PHYTOCHEMICALS OF THE LEAF AND ROOT FRACTIONS OF *COMBRETUM MOLLE* (R.Br. Ex. G. Don), ANTIBACTERIAL EFFECTS AND TOXICITY TO WISTAR RATS

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DEDICATION

This work is dedicated to my parents, for laying the foundation of my academic career and moral upbringing.

DECLARATION

I declare that the research in this thesis entitled ‘Phytochemicals of the leaf and root fractions of *Combretum molle* (R.Br. Ex. G. Don), antibacterial effects and toxicity on Wistar rats ' has been performed by me in the Department of Botany under the supervision of Prof. S. P. Bako, Prof. D. N. Iortsuun and Prof. H. I. Inabo. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at any other University.

…… ……..

Kulawe Darmasu Sign/Date

CERTIFICATION

This thesis entitled "PHYTOCHEMICALS OF THE LEAF AND ROOT FRACTIONS OF *COMBRETUM MOLLE* (R.Br. Ex. G. Don), ANTIBACTERIAL EFFECTS AND TOXICITY ON WISTAR RATS" by Kulawe Darmasu meets the regulations governing the award of the degree of Doctor of Philosophy of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

This work was carried out with the aim of determining the phytochemical compounds present in the aqueous, n-butanol and the ethyl-acetate fractions of the leaves and roots of *Combretum molle*, its biological activities against selected micro-organisms and toxicity to animal tissues. Ethanol was used as solvent for extraction, after which differential fractionization was carried out using distilled water, ethyl acetate and *n*-butanol. Aqueous, ethyl acetate and *n*-butanol fractions of the leaves and roots of *C. molle* were screened for secondary metabolites, using Thin Layer Chromatography (TLC) and Gas chromatography –mass spectrometry (GC-MS). The micro-organisms studied are (*Erwinia chrysanthemi*, *Salmonella* Typhi, *Xanthomonas axonopodis*, *Pseudomonas syringae* and *Klebsiella pneumoniae*). Ciprofloxacin was used as positive control. The preliminary phytochemical screening of aqueous, n-butanol and ethyl-acetate fractions of the leaves and root *Combretum molle* revealed the presence of; Tannins, Flavonoids, Saponins, Triterpenes and Steroids. Glycosides were present in all the fractions except the root aqueous and root n-butanol fractions. Alkaloids were not present in the root ethyl-acetate and root n-butanol fractions. Anthraquinones were present in the leaf and root aqueous and root n-butanol fractions only. The results from the TLC revealed that, all the fractions of *C. molle* contained secondary metabolites as well as higher density of secondary metabolites in the ethyl-acetate root fraction. GC-MS revealed the presence of 17 against 23 compounds in the n-butanol root and leaf fractions respectively, 15 against 18 in the ethyl-acetate root and leaf fractions and 11against 10 in the aqueous root and leaf fractions respectively. *Erwinia chrysanthemi*, *X. axonopodis* and *S.* Typhi were susceptible to the fractions of *C. molle* with minimum inhibitory concentration (MIC) values of as low as 1.5 mg/ml, 3.125 mg/ml and 6.25 mg/ml respectively, *K. pneumoniae* was not susceptible to the ethyl-acetate root and aqueous leaf fractions of the plant. The minimum bactericidal concentration (MBC) had low values such as 0.76 mg/ml of the n-butanol leaf fraction against *S.* Typhi, and 1.5 mg/ml of the n-butanol leaf fraction against *X. axonopodis.* The analysis of variance indicated that there was no significant difference in the performance of the n-butanol root, n-butanol leaf, ethyl-acetate leaf, ethyl-acetate root, aqueous root and aqueous leaf fractions on the microorganisms *E. chrysanthemi*, *S.* Typhi, *X. axonopodis*, *P. syringae* and *K. pneumoniae*. In the acute toxicological investigation, there was no mortality in the experimental animals after orally administering the fractions of *C. molle*, however, there were induced histopathological alterations in the cellular anatomy of the liver and kidney. The analysis of variance also indicated that there was no significant difference in the performance of the roots was not significantly different from that of the leaf. The study indicates that, *C. molle* contains phytochemical compounds which makes it a good inhibitor of microbial growth and could be exploited through further research to determine the active compounds that could be utilized in the treatment of common ailments. The cellular alterations in the liver and kidney could become fatal during prolonged use or unregulated dosages.

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LIST OF ABBREVIATIONS

TLC	Thin layer chromatography
GC-MS	Gas Chromatography Mass Spectrometry
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
CAM	Complementary alternative medicine
CLSI	Clinical and Laboratory Standard Institute
HDL	High density lipoprotein
LC-PUFA	Long chain polyunsaturated fatty acids
DOX-96	Disposable oxygen electrode sensors
MSSA	Methicillin susceptible *Staphylococcus aureus*
MRSA	Methicillin resistant *Staphylococcus aureus*
LPS	Lipolysaccharide
INA	Ice nucleation active
NCPPB	National Collection of Plant Pathogenic Bacteria
CEF	Chloroform ethyl-acetate formic acid
ICMP	International Collection of Microorganisms from Plants
H_2SO_4	Sulphuric acid
ANOVA	Analysis of variance
OECD	Organization for economic cooperation and development
DPX	Dibuthylphthalate xylene
TV	Tubular vacoulation
TD	Tubular distortion
VC	Vascular congestion

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CHAPTER ONE

INTRODUCTION

1.1 Background to the study

Medicinal plants are major sources of obtaining antimicrobial agents (Sofowora, 2006). Plants produce diverse types of bioactive molecules, making them a rich source of different types of medicines (Butak, 2005). Medicinal plants have been used since ancient times in virtually all cultures as a source of drugs, and are of great importance to the health care of individuals and communities (Edeoga *et al*., 2005). Many plants have been confirmed to have a variety of antimicrobial effects (Shai *et al*., 2008) and antidiabetic properties. Many research efforts have been directed towards the provision of empirical proofs to back up the use of plant species in trade and medicinal practices. Many researchers have examined the effects of plants used traditionally by indigenous healers to support treatment of various diseases (Ojo *et al.*, 2005). Determining the antimicrobial activity of many plant species has become the subject of many intensive investigations (Obagwu *et al*., 1997; Tanko *et al*., 2011).

According to Alaribe (2008) majority of Nigerian homes, maintain some sort of private family traditional medicine practitioner. Existing data and contemporary researchers seem to authenticate the assumption for general health improvement of the masses by traditional healers. Ethnobotany is a preliminary method of research, suitable for gathering information on the use of plants. It has been proven, time and time again, that the medical knowledge handed down by the common people constitutes sources of information useful for scientific research and that many plants utilized exclusively in popular tradition, when exposed under scientific investigation, have been found to be useful for different sectors in the industry, therefore science and tradition have

a strong connection between them. Science in fact often has traditional origins (Lentini, 2000). The use of plants in the tropical and subtropical regions is diversified and most of the uses are for medicine, source of food, clothing and shelter. However, the medicinal uses of plants are rapidly declining among the present generation of local people as a consequence of modernization and civilization (Cox, 2005). The younger generation is showing little interest in learning this valuable science of healing. Usage of medicinal plants to cure diseases has also been much influenced by religious practices (Trease and Evans, 1989).

