Original Article

# Comparative Evaluation Of Olive Oil And Coconut Oil With Xylene As A Clearing Agent In Histopathology

**Dr Abinay Ranjan Sinha[1*], Dr Garima Bhatt[2], Dr Sandeep Jain[3], Dr Rajat Varshney[4], Dr Gourab Dutta[5], Dr Madhuparnee Chaudhury[6]**

1. 3rd year PG, Department of Oral Pathology and Microbiology, Darshan Dental College and Hospital, Udaipur, Rajasthan, India
2. Reader, Department of Oral Pathology and Microbiology, Darshan Dental College and Hospital, Udaipur, Rajasthan, India
3. Professor and HOD, Department of Oral Pathology and Microbiology, Darshan Dental College and Hospital, Udaipur, Rajasthan, India
4. Professor, Department of Oral Pathology and Microbiology, Darshan Dental College and Hospital, Udaipur, Rajasthan, India
5. BDS MDS, Consultant Orthodontist, West Bengal, India
6. Reader, Dept. of Orthodontics, KSD Jain Dental College and Hospital, West Bengal, India

*Corresponding author:
Dr Abinay Ranjan Sinha, 3rd year PG, Department of Oral Pathology and Microbiology, Darshan Dental College and Hospital, Udaipur, Rajasthan, India
Email Id: drabhinay2988@gmail.com
DOI: 10.47750/pnr.2022.13.S10.670

## Abstract

**Aim:** The aim of the present study was to evaluate the efficacy of olive oil and coconut oil as clearing agent with regards to Xylene and to compare the efficacy of olive oil and coconut oil as clearing agent in two different histopathological staining methods.

**Methods:** The study was carried out in Department of Oral Pathology & Microbiology, Darshan Dental College & Hospital, Loyara (Udaipur), Rajasthan. A total of 192 tissue specimens were collected, fixed in 10% formalin, and sectioned into 8 equal parts in each group broadly divided in 6 grouped as group I, II, III, IV, V, VI Groups were divided on the clearing agents and stains used respectively. Tissue specimen were taken for routine processing followed by H&E and PAS staining procedure with xylene in group I & II as clearing agent, whereas group III & IV have Coconut oil as clearing agent and group V & VI have Olive oil as clearing agent.

**Results:** Coconut and Olive Oil treated specimen showed better characteristic features than Xylene treated specimen with respect to rigidity, tissue shrinkage and translucency respectively with p (<0.001). Both Coconut and Olive oil treated specimen showed similar features in almost all parameters on both macro and microscopic levels with insignificant p value of (>0.05). Among H&E and PAS-stained slides, coconut and Olive oil sections showed better nuclear staining, cytoplasmic staining and quality of staining compared to Xylene processed sections.

**Conclusion:** Coconut and Olive oil treated specimen showed better characteristic features than xylene treated specimen with respect to rigidity, tissue shrinkage and translucency. Coconut and Olive oil treated sections showed better nuclear staining, cytoplasmic staining, and quality of staining than Xylene treated sections.

**Keywords:** olive oil, coconut oil, histopathological staining methods, xylene, clearing agents

## INTRODUCTION

The term “xylene” is a Greek word meaning “Wood” as it is found in crude wood spirit. It is an aromatic hydrocarbon (dimethylbenzene [C6H4(CH3)2]) derived from petroleum, which occurs naturally in petroleum and coal tar and is

also formed, to a small extent, during forest fires. It is a colorless, flammable liquid with a sweet odor.[1,2] Apart from its various uses, xylene is commonly used in histopathology laboratories as a de-alcoholization agent during tissue processing and in staining and mounting of tissue sections.[3] It has a high solvency factor that helps in maximum displacement of alcohol rendering the tissue transparent and thus enhances paraffin infiltration. In staining procedures, its excellent dewaxing and clearing capabilities contribute to brilliant staining of the tissue sections.[4]

