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Usage of Coconut Oil as a Biofriendly Xylene Substitute in Tissue Processing and Staining

Mamta Tanwar
Department of Oral Pathology and Microbiology, Surendera Dental College and Research Institute, Sri Ganganagar, Rajasthan, India

Manish Sharma
Department of Oral Pathology and Microbiology, Surendera Dental College and Research Institute, Sri Ganganagar, Rajasthan, India

R. Karthikeyan
Department of Oral Pathology and Microbiology, Saveetha Dental College, Chennai, India

Manish Kumar
Department of Oral Pathology and Microbiology, Surendera Dental College and Research Institute, Sri Ganganagar, Rajasthan, India

Vineet Tyagi
MDS Oral Pathology, General Practitioner, New Delhi, India

***Abstract*---**Background: Xylene is colorless flammable liquids that is extensively used in stock pathology, however it is carcinogenic & neurotoxic. Hence, need to identify safer substitute of xylene is necessary. Aim: The aim of study was to determine efficacy of Coconut oil as a biofriendly substitute for Xylene as a clearing and dewaxing agent. Materials and methods: 70 soft tissue specimens were divided into equal halves which were processed simultaneously in xylene and coconut oil as clearing agents. Samples obtained after processing were observed for following parameters: Rigidity, Tissue shrinkage and Translucency. Paraffin blocks were prepared and two serial sections were obtained from each block. During sectioning tissue samples were observed for Nicks and Ribbons. After sectioning, one section was stained by standard hematoxylin and eosin using xylene while other section was stained by standard hematoxylin and eosin using Coconut oil as dewaxing and clearing agent. Tissue were evaluated using following parameters: cytoplasmic staining, Nuclear staining, Uniformity of staining, Clarity of staining, Crispness of staining and

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***Corresponding author**: Sharma, M.; Email: drmanishsharma2007@gmail.com*
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cell morphology criteria by 3 independent oral pathologists. Kappa test and Chi-square test were done.

***Keywords*---**biofriendly, clearing agent, coconut oil, Dewaxing agent, Xylene.

Introduction

Xylene comes from a Greek word XYLO meaning 'wood'. Xylene can exist in three Isomers: Xylene, Xylol or Dimethylbenzene, with the chemical formula $(CH_3)_2$ C_6H_4. Isolated and named by Auguste Cahours in 1850, it was identified as a constituent of wood tar. They are colorless, sweet- smelling hydrocarbons and flammable liquids that are extensively used in stock pathology for tissue processing, staining & cleaning tissue (Harper & Liccione, 1995). Meta-xylene, ortho-xylene, and para-xylene are all variants of xylene that have different methyl groups on the benzene ring (m-,o-, and p-xylene). Xylene is used to make plastic bottles and polyester clothes, whereas Ortho-xylene is used to make phthalic anhydride (Harper & Liccione, 1995). Xylene is utilised in the printing, rubber, and leather industries, among other applications such as Cleaner, thinner for paint, and varnishes (Fabri et al., 2000). Xylene is added to pesticides and disinfectants (Uchida et al., 1993). In dentistry, xylene can be used to dissolve guttapercha, used in root canal treatment (Harper & Liccione, 1995). In light microscopy, xylene is used as a solvent to remove synthetic immersion oil from the objective (Harper & Liccione, 1995). In tissue processing, xylene is used as a clearing agent to remove as much alcohol as possible and make the tissue more transparent (Harper & Liccione, 1995). In histology procedures, xylene is used as clearing agent and dewaxing agent, hence it is the preferred agent to produce crisp stained slides (Harper & Liccione, 1995). Xylene is used as deparaffinizing agent, before and after staining, to remove the paraffin from dried microscope slides; slides are treated in xylene before being mounted with cover slip (Harper & Liccione, 1995).

Hence, xylene exposure is frequently seen in histopathology laboratory technicians. M-xylene has a percutaneous absorption rate of 2 $g/cm^2/min$ in humans when applied to the hands (Uchida et al., 1993). Bardodej (1964), states that in occupational exposure the main route of xylene absorption is the lungs, the retention being 60 to 64% (Bardodej, 1964). Engström et al. (1978), states that Long-term skin contact with organic solvents such as Xylene can induce dermatitis (disease of the skin's natural protective oils), dermatitis (dryness), peeling and cracking (Engström et al., 1978). Exposure to xylene, whether acute (lasting less than 14 days) or long-term (lasting more than 365 days), has health consequences (Harper & Liccione, 1995). Acute exposure can cause: inflammation of the skin, eyes, and respiratory tract, Tiredness, irritation, and a headache Impairment in motor coordination and balance Flushing, a flushed appearance, and an enhanced awareness of one's own heat symptoms such as excessive salivation, tremors, disorientation, and confusion Problems with the digestive system Irritation in the heart Loss of consciousness and severe abdominal aches (Harper & Liccione, 1995; Rajan & Malathi, 2014).

