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An integrated investigation into the antibacterial and antioxidant properties of propolis against *Escherichia coli* cect 515: A dual *in vitro* and *in silico* analysis

Farouk BOUDOU[1], Abdelmadjid GUENDOUZI[2,3], Amal BELKREDAR[1], Mohammed RASHEED[4,5]*

[1]*University of Saïda, Faculty of Sciences, Department of Biology, P.B 138 Ennasr 20000, Saida, Algeria; farouk.boudou@yahoo.fr; belakredar-amel@hotmail.fr*
[2]*Higher Normal School (ENS), Ville Universitaire, Ain El Bey Ali Mendjeli Constantine 25000, Constantine, Algeria; maliabendaman@gmail.com*
[3]*Pharmaceutical Sciences Research Center, (CRSP), Nouvelle Ville, Constantine, 2000, Algeria; belakredar-amel@hotmail.fr*
[4]*University of Technology - Iraq, Applied Sciences Department, P.O. Box 19006, Baghdad, Iraq; rasheed.mohammed40@yahoo.com*
[5]*MOLTECH Anjou, Universite d'Angers/UMR CNRS 6200, 2, Bd Lavoisier, 49045 Angers, France; rasheed.mohammed40@yahoo.com (*corresponding author)*

Abstract

Urinary tract infections resulting from *Escherichia coli*, are prevalent and have become increasingly difficult to treat due to antibiotic resistance. This study aimed to explore the potential of propolis extract from beehives in Algeria's Tiout region as a natural antibacterial agent against *E. coli* CECT_515. The phytochemical composition, antioxidant activity, and antibacterial action of the extract has been evaluated and compared to four synthetic antibiotics, both *in vitro* and *in silico*. The propolis extract had high levels of total polyphenol and total flavonoids, regarding its antioxidant properties, the extract showed an important ability of DPPH radicals scavenging, as determined by its IC50 value of 330.08±13.35 µg/mL. The excerpt showed important antibacterial efficacy against *Escherichia coli*, as demonstrated with important inhibition zones. The extract's inhibitory effects were compared to four synthetic antibiotics, including gentamicin, pipemidic acid, cefazolin, and colistin, molecular docking analysis revealed that the propolis extract interacted with the active site of beta-lactamase, a protein responsible for antibiotic resistance, and showed binding affinities and conformational stability during molecular dynamics simulation for 250 ns. Overall, the results suggest that the propolis has significant antioxidant and antibacterial properties and could serve as a promising natural alternative to synthetic antibiotics for the treatment of *E. coli* infections.

Keywords: antibacterial activity; antioxidant activity; *Escherichia coli; in silico; in vitro* natural products; propolis

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Introduction

Urinary tract infection (UTI) is a prevalent condition among children, with an approximate incidence rate of 6.5% in girls and 3.3% in males during their very first year of life. During the period lasting from infancy to late adolescence, a minimum of 3% to 5% of girls and 1.0% of boys experience the development of at least one urinary tract infection. In 2007, the United States witnessed 8.6 million ambulatory visits for urinary tract infections (UTIs) among both genders (Abraham *et al*., 2015). In the United States in 2007, there were 8.6 million ambulatory visits for UTIs in both men and women (Al-Ani *et al*., 2018). Urinary cystitis is characterized by dysuria (burning or pain when urinating), increased frequency of urination, bedwetting, abdominal and suprapubic pain, urgency, and cloudy (pyuria) or bloody (haematuria) urine (Almuhayaw *et al*., 2020). The recurrence of infection, whether it is asymptomatic bacteriuria, cystitis, or pyelonephritis, is influenced by a complex combination of genetic factors and uropathogenic virulence features (Aukštuolis *et al*., 2017); (Banerjee *et al*., 2022). The diagnosis is based on a correctly obtained urine culture containing more than 100,000 colonies of a single pathogen. *Escherichia coli* is the most frequent pathogen in children, accounting for 90% of the first episodes of acute cystitis and 75% of recurring infections. *E. coli's* pathogenicity in UTI is based on its ability to attach to uroepithelial cells and red blood cells (Becke, 1992). The etiology of acute cystitis involves the ascent of bacteria from the perineum, urethra, and fecal matter into the bladder and renal systems through the urethral pathway. The majority of UTIs acquired within the community, specifically 80%, are attributed to the bacterial strains Escherichia coli and *Staphylococcus saprophyticus* (Becke, 1992). In addition, *E. coli* is the most prevalent uropathogenic in nosocomial situations (Bitencourt-Ferreira *et al*., 2019). *Escherichia coli,* which is the predominant etiological agent of urinary tract infections, have exhibited resistance to a multitude of antibiotics, involving third-generation cephalosporins, carbapenems, and colistin (Bogart *et al*., 2007; Bouras *et al*., 2023). Furthermore, the prevalence of infections resulting from multi-drug-resistant bacteria has emerged as a significant global public health issue. The administration of antibiotics during the early stages of childhood has been associated with a heightened susceptibility to non-communicable ailments such as allergies and obesity. Furthermore, the impact of antibiotics on the development of the neonatal gut resistome, along with the microbiota's function as a repository of resistance genes, is associated with the rising prevalence of antibiotic-resistant pathogens (Bouras *et al*., 2022). However, there is an urgent need to discover novel natural compounds with antibacterial properties capable of combating these pathogens (Bruschi *et al*., 2017; Bussi *et al*., 2007). Propolis, occasionally referred to as bee glue, commonly regarded as a natural antibiotic, is a resinous substance that is produced and utilized by the *Apis mellifera* L. species of bees to reinforce the comb and hive entrance borders, as well as to seal hive walls. It has been used by humans since ancient times due to its pharmaceutical properties, which include antioxidant, anti-inflammatory, and antimicrobial activities (Copp *et al*., 2011). Due to its antiseptic, antimycotic, and bacteriostatic properties, this substance is commonly utilized in cosmetics and self-treatment "natural products". Additionally, it is considered to be relatively safe and nontoxic (De Souza *et al*., 2019). Propolis contains mainly phenolics and flavonoids. The chemical composition of propolis exhibits significant variation, which relies upon the geographical location and time of collection (DeFrees *et al*., 2019). The medicinal properties of propolis have led to its increased utilization in the pharmaceutical, food, and cosmetic sectors, resulting in its growing popularity. Propolis is now incorporated into consumable products, including food additives and beverages, for human consumption (Desai *et al*., 2016).

