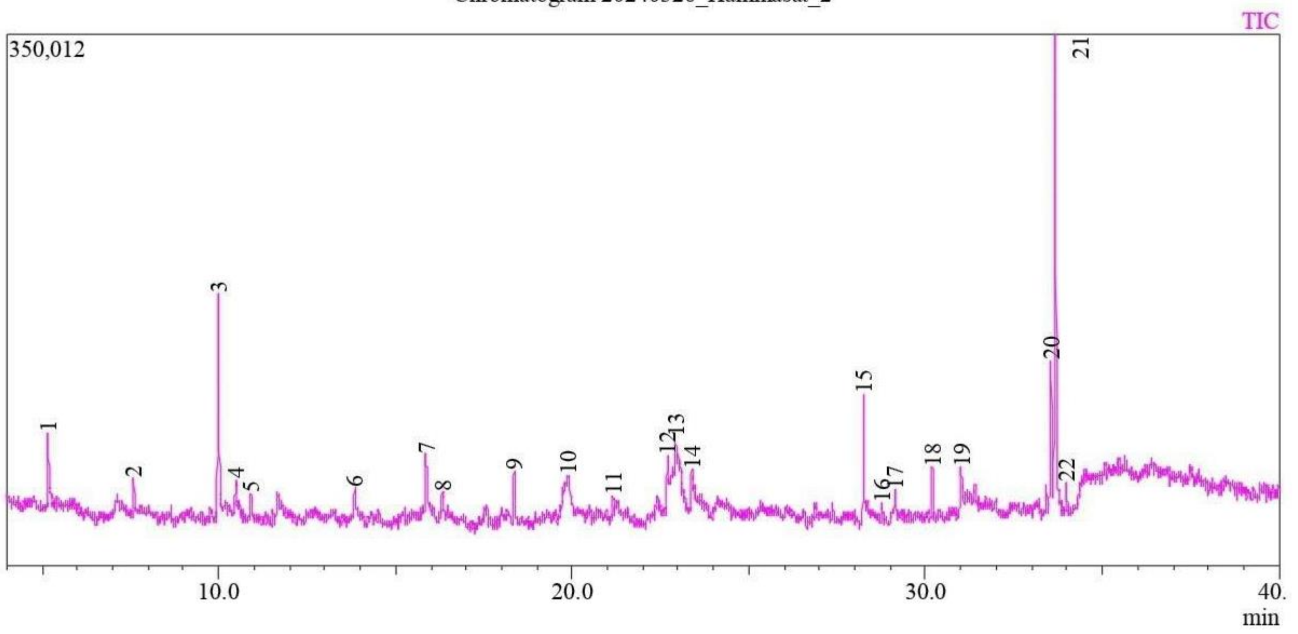
Identification and analysis of *Ziziphus spina-christi* bioactive compounds

A total of 34 unique compounds, together with their retention times (R. Time) and area percentages (Area%), were identified by GC-MS chromatogram. In GC-MS analysis, retention time (RT) helps identify metabolites based on their volatility and interaction with the stationary phase, while peak area reflects their relative concentration. RT aids structural identification by comparison with reference standards, and the peak area provides quantitative insights. Variations in ionization efficiency and matrix effects can affect peak area, requiring normalization techniques for accuracy. Together, RT and peak area enable precise metabolite profiling. Squalene (Peak 24) was the most predominant compound, comprising 46.12% of the area. Squalene serves as a precursor in sterol biosynthesis and exhibits antioxidant properties, contributing to skin protection and antibacterial activity by disrupting bacterial membranes, followed by Vitamin E (Peak 25) at 9.87%. Vitamin E functions as a potent antioxidant, supporting immune function and potentially enhancing antibacterial defense by affecting membrane integrity. The additional metabolite presented in a high concentration was 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (Peak 21) at 8.74%, which plays a role in chlorophyll metabolism and demonstrates antimicrobial activity by disrupting bacterial lipid membranes (Figure 1).

The LC-MS analysis identified 25 metabolites along with their retention times and areas under the peak (Figure 2). The predominant metabolite was Beta-indoleacetic acid (ID 11) with an area of 5.89 \times 10^9. Beta-Indole Acetic Acid (IAA) functions as a plant hormone regulating growth and may influence bacterial quorum sensing and inhibit bacterial growth, followed by N-Isovaleroylglycine (ID 46) with an area of 3.34/times 10^9, known to have antimicrobial effects by altering bacterial membrane stability and inhibiting growth in certain microbial species and Delphinidin-3-O-(6''-O-alpha-rhamnopyranosyl-beta-glucopyranoside; IDs 95 and 96) with an area of 3.19/times 10^9 each, is a potent antioxidant anthocyanin that has antibacterial activity by rupturing bacterial membranes and preventing the formation of biofilms.

Figure 1 shows the predominant metabolite was Beta-indoleacetic acid (ID 11) with an area of (5.89 \times 10^9). Out of a total of 34 compounds, the 2-Methoxy-4-vinylphenol, 2-Formyl-9- (beta -d-ribofuranosyl) hypoxanthine, and 2,4-Di-tert-butylphenol were predominantly recognized for their potent antibacterial properties, shown in Table 1. The detailed results on all 34 compounds are shown in S1 Table.

Results of the LC-MS phytochemical analysis indicated the presence of 53 compounds (Positive mood and negative mood), including cerulenin, Quercetin-3-O-arabinoglucoside, chlorogenic acid, and Esculin, among others that have been identified for their antibacterial properties (Table 2). Further details about the rest of the compounds are presented in S2-S3 Tables.



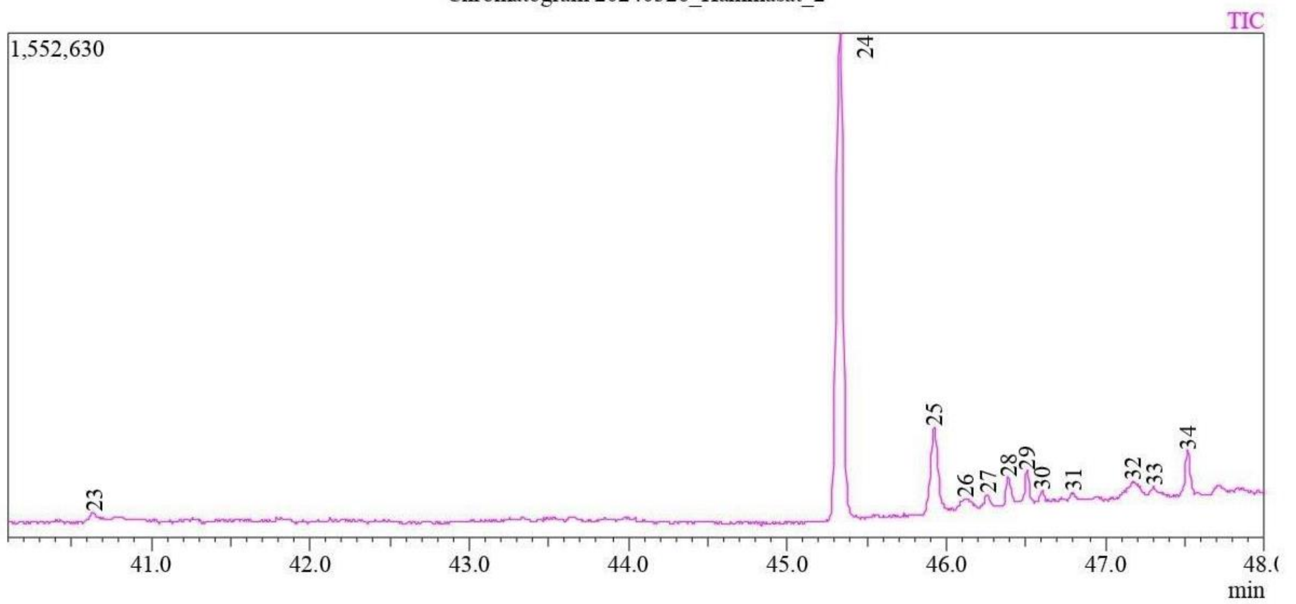


Figure 1. GC-MS chromatogram illustrating the Phyto-compounds present in the methanol-dichloromethane extract of *Ziziphus spina christi*. The peaks show that Squalene (Peak 24) was the most predominant compound, comprising 46.12% of the area.

