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Effectiveness Test of Red Spinach Leaf Extract (Amarathus Tricolor L) as a Substitute for Crystal Violet in Gram Staining of Staphylococcus Aureus Bacteria Morphology

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Abstract

Gram staining is the most common procedure used to identify bacterial morphology, using crystal violet as the primary dye. However, crystal violet is a synthetic dye that has toxic effects on the environment and health. The purpose of this study was to test the effectiveness of red spinach leaf extract (Amaranthus tricolor L) as an alternative natural dye to replace crystal violet in Gram staining of Staphylococcus aureus bacteria morphology. This type of research is a laboratory experiment with a post-test group control design with samples of Staphylococcus aureus bacterial colonies and red spinach leaf extract with concentrations of 20%, 40%, 60%, 80%, and 100%. Bacterial morphology was observed based on color intensity and clarity of cell shape. The results showed that red spinach leaf extract with a concentration of 100% gave very good morphological results and approached staining using crystal violet. The results of the Kruskal-Wallis test showed a significance value of 0.009 ($p < 0.05$), indicating that there is a significant difference between the concentration groups and the color quality of the Staphylococcus aureus bacteria. This study concludes that red spinach leaf extract is effective as an alternative natural dye to replace crystal violet in Gram staining of Staphylococcus aureus bacteria. Suggestions for further researchers are to conduct further research on the use of natural dyes that can be used as natural dyes to replace crystal violet, which are able to color bacterial morphology in Gram staining.

Keywords: Gram Staining, Crystal Violet, Red Spinach Leaf Extract, Staphylococcus Aureus Bacteria.

Introduction

According to the WHO, 25 million deaths worldwide are caused by infectious diseases, one of which is nosocomial infections (Ni Putu Saraswati Kristina, 2023).

According to data from the Indonesian Ministry of Health, the prevalence of nosocomial infections in Indonesia reached 15.74% in 2022, far exceeding the prevalence in developed countries, which ranges from 4.8 to 15.5% (Sophia Hasanah, 2024). The incidence of nosocomial infections in West Sumatra is known to reach 13.21%. One type of pathogenic bacteria frequently found to cause nosocomial infections is the gram-positive bacterium Staphylococcus aureus (Yulika, 2020).

Staphylococcus aureus is a bacterium that frequently causes nosocomial infections. Staphylococcus aureus is a normal microflora in the nose and skin that can become pathogenic in individuals with weakened immune systems and can cause itching, sore throats, abscesses, and serious skin infections (Rianti, Tania, and Listyawati, 2022). The bacteria are very small (microscopic) and, when alive, appear nearly colorless due to the contrast with water, which can only be seen under a microscope. To visualize and observe bacterial cell morphology, a staining method is required. One such staining method is Gram staining (Mirfaidah Nadjamuddin, 2023).

Gram staining is the most common procedure used to identify many bacteria. Gram staining allows for the identification of bacterial cell morphology, including gram-positive and cell shape. Staining facilitates the visualization and observation of bacterial morphology under a microscope. The substances used for Gram staining are the primary dye crystal violet, iodine solution, alcohol solution (bleaching agent), and the counterstain, safranin. Gram-positive bacteria retain the crystal violet dye, appearing purple, while gram-negative bacteria lose the crystal violet color after being washed with alcohol and retain the dye, appearing red (Mirfaidah Nadjamuddin, 2023). Currently, laboratory staining uses synthetic dyes, such as crystal violet (Edyani, 2020).

Synthetic dyes are dyes made through synthesis or chemical reactions. Synthetic dyes, such as crystal violet, are highly favored due to their advantages: brighter colors, resistance to fading, and greater practicality. However, synthetic dyes are hazardous contaminants found in textile factory wastewater, which have toxic effects on the environment and human health (Dafrita and Sari, 2020).

Red spinach (Amaranthus tricolor L.) can replace synthetic dyes because it is environmentally friendly, biodegradable, readily available, relatively inexpensive, and contains anthocyanin pigments (Afida et al., 2020). Anthocyanins are natural pigments found in red spinach that are water-soluble and impart pink, red, purple, blue, and

yellow colors to plants. The anthocyanin content in red spinach leaves is 6350 ppm, while the anthocyanin content in red spinach stems is only 2480 ppm (Eppang et al., 2020).

