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# Comparative Evaluation of Refined Palm Oil as a Non-Toxic Alternative to Xylene for Histological Tissue Processing: A Cross-Sectional Study

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**KEYWORDS**

clearing agent, eco-friendly, haematoxylin and eosin staining, histological tissue processing, palm oil, refined palm oil, tissue processing, xylene

**ABSTRACT:**

**Introduction**: Xylene, commonly used as a clearing agent in histological tissue processing, poses toxic and environmental risks. This study explores refined palm oil as a safer, eco-friendly alternative, evaluating its performance in nuclear staining, cytoplasmic staining, clarity of staining, and tissue distortion.

**Objectives**: The objective of the study was to compare the clearing efficiency of refined palm oil with xylene in histological tissue processing.

**Methods**: A cross-sectional study was conducted with 20 archival human oral soft tissue specimens fixed in 10% neutral buffered formalin. Tissue specimens were processed using xylene (Group A) and refined palm oil (Group B), followed by haematoxylin and eosin staining. Two oral pathologists performed microscopic evaluations to assess nuclear staining, cytoplasmic staining, clarity of staining, and tissue distortion. Statistical analysis was conducted using the Chi-square test and Cohen's Kappa for inter-observer agreement.

**Results**: Both xylene and refined palm oil yielded identical results for nuclear staining and no tissue distortion. For cytoplasmic staining, 80% of the refined palm oil group showed excellent results, compared to 70% in the xylene group. Clarity of staining was excellent in 80% of specimens for both groups. Statistical analysis showed no significant differences ($p > 0.05$) between the two clearing agents for any evaluated parameter.

**Conclusions**: Refined palm oil is as effective as xylene in histological tissue processing, offering comparable staining quality and tissue preservation. Its low cost, availability, non-toxicity, and eco-friendly characteristics make it a valuable alternative, especially in settings aiming to reduce occupational and environmental risks.

## 1. Introduction

Clearing is an essential step in histopathology processing for light microscopy. The purpose of clearing is to remove dehydrating agents from tissues and to prepare the tissues for impregnation with the embedding agent (1). Xylene was substituted as a safe alternative to other hazardous chemicals such as aniline oil, benzene, chloroform, dioxane and toluene in the histology laboratory in the 1950s. However, this proved to be a failure because regular and prolonged exposure to xylene has serious health effects(2). Xylene is the most commonly used clearing agent in histology, providing translucency to tissues and enhancing paraffin infiltration for better sectioning and examination(3).

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Xylene exposure has been linked to various toxic effects across different organ systems, including the central nervous system (causing headaches, dizziness, and memory loss), respiratory system (irritation, chest pain, and potential pulmonary edema), liver and kidneys (causing fat deposition), gastrointestinal tract (nausea, vomiting), musculoskeletal system (muscle weakness), skin (dryness and irritation), eyes (irritation), and the reproductive system (fetal toxicity and potential effects on breast milk). Under the Resource Conservation and Recovery Act (RCRA), xylene is classified as hazardous waste(4). For xylene, a 8-h indicative occupational exposure limit (IOEL) value of 221 mg m−3 (50 ppm) and a short term (15-min) IOEL of 442 mg m−3 (100 ppm) were introduced for the protection of the health and safety of workers(5).

Despite its toxicity to laboratory personnel and the environmental hazards it poses, xylene has remained the preferred clearing agent in histology. These concerns have prompted researchers to explore safer alternatives. Earlier studies on clove oil and orange oil-based clearing agents as alternatives to xylene have shown that they are either ineffective substitutes or relatively expensive(6). Some previous research revealed that vegetable cooking oils such as palm oil are cost-effective and widely accessible. The source of this plant is also abundant and self-cultivated(7).

## 2. Objectives

Hence, the objective of the study was to evaluate the efficacy of refined palm oil as a clearing agent in histological tissue processing compared to conventional xylene. The null hypothesis ($H_0$) states that refined palm oil is not as effective as xylene in histological tissue processing. In contrast, the alternate hypothesis ($H_1$) proposes that refined palm oil is as effective as xylene, offering comparable results across key histological parameters.

## 3. Methods

### Ethical Approval

This study was approved by the Institutional Ethics Committee.

### Study Design

This study employed a cross-sectional, comparative experimental design to evaluate the effectiveness of refined palm oil as an alternative to conventional xylene in histological tissue processing.

### Study Population

A total of 20 archival human oral soft tissue specimens were retrieved from the Department of Oral Pathology and Microbiology. These specimens were formalin-fixed. The demographic data such as age and gender were not recorded to maintain confidentiality.

