Developing a Supplemental Cocktail Therapy for *Staphylococcus epidermidis* Drug-Resistant Infections Using Natural Products

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Abstract

Antibiotic resistance is a rising global health threat, causing over 5 million deaths worldwide annually[43]. This is a particular concern in hospital settings where bacterial infections can spread rapidly amongst patients and give rise to highly drug-resistant strains. *Staphylococcus epidermidis* exemplifies this threat; this pathogen has a 24.4% mortality rate, and 70% of isolates are multidrug resistant (MDR), making it one of the most difficult hospital-acquired infections to treat[44]. These challenges highlight the need for alternative therapeutic strategies beyond conventional antibiotics. Here, this project targets the GraRS two-component system, a central regulator of virulence and drug resistance in this pathogen that is not targeted by current antibiotics, and evaluates the efficacy of GraRS allosteric inhibitors in inhibiting S. epidermidis growth. From ~40 natural compounds with known antibacterial properties, Absorption, Digestion, Metabolism, and Excretion (ADME) filtering selected 10 orally viable candidates for GraRS docking, yielding four top hits: sesamin, sesamolin, guggulsterone, and beta-ecdysterone. These natural products were tested *in vitro* by spectrophotometric growth assays, both individually and in tandem. Furthermore, *Caenorhabditis elegans* cytotoxicity screening was used to confirm antibacterial activity with minimal host toxicity. Strikingly, combination therapy using low doses of these four compounds drastically reduced bacterial viability without impacting host survival. Together, these findings demonstrate how natural products can be leveraged to develop orally bioavailable therapeutics to combat multidrug-resistant bacterial pathogens.

Key Terms: Antibiotic resistance, GraRS, *Staphylococcus epidermidis*, cocktail treatment

Introduction

graRS Structure and Significance

Staphylococcus epidermidis is a Gram-positive, coagulase-negative staphylococcus bacterium (CoNS) that accounts for more than half of staphylococci bacteria isolated from human skin and mucous membranes [1]. It accounts for more than 70% of growth on clinical specimens and has methicillin resistance rates that are 90% greater than its well-recognized cousin, *Staphylococcus aureus.* Treatment options are limited for these infections due to emerging drug-resistant strains against common antibiotics, including methicillin, vancomycin, daptomycin, and linezolid. Notably, methicillin-resistant *S.epidermidis* (MRSE) infections are increasing by 67.5% [2]. Given this rapid emergence of antibiotic resistance, it is vital to develop alternative treatment options as adjunct or salvage therapies to combat infections caused by CoNS like MRSE.

Cationic antimicrobial peptides (CAMPs) are small biomolecules that play a critical role in the innate immune response, disrupting the stability of the bacterial membrane by binding to calcium chains on the *S. epidermidis* extracellular membrane to induce cell death[3].

In response, *S. epidermidis* has developed resistance mechanisms to combat CAMP activity. For example, the signal transduction pathway graRS (glycopeptide-resistance-associated) contributes to host-derived CAMP resistance through its two main components: GraS and GraR.

GraS is a membrane-bound histidine kinase that senses and detects CAMPs via a negatively charged extracellular loop. When CAMPs attempt to access the cell membrane, GraS autophosphorylates, activating the intracellular protein GraR that upregulates the *dltABCD* and *mprF* operons, among others [4]. This upregulation alters the ionic charges present on the cell membrane, contributing to resistance under low-pH conditions. Importantly, the deletion of GraXRS from staphylococci impairs the bacterial response to polymixin B, a positively charged polypeptide antibiotic, demonstrating GraRS's potential to play a role in the biofilm formation and antibiotic resistance of *S. epidermidis* [5]. Given the rising prevalence of MRSE and the central regulatory role of the GraRS two-component system, identifying alternative, natural compounds that can target this pathway represents a potential avenue for new therapeutics. The following literature review synthesizes information to contextualize this gap.

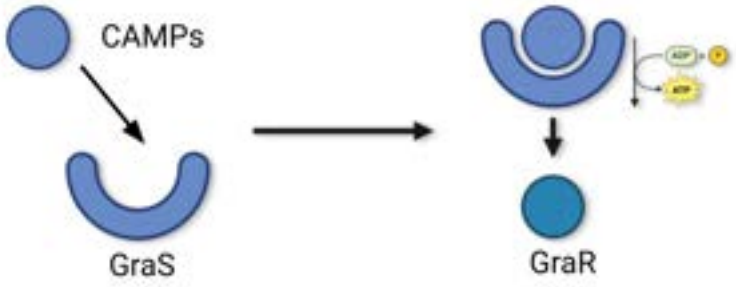

Figure 1: GraRS system operations

Literature Review

Current Treatments and Limitations

Various strategies to inhibit *S. epidermidis* growth and antibiotic resistance in clinical settings have already been employed; however, these medications often have several limitations in terms of efficacy and practicality. Vancomycin remains the primary treatment for drug-resistant *S. epidermidis* infections, yet its poor tissue penetration and reliance on intravenous administration frequently result in prolonged hospital stays and reduced patient convenience. Additionally, vancomycin carries nephrotoxic and hepatotoxic risks that necessitate therapeutic drug monitoring, and its limited penetration into *S. epidermidis* biofilms further compromises efficacy, with emerging resistance posing an increasing clinical concern[39]. Rifampin is often used as an adjunctive agent due to its ability to combat biofilms and its synergy with primary antibiotics; however, its potential for adverse drug-drug interactions, hepatotoxicity, and need for constant monitoring limit its applicability as an effective supplemental medication[40]. These limitations suggest a targeted, less toxic, bioavailable GraRS-centric approach could potentially provide significant advantages to current adjunctive antimicrobials targeting *S. epidermidis.*

The Significance of Combination Therapies

Multiple studies demonstrate that combination therapies, especially those composed of natural compounds, can enhance microbial efficacy. Yet, these investigations vary in their target organisms and mechanisms. For instance, combinations of herbal products with antibiotics can

enhance antimicrobial activity by damaging cell membranes and disrupting protein synthesis and expression [27]. Furthermore, a combination antibiotic therapy of amikacin and levofloxacin produced a synergistic effect against methicillin-resistant *Staphylococcus aureus* (MRSA), leading to anti-biofilm efficacy and delaying the emergence of resistance [6]. While both studies confirm that combining antibiotics with other compounds can inhibit bacterial growth, they do not examine whether natural compound combinations exhibit synergistic antibacterial activity.

