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Histological evaluation of eco-friendly clearing agents as substitutes for xylol in hematoxylin and eosin staining of fibroadenoma mammae tissue

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Abstract: Xylol is widely used as a clearing agent in histopathological tissue processing because of its excellent ability to enhance tissue transparency and staining quality. However, its toxicity, volatility, and potential carcinogenicity pose serious occupational and environmental hazards. This cross-sectional analytical experimental study evaluated the histological performance of natural essential oil based clearing agents as sustainable substitutes for xylol in hematoxylin and eosin (H&E) staining of fibroadenoma mammae tissue. Twenty-eight archival samples were processed using α-pinene, 1,8-cineole, or virgin coconut oil (VCO), with xylol serving as the control. Specimens were fixed in 10% neutral buffered formalin, dehydrated in graded alcohol, and evaluated macroscopically and microscopically using an ordinal scoring system encompassing tissue shrinkage, transparency, color uniformity, nuclear and cytoplasmic clarity, staining quality, and integrity. Data were analyzed descriptively and statistically. Results showed that α-pinene and VCO achieved total scores of 32.57 ± 0.30 ($p = 0.784$) and 32.14 ± 0.26 ($p = 0.453$), respectively, comparable to xylol (33.00 ± 0.00), while 1,8-cineole performed significantly lower (22.43 ± 0.65, $p < 0.05$). These findings highlight the novelty of using α-pinene and VCO as effective, non-toxic, inexpensive, and readily available xylol alternatives, reinforcing their relevance for sustainable histopathology, particularly in resource-limited settings.

Keywords: 1,8-cineole; α-pinene; eco-friendly clearing agents; fibroadenoma mammae; H&E staining; virgin coconut oil; xylol substitute.

INTRODUCTION

Xylol, also known as xylene, is one of the most commonly used clearing agents in histopathological tissue processing because of its ability to displace alcohol, render tissues transparent, and facilitate infiltration of paraffin. However, xylol exhibits high volatility, is neurotoxic, and is considered a potential carcinogen; prolonged exposure can lead to respiratory irritation, central nervous system depression, and organ toxicity.[1] In addition, its persistence in laboratory waste and poor biodegradability contribute to environmental hazards. [2] These health and ecological risks motivate the search for safer and eco-friendly clearing alternatives.

Several recent studies have evaluated plant-based oils and essential oil compounds as xylol substitutes in various tissue types. For example, Bright et al (2024) demonstrated that coconut oil can serve as an effective clearing agent in prostate tissue, showing no significant difference in cellular detail and staining quality compared with xylol when sufficient clearing time is allowed.[3] Cano et al (2024) performed a systematic review showing that oils such as coconut, olive, pine, and eucalyptus oils are comparable to xylol in producing good histological

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architecture and staining across multiple tissue types[4] Jyostna et al (2024) compared carrot oil, olive oil, pine oil, and rose oil to xylol and found that pine oil in particular preserved nuclear and cytoplasmic detail similarly to xylol.[5] Meanwhile, studies using eucalyptus-derived 1,8-cineole reported moderate clearing and satisfactory tissue preservation with reduced toxicity. [6] Other reports have highlighted virgin coconut oil (VCO) as a biodegradable triglyceride-based agent that effectively replaces xylol while maintaining adequate staining intensity.[7]

These three natural compounds : α-pinene, 1,8-cineole, and virgin coconut oil (VCO) represent distinct classes of eco-friendly substances with promising clearing properties. α-Pinene, a terpene hydrocarbon derived from *Pinus merkusii* (Sumatran pine), exhibits strong lipid solubility and a high refractive index (1.46–1.47 at 20 °C) similar to xylol, thereby promoting efficient tissue transparency and paraffin infiltration.[8,9] 1,8-Cineole, an oxygenated monoterpenoid obtained primarily from *Eucalyptus globulus* Labill. oil, demonstrates moderate volatility and low irritancy while maintaining acceptable clearing performance. [10] VCO, a triglyceride-based natural oil extracted from the kernel of *Cocos nucifera* L., contains high concentrations of medium-chain fatty acids, predominantly lauric and myristic acids that ensure superior tissue compatibility, minimal shrinkage, and absence of toxic residue.[11] [12]These agents were selected for their biodegradability, non-toxicity, and solvent compatibility with paraffin embedding media, making them promising natural substitutes for xylol in histological tissue processing.

