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Use of Carrot Oil, Olive Oil, Pine Oil, Rose Oil in Comparison to Xylene as Clearing Agent During Tissue Processing: an in Vitro Study

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Clearing plays a vital role in histological section preparation. Xylene has long been valued for its ability to efficiently deparaffinize and clear tissue samples. However, its hazardous properties have raised health and environmental concerns, leading researchers to seek alternative solvents for tissue processing. This study utilized safer substitutes to address these concerns. Aim: To assess the clearing ability of four different oils i.e., Carrot oil, Olive oil, Pine oil, and Rose oil in comparison with that of xylene. Materials and Methods: Thirty different formalin-fixed tissue samples were cut into five parts and cleared using Carrot oil, Olive oil, Pine oil, Rose oil, and xylene followed by routine processing, sectioning, and staining steps. Presence or absence of shrinkage, ease of cutting, nucleus, cytoplasm details and staining quality were assesed and comparison was done between each of them cleared with the 4 different oils with that cleared using xylene. The results showed that all four oils cleared tissues as effectively as xylene, with Pine oil superior in nucleus and cytoplasm details, as well as staining quality insignificant number of samples. These oils not only more affordable and eco-friendly than xylene but can also serve as effective clearing agents.

Keywords: Bio-friendly; Clearing agents; Deparaffinize; Histopathology; Xylene.

Clearing plays a vital role in histological section preparation. Prior to embedding the tissues in paraffin wax, their primary function is the removal of alcohol and other dehydrating substances. Xylene, a common solvent in histology and pathology laboratories, has long been valued for its ability to efficiently deparaffinize and clear tissue samples.[1] It is used in histology labs for cover sliding, staining, and tissue processing in the field of dentistry.[2] Because of its high solvency factor, which also makes the tissue translucent and permits maximal alcohol displacement, paraffin penetration is enhanced. Its superior dewaxing and clearing qualities throughout staining processes result in a tissue section throughout crucial steps like deparaffinization, rehydration, and dehydration, which made use of xylene and graded alcohol essential.[3] However, concerns about its health and environmental impacts have prompted researchers to explore alternative solvents for tissue processing. Despite the availability of more sophisticated methods, the pathological laboratory still relies on various potentially hazardous chemicals.

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Xylene, a type of hydrocarbon is recognized for its hazardous properties. Technicians working in histopathology laboratories frequently come into contact, with xylene during procedures such as tissue processing, clearing, and staining. The majority of xylene exits the body within 18 hours after exposure. Research has indicated that it may be effectively absorbed by oral, cutaneous, and inhalation routes.[4] Since it dissolves in adipose tissue, it remains for around 1-6 days in the human body. Prolonged exposure, particularly in occupational exposed personnel, leads to xylene getting continuously and increasingly accumulated in our body. Exposure to xylene poses various health risks, ranging from mild symptoms like dizziness to severe conditions such as lung congestion with focal interalveolar hemorrhage. Adenosine triphosphate, a mitochondrial enzyme, is depleted in xylene-affected cells, which results in tissue damage.It causes damage to the heart and kidneys, some deadly blood dyscrasias, and other less serious issues including the skin. Additional harmful consequences of xylene usage include erythema, dryness, scaling, and secondary infections.[5] Chronic exposure commonly causes dizziness, along with irritation of the eyes, nose, and throat.[6] According to studies, people working in laboratories who were exposed for many years were said to have similar symptoms as those who had been poisoned generally, such as pancytopenia from a wound.[7] In this study, four different oils—Carrot oil, Olive oil, Pine oil, and Rose oil— were assessed for their clearing efficacy and how they affect tissues during several regular histopathology procedures, a comparison was conducted for each oil physical and chemical characteristics.

MATERIALS AND METHODS

The study comprised of 30 soft tissue samples obtained from routine biopsies and fixed in 10% formalin. The research was carried out at the Department of Oral & Maxillofacial Pathology and Oral Microbiology, Sharda University, Greater Noida. Ethical permission was obtained.

