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Assessment and Comparison of the Efficacy of Clove Oil versus Xylene as a Deparaffinizing and Clearing Agent during Hematoxylin and Eosin Staining at Muhimbili National Hospital Dar es Salaam, Tanzania

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Abstract

Introduction: Xylene is used as a clearing and deparaffinizing agent during haematoxylin and eosin (H&E) staining in histopathology laboratories. However, in addition to its expensive cost, the risks of xylene exposure are concerning. These risks include flammability, carcinogenicity, and allergic reactions. Many alternatives have been considered as possible replacements, including essential oils. Clove oil, which is widely accessible in Zanzibar, could provide a more affordable and practical substitute. **Aim:** The aim of this study is to evaluate and contrast the deparaffinizing and clearing properties of clove oil and xylene in the course of routine H&E staining at Muhimbili National Hospital. **Study Design:** A prospective, comparative, matched-pairs experimental laboratory design. **Setting:** Muhimbili National Hospital, Central Pathology Laboratory, Dar es Salaam, Tanzania. **Methodology**: During the (February-June 2023) research period, 23 routine biopsy specimens were collected. 23 matched pairs (46 separate tissue samples) were created by splitting each specimen in half. During H&E staining, one half of each pair was processed with xylene as the deparaffinizing and clearing agent (Group A), whereas the other half was processed with clove oil (Group B). A pathologist who was blind

to the clearing chemical employed reviewed each slide at random. Staining quality was evaluated using a standardized binary scoring system for four parameters: background clarity, nuclear staining preservation, cytoplasmic staining preservation, and dewaxing efficacy. McNemar's test was used to analyze the data with 95% confidence intervals for paired proportions. **Results:** For all parameters, including dewaxing [23/23 (100%) vs. 18/23 (78.3%)], nuclear staining [23/23 (100%) vs. 20/23 (87.0%)], cytoplasmic staining [23/23 (100%) vs. 19/23 (82.6%)], and background clearing [23/23 (100%) vs. 20/23 (87.0%)], xylene showed greater percentages of successful outcomes than clove oil. McNemar's test revealed no statistically significant differences between xylene and clove oil for background clearing (p = 0.2500), cytoplasmic staining (p = 0.1250), or nuclear staining (p = 0.2500). For dewaxing, there was a trend toward significance (p = 0.0625). Nuclear staining was 13.0% (−4.5% to 30.1%), cytoplasmic staining was 17.4% (−2.3% to 35.3%), background staining was 13.0% (−4.5% to 30.1%), and dewaxing was 21.7% (0.5% to 40.2%) with 95% confidence intervals. **Conclusion:** Clove oil can perform similarly to xylene in the deparaffinization and clearing stages of standard H&E staining in terms of background clarity, nuclear staining preservation, cytoplasmic staining quality, and dewaxing efficacy.

Keywords

Clove Oil, Xylene, H&E Staining, Deparaffinizing Agent, Clearing Agent, Histopathology

1. Introduction

Xylene is a chemical aromatic hydrocarbon, which has excellent dewaxing and clearing capabilities and hence routinely used in staining of tissue sections. Haematoxylin and eosin (H&E) stain forms the backbone of routine histopathological diagnostic work. It is remarkably robust and is used to discriminate between the cytoplasm, nucleus and extracellular matrix. Despite the remarkable utility of xylene in histological staining, its use is associated with potential occupational hazards [1].

Xylene, a mixture of three aromatic hydrocarbon isomers related to benzene, is widely used in industries and medical technology as a solvent. It is a colourless, sweet smelling liquid or gas occurring naturally in petroleum, coal, and wood tar. It is also worth noting that xylene is present in many household solvents, air fresheners, stainless steel cleaners, floor polishers, and gasoline. It is used in histopathology laboratories for tissue processing, deparaffinising the tissue sections, cover slipping, cleaning tissue processors, and recycling [2].

Its high solvency factor allows maximum displacement of alcohol and renders the tissue transparent, enhancing paraffin infiltration. In staining procedures, its excellent dewaxing and cleaning capabilities contribute to brilliant stained slides. Exposure to alcohol occurs during tissue processing and deparaffinizing the tissue sections before staining [2].

Exposure to xylene can occur via inhalation, ingestion and eye or skin contact. In our histopathology laboratory, we personally experienced a problem with xylene while doing hand-immersion technique. Because of frequent exposure to xylene in the laboratory, the fingers that are in contact revealed vasodilation along with dryness, itching and scaling of that particular area. A closed patch test was done and then it was diagnosed as allergic dermatitis secondary to xylene [3].

Nowadays, many advanced molecular techniques have been introduced in histopathological practice for precise diagnosis. However, the basis for routine diagnostic work is the usage of haematoxylin and eosin (H&E)-stained paraffin sections. H&E stain is used as a gold standard universal stain in the field of pathology. It is used to discriminate nuclear, cytoplasmic, and extracellular features, and the staining procedure has remained constant for the past 150 years. Apart from H&E, components of H&E stain include xylene and different grades of alcohol that are used to carry out intermediate steps such as deparaffinization, rehydration, and dehydration of tissue sections during staining. The pitfalls associated with this H&E staining procedure are the usage and disposal of biohazardous reagents such as xylene, toxicity, cost containment, and working environment pollution.

For almost 150 years, paraffin deparaffinization of the slides with xylene has been the crucial step before the staining procedure because it allows the tissue sections to properly absorb the Haematoxylin and Eosin Stain. Because of its paraffin solvent effect, xylene is an unavoidable chemical in histology. It is the best deparaffinizing agent as it shows the accurate cellular structure of the tissues in H&E, Pap and various other special stains. The cell structure can be analysed using morphometric analysis. Xylene easily displaces the dehydrating agent and the molten wax causing minimal tissue damage. It has a high solvency capacity making it capable of excellent dewaxing and clearing agent [4].

