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Utilization ethanol extract of sappan wood as an alternative in *escherichia coli* staining

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ABSTRACT

In life, sappan wood is used as an ingredient in traditional drinks that has antioxidant, antimicrobial and has stable pigments. The content of red pigment, brazilin in sappan wood can be used as a dye. The purpose of this study was to determine the potential of sappan wood ethanol extract as an alternative to safranin in gram staining. Ethanol extract of sappan wood 6% was added with alum mordant to obtain a red pigment. The bacteria used *Eschericia col,* isolated from water. Staining using ethanol extract of sappan wood was carried out for 30 seconds, 3, 5 and 10 minutes. This stain was compared with safranin. The results showed that staining using sappan wood extract shows clarity of the visual field, shape and good color. Based on the results, the ethanol extract of sappan wood with mordant can be used as an alternative to safranin in the gram-staining.

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1. Introduction

Bacterial staining is needed for cell identification (Risandiansyah et al., 2020)and distinguishing types of bacteria for diagnostic purposes (Vijayakumar *et al.*, 2023). One gram staining method uses crystal violet and safranin dyes. Gram-positive bacteria have thick peptidoglycan cell walls and show a blue color when viewed under a microscope (Prayekti and Sumarsono, 2021). Gram-negative bacteria have a thinner outer membrane and peptidoglycan layer, which does not retain the crystal violet color during the decolorization process. Gram-negative bacteria appear red under a microscope (Hardiansyah, Musa and Jaya, 2020).

Dyes that are widely used for staining bacteria include methylene blue, crystal violet, carbolic fuchsin, safranin, malachite green. Staining using chemicals sometimes causes problems, namely the price is very expensive (Franco-Duarte *et al.*, 2019). Moreover, examination sites in rural or underdeveloped areas are a challenge in providing gram staining kits which is difficult (Risandiansyah et al., 2020). The use of synthetic dyes is also dangerous for the environment and water sources because they tend to decompose (Ayele *et al.*, 2021). In addition, this synthetic material is considered may promote toxicity, mutagenicity and carcinogenicity (Lellis *et al.*, 2019)

The use of natural dyes derived from plants, animals and minerals is a sustainable resource that is environmentally friendly (Alaskar and Hassabo, 2021). Natural dyes serve as an alternative source in biodegradable polymers to replace inorganic pigments (Girdthep *et al.*, 2018). Coloring with natural dye have been applied for textile coloring (Hazmi and Oetopo, 2022), solar cells (Rahul *et al.*, 2019), food (Singh *et al.*, 2023), pharmaceuticals (Novelia A and Asra, 2020). Several studies have revealed the potential of natural dyes for histological studies. Tissue staining can use hibiscus (Okolie, Aa and Ib, 2021), curcumin (Bose *et al.*, 2022), purple knight, grenadine, red cabbage, red spinac (Abu *et al.*, 2019), henna extract (Okolie, Aa and Ib, 2021) and red dragon (Asra *et al.*, 2021).. Staining of sperma can use purple cabbage, sappan wood, beet root (Mamay, Ernawati and Nurisani, 2023) red dragon peel (Heryanto *et al.*, 2023). Identification of gram bacteria using hibiscus (Pratama *et al.*, 2023) and purple sweet potato (Nunki, Titik Mutiarawati and Prayekti, 2020). The use of natural sappan wood dyes in gram staining has not been studied further.

