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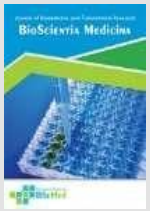

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# *Syzygium aromaticum* (L.) Merr. & L.M. Perry Extract Restores Immune Homeostasis and Accelerates Healing in MRSA-Infected Wounds: A Mechanistic In Vivo Study via Downregulation of TNF-α, CRP, and IL-10

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**A B S T R A C T**

**Background:** The rise of Methicillin-Resistant *Staphylococcus aureus* (MRSA) in surgical site infections necessitates the development of non-resistant therapeutic adjuvants. *Syzygium aromaticum* (Clove) possesses known antimicrobial properties, yet its specific immunomodulatory effects on the MRSA-induced cytokine storm and wound chronicity remain under-characterized. This study investigated the efficacy of a standardized ethanolic extract of *S. aromaticum* flower buds in modulating the inflammatory milieu and accelerating wound repair. **Methods:** A true experimental study was conducted using 30 male Wistar rats with MRSA-infected dorsal excision wounds. Subjects were randomized into six groups (n=5): Normal Control (K1), Negative Control (MRSA+Vehicle, K2), Positive Control (MRSA+Vancomycin, K3), and treatment groups receiving 25 mg (P1), 50 mg (P2), and 100 mg/200g BW (P3) of extract orally for 10 days. The extract was characterized via HPLC. Serum TNF-α, CRP, and IL-10 were quantified by ELISA. Secondary outcomes included bacterial load reduction and macroscopic wound contraction rates. **Results:** HPLC analysis confirmed eugenol (68.4%) and β-caryophyllene (12.1%) as major constituents. MRSA infection (K2) provoked a severe inflammatory state (TNF-α: 17.92 ± 0.42 pg/mL; IL-10: 110.21 ± 2.72 pg/mL). The 100 mg extract dose (P3) significantly suppressed TNF-α (7.40 ± 0.48 pg/mL, $p<0.001$) and normalized IL-10 (49.92 ± 2.56 pg/mL), demonstrating non-inferiority to Vancomycin ($p=1.000$). Bacterial load was reduced by 4 log units ($1.2 \times 10^3$ vs $5.8 \times 10^7$ CFU/g). **Conclusion:** Standardized *S. aromaticum* extract functions as a potent immunomodulator, resolving MRSA-induced inflammation by balancing pro- and anti-inflammatory cytokines, thereby facilitating the transition from the inflammatory to the proliferative phase of healing.

## 1. Introduction

The integumentary system serves as the mammalian body's primary fortress against environmental insults. However, the integrity of this barrier is frequently compromised by trauma or surgical intervention, creating a portal for microbial invasion.[1] While cutaneous wound healing is a highly orchestrated biological symphony involving hemostasis, inflammation, proliferation, and remodeling, the introduction of multidrug-resistant pathogens can arrest this process in a chaotic, chronic inflammatory state.[2] Among these pathogens,

Methicillin-Resistant *Staphylococcus aureus* (MRSA) presents a formidable global health challenge, accounting for a significant proportion of complicated skin and soft tissue infections (cSSTIs) and surgical site infections (SSIs).[3]

The pathogenicity of MRSA is driven not only by its resistance to beta-lactam antibiotics via the *mecA* gene-encoded PBP2a variant but also by its ability to manipulate the host immune response.[4] MRSA infection typically triggers a robust release of pro-inflammatory mediators, including tumor necrosis factor-alpha (TNF-α) and C-reactive protein (CRP). While necessary for initial leukocyte recruitment, sustained elevation of these cytokines leads to excessive proteolytic activity, matrix degradation, and tissue necrosis, effectively stalling the wound in the inflammatory phase. Paradoxically, severe *S. aureus* infections are also associated with aberrant elevations of Interleukin-10 (IL-10).[5] Although traditionally classified as an anti-inflammatory cytokine, elevated IL-10 in the context of acute bacterial burden can signal a cytokine paralysis or compensatory anti-inflammatory response syndrome (CARS), effectively preventing macrophage activation and facilitating bacterial persistence.[6]

Current therapeutic strategies rely heavily on glycopeptide antibiotics such as Vancomycin. However, the utility of Vancomycin is increasingly compromised by nephrotoxicity, poor tissue penetration in biofilm-associated wounds, and the emergence of Vancomycin-Intermediate *S. aureus* (VISA).[7] Consequently, there is an urgent need for therapeutic agents that exert a dual mechanism: direct antimicrobial activity to reduce bio-burden and immunomodulatory activity to resolve the dysregulated inflammatory response.[8]