Xylene is mainly metabolized in the liver via oxidation of methyl groups, followed by conjugation with glycine to yield hippuric acid (Ogata *et al*., 1970; Šedivec and Flek, 1976), which is excreted through urine. But high amount and dose of xylene harm the liver and even its metabolites damage the hepatocyte also. Some amount of xylene is eliminated via exhalation. The kind and intensity of health effects caused by xylene are determined by different parameters, like exposure route, duration and also individual responds differently to various levels of exposure [5] [6].

Therefore, the purpose of this study was to evaluate clove oil's effectiveness as a xylene substitute, particularly for the deparaffinizing and clearing stages of H&E staining procedures at the Histopathology Laboratory at Muhimbili National Hospital. The study assesses staining results solely, not the entire tissue processing cycle, with an emphasis on whether clove oil can successfully substitute xylene in these crucial staining procedures without compromising diagnostic quality.

1.1. Problem Statements

Exposure to xylene can occur via inhalation, ingestion, eye or skin contact. It is

primarily metabolized in the liver by oxidation of a methyl group and conjugation with glycine to yield methyl hippuric acid, which is excreted in the urine. Smaller amounts are eliminated unchanged in the exhaled air [7] [8].

Health hazardous following xylene exposure are a growing problem with increasing incidences of skin, eye, respiratory tract, and mucous membrane irritation. It also affects the central nervous system and may result in headache, weakness, memory loss, irritability, dizziness, and giddiness, loss of coordination and judgment, respiratory depression or difficulty in breathing, loss of appetite, nausea, vomiting, shivering, unconsciousness, coma, and possible death due to respiratory failure. Ingestion of xylene may cause gastrointestinal irritation including abdominal pain, nausea, and vomiting and may also affect the liver and kidneys [9].

However, data on Essential oil as an alternative to xylene exposure in our setting is scarce, posing a challenge in preventing its occurrence and complications that arises after xylene Exposure.

1.2. Rationale

The findings of this study will aid in distinguishing the health hazards caused by xylene when used as deparaffinising and clearing agents prior to mounting during Haematoxylin and Eosin Staining in histopathology laboratories among laboratory personnel's at MNH's Central Pathology Laboratory while using essential oils (clove oils) as an alternative clearing agent during tissue processing.

It will create a suitable environment for the laboratory staff to work in a pleasant and safe environment without posing any health risks at the Central Pathology Laboratory.

1.3. Research Question

What is the effectiveness of essential oils (clove oils) on deparaffinising during Hematoxylin and Eosin staining procedure at the Histopathology Laboratory?

What is the effectiveness of essential oils (clove oils) as clearing agent during Hematoxylin and Eosin staining procedure at Histopathology Laboratory?

How essential oils (clove oils) as dewaxing, clearing agent prior to covesliping attain a staining intensity during Hematoxylin and Eosin staining procedure at Histopathology Laboratory?

1.4. Objectives

1.4.1. Broad Objectives

To evaluate and contrast the effectiveness of xylene and clove oil as clearing and deparaffinizing agents during standard haematoxylin and eosin (H&E) staining at Muhimbili National Hospital from February to June 2023.

1.4.2. Specific Objectives

To evaluate clove oil's efficacy as a deparaffinizing agent in H&E staining processes.

To evaluate clove oil's performance as a clearing agent during H&E staining

techniques.

To compare the staining quality (nuclear resolution, cytoplasmic preservation, and background clarity) obtained after deparaffinizing and cleaning with xylene against clove oil during H&E staining.

1.5. Literature Review

Several studies worldwide have evaluated and compared essential oils (clove oil, coconut oil, cedarwood oil, groundnut oil) as clearing agents during H&E staining procedures in histopathology laboratories.

Historical Context and Foundational Studies

A study by the Department of Oral Pathology and Microbiology, Mahatma Gandhi Post Graduate Institute of Dental Sciences, India demonstrated adequate nuclear staining in 90% and 93.33% of sections cleared with cedarwood oil and xylene, respectively. Sections cleared with cedarwood oil or xylene showed similar (93.33%) cytoplasmic staining and uniformity of staining, with no statistically significant differences ($p = 0.2326$) [1].

Many published literatures have highlighted the importance of substituting xylene due to its toxicity. Xylene continues to be used routinely as a clearing agent despite its known hazards. From an environmental and occupational health perspective, replacing xylene with safer substitutes has been a longstanding concern [3].

Another study comparing dishwashing solution (DWS) found it to be an efficient substitute for xylene, being non-hazardous and inexpensive compared to 95% alcohol. However, the authors emphasized that all substitutes should be thoroughly analyzed before concluding which alternative is better [2].

Research from the Departments of Oral Pathology and Microbiology and Public Health Dentistry, Mamata Dental College, India reported adequate nuclear staining in 90% of sections in Group A and 100% in Groups B and C ($p < 0.05$); adequate cytoplasmic staining in 96.7% in Group A and 100% in Groups B and C ($p > 0.05$); and adequate uniformity of staining in 53.3% of sections in Group A, 70% in Group B, and 83.3% in Group C ($p < 0.05$) [3].

Recent Studies (2021-2025)

The search for safer, cost-effective alternatives to xylene has intensified in recent years, with numerous studies published between 2021 and 2025 evaluating various natural oils and essential oils as clearing agents.

Studies on Clove Oil

Sharma *et al*. (2023) evaluated orange oil as a potential xylene substitute in hematoxylin and eosin staining. In a study of 40 tissue blocks, orange oil demonstrated 92.5% adequacy for nuclear staining compared to 95% for xylene, with no significant difference in overall staining quality ($p = 0.48$). The study highlighted the cost-effectiveness and reduced occupational hazard profile of essential oil alternatives [10].

Nwozor *et al*. (2022) conducted a comparative evaluation of xylene and essen-

tial oil of clove (Syzygium aromaticum) as clearing agents in histopathology. Their study of 30 tissue samples demonstrated that clove oil produced comparable nuclear and cytoplasmic detail to xylene, with no statistically significant differences in staining quality ($p > 0.05$). The authors recommended clove oil as a safer, environmentally friendly alternative for routine histology [11].

