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RESEARCH ARTICLE

The effects of varying in incubation time and temperature of methyl salicylate as a clearing agent on the quality of breast tissue slides

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Abstract

Non-polar clearing agents have the ability to dissolve breast adipose tissue. While fat dissolution occurs during the clearing process, careful monitoring of adipose cell membranes mis crucial due to their impact on the behavior of breast cancer cells and disease progression. Xylol is a widely employed clearing agent. However, its toxicity and prolonged clearing time necessitate the exploration of alternatives. In this context, methyl salicylate emerges as a viable substitute. It is non-polar nature facilitates efficient fat dissolution, leading to quicker tissues clarification. This study adopted a quasi-experimental method, utilizing 24 pieces of breast tissue as the sample. These tissue sample were divided into six groups according to the treatment regimen. The treatment groups involved breast tissue clearing using xylol for 2x60 minutes at room temperature, methyl salicylate for 2x30 minutes at room temperature, and methyl salicylate at 60°C for 20 minutes, 30 minutes, 2x20 minutes, and 2x30 minutes. The quality of breast tissue slides was assessed by analyzing the color contrast between the nucleus and cytoplasm using ImageJ software, along with the clarity of adipose cell cell membrane using microscopy. The results indicated that prolonged exposure at high temperature resulted in poor quality breast tissue slides. Conversely, tissue clearing with methyl salicylate for 2x30 minutes at room temperature exhibited excellent contrast between the nucleus and cytoplasm, as well as clear adipose cell membranes. Further study is warranted to explore the applicability of methyl salicylate as a clearing agent in tissues with lower fat content.

1. INTRODUCTION

Histopathology stands as the gold standard for diagnosing breast cancer, with adipose tissue being a prominent components of the breast, constituting approximately 90% of the tissue (adipose tissue AT) (1). Within the breast, there exist enduring interactions between epithelial cells and adipose cells. Adipose tissue can impede fluid penetration during tissue processing, leading to suboptimal tissue slides quality (2). Civen that lipids, constituting a significant part of adipose tissue, are non-polar organic compounds, their dissolution occurs exclusively in solvents with comparable polarity or those that are similarly non-polar (3,4).

Xylene serves as a non-polar clearing agent, proficient in dissolving adipose tissue and rendering the tissue transparent. Nevertheless, its drawbacks include toxicity and an extended clearing time, prompting the exploration of alternative clearing agents. One such alternative, methyl salicylate, has proven effective in producing transparent tissue slides (5,6).

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In a study conducted by (6), the application of methyl salicylate as a clearing agent in liposarcoma tissue revealed no discernible difference in the quality of color intensity between the nucleus and cell membrane when compared to xylol (6). The suitability of methyl salicylate as a cleaning agent is attributed to its non-polar nature, which enables it to effectively dissolve lipids (7). Methyl salicylate is an organic solvent with a high refractive index (RI) of 1.54, which allows it to effectively infiltrate tissues during the cleaning stage (8).

During the clearing stage, tissue pieces underwent a dual immersion in methyl salicylate for 30 minutes each at room temperature (6). Concurrently, other tissue pieces were immersed in methyl salicylate for 20 and 30 minutes at 60°C (6). As per the protocol outlined by (9), the clearing process involves two immersion in the clearing agent for an hour each. The initial immersion aims to eliminate any residual dehydrating agent retianed by the tissue. Subsequently, a second immersion in the clearing agent is of paramount importance for achieving thorough tissue transparency and preparing it for paraffinization (10).

In this study, the clearing process for breast tissue will involve the use of methyl salicylate, with two immersions lasting 20 minutes each and two immersions lasting 30 minutes each, conducted at an incubation temperature of 60°C. The incubation temperature for methyl salicylate, as a clearing agent, will be applied at both 60°C and room temperature. The choice of a 60°C temperature during the clearing stage is motivated by its ability to eliminate protein within the tissue, thereby enhancing the clarity of the tissue at the clearing stage (11).

The evaluation of tissue slides involved an assessment of the intensity of the nuclear and cytoplasmic color, as well as the clarity of adipose cell membrane. Adipose tissue stores energy and is a factor that contributes to the development of breast cancer cells (12). Interactions between adipose tissue surrounding cancer cells play a pivotal role in modifying the tumor microenvironment, creating conditions favorable for cancer progression (13). Another research (14), demonstrated that adipose tissue in close proximity to both malignant and benign lesions exhibited distinct gene expression profiles and functional characteristics.

