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Evaluation of the Potential of Kiat-Kiat (Citrus Reticulata) Oil D-Limonene as Deparaffinization Agent in Histopathological Preparations

Carcueva, Ed Francis[1], Balucas, Casseopeia Alexandria[2], Pepito, Jeldine[3], Oflas, Melogen II[4], Rodriguez, Rafael Niño[5], De Lara, Sachi Xandria*[6]

[1-6]University of Cebu-Banilad, Gov. M. Cuenco Ave, Banilad, Cebu City, 6000 Cebu, Philippines

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Corresponding Author:
De Lara, Sachi Xandria

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ABSTRACT

This study evaluates the potential of kiat-kiat (Citrus reticulata) peel–derived oil D-limonene as a potential sustainable deparaffinizing substitute to xylene. Specifically, it compared the macroscopic and microscopic performance of Solution 1 (oil D-limonene mixed with 90% ethanol and Polysorbate 80), pure oil D-limonene extract, cedarwood oil (commercial xylene alternative), and xylene (positive control). Macroscopic evaluation included deparaffinization capacity, ease of wax removal, and macroscopic artifacts, while microscopic evaluation assessed nuclear staining, cytoplasmic staining, and clarity of staining. Oil extraction was performed using absolute ethanol and rotary evaporation, and the extract was applied to pig liver tissues processed using Hematoxylin and Eosin (H&E) staining. Data were obtained from three clinical/anatomical pathologists and three registered medical technologists at the University of Cebu Medical Center. Statistical analysis was conducted using IBM SPSS Statistics with the Kruskal-Wallis test and post hoc analysis. Results showed that xylene and cedarwood oil consistently achieved the highest macroscopic (mean rank: xylene = 40.50; cedarwood oil = 45.50) and microscopic quality (mean rank: xylene = 40.50; cedarwood oil = 40.50). Pure oil D-limonene and Solution 1 demonstrated moderate potential, with better microscopic performance for D-limonene (mean rank = 25.50) and better macroscopic performance for Solution 1 (mean rank = 25.50). However, both were inferior to xylene and cedarwood oil in wax removal and artifact reduction. The study concludes that kiat-kiat-derived D-limonene shows limited but observable potential as a natural deparaffinizing agent but requires further optimization. Future studies should focus on formulation refinement and improved extraction methods.

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1.0 INTRODUCTION

Histopathology is essential in disease diagnosis through microscopic examination of tissue structures. A critical preparatory step is deparaffinization, where paraffin wax is removed to allow proper staining and visualization of cellular components [1]. Traditionally, xylene is used due to its efficiency in wax dissolution [2]. However, it poses significant health and environmental hazards, including neurological and respiratory toxicity with prolonged exposure [3]. These concerns have prompted growing interest in safer, eco-friendly alternatives consistent with Green Chemistry principles [4].

Citrus peels, particularly from kiat-kiat (*Citrus reticulata*), are rich sources of D-limonene, a nonpolar monoterpene capable of dissolving paraffin wax through hydrophobic interactions [5]. Studies have shown that D-limonene not only effectively removes paraffin but also preserves tissue morphology and provides improved safety profiles compared to xylene [6]. Its biodegradability, low toxicity, and compatibility with histological processes make it a promising alternative solvent.

Commercial substitutes such as CitriSolv have also demonstrated effectiveness in deparaffinization due to high D-limonene content but are limited by availability, cost, and potential staining inconsistencies [7]. Similarly, cedarwood oil has been reported to gently clear paraffin while preserving tissue structure, though it requires longer processing time and higher cost [8]. Despite extensive research on D-limonene, limited studies have explored kiat-kiat-derived oil specifically as a histopathological deparaffinizing agent. Given the abundance of Citrus reticulata in the Philippines and its high D-limonene content, this study investigates its potential as a locally sourced, sustainable alternative to xylene.

