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Eucalyptus Oil And Telon Oil As A Substitute For Xylene (Clearing Agent) Using Mice Liver And Kidney Samples With A Soaking Time Of 45 Minutes.

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Abstract

Xylene has long been recognized as the standard clearing agent in histopathological tissue processing due to its effectiveness in removing alcohol and promoting paraffin infiltration. Nevertheless, its toxic properties and potential health risks have encouraged the development of

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safer alternative clearing agents. This study investigated the potential use of cajuput oil and telon oil as substitutes for xylene in processing mouse liver and kidney tissues with a 45-minute clearing period. A true experimental laboratory design was applied, involving three groups: xylene as the control, cajuput oil, and telon oil as the experimental treatments. All tissue samples underwent identical fixation, dehydration, paraffin embedding, sectioning, and Hematoxylin-Eosin staining procedures. Histological quality was evaluated microscopically by assessing nuclear clarity, cytoplasmic staining, and staining uniformity. The findings showed that xylene produced the best overall tissue morphology and staining quality. Cajuput oil yielded satisfactory histological features with relatively clear nuclei and cytoplasm, whereas telon oil demonstrated lower staining performance, particularly in kidney tissues. These results indicate that cajuput oil may serve as a promising and safer alternative to xylene, while further optimization is required before telon oil can be considered for routine histopathological tissue processing.

Keywords: Cajuput oil; Telon oil; clearing agent; Histopathology

INTRODUCTION

Histopathology is an important method in the medical field for evaluating changes in tissue structure through microscopic slides. The quality of histological slides is greatly influenced by each stage of the preparation process, from fixation, dehydration, clearing, impregnation, embedding, sectioning, and staining. One crucial stage is tissue clearing, which involves removing alcohol from the tissue and replacing it with a paraffin-compatible material so that the tissue can be processed to the next stage. In histotechnical practice, xylene remains the most commonly used clearing agent due to its ability to dissolve alcohol and facilitate good paraffin penetration. However, the continued use of xylene can cause several problems due to its toxic, volatile nature, and the potential to cause respiratory and skin irritation in laboratory personnel.

As research into safer alternatives advances, several natural oil-based materials are being considered as alternatives to xylene. Essential oils contain nonpolar compounds that allow them to interact with lipid components of tissue and have the ability to dissolve certain substances needed in the clearing process. Eucalyptus oil (cajuput oil) and telon oil are readily available and contain terpenoid compounds, which have lipophilic properties, making them potential alternative clearing agents.

Previous research has shown that the use of alternative oil-based materials has been widely studied in histotechnical processes. Research by Metwally et al. (2020) reported that

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several natural materials can be used as alternative clearing agents because they are able to produce histological slide quality that approaches the use of xylene with a better level of safety. Furthermore, research on natural materials as xylene substitutes also shows that factors such as tissue type, soaking time, and clearing agent characteristics can affect the final slide quality, particularly tissue clarity, hematoxylin-eosin staining quality, and the presence of artifacts in the slide.

Although research into xylene alternatives has been growing, most previous studies have used specific alternatives, such as mineral oil or other essential oils, with varying clearing times. Research on the use of eucalyptus oil and telon oil as clearing agents in liver and kidney tissue in mice with a 45-minute immersion time is still limited. Therefore, this study was conducted to determine the ability of eucalyptus oil and telon oil as xylene substitutes in tissue clearing with the same treatment time.

Based on the description, the problem formulation in this study is whether eucalyptus oil and telon oil can be used as a substitute for xylene as a clearing agent in the preparation of histological preparations of mouse liver and kidney with a soaking time of 45 minutes. The hypothesis of this study is that the use of eucalyptus oil and telon oil as alternative clearing agents can produce quality histological preparations of mouse liver and kidney that are comparable to the use of xylene based on the quality of staining and clarity of the preparation.

The purpose of this study was to determine the effectiveness of eucalyptus oil and telon oil as a substitute for xylene in the clearing process of liver and kidney histology preparations of mice with a 45-minute immersion time and to compare the quality of the resulting preparations between the control group using xylene and the treatment group using eucalyptus oil and telon oil. In this study, the control group used standard laboratory xylene, while the experimental group used eucalyptus oil and telon oil as alternative clearing agents. The clearing process was carried out for 45 minutes without changing the container for each treatment.