Despite these advances, few studies apply these alternative clearing agents specifically to *fibroadenoma mammae* tissue, nor do they systematically compare multiple agents side by side using both macroscopic and microscopic scoring. The *fibroadenoma mammae* model was chosen for this curretnt investigation because it represents one of the most common benign breast lesions, typically composed of both glandular and fibrous stromal components.[13] [14] This dual composition provides an ideal structural diversity for evaluating clearing agent performance, allowing simultaneous observation of epithelial, stromal, and connective tissue preservation.[15] Furthermore, *fibroadenoma* tissue is relatively abundant and uniform, enabling consistent replication across samples without ethical or oncological risks associated with malignant tissue.[16] From a diagnostic standpoint, maintaining morphological integrity and staining precision in fibroadenoma is crucial for distinguishing benign proliferative patterns from atypical hyperplasia or early neoplastic changes. Therefore, assessing alternative clearing agents on this tissue type has both methodological and diagnostic significance.

The gap in knowledge is therefore twofold and rooted in both methodological and contextual limitations of previous investigations. First, there remains a paucity of comparative data assessing multiple eco-friendly clearing agents : particularly α-pinene, 1,8-cineole, and virgin coconut oil (VCO) in *human fibroadenoma mammae* specimens. Although several researchers have reported that essential oils such as coconut, pine, and eucalyptus oils can substitute for xylol [17] [18]in animal or non-breast tissues, most studies have evaluated these agents in isolation rather than within a standardized comparative framework This limitation reduces the translational relevance of prior findings to human diagnostic histopathology, where tissue composition and architectural preservation are more complex.[19] The *fibroadenoma mammae* model, characterized by biphasic epithelial and stromal components, provides a unique opportunity to assess clearing efficiency across glandular and connective domains, yet this model has rarely been utilized in previous clearing studies.

Second, there is an evident methodological gap concerning the absence of an integrated, standardized ordinal scoring framework to evaluate both macroscopic and microscopic tissue characteristics after clearing. Most prior works relied on qualitative visual assessments or single-parameter grading without quantitatively correlating gross tissue transparency, consistency, and coloration with microscopic parameters such as nuclear clarity, cytoplasmic definition, and

staining contrast.[20] [21] The development and application of a unified descriptive scoring system are therefore critical to ensure reproducibility and comparability between natural clearing agents and conventional xylol-based processing. Such an integrated approach enhances the validity of histomorphological evaluations and provides objective evidence to support laboratory transitions toward safer reagents.

The novelty of this research lies in its parallel evaluation of α-pinene, 1,8-cineole, and VCO as eco-friendly clearing agents, applied to human *fibroadenoma mammae* tissue using a standardized ordinal scoring system encompassing both macroscopic and microscopic endpoints. This dual-level assessment not only bridges the methodological gap identified in previous studies but also contributes to establishing a sustainable, non-toxic, and diagnostically reliable clearing protocol.

Accordingly, this research aims to evaluate and compare the histological performance of α-pinene, 1,8-cineole, and virgin coconut oil in hematoxylin and eosin (H&E) staining of *fibroadenoma mammae* specimens. The objective is to determine which natural clearing agent most closely replicates the clearing and staining efficiency of xylol while maintaining tissue morphology, staining uniformity, and structural integrity, thereby supporting safer and environmentally responsible histopathological practice.

MATERIAL AND METHOD

Study location and design

This research received ethical approval from the Health Research Ethics Committee of Universitas Muhammadiyah Purwokerto (Approval No. KEPK/UMP/85/X/2025). All procedures were conducted in accordance with institutional and national ethical standards governing biomedical research involving human-derived materials.

The experimental work was performed at the Department of Anatomical Pathology, Rumah Sakit Islam Surakarta, Central Java, Indonesia, between August and September 2025. A comparative cross-sectional analytical laboratory-based design was employed to evaluate the histological quality of fibroadenoma mammae tissue processed with three eco-friendly clearing agents : α-pinene (derived from *Pinus merkusii*), 1,8-cineole (from *Eucalyptus globulus*), and virgin coconut oil (VCO) (from *Cocos nucifera* L.) in comparison with conventional xylol. All specimens were processed and analyzed using standardized histopathological protocols under controlled laboratory conditions (temperature 28 ± 2 °C) and relative humidity maintained below 60%. to ensure uniform clearing, embedding, and hematoxylin–eosin staining outcomes. This approach allowed a reliable assessment of the clearing efficiency and morphological preservation achieved by each agent across both macroscopic and microscopic parameters.[22]

Sampling technique and tissue samples

A total of 28 paraffin tissue blocks of *fibroadenoma mammae* were obtained purposively from the histopathology archives of the Department of Anatomical Pathology, Rumah Sakit Islam Surakarta, Central Java, Indonesia. The inclusion criteria were *benign fibroadenoma* tissues with intact epithelial and stromal components, absence of necrosis or calcification, and good fixation quality. Each tissue specimen was grossly trimmed into standardized dimensions of approximately 1 × 1 × 0.3 cm.

