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A study on antibacterial activities of RHRet: a formulation of plant-derived compounds

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Abstract

Background Plant-based compounds have gained significant attention as therapeutic agents for managing infections and facilitating tissue repair. RHRet is a plant-derived product composed of bioactive compounds from the extract of different plant species, like *Phyllanthus emblica*, *Curcuma longa*, *Terminalia chebula*, *Swertia chirayita*, *Azadirachta indica*, *Pterocarpus santalinus*, *Adhatoda vasica*, and *Terminalia bellirica*. These medicinal plants are known for their potent antibacterial and immune-modulatory properties. In this study, we evaluated the molecular interactions between major bioactive compounds present in RHRet and bacterial proteins, followed by evaluation of the antibacterial properties of RHRet, its impact on biofilm formation, and oxidative stress responses.

Methods Molecular docking was studied to evaluate the interactions of different bioactive compounds potentially present in RHRet with PerR, SodA, and KatG proteins. The antibacterial activity was evaluated using disc diffusion, growth curve analysis, minimum inhibitory concentration analysis, and biofilm formation. Hyaluronidase inhibitory effect was also evaluated to study the potential of RHRet in restricting bacterial growth. The oxidative stress response was analyzed through lipid peroxidation, superoxide dismutase and catalase activity, and measurement of total thiol content.

Results Docking analysis revealed strong binding interactions between compounds potentially present in RHRet and PerR, SodA, and KatG proteins. RHRet inhibits *Staphylococcus aureus* growth, biofilm formation, and hyaluronidase activity in a concentration-dependent manner. Additionally, RHRet increases lipid peroxidation levels, thiol content, and reducing superoxide dismutase activity. However, no significant changes have been found in catalase activity.

Conclusion RHRet inhibits *Staphylococcus aureus* growth and biofilm formation and inhibits hyaluronidase activity while modulating oxidative stress and interacting with bacterial proteins. Although RHRet showed promising antibacterial potential, further in vivo studies are necessary to thoroughly evaluate its efficacy and safety profile.

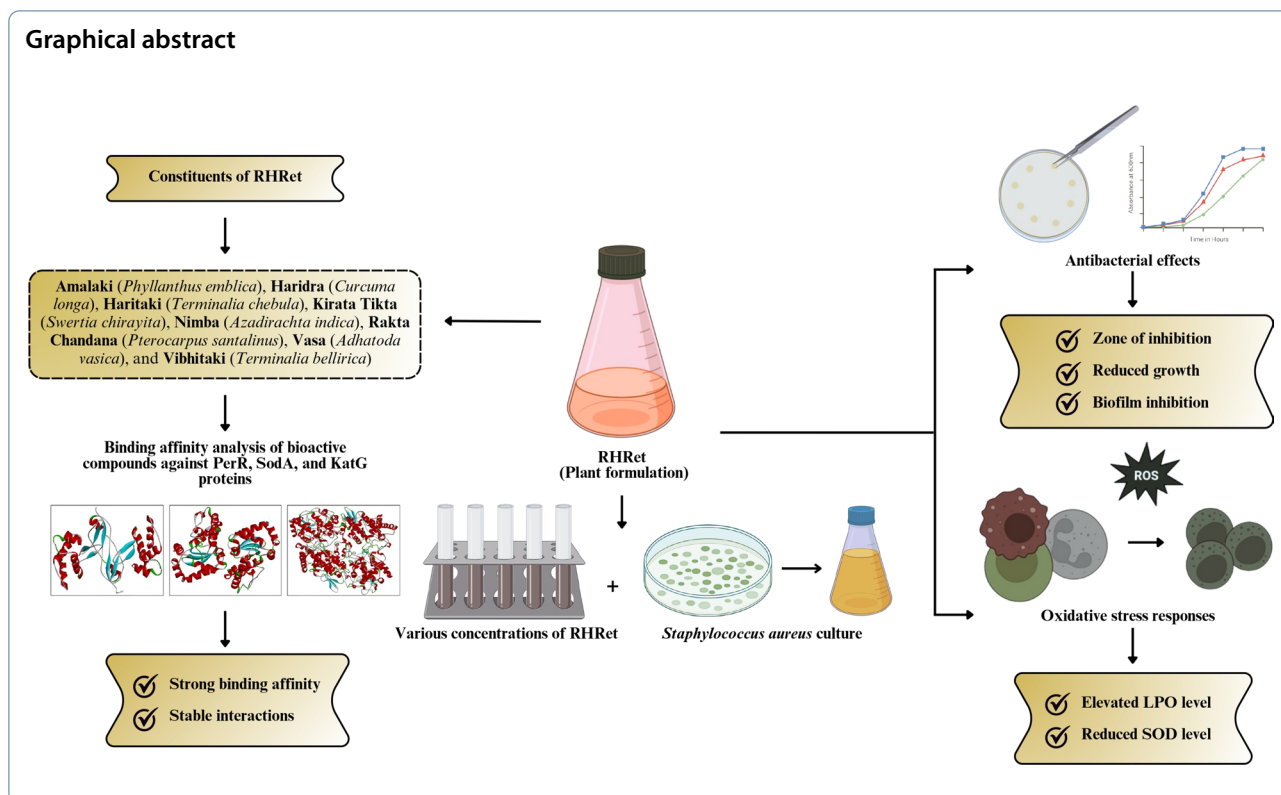
Keywords Antibacterial activity, Bioactive compounds, Biofilm formation, Molecular docking, Oxidative stress, RHRet, *Staphylococcus aureus*

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Graphical abstract



1 Introduction

Infections caused by bacteria and other pathogens are a significant global health concern, particularly in the context of wound healing, where microbial contamination can severely complicate recovery and contribute to prolonged illness [1]. Bioactive compounds like polyphenols, alkaloids, and terpenoids found in natural sources help in combating microbial infections and possess anti-inflammatory and immune-enhancing properties [2–4]. The ability of bioactive compounds to disrupt microbial cell membranes, inhibit biofilm formation, and target specific pathogens without causing significant toxicity to the host highlights their importance in developing alternative therapies against drug-resistant bacteria and offers a promising approach to overcoming microbial resistance and enhancing global health [5, 6]. Recent studies [7–10] further underscore the promising pharmacological activities of plant-derived natural compounds for various diseases. For instance, HPLC-ESI/MS–MS profiling of white pitaya (*Hylocereus undatus*) fruit revealed a wide range of metabolites with significant cytotoxic effects against cervical cancer cells and apoptotic mechanisms [7]. Similarly, *Dolomiaea costus* extract was characterized by its rich bioactive content, which not only induces apoptosis in breast cancer cells but also inhibits viral

replication, offering antiviral and anticancer potential [8]. Plant-derived triterpenoids and polyphenols have shown significant therapeutic potential in breast cancer due to their anti-inflammatory and antioxidant properties, as well as their ability to modulate key pathways involved in carcinogenesis [9, 10]. Previous studies have also shown that plant extracts rich in polyphenols, flavonoids, and alkaloids can inhibit microbial growth by disrupting cell walls and membranes, interfering with DNA replication, inhibiting protein synthesis and enzymatic activity, and generating free radicals, thereby preventing infections and supporting the immune system in combating pathogens [11–13]. Regulating oxidative stress is a key mechanism by which these bioactive compounds exert their antibacterial effects [14]. Oxidative stress is a condition resulting from an imbalance between the production of reactive oxygen species (ROS) and microbial antioxidant defenses, which plays a significant role in microbial pathogenesis and host-microbe interactions [15, 16]. Additionally, recent research suggests that certain combinations of natural compounds exhibit enhanced antibacterial activity compared to individual compounds [17, 18]. For instance, the co-administration of curcumin and quercetin has been shown to exhibit synergistic antibacterial effects by simultaneously