Ocampo-García *et al*. (2024) published a systematic review on plant-based essential oils as eco-friendly alternatives to xylene in Histotechnology. Reviewing 27 studies published between 2000-2023, the authors concluded that essential oils including clove, cedarwood, orange, and coconut oils demonstrate comparable clearing efficacy to xylene while offering significant occupational safety advantages. The review highlighted the need for standardized protocols to facilitate widespread adoption.

A 2021 study by Muhammad *et al*. from the Department of Histopathology, Usmanu Danfodiyo University, Nigeria, specifically evaluated the clearing ability of clove oil alongside olive oil and groundnut oil in comparison with xylene. Using kidney, liver, and heart tissues from Wistar rats, the study demonstrated that sections cleared with natural oils revealed normal nuclear and cytoplasmic features. Among the three oils evaluated, groundnut oil was superior in clearing ability, maintaining good cellular architecture with high-quality staining patterns. Importantly, the study concluded that all three natural oils, including clove oil, have the ability to dealcoholize tissues when compared with xylene-cleared control tissues. The authors advocated for further exploration of natural oils as clearing agents considering their low cost, availability, nature-friendly effects, and health safety [6].

Studies on Cedarwood Oil

A 2024 study by Chaduvula *et al*. published in the *Bulletin of Stomatology and Maxillofacial Surgery* evaluated the efficacy of cedarwood oil (8% concentration) as an alternative clearing agent to xylene. Using twenty paraffin blocks from routine biopsy specimens, the study assessed staining quality based on nuclear and cytoplasmic detail, clarity, and uniformity. Results demonstrated that cedarwood oil showed a significant correlation with xylene concerning all assessed staining parameters. The authors concluded that cedarwood oil is a viable, eco-friendly, and safer alternative to xylene for use as a clearing agent in histopathological laboratories [6].

A 2025 study by Chaduvula *et al*. further explored cedarwood oil as an alternative to xylene, emphasizing the need for upgradation to minimize hazardous effects in histopathological laboratories. The study reinforced that essential oils, including cedarwood oil, can effectively replace xylene while maintaining diagnostic accuracy [12].

Studies on Coconut Oil

Multiple recent studies have evaluated coconut oil as a xylene substitute:

Bright *et al*. (2024) published in the *Journal of Histochemistry and Cytochemistry* evaluated the clearing properties of coconut oil in prostate tissues. The study

compared xylene clearing with coconut oil clearing at varying times (1.5, 3, and 4 hours). Results indicated significant shrinkage in coconut oil-treated specimens compared to xylene, with only tissues cleared for 4 hours achieving rigidity comparable to xylene ($p > 0.05$). Importantly, no significant difference was found in cellular details and staining quality between the two agents ($p > 0.999$). The authors concluded that coconut oil is an efficient substitute for xylene with a minimum clearing time of 4 hours, being environmentally friendly and less expensive, though it may cause tissue shrinkage [10].

A 2025 study by Putri and Zalzabillah published in the *Jaringan Laboratorium Medis* evaluated traditional Mandar coconut oil (MTM) as an alternative deparaffinization agent in H&E staining. Using 40 samples of mouse liver tissue, the study compared xylol (xylene) with MTM. The results showed that both groups achieved 100% good quality staining (score 3) for nucleus, cytoplasm, and color homogeneity. The authors concluded that traditional Mandar coconut oil is effective as an alternative deparaffinization agent in H&E staining, attributing its efficacy to lauric acid content which is non-polar and can dissolve paraffin [10].

Kharomah and Nailufar (2024) conducted a thorough literature review on coconut oil as a green substitute for xylene. The review highlighted that coconut oil contains steroid compounds, tocopherols, and tocotrienols that can effectively replace alcohol in tissue preparations. The findings indicated that coconut oil can efficiently clear tissue preparations, allowing paraffin to permeate tissue pores and enhancing the visibility of cellular structures such as the nucleus and cytoplasm. The study emphasized coconut oil's eco-friendly composition and non-toxic properties, positioning it as a promising choice for advancing histological techniques [10].

Studies on Other Natural Oils

Shivani *et al*. (2025) published in the *Journal of the Scientific Society* compared the efficacy of natural oils (groundnut oil, sesame oil, coconut oil, and castor oil) as clearing agents. Using 30 retained oral soft-tissue specimens (divided into 150 bits), the study evaluated physical properties, ease of section cutting, cellular architecture, and staining quality. Results showed that groundnut oil demonstrated comparable results with xylene across all parameters, followed by coconut oil and sesame oil. Castor oil showed poor results in sectioning and was considered ineffective. The study highlighted that xylene had an unpleasant smell, was more expensive, and non-recyclable compared to natural oils, which were less expensive, less hazardous, and easier to recycle [6] [9] [13].

Comprehensive Reviews

A comprehensive 2025 review by Devendrababu *et al*. published in the *Journal of Applied Toxicology* synthesized findings from past decades on safer and sustainable alternatives to xylene in histopathology. The review emphasized that xylene's toxic emissions pose significant health risks to laboratory personnel, necessitating strict safety protocols. Due to increasing awareness of these health risks, research into safer alternatives has expanded considerably. The review consolidated findings on various natural extracts and synthetic compounds, with special

emphasis on agents that preserve diagnostic accuracy while offering enhanced biosafety, cost-effectiveness, and suitability for routine use. The authors noted that while many alternatives have been explored, only a few have demonstrated comparable efficacy to xylene, highlighting the importance of continued research in this area [11] [13] [14].

2. Methodology

2.1. Study Area

This study was conducted in Histopathology Unit, Muhimbili National Hospital (MNH) Dar es Salaam, Tanzania. MNH is the highest-level institution of the Tanzania health system and receives referred patients from all over the country. MNH serves as a national consultant Hospital as well as teaching hospital for the Muhimbili University of Health and Allied Sciences (MUHAS). The Unit of histopathology is part of the Department of Diagnostic Laboratory Services at MNH and it has the capacity to do routine surgical pathology, cytology, histochemical special stains as well as immunohistochemistry. Paraffin embedded tissue blocks, slides as well as patient's clinical information and morphology reports are kept in the archive of the unit from where they can be retrieved for further studies. This unit receives specimens from MNH and outside MNH, from private and public institutions.

