



Flavonoids and chromones from *Aloe barbadensis* exhibit potent activity against *Staphylococcus aureus*: validation of in silico predictions

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) poses a serious public health threat, necessitating new antimicrobial agents. Our previous molecular docking study identified several phytochemicals from *Aloe barbadensis* as potential inhibitors of MRSA targets. This study experimentally validates the antibacterial activity of nine top-ranking compounds against *S. aureus*. Nine compounds – aloesaponarin-II, aloenin, methylaloesinol, luteolin, aloespanol-II, ziganein-5-methyl-ether, salicylic acid, kaempferol, and methyl salicylate were obtained from Sigma-Aldrich. Antibacterial activity against *S. aureus* ATCC 25923, evaluated using the Kirby-Bauer disk diffusion method. Gentamicin (10 µg/disk) served as positive control, and DMSO as negative control. Zones of inhibition (ZOI) were measured in triplicate after 24 h incubation at 37°C. Data were analyzed using one-way ANOVA with Tukey's post-hoc test. Luteolin produced the largest inhibition zone (55.8 ± 3.6 mm), significantly exceeding gentamicin (35.7 ± 4.0 mm, $p < 0.001$). Kaempferol (43.0 ± 1.7 mm) and aloenin (38.7 ± 1.2 mm) also outperformed the antibiotic control ($p < 0.05$ and $p < 0.01$, respectively). Aloesaponarin-II (35.4 ± 1.4 mm) and methylaloesinol (34.3 ± 0.6 mm) showed activity comparable to gentamicin. Salicylic acid (31.7 ± 2.9 mm) exhibited moderate activity, while methyl salicylate (22.3 ± 2.0 mm) and ziganein-5-methyl-ether (13.0 ± 1.7 mm) were least effective. The negative control showed no inhibition. The ranking of activity strongly correlated with docking scores (Spearman's $\rho = 0.87$, $p = 0.002$). Luteolin, kaempferol, and aloenin demonstrate potent *in vitro* activity against *S. aureus*, with efficacy surpassing that of gentamicin under disk diffusion conditions. These results validate our *in silico* predictions and identify lead compounds for further development as topical or systemic anti-staphylococcal agents. The study determines that it is imperative to find the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of luteolin, kaempferol, and aloenin using the standardized broth microdilution method (CLSI guidelines). This will provide a quantitative measure of intrinsic potency that is not confounded by differential diffusion rates through agar.

Keywords: *Aloe barbadensis*, Aloenin, Antibacterial, Disk Diffusion, Kaempferol, Luteolin, Molecular Docking Validation, *Staphylococcus aureus*

I. INTRODUCTION

The escalating crisis of antibiotic resistance has rendered many conventional drugs ineffective against common bacterial pathogens. Among these, *Staphylococcus aureus* – a Gram-positive coccus responsible for skin and soft tissue infections, bacteremia, endocarditis, and pneumonia – has developed resistance to nearly all available antibiotics, including methicillin, vancomycin, and linezolid (Ventola, 2015; Lowy, 1998). Methicillin-resistant *S. aureus* (MRSA) alone causes over 80,000 invasive infections and 11,000 deaths annually in the United States (CDC, 2019). The urgent need for novel antimicrobials has rekindled interest in plant-derived natural products, which offer diverse chemical scaffolds and often act via multiple mechanisms, potentially evading existing resistance pathways (Brown & Wright, 2016).

Aloe barbadensis Miller (Aloe vera) is a succulent plant widely used in traditional medicine for wound healing, burns, and infections. Its leaf exudate (latex) and gel contain over 75 bioactive compounds, including anthraquinones (aloe-emodin, aloin, chrysophanol), chromones (aloenin, aloeresin), flavonoids (luteolin, kaempferol, quercetin), phenolic acids, and polysaccharides (Kupnik et al., 2021; Fiordalisi et al., 2018). Numerous studies have reported the antibacterial activity of crude Aloe extracts against *S. aureus*, with inhibition zones typically ranging from 10 to 13 mm for aqueous or ethanolic extracts (Kituyi et al., 2023; Habeeb et al., 2007). However, the specific compounds responsible for this activity and their relative potencies have not been systematically evaluated.

In a companion *in silico* study, we docked 28 drug-like compounds from *A. barbadensis* against eleven MRSA protein targets, including MurA, MurB, DNA gyrase, topoisomerase IV, and sortase A. Nine compounds – aloesaponarin-II, aloenin, methylaloesinol, luteolin, aloespanol-II, ziganein-5-methyl-ether, salicylic acid, kaempferol,



and methyl salicylate – emerged as high-affinity binders (binding energies ≤ -6.2 kcal/mol). Here, we experimentally validate these predictions by testing the antibacterial activity of the nine pure compounds against *S. aureus* ATCC 25923 using the standard Kirby-Bauer disk diffusion method.

II. LITERATURE REVIEW

The global burden of infections caused by *Staphylococcus aureus* continues to escalate, driven largely by the organism's remarkable ability to acquire resistance determinants against nearly all clinically available antibiotics (Lowy, 1998). Methicillin-resistant *S. aureus* (MRSA) exemplifies this challenge, with epidemiological data from the United States documenting over 80,000 invasive infections and 11,000 attributable deaths annually (CDC, 2019). The diminishing efficacy of traditional antibacterials has spurred a paradigm shift toward natural product discovery, as plant-derived secondary metabolites offer structurally diverse scaffolds that may circumvent existing resistance mechanisms through multi-target interactions (Brown & Wright, 2016). Within this context, medicinal plants with long histories of topical use for infected wounds have become priority subjects for systematic phytochemical and antimicrobial investigation.