Non-necrotic soft tissue samples were included in the study. Hard, necrotic tissues and samples of inadequate size were excluded. Fixation was followed by grossing. Five equal sections were cut from each tissue specimen (30x5=150 total bits) namely, Group A - xylene, Group B - carrot oil, Group C - olive oil, Group D - pine oil, and Group E - rose oil. For further processing, the individually labeled tissue sections were put into metal cassettes. Different alcohol concentrations (70, 80, 90, and 100%) were used to dehydrate these tissues. Each segment of tissue were then placed in one of five glass jars filled with either Xylene, Carrot oil, Olive oil, Pine oil, or Rose oil. Subsequently, these tissue sections underwent routine processing for paraffin embedding. Then they were sectioned, after which staining was done with Haematoxylin and Eosin (H&E). Using a light microscope, an examination was then finally

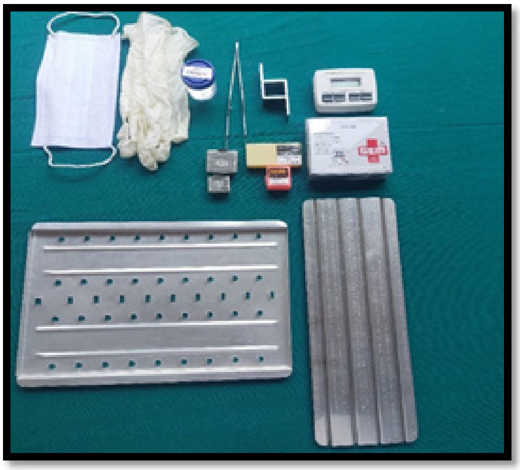


Fig. 1. Armamentarium used in the study

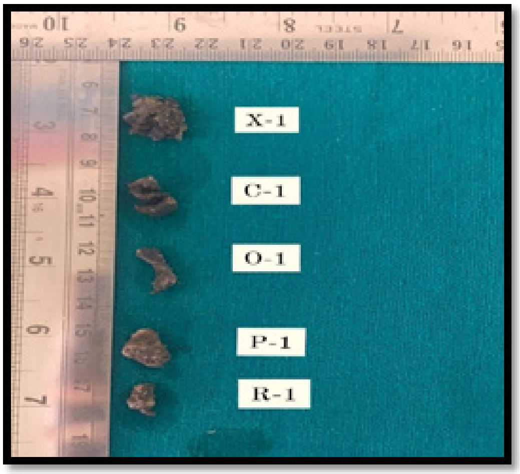


Fig. 2. Soft tissue specimen and labelled

conducted on the stained slides. A comparison was then conducted for various parameters based on the enclosed tables. Photomicrographs of the samples were observed and captured directly after zooming in with a Light microscope.

RESULTS

All the four oils used in this study viz. carrot oil, olive oil, pine oil, rose oil as well as xylene showed shrinkage as well as all the samples processed with all of them had ease of cutting (Table 1). Tissues which were cleared using all the four oils, in terms of nuclear details, cytoplasm details as well as quality of staining, did not show any statistical significance as being poor or inferior to that of xylene whereas that of average or equally good when compared to xylene showed statistical significance. Statistical significance was noted in being good or superior to xylene in pine oil but no such significance was seen in the remaining 3 oils viz., rose oil, carrot oil and olive oil. (Table 3)

All 4 oils had nucleus details, cytoplasm details and quality of staining average and equally good as compared to xylene, pine oil demonstrated good and superior quality as compared to xylene in a statistically significant number of samples while none of the oils were significant in poor or inferior to xylene.

Fig. 3. Reagents used in tissue processing

Fig. 4. Paraffin wax bath where processed tissue is immersed before embedding.

