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In silico antimicrobial activity of *Hypericum perforatum* to inhibit some enzymes of three bacterial species and *in vitro* antimicrobial effects of its extracts

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ABSTRACT

Hypericum perforatum is a medicinal plant of the Family *Hypericaceae* with phenolic and flavonoid compounds with high antimicrobial properties. The present study was aimed to assess the antimicrobial effects of *H. perforatum* ethanolic, acetone, and triethylamine extracts against pathogenic bacteria. The *H. perforatum* aerial parts were prepared, dried, powdered and used to prepare ethanolic, acetone, and triethylamine extracts by maceration method. Phytochemical components were detected using high-performance liquid chromatography (HPLC). The diameter of the growth inhibition zones of bacteria was assessed using disk diffusion. Minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) of extracts were evaluated using an ELISA plate and compared to antibiotics. Hypericin, pseudohypericin and hyperforin derivatives were identified in ethanolic extract. According to molecular docking, hypericin exhibited high binding energy to Beta-Lactamase *Escherichia coli* (-6.88 kj/mol), Glycosyltransferase-*Staphylococcus* bacterial enzymes (-6.47 kj/mol) and Pseudohypericin with Porin D-*Pseudomonas aeruginosa* (-8.31 kj/mol). Pseudohypericin connection was almost higher than the three antibiotics, i.e., Ceftazidime (-7.86 kj/mol), Imipenem (-8.79 kj/mol) and Vancomycin (-5.25 kj/mol) with Porin D. Only 3 components were identified in the acetone and triethylamine extracts, respectively. The growth inhibition zone of bacteria was in the range of 17.72 ± 1.31 to 4.61 ± 0.17 mm. *H. perforatum* antimicrobial effects were dose-dependent up to 50 mg mL^{-1} concentration ($p < 0.05$). Application of 50 mg mL^{-1} *H. perforatum* ethanolic extract exhibited the largest growth inhibition zone of *Staphylococcus aureus* (17.72 ± 1.31 mm), *Escherichia coli* (14.51 ± 1.22 mm), *Pseudomonas aeruginosa* (13.97 ± 1.18 mm), and *Acinetobacter baumannii* (10.20 ± 0.56 mm). The growth inhibition zone of *H. perforatum* was significantly higher than some tested antibiotics ($p < 0.05$). The lowest MIC (12.50 mg mL^{-1}) and MBC (25 mg mL^{-1}) were obtained for the *H. perforatum* ethanolic extract, ceftazidime, imipenem, and vancomycin. *H. perforatum* triethylamine extract displayed the highest MIC and MBC values. Given the high growth inhibition zone, as well as low MIC and MBC levels of *H. perforatum* ethanolic extract (50 mg mL^{-1}) in comparison with the antibiotic agents, it can be recommended as an economical source of antimicrobials.

Keywords: *Hypericum perforatum*, Extract, Antimicrobial effects, Pathogenic bacteria.
Article type: Research Article.

INTRODUCTION

Despite the increasing advances in medical sciences, the control and treatment of infectious diseases face many challenges (Halaji *et al*. 2020; Reina *et al*. 2021). Bacterial infectious diseases have led to 291,162 people illnesses, 102,746 medical visits, 7830 hospitalizations, and 64 deaths among children in the United States in 2013 (Scallan *et al*. 2013; Yuandani Septama *et al*. 2024). It has been estimated that the death rates of infectious diseases in 2030 among low-income, lower-middle-income, upper-middle-income, and high-income countries in 2030 will be around 8.62, 18.11, 11.60, and 300 million respectively (Holmes *et al*. 2017). Therefore, providing appropriate

