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ANTIBACTERIAL EFFICACY OF CLOVE EXTRACT (*SYZYGIUM AROMATICUM*) AGAINST WOUND-ISOLATED STAPHYLOCOCCUS AUREUS AND IN SILICO MECHANISTIC DOCKING OF EUGENOL

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ABSTRACT

This study commenced by the problem of multidrug-resistant *Staphylococcus aureus* (MRSA) infection in wound which was addressed by a study of the antibacterial activity of ethanolic extract of clove (Syzygium aromaticum) and the elucidation of the molecular mechanism by which the major bioactive compound, eugenol, in the extract acts against the tested bacteria by conducting molecular docking simulation. Isolates from the clinical wounds were cultured and sensitivity was determined by agar well diffusion method against the three different concentrations of extracts namely 25mg/dL, 50mg/dL and 75mg/dL. The extract showed significantly strong dose dependent antibacterial activity with the highest conc. 75 mg/dL having greatest inhibition zone (27 mm), 50 mg/dL (23 mm) and 25 mg/dL (18 mm) respectively, while the negative control showed no inhibition effects. To back up the *in vitro* results, molecular docking was carried out on four important protein targets of *S. aureus*, namely penicillin-binding protein 2a (PBP2a, PDB ID: 1VQQ), β-lactamase (PDB ID: 6WGR), dihydrofolate reductase (DHFR, PDB ID: 6PR6), and the CHAP domain of lysin (PDB ID: 11CF). Eugenol exhibited excellent binding affinities to all the targets, having the AutoDock Vina scores ranging from -5.5 to -6.2 kcal/mol. Multiple hydrogen bonds and hydrophobic contacts with β-lactamase (-6.2 kcal/mol) were the most frequent in the interactions. The results obtained here suggest that eugenol is a potent inhibitor of important enzymes in bacterial cell wall biosynthesis, antibiotic resistance and folate metabolism. Overall, the *in vitro* and in silico results suggest that *S. aromaticum* and its active compound eugenol are potential natural therapeutic agents against DRWIs.

Keywords: *Syzygium aromaticum, Staphylococcus aureus*, Beta-lactamase, Molecular docking.

1 INTRODUCTION

In biomedicine, wounds are crucial for the healing of the body, but are also significant sites of microbial entry [1, 2]. Infected wounds have significant difficulties with natural healing, often resulting in non-functional scar tissue, delayed healing, or leading to systemic sepsis, which are extremely detrimental to the wound healing process, whether acute (due to surgical procedures or trauma) or chronic (due to underlying conditions like diabetes) [1, 2].

Staphylococcus aureus is the most important and common skin and soft tissue pathogen [3]. It possesses many virulence factors, such as cytotoxins, cell wall-associated proteins and strong biofilm production which protects the bacteria from immune defense mechanisms and antimicrobial treatment [3]. *S. aureus* infections can range from skin lesions to severe diseases like toxic shock syndrome, bacteremia, and osteomyelitis, causing around 1.1 million deaths due to infection worldwide in 2019 [4].

The emergence of resistant phenotypes, particularly methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA), poses a significant clinical challenge and is threatening the efficacy of conventional antibiotic treatments, increasing healthcare costs, extending hospital stays and driving up mortality and morbidity rates [5]. Therefore, it is urgent to discover novel and effective natural therapeutic options that are effective against infection without the development of resistance [5].

In this context, clove extract (*Syzygium aromaticum*) represents a connection between ancient ethnobotanical uses and modern biomedical research. Clove extract has been used for its healing and medicinal benefits for a long time, and current research suggests that it can enhance wound healing by inhibiting bacterial growth, promoting collagen production and ensuring tissue regeneration [6].

The main bioactive compound of clove oil is eugenol, a phenylpropanoid that is present in 70–90% of the essential oil [7, 8]. The effect of eugenol is a multi-target action in that it causes leakage of cellular components by compromising the integrity of the bacterial membrane, inhibits the synthesis of DNA and proteins, prevents the formation of biofilms, and provides antioxidant and anti-inflammatory activity which helps repair tissues [7]. Moreover it has been proved to be effective against resistant strains, and this makes it a promising candidate for creating novel topical formulations that are specific to infected wounds [9].

