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Natural Dye as an Alternative to Hematoxylin-Eosin Staining on Histological Preparations

Ina Karlina[1], Nur Ainun Oktavia Pusparini[1], Chesa Ekani Maharesi[1], Faisal Saeed[1], Bambang Retnoaji[2], Hendry Saragih[2], Nur Indah Septriani[2], Zuliyati Rohmah[2], Susilo Hadi[2], Ardaning Nuriliani[2*]

[1]Master Study Program of Biology, Faculty of Biology, Universitas Gadjah Mada
Jl. Teknika Selatan, Sendowo, Sinduadi, Kecamatan Mlati, Kabupaten Sleman, Daerah Istimewa Yogyakarta, Indonesia
[2]Laboratory of Animal Structure and Development, Faculty of Biology, Universitas Gadjah Mada
Jl. Teknika Selatan, Sendowo, Sinduadi, Kecamatan Mlati, Kabupaten Sleman, Daerah Istimewa Yogyakarta, Indonesia
Email: ardaning@ugm.ac.id **Corresponding Author*

Abstract

Hematoxylin-eosin is widely utilized in the field of animal microtechniques. However, the need to develop alternative dyes from natural sources such as plants has gained attention. Several studies have shown that many plants contain secondary metabolites with the potential to be developed as natural dyes. *Lonchocarpus cyanescens* and *Syzygium cumini* are promising candidates as alternative dyes for hematoxylin, while *Lawsonia inermis* and *Hibiscus sabdariffa* have shown potential as substitute dyes for eosin. These plants contain various secondary metabolites, including anthocyanins, flavonoids, chlorophyll, betalains, alkaloids, saponins, tannins, carbohydrates, proteins, phenolics, terpenoids, quinones, coumarins, xanthones, and resins. *L. cyanescens* exhibits a strong binding affinity to cells and tissues, particularly testicular tissue. Dyes derived from *Syzygium cumini* have been shown to provide a good staining result for rat liver cells. In contrast, dyes from *Lawsonia inermis* can stain cytoplasmic components and muscle fibers. Additionally, the dye from *Hibiscus sabdariffa* is capable of staining various biological components, including sperm, nerve cells, and blood cells. The dye preparation process involved extraction from different plant organs, such as leaves, flowers, and fruit. These findings suggest that secondary metabolites from these four plants hold significant potential for development as natural dyes to replace hematoxylin-eosin in histological applications.

Keywords: ***Hibiscus sabdariffa, Lawsona inermis, Lonchocarpus cyanescens*, natural dyes, *Syzygium cumini***

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Introduction

The use of colorants is essential in daily life, serving purposes such as enhancing visual appeal, adding decoration, and enhancing aesthetics. However, in the fields of histology and cell biology, dyes play an important role in staining tissues from plants, animals, microorganisms, and spores, allowing for the visual differentiation of their components. Staining refers to the application of dyes to tissues or cells to facilitate microscopic observation (Korade *et al*., 2014; Wahyuni, 2015; Obeta *et al*., 2022). One of the most commonly used staining methods in histology is the hematoxylin-eosin method. Hematoxylin is often used to stain cell nuclei dark blue (Irshad, 2014; Siregar *et al*., 2019, while eosin, a dye that binds to positively charged salt compounds, imparts a pink to red color to the cytoplasmic components of cells and tissues (Suvarna *et al*., 2019).

Hematoxylin-eosin staining is widely favored for its practicality, simplicity, affordability, and effectiveness. It is the

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standard method for histological tissue examination (Chan, 2014; Pujilestari, 2016; Kawilarang & Pohan, 2022). Despite its advantages, hematoxylin-eosin staining has notable drawbacks. Certain types of hematoxylin and eosin dyes are expensive and have limited shelf life (Pujilestari, 2016). Moreover, eosin is classified as a Group 3 carcinogen (not classifiable as to its carcinogenicity to humans) by the International Agency for Research on Cancer (IARC) (Yeti & Hariyanto, 2019). Long-term exposure to hematoxylin and eosin has been reported to have adverse effects, prompting the need to explore natural dyes as a safer alternative (Saraswati & Rahmawati, 2023). Natural dyes offer an environmentally friendly and non-toxic option for histological staining to replace synthetic dyes (Affat, 2021).

