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Utilization of Yam Extract as an Alternative to Crystal Violet for Staining *Streptococcus mutans* Bacteria

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ABSTRACT

Gram staining is a fundamental technique in microbiology, vital for the initial identification of bacteria based on differences in cell wall structure, classifying them as Gram-positive and Gram-negative. This method is crucial for rapid clinical diagnosis. Currently, its primary stain, Crystal Violet, raises serious concerns due to its mutagenic, toxic, and environmentally unfriendly properties, necessitating the search for safer and more sustainable alternatives. This study focuses on the potential of yam tubers (Dioscorea sp.), an abundant local resource, as a substitute for Crystal Violet. Yam tubers are rich in anthocyanins, natural pigments with cationic properties and antibacterial activity, making them a promising dye candidate. The selected test bacterium was Streptococcus mutans, a Gram-positive, coccus-shaped bacterium and a major cause of dental caries, ideal for validating the staining ability of thick cell walls. The aim of this study was to evaluate the effectiveness of yam tuber extract in staining S. mutans and to compare its staining quality with standard Crystal Violet. The expected outcome is the creation of an economical, environmentally friendly, and sustainable staining method.

Keywords: Gram staining, natural dyes, yam tubers, Streptococcus mutans

I. INTRODUCTION

Gram Staining and crystal violet gram staining, introduced by hans christian gram in 1884, remains an indispensable foundation in clinical and research microbiology. This technique fundamentally divides bacteria into two groups : Gram positif (stained purple/blue due to retention of a primary dye) and gram negative (stained pink/red after absorption of a counter dye). This diffenretiation is based on differences in cell wall structure, geam positive bacteria have a thick peptidoglycan layer that effectively traps the crystal violet-yodine complex during decolorization, while gram negative bacteria, with a thin peptidoglycan layer, Lose color (Wu and Yang, 2020; Li et al., 2020). Dispite it's clinically proven affectiveness, the use of Crystal violet (CV) as a primary stain has raised serious concerns. Gentian Violet (KV) is a synthetic cationic dye tahat has been prove to be mutagenic, toxic, and a persistent environmental pollutant. It's high stability in the environment makes it difficult to decompose,

creating seriuos challenges in laboratory waste management (Dedefwin, 2021). Thereofore, there is an urgent need to develop safer and more sustainable alternatives, in line with the principles of green chemistry of green laboratories (Aluf supardi and risandiansyah, 2022). Alternative Natural Dyes And The Role Of Anthocyanins The search for alternative primary dyes for Gram stain has led to the use of natural resources, particularly plant extracts rich in natural pigments. Current research trends focus on anthocyanin compounds.

Anthocyanins are natural pigments responsible for the red, purple, and blue colors in many plants. Chemically, anthocyanins belong to the flavonoid group and, most importantly, have cationic (positively charged) properties (Marbun, 2020). This cationic nature theoretically mimics the function of crystal violet, allowing it to interact and bind strongly with negatively charged components of bacterial cell walls, particularly the thick peptidoglycan layer in Gram-positive bacteria.

Several studies have demonstrated the potential of anthocyanin-rich plant extracts as a substitute for Crystal Purple:

1. Purple Sweet Potato (Ipomoea batatas P.): Studies have shown that purple sweet potato juice or extract can stain Gram-positive bacteria, such as Staphylococcus aureus and Bacillus sp., producing a fairly reddish-purple color, although sometimes leaving a precipitate (Sri Nurul Hidayanti et al., 2021; Marbun, 2020). This strengthens the hypothesis that anthocyanin structures can function as primary dyes in the Gram differential staining scheme.
2. Other Plant-Based Ingredients: In addition to sweet potatoes, ingredients such as henna leaves (Lawsonia inermis), which also contain anthocyanins, have been studied as alternative dyes, particularly for comparison dyes (safranin/Gram-negative), although their primary potential as a primary dye for Gram-positive bacteria is also being explored (Tyasningrum, 2021).

