# PRELIMINARY STUDY : THE POTENCY OF VEGETABLE COOKING OIL AS ALTERNATIVE CLEARING AGENT FOR HISTOLOGICAL PREPARATION

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**Abstract**

**Purpose:** This study aims to explore the potency of vegetable cooking oil as alternative clearing agent for histological preparation.

**Research Methodology:** Two different kind of formaline fixed tissue was taken from 3 rats. Each sample of tissue was cut into approximately 1 cm which were subjected for dehydration in differential alcohol gradients. Later, each cutted tissue was kept in 4 different solution; xylene as original clearing agent, palm oil, corn oil, and coconut oil. Further routine steps of processing, sectioning and staining were done. Those were assessed for gross tissue specimen assessment, staining quality and cellular architecture. Comparison was done among them.

**Results:** This study shows a good overall staining results. Those also shows a good and clear distinct nuclear-cytoplasm and good staining quality which almost equally compared to xylene. The staining intensity also shows a really good result both in nucleus and cytoplasm. Hence, it concludes that all three vegetable cooking oils can be used as alternative clearing agents in histological preparations

**Limitations:** This study is lack of evaluation of stability and longevity of staining after some periods.

**Contribution:** This study is potencial to be used in the field of anatomy, histology, anatomical pathology and other fields related to histological preparation

***Keywords:*** *xylene, cooking oil, clearing agent, histological preparation*

## 1. INTRODUCTION

Histological preparation is one gold standard to prepare tissue or biopsy specimen for research or diagnostic. There are four main steps in histological preparation, which are fixation, dehydration, clearing, and embedding, continued by sectioning and staining step (Rai *et al*., 2016; Ramamoorthy *et al*., 2016). Clearing is an important step in the preparation of histological sections, aiming to remove alcohol and other dehydrants from tissue prior to infiltration of embedding materials. In the field of histotechnique, xylene is used as clearing agent the gives translucency to tissue. Up to now, xylene is revealed as the safest clearing agent compared to other dangerous chemicals such as aniline oil, benzene, chloroform, dioxine, or toluene. Xylene in histological preparation can give a good clearing result. Otherwise, the toxicity grade of xylene is still high enough (Rai *et al*., 2016)

High exposure of xylene cause heart and kidney injuries, some fatal blood dyscrasias, and other less dangerous problems, such as skin erythaema. Literature also reveals that the persons who work with toluene and xylene are at increased risk of developing a vascular condition known as Raynaud's phenomenon. Long exposure of xylene also can cause disability of nervous system (Kandyala *et al*., 2010; Purdie *et al*., 2011; Ramamoorthy *et al*., 2016) . In order to overcome those problems, we need to find other alternative clearing agent whih are less toxic, less economic, easily found and have similar to better results compared to xylene. Some previous research reveals that various vegetable oils such as carrot oil, pine oil, olive oil, rose oil, and cedarwood oil can use as alternative agent for histological preparation (Kunhua *et al*., 2012; Swamy, *et al*, 2015; Madhura *et al*., 2016; Rai *et al*., 2016). They showed relatively similar results as xylene. In Indonesia, vegetable

cooking oil such as coconut oil, palm oil, and corn oil is easily found and economically pricing. The source of those plant is also abundant and self-cultivated. Those also have further less toxicity than xylene. Based on those statement, this present study aims to observe the efficiency of three different cooking oil which are coconut oil, palm oil, and corn oil as alternative clearing agent for histological preparation.

## 2. LITERATURE REVIEW

Histotechnique is a method to prepare tissue specimen in order to be ready for microscopy analyzing. Histotechniques refer to series of chemical procedure through which tissue have to undergo before they are ready to be microscopically examined (Rai, 2016). The principal is maintain the processing tissue integrity to represent the same as living condition. The tissue must be fixed and processed in such an exact process that when microscopically examined, all the structures can be differentiated and lead to a correct diagnosis. Tissue processing includes embed the tissue into a solid medium that firm enough to support and give it sufficient rigidity to be enable cutted into thin section but yet soft enough not to damage the tissue. There are four different major steps of tissue processing (Ofusori *et al*., 2009; Rai *et al,* 2016):

1. Fixation, is the first and foundation step which should be done immediately yet properly after biopsy. This process aim to stop cell death and prevent cell lysis by giving fixating solution.
2. Dehydration, refers to removal of fixative solution and other residual water from tissue. Dehydration procedure is usually done by subjecting the tissue into graded alcohol concentrations.
3. Clearing, is a process of replacing dehidrant with a material or solution that is miscible with the embedding medium. When the tissue is completely infiltrated with clearing agent, it becomes translucent. Thus, is often used to indicate the effectiveness of clearing process.
4. Infilltration, is a process of placing the tissue into paraffin as embedding medium to be enable cutted into thin section.