Traditional medicine has remained the most affordable and easily accessible source of treatment in the primary health care system of resource poor communities and local therapy is the only means of medical treatment for such communities (Yinger and Yewhalaw, 2007). Areas of high biodiversity, including tropical rainforests, are domains of chemical warfare. In the battle for survival, plants have evolved with many chemical defenses as means of survival to ward off attackers such as bacteria, insects, fungi, severe weather and, in some cases, mammals that may threaten their existence. Secondary metabolites, while not essential for growth and development, do promote the spread and dominance of plant species in an ecological setting (Fellows and Scofield, 1995).

Plants have broader uses than just food and genetic reservoirs. Medicinal plants have been used for centuries to treat a wide variety of ailments (Vaidya and Devasagayam, 2007). The presence of secondary metabolites in plants has been associated in most of their therapeutic activities (Ogunleye and Ibitoye, 2003). Herbal medicines are now considered a part of Complementary and Alternative medicine (CAM) and are gaining popularity due to their potent antioxidant effects, minimal side effects and economic viability (Auddy *et al*., 2003). Active

principles from natural sources have contributed significantly to the development of new drugs from medicinal plants (Cox and Balick, 1994).

Thousands of secondary plant products have been identified and it is estimated that thousands of these compounds still exist (Koehn and Carter, 2005). Since secondary metabolites from plants have been elaborated within living systems, they are often perceived as showing more "potentials and are biologically non-toxic than totally synthetic molecules" making most of them, good candidates for further drug development (Koehn and Carter, 2005). Bio molecules of plant origin appear to be one of the alternatives for the control of these antibiotic resistant human pathogens (Drahl *et al*., 2005). These can be derived from barks, leaves, flowers, roots, fruits and seeds. Knowledge of the chemical constituents of plants is desirable because such information will be used for synthesis of complex chemical substances (Criagg and David, 2001) The increased use of conventional chemical pesticides over the years has not only contributed to an increase in food production, but also has resulted in adverse effects on the environment and non-target organisms. In view of these side effects, the necessity for sustainable crop production through eco-friendly pest management technique is being largely felt in the recent times (Stoll, 1998; Burges, 1998). Neem products though extensively used in insect pest management possess bactericidal properties (Emechebe and Alabi, 1997; Bdliya and Dahiru, 2006) have been used in plant disease management.

Determination of optimal solvent for extraction of bioactive compounds from natural materials is also important for maximizing the extraction yield of bioactive compounds because these compounds have different characteristics as well as polarities (Jain *et al*., 2014). According to Kolar *et al*. (2002), the presence of a solvent in the extract may also affect the kinetics of crystallization and the morphology of the crystals, which is an important factor in

determining the product quality. Most of the bioactive components contained in the plant matrices are medium-sized molecules. Due to the presence of aromatic delocalized micro-electrons, the molecules are highly polarizable. Their high polarizability makes the molecules liable to a variety of specific interactions with polar solvents (Vuong *et al*., 2013). According to Shui and Leong (2004) the polarity of the solvents affects the efficiency of the extraction and the activity of the obtained extracts, for example water, methanol, ethanol, acetone, aqueous and ethyl acetate are commonly used as extraction solvents.

1.2 STATEMENT OF RESEARCH PROBLEM

Diseases associated with microbial pathogens are found both in plants and animals. In plants, these microorganisms causes many diseases in both field crops and stored products resulting in extreme losses of produce.

In animals, microbial pathogens cause systematic and significant diseases that result in reduced productivity and even death. The search for medicines that alleviate these conditions and restore health as well as improving productivity and longevity of crops in storage has led to the development of many drugs, fungicides and bactericides that are prescribed today for the control of microbial pathogens. However, prolonged and frequent use of these drugs have led to development of resistance by the pathogens or in some cases have caused disruption of environmental equilibrium.

Despite the existence of potent antibiotic and antifungal agents, resistant or multi-resistant strains are continuously appearing, imposing the need for a continuous search and development of new drugs (Silver and Bostian, 1993).

Traditional plant remedies include crude vegetable drugs (herbs) as well as extracts, fluids, tinctures and infusions prepared from them. Although a number of studies on the antimicrobial effects of plant extracts have been performed, many plants used in different traditional medicinal systems are yet to be evaluated for their antimicrobial effects. For example, in Africa, over 5000 plants are known to be used for ethno-medicinal purposes, but only a small percentage have been described or studied scientifically (Taylor *et al.*, 2001), and different combinations of plant species used in traditional medicines have not been studied (Taylor *et al.*, 2001).

The major problem in investigations on the biological activities of plant extracts lies in the fact that, a variety of plants may be used in a single traditional medicine preparation, and thus possibilities of synergism from the interactions of the compounds in the mixed extract. Such may even result in a loss of activity as the extract is purified (Izhaki, 2002). Eloff and McGaw (2006) pointed out that, biologically active extracts can be extremely useful in their entirety, taking into account synergistic and other effects, and according to them an approval of standard for plant extracts as drugs might be the starting point in developing countries for a successful pharmaceutical industry to be able to compete with Western pharmaceutical companies.

The use of various parts of *Combretum molle* for the treatment of different ailments in communities in Africa is without scientific basis. Thus, the need to investigate it scientifically, so as to draw definite conclusions on the use of the *Combretum molle* plant.

1.3 JUSTIFICATION

Pathogenic infestations affecting agricultural production are a common occurrence. Increases in pesticide use to control pathogens that easily attack commercially grown high yielding varieties have led to an increase in the virulence of many species of crop pathogens due to the destruction of non-target species, which include natural predators of pests and parasites (Litsinger, 1989; Pimentel *et al*., 1992).

Apart from pathogens developing resistance to pesticides there are many harmful effects of pesticides that affect agricultural sustainability, the environment and the health of farmers as well as those living around the farms. Furthermore, it has been pointed out that, pesticides can adversely affect paddy soils (Bdliya and Dahiru, 2006). Declining soil fertility adversely affects productivity and increases the need to apply larger quantities of chemical inputs to maintain productivity, thus further increasing the costs to farmers. Also, according to Reigart and Roberts (1998) for all pesticides to be effective against the pathogens they are intended to control, they must be biologically active, or toxic. Because pesticides are toxic, they are also potentially hazardous to humans, animals, other organisms, and the environment. The acute toxicity of pesticides to humans is generally considered to be low, but pesticides can be irritating to the skin and eyes. Inhalation of spray mist or dust from these pesticides may cause throat irritation, sneezing, and coughing. Chronic exposures to lower concentrations of fungicides can cause adverse health effects. Most cases of human pesticide poisonings have been from consumption of seed grains (Amadioha, 1999). Studies reveal that, plants possess many subtle defenses that interfere with pest growth, development and behaviour and lack toxicity to higher animals. Hence, an alternative, safe and eco-friendly approach for plant pathogens control is the use of extracts of plant origin.

