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Poonam
P.G. Scholar, Department of Veterinary Pathology, CVAS, Navania, Udaipur, RAJUVAS, Rajasthan, India

Mamta Kumari
Assistant Professor, Department of Veterinary Pathology, CVAS, Navania, Udaipur (RAJUVAS). Rajasthan, India

Rashmi
P.G. Scholar, Department of Veterinary Pathology, CVAS, Bikaner, RAJUVAS, Rajasthan, India

Rahul Kumar
Livestock assistant (LSA), RAJUVAS, Bikaner, Rajasthan, India

Kokila Himat
P.G. Scholar, Department of Veterinary Pathology, CVAS, Navania, Udaipur, RAJUVAS, Rajasthan, India

Subhita
P.G. Scholar, AGB, CVAS, Bikaner, RAJUVAS, Rajasthan, India

Corresponding Author:
Poonam
P.G. Scholar, Department of Veterinary Pathology, CVAS, Navania, Udaipur, RAJUVAS, Rajasthan, India

The effect of time on clearing of tissues with natural alternatives to xylene in tissue processing

Poonam, Mamta Kumari, Rashmi, Rahul Kumar, Kokila Himat and Subhita

Abstract
As an aromatic hydrocarbon, the histology lab commonly uses xylene for stains, cover slipping, and tissue processing. Despite being a common clearing agent, it is highly toxic, carcinogenic, and potentially hazardous to histopathology lab technicians. The aim of this study was to evaluate the effectiveness of coconut oil, groundnut oil, liquid paraffin, mustard oil, and sesame oil at various time intervals (Thirty minutes, Sixty minutes, and Ninety minutes) to observe the effect on clearing of tissues in comparison to xylene kept for thirty minutes. 48 tissue specimens of liver was gathered from carcass of goat, fixed in 10% NBS and sectioned into 6 groups, namely Group 1 control (Xylene), Group 2 (Coconut oil), Group 3 (Groundnut oil), Group 4 (Liquid paraffin), Group 5 (Mustard oil), and Group 6 (Sesame oil). Xylene-treated samples and natural alternatives-treated samples were evaluated and compared with xylene for clarity and ease of sectioning, and for staining quality, including nuclear staining, cytoplasmic staining, and overall staining quality. Statistically significant differences were not found in either section when the cellular details and staining quality were examined. At various time intervals (30 minutes, 60 minutes, 90 minutes), the natural alternatives were observed to achieve tissue clearing at par with xylene, so compared with toxic xylene, these alternatives may be used without compromising histopathological details.

Keywords: Clearing agent, coconut oil, groundnut oil, liquid paraffin, mustard oil, sesame oil, tissue processing, xylene, histology, histopathology

Introduction
In order to diagnose diseases in dead animals, histopathological examination is routinely performed. This process involves fixation, dehydration, clearing, and embedding, followed by sectioning and staining of tissues (Rahmawati *et al.*, 2020) [10].

Clearing is a vital step in tissue processing that imparts optical clarity or transparency to tissue by removing dehydrants like alcohol/acetone from tissues before they are embedded in a substance, typically paraffin wax (Kumar *et al*. 2019) [9]. Xylene, chloroform, toluene, methyl salicylate, and methyl benzoate are only a few of the numerous cleaning agents utilized in tissue processing. The most often used clearing agent, xylene, may dissolve in both alcohol and paraffin wax (Chandraker *et al.*, 2018) [3].

Aromatic hydrocarbons such as xylene are generally used as solvents in industries and medicine. As a chemical compound, it has the formula C6 H4 (CH 3)2 and is commonly known as "Dimethyl benzene" and it is a uncoloured, sweet smell, flammable liquid or gas, it naturally present in petroleum, coal, and wood tar, and is named after its presence in crude wood spirit (Gr. Xy'lon- wood) (Kandyala *et al*. 2010) [8]. The Occupational Safety and Health Administration (OSHA) states that the permissible exposure levels are 100 parts per million parts of air (ppm) for 8 hours and 200 ppm over 10 minutes as a short-term exposure limit (Bordoloi, 2018) [2].