Whereas chronic exposure can cause: Dryness of the nose, mouth, and skin, as well as conjunctivitis Damage to the kidney and liver. An increase in the number of miscarriages and birth abnormalities has been linked to irregular menstrual cycles. Leukemia risk is enhanced, as well as a variety of nervous system complications, due to decreased blood platelet counts (Harper & Liccione, 1995; Rajan & Malathi, 2014; Kandyala et al., 2010; Salimi et al., 2017). Xylene's carcinogenicity for humans cannot be classified, International Agency for Research on Cancer (IARC), Environmental Protection Agency (EPA) (EPA). So, there is a need to study the effect of xylene in humans (Uchida et al., 1993). Xylene has an acceptable limit of 100 ppm (parts per million) as an eight-hour average concentration of exposure, according to the Occupational Safety and Health Administration. Levels higher than this could produce toxicity which could be after acute (<2 weeks) or chronic (>1 year) exposure (Langman, 1994). Health risks in histology labs should be reduced. Urinary excretion of xylene's metabolites should be used to track exposure over time. Workers' bodily fluids should be tested for xylene at least once a year to ensure that the chemical is not exceeding acceptable levels (Rajan et al., 2019). So to avoid hazards caused by xylene, alternative to xylene is required. An acceptable replacement to xylene is needed for dewaxing and clearing tissues that is cost-effective, minimally toxic and non-flammable; it should also retain the morphology and staining features of the tissue sections throughout the process. Hence, the present study was conducted using coconut oil as an alternative to xylene as deparaffinising agent, dewaxing agent and clearing agent.

Materials and Method

70 soft tissue samples were retrieved from archive of the Department of Oral & Maxillofacial Pathology and Oral Microbiology of Surendera Dental College and Research Institute, Sriganganagar, Rajasthan. 70 samples included 10 gingival enlargements, 10 odontogenic cysts, 10 benign epithelial lesions, 10 oral squamous cell carcinomas, 10 salivary gland tumours, 10 benign connective tissue tumours, and 10 mucocutaneous lesions. Each tissue sample was divided into two equal halves. One half of the sample was processed in xylene as per standardized protocol (Processing protocol Table 1) and another half of the sample was processed in Coconut oil (100%) {in place of Xylene in previous protocol Coconut oil was used (Processing protocol Table 1)}. Coconut oil used in the procedure during winter was kept in incubator for maintaining its liquid state.

Table 1
Tissue processing procedure using Xylene & Coconut oil

Procedure	Group Xylene	Group Coconut oil
Dehydration		
50% Alcohol	1 Hour	1 Hour
70% Alcohol	1 Hour	1 Hour
90% Alcohol	1 Hour	1 Hour
Absolute Alcohol I	1 Hour	1 Hour
Absolute Alcohol II	Overnight	Overnight
Absolute Alcohol III	1 Hour	1 Hour
Clearing		

	Xylene I	1 Hour	Coconut oil I	1 Hour
	Xylene II	1 Hour	Coconut oil II	1 Hour
Wax Infiltration				
Wax I	1 Hour		1 Hour	
Wax II	1.30 Hour		1.30 Hour	
Wax III	2 Hour		2 Hour	

Tissue samples obtained after processing were observed individually, for the following parameters:- Rigidity (0- Soft, 1- Semi Rigid, 2- Rigid), Tissue shrinkage (0- <10%, 1- <11-20%, 2- <21-30%) and Translucency (0-Opaque, 1- Mild opaque / Mild translucent, 2- Translucent) (Table 2) to assess the quality of the tissue processed using Xylene and Coconut oil as clearing agent. Later paraffin blocks were prepared and 4µ sections were prepared by Leica manual microtome (RM2125 RTS). Two serial sections were obtained from each block. During sectioning xylene (X) and coconut oil (C) processed tissue samples were observed for Nicks (0- Absent, 1- Present) and Ribbons (0- Absent, 1- Present) (Table 3) to assess the quality of the tissue processed while sectioning. After sectioning, one section was stained by standard hematoxylin and eosin (H&E) using xylene as dewaxing and clearing agent as per standardized protocol (Staining protocol Table 4) while the other section was stained by standard hematoxylin and eosin (H&E) using Coconut oil as dewaxing and clearing agent {in place of Xylene in previous Staining protocol Coconut oil was used (Staining protocol Table 4)}.

