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Clove Flower Extract (*Syzygium aromaticum*) enhance Wound Healing Time, Bacterial Count, and IL 10 Levels in Methicillin Resistant Staphylococcus aureus infected Rats

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ABSTRACT

Background: Any disruption to the processes of wound healing causes abnormal wound healing. Infection can prolong the healing process and exacerbate the wound. The high rate of resistance, the need for antibiotics, and the increase in healthcare cost make it a necessity to look for alternative antibiotics from natural ingredients. Clove flower (*Syzygium aromaticum*) is a type of traditional medicine that contains antibacterial effect.
Objective: To determine the effect of clove flower extract on the healing time of incision wounds, bacterial count and IL-10 levels in rats infected with MRSA.
Methods: This research used laboratory experimental method with a post-test-only control group design in rats that were given incisions on their backs. Random allocation was carried out to divide the Sprague Dawley rats into 6 groups (each 5 rats). Healing time was assessed based on the length of days, the bacterial count was examined from the blood and then counted by culture using nutrient agar. IL-10 levels were examined from the venous blood of rats and then measured using an ELISA kit assessed by a certified microbiologist. Data were analyzed and processed using the One Way Anova - Post Hoc hypothesis test.
Results: The bacterial count in the clove flower extract group was lower than the group without antibiotics and there was no difference compared to the group given antibiotics. IL-10 levels in the group given clove flower extract were lower when compared to the group given antibiotics. The wound healing time in the clove flower extract group was faster when compared to the group with or without antibiotics.
Conclusion: Clove flower extract enhance wound healing in MRSA-infected rats.

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1. Introduction

Wounds damage the integrity of biological tissues, such as skin, mucous membranes, muscles, and organs. Wound healing has 3 overlapping phases: inflammation, proliferation, and remodeling. Any disruption to these processes causes abnormal wound healing.[1] Wound healing is classified as primary healing and secondary healing. Uncomplicated healing of an uninfected wound with good predictability is defined as primary healing.[2] Complete closure without complications is an example of primary healing. If the wound-healing course of these wounds is interrupted by infection, hypoxia, or compromise of the immune system, a secondary healing stage may begin. Infection by bacteria inhibits healing wounds on the skin of patients undergoing hospital treatment, such as patients who receive surgery, installation of bone prostheses, and placement of urinary catheters.[3]

Several microbes that cause nosocomial skin infections include *Staphylococcus aureus*, *Streptococcus pyogenes*, *Acinetobacter sp.*, *Pseudomonas sp*. Infection can prolong the healing process and exacerbate the wound, such as infection from S.aureus and MRSA (*Methicillin Resistant Staphylococcus aureus*). S. aureus is a normal flora in the human body that colonizes around 30% – 50% of adults.[4] If left untreated, wounds that are initially shed on the surface of the skin will provide a port de entry for germs and can cause systemic infection, leading to sepsis and death.[5,6] MRSA is a bacteria that is resistant to beta-lactam antibiotics. Infections caused by MRSA can result in the length of hospital stay, increase morbidity and mortality, and thus burden the overall healthcare system.[7] The unwise usage of antibiotics can cause an increase in the resistance patterns, while it is still challenging to obtain antibiotics that are sensitive to inhibit or kill MRSA.[8] The high rate of resistance, the need for antibiotics, and the increase in healthcare cost make it a necessity to look for alternative antibiotics from natural ingredients.

Clove flower (*Syzygium aromaticum*) is a type of traditional medicine. Previous studies tested cloves' antibacterial effect, which showed that Syzygium aromaticum had an antimicrobial effect on MRSA. Eugenol, tannins, saponins, flavonoids, and alkaloids in

cloves play an active role in inhibiting MRSA bacteria in vitro. In addition, it was reported that clove flower extract can reduce IL-6 and IL-10 cytokines, improving wound healing.[9] Based on this background presentation and the lack of further research regarding the effect of clove flower extract on wounds in MRSA-infected rat, the authors wanted to further study the effect of clove flower extract on rats infected with MRSA

2. Methods

Experimental study with a post-test only control group design in rats that were given incisions on their backs. Random allocation was carried out to divide the Sprague Dawley rats into 5 groups (each 5 rats). Healing time was assessed based on the length of days, the bacterial count was examined from the blood and then counted on culture using blood agar and IL-10 levels were examined from the venous blood of the rats and then measured using an ELISA kit assessed by a certified microbiologist. Data were analyzed and processed using the One Way ANOVA and Post Hoc test.