Patents and Innovation: Although still dominated by scientific journal publications, the trend toward natural dyes has created opportunities for patented innovation, particularly in the formulation of stable dye preparations. Current research focuses on pH stability and optimal anthocyanin concentration, as the stability of these natural pigments is strongly influenced by pH, temperature, and light, which are key factors in the maceration and Gram staining processes (Abdulrahman et al., 2020). Therefore, yam tuber (Dioscorea sp.) extract, which is also rich in anthocyanins and a local resource, has the potential to become the next state of the art in the search for low-toxic and sustainable Gram stain formulations.

II. METHODS

1. Extract Preparation and Dyeing Principles

   This study aims to utilize purple yam tuber extract (Dioscorea alata L.) as an alternative dye to crystal violet in a modified Gram staining procedure. Yam tubers were chosen for their high anthocyanin pigment content. These flavonoid compounds can produce a spectrum of colors from red to purple, with a tendency toward purple at pH 4-6. Theoretically, this purple color resembles crystal violet, and the nature of anthocyanins as phenolic compounds that can interact with the cell wall structure of Gram-positive bacteria (Streptococcus mutans) underpins their potential as natural dyes.

2. Rejuvenation and Preparation of Stain

   To ensure the validity of the findings, all equipment was oven-sterilized prior to the study. The yam tuber extract was prepared through a process of washing, weighing, and grinding to obtain the optimal pigment concentration. The yam extract was then prepared in three different concentrations: 60%, 80%, and 100%. Prior to staining, the S. mutans bacteria were rejuvenated on nutrient agar medium to ensure they were in an active growth

phase. Stain preparations were prepared by taking a single bacterial colony, spreading it on a glass slide, drying it, and fixing it over a flame.

3. Modified Gram Staining Procedure

   The Gram staining procedure was modified by replacing the crystal violet solution with yam extract (in three concentrations). Staining began with the application of yam extract for 1 minute, followed by rinsing with running water. Next, the slides were exposed to Lugol's stain for 1 minute, washed, and destained with 96% alcohol for 30 seconds. The final step was the addition of safranin as a counterstain. As a positive control, the standard Gram staining procedure with crystal violet, Lugol's stain, alcohol, and safranin was used. All slides were then dried and observed under a microscope at 100x magnification.

This study aims to validate yam tuber extract as a substitute for Crystal Violet in the Gram staining of *Streptococcus mutans*, focusing on analyzing and comparing staining qualityspecifically color intensity and morphological clarityagainst the standard Crystal Violet stain. This innovation aligns with global Green Chemistry trends and provides significant contributions in the following areas:

1. **Reducing Toxicity:** Replacing carcinogenic synthetic dyes (Crystal Violet) with natural pigments (anthocyanins), which possess antioxidant and antibacterial properties, thereby improving laboratory safety.
2. **Sustainability and Economics:** Utilizing abundant local natural resources (yam tubers) to produce a more economical and environmentally sustainable diagnostic reagent.

This research positions yam tuber extract as an innovative and sustainable solution at the forefront of global efforts to develop environmentally friendly laboratory procedures, ensuring that fundamental diagnostic methods such as Gram staining can continue to be performed safely and efficiently.

III. RESULTS AND DISCUSSION

The study, entitled "Utilization of Yam Extract as a Natural Dye Substitute for Crystal Violet in Gram Staining," was conducted at the Microbiology Laboratory of Panrita Husada Health College, Bulukumba, in August 2025. The aim was to observe the results of staining using Yam Extract. The results are as follows. Figure 1. Positive control for Gram staining of Streptococcus mutans bacteria. In the study, the positive control was found to be purple-colored bacteria with a coccus shape arranged in a chain.

