

## Comparative Evaluation of Clove, Olive and Groundnut Oil's Clearing Ability in Tissue Processing

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### ABSTRACT

**Introduction:** Clearing agents are vital in tissue processing to remove dehydrating agent at the same time be soluble in the impregnating media to be use for final impregnation stage. Xylene is one of the most commonly used clearing agents in Histopathology Laboratory despite its associated health hazards as well as flammable nature. **Aim:** This study was aimed at evaluating clearing ability of Three (3) natural oils namely Clove Oil, Olive Oil and Groundnut Oil in comparison with Xylene cleared tissues as control. **Methods:** The three natural oils alongside xylene were used to clear tissues of kidney, liver and heart of Wistar rats after which thin paraffin sections were obtained and stained using Haematoxylin and Eosin staining method for histological examination. Photomicrographs of the stained sections were taken for comparison with the xylene cleared tissues as control. **Results:** Stained sections that are cleared with natural oils revealed staining pattern with normal nuclear and cytoplasmic features. Out of the three oils used, groundnut oil was superior in its clearing ability as it maintained good cellular architecture with a high-quality staining pattern. **Conclusion:** The three (3) natural oils used in the study have ability to dealcoholized the tissues when compared with xylene-cleared control tissues, Therefore, we advocate for the need to further explore the use of natural oils as clearing agents in comparison with xylene considering their low cost, availability, nature-friendly effects and health safety.

**Key words:** Natural Oils, Xylene, Clearing Ability, Tissue Processing

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### Author's contributions:

This work was conducted and approved in collaboration between all the authors, who takes responsibility for its accuracy and integrity. TRI designed the study; MHT sourced for funding; MMO wrote the protocol; AU contributed in literature search; MHT and AAS did the lab experiments; MI did statistical analysis; TRI drafted the manuscript; TRI supervised the study; MMO and MAT Wrote the final manuscript; All authors proofread the final manuscript for publication.

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## **INTRODUCTION**

The tissues must undergo preparatory treatment entailing fixation, dehydration, clearing, infiltration and embedding before the microscopic examination of tissues. This preparatory treatment is known as tissue processing (1). This process has been designed to remove all extractable water from the tissue followed by replacing it with a support medium which provides sufficient rigidity and a suitable consistency for sectioning of the tissues without damage or distortions (2).

Clearing is a process of removing absolute alcohol from tissue and replacing it with a solvent, which is miscible with both absolute alcohol and paraffin wax. Some clearing agents increases the refractive index of tissue; hence, this stage is called “clearing” (3). The purpose of clearing is to replace the dehydrating fluid with a substance that is miscible with the impregnating medium to be employed. This step has come to be known as clearing stage because most but not all the reagents used for this purpose raise the refractive index of tissue rendering it more transparent (4).

Clearing is an important step in the preparation of histological section, aiming to remove alcohol and other dehydrants from tissue prior to infiltration of the tissues with impregnating media usually paraffin wax. Another important role of the clearing agent is to remove a substantial amount of fat from the tissue which otherwise presents a barrier to wax infiltration (5). There are many clearing agents such as xylene, toluene, chloroform, acetone, kerosene, dioxane, benzene, petrol, cedar wood oil. Most clearing agents are derivatives of aromatic hydrocarbons such as benzene, while others are derived from natural essential oils such as cedar wood oil, olive oil and clove oil (6). Xylene has probably been the most

commonly used chemical in the Histopathology Laboratory despite its associated hazards. It is expensive but work well for short time in clearing small tissue blocks. Its high solvency factor allows maximum displacement of alcohol and enhanced paraffin infiltration. There is a visual clue given by xylene, it causes an increase in the refractive index of tissue as the dehydrants is removed. Eventually, when the clearing stage is completed using xylene, the tissue becomes quite translucent and almost transparent (7).

Clearing agents are among the most noxious and hazardous chemicals found in histopathology laboratories. Most of them are synthetic oils of hydrocarbon origin, with different levels of toxicity (8). For several years, xylene has been widely used as clearing agent of choice in spite of its high cost and the health hazard to personnel and the environment (9,10,11,12). There have been several attempts to substitute xylene as clearing agent using a mixture of vegetable oils with paraffin wax (8,9,11).