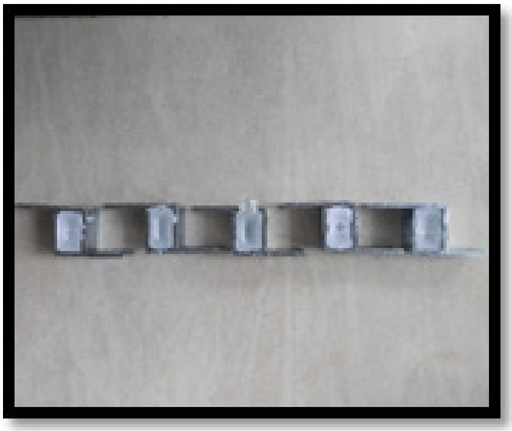


Fig. 5. Embedding

Fig. 6. Tissue Sectioning

DISCUSSION

Xylene emerged as the preferred clearing agent following the discovery of chloroform's carcinogenic properties. Xylene, which is chemically dimethylbenzene whose chemical formula is C6H4(CH3)2, is an aromatic hydrocarbon. In nature, it is found in wood tar, coal and petroleum in the liquid or solid state, which is colorless and has a sweet smell. 'Xylene' comes from 'xylon,' a Greek word which means 'wood,' reflecting its presence in crude wood spirit. According to the Occupational Safety and Health Administration, 100 ppm as an 8-hour time-weighted average concentration is the permissible exposure limit for xylene. Consequently, it is categorized as hazardous waste under the Resource Conservation and Recovery Act.[2] As xylene was classified as a health hazard, the primary aim for researchers and manufacturers became the identification of safer alternatives. Proposed substitutes encompassed terpenes, alkanes, vegetable oils, and more. Therefore, this study was carried out to investigate safer alternatives, such as carrot oil, olive oil, pine oil, and rose oil, considering the hazardous health consequences of xylene. They are used widely for various purposes like cooking oil, natural flavor additives, perfumery, cosmetics, medicine, insecticides, cleaning products, and disinfectants. Clearing agents are used in histopathological laboratories to replace the ethanol within the tissues used during dehydration which is then replaced by molten paraffin wax.

Fig. 7. Sectioned tissue placed on slide and warming

Fig. 8. Hematoxylin and Eosin Staining

Table 1. Sample different oli

Gross changes	Xylene	Carrot oil	Olive oil	Pine oil	Rose oil
Shrinkage	–	–	–	–	–
Ease of cutting	–	–	–	–	–

Grading Scale for Each Criteria
Shrinkage: YES/ NO

Table 2. Presence or absence of gross changes in tissues cleared with the four different oils and xylene

Gross changes	Xylene	Carrot oil	Olive oil	Pine oil	Rose oil
Shrinkage	yes	Yes	Yes	Yes	Yes
Ease of cutting	yes	Yes	Yes	Yes	Yes

In our study, shrinkage and ease of cutting in all the study samples were present for both xylene as well as all four oils. In nucleus details, pine oil had a maximum number of samples (46.7%) with good details followed by carrot oil with 13.3% while olive and pine oil had the least with 10% each. Most of the oils (carrot, olive, and rose) had maximum values in average grade when compared to xylene with 80% each while pine oil had 46.7%. 10% of samples in olive, pine, and rose oil had poor grades, and only 6.7% in carrot oil. In cytoplasm details also, the maximum number of samples (40%) with good details were seen in pine oil followed by olive and rose oil with 13.3% each, and the least in carrot oil with only 10% samples. In average grade maximum value was seen in carrot oil (83.3%) followed by olive and rose oil with 80% each while pine oil had 50%. 6.7% of samples in olive, carrot, and rose oil had poor grades while pine oil had in 10% of samples. Finally, in quality of staining, the maximum number of samples with good quality of staining as compared to xylene were seen in pine oil with 43.3% of samples followed by carrot with 16.6% of samples, olive oil with 10% of samples and rose oil with 6.7% of samples. In average grade maximum value was seen in olive oil and rose oil, each with 83.3% followed by carrot oil with 76.7% and pine oil with 50% of samples. 6.7% of samples in olive, carrot, and pine oil had poor grades while rose oil had in 10% of samples.