solutions for the optimal control and treatment of infectious diseases can prevent millions of deaths. Reports have shown that bacteria have a higher potential to infect the host. Among them, *Staphylococcus aureus*, *Escherichia coli*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* have more been involved in epidemics of the infectious diseases (Sharifipour *et al*. 2020; Kwaengmuang *et al*. 2023; Mohammadrezaei Khorramabadi *et al*. 2022). They are mainly associated with the occurrence of urinary tract infections (UTIs), respiratory tract infections (RTIs), wound, burn, and soft tissue infections, blood infections, food poisoning and food-borne diseases globally (Momtaz *et al*. 2012; Ranjbar *et al*. 2018; Ranjbar *et al*. 2019; Ayoub Moubareck *et al*. 2020; Risvanli *et al*. 2023). The emergence of severe antibiotic resistance among these bacteria has led to common treatment insufficiency, as well as the significant increase in the length of hospital stay and treatment costs (Serwecińska *et al*. 2020). As a result, many efforts have been made to find new antimicrobials sources over the years (Aliasghari Veshareh *et al*. 2023). *Hypericum perforatum* is a perennial herbaceous plant known as St. John's Wort. This plant is a potential source of medicinal compounds with healing effects. It has been considered as an anti-inflammatory, antidepressant, antidiabetic, wound-healing, and antimicrobial agent, containing numerous phytochemicals, including flavonoids, tannins, naphthodianthrones (hypericin and hyperforin), prenylated phloroglucinols, and volatile oil (Suntar *et al*. 2010). Despite the high antimicrobial effects of the *H. perforatum* (Saddiqe *et al*. 2010), most recent surveys have focused on its antidepressant activities, and only lately has its antimicrobial activity been assessed against small numbers of bacteria. Thus, the current survey aimed to assess the antimicrobial effects of *H. perforatum* ethanolic, acetone, and triethylamine extracts compared to diverse antibiotic agents against *S. aureus*, *E. coli*, *A. baumannii*, and *P. aeruginosa* in vitro condition and the docking studies of the compounds identified by HPLC method with Beta-Lactamase *E. coli*, Glycosyltransferase-*Staphylococcus* bacterial enzymes and Porin D-*P. aeruginosa* were also studied.

MATERIALS AND METHODS

Plant materials

The aerial parts of the *H. perforatum* were collected in Shahrekord City, Chaharmahal Va Bakhtiari Province, Iran (Southwest Iran, 32.3282° N, 50.8769° E, and 2,061 masl) in June and July 2020. A voucher specimen (Herbarium No. 1966) was deposited in the Agricultural Research and Training Centre and Natural Resources of Chaharmahal Va Bakhtiari Province. The plant material was air-dried at room temperature (20 ± 2 °C).

Extract preparation

The plant's dried parts were pulverized using an electric grinder (Best 350, Germany). To prepare the extract, 30 g of the crushed aerial parts were macerated for 72 h at room temperature in 100 mL aqueous solvents solutions including ethanol, acetone, and triethylamine (Merck, Germany) diluted 7:3 in water. After filtration through a paper filter (Whatman No.1), the filtrates were recovered and centrifuged at 4000 rpm at 4 °C for 20 min and then stored at -80 °C for further examinations.

Bacteria and growth conditions

Four pathogenic bacteria, including *E. coli* (ATCC 25922), *S. aureus* (ATCC 9144), *A. baumannii* (ATCC 19606), and *P. aeruginosa* (ATCC 25922) were obtained from the Microbiology Research Centre of the Islamic Azad University, Shahrekord Branch, Shahrekord, Iran. Pure culture of the bacteria was cultured separately in tryptic soy broth (Merck, Germany) and incubated at 37 °C for 24 h.

Disk diffusion

The antimicrobial activity of *H. perforatum* extracts was investigated using the disk diffusion method. Briefly, after overnight incubation of bacteria, their concentrations were reached to 1×10^6 colonies per mL (CFU/mL). The bacteria were then cultured superficially on Müller-Hinton agar medium. Then 6-mm blank discs were placed on Müller-Hinton agar medium, then 1000 µL of *H. perforatum* different extracts with concentrations of 100, 50, 25 and 12.5 mg mL^{-1} were gently poured on the blank discs. To compare the antimicrobial effects of the extracts, antibiotic discs including ceftazidime (30 µg/disc), imipenem (10 µg/disc), gentamicin (10 µg/disc), vancomycin (30 µg/disc), penicillin (10 µg/disc), ciprofloxacin (5 µg/disc), tetracycline (30 µg/disc), erythromycin (15 µg/disc), ampicillin (10 µg/disc), and azithromycin (15 µg/disc; Oxoid, UK) were used. Blank and antibiotic discs

were placed at regular intervals on plates containing bacteria and were then incubated at 37 ° C for 24 h. The diameter of the growth inhibition zones around discs was measured and presented in mL (CLSI 2012).

