Original Article

Comparative efficacy of cedarwood oil and xylene in hematoxylin and eosin staining procedures: An experimental study

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Abstract

Background: Xylene is used as a clearing agent in hematoxylin and eosin (H and E) staining of tissue sections in routine histopathology based diagnosis. However, the hazards associated with exposure to xylene are of concern. Numerous solutions mainly essential oils have been evaluated in the past as clearing agents, which can possibly be substituted for xylene during the routine tissue processing. **Aim:** The aim of this study is to compare the efficacy of essential oil (cedarwood oil), as a possible replacement for xylene in H and E staining procedures. **Materials and Methods:** The study was carried out in the Department of Oral Pathology and Microbiology. Thirty paraffin blocks of the routine biopsy specimen were retrieved from the department archives. The cedarwood oil was procured from organic and essential oil dealer in the local market. Two to three paraffin sections of four micron thickness were cut from each of the 30 paraffin blocks of processed tissue specimens, were subjected to different clearing agents: Essential oil (8% cedarwood oil) or xylene and stained with H and E stain. The stained sections were scored based on nuclear and cytoplasmic details, clarity and uniformity of staining. **Results:** Significant correlation was observed between cedarwood oil and xylene in terms of the three staining quality parameters assessed. **Conclusions:** We conclude that cedarwood oil can be an effective, eco-friendly and safe alternative to xylene as a clearing agent in the histopathological laboratory.

Key words: Cedarwood oil, clearing, staining, xylene

INTRODUCTION

Xylene is a chemical aromatic hydrocarbon,[1] which has excellent dewaxing and clearing capabilities and hence routinely used in staining of tissue sections.[2] Hematoxylin and eosin (H and E) stain forms the backbone of routine histopathological diagnostic work. It is remarkably robust and is used to discriminate between the cytoplasm, nucleus and extracellular matrix.[1] Despite the remarkable utility of xylene in histological staining, its use is associated with potential occupational hazards. It can cause debilitating effects on skin, eyes, nervous system, blood, liver and kidneys. In addition, due to its volatility and limitations in complete containment, it can potentially contaminate the working environment.[2] Hence in the quest to eliminate or reduce the use of xylene in histopathology laboratory, numerous substitute chemicals such as limonene reagents, aliphatic hydrocarbons, aromatic hydrocarbons, vegetable oils, olive oil and mineral oil substitutes were used in the past.[1] Moreover, any technique, which can minimize or replace the use of xylene in histopathology laboratory, will be valuable not only for diagnostic reasons but as well as for maintaining a relatively safe laboratory environment. Hence the present study aimed to evaluate the comparative efficacy of 8% cedarwood oil as a non-biohazardous replacement for xylene.

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MATERIALS AND METHODS

The study was carried out in the Department of Oral Pathology and Microbiology. Thirty paraffin blocks of the routine biopsy specimens were retrieved from the department archives. The essential oil (cedarwood oil) was procured from the local commercial market (Bon Appetite-Go Organic Pondicherry, India outlet, to which it was supplied from Chaitanya Agro Herbals, Jayalakshmipuram, Mysore, India).

Two to three paraffin sections of four microns thickness were cut from each of the 30 paraffin blocks of routinely processed tissue specimens. Each of these sections were deparaffinized by the two different clearing agents: Essential oil – 8% cedarwood oil labeled and xylene.

The paraffin sections cleared by cedarwood oil were immersed in 8% cedarwood oil solution for about 4 h at room temperature and then subjected to microwave processing for about 1 min at 60°C. Following this the sections were washed in distilled water and subjected to conventional H and E staining procedure.

The sections cleared by xylene were incubated overnight at 37-40°C in xylene and then subjected to xylene I, II for 15 min each followed by conventional H and E staining procedure.

The details of the protocol are summarized in Table 1 (cedarwood oil) and Table 2 (xylene).

H and E stained sections were graded based on the parameters of nuclear staining (adequate = score 1, inadequate = score 0), cytoplasmic staining (adequate = score 1, inadequate = score 0) and uniformity of staining (present = score 1, absent = score 0).

The scores for each slide were added. A score of <1 was graded as inadequate for diagnosis; slides with score 1-3 were assigned as adequate for diagnosis. Slides were scored for diagnosis based on a similar scoring system used previously.