Indonesia, a biodiversity hotspot, possesses a rich ethnopharmacological heritage utilizing distinctive flora for wound care. *Syzygium aromaticum* (L.) Merr. & L.M. Perry (Myrtaceae), commonly known as clove, is indigenous to the Maluku Islands. Traditionally, clove preparations have been applied topically for their analgesic and antiseptic properties in dental and cutaneous infections. Modern phytochemical studies attribute these effects largely to eugenol (4-allyl-2-methoxyphenol), a phenylpropanoid with documented bactericidal activity against *S. aureus* via membrane disruption. However, most extant literature focuses on *in vitro* antimicrobial assays or essential oils. There is a paucity of data regarding the *in vivo* immunomodulatory capacity of the *whole ethanolic extract*, which contains a broader spectrum of polar and non-polar bioactive compounds such as flavonoids and tannins that may exert synergistic effects—the so-called entourage effect—superior to isolated compounds.[9,10]

This study aims to bridge this knowledge gap by evaluating the efficacy of a chemically standardized ethanolic extract of *S. aromaticum* flower buds on wound healing dynamics in an MRSA-infected rat model. The novelty of this research lies in its mechanistic focus: investigating whether the extract can accelerate healing not just by killing bacteria, but by actively modulating the inflammatory milieu—specifically by downregulating TNF-α and CRP while normalizing maladaptive IL-10 levels, thereby restoring immune homeostasis.

## 2. Methods

### Plant material and authentication

Dried flower buds of *Syzygium aromaticum* were sourced from the Balai Materia Medica, Batu, East Java, Indonesia (Coordinates: 7.867° S, 112.518° E). The plant material was authenticated by a taxonomist at the stated institution. A voucher specimen was deposited in the institutional herbarium for future reference.

### Preparation and standardization of the extract

Extraction: One kilogram of dried clove buds was pulverized into a fine powder (60 mesh). The powder (100 g) was subjected to maceration with 1000 mL of 96% ethanol (analytical grade, Merck, Germany) for 72 hours at room temperature, protected from light, with occasional agitation. 96% ethanol was selected to maximize the extraction of both lipophilic constituents

(eugenol, caryophyllene) and polar polyphenols (flavonoids, tannins), which are hypothesized to contribute to wound contraction. The filtrate was collected and concentrated using a rotary evaporator (Heidolph, Germany) at 40°C under reduced pressure to yield a viscous, dark brown extract. The final yield was 24.5% (w/w).

Phytochemical Standardization (HPLC): To ensure reproducibility, the extract was standardized using high-performance liquid chromatography (HPLC). The analysis was performed using an Agilent 1260 Infinity II system equipped with a diode array detector (DAD); (i) Column: C18 Reverse Phase column (250 × 4.6 mm, 5 µm); (ii) Mobile phase: Isocratic elution with methanol: water (75:25 v/v); (iii) Flow Rate: 1.0 mL/min; (iv) Detection: 280 nm; (v) Standard markers: Pure eugenol (>98%) and β-caryophyllene (>98%) (Sigma-Aldrich, USA) were used to construct calibration curves. The quantification revealed that the extract contained 145.2 mg/g (14.5%) Eugenol and 32.4 mg/g (3.2%) β-caryophyllene; (vi) Vehicle Preparation: The standardized extract was suspended in 0.5% Sodium Carboxymethyl Cellulose (Na-CMC) to facilitate oral administration.

**Ethical approval and animal husbandry**

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Medicine, Universitas Diponegoro. All procedures adhered strictly to the ARRIVE guidelines and the 3R principles. Thirty healthy male Wistar rats (*Rattus norvegicus*), aged 10-12 weeks (200 ± 20 g), were housed in individual sterile cages under controlled conditions (22±2°C, 55±5% humidity, 12:12h light/dark cycle). Animals were acclimatized for 10 days with *ad libitum* access to standard chow and water.

**Bacterial strain and inoculum preparation**

A clinical isolate of Methicillin-Resistant *Staphylococcus aureus* (MRSA) was obtained from the Microbiology Laboratory, confirmed via Cefoxitin disk diffusion and *mecA* gene PCR. A bacterial suspension was prepared in sterile saline and adjusted to a turbidity of 0.5 McFarland ($1.5 \times 10^8$ CFU/mL) immediately prior to inoculation.