The increasing resistance to antibiotics has resulted in the research to identify new organic molecules from plants with antimicrobial properties for treating diseases (Sofowora, 2006). Using local plants will be a welcome alternative as the cost will be minimal. The leaf, stem and bark extracts of *C. molle* have been reported in the treatment of various ailments such as diarrhea and dysentery. It is therefore important to scientifically investigate these plant parts to ascertain their therapeutic potentials. Determination of their chemical composition as well as antimicrobial efficacy against specific pathogens is important in the recognition of this plant as a potent commercial medicinal plant.

1.4 AIM

This study was aimed at evaluating the antibacterial activities of the leaf and root fractions of *Combretum molle* against selected pathogens and its toxicity to Wistar rats.

1.5 SPECIFIC OBJECTIVES

The specific objectives were:

1. To determine the occurrence of bioactive components in the ethyl acetate, aqueous and n-butanol fractions of the leaf and root of *C. molle*.
2. To determine the antibacterial activities of the ethyl acetate, aqueous and n-butanol fractions of *Combretum molle* leaf obtained from the ethanol extract in selected bacteria.
3. To determine the antibacterial activities of the ethyl acetate, aqueous and n-butanol fractions of *Combretum molle* root obtained from the ethanol extract in selected bacteria.

4. To determine the acute toxicity (LD_{50}) of *Combretum molle* fractions to Wistar rats.
5. To evaluate the histological effects of *Combretum molle* fractions to Wistar rats (liver and kidney).

1.6 HYPOTHESES

1. There are no significant differences between the phytochemicals present in the ethyl acetate, aqueous and n-butanol fractions from leaves and roots of *C. molle.*
2. There are no significant differences in the antibacterial activities of the ethyl acetate, aqueous and n-butanol fractions of *Combretum molle* leaf.
3. There are no significant differences in the antibacterial activities between the ethyl acetate, aqueous and n-butanol fractions of *Combretum molle* root.
4. There are no significant differences in the toxicity effect of *Combretum molle* to Wistar rats.
5. There are no significant differences in the histological profile of Wistar rats (kidney and liver) treated with the fractions of *Combretum molle*.

CHAPTER TWO

LITERATURE REVIEW

2.1 Origin and distribution of *Combretum molle*

Combretum molle is indigenous to Nigeria. In Africa, it is found in: Botswana, Central African Republic, Congo, Democratic Republic of Congo, Djibouti, Eritrea, Ethiopia, Gabon, Kenya, Lesotho, Namibia, Senegal, Somalia, South Africa, Sudan, Swaziland, Tanzania, Uganda and Zambia. It is generally a tree of the forest and savannah regions of Africa, often occurring on ant-hills, in semi-evergreen thickets and frequently associated with quartzite formations. It is a common tree in Pretoria and Johannesburg, for it grows freely on the slopes of Magaliersberg and on those of the western Waterberg and Soutpansberg (Wickens, 2000). *Combretum molle* is found throughout Zambia with the exception of Kalabo, Senanga, Sesheke and most of Mongu Districts. It has a wide range, from Senegal to Sudan, down across the whole of Central and Eastern Africa.

In Nigeria, it occurs throughout the north and south in areas where woodlands and wooded grasslands predominate, often forming pure stands on hillsides (Wickens, 2000; Drummond and Palgrave, 2006). Most of the species of *Combretum* growing in southern Nigeria are trees or woody climbers. The trees often grows in woodlands. Climbers can be found in riverine forests, thickets and rain forests (Wickens, 2000). *Combretum* or the bush willows, make up the type genus of the family *Combretaceae*. The genus comprises about 370 species of trees and shrubs, roughly 300 of which are native to tropical and southern Africa, about 5 to Madagascar, some 25 to tropical Asia and approximately 40 species to tropical America (Katende, 1995; Ahmad and Beg, 2001). The genus is absent in Australia. Other species of this genus are a major component

of Southwestern Amazonian moist forests. This genus contains several species that are pollinated by mammals other than bats, which is quite rare indeed, although most species are more conventionally pollinated by insects or birds (Katende, 1995).

2.1.1 Classification

Combretum molle belongs to the Kingdom Plantae; Division: Magnoliophyta, Class: Magnoliopsida, SubClass: Rosids, Order: Myrtales, Family: Combretaceae, Genus: *Combretum*, Species: *molle*. Authority: R.Br. ex G. Don. Synonym (s) *Combretum atelanthum, C. gueinzii* and *C. holosericeum.* Common names for *Combretum molle* are Velvet bush willow (English); basterrooibos (Afrikaans); mlama (Swahili); umbondwe (Zulu); gogen damo, wuyan damo, tsiriri, karya, gooda jiki (Hausa): efekoci (Nupe) (Blench, 1989); damoruhi (Fulani); Anragba (Yoruba) (Gbile, 1984).

2.1.2 Botanical characteristics

Combretum was the name given by Pliny to a particular climbing plant, the identity of which has been lost in time. The specific name is the Latin word 'mollis', meaning 'soft', presumably because of the softness of the leaves. *Combretum molle* is a small, deciduous tree 3-13 m high; trunk is crooked, occasionally swollen at the base, up to 30 cm in diameter. Bark grey and smooth when young, grey-brown to almost black, rough and flaking when older, twigs often with reddish hairs. It is deciduous and yields a gum (Palmer, 1972). Leaves are opposite, simple, leathery, 5-17 cm long, 2.5-9 cm wide, narrowly elliptic, broadly ovate almost circular, with dense, grey, velvety hairs on both sides, especially on the underside (some forms are almost without hairs; margins are entire; apex tapering, often with a short point or sometimes notched; base round, heart-shaped or slightly narrowed) divided by midrib into 2 unequal parts; margins are entire; petiole thickset, 2-3 mm long; net-veining conspicuous, especially on the

lower surface (Palmer, 1972). Individual flowers small, on bare branches; pale cream or greenish-yellow, sometimes with a reddish tinge; heavily scented, borne singly in the axils of the leaves (in dense, axillary, often branched spikes, 4-9 cm long. The short-stalked flowers are borne on a central stalk 3-7 cm long). Bracts present. Fruits 1.4-2 (max. 2.5) x 1.5-2 cm; yellowish-green flushed with red, drying to golden reddish-brown; borne abundantly, some old fruits remaining on the tree into the next flowering season; 4-winged, the wings pale yellow-brown, rounded, with shallow notches at top and bottom (Palmer, 1972).