Clove Flower Extract Preparation

One hundred grams of clove flower powder (*Syzygium aromaticum*) soaked in ethanol up to a volume of 1000 ml, shaken for 24 hours until it settles, and placed into the evaporating flask. The evaporating flask is attached to the evaporator, and fill the water bath with water to the brim. All tools are installed, including the rotary evaporator and water bath heater (set to 80-90°C), connected to the electric current. When the ethanol solution separates from the active substance already in the evaporating flask, leave it until the ethanol flow stops dripping into the holding flask (± 1.5 to 2 hours for one flask). The results are approximately a quarter of the original amount of dried clove flowers. The extraction results are stored in plastic bottles in the refrigerator or freezer.

Animal Model and Study Procedures

Thirty male Wistar rats (*Rattus norvegicus*) aged 2-3 months and weighting 200 grams were used as experimental models. All rats that met the inclusion criteria were then caged individually, adapted and given food (BioFeed pellets) and drink (clean drinkable water) ad libitum for 10 days. Clinical and behavioral status of the rat were assessed routinely to prevent any significant changes. All rats were randomly assigned into 6 groups; healthy rats with incision only (C1), MRSA-infected rats with incision wound and saline (C2), MRSA-infected rats with incision wound and vancomycin (C3), MRSA-infected rats with incision wound and 25 mg/200kgBW clove flower extract (T1), MRSA-infected rats with incision wound and 50 mg/200kgBW clove flower extract (T2), and MRSA-infected rats with incision wound and 100 mg/200kgBW clove flower extract (T3). Before incision, an injection of ketamine and xylazine was given for anesthesia. A 2 cm incision was made in the rat's back until it reached the subcutaneous layer, parallel to the vertebras. All groups except K1 were inducted with MRSA infection. The clove flower extracts were given 1 dose per day for 10 days orally.

Evaluation

Bacterial Count

The bacterial count was examined from the swab on the wound and then calculated from the culture using blood order. A total of 1 mL of S.aureus bacterial suspension was inoculated by pour plate on 15 mL of MHA media. The culture is incubated for 24 hours, turbidity of the substrate is observed. Examined from the blood using BHI (Bacterial Heat Infusion) media and incubated for 24 hours.

IL-10 Level

IL-10 levels were examined from rat vein blood obtained on after the last intervention day and measured using Rat IL-10 ELISA kit ab214566 (Abcam, United State of America)

Healing Period

The wound is made with a 2 cm long incision. The duration of healing is calculated how long and photographed each day, and the data was expressed in the number of days.

Data Analysis

Data analysis was performed using SPSS for Windows ver.21. Univariate analysis results are presented in the form of mean, and standard deviation, median, minimum and maximum value. Bivariate analysis involved One-Way ANOVA test followed by post-hoc analysis for normally distributed data, and Kruskal-Wallis followed by the Mann-Whitney test for not normally distributed data. A p-value of <0.05 was considered as statistically significant with a 95% confidence interval

3. Result

Baseline Characteristics

The sample for this study were 30 adults male Wistar rats weighing 200 ± 50 grams. Data were taken in the form of the bacterial count, IL-10 levels and healing time on the 22nd day.

Bacterial Count

Bacterial count from each group were addressed in table 1. Based on the results, the number of bacteria were lower in the positive control group (C3) and treatment group III (T3). Post Hoc analysis showed significant differences in groups C2 to T2 and T3, C3 and T2 (Figure 1).

IL-20 Levels

IL-10 levels from each group were addressed in table 2. Post Hoc analysis showed that there were significant differences in the C1 group towards C2, C3, T1, T2 and T3; group C2 to C3, T1, T2, and T3; group C3 against T1, T2 and T3; group T1 to T2 and T3; group T2 to T3 there is a significant difference (Figure 2).

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Table 1. Bacterial Count Analysis

Groups	Mean ± SD	Median (min-max)	p£
C1	345,60 ± 282,39	293 (105 – 791)	<0,001*
C2	638,20 ± 124,46	608 (477 – 800)	
C3	552,00 ± 148,33	491 (421 – 800)	
T1	491,60 ± 187,72	480 (338 – 800)	
T2	315,00 ± 105,84	386 (100 – 398)	
T3	332,60 ± 194,52	216 (156 – 558)	

Description: £, One-Way ANOVA test; C1, incision; C2, Incision and MRSA Induction; C3, Incision, MRSA Induction, and Vancomycin; T1, Incision, MRSA and clove flower extract 25 mg/kg/day; T2, Incision, MRSA and clove flower extract 50 mg/kg/day; T3, Incision, MRSA and clove flower extract 100 mg/kg/day

