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Potential of Henna Leaf (*Lawsonia inermis*) Extract as a Natural Alternative to Safranin in the Differential Staining of *Escherichia coli* and *Salmonella typhi*

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Abstract: Increased environmental awareness among commercial dyes and textile exporters is shifting attention to the use of natural dyes pigments in foodstuffs, pharmaceuticals and textile products. This is because synthetic dyes are relatively expensive and cause reactions such as allergies or are carcinogenic which are potentially harmful to the environment and human health. One of the natural dyes that is usually used in staining is henna leaves (*Lawsonia inermis)*. This study aims to determine the potential of henna leaves extract in staining *Escherichia coli* and *Salmonella typhi*. The type of research used is descriptive qualitative to observe the effectiveness of henna leaves extract in staining bacteria with an experimental method that aims to compare the results of staining between safranin control and henna leaves extract. Based on the research conducted, it was found that only henna leaves extract with a concentration of 100% was able to staining *Escherichia coli* and *Salmonella typhi* bacteria like safranin with brown color, while the results of henna leaves extract oxidized using potassium permanganate and sodium carbonate could not dyeing the two bacteria properly and clearly. So further research is needed on Lawsonia inermis leaves extract to be able to dyeing other bacteria.

Keywords: *Alternative Dyes; Henna Leaf Extract; Escherichia coli; Salmonella Typhi.*

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I. INTRODUCTION

Currently, increasing environmental awareness among commercial dye manufacturers and small-scale textile exporters has shifted attention toward the use of natural pigment dyes in food, pharmaceuticals, and textile products as alternatives to synthetic colorants (Venturina et al., 2020). This trend is driven by the harmful effects of synthetic dyes, which are known to cause allergic reactions and possess carcinogenic properties, posing risks to both human health and the environment (Khantamat et al., 2021).

Synthetic dye safranin is commonly used for staining Gram-negative bacteria (Venturina et al., 2020). However, its high cost and instability during storage have led to a decline in its use among commercial dye manufacturers and textile exporters. The price of safranin was reported to reach IDR 3,565,000 per 25 grams in 2022. Additionally, safranin is carcinogenic, toxic, and environmentally unfriendly due to its recalcitrant wastewater residues that are difficult to degrade in soil (Jannah et al., 2023; Niken & Yulia, 2023; Papalanggi & Dwisari, 2023).

The use of natural dyes, which are more affordable, eco-friendly, non-carcinogenic, and biodegradable, represents a promising solution to overcome the limitations of synthetic dyes (Dafrita & Sari, 2020). One plant known to contain natural pigments is the henna plant (*Lawsonia inermis*). According to Prastiwi (2021), henna leaves contain the pigment compound lawsone (2-hydroxy-1,4-naphthoquinone) in concentrations ranging from 1–4%. Ethanolic extracts of henna leaves produce phenolic compounds classified as proteins and exhibit effective pigmenting properties. Furthermore, henna leaves contain 4.5% tannins, flavonoids, coumarins, and steroids (Niken & Yulia, 2023).

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Henna yields the acidic dye molecule hennotannic acid, which imparts an orange-red color. This compound binds to proteins and is traditionally used to stain nails, hair, skin, and textiles. Medicinally, henna leaves have been used prophylactically for boils, burns, bruises, dermatitis, and as a mouthwash for sore throat. The roots are believed to treat burns, leprosy, strangulation, and premature graying of hair (Hafiz et al., 2012). According to a study conducted by Prastiwi (2021), ethanol extract of *Lawsonia inermis* leaves was not able to optimally stain the cell wall of Klebsiella pneumoniae; however, the bacterial morphology remained discernible. This may have been due to the absence of an oxidizing agent, the delay in staining after extraction, or prolonged storage of the extract (Prastiwi, 2021). Virgianti and Luciana (2017) emphasized that natural dyes must be pre-oxidized, likely due to the dissociation of dye–oxidant ion pair complexes (Virgianti & Luciana, 2017).