Natural Compounds

Unlike antibiotics, which often come with toxicity issues, natural compounds are generally less harmful to human tissue. A myriad of natural compounds have supported antibacterial and antiviral activity. For instance, many studies support the antimicrobial activity of natural phytosteroids and ligans like sesamin, sesamolin[28], guggulsterone[29], and beta-ecdysterone[30]. However, the majority of natural compounds currently being investigated are water-soluble flavonoids, phenols, and alkaloids. For instance, flavonoids like catechin [7], luteolin [8], apigenin [9], xanthomunthol [10], baicalein [11], and galangin [12] have been shown to have significant antibacterial activity against Gram-positive bacteria. In addition, phenolic acids/phenylpropanoids have demonstrated similar activity, encompassing compounds like vanillic acid, syringic acid [7], and cinnamaldehyde [13]. The final main category of naturally occurring antimicrobials includes alkaloids and terpenoids, with compounds such as piperine, piperlongumine [14], thymol, and carvacrol [15].

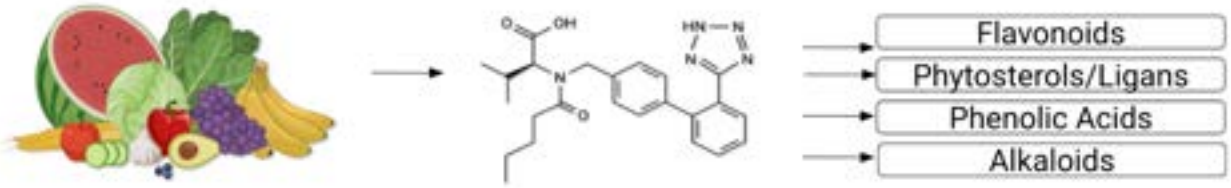

Figure 2: Current Classifications of Natural Compounds with Antibacterial Properties

ADME Analysis

Absorption, Digestion, Metabolism, and Excretion (ADME) testing has emerged as a critical determinant of clinical success. Late-stage attrition analyses in the 1990s and early 2000s reveal that the majority of candidate drug failures stem from inadequate pharmacokinetics or unanticipated toxicities rather than insufficient potency [16]. The development of the software ADMETlab3.0 allows for the analysis of over 119 pharmaceutical properties, reflecting the growing desire for preliminary knowledge of critical drug properties before wet-lab testing and design [17]. Current ADME properties that have been gaining relevance in the clinical community in terms of importance to safety and convenience of medication and dosing include Lipinski's Rule, Caco-2 permeability, the ability of the drug to pass through the blood-brain barrier (BBB), and human liver microsomal (HLM) stability. Lipinski's Rule of 5 approximates the absorptivity and permeability of drugs through analyzing 4 main characteristics: hydrogen bond donors and acceptors, molecular weight, and LogP [18]. These properties are vital for understanding the likelihood of drugs being developed as oral or prophylactic medications, both of which are more convenient for patients compared to intravenous (IV) administration. Caco-2 permeability

similarly predicts the ability of a drug to be absorbed into the human small intestine without having to perform an *in vitro* (using petri dishes or test tubes) study [19]. Furthermore, the ability, or inability, of a drug to pass the BBB is significant in examining potential psychological properties of medications. The BBB refers to a selective barrier that limits the entry of substances into the brain; assessing a drug's ability to cross the BBB is essential to ensure that drugs do not correlate with unexpected central nervous system side effects [19]. Finally, HLM stability reflects how rapidly the liver metabolizes a drug. The faster metabolism occurs, the greater the risk of first-pass metabolism and toxicity, and the decreased overall bioavailability of the medication used [19]. Together, these factors help guide the optimization of a drug's chemical structure and formulation to maximize absorption, maintain adequate systemic exposure, and minimize adverse effects, ultimately increasing the likelihood of successful drug implementation.

Bacterial Growth and Cytotoxicity Assay

To determine drug efficacy *in vitro* and *in vivo,* spectrophotometry is used to measure bacterial growth, while cytotoxicity assays are used to assess undesired toxic effects of each compound. More specifically, spectrophotometry uses light to determine bacterial density at OD~600 nm, and is often applied in time-kill assays, where multiple readings of a bacterial sample are taken over a 2-3 day period [20]. On the other hand, common models for cytotoxicity include human alveolar or intestinal tissues [22], mouse or mammalian models, or invertebrates such as *C. elegans* [23]. Combining both bacterial growth and cytotoxicity assays will allow us to identify compounds with high antibacterial effects and minimal toxicity.

Purpose and Hypotheses

Based on the current scientific literature, no study has explicitly identified naturally derived compounds that can allosterically inhibit the GraRS receptor in *S. epidermidis* without contributing to drug resistance. The goal of this project is to develop a potential adjunct/salvage cocktail therapy, comprised of a combination of natural compounds, to combat drug-resistant *S. epidermidis.* The central research question for this study is: Can we identify specific natural compound inhibitors of the GraRS two-component system in *S. epidermidis* and overcome multi-drug resistance effectively?