Fig. 9. Observation of the slides Under Olympus CH20i Binocular

This study evaluated four different oils individually rather than in mixtures. Previous research by Rasmussen BB, Hjort KN, Mellerup I, Sether G, Christensen N used coconut and olive oil combinations, which resulted in incomplete impregnation and section-cutting difficulties.[3] Similarly, Andre GG, Wenger JB, Rebolloso D, Arrington JB, Mehm WJ tested blend of four oils made of peanut, soybean, coconut, & cotton oil

Table 3. Comparison of nucleus details, cytoplasm details and quality of staining of each of the four oils with xylene

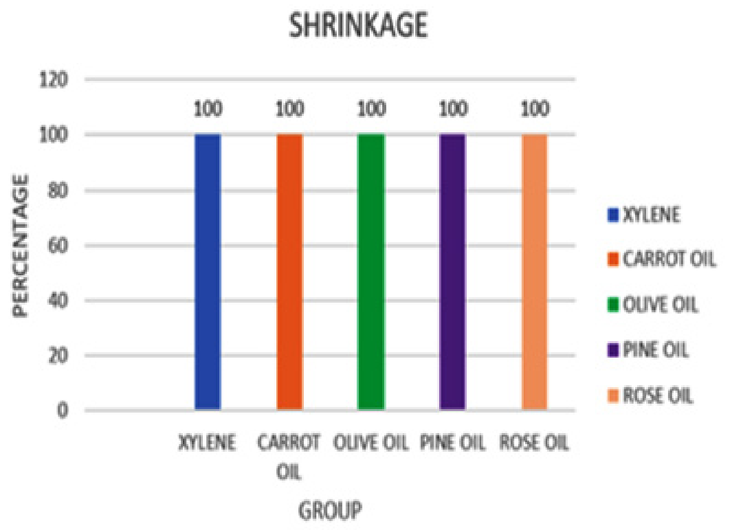
Xylene vs	Grade	p Value Nucleus details	Cytoplasm details	Quality of staining
Carrot oil	Poor	0.211	0.810	0.371
	Average	0.003*	0.031*	0.049*
	Good	0.120	0.471	0.231
Olive oil	Poor	0.086	0.156	0.237
	Average	0.042*	0.012*	0.004*
	Good	0.066	0.081	0.074
Pine oil	Poor	0.077	0.644	0.561
	Average	0.012*	0.002*	0.012*
	Good	0.001*	0.004*	0.010*
Rose oil	Poor	1.000	0.671	1.000
	Average	0.012*	0.021*	0.041*
	Good	0.110	0.084	0.253

*Statistically significant

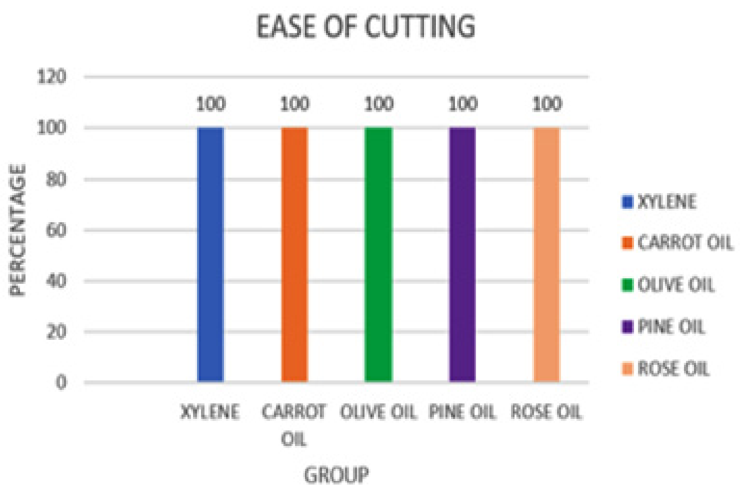
and found it to be inferior to xylene, as the quality of the section deteriorated when these substitutes were mixed with xylene.[6]

In a similar study conducted in 2015 by Swamy SR, Nandan SR, Kulkarni PG, Rao TM, Palakurthy P, they assessed four different oils - rose oil, carrot oil, pine oil, and olive oil - as potential alternatives to xylene for tissue staining. Gross evaluation of tissue specimens, including assessment of shrinkage, ease of cutting, cellular architecture, and staining quality, was conducted for each oil. The morphology of the tissue was effectively maintained in all sections cleared using these oils and the staining quality overall were equivalent with slides that had been treated with xylene.