Several xylene substitutes have been commercially developed in recent years, some being aromatic derivatives of terpene, natural oils and resins produced by some plants and animals. Others are hydrocarbons, cyclic monoterpenes and isoparaffinic hydrocarbons with several trade names, these have been comparatively used in tissue processing (13). As far back as 1978, Maxwell has looked for safer substitutes for xylene in histology, he suggested the use of 1,1,1-trichloroethane as clearing agents. This compound has a high vapour pressure but is nonflammable and less toxic than xylene. However, trichloroethane is an anesthetic agent which preclude it from being used as clearing agent (14).

The purpose of this study is to substitute the use of xylene with natural oil(s) that is less

toxic compound without altering the morphology and staining characteristics of the tissue section. The natural oils are non-carcinogenic, less expensive and readily available thereby if found to have clearing ability of course their usage can reduce cost of tissue processing and staff exposure to hazardous chemicals.

## METHODS

### MATERIALS AND REAGENTS

The reagents and materials for this study include: Clove oil, Olive oil, Groundnut oil, Xylene, Formalin, Absolute alcohol, 70% alcohol, 90% alcohol, 1% acid alcohol, Hematoxylin, Eosin, egg albumin, 20% alcohol, distilled water, Scott's tap water, DPX mountant, Paraffin wax, measuring cylinder, Microscope glass slide, cover slip, water bath, rotary microtome, hot plate, tissue cassettes.

### STUDY DESIGN AND TISSUE PROCESSING

Two (2) healthy Wistar rats weighing 117g and 124g respectively were anaesthetized using chloroform vapour in an enclosed jar and then sacrificed using cervical dislocation. Abdominal incision was made to remove liver, kidney and heart which were washed in normal saline and fixed immediately in 10% formol saline. The organs were grossed into 5mm pieces for processing using manual method. The tissues were loaded into tissue basket for onward manual processing stages of dehydration, clearing, impregnation and embedding as follows:

**Dehydration** - it was carried out in ascending grades of alcohols (70%, 90%) then three changes of absolute alcohol for 1 hour 30 minutes each.

**Clearing** – The dehydrated tissues were divided into four (4) groups and manually transferred into 4 stainless containers each

containing clove oil, olive oil, groundnut oil and xylene. The tissues were cleared for 1 hour 30 minutes, after which they were removed and transferred into molten paraffin wax for infiltration.

**Impregnation** – The tissues were transferred manually into an impregnation oven containing molten paraffin wax at 60<sup>o</sup>c for 1 hour 30 minutes each in two changes.

**Embedding** - The processed tissues were embedded in paraffin wax using Tissue Tek embedding mould filled with the molten paraffin wax which was then allowed to solidify on the cold compartment of the embedding centre.

### TISSUE SECTIONING, STAINING AND MOUNTING

**TISSUE SECTIONING:** Embedded tissue blocks were trimmed at 10 µm and then sectioned at 4 µm using the Rotary Microtome. The ribbons were floated separately on 20% alcohol to straightened macro folds and transferred onto a water bath maintained thermostatically at 55<sup>o</sup>C to further straightened micro folds. The ribbons were separated into individual sections by means of angled forcep and then mounted on clean albuminized microscopic glass slides. The microscopic glass slides were angled to drain and placed on a hot plate maintained thermostatically at 60<sup>o</sup>C for 5 minutes to dry and become strongly affixed to the slide before staining.

