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Dentistry Section

# Bio-Friendly Alternatives for Xylene – Carrot oil, Olive oil, Pine oil, Rose oil

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## ABSTRACT

**Background:** Xylene is a flammable liquid with characteristic petroleum or aromatic odours, it is miscible with most of the organic solvents and paraffin wax. Xylene clears tissues rapidly and renders transparency, facilitating clearing endpoint determination, this made it to be used as a clearing agent in routine histopathological techniques. Even though it is a good clearing agent, it causes damage to the tissues by its hardening effect particularly those fixed in non-protein coagulant fixatives. Apart from these tissue effects, it has severe, long lasting ill effects on health of technicians and pathologists when exposed to longer duration. Hence in order to overcome these effects and replace xylene with a safe alternative agent, the present study was carried out to assess the clearing ability and bio-friendly nature of four different natural oils i.e., Carrot oil, Olive oil, Pine oil and Rose oil in comparison with that of Xylene. According to Bernoulli's principle of fluid dynamics, to decrease viscosity of these oils and increase penetration into tissues for rapid clearing hot-air oven technique was used.

**Aims:** To assess:1) Clearing ability and bio-friendly nature of four different oils i.e., Carrot oil, Olive oil, Pine oil, Rose oil in comparison with that of xylene, 2) Application of Bernoulli's principle of fluid dynamics in rapid clearing of tissues by using hot-air oven.

**Materials and Methods:** Forty different formalin fixed tissue samples were taken. Each sample of tissue was cut into 5 bits (40x5=200 total bits) which were subjected for dehydration in differential alcohol gradients. Later, each bit is kept in 4 different oils such as Carrot oil, Olive oil, Pine oil, Rose oil and xylene and transferred into hot-air oven. Further routine steps of processing, sectioning and staining were done. Individual sections cleared in four different oils were assessed for cellular architecture, staining quality and a comparison was done between them.

**Results:** Results showed that all the four oils had ability to clear the tissues similar to that of xylene. Pine oil was superior in its physical and clearing properties, which also maintained good cellular architecture and distinct staining quality, when compared with other oils and xylene.

**Conclusion:** Carrot oil, Olive oil, Pine oil, Rose oil, are not only bio friendly and economical but can also be used as clearing agent instead of xylene. In this study stability and longetivity of haematoxyllin and eosin staining was evaluated for over a period of one year and no significant difference in staining quality were observed.

**Keywords:** Bernoulli's principle, Clearing agent, Histopathology, Hot-air oven

## INTRODUCTION

Clearing is an important step in the preparation of histological sections, aiming to remove alcohol and other dehydrants from tissues prior to infiltration of the embedding material [1]. In the field of histopathology xylene is used as a clearing agent that gives translucency to the tissues [2]. History reveals that in 1950s xylene was used as the safest alternative to dangerous chemicals such as aniline oil, benzene, chloroform, dioxane, and toluene in the histology laboratory. But this proved to be an example of a failed substitution, by the late 1970s, there were great concerns about its safety with evidence that its acute neurotoxicity was greater than that of benzene or toluene [3].

Technical grade xylene is a combination of the 3 isomers namely, Ortho, Para and Meta. This mixture is referred to as 'Xylol'. Studies have shown that xylene is well-absorbed by inhalational, oral and to some extent by the dermal route [2]. Once entered into the body it is stored in adipose tissue as it is soluble in it. It has a half life of 1 to 6 days in the subcutaneous fat. [4]. Studies have shown that laboratory workers exposed for 1.5 to 18 years were described as having the equivalent of general poisoning disorders including bone marrow toxicity and pancytopenia as caused by a wound contaminated with xylene [5,6].

