Potential of Methanol Extract From Rosella (*Hibiscus Sabdariffa*) as an Alternative Dye in Gram Staining

Mohammad Indra Pratama, Rajun, Faradilla Dahlan, Nurul Anisa, Indri Yudita Ningsih, Khusnul Yumni Bulan Sari, Sri Sela Pratiwi, Niky Aulia Putri, Teguh Pribadi, Didimus Tanah Boleng, and Dora Dayu Rahma Turista(✉)

Biology Education Department Faculty of Teacher Training and Education, Mulawarman University, Samarinda, Indonesia
doraturistaofficial@gmail.com

Abstract. Gram staining is one of the important steps to identify bacteria. This type of staining uses synthetic substances safranin and violet crystals which can result in environmental pollution. Rosella flowers (*Hibiscus sabdariffa*) contain natural dyestuffs, namely anthocyanins that have the potential to color bacterial cells. This study aims to find out whether rosella flower methanol extract (*Hibiscus sabdariffa*) can be used as a substitute for alternative dyestuffs in Gram staining. This study is an experimental study with a descriptive approach that uses three repetitions. The results showed that rosella flower extract (*Hibiscus sabdariffa*) cannot be used as an alternative to natural dyes for staining bacteria *Staphylococcus aureus, Klebsiella Pneumonia* and *E. coli.* The bacterial cell wall has a complex layer so it requires a special type of dye to be able to penetrate it. Different materials need to be carried out so that they can be used as alternative dyes in gram staining.

Keywords: Methanol · Rosella · *E. coli* · *Staphylococus* · *Klebsilla* · Safranin · Violet Crystal

1 Introduction

Gram staining is one of the important stages in the bacterial identification process The type of dye used in this process is a dye that can glue to peptidoglycan in this case Violet and safranin crystals. Safranin can work well in bacteria because it is alkaline (positively charged color-carrying component), while the cytoplasm of bacteria is basophilic (such as alkaline) so that an attractive force of attraction between components occurs [1]. Color carriers in dyestuffs and bacterial cells allow bacteria to easily absorb dyes. Synthetic dyes, more stable, resistant to various environmental factors, strong colors, wide color range (fade resistance, bright) while the disadvantages of using synthetic dyes can cause health and environmental problems. Anthocyanins are color pigments found in rosella flowers (*Hibiscus sabdariffa*). Anthocyanin is a red dye that may be a natural food coloring and can be used as a safer alternative to synthetic dyes. Rosella (*Hibiscus sabdariffa*) can be a source of natural dyes because this flower contains anthocyanins.

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In addition to producing anthocyanin pigments, this flower is also known to have many health benefits. Apart from being a dye, rosella (*Hibiscus sabdariffa* L.) is one of the plants that can be used as a preservative because it can prevent microbial growth [2, 3].

Chemically anthocyanins are derivatives of a single aromatic structure, namely cyanidin, and all of them are formed from cyanidin pigments by addition or reduction of hydroxyl groups, methylation and glycosylation. Anthocyanins are compounds that are amphoteric, which means they have the ability to react with both acids and bases. In acidic media, anthocyanins are red, and in alkaline media they turn purple and blue. Anthocyanins are a group of pigments that cause a reddish color and are found in water-soluble cell fluids. Anthocyanins act as stable food and non-food colorants [4].

2 Methodology

2.1 Rosella Preparation

This study is an experimental study with a descriptive approach that uses three repetitions. How it Works: the first stage is the preparation of rosella (*Hibiscus sabdariffa*) by making maceration extract obtained by soaking with methanol Making maceration extract, the first step is to weigh the sample powder. The weighed sample was then soaked for 3 days with 95% methanol solvent and at acidic pH conditions. 95% methanol solvent is used because methanol is more selective, difficult for fungi and bacteria to grow, has good absorption, does not cause swelling of cell membranes, improves the stability of soluble drugs, has the ability of enzyme inhibitory effect and is very effective in producing optimal amounts of extracts. During daily soaking should be carried out by stirring to facilitate circulation, this is for faster balance of the concentration of active ingredients in the liquid. After 3 days it is then filtered with filter paper. The next stage is evaporation by evaporation to separate the methanol which then drying is done by putting the liquid in the oven for 3 days with a temperature of 60 °C [5].