Reagents and chemicals

All reagents used in this research were of analytical grade and employed either in their pure or precursor forms to ensure reproducibility and consistency of results.The primary reagents included 10% neutral buffered formalin, prepared

from 37% formaldehyde (Merck, Germany); absolute ethanol (≥99.9%, Merck, Germany); and xylol (≥99.5%, Merck, Germany).

The eco-friendly clearing agents evaluated comprised α-pinene (extracted from *Pinus merkusii*), 1,8-cineole (from *Eucalyptus globulus*), and virgin coconut oil (VCO) (from *Cocos nucifera* L.), which was cold-pressed, food-grade, and locally produced. Additional reagents included Mayer's hematoxylin, eosin Y, Entellan mounting medium (Merck, Germany), and laboratory-grade distilled water. All solutions were freshly prepared and used at ambient room temperature unless otherwise specified.

Main Equipment

The main equipment utilized in this research included a tissue processor (Leica TP1020, Germany), embedding station (Leica EG1150H, Germany), rotary microtome (Leica RM2125 RTS, Germany), light microscope (Olympus CX43, Japan; up to 400× magnification), paraffin oven (Memmert UN75, Germany), and hot plate (Stuart HP1, UK).

Fixation

All tissues were immersed in 10% neutral buffered formalin for 24 hours at room temperature to stabilize proteins and prevent autolytic or putrefactive changes before dehydration, in accordance with the standard histological procedure described by Bancroft and Gamble.[22]

Tissue processing (dehydration, clearing, and embedding)

Following fixation, the tissues underwent a series of processing steps consisting of dehydration, clearing, and paraffin infiltration to ensure optimal preservation and support for microtomy. Dehydration was carried out through a graded series of ethanol concentrations to gradually replace water with ethanol. The tissues were sequentially immersed in 70% ethanol for 1 hour, 80% ethanol for 1 hour, 90% ethanol for 1 hour, 96% ethanol for 1 hour, and absolute ethanol (≥99.9%, Merck, Germany) in two changes for 2 hours each. This gradual dehydration process prevented tissue shrinkage and hardening while ensuring complete removal of residual water.[23]

Subsequently, the tissues were cleared to replace ethanol with an organic solvent that is miscible with both ethanol and paraffin wax. Clearing was performed using four agents for comparison: xylol (≥99.5%, Merck, Germany), α-pinene (derived from *Pinus merkusii*), 1,8-cineole (from *Eucalyptus globulus* Labill.), and virgin coconut oil (VCO) (from *Cocos nucifera* L.). Each clearing agent was used in two changes for 1 hour each at room temperature (28 ± 2 °C). [24]
After clearing, the tissues were infiltrated with molten paraffin wax (melting point 56–58 °C; Leica, Germany) for four hours to achieve complete paraffin penetration into the tissue matrix. [25]The infiltrated tissues were then embedded in stainless-steel base molds and allowed to solidify at room temperature, forming paraffin blocks with uniform orientation and consistency suitable for microtomy. [26]

Microtomy

After embedding, the paraffin blocks were trimmed to expose the tissue surface and then sectioned using a rotary microtome (Leica RM2235, Germany) at a uniform thickness of 4 µm. Ribbon sections were floated on a 40 °C water bath to eliminate wrinkles and immediately mounted onto pre-labeled glass slides coated with Mayer's albumin adhesive. The slides were air-dried at room temperature for 12 hours and subsequently oven-dried at 60 °C for 1 hour before hematoxylin and eosin (H&E) staining.

Hematoxylin and Eosin (H&E) Staining

The staining followed the protocol of Bancroft and Gamble [22] with minor modifications. Sequential treatment included deparaffinization with xylol I for 10 minutes and xylol II for 5 minutes, followed by rehydration through absolute ethanol (5 minutes), 96% ethanol (5 minutes), 70% ethanol (5 minutes), and running tap water (5 minutes). The slides were then immersed in Mayer's hematoxylin for 5 minutes, rinsed under running tap water for 5 minutes, and counterstained with eosin Y for 1 minute. The sections were subsequently dehydrated through 70% ethanol (3 minutes), 96% ethanol (3 minutes), and two changes of absolute ethanol for 5 minutes each before clearing and mounting with Entellan mounting medium.[27]

Mounting and Microscopic Observation

Two drops of Entellan mounting medium were applied to each stained section, which was then covered with a cover slip and air-dried at room temperature. Microscopic evaluation was conducted using an Olympus CX43 light microscope under magnifications of 400× to assess cellular and tissue morphology.

