EVALUATION OF SOME ESSENTIAL OILS AS CLEARING AGENTS ON THE HISTOLOGY OF SOME SELECTED ORGANS OF ADULT WISTAR RATS

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DEPARTMENT OF HUMAN ANATOMY,
FACULTY OF MEDICINE,
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JULY, 2015

DECLERATION

I declare that the work in the dissertation entitled Evaluation of some Essential Oils as Clearing Agents on the Histology of some selected organs of adult Wistar rats, has been performed by me in the department of human anatomy Ahmadu Bello university, Zaria Nigeria under the supervision of Prof. J.O. Hambolu and Dr. A.O Ibegbu. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at the university.

……. ……

Peter Stephen Akpulu Date

CERTIFICATION

This Thesis entitled EVALUATION OF SOME ESSENTIAL OILS AS CLEARING AGENTS ON THE HISTOLOGY OF SOME SELECTED ORGANS OF ADULT WISTAR RATS by Peter Stephen AKPULU meets the regulations governing the award of the degree of Masters in Human Anatomy of Ahmadu Bello University, and is approved for its contribution to knowledge and literacy presentation.

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TABLE OF CONTENTS

Contents **Pages**

Cover page i

Fly Page ii

Title Page iii

Declaration iv

Certification v

Acknowledgment vi

Table of Contents vii

List of Tables xii

List of Figures xiii

List of Plate xiv

Abstract xvi

CHAPTER ONE

1.0 Introduction 1

1.1 Statement of the research problem 6

1.2 Justification 7

1.3 Significance of the Study 7

1.4 The Aim of the Study 7

1.5 The Objectives of the Study 7

1.6 Hypothesis 8

CHAPTER TWO

2.0 Literature review 9

2.1 Xylene 10

2.2 Production and properties of Xylene 11

2.3 Applications of Xylene 13

2.3.1 Solvent and general applications 13

2.3.2 Laboratory uses of xylene 13

2.3.3 Health effects (Toxicity of xylene) 14

2.3.4 Xylene substitutes for clearing tissues 15

2.3.5 Limonene Reagents 15

2.3.6 Aliphatic Hydrocarbon 15

2.3.7 Aromatic Hydrocarbon Mixture (BTX) 15

2.3.8 Mineral Oil Mixture 16

2.4 Essential oils 16

2.4.1 Brief History of Essential Oils 18

2.4.2 Production of Essential Oils 19

2.5 Health benefit of essential oils 23

2.5.1 Therapeutic use of Essential Oils 23

2.5.2 Effect of Essential oils on Culinary (Flavouring)/ Ingestion 24

2.5.3 Industrial effect of Essential oils 24

2.5.4 Toxicity of essential oils 25

2.6 List of some essential oils and the part of plant they are gotten from 27

2.7 Eucalyptus oil 28

2.7.1 Origin of eucalyptus oil 28

2.7.2 Citodora 29

2.7.3 Cultivation and Extraction of Eucalyptus oil 30

2.7.4 Chemical Properties and Composition of Eucalyptus Oil 31

2.8 Origin and history of orange 34

2.8.1 Origin and History of Essential Oils 34

2.8.2 Physical and Chemical Characteristics of Orange Oil.....35

2.8.3 Composition of Orange Oil.....37

2.8.4 Propagation and growth of Oranges 39

2.8.5 Harvesting, Storage and Extraction of Oranges Oil.....39

2.8.6 Uses of orange oils.....39

2.8.7 Warnings and Precautions when using orange essential oil 40

2.8.8 Orange peel consumption 40

2.9 Neem oil (*Azadirachta indica*).....40

2.9.1 Origin and History of Neem 41

2.9.2 The physical, Chemical Properties and Composition of Neem Oil.....41

2.9.3 Physical and Chemical Properties of Neem Oil.....42

2.9.4 Chemistry of Neem.....44

2.9.5 Cultivation and Production of Neem Oil 48

2.9.6 Storage of Neem Oil 49

2.9.7 Extraction of Neem Oil.....49

2.9.8 Uses of Neem Oil.....49

2.9.9 Therapeutic uses of Neem Oil.....50

2.9.10 Toxicity of Neem Oil.....51

2.9.11 Safety precautions and warnings when using Neem.....51

CHAPTER THREE

3.0 Materials and methods 52

3.1 Reagents and materials.....52

3.2 Plant materials for the oil.....52

3.2.1 Collection and Identification of Plant.....52

3.2.2 Extractions of the Essential Oils.....53

3.2.3 Eucalyptus oil and Citrus oil.....53

3.2.4 Neem Oil.....53

3.2.5 Determination of the percentage yield of the Oils.....53

3.2.6 Phytochemical Studies of the Essential Oils.....54

3.2.7 Physical Properties of the Essential Oils 56

3.2.8 Gas Chromatography–Mass Spectroscopy (GC-MS) Studies of the Oils 60

3.3 Experimental animals.....61

3.3.1 Experimental Protocol 61

3.3.2 Animals Sacrifice and Fixation.....62

3.3.3 Method of tissue processing.....66

3.4 Tissue processing.....66

3.4.1 Dehydration.....68

3.4.2 Clearing.....68

3.4.3 Infiltration 70

3.4.4 Embedding 70

3.4.5 Sectioning 70

3.5 Staining and mounting 70

3.6 Photomicrograph 71

3.7 Evaluation criteria and method.....71

3.8 Average time, volume and cost of the oils.....72

3.9 Statistical analysis.....72

CHAPTER FOUR

4.0 Results……...73

4.1 Result of phytochemical screening of the oils 73

4.2 Physical properties of the oil.....75

4.3 Refractive index of the oils (RF) at 28 ^{0}C.....77

4.4 Physical observation of the oils 79

4.5 Mean time, volume and estimated cost of the oils……..……81

4.6 GC-MS of the oils.....83

4.7 Gas chromatography–mass spectroscopy (GC-MS) profile 86

4.8 Clearing effect and staining quality on sections 91

4.9 photomicrographs of stained tissue sections.....102

CHAPTER FIVE

Discussion.....129

Physical properties of the oil.....129

Phytochemical study of the oils 133

Gas chromatograph-mass spectrophotometry (GC-MS) study of the oils.....134

Stained tissue sections.....136

CHAPTER SIX

6.0 Summary, Conclusion and Recommendations 139

6.1 Summary.....139

6.2 Conclusion 140

6.3 Recommendations.....141

6.4 Limitations 141

References.....142

Appendices 151

LIST OF TABLES

Table 2.1 Physical and Chemical Properties of *m*-Xylene, *o*-Xylene, *p*-Xylene, and Mixed Xylene (Chemical formula of $C_6H_4(CH\ 3)_2$).....12

Table 2.2: Showing Production quantities of some essential oils.....22

Table: 2.3 Showing oral and dermal LD_{50} of some essential oils.....26

Table 2.4: Chemical and physical properties of citrodora oil.....33

Table2.5: Physical and chemical properties of sweet orange 36

Table2.6: Types and concentrations of some oranges components.....38

Table 2.7: Showing the physical and chemical properties of neem.....43

Table 2.8: Showing the composition of neem fatty acids.....47

Table 3.1: Showing grouping of the various tissues that form a Set 62

Table 3.2: Showing grouping of tissue in sets and their clearing time 65

Table 3.3: Showing protocol for tissue processing, embedding and sectioning……67

Table 3.4: Showing clearing schedules and Time for clearing.....69

Table 4.1: Result of Phytochemical Screening of the Oils 74

Table 4.2: Results of Physical Properties of the Oil 76

Table 4.3: Refractive Index of the Oils (RF) At 28 ^{0}c.....78

Table 4.4: Percent (%) of essential oils yielded of the oils used……..80

Table 4.5: showing the amount time, volume and estimated cost of the clearing oils82

Table 4.6: Percentage composition of citrus oil, citrodora oil, neem oil and xylene.84

Table 4.7: Clearing effect on sections and staining quality of liver 92

Table 4.8: Clearing effect on sections and staining quality of kidney.....94

Table 4.9: Clearing effect on sections and staining quality of Brain.....96

Table 4.10: Clearing effect on sections and staining quality of Testes 98

Table 4.11: Clearing effect on sections and staining quality of Intestine.....100

LIST OF FIGURES

Fig: 2.1: Structural Isomers of Xylene.....10

Fig: 2. 2: Isoprene units in the formulation of terpenes and terpenoids. 18

Fig 2.3: Structural formula of Citrodora 30

Fig 2.4: Octyl ethanoate is responsible for the fragrance of oranges.....35

Fig 2.5: Limonene 37

Fig 2.6: Molecular Formula of neem 42

Fig 3.1: Showing format of the processing technique 66

Fig 3.2: showing the process for the clearing 68

Fig. 4.1 GC-MS profile of citrus oil 87

Fig. 4.2 GC-MS profile of Citrodora oil 88

Fig. 4.3 GC-MS profile of neem oil.....89

Fig. 4.4 GC-MS of xylene.....90

LIST OF PLATES

Plate 1.....103

Plate 2.....104

Plate 3.....105

Plate 4.....106

Plate 5.....107

Plate 6.....108

Plate 7.....109

Plate 8.....110

Plate 9.....111

Plate 10.....112

Plate 11.....113

Plate 12.....114

Plate 13.....115

Plate 14.....116

Plate 15.....117

Plate 16.....118

Plate 17.....119

Plate 18.....120

Plate 19.....121

Plate 20.....122

Plate 21.....123

Plate 22.....124

Plate 23.....125

Plate 24.....126

Plate 25.....127

Plate 26.....128

ABSTRACT

Clearing agents are among the most noxious and hazardous chemicals found in histology laboratories. Xylene has probably been the most commonly used chemical in the histology laboratory despite its hazards. There has been several laboratory tests to replace xylene as clearing agent but most of these commercially available substitutes in some cases are less effective, more expensive, slightly biodegradable and are not readily available than xylene itself. The present study was aimed at evaluating the clearing abilities of some nontoxic concentrated hydrophobic liquids commonly called essential oils namely citrus oil, citrodora oil, and neem oil during histological processing of some selected organs of adults Wistar rats. The three essential oils were used alongside xylene to clear livers, kidneys, Brains, testes and intestinal tissues of Wistar rats. The citrus oil was extracted from the peels of citrus oranges by hydro distillation, while the citrodora oil was extracted from the leaves of eucalyptus plants by hydro distillation method. The neem oil was extracted from the seeds of neem tree by Cold *n*-hexane method. Physical properties, the phytochemical and Gas Chromatography-Mass Spectroscopy (GC-MS) studies of the oils were carried out using the methods of Siddiqui and Alli, (1997); Tresae, (1989) and Ladan, (2011) respectively. The tissues were histologically processed using the method of Bancroft and Stevens (2008). The result showed that citrus oil and xylene were colorless while citrodora oil was pale yellow and neem oil was dark brown. The densities of the oils are 0.80 g/ml for citrus oil, 0.80g/ml for citrodora oil, and 0.89 g/ml for neem oil when compared to 0.84 g/ml of xylene. The viscosity of neem oil was 36cp when compared to that of citrus oil which was 0.35cp, citrodora oil 0.34cp and xylene 0.45cp. It was also demonstrated that neem oil has the highest value of flash point at 108 ^{0}C while citrus and citrodora oils had similar flash points at 34^{0}C and 35^{0}C respectively and xylene had flash point at 39^{0}C. The oils had refractive indexes of 1.46,

1.46, 1.47 and 1.50 for citrus, citrodora, neem oil and xylene respectively. Histological observation showed that the cytoplasmic and nuclear staining ability appeared to give good contrast in citrodora oil followed by citrus oil when compared with xylene. Significant changes in the section and staining ability were observed in the neem oil cleared sections when compared to xylene, citrus and citrodora oil (p□ 0.05). The result of the present study showed that the three essential oils used in this work can clear tissues substantially well. Citrodora oil appeared to be better followed by citrus oil while the neem oil had the least ability to clear tissue properly.

CHAPTER ONE

1.0 INTRODUCTION

Most Histology and Histopathology Laboratories use either aromatic solvents such as xylene, toluene or aliphatic petroleum distillates for the purpose of clearing and de-waxing in the paraffin histological technique.

Paraffin wax is used universally to infiltrate and embed tissues in the final step prior to microtomy (Bancroft and Gamble, 2008). Paraffin wax does not mix with water due to their hydrophobic nature; therefore water present in tissues has to be removed by dehydration using alcohols prior to infiltration and embedding. Following dehydration, the tissues are saturated with alcohol which is also not miscible with paraffin wax (Baker and Silvertons, 1998). Certain agents are used to remove alcohols from the tissue before the tissue can be infiltrated with paraffin wax, these agents are called clearing agents. Clearing agents are sometimes called "de-alcoholization agents" or ante medium. They act as intermediary between the dehydrating and infiltrating solutions. They are miscible with both solutions and have refractive indices similar to proteins with different levels of toxicity (Kiernan, 2010).

There are many clearing agents such as: - xylene, toluene, chloroform, acetone, kerosene, diaxane, benzene, petrol, cedar wood oil etc. 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 anicine, etc (Hans *et al.,* 1995).

Bancroft and Stevens (1990), stated that the physical and chemical properties of clearing agents should include the following; high fluidity, miscibility with both ethanol and paraffin, molecular weight not above 350 g/mol, melting point not below 18^{o} C and

physical and chemical stability. The potential hazard of a compound to health is directly related to its boiling point and vapor pressure, and as such clearing agents should have a very low vapour pressure and high boiling point as well (Baker and Silvertons, 1998).

Clearing agents are among the most noxious and hazardous chemicals found in histology laboratories. They are highly inflammable with the exception of olive oil and cedar wood oil. Most of the other clearing agents are harmful to health and constitute health hazards on inhalation or on contact with the skin (Amdur *et al*., 1991).

Xylene has probably been the most commonly used chemical in the histology laboratory despite its hazards. It is expensive but work well for short time clearing of small tissue blocks. Its high solvency factor allows maximum displacement of alcohol and enhancing 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 completed, the tissue becomes quite translucent, and almost transparent (Carson and Hladik, 2009). Generally only three changes of xylene are required to reach this stage. If the tissue remains opaque it means that either water or ethanol remains in the tissue. If the opacity is very high, then it probably indicates the presence of water (Tardif and Braddeir 1992). Xylene does tend to harden tissues a little, but this does not usually interfere with sectioning qualities. One very distnct defect, though, is that it may reharden decalcified bone excessively. This is not too much of a problem with the softer cancellous bones encountered, but it can make sectioning of decalcified long bones extremely difficult, if not impossible (Stevenson *et al*., 1998; Kieranan, 2010). Long term immersion of tissue in xylene results in tissue distortions (Visfeldt *et al.*, 1982).

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, and others, hydrocarbons, cyclic monoterpenes and isoparaffinic hydrocarbons, with several trade names, these have been comparatively used in tissue processing (Luna, 1992). 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 non flammable and less toxic than xylene. However, its use as anaesthetic agents precludes it being used as serious clearing agent (Maxwell, 1978).

Histo-Clear is another xylene substitutes that is commercially produce and is regarded to be non toxic. It is claimed to consist of essential oils (isoprene derivatives) produced by distillation of citrus and corn oils. Histo-Clear is excellent for preserving fine tissue structure, and can often be used in place of xylene with no alteration of protocol. However, the compound is unstable owing to its double bounds. It also has a high vapour pressure (4.5mm Hg at 25^{o}C) and has a penetrating odour (Hans *et al*., 1995).

The least toxic of all the chemicals used in tissue processing are the paraffin. Although the tissue is ultimately embedded in them, paraffin oil has been mentioned in the literature as 1 out of 12 clearing agents suitable for routine use, with clearing time similar to that of chloroform. There have been recent tests on mixing vegetable oils with paraffin wax and even the use of pure paraffin oil as the clearing agent in a single step after dehydration by alcohols (Luna, 1992., Hans *et al.,* 1995).

Vegetable oils consisting mainly of triglycerides are all liable to decompose. The change may arise as a result of several different types of chemical reactions, including hydrolysis of the glycerides and oxidation at double bonds of unsaturated fatty acids. Olive oil and other similar unsaturated oils are not good clearing agents because they easily go rancid (Hans *et al*., 1995).

It was reported that Toluene is better at preserving tissue structure and is more tolerant of small amount of water left behind in the tissues than xylene. However, toluene is more expensive and toxic than xylene (Thompson *et al.*, 1978),

Isopropyl alcohol has also been recently used as xylene substitute in tissue processing (Falkeholm *et al.*, 2001). It is claimed to be more cost effective, saves time, and improves the laboratory environment. It also causes less shrinkage and hardening of tissue than ethyl alcohol (Dapson *et al*, 2005).

Chloroform has been used in some applications but it has a severe health hazard, acts slowly and may lead to sectioning difficulties (Hans *et al.*, 1995). Other clearing agents include long chain aliphatic hydrocarbon which represent less of a health hazard, but are less tolerated with poorly fixed, dehydrated or sectioned tissues (Hans *et al*., 1995). Most of these commercially available xylene substitutes are less effective, more expensive, and are not readily available than xylene itself (Gosselin *et al.*, 1984; Amdur *et al.*, 1991; Luna, 1992).

Cedar wood oil is perhaps the most well known natural wood oil for clearing tissues. Cedar wood oil is obtained from juniper and cypress species, both of which are within the general descriptions of being “cedar” trees. For histological processing the major advantage of this oil is that it causes almost no damage to the tissue. However it does take significantly longer, and is significantly more expensive than the usually used alternatives (Gurr, 1962; Bancroft and Gamble,, 2008). Some of the other natural oils, such as clove oil, have also been used histologically but they are usually even more expensive than cedar wood oil and, since there is little advantage to using them in preference to cedar wood oil, they have never been popular (Bancroft and Gamble, 2008). Many natural oils and resins belong to a group of chemicals called terpenes which are produced by

numerous plants and some animals. The name is derived from “turpentine” which was one of the first to become available. The basic building block of these oils is isoprene. The isoprene units are combined in various ways to produce the individual compounds making up the oils. Some of these natural oils have also been used as mounting media (Canada balsam). As a group they tend to be gentler in action, but they are not as freely miscible with dehydrant agents (Hans *et al*., 1995).

Most essential oils belong to a family of compounds known as terpenes and terpenoids. Terpenes are small organic hydrocarbon molecules; they may be cyclic or acyclic, saturated or unsaturated. Terpenoids are oxygenated derivatives of terpenes, which may contain hydroxyl groups or carbonyl groups. Regardless of their structural diversity, terpenes and terpenoids share certain structural similarities. They contain multiples of five carbon atoms i.e., hemiterpene contains 5 carbon atoms; monoterpene, 10; sesquiterpene, 15; diterpenes, 20; etc. (Crowell, 1999). Terpenes, such as limonene, and terpenoids, such as neral or geranial may be found in abundance in oil sacs located in the outer, colored portion of the rind of many common citrus fruits (Gilles *et al*., 2010).

Limonene is another example of clearing agent from terpene. It is the natural component found in the skins of citrus fruits, such as lemons or oranges, and in cooking is usually referred to as lemon or orange *zest*. The name is derived from *Citrus limonum*, the lemon tree. Limonene is obtained industrially by the steam distillation of orange peel which is a by product of the orange juice industry. It is a clear, colourless fluid with a distinctly citrus aroma. Although, effective for clearing albeit a little more slowly than xylene, it does have one serious disadvantage. Some people are allergic to it and develop skin rashes when they come in contact with it. When used as the clearant immediately prior to cover slipping there are some reports that the mounting medium, usually dissolved in

either toluene or xylene, does not mix well with the limonene and flow under the coverslip is affected. In such cases, replacing the limonene with xylene or toluene, or quickly dipping the section in either one just prior to cover slipping should be effective. This does, of course, defeat the purpose of the replacement (Rene, 2000).

Essential oils are also known as volatile or ethereal oil, or simply as the "oil of" the plant from which they were extracted. For instance, eucalyptus oil is from eucalyptus plant and neem oil is from neem plant. Essential oil is a concentrated hydrophobic liquid containing volatile aroma compounds from plants (Pino *et al., 2006).* An essential oil is a liquid that is generally distilled most frequently by steam or water from the leaves, stems, flowers, bark, roots, or other elements of a plant.

1.1 STATEMENT OF THE RESEARCH PROBLEM

Clearing agents are among the most noxious and hazardous chemicals found in histology laboratories. Most of them are synthetic oils of hydrocarbon origin, with different levels of toxicity (Gosselin *et al.,* 1984). For several years, xylene has been widely used as clearing agent of choice inspite its cost, and the health hazard to personnel and the environment (Bush, 1977; Maxwell, 1978; Hans *et al.,* 1995). There have been several attempts to substitute xylene as clearing agent. Recently, xylene substitute as clearing agents was developed by mixing vegetable oils with paraffin wax. Most of these commercially available xylene substitutes are less effective, more expensive, and are not as readily available as xylene (Gosselin *et al.,* 1984; Amdur *et al.,* 1991; Luna, 1992).

1.2 JUSTIFICATION

Several xylene substitutes as clearing agents have been commercially developed in recent years, some being aromatic derivatives of terpene, a natural oils and resins produced by some plants and animals. Other clearing agents are hydrocarbons and most of these substitutes are less effective and more expensive (Maxwell, 1978). Therefore, these essential oils (citrus oil and citrodospra oils) may be used as a substitute to xylene thereby reducing exposure to its hazaeds in histology laboratories.

1.3 SIGNIFICANCE OF THE STUDY

Results from this study will extend our knowledge on the uses and availability of some essential oils as clearing agents and as a xylene substitute during histological tissue processing.

1.4 THE AIM OF THE STUDY

The aim of the study was to evaluate the clearing potency of some essential oils namely eucalyptus (citrodora) oil, citrus oil, and neem oil using some tissues such as Brain, liver, kidney, intestine and testes of adult Wistar rats.

1.5 THE OBJECTIVES OF THE STUDY

The objectives of the research are to study:

i. The effectiveness of essential oils namely citrodora oil, citrus oil, and neem oil as clearing agent in tissue processing.
ii. The effect of these oils on the structures of some tissues of adult Wistar rats.
iii. The effects of these oils on the staining qualities of brain, liver, kidney, intestine and testes of adult Wistar rats.

iv. The clearing ability of these essential oils with themselves and the standard xylene.

1.6 HYPOTHESIS

i. Essential oils cannot clear animal tissues during tissue processing.

ii. Essential oils can impair the morphology and staining characteristics of animal tissues.

iii. There is no difference between xylene and these essential oils when using them as clearing agents.

CHAPTER TWO

2.0 LITERATURE REVIEW

Before the microscopic examination of tissues, the tissues must undergo preparatory treatment entailing fixation, dehydration, clearing, infiltration and embedding. This preparatory treatment is known as tissue processing (Bancroft and Gamble 2008). Tissue processing is all the necessary treatments given to tissues from procurement and sacrificing of the animal, excising, fixation and labeling of the tissue. It also include the various stages of histological section preparations; dehydration, clearing, infiltration and embedding to the production of sections for microscopy (Bancroft and Stevens, 1990). This process has been designed to remove all extractable water from the tissue, replacing it with a support medium which provides sufficient rigidity and a suitable consistency for sectioning of the tissues without damage or distortions (Kieranan, 2010).

Paraffin wax is used universally to infiltrate and embed tissues in the final step prior to microtomy. Paraffin wax does not mix with water due to their hydrophobic nature; therefore water present in tissues has to be removed by dehydration using alcohols in ascending grades (Baker, 1966). Following dehydration, the tissues are saturated with alcohols which also are not miscible with paraffin wax. Prior to infiltration and embedding, the alcohols in the tissues have to be removed and replaced by certain chemical agents, a process called clearing. Clearing agents are both miscible with alcohols and paraffin wax. These clearing agents usually leave the tissues translucent after the clearing process. Impregnation is the complete removal of clearing reagents by media that is miscible with both the dehydrating agents and the impregnation. Embedding is the orientation of tissue in melted paraffin which when solidified provides a firm medium for keeping intact all parts of the tissue when sections are cut (Baker, 1966).

According to Kieranan (2010), there are many clearing agents sometimes called “de-alcoholization agents”. Most clearing agents are hydrocarbons with refractive indices similar to proteins. Some are aromatic derivatives of benzene with different levels of toxicity. Others are natural essential oils such as cedar wood oil and anicine (Hans *et al.,* 1995). Common examples of clearing agents include xylene, toluene, chloroform, acetone, kerosene, diaxane, benzene etc

2.1 XYLENE

Xylene is an aromatic hydrocarbon consisting of a benzene ring with two methyl substituent. Xylene also known as xylol is widely used in industry and medical technology as a solvent. It is a colorless, liquid or gas occurring naturally in petroleum, coal and wood tar, and is named because it is found in crude wood spirit. It exists in three isomeric forms namely ortho- meta- and para-xylene (Fabri, *et al*., 2002). This is shown in Figure 2.1

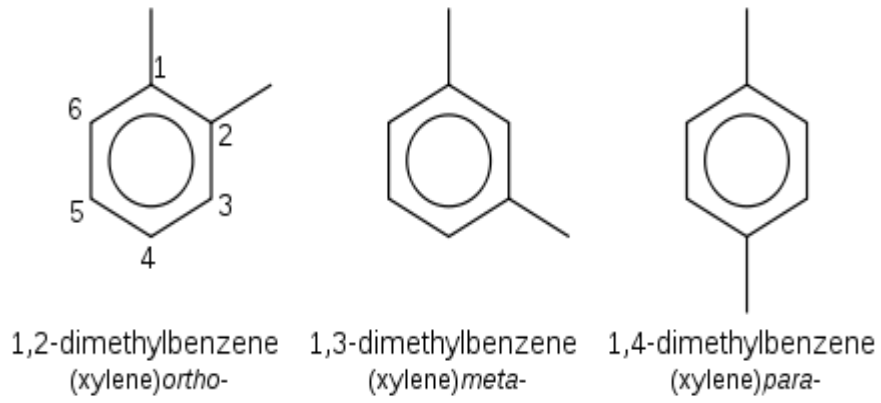


Fig: 2.1: Structural Isomers of Xylene. (Sourcs: Wikipedia, the free encyclopedia)

2.2 PRODUCTION AND PROPERTISE OF XYLENE

Xylene represents about 0.5–1% of crude oil, depending on the source. It is mainly produced from reformate. and from coal carbonization derived from coke ovens. It is also produced by dehydrocyclodimerization and by methylating of toluene and benzene (Martindale and Kuchar2012).

Laboratory-grade xylene is composed of m-xylene (40-65%), p-xylene (20%), o-xylene (20%) and ethyl benzene (6-20%) and traces of toluene, trimethyl benzene, phenol, thiophene, pyridine and hydrogen sulfide (Tremblay and Jean, 2011).). It has a chemical formula of C_6H_4 (CH 3)$_2$ and is referred to as "dimethyl benzene" because it consists of a six-carbon ring to which two methyl groups are bound. Other physical and chemical properties of xylene include molecular weight, color, melting and boiling point, viscosity and density, odor and flash point etc (Table 2.1).

Table 2.1 Physical and Chemical Properties of *m*-Xylene, *o*-Xylene, *p*-Xylene, and Mixed Xylene (Chemical formula of $C_6H_4(CH_3)_2$)

Property	Mixed xylene	*m*-Xylene	*o*-Xylene	*p*-Xylene
Synonyms/trade name	Dimethylbenzene	1,3-Dimethylbenzene	1,2-Dimethylbenzene	1,4-Dimethylbenzene
CAS number	1330-20-7	95-47-6	108-38-3	106-42-3
Molecular weight g/mol	106.16	106.16	106.16	106.16
Color	Clear	Colorless	Colorless	Colorless
Physical state	Liquid	Liquid	Liquid	Liquid
Melting point	No data	-47.8 °C	–25.2 °C	13.2 °C
Boiling point	137–140 °C	139.1 °C	144.5 °C	138.4 °C
Viscosity (20^{0C})	No data	0.62 cP	0.812 cP	0.34 cP
Density (20^{0C})	0.864 g/cm3	0.864 g/cm3	0.880g/cm	0.880g/cm
Odor	Sweet	Sweet	Sweet	Sweet
Flash point	29 °C	27 °C	32 °C	27 °C
Conversion Factors in Air	0.0045 mg/L (1.0 ppm)	0.05 ppm	0.05 ppm	0.05 ppm
Solubility:				
Water at 25 °C	106 mg/L	161 mg/L	178 mg/L	162 mg/L
Organic solvent(s)	Miscible with alcohol and Ether	Miscible with alcohol,ether, and other solvents	Miscible with alcohol and ether	Soluble in alcohol,ether, and other organic solvents
Partition coefficient:				
Log K_{ow}	No data	3.2k	3.12k	3.15k
Log K_{oc}	No data	2.22	2.11	2.31
Vapour pressure	l6.72 mmHg at 21^0 c	8.29 mmHg 25^0 c	l6.61 mmHg 25^0 c	l 8.84 mmHg 25^0 c
Henry's law constant	No data	7.18x10-3 atmm3/mol	5.18x10-3 atmm3/mol	6.90x10-3 atmm3/mol
Auto ignition temperature	464 °C	527 °C	463 °C	528 °C
Flammability limits	No data	No data	1.0–7.0%	1.1–7.0%

Source: Hazardous Substances Data Bank (HSDB) 2007,

2.3 APPLICATIONS OF XYLENE

2.3.1 Solvent and general applications

Xylene is used as a solvent. In this application, the mixture of isomers is often referred to as xylene or xylol. Solvent xylene often contains a small percentage of ethylbenzene. Areas of application include printing, rubber, and leather industries. It is a common component of ink, plastic, adhesive (Tremblay and Jean, 2011). In thinning paints and varnishes, it can be substituted for toluene where slower drying is desired. Similarly it is a cleaning agent used for steel, silicon wafers, and chips (Swedish Chemicals Agency, 2010; Kieranan, 2010). *p*-Xylene is the principal precursor to terephthalic acid and dimethyl terephthalate, both monomers used in the production of polyethylene terephthalate (PET) plastic bottles and polyester clothing. 98% of p-xylene production, and half of all xylene, is consumed in this way (Clark and Luthy, 1955; Stevenson *et al.*, 1998).

2.3.2 Laboratory uses of xylene

Xylene is used in the laboratory to make baths with dry ice to cool reaction vessels, and as a solvent to remove synthetic immersion oil from the microscope objective in light microscopy (Cargille,1985; Carson and Hladik, 2009).

For histological purposes, it does not matter which isomer is used, but histological grade of xylene is often provided as a mixture of all the three isomers. Xylene is used in histological laboratories for tissue processing, staining and cover slipping and also in endodontic retreatment as a guttapercha solvent. In staining procedures, its excellent de.waxing and clearing capabilities contribute to brilliantly stained sections of tissues. Xylene is an efficient fat solvent, and that is one of its major advantages. Triglyceride and

fats in tissues has a distinctly negative effect on the quality of sectioning (Bancroft and Gamble, 2008).

2.3.3 Health effects (Toxicity of xylene)

Histopathological technicians who routinely come in contact with xylene-contaminated solvents in the workplace are the population most likely to be exposed to high levels of xylene. Although, the type and severity of health effects depends on several factors, including the amount of chemical and the length of time you are exposed. Individuals also react differently to different levels of exposure (U.S. EPA. 2000).

Most people begin to smell xylene in air at 0.08-3.7 ppm and begin to taste it in water at 0.53-1.8 ppm (U.S Department of Health and Human Services, 1993). Exposure to xylene can occur via inhalation, ingestion, eye or skin contact. It is primarily metabolized in the liver by oxidation of a methyl group and conjugation with glycine to yield methyl hippuric acid, which is excreted in the urine. Smaller amounts are eliminated unchanged in the exhaled air. There is a low potential for accumulation (Amdur *et al*., 1991; Gagnaire *et al.*, 2001).

Xylene causes health effects from both acute (<14 days) and chronic (>365 days) exposure. Xylene has a high value of LD_{50}, which range between 200 to 4000 mg/kg for animals. This principal mechanism of detoxification is oxidation to methylbenzoic acid and hydroxylation to hydroxylene. The National Institute for Occupational Safety and Health recommended exposure limits for xylene at 100 ppm (parts per million) as a time-weighted average (TWA) concentration for up to a 10-h work shift and a 40-h work week and 200 ppm for 10 minutes as a short-term limit (U.S Department of Health and Human Services, 1993).

2.3.4 Xylene substitutes for clearing tissues

After the hazardous effects of xylene became indisputable in the 1970s; many potential substitutes became available, some with as many if not more hazards. In general, these substitutes fall into four classes and are marketed under various trade names (Bancroft and Gamble, 2008). These four classes include the following: 1. Limonene reagent Aliphatic. 2. Hydrocarbon mixtures. 3. Aromatic hydrocarbon mixtures. 4. Mineral oil mixtures.

2.3.5 Limonene Reagents

Limonene mainly composed of d-limonene, which is a hydrocarbon. It is the major component of citrus peel oils. Limonene is prepared by steam distillation of orange Peels (Tardif *et al*., 1992). It has a strong citrus smell, variously described as pleasant, overwhelming, disgusting and allergenic and cannot be made odorless (Tremblay and Jean, 2011).

2.3.6 Aliphatic Hydrocarbon

The term aliphatic means that these hydrocarbons are arranged in the form of a "chain" instead of being arranged in a "ring" (aromatic). Because of their aliphatic structure, the substitutes generally need more time to exact the same effect on the tissue as does their aromatic counterpart (Tardif *et al.,* 1993).

2.3.7 Aromatic Hydrocarbon Mixture (BTX)

In the petroleum refining and petrochemical industries, the acronym BTX refers to mixtures of benzene, toluene, and the three xylene isomers, all of which are aromatic hydrocarbons. The xylene isomers are distinguished by the designations *ortho* – (o –),

meta – (*m* –), and *para* – (*p* –) . If ethylbenzene is included, the mixture is sometimes referred to as BTEX**.** Most BTX production is based on the recovery of aromatics derived from the catalytic reforming of naphtha in a petroleum refinery. Catalytic reforming usually utilizes a feedstock naphtha that contains non-aromatic hydrocarbons with 6 to 11 or 12 carbon atoms and typically produces a reformate product containing C_6 to C_8 aromatics (benzene, toluene, xylene) as well as paraffin and heavier aromatics containing 9 to 11 or 12 carbon atoms (IEA, 2006; Gagnaire *et al*., 2001).

2.3.8 Mineral Oil Mixture

Mineral (paraffin) oil mixtures look promising in eliminating xylene from most of the procedures. Isopropanol alone or mixed with molten paraffin is a technically acceptable and cost-effective substitute for xylene for tissue processing (Tremblay and Jean, 2012) It has been demonstrated that the best clearing agents from the sectioning quality and diagnostic value point of view, whether with automated or manual protocols, are mixtures of 5:1 and 2:1 isopropanol and mineral oil, followed by undiluted mineral oil, all at 50^{o}C, making them a safer and cheaper substitute than xylene (Martindale *et al*., 2012). Use of a 1.7% dishwasher soap aqueous solution at 90^{o}C to dewax before staining and oven drying the stained sections before cover slipping can eliminate xylene from the staining tasks. These methodologies can make the histology laboratory xylene-free (Gérin *et al.*, 1998; Martindale *et al*., 2012).