In other related research (15), it was found that alterations in the histomorphological parameters of adipocytes reflect alterations in adiposity at body composition analysis. This represents a clinically relevant problem in cancer patients, as early diagnosis and treatment can improve cancer patients' outcomes and quality of life. Therefore, breast tissue was selected for this study, driven by an interest in elucidating the histologic appearance of the adipose cell membrane surrounding breast tissue cancer cells.

The utilization of methyl salicylate as a clearing agent was anticipated to render adipose tissue in the breast visibly clear without causing damage to the adipose tissue itself. This expectation was based on the aim of achieving clarity in the color of the cell nucleus, cytoplasm and adipose cell membrane.

Previous study on the use of methyl salicylate as a clearing agent suggest that the incubation time can significantly impact the preparation result. Studies have shown that varying incubation times can influence the effectiveness of tissue clearing and the overall quality of slides. Different durations of exposure to methyl salicylate have been explored in the literature, and findings indicate that optimizing the incubation time is crucial for achieving the desired results in terms of tissue transparency and preservation of cellular structures. Therefore, understanding and carefully adjusting the incubation time with methyl salicylate is a critical aspect of ensuring successful tissue preparation and accurate assessment of nuclear and cytoplasmic color intensity, as well as the clarity of adipose cell membranes in breast tissue.

Building on the background information provided, the primary objective of this study is to investigate the impact of varying incubation time and temperature during the utilization of methyl salicylate as a clearing agent. Specifically, the study aims to asses how variation in these parameters influence the quality of tissue slides. Additionally, the study endeavors to discern and compare the outcomes achieved with the use of xylol versus methyl salicylate as a clearing agent in terms of producing high-quality tissue slides. By systematically analyzing the effects of incubation time and temperature, as well as comparing different clearing agents, the study aims to contribute valuable insights into optimizing the tissue clearing process for improved clarity of nuclear and cytoplasmic color, along with enhanced visibility of adipose cell membranes in breast tissue.

2. MATERIALS AND METHODS

This study employed a quasi-experimental methodology and was carried out at the Cytohistotechnology Laboratory of the Medical Laboratory Technology Department, Bandung Health Politechnic in May 2023. The sample utilized residual breast tissue obtained from a hospital in Cimahi City. The study was approved by the Health Research Ethics Committee of the Bandung Health Politechnic under the reference number 49/KEPK/EC/V2023.

In this study, two research variables were considered: the duration and temperature of methyl salicylate incubation. The samples sizw was determined using Federer's formula, resulting in the utilization of a total of 24 pieces of breast tissue. The distribution of these samples involved six treatment groups, with each group comprising four pieces of breast tissue. The treatment groups consisted of a control group (FX), utilizing xylol to clear breast tissue for 2x60 minutes at room temperature. Additionally, there were five experimental groups that employed methyl salicylate for clearing breast tissue at varying temperatures and durations: 2x30 minutes at room temperature (MS.I), 20 minutes at an incubation temperature of 60°C (MS.II), 30 minutes at an incubation temperature of 60°C (MS.III), 2x20 minutes at an incubation temperature of 60°C (MS.IV), and 2x30 minutes at an incubation temperature of 60°C (MS.V).

2.1. Fixation

The residual breast tissue was immersed in a 10% Neutral Buffered Formalin (NBF) fixative solution for a duration exceeding 24 hours.

2.2. Gross Examination

Specimens should be cut to no larger than 2.5 x 2.0 x 0.4 cm (16). During this stage, the breast tissue underwent division into 24 pieces, each measuring 2.0 x 2.0 x 0.3 cm. Subsequently, these tissue pieces were carefully placed in a tissue cassette distinctly labeled with yellow cardboard, and assigned a sample number using a 2B pencil. Following this preparation, the cassette, along with the tissue samples, was immersed in 10% NBF solution.

2.3. Tissue Processing

This study used the tissue processing procedure from a previous study (6), introduced variations in incubation time and temperature, specifically incorporating two immersions in methyl salicylate (Table 1). The clearing stage was performed employing xylol as the control and methyl salicylate (Zhenjiang Gaopeng Pharmaceutical Co., Ltd., Jiangsu, China) as an alternative clearing agent during tissue processing. The methyl salicylate used exhibited characteristics of a colorless liquid, possessing an acidity of 0.30 mL, a specific gravity of 1.182, a refractive index of 1.537, soluble in alcohol, and a concentration of 99.75%.