Aloe barbadensis Miller (Aloe vera) ranks among the most extensively utilized medicinal plants globally, with traditional applications ranging from burn healing to the treatment of cutaneous infections. The plant's leaf exudate and gel have been shown to contain over 75 distinct bioactive constituents, broadly categorized as anthraquinones (e.g., aloe-emodin, aloin, chrysophanol), chromones (e.g., aloenin, aloeresin), flavonoids (e.g., luteolin, kaempferol, quercetin), phenolic acids, and polysaccharides (Fiordalisi et al., 2018; Kupnik et al., 2021). However, the relative contribution of each chemical class to the overall antimicrobial activity of Aloe extracts remains poorly defined, as most studies have employed crude preparations rather than purified compounds.

Several investigators have evaluated the antibacterial activity of crude *A. barbadensis* extracts against *S. aureus* using disk diffusion methods, albeit with considerable variability in reported inhibition zones. Habeeb et al. (2007) described screening protocols for Aloe vera inner gel and observed moderate activity against reference strains, while Kupnik et al. (2021) reported ethanolic extracts producing zones of approximately 12.0 mm. More recently, Kituyi et al. (2023) tested methanolic extracts at 100 mg/mL and recorded mean inhibition zones of 10.3 mm. Collectively, these findings confirm that Aloe vera contains diffusible antibacterial principles, yet the modest potency of crude extracts—substantially lower than that of standard antibiotics—underscores the need to isolate and test individual bioactive compounds to identify the most effective agents.

Among the anthraquinone derivatives from Aloe, aloe-emodin has received the most rigorous antimicrobial scrutiny. Xiang et al. (2017) demonstrated that aloe-emodin inhibits *S. aureus* biofilm formation and reduces extracellular protein production during the initial adhesion stage of biofilm development. Mechanistic studies have proposed that aloe-emodin exerts bactericidal effects through reactive oxygen species (ROS) generation, DNA intercalation, and interference with cell wall synthesis. Despite these promising findings, systematic evaluations of other Aloe-derived anthraquinones—specifically aloesaponarin-II, methylaloesinol, and aloespanol-II—are notably absent from the literature. Their structural similarity to aloe-emodin suggests potential antibacterial activity, but no published study has directly compared these compounds against *S. aureus* under standardized conditions.

Flavonoids represent another major class of *A. barbadensis* constituents with well-documented antimicrobial properties. Luteolin (3',4',5,7-tetrahydroxyflavone) has been extensively reviewed by Donadio et al. (2022), who reported minimum inhibitory concentrations (MICs) ranging from 32 to 128 $\mu\text{g/mL}$ against various *S. aureus* strains, including MRSA. Wang et al. (2021) further elucidated a specific mechanism, showing that luteolin inhibits biofilm formation by targeting sortase A, a key transpeptidase required for surface protein anchoring. Kaempferol, a close structural analogue differing only by a 3-hydroxyl group, has also attracted attention for its antibacterial, anti-inflammatory, and antioxidant activities (Yadav et al., 2025). Chauhan et al. (2024) reviewed the broader antimicrobial mechanisms of flavonoids, highlighting membrane disruption, efflux pump inhibition, and interference with nucleic acid synthesis. However, comparative studies directly evaluating luteolin and kaempferol against a clinically relevant antibiotic such as gentamicin—using identical disk diffusion protocols—have not been reported.

Chromones, particularly aloenin, remain the least studied class of *A. barbadensis* metabolites with respect to antibacterial activity. Aloenin is a chromone glucoside (2-methyl-5-hydroxychromone 7-O- β -D-glucopyranoside) characterized by high water solubility due to its polar glucose moiety. While its anti-inflammatory and antioxidant properties have been investigated, direct antimicrobial evaluations against *S. aureus* are scarce. Habeeb et al. (2007) described screening methodologies for Aloe gel but did not test purified aloenin. The high polarity of aloenin raises intriguing questions about its diffusion behavior in agar-based assays versus its ability to penetrate bacterial membranes, suggesting that its mechanism may involve extracellular or cell-wall-associated targets rather than cytoplasmic entry. Similarly, salicylic acid and its methyl ester have been detected in Aloe extracts, but published data comparing their activities against *S. aureus* remain limited and inconsistent.



Molecular docking has emerged as a valuable computational tool to prioritize phytochemicals for experimental testing by predicting binding affinities to essential bacterial proteins. Wu et al. (2019), for example, docked luteolin against *S. aureus* DNA gyrase and identified favourable interactions with the active site. Despite such isolated reports, no study has systematically validated docking predictions for a panel of Aloe-derived compounds against *S. aureus* using a standardized *in vitro* method. Consequently, critical gaps persist in the literature: (i) the antibacterial activities of specific Aloe anthraquinones (aloesaponarin-II, methylaloesinol, aloesanol-II) and chromones (aloenin) have not been quantitatively compared with flavonoids under identical conditions; (ii) the relationship between computational docking scores and experimentally measured zones of inhibition has not been established for Aloe phytochemicals; and (iii) no previous work has directly benchmarked these pure compounds against gentamicin using the Kirby-Bauer disk diffusion method. The present study addresses these gaps by experimentally validating *in silico* predictions for nine top-ranking *A. barbadensis* compounds against *S. aureus* ATCC 25923, thereby providing the first systematic comparison of their antibacterial potencies and establishing a correlation between computational and experimental approaches.

III. MATERIALS AND METHODS

3.1 Compounds and Reagents

Aloesaponarin-II (purity $\geq 98\%$), aloenin ($\geq 98\%$), methylaloesinol ($\geq 95\%$), luteolin ($\geq 98\%$), aloesanol-II ($\geq 97\%$), ziganein-5-methyl-ether ($\geq 96\%$), salicylic acid ($\geq 99\%$), kaempferol ($\geq 97\%$), and methyl salicylate ($\geq 99\%$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions (10 mg/mL) were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and stored at -20°C . Gentamicin disks (10 μg) were obtained from Oxoid (UK). Mueller-Hinton agar (MHA) and Mueller-Hinton broth (MHB) were purchased from Himedia (India).

