Phytochemical Composition and Biological Activities of Unripe *Ricinus communis* Fruits with Protein–Ligand Docking Interaction Study

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Plant-derived metabolites have attracted considerable attention with increasing interest in sustainable, bio-based resources for biological applications. HPLC analysis of unripe *Ricinus communis* fruits revealed a rich profile of phenolic compounds, with hesperetin (42400 µg/g) and chlorogenic acid (14300 µg/g). The extract exhibited antimicrobial activity. It produced inhibition zones of 23 to 31 mm for examined bacteria and 32 mm against *Candida albicans*, with minimum inhibitory concentrations as low as 15.6 µg/mL. Biofilm formation was inhibited by up to 97.8%. Cytotoxic evaluation showed anticancer activity against a human epidermoid carcinoma cell line (A431), with an IC_{50} of 76.8 µg/mL compared to 359 µg/mL for normal HFB4 cells. Antibacterial and anticancer potential of hesperetin (main constituent of unripe *R. communis* fruits) through molecular docking against PBP2a from *S. aureus* (PDB ID: 4CJN) and EGFR kinase domain (PDB ID: 2GS6) were reported. Hesperetin exhibited favorable binding toward both targets, with docking scores ranging from -5.12 to -5.47 kcal/mol for PBP2a and -5.98 to -6.53 kcal/mol for EGFR. Key hydrogen bonding interactions were observed with GLN521 in PBP2a and ASP831 in EGFR, indicating stable ligand-protein complexes. Notably, hesperetin demonstrated stronger binding affinity toward EGFR, suggesting enhanced anticancer potential. Docking-supported hesperetin-rich extract exhibited antimicrobial resistance inhibition and selective anticancer activity.

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INTRODUCTION

Plant bioactive compounds have become increasingly important in modern therapeutics owing to their chemical diversity, biocompatibility, and environmental friendliness (Alsalamah *et al.* 2023; Qanash *et al.* 2023a). These naturally occurring

constituents, often present in low concentrations, exhibit several beneficial biological activities that contribute to public health and disease prevention. Consequently, there has been increasing attention toward optimizing extraction strategies to efficiently recover these valuable metabolites, although no universally standardized method has been established (Abdelghany *et al.* 2019; Al-Rajhi *et al.* 2023a). Increasing resistance to antimicrobial treatments has heightened the need for alternative natural treatment options. Medicinal plants are a vast source of bioactive substances with potential antimicrobial and anticancer effects (Alawlaqi *et al.* 2023; Al-Rajhi *et al.* 2024; Jeong *et al.* 2025). Among these, *Ricinus communis* L. (Euphorbiaceae), commonly designated to as the castor bean plant, is found in tropical areas and has historically been appreciated for its uses in medicine, agriculture, and industry. Different sections of the plant—such as seeds, leaves, roots, and blossoms—have been used for issues including inflammation, tumors, and neurological conditions (Kumar 2017; Abdul *et al.* 2018). Phytochemical studies have shown that *R. communis* contains abundant phenolic compounds, flavonoids, and terpenoids, including quercetin, rutin, gallic acid, and epicatechin, which are recognized for their antioxidant, antimicrobial, and therapeutic effects (Singh *et al.* 2009; Abomughaid *et al.* 2024).

Research findings have revealed that extracts from various plant parts of *R. communis*, *Markhamia obtusifolia*, *R. communis*, *Curtisia dentata*, *Kirkia wilmsii*, *Bauhinia galpinii*, and *Acokanthera oppositifolia* have antibacterial properties against representative bacterial species, including *Staphylococcus aureus*, *Escherichia coli*, and *Streptococcus mutans* (Suurbaar *et al.* 2017), alongside antifungal effects against multiple pathogenic fungi (Dikhoba *et al.* 2019). Additionally, extracts from *R. communis* have demonstrated anticancer effects toward diverse cancer cell lines, such as breast, liver, lung, and cervical cancers, in both *in vitro* and *in vivo* studies (Javanshir *et al.* 2020; Mabasa *et al.* 2021; Herawati *et al.* 2022; Ramothloa *et al.* 2025). Most current research has concentrated on plants' seeds, leaves, and roots, whereas the immature fruits are largely unexamined. Specifically, there have been few thorough studies connecting the phytochemical makeup—particularly phenolic and flavonoid levels—of unripe *R. communis* fruits to their antimicrobial and anticancer properties. This indicates a notable research gap, as the growth stage of plant tissues can greatly affect their chemical composition and biological effectiveness. Simultaneously, molecular docking has emerged as a vital computational method for clarifying the molecular interactions between bioactive substances and biological targets (Al-Rajhi *et al.* 2023a). This method forecasts the binding affinity and interaction patterns of small molecules at active protein sites, offering a mechanistic understanding of their biological functions (Yahya *et al.* 2022). Molecular docking corroborates and elucidates the noted antimicrobial and anticancer effects *via* structure–activity relationships (Al-Rajhi *et al.* 2023b; Qanash *et al.* 2023b). This study explored the biological potential of unripe *R. communis* fruit extract by assessing its antibacterial, antifungal, and anticancer properties. Furthermore, the research emphasized quantitative and qualitative assessments of phenolic and flavonoid compounds, while docking analysis investigated their molecular interactions with specific biological targets. Through phytochemical profiling, biological tests, and computational modeling, this study aimed to address the current literature gap and emphasize the medicinal potential of this lesser-studied plant part as a natural promising provider of bioactive substances.

EXPERIMENTAL

Plant Material Collection and Identification

Unripe fruits of *Ricinus communis* L. were collected at the early developmental stage (green, immature fruits). The plant material was harvested from naturally growing plants in the Al-Baha region, Saudi Arabia, in April 2025. The collected fruits were separated, cleaned to remove dust and debris, and relocated to the laboratory setting in clean polyethylene bags. After collection, the fruits were washed with distilled water and air-dried under shade under ambient temperature to preserve thermolabile bioactive compounds. The dried samples were finely ground using a mechanical grinder and stored in sealed airtight containers at 4 °C pending further analysis.

HPLC Analysis of Phenolic Compounds in Unripe *R. communis* L. Fruit Extract

Phenolic compounds in the unripe *R. communis* L. fruit extract were characterized using High-Performance Liquid Chromatography (HPLC) with an Agilent instrument controlled by ChemStation software. Chromatographic separation was carried out on a reversed-phase C18 column (250 × 4.6 mm, 5 µm particle size). The mobile phase consisted of solvent A (0.1% formic acid in deionized water) and solvent B (acetonitrile), using a gradient elution program: 0 to 5 min (5% B), 5 to 20 min (5 to 25% B), 20 to 30 min (25 to 50% B), followed by re-equilibration to initial conditions, under a flow rate of 1.0 mL/min and a column temperature of 30 °C. The injection volume was 5 µL. Detection was performed using a diode-array detector (DAD) at 280 nm and 320 nm for phenolic acids and related compounds. The extract was prepared by dissolving the dried extract in HPLC-grade methanol, then filtering through a 0.45 µm membrane filter before analysis. Data acquisition and processing were conducted using ChemStation software. Peak identification was based on comparison of retention times and UV spectra with those of authentic standards. Quantification was achieved using external calibration curves. Peaks were manually integrated when necessary to ensure accurate quantification (Bakri *et al.* 2024).

Preliminary Phytochemical Screening

The unripe *R. communis* fruit extract was qualitatively screened for phytochemicals to determine the occurrence of major groups of secondary metabolites using standard qualitative procedures (Harborne 1984; Jean *et al*. 2018). The investigated classes included alkaloids. The extract was acidified, filtered, and treated with Dragendorff's reagent. The development of an orange-red precipitate was considered indicative of alkaloids. To investigate tannins, an aqueous solution of the extract was reacted with ferric chloride solution. Development of a dark green, bluish-green, or purple coloration evidenced the presence of tannins. For phenolic compounds, the extract was mixed with ferric chloride reagent. Development of a blue-black or green coloration suggested the presence of phenolic constituents. To show flavonoids, the extract was treated with alkaline reagent followed by acidification. The appearance of an intense yellow color that diminished after acid addition indicated flavonoids. For terpenoids, the extract was mixed with chloroform and carefully layered with concentrated sulfuric acid. Formation of a reddish-brown interface indicated terpenoid constituents. The intensity of each reaction was recorded qualitatively as absent (−), weak (+), moderate (++), or strong (+++).

Evaluation of Antimicrobial Activity

The antimicrobial behavior of the investigated extract was examined against a selected panel of reference microorganisms, including *Bacillus subtilis* (ATCC 29212), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 8739), *Klebsiella pneumoniae* (ATCC 13883), *Candida albicans* (ATCC 10221), and *Aspergillus terreus* (AUMC 15762). Fresh microbial cultures were prepared under appropriate growth conditions to ensure active and standardized inocula before experimentation. A stock solution was prepared by dissolving the extract in DMSO, followed by serial dilution to obtain the desired working concentrations. DMSO without any extract was included as a negative control to verify the absence of intrinsic antimicrobial effects. Standard antimicrobial agents, Ciprofloxacin for bacterial strains and Fluconazole for tested fungi, were used as reference controls for comparative evaluation (Selim *et al.* 2024).

The primary screening of antimicrobial activity was conducted using an agar diffusion-based approach, where standardized microbial suspensions were uniformly distributed over the solidified agar media surface. Wells (6 mm) loaded with the prepared extract (200 µg/mL) were then introduced onto the inoculated plates and incubated under conditions dependent on the microorganism type. After the incubation period, the diameter of inhibition zones was measured in millimeters to assess the antimicrobial effect and reflect the extent of microbial growth suppression.

To quantify the antimicrobial potency, the minimum inhibitory concentration (MIC) was determined using a broth dilution technique. The technique involved a series of decreasing extract doases (1000 to 31.25 µg/mL) in liquid medium inoculated with the test organisms. Following incubation, the lowest concentration that prevented visible microbial growth was recorded as the MIC. Aliquots from growth-free samples were transferred onto fresh agar plates without the extract to distinguish between inhibitory and lethal effects. The absence of colony formation after incubation was considered indicative of bactericidal or fungicidal activity. The corresponding concentrations were recorded as the minimum bactericidal concentration (MBC) for bacteria or the minimum fungicidal concentration (MFC) for *C. albicans* (Almehayawi *et al.* 2024). Where applicable, the minimum preventive concentration (MPC) was estimated as the lowest concentration inhibiting regrowth upon transfer to extract-free media. Antimicrobial activity was expressed quantitatively using optical density measurements. The percentage growth inhibition was calculated relative to untreated controls according to the standard relationship between treated and control absorbance values.

Biofilm Inhibition Assay

The ability of the tested microorganisms to form biofilms and the inhibitory effect of the plant extract on biofilm development were evaluated using a microtiter plate-based method. Fresh overnight microbial cultures were adjusted to a standardized cell density and inoculated into sterile 96-well flat-bottom microplates containing appropriate growth medium. To assess antibiofilm activity, different quantities of 25, 50, and 75% of detected MBC were added to the wells at the time of inoculation. Control wells included untreated cultures (positive control for biofilm formation) and medium containing DMSO alone (negative solvent control). Following incubation under static conditions (24 to 48 h, depending on the microorganism), the planktonic cells were removed, and the wells were washed with a sterile buffer to eliminate non-adherent cells. The remaining attached biofilm was then fixed and stained using a crystal violet solution. Excess stain was rinsed

off, and the bound dye was solubilized using ethanol (Alsolami *et al.* 2025; Bazaid *et al.* 2025). The absorbance was measured at a suitable wavelength using a microplate reader, providing a quantitative estimate of biofilm biomass. The inhibitory effect of the extract on biofilm formation was calculated relative to the untreated control, as follows:

$$Biofilm\ inhibition\ (\%) = 1 - \left(\frac{Absorbance\ of\ untreated\ cells}{Absorbance\ of\ untreated\ cells}\right) \times 100 \quad (1)$$

Evaluation of Cytotoxic Activity on Cancer and Normal Cell Lines

The inhibitory effect on cell proliferation by the unripe *R. communis* L. fruit extract was investigated using a comparative *in vitro* approach involving a human epidermoid carcinoma cell line (A431) and normal human fibroblast cells (HFB4). Both cell types were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin, and maintained in a humidified incubator at 37 °C with 5% CO_2 atmosphere. For the cytotoxicity assay, cells were seeded in 96-well plates at a density of 1×10^4 cells/well and allowed to adhere overnight. Subsequently, the cells were exposed to varying concentrations of the extract (31.25–1000 µg/mL) and incubated for 24 h prior to performing the MTT assay. After treatment, MTT solution was added and the plates were incubated for an additional 4 h. The resulting formazan crystals were dissolved in DMSO, and absorbance was measured at 570 nm using a microplate reader. Cytotoxicity was determined by comparing the absorbance of treated cells with that of untreated control cells, representing 100% cell viability (Al-Rajhi *et al.* 2022). The half-maximal inhibitory concentration (IC_{50}) values were estimated. The percentage of cytotoxicity was calculated using the following equation:

$$Cytotoxicity\ (\%) = 1\text{-}\left(\frac{Absorbance\ of\ untreated\ cells}{Absorbance\ of\ treated\ cells}\right) \times 100 \quad (2)$$

Molecular Docking Methods

The molecular docking study was conducted using the Molecular Operating Environment (MOE 2019) software (Chemical Computing Group, Montreal, Canada). The crystal structures of PBP2a (PDB ID: 4CJN) and EGFR kinase domain (PDB ID: 2GS6) were retrieved from the Protein Data Bank (www.rcsb.org/pdb). PBP2a from *Staphylococcus aureus* (MRSA) (PDB ID: 4CJN) was selected as the molecular target due to its critical role in mediating β-lactam resistance in *Staphylococcus aureus* and its well-defined active site, making it a validated and widely utilized target in antibacterial drug discovery.

The EGFR kinase domain (PDB ID: 2GS6) was chosen as the anticancer target due to the well-documented overexpression of EGFR in A-431 epidermoid carcinoma cells, its essential role in regulating tumor cell proliferation and survival, and its structurally characterized ATP-binding site suitable for molecular docking studies.

Protein preparation involved the removal of water molecules and co-crystallized ligands, addition of hydrogen atoms, and energy minimization using the MMFF94x force field until the RMS gradient reached 0.01 kcal/mol·Å.

Hesperetin structure was protonated, and energy was minimized.

Docking process: *via* placement: Triangle Matcher, scoring: London dG (Score1), and refinement: GBVI/WSA dG (Score2).

The poses were selected based on: Lowest binding energy, RMSD values (< 2 Å), and interaction with key active site residues.

Statistical Analysis

The experimental findings were statistically processed and presented as mean values with the standard deviation (mean ± SD) derived from three independent replicates to ensure reliability and consistency of the result.

RESULTS AND DISCUSSION

The collected unripe *Ricinus communis* fruit was subjected to HPLC analysis (Fig. 1). The HPLC chromatogram of the extract revealed a complex and diverse composition of phenolic compounds, represented by multiple well-resolved peaks at different retention times (RT). Each peak corresponded to a specific phenolic constituent, indicating the extract's richness in bioactive molecules. The quantitative HPLC analysis confirmed a wide range of phenolic acids and flavonoids with varying concentrations (Table 1). Among the identified compounds, hesperetin was the most abundant (42400 µg/g), followed by chlorogenic acid (14300 µg/g), gallic acid (5060 µg/g), ellagic acid (4630 µg/g), and kaempferol (3780 µg/g). Moderate concentrations were recorded for rutin (1640 µg/g) and coumaric acid (1050 µg/g). Lower amounts were detected for compounds including ferulic acid, vanillin, daidzein, and naringenin. Catechin was not detected in the extract, indicating its absence or presence below the detection limit. The predominance of phenolic acids and flavonoids, particularly hesperetin and chlorogenic acid, suggested a strong contribution of these compounds to the biological activities. The phenolic profile obtained was in strong agreement with previously reported phytochemical investigations of *R. communis* on other plant parts. Earlier work by Ghosh *et al.* (2013) identified key phenolic constituents such as gallic acid, ellagic acid, rutin, quercetin, and kaempferol, along with other compounds including gentisic and ascorbic acids and epicatechin. Similarly, subsequent studies on different plant parts have confirmed the presence of comparable flavonoids and phenolic acids, particularly in the leaves, where rutin, epicatechin, quercetin, and gallic acid were reported as dominant compounds (Rana *et al.* 2016). The detection of these compounds in the current extract—especially gallic acid, ellagic acid, rutin, and kaempferol—supported the consistency of the phenolic fingerprint across different organs of the plant. Although variation in concentration and composition was expected due to differences in plant part, maturity stage, and extraction method, the overall profile reflected a conserved phytochemical pattern. This was further supported by earlier phytochemical screenings, which demonstrated that *R. communis* is rich in flavonoids, tannins, alkaloids, and other secondary metabolites (Rana *et al.* 2012; Srivastava *et al.* 2025). The preliminary phytochemical screening of the unripe *R. communis* fruit extract confirmed the presence of several major classes of secondary metabolites (data not tabulated). Alkaloids, terpenoids and tannins were detected at a moderate level (++). Phenolic compounds showed a strong positive reaction (+++), indicating their high abundance in the extract. Flavonoids similarly exhibited a strong positive response (+++). Generally, the phytochemical profile indicated that phenolic compounds and flavonoids constituted the dominant secondary metabolites in the extract, which is in strong agreement with the HPLC analysis showing high levels of hesperetin, chlorogenic acid, gallic acid, and kaempferol. These findings cooperatively support the role of these ingredients in biological activities.

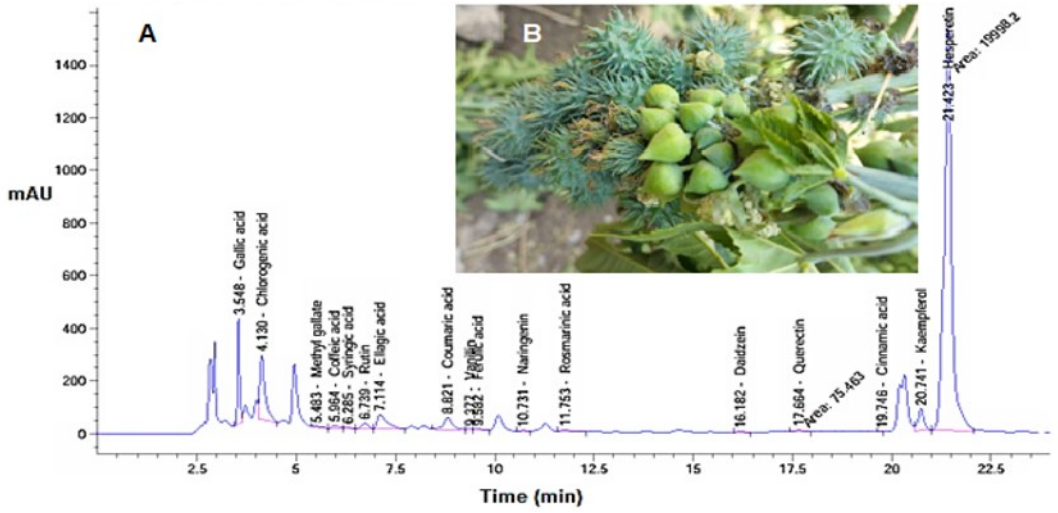


Fig. 1. HPLC chromatographic fingerprint of phenolic constituents (A) in unripe *R. communis* L. fruit (B) extract

The antimicrobial activity of the unripe *R. communis* fruit extract showed effectiveness against both Gram-positive (*B. subtilis* and *S. aureus*) and Gram-negative (*E. coli* and *K. pneumoniae*) bacteria besides fungi (*C. albicans* and *A. terreus*) compared with the standard antibiotic (Fig. 2).

Table 1. Phenolic Compounds in Unripe *R. communis* Fruits Extract

Compound Name	Retention Time (min)	Area	Conc. (µg/g)
Gallic acid	3.548	1558.74	5060
Chlorogenic acid	4.130	2278.16	14300
Catechin	5.483	0.00	0.00
Methyl gallate	5.483	26.92	68
Caffeic acid	5.964	128.33	345
Syringic acid	6.285	59.69	170
Rutin	6.739	250.79	1635
Ellagic acid	7.114	908.01	4630
Coumaric acid	8.821	638.37	1050
Vanillin	9.377	17.43	25
Ferulic acid	9.582	40.50	105
Naringenin	10.731	26.99	117
Rosmarinic acid	11.753	124.66	500
Daidzein	16.182	28.92	70
Quercetin	17.664	75.46	440
Cinnamic acid	19.746	0.97	0.94
Kaempferol	20.741	822.23	3780
Hesperetin	21.423	19998.20	42400

The extract exhibited noticeable inhibitory effects on *E. coli*, *B. subtilis*, *K. pneumoniae*, *S. aureus*, and *C. albicans*, as indicated by clear zones surrounding the wells. In contrast, no inhibition zone was observed against *A. terreus*, suggesting that this fungal strain is resistant to the extract. The inhibition zones produced by the extract were generally comparable to those of the standard treatment, highlighting its strong antimicrobial efficacy. The quantitative data further supported the visual observations. The extract showed inhibition zones ranging from 23 to 31 mm for bacterial strains and reaching 32 mm against *C. albicans* (Table 2). The lowest MIC values (15.6 µg/mL) were registered for *E. coli*, *B. subtilis*, and *C. albicans*, indicating high sensitivity, while *K. pneumoniae* and *S. aureus* required higher concentrations (31.2 µg/mL). Similarly, MBC/MFC values followed the same trend, confirming the potent microbicidal effect.

In terms of antibiofilm activity, the extract exhibited dose-dependent inhibition across all tested microorganisms. At 25% of MBC, inhibition ranged from approximately 64.9% (*S. aureus*) to 89.0% (*E. coli*). Increasing the concentration to 50% and 75% greatly enhanced biofilm inhibition, reaching 97.8% for *E. coli* and over 97% for *B. subtilis*. Biofilm inhibition exceeded 90% at higher concentrations for less sensitive strains such as *S. aureus*. However, *C. albicans* showed a moderate response, with inhibition reaching 84.8% at 50% MBC and slightly increasing to 88.9% at 75% MBC. The microtiter plate image visually confirms the antibiofilm results (Fig. 3). The wells with higher extract concentrations appeared clearer (less purple staining), indicating reduced biofilm formation, while darker wells corresponded with higher biofilm density. Staining intensity gradually decreased as the extract concentration increased, reflecting effective disruption of biofilm formation.

The outcomes of this investigation are strongly aligned with previous reports highlighting the antimicrobial potential of *R. communis*, although activity levels vary on plant part, extraction solvent, and developmental stage. The unripe fruit extract exhibited relatively large inhibition zones (23 to 32 mm) and low MIC values (15.6 to 31.2 µg/mL), indicating high antimicrobial potency. The pronounced antimicrobial and antibiofilm activities of the unripe *R. communis* fruit extract can be ascribed to its rich content of phytochemicals, particularly phenolic ingredients, flavonoids, and fatty acid derivatives. The antimicrobial action of these bioactive constituents is believed to involve multiple cellular targets, including disruption of membrane integrity, enhanced permeability, efflux of intracellular contents, and inhibition of key metabolic enzymes required for microbial survival. Gram-positive bacteria may be particularly susceptible due to the absence of an outer membrane barrier, whereas the activity observed against Gram-negative bacteria suggests that some constituents can penetrate or destabilize the lipopolysaccharide layer. The strong antifungal activity against *C. albicans* may result from interference with membrane sterols and impairment of fungal cell wall synthesis. These results were more pronounced than those reported by Suurbaar *et al.* (2017), where leaf extracts showed MIC values ranging from 3.13 to 25.0 mg/mL and MBC values between 200 and 400 mg/mL.

The present study's substantially lower MIC values suggested that unripe fruits may contain more potent or more bioavailable bioactive compounds than leaves. Furthermore, while Suurbaar *et al.* (2017) documented strong activity particularly against *Pseudomonas aeruginosa* (24 ± 1.8 mm), the present study's results demonstrated comparable or even superior activity against *E. coli*, *B. subtilis*, and *C. albicans*. This highlighted the effectiveness of the tested extract against Gram-positive and Gram-negative bacteria as well as yeast. Similarly, the findings of Rampadarath and Puchooa

(2016) showed moderate antimicrobial activity in mature plant parts, with inhibition zones ranging between 16 and 19 mm for several bacterial strains. In contrast, the larger inhibition zones presently recorded further emphasize the enhanced bioactivity of unripe fruit extracts. This difference may be attributed to variations in phytochemical composition during plant development, as immature tissues are often richer in defensive secondary metabolites such as phenolics and flavonoids. Additional studies on leaf extracts have reported lower inhibition zones. For example, 7 to 13 mm for *S. aureus* and 6 to 9 mm for *E. coli*, with moderate antifungal activity against *C. albicans*, *Aspergillus flavus*, and *Fusarium* species. Compared to these findings, the current results demonstrated markedly higher antibacterial and antifungal efficacy, particularly against *C. albicans* (32 mm). However, the absence of activity against *A. terreus* suggested that antifungal effectiveness may be species-specific, consistent with earlier reports showing variable sensitivity among fungal strains.

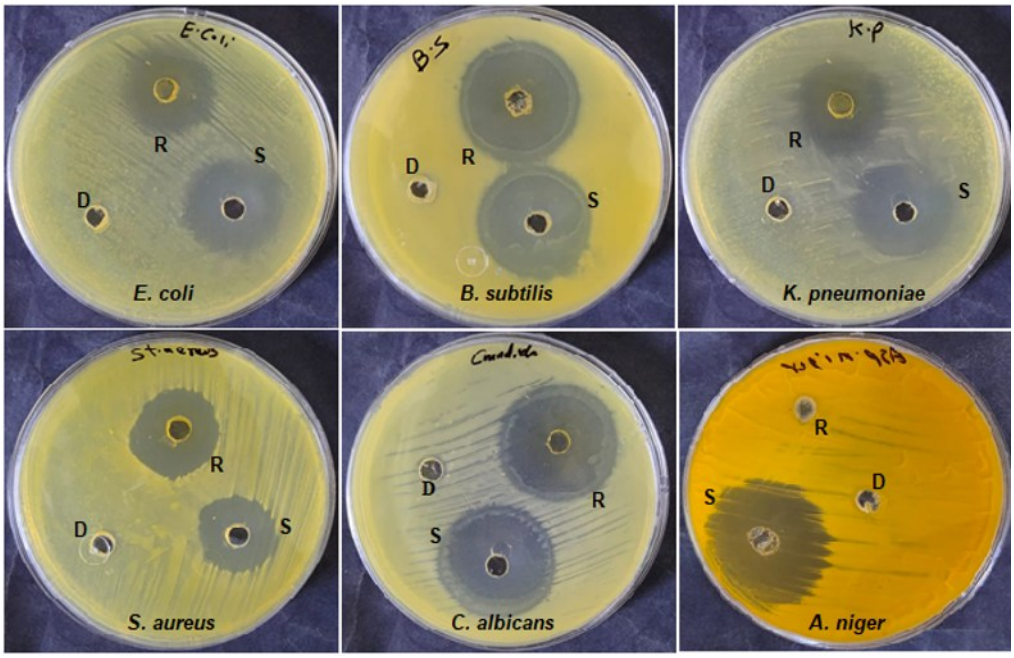


Fig. 2. Comparative antimicrobial efficacy of unripe *R. communis L*. fruit extract (R), standard (S), and DMSO (D) against selected bacterial and fungal pathogens using agar well diffusion assay

Table 2. Antimicrobial Potency and Antibiofilm Efficiency of Unripe *R. communis* L. Fruit Extract Against Selected Microorganisms: Inhibition Zones, MIC/MBC Values, and Biofilm Inhibition Percentages

Assessed microbes	Inhibition zones (mm)		Extract		Anti-Biofilm at different doses of MBC		
	Extract	*Control	MIC	MBC/ MFC	25%	50%	75%
E. coli	25±0.5	25±0.8	15.6	31.25	89.0± 2.1	92.5± 1.3	97.8± 1.8
B. subtilis	31±0.5	30±0.3	15.6	31.25	86.3± 1.3	92.7± 2.0	97.2± 1.3
K. pneumoniae	23±0.3	25±0.4	31.2	62.5	81.2± 3.0	87.8± 1.6	92.9± 3.0
S. aureus	23±0.2	21±0.2	31.2	62.5	64.9± 2.1	83.0± 1.3	92.7± 2.8
C. albicans	32±0.4	33±0.1	15.6	31.25	78.9± 2.0	84.8± 1.2	88.9± 2.0
A. terreus	0.0	34±1.0	--	--	--	--	--

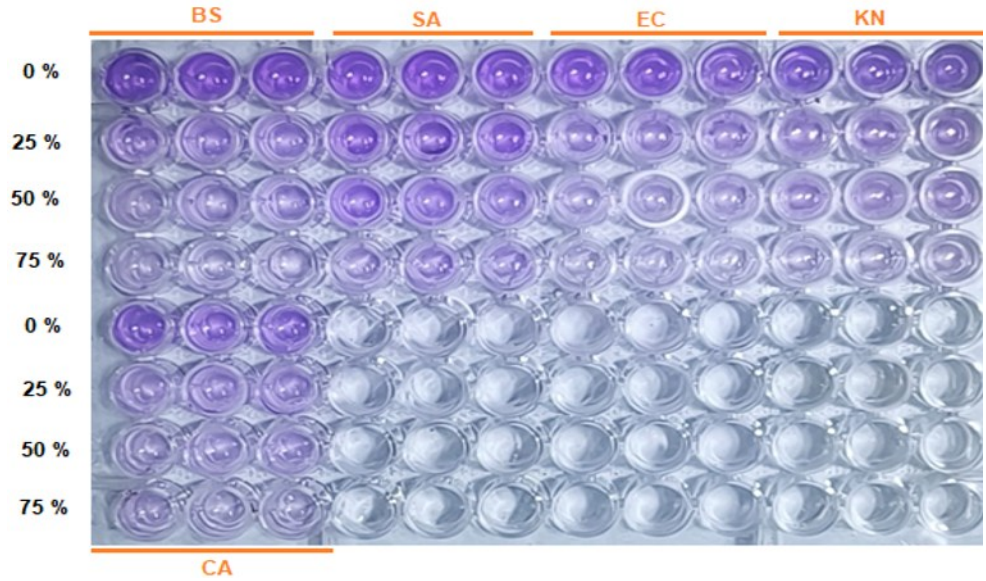


Fig. 3. Dose-dependent antibiofilm activity of unripe *R. communis L.* fruit extract against pathogenic microorganisms *B. subtilis* (BS), *S. aureus* (SA), *E. coli* (EC), *K. pneumoniae* (KN), and *C. albicans* (CA) evaluated by microtiter plate assay

Regarding antibiofilm activity, previous studies primarily focused on planktonic cells. The high inhibition percentages observed in this study (up to 97%) provided an added advantage, as biofilm-associated infections typically resist conventional treatments. This highlights the potential of unripe *R. communis* fruit extract as an antimicrobial agent and a potent antibiofilm compound.

The cytotoxic activity of unripe *R. communis* L. fruit extract was evaluated against A431 and HFB4 across a concentration range of 0 to 1000 µg/mL (Table 3). The results demonstrated a dose-dependent cytotoxic effect on A431 cancer cells. At low concentrations (31.25 µg/mL), the extract exhibited negligible cytotoxicity (0.37 ± 0.05%). However, a sharp increase in cytotoxic activity was observed at 62.5 µg/mL (49.86 ± 0.66%), reaching high levels of inhibition at 125 µg/mL (88.5 ± 2.3%) and exceeding 96% at concentrations of 250 µg/mL and above. Maximum cytotoxicity (97.3%) was achieved at 500 and 1000 µg/mL, indicating strong anticancer potential. In contrast, the extract showed minimal toxicity toward normal HFB4 cells at lower concentrations. Cytotoxicity remained very low up to 125 µg/mL (1.12 ± 0.33%), suggesting good biocompatibility

within this range. A moderate increase in toxicity was observed at 250 µg/mL (49.8 ± 1.5%), which further increased at higher concentrations, reaching 94.3 ± 1.5% at 1000 µg/mL. The calculated IC_{50} values further confirmed the extract's selectivity, with a markedly lower IC_{50} for A431 cells (76.84 ± 0.78 µg/mL) compared to HFB4 cells (358.81 ± 1.93 µg/mL). This indicated that the extract was substantially more toxic to cancer cells than to normal cells. These outcomes suggested that unripe *R. communis* fruit extract possesses promising selective anticancer activity, particularly at moderate concentrations, with relatively low toxicity toward normal cells.

The microscopic images illustrated the morphological alterations induced by unripe *R. communis* L. fruit extract on A431 cancer cells and HFB4 normal fibroblast cells (Fig. 4). In A431 cells, the control group exhibited normal morphology with intact cell membranes and an epithelial-like structure. Progressive morphological changes were observed in a concentration-dependent manner upon treatment. Cells showed minimal changes at 31.2 µg/mL. Early signs of cytotoxicity appeared at 62.5 µg/mL, including slight cell shrinkage and reduced cell density. Clear morphological damage was evident at 125 µg/mL, including cell rounding and detachment. Pronounced cytotoxic effects were noted at elevated concentrations (250 to 1000 µg/mL), characterized by extensive cell shrinkage, membrane blebbing, loss of adherence, and significant reduction in cell population, indicating severe cell death. HFB4 normal cells maintained their typical elongated fibroblast morphology in the control and at lower concentrations (31.2 to 125 µg/mL), with minimal visible damage. At higher concentrations (500 and 1000 µg/mL), noticeable cytotoxic effects were manifested as cell rounding, detachment from the culture surface, and a decline in cell density. However, these effects were less pronounced compared to A431 cells at equivalent concentrations. The results indicated that the morphological observations support the cytotoxicity data, confirming that the extract induces dose-dependent cellular damage with higher sensitivity in cancer cells (A431) than in normal cells (HFB4). *R. communis's* phytochemical richness underpins its reported anticancer potential. The occurrence of flavonoids and phenolic acids in the extract was particularly relevant, as these compounds regulate key cellular pathways involved in cancer progression. Phenolic compounds and flavonoids, including chlorogenic acid, hesperetin, and kaempferol, are known to induce oxidative stress imbalance in cancer cells, leading to mitochondrial dysfunction and activation of the intrinsic apoptotic pathway as mentioned previously (Ahmadi and Shadboorestan 2016; Sezer *et al*. 2019). Previous studies have shown that *R. communis* extracts can reduce tumor aggressiveness by limiting cell migration, invasion, and adhesion, while inducing apoptosis through modulating the Bax/Bcl-2 ratio and activating caspase-dependent pathways (Majumder *et al.* 2019; Megharaj *et al.* 2025). The plant exhibits notable anti-inflammatory activity, which is closely linked to its anticancer effects. Chronic inflammation is a well-established driver of tumor initiation and progression, and targeting inflammatory pathways is considered a key strategy in cancer prevention and therapy. In this context, Hajrah *et al.* (2019) demonstrated that *R. communis* extracts upregulate anti-inflammatory genes, such as TNFAIP6. This is further supported by *in vivo* findings showing a significant reduction in edema, confirming its anti-inflammatory efficacy (Hussain *et al.* 2021). The interplay between these suggests that the *R. communis*'s anticancer effects may be partially mediated through attenuating inflammation and oxidative stress. The high levels of phenolic compounds in the present study, including hesperetin, chlorogenic acid, and kaempferol, likely contribute to a dual mechanism of direct cytotoxic effects on cancer cells and indirect

suppression of inflammation-driven tumor progression. This integrated mode of action emphasizes the therapeutic probable of *R. communis* as a multifunctional natural agent.

Table 3. Cytotoxicity (%) of Unripe *R. communis* L. Fruit Extract against Cancer and Normal Cells

Concentration (µg/mL)	Cytotoxicity (%)	
	A431	HFB4
0	0±0	0±0
31.2	0.37± 0.05	0±0
62.5	49.86± 0.66	0.56± 0.02
125	88.50± 2.3	1.12± 0.33
250	96.81± 1.2	49.79± 1.5
500	97.28± 0.52	60.14± 2.0
1000	97.28± 1.0	94.27± 1.5
IC_{50} µg/mL	76.84 ± 0.78	358.81 ± 1.93

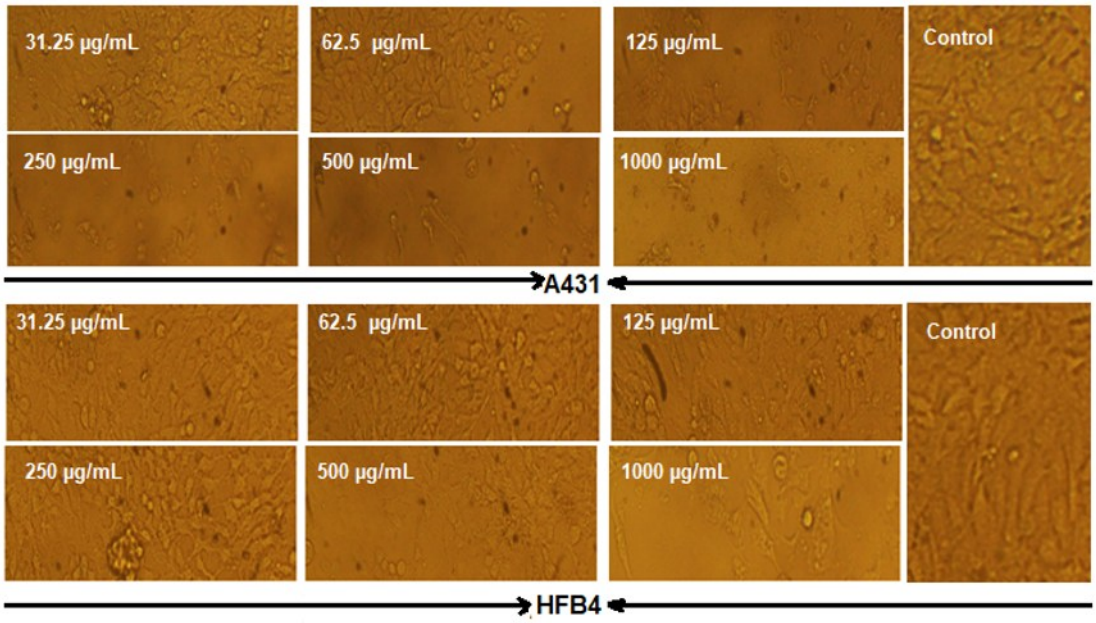


Fig. 4. Morphological changes in A431 and HFB4 cells following treatment with unripe *R. communis* L. fruit extract

The molecular docking analysis of hesperetin (main constituent of unripe R. communis fruits) against PBP2a (PDB ID: 4CJN) and EGFR kinase domain (PDB ID: 2GS6) revealed favorable binding affinities and stable ligand-protein interactions (Fig. 5). Hesperetin exhibited docking scores ranging from -5.12 to -5.47 kcal/mol, with the best binding pose achieving -5.47 kcal/mol and a low RMSD value (0.99 Å) in PBP2a. This indicated good conformational stability (Table 4). The ligand formed key hydrogen bonding interactions. The active site residue GLN521 was at distances of approximately 2.86 to 2.87 Å, suggesting proper accommodation within the binding pocket. Docking against the EGFR kinase domain demonstrated comparatively stronger binding, ranging from -5.98 to -6.53 kcal/mol. The most favorable pose was -6.53 kcal/mol, accompanied by RMSD values between 1.02 and 1.57 Å (Table 5).

The molecular docking analysis revealed that decreasing binding energy scores were directly associated with increased binding affinity and stability of ligand–protein complexes. This enhanced the biological effectiveness of the tested compounds. This agreed with previous studies on phenolic and flavonoid derivatives, which demonstrated pronounced antimicrobial and anticancer activities associated with low docking energies across diverse protein targets (Qanash *et al.* 2022; Qanash *et al.* 2023c; Al-Rajhi *et al.* 2025). A critical hydrogen bond interaction was observed between hesperetin and ASP831 at a distance of 3.07 Å, highlighting stable binding within the ATP-binding site (Table 6). The findings indicated that hesperetin exhibits higher binding affinity toward EGFR than PBP2a, suggesting a potentially stronger anticancer activity while maintaining moderate antibacterial potential. The docking results demonstrate that hesperetin exhibits favorable binding affinity toward both bacterial and cancer-related targets, supporting its multifunctional pharmacological profile. For PBP2a (PDB ID: 4CJN), the observed interactions with GLN521 were particularly weighty, as this residue is located within the transpeptidase active site. Inhibition of PBP2a disrupts peptidoglycan cross-linking, ultimately compromising bacterial cell wall integrity and leading to cell death. This mechanism is well-established for combating methicillin-resistant *S. aureus* (Otero *et al.* 2013).

The moderate docking scores obtained (approximately -5.4 kcal/mol) suggested that hesperetin can occupy the active site, albeit with lower affinity than synthetic β-lactam antibiotics. However, natural flavonoids such as hesperetin use multi-target mechanisms to exert antibacterial activity, including enzyme inhibition and membrane disruption (Cushnie and Lamb 2011). The stronger binding affinity (-6.5 kcal/mol) and interaction of the EGFR kinase domain (PDB ID: 2GS6) with ASP831 highlight the ability of hesperetin to target the ATP-binding pocket of EGFR. This residue plays a crucial role in kinase activity and inhibitor binding. EGFR is highly overexpressed in A-431 cells and drives tumor proliferation and survival signaling pathways (Normanno *et al.* 2006). The stronger interaction with EGFR compared to PBP2a suggested that hesperetin may exert a more pronounced anticancer effect. This observation corroborates previous studies reporting that flavonoids can inhibit tyrosine kinases and induce apoptosis in cancer cells (Yun *et al.* 2008). The docking findings aligned with experimental evidence that natural polyphenols possess dual antimicrobial and anticancer activities, making hesperetin a promising candidate for further pharmacological investigation.

Table 4. Docking Scores and Energies of Hesperetin with the Crystal Structure of PBP2a from *S. aureus* (MRSA) (PDB ID: 4CJN)

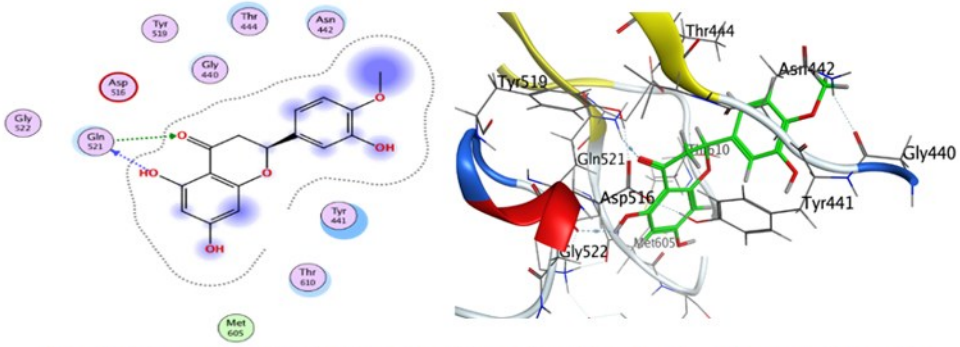
Molecule	S	rmsd_efine	E_conf	E_place	E_score1	E_refine	E_score2
Hesperetin	-5.47	0.99	-26.89	-82.21	-14.02	-29.38	-5.47
Hesperetin	-5.41	2.23	-17.58	-56.07	-11.84	-20.56	-5.41
Hesperetin	-5.31	2.11	-27.01	-70.10	-13.18	-26.41	-5.31
Hesperetin	-5.25	2.09	-28.86	-90.32	-11.96	-26.23	-5.25
Hesperetin	-5.12	1.17	-24.69	-97.77	-12.14	-25.53	-5.12

Table 5. Docking Scores and Energies of Hesperetin with the Crystal Structure of the Active EGFR Kinase Domain (PDB ID: 2GS6)

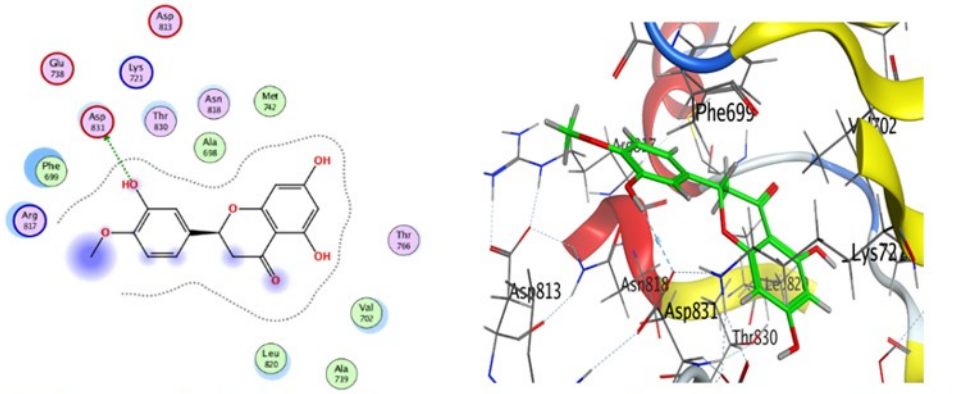
Molecule	S	rmsd_refine	E_conf	E_place	E_score1	E_refine	E_score2
Hesperetin	-6.53	1.16	-24.13	-82.72	-11.39	-36.66	-6.53
Hesperetin	-6.33	1.03	-24.16	-81.27	-11.74	-33.99	-6.33
Hesperetin	-6.13	1.16	-26.22	-105.57	-10.95	-31.44	-6.13
Hesperetin	-6.00	1.21	-24.56	-91.94	-11.56	-34.95	-6.00
Hesperetin	-5.99	1.57	-27.06	-83.50	-11.19	-29.48	-5.99

Table 6. Interaction of Hesperetin with the Crystal Structure of *PBP2a From *S. aureus* (MRSA) (PDB ID: 4CJN) and **EGFR Kinase Domain (PDB ID: 2GS6)

Molecule	Ligand	Receptor	Interaction	Distance	E (kcal/mol)
*Hesperetin	O 3	O GLN 521 (A)	H-donor	2.86	-0.6
	O 2	NE2 GLN 521 (A)	H-acceptor	2.87	-1.0
**Hesperetin	O 6	OD2 ASP 831 (A)	H-donor	3.07	-1.1



2D and 3D diagrams show the interaction between Hesperetin and the active sites of *S. aureus* 4CJN protein



2D and 3D diagrams show the interaction between Hesperetin and the active sites of the EGFR kinase domain 2GS6 protein

Fig. 5. Two- and three-dimensional molecular docking interaction diagrams of hesperetin with target proteins

Study Limitations

Despite the promising phytochemical and biological findings, this study has certain limitations. The investigation was limited to *in vitro* assays and *in silico* molecular docking analysis, which may not fully represent *in vivo* biological behavior. Additionally, only the

unripe fruit extract of *R. communis* was evaluated, without a comparative analysis with other plant parts or different maturity stages.

CONCLUSIONS

1. The high performance liquid chromatography (HPLC) analysis of unripe *R. communis* fruit extract revealed a rich phytochemical profile dominated by phenolic compounds and flavonoids. Hesperetin was recorded as the major constituent (42400 µg/g), followed by chlorogenic acid (14300 µg/g), gallic acid, ellagic acid, and kaempferol, indicating a strong abundance of bioactive secondary metabolites.
2. The extract demonstrated pronounced biological activities. It exhibited strong antimicrobial effects, with inhibition zones up to 32 mm against *C. albicans*, antibiofilm activity reaching up to 97.8% inhibition, and selective anticancer potential against A431 cells with an IC_{50} value of 76.8 µg/mL, confirming its potent multifunctional bioactivity.
3. Hesperetin demonstrated favorable binding interactions with both PBP2a and EGFR targets, indicating its potential as a dual antibacterial and anticancer agent. The compound showed stronger binding affinity toward EGFR, suggesting enhanced anticancer activity compared to antibacterial effects. These findings support the potential of hesperetin as a multi-target bioactive compound.

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