COMPARATIVE STUDY OF EUCALYPTUS (CITRODORA) OIL AND XYLENE ASCLEARING AND DEWAXING AGENTS IN HISTOLOGICAL AND IMMUNOHISTOCHEMICAL TISSUE PROCESSING AND STAINING

BY

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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF DOCTOR OF PHILOSOPHY IN HUMAN ANATOMY

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ZARIA, NIGERIA

APRIL, 2021

DECLARATION

I declare that the work in this thesis entitled "Comparative Study of Eucalyptus Oil (Citrodora) and Xylene as Clearing and Dewaxing Agents in Histological and Immunohistochemical Tissue Processing and Staining" has been performed by me in the Department of Human Anatomy Ahmadu Bello University Zaria. The information derived from the literature has been dulyacknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any otherInstitution.

…….	……	……
Name of Student	**Signature**	**Date**

CERTIFICATION

This thesis entitled "COMPARATIVE STUDY OF EUCALYPTUS OIL (CITRODORA) AND XYLENE AS CLEARING AND DEWAXING AGENTS IN HISTOLOGICAL AND IMMUNOHISTOCHEMICAL TISSUE PROCESSING AND STAINING"by Peter Stephen AKPULU meets the regulations governing the award of the degree of Doctor of Philosophy in Human Anatomy of Ahmadu Bello University, and is approved for its contribution to knowledge and literacy presentation.

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DEDICATION

I dedicate this work to my wife,Mrs Peter, children: Stephen, Daniel, Magdaline, Sarah and my parents, Mr and Mrs Stephen Akpulu.

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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 have 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. The present study was aimed at evaluating the clearing and dewaxing effect of Eucalyptus(citrodora oil), and Xylene during histological and histochemical tissue processing and staining of some selected organs of adults Wistar rats. Citrodora oil was used alongside xylene to clear livers, kidney, brain, and testicular tissues of adult Wistar rats. The Citrodora oil was extracted from the leaves of eucalyptus plants by hydro distillation method. The physical properties, the phytochemical, Gas Chromatography-Mass Spectroscopy (GC-MS) and Nuclear Magnetic Resonancestudies of the oil were carried out using standard procedures. The tissues were also histologically processed using standard method. The resultsshowed that Citrodora oil was pale yellow in color. The densities of theCitrodora oil and xylene were0.80g/ml and 0.84 g/ml, respectively. The viscosity of the Citrodora oil was 0.34cp and that forxylene was 0.45cp. The flash point of Citrodorawas at 35^0C, while that of xylene, was at 39^0C. The refractive indexes ofCitrodora oil and xylene were 1.46 and 1.50,respectively. The GC-MS results of Citrodora oil shows that the predominant constituent in Citrodora oil iscitronellal with relative abundance of 42.20% followed by β-Citronellol (21.71%). Xylene contains 94% of o-xylene, 97% of p-xylene, 98% of m-xylene and 98%p-xylene. The nuclear magnetic resonance showed that all the chemical shifts are positive to the reference and are made up of mainly triglycerides. Histological, histochemical and immunohistochemical observation showed that the section quality, the cytoplasmic and nuclear staining qualities appeared to give good contrast in Citrodora oil when compared with xylene.

There was nostatisticallysignificantdfferences($p \geq 0.05$)in the section quality and staining ability of liver, kidney, brain and testicular tissues of Wistar rats clearedin Citrodora oil and Xylene compared. This present study concludes that Citrodora oil can clear and dewax Wistar rat tissues during tissue and staining processes for histological, histochemical and immunohistochemical tissue processing and staining as xylene.

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CHAPTER ONE

1.0 Introduction

1.1 Background Information

The preparation of tissue to enable the microscopic examination involves a series of treatments of the tissue prior to the cutting of the sections from the tissue specimens, for the staining and mounting of the sections on the microscope slides (Adeniyi *et al.,* 2016). In the preparation of the tissue, it is necessary to immerse the tissue successively in a series of liquid agents for certain periods as follows: First to fix the tissue, washing of tissue to remove the fixative, then to dehydrate the tissue, usually by immersion of the tissue successively in graded alcohols or other dehydrating agents, immerse the tissue in a clearing agent, and thereafter to infiltrate the tissue with an infiltration agent such as, paraffin wax, celloidin, paraplast, gelatin and then embedded usually in the same infiltrating agents (Ankle and Joshi, 2011).

The purpose of treating tissue with a clearing agent before infiltrating the tissue with paraffin is to eliminate from the tissue the dehydrating agents.If the dehydrating agent is not removed, it will decrease the refractive index of the tissue thereby interfering with the thorough infiltration of the infiltrating agent into the tissue. This may result in poorly cut tissue sections. It is also very important that the clearing agent be of such character that does not harden the tissue and does not impair the cytological structures of the tissue. Furthermore, it is desirable that the clearing agent be non-inflammable, nontoxic and biodegradable (Radhika *et al*., 2016)

Most Histological and Histopathological Laboratories use either aromatic solvents, such as xylene, toluene or aliphatic petroleum distillates for the purpose of clearing and de-waxing in paraffin histological technique **(**Ankle and Joshi, 2011). 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 its hydrophobic nature; therefore, water present in tissues has to be removed by dehydration using graded alcohols prior to infiltration and embedding. Following dehydration, the tissues are saturated with alcohol which is also not miscible with paraffin wax (Baker and Silverton, 1998). Clearing agents are used to remove alcohols from the tissue before the tissue can be infiltrated with paraffin wax. 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 (Kieranan, 2010).

There are many clearing agens, such as xylene, toluene, chloroform, acetone, kerosene, diaxane, benzene, petrol, methyl salicylate and cedar wood oil. Most clearing agents are derivatives of aromatic hydrocarbons, such as benzene, while others are derived from natural essential oils, such as cedar wood oil and olive oil (Hans *et al.,* 1995).

1.2 Statement of Research Problem

Clearing agents are among the most noxious and hazardous chemicals found in histology and histochemical 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 of its cost, and the health hazard to personnel and the environment (Bush, 1977; Maxwell, 1978; Hans *et al.,* 1995). The need to reduce laboratory hazard has been a challenge. During tissue processing and staining, most of the clearing agents used are among the most noxious and hazardous chemicals with different levels of toxicity (Dapson and Richard, 2005). Several toxicities believed to be caused by intermediate products of xylene metabolism, such as metylbenzaldehyde have been reported (Indu*et al,* 2014, Ankle *et al.*2011). These include central nervous system disorders, respiratory depression, abdominal

pain, dryness and redness of skin, dermatitis, liver diseases, nephrotoxicity, conjunctivitis, and teratogenic fetotoxic effects. These are in addition to environmental pollution from unsafe disposal of xylene (Ankle *et al.,* 2011) and tissue distortions as a result of long-term immersion in xylene (Hans *et al.,* 1995).

There have been several attempts to substitute toxic clearing agent and provide nontoxic and bio friendly alternative clearing agents. Several attempts have been made to use xylene alternatives as clearing agents, by mixing vegetable oils such as groundnut oil, palm kernel oil and coconut oil, either alone or as mixture with other clearing agents (Adeneyi*et al.,* 2016). Orange based oils as clearing agents have also been reported by Rene (2000). Some essential oil such as olive, Clove, Coconut oil and Cedal wood oil has been reported (Hans *et al.,* 1995). However, most of these commercially available xylene alternatives are less effective, more expensive, and are not as readily available as xylene (Gosselin *et al.*, 1984; Amdur*et al.,* 1991; Luna, 1992). There is little or no report of work on the use of Citrodora oil as tissue clearing and dewaxing agent in histological and immunohistochemical tissue processing and staining techniques.

1.3 Justification

Several attempts to substitute xylene and other hazardous clearing and dewaxing agents have been commercially developed, some being aromatic derivatives of terpene, a natural oils and resins produced by some plants and animals (Jim, 2007). Other clearing agents are hydrocarbons and most of these alternatives are either less effective or more expensive (Kunhua*et al.,* 2012).Citrodora oil may be used as a substitute to xylene thereby, reducing exposure to xylene hazard in histology and histochemical laboratories and the environment. Citrodora oil is cheap, effective, non-toxic, readily available and easy to use Therefore, the need to explore the use of Citrodora oil as xylene alternative as tissue clearing and dewaxing agents, can not be overemphasized.

1.4 Significance of the Study

Results from this study could identify Citrodora oil as a possible tissue clearing agent and as a xylene alternative during histological and immunohistochemical tissue processing and staining which may be effective, non-toxic, readily available, easy to use and environmentally friendly.

1.5 Aim of the Study

The aim of this study was to evaluate the tissue clearing and dewaxing effect of Eucalyptus (Citrodora) oil and xylene, on some selected organs of adult Wistar rat after histological and immunohistochemical tissue processing and staining.

1.6 Objectives of the Study

The objectives of the study were to:

1. Screen the phytochemical and determne thephysical properties of the Citrodora oil
2. Dtermine the quantification and separation of the chemical contents of the Citrodora oil using Gas Chromatography and Mass Spectrometry (GC-MS) technique.
3. Determine the molecular structure, dynamics, reaction state and phase changes of Citrodora oil using Nuclear Magnetic Resonance (NMR) and Fourier transform infrared spectroscopyitrodoratechniques.
4. Evaluate the clearing and dewaxing effect of the Citrodora oil on brain, liver, kidney, and testes of adult Wistar rat when used during tissue processing.
5. Evaluate the histological and immunohistochemical staining qualities of brain, liver, kidney and testes tissues of adult Wistar rat after clearing and de-waxing in the Citrodora oil when compared to xylene.

1.7 Hypothesis

i. Citrodora oil will have a better clearing qualities than xylene in histological and histochemical tissues processing.

ii. There will beno difference between Citrodora oil and xylene when used in animal tissue processing and staining techniques.

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 alcohol 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.Impregnationis 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

2.0.1 Alternative Clearing Agent

Several tissue clearing substitutes have been commercially developed, 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, have been comparatively used in tissue processing (Busa, 2009).Most of these commercially available xylene substitutes are less effective, more expensive, not readily available and are constitute health hazard as or more than xylene itself (Udonkang*et al.,*2014). 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 a clearing agent. This compound has a high vapor pressure but is nonflammable and less toxic than xylene. However, its use as ananaesthetic agent precludes it being used as serious clearing agent (Maxwell, 1978).

Xylene has probably been the most commonly used clearing agent in the histology laboratory despite its hazards. Xylene is an aromatic hydrocarbon consisting of a benzene ring with two methyl substituents (C_6H_4 (CH 3)$_2$). It is costly, but work well for short time clearing of small tissue blocks. Its high solvency factor allows maximum displacement of alcohol and enhancing paraffin infiltration (Tardif and Brodeur1992; Carson and Hladik, 2009). Xylene tends to harden tissues a little, but this does not usually interfere with sectioning qualities (Kieranan, 2010). Long term immersion of tissue in xylene results in tissue distortions (Visfeldt*et al*., 1982).

Xylene has been reported to affect the skin, eyes, nervous system, blood, liver and kidneys of animals exposed to it and can potentially contaminate the working environment (Ankle and Joshi, 2001).

Histo-Clear®is another xylene substitutes that is commercially produced 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 bonds. 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 is paraffin. Although the tissue is ultimately embedded in it, 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 previous works 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 decomposition. 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 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 hydrocarbons 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 oilis 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 agents 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, defeat the purpose of the replacement (Rene, 2000).

2.0.2 Mechanism of Tissue Clearing

It is well known that tissues are densely packed with many types of substances, including scattering particles with higher refractive index (RI), i.e., collagen, elastic fibers, cells and cell

compartments, and surrounding media with lower refractive index, i.e., interstitial fluid and/or cytoplasm. This architecture makes light travel at different speeds and angles because each component has a different refractive index Azaripour*et al.* (2016). Tissue optical clearing (TOC) method, proposed by Tuchin and coworkers, involve using immersion of tissues into optical-clearing agents (OCAs) that reduces the scattering of tissue and make tissue more transparent. Typical OCAs usually have high refractive index and, thus, penetration of OCAs into the extracellular space matches the refractive indices of the scatters and their surrounding media, and then lead to reduction of the light scattering. This process leads to significantly enhancement of the imaging depth as light penetrates deeper into the tissues Poguzhelskaya*et al*. (2013).

The diffusion of optical-clearing agents with higher refractive indices and higher osmolality into tissues will match the refractive indices of tissue components with extracellular fluid, thus reducing the scattering of tissue. This is regarded as the major mechanism of TOC. However, in-vitro experiments demonstrated that optical-clearing efficacy of skin did not correlate with refractive indices of OCAs, but with the molecular structure of OCAs, which was further supported by molecular dynamics simulation Dan e*t al*. (2013). Furthermore, additional mechanisms such as dissociation of collagen fibers and tissue dehydration caused by OCAs have been proposed to explain the mechanism of TOC based on experimental results. However, for in-vivo tissue optical clearing, only the thickness of dermis and the diameter of collagen fiber were reduced, but there was no dissociation of collagen fiber as observed during in vitro skin optical clearing studies Wen *et al.* (2010). Different views on the mechanisms of optical clearing are likely due to different experimental protocols and techniques as well as difference in studied clearing agents and the complex of mechanical and chemical properties for different tissues.

2.0.3 Physical mechanism of optical clearing

In order to eliminate the effect of tissue inhomogeneity, Wen *et al*. (2010) performed studies on a simple tissue-simulating phantom based on Intralipid. Intralipid is a homogeneous fat emulsion consisting of lipid droplets dissolved in water. Even though it has no collagen structure like tissue, it is a commonly used compound that simulates tissue optical properties Poguzhelskaya*et al*. (2013). They mixed six different common OCAs, such as DMSO, glycerol, 1,4-butanediol, 1,2-propanediol, PEG200, PEG400 and water with 10%-Intralipid, respectively, while keeping the concentration of scattering particles of the mixtures constant. The results demonstrated that 5% Intralipid without any OCAs completely conceals a pattern under the sample box, while the addition of OCAs can make the Intralipid transparent at different degrees, and the pattern became visible. Also, after adding PEG200 and PEG400, the mixture becomes more transparent but nonuniform due to aggregation of particles. The other four OCAs can keep the mixture uniform, and the optical-clearing efficacy decreases gradually for DMSO, glycerol, 1,4-butanediol, 1,2-propanediol, respectively, whose refractive indices reduce gradually. Additionally, the reduced scattering coefficients of uniform mixtures were estimated with Mie theory based on the particle-size distribution measured by an electron microscopy and the refractive index of the background medium. Also, a commercially available spectrophotometer. (Lambda 950, PerkinElmer, USA) with an integrating sphere of 150 mm diameter was used to measure the transmittance and the reflectance of the sample and then the inversed adding-doubling (IAD) algorithm was used to calculate the reduced scattering coefficient. The results demonstrated that the theoretical calculations match well to the experimental results for different OCAs, which has a good consistency with direct observation.

Therefore, the mechanisms of TOC could be due to two aspects: one is the interaction between OCAs and tissue phantom, which makes the phantom more transparent but nonuniform from the direct observation; and the other is the enhancement of refractive index of the background

medium caused by OCAs according to the theoretical estimation and measurement for the uniform mixture.

2.0.4 Other mechanism of optical clearing

Besides the ability of OCAs to match the refractive indices of tissue components, tissue dehydration can be an important mechanism of TOCGurjarpadhye*et al.* (2011). Application of a hyperosmotic OCA to the tissue surface induces water flux from the interstitial space to the tissue surface and even out of the tissue, increases the osmolality of interstitial fluid (ISF), and, consequently, draws water from cells and/or collagen fibers. These processes give an additional refractive-index matching effect due to reduction of water content in the interstitial space, and reduce the overall thickness of tissue and make it denser (more ordered). All these effects raise the optical transmittance and reduce diffuse reflectance of the tissue. Indeed, the dehydration of tissue commonly happens during the optical-clearing process Poguzhelskaya*et al.* (2013).

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 so 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

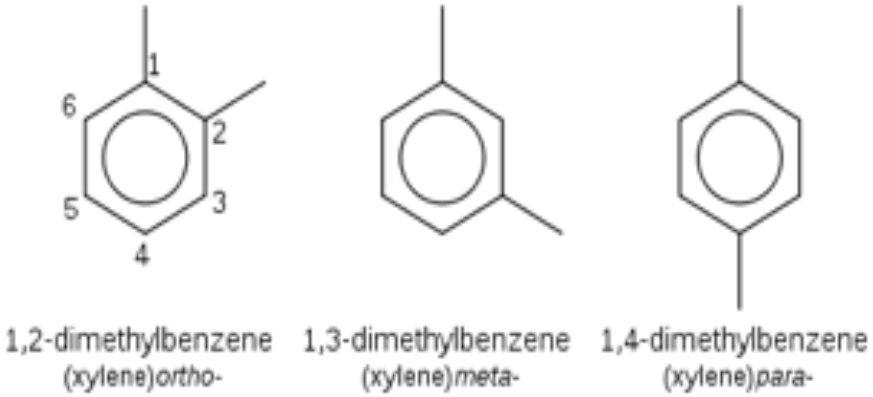


Fig: 2.1: Structural Isomers of Xylene. (Source: Wikipedia, the free encyclopedia, 2019)

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 reformateand from coal carbonization derived from coke ovens. It is also produced by dehydrocyclodimerization and by methylating of toluene and benzene (Martindale and Kuchar, 2012).

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. presented in (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 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 and 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). The *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 dewaxing 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 have 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 (Gagnaire*et al.,* 2001).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.

2.3.4.1*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).

2.3.4.2 *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).

2.3.4.3 *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 carbonatoms 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).

2.3.4.4 *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 as 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

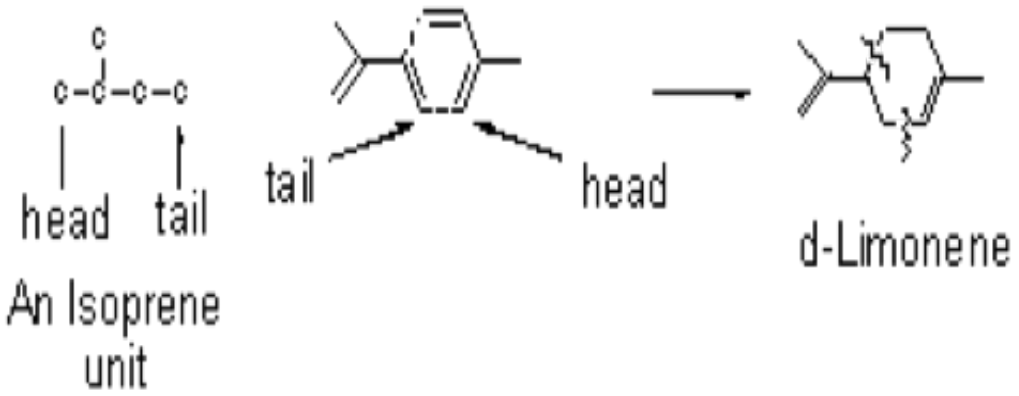


Fig: 2.2: Isoprene units in the formulation of terpenes and terpenoids. (Sourcs: Wikipedia, the free encyclopedia, 2019)

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 Sources of natural essential oil

Essential oils are generally derived from one or more plant parts, such as flowers (e.g. rose, jasmine, carnation, clove, mimosa, rosemary, lavander), leaves (e.g. mint, *Ocimum*spp., lemongrass, jamrosa), leaves and stems (e.g., geranium, patchouli, petitgrain, verbena, cinnamon), bark (e.g., cinnamon, cassia, canella), wood (e.g., cedar, sandal, pine), roots (e.g. angelica, sassafras, vetiver, saussurea, valerian), seeds (e.g., fennel, coriander, caraway, dill, nutmeg), fruits (bergamot, orange, lemon, juniper), rhizomes (e.g., ginger, calamus, curcuma, orris) and gums or oleoresin exudations (e.g., balsam of Peru, *Myroxylonbalsamum*, storax, myrrh, benzoin).

2.4.3 Productionand Extraction of Essential Oils

2.4.3.1 *Uses and extraction of essentionls oils*

Essential oils are used in a wide variety of consumer goods such as detergents, soaps, toilet products, cosmetics, pharmaceuticals, perfumes, confectionery food products, soft drinks, distilled alcoholic beverages (hard drinks) and insecticides. The world production and consumption of essential oils and perfumes are increasing very fast. Production technology is an essential element to improve the overall yield and quality of essential oil. Essential oils are obtained from plant raw material by several extraction methods (Hesham *et al.,* 2016). The techniques and methods first used to produce essential oils was first mentioned by Ibn al-Baitar (1188–1248), an Andalusianphysician, pharmacist and chemist (Standards Australia,2001). Essential oils are used in a wide variety of consumer goods such as detergents, soaps, toilet products, cosmetics, pharmaceuticals, perfumes, confectionery food products, soft drinks, distilled alcoholic beverages (hard drinks) and insecticides. The world production and consumption of essential oils and perfumes are increasing very fast. Production technology is an essential element to improve the overall yield and quality of essential oil. The traditional

technologies pertaining to essential oil processing are of great significance and are still being used in many parts of the globe.

2.4.3.2*Extraction Method*

Water distillation, water and steam distillation, steam distillation, cohobation, maceration and enfleurage are the most traditional and commonly used methods. Maceration is adaptable when oil yield from distillation is poor. Distillation methods are good for powdered almonds, rose petals and rose blossoms, whereas solvent extraction is suitable for expensive, delicate and thermally unstable materials like jasmine, tuberose, and hyacinth. Water distillation is the most favored method of production of citronella oil from plant material (Standards Australia, 2001).

2.4.3.3 *Hydrodistilation*

Hydrodistillation is a traditional method for removal of essential oils. Water or hydrodistillation is one of the oldest and easiest methods (Meyer-Warnod*et al*., 1984). The essential oils industry has developed terminology to distinguish three types: water distillation; water and steam distillation; and direct steam distillation. Originally introduced by Von Rechenberg, these terms have become established in the essential oil industry. All three methods are subject to the same theoretical considerations which deal with distillation of two-phase systems. The differences lie mainly in the methods of handling the material. Some volatile oils cannot be distilled without decomposition and thus are usually obtained by expression (lemon oil, orange oil) or by other mechanical means. In certain countries, the general method for obtaining citrus oil involves puncturing the oil glands by rolling the fruit over a trough lined with sharp projections that are long enough to penetrate the epidermis and pierce the oil glands located within outer portion of the peel (*ecuelle*method). A pressing action on the fruit removes the oil from the glands, and a fine spray of water washes the oil from the mashed peel while the juice is extracted through a central tube that cores the fruit. The resulting oil-water emulsion is separated by centrifugation.

A variation of this process is to remove the peel from the fruit before the oil is extracted. Often, the volatile oil content of fresh plant parts (flower petals) is so small that oil removal is not commercially feasible by the aforementioned methods. In such instances, an odorless, bland, fixed oil or fat is spread in a thin layer on glass plates. The flower petals are placed on the fat for a few hours; then repeatedly, the oil petals are removed, and a new layer of petals is introduced. After the fat has absorbed as much fragrance as possible, the oil may be removed by extraction with alcohol. This process, known as enfleurage, was formerly used extensively in the production of perfumes and pomades. In the perfume industry, most modern essential oil production is accomplished by extraction, using volatile solvents such as petroleum ether and hexane. The chief advantages of extraction over distillation is that uniform temperature (usually 50° C) can be maintained during the process, As a result, extracted oils have a more natural odor that is unmatched by distilled oils, which may have undergone chemical alteration by the high temperature. This feature is of considerable importance to the perfume industry; however, the established distillation method is of lower cost than the extraction process (Wang and Weller, 2006).

Destructive distillation means distilling volatile oil in the absence of air. When wood or resin of members of the Pinaceae or Cupressaceae is heated without air, decomposition takes place and a number of volatile compounds are driven off. The residual mass is charcoal. The condensed volatile matter usually separates into 2 layers: an aqueous layer containing wood naptha (methyl alcohol) and pyroligneous acid (crude acetic), and a tarry liquid in the form of pine tar, juniper tar, or other tars, depending on the wood used. This dry distillation is usually conducted in retorts and, if the wood is chipped or coarsely ground and the heat is applied rapidly, the yield often represents about 10% of the wood weight used (Dick and Starmans,1996).

In order to isolate essential oils by hydrodistilation, the aromatic plant material is packed in a still and a sufficient quantity of water is added and brought to a boil; alternatively, live steam is injected into the plant charge. Due to the influence of hot water and steam, the essential oil is freed from the oil glands in the plant tissue. The vapor mixture of water and oil is condensed by indirect cooling with water. From the condenser (Fig.2.3), distillate flows into a separator, where oil separates automatically from the distillate water (Hesham *et al.,* 2016).

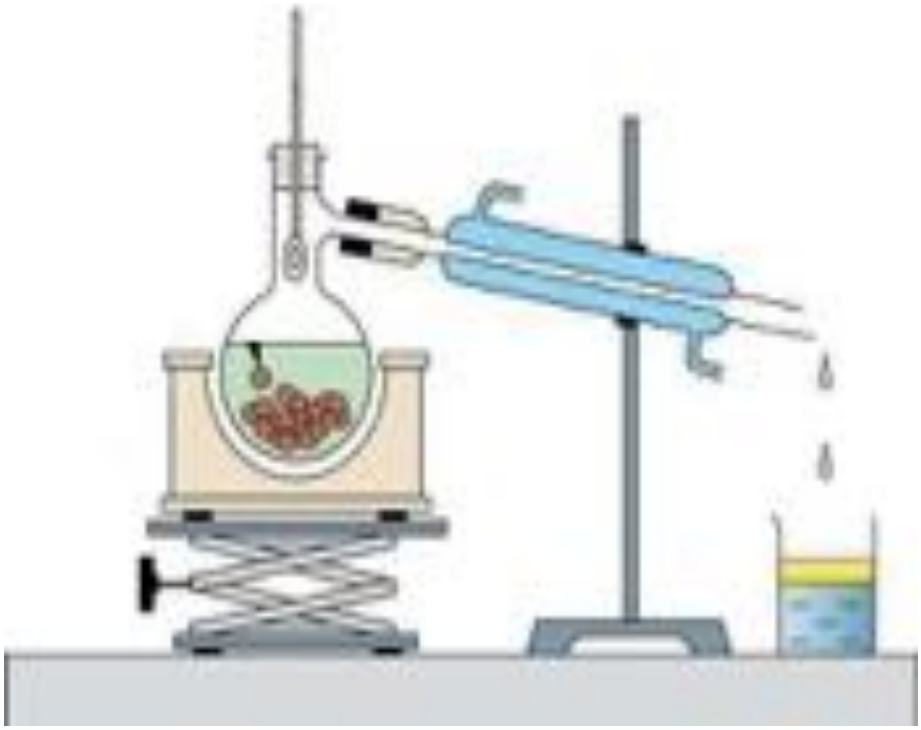


Fig 2.3: hydrodistilation (Hesham *et al.,* 2016).

2.4.3.4 *Mechanism of Distillation*

Hydrodistillation of plant material involves the following main physicochemical processes:

i) Hydrodiffusion

ii) Hydrolysis

iii) Decomposition by heat

i) Hydro diffusion

Diffusion of essential oils and hot water through plant membranes is known as hydro diffusion. In steam distillation (Fahlbusch*et al*., 2003),the steam does not actually penetrate the dry cell membranes. Therefore, dry plant material can be exhausted with dry steam only when all the volatile oil has been freed from the oil-bearing cells by first thorough comminution of the plant material. But, when the plant material is soaked with water, exchange of vapors within the tissue is based on their permeability while in swollen condition. Membranes of plant cells are almost impermeable to volatile oils. Therefore, in the actual process, at the temperature of boiling water, a part of volatile oil dissolves in the water present within the glands, and this oil-water solution permeates, by osmosis, the swollen membranes and finally reaches the outer surface, where the oil is vaporized by passing steam. Another aspect of hydro diffusion is that the speed of oil vaporization is not influenced by the volatility of the oil components, but by their degree of solubility in water. Therefore, the high-boiling but more water-soluble constituents of oil in plant tissue distill before the low boiling but less water-soluble constituents. Since hydro diffusion rates are slow, distillation of uncomminuted material takes longer time than comminuted material (Hesham *et al.,* 2016).

ii) Hydrolysis

Hydrolysis in the present context is defined as a chemical reaction between water and certain constituents of essential oils. Esters are constituents of essential oils and, in the presence of

water, especially at high temperatures, they tend to react with water to form acids and alcohols. However, the reactions are not complete in either direction and the relationship between the molal concentrations of various constituents at equilibrium is written as:

$$K = \frac{(\text{alcohol}) \times (\text{acid})}{(\text{ester}) \times (\text{water})}$$

where K is the equilibrium constant.

Therefore, if the amount of water is large, the amounts of alcohol and acid will also be large, resulting in a decreased yield of essential oil. Furthermore, since this is a time-dependent reaction, the extent to which hydrolysis proceeds depends on the time of contact between oil and water. This is one of the disadvantages of water distillation (Hesham *et al.,* 2016).

iii) Decomposition by Heat

Almost all constituents of essential oils are unstable at high temperature. To obtain the best quality oil, distillation must be done at low temperatures. The temperature in steam distillation is determined entirely by the operating pressure, whereas in water distillation and in water and steam distillation the operating pressure is usually atmospheric. All the previously described three effects, i.e., hydro diffusion, hydrolysis and thermal decomposition, occur simultaneously and affect one another. The rate of diffusion usually increases with temperatures as does the solubility of essential oils in water. The same is true for the rate and extent of hydrolysis. However, it is possible to obtain better yield and quality of oils by: (1) maintaining the temperature as low as possible, (2) using as little water as possible, in the case of steam distillation, and (3) thoroughly comminuting the plant material and packing it uniformly before distillation (Hesham *et al.,* 2016).

2.4.3.5 *Types of Hydro distillation*

There are three types of hydro distillation for isolating essential oils from plant materials:

1. Water distillation

2. Water and steam distillation

3. Direct steam distillation

1. Water Distillation

In this method, the material is completely immersed in water, which is boiled by applying heat by direct fire, steam jacket, closed steam jacket, closed steam coil or open steam coil. The main characteristic of this process is that there is direct contact between boiling water and plant material. When the still is heated by direct fire, adequate precautions are necessary to prevent the charge from overheating. When a steam jacket or closed steam coil is used, there is less danger of overheating; with open steam coils this danger is avoided. But with open steam, care must be taken to prevent accumulation of condensed water within the still. Therefore, the still should be well insulated. The plant material in the still must be agitated as the water boils, otherwise agglomerations of dense material will settle on the bottom and become thermally degraded (Hesham *et al.,* 2016). Certain plant materials like cinnamon bark, which are rich in mucilage, must be powdered so that the charge can readily disperse in the water; as the temperature of the water increases, the mucilage will be leached from the ground cinnamon. This greatly increases the viscosity of the water-charge mixture, thereby allowing it to char. Consequently, before any field distillation is done, a small-scale water distillation in glassware should be performed to observe whether any changes take place during the distillation process. From this laboratory trial, the yield of oil from a known weight of the plant material can be determined. The laboratory apparatus recommended for trial distillations is the Clevenger system. During water distillation, all parts of the plant charge must be kept in motion by boiling water; this is possible when the distillation material is charged loosely and remains loose in the boiling water. For this reason only, water distillation possesses one distinct advantage, i.e. that it permits processing of finely

powdered material or plant parts that, by contact with live steam, would otherwise form lumps through which the steam cannot penetrate. Other practical advantages of water distillation are that the stills are inexpensive, easy to construct and suitable for field operation. These are still widely used with portable equipment in many countries. The main disadvantage of water distillation is that complete extraction is not possible. Besides, certain esters are partly hydrolyzed and sensitive substances like aldehydes tend to polymerize. Water distillation requires a greater number of stills, more space and more fuel. It demands considerable experience and familiarity with the method. The high-boiling and somewhat water-soluble oil constituents cannot be completely vaporized or they require large quantities of steam. Thus, the process becomes uneconomical. For these reasons, water distillation is used only in cases in which the plant material by its very nature cannot be processed by water and steam distillation or by direct steam distillation (Hesham *et al.,* 2016).

Disadvantages of Water Distillation

a. Oil components like esters are sensitive to hydrolysis while others like acyclic monoterpene hydrocarbons and aldehydes are susceptible to polymerization (since the pH of water is often reduced during distillation, hydrolytic reactions are facilitated).

b. Oxygenated components such as phenols have a tendency to dissolve in the still water, so their complete removal by distillation is not possible.

c. As water distillation tends to be a small operation (operated by one or two persons), it takes a long time to accumulate much oil, so good quality oil is often mixed with bad quality oil.

d. The distillation process is treated as an art by local distillers, who rarely try to optimize both oil yield and quality.

e. Water distillation is a slower process than either water and steam distillation or direct steam distillation.

2. Water and Steam Distillation

In water and steam distillation, the steam can be generated either in a satellite boiler or within the still, although separated from the plant material (Chrissie *et al*., 1996). Like water distillation, water and steam distillation is widely used in rural areas. Moreover, it does not require a great deal more capital expenditure than water distillation. Also, the equipment used is generally similar to that used in water distillation, but the plant material is supported above the boiling water on a perforated grid. In fact, it is common that persons performing water distillation eventually progress to water and steam distillation (Fig 2.4). It follows that once rural distillers have produced a few batches of oil by water distillation, they realize that the quality of oil is not very good because of its still notes (subdued aroma). As a result, some modifications are made. Using the same still, a perforated grid or plate is fashioned so that the plant material is raised above the water. This reduces the capacity of the still but affords a better quality of oil. If the amount of water is not sufficient to allow the completion of distillation, a cohobation tube is attached and condensate water is added back to the still manually, thereby ensuring that the water, which is being used as the steam source, will never run out. It is also believed that this will, to some extent, control the loss of dissolved oxygenated constituents in the condensate water because the re-used condensate water will allow it to become saturated with dissolved constituents, after which more oil will dissolve in it (Dawidowicz*et al*., 2008; Hesham *et al.,* 2016).

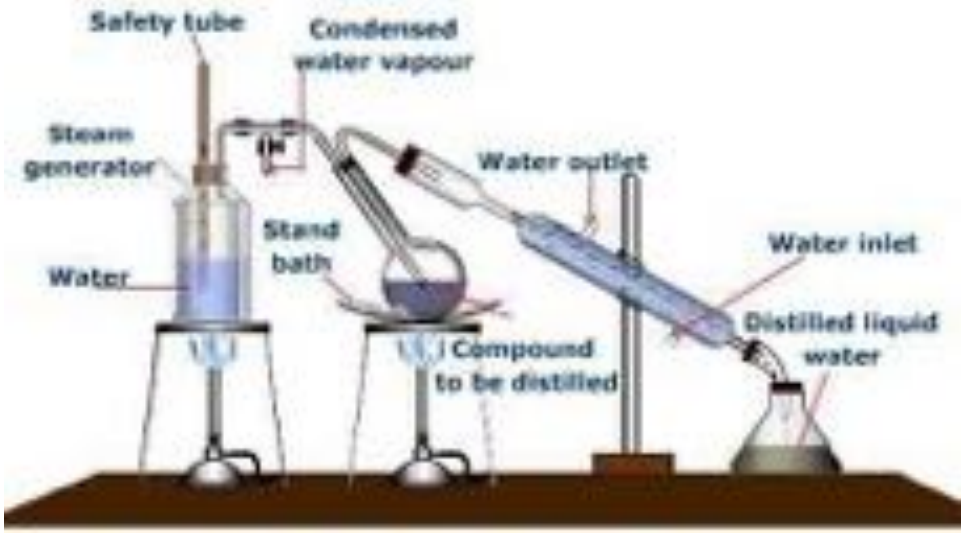


Fig 2.4: Water and Steemdiastilation (Hesham *et al.,* 2016).

Cohobation

Cohobation is a procedure that can only be used during water distillation or water and steam distillation. It uses the practice of returning the distillate water to the still after the oil has been separated from it so that it can be re-boiled. The principal behind it is to minimize the losses of oxygenated components, particularly phenols which dissolve to some extent in the distillate water. For most oils, this level of oil loss through solution in water is less than 0.2%, whereas for phenol-rich oils the amount of oil dissolved in the distillate water is 0.2%-0.7%. As this material is being constantly re-vaporized, condensed and re-vaporized again, any dissolved oxygenated constituents will promote hydrolysis and degradation of themselves or other oil constituents. Similarly, if an oxygenated component is constantly brought in contact with a direct heat source or side of a still, which is considerably hotter than 100° C, then the chances of degradation are enhanced. As a result, the practice of cohobation is not recommended unless the temperature to which oxygenated constituents in the distillate are exposed is no higher than 100° C. In steam and water distillation, the plant material cannot be in direct contact with the fire source beneath the still; however, the walls of the still are good conductors of heat so that still notes can also be obtained from the thermal degradation reactions of plant material that is touching the sides of the still. As the steam in the steam and water distillation process is wet, a major drawback of this type of distillation is that it will make the plant material quite wet. This slows down distillation as the steam has to vaporize the water to allow it to condense further up the still. One way to prevent the lower plant material resting on the grid from becoming waterlogged is to use a baffle to prevent the water from boiling too vigorously and coming in direct contact with the plant material (Dawidowicz*et al*., 2008; Hesham *et al.,* 2016).

Advantages of Water and Steam Distillation over Water Distillation

a. Higher oil yield.

b. Components of the volatile oil are less susceptible to hydrolysis and polymerization (the control of wetness on the bottom of the still affects hydrolysis, whereas the thermal conductivity of the still walls affects polymerization).

c. If refluxing is controlled, then the loss of polar compounds is minimized.

d. Oil quality produced by steam and water distillation is more reproducible.

e. Steam and water distillation is faster than water distillation, so it is more energy efficient.

Many oils are currently produced by steam and water distillation, for example lemongrass is produced in Bhutan with a rural steam and water distillation system.

Disadvantages of Water and Steam Distillation

a. Due to the low pressure of rising steam, oils of high-boiling range require a greater quantity of steam for vaporization -hence longer hours of distillation.

b. The plant material becomes wet, which slows down distillation as the steam has to vaporize the water to allow it to condense further up the still.

c. To avoid that the lower plant material resting on the grid becomes waterlogged, a baffle is used to prevent the water from boiling too vigorously and coming in direct contact with the plant material.

3. Direct Steam Distillation

As the name suggests, direct steam distillation is the process of distilling plant material with steam generated outside the still in a satellite steam generator generally referred to as a boiler. As in water and steam distillation, the plant material is supported on a perforated grid above the steam inlet. A real advantage of satellite steam generation is that the amount of steam can be readily controlled. Because steam is generated in a satellite boiler, the plant material is heated no

higher than 100° C and, consequently, it should not undergo thermal degradation. Steam distillation is the most widely accepted process for the production of essential oils on large scale. Throughout the flavor and fragrance supply business, it is a standard practice. An obvious drawback to steam distillation is the much higher capital expenditure needed to build such a facility. In some situations, such as the large-scale production of low-cost oils (e.g. rosemary, Chinese cedarwood, lemongrass, litseacubeba, spike lavender, eucalyptus, citronella, cornmint), the world market prices of the oils are barely high enough to justify their production by steam distillation without amortizing the capital expenditure required to build the facility over a period of 10 years or more (Dawidowicz*et al*., 2008; Hesham *et al.,* 2016).

Advantages of Direct Steam Distillation

a.Amount of steam can be readily controlled.

b. No thermal decomposition of oil constituents.

c. Most widely accepted process for large-scale oil production, superior to the other two processes.

Disadvantage of Direct Steam Distillation

a. Much higher capital expenditure needed for this activity than for the other two processes.

2.4.3.6 *Hydrolytic Maceration Distillation*

Certain plant materials require maceration in warm water before they release their essential oils, as their volatile components are glycosidically bound. For example, leaves of wintergreen (*Gaultheria procumbens*) contain the precursor gaultherin and the enzyme primeverosidase; when the leaves are macerated in warm water, the enzyme acts on the gaultherin and liberates free methyl salicylate and primeverose. Other similar examples include brown mustard (sinigrin), bitter almonds (amygdalin) and garlic (alliin).

2.4.3.7 *Essential Oil Extraction by Expression*

Expression or cold pressing, as it is also known, is only used in the production of citrus oils. The term expression refers to any physical process in which the essential oil glands in the peel are crushed or broken to release the oil. One method that was practiced many years ago, particularly in Sicily (*spugna*method), commenced with halving the citrus fruit followed by pulp removal with the aid of sharpened spoon-knife (known as a *rastrello*). The oil was removed from the peel either by pressing the peel against a hard object of baked clay (*concolina*) which was placed under a large natural sponge or by bending the peel into the sponge. The oil emulsion absorbed by the sponge was removed by squeezing it into the *concolina*or some other container. It is reported that oil produced this way contains more of the fruit odor character than oil produced by any other method. A second method known as equaling (or the *scodella*method), uses a shallow bowl of copper (or sometimes brass) with a hollow central tube; the equaling tool is similar in shape to a shallow funnel. The bowl is equipped with brass points with blunt ends across which the whole citrus fruit is rolled by hand with some pressure until all of the oil glands have burst. The oil and aqueous cell contents are allowed to dribble down the hollow tube into a container from which the oil is separated by decantation. Obviously, hand pressing is impractical because it is an extremely slow process, e.g. on average only 2-4 lbs oil per day can be produced by a single person using one of these hand methods. As a result, over the years a number of machines have been designed to either crush the peel of a citrus fruit or crush the whole fruit and then separate the oil from the juice (Arnould*et al*., 1981).

2.4.3.8 *Hot Maceration Process*

In this process, the long enfleurage time is reduced by the immersion of petals in molten fat heated at 45°-60° C for 1 to 2 h, depending upon the plant species. After each immersion, the fat

is filtered and separated from the petals. After 10 to 20 immersions, the fat is separated from waste flowers and water. Absolute of maceration is then produced from fat containing oil through the process of extraction and concentration under reduced pressure. It is mainly used for highly delicate flowers whose physiological activities are lost rapidly after their harvest, such as lily of valley (Schantz *et al*., 1998).

2.4.3.9*Solvent extraction*

Solvent extraction, also known as Liquid–liquid extraction or partitioning, is a method to separate a compound based on the solubility of its parts. This is done using two liquids that don't mix, for example, water and an organic solvent (Fig 2.5). In the Solvent-Extraction method of Essential Oils recovery, an extracting unit is loaded with perforated trays of essential oil plant material and repeatedly washed with the solvent. Solvent extraction is used in the processing of perfumes, vegetable oil, or biodiesel. Solvent extraction is used on delicate plants to produce higher amounts of essential oils at a lower cost (Chrissie *et al*., 1996). The most frequently applied sample preparation procedure in plant material analysis. The quality and quantity of extracted mixture are determined by the type of extra heat applied because of the method is limited by the compound solubility in the specific solvent used. Although the method is relatively simple and quite efficient, it suffers from such disadvantages as long extraction time, relatively high solvent consumption and often unsatisfactory reproducibility (Dawidowicz*et al*., 2008).