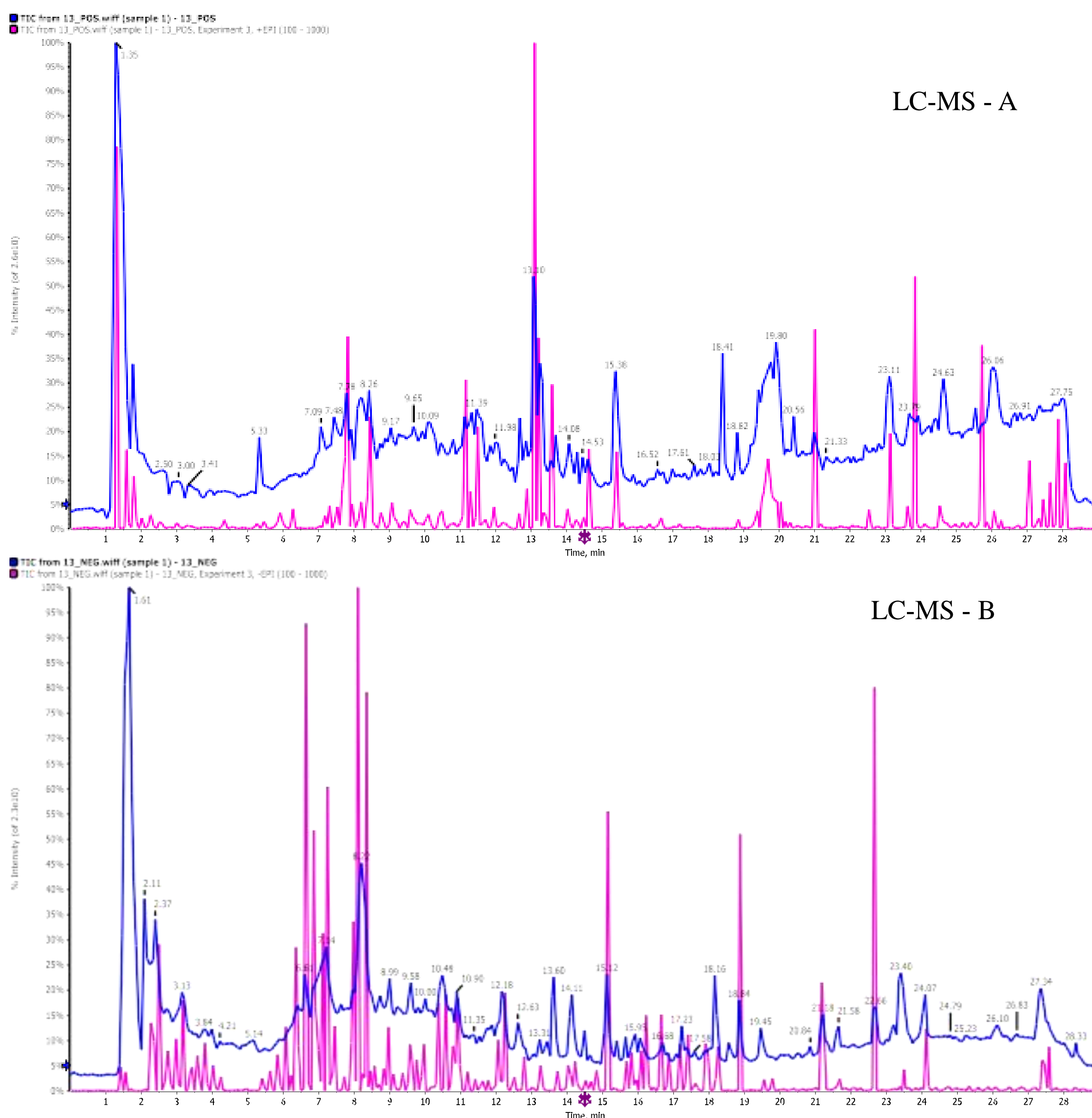


Figure 2. LC-MS chromatogram illustrating the chemical constituents found in *Ziziphus spina-christi* water extract. Presentations of both positive (LC-MS A) and negative (LC-MS B) moods of compounds from water extracts.

Table 1. The GC-MS identified compounds from *Ziziphus spina christi* methanol-dichloromethane extract

Peak Number	R. time (min)	Compound identified	MW	MF	Structure type	Function
8	16.320	2-Methoxy-4-vinylphenol	150.07	C9H10O2	Phenoxy compound	Antimicrobial and antioxidant Agent
10	19.878	2-Formyl-9-[.beta.-d-ribofuranosyl]hypoxanthine	296.08	C11H12N4O6	Tetrahydrofurans	Antiviral and Antimicrobial agent
11	21.149	2,4-Di-tert-butylphenol	206.17	C14H22O	Benzenoids	Antimicrobial agent

MW: Molecular weight; MF: Molecular formula; RT: Retention time

Table 2. The LC-MS identified compounds in *Ziziphus spina-christi* water extract

ID	RT (min)	Metabolite name	MW	MF	Structure Type	Function
56	2.328	Quercetin-3-O-arabinoglucoside	596.49	$C_{26}H_{28}O_{16}$	Flavonoids	Antimicrobial and antioxidant agent
500	15.780	Chlorogenic acid	354.31	$C_{16}H_{18}O_9$	Cyclic alcohol	Antimicrobial and antioxidant agent
549	16.767	Esculin	340.28	$C_{15}H_{16}O_9$	Coumarins glycosides	Antimicrobial and antioxidant Agent

MW: Molecular weight; MF: Molecular formula; RT: Retention time

Antimicrobial activities of *Z. spina-christi* leaves extracts against *Staphylococcus aureus*, *Escherichia coli,* and *Shigella flexneri*

Well diffusion assay

Mean zone of inhibition in millimeters ± standard error of the mean beyond the good diameter (11 mm) generated on *E. coli* (ATCC25922), *S. aureus* (ATCC25923), and *S. flexneri* (ATCC12522). The methanol-dichloromethane extract exhibited the highest antibacterial activity against *S. aureus*, with a zone of inhibition measuring (22.3 ± 0.6) mm at a concentration of 400 mg/mL. This was followed by *S. flexneri* (21.3 ± 1 mm) and *E. coli* (19.6 ± 0.6 mm) at the same concentration. In comparison, the water extract demonstrated strong activity against *S. aureus* (21.6 ± 0.5 mm) at 400 mg/mL but showed lower activity against *S. flexneri* and *E. coli*, with mean inhibition zones of (15 ± 0) mm and (14.6 ± 0.5) mm, respectively, as shown in Figure 3. The methanol dichloromethane extracts consistently exhibited superior antibacterial activity compared to the water extract, particularly against *S. aureus.*

In the present study, Methanol-dichloromethane extracts frequently demonstrate enhanced antibacterial efficacy relative to aqueous extracts, attributable to variances in their capacity to extract bioactive components. Organic solvents like methanol and dichloromethane efficiently dissolve both polar and non-polar bioactive chemicals, including alkaloids, flavonoids, terpenoids, and phenolics, which are recognized for their potent antibacterial effects. Conversely, water, being a highly polar solvent, predominantly removes hydrophilic substances like tannins, carbohydrates, and proteins, which may lack substantial antibacterial properties (Truong et al., 2019).