Clearing is an important step in preparing for histological preparation. Most clearing process use xylene as clearing agent which offer good clearing results but unfortunately is considered to be highly toxic (Rai *et al*., 2016). In the field of histopathology, xylene was commonly used as clearing agent that gives a good tissue translucency. High and long exposure of xylene can affect nervous tissue, respiratory tract, gastrointestinal tract, eye, and skin. Long term exposure may lead to headaches, irratibility, depression, insomnia, agitation, extreme tiredness, tremors, impaired concentration, and short-term memory. Exposure of xylene at levels greater to 200ppm can irritate lungs, causing chest pain and shortness of breathing. Furthermore, extreme exposure can cause pulmonary edema (Rajan & Malathi, 2014; Rai *et al*., 2016).

A solution can be considered as a good clearing agent if it can rapidly penetrate the tissue. It should be fully miscible with both ethanol and paraffin wax as well. This should be displace the ethanol and turn to displaced by molten paraffin wax. Various xylene substitues were tried such as limonene, aliphatic hydrocarbons and mineral oils to replace xylene in laboratory using. However, those substitues were found to be less effective yet more expensive than xylene. Various natural products also were known to be alternative clearing agents, such as cedarwood oil, pine oil, rose oil, carrot oil, coconut oil, bleached palm oil and even a lemon water (Swamy, *et al*, 2015; Madhura *et al*., 2016; Rai *et al*., 2016; Ramamoorthy *et al*., 2016; Chandraker *et al*., 2018 ). Esan *et al*. (2015) in Rai *et al*. (2016) did a study by using groundnut oil as an alternative clearing agent to xylene in histological tissue processing. The results showed that there was no difference between tissue sections cleared in groundnut oil and those cleared in xylene. Indu *et al* (2014) compared the efficacy of cedarwood oil and xylene for H&E staining. They observed adequate nuclear staining in 90% sections clearing using cedarwood oil and 93.33% with xylene. These qualities considered cedarwood oil as an uncomprimes alternative to xylene as a clearing agent.

Alwahaibi *et al*. (2018) tried to use UltraClear™ as a substitute of xylene. UltraClear™ is a colorless, odorless isoparaffin-based liquid that contains C11-12 hydocarbons that are derived from crude oil fractionations and cracking operations. It is enviromentally friendly and have no toxic in humans. The study showed a good nuclear staining, cytoplasmic staining, cell morphology, clarity of

staining, and uniformity of staining, respectively. They also found that the tissue process using UltraClear™ were easy to cut and worked well for H&E staining. But, it two times more expensive than xylene. On the other hand, Pramalatha *et al*. (2013) in previous study is recommended refined mineral oil as biofriendly and effective xylene substitutes. Those study showed that staining quality provided by xylene free method, using mineral oil is equally effective as the conventional method. Digala *et al*. (2013) in previous study also reported that edible oil such as coconut and groundnut oil revealed better results for tissue processing and suggests its use as an alternative to xylol to avoid occupational health hazard.

Swamy *et al*. (2015) recently used carrot oil, olive oil, pine oil, and rose oil as an efficient substitute to xylene. Those showed all the four oils had the ability to clear tissue similar to xylene. Among all those oils, pine soil stands good with physical and chemical properties of xylene. Those study also evaluated the stability and longevity of H&E staining for over a one year period and there are no significant difference in staining quality observed. In contrast, Andre *et al* in Swamy *et al*. (2015) substituted xylene with a mixture of peanut oil, soybean oil, coconut oil, and cotton oil and found that it was a poor alternative. From the previous researches, it is assumed that natural products such as extracted oils can be used as substitute of xylene.