The current study aims to elucidate the specific molecular mechanisms underlying the multifaceted inhibitory activities of eugenol against *Staphylococcus aureus*, following the observation of its ability to control the virulence factors, inhibit the development of biofilm and disrupt cell wall integrity of *S. aureus* in previous studies [10]. In silico molecular docking was carried out to understand the binding interactions for eugenol with four important therapeutic targets of *S. aureus*, namely: penicillin-binding protein 2a (PBP2a, PDB ID: 1VQQ), β-lactamase (PDB ID: 6WGR), dihydrofolate reductase (DHFR, PDB ID: 6PR6) and CHAP domain of lysin L1 (PDB ID: 11CF), which are involved in cell wall biosynthesis, antibiotic resistance, folate biosynthesis, and peptidoglycan hydrolysis, respectively.

2 MATERIAL AND METHODS

2.1 Isolation and Identification of *Staphylococcus aureus*

Wound infection samples were taken and streaked onto Blood Agar, MacConkey Agar and Mannitol Salt Agar and incubated at 37 °C for 24 hours. The isolates of *S. aureus* were recovered and kept for additional evaluations for antibacterial assay.

2.2 Preparation of Ethanolic Clove Extract

The buds of *Syzygium aromaticum* were manually separated, washed and shade dried. The dried plant material was powdered, and then macerated in 70% ethanol for 8-10 days, stirring with a sterile glass rod every 10 hours. The mixture was filtered through Whatman No. 1 filter paper (Whatman Ltd., England) and the ethanolic filtrate was condensed with water bath condenser at 40 °C till the filtrate becomes semisolid. The concentrated extract was kept in the refrigerator at 4 °C. To prepare the stock solution, the required amount of dry extract was dissolved in dimethyl sulfoxide (DMSO) to obtain a stock solution of 100 mg/mL, according to [11].

2.3 Assessment of Antibacterial Activity

The antibacterial potency of the *S. aromaticum* extract was evaluated using the agar well diffusion assay, following the methodology of [12]. Bacterial suspensions of *S. aureus* adjusted to a standard concentration (1.5×10^8 CFU/mL) were evenly spread over agar plates using sterile swabs. Four 8 mm wells were punched into each inoculated agar plate with a sterile borer, and the bottom of each well was sealed using a small volume of molten agar. Aliquots (0.04 mL) of the

extract at three distinct concentrations (25, 50, and 75 mg/dL) were loaded into separate wells. The fourth well was the negative control, and was filled with sterile distilled water. After 10 min at room temperature, for allowing some diffusion of extract in the plates, they were then incubated at 37 °C for 24 hours. The presence of clear zone of inhibition around each well indicated the antibacterial efficiency and the diameter of inhibition zone (DIZ) was determined in mm and mean values were calculated.

2.4 *In Silico* Molecular Docking Simulations

Structures of four proteins from *S. aureus* were retrieved from RCSB PDB: penicillin-binding protein 2a (PBP2a, PDB ID: 1VQQ), beta-lactamase (PDB ID: 6WGR), dihydrofolate reductase (DHFR, PDB ID: 6PR6), and the CHAP domain of lysin L1 (PDB ID: 11CF). To remove the crystallographic water molecules, the co-crystallised ligands and unwanted ions, the polar hydrogen atoms were added and the Gasteiger partial charges were calculated during the protein preparation in Discovery Studio Visualizer v21.1.

To define the search space within the active binding site (Pocket C1) of each target receptor, specific grid center coordinates and box sizes were configured as detailed in Table 1.

Table 1: Grid box center coordinates and spatial dimensions applied for molecular docking against *S. aureus* protein targets.

Receptor Target	PDB Entry	Active Site	Grid Center (x, y, z) (Å)	Box Size (x × y × z) (Å)
β-lactamase	6WGR	Pocket C1	19, 29, 43	35 × 34 × 30
Dihydrofolate reductase (DHFR)	6PR6	Pocket C1	0, 32, -8	30 × 18 × 18
Penicillin-binding protein 2a (PBP2a)	1VQQ	Pocket C1	8, 38, 31	35 × 29 × 32
CHAP domain of lysin L1	11CF	Pocket C1	22, 5, -45	24 × 18 × 32

The 3D structure of eugenol was obtained from PubChem in SDF format and energy-minimized using the MMFF94 force field. Since eugenol is the primary antibacterial component (70–90%) of clove essential oil (CEO) and it also leads the antibacterial action against *S. aureus* [7, 8, 10], its molecular docking was performed. The binding site identification and docking simulations were performed using AutoDock Vina, embedded in the CB-Dock2 server with an exhaustiveness value of 8. The top-ranking conformations were chosen, based on Vina binding affinity scores (kcal/mol) and the non-covalent interaction profile. Discovery Studio Visualizer v21.1 was used for comprehensive visualization and interaction mapping.