2.2. Study Design

Using a prospective, comparative, matched-pairs experimental laboratory approach, this investigation was carried out between February and June of 2023. Not the entire tissue processing cycle, but the deparaffinization and clearing procedures during H&E staining were explicitly assessed in this study. The Muhimbili National Hospital Histopathology Unit divided each tissue sample it received in half to make a matching pair. During H&E staining, xylene was used as a deparaffinizing and clearing agent in one half (Group A), and clove oil was used in the other half (Group B). Every section treated with clove oil had a directly equivalent control from the same tissue specimen, allowing this paired design to account for tissue heterogeneity as a confounding factor. The pathologist who assessed the stained sections was blinded to the clearing agent applied to each slide.

2.3. Study Population

Some of the tissue samples received at Muhimbili National Hospital's histology unit were included in the study population

2.4. Inclusion Criteria

All large tissue biopsy specimens received at the Histology laboratory in the Histology unit at Muhimbili National Hospital's CPL. (Mastectomy Biopsy, Total Ab-

dominal Hysterectomy biopsy, Liver Biopsy, Cervical Biopsy).

2.5. Exclusion Criteria

All core needle tissue biopsy specimens received at the Histology laboratory in the Histology unit at Muhimbili National Hospital's CPL.

2.6. Sample Size

$$N = \left[Z^2 \times P \times (1-P)\right] / d^2$$

where:

Z = 1.96 (for 95% confidence level)

P = 0.90 (proportion of successful nuclear staining with essential oils from a similar study by Indu *et al.*, 2014)

d = 0.10 (margin of error)

Calculation:

$N = [(1.96)^2 \times 0.90 \times (1 - 0.90)]/(0.10)^2$

$N = [3.8416 \times 0.90 \times 0.10]/0.01$

$N = [3.8416 \times 0.09]/0.01$

$N = 0.345744/0.01$

$N = 34.57 \rightarrow 35$ matched pairs (70 individual samples)

Logistical limitations and specimen availability during the February-June 2023 research period led to the enrolment of 23 consecutive eligible specimens that met inclusion criteria, resulting in 23 matched pairs (46 individual samples). This amounts to 66% of the computed minimal sample size. The obtained sample of 23 pairs is comparable to other published studies assessing xylene substitutes in histopathology laboratories [1] [4].

2.7. Laboratory Methods

Note: Only the deparaffinization, staining, dehydration, and clearing procedures carried out during H&E staining are covered in the following methods. Prior to this investigation, all specimens underwent identical tissue fixation, processing, embedding, and sectioning; these procedures are not included in the experimental comparison.

2.7.1. Reagent Preparation

Harris' Haematoxylin

2.5 g of Haematoxylin will be dissolved in 25 mls of absolute alcohol. 50 g of Potassium alum will be dissolved in 500 mls of warm distilled water. Then, the solution of Haematoxylin will be added to the solution of Potassium alum in a 2 L flask. The mixture will be brought to boiling, and then mercuric chloride will be added slowly and carefully. After cooling, 20 mls of Glacial acetic acid will be added.

I) Eosin

0.5 g of Eosin Y powder will be dissolved with 100 ml of distilled water. Then the solution of eosin and distilled water is heated over a low flame until the powder is dissolved. The 1 ml of glacial acetic acid is added to the solution of eosin and distilled water and mix well. The reagent is stored in a brown bottle in the dark room temperature.

II) 95% Alcohol/Ethanol

Measure out 95 ml of ethanol and add it to clean container. Then the 5 ml of distilled water will be added to the container with ethanol and also carefully mix the two liquid thoroughly.

III) 80% Alcohol/Ethanol

Measure out 80 ml of ethanol and add in to clean container. Then the 20 ml of distilled water will be added to the container with ethanol and also carefully mix the two liquid thoroughly.

IV) 70% Alcohol/Ethanol

Measure out 70 ml of ethanol and add in to clean container. Then the 30 ml of distilled water will be added to the container with ethanol and also carefully mix the two liquid thoroughly.

V) 1% acid Alcohol

Measure out 1 ml of glacial acetic acid/I ml of hydrochloric acid and add it to a clean container. Then measure out 99 ml of ethanol/ alcohol and add to the container with acetic acid. Carefully mix the two liquid thoroughly.

2.7.2. Cassettes/Slide Labelling

The cassettes to be sectioned and stained with xylene as the clearing and deparaffinizing agent were given a unique study number starting with the letter A followed by a number in ascending order (A-001 to A-023), while those to be sectioned and stained with clove oil were given the letter B followed by a number in ascending order (B-001 to B-023). Pairs from the same original specimen received matching numbers (e.g., A-004 and B-004 originated from the same parent tissue block).

2.7.3. Staining Techniques

The Zanzibar Clove Distillers (Lot# ZSTC2023-01, Zanzibar, Tanzania) provided the clove oil used in this investigation. With an 85% - 88% validated eugenol concentration, the oil was confirmed to be 100% pure and undiluted essential oil. There was no alteration or dilution done. The oil was kept out of direct sunlight and at room temperature (between 22˚C and 25˚C) in amber glass bottles.

Each phase in the deparaffinization and clearing process involved two changes of fresh clove oil, and each bath lasted five minutes at room temperature (a total of ten minutes for clearing and ten minutes for deparaffinization). To guarantee consistent exposure of tissue sections, mild manual agitation was used for the first ten seconds of every wash. To avoid solution saturation and guarantee constant cleaning effectiveness, a maximum of 10 slides were processed each batch of fresh

clove oil (50 ml per staining plate).

The detailed steps for both staining protocols are summarized below in **Table 1**.

Table 1. The summary of procedure undertaken in H and E staining with Essential oil (clove oils) verses Xylene for deparaffizing and clearing agents.