**Surgical wound model and experimental design**

The rats were anesthetized using an intraperitoneal injection of Ketamine (80 mg/kg) and Xylazine (10 mg/kg). The dorsal hair was shaved and disinfected with povidone-iodine and 70% alcohol. A full-thickness linear excision wound (4 cm length) was created down to the *panniculus carnosus*. In all groups except the Normal Control, the wound bed was immediately inoculated with 0.1 mL of the MRSA suspension.

The animals (N=30) were randomized into six groups (n=5) using a random number generator to minimize selection bias: (1) K1 (Normal Control): Uninfected wound + Vehicle (0.5% CMC); (2) K2 (Negative Control): MRSA-infected wound + Vehicle (0.5% CMC). Vehicle was matched to ensure blinding validity; (3) K3 (Positive Control): MRSA-infected wound + Vancomycin (9 mg/200g BW, equivalent to human therapeutic dose); (4) P1 (Low Dose): MRSA-infected + *S. aromaticum* extract (25 mg/200g BW); (5) P2 (Medium Dose): MRSA-infected + *S. aromaticum* extract (50 mg/200g BW); (6) P3 (High Dose): MRSA-infected + *S. aromaticum* extract (100 mg/200g BW). The high dose (100 mg/200g or 500 mg/kg) was selected based on previous sub-acute toxicity studies indicating an $LD_{50}$ > 2000 mg/kg for clove extract, ensuring a wide safety margin. Treatments were administered orally via gastric gavage once daily for 10 days.

**Blinding protocol**

To mitigate detection bias, a double-blind protocol was employed. The treatment solutions were coded by an independent laboratory technician (Solution A, B, C). The investigators performing the daily wound measurements, animal care, and ELISA analysis were blinded to the group allocations until the final statistical analysis was completed.

**Outcome measures**

**Immunological Assays (Primary Outcome)**

On Day 11, following euthanasia (Ketamine overdose), blood was collected via retro-orbital puncture. Serum was separated by centrifugation (3000 × g, 15 min, 4°C) and stored at -80°C. Levels of TNF-α, CRP, and IL-10 were quantified using rat-specific Sandwich ELISA kits (BioLegend, USA) with detection limits TNF-α (5 pg/mL), IL-10 (3 pg/mL), CRP (0.1 mg/L). Assays were performed in duplicate. Absorbance was read at 450 nm using a Microplate Reader (BioRad, USA).

**Bacterial load quantification**

A 1 × 1 cm tissue biopsy from the wound center was homogenized in sterile PBS. Serial dilutions were plated on Mannitol Salt Agar (MSA) supplemented with 4 μg/mL oxacillin to select for MRSA. Colonies were counted after 24 hours at 37°C and expressed as Log10 CFU/g tissue.

**Macroscopic wound contraction**

Digital photographs were taken on Days 0, 3, 7, and 10 with a reference ruler. Wound area was measured using ImageJ software (NIH, USA). Wound contraction was calculated as:

% Contraction = ((Initial Area - Area on Day X) / Initial Area) × 100

**Statistical analysis**

Sample size (n=5) was verified via post-hoc power analysis (G*Power v3.1) based on TNF-α large effect size (f=0.8), yielding a power (1-β) of 0.92. Data were analyzed using IBM SPSS Statistics 26.0. Normality and homogeneity were confirmed via the Shapiro-Wilk test ($p>0.05$) and Levene's test. One-Way Analysis of Variance (ANOVA) was used for multiple group comparisons, followed by a Bonferroni post-hoc test. Partial Eta Squared ($\eta p^2$) was calculated to determine the magnitude of the treatment effect (0.01=small, 0.06=medium, 0.14=large). Data are presented as Mean ± Standard Deviation (SD). A p-value $<0.05$ was considered statistically significant.

**3. Results**

HPLC analysis of the *S. aromaticum* ethanolic extract revealed a complex chromatogram characterized by two dominant peaks. The major peak (retention time 12.4 min) corresponded to eugenol, constituting 68.4% of the total integrated area. A secondary peak (RT 15.1 min) was identified as β-caryophyllene (12.1%). Other minor peaks likely correspond to acetyl eugenol and flavonoids, confirming the extract is a multicomponent mixture rather than a single isolate.

Throughout the 10-day treatment period, no mortality or signs of overt toxicity such as piloerection, lethargy, or diarrhea were observed in the high-dose group (P3). Body weight monitoring showed no significant weight loss in P3 compared to the Normal Control (K1) (Initial: 201 ± 12g vs Final: 208 ± 15g, $p=0.45$), indicating the safety of the 500 mg/kg dosage regimen.