METHOD

This study used a true experimental laboratory design with a quantitative approach to evaluate the effectiveness and functionality of natural ingredients as alternative clearing agents in the preparation of histological preparations. This experiment compared the use of three types of clearing agents: xylene as a positive control (gold standard), cajuput oil, and telon oil as treatment/experimental groups. The independent variables in this study were the type of clearing agent with a constant soaking duration of 45 minutes. Meanwhile, the objectively measured

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dependent variables included the quality of microanatomic visualization of the tissue preparations, which was assessed by the level of nuclear clarity, cytoplasm clarity, and uniformity of Hematoxylin-Eosin staining intensity (Giri et al. (2022).

The objects or samples in this study used the liver and kidney organs from healthy male mice (Mus musculus) with inclusion criteria of body weight ranging between 25-30 grams and 2-3 months of age. Organ sampling was carried out after euthanasia of the test animals in accordance with ethical guidelines for laboratory experiments. The sampling technique used was purposive sampling to ensure that the organs taken were in normal condition, free from macroscopic lesions, and representative for histopathological analysis. From the obtained liver and kidney organs, macroscopic cutting was carried out to produce several homogeneous tissue fragments that were randomly distributed into the control group (xylene) and treatment groups (eucalyptus oil and telon oil).

Data collection techniques were conducted through a series of microtechnical laboratory experiments that adhered to the standard operating procedures of anatomical pathology laboratories and adjusted to the optimized immersion time of 45 minutes (Suvarna et al., 2022). The stages of histology preparation were carried out sequentially and precisely as follows:

1. **Tissue Fixation:**Immediately after organ isolation, tissue fragments were immersed in 10% Neutral Buffered Formalin (NBF) solution. The 10% NBF composition was prepared by dissolving 6.5g of Disodium phosphate and 4.0g of Monosodium phosphate in 100ml of 40% Formaldehyde, then diluting with distilled water until the volume reached 1000ml (balanced pH ~7.0). The ratio of tissue volume to fixative solution was strictly maintained at a minimum ratio of 1:10 to ensure complete penetration of protein cross-linking. The tissue was left in the fixative solution for 24 hours to prevent autolysis and post-mortem degradation.
2. **Macroscopic Cutting (Grossing):**The dimensions of the fixed organ fragments (length, width, and height) were then measured using a ruler. The tissue was neatly sectioned to a maximum thickness of 0.5 cm (total dimensions adjusted to the capacity of the 2 x 3 cm tissue cassette) using a sharp scalpel to avoid mechanical damage (crush artifact). Representative tissue sections were then placed into tissue cassettes labeled with a special pencil code.
3. **Tissue Processing:**This process includes three crucial sub-stages in sequence:
   A. Dehydration: Removing water content from tissue using a graded alcohol series to prevent extreme cellular shrinkage. Tissue tapes are immersed successively in 70%

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Alcohol (I), 70% Alcohol (II), 80% Alcohol, 85% Alcohol, 90% Alcohol, 96% Alcohol (I), and 96% Alcohol (II) for 60 minutes each.

B. Clearing: Removing alcohol from the tissue and replacing it with a paraffin-compatible material. At this stage, the samples were divided into three treatment groups with a constant soaking duration of 45 minutes without changing the container: Group I (pure Xylene as a control), Group II (pure Eucalyptus Oil rich in the monoterpene compound 1,8-cineole), and Group III (commercial Telon Oil which is a mixture of eucalyptus oil, fennel oil, and coconut oil).
C. Infiltration/Impregnation: Removing the clarifying agent from the tissue and replacing it with paraffin. The tissue is immersed in three consecutive baths of molten paraffin (Paraffin 1, Paraffin 2, and Paraffin 3) in an oven maintained at a temperature of 60°C for 60 minutes each.

4. **Rough and Fine Cutting (Trimming and Cutting):**The paraffin block is firmly mounted in the rotary microtome holder. The initial stage involves trimming (rough cutting) at a thickness of 5–10 µm until the tissue surface is completely exposed. Next, rhythmic cutting (fine cutting) is performed to an ideal thickness of 3–5 µm.µmto obtain a continuous single-layer tissue strip. The paraffin strip was carefully transferred using a brush into a water bath containing warm water (temperature 50–56(µm)so that it expands perfectly without creases. The tissue tape is then attached (mounted) to a clean, grease-free glass object.(Shrestha et al., 2024).
5. **Hematoxylin-Eosin (HE) Staining:**The object glass is deparaffinized with a hotplate and then immersed in a xylene solution.(I), xylol (II) with 5 minutes each,followed by rehydration through a series of decreasing alcohols (absolute to 50%) for 1 minute each, then rinsed with running water. The preparation was stained with Harris Hematoxylin for 3 minutes to stain the nucleus, then rinsed with running water, differentiated with acid alcohol for 1 minute, rinsed, and continued with counterstaining using Eosin for 1 minute to stain the cytoplasm. After being rehydrated through 96% alcohol for 2 minutes and final clearing with xylol (I, II, III) 1 minute, then the preparation is driedand covered with a cover glass using entelan (mounting agent) precisely and free of air bubbles.

After the entire staining sequence was completed, the liver and kidney histological slides were observed under a light microscope. Observations began with low magnification (100x) for

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general field assessment, followed by high magnification (400x) for specific cellular microanatomy evaluation. To avoid subjective bias and maintain the validity of the data obtained, the entire visual observation process was conducted in a blinded manner by two experts (biomedical/pathologists) independently (inter-observer variability).

The accuracy of the assessed microanatomy visualization results is inseparable from the quality of the biological samples obtained from the beginning of maintenance. Therefore, all procedures for handling, care, and euthanasia of mice (Mus musculus) in this study were carried out in accordance with animal welfare principles to minimize pain. The entire series of experiments has also been legally reviewed and received ethical approval from the Health Research Ethics Committee of Pelita Harapan University with Ethics Registration Number: 0071/PE.KEPK-FIKes-UPH/VI/2026.

RESULTS AND DISCUSSION

Results and Discussion 1

Functional assessment of the effectiveness of alternative clearing agents is measured objectively through the quality of microanatomical visualization of preparations stained with Hematoxylin-Eosin (HE). The standard for the success of this differential staining rests on its ability to express the details of cellular architecture in sample organs, which in this study focused on histopathological preparations of the liver and kidneys of mice (Mus musculus). Microscopic evaluation parameters were carried out comparatively by observing the sharpness of cell nucleus visualization, the level of cytoplasmic color brilliance, and the homogeneity of dye absorption throughout the tissue field of view. The viewing criteria are presented in the table below:

Table 1. Results of Evaluation of Histological Quality and Clearing Agent Effectiveness

No	Criteria	Description	Quality
1.	Cell Nucleus	There is no clear blue color in the cell nucleus.	Not good
		The blue color is less clear on cell nucleus	Good
		The blue color is clearly visible on cell nucleus	Very good
2.	Cytoplasm	The red color is not clearly visible young in the cytoplasm	Not good

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No	Criteria	Description	Quality
		Less clear pink color in the cytoplasm	Good
		The pink color is clearly visible in the cytoplasm	Very good
3.	Color uniformity	Staining of the preparation is not uniform	Not good
		Insufficient staining of the preparation uniform	Good
		Uniform staining of the preparation	Very good

(Source:(Sofyanita et al., 2022), data processed)

The quality of histological preparations in this study was evaluated through microscopic observation of liver and kidney tissues from mice (Mus musculus) processed using three types of clearing agents: xylene as a control, eucalyptus oil, and telon oil with a soaking time of 45 minutes. All preparations were processed using the same histotechnical procedure and stained using the Hematoxylin-Eosin (HE) method. Microscopic evaluation was performed to assess the quality of the preparations based on the parameters of nuclear clarity, cytoplasmic clarity, uniformity of staining intensity, and microscopic appearance of the tissue. The results showed that all preparations maintained the basic morphology of the tissue so that the liver and kidney structures were still recognizable. However, there were variations in the quality of the preparations in each clearing agent group, especially in nuclear clarity, cytoplasmic staining intensity, and uniformity of color distribution. The data from the histological quality evaluation are summarized comparatively in Table 2.

Table 2. Results of Evaluation of Histological Quality and Clearing Agent Effectiveness

Types of Organs	Clearing Agent (45 Minutes)	Quality	Core Clarity f (%)	Cytoplasm Clarity f (%)	Color Uniformity f (%)
Liver(n=12)	**Xylene**(Control)	Not good	0 ()0,0%	0 ()0,0%	0 ()0,0%
		Good	2 (16.6)%	2 (16.6)%	2 (16.6)%
		Very good	10 ()83,3%	10 ()83,3%	10 ()83,3%

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Types of Organs	Clearing Agent (45 Minutes)	Quality	Core Clarity f (%)	Cytoplasm Clarity f (%)	Color Uniformity f (%)
	Eucalyptus oil	Not good	0 ()0,0%	0 ()0,0%	0 ()0,0%
		Good	4 (33.3)%	4 (33.3)%	4 (33.3)%
		Very good	8 ()66,6%	8 ()66,6%	8 ()66,6%
	Telon Oil	Not good	0 ()0,0%	2 ()16,6%	2 ()16,6%
		Good	6 (50)%	4 ()33,3%	4 ()33,3%
		Very good	6 ()50%	6 (50)%	6 (50)%
Kidney (n=12)	**Xylene**(Control)	Not good	0 ()0,0%	0 ()0,0%	0 ()0,0%
		Good	2 (16.6)%	2 (16.6)%	2 (16.6)%
		Very good	10 ()83,3%	10 ()83,3%	10 ()83,3%
	Eucalyptus oil	Not good	0 ()0,0%	0 ()0,0%	0 ()0,0%
		Good	4 (33.3)%	4 (33.3)%	4 (33.3)%
		Very good	8 ()66,6%	8 ()66,6%	8 ()66,6%
	Telon Oil	Not good	1 (8.3)%	1 (8.3)%	1 (8.3)%
		Good	4 (33.3)%	4 (33.3)%	4 (33.3)%
		Very good	7 (58.3)%	7 (58.3)%	7 (58.3)%
Total			100%	100%	100%

(Source: Author, 2026, processed data)

Information :$Persentase\ (\%) = \frac{Jumlah\ data\ per\ kategori}{Total\ jumlah\ preparat\ yang\ diamati} x100\%$

Results and Discussion 2

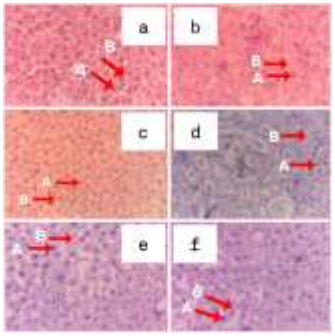


Figure 1.Microscopic image of liver and kidney tissue of mice (Mus musculus) with

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Hematoxylin-Eosin (HE) staining after the clearing process using xylene, eucalyptus oil, and telon oil for 45 minutes.

(Source: Author, 2026, processed data)

Image Caption 1. Microscopic images of liver and kidney tissue preparations of mice (Mus musculus) after the clearing process using xylene, eucalyptus oil, and telon oil with a soaking time of 45 minutes and Hematoxylin-Eosin (HE) staining. (a) Kidney–Xylene; (b) Liver–Xylene; (c) Liver–Eucalyptus Oil; (d) Kidney–Telon Oil; (e) Kidney–Eucalyptus Oil; (f) Liver–Telon Oil. The letter A indicates the nucleus, while the letter B indicates the cytoplasm. Microscope magnification: ×400 (adjust to the magnification used).

The success of the clearing process is one of the factors that determine the quality of histological preparations, because this stage serves to replace the alcohol in the tissue with a substance compatible with the paraffin infiltration medium. An optimal clearing process will result in homogeneous paraffin infiltration so that the tissue structure, especially the nucleus, is maintained during the cutting and staining process. In Hematoxylin-Eosin (HE) staining, the nucleus has a high affinity for hematoxylin so it will appear blue to purplish blue with clear nuclear boundaries if the preparation is of good quality (Bancroft & Gamble, 2020).

Based on the observations in Figure 1, the preparations processed using xylene as a clearing agent produced the best nuclear clarity compared to the other treatment groups. In the kidney preparation (Figure 1a), the nuclei of the tubular epithelial cells appeared purplish blue with distinct nuclear boundaries and were evenly distributed following the arrangement of the tubules. Similarly, in the liver preparation (Figure 1b), the hepatocyte nuclei still appeared intact with a relatively uniform distribution so that the contrast between the nucleus and cytoplasm could be clearly observed. These findings indicate that the use of xylene is still capable of producing preparations with optimal nuclear morphology quality.

The excellent nuclear staining quality of the xylene group is related to xylene's characteristics as a nonpolar organic solvent with a high capacity to replace alcohol after dehydration. Furthermore, xylene has excellent compatibility with paraffin, allowing for a more homogeneous infiltration process. Optimal paraffin infiltration maintains the chromatin structure and nuclear membrane during microtome sectioning, thus maintaining hematoxylin's affinity for the cell nucleus. Therefore, xylene remains the gold standard for histological tissue clearing (Carson & Hladik, 2021).

The results of this study are consistent with those of Sofyanita et al. (2022) who reported that mouse tissue preparations processed using xylene produced excellent nuclear staining quality

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with clear nuclear boundaries and optimal contrast to the cytoplasm. The study also stated that the control group using xylene provided consistent preparation quality, making it useful as a comparison in evaluating various alternative clearing agents. Similar results were also reported by Lamsudiansyah et al. (2023) who compared the use of cinnamon leaf oil with xylene as a clearing agent. In that study, the xylene group showed clear, homogeneous nuclear staining and no significant morphological changes.

In contrast to the xylene group, the preparations processed with eucalyptus oil (Figures 1c and 1e) still showed clearly observable nuclei in both liver and kidney tissue. Nuclear boundaries remained distinct with relatively good hematoxylin staining intensity, although in some areas the contrast appeared slightly lower than in the control group. Nevertheless, the nuclear structure was still easily recognizable, allowing for morphological identification of the tissue without significant difficulty. These findings indicate that eucalyptus oil is capable of maintaining tissue integrity during the clearing process, although its effectiveness is not yet fully comparable to that of xylene.

The ability of eucalyptus oil to maintain the quality of the nucleus is thought to be related to its main content, 1,8-cineole (eucalyptol), which is the dominant component of eucalyptus oil. This compound belongs to the monoterpene group and is lipophilic, allowing it to dissolve lipid components in tissue and replace alcohol after dehydration. In addition to 1,8-cineole, eucalyptus oil also contains α-pinene, limonene, and α-terpineol, which contribute to the oil's penetration into tissue.(Novi Yuniar et al., 2024)This combination of compounds allows the paraffin infiltration process to continue, allowing the nuclear structure to maintain its affinity for hematoxylin. This finding is supported by research by Lamsudiansyah et al. (2023), who reported that essential oils rich in terpenoid compounds can produce nuclear staining quality close to that of the xylol group, thus potentially being used as an alternative clearing agent.

Meanwhile, preparations processed using telon oil (Figures 1d and 1f) exhibited varying results across organs. In liver tissue (Figure 1f), hepatocyte nuclear clarity was observed to be intact and stable in the "Good" category. However, a significant decrease in nuclear contrast quality occurred in kidney tissue (Figure 1d), which fell into the "Poor" category, where nuclear boundaries in some areas appeared blurred due to decreased hematoxylin staining intensity. This decrease in nuclear contrast quality in the telon oil treatment stems from the physical characteristics of the mixture formulation contained in the telon oil. The composition of telon oil includes eucalyptus oil (Oleum cajuputi), fennel (Oleum foeniculi), and coconut oil (Oleum cocos) (Sari et al., 2021).

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The long-chain triglyceride content of coconut oil causes telon oil to have a significantly higher viscosity than pure eucalyptus oil. This high viscosity slows fluid penetration into the tissue, thus inhibiting maximum hematoxylin absorption and reducing the visual contrast of the nuclei in some sections. In addition to nuclear clarity, another equally important parameter in assessing the quality of histological slides is cytoplasmic clarity. This parameter relates to the tissue's ability to optimally absorb eosin after the clearing process, allowing for clear contrast between the cytoplasm and nucleus in hematoxylin-eosin (HE) stained slides.

Based on the observations in Figure 1, the preparations processed using xylene showed more optimal cytoplasmic clarity compared to the eucalyptus oil and telon oil groups. In kidney tissue, the cytoplasm of tubular epithelial cells appeared pink with even eosin intensity, allowing for clear cell boundaries. Meanwhile, in liver tissue, the cytoplasm of hepatocytes showed homogeneous pink staining with good contrast to the nucleus. This condition indicates that the xylene group was able to produce a balance of staining between the nuclear and cytoplasmic components in Hematoxylin-Eosin (HE) staining. Cytoplasmic clarity in histological preparations is primarily influenced by the tissue's ability to accept eosin staining. Eosin is an acidic dye that binds to basic cytoplasmic components, particularly cytoplasmic proteins, resulting in a pink color in the cytoplasm. In the xylene group, the more even distribution of eosin indicates that the tissue structure was well maintained during the preparation process, allowing optimal staining of the cytoplasmic components (Bancroft & Gamble, 2020).

The results of this study align with those of Sofyanita et al. (2022) who reported that mouse tissue preparations cleared with xylene produced a homogeneous distribution of cytoplasmic staining and good contrast between the nucleus and cytoplasm. Furthermore, research by Lamsudiansyah et al. (2023) showed that xylene as a control still provided the best cytoplasmic staining quality compared to several essential oil-based alternatives.

In the eucalyptus oil group, the cytoplasm of both liver and kidney tissues still showed clear eosin staining, although the color intensity was slightly lower than in the xylene group. The boundary between the cytoplasm and the nucleus was still observable, allowing for proper identification of tissue structures. These results indicate that the use of eucalyptus oil maintained cytoplasmic staining quality close to that of standard materials. Eucalyptus oil's ability to produce adequate cytoplasmic staining is thought to be related to its ability to maintain tissue integrity during processing. Terpenoid compounds, particularly 1,8-cineole (eucalyptol), have the potential to support the clearing process due to their nonpolar nature, which allows interaction with specific tissue components. This allows for continued paraffin infiltration, preventing significant damage

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to the cytoplasmic structure during preparation.

In contrast, a significant decrease in cytoplasmic staining and color uniformity was observed in the telon oil group. In the hepatic stroma (Figure 1f) and kidneys (Figure 1d), cytoplasmic color intensity tended to weaken and drop to the "Poor" level, characterized by a pale pink color and less homogeneous color distribution in some areas of the visual field. This difference in visual quality is directly correlated with the telon oil formula, which uses a viscous carrier. This mixture composition can affect the material's ability to clear, resulting in less than optimal replacement of residual components from the previous stage with xylene or eucalyptus oil. The imperfection in the removal of residual alcohol components from the previous dehydration stage leaves hydrophobic barriers in the cytoplasm, preventing the eosin from being evenly distributed.

The variation in microscopic quality of the final results in telon oil treatment, where the liver organ still recorded a "Good" nuclear clarity value while the kidney tissue decreased in all parameters to "Poor" proves the influence of differences in absorption capacity based on the microanatomical characteristics of the target organ. Kidney tissue histologically has a much higher level of stromal density compared to liver tissue. The kidney architecture is tightly composed of millions of complex tubule systems and renal corpuscles that are closely packed together, which creates greater spatial resistance to external fluids. When a substance with a high viscosity such as telon oil is applied, the dense saturated fatty acid molecules within it experience massive diffusion resistance to be able to pass through the narrow gaps of the kidney stroma within 45 minutes. In contrast, the liver stroma has a relatively more permeable structure because it is surrounded by a network of sinusoidal blood vessels that loosen its cellular density, allowing telon oil molecules to partially penetrate and maintain the quality of the cell nucleus. This phenomenon confirms that the viscosity factor of alternative clearing agents has a much more destructive negative domino effect when applied to organs with dense connective tissue and stroma such as the kidneys.

CONCLUSION

Based on the research results, eucalyptus oil has strong potential as an alternative clearing agent to xylene because it is safer for health. With a 45-minute soaking time, eucalyptus oil was able to produce clear microanatomical visualization of liver and kidney tissue in mice, where the structure of the cell nucleus and cytoplasm remained well preserved. On the other hand, xylene still provided the most optimal staining and morphological results as the main standard (gold

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standard). Meanwhile, telon oil showed low effectiveness, especially in dense tissues such as the kidney, due to viscosity constraints. Thus, eucalyptus oil can be recommended as an alternative to xylene in histopathology laboratories, while telon oil cannot yet be used routinely and still requires further optimization.

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