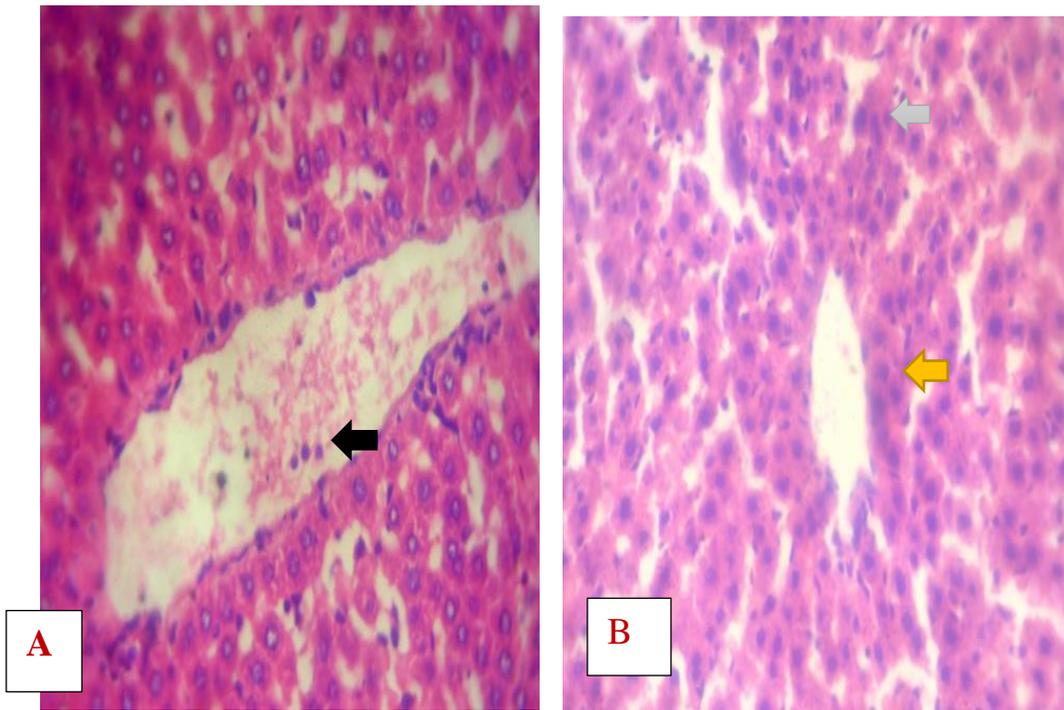
**STAINING AND MOUNTING:** The sections were dewaxed in two changes of xylene for 10 minutes and hydrated through descending grades of alcohol each for 2 minutes (100%, 90% and 70%) then finally washed in tap water for 2 minutes. The sections were stained with Harris Hematoxylin 10 minutes after which they were washed in running tap water for 2 minutes. Differentiation was done briefly

with 1% acid alcohol (1 dip) and they were washed in running tap water. The sections were blued in a Scott's tap water for 1 minute and counter stained with 1% eosin for 1 minutes. Finally, the sections were dehydrated, cleared and mounted with DPX mountant.

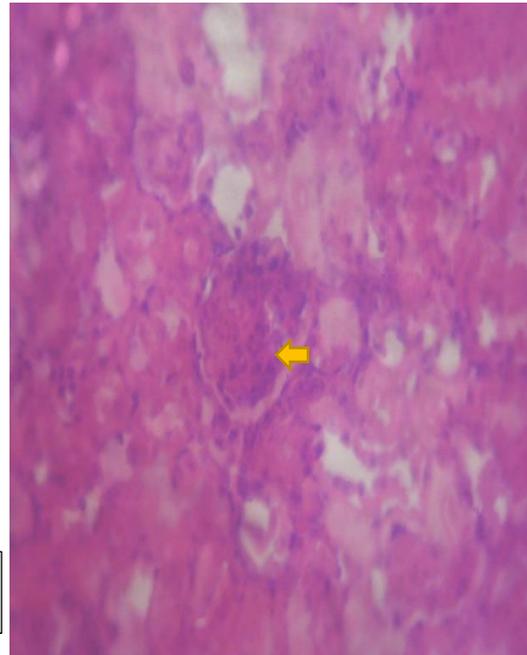
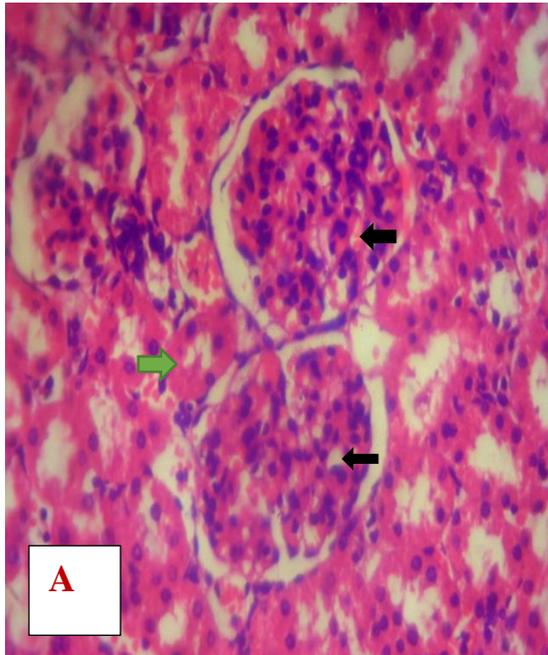
### HISTOLOGICAL EXAMINATION

The stained sections were examined using x10 and x40 objectives. Photomicrographs of the interested areas were taken using model BUC2-500C camera.

