Journal of Health and Nutrition Research
Vol. 5, No. 2, 2026, pg. 685-692, https://doi.org/10.56303/jhnresearch.v5i2.1353
Journal homepage: https://journalmpci.com/index.php/jhnr/index

e-ISSN: **2829-9760**

Effect of Temperature on the Thermal Stability and Staining Quality of Parijoto Fruit Extract as a Counterstain in Gram Staining

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ORIGINAL ARTICLES

Submitted: 18 March 2026

Accepted: 02 June 2026

Published: 01 August 2026

Keywords:
Counterstain, Gram Stain, Parijoto Fruit Extract, Stability Test, Temperature Variations

ABSTRACT

Gram staining is a differential staining procedure that distinguishes between Gram-positive and Gram-negative bacteria. Toxicity and health hazards arising from the use of safranin dye have been widely reported, especially the danger of dye contamination originating from synthetic dye waste. Safranin is one of the dyes in Gram staining. Anthocyanin is a natural pigment that has the potential to be used as a dye, including applications in the laboratory such as Gram staining. Parijoto is a red fruit and contains anthocyanin. To evaluate the stability of parijoto fruit extract as a contrast dye for Gram staining based on temperature variations. The research design used was a true experimental design. Samples were E. coli bacteria to see the results of microscopic examination using alternative parijoto fruit extracts and safranin as a control in Gram staining. Data were processed using descriptive analysis based on the results of Gram staining. All treatment groups showed clear background contrast, good color, optimally stained bacteria, and clean preparations without debris. The best results were obtained with safranin (100%), followed by parijoto extract without heating (85%), heating at 40°C (80%), and heating at 80°C (75%), with a tendency to decrease in quality as the temperature increases. Parijoto fruit extract can be used as an alternative contrast dye in Gram staining, with the best results in conditions without heating, while increasing the temperature decreases the staining quality. Parijoto fruit extract has the potential to be an alternative natural contrast dye that is safer and more environmentally friendly. Use without heating is recommended because it provides optimal results and can be further developed in laboratory tests.

Key Messages:

- Parijoto fruit extract has the potential to be used as an alternative counterstain in Gram staining, but its use requires careful attention to storage temperature conditions to maintain optimal staining quality. Storage at lower, controlled temperatures is recommended to maintain pigment stability, especially in tropical environments with relatively high temperatures.

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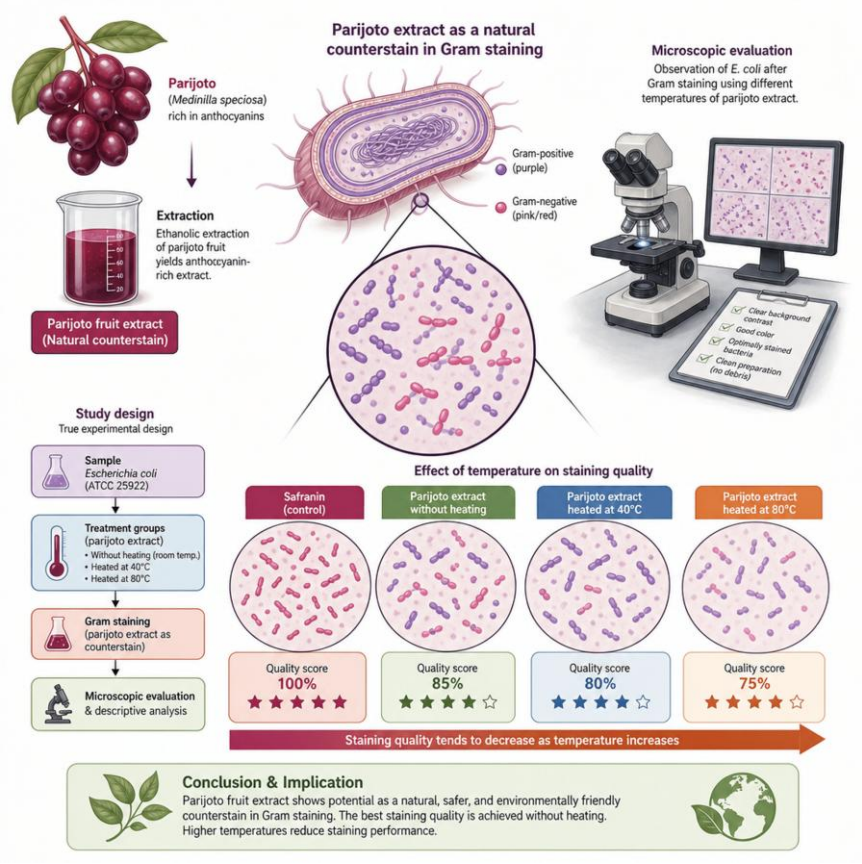
GRAPHICAL ABSTRACT