### Inclusion and Exclusion Criteria

Archival, formalin-fixed human oral soft tissues specimens were included. Specimens previously subjected to special stains or immunohistochemistry and poorly preserved tissues were excluded from the study.

### Tissue Processing

Each specimen was bisected into two groups: Group A was processed using xylene, and Group B using refined palm oil (Brand: Ruchi Gold; Manufacturer: Ruchi Soya, Patanjali Foods Ltd, India; Ingredient: Refined Palmolein Oil). Standard tissue processing was followed, including dehydration in ascending grades of ethanol (70%, 90%, 95%, and 100%) with two changes of each concentration for one hour. Clearing was performed in two changes of the respective agents for 30 minutes each. Xylene was used at room temperature, while refined palm oil was heated to 45°C to ensure complete liquefaction. Specimens were then infiltrated with molten paraffin wax at 60°C and embedded in paraffin blocks.

### Sectioning and Staining

Tissue blocks were sectioned at 4-5 µm thickness using a semi-automated rotary microtome (Thermo Scientific Microm HM 340 E). Sections were mounted on glass slides, deparaffinized using the respective clearing agents (two changes, 5 minutes each). Haematoxylin and eosin (H&E) staining was performed using Harris hematoxylin and eosin reagents (Thermo Fisher Scientific India Pvt. Ltd., Mumbai, India) according to standard histological protocols.

### Study Parameters and Scoring

Microscopic analysis was performed using an Olympus CX43 Research Microscope with JENOPTIK GRYPHAX® software at 10X and 40X magnifications.

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Two experienced oral pathologists, blinded to the group allocation, independently evaluated the stained slides. The evaluated parameters included nuclear staining (clarity of nuclear borders), cytoplasmic staining (quality of cytoplasmic detail) and clarity of staining (contrast between stained components). Staining quality was scored on a 0-3 scale: 0 = poor, 1 = satisfactory, 2 = good, and 3 = excellent. Tissue distortion was also assessed to determine structural preservation.

**Statistical Analysis**

To ensure statistical validity, a power analysis was conducted using standard assumptions for comparative studies. The analysis aimed to detect a minimum 20% difference in staining quality between the two groups, with a statistical power of 80% ($\beta = 0.20$) and a significance level of 5% ($\alpha = 0.05$). Descriptive statistics were used to summarize the data, reporting frequencies and percentages for each staining category. Inter observer agreement was assessed using Cohen's Kappa ($\kappa$), interpreted as follows: >0.80 = excellent, 0.60 0.80 = good, 0.40-0.60 = fair, and 0.05 was regarded as non-significant (NS). All statistical analyses were conducted using SPSS version 24.0 (IBM Corporation, USA).

## 4. Results

The comparative evaluation of refined palm oil and xylene as clearing agents in histological tissue processing was conducted across four key parameters: nuclear staining, cytoplasmic staining, clarity of staining, and tissue distortion. Nuclear staining showed identical outcomes in both groups, with 100% of specimens rated as 'Excellent'. This indicates that refined palm oil is equally effective as xylene in preserving nuclear morphology. Similarly, tissue distortion was absent in all specimens across both groups, confirming that neither clearing agent compromised tissue architecture. In terms of cytoplasmic staining, refined palm oil demonstrated slightly better performance, with 80% of specimens rated 'Excellent' compared to 70% in the xylene group. The remaining specimens were rated 'Good', with no cases of poor or satisfactory staining in either group. For clarity of staining, both groups showed identical results, with 80% of specimens rated 'Excellent' and 20% rated 'Good', indicating comparable contrast and visibility between stained components. Statistical analysis using Fisher's Exact Test and Chi-square Test revealed no significant differences between the two groups for any of the evaluated parameters ($p > 0.05$). These findings are summarized in Table 1, which illustrates the distribution of staining quality and tissue distortion across both groups.

| Parameter | Category | Xylene (n=20) | Refined Palm Oil (n=20) | P-value |
|---|---|---|---|---|
| **Nuclear Staining** | Poor | 0 (0%) | 0 (0%) | 1.0* |
| | Satisfactory | 0 (0%) | 0 (0%) | |
| | Good | 0 (0%) | 0 (0%) | |
| | Excellent | 20 (100%) | 20 (100%) | |
| **Cytoplasmic Staining** | Poor | 0 (0%) | 0 (0%) | 0.716* |
| | Satisfactory | 0 (0%) | 0 (0%) | |
| | Good | 6 (30%) | 4 (20%) | |
| | Excellent | 14 (70%) | 16 (80%) | |
| **Clarity of Staining** | Poor | 0 (0%) | 0 (0%) | 1.0* |
| | Satisfactory | 0 (0%) | 0 (0%) | |
| | Good | 4 (20%) | 4 (20%) | |
| | Excellent | 16 (80%) | 16 (80%) | |
| **Tissue Distortion** | No Distortion | 20 (100%) | 20 (100%) | 1.0* |
| | Distortion | 0 (0%) | 0 (0%) | |

