

Cedarwood oil as an alternative to xylene as a clearing agent in histopathological tissue processing – A comparative study

Sneha Thamilselvan, Herald J. Sherlin, Gifrina Jayaraj, K. R. Don, Archana Santhanam

Department of Oral Pathology and Microbiology, Saveetha Institute of Medical and Technical Sciences, Saveetha Dental College and Hospitals, Saveetha University, Chennai, Tamil Nadu, India

Abstract

Background: Clearing in histopathological tissue processing should be able to make the tissues translucent and clear for the tissues to be visible under light microscopy and should render the clearing agent to be miscible with the dehydrant and the impregnation wax in the preceding and following processing steps. Xylene is a gold standard clearing agent but increasing concerns about the potential carcinogenicity, implementing eco-friendly agents in routine histopathology is necessary. **Aim:** The aim of the study is to assess the clearing ability of Cedarwood oil as an alternative to Xylene in routine tissue processing. **Materials & Methods:** The study was carried out in the Department of Oral Pathology and Microbiology. Formalin fixed 50 tissue samples of size 3-7mm were taken and subsequent dehydration done with acetone and alcohol. The dehydrated tissue is later processed using 90ml of Cedarwood oil with few drops of Xylene and Thymol. After clearing the tissues were subjected to impregnation and embedded in paraffin wax, later which sections were made and stained using H & E stain. **Results:** The results of our study on comparison showed better outcome in tissues processed with cedarwood oil than xylene. Statistical Significant correlation was observed in nuclear staining (p value = 0.001); cytoplasmic staining (p value = 0.08) and background staining (p value = 0.045) indicating a positive results on using cedarwood oil as clearing agent. **Conclusion:** The cedarwood oil can be considered as a safer natural alternative to xylene in laboratories. The cedarwood oil is eco - friendly and easily available with enhanced tissue processing qualities.

Keywords: Cedarwood oil, clearing agent, substitute, tissue processing, xylene

Address for correspondence: Dr. Sneha Thamilselvan, Department of Oral Pathology and Microbiology, Saveetha Dental College and Hospitals, 162, Poonamalle High Road, Velappanchavadi, Chennai 600 077, Tamil Nadu, India.
E-mail: drherald888@rediffmail.com

Submitted: 30-Sep-2020, **Revised:** 07-Jun-2021 **Accepted:** 05-Jul-2021, **Published:** 31-Aug-2021

INTRODUCTION

Clearing is an essential step in histopathological tissue processing for light microscopy. The purpose of clearing is to remove the dehydrating agents from the tissue in the preceding step and to prepare the tissues for impregnation with the embedding agent in the following step.^[1] Clearing should be able to make

the tissue sections translucent and clear for the tissue sections to be visible under a light microscope. Xylene is a gold standard chemical that has been widely used as a clearing agent due to its ease of use, economic viability, effortless processing and an array of histological techniques that can be performed.^[2]

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

For reprints contact: WKHLRPMedknow_reprints@wolterskluwer.com

How to cite this article: 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. J Oral Maxillofac Pathol 2021;25:299-305.

Access this article online

Quick Response Code:



Website:

www.jomfp.in

DOI:

10.4103/0973-029X.325232

Xylene is a chemical aromatic hydrocarbon, being a highly flammable colorless liquid with aromatic odors.^[3] It is highly toxic and carcinogenic and causes severe long-lasting ill effects on the health of technicians and pathologists when exposed for a longer duration.^[4] The main effects of inhaling xylene vapor are depression of the central nervous system with symptoms such as headache, dizziness, nausea and vomiting. Long-term exposure may lead to irritability, insomnia, agitation, extreme tiredness, tremors, impaired concentration and short-term memory.^[4] As a result of increasing concerns about the potential carcinogenicity and other long-lasting ill effects on health, implementing eco-friendly naturally sourced agents in routine histopathological tissue processing is necessary.^[5] The other naturally available substitutes for xylene are limonene reagents, aliphatic hydrocarbon mixtures, mineral oils and aromatic hydrocarbon mixture having lower volatility than xylene.^[6] One such agent is cedarwood oil which is a safe, natural alternative, eco-friendly, nonflammable, readily available, easy to handle and specifically it is noncarcinogenic.^[7] Cedarwood oil is an essential oil derived from various types of conifers, most in the pine or cypress botanical families. It is produced from the foliage, and sometimes the wood, roots and stumps.^[8] The health benefits of cedarwood oil can be attributed to its properties as an antiseptic, antiseborrheic, antifungal, antibacterial, antiplasmodic, astringent and also used in aromatherapy applications.^[9] Cedarwood oil has clearing properties as well. It has gentle actions on the tissues and has nondamaging effects on the tissues. It can also be used as a deparaffinization agent in laboratories for H & E staining procedures. All these factors are attributed positively to cedarwood oil as a clearing agent in comparison with any other gold standard clearing agent in histopathological laboratories. Cedarwood oil quantifies the relevance of one's health more than cost-effectiveness of a product. There are only a few researches have been conducted in the past to determine the effectiveness of cedarwood oil. Ankle and Joshi identified that H & E stain without xylene to be a better alternative to traditional H & E staining.^[9] There have also been few studies with cedarwood oil as a clearing agent in tissue processing but none with different types of tissues to test its efficacy. Hence, the aim of the present study was to compare the efficacy of cedarwood oil for different tissue types as an eco-friendly natural substitute to xylene as a clearing agent in histopathological tissue processing.

MATERIALS AND METHODS

The study was conducted using the tissue samples obtained from the oral squamous cell carcinoma resection specimens received in the department of Oral Pathology and Microbiology from Saveetha Dental College and Hospitals. The study included 50 tissue samples which were divided into two groups. Of these, 25 tissue

specimens were processed with cedarwood oil (Group A) as xylene alternative and 25 tissue specimens with routine xylene (Group B). Each group was categorized into five subgroups based on the different types of tissues such as skin, muscle, adipose tissue, gland and mucosa with five tissue specimens in each subgroup.

The selected tissue specimens were dehydrated initially using 99.9% 2-isopropyl alcohol (1change) and acetone (2 changes) for 30 min each. The dehydrated tissues were later cleared using 100 ml of cedarwood oil solution. The cedarwood oil which is 99.9% pure was purchased from the local commercial organic market (Allin exporters, Noida, India outlet). Cedarwood oil solution was prepared by adding 5 ml of xylene to 95 ml of cedarwood oil, to avoid the formation of crystalline cedrol. Few crystals of thymol, a component of the botanical thyme oil, were also added which acts as a disinfectant. The tissues were then cleared in cedarwood oil solution overnight at room temperature (37°–39°), whereas the tissue specimens cleared using xylene were subjected to 40°–45° for 30 min (2 changes) [Table 1]. Post clearing, the tissues were immersed in paraffin wax for overnight infiltration and paraffin-embedded tissue blocks were made. Tissue sections of 3 µm were made using semi-automated tissue microtome (Leica, Germany), deparaffinized using xylene, rehydrated using alcohol and subsequently stained with H & E stain to assess tissue morphology. The slides were evaluated for the quality of clearing during tissue processing. The tissue sections were assessed for various histomorphological criteria such as nuclear staining, cytoplasmic staining, background staining and artifacts by two independent pathologists who were blinded to the type of processing. The histomorphological criteria were graded in the score of 1–4 for nuclear and cytoplasmic staining, 0–3 for background staining and 0–1 for artifacts as follows.

Scoring criteria

Nuclear and cytoplasmic staining: (1) Poor Staining with poor morphology, (2) fair staining, (3) good staining and (4) excellent staining.