2.1.3 Uses

Several species are used in African or Indian traditional medicine. *Combretum molle* is also recorded to contain large quantities of punicalagins, the antioxidant well-known from the Pomegranate (*Punica granatum*), a somewhat related plant. These phytochemicals, are suspected to suppress cancer growth (Bein, 1996). Flowers attract bees and make good forage for honey production. Wood burns slowly, giving intense heat, and is suitable for firewood and production of high quality charcoal. *Combretum* wood is yellow, hard, coarse, and brittle when dry and rots easily. It is said to be reasonably termite resistant and is suitable for implement handles, poles, stools, construction and fence posts (Kokwaro, 2000). Tannin or dyestuff; a red dye can be obtained from the leaves and yellow dye from the roots. An infusion of the inner bark is taken orally or as an enema to relieve various stomach ailments. The bark exudes a gum that can be used to treat wounds, the crushed dried or fresh leaves can also be used for the same purpose. It can also be used as soil improver; leaf fall is a source of mulch and green manure for the soil (Oerke, 2006). *Combretum molle* is widely used in African traditional medicine for treatment of various ailments and diseases. Various organs such as the leaves, roots, and to a lesser extent the fruits and stem bark have applications in traditional medicine. The most commonly used parts

are the roots and the leaves of this species for medicinal purposes. The inner part of the root is powdered and used in wound dressing (Wickens, 2000; Drummond and Palgrave, 2006).

Very often *C. molle* is mixed with other species of *Combretum* as well as with other plant species since remedies made from mixtures of plant species are thought to be more powerful in their healing effects (Wickens, 2000). There are only two reports on the medicinal use of the stem bark of *C. molle*: an aqueous suspension of the stem bark is used for gargling and decoctions of the inner bark used for treatment of stomach problems (Wickens, 2000). The leaves of *C. molle* are used in various ways for medicinal purposes. Decoctions of dried leaves are boiled with salt and water and sprinkled on wounds. Fresh leaves, or moistened dry leaves, are used as dressings for wounds (Drummond and Palgrave, 2006). The juice of the leaves of *C. molle* is mixed with a decoction of the roots to treat fevers and as an antidiarrheal (Haerdi, 2001). Aqueous extracts of the leaves are used to treat chest complaints, as anthelmintics and as inhalants in steam baths. Leaves of *C. molle* are used externally together with the roots of *Senecio lyratipartitus* to heal wounds (Kokwaro, 2000). Leaf decoctions are used for the treatment of dropsy. The dried leaves are mixed with food for the same purpose (Kokwaro, 2000). An infusion of the leaves is drunk as an aid in child birth and a hot application is applied to the vulva and abdomen. Although, fruits of *Combretum* species in general are reported to be toxic, the fruits of this species are used as an aid in child birth in the same way as the leaves (Drummond and Palgrave, 2006).

Combretum molle has been found to possess antibacterial (Chhabra *et al.*, 1999) and antifungal effects (Pegel and Rogers, 1985), which is in accordance with the uses of this plant for treatment of infectious diseases and wounds in African traditional medicine. Eloff *et al.* (2006) found that a leaf extract of *Combretum molle* was especially active against *Enterococcus faecalis*

(MIC < 0.2 mg/ml). Pegel and Rogers (1985) found that the compound responsible for the antifungal effects of the leaf extract was the triterpenoidmollic acid-3-β-D-glucoside. Also the stem bark of *C. molle* contains antibacterial compounds, and its acetone fractions have been found to significantly inhibit the growth of *Mycobacterium tuberculosis* typus humanus (ATCC 27294) (Chhabra *et al.*, 1999; Kokwaro, 2000). Hydrolysable tannin, punicalagin, was found to be responsible for the antibacterial effect of the stem bark, whereas the isolated saponins were found to be inactive (Chhabra *et al*., 1999).

The extracts of the roots of *Combretum* and *Terminalia* species generally gave the best antibacterial effects, although extracts of leaves were also effective. Stem extracts were less active than the other parts of the plants (Fyhrquist *et al.*, 2002). Interestingly, none of the extracts, with the exception of a methanol leaf extract of *T. kaiserana*, were active against *Escherichia coli*, although Eloff (1999) reported good effects of many African species of *Terminalia* and *Combretum* against *E. coli.* Root extracts of *Combretum padoides* and of *C. molle* gave the most outstanding antifungal effects of all the investigated extracts, whereas some other extracts, such as a leaf extracts of *C. fragrans* and *C. molle* showed only slight or no activity against the fungal species used (Fyhrquist *et al*., 2002). Arses *et al.* (2001) investigated the antimicrobial activity of the bark extract of *C. molle* against Gram positive and Gram negative bacteria. The acetone fraction exhibited a powerful activity and was therefore further tested against twenty-one bacterial and six fungal strains. The highest antibacterial activity of the acetone fraction was against the Gram negative organisms such as *E. coli* and *Shigella dyesentriae* with an MIC value of 50 mg/ml. Among the fungal strains tested *Candida albicans* showed high susceptibility to the acetone extract and growth was completely inhibited at a

concentration of 400 mg/ml. The antimicrobial activity of the extract was attributed to the high amount of hydrolysable tannins present in the bark of the plant (Arses *et al.*, 2001).

2.2 Secondary plant metabolites

Higher plants produce a great diversity of chemicals that have antimicrobial activity *in vitro* (Mason and Wasserman, 1987). Most of these secondary metabolites are defense molecules, of which at least 12 000 have been isolated (Cantrell *et al.*, 2001). Secondary metabolites are chemicals produced by plants for which no role has yet been found in growth, photosynthesis, reproduction, or other "primary" functions (Cushnie, 2005). These chemicals are extremely diverse; many thousands have been identified in several major classes. Each plant family, genus, and species produces a characteristic mix of these chemicals, and they can sometimes be used as taxonomic characters in classifying plants. Humans use some of these compounds as medicines, flavourings, or recreational drugs (Spencer, 2008).

Secondary metabolites can be classified on the basis of chemical structure (for example, sugar moiety), composition (containing nitrogen or not), their solubility in various solvents (Tsushida and Suzuki, 1996), or the pathway by which they are synthesized (e.g., phenylpropanoid, which produces tannins). A simple classification includes three main groups: the terpenes (made from mevalonic acid, composed almost entirely of carbon and hydrogen), phenolics (made from simple sugars, containing benzene rings, hydrogen, and oxygen), and nitrogen-containing compounds (Mangan, 2004).