The aim of this research is to determine the potential of original natural dyes derived from sappan wood as counterstains that have not been studied in gram staining. Sappan wood with a scientific name *Caesalpinia sappan* L. contains brazilin red pigment (Girdthep *et al.*, 2018). In everyday use, sappan wood powder is commonly used as an ingredient in traditional health drinks. It has previously been reported to have antioxidant, antimicrobial abilities and has a stable color (Yim *et al.*, 2019). The color of sappan wood is influenced by pH and also mordant (Nathan and Rani, 2021) so that the type of color can be adjusted according to what is expected. By using environmentally friendly natural dyes, sperm examination will minimize dangerous waste

2. Methods

Sappan wood extract preparation

100 grams of dry sappan wood is ground until a coarse powder is obtained. Extraction was carried out by maceration using 1000 ml of 96% ethanol. Maceration was carried out for approximately 24 hours at room temperature, with occasional stirring and filtering to obtain the filtrate. The filtrate was concentrated using a rotary vacuum evaporator at a temperature of 60 ^{o} C until a thick filtrate was obtained.

Dye preparation

The thick extract of sappan is made by weighing 0.6 grams in 96% alcohol solvent to a volume of 10 ml plus 240 ul of 10% $KAl(SO_4)_2 \cdot 12H_2O$, 300 ul of concentrated acetic acid, 300 ul of 0.1 M NaOH.

Preparation of bacterial preparations

The material used in the research was *Escherichia coli culture* isolated from water samples in coliform testing. Bacteria were grown in EMBA media. A single colony of bacteria was smeared on a glass object and then fixed by heating it slowly in a spirit lamp.

Bacterial gram-staining

Bacterial smears on glass objects were soaked in crystal violet primary dye for 1 minute. After that, Lugol's reagent was added for 1 minute. Decolorization was carried out with 96% aclohol. The final reagent as a counter stain was added with safranin for 30 seconds. Every time the reagent is changed, it is washed with running water. The experimental group replaced the safranin dye with a sappan extract solution with varying times of 30 seconds, 1, 3, 5, 7 and 10 minutes.

Data analysis

The staining results were viewed with a total magnification of 1000x under a microscope. The parameters observed were visual field clarity, visual field cleanliness, contrast, bacterial shape and bacterial color. For data analysis, observations were made at 30 seconds, 3 minutes, 5 minutes and 10 minutes of staining time compared to the control

3. Results and Discussion

The results of gram staining of Escherichia coli bacteria using the safranin dye control can be seen in Figure 1. The staining results show red, rod-shaped bacteria. The coloring results obtained were based on the length of soaking using sappan wood ethanol extract dye with Al mordant. For clarity of the field of view and the shape of the rod bacteria, it is clearly visible when staining the extract for 30 seconds, 3 minutes, 5 minutes and 10. The contrast in the color of the bacteria is clearly visible when staining for 5 and 10 minutes, as seen in Figure 2 a-c

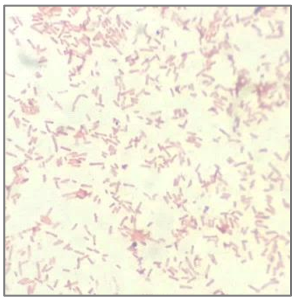
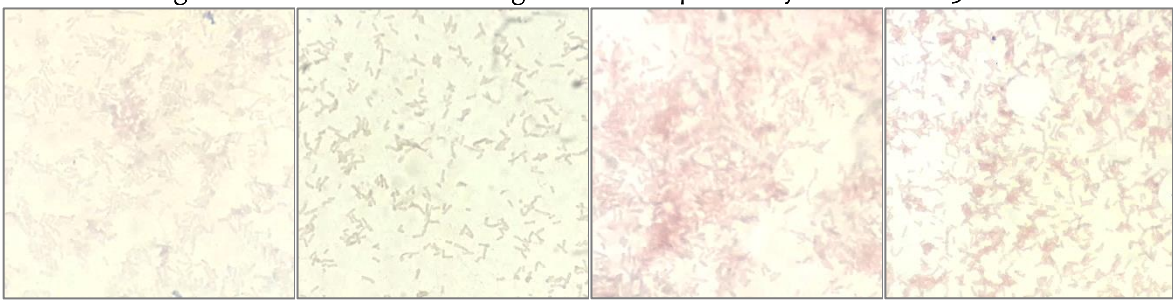