Evaluation and data analysis

Both macroscopic and microscopic evaluations were performed using an ordinal scale (0–3) adapted from Bright et al.[3] Macroscopic parameters included tissue shrinkage, transparency, color uniformity, consistency, and surface smoothness, whereas microscopic assessment covered cellular boundary clarity, cytoplasmic detail, nuclear visibility, staining uniformity, and tissue integrity. All evaluations were independently performed by two experienced histopathologists who were blinded to the treatment groups. Inter-observer reliability was assessed using Cohen's kappa coefficient for ordinal scoring and the intraclass correlation coefficient (ICC) for averaged quantitative data, both showing strong agreement ($\kappa = 0.89$; ICC = 0.92). Each clearing agent was tested on three representative tissue sections per group ($n = 28$ in total), and data were analyzed descriptively and statistically to compare average ordinal scores and qualitative differences among agents. Statistical analyses were performed using SPSS version 26.0 (IBM Corp., Armonk, NY, USA). The Kruskal–Wallis test was applied to assess overall differences among the four clearing agents for both macroscopic and microscopic scores, as the data were ordinal and non-normally distributed.

RESULTS AND DISCUSSION

Macroscopic evaluation of fibroadenoma mammae tissue using various clearing agents

Macroscopic Scoring Rubric

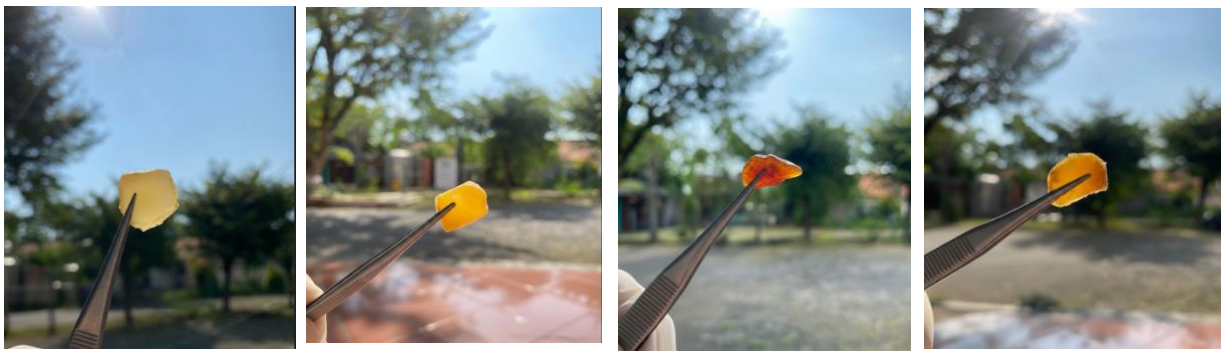
The macroscopic quality of the processed *fibroadenoma mammae* tissue was assessed using an ordinal scale (0–3) adapted from Bright et al. (2024) [3]The evaluation criteria included tissue shrinkage, transparency, color uniformity, consistency, and surface smoothness (Table 1). The highest score (3) indicates optimal tissue appearance with clear transparency, uniform coloration, and intact consistency, while a score of 0 reflects severe damage or poor morphological quality.

Table 1. Ordinal scoring rubric for macroscopic tissue morphology

No	Evaluation Criteria	Score			
		0	1	2	3
1	Tissue shrinkage	Severe shrinkage; tissue markedly smaller, wrinkled, distorted	Moderate shrinkage; smaller, wrinkled surface	Mild shrinkage; shape relatively maintained	No shrinkage; original size preserved
2	Tissue transparency	Completely opaque, non-translucent	Low transparency, slightly translucent but cloudy	Moderate transparency, internal details partly visible	High transparency, clear and well-defined structure
3	Color uniformity	Uneven color, many dark patches	Slightly uneven color, few patches	Fairly uniform color, minor variations	Highly uniform color, bright and patch-free
4	Tissue consistency	Extremely fragile, disintegrated tissue	Partially fragile, soft, with fragmented areas	Moderately firm, slightly fragile but processable	Intact, firm, and resilient tissue
5	Tissue surface	Severely irregular surface, torn or cracked	Slightly uneven, many wrinkles and irregularities	Fairly smooth surface, few wrinkles	Smooth, even surface without cracks or wrinkles