2.2.1 Phenolics

Simple phenolics, such as caffeic acid and cinnamic acid are known to possess antimicrobial properties (Cowan, 1999). Catechol and pyrogallol are hydroxylated phenols shown to be toxic against microorganisms. Increased hydroxylation of the phenol group has been found to result in increased toxicity to microorganisms (Cowan, 1999). On the contrary, it has been found that highly oxidized phenolics are more inhibitory (Haslam, 2003). Phenolic compounds are thought to inhibit microbial enzymes possibly through reaction with sulfhydryl groups (the oxidized phenols) or through nonspecific interactions with the protein synthesis as a result of infection more in infected parts of plant (Mason and Wasserman, 1987).

2.2.2 Quinones

The potential range of quinone antimicrobial effects seems to be great. Probable targets for the quinones in the microbial cell are the surface –exposed adhesins, cell wall polypeptides and 37 enzymes bound to the membranes. Quinones are known to be complex irreversible with nucleophilic amino acids in proteins (Cowan, 1999) thus leading to inactivation of the protein and loss of its function. Derivatives of anthraquinone include many important drugs (collectively called anthracenediones). They include laxatives such as dantron, emodin, and aloe emodin, and some of the senna glycosides antimalarials such as rufigallol antineoplastics used in the treatment of cancer, such as mitoxantrone, pixantrone, and the anthracyclines (James, 2008). Anthraquinone is used as a bird repellant on seeds and as a gas generator in satellite balloons. Natural anthraquinone derivatives tend to have laxative effects (Foerster, 2006)

2.2.3 Flavonoids

Flavonoids are constitutive compounds but are also synthesized by plants in response to microbial infection. Nearly half of the 200 phytoalexins characterized up to now belong to the flavonoids (Harborne, 1999). Flavonoids have been found to show *in vitro* antimicrobial activity against a wide range of microorganisms, some showing potent activity against Methicillin resistant *Staphylococcus aureus* (Iinuma *et al*., 1994). Their activity has been attributed to their ability to form a complex with extracellular and soluble proteins and to complex with bacterial cell walls (Cushnie, 2005). Lipophilic flavonoids may also disrupt microbial membranes (Tsushida and Suzuki, 1996). There are conflicting findings on the kind of molecular substitutions needed for a flavonoid in order to show antimicrobial activity. Some authors have found that, flavonoids lacking hydroxyl groups on their β-rings are more active against microorganisms than flavonoids containing these groups and this finding supports the idea that, their microbial target is the membrane (Chabot *et al*., 1992). Several authors have, however, also found the opposite effect; the more hydroxyl groups the greater antimicrobial activity. The low toxic potential of flavonoids makes them ideal as antimicrobial medicines (Cushnie, 2005).

2.2.4 Tannins

Tannins are a large group of polyphenolic compounds which have received attention in recent years due to their claimed ability to cure a variety of diseases (Chabot *et al*., 1992). Tannins are subdivided into two groups: *hydrolysable tannins* and *proantocyanidins* (condensed tannins). Hydrolysable tannins are gallic acid and ellagic acid esters of core molecules that consist of 38 polyols such as sugars. Proantohocyanidins are polymers of flavan-3-ols (for example catechin) and flavan-3,4-diols linked through an interflavan bond that is not susceptible to hydrolysis

(Kumar *et al*., 2005). A wide range of anti-infective actions have been assigned to tannins (Haslam, 2003). Tannins have the ability to form a complex with proteins through nonspecific forces such as hydrogen bonding and hydrophobic effects and also through covalent binding (Stern *et al.,* 1996). Latté and Kolodziej, (2000) found that a panel of different hydrolysable tannins had low antibacterial effects, but that they possessed fairly high anticryptococcal effects. Some research have been performed on the relationship between tannin structure and antimicrobial activity. The pattern of B-ring hydroxylation of monomeric flavonols in condensed tannins has been shown to affect the level of growth inhibition in *Streptococcus sobrinus* and *S. mutans* (Chabot *et al*., 1992).

The synthesis of red beet β- glucan synthase was found to be strongly inhibited by various oxidized phenols, but the effect of oxidation was less marked for tannic acid (hydrolysable tannin) than for smaller phenols (Izhaki, 2002). It has also been proposed that tannin toxicity would be related to molecular size, since the larger the molecule the more effectively it binds to proteins. Zhu *et al*. (1997) found that, some of the tannins inhibited ligand binding to specific receptors. The anti-inflammatory effect of tannins help control all indications of gastritis, esophagitis, enteritis and irritating bowel disorders. Tannins not only heal burns and stop bleeding, but they also stop infection while they continue to heal the wound internally (Wood, 2005). The ability of tannins to form a protective layer over the exposed tissue keeps the wound from being infected even more. Tannins can also be effective in protecting the kidneys. Tannins have been used for immediate relief of sore throats, diarrhea, dysentery, haemorrhaging, fatigue, skin ulcers and as a cicatrizant on gangrenous wounds (McAllan, 1992). Tannins can cause regression of tumors that are already present in tissue, but if used excessively over time, they can cause tumors in healthy tissue. Tannins are used indirectly as molluscides to interrupt the

transmission cycle of schistosomiasis. They have also been reported to have anti-viral effects. When incubated with red grape juice and red wines with a high content of condensed tannins, the poliovirus, herpes simplex virus, and various enteric viruses are inactivated (Bajaj,1988). Tannins are distributed in species throughout the plant kingdom. They are commonly found in both gymnosperms as well as angiosperms. Botanically, tannins are mainly physically located in the vacuoles or surface wax of plants. These storage sites keep tannins active against plant predators, but also keep some tannins from affecting plant metabolism while the plant tissue is alive; it is only after cell breakdown and death that the tannins are active in metabolic effects (Scalbert, 2002).

2.2.5 Terpenes

Terpenes are a large group of compounds responsible for the fragrance of plants and comprise the so called essential oil fraction. They are synthesized from isoprenoid units, and share origins with fatty acids. They differ from fatty acids in that they are branched and cyclic. Terpenes occur as monoterpenes, diterpenes, triterpenes, tetraterpenes, hemiterpenes and sesquiterpenes. When additional elements, such as oxygen, are added they are called terpenoids (Cowan, 1999). The mechanism of action of terpenes on microbes is not yet fully understood, but it is speculated to involve membrane disruption by the lipophilic compounds. Accordingly, Mendoza *et al*. (1997) found that, the addition of a methyl group on kaurene diterpenoids drastically reduced their antimicrobial activity. When different series of terpenoids were investigated for their antimicrobial effects it was found that, more lipophilic compounds were significantly more antibacterial than their more polar analogues (Cantrell *et al*., 2001).