Propolis, a bee-collected resin rich in polyphenols and flavonoids, exhibits potent antimicrobial, anti-inflammatory, antioxidant, and immunomodulatory effects, driving its growing utilization in pharmaceuticals, dietary supplements, cosmetics, and the food and beverage industry, though caution is advised due to potential individual variations necessitating consultation with healthcare professionals before medicinal use. The chemical composition of propolis is influenced by factors such as floral sources, climate, and environmental

conditions. Its geographical origin impacts medicinal properties due to variations in plant species, leading to diverse bioactive compounds and therapeutic effects. The propolis used in this study was obtained from apiaries located in the Tiout region of Algeria, where the major vegetation is that which surrounds the palm steppe. *Remt Hammada scoparia*, *Alfa Stipa tenacissima*, and *Hamada psammophytes schmittiana* dominate the steppes, whereas *Retama retam*, *Ziziphus lotus*, and *Thy nelaea microphylla* inhabit the silted wadis, and a few feet and *Gymnocarpos decander* Anabasis aretioides populate Thalwegs wadis. The banks of the major wadis are populated by a Gypso-halophilic vegetation, which includes tree strata such as *Tamarix gallica*, *Salsola vermiculara*, *Traganum nuudatum*, and others. Along the wadis claws, look for Betoum (*Pistacia atiantica*), a protected *Spezia*, and *Rhus tripartitum* (Enneffatia *et al*., 2011). However, the evaluation of the antibacterial characteristics of propolis has been limited to a small number of investigations, primarily targeting the reference strains of the American Type Culture Collection (ATCC) (Freires *et al*., 2018; Gharbi *et al*., 2021). To the very best of the present knowledge, there is a lack of documentation regarding the antibacterial efficacy of Tiout ain sefra propolis from Algeria against the reference strains of microorganisms (CECT) belonging to the Spanish type culture collection. The objective of this investigation is to assess the phytochemical composition and antioxidant potential of a hydroethanolic extract of propolis. Additionally, the study aims to conduct a comparative analysis of the extract's *in vitro* antibacterial efficacy against *Escherichia coli* CECT 515 with that of four synthetic antibiotics, namely gentamicin (GEN), cefazolin (CZ), colistin (CT), and pipemidic acid (PI). Additionally, the utilization of *in silico* techniques such as molecular docking and molecular simulation for a duration of 250 nanoseconds has been documented. The Tiout region in Algeria is surrounded by vegetation dominated by *Remt Hammada scoparia, Alfa Stipa tenacissima*, and *Hamada psammophytes schmittiana* in the steppes, *Retama retam*, *Ziziphus lotus*, and *Thy nelaea microphylla* in the silted wadis, and Gypso-halophilic species like *Tamarix gallica*, *Salsola vermiculara*, and *Traganum nuudatum* along the banks of major wadis. The propolis utilized in the study was sourced from beehives located in the Tiout region, characterized by this diverse array of vegetation. The propolis from the Tiout region in the Algerian desert remains relatively underexplored in scientific studies. The bees in this area belong to the Saharan *Apis mellifera* type, and despite their unique habitat, there has been limited research on the specific properties of the propolis they produce. This study seeks to address this gap in knowledge by conducting a thorough investigation into the antibacterial properties of propolis sourced from the Tiout region, shedding light on its potential medicinal significance. The primary goals of this investigation on Tiout region propolis are to evaluate its antibacterial potential against specific strains and understand its chemical composition. Techniques employed include antibacterial assays against *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Bacillus cereus*, along with molecular docking and dynamics to assess interactions with target proteins.

Materials and Methods

Preparation of propolis extract

The hydroalcoholic extract of propolis from the Tiout region in Algeria was prepared following the method described by Mohdaly *et al*. (2015). Propolis powder samples (10 g) were extracted with 100 ml of 70% hydroethanolic solution at room temperature overnight. The extract was filtered, the solvent was evaporated using a rotary vacuum evaporator, and the dry residue was stored at 4 °C for further analysis.

Phytochemical analysis

Thin-layer chromatography

Chromatographic analysis by TLC (thin layer chromatography) was performed using MilliporeSigma TM TLC Silica Gel 60 F254: 20 × 20 cm, according to (Gordon *et al*., 1982). One drop of propolis extract

solution and 5 drops of each standard used (quercetin, catechin, rutin, gallic acid, or vanillin) were placed at a distance of 1.5 cm from the bottom edge of the plate. The development of spots on the stationary phase required the use of a mobile phase consisting of the mixture hexane, ethylacetate, and acetic acid (60:40:1, v/v). All solvents were purchased from Sigma-Aldrich or Merck (Darmstadt, Germany). Plates were visualized with vanillin, sulfur, or iodine vapor. The retention factor (Rf) is used to compare and help identify compounds. The Rf value of a compound is equal to the distance traveled by the compound divided by the distance travelled by the solvent front.

Total phenolic content (TPC)

The total phenolic content of the samples was determined using the Folin-Ciocalteu reagent, following the method described by Guendouzi *et al*. (2023). An aliquot of the diluted sample was mixed with distilled water, Folin-Ciocalteu reagent, and Na_2CO_3 (7%). After incubation, the solution was diluted, and its absorbance was measured at 760 nm. The total phenolic content was expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g d.w.) using a calibration curve with gallic acid. Three replicates were performed for each sample.

Hormonal total flavonoid content (TFC)

The total flavonoid content of the extracts was determined using the colorimetric method described by Hadidi *et al*. (2016). The extract (100 µL) was mixed with distilled water, sodium nitrite solution, and $AlCl_3$ solution. After incubation, Na_2CO_3 solution and water were added. The mixture was vortexed, and the absorbance was measured at 510 nm. The total flavonoid content was expressed as milligrams of catechin equivalents per gram of dry weight (mg CE/g d.w.) using a catechin calibration curve. Triplicate samples were tested.

DPPH radical-scavenging activity

The DPPH radical-scavenging activity was assessed using the method described by Hayouni *et al*. (2007). Propolis extract or ascorbic acid at various concentrations (4.88 to 5000 µg/mL) was combined with a methanolic solution of DPPH. A negative control was prepared by mixing methanol with DPPH solution. After 30 minutes of incubation in the dark, the absorbance of the solution was measured at 515 nm. The scavenging activity was quantified as IC50 (mg/mL), representing the dose required to inhibit DPPH by 50%. A lower IC50 value indicates stronger antioxidant activity. The percentage of radical scavenging was calculated using the equation: $I\% = (A_o - A) / A_o \times 100$, where A0 is the absorbance of the control (DPPH solution without extract) and A is the absorbance in the presence of the extract.

Antibacterial activity

For the antimicrobial analysis, the microorganisms were cultured in nutrient broth at 37 °C for 12 hours to prepare the inoculum. The bacterial suspension with a concentration of approximately 108 colony-forming units (CFU/ml) was used (Hohenberg *et al*., 1964). The Agar-well diffusion method was employed by spreading the bacterial suspension on Mueller-Hinton agar plates, each containing four 6 mm diameter wells. Different concentrations of the extract (625, 1250, 2500, 5000 µg/mL) were added to the wells, while ethanol served as the control. Additionally, the disc diffusion method was used for the antibiogram analysis using commercial antibiotic discs (Gentamicin, Cefazolin, Colistin and Pipemidic Acid). The Petri dishes were incubated at 37 °C overnight, and the results were evaluated by measuring the diameter of the inhibition zone. Sterile water was used as the control [36].