Table 1: Depicting comparison of steps in tissue processing between xylene and cedarwood oil

Xylene processing	Cedarwood oil
10% formalin - Fixation	10% formalin-fixation
IsoPropyl alcohol - 45°C × 30 min	IsoPropyl alcohol - 45°C × 30 min
Acetone - 45°C × 30 min	Acetone - 45°C × 30 min
Acetone - 45°C × 30 min	Acetone - 45°C × 30 min
Xylene - 45°C × 30 min	Cedarwood oil - 37-39°C × 1
Xylene - 45°C × 30 min	overnight
Paraffin wax-overnight impregnation	Paraffin Wax-overnight impregnation

Background staining: (0) Nil, (1) mild, (2) moderate and (3) sever background staining.

Artifacts: Presence/absence of artifacts.

Statistical analysis

The scores obtained were tabulated in excel sheets and assessed for statistical significance using IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp. The scores of the samples processed by the clearing method between xylene and cedarwood oil were compared with Mann–Whitney U-test. The subgroup analysis of individual tissues was done using Kruskal–Wallis ANOVA. For both the tests, $P < 0.05$ was considered to be statistically significant.

RESULTS

The percentage values obtained for the parameters were analyzed in comparison between the xylene and cedarwood oil tissue processing. The percentage value was calculated based on the scores obtained for each category. The cedarwood oil tissue processing and xylene tissue processing were compared based on different parameters, nuclear staining, cytoplasmic staining, background staining and artifacts.

The skin samples processed using cedarwood oil revealed better results in nuclear staining and cytoplasmic staining. The Kruskal–Wallis test results showed $P = 0.012$ ($P < 0.05$ statistically significant) for nuclear staining and $P = 0.496$ for cytoplasmic staining. Background staining was more in xylene-processed samples than cedarwood oil tissue processing. However, the difference was not found to be statistically significant ($P = 0.740$). Artifacts were appreciated more in xylene tissue processing ($P = 0.513$) [Table 2 and Figure 1].

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; $P = 0.042$). There was no difference in the background staining between xylene and cedarwood processing ($P = 0.439$). Although artifacts were seen more in xylene-processed tissues, the difference was not statistically significant ($P = 1$) [Table 3 and Figure 2].

The gland samples in cedarwood oil processing revealed better results in nuclear staining and cytoplasmic staining than routine xylene processing. However, the results were not found to be statistically significant.

(Kruskal–Wallis nuclear staining $P = 0.053$, cytoplasmic staining, $P = 0.093$). Here again, xylene process tissues had more background staining compared to cedarwood oil ($P = 0.093$). The artifacts were seen more in xylene tissue processing ($P = 0.134$) [Table 4 and Figure 3].

The adipose tissue and mucosa showed similar results. Nuclear staining of the tissues processed with cedarwood was better compared to xylene. The results were not statistically significant (Kruskal–Wallis, $P = 0.317$; $P = 0.371$). The cytoplasmic staining was better appreciated in cedarwood oil tissue processing. This was also found to be statistically not significant ($P = 0.513$; $P = 0.381$). The background staining was more in xylene-processed tissues than cedarwood oil ($P = P = 0.197$; 0.371). The artifacts were more appreciated in xylene-processed tissues ($P = 0.317$) [Tables 5 and 6 and Figures 4 and 5].

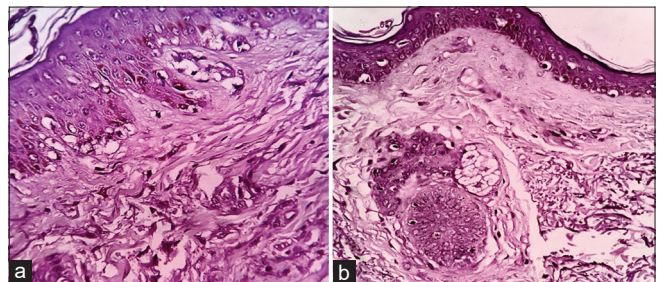


Figure 1: Photomicrograph showing the H & E staining of skin processed with (a) Cedarwood oil and (b) Xylene. (Mag: x40)