Steps	Clove oil	Xylene
Deparaffinization	Bath 1 Clove oil (fresh) - 5 minutes with gentle agitation	Bath 1 Xylene (fresh) - 5 minutes with gentle agitation
	Bath 2 Clove oil (fresh) - 5 minutes with gentle agitation	Bath 2 Xylene (fresh) - 5 minutes with gentle agitation
	100% alcohol - 3 minutes	100% alcohol - 3 minutes
	95% alcohol - 3 minutes	95% alcohol - 3 minutes
	80% alcohol - 3 minutes	80% alcohol - 3 minutes
	70% alcohol - 3 minutes	70% alcohol - 3 minutes
	Distilled water - 2 minutes	Distilled water - 2 minutes
Staining	Harris Hematoxylin - 8 minutes	Harris Hematoxylin - 8 minutes
	Running tap water - 5 minutes (blueing)	Running tap water - 5 minutes (blueing)
	1% acid alcohol - 2 quick dips (differentiation)	1% acid alcohol - 2 quick dips (differentiation)
	Running tap water - 10 minutes (complete blueing)	Running tap water - 10 minutes (complete blueing)
	Eosin solution - 3 minutes	Eosin solution - 3 minutes
	Distilled water - quick dip	Distilled water - quick dip
Clearing	70% alcohol - 2 minutes	70% alcohol - 2 minutes
	80% alcohol - 2 minutes	80% alcohol - 2 minutes
	95% alcohol - 2 minutes	95% alcohol - 2 minutes
	100% alcohol - 3 minutes	100% alcohol - 3 minutes
	100% alcohol - 3 minutes (second bath)	100% alcohol - 3 minutes (second bath)
	Bath 1: Clove oil (fresh) - 5 minutes with gentle agitation	Bath 1: Xylene (fresh) - 5 minutes with gentle agitation
	Bath 2: Clove oil (fresh) - 5 minutes with gentle agitation	Bath 2: Xylene (fresh) - 5 minutes with gentle agitation
Mounting	Drain excess oil by touching slide edge to absorbent paper	Drain excess oil by touching slide edge to absorbent paper
	Apply 1 - 2 drops DPX mounting medium	Apply 1 - 2 drops DPX mounting medium
	Apply coverslip gently to avoid air bubbles	Apply coverslip gently to avoid air bubbles
	Dry horizontally at room temperature for 24 - 48 hours or in 37˚C oven overnight	Dry horizontally at room temperature for 24 - 48 hours or in 37˚C oven overnight

2.7.4. Quality Control

For each staining run, new solutions of clove oil were made. Throughout the study, the room temperature was kept between 22 and 25 degrees Celsius, with daily readings. Each clove oil staining run included one control slide that had been treated with xylene to ensure staining quality. The same skilled technician carried out every process to reduce operator variability.

2.8. Data Collection Methods

Each prepared slide section was given its own study number. The temporary codes A-001 through A-023 were applied to the slides from Group A (xylene) after staining, while B-001 through B-023 were applied to the slides from Group B (clove oil).

There was a strict blinding and randomization procedure in place to reduce observer bias. Not participating in the scoring process, a lab assistant carried out the following actions:

On Randomization: A random number generator (the RAND function in Microsoft Excel) was used to pool all 46 slides and randomly assign them.

Re-Coding: Three-digit random codes (R-001 through R-046) were sent to every slide. The initial group labels on the slides were entirely covered by new labels with these arbitrary codes. There was no discernible information that connected the random codes to the first groups.

Master Code Key: Each random code was associated with its initial group assignment (A or B) using a master code key that was made, sealed in an envelope, and kept in a locked cabinet that the lab assistant alone could access.

Slide Arrangement by Using a random coding order, the slides were placed in slide holders.

An experienced pathologist received the re-coded, randomised slides for microscopic examination. The pathologist did not have access to the master code key and was totally blind to the clearing agent used on each slide. Using the standardised scoring method outlined in Section 2.10, the pathologist assessed each slide, recording each score using just the random code as an identifier.

2.9. Variables

Variables in this study are to find out the scientific alternative essential oil (clove oils) as a clearing, deparafizing agent during routine Histological staining by comparing of nuclear staining, cytoplasmic staining, and, clarity of staining and uniformity of staining. Therefore, quality involves all the policies, practices and procedures which take part to the production of the stained slides that will be observed in bright field microscope. The samples will be preceded to the staining.

Independent variables include, concentration of the clove oils used and the type of tissue used.

2.10. Investigation Tools and Validity and Reliability Issues

A standardised grading system was created to guarantee an impartial and repeat-

able evaluation of staining quality. A lab assistant who was not engaged in the scoring process randomly issued fresh code numbers to all slides (both Group A and Group B). A skilled pathologist conducted all microscopic analyses while blind to the cleaning agent.

2.10.1. Sample Processing and Scoring

Following management approval, the tissue sample were received, documented in the sample register book, and grossed to check the quality of fixation. Once the grossing is completed, the tissue samples will be processed and staining by H&E staining procedures, with one group using essential oils (clove oils) as a clearing agent and the other using xylene as a clearing agent at the MNH histology laboratory unit in accordance with SOP.

At the slide level, scoring was carried out by evaluating several typical fields. To determine representative areas and evaluate overall quality, the pathologist initially scanned the full part of each slide at low power (40× magnification). In order to guarantee thorough coverage, ten representative fields were then methodically chosen, five from the section's center and five from its periphery, following a standardized meander pattern. Every field was assessed at both 100 and 400 magnification.

2.10.2. Examination and Reporting Scoring Parameter Criteria

Four parameters were assessed for each field, using binary criteria:

Parameter	Well preserved/Cleared	Not preserved/Not cleared
Dewaxing	No visible wax crystals; tissue fully exposed; crisp appearance	Visible wax remnants; refractive artefacts, patchy reagent penetration
Nuclear staining	Deep blue/purple-black; chromatin pattern visible; distinct membranes; uniform	Pale/faint; chromatin obscured; indistinct membranes, uneven staining
Cytoplasmic staining	Appropriate pink/red; uniform; good nuclear contrast; details visible	Too pale or too dark, uneven; poor contrast; precipitate present
Background	Crystal clear; no precipitate; no debris; no haze	Hazy/cloudy, precipitate present; debris; oily residue

2.10.3. Slide Level Classification

The pathologist noted if each of the ten fields assessed on a slide satisfied the requirements for being "well preserved/cleared" (score = 1) or "not preserved/not cleared" (score = 0) for each parameter. If at least 8 out of 10 fields (≥80%) scored 1, the slide was considered "well preserved/cleared" for that parameter. For that parameter, the slide was categorised as "not preserved/not cleared" if seven or fewer fields (≤70%) scored 1.