Statistical analysis

The statistical analysis conducted on the experimental results involved performing the Tukey test for group comparisons. The significance level used in the Tukey test was $p < 0.05$. Results were presented as mean ± standard deviation based on experiments performed in triplicate (n = 3), and groups with different letters (a-c) were considered to have significant differences at the specified significance level. All experiments were performed in triplicate (n = 3) and the results were presented as the mean ± standard deviation. Statistical analysis was conducted using SigmaPlot for Windows version 11.0. Group comparisons were made using the Tukey test. Groups with different letters (a-c) indicate significant differences at a significance level of $p < 0.05$ (Tukey test).

Molecular docking

The structures of the ligands were drawn using ChemDraw software and optimized with the B3LYP/DFT method (Jorgensen *et al*., 1983: Kim *et al*., 2003; Kaushik *et al*., 2010; Krisyuk *et al*., 2016; Kadri *et al*., 2021) using the basis set 6-31G** (Lafitte *et al*., 2002; Laskowski *et al*., 2011) implemented in the GAUSSIAN16 package (Lee *et al*., 1988). The docking of the ligands (val, gal, cat, que, and rut) in proteins was carried out using the Molegro program (Lengauer *et al*., 1996).

The studies were conducted to investigate the binding of the ligand with three target proteins: beta-lactamase (PDB ID: 1KZN Resolution: 2.30 Å (Lestari *et al*., 2020; Lustri *et al*., 2023), (PDB ID: 6OOH Resolution: 1.50 Å (Mihai *et al*., 2010; Minardi *et al*., 2011), and (PDB ID: 1TEM Resolution: 1.70 Å) (Mohdaly *et al*., 2015; Nabti *et al*., 2019). The RCSB Protein Data Bank (*www.rcsb.org*) was the source of the receptor structure utilized in the study.

The water molecules, co-crystallized ligands, and heteroatoms in the receptor were eliminated, and the active site grid parameters were recorded for each complex. The docking protocol was confirmed using an RMSD value of less than 2 Å (Ness *et al*., 2000), which was obtained by aligning the bioactive conformation of the X-ray ligand with its docked conformation. The docking interactions were analyzed using Ligplot software (Nicolle *et al*., 2001). In the molecular docking analysis, the following ligands were docked with three beta-lactamase target proteins: vanillin (CID: 1183), gallic acid (CID: 370), catechin (CID: 73160), quercetin (CID: 5280343), and rutin (CID: 5280805). Beta-lactamase target proteins: PDB ID: 1KZN (resolution: 2.30 Å), PDB ID: 6OOH (resolution: 1.50 Å), and PDB ID: 1TEM (Resolution: 1.70 Å). The ligands' structures were drawn and optimized using ChemDraw software and the B3LYP/DFT method with a 6-31G** basis set in the GAUSSIAN16 package. The Molegro program was employed for the docking procedure. Protein structures were obtained from the RCSB Protein Data Bank, and ligands were sourced from the PubChem database. The docking protocol was validated by ensuring a root-mean-square deviation (RMSD) value below 2 Å, comparing the X-ray ligand's bioactive conformation with its redocked conformation. Ligplot software was utilized for analyzing the docking interactions.

Molecular dynamics simulations

The docked complexes were subjected to molecular dynamics simulations using the GROMACS-2023-GPU package (Nogacka *et al*., 2018) with the CHARMM-36-2019 force field (O'Connell *et al*., 2013). To solvate the protein-ligand complex, TIP3P water models (Parrinello *et al*., 1981) were used to fill a cubic box. Na^+ and Cl^- ions were added to neutralize the systems. An initial energy minimization process was carried out on the solvated complex. The systems were then equilibrated through NVT and NPT (Pople *et al*., 1978; Pascoal *et al*., 2014) ensemble for 2 ns each. Finally, all systems were subjected to production MD simulations for 250 ns at 300 K and 1 bar pressure. Trajectory files were collected, and root mean square deviation (RSMD), root mean square fluctuation (RMSF), and radius of gyration (Rg) were used to gauge the dynamics and stability of each system.

Results

Phytochemical analysis

The development of TLC using the mixture of hexane, ethylacetate, and acetic acid (60:40:1, v/v) as a mobile phase has allowed us to observe five different spots from the propolis extracts migrating in parallel with those from the five different standards used. The Rf values are 0.35±0.028, 0.74±0.026, 0.06±0.026, 0.98±0.028, and 0.95±0.026 for quercetin, catechin, rutin, gallic acid, or vanillin respectively (Table 1). The results in Table 2 demonstrate that propolis extract has a significantly higher total phenol content (272.45±11.90 mg GAE/mg DS) than flavonoid content (8.64±1.79 mg CE/g RS). According to our findings, flavonoids make up only 3.17 percent of the total phenolic compounds present in the ethanolic extract of propolis. These results align with the study conducted by Ragnarsdóttir *et al*. (2012) which investigated the phenolic composition and antioxidant activity of propolis from different regions of Poland. Their findings revealed that the total phenolic content of propolis samples ranged from 150.05 to 197.14 mgGAE/g, while the total flavonoid content ranged from 35.64 to 62.04 mgQE/g. The dominant phenolic acids identified were p-coumaric acid and ferulic acid, while chrysin, naringin, and galanin were found to be the dominant flavonoids. The study by Rahman *et al*. (2010), after analyzing two samples of propolis from Algerian and Turkish origins, discovered that the levels of phenolic compounds were lower compared to our findings. The total phenol content ranged from 19.51 ± 0.86 to 219.66 ± 1.23 mg GAE/g, while the flavonoid content varied from 5.27 ± 0.07 to 74.57 ± 1.03 QE/g. Another study conducted on Malaysian propolis investigated the impact of different ethanol concentrations (20%, 50%, and 80%) on the total phenolic and flavonoid content. The results from that study also indicated lower levels compared to our research, with the highest total phenolic content (TPC) and total flavonoid content (TFC) recorded at 80% ethanol concentration, specifically 8.898 mg GAE/ml and 0.034 mg QE/ml, respectively (Rai *et al*., 2013). According to Rasheed *et al*. (2021), propolis is primarily composed of phenolics and flavonoids. However, the composition of propolis can vary significantly depending on factors such as the geographical region it is sourced from and the season of collection (see Table 1 and Table 2).