2.2 Gram Staining Experiment

Preparations are made using pure cultures of *Escherichia coli, Staphylococcus aureus,* and *Klepsiela pneumonia* by taking it using ose needles and flattened on the glass of the object that has been dripped with aquades and then fixed using Bunsen, then the preparations are dripped with violet crystals for 20 s and rinsed under running water. Next, it is dripped with mordant solution for 1 min and rinsed under running water then rinsed with alcohol for 20 s. And finally, dripped with rosella extract for 1 min, 2 min, and 3 min. This also applies to the coloring of violet crystal substitutes. After fixation, the preparations were stained with rosella extract for 1 min, 2 min, and 3 min. Then rinsed and dripped mordant solution. After which it is rinsed and dripped with alcohol for 20 s and dripped with safranin dye for 20 s. Then rinsed and covered with glass objects [6]. And the preparations are observed using a microscope. Preparations are made using pure cultures of Escherichia coli, Staphylococcus aureus, and Klepsiela pneumonia by taking it using ose needles and flattened on the glass of the object that has been dripped with aquades and then fixed using Bunsen, then the preparations are dripped with violet

crystals for 20 s and rinsed under running water. Next, it is dripped with mordant solution for 1 min and rinsed under running water then rinsed with alcohol for 20 s. And finally, dripped with rosella extract for 1 min, 2 min, and 3 min. This also applies to the coloring of violet crystal substitutes. After fixation, the preparations were stained with rosella extract for 1 min, 2 min, and 3 min. Then rinsed and dripped mordant solution. After which it is rinsed and dripped with alcohol for 20 s and dripped with safranin dye for 20 s. Then rinsed and covered with glass objects. And the preparations are observed using a microscope [5].

3 Results and Discussion

The coloring component used is violet crystals as the primary dye, then iodine solution as mordant which increases the affinity in the cells and stains so that it forms a complex with violet crystals. Then 95% alcohol functions as a lipid solvent that dissolves fats in the cell wall and causes decolorization. Then, safranin serves as a counterstain that stains bacteria that have been decolorized (Table 1 and Fig. 1).

The first table is a control table where the controls use staining with synthetic dyestuffs. In the control staining using 2 min, in the first control of *Staphylococus aureus* bacteria, a blue appearance was obtained at a magnitude of 100 × 10 on its cells. This is because *staphylococus aureus* is a gram-positive bacterium that has 1 layer of cell wall in the form of tightly arranged peptidoglycan which makes violet crystal staining can enter and be bound by mordans. Then when decolorizing the dye remains attached and the peptidoglycan will shrink so that the safranin staining will not be able to enter and color the cells. In the next bacteria *E. coli,* and *Klepsillia pneumonia*, it was found that these two bacteria are gram-negative bacteria, because they absorb safranin dyes. This happens because when given a primary dye it will seep into the cell wall whose components are a large amount of lipids. When decolorization using 95% alcohol, the

Table 1. Staining of *bacteria E. coli, Staphylococus aureus,* and *Klepsiella pneumonia* as substitutes for safranin.

Bacterias	Time (minutes)	Result	
		Safranin	**Rosella**
E. coli	1	Redness	Blue
	2	Redness	Colorless
	3	Redness	Colorless
Staphylococus aureus	1	Purplish	Colorless
	2	Purplish	Blue
	3	Purplish	Blue
Klepsiella pneumonia	1	Redness	Colorless
	2	Redness	Colorless
	3	Redness	Colorless