Three hypotheses will be tested through this paper. The first hypothesis dictates that a combination of any of the natural compounds mentioned above will lead to decreased bacterial growth compared to a negative control, indicating that the drug therapy is effective. The second hypothesis states these drugs will comply with the ADME guidelines previously discussed: passing Lipinski's Rule of 5, having strong Caco-2 permeability, failing to pass the BBB, and having moderate to high HLM stability. Finally, the third hypothesis is that the drugs selected through computational modeling will exhibit low cytotoxicity when used in a *C. elegans* model system, suggesting that this cocktail will not be acutely toxic to human tissues and will likely be safe to use in clinical settings.

Methods

Literature Review and ADME Analysis

To analyze past research and the pharmacological properties of the natural compounds collected, a literature review was performed. Due to the lack of prior research investigating natural compounds' antibacterial efficacy against *S. epidermidis*, compounds were collected based on the hypothesized efficacy of the GraRS system's extracellular loop and prior antibacterial research conducted across different staphylococci and general bacteria. In particular, the significance of D35, an aspartic acid residue recently discovered to play a critical role in upregulating the activity of the GraRS system, was a vital factor in selecting the 46 compounds for ADME testing[31].

To evaluate the ADME properties of the compounds collected, the computational screening tool ADMETlab 3.0 was used[32]. By extracting the chemical structure of each compound through its SMILES code, the software was able to provide a comprehensive overview of over 119 different properties associated with each ADMETlab3.0 sector. Of these properties, each drug was analyzed based on whether it passed Lipinski's Rule of 5, Caco-2 permeability value, HLM stability, and whether the drug passed the BBB (see Appendix A). These interpretations informed the selection and prioritization of compounds for further docking and experimental analyses.

Then, molecular docking was performed using AutoDock Vina (version 1.5.7)[33] to evaluate the binding affinity of ADMET-selected natural compounds against the GraS sensor kinase. The three-dimensional structure of GraS was obtained from UniProt[34], an open-access protein database, and the ligand structures were downloaded from RCSB PDB and converted into .pdbqt files using Open Babel. Protein preparation involved removing water molecules, adding polar hydrogen residues, and assigning Kollman charges. The ligand files were energy-minimized before docking. A grid box was defined around the predicted binding site of the extracellular loop region, with parameters set to ensure accurate conformational sampling within the energy range and exhaustiveness of the software (see Appendix B). Results were recorded as binding affinities (kcal/mol), and the top-ranked poses were visualized using PyMOL[35] to assess the orientation of the ligands.

Figure 3: *In silico* Methodology Flow Chart

Confirming GraRS Similarity in Pathogenic and Non-Pathogenic Strains

Due to lab and BSL restrictions, *in vitro* pathogenic or biofilm-forming strains of *S. epidermidis* were not used. Instead, I used a model non-pathogenic strain: *S. epidermidis* ATCC 12228. To confirm that the GraRS system is similar across pathogenic and model strains, I aligned the structures of GraRS from both strains and used Root Mean Square Deviation (RMSD) testing to assess similarity[36].RMSD values<1 demonstrated significant similarities in protein structure.

Wet Lab Methodology

First, commercial concentrations of the top-scoring compounds, sesamin, sesamolin, guggulsterone, and beta-ecdysterone in ADME and AutoDock Vina (see Results) were selected for the experiment. The experiment was conducted on *S. epidermidis* in 2 phases: spectrophotometry (Phase 1) and cytotoxicity screening (Phase 2).

Phase 1: Spectrophotometry

All cultures were grown at 37°C aerobically. Phase 1 was carried out by first preparing LB^+ broth. This broth was specifically formulated to have a significantly higher salt (NaCl) concentration compared to traditional LB broth (7.5%). The excess salt served to indirectly stimulate the GraRS system, because salt has shown to indirectly stimulate oxidative and osmotic stress in halotolerant *S. epidermidis*, which activates the GraRS system[37]. After this broth was prepared, *S. epidermidis* ATCC 12228 was inoculated into the broth and incubated overnight. Stock solutions were created with individual compounds and combinations of all 4 compounds, and 3 sets of two-fold dilutions were created for each experimental group to test the Minimum Inhibitory Concentration (MIC) (note: sesamin and sesamolin were tested as individual treatments despite being 2 separate compounds due to limited commercial availability of sesamolin and sesamin as separate compounds). Each dilution was added in specific amounts(see figure below) in combination with sterile water and the *S. epidermidis* culture to create 3 two-fold dilutions of each treatment. Then, each dilution was allowed to incubate for over 72 hours, and OD600 readings were taken every 24 hours to observe growth.

Working Drug Stock	Sterile Water	LB^+ Broth Culture	Final Volume
1 mL	–	1 mL	2 mL
500 µL	500 µL	1 mL	2 mL
250 µL	750 µL	1 mL	2 mL
125 uL	875 µL	1 mL	2 mL

Table 1: Standard Curve of Natural Compound Bacterial Dilutions

Phase 2: Cytotoxicity Assay

Phase 2 was carried out by obtaining commercially available Nematode Growth Medium and a *C. elegans* parent culture from the Carolina Biological Society. 20 ml of nematode growth media was cooled in each of 6 separate petri dishes. *E. coli* OP50 was inoculated overnight, and 1 mL

of the previously prepared cocktail treatment was combined with 9 mL of the OP50. Half of the cooled petri dishes received OP50 with no cocktail treatment added, while the other dishes received the cocktail OP50. *C. elegans* was then synchronized to the egg/L1 stage by washing with HCl and NaOH as well as M9 Buffer (0.6 g KH_2PO_4, 1.2 g Na_2HPO_4, 1 g NaCl, 0.024076 g $MgSO_4$, H_2O to 200 mL)[38]. All 6 plates were then incubated at room temperature. Finally, using a digital microscope to perform live cell microscopy, *C. elegans* were observed at 40x magnification for their number, size, and motility at 24 and 48 hours. High-resolution images were also taken with the digital microscope, and worms were manually counted at both time points to approximate the % of motile worms.