Table 1. TLC results for the separation of propolis extract using hexane, ethylacetate and acetic acid (60:40:1) as mobile phase

Number of spots	Standards	Rf values
1	Vanillin	0.95±0.026
2	Gallic Acid	0.98±0.028
3	Catechin	0.74±0.026
4	Quercetin	0.35±0.028
5	Rutin	0.06±0.026

Table 2. Results of total phenol, total flavonoid contents, and antioxidant activity of the propolis extract

Parameters	Total phenol (mg GAE/g RS)	Total flavonoid (mg CE/g RS)	IC50% (µg/mL)
Propolis extract	272.45±11.90[a]	8.64±1.79 [b]	330.08±13.35a
Ascorbic acid (Vit. C)	/	/	19.24 ± 0.98b

Data are expressed as means ± SD (n = 3). A comparison between parameters was made using the Tukey test. Rows and columns not sharing a common letter (a–b) differ significantly at LSD ($p < 0.05$) (Tukey test).

The DPPH free radical scavenging test

Figure 1 and Table 2 show that the hydroethanolic extract of propolis has a significant capacity to scavenge the DPPH radical, with an inhibitory concentration of IC50=330.08±13.35 µg/mL but which remains significantly lower than that of ascorbic acid (EC50%= 19.24 ± 0.98 µg/mL). The results obtained in

our study align with those reported by Rasheed *et al*. (2021), who found that the antioxidant capacity of Transylvanian propolis ranged from 1.67 mg/ml to 3.28 mg/ml. However, our findings indicate relatively lower antioxidant activity compared to a separate investigation of Cameroonian propolis. In that study, the DPPH radical scavenging activity of different propolis extracts was examined, and the results revealed varying levels of activity. For example, the hexane extract of Foumban propolis exhibited an IC50 value of 5.6 mg/mL, while the methanol extract of Foumban propolis showed an IC50 value of 1.07 mg/mL. Additionally, the compound 3β-hydroxylanostan-9,24-dien-21-oic acid exhibited an IC50 value of 1.22 mg/mL. These values were compared to the IC50 values of the standard antioxidants gallic acid (0.30 mg/mL) and vitamin C (0.80 mg/mL) (Rasheed *et al*., 2021).

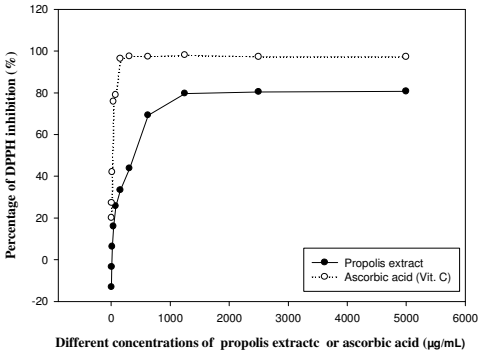

Figure 1. Results of the DPPH radical-scavenging activity of propolis extract compared to ascorbic acid (Vit. C)

Antibacterial activity

Figures 2-4 show inhibition zones (mm) for different propolis extract concentrations, antibiotics, and bacterial strains. Algerian propolis extract (5 mg/mL) had higher inhibition zones than antibiotics against *E. coli*, *K. pneumoniae*, and *S. aureus*. Gentamicin was the most effective against *Bacillus cereus*. At 2.5 mg/mL, propolis extract still showed considerable inhibition. At 1.25 mg/mL, inhibition decreased. Gentamicin and cefazolin were effective against *Escherichia coli* and *Staphylococcus aureus*, respectively. At 0.630 mg/mL, propolis extract had reduced inhibition. Gentamicin remained effective against *Escherichia coli* and *Bacillus cereus*. Colistin showed no inhibition. Overall, higher propolis extract concentrations had larger inhibition zones, while antibiotics, especially gentamicin, were consistently effective. The antibacterial activity observed in our findings surpasses that reported in the study conducted by Rasheed *et al*. (2023), regarding the antibacterial effects of propolis against *S. aureus* and *E. coli*. A study by Sanchez-Moreno *et al*. (1998) found that a concentration of 5.48 mg/ml had moderate zone inhibition ranging from 13.0 ± 0.09 to 15.0 ± 0.11 mm against these two bacteria. They also suggested that *S. aureus* exhibited greater sensitivity to propolis compared to the gram-negative bacterium *E. coli*. A study by Sawaya *et al*. (2004) looked at how propolis affected the growth of *S. aureus* and *E. coli* at different concentrations (30%, 50%, 70%, and 90%). They found that propolis did stop the growth of *S. aureus* colonies, with 90% being the most effective concentration. However, they did not observe any significant effect on *E. coli* colonies. The propolis extract we used in our study demonstrated notable efficacy against the gram-negative strain *E. coli*, contradicting our findings.

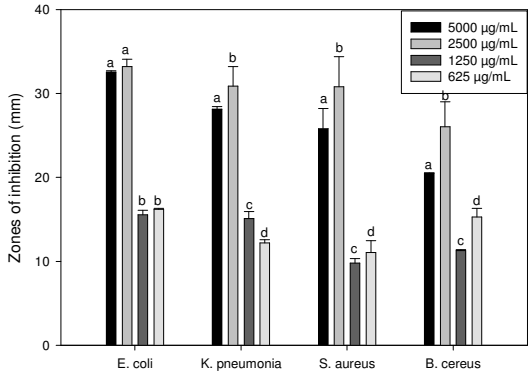

Figure 2. Antibacterial activity results of propolis extract expressed in millimeters of inhibition zone
Data are expressed as means ± SD (n = 3). Comparison between groups was performed using the Tukey test. Bars that do not share a common a common letter (a-d) differ significantly at $p < 0.05$ (Tukey test).

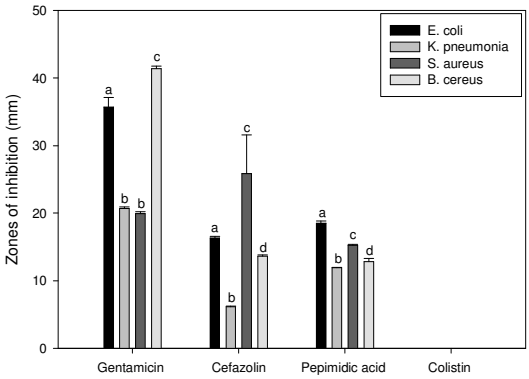

Figure 3. Antibacterial activity results from standard antibiotics expressed in millimeters of inhibition zone
Data are expressed as means ± SE (n = 3). A comparison between groups was performed using the Tukey test. Bars that do not share a common letter (a-e) differ significantly at $p < 0.05$ (Tukey test).

