Antibacterial Activity of *Rhinacanthus nasutus* L. Kurz ointment to Inhibit *Staphylococcus aureus* Growth Using In Vitro Dilution Method

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**Abstract:** This study aimed at examining the antibacterial activity of *R. nasutus* ointment in different concentration of *S. aureus* bacteria growth by in vitro dilution method. The antibacterial activity of *R. nasutus* ointment was determined based on minimum inhibitory concentration (MIC) value and minimum bactericidal concentration (MBC) from *R. nasutus* ointment against *S. aureus* bacteria. The results showed *R. nasutus* ointment that the MIC value was 250,000 µg/mL up, whereas the MBC was 125,000 µg/mL. The variation of *R. nasutus* ointment concentration significantly has a relation with *S. aureus* bacterial growth. These results indicated that *R. nasutus* ointment potentially used as an alternative medicine for skin disease because it has an ability as antibacterial.

**Keywords:** Antibacterial activity, *Rhinacanthus nasutus*, antibiotic resistance, *Staphylococcus aureus*, skin disease.

1. **Introduction**

*Staphylococcus aureus* is one of the bacterial floras on human skin which can be a pathogen and causes diseases by the environment change. Approximately 50%-60% of people are frequently or permanently colonized by *S. aureus* and finally get infection [1]. The patients who have soft tissue infection by *S. aureus* have a higher risk for another infection either caused by *S. aureus* or other bacteria [2]. Setiawati et al. reported that there is an increase in the number of patients infected by bacteria. This phenomenon is caused by a mutational mechanism in bacteria and environmental change. Besides, utilization and increasing antibiotic dosage cause bacterial resistance towards certain antibiotics [3]. One of the solutions for antibiotic resistance is using natural ingredient as an alternative for antibiotic medicine. The natural ingredients can be from the plant which has antibacterial activity.
Rhinacanthus nasutus L. Kurz or snake jasmine is one of a medicinal plants widely used in Indonesia. A phytochemical study revealed that R. nasutus contains secondary metabolites such as flavonoids, steroids, terpenoids, anthraquinones and especially naphthoquinone as the significant compound [4]. Several pharmacological types of research on R. nasutus indicated that R. nasutus has anti-fungal activities to inhibit C. albicans and Aspergillus niger growths [5]. Sattar et al. reported that R. nasutus leaves and stems extract has anti-bacterial activities inhibit Pseudomonas aeruginosa, Escherichia coli [6], and Staphylococcus aureus bacterial growth [7–9]. Some different parts of R. nasutus were used as traditional medicine for eczema and other skin diseases [10].

R. nasutus Ointment is one of the utilizations as an alternative medicine for skin diseases. The ointment is semi-solid preparations for external uses. Ointment prevents direct contact to the wounded skin with air, and it is easy to store and use [11]. The research conducted to produce skin disease ointment made from a mixture of R. nasutus leaf ethanol extract, chicken feet skin gelatin and white Vaseline as the basic material. Previous research reported that chicken feet skin gelatin has inhibitory activity on S. aureus growth [3,12]. This research aimed to determine the effective concentration of R. nasutus leaf ointment to inhibit S. aureus bacterial growth based on minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

2. Methods

2.1. R. nasutus making the ointment

The extraction of R. nasutus used recurrent maceration method and ethanol 96% as a solvent. Ointment obtained from a mixture of 33.33% R. nasutus leaf ethanol extract, 0.0067% chicken feet skin gelatin, and 66.66% white Vaseline as a base. The ointment was diluted using DMSO 10% in various concentrations i.e. 31,250 µg/mL, 62,500 µg/mL, 125,000 µg/mL, 250,000 µg/mL, and 500,000 µg/mL. Previous research reported that chicken feet skin gelatin has inhibitory activity on S. aureus growth [3,12]. This research aimed to determine the effective concentration of R. nasutus leaf ointment to inhibit S. aureus bacterial growth based on minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