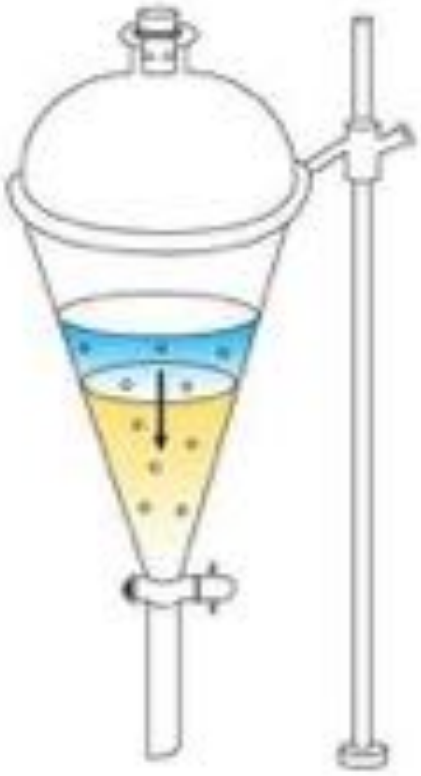


Fig 2.5: (Hesham *et al.,* 2016).

2.4.3.10*Soxhlet extraction*

Typically, a Soxhlet extraction is used when the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent. It allows for unmonitored and unmanaged operation while efficiently recycling a small amount of solvent to dissolve a larger amount of material. Soxhlet extraction (Fig 2.6) involves solid-liquid contact for the removal of one or several compounds from a solid by dissolution into a refluxing liquid phase. In a conventional soxhlet device, the solid matrix is placed in a cavity that is gradually filled with the extracting liquid phase by condensation of vapors from a distillation flask. When the liquid reaches a preset level, a siphon pulls the contents of the cavity back into the distillation flask, thus carrying the extracted analytes into the bulk liquid. This procedure is repeated until virtually complete extraction is achieved. There are several advantages of Soxhlet extraction (Soxhlet *et al*., 1879). The most important are that the sample is repeatedly brought into contact with fresh portions of the solvent. This procedure prevents the possibility of the solvent becoming saturated with extractable material and enhances the removal of the analyte from the matrix. Moreover, the temperature of the system is close to the boiling point of the solvent. This excess energy in the form of heat helps to increase the extraction kinetics of the system. Soxhlet extraction has several disadvantages, including it requires several hours or days to perform; the sample is diluted in large volumes of solvent, and due to the heating of the distillation flask losses due to thermal degradation and volatilization have been observed. A Soxhlet extractor is a piece of laboratory apparatus (Harwood, *et al.,*1989) invented in 1879 by Franz von Soxhlet. It was originally designed for the extraction of a lipid from a solid material.

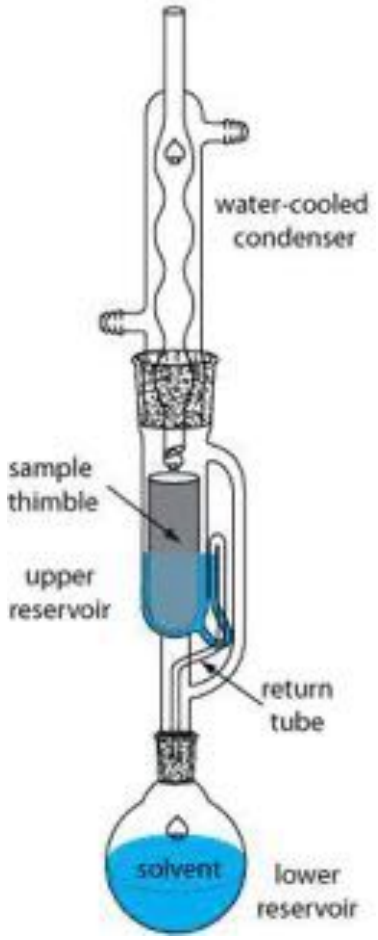


Fig 2.6: Soxhlet extractor (Hesham *et al.,* 2016).

2.5 Modern (Non-traditional) Methods of Extraction of Essential Oils

Traditional methods of extraction of essential oils have been discussed and these are the methods most widely used on commercial scale. However, with technological advancement, new techniques have been developed which may not necessarily be widely used for commercialproduction of essential oils but are considered valuable in certain situations, such as the production of costly essential oils in a natural state without any alteration of their thermosensitive components or the extraction of essential oils for micro-analysis. These techniques are as follows:

2.5.1 Supercritical Fluid Extraction (SFE)

Supercritical Fluid Extraction (SFE) is the process of separating one component (the extractant) from another (the matrix) using supercritical fluids as the extracting solvent. Extraction is usually from a solid matrix, but can also be from liquids. Supercritical fluids (Fig 2.7) have been used as solvents for a wide variety of applications such as essential oil extraction and metal cation extraction. In practice, more than 90% of all analytical supercritical fluid extraction (SFE) is performed with carbon dioxide (CO2) for several practice reasons. Apart from having relatively low critical pressure (74 bars) and temperature (32Co), CO2 is relatively non-toxic, nonflammable, noncorrosive, safe, available in high purity at relatively low cost and is easily removed from the extract (Rozzi*et al*., 2002). The main drawback of CO2 is its lack of polarity for the extraction of polar analytes (Pourmortazavi*et al*., 2007). These essential oils can include limonene and other straight solvents.Carbon dioxide (CO2) is the most used supercritical fluid, sometimes modified by co-solvents such as ethanol or methanol. It was found that extracts prepared by SFE yielded a higher antioxidant activity than extract prepared by other methods (Fadel *et al*., 1999). This extraction method produces higher yield, higher diffusion coefficient, and lower viscosity. Many essential oils that cannot be extracted by steam distillation can be

obtainable with carbon dioxide extraction. Nevertheless, this technique is very expensive because of the price of this equipment for this process is very expensive and it is not easily handled. Supercritical extracts proved to be of superior quality, with better functional and biological activities (Capuzzo*et al*., 2013). Furthermore, some studies showed better antibacterial and antifungal properties for the supercritical product.

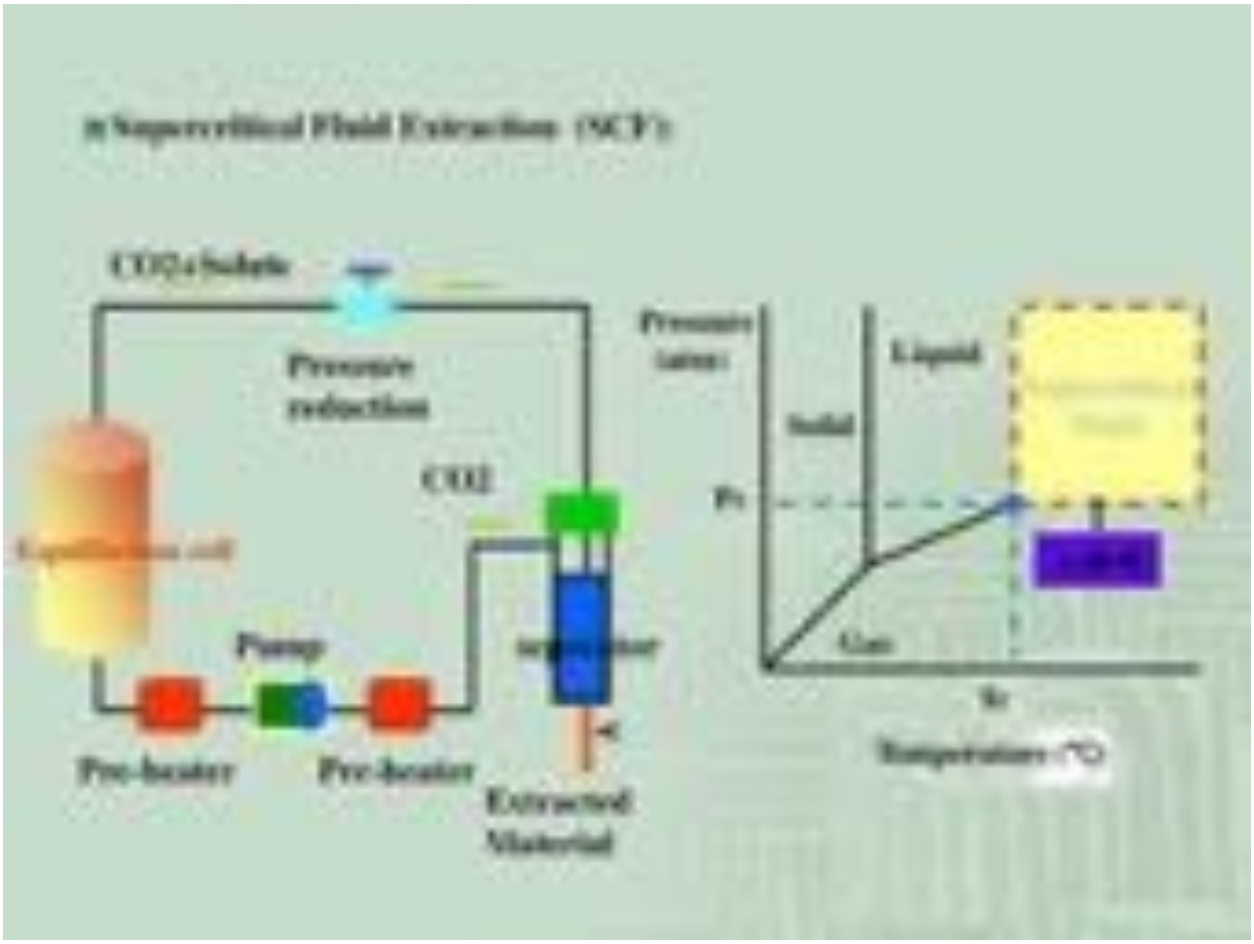


Fig 2.7: Supercritical Fluid Extraction (Hesham *et al.,* 2016).

2.5.2 Microwave-Assisted Hydrodistillation (MAHD):

Microwave-assisted hydrodistillation is an advanced hydrodistillation technique utilizinga microwave oven in the extraction process (Fig 2.8). (Golmakani*et al*., 2008) reported some recently published studies have successfully utilized a microwave oven for the extraction of active components from plants. The efficiency of Microwave-assisted hydrodistillation is strongly dependent on the dielectric constant of water and the sample (Brachet*et al*., 2002).

Conventional techniques for the extraction of active constituents are time and solvent consuming, thermally unsafe and the analysis of numerous constituents in plant material is limited by the extraction step (Mandal *et al*., 2007). High and fast extraction performance ability with less solvent consumption and protection offered to thermolabile constituents are some of the attractive features of this new promising microwave-assisted hydrodistilation technique. Application of Microwave-assisted hydrodistilation in separation and extraction processes has shown to reduce both extraction time and volume of solvent required, minimizing environmental impact by emitting less CO2 in atmosphere (Lucchesi*et al*., 2004; Ferhat *et al*., 2006) and consuming only a fraction of the energy used in conventional extraction methods (Farhat *et al*., 2009). The use of Microwave-assisted hydrodistilation in industrial materials processing can provide a versatile tool to process many types of materials under a wide range of conditions. Microwave-assisted hydrodistilation is a current technology to extract biological materials and has been regarded as an important alternative in extraction techniques because of its advantages which mainly are a reduction of extraction time, solvents, selectivity, volumetric heating and controllable heating process.

The principle of heating using Microwave-assisted hydrodistilation is based upon its direct impact with polar materials/solvents and is governed by two phenomenon's: ionic conduction and dipole rotation, which in most cases occurs simultaneously (Letellier*et al*., 1999).

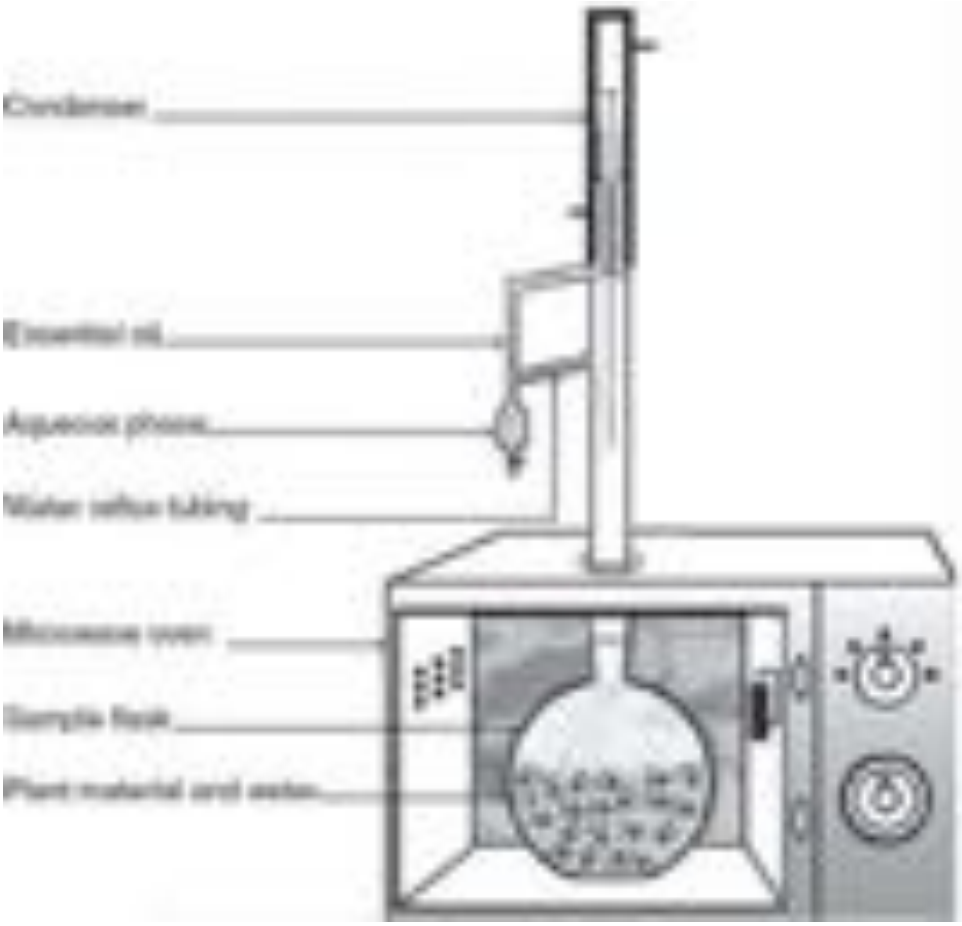


Fig 2.8: Microwave-Assisted Hydrodistillation(Lucchesi*et al*., 2007).

2.5.3 Ultrasound-assisted extraction (UAE):

Ultrasound-assisted extraction (UAE) is a good process to achieve high valuable compounds and could involve to the increase in the estimate of some food by-products when used as sources of natural compounds or plant material (Bhaskaracharya*et al*., 2009). The major importance will be a more effective extraction, so saving energy, and also the use of mean temperatures, which is beneficial for heat-sensitive combinations. This technique was developed in 1950 at laboratory apparatus (Vinatoru*et al*., 2001). Ultrasound allows selective and intensification of essential oils extraction by release from plant material when used in combination with other techniques for example solvent extraction and hydrodistilation (Fig 2.9). Ultrasound technology has been featured as a valuable method in food engineering processes and plants (Bhaskaracharya*et al*., 2009), and become this field from the techniques active. In these applications the power ultrasound increases the surface wetness evaporation average and causes oscillating velocities at the interfaces, which may affect the diffusion boundary layer and generate rapid series of alternative expansions of the material, affecting cluster transfer (Garcı´a-Pe´rez*et al*., 2006).

The plants raw material is immersed in water or another solvent (Methanol or ethanol or anyone from the solvents) and at the same time, it is subjected to the work of ultrasound (Karim *et al*., 2012). This technique has been used for the extraction of many essential oils especially from the flower, leaves or seeds (Sereshti*et al*., 2012).

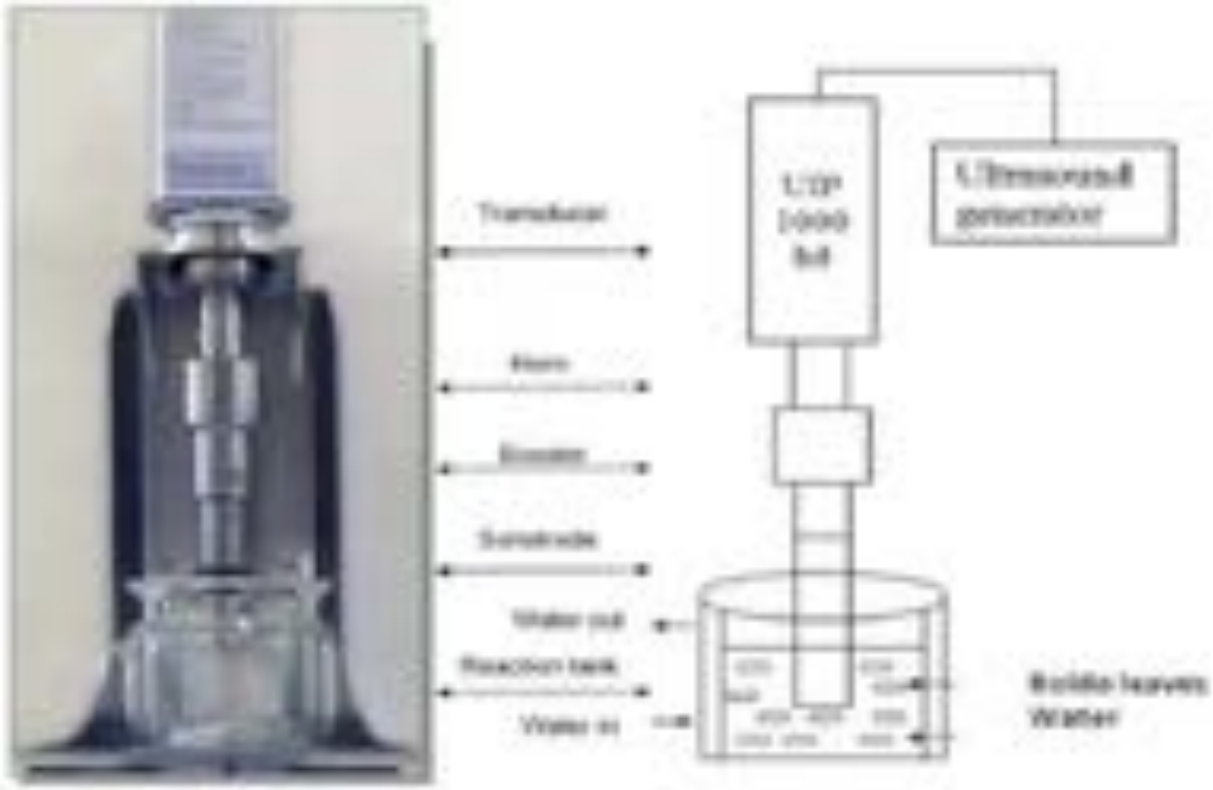


Fig 2.9: Ultrasound-assisted extraction (Lucchesi*et al*., 2007).

2.5.4 Solvent-free microwave extraction (SFME)

Solvent-free microwave extraction (SFME) is in the extraction procedure of essential oil which is cloaca by the in site water of the plant material without added any solvent (Lucchesi*et al*., 2007). Based on the integration of dry distillation and microwave heating energy, (Figure 10). It consists on the microwave dry-distillation at atmospheric pressure of plant without adding water or any organic solvent (Filly *et al*., 2014). In a model SFME procedure, the plant material was moistened before to extraction by soaking in a certain amount of water for 1 to 2 h and then draining off the excess water. After that, the moistened materials were subjected to the microwave oven cavity and a condenser was used to collect the extracted essential oils in a presetting procedure. The irradiation power, temperature, and extraction time were controlled by the panel in the instrument. The separated essential oil was dried over anhydrous sodium sulfate and stored at 4^0 C in the dark. The extraction yield of essential oil was calculated as follows:

Extraction yield (ml/kg) = V/M where V is the volume of essential oil in herb samples (ml), and M is the mass of the herb samples (kg).

Fig 2.10: Solvent-free microwave extraction (Lucchesi*et al*., 2007).

2.5.5 Microwave hydro diffusion and gravity (MHG)

Is a new green technique for the extraction of essential Oils? This green extraction technique is an original microwave blend microwave heating and earth attraction at atmospheric pressure. MHG was conceived for experimenter and processing scale applications for the extraction of essential oils from different kind of material plants (Abert*et al*, 2008). Microwave hydro diffusion and gravity (MHG) become clear not only as economic and efficient but also as environment-friendly, not require solvent or water and as it does require less energy (Chemat*et al*., 2004). The performances and advantages of this technique are a reduction of extraction time (in the case of hydro-distillation it takes 90min or more but in this technique only 20 min) and reducing environmental impact and power saving (Vian*et al*., 2008).

2.6 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.

2.6.1 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).

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
Litseacubeba	2,000
Clove (leaf)	2,000

(Sourcs: Wikipedia, the free encyclopedia, 2018)

2.7 HEALTH BENEFIT OF ESSENTIAL OILS

2.7.1 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).

2.7.2 Effect of Essential Oils on Culinary (Flavoring)/ Ingestion

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

2.7.3 Industrial effect of Essential Oils

Research shows that cineole based eucalyptus oil (5% of mixture) prevents the separation problem with ethanol and petrolfuel 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).

2.7.4 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 2.3 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).

2.8 LIST OF SOME ESSENTIAL OILS AND THE PART OF PLANT WHERE THEY WERE OBTAINED FROM

Essential oils are derived from various sections of plants (Table 2.4). Some are from berries, leaves, flowers, bark, wood, peel, resin, rhizome and root(Julia, 1995).

Table 2.4 Some Essential Oils their uses and the Parts of plant they were derived

Essential oil	Parts of Plants	Uses
Agar oil	Agarwood (*Aquilariamalaccensis*)	It is for fragrance
Ajwain oil	leaves of Bishop's weed (*Carumcopticum*).	for preservatives
Frankincenseoil	from resin is	aromatherapy/ perfumes
Grapefruitoil	extracted from the peel of the fruit.	used in aromatherapy
Lavender oil	from flowers	fragrance and medicinally
Orris oil	roots of the Florentine iris (*Iris florentina*)	flavouring agent/ perfume, and medicinally
Rose oil	distilled from rose petals	Fragrance
Sandalwood oil	derived from the wood.	Fragrance
Valerian oil**:**	Obtained from the root.	Aromatherapy/medicinal

Source: Hesham *et al.*, 2016

2.9 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).

2.9.1 Origin of Eucalyptus Oil

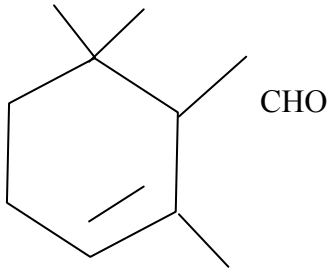
There are over 500 species of Eucalyptus trees, with tough long and narrow blue-green leaves, creamy white flowers and smooth pale bark (Burke *et al.,* 2004). In Pre 1788, The Australian Aboriginal people were among the first to use *Backhousiacitriodora*(*B.citriodora)* for medicine and flavouring. 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 *Backhousiacitriodora* 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

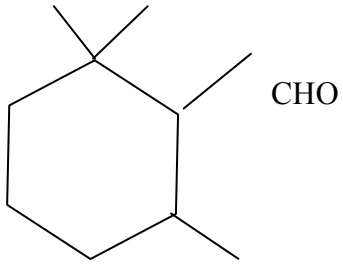
Essential Oils Pty Ltd, and subsequent commercial supply of plantation produced *B.citriodora* oil in 1993. In 2001, Standard for oil of *B.citriodora* established by the essential oils unit, Wollongbar, and Standards Australia was developed (Zhao and, Agboola 2007).

2.9.2 Citodora

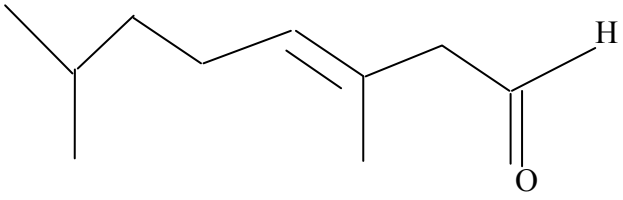
Citrodora oil is extracted from the Eucalyptus plant which belongs to the Kingdom: Plantae (unranked). Angiospermss (unranked). Eudicots (unranked). Rosids**.** Order: Myrtales. Family: Myrtaceae. Genus: *Backhousia* .Species:.*Backhousiacitriodora* (common names lemon myrtle, lemon scented myrtle, lemon 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. 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 *Backhousiacitriodora* 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 seen below in Figure 2.11.



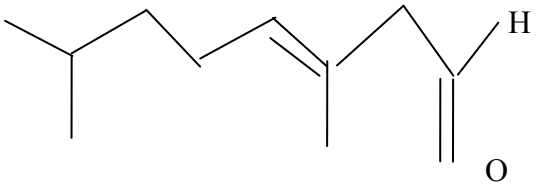
alpha-cyclocitral



beta-cyclocitral



Isocitral



Citral **Figure 2.11:** Structural formula of Citrodora (Sourcs: www.cropwatch.org, 2019)

2.9.3 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).

Figure 2:12. Eucalyptus plant obtained from Zaria, Nigeria

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

2.9.4 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 citralchemotype 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 2.5: 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

2.9.5 Types of Eucalyptus Oil

The most common Eucalyptus oil is Eucalyptus Globulus, the other lesser known ones include Eucalyptus Citriodora, Eucalyptus Radiata (this one I am particularly fond of), Eucalyptus Staigerina and Eucalyptus Smithii. They all have similar properties although some are better at one thing than another (Price and Price, 2012).

Eucalyptus radiata: This oil is commonly known as Black Peppermint Oil. It contains 60-75% 1, 8 cineol and is native to Australia. *Radiata* is less intense and milder, making it sometimes chosen over *E. globulus*. Only two out of the six known chemotypes are harvested, CT 1, 8 cineol and CR peperitone. It has a similar composition to that of the essential oils from the Melaleuca family (e.g. tea tree). It stimulates the immune system making it beneficial to people who are tired, run down, and/or prone to frequent colds and infections. It is less likely to cause skin irritation compared with Eucalyptus Globulus. Eucalyptus Radiata is excellent for reducing inflammation helping with arthritis, sprains, strains, torn ligaments and tendons. It can also be beneficial in cases of Endometriosis. It is said to be absorbed by the skin better than the other Eucalyptus oils.Eucalyptus radiate, has a sweet and fruity aroma. Dilute 50:50. Cannot be used as a dietary supplement (Price and Price, 2012).

Eucalyptus Citrodora: This oil is commonly known as Lemon Eucalyptus Oil. It contains 40-80% citronellal and has several chemotypes. Although originally from Australia, the trees grow in Guatemala, Colombia, Egypt South Africa, India and China. The trees of Madagascar are high in phenols and the Brazilian trees contain a high percentage of aldehydes. Its fresh and lemony aroma is uplifting. It is a very rich source of citronellal making it a powerful insect repellent, it is hypotensive, calming to the nervous system, and a febrifuge (lowers body temperature to prevent or alleviate fever). It has a similar strength anti-inflammatory property as Eucalyptus Radiata (Price and Price, 2012).

Eucalyptus Smithii: is anti-viral and an expectorant. It is considered to be the mildest of the Eucalyptus essential oils making it more favourable for use with children and the elderly. It is less likely to cause skin irritation and the aroma isn't quite as strong. Eucalyptus smithii *(Eucalyptus Smithii)* is a tall tree with gray-green leaves. The essential oil is distilled from the leaves and twigs and has a camphoraceous aroma. In aromatherapy practice, it is used for colds, asthma, headaches and muscle pain. The main chemical components of eucalyptus smithiii essential oil are oxides. Eucalyptus smithii essential oil is not contra-indicated for use with babies and children, even though it contains similar chemical components to blue gum eucalyptus essential oils (Price and Price, 2012).

Eucalyptus dives*:* This oil is commonly known as Peppermint Eucalyptus Oil. It contains 35-50% piperitone and 23-30% phellandrene. It has three chemotypes that have different uses: CT cineole, CT piperitone has a minty fragrance and CT phellandrene is traditionally used for insect repellant. The broad-leaved peppermint eucalyptus *(Eucalyptus dives)* is also known as the blue peppermint tree. This particular eucalyptus tree is medium-sized and has blue, heart-shaped leaves when young, turning to thick, aromatic leaves in maturity.This essential oil is distilled from the leaves and twigs of the tree; it has a camphoraceous-minty aroma. In aromatherapy practice, broad-leaved peppermint eucalyptus essential oil is used for asthma, coughs and colds, arthritis and nervous exhaustion. The main chemical components of this essential oil are ketones and monoterpenes. Avoid in use with babies and children, and in pregnancy (Price and Price, 2012).

Eucalyptus polybractea: This oil is commonly known as Blue Mallee Oil. This tree is grown in France, but native to Australia. It contains 85-95% 1, 8 cineol. The chemotype from the French Tree contains cryptone and smells similar to cumin. Its fresh and earthy aroma is uplifting. Dilute 50:50. Cannot be used as a dietary supplement (Price and Price, 2012).

Eucalyptus bicostata*:* This oil is commonly known as Eucalyptus Blue. The plant is grown in Ecuador (Eucalyptus Blue) and Australia (Southern Blue Gum). It contains the high amount of alpha-pinene. It also contains eucalyptol (1, 8 cineol). Some botanists consider this a subspecies of *E. globulus*; however, this does not have global support at this time. Its fresh aroma is very calming to the mind. Dilute 50:50. Use in humidifier. Cannot be used as a dietary supplement (Price and Price, 2012).

Eucalyptus staigeriana: This oil is commonly known as Lemon Ironbark (not to be confused with lemon myrtle). This oil contains a high amount of aldehydes (about 51%) (Price and Price, 2012).

2.10 GAS CHROMATOGRAPHY MASS SPECTROMETRY (GC/MS)

Gas Chromatography Mass Spectrometry (GC/MS) Gas chromatography mass spectrometry (GC/MS) is an instrumental technique, comprising a gas chromatograph (GC) coupled to a mass spectrometer (MS), by which complex mixtures of chemicals may be separated, identified and quantified.

2.10.1 Gas Chromatography (GC)

The GC analysis is a common confirmation test. Among its uses are drug testing and environmental contaminant identification. GC analysis separates all of the components in a sample and provides a representative spectral output. The technician injects the sample into the injection port of the GC device. The GC instrument vaporizes the sample and then separates and

analyzes the various components. Each component ideally produces a specific spectral peak that may be recorded on a paper chart or electronically. The time elapsed between injection and elution is called the "retention time." The retention time can help to differentiate between some compounds. The size of the peaks is proportional to the quantity of the corresponding substances in the specimen analyzed. The peak is measured from the baseline to the tip of the peak (Adam, 2007).

GC analysis depends on similar phenomena to separate chemical substances. A mixture of chemicals present in a specimen can be separated in the GC column. Some chemical and physical characteristics of the molecules cause them to travel through the column at different speeds. If the molecule has low mass it may travel more swiftly. Also, the molecule's shape may affect the time needed to exit the column. How the different substances relate to each other may cause the time needed to travel the column to increase or decrease. Interactions between the sample's molecule and the column surface may cause the molecule to be retained inside the column for a different amount of time than similar molecules that interact with the column differently (Sloan *et al.,* 2001).

2.10.2 Description of the Process of Gas Chromatography

The equipment used for gas chromatography generally consists of an injection port at one end of a metal column packed with substrate material and a detector at the other end of the column. A carrier gas propels the sample down the column. The technician uses flow meters and pressure gauges to maintain a constant gas flow. A gas that does not react with the sample or column is essential for reliable results. For this reason, carrier gases are usually argon, helium, hydrogen, nitrogen, or hydrogen. Many analysts use helium because it does not react. Hydrogen usually is a good carrier gas but it may react and convert the sample into another substance. The ultimate

choice for a carrier gas may depend on the type of detector used (Formaceck and Kubeczka, 1982).

To ensure proper separation, the sample must enter the column in a discreet, compact packet. Normally the sample is injected into the injection port with a hypodermic needle and syringe capable of measuring the specimen amount. The needle is stuck into a replaceable neoprene or silicone rubber septum that covers the injection port. The injection port is maintained at a temperature at which the sample vaporizes immediately. Ideally, the sample spreads evenly along the cross section of the column, forming a plug. The column is a metal tube, often packed with a sand-like material to promote maximum separation. Columns are commonly obtained pre-packed by vendors. As the sample moves through the column, the different molecular characteristics determine how each substance in the sample interacts with the column surface and packing. The column allows the various substances to partition themselves. Substances that do not like to stick to the column or packing move through the column rapidly. Substances that do not like to stick to the column or packing are impeded but eventually elute from the column. Ideally, the various components in the sample separate before eluting from the column end (Adams, 2007).

The GC instrument uses a detector to measure the different compounds as they emerge from the column. Among the available detectors are the argon ionization detector, flame ionization detector, flame emission detector, cross section detector, thermal conductivity detector, and the electron capture detector. Choosing the proper detector depends upon the use. Some considerations are that the flame detectors destroy the sample, the thermal conductivity detector is universally sensitive, and the argon ionization detector requires argon as a carrier gas. The spectral output is usually stored electronically and displayed on a monitor. The technician can

produce a hard copy record. The argon ionization detector does not detect water, carbon tetrachloride, nitrogen, oxygen, carbon dioxide, carbon monoxide, ethane, or compounds containing fluorine. The flame ionization detector does not respond to water, nitrogen, oxygen, carbon dioxide, carbon monoxide, helium, or argon. If a specimen contains water, a flame ionization detector should be used. The electron capture detector cannot detect simple hydrocarbons but does detect compounds containing halides, nitrogen, or phosphorus (Sloan 2001; Adams, 2007).

2.10.3 Retention Timeof Gas Chromatography

The amount of time that a compound is retained in the GC column is known as the retention time. The technician should measure retention time from the sample injection until the compound elutes from the column. The retention time can aid in differentiating between some compounds. However, retention time is not a reliable factor to determine the identity of a compound. If two samples do not have equal retention times, those samples are not the same substance. However, identical retention times for two samples only indicate a possibility that the samples are the same substance. Potentially thousands of chemicals may have the same retention time, peak shape, and detector response. For example, under certain conditions, DDT has the same retention time as PCBs (polychlorinated biphenyls). Some believe that environmental testing showed erroneously high amounts of DDT. GC instruments showed only one peak for what is believed to be a mixture of DDT and PCBs. This experimental data led to the banning of DDT in the U.S. Bluntly, GC is one of the quickest ways of getting the wrong answer in qualitative analysis," (Sloan 2001; Adams,2007).

2.10.4 Quality Assurance/Quality Control ProceduresGC/MS

Before analyzing a sample, the technician should tune and calibrate the instrument. Tuning can be accomplished using specific concentrations of Decafluorotriphenylphosphine and p-

Bromofluorobenzene. A technician can process a spiked sample (containing a known concentration of a substance) to check calibration and tuning. If the GC/MS instrument does not detect the substance or shows a greater or lesser concentration than the known concentration, the technician must recalibrate the instrument. Also, the technician can use a blank sample (containing no detectable compounds) to test the GC/MS instrument's data reporting accuracy. If the device indicates the presence of a substance in the blank sample, the device may contain residue the technician must retune and recalibrate the GC/MS instrument (Sloan 2001; Adams, 2007).

Proper scientific practice requires that the GC technician compare the spectral output with a known standard sample of the suspected substance. The standard sample must be analyzed with the same instrument, under the same conditions, immediately before and immediately after analyzing the unknown specimen. If the resulting three spectral outputs do not agree, the technician cannot make a reliable identification of the specimen based on the GC analysis (Sloan 2001; Adams, 2007).

2.10.5 Analysis of Outputof Gas Chromatography

Less than ideal spectral peaks may indicate less than ideal analytical procedures or equipment. The technician can readily observe whether the output exhibits unsatisfactory results. Ideally, the spectral peaks should be symmetrical, narrow, separate (not overlapping), and made with smooth lines. GC evidence may be suspect if the peaks are broad, overlapping, or unevenly formed. If a poorly shaped peak contains a steep front and a long, drawn-out tail, this may indicate traces of water in the specimen (Sloan 2001; Adams, 2007).

The GC technician should inject the specimen into the septum rapidly and smoothly to attain good separation of the components in a specimen. If the technician injects the specimen too

slowly, the peak may be broad or overlap. A twin peak may result from the technician hesitating during the injection. A smoothly performed injection, without abrupt changes, should result in a smoothly formed peak. A twin peak may also indicate that the technician injected two specimens consecutively (Sloan 2001; Adams, 2007).

2.10.6 Limitationsof Gas Chromatography

a. **Response Factor**: The size of a spectral peak is proportional to the amount of the substance that reaches the detector in the GC instrument. No detector responds equally to different compounds. Results using one detector will probably differ from results obtained using another detector. Therefore, comparing analytical results to tabulated experimental data using a different detector does not provide a reliable identification of the specimen (Stein and Scott, 1994).

b. A "response factor" must be calculated for each substance with a particular detector. A response factor is obtained experimentally by analyzing a known quantity of the substance into the GC instrument and measuring the area of the relevant peak. The experimental conditions (temperature, pressure, carrier gas flow rate) must be identical to those used to analyze the specimen. The response factor equals the area of the spectral peak divided by the weight or volume of the substance injected. If the technician applies the proper technique, of running a standard sample before and after running the specimen, determining a response factor is not necessary (Stein and Scott, 1994).

c. **Worn Septum:** An injection port septum should last between 100 and 200 injections. Higher injection port temperatures shorten the septum's lifespan. A leaking septum adversely affects the GC instrument's sensitivity. If a portion of the specimen leaks

back out of the septum, the amount of the specimen is not recorded. This event makes any eventual quantitative result erroneous. If air should leak into the injection port through a worn septum, the oxygen and water contained in air may skew the results. Any oxygen may react with the specimen components. If this happens, the GC instrument will provide results indicating the presence of this unintended reaction product, instead of the original compounds present in the specimen vial. Any water in the column adversely affects the GC instrument's ability to separate components (Stein and Scott, 1994).

d. **Injection Port Temperature**: The temperature of the GC injection port must be high enough to vaporize a liquid specimen instantaneously. If the temperature is too low, separation is poor and broad spectral peaks should result or no peak develops at all. If the injection temperature is too high, the specimen may decompose or change its structure. If this occurs, the GC results will indicate the presence of compounds that were not in the original specimen (Stein and Scott, 1994).

e. **Residual Impurities:** Ideally, all components of a specimen elute completely from the GC column. If any substance remains inside the column, the substance may elute during subsequent analyses with other specimens. This may result in an unexpected peak in the output. The peak produced should be broad (Stein and Scott, 1994).

f. **Carrier Gas:** If the GC instrument uses hydrogen for the carrier gas, the technician must consider whether the hydrogen may react with any of the compounds present in the specimen. If the hydrogen does react, a broad peak will result. When using a thermal conductivity detector, care should be taken as a false peak may occur if the carrier gas's thermal conductivity is in the range of the thermal conductivity of any compound in the specimen. An unstable carrier gas flow rate may produce a drifting baseline and false broad

peaks. A carrier gas should be pure. Regular changing of the gas filter should prevent significant impurities (Stein and Scott, 1994).

2.10.7 Crucial Factorsof Gas Chromatography

The GC analysis is highly reliable if the instrument is properly maintained, the technician follows proper procedures, and the interpretation of the results is competent. While some factors rarely affect GC analysis, some factors are absolutely essential for the use of reliable GC evidence. In all cases a technician must process a standard sample containing a verified composition identical to the presumed contents of the collected specimen. This standard sample must be processed before and after the collected specimen under identical conditions. Any output from the collected specimen that does not match the standard sample is inconclusive. If tabulated reference data exists for the relevant conditions, the specimen data must match the reference data (Sloan 2001; Adams, 2007).

If advance notice of GC testing is available, an adverse party should observe the procedure. If a retained consultant or the knowledgeable attorney observes the technician's use of the GC instrument, important information can be recorded. The technician's preparation of the specimen and the subsequent injection can be observed for errors or malfunctioning equipment. The observer should record the instrument's make, model, serial number, injection temperature, column temperature, carrier gas flow rates and pressure, identify the type of detector used, and observe any manipulation of the data by use of a computer. Ensure that the technician properly starts measuring the time at injection and records the time of elution. Any discrepancy in the time will produce an erroneous retention time. If the procedure cannot be observed, the adverse party should seek all pertinent information (experimental conditions, measurements, instrument identification) and hard copy output (Sloan 2001; Adams, 2007).

2.10.8 Mass Spectrometry (MS)

The MS analysis is commonly used in arson investigations, engine exhaust analysis, petroleum product analysis, and for blood monitoring in surgery. MS identifies substances by electrically charging the specimen molecules, accelerating them through a magnetic field, breaking the molecules into charged fragments and detecting the different charges. A spectral plot displays the mass of each fragment. A technician can use a compound's mass spectrum for qualitative mass of the original molecule, the "parent mass," (Stein and Scott, 1994).

The parent mass is analogous to the picture on top of a puzzle box, a guide to the end result obtained by putting together the fragment masses, or puzzle pieces. From the molecular mass and the mass of the fragments, reference data is compared to determine the identity of the specimen. Each substance's mass spectrum is unique. Providing that the interpretation of the output correctly determines the parent mass, MS identification is conclusive (Stein and Scott, 1994).

2.10.9 Description of Processof Mass Spectrometry

Today many different types of MS instruments exist, each one using a different apparatus and process for producing mass spectra. This article's description of the MS process will limit itself to a basic description of a conventional large magnet mass spectrometer. Such a MS instrument contains a sample inlet, an ionization source, a molecule accelerator, and a detector (Abragam 1961; Stein and Scott, 1994).The MS analysis requires a pure gaseous sample. The sample inlet is maintained at a high temperature, up to 400° C (752° F), to ensure that the sample stays a gas. Next the specimen enters the ionization chamber. A beam of electrons is accelerated with a high voltage. The specimen molecules are shattered into well-defined fragments upon collision with the high voltage electrons. Each fragment is charged and travels to the accelerator as an individual practice. In the acceleration chamber the charged particle's velocity increases due to the influence of an accelerating voltage. For one value of voltage only one mass accelerates

sufficiently to reach the detector. The accelerating voltage varies to cover a range of masses so that all fragments reach the detector (Stein and Scott, 1994; Tyszka *et al.,* 2005).