INTRODUCTION

A frequently used staining method in bacteriology is Gram staining. Gram staining is a differential staining procedure that divides bacteria into Gram-positive and Gram-negative groups (1)(2). Differential staining requires more than one type of stain and is used to differentiate between different types of bacterial cells. Differential staining generally consists of three stages: the first stage, a primary stain, is used to stain all cells on the slide (3). The second stage, decolorization, is used to remove the stain from certain types of cells. The third stage, a counterstain, stains cells whose paint was removed during decolorization but does not affect cells still stained with the primary stain (4)(5). Counterstain is a comparative paint, so the results of differential painting will produce 2 types of cells with different colors, namely cells that are colored with the main paint and those that are colored with the comparative paint (6). Diagnosis of bacterial infections can be confirmed through laboratory tests by identifying bacteria using bacterial staining methods (7)(8).

Escherichia coli (*e. coli*) is a group of gram-negative bacteria that are part of the normal flora in the body. Under certain conditions, these bacteria can become pathogenic, producing enterotoxins that can cause serious infections such as diarrhea (9)(10). The most widely used bacterial stains are synthetic dyes such as safranin. Safranin, a Gram staining dye, can be carcinogenic and has long-term negative health effects (11)(5). To overcome this problem, it is necessary to innovate natural dyes that can be used as alternative dyes (12).

Parijoto (*Medinilla speciosa*) is a red fruit and contains anthocyanin (13)(14). Anthocyanin is a natural pigment that has the potential to be used as a coloring agent, including in laboratory applications such as Gram staining (15)(16)(17). The use of natural dyes can reduce synthetic dye waste in simple bacterial staining (18)(19). This research is a follow-up to the 2024 Ministry of Research, Technology, and Higher Education PKM-RE Research Grant on the Utilization of Parijoto Fruit Extract as a Gram Stain Counterstain for *E. coli* Bacteria, which produced 75% good quality preparations based on microscopic examination results. The results indicate that parijoto fruit extract can be used as an alternative to replace

https://doi.org/10.56303/jhnresearch.v5i2.1353

safranin as a counterstain due to its environmentally friendly properties (20).

The successful implementation of an innovation in the laboratory depends not only on technical effectiveness but also on the stability of the reagents so that they can be used effectively. The anthocyanin contained in parijoto fruit extract is an unstable compound (21)(22). Natural reagents such as fruit extracts like parijoto are highly influenced by various environmental factors and storage conditions. Temperature is a major factor because it can accelerate or slow down the pigment degradation process. High temperatures tend to damage the color structure, while too low temperatures can disrupt the solubility of the active components (23). Anthocyanins have low stability due to the effects of heating (14).

Many studies have reported the use of anthocyanins as natural dyes, including as an alternative counterstain in Gram staining. However, most of these studies have focused solely on the potential use and effectiveness of the dye, without paying particular attention to its stability under specific temperature conditions (24). The studies specifically evaluating the thermal stability of anthocyanin extracts, particularly from parijoto fruit, at varying temperatures, such as 40°C and 80°C, are still very limited. Understanding stability under these temperature conditions is crucial for assessing their feasibility in laboratory practice, particularly in high-temperature environments or uncontrolled storage conditions (25). The extraction time of 20 minutes produced the highest yield, namely 27.89% (26). This study aims to evaluate the effect of temperature variations on the thermal stability and staining quality of parijoto fruit extract as a counterstain in Gram staining focuses on the aspects of color intensity changes and the differentiation ability of Gram bacteria at temperature conditions of 4°C, 40°C, and 80°C. The novelty of this study lies in the specific study of the thermal stability of anthocyanin extract from parijoto fruit at certain temperatures, which is still very limited in previous studies, especially in its application as a natural dye in Gram staining in the field of microbiology laboratories.