2.2. Experimental design and antibacterial activity assay

The S. aureus bacterial cultures were used in this research 10⁶ CFU/mL in cell density based on spectrophotometry (625 nm) absorbance values. One isolate of S. aureus of 10⁶ CFU/mL was inoculated in Mueller-Hinton Agar (MHA). Experimental designs were divided into 7 groups: (K1) R. nasutus ointment 500,000 µg/mL in concentration, (K2) R. nasutus ointment 250,000 µg/mL concentration, (K3) R. nasutus ointment 125,000 µg/mL concentration, (K4) R. nasutus ointment 62,500 µg/mL concentration, (K5) R. nasutus ointment 31,250 µg/mL concentration, (K+) without ointment as negative control, (K-) erythromycin antibiotic 100 µg/mL concentration as positive control group. These experiments repeat 4 times.

The antibacterial activity assay of R. nasutus ointment was conducted using dilution method with MIC and MBC as parameters. MIC test was conducted by observing turbidity in each test tube [13]. Each tested material that had been observed its turbidity was inoculated about 0.1 mL in MHA (Mueller-Hinton Agar) medium and incubated at room temperature for 24 hours. The determination of MBC was conducted by calculating the number of bacterial cells grown in MHA medium using a colony counter. MBC determined by the lowest concentration with the bacteria cell of < 0.1% of the number of bacterial cells grown in negative control treatment [14].

2.3. Statistical analysis

The data were analyzed using one-way ANOVA following with post hoc test Duncan (P < 0.05). Simple linear regression analysis was conducted determined correlation between variation in R. nasutus ointment concentration and the number of bacterial cells.
3. Results and Discussion
The results of turbidity observation using *S. aureus* bacteria is shown in Table 1.

**Table 1.** The Results of Turbidity Observation of Test Materials Inoculated in *Staphylococcus aureus* Bacteria

| Treatments | K1 | K2 | K3 | K4 | K5 | K- | K+ |
|------------|----|----|----|----|----|----|----|
| Result     | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) | ![Image](image7.png) |
| Description| Not turbid | Not turbid | Less turbid | Less turbid | Turbid | Very turbid | Undetermined |

Based on Table 1, the K2 group (*R. nasutus* ointment of 250,000 µg/mL) is not turbid. The MIC value of *R. nasutus* ointment on *S. aureus* bacteria is 250,000 µg/mL. The observation of *S. aureus* bacterial cells are grown in MHA medium as shown in Figure 1. The calculation results of the mean number of *S. aureus* bacterial cell grown in the MHA medium in each treatment is presented in Table 2.

**Figure 1.** *S. aureus* bacterial cells grown in MHA medium for 24 hours after treated with various concentrations of *R. nasutus* ointment. K-, Negative control without ointment; K1, *R. nasutus* ointment 500,000 µg/mL; K2, *R. nasutus* ointment 250,000 µg/mL; K3, *R. nasutus* ointment 125,000 µg/mL; K4, *R. nasutus* ointment 62,500 µg/mL; K5, *R. nasutus* ointment 31,250 µg/mL; and K+, Erythromycin antibiotic 100 µg/mL as positive control.

**Table 2.** Calculation of *Staphylococcus aureus* bacterial cells grown in MHA Medium with Dilution of 10² times of Bacterial Suspense that has been observed its Turbidity

| Treatments                        | Mean of Colony-forming Unit (CFU)±Std. error |
|-----------------------------------|---------------------------------------------|
| K1 (Ointment concentration of 500,000 µg/mL) | 15⁵±4                                       |
| K2 (Ointment concentration of 250,000 µg/mL) | 246⁴±24                                     |
| K3 (Ointment concentration of 125,000 µg/mL) | 1,198⁸±185                                  |
| K4 (Ointment concentration of 62,500 µg/mL) | 2,423⁷±121                                  |
| K5 (Ointment concentration of 31,250 µg/mL) | 5,238⁸±155                                  |
| K- (without treatment)            | 6,828±146                                   |

