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Original Research Article

To evaluate the efficacy of dishwashing soap (DWS) solution, coconut oil, cedarwood oil and limonene as a substitute to xylene in routine Hematoxylin and Eosin (H & E) staining procedure

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ABSTRACT

Context: The components in the H and E staining procedure are xylene and graded alcohol which are used to carry out the intermediate steps of deparaffinization, rehydration and dehydration of tissue sections during the staining. Xylene causes health effects.

Aims: To evaluate the efficacy of dishwashing soap (DWS) solution, Coconut oil, Cedarwood oil and Limonene as a substitute to xylene in routine and Eosin (H & E) Staining Procedure.

Materials and Methods: The paraffin blocks of normal oral mucosa were retrieved. The biopsied tissues of the study samples were fixed in 10 percent buffered formalin, manually processed, embedded in paraffin and was sectioned from the samples. All the stained sections were evaluated by three oral pathologists independently for the following parameters like nuclear staining, cytoplasmic staining, clarity of staining, uniformity of staining and crispiness of staining.

Statistical Analysis Used: Data were summarized as Mean ± SD (standard deviation). Groups were compared one factor analysis of variance (ANOVA) and the significance of mean difference between (inter) the groups were done by Tukey's HSD (honestly significant difference) post hoc test after ascertaining normality by Shapiro-Wilk's test and homogeneity of variance between groups by Levene's test. Inter observer variability was tested by Kappa test. A two-tailed (α=2) P<0.05 was considered statistically significant. Analysis was performed on SPSS software (Windows version 17.0).

Result: The results of the present study infer that coconut oil is an efficient substitute for Xylene.

Conclusion: Coconut oil is an efficient substitute for Xylene, as it is non-hazardous, and causes less shrinkage of the tissue. It can be used as a de-alcoholization agent in the histopathological laboratory, without losing the quality of the histological details.

1. Introduction

In addition to H and E, the components in the H and E staining procedure are xylene and graded alcohol which are used to carry out the intermediate steps of deparaffinization, rehydration and dehydration of tissue sections during the staining.

In dentistry, xylene its high solvency factor allows maximum displacement of alcohol and renders the tissue transparent, enhancing paraffin infiltration. In staining procedures, its excellent DEWAXING and CLEARING capabilities contribute to brilliantly stained slides.[1]

Xylene is a volatile compound with its low flash point of 28.9°C makes it a flammable solvent. It is potentially neurotoxic to humans can cause skin irritation after even mild exposures. Xylene causes health effects from both

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acute (365 days) exposure. Individuals also react differently to different levels of exposure.

The efficacy of 1.7% dishwashing soap (DWS) solution as a deparaffinizing agent for hematoxylin and eosin (H and E) staining and compared it with xylene. 1.7% DWS was found to be an effective alternative deparaffinizing agent to xylene and meanwhile facilitating as less biohazardous, economical and a faster deparaffinizing agent.[2]

Vegetable and coconut oil as a clearing agent was efficient substitute for xylene. They also suggested that it can be used as a substitute agent in the histopathological laboratory, without losing the quality of the histological details.[3] Cedarwood oil as a clearing agent produced quality staining with sufficient clarity and uniformity of staining.[4]

Hence, this in-vitro study has been planned to evaluate the efficacy of the dish washing soap solution, coconut oil, Cedarwood oil and limonene as a substitute to xylene in routine hematoxylin and eosin (H& E) staining procedure in histopathological labs.

2. Materials and Methods

2.1. Study design

The present prospective case control study was conducted by retrieving the paraffin blocks of normal oral mucosa from the department of oral pathology and microbiology, Career Post graduate Institute of Dental Sciences and Hospital, Lucknow. The biopsied tissues of the study samples was fixed in 10 percent buffered formalin, manually processed, embedded in paraffin and was sectioned from the samples. All the stained sections were evaluated by three oral pathologists independently for the following parameters like nuclear staining, cytoplasmic staining, clarity of staining, uniformity of staining and crispiness of staining.

2.2. Sample size

1. Paraffin blocks of routine biopsy specimens were taken for study.

These will be divided into five groups as follows:

Sample	Reagent	Size
Group A	Xylene	100
Group B	1.7% Dish Washing Soap	100
Group C	Coconut Oil	100
Group D	Cedarwood Oil	100
Group E	Limonene	100

2.3. Reagents

2.3.1. Dish washing soap solution

Dish washing soap solution of 1.7% was used as an alternative to xylene. Vim dishwashing soap was used for preparation of 1.7% dish washing soap solution. A conical volumetric flask was filled with 1500 ml of distilled water in which 25 ml of dish washing soap was added. The solution was mixed using glass rod. The working solution was heated at 90°C for used as deparaffinizing agent.