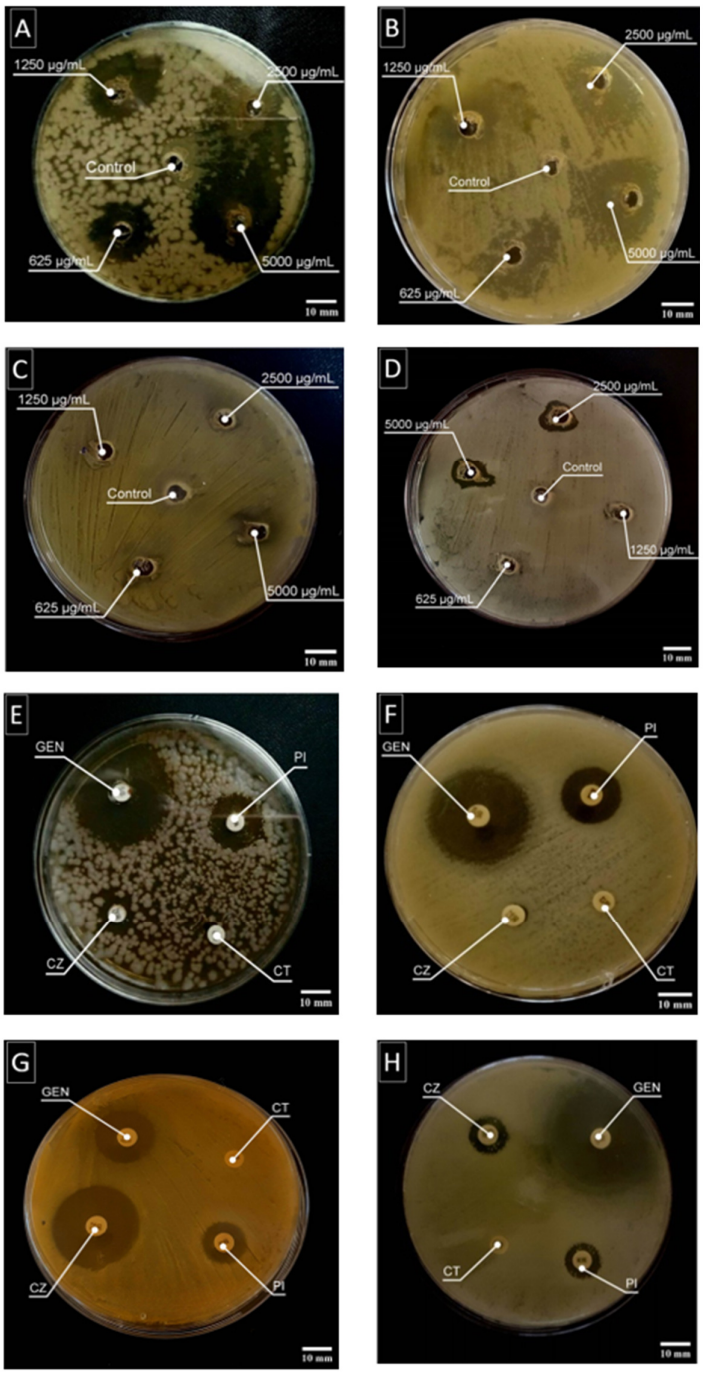

Figure 4-1. Antibacterial activity of standard antibiotics expressed in millimetres of the inhibition zone
The data are expressed as means ± SE (n = 3). A comparison between groups was performed using the Tukey test. Bars that do not share a common letter (a–e) differ significantly at $p < 0.05$ (Tukey test).

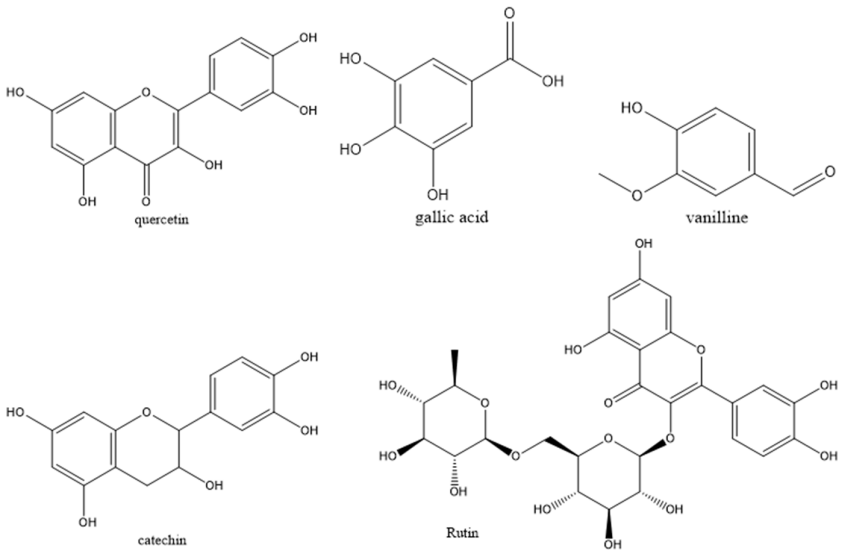

Figure 4-2. Initial modeled 2-D structures of ligands

Molecular docking

Molecular docking studies were carried out to obtain some information about the binding mode of the extract of propolis compounds (que, gal, rut, cat and val) **(**Figure 4) and the multi-target of beta-lactamase (Figure 5)**.**

Before proceeding with molecular docking analysis, it is essential to validate the docking process to ensure its reliability and accuracy. Docking validation can be achieved through re-docking experiments, in which the protein structure remains fixed, and the ligand is re-docked into its original binding pocket. The docked poses are then compared with the crystal structure pose of the ligand, and the comparison is based on the root mean square deviation (RMSD) between the docked and crystal structure poses.

To validate the docking process, a re-docking experiment was performed for each of the three protein structures (1KZN, 6OOH and 1ERM), as shown in Figure 6. The best pose obtained gave RMSD values of 1.6 Å, 0.81 Å, and 1.57 Å, respectively. The RMSD values for all the ligands were less than 2 Å (Schmiemann *et al*. 2010), indicating that the Molegro software was able to reproduce the crystal structure poses of the ligands with high accuracy.

Following the validation of the docking process, molecular docking simulations were performed for extracted compounds of propolis (Table 3).

The resulting data, including docking scores (kcal/mol) and hydrogen bond energies (kcal/mol), were tabulated in Table 3 and Figures S1-S3, in supplementary data for the extracted compounds. These results provide insight into the compounds' binding potential to the protein target and can guide further experimentation and optimization of potential drug candidates.

The analysis of the docking results presented in Table 3 revealed that the ligands exhibited good interactions with the target proteins in the following molecular dock score order (kcal/mol):

KZN1 : rut (-150.051) > cat (-115.487) >que (-109.668) > gal (-74.4997) > val (-72.96[)

6OOH : rut (-156.427) > cat (-98.5546) > que (-94.0593) > val (-68.2721) > gal (-58.9677)

1ERM: rut (-134.252) > val (-51.6841) > cat (-74.7785) > que (-73.2711) > gal (-48.6524)

All the complexes strongly interacted with beta lactamase receptors via hydrogen bonds and steric interactions.

Based on the binding free energy values, the rut (rutin) ligand is more stable than all proteins with a lower MolDock Score, a significant H-bond, and the highest inhibition.