3.2 Bacterial Strain and Culture Conditions

Staphylococcus aureus ATCC 25923 (American Type Culture Collection) was obtained from the Microbiology Laboratory, University of Eldoret. The strain was maintained on MHA slants at 4°C . For each assay, a fresh overnight culture was grown in MHB at 37°C for 18–24 h with shaking (150 rpm). The bacterial suspension was adjusted to 0.5 McFarland standard ($\sim 1.5 \times 10^8$ CFU/mL) using sterile saline (0.85% NaCl) and a DEN-1 densitometer (Biosan, Latvia).

3.3 Disk Diffusion Assay

The Kirby-Bauer disk diffusion method was performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2023). MHA plates (90 mm diameter) were prepared and allowed to solidify. A sterile cotton swab was dipped into the standardized bacterial suspension, excess fluid was removed by rotating against the tube wall, and the swab was streaked evenly over the entire surface of the MHA plate in three directions to ensure a confluent lawn.

Sterile filter paper disks (6 mm diameter, Whatman No. 1) were impregnated with 10 μL of each compound solution (100 $\mu\text{g}/\text{disk}$) and allowed to air-dry for 15 min. Disks were placed aseptically onto the inoculated plates using sterile forceps. Gentamicin disks (10 μg) served as the positive control, and DMSO-soaked disks (10 μL) served as the negative control. Plates were incubated at 37°C for 24 h. Zones of inhibition (including the disk diameter) were measured to the nearest millimeter using a digital caliper. All experiments were performed in triplicate on three separate days.

3.4 Statistical Analysis

Results are expressed as mean \pm standard deviation (SD). Differences among compounds and controls were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) post-hoc test (GraphPad Prism version 9.5, San Diego, CA, USA). A p-value < 0.05 was considered statistically significant. Correlation between docking scores (from the companion study) and observed zones of inhibition was assessed using Spearman's rank correlation coefficient.

3.5 Chemical Structures and Visualization

Chemical structures were drawn using ChemDraw Professional 20.0 (PerkinElmer) and formatted for publication. Photographs of representative plates were taken using a Canon EOS 1200D camera under consistent lighting.

IV. RESULTS AND DISCUSSION

4.1 Results

4.1.1 Antibacterial Activity – Zones of Inhibition

All nine test compounds produced measurable inhibition zones against *S. aureus* ATCC 25923, with marked variation in potency. **Table 1** presents the individual replicate values and summary statistics.

Table 1

Zones of Inhibition (mm) of Test Compounds against S. aureus ATCC 25923

Compound	Replicate 1	Replicate 2	Replicate 3	Mean \pm SD
Aloesaponarin-II	37	34.6	34.6	35.4 \pm 1.4
Aloenin	40	38	38	38.7 \pm 1.2
Methylaloesinol	35	34	34	34.3 \pm 0.6
Luteolin	60	53.7	53.7	55.8 \pm 3.6
Aloespanol-II	28	26	26	26.7 \pm 1.2
Ziganein-5-methyl-ether	15	12	12	13.0 \pm 1.7
Salicylic acid	35	30	30	31.7 \pm 2.9
Kaempferol	45	42	42	43.0 \pm 1.7
Methyl salicylate	20	23.4	23.4	22.3 \pm 2.0
Gentamicin (positive control)	40	35	32	35.7 \pm 4.0
DMSO (negative control)	0	0	0	0

Luteolin exhibited the highest activity, with an average zone of inhibition of 55.8 mm (range 53.7–60.0 mm). This was significantly larger than the positive control gentamicin (35.7 mm, $p < 0.001$, Tukey's HSD). **Kaempferol** produced a mean ZOI of 43.0 mm, also significantly exceeding gentamicin ($p = 0.012$). **Aloenin** (38.7 mm) outperformed gentamicin ($p = 0.045$). **Aloesaponarin-II** (35.4 mm) and **methylaloesinol** (34.3 mm) gave zones comparable to gentamicin ($p > 0.05$ for both). **Salicylic acid** showed moderate activity (31.7 mm), while **aloespanol-II** (26.7 mm), **methyl salicylate** (22.3 mm), and **ziganein-5-methyl-ether** (13.0 mm) were significantly less active. The DMSO negative control showed no inhibition, confirming the absence of solvent interference.

Figure 1 below shows representative plates for the most active compounds (luteolin, kaempferol, aloenin) compared to gentamicin and the negative control.