Based on the above background, it is known that red spinach leaves contain anthocyanin pigments, and there has been no research on whether anthocyanin extract from red spinach leaves is effective as an alternative dye to replace crystal violet in Gram staining. Therefore, researchers are interested in conducting research related to the effectiveness of red spinach leaf extract with concentrations of 20%, 40%, 60%, 80% and 100% to replace crystal violet dye in Gram stain morphology staining of Staphylococcus aureus bacteria. Therefore, based on this, it is necessary to conduct research on "Test of the Effectiveness of Red Spinach Leaf Extract (Amarathus Tricolor L) as a Substitute for Crystal Violet in Gram Morphology Staining of Staphylococcus Aureus Bacteria". In this study, red spinach leaves will be processed by maceration and extraction. This method was chosen because the process is more practical and simple (Khairuddin et al., 2020).

Method

This This type of research is Experimental Laboratory by testing the effectiveness of red spinach leaf extract (Amarathus tricolor L) with concentrations of 20%, 40%, 60%, 80% and 100%, as a substitute for crystal violet dye in gram staining of Staphylococcus aureus bacteria as a group given treatment and gram staining of Staphylococcus aureus bacteria using crystal violet dye as a control group. The design used is a post-test control group design, by observing the morphology of Staphylococcus aureus bacterial cells in the group given treatment and then seeing the effectiveness of staining by comparing the results of the treatment group and the control group.

The population in this study was Staphylococcus aureus bacteria. The samples in this study were red spinach leaf extract (Amaranthus tricolor L) with concentrations of 20%, 40%, 60%, 80% and 100% Staphylococcus aureus bacteria. After the data is collected, it will be processed. The results will be analyzed using univariate and bivariate methods.

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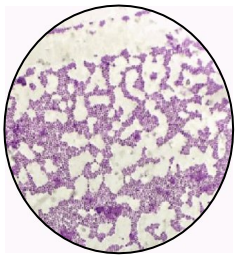
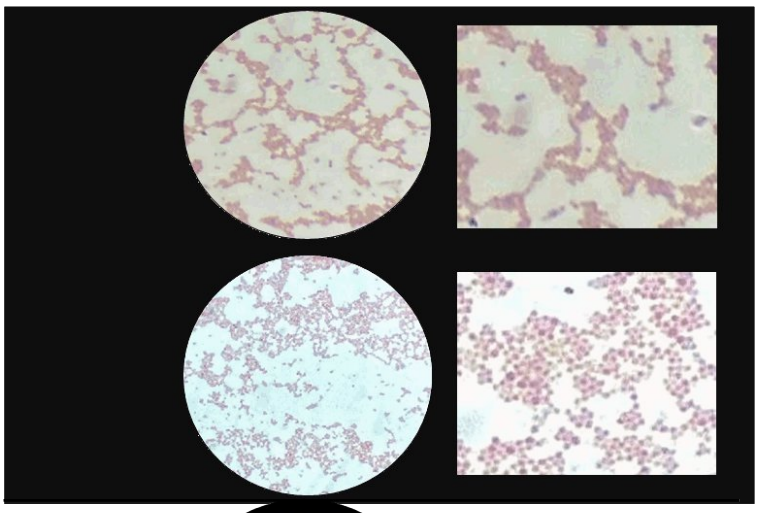
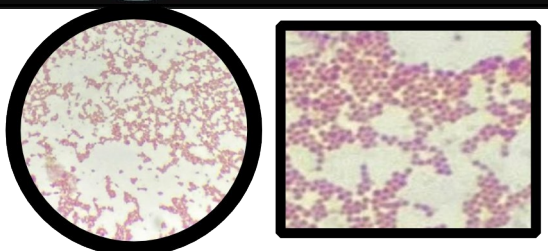
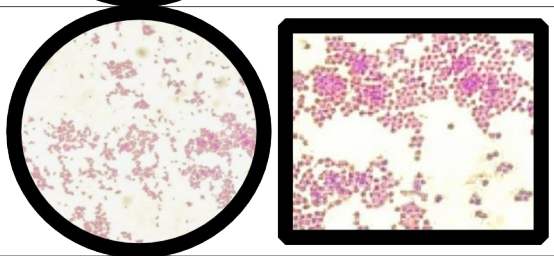
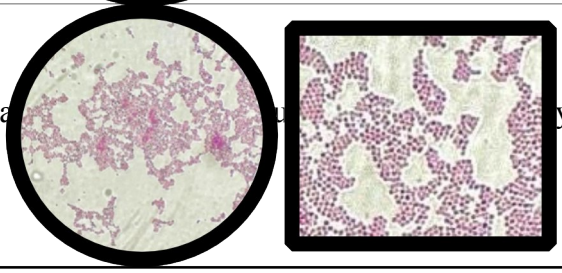
Results