Xylene was substituted as a safe alternative to other hazardous chemicals such as aniline oil, benzene, chloroform, dioxane and toluene in the histology laboratory in the 1950s. However, this proved to be a failure because of its toxicity which ranges from acute neurotoxicity, cardiac and kidney injury, cancer, blood dyscrasias, skin diseases, gastrointestinal disturbances, musculoskeletal system disorders, fetotoxicity and tissue distortions as a result of long-term immersion of tissue in xylene.[5] By the late 1970s, histopathologists started raising concerns about its safety, with evidence of its acute neurotoxicity being greater than that of benzene or toluene.[6] Since then, the search for a safe xylene substitute has been going on. With that aim, various xylene substitutes such as limonene reagents, aliphatic hydrocarbons, aromatic hydrocarbon mixtures, vegetable oils and mineral oils were tried in the past, but they did not receive much popularity as they were found to be either equally toxic or less effective or expensive.[3,4]

Clearing agents are among the most noxious and hazardous chemicals found in histology laboratories. Toxic effects of xylene includes acute neurotoxicity, cardiac and kidney injuries, some fatal blood dyscrasias, and other less dangerous problems, such as skin erythema, drying and scaling, and secondary infections, all associated with its use and caused by depletion of mitochondrial adenosine triphosphate in the affected cells. Because of new regulations from Occupational Safety and Health Administration (OSHA) and the Environmental Protection Agency (EPA),[3,4,7,8] several xylene substitutes such as limonene reagents, aliphatic hydrocarbons, vegetable oils and mineral oils have been commercially developed in recent years.[9-11] However these commercially available xylene substitutes are less effective, more expensive, and not much less hazardous than xylene itself. Coconut and olive oil are commonly used vegetable oils available throughout the tropical world. They are non-toxic, heat stable, slow to oxidize and have highest resistant to rancidity.[12-14]

The aim of the present study was to evaluate the efficacy of olive oil and coconut oil as clearing agent with regards to Xylene and to compare the efficacy of olive oil and coconut oil as clearing agent in two different histopathological staining methods.

## MATERIALS AND METHODS

The study was carried out in Department of Oral Pathology & Microbiology, Darshan Dental College & Hospital, Loyara (Udaipur), Rajasthan. A total of 192 tissue specimens were collected, fixed in 10% formalin, and sectioned into 8 equal parts in each group broadly divided in 6 grouped as group I, II, III, IV, V, VI Groups were divided on the clearing agents and stains used respectively. Tissue specimen were taken for routine processing followed by H&E and PAS staining procedure with xylene in group I & II as clearing agent, whereas group III & IV have Coconut oil as clearing agent and group V & VI have Olive oil as clearing agent.

- Group I –Tissue samples undergoing conventional Hematoxylin and eosin histo- pathological procedure with Xylene.

- Group II –. Tissue samples undergoing conventional Periodic acid – Schiff histo- pathological procedure with Xylene.

- Group III- Tissue samples undergoing conventional Hematoxylin and eosin histo- pathological procedure replacing Xylene with coconut oil.

- Group IV- Tissue samples undergoing conventional Periodic acid- Schiff histo- pathological procedure replacing Xylene with coconut oil.

• Group V - Tissue samples undergoing conventional Hematoxylin and eosin histo- pathological procedure replacing Xylene with Olive oil.

• Group VI - Tissue samples undergoing conventional Periodic acid- Schiff histo- pathological procedure replacing Xylene with Olive oil.

Selection of Specimens:

Soft Tissue specimens were collected from goat from buccal mucosa, lymph node, liver, and salivary gland.

Inclusion criteria:

1) Only Soft tissue specimens will be considered.

2) Specimens with size of 0.5cm X 1cm or greater and thickness of 3-5mm will be considered.

3) Tissue specimens which are adequately formalin fixed.

4) Freshly Cut Tissue.

Exclusion criteria:

1) Inadequate tissue specimen.

2) Tissue specimens which are not adequately formalin fixed.

3) Tissue which are Old/Previously Extracted.

Procedure in details: -

• Soft tissue specimen from the goat fulfilling the inclusion and exclusion criteria of the study were taken and studied in the department of Oral and Maxillofacial Pathology and Microbiology of Darshan Dental College and Hospital. The sample included a total of 192 tissue specimens.

• Each of 192 specimens collected were fixed with 10% buffered formalin for 48 hours. Tissue specimens were cut into six parts and were divided in experimental groups namely Group I, Group II, and Group III, Group IV, Group V, Group VI in which each group consisting of 32 tissue bits.