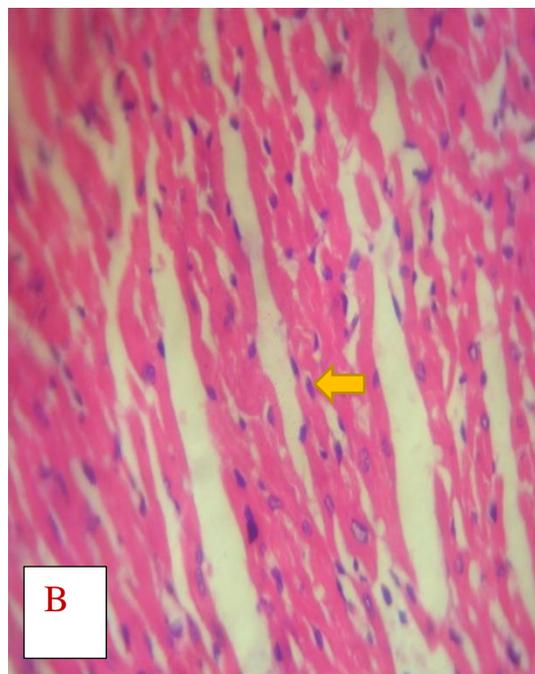
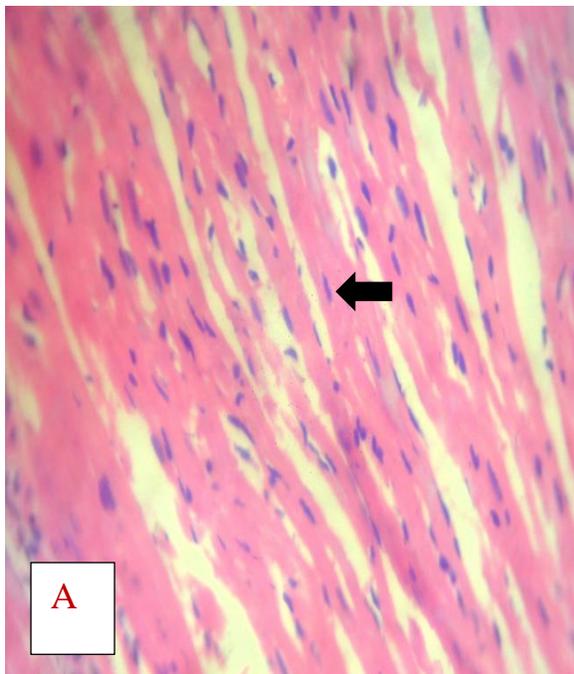
### RESULTS



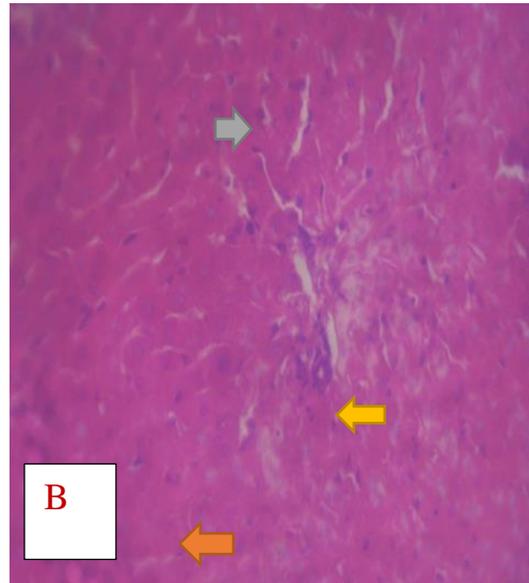
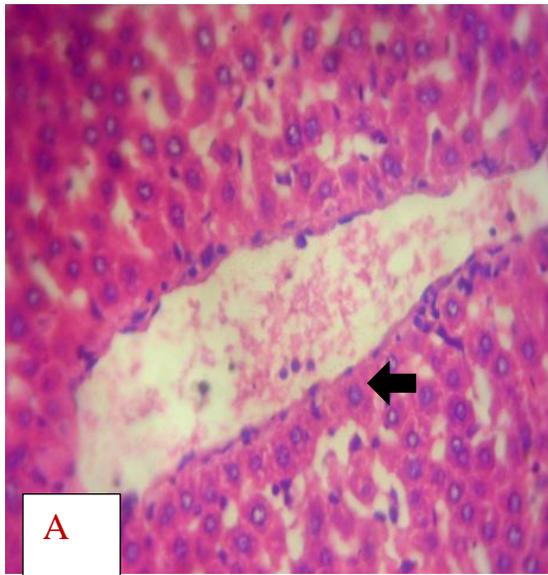
**PLATE1:** Photomicrograph of Liver Section A cleared with Xylene showed a central vein (black arrow) and Liver Section B cleared with Clove oil showed a central vein (yellow arrow), hepatocytes (orange arrow) and sinusoids (ash arrow), stained with H and E method. X400