The following targets exhibit hydrogen bonding between ligands and amino acid residues:

- In the 1KZN target, all ligands interact with Asn46, except gal, which forms hydrogen bonds with Val71 and Thr165. Ligand que forms hydrogen bonds with Asn46, Gly77, Asp73, and Val71, resulting in a binding energy of -8.09 kcal/mol. Ligands gal, rut, and cat also engage in hydrogen bonding with amino acid residues, showing similarities in their binding mechanisms.
- In the 6OOH target, all ligands interact with Asn46, Ser130, and Ser237 through hydrogen bonding. The compounds gal, val, que, and cat share a hydrogen bonding pattern with Asn132, Ser130, Ser237, Thr216, and Arg276 amino acids. Ligands queue and rut form additional hydrogen bonds with Asn170 and Pro167, Asn170 and Asn104, and Asn104 and Ser70, respectively.
- In the 1ERM target, all ligands interact with Asn46, Ser70, and Asn132 through hydrogen bonding, ligands que, cat, and rut form additional hydrogen bonds with Ser130, Asn170, and Met69, respectively.

After analyzing the complexes formed between the extracted compounds and the active residues of the beta-lactamase targets, it was observed that all the complexes exhibited negative and low binding energies. This indicates that the binding between the compounds and the target residues is thermodynamically favorable, meaning that the complexes are stable and energetically favorable (Sham *et al*., 1966; Vanommeslaeghe *et al*., 2012; Socha *et al*., 2015; Talla *et al*., 2017; Serairi-Beji *et al*., 2018; Yusof *et al*., 2020; Zakaria *et al*., 2020; Segueni *et al*., 2021; Yadav *et al*., 2023). On the other hand, the low binding energies indicate that the compounds have a strong affinity for the active residues of the beta-lactamase targets, suggesting that they could effectively inhibit the enzyme. Therefore, these results confirm that the proposed compounds have the potential to act as promising beta-lactamase inhibitors, which could be explored further for their use in combating bacterial infections caused by beta-lactamase-producing bacteria.

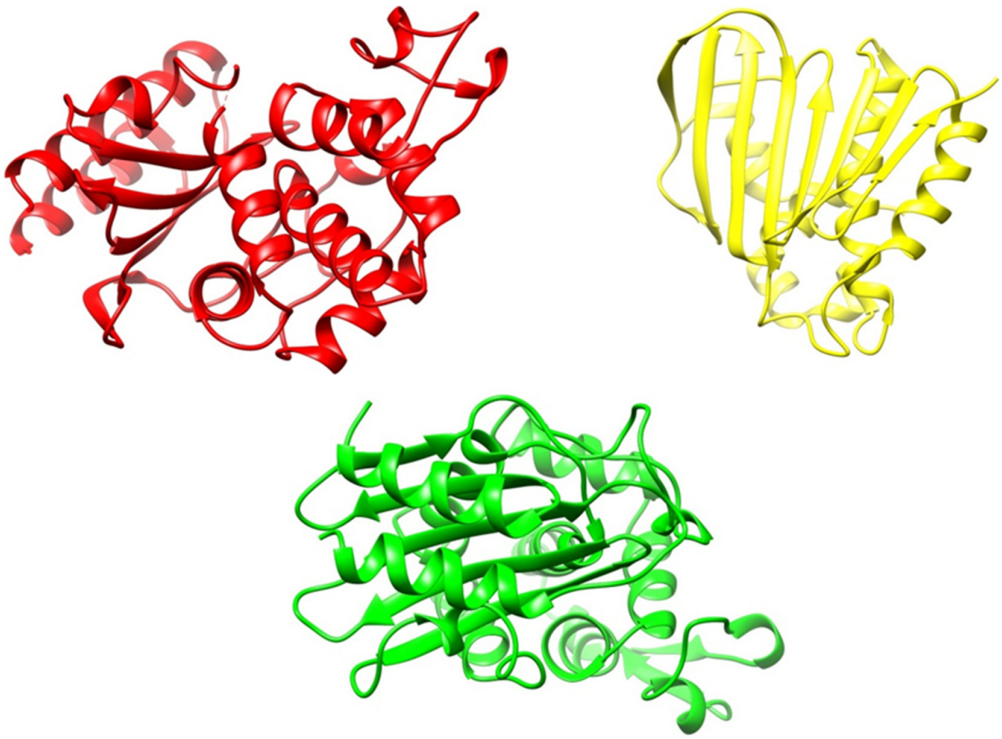

Figure 5. Initial modeled 3-D structures of proteins (1ERM "Red", 1KZN "Yellow", 6OOH "Green")

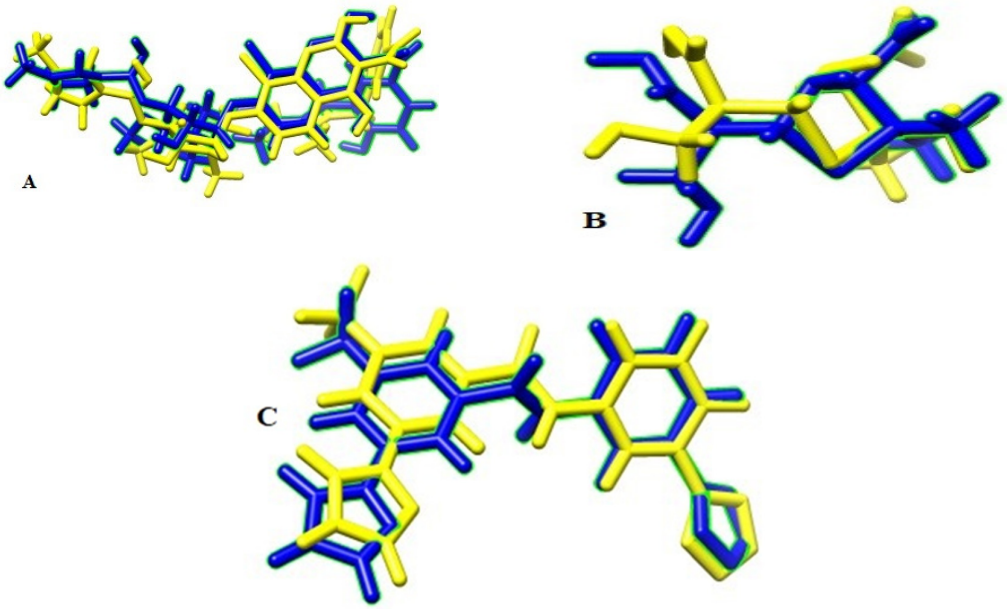

Figure 6. The conformational relationship between the pose and its reference ligand in the inhibitors active site, A: 1KZN target RMSD 1.6 Å; B: 6OOH target RMSD 0.81 Å C: 1ERM target 1.57 Å with the blue representing the original and the yellow representing the docked poses

Table 3. Molecular docking results of extracted ligands with receptors with docking score (kcal/mol) and hydrogen bond (kcal/mol) interactions