METHODS

The type of research is experimental. The sampling technique used in this study is non-probability purposive sampling, a sampling technique based on specific considerations. Sampling is done intentionally to align with the research objectives (27). The research design used was a true experiment design. The samples were *e. coli* bacteria to observe the results of Gram staining microscopically using alternative reagents of parijoto fruit extract at temperatures of 40°C and 80°C and safranin as a control. The bacterial counts used in this study were prepared based on the McFarland turbidity standard to ensure uniform cell concentration. The rejuvenated bacterial culture was then suspended in sterile physiological saline (0.85% NaCl) and homogenized to achieve a turbidity equivalent to the 0.5 McFarland standard, which is equivalent to approximately 1.5×10^8 CFU/mL. This suspension was then diluted as needed to achieve the desired working concentration. To prepare slide slides, a drop of the bacterial suspension was placed on a clean glass slide and spread evenly using a sterile loop to form a thin layer (smear). The slide was then dried at room temperature and fixed using a heat fixation method to adhere the bacteria to the slide surface. The slide was then ready for Gram staining using parijoto fruit extract as a counterstain (21).

Data were processed using descriptive analysis based on the results of Gram staining. The study was conducted in the microbiology laboratory of Muhammadiyah Ahmad Dahlan University, Palembang in January 2026. The independent variable was the parijoto fruit extract reagent based on temperature variations while the dependent variable was the results of Gram staining. The results of the examination were analyzed descriptively. The process of making parijoto Fruit Extract Reagents: Weigh 800 g of parijoto fruit, wash, dry, and then blend. Blend the fruit only without adding water or distilled water. 100% parijoto fruit extract is obtained without a mixture of distilled water using filter paper. Then, pipette 75 ml of parijoto fruit juice and add 25 ml of distilled water until it reaches a total volume of 100 ml. The parijoto fruit extract dye with a concentration of 75% is ready to use (28).

Temperature Stability Testing: Parijoto fruit extract was made into two solutions, namely the first solution of 2 ml of parijoto fruit extract added with 50 ml of distilled water, then homogenized and heated on a hotplate at a temperature of 40°C for 20 minutes. The second solution was made by adding 2 ml of parijoto fruit extract with 50 ml of distilled water, then homogenized and heated on a hotplate at a temperature of 80°C for 20 minutes. Then a stability test was carried out by reading the staining results

https://doi.org/10.56303/jhnresearch.v5i2.1353

microscopically (24). 40°C was chosen to represent realistic hot environmental conditions, such as storage temperatures in tropical regions or uncontrolled transportation conditions, where biological materials can be exposed to temperatures above normal room temperature. Meanwhile, 80°C was used as an accelerated thermal test condition to evaluate the extract's resistance to short-term exposure to extreme heat, thereby more quickly demonstrating the limits of pigment degradation (26).

The process of making *e-coli* bacterial preparations is carried out by heating a loop ring on a spirit lamp. Take *E. coli* and place it on a glass object. Then, a drop of NaCl solution is added and flattened to form an oval. The preparation is allowed to dry after which it is fixed on the spirit lamp. The Gram Staining Procedure is that the fixed bacterial preparation is flooded with crystal violet solution for one minute, then rinsed with distilled water (24). After that, the preparation is flooded with Lugol's solution for one minute and rinsed with distilled water. Then the preparation is flooded with an alcohol solution until the color fades and rinsed with distilled water. Then the preparation is flooded with a safranin solution for 30 seconds as a control, while as an experiment, the safranin is replaced with a dye solution from parijoto fruit extract at a temperature of 40°C and 80°C, then the preparation is dried. After drying, it is observed using a microscope at 100x magnification (29).

Microscopic observations were conducted by three laboratory technicians. In descriptive observations, the following were observed: Contrast to the background on the preparation is clearly visible or not (2 = contrast is clearly visible, 1 = contrast is less visible, 0 = no contrast), Having a clear color (2 = clear color, 1 = dull color, 0 = no color), The shape of the bacteria that were successfully stained (2 = good quality, 1 = less good, 0 = no visible shape), and Debris (2 = no debris/clean, 1 = debris is visible but does not cover the entire bacterial body, 0 = debris covers the entire bacterial body)(18).