2.3.2. Limonene oil

Limonene oil was obtained as such in its purest form by organic and essential oil shop. It is colorless liquid hydrocarbon. The working oil was heated at 60°C for used as deparaffinizing agent.

2.3.3. Cedarwood oil

In our study we have used the 100% pure form of Cedarwood oil that is commercially available. The working oil was heated at 60^0C for used as deparaffinizing agent.

2.3.4. Coconut oil

We have used 100% pure coconut oil. It is a colorless liquid which is easily available, cheap, non-toxic also used as edible oil. We have used brand Parachute that is easily available.

2.3.5. Histological procedure

The tissue specimens were routinely processed. Total 100 numbers of wax blocks were made in our department laboratory. Five sections of 4 microns thick were prepared from each wax block. One section was stained with conventional hematoxylin and Eosin method where xylene was used as deparaffinizing agent. The other four sections were stained with xylene free hematoxylin & Eosin, where 1.7% Dish washing soap solution, limonene oil, Cedarwood oil, and coconut oil were used as deparaffinizing agent.

1. **Group A**: Tissue sections were stained with conventional hematoxylin & Eosin method.
2. **Group B**: Tissue sections were stained with xylene free hematoxylin & Eosin where 1.7% Dish washing soap solution was used as a deparaffinizing agent.
3. **Group C**: Tissue sections were stained with xylene free hematoxylin & Eosin where Coconut oil was used as a deparaffinizing agent.
4. **Group D**: Tissue sections were stained with xylene free hematoxylin & Eosin where Cedarwood oil was used as a deparaffinizing agent.
5. **Group E**: Tissue sections were stained with xylene free hematoxylin & Eosin where Limonene was used as a deparaffinizing agent.

2.3.6. Deparaffinization with xylene

Staining of the conventional tissue sections was preceded by rehydration, followed by dehydration in alcohol and clearing with xylene before mounting in DPX.

2.3.7. Deparaffinization with 1.7% dish washing soap solution

The section obtained from the paraffin wax block was placed on slide warming table at 60°C for 30 minutes, then they were immersed in prewarmed 1.7% dish washing soap solution at 90°C for 2minutes with two consecutive changes at 90°C were given. The slides were kept upright in a slide stand for 1minute so that 1.7% dish washing soap solution was properly rinsed out.

Following this the section were kept in distilled water at 90°C for 30 seconds with two consecutive changes. Now the slides were separately washed with distill water at 45°C and at room temperature for 30 seconds. Hydrated sections were stained with harries hematoxylin for eight minutes and washed in running tap water for two minutes.

The nuclear staining was differentiated by 1% acid alcohol dipped once and washed them for 10 minutes. The slides were subjected for bluing in 0.5% lithium carbonate at room temperature for 1 minute. The sections were water washed at room temperature for 10 minutes. The sections were counterstained in 1% eosin solution at RT and washed in running tap water for 1 minute.

The section were over dried at 60°C for 5 minutes and was mounted using DPX. The total approximate time were taken by us in this procedure was about 40-45 minutes as compared to conventional hematoxylin & eosin method of staining the section. The stained slides were viewed in a low power and high power magnification of a light microscope.

Photographs were made used using Olympus E330-ADU1.2X attached to research microscope.

2.4. Deparaffinization with coconut oil/cedarwood oil/ limonene oil

The section obtained from the paraffin wax block was placed on slide warming table at 60°C for 30 minutes, and then they were separately immersed in prewarmed coconut oil, cedar wood oil, limonene oil for dewaxing at 60°C. The sections were taken out two stands upright for 1 minute to drain off excessive oil and rinsed in two changes of 1.7% dish washing soap solution prewarmed at 60°C for 10 minutes each. Sections were degreased before rinsing in distilled water.

Hydrated section were stained with Harris hematoxylin at room temperature for 8 minutes and washed in running tap water for 2 minutes. The nuclear stain was differentiation by 1% acid alcohol dipped once and washed them for 10 minutes. The color changes blue to red of stained sections.

The slides were subjected for bluing in 0.5% lithium carbonate at room temperature for 1 minute. The sections were water washed at room temperature for 10 minutes. The sections were counterstained in 1% eosin solution at room temperature and washed in running tap water for 1 minute.

The section were over dried at 60°C for 5 minutes and was mounted using DPX. The total approximate time were taken by us in this procedure was about 40-45 minutes as compared to conventional hematoxylin & eosin method of staining the section. The stained slides were viewed in a low power and high power magnification of a light microscope.

Photographs were made used using Olympus E330-ADU1.2X attached to research microscope.