The charged particles travel in a curved path towards the detector. When an individual charged particle collides with the detector surface, several electrons (also charged particles) emit from the detector surface. Next, these electrons accelerate towards a second surface, generating more electrons, which bombard another surface. Each electron carries a charge. Eventually, multiple collisions with multiple surfaces generate thousands of electrons which emit from the last surface. The result is an amplification of the original charge through a cascade of electrons arriving at the collector. At this point the instrument measures the charge and records the fragment mass as the mass is proportional to the detected charge. The MS instrument produces the output by drawing a array of peaks on a chart, the "mass spectrum." Each peak represents a value for a fragment mass. A peak's height increases with the number of fragments detected with one particular mass. As in the case of the GC detectors, a peak may differ in height with the sensitivity of the detector used (Haner and Keifer, 2009)

2.10.10Analysis of Output of Mass Spectrometry

Each substance has a characteristic mass spectrum under particular controlled conditions. A technician can identify a specimen by comparing the specimen's mass spectrum with known compounds. Quantitative analysis is possible by measuring the relative intensities of the mass spectra. Usually a mass spectrum will display a peak for the unfragmented molecule of the specimen. This is commonly the greatest mass detected, called the "parent mass." Like the picture on a puzzle box, the parent mass is used to fit the pieces together from the other peaks in the mass spectrum. The parent mass reveals the mass of the molecule while the other peaks indicate the molecule's structure (Clore and Gronenborn, 1991).

Determining the parent peak and consequently the molecular mass of the specimen is the most difficult part of MS analysis. Identifying the parent mass is outside the scope of this article. Assuming that a technician can correctly determine the molecular mass, the technician makes an educated guess of the specimen's identity and compares the mass spectrum to reference spectra for confirmation. The mass spectra for larger molecules containing carbon are complicated and require tedious calculations that are subject to error. Computers are commonly used for spectral analysis (Clore and Gronenborn, 1991).

2.10.11 Limitations of Mass Spectrometry

a. Resolution: The "resolution" is a value that represents the instrument's ability to distinguish two particles of different masses. The greater the MS instrument's resolution, the greater its usefulness for analysis. An MS instrument provides more accurate results for larger molecules when the instrument has a high resolution. A high resolution MS instrument is advisable for analyzing body fluids because they have high molecular masses. A low resolution MS instrument may not sufficiently characterize a large mass substance (Clore and Gronenborn, 1991; Haner and Keifer, 2009).

b. Pressure: If the interior pressure in an MS instrument is too high, erroneous results may occur. As the specimen molecule breaks up, the fragments accelerate. If a fragment collides with another fragment, then these two fragments may combine to make a new particle. In this event, the detector will register the mass of this new particle on the mass spectrum. The reference spectra for comparison are produced under low pressure conditions which minimize collisions between fragments. A technician would find a spectral peak where one is not expected. In the puzzle analogy, this is similar to finding pieces from a different puzzle in your box and trying to make these extraneous pieces fit. As this is impossible, any MS

analysis under high pressure conditions would depend greatly on guesswork by the technician (Clore and Gronenborn, 1991; Haner and Keifer, 2009).

c. Parent Mass: Finding the correct parent peak in the mass spectra may be difficult. Finding the parent peak helps to determine the parent mass, which should lead to determining the specimen's molecular mass. For high molecular mass compounds, like drugs and body fluids, a parent peak is often not observed. This makes qualitative identification difficult. A special type of MS, chemical ionization MS, reduces the likelihood of missing the parent mass Haner and Keifer, 2009). .

d.High Speed Scanning: High speed scanning MS instruments are able to rapidly analyze specimens. However, the increased speed is a tradeoff for decreased resolution. Quantitative measurements are unreliable with high speed scanning (Clore and Gronenborn, 1991; Haner and Keifer, 2009).

e.Technician's Skills: As in the puzzle analogy, knowing the shape of a piece of the molecule helps to join the pieces together. To determine the specimen's molecular structure before fragmentation, the technician needs to employ skill and art to determine the molecular structure from mass spectra patterns. Computers and databases can assist, but a human expert is necessary to distinguish between likely and unlikely answers. Alone, a computer cannot determine molecular structures as well as a competent human. This causes the weight of MS evidence to depend greatly on the technician's qualifications and proficiency with MS spectrum analysis (Clore and Gronenborn, 1991; Haner and Keifer, 2009).

2.10.12 Gas Chromatography and Mass Spectrometry (GC-MS) Combination

The GC device is generally a reliable analytical instrument. The GC instrument is effective in separating compounds into their various components. However, the GC instrument cannot be used for reliable identification of specific substances. The MS instrument provides specific

results but produces uncertain qualitative results. When an analyst uses the GC instrument to separate compounds before analysis with an MS instrument, a complementary relationship exists. The technician has access to both the retention times and mass spectral data. Many scientists consider GC/MS analysis as a tool for conclusive proof of identity. GC/MS analysis, where the effluent to the GC instrument is the feed to the MS instrument, is in wide use for confirmation testing of substances. Drug testing, manufacturing quality control, and environmental testing are some typical uses (Robert and Adams, 2007).

Gas Chromatography Mass Spectrometry (GC/MS) Gas chromatography mass spectrometry (GC/MS) is an instrumental technique, comprising a gas chromatograph (GC) coupled to a mass spectrometer (MS), by which complex mixtures of chemicals may be separated, identified and quantified. Among its uses are drug testing and environmental contaminant identification. GC analysis separates all of the components in a sample and provides a representative spectral output according to the various components. Each component ideally produces a specific spectral peak that may be recorded on a paper chart or electronically. The size of the peaks is proportional to the quantity of the corresponding substances in the specimen analyzed. The peak is measured from the baseline to the tip of the peak. A mixture of chemicals present in a specimen can be separated in the GC column. Some chemical and physical characteristics of the molecules cause them to travel through the column at different speeds. If the molecule has low mass it may travel more swiftly. Also, the molecule's shape may affect the time needed to exit the column. How the different substances relate to each other may cause the time needed to travel the column to increase or decrease. Interactions between the sample's molecule and the column surface may cause the molecule to be retained inside the column for a different amount of time than similar molecules that interact with the column differently. Mass Spectrometry (MS) analysis is commonly used in arson investigations, engine exhaust analysis, petroleum product analysis, and

for blood monitoring in surgery. MS identifies substances by electrically charging the specimen molecules, accelerating them through a magnetic field, breaking the molecules into charged fragments and detecting the different charges. A spectral plot displays the mass of each fragment (Robert and Adams, 2007).

2.10.13 Limitations Gas Chromatography and Mass Spectrometry

Although many consider GC/MS to be the "gold standard" in scientific analysis, GC/MS does have some limitations. Because great faith is maintained in GC/MS analysis, erroneous results are not expected and hard to dispute. However, false positives and false negatives are possible. Some problems with GC/MS originate in improper conditions in the GC portion of the analysis. If the GC instrument does not separate the specimen's compounds completely, the MS feed is impure. This usually results in background "noise" in the mass spectrum. If the carrier gas in the GC process is not correctly deflected from entering the MS instrument, similar contamination may occur (Robert and Adams, 2007).

Also, the MS portion suffers from the inexact practice of interpreting mass spectra. An analyst must correlate computer calculations with system conditions. The typical memory bank for MS identification contains about 5000 spectra for a particular group of compounds. Even if a competent analyst could find conclusive results pointing to one substance out of 5000 substances, this does not rule out the remaining over 200,000 known existing chemicals. For the 5000-spectra memory bank, the typical computer result is limited to as many as six possible identifications (Robert and Adams, 2007).

2.12 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Nuclear magnetic resonance spectroscopy, most commonly known as NMR spectroscopy, is a research technique that exploits the magnetic properties of certain atomic nuclei. This type of spectroscopy determines the physical and chemical properties of atoms or the molecules in

which they are contained. It relies on the phenomenon of nuclear magnetic resonance and can provide detailed information about the structure, dynamics, reaction state, and chemical environment of molecules. The intramolecular magnetic field around an atom in a molecule changes the resonance frequency, thus giving access to details of the electronic structure of a molecule and its individual functional groups. Most frequently, NMR spectroscopy is used by chemists and biochemists to investigate the properties of organic molecules, although it is applicable to any kind of sample that contains nuclei possessing spin. Suitable samples range from small compounds analyzed with 1- 4-dimensional techniques. The impact of NMR spectroscopy on the sciences has been substantial because of the range of information and the diversity of samples, including solutions and solids (Abragam, 1961).

The NMR spectra are unique, well-resolved, analytically tractable and often highly predictable for small molecules. Thus, in organic chemistry practice, NMR analysis is used to confirm the identity of a substance. Different functional groups are obviously distinguishable, and identical functional groups with differing neighboring substituents still give distinguishable signals. NMR has largely replaced traditional wet chemistry tests such as color reagents or typical chromatography for identification. A disadvantage is that a relatively large amount, 2–50 mg, of a purified substance is required, although it may be recovered through a workup. Preferably, the sample should be dissolved in a solvent, because NMR analysis of solids requires a dedicated MAS machine and may not give equally well-resolved spectra. The timescale of NMR is relatively long, and thus it is not suitable for observing fast phenomena, producing only an averaged spectrum. Although large amounts of impurities do show on an NMR spectrum, better methods exist for detecting impurities, as NMR is inherently not very sensitive - though at higher frequencies, sensitivity narrows (Robert and Adams, 2007).

The NMR spectrometers are relatively expensive; universities usually have them, but they are less common in private companies. Modern NMR spectrometers have a very strong, large and expensive liquid helium-cooled superconducting magnet, because resolution directly depends on magnetic field strength. Less expensive machines using permanent magnets and lower resolution are also available, which still give sufficient performance for certain application such as reaction monitoring and quick checking of samples. There are even benchtop NMR spectrometers(Robert and Adams, 2007).

2.12.1 History Nuclear Magnetic Resonance

Nuclear magnetic resonance was first described and measured in molecular beams by Isidor Rabi in 1938, by extending the Stern–Gerlach experiment, and in 1944, Rabi was awarded the Nobel Prize in Physics for this work Shan *et al.,* (2006) In 1946, Felix Bloch and Edward Mills Purcell expanded the technique for use on liquids and solids, for which they shared the Nobel Prize in Physics in 1952. Neufeld and Stalke, (2015)**.** Yevgeny Zavoisky likely observed nuclear magnetic resonance in 1941, well before Felix Bloch and Edward Mills Purcell, but dismissed the results as not reproducible. Russell H. Varian filed the "Method and means for correlating nuclear properties of atoms and magnetic fields", U.S. Patent 2,561,490 on July 24, 1951. Varian Associates developed the first NMR unit called NMR HR-30 in 1952 Jeener (2007)

Purcell had worked on the development of radar during World War II at the Massachusetts Institute of Technology's Radiation Laboratory. His work during that project on the production and detection of radio frequency power and on the absorption of such RF power by matter laid the foundation for Rabi's discovery of NMR. Rabi, Bloch, and Purcell observed that magnetic nuclei, like 1H and P 31 could absorb RF energy when placed in a magnetic field and when the RF was of a frequency specific to the identity of the nuclei. When this absorption occurs, the nucleus is described as being in resonance. Different atomic nuclei within a molecule resonate at

different (radio) frequencies for the same magnetic field strength. The observation of such magnetic resonance frequencies of the nuclei present in a molecule allows any trained user to discover essential chemical and structural information about the molecule. The development of NMR as a technique in analytical chemistry and biochemistry parallels the development of electromagnetic technology and advanced electronics and their introduction into civilian use (Robert and Adams, 2007).

2.12.2 Basic Nuclear magnetic resonance(NMR) techniques

2.12.2.1 *Resonant frequency*

The NMR sample is prepared in a thin-walled glass tube - an NMR tube. When placed in a magnetic field, NMR active nuclei (such as ^{1}H or ^{13}C) absorb electromagnetic radiation at a frequency characteristic of the isotope.Shah, (2006). The resonant frequency, energy of the absorption, and the intensity of the signal are proportional to the strength of the magnetic field. For example, in a 21 Tesla magnetic field, protons resonate at 900 MHz. It is common to refer to a 21 T magnet as a 900 MHz magnet, although different nuclei resonate at a different frequency at this field strength in proportion to their nuclear magnetic moments. Neufeld and Stalke (2015).

2.12.2.2 *Sample handling*

A NMR spectrometer typically consists of a spinning sample-holder inside a very strong magnet, a radio-frequency emitter and a receiver with a probe (an antenna assembly) that goes inside the magnet to surround the sample, optionally gradient coils for diffusion measurements and electronics to control the system. Spinning the sample is necessary to average out diffusional motion. Whereas, measurements of diffusion constants (diffusion ordered spectroscopy or DOSY) (Johnson, 1999 and Neufeld and Stalke, 2015) are done the sample stationary and

spinning off, and flow cells can be used for online analysis of process flows. Neufeld and Stalke (2015).

2.12.2.3*Deuterated solvents*

The vast majority of nuclei in a solution would belong to the solvent, and most regular solvents are hydrocarbons and would contain NMR-reactive protons. Thus, deuterium (hydrogen-2) is substituted (99+%). The most used deuterated solvent is deuterochloroform ($CDCl_3$), although deuterium oxide (D_2O) and deuterated DMSO (DMSO-d_6) are used for hydrophilic analytes. The chemical shifts are slightly different in different solvents, depending on electronic solvation effects. NMR spectra are often calibrated against the known solvent residual proton peak instead of added tetramethylsilane, Neufeld and Stalke (2015).

2.12.2.4*Shim and lock*

To detect the very small frequency shifts due to nuclear magnetic resonance, the applied magnetic field must be constant throughout the sample volume. High resolution NMR spectrometers use shims to adjust the homogeneity of the magnetic field to parts per billion (ppb) in a volume of a few cubic centimeters. In order to detect and compensate for inhomogeneity and drift in the magnetic field, the spectrometer maintains a "lock" on the solvent deuterium frequency with a separate lock unit. The operator usually has to optimize the shim parameters manually to obtain the best possible intensity and prevent artifacts. Neufeld and Stalke (2015).

2.12.2.5*Acquisition of spectra*

Upon excitation of the sample with a radio frequency (60–1000 MHz) pulse, a nuclear magnetic resonance response - a free induction decay (FID) - is obtained. It is a very weak signal, and requires sensitive radio receivers to pick up. A Fourier transform is carried out to extract the frequency-domain spectrum from the raw time-domain FID. A spectrum from a single FID has a low signal-to-noise ratio, but fortunately it improves readily with averaging of repeated

acquisitions. Good ^{1}H NMR spectra can be acquired with 16 repeats, which takes only minutes. However, for elements heavier than hydrogen, the relaxation time is rather long, e.g. around 8 seconds for ^{13}C. Thus, acquisition of quantitative heavy-element spectra can be time-consuming, If the second excitation pulse is sent prematurely before the relaxation is complete, the average magnetization vector still points in a nonparallel direction, giving suboptimal absorption and emission of the pulse. In practice, the peak areas are then not proportional to the stoichiometry; only the presence, but not the amount of functional groups is possible to discern. An inversion recovery experiment can be done to determine the relaxation time and thus the required delay between pulses. A 180° pulse, an adjustable delay, and a 90° pulse is transmitted. When the 90° pulse exactly cancels out the signal, the delay corresponds to the time needed for 90° of relaxation (Rahmani, 2013). Inversion recovery is worthwhile for quantitive^{13}C, 2D and other time-consuming experiments (Robert and Adams 2007).

2.12.2.6 *Chemical shift*

A spinning charge generates a magnetic field that results in a magnetic moment proportional to the spin. In the presence of an external magnetic field, two spin states exist (for a spin 1/2 nucleus): one spin up and one spin down, where one aligns with the magnetic field and the other opposes it. The difference in energy (ΔE) between the two spin states increases as the strength of the field increases, but this difference is usually very small, leading to the requirement for strong NMR magnets (1-20 T for modern NMR instruments). Irradiation of the sample with energy corrsponding to the exact spin state separation of a specific set of nuclei will cause excitation of those set of nuclei in the lower energy state to the higher energy state (Rahmani, 2013).

For spin 1/2 nuclei, the energy difference between the two spin states at a given magnetic field strength is proportional to their magnetic moment. However, even if all protons have the same magnetic moments, they do not give resonant signals at the same frequency values. This

difference arises from the differing electronic environments of the nucleus of interest. Upon application of an external magnetic field, these electrons move in response to the field and generate local magnetic fields that oppose the much stronger applied field. This local field thus "shields" the proton from the applied magnetic field, which must therefore be increased in order to achieve resonance (absorption of rf energy). Such increments are very small, usually in parts per million (ppm). For instance, the proton peak from an aldehyde is shifted ca. 10 ppm compared to a hydrocarbon peak, since as an electron-withdrawing group, the carbonyl deshields the proton by reducing the local electron density. The difference between 2.3487 T and 2.3488 T is therefore about 42 ppm. However a frequency scale is commonly used to designate the NMR signals, even though the spectrometer may operate by sweeping the magnetic field, and thus the 42 ppm is 4200 Hz for a 100 MHz reference frequency (rf) (Rahmani, 2013).

However given that the location of different NMR signals is dependent on the external magnetic field strength and the reference frequency, the signals are usually reported relative to a reference signal, usually that of TMS (tetramethylsilane). Additionally, since the distribution of NMR signals is field dependent, these frequencies are divided by the spectrometer frequency. However, since we are dividing Hz by MHz, the resulting number would be too small, and thus it is multiplied by a million. This operation therefore gives a locator number called the "chemical shift" with units of parts per million,(Fürtig*et al.,* 2003). In general, chemical shifts for protons are highly predictable since the shifts are primarily determined by simpler shielding effects (electron density), but the chemical shifts for many heavier nuclei are more strongly influenced by other factors including excited states ("paramagnetic" contribution to shielding tensor). Example of the chemical shift: NMR spectrum of hexaborane B_6H_{10} showing peaks shifted in frequency, which give clues as to the molecular structure is shown in figure 2.13.

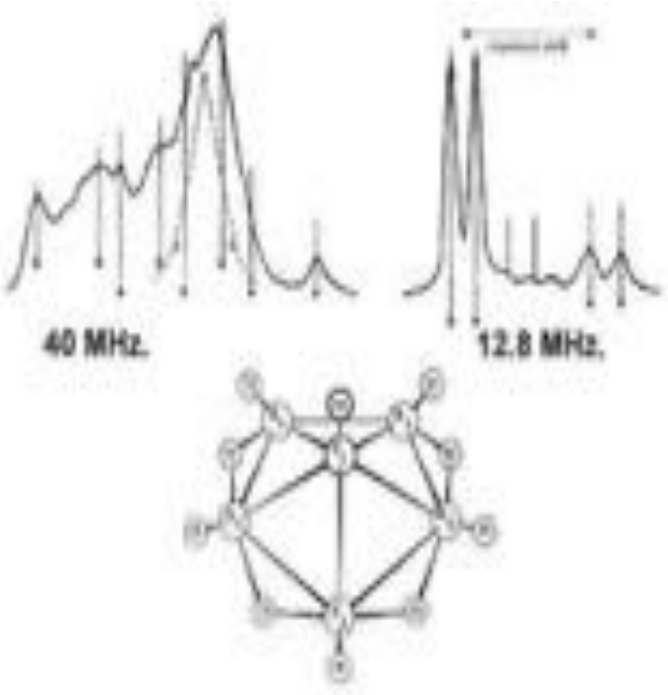
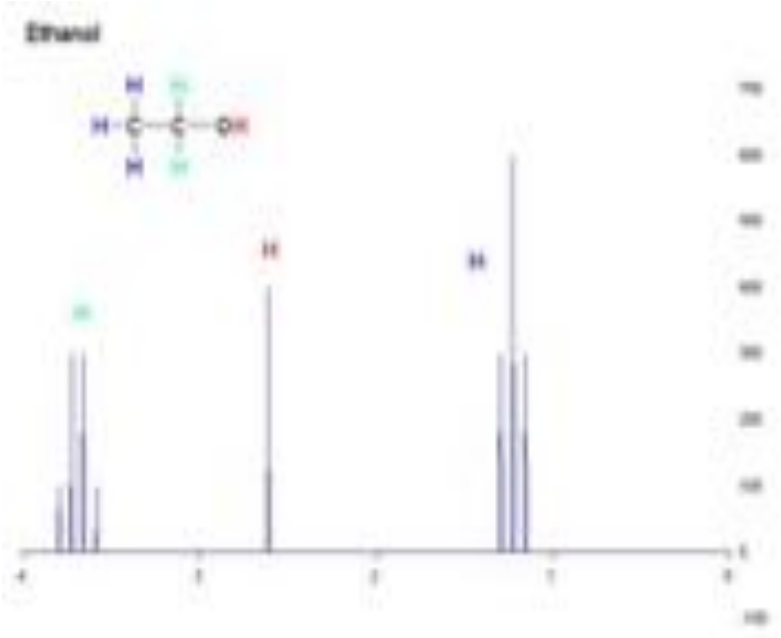


Fig 2.13 Chemical shift: NMR spectrum of hexaborane B_6H_{10}. (Rahmani, 2013).

The chemical shift provides information about the structure of the molecule. The conversion of the raw data to this information is called assigning the spectrum. For example, for the ^{1}H-NMR spectrum for ethanol (CH_3CH_2OH), one would expect signals at each of three specific chemical shifts: one for the C*H*$_3$ group, one for the C*H*$_2$ group and one for the O*H* group. A typical CH_3 group has a shift around 1 ppm, a CH_2 attached to an OH has a shift of around 4 ppm and an OH has a shift anywhere from 2–6 ppm depending on the solvent used and the amount of hydrogen bonding. While the O atom does draw electron density away from the attached H through their mutual sigma bond, the electron lone pairs on the O bathe the H in their shielding effect (Rahmani, 2013).

In Paramagnetic NMR spectroscopy, measurements are conducted on paramagnetic samples. The paramagnetism gives rise to very diverse chemical shifts. In 1H NMR spectroscopy, the chemical shift range can span 500 ppm. Because of molecular motion at room temperature, the three methyl protons average out during the NMR experiment (which typically requires a few ms). These protons become degenerate and form a peak at the same chemical shift. The shape and area of peaks are indicators of chemical structure too. In the example above—the proton spectrum of ethanol—the CH_3 peak has three times the area as the OH peak. Similarly the CH_2 peak would be twice the area of the OH peak but only 2/3 the area of the CH_3 peak (Rahmani, 2013).

Software allows analysis of signal intensity of peaks, which under conditions of optimal relaxation, correlate with the number of protons of that type. Analysis of signal intensity is done by integrationthe mathematical process that calculates the area under a curve. The analyst must integrate the peak and not measure its height because the peaks also have *width*and thus its size is dependent on its area not its height. However, it should be mentioned that the number of protons, or any other observed nucleus, is only proportional to the intensity, or the integral, of the NMR signal in the very simplest one-dimensional NMR experiments. In more elaborate experiments, for instance, experiments typically used to obtain carbon-13 NMR spectra, the integral of the signals depends on the relaxation rate of the nucleus, and its scalar and dipolar coupling constants. Very often these factors are poorly known - therefore, the integral of the NMR signal is very difficult to interpret in more complicated NMR experiments (Rahmani, 2013).



Multiplicity	Intensity Ratio
Singlet (s)	1
Doublet (d)	1:1
Triplet (t)	1:2:1
Quartet (q)	1:3:3:1
Quintet	1:4:6:4:1
Sextet	1:5:10:10:5:1
Septet	1:6:15:20:15:6:1

Fig 2.14 Example of ^{1}H NMR spectrum (1-dimensional) of ethanol plotted as signal intensity against chemical shift. (Rahmani, 2013).

There are three different types of H atoms in ethanol regarding NMR. The hydrogen (H) on the -OH group is not coupling with the other H atoms and appears as a singlet, but the CH_3- and the -CH_2- hydrogens are coupling with each other, resulting in a triplet and quartet respectively.

2.12.2.7 *J-coupling*

Some of the most useful information for structure determination in a one-dimensional NMR spectrum comes from **J-coupling** or **scalar coupling** (a special case of spin-spin coupling) between NMR active nuclei. This coupling arises from the interaction of different spin states through the chemical bonds of a molecule and results in the splitting of NMR signals. These splitting patterns can be complex or simple and, likewise, can be straightforwardly interpretable or deeptive. This coupling provides detailed insight into the connectivity of atoms in a molecule (Rahmani, 2013).

Coupling to *n* equivalent (spin ½) nuclei splits the signal into a *n*+1 **multiplet** with intensity ratios following Pascal's triangle as described on the right. Coupling to additional spins will lead to further splittings of each component of the multiplet e.g. coupling to two different spin ½ nuclei with significantly different coupling constants will lead to a doublet of doublets (abbreviation: dd). Note that coupling between nuclei that are chemically equivalent (that is, have the same chemical shift) has no effect on the NMR spectra and couplings between nuclei that are distant (usually more than 3 bonds apart for protons in flexible molecules) are usually too small to cause observable splittings. Long-range couplings over more than three bonds can often be observed in cyclic and aromatic compounds, leading to more complex splitting patterns (Rahmani, 2013).For example, in the proton spectrum for ethanol described above, the CH_3

group is split into a triplet with an intensity ratio of 1:2:1 by the two neighboring CH_2 protons. Similarly, the CH_2 is split into a quartet with an intensity ratio of 1:3:3:1 by the three neighboring CH_3 protons. In principle, the two CH_2 protons would also be split again into a doublet to form a doublet of quartets by the hydroxyl proton, but intermolecular exchange of the acidic hydroxyl proton often results in a loss of coupling information (Rahmani, 2013).

Coupling to any spin ½ nuclei such as phosphorus-31 or fluorine-19 works in this fashion (although the magnitudes of the coupling constants may be very different). But the splitting patterns differ from those described above for nuclei with spin greater than ½ because the spin quantum number has more than two possible values. For instance, coupling to deuterium (a spin 1 nucleus) splits the signal into a 1:1:1 triplet because the spin 1 has three spin states. Similarly, a spin 3/2 nucleus splits a signal into a 1:1:1:1 quartet and so on (Haner and Keifer, 2009; Rahmani, 2013).

Coupling combined with the chemical shift (and the integration for protons) tells us not only about the chemical environment of the nuclei, but also the number of neighboring NMR active nuclei within the molecule. In more complex spectra with multiple peaks at similar chemical shifts or in spectra of nuclei other than hydrogen, coupling is often the only way to distinguish different nuclei.

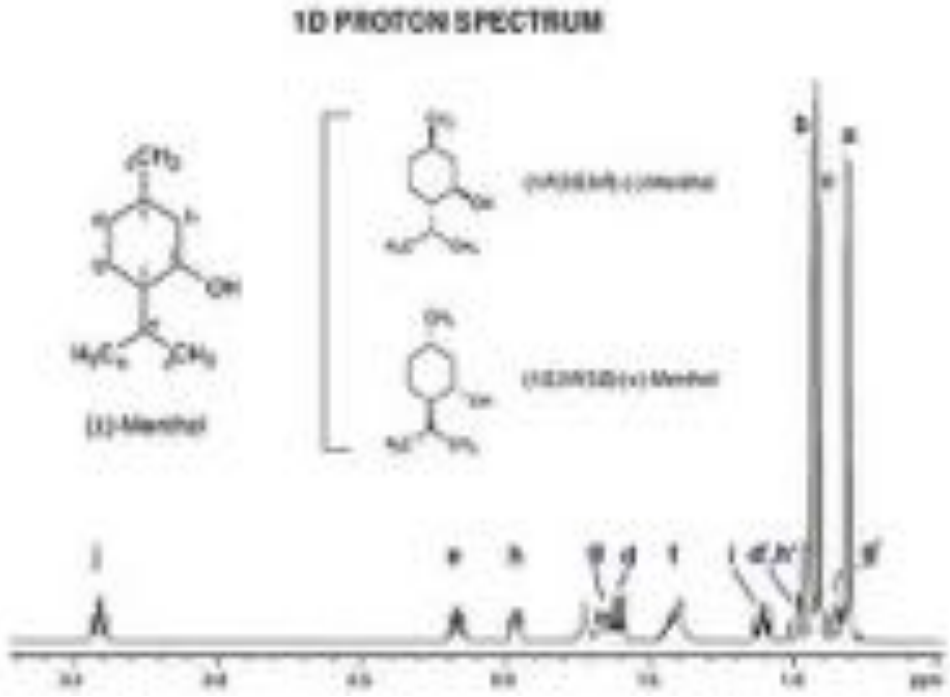


Fig 2.15 ^{1}H NMR spectrum of menthol with chemical shiftin ppm on the horizontal axis.(Rahmani, 2013).

Each magnetically inequivalent proton has a characteristic shift, and couplings to other protons appear as splitting of the peaks into multiplets: e.g. peak *a*, because of the three magnetically equivalent protons in methyl group *a*, couple to one adjacent proton (*e*) and thus appears as a doublet (Haner and Keifer, 2009; Rahmani, 2013).

2.12.2.8 *Second-order (or strong) coupling*

The above description assumes that the coupling constant is small in comparison with the difference in NMR frequencies between the inequivalent spins. If the shift separation decreases (or the coupling strength increases), the multiplet intensity patterns are first distorted, and then become more complex and less easily analyzed (especially if more than two spins are involved). Intensification of some peaks in a multiplet is achieved at the expense of the remainder, which sometimes almost disappear in the background noise, although the integrated area under the peaks remains constant. In most high are usually modest and the characteristic distortions (roofing) can in fact help to identify related peaks (Haner and Keifer, 2009; Rahmani, 2013).

Some of these patterns can be analyzed with the method of John Pople as reported by Abragam**,** **(**1961). Though it has limited scope. Second-order effects decrease as the frequency difference between multiplets increases, so that high-field (i.e. high-frequency) NMR spectra display less distortion than lower frequency spectra. Early spectra at 60 MHz were more prone to distortion than spectra from later machines typically operating at frequencies at 200 MHz or above (Haner and Keifer, 2009; Rahmani, 2013).

2.12.3 Magnetic inequivalence

More subtle effects can occur if chemically equivalent spins (i.e., nuclei related by symmetry and so having the same NMR frequency) have different coupling relationships to external spins. Spins that are chemically equivalent but are not indistinguishable (based on their coupling relationships) are termed magnetically inequivalent. For example, the 4 H sites of 1,2-

dichlorobenzene divide into two chemically equivalent pairs by symmetry, but an individual member of one of the pairs has different couplings to the spins making up the other pair. Magnetic inequivalence can lead to highly complex spectra which can only be analyzed by computational modeling. Such effects are more common in NMR spectra of aromatic and other non-flexible systems, while conformational averaging about C-C bonds in flexible molecules tends to equalize the couplings between protons on adjacent carbons, reducing problems with magnetic inequivalence (Haner and Keifer, 2009; Rahmani, 2013).

2.12.4 Correlation spectroscopy

Correlation spectroscopy is one of several types of two-dimensional nuclear magnetic resonance (NMR) spectroscopy or 2D-NMR. This type of NMR experiment is best known by its acronym, COSY. Other types of two-dimensional NMR include J-spectroscopy, exchange spectroscopy (EXSY), Nuclear Overhauser effect spectroscopy (NOESY), total correlation spectroscopy (TOCSY) and heteronuclear correlation experiments, such as HSQC, HMQC, and HMBC. In correlation spectroscopy, emission is centered on the peak of an individual nucleus; if its magnetic field is correlated with another nucleus by through-bond (COSY, HSQC, etc.) or through-space NOE) coupling, a response can also be detected on the frequency of the correlated nucleus. Two-dimensional NMR spectra provide more information about a molecule than one-dimensional NMR spectra and are especially useful in determining the structure of a molecule, particularly for molecules that are too complicated to work with using one-dimensional NMR (Haner and Keifer, 2009; Rahmani, 2013).

2.12.5 Solid-state nuclear magnetic resonance

A variety of physical circumstances do not allow molecules to be studied in solution, and at the same time not by other spectroscopic techniques to an atomic level, either. In solid-phase media, such as crystals, microcrystalline powders, gels, anisotropic solutions, etc., it is in particular the

dipolar coupling and chemical shift anisotropy that become dominant to the behaviour of the nuclear spin systems. In conventional solution-state NMR spectroscopy, these additional interactions would lead to a significant broadening of spectral lines. A variety of techniques allows establishing high-resolution conditions that can, at least for ^{13}C spectra, be comparable to solution-state NMR spectra (Haner and Keifer, 2009; Rahmani, 2013).

Two important concepts for high-resolution solid-state NMR spectroscopy are the limitation of possible molecular orientation by sample orientation, and the reduction of anisotropic nuclear magnetic interactions by sample spinning. Of the latter approach, fast spinning around the magic angle is a very prominent method, when the system comprises spin 1/2 nuclei. Spinning rates of ca. 20 kHz are used, which demands special equipment. A number of intermediate techniques, with samples of partial alignment or reduced mobility, is currently being used in NMR spectroscopy. Applications in which solid-state NMR effects occur are often related to structure investigations on membrane proteins, protein fibrils or all kinds of polymers, and chemical analysis in inorganic chemistry, but also include "exotic" applications like the plant leaves and fuel cells. For example, Rahmani et al. studied the effect of pressure and temperature on the bicellar structures' self-assembly using deuterium NMR spectroscopy (John *et al.,* 1965; Haner and Keifer, 2009; Rahmani, 2013).

2.12.6 Biomolecular NMR spectroscopy

2.12.6.1*Proteins*

Much of the innovation within NMR spectroscopy has been within the field of proteinNMR spectroscopy, an important technique in structural biology. A common goal of these investigations is to obtain high resolution 3-dimensional structures of the protein, similar to what can be achieved by X-ray crystallography. In contrast to X-ray crystallography, NMR spectroscopy is usually limited to proteins smaller than 35 kDa, although larger structures have

been solved. NMR spectroscopy is often the only way to obtain high resolution information on partially or wholly intrinsically unstructured proteins. It is now a common tool for the determination of Conformation Activity Relationships where the structure before and after interaction with, for example, a drug candidate is compared to its known biochemical activity. Proteins are orders of magnitude larger than the small organic molecules discussed earlier in this article, but the basic NMR techniques and some NMR theory also applies. Because of the much higher number of atoms present in a protein molecule in comparison with a small organic compound, the basic 1D spectra become crowded with overlapping signals to an extent where direct spectral analysis becomes untenable. Therefore, multidimensional (2, 3 or 4D) experiments have been devised to deal with this problem. To facilitate these experiments, it is desirable to isotopically label the protein with ^{13}C and ^{15}N because the predominant naturally occurring isotope ^{12}C is not NMR-active and the nuclear quadrupole moment of the predominant naturally occurring ^{14}N isotope prevents high resolution information from being obtained from this nitrogen isotope. The most important method used for structure determination of proteins utilizes NOE experiments to measure distances between pairs of atoms within the molecule. Subsequently, the distances obtained are used to generate a 3D structure of the molecule by solving a distance geometry problem. NMR can also be used to obtain information on the dynamics and conformational flexibility of different regions of a protein (John *et al.,* 1965; Haner and Keifer, 2009; Rahmani, 2013).

2.12.6.2 *Nucleic acids*

"Nucleic acid NMR" is the use of NMR spectroscopy to obtain information about the structure and dynamics of polynucleic acids, such as DNA or RNA. As of 2003, nearly half of all known RNA structures had been determined by NMR spectroscopy (John *et al.,* 1965). Nucleic acid and protein NMR spectroscopy are similar but differences exist. Nucleic acids have a smaller

percentage of hydrogen atoms, which are the atoms usually observed in NMR spectroscopy, and because nucleic acid double helices are stiff and roughly linear, they do not fold back on themselves to give "long-range" correlations. The types of NMR usually done with nucleic acids are ^{1}H or proton NMR, ^{13}C NR, ^{15}N NMR, and ^{31}P NMR. Two-dimensional NMR methods are almost always used, such as correlation spectroscopy (COSY) and total coherence transfer spectroscopy (TOCSY) to detect through-bond nuclear couplings, and nuclear Over Hauser effect spectroscopy (NOESY) to detect couplings between nuclei that are close to each other in space John *et al.,* 1965; Haner and Keifer, 2009; Rahmani, 2013).

Parameters taken from the spectrum, mainly NOESY cross-peaks and coupling constants, can be used to determine local structural features such as glycosidic bond angles, dihedral angles (using the Karplus equation), and sugar pucker conformations. For large-scale structure, these local parameters must be supplemented with other structural assumptions or models, because errors add up as the double helix is traversed, and unlike with proteins, the double helix does not have a compact interior and does not fold back upon itself. NMR is also useful for investigating nonstandard geometries such as bent helices, non-Watson–Crick basepairing, and coaxial stacking. It has been especially useful in probing the structure of natural RNA oligonucleotides, which tend to adopt complex conformations such as stem-loops and pseudoknots. NMR is also useful for probing the binding of nucleic acid molecules to other molecules, such as proteins or drugs, by seeing which resonances are shifted upon binding of the other molecule (John *et al.,* 1965; Haner and Keifer, 2009; Rahmani, 2013).

2.13 CHALLENGES AND APPROACHES TO TISSUE CLEARING METHODS

Biologists have long appreciated that it is easier to see things in thin sections than thick volumes—hence, the pervasive use of microtomes, the indispensable tools that cut thin sections

of tissue samples and provide information about cellular constituents within two-dimensional sections of biological tissues. Now, however, there is a growing trend to inquire about structure in three dimensions, requiring biologists to contend with volumes rather than sections. The need for volumetric imaging is related to the inherent three-dimensional structure of cells and organs. The

nervous system is the most obvious example, given that most individual neurons extend in many directions and their true nature cannot be ascertained by a thin section. Also, much of developmental biology requires understanding morphogenesis of organs and even whole animals in the context of three dimensions. How does one obtain such three-dimensional information? One possibility is to reconstruct three-dimensional information by putting into register a series of serial thin sections. This approach is technically challenging due to loss or distortion of individual sections that become torn, folded, compressed, or stretched. With imperfect sections, the final volumetric reconstruction can be unsatisfactory. However, if done under sufficient control, serial sectioning can give rise to very useful results (Toga *et al.,* 1997; Oh *et al.,* 2014). Another possibility is to image the surface of a block of tissue and then sequentially shave off the surface.Such ‘‘blockface’’ methods are used in both light (Toga *et al.,* 1994) and electron microscopy (Denk and Horstmann, 2004; Ichimura*et al.,* 2015). Blockface approaches eliminate the loss and alignment issues of sections but are destructive in the sense that, once each section is imaged, it is destroyed to reveal the next block surface. The other possibility is to image the volume without sectioning. Non-sectioning approaches avoid the demanding alignment issues, and the same tissue sample in principle can be imaged multiple times. One problem, however, with imaging a volume is that out of focus information from regions above and below the plane of focus contaminates information from any one plane. This problem led Marvin Minsky to invent the first confocal microscope to filter the out-of-focus light (Minsky, 1988) and led to the

revolution of ‘‘optical sectioning’’techniques-most notably, commercial laser-scanning confocal microscopes, laser scanning two-photon microscopy, parallelized confocal (i.e., spinning disk), computational image deconvolution methods, and lightsheet approaches (Conchello and Lichtman, 2005; Reynaud *et al.,* 2008; Mertz, 2011). All of these microscopy methods provide information about single planes of a volume by minimizing contributions from other parts of the volume and do so without physical sectioning. These methods thus allow access to image data from any arbitrary thin section in a thick sample. By creating a ‘‘stack’’ of such optically sectioned images, a full reconstruction of the three-dimensional features of a tissue volume can be ascertained. But even with the advent of optical sectioning microcopies, there remain major obstacles facing a microscopist looking at biological tissues that are thick. First, in some tissues, a pigment gives the tissue a color. Second, inherently fluorescent molecules may be present in the tissue or introduced during processing, giving rise to autofluorescence that masks fluorescently labeled structures of interest. The final problem, and the one that we focus on here, is that most biological tissues have an intrinsic milky appearance. This property gives tissues the look of frosted glass or translucence. The lack of clarity undermines sharp images and becomes progressively more of an impediment the deeper one tries to look into a tissue volume. This translucency is caused by light scattering. Light rays that should travel in straight lines are deviated many times as light is reflected off of molecules, membranes, organelles, and cells in the tissue. It is useful to delve a bit into the underlying physics of scattering in order to understand the kinds of strategies that have been used to clear tissues (John *et al.,* 1965; Haner and Keifer, 2009; Rahmani, 2013).