The methanol-dichloromethane extract demonstrated superior antibacterial activity, particularly against Gram-negative bacteria, due to its higher concentration of flavonoids, alkaloids, and terpenoids, which are well-documented for their antimicrobial properties. In contrast, the aqueous extract, which contained coumarins, flavonoids, alkaloids, terpenoids, and cinnamic acids, exhibited lower activity against Gram-negative bacteria, possibly due to structural differences affecting their interaction with bacterial cell walls.

A key observation in this study was that the zones of inhibition for both extracts against *S. aureus* (at 400 mg/mL) were relatively close to that of ceftriaxone (25 mm). This suggests that both extracts have significant antibacterial activity against Gram-positive bacteria (P = 0.0001 However, in the case of Gram-negative bacteria, the zones of inhibition for ceftriaxone were generally larger than those of the extracts, particularly for *S. flexneri* (31 mm for ceftriaxone versus the extract's inhibition zone). Interestingly, the inhibition zones for *E. coli* were relatively closer between ceftriaxone and the methanol-dichloromethane extract, indicating a moderate yet promising activity against this strain.

The differences in activity could be attributed to the structural complexity of Gram-negative bacterial cell walls, which include an outer membrane with lipopolysaccharides that act as a barrier against many antibacterial agents. The methanol-dichloromethane extract likely contained more lipophilic compounds, facilitating better penetration through this outer membrane, whereas the aqueous extract, despite containing known antibacterial compounds, exhibited lower efficacy due to possible limited permeability or compound stability in bacterial environments. Both extracts exhibit maximal efficacy against Gram-positive *S. aureus*, whereas their activity against Gram-negative bacteria *S. flexneri* and *E. coli* is comparatively diminished. This finding corroborated the antibacterial activity observed with methanol dichloromethane extract and water extract (Figure 4). Additional detailed results are provided in Table S4.

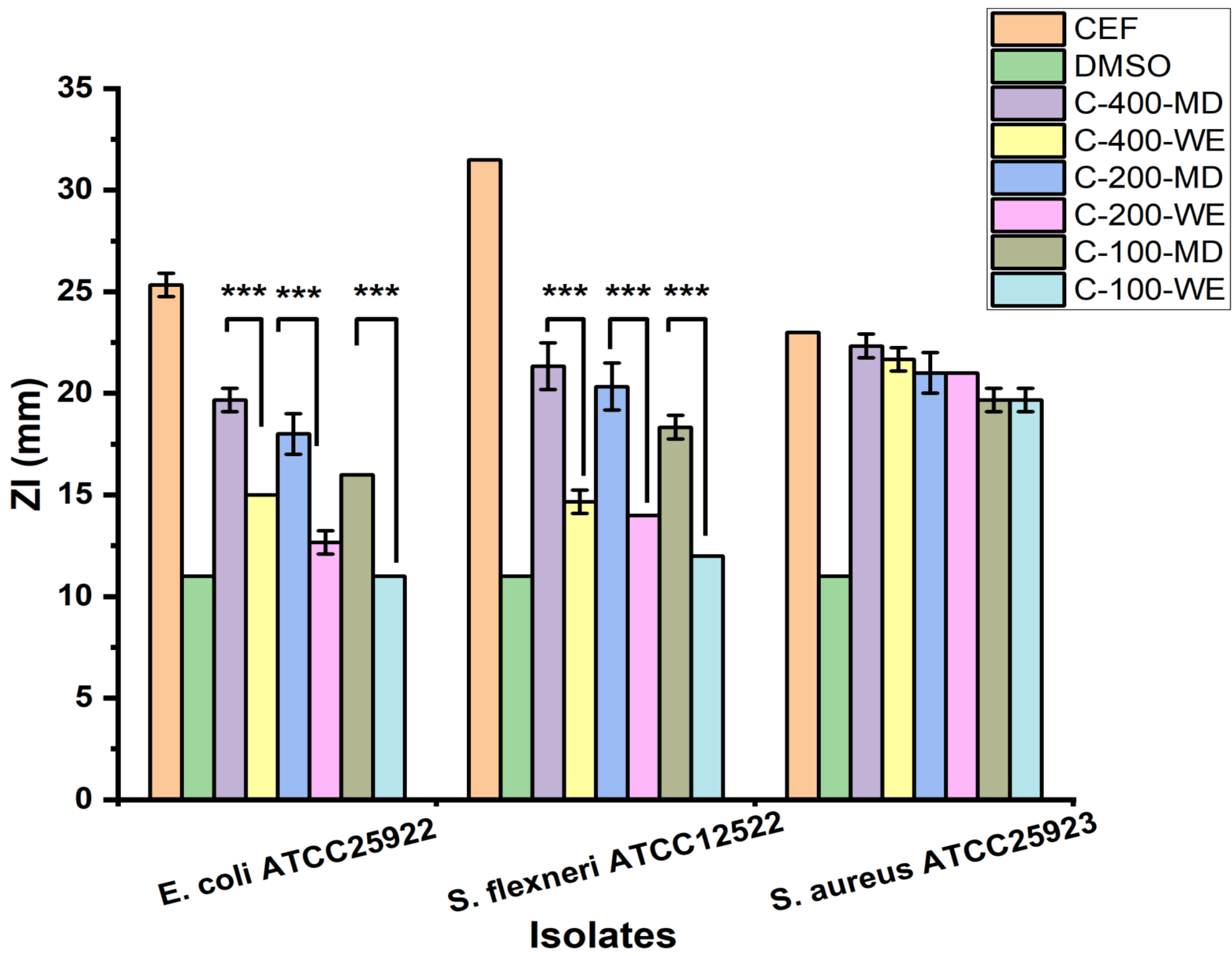


Figure 3. Antimicrobial activities (well diffusion assay) of *Ziziphus spina-christi* extracts with bacterial isolates

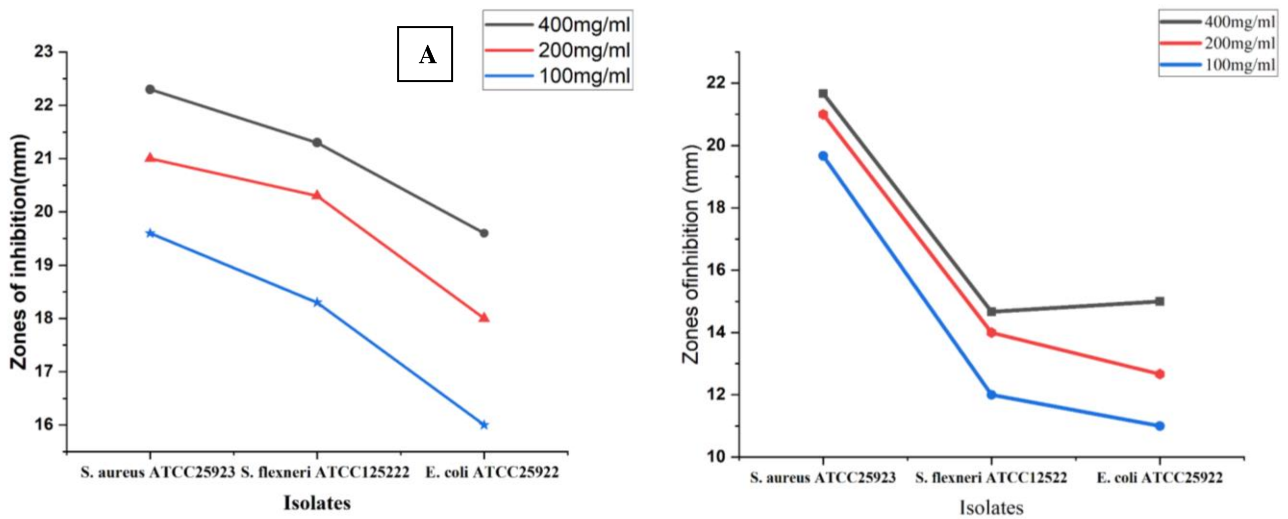


Figure 4. The performance of the *Ziziphus spina-christi* extracts against the bacterial isolates. **A**: Methanol-dichloromethane extract, **B**: Water extract

Minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentration (MIC) results indicated that both extracts demonstrated high potency against *S. aureus*, with a MIC of 12.5 mg/mL for each extract. The methanol dichloromethane extract demonstrated greater inhibitory activity of 12.5 against the tested isolates, effectively inhibiting *S. aureus* and S. *flexneri* at a concentration of 12.5 mg/mL, while *E. coli* showed inhibition at a higher concentration of 50 mg/mL (Table 3).