3. Results and Observations

3.1. Inter observer variability

Before conducting the analysis on staining scores observed by observer 1 (AA), the validity of data of observer 1 were retested by two another observers (AK and SSK) on same day in random order and compared by Kappa test and summarised in Table 1. Kappa test showed good (Kappa value 0.61-0.80) to very good (kappa value 0.81-1.00) agreement between the observers thus suggesting high reliability of on staining scores observed by observer 1. Hereafter, the analysis was done on data observed by observer 1.

3.2. Outcome measures

3.2.1. Nuclear staining

The nuclear staining score of five groups is summarised in Table 2. The mean nuclear staining score of Group A was highest followed by Group C, Group E, Group D and Group B the least (Group B < Group D < Group E < Group C < Group A).

Comparing the mean nuclear staining score of five groups, ANOVA showed significantly different nuclear staining score among the groups ($F=46.88$, $P<0.001$) (Table 2).

3.2.2. Cytoplasmic staining

The cytoplasmic staining score of five groups is summarised in Table 4. The mean cytoplasmic staining score of Group A was highest followed by Group C, Group E, Group D and Group B the least (Group B < Group D < Group E < Group C < Group A).

Comparing the mean cytoplasmic staining score of five groups, ANOVA showed significantly different cytoplasmic staining score among the groups ($F=45.85$, $P<0.001$) (Table 4).

3.2.3. Clarity of staining

The clarity of staining score of five groups is summarised in Table 6 . The mean clarity of staining score of Group A was highest followed by Group C, Group E, Group D and Group B the least (Group B < Group D < Group E < Group C < Group A).

Table 1: Inter observer variability of staining scores by Kappa test (n=100)

Group	Nuclear staining			Cytoplasmic staining			Clarity of staining			Uniformity of staining			Crispiness of staining		
	Obs 1 vs. Obs 2	Obs 1 vs. Obs 3	Obs 2 vs. Obs 3	Obs 1 vs. Obs 2	Obs 1 vs. Obs 3	Obs 2 vs. Obs 3	Obs 1 vs. Obs 2	Obs 1 vs. Obs 3	Obs 2 vs. Obs 3	Obs 1 vs. Obs 2	Obs 1 vs. Obs 3	Obs 2 vs. Obs 3	Obs 1 vs. Obs 2	Obs 1 vs. Obs 3	Obs 2 vs. Obs 3
Group A	0.917	0.895	0.943	0.953	0.904	0.911	0.768	0.879	0.750	0.932	0.767	0.879	0.794	0.921	0.753
Group B	0.856	0.924	0.781	0.784	0.837	0.854	0.896	0.814	0.916	0.804	0.914	0.793	0.936	0.883	0.714
Group C	0.769	0.852	0.877	0.855	0.916	0.767	0.932	0.909	0.794	0.735	0.857	0.918	0.752	0.870	0.856
Group D	0.764	0.859	0.854	0.863	0.895	0.773	0.753	0.871	0.713	0.796	0.923	0.745	0.871	0.769	0.910
Group E	0.776	0.737	0.743	0.855	0.837	0.885	0.859	0.743	0.892	0.834	0.748	0.893	0.776	0.834	0.881

Group A: Xylene, Group B: Dish washing soap 1.7%, Group C: Coconut oil, Group D: Cedar wood oil, Group E: Limonene

Table 2: Nuclear staining score (Mean ± SD, n=100) of five groups

Group	Nuclear staining (score)	F value	P value
Group A	1.90 ± 0.30		
Group B	0.91 ± 0.51		
Group C	1.65 ± 0.59	46.88	<0.001
Group D	1.38 ± 0.66		
Group E	1.50 ± 0.54		

Group A: Xylene, Group B: Dish washing soap 1.7%, Group C: Coconut oil, Group D: Cedar wood oil, Group E: Limonene

Table 3: Comparison of difference in mean nuclear staining score between groups by Tukey test

Comparison	Mean Diff.	q value	P value	95% CI of diff
Group A vs. Group B	0.99	18.45	P < 0.001	0.7802 to 1.200
Group A vs. Group C	0.25	4.66	P < 0.05	0.04025 to 0.4598
Group A vs. Group D	0.52	9.69	P < 0.001	0.3102 to 0.7298
Group A vs. Group E	0.40	7.46	P < 0.001	0.1902 to 0.6098
Group B vs. Group C	-0.74	13.79	P < 0.001	-0.9498 to -0.5302
Group B vs. Group D	-0.47	8.76	P < 0.001	-0.6798 to -0.2602
Group B vs. Group E	-0.59	11.00	P < 0.001	-0.7998 to -0.3802
Group C vs. Group D	0.27	5.03	P < 0.01	0.06025 to 0.4798
Group C vs. Group E	0.15	2.80	P > 0.05	-0.05975 to 0.3598
Group D vs. Group E	-0.12	2.24	P > 0.05	-0.3298 to 0.08975

Group A: Xylene, Group B: Dish washing soap 1.7%, Group C: Coconut oil, Group D: Cedar wood oil, Group E: Limonene