Table 2
Rigidity, tissue shrinkage & translucency among Xylene and Coconut Oil processed tissues

	Xylene processed tissues	Coconut oil processed tissues	Chi square	P value
Rigidity				
Soft	1.4%	23%	27.53	<0.01*
Semi- rigid	4.3%	32.9%		
Rigid	94.3%	65.7%		
Tissue shrinkage				
<10%	35.7%	90.0%	94.37	<0.01*
<11-20%	54.3%	10.0%		
<21-30%	10.0%	0.0%		
Translucency				
Opaque	0.0%	1.4%	1.14	0.54
Mild Opaque / Mild Translucent	5.7%	4.3%		
Translucent	94.3%	94.3%		

*: statistically significant

Table 3
Nicks and ribbons among Xylene and Coconut Oil processed tissues while sectioning

	Xylene processed tissues	Coconut oil processed tissues	Chi square	P value
Nicks				
Absent	94.3%	85.7%	2.86	0.09
Present	5.7%	14.3%		
Ribbons				
Absent	90.0%	88.6%	0.08	0.79
Present	10.0%	11.4%		

Table 4
Staining procedure protocol using Xylene & Coconut oil

Procedure	(X-X)	(C-C)	(C-X)	(X-C)
Type of tissue section	Xylene processed tissue section	Coconut oil processed tissue section	Coconut oil processed tissue section	Xylene processed tissue section
Deparaffinization	Xylene I (5 min)	Coconut oil I (5 min)	Xylene I (5 min)	Coconut oil I (5 min)
	Xylene II (5 min)	Coconut oil II (5 min)	Xylene II (5 min)	Coconut oil II (5 min)
Rehydration				
Absolute Alcohol I	3 min	3 min	3 min	3 min
Absolute Alcohol II	3 min	3 min	3 min	3 min
80% Alcohol	3 min	3 min	3 min	3 min
70% Alcohol	3 min	3 min	3 min	3 min
60% Alcohol	3 min	3 min	3 min	3 min
Water wash	3 min	3 min	3 min	3 min
Nuclear staining				
Harris' hematoxylin	5 min	5 min	5 min	5 min
Running tap water	30 min	30 min	30 min	30 min
Differentiation				
1% Acid alcohol	10 sec	10 sec	10 sec	10 sec
Bluing				
Tap water wash	15 min	15 min	15 min	15 min
Dehydration				
60% Alcohol	2 min	2 min	2 min	2 min
80% Alcohol	2 min	2 min	2 min	2 min
Absolute Alcohol	2 min	2 min	2 min	2 min
Cytoplasmic staining				
Eosin	1 min	1 min	1 min	1 min
Absolute Alcohol I	3 min	3 min	3 min	3 min
Absolute Alcohol II	3 min	3 min	3 min	3 min
Clearing	Xylene I (5 min)	Coconut oil I (5 min)	Xylene I (5 min)	Coconut oil I (5 min)
	Xylene II (5 min)	Coconut oil II (5 min)	Xylene II (5 min)	Coconut oil II (5 min)

Slide mounted with DPX

Note: X-X – Xylene processed & Xylene stained tissues
C-C – Coconut oil processed & Coconut oil stained tissues
C-X – Coconut oil processed & Xylene stained tissues
X-C – Xylene processed & Coconut oil stained tissues
Slides cleared using coconut oil were dapped with commercially available tissue paper, for absorbing extra coconut oil present on the slides.

Each sample was coded (from 1 to 70) and two halves was coded from 1C to 70C (for Coconut oil processing) and from 1X to 70X (for Xylene processing). The sections produced from Coconut oil processing & Coconut oil dewaxing and clearing were coded from 1CC to 70CC and the sections produced from Coconut oil processing & Xylene dewaxing and clearing were coded from 1CX to 70CX where as the sections produced from Xylene processing & Xylene dewaxing and clearing were coded from 1XX to 70XX and the sections produced from Xylene processing & Coconut oil dewaxing and clearing were coded from 1XC to 70XC. 280 sections were blindly examined and decoded by three histopathologists (Dr Manish Sharma, Dr R Karthikeyan and Dr Manish Kumar). Following criterias were used:- Cytoplasmic staining (0- Inadequate, 1- Adequate), Nuclear staining (0- Inadequate, 1- Adequate), Uniformity of staining (0- Inadequate, 1- Patchy, 2- Uniform), Clarity of staining (0- Inadequate, 1- Adequate), Crispness of staining (0- Inadequate, 1- Adequate) and cell morphology (0- Inadequate, 1- Adequate) (Table 5). Kappa test and Chi-square test were applied using SPSS software version 22.0. The data was analyzed and the test results tabulated and evaluated.

Table 5
Criterias used for analysis of tissue sample after staining

Parameters		C-C (%)	X-X (%)	C-X (%)	X-C (%)	X-X vs C-C p value	X-X vs C-C k Value	X-X vs C-X p value	X-X vs C-X k Value	X-X vs X-C p value	X-X vs X-C k Value
Cytoplasmic staining	0- Inadequate	0	0	1.43	2.86	1	1	0.87	0.92	0.71	0.86
	1- Adequate	100	100	98.57	97.14						
Nuclear staining	0- Inadequate	4.3	1.43	1.43	5.7	0.56	0.88	1	1	0.43	0.93
	1- Adequate	95.7	98.57	98.57	94.3						
Uniformity of staining	0- Inadequate	0	0	0	0	0.69	0.90	0.24	0.87	0.38	0.90
	1- Patchy	5.7	4.3	10	7.1						
	2- Uniform	94.3	95.7	90	92.9						
Clarity of staining	0- Inadequate	0	2.86	4.3	4.3	0.71	0.91	0.77	1	0.77	0.96
	1- Adequate	100	97.14	95.7	95.7						
Crispness of staining	0- Inadequate	1.43	0	7.15	1.43	0.87	0.91	0.74	0.86	0.87	0.90
	1- Adequate	98.57	100	92.85	98.57						
Cell morphology	0- Inadequate	0	0	1.43	2.86	1	0.94	0.87	0.89	0.82	0.88