Figure 4: Lab procedures performed by student researcher

Results

ADME and Molecular Docking Confirm Allosteric Inhibitors

Of the ~40 natural compounds tested, the top 4 that met the ADME analysis parameters were sesamin, sesamolin, guggulsterone (isomers E and Z), and beta-ecdysterone. They are non-harmful to the BBB and liver and could potentially be developed as oral medications, as demonstrated by their passing of Lipinski's Rule of 5 and high Caco-2 permeability. The only exception to these successes was Guggulsterone Z, one of the 2 isomers of the natural compound guggulsterone, which demonstrated unstable HLM stability. However, given its other successful values, it was still moved past the molecular docking stage, with the caveat that lower amounts of this compound could be included in hypothetical cocktail treatments

Drug Name	Lipinski's Rule	HLM Stability	CaCo-2	BBB	Natural Source
Sesamin	Accepted	Stable	-4.886	Good	Sesame Seeds
Sesamolin	Accepted	Stable	-4.887	Good	Sesame Seeds
Guggulsterone E*	Accepted	Stable	-4.513	Good	Guggul Tree
Guggulsterone Z*	Accepted	Unstable**	-4.611	Moderate	Guggul Tree
Beta-Ecdysterone	Accepted	Moderate	-4.718	Good	Insects, Spinach

Table 2: ADME Analysis Table for Top Compounds

The binding affinities of the top 9 natural compounds to the GraS receptor protein of the GraRS system were then analyzed computationally using AutoDock Vina. More negative binding affinities indicate a stronger likelihood to disrupt the receptor. Although the standard for what constitutes a strong binding affinity can vary, in prior literature, typical compounds that resulted in high inhibition efficacy had binding affinities ranging less than -7.1 kcal/mol, so this binding affinity was used to assess the top-scoring compounds in the docking analysis[40]. The top-scoring compounds were sesamin, sesamolin, guggulsterone E and Z, and beta-ecdysterone (Fig. 5), all of which have binding energies <-7.4 kcal/mol. These results indicated that all compounds had a strong likelihood to alter the structure of the GraS protein, inhibiting the resistance mechanism and blocking a key system for *S. epidermidis* antibiotic resistance.

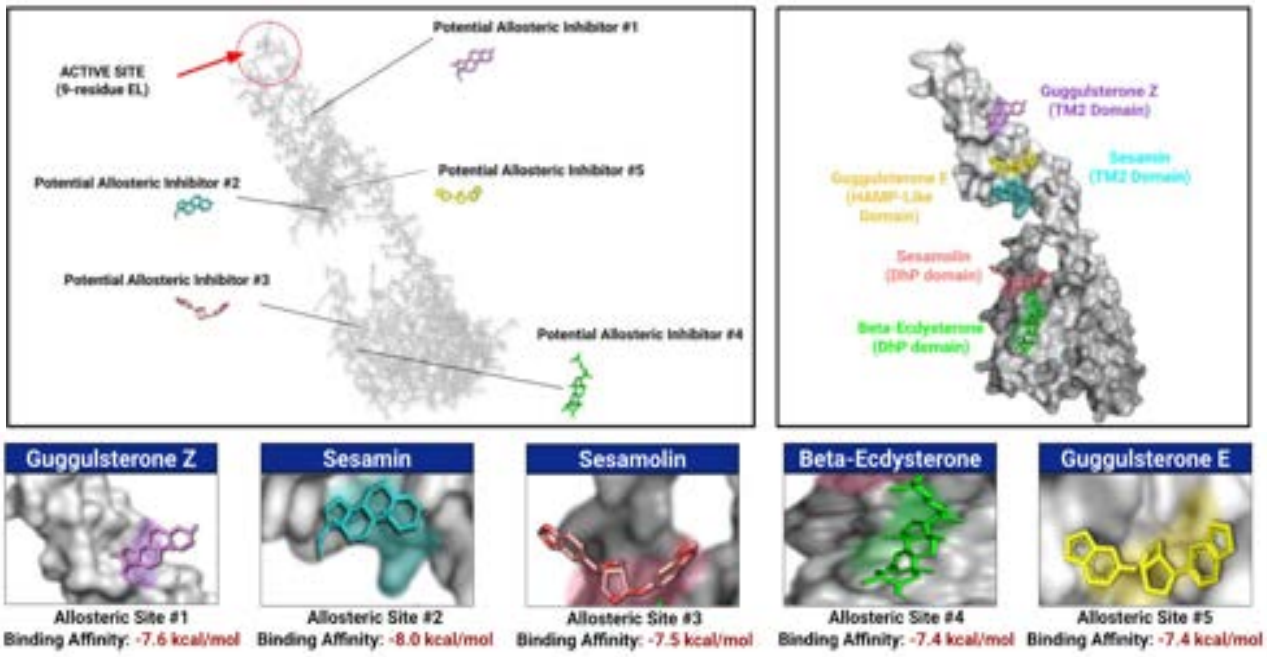

Figure 5: Identification of allosteric binding sites and binding affinities for top-scoring compounds

RMSD Confirms GraRS Across Pathogenic/Non-Pathogenic Strains

Due to laboratory constraints for working with the BSL-2 pathogenic strain of *S. epidermidis*, *in vitro* verification of the computational results was performed with the BSL-1 *S. epidermidis*

non-pathogenic strain ATCC 12228. Toensure the GraRS system tested was similar across pathogenic and non-pathogenic strains, and that wet-lab results could be applied to clinical settings, RMSD comparisons were done between GraRS in ATCC 12228 and the biofilm-forming pathogenic strain ATCC 35984 using PyMOL. The results of the analysis indicated high degrees of similarity (RMSD <1) between both the GraS and GraR proteins, suggesting that the BSL-1 laboratory strain serves as a good model system for the study of GraRS inhibition by natural compounds.