Univariat Analysis

The effectiveness of red spinach leaf extract solution (Amaranthus Tricolor L) with concentrations of 20%, 40%, 60%, 80% and 100% as a substitute for crystal violet in Gram staining of Staphylococcus aureus bacteria morphology.

Table 1

Morphological Description of Staphylococcus Aureus Bacteria Using Red Spinach Leaf Extract Solution Staining (Amarathus Tricolor L)

Concentration Variation	Morphology
Crystal Violet	
	
60%	
80%	
100%	

Based on Ta ylococcus aureus bacteria in the

control specimen using crystal violet. The bacteria were clearly visible as round cocci clustered in a deep purple color. Using a 20% concentration of red spinach leaf extract, the bacteria appeared shapeless or indistinct, with a slightly faded reddish-brown color. At a concentration of 40%, the bacteria were clearly visible as round clusters in a purplish-red color. At a concentration of 60%, the bacteria were clearly visible as round clusters in a purplish-red color. At a concentration of 80%, the bacteria were clearly visible as round clusters in a purplish-red color. At a concentration of 100%, the bacteria were clearly visible as round clusters in a purple color.

Table 2 Color Quality Score of Staphylococcus aureus Bacteria Morphology Examination Results with Red Spinach Leaf Extract Solution (Amarathus Tricolor L)

Concentration Variation	Bacterial Morphology			
	Slide	Cell Color Intensity	Cell Shape	Score
Violet Crystal	1	Strong	Very Clear	(3) Very Good
	2	Strong	Very Clear	(3) Very Good
	3	Strong	Very Clear	(3) Very Good
Concentration 20%	1	Weak	Faint	(1) Very Good
	2	Weak	Faint	(1) Not Good
	3	Weak	Faint	(1) Not Good
Concentration 40%	1	Medium	quite clear	(2) Good
	2	Medium	quite clear	(2) Good
	3	Medium	quite clear	(2) Good
Concentration 60%	1	Medium	quite clear	(2) Good
	2	Medium	quite clear	(2) Good
	3	Medium	quite clear	(2) Good
Concentration 80%	1	Medium	quite clear	(2) Good
	2	Medium	quite clear	(2) Good
	3	Medium	quite clear	(2) Good
Concentration 100%	1	Medium	quite clear	(2) Good
	2	Strong	Very Clear	(3) Very Good
	3	Strong	Very Clear	(3) Very Good

Based on Table 2, it can be seen that the staining quality score using crystal violet as a control of color intensity is strong, and the shape of bacterial cells is very clearly visible with a score of (3) very good. The results using a solution of red spinach leaf extract (Amarathus Tricolor L) with a concentration of 20% color intensity look weak, and the shape of bacterial cells looks faint with a score of (1) not good. At a concentration of 40%, the color intensity is moderate, and the shape of the cells is quite clear with a score of (2) good. At a concentration of 60%, the color intensity is moderate, and the shape of the cells is quite clear with a score of (2) good. At a concentration of 80%, the

color intensity is moderate, and the shape of the cells is quite clear, with a score of (2) good. At a concentration of 100%, the color intensity is strong and the shape of the cells is very clear, with a score of (3) very good.