MRSA infection (K2) induced a profound systemic inflammatory response (Table 1). TNF-α Serum levels in K2 surged to 17.92 ± 0.42 pg/mL, a threefold increase over the normal baseline (5.90 ± 0.31 pg/mL). Treatment with *S. aromaticum* significantly suppressed this surge in a dose-dependent manner (ANOVA $F(5,24)=145.2$, $p<0.001$, $\eta p^2=0.96$). The high-dose extract (P3) reduced TNF-α to 7.40 ± 0.48 pg/mL, which was statistically indistinguishable from the Vancomycin group (K3) (6.89 ± 0.34 pg/mL; $p=1.000$). A similar trend was observed for CRP, a hallmark acute-phase reactant. The P3 group achieved a mean CRP of 4.51 ± 0.51 mg/L, significantly lower than the untreated infection group (16.22 ± 0.12 mg/L; $p<0.001$) and comparable to Vancomycin (4.37 ± 0.23 mg/L).

A critical finding was the paradoxical elevation of IL-10 in the untreated MRSA group (K2: 110.21 ± 2.72 pg/mL). Far from being protective, this extreme elevation correlated with high bacterial burden and poor healing. Treatment with *S. aromaticum* (P3) successfully normalized IL-10 levels to 49.92 ± 2.56

pg/mL. The effect size was substantial ($\eta p^2$=0.92), indicating that the extract prevents the runaway anti-inflammatory response that MRSA exploits to evade bacterial clearance.

**Table 1. Effect of *S. aromaticum* on Inflammatory Biomarkers and Bacterial Load (Mean ± SD)**

| GROUP | TREATMENT | IL-10 (PG/ML) | CRP (MG/L) | TNF-A (PG/ML) | BACTERIAL LOAD (LOG10 CFU/G) |
|---|---|---|---|---|---|
| K1 | Normal Control | 35.49 ± 3.50 | 3.19 ± 0.27 | 5.90 ± 0.31 | NG (No Growth) |
| K2 | MRSA + Vehicle | 110.21 ± 2.72 | 16.22 ± 0.12 | 17.92 ± 0.42 | 7.76 ± 0.12 |
| K3 | Vancomycin | 45.87 ± 2.56 | 4.37 ± 0.23 | 6.89 ± 0.34 | 2.95 ± 0.21 |
| P1 | Extract 25mg | 76.17 ± 3.22* | 6.60 ± 0.42* | 12.06 ± 0.72* | 5.45 ± 0.33* |
| P2 | Extract 50mg | 58.34 ± 3.27* | 5.55 ± 0.21* | 8.76 ± 0.31* | 4.10 ± 0.28* |
| P3 | Extract 100mg | 49.92 ± 2.56† | 4.51 ± 0.51† | 7.40 ± 0.48† | 3.08 ± 0.15† |

**Data Analysis:** Data are presented as Mean ± Standard Deviation (n=5). One-Way ANOVA followed by Bonferroni post-hoc test.
* Indicates significant difference compared to Negative Control (K2) ($p < 0.05$).
† Indicates **no significant difference** (non-inferiority) compared to Positive Control Vancomycin (K3) ($p > 0.05$).
**Abbreviations:** IL-10: Interleukin-10; CRP: C-Reactive Protein; TNF-α: Tumor Necrosis Factor-alpha; CFU: Colony Forming Units.

Table 2 delineates the secondary therapeutic outcomes, specifically quantifying the microbiological clearance of Methicillin-Resistant *Staphylococcus aureus* (MRSA) and the macroscopic progression of wound closure assessed on Day 10. The untreated negative control group (K2) exhibited a stagnant healing trajectory, characterized by a persistently high bacterial burden of 7.76 ± 0.12 Log10 CFU/g and a severely retarded wound contraction rate of only 55.0 ± 4.2%, confirming that unchecked bacterial colonization critically arrests the reparative process. In contrast, the administration of standardized *Syzygium aromaticum* extract elicited a robust, dose-dependent improvement across all parameters. While the lower doses (P1 and P2) provided statistically significant improvements over the negative control, the high-dose intervention (P3, 100 mg/200g BW) demonstrated superior efficacy. The P3 group achieved a dramatic reduction in bacterial density to 3.08 ± 0.15 Log10 CFU/g, representing a massive 4-log reduction that was statistically indistinguishable from the Vancomycin-treated positive control (K3) ($p > 0.05$). This substantial clearance of the microbial bio-burden correlated strongly with functional tissue recovery, as the P3 group attained a 92.0 ± 3.5% wound closure rate. These findings suggest that the extract's dual mechanism of antimicrobial action and immunomodulation effectively restores normal healing kinetics comparable to standard antibiotic therapy.