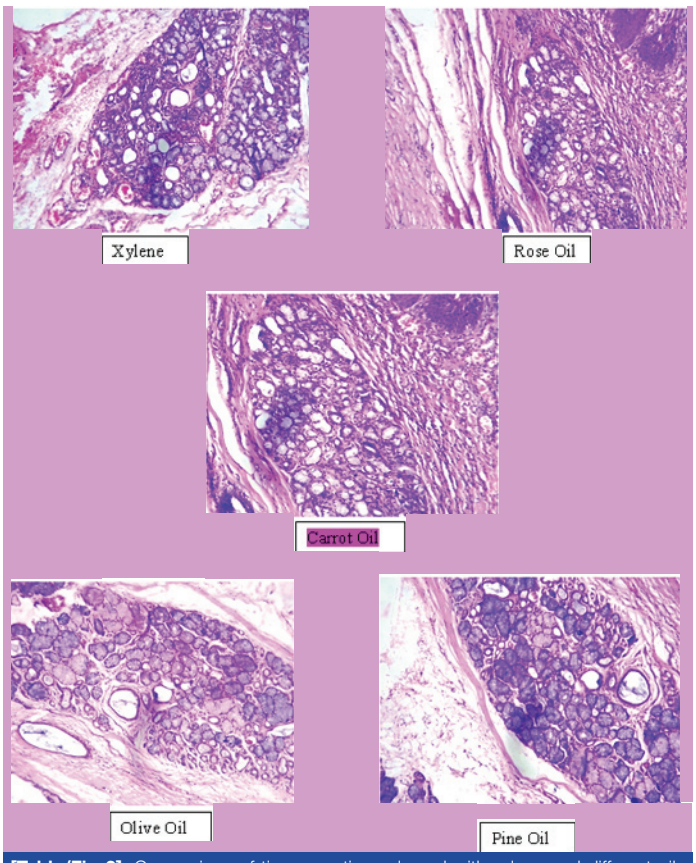
Effects of xylene on the tissues are due to depletion of mitochondrial enzyme adenosine triphosphate in the affected cells. Heart and kidney injuries, some fatal blood dyscrasias, and other less dangerous problems, such as skin erythaema, drying, scaling and secondary infections are other toxic effects seen to be associated with use of xylene. [3].

Various xylene substitutes were tried in the past such as limonene reagents, aliphatic hydrocarbons, vegetable oils and mineral oils to avoid xylene in the laboratory. However, these substitutes were found to be less effective as clearing agents and more expensive than xylene [7]. In the present study in order to overcome these effects and replace xylene with a safe alternative, 4 different oils like Carrot oil, Olive oil, Pine oil and Rose oil were evaluated for their clearing ability and bio-friendly nature. A comparsion was done to asses individual physical, chemical properties and their effects on tissues during different procedures employed in routine histopathology. Apart from this, stability and longevity of haematoxyllin and eosin staining was evaluated for over a period of one year and no significant difference in staining quality were observed.

## MATERIALS AND METHODS

Forty different tissue samples were taken and fixed in 10% formalin. After fixation grossing was done. Each tissue specimen was cut into 5 equal sections (40x5=200 total bits) and each section approximately measuring 0.5cm x 0.8cm and thickness ranging from 1.5mm-3mm and then each section was labeled. Labeled individual cut tissue sections were placed in metal cassettes for further processing. These tissues were subjected to different grades of alcohol (70%, 80%, 90% and absolute alcohol) for dehydration. After dehydration each cut tissue section were placed in 5 glass jars containing Carrot oil, Olive oil, Pine oil, Rose oil and xylene for comparsion of different physical, chemical and clearing properties. The 5 glass jars containing different oils and xylene were placed in a Hot-air oven at 60°C for two cycles 45 minutes each [Table/Fig-1]. Further these tissue sections were routinely processed paraffin


**[Table/Fig-1]:** Clearing of tissues using different oils and xylene using Hot-air oven.




**[Table/Fig-2]:** Comparison of tissue sections cleared with xylene and different oils after staining with (H&E, 10X)

embedded, sectioned and stained with Haematoxylin and Eosin (H&E) for examining under Light microscope [Table/Fig-2].

## Criteria for evaluation

1. **Physical properties of oils:** Colour, odour, viscosity, volatility, potential health hazards, disposal after use.
2. **Gross tissue specimen:** The gross tissue features such as translucency, rigidity after wax impregnation, shrinkage after clearing and ease in section cutting, were noted down for each specimen cleared in 5 different solvents separately at 60°C for two cycles 45 minutes each.
3. **Cellular architecture:** Distinct nuclear-cytoplasmic contrast is considered as score 1 and indistinct/blurred nuclear-cytoplasmic contrast as score 0.
4. **Quality of staining [7]:** The staining of tissues was evaluated and designated as poor, satisfactory and good. The criteria used for evaluation are given by Sermadi Wajjid et al. Poor indicated that the tissue failed to take up the stain adequately, stained unevenly (score = 0). 'Satisfactory' pointed toward details like not visualized up to the mark (score = 1). 'Good' designated good contrast between the nucleus and cytoplasm and visibility of details, along with brilliance of staining (score = 2).