Bivariate Analysis

Bivariate analysis in this study used the Kruskal-Wallis test with a significance value of less than 0.05. The Kruskal-Wallis test is part of the non-parametric statistics alternative to the one-way ANOVA test used when one or all of the data distributions are not normally distributed. From the results of the Kruskal-Wallis test, it can be seen whether or not there is a difference in the effectiveness of red spinach leaf extract as a natural dye to replace crystal violet in the Gram staining of Staphylococcus aureus bacteria morphology.

Table 3
Kruskal-Wallis Test Results

Variable	Concentration	N	Mean Rank	*P Value*
Control	Crystal Violet	3	16,00	0,009
Red Spinach Leaf Extract *(Amarathus Tricolor L)*	Concentration 20%	3	2,00	
	Concentration 40%	3	8,50	
	Concentration 60%	3	8,50	
	Concentration 80%	3	8,50	
	Concentration 100%	3	13,50	

Based on the Kruskal-Wallis test results in Table 4.3, each of the five treatment groups obtained a mean rank value, which was used as a reference to determine the differences in effectiveness of red spinach leaf extract (Amarathus Tricolor L.) as a natural dye substitute for crystal violet in Gram staining of Staphylococcus aureus bacteria. The control treatment (crystal violet) had the highest mean rank of 16.00, indicating the best color quality. The treatment using red spinach leaf extract showed the lowest color quality at a concentration of 20%, with a mean rank of 2.00. At concentrations of 40%, 60%, and 80%, the color quality was moderate, with a mean rank of 8.50. At 100%, the color quality was good, approaching the control, with a mean rank of 13.50. Based on this, a significance value (p-value) of 0.009 was obtained, which is less than 0.05, indicating a significant difference in effectiveness between the extract concentration variations on the Gram staining results of Staphylococcus aureus bacteria. Therefore, Ha was accepted and Ho was rejected. The Kruskal-Wallis test is

omnibus, meaning it provides information on whether there is a significant difference in overall effectiveness between treatment groups, without identifying which groups differ. Therefore, a post-hoc Dunn test is necessary to determine which pairs of groups have statistically significant differences.

Table 4
Post Hoc Dunn Test Results

Comparison of concentration with control	Adj. Sig.[a]
Concentration 20% - Control (crystal violet)	.005
Concentration 40% - Control (crystal violet)	.827
Concentration 60% - Control (crystal violet)	.827
Concentration 80% - Control (crystal violet)	.827
Concentration 100% - Control (crystal violet)	1.000

Based on the results of the Dunn Post Hoc Test in Table 4.4, each concentration was compared with the control (crystal violet). The mean value (adj. sig.) was obtained as a reference for determining significant differences between the treatment and control groups (crystal violet). The comparison of the 20% concentration with the control resulted in a sig. 0.005 (<0.05), indicating a significant difference between the 20% concentration and the control. The comparison of the 40% concentration with the control resulted in a sig. 0.827 (>0.05), indicating no significant difference between the 40% concentration and the control. The comparison of the 60% concentration with the control resulted in a sig. 0.827 (>0.05), indicating no significant difference between the 60% concentration and the control. The comparison of the 80% concentration with the control resulted in a sig. 0.827 (>0.05), indicating no significant difference between the 80% concentration and the control. In the comparison of 100% concentration with the control, a significance value (p-value) of 1,000 > 0.05 was obtained, which means there was no significant difference between the 100% concentration and the control.