The methanol dichloromethane extract demonstrated killing activity against the tested isolates at 100 mg/mL concentration for *S. aureus, E. coli,* and *S. flexneri*. The water extract demonstrated killing activity against *S. aureus* at 100 mg/ml concentration, while both *E. coli* and *S. flexneri* showed resistance at this concentration.

Table 3. Minimum inhibitory concentrations of *Z. spina-christi* leaf extracts

Microorganism	Water Extract MIC (mg/mL)	Methanol- Dichloromethane Extract MIC (mg/mL)	Mean	Standard Deviation	CV (%)
Staphylococcus aureus **ATCC25923**	12.5	12.5	12.5	0	0%
Shigella flexneri **ATCC12522**	50	12.5	31.25	26.53	84.9%
Escherichia coli **ATCC25922**	50	50	50	0	0%

CV: Coefficient of variance, MIC: Minimum inhibitory concentration

In silico **evaluation**

Screening for pharmacologically viable chemicals in Ziziphus spina-christi leaf extract

Out of 34 chemicals extracted from *Z. spina-christi* using dichloromethane-methanol and identified using GC-MS analysis, 14 were deemed optimal drug-like candidates based on their compliance with Lipinski's Rule of Five and exhibited favourable ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) profiles, positioning them as viable candidates for drug development. Compounds molecular weight was under 500 Dalton, a logP value below 5, fewer than 5 hydrogen bond donors, and fewer than 10 hydrogen bond acceptors and they demonstrated favourable oral bioavailability and potential systemic distribution through Total polar surface area. In addition, the compounds were not permeable to the blood-brain barrier, and they do not inhibit Cytochrome P2D6, Lipinski's RO5, ADMET analysis offered insights into the pharmacokinetic properties of the selected compounds, confirming their optimal absorption, distribution, and metabolic stability, alongside low toxicity and minimal side effects. The favourable ADMET profiles indicate a reduced likelihood of rapid metabolism or elimination, potentially resulting in prolonged antibacterial effects. In the aqueous extract of *Z. spina-christi*, LC-MS analysis found 52 chemicals, of which 38 were deemed suitable drug-like candidates based on the same previous criteria. The presence of these bioactive compounds in both the methanol-dichloromethane and aqueous extracts aligns with the observed antibacterial effects shown by both extracts. The prioritized compounds are delineated in Tables 4 and 5.

Table 4. *Ziziphus spina-christi* dichloromethane-methanol compounds with the ideal drug candidate qualities

N	Compounds	ID	MW (g/mol)	BBB	Lipinski's Rule (Violation)	CYP2D6	HA	HD	TPSA
1	DL-Glyceraldehyde	751	93.03	No	Yes;0	No	2	3	20.23
2	2-Hydroxy-gamma-butyrolactone	545831	102.09	No	Yes;0	No	3	1	46.53
3	Coumaran	10329	120.06	No	Yes;0	No	0	1	13.14
4	8-Methyl-8-azabicyclo [3.2.1] octane-3-carbonitrile	15405279	136.1	No	Yes;0	No	2	1	35.82
5	2-Methoxy-4-vinylphenol	332	150.07	No	Yes;0	No	2	1	29.46
6	2-Formyl-9-[beta-d- ribofuranosyl] hypoxanthine	135599166	296.08	No	Yes;0	No	8	4	150.56
7	2,4-Di-tert-butylphenol	7311	206.17	No	Yes;0	No	8	4	150.56
8	cis-1,4-Cyclohexanediol	11162	116.08	No	Yes;0	No	2	2	40.46
9	Arachidic Acid	10467	312.3	No	Yes;0	No	2	1	37.3
10	Octadecanoic acid	5281	284.27	No	Yes;0	No	2	1	37.3
11	Tetraprenol	5281365	290.26	No	Yes;0	No	0	0	0
12	1-Heptacosanol	74822	396.43	No	Yes;0	No	1	1	20.23
13	1,2-Benzenediol	289	110.4	No	Yes;0	No	2	2	40.46
14	Undec-10-ynoic acid	91692467	378.35	No	Yes;0	No	2	0	26.3

ID: PubChem ID, MW: Molecular weight, BBB: Blood-brain barrier, CYP2D6: Cytochrome. P450 2D6, HA: Number of hydrogen bond acceptors, HD: Number of hydrogen bond donors; TPSA: Total polar surface area

Table 5. *Ziziphus spina-christi* water compounds with ideal drug candidate qualities

N	Compounds	ID	MW (g/mol)	BBB	Lipinski''s Rule (Violation)	HA	HD	CYP2D6	TPSA
1	Thiamine	1130	265.35	No	Yes;0	3	2	No	104.15
2	4-hydroxy-3-Methoxyphenylacetic acid (2,5,6-d3,alpha,alpha-d2)	1738	182.17	No	Yes;0	4	2	No	66.76
3	4-hydroxy-3-methoxyphenylacetic acid (2,5,6-d3,alpha,alpha-d2)	1738	182.17	No	Yes;0	4	2	No	66.76
4	N-Isovaleroylglycine	546304	159.18	No	Yes;0	3	2	No	66.4
5	3-Methylxanthine	70639	166.14	No	Yes;0	3	2	No	83.54
6	Robinetin	5281692	302.24	No	Yes;0	7	5	No	131.36
7	L-Saccharopine	160556	276.29	No	Yes;0	8	5	No	149.95
8	L-Saccharopine	160556	276.29	No	Yes;0	8	5	No	149.95
9	L-Saccharopine	160556	276.29	No	Yes;0	8	5	No	149.95
10	Chlorogenic acid	1794427	354.31	No	Yes;0	9	6	No	164.75
11	Esculin	5281417	340.28	No	Yes;1	9	5	No	149.82
12	Acadesin	17513	258.23	No	Yes;0	6	5	No	156.85
13	Acadesin	17513	258.23	No	Yes;0	6	5	No	156.85
14	Sinapoyl malate	14605050	340.28	No	Yes;0	9	3	No	139.59
15	Sinapoyl malate	14605050	340.28	No	Yes;0	9	3	No	139.59
16	Sinapoyl malate	14605050	340.28	No	Yes;0	9	3	No	139.59
17	trans-Cinnamate	444539	184.16	Yes	Yes;0	2	1	No	37.3
18	Beta-indoleacetic acid	802	175.06	No	Yes;0	2	3	No	53.09
19	Beta-indoleacetic acid	802	175.06	No	Yes;0	2	3	No	53.09
20	3,4-Dihydroxy-L-phenylalanine	6047	197.07	No	Yes;0	5	5	No	103.78
21	3,4-Dihydroxy-L- phenylalanine	6047	197.07	No	Yes;0	5	5	No	103.78
22	Scoulerin	439654	327.15	No	Yes;0	5	2	No	62.16
23	Scoulerin	439654	327.15	No	Yes;0	5	2	No	62.16
24	3-Hydroxyanthranilic acid	86	153.04	No	Yes;0	4	4	No	83.55
25	3-Hydroxyanthranilic acid	86	153.04	No	Yes;0	4	4	No	83.55
26	4-Hydroxyphenylpyruvic acid	979	180.04	No	Yes;0	4	2	No	74.6
27	4-Hydroxyphenylpyruvic acid	979	180.04	No	Yes;0	4	2	No	74.6
28	3,4-Dihydroxymandelate	85782	184.04	No	Yes;0	5	4	No	97.99
29	Genistein	5280961	270.05	No	Yes;0	5	3	No	90.9
30	Genistein	5280961	270.05	No	Yes;0	5	3	No	90.9
31	Glutamine	5961	146.07	No	Yes;0	5	5	No	106.41
32	L-(+)-Lysine	5962	146.11	No	Yes;0	4	5	No	89.34
33	Guanosine-3',5'-cyclic monophosphate	135398570	345.05	No	Yes;0	12	5	No	184.62
34	Guanosine-3',5'-cyclic monophosphate	135398570	345.05	No	Yes;0	12	5	No	184.62
35	4-aminophenol	403	109.05	No	Yes;0	2	3	No	46.25
36	Cerulenin	5282054	223.12	No	Yes;0	4	2	No	72.69
37	Cerulenin	5282054	223.12	No	Yes;0	4	2	No	72.69
38	Cerulenin	5282054	223.12	No	Yes;0	4	2	No	72.69

