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The Republic of Sudan

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Evaluation of Sudanese Hibiscus sabdariffa (Roselle) Extract as an Alternative Counterstain to Eosin in Histopathology

"A Research Proposal Submitted in Partial Fulfillment of the Requirements for the Award of the Bachelor of Science (B.Sc.) Degree in Medical Laboratory Sciences (Histopathology)"

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CHAPTER I

Introduction and Literature Review

1. Introduction and literature review

6 1. Introduction Recent studies have demonstrated that the aqueous extract of Hibiscus sabdariffa is a promising natural substitute for synthetic eosin in routine histopathological staining. The aqueous flower extract has been shown to function effectively as a cytoplasmic stain, providing clear visualization of cellular components and tissue morphology comparable to conventional eosin, thereby supporting its application in routine tissue analysis [4]. Similarly, aqueous extracts obtained from selected floral petals have exhibited satisfactory staining characteristics in tissue sections. When compared with conventional eosin, these natural extracts showed no statistically significant differences in cytoplasmic clarity, staining uniformity, or background cleanliness, confirming their effectiveness and suitability as alternative cytoplasmic stains [5]. Furthermore, the aqueous extract of Hibiscus sabdariffa possesses natural acidic properties that enable it to function effectively as a cytoplasmic counterstain in paraffin-embedded tissue sections, highlighting its potential as a sustainable and environmentally friendly alternative to synthetic eosin [6]. Moreover, comparative investigations evaluating different extracts of Hibiscus sabdariffa, including the aqueous extract, demonstrated no statistically significant difference in staining quality compared with conventional eosin. These findings indicate that the aqueous extract is a reliable alternative for routine H&E staining [7]. Earlier studies also demonstrated the successful use of Hibiscus sabdariffa extract as a cytoplasmic stain in formalin-fixed paraffin-embedded skin tissue sections. Although satisfactory cytoplasmic staining was achieved, further optimization of staining conditions, including temperature, staining duration, and pH, was recommended to improve staining intensity and overall tissue clarity [8]. 1.1 Background of the Study

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y Histopathological diagnostics rely fundamentally on routine staining methods to achieve adequate contrast between cellular structures. For over a century, the combination of Hematoxylin and Eosin (H\&E) has remained the global gold standard for routine tissue sections. Interestingly, the historical origins of these two chemical entities are contrastingly distinct. Hematoxylin is a naturally occurring chemical compound extracted from the logwood tree, Haematoxylum campechianum, which was discovered in 1502 and later introduced to histological staining in the mid-1800s to demonstrate nuclear components [1]. Conversely, Eosin is a purely synthetic acid dye belonging to the xanthene chemical class, derived as a tetrabromo-

derivative of fluorescein, which was first synthesized in 1871 by Heinrich Caro and applied as a cytoplasmic counterstain in 1876 [2]. Despite its efficiency, the ongoing reliance on synthetic Eosin presents notable drawbacks. Synthetic dyes involve complex chemical synthesis, are non-biodegradable, expensive, and present eco-toxicological concerns as well as health risks to laboratory personnel upon chronic exposure. Consequently, modern histopathology faces a compelling drive to transition toward sustainable, biocompatible, and economically viable plant-derived alternatives. Among potential natural colorants, Roselle (Hibiscus sabdariffa) stands out due to its unique phytochemical profile. Phytochemical investigations have confirmed that Hibiscus sabdariffa calyces contain exceptionally high concentrations of bioactive phenols, flavonoids, and anthocyanins [3]. These natural pigments display a strong affinity for basic cellular elements, offering a viable, non-toxic, and locally available substitute for synthetic chemical counterstains.