The data represent a mean ± standard error for 4 repeated per group. "a,b" showed a significant difference between the groups.
The ANOVA analysis indicated that *R. nasutus* ointment in various concentration significantly decreased *S. aureus* bacteria growth compared with the negative control group (*p*<0.05). The lowest number of *S. aureus* growth was K1, but it’s not significantly different from the K2 group. *S. aureus* bacterial cells grown in MHA medium resulted from dilution of 2 x 10⁴ times from negative control group was 6,828 cells. Therefore, the number of cells in the negative control group was estimated at 13.66 x 10⁷ CFU/mL (6,828 cell × 1/17,000). The MBC of *R. nasutus* ointment against *S. aureus* was 125,000 µg/mL. This result considered based on the lowest concentration of *R. nasutus* ointment with the mean number of cells < 1,366 cell (0.1 x 13.66 x 10⁷ CFU/mL × 1/100).

Based on simple linear regression analysis showed that the correlation coefficient (rₓᵧ) was negative and larger than rtable1% with a significance level of 0.000 (*p*<0.05). This result indicated that there was a negative and significantly has a relation between variation in *R. nasutus* leaf ointment concentration and *S. aureus* bacteria growth. The higher of the ointment concentration resulted in less the number of bacterial cells growth. Based on Figure 2, regression equation obtained: y = 5,611.13 – 1,262.43 x. The regression equation indicates that the relationship between *R. nasutus* ointment concentration and the number of *S. aureus* bacterial cells was linear. The determination coefficient obtained a value of 0.867. It means that the contribution of *R. nasutus* leaf ointment concentration variable to the number of *S. aureus* bacterial cells was 86.7%, and the remaining 13.3% was explained by other variables, such as bacterial properties, the solubility of test materials, external factors, and such.

![Figure 2. Linear Regression Graphic of the Correlation between *R. nasutus* Ointment Concentration and the CFU of *Staphylococcus aureus* Bacterial Cells](image)

Erythromycin at 100 µg/mL concentration is an antibiotic which used as positive control. Based on the observation, MIC of erythromycin antibiotic against *S. aureus* in the research was undetected due to the turbidity in the K+ group causing the inhibition of bacterial growth. This result determined by the non-existence of bacterial growth in MHA medium of K+ group. The turbidity in K+ treatment was caused by antibiotic particles dissolved in Liquid Nutrient medium instead of bacteria cells. MIC and MBC of erythromycin antibiotic against *S. aureus* bacteria about 0.06-128 µg/mL [15] and 32 µg/mL, respectively [16]. Erythromycin can interact directly and irreversibly with 50S ribosomal subunit in bacteria and causes disruption in the translation process. Erythromycin inhibits translocation stage since the polypeptide chain stops at A site of peptidyl transferase enzyme and cannot move to P site (donor site). Thus, it disturb protein synthesis [17]. Long term in the utilization of various antibiotics can cause antibiotic resistance. *S. aureus* develops resistance in a different mechanism to various antimicrobial. Yilmaz and Aslantas reported that *S. aureus* had various rates of resistance in antibiotic which the resistance towards erythromycin was 63.9% based on disk diffusion method [18].
Based on the data, *R. nasutus* ointment has an antibacterial activity against *S. aureus* bacteria. The MIC of *R. nasutus* leaf ointment against *S. aureus* was K2 group (250,000 µg/mL concentration). K2 group contains 83.33 µg/mL *R. nasutus* ethanol extract, 16.75 µg/mL chicken feet skin gelatin, and 166.65µg/mL Vaseline. *R. nasutus* leaf extract contained chemical compounds from polyphenol, steroid, terpene, and alkaloid groups. Those chemical compounds have antibacterial activity [5,7–9]. Polyphenol group included flavonoid and tannin. Flavonoid is a polar phenolic compound widely distributed in *R. nasutus* leaves [19]. Previous research was done by Bukke *et al.* found that extraction using methanol as solvent produced the highest total phenol content (TPC) in *R. nasutus* leaf extract [4]. Puttarak *et al.* reported that *R. nasutus* leaf methanol extract had antibacterial activity against *S. aureus* with MIC 500 µg/mL and MBC 256 µg/mL [20]. The results from Puttarak *et al.* showed that treatment using *R. nasutus* ointment has greater MIC and MBC compared with treatment using *R. nasutus* leaf methanol extract.