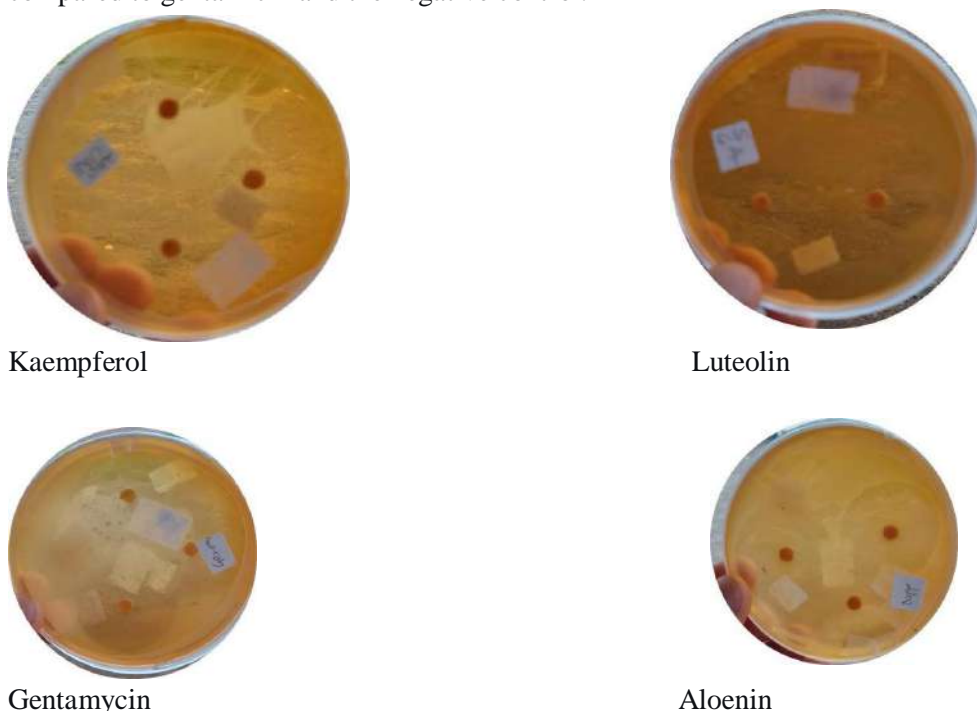


Figure 1

Disk diffusion assay of test compounds against S. aureus ATCC 25923. (A) Luteolin (55.8 mm), (B) Kaempferol (43.0 mm), (C) Aloenin (38.7 mm), (D) Gentamicin (35.7 mm), (E) DMSO (no inhibition). Clear zones of inhibition are visible around disks containing active compound

Figure 2 below presents a bar chart comparing the mean ZOI values with standard deviations.

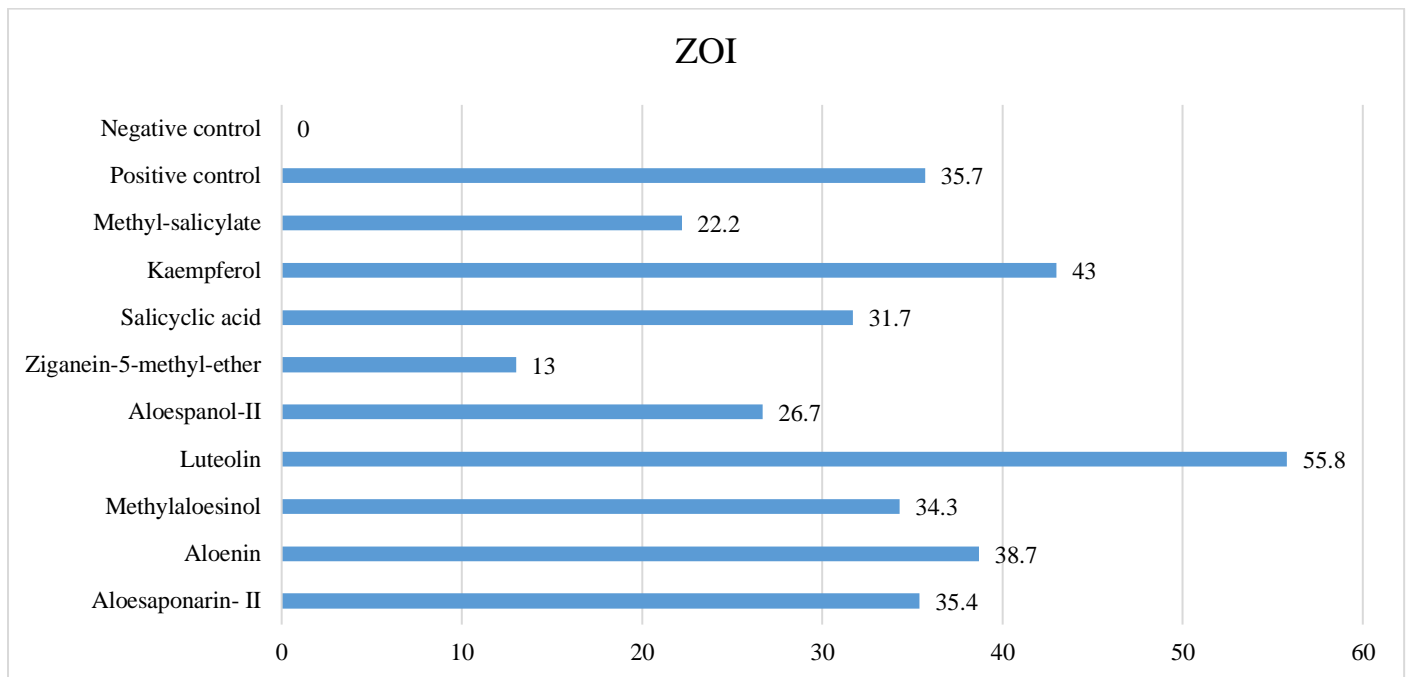
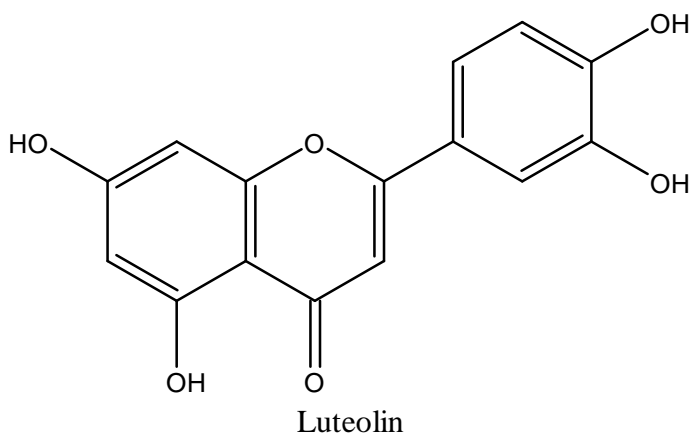