## RESULTS

Among the 4 different oils used pine oil has similar colour, viscosity properties with that of xylene and is very economical as compared to xylene. Rose, Carrot and Pine oils are more viscous than xylene but when subjected to heat they showed equal penetration to that of xylene [Table/Fig-3]. Tissues cleared in different oils showed similar gross changes compared to that of xylene. Pine oil was superior in translucency and caused less shrinkage of tissue compared with that of xylene. On other hand carrot and rose oils showed less shrinkage of tissue compared to xylene. Translucency of the tissues cleared in carrot and rose oils was similar to tissues cleared in xylene [Table/Fig-4]. Tissue cellular architecture was preserved in all the sections cleared with the four different oils and a clear distinction was observed between nucleus and cytoplasm. The overall staining quality almost equivalent with that of xylene [Table/ Fig-5].

| Physical Properties | Xylene | Rose Oil | Carrot Oil | Pine Oil | Olive Oil |
|---|---|---|---|---|---|
| Odour | Strong-unpleasant | Pleasant | Pleasant | Pleasant | Pleasant |
| Viscous | Less | More | More | Less | More |
| Volatility | - | 1 | 1 | 1 | 1 |
| Cost | - | **1 | *2 | ***0 | **1 |
| Health Hazards | Yes | *No | *No | *No | *No |
| Disposal | Not Recycled | *Recycled | *Recycled | *Recycled | *Recycled |

**[Table/Fig-3]:** Shows different physical properties of oils in comparsion with xylene
*Volatility scoring: Score-0= less volatile than xylene, Score-1= Equivalent to xylene, Score-2= more volatile than xylene
*Cost comparison per 1000ml of xylene: Score-0= Very economical compared to xylene; Score-1= Approximately comparable to xylene, Score-2= Costly than xylene

| Gross changes | Rose Oil | Carrot Oil | Pine Oil | Olive Oil |
|---|---|---|---|---|
| Translucency | 1 | 1 | 2 | 1 |
| Rigidity | 0 | 0 | 0 | 0 |
| Shrinkage | 0 | 0 | 0 | 1 |
| Section cutting | 1 | 1 | 1 | 1 |

**[Table/Fig-4]:** Comparison of gross changes in tissues cleared with different oils and xylene
*Score-0= Inferior to xylene, Score-1= Equivalent to xylene, Score-2= Superior to xylene

| Clearing solutions | Cellular architecture | Quality of staining |
|---|---|---|
| Rose oil | 1 | 1 |
| Carrot oil | 1 | 1 |
| Pine oil | 1 | 2 |
| Olive oil | 1 | 1 |

**[Table/Fig-5]:** Showing Cellular architecture and staining quality
*Cellular architecture: Score-1: Distinct nucleus-cytoplasm, Score-0: Indisctinct Nucleus-cytoplasm
*Quality of staining: Score-0= Poor, Score-1= Satisfactory, Score-2= Good

## DISCUSSION

Dimethylbenzene (Xylene) is an aromatic hydrocarbon with a chemical formula of $C_6H_4(CH3)_2$. It is a colourless sweet- smelling liquid or gas which is present naturally in petroleum, coal and wood tar. Term 'xylene' is derived from greek (in Greek xylon means 'Wood') as it is found in crude wood spirit [8]. According to Occupational Safety and Health Administration (OSHA), the permissible exposure limit of xylene is 100ppm as an 8-hour time-weighted average (TWA) concentration. Under RCRA: Resource Conservation and Recovery Act, it is considered to be a hazardous waste. [9]. Various toxic effects of xylene on different oragan systems in humans are listed in [9] [Table/Fig-6].