Statistical analysis

Percentage adequacy of the staining method between cedarwood and xylene was compared using Chi-square test. In the above tests, $P < 0.05$ was taken to be statistically significant.

RESULTS

Adequate nuclear staining was observed in 90% and 93.33% of the sections cleared with cedarwood oil or xylene respectively [Table 3]. Sections cleared with cedarwood oil or xylene had similar (93.33%) cytoplasmic staining and uniformity of staining [Table 3]. Moreover, the scorings from both groups were not statistically ($P = 0.2326$) different [Table 4].

Our results suggest that of all the 30 sections, the sections which had undergone clearing with cedarwood oil had comparable results to that of xylene, in terms of adequacy of nuclear and cytoplasmic staining and overall uniformity of staining [Graph 1 and Figure 1].

Table 1: The summary of procedure undertaken in H and E staining with clearing agent cedar wood oil as deparaffinizing agent

Steps	Solution	Temperature	Duration
Deparaffinization	Clearing agent cedarwood oil	At room temperature	2-4 h
Microwave application	Clearing agent	60°C	1 min
Washing	In distilled water	At room temperature	5 min
Rehydration	100%, 100%, 100%, 80%, 60% alcohol	At room temperature	2 min each
Nuclear staining	Hematoxylin	At room temperature	25 min
	Washing in running tap water	At room temperature	5 min
Differentiation	2% Acid alcohol	At room temperature	2 dips
	Washing in running tap water	At room temperature	30 min
Dehydration	60%, 80%, 100% alcohol	At room temperature	2 min each
Cytoplasmic staining	1% eosin	At room temperature	1 min
Dehydration	100%, 100%, 100% alcohol	At room temperature	5 min

Dry and mount in DPX, H and E: Hematoxylin and eosin

Table 2: The summary of procedure undertaken in H and E staining with clearing agent xylene as deparaffinizing agent

Steps	Solution	Temperature	Duration
Incubation	-	37-40°C	Overnight
Deparaffinization	Clearing agents-xylene-I, II	At room temperature	15 min each
Drying	-	At room temperature	30 min
Rehydration	100%, 100%, 100%, 80%, 60% alcohol	At room temperature	2 min each
Nuclear staining	Hematoxylin	At room temperature	25 min
	Washing in running tap water	At room temperature	5 min
Differentiation	2% Acid alcohol	At room temperature	2 dips
	Washing in running tap water	At room temperature	30 min
Dehydration	60%, 80%, 100% alcohol	At room temperature	2 min each
Cytoplasmic staining	1% eosin	At room temperature	1 min
Dehydration	100%, 100%, 100% alcohol	At room temperature	5 min

Dry and mount in DPX, H and E: Hematoxylin and eosin

DISCUSSION

Xylene is an integral component of histopathological laboratory for the last six decade and is an effective replacement to other toxic clearing agents such as benzene and chloroform. However, xylene has inflammable properties and can manifest skin reactions, cardiac and blood ailments, neural and renal toxicities.[4] Xylene is a volatile compound and its disposal are a major problem for the laboratories were it is being extensively used due to its potential environmental hazards. Hence, through our study an attempt was made to evaluate essential oils (cedarwood oil) as possible substitutes to xylene as clearing agents. Importantly essential oils are non-toxic, non-biohazardous and eco-friendly. Our results are in concurrence to previous reports where cedarwood oil has shown to be a better clearing agent over xylene in tissue processing (perfect ribboning).[5] This observation also brought out desirable results where use of essential oils can also be used at tissue processing stage before the wax impregnation and tissue embedding.