2.4 ESSENTIAL OILS

As defined by the International Organization for Standardization (ISO 2002), the term "essential oil" is reserved for a "product obtained from vegetable raw material, either by distillation with water or steam or by other mechanical/physical process. Essential oil is a concentrated hydrophobic liquid containing volatile aroma compounds from various parts

of plants. When they are exposed to air at ordinary temperatures they evaporate and such, they are called volatile oils or ethereal oils. The term essential is applied because the oils represent the "essences" or odor constituents of the plants. As a result, they are usually colorless, particularly when fresh, but with age they may oxidize and resinify, thus becoming darker. There are some that may be colored brown, pale blue yellow or green. Therefore, storage should be in a cool, dry place, tightly stoppered, preferably full in amber glass containers (Julia, 1995; Nordin *et al*., 2004)

They mix in all proportions with the fixed oils, dissolve freely in both alcohol and ether, and are sparingly soluble in water, forming perfumed or medicated waters. Their boiling point usually ranges between 310° and 325° Fahrenheit, and is always considerably higher than water. They resist saponification and (excepting oil of cloves) do not combine with the salifiable bases. Their density fluctuates a little on either side of water. The lightest oil is that of citrons (specific gravity 0.847), and the heaviest, that of sassafras (specific gravity 1.096). When cooled sufficiently they all soldify. By exposure to the air they rapidly absorb oxygen, and become partially converted into resin. This is the cause of the deposit that usually forms in them especially in the expressed oil of orange when kept in an imperfectly stopped bottle (Bischoff and Guale, 1998; Juergens *et al*., 2003; Gilles *et al*., 2010).

Many of these essential oils belong to a family of compounds known as terpenes and terpenoids. Terpenes are small organic hydrocarbon molecules; they may be cyclic or acyclic, saturated or unsaturated. Terpenoids are oxygenated derivatives of terpenes, which may contain hydroxyl groups or carbonyl groups. Regardless of their structural diversity, terpenes and terpenoids share certain structural similarities. They contain multiples of five carbon atoms such as; hemiterpene contains 5 carbon atoms;

monoterpene10 carbon atoms; sesquiterpene15 carbon atoms; diterpenes carbon atoms 20 etc. Rather than having a haphazard arrangement of these carbon atoms, terpenes and terpenoids may be thought to be assembled from isoprene units linked together in a head-to-tail fashion (Doran *et al*., 2001). This is shown in figure 2.2 below.

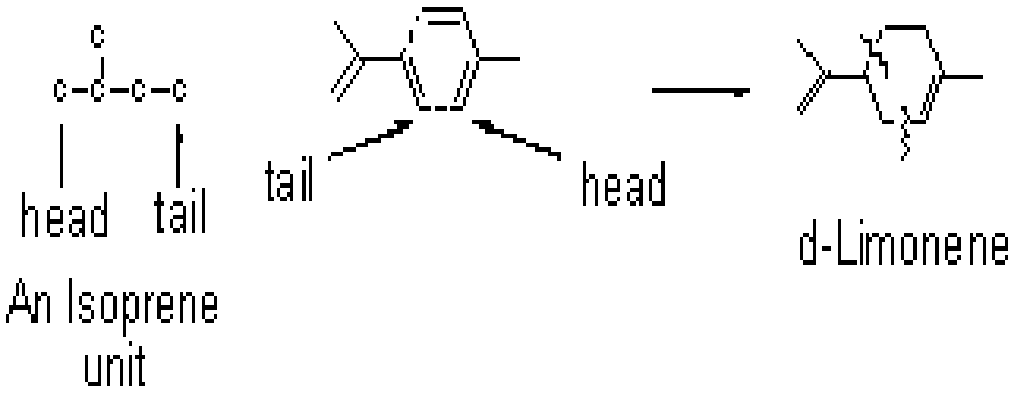


Fig: 2. 2: Isoprene units in the formulation of terpenes and terpenoids. (Sourcs: Wikipedia, the free encyclopedia)

Terpenes, such as limonene, and terpenoids, such as neral or geranial may be found in abundance in oil sacs located in the outer, colored portion of the rinds of many common citrus fruits (Doran *et al*., 2001). These essential oils may be readily isolated through a variety of methods, such as cold pressing, steam distillation, extraction etc (Burke *et al*., 2004).

2.4.1 Brief History of Essential Oils

Essential oils have been used throughout recorded history for a wide variety of wellness application ranging from skin treatments to remedies for cancer. The Egyptians were some of the first people to use aromatic essential oils extensively in medical practice, beauty treatment, food preparation, and in religious ceremony. Frankincense, sandalwood,

myrrh and cinnamon were considered very valuable cargo along caravan trade routes and were sometimes exchanged for gold (Julia, 1995; Doran *et al.*, 2001).

The Romans also used aromatic oils to promote health and personal hygiene. Influenced by the Greeks and Romans, as well as Chinese and Indian Ayurvedic, the Persians began to refine distillation methods for extracting essential oils from aromatic plants. Essential oil extracts were used throughout the dark ages in Europe for their anti-bacterial and fragrant properties (Julia, 1995; Doran, 2001; Balch, 2002).

Interest in essential oils has been revived in recent decades with the popularity of aromatherapy, a branch of alternative medicine that claims that essential oils and other aromatic compounds have curative effects. For these plant extracts to be effective, they must be grown in the right place at the right time and in the right way. Pure essential oils will not contain additives, fillers or synthetics (Lassak and McCarthy1983; Burke *et al*., 2004).

2.4.2 Production of Essential Oils

The techniques and methods first used to produce essential oils was first mentioned by Ibn al-Baitar (1188–1248), an Andalusian physician, pharmacist and chemist (Standards Australia,2001). Some of the methods used in the production include the following:

2.4.2.1 Distillation of Essential Oils

Today, most common essential oils, such as lavender, peppermint, and eucalyptus, are distilled. Raw plant material, consisting of the flowers, leaves, wood, bark, roots, seeds, or peel, is put into an alembic (distillation apparatus) over water. As the water is heated, the steam passes through the plant material, vaporizing the volatile compounds. The

vapors flow through a coil, where they condense back to liquid, which is then collected in the receiving vessel (Salari *et al*., 2004). The recondensed water is referred to as a hydrosol, hydrolat, herbal distillate or plant water essence, which may be sold as another fragrant product. Popular hydrosols include rose water, lavender water, lemon balm, clary sage and orange blossom water. The use of herbal distillates in cosmetics is increasing. Some plant hydrosols have unpleasant smells and are therefore not sold (Jones, 1986).

2.4.2.2 Expression of Essential Oils

Prior to the discovery of distillation, all essential oils were extracted by pressing. Most citrus peel oils are expressed mechanically, or cold pressed (similar to olive oil extraction). Due to the relatively large quantities of oil in citrus peel and low cost to grow and harvest the raw materials, citrus-fruit oils are one of the most readily available essential oils. Lemon or sweet orange oils that are obtained as byproducts of the citrus industry are even cheaper (Zhao and Agboola, 2007).

2.4.2.3 Solvent extraction of Essential Oils

Some flowers contain too little volatile oil to undergo expression and their chemical components are too delicate and easily denatured by the high heat used in steam distillation (Doran *et al*., 2001; Hayes and Markovic, 2002). In order to maintain their chemical constituents, a solvent such as hexane or supercritical carbon dioxide is used to extract the oils. Extracts from hexane and other hydrophobic solvent are called concretes, which are a mixture of essential oil, waxes, resins, and other lipophilic (oil soluble) plant material (Hayes and Mark, 2003). Although highly fragrant, concretes contain large quantities of nonfragrant waxes and resins. Often, another solvent, such as ethyl alcohol, which is more polar in nature, is used to extract the fragrant oil from the concrete. The

alcohol is removed by evaporation, leaving behind the absolute (Lassak and McCarthy, 1983; Burke *et al*., 2004).

Supercritical carbon dioxide is used as a solvent in supercritical fluid extraction. This method has many benefits including avoiding petrochemical residues in the product and the loss of some "top notes" when steam distillation is used. It does not yield an absolute directly. The supercritical carbon dioxide will extract both the waxes and the essential oils that make up the concrete. Subsequent processing with liquid carbon dioxide, achieved in the same extractor by merely lowering the extraction temperature, will separate the waxes from the essential oils. This lower temperature process prevents the decomposition and denaturing of compounds. When the extraction is complete, the pressure is reduced to ambient and the carbon dioxide reverts to a gas, leaving no residue (Burke *et al*., 2004).

2.4.2.4 Production Quantities of Essential Oils

Estimates of total production of essential oils are difficult to obtain. One estimate, compiled from data in 1989, 1990 and 1994 from various sources, gives the following total production, in tones, of essential oils for which more than 1,000 tones were produced (SAO, 2004). See table 2.2 below.

Table: 2.2 Showing Production quantities

Oils	Tones
Sweet orange	12,000
Mentha arvensis	4,800
Peppermint	3,200
Cedarwood	2,600
Lemon	2,300
Eucalyptus globulus	2,070
Litsea cubeba	2,000
Clove (leaf)	2,000

(Sourcs: Wikipedia, the free encyclopedia)

2.4.2.5 Handling /Precautions of Essential oils

Because of their concentrated nature, essential oils generally should not be applied directly to the skin in their undiluted or "neat" form. Some can cause severe irritation, provoke an allergic reaction and, over time, prove hepatotoxic. They must be diluted with passive carrier oils before ingestion or topical application. Water cannot be used in this case as all essential oils are not miscible with water (Schiestl and Roubik, 2004). People with high blood pressure and epilepsy should avoid it. Excessive use of this oil may cause headaches. Essential oils can be aggressive toward rubbers and plastics, so care must be taken in choosing the correct handling equipment (Hong and Shellock, 1991; Salari *et al*., 2006).

2.5 HEALTH BENEFIT OF ESSENTIAL OILS

2.5.1 Therapeutic use of Essential Oils

The therapeutic properties of eucalyptus oil are analgesic, anti-bacterial, anti-inflammatory, anti-neuralgic, anti-rheumatic, antiseptic, antispasmodic, antiviral, astringent, balsamic, cicatrisant, decongestant, deodorant, depurative, diuretic, expectorant, febrifuge, hypoglycaemic, rubefacient, stimulant, vermifuge and vulnerary (Nagata *et al*., 2008).

Eucalyptus oil has a cooling and deodorizing effect on the body, helping with fevers, migraine and malaria. In the respiratory tract, it helps with coughs, asthma, throat infections, sinusitis and catarrhal conditions. Eucalyptus oil soothes inflammation and eases mucus, clearing the head from the stuffiness of colds and hay fever (Juergens *et al.*, 2003).

Eucalyptus oil is useful as warming oil when used for muscular aches and pains, rheumatoid arthritis, sprains and poor circulation (Hong and Shellock, 1991). In skin care, it can be used for burns, blisters, herpes, cuts, wounds, skin infections and insect bites. It can furthermore boost the immune system and is helpful in cases of chicken pox, colds, flu and measles. Lemon myrtle essential oil possesses antimicrobial properties; The dried leaf has free radical scavenging ability (Singh *et al*., 2002). The use of essential oils in pregnancy is not recommended due to inadequate published evidence to demonstrate evidence of safety. Pregnant women often report an abnormal sensitivity to smells and taste (Nordin *et al.,* 2004; Sfara 2009).

2.5.2 Effect of Essential oils on Culinary (Flavouring)/ Ingestion

Essential oils are used extensively as flavoring agents in foods, beverages and confectioneries. Lemon myrtle is one of the well known bushfood flavours and is sometimes referred to as the "Queen of the lemon herbs" (Moteki, 2002; Muell, 2003). The leaf is often used as dried flakes, or in the form of an encapsulated flavour essence for enhanced shelf-life (Wong *et al*., 2006).

2.5.3 Industrial effect of Essential oils

Research shows that cineole based eucalyptus oil (5% of mixture) prevents the separation problem with ethanol and petrol fuel blends. Eucalyptus oil also has a respectable octane rating and can be used as a fuel in its own right (Jones, 1986; Doran *et al*., 2001). Phellandrene and piperitone based eucalyptus oils have been used in mining to separate sulfide minerals via flotation (Zhao and Agboola, 2007). Eucalyptus oil is also used as a fragrance component to impart a fresh and clean aroma in soaps, detergents, lotions and perfumes (Möllenbeck *et al.,* 1997).

2.5.4 Toxicity of essential oils

The median lethal dose (LD_{50}) is the dose required to kill half the members of a tested population. The risks of sensitisation, irritation, phototoxicity or being an abortifacient are fact. The use of essential oils can be safe provided that the percentage use, product application, target consumer and all of the toxicology data have been carefully evaluated and considered. It is never wise to use an essential oil without first diluting it in carrier oil (Allardice, 1994). For instance, Almond oil bitter (*Prunus amygdalus*) contains hydrocyanic acid which may be dangerous in aromatherapy. Clove oil (stem, leaf, bud) has high linalool content Oils that may cause irritation. An important consideration in tissue processing relates to reagent handling; clearly the less toxic the reagent the lower the risk to health, especially over the longer term (Jim, 2007). The LD_{50} of Some common clearing agents and essential oils is shown in Table 2.2 below.

Table 2.3: Showing oral and dermal LD_{50} of some essential oils

CLEARING AGENTS	ORAL	DERMAL
Xylene	2,840 mg/kg	4,500 mg/kg
Sweet orange (citrus)	4,400 mg/kg	
Eucalyptus (Myrtle)	2,430 mg/kg	2,250 mg/kg
Neem	14,000 mg/kg	>2,000 m/kg
Toluene	636 mg/kg	12,124 mg/kg
Chloroform	908 mg/kg	-
Isopropanaol	5,045 mg/kg	12,800 mg/kg
Frankincense	>5,000 mg/kg	>5,000 mg/kg
Cassia	2,800 mg/kg	320 mg/kg
Cedarwood	>5,000 mg/kg	>5,000 mg/kg
Roman chamomile	>5,000 mg/kg	>5,000 mg/kg
White camphor	>5,000 mg/kg	>5,000 mg/kg

Isopropanol has the highest LD_{50} figures, for both oral and dermal; it is considerably less toxic than the other reagents. (Sourcs: Personal care September, 2009)

2.6 LIST OF SOME ESSENTIAL OILS AND THE PART OF PLANT THEY ARE GOTTEN FROM.

Essential oils are derived from various sections of plants. Some are from berries, leaves, flowers, bark, wood, peel, resin, rhizome and root (Julia, 1995).

Agar oil: distilled from Agarwood (*Aquilaria malaccensis*). It is for fragrance.

Ajwain oil: distilled from the leaves of Bishop's weed (*Carum copticum*). Oil contains 35-65% thymol for preservatives.

Frankincense oil: from resin is used for aromatherapy and in perfumes.

Grapefruit oil: extracted from the peel of the fruit. It is used in aromatherapy Contains 90% limonene.

Lavender oil: from flowers, is used primarily as a fragrance and medicinally.

Orris oil: is extracted from the roots of the Florentine iris (*Iris florentina*) and used as a flavouring agent, in perfume, and medicinally.

Rose oil: distilled from rose petals, Used primarily as a fragrance.

Sandalwood oil: is derived from the wood. It is used primarily as a fragrance, for its pleasant, woody fragrance.

Valerian oil: is gotten from the root. It is used for insomnia, migraines, nervous dyspepsia, and dandruff treatment.

2.7 EUCALYPTUS (CITRODORA OIL)

Eucalyptus oil is the generic name for distilled oil from the leaf of *Eucalyptus*, a genus of the plant family Myrtaceae native to Australia and cultivated worldwide. Eucalyptus oil could be Citrodora (lemon scented) or Camaldulensis (menthol scented) (Seenivasan *et al*., 2006).

2.7.1 Origin of eucalyptus oil

There are over 500 species of Eucalyptus trees, with tough long and narrow blue-green leaves, creamy white flowers and smooth pale bark (Burke *et al.,* 2004). In Pre 1788, The Australian Aboriginal people were among the first to use *Backhousia citriodora* (*B.citriodora)* for medicine and flavouring. The Australian Aborigines calls it 'kino' and they use the leaves to cover serious wounds. Eucalyptus oil was introduced to Europe in 1788, and the first oil exported to England was called 'Sydney peppermint'. It was extracted from Eucalyptus peperita which is a more industrial type of oil. Lemon myrtle was given the botanical name *Backhousia citriodora* in 1853 after the English botanist, James Backhouse (Standards Australia, 2001).

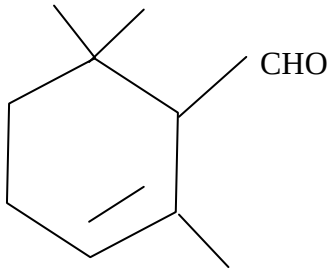
Discovery of antimicrobial qualities of steam-distilled *B.citriodora* oil was done in the 1920s by A.R. Penfold and R.Grant, Technological Museum, Sydney. In the 1940s, Tarax Co. use *B.citriodora* oil as a lemon flavouring during World War II. In the 1950s, some production of oil was carried out in the Maryborough and Miriam Vale areas from bush stands by JR Archibald, (Jones, 1986). But the small industry falls into decline. By 1989, *B.citriodora* was investigated as a potential leaf spice and commercial crop by Peter Hardwick (Doran *et al.,* 2001).

By late 1990s, *B.citriodora* begins to be supplied internationally for a range of flavouring, cosmetic and anti-microbial products. Agronomic production of *B.citriodora* starts to exceed demand. In 1991 – *B.citriodora* plantation established by Dennis Archer and Rosemary Cullen-Archer, Toona Essential Oils Pty Ltd, and subsequent commercial supply of plantation produced *B.citriodora* oil in 1993. In 2001, Standard for oil of *B.citriodora* established by the essential oils unit, Wollongbar, and Standards Australia was developed (Zhao and, Agboola 2007). Example of eucalyptus oil species includes is grouped into two which include the commercial cineole base such as eucalyptus cineorifolia, eucalyptus globules, etc and non-cineole producing species such as eucalyptus dives –piperitone variant.

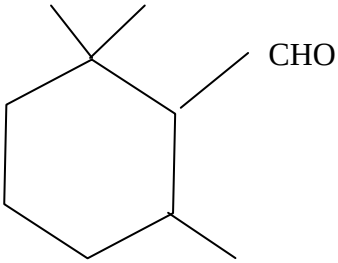
2.7.2 Citodora

Kingdom: Plantae (unranked). Angiospermss (unranked). Eudicots (unranked). Rosids**.** Order: Myrtales. Family: Myrtaceae. Genus: *Backhousia* .Species: ***B.*** *citriod.Backhousia citriodora* (common names lemon myrtle, lemon scented myrtle, lemon scented ironwood) is a flowering plant in the family Myrtaceae, genus *Backhousia*. It is endemic to subtropical rainforests of central and south-eastern Queensland, Australia, with a natural distribution from Mackay to Brisbane (Muell, 2003). Other common names are sweet verbena tree, sweet verbena myrtle, lemon scented verbena, and lemon scented backhousia. Lemon myrtle has been the common name. It was given the botanical name *Backhousia citriodora* in 1853 after the English botanist; James Backhouse (Julia, 1995). The common name reflects the strong lemon smell of the crushed leaves. “Lemon scented myrtle” was the primary common name until the shortened trade name, “lemon myrtle”, was created by the native foods industry to market the leaf for culinary use. Eucalyptus oil has a clear, sharp, fresh and very distinctive smell, is pale yellow in color and watery

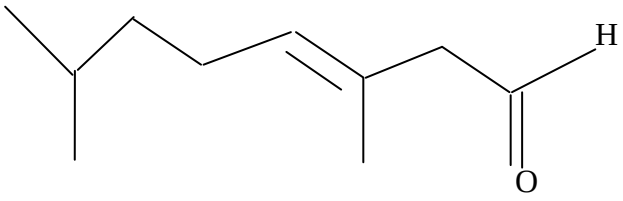
in viscosity (Julia, 1995). It has molecular weight of 154.25 and the structural formula of C10H180 as shown in Figure 2.3.



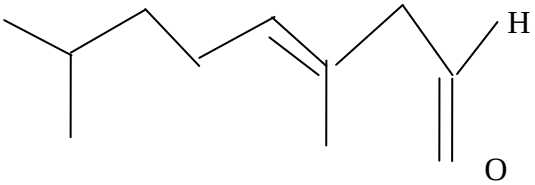
alpha-cyclocitral



beta-cyclocitral



Isocitral



Citral

FIG 2.3 Structural formula of Citrodora (Sourcs: www.cropwatch.org)

2.7.3 Cultivation and Extraction of Eucalyptus oil

B.citriodora is a cultivated ornamental plant. It can be grown from tropical to warm temperate climates, and may handle cooler districts provided it can be protected from frost when young. In cultivation it rarely exceeds about 5 meters (16 ft) and usually has a dense canopy. But some species such as the Australian Blue-gum can sometimes be as high as100 meters (300 feet), making it one of the highest trees in the world. Seedlings of *B.citriodora* go through a shrubby, slow juvenile growth stage, before developing a dominant trunk. Lemon myrtle can also be propagated from cutting, but is slow to strike. Growing cuttings from mature trees bypasses the shrubby juvenile stage. Cutting

propagation is also used to provide a consistent product in commercial production (Zhao and Agboola, 2007).

The tree requires a lot of water while growing and has been used to clear water-logged land, draining the water from swamps where malaria mosquito may be found. The tree was thought to prevent malaria in the past, due to this draining action (Jones, 1986). The leaves are evergreen, opposite, lanceolate, 5–12 cm (2.0–4.7 in) long and 1.5–2.5 cm (0.59–0.98 in) broad, glossy green, with an entire margin. The flowers are creamy-white, 5–7 mm diameter, produced in clusters at the ends of the branches from summer through to autumn, after petal fall the calyx is persistent. The principal attraction to gardeners is the lemon smell which perfumes both the leaves and flowers of the tree. Lemon myrtle is a hardy plant which tolerates all but the poorest drained soils. It can be slow growing but responds well to slow release fertilizers (Zhao and Agboola, 2007). In plantation cultivation the tree is typically maintained as a shrub by regular harvesting from the top and sides. Mechanical harvesting is used in commercial plantations. It is important to retain some lower branches when pruning for plant health. The harvested leaves are dried for leaf spice, or distilled for the essential oil (Julia, 1995).

Eucalyptus oil is extracted from the fresh or partially dried leaves and young twigs eucalyptus plant mostly by hydrodistilation

2.7.4 Chemical Properties and Composition of Eucalyptus Oil

Eucalyptus has a clear, sharp, fresh and very distinctive smell, is pale yellow in color and watery in viscosity. The main chemical components of eucalyptus oil are a-pinene, b-pinene, a-phellandrene, 1,8-cineole, limonene, terpinen-4-ol, aromadendrene, epiglobulol, piperitone and globulol. Eucalyptol has a fresh camphor-like smell and a spicy, cooling

taste. It is insoluble in water, but miscible with ether, ethanol and chloroform. The boiling point is 176 °C and the flash point is 49 °C. Eucalyptol forms crystalline adducts with halogen acids, o-cresol, resorcinol, and phosphoric acid. Formation of these adducts are useful for purification (Jones, 1986; Zhao and Agboola, 2007). The composition of the leaf oil is 90-95% citral, with some variable presence of citronellal, myrcene, methyl heptenone, linalol, and a- and b-cyclocitrals (Doran *et al*., 2001). *B.citriodora* has two essential oil chemotypes:

I. The citral chemotype is more prevalent and is cultivated in Australia for flavouring and essential oil. Citral as an isolate in steam distilled lemon myrtle oil is typically 90–98%, and oil yield 1–3% from fresh leaf. It is the highest natural source of citral.
II. The citronellal chemotype is uncommon, and can be used as an insect repellent (Doran *et al*., 2001). More of the physical and chemical properties are shown in table 2.4 below.

Table 2.4: CHEMICAL AND PHYSICAL PROPERTISE OF CITRODORA OIL

Chemical name	**Eucalyptus oil**
Molecular Formula	C10H18O
Formula Weight	154.25
Physical State	Liquid, Oil
Color	Clear colorless to pale yellow
Flash Point	>74°C Tag Closed Cup
Boiling Point	200.00 °C. @ 760.00 mm Hg
Specific Gravity (H2O = 1) 20°C	0.86-0.87
Refractive Index @ 20°C	1.4480 to 1.4600
Density	0.909 g/ml at 25 °C
Solubility	Insoluble in water, soluble in solvent and alcohol
Extinguishing Media	Dry Chemical, Carbon Dioxide, Foam
Stability	stable under normal conditions
Reactivity	does not react with water, undergo explosive decomposition.
Incompatibility (Materials to Avoid)	Avoid contact with strong oxidizing agents, hot work and sources of ignition.
Conditions to Avoid	Avoid contact with sparks, flame and heat.
Acute Toxicity Data	Oral LD50: None established, Dermal LD50: None established, Inhalation LC50: None established
Health Hazards (Acute & Chronic)	Ingestion of large quantities: None established Inhalation: None established Skin Contact: Liquid may be irritating Eye Contact: Liquid may be irritating
Ecological data	Degradable product, however, do not release directly into the environment.
Shelf Life	12.00 month(s) or longer if stored properly. Storage :store in cool, dry place

Source: Material Safety Data Sheet for Eucalyptus Citriodora Oil, 2010

2.8 CITRUS (ORANGE) OIL

The word orange is derived from Sanskrit, and is botanically called Citrus Sinensis (NPGS/GRIN, 2011). The name "orange" applies primarily to the sweet orange, *citrus sinensis*, which accounts for about 70% of world citrus production (Nicolosi *et al.*, 2000). Other citrus species known as oranges include: The bergamot orange (*Citrus bergamia Risso*), The mandarin orange (*Citrus reticulata*), The trifoliate orange (*Poncirus trifoliata*), Navel oranges, Cara cara oranges, Blood oranges, Acid less oranges etc (Alfred *et al*., 2010).

2.8.1 Origin and History of Essential Oils

The orange is a hybrid of ancient cultivated origin, possibly between pomelo (*Citrus maxima*) and mandarin (*Citrus reticulata*) (Nicolosi *et al*., 2000). Oranges probably originated in Southeast Asia (John, 1967; Nicolosi *et al.*, 2000). It is cultivated in China by 2500 BC but are now cultivated extensively in America and other parts of the world. Sweet orange esential oil is most often referred to simply as Orange Oil. Orange oil is extracted from Citrus sinensis of the Rutaceae family and is also known as Portugal or China orange. All citrus trees are of the single genus, *Citrus*, and remain almost entirely interfertile; that is, there is only one "superspecies" which includes grapefruits, lemons, limes, oranges, and numerous other types and hybrids (Verzera *et al*., 2004). It is an evergreen flowering tree generally growing to 9–10 m in height although very old specimens have reached 15 m. The leaves are arranged alternately, are ovate in shape with crenulate margins and are 4–10 cm long. The orange fruit is a hesperidium, a type of berry (Webber *et al.,* 1903). There are typically two types of orange essential oil: sweet and bitter. This quality determines the purpose of the oil. The fruit of *Citrus sinensis* is called *sweet orange* to distinguish it from *Citrus aurantium*, the bitter orange.

2.8.2 Physical and Chemical Characteristics of Orange Oil

Orange seed oil is pale yellow to orange in color and watery in viscosity. It has a slightly sweet aroma, but is generally flavorless. It contains Octyl ethanoate which is responsible for the fragrance of oranges (fig 2.4). The shelf life is approximately 6 months. Orange seed oil does not freeze solid, but congeals at about 10 degrees Fahrenheit and gels at -4 degrees. Orange seed oil begins to smoke at 300 degrees Fahrenheit and catches fire at 343 degrees Fahrenheit (Pino *et al*., 2006).

Orange seed oil is composed of three primary fatty acids: palmitic acid, stearic acid, and oleic acid. Trace amounts of linoleic acid are also present. These acids all make excellent base ingredients for a variety of personal care items. Additionally, linoleic acid is one of two fatty acids that the human body cannot produce for itself (Pino *et al*., 2006; Kegley *et al*., 2012). The molecular formula of sweet orange is C15H24O. Other physical properties are shown in table 2.5 below.

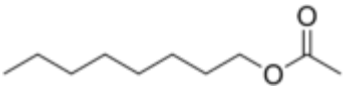


Fig 2.4: Octyl ethanoate is responsible for the fragrance of oranges. (Sourcs: Wikipedia, the free encyclopedia)

Table2.5: PHSICAL AND CHEMICAL PROPERTIES OF SWEET ORANGE

Chemical Name	**Orange sweet oil**
Molecular Formula	C15H24O
Odour	Pungent Orange Aroma
Color	Yellow. Orange. Deep Orange
PH	3.69
Vapour Pressure (mm Hg @14°C)	1.0mmHg
Boiling point	347.9 to 352.4°F
Melting Point/Range	-89°C to –96.9°C
Specific Gravity: @ 20 to 25°C	0.838 to 0.850 g/ml
Refractive Index: (@ 20°C)	1.472
Density	0.845 g/ml at 25 °C
Vapor Density:	(Air = 1), (Ether = 1) <1.0
Flash point	46.111°C (115°F) Closed up
Solubility in Water	Insoluble in cold water
Viscosity @ 20°C	1.28 cST
Heat of Combustion	1.471 Kcal/mol
Stability	Stable under ordinary conditions of use and storage.
Incompatibilities	Avoid strong oxidizing agents, exposure to sparks, heat and flames.
Acute Effects	LD50, Oral (rat): 4,400 mg/kg. LD50, Dermal (rabbit): >2,000 mg/kg. LD50, Dermal (mice): 5,600 to 6,600 mg/kg.
Global Warming Potential	Zero
Biodegradability	D-Limonene is a biodegradable solvent occurring in nature as the main component of peel oil.

Source: Material Sfety Data Sheet Orange Oil

2.8.3 Composition of Orange Oil

The composition of orange oil varies due to regional and seasonal changes as well as the method used for extraction lead to these variations (Table 2.6). It is composed of mostly d-limonene, other main chemical components of orange oil are a-pinene, sabinene, myrcene, linalool, citronellal, neral and geranial (Verzera *et al*., 2004; Pino *et al*., 2006).

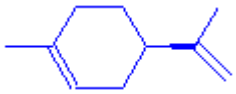


Fig 2.5: Limonene. Source: Wikipedia, the free encyclopedia

Limonene (fig 2.5) is what gives citrus fruit their familiar aroma. This is natural oil found in the skins of citrus fruits, such as lemons or oranges, and it is used in perfume and household cleaners for its fragrance. It is also an effective, environmentally friendly, and relatively safe solvent, which makes it an active ingredient of choice in many applications, such as, but not limited to, adhesive and stain removers, cleaners of various sorts, and strippers (Allen, 1997). It is very useful in agriculture. The name is derived from *Citrus limonum*, the lemon tree. Limonene is obtained industrially by steam distillation of orange peel which is a byproduct of the orange juice industry. Since xylene has been seen as an undesirable and possibly toxic chemical, one of the substitutes recommended is limonene, which goes by several trade names. . Although, limonene is effective for clearing, however it is a little slower in cleaning than xylene. Some people are allergic to limonene and develop skin rashes when they come in contact with it (Kegley *et al.*, 2010).

Table 2 6: Types and concentrations of some oranges compnents

Compound	Italian Orange oil Concentration [%]	Valencia Orange oil Concentration [%]	Valencia Orange oil Concentration [%]	Valencia Orange oil Concentration [%]
Limonene	93.67	91.4	95.17	97.0
α-Pinene	0.65	1.4	0.42	–
Sabinene and β-Pinene	1.00	0.4	0.24	–
Myrcene	2.09	4.3	1.86	0.03
Octanal	0.41	–	–	–
Linalool	0.31	0.8	0.25	0.3
δ-3-Carene	0.31	–	–	–
Decanal	0.27	0.4	0.28	–

Source: Food and Agriculture Organization (FOA) (Thomas, 2010)

2.8.4 Propagation and growth of Oranges

Oranges can be grown outdoors in warmer climates, and indoors in cooler climates. Like most citrus plants, oranges will not do well unless kept between 15.5 °C – 29 °C (60 °F – 85 °F). Propagation of orange trees is deceptively difficult, because hardy edible oranges are not generally grown from seed. Cultivars that produce good quality fruit are highly susceptible to root diseases. Grafted trees also begin bearing fruit many years earlier than trees reproduced by seed (FAO, 2008; Thomas, 2010). Oranges require a huge amount of water and the citrus industry in the Middle East is a contributing factor to the desiccation of the region (Thomas, 2010).

2.8.5 Harvesting, Storage and Extraction of Oranges Oil

Canopy-shaking mechanical harvesters are increasingly being used in Florida to harvest process oranges. Current canopy shaker machines use a series of six- to seven-foot long tines to shake the tree canopy at a relatively constant shaking stroke and frequency (Alfred, 2010). After harvesting, oranges have a shelf life of about one week at room temperature and one month refrigerated. In either case, they are optimally stored loosely in an open or perforated plastic bag. Oranges produce odours that are absorbed by meat, eggs and dairy products (Kimball, 1999). Unlike most essential oils that are extracted using steam distillation, most citrus oils are extracted by cold pressing the rinds of the orange (Katzer, 1999, Gottwald *et al*., 2002; Alfred, 2010).

2.8.6 Uses of orange oils

Orange essential oils offer a magnitude of health benefits, some of these include: as anti-depressant (CDC, 1999), as anti-inflammatory (Crowell, 1999), as anti-septic (Crowell, 1999), It improves digestion (Tsuda *et al*., 2004), as anti-Cancer (Tsuda *et al*. (2004), It

encourages detoxification (Tsuda *et al*. (2004), use as skin treatment (Nutrition, 2011), as biological pest control (Kegley *et al.,* 2010), as industrial and household uses, (Allen, 1997).

2.8.7 Warnings and Precautions when using orange essential oil

Orange Essential Oil shows photo-toxicity; do not apply before going out in the sun. If ingested in excess quantity, it can result in vomiting and nausea. If you are pregnant, epileptic, have liver damage, have cancer, or have any other medical problem, use essential oils only under the guidance of a qualified aromatherapy practitioner (Crowell, 1999; CGD, 2009; Nutrition, 2013;).

2.8.8 Orange peel consumption

Although not as juicy or delicious as the inside of an orange, the peel is edible, and has been consumed particularly in environments where there is scarcity of resources and where maximum nutritional value must be derived and minimal waste generated for example, on a submarine. The peel of an orange has increased vitamin C and fiber, however one should only consume the peels of organically grown and processed oranges, where chemical pesticides or herbicides would not have been used on the peel Orange peel contains citral, an aldehyde that antagonizes the action of vitamin A. Therefore, anyone eating orange peels should make certain that their dietary intake of Vitamin A is sufficient (CDC, 1999).

2.9 NEEM OIL (*Azadirachta indica*)

Neem oil is a vegetable oil pressed from the fruits and seeds of the neem (*Azadirachta indica*), an evergreen tree which is part of the mahogany family – Meliaceae and it is one

of two species in the genus Azadirachta, native to India, Sri Lanka, Malaysia, Bangladesh and Pakistan. It is classified under the order Sapindales, from the family Meliaceae, with the genus Azadirachta and the specie A. indica (Neem Foundation, 2007).

2.9.1 Origin and History of Neem

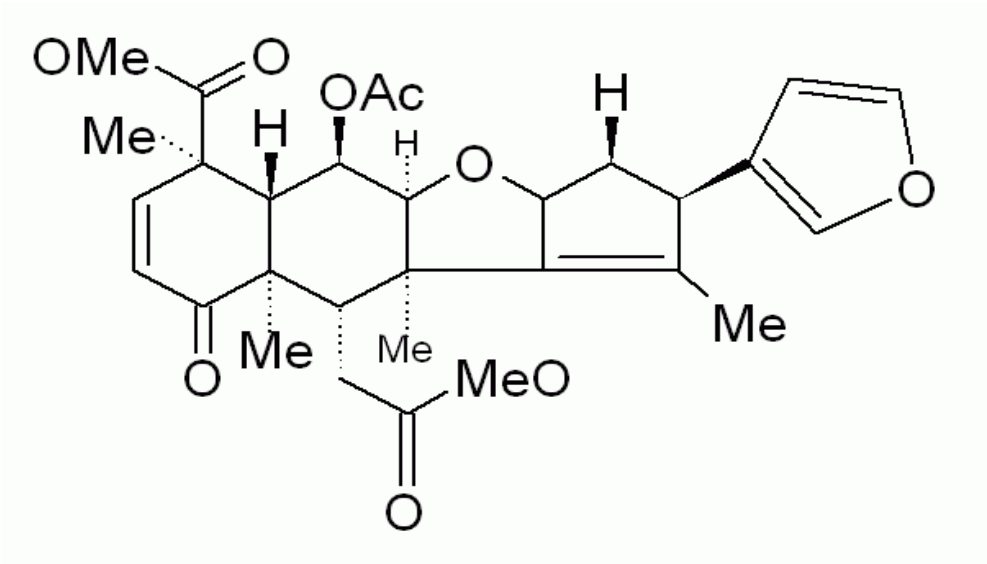
The word Neem is derived from Sanskrit Nimba which means 'bestower of good health'. Neem was introduced to Africa earlier this century. It was brought from the Indian subcontinent and has been introduced to many other areas in the tropics (Puri, 1999). It has also been known as Ravisambha – sun ray like effects in providing health.. Now it is planted extensively in the tropical regions of Africa, particularly in the regions along the Sahara's southern fringe (Puri, 1999).The tree is native to South Asia, and is grown on a large scale in India although the tree has been successfully grown in Laos, Burma, Thailand, Africa, Fiji, Mauritius, Malaysia, Indonesia, Cambodia, as well as Latin America (Vietmeyer, 1992).

