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Evaluation of Comparative Performance of Xylene and Fresh Lemon Fruits Extract as Dewaxing Agent in Histopathology Staining for Liver, Kidney, and Lung in Sectioned Wistar Rats

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ABSTRACT: Xylene is a significant material in histopatholoy staining, however, it poses health risks to people and therefore, the histopathologist requires a substitute which could be less costly with zero or to minimal health risk if ingested, inhaled or in direct contact with eyes or skin. Therefore, the objective of this paper is to evaluate the comparative performance of xylene and fresh lemon fruits extract as dewaxing agent in histopathology staining for liver, kidney, and lung in sectioned Wistar rats using appropriate standard techniques. Data obtained from both lemon water and using Haematoxylin and Eosin (H&E) and a photomicrograph revealed that concentrated lemon water could serve as a bio-safe substitute for xylene in deparaffinization process during sectioning.

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In the majority of surgical instances, histopathology is the gold standard for diagnosis. Tissues must first be preserved and treated before being cut into sections and stained for histopathology diagnosis. The most used deparaffinization agent is xylene because of its superior clearing capabilities. Commercial Xylene has an aromatic odor and is a clear, colorless liquid (Swaroop *et al.*, 2018). Xylene, however, not only costs a lot of money but also poses health risks to people. Xylene exposure can happen through ingesting, inhaling, or direct contact with the eyes or skin. Longterm exposure to Xylene can cause cells impacted by it to produce less mitochondrial ATP, which can result in irreversible impairment (Ghosh *et al.*, 2016). Xylene exposure has been linked to 3 disease conditions like hepatitis, chemical pneumonitis, depression, anaemia, and skin irritation (Kandyala *et al.*, 2010). The National Institute for Occupational Safety and Health advised that exposure limits for xylene be set at 100 parts per million (ppm) as a time-weighted average for up to a 10-hour work shift and a 40-hour work week, and 200 ppm for 10 minutes as a short-term limit. By eliminating Xylene from tissue processing, prices are reduced, time is saved, and the laboratory environment is improved (Kandyala *et al.*, 2010; *Buesa*, 2000). To combat Xylene's toxicity, a number of alternatives have been researched, including alcohols (provide reference here), kerosene (provide reference here), liquid dishwashing solution (provide reference here), natural vinegar (provide reference here), mineral oils (provide reference here), and lemon water (provide reference here). The most affordable, easily accessible, cost-effective, nontoxic, and non-flammable substitute for

Xylene is lemon water. Citric acid, a weak organic acid, is abundant in lemon fruit. It is a natural preservative that is also used to give meals and soft drinks an acidic (sour) flavour. Glass stains from hard water can be eliminated without cleaning using a 6% citric acid solution (provide reference here). Additionally, it serves as an antioxidant and a safe cleaning agent for the environment (Simonne and Ritenour, 2011). The goal of this study was to determine whether environmentally friendly deparaffinization substitutes, like lemon water, could replace the industry standard Xylene in histopathological staining procedures. Hence, the objective of this paper was to evaluate the comparative performance of xylene and fresh lemon fruits extract as dewaxing agent in histopathology staining for liver, kidney, and lung in sectioned Wistar rats.

MATERIALS AND METHOD

Study area: This study was carried out at the Histology Unit, Anatomy Department, Olabisi Onabanjo University, Sagamu, Ogun State, geographically located at latitude: N 6° 55.5201' and Longitude: E 3° 52.6013' (Zwiefelhofer, 2022).

Materials: Wistar rats, Glass coupling jar, slides, coverslips, slide racks, oven, incubator, pencil, Distilled water (used in the dissolving and mixing of extract 95% lemon water), sellotape, hematoxylin, and Eosin staining jars.

Study design: This is a descriptive comparative study between two deparaffinization agents, standard Xylene and 95% concentrated lemon extract (lemon water), in histopathology. Two sets of tissue samples (lung, liver, and kidney samples) were obtained from Wistar rats and sectioned. Xylene was used to dewax one set of liver, kidney, and lung samples. The slides were placed in two changes of Xylene for 15 minutes each. 95% concentrated lemon water was used to dewax the other liver, kidney, and lung samples. The slides were placed in two changes of lemon water and heated to 70 OC for 15 minutes each. Both the slides dewaxed with Xylene and those dewaxed with lemon water were stained afterwards using Haematoxylin and Eosin (H&E), and a photomicrograph was taken of the slides.