ID: PubChem ID, MW: Molecular weight, BBB: Blood-brain barrier, CYP2D6: Cytochrome. P450 2D6, HA: Number of hydrogen bond acceptors, HD: Number of hydrogen bond donors; TPSA: Total polar surface area

Molecular docking

Chemical formulas of the selected phytocompounds (ligands) from both extracts were retrieved from the PUB Chem database and prepared for docking using Chimera. Docking results showed that compounds from water extract such as Quercetin-3-O-arabinoglucoside with a docking score of -8.9 kcal/mol, Esculin with a docking score of -8.1 kcal/mol, and Chlorogenic acid with docking score -7.8 kcal/mol exhibited a strong interaction with the enzyme Peptidoglycan D, D-Transpeptidase (6G9F) from *E. coli*, in contrast, 2-Formyl-9-(beta-d-ribofuranosyl) hypoxanthine, 2,4-Di- tert-butylphenol and 2-Methoxy-4-vinylphenol from methanol dichloromethane demonstrated lower affinity energy with docking score -7 kcal/mol, - 6.1 kcal/mol and -5.3 kcal/mol, respectively. Within this framework, of the selected microbial enzymes considered in the docking analysis, Esculin, Quercetin-3-O-arabinoglucoside, Chlorogenic acid, and 2-Formyl-9-(beta.-d-Ribofuranosyl) hypoxanthine exhibited interactions with the TEM-1 Beta-Lactamase (8GII), demonstrating enhanced antibacterial activity with docking scores of (-8.5, -8.4, -8.1 and -7.6) kcal/mol, respectively. While, 2,4-Di-tert-butylphenol and 2-Methoxy-4-vinylphenol with docking scores -6.3 kcal/mol and -5.4 kcal/mol, respectively, demonstrated lower affinity energy compared to 2-Formyl-9- (beta-d-Ribofuranosyl) hypoxanthine from the same extract and compounds from water extract. Quercetin-3-O-arabinoglucoside and Chlorogenic acid demonstrated a binding affinity with the Beta-Lactamase (1BLH), attaining a docking score of -8.8 kcal/mol and -8.1 kcal/mol, respectively. The rest Chlorogenic acid and 2-Formyl-9- (beta -d-ribofuranosyl) hypoxanthine, 2,4-Di-tert-butylphenol and 2-Methoxy-4-vinylphenol demonstrated lower binding affinity with docking score -7.5 kcal/mol, 6.8 kcal/mol, 6.4 kcal/mol and -5.5 kcal/mol, respectively (Table 6).

Table 6. The predicted docking scores of the different ligands for inhibitor binding with the tested proteins

Extract type	**Extract phytochemical**	**Targets binding affinity (kcal/mol)**		
		Peptidoglycan D, D-Transpeptidase (6G9F)	**TEM-1 Beta-Lactamase (8GII)**	**Beta Lactamase (1BLH)**
Methanol-dichloromethane extract	2-Methoxy-4-vinylphenol	-5.3	-5.4	-5.5
	2,4-Di-tert-butylphenol	-6.1	-6.3	-6.4
	2-Formyl-9-beta-d- ribofuranosyl hypoxanthine	-7	-7.6	-6.8
Water extract	Quercetin-3-O- arabinoglucoside	-8.9	-8.4	-8.8
	Chlorogenic Acid	-7.8	-8.1	-8.1
	Esculin	-8.1	-8.5	-7.5

Quercetin-3-O-arabinoglucoside, Esculin, and Chlorogenic acid from water extract demonstrated a higher interaction with the docking enzymes than the compounds from methanol, dichloromethane extract. The 2D diagram and the ligand interactions with the distance in Angstrom and the amino acid incorporated in the interaction for the three compounds are represented in figures 5 , 6, and 7, while detailed results on the methanol dichloromethane compounds are shown in S1, S2, and S3 Figures.