2.9.2 The physical, Chemical Properties and Composition of Neem Oil

Neem oil is generally light to dark brown depending on the time of harvest as well as growing conditions before harvesting. It is bitter and has a strong odor – described by some as a combination of the smell of peanut and garlic (Vietmeyer, 1992). Neem has a molecular formula of $C_{30}H_{36}0_6$ and a large structure as shown in fig 2.6 below. Other physical and chemical properties are shown in table 2.8.

The oil comprises mainly of triglycerides and large amounts of triterpenoid compounds. It furthermore contains steroids (campesterol, beta-sitosterol, stigmasterol) and triterpenoids of which Azadirachtin is the most well known and studied. The oil normally solidifies at

room temperature, and to get it in liquid form, place the container in hot water (not boiling) and wait for the oil to liquefy (Vietmeyer, 1992).



CA index Name: 2H-Cyclopenta [b] naphtha [2,3-d] furan-10-acetic acid.

Fig 2.6: Molecular Formula of neem. Source:www.neemfoundation.org/growing.2007

2.9.3 Physical and Chemical Properties of Neem Oil

The physical and chemical properties of neem oil is shown in Table 2.7 below:

Table 2.7: showing the physical and chemical properties of neem

Chemical Name	Neem Oil
Molecular Formula	C30H36O6
Physical State	Liquid
Appearance	Brown Viscous Liquid
Odor	fragrant odor
PH	7+- 0.02
Vapur Pressure	38 at 200C
Boiling Point	More than 100 ^{o}C
Viscosity	Viscous Material
Freezing/Melting Point	14 ^{o}C
Auto ignition Temperature	Above 200 ^{o}C
Flash Point	More than 170C
Solubility in Water	Emulsifies with water
Specific Gravity Density	0.84 g/ml
Chemical Stability	Stable at ordinary condition of use and storage.
Acute Toxicity	Acute oral Rat LD 50 >500mg/kg
	Acute Dermal Rat LD 50> 2000 mg/kg
	Inhalation - Rat LC 50 > 5.4 mg/l

Source: organicneem.com. 2008

2.9.4 Chemistry of Neem

Chemical investigations of neem were undertaken by Indian pharmaceutical chemists in 1919, they isolated acidic principle in neem oil, which they named as ‘margosic acid”. However, real chemical research originated in 1942 with isolation of three active constituents, viz, nimbin, nimbidin and nimbinene .Nimbin is the first bitter compound isolated from neem oil, more than 135 compounds have been found, which are divided into two major classes: isoprenoids and the nonisoprenoids. The isoprenoids include diterpenoids and triterpenoids containing protomeliacins, limonoids, azadirone and its derivatives, gedunin and its derivatives, vilasinin type of compounds and Csecomeliacins such as nimbin, salanin and azadirachtin. The nonisoprenoids include proteins (amino acids) and carbohydrates (polysaccharides), sulphurous compounds, polyphenolics such as flavonoids and their glycosides, dihydrochalcone, coumarin and tannins, aliphatic compounds, etc (Kaushik and Vir, 2006).

Neem leaves mainly yield quercetin (flavonoid) and nimbosterol (β- sitosterol) as well as number of liminoids (nimbin and its derivatives). Quercetin, a polyphenolic flavinoid is known to have antibacterial and antifungal properties. This may perhaps account for the curative properties of leaves for sores and scabies. The principal constituents of neem leave include protein (7.1%), carbohydrates (22.9%), minerals, calcium, phosphorus, vitamin C, carotene etc. But they also contain glutamic acid, tyrosine, aspartic acid, alanine, praline, glutamine and cystine like amino acids, and several fatty acids (dodecanoic, tetradecanoic, elcosanic, etc.) (Schmutterer, 2002).

The essential oil of neem consist of sesquiterpene derivatives, the flowers contain nimbosterol and flavonoids like kaempferol, melicitrin etc. Flowers also yield a waxy material consisting of several fatty acids, viz., behenic (0.7%), arachidic (0.7%), stearic

(8.2%), palmitic (13.6%), oleic (6.5%) and linoleic (8.0%). The pollen of neem contains several amino acids like glumatic acid, tyrosine, arginine, methionion, phenylalanine, histidine, arminocaprylic acid and isoleucine (Kaushik and Vir, 2006).

The trunk bark contains nimbn (0.04%), nimbinin (0.001%), nimbidin (0.4%), nimbosterol (0.03%), essential oil (0.02%), tannins (6.0%), a bitter principle margosine and 6-desacetyl nimbinene. It also contains tannins (12-16%) and non-tannin (8-11%). The bark contains anti-inflammatory polysaccharide consisting of glucose, arabinose and fructose at a molar ratio 1:1:1 with molecular weight of 8,400. The bark also yields an antitumor polysaccharide. Besides polysaccharides, several diterpenoids, viz., nimbinone, nimbolicin, margocin, nimbidiol, nimbione, etc. have been isolated from stem bark and root bark (Schmutterer, 2002).

The heartwood contains β- sitosterol, 24-methylenelophenol, nimatone, calcium, potassium and iron salts. The heartwood on destructive distillation gives charcoal (30%) and pyroligeneous acid (38.4%). Neem wood contains cellulose, hemicellulose (14.00%) and lignin (14.63%), while wood oil contains β-sitosterol, cycloeucalenol and 24-methylenecyceloartenol (Caboni *et al.,* 2006).

Neem tree exudes a gum, which on hydrolysis yields, L-arabinose, L-fucose, D-galactose and D-glucoronic acid. The older tree exudes a sap containing free sugars, glucose, fructose, mannose and xylose, amino acids alanine, aminobutyric acid, arginine, asparagines, aspartic acid, glycine, norvaline, praline, etc and organic acids citric, malonic, succinic and fumaric. The sap is reported to be useful in the treatment of general weakness and skin diseases.

The Seed is very important both because of its high lipid content as well as the occurrence of a large number of bitter principles azadirachtin, azadiradione, fraxinellone, nimbin, salannin, salannol, vepinin, vilasinin, etc. in considerable quantities. Azadirachtin has proven effectiveness as a pesticide against about 200 insect species and is reported as non-toxic to humans (Hallur *et al.*, 2007).

Neem kernel lipids are similar to the normal glycerides from other oilseeds .Neem oil contains oleic acid (50-60%), palmitic acid (13-15%), stearic acid (14-19%), linoleic acid (8-16%) and arachidic acid (1-3%). It is brownish yellow, non-drying oil with an acrid taste and unpleasant odour. The quality of the oil differs with the method of processing (Kaushik and Vir, 2000; Hallur *et al.*, 2007). Neem oil also contains some fatty acids as shown in the table below.

Table 2.8: Showing the composition of neem fatty acids

Common Name	Acid Name	Composition range
Omega-6	Linoleic acid	6-16%
Omega-9	Oleic acid	25-54%
Palmitic acid	Hexadecanoic acid	16-33%
Stearic acid	Octadecanoic acid	9-24%
Omega-3	Alpha-linolenic acid	?%
Palmitoleic acid	9-Hexadecenoic acid	?%

From Wikipedia, the free encyclopedia, 2013

2.9.5 Cultivation and Production of Neem Oil

Neem tree grows slowly during the first year of planting. Young neem plants cannot tolerate intensive shade, frost or excessive cold. A Neem tree normally begins to bear fruit between 3 and 5 years and becomes fully productive in 10 years. Mature trees produce 30 – 50 kg fruit every year. The Neem tree can reach a height of 12-15 m (40 – 50 feet) and is evergreen, but in severe drought it may shed most or nearly all of its leaves. The branches are wide spread and the diameter of the tree is about 12 m (40 feet) (Caboni *et al.*, 2006).

The Neem grows on almost all types of soils including clayey, saline and alkaline soils, with pH upto 8.5, but does well on black cotton soil and deep, well-drained soil with good sub-soil water. Unlike most other multipurpose tree species, it thrives well on dry, stony, shallow soils and even on soils having hard calcareous or clay pan, at a shallow depth. The tree improves the soil fertility and water-holding capacity as it has a unique property of calcium mining, which changes the acidic soils into neutral. Neem tree needs little water and plenty of sunlight. The tree grows naturally in areas where the rainfall is in the range of 450 to 1200 mm. However, it has been introduced successfully even in areas where the rainfall is as low as 200 – 250 mm. It cannot withstand water-logged areas and poorly drained soils (Kaushik and Vir, 2000)

Neem tree has adapted to wide range of climates, it thrives well in hot and cold weather where the maximum temperature is high as 49 ° C and tolerates cold up to 0° C on altitudes up to 1500m. Neem grows almost all types of soils with pH up to 8.5. The Neem has adapted to a wide range of climates. It thrives well in hot weather, where the maximum shade temperature is as high as 49° C and tolerates cold upto 0° C on altitudes upto 1500 m. Today, the Neem is well established in at least 30 countries world-wide, in

Asia, Africa and Central and South America. Some small scale plantations are also reportedly successful in the United States of America (Caboni *et al.,* 2006).

2.9.6 Storage of Neem Oil

It is estimated that a Neem tree has a productive life span of 150 – 200 years .Store Neem oil in a cool dark place, away from sunlight. Neem oil can easily solidify - even at low room temperatures. Schmutterer, 2002 suggested that the bottle should be placed in warm water (below 95 degree F) to liquefy. He also suggested that it should not be place in near boiling water, as it may reduce the effectiveness of the oil (Schmutterer, 2002).

2.9.7 Extraction of Neem Oil

The method of processing and extracting could be methods, such as pressing (expelling) or solvent extraction. The neem oil yield that can be obtained from neem seed kernels also varies widely in literature from 25% to 45%. The oil can be obtained through pressing (crushing) of the seed kernel both through cold pressing or through a process incorporating temperature controls (Caboni *et al.,* 2006).

Neem seed oil can also be obtained by solvent extraction of the neem seed, fruit, oil, cake or kernel. A large industry in India extracts the oil remaining in the seed cake using hexane. This solvent-extracted oil is of a lower quality as compared to the cold pressed oil and is mostly used for soap manufacturing (Mishra *et al.,* 1995).

2.9.8 Uses of Neem Oil

Bark: It is a bitter, cool, acrid, astringent, and refrigerant herb. It is useful for fever, loss of appetite, tiredness, coughs and intestinal worm infestation. It is also helpful for healing wounds and to combat vomiting, excessive thirst and skin diseases (Schmutterer, 2002).

Leaves: Are used in the treatment of Vatik disorders that is neuro-muscular pains and is also reported to remove toxins, preventing damage from free radicals (Caboni *et al.,* 2006).

Fruits: The fruit is bitter, purgative, anti-hemorrhodial and anthelmintic in nature.

Flowers: The flowers are used in conditions of Pitta balancing the body heat and Kapha cough formation and by nature are astringent and anthelmintic

Seeds: They are bitter and have anthelmintic (vermifuge – destroys and expels intestinal worms) properties, as well as being anti-bacterial (Mishra *et al.,* 1995)

Oil: The oil derived from crushing the seeds is a powerful anthelmintic compound and has a very wide spectrum of action and is highly medicinal in nature, which includes being a spermicidal compound (this action is because of the volatile fraction coded as NIM-76) s as well as anti-bacterial, anti-fungal, etc (Caboni *et al.,* 2006).

Mixture: A mixture of all five parts of the tree - bark, root, fruit, flowers and leaves – are used in diseases of the blood. It is also used to alleviate conditions of excess heat, itching, helps with wound healing, reduces burning sensation in the body as well as in skin diseases (Drugs information online, 2007).

To keep insects, silverfish, beetles and moths away from your books - place a couple of fresh leaves in your books. This can also be used in herbaria to protect against insect damage (Mishra *et al.,* 1995).

2.9.9 Therapeutic uses of Neem Oil

Neem oil should only be used externally on the skin and has been therapeutically used as folk medicine to control respiratory disorders, constipation, leprosy, as well as a general

tonic. It has been used for the topical treatment of rheumatism, eczema, ringworm, athlete's foot, cold sores, psoriasis, warts, chronic syphilitic sores, infected burn wounds and slow-healing skin ulcers as well as controlling various skin infections It has anti-inflammatory, antipyretic relieving fever and analgesic relieving pain activity and possesses immunostimulant activity increasing the body's defense mechanism to fight infectious organisms and other foreign material by selectively activating the cell-mediated immune mechanisms to elicit an enhanced response to subsequent mitogenic or antigenic challenge (Mishra *et al.*, 1995).

2.9.10 Toxicity of neem Oil

Studies done when Azadirachtin the primary active pesticide ingredient in neem oil was approved as a pesticide showed that when neem leaves were fed to male albino rats for 11 weeks, 100% (reversible) infertility resulted (Rajeev, 2009). Neem oil and other neem products such as neem leaves and neem tea should not be consumed by pregnant women, women trying to conceive, or children. There is some evidence that heavy use may be associated with liver damage in (Kaushik and Vir, 2006).

2.9.11 Safety precautions and warnings when using neem

Neem is considered generally safe, and neem oil, when applied topically on the skin, has no side effects. Some animal studies have shown side effects on internal ingestion of neem extracts (Mishra *et al*., 1995)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 REAGENTS AND MATERIALS

The reagents and materials for this study include xylene, ethanol, neutral buffered formalin, chloroform, paraffin wax, egg albumen, Oven, Rotary microtome made in England Search Tech Instrument, Duplex Shandon Procesor serial number 02030, glass slides and microscope. The reagents were manufactured by Mayer and Baker Limited Dagenham England with product number 28304. They were purchased from Cardinal Scientific Supply by Zaria Hotel, Kwangila Zaria.

3.2 PLANT MATERIALS FOR THE OIL.

Orange (*Citrus Sinensis)* peels of ripened fruits of Citrus (sweet orange) plant, leaves of Eucalyptus (*Eucalyptus Citrodora. Hook*) plants and the seed of Neem trees were used in this study. These plant materials were all sourced within Zaria metropolis.

3.2.1 Collection and Identification of Plant

The Citrus (sweet orange) fruits were purchased from the Orange market in Zaria, Kaduna State, while the leaves and seeds of the Eucalyptus and Neem (*Azadirachta indica*) plants were obtained from National Research Institute for Chemical Technology (NARICT) Zaria. The plants were taxonomically identified and authenticated by Mr. U.S Gallah of the Department of Biological Science herbarium, Ahmadu Bello University Zaria with the following voucher number:

1. Famliy: RUTACEAE. *Citrus Sinensis* (L) Osb. voucher number: 1432
2. Famliy: MYRTACEAE. *Eucalyptus Citrodora. Hook.* voucher number: 2767

3. Famliy: MELIACEAE. *Azadirachta indica. AJussi.* voucher number: 900151

3.2.2 Extractions of the Essential Oils

The essential oil of eucalyptus and citrus plants was extracted in National Istitute for Chemical Research Technology (NARICT) by hydro distillation method, while that of the neem oil was by the Cold *n*-hexane extraction as described by Ladan (2011)

3.2.3 Eucalyptus oil and Citrus oil

500g of the fresh leaves of eucalyptus and citrus peel was separately weighed and packed into a distillation flask fitted with condensers. Heat was supplied to the flask through a steam generator at constant flow. The essential oil which vaporizes with the steam was condensed into a collecting funnel. The oil was then separated by gravity, dried over anhydrous sodium sulphate, measured, labeled and stored in a brown bottle.

3.2.4 Neem Oil

Dried seeds of the neem were collected and cracked to remove the kernel, the dried kernel were grounded using mortar and pestle and packaged in a container for oil extraction. About 28g of finely grounded sample of neem seeds was dissolved in 140ml normal hexane in a 500ml conical flask and covered with aluminium paper foil for 24hrs with continuous shaking on a shaker after which it was filtered. The filtrate was concentrated on a water bath at 40^0C, measured, labeled and stored in a brown bottle labelled.

3.2.5 Determination of the percentage yield of the Oils

The percent yield of each of the oil was determined using the formula

$$\text{Essential oils yield (\%)} = W_1 / W_2 \times 100$$

W_1 = net weight of oils (grams)

W_2 = total weight of fresh leaves (grams)

3.2.6 Phytochemical Studies of the Essential Oils

The following test were carried out to detect the presence of some active chemical constituents like alkaloids, tannins, glycosides, Flavonoids, terpenes, saponins and carbohydrates .

3.2.6.1 Alkaloids

Mayer's Test:

Few drops of Mayer's reagent (Potassium Mercuric Iodide) were added to the extract. Formation of a yellow cream coloured precipitate indicates the presence of alkaloids (Siddiqui and Ali, 1997).

3.2.6.2 Carbohydrates

Fehlings test

Fehling test was conducted by adding 2m1 of extract, 5 ml of a mixture of Fehlings solution A and B in the ratio of 1:1 and the mixture allowed to boiled for 5 minutes. A brick-red precipitate indicates the presence of free reducing sugar (Trease and Evans, 1989).

3.2.6.3 Cardaic Glycosides

Kella-Killiani Test for cardiac glycosides

To 2ml extract glacial acetic acid, few drops of 5% $FeCl_3$ and concentrated sulphuric acid was added. Formation of reddish brown color at the junction of the two liquid and upper layer appears bluish green indicates the presence of glycosides (Trease and Evans,1989).

3.2.6.4 Saponins

Frothing test for saponins

150mg of **e**xtracts were diluted with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins (Siddiqui and Ali, 1997).

3.2.6.5 Terpenes

To 2ml of extract 5ml chloroform and 2ml concentrated sulphuric acid ($H_2SO_{4)}$ was added. Reddish brown coloration of interface indicates the presence of terpenes (Harbon, 1973)

3.2.6.6 Flavonoids

Sodium hydroxide (NaOH) test for flavinoid

Few drops of aqueous NaOH were added to 5m1 of BM extract, a yellow coloration was observed indicating the presence of flavinoid (Trease and Evans, 1989).

3.2.6.7 Tannins

Ferric chloride test

1ml of 5% $FeCl_3$ is added to the extract presence of tannin is indicated by the formation of bluish black or greenish black precipitates (Siddiqui and Ali, 1997).

3.2.6.8 Antraquinones Derivatives

Modified Borntrager's test for combined anthracene

Sample was boiled with 5 ml of 10 % hydrochloride acid for 3 minutes. This will hydrolyze the glycosides to yield aglycones which are soluble in hot water only. The solution was filtered hot. The filtrate was cooled and extracted with 5 m1 of benzene. The benzene layer was filtered off and shaken gently with half volume of 10 % ammonia solution. A rose-pink or a cherry red color indicates combined anthracene (Trease and Evans, 1989).

3.2.7 Physical Properties of the Essential Oils

3.2.7.1 Specific gravity (SG)

An improvised specific gravity bottle was washed, rinsed with acetone and dried in the oven. The bottle was cooled at room temperature in a Desiccators and the weight of the empty bottle determined using an electronic weighing balance. The weight of the bottle filled with water was recorded. Then the water poured out and rinsed with acetone and dried in the oven. The same procedure was repeated with the oil and the specific gravity was calculated as follows:

$$\text{Specific gravity} = \frac{W2-W1}{W3-W1}$$

Where W1=weight of empty container

W2= weight of bottle + oil

W3=weight of bottle +water

3.2.7.2 Density (S)

The weight of a density bottle was determined using an electronic weighing balance. The bottle was then filled to the brim with oil and the weight of the bottle and the oil determined. This procedure was repeated for each of the oil and the density calculated using the formula:

$$\text{Density} = \frac{W2-W1}{V}$$

Where W1=weight of empty bottle

W2= weight of bottle + oil

V = volume of oil

3.2.7.3 Free Fatty Acid (FFA)

The method use for the determination was that of British standard institute no 684

1g of the oil was placed in a 250 conical flask and warmed. 25 ml of methanol was added and mixwd well, followed by 2 drops of phenolphthalein indicator and a drop of 0.1m NaOH solution. The contents were then titrated with 0.1M NaOH solution until a light pink color which persisted for 1 minute was seen. The end point was recorded and used to calculate as follow:

$$\text{FFA} = \frac{\text{titre} \times M \times 28.2}{\text{Weight of sample}}$$

Where M = molarity of base

3.2.7.4 Acidic Value (AV)

1g of the oil was placed a 250 conical flask and warmed. .25ml of methanol was added with through stirring followed by 2 drops of phenolphthalein indicator and a drop of 0.1m

KOH solution. The contents were then titrated with 0.1M KOH solution until a light pink color which persisted for 1 minute was seen. The end point was recorded and used to calculate the AV as:

$$AV = \frac{\text{titre x M x 28.2}}{\text{Weight of sample}}$$

Where M = molarity of base.

3.2.7.5 Saponification Value (SV)

The method used was that of the British Standard Institute 1995. 2g of the oil was place in a 250ml conical flask and 25ml of 0.5M ethanol potassium hydroxide solution was added. A reflux condenser was attached and the flask content refluxed for 30 minute on a water bath while swirling until it simmered. The mixture was then titrated against 0.5M HCl using phenolphlalein indicator while still hot. A blank determination was also carried out under the same condition and the Saponification value calculated as :

$$SV = \frac{(B-S) \times 28.05}{W}$$

Where B = titre value of blank

S = titer value of sample

W = weight of oil

3.2.7.6 Iodine Value (IV)

Hamus method of association of analytical chemist 1975 was used for the determination of the iodine value.

1g of the oil was place in a 250ml conical flask followed by 30ml Hamus solution and the flask Stoppard. The content was mixed and place in the drawer for exactly 30 minute. It was then titrated against 0.1N $Na_2S_2O_3$ until the solution became light yellow, 2ml of 1%

starch indicator was added and the titration continued until the blue color just disappeared. A blank determination was also carried out under the same condition and the I.V calculated as:

$$IV = \frac{(B-S) \times 12.69 \times N}{W}$$

Where B = titre value of blank

S = titer value of sample

N = normality of $Na_2S_2O_3$

W = weight of oil

3.2.7.7 Pour point (PP)

An improvised thermocouple method was used for the determination. The cylindered test tube was filled with the oil to a specific level (5ml) and clamped with a wooden clamp bearing the thermometer. The sample was then allowed to cool below 0% in the ice/salt both. At this point it was removed and tilted on the clamp and the set up observed at interval. The lowest temperature at which the oil was observed to flow was recorded as the pour point.

3.2.7.8 Cloud Point (CP)

An improvised method was used for this determination. The cylindered test tube was filled with the oil to a 5ml level and clamped with a wooden clamp bearing the thermometer. The test tube was placed in the ice/salt bath and the set up inspected at intervals for cloud formation. The temperature at which a distinct cloudiness appeared at the bottom of the test tube was observed and recorded as the cloud point.

3.2.7.9 Flash Point (FP)

20ml each of the oil was poured into a beaker and placed on the pot of the flash point machine. The oil was aspirated through a 20 ml capacity spindle connected to a Bunsen burner and a thermometer. As the machine hose was opened, the oil began to burn giving out yellow flame. The temperature at which the color of the yellow flame turned to blue was noted and recorded as the end point of FP.

3.2.7.10 Viscosity (V)

Using the Oswald type viscometer, 5ml of each oil was aspirated using the smallest spindle. The value displayed on the monitor was noted and recorded as the viscosity of the oil.

3.2.7.11 Refractive index

A refractometer with model RFM311 RFM 340 of Bellingham and Stanley LTD was cleaned and reset with a light compensator (water at 28^0c). A drop of each of the oil was smeared on a clean glass prism of the machine and closed. A light was allowed to pass thorough by putting on of the machine and the displayed reading recorded.

3.2.8 Gas Chromatography–Mass Spectroscopy (GC-MS) Studies of the Oils

The GC-MS of the essential oils was analyzed using a made in Japan Shimadzu QP-2010 instrument at 70 eV and 250^0C GC Column : ULBON HR-1 equivalent to OV -1, fused silica capillary -0.25 mm-50 M with film thickness 0.25µ. The GC-MS was operated under the following conditions: the initial temperature was 60^0C for 5minutes and then heated at the rate of 5^0C per minutes to 250^oC. Carrier gas (Helium) flow was 2ml per minutes. The identification of components was based on composition of their mass

spectra with those present in the National Institute for Standard Technology computer data bank (Adams, 2001; Ladan *et al.,* 2011).

3.3 EXPERIMENTAL ANIMALS

A total number of 20 adult male Wistar rats were obtained from the Department of Pharmacology, Ahmadu Bello University Zaria. The animals were housed in plastic cages in the animal house of the Department of Human Anatomy, Ahmadu Bello University Zaria. The animals were between the ages of six to seven weeks and weighed between 130g-180g. The animals were kept and maintained on standard laboratory condition of room temperature, humidity and under twelve hours dark – light cycle. The animals were fed with standard pellet diet and water was allowed adbilitum. The animals were allowed to acclimatize to their new condition for two weeks before the commencement of the experiment. The cages were cleaned daily and washed weekly.

3.3.1 Experimental Protocol

The 20 adult male Wistar rats were randomly grouped into four groups (A, B, C, and D) with 5 animals per group. From each of these animals in a group 5 organs (brain, liver, kidney, small intestine and testes) were taken. From each of these 5 organs in a group, five tissues were taken to give a total of 25 tissues each in a group. All the tissues were assigned numerical values within the groups. For instance all brains were assigned number 1; all the livers as 2; kidney as 3; small intestine as 4; and testes as 5. This means that in group A, the 5 brain tissues were assigned A1; while Liver tissues A2; Kidney tissues A3; small intestine A4; and testes A5 as shown below.

3.3.2 Animals Sacrifice and Fixation

All the animals were anaesthetized by making them to inhale small amount of chloroform until they were unconscious. Their brains, livers, kidneys, small intestines and testes were excised and immediately fixed in neutral buffered formalin for 72 hours before processing. This was done separately for each of the animal from the different study groups and labeled appropriately as shown in Table 3.1 below.

Table 3.1: Showing grouping of the various tissues that form a set

Groups	A	B	C	D
Brain	A1	B1	C1	D1
Liver	A2	B2	C2	D2
Kidney	A3	B3	C3	D3
Small intestine	A4	B4	C4	D4
Testes	A5	B5	C5	D5

The tissues were randomly picked from each group to make up 4 sets. A set represent the different types of tissues from all the groups making a total of 20 tissues in a set. This is represented bellow in Table 3.2:

Table 3.2: Showing grouping of tissue in sets and their clearing time

SETS	TISSUE GROUPS	CLEARING TIME
SET1	A1, A2, A3, A4, A5	15 MINUTES
	B1, B2, B3, B4, B5	
	C1, C2, C3, C4, C5	
	D1, D2, D3, D4, D5	
SET2	A1, A2, A3, A4, A5	30 MINUTES
	B1, B2, B3, B4, B5	
	C1, C2, C3, C4, C5	
	D1, D2, D3, D4, D5	
SET3	A1, A2, A3, A4, A5	1 HOUR
	B1, B2, B3, B4, B5	
	C1, C2, C3, C4, C5	
	D1, D2, D3, D4, D5	
SET4	A1, A2, A3, A4, A5	2 HOURS
	B1, B2, B3, B4, B5	
	C1, C2, C3, C4, C5	
	D1, D2, D3, D4, D5	

A set of tissue from each group were processed, cleared at different time interval in each of the clearing oil, sectioned and stained respectively.

3.3.3 Method of tissue processing

The tissue processing techniques employed were both manual and automated as outlined by Gurr, (1962), Culling (1993), Drury and Wallington (1973), Bancroft and Gamble, (2008) and Kieranan (2010). All the tissues were subjected to the same treatment except for the clearing, where tissues were cleared in the different oil at different time interval. The processing technique is as represented in Figure 3.1 below.

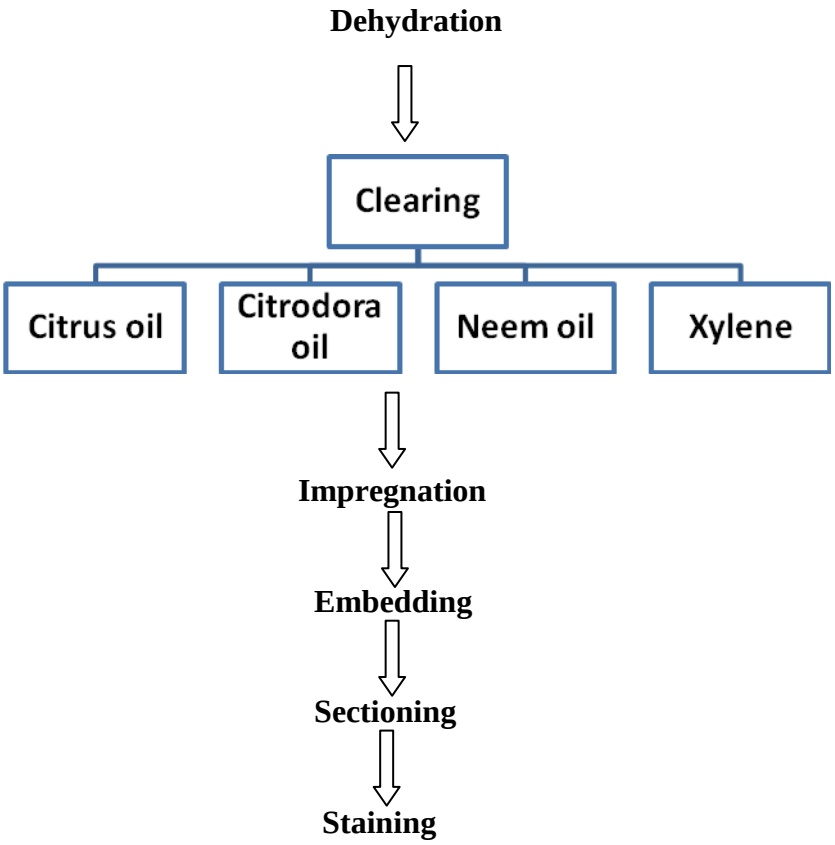


Fig: 3.1 showing format of the processing technique

3.4 TISSUE PROCESSING, EMBEDDING AND SECTIONING PROTOCOL

Five samples of 5mmx5mm x3mm thick from each excised tissues were tagged according to their groups and set. The tissues loaded into four tissue baskets respectively. The tissue baskets were then loaded onto the automatic tissue processor for onward processing, embedding, sectioning before staining as outline in the protocol below.

Table 3.3 showing protocol for tissue processing, embedding and sectioning

Procedure	**Reagent**	**Time (Min)**	**Temprature (^{O}C)**
Fixation	10% Buffered formalin	43200 (72hr)	25
Dehydration	70% propanol	30	25
	80% propanol	30	25
	95% propanol	60	25
	95% propanol	60	25
	100% propanol	60	25
	100% propanol	60	25
Clearing	Citrus oil	15, 30, 60 and 120	25
(for each oil)	Citrodora oil	15, 30, 60 and 120	25
	Neem oil	15, 30, 60 and 120	25
	Xylene	15, 30, 60 and 120	25
Infiltration	Paraffin wax	60	58
	Paraffin wax	60	58
Embedding	Paraffin wax	Not determined	58
Sectioning		Not determined	25
Dewaxing	Citrus oil	10	25
(for each oil)	Citrodora oil	10	25
	Neem oil	10	25
	Xylene	10	25
Rehydration	100% propanol	2	25
	100% propanol	2	25
	95% propanol	2	25
	70% propanol	2	25
	70% propanol	2	25
	Water	Not determined	25

3.4.1 Dehydration

The dehydration was initiated in 70% propanol, progressing through 80%-95% propanol, then two changes of absolute propanoll for 1hour each before proceeding to the clearing stage.

3.4.2 Clearing

Tissues after dehydration were manually transferred into 200 ml of each oil separately in a clean 500ml beakers. The tissues were cleared for 15minutes, 30minutes, 1hour and 2 hours respectively. At the end of 15minutes clearing, the sets of tissue for 15 minutes were removed and transfer for infiltration leaving behind the other sets. The remaining sets were then transfer into fresh beaker of oil for 30 minutes after which they removed and transfer into molten paraffin wax for infilteration. The same process was repeated for other sets according to their various timing. The clearing process and time for clearing is shown below in Figure 3.2 and Table 3.4 respectively.

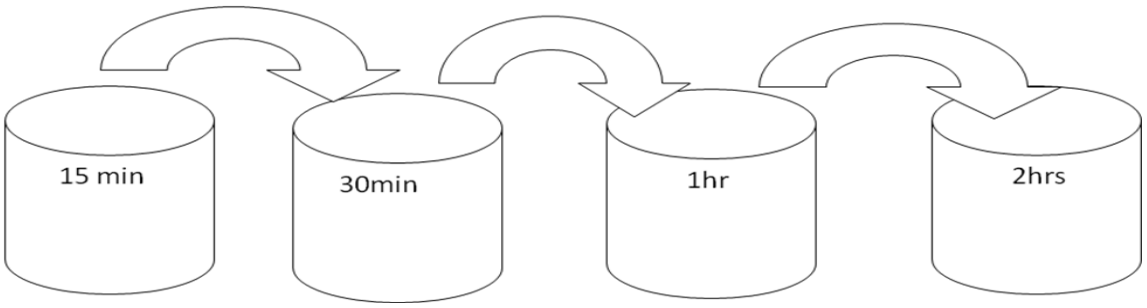


Fig 3.2: showing the process of clearing for each oil

Table 3.4: Showing clearing schedules and Time for clearing

	CITRUS	CITRODORA	NEEM	XYLENE
SET 1	15 minutes	15 minutes	15 minutes	15 minutes
SET 2	30 minutes	30 minutes	30 minutes	30 minutes
SET 3	1hour	1hour	1hour	1hour
SET 4	2hours	2hours	2hours	2hours

3.4.3 Infiltration

Impregnation is the saturation of tissue cavities and cells by a supporting substance which is generally, but not always, the medium in which they are finally embedded.

Tissues were transferred back to the tissue processor for impregnation in a molten paraffin wax for 1 hour each, two changes.

3.4.4 Embedding

Embedding is the process by which tissues are surrounded by a medium such as agar, gelatine, or wax which when solidified will provide sufficient external support during sectioning. Tissues were embedded in paraffin wax by placing them in a mould filled with the molten embedding medium which is then allowed to solidify.

3.4.5 Sectioning

Embedded tissues were sectioned using the rotary microtome at 15 microns for the brain and 5 microns for the others tissues. The ribbons were floated separately on water maintained thermostatically at 55^{o} C in water bath by means of 20% alcohol. The ribbon was separated into individual sections by means of an angled forceps and then mounted on clean slides. The slides were angled to drain and placed on a hot plate maintained thermostatically at 60^{o}C for 30minutes to dry and become strongly affixed to the slide before staining.