Preparation and extraction of lemon: Fresh lemon fruits (Bearss lemon) were purchased from Sagamu Market, Ogun State. The lemons were extracted using manual methods. The lemons were washed with tap water, rubbed on a clean, flat surface to soften, and then cut horizontally into four pieces. Afterwards, each cut lemon piece was squeezed into a jug using a filter to remove the pulp, seed, and any lemon particles. The lemon extract was then placed into two glass coupling jars and placed in an oven to increase the temperature of the lemon water to a steady 70 OC immediately before use.

Experimental animal preparation: Six Wistar rats were obtained from the animal house of the department of veterinary medicine at Olabisi Onabanjo University, Sagamu, Ogun State. They were acclimatised for two weeks in the animal house. The animals were housed in standard plastic cages, fed a standard pellet diet (Bendel Feeds and Flour Mill, Limited, Ewu, Nigeria), and allowed free access to water. Three rats each were assigned to two groups: the xylene group (group 1) and the Lemon water group (group 2). The animals were anaesthetized in a chloroform chamber, after which the required tissues (liver, lung, and kidney) were obtained, processed, and sectioned. Animal care and treatment were in conformity with the institutional guidelines, which comply with international laws and policies (National Research Council of the National Academics, 2011).

Histological assessment: The tissue samples were processed using the manual tissue processing method after being fixed in neutral buffered formalin. The tissues were first treated for 30 minutes each in three baths of 50% alcohol, then moved to three baths of 80/20 ethanol/isopropyl alcohol (IPA) for 30 minutes each, and finally treated for an hour each in three baths of plain IPA. The samples were allowed to drain for 2 hours before being immersed in two baths of cell path wax at 56°C for 112 hours each (Adediran and Ibikunle, 2016). The tissues were embedded in cell-path paraffin wax, divided into sections, and later stained with hematoxylin and eosin.

Sixteen sections, two each for the liver, three each for the kidney, and lung samples for groups 1 and 2, were prepared and labeled accordingly, making two sets of slides**.** The sixteen sections will be placed in an incubator at a temperature of 95 OC for 30 minutes until they are ready for use.

RESULTS AND DISCUSSION

Table 1 shows the evaluation of the lung slides between specimens treated with xylene and lemon, in which for the nucleus, background staining and artefact staining were high, with a prevalence of 66.6%. Table 2 shows the evaluation of the liver slides between specimens treated with xylene and lemon, in which, for the adhesion of sections to slides, nucleus staining and artifacts were least prevalent with a prevalence of 33.3%. Table 3 shows the evaluation of the kidney slides between specimens treated with xylene and lemon, in which the adhesion to slides was

rated as 'inferior to xylene and same as xylene, with prevalences of 33.3% and 66.6%, respectively. The cytoplasm staining was as high as "same as xylene," with a prevalence of 66.6%. For the nucleus staining and artefacts, a mild, good, or very good report was recommended.

Table 1: Evaluation of lungs slides between specimens treated with xylene and Lemon

Lungs Group	Inferior to xylene (nil)		Same as xylene (mild)		Superior to xylene (good)		Very superior to xylene (Very good)	
	Number	%	Number	%	Number	%	Number	%
Adhesion of sections to slides	1	33.3	2	66.6	0	00.0	0	00.0
Nucleus staining	0	00.0	0	00.0	2	66.6	1	33.3
Cytoplasmic staining	2	66.6	1	33.3	0	00.0	0	00.0
Background staining	0	00.0	0	00.0	2	66.6	1	33.3
Artefacts	0	00.0	0	00.0	2	66.6	1	33.3

Table 2: Evaluation of liver slides between specimens treated with xylene and Lemon

Lungs Group	Inferior to xylene (nil)		Same as xylene (mild)		Superior to xylene (good)		Very superior to xylene (Very good)	
	Number	%	Number	%	Number	%	Number	%
Adhesion of sections to slides	1	33.3	2	66.6	0	00.0	0	00.0
Nucleus staining	0	00.0	1	33.3	2	33.3	1	33.3
Cytoplasmic staining	0	00.0	1	33.3	2	66.6	0	00.0
Background staining	0	00.0	0	00.0	2	66.6	1	33.3
Artefacts	0	00.0	0	33.3	1	33.3	1	33.3

Table 3: Evaluation of Kidney slides between specimens treated with xylene and Lemon

Lungs Group	Inferior to xylene (nil)		Same as xylene (mild)		Superior to xylene (good)		Very superior to xylene (Very good)	
	Number	%	Number	%	Number	%	Number	%
Adhesion of sections to slides		33.3	2	66.6	0	00.0	0	00.0
Nucleus staining	0	00.0	1	00.0	2	33.3	1	33.3
Cytoplasmic staining	1	66.6	2	66.6	0	00.0	0	00.0
Background staining	0	00.0	0	00.0	2	66.6	1	33.3
Artefacts	0	00.0	0	33.3	1	33.3	1	33.3