The study aimed to evaluate the potential of D-limonene, a constituent of kiat-kiat (*Citrus reticulata*) oil, at room temperature as a deparaffinization agent for Hematoxylin and Eosin [H&E] staining in histopathological preparations. Specifically, this study sought to do the following:

1. To evaluate and compare the macroscopic effects of oil D-limonene mixed with 90% ethanol and Polysorbate 80 (Solution 1), oil D-limonene extract, cedarwood oil, and xylene as deparaffinization agents in Hematoxylin and Eosin [H&E] stained liver tissue, focusing on:
   - 1.1. deparaffinization capacity;
   - 1.2. ease of paraffin wax removal; and
   - 1.3. macroscopic artifacts?
2. To evaluate and compare the microscopic effects of oil D-limonene mixed with 90% ethanol and Polysorbate 80 (Solution 1), oil D-limonene extract, Cedarwood oil, and xylene as a deparaffinization agent in Hematoxylin and Eosin [H&E] stained liver tissue in terms of:
   - 2.1. nuclear staining;
   - 2.2. cytoplasmic staining; and
   - 2.3. clarity of staining?
3. To determine if there is a significant difference between oil D-limonene mixed with 90% ethanol and Polysorbate 80 (Solution 1), oil D-limonene extract, Cedarwood oil, and xylene as a deparaffinization agent on liver tissue in its macroscopic and microscopic evaluation.
4. To determine if there is a significant difference between oil D-limonene mixed with 90% ethanol and Polysorbate 80 (Solution 1), oil D-limonene extract, Cedarwood oil, and xylene in terms of deparaffinization capacity, ease of paraffin wax removal, and macroscopic artifacts in Hematoxylin and Eosin [H&E] staining.

Hypothesis of the Study

The following hypotheses were formulated for this study:

H_{01}: There was no significant difference in the macroscopic effects—including deparaffinization capacity, ease of paraffin wax removal and macroscopic artifacts —among oil D-limonene mixed with 90% ethanol and Polysorbate 80 (Solution 1), oil D-limonene extract, cedarwood oil, and xylene as deparaffinization agents in Hematoxylin and Eosin [H&E] stained liver tissue.

H_{02}: There was no significant difference in the microscopic effects — nuclear staining, cytoplasmic staining, and clarity of staining — among oil D-limonene mixed with 90% ethanol and Polysorbate 80 (Solution 1), oil D-limonene extract, cedarwood oil, and xylene as deparaffinization agents in Hematoxylin and Eosin [H&E] stained liver tissue.

H_{03}: There was no significant difference in the overall macroscopic and microscopic evaluations of liver tissue deparaffinized using oil D-limonene mixed with 90% ethanol and Polysorbate 80 (Solution 1), oil D-limonene extract, cedarwood oil, and xylene.

H_{04}: There was no significant difference among oil D-limonene mixed with 90% ethanol and Polysorbate 80 (Solution 1), oil D-limonene extract, cedarwood oil, and xylene in terms of deparaffinization capacity, ease of paraffin wax removal, and macroscopic artifacts in Hematoxylin and Eosin [H&E] stained liver tissue.

2.0 MATERIALS AND METHODS

This study utilized a comparative experimental research design. The performance of kiat-kiat (*Citrus reticulata*) oil D-limonene as a deparaffinization agent was compared to that of xylene and cedarwood oil. Researchers assessed processed pig liver tissue based on six criteria: deparaffinization capacity, ease of paraffin wax removal, macroscopic artifacts, nuclear staining, cytoplasmic staining, and clarity of staining.