2.13.1 How Does Tissue Scatter Light?

The purpose of this short analysis is to understand that clearing tissues is not aimed at preventing scattering (only a vacuum has no scattering) but, rather, assuring that there is a high uniform

density of scatterers so that lateral scattering is minimal and that all wavelengths of light pass ‘‘through’’ the tissue. The ways to think about light range from light as rays, light as waves, or light as photons. Although rays are easiest to talk about and photons seem closest to the true essence of light, it is the wave framework that is most helpful when thinking about light scattering. Light waves of a particular color (wavelength) vibrate at a particular frequency and have an electrical and magnetic component. The electrical component is the one that for the most part interacts with the atoms in biological tissues. The wave vibration is extraordinarily fast. For example, red light (600 nm wavelength) vibrates at the rate of 0.5 3 1015 oscillations per second. Let’s imagine this wave is a plane that passes an atom or molecule from a particular direction (plane waves are basically light that propagates in a single direction without converging or diverging, like a laser beam). As the plane wave reaches the atoms, it may impart some of its energy from its electrical component to the atom or molecule, typically to an outer electron which is more susceptible to absorbing energy than electrons closer to the nucleus or protons or neutrons in the nucleus. For most molecules, the energy absorbed by an electron is not sufficient to cause an electron to jump to a new orbital. Therefore, neither fluorescence (re-emission of lower-energy

light; nor ionization (removal of the electron from the atom or molecule) occurs (Lichtman and Conchello, 2005). Rather, the light wave’s vibrational energy momentarily causes the electron to vibrate (as if it were connected to its nucleus by a spring that was stretched a bit by the incoming light wave). The electron vibration is short lived, and all of the energy absorbed is quickly released again in the form of another light wave. There are a few differences between the incoming light wave and the outgoing light wave emitted by the atom or molecule. First, the incident light is coming from a particular direction, but the outgoing light is sent in all directions as a spherical wave. Thus, the light is scattered. Because the whole process occurs without any

energy loss, the light is said to be elastically scattered. Elastic scattering means that the vibrational frequency (and hence the wavelength) of the scattered light is unchanged from the incoming light. The second difference is that the interaction between the incident light wave and the electron cloud of the scatterer, although brief, causes a momentary pause in the light's progression, evidenced by the fact that the new scattered wave is delayed (usually one-half of a wavelength). The duration of the delay is only about a femtosecond (10-15 s) for visible light. But as light passes through a material, it interacts with many molecules and these little delays associated with each interaction add up. As a result, light's propagation through the material is slowed down. This reduced velocity is the basis of the so-called refractive index of the medium (literally, the ratio of the speed of light in a vacuum divided by the speed of light in the medium). The amount of slowdown per unit volume is proportional to the density of molecules and hence the number of electrons that the light wave can interact with. But density is only one variable that affects refractive index. Some materials such as the hydrophobic molecules in the plasma membrane have electrons that are more susceptible to absorbing light energy than other molecules, such as the hydrogen atoms in water. Thus, even though the density of water surrounding a cell is higher than the density of the fatty material in cell membranes (fat, after all, floats in water), the membrane has a higher index of refraction than water (1.45 versus 1.33). Although it is commonly explained that scattering occurs due to the mismatch of the index of refractions at interfaces between different substances in a tissue, this is not the whole story. As we will explain below, scattering occurs everywhere that there are molecules, not just at sites of refractive index mismatches. It is more accurate to say that heterogeneity in the amount of scattering between different regions in biological material is actually what gives rise to the scattering and milky appearance. The light energy absorbed by an electron is re-radiated in all directions as an expanding spherical wave. This spherical wave, sometimes called a ‘‘wavelet,’’

is a wave that diverges from a source as if that source were a luminous object sending wave energy along the expanding surface of a sphere. The two-dimensional analog of a spherical wave would be the circular wave that originates on the surface of a pool of water from the site where a pebble is dropped. The emitted spherical wave, just like the original incident light, can and will interact with electrons in other atoms or molecules re-emerging over and over again as new spherical waves at different sites. The wave conception of light is powerful because it explains how light waves can add their amplitudes and give rise to brighter light though constructive interference when they crest or trough in phase. The intensity of light is actually the square of the summed amplitudes integrated over time, so both troughs and crests give light energy. The wave conception also explains why light may sometimes not be present if two waves reach the same place half a wavelength out of phase (i.e., while one wave is in the crest and another is in the trough at the same place). In this situation, the sum of their amplitudes is zero, and no light from those wavelets can appear at that site; this is known as destructive interference. Destructive interference explains why homogenous materials, such as air, water, and glass, appear clear even though the molecules in these substances are scattering light. In such materials, the density of scattering molecules is so high that many scatterers exist even over dimensions much smaller than the wavelength of visible light (i.e., between 400 and 700 nm). Air molecules, for example, are about 3 nm apart, and liquid water molecules are about ten times closer together (Hecht, 2001). When a plane wave of light passes through such materials, all of the molecules in a plane are set into vibration simultaneously and give rise to densely packed spherical waves. The consequence of such a high density of scatterers is nearly complete destructive interference in the axes of the plane. This cancellation occurs because when wavelets are uniformly distributed at high density, each point in the plane is bombarded by wavelets at every phase. For example, for every cresting wave, another is in the trough at the same place. As a result, the sum amplitude is

zero in the plane, preventing any light from escaping in any lateral direction out of the plane (i.e., hardly any light propagates perpendicular to the direction of the impinging light wave. We know, of course, that light continues in the forward direction in media like air, water, or glass without difficulty, raising the question of why the scatterers in front of each molecule don't also give rise to destructive interference by the same argument. Distinct from the simultaneous vibrations of all the scatterers in a plane, the molecules in front of the plane are activated later, when the primary plane wave reaches them. The scattered light from the molecules that were previously acted on by the plane wave reach this forward direction slightly later due to the additional half wavelength phase delay of scattered light. The scattered light from the earlier illuminated molecules always constructively interferes with the scattered light originating from molecules at more forward sites. Thus, in the forward direction, the amplitudes sum constructively and light propagates. Given that dense materials like water don't scatter in lateral directions, why do dense cellular tissues scatter? The important point here is the inhomogeneity of scatterers. The prevention of lateral scattering requires that each scattering molecule is equidistant from other scatterers at every distance in the plane. For example, in living tissue, a physiological saline solution surrounds the membrane of a cell. This means that the amount of scattering in the membrane is likely to be different than the amount of scattering in the saline solution. Thus, scattering from the water molecules near the membrane may not be completely cancelled by the scattering from the membrane itself; thus, both materials will generate light scattered perpendicular to the direction of light impinging on the sample. If a tissue is sufficiently thick, then most of the incoming light will be scattered and the tissue will behave as if it contains a multitude of little luminous sources, each sending light in every direction. This multiple scattering is the property that generates the whitish translucency of tissues. The whitish color implies that all wavelengths of visible light are scattered, and this is due to the intrinsic inhomogeneities of scatterers in the

tissue. The sizes of the inhomogeneities affect the wavelength of the light that is scattered. For particles that are much smaller than the wavelength of visible light, short-wavelength light has a greater probability of being absorbed and re-emitted (in proportion to the wavelength to the fourth power, Rayleigh scattering). As a result, there is some tendency for short wavelengths to scatter more than long ones. This is sometimes mentioned as an advantage of using the long-wavelength infrared-light-based two-photon excitation on thick samples, as relatively less of the exciting light is scattered compared to visible or ultraviolet light. For particles larger than about one-tenth of the wavelength, such as organelles and large protein complexes, the wavelength dependence of scattering is not evident (Mie scattering). Older Clearing Techniques It has been known for more than a century that biological samples can be stably maintained in a somewhat transparent hardened material resin. Indeed, ancient flies in amber show that resins can stably maintain biological samples for 50 million years. Many of the resins used by biologists are hydrophobic, requiring that the sample be dehydrated. Following dehydration (in a series of alcohol-water mixtures with progressively less water), samples are put into solvents that dissolve lipids and act to remove one of the main sites of tissue inhomogeneity: the membranes. Canada Balsam is such a resin that provides a transparent mountant for tissue that is dehydrated and cleared with xylene. Such resins, however, are intrinsically fluorescent and are best used with absorbance dyes like the Golgi stain rather than fluorophores due to high background (Rost, 1992). Moreover, most fluorescent proteins require an aqueous environment, so this kind of clearing quenches the signal. At the beginning of the 20th century, Spalteholz described a clearing technique for large (entire organ and organ system) tissues using organic solvents (Spalteholz, 1914). The method was intensive, requiring various dehydration, tissue bleaching, and clearing steps. However, it produced samples that were unprecedented at the time and helped to push forward the field of anatomy (Spalteholz, 1898). Unfortunately, this approach damaged

the superficial few centimeters of a tissue and therefore was useful only for clearing the largest samples (Steinke and Wolff, 2001). Modern Clearing Techniques. The optical sectioning advances mentioned above (i.e., confocal, two-photon, and image deconvolution) led to fluorescence volume imaging becoming the contrast method of choice for microscopy at the end of the 20th century. Most notably, two photon detection pushed microscopy from imaging depths of tens of microns to fractions of a millimeter (Denk*et al.,* 1990). Genetically encoded fluorescent proteins provided a labeling method with high specificity that did not require antibodies to diffuse through the entire sample to their target and hence motivated ever deeper imaging (Chalfie*et al.,* 1994). However, the scatter of light in heterogeneous tissue remained a limiting factor for these techniques, preventing researchers from achieving high-resolution three-dimensional renderings of thick tissue.

The source of the scattering is a diverse set of cellular constituents, including ribosomes, nuclei, nucleoli, mitochondria, lipid droplets, membranes, myelin, cytoskeletal components, and extracellular matrix components such as collagen and elastin. Therefore, techniques that can alter the scattering properties of cellular components hold the key for unlocking the full potential of today's best optical sectioning light microscopes. As volume fluorescence microscopy gained in popularity and advances in data acquisition and storage made large volume imaging possible, a number of attempts were made to revisit the tissue clearing techniques that had originally been developed by Spalteholz. All tissue clearing techniques focus on equilibrating the refractive index throughout a sample to reduce inhomogeneities in light scatter. However, the field was quickly split into two approaches: (1) those that followed Spalteholz's historical reliance on tissue dehydration and solvent-based clearing and (2) emerging aqueous-based techniques.

2.13.2 Solvent-based clearing techniques

Solvent-based clearing techniques are most commonly comprised of two steps: (1) dehydration with lipid solvation and (2) additional lipid solvation and clearing by refractive index matching to the remaining dehydrated tissue's index. A number of solvents have been tested for use in either the dehydration or clearing steps (Peters, 1961; Becker *et al.,* 2012). Most commonly, dehydration is now performed using methanol with or without hexane or with tetrahydrofurane (THF) alone (Spalteholz, 1914; Dodt*et al.,* 2007; Ertu¨ rk*et al.,* 2012a, 2012b; Renier*et al.,* 2014). While these agents remove water, they also solvate and remove some of the lipids. Removal of water and lipid results in a fairly homogenous, primarily proteinaceous, dense (i.e., high index of refraction) sample. Dehydrated protein has a refractive index of >1.5 (higher than water or lipid). Therefore, the dehydration step must be followed by a second set of agents that solvate additional lipid and intercalate homogeneously throughout the sample to clear it by matching the higher refractive index of the defatted and dehydrated tissue. To date, methylsalicilate, benzyl alcohol, benzyl benzoate, dichloromethane, and dibenzyl ether have been used as final clearing solutions (Spalteholz, 1914; Steinke and Wolff, 2001; Becker Dodt*et al.,* 2007; Ertu¨ rk*et al*., 2012a, 2012b; Renier*et al.,* 2014)

The ideal organic solvent for clearing possesses two characteristics. First, it must have a high lipid-solvating capacity and, second, it must possess a high (R1.5) refractive index. As alluded to above, low-density organic molecules can have surprisingly high refractive indices if a number of loosely held electrons, such as those found in the pi bonds of an aromatic ring, are present. Many of the most successful clearing solvents contain one or two aromatic rings in their structures. Oscillations can easily be imparted to these electrons when they are placed in the electric field of a light wave. When the clearing solvent's refractive index is well matched to the refractive index of the dehydrated tissue sample, all non-forward scatter is destructive, as described above, and the tissue becomes clear. Importantly, these ring structures do not generally

allow for electrons to be promoted to excited states and yield fluorescence with the moderate energy levels available at visible light wavelengths. One major limitation to solvent-based clearing methods is that dehydration removes water molecules from the sample that are necessary to maintain emission from most fluorescent protein chromophores. This limitation was partially addressed in two of the most recent advances in solvent clearing, 3DISCO (Ertu¨ rk*et al.,* 2012a, 2012b) and iDISCO (Renier*et al*., 2014). These techniques allow for solvent-based clearing that can maintain fluorescent protein emission for a few days. 3DISCO replaces the methanol dehydration step with incubation in THF. iDISCO presents two options. First, a traditional methanol dehydration step can be performed that results in the quenching of fluorescent proteins. These molecules are then visualized by standard immunofluorescence with an antibody directed against the fluorescent protein. The second approach involves replacing the methanol dehydration step with a number of aqueous solutions containing phosphate-buffered saline (PBS), detergent, and dimethyl sulfoxide (DMSO). This combination of aqueous solutions and solvents sustained GFP expression longer than the traditional BABB or 3DISCO protocols, presumably due to residual water in the sample; however, by 2–4 days, the fluorescence had dissipated. Alternative labeling options include fluorescent proteins optimized for correlative electron and light microscopy that are able to survive dehydration (Paez-Segala*et al.,* 2015) or the use of immunofluorescent labeling with antibodies raised against the fluorescent proteins and conjugated to dehydration-resistant organic dyes (Cai *et al.,* 2013; Renier*et al.,* 2014). The solvent-based clearing techniques are robust and work on a number of different tissue types. However, the toxic nature of many solvents, their capacity to dissolve glues used in the construction of objective lenses, substantial shrinkage of tissue during dehydration (up to 50%; Becker *et al*., 2012), and the quenching of fluorescent protein emission reduce their utility.

2.13.3 Aqueous-Based Clearing

The inability to preserve fluorescent protein emission in many of the solvent-based techniques and a desire to prevent changes in tissue architecture (primarily dehydration-induced shrinkage) led a number of researchers to pursue aqueous-based clearing solutions. All aqueous-based techniques to date utilize one of three mechanistic approaches for homogenizing the scattering throughout a sample: (1) passive immersion in a solution that is refractive index matched to the tissue, (2) removal of lipid followed by hydration of the sample to lower the refractive index of the remaining tissue components or, (3) active or passive removal of lipid followed by immersion in a refractive index matched medium. Simple Immersion Passive clearing by immersion in high refractive index solutions is effective especially for smaller samples. Most commercially available microscopy mounting media are glycerol based with refractive indices between 1.40 and 1.44 and are able to impart a slight clearing effect on thin cells and tissues. However, because the refractive index of these mountants are not well matched to that of coverslip glass and immersion oil (1.51), reduced image quality due to spherical aberration is often noticeable in samples greater than a few cell layers thick, and additional clearing and/or index-matching approaches are required (Staudt *et al.,* 2007). In simple immersion, a tissue sample is placed in an aqueous solution containing a dissolved, high refractive index molecule and is allowed to gradually clear. Sucrose (Tsai *et al.,* 2009b), fructose (Ke*et al.,* 2013; Costantini*et al.,* 2015), glycerol (Meglinski*et al.,* 2002), 2,20-thiodiethanol (TDE) (Staudt *et al.,* 2007; Aoyagi *et al.,* 2015; Hou *et al*., 2015), and formamide (Kuwajima*et al*., 2013) have all been used for this purpose. Generally, a refractive index greater than 1.45 needs to be reached to achieve adequate clearing of hydrated samples that still contain lipids. Highly concentrated sugar or glycerol solutions can be difficult to work with due to their high viscosity. At the working concentrations required for index matching, there is potential for precipitation at room temperature, air bubbles can easily be introduced, and movement of the sample through the

clearing solutions during imaging may not be possible. However, a number of alternative simple immersion clearing solutions with lower viscosities exist to avoid these issues. The proprietary Focus Clear (Chiang *et al.,* 2002) and published Refractive Index Matched solution (RIMs, Yang *et al.,* 2014) utilize low-viscosity, high refractive index contrast reagents often used in biomedical imaging as their key components. Both diatrizoic acid (Hypaque) used in FocusClear and Histodenz used in RIMs are complex molecules that contain an aromatic ring and three iodine atoms. This chemistry provides a large number of electrons for interaction with passing light waves (i.e., high refractive index) but in a relatively low-concentration, low-viscosity solution. In both cases, the high expense of these reagents may limit their use. Two lower-cost options are TDE and the FRUIT technique. TDE is a water-soluble, low-viscosity liquid that can be tuned over a range of refractive indices by diluting it in water. It was first demonstrated as a mounting media for super-resolution microscopy (Staudt *et al.,* 2007) but can also clear large tissues (Aoyagi *et al.,* 2015; Costantini*et al.,* 2015). One drawback to TDE is that, at high concentrations, the brightness of a number of fluorophores is reduced (Staudt *et al.,* 2007). FRUIT is a combination of the SeeDB and Scale techniques (Hou *et al.,* 2015). FRUIT mixes urea with fructose to lower the overall viscosity of the SeeDB fructose solution and improve tissue penetration and clearing. Although the techniques of passive clearing in simple aqueous solutions does not clear as well as the solvent-based methods or other aqueous-based clearing methods discussed below, they are economical and easy to implement and retain compatibility for use in samples with a wide range of florescent dyes and proteins, including lipid targeting dyes. Hyperhydration Most simple immersion techniques do not remove lipid and simply try to match the average refractive index of a tissue (>1.45) by replacing the liquid in and around a tissue with a high refractive index solution. An alternative approach is to remove lipid and reduce the refractive index of tissue samples during the clearing process. The first technique to

take advantage of this mechanism was Scale (Hama *et al*., 2011). Scale utilizes detergent-based removal of lipid in conjunction with urea-mediated hydration (in the presence of glycerol) of the remaining tissue to produce a cleared sample. The strategy to remove the lipid but without hydrophobic solvents (in order to maintain an aqueous environment for fluorescent proteins) is by extensive incubation lasting days to months with detergent (e.g., Triton X-100) and multiple solution changes. At the same time, urea is used to penetrate, partially denature, and thus hydrate even the hydrophobic regions of high refractive index proteins (Hua *et al*., 2008). This hyperhydration reduces the overall refractive index to 1.38 (Hama *et al.,* 2011). The atomic-level explanation for this reduction in refractive index is not well understood. However, the reduced refractive index may be due to a spreading apart of dense, high refractive index scattering sites within protein complexes by partial denaturation and hydration. This view is supported by the observation that these samples have an expanded volume. This expansion can be controlled by adjusting glycerol levels in the clearing solution, which may act to lower the concentration of water that has access to protein moieties. If the relative placements or absolute sizes of objects in the tissue are important, then care must be taken, as greater hydration produces better clearing but also creates greater expansion. In addition, a larger sample will take longer to image. Interestingly, one recent approach that intentionally enlarges samples for the purpose of imaging diffraction-limited structures (see below) also has the effect of clearing the tissue. ClearT, a simple immersion method that utilizes a urea-like molecule (formamide) as the active clearing reagent, may partially act by this mechanism as well (Kuwajima*et al.,* 2013). This potential hyperhydration may help to explain ClearT and FRUIT's ability to clear tissue thicker than most simple immersion techniques. The urea-based CUBIC method utilizes a similar hyperhydration mechanism. However, a high refractive index sucrose based clearing solution was proposed as an optional second step to expedite the clearing process (Susaki*et al.,* 2014; Tainaka*et al.,* 2014).

CUBIC also uses very high triton levels (50%) to maximize lipid removal. This lipid removal process can result in a high degree of protein loss (24%–41%), which lowers epitope concentrations and potentially weakens immunostaining (Chung *et al*., 2013).

2.13.4 Hydrogel Embedding

The aqueous-based clearing methods discussed thus far are either limited to clearing small samples (simple immersion) or are slow (hyperhydration). In addition, any technique utilizing harsh solvents or high concentrations of detergent risks removal of large percentages of the protein content of the tissue. The CLARITY and PACT/PARS methods attempt to address these issues by first embedding the tissue in hydrogel (Chung *et al.,* 2013; Tomer *et al*., 2014; Yang *et al.,* 2014).

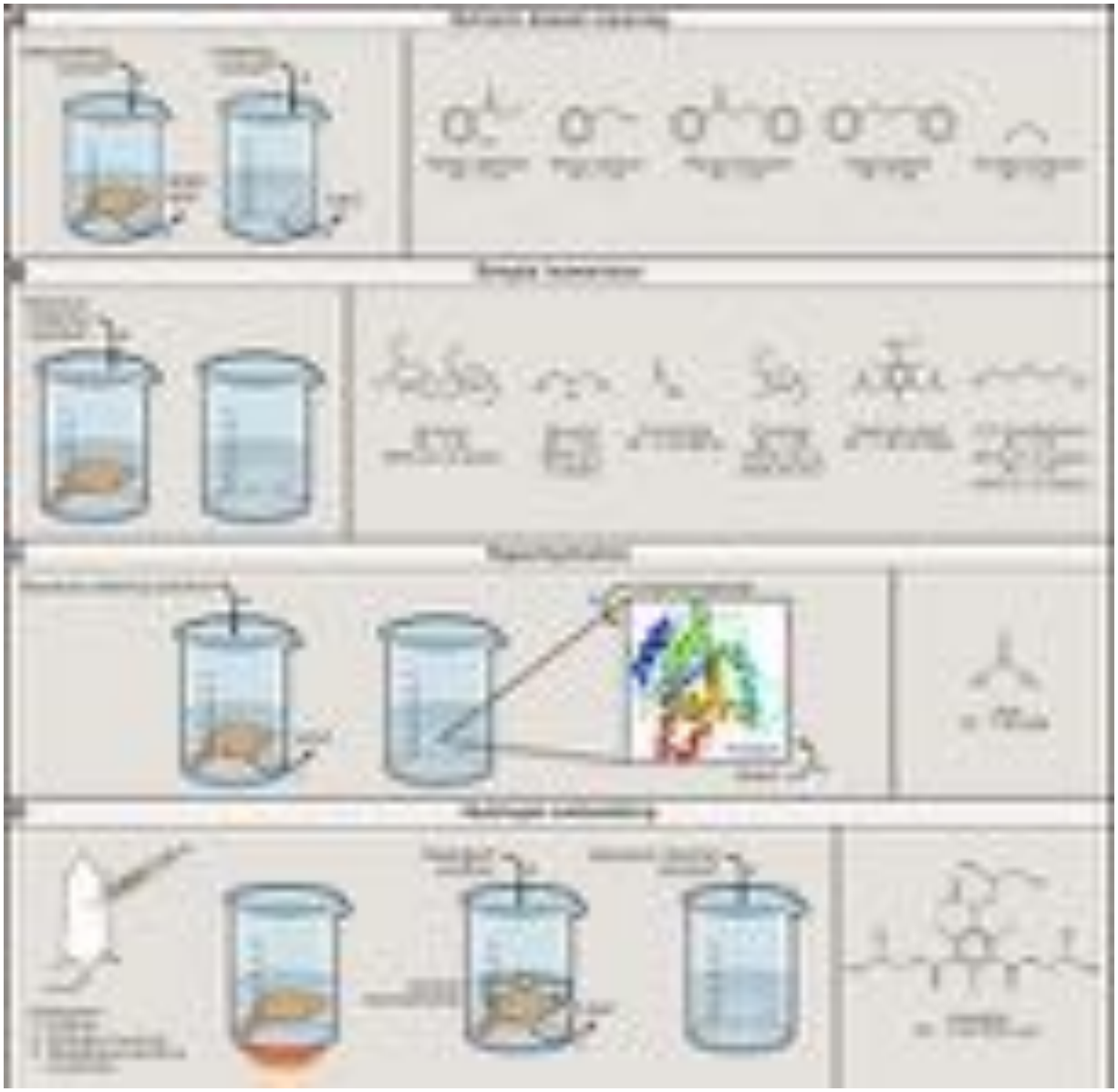


Fig 2.16 Methodology of Different Tissue Clearing Techniques (Douglas et al, 2015)

(A) (Left) Solvent-based clearing is a two-step process. First, the tissue is dehydrated and lipid is removed. Second, the tissue is moved to a high refractive index solvent where additional lipid solvation and clearing occurs. (Right) Molecules commonly used for solvent-based clearing along with the refractive indices (RI) of the pure chemical.

(B) (Left) For simple immersion, the tissue to be cleared is placed in an aqueous clearing solution for days to months. During this time, the solution is exchanged repeatedly. (Right) Molecules commonly used for simple immersion along with the refractive indices (RI) at the commonly used concentration.

(C) Hyperhydration involves submerging the sample in an aqueous solution and allowing it to passively clear. During this clearing step, urea or formamide in the clearing solution can enter tightly folded regions of high refractive index proteins, creating an osmotic gradient that pulls in water as well. This partially denatures the protein, hydrates it, and decreases its overall refractive index. Some hyperhydration methods contain detergent that is used to disrupt membranes and remove lipid from the sample.

(D) (Left) Hydrogel embedding is most often performed on an entire animal by perfusing with a fixative, a temperature-sensitive crosslinker, and the hydrogel monomer. Alternatively, these chemicals can be passively diffused into an isolated tissue sample. Once fixed, the tissue of interest is warmed to induce hydrogel

Table 2.6 Comparismof Tissue Clearing Techniques

(A) Solvent Based	Final RI	Key Components	Time to Clear	Immunostaining Demonstrated	Alterations in Tissue Morphology	fluorescent protein Emission	Lipid Preserved	Toxic
Spatleholz	1.55	benzylbenzoate/ methylsalicilate	Months	No	shrinkage	No	no	Yes
BABB	1.55	benzylalcohol/ benzylbenzoate	Days	Yes	shrinkage	yes, but only half day	no	Yes
(B) Simple Immersion								
Sucrose	1.44	Sucrose	1 day	Yes	no	Yes	no	No
ClearTa	1.44	Formamide	hours–days	Yes	no	No	yes	No
(C) Hyperhydration								
Scale A2	1.38	4M urea, 10% glycerol	Weeks	No	expansion	Yes	no	no
CUBIC	1.38	4M urea/50% sucrose	Days	Yes	expansion	Yes	no	yes
(D) Hydrogel Embedding								
CLARITY	1.45	FocusClear/80% glycerol	Days	Yes	slight expansion	Yes	no	No
PARS	1.38–1.4	Histodenz	Days	Yes	no	yes	no	No

Source: Cell 162, 2015 Elsevier Inc.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

The materials and reagents used for this study include xylene (BDH England), ethanol (BDH England), neutral buffered formalin, chloroform, paraffin wax, egg albumen, Oven, Rotary microtome (Leica RM2 125 RTS) made in England Search Tech Instrument, Tissue processor (Leica TP 1020, England) and binocular light microscope (Olympus). The ethanol and xylene (Sigma –Aldrich) were purchased from Cardinal Scientific Supply, Zaria, Nigeria.

Mayer's reagent (Potassium Mercuric Iodide), Acetone, Methanol, phenolphthalein indicator

Fehling's solution A and B, glacial acetic acid,concentratedsulphuric acid (H_2SO_4), Sodium hydroxide (NaOH), 10% hydrochloride acid, benzene,10 % ammonia solution, specific gravity bottle, Desiccators, Hot air Oven, electronic weighing balance., electronic calculator, 250 conical flask, measuring cylinders, thermometer, test tubes, Bunsen burner, Oswald type viscometer, refractometer with model RFM311 RFM 340 of Bellingham and Stanley LTD, water bath, GC-MS QP-2010 instrument at 70 eV and 250^0C GC Column, made in Japan, Bruker DRX500 spectrophotometer (Bruker Biospin, Rheinstetten, Germany)

3.1.1 Plant Materials for the Oil

Fresh leaves of Eucalyptus (Famliy: Myrtaceae. *Eucalyptus Citrodora. Hook*) plants were obtained from National Research Institute for Chemical Technology (NARICT) farm, Zaria. The leaves were taxonomically identified and authenticated in the Department of Biological Science herbarium, ABU Zaria, with the following voucher number: 2767

Famliy: MYRTACEAE. *Eucalyptus Citrodora. Hook.*

3.1.2 Ethical clearance/Approval

Ethical approval (ABUCAUC/20/58) was obtained from Ahmadu Bello University (ABU) Committee on Animal Use and Care (ABUCAUC), copy attached in appendix III.

3.1.3 Experimental Animals

Fiveapparently healthy adult male Wistar rats, weighing between 120g-140g were obtained from the Department of Human Anatomy, ABU Zaria. The Wistar rats were kept and maintained on standard laboratory condition of room temperature, two weeks for acclimatization before the experiments. The Wistar rats were feed with standard pellet diet and water and allowed *ad libitum*

3.2 Methods

3.2.1 Extractions of the CitrodoraOil

The Citrodora oil was extracted in NARICT by Hydro distillation method, as described by Ladan, *et al,*(2011). 500 g of the fresh leaves of eucalyptus was 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 to avoid rapid oxidation that will lead to color change.

3.2.2 Determination of the percentage yield, mean cost of the Oils

The percentage yield of each of the oil when the total weight of the eucalyptus leaves used and the weight of the Citrodora oil obtained were determined, using the formula

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

W_1 = net weight of oils (grams).The weight of oil alone without the weight of its container

W_2 = total weight of fresh leaves (grams)
(Nkafamiya*et al.,* 2010)

The cost of the oil was determined by adding all the amount spent to produce 500ml of the oil, this included the amount charged to get the eucalyptus leaves and the laboratory production cost.

3.2.3 Phytochemical Screening of theCitrodoraOils

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

Alkaloids

Mayer's Test:

Few drops ofMayer's reagent (Potassium Mercuric Iodide) were added to 2 ml of the extract. Formation of a yellow cream colored precipitate indicates the presence of alkaloids (Siddiqui and Ali, 1997).

Carbohydrates

Fehling's test

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

Cardaic Glycosides

Kella-KillianiTest for cardiac glycosides

To 2ml of the extract glacial acetic acid, few drops of 5% $FeCl_3$ andconcentrated 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 (Treaseand Evans,1989).

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).

Terpenes

To 2ml of extract 5ml chloroform and 2ml concentratedsulphuric acid (H_2SO_4)was added.Reddish brown coloration of interface indicates the presence of terpenes (Harbon, 1973)

Flavonoids

Sodium hydroxide (NaOH) test for flavonoid

Few drops of aqueous NaOH were added to 5ml of BM extract, there was no yellow coloration observed indicating the absenceof Flavinoid(Treaseand Evans,1989).

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).

Antraquinones Derivatives

Modified Borntrager's test for combined anthracene

2 ml of the sample was boiled with 5ml of 10% hydrochloride acid for 3minutes. 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 5m1 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).

3.2.4 Physical Properties of the Essential Oils

3.2.4.1 *Specific gravity (SG)*

An improvised specific gravity bottle was washed, rinsed with acetone and dried in the oven. The bottle was allowed 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 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

(Munson *et al.,* 2016).

3.2.4.2 *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. 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 (Dana, 1992)

3.2.4.3 *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 mixed 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

(British Standard Institute, 1995)

3.2.4.4 *Acidic Value (AV)*

1ml 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:

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

Where M = molarity of base. (Kardash, and Tur'yan, 2005)

3.2.4.5 *Saponification Value (SV)*

The method used was that of the British Standard Institute, 1995. Two grams 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
(British Standard Institute, 1995)

3.2.4.6*Iodine Value (IV)*

1ml 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 (Association of Official Analytical chemists, 1975)

3.2.4.7 *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(Association of Official Analytical chemists, 1975)

3.2.4.8 *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(Association of Official Analytical chemists, 1975).

3.2.4.9 *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 (Association of Official Analytical chemists, 1975).

3.2.4.10 *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.

3.2.4.11*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.

3.2.5 Gas Chromatography–Mass Spectroscopy (GC-MS) Studies of the Oil

The GC-MS of the Citrodora 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).

3.2.6 Nuclear Magnetic Resonance (NMR) technique

NMR spectra were recorded on a Bruker DRX500 spectrophotometer (Bruker Biospin, Rheinstetten, Germany) at 400MHz(1H) and 100 MHz (13C). Samples were prepared in CDCL3 with tetra ethyl silage (TMS) as an internal standard. The chemical shift values (sigma sign here) we're measured relative to the internal standard in ppm.

3.2.7 Animals Sacrifice and Fixation

Five Wistar rats were anaesthetized with ketamine hydrochloride (0.5 mg/kg) administratedintraperitoneally. The rats were perfused with the neutral buffered formalin via the cardiac artery under anaesthesia and then their livers and kidneys were excised and immediately fixed in 10 % neutral buffered formalin,while the brains and testes were fixed in Bouin fluid for 72 hours before processing.

3.2.8 Experimental Protocol

Duplicates tissues from each animal were collected by taking about 5mmx5mm thick from each organ. One set of the duplicate were labelled as C1, C2, C3, C4 and C5 (Group A). They represented the tissues that were cleared in Citrodora oil during the tissue processing and dewaxing prior to staining. The other set of tissue duplicate were labelled X1, X2, X, X4 and X5 (Group B) respectively. Tissues in this group were cleared in xylene during the tissue processing and dewaxing prior to staining.

3.2.9 Method of tissue processing

The tissue processing technique employed was as outlined by Bancroft and Gamble (2008) and Kieranan (2010). Briefly, tissues were dehydrated in graded ethanol in ascending grades (70%, 80%, 90%, and 100%), cleared I Citrodora oil or xylene, impregnated and embedded in paraffin wax respectively. All the tissues were subjected to the same treatment during the tissue processing except for the clearing step, where tissues were separated into the two groups (Group A and B). The group A tissues were cleared in the Citrodora oil, while the group B tissues in xylene using the same time interval. The tissue processing protocol is represented in Figure 3.1 and in Table 3.1 below:

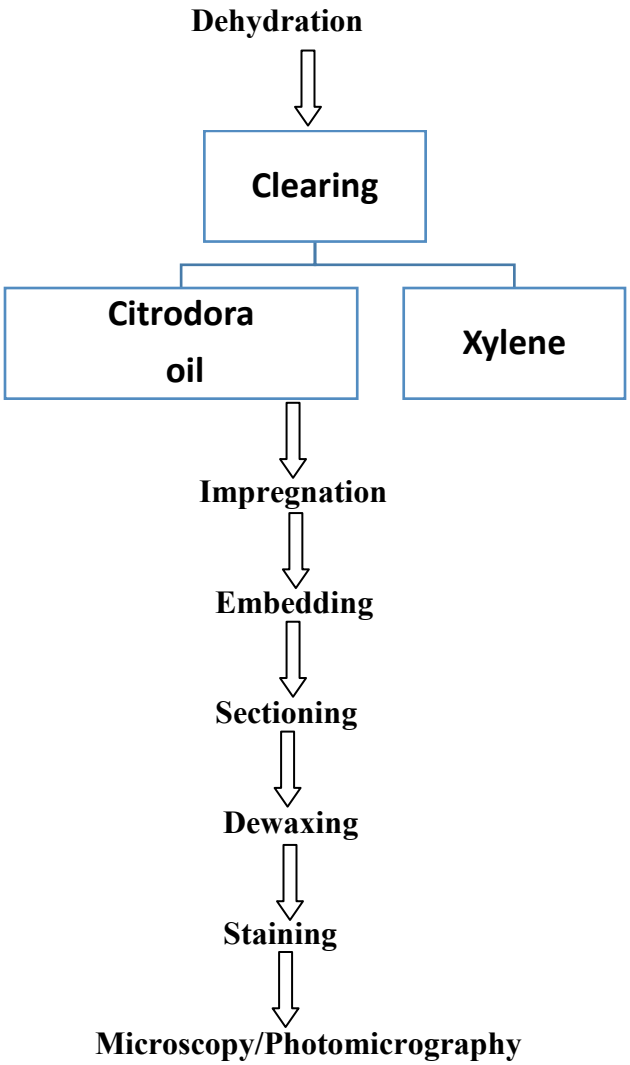


Figure 3.1: Tissue processing technique

All the embedded tissues were surface trimmed to remove excess wax and exposed tissues. The tissues blocks were all cooled on ice for 30 minutes before sectioning with Rotary microtome (Leica RM2 125 RTS) made in England at four microns (4µ) for the liver, kidney and testes tissues and 7 microns (7µ) for the brain tissues. Sections were floatedout on water (52^0C) for 2 minutes each, drained for 3 minutes then dried for 2 hours at 62^0C before staining.

Table 3.1 The protocol for tissue processing, embedding, and sectioning

PROCEDURE	REAGENT	TIME (min)	TEMPERATURE (°C)
Fixation	10 % neutral buffered formalin and Bouin fluid	4320	Room Temprature
Dehydration	Ethanol 70%	30	,,
	Ethanol 80%	30	,,
	Ethanol 90%	30	,,
	Ethanol 100%	30	,,
	Ethanol 100%	30	,,
	Ethanol 100%	30	,,
Clearing	Citrodora oil or xylene	30	,,
	Citrodora oil or xylene	30	,,
Infiltration	Paraffin wax	30	,,
	Paraffin wax	30	,,
	Paraffin wax	30	,,
Embedding	Paraffin wax	Not applicable	,,
Sectioning	Paraffin wax	Not applicable	,,

Kunhua*et al.,* 2012.

3.2.10 Staining Tissue Section

All the group A tissue sections (C1, C2, C3, C4 and C5) were dewaxed in the Citrodora oil and the group B tissue sections (X1, X2, X, X4 and X5) in xylene separately, then hydrated prior to staining as shown in Table 3.2 bellow:

Table 3.2 Dewaxing and hydration of sections before Staining

PROCEDURE	REAGENT	TIME (min)	TEMPERATURE (°C)
Dewaxing	Citrodora oil or xylene	3	Room Temprature
	Citrodora oil or xylene	3	,,
	Citrodora oil or xylene	3	,,
			,,
Rehydration	Ethanol 100%	3	,,
	Ethanol 100%	3	,,
	Ethanol 90%	3	,,
	Ethanol 80%	3	,,
	Ethanol 70%	3	,,
	Water	Not determined	,,
Staining		Acording to manuals	,,

Source: Kunhua*et al.,* 2012 (modified).

3.2.11 Demonstration and Photomicrograph of Stained Tissue Sections

The histological and histochemical staining includes haematoxylin and eosin (H and E) method for general tissue structures, Gordon and Sweet staining for reticulin fibers (Puchtler and

Waldrop, 1978; Bralet*et al.,* 2000); Golgi silver staining for nerve fibers (Bancroft and Stevens,2008); Perodic acid-Schiff (PAS) staining for glycogen (Doyle and Campbell, 1976) and Immunohistochemical staining for astrocytes using Glial fibrillar acidic protein (GFAP) andCD 34 for ependymal cells antigens were also carried. All the staining procedures were done in the Department of Human Anatomy Histology and Histochemical Laboratories, Ahmadu Bello University Zaria, except for the GFAP and CD 34 Immunohistochemical staining which was carried out in Ahmadu Bello University Teaching Hospital (ABUTH) Zaria, using GFAP and CD34 antibodies (DBBiotech, Slovakia).

From each of the cleared tissues in Citrodora oil and Xylene five sections were made. One was stained using the routine Haematoxylin and Eosin stain for demonstrating general tissue structures, while the others were stained using special stains and immunohistochemical stains, which include:Gordon and Sweets, for demonstrating reticular fibres, Periodic Acid Schiff for glycogen and basement membrane and Golgi Cox stain for nerve cells as described by Bancroft and Stevens (2010).The immunohistochemical stains are the Glial fibrillary acidic protein (GFAP) and the CD34 markers. Stained sections were examined microscopically and photomicrographed as shown in plate 1 to 24.

3.2.12 Assessment of stained sections

The stained sections were examined microscopically (light microscope Olympus) and photomicrographed using the Amscope 3.0 digital camera for microscope and telescope made in China (Amscope and Microscope Accessory supplier).

3.2.13 Evaluation Criteria and Method

All tissue sectionswere coded and evaluated by ten researchersindependently using certain criteria as guide. All the slides were coded to minimise bias. The criterial used for the assessment

of section quality were the presence of folds, cracks, and artefacts. While the parameters considered to determine the staining quality were the nuclear staining, cytoplasmic details, staining intensity, uniformity and contrast/clarity. The section and staining quality were evaluated by direct microscopic observation and graded (scored). Each of the parameter were given a score of Excellent (5 scores), satisfactory (4 scores), Good (3 scores) fair (2 scores) or poor (1 score)based on the ability to appreciate the stained section. The scoring criteria were modified from that employed by Marin *et al.* (2017), where they used Poor (p=0-1), Satisfactory (S=2), Good (G=3) or Excellent (E=4) to score and grade the stained slide sections.

3.2.14 Data Analysis

Data generated were analyzed using SPSS version 20. 0. Values were expressed as mean plus or minus (±) standard error of mean (SEM). Student' t- test, were used to compare the parameters of the oil with that of the standard xylene and Chi-square test was used to check the association. P-value less than or equal to ($P < 0.05$) was considered statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1 PHYTOCHEMICAL SCREENING OF THE CITRODORA OIL

The phytochemical screening showed positive result for carbohydrate inCitrodora oil. Citrodora oil also showed positive result for glycosides, steroid and Terpenoids. Citrodora Oil shows the absence ofanthraquinones, anthracene, saponins,flavonoid, tannins and alkaloid were observed (Table 4.1).

4.2 Physical Properties of Citrodora Oil and Xylene

In this study it was observed that, Citrodora oil has a lesser value of 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),flash point (35^{o} C) and viscosity (0.34%). This study also observed that, xylene has slightly higher value of 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), flash point (39 ^{o} C), and viscosity (0.45%) (Table 4.2).

4.3Refractive Index (RF) of Citrodora oil and xylene at 28 ^{0}C

In the present study xylene have the highest refractive index of 1.496 followed by Citrodora (1.457), and when compared to that of water (1.33), they have a higher RF as shown in Table 4.3

Table 4.1: Results of Phytochemical Screening of the Citrodora oil

S/N	Phytoconstituents	**Inference**
1	Saponins	Positive (+)

2	Terpenoid/steroids	Positive (+)
3	Anthraquinones	Negative (-)
4	Cardiac glycosides	Deoxy sugar present(+)
5	Flavonoids	Negative (-)
6	Carbohydrates	Positive (+)
7	Alkaloids	Negative (-)
8	Tannins	Negative (-)

Key: Negetive (-)=Phytoconstituientsabsent, Positive (+) = Phytoconstituientspresent

Table 4.2: Results of Physical Properties of Citrodora oil and Xylene

S/N	Parameters	Citrodora Oil	Xylene
1	Color	Pale yellow	Colorless
2	Specific Gravity	8.3g/ml	8.6 g/ml
3	Density	0.801g/ml	0.804 g/ml
4	Free Fatty Acid	5.6	10.9
5	Acid Value	11.8	16.9
6	Saponification Value	98	112
7	Iodine Value	18	19
8	Pour Point	-37^0C	-35^0C
9	Cloud Point	-69^0C	-65^0C
10	Flash point	35^0C	39^0C
11	Viscosity	0.34cp at 28^0C	0.45cp at 28^0C

Table 4.3Refractive Index of Citrodora oil and xylene at 28 ^{0}C

Sample	Refractive Index
Water	1.335
Citrodora oil	1.457
Xylene	1.496

4.4 COST OF CITRODORA OIL AND XYLENE USED IN THE TISSUE CLEARING AND DWAXING PROCESS

In Table 4.4,the estimated cost of the 500 ml of xylene was ₦1,600.00 (Naira) while that of the Citrodora oil was ₦2, 100.00 (Naira) respectively. The total time used for all the tissue clearing process using the Citrodora oil and xylene was 60 minutes. The volume used for the Citrodora oil and xylene was 500ml each.

Table 4.4: Cost of Citrodora oil and the Time used in the clearing process

	Citrodora	Xylene
Volume (ml) used in clearing	500	500
Total Time (minutes)of clearing	60	60
Cost of Citrodora leaves (50kg)	₦300.00	Not applicable
Cost of Laboratory production of Citrodora oil (500ml)	₦1,800.00	Not applicable
Cost of 500ml xylene	Not applicable	₦1,600.00

4.5 COMPOSITION OF CITRODORA OIL AND XYLENE ANALYSIS

The GC-MS analysis showed that the predominant constituents in the Citrodora oil isCitronellal 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.

4.6 GAS CHROMATOGRAPHY–MASS SPECTROSCOPY (GC-MS) PROFILE OF CITRODORA OIL AND XYLENE

The GC-MS profile for the Citrodora oil showing the highest peak at 5 and closely followed by peak 7. (Fig 4.1). Peak 5 represented Citronellal (42.20 %) which had the highest as shown in Table 4.5. On the other hand, xylene GC-MS profile showing peak 2 to be the highest, and this represent p-xylene (97%) as shown in Table 4.5.