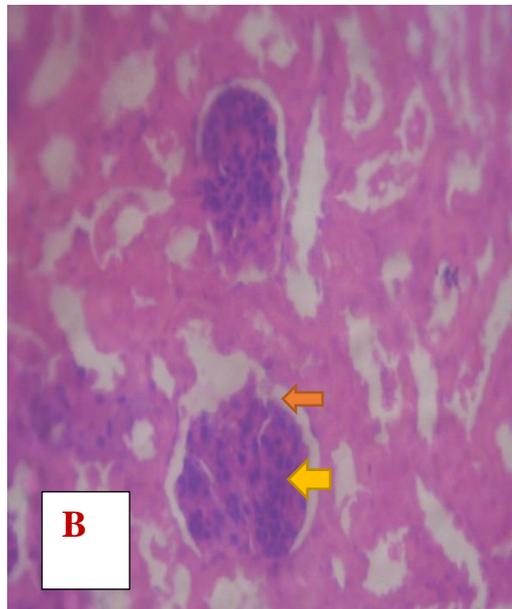
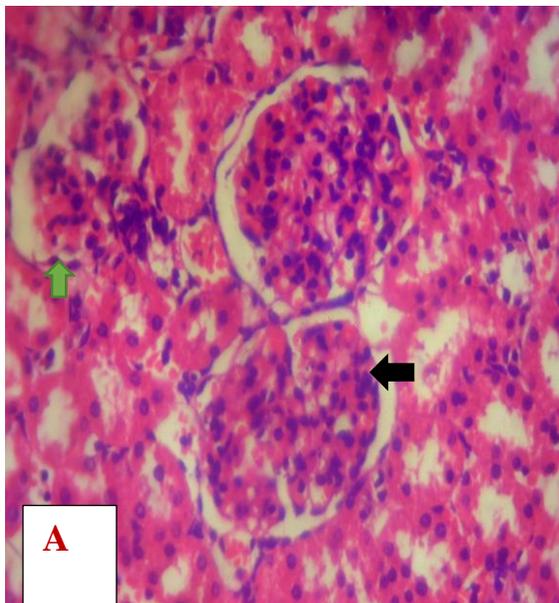
**PLATE 2:** Photomicrograph of Kidney Section A cleared with Xylene showed glomeruli (black arrow) and convoluted tubules (green arrow) and Kidney Section B cleared with Clove oil showed glomerulus (yellow arrow), stained with H and E method. X400