Results

The results of Rigidity, Tissue shrinkage & Translucency among Xylene and Coconut oil processed tissues are shown in Table 2. It was found that there is statistical difference in rigidity ($p < 0.01$) and tissue shrinkage ($p < 0.01$). However, there was no difference in Transluceny among the Xylene and Coconut

oil processed tissue samples ($p > 0.05$) (Table 2). The results of Nicks and Ribbons as shown in Table 3, it was found that there was no statistical difference in presence of Nicks ($p > 0.05$) (Table 3) and Ribbons ($p > 0.05$) (Table 3) among the Xylene and Coconut oil processed tissue samples. The results of Cytoplasmic staining, Nuclear staining, Uniformity of staining, Clarity of staining, Crispness of staining and Cell morphology criteria are shown in Table 5. The results showed no significant difference in Xylene processed & Xylene stained samples (X-X) and Coconut processed & Coconut stained samples (C-C), Xylene processed & Xylene stained samples (X-X) and Coconut processed & Xylene stained samples (C-X), Xylene processed & Xylene stained samples (X-X) and Xylene processed & Coconut stained samples (X-C) (Table 5). This indicates that Coconut oil is equivalent to Xylene as clearing agent and dewaxing agent when compared to that of Xylene in routine tissue processing and hematoxylin and eosin (H&E) staining (Ravindran et al., 2018; Alwahaibi et al., 2018; Ab & Br, 2018; Sravya et al., 2018).

Discussion

Xylene is a benzene homologues of general formula C8H10 (Fabri et al., 2000). Xylene available in the three isomers forms i.e. ortho-, para- & meta-dimethylbenzenes and ethylbenzene. It is extensively used in rubber painting, printing and leather industries (Bardodej, 1964). Xylene exposure can result in acute and chronic toxicity. It exhibits pre narcotic and narcotic activity. Exposure to xylene vapours can result in irritation of mucous membrane of eye, respiratory passage, nausea, vomiting, headaches. However long term exposure results in dizziness, irritability, disturbances in sleep, breathlessness, shaking of limbs, impaired concentration, short term memory loss, confusion, fatal blood dyscrasias, skin erythema, and cardiac & kidney failure. The cell toxicity of xylene has been linked to the induction of mitochondrial uncoupling and oxidative stress (Salimi et al., 2017). An 8-hour time-weighted average concentration of xylene of 100 ppm is permitted by the occupational safety and health administration (OSHA). Levels higher than this could produce toxicity which could be after acute (<2 weeks) or chronic (>1 year) exposure (Langman, 1994).

The histopathologist and technicians are constantly exposed to xylene. Due to constant risk to health there is need to find a suitable substitute for xylene. The following substitute had been used as an alternative to xylene in literature:- Sesame oil, Limonene oil, Cedarwood oil, Mineral oil, Carrot oil, Olive oil, Pine oil, Rose oil, Dish washing solution, Diluted lemon water, Distilled water, UltraclearTM, Polychem, coconut oil etc. Coconut oil is commercial easily available, non toxic, non harzardous solvent. It is non polar in nature and dissolves wax, which is also a non polar compound (Yadav et al., 2019). In our present study, 70 samples were divided into two equal halves and processed in xylene and coconut oil as per standardized processing protocol (Processing protocol Table 1). Xylene processed tissue samples were found to be more rigid than Coconut oil processed tissue samples ($p < 0.01$) (Table 2). Xylene processed tissue samples showed more tissue shrinkage than Coconut oil processed tissue samples ($p < 0.01$) (Table 2) and similar to the study done Sermadi W et al(12), they conducted study on 60 tissue samples and found that shrinkage was

relatively less in Coconut oil processed tissue sample than that of Xylene processed tissue sample(P = 0.0006).

Results were similar to Chandraker et al. (2019), they studied 25 oral soft tissue specimens and found that significant shrinkage was found in xylene processed samples as compared to that of coconut oil processed samples (P <0.05) and they found that xylene processed tissue samples were more rigid than coconut oil processed tissue samples (P < 0.05). However, there was no difference in Transluceny among the Xylene and Coconut oil processed tissue samples (p > 0.05) (Table 2), which was in contrast to study done by Sermadi et al. (2014), they conducted study on 60 tissue samples and found that translucency was better in all Coconut oil processed tissue sample than that of Xylene processed tissue sample (p <0.05) (Rai et al., 2016; Ramaswamy & Dayasagar, 2017; Muddana et al., 2017).