Natural dyes, derived from minerals, animals, and plants, are extensively used in industries such as textiles, food, cosmetics, and medicine (Nilgun & Ferda, 2022). In the context of histology, plant-based natural dyes can be extracted from various parts of plants, including stems, leaves, fruits, roots, and seeds. These dyes are considered economical, readily available, and environmentally sustainable (Agbede *et al*., 2017; Wulandari *et al*., 2019). However, a major limitation of natural dyes is their tendency to fade quickly (Anzani *et al*., 2016). Therefore, this literature review focuses on the potential of natural dyes as an alternative to hematoxylin-eosin staining of histological preparations. It explores the advantages and disadvantages of hematoxylin-eosin staining, while highlighting the potential benefits and challenges associated with the use of natural dyes in histology.

Advantages of Hematoxylin-Eosin Staining

Hematoxylin-eosin staining is the most commonly used method for tissue staining for analyzing cell and tissue conditions. This technique highlights the unique morphology of cells and tissues (Ozawa & Sakaue, 2020). Hematoxylin-eosin differentiates between acidophilic and basophilic components (Sunarno *et al*., 2015). Acidophil cells absorb the acidic dye of eosin, appearing bright pink, whereas basophil cells absorb the basic dye hematoxylin, resulting in a purplish or bluish hue. The hematoxylin-eosin method reveals the fine structural details of cells and tissues, such as eosinophilic elements (e.g., mitochondria, secretory granules, and collagen) and basophilic elements (e.g., rough endoplasmic reticulum, ribosomes, and nuclei) (John & Chan, 2014; Mayangsari *et al*., 2019).

Hematoxylin-eosin also demonstrates high specificity for keratin staining (Kakkar *et al*., 2022). According to Zhang *et al*. (2014), hematoxylin-eosin staining is widely regarded as one of the most reliable staining protocols in clinical practice. The procedure is straightforward to prepare and yields consistent results, making it the most frequently used staining protocol in histopathology and cytopathology. Hong *et al*. (2014) stated that hematoxylin-eosin staining is a practical and cost-effective method for detecting biofilm, which can help predict endoscopic sinus surgery (ESS) prognosis. The hematoxylin-eosin staining method is user-friendly, widely accessible (Qu *et al*., 2015; Silvia *et al*., 2017), and does not require special filters or fluorescent microscopy for interpreting results. In developing countries, hematoxylin-eosin remains a preferred option due to limited financial resources for advanced and more expensive technologies, offering a cost-effective solution (Zhang *et al*., 2014; Setadi *et al*., 2017). Zhang *et al*. (2013) compared hematoxylin-eosin and methyl violet staining for identifying ghost cells in vitreous or aqueous humor. While methyl violet staining was faster and simpler, it faded easily. In contrast, hematoxylin-eosin staining required more time but provided superior clarity in cell morphology and component distinction. Hematoxylin-eosin staining outperformed methyl violet in detecting ghost cells at concentrations below $8x10^4$ cells/mL. Alkhamiss (2020) employed hematoxylin-eosin, Giemsa, and periodic acid-Schiff (PAS) stains to detect *Helicobacter pylori* in gastric mucosal biopsies. The results showed that hematoxylin-eosin staining provided good specificity, highlighting its value in diagnostic histology.

Disadvantages of Hematoxylin-Eosin Staining

Hematoxylin-eosin is a synthetic dye that plays a crucial role in cellular or histological examination under a microscope (Saraswati & Rahmawati, 2023). Its critical role in histopathological analysis has driven a

high demand for hematoxylin-eosin, contributing to its relatively high cost. Moreover, prolonged storage of hematoxylin-eosin can lead to degradation of the dye, and its chemical components also pose potential health risks to users (Asyah *et al*., 2024). The use of synthetic dyes potentially triggers risks of cancer and damage to vital organs, including the liver and kidneys (Pujilestari, 2016).

Hematoxylin-eosin staining involves a relatively long procedure, with a single slide preparation requiring 3-5 minutes, depending on the protocol used. Additionally, hematoxylin-eosin is less effective in staining certain structures, such as reticular fibers, the basal membrane, and lipids (Mendoza & Bishop, 2020). While hematoxylin-eosin staining can distinguish basic tissue structures, it is not always able to differentiate between cell types with similar morphological features. Furthermore, hematoxylin-eosin staining cannot detect specific protein expression patterns or identify localization within tissues. Additionally, hematoxylin-eosin staining lacks specificity in identifying certain cellular components or molecular markers associated with specific diseases (Histowiz, 2024).