*P-value>0.05: Not significant, P-value<0.05: Statistically Significant

**Table 1: Distribution of nuclear staining, cytoplasmic staining, clarity of staining, and tissue distortion between the two study groups (Xylene and Refined Palm Oil) using Fisher's Exact Test and Chi-square Test**

Figures 1 through 4 collectively illustrate the comparative performance of xylene and refined palm oil across key histological parameters. Figure 1 shows that

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both clearing agents achieved 100% excellent nuclear staining, indicating their equal effectiveness in preserving nuclear morphology. In Figure 2, cytoplasmic staining results reveal a slight advantage for refined palm oil, with 80% of specimens rated as excellent compared to 70% in the xylene group. Figure 3 demonstrates that clarity of staining was identical in both groups, with 80% of specimens rated excellent and 20% rated good, confirming comparable contrast between stained components. Finally, Figure 4 highlights that no tissue distortion was observed in either group, further validating the structural preservation capabilities of both clearing agents.

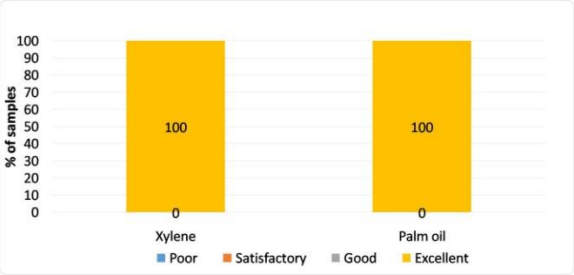


FIGURE 1: Distribution of nuclear staining between the two study groups (Xylene and Refined Palm Oil)

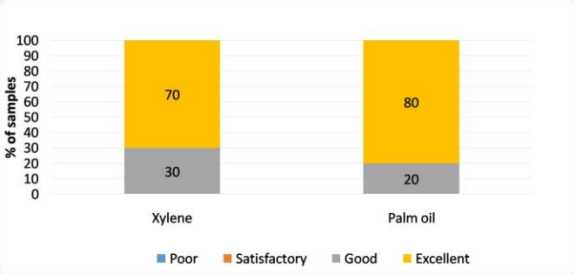


FIGURE 2: Distribution of cytoplasmic staining between the two study groups (Xylene and Refined Palm Oil)

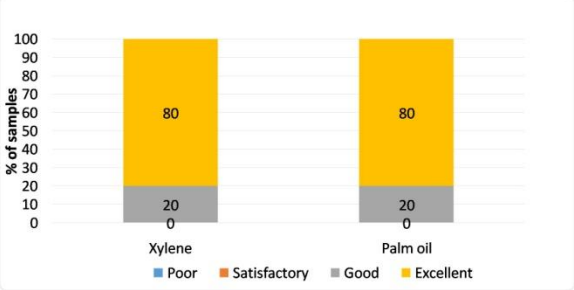


FIGURE 3: Distribution of clarity of staining between the two study groups (Xylene and Refined Palm Oil)

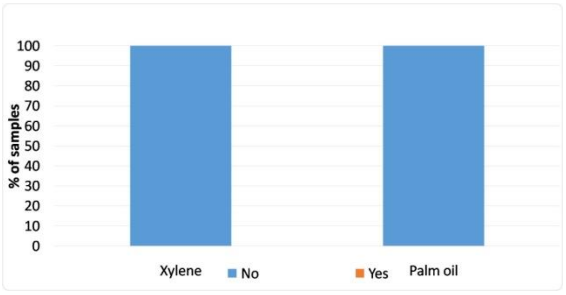


FIGURE 4: Distribution of tissue distortion between the two study groups (Xylene and Refined Palm Oil)

Figures 5 to 8 present photomicrographs that support the quantitative findings of the study. Figure 5 shows Haematoxylin and eosin (H&E) stained connective tissue sections processed with xylene and refined palm oil. Both clearing agents preserved nuclear morphology effectively, as evidenced by the well-defined nuclear borders. In Figure 6, the oral epithelium demonstrates distinct nuclear-cytoplasmic contrast and visible intercellular bridges. The refined palm oil-processed section appears slightly more defined, suggesting enhanced cytoplasmic clarity. Figure 7 highlights the clarity of staining in oral epithelium and connective tissue. Refined palm oil consistently produced well-demarcated tissue structures, reinforcing its effectiveness as a clearing agent. Finally, Figure 8 displays striated muscle tissue sections, where both xylene and refined palm oil preserved tissue integrity without any signs of distortion.