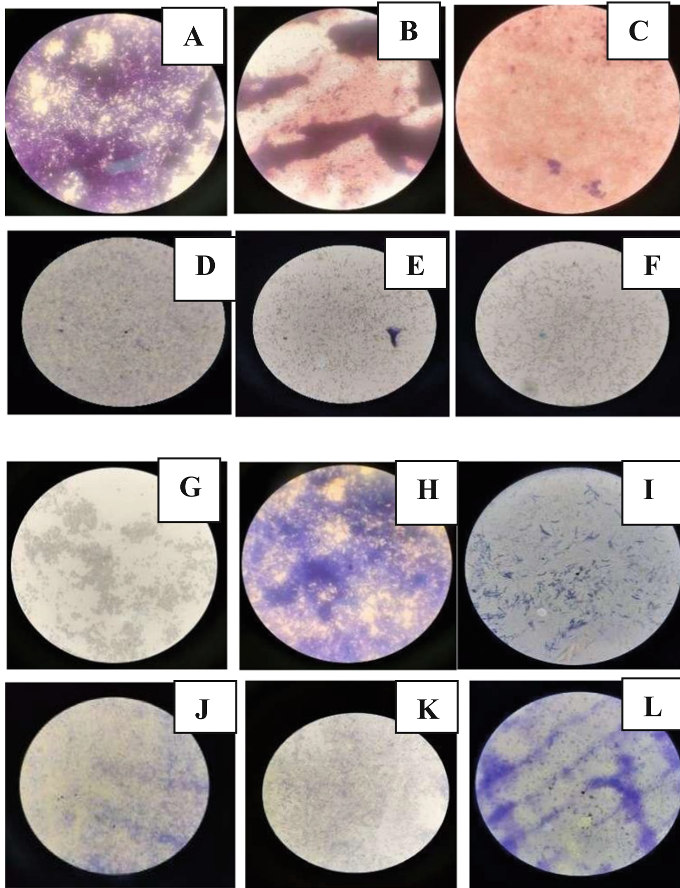

Fig. 1. Safranin staining of bacteria *E. coli, Staphylococus aureus, and Klepsiella pneumonia.* a. stained safranin bacteria E. coli, b. stained safranin bacteria *Staphylococus aureus*, c. dyed using bacterial safranin *Klepsiella pneumonia*, d. staining of *E. coli* bacteria with rosella extract (*Hibiscus sabdariffa*) 1 min, e. staining of *E. coli bacteria* with rosella extract (*Hibiscus sabdariffa*) 2 min, f. staining of *E. coli* bacteria with rosella extract (*Hibiscus sabdariffa*) 3 min, g. staining of *Staphylococus aureus* bacteria with rosella extract (*Hibiscus sabdariffa*) 1 min, h. staining of *Staphylococus aureus* bacteria with rosella extract (*Hibiscus sabdariffa*) 2 min, i. staining of *Staphylococus aureus* bacteria with rosella extract (*Hibiscus sabdariffa*) 3 min, j. staining of bacteria *Klepsiella pneumonia* with rosella extract (*Hibiscus sabdariffa*) 1 min, k. staining of bacteria *Klepsiella pneumonia* with ekstrak rosella *(*Hibiscus sabdariffa) 2 min, l. staining of bacteria *Klepsiella pneumonia* with rosella extract (*Hibiscus sabdariffa*) 3 min. Source: Indra, dkk (2022)

lipids will melt and melt the primary dye that has been bound by mordant. This makes the peptidoglikan that composes the cell wall open, causing a counter-pewaarnaan in the form of safranin to enter. In the appearance, it can be seen that preparations B and C have deposits due to poorly clean decolorization washing so that the rest of the primary dyeing is still visible and reduces the quality of the preparations.

The 1st table is a table of staining results using natural materials, namely rosella (*Hibiscus sabdariffa*) with 3 kinds of different time treatments, namely 1 min, 2 min, and 3 min treatments. In the table, it can be clearly observed that in gram-negative bacteria cannot absorb the red dye in the form of anthocyanins present in Rosella solution (H*ibiscus sabdariffa*), thus causing the bacteria to be unstained. This is because the solution used is acidic, and it is proven during the pH test where the Ph of the Rosella extract (*Hibiscus sabdariffa*) is at pH 3 which indicates that the solution is acidic so that there is no reaction with peptidoglycan which causes anthocyanin dyes to not enter peptidoglycan. In addition, the properties of natural dyes that are easily soluble and easily lost when rinsed with water, unlike synthetic dyes that stubbornly color each bacterial cell.

The gram-positive staining of *Staphylococus aureus* is obtained blue because it absorbs violet crystals which are the primary color and traps it after the mordant administration process and also decolorization using 95% alcohol. This causes safranin to be unable to enter and color gram-positive bacteria. In gram-negative bacteria, a blue color is visible due to the incomplete decolorization process, causing the previous staining deposits and disrupting the second staining process (Fig. 2 and Table 2).

The next coloring uses rosella (*Hibiscus sabdariffa*) instead of violet crystals in hopes of coloring gram-positive bacteria. Based on the results obtained, it was concluded that gram-negative bacteria will absorb the existing safranin due to the decolorization process during rinsing with 95% alcolol, so that in samples of *E.Colli bacteria,* and *Klepsilia pneumonia* is stained with safranin. Then in the gram-positive bacteria, namely *Staphylococus aureus*, a red color was obtained but after a single staining test, it was found that the red color was not from rosella extract, but from safranin staining. This is in accordance with table 8 which shows that a single staining of rosella extract is not capable of coloring cells present in gram-positive bacteria. This is because anthocyanins or substances present in rosella flowers are not able to enter and color peptidoglycan because it is acidic and cannot bind to each other between peptidoglycan and anthocyanins which causes the colorlessness of bacterial cells.