Tarum Afrika (*Lonchocarpus cyanescens*)

Lonchocarpus cyanescens, commonly known as the African tarum plant and belonging to the Fabaceae family, is predominantly found in coastal and forested regions (World Flora Online, 2022). Native to West Africa, *Lonchocarpus cyanescens* Benth, a member of the Fabaceae family, is extensively cultivated in countries like Nigeria, Ghana, Cameroon, Ivory Coast, Togo, Sierra Leone, Benin, and Guinea (Amit *et al*., 2023). The leaves of *Lonchocarpus cyanescens*, used for staining testicular tissue, contain various compounds, including saponins, tannins, flavonoids, and alkaloids. Among these, tannins and flavonoids are the primary compounds responsible for the blue staining of testicular tissue (Hartika *et al*., 2021). Optimal staining results with *Lonchocarpus cyanescens* leaves are achieved with a staining time of 5 minutes (Hartika *et al*., 2021).

Lonchocarpus cyanescens is a woody climbing or straggling shrub that can reach a height of approximately 2.5 meters. It features large panicles of reddish flowers that turn blue and possesses pinnately compound leaves. In West Africa, a dark dye extract obtained from the leaves, fruits, and young twigs is traditionally used for dyeing human hair, clothing, mats, and leather. Additionally, the plant has applications in herbal remedies. The dye solution derived from its flowers and other parts of the plant contains components such as anthocyanins, flavonoids, chlorophyll, quinones, and betalains (Onah *et al*., 2020). The secondary metabolites present in this plant, such as alkaloids, saponins, flavonoids, and tannins, serve as staining agents for tissues, with tannins and flavonoids being the primary contributors to the natural staining process (Asra *et al*., 2021).

Blue pigments are usually extracted from the leaves, which involves transferring dye components from the solid phase to the liquid phase. The extraction process can be conducted using either conventional or non-conventional methods. Conventional methods include Soxhlet extraction and maceration, which require a large volume of solvent and prolonged extraction times. In contrast, non-conventional methods, such as Ultrasound Assisted Extraction (UAE), leverage ultrasonic waves (18 kHz to 100 MHz) to disrupt plant cell walls via cavitation, thereby enhancing the extraction process (Nurandriea & Azmi, 2017). Research by Bassey *et al*. (2012) found that the leaf extract of the *Lonchocarpus cyanescens* used to stain testicular tissue contains several compounds, including saponins, tannins, flavonoids, and alkaloids. However, tannins and flavonoids are the primary contributors to the blue coloration observed in the testes (Hartika *et al*., 2021). *Lonchocarpus cyanescens* is widely used as a natural dye due to its cost-effectiveness and strong affinity for cells and tissues, allowing observation and identification of histological preparations (William & Festus, 2017).

However, the plant also has notable limitations. The extraction process is complex and costly, requiring specialized techniques such as fermentation or the addition of specific chemicals, which limits large-scale, commercial production (Asra *et al*., 2021). Natural dyes from *Lonchocarpus cyanescens* are prone to degradation when exposed to UV light, humidity, or high temperatures, indicating limited long-term stability. This reduces their industrial value (Nurandriea & Azmi, 2017). *Lonchocarpus cyanescens* grows

in certain tropical regions, making its production highly dependent on geographic location and limiting the availability of global raw materials. Additionally, if not properly processed, the chemical compounds in *L. cyanescens* can be toxic to animals and humans. Furthermore, predominantly blue, thus limiting their applicability in industries that demand a broader color spectrum (Amit *et al*., 2023).

Black Plum (*Syzygium cumini*)

Syzygium cumini, commonly known as Java Plum or Duhat, is a fruit-bearing tree native to the Philippines, renowned for its wide range of therapeutic and clinically significant health benefits. The fruit of *S. cumini* is of particular interest in research due to its phytochemical composition, particularly its anthocyanin content, which allows it to function as a potential natural dye for cytological staining (Khasandra *et al*., 2023). Recent studies have explored the potential use of *Syzygium cumini* leaf extract as an alternative staining agent in cytological applications, including the staining of buccal and urine samples (Khasandra *et al*., 2023). Several studies have associated *Syzygium cumini* with various beneficial biological activities, including cardioprotective, anti-inflammatory, hepatoprotective, antineoplastic, hypoglycemic, hypolipidemic, antibacterial, and antiallergic properties (Srivastava *et al*., 2020).

Notably, natural dyes derived from *Syzygium cumini* have been shown to effectively stain rat liver tissue, particularly the nuclei and cytoplasm of liver cells (Hartika *et al*., 2021). The advantages of using *Syzygium cumini* as a natural dye include its cost-effectiveness and availability in Indonesia. However, some limitations must be addressed, primarily their susceptibility to environmental factors and their instability, especially during extraction, such as temperature and duration. Optimizing these parameters is essential for effective dye powder production, including increasing both the extraction temperature and time. The most effective staining conditions involve extracting dried black plum dye using a 1:5 fruit-to-solvent ratio (w/v) in distilled water for 15 minutes, without the addition of a mordant. This natural black plum fruit dye exhibits potential as an alternative natural dye for histological application and cytotoxicity testing in cosmetics and related fields (Syamsul & Jalaluddin, 2017).