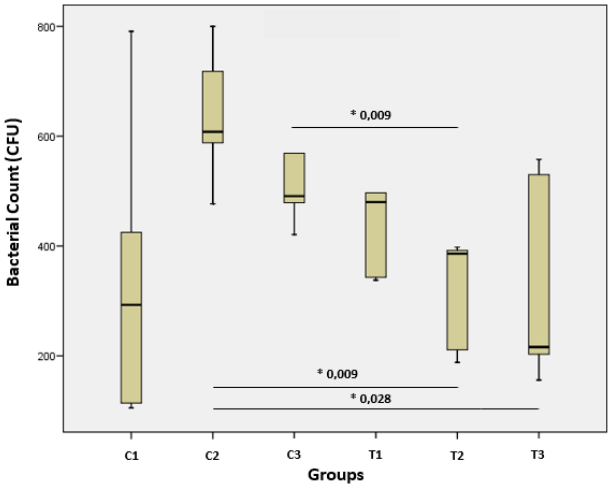

Figure 1. Boxplot graph of bacterial count

Description: C1, incision; C2, Incision and MRSA Induction; C3, Incision, MRSA Induction, and Vancomycin; T1, Incision, MRSA and clove flower extract 25 mg/kg/day; T2, Incision, MRSA and clove flower extract 50 mg/kg/day; T3, Incision, MRSA and clove flower extract 100 mg/kg/day

Table 2. IL-10 Levels Analysis

Groups	Mean ± SD	Median (min-max)	p£
C1	35,49 ± 3,50	35,33 (31,28 – 39,38)	<0,001*
C2	110,21 ± 2,72	110,70 (106,65 – 113,13)	
C3	45,87 ± 2,56	45,87 (42,63 – 49,11)	
T1	76,17 ± 3,22	76,66 (71,80 – 79,90)	
T2	58,34 ± 3,27	58,83 (54,78 – 62,88)	
T3	49,92 ± 2,56	49,92 (46,68 – 53,16)	

Description: £, One-Way ANOVA test; C1, incision; C2, Incision and MRSA Induction; C3, Incision, MRSA Induction, and Vancomycin; T1, Incision, MRSA and clove flower extract 25 mg/kg/day; T2, Incision, MRSA and clove flower extract 50 mg/kg/day; T3, Incision, MRSA and clove flower extract 100 mg/kg/day

Healing Period

Table 3 addressed the healing period of each group. Mann-Whitney test was carried out and the results showed that there were significant differences in C1 to C2, C3, T1, T2 and T3; group C2 to C3, T1, T2 and T3; C3 group towards T1 and T2; group T1 to T2 and T3; group T2 to T3 (Figure 3).

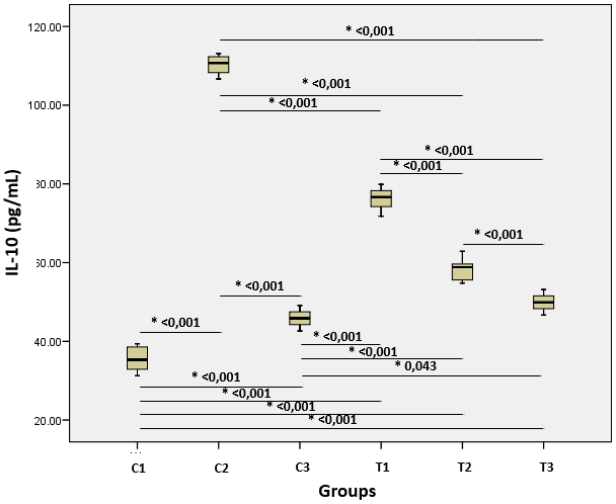

Figure 2. Boxplot graph of IL-20 level

Description: C1, incision; C2, Incision and MRSA Induction; C3, Incision, MRSA Induction, and Vancomycin; T1, Incision, MRSA and clove flower extract 25 mg/kg/day; T2, Incision, MRSA and clove flower extract 50 mg/kg/day; T3, Incision, MRSA and clove flower extract 100 mg/kg/day

Table 3. Healing Period Analysis

Groups	Mean ± SD	Median (min-max)	pδ
C1	8,00	8,00	<0,001*
C2	10,00	10,00	
C3	5,00	5,00	
T1	7,00	7,00	
T2	6,00	6,00	
T3	5,00	5,00	

Description: δ, Kruskal-Wallis test; C1, incision; C2, Incision and MRSA Induction; C3, Incision, MRSA Induction, and Vancomycin; T1, Incision, MRSA and clove flower extract 25 mg/kg/day; T2, Incision, MRSA and clove flower extract 50 mg/kg/day; T3, Incision, MRSA and clove flower extract 100 mg/kg/day.