While sectioning, in our study it was found that there was no difference in presence of Nicks among the Xylene and Coconut oil processed tissue samples (p > 0.05) (Table 3) and there was no difference in presence of Ribbons among the Xylene and Coconut oil processed tissue samples (p > 0.05) (Table 3). While staining (as per Staining procedure protocol Table 4), in our study it was found that Cytoplasmic staining (figure 1), Nuclear staining (figure 2), Uniformity of staining (figure 3), Clarity of staining (figure 4), Crispness of staining (figure 5) and Cell morphology (figure 6) criteria showed no significant difference in Xylene processed & Xylene stained samples (X-X) and Coconut processed & Coconut stained samples (C-C), Xylene processed & Xylene stained samples (X-X) and Coconut processed & Xylene stained samples (C-X), Xylene processed & Xylene stained samples (X-X) and Xylene processed & Coconut stained samples (X-C) (Table 5). The results was similar to study done by Sermadi et al. (2014), they studied 60 tissue specimen and found that there was no change in cellular, nuclear & cytoplasmic staining, when xylene and coconut oil groups were compared (P >0.05). Chandraker et al. (2019), studied 25 oral soft tissues and processed them in xylene and coconut oil respectively. They found that there was no difference in cellular details and staining quality (P >0.05), the results were similar to results of present study. The results were in contrast to Yadav et al. (2019), they studied 50 samples, cytoplasmic staining, clarity, and crispness of section stained with dish washing solution were found superior to that of routine xylene procedure (P <0.05). Premalatha et al. (2013), in their study found that Refined mineral oil stained samples showed better nuclei details than routine xylene stained samples (P <0.05).

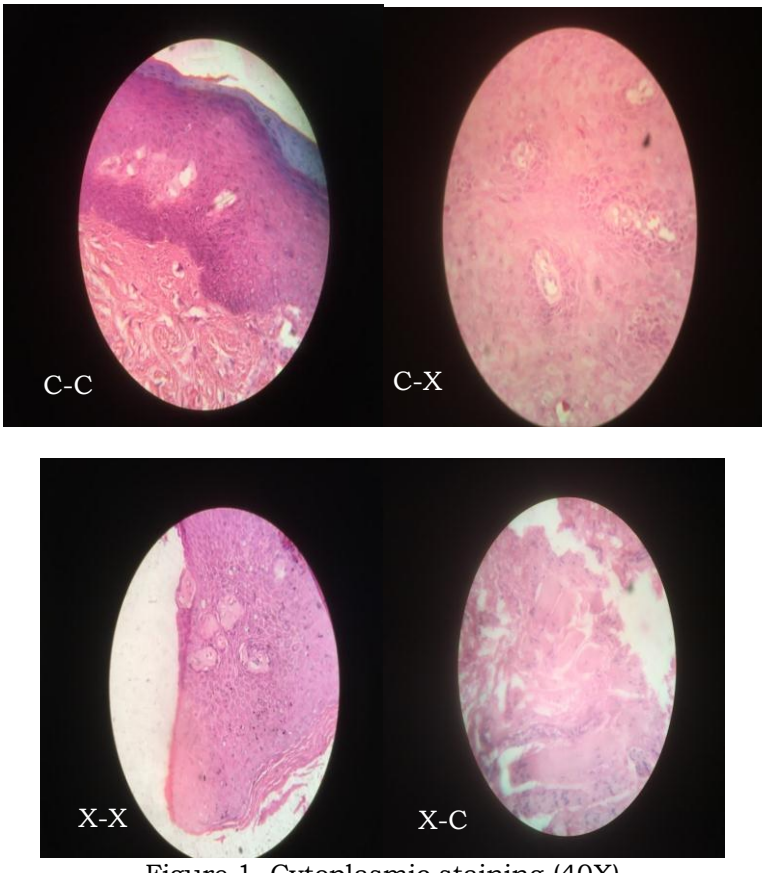
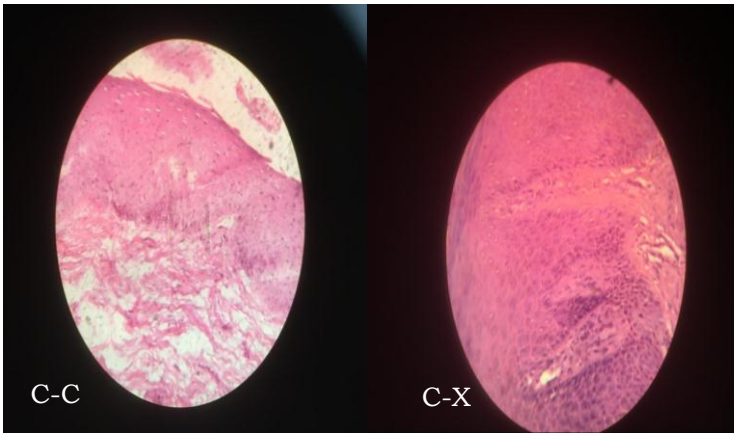


