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RESEARCH ARTICLE

Eco-friendly deparaffinizing agents Vs Xylene in routine hematoxylin and eosin staining – A Comparative study

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Abstract: ***Aim:*** Our study presents a new deparaffinizing agents in routine hematoxylin and eosin staining method. These agents are easily available, nontoxic and eco-friendly by completely eliminating expensive and hazardous xylene in staining protocol. The current study compares the staining characteristics of lemon skin oil, liquid dishwashing solution, and liquid detergent solution with those of xylene in order to evaluate the efficacy of these solutions as de-paraffinizing agents during standard hematoxylin and eosin procedures. ***Materials and Methods***: 20 paraffin-embedded tissue blocks were taken for preparing the slide sections. The slides were divided into four groups based on the reagent being used for deparaffinization and then stained with hematoxylin and eosin. Xylene (group 1), lemon skin oil (group 2), liquid dish wash solution (group 3), liquid detergent solution (group 4). The following parameters were used to score and assess each section: cytoplasmic and nuclear staining, uniformity and clarity of staining and retention of wax. The results were tabulated and statistically analyzed using Chi-square test. ***Results:*** In all four groups, nuclear and cytoplasmic staining were 100% adequate. Uniformity and clarity of staining was superior in group 1 using xylene followed by liquid dish wash. Again xylene-stained slides showed no residual wax, similarly very minimal retention of wax was seen in group 2 and 3. ***Conclusion:*** In regular hematoxylin and eosin process, xylene can be substituted with dishwashing solution or lemon skin oil as the deparaffinizing agent. ***Clinical significance:*** To reduce the harmful subjection of xylene by substituting with economical and non-hazardous agents like lemon skin oil, liquid dish wash solution, and liquid detergent solution.

Keywords: Deparaffinization, Liquid detergent, Lemon skin oil, Liquid dishwash, Xylene.

INTRODUCTION

Xylene is a synthetic aromatic hydrocarbon and also termed dimethyl-benzene or xylol. It plays a crucial role in the pathological laboratory for years.[1]It is a colorless, combustible gas or liquid or gas with a sweet smell and its name is derived from crude wood spirit (Greek xylon- wood). Xylene is used in dentistry in histology labs for tissue processing, staining, and mounting. It is also utilized as a gutta-percha solvent during endodontic retreatment. [2]Its high solvency factor facilitates maximum alcohol displacement, makes the tissue clear, and improves paraffin penetration during tissue processing. Its superior dewaxing and clearing qualities throughout staining processes result in properly stained slides. Despite its great utility, there are a few disadvantages, including the potential for harm to the skin, eyes, nose, nerve system, and musculoskeletal system, as well as other health risks. Xylene exposure shows both short term (<14 days) and long terms effects on health (>365 days). When xylene vapour is inhaled, the central nervous system is depressed, resulting in symptoms such as headache, nausea, vomiting, disorientation, and confusion.[3]Eye surface injury could result from an accidental splash with xylene. Being exposed to 200 parts per million or more of xylene can cause irritation of nose, throat and lungs leading to shortness of breath and chest pain. Long term exposure may lead to pulmonary edema, also injure liver and kidney. If a woman breathes in xylene, it can contaminate her breast milk and reach the unborn foetus. Avoiding as much xylene as possible is advised for pregnant and nursing mothers.[3]Ordinary gloves and clothing are easily penetrated by xylene, which can lead to skin irritation, dermatitis, dryness, flaking, blistering, and cracking.[4,5]

The National Institute of Occupational Safety and Health advised against exposure to xylene at time-weighted averages (TWAs) of 100 ppm for up to a 10-hour work shift, 40 hours per week, and short-term limits of 200 ppm for 10 minutes.[6]To overcome the harmful exposure to xylene, an alternative must be established without compromising the routine hematoxylin staining. Many xylene alternatives, like Hexane, EZ-Dewax, Skip Dewax, Aqua-Depar, Ultraclear, and Clearium have been tested for dewaxing of tissues in order to provide a xylene-free environment in laboratories.[7] Thus, the current research aims to substitute xylene with an eco-friendly deparaffinizing agents such as lemon skin oil, liquid dish wash solution and liquid detergent during standard hematoxylin and eosin procedures.