Furthermore, pine oil group was found to have better results than the other 3 oils and was concluded as having superior characteristics.[8] Our study results were in strong agreement as in our study also shrinkage, ease of cutting, quality of staining, and tissue morphology in terms of nucleus and cytoplasm details of all the oils were equivalent to that of xylene. Similarly, superior characteristics



Graph 1. Descriptive Statistics of Shrinkage

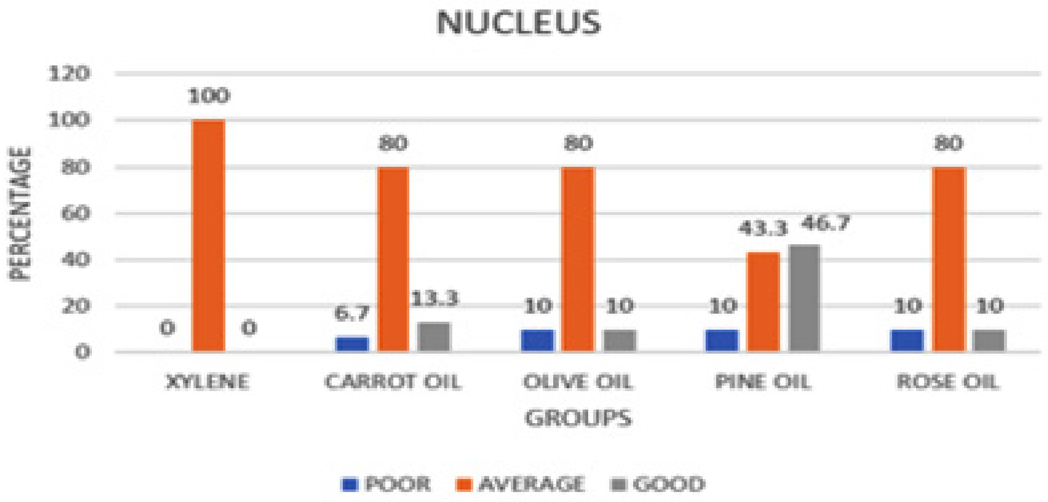


Graph 2. Descriptive Statistics Ease of Cutting

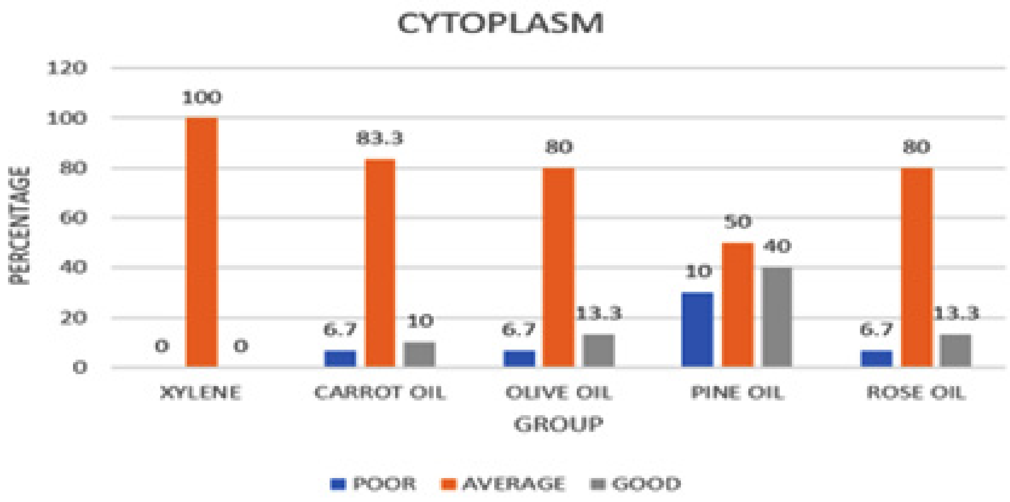
in terms of quality of staining, nucleus, and cytoplasm details in pine oil were also seen.