Figure 1. Cytoplasmic staining (40X)



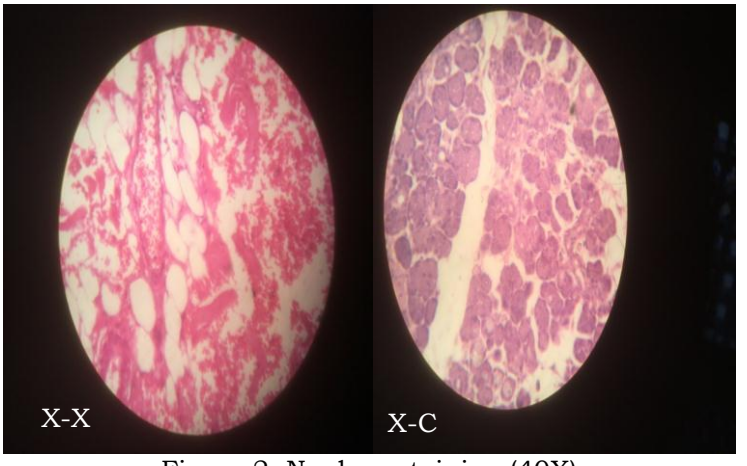


Figure 2. Nuclear staining (40X)

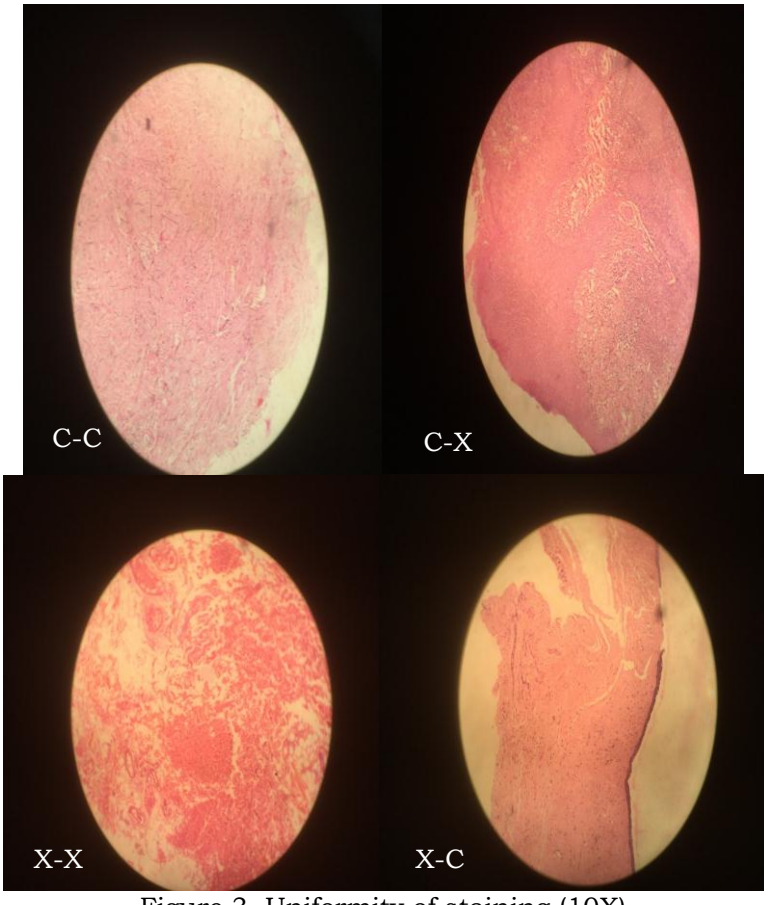


Figure 3. Uniformity of staining (10X)

