Antimicrobial Effects of Annona squamosa Leaf Extract on Staphylococcus aureus Growth: An In Vitro Study

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ABSTRACT

Introduction: Mastitis is inflammation of the breast tissue due to infection with the Staphylococcus aureus bacteria causing local infections. It takes the natural ingredient Annona squamosa extract as an antimicrobial. Purpose: to determine the effectiveness of Annona squamosa leaf extract on the inhibition and killing power of Staphylococcus. Method: Experimental laboratory research with antibacterial activity test. Tube dilution method by incorporating bacteria and extracts with concentrations of 20%, 22.5%, 25%, 27.5%, 30%, and 32.5% and then measuring the MIC. The diffusion method is carried out by planting bacteria in a Nutrient Agar Plate and then giving the extract with 6 concentrations to calculate the MBC. Results: The results of the One-Way ANOVA test obtained a significance value of (p<0.001), indicating that changes in the concentration of Annona squamosa made a significant difference to Staphylococcus. The correlation test showed a significant p-value of (p<0.001), which means that there was a significant relationship between the administration of extract and the number of Staphylococcus. Conclusion: Annona squamosa leaf extract is able to significantly inhibit the growth of Staphylococcus bacteria and the higher the concentration of Annona squamosa leaf extract, the fewer the number of bacterial colonies that grow.

INTRODUCTION

The Indonesian government has enacted Government Regulation Number 33 of 2012 on Exclusive Breastfeeding, which defines exclusive breastfeeding as the provision of breast milk to infants from birth up to 6 months of age, without adding any other food or drink (Kementerian Kesehatan, 2012). Exclusive breastfeeding has been found to reduce under-five mortality by approximately 13%, and 16% of neonatal deaths can be prevented if infants are breastfed from birth and receive colostrum (Ade Harshindy & Budi Raharjo, 2022). However, the coverage rate of exclusive breastfeeding in Indonesia tends to decline, with the Ministry of Health setting a target coverage rate of 80%.

Common issues that occur in breastfeeding mothers include sore nipples, breast engorgement, breast milk stasis, and mastitis. These problems can disrupt the continuity of breastfeeding and pose health risks to mothers if left untreated (Bambang Ari Purwoko, 2019). Mastitis is an inflammation or infection of the breast characterized by hardening, redness, and pain, often accompanied by a fever >38°C,
caused by a Staphylococcus aureus bacterial infection (Ika Tristanti dan Nasriyah, 2019). According to the Indonesian Demographic and Health Survey in 2015, 37.12% of postpartum mothers experienced breastfeeding issues such as breast milk stasis and mastitis (Jamaruddin S, Ferawati Tahirong, & Syatirah, 2022). Staphylococcus aureus is one of the gram-positive bacteria that is part of the normal flora of the mucous membranes and skin in humans. This opportunistic bacterium can cause local infections in various parts of the body. Examples of problems that can arise from Staphylococcus aureus infection include bacteremia, pneumonia, meningitis, osteomyelitis, sepsis, nosocomial infections, and others. Staphylococcus aureus can cause both local infections on the skin and nasal mucosa and endocarditis (Rahmadani, Budiyono, 2017).

According to the Centers for Disease Control and Prevention, Staphylococcus aureus is currently a serious threat due to its resistance to various antibiotics. Hence, many studies are now focusing on the development and utilization of the antibacterial potential of natural substances (Centers For Disease Control and Prevention, 2013). Annona squamosa, commonly known as Sugar Apple, is one of the medicinal plants that show potential as an antibacterial agent. The leaves of Annona squamosa are known to possess significant health benefits due to the presence of various phytochemical compounds such as alkaloids and flavonoids (Kumar et al., 2021). Research has shown that Annona squamosa leaf extract exhibits biological activities such as anticancer, antidiabetic, antioxidant, antifungal, and antimicrobial effects (Anaya-Esparza et al., 2020). The leaf extract of Annona squamosa has demonstrated potential for pharmaceutical development (Sundaramahalingam et al., 2021).

