Biofriendly Substitutes for Xylene in Deparaffinization

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Background: Xylene is an aromatic hydrocarbon that is routinely used in histopathological laboratories. It is biohazardous and produces many toxic effects like carcinogenesis. A safer substitute for xylene is necessary to minimize its usage. Aim: The aim of this study was to evaluate the effectiveness of 1.7% dishwashing solution, 95% lemon water, and 100% coconut oil when compared to xylene as a deparaffinizing agent during hematoxylin and eosin (H&E) staining. Materials and Methods: Fifteen paraffin-embedded tissue blocks were selected. Four sections were made from each block. One section was stained with conventional H&E method using xylene (group A) as deparaffinizing agent and other three sections were stained with xylene-free H&E method using 1.7% dishwashing solution (group B), 95% lemon water (group C), and 100% coconut oil (group D), respectively. Slides were scored blindly by a single pathologist considering the parameters such as nuclear and cytoplasmic staining; uniformity, clarity, and crispness of staining; and presence or absence of wax retention.

Results: Adequate nuclear staining was noted in 100% of sections of groups A, B, C, and D (P < 0.001), whereas adequate cytoplasmic staining was noted in 93.33% each in groups A, C, and D when compared with 100% in group B (P > 0.05). Uniform staining was present in 80% of groups A and B and in 73.33% of groups C and D (P > 0.05). Clarity of staining was present in 86.66% of groups A and B and in 80% of groups C and D (P > 0.05), whereas crispness of staining was seen in 73.33% of groups A and D, 86.66% of group B, and 80% of group C (P > 0.05). Wax retention was noted in 20% of groups A and B, and 26.66% of groups C and D (P > 0.05). Adequate staining for diagnosis was noted in 100% of group A sections followed by 93.33% in group B, 86.66% in group C, and 80% in group D as compared with 90% in group B (P > 0.05). Conclusion: Dishwashing solution, lemon water, and coconut oil can be used as safer and cost-effective substitutes to xylene for deparaffinization in H&E staining procedure.

Keywords: Deparaffinization, hematoxylin and eosin, xylene, xylene-free staining

INTRODUCTION

Nowadays many advanced molecular techniques have been introduced in histopathological practice for precise diagnosis. However, the basis for routine diagnostic work is the usage of hematoxylin and eosin (H&E)–stained paraffin sections. H&E stain is used as a gold standard universal stain in the field of pathology. It is used to discriminate nuclear, cytoplasmic, and extracellular features, and the staining procedure remains constant for past 150 years. Apart from H&E, components of H&E stain include xylene and different grades of alcohol that are used to

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carry out intermediate steps such as deparaffinization, rehydration, and dehydration of tissue sections during staining. The pitfalls associated with this H&E staining procedure are the usage and disposal of biohazardous reagents such as xylene, toxicity, cost containment, and working environment pollution.[1-2]

Xylene is an aromatic hydrocarbon that is biohazardous in nature. Exposure to xylene in laboratory occurs during tissue processing, deparaffinizing, cover slipping, and cleaning tissue processors. Inhalation of xylene vapors produces symptoms such as dizziness, headache, nausea, and vomiting. Long-term exposure leads to insomnia, tremors, irritability, impaired concentration, and short-term memory loss.[3-4] Occupational safety and health administration (OSHA) regulation identified xylene as a biohazardous chemical and the need for a safer nontoxic substitute increases.[5]

Go green is a challenging arena that seeks various safer and biofriendly alternative to toxic chemicals. Many substitutes such as aliphatic and aromatic hydrocarbon, mineral oil mixtures, and limonene reagents have been used as a clearing agent to substitute the xylene during tissue processing.[1-2]

Studies by Falkeholm et al.,[6] Ankle and Joshi,[2] and Buesa and Peshkov[7] showed the advantages of using hot dishwashing soap (DWS) solution for deparaffinization of tissue sections for H&E staining and some special staining procedures such as Van Gieson staining and periodic acid-Schiff staining. Henwood[8] also used hot DWS solution for deparaffinizing in immunohistochemistry. Premalatha et al.[5] also used mineral oil as a deparaffinizing agent and Ananthaneni et al.[9] attempted to use lemon water as a dewaxing agent.

Thus, this study was designed to determine the efficacy of dishwash liquid, lemon water, and coconut oil as an alternative to xylene for deparaffinizing agent in H&E staining.