Niken and Yulia (2023) demonstrated that *Lawsonia inermis* leaf extract could serve as a substitute for safranin in Gram staining of *Escherichia coli*. The extract was prepared at concentrations of 25 mg/mL, 50 mg/mL, 75 mg/mL, and 100 mg/mL. However, staining results using 25–75 mg/mL concentrations were suboptimal, yielding poorly visible, low-contrast, reddish-brown bacterial images. In contrast, staining with a 100 mg/mL concentration produced significantly improved results with clearly visible bacterial morphology (Niken & Yulia, 2023).

Based on the foregoing, further investigation is warranted to evaluate the potential of *Lawsonia inermis* leaf extract as an alternative staining agent for *Escherichia coli* and *Salmonella typhi*. Therefore, this study aims to assess the efficacy of henna leaf extract in staining *Escherichia coli* and *Salmonella typhi*.

II. METHODOLOGY

A. Research Location and Period

Sample collection was conducted in Tanjung Sari Village, Medan Selayang District, Medan City, North Sumatra. The samples were subsequently processed and identified at the Integrated Laboratory of Poltekkes Medan, located in Medan Tuntungan District, Medan City, North Sumatra. The study was conducted from December 2023 to May 2024.

B. Instruments and Materials

The instruments used included an analytical balance, oven, laboratory blender, rotary evaporator, digital pH indicator, microscope, and sterile bottle containers. Materials included henna leaves (*Lawsonia inermis*), 96% ethanol, distilled water (aquadest), safranin, 0.1 N potassium permanganate, 10% sodium carbonate, and pure bacterial cultures of *Escherichia coli* and *Salmonella typhi* obtained from the Integrated Laboratory of Poltekkes Medan.

C. Sample Extraction

A total of 750 grams of henna leaves were weighed and washed with distilled water. The cleaned leaves were drained and dried in an oven at 60°C for 12 hours. The dried leaves were then ground into a fine powder using a laboratory blender. A 200-gram portion of this powder was placed in a sterile bottle, followed by the addition of 1 liter of 96% ethanol for maceration, with periodic agitation. The mixture was left to stand for 24 hours. Remaceration was conducted similarly until the extract was depleted of color. The resulting filtrate was collected, combined, and evaporated using a rotary evaporator at 40°C until a concentrated extract was obtained (Chukwu et al., 2011; Singh et al., 2015).

D. Preparation of Extract Variants

A 2.5-gram portion of the concentrated extract was weighed and mixed with 50 mL of distilled water. The solution was stirred until homogeneous and divided into two separate sterile bottles (Chukwu et al., 2011). The pH of each solution was measured using a digital pH indicator.

For oxidation, one bottle was treated with potassium permanganate until a neutral pH was achieved. The second bottle was oxidized using sodium carbonate. A final extract sample, undiluted and non-oxidized, was prepared as a 100% extract (Chukwu et al., 2011; Yusuf & Mohammad, 2017).

E. Staining of Escherichia coli and Salmonella typhi

A loop (inoculating wire) was flame-sterilized until red-hot, and 1–2 colonies of each bacterial culture were aseptically collected and evenly smeared onto microscope slides. The smears were heat-fixed until dry. The slides were flooded with henna extract solution for 10 minutes, followed by fixation over a Bunsen burner for 30 seconds. The slides were rinsed with running water and air-dried at room temperature. Microscopic observation was conducted at 100× magnification using an oil immersion lens (Prastiwi, 2021; Indrawati et al., 2022).