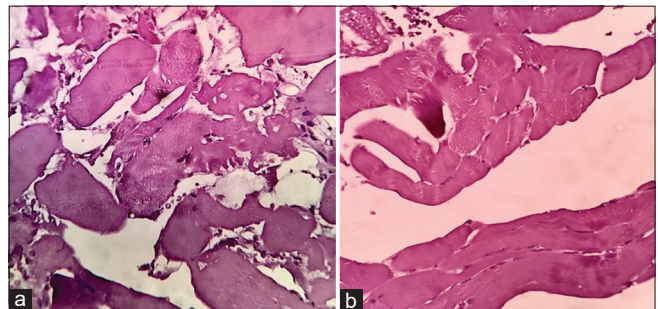


Figure 2: Photomicrograph showing the H & E staining of muscle processed with (a) cedarwood oil and (b) Xylene (Mag: x40)

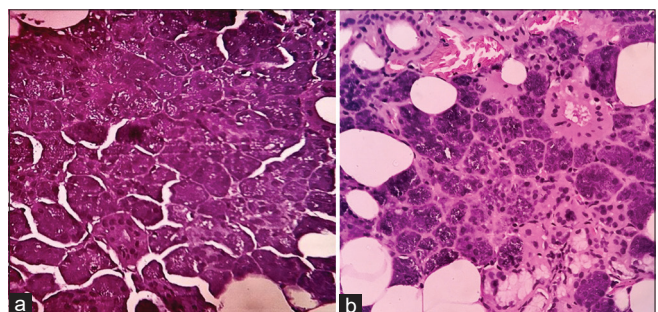


Figure 3: Photomicrograph showing the H & E staining of gland processed with (a) Cedarwood oil and (b) Xylene (Mag: x40)

Table 2: The percentage values of the parameters analyzed between xylene and cedarwood oil processing for skin and with its respective P values (Kruskal-Wallis $P < 0.05$ = statistically significant)

Tissue	Parameters	Types of processing	Weak (%)	Intense (%)	Good (%)	Excellent (%)	P
Skin	Cytoplasmic staining	Xylene processing	40	20	40	0	0.496
		CO processing	20	20	40	0	
	Nuclear staining	Xylene processing	80	20	0	0	0.012*
		CO processing	0	40	40	20	
	Background staining		Nil (%)	Mild (%)	Moderate (%)	Severe (%)	0.740
		Xylene processing	40	20	40	0	
	Artifacts		Present (%)			Absent (%)	0.513
		Xylene processing	80			20	
		CO processing	60			40	

P value < 0.05, CO: Cedarwood oil

Table 3: The percentage values of the parameters analyzed between xylene and cedarwood oil processing for muscle and with its respective P values (Kruskal-Wallis $P < 0.05$ = statistically significant)

Tissue	Parameters	Types of processing	Weak (%)	Intense (%)	Good (%)	Excellent (%)	P
Muscle	Cytoplasmic staining	Xylene processing	20	80	0	0	0.042*
		CO processing	0	40	60	0	
	Nuclear staining	Xylene processing	0	80	20	0	0.015*
		CO processing	0	80	20	0	
	Background staining		Nil (%)	Mild (%)	Moderate (%)	Severe (%)	0.439
		Xylene processing	20	40	40	0	
	Artifacts		Present (%)			Absent (%)	1.00
		Xylene processing	100			0	
		CO processing	80			20	

P value < 0.05, CO: Cedarwood oil

Table 4: The percentage values of the parameters analyzed between xylene and cedarwood oil processing for gland and with its respective P values (Kruskal-Wallis $P < 0.05$ = statistically significant)

Tissue	Parameters	Types of processing	Weak (%)	Intense (%)	Good (%)	Excellent (%)	P
Gland	Cytoplasmic staining	Xylene processing	0	40	60	0	0.093
		CO processing	0	0	80	20	
	Nuclear staining	Xylene processing	0	100	0	0	0.053
		CO processing	0	40	40	20	
	Background staining		Nil (%)	Mild (%)	Moderate (%)	Severe (%)	0.093
		Xylene processing	0	80	20	0	
	Artifacts		Present (%)			Absent (%)	0.134
		Xylene processing	40	60	0	0	
		CO processing	100			0	
			60			40	