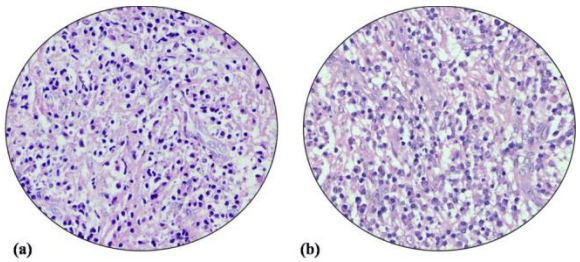


**Figure 5: Photomicrograph of haematoxylin and eosin (H&E) stained connective tissue section (a) Xylene processed (b) Refined palm oil processed (40×)**

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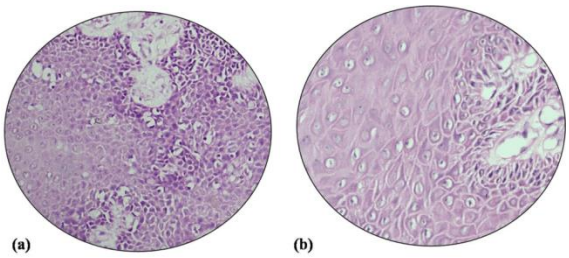


**Figure 6: Photomicrograph of H&E stained oral epithelium showing intercellular bridges, distinct nuclear cytoplasmic contrast and distinct nuclear boundaries (a) Xylene processed (b) Refined palm oil processed (40×)**

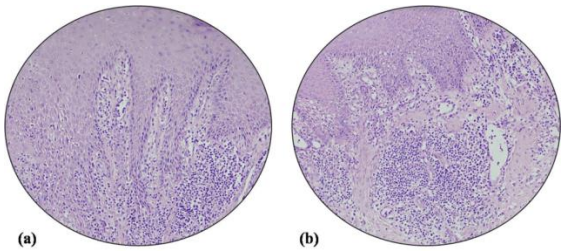


**Figure 7: Photomicrograph of H&E stained oral epithelium and connective tissue section (a) Xylene processed (b) Refined palm oil processed (10×)**

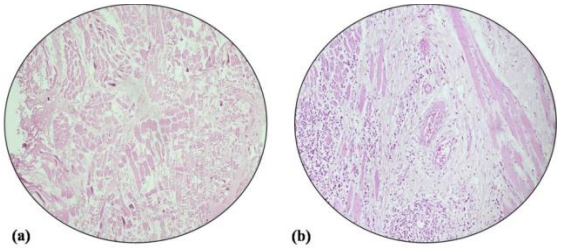


**Figure 8: Photomicrograph of H&E stained striated muscle tissue section (a) Xylene processed (b) Refined palm oil processed (10×)**

Inter-observer agreement was assessed using Cohen's Kappa (κ). For nuclear staining and tissue distortion, κ was undefined due to identical scores from both observers. For cytoplasmic staining, xylene showed excellent agreement (κ = 0.875), while refined palm oil showed good agreement (κ = 0.6875). In clarity of staining, refined palm oil demonstrated excellent agreement (κ = 0.875), and xylene showed good agreement (κ = 0.6875), as detailed in Table 2.

| Parameter | Group | Cohen-Kappa (κ) | Interpretation (Inter-Observer Agreement) |
|---|---|---|---|
| **Nuclear staining** | Xylene | κ = Undefined (identical scores) | κ is not applicable since both observers gave the same scores for all slides, leading to undefined Kappa. |
| | Refined Palm oil | κ = Undefined (identical scores) | |
| **Cytoplasmic staining** | Xylene | 0.875 | Excellent agreement |
| | Refined Palm oil | 0.6875 | Good agreement |
| **Clarity staining** | Xylene | 0.6875 | Good agreement |
| | Refined Palm oil | 0.875 | Excellent agreement |
| **Tissue Distortion** | Xylene | κ = Undefined (identical scores) | κ is not applicable since both observers gave the same scores for all slides, leading to undefined Kappa. |
| | Refined Palm oil | κ = Undefined (identical scores) | |
| Statistical test: Cohen's Kappa (κ); interpreted as >0.80 = excellent, 0.60–0.80 = good, 0.40–0.60 = fair, <0.40 = poor. | | | |