**MATERIALS AND METHODS**

Fifteen paraffin-embedded tissue blocks were retrieved from the department archives. The study group was divided into 4 groups, namely.

Group A—tissue sections stained with routine H&E stain using xylene as deparaffinizing agent [Table 1]

Group B—tissue sections stained with xylene-free H&E stain using 1.7% dishwash solution as deparaffinizing agent [Table 2]

| Table 1: Routine (conventional) H&E staining procedure using xylene as a deparaffinizing agent |
|-----------------------------------------------|
| Procedure | Reagents | Time |
| Dепaraffinization and rehydration | Xylene I | 5 min |
| | Xylene II | 5 min |
| | 90% alcohol | 5 min |
| | 70% alcohol | 5 min |
| | Water wash | 10 min |
| Nuclear staining | Harris hematoxylin | 8 min |
| | Water wash | 2 min |
| Differentiation | Differentiation in 1% acid alcohol | 1 dip |
| | Water wash | 10 min |
| Bluing | 1% lithium carbonate | 1 min |
| | Water wash | 10 min |
| Cytoplasmic staining | 1% eosin | 1 min |
| | 90% alcohol | 30 s |
| | 70% alcohol | 30 s |
| | Xylene I | 5 min |
| | Xylene II | 5 min |

Approximate time required = 70–75 min

| Table 2: Xylene-free hematoxylin and eosin staining procedure using 1.7% dishwashing soap as a deparaffinizing agent |
|-----------------------------------------------|
| Procedure | Reagents | Temperature | Time |
| Dепaraffinization | Diluted dishwashing soap 1.7% I* | At 90°C | 1 min |
| | Diluted dishwashing soap 1.7% II | At 90°C | 1 min |
| | Distilled water I | At 90°C | 30 s |
| | Distilled water II | At 90°C | 30 s |
| | Wash slide in distilled water | At 45°C | 30 s |
| | Wash slide in distilled water | At room temp | 30 s |
| Nuclear staining | Harris hematoxylin | At room temp | 7 min |
| | Tepid water wash | | 4 min |
| Bluing | 0.5% Lithium carbonate | At room temp | 1 min |
| | Water wash | At room temp | 5 min |
| Cytoplasmic staining | Eosin 1% | At room temp | 1 min |
| | Tepid water wash | At room temp | 1 min |
| Dehydration | Over drying the sections | At 60°C | 10 min |

*1.7% diluted dishwashing soap solution—25 mL of liquid dishwashing solution added to 1500 mL of distilled water. Approximate time required = 30–35 min
Group C—tissue sections stained with xylene-free H&E stain using 95% diluted lemon water as deparaffinizing agent [Table 3]

Group D—tissue sections stained with xylene-free H&E stain using 100% coconut oil as deparaffinizing agent [Table 4]

Four sections of 4 μm thickness were made from each block. One section was stained with conventional H&E method, where xylene was used as deparaffinizing agent. The other three sections were stained by xylene-free H&E staining using 1.7% dishwash solution, 95% diluted lemon water, and 100% coconut oil as deparaffinizing agent, respectively.

Thus, a total of 60 sections were made and each section was evaluated and scored blindly by a single oral pathologist. The scoring was based on the following parameters:

1. Nuclear staining (adequate = score 1, inadequate = score 0)
2. Cytoplasmic staining (adequate = score 1, inadequate = score 0)
3. Clarity of staining (present = score 1, absent = score 0)
4. Uniformity of staining (present = score 1, absent = score 0), and
5. Crispness of staining (present = score 1, absent = score 0).

The scores for each slide were added. A score of ≤2 was graded as inadequate for diagnosis and slides with scores 3–5 were graded as adequate for diagnosis.

Inadequate deparaffinization of the sections was also noted.