Table 4: Cytoplasmic staining score (Mean ± SD, n=100) of five groups

Group	Cytoplasmic staining (score)	F value	P value
Group A	1.89 ± 0.31		
Group B	1.02 ± 0.32		
Group C	1.59 ± 0.49	45.85	<0.001
Group D	1.46 ± 0.61		
Group E	1.50 ± 0.50		

Group A: Xylene, Group B: Dish washing soap 1.7%, Group C: Coconut oil, Group D: Cedar wood oil, Group E: Limonene

Table 5: Comparison of difference in mean cytoplasmic staining score between groups by Tukey test

Comparison	Mean Diff.	q value	P value	95% CI of diff
Group A vs. Group B	0.87	18.82	P < 0.001	0.6893 to 1.051
Group A vs. Group C	0.30	6.49	P < 0.001	0.1193 to 0.4807
Group A vs. Group D	0.43	9.30	P < 0.001	0.2493 to 0.6107
Group A vs. Group E	0.39	8.44	P < 0.001	0.2093 to 0.5707
Group B vs. Group C	-0.57	12.33	P < 0.001	-0.7507 to -0.3893
Group B vs. Group D	-0.44	9.52	P < 0.001	-0.6207 to -0.2593
Group B vs. Group E	-0.48	10.38	P < 0.001	-0.6607 to -0.2993
Group C vs. Group D	0.13	2.81	P > 0.05	-0.05072 to 0.3107
Group C vs. Group E	0.09	1.95	P > 0.05	-0.09072 to 0.2707
Group D vs. Group E	-0.04	0.87	P > 0.05	-0.2207 to 0.1407

Group A: Xylene, Group B: Dish washing soap 1.7%, Group C: Coconut oil, Group D: Cedar wood oil, Group E: Limonene

Comparing the mean clarity of staining score of five groups, ANOVA showed significantly different clarity of staining score among the groups (F=49.11, P<0.001) (Table 6).

3.2.4. Uniformity of staining

The uniformity of staining score of five groups is summarised in Table 8. The mean uniformity of staining score of Group A was highest followed by Group C, Group E, Group D and Group B the least (Group B < Group D < Group E < Group C < Group A).

Comparing the mean uniformity of staining score of five groups, ANOVA showed significantly different uniformity of staining score among the groups (F=51.75, P<0.001) (Table 8).

Table 6: Clarity of staining score (Mean ± SD, n=100) of five groups

Group	Clarity of staining (score)	F value	P value
Group A	1.80 ± 0.40		
Group B	0.87 ± 0.46		
Group C	1.64 ± 0.48	49.11	<0.001
Group D	1.40 ± 0.60		
Group E	1.43 ± 0.54		

Group A: Xylene, Group B: Dish washing soap 1.7%, Group C: Coconut oil, Group D: Cedar wood oil, Group E: Limonene

Table 7: Comparison of difference in mean clarity of staining score between groups by Tukey test

Comparison	Mean Diff.	q value	P value	95% CI of diff
Group A vs. Group B	0.93	18.52	P < 0.001	0.7337 to 1.126
Group A vs. Group C	0.16	3.19	P > 0.05	-0.03634 to 0.3563
Group A vs. Group D	0.40	7.96	P < 0.001	0.2037 to 0.5963
Group A vs. Group E	0.37	7.37	P < 0.001	0.1737 to 0.5663
Group B vs. Group C	-0.77	15.33	P < 0.001	-0.9663 to -0.5737
Group B vs. Group D	-0.53	10.55	P < 0.001	-0.7263 to -0.3337
Group B vs. Group E	-0.56	11.15	P < 0.001	-0.7563 to -0.3637
Group C vs. Group D	0.24	4.78	P < 0.01	0.04366 to 0.4363
Group C vs. Group E	0.21	4.18	P < 0.05	0.01366 to 0.4063
Group D vs. Group E	-0.03	0.60	P > 0.05	-0.2263 to 0.1663

Group A: Xylene, Group B: Dish washing soap 1.7%, Group C: Coconut oil, Group D: Cedar wood oil, Group E: Limonene

Table 8: Uniformity of staining score (Mean ± SD, n=100) of five groups

Group	Uniformity of staining (score)	F value	P value
Group A	1.81 ± 0.39		
Group B	0.87 ± 0.39		
Group C	1.64 ± 0.48	51.75	<0.001
Group D	1.36 ± 0.59		
Group E	1.42 ± 0.57		

Group A: Xylene, Group B: Dish washing soap 1.7%, Group C: Coconut oil, Group D: Cedar wood oil, Group E: Limonene

Table 9: Comparison of difference in mean uniformity of staining score between groups by Tukey test