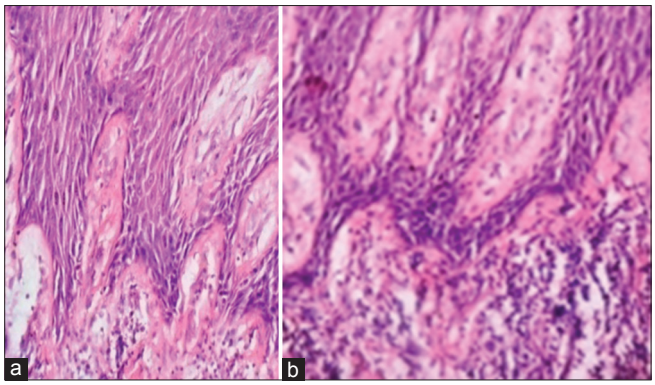


Figure 1: Photomicrograph showing adequacy and uniformity of staining. (a) Section which underwent clearing in cedarwood oil. (b) Section which underwent clearing in xylene (H and E, ×40)

Table 3: Comparison of adequacy of staining between cedarwood oil and xylene (*n*=30 comparisons)

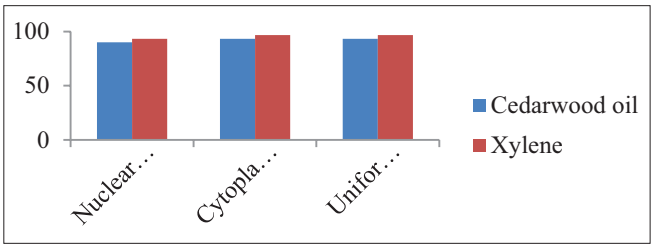
Staining characteristics	Adequacy *N* (%)				*P* value on Chi-square test
	Cedarwood oil		Xylene		
	N	%	*N*	%	
Nuclear staining	27	90.0	28	93.3	0.640
Cytoplasmic staining	28	93.3	29	96.7	0.554
Uniformity	28	93.3	29	96.7	0.554

Table 4: Overall comparison of cedarwood oil with xylene (*n*=90 comparisons)

Grading	Cedarwood oil		Xylene		Chi-square value	*P* value
	N	%	*N*	(%)		
Adequate	82	91.11	86	95.55		
Inadequate	8	0.88	4	0.44	1.429	0.233

As a natural product obtained from several sources, cedarwood oil differs in characteristics and quality. As per the literature review, for histological processing a minimally viscous variety is required. The clearing time will vary, with more viscous oils taking longer than less viscous oils. The major advantage of this oil is that it causes almost no damage to the tissue. However, it does take significantly longer time to process and is significantly expensive than the usually used alternatives.[6] Nevertheless the added cost versus work environment safety can be balanced out with optimal procurement plans. Moreover, tissue cleared with cedarwood oil were shown to give much favorable staining results[7] due its gentle and non-damaging effects on tissues[8] and are in agreement with our results where cedarwood oil cleared tissue sections had consistent good uniformity of staining.

Although other agents like the home use dish washing soap water solution as a replacement for xylene in clearing of tissue sections has shown some promise, its potential effects on some tissue structure is a concern.[3] It is essential to note that in our study, chemically ripened Ehrlich's hematoxylin was used to stain the nuclear components, whereas in previous studies Harris's and Mayer's hematoxylin were used.[2,3] Being chemically ripened, this Ehrlich's hematoxylin has a longer duration of stability and hence better staining quality of the sections can be achieved. Nevertheless, Harris's hematoxylin, which is widely used in Indian laboratories has potential of being used progressively and regressively.[9] There are a good amount of literature reviews, which have reported the use of cedarwood oil and lemon oil as clearing agents.[10] However, research trials using these solutions in tissue processing, have reported mixed results. The results of the present study revealed that at least 8% cedarwood oil provided appreciable tissue



Graph 1: Graphical representation of comparison of adequacy of staining between cedarwood oil and xylene

Characteristics	Cedarwood oil	Xylene
Nuclear staining	90	93.3
Cytoplasmic staining	93.3	96.7
Uniformity of staining	93.3	96.7

staining in terms of clarity and uniformity although this was expensive than the commonly used counterparts, but taking into consideration the safety aspects these essential oils should be recommended for clearing and staining of tissue sections.

CONCLUSION

The present study shows that xylene free H and E staining procedure carried out using an essential oil (8% cedarwood oil) produced quality staining with sufficient clarity and uniformity of staining. It also has added advantages of being non-toxic, non-inflammable and non-hazardous and is easy to handle. Nevertheless the xylene free H and E-stained sections should also be evaluated over a considerable period of time to ascertain its suitability for stability in the staining of tissue sections.

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