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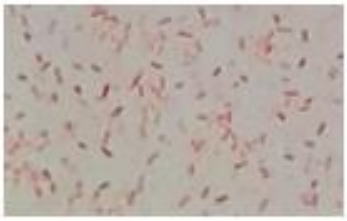
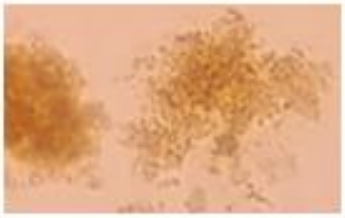
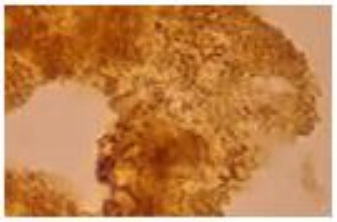
Table 1 Microscopic Staining Results of E. coli and S. typhi Using Safranin and L. Inermis Leaf Extract

Variable		*Escherichia coli*	*Salmonella typhi*
Safranin	Appearance under the microscope		
	Cytoplasmic color	Red	Red
	Color brightness	Bright	Bright
	Background of preparation	Clean and Contrasting	Clean and Contrasting
	Quality of smear	Good	Good
100% Extract	Appearance under the microscope		
	Cytoplasmic color	Brown	Brown
	Color brightness	Bright	Bright
	Background of preparation	Clean and Contrasting	Clean and Contrasting
	Quality of smear	Good	Good
5% Extract + 0.1 N $KMnO_4$	Appearance under the microscope		
	Cytoplasmic color	Brown	Brown
	Color brightness	Poor	Poor
	Background of preparation	Unclean, Clumped	Unclean, Clumped
	Quality of smear	Poor	Poor
5% Extract + 10% Na_2CO_3	Appearance under the microscope		
	Cytoplasmic color	Transparent	Transparent
	Color brightness	Dull	Dull
	Background of preparation	Unclean	Unclean
	Quality of smear	Poor	Poor

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For the control group using safranin, a similar procedure was followed: bacterial smears were heat-fixed and flooded with safranin for 1 minute, rinsed gently with water, air-dried, and examined under a microscope at 100× magnification (Saputra et al., 2022).

F. Data Analysis

Data collected included the pH of each henna extract solution, the cytoplasmic staining characteristics of *Escherichia coli* and *Salmonella typhi*, the intensity of bacterial coloration, and the background coloration of the slides. Data were analyzed using a qualitative descriptive approach. Staining outcomes using *Lawsonia inermis* extract were compared to those achieved using safranin. The data were categorized according to each variable and presented in tabular form to facilitate comparison of the staining effectiveness of each henna extract preparation on *Escherichia coli* and *Salmonella typhi*.

III. RESULTS AND DISCUSSION

The pH values of various *Lawsonia inermis* (henna) leaf extract solutions were measured using a digital pH meter. These three extract solutions were subsequently used to stain *Escherichia coli* and *Salmonella typhi* and then compared to safranin as a control stain. The pH measurements are presented in the following table:

Table 2 pH Values of Various *Lawsonia inermis* Leaf Extract Solutions and Safranin

Variant of Henna Leaf Extract	pH Value
Safranin	6,85
100% Extract	3,03
5% Extract + 0.1 N Potassium Permanganate	7,00
5% Extract + 10% Sodium Carbonate	7,02

The results indicated that the addition of oxidizing agents to diluted henna extract shifted the pH towards neutrality. The 100% concentrated extract exhibited a more acidic pH compared to the oxidized extract variants.

According to Hafiz et al. (2012), henna leaf extract with a neutral pH exhibited greater staining efficacy for *Escherichia coli* due to its similarity to the alkaline nature of safranin. Alterations in pH destabilize lawsone, the active dye compound, and induce structural changes in its chromophore. Acidic dyes exhibit affinity for basic cellular structures such as the cytoplasm, while basic dyes preferentially bind to acidic components like bacterial nucleic acids (Hafiz et al., 2012; Adisa et al., 2017).