Comparison	Mean Diff.	q value	P value	95% CI of diff
Group A vs. Group B	0.94	19.00	P < 0.001	0.7466 to 1.133
Group A vs. Group C	0.17	3.44	P > 0.05	-0.02339 to 0.3634
Group A vs. Group D	0.45	9.10	P < 0.001	0.2566 to 0.6434
Group A vs. Group E	0.39	7.88	P < 0.001	0.1966 to 0.5834
Group B vs. Group C	-0.77	15.57	P < 0.001	-0.9634 to -0.5766
Group B vs. Group D	-0.49	9.91	P < 0.001	-0.6834 to -0.2966
Group B vs. Group E	-0.55	11.12	P < 0.001	-0.7434 to -0.3566
Group C vs. Group D	0.28	5.66	P < 0.001	0.08661 to 0.4734
Group C vs. Group E	0.22	4.45	P < 0.05	0.02661 to 0.4134
Group D vs. Group E	-0.06	1.21	P > 0.05	-0.2534 to 0.1334

Group A: Xylene, Group B: Dish washing soap 1.7%, Group C: Coconut oil, Group D: Cedar wood oil, Group E: Limonene

3.3. Crisping of staining

The crisping of staining score of five groups is summarised in Table 10. The mean crisping of staining score of Group A was highest followed by Group C and Group E, Group D and Group B the least (Group B < Group D < Group E = Group C < Group A).

Comparing the mean crisping of staining score of five groups, ANOVA showed significantly different crisping of staining score among the groups (F=23.54, P<0.001) (Table 10).

4. Discussion

The biopsied tissues of the study samples was fixed in 10 percent buffered formalin, manually processed, embedded in paraffin and was sectioned from the samples.

Table 10: Crisping of staining score (Mean ± SD, n=100) of five groups

Group	Crisping of staining (score)	F value	P value
Group A	1.85 ± 0.36		
Group B	1.14 ± 0.65		
Group C	1.59 ± 0.49	23.54	<0.001
Group D	1.42 ± 0.61		
Group E	1.59 ± 0.53		

Group A: Xylene, Group B: Dish washing soap 1.7%, Group C: Coconut oil, Group D: Cedar wood oil, Group E: Limonene

Table 11: Comparison of difference in mean crisping of staining score between groups by Tukey test

Comparison	Mean Diff.	q value	P value	95% CI of diff
Group A vs. Group B	0.71	13.19	P < 0.001	0.4995 to 0.9205
Group A vs. Group C	0.26	4.83	P < 0.01	0.04949 to 0.4705
Group A vs. Group D	0.43	7.99	P < 0.001	0.2195 to 0.6405
Group A vs. Group E	0.26	4.83	P < 0.01	0.04949 to 0.4705
Group B vs. Group C	-0.45	8.36	P < 0.001	-0.6605 to -0.2395
Group B vs. Group D	-0.28	5.20	P < 0.01	-0.4905 to -0.06949
Group B vs. Group E	-0.45	8.36	P < 0.001	-0.6605 to -0.2395
Group C vs. Group D	0.17	3.16	P > 0.05	-0.04051 to 0.3805
Group C vs. Group E	0.00	0.00	P > 0.05	-0.2105 to 0.2105
Group D vs. Group E	-0.17	3.16	P > 0.05	-0.3805 to 0.04051

Group A: Xylene, Group B: Dish washing soap 1.7%, Group C: Coconut oil, Group D: Cedar wood oil, Group E: Limonene

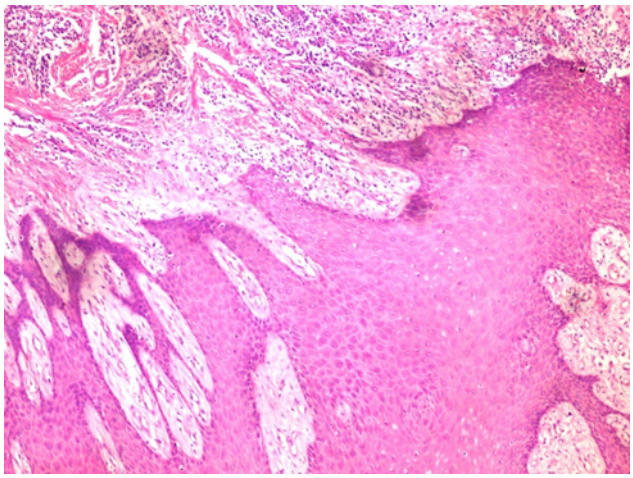

Figure 1: Xylene as a clearing agent in 20x (Group A)