1.2 Problem Statement

Although eosin is widely used as a cytoplasmic counterstain in routine Hematoxylin and Eosin (H&E) staining, its synthetic nature, cost, and environmental concerns have encouraged the search for natural alternatives. Hibiscus sabdariffa contains natural pigments, particularly anthocyanin, which have shown potential as histological stains. However, there is still insufficient evidence regarding the standardization and evaluation of Hibiscus sabdariffa extract as a reliable alternative counterstain to eosin in routine histopathological staining. Therefore, this study aims to evaluate the staining performance of Sudanese Hibiscus sabdariffa extract compared with conventional eosin

1.3 Literature Review

Recent studies have investigated the use of Hibiscus sabdariffa as a natural alternative to synthetic eosin. One study demonstrated that the aqueous extract of Hibiscus sabdariffa flowers could serve as a promising natural substitute for synthetic eosin in routine histopathological staining. The extract produced effective cytoplasmic staining with clear visualization of cellular components and tissue morphology comparable to conventional eosin, supporting its suitability for routine tissue analysis [4]. Similarly, aqueous extracts

obtained from selected floral petals have exhibited satisfactory staining characteristics in tissue sections. When compared with conventional eosin, these natural extracts showed no statistically significant differences in cytoplasmic clarity, staining uniformity, or background cleanliness, confirming their effectiveness and suitability as alternative cytoplasmic stains [5]. Another study reported that the aqueous extract of Hibiscus sabdariffa possesses natural acidic properties that enable it to function effectively as a cytoplasmic counterstain in paraffin -embedded tissue sections. The authors emphasized that these characteristics make the extract a sustainable and environmentally friendly alternative to synthetic eosin [6]. Moreover, comparative investigations evaluating different extracts of Hibiscus sabdariffa, including the aqueous extract, demonstrated no statistically significant difference in staining quality compared with conventional eosin. These findings indicate that the aqueous extract is a reliable alternative for routine H&E staining [7]. Earlier studies also demonstrated the successful use of Hibiscus sabdariffa extract as a cytoplasmic stain in formalin-fixed paraffin-embedded skin tissue sections. Although satisfactory cytoplasmic staining was achieved, further optimization of staining conditions, including temperature, staining duration, and pH, was recommended to improve staining intensity and overall tissue clarity [8].

1.3 Justification (Rationale)

Histopathological staining is an essential step in tissue diagnosis because it enables clear visualization of cellular and tissue structures. Hematoxylin and eosin (H&E) staining is the standard method used in routine histopathology laboratories, with eosin serving as the principal cytoplasmic counterstain. However, eosin is a synthetic dye that is relatively expensive, non-biodegradable, and may pose environmental and occupational health risks with continuous use.

Natural plant-based dyes have attracted increasing interest as environmentally friendly, biodegradable, inexpensive, and readily available alternatives to synthetic stains. Hibiscus sabdariffa (Roselle) is rich in natural pigments and previous studies have demonstrated its ability to stain histological tissues with promising results. Nevertheless, further evaluation is required to determine its staining quality and suitability as a reliable alternative counterstain to eosin under routine histopathological conditions.

Therefore, this study is justified to evaluate the staining performance of Hibiscus sabdariffa extract as an alternative counterstain to eosin, aiming to provide a safer, more economical, and environmentally sustainable staining method for histopathology laboratories.

1.4 Research Question

Can Hibiscus sabdariffa (Roselle) extract be used as an effective alternative counterstain to eosin in routine histopathological staining?

1.5 General Objective

To evaluate the effectiveness of Hibiscus sabdariffa (Roselle) extract as an alternative counterstain to eosin in histopathology.

1.6 Specific Objectives (SMART)

To evaluate the staining performance of an aqueous extract of Hibiscus sabdariffa.

To use the Roselle extract as a counterstain for formalin-fixed paraffin-embedded tissue sections.

To compare the staining quality of Roselle extract with the conventional eosin stain.

To assess the cytoplasmic staining characteristics of Roselle extract.

To determine the suitability of Hibiscus sabdariffa extract as a safe, cost-effective, and eco-friendly alternative to eosin in routine histopathology.