Inhalation, ingestion, and contact with the eyes or skin are the main ways that "xylene" can be hazardous (Rahmawati *et al*., 2020) [10]. It is soluble in adipose tissue, where it is kept after being taken into the body. Its half-life in subcutaneous fat ranges from 1 to 6 days (Erikson *et al*., 1994). Hazardous effects of xylene include severe neurotoxicity, cardiovascular disturbances, cancer, kidney disease, blood dyscrasias, musculoskeletal system disorders, skin infections, gastric disturbances, and fetotoxicity. There is also evidence that workers who are exposed to toluene or xylene are also at more risk to develop a vascular disorder called Raynaud's phenomenon (Rahmawati *et al.*, 2020) [10]. Researchers have found that laboratory workers who worked with xylene for 1.5 to 18 years had symptoms similar to general

poisoning disorders including pancytopenia and bone marrow toxicity. The health hazards associated with xylene exposure should be minimize to enhance the safety of working environments in histology laboratories (Dapson, 2005) [4].

There was huge concern over its safety, with evidence that its acute neurotoxicity was higher in comparison to that of benzene and toluene In mild form, it enacts as an irritant, but in severe form, it can cause cancer.

Alternatives to "xylene," such as limonene reagents, aliphatic hydrocarbons, mineral oils, and vegetable oils, have also been researched for their effects on the skin, buccal mucosa, salivary glands, tendon, muscle, and lymph nodes. Good histology features were discovered using these alternate clearing agents (Sermadi *et al*., 2014; Saravanakumar *et al*., 2019) [12, 11]. Because they are secure, easily accessible, and less expensive than common chemical cleansing agents, natural oils (such as vegetable and mineral oil) may be a superior option. The goal of the current study is to contrast the effectiveness of natural oils against chemicals for tissue cleaning during the preparation of animal tissues for histopathological investigation.

Materials and Methods

Tissue sample collection

- At the College of Veterinary and Animal Science, Navania, Vallabhnagar, Udaipur, the department of Veterinary Pathology gathered a total of 48 tissue samples of liver from a goat carcass that had been submitted for post-mortem analysis. They were separated into six equal parts for each group, frozen in 10% formalin, taken in triplicates, and categorized as group 1, 2, 3, 4, 5, and 6.
- Tissues were prepared for routine paraffin embedding technique while clearing of tissues was done as follows - Group 1- control (Xylene), Group 2-Coconut oil, Group 3- Groundnut oil, Group 4 -Liquid paraffin, Group 5- Mustard oil and Group 6- Sesame oil.

Tissue processing

The formalin-fixed tissues were cleaned in running water for an entire night before being prepared for paraffin embedding (Lillie, 1965). After being washed, tissues were dehydrated for 1 hour in 70% alcohol, followed by 1 hour in acetone for each of the three changes (I, II, and III). After dehydration, Group 1 tissue specimen (in triplicates) undergoing conventional tissue processing with xylene as clearing agent (I, II, and III changes) for 30 minutes in each change, whereas tissues (in triplicates) of other groups treated with natural alternatives as clearing agents were further divided into three different time treatments (30, 60, 90 minutes) for clearing. The tissue specimens were treated with different oils (as per the group category) for 30 minutes in subgroup I (3 changes), 60 minutes in subgroup II (3 changes), and 90 minutes in subgroup III (3 changes) rather than xylene as a clearing agent (Table 1).

- **Group 1:** Tissue undergoing conventional clearance histopathological procedure with xylene, 3 changes (I, II, and III) for 30 minutes in each change (Table 1).
- **Group 2:** Tissue undergoing conventional clearance histopathological procedure replacing xylene with coconut oil. Three changes of coconut oil (I, II, and III) for 30 minutes subgroup I, 60 minutes in subgroup II, and 90 minutes in subgroup III (Table 1).
- **Group 3:** Tissue undergoing conventional clearance histopathological procedure replacing xylene with three changes of groundnut oil in three different time groups as 30 minutes, 60 minutes, and 90 minutes (Table 1).
- **Group 4:** Tissue undergoing conventional clearance histopathological procedure replacing xylene with liquid paraffin (I, II, and III changes) for 30 minutes, 60 minutes, and 90 minutes in each change time subgroup (Table 1).
- **Group 5:** Tissue undergoing conventional clearance histopathological procedure replacing xylene with mustard oil (I, II, and III changes) for 30 minutes, 60 minutes, and 90 minutes in different time subgroup change (Table 1).
- **Group 6:** Tissue undergoing conventional clearance histopathological procedure replacing xylene with sesame oil (I, II, and III change) for 30 minutes, 60 minutes, and 90 minutes in various time subgroup change (Table 1).
- Tissues were evaluated for ease of sectioning at different time intervals and compared with group 1 as the control.