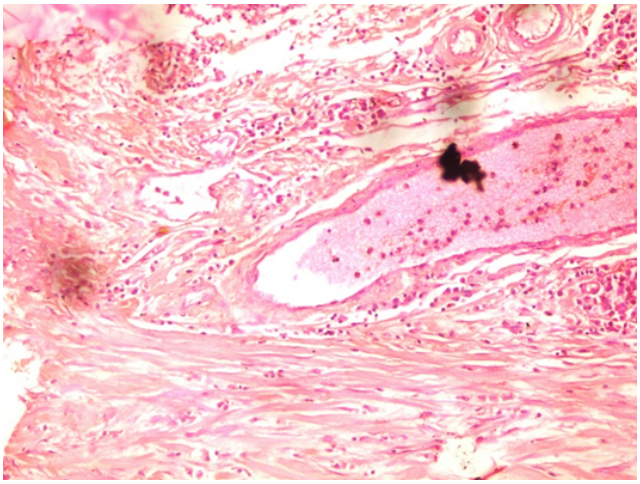

Figure 2: DWS as a clearing agent in 20x (Group B)

All the stained sections were evaluated by three oral pathologists as shown in Table 1 independently for the following parameters like nuclear staining, cytoplasmic staining, clarity of staining, uniformity of staining and crispiness of staining.

The kappa statistical analysis was conducted for assessment of agreement between observers. The statistical analysis shows good correlation varying from 0.61 -0.80 to 0.81-1.00 thus high agreement was suggested between observers and high reliability of data collected during this study.

Table 1 shows nuclear staining of coconut oil and limonene were closest to our gold standard H & E staining.

In Table 3 mean nuclear staining of different groups was assessed and significant P value (P<0.001) were obtained for routine staining with DWS, Cedarwood oil, Limonene. Nuclear staining of DWS shows significant P value (P<0.001) with coconut oil, cedarwood oil, and limonene. The coconut oil showing significant P value (P<0.01) with Cedarwood oil, While no significant P value (P>0.05) with limonene and Cedarwood oil.

Wajid Sermadi et al. compared the efficacy of coconut oil and xylene in histopathology laboratory as clearing agent . They concluded that coconut oil is an efficient substitute for Xylene. Coconut oil causes less shrinkage of tissue. Coconut oil is also non hazardous, less expensive.

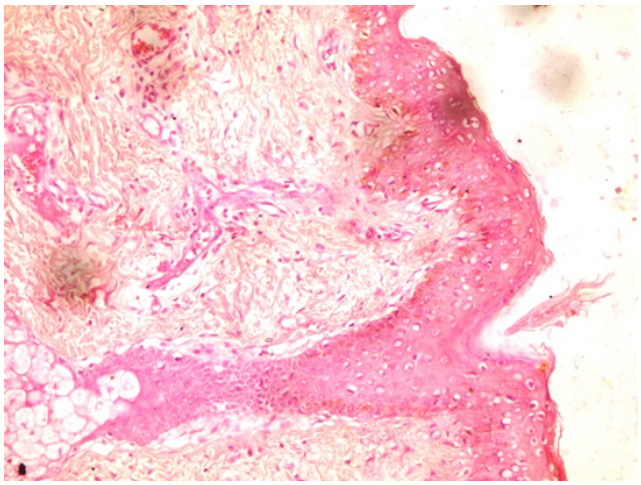

Figure 3: Coconut as a clearing agent in 20x (Group C)

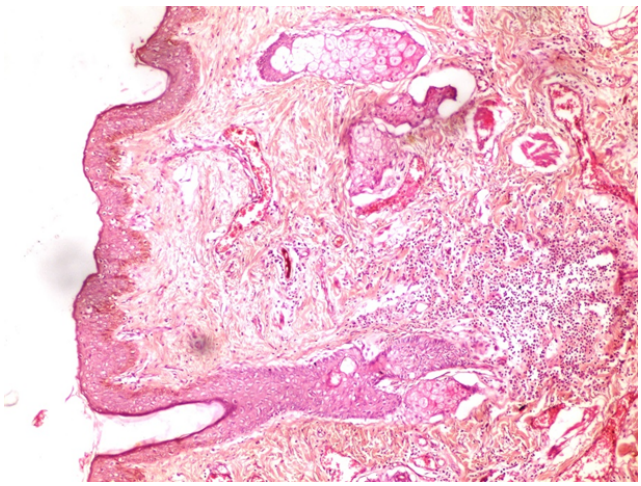

Figure 4: Cedarwood oil as a clearing agent in 20x (Group D)