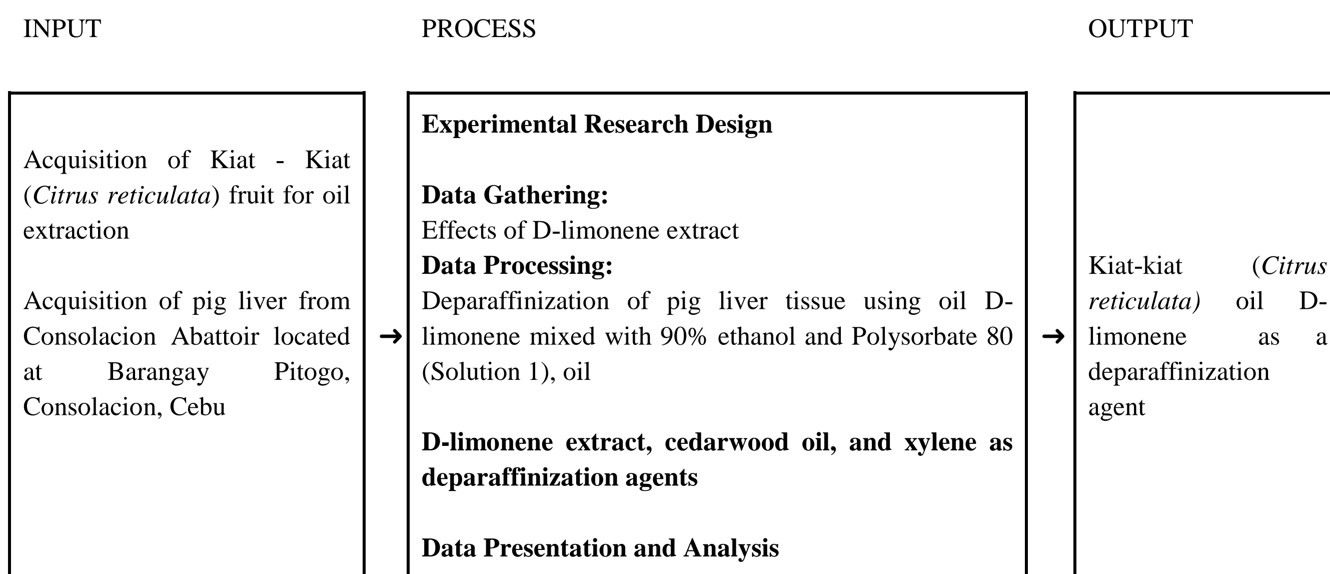

Figure 1. Research Flow Process

The research was conducted in multiple locations, with tissue processing primarily performed at the University of Cebu Medical Center. *Citrus reticulata* peels were obtained from a local farm in Danao, Cebu, and their authenticity was verified by the Department of Agriculture – Regional Field Unit VII. The peels were immersed in 100% ethanol, and rotary evaporation was used to remove the solvent and concentrate the extracted oil, which was carried out at the Physiology Laboratory of Southwestern University PHINMA. After extraction, gas chromatography analysis was performed, with samples sent to Philippine Institute of Pure and Applied Chemistry [PIPAC] to validate and quantify the D-limonene content. Pig liver tissue was obtained from Consolacion, Cebu. Fixation was performed at the Histopathology Laboratory of the University of Cebu – Banilad Campus, while all subsequent tissue processing procedures were conducted at the University of Cebu Medical Center. This was followed by the standard histopathological processes and protocols at the University of Cebu – Medical Center to minimize bias and ensure reliability, using a Leica Automatic Benchtop Tissue Processor (semi-enclosed) for tissue processing samples. Pathologists from the same institution were consulted for guidance and evaluation of samples. Most reagents used in the study were locally sourced from suppliers within Cebu.

For the experimental setup, fifty (50) tissue slides were prepared from pig liver samples and divided into five groups. Each group was treated with different deparaffinization conditions, including xylene (control), cedarwood oil, Solution 1 (Oil D-limonene with 90% Ethanol and Polysorbate 80), D-limonene extract, and a negative control without a deparaffinization agent (as shown in Table 1).