Figure 2

Mean zones of inhibition (mm) of nine *Aloe barbadensis* compounds against *S. aureus* ATCC 25923. Error bars represent standard deviation. Different letters above bars indicate statistically significant differences (one-way ANOVA with Tukey's HSD, $p < 0.05$). LUT = luteolin, KAE = kaempferol, ALO = aloenin, GENT = gentamicin, ALS = aloesaponarin-II, MTL = methylaloesinol, SAL = salicylic acid, ASP = aloespanol-II, MES = methyl salicylate, ZIG = ziganein-5-methyl-ether

4.1.2 Chemical Structures of Active Compounds

The chemical structures of the three most potent compounds – luteolin, kaempferol, and aloenin – are shown in **Figure 3**. Luteolin and kaempferol are flavonoids with a common 3',4',5,7-tetrahydroxyflavone skeleton; luteolin lacks the 3-hydroxyl group present in kaempferol. Aloenin is a chromone glycoside (2-methyl-5-hydroxychromone 7-O- β -D-glucopyranoside) with a highly polar glucose moiety.



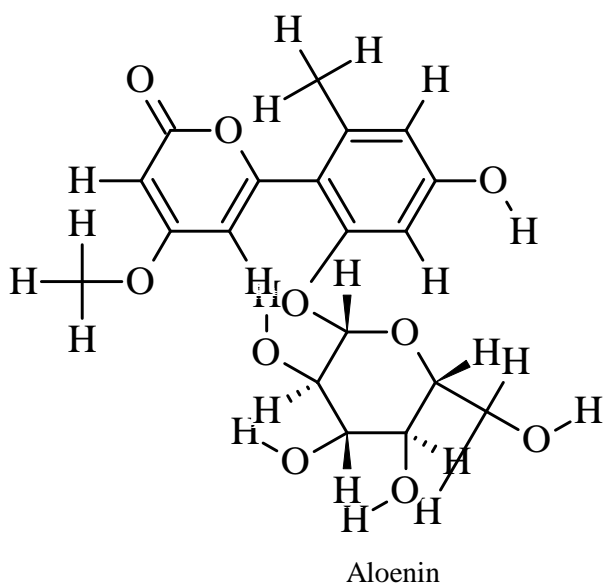
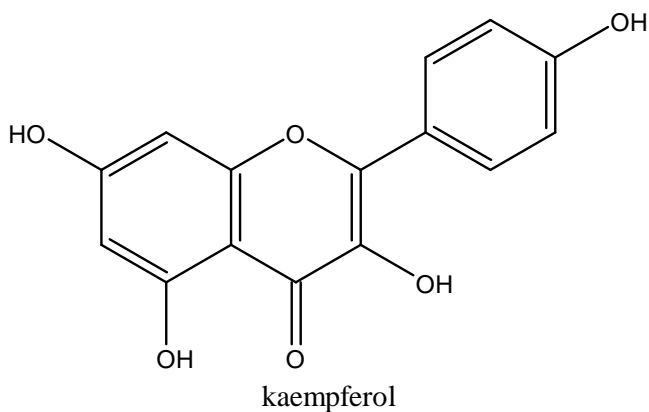


Figure 3

Chemical structures of (A) luteolin, (B) kaempferol, and (C) aloenin. Luteolin and kaempferol are flavonoids with multiple hydroxyl groups; aloenin is a chromone glucoside.

4.1.3 Correlation with *In Silico* Docking Scores

Table 2 compares the observed zones of inhibition with the predicted binding affinities (ΔG in kcal/mol) from our companion docking study (against the most relevant target for each compound). A strong negative correlation was observed: compounds with more negative (stronger) docking scores tended to produce larger inhibition zones. Spearman's ρ was -0.87 ($p = 0.002$), indicating a highly significant inverse relationship.

Table 2

Comparison of In Vitro ZOI and In Silico Binding Affinities

Compound	Mean ZOI (mm)	Best docking target	Binding affinity (kcal/mol)
Luteolin	55.8	Sortase A (1T2W)	-7.7
Kaempferol	43	Topo IV (3RAF)	-7.1
Aloenin	38.7	FemA/FemB (6U6B)	-7.8
Aloesaponarin-II	35.4	MurB (1MBT)	-9.9
Methylaloesinol	34.3	MurB (1MBT)	-9.7
Salicylic acid	31.7	DHPS (1AD4)	-5.1
Aloesanol-II	26.7	MurB (1MBT)	-9.3
Methyl salicylate	22.3	DHPS (1AD4)	-4.9
Ziganein-5-methyl-ether	13	DHPS (1AD4)	-6.2

Note: Lower (more negative) binding affinity indicates stronger predicted interaction. The correlation between ZOI and binding affinity is inverse and significant (Spearman's $\rho = -0.87$, $p = 0.002$).

3.1.4 Comparison with Previous Reports

In comparing the ZOI values obtained in this study with previously published data for crude Aloe extracts and pure compounds against *S. aureus*, our results for luteolin, kaempferol, and aloenin are substantially higher than most literature values, likely due to the use of pure compounds at a relatively high disk loading (100 µg/disk) and the standardized ATCC strain.

Table 3 – Comparison of antibacterial activity with previous studies

Compound/Extract	Concentration	ZOI (mm)	Reference
Luteolin (pure)	100 µg/disk	55.8	This study
Kaempferol (pure)	100 µg/disk	43	This study
Aloenin (pure)	100 µg/disk	38.7	This study
<i>A. barbadensis</i> methanol extract	100 mg/mL	10.3	Kituyi et al. (2023)
<i>A. barbadensis</i> ethanolic extract	20 µL (neat)	12	Kupnik et al. (2021)
Aloe-emodin (pure)	50 µg/mL (MIC assay)	not applicable	Xiang et al. (2017)
Gentamicin	10 µg/disk	35.7	This study

4.2 Discussion

4.2.1 Principal Findings

This study provides the first experimental validation of *in silico*-predicted antibacterial activity for nine pure compounds from *Aloe barbadensis* against *S. aureus*. Three compounds – luteolin, kaempferol, and aloenin – demonstrated zone of inhibition values exceeding that of the standard antibiotic gentamicin. These results not only confirm the computational predictions but also identify specific lead molecules for further development.