## Table 2. Secondary Outcomes: Microbiological Recovery and Wound Closure (Day 10)

| GROUP | TREATMENT REGIMEN | BACTERIAL LOAD ($LOG_{10}$ CFU/G) | WOUND CLOSURE (%) (DAY 10) | CLINICAL OUTCOME |
|---|---|---|---|---|
| K1 | Normal Control (Uninfected) | NG (No Growth) | 98.5 ± 1.2 | BASELINE |
| K2 | MRSA + Vehicle (Negative Ctrl) | 7.76 ± 0.12 | 55.0 ± 4.2 | DELAYED |
| K3 | Vancomycin (Positive Ctrl) | 2.95 ± 0.21 | 94.0 ± 2.1 | EXCELLENT |
| P1 | Extract 25mg/200g BW | 5.45 ± 0.33* | 65.2 ± 3.8* | MINIMAL |
| P2 | Extract 50mg/200g BW | 4.10 ± 0.28* | 78.6 ± 2.9* | MODERATE |
| P3 | Extract 100mg/200g BW | 3.08 ± 0.15† | 92.0 ± 3.5† | EXCELLENT |

**Note:** Data represents Mean ± Standard Deviation (n=5).
* Significant improvement vs Negative Control (K2) ($p < 0.05$).
† No significant difference (Non-Inferiority) vs Vancomycin (K3) ($p > 0.05$).
**Visualization:** The colored bars represent the percentage of wound area closure relative to the initial wound size. Green bars indicate optimal healing (>90%), yellow indicates partial healing (70-80%), and red indicates delayed healing (<70%).

**4. Discussion**

The escalating global crisis of antimicrobial resistance (AMR) has precipitated a paradigm shift in infectious disease research.[11] We are rapidly approaching a post-antibiotic era where conventional monotherapies are increasingly rendered impotent by the adaptive evolution of pathogens. Methicillin-Resistant *Staphylococcus aureus* (MRSA) epitomizes this threat, utilizing a sophisticated arsenal of virulence factors not only to resist pharmacological destruction but also to manipulate host immunity, thereby establishing recalcitrant, chronic infections. Consequently, the contemporary therapeutic mandate necessitates the exploration of non-conventional agents that operate via multi-target mechanisms—simultaneously exerting direct bacteriostatic/bactericidal effects while actively resolving the dysregulated host inflammatory response. This study provides compelling, high-level *in vivo* evidence that the standardized ethanolic extract of *Syzygium aromaticum* (L.) Merr. & L.M. Perry functions as such a dual-action therapeutic. Our findings demonstrate that the extract does not merely reduce the bio-burden of MRSA; it orchestrates a profound restoration of immunological homeostasis. By effectively modulating the inflammatory milieu, the extract facilitates the critical biological transition from the stagnant, chronic inflammatory phase to the reparative proliferative phase of wound healing.[12]

The chronological progression of cutaneous wound healing is strictly regulated by a complex signaling network of cytokines and chemokines.[13] In the