## 3. RESEARCH METHODOLOGY

This study is analytical observational study with cross-sectional design. This study was conducted in the Laboratory of Histology and Anatomical Pathology, Faculty of Medicine, Universitas Lampung. The subjects were 4 male white rats (*Rattus norvegicus* Berkenhout, 1769) strain Sprague-Dawley without any intervention given. The sample used were liver and kidney from each rat. The inclusion and exclusion criteria as stated below:

Inclusion criteria:

1. Good fixated tissue
2. Good gross specimen appearance

Exclusion criteria:

1. Tissue with not enough section for histological preparation
2. Tissue from rat with given treatment

Samples will be divided into 4 groups of clearing processed. First group was processed with xylene as control; second group was processed with palm oil; third group was processed with corn oil and the last group was processed with coconut oil. The specimen in each group will be processed by ratio organ : reagent = 1 : 10. The detail for each group clearing processed as given in table 1.

Table 1. Groups of clearing process

| Reagent | | | | Time |
|---|---|---|---|---|
| **Group 1** | **Group 2** | **Group 3** | **Group 4** | |
| Xylene I, II, III (RT) | Palm oil I, II, III (60°C) | Corn oil I, II, III (60°C) | Coconut oil I, II, III (60°C) | @30 minute |

After clearing processed, the next part was embedding and sectioning. Specimen will be cutted in 4-6 µm thick using rotary microtome, then stained using routine staining, Hematoxylin-Eosin.

The processed specimen will be evaluated based on criteria below:

1. Gross tissue specimen evaluation included transparency, rigidity after infiltration process, tissue shrinkage after clearing, and easiness cutting. Evaluation score as stated in Swamy *et al* (2015) which are score 0 = worse than xylene; score 1 = equivalent compare to xylene; score 2 = better than xylene
2. Cellular architecture after staining included distinct nuclear-cytoplasmic contrast was evaluated based on Swamy *et al*. (2015) which are score 1 = indistinct nuclear-cytoplasmic contrast; score 2 = distinct nuclear-cytoplasmic contrast
3. Staining quality evaluated based on Sermadi *et al*., (2014) which are score 0 = bad; score 1 = adequate; score 2 = good.

Staining intensity for nucleus and cytoplasm will be analyzed using Image J software.

## 4. RESULTS AND DISCUSSIONS

Among 4 different groups, xylene is the only one giving strong and unpleasant odor, while the other clearing solutions give pleasant odor. Besides, the viscosity of three different oils is more than xylene (Table 2). Tissue cleared with three different oils showed similar gross changes compared to that of xylene. Those showed rigidity, tissue shrinkage and easiness in section cutting almost equivalent to xylene, while the translucency giving by xylene is still the best one (Table 3).

Table 2. Different physical properties of oils in comparison with xylene

| Physical properties | Xylene | Palm oil | Corn oil | Coconut oil |
|---|---|---|---|---|
| Odour | Strong-unpleasant | Pleasant | Pleasant | Pleasant |
| Viscosity | Less | More | More | More |
| Cost* | - | 0 | 0 | 0 |
| Health hazard | Yes | No | No | No |

*Cost comparison per 1000ml of xylene: score 0 = very economical compared to xylene, score 1 = approximately comparable to xylene, score 2 = costly than xylene

Table 3. Comparison of gross changes in tissue cleared with different oils compared to xylene

| Physical properties | Palm oil | | Corn oil | | Coconut oil | |
|---|---|---|---|---|---|---|
| | Kidney | Liver | Kidney | Liver | Kidney | Liver |
| Translucency | 0 | 0 | 0 | 0 | 0 | 1 |
| Rigidity | 1 | 1 | 1 | 1 | 1 | 1 |
| Shrinkage | 1 | 1 | 1 | 1 | 1 | 1 |
| Section cutting | 1 | 1 | 1 | 1 | 1 | 1 |

*score 0 = inferior to xylene, score 1 = equivalent to xylene, score 2 = superior to xylene

Tissue cellular architecture preserved in all clearing solutions showed a clear distinction between nucleus and cytoplasm (Figure 2 and 3). Furthermore, overall staining quality is almost equivalent with those in xylene (Table 4/Figure 1). Among all those groups, only coconut oil which giving “satisfactory” quality of staining, while he others give “very good” results. Staining intensity analysis using image J aslo shows a good results for all clearing solutions (Figure 4). Both in nucleus and cytoplasm, compared to xylene, is almost no differences of staining intensity in all different oils which shows that the stainer was well absorbed.