4.7 NUCLEAR MAGNETIC RESONANCE (NMR) OF THE CITRODORA OIL

The IR spectrum of Citrodora oil showed characteristic absorption frequencies at 3444cm-1 which was consistent with C-H vibration for free H- bonded carboxylic acids, vibrations at 1463cm-1 and 1379 cm-^{1} were observed to be associated with C-H vibrations of CH2 and CH3 bending respectively. Furthermore, vibrations at 2713cm^{-1} and 1722 cm^{-1} with absorption peaks of medium intensities were observed to be for C-H vibrations for aliphatic aldehydes. Symmetric and asymmetric C-H vibrations due to methyl groups were recorded at 2877cm^{-1} and 2959cm^{-1} respectively and the extended conjugation.

Table 4.5: Percentage composition of Citrodora oil and xylene

	Peak No.	Component	%
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%
Xylene	1	o-xylene	94%
	2	p-xylene	97%
	3	m-xylene	98%
	4	p-xylene	98%

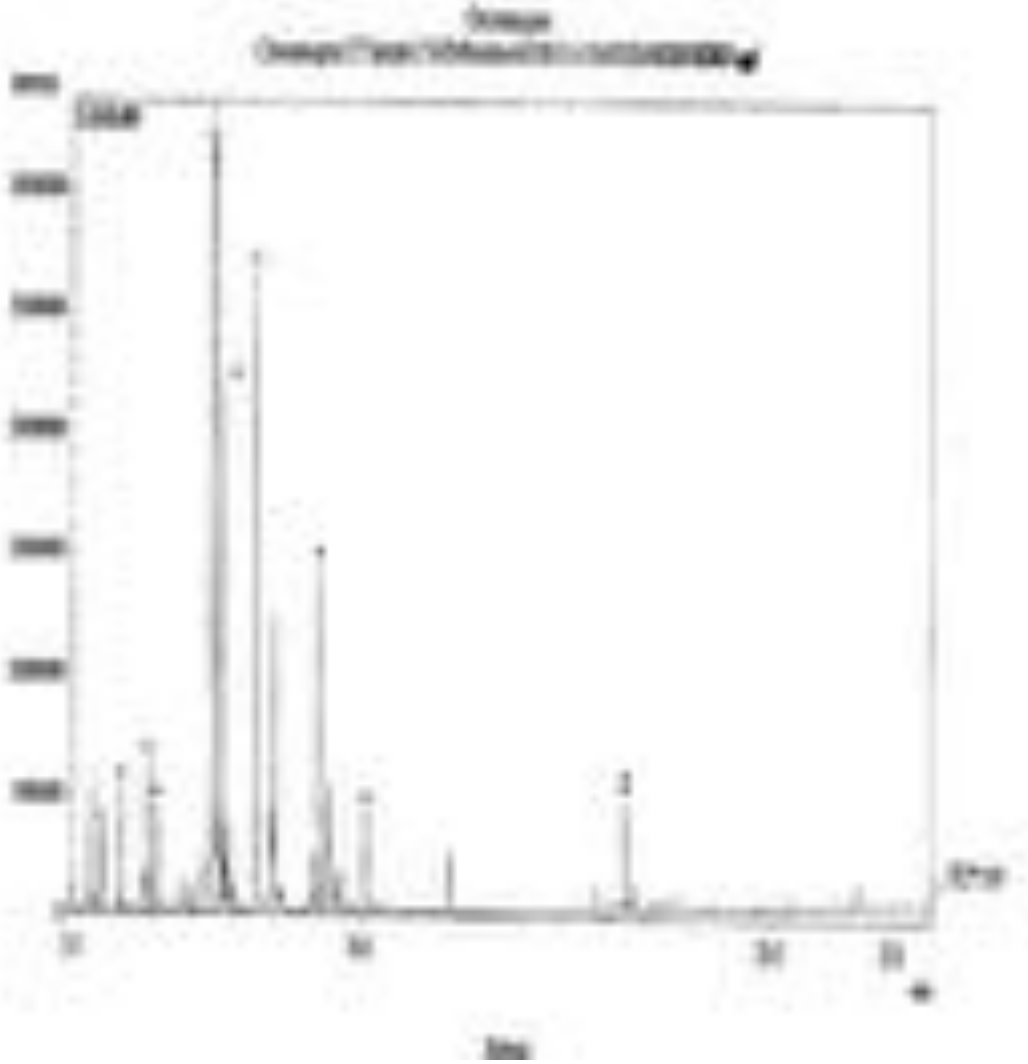


Figure 4.1Gas Chromatography–Mass Spectroscopy (Gc-Ms)Profile of Citrodora Oil

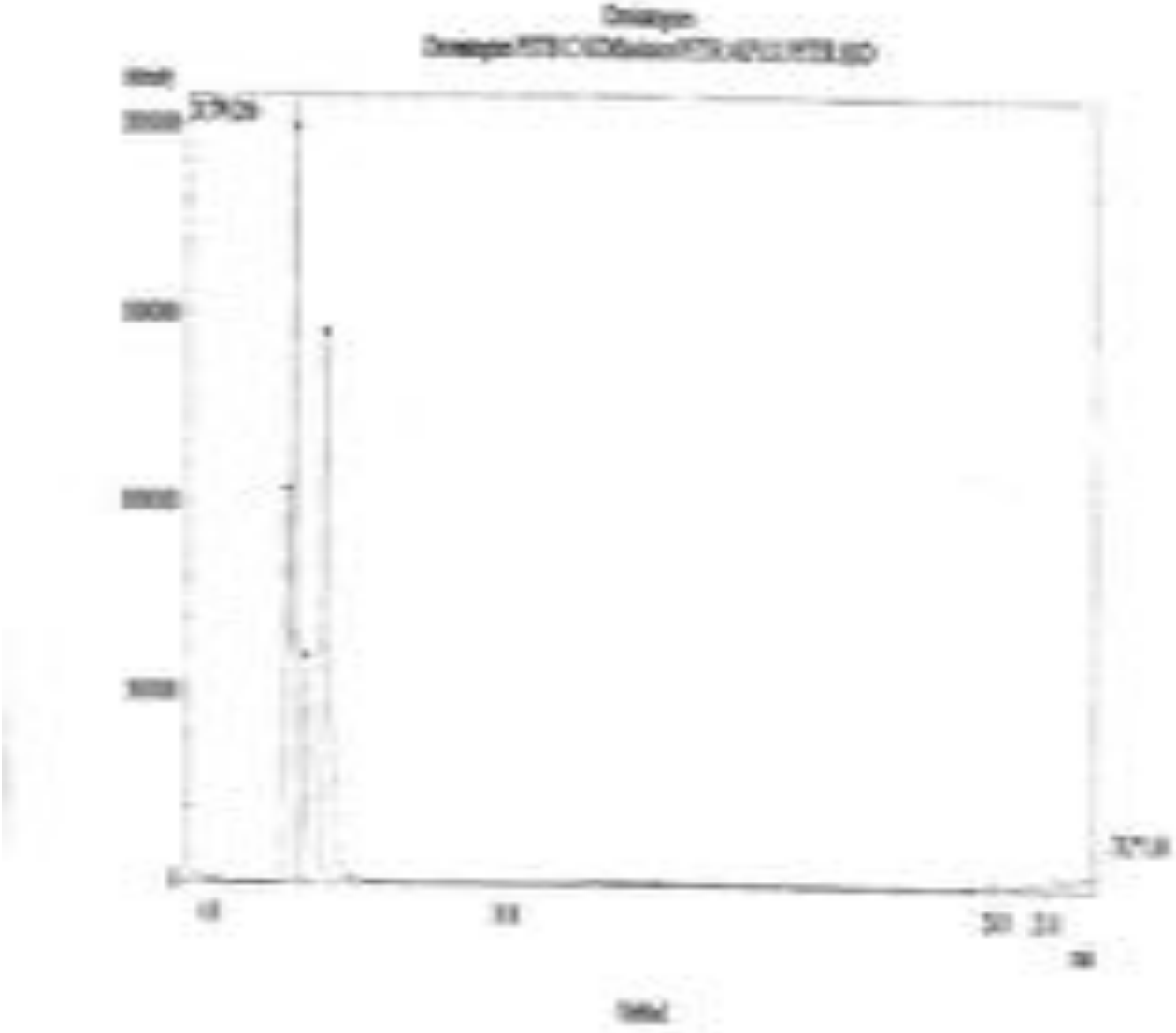


Figure 4.2Gas Chromatography–Mass Spectroscopy (Gc-Ms)Profile of xylene

Table 4.6: Assignment of ^{1}H NMR Signal for Citrodora Oil

S/N	Chemical shift value (δ)	Functional group	Attribution
1	0.87 (m)	-CH3 (acyl)	Sat. Oleic and linoleic
2	0.92 (td)	-CH3 (acyl)	Sat. Oleic and linoleic
3	1.22 (dddd)	-(CH2)n	Aliphatic chain
4	1.32 (ddt)	-(CH2)n	Aliphatic chain
5	1.55 (d)	-OCO-CH2-CH2-	Oleyl acyl chain
6	1.63 (q)	-CH2-C-CO2-	
7	1.98 (m)	-CH2-CH=CH=	Unsaturated fatty acid
8	2.18 (ddd)	-CH2-CH=CH=	Unsaturated fatty acid
9	2.35 (ddd)	-O-CO-CH2	Oleyl acyl chain
10	5.04 (tdq)	-CH2-O-COR	*sn*-1,3 Diacylglycerol
11	9.69 (t)	-R-CHO	Aldehydes

Table 4.7: Fourier transform infrared spectroscopy (FTIR) frequency vibrations for Citrodora oil

S/N	Frequency vibrations (cm^{-1})	Attribution
1	1379	CH3 bending (rocking)
2	1463	CH2 bending
3	1647	C=C extended conjugation
4	1722	C-H overtune
5	2713	C-H aliphatic aldehyde
6	2877	
7	2914	CH3 symmetric
8	2959	CH3 asymmetric
9	3444	C-H free H-bonded carboxylic acid

4.8 THE EFFECT OF CITRODORA OIL AND XYLENE ON LIVER AND KIDNEY SECTIONS AND STAINING QUALITY SCORES

The study revealed that there was no statistically significant differences ($p > 0.05$) between Citrodora oil and xylene as clearing and dewaxing agents on liver section quality scores (Table 4.8). Similarly, it was observed that the scores of nuclear staining, cytoplasmic contrast and clearity as well as staining intensity of liver sections stained with Hand E dis not vary significantly ($p > 0.05$) with the use of xylene or Citrodora oil as clearing and dewaxing agents (Table 4.9).

Table 4.8: Effect of Citrodora oil and Xylene on liver section quality score under H and E stain

Section Quality	**Type of Clearing Agent (Liver H andE)**			
Folds	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 0.277$, df = 2, p = 0.871
Good	0(0.0%)	0(0.0%)	0(0.0%)	
Satisfactory	3(15.0%)	2(10.0%)	5(25.0%)	
Excellent	6(30.0%)	7(35.0%)	13(65.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
Cracks	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 2.291$, df = 2, p = 0.514
Good	0(0.0%)	2(10.0%)	2(10.0%)	
Satisfactory	3(15.0%)	2(10.0%)	5(25.0%)	
Excellent	6(30.0%)	5(25.0%)	11(55.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
Artefacts	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 2.077$, df = 3, p = 0.557
Good	0(0.0%)	1(5.0%)	1(5.0%)	
Satisfactory	3(15.0%)	1(5.0%)	4(20.0%)	
Excellent	6(30.0%)	7(35.0%)	13(65.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	

Table 4.9: Effect of Citrodora oil and Xylene on liver staining quality score under H and E stain

Staining Quality	**Type of Clearing(Liver H and E)**			
NS	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	1(5.0%)	1(5.0%)	$x^2 = 4.485$, df = 3, p = 0.214
Good	1(5.0%)	1(5.0%)	2(10.0%)	
Satisfactory	5(25.0%)	1(5.0%)	6(30.0%)	
Excellent	4(20.0%)	7(35.0%)	11(55.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
CCC	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 6.171$, df = 3, p = 0.104
Good	0(0.0%)	1(5.0%)	1(5.0%)	
Satisfactory	6(30.0%)	1(5.0%)	7(35.0%)	
Excellent	3(15.0%)	7(35.0%)	10(50.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
SIU	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	1(5.0%)	1(5.0%)	$x^2 = 6.171$, df = 3, p = 0.104
Good	1(5.0%)	1(5.0%)	2(10.0%)	
Satisfactory	6(30.0%)	1(5.0%)	7(35.0%)	
Excellent	3(15.0%)	7(35.0%)	10(50.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	

Key: nuclear staining (NS), Cytoplasmic contrast and clarity (CCC),Staining intensity and uniformity (SIU)

The scores of section quality (Table 4.10) of liver sections satined with CD34 immunohistochemical techniques showed no significant difference ($p > 0.05$) when Citrodora oil or Xylene were used as clearing and dewaxing agents. In the same way, comparison of scores of staining qualities (Table 4.11) carried out on liver sections did not vary significantly ($p > 0.05$) when Citrodora oil and xylene were used as clearing and dewaxing agents under CD34 immunohistochemical stains.

Table 4.10: Effect of Citrodora oil and Xylene on liver section quality score under CD34 immunohistochemical stain

Section Quality	Type of Clearing(Liver CD34)			
Folds	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 0.000$, df = 1, p = 1.000
Good	0(0.0%)	0(0.0%)	0(0.0%)	
Excellent	9(45.0%)	9(45.0%)	18(90.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
Cracks	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 0.000$, df = 1, p = 1.000
Good	0(0.0%)	0(0.0%)	0(0.0%)	
Excellent	9(45.0%)	9(45.0%)	18(90.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
Artefacts	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 0.000$, df = 1, p = 1.000
Good	0(0.0%)	0(0.0%)	0(0.0%)	
Excellent	9(45.0%)	9(45.0%)	18(90.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
NS	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	0(0.0%)	0(0.0%)	
Good	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 2.250$, df = 2, p = 0.325
Satisfactory	0(0.0%)	2(10.0%)	2(10.0%)	
Excellent	9(45.0%)	7(35.0%)	16(80.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	

Table 4.11: Effect of Citrodora oil and Xylene on liver staining quality score under CD34 immunohistochemical stain

Staining Quality	**Type of Clearing(Liver CD34)**			
NS	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	0(0.0%)	0(0.0%)	
Good	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 2.250$, df = 2, p = 0.325
Satisfactory	0(0.0%)	2(10.0%)	2(10.0%)	
Excellent	9(45.0%)	7(35.0%)	16(80.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
CCC	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	0(0.0%)	0(0.0%)	
Good	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 2.250$, df = 2, p = 0.325
Satisfactory	0(0.0%)	2(10.0%)	2(10.0%)	
Excellent	9(45.0%)	7(35.0%)	16(80.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
SIU	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	0(0.0%)	0(0.0%)	
Good	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 3.600$, df = 2, p = 0.165
Satisfactory	0(0.0%)	3(15.0%)	3(15.0%)	
Excellent	9(45.0%)	6(30.0%)	15(75.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	

Key: nuclear staining (NS), Cytoplasmic contrast and clarity (CCC),Staining intensity and uniformity (SIU)

It was observed that liver sections stained with Gordon and Sweet for reticular fibers showed no significant difference ($p > 0.05$) in section quality (Table 4.12) and staining quality(Table 4.13) scores between the use of Citrodora oil and xylene as clearing and dewaxing agents. Similarly, sections of kidneys stained with H and E and scored for section quality (Table 4.14) and staining quality (Table 4:15) showed no significant difference ($p > 0.05$) between Citrodora oil and xylene used as clearing and dewaxing agents. Staining with CD34 immunohistochemical technique did not reveal any significant difference ($p > 0.05$) in the section (Table 4.16) and section (Table 4.17) qualities of Citrodora oil and xylene cleared kidney sections.

Table 4.12: Effect of Citrodora oil and Xylene on liver section quality score under Gordon and Sweet histochemical stain for reticular fibers

Section Quality	**Type of Clearing (Liver Gordon and Sweet)**			
Folds	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 1.059$, df = 2, p = 0.589
Good	0(0.0%)	0(0.0%)	0(0.0%)	
Satisfactory	0(0.0%)	1(5.0%)	1(5.0%)	
Excellent	9(45.0%)	8(40.0%)	17(85.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
Cracks	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 1.059$, df = 2, p = 0.589
Good	0(0.0%)	0(0.0%)	0(0.0%)	
Satisfactory	0(0.0%)	1(5.0%)	1(5.0%)	
Excellent	9(45.0%)	8(40.0%)	17(85.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
Artefacts	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 6.923$, df = 2, p = 0.031
Good	0(0.0%)	0(0.0%)	0(0.0%)	
Satisfactory	0(0.0%)	5(25.0%)	5(25.0%)	
Excellent	9(45.0%)	4(20.0%)	13(65.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	

Table 4.13: Effect of Citrodora oil and Xylene on liver staining quality score under Gordon and Sweet histochemical stain for reticular fibers

Staining Quality	**Type of Clearing (Liver Gordon and Sweet)**			
NS	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	0(0.0%)	0(0.0%)	
Good	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 2.250$, df = 2, p = 0.325
Satisfactory	0(0.0%)	2(10.0%)	2(10.0%)	
Excellent	9(45.0%)	7(35.0%)	16(80.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
CCC	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	0(0.0%)	0(0.0%)	
Good	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 3.600$, df = 2, p = 0.165
Satisfactory	0(0.0%)	3(15.0%)	3(15.0%)	
Excellent	9(45.0%)	6(30.0%)	15(75.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
SIU	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	0(0.0%)	0(0.0%)	
Good	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 2.250$, df = 2, p = 0.325
Satisfactory	0(0.0%)	2(10.0%)	2(10.0%)	
Excellent	9(45.0%)	7(35.0%)	16(80.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	

Key: nuclear staining (NS), Cytoplasmic contrast and clarity (CCC),Staining intensity and uniformity (SIU)

Table 4.14: Effect of Citrodora oil and Xylene on kidney section quality score under H and E stain

Section Quality	**Type of Clearing (Kidney H andE)**			
Folds	**Citrodora Oil**	**xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 0.000$, df = 2, p = 1.000
Good	0(0.0%)	0(0.0%)	0(0.0%)	
Satisfactory	1(5.0%)	1(5.0%)	2(10.0%)	
Excellent	8(40.0%)	8(40.0%)	16(80.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
Cracks	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 0.000$, df = 2, p = 1.000
Good	0(0.0%)	0(0.0%)	0(0.0%)	
Satisfactory	1(5.0%)	1(5.0%)	2(10.0%)	
Excellent	8(40.0%)	8(40.0%)	16(80.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
Artefacts	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 0.000$, df = 2, p = 1.000
Good	0(0.0%)	0(0.0%)	0(0.0%)	
Satisfactory	1(5.0%)	1(5.0%)	2(10.0%)	
Excellent	8(40.0%)	8(40.0%)	16(80.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	

Table 4.15: Effect of Citrodora oil and Xylene on kidney staining quality score under H and E stain

Staining Quality	**Type of Clearing (Kidney Hand E)**			
NS	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Good	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 1.000$, df = 2, p = 0.607
Satisfactory	4(20.0%)	2(10.0%)	6(30.0%)	
Excellent	5(25.0%)	7(35.0%)	12(60.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
CCC	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	0(0.0%)	0(0.0%)	
Good	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 2.104$, df = 2, p = 0.349
Satisfactory	5(25.0%)	2(10.0%)	6(35.0%)	
Excellent	4(20.0%)	7(35.0%)	12(55.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
SIU	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	0(0.0%)	0(0.0%)	
Good	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 1.000$, df = 2, p = 0.607
Satisfactory	4(20.0%)	2(10.0%)	6(30.0%)	
Excellent	5(25.0%)	7(35.0%)	12(60.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	

Key: nuclear staining (NS), Cytoplasmic contrast and clarity (CCC),Staining intensity and uniformity (SIU)

Table 4.16: Effect of Citrodora oil and Xylene on kidney section quality score under CD34 immunohistochemical stain

Section Quality	**Type of Clearing (Kidney CD34)**			
Folds	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 1.067$, df = 3, p = 0.785
Good	1(5.0%)	1(5.0%)	2(10.0%)	
Satisfactory	1(5.0%)	0(0.0%)	1(5.0%)	
Excellent	7(35.0%)	8(40.0%)	15(75.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
Cracks	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 2.410$, df = 3, p = 0.492
Good	1(5.0%)	2(10.0%)	3(15.0%)	
Satisfactory	2(10.0%)	0(0.0%)	2(10.0%)	
Excellent	6(30.0%)	7(35.0%)	13(65.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
Artefacts	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 1.067$, df = 3, p = 0.785
Good	1(5.0%)	1(5.0%)	2(10.0%)	
Satisfactory	1(5.0%)	0(0.0%)	1(5.0%)	
Excellent	7(35.0%)	8(40.0%)	15(75.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	

Table 4.17: Effect of Citrodora oil and Xylene on kidney staining quality score under CD34 immunohistochemical stain

Staining Quality	**Type of Clearing (Kidney CD34)**			
NS	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	1(5.0%)	1(5.0%)	$x^2 = 5.067$, df = 3, p = 0.167
Good	3(15.0%)	0(0.0%)	3(15.0%)	
Satisfactory	0(0.0%)	1(5.0%)	1(5.0%)	
Excellent	7(35.0%)	8(40.0%)	15(75.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
CCC	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	1(5.0%)	1(5.0%)	$x^2 = 3.619$, df = 3, p = 0.306
Good	2(10.0%)	1(5.0%)	3(15.0%)	
Satisfactory	2(10.0%)	0(0.0%)	2(10.0%)	
Excellent	6(30.0%)	8(40.0%)	14(70.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
SIU	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	1(5.0%)	1(5.0%)	$x^2 = 5.067$, df = 3, p = 0.167
Good	3(15.0%)	0(0.0%)	3(15.0%)	
Satisfactory	0(0.0%)	1(5.0%)	1(5.0%)	
Excellent	7(35.0%)	8(40.0%)	15(75.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	

Key: nuclear staining (NS), Cytoplasmic contrast and clarity (CCC),Staining intensity and uniformity (SIU)

Hematoxylin and Eosing stained sections of testes fromWistar rats evaluated for sectioning (Table 4.18) and staining (Table 4.19) qualities revealed no significant difference ($p > 0.05$) between tissues cleared in Citrodora oil and xylene. When the PAS stain was used on testicular sections, it was observed that both sectiing (Table 4.20) and staining (Table 4.21) qualities did not vary significantly ($p > 0.05$) between xylene and Citrodora oil cleared tissues.

Similarly, brain section quality score (Table 4.22) showed no significant differenceand($p > 0.05$) under H and E stain, but in staining quality score (Table 4.23) significant difference ($p < 0.05$) was observed between the staining ability of tissues cleared and dewaxed inCitrodora oil and xylene.

Comparison of GFAP stained sections (Table 4.24 and Table 4.25) and Golgi Silver staings (Table 4.26 and Table 4.27) of Wistar rats brains followed the above pattern of insignificant ($p > 0.05$) variation in the section and atining qualities of xylene and Citrodora oil cleared tissues.

Table 4.18: Effect of Citrodora oil and Xylene on testes section quality score under H and E stain

Section Quality	**Type of Clearing (Testes H and E)**			
Folds	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 1.333$, df = 3, p = 0.721
Good	1(5.0%)	0(0.0%)	1(5.0%)	
Satisfactory	1(5.0%)	2(10.0%)	3(15.0%)	
Excellent	7(35.0%)	7(35.0%)	14(70.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
Cracks	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 1.333$, df = 3, p = 0.721
Good	1(5.0%)	0(0.0%)	1(5.0%)	
Satisfactory	1(5.0%)	2(10.0%)	3(15.0%)	
Excellent	7(35.0%)	7(35.0%)	14(70.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
Artefacts	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 1.543$, df = 3, p = 0.672
Good	1(5.0%)	0(0.0%)	1(5.0%)	
Satisfactory	4(20.0%)	3(15.0%)	7(35.0%)	
Excellent	4(20.0%)	6(30.0%)	10(50.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	

Table 4.19: Effect of Citrodora oil and Xylene on testes staining quality score under H and E stain

Staining Quality	Type of Clearing (Testes H and E)			
NS	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	1(5.0%)	1(5.0%)	$x^2 = 4.077$, df = 3, p = 0.253
Good	2(10.0%)	0(0.0%)	2(10.0%)	
Satisfactory	1(5.0%)	3(15.0%)	4(20.0%)	
Excellent	7(35.0%)	6(30.0%)	13(65.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
CCC	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	1(5.0%)	1(5.0%)	$x^2 = 1.744$, df = 3, p = 0.627
Good	2(10.0%)	1(5.0%)	3(15.0%)	
Satisfactory	1(5.0%)	2(10.0%)	3(15.0%)	
Excellent	7(35.0%)	6(30.0%)	13(65.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
SIU	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	1(5.0%)	1(5.0%)	$x^2 = 3.333$, df = 3, p = 0.343
Good	2(10.0%)	0(0.0%)	2(10.0%)	
Satisfactory	1(5.0%)	2(10.0%)	3(15.0%)	
Excellent	7(35.0%)	7(35.0%)	14(70.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	

Key: nuclear staining (NS), Cytoplasmic contrast and clarity (CCC),Staining intensity and uniformity (SIU)

Table 4.20: Effect of Citrodora oil and Xylene on testes section quality score under PAS histochemicalstain

Section Quality	**Type of Clearing (Testes PAS)**			
Folds	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 0.000$, df = 2, p = 1.000
Good	0(0.0%)	0(0.0%)	0(0.0%)	
Satisfactory	1(5.0%)	1(5.0%)	2(10.0%)	
Excellent	8(40.0%)	8(40.0%)	16(80.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
Cracks	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 0.000$, df = 2, p = 1.000
Good	0(0.0%)	0(0.0%)	0(0.0%)	
Satisfactory	1(5.0%)	1(5.0%)	2(10.0%)	
Excellent	8(40.0%)	8(40.0%)	16(80.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
Artefacts	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 0.000$, df = 2, p = 1.000
Good	0(0.0%)	0(0.0%)	0(0.0%)	
Satisfactory	1(5.0%)	1(5.0%)	2(10.0%)	
Excellent	8(40.0%)	8(40.0%)	16(80.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	

Table 4.21: Effect of Citrodora oil and Xylene on testes staining quality score under PAS histochemical stain

Staining Quality	Type of Clearing (Testes PAS)			
NS	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	0(0.0%)	0(0.0%)	
Good	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 2.250$, df = 2, p = 0.325
Satisfactory	2(10.0%)	0(0.0%)	2(10.0%)	
Excellent	7(35.0%)	9(45.0%)	16(80.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
CCC	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	0(0.0%)	0(0.0%)	
Good	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 1.286$, df = 2, p = 0.526
Satisfactory	3(15.0%)	1(5.0%)	4(20.0%)	
Excellent	6(30.0%)	8(40.0%)	14(70.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
SIU	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	1(5.0%)	1(5.0%)	$x^2 = 2.277$, df = 3, p = 0.517
Good	1(5.0%)	0(0.0%)	1(5.0%)	
Satisfactory	3(15.0%)	2(10.0%)	5(25.0%)	
Excellent	6(30.0%)	7(35.0%)	13(65.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	

Key: nuclear staining (NS), Cytoplasmic contrast and clarity (CCC),Staining intensity and uniformity (SIU)

Table 4.22: Effect of Citrodora oil and Xylene on brain section quality score under H and E stain

Section Quality	**Type of Clearing (Brain H and E)**			
Folds	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 0.000$, df = 2, p = 1.000
Good	0(0.0%)	0(0.0%)	0(0.0%)	
Satisfactory	1(5.0%)	1(5.0%)	2(10.0%)	
Excellent	8(40.0%)	8(40.0%)	16(80.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
Cracks	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 1.059$, df = 2, p = 0.589
Good	0(0.0%)	0(0.0%)	0(0.0%)	
Satisfactory	0(0.0%)	1(5.0%)	1(5.0%)	
Excellent	9(45.0%)	8(40.0%)	17(85.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
Artefacts	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 3.600$, df = 2, p = 0.165
Good	0(0.0%)	0(0.0%)	0(0.0%)	
Satisfactory	0(0.0%)	3(15.0%)	3(15.0%)	
Excellent	9(45.0%)	6(30.0%)	15(75.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	

Table 4.23: Effect of Citrodora oil and Xylene on brain swection quality score under H and E stain

Staining Quality	Type of Clearing (Brain H and E)			
NS	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 11.455$, df = 2, p = 0.003
Good	0(0.0%)	0(0.0%)	0(0.0%)	
Satisfactory	0(0.0%)	7(35.0%)	7(35.0%)	
Excellent	9(45.0%)	2(10.0%)	11(55.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
CCC	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	1(5.0%)	1(5.0%)	$x^2 = 16.400$, df = 3, p = 0.001
Good	1(5.0%)	0(0.0%)	1(5.0%)	
Satisfactory	0(0.0%)	8(40.0%)	8(40.0%)	
Excellent	9(45.0%)	1(5.0%)	10(50.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
SIU	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	1(5.0%)	1(5.0%)	$x^2 = 14.400$, df = 3, p = 0.002
Good	1(5.0%)	1(5.0%)	2(10.0%)	
Satisfactory	0(0.0%)	7(35.0%)	7(35.0%)	
Excellent	9(45.0%)	1(5.0%)	10(50.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	

Key: nuclear staining (NS), Cytoplasmic contrast and clarity (CCC),Staining intensity and uniformity (SIU)

Table 4.24: Effect of Citrodora oil and Xylene on brain section quality score under GFAP immunohistochemical stain

Section Quality	Type of Clearing (Brain GFAP)			
Folds	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 2.250$, df = 2, p = 0.325
Good	0(0.0%)	0(0.0%)	0(0.0%)	
Satisfactory	0(0.0%)	2(10.0%)	2(10.0%)	
Excellent	9(45.0%)	7(35.0%)	16(80.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
Cracks	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 2.250$, df = 2, p = 0.325
Good	0(0.0%)	0(0.0%)	0(0.0%)	
Satisfactory	0(0.0%)	2(10.0%)	2(10.0%)	
Excellent	9(45.0%)	7(35.0%)	16(80.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
Artefacts	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 2.250$, df = 2, p = 0.325
Good	0(0.0%)	0(0.0%)	0(0.0%)	
Satisfactory	0(0.0%)	2(10.0%)	2(10.0%)	
Excellent	9(45.0%)	7(35.0%)	16(80.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	

Table 4.25: Effect of Citrodora oil and Xylene on brain staining quality score under GFAP immunohistochemical stain

Staining Quality	**Type of Clearing (Brain GFAP)**			
NS	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	0(0.0%)	0(0.0%)	
Good	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 4.000$, df = 2, p = 0.135
Satisfactory	1(5.0%)	5(25.0%)	6(30.0%)	
Excellent	8(40.0%)	4(20.0%)	12(60.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
CCC	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	1(5.0%)	1(5.0%)	$x^2 = 6.000$, df = 3, p = 0.112
Good	1(5.0%)	0(0.0%)	1(5.0%)	
Satisfactory	1(5.0%)	5(25.0%)	6(30.0%)	
Excellent	8(40.0%)	4(20.0%)	12(60.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
SIU	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	1(5.0%)	1(5.0%)	$x^2 = 4.104$, df = 3, p = 0.250
Good	1(5.0%)	0(0.0%)	1(5.0%)	
Satisfactory	2(10.0%)	5(25.0%)	7(35.0%)	
Excellent	7(35.0%)	4(20.0%)	11(55.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	

Key: nuclear staining (NS), Cytoplasmic contrast and clarity (CCC),Staining intensity and uniformity (SIU)

Table 4.26: Effect of Citrodora oil and Xylene on brain section quality score under Golgi histochemical stain

Section Quality	**Type of Clearing (Brain Golgi Silver)**			
Folds	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 0.277$, df = 2, p = 0.871
Good	0(0.0%)	0(0.0%)	0(0.0%)	
Satisfactory	2(10.0%)	3(15.0%)	5(25.0%)	
Excellent	7(35.0%)	6(30.0%)	13(65.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
Cracks	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 0.277$, df = 2, p = 0.871
Good	0(0.0%)	0(0.0%)	0(0.0%)	
Satisfactory	2(10.0%)	3(15.0%)	5(25.0%)	
Excellent	7(35.0%)	6(30.0%)	13(65.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
Artefacts	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 0.277$, df = 2, p = 0.871
Good	0(0.0%)	0(0.0%)	0(0.0%)	
Satisfactory	2(10.0%)	3(15.0%)	5(25.0%)	
Excellent	7(35.0%)	6(30.0%)	13(65.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	

Table 4.27: Effect of Citrodora oil and Xylene on brain staining quality score under Golgi histochemical stain

Staining Quality	**Type of Clearing (Brain Golgi Silver)**			
NS	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	0(0.0%)	0(0.0%)	
Good	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 3.600$, df = 2, p = 0.165
Satisfactory	2(10.0%)	6(30.0%)	8(40.0%)	
Excellent	7(35.0%)	3(15.0%)	10(50.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
CCC	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	0(0.0%)	0(0.0%)	
Good	1(5.0%)	1(5.0%)	2(10.0%)	$x^2 = 5.556$, df = 2, p = 0.062
Satisfactory	2(10.0%)	7(35.0%)	9(45.0%)	
Excellent	7(35.0%)	2(10.0%)	9(45.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	
SIU	**Citrodora Oil**	**Xylene**	**Total**	**Chi-square**
Fair	0(0.0%)	0(0.0%)	0(0.0%)	
Good	1(5.0%)	3(15.0%)	4(20.0%)	$x^2 = 5.063$, df = 2, p = 0.080
Satisfactory	2(10.0%)	5(25.0%)	7(35.0%)	
Excellent	7(35.0%)	2(10.0%)	9(45.0%)	
Total	10(50.0%)	10(50.0%)	20(100.0%)	

Key: nuclear staining (NS), Cytoplasmic contrast and clarity (CCC),Staining intensity and uniformity (SIU).

4.9 COMPARISON OF PHOTOMICROGRAPHS OF CITRODORA OIL AND XYLENE CLEARED TISSUES WITH DIFFERENT STAINS

Photomicrographs of H and E stained sections of livers (Plate I and II), kidneys (Plate III and IV), testes (Plate V and VI), and cerebellar cortex (Plate VII and VIII) of Wistar rats adequately showed all the relevant histological features of both the Citrodora oil and xylene cleared and dewaxed tissues during the present study. Similarly, the use of Anti-GFAP antibody staining on section of cerebral cortex from Wistar rats (Plate IX to XI) showed that tissue cleared and dewaxed in Citrodora oil compared well with those cleared and dewaxed in xylene.

Staining with anti-CD34 antibody techniques on sections on intestine (Plate XII), kidney (Plate XIII and XIV)and liver (Plate XV and XVI) showed that clearing and dewaxing in Citrodora oil and xylene were equally good. Also, Gordon and Sweet staining for reticular fibers in liver (Plate XVII and XVIII), PAS on testicular sections (Plate XIX and XX) as well as Golgi Cox on cerebral cortex (Plate XXI and XXII) all revealed Citrodora oil as a clearing and dewaxing agent did not have any adverse effect on histological structures as compared to the use of xylene on the tested tissues of Wistar rats.

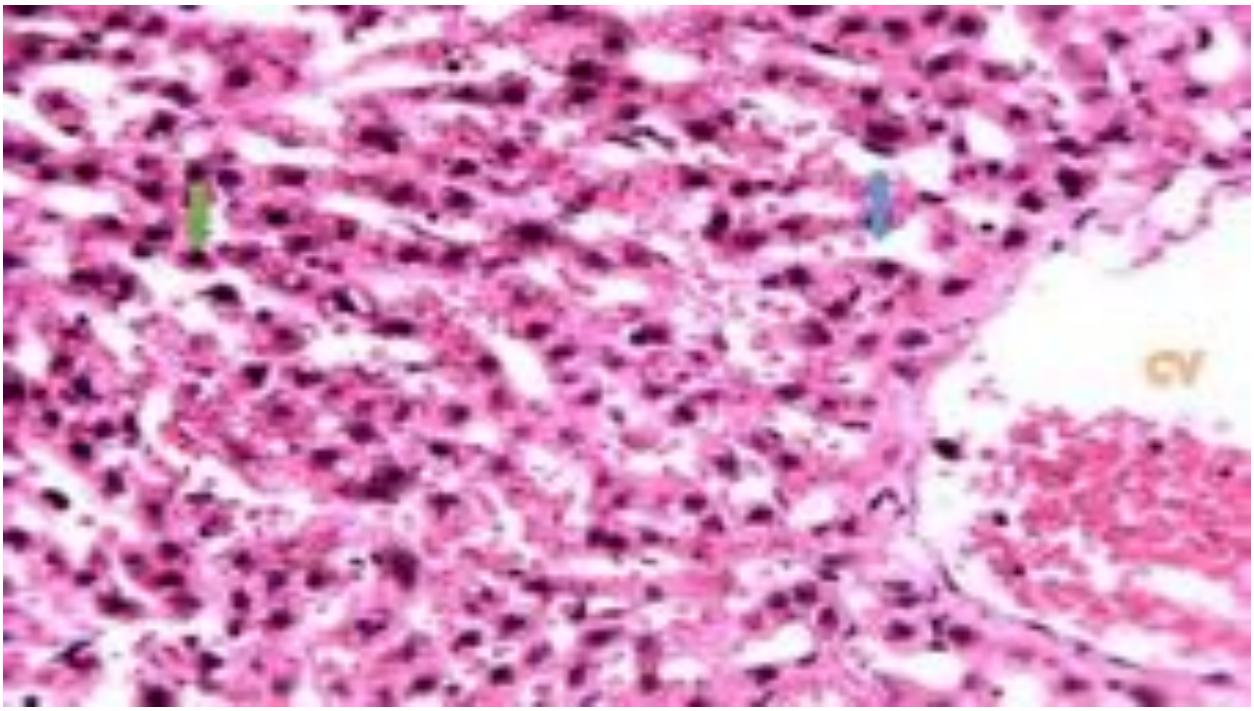


Plate I:Photomicrograph of liver section of Wistar rat cleared in Citrodora oil showing central vein (CV) and sinusoid (blue arrow). The hepatocytes (green arrow) appeared normal. H&E x400

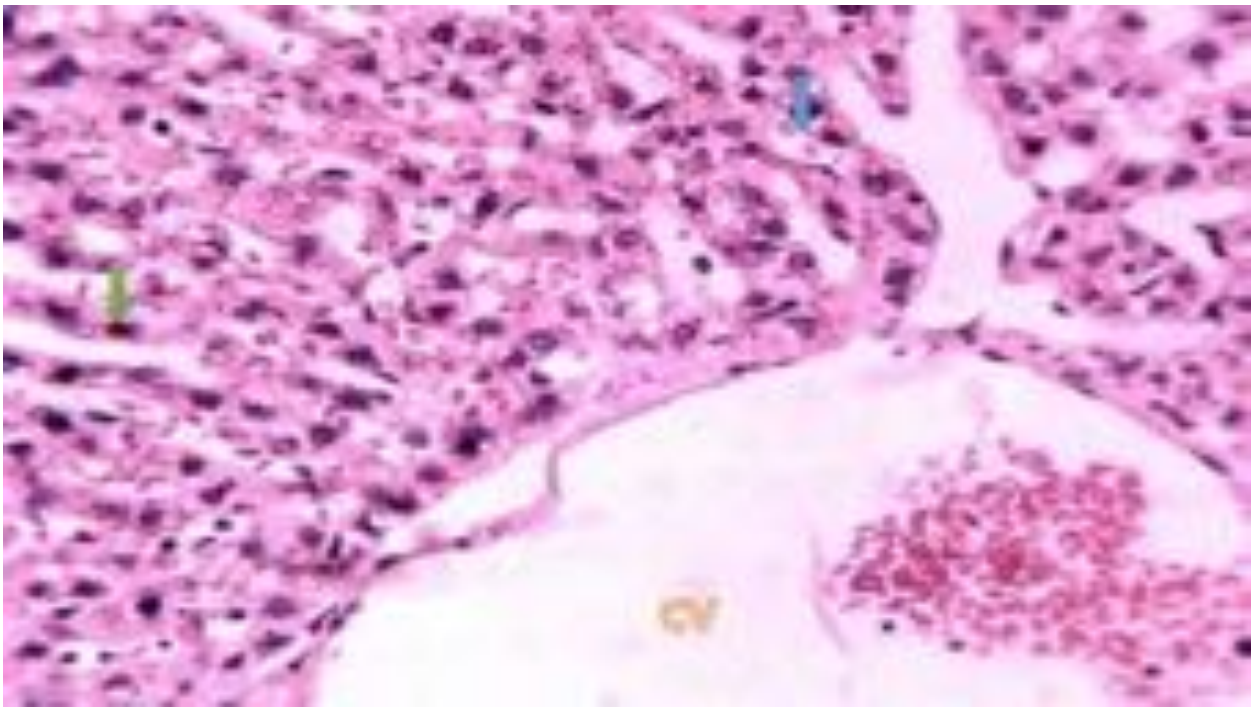


Plate II: Photomicrograph of liver section of Wistar rat cleared in xyleneshowing central vein (CV) and sinusoid (blue arrow). The hepatocytes (green arrow) appearing normal. H&E x400