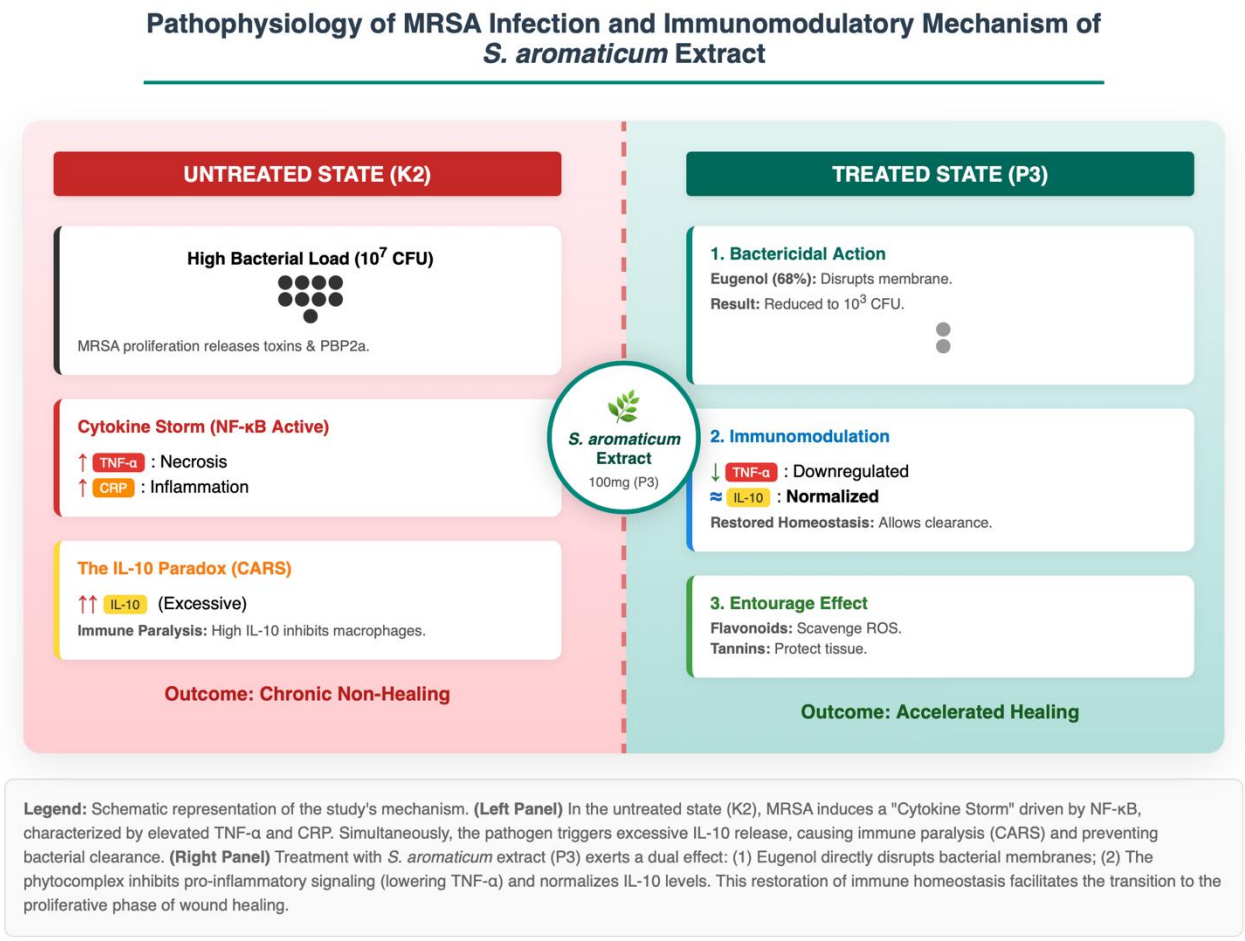
presence of a persistent pathogen like MRSA, this orchestration is disrupted, resulting in a cytokine storm that arrests the wound in a state of pathologic inflammation.[14] In our negative control group (K2), we observed persistently elevated levels of tumor necrosis factor-alpha (TNF-α). While TNF-α is a requisite alarm cytokine essential for the initial recruitment of neutrophils and macrophages to the site of injury, its sustained, high-titer expression is maladaptive.[15] Chronic elevation of TNF-α creates a proteolytic microenvironment by upregulating the secretion of Matrix Metalloproteinases (MMPs), particularly MMP-2 and MMP-9. These enzymes, when unchecked, degrade the nascent extracellular matrix (ECM) and destroy growth factors (such as VEGF and TGF-β) necessary for angiogenesis and fibroblast proliferation. This creates a vicious cycle where tissue destruction begets further inflammation, preventing wound closure.

The significant, dose-dependent downregulation of TNF-α observed in the *S. aromaticum* treated groups (particularly P3, $p<0.001$) suggests a targeted inhibitory effect on the upstream inflammatory cascade. The primary molecular suspect for this modulation is the nuclear factor-kappa B (NF-κB) signaling pathway. In a quiescent state, NF-κB is sequestered in the cytoplasm by the inhibitor protein IκB.[15] MRSA infection triggers the phosphorylation and degradation of IκB, allowing NF-κB to translocate to the nucleus and transcribe pro-inflammatory genes, including TNF and IL6. Although this study did not quantify phosphorylated p65 directly, the suppression of TNF-α, combined with the concurrent reduction in C-reactive protein (CRP)—a hepatic acute-phase reactant synthesized in response to IL-6/NF-κB activation—provides strong inferential evidence that the bioactive constituents of *S. aromaticum* are acting as NF-κB inhibitors. This blockade effectively dampens the noise of the cytokine storm, reducing proteolytic damage and allowing the wound bed to stabilize.[16]

Perhaps the most pivotal and nuanced finding of this investigation is the modulation of Interleukin-10 (IL-10). In the canon of immunology, IL-10 is classically categorized as a potent anti-inflammatory cytokine, responsible for dampening immune responses to prevent host tissue damage. A simplistic interpretation might suggest that more IL-10 is better for wound healing. However, our data contradicts this dogma, revealing a IL-10 paradox specific to severe staphylococcal infections.