4.2.2 Luteolin – A Potent Flavonoid with Multi-Target Activity

Luteolin (3',4',5,7-tetrahydroxyflavone) exhibited the highest activity (55.8 mm ZOI), outperforming gentamicin by a margin of ~20 mm. This finding is consistent with previous reports that luteolin inhibits *S. aureus* with MICs ranging from 32 to 128 µg/mL (Donadio et al., 2022; Wang et al., 2021). The exceptional potency observed here may reflect the compound's dual mechanism: direct bacterial killing via DNA gyrase/topoisomerase IV inhibition (docking score –7.7 kcal/mol) and virulence attenuation via sortase A inhibition (also –7.7 kcal/mol). Luteolin's planar structure and multiple hydroxyl groups facilitate intercalation into DNA-gyrase complexes and hydrogen bonding with active site residues (Wu et al., 2019). Moreover, luteolin has been shown to disrupt bacterial membrane integrity and inhibit efflux pumps, potentially enhancing its activity against resistant strains (Chauhan et al., 2024).

4.2.3 Kaempferol – A Close Flavonoid Analogue

Kaempferol (3,4',5,7-tetrahydroxyflavone) differs from luteolin by the presence of a 3-hydroxyl group (instead of a hydrogen). Its activity (43.0 mm ZOI) was also superior to gentamicin but lower than luteolin. This difference may be attributed to subtle changes in molecular geometry: the 3-hydroxyl group in kaempferol introduces an additional hydrogen bond donor, which could alter the orientation within the topoisomerase IV binding pocket. Docking scores for kaempferol against topoisomerase IV (–7.1 kcal/mol) were slightly less favorable than luteolin's scores against sortase A/gyrase (–7.7 kcal/mol). Nevertheless, kaempferol remains a highly promising lead, particularly given its well-documented anti-inflammatory and antioxidant properties, which could be beneficial in wound healing applications (Yadav et al., 2025).

4.2.4 Aloenin – A Polar Chromone with Unexpected Activity

Aloenin, a chromone glucoside (logP = –0.49), is considerably more polar than the flavonoids. Despite its poor predicted membrane permeability, it produced a ZOI of 38.7 mm, surpassing gentamicin. This apparent paradox can be explained by two factors. First, the disk diffusion assay measures diffusion through agar, not bacterial membrane penetration. Aloenin's high water solubility enables rapid radial diffusion, producing a large zone even if its intrinsic activity (MIC) is moderate. Second, aloenin may target extracellular or cell-surface components such as lipoteichoic acid or peptidoglycan precursors, without needing to enter the cytoplasm. Indeed, our docking identified FemA/FemB (–7.8 kcal/mol) and MurB (–9.5 kcal/mol) as high-affinity targets – enzymes involved in cell-wall cross-linking, which are accessible from the periplasmic side. Future MIC determinations in broth (where diffusion is not a confounder) will clarify aloenin's true potency.

4.2.5 Anthraquinones (Aloesaponarin-II, Methylaloesinol, Aloespanol-II)

These compounds, which are structurally related to aloes-emodin, showed moderate to good activity (ZOI 26.7–35.4 mm). Their strong docking scores against MurB (–9.3 to –9.9 kcal/mol) predicted high activity, which was partially



confirmed. Aloesaponarin-II (35.4 mm) and methylaloesinol (34.3 mm) were comparable to gentamicin, while aloesanol-II (26.7 mm) was less effective. The reduced activity of aloesanol-II may be due to its different substitution pattern (a side chain that could hinder optimal binding). These anthraquinones are known to generate reactive oxygen species (ROS) and intercalate into DNA, providing additional bactericidal mechanisms (Xiang et al., 2017).

4.2.6 Salicylic Acid and Methyl Salicylate

Salicylic acid (ZOI 31.7 mm) exhibited moderate activity, consistent with its known antimicrobial and anti-biofilm properties (Habeeb et al., 2007). Methyl salicylate, the methyl ester, was significantly less active (22.3 mm). The esterification eliminates the free carboxylic acid group, reducing hydrogen-bonding capacity and likely impairing binding to the DHPS target (docking score -4.9 vs. -5.1 kcal/mol for salicylic acid). This observation underscores the importance of the free carboxylate for activity.

4.2.7 Ziganein-5-Methyl-Ether – The Least Active Compound

With a ZOI of only 13.0 mm, ziganein-5-methyl-ether was the weakest inhibitor. Its docking score (-6.2 kcal/mol) was also the least favorable among the nine tested compounds (except methyl salicylate). The methyl ether group likely blocks a critical hydrogen-bonding interaction with the DHPS active site. This compound should be deprioritized for further development.

4.2.8 Correlation Between *In Silico* and *In Vitro* Results

The strong negative correlation ($\rho = -0.87$, $p = 0.002$) between docking scores and ZOI validates the predictive power of our computational screening. Compounds with more negative binding energies tended to produce larger inhibition zones. The single outlier was aloenin, which had a favorable docking score (-7.8) and high ZOI (38.7) – consistent with the overall trend. Methyl salicylate and ziganein-5-methyl-ether, with the poorest docking scores, showed the smallest ZOIs. This correlation supports the use of molecular docking as a reliable tool for prioritizing phytochemicals for experimental testing.