Table 1. A summary of steps in breast tissue processing

Steps	Placed in	Duration					
		FX	MS.I	MS.II	MS.III	MS.IV	MS.V
Fixation	NBF 10%	24 hours	24 hours	60 minutes	60 minutes	60 minutes	60 minutes
Tap water	Aquadest	15 minutes	15 minutes	15 minutes	15 minutes	15 minutes	15 minutes
Dehidration	Alcohol 70%	60 minutes	60 minutes	30 minutes	30 minutes	30 minutes	30 minutes
	Alcohol 85%	60 minutes	60 minutes				
	Alcohol 100%	60 minutes	60 minutes	30 minutes	30 minutes	30 minutes	30 minutes
	Alcohol 100%			30 minutes	30 minutes	30 minutes	30 minutes
	Ethanol I	60 minutes	60 minutes				
	Ethanol II	60 minutes	60 minutes				
Clearing	Xylol II	60 minutes					
	Xylol II	60 minutes					
	Methyl Salicylates I		30 minutes	20 minutes	30 minutes	20 minutes	30 minutes
	Methyl Salicylates II		30 minutes			20 minutes	30 minutes
Infiltration	Paraffin	18 hours	18 hours	1 hour	1 hour	1 hour	1 hour

2.4. Tissue Embedding

The finalized tissue pieces were positioned within a base mold filled with liquid paraffin. Carefully situating the tissue pieces at the base and center of the mold, the cassette lid was then applied and gently pressed into position. Ensuring that the cassette was appropriately labeled with the labelling facing upward. The base mold should be transferred to the cold plate and allowed to fully solidify, ideally for a period of up to 30 minutes. Thereafter, the mold should be separated from the cassette (10).

2.5. Paraffin Block Sectioning

Paraffin blocks were sectioned using a microtomeand it was necessary to ensure that the paraffin blocks were in a frozen state when sectioned (10). The first step in paraffin block cutting was rough cutting (trimming) with a thickness of 15-30 µm. Subsequently, the paraffin blocks were then sectioned at a thickness of 3-4 µm (17). The tissue sections were placed in warm water and then mounted on a slide.

2.6. Heating of Tissue Slides

The tissue slides that had been drained were heated on a hot plate set at 60°C until the remaining paraffin was dissolved (18).

2.7. Hematoxylin Eosin (HE) Staining

Breast tissue staining with hematoxylin and eosin proceeds through 13 steps. It begins with the deparaffinization step by immersing the tissue slides in xylol I and O II for 5 minutes each. This is followed by rehydration with 90%, 80%, and 70% alcohol for 5 minutes at each concentration. Then, the slides were immersed in hematoxylin as a nuclear stain for 5 minutes. The slides were washed with tap water and then immersed twice in 0.5% HCl alcohol. After washing the sample in tap water, the cytoplasm is stained with eosin for one minute. Dehydration were performed with 70%, 80% and 90% alcohol, each immersed 10 times. The sample was then cleared using xylene I and xylene II for two minutes each. Finally, the breast tissue slides were mounted and covered with a cover glass, which was glued with the addition of entellan (19).

2.8. Quality Assessment of Breast Tissue Slides

Tissue slides that had been stained were then subjected to digital image processing using ImageJ software to obtain the color intensity (Optical Density/OD) of the nucleus and cytoplasm. Digital images were captured three times with different fields of view for each slide. The data were processed in Excel to obtain the mean values of nucleus and cytoplasm color intensity across treatments. Subsequently, the mean intensity values of the nucleus and cytoplasm were compared, and the resulting difference determined the contrast value. If the contrast value of the methyl salicylate group was greater than the contrast value of xylol, it is interpreted that this group has good tissue slides.

In addition to the color intensity parameter, the quality assessment of breast tissue slides in this study was also based on the clarity parameter of the fat cell membrane. The scoring system was grounded in the theory that interactions between breast adipose tissue surrounding cancer cells modify the tumor microenvironment to support cancer (13). Therefore, the quality of breast tissue slides was evaluated based on the clarity of the adiposyte cell membrane. Adipose cell membrane clarity was observed by an anatomic pathology specialist, an anatomic pathology laboratory practitioner, and the author under a microscope with 10x40 magnification. A score of 0 indicated poor quality when the adipocyte membrane was not clearly visible, while a score of 1 indicated good quality when the fat cell membrane was clearly visible.