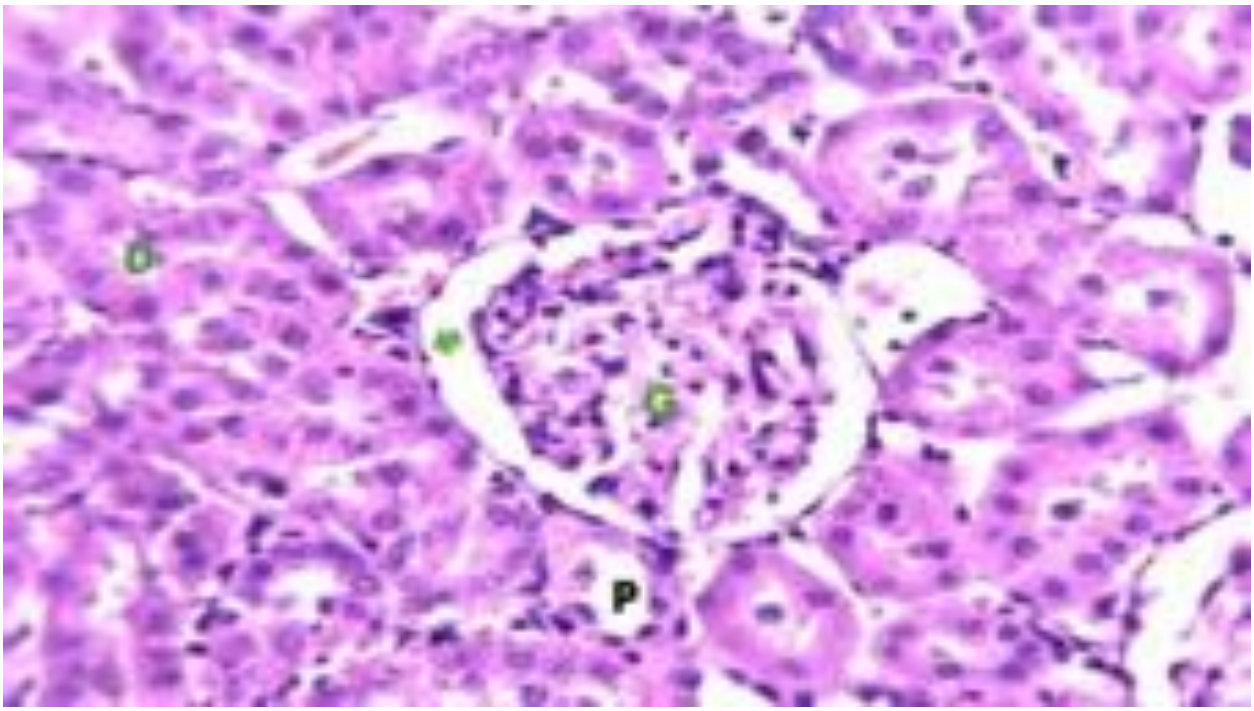


Plate III: Photomicrograph of section of kidney of Wistar rat cleared in Citrodora oil. The renal cortex shows Malpighian renal corpuscle containing glomerulus (G) and Bowman's space (green arrowhead) lined with squamous epithelium. The proximal convoluted tubules (P) have narrow lumina and are lined with cuboidal cells with rounded vesicular basal nuclei. The distal convoluted tubules (D) have wider lumina and are lined with rounded vesicular central nuclei. . H&E x250

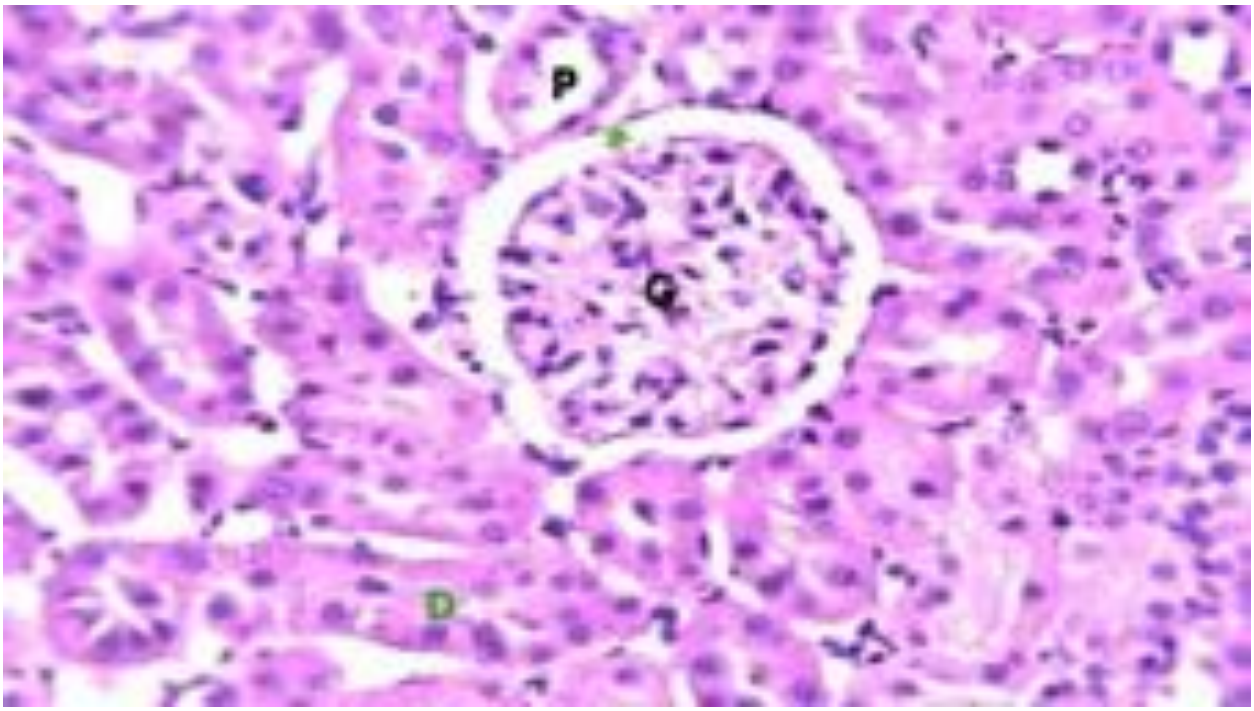


Plate IV: Photomicrograph of section kidney of Wistar rat cleared in xylene. The renal cortex shows Malpighian renal corpuscle containing glomerulus (G) and Bowman's space (green arrowhead) lined with squamous epithelium. The proximal convoluted tubules (P) have narrow lumina and are lined with rounded vesicular basal nuclei. The distal convoluted tubules (D) have wider lumina and are lined with rounded vesicular central nuclei. H&E x250

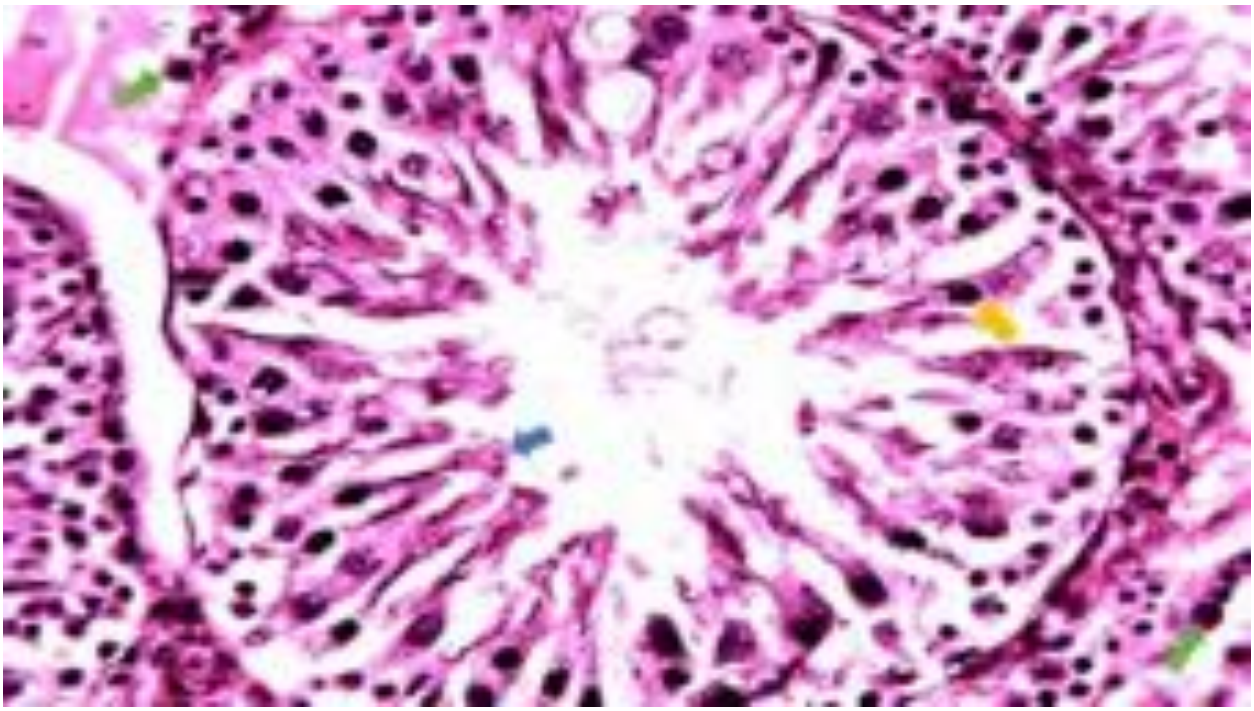


Plate V: Photomicrograph of section of testes of Wistar rat cleared in Citrodora oil; illustrating the structure of the seminiferous tubule, showing the stages of spermatogenesis, spermatogonia (blue arrows), spermatocytes (yellow arrows), and the interstitial cells of Leydig (green arrow) (H & E x250).

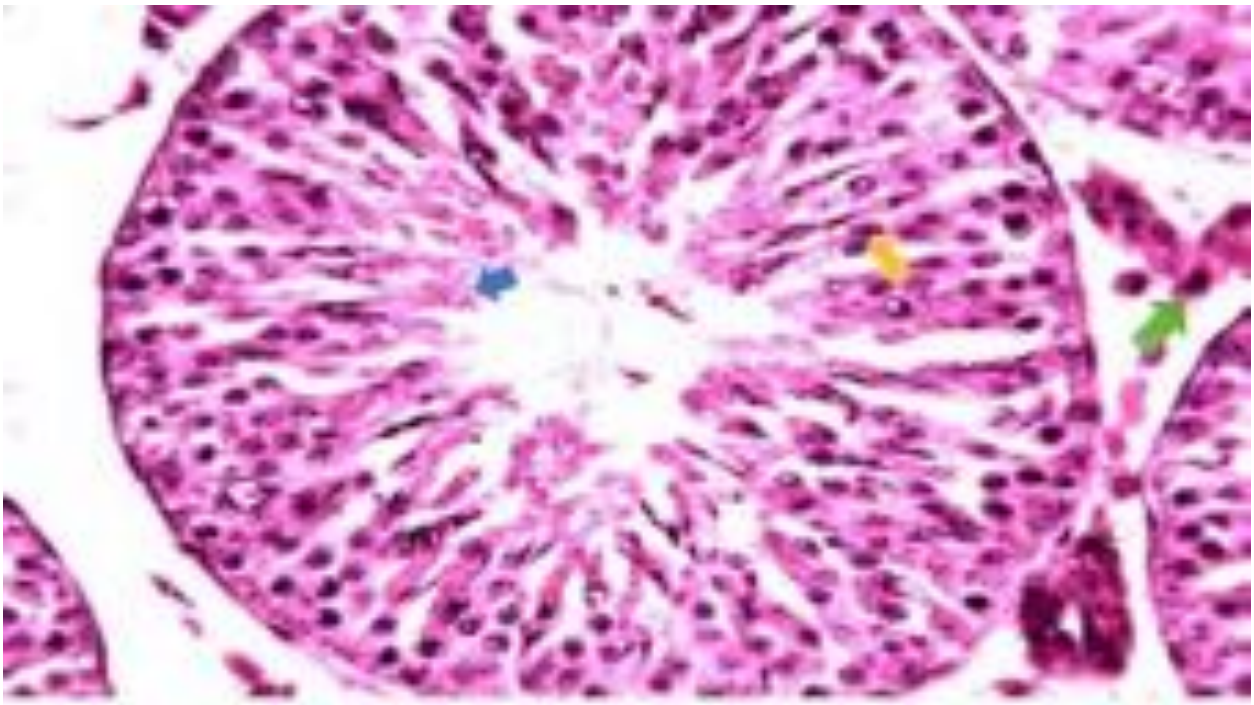


Plate VI: Photomicrograph of section of testes of Wistar rat cleared in Xylene; illustrating the structure of the seminiferous tubule showing the stages of spermatogenesis, spermatogonia (light blue arrows), sperm cells (yellow arrows), and the interstitial cells of Leydig (green arrow) (H & E x250).

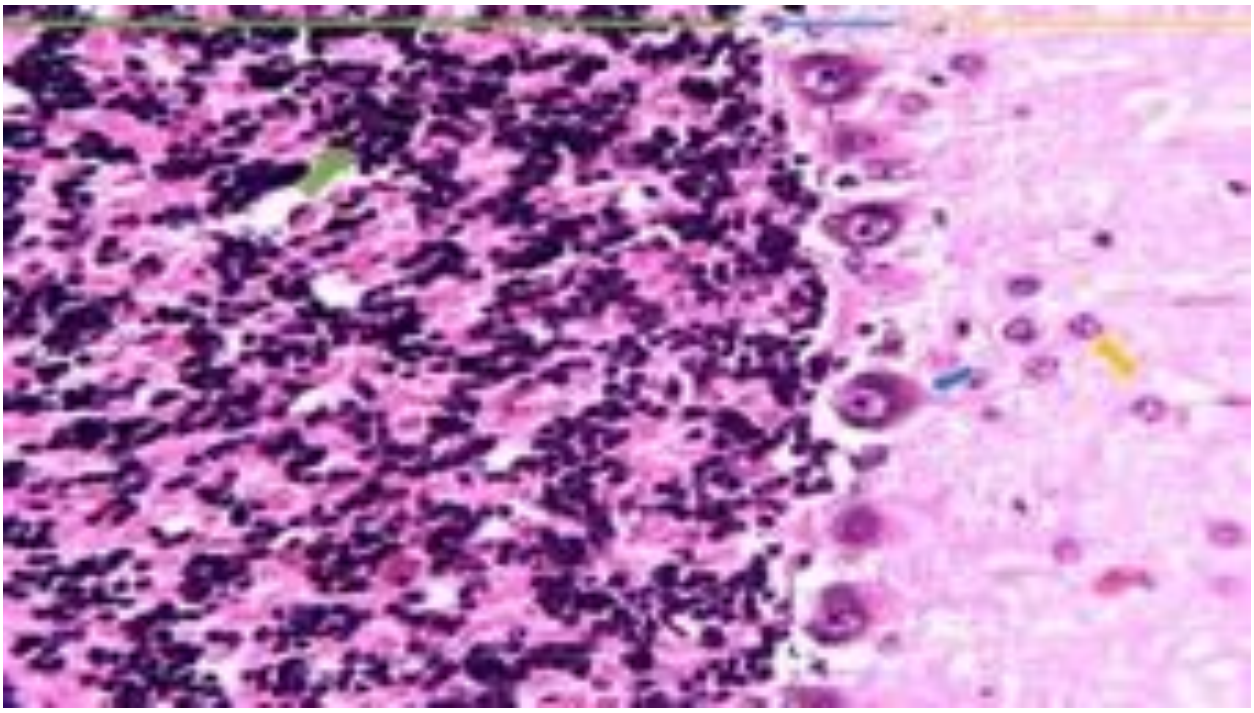


Plate VII: Photomicrograph of section of cerebellar cortex of Wistar rat brain tissue cleared in Citrodora oil; illustrating the typical three layered structure showing the stellate cells of the molecular layer (yellow arrow and line), Purkinje cell in the Purkinje cell layer (blue arrows and line) and the granule cells of the granular layer (green arrows and line), (H & E x250).

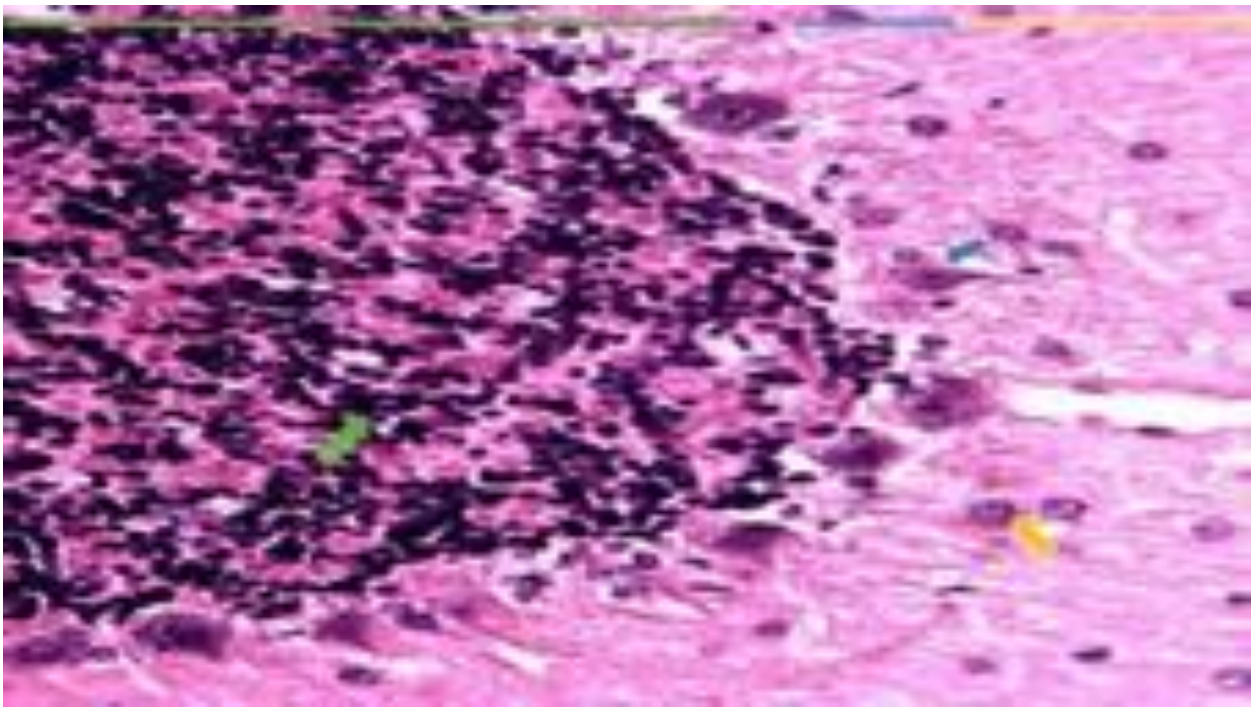


Plate VIII: Photomicrograph of section of cerebellar cortex of Wistar rat brain tissue cleared in xylene; illustrating the typical three layered structure showing the stellate cells of the molecular (yellow arrow) Purkinje cell in the Purkinje cell layer (blue arrows) and the granule cells of the granular layer (green arrows), (H & E x250).

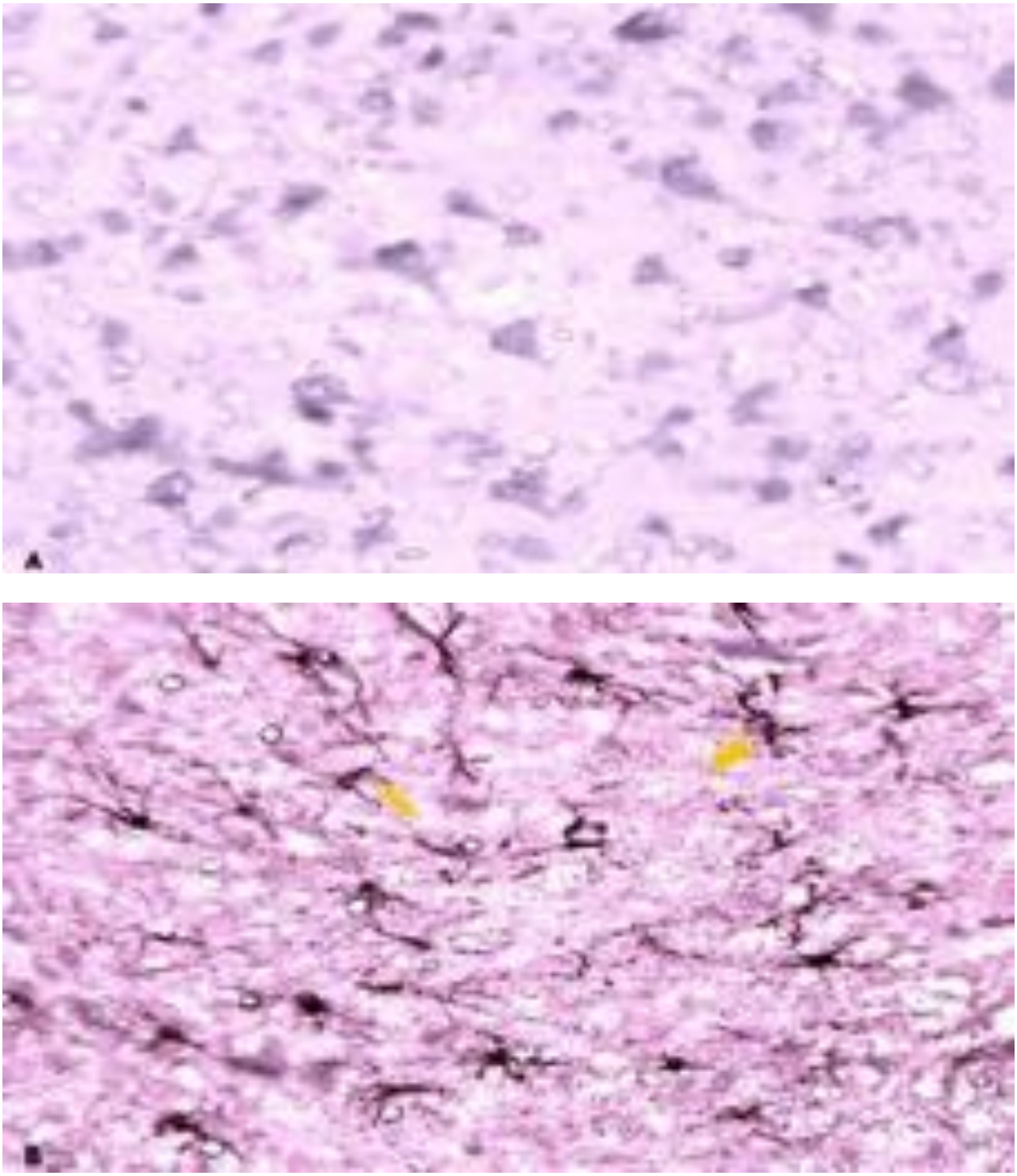


Plate IX: Photomicrograph of section of cerebral cortex of Wistar rat brain stained with anti-GFAP antibody. Slide A is a negetivecontrol and slide B is a positive control section showing astrocytes immunoreactivity as indicated by the yellow arrows.(Anti-GFAP antibodyx250)

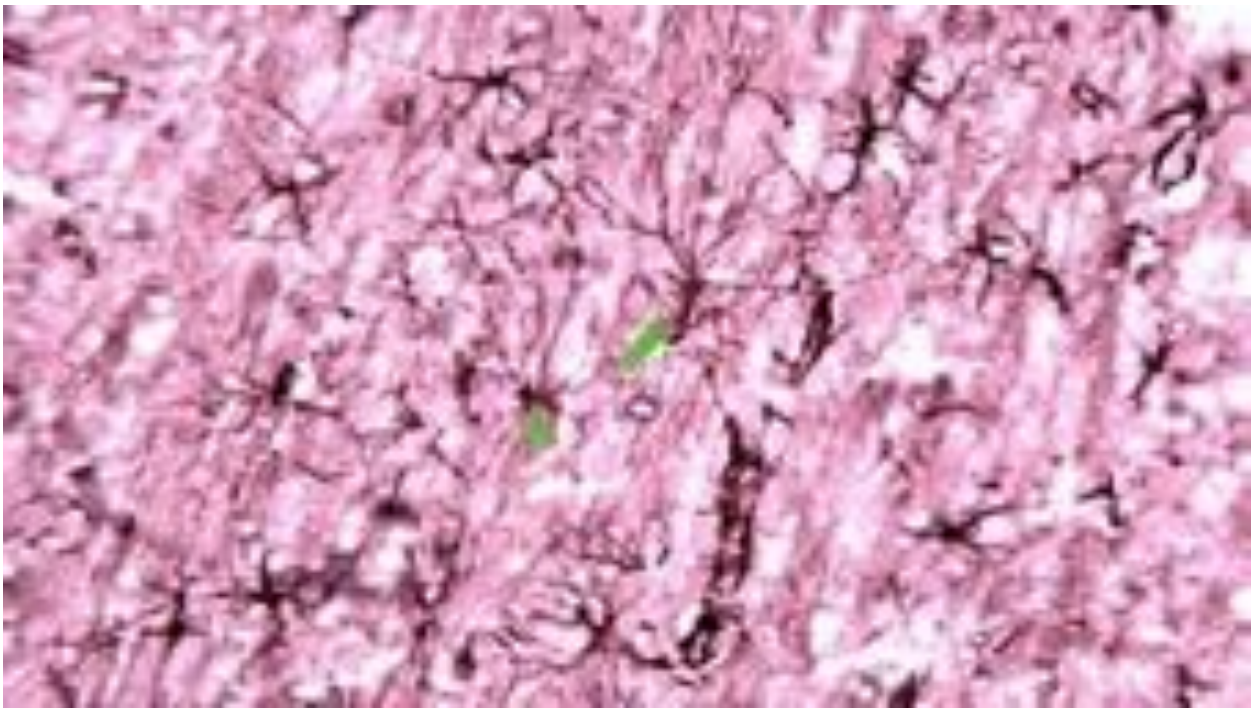


Plate X: Photomicrograph of section of cerebral cortex of Wistar rat braintissue cleared in Citrodora oil and stained with anti-GFAP antibody showing a positive astrocytes immunoreactivity as indicated by the green arrows.(Anti-GFAP antibody x250)

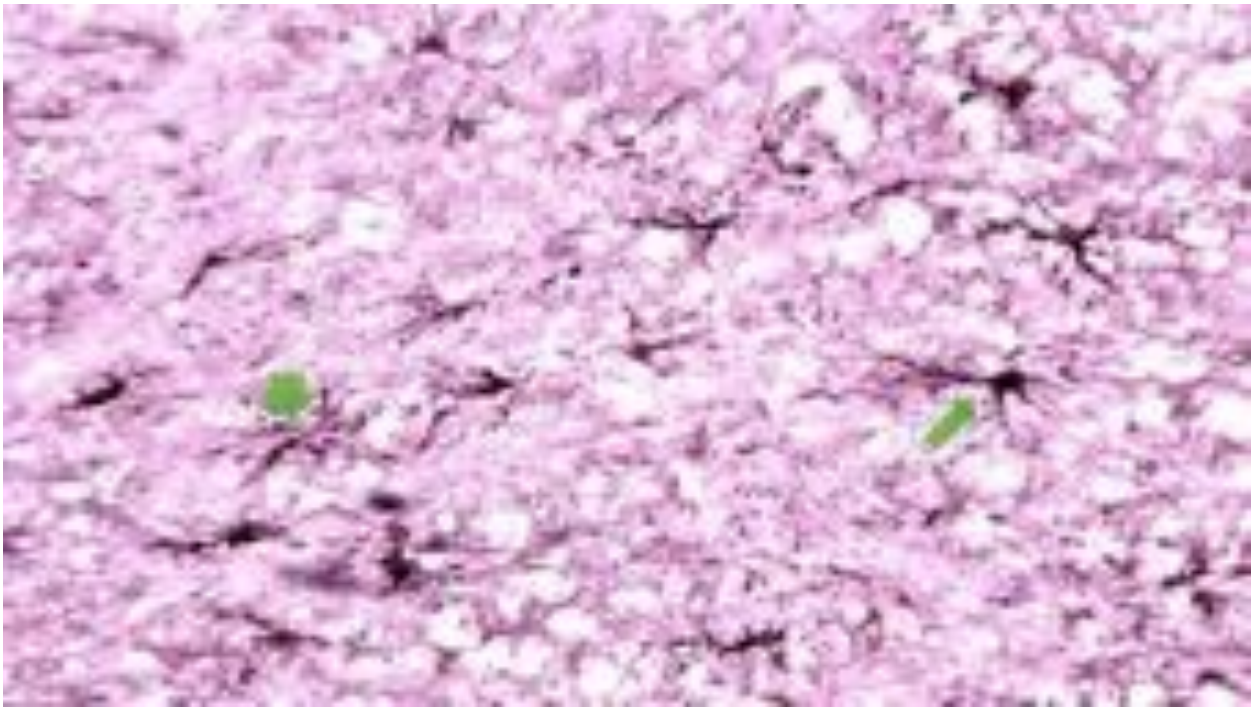


Plate XI: Photomicrograph of section of cerebral cortex of Wistar ratbrain tissue cleared in Xylene and stained with anti-GFAP antibody showing a positive astrocytes immunoreactivity as indicated by the green arrows.(Anti-GFAP antibody x250)

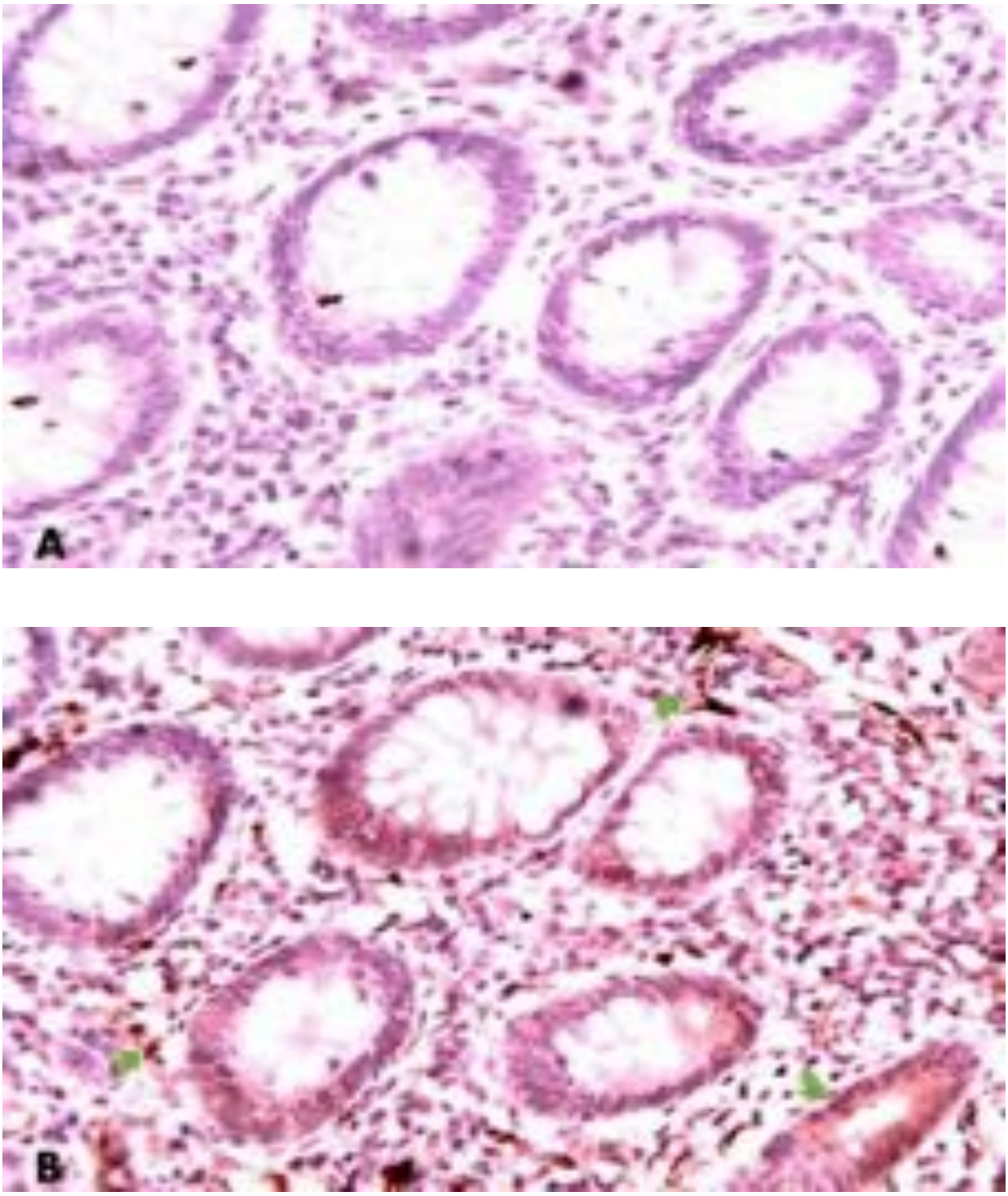


Plate XII: Photomicrograph of Intestinalsections stained with anti-CD34 antibody. Slisd A is a negative control and slide B is a positive control section showing positive immunoreactivity as indicated by the brown coloration (green arrows).(Anti-CD34 antibody x250)

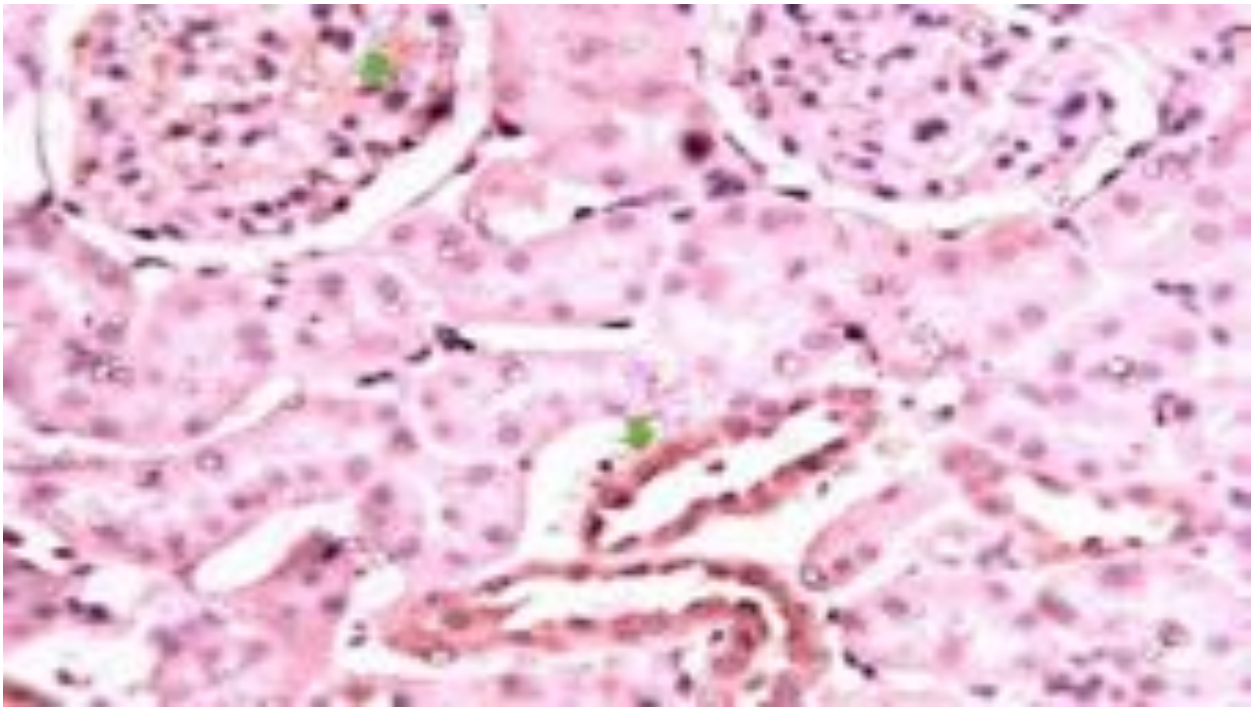


Plate XIII: Photomicrograph of Kidney section of Wistar rat cleared in Citrodora oil and stained with anti-CD34 antibody, showing positive immunoreactivity as indicated by the brown coloration (green arrows).(Anti-CD34 antibody x250)

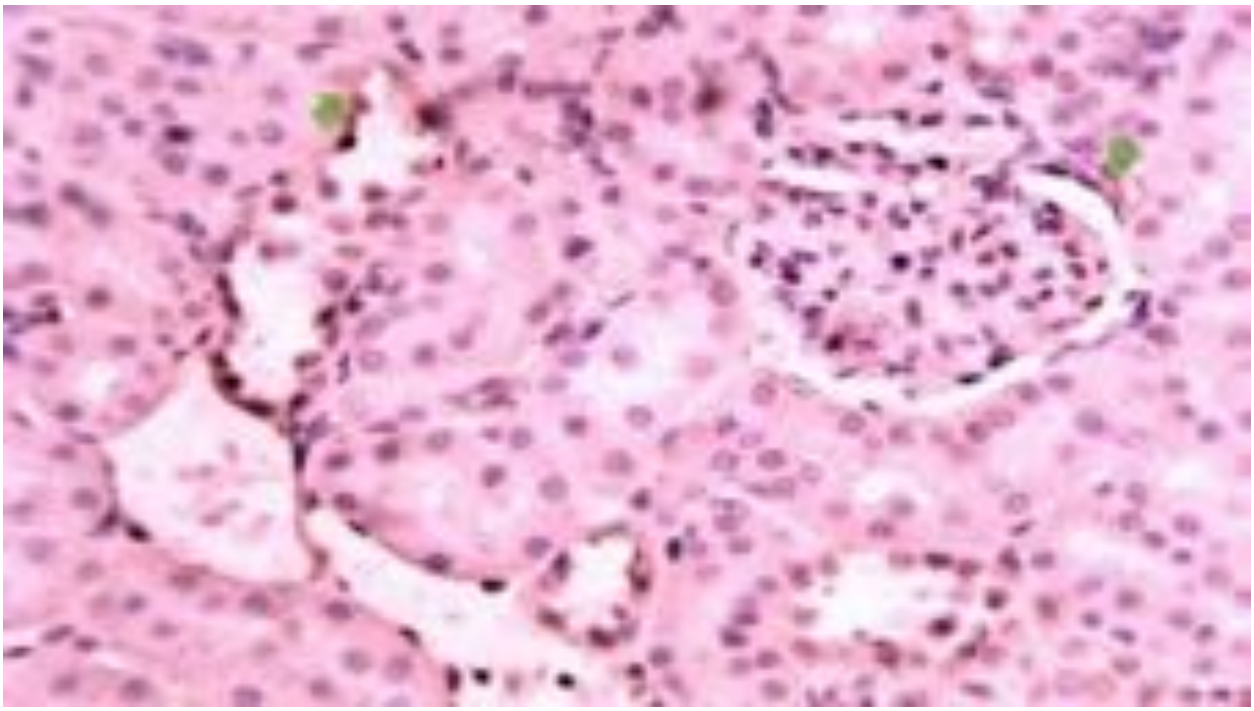


Plate XIV: Photomicrograph of kidney section of Wistar rat cleared in Xylene and stained with anti-CD34 antibody, showing positive immunoreactivity as indicated by the brown coloration (green arrows).(Anti-CD34 antibody x250)

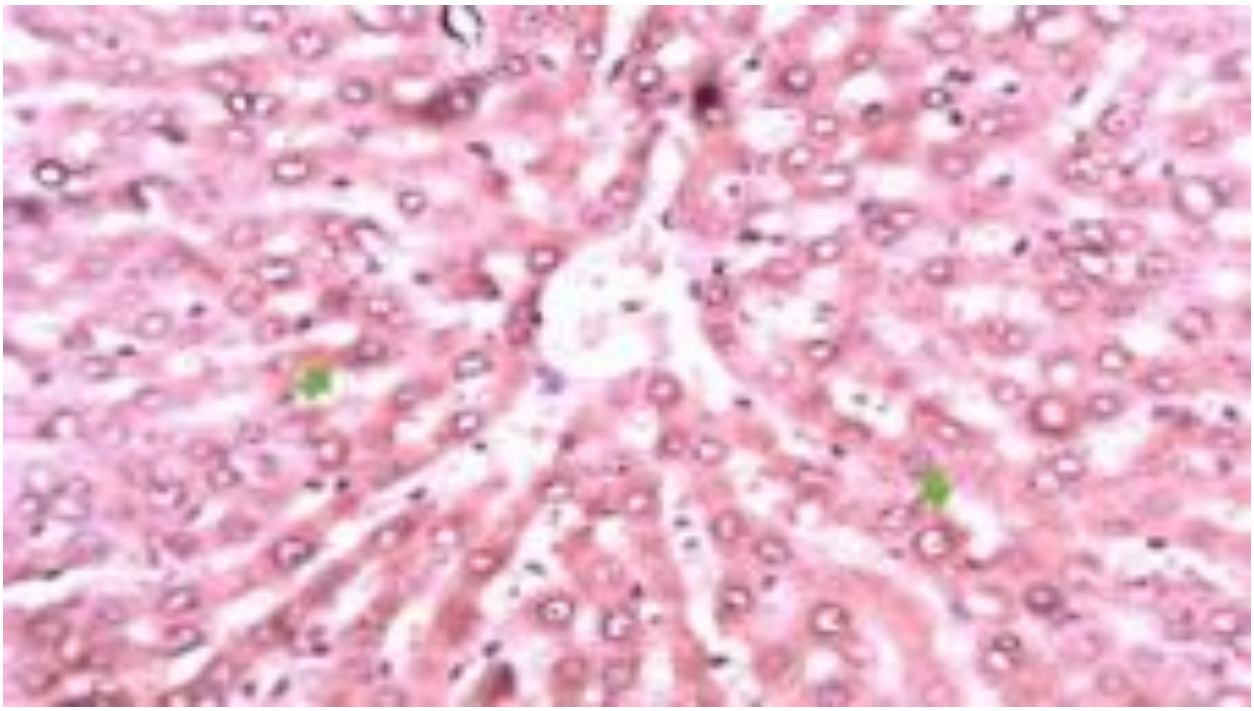


Plate XV: Photomicrograph of Liver section of Wistar ratcleared in Citrodora oil and stained with anti-CD34 antibody, showing positive immunoreactivity as indicated by the brown coloration (green arrows).(Anti-CD34 antibody x250)

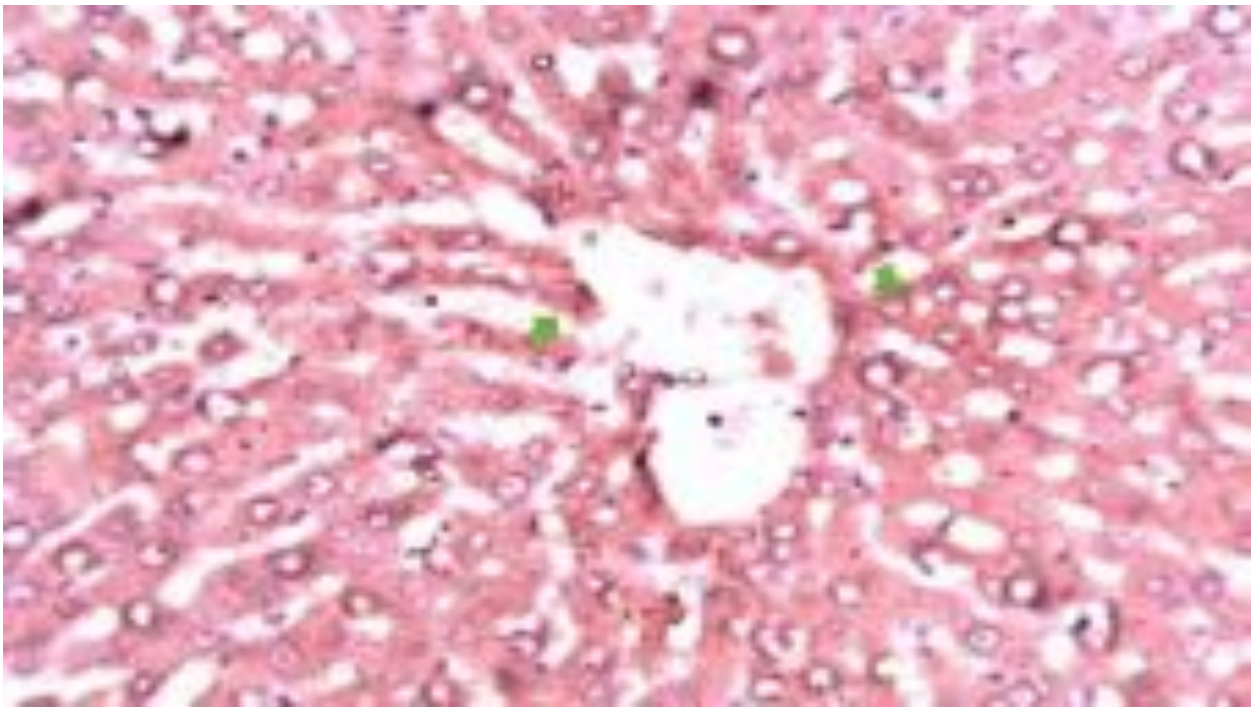


Plate XVI: Photomicrograph of Liver sectionof Wistar rat cleared in Xylene and stained with anti-CD34 antibody, showing positive immunoreactivity as indicated by the brown coloration (green arrows).(Anti-CD34 antibody x250)