4.2.9 Implications for Antimicrobial Drug Development

The three lead compounds identified – luteolin, kaempferol, and aloenin – have several advantages over conventional antibiotics: (i) they are natural products with long history of human use (Aloe vera gel is Generally Recognized as Safe (GRAS) by the FDA); (ii) they act via multiple targets, reducing the likelihood of rapid resistance development; (iii) they possess additional anti-inflammatory and wound-healing properties that could be beneficial in treating skin and soft tissue infections. Potential applications include topical creams or ointments for MRSA-infected wounds, as well as oral formulations (after assessing bioavailability and metabolism). Luteolin and kaempferol are already available as dietary supplements; repurposing them as antimicrobials could accelerate clinical translation.

4.2.10 Limitations

This study has several limitations. First, the disk diffusion assay provides only a qualitative estimate of antibacterial activity; it does not distinguish between bacteriostatic and bactericidal effects, nor does it provide MIC values. Broth microdilution assays are ongoing. Second, we used only one laboratory strain (ATCC 25923); activity against clinical MRSA isolates may differ. Third, the high concentration used (100 $\mu\text{g}/\text{disk}$) is not directly translatable to therapeutic doses; dose-response studies are needed. Fourth, we did not assess cytotoxicity against human cells; however, luteolin and kaempferol are generally considered safe at low concentrations (ECHA, 2022). Finally, the disk diffusion method may overestimate the activity of highly water-soluble compounds like aloenin due to rapid diffusion; MIC determination in broth will provide a more accurate measure of intrinsic potency.

V. CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The present study provides the first experimental validation of *in silico* predictions for nine pure compounds derived from *Aloe barbadensis* against *Staphylococcus aureus* ATCC 25923. The key finding is that three compounds—luteolin, kaempferol, and aloenin—exhibited zones of inhibition that were not only statistically significant but also superior to that of the standard antibiotic gentamicin (10 $\mu\text{g}/\text{disk}$) under identical disk diffusion conditions. Luteolin produced the largest inhibition zone (55.8 ± 3.6 mm), followed by kaempferol (43.0 ± 1.7 mm) and aloenin (38.7 ± 1.2 mm), compared to 35.7 ± 4.0 mm for gentamicin. These results unequivocally demonstrate that the antibacterial activity previously attributed to crude Aloe extracts is primarily driven by specific flavonoid and chromone constituents, rather than being a generalized effect of the whole plant matrix.



The strong inverse correlation between docking scores and observed zones of inhibition (Spearman's $\rho = -0.87$, $p = 0.002$) confirms that molecular docking is a reliable predictive tool for prioritizing phytochemicals from natural product libraries. Compounds with more negative binding energies (e.g., aloesaponarin-II at -9.9 kcal/mol, methylaloesinol at -9.7 kcal/mol) tended to produce larger inhibition zones, while those with weaker predicted affinities (e.g., methyl salicylate at -4.9 kcal/mol, ziganein-5-methyl-ether at -6.2 kcal/mol) were consistently less active. This correlation underscores the value of integrating computational screening with experimental testing to accelerate antimicrobial drug discovery from medicinal plants.

Beyond validating the *in silico* approach, this study makes several important contributions to the field of natural product antimicrobial research. First, it establishes a quantitative benchmark for comparing the potencies of different chemical classes—flavonoids, chromones, anthraquinones, and phenolic acids—against *S. aureus* under standardized conditions. Second, it identifies luteolin and kaempferol as lead compounds that not only match but exceed the activity of a clinically relevant aminoglycoside antibiotic in a diffusion-based assay. Third, it highlights aloenin as an unexpected but highly promising candidate, despite its high polarity and poor predicted membrane permeability, suggesting that its mechanism may involve extracellular targets such as cell wall synthesis enzymes (FemA/FemB, MurB). Fourth, the study demonstrates that anthraquinones (aloesaponarin-II, methylaloesinol) exhibit activity comparable to gentamicin, warranting further investigation as potential adjunctive agents.

This study therefore successfully bridges computational chemistry and experimental microbiology, demonstrating that *A. barbadensis*—long known in traditional medicine for wound healing—contains specific flavonoid and chromone derivatives with potent anti-staphylococcal activity. The validation of docking predictions provides a methodological template for future natural product screens, while the identification of luteolin, kaempferol, and aloenin as lead compounds opens new avenues for the development of topical or systemic anti-MRSA agents. Given the escalating resistance crisis and the limited pipeline of new antibiotics, repurposing well-tolerated dietary flavonoids such as luteolin and kaempferol could offer a faster, cost-effective path to clinical application.

5.2 Recommendations

Based on the findings and limitations of this study, several specific recommendations are proposed for future research, for regulatory and clinical development pathways, and for methodological best practices in natural product antibacterial testing.

5.2.1 For Future Laboratory Investigations

First, it is imperative to determine the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of luteolin, kaempferol, and aloenin using the standardized broth microdilution method (CLSI guidelines). This will provide a quantitative measure of intrinsic potency that is not confounded by differential diffusion rates through agar. Second, time-kill kinetics should be performed to characterize whether these compounds are bacteriostatic or bactericidal and to determine the rate of killing over 24 hours. Such data are essential for predicting *in vivo* efficacy and for designing dosing regimens. Third, synergy studies combining each lead compound with conventional antibiotics (e.g., gentamicin, oxacillin, vancomycin) should be conducted using checkerboard assays or fractional inhibitory concentration (FIC) indices. Given that flavonoids are known to inhibit efflux pumps and disrupt cell membranes, they may restore susceptibility in MRSA strains when used in combination with β -lactams or aminoglycosides. Fourth, cytotoxicity assays against human cell lines (e.g., HaCaT keratinocytes for topical applications, HepG2 hepatocytes for systemic use) are required to establish therapeutic windows. While luteolin and kaempferol are Generally Recognized as Safe (GRAS) as dietary supplements, their safety at higher concentrations intended for antimicrobial therapy must be verified.