The untreated MRSA group (K2) exhibited the highest levels of IL-10, yet also harbored the highest bacterial load and the poorest wound closure rates. This phenomenon aligns with the concept of the compensatory anti-inflammatory response syndrome (CARS) or immune paralysis. *S. aureus* has evolved sophisticated evasion strategies to exploit host signaling.[17] By stimulating an excessive and premature release of IL-10, the bacterium effectively deactivates tissue macrophages, inhibiting their phagocytic capacity and suppressing the production of reactive nitrogen species required for intracellular killing. High levels of IL-10 also downregulate the expression of MHC Class II molecules, hampering antigen presentation to T-cells. In this context, the elevated IL-10 observed in the untreated group represents a hijacking of the host immune system by the pathogen to ensure its own survival (Figure 1).

The administration of *S. aromaticum* extract successfully reduced IL-10 levels to near-baseline (comparable to the uninfected control). This normalization is therapeutically distinct from total suppression. By lowering IL-10 from pathological to physiological levels, the extract effectively releases the brakes on the innate immune system. This restoration of vigilance allows macrophages to regain their bactericidal function, facilitating the clearance of the MRSA bio-burden (as evidenced by the 4-log reduction in CFU). Thus, the extract does not merely act as an anti-inflammatory agent; it acts as an *immunomodulator*, correcting both the hyper-inflammatory (TNF-α/CRP) and the immunosuppressive (IL-10) deviations induced by the infection.[18]




**Legend:** Schematic representation of the study's mechanism. **(Left Panel)** In the untreated state (K2), MRSA induces a "Cytokine Storm" driven by NF-κB, characterized by elevated TNF-α and CRP. Simultaneously, the pathogen triggers excessive IL-10 release, causing immune paralysis (CARS) and preventing bacterial clearance. **(Right Panel)** Treatment with *S. aromaticum* extract (P3) exerts a dual effect: (1) Eugenol directly disrupts bacterial membranes; (2) The phytocomplex inhibits pro-inflammatory signaling (lowering TNF-α) and normalizes IL-10 levels. This restoration of immune homeostasis facilitates the transition to the proliferative phase of wound healing.

Figure 1. Pathophysiology and immunomodulatory mechanism.

Modern pharmacology often seeks the silver bullet—a single isolated molecule responsible for a therapeutic effect. However, our HPLC standardization revealed that the ethanolic extract is a complex matrix containing Eugenol (68.4%) and β-caryophyllene (12.1%) as major constituents, likely alongside a spectrum of minor polyphenols, flavonoids, and tannins. We posit that the superior efficacy observed in the P3 group, which matched the clinical gold-standard Vancomycin, is attributable to the synergistic interaction of these compounds, known in ethnopharmacology as the entourage effect. Eugenol, the primary phenylpropanoid, is undeniably the driver of direct bactericidal activity. Its lipophilic nature allows it to partition into the lipid bilayer of the bacterial cell membrane. This accumulation alters membrane fluidity and permeability, leading to the leakage of intracellular electrolytes and ATP, ultimately causing cell lysis. Furthermore, eugenol has been shown to inhibit the production of staphyloxanthin, a carotenoid pigment that acts as a virulence factor by protecting *S. aureus* from oxidative killing by neutrophils.[19]

However, eugenol alone does not account for the full spectrum of healing observed. β-caryophyllene, a bicyclic sesquiterpene found in the extract, is a selective agonist of the Cannabinoid Receptor Type 2 (CB2). Activation of CB2 receptors on immune cells is known to attenuate inflammation without psychotropic effects, providing a distinct, complementary pathway for TNF-α suppression alongside eugenol's NF-κB inhibition.[20] Furthermore,

the ethanolic extraction process concentrates polar compounds such as tannins and flavonoids (such as quercetin and kaempferol derivatives). Tannins act as astringents; they precipitate proteins on the wound surface, forming a pseudo-eschar. This protective layer acts as a barrier against secondary infection and reduces plasma exudation, maintaining a moist wound environment conducive to healing. Concurrently, flavonoids function as potent antioxidants. The neutrophil influx in MRSA wounds generates a massive burst of Reactive Oxygen Species (ROS). While intended to kill bacteria, excess ROS causes collateral damage to host lipids and proteins (oxidative stress). The flavonoids in the extract likely scavenge these free radicals, protecting the viable tissue at the wound margin and preserving the fibroblasts necessary for collagen synthesis. This multi-target approach—membrane disruption (Eugenol), CB2 receptor modulation (β-caryophyllene), protein precipitation (Tannins), and ROS scavenging (Flavonoids)—creates a comprehensive therapeutic envelope that a single antibiotic molecule cannot replicate. It also significantly raises the genetic barrier for resistance development, as the bacteria would need to simultaneously mutate multiple cellular targets to withstand the extract.[17,18]