A study by Jangnga (2018) revealed that ethanol extract of Annona squamosa leaves has inhibitory effects on the growth of Enterococcus faecalis bacteria in vitro (Jangnga & Kambaya 2018). Another study demonstrated that flavonoid compounds in sugar apple leaf extract can inhibit the growth of Staphylococcus aureus bacteria using the well diffusion method (Karunia & Supartono, 2017). These findings indicate the need for further research on the effectiveness of ethanol extract of Annona squamosa leaves in inhibiting the growth of Staphylococcus aureus bacteria using the tube dilution and diffusion methods. The aim of this study is to determine the inhibitory and bactericidal effects of Annona squamosa leaf extract on Staphylococcus aureus, thereby providing an alternative natural treatment solution for mastitis caused by Staphylococcus aureus bacteria.

**METHODS**

This study utilized laboratory experimental research using an in vitro approach with the tube dilution and diffusion methods for antibacterial activity testing. The research was conducted from January to February 2023 at the Biology Laboratory of Muhammadiyah University of Lamongan. The research sample consisted of Staphylococcus aureus bacteria on Nutrient Agar (NA).

The Annona squamosa leaf sample, obtained from Palang District, Tuban Regency, was extracted using the maceration method. The leaves were thoroughly washed with running water, then cut into small pieces and dried in an oven before being blended into a powdered form. The maceration process involved soaking the powdered plant material in 96% ethanol solution for three cycles of 24 hours each, with intermittent stirring. Afterward, the extract was filtered and subjected to rotary vacuum evaporation at a temperature of 60-70°C to reduce the extraction volume. The resulting concentrated extract was then diluted with 96% ethanol.
Staphylococcus aureus bacteria were prepared in the laboratory and adjusted to a McFarland standard of 0.5. The bacteria were diluted twice to achieve a bacterial concentration of 1x10^6. The tube dilution method was performed by adding the ethanol extract of sugar apple leaves to each reaction tube labeled A-F, with concentrations of 20%, 22.5%, 25%, 27.5%, 30%, and 32.5%, respectively. Tube KB served as the Bacterial Control. Each tube contained different series of test substance concentrations, with sterile aquadest used as the diluent. The bacterial suspension was added to each test tube. All tubes were incubated at 37°C for 18-24 hours, and the turbidity was observed to determine the Minimum Inhibitory Concentration (MIC). Subsequently, a volume of 0.01 ml was taken from each tube and streaked onto NA medium. The plates were incubated again at 37°C for 18-24 hours. After incubation, the colonies grown on the NA plates were counted to determine the Minimum Bactericidal Concentration (MBC).

The obtained data were subjected to One-Way ANOVA statistical analysis to determine differences, and Pearson correlation analysis was conducted to assess the relationship between the administration of Annona squamosa leaf extract and Staphylococcus aureus bacteria.

**RESULT**

The turbidity level of the sugar apple leaf extract solution was observed to determine the Minimum Inhibitory Concentration (MIC). The Minimum Inhibitory Concentration (MIC) is the lowest concentration of an antimicrobial agent that can inhibit the growth of bacteria. The comparison of turbidity levels at each concentration is shown in Figure 1.

From the observations, it can be observed that the higher the treatment concentration, the lower the turbidity level in the tubes. It can be seen that the 30% concentration is the lowest concentration that does not show turbidity in the tube, indicating that the MIC in this study is at a concentration of 30% as shown in Figure 1.
The Minimum Bactericidal Concentration (MBC) is the lowest concentration of an antimicrobial agent that can kill bacteria, indicated by the absence of bacterial growth on the Nutrient Agar Plate (NAP) medium. From the observations in Figure 2, it can be seen that as the concentration of sugar apple leaf extract increases, the colony count of Staphylococcus aureus decreases. The highest colony growth is observed at the KB concentration, while a decrease in bacterial growth is observed at concentrations of 32.5%, 30%, 27.5%, 25%, 22.5%, and 20%. The results show no growth of Staphylococcus aureus bacteria at a concentration of 32.5%. Thus, it can be concluded that the MBC in this study is obtained at a concentration of 32.5%. For a clearer presentation of the colony counts on each plate, Table 1 is provided.

