ASSESSMENT OF NEPHELIUM LAPPACEUM PEEL EXTRACT AS A POTENTIAL PRIMARY STAIN FOR GRAM STAINING IN BACTERIAL IDENTIFICATION

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ABSTRACT: The study determines the potential of *Nephelium lappaceum* peel extract as an alternative to the standard Crystal violet in bacterial identification. This study utilized a quasi-experimental design to assess the staining efficacy of *Nephelium lappaceum* peel extract. Conventional ethanolic extraction is the method for obtaining the rambutan peel extract. The extract was then turned into a powdered form and portioned to produce concentrations of 25%, 50%, and 75%. The staining process employed was the same as the routine gram staining procedure. The stained specimens were then examined under a light microscope and photos were taken. The photos were subjected to RGB analysis using Adobe Photoshop software. Findings revealed that all dependent variables exhibit significant differences among the groups, as indicated by the provided F-values and significance levels ($p = 0.0001425$, below the 0.05 threshold). The 75% concentration of *Nephelium lappaceum* peel extract was the most effective, producing a color closely resembling standard Crystal Violet. This suggests its potential as an alternative primary stain in Gram staining. However, the rambutan peel stain did not effectively stain all Gram-positive bacteria across all tested concentrations. Therefore, to increase the yield of anthocyanins, it is recommended to explore non-conventional extraction methods.

KEYWORDS: *Nephelium lappaceum, bacteriologic stain, anthocyanin, rambutan peel extract, gram-staining alternative*

1.0 INTRODUCTION

Nephelium lappaceum, commonly known as rambutan, is a delicious tropical fruit that belongs to the *Sapindaceae* family. The peels of *Nephelium lappaceum* fruit possess anthocyanin and flavonoid compounds suitable for fabric dyeing. The *Nephelium lappaceum* peel contains valuable components such as ascorbic acid, flavonoids, anthocyanin, tannin, ellagic acid, corilagin, and geraniin [1]. Anthocyanins are a multitudinal group of about 600 water-soluble phenolic pigments that are responsible for the blue, purple, and red hue of different fruits, flowers, and vegetables for example, beetroot, pomegranate, grapes, roses, and red cabbage [2]. Gram staining is one of the most crucial staining techniques in microbiology allowing the differentiation of bacterial cells into two major groups based on their cell wall characteristics: Gram-positive and Gram-negative. The primary stain used in this process is crystal violet. However, there are growing concerns about the environmental impact, health impact, cost, and accessibility of synthetic dyes. These challenges highlight the need for an alternative primary stain that is cost-effective, readily available, environmentally, and health-friendly.

The Philippine Textile Research Institute's [PTRI] involvement in the 'Colorimetric and Performance Standardization of NatDyes Produced in Various Natdyes Hubs in the Philippines' project. The project addressed environmental concerns about synthetic colorants by encouraging the use of natural alternatives to reduce risks. Researchers at Cebu Doctors' University, Mandaue, Cebu, Philippines, studied the extraction of natural dye using Syzygium jambolanum (Java Plum) extract as an alternative to Crystal violet dye for gram- staining bacteria which presented an important contribution to the search for eco-friendly staining methods in biological research [3]. In addition, a study at Ahmadu Bello University Funtua, Nigeria investigated the potential of a plant extract as an alternative counterstain in gram staining reactions using Lawsonia inamis L. (henna plant) leaf extracts [4]. A study also investigated the potential of Ipomoea batatas L (purple sweet potato) peel extract as an alternative for the primary stain in Gram staining of bacteria [5]. *Nephelium lappaceum* peel have underwent extraction through methods involving heat-assisted extraction and ultrasound-assisted extraction in a previous study [6]. An increasing pH caused greater destruction of anthocyanin in samples. The concentration of the amount of anthocyanin is variable in plants [7]. In a study conducted on Lycium ruthenicum the total

anthocyanin content in the green fruit stage, or at the juvenile stage was relatively low but significantly increased during fruit development and maturation was observed [8].

In recent times, there has been an increasing focus on creating environmentally friendly and sustainable alternatives for the chemical reagents employed in laboratory techniques, such as Gram staining. However, no prior research has specifically delved into investigating alternative plant extracts, specifically utilizing *Nephelium lappaceum* peel extract as a primary stain in Gram staining, subsequently, most studies focus only on investigating the antioxidant properties of *Nephelium lappaceum*. In this study, Staphylococcus aureus is subjected to staining, serving as the control for the study. Also, this study determined the concentration for the Gram staining process. The purpose of this study was to assess the efficiency of utilizing *Nephelium lappaceum* peel extract as the primary stain in Gram staining. Successful outcomes from this research could potentially lead to a more economical and environmentally friendly microbiological staining procedure, promoting health-conscious practices.

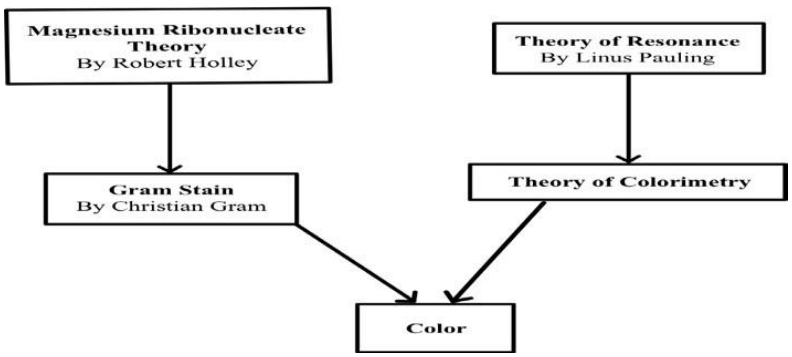

Figure 1. Theoretical Framework of the Study

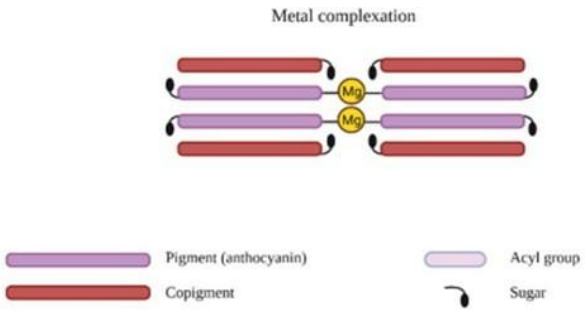

Figure 2. Affinity of Magnesium and Anthocyanins [9]

Colorimetry is the science of the measurement of color. It involves the replacement of subjective responses, such as 'light blue', rich dark purple', and 'bright gold', with an objective numerical system. Colorimetry quantitates these aspects and introduces the concepts of standard illumination and observers, leading to color representations such as RGB, XYZ, L*a*b*, and L*C*h to unambiguously define color [10]. The Magnesium Ribonucleate Theory plays a significant role in the field of Gram Staining, a fundamental technique in microbiology. This theory focuses on the differential staining characteristics of Gram- positive and Gram-negative bacteria. Moreover, the main staining component to be experimented is the anthocyanin that is present in the peel of *Nephelium lappaceum*. The color production from the peel extract is based on the Theory of Resonance by Linus Pauling which explained that the actual normal state of a molecule is represented not by a single valence-bond structure but by a combination of several alternative distinct structures [11]. The difference between the energies of any one of the alternative structures and the energy of the resonance hybrid was designated resonance energy. This theory greatly supports how our main staining component, anthocyanin, produces its color.

This study aimed to investigate the capability of *Nephelium lappaceum* peel extract to effectively act as a primary stain in gram- staining Staphylococcus aureus. The study aimed to answer the following research sub-questions:

1. Is there a significant difference between the staining results of the *Nephelium lappaceum* peel extract at different concentrations as a primary stain and the standard crystal violet in the Gram staining method?

2. What is the optimal concentration of *Nephelium lappaceum* peel extract needed to adequately stain the bacterial control (Staphylococcus aureus)?

2.1. 25% stain concentration

2.2. 50% stain concentration

2.3. 75% stain concentration

3. Is there a difference in cost between the *Nephelium lappaceum* peel extract stain and commercially purchased crystal violet for Gram staining?

Hypothesis of the Study

The following hypotheses were formulated for this study:

H_0: The use of *Nephelium lappaceum* peel extract does not result in a statistically significant difference in effectiveness as an alternative primary stain compared to the standard crystal violet stain in the gram staining method.

H_a: The use of *Nephelium lappaceum* peel extract resulted in a statistically significant difference in effectiveness as an alternative primary stain compared to the standard crystal violet stain in the gram staining method.

This study is significant as it proposes the use of *Nephelium lappaceum* peel as a novel and environmentally friendly method of staining bacteria in laboratories. The research aimed to determine whether this natural material can be used in place of more expensive and environmentally harmful staining techniques. The study, taken as a whole, is in favor of the use of ecologically friendly substitutes in scientific research.

2.0 METHODOLOGY

2.1 Research Design, Environment, Instruments

This study utilized a quasi-experimental design to assess the staining efficacy of *Nephelium lappaceum* peel extract compared to traditional Gram stains. The experiment was conducted in a controlled environment, with a focus on maintaining consistency in bacterial cultures, staining procedures, and environmental conditions.

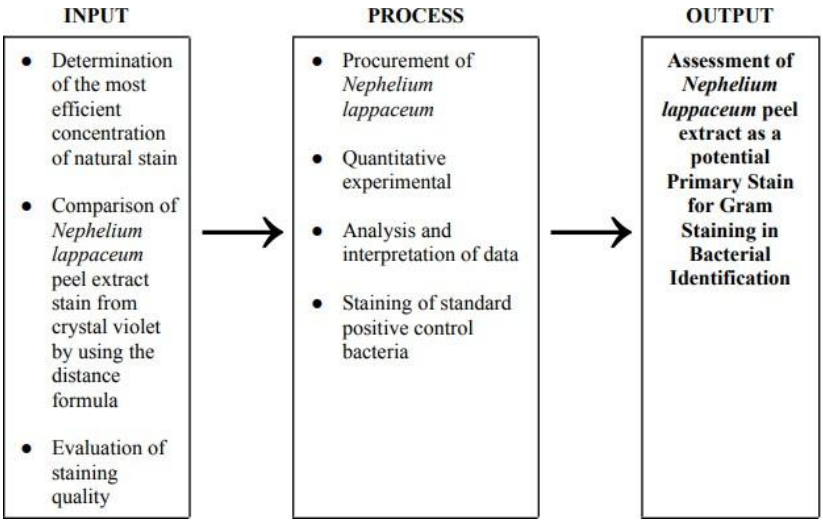

Figure 3. Overall Research Flow

The research was conducted in the Microbiology-Parasitology Laboratory at the University of Cebu - Banilad Campus. The strain of bacteria, Staphylococcus aureus, were carefully obtained from the University of Cebu Medical Center. *Nephelium lappaceum* peels were responsibly sourced from a local and pesticide-free origin. The extraction process was conducted within the laboratory using well-established and validated protocols. The research began with fruit collection from the Carbon public market, and taxonomic identification was verified by Community Environmental and Natural Resources Officer (CENRO) and the Dean of the College of Agriculture, Forestry, and Environment (CAFE). Fruit peels were soaked in absolute ethanol, extracted using a mortar and pestle, dehydrated in an oven, and filtered with Whatman paper; pH was adjusted using 0.1 N NaOH. A Gram staining setup was conducted with Staphylococcus aureus as the control, under aseptic conditions in a biosafety cabinet, with incubation and PPE protocols strictly followed. Bacterial smears were examined using light microscopes, with standard glassware and staining dishes. RGB charts, prior studies, and literature guided stain analysis, while photos of each specimen were taken using a 12MP Apple iPad Pro (f1.8, 29mm lens) to determine RGB codes. Quantitative data were analyzed using the distance formula to compare staining efficacy between traditional Gram stains and *Nephelium lappaceum* peel extract.

2.2 Research Procedure and Analysis

Prior to conducting the study, the researchers have secured an approval sheet from the University of Cebu Academic Research Ethics Committee (UCAREC) to make sure that the study adheres to ethical standards. To investigate the efficacy of *Nephelium lappaceum* as an alternative primary stain in Gram staining and identify the optimal time and concentration for optimal results, the researchers prepared instruments, extracted peel content by soaking in ethanol, oven-drying at 40°C, and pulverizing the peels. Powdered Extract Preparation involved weighing 50 grams of powdered peel extract. Mix weighed powdered peel extract with 300 mL absolute alcohol.

With the use of filter paper, filter mixture. Add 0.1 N Sodium hydroxide until the solution reaches a pH of 7.3. Oven dry again until completely powdered. Concentration variation includes weighing 6.25 grams, 12.5 grams, and 18.75 grams of powdered extract. Place each amount into containers labeled with 25% concentration, 50% concentration, and 75% concentration, respectively. Add 50 mL of distilled water to each container with powdered extract. Mix until the powder is completely dissolved. Prepare microorganisms for the experiment, ensuring a total of 31 slides. Use 10 slides of Staphylococcus aureus bacterium (gram- positive) for each concentration (25%, 50%, and 75%). Utilize one slide for Staphylococcus aureus as control by employing standard gram staining. Thirty (30) slides were stained using three different concentrations, with 10 slides per concentration (25%, 50%, 75%) and one slide was stained with the standard crystal violet. Thirty-one (31) slides were stained overall. Compare each slide based on their colors and staining intensity at different times and concentrations. Acquire RGB codes to the bacteria that were stained using Adobe Photoshop 2024, with the use of the distance formula, to compare the alternative stain with the standard Crystal violet. Record and analyze the results for further interpretation and comparison. To assess the potential of *Nephelium lappaceum* peel extract as a primary stain, the researchers employed high-quality light microscopes to examine the morphology and staining patterns of Staphylococcus aureus treated with both crystal violet and the peel extract. RGB coding and color analysis software were used to quantitatively compare staining intensity and distribution, guided by prior studies and relevant literature. The distance formula facilitated precise comparisons between the standard

and alternative stains. Statistical analyses were conducted to evaluate any significant differences, with findings highlighting the potential efficacy of *Nephelium lappaceum* extract as a Gram staining agent.

Data Analysis

1. To determine the concentration of the *Nephelium lappaceum* peel extract that was used in the experiment, the percent weight of the extract in the total volume of water is utilized:

$$\text{Concentration \% (w/v)} = \frac{\text{mass of solute (g)}}{\text{volume of solution}} \times 100$$

where:
w = weight of *Nephelium lappaceum* peel extract in grams v = volume of distilled water in milliliters

2. To compare the significant difference in the results of the different concentrations of the sample, a one-way ANOVA test with SPSS instrument version 23.0 will be used.

3. In order to determine the percent difference between the two RGB codes, the researchers utilized the distance formula, which took into account the variations in color values across the red, green, and blue channels. This formula allowed the researchers to quantify the degree of dissimilarity between the colors represented by the RGB codes. It's important to note that the higher the percentage difference calculated using this method, the more dissimilar it is between the colors, providing a clear indication of their distinctiveness.

$$d = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2 + (z_2 - z_1)^2}$$

$$d\% = \frac{d1}{d2} \times 100$$

where:

d = distance of two RGB codes
d% = distance percentage of dissimilarity of both RGB codes
x1 = first value of red in RGB
x2 = second value of red in RGB
y1 = first value of green in RGB
y2 = second value of green in RGB
z1 = first value of blue in RGB
z2 = second value of blue in RGB

The data that were gathered from the study will be presented through tabular and graphical presentations. The significant differences of the

different concentrations gathered from the one-way ANOVA will be presented in a histogram for comparison of the 1-minute time of staining in both bacterial controls and crystal violet control. The variation in hues of the colors will also be detected and compared with the crystal violet control which is presented in a tabular form.

3.0 RESULTS AND DISCUSSION

This chapter contains four parts. The first (1st) part reveals the summary of the one-way ANOVA results and its quantitative significance to the study. The second (2nd) part shows the RGB results and its corresponding RGB integer with the 3 different concentrations of the *Nephelium lappaceum* peel extract as a primary stain that represents the intensity of color in comparison to Crystal Violet. The third (3rd) part pertains to the breakdown of cost in order to compare and to determine the cost-effectiveness of the *Nephelium lappaceum* peel extract as a primary stain of Gram's stain. The fourth (4th) part showcases the microscopic visualization of the different slides per concentration to appreciate the outcome of the experiment.

3.1 Determination of Statistical Significance Between Concentrations

To statistically represent the color from the gathered RGB code, RGB Integer is utilized for the purpose of analysis with SPSS One-way ANOVA. In the RGB color model, colors are represented by combining different intensities of red, green, and blue color. Each color channel (R, G, B) uses 8 bits, allowing values from 0 to 255. The combination of these three channels creates a wide spectrum of colors. To convert an RGB color to an integer, we use bitwise shifting and logical OR operations. By left-shifting the alpha value (for opacity) by 24 bits, and similarly shifting the red, green, and blue values by 24, 8, and 0 bits, respectively, we create a single integer. This integer represents the color in the RGB format, commonly used in programming languages for color manipulation and visualization (Using Data Types, 2020). Findings from the analysis show p-values a statistically significant difference in stain intensity among the concentrations. The p-value of 0.0001245 indicates strong evidence against the null hypothesis, and the large effect size emphasizes the practical significance of this difference. As these p-value than the common significance level of α =

0.05, we reject the null hypothesis for the variable. In other words, the difference between the sample averages of some groups is big enough to be statistically significant. F-statistic of 17.6641 suggests that there are significant differences among the groups you're comparing. Further research with larger sample sizes or different conditions may be needed to detect subtle impacts or other potential influences.

3.2 Concentrations of *Nephelium lappaceum* Peel Extract

The gram-positive bacteria Staphylococcus aureus was gram-stained with the 3 different concentrations of the *Nephelium lappaceum* peel extract as a primary stain. Table 1 shows a summary of the results gathered from the different concentrations of the peel extract and its corresponding RGB code that represents the intensity of color in comparison to the standard control, Crystal Violet.

The gram staining technique was followed in all of the samples regardless of concentration to maintain consistency. Photos of the slides under the microscope were taken and observations were noted. The photos of the bacteria were subjected to RGB analysis using Adobe Photoshop. The RGB code represents a color, as seen in Table 1 [10]. The mean of the RGB code was determined and compared with the control using the Euclidean distance formula and percentage difference. It was observed that the percentage difference of the 25%, 50%, 75% concentrations are 28.95%, 19.42%, 14.18%, respectively, as seen in Table 2.

Table 1. Results of the Hypothesis Test on Significant Differences in Staining Results of *Nephelium lappaceum* peel extract

Source	Sum of Squares	Mean Square	df	F	P-value	Significant	Decision
Groups	5.27 x 10^13	2.63 x 10^13	2	17.6641	0.00001245	Significant	Reject H_0
Within Groups	4.03 x 10^13	1.49 x 10^12	27				
Total	9.30 x 10^13	3.21 x 10^12	29				

Table 2. RGB Results for *Nephelium lappaceium* peel extract

Concentration	RGB Code Mean			Color	Euclidean distance	Percentage difference	RGB Int	RGB Hex
	R	G	B					
25%	128	68	159		127.85	28.95%	8406175	80449F
50%	98	37	143		85.85	19.42%	6432143	62258F
75%	79	15	133		62.72	14.18%	5181317	4F0F85
Control	30	18	94				1970782	1E125E

The decrease in percentage indicates a closer distance in color between the certain peel concentration and crystal violet. Based on the results, the 25% Rambutan peel extract was inadequate to penetrate the gram-positive bacteria and some bacteria were not completely stained with the extract. With the use of the distance formula to quantify the degree of dissimilarity between the extract and the commercial Crystal Violet, the researchers were able to come up with a value of 28.95% difference. The results also indicate that the 50% Rambutan peel extract was insufficient in penetrating gram-positive bacteria, and some bacteria were not fully stained by the extract. The percent difference of the extract and the commercial Crystal Violet was lower compared to the 25% concentration which indicates that the color produced in this concentration was closer to the commercial stain compared to the previous concentration. The 75% peel extract concentration showed the lowest percentage difference, 14.18%, out of the 3 concentrations (Table 2). This would indicate that this concentration had the closest staining color as the control which is the Crystal Violet. The color intensity of this concentration can be explained by the self-association phenomenon of anthocyanins where each anthocyanin molecule can form bonds with other anthocyanin molecules [9]. However, as for microscopic observation, it did not perform as well as the Crystal Violet as it was not able to stain all of the Staphylococcus aureus in the slide (Figure 4).

3.3 Total Cost and Breakdown

Table 3. Breakdown of Total Cost

Materials	Quantity	Used	Cost (in pesos)	Actual Cost
NaOH	1 kg	4 g	300.00	1.20
Distilled	6 L	6 L	80.00	80.00
Ethanol	2.5 L	1 L	1,450.00	580.00
Filter paper	1	1	50.00	50.00
			Total cost	**711.20**

Table 4. Cost Per Slide Comparison of Standard Crystal Violet Stain and Rambutan Peel Stain

	Quantity	Used	Cost (in pesos)	Actual Cost	Cost per slide (assuming 1 mL per slide)
Crystal Violet	500 mL	100 mL	400.00	400.00	4.00
Rambutan peels stain	150 mL	100 mL	711.2	474.00	4.74

To determine the cost-effectiveness of the Rambutan peel extract as a stain compared to the commercial crystal violet, the researchers identified the cost of the product and control per slide assuming that a volume of 1ml is used. Presented on Table 4, the Rambutan peel stain cost 74 Philippine centavos more than the commercial crystal violet. Although the resultant Rambutan peel stain was not cost-effective as compared to the standard crystal violet, taking into account that crystal violet is still regarded as a toxic biohazard substance that causes serious environmental and health problems makes natural dyes a more practical option [12].

Morphologic and color examination of Staphylococcus aureus under 1000x magnification of the microscope are carried out. Staphylococcus aureus is a gram-positive clustered round (cocci) bacteria that stain purple with a Gram-staining technique [13]. When stained with rambutan peel extract, the morphologic characteristics of the bacteria are clearly visualized and fairly similar to the control (Figure 4). As aforementioned, the bacteria were stained, showing colors ranging from pink to purple. The Gram stain procedure involves a primary stain, a mordant, a decolorizer, and the addition of the secondary stain or counterstain safranin. The colorless gram- negative bacteria will then turn pink or red when stained with safranin [14]. Because there is

scant anthocyanin in rambutan, 16.2 mg/g [6], some of the gram-positive bacteria are tinted pink by safranin, rather than violet. All three concentrations generally exhibit minimal Gram-positive color violet. Upon collection using maceration of the extract, it is noted that it exhibits a yellow-brown color. The addition of NaOH for the adjustment of pH did not affect the color of the extract. The yellow-brown color of the extract may be due to the color of the peels of the Rambutan or due to its ripeness. A factor that may have caused the minimal gram-positive violet coloration is the instability of the anthocyanins found in the rambutan peels. Anthocyanins are sensitive to light, pH, and temperature [15]. Under these circumstances, anthocyanins lose their color and become colorless or yellow degradation products [16].

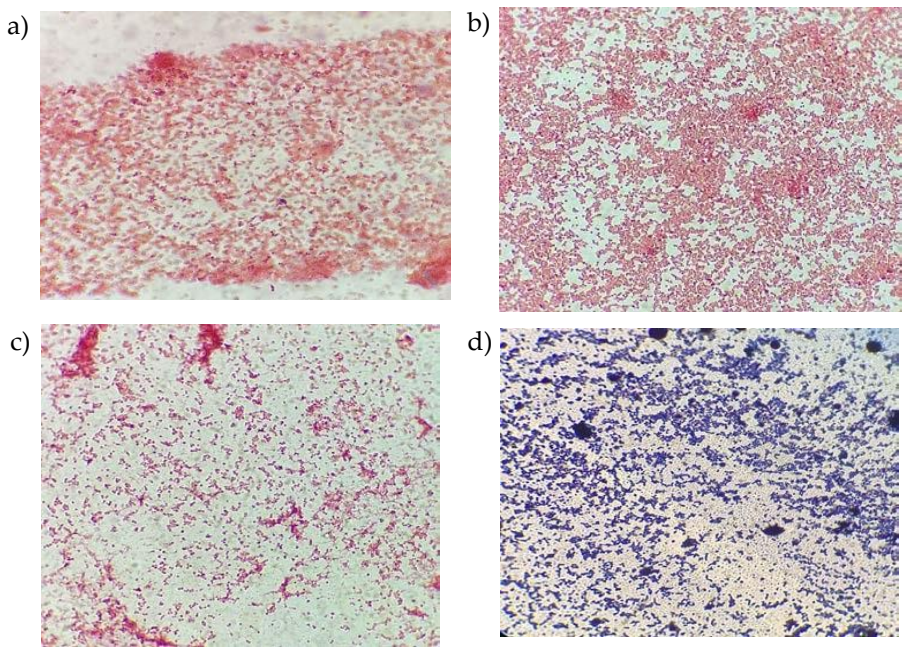

Figure 4. *S.aureus* Stained with a) 25% peel extract b) 50% peel extract c) 75% peel extract d) Crystal violet (control)

4.0 CONCLUSION

The researchers concluded that the p-value in the statistical tests suggests that there is a significant difference between the groups in any of the dependent variables. As a result, even though the extract had the potential to stain, it still fell short of Crystal violet in staining. The analysis showed that a 75% concentration of the extract produced the closest color similarity to Crystal violet. The *Nephelium lappaceum* peel extract is not as efficient as Crystal violet for bacterial identification since some of the Gram-positive bacteria were not sufficiently stained by the peel extract at all of the quantities examined. Based on a cost analysis, it was found that the peel extract was slightly more costly than Crystal Violet, yet environmentally preferable due to Crystal Violet's hazardous carcinogenic nature. Microscopic observations revealed a range of staining results, which may be related to the anthocyanin instability of the extract.

5.0 ACKNOWLEDGMENTS

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