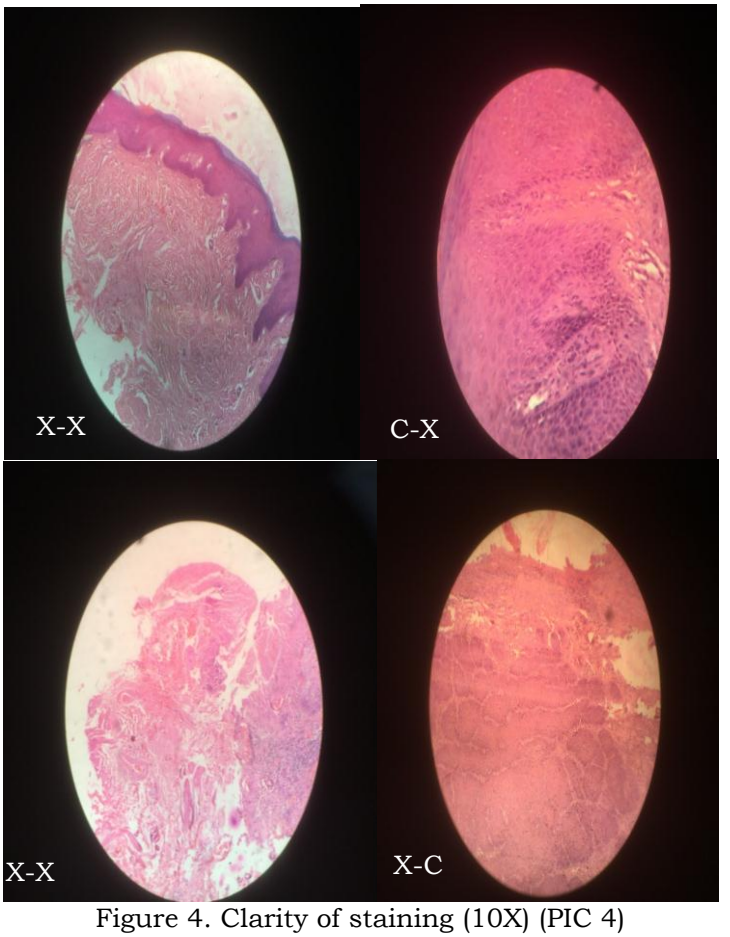
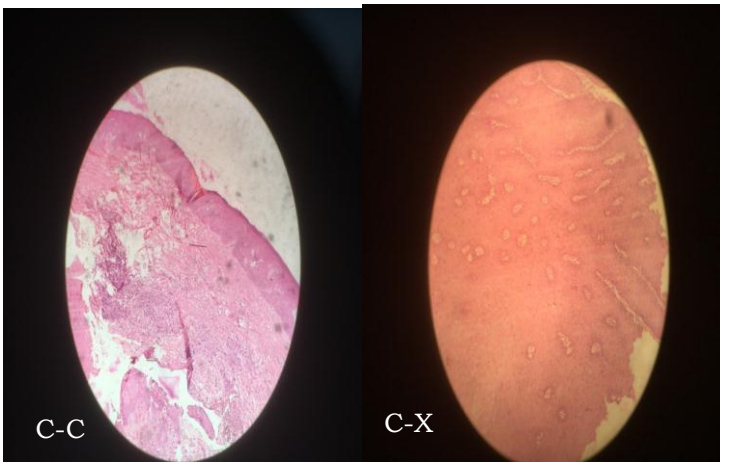


Figure 4. Clarity of staining (10X) (PIC 4)



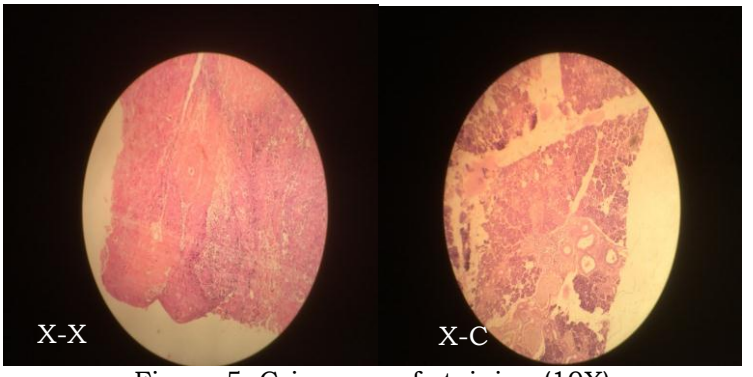


Figure 5. Crispness of staining (10X)