Table 1: Clearing of tissues by using different clearing agents at different time

Groups	Reagents	Subgroups of different time treatment		
		Time 1	Time 2	Time 3
Group-1 (Control)	Xylene	30 minutes	-	-
Group-2	Coconut oil	30 minutes	60 minutes	90 minutes
Group-3	Groundnut oil	30 minutes	60 minutes	90 minutes
Group-4	Liquid paraffin	30 minutes	60 minutes	90 minutes
Group-5	Mustard oil	30 minutes	60 minutes	90 minutes
Group-6	Sesame oil	30 minutes	60 minutes	90 minutes

The cleared tissues were then embedded in 4 changes of molten paraffin wax (wax I, II for 45 minutes and wax III, IV for 30 minutes) and blocks were maked.

Histopathological examination

Tissue blocks were segmented (4-5µm), and stained using Hematoxylin and Eosin (H&E) and were assessed for evaluation of nuclear staining, cytoplasmic staining, clarity and overall quality of stained slide under low and high power of microscope. The following criteria were used to assess the staining quality of the slides:

Features	Score and criteria
Nuclear staining	0. Out of focus and unclear nuclear membrane 1. Clear staining and nuclear membrane 2. Smooth and clear staining with good contrast
Cytoplasmic staining	0. Out of focus

	1. Cytoplasm clear 2. Clear smooth cytoplasm, clear nuclear membrane, good contrast
Clarity of staining	0. Not clear, hazy, out of focus 1. Clear staining throughout the slide 2. Crisp and contrast staining

The data was examined and elucidated by using ANOVA and significant differences between groups were assessed using Tukey post hoc test.

Results and Discussion

In the current investigation, it was found that these natural substitutes have the same ability as xylene to clean tissue while maintaining cellular architecture. These oils can be recycled and are non-toxic. Group 1 tissue specimen undergoing conventional tissue processing with xylene as clearing agent (I, II, and III changes) for 30 minutes in each change, whereas tissues of other groups treated with natural alternatives as clearing agents were further divided into three different time treatments (30, 60, 90 minutes) for clearing. In other groups tissue specimens were treated with different oils (as per the group category) for 30 minutes in subgroup I (3 changes), 60 minutes in subgroup II (3 changes), and 90 minutes in subgroup III (3 changes) instead of xylene as a clearing agent.

At various points in time, the specimen's ease of sectioning was compared. It was noted that tissues cleared in sesame oil, groundnut oil and mustard oil for 90 minutes in each change showed difficulty in sectioning as compared to control.

At 30 minutes

When all the stained slides were evaluated for quality of staining and compared with group 1 as a control group, group 2 (coconut oil) and group 6 (sesame oil) showed statistically significant decrease in quality of slides when cleared for 30 minutes in each change, whereas quality of slides of group 3, 4, and 5 was similar to xylene (Table 03 and Fig. 01).

At 60 minutes

Quality of staining of slides was similar to control when they were cleared for 60 minutes. The quality of group 3 and 5 slides was better than control although significant differences were absent (Table 03 and Fig. 02).

At 90 minutes

All natural alternatives displayed high-quality slides when the staining on all stained slides was evaluated for clarity and compared to group 1 as the control group. The slides of group 2 were even better than xylene although statistically significant difference was absent (Table 03 and Fig. 03).

Table 3: Mean score of Quality of H & E stained sections cleared with natural alternatives at different time intervals

Groups	Reagents	Quality of staining at 30 minutes T1 (Mean ± SE)	Quality of staining at 60 minutes T2 (Mean ± SE)	Quality of staining at 90 minutes T3 (Mean ± SE)	Quality of staining at 30 minutes T1 (Mean ± SE) Group 1 (Xylene)
Group 2	Coconut oil	1.06 ± 0.04^{a}	1.44 ± 0.09^{b}	1.57 ± 0.08^{b}	1.37 ± 0.07^{b}
Group 3	Groundnut oil	1.29 ± 0.08^{a}	1.51 ± 0.09^{a}	1.33 ± 0.08^{a}	1.37 ± 0.07^{a}
Group 4	Liquid paraffin	1.26 ± 0.09^{a}	1.33 ± 0.08^{a}	1.35 ± 0.07^{a}	1.37 ± 0.07^{a}
Group 5	Mustard oil	1.46 ± 0.10^{a}	1.51 ± 0.10^{a}	1.33 ± 0.10^{a}	1.37 ± 0.07^{a}
Group 6	Sesame oil	1.08 ± 0.05^{a}	1.19 ± 0.06^{ab}	1.40 ± 0.10^{b}	1.37 ± 0.07^{b}