In 2016, Ghosh S, Rao RS, Nambiar S, Haragannavar VC, Augustine D, Sowmya SV, Patil S examined the effectiveness of refined sunflower oil and extra virgin olive oil as clearing agents to xylene in histopathological staining and concluded that both these oils can serve as environmentally friendly alternatives to xylene in histopathological laboratories.[9] Similarly, Saravanakumar P, Bharanidharan R, Ramadoss R, Kumar AR conducted a study in 2019 that inferred that pure Groundnut and Coconut oil, which are nonhazardous and affordable, can be utilized as an effective replacement.[10]

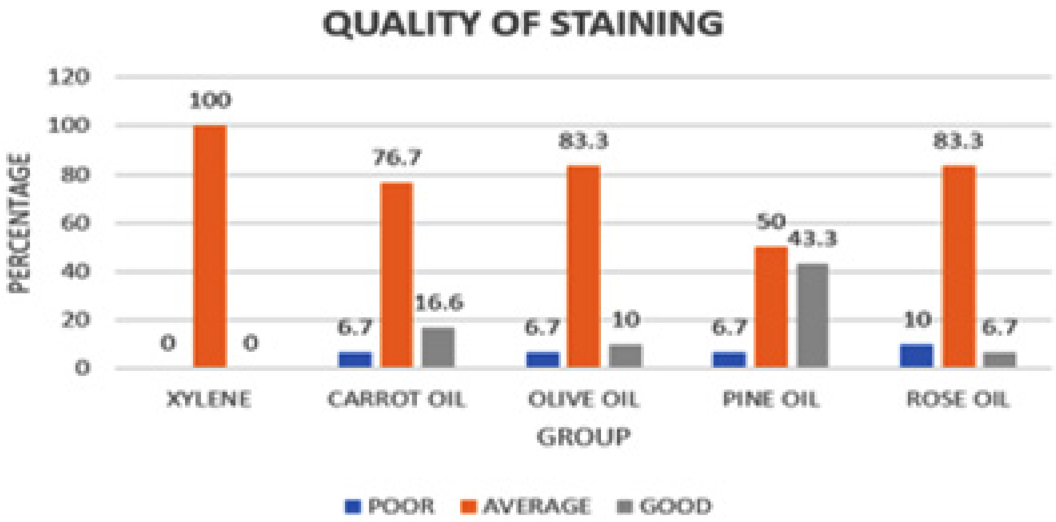
Bordoloi B, Jaiswal R, Tandon A, Jayaswal A, Srivastava A, Gogoi N 2022 selected 45 tissue specimens preserved in formalin and coconut oil's efficacy as a clearing agent was evaluated, compared it with xylene and it was found that it can be used as a clearing agent without the loss of information while recognizing that it may be necessary to investigate a broader range of tissues before making any general recommendation.[12] Our study also holds a similar conclusion that the four oils can be used as an effective clearing agent