2.11. Quality Assurance

A random selection of 10% of the slides (5 slides) were re-scored by the same

pathologist two weeks following the original evaluation, with the pathologist once more blinded to slide identification, in order to evaluate intra-observer reliability. According to Landis and Koch (1977), the agreement between the first and second scores was "almost perfect" with a kappa statistic of 0.85, demonstrating the scoring system's reproducibility.

2.12. Data Analysis

After being recorded on data collecting sheets, the raw data was transferred into SPSS 23.0 for analysis and data cleaning. Frequency tables and proportions were used to summarize the data. Microsoft Excel 2013 was used to create the graphics and charts.

Given the study's matched-pairs design and the binary outcome variables (such as "well preserved" versus "not preserved"), the efficacy of xylene and clove oil was compared using McNamara's test with continuity correction for the inferential analysis. This test is the proper statistical approach for this study design and was created especially for paired nominal data. A two-tailed p-value of less than 0.05 was deemed statistically significant. In order to provide a range of reasonable values for the actual difference between the two clearing agents, 95% confidence intervals for the proportional difference were also computed.

2.13. Ethical Consideration

This study proposal was submitted to Pathology Department at MUHAS for approval by the University Ethical Clearance Committee. When the ethical clearance were obtained, was also presented to the management of MNH to get permission for study conduction at MNH facilities. The results are therefore confidential and for positive use only. This study is expected to have no adverse effects on subject's health.

2.14. Study Limitations and Mitigation

Failure to have standard quality control slides and reliance on experienced personnel has a significant impact on the study. The lack of research done in Tanzania pertaining to the evaluation and comparison of the efficacy of essential oils (clove oils) as clearing agents in tissue processing analysis has deprived Tanzania of knowledge gained from other studies. The use of a bright field microscope instead of a phase contrast microscope for tissue visualization may result in insufficient results, which may introduce bias.

3. Results

Comparative Analysis of Deparaffinization and Clearing Efficacy during H&E Staining: Xylene vs. Clove Oil

These findings demonstrate the relative effectiveness of clove oil and xylene, particularly for the deparaffinization and clearing stages of H&E staining. For both groups, the remaining phases in the staining protocol dehydration, haema-

toxylin staining, eosin staining, and rehydration were carried out exactly the same.

The analysis compared the effectiveness of staining techniques using xylene and clove oil on 23 matched pairs of tissue samples (46 individual slides). For each parameter, the number of discordant pairs where outcomes differed between the two agents was examined using McNemar's test.

Dewaxing Efficacy among the 23 pairs, there were 5 discordant pairs where xylene successfully removed wax (absence of wax) but clove oil failed to do so (presence of wax). There were no discordant pairs where clove oil succeeded and xylene failed. McNemar's test with continuity correction showed a trend toward significance but did not reach the conventional threshold ($\chi^2 = 3.20$, $p = 0.0625$). The difference in proportions was 21.7% (95% CI: 0.5% to 40.2%), indicating that xylene had a net advantage of 21.7% over clove oil in successful dewaxing. The confidence interval does not include zero, suggesting a trend toward significance for this parameter.

On Nuclear staining there were 3 discordant pairs where xylene achieved well-preserved nuclear staining while clove oil did not, and no discordant pairs where the reverse occurred. McNemar's test showed no significant difference ($\chi^2 = 1.33$, $p = 0.2500$). The difference in proportions was 13.0% (95% CI: −4.5% to 30.1%). The confidence interval includes zero, indicating no significant difference.

Cytoplasmic staining Xylene produced well-preserved cytoplasmic staining in 23 out of 23 samples (100%), while clove oil produced it in 19 out of 23 samples (82.6%). There were four discordant pairs (17.4%) in which clove oil failed and xylene succeeded, and there were none in which the opposite was true. Using McNemar's test, no significant difference was discovered ($\chi^2 = 2.25$, $p = 0.1250$). The proportional difference was 17.4% (95% CI: −2.3% to 35.3%). A difference that is not statistically significant is shown with a confidence interval that includes zero.

Background Staining There were 3 discordant pairs where xylene produced well-cleared backgrounds while clove oil did not, and no discordant pairs where the reverse occurred. McNemar's test showed no significant difference ($\chi^2 = 1.33$, $p = 0.2500$). The difference in proportions was 13.0% (95% CI: −4.5% to 30.1%). The confidence interval includes zero, indicating no significant difference.

In summary, while xylene showed numerically higher success rates across all parameters, McNemar's test revealed no statistically significant differences between xylene and clove oil at the $\alpha = 0.05$ level, except for a trend toward significance for dewaxing ($p = 0.0625$). The wide confidence intervals reflect the sample size and indicate that larger studies may be needed to definitively establish equivalence or difference between these agents.

The comparative analysis of staining quality between xylene and clove oil is summarized in **Table 2**.

Figure 1 quantifies the Percentage of the performance of two technique for H&E staining (Xylene vs. Clove oils) across four parameter.

Table 2. Comparative analysis of staining quality between xylene and clove oil in H&E staining (N = 23 matched pairs).