Minimum inhibitory concentrations (MIC) and Minimum bacterial concentrations (MBC)

First, turbidity of 0.5 McFarland was obtained from fresh cultures of bacteria in a Mueller–Hinton broth medium (Merck, Germany). The turbidity prepared from each bacterium was then diluted to a ratio of 1 to 100 to give a concentration of 1×10^6 CFU/mL. Then, 8 µL of different dilutions of the extract containing 2 µL of bacterial suspension were poured into the polystyrene plate. In addition, wells containing 4 µL of broth medium were considered as negative control, while wells containing culture medium and bacteria were considered as positive control. Wells were also considered as control of turbidity containing 2 µL medium and 1 µL of each dilution. Tests were performed in triplicates. The surface of the plates was then covered and incubated at 4 °C for 4 h. Afterward, the turbidity was read at 630 nm using the ELISA reader (Statfax 2100, USA). The lowest concentration of extracts that reduced the 90% of turbidity compared to the control group was considered as MIC, while the lowest concentration that caused complete turbidity removal was considered as MBC (Etame *et al*. 2018). In order to compare, the MIC and MBC of antibiotics were also determined (Al-Mariri, 2014). So that, ceftazidime, imipenem, gentamicin, vancomycin, penicillin, ciprofloxacin, tetracycline, erythromycin, ampicillin, and azithromycin (Oxoid, UK) were prepared in powder forms. Serial concentrations of antibiotics were prepared from 100, 50, 25, and 12.5 mg mL^{-1} using dilution in sterile water. A negative control experiment was conducted using only sterile water.

Numerical evaluation

Data collected from the experiment were numerically evaluated by the SPSS 21.0 software (SPSS Inc., Chicago, IL). The results were analysed using MiniTab19 software in a completely randomized design. Qualitative data taken from the tests were examined using the chi-square test and Fisher's exact two-tailed test. p-value less than 0.05 was determined as a significance level.

Molecular Docking

The molecular docking study was performed using Autodock software version 4.2. All the above-mentioned ligands were docked on the simulated Beta-Lactamase *Escherichia coli* with ID pdb: 7u48, Glycosyltransferase-*Staphylococcus* with ID: 7ec1 and Porin D-*Pseudomonas aeruginosa* with ID: 3sy7 as receptor to find the best binding sites for the ligand-receptor and to determine the most stable free energy state of ligand-receptor. In the present study, a grid box including the entire receptor and blind docking was created for receptor and ligands docked to it. For docking 200 runs of molecular docking on ligands, the Genetic Algorithm and Lamarckian GA parameters were used. The autodock4 version Linux was used to generate the results file (dlg). The obtained data from the dlg file were analysed (*).

RESULTS

Phytochemical analysis of extracts

The *Hypericum* extracts were analysed using HPLC system (model Agilent 1090). The HPLC elution method has been used previously by Sarfaraz *et al*. (2021). Hypericin (Sigma-Aldrich 56690) was used as reference compound. A 0.22 µm nylon acro-disk filter and 20 µL of the extract were used for injection. The stationary phase had a 250 mm × 4.6 mm (5 µm) symmetry C18 column (Waters Crop., Milford, MA, USA; 10 mm × 4 mm ID), and the mobile one included 0.1% formic acid in acetonitrile (flow rate of 0.8 mL min^{-1}) with the wavelength between 200-400 nm. The gradient conditions were also performed as follows: a linear step from 10% to 26% solvent B (v/v) for 40 min, 65% solvent B for 70 min, and finally to 100% solvent B for 75 min. The hypericin concentration was calculated based on the peak areas and their retention times. Finally, the amount was calculated based on mg /100 g of the sample dry weight. All reagents were analytical grade (Sigma–Aldrich, USA) and the solvents used for HPLC were from Merck (Germany).

Findings of the disk diffusion

Tables 1 and 2 depict the diameter of the growth inhibition zone of examined bacteria toward *H. perforatum* ethanolic, acetone, and triethylamine extracts and antibiotic discs. The mean diameter of the tested bacteria's growth inhibition zone increased by elevating the extracts concentrations up to 50 mg mL^{-1}. *H. perforatum*