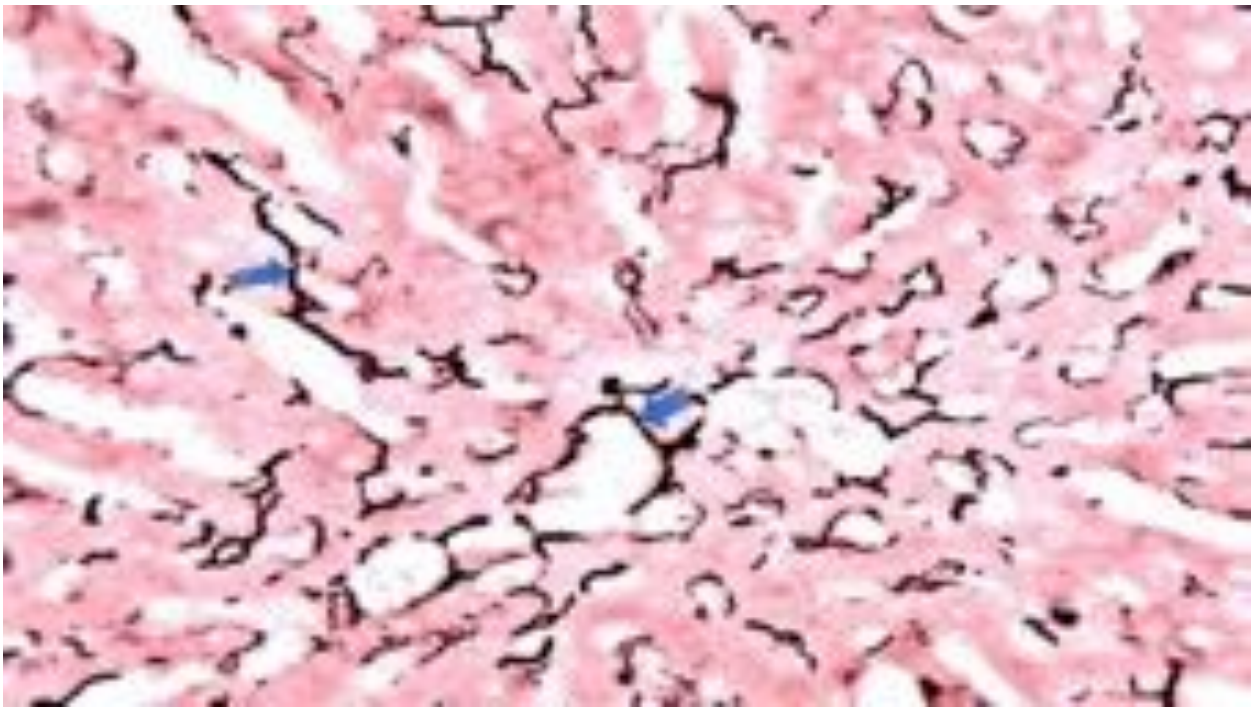


Plate XVII: Photomicrograph of Liversection of Wistar rat cleared in Citrodora oil showing clear demonstration of black reticular fibers, (blue arrow). (Gordon and Sweet x250)

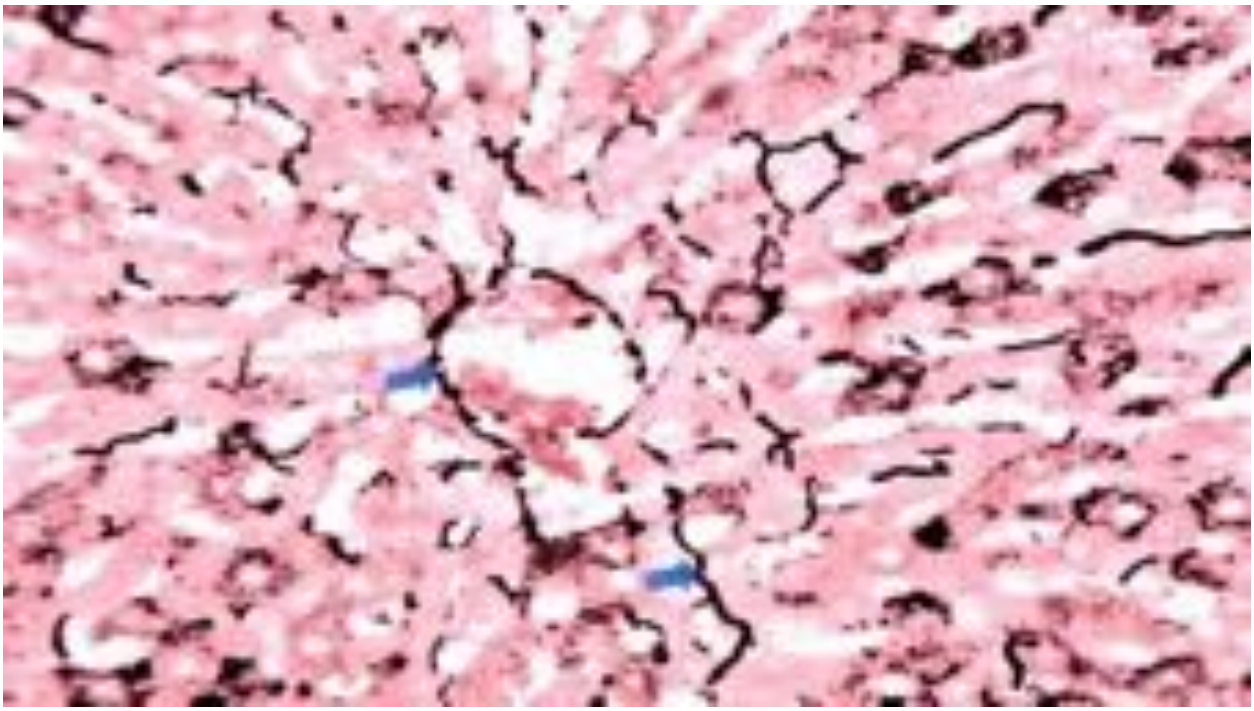


Plate XVIII: Photomicrograph of Liversection of Wistar rat cleared in Xylene showing clear demonstration of black reticular fibers, (blue arrow). (Gordon and Sweet x250)

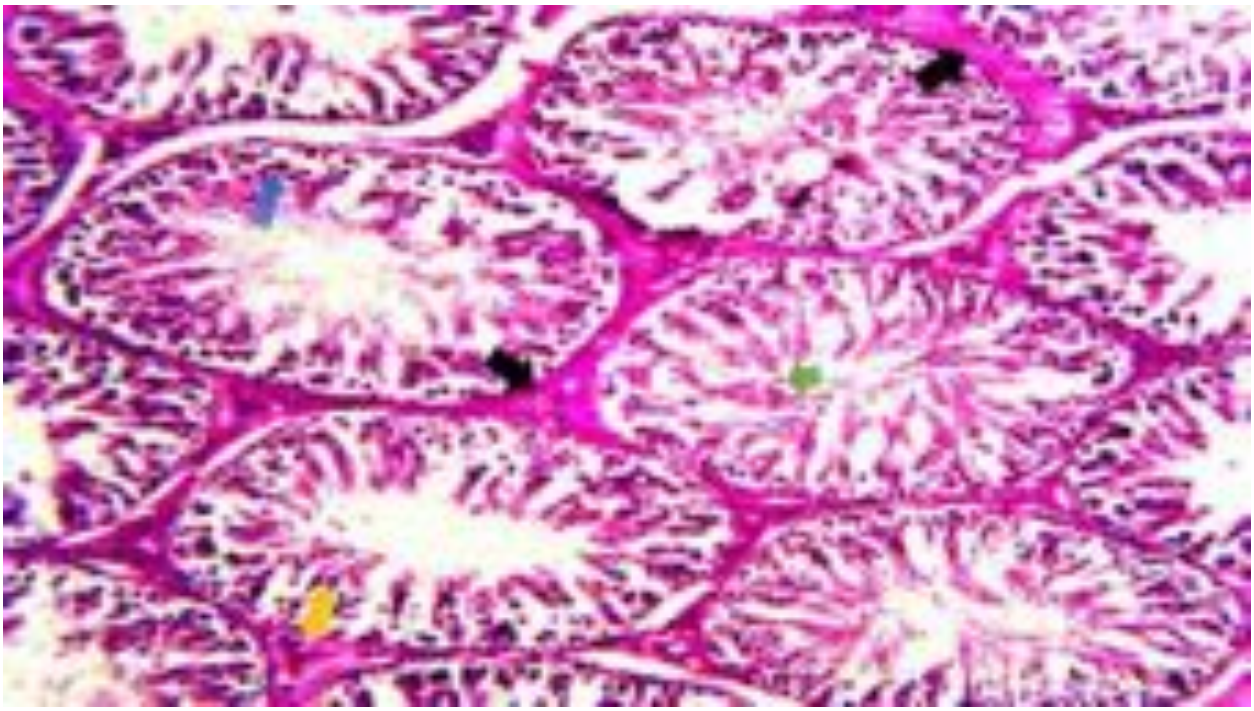


Plate XIX:Photomicrograph of transverse section of testes of Wistar rat cleared in Citrodora oil; illustrating the structure of the seminiferous tubule showing the stages of spermatogenesis, spermatogonia (blue arrows), sperm cells (green arrows), and the interstitial cells of Leydig (yellow arrow).Note the strong PAS positive reaction on the basement membrane and interstitial cells of Leydig (black arrow). PAS x250

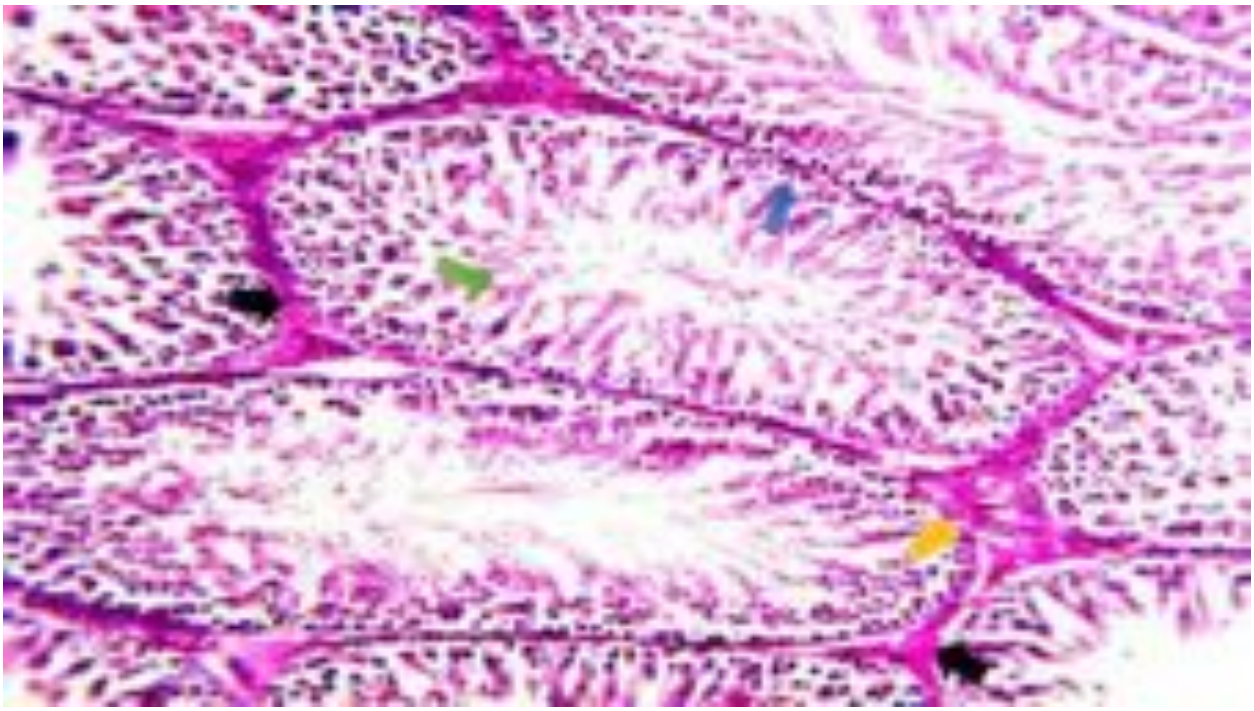


Plate XX: Photomicrograph of transverse section of testes of Wistar rat cleared in Xylene; illustrating the structure of the seminiferous tubule showing the stages of spermatogenesis, spermatogonia (blue arrows), spermatocytes (green arrows), and the interstitial cells of Leydig (yellow arrow).Note the strong PAS positive reaction on the basement membrane and interstitial cells of Leydig (black arrow). PAS x250

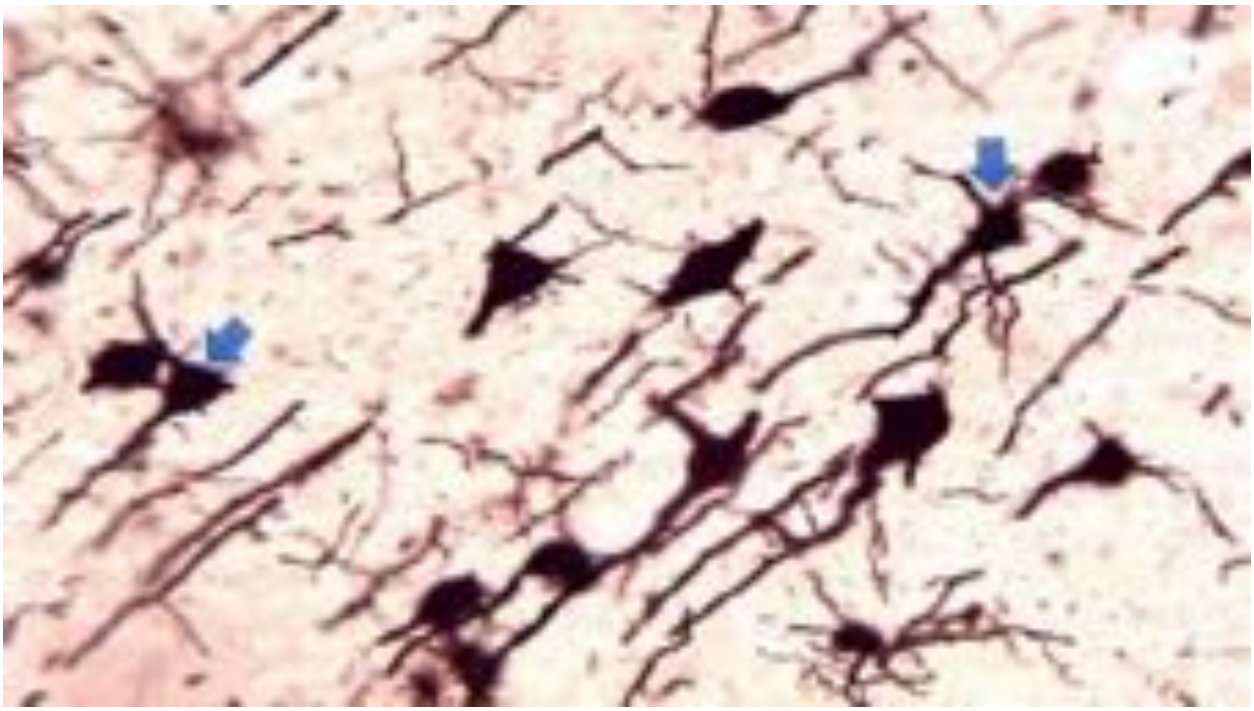


Plate XXI: Photomicrograph of transverse section of cerebral cortex of Wistar rat brain cleared in Citrodora oil showing clear demonstration of black neuronal cells, (blue arrow). (Golgi Cox x250)

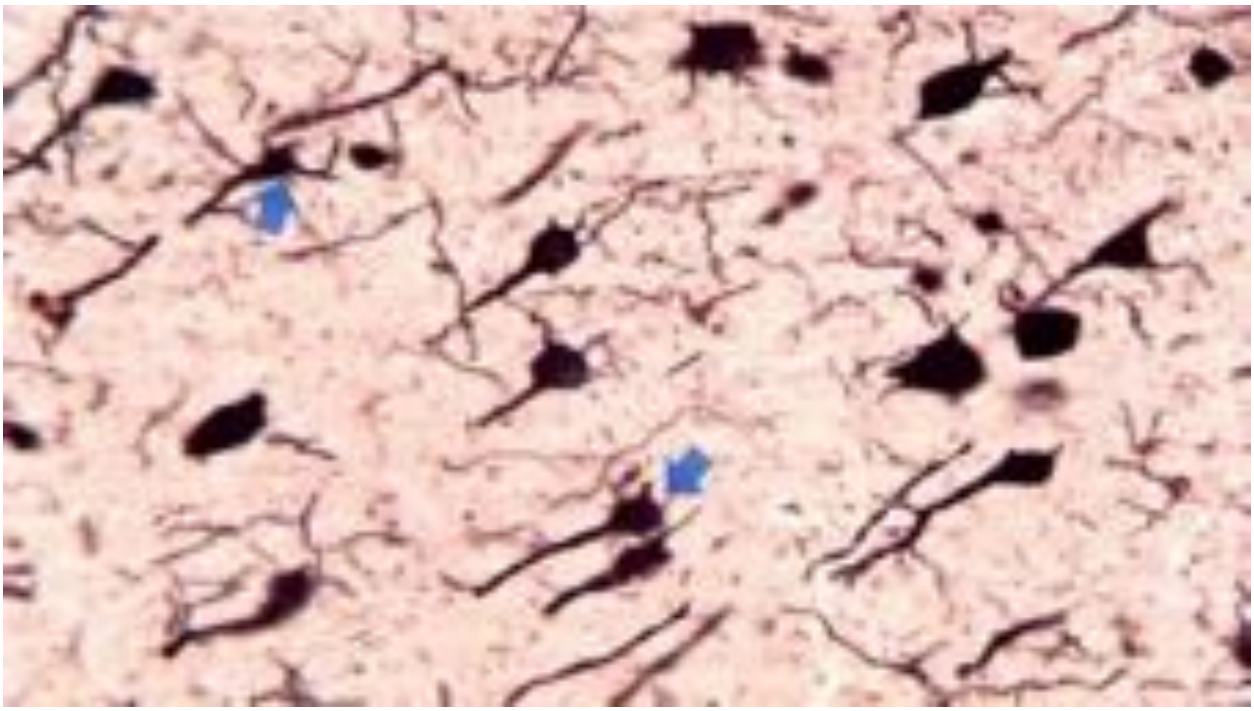


Plate XXII: Photomicrograph of transverse section of cerebral cortex of Wistar rat brain tissue cleared in Xylene showing clear demonstration of black neuronal cells, (blue arrow). (Golgi Cox x250)

CHAPTER FIVE

5.0 DISCUSSION

Xylene traditionally has been employed as a clearing, dewaxing, and mounting agent for histology (Buesaand Peshkov, 2009; Chen *et al.,* 2010). It is well documented, however, that xylene is an environmental hazard andhighly toxic to humans. Accumulating evidences indicate that being repeatedly or excessively exposed to xylenecan do harm to the nervous system, skin, liver, kidney, and lung tissues of human and animals (Gamberale*et al.,* 1978; Hans *et al.,* 1995;Kum *et al.,* 2007a; Chatterjee *et al.,* 2005; Kum *et al.,* 2007b; Sandikci*et al.,* 2009). In addition, the xylene is also reported to have many shortcomings, such as being highly flammable and volatile. It has a much lower boiling. Point(137-143°C), a flash point(25°C) and an ignition point (25°C). Therefore, xylene is regarded as a toxic as a clearingagent in histology. Although many xylene substitutes have been commercially developed (Buesa, 1997), they fail tocompletely replace it, partially due to the variable effectiveness and high costs. This study has demonstrated the use of Citrodora oil as alternative clearing agent with potentials to xylene in tissue processing, dewaxing and mounting agent for histology.

The pale yellow color of Citrodora oil observed in this study is 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 works on colors of essential oils reported have to do with mixing of essential oils based on color for acupuncture and aromatherapy (Chiazzari 1998; Mary 2005). This study was not able to establish a direct link between the Citrodora oil colour and tissue clearing ability. However, we observed that Citrodora oilin this was positive for saponins, terpinoids, flavonoids-polyphenolic compounds and carbohydrates.Kumar (2014) reported thatflavonoids are the substances that impart colour,

and are the most important ingredients necessary for dyeing. Saponins have the ability to lower the surface tension that benefits staining efficacy (Kumar, 2014).Oils are made up of mainly triglycerides, with different degree and kind of unsaturation of the acyl groups. (Guillem and Ruiz, 2001). This may account for the presence of the carbohydrates.This could probably be a contributing factor for the good quality staining observed in this study. The intensity of the staining quality of Citrodoraoil and xylene cleared tissues observed in this study appeared similar, with no significant difference, this observation is similar to the finding of Kurien*et al.* (2012).

Specific gravity is the heaviness of a substance compared to that of water. In this present study it was observed that the specific gravity of xylene is slightly higher than that ofCitrodora oil.Awolu*et al.* (2013), reported the same density value for neem oil, while Shabnam*et al.* (2014), reported that the specific gravity of five different species of *Citrus* essential oils ranged from 0.842 to 0.858 g/ml. The present result is 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.These values are similar to the values of Citrodora oil (0.801g/ml) and xylene (0.804 g/ml) obtained in this study. High density oil may mean high molecular weight oil which in most cases has slower tissue penetration ability, since Citrodora oil and xylene have very close specific gravity and density, this may be the reason for similar tissue clearing ability in the present study. This observation is similar to the report ofSermadi*et al.* (2014) and Adeniyi*et al.* (2016) who used different vegetable oils as tissue clearing agents.

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 higherviscosity 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 Citrodora (0.34cp) and xylene (0.45cp) recorded in this study, but similar to what was recorded byAkpulu*et al.* (2015) who,after a similar work on tissue clearing ability of some essential oils, reported viscosity of citrus oil to be 0.35cp at 28^0 C. It can therefore, be said that any clearing agent with viscosity value that is closer in value to that of xylene may be a good clearing agent as demonstrated in this study. 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 dependent constants for specific substances. This imply that xylene with higher viscosity have a higher molecular structure than Citrodora oil, leading to higher attraction between particles, and as such may flow slower in tissue than the Citrodora oil with lesser value of viscosity.

Flash point (FP) 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,Citrodora oil has a FP of 35^0C and Xylene 39^0C. Similar reports on some essential oils gave inconsistent values of FP. Hans (1995) had reported that the FP of olive oil was 60^0C. Similarly, Kunhua*et al.* (2012) had reported xylene to have 25^0C FP and a mixture of white oil (an essential oil) with N-heptane to be 144^0C. The lower FP of xylene and Citrodora in this work though slightly higher than the value from other works suggest that they may be highly flammable as reported by previous authors (Chatterjee *et al*., 2005; Kum*et al*., 2007a; Sandikci*et al*., 2009). It is also possible that oils with lower flash point have better tissue clearing and

dwaxingabilitiesas reported by Akpulu*et al.* (2015)where they reported that citrus oil, Citrodora oil and xylene seemed to be better clearing and dewaxing agents than neem oil, which have very high FP.

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, Citrodora oil (11.8) has the lowest AV when compared to xylene (16.9). This may mean less activity of lipase enzyme in Citrodora oil which may suggest less damaging effect on tissues during clearing. The AV recorded in this work is higher when compared to the report from other similar oils, as reported by Kardash (2005) and Akpulu*et al*. (2015) who reported the AV of neem oil and citrus oil to be 37.8 and 34.4, respectively

The cloud point (CP) 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. In this present study, the CP of Citrodora oil (-69%) and xylene (-65%) are very close in value. Akpulu*et al.* (2015) reported a similar value for citrus oil (-67%), and a very high value of CP for neem oil (2%). They surmised that neem oil will cloud faster,

therefore may be the reason why neem oil seems to be the poorest in tissues clearing. From this report, Citrodora oil can clearand dewax Wistar rats' tissue comparably well as xylene because they both have close values of CP values, meaning that they have less tendency to clog tissue pores, thereby allowing free flow during tissue penetration leading to better tissue clearing.

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. Refractive index (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 (Bancroftand 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 Citrodoraand xylene were 1.457 and 1.496 respectively.It is also in agreement with the work of Akpulu*et al.* (2015)who reported the RI of neem oil and citrus oil to be 1.47 and 1.455 respectively. It should be noted that these values recorded are very close to that of the RI of tissue (1.4) as indicated by Delpy*et al.* (1988) and Zocchi (1995), this may be one of the reasons why Citrodora oil and xylene can clear and dewax tissues comparably well. Swamy *et al.,* (2015) observed that translucency of the tissues cleared in carrot and rose oils was similar to tissues cleared in xylene.Tissue cellular architecture was preserved in all the sections cleared with different oils and a clear distinction was observed between nucleus and cytoplasm. The overall staining quality was almost equivalent to that of xylene (Kunhua*et al.* (2011). Udonkang*et al.*(2014) used bleached palm oil as tissue clearing agent and reported 93.3% success, which he attributed to the RI of the bleached palm oil (1.455 at 50^{o}C), closer to that of tissue proteins (varying between 1.33-1.4) enabling it to infiltrates the intercellular spaces of tissues. This leads to reduction in the light

scattering properties and increase in optical clearance of the tissue making them appear transparent. The German anatomist Werner Spalteholz(1911) found out that transparency of tissue was highest when light is not reflected on the surface of the tissue, which is the case when the RI of the tissue is the same as the RI of the medium or the solution that holds the tissue.All tissue clearing techniques focus on equilibrating the refractive index throughout a sample to reduce inhomogeneities in light scatter. When the clearing solvent's refractive index is well matched to the refractive index of the dehydrated tissue sample, all non-forward scatter is destructive, and the tissue becomes clear(Spalteholz, 1911, 1914). From this study, it can be assumed that the RT of Citrodora oil may be similar to the RI cleared tissues, equilibrating the refractive indexes and reducing the inhomogeneities in light scatter, enhancing section and staining qualities.

The phytochemical properties of Citrodora oil in this present study was positive for carbohydrates, cardiac glycosides and saponins (steroid and triterpenes) but however was negative for antraquinones derivatives, flavonoids, tannins, and alkaloids. On the other hand, xylene indicated the absence of all other phytochemical properties except for carbohydrates. This finding is similar to that of citrus oil as reported by Akpulu*et al.* (2015). The presence of saponins could be one of the reasons for the therapeutic use of it essential oil as documented by previous authors (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. Is is however, not clear yet its role in tissue clearing. Mosad*et al.*(2018) also documented that isolation and identification of some chemical ingredients from different parts of eucalyptus camaldulensisincludedeucalyptanoic acid, flavonoids, acylated pentacyclic triterpenoids, andessential oils.However, the presence of carbohydrate in both the Citrodora oil ($C_{10}H_{18}O$), and xylene (C_6H_4 $(CH_3)_2$), may be connected to their structural composition and

weight. 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 the reason for the better clearing ability of the Citrodora oil when compared to that of Citrus oil ($CH_{15}H_{24}O$) and Neem oil ($C_{30}H_{36}O_6$) with higher molecular weight as reported byAkpulu*et al.* (2015).In this study it was observed thatCitrodora oil contains saponins, tannins, flavonoids-polyphenolic compounds and alkaloids. The tannins and flavonoids are the substances that impart colour. Tannins are the most important ingredients necessary for dyeing. Saponins have the ability to lower the surface tension that benefits the staining efficacy and flavonoids present in curcumin are primarily the pigments that provide a brightened tinge (Kumar, 2014). It was observed that both the sectioning and staining qualities of Citrodora oil cleared tissues and xylene cleared tissues in all the stains used in this study appeared similar, with no significant difference, this observation is similar to the finding of Kurien*et al*.(2012). We are yet to find a direct correlation between the tannins and flavonoids presence in Citrodora oil to better staining quality after tissue clearing in Citrodora oil.

The GC-MS results of Citrodora oil shows that the predominant constituent in Citrodora oil isCitronellal with relative abundance of 42.20% followed by β-Citronellol (21.71%). This agree with the report of Doran *et al.* (2001). The presence of the citronellal as dominant constituent in Citrodora oil may therefore, be the reason for the good clearing effect observed with tissues cleared in Citrodora oil when compared to xylene. 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,Fabri, *et al.*in (2002) found 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 study. 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). Xylene is an efficient fat solvent, and that is one of its major advantages. This may be the reasons for the better clearing qualities observed in this study. However, Stevenson *et al*. (1998) andKieranan (2010) had found 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.

From the results of theNuclear magnetic resonance(NMR) spectroscopy, Citrodora oil were recorded on the basis of their chemical shift with respect to Tetramethylsilane(TMS) as an internal standard. Protons under the same chemical environment produced signals at the same frequency and the chemical shift recorded in part per million (ppm), whereas all chemical shifts are positive to the reference TMS. The infrared spectrum of Citrodora oil showed characteristic absorption frequencies at 3444 cm^{-1} which was consistent with C-H vibration, a free H-bonded carboxylic acid, vibrations at 1463cm^{-1} and1379cm^{-1} were observed to be associated with C-H vibrations of CH_2 bonding and CH_3 bonding (rocking) respectively. Furthermore, vibrations at 2713cm^{-1} and 1722 cm^{-1} with absorption peaks of medium intensities were observed to be for C-H vibrations for aliphatic aldehydes. Symmetric and asymmetric C-H vibrations due to methyl groups were recorded at 2877cm^{-1} and 2959cm^{-1},respectively and the extended conjugation.This suggest that the CitrodoraOil is made up of mainly triglycerides, with different substitution patterns due to length, degree and kind of unsaturation of the acyl groups similar to what was documented by Guillem and Ruiz(2001).

The percentage yield of Citrodora oil (5.2%) in this study was similar to the report of study carried out on some essential oil by Ladan (2011), who reported that neem oil has the highest yield of 7.8% followed by citrus oil (5.3%). However, since the neem oil was extracted from the seed, it can be argued that the neem oil is higher in yield because its extraction was from the seed and not the leaves. In general, this yield is similar to the ones reported by some authors on essential oils (Essential Oil company, 1996). This little quantity of the Citrodora oil may be attributed to the high volatility of the essential oils during extraction.

The cost of producing 500 ml of Citrodora oil is two thousand, one hundred naira (₦2, 100.00) only against that of xylene of same volume (₦1, 600.00). The cost in this present study included total cost of production from sourcing of the eucalyptus leaves to the laboratory production charges. It should be noted that cost of producing larger quantities of the oil may be cheaper. Besides, it is far cheaper when compared 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 essential oil especially Citrodora oil. There was no transfer of the tissues from one container to another of the same clearing agent used as against the twoseparate changes commonly used in xylene tissue clearing. This implies thatCitrodoraoil will require less frequent changing than xylene, thereby reducing the quantity of clearing agent used. It was also observed that the clearing time was 1hour which is a gain in time when compared to standard xylene clearing time of 2hours two changes each (4hours) recommended by Bancroft and Stevens (2010). The cost of 2.5litres of xylene was cheaper when compared to that Citrodora oil but may be far cheaper in a very large production. 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.

In the demonstration of stained tissue sections, xylene traditionally has been employed as a clearing, dewaxing, and mounting agent for histology (Chen *et al.,* 2010).The results of photomicrographs from the present study showed that the general structures of most of the tissues cleared with Citrodora oil appeared similar to that cleared with xylene both in the section and staining quality using the H and E stain. The results in this study are in agreement with previous reports by Indu, *et al*. (2013) and Sudip *et al.* (2014), whereit was reported that xylene free H and E staining procedure carried out using an essential oil (8%cedarwood oil) produced quality staining with sufficientclarity and uniformity of staining when compared to xylene. Similarly, Premalatha*et al.* (2013),reported that the staining quality provided by xylene free methods using refined mineral oil is equally effective as the conventional methods. This observation also brought out desirable results where the use of essential oils can also be useful at tissue processing stage before the wax impregnation and tissue embedding. As a natural product obtained from several sources, cedar wood oil differs in characteristics and quality. The major advantage of cedar oil is that it causes almost no damage to the tissue. However, it does take significantly longer time to process and is significantly expensive than the usually used alternatives. Nevertheless, the added cost versus work environment safety can be balanced out with optimal procurement plans. Adeniyi*et al.,* (2016) reported that palm kernel oil, groundnut oil and coconut oil can replace the commonly used de-alcholization agents. The result obtained from coconut oil-cleared sections was inaccordance with an earlier study by Sermadi*et al.* (2014). Groundnut oil can also be employed in wood histology as sections processed in it presented distinctfeatures. This is in agreement with the work of Esan *et al*. (2015) in which groundnut oil was stated as a suitable alternativeto xylene in histological tissue processing. The alternative oils used in this study are non-toxic and therefore do not pose a threatto health or the

environment during the clearing process.Furthermore, Kunhua*et al.*(2012), in their work on non-toxic xylene substitute reported that all the tissue sections cleared were stained with various histological procedures including H and E, Van Gieson staining, Aldehyde-fuchsin staining, Gordon Sweet's staining, Alcian blue staining, PAS staining, Victoria blue staining, and immunostaining.The staining results were comparable or superior to those achieved by using xylene.

Taneeru*et al.* (2013) used Limonene oil and Sesame oil as a substitute for xylene to deparaffinize tissue sections during hematoxylin and eosin (H and E) staining and compared them with conventionally deparaffinized H and E sections. The study revealed better results with sesame oil in tissueprocessing and suggested its use as an alternative to xylene. A study by Andre *et al.,* (1994) substituted xylene with amixture of peanut oil, soyabean oil, coconut oil and cotton oiland concluded that it was a poor alternative, as the quality ofsections cleared with xylene were better. Sermadi*et al.*(2014) compared the efficacy of coconut oil with that of xylene,as a clearant. The results proved coconut oil to be an efficientsubstitute for xylene, as it is non-hazardous, less expensive andcaused less shrinkage of the tissue.

The photomicrographs of liver sections of Wistar rat cleared in Citrodora oil and xylene appeared similar with no significant difference ($p > 0.05$). The sections show some histological features of the liver such as the central vein, sinusoid, and the hepatocytes appearing normal with clear nuclear and cytoplasmic details. Comparing the section and staining quality using the H and E, the xylene cleared section appeared to be more in intensity than that of the Citrodora oil cleared section which presented a better cytoplasmic contrast and clarity (Plates I and II). Similarly, the photomicrograph of kidney sections cleared in Citrodora oil and xylene clearly shows the nuclear and cytoplasmic details as evident in the renal cortex, with the

Malpighianrenal corpuscle containing glomerulus and Bowman's space lined with squamous epithelium. The proximal convoluted tubules have narrow lumina and are lined withcuboidal cells with rounded vesicular basal nuclei. The distal convoluted tubules have wider lumina and arelined with cubical cells with rounded vesicular central nuclei (Plates III and IV).

The photomicrograph of transverse sections of the testis of Wistar rat cleared in Citrodora oil and xylene presented a similar feature with H and E stain using the section and staining quality. The sections illustrate the typical structure of the seminiferous tubule showing the stages of spermatogenesis, spermatogonia, sperm cells, and the interstitial cells of Leydig. The staining intensity and the cytoplasmic contrast and clarity of both the Citrodora oil and xylene cleared section presented no significant difference (Plates V and VI). The photomicrograph of section of the cerebellar cortex of Wistar rat cleared in Citrodora oil and xylene is similar; illustrating the typical three layered structure showing the stellate cells of the molecular, Purkinje cell in the Purkinje cell layer and the granule cells of the granular layer. Comparing the staining quality using the H and E, the Xylene cleared section appeared to be more in staining intensity than that of the Citrodora oil cleared and dewaxed section with a better cytoplasmic contrast and clarity (Plates VII and VIII).

The low density and viscosity of Citrodora oil may be the reason for the better section quality observed 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, it is likely the reason why the penetrating ability of the Citrodora oil within one hour was good enough to effect better section and staining qualities observed. 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 found that the xylene processed tissues and the vegetable oils' processed tissues showed only minor or insignificant difference in staining and section quality.

Rene *et al.* (2009), used citrus oil to clear similar tissues with similar results. 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 results obtained in this 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 reported 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).

Histochemical observation using PAS stain shows a strong PAS positive reaction in both the Citrodora oil and xylene cleared and dewaxed testicular tissues, illustrating a well outlined structure of the seminiferous tubule showing concentration of the PAS reaction on the base the basement membrane and interstitial cells of Leydig. The cytoplasmic differentiation and clarity appeared better with the xylene cleared section than with the appearance of the Citrodora oil cleared section (Plates XIX and XX). This finding is similar to the work of Falkeholm*et al.* (2001) and Buesa*et al.* (2009) who reported the advantages of hot dishwashingsoap (DWS) solution for deparaffinization of tissue sections forH and E staining and some special staining procedures like periodic acid-Schiff (PAS) staining and Van Gieson staining.PAS can demonstrate basement membrane (normal and in tumors), glycogen, some mucins and mucopolysaccharides. Based on the mechanism of their glycol groups or their amino or alkylamino derivatives are oxidized by periodic acid to form dialdehydes, which combine with Schiff's reagent to form an insoluble magenta compound (Officer, 2009). The concentration of

any of these metabolites in the basement membrane and interstitial cells of Leydig may contribute to the PAS positive reaction observed.Adibmoradi*et al.* (2015), have demonstrated PAS positive reaction on cytoplasm of Leydig cells, peritubular interstitial tissue and basement membrane testes in a study on the protective effects of wheat sprout on testicular toxicity in male rats exposed to lead.The Citrodora oil in this study is positive for carbohydrate in the phytochemical screening, and the MNR results show mainly triglycerides, with different substitution patterns due to length, degree and kind of unsaturation of the acyl groups. These may be some of the possible reasons for the strong PAS positive reaction in the basement membrane and the cytoplasm of the interstitial cells of Leydig.It can therefore mean that tissues cleared in Citrodora oil and stained hisochemically can demonstrate PAS reaction.

The result from the tissue section of liver in Gordon and Sweet staining method for reticular fibersshowed that the liver tissues cleared and dewaxed withCitrodora oil and xylene both demonstrated clearly reticular fibers with no significant difference in the staining intensity, even though the cytoplasmic differentiation and clarity appeared better with the Citrodora oil cleared and dewaxed section and with the appearance of artifacts on the xylene cleared section. This may mean that Citrodora oil as clearing and dewaxing agents support the demonstration of reticular fibers.Maresch (1905) introduced Bielschowsky's silver impregnation technique for neurofibrils as a stain for reticulum fibers, but emphasized the nonspecifity of such procedures. This lack of specifity has been confirmed repeatedly. (Montes, *et al*, 1980). In this study, it was observed that Citrodora oil contain carbohydrate, and mainly triglycerides in this study, this may be the reason for the good demonstration of the reticular fibers as observed in this study (Plates XVII and XVIII).

The brain sections stained with Golgi Cox silver stain to demonstrate nerve fibers, showed that the tissues cleared in both the Citrodora oil and xylene can demonstrate nerve fibers well.The staining intensity is similar, but the cytoplasmic contrast and clarity of the Citrodora oil cleared and dewaxed section appeared better than that of the xylene (Plates XXI and XXII), though there was no significant difference (0.165). This may mean that Citrodora oil as clearing agents support the demonstration of nerve fibers.

The photomicrographs of formalin-fixed paraffinembedded sections of cerebral cortexcleared and dewaxed in Citrodora oil and xylene stained with anti-GFAP (glial fibrillary acidic protein) antibody showed a positive astrocytes immunoreactivity(Plates X and XI). Comparing both clearing and dewaxing agents with the positive GFAP control, their staining intensity is similar, but the cytoplasmic contrast and clarity of the xylene cleared section appeared better.This may imply that Citrodora oil do not have negative interference with the immunoactivity of the anti-GFAP antibody in brainsections of Wistar rats.

The photomicrographs of formalin-fixed paraffinembedded sections of kidney and livercleared in Citrodora oil and xylene stained with anti-CD34 antibody, showed positive immunoreactivity as indicated by the brown coloration (Plates XIIIXIV, XV and XVI). The Citrodora oil cleared and dewaxed sections showed a better cytoplasmic contrast and clarity than that of the xylene when compared to the positive control with anti-CD34 antibody. This may imply that Citrodora oil do not have negative interference with the immunoactivity of the anti-CD34 antibody in kidney and liver sections of Wistar rats.

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 Citrodora oil, can clear and dewax tissue during processing and staining in their undiluted form. The Citrodora essential oil may be viable alternative clearing agent to xylene.Citrodora oil is naturally extracted from plant with no or little known toxicity.Citrodora oil has similar physical properties, such as low specific gravity and density, viscosity, flash point and refractive index to that of xylene, placing it in the same clearing group. Compared with xylene, Citrodora oil have a sweet smell unlike xylene that have a pungent offensive smell. The phytochemical properties ofCitrodora oils in this present study was positive for carbohydrates, cardiac glycosides and saponins (steroid and triterpenes) but wasnegative for antraquinones derivatives, flavonoids, tannins, and alkaloids. On the other hand, xylene indicated the absence of all other phytochemical properties except for carbohydrates.The presence of carbohydrate in both the Citrodora oil ($C_{10}H_{18}O$), and xylene (C_6H_4 $(CH_3)_2$), may be connected to their structural composition and weight. 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 the reason for the

better clearing ability of the Citrodora oil.The GC-MS results of Citrodora oil shows that the predominant constituent in Citrodora oil isCitronellal with relative abundance of 42.20% followed by β-Citronellol (21.71%).Xylene contains 94% of o-xylene, 97% of p-xylene, 98% of m-xylene and 98%p-xylene.This therefore can be said that the citronellal which serves as dominant constituent in Citrodora oil may be the reason for the good clearing effect observed with tissues cleared in Citrodora oil when compared to xylene. The use of ^{1}HNMR has been demonstrated to be very useful in determining acyl composition in oils.From the results of the ^{1}HNMR spectroscopy, Citrodora oil were recorded on the basis of their chemical shift with respect to TMS as an internal standard. Protons under the same chemical environment produced signals at the same frequency and the chemical shift recorded in PPM, whereas all chemical shifts are positive to the reference TMSOils are made up of mainly triglycerides, with different substitution patterns due to length, degree and kind of unsaturation of the acyl groups.