2.9. Data Analysis

A total of six treatment groups were established, with each group comprising four distinct preparations. The color intensity was measured on three occasions for each preparation. Thus, the study yielded 72 data for the nucleus color intensity value and 72 data for the cytoplasm color intensity value. Subsequently, the data were subjected to statistical analysis using SPSS version 24.0.

The Friedman test was applied to the data regarding the color intensity of the nucleus and cytoplasm. The Friedman test is a non-parametric test used for paired data that is not normally distributed in repeated measurements. If a statistically significant difference was identified through the Friedman test, a post hoc analysis was conducted using the Wilcoxon test with paired Bonferroni adjustments. This was employed to determine which pairs of conditions exhibited a statistically significant difference.

3. RESULTS AND DISCUSSION

3.1. Color Intensity of Nucleus and Cytoplasm

The normality test was carried out on the data pertaining to the nucleus and cytoplasm color intensity. The normality test of nucleus and cytoplasm color intensity both showed a Sig value of 0.000, which is less than 0.05.

Consequently, the data on nucleus and cytoplasm color intensity were not normally distributed. As a result, the non-parametric statistical test, namely the Friedman test, will be employed to continue the analysis.

The Friedman test on the data of nucleus and cytoplasm color intensity both showed an Asymp. Sig value of 0.000, which is less than 0.05. Therefore, there is a significant difference in both nucleus and cytoplasmic color intensity values.

It can be concluded that the use of methyl salicylate as a breast tissue clearing agent, with variations in time and temperature at the tissue clearing stage, can produce significant differences in nucleus and cytoplasm color intensity. To identify which group exhibited the p-value difference, post-hoc analysis was conducted using the Wilcoxon Test.

Table 2. Wilcoxon test of nucleus and cytoplasm color intensity

	Asymp. Sig of Wicoxon Test				
	MS.I-FX	**MS.II-FX**	**MS.III-FX**	**MS.IV-FX**	**MS.V-FX**
Nucleus color intensity	0.034	0.480	0.002	0.002	0.008
Cytoplasm color intensity	0.272	0.004	0.530	0.003	0.084

In Table 2, the Wilcoxon test of nucleus color intensity, the asymptotic The significance level was more than 0.05 in the MS.II group in comparison to the FX (control) group. This indicates that there was no significant difference in nuclear color intensity between the use of xylol and methyl salicylate for 20 minutes at a temperature of 60°C (MS.II) for tissue clearing process.

The Wilcoxon Test of cytoplasm color intensity in Table 2 revealed that the Asymp. Sig value was greater than 0.05 in the MS.I and MS.III groups. This indicates that there is no significant difference in cytoplasmic color intensity between the use of xylol with methyl salicylate for 2x30 minutes at room temperature incubation (MS.I) and for 30 minutes at 60°C incubation temperature (MS.III) for tissue clearing.

3.2. Color Contrast of Nucleus and Cytoplasm

In Table 3, the optical density of the cytoplasm was higher than that of the nucleus, attributed to the larger surface area of the cytoplasm. The contrast value was determined by the difference in mean optical density between the nucleus and cytoplasm. The greater the difference in mean OD, the better the contrast between the nucleus and the cytoplasm. A high contrast value facilitates easier differentiation between the nucleus and cytoplasm when observed under the microscope. Among the breast tissue clearing treatment groups, the highest contrast value was observed when methyl salicylate was used for 2x30 minutes at 60°C.

Table 3. Color contrast values between of nucleus and cytoplasm

Treatment Group	Mean OD		Difference of Mean OD
	Nucleus	Cytoplasm	
FX	215.386	245.547	30.162
MS.I	207.545	246.770	39.225
MS.II	211.706	229.500	17.793
MS.III	181.055	246.234	65.179
MS.IV	177.233	250.719	73.486
MS.V	201.149	248.280	47.131

A reliable method for assessing breast tissue slide quality involves ensuring that the contrast value of methyl salicylate is greater than or equal to that of xylol. According to Table 4, clearing breast tissue with methyl salicylate for 2x30 minutes at room temperature (MS.I), 30 minutes at 60°C (MS.III), 2x20 minutes at 60°C (MS.IV), and 2x30 minutes at 60°C (MS.V) resulted in good slide quality, as the color contrast between the nucleus and cytoplasm surpassed that of xylol. Elevated color contrast aids in distinguishing the nucleus and cytoplasm during microscopic observation, leading to more accurate diagnoses.