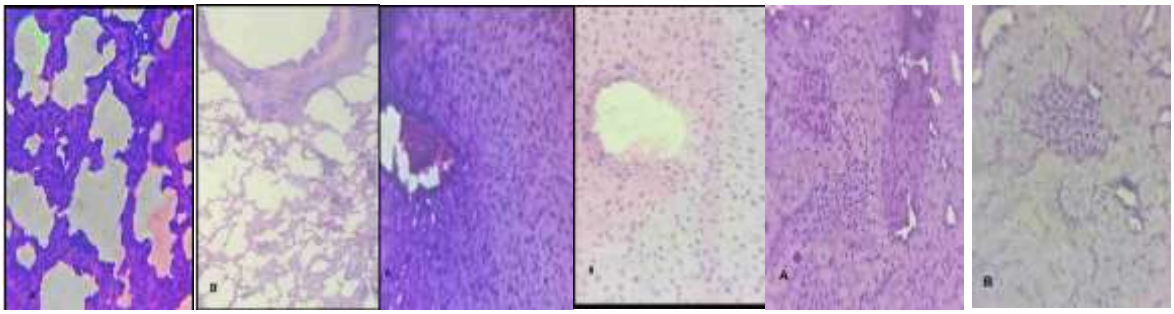

Plate 1: A lungs section dewaxed with (A) lemon, (B) lungs section dewaxed with xylene, (C) liver section cleared with lemon, (D) liver section cleared with xylene, (E) liver section cleared with lemon, liver section cleared with xylene, kidney section cleared with lemon, kidney section cleared with xylene. H and E stanning (×400)

For a number of years, xylene has been widely used in histology laboratories in spite of its toxicity to personnel and the danger it poses to the environment (Pandey *et al.*., 2014). The historical use of xylene in the histology laboratory is an example of futile substitution. Due to new regulations from Occupational Safety and Health Administration, several xylene substitutes have been commercially developed in recent years (Zhang *et al.*, 2018). However, most of the commercially available xylene substitutes are less effective, more expensive, and not that much less hazardous than xylene itself (Tao *et*

aldevise., 2020). Any technique minimizes the use of xylene by using non-biohazardous substitutes, reduces staining time with unequivocal cell morphology will be indispensable for diagnostic reasons as well as for maintaining a healthy laboratory environment. Thus, our study presents the alternatives of deparaffinizing agent prior to H and E staining method that involves the use of easily available, nontoxic, and eco-friendly liquid diluted lemon water by completely eliminating expensive and hazardous xylene from deparaffinizing prior to H and E staining, so as to device an optimal staining technique that is easily available, less toxic, time-saving, and cost-effective.

Lemon juice is customarily used to brighten up copper cookware, as a sanitary kitchen deodorizer, to remove grease, polish, and wood cleaner, and so forth. A review of the literature showed very few studies to date where diluted Lemon Water was used as a deparaffinizing agent. The novel concept of using diluted lemon water as a deparaffinizing agent came from its solvent property, which was used to dissolve old wax (Abbas *et al*., 2022).
In this present study, Lemon water was more viscous when compared with xylene.

Hence, in order to decrease the viscosity of lemon, increase its penetration into tissues (liver, kidney, and lungs), and clear them at a faster rate, the lemon water was diluted with distilled water (95% concentrated lemon water), and dewaxing of tissue during staining was carried out using an incubator at a temperature maintained at 70°C or 80°C. It was reported from this study that lemon water could not deparaffinize the tissue (liver, kidney, and lungs) sections at these temperatures. The sections were deparaffinized at 95 °C and immediately hydrated by taking them through descending grades of alcohol.

Routine paraffin wax tissue processing requires properly timed dehydration, clearing (de-alcoholization), infiltration, and embedding procedures. But in this study, we skipped the clearing process, hence the use of isopropyl alcohol, and drained for 2 hours before infiltrating with wax just to minimise the use of xylene. On microtomy, sections were cut, dewaxed with both 95% diluted lemon water and xylene for the two groups, and further hydration was done before the staining procedure.