Within this context, the objective of this study was to limit the harmful subjection to xylene by substituting with economical and non-hazardous agents like lemon

skin oil, liquid dish wash solution, and liquid detergent solution as deparaffinizing agents.

MATERIAL AND METHODS

The study comprised of 20 random neutral buffered formalin fixed paraffin embedded (FFPE) tissue blocks which were procured from the Department of Oral Pathology, SRM Dental College, Ramapuram, Chennai containing oral epithelium. From each tissue wax block, four slides were made with two sections of 4µm thickness using Leica Semiautomatic microtome. The slides were divided into four groups based on the reagent being used for de-paraffinization and then stained using hematoxylin and eosin[8]. The results were tabulated and statistically analyzed using Chi-square test.

Group 1: Using xylene as a de-paraffinizing agent, sections were stained using the standard hematoxylin and eosin protocol. (Table 1)

Group 2: Using 100% lemon skin oil [Aseschem brand – 100 ml, Component – Limonene] as a de-paraffinizing agent, sections were stained using the standard hematoxylin and eosin protocol (Table 1)

Group 3: Using 90% Liquid dish wash solution [Pril – 50 ml, component - alkylbenzene sulfonates, 90 ml in 10 ml distilled water] as a de-paraffinizing agent, sections were stained using the standard hematoxylin and eosin protocol. (Table 1)

Group 4: Using 95% Liquid detergent [Surf excel – 50 ml, component - alkylbenzene sulfonates, 95 ml in 5 ml distilled water] as a de-paraffinizing agent, sections were stained using the standard hematoxylin and eosin protocol. (Table 1)

Each section was evaluated and scored by two oral pathologists with 10 years of experience. The following parameters were examined on the slides by single blinded method:

- Nuclear staining: Nuclear contrast that was distinct was rated as a 1, whereas nuclear contrast that was blurry or indistinct was rated as a 0.
- Cytoplasmic staining: Adequate cytoplastic staining was scored as 1, inadequate staining was scored as 0.
- Uniformity and clarity of staining: The tissues' staining was assessed using Sermadi Wajjid et al.'s criteria, and rated as good, satisfactory, or poor[9]. "Poor" meant that the tissue had an irregular staining pattern and had not sufficiently taken up the stain(score = 0). "Satisfactory" denoted details such as inadequate visualisation (score = 1). "Good" signified brilliant staining, good detail visualisation, and a good contrast between the nucleus and cytoplasm. (score = 2).
- Retention of wax: Retained and not retained.

RESULTS AND OBSERVATIONS:

It was observed that the typical staining duration for hematoxylin and eosin using xylene ranged from 75-80 min for Group 1 (Table 1), for Group 2 samples using 100% Lemon skin oil as deparaffinizing agent it took 48-50 min (Table 1), and for Group 3 using 90% Liquid dish wash solution as deparaffinizing agent it took approximately 35-40 min (Table 1) and for group 4 samples using 95% Liquid detergent as deparaffinizing agent the time taken was approximately 48-50 min (Table 1). Cytoplasmic staining, nuclear staining, uniformity and clarity of staining, and retention of wax for each slide were checked by two observers for inter- and intra-observer reliability. In all sections across the four groups, nuclear and cytoplasmic staining were 100% adequate for diagnosis (Table 2).

To compare the groups' staining uniformity and clarity, the Chi Square test was used. The Uniformity and clarity of staining in group 1 using xylene as de-paraffinizing agent was 90% good, for group 2 using 100% Lemon skin oil as de-paraffinizing agent was 35% good and 65% was satisfactory. For group 3 using 90% Liquid dish wash solution as deparaffinizing agent it was 55% good and 45% was satisfactory whereas for group 4 using 95% Liquid detergent as deparaffinizing agent it was 35% good and 65% was satisfactory. Chi Square value of the test is 16.24 and the comparison was Statistically Significant ($P < 0.05$) (Table 3).

Chi Square test was done to compare retention of wax among the four groups. Group 1 showed no retention of wax in 85% of slides, whereas the other three groups showed maximum retention of wax in almost all the slides. Chi Square value of the test is 8.48 and the comparison is statistically significant ($P < 0.05$) (Table 4).