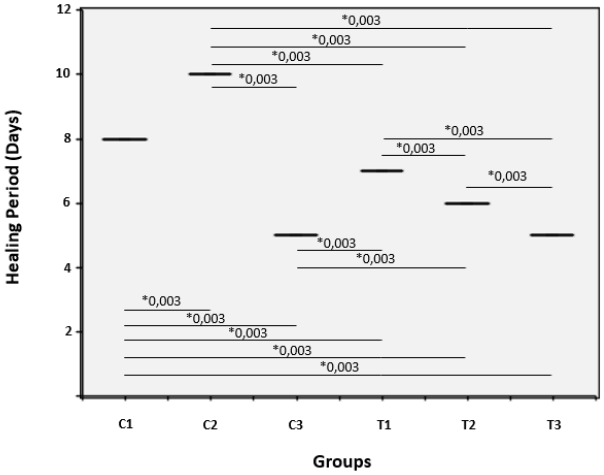

Figure 3. Boxplot graph of Healing Period

Description: C1, incision; C2, Incision and MRSA Induction; C3, Incision, MRSA Induction, and Vancomycin; T1, Incision, MRSA and clove flower extract 25 mg/kg/day; T2, Incision, MRSA and clove flower extract 50 mg/kg/day; T3, Incision, MRSA and clove flower extract 100 mg/kg/day

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4. Discussion

The results of this study were obtained from swab examination of the wound and bacterial counts were carried out in cultures using blood agar and showed that the number of post-treatment germs was higher in the control group compared to the treatment group with clove flower extract. In the treatment group with clove flower extract, the bacterial count tended to decrease if given the extract at higher doses. There is a significant difference between the negative control group and the treatment. However, based on statistical analysis, there was no significant difference between the doses of 25, 50 and 100 mg/kgBW. The decrease in the number of bacteria in the treatment and positive control groups was due to antibacterial activity. In the group given clove flower extract, its antibacterial activity was due to the eugenol, flavonoids, tannins, saponins, alkaloids and phenols contained in clove flower for which can damage the bacterial cell structure.[10-12]

In this study, IL-10 levels were also measured. In the C2 group, the average level was higher when compared to the C3 group and groups T1, T2, and T3. This proves that MRSA infection without given antibiotics can increase IL-10 levels. Previous studies have suggested that too much IL-10 can suppress protective T-cell responses, thereby facilitating bacterial persistence.[13-15] MRSA induction increases the number of bacteria in the body so that a high number of bacteria is obtained in the C2 group. Whereas in the treatment group which was given clove flower extract, the value was lower, proving that clove flower has some antibacterial benefits.

One of the stages in wound healing is wound epithelialization. Wound inoculation with MRSA significantly reduced the period of epithelialization.[16] This ongoing bacterial activity tends to increase as seen in the healing time observed in this experiment. Clove flower extract contains 16-23% of essential oil consisting of 64-85% eugenol. Eugenol contains active compounds that play an active role in inhibiting bacterial growth, namely tannins, saponins, flavonoids, and alkaloids.[17,18] Tannin inhibits the reverse transcriptase and DNA topoisomerase enzymes, so bacterial cells cannot form. Saponins will reduce the surface tension of bacterial cell walls, thereby increasing their permeability and causing leakage of intracellular components. Meanwhile, flavonoids produce antibacterial effects by inhibiting nucleic acid synthesis, cytoplasmic membrane function, energy metabolism, porins on cell membranes, changing membrane permeability, and reducing pathogenicity.[19]

5. Conclusion

There was an improved and accelerated wound healing using clove flower extract in rats that were infected by MRSA. However, our study had some limitations, as it requires in-depth phytochemical tests on clove flower extracts to determine the ingredients that have the most significant influence on antibacterial activity. In future research, in vitro testing of MRSA bacteria can also be carried out by measuring the inhibition zone, minimum inhibitory concentration, and minimum kill concentration.

Ethical Approval

This research was approved and declared ethically feasible by the Ethics Commission of the Public Health Faculty, Diponegoro University Semarang, with ethical clearance number No.06/EC/H/FK-UNDIP/I/2023. All efforts were made to alleviate harm to animals by administering anesthesia to all of the study animals before procedures, keeping the animals in a well-maintained cage, and ensuring graceful termination of animals before we took samples.

Conflicts of Interest

The authors verify that they have no competing financial interest or personal relationship that could influence the work reported this paper.

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Author Contributions

Conceptualization, MIF; methodology, MIF, DP DAI; software MIF; formal analysis, MIF, DP, DAI, HI; investigation, HI; resources, MIF,DP; data curation, MIF, HI; writing—original draft preparation, MIF; writing—review and editing,DP, DAI, APW; visualization, MIF; supervision, DP, DAI, APW; project administration, MIF; funding acquisition, MIF".

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