The mechanism by which lemon extract serves as a deparaffinization agent involves the use of citric acid to soften and dissolve paraffin wax. Paraffin wax is a complex mixture of long-chain hydrocarbons that is solid at room temperature (Aswani *et al*., 2020). Citric acid is a weak acid that is able to soften and dissolve the wax through a process known as saponification. Saponification is a chemical reaction in which an acid and a base react to form salt and water. In the case of lemon extract and paraffin wax, the citric acid in the lemon extracts acts as the acid and the hydrocarbons in the paraffin wax act as the base. The reaction between the citric acid and the hydrocarbons produces salt and water, effectively breaking down the paraffin wax and making it easier to remove the wax (Hassa *et al*., 2018). As seen in plates 1, when comparing A to B, the lemon water showed a better Photomicrograph when compared to xylene, which is in agreement with the study of Hassa *et al*. (2018). It is important to note that the citric acid in lemon extract is not the only component that can contribute to its effectiveness as a deparaffinization agent. Lemon extract also contains other compounds, such as essential oils and flavonoids, which may contribute to its ability to soften and dissolve paraffin wax. However, the exact mechanism by which these compounds contribute to the deparaffinization process is not well understood. Rasmussen *et al.* (1992) made use of a mixture of lemon water as a clearing agent before routine hematoxylin and eosin histopathological staining procedures. In their study, incomplete impregnation of wax was noted, leading to problems while sectioning and they concluded that the mixture was ineffective as a clearing agent.
A study by Taneeru *et al.* (2013) evaluated the efficiency of lemon water and sesame oil in terms of nuclear staining, cytoplasmic staining, uniformity, clarity, and intensity of staining, which was also similar to this present study. Their study concluded that specimens cleared with lemon water had all the staining features that were adequate for the diagnosis aspect and were consistent with our study results. Sermadi *et al*. (2014) checked the efficiency of coconut oil as a deparaffinizing agent and concluded that xylene-treated tissue specimens were more rigid (73% rigidity) than lemon water-treated specimens. The transparency of the specimen was also evaluated in their study, which showed 100% better features in lemon water-treated specimens. 100% of lemon-treated specimens showed the same features as those of xylene-treated specimens. In their study, there was not much difference in staining quality and tissue architecture in both lemon water and xylene-treated slides (Sermadi *et al.* 2014).
Our study results are in agreement with another study by Udonkang *et al.* (2014) using bleached palm oil, and their results showed 93% of palm oil-processed tissues appeared transparent after clearing as compared with xylene-processed specimens. Ease of microtomy was also evaluated, and they concluded that easy microtomy was found in 100% of xylene-exposed specimens and 73.7% of palm oil-exposed

specimens. Hematoxylin and eosin-stained slide evaluation was done and evaluated based on nuclear staining, cytoplasmic staining, and clarity of staining, in which 100% of all specimens showed normal nuclear and cytoplasmic staining in both groups (Udonkang *et al.,* 2014).

Indu *et al*.-(2014)4 concluded that adequate nuclear staining was observed in 90%ofncedarwoodd oil and 93%ofnxylene,e similar to this present study. Also, adequate cytoplasmic staining and overall uniformity of staining were noted in 93.33% of cedarwood oil treated slides (Indu *et al*., 2014). All the samples in their study had appreciable results when treated with cedarwood oil as compared with that of xylene according to the grossing and microscopic features, and this also was in quite similarity with our study.

Sugunakar Raju *et al.* (2015) evaluated four different oils, namely rose oil, carrot oil, pine oil, and olive oil, as deparaffinizing agents instead of xylene. Gross tissue specimen evaluations such as translucency, rigidity, shrinkage, section cutting, cellular architecture, and staining quality were evaluated for all the oils. Pine oil had superior characteristic features when compared with the other three oils.

Tissue morphology was well preserved in all of the tissue sections deparaffinized by using these four different oils, and a clear demarcation was noticed in the nucleus and cytoplasm. Their results concluded that the overall staining quality was equivalent to that of xylene-treated slides, and these were also similar to our study as lemon water was used. Our study results with respect to nuclear and cytoplasmic clarity in hematoxylin and eosin-stained slides were in agreement with this study. In another study conducted by Buesa in 2000, mineral oil was used in proportion with ethanol and isopropyl alcohol as a clearing agent for tissue specimens (Buesa, 2000). In this study, processed tissue with isopropyl alcohol showed equivalent qualities when compared with processed tissue with xylene.

Conclusion: The hazardous and harmful effects of exposure to Xylene have been well documented. In addition, its high cost makes it a financial burden for laboratories. It therefore becomes necessary to consider safer alternatives, essential to decreasing the price and hazards of unsafe Xylene and suitable for laboratory use. A study of eco-friendly deparaffinization agents that may be more efficient than Xylene in the H&E (Haematoxylin and Eosin) staining procedure and harmless, quicker, and more cost-effective will be beneficial to the scientific community.

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