Macroscopic Comparison

The macroscopic evaluation of *fibroadenoma mammae* tissue processed with xylol, α-pinene, 1,8-cineole, and virgin coconut oil (VCO) revealed distinct differences in tissue morphology (Table 2). Tissues cleared with xylol, α-pinene, and VCO showed excellent macroscopic quality with no visible shrinkage, high transparency, uniform color, intact consistency, and smooth surface texture. In contrast, tissues processed with 1,8-cineole exhibited moderate shrinkage, lower transparency, and less color uniformity. The surface of cineole-cleared tissues appeared slightly irregular, and the overall consistency was softer compared to the other groups. Representative macroscopic appearances of the fibroadenoma samples processed with each clearing agent are shown in Figure 1.



(a) (b) (c) (d)

Figure 1. Macroscopic appearance of *fibroadenoma mammae* tissue processed with various clearing agents.
Description: (a) Xylol (control), (b) α-pinene, (c) 1,8-cineole, (d) Virgin Coconut Oil (VCO).

Macroscopic comparison of fibroadenoma mammae tissue showing differences in shrinkage, transparency, color uniformity, and surface texture among clearing agents.

Table 2. Macroscopic evaluation of *fibroadenoma mammae* tissue using different clearing agents

Evaluation Criteria	Mean Score ±SD			
	Xylol (control)	α-Pinene (*Pinus merkusii*)	1,8-Cineole (*Eucalyptus globulus*)	Virgin Coconut Oil (*Cocos nucifera* L.)
Tissue shrinkage	3.00±0.00	2.86±0.14	2.43±0.20	2.86±0.14
Transparency	3.00±0.00	3.00±0.00	2.14±0.14	3.00±0.00
Color uniformity	3.00±0.00	3.00±0.00	1.86±0.14	2.86±0.14
Tissue consistency	3.00±0.00	2.86±0.14	2.57±0.20	2.86±0.14
Surface smoothness	3.00±0.00	3.00±0.00	2.00±0.22	2.86±0.14

Values are presented as mean ± SD based on ordinal scoring (0–3). No statistical comparison is shown in this table; overall significance values are presented in Table 5.

The macroscopic observations showed that α-pinene and VCO achieved the most satisfactory clearing outcomes comparable to xylol. Both agents maintained high transparency and preserved the tissue's natural morphology, indicating efficient alcohol displacement and compatibility with paraffin infiltration. The superior performance of α-pinene may be attributed to its high refractive index (1.46–1.47 at 20 °C) and hydrophobic terpene composition, which facilitate optimal tissue clarification without inducing excessive hardening. These finding aligns with Bright et al. (2024), who reported that terpene-based agents such as α-pinene provide optical clarity similar to xylol with reduced toxicity [3]

Similarly, VCO, a lipid-based natural oil derived from *Cocos nucifera* L., demonstrated exceptional clearing potential. Its triglyceride composition enables gentle dehydration and reduces mechanical stress during processing, thereby preventing tissue shrinkage or cracking. The mild lipid-soluble property of VCO allows for slow and uniform penetration through the tissue matrix, minimizing distortion and maintaining elasticity, which explains the superior macroscopic integrity observed in this current investigation. These characteristics are consistent with the findings of Tian et al. (2021), who emphasized VCO's potential to maintain tissue pliability while ensuring sufficient transparency.[28] These characteristics are consistent with observations in the oral soft tissue specimens comparing coconut oil and xylol (Chandraker et al., 2019).[30]

Meanwhile, 1,8-cineole produced only moderate transparency and color uniformity, likely due to its lower viscosity and limited paraffin miscibility. These physicochemical properties may lead to incomplete replacement of ethanol residues, which can reduce light transmission and affect tissue consistency. Nevertheless these shortcomings, the low toxicity, pleasant odor, and biodegradability of 1,8-cineole still make it a promising biosafe alternative for laboratories aiming to minimize volatile organic solvent exposure. [29]

Comparisons in the literature support such differential clearing performance. Some studies comparing natural oils (coconut, cedarwood, limonene) found that coconut oil maintained high transparency and structural detail comparable to xylene, while more viscous oils or those with less favorable refractive indices underperformed. [31]Similarly, investigations of cedarwood oil as a clearing agent in oral tissues yielded good gross morphology and staining uniformity when compared with xylol, albeit requiring longer processing times.[32]

Collectively, the macroscopic findings indicate that α-pinene and virgin coconut oil (VCO) not only preserve tissue morphology with high fidelity but also serve as practical, lower-toxicity substitutes for conventional xylol in routine histopathological workflows. Beyond their comparable clearing performance, both agents offer distinct operational advantages, being readily available, cost-effective, and derived from renewable natural sources. These attributes make α-pinene and VCO particularly appealing for implementation in resource limited or high throughput laboratory settings, where maintaining quality, safety, and affordability are equally critical.