Methods of Observation:

i. Evaluation of Rigidity: The tissue samples after clearing in three different clearing agents like xylene, coconut oil and olive oil respectively according to their groups are assessed by palpation between two fingers to observe the rigidity level of each specimen. The scoring is done as (0- Soft, 1- Semi- rigid, 2 – Rigid).

ii. Evaluation of Tissue Shrinkage: The tissue samples are measured individually before and after clearing with xylene, coconut oil and olive oil respectively according to the groups that they are divided. The change in measurements before and after clearing are noted and scored as (0 - < 10%, 1 – 11-25% ,2- >25%).

iii. Evaluation of Translucency: The tissue samples after clearing in three different clearing agents like xylene, coconut oil and olive oil respectively according to their groups are observed using reflected light and scored as (0 – Opaque, 1 – Mild Opaque/ Mild Translucent, 2 – Translucent).

iv. Evaluation of Nuclear Staining: The stained slides in each group processed by xylene, coconut oil and olive oil respectively are observed under 40X magnification of Light microscope for nuclear staining and is scored as (0 –

Poor indistinct smudging and pyknosis of nuclei, 1 – Good distinct chromatin condensation, prominent nuclear membrane).

v. Evaluation of Cytoplasmic Staining: The stained slides in each group processed by xylene, coconut oil and olive oil respectively are observed under 40X magnification of Light microscope for cytoplasmic staining and is scoredas (0 - indistinct / blurred nuclear-cytoplasmic contrast., 1 – distinct architecture and good nuclear-cytoplasmic contrast).

vi. Evaluation of Quality of Staining: The stained slides in each group processed by xylene, coconut oil and olive oil respectively are observed under 10X magnification of Light microscope for cytoplasmic staining and is scored as (0 - Poor indicates that the tissue failed to take up stain adequately, stained unevenly, 1 – Satisfactory indicates details not visualized up to the mark, 2 - Good designates good contrast between the nucleus and cytoplasmand visibility of details along with brilliance of staining).

## RESULTS

Data was tabulated and subjected to statistical analysis for interpretation of results. One way analysis of variance followed by post hoc Bonferroni test was applied for pair wise comparison. Conclusions were drawn based on the statistical analysis.

Table 1: One Way ANOVA for Rigidity in three groups of tissues processed with Xylene, Coconut oil and Olive oil

| **Variance** | **N** | **Mean** | **Std. Deviation** | **Std. Error** |
|---|---|---|---|---|
| Xylene (Gr – I & II) | 64 | 1.52 | .534 | .067 |
| Coconut Oil (Gr – III & IV) | 64 | .66 | .597 | .075 |
| Olive Oil (Gr – V & VI) | 64 | .33 | .473 | .059 |
| Total | 192 | .83 | .733 | .053 |

Statistical analysis:

One Way ANOVA for Rigidity in three groups of tissues processed with Xylene, Coconut oil and Olive oil. On subjecting the values of rigidity to the One-way ANOVA, the p was 0.000 indicating that there is statistically significant difference between the study groups. ($p<0.05$).

Table 2: Descriptive statistics for Tissue Shrinkage in three groups of tissues processed with Xylene, Coconut oil and Olive oil

| **Variance** | **N** | **Mean** | **Std. Deviation** | **Std. Error** |
|---|---|---|---|---|
| Xylene (Gr – I & II) | 64 | 1.20 | .739 | .092 |
| Coconut Oil (Gr – III & IV) | 64 | .78 | .766 | .096 |
| Olive Oil (Gr – V & VI) | 64 | .44 | .560 | .070 |
| Total | 192 | .81 | .758 | .055 |

One Way ANOVA for Tissue Shrinkage in three groups of tissues processed with Xylene, Coconut oil and Olive oil. On subjecting the values of Tissue Shrinkage to the One-way ANOVA, the p was 0.000 indicating that there is statistically significant difference between the study groups. ($p<0.05$).