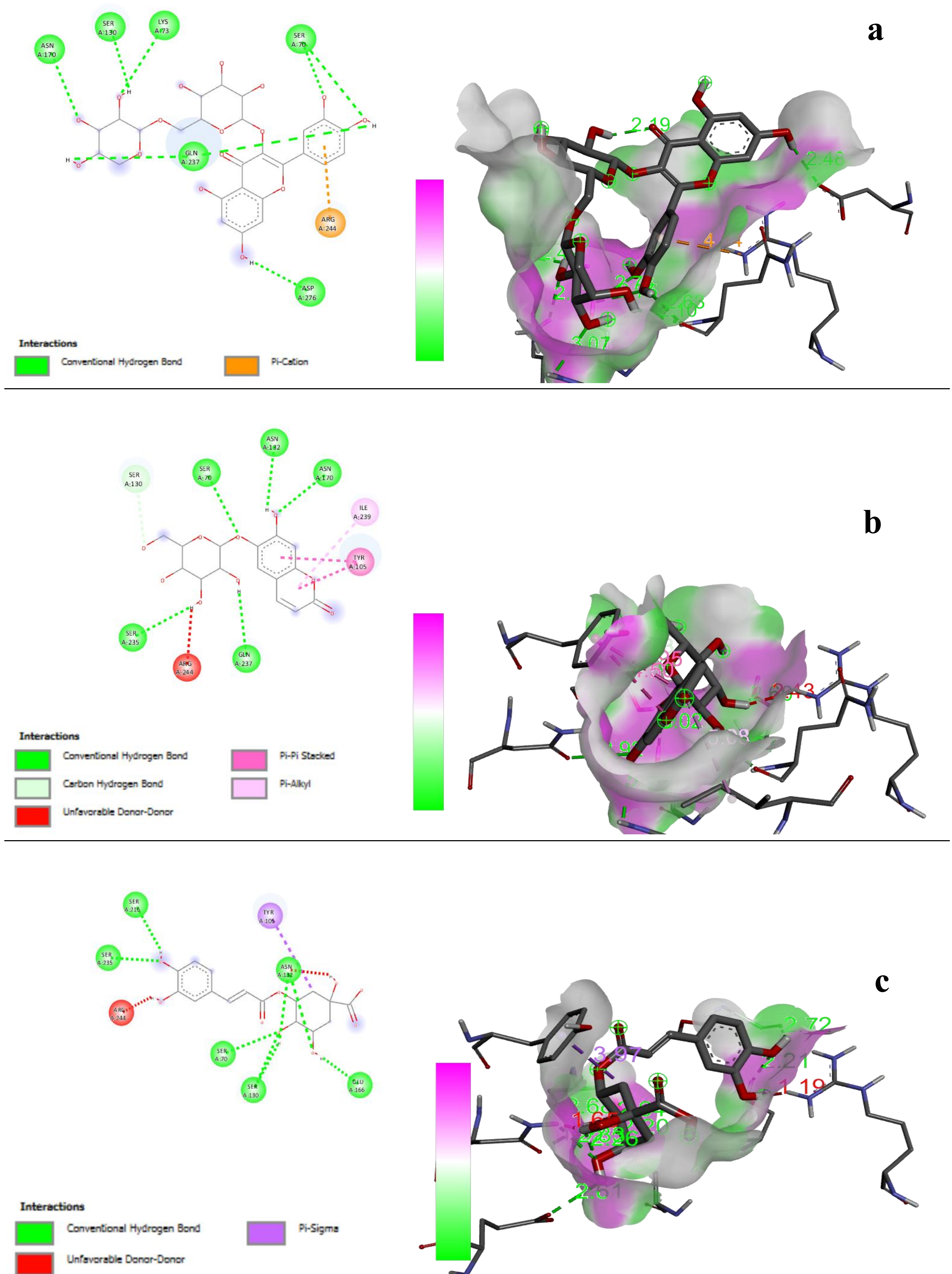


Figure 5. 2D diagrams and ligand interaction profiles of Quercetin-3-O-arabinoglucoside (a), Esculin (b), and Chlorogenic acid (c) with the 1BLH enzyme

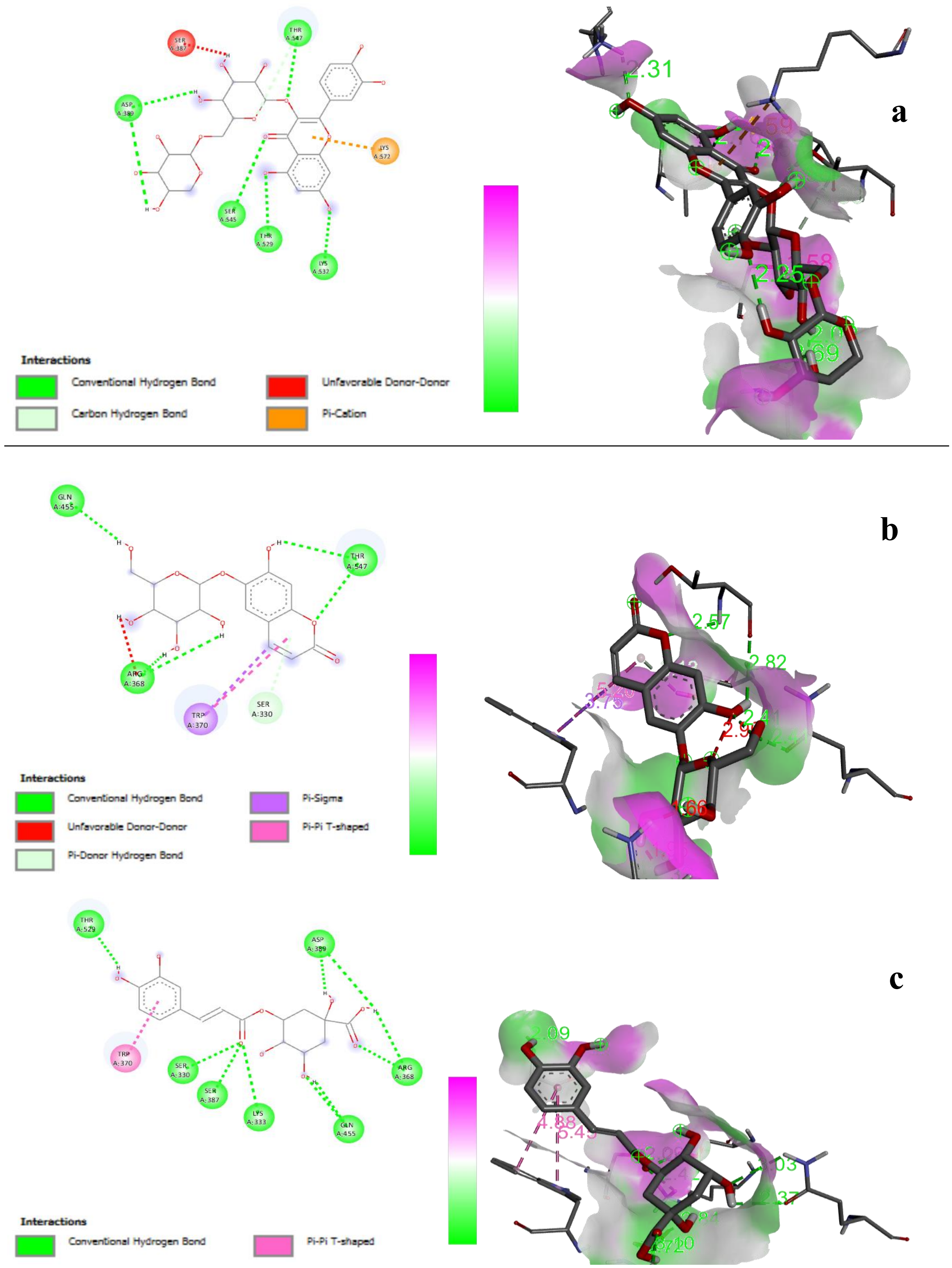


Figure 6. 2D diagrams and ligand interaction profiles of Quercetin-3-O-arabinoglucoside (a), Esculin (b), and Chlorogenic acid (c) with 6G9F enzyme