**RESULTS**

The scores obtained from the different groups were tabulated and statistically analyzed using Pearson

| Table 3: Xylene-free H&E staining procedure using 95% lemon water as deparaffinizing agent |
|---------------------------------------------|-----------------------------------------------|
| **Procedure**                               | **Reagents**                                  | **Temperature** | **Time** |
| Deparaffinization                           | 95% diluted lemon water I                     | At 94°C         | 5 min    |
|                                             | 95% diluted lemon water II                    | At 94°C         | 5 min    |
|                                             | Distilled water I                             | At 94°C         | 5 min    |
|                                             | Distilled water II                            | At 94°C         | 5 min    |
|                                             | Wash slide in distilled water                 | At 45°C         | 30 s     |
|                                             | Wash slide in distilled water                 | At room temp    | 30 s     |
| Neutralizing the acidity effect in lemon    | Lithium carbonate                            |                   |          |
| water                                       | Tap water wash                               |                   |          |
| Nuclear staining                            | Harris hematoxylin                            | At room temp     | 2 min    |
|                                             | Tap water wash                               |                   |          |
| Differentiation                             | Differentiation in 1% acid alcohol            | At room temp     | 1 dip    |
| Bluing                                      | Tap water wash                               |                   |          |
|                                             | 1% eosin                                     | At room temp     | 1 dip    |
|                                             | Tap water wash                               |                   |          |
|                                              | Wash slide in distilled water                 |                   |          |
|                                              | Over drying the sections                      | At 60°C          | 5 min    |

**Approximate time required = 54 min**

| Table 4: Xylene- and alcohol-free H&E staining procedure using 100% coconut oil |
|---------------------------------------------|-----------------------------------------------|
| **Procedure**                               | **Reagents**                                  | **Temperature** | **Time** |
| Deparaffinization and rehydration           | Coconut oil 100% I                            | At 90°C         | 2 min    |
|                                             | Coconut oil 100% II                           | At 90°C         | 2 min    |
|                                             | Distilled water I                             | At 90°C         | 2 min    |
|                                             | Distilled water II                            | At 90°C         | 2 min    |
|                                             | Wash slides in distilled water                 | At 45°C         | 1 min    |
|                                              | Wash slides in distilled water                 | At room temp    | 1 min    |
| Nuclear staining                            | Harris hematoxylin                            | At room temp     | 15 min   |
|                                              | One dip in tap water and 1% acid alcohol      |                   |          |
|                                              | Running tap water                             |                   |          |
|                                              | Eosin 1%                                     | At room temp     | 2 min    |
|                                              | Wash slides in distilled water                 |                   |          |
|                                              | Over drying the sections                       |                   |          |
|                                              | Mounting                                      |                   |          |

**Approximate time required = 45 min**
chi-square test [Table 5]. Adequate nuclear staining was noted in 100% of group A, group B, group C, and group D sections ($P < 0.001$). Adequate cytoplasmic staining was noted in 93.33% each in group A, group C, and group D as compared with 100% in group B ($Z = 1.053, P > 0.05$). Uniform staining was present in 80% of group A and group B and in 73.33% of group C and group D ($Z = 0.480, P > 0.05$). Clarity of staining was noticed in 86.66% of group A and group B, and in 80% of group C and group D ($Z = 0.480, P > 0.05$). Crispness of staining was seen in 73.33% of group A and group D, 86.66% of group B, and 80% of group C ($Z = 1.080, P > 0.05$). We also noticed wax retention in 20% of group A and group B, and 26.66% of group C and group D sections ($Z = 0.978, P > 0.05$) [Graph 1]. The staining was found to be adequate for diagnosis in 100% of group A sections followed by 93.33% in group B, 86.66% in group C, and 80% in group D ($Z = 3.704, P > 0.05$) [Graph 2 and Figures 1-4].

**Discussion**

Xylene or dimethylbenzene is an aromatic hydrocarbon with a chemical formula of $C_6H_4(CH_3)_2$. It is a colorless, sweet-smelling liquid or gas that occurs naturally in petroleum, wood tar, and coal. Xylene is so called because it is present in crude wood spirit (in Greek, xylon means wood). Laboratory grade xylene is composed of three isomers namely ortho, para, and meta, and this mixture is known as xylol. Few traces of ethyl benzene, trimethylbenzene, phenol, toluene, thiophene, pyridine, and hydrogen sulfide are also seen.[4,10]

Xylene had been declared as biohazardous by National Institute for Occupational Safety and Health (NIOSH), and the permissible exposure limit is 100 parts per million (ppm) as time-weighted average for 10-h work shift and 40-h work week, and 200 ppm for 10 min as a short-term limit. Xylene is a volatile compound and it cannot be disposed easily by poured down into the drain. It has a low flash point of 28.9°C, which makes it an inflammable solvent.[11]