Table 4. Comparison of cellular architecture and quality of staining between groups

| Clearing solution | Cellular architecture | | Quality of staining | |
|---|---|---|---|---|
| | Kidney | Liver | Kidney | Liver |
| Xylene | 1 | 1 | 2 | 2 |
| Palm oil | 1 | 1 | 2 | 2 |
| Corn oil | 1 | 1 | 2 | 2 |
| Coconut oil | 1 | 1 | 1 | 1 |

*cellular architecture: score 0 = indistinct nucleus-cytoplasm, score 1 = distinct nucleus-cytoplasm
*quality of staining: score 0 = poor, score 1 = satisfactory, score 2 = very good

Among those groups, palm oil gives brighter staining results than other groups, even those from xylene. The cellular architecture also very clear with distinct nucleus-and cytoplasm. Staining intensity showed that it almost equal compared to xylene. Corn oil also shows bright staining results, slightly more than xylene. The distinct nucleus and cytoplasm also very clear and the staining intensity slightly higher than xylene.

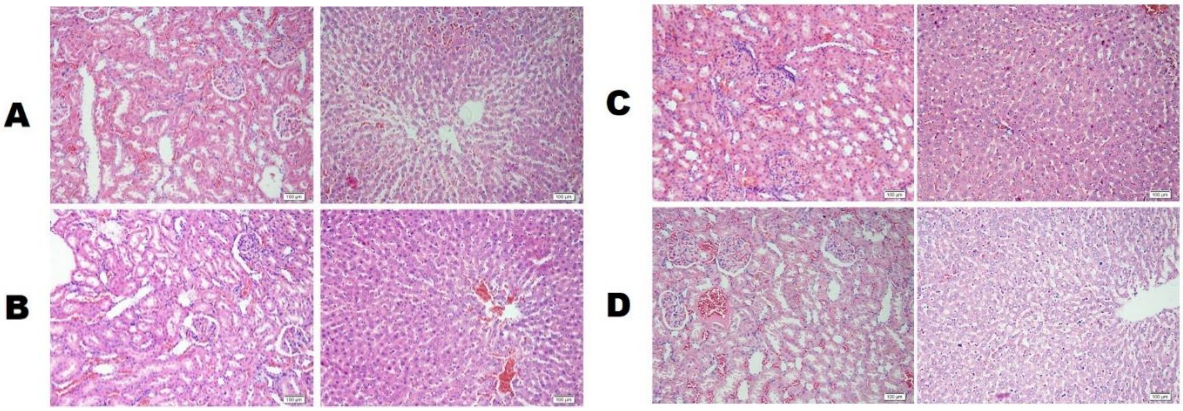


Figure 1. Comparison of kidney and liver tissue sections cleared with (A) xylene, (B) palm oil, (C) corn oil and (D) coconut oil after staining with (H&E, 20X)