CO: Cedarwood oil

Table 5: The percentage values of the parameters analyzed between xylene and cedarwood oil processing for adipose tissue and with its respective P values (Kruskal-Wallis $P < 0.05$ = statistically significant)

Tissue	Parameters	Types of processing	Weak (%)	Intense (%)	Good (%)	Excellent (%)	P
Adipose tissue	Cytoplasmic staining	Xylene processing	0	0	80	20	0.317
		CO processing	0	0	80	20	
	Nuclear staining	Xylene processing	0	60	40	0	0.513
		CO processing	0	80	20	0	
	Background staining		Nil (%)	Mild (%)	Moderate (%)	Severe (%)	0.197
		Xylene processing	0	0	100	0	
	Artifacts		Present (%)			Absent (%)	0.317
		Xylene processing	80			20	
		CO processing	100			0	

CO: Cedarwood oil

Of all the 25 matched samples processed, nuclear staining was better with cedarwood processed tissue than xylene (U = 158.50; P = 0.001). Cytoplasmic

staining of samples processed with cedarwood oil was superior to that of xylene. The difference was found to be statistically significant (U = 203.05; P = 0.018).

Table 6: The percentage values of the parameters analyzed between xylene and cedarwood oil processing for skin and with its respective P values (Kruskal-Wallis $P < 0.05$ = statistically significant)

Tissue	Parameters	Types of processing	Weak (%)	Intense (%)	Good (%)	Excellent (%)	P
Mucosa	Cytoplasmic staining	Xylene processing	20	20	60	0	0.371
		CO processing	0	0	80	20	
	Nuclear staining	Xylene processing	60	20	0	20	0.381
		CO processing	20	40	40	0	
	Background staining	Xylene processing	Nil (%)	Mild (%)	Moderate (%)	Severe (%)	0.371
			0	80	20	0	
		CO processing	60	20	20	0	
Artifacts	Xylene processing		Present (%)		Absent (%)	0.317	
			100		0		
		CO processing	60		40		

CO: Cedarwood oil

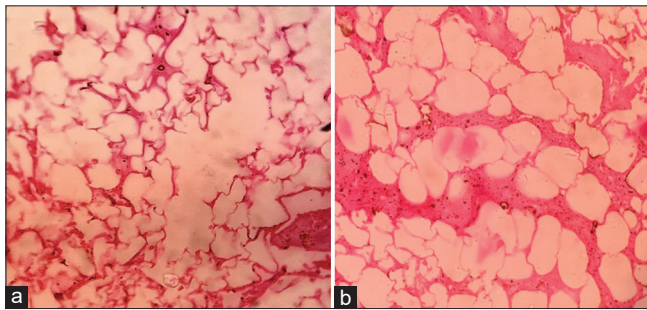


Figure 4: Photomicrograph showing the H & E staining of adipose tissue processed with (a) Cedarwood oil and (b) Xylene (Mag: x40)

Although artifacts and background staining were more in xylene-processed samples, the difference was not statistically significant ($P = 0.45$ -background staining; $P = 0.68$ -artifacts) [Table 7].

DISCUSSION

Xylene is a universal gold standard clearing agent and is routinely used in histopathological laboratories for tissue processing in the last six decades.^[9] However, serious issues were raised because it poses several health and environmental hazards. The vapors of xylene are heavier than air and have a tendency to accumulate which is rapidly absorbed by the body after inhalation and ingestion.^[10] Exposure above 200 ppm through inhalation or ingestion causes systemic toxicity since the most common route of exposure is via inhalation. Xylene’s odor threshold is about 1 ppm where the permissible exposure limit is 100 ppm above which it causes irritation to skin, eyes and respiratory tract.^[10] Along with its toxicity, disposal of xylene is a major problem for the laboratories. So taking into consideration of all these pitfalls of xylene, this study was attempted to opt for an alternative safe clearing agent. There are few similar studies done using cedarwood oil as a deparaffinization agent before staining of histopathological sections. No other studies were reported with cedarwood oil (99.9%) as a clearing agent in histopathological tissue processing.