There was no change of volume of the clearing agent used as against the standard two changes of xylene. This implies thatCitrodoraoil will require less frequent changing than xylene, thereby reducing the quantity of clearing agent used. It was also observed that the clearing time was 1hour which is a gain in time when compared to standard xylene clearing time of 2hours two changes each (4hours). The cost of 2.5litres of xylene was cheaper when compared to that Citrodora oil. 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.

Microscopic examination showed no significant changes both in section and staining quality (H and E) between the sections cleared with Citrodora oil and xylene at one hour, 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 comparably well with no significant changes in staining quality and cytoplasmic contrast and clarity. In the immunohistochemical studies, Citrodora oil do not have any negative interference with the immunoactivity of the anti GFAP and CD34 antibody used respectively.

6.2 CONCLUSION

This present study concludes that:

i. Citrodora oil can clear and dewax Wistar rat tissues (liver, kidney, testes and brain) during tissue processing and staining.
ii. There was no statistical difference both in the tissue section quality and staining abilityin the tissues cleared between Citrodora oil and xylene when used as clearing agent during tissue processing and dewaxing of Wistar rat tissues at one hourtwo changes respectively.
iii. Citrodora oil can comparably demonstrate general tissue structure well as xylene in Hand E stain, reticulin fibers in Gordon and Sweet and nerve fibers in Golgi Cox stain. Citrodora oil also support the positive activity of anti GFAP and CD34 immunoactivity in kidney and liver tissues of Wistar rats.
iv. It therefore can be said that by substituting clearing and dewaxing agent using Citrodora oil, 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 recommended:

1. Study on the effect of storage duration of staining on tissues cleared and dewaxed in Citrodora oil should be carried out.

2. Study on the effect of different mounting media on tissues cleared and dewaxed in Citrodora oil should be carried out.
3. Study on the mixture of Citrodora oil with other clearing agent should be carried out.
4. The use of electron and florescence microscope can be employed to compare tissue structures
5. Study on the use of human and other animal tissues in place of Wistar rat can be employed in a similar study.
6. Further study on the effect of heat and shorter duration on tissues cleared and dewaxed in Citrodora oil should be carried out.
7. Study of the use of citronellal extract from the Citrodora oil as tissue clearing and dewaxing agents should be carried out.

6.4.1 LIMITATIONS OF THE STUDY

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, it however, observed that:

i. During the tissue processing, it was difficult to use the complete automation system.
ii. The criteria used for the assessment of tissue section and staining qualities were subjective.
iii. It was difficult to state the exact end point of tissue clearing in this study.
iv. Ultra-structural details of the tissues cleared in Citrodora oil could not be studied using only bright field.

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APPENDIX I

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

SG of xylene $= \frac{44.0290-23.1026}{}$

47.4091-23.1026 =8.6g/ml

SG of Citrodora = 43.3708-23.1026

47.4091-23.1026 =8.3g/ml

SG of Citrus = 43.1325-23.1026

47.4091-23.1026 =8.2g/ml

DENSITY

Density = 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 = 45.3585-23.1026

25 = 0.89g/ml

Density of xylene = 44.0290-23.1026

25 =0.84g/ml

Density of Citrodora = 43.3708-23.1026

25 =0.81g/ml

Density of citrus = 43.1325-23.1026

25 =0.80g/ml

FREE FATTY ACID (FFA)

FFA = titre x M x 28.2

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 = $\frac{3.8 \times 0.1 \times 28.2}{1}$ =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 = $\frac{\text{titre} \times M \times 28.2}{\text{Weight of sample}}$

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) and W2 = total weight of fresh leaves (grams)

APPENDIX II

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) 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

(D) GOLGI SILVER STAIN (MODIFIED)

Purpose: Forthe 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

(E) IMMUNOHISTOCHEMICAL STAIN

Purpose:to express a tissue antigen

Reagents: hydrogen peroxide, anti GFAP, anti CD34, Biotin

Procedure:

1. Sections were deparaffinized in Citrodora oil and rehydrated in graded alcohol series.

2. Sections were placed in epitope retrieval solution Ph 6.0 for 30 minutes

3. The sectionswere then washed in distilled water.

4. The sectionswere then washed in PBS

5. Sections wereincubated in apprioperate antibody (GFAP and CD34) for 60 min at RT.

6. The sectionswere then washed in PBS.

7.Sectionswere then incubated in Mouse + Rabbit HRP for 30 minutes

8. The sectionswere then washed in PBS.

9.Sectionswere then incubated in DAB + substrate mixture for 5 minutes (1/100 dilution).

10. The sectionswere then washed in distilled water.

11. The sectionswere counter stained in Hematoxylin for 2 minutes.

12. The sections were washed.

13. The sectionswere then dehydrated, cleared and mounted in DPX

Results:

GFAP positive for astrocytes is purplish black in Wistar rat brain section

CD34 positive is brownish in Wistar rat intestinal tissues

APPENDIX III

SCORING SHEET OF STAINED SLIDE

Instruction: Thick the appropriate boxes in front of the criteria as observed using the score description on the top of the table.

SLIDE NO: .....

..... TISSUE	**Excellent**		**Satisfactory**		**Good**		**Fair**		**Poor**	
	A	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
Section quality:										
Folds										
Cracks										
Artefacts										
Staining quality:										
Nuclear staining										
Cytoplasmic contrast/clarity										
staining intensity/uniformity										

Key: (A and B are the clearing agents used)

Excellent	**(5 marks)**
Very good	**(4 marks)**
Good	**(3 marks)**
Fair	**(2 marks)**
Poor	**(1mark)**

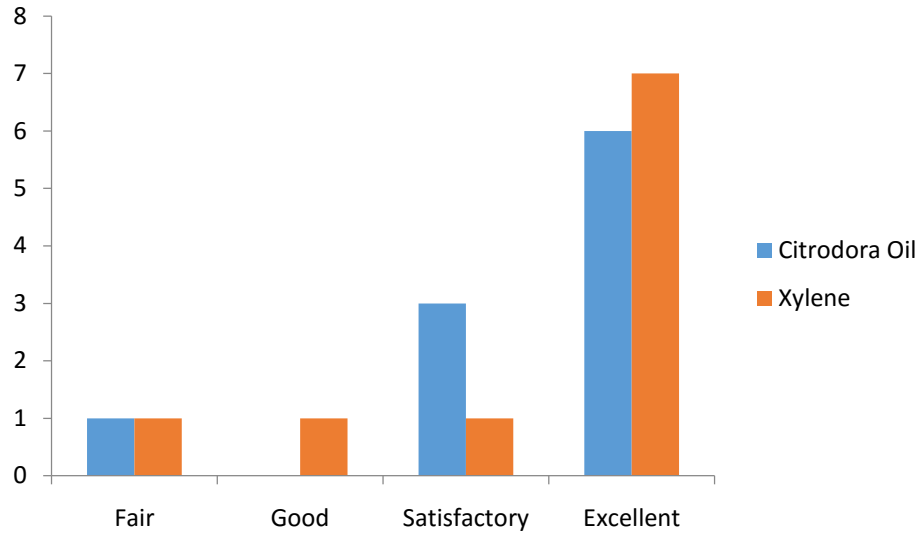
APPENDIX IV

TOTAL PERCENT SCORES OF STAINED SLIDE

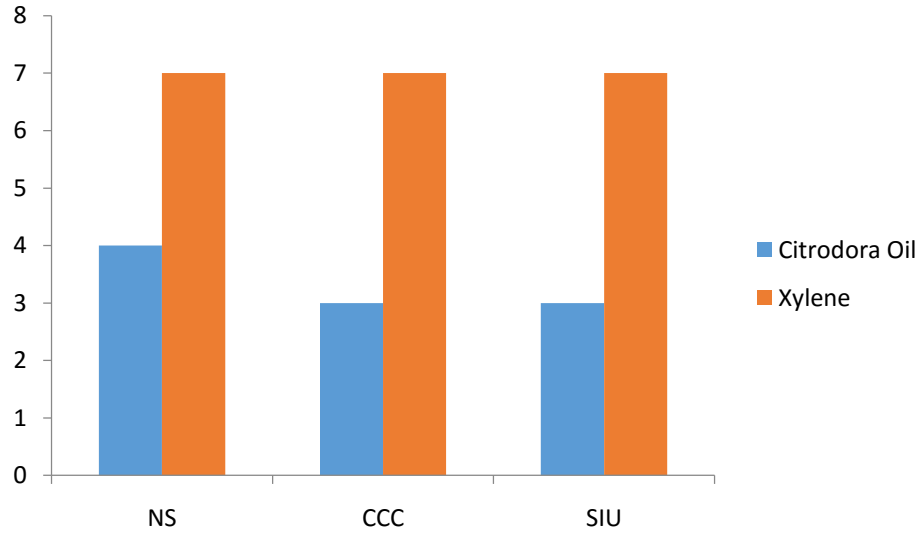
	LIVER (H&E)		**KIDNEY (H&E)**		**TESTES (H&E)**		**BRAIN (H&E)**		**TESTES (PAS)**		**BRAIN (GFAP)**		**KIDNEY (CD34)**		**LIVER (CD34)**		**LIVER (RETIC)**		**BRAIN (GOLGI)**	
	Citr oil	Xyl oil	Citr oil	Xyl oil	Citr oil	Xyl oil	Citr oil	Xyl oil	Citr oil	Xyl oil	Citr oil	Xyl oil	Citr oil	Xyl oil	Citr oil	Xyl oil	Citr oil	Xyl oil	Citr oil	Xyl oil
Section quality:																				
Folds	44	45	46	46	44	45	47	46	46	46	47	45	44	45	47	47	47	46	45	44
Cracks	44	41	46	46	44	45	47	44	46	46	47	45	43	43	47	47	47	46	45	44
Other Artefacts	44	44	46	46	41	44	46	46	46	46	47	45	44	45	47	47	47	42	45	44
Total section quality score	132	130	138	138	129	134	140	136	138	138	141	135	131	133	141	141	141	134	135	132
Percent (%)	**50.3**	**49.6**	**50.0**	**50.0**	**49.0**	**50.9**	**50.7**	**49.3**	**50.0**	**50.0**	**51.1**	**48.9**	**53.3**	**46.7**	**50.0**	**50.0**	**51.3**	**48.7**	**50.6**	**49.4**
Staining quality:																				
Nuclear staining	43	44	44	46	45	44	48	41	46	48	47	43	44	46	48	46	48	46	46	43
Cytoplasmic contrast/clarity	41	44	43	42	45	43	48	45	45	47	47	42	41	45	48	46	48	45	46	41
staining intensity/ uniformity	42	44	44	46	45	45	48	44	41	45	46	42	44	46	48	45	48	46	46	40
Total staining quality score	126	132	131	134	135	132	144	130	132	140	140	127	131	137	144	137	144	137	138	124
Percent (%)	**48.8**	**51.2**	**49.4**	**50.6**	**50.6**	**49.4**	**52.6**	**47.4**	**48.5**	**51.5**	**52.4**	**47.6**	**48.9**	**51.1**	**51.2**	**48.8**	**51.2**	**48.8**	**52.7**	**47.3**

KEY: Citr Oil = CitrodoraOil, Xyl Oil = Xylene

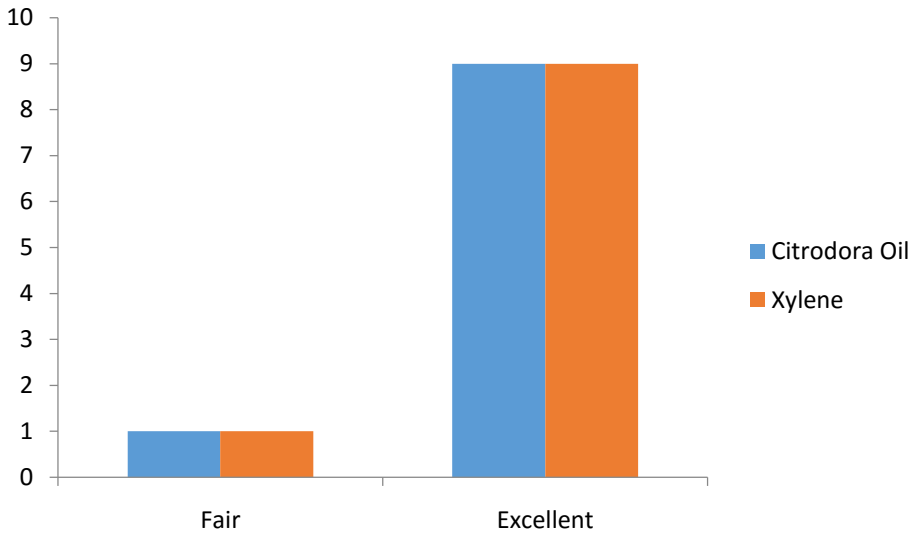
APPENDIX V
BAR CHATS OF STAINED TISSUES SECTIONS



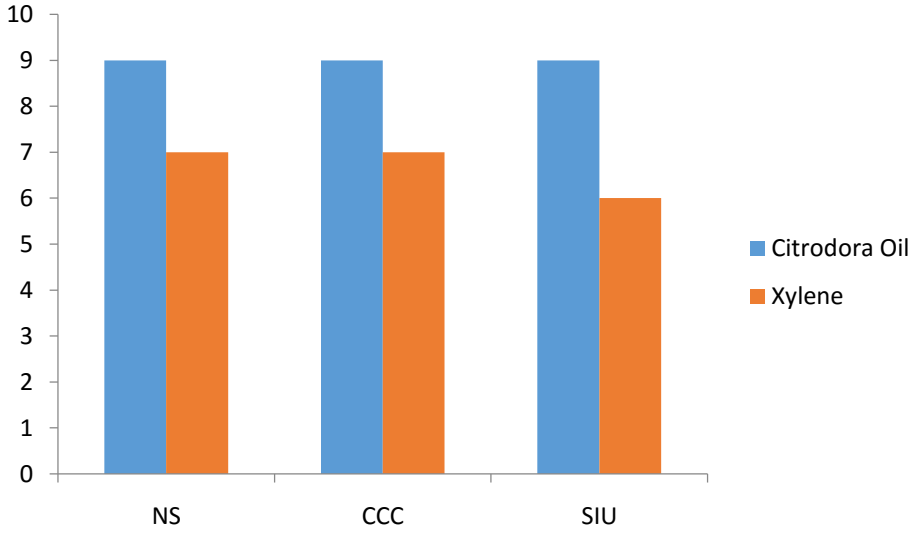
Effect of Citrodora oil and Xylene on liver section quality score under H and E stain



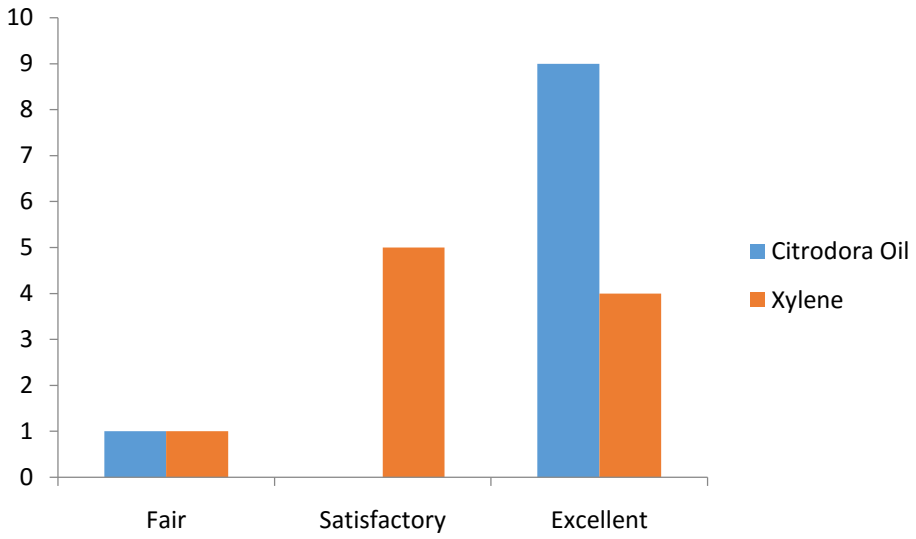
Effect of Citrodora oil and Xylene on liver staining quality score under H and E stain



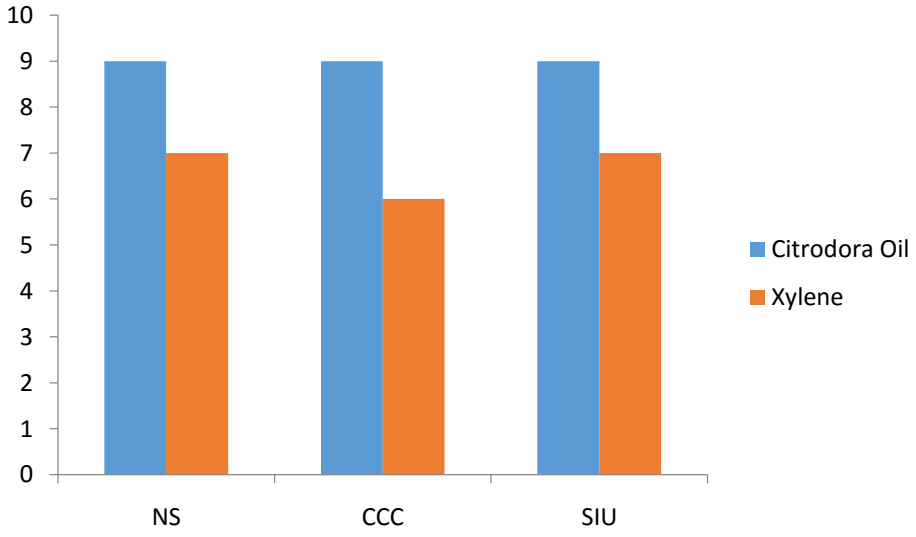
Effect of Citrodora oil and Xylene on liver section quality score under CD34 immunohistochemical stain



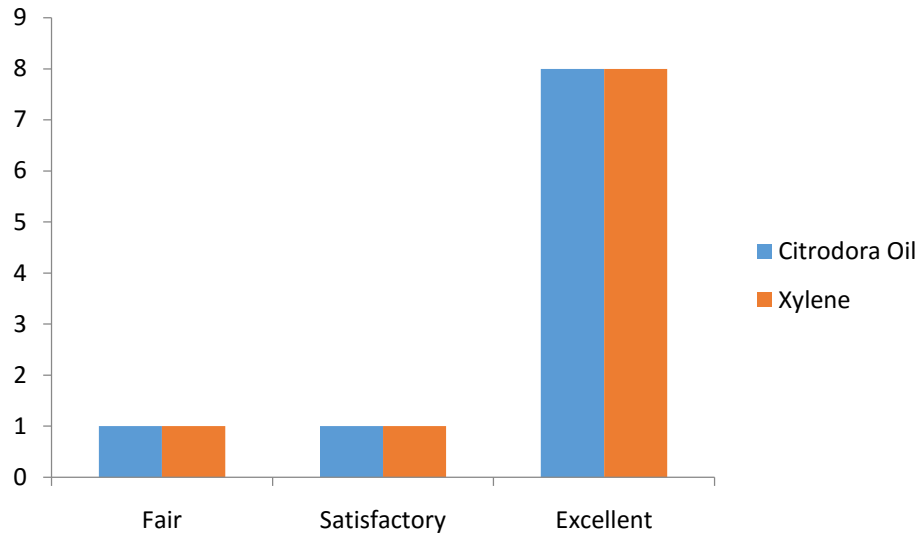
Effect of Citrodora oil and Xylene on liver staining quality score under CD34 immunohistochemical stain



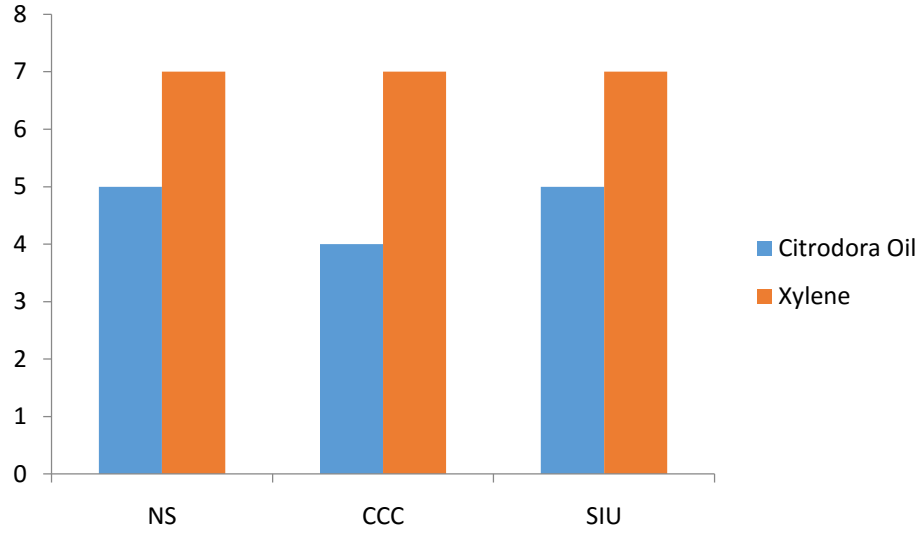
Effect of Citrodora oil and Xylene on liver section quality score under Gordon and Sweet histochemical stain for reticular fibers



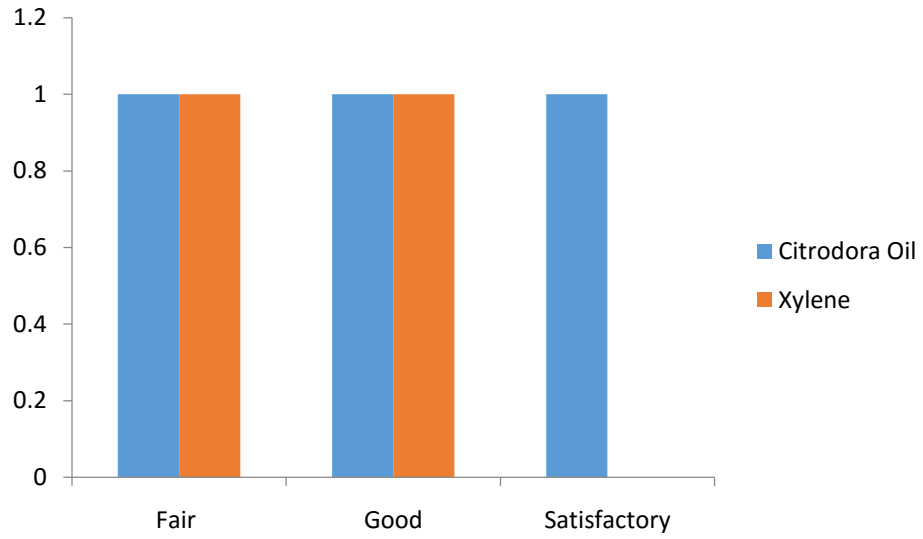
Effect of Citrodora oil and Xylene on liver staining quality score under Gordon and Sweet histochemical stain for reticular fibers



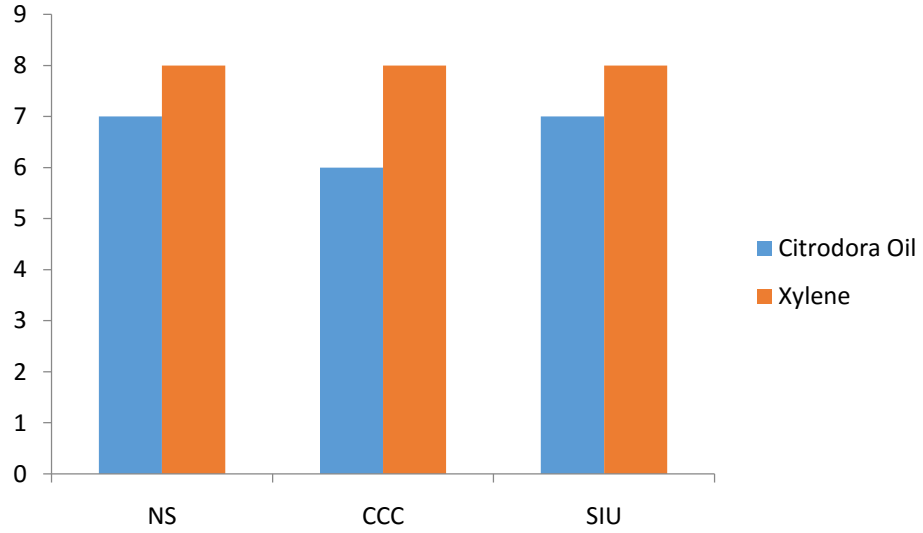
Effect of Citrodora oil and Xylene on kidney section quality score under H and E stain



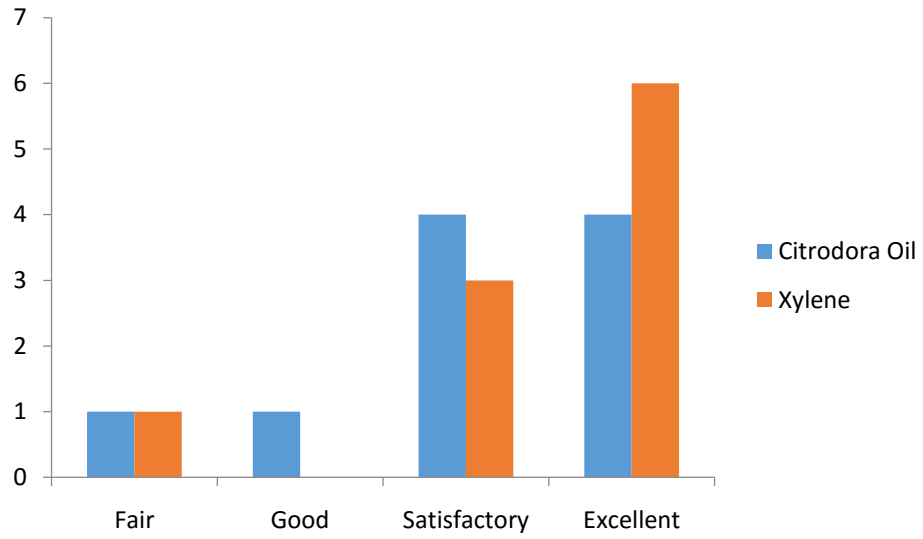
Effect of Citrodora oil and Xylene on kidney staining quality score under H and E stain



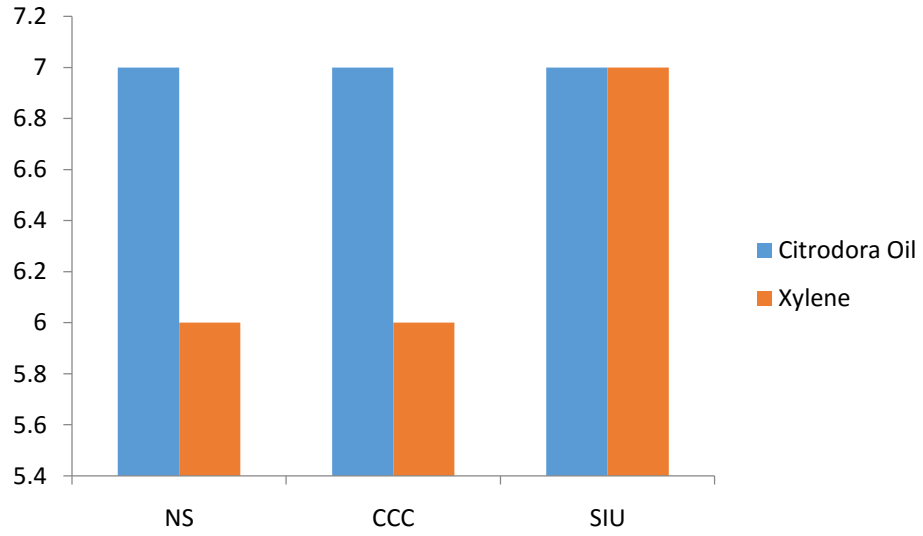
Effect of Citrodora oil and Xylene on kidney section quality score under CD34 immunohistochemical stain



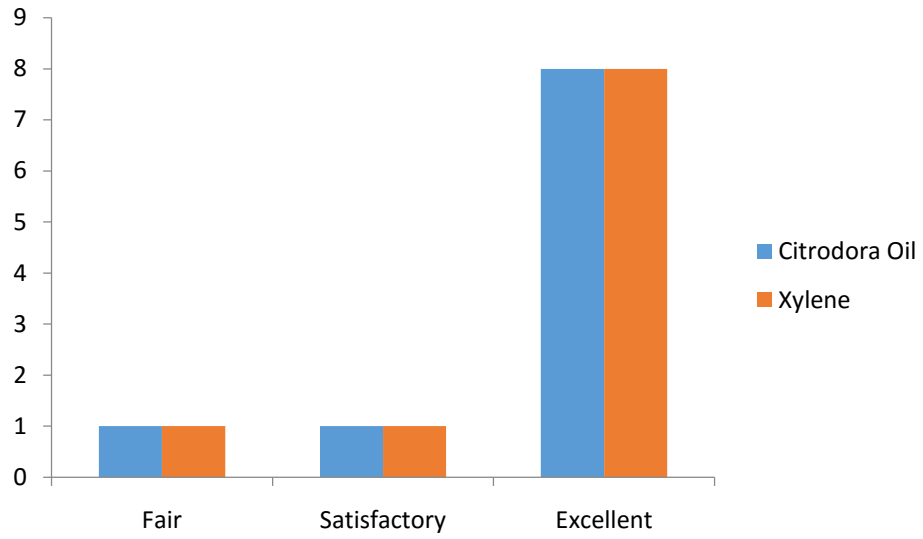
Effect of Citrodora oil and Xylene on kidney staining quality score under CD34 immunohistochemical stain



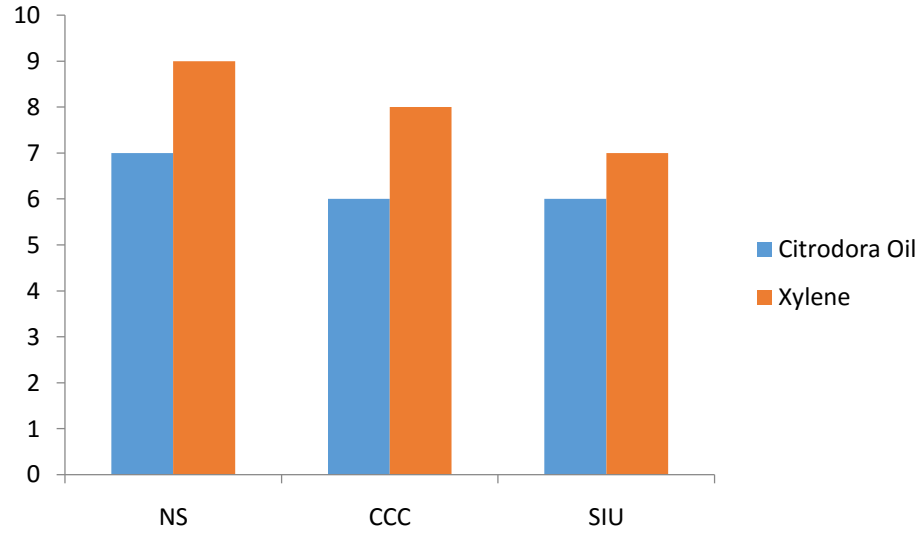
Effect of Citrodora oil and Xylene on testes section quality score under H and E stain



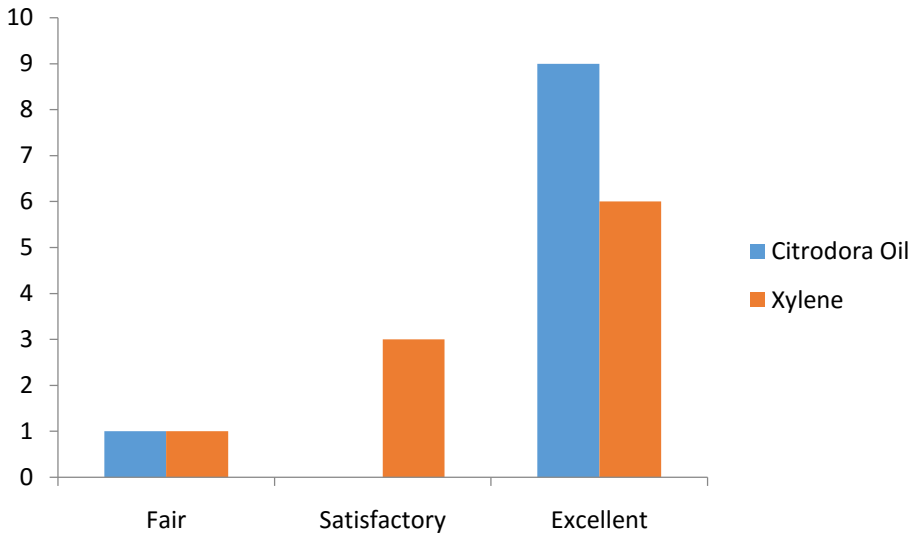
Effect of Citrodora oil and Xylene on testes staining quality score under H and E stain



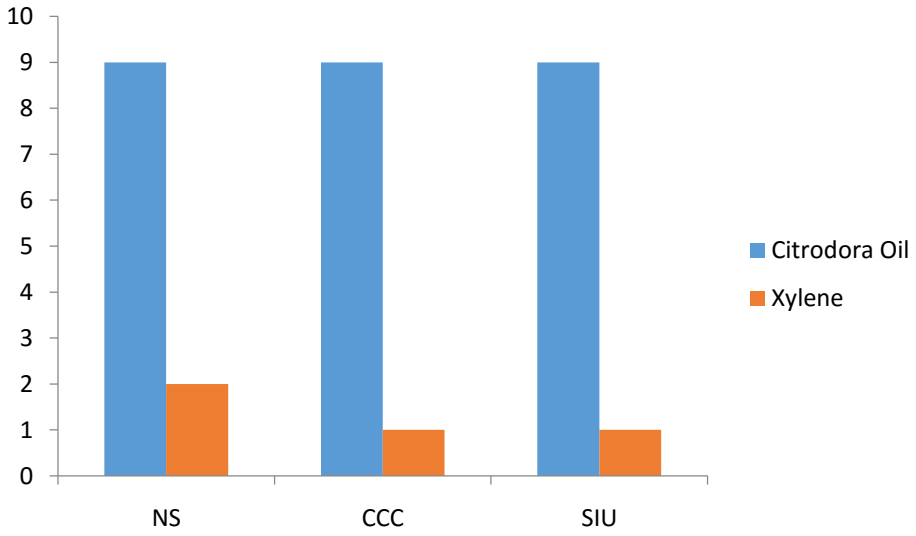
Effect of Citrodora oil and Xylene on testes section quality score under PAShistochemicalstain



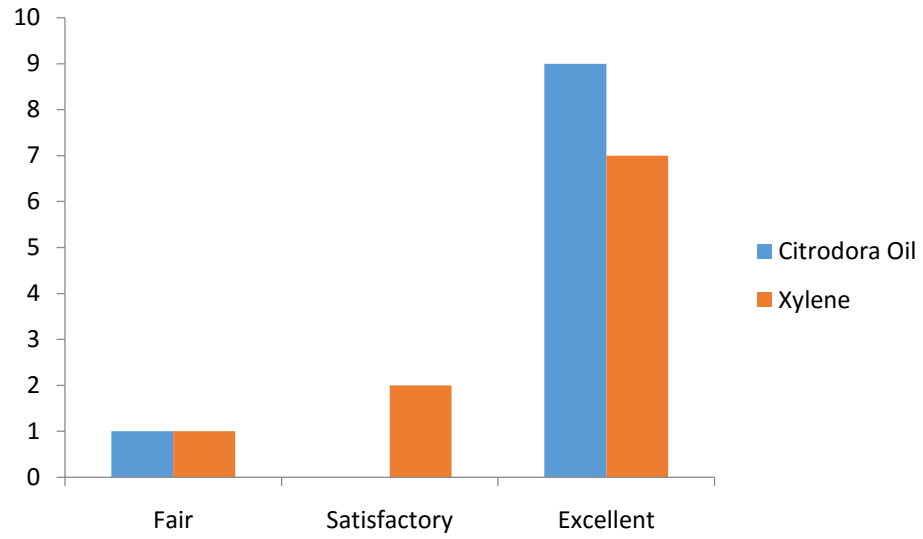
Effect of Citrodora oil and Xylene on testes staining quality score under PAShistochemicalstain



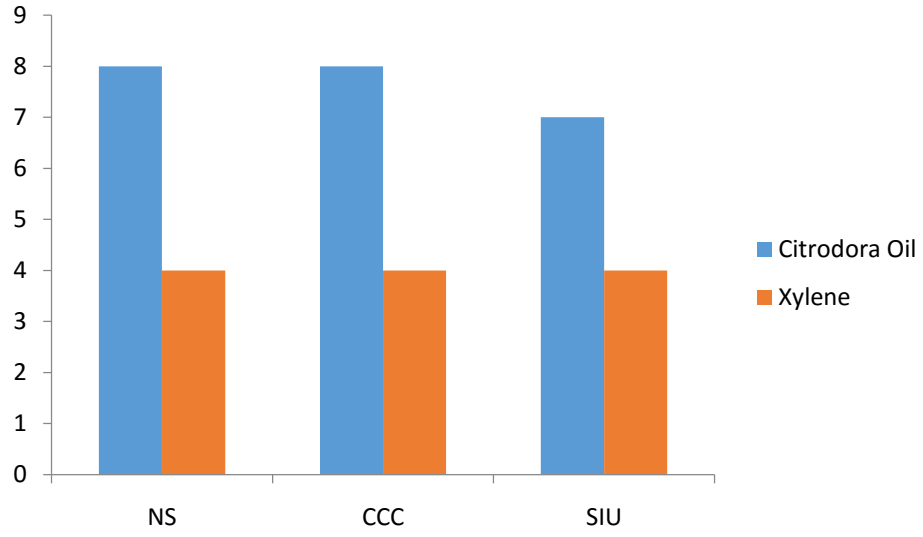
Effect of Citrodora oil and Xylene on brain section quality score under H and E stain



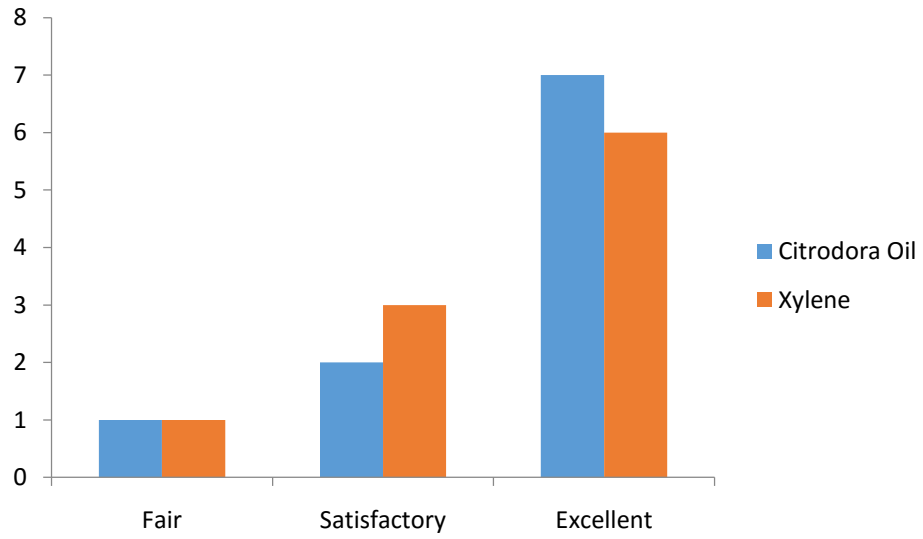
Effect of Citrodora oil and Xylene on brain staining quality score under H and E stain



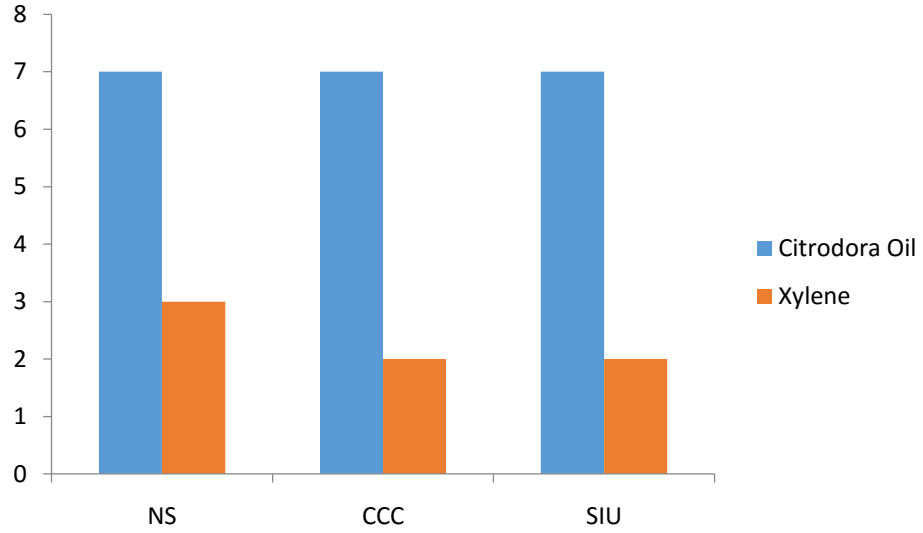
Effect of Citrodora oil and Xylene on brain section quality score under GFAP immunohistochemical stain



Effect of Citrodora oil and Xylene on brain staining quality score under GFAP immunohistochemical stain



Effect of Citrodora oil and Xylene on brain section quality score under Golgi histochemical stain



Effect of Citrodora oil and Xylene on brain staining quality score under Golgi histochemical stain

APPENDIX VI

Ethical Approval Letter