2.5 Statistical Analysis

All experimental assays were performed in triplicate. The recorded inhibition zone diameters (DIZ) were analyzed using Microsoft Excel / GraphPad Prism (v9.0) and expressed as mean ± standard deviation (Mean ±SD). Comparative evaluation of the antibacterial activity across different extract concentrations was conducted to determine mean differences.

3 RESULTS

3.1 Antibacterial Efficacy of Ethanolic Clove Extract

Evaluation of the ethanolic extract of *Syzygium aromaticum* demonstrated a pronounced, dose-dependent antibacterial activity against clinical isolates of *Staphylococcus aureus* (Figure 1). The maximum antibacterial inhibition was produced by the highest extract concentration of 75 mg/dL, achieving a diameter of inhibition zone (DIZ) of 27 mm. Intermediate and lower concentrations of 50 mg/dL and 25 mg/dL yielded inhibition zones measuring 23 mm and 18 mm, respectively. Conversely, the negative control (sterile distilled water) exhibited no observable zone of inhibition (0 mm), confirming that the inhibitory effect was entirely attributable to the plant extract constituents.

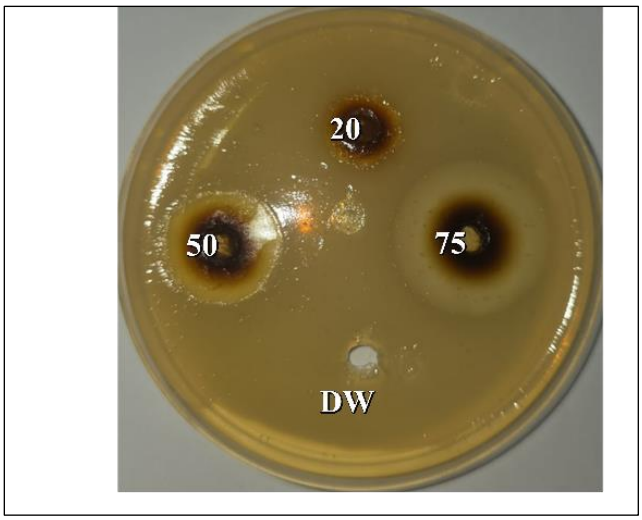


Figure 1: Clove extract inhibition of *S. aureus* in Mueller-Hinton agar.

3.2 Molecular Docking Analysis of Eugenol

Moderate to strong binding affinities of eugenol were achieved for all four targeted *S. aureus* proteins by computational docking simulations conducted on CB-Dock2 (AutoDock Vina) resulting in the binding energy score of the protein-ligand complex being between -5.5 and -6.2 kcal/mol. Of the assessed targets, eugenol had the highest thermodynamic binding affinity towards Beta-lactamase (PDB ID: 6WGR) of -6.2 kcal/mol. The CHAP domain of lysin L1 (PDB ID: 11CF) with a binding affinity of -6.0 kcal/mol followed closely and Dihydrofolate Reductase (DHFR, PDB ID: 6PR6) at -5.9 kcal/mol. The lowest binding affinity was found for Penicillin-binding protein 2a (PBP2a, PDB ID: 1VQQ) with a binding score of -5.5 kcal/mol. Two-dimensional interaction topology diagrams and three-dimensional diagrams illustrating the interactions between the receptors and the ligands are shown in detail for each receptor-ligand interaction pair in Figures 2-5.