Means with unlike superscript in each row show significant differences at $p<0.05$

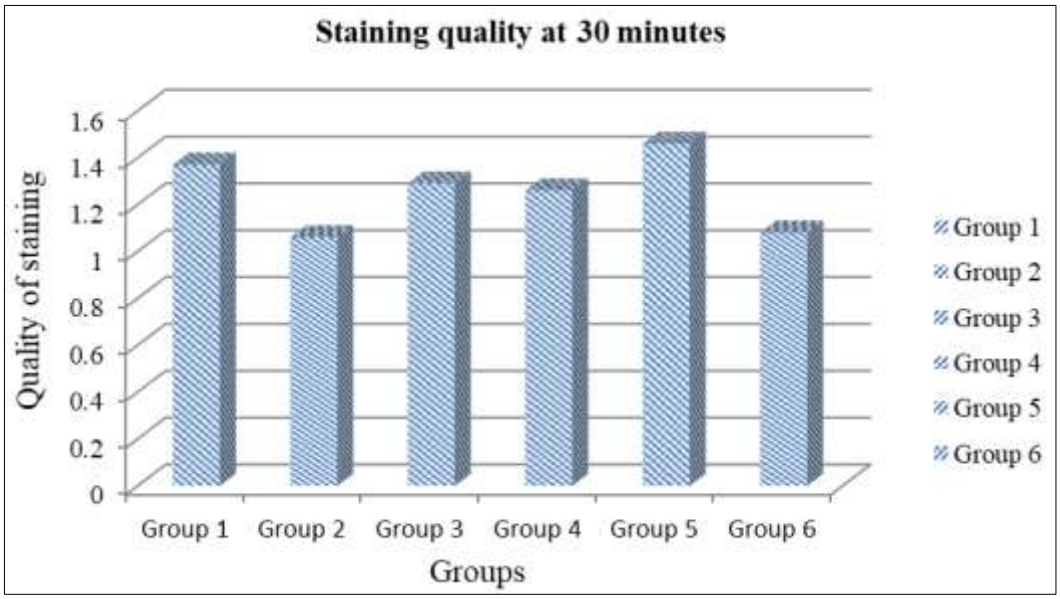


Fig 1: Histogram manifesting comparison of mean score of quality of staining between xylene and other groups (when cleared for 30 minutes in each change)

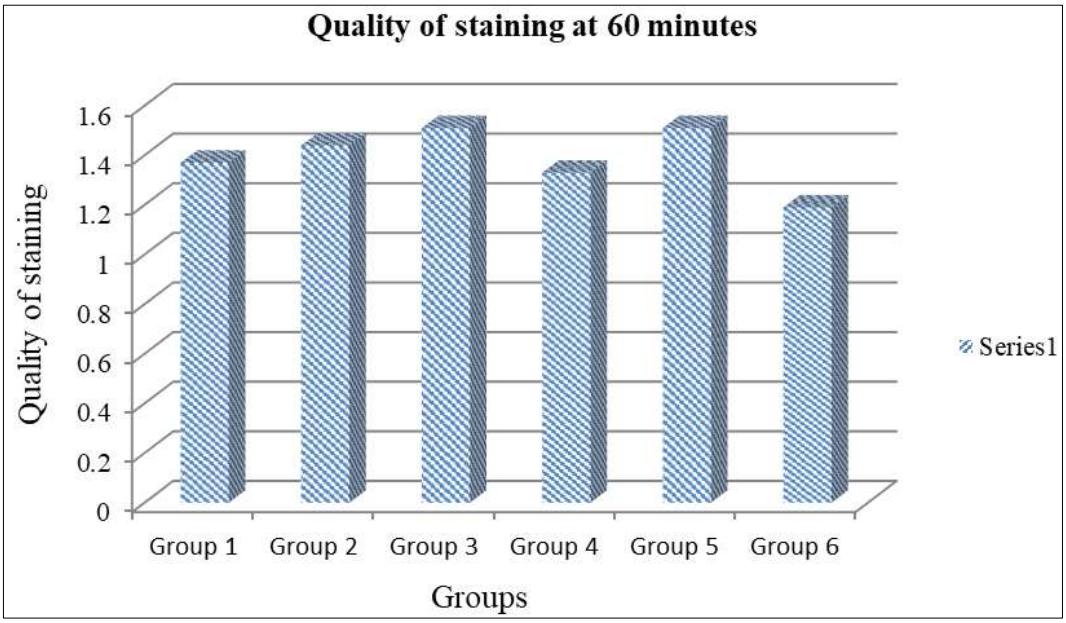


Fig 2: Histogram exhibiting comparison of Mean score of quality of staining between xylene and other groups (when cleared for 60 minutes in each change)