Table 3: Descriptive statistics for Translucency in three groups of tissues processed with Xylene, Coconut oil and Olive oil

| Variance | N | Mean | Std. Deviation | Std. Error |
|---|---|---|---|---|
| Xylene (Gr – I & II) | 64 | .67 | .778 | .097 |
| Coconut Oil (Gr – III & IV) | 64 | 1.19 | .732 | .091 |
| Olive Oil (Gr – V & VI) | 64 | 1.47 | .563 | .070 |
| Total | 192 | 1.11 | .768 | .055 |

One Way ANOVA for Translucency in three groups of tissues processed with Xylene, Coconut oil and Olive oil. On subjecting the values of Translucency to the One-way ANOVA, the p was 0.000 indicating that there is statistically significant difference between the study groups. ($p<0.05$).

Table 4: Descriptive statistics for Nuclear Staining using H&E in three groups of tissues processed with Xylene, Coconut oil and Olive oil

| Variance | N | Mean | Std. Deviation | Std. Error |
|---|---|---|---|---|
| Xylene (Gr – I) | 32 | .38 | .492 | .087 |
| Coconut Oil (Gr – III) | 32 | .72 | .457 | .081 |
| Olive Oil (Gr – V) | 32 | .63 | .492 | .087 |
| Total | 96 | .57 | .497 | .051 |

It shows the xylene processed samples with mean and standard deviation of .38 ± .492 had more poor nuclear staining when seen in Light Microscope under 40X magnification, compared to samples of Coconut oil and Olive oil with Mean of .72 ± .457 and .63 ± .492 shows good nuclear staining. There is a statistically significant difference with p value (<0.001). In samples cleared with xylene to that of coconut and olive oil.

Table 5: Descriptive statistics for Cytoplasmic Staining using H&E in three groups of tissues processed with Xylene, Coconut oil and Olive oil

| Variance | N | Mean | Std. Deviation | Std. Error |
|---|---|---|---|---|
| Xylene (Gr – I) | 32 | .31 | .471 | .083 |
| Coconut Oil (Gr – III) | 32 | .53 | .507 | .090 |
| Olive Oil (Gr – V) | 32 | .72 | .457 | .081 |
| Total | 96 | .52 | .502 | .051 |

Descriptive statistics for Cytoplasmic Staining using H&E stain in three groups of tissues processed with Xylene, Coconut oil and Olive oil. It shows the xylene processed samples with mean and standard deviation of .31 ± .471 showed more indistinct cytoplasmic staining when seen in light microscope under 40X magnification, compared to samples cleared with Coconut and Olive oil with Mean of .53 ± .507 and .72 ± .457 shows distinct cytoplasmic staining. There is a statistically significant difference with p value (<0.001). In samples cleared with xylene to that of olive oil and no such difference with coconut oil cleared samples.

Table 6: Descriptive statistics for Nuclear Staining using PAS in three groups of tissues processed with Xylene, Coconut oil and Olive oil

Descriptive statistics for Nuclear Staining using PAS stain in three groups of tissues processed with Xylene, Coconut oil and Olive oil. It shows the xylene processed samples with mean and standard deviation of .44± .504 had more poor nuclear staining when seen in Light Microscope under 40X magnification, compared to samples of Coconut oil and Olive oil with Mean of .75 ± .440 and .78 ± .420 shows good nuclear staining. There is a statistically significant difference with p value (<0.001). In samples cleared with xylene to that of coconut and olive oil.

| Variance | N | Mean | Std. Deviation | Std. Error |
|---|---|---|---|---|
| Xylene (Gr – II) | 32 | .44 | .504 | .089 |
| Coconut Oil (Gr – IV) | 32 | .75 | .440 | .078 |
| Olive Oil (Gr – VI) | 32 | .78 | .420 | .074 |
| Total | 96 | .66 | .477 | .049 |

## DISCUSSION

Xylene acts as a clearing agent, with the purpose of making the histological tissue sections clear or transparent so that the detailed morphological structure of the tissues can be examined.[15] According to Occupational Safety and Health Administration (OSHA), the permissible exposure limit of xylene is 100 parts of xylene per million parts of air (ppm). The type and severity of health effects depends on several factors, including the amount of chemical one is exposed to, duration of exposure, individual response, and the route of exposure.[16] Considering the toxicity of xylene, it is desirable to minimize its use in histopathology laboratory without compromising the staining quality and hence the appropriate diagnosis.[17] Any experiment reducing health hazards in histology laboratories deserves to be tried.[18]