ethanolic extract exhibited the highest dimeter against all examined bacteria. *H. perforatum* triethylamine extract displayed the lowest dimeter against all examined bacteria. All examined extracts revealed the higher antimicrobial effects against *S. aureus*. The growth inhibition zone of examined bacteria was in the range between 17.72 ± 1.31 and 4.61 ± 0.17 mm. The highest diameter of *P. aeruginosa* was observed by the 50 mg mL^{-1} of *H. perforatum* ethanolic extract (13.97 ± 1.18 mm) and imipenem (12.30 ± 0.50 mm); while 100 mg mL^{-1} of its ethanolic extract (11.74 ± 0.82 mm), and ceftazidime (11.51 ± 0.64 mm). In the case of *E. coli*, the highest diameter was recorded by the 50 mg mL^{-1} of *H. perforatum* ethanolic extract (14.51 ± 1.22 mm), imipenem (12.93 ± 0.48 mm), vancomycin (12.86 ± 0.63 mm), and azithromycin (12.26 ± 0.18 mm). In the case of *S. aureus*, the highest diameter was achieved by 50 mg mL^{-1} of *H. perforatum* ethanolic extract (17.72 ± 1.31 mm), imipenem (14.61 ± 0.29 mm), while by 100 mg mL^{-1} of *H. perforatum* ethanolic extract (14.30 ± 1.10 mm), vancomycin (13.97 ± 0.49 mm), ceftazidime (13.42 ± 0.93 mm), and by 50 mg mL^{-1} of *H. perforatum* acetone extract (13.39 ± 0.83 mm). In the case of *A. baumannii*, the highest diameter was obtained by 50 mg mL^{-1} of *H. perforatum* ethanolic extract (10.20 ± 0.56 mm), imipenem (10.01 ± 0.31 mm), while by 100 mg mL^{-1} of its ethanolic extract (10.07 ± 0.62 mm), vancomycin (9.92 ± 0.71 mm), and azithromycin (9.19 ± 0.14 mm). Statistically, significant differences were observed in the diameter of the examined bacteria between different extracts of *H. perforatum* and antibiotic agents ($p < 0.05$).

Table 1. The phytochemical components identified in *H. perforatum* ethanolic, acetone, and triethylamine extracts.

HPLC	Hypericin (mg mL^{-1})	Pseudohypericin (mg mL^{-1})	Hyperforin (mg mL^{-1})
Ethanol	0.29048	0.18904	0.75766
3-Amine acetate	0.39335	0.41549	1.32806
Acetone	0.39544	0.04144	1.01894

MIC and MBC values

Table 3 presents the MIC and MBC indexes of *H. perforatum* ethanolic, acetone, and triethylamine extracts and antibiotic agents against examined bacteria. The lowest MIC and MBC values were obtained by the *H. perforatum* ethanolic extract, ceftazidime, imipenem, and vancomycin. However, the highest MIC and MBC values were obtained by the *H. perforatum* triethylamine extract, gentamicin, penicillin, tetracycline, and ampicillin. The MIC values of the *H. perforatum* triethylamine extract, gentamicin, penicillin, tetracycline, and ampicillin were not detected. It was true for the MBC values of *H. perforatum* triethylamine extract, gentamicin, penicillin, tetracycline, erythromycin, azithromycin, and ampicillin. The lowest MIC and MBC values were recorded for *S. aureus*, while the highest for *A. baumannii*.

Table 2. The growth inhibition zone diameter of examined bacteria toward *H. perforatum* ethanolic, acetone, and triethylamine extracts and antibiotic discs.

Extracts and antibiotics/concentrations		Diameter of the growth inhibition zone (mm)			
		P. aeruginosa	***E. coli***	***S. aureus***	***A. baumannii***
Ethanolic	100*	11.74 ± 0.82 ^{b***}	11.92 ± 0.43 ^{b}	14.30 ± 1.10 $_{b}$	10.07 ± 0.62 $_{a}$
	50	13.97 ± 1.18 $_{a}$	14.51 ± 1.22 $_{a}$	17.72 ± 1.31 $_{a}$	10.20 ± 0.56 $_{a}$
	25	11.21 ± 0.82 ^{b}	11.76 ± 0.88 ^{b}	12.25 ± 0.68 ^{c}	8.15 ± 0.24 ^{c}
	12.5	9.14 ± 0.37 ^{c}	8.35 ± 0.57 ^{c}	10.61 ± 0.74 ^{d}	7.21 ± 0.18 ^{c}
Acetone	100	10.51 ± 0.28 ^{b}	10.88 ± 0.32 ^{b}	12.55 ± 0.86 ^{c}	7.71 ± 0.28 ^{c}
	50	11.15 ± 0.54 ^{b}	11.39 ± 0.43 ^{b}	13.39 ± 0.83 ^{c}	8.17 ± 0.61 ^{c}
	25	9.14 ± 0.33 ^{c}	9.53 ± 0.41 ^{c}	11.15 ± 0.27 ^{d}	6.98 ± 0.19 ^{c}
	12.5	7.25 ± 0.21 ^{d}	7.11 ± 0.30 ^{d}	9.20 ± 0.44 ^{e}	5.32 ± 0.24 ^{d}
Triethylamine	100	10.61 ± 0.16 ^{b}	9.96 ± 0.04 ^{c}	12.09 ± 0.23 ^{c}	6.64 ± 0.52 ^{c}
	50	10.87 ± 0.64 ^{b}	10.18 ± 0.18 ^{c}	13.01 ± 0.09 ^{c}	7.18 ± 0.36 ^{c}