2.2.6 Fatty acids

Fatty acids are a class of compounds containing a long hydrocarbon chain and a terminal carboxylate group (-COOH). They have the general structure $CH_3(CH_2)_nCOOH$. Fatty acids belong to a category of biological molecules called lipids, which are generally water-insoluble but highly soluble in organic solvents (Stryer, 1995).

Fatty acids function as fuel molecules and serve as components of many other classes of lipids, including triglycerides (commonly known as "fats") and phospholipids, which are important building blocks of biological membranes. Fatty acid derivatives also function as hormones and intracellular messengers.

Fatty acids can be either saturated or unsaturated, a distinction that has important consequences for their chemical properties as well as the properties of other lipids with fatty acid components:

- Saturated fatty acids have no double bonds between the carbon atoms of the fatty acid chain (as the carbons are fully "saturated" with hydrogen atoms)
- Unsaturated fatty acids have one or more double bonds between carbon atoms

Fatty acids are distinguished by two important characteristics: (1) chain length and (2) degree of unsaturation (Beermann *et al*., 2003). Fatty acid chains in naturally occurring triglycerides (fat) are typically unbranched and range from 14 to 24 carbon atoms, with 16- and 18-carbon lengths being the most common. Fatty acids found in plants and animals are usually composed of an even number of carbon atoms, because their biosynthesis in these organisms involves acetyl-CoA, a coenzyme carrying a two-carbon-atom group. Bacteria, however, possess the ability to synthesize odd- and branched-chain fatty acids. Consequently, ruminant animal fat, such as in

cattle, contains significant proportions of branched-chain fatty acids, due to the action of bacteria in the rumen.

Fatty acids with long chains are more susceptible to intermolecular forces of attraction (in this case, van der Waals forces), raising their melting point. Long chains also yield more energy per molecule when metabolized (Tsuji, 2005).

Saturated fatty acids

When each carbon atom in the chain is bonded to two hydrogen atoms, the fatty acid is said to be saturated. Saturated fatty acids do not contain any double bonds between carbon atoms, because the carbon molecules are "saturated" with hydrogen; that is, they are bonded to the maximum number of hydrogen atoms. Saturated fatty acids form straight chains and, as a result, can be packed together very tightly, allowing living organisms to store chemical energy very densely (Vijay and Morris, 2014).

Unsaturated fatty acids

Monounsaturated fatty acids contain one double bond near the middle of the chain, creating a "kink" in the chain. One of the carbon atoms, bonded to only one hydrogen atom, forms a double bond with a neigbhouring carbon atom.

Polyunsaturated fatty acids may contain between two and six double bonds, resulting in multiple "kinks." As the degree of unsaturation increases, the melting points of polyunsaturated fatty acids become lower.

The double bonds in unsaturated fatty acids may occur either in a *cis* or *trans* isomer, depending on the geometry of the double bond. In the *cis* conformation, the hydrogens are on the same side of the double bond, whereas in the *trans* conformation, they are on the opposite side. A *trans* double bond between carbon atoms makes the molecule less 'kinked' in comparison to fatty acids with *cis* double bonds (Ferre and Foufelle, 2007).

The differences in geometry between the various types of unsaturated fatty acids, as well as between saturated and unsaturated fatty acids, play an important role in the construction of biological structures. For example, the presence of short and/or unsaturated fatty-acid chains in phospholipids and glycolipids generally enhances the fluidity of biological membranes, which are barriers that differentiate the cell from its environment, or define compartments within the cell (called organelles). At reduced temperatures, some organisms may vary the type and relative amounts of fatty acids to maintain the flexibility of their membranes. These changes in membrane lipid components contribute to the survival of plants, bacteria and hibernating animals during winter (Zechner *et al*., 2005).

Triglyceride is the storage form of fatty acids

Fatty acids are also important components of another type of lipid, triglyceride (commonly known as *fat*), which consist of three fatty acid chains bonded to a glycerol backbone. A triglyceride is an ester of glycerol; i.e., a molecule formed from a condensation (water-releasing) reaction between the three hydroxyl (-OH) groups of glycerol and the carboxyl groups of the three fatty acid molecules.

Triglycerides play an important role in metabolism as highly concentrated energy stores; when metabolized, they yield more than twice as much energy as carbohydrates and proteins (approximately 9 kcal/g versus 4 kcal/g). Some plant species, such as avocados, olives and nuts, have substantial amounts of triglycerides in seeds or fruits that serve as energy reserves for the next generation (Breuer *et al.*, 1987).

2.2.6.1 Caprylic acid

Caprylic acid is the common name for the eight-carbon saturated fatty acid known by the systematic name octanoic acid. Its compounds are found naturally in the milk of various mammals and as a minor constituent of coconut oil and palm kernel oil. It is an oily liquid that is minimally soluble in water with a slightly unpleasant rancid-like smell and taste (Papamandjaris *et al*., 1998).

Uses of Caprylic acid

Caprylic acid is used as an antimicrobial pesticide and as a food contact surface sanitizer in commercial food handling establishments, on dairy equipment, food processing equipment, breweries, wineries and beverage processing plants (Jones, 2002). It is also used as disinfectant in health care facilities, schools/colleges, animal care/veterinary facilities, industrial facilities, office buildings, recreational facilities, retail and wholesale establishments, livestock premises, restaurants, and hotels/motels. In addition, caprylic acid is used as an algaecide, bactericide, and fungicide in nurseries, greenhouses, garden centers, and on ornamentals. Products containing caprylic acid are formulated as soluble concentrate/liquids and ready-to-use liquids (Beare-Rogers *et al*., 2001).

2.2.6.2 Lauric acid

Lauric acid or systematically dodecanoic acid, is a saturated fatty acid with a 12-carbon atom chain, thus falling into the medium chain fatty acids, is a white, powdery solid with a faint odor of baby oil or soap. The salts and esters of lauric acid are known as laurates. Lauric acid, as a component of triglycerides, comprises about half of the fatty acid content in coconut milk, coconut oil, laurel oil, and palm kernel oil (not to be confused with palm oil), Otherwise, it is relatively uncommon (Yang *et al*., 2009)

Uses of Dodecanoic acid

In vitro experiments have suggested that some fatty acids including lauric acid could be a useful component in a treatment for acne, but no clinical trials have yet been conducted to evaluate this potential benefit in humans. Lauric acid increases total serum cholesterol more than many other fatty acids. But most of the increase is attributable to an increase in high-density lipoprotein (HDL) (the "good" blood cholesterol). As a result, lauric acid has been characterized as having "a more favourable effect on total HDL cholesterol than any other fatty acid [examined], either saturated or unsaturated" (Mensink *et al*., 2003).