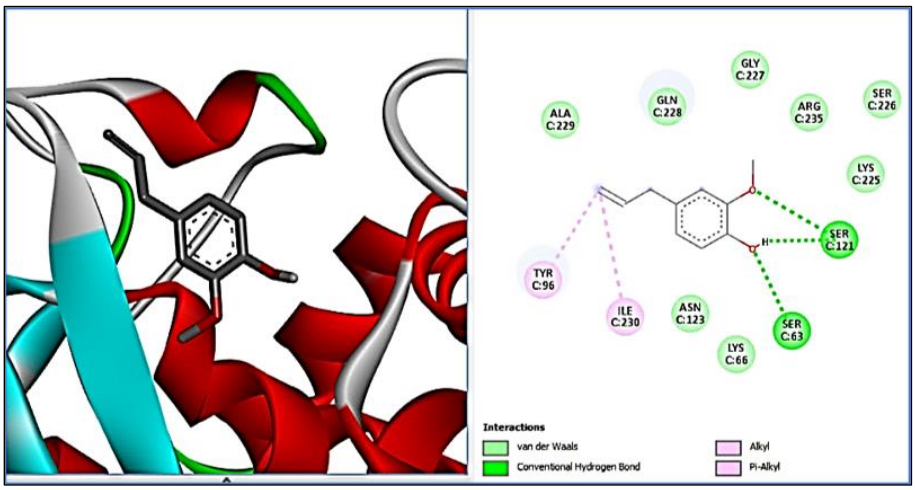


Figure 2: Molecular docking of eugenol in the *β*-lactamase active site (PDB ID: 6WGR) showing high binding affinity, illustrated via the 3D binding pose (A) and 2D interaction map (B) with key amino acid residues.

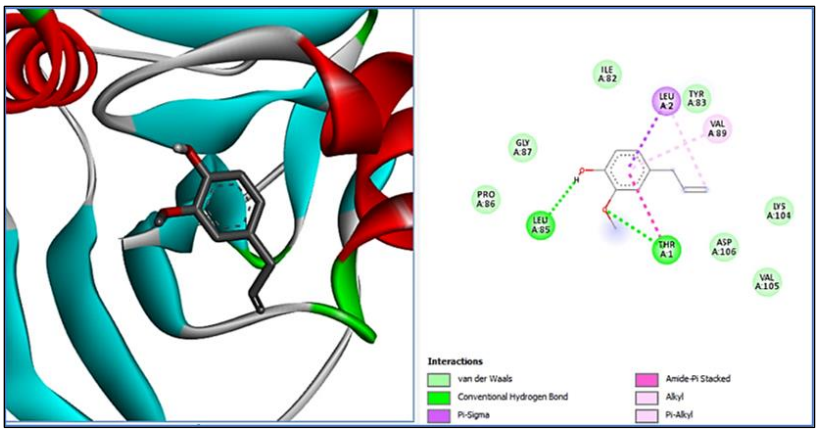


Figure 3: Molecular docking interaction of eugenol inside the binding pocket of the dihydrofolate reductase (DHFR; PDB ID: 6PR6). (A) 3D spatial representation of the docked ligand, (B) 2D interaction plot showing important amino acid contacts.

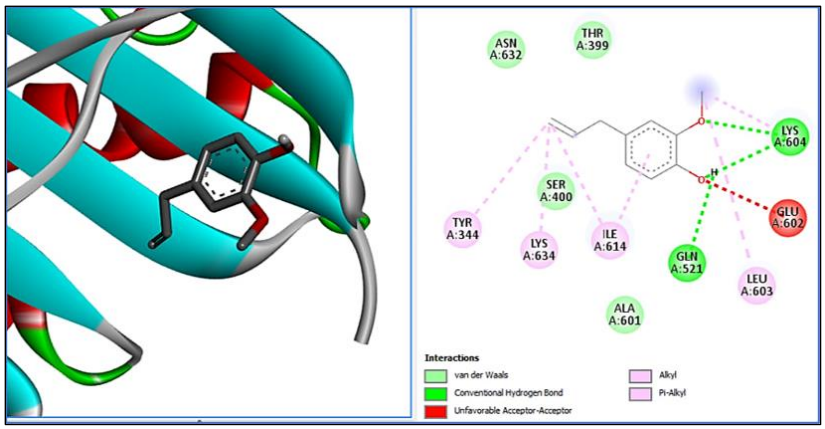


Figure 4: Molecular docking image of eugenol in the active site of penicillin-binding protein 2a (PBP2a; PDB ID: 1VQQ). (A) 3D representation of the predicted binding pose and (B) 2D binding interaction diagram showing important interactions between residues and ligand.