	25	8.75 ± 0.33 ^{c}	8.82 ± 0.44 ^{d}	10.53 ± 0.27 ^{d}	5.73 ± 0.54 ^{d}
	12.5	7.01 ± 0.14 ^{d}	6.60 ± 0.21 ^{e}	8.14 ± 0.16 ^{e}	4.61 ± 0.17 ^{e}
	Cef30**	11.51 ± 0.64 $_{b}$	12.17 ± 0.35 $_{b}$	13.42 ± 0.93 $_{c}$	9.13 ± 0.29 $_{a}$
	Imp10	12.30 ± 0.50 ^{a}	12.93 ± 0.48 ^{b}	14.61 ± 0.29 ^{b}	10.01 ± 0.31 ^{a}
	G10	10.41 ± 0.60 ^{b}	10.53 ± 0.28 ^{c}	11.14 ± 0.22 ^{d}	7.20 ± 0.35 ^{c}
	V30	11.90 ± 0.75 ^{b}	12.86 ± 0.63 ^{b}	13.97 ± 0.49 ^{b}	9.92 ± 0.71 ^{a}
	P10	10.65 ± 0.39 ^{b}	11.51 ± 0.40 ^{b}	12.08 ± 0.27 ^{c}	8.23 ± 0.45 ^{c}
Antibiotics	Cip5	10.96 ± 0.27 ^{b}	11.95 ± 0.57 ^{b}	12.58 ± 0.39 ^{c}	8.77 ± 0.41 ^{c}
	Tet30	10.37 ± 0.56 ^{b}	10.60 ± 0.14 ^{c}	11.02 ± 0.18 ^{d}	7.07 ± 0.27 ^{c}
	Er15	10.89 ± 0.44 ^{b}	11.72 ± 0.48 ^{b}	12.39 ± 0.26 ^{c}	8.61 ± 0.53 ^{c}
	Am10	10.77 ± 0.42 ^{b}	11.25 ± 0.56 ^{b}	11.83 ± 0.36 ^{d}	8.05 ± 0.20 ^{c}
	Az15	11.30 ± 0.55 ^{b}	12.26 ± 0.18 ^{b}	13.22 ± 0.46 ^{c}	9.19 ± 0.14 ^{a}

*mg/mL; **Cef30: ceftazidime (30 µg/disc), Imp10: imipenem (10 µg/disc), G10: gentamicin (10 µg/disc), V30: vancomycin (30 µg/disc), P10: penicillin (10 µg/disc), Cip5: ciprofloxacin (5 µg/disc), Tet30: tetracycline (30 µg/disc), Ertt15: erythromycin (15 µg/disc), Am10: ampicillin (10 µg/disc), Az15: azithromycin (15 µg/disc). ***Dissimilar letters in each column show statistically significant differences about $p < 0.05$.

Table 3. The MIC and MBC indexes of *H. perforatum* ethanolic, acetone, and triethylamine extracts and antibiotic agents

Extracts/antibiotics	**MIC and MBC (mg mL^{-1})**							
	P. aeruginosa		***E. coli***		***S. aureus***		***A. baumannii***	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Ethanolic	25	50	25	50	12.5	25	50	100
Acetone	50	100	50	100	25	50	50	100
Triethylamine	50	100	50	100	25	50	-	-
Cef30*	25	50	25	50	12.5	25	50	100
Imp10	25	50	25	50	12.5	25	50	100
G10	50	100	50	100	50	100	-	-
V30	25	50	25	50	12.5	25	50	100
P10	50	100	50	100	50	100	-	-
Cip5	50	100	50	100	25	50	50	100
Tet30	50	100	50	100	50	100	-	-
Er15	50	100	50	100	25	50	100	-
Am10	50	100	50	100	50	100	-	-
Az15	50	100	50	100	25	50	100	-

Note: *Cef30: ceftazidime (30 µg/disc), Imp10: imipenem (10 µg/disc), G10: gentamicin (10 µg/disc), V30: vancomycin (30 µg/disc), P10: penicillin (10 µg/disc), Cip5: ciprofloxacin (5 µg/disc), Tet30: tetracycline (30 µg/disc), Ertt15: erythromycin (15 µg/disc), Am10: ampicillin (10 µg/disc), Az15: azithromycin (15 µg/disc).