**Table 2: Inter-observer agreement between two observers in each study group (Xylene and Refined Palm Oil)**

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## 5. Discussion

The findings of this study indicate that refined palm oil performs comparably to xylene across four critical histological parameters: nuclear staining, cytoplasmic staining, clarity of staining, and tissue distortion. Both clearing agents achieved 100% excellent nuclear staining, and no distortion of tissue architecture was observed. These findings are consistent with previous studies exploring plant-based alternatives to xylene. Ghosh et al. (2016) evaluated the efficacy of extra virgin olive oil and refined sunflower oil as clearing agents on animal tissues and reported that they could replace xylene without compromising diagnostic quality. Similarly, the present results show that refined palm oil is an effective alternative, supporting the trend toward safer and more sustainable laboratory practices(8). The present study strengthens the evidence by using archival human oral soft tissues. This also enhances its relevance to diagnostic pathology and laboratory practices. Pinto et al. (2024), in a systematic review, analyzed natural substitutes for xylene and identified oils such as coconut, cedar, carrot, rose, palm, pine, and olive oil as viable alternatives. Out of 2,614 screened studies, only seven met PRISMA guidelines, underscoring the limited research in this area(9). The present study contributes to this by demonstrating that refined palm oil can serve as an effective xylene substitute while maintaining diagnostic integrity. Bright et al. (2024) reported that coconut oil caused shrinkage in prostate tissues, despite being a viable clearing agent. In contrast, no tissue shrinkage or distortion was observed in the present study with refined palm oil, underscoring its suitability for routine diagnostic use(10). Thamilselvan et al. (2021) assessed the clearing ability of cedarwood oil in routine tissue processing and concluded that it is an eco-friendly, easily available, and safer natural alternative to xylene in laboratories. However, their preparation involved adding xylene to prevent cedrol crystallization(11). The inclusion of xylene undermines its claim of being a completely safe and xylene-free alternative, as even small amounts of xylene pose health risks. In contrast, the present study used refined palm oil, offering a truly non-toxic alternative. Refined palm oil, like olive oil, is a neutral, non-polar liquid miscible with alcohol and paraffin wax. Its physical characteristics-such as a density similar to human fat-allow it to eliminate fat by displacement, rather than dissolution as xylene does, thereby preserving tissue structure more effectively(12–14). Killedar et al. (2019) highlighted the need to shift away from xylene due to its toxic effects and environmental risks, advocating for safer, eco-friendly alternatives. They concluded that olive oil could effectively replace xylene without compromising diagnostic quality(14). Similarly, the present study demonstrated that refined palm oil offers comparable performance to xylene with the additional advantage of its lower cost. Furthermore, while some xylene alternatives like coconut oil, olive oil, and cedarwood oil have shown promise, their higher costs compared to refined palm oil pose a significant economic challenge for histology laboratories. Refined palm oil, on the other hand, presents a cost-effective and widely available alternative, making it a practical substitute for laboratories seeking safer, eco-friendly clearing agents(15). While previous studies have investigated plant-based oils as xylene alternatives, this study offers distinct contributions. It utilized archival human oral soft tissues, enhancing clinical relevance compared to animal models. A direct comparison between refined palm oil and xylene was conducted using a standardized scoring system and blinded evaluation across four key histological parameters. Robust statistical methods, including Cohen's Kappa and Chi-square/Fisher's exact tests, validated inter-observer agreement and group differences. Additionally, the study highlights cost-effectiveness, non-toxicity, and eco-friendly nature of refined palm oil, making it a practical option for resource-limited settings. These strengths underscore the novelty and contextual relevance of the findings. Despite its promise, the study has limitations. It did not assess the potential rancidity of heated palm oil, which future research should address through chemical analysis. The evaluation was limited to routine H&E staining; further studies are needed to test compatibility with special stains and immunohistochemistry. Additionally, palm oil's semi-solid state at room temperature necessitates heating, which may be perceived as an extra step in laboratory workflows. However, considering the hazardous effects of xylene, this minor inconvenience is justified, as it allows laboratories to prioritize staff safety without compromising histological quality. Although the findings of this and previous studies are promising, refined palm oil has yet to gain widespread adoption in

routine histological practice. This is likely due to limited awareness and adherence to conventional protocols. Therefore, large-scale validation studies are essential to build confidence in its routine use.

Thus, the present study demonstrated that refined palm oil is as effective as xylene for histological tissue processing, with no statistically significant differences observed across nuclear staining, cytoplasmic staining, clarity of staining, and tissue distortion. Its comparable performance, low cost, and environmental friendliness make it a strong candidate for adoption in routine histopathology protocols, especially in settings that prioritize laboratory safety and sustainability.

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