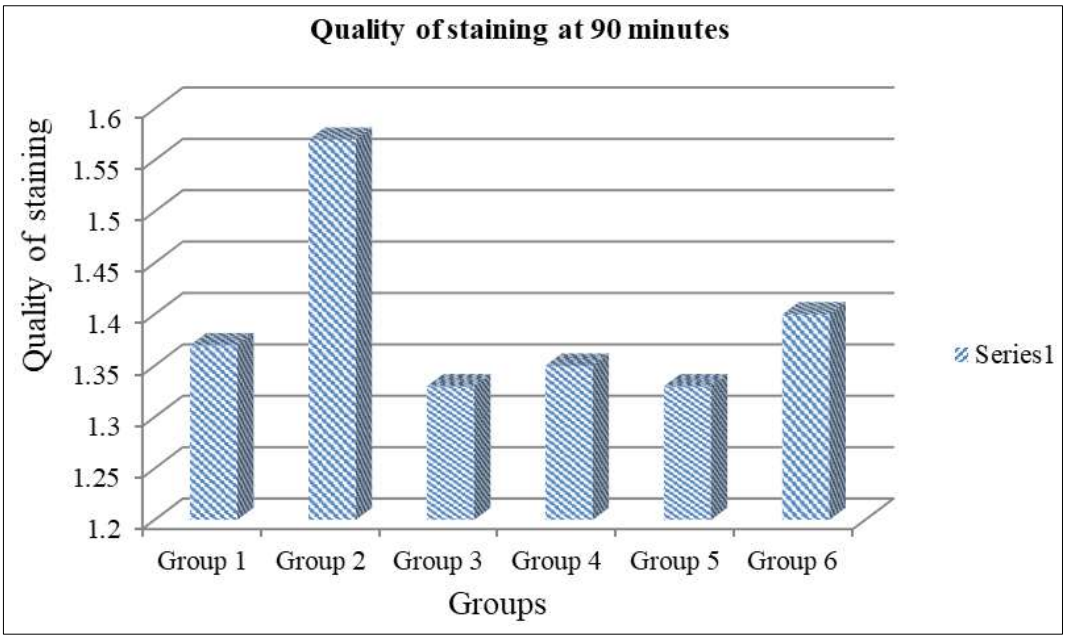
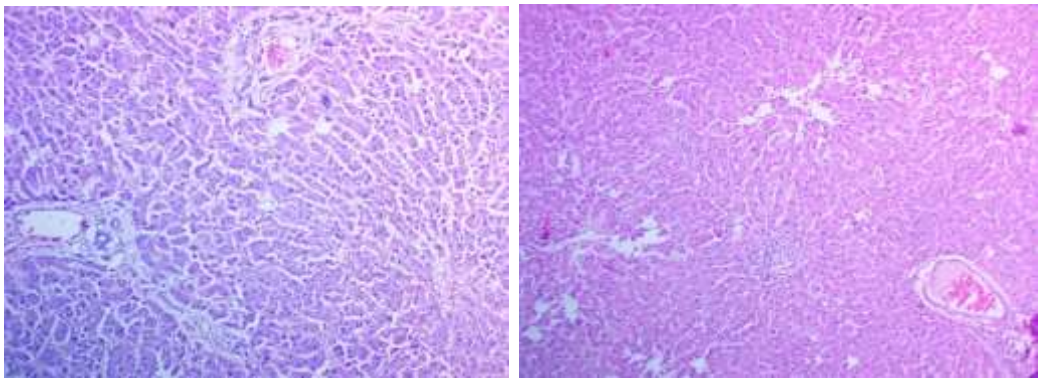


Fig 3: Histogram manifesting Comparison of Mean score of quality of staining between xylene and other groups (when cleared for 90 minutes in each change)



(1) (2)