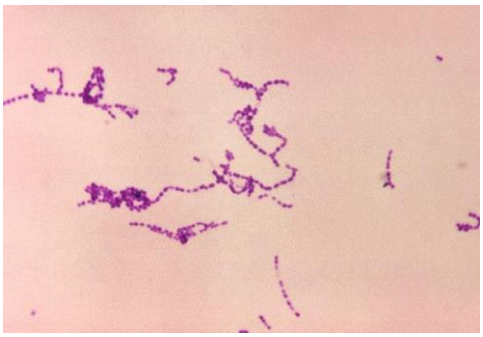
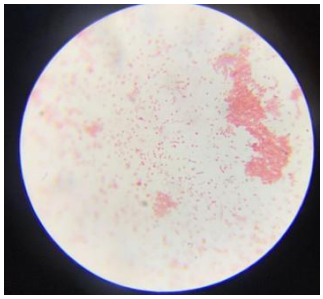
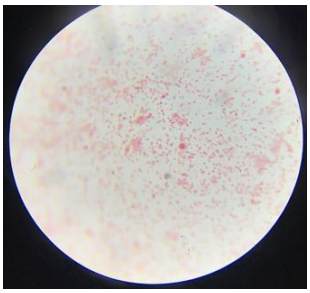
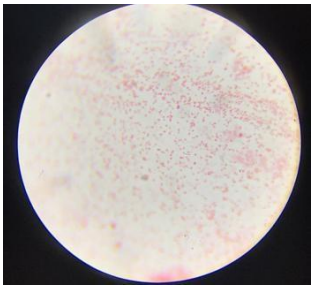
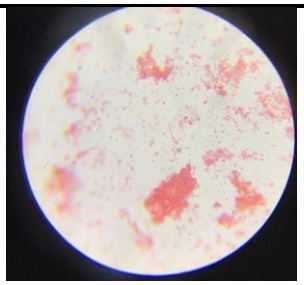
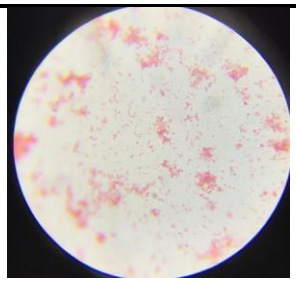
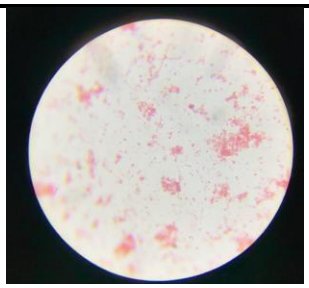
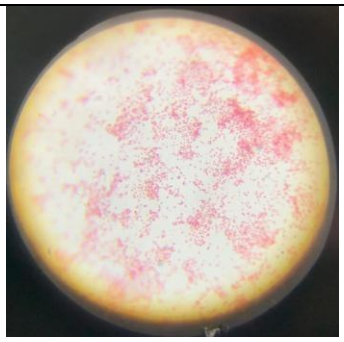
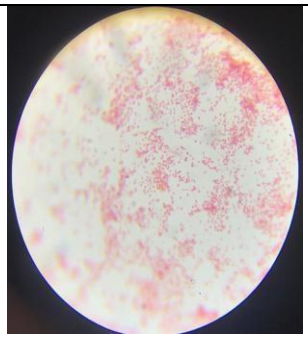
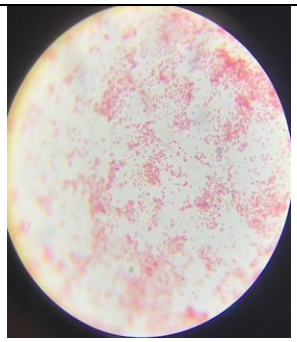


Figure 1. Positive control for Gram staining of Streptococcus mutans bacteria

Table 1. **Results of gram staining of *Streptococcus mutans* bacteria using yam tuber extract**

Concentration	Repetition 1	Repetition 2	Repetition 3	Description

60%				Coccus-shaped bacteria, red in color
80%				Coccus-shaped bacteria, red in color
100%				Coccus-shaped bacteria, red in color