Microscopic Comparison

Following the macroscopic assessment, a comprehensive microscopic evaluation was conducted to further elucidate the histological integrity and staining performance of fibroadenoma mammae tissues processed with the various clearing agents. This microscopic examination provided a more nuanced appraisal of each agent's capacity to preserve fine cellular architecture, nuclear morphology, and chromatic definition under hematoxylin and eosin (H&E) staining.

The evaluation incorporated six standardized parameters: cellular outline, cytoplasmic detail, nuclear clarity, staining quality, optical clarity, and overall tissue integrity, each representing a critical determinant of histoprocessing fidelity. Together, these indices provided an integrated framework for assessing how effectively the clearing agents maintained both structural preservation and staining precision, thereby bridging gross morphological findings with microscopic evidence of tissue preservation.

Table 3. Ordinal Scoring Rubric for Microscopic Evaluation of Tissue Sections

No	Evaluation Criteria	Score			
		0	1	2	3
1	Cellular outline	Not visible	Partially visible, indistinct	Clearly visible in some areas	Very distinct and sharp throughout the section
2	Cytoplasmic detail	Not visible or very obscure	Partially visible, poor detail	Clear in most cells	Very clear with visible subcellular structures
3	Nuclear detail	Not visible or blurred	Partially visible, chromatin indistinct	Clear chromatin in some nuclei	Very clear with well-defined chromatin and nucleoli
4	Staining quality	Uneven staining, poor contrast	Fairly even staining with moderate contrast	Even staining, moderate clarity	Even staining with strong contrast
5	Clarity	Opaque, difficult to observe	Partly opaque, some details indistinct	Clear with slightly soft details	Very clear, all details easily observed
6	Tissue integrity	Severely damaged, torn	Partially damaged, some missing areas	Mostly intact with minor artifacts	Fully intact, overall structure well preserved