Routine paraffin wax tissue processing requires properly timed dehydration, clearing (de-alcoholization), infiltration and embedding procedures. On microtomy, sections are dewaxed and further dehydration is done before staining procedure. In this study, tissue specimens are processed in parallel with Xylene, Coconut oil and Olive oil as a clearing agent. Staining is done with two stains H&E staining and PAS Stain. In our present study, 192 samples taken from 4 different sites were divided into 6 groups and processed in xylene and coconut oil and Olive Oil as per standardized processing protocol. Xylene processed tissue samples were found to be more rigid than Coconut oil and Olive Oil processed tissue samples ($p < 0.01$). Xylene processed tissue samples showed more tissue shrinkage than Coconut oil and Olive oil processed tissue samples ($p < 0.01$) and similar to the study done by Sermadi W et al, they conducted study on 60 tissue samples and found that shrinkage was relatively less in Coconut oil processed tissue sample than that of Xylene processed tissue sample.[3]

Cellular architecture and staining parameters like Nuclear, Cytoplasmic and Quality of staining in PAS stain between cleared samples of coconut oil and Olive oil shows no statistically significant difference which is in line to study done by Sermandi W et al found that histochemical and immunohistochemical stains showed identical features in both xylene and olive oil processed samples. Periodic acid Schiff's reagent when used on submandibular gland-stained mucous cells with magenta color indicating same stain intensity and specificity as that of xylene processed tissue samples.[19] In a study done by Sermandi W et al there was no difference in staining quality and tissue architecture in both kinds of specimens. CO-S, when stained with Periodic acid-Schiff (PAS), showed similar details as seen in XY-S.[3]

Cellular architecture and staining parameters like Nuclear, Cytoplasmic and Quality of staining in H & E stain between cleared samples of coconut oil and Olive oil with that of Xylene processed samples shows statistically significant difference which is in contrast to the finding of study done by Ghosh et al that the H & E staining quality of sections of four different types of tissues cleared and deparaffinized using extra virgin olive oil were at par with xylene cleared and deparaffinized tissues. Although the H and E staining quality of tissue sections cleared and deparaffinized using RSO were inferior to xylene and extra virgin olive oil, they were adequate for histopathological interpretation. The results obtained were statistically significant ($P < 0.001$) in all the three groups.[20] Tanwar M et al found that Coconut oil is equivalent to Xylene as clearing agent and dewaxing agent when compared to that of Xylene in routine tissue processing and hematoxylin and eosin (H&E) staining.[21] Sermandi W et al found olive oil have no adverse effects on the quality of staining, cellular and nuclear details during clearing as compared to xylene cleared tissue samples.[19]

In line with our results a study of Rasmussen et al. used olive oil for the clearing process and coconut oil for deparaffinization before staining. They found that in 91% of the cases, there were no differences in the quality of the two slides. The oil-prepared tissue was evaluated as identical to or better than the xylene-prepared tissue in 94% of the cases. They concluded that vegetable oil may be substituted for xylene without loss of information using H & E stain.[22] In accordance to our study findings using a different Oil Thamilselvan et al 2021 found that the muscle samples processed with cedarwood oil and xylene yielded a better nuclear staining and cytoplasmic staining of samples processed in cedarwood oil. The difference was found to be statistically significant (nuclear staining; $P = 0.015$, cytoplasmic staining; $P = 0.042$). In adipose tissue and mucosa showed similar results. Nuclear and cytoplasmic staining of the tissues processed with cedarwood was better compared to xylene using H&E stain.[23]

## CONCLUSION

In conclusion, we can state that in quality of tissue processing and staining coconut oil and olive oil can be used to almost same effect and has better properties than xylene. Hence, they are a good natural replacement of xylene because they are free of health hazards to laboratory technicians without compromising the quality of staining for a good diagnosis. Further studies need to be done in this field with other type of stains and tissues.

## REFERENCES


1. Toxicological Profile for Xylene. U.S Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry (ATSDR); 2007.
2. Occupational Health Guideline for Xylene. National Institute for Occupational Safety and Health (NIOSH).
3. 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: JOMFP. 2014 Sep;18(Suppl 1):S49.
4. Kandyala R, Raghavendra SP, Rajasekharan ST. Xylene: An overview of its health hazards and preventive measures. Journal of oral and maxillofacial pathology: JOMFP. 2010 Jan;14(1):1.