In addition to the chemical factors previously described, methodological aspects in the extraction and stabilization process of the pigment must also be considered in understanding the failure of yam extract as a substitute for crystal violet. The preparation of yam extract that is not standardized—such as variations in heating temperature, drying method, or type of solvent—may reduce the effective concentration of anthocyanins. Anthocyanin pigments are known to be highly susceptible to oxidation when exposed to air, light, or heat, which can alter their color before they are applied to bacterial preparations. If pigment degradation occurs, the ability of anthocyanins to bind strongly to the peptidoglycan layer will be significantly weakened. Furthermore, the presence of impurities in the extract, such as polysaccharides, proteins, or other phenolic compounds, may interfere with the pigment's affinity for the cell wall. Such impurities could result in uneven staining distribution across the bacterial sample. Overall, these technical factors highlight the importance of optimizing extraction procedures before assessing the effectiveness of yam extract as a microbiological stain.

From a chemical standpoint, the fundamental difference between anthocyanin and crystal violet lies not only in their molecular structures but also in their interaction with bacterial cell wall components. Crystal violet is a basic (cationic) dye with a strong affinity for negatively charged groups in the thick peptidoglycan layer of Gram-positive bacteria. Anthocyanins, however, exhibit varying properties depending on pH, showing red

coloration in acidic environments and shifting to purple or becoming unstable in neutral to alkaline conditions. During Gram staining, exposure to iodine and alcohol introduces pH changes that can rapidly alter the chemical form of anthocyanins. This instability prevents the pigment from maintaining its color during the decolorization step. As a result, Gram-positive bacteria lose the primary stain and subsequently absorb safranin, appearing red. This phenomenon provides strong evidence that yam extract cannot function as a differential stain component.

The results of this study also highlight the importance of understanding the complete mechanism of Gram differentiation before developing alternative natural dyes. Gram staining is not merely a visual coloring process; it involves complex chemical interactions between dyes, solvents, and the microscopic structure of bacterial cell walls. The inability of yam extract to retain color in Gram-positive bacteria suggests that the natural pigment fails to perform one of the most critical steps—the formation of a stable dye-iodine complex. To serve as a substitute for crystal violet, a dye must be capable of forming large complexes that can precipitate within the peptidoglycan matrix. Without fulfilling this requirement, the distinction between Gram-positive and Gram-negative bacteria cannot be observed. Therefore, although yam extract demonstrates the capacity to color bacterial cells, its function resembles that of a simple stain rather than a differential stain. This understanding is essential for guiding future research toward more appropriate applications.

Although yam extract did not succeed as a replacement for crystal violet, the findings of this study still contribute significantly to the development of natural dyes in microbiology. The use of natural colorants is an important trend due to their environmental friendliness, safety, and potential to reduce reliance on synthetic chemicals. Excluding its role in differential staining, yam extract still has promising potential as a simple stain for basic microbiology practices aimed at visualizing cell morphology. Further research could investigate the effects of pH adjustments, solvent variations, pigment purification techniques, or the addition of natural stabilizers to enhance color stability. Studies on the extract's toxicity and compatibility with biological preparations are also needed. Such optimization efforts may lead to a more consistent and economical natural dye. These developments could eventually support the creation of environmentally friendly staining kits suitable for educational or research settings.

IV. CONCLUSION

This study aimed to test the potential of yam tuber extract as a substitute for crystal violet in Gram staining procedures. Based on the data obtained, it can be concluded that yam tuber extract at concentrations of 60%, 80%, and 100% is ineffective as a substitute for crystal violet in distinguishing Gram-positive from Gram-negative bacteria. The results showed that Gram-positive bacteria (Streptococcus mutans), which should retain a purple color, were either colorless or absorbed the counterstain safranin (red). Therefore, yam tuber extract, with the formulation and procedure used in this study, cannot be used as a differential stain to distinguish bacterial types based on their cell wall structure.

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