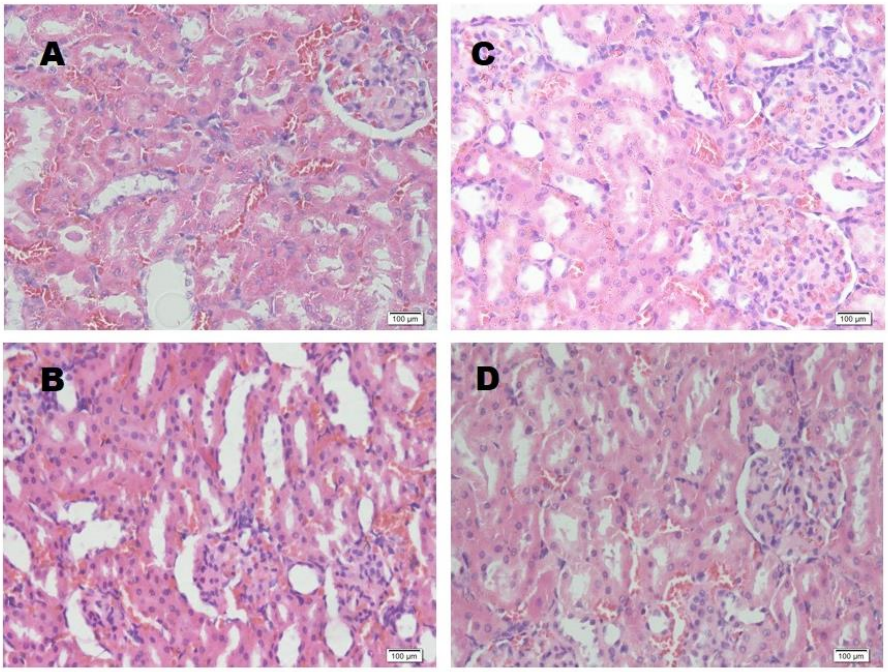


Figure 2. Comparison of kidney tissue sections cleared with (A) xylene, (B) palm oil, (C) corn oil and (D) coconut oil after staining with (H&E, 40X)

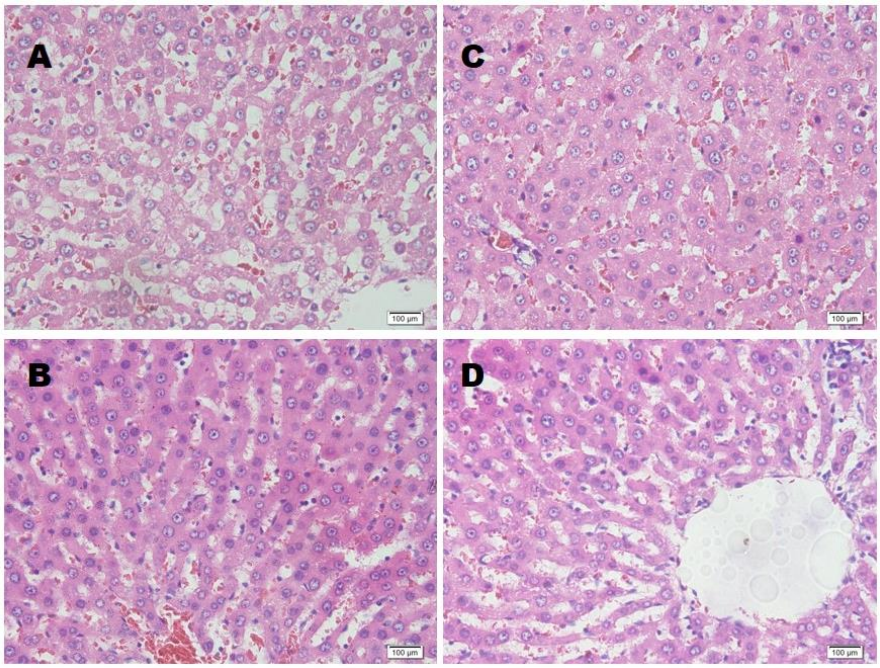


Figure 3. Comparison of liver tissue sections cleared with (A) xylene, (B) palm oil, (C) corn oil and (D) coconut oil after staining with (H&E, 40X)

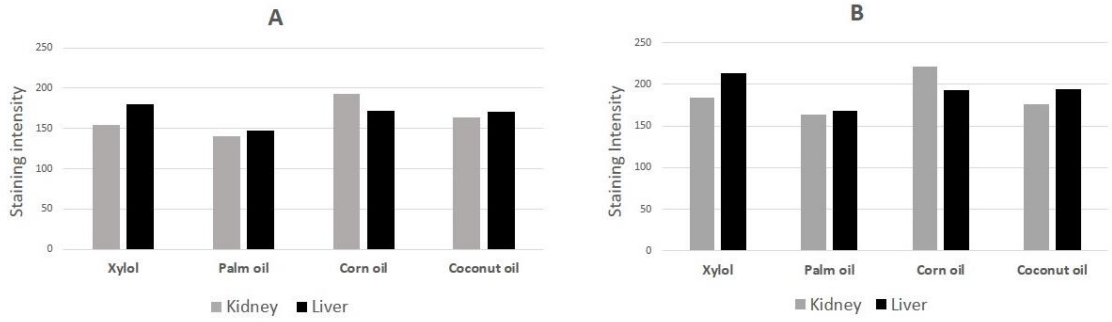


Figure 4. Comparison of (A) nucleus and (B) cytoplasm staining intensity between groups

Xylene or dimethylbenzene is a colorless aromatic hydrocarbon which has a strong to unpleasant odor. According to Occupational Safety and Health Administration (OSHA), the permissible exposure of xylene is 100ppm as an 8-hour time-weighed averaged concentration. It is considered as hazardous material. Various toxic of xylene are listed in table 5.