Other than occupational exposure, humans get exposure for xylene primarily via soil contamination. Xylene can leak into soil or ground water, or surface water, where it remains for months before it gets broken down into other chemicals. It can be smelled in air at 0.08–3.7 ppm and can be tasted in water at 0.53–1.8 ppm.[11]

Exposure to xylene can occur via inhalation, ingestion, eye, and skin contact. It is primarily metabolized in liver by oxidation of methyl group and conjugation with glycine to form methyl hippuric acid, which is excreted in urine. Very small amount is exhaled as such and usually there is less chance for accumulation within the body. Xylene can cause toxic effects from both acute (<14 days) and chronic (>365 days) exposure.[1]

In developed countries, there are provisions for monitoring the xylene exposure, disposal, and recycling. However, in India, no such provisions for monitoring of xylene in private pathology laboratories exist.[2]

Nowadays, several xylene substitutes, which are not biohazardous, which do not compromise the staining

| Table 5: Staining pattern in group A, group B, group C, and group D |
|-----------------------------------------------|
| Score                                      |
| Nuclear staining                            |
| Adequate                                   |
| Group A | 15 | 15 | 15 | 15 | 60 | — | <0.001 | S |
| Inadequate                                  |
| 0 | 0 | 0 | 0 | 0 | 0 |
| Cytoplasmic staining                        |
| Adequate                                   |
| Group A | 14 | 15 | 14 | 14 | 57 | 1.053 | 0.789 | NS |
| Inadequate                                  |
| 1 | 0 | 1 | 1 | 3 | 0 |
| Uniformity of staining                      |
| Adequate                                   |
| Group A | 12 | 12 | 11 | 11 | 46 | 0.373 | 0.946 | S |
| Inadequate                                  |
| 3 | 3 | 4 | 4 | 14 | 0 |
| Clarity of staining                         |
| Adequate                                   |
| Group A | 13 | 13 | 12 | 12 | 50 | 0.480 | 0.923 | NS |
| Inadequate                                  |
| 2 | 2 | 3 | 3 | 10 | 0 |
| Crispness of staining                       |
| Adequate                                   |
| Group A | 11 | 13 | 12 | 11 | 47 | 1.080 | 0.782 | NS |
| Inadequate                                  |
| 4 | 2 | 3 | 4 | 13 | 0 |
| Wax retention                               |
| Absent                                      |
| Group A | 12 | 12 | 11 | 10 | 45 | 0.978 | 0.807 | NS |
| Present                                     |
| 3 | 3 | 4 | 5 | 15 | 0 |
| Adequacy for diagnosis                      |
| Present                                     |
| Group A | 15 | 14 | 13 | 12 | 54 | 3.704 | 0.295 | NS |
| Absent                                      |
| 0 | 1 | 2 | 3 | 6 | 0 |

S = significant, NS = not significant

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quality, and which help maintain a healthy laboratory environment, are being tried out. We investigated the cheap, less toxic, and easily available diluted dishwash solution, lemon water, and coconut oil as deparaffinizing agents for routine H&E staining.

Liquid dishwash solution is composed of sodium laureth sulfate, cocamidopropyl betaine, sodium dodecyl benzene sulfonate, and nonionic surfactants. These substances are anionic surfactants that are commonly used in detergent soaps and shampoos. Their concentration in these products is monitored already by the manufacturers. It is primarily used for washing cutlery, glasses, and cooking utensils. They are easily available and cheaper products. We used diluted DWS by mixing only 25 mL of liquid DWS in 1500 mL of distilled water, in our study. Hence, there is a less chance of it being toxic to human beings. Lemon water is used to brighten the copper utensils, to remove grease and polish, and as sanitary kitchen deodorizer. Coconut oil is an easily available colorless solution generally used in lotions and ointments, as hair oil, in laxatives, and for cooking.

In our study and in studies conducted by Ramulu et al.,[11] Ananthaneni et al.,[9] Sravya et al.,[12] Premalatha et al.,[3] and Negi et al.,[1] Harris hematoxylin, a regressive stain, was used. However, Ankle and Joshi[2] used Mayer’s hematoxylin, a progressive stain.