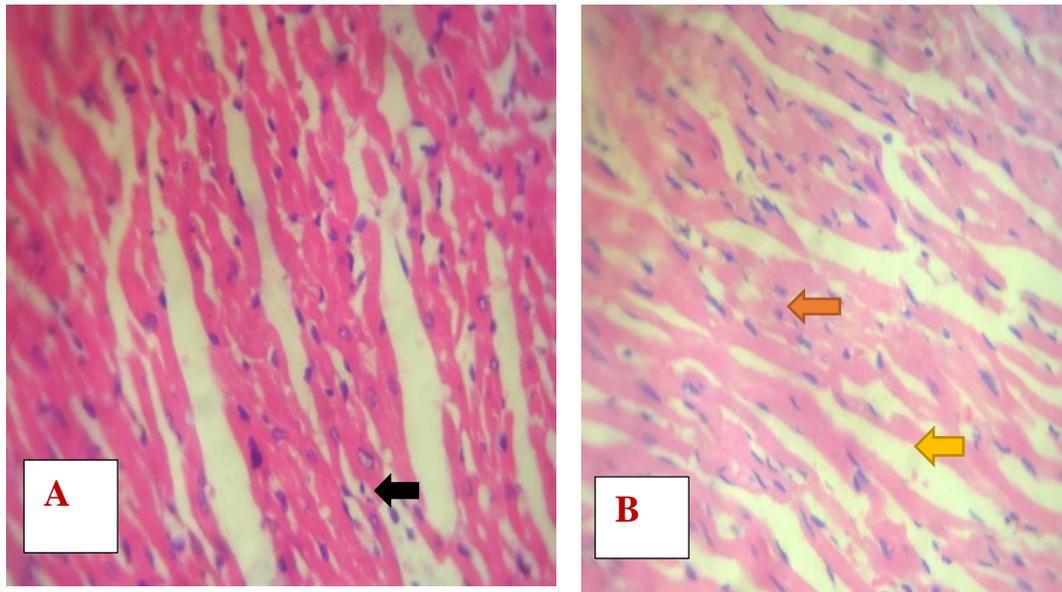
**PLATE 3:** Photomicrograph of Heart Section A cleared with Xylene showed nuclei (black arrow) and Heart Section B cleared with Clove oil showed nuclei (orange arrow) and muscles (yellow arrow), stained with H and E method. X400



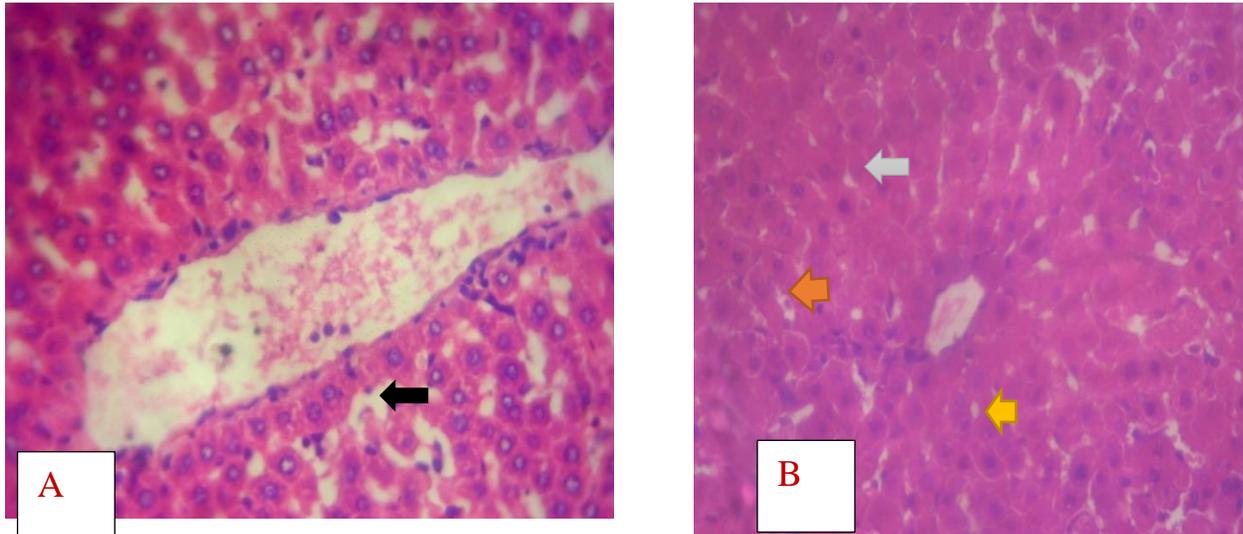
**PLATE 4:** Photomicrograph of Liver Section A cleared with Xylene showed a central vein (black arrow) and Liver Section B cleared with Olive oil showed portal triad (yellow arrow), hepatocytes (orange arrow) and sinusoids (ash arrow), stained with H and E method. X400