3.5 STAINING AND MOUNTING

Different staining techniques were employed, both routine staining and special staining techniques. The routine staining technique used was the haematoxylin and eosin (H and E) method by Bancroft (2008). The tissues were dewaxed with their respective clearing

oils. Citrus oil was used to dewax all the sections cleared with citrus oil, while citrodora oil, neem oil and xylene were all used to dewax the sections respectively according to how they were previously cleared during the processing. After staining the sections were washed, drained off and oven dried at 60^0C without chemical dehydration or xylene clearing, and the mounted with DPX mountant. The special stains used include: Gordon and Sweets, (1936) for demonstrating reticular fibers, Rafferty's Stain (Light Green-Haematoxylin) for general and connective tissue stain, Periodic Acid Schiff for glycogen and Golgi Cox stain for nerve cells. Staining procedures are outlined in Appendix I.

3.6 PHOTOMICROGRAPH

The stained sections were examined microscopically and photomicrographed using the Amscope 3.0 digital camera for microscope and telescope.

3.7 EVALUATION CRITERIA AND METHOD

1. Conventional tissue processing using xylene for clearing and essential oils were run in parallel; the same type of tissues specimen, essential oil and timing were used.

2. Section and staining quality were evaluated by direct microscopic observation and graded (scored) adopted and modified from the works of Kunhua (2012) and Hardisty and Eustis (1990). Excellent (5 scores), Very good (4 scores), Good (3 scores) fair (2 scores) or poor (1 score) in each case, using section folds ,crack and artifacts to score for the section quality and color brightness, nuclear/cytoplasmic contrast for the staining quality.

3.8 AVERAGE TIME, VOLUME AND COST OF THE OILS

The average time to clear tissues in each of the oil was documented. This was obtained by finding the mean of the total time used to clear tissue with each oil. The volume of each of the oil used, the estimated total cost of production for each oil was documented and compared to the time, volume and cost of xylene as conventional clearing agents used in the histology laboratory. Each cost/2.5L of oil was estimated by multiplying 2.5L by the estimated amount and dividing it by 200ml (volume of oil used).

3.9 STATISTICAL ANALYSIS

Data generated from the study were expressed as mean plus or minus (±) standard deviation (SD). Student t test and Analysis of Variance (ANOVA) was used to compare the parameters of these oils with that of the standard xylene. Data was analyzed by SPSS 20.0. P value less than or equal to ($P \leq 0.05$) were considered statistically significant.

CHAPTER FOUR

RESULTS

4.1 RESULT OF PHYTOCHEMICAL SCREENING OF THE OILS

The phytochemical screening showed positive result for carbohydrate in citrus oil, citrodora oil, neem oil and xylene and also showed positive result for glycosides, steroid and tripenes in citrus and citrodora. Absence of anthraquinones, anthracene, saponins, flavonoid, tannins and alkaloid were observed (Table 4.1).

Table 4.1: Result of Phytochemical Screening of the Oils

PHYTOCHEMICAL COMPOUNDS AND TESTS	CITRUS OIL	CITRODORA OIL	NEEM OIL	XYLENE
Fehlings test for carbohydrates	+	+	+	+
Kella-Killiani Test for cardiac glycosides	+	+	-	-
Kadde Test for cardenolides	+	-	-	-
Salkowsk test for steroidal ring	+	+	-	-
Modified Borntrager's test for Antraquinones	-	-	-	-
Frothing test for saponins	+	+	-	-
Sodium hydroxide Test for flavinoid	-	-	-	-
Shinoda Test for Flavinoid	-	-	-	
Ferric chloride test for Tanins	-	-	-	-
Lead sub-acetate test for tannins	-	-	-	
Meyers Test for alkaloids	-	-	-	-
Dragendoff's test for alkaloids	-	-	-	-

KEY: - = negative is absent, + = positive is present

4.2 PHYSICAL PROPERTIES OF THE OIL

In this study it was observed that, neem oil has the highest specific gravity (9.1g/ml), density (0.89 g/ml), free fatty acid (23.4), acid value (37.8), saponification value (187), iodine value (27), flash point (108^{o} C), and viscosity (36%) but has lowest value of pour point (5 ^{o} C) and cloud point (2 ^{o} C) (Table 4.2).

It was also observed that, xylene oil has the specific gravity (8.6 g/ml), density (0.84 g/ml), free fatty acid (10.9), acid value (16.9), saponification value (112), iodine value (19), pour point (35 ^{o} C), cloud point (65 ^{o} C) flash point (39 ^{o} C), and viscosity (0.45%) (Table 4.2).

This study observed that, citrus oil has the specific gravity (8.8.2 g/ml), density (0.80 g/ml), free fatty acid (5.9), acid value (34.4), saponification value (84), iodine value (21), pour point (34 ^{o}c), cloud point (67 ^{o} C) flash point (34^{o}c), and viscosity (0.35%) (Table 4.2).

In this study it was observed that, citrodora oil has the specific gravity (8.3 g/ml), density (0.801 g/ml), free fatty acid (5.6), acid value (11.8), saponification value (98), iodine value (18), pour point (37 ^{o} C), cloud point (69 ^{o} C) flash point (35^{o} C), and viscosity (0.34%) (Table 4.2)

Table 4.2: Results of Physical Properties of The Oil

Parameters	**Citrus Oil**	**Citrodora Oil**	**Neem Oil**	**Xylene**
Color	Colorless	Pale yellow	Dark brown	Colorless
Specific Gravity	8.2g/ml	8.3g/ml	9.1g/ml	8.6 g/ml
Density	0.80 g/ml	0.801g/ml	0.89 g/ml	0.84 g/ml
Free Fatty Acid	5.9	5.6	23.4	10.9
Acid Value	34.4	11.8	37.8	16.9
Saponification Value	84	98	187	112
Iodine Value	21	18	27	19
Pour Point	-34^0C	-37^0C	5^0C	-35^0C
Cloud Point	-67^0C	-69^0C	2^0C	-65^0C
Flash point	34^0C	35^0C	108 ^{0}C	39^0C
Viscosity	0.35cp at 28%	0.34cp at 28%	36cp at 28%	0.45cp at 28%

4.3 REFRACTIVE INDEX OF THE OILS (RF) AT 28 ^{0}C

In the present study neem had the highest refractive index of 1.470 followed by citrodora (1.457) and citrus oil (1.455). When compared to that of xylene (1.496) used in this work, they all have a lower RF, and when compared to that of water (1.33), they have a higher BF as shown in Table 4.3

Table 4.3 Refractive Index of the Oils (RF) At 28 ^{0}c

Sample	Refractive Index
Water	1.335
Citrus oil	1.455
Citrodora oil	1.457
Neem	1.470
Xylene	1.496

4.4 PHYSICAL OBSERVATION OF THE OILS

Color was one of the physical properties observed macroscopically. Color here means the visual perceptual property of the oil. In this study, it was observed that the citrus oil and xylene were colorless; citrodora oil was pale yellow while the neem oil was dark brown. This is shown in Table 4.4 below.

PERCENTAGE YIELD OF THE ESSENTIAL OILS EXTRACTED

The oil from each plant was measured and calculated to get their various yield in percentages as shown in Table 4.4 below. 500g was the quantity of orange peels and eucalyptus leaves to extract 5.3% and 2.2% of citrus oil and citrodora oil. 28g of neem seen was used to extract 7.8% of neem oil.

Table 4.4: Percent (%) of essential oils yielded of the oils used

OIL	QUANTIY (g)	COLOR	YIELD (%)
CITRUS	500	colorless	5.3
CITRODORA	500	Pale yellow	2.2
NEEM	28	Dark brown	7.8

4.5 MEAN TIME, VOLUME AND ESTIMATED COST OF THE OILS

In Table 4.5, it was observed that the mean time for all the oils used in the clearing was 56 minutes as against the 120 minutes of the conventional xylene clearing agent. The same volume of 200ml of each of the oils was used as against the conventional clearing agent of 1000ml recommended. The cost of 2,5liters of xylene is about ₦8600.00 naira. The result showed that the estimated cost for 2.5litres citrodora oil is less (₦3125.00) naira followed by citrus oil (₦8125.00 naira) while neem oil cost more (₦13,250.00 naira).

The total average time was 56minutes in all for the tissues cleared in citrus oil, citrodora oil, neem oil and xylene. 200ml of each of the oil was used at various time intervals while the cost estimate was based on amount spent to extract 200ml of each of the oil. Citrus oil cost about ₦650.00 naira, citrodora oil about ₦250.00 naira and neem was ₦1060.00 naira respectively. The ₦688.00 naira for xylene was obtained by multiplying cost of 2.5liters (₦8600.00 naira) by the volume (200ml) and dividing by 2500ml. This estimate did not include the production cost of ₦11,000.00 charged by NARICT for the three oils.

Table 4.5: showing the amount time, volume and estimated cost of the clearing oils

	Time (Mean/Minutes)	Volume (ml)	Cost (Naira/200ml)	Cost (Naira/2.5L)
Citrus	56	200	650.00	8,125.00
Citrodora	56	200	250.00	3,125.00
Neem	56	200	1060.00	13,250.00
Xylene	56	200	688.00	8600.00

4.6 GC-MS OF THE OILS

The GC-MS results of the citrus oil, citrodora oil, neem oil and xylene analyzed is

Shown in Table 4.6 below:

Table 4.6: Percentage composition of citrus oil, citrodora oil, neem oil and xylene

	Peak No.	Component	%
Citrus oil	1	Dimethyl-3-ethylpentene	7.15
	2	α-pinene	1.92
	3	β-Myrcene	5.34
	4	D-Limonene	**62.23**
	5	β-Linalool	6.42
	6	P-Mentha2,8-dienol	2.81
	7	7-Oxabicyclo (4.1.0) heptane	4.48
	8	Cix-cerveol	5.03
	9	(R)-Carvone	4.60
			100%
Citrodora oil	1	α-pinene	2.40
	2	β -pinene	2.28
	3	Cineole (Eucalyptol)	4.99
	4	Cyclopropane	2.33
	5	Citronellal	**42.20**
	6	Isopulegol	11.76
	7	β-Citronellol	21.71
	8	Citronellol acetate	7.79
	9	Caryophyllene	2.22
	10	Cyclopropane methanol	2.33
			100%
	Peak No.	**Component**	**%**
Neem oil	1	Myristic acid	0.95
	2	Palmitic acid	0.77
	3	Hexadecanoic acid	21.18
	4	Oleic acid	3.59
	5	6-Octadenol	**47.45**
	6	Stearic acid	18.18
	7	9-Octadecenoic acid	7.89
			100%
xylene	1	o-xylene	94%
	2	p-xylene	97%
	3	m-xylene	98%
	4	p-xylene	98%

The GC-MS analysis in Table 4,6 showed the predominant constoituents in citrus oil is D-Limonene with concentration (relative abundance) of 62.23% followed by Dimethyl-3-ethylpentene with relative abundance of 7.15% and β-Linalool6 (42). Other components present have lower relative abundance of 1.9% α-pinene, β-Myrcene (5.34%), P-Mentha 2,8-dienol (2.81%), Oxabicyclo (4.1.0) heptanes (4.48%), and (R)-Carvone (4.60%) respectively. This is shown in the chromatogram of Fig 4.1 below where the highest peak is line 4.

The predominant constituent in citrodora oil is Citronellal with relative abundance of 42.20% followed by β-Citronellol (21.71%), Isopulegol (11.76%) and Citronellol acetate (7.79%). The other components with lower relative abundance include α-pinene (2.40%), β –pinene (2.28%), Cineole (Eucalyptol) (4.99%), Cyclopropane (2.33%), Caryophyllene (2.22%) and Cyclopropane methanol (2.33%) respectively. This can be seen in Fig 4.2 below where the highest peak is in line 5.

The predominant constituent in neem oil is 6-Octadenol with relative abundance of 47.45 % followed by Hexadecanoic acid (21.18%), Stearic acid (18.18%) and 9-Octadecenoic acid (7.89%). The other components with lower relative abundance include myristic acid (0.95%), Palmitic acid (0.77%), and Oleic acid (3.59%) respectively. This is seen in Fig 4.3 below.

The xylene contains 94% of o-xylene, 97% of p-xylene, 98% of m-xylene and 98%p-xylene respectively (Fig 4.4).

4.7 GAS CHROMATOGRAPHY–MASS SPECTROSCOPY (GC-MS) PROFILE

The GC-MS profile is a chromatogram representing the concentration of each constituent present in the oil. The concentration is represented by peaks, and the higher the peak, the more the concentration in the oil. The GC-MS profile for each oil and xylene is shown in the Figures below (Fig.4.1, Fig.4.2, Fig.4.3, and Fig.4.4),

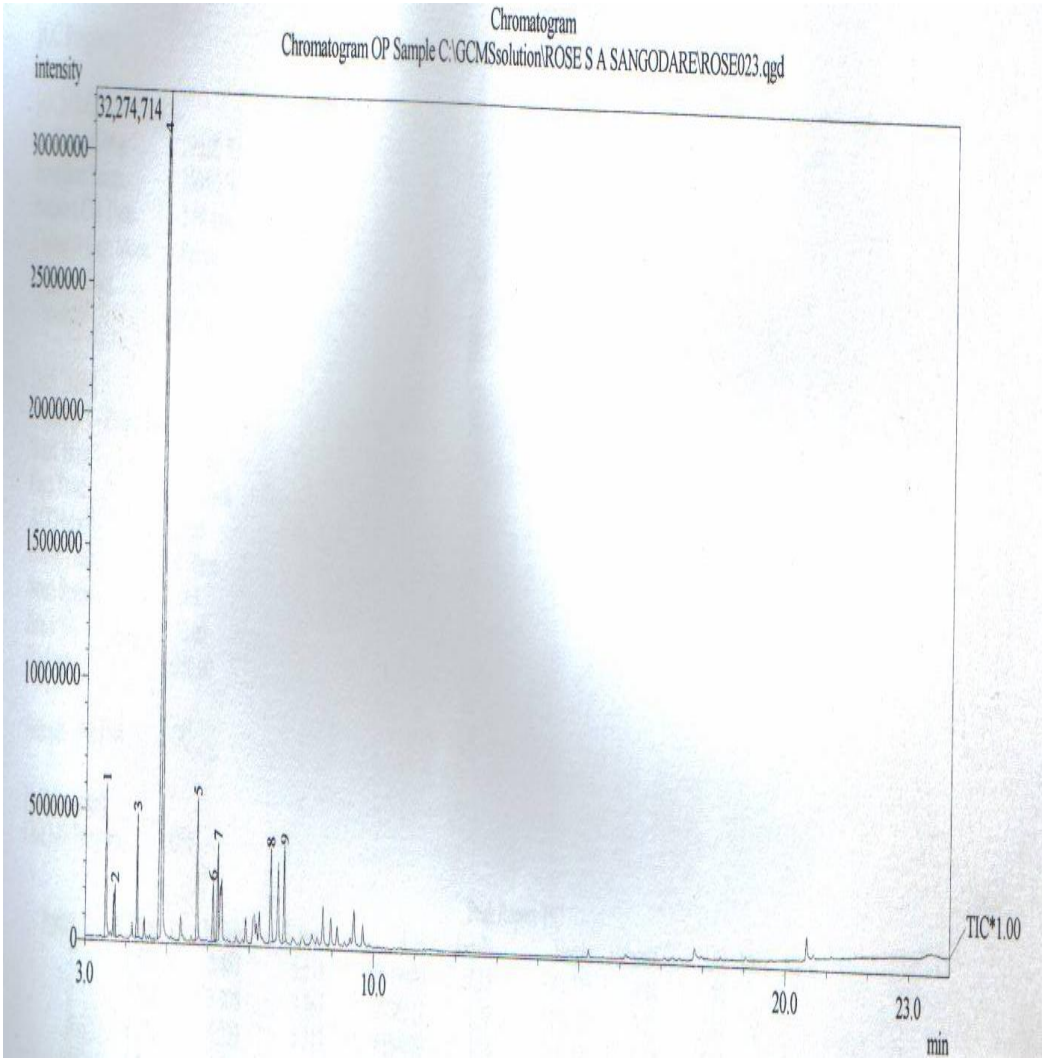


Fig. 4.1 GC-MS profile of citrus oil

Fig. 4.1 GC-MS profile of citrus oil showing the highest peak value at peak 4 which represent D-limonene (62.23%) in Table 4.6.This imply that D-limonene is the highest constituent in the citrus oil.

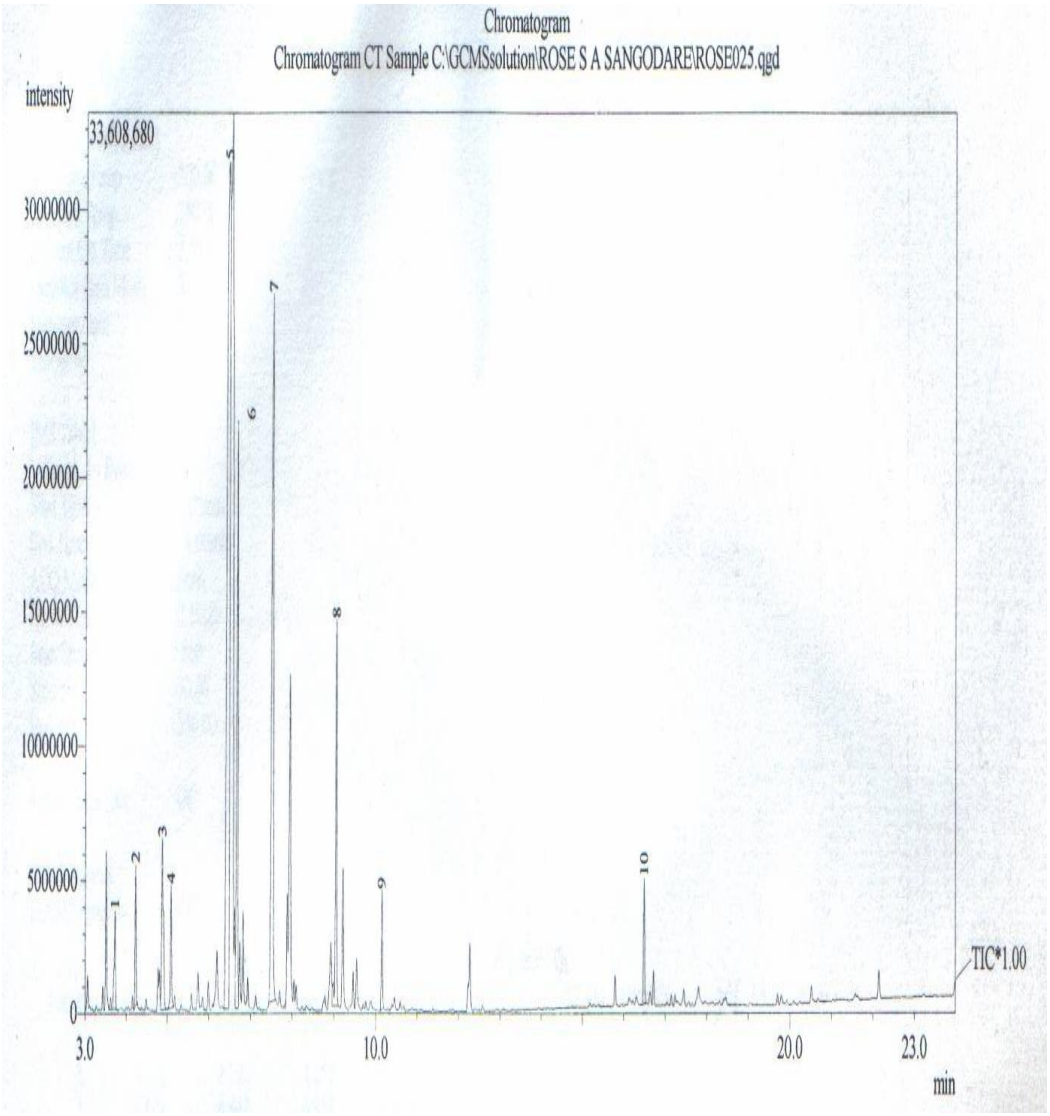


Fig. 4.2 GC-MS profile of Citrodora oil

Fig. 4.2 GC-MS profile of Citrodora oil showing the highest peak at 5 and closely followed by peak 7. Peak 5 represented Citronellal (42.20 %) which was the highest as shown in Table 4.6 meaning that citronellal is the highest constituents.

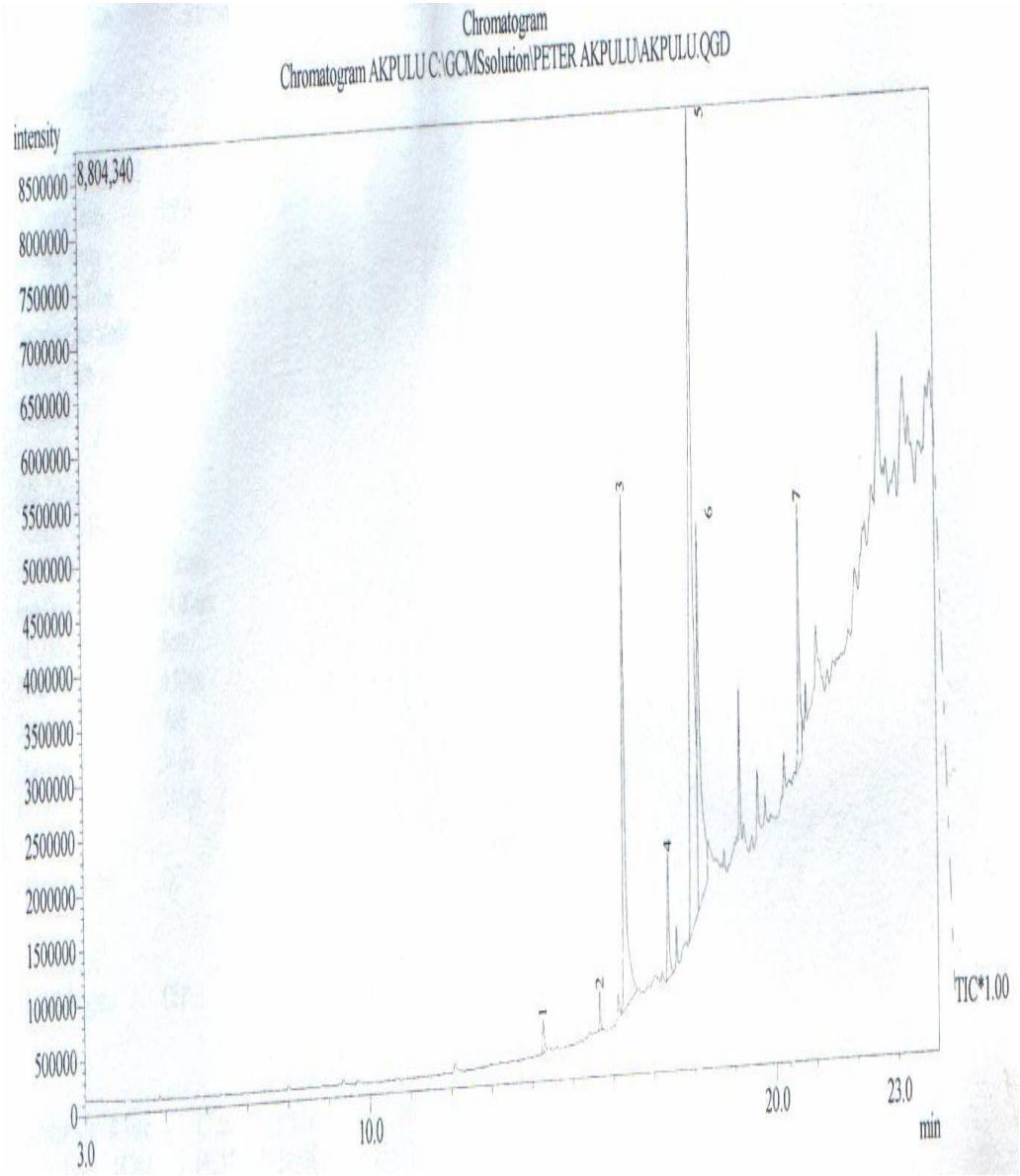


Fig. 4.3 GC-MS profile of neem oil

Fig. 4.3 GC-MS profile of neem oil showing highest peak at 5 which represent 6-Octadenaol of 47.45% to be the highest constituents (Table 4.6). Note the shift of all the peaks toward the right.

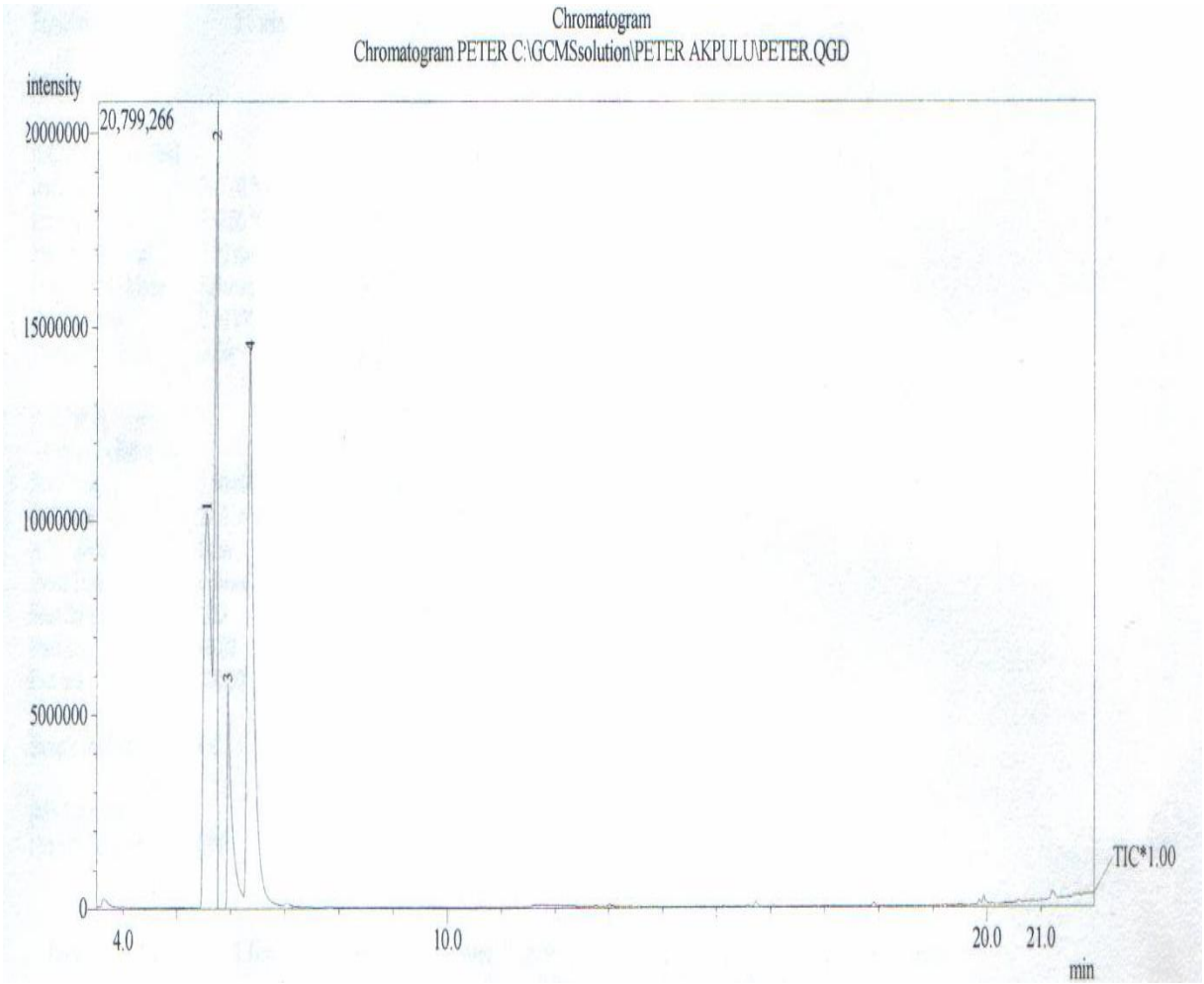


Fig. 4.4 GC-MS of xylene

Fig. 4.4 GC-MS of xylene profile showing peak 2 to be the highest, and this represent p-xylene (97%) as shown in Table 4.6.

4.8 CLEARING EFFECT AND STAINING QUALITY ON SECTIONS

The result from the photomicrographs were studied and analyzed based on time of clearing of the various tissues. The clearing effect of each of the oil on the section quality and the staining quality of each tissue at 15minutes, 30minutes, 1 hour and 2 hours were scored and presented in Table 4.7, Table 4.8, Table 4.9, Table 4.10 and, Table 4.11 below. From the result, kidney sections at 30minutes and intestinal sections at 15minutes had P value less than or equal to 0.05 and were considered statistically significant. All the others were considered statistically insignificant.

Table 4.7: Clearing effect on sections and staining quality of liver

	TIME			
SQ (LIVER)	15 MINUTE	30 MINUTES	1 HOUR	2 HOURS
CIT OIL	1.833±0.408	2.500±0.548	3.667±0.816	3.667±1.033
CTRO OIL	1.500±0.543	2.667±1.211	3.333±1.211	3.500±1.049
NM OIL	1.333±0.516	2.000±0.894	2.500±1.643	3.167±.0753
XYL	1.167±0.408	2.167±0.408	3.500±0.837	4.167±0.753
STQ (LIVER)				
CIT OIL	2.167±0.408	2.667±0.516	3.500±1.049	3.000±0.894
CTRO OIL	1.835±0.753	3.167±0.983	3.500±1.049	3.333±0.816
NM OIL	2.000±0.632	2.000±0.894	2.500±1.517	2.667±1.033
XYL	1.500±0.348	2.833±1.169	3.333±0.816	3.333±0.516

P ≥ 0.05: this indicated there were no statistically significant sections across the groups.

KEY: SQ –Section quality, STQ-staining quality, CIT OIL-citrus oil, CITRO OIL-citrodora oil, NM OIL- neem oil and XYL-xylene.

In Table 4.7, the section and staining quality of liver showed no statistical significant difference across the groups. Tissues cleared in citrus oil at 15minutes showed better section quality and staining ability (with mean increased of 1.833±0.408 and 2.167±0.408) when compared with citrodora, neem and xylene.

The section and staining quality of liver at 30 minutes showed better with citrodora oil having a mean score of 2.667±1.211 and 3.167±0.983, followed by citrus (2.500±0.548) and (2.667±0.516). Neem oil showed the least liver section quality (2.000±0.894) at 30 minutes among the clearing oils used. At 1 hour the section and staining quality of liver gave better result with citrus oil having 3.667±0.816 and 3.500±1.049 followed by xylene and citrodora. Neem had the least section quality (2.500±1.643) and staining quality (2.500±1.517) of liver when compared to the other clearing agents at 1hour. At 2 hours xylene gave better section quality (4.167±0.753) of liver when compared to citrus (3.667±1.033), Citrodora (3.500±1.049) and neem (3.167±.0753). Citrodora oil gave better staining ability (3.333±0.816) than xylene, citrus oil and neem oil.

Table 4.8: **Clearing effect on sections and staining quality of kidney**

	TIME			
SQ (KIDNEY)	15 MINUTE	30 MINUTES	1 HOUR	2 HOURS
CIT OIL	1.667±0.516	3.167±0.408	3.333±1.033	2.833±0.983
CTRO OIL	2.167±0.408	3.000±0.000	3.667±0.816	3.000±0.894
NM OIL	1.500±0.548	1.833±0.753*	2.500±1.517	3.167±0.753
XYL	1.833±1.169	2.833±0.753	3.667±0.816	3.500±0.837
STQ (KIDNEY)				
CIT OIL	1.833±0.408	2.833±0.753	3.667±0.816	2.667±1.211
CTRO OIL	2.333±0.816	3.333±0.516	3.833±0.753	2.833±0.753
NM OIL	1.833±0.408	2.500±0.837	2.500±1.517	2.667±1.033
XYL	2.167±0.753	2.667±0.816	3.667±0.816	3.833±0.753

*** P < 0.003 meaning section of tissues cleared with neem oil was statistically significant when compared to the other groups at 30minutes.**

In Table 4.8 the effect of citrodora oil on kidney section quality (2.167±0.408) and staining quality (2.333±0.816) at 15minutes showed no statistical difference but appeared to be better than citrus oil, neem oil and xylene. The effect of essential oil (citrus, citrodora and neem) and xylene at 30 minutes on the section quality of kidney (1.833±0.753) showed a statistical significant decrease of $P \leq 0.003$ with neem oil cleared tissues when compared with the other oils, even though citrus oil seem to be better (3.167±0.408) at 30minutes. At 1 hour section quality of kidney gives better result with citrodora (3.667±0.816) and xylene (3.667±0.816) followed by citrus (3.333±1.033). Neem has the least section quality (2.500±1.517) of kidney when compared to the other clearing agents. At 2 hours xylene gives better section quality (3.500±0.837) of kidney when compared to citrus (2.833±0.983), Citrodora (3.000±0.894) and neem (3.167±.0753). At 15 citrodora showed good staining quality with mean increased (2.333±0.816) when compared with other clearing agents (neem, citrodora and xylene).We also observed that, at 15 minutes xylene showed better kidney staining quality (2.167±0.753) when compared to citrus and neem (1.833±0.408) and (1.833±0.408. The staining quality of kidney at 30 minutes showed citrodora (3.333±0.516) to be better followed by citrus (2.833±0.753) and xylene (2.667±0.816). Neem shows the least kidney staining quality (2.500±0.837) at 30 minutes among the clearing agents. At 1 hour staining quality of kidney gives better result with citrodora (3.833±0.753) followed by xylene (3.667±0.816) and citrus (3.667±0.816). We observed that neem has the least section quality (2.500±1.517) of kidney when compared to the other clearing agents. At 2 hours, xylene gives best staining quality (3.833±0.753) followed by citrodora (2.833±0.753). Citrus (2.667±1.211) and neem (2.667±1.033) showed the least staining quality.

Table 4.9: Clearing effect on sections and staining quality of B**rain**

	TIME			
SQ (BRAIN)	15 MINUTE	30 MINUTES	1 HOUR	2 HOURS
CIT OIL	1.833±0.753	2.833±0.753	3.000±1.265	2.500±0.548
CTRO OIL	2.667±0.516	3.000±0.632	3.500±0.837	3.333±0.816
NM OIL	2.167±0.408	3.000±0.000	3.167±0.983	3.000±0.894
XYL	2.167±0.753	2.833±0.753	3.333±1.033	3.500±1.225
STQ (BRAIN)				
CIT OIL	2.667±0.816	3.000±0.000	3.167±1.169	2.833±0.753
CTRO OIL	2.167±0.753	3.167±0.408	3.167±1.329	3.333±1.033
NM OIL	2.833±0.408	2.167±1.169	2.167±1.472	2.000±0.894
XYL	3.167±1.169	3.333±1.033	3.833±0.983	3.000±0.894

P ≥ 0.05: this indicated there were no statistically significant section across the groups

In Table 4.9, Brain section and staining quality showed no statistical significant difference across the groups but however, at 15 minutes Citrodora showed better mean value section quality (2.667±0.516) when compared with other clearing agents (Citrus, neem and xylene).We also observed that, at 15 minutes neem and xylene showed the same brain section quality (2.167±0.408 and 2.167±0.753). However, citrus had the least mean value for section quality (1.833±0.753). Staining quality of essential oils (citrus, citrodora and neem) and xylene at 15 minutes showed high mean value of staining quality with xylene (3.167±1.169) when compared with neem (2.833±0.408), citrus (2.667±0.816) and citrodora (2.167±0.753). The section quality of brain at 30 minutes showed good section quality with citrodora and neem (3.000±0.632 and 3.000±0.000). Citrus (2.833±0.753) and neem (2.833±0.753) showed similar mean value of brain section quality at 30 minutes.