In this study, all sections showed adequate nuclear staining, suggesting that there was no difference between these staining procedures. When cytoplasmic staining was evaluated, 100% of group B showed adequate cytoplasmic staining when compared to 93.33% of group A, group C, and group D sections. Of group A and group B sections, 80% showed uniformity in staining whereas 73.33% of

![Graph 1: Comparison of various staining parameters between group A, group B, group C, and group D](image1)

![Graph 2: Adequacy of H&E-stained sections for diagnosis in different groups](image2)
Figure 1: Photomicrograph of H&E-stained sections deparaffinized using xylene. (A) Uniformity and clarity of staining (×10). (B) Nuclear and cytoplasmic staining (×20). (C) Muscle and fat tissue (×10). (D) Glandular tissue (×10)

Figure 2: Photomicrograph of H&E-stained sections deparaffinized using 1.7% dishwash solution. (A) Uniformity and clarity of staining (×10). (B) Nuclear and cytoplasmic staining (×20). (C) Muscle and fat tissue (×10). (D) Glandular tissue (×10)
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**Figure 3:** Photomicrograph of H&E-stained sections deparaffinized using 95% diluted lemon water. (A) Uniformity and clarity of staining (×10). (B) Nuclear and cytoplasmic staining (×20). (C) Muscle and fat tissue (×10). (D) Glandular tissue (×10)

**Figure 4:** Photomicrograph of H&E-stained sections deparaffinized using 100% coconut oil. (A) Uniformity and clarity of staining (×10). (B) Nuclear and cytoplasmic staining (×20). (C) Muscle and fat tissue (×10). (D) Glandular tissue (×10)
group C and group D sections showed uniform staining. Out-of-focus areas were seen in some of the sections, which compromised uniformity of staining. This could be due to section tear, dirty microscopic lenses, blade defect, thick section, and moisture in cover slip. All these factors should be ruled out.[3]

The clarity of staining in group A and group B sections was 86.66% as compared with 80% in group C and group D sections. Of group A and group D, 73.33% revealed a crisp staining whereas group B showed 86.66% and group C showed 80% crispness in staining. Wax retention was highest in 33.33% of group D sections followed by 26.66% of group C and 20% in group A and group B sections. Analysis of previous studies showed that xylene-free H&E staining is temperature sensitive. A reduction in standardized temperature results in failure of complete wax removal. This results in residual wax retention on tissue sections.[2] On the other hand, increase in temperature of solution leads to dislodgement of sections from the slides.

When all the individual scores were added, 100% of group A sections showed adequate staining for diagnosis followed by 93.33% of group B, 86.66% of group C, and 80% of group D sections. The time taken for deparaffinizing the sections using xylene-free technique is less compared to conventional H&E staining. Apart from this, they are nontoxic, easy to dispose, and are cost-effective.

Falkholm et al.[6] conducted a study and found that 74% of sections were good for diagnosis and 26% were inadequate when compared to conventional xylene sections. Ankle and Joshi,[3] Ramulu et al.[11] and Negi et al.[4] conducted a study using 1.7% dishwash solution as a substitute for alcohol and xylene in conventional H&E procedure. Their results showed that liquid dishwash solution can be used as an effective substitute to xylene and alcohol in routine H&E staining. Premalatha et al.[5] used refined mineral oil for deparaffinization in H&E staining and found that 93.3% sections were adequate for diagnosis.

Ananthaneni et al.[9] conducted a study using 1.5% dishwash solution and 95% diluted lemon water as deparaffinizing agents in routine H&E staining and they found that 1.5% dishwash solution showed superior uniform staining and less wax retention compared to 95% lemon water. They also found that 100% of diluted lemon water sections and 95% of dishwash liquid sections showed adequate staining for diagnosis.

Sravya et al.[12] used sesame oil for tissue processing and stained using 95% diluted lemon water and 1.5% dishwash solution as an alternative to xylene in routine H&E staining, and they found that 88.7% of sections showed adequate for diagnosis using 1.5% dishwash solution whereas sections deparaffinized by 95% dilute lemon water revealed 78% of adequate for diagnosis.[12]

**Conclusion**

On the basis of the findings of our study, we conclude that 1.5% dishwash solution, 95% diluted lemon water, and 100% coconut oil can be used as an effective alternative to xylene in routine H&E staining. They also had an added advantage of being eco-friendly, nonhazardous, cost-effective, less time-consuming, easy to handle, and disposable. Further studies with larger sample sizes must be performed for validating our findings.

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**Conflicts of interest**

There are no conflicts of interest.

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