Graph 3. Descriptive Statistics of Nucleus Details



Graph 4. Descriptive Statistics of Cytoplasm



Graph 5. Descriptive Statistics of Quality of Staining

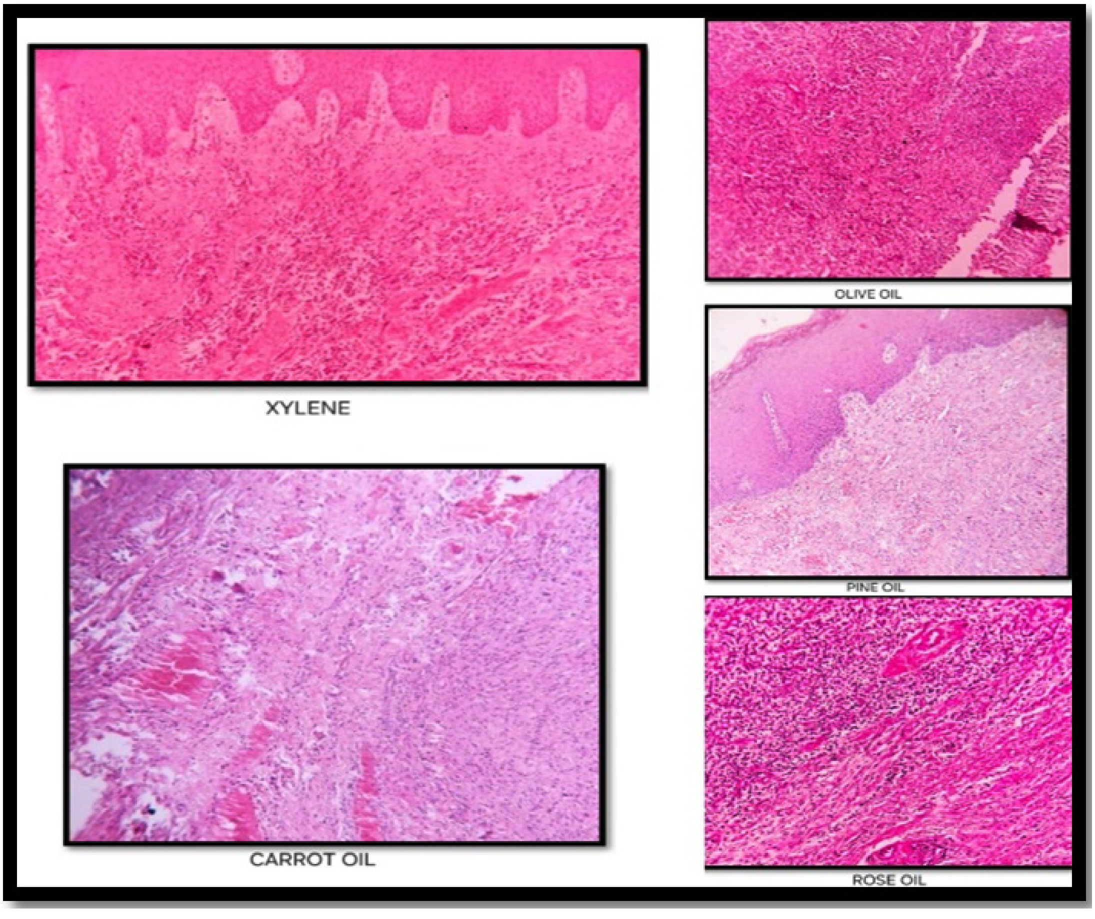


Fig. 10. Photomicrograph of H&E stained sections with Xylene, Carrot oil, olive, Pine oil, Rose oil

substituted for xylene but further research with more parameters and sample size is necessary to come to a more definitive conclusion.

Various researchers have explored different oils as substitutes for xylene. In our study, we examined four specific oils: carrot oil, olive oil, pine oil, and rose oil. Also, olive oil has been used alone and as well as mixed with other oils in previous studies.[11] We evaluated and compared the clearing effects of each of these four oils with those of xylene on gross changes and staining quality in 30 tissue sections. This assessment was carried out at different stages of histopathological procedures and during the microscopic evaluation of the sections, making this study a valuable and distinctive contribution.

CONCLUSION

The hazardous effects of xylene are undeniable, making it imperative for decision-makers to eliminate its use in histological laboratories. This includes removing xylene not only from tissue processing but also from any activity where personnel may come into contact with it. As xylene was classified as a health hazard, the primary aim for researchers and manufacturers became the identification of safer alternatives. Proposed substitutes encompassed terpenes, alkanes, vegetable oils, and more. Therefore, given the hazardous health implications of xylene, this study was conducted to explore safer alternatives, including carrot oil, olive oil, pine oil, and rose oil. Our study was done to establish whether the usage of these oils as a clearing agent has any effect on the shrinkage of tissue, ease of sectioning, nucleus details, cytoplasmic details, and quality of staining when compared to xylene. Our study results suggest that all four oils showed significant efficacy as compared to xylene, especially pine oil which showed better characteristic features than xylene in nucleus details, cytoplasm details, and quality of staining thus concluding that all four oils can be used as safer, non-hazardous, and more economical alternatives to xylene as a clearing agent. However, there is a need for further studies with larger sample sizes and more parameters to validate the usage of bio-friendly xylene alternatives.

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Conflict of Interest

The authors do not have any conflict of interest.

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

The ethical approval to conduct this research was obtained from Institutional Ethics Committee,School Of Medical Sciences And Research,Sharda University,Greater Noida (U.P), REF NO. SU/SMS&R/76-A/2022/134

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Authors' Contribution

Ningombam Jyostna: Conceptualization, Methodology, Writing – Original Draft. Ningombam Jyostna: Data Collection, Analysis. Deepak Bhargava : Review; Editing. Deepak Bhargava, Puja Bansal: Visualization, Supervision, Project Administration. Ningombam Jyostna: Funding Acquisition, Resources.

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