Henna (*Lawsonia inermis*)

Lawsonia inermis Linn., commonly known as henna, is an ornamental plant widely cultivated for its use as a coloring agent and decorative shrub. It is extensively applied in cosmetics, particularly as a pigment for coloring hair and nails in reddish-yellow hues. Belonging to the family Lythraceae, *Lawsonia inermis* thrives in subtropical and tropical regions, including East Africa, North Africa, Asia, and northern Australia. It grows naturally in tropical areas of America, Egypt, India, and parts of the Middle East (Babili *et al*., 2013). Henna dye has a long history of use as both a cosmetic and textile dye. However, its potential as an alternative natural dye for histological application, particularly for staining animal tissues, remains underexplored (Joshua *et al*., 2021).

Notably, *Lawsonia inermis* can serve as a substitute for eosin dye due to its ability to bind tissues and produce staining results comparable to eosin. The primary coloring component in henna is 2-hydroxy-1,4-naphthoquinone (lawsone), which is complemented by other bioactive compounds, saponins, carbohydrates, proteins, alkaloids, phenolics, flavonoids, terpenoids, quinones, coumarins, xanthones, and resins (Raju *et al*., 2018). *Lawsonia inermis* contains naphthoquinone with high reactivity to amino acids, leading to the formation of brown to purple pigments upon reacting with amino acids, the building blocks of proteins in biological tissues in biological tissues. This reactivity underscores its potential to serve as a natural dye in histology, either as a complement to eosin or as a replacement (Raju *et al*., 2018). Despite its potential, *Lawsonia inermis* faces limitations, such as poor color fastness, which results in fading under exposure to sunlight or washing. Additionally, natural dyes often exhibit lower affinity for tissues compared to synthetic dyes, leading to reduced staining intensity. The efficiency of dye extraction also depends on factors such as solvent type and extraction conditions (Raju *et al*., 2018).

To mitigate these challenges, several strategies can be implemented. For example,

mordanting with agents like alum or tannin can enhance dye adherence and improve resistance to fading. Optimizing dyeing conditions, such as maintaining specific temperatures and durations, can also maximize dye uptake. Pre-treatment of biological tissues or the use of plasma treatment can increase dye affinity, while blending natural dyes with synthetic dyes may produce brighter, more durable colors while maintaining environmental sustainability. Standardizing extraction techniques, including solvent concentration control, can ensure consistent yields and dye quality (Raju *et al*., 2018). The extraction process is the first step in utilizing henna as a coloring agent. Henna dye is primarily obtained from the leaves of *Lawsonia inermis* with water proving to be the most effective solvent for extraction. Water-based extraction yields approximately 18.6%, compared to 8.1% with alcohol-based methods (Adisa *et al*., 2017).

The common extraction methods used for obtaining lawsone from *Lawsonia inermis* (henna) leaves include maceration, Soxhlet extraction, and various solvent extraction methods. The maceration method involves soaking dried, powdered leaves in a solvent (e.g., water or alcohol) for extended periods to extract active compounds. For example, soaking 100 grams of dried leaves in 1,000 mL of water for 24 hours has been shown to yield a concentrated lawsone extract (Adisa *et al*., 2017). The Soxhlet method, by contrast, is another effective technique where the powdered leaves are placed in a Soxhlet apparatus and continuously extracted with a solvent over several hours. This method has been shown to produce higher yields of lawsone compared to maceration due to its efficiency in extracting compounds with less solvent. Additionally, solvent extraction using organic solvents such as ethyl acetate, toluene, and chloroform has been employed to optimize the yield and purity of lawsone. Each method has its advantages and can be chosen based on the desired outcome regarding yield and purity of the extracted dye (Adisa *et al*., 2017).

In histology, eosin dye is commonly used as a counterstain with hematoxylin to enhance contrast. Similarly, *Lawsonia inermis* possesses physicochemical properties analogous to those of eosin, such as the presence of flavonoids (luteolin, apigenin, and glycosides), coumarins (esculatin, fraxetin, and scopoletin), tannins, and saponins. These compounds facilitate the staining of elastin, collagen, keratin, and cytoplasm in connective tissues and muscle fibers (Raju *et al*., 2018). According to Meenakshi (2021) said that cytoplasmic staining using henna produces very clear cytoplasmic details and is comparable to the conventional eosin method.