Figure 6: Similar RMSD values across GraS and GraR proteins in pathogenic/model strains

Spectrophotometry: Treatments Reduced Total Bacterial Growth

In bacterial spectrophotometry, a lower OD600 (Optical Density, 600 nm) indicates a lower bacterial cell density and increased microbial death. Results showed the negative control *S. epidermidis* culture had an OD600 of ~4.55 at 24 hours, 3.56 at 48 hours, and 1.30 at 72 hours (averaged across triplicates). The turbidity (cloudiness) of the negative control cultures across 72 hours was visibly more prominent compared to most experimental groups treated with natural compounds. Of all individual treatments, beta-ecdysterone exhibited the greatest inhibitory effect on bacterial growth [Figure 7]. The combination of all 4 compounds exhibited the lowest OD600 values, with its lowest concentration tested, 192 µg/mL, measuring an OD600 of ~0.35 at 72 hours. This represents a 59% decrease in bacterial growth. Overall, these results suggest the **combined** treatment was most effective at inhibiting *S. epidermidis* growth, with inhibition ranging from approximately 59% to 99% across the 3 tested concentrations (lowest-highest).

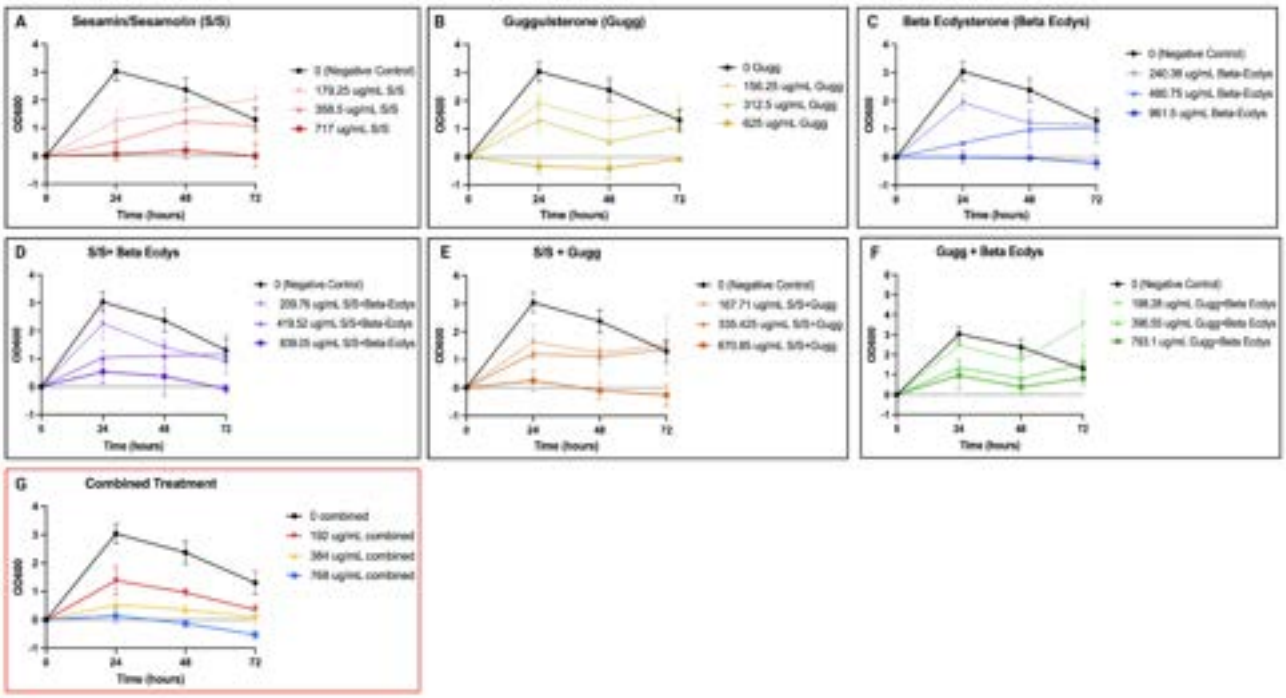

Figure 7a-g: Average OD600 values (with standard deviation included) of various concentrations of treatments of sesamin/sesamolin, guggulsterone, and beta-ecdysterone. The red outline around box G represents the improved bacterial inhibition efficacy of the combined treatment of all 4 natural compounds.

All individual and in tandem treatments were tested at a range of concentrations to determine the Minimum Inhibitory Concentration (MIC) of a specific treatment that produced the greatest *S. epidermidis* inhibition. This was demonstrated effectively with the combined treatment: not only did it demonstrate the lowest bacterial growth across 72 hours of all other treatments tested, but it also had significant inhibition at its lowest concentration tested compared to the other experimental groups.

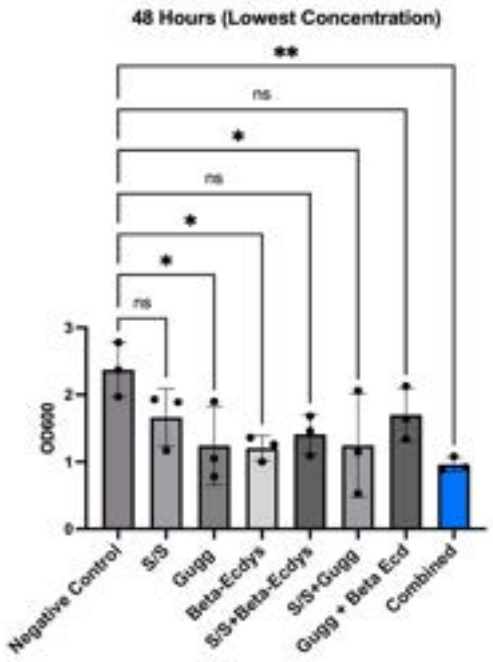

Figure 8: Average OD600 of sesamin/sesamolin, guggulsterone, and beta-ecdysterone combinations at the lowest tested concentrations. **ns:** not statistically significant ***:** statistically significant ($p<0.05$) ****:** very statistically significant ($p<<0.05$). Note that the data from 48 hours was taken due to it most accurately representing the effects of the individual and combination treatments.