Discussion

Univariate analysis

1. **Morphology of Staphylococcus Aureus Bacteria Using Red Spinach Leaf Extract Solution (Amarathus Tricolor L) Staining**

Based on the results of microscopic bacterial studies conducted using natural dyes

from red spinach leaf extract solutions (Amarathus Tricolor L.) at concentrations of 20%, 40%, 60%, 80%, and 100%, Table 1 shows that at a concentration of 20%, the bacterial cell color intensity was weak, with the bacteria appearing slightly faded brownish-red, and the bacterial cell shape was vaguely amorphous. At a concentration of 40%, the bacterial cell color intensity was moderate, with the bacteria appearing purplish-red, and the spherical, clustered bacterial cells were quite clearly visible. At a concentration of 60%, the bacterial cell color intensity was moderate, with the spherical, clustered bacterial cells being quite clearly visible. At a concentration of 80%, the bacterial cell color intensity was moderate, with the spherical, clustered bacterial cells being quite clearly visible. At a concentration of 100%, the bacterial cell color intensity was strong, with the purple and spherical, clustered bacterial cells being very clearly visible. This research is in line with previous research on the use of rose extract (Rosa Hybrida) as a substitute for crystal violet in gram staining of Staphylococcus Aureus bacteria with concentrations of 20%, 40%, 60% and 80%. The results obtained at a concentration of 80% rose extract effectively replaced crystal violet because bacteria with a purplish blue color intensity were found to be round in clusters, but at concentrations of 20%, 40%, 60% it was less effective because the bacteria were not stained clearly (Suriany, Lasmawati and Sitepu, 2024).

Red spinach leaves are known to contain 6350 ppm of anthocyanin. Anthocyanins are natural plant pigments that impart red, purple, blue, and yellow hues to plants. Therefore, red spinach leaves have great potential as a natural dye source (Najmawati Sulaiman, 2022).

The cell wall of Staphylococcus aureus bacteria has a thick and dense peptidoglycan layer. This thickness and composition of peptidoglycan allow the crystal violet dye to adhere firmly, making the bacteria appear deep purple under the microscope after the Gram staining process (Hayati et al., 2019).

The researchers assumed that this study differed from previous research, which used red spinach leaf extract as a natural dye substitute for crystal violet. Previous research using a solution of red spinach leaf extract (Amarathus Tricolor L.) at pH 6.7 yielded results.

There were differences in bacterial color intensity at each concentration, as the

higher the concentration, the greater the amount of anthocyanin pigment produced. Red spinach leaf extract can color bacteria because the pH of the red spinach leaf extract solution is acidic, and it is known that anthocyanins will be stable at acidic pH. Anthocyanin pigments from red spinach leaf extract can color Staphylococcus aureus bacteria. Based on this, it can be concluded that red spinach leaf extract with a concentration of 40%, 60%, 80% is considered quite effective to be used as a natural dye to replace crystal violet in gram staining because the shape of bacterial cells is clearly visible in the form of round clusters but produces a purplish red color intensity that is different from the control color of deep purple. And a concentration of 100% is considered effective to be used as a natural dye to replace crystal violet in Gram staining because the shape of the bacteria that are seen very clearly in the form of round clusters and produces a purple color intensity close to the control color.

Bivariate analysis

Based on the results of microscopic bacterial studies conducted using natural dyes from red spinach leaf extract solutions (Amarathus Tricolor L.) at concentrations of 20%, 40%, 60%, 80%, and 100%, Table 1 shows that at a concentration of 20%, the bacterial cell color intensity was weak, with the bacteria appearing slightly faded brownish-red, and the bacterial cell shape was vaguely amorphous. At a concentration of 40%, the bacterial cell color intensity was moderate, with the bacteria appearing purplish-red, and the spherical, clustered bacterial cells were quite clearly visible. At a concentration of 60%, the bacterial cell color intensity was moderate, with the spherical, clustered bacterial cells being quite clearly visible. At a concentration of 80%, the bacterial cell color intensity was moderate, with the spherical, clustered bacterial cells being quite clearly visible. At a concentration of 100%, the bacterial cell color intensity was strong, with the purple, and spherical, clustered bacterial cells being very clearly visible. Based on the results of the hypothesis test using the Kruskal-Wallis test, as seen in Table 3, the control treatment (crystal violet) had the highest mean rank of 16.00, indicating the best color quality. The 20% concentration treatment had the lowest staining quality, with a mean rank of 2.00. At concentrations of 40%, 60%, and 80%, moderate staining quality was achieved with a mean rank of 8.50. At 100% concentration, good color quality was achieved, approaching the control, with a mean rank of 13.50. The Kruskal-Wallis test yielded a significance value (p-value) of 0.009,