targeting bacterial membrane integrity and oxidative stress pathways [17]. Skroza et al., (2019) reported that combining resveratrol with kaempferol resulted in synergistic antibacterial activity against *Staphylococcus aureus*, *Bacillus cereus*, and *Escherichia coli*, while its combination with rutin was effective against *Staphylococcus aureus*, underscoring the potential of targeted phenolic combinations to enhance antibacterial efficacy [18]. Similarly, various natural compounds with rich ethnopharmacological background have also shown promising antimicrobial properties, such as Haritaki (*Terminalia chebula*), a traditional medicinal plant, and its fruit extract has been reported to exhibit antibacterial activity against bacteria-induced autoimmune inflammation. It also shows antibacterial activity against *Klebsiella pneumoniae* [19]. Haritaki extract is reported to have compounds like corilagin, rutin, chebulinic acid, casuarinin, chebulagic acid, and phenolic acids, which exhibit various properties including antibacterial activity, anti-inflammatory activity, and aid in digestion [20]. The extract of Vibhitaki (*Terminalia bellirica*) shows promise as a source for developing broad-spectrum antibacterial drugs effective against multidrug-resistant (MDR) bacteria [21]. These compounds are non-toxic to mammalian cells and offer additional health benefits due to their high antioxidant activity [21]. Li et al., (2022) have reported that Amalaki (*Phyllanthus emblica*) extract has shown antibacterial activity against gram-positive, negative, and MDR bacteria. It also exhibits anti-inflammatory, immunomodulatory, anti-diabetic, hepatoprotective, and cardioprotective activities [22]. Rakta Chandana (*Pterocarpus santalinus*), or red sandalwood, exhibits potent antibacterial properties, effectively preventing infections by inhibiting microbial growth [23]. The antibacterial activity of leaf and bark extracts of red sandalwood was evaluated against gram-positive bacteria, including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, and *Enterococcus faecalis*, as well as gram-negative bacteria like *Escherichia coli* and *Pseudomonas aeruginosa* [23]. Haridra (*Curcuma longa*), or turmeric, is a potent anti-inflammatory, antimicrobial, and anticancer agent. It accelerates wound closure, reduces healing time, prevents infections, and inhibits bacterial protein synthesis and biofilm formation [24, 25]. Vasa (*Adhatoda vasica*), Kirata tika (*Swertia chirayita*), and Nimba (*Azadirachta indica*) possess strong antimicrobial activity, which interferes with bacterial DNA replication and enzyme function [26–28]. This work was undertaken to understand the potential antibacterial activity of RHRet, a formulation of combination of bioactive compounds. The findings of the present study aim to provide scientific validation for

the ethnopharmacological use of this formulation and may encourage further studies using in vivo models to assess its efficacy and safety.

2 Materials and methods

2.1 Constituents of the novel formulation – RHRet

RHRet is a herbal formulation containing extracts from various plants, including Amalaki (*Phyllanthus emblica*), Haridra (*Curcuma longa*), Haritaki (*Terminalia chebula*), Kirata Tikta (*Swertia chirayita*), Nimba (*Azadirachta indica*), Rakta Chandana (*Pterocarpus santalinus*), Vasa (*Adhatoda vasica*), and Vibhitaki (*Terminalia bellirica*). The formulation was kindly gifted by Retort Pharmaceuticals Pvt. Ltd., Chennai, Tamil Nadu, for evaluating its antibacterial activity. A comprehensive review of existing literature indicates that the plant extracts used in RHRet possess diverse pharmacological properties, suggesting that the formulation likely contains multiple bioactive compounds with significant therapeutic potential [23, 29–35]. The selected bioactive compounds present in RHRet and their respective plant sources are summarized in Table 1.

2.2 Molecular docking studies

Protein structures of Peroxide-responsive repressor (PerR) (PDB ID: 3F8N), Superoxide dismutase A (SodA) (PDB ID: 5N56), and Catalase-peroxidase (KatG) (PDB ID: 1SJ2) (Fig. 1a-c) were retrieved from the Protein Data Bank (PDB) (<https://www.rcsb.org/>). These proteins were chosen due to their essential role in oxidative stress response and bacterial survival. The BIOVIA Discovery Studio software (Discovery Studio Visualizer v.25.1.0.24284) was used for preprocessing, including the removal of heteroatoms, water molecules, and pre-bound ligands from the protein structures. The selected bioactive compounds were retrieved in SDF 3D format from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>). The active site of the proteins is analyzed using the Computed Atlas of Surface Topography of proteins (CASTp) web-server (<http://sts.bioe.uic.edu/castp/index.html?1bxw>), and the Gridbox for the docking was constructed based on the data obtained from CASTp. Molecular docking was performed with the PyRx Virtual Screening Tool (PyRx—Python Prescription 0.8) to assess the binding affinity between the identified ligands and target proteins. The docked complexes were further analyzed using BIOVIA Discovery Studio software, enabling visualization of molecular interactions and interpretation of docking results [36]. The selected bioactive compounds from each constituent plant extract, as listed in Table 1, will be subjected to in silico molecular docking studies with bacterial proteins (PerR, SodA, and KatG) to evaluate their potential interactions and binding affinities.

Table 1 The plant sources and their key bioactive compounds present in RHRet

Plant Source	Bioactive Compounds	Molecular Formula	Molecular Weight (g/mol)	Pub Chem CID	Ref
Haritaki (<i>Terminalia chebula</i>)	Chebolic acid	C ₁₄ H ₁₂ O ₁₁	356.24	71,308,174	[29]
	Chebulinic acid	C ₄₁ H ₃₂ O ₂₇	956.7	72,284	
	Ellagic acid	C ₁₄ H ₆ O ₈	302.19	5,281,855	
Vibhitaki (<i>Terminalia bellirica</i>)	Corilagin	C ₂₇ H ₂₂ O ₁₈	634.5	73,568	[30]
	Gallic acid	C ₇ H ₆ O ₅	170.12	370	
	Luteolin	C ₁₅ H ₁₀ O ₆	286.24	5,280,445	
Amalaki (<i>Phyllanthus emblica</i>)	Geranin B	C ₃₀ H ₂₄ O ₁₁	560.5	44,566,353	[31]
	Phyllantidine	C ₁₃ H ₁₅ NO ₃	233.26	12,314,211	
	Quercetin	C ₁₅ H ₁₀ O ₇	302.23	5,280,343	
Rakta Chandana (<i>Pterocarpus santalinus</i>)	Calocedrin	C ₂₀ H ₁₆ O ₇	368.3	9,999,082	[23]
	Santalin A	C ₃₃ H ₂₆ O ₁₀	582.6	5,490,179	
	Savinin	C ₂₀ H ₁₆ O ₆	352.3	5,281,867	
Haridra (<i>Curcuma longa</i>)	Curcumin	C ₂₁ H ₂₀ O ₆	368.4	969,516	[32]
	Curlone	C ₁₅ H ₂₂ O	218.33	196,216	
	Carvacrol	C ₁₀ H ₁₄ O	150.22	10,364	
Vasa (<i>Adhatoda vasica</i>)	Borneol	C ₁₀ H ₁₈ O	154.25	64,685	[33]
	Morin	C ₁₅ H ₁₀ O ₇	302.23	5,281,670	
	Naringenin	C ₁₅ H ₁₂ O ₅	272.25	439,246	
Kirata Tikta (<i>Swertia chirayita</i>)	Enicoflavine	C ₁₀ H ₁₃ NO ₄	211.21	5,281,564	[34]
	Mangiferin	C ₁₉ H ₁₈ O ₁₁	422.3	5,281,647	
	Swerchirin	C ₁₅ H ₁₂ O ₆	288.25	5,281,660	
Nimba (<i>Azadirachta indica</i>)	Germacrene B	C ₁₅ H ₂₄	204.35	5,281,519	[35]
	Nimbin	C ₃₀ H ₃₆ O ₉	540.6	108,058	
	Nimonol	C ₂₈ H ₃₆ O ₅	452.6	73,356,511	

For each bioactive compound, molecular weight, chemical formula, and PubChem ID are provided

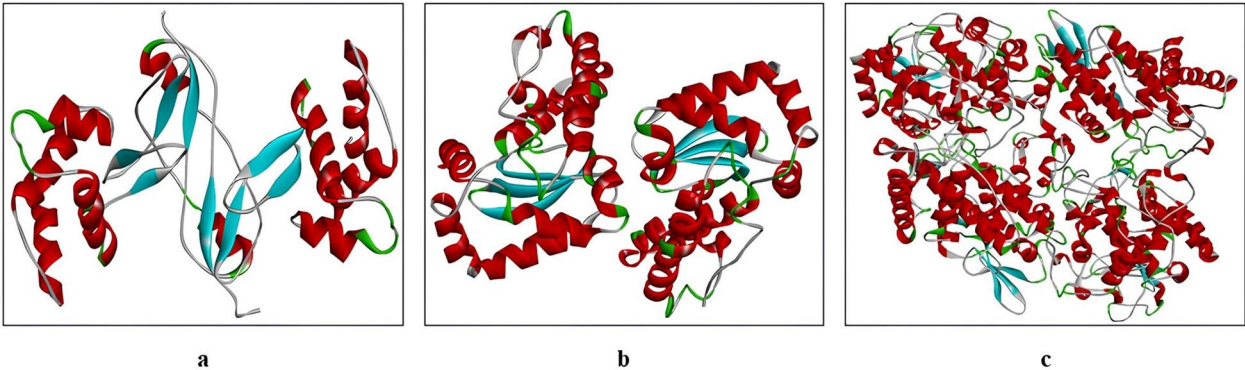


Fig. 1 3D structures of protein (a) PerR (b) SodA and (c) KatG after the removal of heteroatoms, water molecules, and pre-bound ligands to prepare the protein structures for further analysis using BIOVIA Discovery Studio

2.3 Bacterial culture

The *Staphylococcus aureus* (ATCC 25923), was used in the experiments, was a kind gift from the Department of Microbiology, Chettinad Hospital and Research Institute, India. Bacteria were streaked onto a petri plate containing a nutrient agar medium. The plate was

incubated at 37 °C for 24 h, allowing the bacterial colonies to form. The single colony of *Staphylococcus aureus* from the culture was inoculated into a nutrient broth medium and incubated overnight at 37 °C. The bacterial culture was monitored by measuring the optical density (OD) at 600 nm in a 96-well microtiter plate to assess

bacterial concentration. Once the culture reached an OD of approximately 0.5, indicating the exponential growth phase, then the bacteria were sub-cultured for further experimental purposes [37].