The MBC of *R. nasutus* ointment was 125,000 µg/mL with the number of bacterial cells from the inoculation of the treatment in MHA medium was 1198 cells. These results indicated based on bacterial growth after treatment using *R. nasutus* ointment in K3 group did not exceed from the threshold of cell number (1,366 cells). *R. nasutus* active compounds have antibacterial activity. The mechanism of polyphenol which contained in *R. nasutus* as antibacterial was by disrupted cell membrane permeability. Hydroxyl group (-OH) in polyphenol could form a hydrogen bond with positively charged oxygen and nitrogen in a cell membrane protein. Hydroxyl group also formed an ionic bond with positively charged amine (-NH2) in cell membrane protein [21]. The formation of these protein complex bonds causes essential proteins in the cell membrane, such as receptor, transport, and ion channel become inactive thus cause membrane damage and integrity. Membrane damage caused increase permeability and cell leak which are followed by intracellular material discharge. This damage causes obstructed cell growth or cell death [22]. Steroid and polyphenol also reported antihistamines activity [23]. Antihistamine activity by inhibited the binding of histamine to its receptors [24]. Previous research reports that antihistamine substance could treat chronic eczema [25]. Based on its steroid and polyphenol compound, *R. nasutus* leaf ointment can be used as external medicine, particularly for eczema and itching.

Another active compound in *R. nasutus* was terpene. Antibacterial mechanism of terpene was causes disrupted in cell membrane permeability. Terpene group bonds to lipophilic cell membrane components thus changes membrane density and disrupted cell membrane permeability. Saponin also disrupted membrane permeability by a formed complex bond with cholesterol in membranes [22]. Alkaloid has antibacterial activity by breakage the cell walls and disrupted nucleic acid synthesis. Nitrogen base in alkaloid could react to an amino acid of a cell wall components and then penetrated DNA arrangement of bacteria, thus causing changes in the structure and arrangement of a cell wall and bacterial DNA. The alterations cause damage and mutation in cell walls [22].

Chicken feet skin gelatin contained in *R. nasutus* ointment also has antibacterial activity. Gelatin is a protein conversion obtained from hydrolysis of collagen tissue in animal skins or bones. Gelatin is a single α chain with most of its amino acid composed of glycine, proline, and hydroxyproline [26]. The mechanism of gelatin as antibacterial was by damaging the cell membrane. Gelatin releases a peptide bond (-NH-) and react with protein in the membrane. This reaction causes damage in cell membranes that inhibits cell growth or triggered cell death [12]. Fadillah *et al.* reported that chicken feet gelatin able to inhibit bacterial growth at 150,000 µg/mL concentration, whereas at 200,000 µg/mL concentration would kill up to 75% of the number of bacteria [12]. However, the addition of gelatin in the ointment is in the low level (16.75 µg/mL) which is considered unable to increase both MCI and MBC of *R. nasutus* ointment against *S. aureus* bacteria.

4. Conclusion

*R. nasutus* ointment has an antibacterial activity against *S. aureus*. The MIC of *R. nasutus* ointment against *S. aureus* bacteria was 250,000 µg/mL, whereas the MBC was 125,000 µg/mL. Based on simple linear correlation, there was a significant correlation between variation in *R. nasutus* ointment
concentration and *S. aureus* bacterial growth. Ointment concentration was inversely proportional to bacterial growth, the higher the concentration, the lower the number of bacterial cells. The effective concentration which was used against *S. aureus* bacteria was 250,000 µg/mL.

**Acknowledgments**

This research is supported by PNBP FMIPA UM grant no. 16.7.11/U.N32.3.2/PL/2018. We would like to thank Universitas Negeri Malang for providing the research analysis.

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