Rosella (*Hibiscus sabdariffa*)

Hibiscus sabdariffa is a eudicot species belonging to the *Magnoliopsida* class and *Malvaceae* family. Commonly referred to as rosella, *Hibiscus sabdariffa* thrives in tropical and subtropical environments. It contains various bioactive compounds with numerous applications. One notable application of *Hibiscus sabdariffa* is its use as a natural dye in histological preparations (Joshua *et al*., 2021). Natural dyes derived from rosella are also utilized in hair, skin, and nail coloring, producing shades ranging from reddish-orange to brownish tones, which are attributed to the presence of anthocyanins in its petals (Joshua *et al*., 2021).

The most commonly used parts of the *H. sabdariffa* are its red, dark red, and green petals. Each petal color indicates the presence of specific bioactive compounds. For instance, the red petals are rich in anthocyanins, which include derivatives such as delphinidin 3-sambubioside and cyanidin 3-sambubioside. Despite its potential as a natural dye, *Hibiscus sabdariffa* faces several limitations that affect its application.

One significant limitation is its poor stability and solubility in water, which can result in color loss during washing or environmental exposure. Additionally, studies have shown that anthocyanins in rosella extracts have limited ability to penetrate bacterial cell walls, restricting their effectiveness in applications like Gram staining, where specific binding to peptidoglycan is essential (Pratama *et al*., 2023). The acidic nature of rosella extracts (with a pH of approximately 3) further hinders the effective binding of anthocyanins to certain substrates, leading to suboptimal staining results.

To overcome these limitations, several strategies have been proposed. Mordanting with suitable agents can enhance color fixation

on fabrics and improve the dye's resistance to washing and light exposure (Pratama *et al*., 2023). Optimizing the extraction process, such as using different solvents or adjusting extraction conditions, may also increase anthocyanin yield and stability. Suboptimal staining results with other stabilizing agents or synthetic dyes could further enhance color retention and broaden the applications. Additionally, modifying the pH of the dye solution or using additives that can facilitate better binding to target materials could improve its effectiveness in various dyeing processes. By addressing these challenges, rosella can be more effectively utilized as a sustainable alternative to synthetic dyes across multiple industries (Pratama *et al*., 2023).

The petals of *Hibiscus sabdariffa* also contain organic acids, minerals, amino acids, carotene, vitamin C, and sugars (Kumar *et al*., 2022). *Hibiscus sabdariffa* has shown potential as an alternative natural dye to eosin (Joshua *et al*., 2021), with physical and chemical properties similar to those of eosin dyes (Eman, 2006). *Hibiscus sabdariffa* can effectively stain a variety of biological tissues, including fungi, testicular tissue, parasites, sperm cells, nerve tissue, blood cells, appendix, lymph, liver, kidney, brain, hippocampus, and bacteria (Jabeur *et al*., 2017). An alternative dye, *Hibiscus sabdariffa* offers several advantages due to its affordability, availability, and environmentally friendly properties compared to eosin dye. Unlike eosin, it poses fewer health and environmental hazards, making it a more sustainable option (Agbede *et al*., 2017).

Conclusion

Plants possess significant potential as sources of natural dyes due to their rich content of secondary metabolites. The production of these dyes typically involves the extraction of pigments from specific plant organs, each reflecting the dominant color characteristic of the species. *Lonchocarpus cyanescens*, for instance, yields a predominantly blue dye extracted from its leaves. This plant contains high concentrations of secondary metabolites such as anthocyanins, flavonoids, chlorophyll, quinine, and betalains. Among these, tannins and flavonoids play a central role in the natural staining process due to their strong binding affinity to biological tissues.

The dye from *Syzygium cumini* is derived from its fruit, which contains both blue and red pigments. The dominant secondary metabolite in this species is tannin, which significantly contributes to its staining capabilities. Similarly, *Lawsonia inermis* produces dyes ranging from brown to purple, primarily due to the presence of 2-hydroxy-1,4-naphthoquinone (lawsone) found in its leaves. Other active compounds, such as coumarins, resins, and alkaloids, further support its staining potential. In the case of *Hibiscus sabdariffa*, dyes are extracted from the petals, yielding reddish-orange to brownish tones. The key active compound in this species is anthocyanin, which is responsible for its staining effectiveness. To fully optimize the use of plant-based natural dyes, future research should prioritize three key areas. First, the development of environmentally friendly and efficient extraction methods should be emphasized through the optimization of extraction parameters. Second, efforts must be directed toward improving the stability and durability of natural dyes, particularly their resistance to environmental factors and compatibility with other dye agents. Third, advancements in dye technology should focus on color control, including the use of biotechnology, such as microbial modification, to enhance pigment yield, improve color consistency, and tailor dye characteristics for broader applications.

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