| Different Organ System | Toxic Effects of xylene |
|---|---|
| CNS | Headaches, dizziness, irritability, depression, insomnia, agitation, extreme tiredness, tremors, loss of concentration and decreased memory. |
| Respiratory system | Irritation to nose and throat, Chest pain, shortness of breath (exposure ≥ 200 ppm), pulmonary edema (over exposure at a confined space). Because of severe lung congestion and interalveolar hemorrhage death may occur |
| Liver and kidney | In rats, it is proved that increased exposure to xylene(>300 ppm) causes injury and fat deposition in liver and kidney. |
| GIT | Nausea, vomiting and gastric discomfort, anorexia |
| Musculoskeletal system | Decrease in grasping and muscle power in extremities. |
| Skin | Skin erythaema, urticaria, vasodilation of skin, dryness and scaling of skin. |
| Eye | Irritation to eye. |
| Reproductive system | Crosses placental barrier and shows feto-toxic effects. Evidence of entry into breast milk. Immediate abortions. |
| Cancer | Not much data found to cause cancer. |

**[Table/Fig-6]:** Toxic effects of Xylene on different organ systems [2,9].

Considering the hazardous effects of xylene, this study was done in search of a safe alternative to xylene using carrot oil, rose oil, pine oil and olive oil. In general these essential oils are used widely as natural flavour additives for food, as fragrances in perfumery, and in medicine and alternative medicines such as aromatherapy. Clearing agent is used as an intermediate solvent that is fully miscible with both ethanol and paraffin wax. This solvent will displace the ethanol in the tissue, then this in turn will be displaced by molten paraffin wax. In order to consider any solution as a clearing agent, it has to penetrate into tissues rapidly to clear them. For easy penetration of any solution, the viscosity of the solution plays an important role, for example a less viscous solution penetrates faster compared to that of high viscous solutions. According to Bernoulli's principle of fluid dynamics viscosity depends on the temperature i.e., viscosity of the fluid is indirectly proportional to temperature, this means as the temperature increases viscosity of the fluid decreases and penetration of fluid increases. In the present study, except pine oil, the other three oils used were more viscous compared to that of xylene. Hence to decrease viscosity of these oils and increase penetration into tissues and clear them at a faster pace, clearing of tissues after dehydration was carried out using a hot-air oven at 60°C temperature.

This study was carried out using 4 different oils individually and not as mixtures, because a mixture of coconut oil and olive oil was used by Ramussen et al., and they noticed incomplete impregnation, leading to problems in section cutting [10]. Andre et al., used a mixture of peanut oil, soyabean oil, coconut oil and cotton oil and concluded that it was a poor alternative, as the quality of sections with respect to xylene were better because these substitutes were used as mixtures along with xylene [11].

In the present study, all the four oils have the property of clearing tissue and maintaining their cellular architecture which is almost equally comparable with that of xylene. They are non hazardous and can be recycled. Pine oil was superior in its physical and clearing properties. It also maintained good cellular architecture and distinct staining quality, when compared with other oils and is also very economical. Sermadi et al., used coconut oil as a clearing agent which showed similar cellular architecture and better staining quality than xylene [7].

Different researchers used different oils as substitute for xylene but pine oil, carrot oil and rose oil were used for first time in this study. Olive oil was previously used alone or as a mixture with other oils [10]. Application of Bernoulli's principle and use of hot-air oven renders easy penetration of oils into tissues, thereby decreasing the time for clearing tissues. Effects of individual oils on physical and histological properties of 200 tissue sections were assessed. This assessment was not only done during different stages of histopathological procedures but also done during microscopic evaluation of the sections, which makes this study as a unique asset. In this study stability and longevity of haematoxylin and eosin staining was evaluated for over a period of one year and no significant difference in staining quality was observed.

## CONCLUSION

The present study concludes that all the four oils can be used as clearing agents in histopathological procedures as they not only de-alcholize tissues but are also economical, non hazardous, causes less shrinkage of tissues, maintain cellular architecture and staining quality of tissue sections. This study also concludes that amongst 4 different oils, pine oil was the best alternative for xylene in every aspect. In order to consider this agent as a better and safer substitute for xylene further studies needed to be carried out, where these 4 different oils treated specimens can be subjected to all stains and advanced histological techniques like immunohistochemical procedures.

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