CODE OF HEALTH ETHICS

This study has been approved by research ethics committee of Universitas Muhammadiyah Ahmad Dahlan Palembang with the number: 000193/KEP Universitas Muhammadiyah Ahmad Dahlan Palembang/2026.

RESULTS

Table 1 shows, the number of samples and percentage of Gram staining success for each treatment. The control group using safranin showed the best results with all 16 samples successful (100%). Parijoto extract without heating was successful in 14 of 16 samples (85%), while heating at 40°C and 80°C reduced the success to 13 samples (80%) and 12 samples (75%), respectively. These data show a decrease in staining effectiveness as the heating temperature increases, indicating that Parijoto extract is most optimal when used without heating.

Table 1. Number of Samples and Percentage of Success (n=64)

Gram Staining Treatment Group	n	Successful Sample	%
Safranin	16	16	100
Parijoto without heating	16	14	85
Parijoto heating temperature 40°C	16	13	80
Parijoto heating temperature 80°C	16	12	75

Table 2. Gram Staining Observation Quality (n=64)

Gram Staining Treatment Group	Background Contrast	Color	Stained Bacteria	Clean Preparation (Without Debris)
Safranin	Very clear	Very clear	Very good	Very clear
Parijoto without heating	Clear	Very clear	Good	Clear
Parijoto heating temperature 40°C	Clear	Very clear	Good	Clear
Parijoto heating temperature 80°C	Clear	Very clear	Good	Clear

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Table 2 showed that, the quality of Gram staining observations for each treatment. All groups maintained clear background contrast and good color. Bacteria on safranin slides were optimally stained, and the slides were clean and free of debris. In the Parijoto group, both without heating and with heating at 40°C and 80°C, staining quality decreased slightly with increasing temperature. However, overall, the bacteria remained well stained, and the slides were relatively clean. This indicates that Parijoto extract still has potential as a natural counterstain, although its effectiveness is slightly reduced at higher heating temperatures.

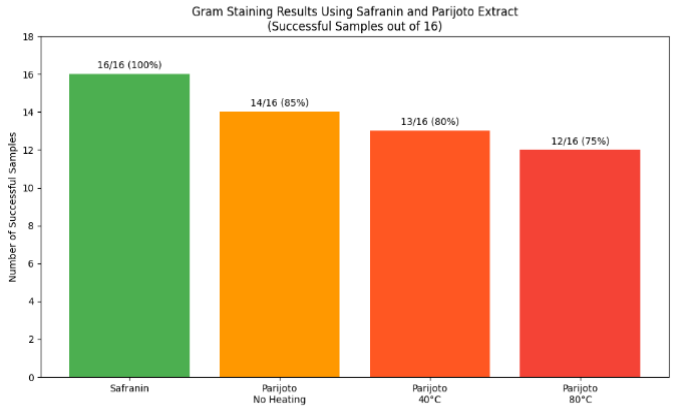

Figure 1. Gram Staining Result Using Safranin and Parijoto Extract

Figure 1 shows the number of successful Gram staining results for each treatment, with safranin achieving 100% success (16/16) as a control. Parijoto extract without heating was successful in 14 samples (85%), while heating at 40°C and 80°C resulted in success in 13 samples (80%) and 12 samples (75%), respectively. This graph shows a decrease in staining success as the temperature increases, indicating that Parijoto extract is most effective when used without heating, but still has potential as an alternative counterstain.

DISCUSSION

The problem-solving approach in this study specifically focused on testing the effect of temperature, namely 40°C and 80°C for 20 minutes on the stability of parijoto fruit extract as a Gram staining reagent. These two temperatures were chosen because they are able to simulate heating conditions that can cause rapid degradation of natural pigments. The effectiveness of parijoto fruit extract as a counterstain was examined through Gram staining to determine whether increasing temperature causes a decrease in the extract's ability to provide color contrast to Gram-negative bacteria. The study can identify the thermal resistance limits of parijoto fruit pigments and determine whether high temperatures still allow the extract to be used as a reagent or actually cause significant degradation making it unsuitable for use. This approach provides a solution in determining safe storage conditions and determining the extent to which high temperatures affect the stability of natural reagents.