Proteins	Ligands	MolDock Score	HBond	Amino acids & H-bond interactions
KZN1	que	-109.67	-8.09	Gly77, Asp73, Val71,Asn46
	gal	-74.50	-6.14	Val71, Thr165
	rut	-150.05	-11.10	Gly77, Arg136, Val71,Asn46
	cat	-115.49	-9.58	Gly77, Thr165, Val71, Val167, Asn46
	val	-72.97	-0.72	Asn46
6OOH	que	-94.06	-12.53	Asn132,Asn170, Ser70, Ser237, Thr216, Arg276
	gal	-58.97	-15.65	Asn132, Ser130, Ser237,Thr216, Arg276
	rut	-156.43	-20.72	Prot167, Asn170, Asn104, Asn104, Ser274, Ser70, Ser130, Thr216, Arg276
	cat	-98.55	-9.23	Asn132, Ser130, Ser237,Thr216, Arg276
	val	-68.27	-8.24	Ser70, Ser130, Thr216, Arg276
1ERM	que	-73.27	-11.32	Asn170, Asn132, Ser70, ser130
	gal	-48.65	-5.01	Ser70, Lys73, Asn132
	rut	-134.25	-8.45	Asn132, ser130, Ser70, Met69
	cat	-74.78	-7.40	Lys73, Asn132, Ser70, ser130
	val	-51.68	-5.07	Ser70, Asn132, Lys73

Molecular dynamics

After performing molecular docking studies of the extracted compounds, MD simulations were carried out for the candidate ligands val, gal, rut, and que, analyzing the stability of the target proteins using RMSD, RMSF, and Rg parameters. The stability of the X-1KZN, X-6OOH, and X-1ERM complexes with ligands was assessed over a period of 250 ns by analyzing the parameters, which are presented in Figures 7-9. The RMSD of the protein-ligand X-target complexes was calculated relative to their initial conformations and plotted in Figure 7. Initially, the data indicate that the complexes of X-1KZN were generally stable after a certain period

of simulation time in an aqueous medium, except for the val-1KZN complex, which stabilized after 140 ns. The average RMSD values for the ligands-1KZN complexes were 0.41, 0.22, 0.48, 0.50, and 0.75 nm for the val, gal, cat, que, and rut ligands, respectively. At the same time, the RMSD values for the 6OOH complexes showed some variation, but they eventually reached stability after 5, 10, 15, and 20 ns of simulation, with the exception of the val-6OOH complex, which took longer to stabilize and did so after 150 ns of simulation. The average RMSD values for val-6OOH, gal-6OOH, cat-6OOH, que-6OOH, and rut-6OOH complexes were 0.32, 0.35, 1.0, 0.23, and 0.67 nm, respectively.

Finally, all complexes in the 1ERM target were stable from the beginning, except for the que-1erm complex, which showed delayed stabilization of up to 160 ns with an average RMSD of 0.23 nm. These results suggest that the molecular dynamics simulations of these structures in an aqueous medium eventually reach stability.

Root mean square fluctuation (RMSF) is a widely used metric to assess the stability and flexibility of protein-ligand complexes. It is similar to root mean square deviation (RMSD) in that it is used to understand the structural changes that occur during molecular dynamics simulations. RMSF calculates the deviation of each coordinate of an individual amino acid residue from its average position during the simulation.

The results of the RMSF calculation presented in Figure 8 show how each coordinate of individual amino acid residues deviated from its equilibrium position during the simulation. By analyzing the RMSF values for each residue, we can identify the flexible and rigid regions in the complexes, which can provide insight into the conformational changes that occur during the simulation. This information is valuable for understanding the stability and interactions of protein-ligand complexes.

From Figure 8, the RMSF values for all ligand targets were low, with values ranging from 0.10 to 0.23 nm. These low values indicate that the ligands maintained their conformations without significant conformational changes during the molecular dynamics' simulations. It was also surprising to note that, despite the presence of different amino acids in the systems, the RMSF values were similar across all systems. This suggests that the ligands were able to maintain stable interactions with the protein targets, regardless of the specific amino acids present.

In addition, it is worth noting that the results obtained from the radius of gyration (Rg) calculation, as illustrated in Figure 9, consistently yielded Rg values less than 1.86 nm across all systems. These Rg values suggest that the protein-ligand complexes retained their shape and conformation throughout the simulation without any significant conformational changes, indicating that the ligands remained stably bound to the proteins and did not induce any noticeable structural alterations. Overall, these findings provide further evidence that the protein-ligand interactions were stable and did not result in any major changes to the protein structure.

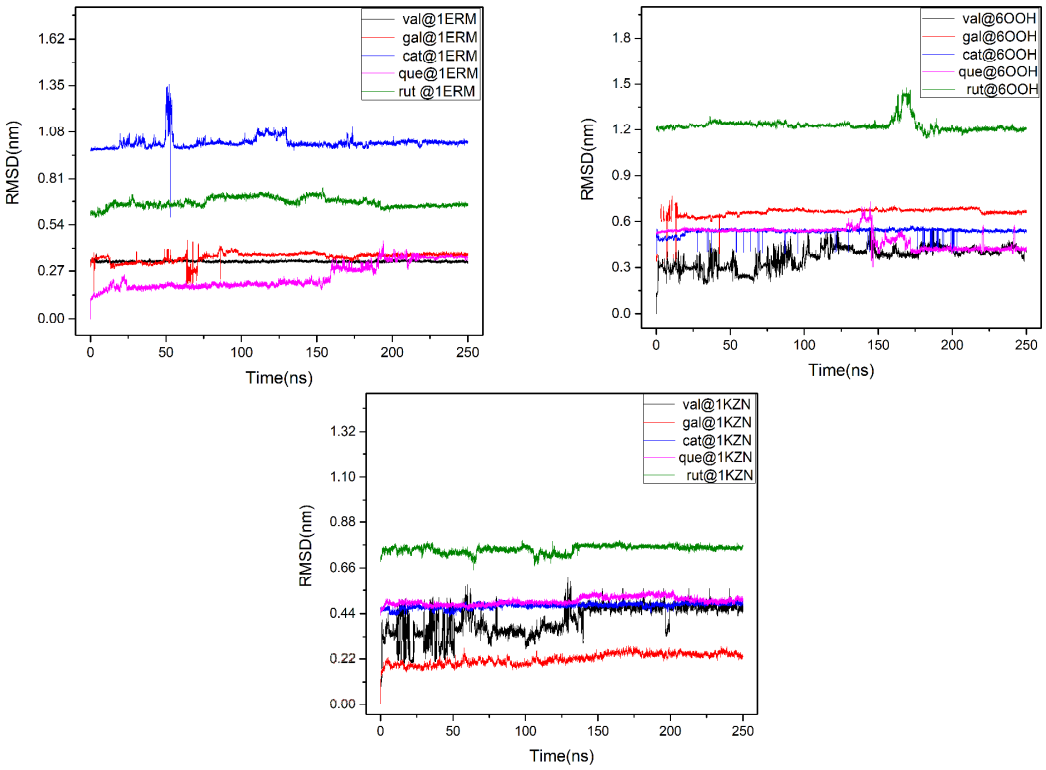

Figure 7. Time evolution of the root mean square deviation (RMSD) proteins of 1KZN, 6OOH and 1ERM with ligands during the 250 ns of simulation by the CHARMM-36 force field