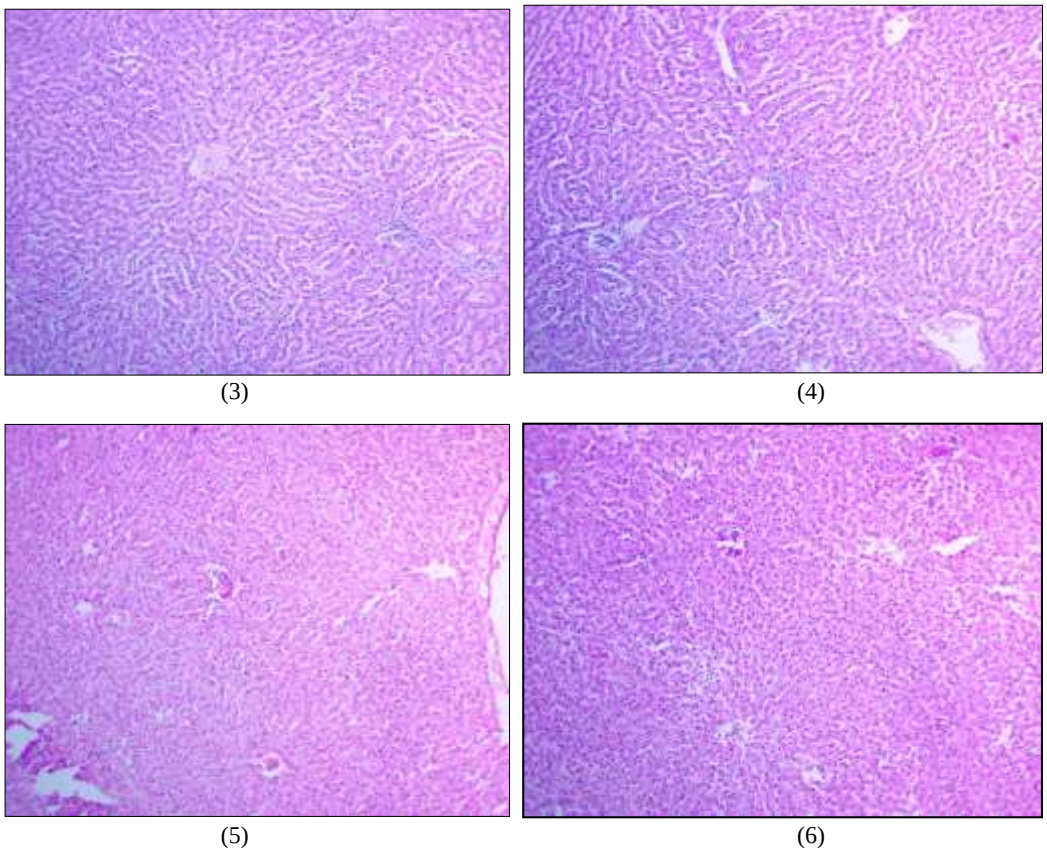


Fig 4: Comparison of microphotographs of Liver tissue sections cleared (at 6o minutes) with (1) Group 1- xylene, (2) Group 2- Coconut oil, (3) Group 3- Groundnut oil, (4) Group 4- Liquid paraffin, (5) Group 5- Mustard oil and (6) Group 6- Sesame oil. H & E (100x)

The results of the experiment done to assess the time required for clearing of tissues with natural alternatives revealed that these oils were capable of clearing the tissues and good quality H & E stained sections at par with xylene were achieved when clearing was done for 60 and 90 minutes in each change for 3 changes, whereas group 2 (coconut oil) showed statistically significant decrease in quality of slides when cleared for 30 minutes. The results revealed that the ease of sectioning was easy at 60 minutes time. For clearing the tissues, the viscosity of a solution plays an important role in the ease of penetration. Less viscous solutions penetrate faster than those with a high viscosity. This could be due to the reason that these natural alternatives have low viscosity so they are able to penetrate the tissues and displace the dehydrating agent (Swamy *et al.* 2015) [13].

Sermadi *et al.* (2014) [12] compared efficacy of coconut oil (CO) as a clearance agent with "xylene." and found that "CO" is an effective alternative to "xylene" since it's nonhazardous, cheaper, and results in less tissue shrinkage. So it can be used as a de-alcoholizing agent in histopathology laboratories without affecting the histological information.

However, Esan *et al.* (2015) [5] used groundnut oil instead of Xylene as a clearing agent on Wistar rat tissues, and used different kinds of special stains which revealed no significant differences from tissues cleared with Xylene.