Meanwhile, the treatment group with 20 minutes at 60°C (MS.II) exhibited contrast values that were not higher than the control. In this particular treatment group, the resulting slides displayed less distinguishable colors as the hues of the nucleus and cytoplasm were similar.

Table 4. Contrast values comparison between methyl salicylates and xylol

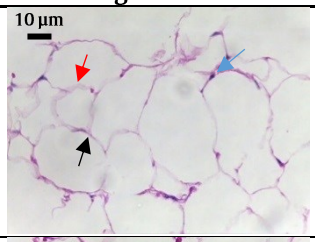
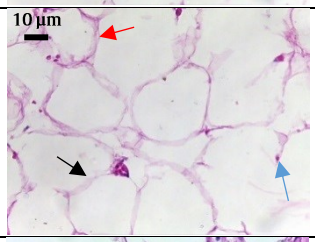
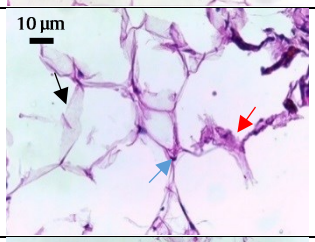
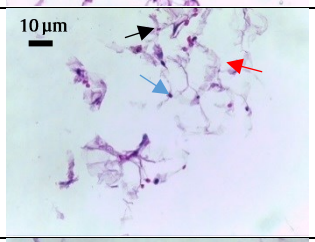
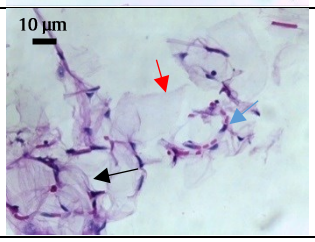
	Comparison of Methyl Salicyllate Contrast Value to Xylol	Interpretation*
MS.I - FX	39.225 > 30.162	Good
MS.II - FX	17.793 < 30.162	Poor
MS.III - FX	65.179 > 30.162	Good
MS.IV - FX	73.486 > 30.162	Good
MS.V - FX	47.131 > 30.162	Good

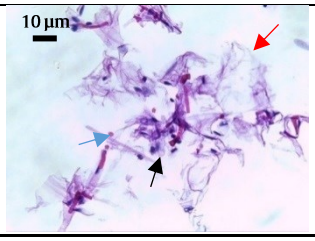
*Intepretation based on the principle of color contrast (20). The color contrast between the blue nucleus and the red cytoplasm should be clearly visible to be considered good, while it was considered poor if the contrast was not clear.

3.3. The Clarity of Adipose Membrane Cells by Microscopy

In addition to OD values of nucleus and cytoplasm, microscopic observations of tissue slides at 10x40 magnifications were conducted. Microscopic interpretation was carried out by three panelists: an anatomic pathology specialist, an anatomic pathology laboratory practitioner, and the authors. This is reported in Table 5.

Table 5. Microscopic image of breast tissue with HE staining

Sample Code	Microscopic Image of Breast Tissue with HE Staining at 10x40 Magnification	Information
FX		The nucleus appeared blue (blue arrow) and the cytoplasm appeared red (red arrow). The adipose cell membrane was clearly visible (black arrow).
MS.I		The nucleus appeared blue (blue arrow) and the cytoplasm appeared red (red arrow). The adipose cell membrane was clearly visible (black arrow).
MS.II		The nucleus appeared blue (blue arrow) and the cytoplasm appeared red (red arrow). The adipose cell membrane was clearly visible (black arrow).
MS.III		The nucleus appeared blue (blue arrow) and the cytoplasm appeared red (red arrow), while the adipose cell membrane was not clearly visible. The tissue exhibited signs of cracking and accumulation (black arrow).
MS.IV		The nucleus appeared blue (blue arrow) and the cytoplasm appeared red (red arrow), while the adipose cell membrane was not clearly visible. The tissue also appeared cracked and accumulated (black arrow).

MS.V		The nucleus appeared blue (blue arrow) and the cytoplasm appeared red (red arrow), while the adipose cell membrane was not clearly visible. The tissue also appeared cracked and accumulated (black arrow).