Table 1. Proportion of Tissue Samples

Steps	Group 1	Group 2	Group 3	Group 4
Clearing	Xylene	Xylene	Xylene	Xylene
Deparaffinization	Xylene	Cedarwood oil	Solution 1 (Oil D-limonene, 90% Ethanol; Polysorbate 80)	D-limonene extract

The prepared slides were evaluated by three pathologists and three medical technologists using a standardized rating system [9] (as shown in Figure 2). A single-blind method was applied during assessment. The collected data were encoded and analyzed using IBM SPSS Statistics. Macroscopic parameters, including deparaffinization capacity, ease of paraffin wax removal, and presence of artifacts, and microscopic parameters, including nuclear staining, cytoplasmic staining, and staining clarity, were evaluated for all samples. Statistical analysis was performed using the Kruskal-Wallis H test, a non-parametric method used for comparing three or more independent groups [10]. This test was selected as an alternative to one-way ANOVA when the assumption of normality is not

satisfied. Post hoc pairwise comparisons were conducted to determine differences between specific groups following the Kruskal-Wallis test. All statistical analyses were performed at a 0.05 level of significance.

Macroscopic Assessment				
Parameters	**Criteria**			
Deparaffinization capacity (*degree of tissue's structural identity)*	0 = None (Hard to get sections of tissue deparaffinized)	1 = Mild (Partially deparaffinized)	2 = Good (Tissue completely maintained its structural identity)	
Ease of Paraffin Removal (*degree of tissue disturbance)*	0 = None (Poor; Difficult to remove; requires repeated immersion or heating)	1 = Mild (Satisfactory; Moderately removed)	2 = Moderate (Good; Completely removed, Easily removed with minimal effort; smooth and quick dissolution)	
Macroscopic Artifacts (*amount of artifacts present)*	0 = None (No visible artifact present)	1 = Mild (Minimal or slight artifact)	2 = Moderate (Clearly visible artifact)	3 = Severe (Severe artifacts present, significantly interfere with accurate evaluation)
Microscopic Assessment				
Parameters	**Criteria**			
Nuclear Staining (*clarity and sharpness of the nuclear membrane)*	0 = Bad (Poor; granular, disintegrated, and out of focus)	1 = Mild (Satisfactory; Nuclei are visible but slightly uneven or blurred; partial membrane integrity)	2 = Good (smooth, and clear nuclear membrane)	3 = Very Good (Nuclear membranes are clear and sharp; nuclei are round, smooth, and well-demarcated)
Cytoplasmic staining (*clarity of the cytoplasmic membrane and the cytoplasm)*	0 = Bad (Poor; disintegrated cytoplasmic membrane, granular cytoplasm, and out of focus)	1 = Mild (Satisfactory; Cytoplasmic boundary moderately preserved; slight granularity or uneven staining)	2 = Good (Intact cytoplasmic membrane and transparent cytoplasm)	3 = Very Good (Cytoplasm is uniformly and clearly stained with good contrast and crisp cellular detail)
Clarity of Staining (distinctiveness of the structure, background staining, etc.)	0 = Bad (Poor; obliterates the nucleus and cytoplasm)	1 = Mild (Satisfactory; Partially stained)	2 = Good (Crispness in staining is present, also transparency is completely witnessed)	3 = Very Good (Structures are sharply defined with excellent contrast; background staining is minimal or absent)

Figure 2. Scoring Criteria

3.0 RESULT AND DISCUSSION

This section is divided into four parts. The first (1st) part tests the potential of kiat-kiat *(Citrus reticulata)* oil D-limonene as a deparaffinization agent, used either as a solution (oil D-limonene with 90% ethanol and Polysorbate 80) or as an extract. Three medical technologists assessed its macroscopic performance (deparaffinization capacity, ease of paraffin wax removal, and presence

of macroscopic artifacts) and compared it with xylene and cedarwood oil. The second (2nd) part presents microscopic results (nuclear staining, cytoplasmic staining, and staining clarity) evaluated by three clinical/anatomical pathologists. The third (3rd) part analyzes significant differences among xylene, cedarwood oil, oil D-limonene solution, oil D-limonene extract, and the untreated (negative control) sample as deparaffinization agents in pig liver tissue, using both macroscopic and microscopic evaluation. The fourth (4th) part determines significant differences in deparaffinization capacity, paraffin wax removal, and macroscopic artifact formation among xylene, cedarwood oil, Solution 1, and oil D-limonene extract.