Moreover, Results of a study conducted by Alwahaibi *et al.* (2018) [1] are also in agreement with our study. They stated that nuclear staining, cytoplasmic staining, and quality of staining of all samples cleared with ultra-clear™ as a clearing agent instead of xylene was at par with xylene.

Similar findings were obtained by Tsamiya *et al.* (2021) [14], they used clove oil, olive oil, and groundnut oil in contrast with xylene treated tissues and found that there was a closer comparison between these oils and xylene in terms of their ability to clear tissue and maintain their cellular structure. They also revealed that groundnut oil was prefer in clearing ability as compared to other oils used.

Conclusion

Based on the results of this study, We draw the conclusion that all natural alternatives tested in the current investigation are suitable for use as clearing agents in histological preparations. They are safer substitutes for xylene in the processing of tissues because they are less expensive, easier to find, and don't pose a threat. They also have lovely smells. In addition, they preserve good staining quality and cell architecture.

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References

1. Alwahaibi N, Aljaradi S, Alazri H. Alternative to xylene as a clearing agent in histopathology. Journal of laboratory physicians. 2018;10(02):189-193.
2. Bordoloi B. Xylene Its Health Hazards and Biocompatible Substitutes. World Journal of Pharmaceutical Research. 2018;7(15):1017-1027.
3. Chandraker R, C Rathod V, K Chandraker N, Pundir S, Dixit S, Desai V. Comparison between Xylene and Coconut Oil in Tissue Processing. Modern Medical Laboratory Journal. 2018;2(1):96-99.
4. Dapson JC, Dapson RW. Hazardous materials in the histopathology laboratory: regulations, risks, handling

and disposal. 4th ed. Battle Creek: Anatech; c2005. p. 65-78.
5. Esan EO, Ajileye AB, Fasogbon SA. Groundnut oil as an alternative clearing agent to xylene in histological tissue processing. Advance Laboratory Medicine International. 2015;5(3):24-31.
6. Erickson T, Amed V, Leibach SJ, Bushnik P, Saxon A, Hryhorczuk DO, *et al*. Acute bone marrow toxicity and pancytopenia following exposure to lead chromate, xylene, and ethylbenzene in a degloving injury. American journal of hematology. 1994;47(4):257-261.
7. Lillie RD. Histopathological techniques and practical histochemistry Mc Graw Hill Book Company., New York; c1965. p. 176.
8. Kandyala R, Raghavendra SPC, Rajasekharan ST. Xylene: An overview of its health hazards and preventive measures. Journal of oral and maxillofacial pathology. 2010;14(1):1-5.
9. Kumar P, Dar Y, Gedam PM, Kumar A. Study on the use of virgin coconut oil as clearing agent in tissue processing [Paper presentation]. XXXIV Annual Convention of Indian Association of Veterinary Anatoimists & National Symposium on Recent Advances in Veterinary Anatomy and its Application in Clinical Sciences: Bengaluru, Karnataka, India. 2019.
10. Rahmawati S, Janar Wulan A, Utami N, Jaya BPD. Preliminary Study: The Potency of Vegetable Cooking Oil as Alternative Clearing Agent for Histological Preparation. International Confrence on Agromedicine and Medical Sciences; c2020. p. 01-08.
11. Saravanakumar P, Bharanidharan R, Ramadoss R, Kumar AR. Efficacy of "Groundnut oil" and "coconut oil" as a substitute for "xylene" in clearing tissues samples: A comparative study. SRM Journal of Research in Dental Sciences. 2019;10(4):194-196.
12. Sermadi W, Prabhu S, Acharya S, Javali SB. Comparing the efficacy of coconut oil and xylene as a clearing agent in the histopathology laboratory. Journal of oral and maxillofacial pathology. 2014;18(4):49-53.
13. Swamy SRG, Nandan SRK, Kulkarni PG, Rao TM, Palakurthy P. Bio-friendly alternatives for xylene–Carrot oil, olive oil, pine oil, rose oil. Journal of clinical and diagnostic research. 2015;9(11):ZC16.
14. Tsamiya RI, Muhammad HT, Mohammed MO, Abubakar U, Mohammed I, Muhammad AT, Ajayi AS. Comparative Evaluation of Clove, Olive and Groundnut Oil's Clearing Ability in Tissue Processing. Journal of Medical Laboratory Science. 2021;31(1):43-53.