2.4 Experimental groups

Staphylococcus aureus was treated with various doses of RHRet (10% (v/v) to 100% (v/v)) to confirm its antibacterial activity. Group 1 is the control group consisting of an overnight culture of *Staphylococcus aureus* at 37 °C without any treatment. Group 2 is the positive control, where the *Staphylococcus aureus* culture was treated with 20 µL of 0.1% (w/v) gentamicin. Groups 3 – 12 involve *Staphylococcus aureus* treated with increasing concentrations of RHRet, ranging from 10% (v/v) to 100% (v/v), and incubated for 2 h at 37 °C. Specifically, in group 3, *Staphylococcus aureus* was treated with 10% (v/v) RHRet, while groups 4, 5, 6, and 7 were treated with 20% (v/v), 30% (v/v), 40% (v/v), and 50% RHRet (v/v), respectively. The concentration of RHRet was further increased in groups 8, 9, 10, and 11, receiving 60% (v/v), 70% (v/v), 80% (v/v), and 90% (v/v) RHRet, respectively, and group 12 was subjected to 100% (v/v) RHRet treatment. To further verify the activity of RHRet in oxidative stress responses, bacteria were treated with 30 mM H₂O₂ to induce oxidative stress and with various concentrations of RHRet. In group 13, *Staphylococcus aureus* was treated with 30 mM H₂O₂ at 37 °C to induce oxidative stress. Based on the result of antibacterial assays, three doses of RHRet, a low dose of 10%, a medium dose of 50%, and a high dose of 100%, were used for further experiments. Hence, in group 14, *Staphylococcus aureus* was treated with 10% (v/v) RHRet + 30 mM H₂O₂, whereas group 15 had 50% (v/v) RHRet + 30 mM H₂O₂, and group 16 was subjected to 100% (v/v) RHRet + 30 mM H₂O₂. After treatment, the antioxidant enzyme activities were tested. The experimental groups are summarized in Table 2.

2.5 Kirby-Bauer disk diffusion assay

The Kirby-Bauer disk diffusion test is a standard method used to assess antibacterial activity. The test was carried out based on the protocol mentioned by Drew et al, (1972) and Yao et al. (2021) with minor modifications [38, 39]. To ensure active bacterial growth, *Staphylococcus aureus* was cultured in 20 mL of nutrient broth to the logarithmic growth phase. Paper discs (4 mm/diameter) soaked with different dosages of RHRet, 10% (v/v), 20% (v/v), 30% (v/v), 40% (v/v), 50% (v/v), 60% (v/v), 70% (v/v), 80% (v/v), 90% (v/v) and 100% (v/v) were placed on the agar plate using sterile forceps. 0.1% (w/v) gentamicin served as a positive control to compare the efficacy of RHRet. The plates were then incubated at 37 °C for 24 h,

Table 2 Various treatment groups tested to evaluate the antibacterial efficacy and oxidative stress response of RHRet in *Staphylococcus aureus*

Treatment Groups	Compositions
1	Control (overnight culture of <i>Staphylococcus aureus</i> at 37 °C)
2	Positive control (<i>Staphylococcus aureus</i> treated with 0.1% (w/v) gentamicin)
3	<i>Staphylococcus aureus</i> + 10% (v/v) RHRet
4	<i>Staphylococcus aureus</i> + 20% (v/v) RHRet
5	<i>Staphylococcus aureus</i> + 30% (v/v) RHRet
6	<i>Staphylococcus aureus</i> + 40% (v/v) RHRet
7	<i>Staphylococcus aureus</i> + 50% (v/v) RHRet
8	<i>Staphylococcus aureus</i> + 60% (v/v) RHRet
9	<i>Staphylococcus aureus</i> + 70% (v/v) RHRet
10	<i>Staphylococcus aureus</i> + 80% (v/v) RHRet
11	<i>Staphylococcus aureus</i> + 90% (v/v) RHRet
12	<i>Staphylococcus aureus</i> + 100% (v/v) RHRet
13	<i>Staphylococcus aureus</i> + 30 mM H ₂ O ₂
14	<i>Staphylococcus aureus</i> + 10% (v/v) RHRet + 30 mM H ₂ O ₂
15	<i>Staphylococcus aureus</i> + 50% (v/v) RHRet + 30 mM H ₂ O ₂
16	<i>Staphylococcus aureus</i> + 100% (v/v) RHRet + 30 mM H ₂ O ₂

The concentrations of RHRet are expressed as volume/volume (v/v), representing the ratio of the solute volume to the total solution volume. *Staphylococcus aureus* without treatment served as the control, while gentamicin at a concentration of 0.1% (w/v) was used as a positive control

and the diameter of the clear zones was measured the following day.

2.6 Growth curve analysis

The growth curve of *Staphylococcus aureus* was assessed based on the method described by Ortega et al., (2015) [40], with slight modifications. *Staphylococcus aureus* was inoculated in the nutrient broth at an initial concentration of 1×10⁶ CFU/mL until it reached the logarithmic phase. After treating with different doses of RHRet ranging from 10% (v/v) to 100% (v/v) in the cultured broth and then incubating overnight at 37 °C, the bacterial growth was monitored by measuring the optical density (OD) at 600 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Rad PR-4100). The *Staphylococcus aureus* cultured without RHRet was the control, while the culture treated with 0.1% (w/v) gentamicin served as the positive control.

2.7 Minimum inhibitory concentration (MIC) determination

To determine the MIC of RHRet in *Staphylococcus aureus*, the broth microdilution method was followed according to the protocol mentioned by Weigand et al, (2008) [41]. RHRet (10% (v/v) – 100% (v/v)) was serially diluted in a 96-well microtiter plate containing nutrient

broth and bacterial suspension. The *Staphylococcus aureus* culture without RHRet was the control, while the culture treated with 0.1% (w/v) gentamicin was the positive control. The plates were incubated at 37 °C for 24 h, and inhibitory effects were assessed by measuring the optical density at 600 nm using an ELISA reader (Bio-Rad PR-4100).

2.8 Determination of biofilm formation

To evaluate biofilm inhibition by RHRet, the Crystal Violet assay was followed according to the protocol defined by Xu et al., (2016) [42] with minor modifications. Briefly, after an overnight culture of *Staphylococcus aureus*, the culture was diluted in a ratio of 1:100 in a fresh nutrient broth, and 20 µL of the diluted culture was inoculated into each well of a sterile 96-well microtiter plate. 100 µL of various concentrations of RHRet, ranging from 10% (v/v) to 100% (v/v), were added to the wells. The *Staphylococcus aureus* culture without RHRet served as the control, while the culture treated with 0.1% (w/v) gentamicin served as the positive control. The plate was incubated at 37 °C for 48 h to facilitate biofilm formation. After 48 h, the plate was washed thrice with 200 µL of PBS to remove the supernatant. Later, 100 µL of 0.01% (w/v) Crystal Violet solution was added to each well. Following a 15-min staining, the wells were rinsed twice with sterile water to remove excess Crystal Violet. Finally, Crystal Violet was dissolved with 95% (v/v) ethanol, and the absorbance was measured at 600 nm using an ELISA reader (Bio-Rad PR-4100).

2.9 Preparation of bacterial cell lysate

Staphylococcus aureus was treated with various concentrations of RHRet (as explained in Table 1) in test tubes. Subsequently, 2 mL from each group was transferred to Eppendorf tubes and incubated at 37 °C for 4 h. It was centrifuged at 8000 rpm for 10 min at a temperature of 4 °C. After discarding the supernatant, the pellet is washed and resuspended in an ice-cold lysis buffer (50 mM Tris–HCl, pH 7.5, 1% Triton X-100, 50 mM Ethylenediaminetetraacetic Acid (EDTA), 0.2 M NaCl) [43]. The tubes were kept in an ice bucket for 30 min, followed by sonicating the mixture thrice for 20 s in cold water. The mixture was then centrifuged for 15 min at 5000 rpm. After collecting the supernatant, the cell lysate was stored at –20°C until further use.