Research into the use of natural dyes as alternative reagents in microbiological staining has grown rapidly, primarily in response to increasing concerns about chemical safety, cost, and environmental issues. Several previous studies have utilized sappanwood solutions (30), methanol extract of rosella flowers (18), teak leaf extract (31) and Lawsonia inermis extract to replace safranin which is stable at 40°C (24). The best time to maintain the detected compounds was in the extraction treatment with a time of 20 minutes (26) . Parijoto fruit (*Medinilla speciosa*), known to contain anthocyanin pigments and phenolic compounds, has been studied as a source of natural dyes, but its use as a counterstain in Gram staining has not been widely explored and is generally only tested under normal temperature conditions or cold storage. Research on the effect of high temperatures on the stability of parijoto extracts remains a rarely explored area and requires a more in-depth scientific approach.

The novelty of this research lies in the effectiveness of staining on Gram-negative bacteria based on temperature variations. This approach provides a novel contribution by revealing the thermal resistance

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limits of parijoto pigments and evaluating whether high heating can damage the extract's ability to produce adequate staining contrast. The results of this study are expected to provide new scientific insights in the development of more stable natural dye reagents, as well as serve as a basis for natural dye formulations that are resistant to environmental conditions.

The results showed that Parijoto fruit extract can function as an alternative counterstain in Gram staining, although its effectiveness is slightly lower than the standard stain safranin. Safranin as a control produced 100% success, indicating optimal staining quality, while Parijoto without heating reached 85%, still good enough to detect bacteria with clear contrast and color. The decrease in staining effectiveness with increasing temperature can be explained by changes in the chemical structure and stability of the anthocyanin pigments found in parijoto fruit extract. Anthocyanins are heat-sensitive flavonoid compounds. Increasing temperature can accelerate the thermal degradation reaction, changing their active structure from the flavylium cation (colored) form to a chalcone, or colorless, degraded derivative. This process causes a decrease in color intensity, thus diminishing their ability as a counterstain. Furthermore, high temperatures can accelerate the oxidation and hydrolysis of anthocyanin compounds, resulting in the destruction of the electron conjugation system responsible for light absorption and staining. The higher the temperature (e.g., 40°C and especially 80°C), the greater the kinetic energy of the molecules that accelerate these degradation reactions, significantly reducing pigment stability.

In the context of Gram staining, this decrease in staining quality impacts the extract's ability to provide contrast between Gram-negative bacteria and the background. This results in less clear morphological differentiation of bacteria, primarily because the counterstain color intensity is not strong enough to highlight cells that have lost the primary stain. Thus, the decrease in dyeing effectiveness is not only caused by physical changes in color, but also by chemical degradation of the pigment, which affects the dye's interaction with the bacterial cell structure. Overall, these findings confirm that thermal stability is a critical factor in determining the effectiveness of parijoto extract as a natural dye, particularly in microbiological applications that require color consistency and sharp cell differentiation.

Despite the decrease in effectiveness, all parijoto treatments still produced clean preparations and well-stained bacteria, thus demonstrating the potential of this extract as an environmentally friendly natural counterstain. These findings align with previous research on the use of plant-based materials as laboratory dyes, which showed that dye stability and quality are significantly affected by processing conditions such as temperature.

CONCLUSION

Parijoto fruit extract can be used as an alternative counterstain in Gram staining with good results and clean preparations without debris. The decrease in staining effectiveness with increasing temperature is mainly due to the thermal degradation of anthocyanins in parijoto extract, which changes its chemical structure, thereby reducing color intensity and ability as a counterstain. Thus, Parijoto extract has the potential to be a safe and environmentally friendly natural dye for laboratories. Based on the results, further research can be conducted to standardize the extract concentration, test its long-term stability, and explore other safe and environmentally friendly natural dyes as alternative laboratory dyes.

DECLARATION OF THE USE OF AI

The authors declare that no artificial intelligence (AI), AI-assisted technologies, or large language models (LLMs) were used in the conception of the study, data analysis, or the drafting, writing, and editing of this manuscript. The only exception is the graphical abstract, which was created using the design platform Illustrae (https://illustrae.co/). The authors take full responsibility for the content and accuracy of the graphical abstract and the entire manuscript.

FUNDING

Funding for this research was supported by Muhammadiyah Ahmad Dahlan University, Palembang.

https://doi.org/10.56303/jhnresearch.v5i2.1353

ACKNOWLEDGMENTS

The author would like to thank Muhammadiyah Ahmad Dahlan University Palembang and the research team who supported the data collection process.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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