Based on Table 5, microscopic analysis indicated that the blue color observed in the nucleus and the red color in the cytoplasm were very clear in all treatment groups. However, the clarity of the adipose cell membrane was not satisfactory in any of the treatment groups. Therefore, data processing for adipose membrane clarity was carried out with Excel to obtain the mode of each treatment group. The following results were obtained.

Table 6. Evaluation of adipose cell membranes clarity

Treatment Groups	The Clarity Value of Adipose Cell Membranes*			Mode	Interpretation
	Panelist 1	Panelist 2	Panelist 3		
FX	1	1	1	1	Good
MS.I	1	1	1	1	Good
MS.II	1	1	1	1	Good
MS.III	0	0	0	0	Poor
MS.IV	0	0	0	0	Poor
MS.V	0	0	0	0	Poor

*Adipocyte membrane clarity was assessed microscopically by three panelists: an anatomic pathology specialist (panelist 1), an anatomic pathology laboratory practitioner (panelist 2), and the author (panelist 3).

According to Table 6, the tissue clearing treatment group that used xylol for 2x60 minutes at room temperature, methyl salicylate for 2x30 minutes at room temperature and methyl salicylate for 20 minutes at 60°C received a score of 1 (indicating good). This implies that the adipose cell membranes in these three groups are clearly visible. Conversly, the tissue clearing treatment groups that used Methyl salicylate for 30 minutes at 60°C, 2x30 minutes at 60°C, and 2x30 minutes at 60°C received a score 0 (indicating poor), signifying that the adipose cell membranes in these three groups appeared unclear.

3.4. The Quality of Breast Tissue Slides

In this study, tissue slide quality was evaluated based on favorable values for color contrast between the nucleus and cytoplasm, as well as the clarity of the adipose cell membrane. Table 7 reveals that the breast tissue clearing treatment group, which utilized methyl salicylate for 2x30 minutes at room temperature, performed well on both parameters of breast tissue slide quality assessment.

Table 7. The quality assessment of H&E stained breast tissue slides

Treatment Group of Methyl Salicyllate	The Quality Assessment Parameters of Breast Slides		Interpretation
	Contrast Value of Nucleus and Cytoplasm	Clarity of Adipose Cell Membranes	
MS.I	Good	Good	Good
MS.II	Poor	Good	Poor
MS.III	Good	Poor	Poor
MS.IV	Good	Poor	Poor
MS.V	Good	Poor	Poor

Histopathology is crucial in diagnosing breast cancer, with high-quality tissue slides being imperative for accurate assessments. The clearing stage in tissue processing plays an important role in achieving quality tissue slides by effectively removing alcohol and facilitating paraffin infiltration into the tissue (10).

In this study, the utilization of methyl salicylate as an alternative clearing agent yileded clear breast tissue in all treatment groups under microscopic observation. Methyl salicylate is an organic solvent that is non-polar, rendering it capable of dissolving fat and thus acting as a good tissue clearing agent (7).

Tissues are filtered with a multitude of substances, including scattering particles with a higher refractive index (1.39–1.52), such as fibers, cell membranes, cell nucleus, and cell organelles, and surrounding media with a lower refractive index (1.33–1.37), such as interstitial fluid and/or cytoplasm. However, methyl salicylate exhibits

a high RI, with a value of 1.54. The presence of methyl salicylate with a high RI will result in an increased RI in the background medium. The removal of high RI scattering particles, such as lipids and proteins, can also enhance the RI matching in tissue, thereby reducing the scattering of tissue (8).

The matching of the refractive index (RI) between scattering particles and the background medium can reduce the scattering of tissue and render it transparent. This has been regarded as the essential foundation of tissue clearing (8).

In this study, the assessment of breast tissue slides quality involved the observation of the clarity of the blue color in the nucleus, the red color in the cytoplasm, and the distinctiveness of the adipose cell membrane. The quality of a breast tissue slide can be visualized through hematoxylin and eosin staining, where the nucleus is stained blue and the cytoplasm is stained red (21). The effective observation of these two colors occurs when there is a notable contrast in the color intensity between the nucleus and cytoplasm.

Clearing breast tissue with xylene at room temperature (± 24°C) for 2x60 minutes (FX) serves as a standard in evaluating the quality of breast tissue preparations in this study. The results of the FX treatment group exhibited a contrast value of 30.162 for the nuclear and cytoplasmic colors. With this contrast value , the blue nucleus color of the nucleus and red color of the cytoplasm can be easily identified under microscopic observation. In addition, the adipose cell membrane is clearly visible in this treatment group.