Tables:

Table 1: Hematoxylin and Eosin staining protocol using Xylene, 100% Lemon skin oil, 90% Liquid dish wash and 95% Liquid detergent as deparaffinizing agent

Table 2: Comparison of nuclear and cytoplasmic staining among the groups

GROUPS	NUCLEAR STAINING – PERCENTAGE OF RESULTS		CYTOPLASMIC STAINING - PERCENTAGE OF RESULTS	
	Adequate	**Inadequate**	**Adequate**	**Inadequate**
Xylene	100%	0%	100%	0%
100% Lemon skin oil	100%	0%	100%	0%
90% Liquid dish wash solution	100%	0%	100%	0%
95% Liquid detergent	100%	0%	100%	0%

Staining using Xylene		**Xylene free staining**			
Deparaffinization		**Deparaffinization**	**100% Lemon skin oil**	**90% Liquid dish wash**	**95% Liquid detergent**
Xylene I	5 min	Reagent - I	5 min	2 min	5 min
Xylene II	5 min	Reagent - II	5 min	2 min	5 min
Hydration		Distilled water-I	5 min	30 s	5 min
90% alcohol	5 min	Distilled water-II	5 min	30 s	5 min
70% alcohol	5 min	Distilled water - III	30 s	30 s	30 s
Water wash	10 min	Wash slides in distilled in room temperature	30 s	30 s	30 s
Nuclear staining		**Nuclear staining**			
Harris hematoxylin	8 min	Harris hematoxylin	8 min	8 min	8 min
Water wash	2 min	Water wash	2 min	2 min	2 min
Differentiation		**Differentiation**			
1% acid alcohol	1 dip	1% acid alcohol	1 dip	1 dip	1 dip
Water wash	10 min	Water wash	10 min	2 min	10 min
Bluing		**Bluing**			
1% lithium carbonate	1 min	1% lithium carbonate	1 min	1 min	1 min
Water wash	10 min	Water wash	10 min	10 min	10 min
Cytoplasmic staining		**Cytoplasmic staining**			
1% eosin	2 min	1% eosin	1dip	1 min	1dip
Water wash at room temperature	1min	Water wash at room temperature	1min	1 min	1min
Dehydration		**Dehydration**			
70% alcohol	30 sec	Over drying at 60°C	5 min	5 min	5mins
90% alcohol	30 sec				
Absolute alcohol	30 sec				
Xylene I	5 min				
Xylene II	5 min				
Approximate time: 75-80 min		***Approximate time***	***48-50 mins***	***35- 40 mins***	***48-50 mins***

Table 3: Comparison of uniformity and clarity of staining among the groups

GROUPS IN CLARITY	PERCENTAGE OF RESULTS		CHI SQUARE TEST	
	Satisfactory	Good	Test Value	P Value
Xylene	10%	90%	16.24	.000
100% Lemon skin oil	65%	35%		
90% Liquid dish wash solution	45%	55%		
95% Liquid detergent	65%	35%		

Table 4: Comparison of retention of wax between the four groups

GROUPS IN RETENTION	PERCENTAGE OF RESULTS		CHI SQUARE TEST	
	Retained	Not Retained	Test Value	P Value
Xylene	15%	85%	8.48	.000
100% Lemon skin oil	100%	0%		
90% Liquid dish wash solution	100%	0%		

Figure 1: Photomicrograph of Group 1 stained section with H&E de-paraffinized using xylene solution having good quality staining with no retention of wax. (4x & 20x)