Microscopic staining outcomes using the different extract solutions on E. coli and S. typhi are presented in Table 2. When stained with safranin, both bacteria appeared red against a clear and contrasting background. Similarly, when stained with the 100% extract, both organisms appeared brown with a clean and contrasting background. However, staining with the $KMnO_4$-oxidized extract failed to adequately color either bacterium and produced noticeable clumping. In the Na_2CO_3-treated extract, neither bacterium was stained, and the preparations showed poor clarity.

This study utilized L. inermis leaf extract as a potential natural substitute for safranin in staining E. coli and S. typhi. Based on Table 2, safranin produced clear and contrast-rich staining of both bacterial species. Similarly, the 100% henna leaf extract yielded satisfactory staining with distinct contrast. In contrast, staining results with oxidized extracts ($KMnO_4$ or Na_2CO_3) demonstrated suboptimal performance, with inadequate bacterial staining and poor smear quality.

In line with the experimental findings of Niken and Yulia (2023), the 100% henna extract showed superior staining capacity for E. coli compared to lower concentrations (25%, 50%, and 75%) (Niken & Yulia, 2023). This observation contrasts with the report by Hafiz et al. (2012), which suggested that oxidized henna extract using potassium permanganate provided better staining than non-oxidized extracts for E. coli. This discrepancy may be attributed to pH shifts and color enhancement by $KMnO_4$, facilitating better penetration in Gram-negative bacteria (Hafiz et al., 2012).

The 100% extract was prepared by maceration for 3 × 24 hours with solvent replacement every 24 hours, followed by evaporation using a rotary evaporator. The resulting concentrated extract, acidic in pH and rich in stable lawsone pigment, likely contributed to more effective staining compared to the 5% diluted extract. The inability of the oxidized 5% extract to stain E. coli and S. typhi cytoplasm may be due to the low pigment concentration and inadequate solvent-to-solute ratio, thereby limiting lawsone's ability to penetrate bacterial cell walls. Supporting this, Virgianti and Luciana (2017) reported effective bacterial staining using 50% extracts of Monascus purpureus (angkak) and teak leaves oxidized with potassium permanganate. The 50% concentration likely retained pigment stability across various pH levels (Virgianti & Luciana, 2017).

Lawsone dye stability is influenced by multiple environmental parameters such as temperature, light exposure, pH, concentration, and storage duration. Adisa et al. (2017) demonstrated that lawsone remains most stable at ambient temperature. Storage at extreme temperatures adversely affects the staining potential. Similarly, exposure to light and prolonged storage may lead to pigment degradation or precipitation. Acidic dyes exhibit affinity for basic structures, while alkaline dyes bind more effectively to acidic components (Adisa et al., 2017).

According to Adisa et al. (2017), room temperature is the most effective for extracting dyes, producing a clearer contrast between nuclei, cytoplasm, and connective tissue in histological specimens. This aligns with Asfiya et al. (2024),

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who found that extreme temperatures negatively impact interactions between bacterial cells, stains, and reagents. Dye contamination can also disrupt staining processes, resulting in inaccurate outcomes. Hence, the optimal temperature range for henna leaf extract stability is 35–45°C (Asfiya et al., 2024).

The immersion time of the bacterial smear in the extract significantly influences staining quality. Adisa et al. (2017) concluded that a 10-minute staining duration yields more intense and sharply defined results. Prolonged staining beyond this point does not enhance dye differentiation (Adisa et al., 2017). Similarly, Virgianti and Luciana (2017) reported that 1-minute and 5-minute staining durations produced dull, non-contrasting images, whereas a 10-minute exposure resulted in sharp, bright, and clearly distinguishable bacterial cells (Virgianti & Luciana, 2017).

IV. CONCLUSION

Based on the results of this study, it can be concluded that 100% extract of henna leaves (*Lawsonia inermis*) can serve as an effective alternative staining agent to safranin for the microscopic visualization of *Escherichia coli* and *Salmonella typhi*, in contrast to oxidized extracts treated with potassium permanganate or sodium carbonate, which demonstrated inferior staining performance.

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