2.2.7 Antimicrobial effects of plant extracts

In traditional and alternative medicine it is common to use medicinal plants as such, without isolating the active ingredients from them. Using crude extracts might be a more important way to use medicinal plants than has been realized in Western medicine, since plants contain numerous secondary metabolites, and pathogens in nature interact with many chemicals simultaneously (Izhaki, 2002). The antimicrobial activity of crude ethanolic extracts of

Achyranthes bidentata Blume, *Belamcanda chinensis* (L.) DC., *Chelidonium majus* L., *Houttuynia cordata* Thunb., *Platycodon grandiflorum* (Jacq.)A.DC., *Rehmania glutinosa* (Gaertn) Steud., *Sanguisorba officinalis* L., *Schizandra chinensis* (Turcz.) Baill., *Tribulus terrestris* L., and *Tussilago farfara* L. used in traditional Chinese medicine were tested against five species of microorganisms: *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*. Of the 10 plants tested, 5 showed antimicrobial activity against one or more species of microorganisms. The results showed that, the extracts from *Sanguisorba officinalis*, *Tussilago farfara* (aerial part; rhizome), *Chelidonium majus* (root), *Tribulus terrestris* (aerial part) and *Schisandra chinensis* (leaves) possessed antimicrobial activity. Although, the plants differ significantly in their activities against the micro-organisms tested, more of the extracts showed antimicrobial activity against *B. cereus* and *S. aureus* than against *E. coli*, *P. aeruginosa* and *C. albicans* (Janovska, 2003).

Kamo *et al*. (2015) investigated the acute and subacute toxicity effects of hydro-alcoholic extract of *Terminalia mantaly* in rats. Haematological assessments as well as the body weights of the rats were measured. The hydro-alcoholic extract were administered orally at a single dose of 300, 2000 and 5000 mg/kg body weight to the rats and then observed individually 1 h post-administration, and at least once daily for 14 days. Subacute toxicity was evaluated after administering daily oral doses of 150, 300 and 600 mg/kg body weight, for 28 days to the rats. Haematological assessment as well as body weights of the rats were carried out. They observed that, the limit dose of 5000 mg/kg did not cause any mortality or signs of acute toxicity in the rats tested during the observation period. They concluded that, the hydro-alcoholic extract of *T. mantaly* is relatively safe when administered orally in rats (Kamo *et al*., 2015).

Doughari *et al*. (2007) investigated the aqueous and organic leaves extracts of *Balanites aegyptiaca* and *Moringa oleifera* traditionally used for the treatment of infectious disease were tested for their activity against *Salmonella* Typhi isolated from blood clot culture using the disc diffusion method. Extracts of *B. aegyptiaca* demonstrated higher activity (16 mm zone of inhibition) than those of *Moringa oleifera* (8 mm zone of inhibition) at 100 mg/ml. Of the three solvents used, ethanolic extracts of both plants demonstrated the highest activity, while the aqueous extracts showed the least activity at 100 mg/ml. The activities of these plant extracts were comparable to those of antibiotics, ciprofloxacin, cotrimoxazole and chloramphenicol, commonly used for treating typhoid fever (Doughari *et al*., 2007). The antibacterial activity appears to increase when extracts of the two plants were used in combination at 100 mg/ml each (18 mm zone of inhibition). Preliminary phytochemical screening showed that, both plant extracts contains saponins, tannins and phenols while only *M. oleifera* possesses alkaloids and *B. aegyptiaca* possesses anthraquinones. The antibacterial activities of the extracts on *S.* Typhi was reasonably stable when treated at 4, 30, 60 and 100^{o} C for one hour, however it reduces significantly when the pH was altered towards alkalinity (Doughari *et al*., 2007).

Pieter *et al*. (2008) investigated the organic and aqueous solvent extracts of *Arctotis auriculata* Jacq., *Eriocephalus africanus* L., *Felicia erigeroides* DC., and *Helichrysum crispum* (L.) D. Don, for selective antimicrobial activities. Organic extracts of *A. auriculata* and *H. crispum* inhibited the growth of *Mycobacterium smegmatis*. The same extracts, together with organic extracts of *F. erigeroides*, were active against *Pseudomonas aeruginosa*. Antifungal activities against *Candida albicans* were exhibited by organic extracts of *E. africanus*, *F. erigeroides*, and *H. crispum*. Organic extracts of *A. auriculata* and *E. africanus*, as well as the

aqueous extract of the latter plant, were active against *Staphyllococcus aureus* (Pieter *et al*., 2008).

Extracts from 50 plant parts obtained from 39 different plants belonging to 22 families used to treat infectious diseases in Bunda district, Tanzania, were screened against twelve microorganisms, including the bacteria *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Salmonella* Typhi, the fungi *Aspergillus niger*, *Candida albicans*, and the viruses *Herpes simplex* Virus type 1, *Vesicular stomatitis* Virus T2, *Coxsackie* B2 and *Semliki Forest* A7 (Salie *et al.*, 1996). The highest activity was obtained for the *n*-hexane extract of *Elaeodendron schlechteranum* root bark against the Gram-positive bacteria *Bacillus cereus* (MIC 0.97 μg/ml and MBC 1.95 μg/ml) and *Staphylococcus aureus* (MIC 3.90 μg/ml and MBC 31.25 μg/ml). Gram-negative bacteria were less sensitive. Only *Balanites aegyptiaca* stem bark exhibited a high antifungal activity against *Candida albicans* (MIC 125 μg/ml and MFC 250 μg/ml). Extracts from four plants; *Lannea schweinfurthii*, *Combretum adenogonium*, *Ficus sycomorus* and *Terminalia mollis* showed strong antiviral activity with RF values of 10^3 and 10^4 against *Herpes simplex* Virus type 1 at various concentrations. The results support, at least in part, the use of most plants as claimed by traditional healers/informants especially against the Gram-positive bacteria *Bacillus cereus* and *Staphylococcus aureus* (Salie *et al.*, 1996).

Kubmarawa *et al.* (2009) evaluated the ethanolic and water extracts of *Ficus platyphylla.* The plant materials were screened for their phytochemical and antimicrobial activity against *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus spp*. and *Salmonella spp*. The results indicated that, saponins, tannins, and volatile oils were present

while flavonoids, glycosides, alkaloids, and resin were absent. The roots and stem-bark of the ethanol extract was active on most of the pathogens. But for *Staphylococcus aureus,* all the other microbes developed resistance against the water extract. This attests to the fact that, *Ficus platyphylla* contains bioactive compounds of potentially therapeutic and prophylactic significance and thus could be a promissory candidate for drug development and validates folkloric claim, as a cure for tuberculosis and other bacterial and fungal infections (Kubmarawa *et al.,* 2009).