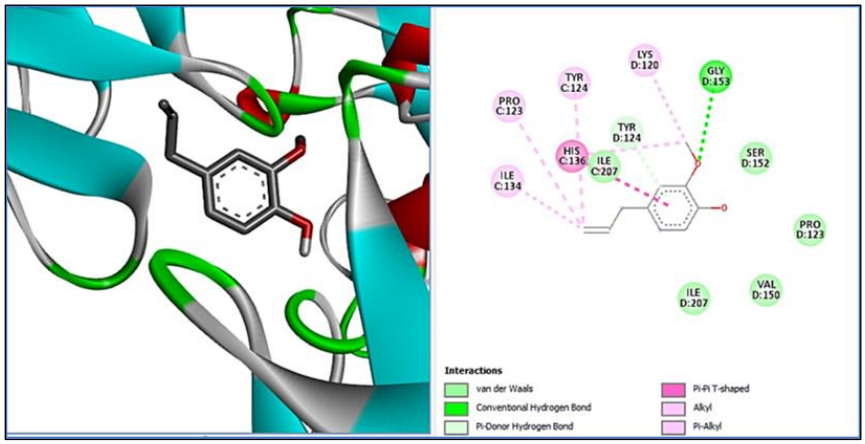


Figure 5: Molecular docking simulation of eugenol within the catalytic pocket of the lysin L1 CHAP domain (PDB ID: 11CF). (A) 3D representation of the predicted binding orientation; (B) 2D interaction plot depicting key amino acid–ligand contacts.

3.2.1 Interaction Profile with Beta-lactamase (PDB ID: 6WGR)

Eugenol showed highest binding affinity in the active pocket of β-lactamase with a docking score of -6.2 kcal/mol (Figure 2). A dual hydrogen-bonding network is established through hydrophilic anchoring with the functional oxygen atom of eugenol which establishes strong conventional hydrogen bonds with residues SER C:121 and SER C:63. The hydrophobic pocket provides stabilization to the ligand via alkyl and π – alkyl interactions with TYR C:96 and ILE

C:230, and via allyl and aromatic interactions with eugenol. Moreover, the cooperative van der Waals forces from the neighboring amino acid microenvironment (ALA C:229, GLN C:228, GLY C:227, ARG C:235, SER C:226, LYS C:225, ASN C:123, and LYS C:66) reinforce the complex.

3.2.2 Interaction Profile with Dihydrofolate Reductase (DHFR, PDB ID: 6PR6)

Eugenol exhibited a strong binding affinity toward the catalytic site of DHFR, with a docking score of -5.9 kcal/mol (Figure 3). The ligand is anchored within the binding pocket via two conventional hydrogen bonds with LEU A:85 and THR A:1. Furthermore, the aromatic core of eugenol forms dual non-covalent interactions with LEU A:2, comprising both a π – sigma and an amide–π stacked interaction that tightly stabilize the ring system. Additional hydrophobic stabilization is provided by alkyl and π – alkyl interactions involving TYR A:83 and VAL A:89, while an extensive network of van der Waals forces with surrounding shell residues (ILE A:82, GLY A:87, PRO A:86, ASP A:106, LYS A:104, and VAL A:105) further secures the ligand's orientation.

3.2.3 Interaction Profile with PBP2a (PDB ID: 1VQQ)

The binding affinity of Eugenol to PBP2a was -5.5 kcal/mol with net balance of stabilizing forces and localized energetic penalties (Figure 4). In the binding complex, the usual hydrogen bonds are established with the residues in the pocket of the binding site, viz. LYS A:604 and GLN A:521. Also, a network of hydrophobic interactions involving the hydrophobic allyl and phenyl groups with TYR A:344, LYS A:634, ILE A:614 and LYS A:604 exists, the latter providing both polar and non-polar stabilization. The docking profile, however, shows some localized conformational strain due to the unwanted acceptor–acceptor repulsion with GLU A:602, which is attributed to the close proximity of electronegative atoms. Although this is a repulsion, surrounding van der Waals contacts between ASN A:632, THR A:399, SER A:400, ALA A:601 and LEU A:603 help to maintain steric equilibrium around the active site.