Figure 1. Results of Gram staining with the comparison dye safranin for 30 seconds



(a) (b) (c) (d)

Figure 2. Results of Gram staining with the comparison dye ethanol extract of sappan wood for (a) 30 seconds (b) 3 minutes (c) 5 minutes (d) 10 minutes

In a modified gram stain using sappan extract as a substitute for safranin as a counterstain was carried out. The concentration of 6% sappan extract with Al mordant showed that the cells started to color slightly at 3 minutes. The extract does not have enough time to penetrate the bacteria so the bacteria are not completely stained and the red color is not clearly visible. The concentration of 6% sappan extract with Al mordant showed good staining results at 5 and 10 minutes. With this time, the dye has enough time to penetrate the bacterial cell walls, so that the results of the bacterial staining appear more red.

Discussion

The process of extracting dye from sappan wood uses the maceration method with 96% alcohol. With this method, the process of extracting the dyes contained in natural ingredients is more optimal (Sofiah *et al.*, 2023). The use of sappan wood alcohol extract is expected to resemble the composition of the safranin solution which is made using 96% alcohol solvent. The color of the ethanol extract of sappan wood is brownish yellow. The color of sappan wood pigment is influenced by its acidity, yellow in acidic conditions and red in neutral and alkaline pH (Ngamwonglumlert, Devahastin and Chiewchan, 2020)Most of the dye isolated from sappan wood is brazilin (Nathan & Rani, 2021). Brazilin is a homoisoflavonide compound which can be oxidized by air in the atmosphere or oxidized compounds to become brazilein (Handayani *et al.*, 2022). The color of sappan extract varies depending on the chelating compound as well as the pH (Nathan and Rani, 2021). The addition of Aluminum Potassium suphate or alum as a mordant changes the color of the ethanol extract of sappan wood from brownish yellow to red. The addition of Al mordant to the brazilein compound forms a branzalum complex. The presence of this metal facilitates interaction between brazilin dye and cell walls through ionic bonds (Budiasih *et al.*, 2023).

This is in line with research, coloring using a combination of red rice and teak leaves oxidized by $KMnO_4$ as a covering dye in gram staining. The positive charge of potassium and dye complexes forms dissociates which can bind to bacterial cell components that have a negative charge. Bacterial cells contain nucleic acids with negatively charged phosphate groups, so they will bind to alkaline dyes which are positively charged (Virgianti and Luciana, 2017).

This research has limitations, namely that it does not use variations in the concentration of sappan extract and certain mordant ratios. Furthermore, research is needed to optimize the concentration of sappan wood ethanol extract with alum mordant. This is done so that the penetration of the dye into the bacterial cell walls can be maximized

3. Conclusion

The present study investigated the potential of sappan wood ethanol extract as an alternative to safranin in gram staining procedures. Utilizing a 6% ethanol extract of sappan wood with alum mordant, we successfully obtained a red pigment, primarily composed of brazilin. The staining process was applied to Escherichia coli bacteria isolated from water, and various durations (30 seconds, 3 minutes, 5 minutes, and 10 minutes) were explored. The results of this investigation revealed that the ethanol extract of sappan wood, when used in gram staining, exhibited notable advantages. The stained samples demonstrated a clear visual field, well-defined bacterial shapes, and vibrant coloration. The efficacy of the sappan wood extract was comparable to traditional safranin staining. These findings suggest that the ethanol extract of sappan wood, in conjunction with alum mordant, holds promise as a viable alternative to safranin in gram staining procedures. The natural red pigment derived from sappan wood not only imparts favorable staining characteristics but also aligns with the increasing interest in utilizing natural and sustainable sources in laboratory applications. Further research and validation across diverse bacterial strains and laboratory settings are warranted to establish the broader applicability and reliability of sappan wood extract as a gram-staining agent. If substantiated, this alternative could contribute to the reduction of synthetic dye usage in microbiological laboratories, promoting environmentally friendly and cost-effective staining practices.

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