The staining quality of brain at 30 minutes showed good stained quality with xylene (3.333±1.033) followed by citrodora (3.167±0.408) and citrus (3.000±0.000). Neem shows the least mean value staining quality (2.167±1.169) at 30 minutes among the clearing agents. At 1 hour section quality of brain mean value of citrodora (3.500±0.837) gives good section quality when compared to xylene (3.333±1.033), neem (3.167±0.983) and citrus (3.000±1.265). At 1 hour staining quality of brain gives good result with xylene (3.833±0.983) followed by citrus and citrodora (3.167±1.329 and 3.167±1.329). We observed that neem has the least section quality (2.167±1.472) of brain when compared to the other clearing agents. Xylene has the highest mean value of section quality (3.500±1.225) of brain at 2 hours when compared to citrodora (3.333±0.816), neem (3.000±0.894) and citrus (2.500±.0548). At 2 hours, citrodora and xylene gives good staining quality (3.333±1.033 and 3.000±0.894) of brain when compared to citrus (2.833±0.753) and neem (2.000±0.894).

Table 4.10: Clearing effect on sections and staining quality of Testes

	TIME			
SQ (TESTES)	15 MINUTE	30 MINUTES	1 HOUR	2 HOURS
CIT OIL	1.667±0.816	3.000±0.632	2.833±1.329	3.333±0.816
CTRO OIL	1.667±0.816	2.667±0.816	3.833±0.753	3.667±1.366
NM OIL	1.667±0.816	1.833±0.983	2.667±1.366	2.167±0.753
XYL	2.167±0.753	2.833±0.408	3.667±1.033	3.833±1.169
STQ (TESTES)				
CIT OIL	2.500±0.837	3.000±0.000	3.000±1.095	3.333±0.816
CTRO OIL	1.833±0.753	2.500±0.548	3.667±0.816	3.500±0.837
NM OIL	1.500±0.837	2.000±0.849	2.833±1.472	2.333±1.033
XYL	2.333±0.516	2.667±0.816	3.667±31.033	3.500±1.049

$P \geq 0.05$: this indicated there were no statistically significant section across the groups

There was statistical significant difference in the effect of essential oil (citrus, citrodora and neem) and xylene (Table 4.10) across the groups. However, at 15minutes, xylene showed better section quality with mean increased (2.167±0.753) when compared with other clearing agents (Citrodora, neem and xylene) which had the same mean score (1.667±0.816). The section quality of testes at 30 minutes showed good section quality with citrus (3.000±0.632) followed by xylene (2.833±0.408) and citrodora (2.667±0.816). Neem showed the least testes section quality (1.833±0.983) at 30 minutes among the clearing agents. At 1 hour the section quality of testes gave good result with citrodora (3.833±0.753) followed by xylene (3.667±1.033) and citrus (2.833±1.329). We observed that neem had the least section quality (2.667±1.366) of testes when compared to the other clearing agents. At 2 hours xylene gave good section quality (3.833±1.169) of testes when compared to citrus (3.333±0.816), Citrodora (3.667±1.366). Neem (2.167±0.753) had the least section quality at 2 hours.

The staining quality of testes tissue cleared with citrus (3.333±0.816) was better at 2 hours than that of citrodora (3.667±0.816), neem (2.833±1.472) and xylene (3.667±31.033)..

Table 4.11: **Clearing effect on sections and staining quality of Intestine**

	TIME			
SQ (INTESTINE)	15 MINUTE	30 MINUTES	1 HOUR	2 HOURS
CIT OIL	1.833±0.408	2.167±0.753	3.833±0.753	3.000±0.894
CTRO OIL	2.333±0.516	2.833±0.753	3.667±0.816	2.833±0.753
NM OIL	1.500±0.548	2.833±0.753	2.667±1.366	2.667±0.816
XYL	1.667±0.816	2.500±1.049	3.667±0.816	3.667±0.816
STQ (INTESTINE)				
CIT OIL	2.667±1.033	2.833±0.983	4.000±0.632	2.667±1.211
CTRO OIL	2.667±0.816	2.667±0.816	4.000±0.632	3.167±1.329
NM OIL	1.167±0.408*	3.000±0.632	2.667±1.366	2.500±1.049
XYL	2.333±1.211	2.667±0.816	3.833±1.169	3.500±1.049

*** $P \leq 0.05$ meaning section of tissues cleared with neem oil was statistically significant when compared to the other groups at 15minutes.**

The section quality showed no significant difference across the groups, it appeared to be better with citrodora (2.333±0.516) at 15minutes when compared to citrus and xylene (1.833±0.408) and (1.667±0.816). Neem showed the least intestine section quality (1.500±0.548) at 15 minutes. The section quality of liver at 30 minutes showed good section quality with citrodora (2.833±0.753) and Neem (2.833±0.753) followed by xylene (2.500±1.049). Citrus oil showed the least intestine section quality (2.167±0.753) at 30 minutes among the clearing agents. At 1 hour section quality of intestine gave good result with citrus (3.667±0.816) followed by xylene (3.667±0.816) and citrodora (3.667±0.816). We observed that neem had the least section quality (2.667±1.366) of intestine when compared to the other clearing agents. At 2 hours xylene gave good section quality (3.667±0.816) of intestine followed by citrus (3.000±0.894), Citrodora (2.833±0.753) and neem (2.667±0.816).

There was a statistical significant difference across the group (p=0.031) with the effect of neem oil (Table 4.11) on the staining ability of intestine at 15 minutes with a mean value of 1.167±0.408. At 15 citrus showed good staining quality with mean increased (2.667±1.033) when compared with other clearing agents (neem, citrodora and xylene).This was followed by citrodora oil (2.667±0.816).

The staining quality of intestine at 30 minutes showed good stained quality with neem oil (3.000±0.6323) followed by citrus oil (2.833±0.983) and citrodora oil (2.667±0.816) and xylene having the same mean. At 1 hour staining quality of intestine gave good result with citrus and citrodora having the same mean (4.000±0.632) followed by neem oil (3.833±1.169). At 2 hours, xylene gave better staining quality (3.500±1.049) followed by citrodora (3.167±1.329) and citrus oil (2.667±1.211). Neem (2.500±1.049) had the least staining quality at 2hours.

4.9 PHOTOMICROGRAPHS OF STAINED TISSUE SECTIONS

From each of the cleared tissues in citrus oil, citrodora oil neem oil and xylene five sections were made. One was stained using the routine Haematoxylin an Eosin stain for demonstrating general tissue structures while the others were stained using special stains which include: Gordon and Sweets, (1936) for demonstrating reticular fibres, Rafferty's Stain (Light Green-Haematoxylin) for general and connective tissue stain, Periodic Acid Schiff for glycogen and Golgi Cox stain for nerve cells as described by Bancroft and Stevens (2010). The stained sections were examined microscopically and photomicrographed as shown in plate 1 to plate 26 below:

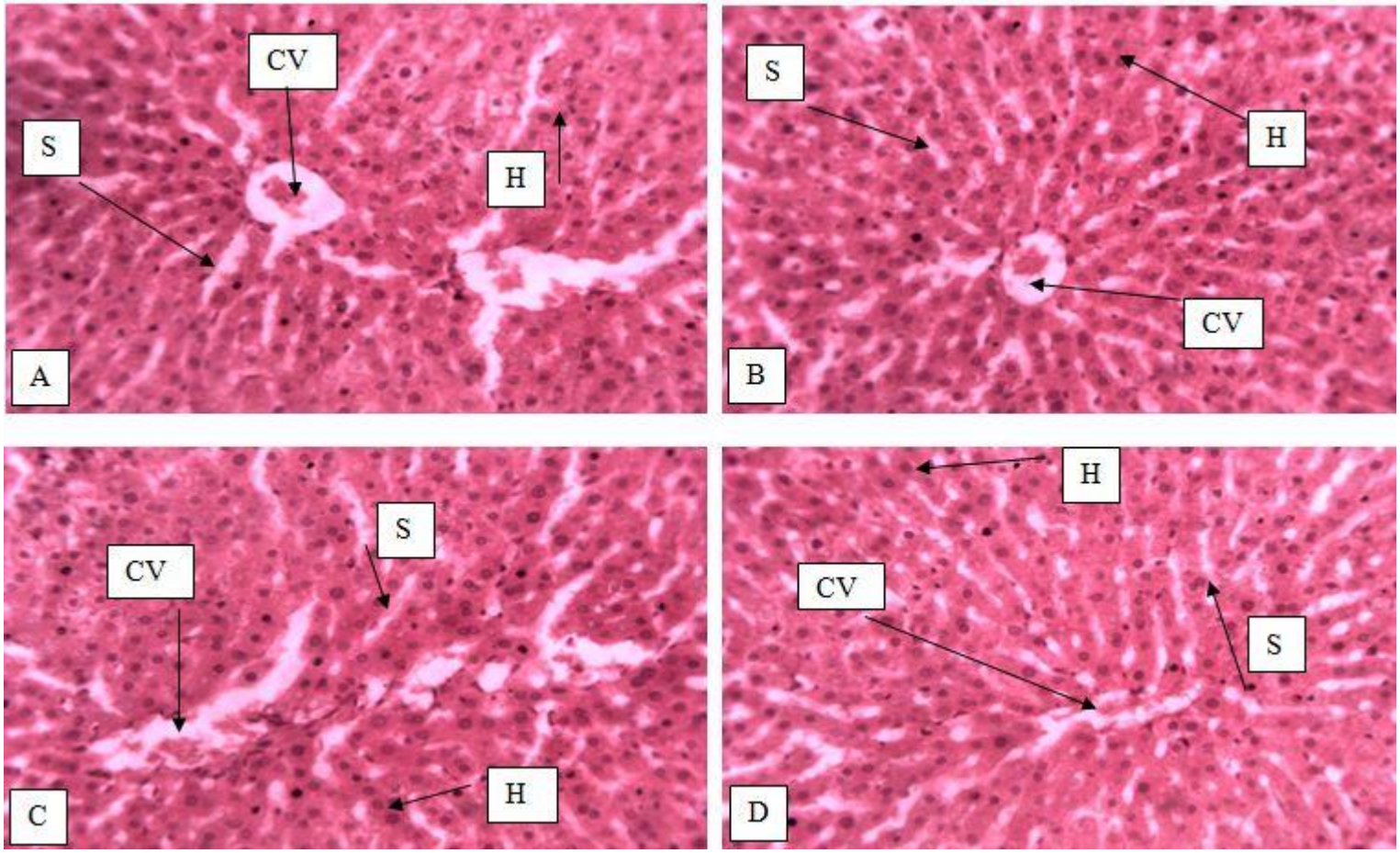


Plate 1: Shows a transverse section of liver tissue cleared for 15minutes with citrus oil (A), citrodora oil (B), neem oil (C) and xylene (D). The section and staining quality appeared good showing clarity of the hepatic cells (H), the sinusoids (S), and the central vein (CV). Neem oil and citrus oil showed more section quality artifacts compared to that of citrodora oil and xylene at 15 minutes (H and E X 100).

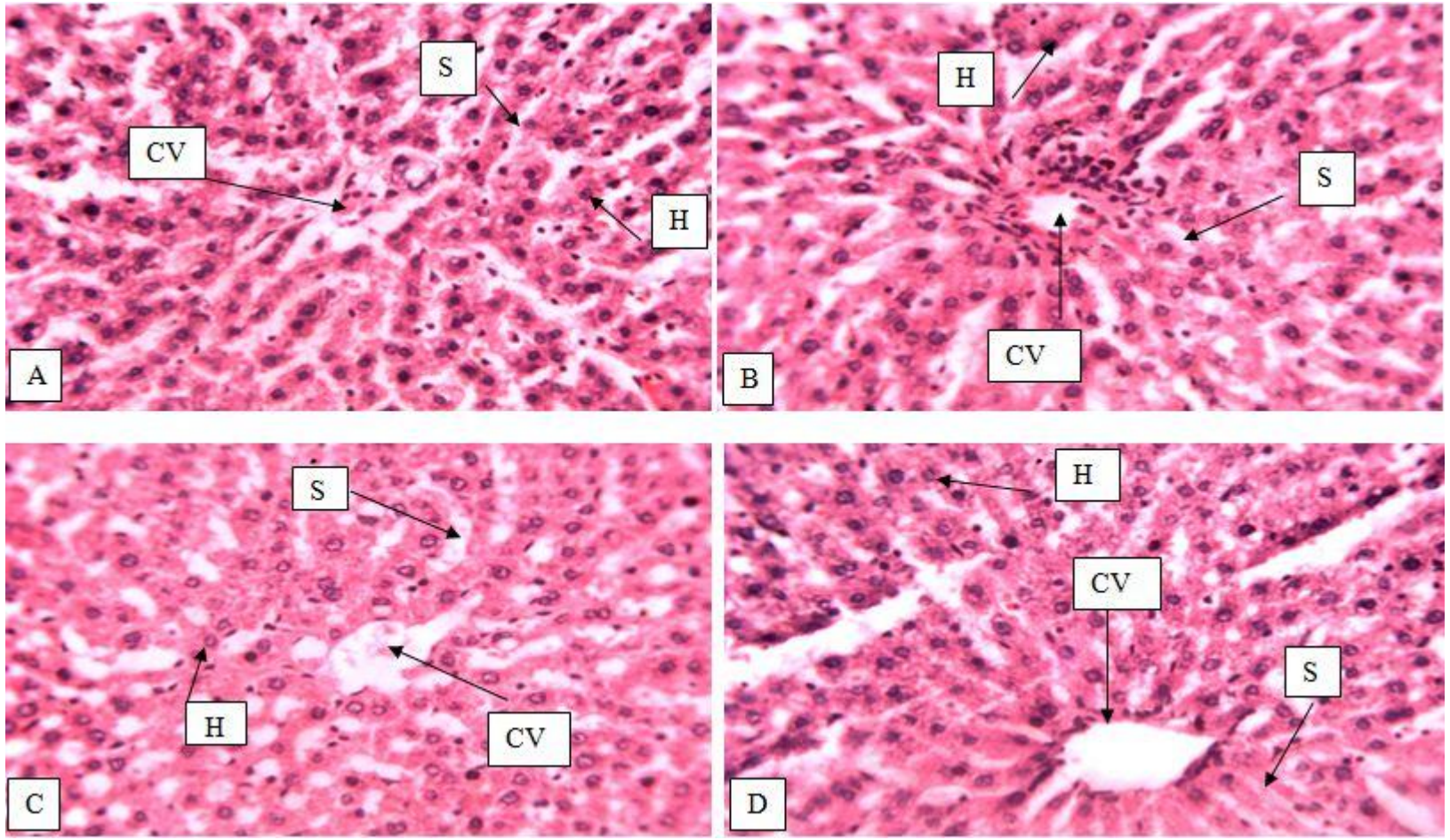


Plate 2: Shows transverse sections of liver tissues cleared for 30 minutes with citrus oil (A), citrodora oil (B), neem oil (C) and xylene (D). The staining quality appeared good showing clearly the hepatic cells (H), the sinusoids (S), and the central vein. There was no difference in histological changes observed in the section quality between the citrus oil (A), citrodora oil (B) and neem oil sections when compared to xylene (D) at 30 minutes (H and E X 250).

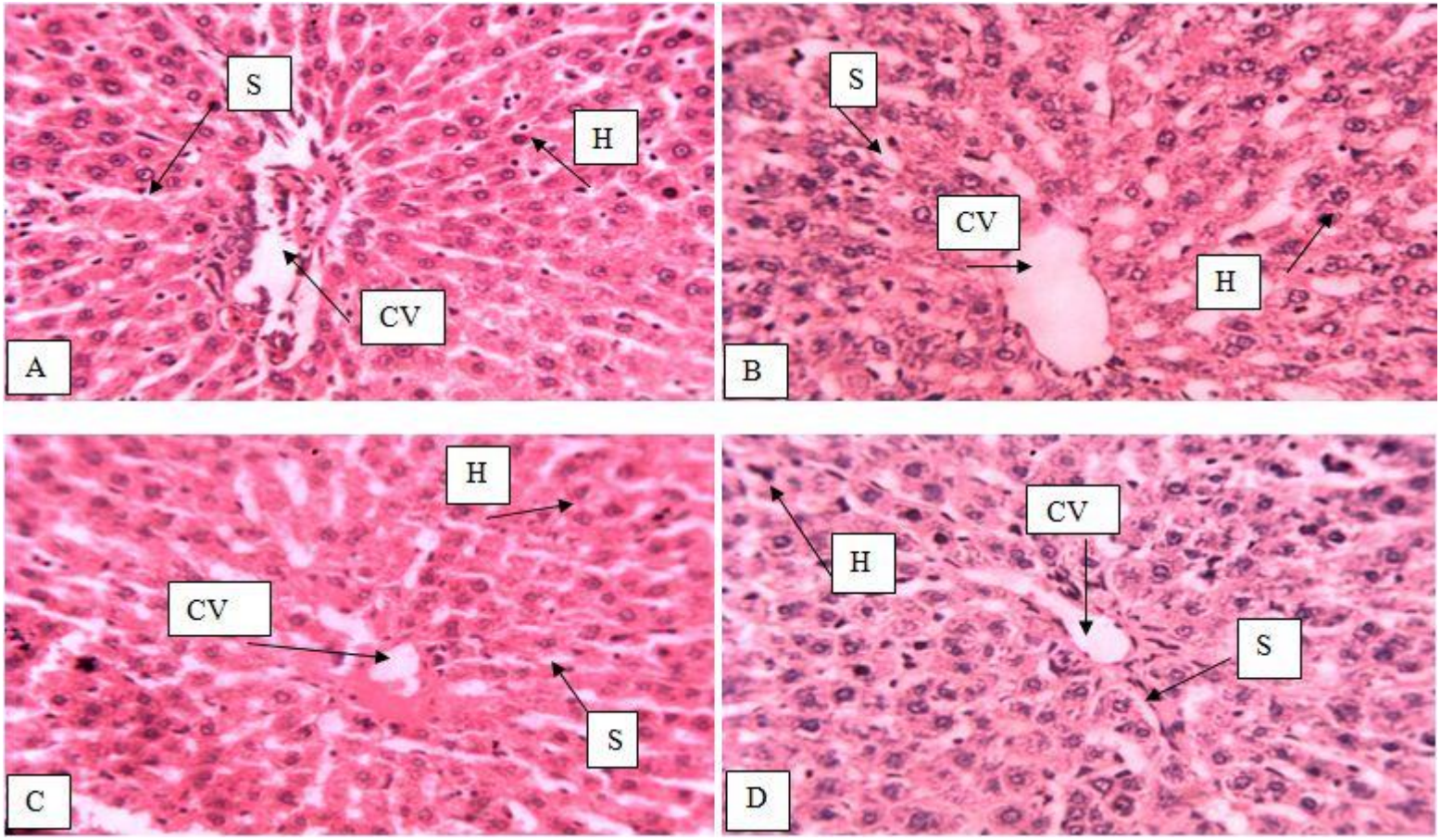


Plate 3: Show a transverse section of liver tissues cleared for 1hour with citrus oil (A), citrodora oil (B), neem oil (C) and xylene (D). The section and staining quality appeared good showing clearly the hepatic cells (H), the sinusoids (S), and the central vein CV). There was no difference in histological changes observed between the tissue sections cleared in all the oils and xylene at 1hour (H and E X 250).

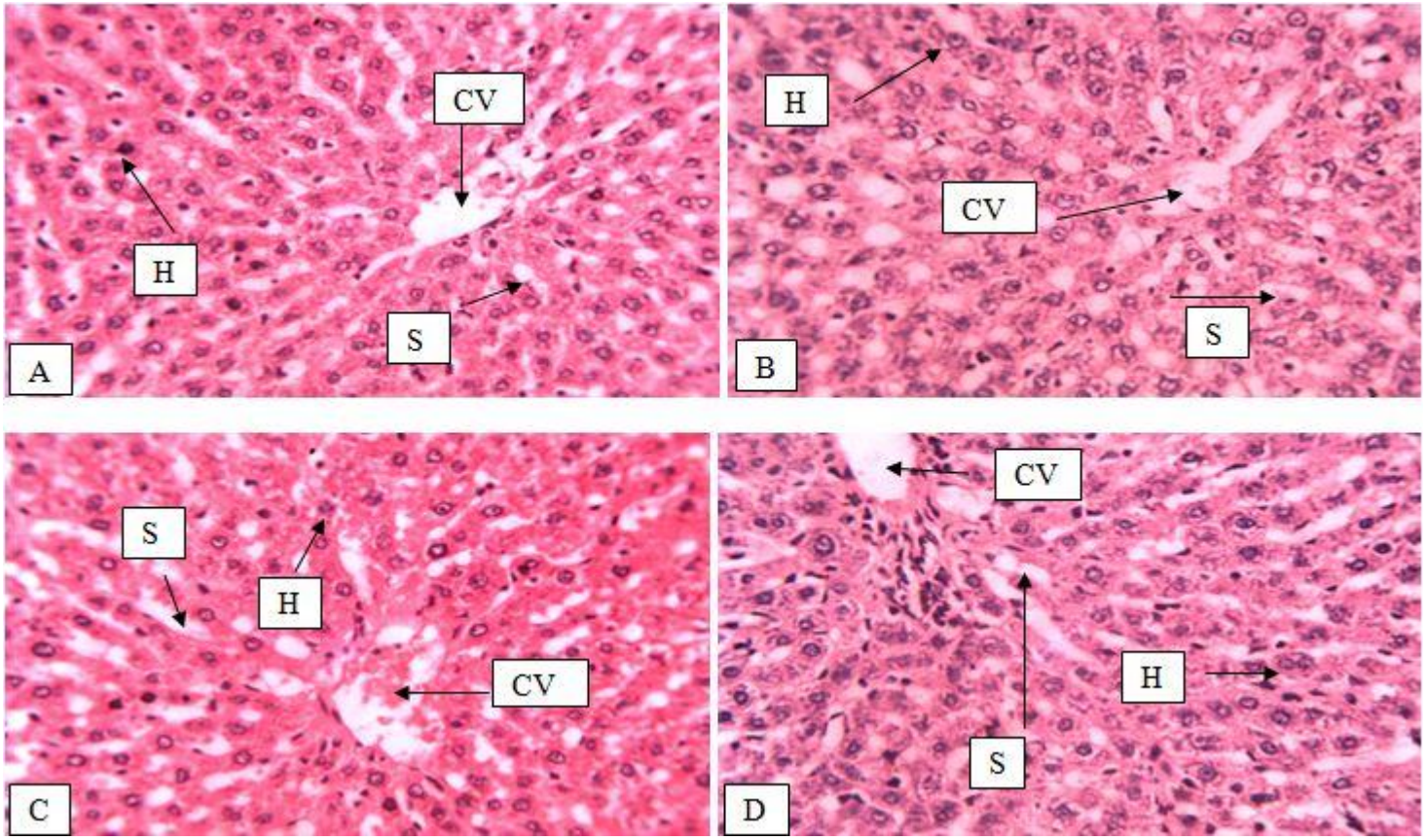


Plate 4: shows transverse sections of liver cleared for 2hours with citrus oil (A), citrodora oil (B), neem oil (C) and xylene (D). The section and staining quality appeared good showing clearly the hepatic cells (H), the sinusoids (S), and the central vein. There was no histological change observed between the citrus oil, citrodora oil and neem oil in section quality when compared to xylene (H and E X 250).

COMPARISON OF KIDNEY SECTIONS CLEARED FOR 15 MINUTES

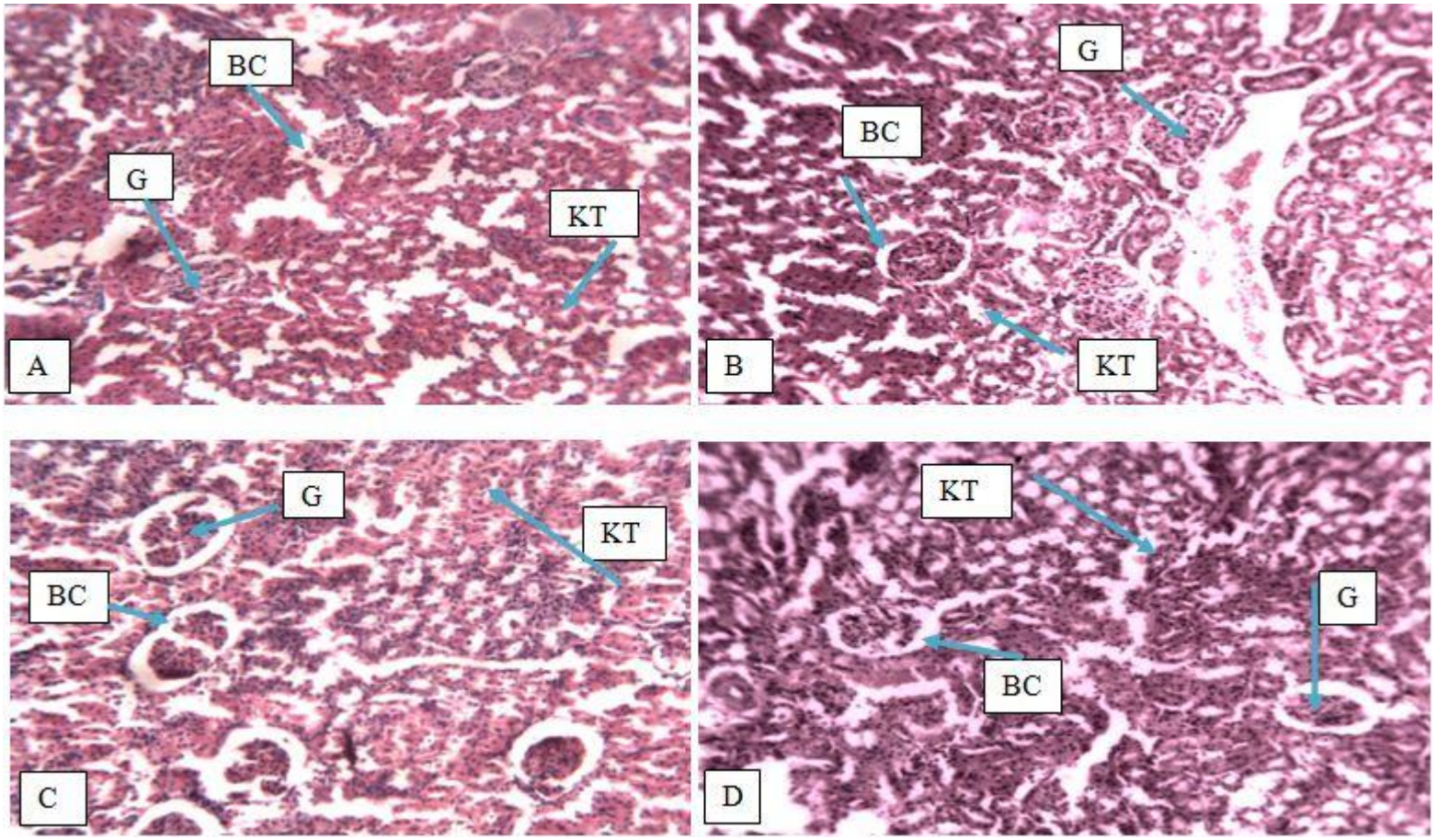


Plate 5:Shows longitudinal sections of kidney tissues cleared for 15minutes with citrus oil (A), citrodora oil (B), neem oil (C) and xylene (D). The kidney tubules (KT), Bowman's capsules (BC) and the glomerulus (G) were not well differentiated because of numerous section artifacts in all the sections. There was no significant changes among the oils and when compared to that of xylene cleared section at 15minutes (H and E X 100).

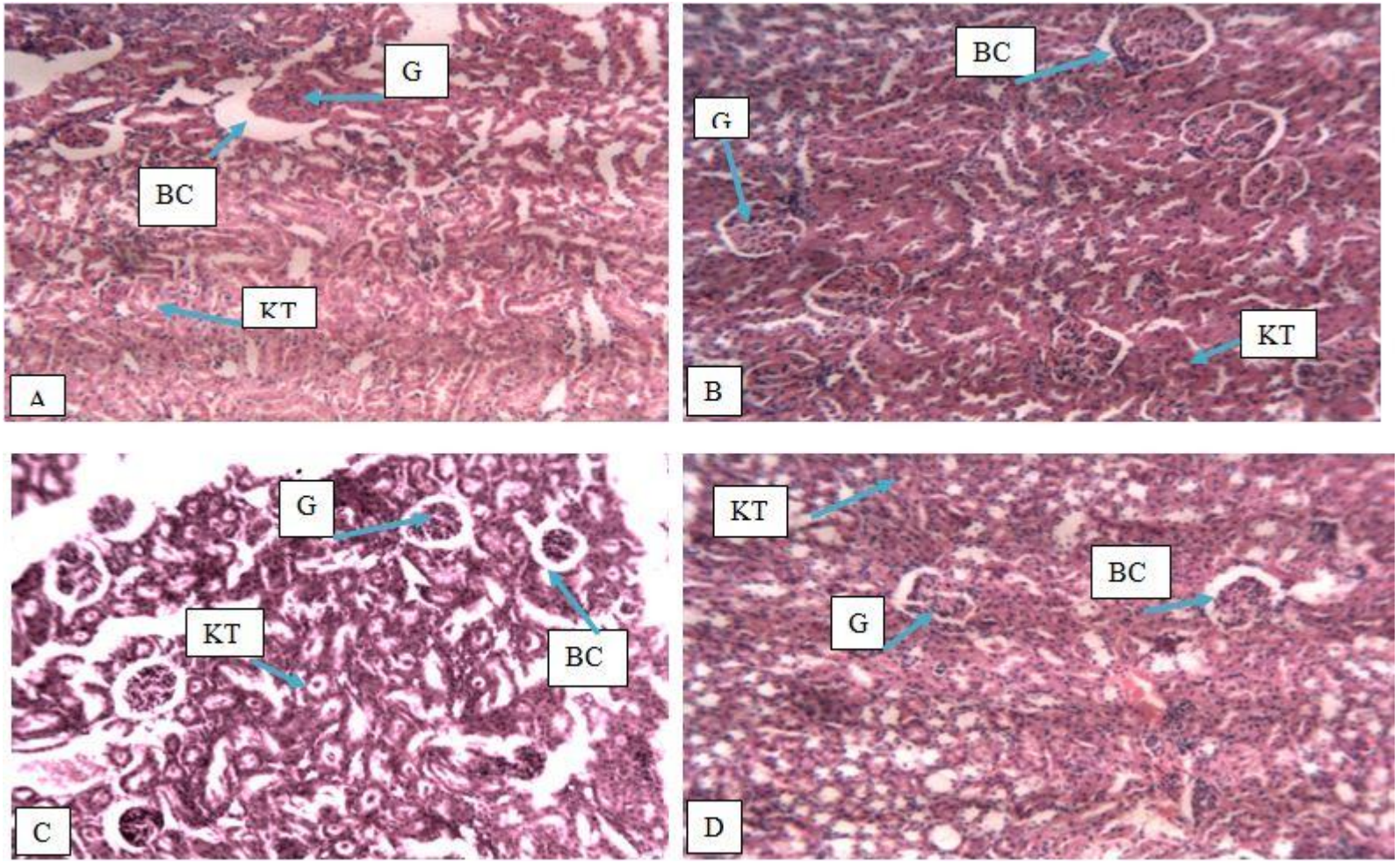


Plate 6: Shows longitudinal sections of kidney tissues cleared for 30minutes with citrus oil (A), citrodora oil (B), neem oil (C) and xylene (D). The kidney tubules (KT), Bowman's capsules (BC) and the glomerulus (G) were well differentiated at 30 minutes. There was significant decrease in the quality of the tissue architectures as can be shown by numerous artficts present in the neem oil cleared section C, when compared to citrus oil, citrodora oil and xylene cleared sections (H and E X 100).

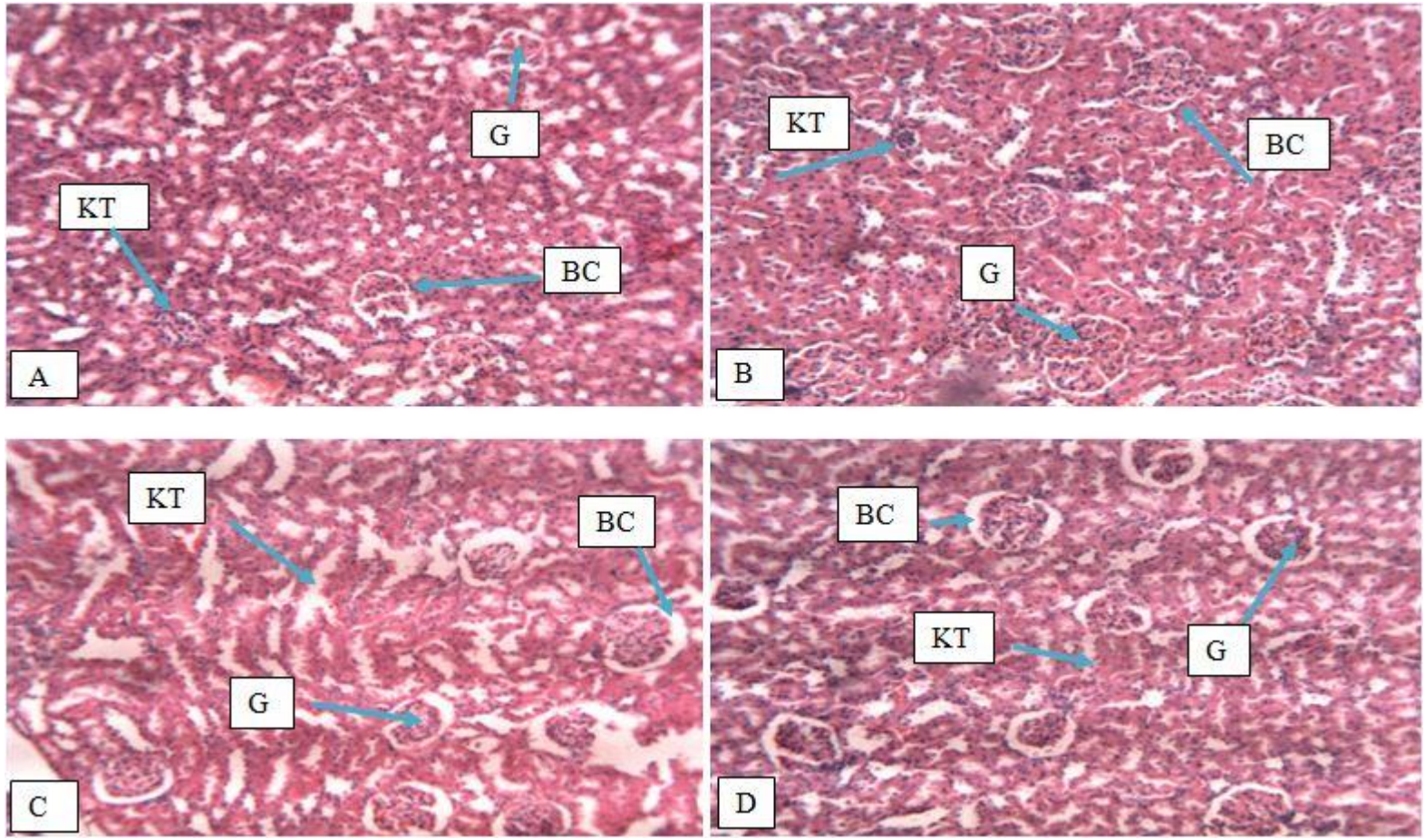


Plate 7:Shows longitudinal sections of kidney tissues cleared for 1hour with citrus oil (A), citrodora oil (B), neem oil (C) and xylene (D). The kidney tubules (KT), Bowman's capsules (BC) and the glomerulus were well differentiated. The section quality of the citrus oil (A) and citrodora oil (B) appeared better than that of neem oil (C) cleared sections when compared to xylene at 1hour (H and E X 100).