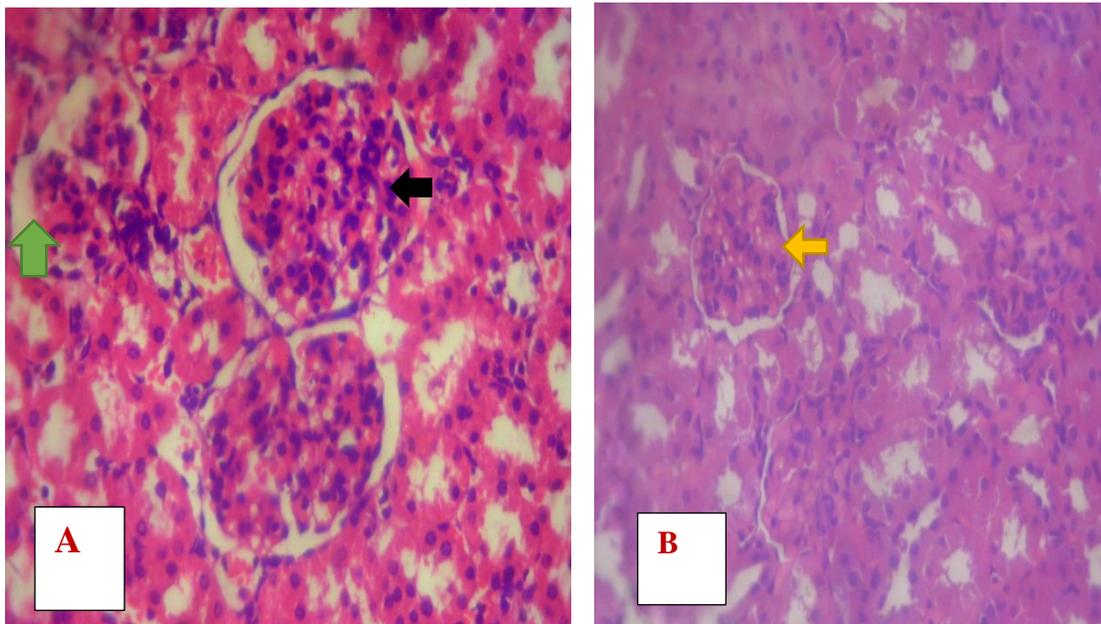
**PLATE 5:** Photomicrograph of Kidney Section A cleared with Xylene showed glomeruli (black arrow) and Bowman's capsule (green arrow) and Kidney Section B cleared with olive oil showed glomerulus (yellow arrow) and Bowman's capsule (orange arrow), stained with H and E method. X400



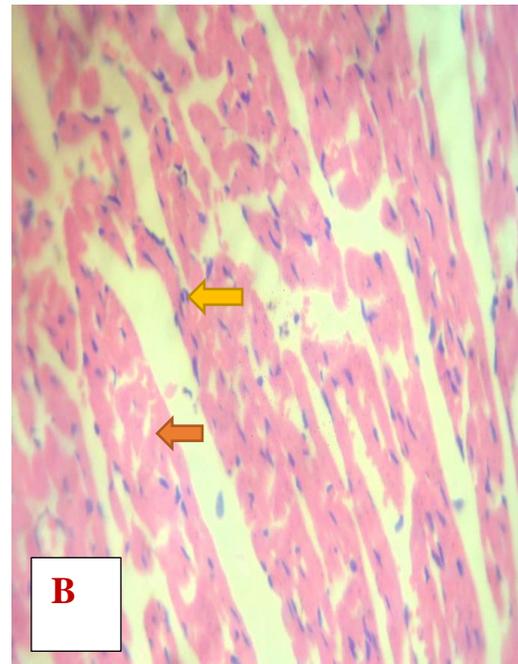
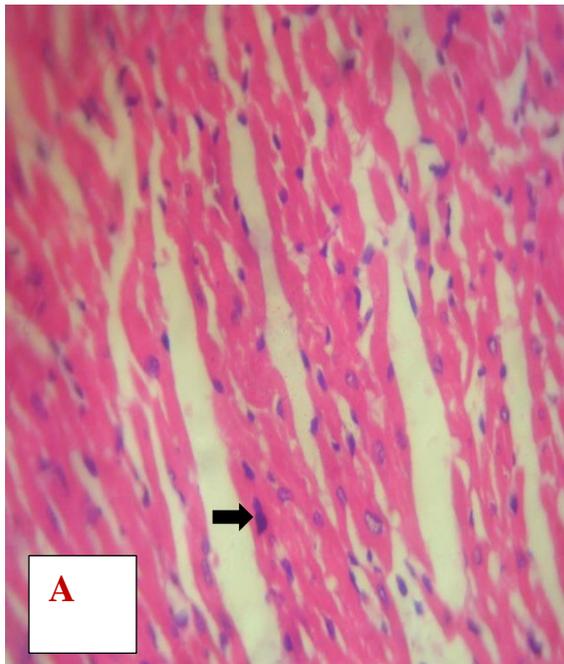
**PLATE 6:** Photomicrograph of Heart Section A cleared with Xylene showed nuclei (black arrow) and Heart Section B cleared with olive oil showed nuclei (orange arrow) and muscles (yellow arrow), stained with H and E method. X40