3.1 Macroscopic Findings and Evaluation of Kiat-kiat *(Citrus reticulata)* Oil D-limonene as a Deparaffinization Agent

Statistical analysis using the Kruskal–Wallis H test confirmed significant differences among all groups in both macroscopic ($H = 47.943$, $p < 0.001$) and microscopic ($H = 48.654$, $p < 0.001$) outcomes, indicating that the choice of deparaffinization agent significantly affects tissue quality. Cedarwood oil and xylene consistently achieved the highest mean ranks in both evaluations (macroscopic = 45.50 vs 40.50; microscopic = 40.50 each), demonstrating statistically comparable performance and confirming their equivalence in both gross and histological preservation. Solution 1 showed moderate effectiveness, while oil D-limonene extract and the negative control demonstrated significantly lower performance, particularly in staining clarity and tissue integrity as seen in Table 2.

These findings reinforce the strong performance of cedarwood oil as a viable alternative to xylene, particularly in its ability to preserve both macroscopic structure and microscopic detail. Cedarwood oil-based protocols produce high-quality staining with minimal tissue distortion, supporting its use as a safer alternative clearing agent [11]. The reduced performance of D-limonene-based systems may be due to variability in concentration, extraction method, or processing conditions, which may significantly influence outcomes [1]. The present findings emphasize that while D-limonene has potential, its current formulation requires optimization to achieve consistent performance comparable to conventional agents.

Table 2. Macroscopic Findings and Evaluation of Kiat-kiat *(Citrus reticulata)* Oil D-limonene as a Deparaffinization Agent

Parameters	xylene (Positive Control)	Interpretation	cedarwood oil	Interpretation	oil D limonene + 90% Ethanol + Polysorbate 80 (Solution 1)	Interpretation	D limonene extract	Interpretation	No Deparaffinization Agent (Negative Control)	Interpretation
A. **Deparaffinization capacity** (degree of tissue's structural identity)	2.00	Good	2.00	Good	1.37	Good	1.20	Mild	0.00	None
B. **Ease of Paraffin Removal** (degree of tissue disturbance)	2.00	Good	2.00	Good	1.33	Mild	1.17	Mild	0.00	None
C. **Macroscopic Artifacts** *(amount of artifacts present)*	0.00	None	0.00	None	0.33	None	2.20	Moderate	2.80	Severe

Rating Scales: Deparaffinization [0.00-0.66=None; 0.67-1.33=Mild; 1.34-2.0=Good] / Ease of Paraffin Removal [[0.00-0.66=None; 0.67-1.33=Mild; 1.34-2.0=Good] / Macroscopic Artifacts [0.00-0.74=None; 0.75-1.49=Mild; 1.50-2.24=Moderate; 2.25-3.00=Severe]

3.2 Microscopic Findings and Evaluation of Kiat-kiat *(Citrus reticulata)* Oil D-limonene as a Deparaffinization Agent

Microscopic evaluation revealed that deparaffinization agents significantly influenced staining quality across nuclear preservation, cytoplasmic integrity, and overall clarity. Xylene and cedarwood oil consistently demonstrated superior and equivalent performance, achieving "very good" scores for nuclear staining, cytoplasmic staining, and staining clarity (mean = 3.00 each; n = 10). This indicates optimal preservation of histomorphological detail and minimal interference with staining reactions. Oil D-limonene extract and Solution 1 both showed reduced but acceptable microscopic quality, with nuclear staining generally maintained (mean = 2.00), though cytoplasmic detail and overall clarity were less consistent. The negative control showed the poorest staining preservation across all parameters (as shown in Table 3).