Table 5. Toxic effect of xylene in different organ system (Purdie *et al*, 2011; Premalatha *et al*., 2016).

| Organ system | Toxic effect of xylene |
|---|---|
| CNS | Headaches, dizziness, irritability, depression, insomnia, agitation, extreme tiredness, tremors, loss of concentration and decreased memory. |
| Respiratory system | Irritation to nose and throat, Chest pain, shortness of breath (exposure ≥ 200 ppm), pulmonary edema (over exposure at a confined space). |
| Liver and kidney | In rats, it is proved that increased exposure to xylene(>300 ppm) causes injury and fat deposition in liver and kidney. |
| Gastrointestinal tract | Nausea, vomiting and gastric discomfort, anorexia |
| Skin | Skin erythaema, urticaria, vasodilation of skin, dryness and scaling of skin. |
| Reproduction system | Crosses placental barrier and shows feto-toxic effects. Evidence of entry into breast milk. Immediate abortions. |
| Eye | Irritation |

Considering the hazardous effects of xylene, this study was done to find of a safe alternative clearing agent compare to xylene, by using different vegetable cooking oils which are also less economic yet easier to find. In order to consider as a good clearing agent, the solution has to penetrate into tissue rapidly in order to clear them. Clearing agent should be a solvent that fully miscible with both ethanol and paraffin. Because it has to displace ethanol and other dehidrant from tissue and will be displaced by molten paraffin. To be easy and fully penetrate, the viscosity of a solution is play an important role. A less viscous solution will penetrate faster than a high one. According to Bernoully's principles, fluid dynamics viscosity depends on temperature. As a temperature increases, viscosity will decrease, and the fluid penetration increases. In this study, the viscosity of those three oils were higher than xylene (Table 2). Hence, to decrease the viscosity, clearing process was carried out in 60°C temperature.

As we can see in Figure 1, overall staining results are good. Palm oil even shows a brighter staining results compared to others. Those also shows a good and clear distinct nuclear-cytoplasm and staining quality (Figure 2 and 3). This study shows that those three different oils provide the property of good clearing tissue and they could maintain the cellular architecture of tissue almost equally compare to xylene. Furthermore, the staining intensity shows a really good result both in nucleus and cytoplasm. Corn oil even shows a little higher staining intensity compare to xylene.

This present study shows that palm oil, corn oil, and coconut oil are potencial to be used as an alternative clearing agents. Besides, they are non hazardous, have a pleasant odor, easy to find and less economic compared to xylene. Palm oil and corn oil was superior in maintaining cellular architecture, bright and good quality staining. Previous research by Swamy *et al*. (2015) was using pine oil, carrot oil, and rose oil as a substitute to xylene, but the use of palm oil, corn oil, and coconut oil were compared first time in this study. Those oils also are abundant and easier to find in tropical country like Indonesia.

## 5. CONCLUSION

This study concludes that all three vegetable cooking oils can be used as alternative clearing agents in histological preparations as they not only good in clearing tissue but also non-hazardous, has pleasant odor, easier to find, less economical, and can maintain good cellular architecture and good quality of staining.

## LIMITATION AND STUDY FORWARD

This study is lack of evaluation of stability and longevity of staining after some periods. Hence, further studies need to be carried out to evaluate the stability and longevity of haematoxyline and eosin staining for over some period. It is also important to observe whether those three different oils can be subjected to all kind of stains and advanced histological techniques such as immunohistochemical of immunofluorescence procedure.


## ACKNOWLEDGEMENT

The authors thank Faculty of Medicine, Universitas Lampung for the supporting facility and funding. This research study was supported and funded by DIPA Funding 2020.


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