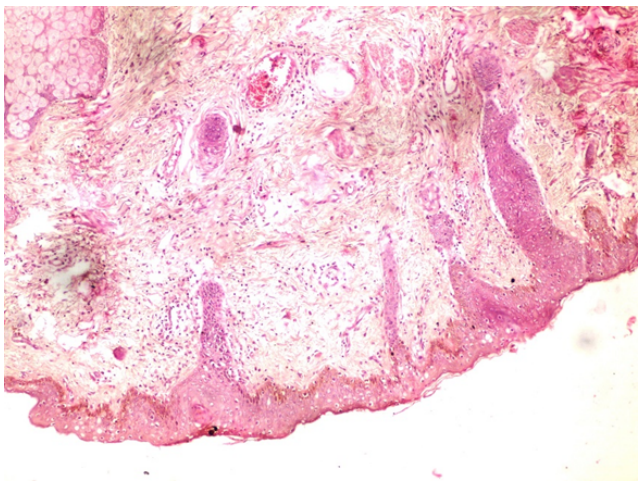

Figure 5: Limonene as a clearing agent in 20x (Group E)

Coconut oil could also be used as the dealcolization agent in histopathology laboratory without losing the quality of histological details. Similar results in our study could be attributed to the less mormhometric shrinkage in coconut oil as compared to xylene.[3]

DWS didn't showed appropriate nuclear staining in a study conducted by Anuradha Ananthaneni et al. there was a adequate result of clearing with DWS. They also stated the disadvantages of DWS as temperature sensitive technique and require electricity. A slight drop in temperature leads to improper removal of wax from sections, and on the other hand increase in temperature would lead to lifting up and loss of sections from slides. As it is known that clearing agent has to be miscible with both alcohol and wax but since the Xylene Free method employs hydrophilic agents it cannot be used as a preferable clearing agent for substituting xylene.[5]

In Table 4, cytoplasmic staining between different groups shows significant P values ($P<0.001$) while in table 5 there was significant P value ($P<0.001$) on comparison with gold standard H & E with DWS, Coconut oil, Cedarwood oil and limonene. There was significant P value ($P<0.001$) on comparison with DWS and coconut oil, Cedarwood oil and limonene. A similar study was done by Mfoniso Udonkange et al. where they processed tissues in parallel with xylene and bleached palm oil at 60 0C as clearing and dewaxing agents respectively. The cytoplasmic staining as observed in 100% of the bleach palm oil- processed sections similar with 100% seen in the xylene-processed tissues. As palm oil and coconut oil combinable grouped under vegetable oils of thus the reason for our findings could be attributed to the ability of coconut oil at 60°C to dewax (de-paraffinize) the sections allowing the penetration of stains during staining. Two factors may contribute to this result. First, at 60°C, paraffin wax becomes molten and is displaced by the coconut oil through diffusion in line with Fick's Law which states that, the rate of solution diffusion through tissues is proportional to the concentration gradient (the difference between the concentrations of the fluids inside and outside the tissue). In addition, the use of 1.7% dish washing soap solution at 60°C for degreasing the sections after dewaxing in coconut oil contributed to clarity of staining observed. First, at 60°C, the viscosity of coconut oil is reduced to 10.69 Pa.s from 16.36 Pa.s at 35°C. This increased the fluidity of the coconut oil and allowed its easy emulsification and removal by the soap solution. During emulsification, the soap forms an interface (micelle) between the water and oil resulting in gradual dissolution of the oil into water.[6]

In the present study table 5 shows no significant difference ($p > 0.05$) was seen between coconut oil and limonene. Irwin L et al. in the year 1986 have used Hemo- D, Histo-clear and AmeriClear - Organic solvents with low toxicity level. All three and xylene were used alternatively over a six-month period in tissue processing

for light microscopy. Hemo-D showed minimal shrinkage of tissue during processing and tissue cleared in Hemo-D after staining show distinct nuclear and cytoplasmic details

Table 6 shows significant P value (P0.001) among all groups for parameter of clarity of staining.

In Table 7 inter comparison between Xylene, DWS, Coconut oil, Cedarwood oil and limonene was done and significant P value were obtained (P>0.001) for Xylene v/s DWS, Cedarwood oil and limonene. Similarly, there was a significant P value (P<0.001) obtained with DWS v/s coconut oil, Cedarwood oil and limonene. Comparatively less significant P values (P<0.01) were obtained when coconut oil and Cedarwood oil and a P value of a (P>0.05) was observed with coconut oil and limonene. In a similar study had done by Ehson EO et al. by using groundnut oil a vegetable oil with that of tissue processed with xylene as a clearing agent. They concluded that groundnut oil is effective clearing agent. As the coconut oil and ground nut oil grouped in vegetable oil of thus the reason by our findings could be credit to the refractive index other tissues i.e., refractive index at 600C is 1.44 closer to the tissue proteins (ranging from 1.33 to 1.4) which allowed easy infiltration spaces of tissues. A similar refractive index also causes reduction in scattering of light and enhances the optical clearance of the tissues, making them more transparent.

Table 8 shows significant P value for the parameter of uniformity of staining, indicating differences between uniformity of staining of all groups.