CHAPTER II

Methodology of the Study

3.1 Methodology

2.1.1 Study Design

This study will be an experimental laboratory-based comparative study that will be conducted

to evaluate the staining ability of Sudanese Hibiscus sabdariffa (Roselle) aqueous extract as

an alternative counterstain to eosin in routine histopathological staining. The staining performance of Hibiscus sabdariffa extract will be compared with the

conventional eosin counterstain used in Hematoxylin and Eosin (H&E) staining. The

effectiveness of the natural extract will be evaluated based on microscopic assessment and

staining quality scores. 2.1.2 Study Area

The study will be conducted at the Histopathology Laboratory, Department of Medical

Laboratory Sciences, College of Medicine and Health Sciences, Abdalatif Alhamad University

of Technology

2.1.3 Study Duration

The study will be conducted over a period of eight weeks (two months), from July 2026 to

September 2026. During this period, sample preparation, extraction of Hibiscus sabdariffa, staining procedures, microscopic evaluation, data collection, statistical analysis, and report

writing

2.1.4 Study Variables

The study variables will include: Independent Variable: Type of counterstain used: Sudanese Hibiscus sabdariffa (Roselle) aqueous extract.

Conventional eosin stain. Dependent Variables:

Cytoplasmic staining intensity. Tissue contrast. Clarity of tissue morphology. Overall staining quality. Staining score obtained from microscopic evaluation

2.1.5 Study Population

The study population will include archived paraffin-embedded Histopathological tissue sections available at the Histopathology Laboratory of Abdalatif Alhamad University of Technology

Different types of tissue sections will be included to evaluate the staining performance of Hibiscus sabdariffa extract on various tissue structures

2.1.6 Sampling and Sample Size

A total of 284 archived formalin-fixed paraffin-embedded (FFPE) tissue

sections will be included in this study using a convenience sampling

technique. The tissue sections will be selected from available archived

samples in the Histopathology Laboratory based on the inclusion criteria and sample availability. The selected tissue sections will be allocated into two equal groups. The control group will include 142 tissue sections stained using the conventional Hematoxylin and Eosin (H&E) staining method, while the test group will include 142 tissue sections stained with Hematoxylin followed by Hibiscus sabdariffa aqueous extract as an alternative counterstain. Different tissue types will be included to evaluate the staining performance of the natural extract across various histological specimens. 2.1.7 study criteria

2.1.7.1 Inclusion Criteria

The study will include:

Archived FFPE tissue sections with adequate tissue quantity for evaluation. Sections with satisfactory preservation and minimal technical artifacts. Archived paraffin-embedded tissue sections

available in the histopathology laboratory. Tissue sections with good preservation and adequate morphology. Different tissue types suitable for routine histopathological

2.1.7.2 Exclusion Criteria

The study will exclude:

Sections with insufficient tissue for microscopic evaluation. Sections showing severe autolysis or processing artifacts. Broken or damaged slides. Poorly preserved tissue sections. Sections with technical artifacts affecting staining interpretati

2.1.8 Data Collection Tools

Data will be collected using a standardized evaluation form to record

microscopic findings and staining quality. All stained slides will be independently

evaluated by two or more trained observers using a light microscope and a

predefined staining scoring system. The assessment will include nuclear staining

quality, cytoplasmic staining intensity, tissue contrast, clarity of tissue structures, and overall staining performance. The obtained scores will be recorded and

compared between the Hibiscus sabdariffa aqueous extract group and the

conventional eosin control group. 3. STEP-BY-STEP PREPARATION PROTOCOL

Preparation of Unmordanted Acidified Hibiscus Cytoplasmic Counterstain – 800

mL

The working volume was calculated based on 142 tissue sections. Assuming 5

mL of stain per section = 710 mL, with an additional 10–15% to compensate for

losses during extraction, filtration, and handling. Final preparation volume: 800

mL. Phase A: Aqueous Extraction

Weighing: Accurately weigh 80.0 g of dried, finely ground Hibiscus sabdariffa

calyces using an analytical balance. Extraction: Transfer the ground material into a heat-resistant 1 L flask. Add 800.0