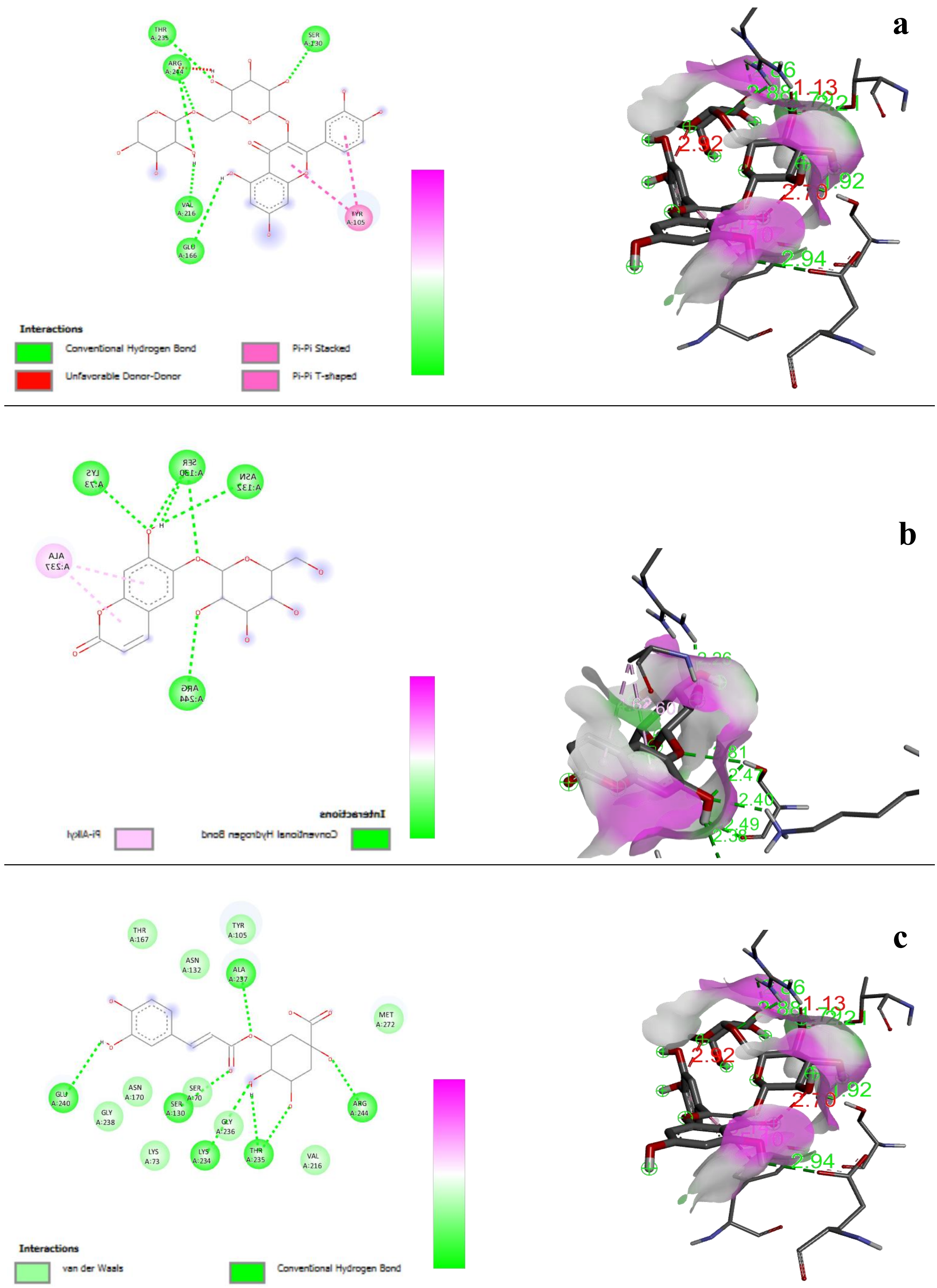


Figure 7. 2D diagrams and ligand interaction profiles of Quercetin-3-O-arabinoglucoside (a), Esculin (b), and Chlorogenic acid (c) with 1BLH enzyme

DISCUSSION

Antibiotic resistance impacts all categories of antibiotics. Multidrug-resistant bacteria (MDR) have emerged as a significant world health concern. Medicinal plants are abundant in secondary metabolites that confer antibacterial properties against many diseases while demonstrating few side effects (Mustapha et al., 2024). This study highlighted *Z. spina-christi* as a source of phytocompounds, such as flavonoids, coumarins, cinnamic acid, alkaloids, saponins, tannins, phenolic compounds, and terpenoids, which have been empirically shown to exhibit activity against both Gram-positive and Gram-negative (Kuete, 2010). Nevertheless, the safety and efficacy of Z. spina have not been fully evaluated in people, necessitating more rigorously conducted clinical trials to validate preclinical results. The mechanism of action of the leaf extract requires examination. The standard dosage and safety of the leaf must be determined (Abdulrahman et al., 2022). The phytochemical analysis results elucidated the antimicrobial activity observed in *Z. spina-christi* leaf extracts. Similar findings have been documented by El-Shahir et al. (2022), who illustrated the existence of diverse compounds in *Z. spina-christi* from Saudi Arabia, encompassing amino flavonoids, saponins, alkaloids, tannins, phenolic compounds, terpenoids, and fatty acids. Abdulrahman et al. (2022) reported that, chemically, polyphenols and flavonoids were the most reported compounds in *Z. spina-christi* leaves, with a composition of 66 compounds out of the total 193 compounds reported from different parts of the plant.

Based on the antibacterial activities observed in this study, both extracts demonstrated minimal efficacy at a concentration of 100 mg/ml while exhibiting significant effectiveness at 400 mg/ml. Results indicated the antibacterial effectiveness of both extracts in combating the studied pathogens was enhanced at higher concentrations, as demonstrated in the study of Motamedi et al. (2009).

Methanol-dichloromethane extract demonstrated antibacterial activities against the three isolates, aligning with Bukar et al. (2015) findings, indicating that *Z. spina-christi* demonstrates antibacterial activity against *Pseudomonas aeruginosa*, *E. coli, Shigella* species, and *S. aureus*. The extract derived from methanol and dichloromethane demonstrated remarkable antibacterial efficacy against both bacterial species, with inhibition zones measuring 19.6 ± 0.5 mm for *S. aureus*, closely followed by *S. flexneri* at 18.3 ± 0.5 mm and *E. coli* at 16.6 ± 0 mm. The water extract demonstrated significant activity solely against gram-positive bacteria, specifically *S. aureus*, which exhibited an inhibition zone of 20.3 ± 0.5 mm. In contrast, *E. coli* and *S. flexneri* displayed a diminished response to the extract 12 ± 0 and 11 ± 0 mm, respectively. The findings align with earlier research indicating that Gram-negative bacteria demonstrate heightened resistance to most plant extracts in comparison to Gram-positive bacteria, in concurrence with the findings of Suliman and Mohammed (2018) have demonstrated that in their study, water extracts derived from the *Z. spina-christi* leaves demonstrate notable antibacterial activity against *S. aureus*, with an inhibition zone measuring 14.2 mm, closely followed by 14.1 mm and 13.3 mm for *E. faecalis* and *B. subtilis,* respectively. In contrast, all tested Gram-negative bacteria, such as *S. typhi*, *P. aeruginosa*, and *E. coli,* exhibited complete resistance to the extracts, resulting in the absence of inhibition zones.

The results suggest that methanol extracts may possess more potent or higher concentrations of active antibacterial compounds than aqueous extracts, and a presence of a potential disparity in susceptibility between Gram-positive *(S. aureus*) and Gram-negative (*E. coli* and *S. flexneri*) bacteria to the extract.

The minimum inhibitory concentration for methanol-dichloromethane extract was 12.5 mg/ml for both *S. aureus* and *S. flexneri*, and 50 mg/ml for *E. coli*. While aqueous extract MIC was 12.5 mg/ml for *S. aureus* and 50 mg/ml for *S. flexneri* and *E. coli*, respectively. Conversely, Alhassan et al. (2019) indicated that the water extract MIC was 31.25 and 62.5 mg/ml for *E. coli* and *S. aureus,* respectively. Regarding those two studies, the aqueous extract of the present study has more potency towards gram-positive bacteria compared to gram-negative ones. Concerning the MBC, the methanol-dichloromethane extract had a lethal activity of 100 mg/ml against the test organisms, whereas the water extract exhibited a lethal activity of 100 mg/ml against *S. aureus,* while *E. coli* and *S. flexneri* were resistant at this concentration. There is a notable inconsistency in the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) results between the present study and the previous findings. These inconsistencies could be attributed to differences in experimental conditions, such as extraction techniques, bacterial strains used, solvent polarity, or differences in phytochemical composition due to variations in plant origin or environmental factors as these bioactive compounds are often synthesized in response to environmental stimuli such as temperature, light intensity, soil composition, and water availability.

Penicillin-binding proteins (PBPs) function as the primary targets for β-lactams, which represent the most potent class of antibiotics developed to address bacterial infections (Freischem et al., 2021). The rapid and widespread emergence of broad-spectrum β-lactam resistance genes, including Carbapenemases, significantly undermines the effectiveness of antibiotics within this class (Sauvage and Terrak, 2016).

There is a pressing need for novel and potent PBP inhibitors, particularly those that demonstrate resistance to β-lactamase hydrolysis. Following the revolutionary finding of penicillin and its extensive application in healthcare throughout the 1940s, numerous additional classes and genera of Beta-lactam antibiotics have been formulated to effectively combat the increasing resistance of bacteria (Sauvage and Terrak, 2016). The considerable resistance to β-lactams, mostly attributed to β-lactamases, limits their effectiveness unless they are administered alongside β-lactamase inhibitors, which are predominantly non-β-lactams (Sauvage and Terrak, 2016).