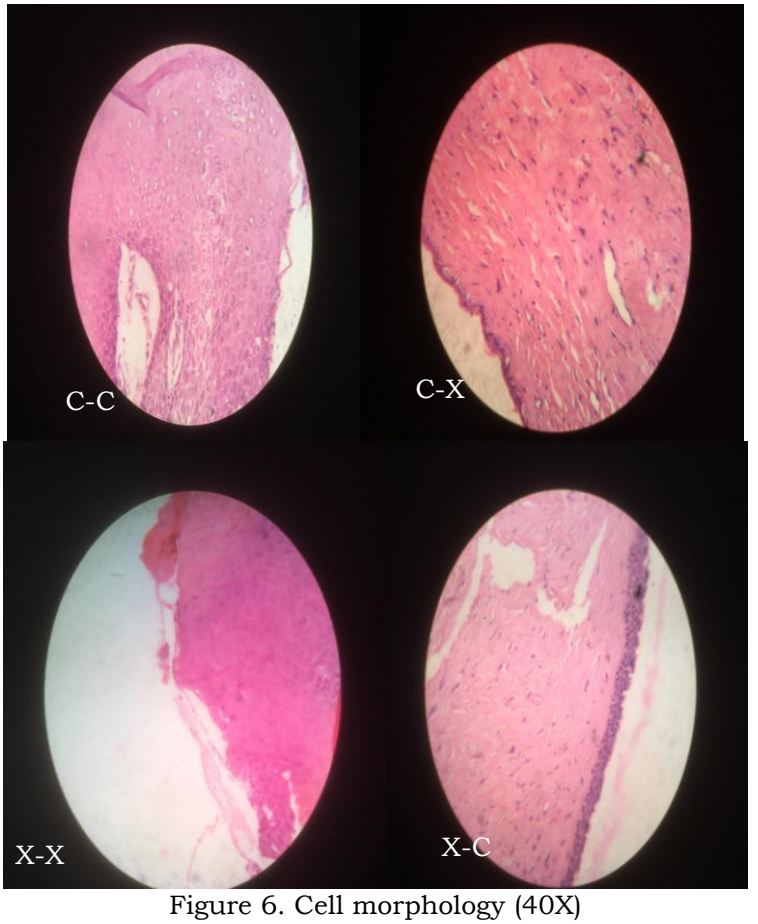


Figure 6. Cell morphology (40X)

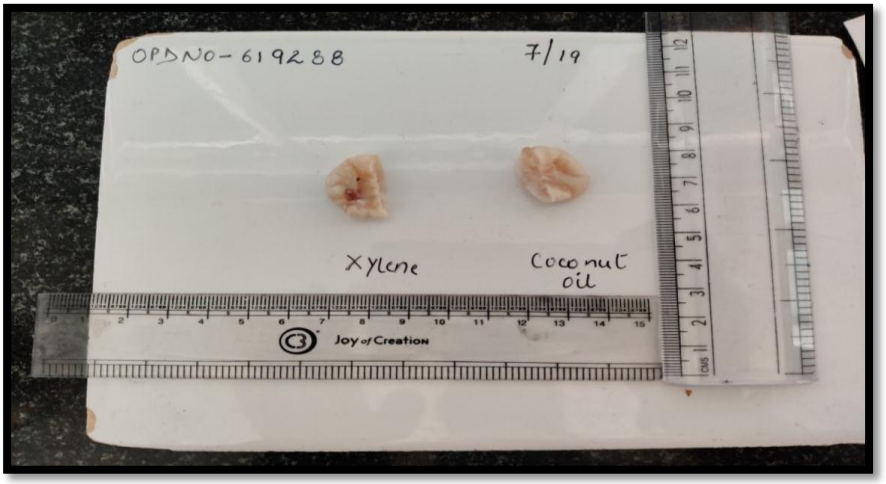


Figure 7. Biopsied soft tissue sample cut into two halves(one processed in xylene while other processed in Coconut oil) used in the study.

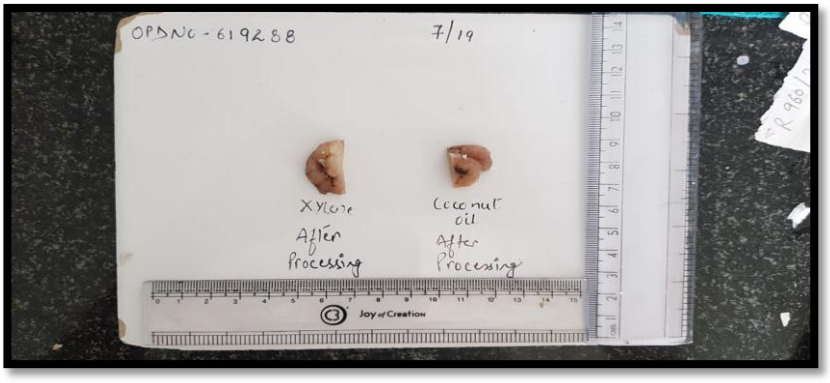


Figure 8. Biopsied soft tissue sample after processing in xylene while other after processing in Coconut oilused in the study to evaluate Tissue shrinkage, Rigidity & Translucency.

Figure 9. Xylene processed Paraffin-Embedded Tissue Blocks used in the study

Figure 10. Coconut oil processed Paraffin-Embedded Tissue Blocks used in the study

Limitation

The only drawback of using coconut oil is its property to solidify at lower temperature. However, this drawback was overcome by placing coconut oil in incubator while clearing procedure during winters. And while staining, slides cleared using coconut oil were dapped with commercially available tissue paper, for absorbing extra coconut oil present on the slides. This was done to remove excess coconut oil present on slide prior coverslipping (Ananthaneni et al., 2014; Ghosh et al., 2016; Gayathri et al., 2016).

Conclusion

We can infer that Coconut oil is an excellent and efficient replacement for Xylene in tissue processing and staining after doing this investigation. As a dewaxing and clearing agent for H&E staining Coconut oil can be as effective as Xylene and can be used in normal tissue processing in the same manner. Coconut oil can be used as clearing agent, without losing the quality of histological details of tissue samples, furthermore it does not produce recognized tissue shrinkage while processing so tissue details are not lost. Coconut oil is a natural product, commercial available and cost effective. It does not produce hazardous health affects that produced by the use of xylene. Hence, Coconut oil is an efficient substitute for Xylene.

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