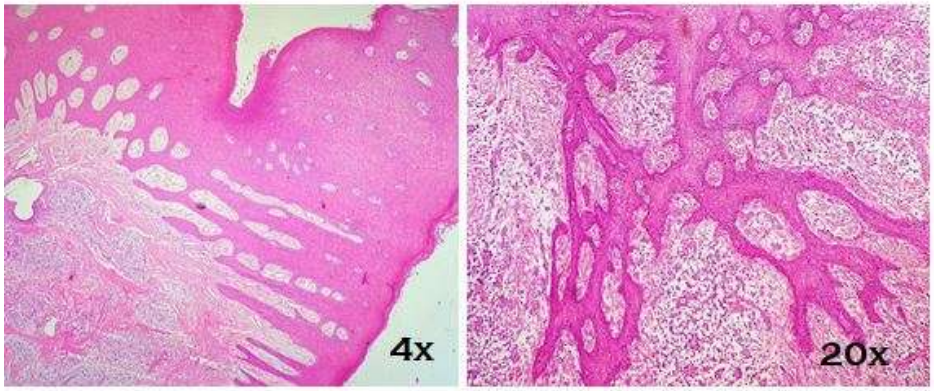


Figure 2: Photomicrograph of Group 3 stained sections with H&E de-paraffinized using liquid dish wash. Staining quality at par with xylene, and no residual wax seen in this section. (4x & 20x)

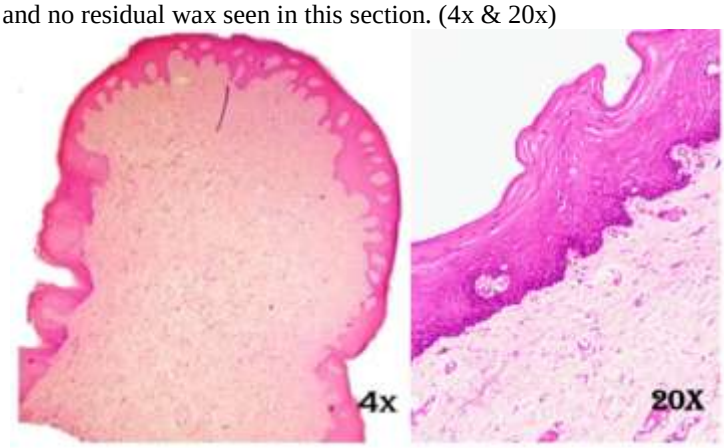


Figure 3: Photomicrograph of Group 2 stained sections with H&E de-paraffinized using lemon skin oil. Red arrow shows residual wax in the superficial epithelium. (4x & 20x)