Oyewo *et al*. (2012) investigated the effects of aqueous extract of *Citrullus lanatus* on the histology of the kidney of adult Wistar rats. The *Citrullus lanatus* aqueous extract was administered as follows: Group A: 1.5 ml/kg, Group B: 3 ml/kg, Group C: 6 ml/kg and Group O: Control (Water). The aqueous extract of *Citrullus lanatus* was given once a day to the animals for 27 days. The results revealed that, aqueous extract of *Citrullus lanatus* on the histology of the kidney in all the groups were normal when compared with the control group. Group B (3 ml/kg) showed significant ($p<0.0001$) difference in weight gain when compared with the control. The evaluation of the possible effects of *Citrullus lanatus* on the kidney did not record any histological distortion of the kidney using H and E stain (Oyewo *et al*., 2012).

Adebayo and Ishola (2009) investigated the root, stem-bark and leaves extracts of *Terminalia glaucescens'* activity against some pathogenic organisms. Phytochemical screening revealed the presence of alkaloids, tannins, saponins, steroids, flavonoids, anthraquinones and phlobatannins (mostly in root and stem-bark). The results of *in vitro* antimicrobial screening of the crude methanol extract exhibited a wide range of activity on *Salmonella* Typhi, *Escherichia coli*, *Staphylococcus aureus, Proteus mirabilis* and *Candida albicans.* The crude leaf extracts inhibited the growth of *E. coli, S. aureus, S.* Typhi *and C. albicans* at a concentration of 50, 80,

40 and 60 mg/ml respectively, while the stem-bark extract had minimum inhibitory concentration (MIC) of 40 mg/ml on *K. pneumoniae, S. aureus, S.* Typhi and 30 mg/ml on *C. albicans.* The extract from the root inhibited the growth of *E. coli* at a concentration of 10 mg/ml while *K. pneumoniae, P. mirabilis* and *C. albicans* were inhibited at a concentration of 60 mg/ml. They concluded that, the extracts from *T. glaucescens,* contained bioactive components that have antimicrobial properties (Adebayo and Ishola, 2009).

Misrak *et al*. (2013) investigated the potency of mustard and ginger rhizome extracts, lemon juice, Atella (residue of traditional Ethiopian beer) and cow urine in controlling tomato seed borne pathogen, *Xanthomonas campestris* pv. *vesicatoria* (Xcv) was evaluated. Artificially inoculated seeds were treated by dipping for 30 min, 3 h, 12 h or 24 h. Streptomycin (0.5%), sodium hypochlorides (1%) and control treatments were similarly applied. The test organism was isolated from tomato fruit collected from central Rift Valley of Ethiopia; it belonged to T1P2 race group of Xcv. Tomato seeds were inoculated with Xcv suspensions, dried and dipped at room temperature in the treatments. Atella at all treatment durations completely inactivated (0 cfu/ml) Xcv from inoculated tomato seeds and induced 92 to 98% seed germination. Ginger and mustard extracts showed 0 cfu/ml on Xcv with 24 h soaking, 97 to 81% germination and 2303 to 2270 vigour indexes of tomato seed. A promising bactericidal effect of Atella is an excellent clue of using other traditional alcoholic drink residues in controlling Xcv on tomato seeds for small scale farming (Misrak *et al*., 2013).

Similarly, the *in vitro* antimicrobial activity of crude ethanolic, methanolic and water extracts of the stem bark of *Jatropha curcas* were investigated. The extracts exhibited antimicrobial activities with zones of inhibition ranging from 5 to 12, 8 to 20 and 0 to 8 mm for ethanol, methanol and water extracts respectively. The minimum inhibitory concentration (MIC) of the

ethanol extract was between 0.5 and 6.25 mg/ml while that of methanol extract ranged from 0.5 to 10 mg/ml. The minimum bactericidal concentration (MBC) for ethanol extract ranged between 2.0 and 12.50 mg/ml, while that of methanol ranged from 2.0 to 20 mg/ml. Again, all the extracts exhibited appreciable activity against all the fungal species investigated. The zones of inhibition exhibited by the extracts against the test fungal species ranged between 15 and 18, 15 and 20 and 5 and 10 mm for ethanol, methanol and water extracts respectively. Phytochemical screening revealed the presence of saponins, steroids, tannins, glycosides, alkaloids and flavonoids in the extracts. The ability of the crude stem extracts of *J. curcas* to inhibit the growth of bacteria and fungi is an indication of its broad spectrum antimicrobial potential which may be employed in the management of microbial infections (Igbinosa *et al*., 2009).

Ernest *et al*. (2012) evaluated crude extracts from 84 plant species *in vitro* and *in planta* assays for antibacterial activity against bacterial leaf spot of tomato. In the *in vitro* assays, 20.2 % of the tested plant extracts totally inhibited growth of bacteria when seed washings from treated seeds were plated on nutrient agar medium. In the *in planta* assays, 17.8 % of the tested plant extracts reduced BLS incidence by 100 % in tomato seedlings. Extracts from *A. vera, C. arabica* and *Y. schidigera* were tested three times using tomato seeds of cultivars Tanya, Cal-J and Moneymaker in Tanzania. Treatment of tomato seeds with these extracts had a positive effect on the number of normal seeds and had no effect on seedling vigor, height and weight. These results indicate that plant extracts from *A. vera*, *C. arabica* and *Y. schidigera* are potential candidates for seed treatment against seed-borne xanthomonas of tomato in Tanzania.

Nandagopalan *et al*. (2015) studied the GC-MS profiles of *Grewia emarginata*. The study was carried out to determine the possible phyto-compounds of *Grewia emarginata* (Tiliaceae) using gc-ms analysis. Sixteen compounds were detected from the investigation. The prevailing

compounds in the methanol extract of *Grewia emarginata* were 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (14.76%), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (10.80%), Alpha-monopropionin (9.83%), Sucrose (8.52%), All-trans-Squalene (8.38%), n Hexadecanoic acid (7.64%), Oxazole, 5-hexyl-2,4-dimethyl- (6.10%), N-Methoxy-N-methylacetamide (5.47%) and 2-Methyl-l methylmannopyranoside (5.30%). The presence of some of these phyto-compounds corresponds to varied medicinal properties that can be exploited for the treatment of various diseases (Nandagopalan *et al*., 2015).

Kalimuthu and Prabakaran (2013) studied the preliminary phytochemical screening and gc-ms analysis of methanol extract of *Ceropegia pusilla*. Qualitative phytochemical analysis of these plants confirm the presence of various secondary metabolites like saponins, triterpenoids, steroids, tannins, lignins, alkaloids, glycosides, flavonoids and phenols. The results suggest that, the plant has phytochemical properties for curing various ailments and possess potential anti-inflammatory, antimicrobial and antioxidant. This can lead to the isolation of new and novel compounds. Gc-ms analysis showed the existence of various compounds with different chemical structures (Kalimuthu and Prabakaran, 2013).

Saradhai *et al*. (2015) carried out the gc-ms analysis of methanolic extract of *Phyllanthus amarus* leaves collected from Salem region. