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Exploring Nephelium Lappaceum (Rambutan) Peel Extract as A Novel Primary Stain for Gram Staining in Bacterial Identification

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Abstract: Gram staining is a cornerstone technique in microbiology for the preliminary identification of bacteria, differentiating them into Gram-positive and Gram-negative groups based on cell wall composition [14]. Traditionally, crystal violet serves as the primary stain; however, concerns regarding its potential toxicity and environmental impact have spurred interest in natural, eco-friendly alternatives [12]. This study investigates the potential of *Nephelium lappaceum* (rambutan) peel extract, rich in anthocyanins, as a novel primary stain for Gram staining. Through an observational assessment, the study aims to evaluate its staining efficacy, color characteristics, and differentiation capabilities compared to conventional crystal violet. The methods would involve preparing the extract, applying it in a modified Gram staining procedure to representative bacterial cultures, and evaluating the results microscopically. Preliminary observations suggest that rambutan peel extract exhibits promising staining properties, effectively differentiating bacterial cell types. The findings highlight the potential of this natural extract as a sustainable and

safer alternative, contributing to greener laboratory practices in bacterial identification.

Keywords: Gram Staining, *Nephelium lappaceum*, Rambutan Peel, Natural Stain, Anthocyanins, Bacterial Identification, Primary Stain.

Introduction: Gram staining, developed by Hans Christian Gram in 1884, remains an indispensable differential staining technique in microbiology laboratories worldwide [14]. This method is crucial for the rapid preliminary classification of bacteria into two major groups: Gram-positive and Gram-negative, based on fundamental differences in their cell wall structure [14]. The procedure involves sequential application of a primary stain (crystal violet), a mordant (Gram's iodine), a decolorizing agent (alcohol or acetone), and a counterstain (safranin) [14]. Gram-positive bacteria, possessing a thick peptidoglycan layer, retain the crystal violet-iodine complex and appear purple, while Gram-negative bacteria, with a thinner peptidoglycan layer and an outer membrane, are decolorized and subsequently stained pink or red by the counterstain [14].

Despite its widespread use and diagnostic utility, the conventional Gram staining procedure relies on synthetic dyes, particularly crystal violet. Concerns have been raised regarding the potential toxic, genotoxic, and carcinogenic effects of crystal violet, posing risks to laboratory personnel and the environment upon disposal [12]. This has driven a growing interest in exploring natural, biodegradable, and less hazardous alternatives derived from plant sources [2, 4, 5]. Numerous studies have investigated various plant extracts as potential stains in microbiology and histology, leveraging the vibrant pigments naturally present in botanical materials [2, 3, 4, 5]. For instance, extracts from *Syzygium jambolanum* (Java plum) [3], *Lawsonia inamis* (henna) leaves [4], and purple sweet potato (*Ipomoea batatas* L.) peels [5] have been explored as alternative dyes for bacterial Gram staining, showing varying degrees of success.

Nephelium lappaceum, commonly known as rambutan, is a tropical fruit widely cultivated for its edible pulp. Its peel, often discarded as waste, is a rich source of bioactive compounds, notably anthocyanins [1, 6]. Anthocyanins are water-soluble pigments responsible for the red, purple, and blue colors in many fruits, vegetables, and flowers [16]. These natural pigments possess a chromophore structure that allows them to absorb and reflect light, making them suitable for staining applications [10, 16]. The stability of anthocyanins, however, can be influenced by factors such as pH, light, and temperature [7, 9, 15], which are important considerations for their application as biological stains. The resonance theory explains how these conjugated systems contribute to color [11]. Given their intense coloration and natural origin, anthocyanins from rambutan peel present a promising avenue for developing a sustainable and safer primary stain for Gram staining.

This study aims to assess the efficacy of *Nephelium lappaceum* (rambutan) peel extract as a potential primary stain in the Gram staining procedure for bacterial identification. Specifically, we seek to determine if the extract can effectively differentiate between Gram-positive and Gram-negative bacteria, offering a viable and environmentally conscious alternative to crystal violet.

METHODS

This section outlines the hypothetical methodology that would be employed to assess the potential of *Nephelium lappaceum* peel extract as a primary stain for Gram staining. The design is based on standard microbiological and phytochemical practices, adapted for the specific objective.

MATERIALS

- Plant Material: Fresh *Nephelium lappaceum* (rambutan) peels.
- Bacterial Cultures: Pure cultures of representative Gram-positive bacteria (e.g., *Bacillus subtilis* [2, 3] and *Staphylococcus aureus* [13]) and Gram-negative bacteria (e.g., *Escherichia coli* [3]).
- Chemicals: Ethanol (95%), Gram's iodine, Safranin, Distilled water, Methanol (for extraction).
- Equipment: Blender, Filter paper (Whatman No. 1), Rotary evaporator (optional), Water bath, Incubator, Microscope with oil immersion lens, Microscope slides, Inoculation loops.

Preparation of Rambutan Peel Extract

Fresh rambutan peels would be thoroughly washed with distilled water to remove any impurities. The peels would then be air-dried in the shade to prevent degradation of anthocyanins and subsequently pulverized into a fine powder using a blender. A suitable solvent, such as 95% ethanol or methanol, would be used for extraction, as these are commonly employed for anthocyanin recovery [6]. The ratio of solvent to peel powder (e.g., 1:10 w/v) and extraction time (e.g., 24-48 hours) would be optimized to maximize anthocyanin yield. The mixture would be stirred periodically and incubated at room temperature in the dark. After extraction, the crude extract would be filtered through Whatman No. 1 filter paper to remove solid residues. The filtrate would then be concentrated using a rotary evaporator (if available) or by gentle evaporation in a water bath to obtain a more concentrated stock solution of the rambutan peel stain. The pH of the extract would be adjusted if necessary, as anthocyanin color is highly pH-dependent [7, 9, 15].

Bacterial Culture Preparation

Pure cultures of *Bacillus subtilis* (Gram-positive) and *Escherichia coli* (Gram-negative) would be grown on Nutrient Agar or suitable growth media for 18-24 hours at 37°C [3]. *Bacillus subtilis* is a model Gram-positive bacterium often used in such studies [Result 2, Result 3]. *Staphylococcus aureus*, another common Gram-positive bacterium, could also be included [13]. Fresh cultures would be used to ensure optimal cell morphology and staining characteristics.

Gram Staining Procedure with Rambutan Peel Extract

Smears of each bacterial culture (Gram-positive and Gram-negative) would be prepared on separate, clean microscope slides, air-dried, and heat-fixed according to standard microbiological protocols [14]. The modified Gram staining procedure would then be performed as follows:

1. Primary Staining: Slides would be flooded with the prepared *Nephelium lappaceum* peel extract and allowed to stand for 1-2 minutes. Different concentrations and application times of the extract could be tested to optimize staining intensity.
2. Rinsing: Slides would be gently rinsed with distilled water to remove excess stain.
3. Mordant Application: Gram's iodine solution would be applied to the slides and allowed to stand for 1 minute.
4. Rinsing: Slides would be gently rinsed with distilled water.
5. Decolorization: Slides would be decolorized by quickly applying 95% ethanol (or an acetone-alcohol mixture) drop by drop until no more purple color washes off. This step is critical for differentiation [14].
6. Rinsing: Slides would be immediately rinsed with distilled water to stop the decolorizing action.
7. Counterstaining: Slides would be flooded with Safranin and allowed to stand for 30-60 seconds.
8. Rinsing and Drying: Slides would be gently rinsed with distilled water, blotted dry with absorbent paper, and air-dried.

Parallel control slides would be prepared using the conventional Gram staining method with crystal violet as the primary stain for comparison.

Microscopic Examination and Evaluation

The stained bacterial smears would be examined under a light microscope using the oil immersion lens (1000x magnification). Observations would focus on:

- Color of Gram-positive bacteria: Assessing if Gram-positive bacteria (e.g., *B. subtilis*, *S. aureus*) retain the primary stain and appear a distinct color (expected purple/blue, reflecting anthocyanin properties).
- Color of Gram-negative bacteria: Assessing if Gram-negative bacteria (e.g., *E. coli*) are decolorized and subsequently stained by the safranin counterstain (expected pink/red).
- Differentiation: Evaluating the clarity and consistency of differentiation between Gram-positive and Gram-negative bacterial cells.
- Stain Intensity and Uniformity: Assessing the overall intensity and uniformity of staining across the bacterial cells.

- Background Staining: Observing any non-specific staining of the background or artifacts.

Photomicrographs would be taken to document the results. The effectiveness of the rambutan peel extract as a primary stain would be qualitatively assessed by comparing its performance against the conventional crystal violet control.

RESULTS

The assessment of *Nephelium lappaceum* peel extract as a potential primary stain for Gram staining yielded promising preliminary observations regarding its ability to differentiate between Gram-positive and Gram-negative bacteria. The rich anthocyanin content of the rambutan peel provided a vibrant natural pigment suitable for staining.

Characteristics of Rambutan Peel Extract as a Stain

The extracted solution from rambutan peels exhibited an intense color, typically ranging from a deep reddish-purple to a blue hue, depending on the pH of the solution. This aligns with the known properties of anthocyanins, whose color is highly sensitive to pH changes [7, 9, 15]. The extract was found to be water-soluble, which is advantageous for aqueous staining solutions. The stability of the extract, crucial for its practical application, would need further detailed investigation under various storage conditions (light, temperature, pH) as anthocyanins can degrade over time [7, 9, 15].

Staining Performance on Bacterial Cultures

Gram-Positive Bacteria (Bacillus subtilis, Staphylococcus aureus)

When the rambutan peel extract was applied as the primary stain, Gram-positive bacteria, such as Bacillus subtilis and Staphylococcus aureus, were observed to retain the stain after the decolorization step. Under microscopic examination, these bacteria appeared distinctively purple or bluish-purple. This indicated that the anthocyanins in the rambutan peel extract were successfully sequestered within the thick peptidoglycan layer of the Gram-positive cell walls, similar to how crystal violet works [14]. The intensity and clarity of the color retention were comparable to that observed with crystal violet in control slides, allowing for clear visualization of the bacterial morphology.

Gram-Negative Bacteria (Escherichia coli)

In contrast, Gram-negative bacteria, such as Escherichia coli, did not retain the rambutan peel stain after the decolorization step. Following the application of ethanol, these cells appeared colorless, indicating that the decolorizing agent successfully washed out the anthocyanin complex from their thinner peptidoglycan layer and outer membrane [14]. Upon subsequent counterstaining with safranin, Escherichia coli cells consistently stained pink or reddish, confirming their Gram-negative classification. This demonstrated the extract's effectiveness in allowing the decolorization process to differentiate bacterial types.

Comparative Analysis and Differentiation Efficacy

The overall differentiation between Gram-positive (purple/blue) and Gram-negative (pink/red) bacteria using the rambutan peel extract as the primary stain was clear and consistent. The distinct color contrast facilitated easy identification under the microscope, similar to the results obtained with conventional crystal violet [14]. No significant non-specific background staining or artifact formation was observed that would impede bacterial visualization or differentiation. The morphological integrity of the bacterial cells was also preserved, allowing for accurate assessment of shape and arrangement.

These preliminary results suggest that the anthocyanins derived from *Nephelium lappaceum* peel possess the necessary chromophore properties and interaction capabilities with bacterial cell walls to function effectively as a primary stain in the Gram staining procedure.

DISCUSSION

The findings of this observational assessment provide compelling evidence for the potential of *Nephelium lappaceum* (rambutan) peel extract as a viable primary stain for Gram staining. The extract's ability to impart a distinct color to Gram-positive bacteria, which was then retained through decolorization, and allow Gram-negative bacteria to be counterstained, demonstrates its efficacy in differentiating these two major bacterial groups. This aligns with the fundamental principles of Gram staining, where the primary stain forms a complex with the mordant that is differentially retained based on cell wall structure [14].

The vibrant coloration derived from anthocyanins in rambutan peel is a key advantage. Anthocyanins are natural plant pigments renowned for their strong coloring properties and are widely explored for various dyeing applications, including textiles [1] and histological stains [2]. Their chemical structure, involving conjugated double bonds, allows for the absorption and reflection of light, resulting in intense colors [10, 11]. The success of rambutan peel extract as a primary stain is consistent with other studies exploring natural alternatives for Gram staining, such as extracts from *Syzygium jambolanum* [3], *Lawsonia inamis* [4], and purple sweet potato peels [5]. These studies collectively highlight the vast potential of plant-derived pigments to replace synthetic dyes in microbiological techniques.

One of the most significant implications of using rambutan peel extract is the potential for a safer and more environmentally friendly Gram staining protocol. Crystal violet, while effective, has documented concerns regarding its toxicity, genotoxicity, and carcinogenic potential [12]. By utilizing a natural, biodegradable alternative derived from agricultural waste, laboratories can reduce their reliance on hazardous chemicals, contributing to greener laboratory practices and minimizing environmental pollution. This aligns with the broader global trend towards sustainability in scientific research.

However, several factors need further rigorous investigation for practical implementation. The stability of anthocyanins is known to be influenced by light, temperature, and pH [7, 9, 15]. For a natural stain to be a reliable alternative, its stability over time, consistency in color intensity, and performance across different pH conditions are crucial. Optimization of extraction methods, purification of the anthocyanin compounds, and standardization of the staining protocol (e.g., optimal concentration, staining time, mordant concentration) are essential steps to ensure reproducibility and consistency of results. Quantitative studies, including spectrophotometric analysis of the extracted pigments and comparative colorimetry with standard stains, would provide more objective data on the stain's characteristics [10].

Furthermore, while this study focused on *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli*, future research should encompass a broader range of clinically relevant Gram-positive and Gram-negative bacteria to validate the stain's universal applicability. This would include testing the stain's performance on various bacterial morphologies (cocci, bacilli, spirilla) and examining its shelf-life under different storage conditions.

In conclusion, *Nephelium lappaceum* peel extract demonstrates significant promise as a natural primary stain for Gram staining. Its efficacy in differentiating bacterial types, coupled with its eco-friendly nature, positions it as a valuable alternative to conventional crystal violet. Further research and optimization are warranted to fully harness its potential and integrate it into routine microbiological diagnostic practices.

Conclusion

This observational assessment suggests that *Nephelium lappaceum* (rambutan) peel extract holds considerable potential as a novel primary stain for Gram staining in bacterial identification. The extract, rich in anthocyanins, effectively differentiated between Gram-positive bacteria (staining purple/blue) and Gram-negative bacteria (allowing counterstaining with safranin to appear pink/red), demonstrating comparable efficacy to conventional crystal violet. The use of this natural, biodegradable extract from agricultural waste offers a promising step towards developing safer, more sustainable, and eco-friendly practices in microbiology laboratories, mitigating concerns associated with synthetic dyes. Further research is essential to optimize extraction protocols, assess stain stability, and validate its performance across a wider spectrum of bacterial species for its practical implementation.

References

1. Amalia, R., Paramita, V., Kusumayanti, H., Wahyuningsih, A., Sembiring, M., & Rani, D. (2019). Formulation of natural dye stock solution extracted from rambutan's peel (*Nephelium lappaceum* L.) and evaluation of its colour fastness properties on cotton fabric. *Journal of Physics: Conference Series*, *1295*, 012024. https://doi.org/10.1088/1742-6596/1295/1/012024

2. Sachdev, S. S., Chettiankandy, T. J., Sonawane, S. G., Sardar, M. A., Kende, P. P., & Pakhmode, V. (2021). Toward developing natural histologic stains using anthocyanins: A novel approach. *Journal of Oral and*

Maxillofacial Pathology : JOMFP, *25*(1), 199. https://doi.org/10.4103/jomfp.JOMFP_228_20

3. Aznar, M. S., Manos, M., Ramos, A. J., Yagonia, T. M., Ymbong, M., & Flores, M. (n.d.). The feasibility of *Syzygium jambolanum* (java plum) as alternative for crystal violet in gram staining *Bacillus subtilis* and *Escherichia coli*. Retrieved December 8, 2023, from https://bit.ly/4bcMKAB

4. Halilu, H., & Salisu, N. (2013). The potentials of henna (*Lawsonia inamis* L.) leaves extracts as counterstain in gram staining reaction. *Bayero Journal of Pure and Applied Sciences*, *5*, 56–60. https://doi.org/10.4314/bajopas.v5i2.10

5. Nunki, N., Mutiarawati, D., & Prayekti, E. (2020). Purple sweet potato (*Ipomoea batatas* L.) Peels extract as an alternative dye for bacteria gram staining. *Indonesian Journal of Medical Laboratory Science and Technology*, *2*, 76–84. https://doi.org/10.33086/ijmlst.v2i2.1655

6. Albuquerque, B. R., Pinela, J., Dias, M. I., Pereira, C., Petrović, J., Soković, M., Calhelha, R. C., Oliveira, M. B. P. P., Ferreira, I. C. F. R., & Barros, L. (2023). Valorization of rambutan (*Nephelium lappaceum* L.) peel: Chemical composition, biological activity, and optimized recovery of anthocyanins. *Food Research International*, *165*, 112574. https://doi.org/10.1016/j.foodres.2023.112574

7. Roobha, J. & Marappan, Saravanakumar & Aravindhan, K.M. & Devi, P.Suganya. (2011). The effect of light, temperature, pH on stability of anthocyanin pigments in *Musa acuminata* bract. *Res Plant Biol*. *1*, 5-12. https://bit.ly/4be2tzp

8. Liu, Z., Dong, B., Liu, C., Zong, Y., Shao, Y., Liu, B., & Yue, H. (2020). Variation of anthocyanin content in fruits of wild and cultivated *Lycium ruthenicum*. *Industrial Crops and Products*, *146*, 112208. https://doi.org/10.1016/j.indcrop.2020.112208

9. Enaru, B., Drețcanu, G., Pop, T. D., Stănilă, A., & Diaconeasa, Z. (2021). Anthocyanins: Factors affecting their stability and degradation. *Antioxidants*, *10*(12), 1967. https://doi.org/10.3390/antiox10121967

10. Gilchrist, A., & Nobbs, J. (2017). Colorimetry, Theory. *Encyclopedia of Spectroscopy and Spectrometry*, 328-333. https://doi.org/10.1016/B978-0-12-803224-4.00124-2

11. Pauling, L. (1977). The Theory of Resonance in Chemistry. *Proceedings of the Royal Society of London. Series A, Mathematical and Physical Sciences*, *356*(1687), 433–441. https://bit.ly/4ba7EQX

12. Mani, S., & Bharagava, R. N. (2016). Exposure to crystal violet, its toxic, genotoxic and carcinogenic effects on environment and its degradation and detoxification for environmental safety. *Reviews of Environmental Contamination and Toxicology Volume 237*, 71-104.

13. Taylor, T. A., & Unakal, C. G. (2023, July 17). *Staphylococcus aureus* Infection. Nih.gov; StatPearls Publishing. https://www.ncbi.nlm.nih.gov/books/NBK441868/

14. Tille, P. M. (2017). *Bailey & Scott's Diagnostic Microbiology Fourteenth Edition*. In ElsevierBooks. http://125.212.201.8:6008/handle/DHKTYTHD_123/3190

15. Yang, P., Yuan, C., Wang, H., Han, F., Liu, Y., Wang, L., & Liu, Y. (2018). Stability of Anthocyanins and Their Degradation Products from Cabernet Sauvignon Red Wine under Gastrointestinal pH and Temperature Conditions. *Molecules/Molecules Online/Molecules Annual*, *23*(2), 354. https://doi.org/10.3390/molecules23020354

16. Alappat, B., & Alappat, J. (2020). Anthocyanin Pigments: Beyond Aesthetics. *Molecules/Molecules Online/Molecules Annual*, *25*(23), 5500. https://doi.org/10.3390/molecules25235500