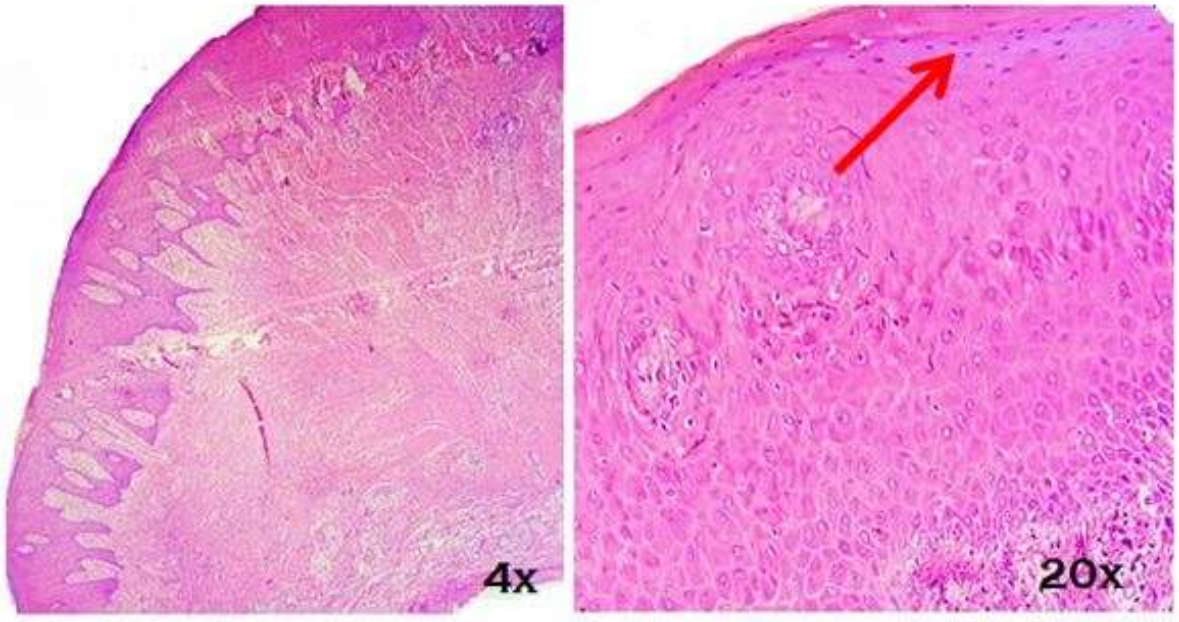
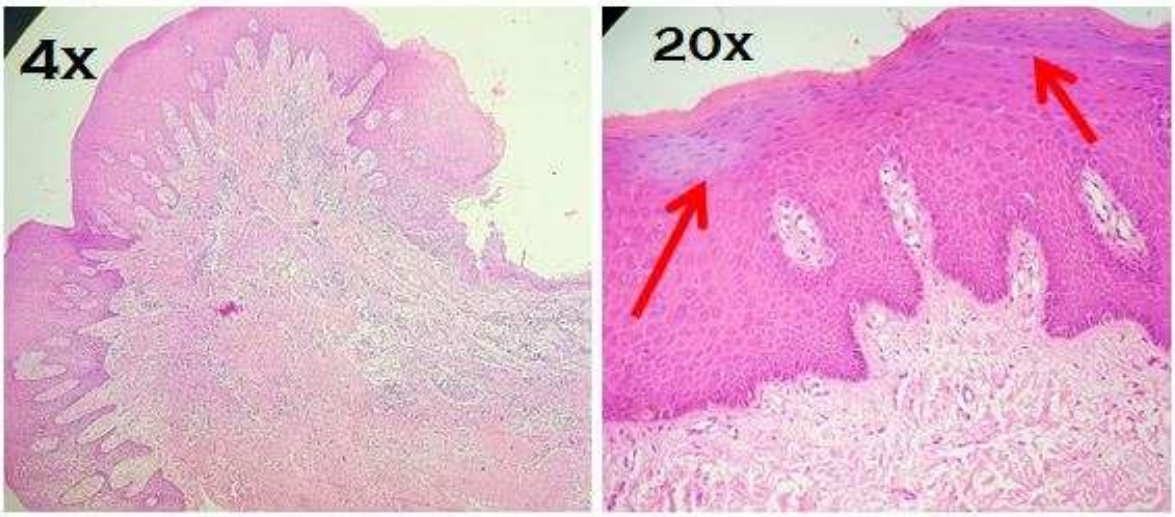


Figure 4: Photomicrograph of Group 4 stained sections with H&E de-paraffinized using liquid detergent solution. Red arrow shows residual wax in the superficial epithelium. (4x & 20x)



DISCUSSION

Clearing is a crucial stage in the histology section preparation process in the histopathological laboratory. Its goal is to eliminate alcohol and other dehydrants from tissues prior to infiltration of the embedding material.9 In the study of histopathology, xylene is employed as a cleaning agent to provide translucency to the tissues. History shows that in the histology lab in the 1950s, xylene was the safest substitute for harmful substances including aniline oil, benzene, chloroform, dioxane, and toluene. However, this proved to be an unsuccessful substitute, and by the late 1970s, there was evidence that its acute neurotoxicity was higher than that of toluene or benzene, raising serious worries about its safety.10 Because xylene is a volatile substance, laboratories that employ it face significant disposal challenges. Because it is a flammable solvent with a low flash point of 28.9°C, it cannot be disposed of by pouring it down drains. After a prolonged exposure, it may be neurotoxic to humans and, in moderate cases, it may irritate the skin.11

Once the dangers of xylene were established beyond dispute, a wide range of viable alternatives became accessible. The goal of the xylene-free approach is to create histological sections that are comparable to those

made the normal way, but with more benefits for the environment and the economy.12

Therefore, the current study's goal was to use substances, which is readily accessible and safe, ecofriendly, time saving, and cost-effective rather than xylene as the dewaxing agent throughout the H and E staining process in order to create the best staining method. So, we used substances like lemon skin oil, liquid dish wash solution and liquid detergent solution in different concentrations.