Molecular Docking

In this study, to investigate the behavior and interaction of three extracts by HPLC, i.e., hypericin, pseudohypericin and hyperfori as ligand with Beta-Lactamase *E. coli*, Glycosyltransferase-*Staphylococcus* bacterial enzymes and Porin D-P. *aeruginosa* were used as receptor from Autodock 1.5.7 software. This bacterial enzymes and protein structures were chosen as the binding target, since the antibiotics Ceftazidime, Imipenem, and Vancomycin were used as positive controls in this *in vitro* study, and these receptors have been experimentally determined to be the

target of these antibiotics. Therefore, they were chosen to compare the binding of these receptors with the three identified compounds. The three-dimensional structures of the ligands were downloaded from the PubChem site with the extension sdf and were energy optimized with Chimera UCSF software, in addition, saved in pdb format for docking. The pdb structure of beta-lactamase, glycotransferase and protein d were downloaded from rcsb.org server. In step, docking was done for 200 runs with genetic algorithm. Docking results of three compounds, i.e., hypericin, pseudohypericin, hyperforin and three antibiotics, Ceftazidime, Imipenem and Vancomycin with 3 target proteins of these three antibiotics (Beta-Lactamase *Escherichia coli*, Glycosyltransferase-*Staphylococcus* and Porin D-*Pseudomonas aeruginosa*) are shown in Table 3 and Fig. 1. The binding energy of pseudohypericin with Porin D-*Pseudomonas aeruginosa* was higher than other dockings (-8.31 kJ/mol; Table 4).

Table 4. Docking results in the form of Binding Affinity of Hypericin, Pseudohypericin, Hyperforin Ceftazidime, Imipenem and Vancomycin used for in silico screening against Beta-Lactamase *Escherichia coli*, Glycosyltransferase-*Staphylococcus* and Porin D-*Pseudomonas aeruginosa* (AutoDock scores are in kcal/mol).

Receptor name (ID)	**Ligand-receptor**	**Estimated Free Energy of Binding (kcal/mol)**	**Final Intermolecular Energy kcal/mol**	**Electrostatic Energy kcal/mol**
Beta-Lactamase Escherichia coli (7u48)	Hypericin	**-6.88**	-9.27	-0.17
	Pseudohypericin	-5.62	-5.01	+0.15
	Hyperforin	-5.25	-9.13	+0.02
	Ceftazidime	-4.72	-8.60	-2.39
	Imipenem	-3.62	-6.30	-0.67
	Vancomycin	-1.93	-11.7	-1.59
Glycosyltransferase-Staphylococcus (7ec1)	Hypericin	**-6.85**	-9.23	-0.28
	Pseudohypericin	-6.47	-8.86	-0.30
	Hyperforin	-5.00	-8.88	-0.04
	Ceftazidime	-4.83	-8.71	-2.00
	Imipenem	-6.30	-8.98	-4.62
	Vancomycin	-0.64	-9.89	-2.30
Porin D-Pseudomonas aeruginosa (3sy7)	Hypericin	**-6.94**	-9.23	-0.34
	Pseudohypericin	**-8.31**	**-10.70**	**-0.47**
	Hyperforin	-6.89	-10.77	-1.84
	Ceftazidime	-7.86	-11.74	-1.84
	Imipenem	**-8.79**	-11.48	-4.86
	Vancomycin	-5.25	-14.50	-0.03

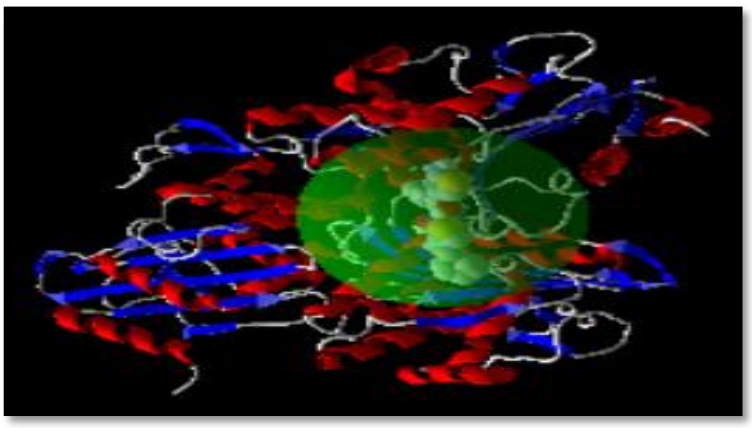

Fig. 1. Molecular simulation of Ceftazidime and Beta-lactamase-*Escherichia coli*.