The equivalence between xylene and cedarwood oil suggests similar clearing mechanisms that preserve protein and lipid structures essential for hematoxylin and eosin uptake. The structural similarity of cedarwood oil to xylene as a key factor in its effective clearing ability [8]. The cedarwood oil maintains staining integrity comparable to xylene in routine histological processing [12]. In contrast, the moderate performance of D-limonene extract shows acceptable nuclear staining but variable cytoplasmic clarity in D-limonene-based systems [1]. However, the reduced staining uniformity observed in Solution 1 suggests that although additives such as ethanol and Polysorbate 80 may enhance solubility, they may also interfere with consistent dye-tissue interaction, resulting in uneven cytoplasmic staining. This partially contrasts with reports of more consistent D-limonene performance, suggesting that formulation differences and protocol standardization significantly affect microscopic outcomes [13].

Table 3. Microscopic Findings and Evaluation of Kiat-kiat *(Citrus reticulata)* Oil D-limonene as a Deparaffinization Agent

Parameters	xylene (Positive Control)	Interpretation	cedarwood oil	Interpretation	oil D limonene + 90% Ethanol + Polysorbate 80 (Solution 1)	Interpretation	oil D limonene extract	Interpretation	No Deparaffinization Agent (Negative Control)	Interpretation
A. **Nuclear Staining** *(clarity and sharpness of the nuclear membrane)*	3.00	Very Good	3.00	Very Good	2.00	Good	2.00	Good	1.57	Good
B. **Cytoplasmic staining** *(clarity of the cytoplasmic membrane and the cytoplasm)*	3.00	Very Good	3.00	Very Good	1.33	Mild	2.00	Good	1.53	Good
C. **Clarity of Staining** *(distinctiveness of the structure, background staining, etc.)*	3.00	Very Good	3.00	Very Good	1.00	Mild	2.00	Good	2.00	Good

Rating Scales: 0.00-0.74=Bad; 0.75-1.49=Mild; 1.50-2.24=Good; 2.25-3.00=Very Good

3.3 Differences in Macroscopic and Microscopic Findings Among Xylene, Cedarwood Oil, Solution 1 (Oil D-limonene + 90% Ethanol + Polysorbate 80), and Oil D-limonene Extract as Deparaffinization Agents

Statistical analysis using the Kruskal–Wallis H test confirmed significant differences among all groups in both macroscopic (H = 47.943, p < 0.001) and microscopic (H = 48.654, p < 0.001) outcomes, indicating that the choice of deparaffinization agent significantly affects tissue quality. Cedarwood oil and xylene consistently achieved the highest mean ranks in both evaluations (macroscopic = 45.50 vs 40.50; microscopic = 40.50 each), demonstrating statistically comparable performance and confirming their equivalence in both gross and histological preservation. Solution 1 showed moderate effectiveness, while oil D-limonene extract and the negative control demonstrated significantly lower performance, particularly in staining clarity and tissue integrity (as shown in Table 4).

These findings reinforce the strong performance of cedarwood oil as a viable alternative to xylene, particularly in its ability to preserve both macroscopic structure and microscopic detail. Cedarwood oil-based protocols produce high-quality staining with minimal tissue distortion, supporting its use as a safer alternative clearing agent [11]. The reduced performance of D-limonene-based systems contrasts with reports showing no significant difference from xylene, suggesting that concentration, extraction method, and processing conditions can influence outcomes [1]. The present findings emphasize that while D-limonene has potential, its current formulation requires optimization to achieve consistent performance comparable to conventional agents.

Table 4. Test of Significant Differences in Macroscopic Evaluation of Xylene, Cedarwood Oil, Solution 1 (Oil D-limonene + 90% Ethanol + Polysorbate 80), and Oil D-Limonene Extract

Macroscopic Parameters	n	Mean Rank	Kruskal Wallis H Test Value	df	p-Value	Decision	Remark
A. xylene (Positive Control)	10	40.50	47.943	4	< 0.001	Reject Ho	Significant Difference
B. cedarwood oil	10	45.50					
C. oil D limonene + 90% Ethanol + Polysorbate 80 (Solution 1)	10	25.50					
D. oil D limonene extract	10	15.50					
E. no Deparaffinization Agent (Negative Control)	10	5.50					
Total	*50*						