Cytotoxicity Assay Confirms Low Acute Cytotoxicity

C. elegans eggs and L1 larvae were implanted on seeded nematode growth media (NGM) plates (in triplicate), with experimental plates having *E. coli* OP50 treated with the cocktail. The difference in the percent of viable worms between the negative control and experimental groups across 48 hours was not statistically significant [see Figure 9], suggesting the natural compound cocktail was not acutely cytotoxic. However, further testing performed on human cells for longer time periods could confirm a lack of chronic cytotoxicity.

Treatment Group	24 hours	48 hours
Without cocktail treatment (negative control)	Motility: Strong Approx. Motility % - ~30% Size: large, dark, thick-bodied	Motility: Strong, remains the same as 24 hrs Size: same as 24 hours
With cocktail treatment	Motility: Strong Approx. Motility % - ~25% Size: some worms slightly thinner, some dark and thick	Motility: Strong, remains the same as 24 hrs Size: same as 24 hours

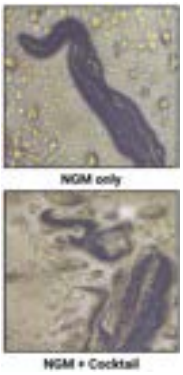

Table 3: *C. elegans* motility and size across negative and experimental control.

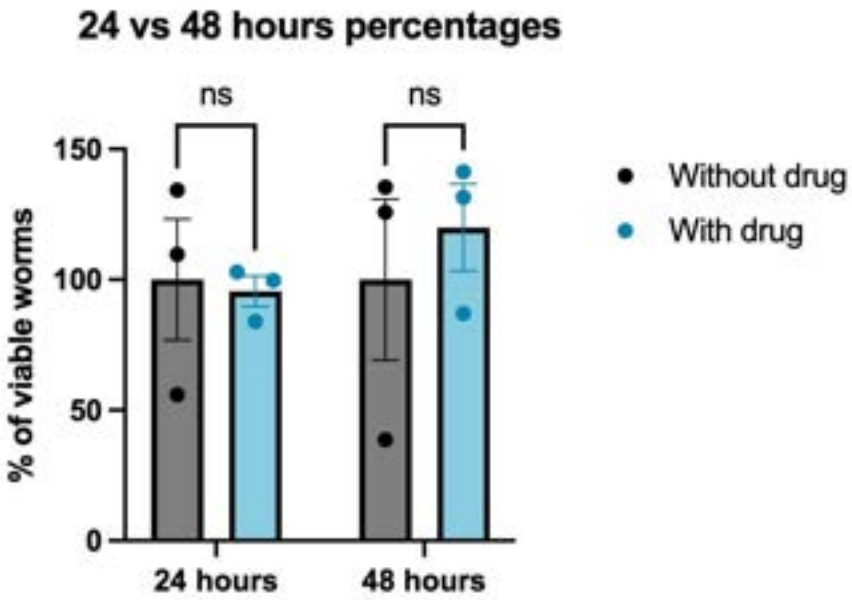

Figure 9: Changes in % of viable C. elegans after 24 and 48 hours

Statistical Analysis: Spectrophotometry

A one-way ANOVA test was conducted on each experimental group (for a total of 7 tests) to determine whether the differences between the means of each treatment group and the negative control were statistically significant at the treatments' lowest concentrations. For each test, the null hypothesis was that there is no difference between the mean OD600 of the treatment group and the negative control, and the alternative hypothesis was that there is some difference between the mean OD600 of the negative vs the respective experimental control. 5 out of the 7 treatments tested had p-values less than 0.05; thus, the null hypothesis was rejected, and the alternative hypothesis was accepted. By conventional criteria, it can be determined that the combined cocktail therapy is statistically significant.

Comparison	Mean Diff	95% Difference	Significant?	P Value
Negative Control vs. S/S	0.710	-0.349 to 1.769	No (ns)	0.2777
Negative Control vs. Gugg	1.130	0.071 to 2.189	Yes (*)	0.0341
Negative Control vs. Beta-Ecdys	1.167	0.108 to 2.226	Yes (*)	0.0279
Negative Control vs. S/S + Beta-Ecdys	0.963	-0.096 to 2.022	No (ns)	0.0827
Negative Control vs. S/S + Gugg	1.127	0.068 to 2.186	Yes (*)	0.0347
Negative Control vs. Gugg + Beta-Ecdys	0.670	-0.389 to 1.729	No (ns)	0.3288
Negative Control vs. Combined	**1.413**	**0.354 to 2.472**	**Yes (**)**	**0.0071**

Table 4: One-Way ANOVA results of individual/combination treatments

Statistical Analysis: Cytotoxicity Assay

Additionally, a two-way ANOVA test was conducted to determine whether the means of the control and experimental *C. elegans* groups were statistically significant across 48 hours. The null hypothesis was that the cocktail treatment did not have an effect on *C. elegans* counts over time, while the alternative hypothesis was that there was a difference between the counts of *C. elegans* in both groups. The p-value reported was greater than 0.05. Thus, the null hypothesis was accepted, and the alternative hypothesis was rejected. This demonstrated that *C. elegans* growth demonstrated similar growth trends whether the cocktail was applied or not, indicating the lack of potential acute toxic or unintended side effects of the treatment over 48 hours.