In Table 9 shows uniformity of staining comparison of xylene with coconut oil and Cedarwood oil with limonene show non-significant P value (P >0.05) while other shows significant P value (P>0.05) while others show significant P value (P < 0.001) correlation. A similar study was done by Sravya Taneeru, Venkateswara Rao Guttikonda et al. with limonene and sesame oil founded that there was reduced uniformity of staining in sections as compared to H & E sections.[7] In another similar study was done by Madhuri R Ankle, Priya S Joshi with DWS as clearing and deparaffinizing agent as a substitute to xylene in H & E staining sections founded that only 50% of the Xylene-ethanol free (XEF) sections to be uniformly stained. The reason for the above study could be due because at the time of processing, tissue became hard resulting in thick and uneven sections. Out of focus areas seen in the section compromised the uniformity of staining. The clarity of the sections showed statistically significant difference to Outof-focus areas seen in the section compromised the uniformity. Out-of focus areas can be due to the reasons like tear or rip of section, introduction of extraneous tissue, unclean blade, dirty microscopic lenses, thick section, and moisture on coverslip. All of these reasons were ruled out. Careful scrutiny and retrospective analysis of the staining procedure led to an important conclusion that the diluted 1.7% liquid DWS-I and II and the distilled water I and II had to be strictly maintained at 90°C. A slight drop in the temperature failed to deparaffinize the sections completely.[8]

In the present study Table 10 crispiness of staining shows maximum comparable values were obtained between coconut oil and limonene, while Cedarwood oil and Limonene comparatively less crispier staining.

In Table 11 the significant P value (P<0.001) was observed among the H & E and all other groups. Similarly, P value was observed between DWS v/s Coconut oil, Cedarwood oil and Limonene. The above results indicate that Coconut oil is showing maximum crispiness the reason could be as coconut oil shows least morphological shrinkage as shows by morphometric analysis done by Sermadi W et al. on comparing the efficacy of coconut oil and xylene as a clearing agent in the histopathology laboratory their results showed less shrinkage in Coconut oil-treated specimen (COS), compared to Xylene-treated specimen(XYS), they would suggest that this would be a preferred procedure, where morphometric studies have to be carried out.[3] While DWS shows least crispiness of staining because DWS is technique sensitive and temperature plays an important role in clearing using DWS. Another reason could be the crispiness of DWS is better when water-soluble Mayer's hematoxylin is used.[8]

In the 1970s, numerous viable alternatives to xylene emerged, making it possible to create an environment free of xylene in laboratories. These alternatives included limonene reagents, aliphatic and aromatic hydrocarbons, olive oil, vegetable oils, and mineral oil substitute. The maximum exposure and handling of xylene occurs during the deparaffinization of the tissue sections, however these compounds were utilised to replace xylene as a clearing agent during normal processing.[9–11]

Exposure to xylene for longer than 365 days can have long-term (>365 days) impacts on health. Additionally, people respond differently to varying degrees of exposure. Nevertheless, because it evaporates readily, the majority of it enters the atmosphere where sunlight breaks it down into other, less dangerous compounds. Most individuals detect xylene in air at 0.08–3.7 ppm (parts per million) and in water at 0.53–1.8 ppm. In response to the Occupational Safety and Health Administration's new restrictions, a number of xylene replacements have entered the commercial market recently. Nevertheless, the majority of xylene replacements that are sold commercially are more costly, less efficient, and not much less dangerous than xylene.[12]

Maintaining the tissue's integrity throughout the entire histotechnique process is crucial. The tissue needs to be preserved and handled carefully so that, upon microscopic examination, every structure can be distinguished, resulting in an accurate diagnosis. The goal of tissue processing is to embed the tissue in a solid medium that is both soft and strong enough to protect the tissue and give it

enough rigidity to allow for the cutting of thin sections without damaging the knife or the tissue itself. Fixation, dehydration, clearing, and embedding are the four main processes that make up the tissue processing process. [13]

Throughout the years, researchers have attempted to replace xylene in histopathology labs with a variety of viable alternatives, including isopropanol, vegetable oils (olive, palm, and coconut oil), hexanes, dishwashing solutions, propylene glycol, and ethyl ether. As with clearing and deparaffinization, each of them has benefits and drawbacks of its own. [14]

5. Conclusion

The results of the present study infer that coconut oil is an efficient substitute for Xylene, as it is nonhazardous, and causes less shrinkage of the tissue. It can be used as a de-alcoholization agent in the histopathological laboratory, without losing the quality of the histological details.

All the Xylene substitutes have to be analyzed thoroughly, before concluding which alternative is better. Further research in this area is expected, where the coconut oil–treated specimen can be subjected to all stains and advanced histological procedures like immunohistochemistry, in order to consider this agent as a better and safer substitute for Xylene.

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7. Conflict of Interest

None.

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