DISCUSSION

Plant antimicrobial agents act by targeting specific sites of bacteria. Among the essential antimicrobial compound's mechanisms are interference with cell wall synthesis, inhibition of protein synthesis, interference with nucleic acid synthesis, inhibition of metabolic mediated pathways, and disruption of cell cytoplasmic membrane (Khameneh *et al*. 2019). More than one compound with antimicrobial properties in plant agents exerts its antimicrobial effects more effectively in different pathways (Khodadadi *et al*. 2015). The present study was performed to assess the antimicrobial effects of *H. perforatum* ethanolic, acetone, and triethylamine extracts against bacterial species. Other investigations were focused on the phytochemical analysis of *H. perforatum* methanolic extract and its essential oils. Camas *et al*. (2014) stated that 2,4-dihydroxybenzoic acid, neochlorogenic acid, chlorogenic acid, caffeic acid, rutin, epicatechin, hyperoside, isoquercetin, avicularin, quercitrin, catechin, amentoflavone, hyperforin, adhyperforin, pseudohypericin, and hypericin were detected in the *Hypericum* methanolic extract. Different chemical agents were also detected in their *Hypericum*'s stem, leaf, and flower. Predominant phytochemical agents of the study conducted by Chatzopoulou *et al.* (2006) were 2-methyl-octane (10-18%), germacrene D (18-23%), βcaryophyllene (6-10%), α-pinene (5-10%), and bicyclogermacrene (4-5%). Caffeic acid and quercetin were detected in the *H. perforatum* extracts later by Božin *et al*. (2013) and Tusevski *et al*. (2014). However, several differences have been reported in the phytochemical profile of *H. perforatum* in numerous studies. The differences in phytochemical contents could have been driven by the different proportions of leaves and flowers in the samples, as it has been shown that some phytochemicals are significantly higher in the leaves. The plant phenological stage at the sampling time also plays a substantial role in the phytochemical profile as most phytochemicals present during full flowering. In keeping with this, differences in the geographical area, climate, altitude, soil type, day and night duration, irrigation rate, and extraction method cause alterations in the chemical composition of *H. perforatum* (Li *et al*. 2020; Yuan *et al*. 2020). *H. perforatum* extracts showed different antimicrobial activities against the tested bacteria. Findings showed that 50 mg mL^{-1} *H. perforatum* ethanolic extract exhibited the highest antimicrobial effects against the tested bacteria. The diameter of the growth inhibition zone of *P. aeruginosa* treated by the 50 mg mL^{-1} *H. perforatum* ethanolic extract was significantly higher than those of ceftazidime, gentamicin, vancomycin, penicillin, ciprofloxacin, tetracycline, erythromycin, ampicillin, and azithromycin ($p < 0.05$). In addition, the diameter of *S. aureus* and *E. coli* treated by the 50 mg mL^{-1} *H. perforatum* ethanolic extract was significantly higher than those of all texted antibiotic disks ($p < 0.05$). However, the diameter of *A. baumannii* treated by the 50 mg mL^{-1} *H. perforatum* ethanolic extract was only significantly higher than those of gentamicin, penicillin, ciprofloxacin, tetracycline, erythromycin, and ampicillin ($p < 0.05$). The lowest MIC and MBC were also detected by *H. perforatum* ethanolic extract, particularly against *S. aureus*. It seems that 50 mg mL^{-1} *H. perforatum* ethanolic extract displayed the highest antimicrobial effects against *S. aureus*, followed by *E. coli*. The presence of more phytochemicals that may have potential antimicrobial effects in the *H. perforatum* ethanolic extract could explain this finding. So that, as the *H. perforatum* triethylamine extract revealed the lowest chemical components, it exhibited the lowest antimicrobial effects. the elevated concentrations of some inhibitory compounds in the *H. perforatum* extracts have been a possible reason for reducing the antimicrobial effects of its extracts at concentrations above 50 mg mL^{-1}. Findings also exhibited the higher susceptibility of the Gram-positive bacteria (*S. aureus*) than Gram-negatives (*E. coli*, *P. aeruginosa*, and *A. baumannii*) against *H. perforatum* extracts. Studies have shown that Gram-positive bacteria's cell wall is more susceptible to many antimicrobial agents, chemical compounds, and even herbal medicines. This may be due to lipopolysaccharides in the outer membrane and the periplasmic space of Gram-negative bacteria, making them inherently resistant to external factors (Masoumian & Zandi 2017). Literature review revealed that *H. perforatum* extracts are reported to be more active against *S. aureus*, *Shigella*, and *E. coli* (Lyles *et al*. 2017; Mazandarani *et al*. 2007). Okmen & Balpınar (2017) reported that the growth inhibition zone caused by *H. perforatum* extract against *S. aureus* strains had a range between 13 to 17 mm. Dordevic *et al.* (2013) and Đorđević *et al*. (2013) reported that the MIC of *H. elegans* and *H. annulatum* essential oils against *S. aureus* was 3.13 mg mL^{-1}, lower than that found in the present study. Lower *H. perforatum* MIC values were reported against *S. aureus* (1.00 µg mL^{-1}) and *E. coli* (400 µg mL^{-1}; Reichling *et al*. 2001). Bahmani *et al*. (2019) reported a 12.66-mm inhibition zone and 625 µg mL^{-1} MIC of the *S. aureus* treated by *H. perforatum* hydroalcoholic extract. Grafakou *et al.* (2020) reported that the mean MIC and MBC values of *S. aureus*, *P. aeruginosa*, and *E. coli* bacteria toward the *Hypericum* essential oil ranged between 0.0015-0.030 and 0.0030-0.060 mg mL^{-1}, respectively. Inefficient antimicrobial effects of the