*Significant @=0.05 level

3.4 Difference Between Solution 1, Oil D-limonene Extract, Cedarwood oil, Xylene in Terms of Deparaffinization Capacity, Ease of Paraffin Wax Removal, and Artifacts

The Kruskal–Wallis test revealed statistically significant differences among groups in deparaffinization capacity (H = 46.37, p < 0.001), ease of paraffin removal (H = 46.30, p < 0.001), and artifact formation (H = 47.91, p < 0.001). Xylene and cedarwood oil consistently demonstrated identical and highest performance across all parameters, with mean ranks of 40.50, indicating efficient paraffin removal and minimal artifact formation. In contrast, Solution 1 and oil D-limonene extract showed significantly lower mean ranks, reflecting reduced clearing efficiency and increased artifact presence, while the negative control consistently performed the poorest.

The equivalence between xylene and cedarwood oil further confirms the strong solvent capacity of cedarwood oil in effectively dissolving paraffin while preserving tissue morphology. This supports findings that cedarwood oil produces histological results comparable in quality to xylene [11,8]. Conversely, the moderate to poor performance of D-limonene-based formulations suggests incomplete paraffin dissolution, which likely contributes to residual wax and increased tissue artifacts. This partially contradicts reports of comparable efficiency between xylene and D-limonene, indicating that formulation differences and processing variability may be key determinants of performance [1]. Overall, these results highlight cedarwood oil as the most reliable natural alternative among the tested agents, while D-limonene formulations require further optimization before routine histological application.

4.0 CONCLUSIONS

The findings show that cedarwood oil (Group 2) performed comparably to xylene (Group 1) across all evaluated parameters, including deparaffinization capacity, ease of wax removal, artifact presence, and staining quality. Both agents consistently maintained tissue structural integrity and produced reliable macroscopic and microscopic results.

Macroscopic assessment demonstrated that Solution 1 (oil D-limonene + 90% ethanol + Polysorbate 80; Group 3) achieved adequate deparaffinization and effectively minimized artifacts, although its wax removal efficiency was lower than that of xylene and

cedarwood oil. Oil D-limonene extract (Group 4) showed limited effectiveness, with reduced wax removal and increased artifact formation. Overall, xylene and cedarwood oil remained the most consistent agents for macroscopic deparaffinization.

Microscopic evaluation showed that both oil D-limonene extract (Group 4) and Solution 1 (Group 3) preserved nuclear morphology; however, xylene and cedarwood oil maintained superior nuclear clarity and sharpness. Cytoplasmic staining was optimal with xylene and cedarwood oil, while Solution 1 showed reduced clarity and oil D-limonene extract demonstrated acceptable but less consistent results. Despite this, oil D-limonene extract produced better staining outcomes than the negative control, indicating measurable effectiveness.

Quantitatively, xylene (mean rank = 40.50) and cedarwood oil (mean rank = 45.50 macroscopic; 40.50 microscopic) consistently achieved the highest performance across parameters, with comparable results. Oil D-limonene extract (mean rank = 25.50 microscopic) and Solution 1 (mean rank = 25.50 macroscopic) showed moderate performance, suggesting partial effectiveness in both wax removal and tissue preservation.

Taken together, these findings indicate that D-limonene's limitations were not due to a complete lack of deparaffinizing ability, but rather to inconsistent wax-removal efficiency and artifact control compared with established agents. The study therefore demonstrates that D-limonene's value lies not in immediate equivalence to xylene, but in its demonstrated capacity to preserve tissue morphology and staining quality under specific conditions. In this context, the use of the term 'potential' is justified. Although D-limonene did not surpass xylene or cedarwood oil, its measurable macroscopic and microscopic performance aligns with the concept of potential as the capacity to develop or become effective under appropriate conditions. Its natural origin, lower toxicity profile, and consistent microscopic outcomes support its consideration as a developing alternative rather than a direct substitute at this stage.

The researchers expressed gratitude to the individuals who contributed, noting that their efforts enabled comparison and evaluation of study outcomes.

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