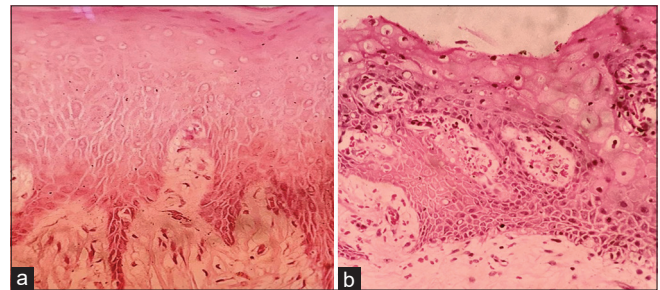


Figure 5: Photomicrograph showing the H & E staining of mucosa processed with (a) Cedarwood oil and (b) Xylene (Mag: x40)

In our study, cedarwood oil tissue processing has brought about almost superior results in overall nuclear and cytoplasmic staining of all subgroups when compared with the routine xylene tissue processing. The adequacy of clearing the tissue specimens with a clearing agent is as essential as other steps of tissue processing because that makes tissues to be transparent and visible under a light microscope. The clarity in the morphological details is much important to arrive at a diagnosis.

The background staining is appreciated in all the slides in tissues processed with xylene. This is in concordance with Indu *et al.*,^[11] who used cedarwood oil as a deparaffinization agent in H & E staining procedure and revealed that 8% cedarwood oil provided appreciable tissue staining in terms of clarity and uniformity in terms of staining quality with appreciable background staining. The deparaffinization agent is used to remove paraffin from the slides after tissue sections have been made. Xylene is also used as a primary deparaffinization agent.^[12]

The artifacts were subjectively higher in tissue cleared with xylene than cedarwood oil. Prolonged treatment of xylene may cause the tissues to become brittle, leading to crumbling and wrinkle formation of tissue sections during sectioning and this is because of the chemical alterations caused by xylene.^[13] Cedarwood oil, being an essential oil, differs in characteristics and quality which causes almost no

Table 7: Mann-Whitney U-test comparing the parameters between samples processed by cedarwood oil and xylene

Processing	n	Mean rank	Sum of ranks	P
Nuclear staining				
Cedarwood processing	25	31.66	791.50	158.500
Xylene processing	25	19.34	483.50	0.001*
Cytoplasmic staining				
Cedarwood processing	25	29.86	746.50	203.500
Xylene processing	25	21.14	528.50	0.018*
Background staining				
Cedarwood processing	23	20.54	472.50	196.500
Xylene processing	25	28.14	703.50	0.045*
Artifacts				
Cedarwood processing	25	23.00	575.00	250.000
Xylene processing	25	28.00	700.00	0.068

*P value <0.05

damage or minimum damage to the tissue specimens. This is well proved in this study that the tissue artifacts are more appreciated in tissue specimens processed with xylene.

The statistical test results revealed significance ($P < 0.05$) in nuclear staining of skin and muscle, also significant in cytoplasmic staining of muscle. This reveals that cedarwood oil possesses good clearing efficacy in skin and muscle tissue. Although the results in other subgroups were found to be statistically insignificant ($P > 0.05$), cedarwood oil is able to produce much better results to that of xylene. The overall nuclear staining, cytoplasmic staining and background were also found to be statistically significant which reassures that the usage of cedarwood oil as a clearing agent in laboratories is beneficial.

Interestingly, we have noticed a similarity between cedarwood oil and xylene in terms of chemical structure. Similar to xylene which is an aromatic hydrocarbon, cedarwood oil also has 50%–65% of sesquiterpene hydrocarbons in its structure.^[14] The general property of hydrocarbons is breaking down of heavy organic molecules into lighter molecules which might contribute to the clearing effect of tissues. The authors acknowledge that implementing cedarwood oil as a clearing agent has limits, such as a much longer processing time and also not cost-effective, but the extra expense vs work environment safety can be balanced out. Essential oils have high viscosity that permits only less flow, which further restricts the penetration of oils into the tissue specimens, but comparatively, cedarwood oil has minimal viscosity than other essential oils. However, it has been widely used because of its ease of availability, cost-effectiveness and long-standing traditional usage.