mL of distilled water. Ratio 1:10 w/

Heating: Place the flask on a hot plate with a magnetic stirrer. Heat the mixture

at 80–90°C with continuous stirring for 15–20 minutes until a deep ruby-red

extract is obtained. Cooling & Filtration: Allow the extract to cool to room temperature (20–25°C). Filter the mixture through Whatman No. 1 filter paper into a clean volumetric

flask to obtain a clear, particulate-free dye solution. Volume Adjustment: Adjust the final volume to 800 mL with distilled water if

necessary. Phase B: Compounding the Cytoplasmic Counterstain Solution

Measure Extract: Measure 800.0 mL of the filtered hibiscus extract. Acidification: Add 16.0 mL of Glacial Acetic Acid gradually with stirring. This

lowers the solution pH to approximately 2.5 – 3.0. Acidification increases the

positive charge on cytoplasmic proteins, ensuring selective binding of the dye. Stabilization: Add 40.0 mL of Glycerol. Mix thoroughly. Glycerol enhances color

retention and solution stability. Storage & Labeling: Pour the finalized solution into an amber glass bottle. Label

clearly as Unmordanted Acidified Hibiscus Cytoplasmic Counterstain with the

preparation date. Storage Conditions: Store at 4°C. Use within one week of preparation. Measure

and record the pH of the final staining solution before use. Note: If preliminary staining results indicate insufficient staining intensity, the

concentration may be adjusted to 1:5 w/v. HISTOPATHOLOGICAL STAINING PROCEDURES

□ Routine Hematoxylin & Eosin (H&E) Staining – Control Group

For formalin-fixed paraffin-embedded (FFPE) tissue sections cut at 3–5 µm:

Deparaffinization: Clear slides in 2 changes of Xylene, 2 minutes each. Rehydration: Hydrate in Absolute Alcohol for 10 dips. Then in 2 changes of 95%

Alcohol, 10 dips each. Rinsing: Rinse in running tap water until the water runs off evenly. Nuclear Staining: Stain nuclei in Mayer's Hematoxylin for 15 minutes. Rinsing: Rinse in 2 changes of tap water, 10 dips each. Blueing: Blue in 0.25% Ammonia Water or 0.5% Lithium Carbonate until nuclei

turn blue. Rinsing: Rinse again in 2 changes of tap water, 10 dips each. Cytoplasmic Counterstaining: Counterstain in 1% Alcoholic Eosin for 10–20 dips. Differentiation: Differentiate in 70% Alcohol for 10–15 dips. Dehydration: Dehydrate in 2 changes of 95% Alcohol, followed by 2 changes of

Absolute Alcohol, 10 dips each. Clearing: Clear in 2 changes of Xylene, 2 minutes each.

Mounting: Mount with DPX mounting medium and apply a glass coverslip. Hematoxylin & Hibiscus (H&H) Staining – Test Group

The same procedure as H&E, except that the Hibiscus sabdariffa aqueous

extract is used instead of Eosin as the cytoplasmic counterstain. Deparaffinization: 2 changes of Xylene, 2 minutes each. Rehydration: Absolute Alcohol 10 dips. Then 2 changes of 95% Alcohol, 10 dips

each. Rinsing: Running tap water until water runs off evenly. Nuclear Staining: Mayer's Hematoxylin for 15 minutes, or Acidified Harris

Hematoxylin for 1–3 minutes. Rinsing: 2 changes of tap water, 10 dips each. Blueing: 0.25% Ammonia Water until nuclei turn blue. Rinsing: 2 changes of tap water, 10 dips each. Cytoplasmic Counterstaining: Immerse slides in the prepared Hibiscus

Counterstain for 2 to 5 minutes. Start with 3 minutes and adjust according to

desired intensity. Quick Rinsing: Quick rinse in distilled water, 2–3 dips only. Do not over-rinse. Differentiation & Fixation: Differentiate and fix the stain in 70% Alcohol, 10–15

dips. Dehydration: Dehydrate rapidly in 2 changes of 95% Alcohol, then 2 changes of Absolute Alcohol. 10–15 seconds each to prevent excessive dye leaching.