Quercetin, a natural flavonoid antioxidant, possesses anti-carcinogenic, anti-inflammatory, and antibacterial characteristics (Mu et al. 2021). It may also impede biofilm formation by reducing Extracellular Polymeric Substances (EPS) synthesis and modifying the EPS composition in *Staphylococcus epidermidis* (Mu et al., 2021).

Esculin exhibits antibacterial efficacy against *S. aureus* and *E. faecalis*, as well as *E. coli*, *S. enteritidis*, *S. typhimurium*, and several multi-resistant strains of *E. coli* (Cai and Cai, 2023). It has also demonstrated antibiosis effects against certain fungi, including *T. interdigitale*, *T. mentagrophyte*, *Scopulariopsis brevicaulis, Microsporum canis,* and *Aspergillus fumigatus* (Cai and Cai, 2023). Conversely, chlorogenic acid significantly increased the permeability of the exterior and inner plasma membranes, resulting in impaired barrier integrity and the efflux of cytoplasmic contents, which led to cellular apoptosis (Lou et al., 2011).

To eliminate bias, TEM-1 Beta-Lactamase (8GII) from *Escherichia coli*, which is predominantly conserved among gram-negative bacteria, was chosen to represent the gram-negative source, while 1BLH Beta-Lactamase (1BLH) from *S. aureus* was selected for the gram-positive origin in the docking activity. The glycosyltransferase and transpeptidase functions of multimodular penicillin-binding proteins within complexes of multiple proteins are crucial for the formation of a functioning, stress-resistant peptidoglycan layer (Edoo et al., 2017). D, D-Transpeptidase catalyzes the synthesis of peptidoglycan cross-links in Gram-positive and Gram-negative bacteria (Edoo et al., 2017). Peptidoglycan D, D-Transpeptidase (6G9F) from *E. coli* was obtained from the PDB for docking purposes.

The docking results indicated that both extracted phytocompounds exhibited substantial interactions with the identified enzymes during docking. Quercetin-3-O-arabinoglucoside exhibits the highest binding affinity for Peptidoglycan D, D-Transpeptidase active site, with a binding score of -8.9 kcal/mol, followed by Esculin at -8.1 kcal/mol and Chlorogenic acid at -7.8 kcal/mol. The Methanol-dichloromethane phytocompounds exhibit a reduced binding affinity to PBP, with a maximum binding affinity of -7 associated with 2-Formyl-9-(beta-d-ribofuranosyl) hypoxanthine.

Within the framework of Beta lactamases (8GII and 1BLH), Esculin demonstrated the greatest degree of binding to 8GII, measured at -8.5 kcal/mol, followed by Quercetin-3-O-arabinoglucoside at -8.4 kcal/mol and Chlorogenic acid at -8.1 kcal/mol. In the methanol dichloromethane extract, 2-Formyl-9-(beta -d-ribofuranosyl) hypoxanthine exhibited the greatest binding score at -7.8 kcal/mol. Quercetin-3-O-arabinoglucoside, with a binding affinity of -8.8 kcal/mol, and Chlorogenic acid, with -8.1 kcal/mol, exhibited the best affinity for inhibiting 1BLH Beta-Lactamase.

Molecular docking analysis revealed that Quercetin, Esculin, Chlorogenic acid, and 2-Formyl-9-(β-d-ribofuranosyl) hypoxanthine exhibited significant binding affinity toward the active sites of penicillin-binding proteins (PBPs) and β-lactamases, which were identified using XYZ coordinates from the Protein Data Bank (PDB). Quercetin-3-O-arabinoglucoside exhibited the most significant affinity for peptidoglycan D, D-transpeptidase (6G9F) at -8.9 kcal/mol, presumably establishing hydrogen bonds and van der Waals interactions with essential active site residues, including Ser294, Lys386, and Thr410, which are vital for enzymatic activity. Esculin exhibited significant affinity for TEM-1 β-lactamase (8GII) (-8.5 kcal/mol), forming hydrogen bonds and π-π stacking interactions with Glu166 and Ser70, which are crucial residues in the hydrolysis of β-lactam antibiotics, thereby potentially impeding bacterial resistance. Chlorogenic acid demonstrated notable binding affinity to β-lactamases (-8.1 kcal/mol), highlighting its potential function in β-lactamase inhibition via interactions with Asp132 and Lys73.

Conversely, the compounds extracted using methanol-dichloromethane, including 2-Formyl-9-β-d-ribofuranosyl hypoxanthine, exhibited moderate binding affinities, achieving a peak score of -7.6 kcal/mol for TEM-1 β-lactamase (Bush and Bradford, 2020). This compound presumably engages in interactions via hydrogen bonds and electrostatic forces with Asn132 and Glu166; however, its affinity appears to be less robust in comparison to Quercetin-3-O-arabinoglucoside and Esculin. The comprehensive docking results correspond with the *in vitro* observations, wherein the water extract exhibited superior activity against Gram-positive bacteria, likely attributable to enhanced interactions with PBPs and β-lactamases. Conversely, the methanol-dichloromethane extract proved more efficacious against Gram-negative bacteria, potentially owing to the presence of nonpolar phytochemicals that aid in membrane penetration. The results underscore the necessity for additional *in vitro* validation and structural refinement to improve antibacterial effectiveness (Bush and Bradford, 2020). Additionally, the comparison of water versus methanol-dichloromethane extracts aligns with phytochemical profiling studies showing that flavonoids, coumarins, and polyphenols (such as

Quercetin-3-O-arabinoglucoside and Esculin) have strong β-lactamase inhibition properties (Cushnie and Lamb, 2011). Meanwhile, terpenoids and alkaloids, dominant in the methanol-dichloromethane extract, exhibit antibacterial activity through membrane disruption rather than enzyme inhibition.

The present study is unprecedented, as no prior research has examined the docking of identical compounds from *Z. spina christi* leaf extracts with these enzymes. The molecular docking results corroborate the *in vitro* antibacterial activity, establishing *Z. spina christi* leaf extract as a potent agent against bacterial infections by interfering with cellular wall synthesis through interaction with PBPs. Additionally, it may serve as a beta-lactamase inhibitor in conjunction with beta-lactam antibiotics to combat resistance to these antibiotics.

CONCLUSION

In conclusion, both methanol and aqueous extracts possess significant antibacterial properties, with methanol extracts being more potent. The results underscored the potential application of these extracts as natural antibacterial agents. *Z. spina christi* leaf extract serves as a strong treatment against bacterial infections by inhibiting cell wall synthesis through interaction with penicillin-binding proteins (PBPs). Moreover, it may function as a beta-lactamase inhibitor alongside beta-lactam antibiotics to address resistance to these agents. Future research should concentrate on isolating and characterizing active compounds in *Z. spina-christi* extracts using bioassay-guided fractionation and advanced spectroscopic techniques. Mechanistic studies, including proteomic and transcriptomic analyses, are needed to understand the mode of action. Synergistic interactions with conventional antibiotics should also be explored.

DECLARATIONS

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Authors' contributions

Hamasat Mohammed Musa Abdallah conducted the methodology and data curation and prepared the original draft. Eddy Okoth Odari and John Benjamin Ochieng supervised, reviewed, and edited the study. All authors have read and agreed to the published the last edition of the manuscript.

Competing interests

The authors acknowledge having no conflict of interest regarding this research.

Availability of data and materials

The datasets generated during the current study are available from the corresponding author upon reasonable request.

Ethical considerations

All authors considered and confirmed the ethical issues, including plagiarism, consent for publication, misconduct, fabrication of data, and duplicate publication or submission.

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