CONCLUSION

Considering the toxicity of xylene, it is desirable to minimize its use in histopathology laboratory without

compromising the processing quality and hence the appropriate diagnosis. This study shows that the clearing ability of cedarwood oil is equivalent to that of the xylene in routine histopathological tissue processing. This will help us in maintaining a favorable and healthy histopathological laboratory environment. Although we have significant positive results in our hands, further studies are required including larger sample size.

Acknowledgment

The authors would like to acknowledge the help and support rendered by the Department of Oral Pathology and Microbiology of Saveetha Dental College and Hospitals and the management for their constant assistance with the research.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

- Alwahaibi N, Aljaradi S, Alazri H. Alternative to xylene as a clearing agent in histopathology. *J Lab Physicians* 2018;10:189-93.
- Shah AA, Kulkarni D, Ingale Y, Koshy AV, Bhagalia S, Bomble N. Kerosene: Contributing agent to xylene as a clearing agent in tissue processing. *J Oral Maxillofac Pathol* 2017;21:367-74.
- Ilango J. Ecofriendly, economic surrogate for xylene. *IOSR J Dent Med Sci* 2017; 16:102-5.
- Negi A, Puri A, Gupta R, Chauhan I, Nangia R, Sachdeva A. Biosafe alternative to xylene: A comparative study. *J Oral Maxillofac Pathol* 2013;17:363-6.
- Dineshshankar J, Saranya M, Tamilthangam P, Swathiraman J, Shanmathee K, Preethi R. Kerosene as an alternative to xylene in histopathological tissue processing and staining: An Experimental Study. *J Pharm Bioallied Sci* 2019;11:S376-9.
- Kandyala R, Raghavendra SP, Rajasekharan ST. Xylene: An overview of its health hazards and preventive measures. *J Oral Maxillofac Pathol* 2010;14:1-5.
- Sumathy G, Sathyapriya B, Chandrakala B, Koshy JM, Govindaraj J, Padmavathy K. Occupational hazards of xylene and its preventive measures. *Indian J Public Health Res Develop* 2019;10:2260.
- Website. [4]Corey Whelon. What You Need to Know About Cedarwood Essential Oil. Available from: <https://www.healthline.com/health/cedarwood-essential-oil>. [Last accessed on 2020 Jul 01].
- Ankle MR, Joshi PS. A study to evaluate the efficacy of xylene-free hematoxylin and eosin staining procedure as compared to the conventional hematoxylin and eosin staining: An experimental study. *J Oral Maxillofac Pathol* 2011;15:161-7.
- Website.[5] Medical Management Guidelines for Xylene (C 6 H 4) CH 3 2. Agency for Toxic Substances and Disease Registry. Available from: <https://www.atsdr.cdc.gov/MMG/MMG.asp?id=291 and tid=53>. [Last accessed on 2020 Jul 01].
- Indu S, Ramesh V, Indu PC, Prashad KV, Premalatha B, Ramadoss K. Comparative efficacy of cedarwood oil and xylene in hematoxylin and eosin staining procedures: An experimental study. *J Nat Sci Biol Med* 2014;5:284-7.
- Premalatha BR, Patil S, Rao RS, Indu M. Mineral oil – A biofriendly

- substitute for xylene in deparaffinization: A novel method. *J Contemp Dent Pract* 2013;14:281-6.
13. Taqi SA, Sami SA, Sami LB, Zaki SA. A review of artifacts in histopathology. *J Oral Maxillofac Pathol* 2018;22:279.
 14. Adams RP. Cedar Wood Oil — Analyses and Properties. In: Linskens H.F, Jackson J.F (eds) *Essential Oils and Waxes. Modern Methods of Plant Analysis*, Springer, Berlin, Heidelberg. 1991; 12. doi.org/10.1007/978-3-642-84023-4_8.