P value	P value summary	Significant?
0.5793	ns	No
0.5793	ns	No
0.7236	ns	No

Table 5: Summary of cytotoxicity assay results. Lack of statistical significance indicates the cocktail's lack of toxicity when observed acutely.

Cost-Benefit Analysis

A cost–benefit analysis was conducted with the combined cocktail treatment at 192 µg/mL total concentration, with each compound consisting of 48 µg/mL, to evaluate the feasibility of the proposed compounds at microgram-scale dosages. The lowest concentration of the cocktail was specifically used as it was the lowest concentration tested that exhibited significant bacterial growth, especially since the compounds were crude in nature. Approximate material costs were estimated by scaling publicly available retail supplement prices to the quantities used in this study [see Appendix C]. These estimates indicate that, at the microgram level, the direct cost of the compounds is negligible, particularly when compared to other considerations such as

formulation, delivery, and experimental or regulatory constraints. As a result, cost was decided to be a significant factor in the efficacy and accessibility of this medication.

Drug Name	Approximate Cost (48 µg/mL each; 192 µg/mL total)
Sesamin	~$0.02–$0.03
Sesamolin	~$0.07
Guggulsterone (E/Z)	~$0.03
Beta-Ecdysterone	~$0.004–$0.01
Combined	~$0.12–$0.14

Table 6: Cost-Benefit Analysis Table for Combined Treatment at Lowest Concentration

Discussion

Summary of Key Findings

This study demonstrates how natural compounds can be rationally selected, computationally validated, and experimentally tested to disrupt a central antibiotic resistance-regulating pathway in *S. epidermidis.* By targeting the GraRS two-component system rather than exerting bactericidal pressure, this work explores an alternative supplemental or salvage therapy for mitigating drug resistance. The results support all 3 initial hypotheses: 1) ADME-filtered natural compounds exhibited strong predicted oral viability, 2) *in silico* molecular docking suggested effective allosteric inhibition of GraRS, and 3) *in vitro* experiments confirmed reduced bacterial growth with minimal acute cytotoxicity.

One key finding of this study is the enhanced efficacy observed with the combination therapy of natural compounds compared to individual treatments. While most individual and dual combinations of sesamin/sesamolin, guggulsterone, and beta-ecdysterone demonstrated levels of inhibitory effects in the spectrophotometry assays, the four-compound cocktail consistently, across 3 replicates, produced the greatest reduction in bacterial proliferation when *S. epidermidis* was grown in a highly stressful, high salt environment. This suggests a synergistic mechanism by which multiple ligands collectively disrupt GraRS signalling, weakening the bacteria's ability to adapt to environmental and antimicrobial stressors. Thus, targeting regulatory systems, including GraRS, may be particularly advantageous, as the cocktail can interfere with virulence-associated pathways without increasing antibiotic resistance.

The computational docking results provide further support for this regulatory approach. All 4 lead compounds demonstrated strong binding affinities to the GraRS receptor protein beyond the active site, consistent with allosteric inhibition rather than competitive, active-site inhibition. This is important because two-component systems are highly conserved across staphylococci and are essential for bacterial adaptation, yet are not targeted by current frontline antibiotics[41]. By altering GraS conformation and downstream signaling, these compounds may prevent

activation of operons responsible for membrane charge modification and CAMP resistance, thereby sensitizing *S. epidermidis* to environmental stress and antimicrobial agents.

Importantly, the ADME profiles of the identified compounds highlight their translational potential. All lead compounds passed Lipinski's Rule of Five and exhibited high predicted Caco-2 permeability, suggesting feasibility for oral administration. This represents a meaningful clinical advantage when compared to commonly used adjunctive therapies such as rifampin. Although rifampin is orally administered, its clinical utility is limited by hepatotoxicity, extensive drug–drug interactions, and the need for careful therapeutic monitoring[42]. In contrast, an orally bioavailable GraRS-targeting cocktail could potentially be combined or supplemented alongside an intravenous antibiotic and improve patient adherence by simplifying treatment regimens. The observed efficacy of low-dose combination therapy further supports the possibility of minimizing systemic toxicity while maintaining antibacterial activity.

Finally, the cytotoxicity assay using *C. elegans* provided preliminary evidence that the cocktail treatment does not induce acute host toxicity. The lack of statistically significant differences in worm motility or survival between control and treated groups suggests that the antibacterial effects observed are not accompanied by immediate off-target toxicity, supporting the potential safety of the approach in early-stage development.

Limitations and Implications

Due to BSL limitations, experiments were conducted using a non-pathogenic strain of *S. epidermidis*, which may encode differences in bacterial response and virulence pathways compared to clinical isolates. However, RMSD analysis confirmed high structural similarity of GraRS proteins between model and pathogenic strains, supporting the relevance of the findings. Additionally, the GraRS system was not measured directly in the *in vitro* verification. Although stressing the bacteria with salt[37] is a known activator of the GraRS system, further genetic and protein analysis should be conducted with this cocktail to specifically measure the extent to which the GraRS system is affected by these natural compounds. Finally, cytotoxicity was assessed only acutely and in an invertebrate model; longer-term studies using mammalian cell lines or animal models would be necessary to fully evaluate safety.

Further work should aim to validate GraRS inhibition directly through gene expression analysis, *graRS* knockout strains, or procedures like an SDS-Page or Western blot, as well as assess efficacy against clinical isolates of biofilm-forming *S. epidermidis.* Investigating the use of this cocktail as an adjunct to existing antibiotics could further clarify its clinical utility, especially in reducing drug resistance. Particularly, the cost-benefit analysis indicates this cocktail treatment could be used as an adjunct or substitutional medication in countries with limited access to primary antibiotics, allowing for temporary treatment until primary antibiotics like vancomycin can be accessed. Overall, this study highlights the promise of combining computational drug screening with natural product synergy to develop orally bioavailable, resistance-targeting therapies for multidrug-resistant bacterial infections.

Conclusion

Overall, the treatment with multiple natural products (sesamin, sesamolin, guggulsterone, and beta-ecdysterone) showed strongly significant inhibition of *S. epidermidis* growth through allosterically targeting the GraRS two-component system. In addition, the cytotoxicity assay and ADME analysis results indicate that the cocktail treatment could be acutely non-toxic for human use. These findings can be translated into the development of an orally available supplemental or salvage medication to current antibiotics, providing a more convenient treatment for patients compared to traditional intravenous drugs. Further clinical testing of this combination treatment can be developed to verify its efficacy, thus reducing mortality from infections that account for a significant amount of hospital-acquired infections, clinical contamination, and drug resistance.

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Appendices

Appendix A: ADME Analysis

Drug Name	Lipinski's Rule	Pfizer Rule	Blood-Brain Barrier (BBB)	HLM	CaCo-2 Permeability (GI)
Catechin	Accepted	Accepted	Good	Unstable	-6.346 (BAD)
Querticin	Accepted	Accepted	Good	Stable	-6.177 (BAD)
Vanillic Acid	Accepted	Accepted	Good	Unstable	-5.096 (Good)
Gallic Acid	Accepted	Accepted	Good	Unstable	-5.604 (BAD)
Hesperetin	Accepted	Accepted	Good	Stable	-5.069 (Good)
Ursolic acid	Accepted	Rejected	Bad	Unstable	-5.462 (BAD)
Betulinic acid	Accepted	Rejected	Bad	Unstable	-5.563 (BAD)
Aurantiamide acetate	Accepted	Accepted	Medium	Stable	-4.962 (Good)
Piperlongumine	Accepted	Accepted	Bad	Stable	-4.691 (Good)
Xanthohumol	Accepted	Accepted	Good	Stable	-5.056 (Good)
Guggelsterone (Z)	Accepted	Rejected	Medium	Unstable	-4.611 (GOOD)
Guggulsterone (E)	Accepted	Accepted	Good	Stable	-4.513 (Good)
Baicalein	Accepted	Accepted	Good	Moderate	-4.774 (Good)
Luteolin	Accepted	Accepted	Good	Stable	-5.192 (Bad)
Apigenin	Accepted	Accepted	Good	Moderate	-5.129 (Good)
Galangin	Accepted	Accepted	Good	Stable	-5.353 (Bad)
Ellagic Acid	Accepted	Accepted	Good	Moderate	-5.167 (Bad)
Tangeretin	Accepted	Accepted	Good	Stable	-4.5 (Good)
Rosmaranic Acid	Accepted	Accepted	Good	Unstable	-6.513 (BAD)
Carnosic Acid	Accepted	Accepted	Good	Unstable	-4.88 (good)
Emodin	Accepted	Accepted	Good	Stable	-5.141 (good)
Naringenin	Accepted	Accepted	Good	Stable	-4.987 (good)

Berberine	Accepted	Rejected	Good	Moderate	-5.01 (good)
Eugenol	Accepted	Accepted	Good	Unstable	-4.57 (good)
Betullic Acid	Accepted	Rejected	Bad	Unstable	-5.353 (bad)
Allicin	Accepted	Accepted	Bad	Stable	-4.495(good)
Vancomycin	Rejected	Accepted	Good	Stable	-6.291 (bad)
Daptomycin	Rejected	Accepted	Good	Unstable	-6.294 (bad)
Linezolid	Accepted	Accepted	Good	Unstable	-4.875 (good)
Tigecycline	Rejected	Accepted	Good	Unstable	-6.038 (bad)
Rifampin	Rejected	Accepted	Good	Unstable	-5.535 (bad)
Ampicillin	Accepted	Accepted	Good	Stable	-5.688 (bad)
Methicillin	Accepted	Accepted	Good	Unstable	-5.766 (bad)
Penicillin	Accepted	Accepted	Good	Stable	-6.071 (bad)
Silidiadin	Accepted	Accepted	Good	Unstable	BAD
Sesamin	Accepted	Accepted	Bad*	Stable	-4.886(GOOD)
Sesamolin	Accepted	Accepted	Bad*	Stable	-4.887 (GOOD)
			Sesamin/sesamolin has been published to have neuroprotective effects, meaning they pass the BBB but remain stable and have positive effects on brain activity. Therefore, these are considered stable in Table 2		
Artemisinin	Accepted	Accepted	Good	Stable	-4.553 (GOOD)
Beta-Ecdysterone	Accepted	Accepted	Good	Unstable	-4.788 (GOOD)

Appendix B: AutoDock Vina Parameters

Receptor = protein_model1.pdbqt

Ligand = ligand.pdbqt

Center_x = 1.826

Center_y = 3.290

Center_z = -8.192

Size_x = 126

Size_y = 82

Size_z = 126

Energy_range = 4

Exhaustiveness = 8

Appendix C: Cost-Benefit Analysis Links and Retail Commercial Sources

Sesamin / Sesamolin

- Lignans for Life Sesame Extract (retail supplement listing): https://www.truegether.com/sesamin-sesame-seed-lignans-90-capsules-dietary-supplements-natural-heal/USER.b037a468-82e3-4a12-98f3-c730d503f84a/listing.html

Guggulsterone

- VemoHerb Guggulsterone (retail supplement listing): https://vemoherb.com/us/product/vemoherb-guggulsterone-90-caps/

Beta-Ecdysterone

- Nutricost Beta-Ecdysterone (retail supplement listing): https://nutricost.com/products/nutricost-beta-ecdysterone

These prices vary by supplier, formulation, concentration, and time; listed sources are provided as representative examples to support approximate cost estimation for application purposes discussed in the research paper.