2.10 Determination of hyaluronidase inhibition

To evaluate the hyaluronidase activity in *Staphylococcus aureus*, the hyaluronidase inhibition assay was performed based on the Xu et al., (2024) [44] protocol with slight modifications. 10 µL of hyaluronidase enzyme and 20 µL of CaCl₂ (12.5 mM) were added to the 25µL of cell lysate

and incubated at 37 °C for 20 min. After this, 50 µL of 2.4 mg/mL hyaluronic acid was added as the substrate, and the sample was incubated again at 37 °C for 40 min. Following incubation, 2 µL of 0.4 N NaOH and 20 µL of 0.4 N potassium tetraborate tetrahydrate were added, and the sample was heated in a water bath at 100 °C for 3 min. After cooling, 150 µL of *p*-Dimethylamino benzaldehyde (DMAB) solution was added, and the mixture was incubated at 37 °C for 20 min. Finally, the optical density of the solution was measured at 600 nm, and the inhibitory activity of hyaluronidase was calculated.

2.11 Lipid peroxidation (LPO) activity

The LPO activity was measured using the Zeb et al., (2016) [45] protocol with minor modifications. In this assay, 0.375% (v/v) Thiobarbituric Acid (TBA) and 15% (v/v) Trichloroacetic Acid (TCA) were dissolved in 0.25 N HCl and gently heated to facilitate the reaction. The sample, which contains the cell lysate, is mixed with the prepared TCA-TBA-HCl solution in a 1:2 ratio. This mixture is incubated in a water bath for 15 min to allow the reaction between TBA and lipid peroxidation by-products. After incubation, the mixture is cooled and centrifuged at 1000 rpm for 10 min to separate the supernatant containing the formed colored complex. The absorbance is measured at 535 nm using a spectrophotometer, corresponding to the TBA-MDA complex's intensity.

2.12 Superoxide dismutase (SOD) activity

To assess the activity of SOD, the Wood et al., (2001) [46] protocol was used with slight modifications. A sodium phosphate buffer (0.05 M, pH 8.0) and an EDTA stock solution (0.01 M) were prepared to stabilize the reaction conditions. Pyrogallol, a substrate that auto-oxidizes in the presence of superoxide, was prepared by dissolving 0.27 mM of pyrogallol in 100 mM HCl. Additionally, 0.5 M EDTA was prepared by dissolving 1.46 g of EDTA in distilled water. A mixture of 500 µL sodium phosphate buffer, 20 µL EDTA, 270 µL of pyrogallol, 50 µL of the sample, and 160 µL of distilled water was made. The reaction was initiated by adding pyrogallol, and the change in absorbance due to the oxidation of pyrogallol was measured against Tris–EDTA buffer 3 times at a time interval of 1 min each at 560 nm.

2.13 Catalase activity

The catalase activity was estimated using the spectrophotometric method by Hadwan et al., (2015) [47]. Phosphate-buffered Saline (PBS) was prepared by dissolving 0.26 g of monosodium phosphate and 0.82 g of disodium phosphate in 100 mL of distilled water to maintain a stable pH of 7. For the assay, a reaction mixture of 3 mL was prepared by combining 100 µL of cell lysate, 450 µL

of H_2O_2 (30 mM) as the substrate, and 50 mM (pH 7.0) PBS. Using a spectrophotometer, the breakdown of H_2O_2 by catalase was monitored by measuring the decrease in absorbance at 240 nm for up to 3 min with an interval of 30 s, and the catalase activity was calculated.

2.14 Measurement of total thiol content

The total thiol content was measured using 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), a reagent that reacts with free thiol groups to form a yellow-colored product according to the protocol mentioned by Sedlak et al., (1968) [48] with slight modifications. 360 μL of the prepared cell lysate was mixed with 60 μL of 1% sulfosalicylic acid and 300 μL of 0.1 M sodium phosphate buffer. The mixture was then made up to 1.5 mL with distilled water. Later, 6.6 μL of DTNB was added to the reaction mixture. After a 75-min incubation period, the samples were vortexed, and the absorbance was measured at 412 nm, corresponding to the yellow product formed from the reaction of DTNB with thiol groups.

2.15 Statistical analysis

In all experiments, data from the treatment groups were compared to the corresponding control groups,

and statistical significance was determined using an unpaired t-test. The analysis was performed with GraphPad Prism 8 software (online version). Statistical significance was indicated on the graphs by asterisks, with * representing a p -value < 0.05, ** representing a p -value < 0.01, and *** representing a p -value < 0.001.

3 Results

3.1 Molecular docking analysis

Chebulinic acid exhibited the highest binding affinity across all target proteins, with the strongest interaction observed with KatG (-11.4 kcal/mol), followed by PerR (-10 kcal/mol), suggesting a role in oxidative stress responses. Similarly, Savinin and Calocedrin showed strong binding, particularly with KatG and PerR. Swerchirin displayed moderate affinity, while Enicoflavine exhibited weak interactions across all targets. The 3D docking interactions (Fig. 2, 3, 4) illustrate the binding orientation and molecular interactions observed in the docking analysis. The binding affinities (in kcal/mol) of the selected bioactive compounds with target proteins are summarized in Table 3, highlighting the interactions found.

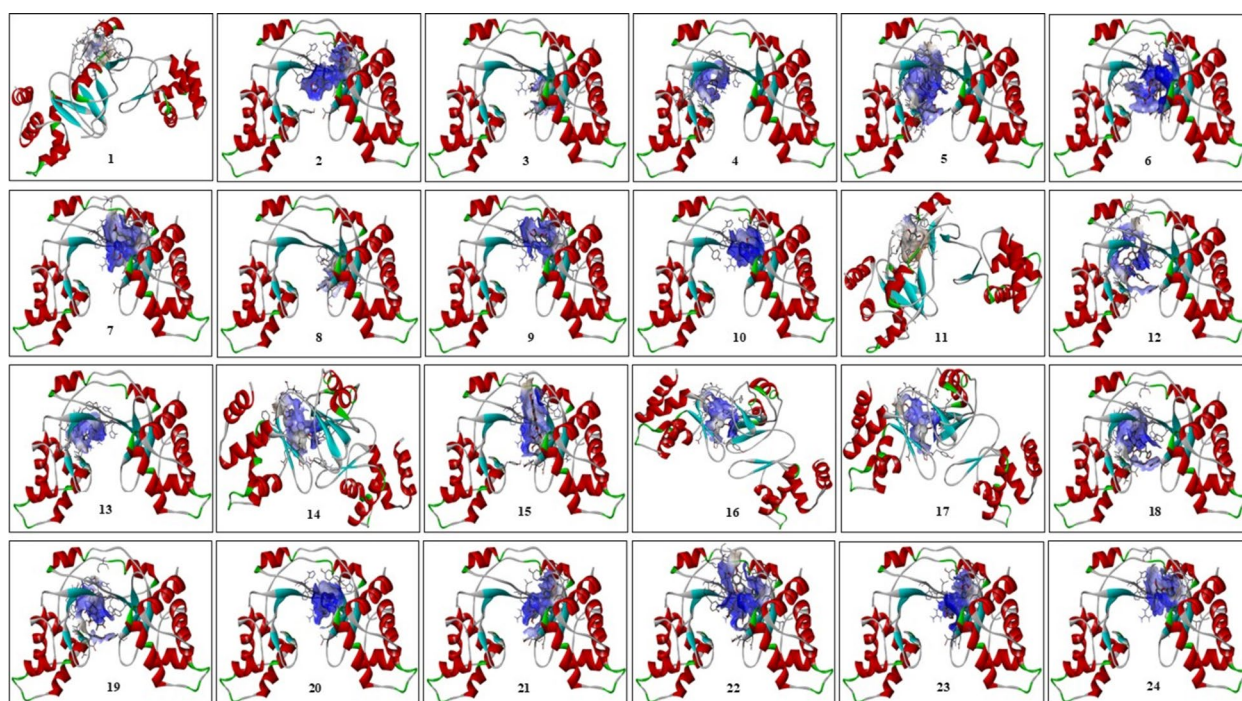


Fig. 2 The molecular docking analysis illustrates the 3D binding interactions between the selected bioactive compounds (1). Borneol, (2). Calocedrin, (3). Carvacrol, (4). Chebulic acid, (5). Chebulinic acid, (6). Corilagin, (7). Curlone, (8). Curcumin, (9). Ellagic acid, (10). Enicoflavine, (11). Gallic acid, (12). Geranin B, (13). Germacrene B, (14). Luteolin, (15). Mangiferin, (16). Morin, (17). Naringenin, (18). Nimbin, (19). Nimanol, (20). Phyllanthidine, (21). Quercetin, (22). Santalin A, (23). Savinin, (24). Swerchirin and the PerR protein

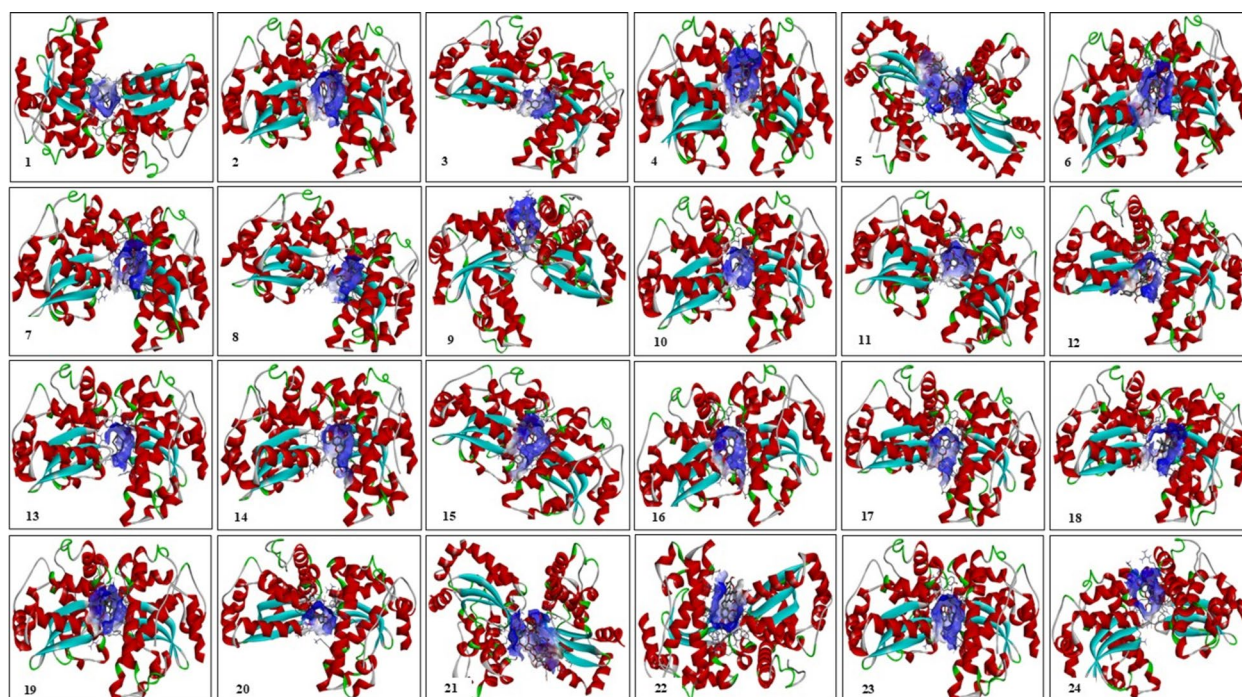


Fig. 3 The molecular docking analysis illustrates the 3D binding interactions between the selected bioactive compounds (1). Borneol, (2). Calocedrin, (3). Carvacrol, (4). Chebulic acid, (5). Chebulinic acid, (6). Corilagin, (7). Curlone, (8). Curcumin, (9). Ellagic acid, (10). Enicoflavine, (11). Gallic acid, (12). Geranin B, (13). Germacrene B, (14). Luteolin, (15). Mangiferin, (16). Morin, (17). Naringenin, (18). Nimbin, (19). Nimanol, (20). Phyllanthidine, (21). Quercetin, (22). Santalin A, (23). Savinin, (24). Swerchirin and the SodA protein

3.2 Antibacterial activity

After 24 h of incubation, the gentamicin control group consistently exhibited clear inhibition zones (Fig. 5a-d). In contrast, no inhibition zones were observed for RHRet concentrations ranging from 10% (v/v) to 30% (v/v) (Fig. 5a). At higher concentrations of RHRet 40% (v/v) to 100% (v/v), small inhibition zones were observed (Fig. 5b-d). These zones showed a gradual increase in size as the RHRet concentration increased, with the largest inhibition zone observed at 100% RHRet (Fig. 5d). The overall inhibitory effects of various treatment groups are summarized in Table 4.

3.3 Growth curve

After 24 h of incubation, absorbance was monitored soon after the treatment, and bacterial growth was subsequently measured at 2, 4, 6, 8, and 24 h (Fig. 6a). No significant changes in growth were observed up to 40% (v/v) RHRet treatment groups. However, a significant decline in growth occurred starting at the 50% (v/v) RHRet concentration treatment group, with a clear decline in the growth curve at the 100% (v/v) RHRet treatment group compared to the untreated control (Fig. 6a).

3.4 Minimum inhibitory concentration (MIC)

The MIC result shown in Fig. 6 b evaluates the inhibitory effect of RHRet on *Staphylococcus aureus* growth. The absorbance at 600 nm reflects the *Staphylococcus aureus* growth, with the control showing the highest absorbance (p -value < 0.01). As the concentration of RHRet increases from 10% (v/v) to 100% (v/v), there is a decline in absorbance, indicating reduced microbial growth. 10% (v/v) and 20% (v/v) RHRet show mild inhibition with no statistical significance compared to the control. While 30% (v/v) to 100% (v/v) RHRet treatment demonstrated significant suppression of growth, with a p -value < 0.05, achieving inhibition at higher concentrations.

3.5 Biofilm formation

The biofilm inhibition shown in Fig. 7 demonstrates a clear concentration-dependent effect of RHRet on biofilm formation. At lower concentrations of RHRet 10% (v/v) and 20% (v/v), there is no significant difference in biofilm formation compared to the control. However, in 30% (v/v) to 60% (v/v) RHRet, a significant reduction in biofilm formation is observed (p < 0.05). The inhibitory effect becomes more pronounced at higher concentrations, particularly from 70% (v/v) RHRet to 100% (v/v),

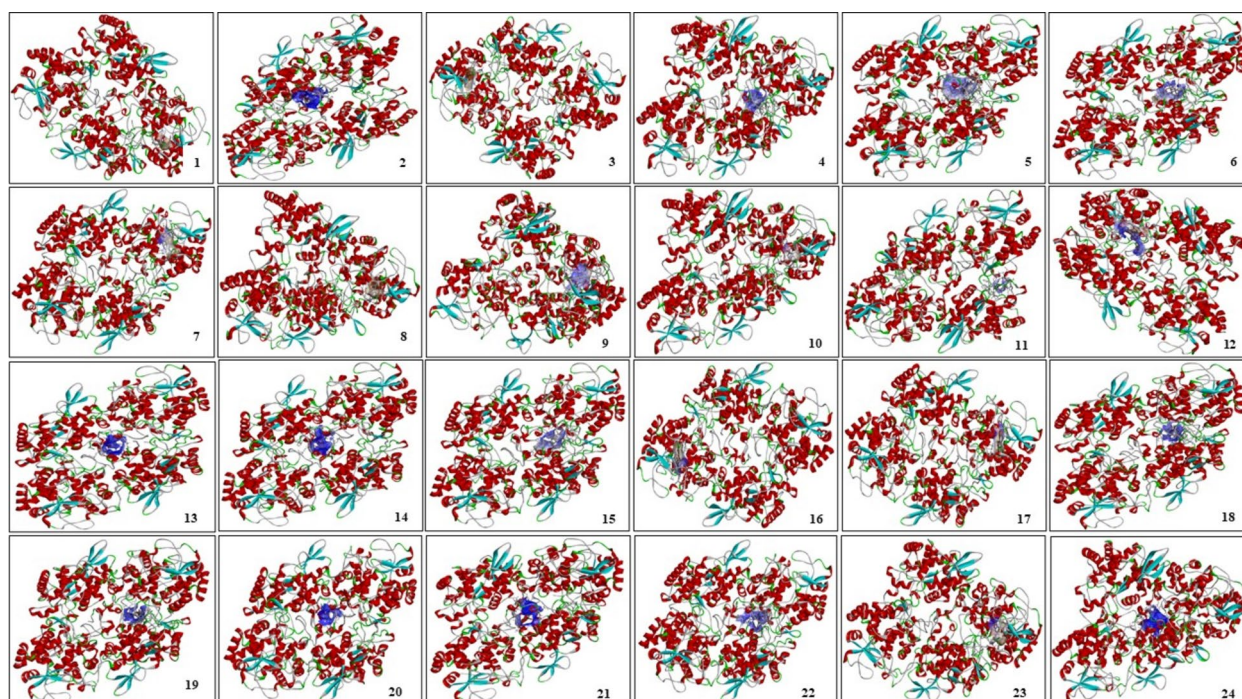


Fig. 4 The molecular docking analysis illustrates the 3D binding interactions between the selected bioactive compounds (1). Borneol, (2). Calocedrin, (3). Carvacrol, (4). Chebulic acid, (5). Chebulinic acid, (6). Corilagin, (7). Curlone, (8). Curcumin, (9). Ellagic acid, (10). Enicoflavine, (11). Gallic acid, (12). Geranin B, (13). Germacrene B, (14). Luteolin, (15). Mangiferin, (16). Morin, (17). Naringenin, (18). Nimbin, (19). Nimonol, (20). Phyllanthidine, (21). Quercetin, (22). Santalin A, (23). Savinin, (24). Swerchirin and the KatG protein

where a more significant reduction ($p < 0.01$) in biofilm production is evident.

3.6 Hyaluronidase activity

No significant change in hyaluronidase activity was observed in *Staphylococcus aureus* upon 10% (v/v) RHRet-treated group compared to the untreated control. However, the hyaluronidase activity significantly decreased in 50% (v/v) and 100% (v/v) RHRet treatment groups (p -value < 0.01 and < 0.05 respectively) (Fig. 8).

3.7 Lipid peroxidation

A significant increase in lipid peroxidation has been observed when *Staphylococcus aureus* treated with H_2O_2 compared to the untreated control (p -value < 0.01). However, no significant changes have been observed in the lipid peroxidation in RHRet 10% (v/v) and 50% (v/v) treatment groups compared to the H_2O_2 -treated group. Lipid peroxidation is significantly increased in *Staphylococcus aureus* + 100% (v/v) RHRet when compared to *Staphylococcus aureus* + H_2O_2 -treated group (p -value < 0.05) and *Staphylococcus aureus* + 100% RHRet + H_2O_2 when compared to *Staphylococcus aureus* + H_2O_2 -treated group (p -values < 0.05) (Fig. 9a).

3.8 Superoxide dismutase activity

The estimation of SOD activity revealed no significant differences in the 10% (v/v) RHRet + H_2O_2 treatment group compared to the *Staphylococcus aureus* + H_2O_2 -treated group. In the 50% (v/v) RHRet + H_2O_2 treatment group, the SOD level significantly decreased with p -values < 0.05 when compared to *Staphylococcus aureus* + H_2O_2 -treated group. Similarly, a more significant decrease in SOD levels was noted in 100% RHRet + H_2O_2 when compared to *Staphylococcus aureus* + H_2O_2 -treated group (p -values < 0.01) and between *Staphylococcus aureus* + 100% RHRet and *Staphylococcus aureus* + H_2O_2 -treated group (p -values < 0.01) (Fig. 9b).

3.9 Catalase activity

The treatment of *Staphylococcus aureus* with RHRet at varied doses showed mild increase in catalase activity after treatment (*Staphylococcus aureus* + 50% RHRet + H_2O_2 and *Staphylococcus aureus* + 100% RHRet + H_2O_2 treated groups). However, the changes were not statistically significant (Fig. 9c).

Table 3 The binding affinities (in kcal/mol) of the selected bioactive compounds of RHRet with bacterial proteins PerR, SodA, and KatG. Lower values represent stronger binding affinities

Ligands	PerR (3F8N)	SodA (5N56)	KatG (15J2)
Binding Affinity (kcal/mol)			
Borneol	−6.3	−5.9	−6.4
Calocedrin	−9.2	−8.8	−9.7
Carvacrol	−5.9	−5.6	−7.6
Chebulic acid	−6.8	−8.3	−8.2
Chebulinic acid	−10	−7.7	−11.4
Corilagin	−8.6	−8.9	−10.2
Curlone	−6.6	−7.1	−8.1
Curcumin	−7.4	−6.8	−9
Ellagic acid	−8.2	−9.3	−9.7
Enicoflavine	−5.2	−6.3	−5.9
Gallic acid	−5.9	−6	−6.5
Geranin B	−8.6	−7.9	−9.4
Germacrene B	−6.6	−6.8	−6.6
Luteolin	−8	−8.1	−9
Mangiferin	−8	−8.4	−9.3
Morin	−8.2	−8	−9.8
Naringenin	−7.8	−7.9	−10.1
Nimbin	−8	−8.2	−7.6
Nimanol	−8.1	−7.7	−8.2
Phyllanthidine	−7	−7.8	−8.5
Quercetin	−8.2	−8.1	−7.4
Santalal A	−8.4	−8.7	−9.7
Savinin	−8.7	−8.9	−11
Swerchirin	−7.5	−7.6	−8

3.10 Measurement of total thiol content

A significant reduction in total thiol content was observed when *Staphylococcus aureus* treated with H_2O_2 compared to untreated control, with a p -value < 0.01 , indicating oxidative stress. Treatment with RHRet at 10% (v/v) and 50% (v/v) with

H_2O_2 partially restored thiol levels, but the changes were not statistically significant. However, a significant increase in total thiol content was noted in 100% RHRet + H_2O_2 when compared to *Staphylococcus aureus* + H_2O_2 -treated group (p -values < 0.05) and *Staphylococcus aureus* + 100% RHRet when compared to *Staphylococcus aureus* + H_2O_2 -treated group (p -values < 0.01) (Fig. 9d).

4 Discussion

Staphylococcus aureus is responsible for a wide range of infections, particularly in wounds and scar sites [49, 50]. The increasing prevalence of drug-resistant strains has necessitated the search for alternative antibacterial agents, including those derived from natural sources [51]. In this study, RHRet, showed significant antibacterial activity in *Staphylococcus aureus*, highlighting its potential as a natural antibacterial agent. The Kirby-Bauer disk diffusion test was conducted to assess the antibacterial activity of RHRet by measuring the size of the zone of inhibition produced at various concentrations. The radius of the zones is directly proportional to the antibacterial properties of the compound [52]. The disc diffusion test showed no inhibition zones for RHRet at 10% (v/v) to 30% (v/v) in *Staphylococcus aureus*, while small zones appeared at higher concentrations 40% (v/v) to 100% (v/v), ranging from 5.3 to 6.6 mm. This zone of inhibition exhibits that RHRet shows antibacterial effects on *Staphylococcus aureus*. However, growth curve analysis revealed a significant reduction in the growth patterns of *Staphylococcus aureus* between the control and the treated groups with higher doses of RHRet. During the lag phase (0–2 h), no significant growth differences were observed across all treatments. In the log phase (2–8 h), bacterial growth increased rapidly in untreated controls and 10% (v/v) to 40% (v/v) RHRet-treated groups. However, growth inhibition became evident at 50% (v/v) RHRet group and above, with the most significant reduction observed at 100% (v/v). The MIC determines the

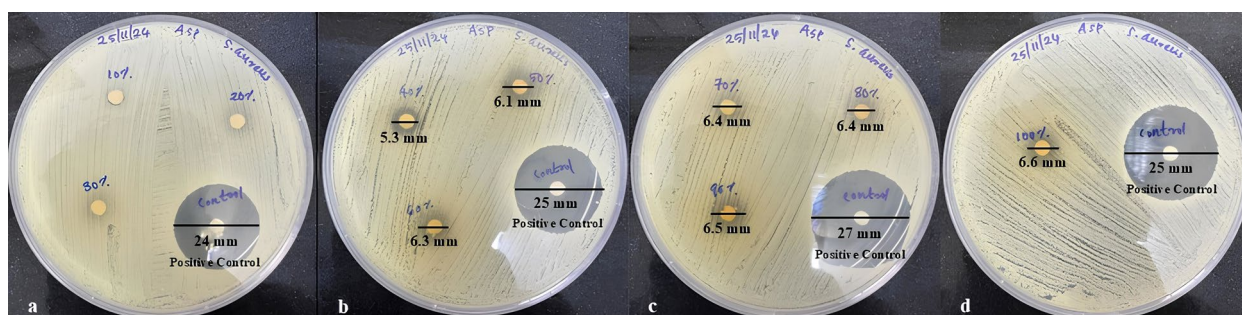
**Fig. 5** Inhibition zones of *Staphylococcus aureus* in the disc diffusion assay using RHRet and gentamicin measured in millimeters (mm) at different concentrations: **a** 10% (v/v)—30% (v/v), **b** 40% (v/v)—60% (v/v), **c** 70% (v/v)—90% (v/v), and **d** 100% (v/v), along with gentamicin

Table 4 The inhibitory zone diameters (in mm) of gentamicin and different concentrations of RHRet treatment in *Staphylococcus aureus*, indicating their antibacterial activity

Treatment groups	Inhibitory zone diameter (mm)
Positive control (<i>Staphylococcus aureus</i> treated with 0.1% (w/v) gentamicin)	25 ± 2
<i>Staphylococcus aureus</i> + 10% RHRet (v/v)	–
<i>Staphylococcus aureus</i> + 20% RHRet (v/v)	–
<i>Staphylococcus aureus</i> + 30% RHRet (v/v)	–
<i>Staphylococcus aureus</i> + 40% RHRet (v/v)	5.3 ± 0.03
<i>Staphylococcus aureus</i> + 50% RHRet (v/v)	6.1 ± 0.02
<i>Staphylococcus aureus</i> + 60% RHRet (v/v)	6.3 ± 0.01
<i>Staphylococcus aureus</i> + 70% RHRet (v/v)	6.4 ± 0.02
<i>Staphylococcus aureus</i> + 80% RHRet (v/v)	6.4 ± 0.01
<i>Staphylococcus aureus</i> + 90% RHRet (v/v)	6.5 ± 0.03
<i>Staphylococcus aureus</i> + 100% RHRet (v/v)	6.6 ± 0.04

Larger inhibitory zones correspond to stronger antibacterial effects

lowest concentration of an antibacterial agent that effectively inhibits the growth of microorganisms [53]. The MIC demonstrates that RHRet inhibits the growth of *Staphylococcus aureus* in a concentration-dependent manner. Lower concentrations of RHRet 10% (v/v) to 40% (v/v) show mild to moderate inhibition, while higher concentrations of RHRet 50% (v/v) to 100% (v/v) result in significant reduction, with maximum inhibition at the highest concentrations. This implies that RHRet may significantly decrease or inhibit the growth of *Staphylococcus aureus*. Biofilm formation is a well-known defense mechanism developed by bacteria, allowing them to

survive in unfavorable conditions [54, 55]. The present study demonstrates that higher concentrations of RHRet (50% (v/v) to 100% (v/v)) significantly reduce biofilm formation compared to the control. RHRet significantly reduced biofilm formation in *Staphylococcus aureus*, potentially by interfering with bacterial adhesion or disrupting the extracellular matrix. This disruption of biofilm formation could enhance the effectiveness of antibacterial properties, making it easier to target and eliminate the bacteria [56]. Hyaluronidase is an enzyme capable of degrading hyaluronic acid, a key component of the extracellular matrix (ECM). Its activity was initially described as a "spreading factor" due to its ability to enhance the spread of pathogens within host tissues by breaking down hyaluronic acid [57]. Among the Staphylococci, *Staphylococcus aureus* and *Staphylococcus hyicus* are the only known species that produce hyaluronidase. Hyaluronidase contributes to bacterial virulence by degrading hyaluronic acid in biofilms, thereby aiding in bacterial invasion and colonization [58]. Inhibiting the hyaluronidase enzyme reduces bacterial virulence [59]. The present study reports that higher concentrations of RHRet, i.e., 50% (v/v) and 100% (v/v), treated groups significantly inhibit the hyaluronidase activity. Therefore, RHRet's ability to inhibit hyaluronidase highlights its potential for infection control [60]. Lipid peroxidation refers to a chain reaction of free radicals in which bacterial reactive oxygen species accelerate the oxidation of fatty acids containing carbon–carbon double bonds, particularly polyunsaturated fatty acids (PUFAs), leading to the formation of lipid peroxides [61]. This process compromises the integrity of the cell membrane and

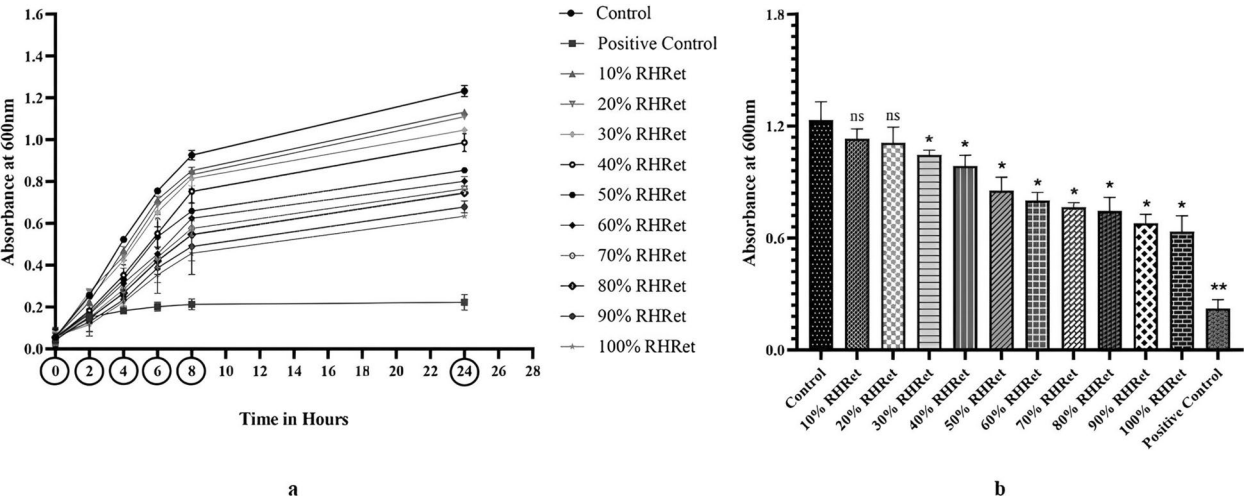


Fig. 6 **a** The growth curve of *Staphylococcus aureus* monitored at 2-h intervals over 24 h of incubation with varying concentrations of RHRet. **b** MIC assay illustrating the dose-dependent inhibitory effect of RHRet on *Staphylococcus aureus*. **p*-value < 0.05 and ***p*-value < 0.01 were considered as statistically significant. ns = non significant

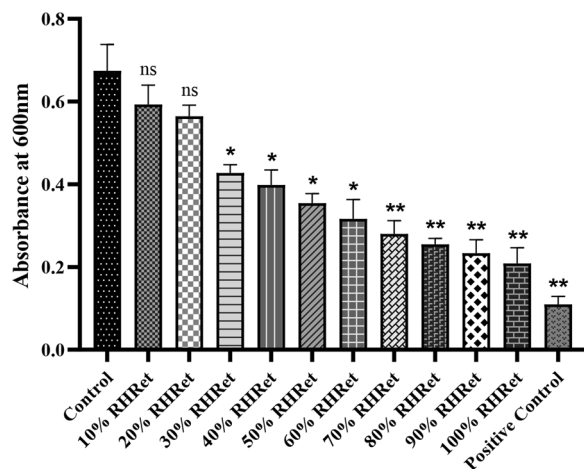


Fig. 7 The biofilm formation assay evaluating the effect of varying concentrations of RHRet on biofilm biomass reduction. * p -value < 0.05 and ** p -value < 0.01 were considered as statistically significant. ns = non significant

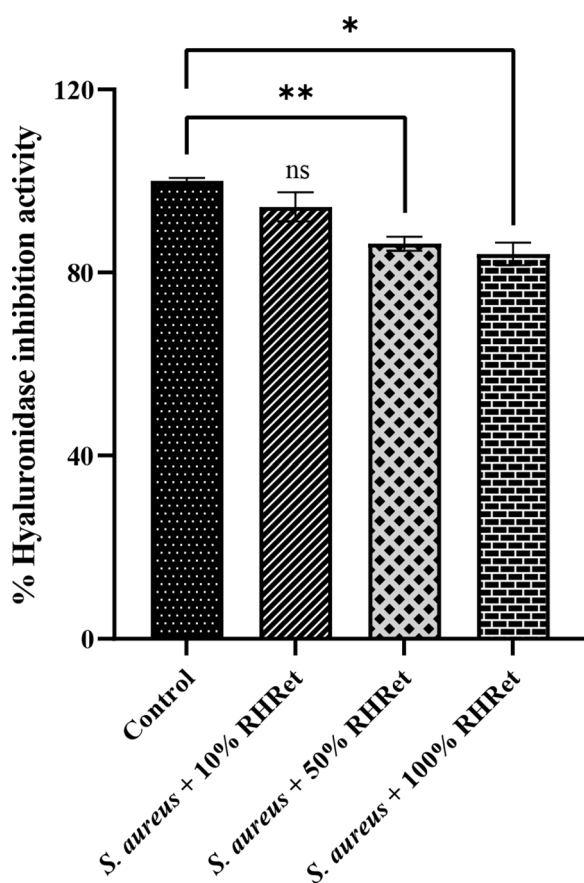


Fig. 8 The hyaluronidase inhibition assay evaluating the effect of RHRet on *Staphylococcus aureus*. * p -value < 0.05 and ** p -value < 0.01 were considered statistically significant. ns = non significant

ultimately causes bacterial cell death [61]. In the present study, lipid peroxidation has been induced by using H_2O_2 in *Staphylococcus aureus* culture. The peroxidation of lipids was elevated even more upon the administration of RHRet. Even in the non-induced group, the lipid peroxidation was significantly higher when treated with RHRet than when *Staphylococcus aureus* was treated with H_2O_2 . The increased lipid peroxidation with higher RHRet concentrations suggests that RHRet induces oxidative stress in *Staphylococcus aureus*. This is further supported by a reduction in SOD expression, indicating diminished bacterial defense against oxidative stress. Superoxide dismutase (SOD) is an enzyme that catalyzes the conversion of superoxide radicals into hydrogen peroxide and oxygen. [62]. In the present study, the reduction of SOD activity in the group treated with a high concentration of RHRet and the significant reduction of SOD activity in the 100% (v/v) RHRet-treated group of *Staphylococcus aureus* confirms that RHRet can reduce the pathogenicity of *Staphylococcus aureus* by compromising its defense mechanism. Catalase is an important enzyme that helps to decompose hydrogen peroxide, a reactive oxygen species, into water and oxygen, thereby protecting cells from oxidative damage [63]. Any alteration in catalase activity impairs an organism's capacity to neutralize H_2O_2 and alter cellular damage caused by ROS [64]. The present study found no significant change in catalase activity in *Staphylococcus aureus* cultures treated with RHRet compared to those treated with H_2O_2 . This suggests that the catalase defense mechanism does not significantly impact RHRet-induced oxidative stress in *Staphylococcus aureus*. Total thiol content refers to the concentration of sulfhydryl groups (-SH) present in proteins and other molecules, which play a vital role in cellular redox balance. Measuring thiol content is commonly used to assess oxidative stress and the antioxidant capacity of cells or organisms [65]. The results show that H_2O_2 treatment significantly reduced total thiol content in *Staphylococcus aureus*, indicating oxidative stress. At lower concentrations of RHRet 10% (v/v) and 50% (v/v), there was partial recovery in thiol levels. However, RHRet at 100% (v/v) significantly increased thiol content in both H_2O_2 -treated and untreated groups, suggesting that higher concentrations of RHRet effectively counteract oxidative stress and restore thiol levels. Furthermore, molecular docking studies were performed [36] to evaluate the interaction of bioactive compounds with the proteins involved in oxidative stress response. Specifically, the binding affinity of the bioactive compounds was assessed with PerR (3F8N), a peroxide-sensing regulator, SodA (5N56), a superoxide dismutase, and KatG (1S2), a catalase-peroxidase enzyme. These enzymes play crucial roles in bacterial defense mechanisms, making them potential targets for

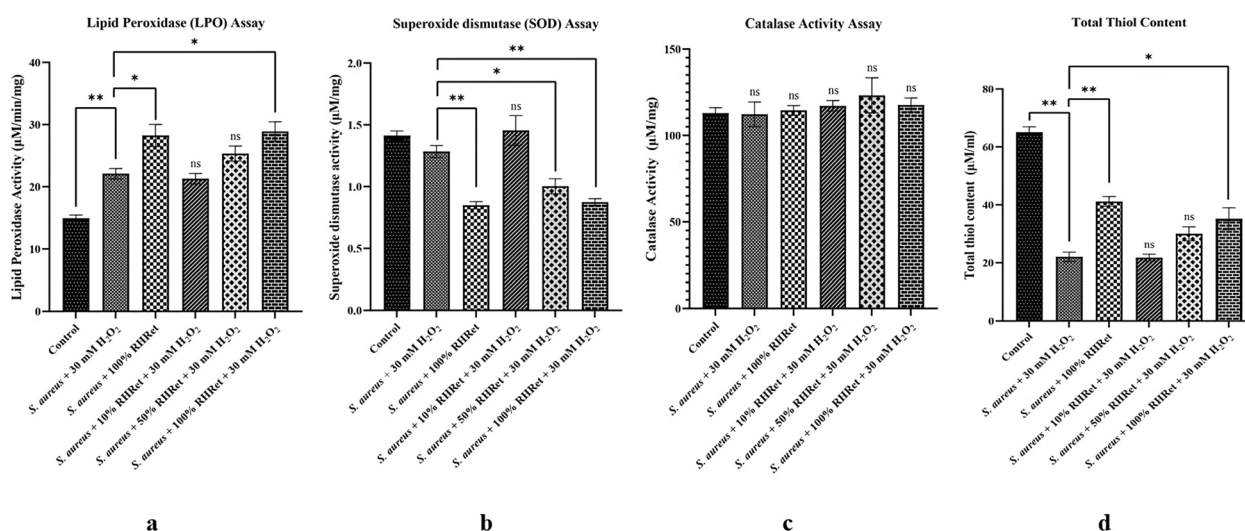


Fig. 9 Oxidative stress responses in *Staphylococcus aureus* treated with RHRet, measured through various enzymatic and non-enzymatic biomarkers: **a** Lipid peroxidation (LPO) activity, **b** Superoxide dismutase (SOD) activity, **c** Catalase activity, and **d** Total thiol content. **p*-value < 0.05 and ***p*-value < 0.01 were considered as statistically significant. ns = non significant

anti-bacterial intervention [66]. Among all interactions, Chebulinic acid exhibited the highest binding affinity across all the studied proteins, with the strongest interaction observed with KatG (−11.4 kcal/mol), suggesting a potential role in oxidative stress responses. Chebulinic acid also showed a strong binding affinity with PerR (−10 kcal/mol). Similarly, savinin also demonstrated strong binding affinity, particularly with KatG (−11 kcal/mol) and SodA (−8.9 kcal/mol). Likewise, Calocedrin also showed a strong binding affinity with KatG (−9.7 kcal/mol) and PerR (−9.2 kcal/mol), which all suggest that they have a key role in oxidative stress responses. Swerchirin showed a moderate binding affinity with all proteins, particularly PerR (−7.5 kcal/mol), SodA (−7.6 kcal/mol), and KatG (−8 kcal/mol). Conversely, enicoflavine exhibited the lowest binding affinities, with values ranging from −5.2 to −6.3 kcal/mol, indicating comparatively weaker interactions with the selected proteins. Overall, the docking analysis suggests that chebulinic acid, calocedrin, savinin, and corilagin may be the most promising bioactive compounds in RHRet due to their high binding affinity with the PerR, SodA, and KatG proteins. The study reveals that the anti-bacterial efficacy of RHRet appears to be more pronounced than that of individual bioactive compounds, indicating a potential synergistic effect. The synergistic effect of bioactive compounds occurs when two or more compounds work together, producing a combined effect that is substantially greater than their individual effects when used separately [67]. These interactions between multiple compounds can enhance the antibacterial potency of RHRet by targeting different bacterial defense

mechanisms simultaneously. Unlike treatments relying on a single compound, which may be less effective due to bacterial resistance, formulations like RHRet take a multi-target approach to enhance their effects. Additionally, the combined effects of these compounds can enhance stability and overall efficacy, making RHRet a promising natural agent for reducing *Staphylococcus aureus* infections.

5 Conclusion and future directions

Numerous medicinal plants, alone or in combination, exhibit promising anti-microbial and antioxidant properties. This study demonstrated that RHRet exhibits significant antibacterial effects in *Staphylococcus aureus*, particularly at higher concentrations. It effectively inhibited bacterial growth, reduced biofilm formation, and inhibited hyaluronidase activity, thereby limiting bacterial adhesion, colonization, and growth. While the current study has established the significant antibacterial potential of RHRet, further in vivo studies could help to evaluate its efficacy and safety within biological systems, providing deeper insights into its therapeutic applicability. Future research may also explore optimized formulations and delivery systems to enhance its stability and efficacy, as well as evaluate its synergistic application with conventional antibiotics in different bacterial infections.

Abbreviations

CASTp	Computed atlas of surface topography of proteins
DMAB	P-Dimethylaminobenzaldehyde
DTNB	5,5'-Dithiobis-2-nitrobenzoic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay

KatG	Catalase-peroxidase
LPO	Lipid peroxidation
MDR	Multidrug-resistant
MIC	Minimum inhibitory concentration
OD	Optical density
PBS	Phosphate-buffered saline
PDB	Protein data bank
PerR	Peroxide-responsive repressor
PUFAs	Polyunsaturated fatty acids
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SodA	Superoxide dismutase A
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid

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Author contribution

AB was involved in the conception and design of the study. ASP, AD, HG, MPK, ERB, and CV performed all the experiments, analyzed the data, prepared the images, and wrote the manuscript. AB and SP wrote parts of the manuscript and critically interpreted the results. AB, SP, and NT reviewed the manuscript. All authors read, reviewed, and approved of the final manuscript. P Ashiq Shibili and Amit Dey are equal contributing authors.

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Data availability

All datas are presented in the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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