Table 1. Number of Bacterial Colonies in Each Replication

<table>
<thead>
<tr>
<th>Number</th>
<th>Concentration</th>
<th>Number of bacterial colonies per replication</th>
<th>Mean</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P 1</td>
<td>P 2</td>
<td>P 3</td>
</tr>
<tr>
<td>1</td>
<td>32.5%</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>30.0%</td>
<td>89</td>
<td>68</td>
<td>45</td>
</tr>
<tr>
<td>3</td>
<td>27.5%</td>
<td>122</td>
<td>156</td>
<td>168</td>
</tr>
<tr>
<td>4</td>
<td>25.0%</td>
<td>315</td>
<td>231</td>
<td>256</td>
</tr>
<tr>
<td>5</td>
<td>22.5%</td>
<td>432</td>
<td>407</td>
<td>414</td>
</tr>
<tr>
<td>6</td>
<td>20.0%</td>
<td>633</td>
<td>580</td>
<td>473</td>
</tr>
<tr>
<td>7</td>
<td>KB (0%)</td>
<td>291000</td>
<td>283000</td>
<td>301000</td>
</tr>
</tbody>
</table>

The One-Way ANOVA test yielded a significance value of (p<0.001) as shown in Table 2. This indicates that the change in sugar apple leaf extract concentration has a significant effect on Staphylococcus aureus. The correlation test, as shown in Table 3, yielded a significance value of (p<0.001), indicating a significant relationship between the administration of sugar apple leaves and the number of S. aureus colonies. The Pearson correlation coefficient is r=−0.919. The negative sign indicates an inverse relationship, where a higher concentration of sugar apple leaf extract results in a lower number of bacterial colonies, and vice versa.
Table 2. Results of One-Way ANOVA Statistical Test

<table>
<thead>
<tr>
<th>Data Name</th>
<th>Sum of Square</th>
<th>DF</th>
<th>Mean square</th>
<th>F</th>
<th>Sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of colonies</td>
<td>3.01×11</td>
<td>6</td>
<td>5.015×10</td>
<td>2377.363</td>
<td>.000</td>
</tr>
<tr>
<td></td>
<td>4.43×108</td>
<td>21</td>
<td>2.1096300</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.01×11</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Results of Pearson Correlation Statistical Test

<table>
<thead>
<tr>
<th>Data Name</th>
<th>Concentration</th>
<th>Number of Colonies</th>
<th>Pearson Cor</th>
<th>Sig. (2-tailed)</th>
<th>N</th>
<th>Sig. (2-tailed)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td></td>
<td></td>
<td>-.919</td>
<td>.000</td>
<td>28</td>
<td></td>
<td>28</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of Colonies</td>
<td></td>
<td>-.919</td>
<td>.000</td>
<td></td>
<td>28</td>
<td></td>
<td>28</td>
</tr>
</tbody>
</table>

DISCUSSION

This study aims to determine the effectiveness of Annona squamosa leaf extract against the inhibitory and bactericidal activity on Staphylococcus aureus. The results of the study showed that Annona squamosa leaf extract was able to inhibit the growth of Staphylococcus aureus bacteria, as indicated by the obtained Minimum Inhibitory Concentration (MIC) value at a concentration of 30%. The results also showed that Annona squamosa leaf extract was able to kill Staphylococcus aureus bacteria, as indicated by the obtained Minimum Bactericidal Concentration (MBC) value at a concentration of 32.5%.