Liquid dishwash solution and liquid detergent are highly foamy surfactant mixture, primarily composed of alkylbenzene sulfonates, that causes little irritation to the skin; primarily used for hand washing garments, cutlery, and kitchen utensils. Liquid dishwashing detergent has proven to be an effective substitute for xylene in tissue section deparaffinization in past research.13,14,15 Lemon juice is commonly used as a hygienic kitchen deodorizer, to brighten copper cookware, and to remove wood cleaning, polish, and grease, among other things. A review of the literature revealed that there has never been a study using lemon skin oil as a de-paraffinizing agent. Lemon juice is commonly used to clean kitchens, brighten copper cookware, remove oil, polish, wood cleaner, and other residue. Lemon skin oil mainly is comprised of D-limonene which is biodegradable, with no benzene or toluene, soluble in alcohol and mounting media, shows less tissue shrinkage, does not evaporate fast. Though this limonene shows few disadvantages like it is comparatively little expensive, it is oily and has an offensive odor, and samples take more time to dry thoroughly.
Four different parameters were considered for evaluating the stained slides in this study, cytoplasmic staining, nuclear staining, cytoplasmic staining, uniformity and clarity of staining, and retention of wax.
Taking into account the time of the entire staining process with 4 reagents, the dewaxing process achieved using liquid dishwashing liquid is only 6 minutes, while in the conventional technique using xylene it is about 25 to 30 minutes and for both lemon skin oil and liquid detergent solution it was approximately 20 min. The entire staining procedure was completed in 35–40 min using liquid dishwash when compared to the conventional H and E that grabbed 75–80 min and again for lemon skin oil and liquid detergent solution it took 48-50 mins. Therefore, the liquid dishwash solution was more effective in saving considerable amount of time.

The nuclear and cytoplasmic staining exhibited 100% adequacy among all the groups suggesting that there was no difference between these staining procedures. On comparing uniformity and clarity of staining among the groups, xylene slides showed 90% crispness in staining followed by liquid dishwash which had 55% (Figure 1&2). Lemon skin oil and liquid detergent solution showed about only 35% good clarity of staining. Based on the percentage of uniformity and clarity of staining, it is found that liquid dishwash is superior to the other non-xylene agents and can be used as an alternative for xylene in routine H & E staining.
85% of the xylene-stained slides showed almost no wax retention, however when considering liquid dishwash, lemon skin oil and liquid detergent solution-stained slides almost all slides showed retention of wax in few areas of the section. The wax retention was mostly seen only in a few areas of the superficial epithelium. As a result of wax retention, the stains were not able to penetrate into the tissues and cellular structures were not clearly visible. This retention of wax may be due to the thickness of the sections or the time factor (Figure 3&4).

In the present study, all four reagents had tissue clearing properties and maintained their cell structure almost equivalent to that of xylene. They are not dangerous and can be recycled.

Furthermore, this study suggests that liquid dishwash was the most effective substitute for xylene in all respects out of the three reagents that were utilized; however, lemon skin oil which was used for the first time was almost at par with liquid dishwash but only needs proper standardization of the full staining process using larger sample size.

Nevertheless, because of the small sample size and short study period, it is strongly advised to carry out additional research on a variety of tissues using larger sample sizes. Since many tissues were not examined in this pilot investigation, caution is suggested when interpreting our findings. Scope for further studies involves evaluation of long-term stability of stained sections cleared and deparaffinized without xylene.

CONCLUSION

The present study concludes that Lemon skin oil, Liquid dishwash solution and Liquid detergent can be used as clearing agents in histopathological procedures and are also economical, non-hazardous, cause little tissue shrinkage, preserve cellular structures and staining quality of tissue sections. This study also concluded that of the three different reagents used, dishwashing liquid was the best alternative to xylene in all aspects. To consider this agent as a better and safer alternative to xylene, additional studies have to be conducted using different types of tissue samples such as salivary glands, lipomas, and epithelial pathologies. The understanding that xylene can be naturally replaced with dishwashing solution, lemon skin oil, and liquid detergent solution is a tiny step towards the development of xylene-free histopathology labs in the future.

CONFLICT OF INTEREST:

The authors have no conflicts of interest regarding this investigation.

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