Clearing: 2 changes of Xylene, 2 minutes each. Mounting

Microscopic Evaluation

All stained slides will be examined under a light microscope by multiple trained observers using the predefined staining scoring system. The evaluation will focus on cytoplasmic staining intensity, tissue contrast, preservation of tissue morphology, and overall staining quality. Mean staining scores will be calculated and statistically compared between the test and control groups.

3.1.9 Data Analysis

The collected data will be entered, coded, and analyzed using the Statistical Package for Social

Sciences (SPSS), version 22. Descriptive statistics, including frequencies, percentages, means, and standard deviations, will be used to summarize the data. The staining quality scores of the

Hibiscus sabdariffa extract and the conventional eosin stain will be compared using the

Independent Samples t-test. A p-value of ≤ 0.05 will be considered

3.1.10 Ethical Considerations

Ethical approval will be obtained from the relevant ethical committee before starting the study.

Permission will be obtained from the responsible laboratory authorities to use archived tissue sections.

3.4 Limitation of the Study

The possible limitations of this study may include:

Variation in staining response among different tissue types.

Limited availability of archived tissue sections.

Possible differences in microscopic evaluation between observers.

Limited study duration.

Variation in pigment concentration obtained from plant extraction.

3.5 Research Outcome (Expected Results)

The study is expected to demonstrate whether Sudanese Hibiscus sabdariffa aqueous extract can be used as an effective natural alternative counterstain to eosin in histopathological staining.

The extract is expected to provide acceptable cytoplasmic staining, good tissue contrast, and clear visualization of tissue morphology.

The findings may support the use of plant-based dyes as accessible, environmentally friendly, and cost-effective alternatives to synthetic histological stains.

Timeline and Activities:

Activities	1 week	2 week	3week	4week	5week	6week	7week	8week
ELiterature Review								
Proposal Finalization								
Ethical Approval								
Collection of								

Hibiscus sabdariffa Samples								
Preparation of Plant Extract								
Tissue Staining								
Microscopic Evaluation								
Data Analysis								
Report Writing								

Budget Table:

Budgeting category	**Unit Cost (SDG)**	**Quantity**	Total Cost (SDG)
Hibiscus sabdariffa (Roselle/Karkadeh	**1800**	**100 g of powder**	**1800**
Glass Slides	**7000**	**Six boxes**	**42000**
Cover Slips	13000	**ONE BOX**	**13000**
Transportation	**50000**		**50000**
Printing and Photocopying			
Internet	**75000**	**75000**	**75000**
Miscellaneous Expenses			
EOSIN	**60000**	**10G**	**60000**

Total			**241800**

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Embedded Tissues. International Journal of Medical Science and Applied Research. 2021;2(2).

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Histopathological Staining Evaluation Form

Section 1: Slide Information

Slide Number: _____

Tissue Type: _____

Fixative Used: _____

Staining Method: ☐Conventional H&E ☐Hibiscus (Hibiscus sabdariffa) Extract

pH of Extract: _____

Section 2: Microscopic Evaluation

Nuclear staining quality ☐Excellent ☐Good ☐Fair ☐Poor

Cytoplasmic staining quality ☐Excellent ☐Good ☐Fair ☐Poor

Staining intensity ☐Excellent ☐Good ☐Fair ☐Poor

Staining uniformity ☐Excellent ☐Good ☐Fair ☐Poor

Contrast between nucleus and cytoplasm ☐Excellent ☐Good ☐Fair ☐Poor

Background clarity ☐Excellent ☐Good ☐Fair ☐Poor

Overall staining quality ☐Excellent ☐Good ☐Fair ☐Poor

Section 3: Overall Assessment

Is the Hibiscus extract suitable as an alternative counterstain to eosin?

☐Yes

☐No

☐Partially

Comments:

Evaluator's Name: _____

Qualification: _____

Signature: _____