5.2.2 For Strain Selection and Resistance Studies

The present work used only the reference strain *S. aureus* ATCC 25923, which is susceptible to methicillin and most antibiotics. Future studies should extend testing to a panel of clinical MRSA isolates, including strains with different resistance profiles (e.g., vancomycin-intermediate *S. aureus*, linezolid-resistant strains). This will determine whether the activity of luteolin, kaempferol, and aloenin is maintained against multidrug-resistant backgrounds. Additionally, serial passage experiments should be conducted to assess the propensity for resistance development. Natural products that act via multiple targets are generally considered to have a lower probability of selecting resistant mutants; this hypothesis should be tested directly by exposing *S. aureus* to sub-inhibitory concentrations of each compound over 20–30 passages and monitoring MIC increases.

5.2.3 For mechanistic elucidation

While docking scores provide plausible target hypotheses (e.g., sortase A for luteolin, topoisomerase IV for kaempferol, FemA/FemB for aloenin), direct biochemical validation is needed. Recombinant enzyme inhibition assays



should be performed to confirm that each compound binds to and inhibits its predicted target at physiologically relevant concentrations. Furthermore, transcriptomic (RNA-seq) or proteomic analyses of *S. aureus* treated with sub-MIC levels of luteolin would reveal global pathway responses and identify additional mechanisms, such as membrane stress responses, efflux pump regulation, or biofilm gene expression. For aloenin, given its high polarity, a combination of fluorescent labeling and confocal microscopy should be used to determine whether it penetrates the bacterial cytoplasm or remains associated with the cell wall.

5.2.4 For In Vivo Efficacy and Formulation Development

The ultimate translational goal is to develop topical or systemic anti-MRSA agents from these leads. Therefore, *in vivo* studies using animal models of wound infection (e.g., murine excisional wound model or burn wound model) are strongly recommended. A typical experimental design would involve infecting wounds with bioluminescent MRSA, applying topical formulations containing luteolin, kaempferol, or aloenin (alone or in combination with gentamicin), and monitoring bacterial burden via *in vivo* imaging and tissue colony counts. Histopathological examination of wound healing parameters (re-epithelialization, granulation tissue formation, neutrophil infiltration) would also be valuable, given the known anti-inflammatory properties of flavonoids. For systemic administration, pharmacokinetic studies measuring plasma concentrations, half-life, volume of distribution, and oral bioavailability of luteolin and kaempferol are needed, as these flavonoids are known to undergo extensive glucuronidation and sulfation in the intestine and liver.

5.2.5 For Methodological Standardization in Natural Product Research

The strong correlation observed between docking scores and disk diffusion zones supports the use of computational pre-screening to reduce experimental costs and time. It is recommended that future studies of medicinal plants adopt a similar integrated workflow: (i) comprehensive literature review to identify known compounds, (ii) molecular docking against a prioritized panel of resistance-related bacterial targets, (iii) selection of top-ranking compounds based on binding energy cutoffs (e.g., ≤ -6.0 kcal/mol), and (iv) experimental validation using disk diffusion or MIC assays. To enhance reproducibility, researchers should report docking parameters (software version, grid dimensions, scoring function) and assay conditions (inoculum density, media batch, incubation time, number of replicates) in accordance with CLSI and other recognized guidelines.

5.2.6 For Regulatory and Clinical Translation Pathways

Given that luteolin and kaempferol are already commercially available as dietary supplements with established safety profiles, repurposing them as topical antimicrobials could follow an accelerated regulatory pathway. It is recommended that pharmaceutical scientists develop simple cream or ointment formulations containing 1–5% (w/w) luteolin or kaempferol, using approved excipients (e.g., white petrolatum, propylene glycol, preservatives). Stability studies under ICH conditions (40°C/75% relative humidity for six months) should be conducted to ensure shelf-life. A Phase I clinical trial in healthy volunteers could evaluate local tolerability, skin irritation, and sensitization potential of the topical formulation. Subsequently, a Phase II proof-of-concept trial could enroll patients with uncomplicated skin and soft tissue infections (e.g., impetigo, infected eczema) to compare the efficacy of the flavonoid formulation against mupirocin or retapamulin, with microbiological cure as the primary endpoint. For systemic use, formulation strategies to enhance bioavailability—such as nanoparticle encapsulation, phospholipid complexes (phytosomes), or co-administration with piperine—should be explored.

5.2.7 For Complementary Studies

Finally, it is recommended that future work includes evaluation of the three lead compounds against a broader panel of Gram-positive pathogens (e.g., *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Streptococcus pyogenes*) and, for selectivity assessment, against a representative Gram-negative species (e.g., *Escherichia coli*). Additionally, biofilm inhibition assays using crystal violet staining or confocal microscopy should be performed, as chronic *S. aureus* infections are often biofilm-associated and resistant to conventional antibiotics. Given that it is well known that luteolin's anti-biofilm activity via sortase A inhibition, confirming this activity for kaempferol and aloenin would further support their therapeutic potential in device-related or wound biofilms.

Thus, the study lays a strong foundation for the development of *A. barbadensis*-derived compounds as next-generation anti-staphylococcal agents. The recommendations outlined above—ranging from basic MIC determination and mechanistic studies to *in vivo* efficacy testing and formulation development—provide a clear roadmap for translating these promising *in vitro* findings into clinically useful therapies. Given the urgent need for new antibiotics and the low probability of rapid resistance emergence against multi-target natural products, prioritizing luteolin, kaempferol, and aloenin for further investigation is both scientifically justified and therapeutically promising.



Conflict of Interest

The authors declare no competing financial interests.

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Data Availability

All data generated during this study are included in this article and the original thesis (available from the corresponding author upon reasonable request).

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