3.2.4 Interaction Profile with the CHAP Domain of Lysin L1 (PDB ID: 11CF)

Docking analysis of eugenol against the CHAP domain yielded a binding energy of -6.0 kcal/mol, indicating a robust receptor–ligand affinity (Figure 5). Stabilization within the catalytic site is primarily driven by conventional hydrogen bonds linking GLY D:153 to both the hydroxyl and methoxy moieties of eugenol. Furthermore, the ligand's benzene ring engages HIS C:136 through a π – π T-shaped stacking interaction, while its aliphatic and aromatic segments form multiple alkyl and π – alkyl contacts with TYR C:124, LYS D:120, PRO C:123, and ILE C:134. Structural stability is further consolidated by surrounding van der Waals forces involving TYR D:124, ILE C:207, SER D:152, PRO D:123, and VAL D:150, which optimize steric and energetic fit inside the binding pocket

4 DISCUSSION

Clove (Syzygium aromaticum) is one of the most clinically effective natural botanical remedies that has been clinically proven to have an antibacterial effect. Eugenol is the main volatile component of *S. aromaticum*'s clove oil and is mainly responsible for its therapeutic versatility and wide-range of bioactivity [13, 14].

In accordance with previous studies, the current study revealed high sensitivity of clinical strains of *Staphylococcus aureus* to ethanolic clove extract. Eugenol is reported to have a strong growth inhibitory effect on *S. aureus* [15] and it is also found to affect the structural integrity of biofilms and reduce the cell adhesion in methicillin-resistant *S. aureus* (MRSA) strains [16]. The results are consistent with previous studies by [17] that demonstrated strong antibacterial and antivirulence properties of CLO and eugenol against *Staphylococcus* spp. and Streptococcus mutans. The antibacterial activity of purified bioactive components, like eugenol, is often higher than that of crude essential oils [18], which is why thorough investigations have been carried out on the antimicrobial activity of *S. aromaticum* extracts and their derivatives [19].

In addition, eugenol exhibits a remarkable antimicrobial activity against the clinical MRSA strains that form biofilms, eliminating pre-established biofilms by reducing the expression of genes involved with biofilm development [20]. Eugenol is also known to be safe by the US Food and Drug Administration (FDA) and European Union for preserving food, and has great potential for therapeutic use in treating recalcitrant MRSA biofilm infections [20].

The computational docking simulations also provided mechanistic insights into the above mentioned phenomenological observations showing moderate to strong binding affinity of eugenol towards key proteins and resistance conferring proteins of *S. aureus* ranging from -5.5 to -6.2 kcal/mol with AutoDock Vina docking software. The best binding interaction was found with Beta-lactamase (PDB ID: 6WGR, -6.2 kcal/mol), followed by the CHAP domain of lysin L1 (-6.0 kcal/mol) and Dihydrofolate Reductase (DHFR-5.9 kcal/mol). The in silico results are in agreement with earlier studies which had suggested eugenol as the major bioactive phytochemical responsible for the antibacterial effect of the clove extract [7, 8, 10].

The binding to Penicillin-binding protein 2a (PBP2a, PDB ID: 1VQQ) is of clinical importance, since PBP2a is the major factor responsible for β-lactam resistance in MRSA. Eugenol forms a hydrogen bond network and hydrophobic interactions in the binding site that can hinder peptidoglycan cross-links, reverting the resistant MRSA phenotype and re-sensitizing MRSA to the use of conventional β-lactam antibiotics. At the same time, affinity for β-lactamase indicates that eugenol could have a protective effect against the enzymatic cleavage of β-lactam drugs. Moreover, the interactive interactions with DHFR suggest the possibility of disruption of bacterial folate biosynthesis, while the targeting of the CHAP domain implies that there is potential for disruption of cell wall remodeling processes. The multi-target activity is a likely molecular basis for the strong antibacterial and anti-biofilm effects seen in *in vitro* models [10]. While there are some computational constraints of molecular docking, including the assumption of a rigid receptor and simplified representation of solvent, the findings strongly support future biochemical testing via enzymatic inhibition assays, as well as synergic drug combination studies.

5 CONCLUSION

The ethanolic *Syzygium aromaticum* extract shows good antibacterial activity against wound isolated *Staphylococcus aureus* bacteria at higher concentration. In accordance with these empirical observations, molecular docking studies confirm the ability of eugenol to form favourable non-covalent interactions with essential bacterial targets that play a role in cell wall integrity, resistance and bacterial metabolism. In conclusion, the results indicated that *S. aromaticum* and its major constituent eugenol could be promising natural antibacterial agents against MDR wound pathogens.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

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Disclosure of conflict of interest

"The authors declare no conflicts of interest regarding this manuscript."

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