Parameter	Scoring Category	Xylene n (%)	Clove Oil n (%)	Discordant Pairs*	McNemar's χ^2	p-value	Difference in proportions (95% CI)
Dewaxing	Absence of wax	23 (100%)	18 (78.3%)	b = 5, c = 0	3.20	0.0625	21.7% (0.5% to 40.2%)
	Presence of wax	0 (0%)	5 (21.7%)				
Nuclear staining	Well preserved	23 (100%)	20 (87.0%)	b = 3, c = 0	1.33	0.2500	13.0% (−4.5% to 30.1%)
	Not preserved	0 (0%)	3 (13.0%)				
Cytoplasmic staining	Well preserved	23 (100%)	19 (82.6%)	b = 4, c = 0	2.25	0.1250	17.4% (−2.3% to 35.3%)
	Not preserved	0 (0%)	4 (17.4%)				
Background staining	Section cleared	23 (100%)	20 (87.0%)	b = 3, c = 0	1.33	0.2500	13.0% (−4.5% to 30.1%)
	Section not clear	0 (0%)	3 (13.0%)				

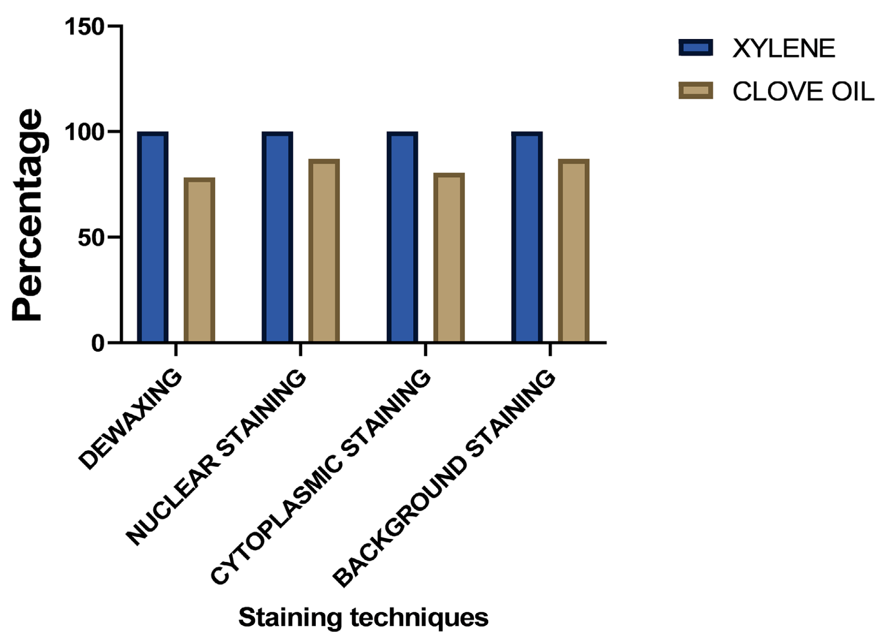
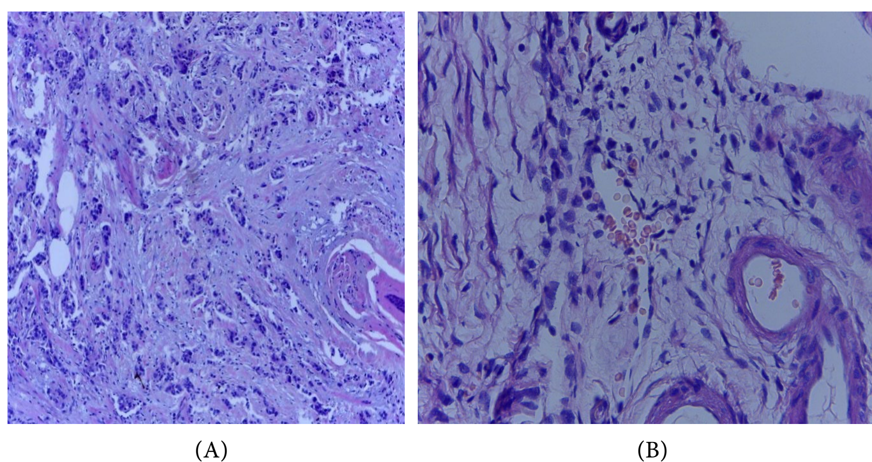


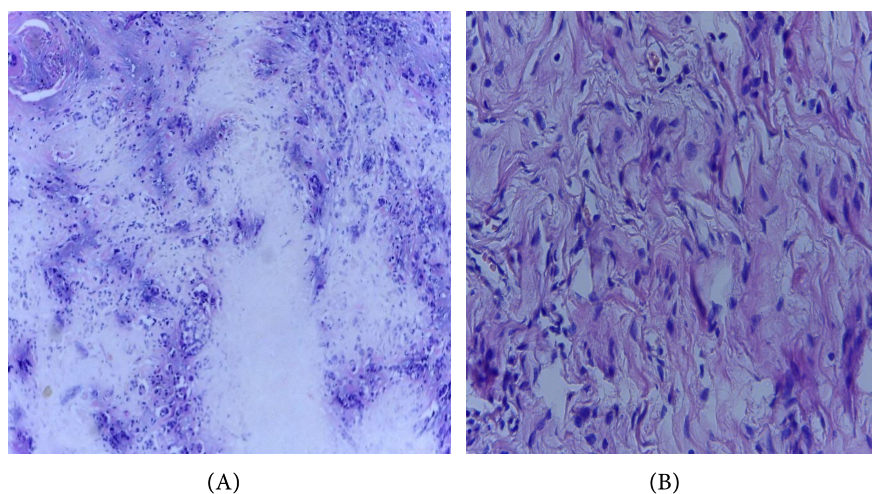
Figure 1. Percentage performance comparison of Xylene and Clove oil across four H&E staining parameters (dewaxing, nuclear staining, cytoplasmic staining, and background clearing).

Photomicrographs of H&E-stained sections deparaffinized with Xylene and clove oil as shown in Figure 2 and Figure 3.



(A) (B)

Figure 2. Photomicrographs of H&E-stained sections deparaffinized with Xylene: (A) Dewaxing staining (×100) and (B) Nuclear and cytoplasmic staining (×400).



(A) (B)

Figure 3. Photomicrographs of H&E-stained sections deparaffinized with clove oil: (A) Dewaxing staining (×100) and (B) Nuclear and cytoplasmic staining (×400).

4. Discussion

The purpose of this study was not to evaluate the entire tissue processing cycle, but to compare and evaluate the effectiveness of clove oil against xylene only for the deparaffinizing and clearing phases during regular H&E staining. Finding safer alternatives for these crucial staining procedures is especially important in resource-constrained regions where occupational safety regulations may be laxer because to the well-established health concerns linked to long-term xylene exposure [3]-[5].

Our results indicate that while xylene demonstrated a higher proportion of successful outcomes across all parameters dewaxing (100% vs. 78.3%), nuclear staining (100% vs. 87.0%), cytoplasmic staining (100% vs. 82.6%), and background clearing (100% vs. 87.0%)—these differences did not reach statistical significance when analyzed with the appropriate paired statistical test (McNemar's test), except for a trend toward significance for dewaxing ($p = 0.0625$). This suggests that

clove oil can produce histologically interpretable sections of comparable quality to xylene, aligning with the core findings of previous research.

The Department of Oral Pathology and Microbiology at Mahatma Gandhi Post Graduate Institute of Dental Sciences, India demonstrated adequate nuclear staining in 90% and 93.33% of sections cleared with essential oil or xylene respectively [1]. Our findings of 87.0% for clove oil nuclear staining are slightly lower but still comparable. Similarly, our cytoplasmic staining results (82.6% for clove oil) align with previous studies reporting 93.33% - 100% adequacy [1] [3].

Recent studies have reinforced these findings. Muhammad *et al.* (2021) evaluated clove oil alongside other natural oils and demonstrated that sections cleared with natural oils revealed normal nuclear and cytoplasmic features, concluding that clove oil has the ability to dealcoholize tissues effectively [8]. Chaduvula *et al.* (2024, 2025) showed that cedarwood oil (another essential oil) demonstrated significant correlation with xylene concerning all assessed staining parameters, supporting the viability of essential oils as alternatives [1] [4] [6].

The non-significant trend observed in our study for dewaxing ($p = 0.0625$) suggests that clove oil might be slightly less efficient at wax removal under the specific conditions used. This could potentially be mitigated by optimizing the protocol, such as increasing the time in clove oil baths or using gentle agitation. Bright *et al.* (2024) found that coconut oil required 4 hours of clearing to achieve rigidity comparable to xylene, suggesting that natural oils may require longer processing times [15]. Similarly, Putri and Zalzabillah (2025) successfully used coconut oil as a deparaffinization agent, attributing its efficacy to lauric acid content which dissolves paraffin [10].

The lack of statistical significance, despite the numerical differences, is likely influenced by the study's primary limitation: a smaller than calculated sample size. This reduces the statistical power to detect a true difference (a Type II error). The wide 95% confidence intervals for the differences in proportions underscore this uncertainty, meaning the true efficacy of clove oil could be closer to that of xylene than our point estimates suggest.

From a safety perspective, the advantages of clove oil are clear. Devendrababu *et al.* (2025) emphasized that xylene's toxic emissions pose significant health risks to laboratory personnel, necessitating strict safety protocols [11]. Natural oils offer significant safety benefits, being non-toxic, non-carcinogenic, and environmentally friendly. Shivani *et al.* (2025) highlighted that natural oils are less expensive, less hazardous, and easier to recycle compared to xylene [9] [13].

Our findings contribute to the growing body of evidence supporting essential oils as xylene substitutes in resource-limited settings. The availability of clove oil in Zanzibar makes it particularly attractive for Tanzanian laboratories, potentially reducing both health risks and costs associated with xylene use.

Study Limitation

Although McNemar's test was suitable for paired binary data, the precision of our estimations was limited due to the relatively small sample size (23 pairs), which

produced broad confidence ranges for the differences in proportions. The absence of statistical significance should be regarded cautiously because the study lacked the power to identify subtler differences between the two clearing agents. More accurate assessments of the actual distinctions between xylene and clove oil will require further research with bigger sample.

Only the deparaffinization and clearing stages of H&E staining were assessed in this work, the entire tissue processing cycle was not. Clove oil is a viable substitute for these staining procedures based on our results, but we are unable to comment on how well it works as a clearing agent during tissue processing (*i.e.*, prior to embedding). The effectiveness of clove oil throughout the whole histopathological workflow requires more research.

5. Conclusions

The current study's findings show that clove oil works well as a xylene alternative, particularly for the deparaffinizing and clearing processes involved in standard H&E staining. Clove oil yielded interpretable histological sections that were comparable to those handled with xylene in terms of background clarity, cytoplasmic preservation, and nuclear detail for the purposes of this evaluation. Nuclear staining, cytoplasmic staining, and background clearing did not show statistically significant changes between the two drugs.

Clove oil's decreased dewaxing efficacy ($p = 0.0625$) indicates that protocol optimisation, including extending exposure duration or employing mild agitation, may enhance its effectiveness even further.

For laboratories with limited resources looking to lessen the occupational health risks associated with the use of xylene, clove oil is especially appealing due to its non-toxic, non-flammable, and ecologically friendly characteristics as well as its local availability in Zanzibar.

It is crucial to remember that these results only pertain to the deparaffinization and clearing processes involved in H&E staining, not the entire tissue processing cycle. Before determining which option is best for certain laboratory uses, all xylene alternatives must be carefully examined.

6. Recommendations

Based on staining quality, safety profile, cost, and local availability, clove oil can be considered a viable alternative to xylene for deparaffinization and clearing during H&E staining in resource-limited settings.

Laboratories adopting clove oil should consider optimizing the protocol particularly increasing exposure time for dewaxing to achieve maximum efficacy.

Further research is needed to a) evaluate clove oil's performance with larger sample sizes, b) assess its compatibility with special stains and immunohistochemistry, and c) determine its efficacy as a clearing agent during the full tissue processing cycle (pre-embedding).

The Ministry of Health should consider supporting research into alternative

clearing agents at the Central Pathology Laboratory, Muhimbili National Hospital, to develop standardized protocols that are cost-effective, accessible, and environmentally sustainable.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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