Hypericum compounds against *P. aeruginosa*, *E. coli*, and *A. baumanii* were reported by Sarkisian *et al*. (2012). A low anti-*P. aeruginosa* effects of *Hypericum* compounds were reported by Alam *et al*. (2019). Kalaba *et al*. (2015) stated that the diameter of the growth inhibition zones of *S. aureus*, *Salmonella typhimurium*, and *P. aeruginosa* against *H. perforatum* compounds were 2.33, 0.00, and 20.00 mm, respectively. Probable reasons for differences in antimicrobial effects of *H. perforatum* in different studies may be due to differences in the geographical area, climate, temperature, altitude, duration of shade, soil type, use of fertilizers, and finally, the type of extract or essential oil used. However, all studies confirmed the antimicrobial effects of different *H. perforatum* extracts or essential oils (Süntar *et al*. 2016; Rahnavard 2016). Hammer *et al*. (2007) and Karioti & Bilia (2010) reported that light-activated pseudohypericin inhibits the production of prostaglandin E_2, while hypericin has been reported to decrease Croton oil-induced ear oedema in mice in comparison with Indometacin (Sosa *et al*. 2007; Karioti & Bilia 2010). Medicinal plants are rich in antioxidants and effective medicinal compounds such as tannin, polyphenol, flavonoid, phenol and anthocyanin, and many medicinal effects are due to the presence of these compounds (Najafi *et al*. 2018; Magbool Alrekaby *et al*. 2023; Ahmadi *et al*. 2023; Razmjoue *et al*. 2023; Habibi Mirzaei *et al*. 2023; Khosravi *et al*. 2024; Bozorgi *et al*. 2024).

CONCLUSION

This study showed that *H. perforatum* ethanolic extract, particularly at a 50 mg mL^{-1} concentration, has exhibited antimicrobial effects against *S. aureus*, *E. coli*, and *P. aeruginosa*. Its antimicrobial effects were also higher than the majority of the tested antibiotics. Low *H. perforatum* ethanolic extract MIC and MBC levels can indicate the specific antimicrobial effects of the plant in low concentrations, which makes it potential as an economic source of antimicrobials. It is recommended to use *H. perforatum* ethanolic extract as an oral antimicrobial compound in the industry. However, further studies are needed in this area. According to the results of this in silico, pseudohypericin can be introduced as a candidate with antibiotic properties, and due to its high binding energy with Porin D-*Pseudomonas aeruginosa*, it can be a strong inhibitor of this receptor.

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