which is less than 0.05, indicating a significant difference in effectiveness between the extract concentration variations on the gram-morphology staining results of Staphylococcus aureus bacteria. A post-hoc Dunn test was then used to identify which group pairs had statistically significant differences. The results of the Dunn Post Hoc Test can be seen in Table 4. The comparison of the 20% concentration with the control yielded a p-value of 0.005, less than 0.05, indicating a significant difference between the 20% concentration and the control. The comparison of the 40%, 60%, and 80% concentrations with the control yielded a p-value of 0.827, greater than 0.05, indicating no significant difference between the 40%, 60%, and 80% concentrations and the control. The comparison of the 100% concentration with the control yielded a p-value of 1.000, greater than 0.05, indicating no significant difference between the 100% concentration and the control.

The Kruskal-Wallis test method was used to collect and analyze data to determine the overall effectiveness of the treatment groups. The hypothesis test used the Kruskal-Wallis test to obtain a mean rank value reflecting the quality of Gram staining at each concentration of red spinach leaf extract (Amaranthus tricolor L). The highest mean rank value in the Kruskal-Wallis test was used as a reference for improved staining quality, as indicated by the strong color intensity in the bacterial cells and the clear appearance of the bacterial cell shape. Further testing was then conducted using the Dunn Post Hoc Test to identify which pairs of groups had statistically significant differences, with a significance value lower than 0.05 (Prihandaru, 2022).

The researchers assumed that, based on previous research, red spinach leaf extract (Amarathus Tricolor L.) was considered effective as a natural dye to replace crystal violet in Gram staining. This finding aligns with the results of staining using red spinach leaf extract, which stains bacterial cells and maintains their characteristic spherical, clustered shape. This is because the anthocyanin pigment produced from red spinach leaf extract interacts with bacterial cells, allowing them to be observed under a microscope. So based on the results of the Post Hoct Dunn Test at concentrations of 40%, 60%, 80% is considered quite effective in coloring Staphylococcus aureus bacterial cells because it is able to maintain the shape of bacterial cells that are round in clusters but produces a purplish red color intensity that is different from the control color which is dark purple, And at a concentration of 100% is considered effective in

coloring Staphylococcus aureus bacteria because it produces a purple color intensity that is close to the control color and can maintain the shape of bacterial cells that are round in clusters. So it can be concluded that in the tests carried out, there is significant effectiveness between variations in the concentration of red spinach leaf extract (Amarathus Tricolor L) with the quality of the morphological color of Staphylococcus aureus bacteria.

Conclusion

Based on the results of the research conducted with the title "Test of the Effectiveness of Red Spinach Leaf Extract (Amarathus Tricolor L.) as a Substitute for Crystal Violet in Gram Staining of Staphylococcus Aureus Bacteria Morphology," it can be concluded that:

1. The effectiveness of red spinach leaf extract (Amarathus Tricolor L.) at a concentration of 20% is considered ineffective as a natural dye to replace crystal violet because the color intensity of bacterial cells appears weak and the shape of the bacterial cells is not visible. Concentrations of 40%, 60%, and 80% are considered quite effective because the shape of the bacterial cells is quite clearly round and clustered. However, the color intensity of the bacterial cells appears moderate with a purplish red color different from the color of crystal violet. Meanwhile, at a concentration of 100%, the color intensity of the bacterial cells is considered effective because the color intensity of the bacterial cells appears strong with a purple color close to the color of crystal violet and the shape of the bacterial cells is very clearly visible.
2. The morphology of bacterial cells at a concentration of 20% showed that the bacteria were not spherical in shape, but rather in clusters, with a faint reddish-brown color. At a concentration of 40%, the bacteria were clearly spherical in shape, with a purplish-red color. At a concentration of 60%, the bacteria were clearly spherical in shape, with a purplish-red color. At a concentration of 80%, the bacteria were clearly spherical in shape, with a purplish-red color. At a concentration of 100%, the bacteria were clearly spherical in shape, with a purple color.

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