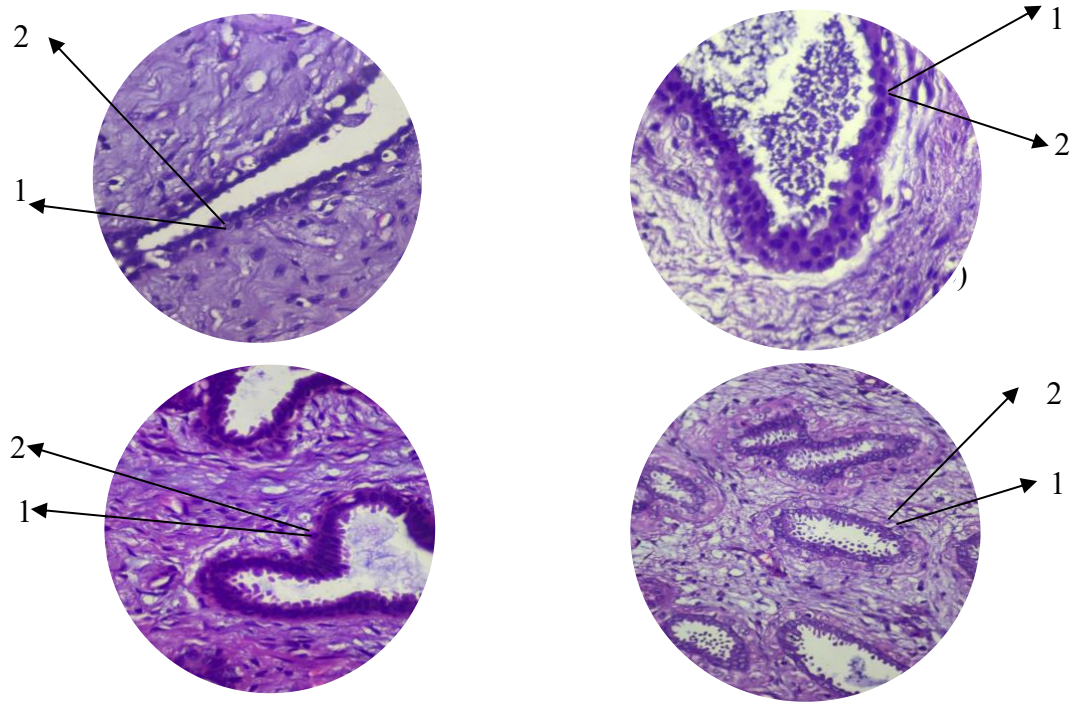


Figure 2. Microscopic observation of hematoxylin and eosin (H&E)–stained fibroadenoma mammae tissue at 400× magnification.
Description: (a) Xylol (control), (b) α-pinene, (c) 1,8-cineole, (d) Virgin Coconut Oil (VCO). Arrows indicate key morphological structures used for evaluation: (1) nucleus showing hematoxylin affinity and chromatin detail; (2) cytoplasm with eosin uptake illustrating clarity and definition.

Microscopic comparison of *fibroadenoma* mammae tissue processed with different clearing agents, showing variations in nuclear detail, cytoplasmic clarity, and staining uniformity among treatment groups.

Table 4. Comparison of Microscopic Quality of H&E-Stained Fibroadenoma Mammae Tissue Using Different Clearing Agents

Evaluation Criteria	**Mean Score ± SD**			
	Xylol (control)	α-Pinene (*Pinus merkusii*)	1,8-Cineole (*Eucalyptus globulus*)	Virgin Coconut Oil (*Cocos nucifera* L.)
Cellular outline	3.00±0.00	3.00±0.00	1.86±0.14	3.00±0.00
Cytoplasmic detail	3.00±0.00	2.86±0.14	1.71±0.18	3.00±0.00
Nuclear detail	3.00±0.00	3.00±0.00	1.43±0.20	3.00±0.00
Staining quality	3.00±0.00	3.00±0.00	2.00±0.00	3.00±0.00
Clarity	3.00±0.00	3.00±0.00	2.00±0.22	2.71±0.18
Tissue integrity	3.00±0.00	3.00±0.00	2.43±0.20	3.00±0.00

Values are presented as mean ± SD based on ordinal scoring (0–3). No statistical comparison is shown in this table; overall significance values are presented in Table 5.

The macroscopic observations revealed that α-pinene and VCO achieved the most satisfactory clearing results comparable to xylol. Both agents maintained high transparency and preserved the tissue's natural morphology, indicating efficient alcohol displacement and compatibility with paraffin infiltration. The high refractive index and hydrophobicity of α-pinene likely contributed to its ability to

enhance tissue clarity without excessive hardening. This finding aligns with Bright et al. (2024), who reported that terpene-based agents such as α-pinene provide optical clarity similar to xylol with reduced toxicity.

Integrated Analysis and Mechanistic Interpretation

Integration of macroscopic and microscopic scores provided a comprehensive view of each agent's performance (Table 5). Both α-pinene and VCO achieved total scores statistically similar to xylol ($p > 0.05$), whereas 1,8-cineole showed a significantly lower score ($p < 0.05$).

Table 5. Mean macroscopic and microscopic scores of fibroadenoma mammae tissue processed using various clearing agents

Clearing Agent	Mean Macroscopic Score ± SD	Mean Microscopic Score ± SD	Total Score	p-value vs Xylol
Xylol (control)	15.00 ± 0.00	18.00 ± 0.00	33.00 ± 0.00	–
α-Pinene (*Pinus merkusii*)	14.71 ± 0.29	17.86 ± 0.14	32.57 ± 0.30	0.784
1,8-Cineole (*Eucalyptus globulus*)	11.00 ± 0.31	11.43 ± 0.53	22.43 ± 0.65	0.000*
Virgin Coconut Oil (*Cocos nucifera* L.)	14.43 ± 0.20	17.71 ± 0.18	32.14 ± 0.26	0.453

* $p < 0.05$ indicates significant difference vs xylol

The integrated macroscopic and microscopic assessments revealed a clear hierarchy in clearing performance among the evaluated agents (Table 5). Both α-pinene and virgin coconut oil (VCO) achieved total scores of 32.57 ± 0.30 and 32.14 ± 0.26, respectively, which were statistically comparable to xylol ($p = 0.784$ and $p = 0.453$). These results indicate that both agents effectively preserved tissue morphology and staining fidelity at both gross and cellular levels. In contrast, 1,8-cineole yielded a markedly lower overall score (22.43 ± 0.65, $p < 0.05$), reflecting suboptimal clearing and reduced histological definition.