Plants contain various metabolite compounds with different chemical properties such as polarity and solubility. Several solvents have been developed for the extraction of plant metabolites, including methanol, ethanol, ethyl acetate, and other solvents (Kamoda, Lelyana, & Sugiaman, 2020). In this study, Annona squamosa extract was prepared using 96% ethanol as the solvent, as ethanol is relatively non-destructive to active chemical compounds. Additionally, 96% ethanol contains a small amount of water, allowing it to absorb more active substances. Ethanol is also a universal solvent that can dissolve both polar and nonpolar compounds, ensuring that the required active compounds are fully extracted (Al-Judaibi, Al-Zahrani, Altammar, Ismail, & Darweesh, 2014).

The different concentrations of Annona squamosa leaf extract showed a significant difference in the inhibitory activity against Staphylococcus aureus bacteria. This result is consistent with the study conducted by Santhoshkumar & Kumar (2016) on the phytochemical activity and antibacterial effect of Annona squamosa leaf extract, which found that higher concentrations of Annona squamosa leaf extract corresponded to higher antibacterial activity. The inhibitory activity of this extract may be attributed to the higher concentration of antibacterial compounds capable of inhibiting the growth of Staphylococcus aureus colonies (Santhoshkumar & Kumar, 2016).

As the concentration of Annona squamosa leaf extract increased, the number of bacterial colonies decreased, and vice versa. Gram-positive bacteria, such as Staphylococcus aureus, have a membrane structure that contains more peptides, fewer lipids, and a cell wall composed of polysaccharides (teichoic acids). Teichoic acids are polymers that can dissolve in water, making the cell wall of gram-positive bacteria more polar (Vijayalakshmi & Nithiya, 2015). Flavonoids present in Annona...
squamosa leaf extract are polar compounds, allowing them to penetrate the cell wall more easily. Flavonoids act as antibacterial agents by disrupting cell wall synthesis, interfering with the cytoplasmic membrane, and inhibiting energy metabolism (Xiao, Zhu, & Zhang, 2014). This is in line with the research conducted by Alberta (2016) on the inhibitory effect of ethanol extract of Annona squamosa leaves on the growth of E. coli and Staphylococcus aureus bacteria, which found that Annona squamosa leaf extract exhibit higher antibacterial and inhibitory effects on Staphylococcus aureus compared to E. coli (Tansil et al., 2016).

Annona squamosa has long been recognized for its medicinal properties, and different parts of the plant, such as fruits, seeds, bark, twigs, and leaves, possess their own unique benefits. Annona squamosa leaves are known to have extensive pharmacological properties and biological activities such as antioxidant, antimicrobial, antidiabetic, antiviral, and anticancer effects (Mohammad Zahid, 2018). This is due to the presence of phytochemical compounds in Annona squamosa leaves, including glycosides, phytosterols, saponins, tannins, phenols, and the two most important compounds, alkaloids and flavonoids (Hosseinabadi, 2021). Alkaloids and flavonoids are the most significant compounds contributing to the antimicrobial activity of Annona squamosa leaves (Santhoshkumar & Kumar, 2016). Flavonoids can induce disturbances in microbial membranes, coagulate cytoplasmic components and cause leakage, as well as interfere with bacterial cell metabolism (Kumar et al., 2021). This is consistent with the research conducted by Swantara (2022), who performed phytochemical screening of Annona squamosa leaves and found the presence of flavonoid compounds, particularly flavonol, in the extract. These compounds exhibited inhibitory effects on the growth of Staphylococcus aureus bacteria (Swantara, Damayanti, & Suirta, 2022).

CONCLUSION AND RECOMMENDATION

The conclusion of this study is that Annona squamosa leaf extract at a concentration of 30% is capable of significantly inhibiting the growth of Staphylococcus aureus bacteria, and the higher the concentration of Annona squamosa leaf extract, the fewer bacterial colonies are observed. Furthermore, Annona squamosa leaf extract also exhibits significant bactericidal activity against Staphylococcus aureus at a concentration of 32.5%. A suggestion for further research is to conduct in vivo studies to investigate the pharmacological effects of Annona squamosa leaves on the human body.

REFERENCES