5. Adeniyi IM, Adejoba OR, Akinlabi FM, Alao OJ. Vegetable oils as clearing agents. Achievements in the Life Sciences. 2016 Jun 1;10(1):1-4.
6. Buesa RJ, Peshkov MV. Histology without xylene. Annals of diagnostic pathology. 2009 Aug 1;13(4):246-56.
7. Langman JM. Xylene: its toxicity, measurement of exposure levels, absorption, metabolism and clearance. Pathology. 1994 Jan 1;26(3):301-9.
8. Engström K, Husman K, Pfäffli P, Riihimäki V. Evaluation of occupational exposure to xylene by blood, exhaled air and urine analysis. Scandinavian Journal of Work, Environment & Health. 1978 Jun 1:114-21.
9. Buesa RJ. Mineral oil: the best xylene substitute for tissue processing yet?. Journal of histotechnology. 2000 Jun 1;23(2):143-9.
10. RASMUSSEN BB, HJORT KN, MELLERUP I, SETHER G, CHRISTENSEN N. Vegetable oils instead of xylene in tissue processing. Apmis. 1992 Jul;100(7-12):827-31.
11. Lyon H, Holm I, Prentø P, Balslev E. Non-hazardous organic solvents in the paraffin-embedding technique: a rational approach: Aliphatic monoesters for clearing and dewaxing: butyldecanoate. Histochemistry and cell biology. 1995 Apr;103:263-9.
12. Kenan Tanılgana, Musa Özcanb K and Ünver A. Physical and chemical characteristics of five Turkish olive (Olea europea L.) varieties and their oils. Grasasy Aceites 2007;58(2):142-7.
13. Okoro L N, Sambo F I, Lawal M Nwaeburu C. Thermodynamic and Viscometric Evaluation of Biodiesel and Blends from Olive Oil and Cashew Nut Oil. Res J Chem Sci 2011;1(4):90-7.
14. Waterman E, Lockwood B. Active Components and Clinical Applications of Olive Oil. Altern Med Rev 2007;12(4):331-42.
15. Suaidi NA, Alshawsh MA, Hoe SZ, Mokhtar MH, Zin SR. Toxicological Effects of Technical Xylene Mixtures on the Female Reproductive System: A Systematic Review. Toxics. 2022 May 4;10(5):235.
16. Bordoloi B. Xylene-its health hazards and biocompatible susbtitutes. 2018; 7(15): 1017-1027.
17. Negi A, Puri A, Gupta R, Chauhan I, Nangia R, Sachdeva A. Biosafe alternative to xylene: A comparative study. Journal of oral and maxillofacial pathology: JOMFP. 2013 Sep;17(3):363.
18. Madhura MG, Bhavana VS, Kumar BV, Suma S, Sarita Y. Bleached vegetable oil as a suitable bio-safe alternative to xylene: An exploratory study. Journal of Advanced Clinical and Research Insights. 2016 Nov 1;3(6):185-9.
19. Sermadi WZM, Niranjan KC, Acharya S, Prabhu S, Killedar S. Olive oil as xylene substitute. J Oral Med, Oral Surg, Oral Pathol, Oral Radiol. 2019; 5(2): 46- 51.
20. Ghosh S, Rao RS, Nambiar S, Haragannavar VC, Augustine D, Sowmya SV, Patil S. Quest for biofriendly Xylene substitutes in histopathology: a comparative study. Journal of International Oral Health. 2016 Dec 1;8(12):1101.
21. Tanwar, M., Sharma, M., Karthikeyan, R., Kumar, M., & Tyagi, V. Usage of coconut oil as a biofriendly xylene substitute in tissue processing and staining. International Journal of Health Sciences. 2022; 6(S1): 660-675.
22. Rasmussen Bb, Hjort Kn, Mellerup I, Sether G, Christensen N. Vegetable oils instead of xylene in tissue processing. Apmis. 1992;100 (7-12): 827-31.
23. Thamilselvan S, Sherlin HJ, Jayaraj G, Don KR, Santhanam A. Cedarwood oil as an alternative to xylene as a clearing agent in histopathological tissue processing– A comparative study. Journal of Oral and Maxillofacial Pathology: JOMFP. 2021; 25(2): 299.