The high-quality breast tissue slides in this study were achieved through the application of breast tissue clearing with methyl salicylate for 2x30 minutes at room temperature (MS.I). This was substantiated by the meticulous data processing of the color intensity in the nucleus and cytoplasm, derived from digital images utilizing the ImageJ software. The outcome revealed a contrast value of MS.I ≥ the contrast value of FX, thereby affirming the superior quality of the breast tissue slides. Furthermore, microscopic examination or the breast tissue slides in the MS.I group exhibited clear and contrasting colors of the nucleus and cytoplasma, with the adipose cell membrane distinctly visible. This collective evidence underscores the overall excellence of the breast tissues slides in the MS.I group.

The breast tissue clearing treatment group using methyl salicylate for 20 minutes at 60°C (MS.II) resulted in poor-quality breast tissue slides. The classification of MS.II treatment group slides as subpar was based on the contrast value analysis, revealing that the contrast value of MS.II was lower than that of the control group, FX, despite successful adipose cell membrane clearance. The reduced contrast value observed in the MS.II treatment group can be attributed to the nearly identical coloration of the nucleus and cytoplasm, as evidenced by microscopic observations where both exhibit a blue color. This color similarity is a consequence of the lack of differentiation at the time of staining. In hematoxylin and eosin (HE) staining, differentiation is achieved through the use of 0.5% hydrochloric acid (HCl) to remove non-specific background and enhance the color contrast between the nucleus and cytoplasm. The lack of differentiation may be attributed to the thickness of the tissue sections or the duration of the differentiation process (22).

The distinction between the blue color of the nucleus and the red color in the cytoplasm is readily discernible under a microscope. However, when utilizing ImageJ software, the color intensity of both the nucleus and cytoplasm can be influenced by various factors, one of which is the illumination from the microscope light source. Images captured with a light microscope rely on the absorption of light by the dye used for tissue slides staining. Another contributing factor is the absence of clear demarcation, wherein the absorption spectra of hematoxylin and eosin overlap across a substantial portion of the visible spectrum (23).

The breast tissue clearing treatment group employing methyl salicylate for durations of 30 minutes at 60°C (MS.III), 2x20 minutes at 60°C (MS.IV), and 2x30 minutes at 60°C (MS.V) yielded suboptimal breast tissue slides. In each of these treatment groups, the contrast value fell below that of the control group, FX. However, the adipose cell membrane exhibited fragmentation and accumulation in all three treatment groups. This phenomenon can be attributed to the inherent brittleness of the breast tissue paraffin blocks generated by MS.III, MS.IV, and MS.V treatment. Consequently, during microtome sectioning, these brittle paraffin blocks impeded the formation of cohesive tissue bands, resulting in fragmented and stacked tissue under microscopic scrutiny. The brittleness is likely induced by prolonged immersion in clearing agents (23).

Furthermore, it is essential to consider the temperature and duration of immersion during clearing agent application. Prolonged immersion at 60°C can exacerbate tissue brittleness, necessitating a careful balance between temperature and time. Elevated temperatures can enhance penetration and fluid exchange rates, expediting the clearing process, but excessive durations may lead to detrimental effects on tissue integrity (23). Thus, optimizing the parameters of temperature and immersion duration is crucial for achieving successful breast tissue clearing without compromising slide quality.

4. CONCLUSIONS

In conclusion, prolonged clearing times for breast tissue with methyl salicylate at 60°C lead to substandard tissue preparations. The elevated incubation temperature of methyl salicylate correlates with increased destruction of breast adipose tissue, consequently yielding unqualified breast tissue slides. Notably, the breast tissue clearing treatment group, which utilized methyl salicylate for 2x30 minutes at room temperature, demonstrated the production of high-quality breast tissue slides. This underscores the critical importance of optimizing clearing parameters to achieve optimal results. Future research endeavors should explore the application of methyl salicylate as a clearing agent on tissues with lower fat content, necessitating a comprehensive understanding of its effects beyond adipose-rich tissues.

Author contributions: FF: Conceptualization, methodology, validation, formal analysis, investigation, resources, writing—original draft preparation, writing—review and editing, visualization, project administration. WW: Conceptualization, methodology, validation, supervision, funding acquisition. AD, MR: Methodology, validation. Mamat Rahmat: Methodology, validation. All authors have read and agreed to the published version of the manuscript.

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