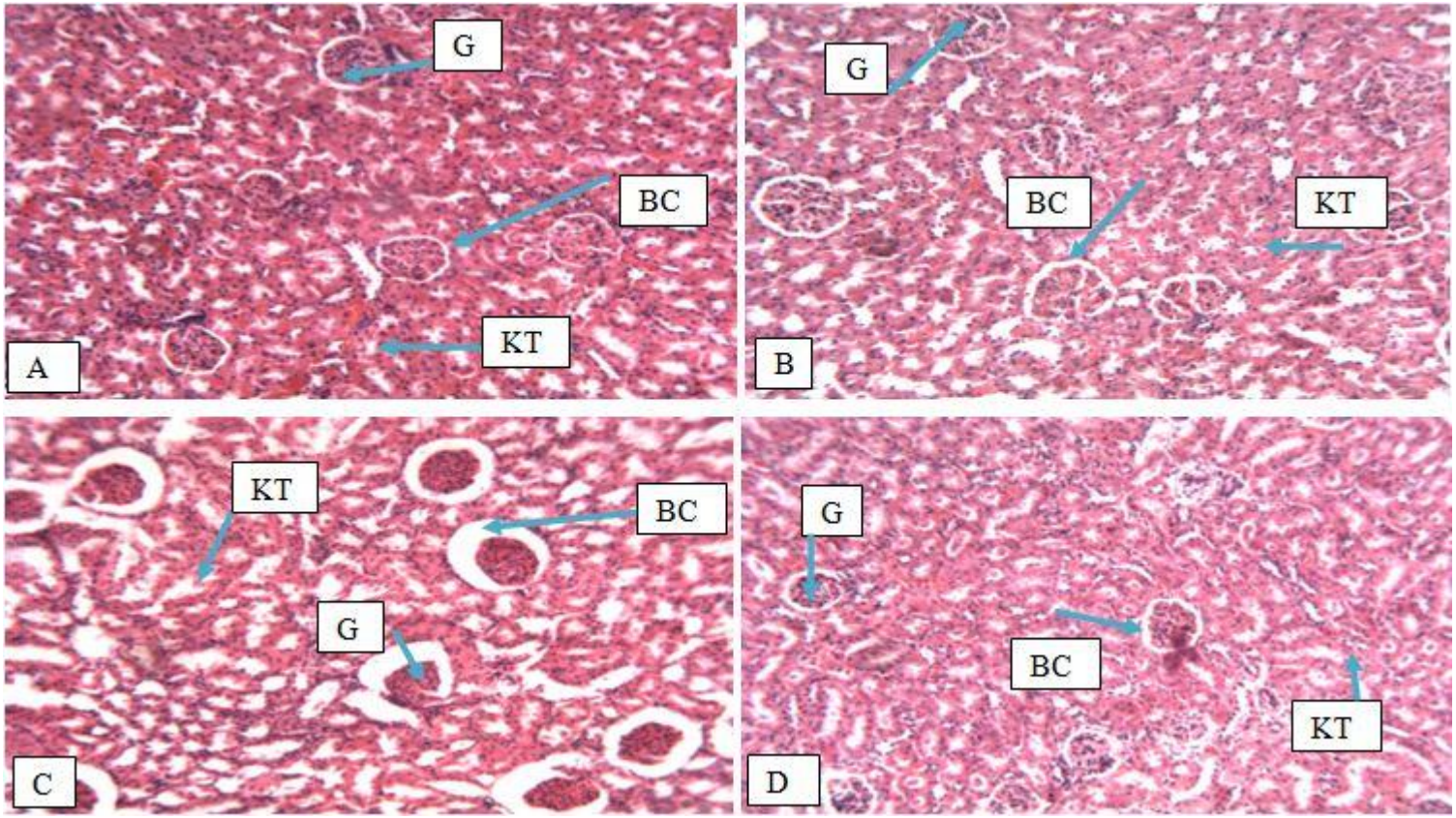


Plate 8: Shows longitudinal sections of kidney tissues cleared for 2hours with citrus oil (A), citrodora oil (B), neem oil (C) and xylene (D). The kidney tubules (KT), Bowman's capsules (BC) and the glomerulus were well differentiated in all the sections. However, there was significant decrease in the quality of the tissue architectures of the neem oil cleared section as can be shown by the shrinkages of the glomerulus from the bowman's capsules when compared to that of xylene which could be as result of longer clearing time of 2 hours (H and E X 100)

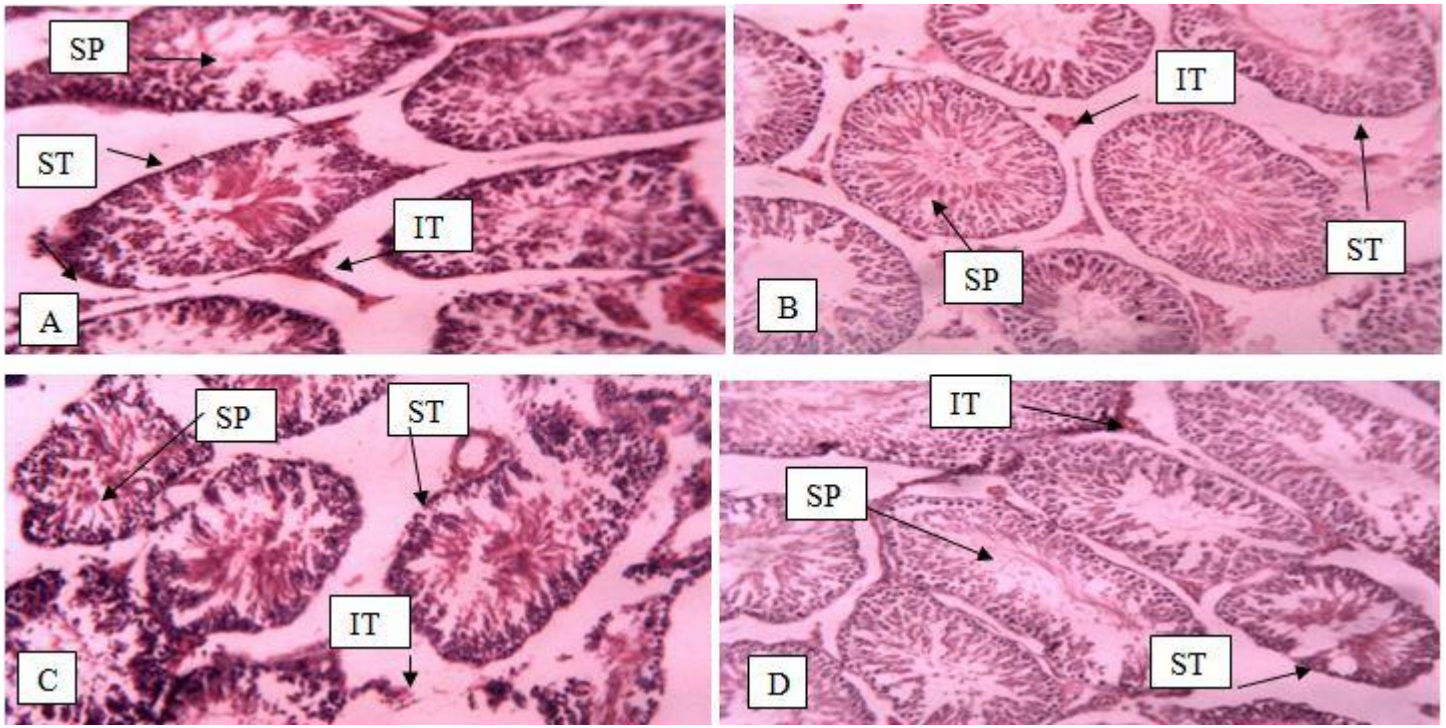


Plate 9: Shows transverse sections of testicular tissues cleared for 15minutes showing poor section and staining quality in the section cleared with citrus oil (A) and neem oil (C) when compared to that of citrodora oil (B), and xylene (D). The interstitial connective tissue (IT) where the Leydig cells are suppose to be found, the seminiferous tubules (ST) and the presence of spermatozoa (SP) in the ST appeared better in the xylene section D and citrodora section B. There was significant decrease in the quality of the tissue architectures of the neem oil (C) and citrus oil (A) cleared sections as can be shown by the absence of interstitial connective tissue (IT) at 15 minutes ((H and E X 100).

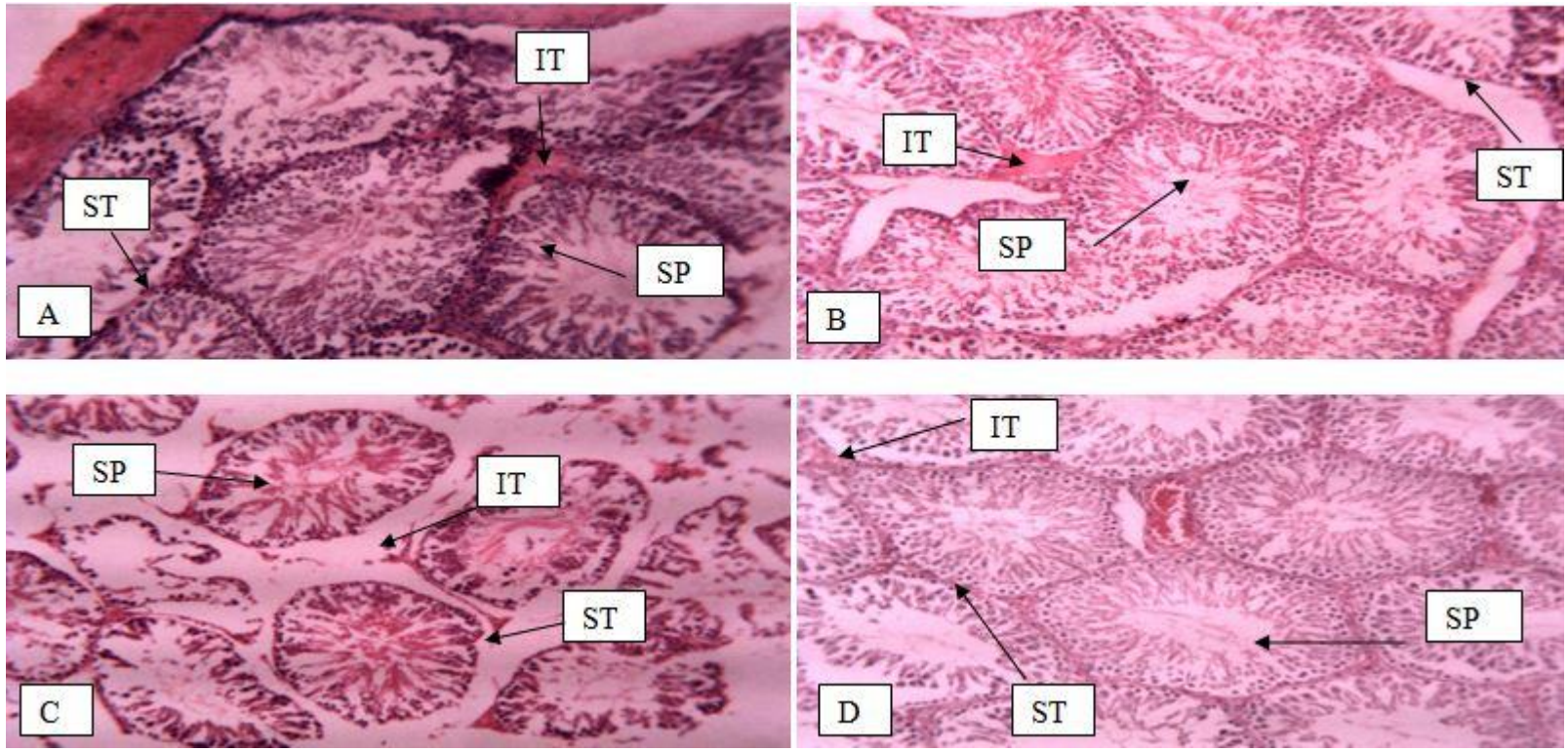


Plate 10:Shows transverse sections of testicular tissues cleared for 30minutes showing poor section and staining quality in the section cleared with citrus oil (A) and neem oil (C) when compared to that of citrodora oil (B), and xylene (D) at 30minutes. There was no significant changes in section and staining quality of the citrodora oil cleared section (B) with the interstitial connective tissue (IT), the seminiferous tubules (ST) and spermatozoa (SP) appearing better than section A and C when compared to xylene cleared section (D) (H and E X 100).

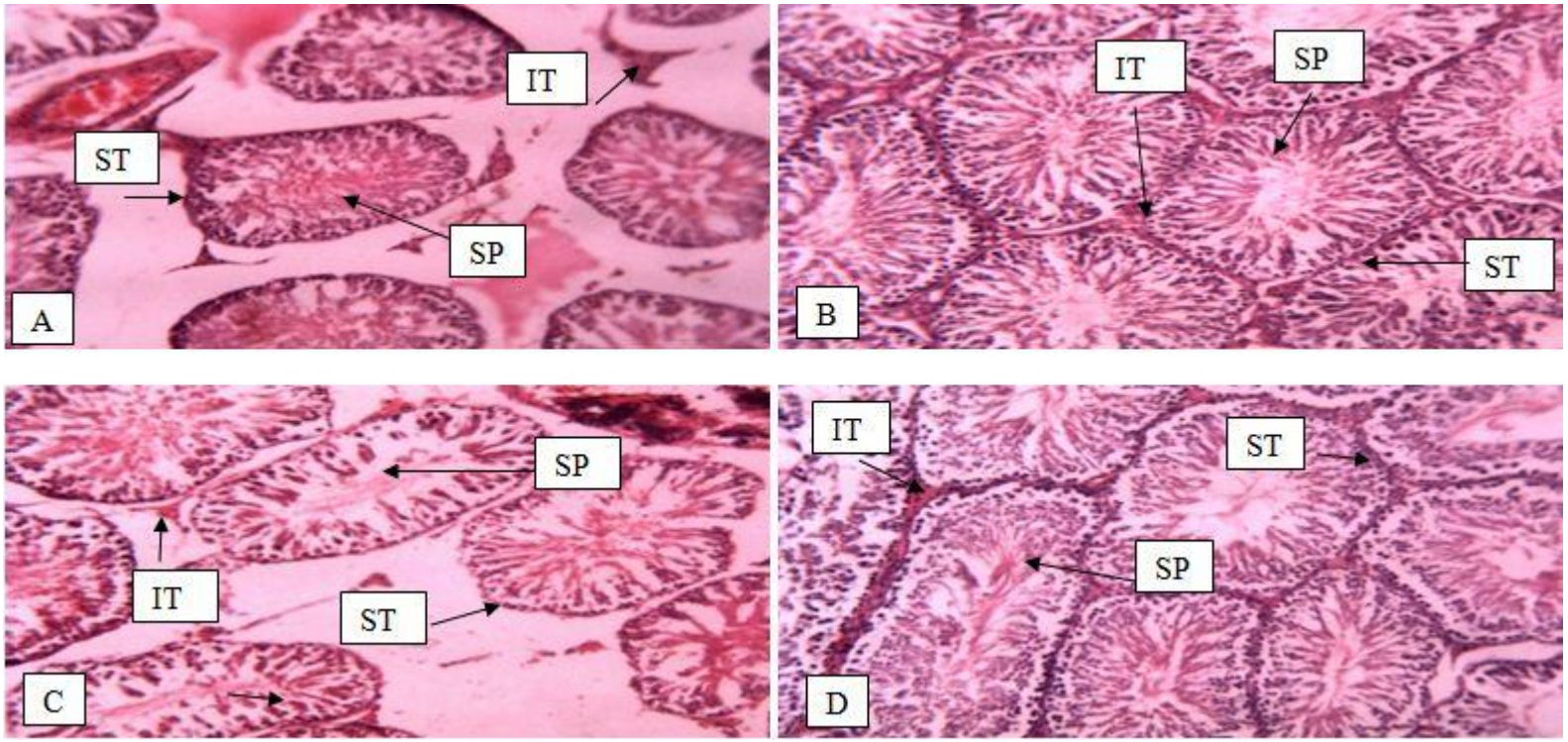


Plate 11: Shows transverse sections of testicular tissues cleared for 1hour. Citrus (A) and neem oil (C) shows poor section quality as evidence by the presence of artifacts when compared to that of citrodora oil (B), and xylene cleared section (D). There was no significant change in section and staining quality between the citrodora oil cleared section (B) and that of xylene (D) at 1 hour as shown by the normal appearance of interstitial connective tissue (IT), the seminiferous tubules (ST) and spermatozoa (SP) (H and E X 100).

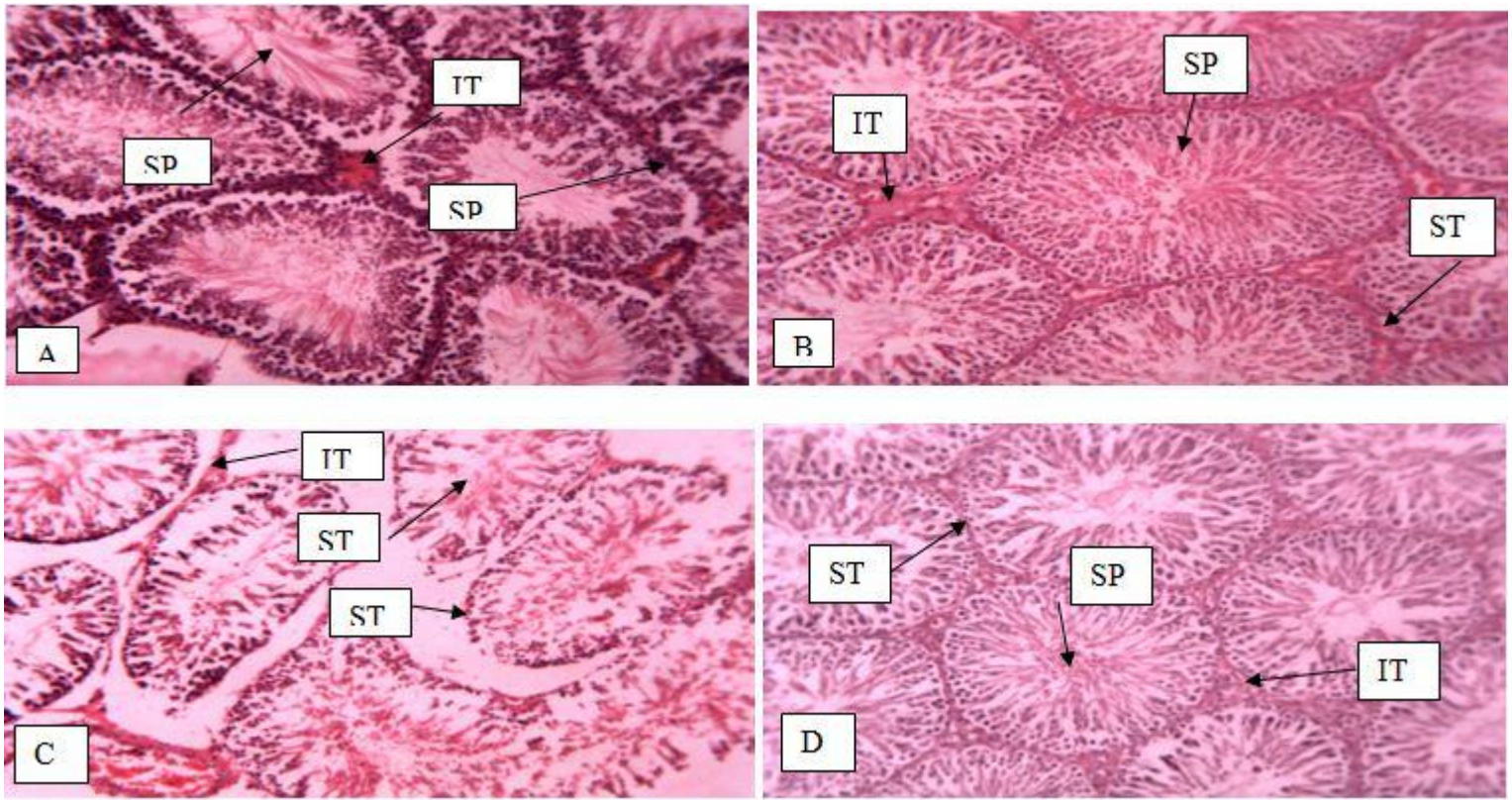


Plate 12:Shows transverse sections of testicular tissues cleared 2hour with citrus oil (A), citrodora oil (B), neem oil (C) and xylene (D). The interstitial connective tissue (IT), the seminiferous tubules (ST) and spermatozoa (SP) in the citrodora oil section (B) and the xylene section (D) appeared better followed by that of the citrus oil section (A). There was many artifacts in the neem oil cleared section (C) with no clear SP and IT differentiation when compared to section A, B and D (H and E X 100)

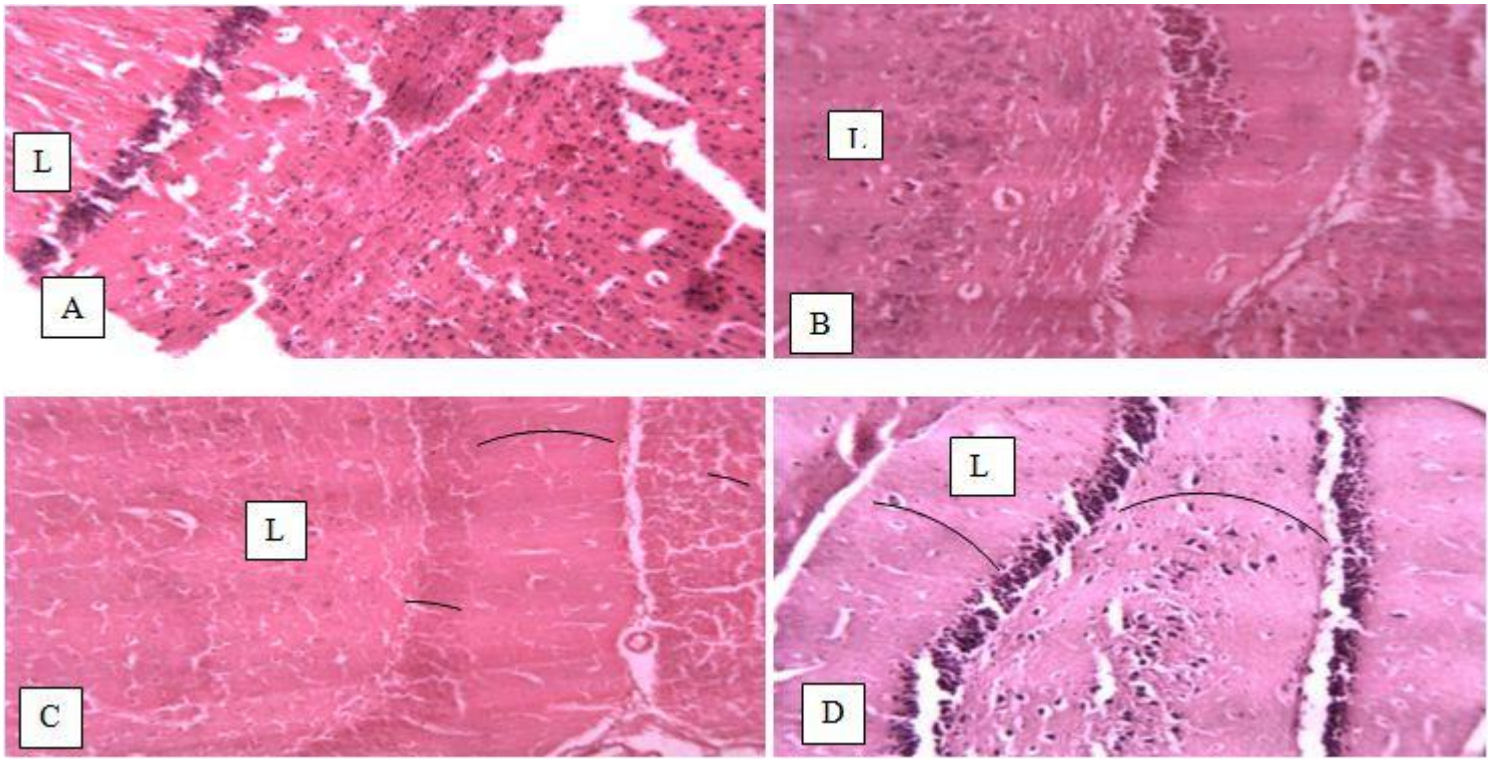


Plate 13: Shows transverse sections of the cerebral cortex cleared for 15minutes with citrus oil (A), citrodora oil (B), neem oil (C) and xylene (D) at 15 minutes. The cerebral cortex layer (L) around the hippocampus region were better differentiated in the citrus oil (A), followed by citrodora section (B), than section (C) when compared to xylene section (D). There was more section quality artifact in the citrus oil section (A) and poor nuclear staining ability in the neem oil section (C) when compared to citrodora oil section (B) and xylene (D) (H and E X 100)

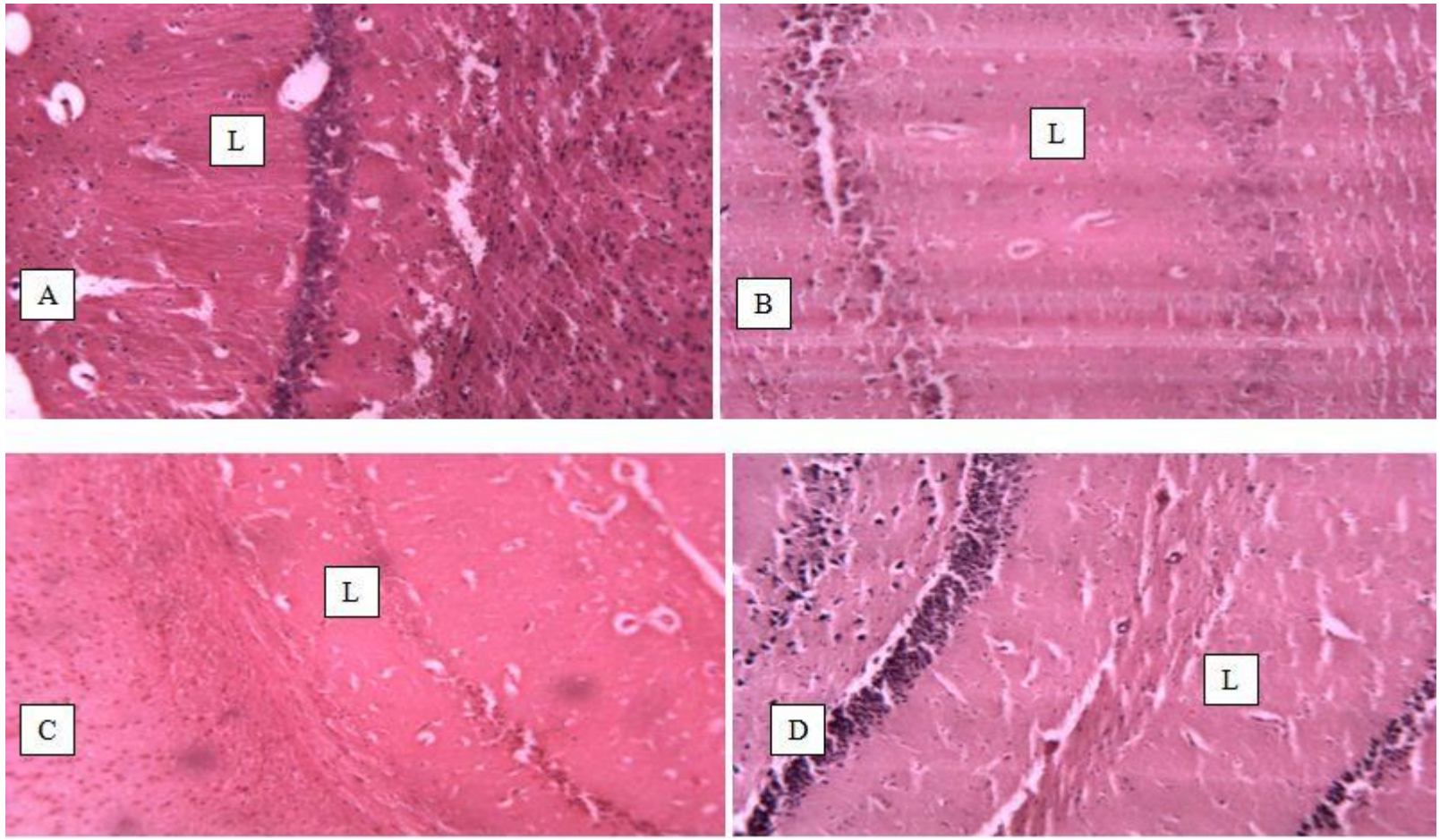


Plate 14: Shows transverse sections of the cerebral cortex cleared for 30minutes with citrus oil (A), citrodora oil (B), neem oil (C) and xylene (D) at 30 minutes. The cerebral cortex layers (L) around the hippocampus region were clearly differentiated by the staining ability of the section cleared with citrus oil (A) and citrodora oil (B) when compared to that of xylene (D). The staining quality of neem oil cleared section C, was poor when compared to xylene cleared section D, which could be due to poor staining ability of neem oil cleared section at 30 minutes (H and E X 100)

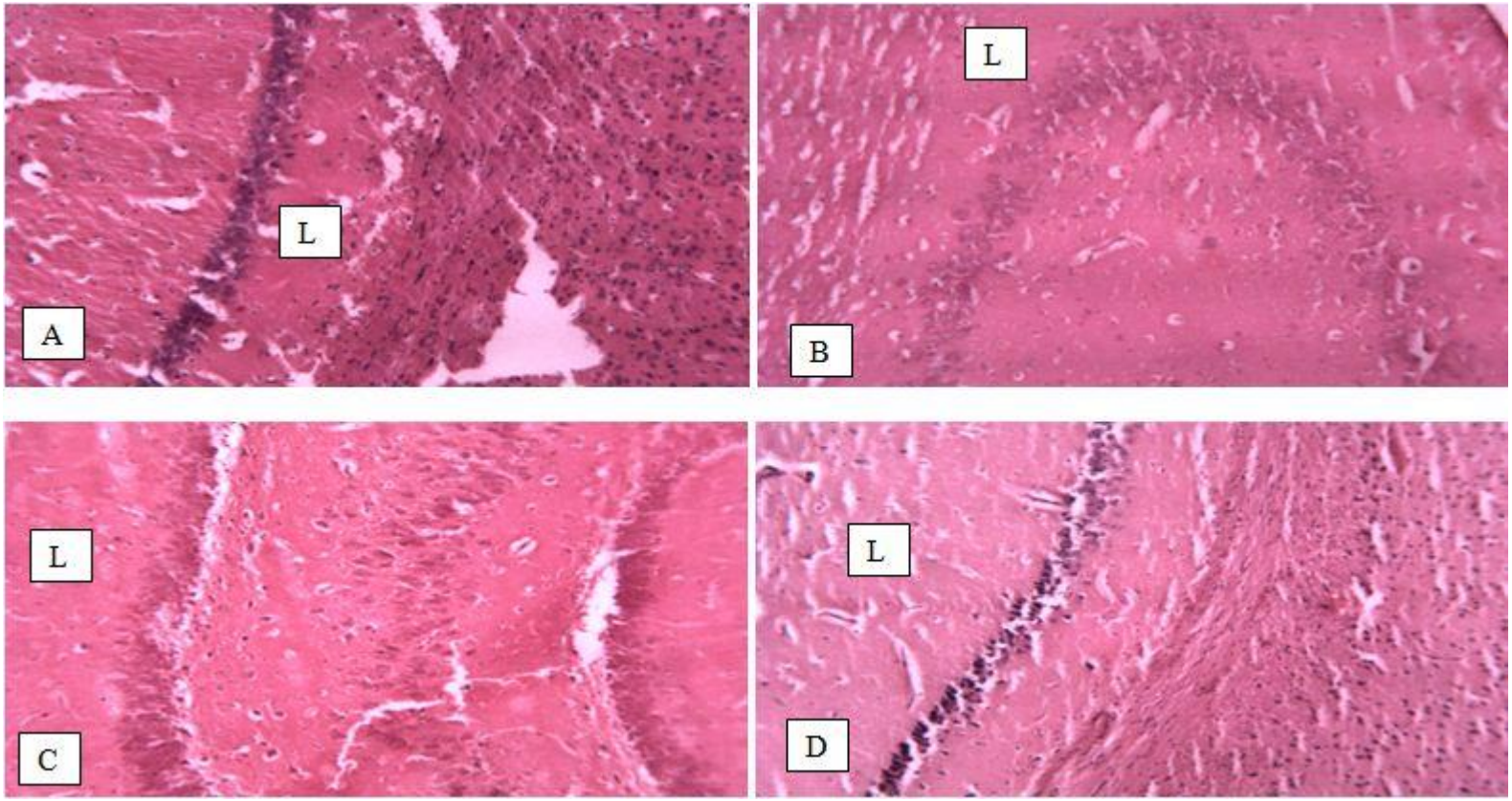


Plate 15: Shows transverse sections of the cerebral cortex cleared for 1 hour. The layers (L) were clearly demonstrated in all the sections cleared with citrus oil (A), citrodora oil (B), neem oil (C) and xylene (D). Note that section C (neem oil) showed better contrast of layer when compared to sections cleared at 15 and 30 minutes. There was no significant change in the section and staining quality among the sections cleared with citrus oil section A, citrodora oil section B, neem oil C and xylene section D (H and E X 100)

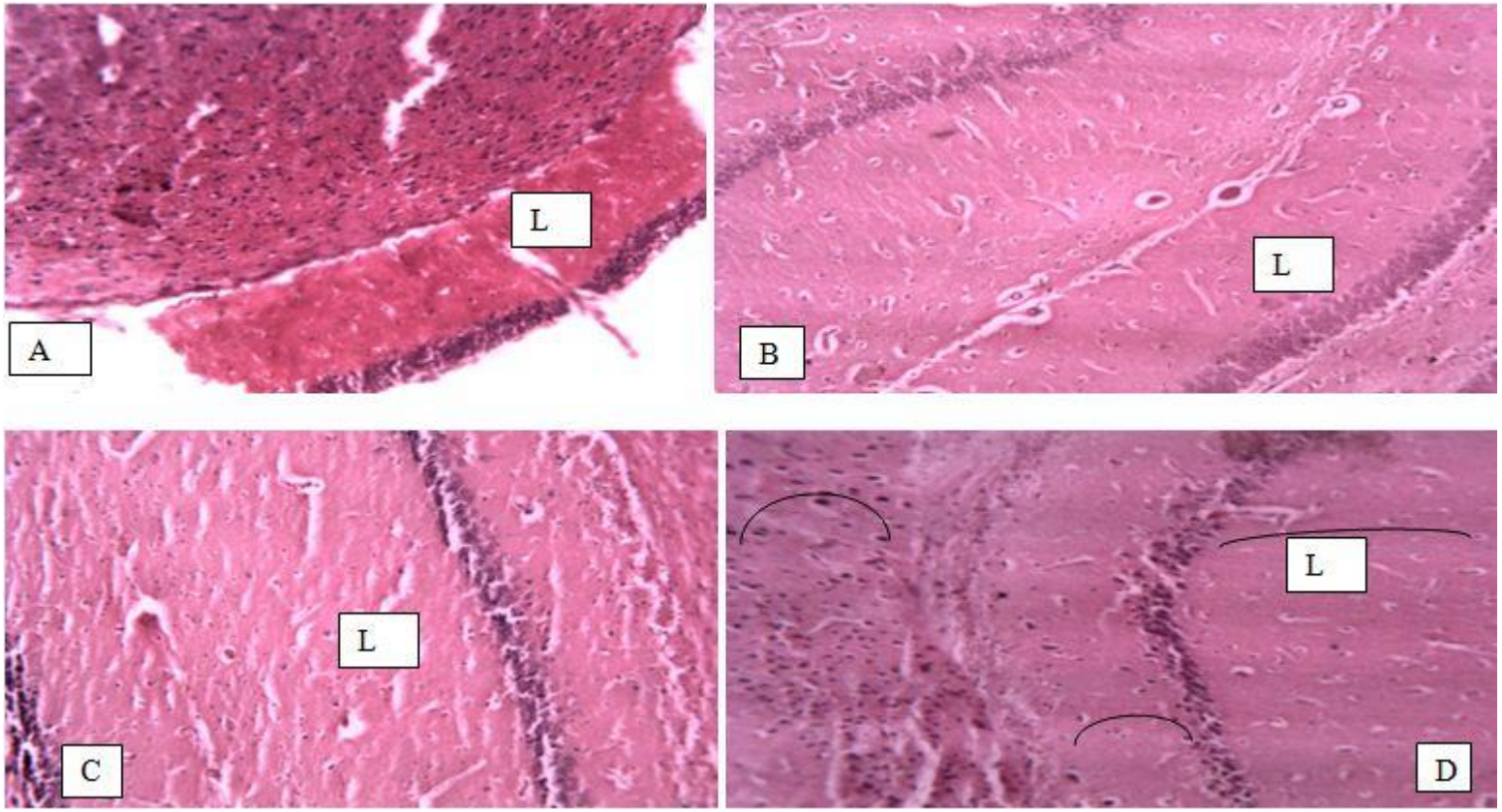


Plate 16: Shows transverse sections of the cerebral cortex cleared for 2 hours using citrus oil (A), citrodora oil (B), neem oil (C) and xylene (D). The cortical layers (L) were clearly demonstrated in all the sections. There was no significant change in the section and staining quality among the three oils when compared to that of xylene (H and E X 100)