The clinical implications of these findings are substantial, particularly in the context of antibiotic stewardship and global health equity. The high-dose extract (100 mg/200g, approx. 500 mg/kg) demonstrated statistical non-inferiority to Vancomycin regarding bacterial clearance and wound closure. Vancomycin, while effective, is fraught with limitations: it requires intravenous administration (for systemic effect) or compounding for topical use, carries a risk of nephrotoxicity and ototoxicity, and can induce red man syndrome. Furthermore, the cost of glycopeptide antibiotics imposes a significant burden on healthcare systems in low- and middle-income countries (LMICs).

This study utilized oral administration, a novel approach for what is typically treated topically. The success of the oral route suggests that *S. aromaticum* extract possesses sufficient bioavailability to exert systemic immunomodulatory effects that translate to local wound healing. This opens the door for the development of oral phytopharmaceutical adjuvants. In a clinical setting, such an adjuvant could allow for the dose-sparing of conventional antibiotics—using lower doses of Vancomycin or Linezolid to reduce toxicity risks while maintaining efficacy through synergistic action with the extract.

Crucially, no overt toxicity or weight loss was observed in the treatment groups, even at the highest dose (500 mg/kg). While this dose appears high, it is well below the established LD50 for clove oil in rodents (>2000 mg/kg). When converted to a Human Equivalent Dose (HED) based on Body Surface Area (BSA), the dose translates to approximately 80 mg/kg. For a 60 kg adult, this represents ~4.8 grams of extract per day—a feasible quantity for encapsulation or incorporation into functional foods. This wide therapeutic index suggests that *S. aromaticum* is a safe candidate for translational research.

Despite the robust findings, this study is not without limitations, which offer fertile ground for future investigation. Firstly, while HPLC provided a chemical fingerprint, we did not isolate the specific fractions to conduct a knock-out study. Therefore, we cannot definitively assign the IL-10 modulation to a specific molecule versus the whole extract. Secondly, while the downregulation of TNF-α strongly implies NF-κB inhibition, we did not perform Western Blotting for phosphorylated p65 or IκBα to molecularly confirm this pathway blockade. Additionally, the study utilized a 0.5% CMC vehicle for the extract, while the negative control utilized the same vehicle. While this controls for the vehicle effect, the pharmacokinetics of eugenol absorption in the presence of CMC versus a lipid carrier warrant further study. Future research should prioritize pharmacokinetic profiling to determine serum concentrations of eugenol and its glucuronide metabolites. Furthermore, docking studies (in silico) could help elucidate the precise binding affinity of clove phytocompounds to bacterial PBP2a or host cytokine receptors, providing a deeper molecular

validation of the mechanisms proposed here. Finally, while the rat model is a standard proxy for human wound healing, the anatomical differences (panniculus carnosus-driven contraction in rats vs. re-epithelialization in humans) necessitate validation in porcine models or clinical trials to fully ascertain translational potential.[19,20]

**5. Conclusion**

In summary, this study establishes the standardized ethanolic extract of *Syzygium aromaticum* as a potent, dual-action therapeutic agent capable of addressing the complex pathology of MRSA-infected wounds. The extract transcends the limitations of simple germicides by acting as a sophisticated immunomodulator. It successfully resolves the MRSA-induced cytokine storm by downregulating pro-inflammatory mediators (TNF-α and CRP) while simultaneously correcting the maladaptive immunosuppression characterized by elevated IL-10. This restoration of immune homeostasis, combined with the direct bactericidal activity of its eugenol-rich phytocomplex, breaks the cycle of chronic inflammation and accelerates the restoration of tissue integrity. The observation that the 100 mg/200g dose exhibits therapeutic efficacy comparable to the gold-standard antibiotic Vancomycin is clinically transformative. It positions *S. aromaticum* not merely as a traditional remedy, but as a viable, scientifically validated candidate for the development of novel phytopharmaceuticals. As the global medical community seeks sustainable solutions to the antibiotic resistance crisis, this study highlights the immense potential of revisiting ethnopharmacological sources with rigorous scientific methodology to discover the next generation of host-directed infection therapies.

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