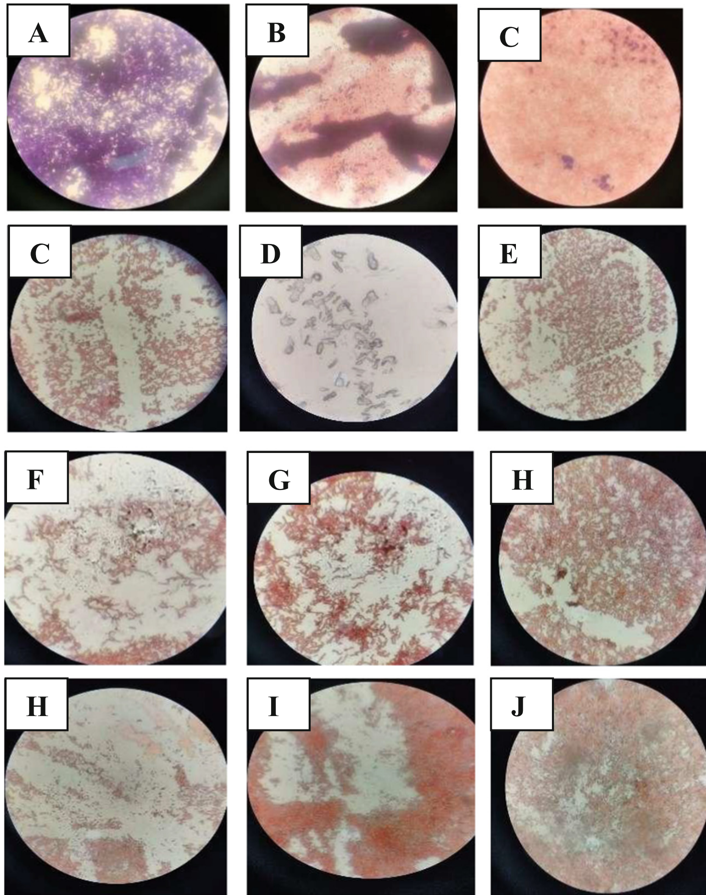

Fig. 2. Coloring of violet crystals of *E. coli* bacteria, *Staphylococus aureus*, and *Klepsiella pneumonia*. a. stained crystalline violet bacteria *E. coli,* b. stained crystal violet bacteria *Staphylococus aureus*, c. dyed using crystal violet bacteria *Klepsiella pneumonia*, d. staining of *E. coli* bacteria with rosella extract (*Hibiscus sabdariffa*) 1 min, e. staining of *E. coli* bacteria with rosella extract (*Hibiscus sabdariffa*) 2 min, f. staining of *E. coli* bacteria with rosella extract (*Hibiscus sabdariffa*) 3 min, g. staining of *Staphylococus aureus* bacteria with rosella extract (*Hibiscus sabdariffa*) 1 min, h. staining of *Staphylococus aureus* bacteria with rosella extract (*Hibiscus sabdariffa*) 2 min, i. staining of *Staphylococus aureus* bacteria with rosella extract (*Hibiscus sabdariffa*) 3 min, j. staining of bacteria *Klepsiella pneumonia* with rosella extract (*Hibiscus sabdariffa*) 1 min, k. staining of bacteria *Klepsiella pneumonia* with rosella extract (*Hibiscus sabdariffa*) 2 min, l. staining of bacteria *Klepsiella pneumonia* with rosella extract (*Hibiscus sabdariffa*) 3 min. Source: Indra, dkk (2022)

Table 2. Staining of *bacteria E. coli, Staphylococus aureus,* and *Klepsiella pneumonia* with staining rosella extract (*Hibiscus sabdariffa*) instead of crystalline violets

Bacterias	Time (minutes)	Result	
		Kristal violet	Rosella
E. coli	1	Redness	Red
	2	Redness	Colorless
	3	Redness	Red
Staphylococus aureus	1	Purplish	Red
	2	Purplish	Red
	3	Purplish	Red
Klepsiella pneumonia	1	Redness	Red
	2	Redness	Red
	3	Redness	Red

4 Conclusions

After staining trials on pure cultures of *E.coli* bacteria*, Staphylococus aureus* and *Klepsilea pneumonia* using different time spans, namely 1 min, 2 min and 3 min in each treatment and observations were made under a microscope with a magnification of 100x10 and compared with staining control of safranin and violet crystals. So, it can be concluded that the extract of rosella flower methanol (*Hibiscus sabdariffa*) is not able to color bacterial cells due to anthocyanins that cannot penetrate peptidoglycan and cannot bind color, this is because rosella flower extract (*Hibiscus sabdariffa*) which has acidic properties and is easily soluble in water so that it cannot stick to the bacterial cell wall, both gram-negative bacteria and gram-positive bacteria.

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