The p-values presented in Table 5 represent the degree of deviation from the xylol control. A non-significant value ($p > 0.05$) indicates comparable performance to xylol, demonstrating adequate preservation of transparency, color uniformity, and nuclear clarity. Conversely, a significant result ($p < 0.05$) denotes a measurable decline in one or more histological parameters. Accordingly, α-pinene and VCO demonstrate high compatibility with paraffin infiltration and superior tissue preservation, while offering added advantages of lower toxicity, wide availability, and reduced cost.

In contrast, the inferior performance of 1,8-cineole is likely due to its lower viscosity and limited paraffin miscibility, which hinder ethanol displacement and result in partial opacity and inconsistent staining.

When the macroscopic and microscopic findings are considered together, a clearer understanding of this investigation's significance emerges. At the gross level, α-pinene and VCO preserved tissue shape, transparency, and surface integrity, features essential for accurate orientation, cassette embedding, and microtomy.

Microscopically, both agents maintained distinct cellular boundaries, clear nuclear and cytoplasmic detail, uniform staining, and intact architecture comparable to xylol. The coherence between these two levels of assessment reinforces the conclusion that α-pinene and VCO provide a comprehensive and reliable replacement for xylol, ensuring structural fidelity from organ morphology down to the cellular level.

Furthermore, the strong correspondence between macroscopic and microscopic outcomes validates the integrated ordinal scoring framework employed in this research. By linking macroscopic cues (e.g., transparency, shrinkage, surface smoothness) with microscopic indicators (e.g., nuclear and cytoplasmic definition), this approach demonstrates that gross tissue characteristics can predict microstructural preservation, offering a practical model for future comparative histotechnology studies.

Beyond methodological value, the practical advantages of α-pinene and VCO enhance their applicability across diverse laboratory settings. Their renewable natural sources, affordability, and accessibility make them particularly suitable for low- and middle-income regions, where reducing toxic solvent exposure without compromising diagnostic quality remains a key challenge.

In contrast, the limitations observed with 1,8-cineole highlight the need to balance physicochemical factors such as viscosity and paraffin solubility when selecting eco-friendly xylol substitutes.

Nevertheless, this investigation has limitations. The descriptive experimental design and the absence of quantitative measurements, such as refractive index or chemical interaction analyses, limit the depth of mechanistic interpretation.

Future studies incorporating spectroscopic, colorimetric, or image-based quantification are recommended to further validate these findings and elucidate the optical mechanisms of natural solvents in paraffin infiltration.

In addition, assessing the long-term stability and archival quality of tissues processed with α-pinene and VCO will be essential to confirm their feasibility for routine diagnostic and research applications.

CONCLUSION

The current investigation shows that α-pinene and virgin coconut oil (VCO) can effectively replace xylol as clearing agents in the histopathological processing of fibroadenoma mammae tissue.

Both agents preserved tissue morphology, nuclear and cytoplasmic detail, and staining quality comparable to xylol, with total scores of 32.57 ± 0.30 ($p = 0.784$) for α-pinene and 32.14 ± 0.26 ($p = 0.453$), showing no significant difference from the control (33.00 ± 0.00).In contrast, 1,8-cineole showed a significantly lower performance (22.43 ± 0.65, $p < 0.05$) due to limited paraffin miscibility and lower clearing strength.

Beyond their histological equivalence to xylol, both α-pinene and VCO offer practical advantages due to their low toxicity, affordability, and local availability, making them viable for implementation in diagnostic and teaching laboratories in resource-limited settings

AUTHORS' CONTRIBUTIONS

Wimpy: Conceptualization, Methodology, Supervision, Project administration, Writing Original Draft, Writing, Review & Editing, Funding acquisition
Endang Widhiyastuti: Validation, Resources, Writing, Review & Editing
Dr. Astri Aditya Wardhani: Investigation, Validation, Writing, Review & Editing
Eva Ocha Pratama: Investigation, Data curation, Visualization
Khoirunisa Saputri: Investigation, Data curation

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DATA AVAILABILITY STATEMENT

The data supporting the findings of this present work are available from the corresponding author upon reasonable request. The dataset may also be shared with the journal and made accessible to the broader research community to ensure transparency and reproducibility

DISCLOSURE STATEMENT

The views and opinions expressed in this article are solely those of the authors and do not necessarily reflect the official policies or positions of any affiliated institution or funding agency. All data presented represent the original findings of the authors and have not been previously published or submitted elsewhere.

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