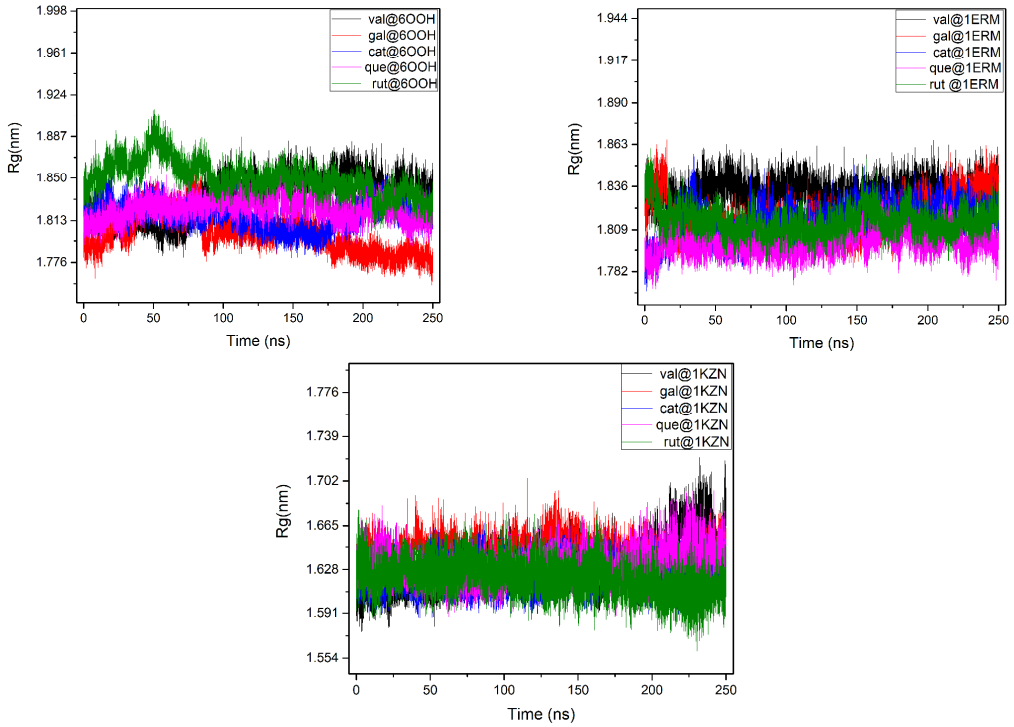

Figure 8. Time evolution of the root mean square fluctuation (RMSF) proteins of 1KZN, 6OOH and 1ERM with ligands during the 250 ns of simulation by the CHARMM-36 force field

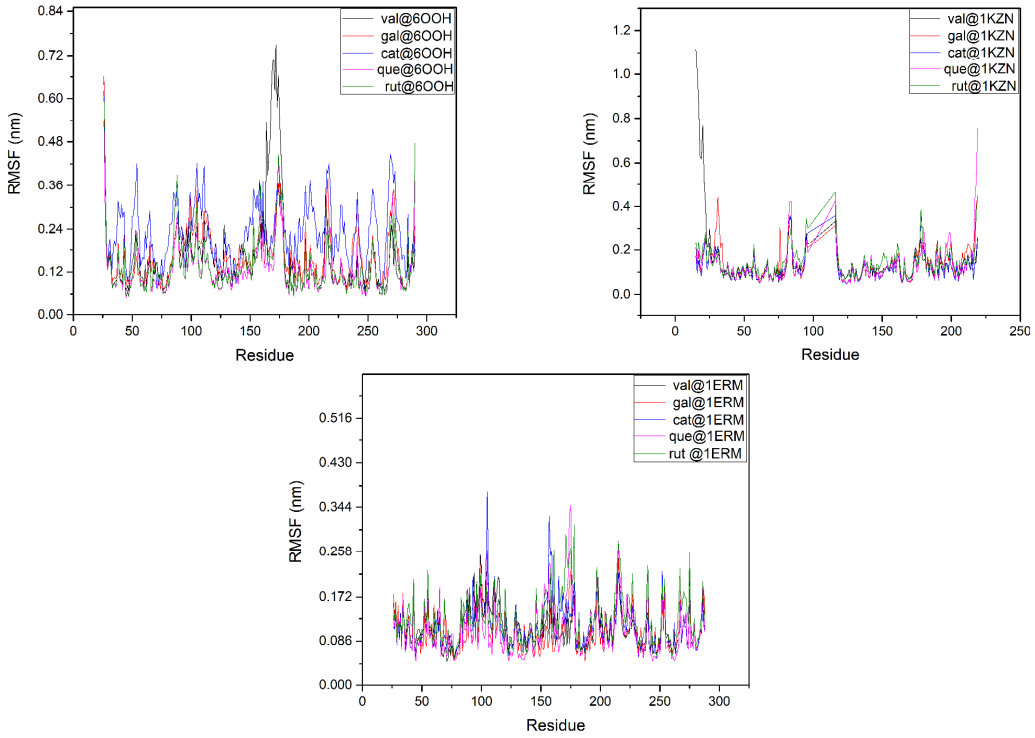

Figure 9. Time evolution of the radius of gyration (Rg) of proteins of 1KZN, 6OOH and 1ERM with ligands during the 250 ns of simulation by the CHARMM-36 force field

Conclusions

This study demonstrates that the increasing bacterial resistance to synthetic antibiotics and the potential risk of urinary tract infections caused by Escherichia coli has led to a search for alternative antibacterial agents. The research suggests that Algerian propolis, due to its high phenolic acid content, may be a suitable substitute for synthetic drugs as it possesses potent antioxidant and antibacterial effects, particularly against gram-negative bacteria. In addition, the study utilized molecular docking and dynamics, to investigate the interaction and the stability of the protein-ligand complexes during a 250 ns for molecular dynamics simulation of the extracted propolis (val, gal, cat, que, and rut) compounds. The results indicate that the complexes remained stable, as evidenced by low RMSD and RMSF values and consistent average Rg values less than 1.86 nm for all systems. The ligands maintained their conformations without significant conformational changes and remained stably bound to the proteins without inducing any significant structural alterations. These findings suggest that the protein-ligand complexes hold promising potential for further study and development.

Authors' Contributions

F. Boudou, A. Guendouzi and A. Belkredar; methodology, M. Rasheed, F. Boudou and A. Guendouzi; planned and conducted the tests, A. Guendouzi and A. Boudou; data analysis and interpretation, and M. Rasheed and F. Boudou prepared the manuscript. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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