**PLATE 7:** Photomicrograph of Liver Section A cleared with Xylene showed a central vein (black arrow) and Liver Section B cleared with Groundnut oil showed a central vein (yellow arrow), hepatocytes (orange arrow) and sinusoids (ash arrow), stained with H and E method. X400



**PLATE 8:** Photomicrograph of Kidney Section A cleared with Xylene showed glomeruli (black arrow), bowman's capsule (green arrow) and Kidney Section B cleared with Groundnut oil showed glomerulus (yellow arrow), stained with H and E method. X400



**PLATE 9:** Photomicrograph of Heart Section A cleared with Xylene showed nuclei (black arrow) and Heart Section B cleared with Groundnut oil showed nuclei (yellow arrow) and muscles (orange arrow), stained with H and E method. X400

## DISCUSSIONS

In the present study, all the three oils showed the property of clearing tissue and maintaining their cellular architecture which is a closer comparable with that of xylene. They are non-hazardous and can be recycled. The result obtained from liver section (Plate 1) showed clove oil cleared the tissue properly with normal hepatocytes, sinusoid and central vein when compared with the control xylene. These findings were in agreement with the report of Peter (2015) that also showed appreciable tissue architecture and their staining quality appeared good (15). Clove oil did not clear the kidney tissue properly when compared with the control xylene (Plate 2). These findings were in line with the report of Peter (2015), because there was decreased appearance of tissue architecture and poor quality of staining (15). But in contrast to what we obtained on kidney tissue, clove oil cleared the heart tissue

properly with normal muscles and nuclei when compared with the control xylene (Plate 3). These findings were in agreement with the report of Peter (2015) that also obtained a good tissue architecture and the staining quality appeared was close that of xylene-stained sections (15).

Olive oil cleared the liver tissue properly with normal hepatocyte, sinusoid and portal triad when compared with the control xylene (Plate 4) while the same oil did not clear the kidney section very well when compared with the xylene control section (Plate 5). These findings were similar with the report of Peter (2015) that obtained heart tissue architecture and the staining quality appeared good and poor kidney architecture when using olive oil (15). The result obtained from heart section (Plate 6) showed olive oil cleared the tissue properly with normal nuclei and muscles when compared with that of the control xylene which the quality is far better

than what we obtained from the kidney cleared tissues using the same olive oil.

From the result obtained liver section (Plate 7), Groundnut oil cleared the liver, kidney and heart tissues properly (Plate 7,8 and 9) when compared with the control xylene. These findings were in line with the report of Peter (2015). The respective structures of liver, kidney and heart were well stained and the appearance was quite appreciable when compared with the xylene control slides.

Groundnut oil was superior in its physical and clearing properties. It also maintained good cellular architecture and distinct staining quality, when compared with other oils and is also very economical. Although we did not use coconut oil in this present study, a similar result of good quality was obtained by Sermadi *et al.*, (2014) when they used coconut oil as a clearing agent which showed similar cellular architecture and better staining quality than xylene (16).

At the same time, results obtained from present study are also in agreement with the work of Rasmussen *et al.*, (1992) on the use of vegetable oils (olive and coconut oils) instead of xylene in tissue processing, where it was stated that the xylene processed tissues and the vegetable oils processed tissues showed only minor or insignificant difference in staining and section quality (13).

## CONCLUSION

The present study concludes that the three oils have clearing ability in histopathological procedures apart from de-alcoholising the tissues they are also economical, non-hazardous, causes less shrinkage of tissues, maintain cellular architecture and staining quality of tissue sections. Amongst the three different oils used in this study, groundnut oil was the best alternative for xylene in terms of quality of tissue architecture and staining quality. There is further need to subject

groundnut oil cleared tissues with other histological stains to further substantiate the efficacy.

## CONFLICT OF INTEREST

The authors declared that they have no conflict of interest

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