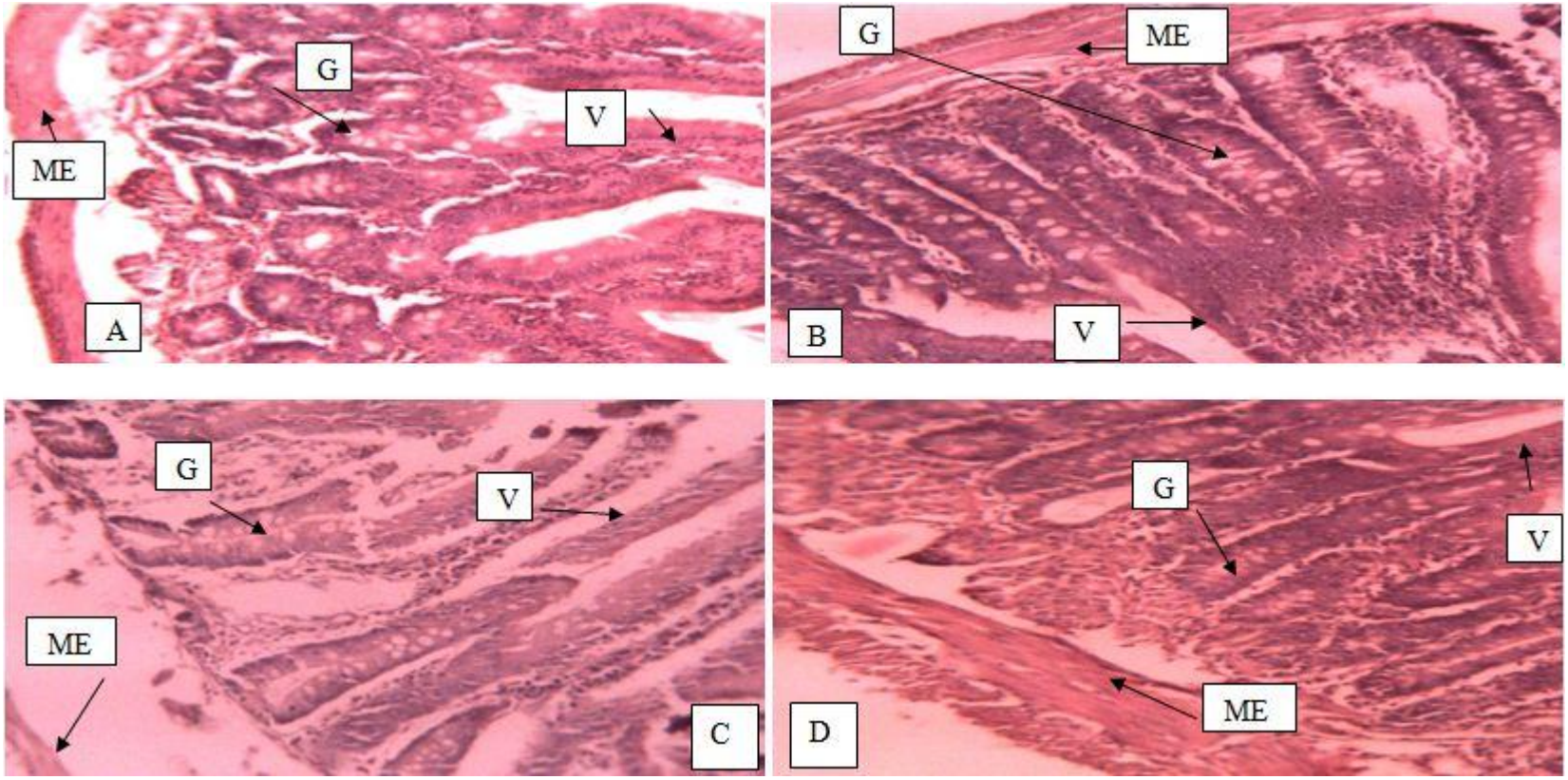


Plate 17: Shows transverse sections of the intestine (jejunum) cleared for 15minutes showing the muscularis externa (ME), the villi (V) and some glands (G) in all the intestinal sections cleared with citrus oil (A), citrodora oil (B), neem oil (C) and xylene (D). Section C (neem oil) showed significant decrease in the staining and section quality when compared citrus oil section (A), citrodora oil section (B) and xylene section (D). Note also the poor clearing effect on the muscularis externa (ME) in section C at 15minutes when compared to the other oils used (H and E X 100)

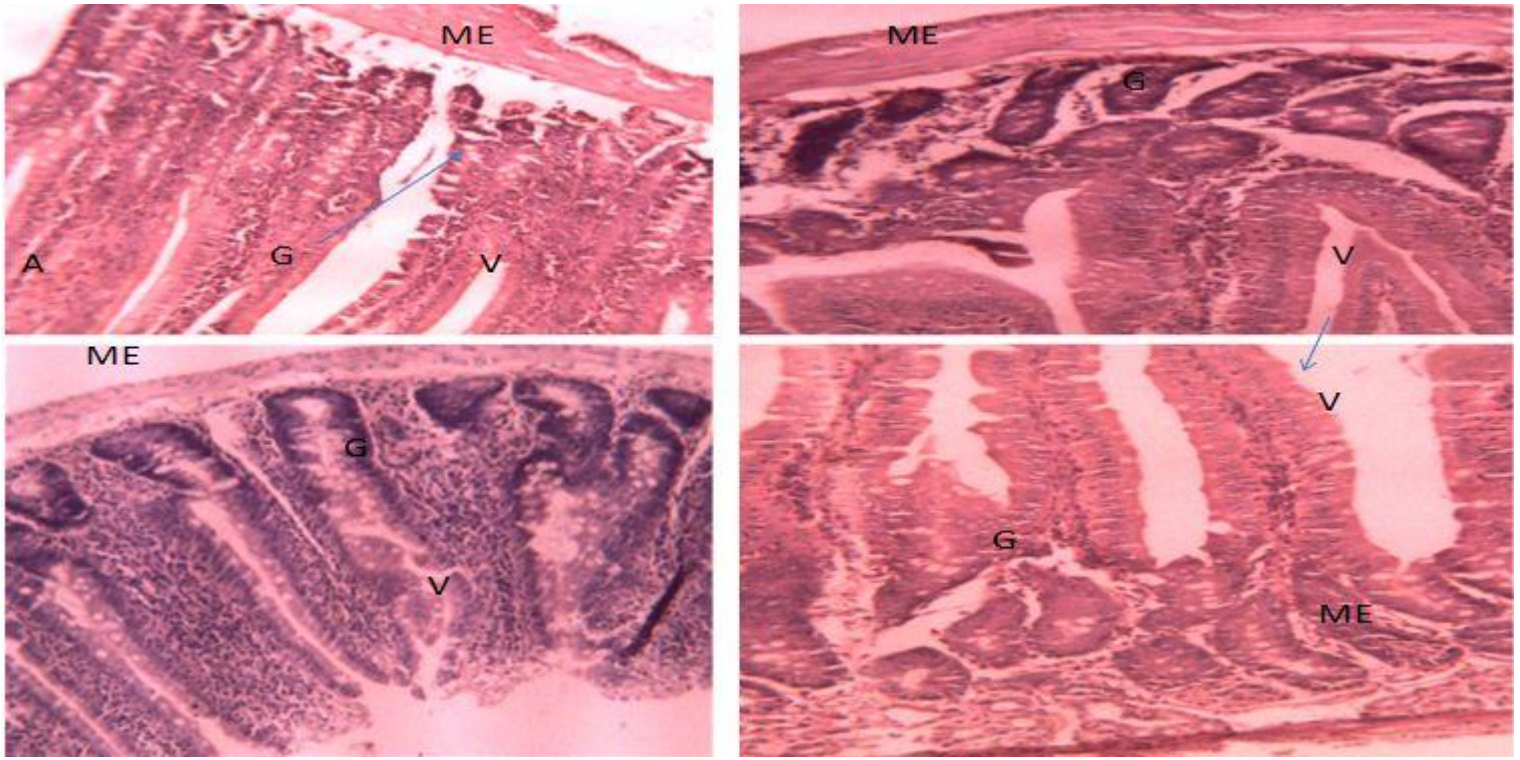


Plate 18: Shows transverse sections of the intestine cleared for 30minutes showing the muscularis externa (ME), the villi (V) and some glands (G) in sections cleared with citrus oil (A), citrodora oil (B), neem oil (C) and xylene (D). The section and staining quality at 30 minutes shows no significant change in the citrus oil section (A), citrodora oil section (B) and the neem oil section (C) when compared to that of xylene section (D) (H and E X 100)

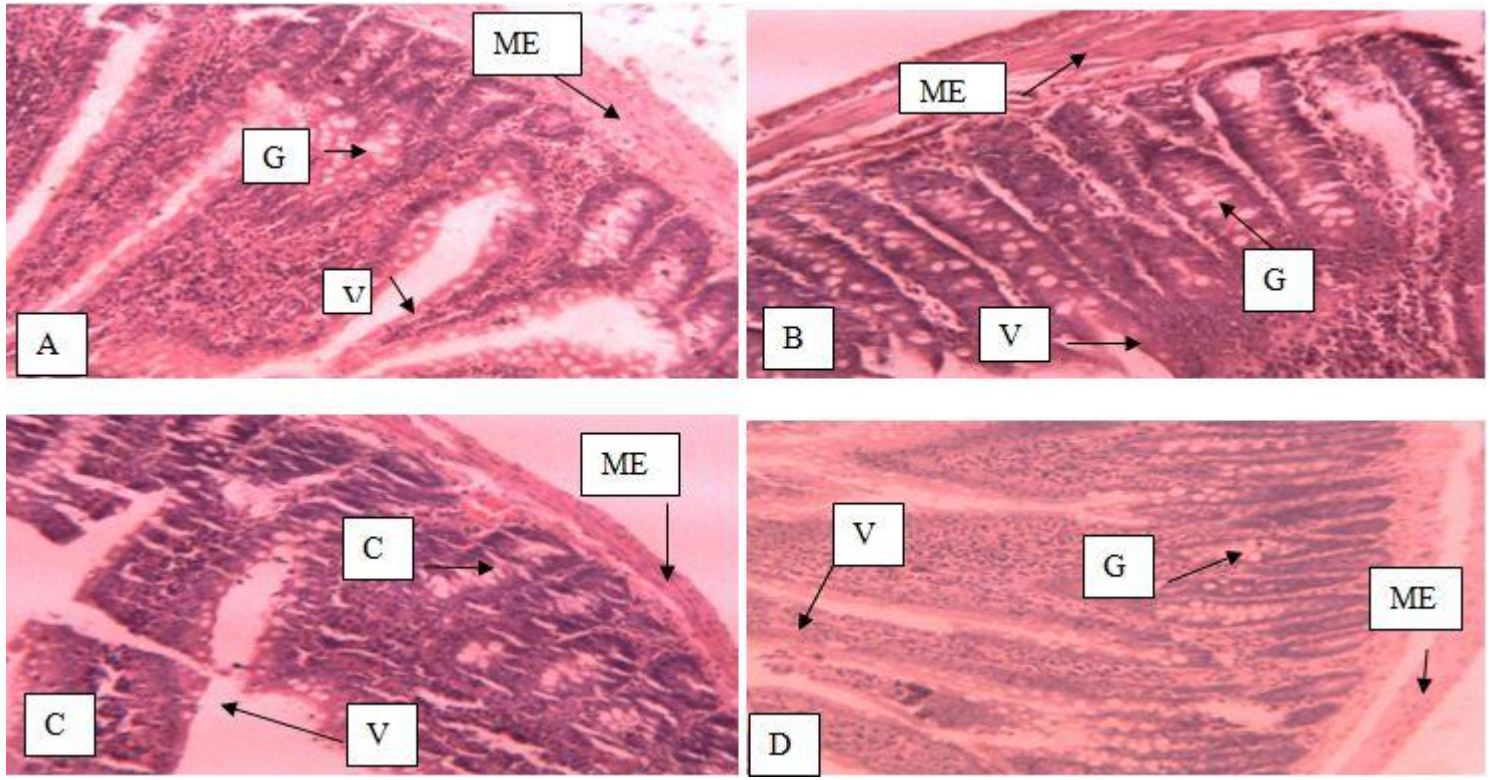


Plate 19: Shows transverse sections of the intestine cleared for 1hour showing the muscularis externa (ME), the villi (V) and some glands (G) in all the intestinal sections cleared with citrus oil (A), citrodora oil (B), neem oil (C) and xylene (D). The section and staining quality shows no significant changes in the citrus oil section, citrodora oil section and neem oil section when compared to xylene section. However, there was more of section quality artifact in section C (neem oil) than xylene section (D) (H and E X 100).

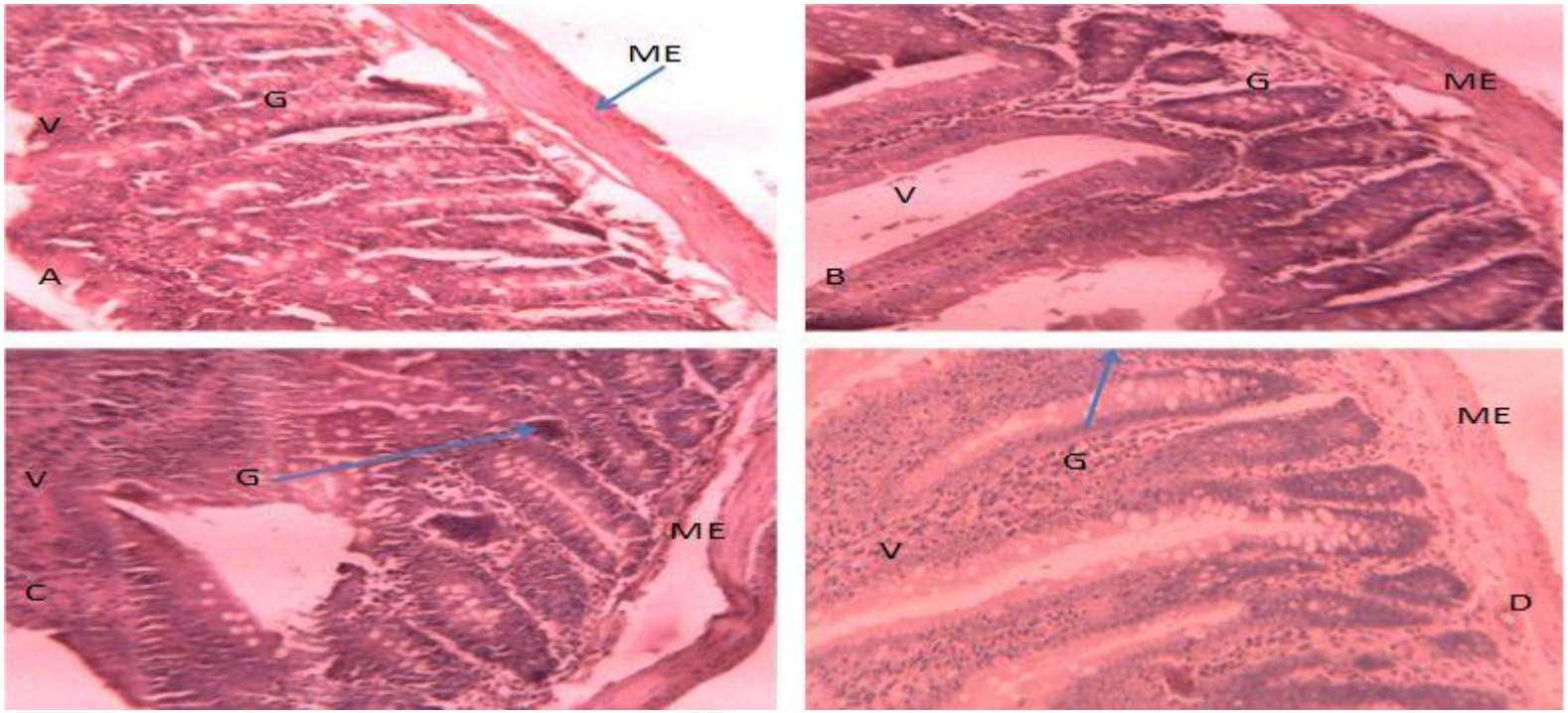


Plate 20: Shows transverse sections of the intestine cleared for 2hours showing the muscularis externa (ME), the villi (V) and some glands (G) in all the intestinal sections cleared with citrus oil (A), citrodora oil (B), neem oil (C) and xylene (D). The section and staining quality shows no significant changes at 2 hours between the citrus oil section, citrodora oil section and neem oil section when compared to that of xylene section (H and E Mg X 100)

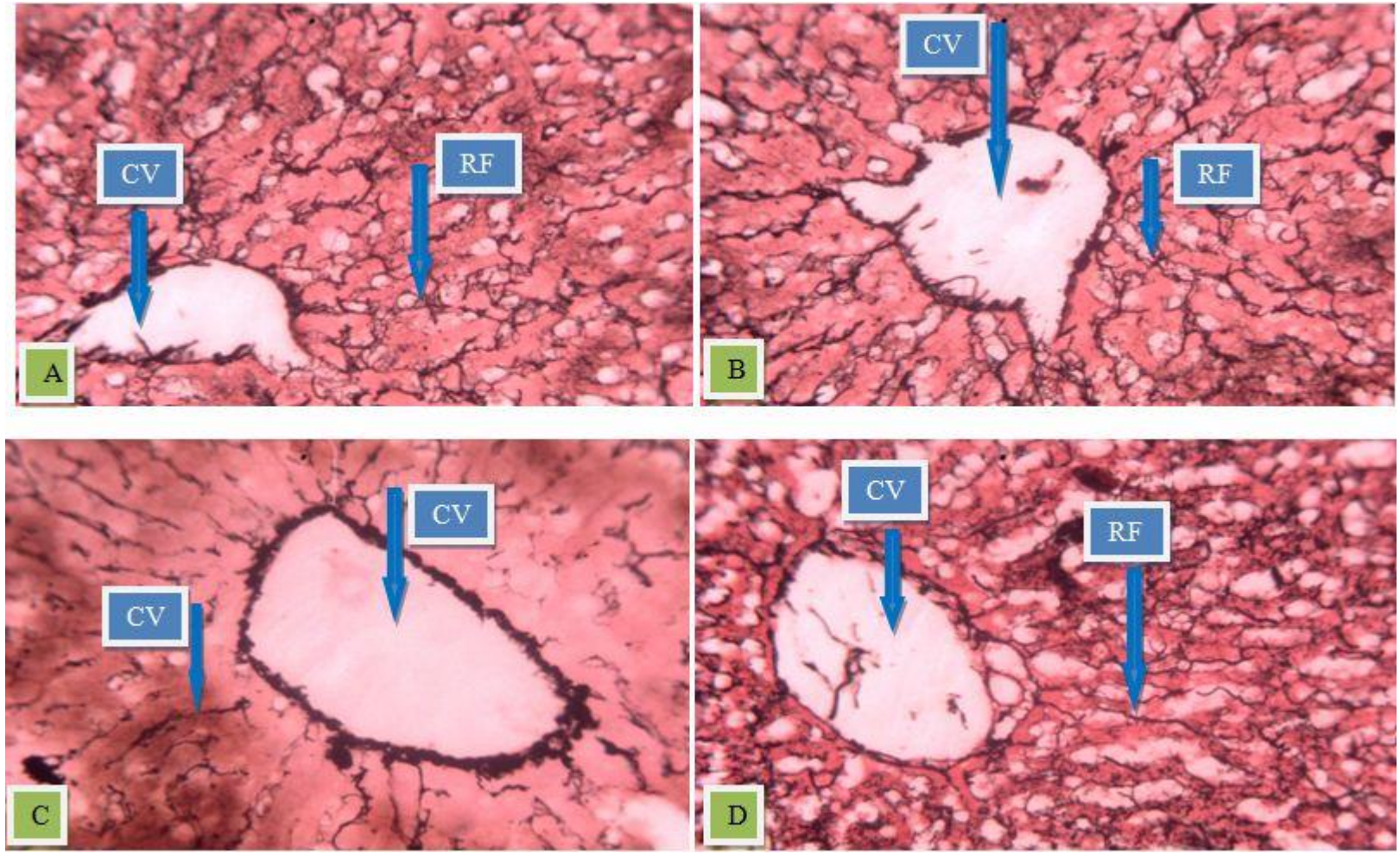


Plate 21: Shows transverse section of liver cleared for 1hour with citrus oil (A), citrodora oil (B), neem oil (C) and xylene (D). The central vein (CV) and reticular fibers (RF) of the citrus oil (A) and citrodora oil (B) were clearly demonstrated black, while that of the neem oil cleared section (C) showed less differentiation of the RT when compared to xylene section (D).Gordon and Sweet X250).

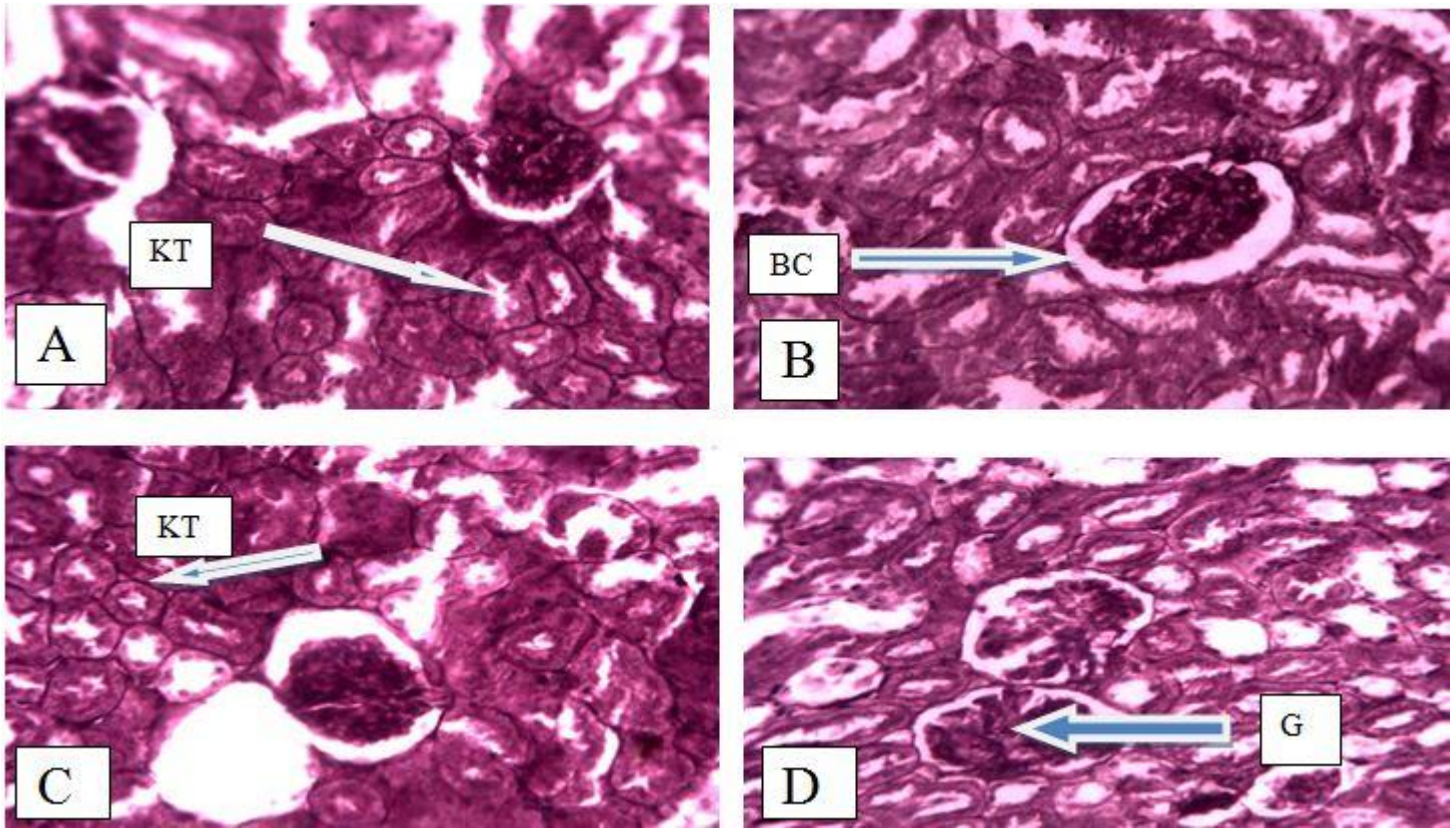


Plate 22: Shows longitudinal sections of kidney cleared for 1hour and stained by PAS method to demonstrate basement membranes. All the sections cleared with citrus oil (A), citrodora oil (B), neem oil (C) and xylene (D) are PAS positive. The kidney tubules (KT) and epithelium of the Bowman's capsules (BC) were sharply stained which could mean the PAS staining ability of the three oils is good. (Periodic Acid Schiff X 250)

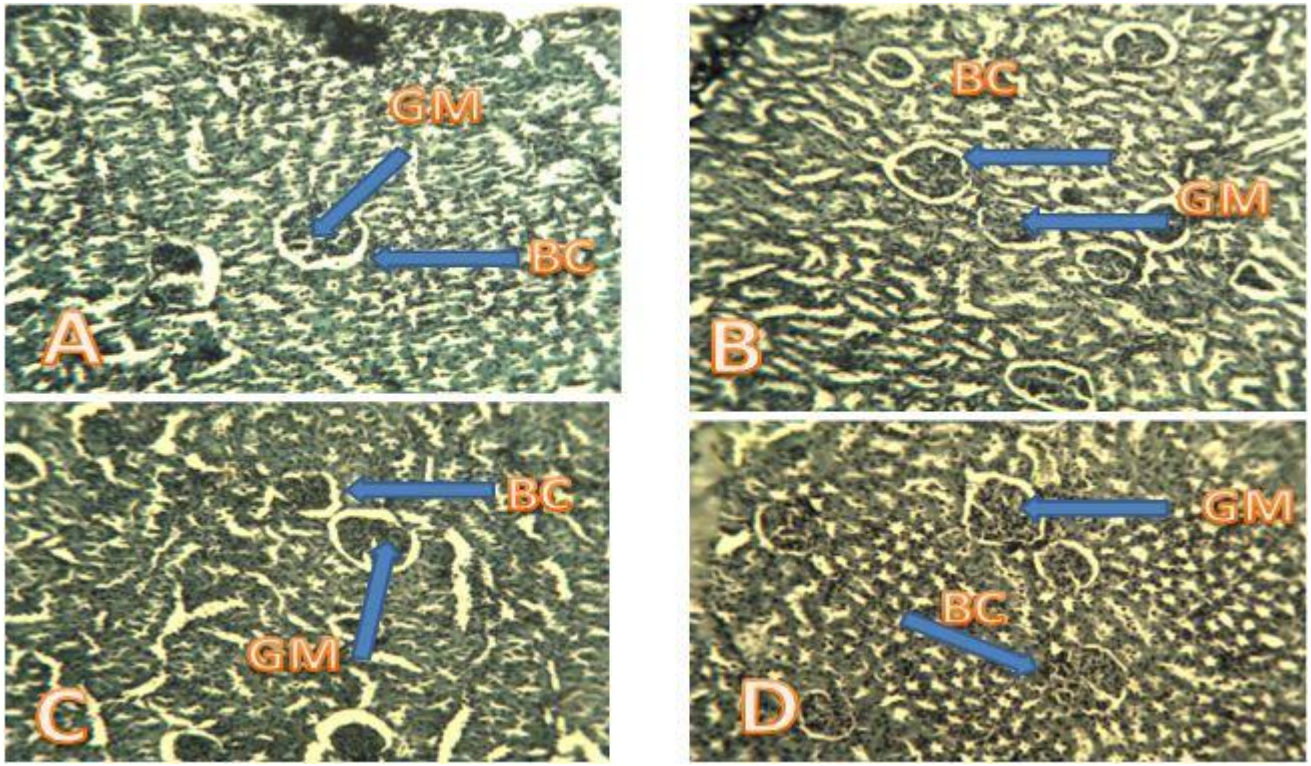


Plate 23: Shows transverse section of kidney stained by Raffaty's method to demonstrate connective tissue and general tissue structures. All the sections cleared with citrus oil (A), citrodora oil (B), neem oil (C) and xylene (D) are positive showing clearly the Bowman's capsule (BC) and the glomerulus. This could mean the staining ability of the three oils with Raffaty's stain is good (Raffaty's X 100)

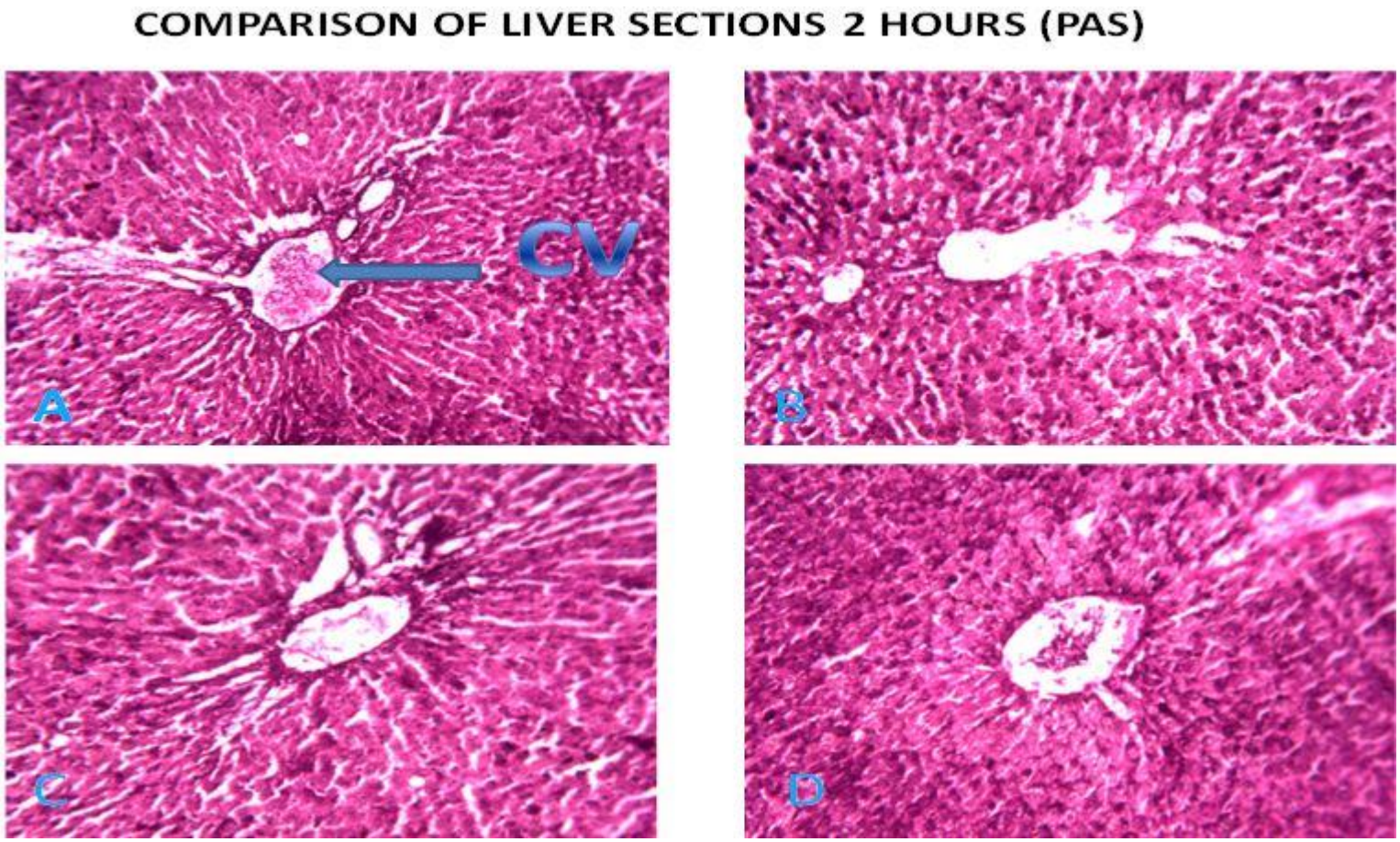


Plate 24: Shows transverse section of liver tissues stained by PAS method to demonstrate carbohydrates. All the sections are PAS positive, this is evidence by the magenta color reaction demonstrated by all the Section cleared with citrus oil (A), citrodora oil (B), neem oil (C) and xylene (D) (Periodic Acid Schiff X 100).

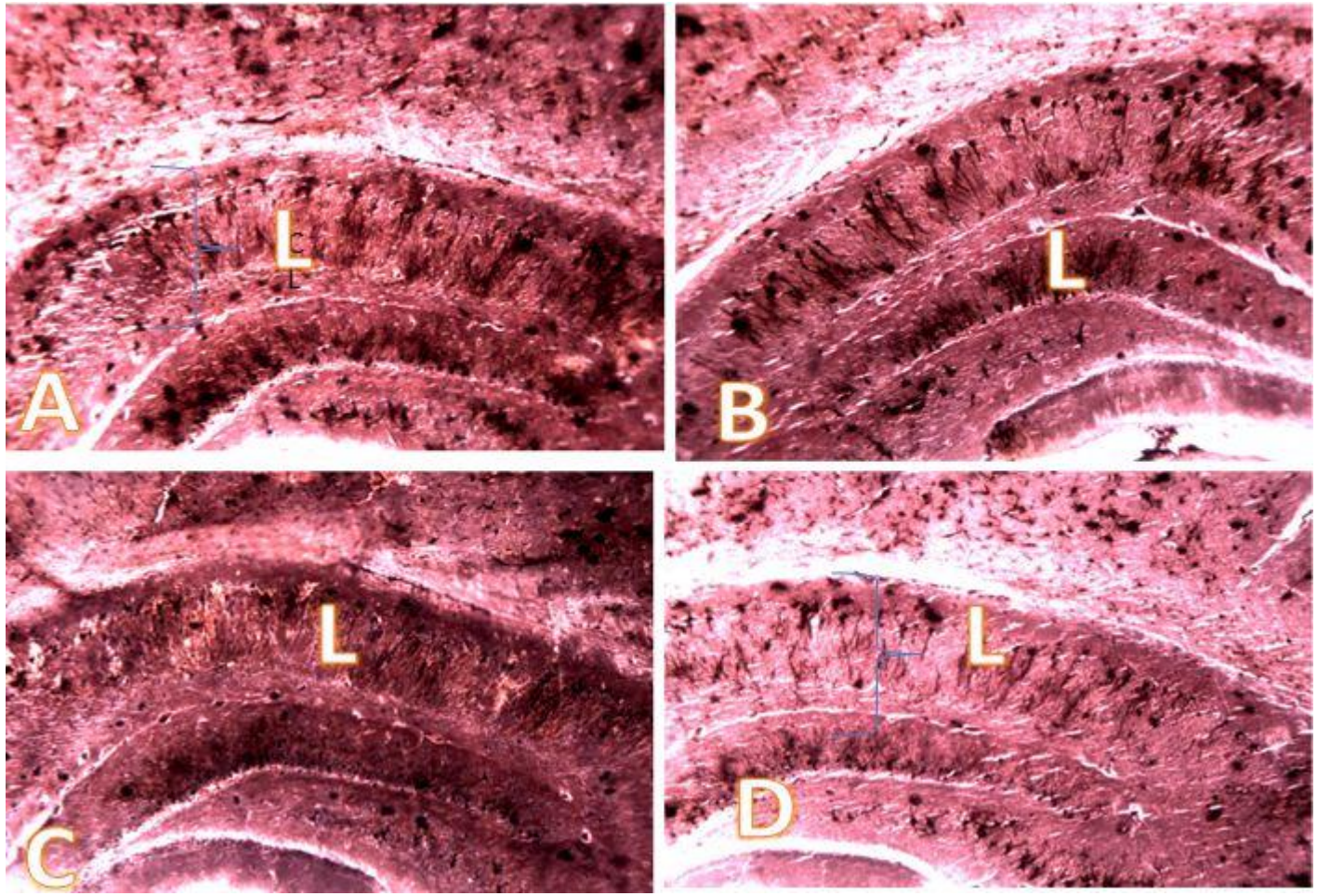


Plate 25: Shows transverse section of the brain cleared for 1hour showing the cortical layers of cerebrum. Note the clear outline of the layers in section cleared with citrus oil (A), citrodora oil (B), neem oil (C) and xylene (D). (Golgi Cox silver stain X40)

DEMONSTRATION OF NEURONS IN BRAIN SECTIONS

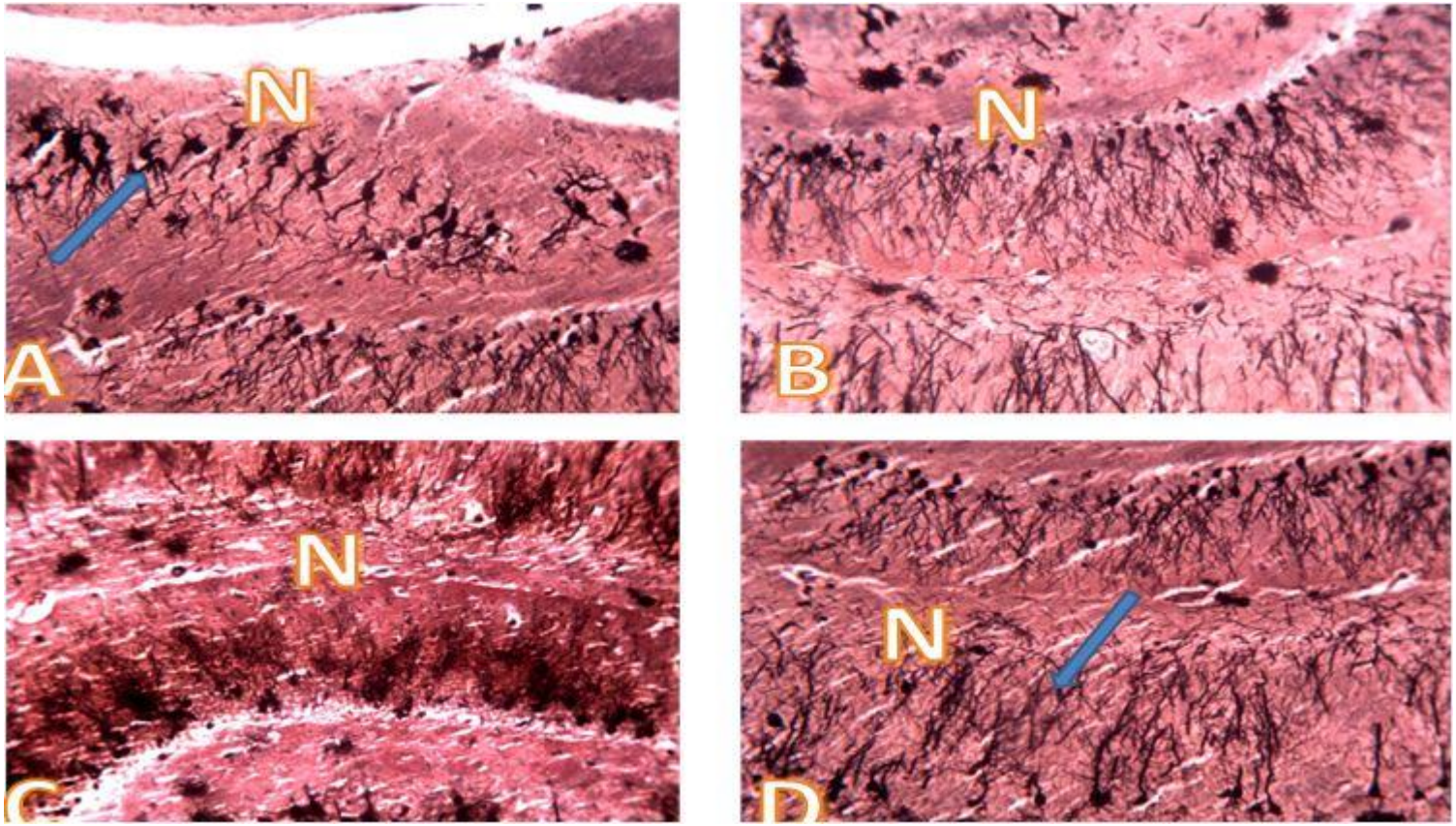


Plate 26: Shows transverse sections of the Brain tissues cleared for 1hour in citrus oil (A), citrodora oil (B), neem oil (C) and xylene (D). The cerebral cortex layers (L) and the neuronal cells (N) in citrus section (A), and citrodora section (B), showed no significant changes when compared to xylene section (D). Section D (Neem oil) appeared masked with artifacts and no clear neuronal structural differentiation as compared to that of xylene section (D) (Golgi Cox silver stain X100).

CHAPTER FIVE

DISCUSSION

Color of the oils

The colors observed were similar to what has been widely documented. Gbenou, (2013) reported that perfectly pure, essential oils can be colorless; some are brown, blue, or green or may have a pale yellow tint. Most of the work on colors of essential oils reported has to do with mixing of essential oils based on color for acupuncture and aromatherapy (Chiazzari 1998; Mary 2005).

Specific Gravity (SG) and Density of the oils

Specific gravity is the heaviness of a substance compared to that of water. In this present study it was observed that, neem oil had the highest specific gravity and density followed by that of xylene, citrodora and citrus oil respectively. Findings from other literature gave similar values. Awolu *et al* (2013), reported the same density value for neem while Shabnam *et al* (2014), reported that the specific gravity of 5 different species of *Citrus* essential oils ranged from 0.842 to 0.858 g/ml. The present results were in agreement with the work on essential oils by Guenther (1948), but however do not agree with the work of Awolu *et al* (2001), who reported a higher density value for most vegetable oil to be between 0.8767 and 0.8811 kg/m3. The high density value of neem recorded in this work may be the reason for the poor clearing as observed on tissues. High density oil may mean high molecular weight oil which in most cases has slower tissue penetration ability.

Viscosity of the oils

Viscosity is oil’s resistance to flow and shear. A liquid's viscosity depends on the size and shape of its particles and the attractions between the particles which may eventually affect

the rate of penetration in tissue. According to Kinast (2003), most vegetable oils have higher viscosity between 2 and 5.7 cp at 28% which is reduced by transesterification processes. These values are higher when compared to the viscosity values of xylene (0.45cp), citrus (0.35cp), and citrodora (0.34cp) in this present study. However the value of viscosity of neem oil was 36.0 cp at 28% in this present study and this is similar, to the work of Awolu *et al* (2013) who reported viscosity of neem to be 36.67 cp at 28%. The high viscosity value of neem oil may mean that neem is thicker or heavier or have a higher molecular structure ($C_{30}H_{36}O_6$) leading to higher attraction between particles, and as such flows slower. This also may be another reason for the poor clearing effect observed on tissue, since tissue clearing is also dependent on rate of penetration of the clearing oils. This can be said to generally conform to Fick's Law: 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) as a multiple of temperature dependant constants for specific substances.

Flash Point (FP) of the Oils

Flash point is the lowest temperature at and above which a liquid gives off enough flammable vapor to form a mixture with air that can be ignited by contact with a hot surface, spark, or flame. In other word the lower the FP, the more flammable the liquid. In the present study, neem has the highest FP of 108 ^{0}C, followed by citrodora oil (35^{0}C), citrus oil (34^{0}C) and Xylene 39^{0}C. Similar reports indicated that some essential oils have higher FP than Xylene. Hans (1995) had reported that the FP of olive oil which is an essential oil to be 60^{0}C and that of xylene, 25^{0}C. Similarly, Kunhua *et al*, (2012) had reported xylene to have 25^{0}C FP and a mixture of white oil (an essential oil) with N-heptane to be 144^{0}C. The lower FP of xylene (39^{0}C) in this work though slightly higher

than the value from other works confirmed that xylene is highly flammable (Chatterjee *et al*., 2005; Kum *et al*., 2007a; Sandikci *et al*., 2009). In this study, citrus and ciyrodora oils had flash point value similar to xylene, meaning that they are also as flammable if not more than xylene. It is also possible that oils with lower flash point have better tissue clearing abilities as shown from the present study where citrus oil, citrodora oil and xylene seemed to be better clearing agents than neem.

Acid value (AV)

The acid value (AV) is defined as the weight of KOH in mg needed to neutralize the organic acids present in 1g of fat and it is a measure of the free fatty acids (FFA) present in the fat or oil. An increment in the amount of FFA in a sample of oil or fat indicates hydrolysis of triglycerides by the action of lipase enzyme. The source of the enzyme can be the tissue from which the oil or fat was extracted or it can be a contaminant from other cells including microorganisms. In the present study, the AV neem has the highest acid value of 37.8, followed by citrus (34.4) oil, xylene (16.9) and citrodora oil (11.8). This may mean that the activity of lipase enzyme in neem oil is higher followed by that of citrus oil and may be responsible for the poor section and staing qualities observed in some of the tissues. The AV recorded in this work is higher when compared to to the report from other oils such as reported by Kardash (2005).

Cloud point (CP)

The cloud point of a fluid is the temperature at which dissolved solids are no longer completely soluble, precipitating as a second phase giving the fluid a cloudy appearance. This term is relevant to several applications with different consequences.In the petroleum industry, cloud point refers to the temperature below which wax in diesel or biowax in

biodiesels form a cloudy appearance. The presence of solidified waxes thickens the oil and clogs fuel filters and injectors in engines. Therefore, cloud point indicates the tendency of the oil to plug filters or small orifices (tissue pores) at cold operating temperatures there by reducing tissue penetration, and affecting clearing. This may be the reason why in the present study neem oil with higher CP of 2%, seems to be the poorest in clearing when compared to citrus oil (-67%), citrodora oil (-69%) and xylene (-65%). This also means neem will cloud faster than the rest oils used.

Refractive Index (RI) of the oils

Refraction is the change of direction of light rays as it travels through one substance to another. This occurs when the light travels at different speeds through materials with different densities. RI is important in the application of evanescent (rate of disappearance of fluid) this may enhance the rate of penetration of the oils into the tissues during the clearing process (Zocchi, 1996). Clearing makes tissue RI as close to that of proteins (Bancroft and Gamble, 2008). Delpy *et al*, (1988) stated that the overall RI of tissue is considered to be around 1.4 for most tissue types. Shyam (2002) reported the RI of orange oil, corn oil and vegetable oil to be 1.47 while safflower oil was 1.466. This was closely related to the finding of the present study where the RI of xylene is 1.496 followed by that of neem oil (1.47), citrodora (1.457) and citrus oil (1.455). Since the RI of the oil used in this study were closely related to that of tissue (1.4) as indicated by Delpy *et al*, (1988), this may be one of the reasons why the oils can clear tissues as stated by Zocchi (1995).

The presence of some active chemical constituents like alkaloids, tannins, glycosides, flavonoids, terpenes, saponins and carbohydrates were determined by phytochemical studies. The phytochemical properties of citrus and citrodora oils have similar properties. They were positive for Carbohydrates, Cardiac Glycosides and Saponins (Steroid and Triterpenes). However, they were negative for Antraquinones derivatives, Flavonoids, Tannins, and Alkaloids. The presence of saponins could be one of the reasons for the therapeutic use of these essential oils as documented (Hong and Shellock, 1991; Singh *et al*., 2002; Juergens *et al.*, 2003 and Nagata *et al*., 2008). Francis *et al* (2002) reported that saponins may serve as antimicrobial and antifungal agent.

On the other hand, the neem oil and xylene indicated the absence of all other phytochemical properties except for carbohydrates which was also noted to be positive in all the oils used. This may be connected to their structural composition and weight as shown for xylene (C_6H_4 $(CH_3)_2$), Citrodora oil ($C_{10}H_{18}O$), Citrus oil ($CH_{15}H_{24}O$) and Neem oil ($C_{30}H_{36}O_6$). Since the higher the structural weight, the lower the movement of molecules in the liquid which can lead to lower tissue penetration, this may be connected to poor tissue penetration and eventual poor clearing ability of neem as observed in the present study.

The GC-MS results of the citrus oil, citrodora oil, neem oil and xylene analysis showed that the predominant constituent in citrus oil was D-Limonene with concentration (relative abundance) of 62.23%. This is in agreement with several works on orange peel where the major component was determined to be limonene (Piotrowska *et al*., 2002; Lyon *et al*., 1995 and Chen, *et al*., 2010). This confirmed that the major component in citrus oil could be d-limonene. Limonene is what gives citrus fruit their familiar aroma. Limonene is effective for clearing; however it is a little slower in clearing than xylene

(William, 1900; Kegley *et al.*, 2010). This could be the reason for the poor section quality recorded with tissues cleared with citrus oil in the present study when compared to that of tissues cleared with xylene.

The predominant constituent in citrodora oil is Citronellal with relative abundance of 42.20% followed by β-Citronellol (21.71%). This also agreed with several authors especially, Doran *et al* (2001). This may mean that citrodora oil have citronellal as dominant constituent. This may be the reason for the good clearing effect observed with tissues cleared in citrodora oil when compared to xylene.

The predominant constituent in neem oil is 6-Octadenol with relative abundance of 47.45 % followed by Hexadecanoic acid (21.18%).This agreed with the work of Schmutterer, (2002) which also reported the presence of similar constituents. However, the percentage abundance recorded in our study slightly varied with that of Kaushik and Vir, (2006) who reported stearic (8.2%), palmitic (13.6%), oleic (6.5%) and linoleic (8.0%). Hallur *et al.*, (2007), also reported that neem oil contains oleic acid (50-60%), palmitic acid (13-15%), stearic acid (14-19%), linoleic acid (8-16%) and arachidic acid (1-3%), and that the quality of the oil differs with the method of processing. The processing method may be the reason for the variation in values recorded. Hallur *et al (*2007) further stated that neem kernel lipids are similar to the normal glycerides from other oil seeds which have octadenol as one of their components. This may then mean that neem oil is similar to other lipids and as such may not be ideal for tissue clearing process as observed in the present study.

Xylene contains 94% of o-xylene, 97% of p-xylene, 98% of m-xylene and 98%p-xylene. This finding is similar to that of several authors. For example, in 2002, Fabri, *et al* stated that xylene exists in three isomeric forms namely ortho- meta- and para-xylene.

Laboratory-grade xylene is composed of m-xylene (40-65%), p-xylene (20%), o-xylene (20%) and ethyl benzene (6-20%) and traces of toluene, trimethyl benzene, phenol, thiophene, pyridine and hydrogen sulfide (Tremblay and Jean, 2012). This value is lower compared to the findings of the present work. Xylene has wide range of application because of its high solvency factor which allows maximum displacement of alcohol and enhancing paraffin infiltration. It causes an increase in the refractive index of the tissue during clearing. Eventually, when completed, the tissue becomes quite translucent, and almost transparent (Carson and Hladik, 2009). Staining procedures, its excellent dewaxing and clearing capabilities contribute to brilliantly stained sections of tissues (Bancroft and Gamble, 2008). Xylene is an efficient fat solvent, and that is one of its major advantages. Triglyceride and fats in tissues has a distinctly negative effect on the quality of sectioning (Bancroft and Gamble, 2008). These may be the reasons for the better section qualities observed in this study. However, Stevenson *et al* (1998) and Kieranan (2010) had stated that xylene does tend to harden tissues a little, especially when over stayed in it. This may be one of the reasons why there was no any significant superiority of xylene over citrodora oil in section quality recorded in this study.

There were no statistical differences across the groups except for the significant decrease in section quality of kidney tissue cleared with neem at 30minutes and staining quality of intestine cleared with neem oil at 15 minutes.

The results of photomicrographs from the present study showed that most of the tissues cleared with citrodora oil appeared better at 1 hour time of clearing, while that of xylene was 2 hours meaning that citrodora oil can clear tissue better with I hour difference when compared to xylene. This may be because of their close physical properties such as density and viscosity values, which is a factor for rapid tissue penetration. This time gain

by citrodora oil seemed to be better when compared to several works where the general average clearing time for most routine tissue processing except for rapid processing is about 4 hours, 2 hours of clearing for 2 changes (Baker, 1966; Kieranan, 2010). Though Maxwell (1978), documented that xylene is a fast tissue clearing agent, but in the present study, citrodora seemed to give better results in the time of clearing. The poor clearing effect observed at 15minutes and 30minutes in this study agreed with what Bancroft and Steven (2010) reported and that inadequate time for clearing may lead to tissue cracking and poor staining ability and quality. The present study is also in agreement with the work of Rasmussen *et al* (1992) on the use of vegetable oils mainly 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.

Though, citrus oil has similar physical properties with xylene and citrodora, the general tissue appearance of tissue section cleared with citrus oil seemed to be poor when compared to the other oils used in the present study. This may be due to its limonene content which is difficult to remove from paraffin and the stain tend to fade when exposed to strong light as stated by Rene *et al* (2009). This may be the reason why most studies conducted using citrus oil as clearing agent was usually in combination with other clearing agents (aliphatic hydrocarbon) and sometimes diluted with water Rene *et a*l (2009). Another reason for this poor citrus effect may be due to its high acid value as discussed above.

The low density and viscosity of citrodora oil may be the reason for the better section quality observed at 1hour and 2hours in the present study. Lower density and viscosity means lower molecular weight of particles present in the oils and since tissue clearing is

dependent on rate of penetration of particles of the oils into the tissues, Neem oil with higher density and viscosity in the present study may mean lower tissue penetration leading to the poor section quality as observed in the present study.

Percentage yield, Cost and Toxicity of the Oils

The percentage yield of the oils indicated that neem oil has the highest yield of 7.8% followed by citrus oil (5.3%) and citrodora oil (2.2%). Neem oil is higher in yield because its extraction was from the seed and not the leaves. This yield is similar to the ones reported by some authors on essential oils (Essential Oil company, 1996; Ladan, 2011). This little quantity of the citrus oil and citrodora oil may be attributed to the high volatility of the essential oils during extraction.

The cost of producing 200 ml of Neem oil is higher, followed by citrodora oil and citrus oil. The neem seeds are more expensive than the leaves of the citrus and eucalyptus plant, and the cost in this present study included total cost of production which may be cheaper when larger quantity of the oil is produce. Besides, it is far cheaper when compaered to the price of managing the side effect of xylene. The toxicity of xylene has long been reported by Hans (1995) to be more than most of the eseential oil especially the ones in this study.

Comparison of the different clearing oils using special stains

The result from the tissue section of liver in Gordon and Sweet staining method for reticular fibers (RF) showed that the liver tissues cleared with, citrus oil and citrodora oil demonstrated clearly RF than the neem oil when compared to xylene. This could be due to the high acid value of the neem oil which probably has affected some of the RF.

The result of tissue section of kidney stained with Raffaty stain to demonstrate general tissue structure showed that the general structural outline was well demonstrated with all

oils used for the clearing. This may mean that the all the oils used in this study did not have any adverse effect on the general tissue stain.

The liver section stained with Periodic Acid Schiff stain to demonstrate glycogen, showed that these clearing oils used in this study support PAS reaction and did not significantly affect glycogen storage in the liver tissues. This means that like the H and E and the Raffaty stain, glycogen can be demonstrated using any of these oils as clearing agent.

The brain sections stained with Golgi Cox silver stain to demonstrate nerve fibers, showed that the tissues cleared in all the oils used can demonstrate nerve fibers except for neem oil where the neurons were masked by numerous artifacts and shows no clear differentiation of the nerve fibers. Perhaps the neurons are also affected by the high acid content of the neem oil, resulting in similar effect as seen in the reticular fibers of the liver sections.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 SUMMARY

The present study have demonstrated that in tissue processing, conventional clearing and dewaxing agents (aromatic and aliphatic hydrocarbon) may be substituted by non-hazardous compounds with no loss of quality. The results from the present study indicated that essential oil namely citrus oil, citrodora oil and neem oil can clear and dewax tissue during processing and staining in their undiluted form. These essential oils may be viable alternative clearing agents to xylene especially the citrodora oil. With the exception of the neem oil, the citrus oil and citrodora oil has similar physical properties such as low specific gravity and density, viscosity, flash point and refractive index to that of xylene, placing them in the same clearing group. Citrus oil and citrodora oil are naturally extracted from plant with no or little known toxicity; however, citrodora oil seemed to be a better clearing agent than citrus oil and neem oil. Compared with xylene, citrus oil and citrodora oil have a sweet smell unlike xylene that have a pungent offensive smell. Citrus oil, citrodora oil and xylene had lower flash point values, meaning they are more flammable than neem oil which had a very high flash point. Microscopic examination showed no significant changes in section and staining quality between the sections cleared with citrodora oil and xylene at I and 2 hours respectively. Artifacts observed normally in tissue cleared using xylene were the same as that using citrodora oil. The sections of citrodora oil and xylene stained with all the special stains demonstrated the respective structures well.

There was no change of volume of the clearing agents used as against the standard two changes of xylene. This implies that these essential oils citrus, citrodora and neem will require less frequent changing than xylene, thereby reducing the quantity of clearing agent used. It was also observed that the better mean clearing time was 1hour with citrodora oil. This is a gain in time when compared to standard xylene clearing time of 2hours two changes each (4hours). The cost of 2.5litres of citrus oil, citrodora oil was cheaper when compared to that of neem oil and xylene is about. The high cost of this essential oil is species dependent in terms of oil yield; however, it is still better than xylene when the safety is considered.

6.2 CONCLUSION

This present study concludes that citrus oil, citrodora oil and neem oil can clear and dewax Wistar rat tissues during tissue processing and staining. There was significant difference in some of the tissues between citrus oil and neem oil when compared to xylene especially at 15 and 30 minutes respectively. There was no significant difference between citrodora oil and xylene when used as clearing agent for Wistar rat tissues at 1 and 2 hours respectively. Citrodora oil is a better clearing agent than citrus oil while neem oil is the poorest of clearing oil used in this present study.

Although the long time stability of these oil in clearing tissues still remain to be clarified, it can be said that by substituting the clearing and dewaxing agent using citrodora oil, and modifying the mounting procedure, xylene or similar organic solvent may be removed completely from the repertoire of reagents used in histology laboratories without losing valuable information.

6.3 RECOMMENDATIONS

The following were recommendations:

1. Studies using advance techniques such as immunohistochemistry, Molecular and enzymatic study, Flurescence and electron microscopy techniques of the effects of these essential oils in tissue clearing.
2. The study on the long time effects of this oil on tissues sections.
3. The long time mounting effect of these oils using different mounting media.
4. Study on the mixture of these oils with other clearing agent.

6.4 LIMITATIONS

Although, the data obtained from the present study suggest that a comparable section quality and staining result can be achieved with citrodora oil and xylene, the criteria for assessment were somewhat subjective. In the present study, the major focus was on the general appearance of the tissue. For it is usually difficult to give a clear definition of the ideal appearance of a tissue section by using only bright field microscope.

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APPENDICES
APPENDIX I

STAINING TECHNIQUES

(A) HAEMATOXYLEN AND EOSIN

Purpose: for demonstrating general tissue structures

Reagents

A. Haematoxylin solution (Mayer)

B. 1% acid alcohol (differentiator)

C. 2% aqueous sodium bicarbonate (Scott bluing agent)

D. 1% aqueous eosin

E. alcohols (70%, 90%, 100%)

Procedure

1. Deparaffinize or dewax in xylene for 2 to 3min, two changes; wash briefly in descending grades of alcohol, then in water.
2. Stain in Harris haematoxylin solution for 5 minutes, Wash briefly in water and
3. Differentiate in acid alcohol (solution B) briefly.
4. Wash well in water and
5. Blue in solution C briefly
6. wash well in water and stain in eosin for 1-3 min
7. wash fairly quickly in water then
8. dehydrate, clear and
9. Mount in a synthetic resin medium

Result

Nuclei	blue to blue black
Cytoplasm	shades of pink

(B) GORDON AND SWEET

Purpose: for the demonstration of Reticular fibres

Reagents:

a. Acidified potassium permanganate

-0.5% potassium permanganate - 475ml

-3% sulphuric acid - 2.5 ml

b. 1% oxalic acid
c. 2.5 % iron alum
d. 10% neutral formalin
e. 0.2% gold chloride
f. 5% sodium thiosulphate
g. 1% neutral red

-10% aqueous silver solution - 5ml

-Add concentration ammonium sulphate drop wise until precipitate re-dissolve

-Then add 5ml of 3.1% aqueous sodium hydroxide, mix, a precipitate will be formed which will dissolve upon the addition of ammonium sulphate.

-stop when only few precipitate remain

-make up the final volume to 50 ml.

Procedure

1. Deparaffinize and take section to water.
2. Stain in acidified potassium permanganate
3. Wash with water briefly
4. Bleach with oxalic acid 1 minute
5. Wash in water and then repeat washing in distilled water briefly
6. Stain with iron alum 5 minutes
7. Wash in distilled water several changes
8. Stain in 10% ammoniacal silver solution for about 4-5 seconds until section is clear
9. Wash in distilled water briefly
10. Reduce in 10% formalin 1 minute
11. Wash in water
12. Tone in 0.1 % gold chloride 2 minutes
13. Wash in water
14. Stain in 5% sodium thiosulphate
15. Wash in water
16. Counter stain in 1% neutral red 5 minutes
17. Wash in water
18. Dehydrate, clear and mount in DPX

Result

Reticulin	- black
Collagen	- yellow- brown if un-toned
Nuclei	- as counterstained
Background	- clear if not counterstained

(C) RAFFERTY STAIN (LIGHT GREEN HAEMATOXYLEN)

Purpose: for the demonstration of connective and general tissue structure

Reagents: A. Haematoxylin (Ehrlich)

B. Light green - 0.125g

Water - 100ml

Procedure

1. Deparaffinise and take section to water.
2. Stain section in solution A 5 to 10 minutes
3. Wash in tap water until blue
4. Stain in solution B 5 minutes
5. Wash in water
6. Dehydrated through graded alcohols
7. Clear in xylene
8. Mount in DPX

Result

Cellular elements	-purple to dark brown
Connective tissue fibres	- green

(D) PERIODIC ACID SCHIFF (PAS)

Purpose: for the demonstration of tissue glycogen

Reagent:

Basic Fuchsin	1g
Sodium metabisufite	1.9g
0.15N Hydrochloric acid	100ml
Activated charcoal	500mg

Procedure

1. Deparaffinise in xylene and take section to water (deionised)
2. Oxidised with periodic acid for 5minutes
3. Rinse in several changes of deionised water
4. Cover the sections with Schiff reagent for 15 minutes
5. Rinse in running tap water 5 to 10 minutes
6. Stain with Harris's or Mayer's haematoxylin
7. Differentiate and blue
8. Dehydrate and Clear
9. Mount

Result

Glycogen	magenta
Nucleus	blue

(E) GOLGI SILVER STAIN (MODIFIED)

Purpose: For the demonstration of neurons and their processes.

Reagents: 3% potassium dichromate 60ml

2% silver nitrate solution 20ml

Procedure

1. Immerse approximately 10x5mm of fixed tissue in 2% potassium dichromate solution for 48 hours.
2. Remove tissue and blot dry with filter paper
3. Transfer the tissue into 2% silver nitrate solution for 48hours avoiding light.
4. Process the tissue as usual (dehydrate, clear, infiltrate and embed)
5. Section tissue at not less than 15microns.
6. Dehydrate, clear and mount

Result

Neurons and processes	black

APPENDIX II

CALCULATION OF RESULTS OF PHYSICAL PROPERTIES OF THE OIL

SPECIFIC GRAVITY (SG)

$$SG = \frac{W2-W1}{W3-W1}$$

Where:

Weight of empty bottle (W1) = 23.1026g

Weight of bottle + oil = (W2)

Weight of bottle + neem oil = 45.3585g

Weight of bottle + xylene oil = 44.0290g

Weight of bottle + Citrodora oil = 43.3708

Weight of bottle + Citrus oil = 43.1325g

Weight of bottle +water (W3) = 47.4091g

SG of neem $= \frac{45.3585-23.1026}{47.4091-23.1026} = 9.1g/ml$

SG of xylene $= \frac{44.0290-23.1026}{47.4091-23.1026} = 8.6g/ml$

SG of Citrodora $= \frac{43.3708-23.1026}{47.4091-23.1026} = 8.3g/ml$

SG of Citrus $= \frac{43.1325-23.1026}{47.4091-23.1026} = 8.2g/ml$

DENSITY

$$\text{Density} = \frac{W2-W1}{V}$$

Where weight of empty bottle (W1) =23.1026

Volume of oil (V) = 25ml

W2= weight of the + oil

Weight of bottle + neem oil = 45.3585g

Weight of bottle + xylene oil = 44.0290g

Weight of bottle + Citrodora oil = 43.3708

Weight of bottle + Citrus oil = 43.1325g

Density of neem = $\frac{45.3585-23.1026}{25}$ = 0.89g/ml

Density of xylene = $\frac{44.0290-23.1026}{25}$ =0.84g/ml

Density of Citrodora = $\frac{43.3708-23.1026}{25}$ =0.81g/ml

Density of citrus = $\frac{43.1325-23.1026}{25}$ =0.80g/ml

FREE FATTY ACID (FFA)

FFA = $\frac{\text{titre x M x 28.2}}{\text{Weight of sample}}$

Where molarity of base (M) = 0.1

Weight of sample = 1g

Titre of neem = 8.3

Titre of xylene =3.9

Titre of citrodora =2.0

Titre of citrus =2.2

FFA of neem = $\frac{8.3 \times 0.1 \times 28.2}{1}$ = 23.4

FFA of xylene = $\frac{3.8 \times 0.1x 28.2}{1}$ =10.9

FFA of citrodora = $\frac{2.0 \times 0.1 \times 28.2}{1}$ =5.6

FFA of citrus = $\frac{2.1 \times 0.1 \times 28.2}{1}$ =5.9

ACID VALUE (AV)

AV = $\frac{\text{titre x M x 28.2}}{\text{Weight of sample}}$

Where molarity of base (M) = 0.1

Weight of sample = 1g

Titre of neem = 13.4

Titre of xylene = 6.0

Titre of citrodora = 4.2

Titre of citrus = 12.2

AV of neem = $\frac{13.4 \times 0.1 \times 28.2}{1}$ = 37.8

AV of xylene = $\frac{6.0 \times 0.1 \times 28.2}{1}$ =16.9

AV of citrodora = $\frac{4.2 \times 0.1 \times 28.2}{1}$ =11.8

AV of citrus = $\frac{12.2 \times 0.1 \times 28.2}{1}$ =34.4

SAPONIFICATION VALUE (SV)

$$SV = \frac{(B-S) \times 28.05}{W}$$

Where B = titre value of blank, S = titre value of sample, W = weight of oil

Titre value of blank = 78

Titre value of neem = 65

Titre value of xylene = 70

Titre value of citrodora = 71

Titre value of citrus = 72

SV of neem = $\frac{(78 - 65) \times 28.05}{2}$ = 187

SV of xylene = $\frac{(78 - 70) \times 28.05}{2}$ = 112

SV of citrodora = $\frac{(78 - 71) \times 28.05}{2}$ = 9

SV of citrus = $\frac{(78 - 72) \times 28.05}{2}$ = 84

IODINE VALUE (IV)

$$IV = = \frac{(B-S) \times 12.69 \times N}{W}$$

Where B = titre value of blank, S = titre value of sample, N = normality of $Na_2S_2O_3$

W = weight of oil. Titre value of blank =65. Normality of $Na_2S_2O_3$ = 0.1

Weight of oil = 1

Titre value of neem = 42

Titre value of xylene = 50

Titre value of citrodora = 52

Titre value of citrus = 47

IV of neem = $\frac{(65 -42) \times 12.69 \times 0.1}{1} = 29$

IV xylene = $\frac{(65-50) \times 12.69 \times 0.1}{1} = 19$

IV of citrodora = $\frac{(65\ 52) \times 12.69 \times 0.1}{1} = 18$

IV of citrus = $\frac{(65 -48) \times 12.69 \times 0.1}{1} = 21$

POUR POINT (PP)

PP of neem = 5%**,** PP of xylene = -35%**,**

PP of citrodora = -37%, PP of citrus = -34%

CLOUD POINT (CP)

CP of neem = 2%**,** CP of xylene = -65%**,**

CP of citrodora = -69%**,** CP of citrus= -67%

FLASH POINT (FP)

Neem = 108 ^{0}C**,** Xylene = 39^{0}C**,**

Citrodora = 35^{0}C**,** Citrus = 34^{0}C

VISCOSITY (V)

Neem = 36cp at 28%**,** Xylene = 0.45cp at 28%**,**

Citrodora = 0.34cp at 28%**,** Citrus= 0.35cp at 28%

Cp is centipoises.

CALCULATION OF PERCENTAGE YIELD OF THE OILS

Essential oils yield (%) = W_1 / W_2 X 100

W1 = net weight of oils (grams)

W2 = total weight of fresh leaves (grams)

APPENDIX III

SCORING SHEET OF STAINED SLIDE

Instruction: The type of oil and tissue examined should be scored and entered using the score marks below for each slide.

..... OIL

..... TISSUE	15min	30min	1 hours	2 hours
Section quality (folds ,crack, artefacts)				
Staining quality ((colour brightness, nuclear/cytoplasmic contrast)				

..... TISSUE	15min	30min	1 hours	2 hours
Section quality (folds, crack, artefacts)				
Staining quality ((colour brightness, nuclear/cytoplasmic contrast)				

..... TISSUE	15min	30min	1 hours	2 hours
Section quality (folds ,crack, artefacts)				
Staining quality (colour brightness, nuclear/cytoplasmic contrast)				

Key

IIIII	-	Excellent	(5 marks)
IIII	-	Very good	(4 marks)
III	-	Good	(3 marks)
II	-	Fair	(2 marks)
I	-	Poor	(1mark)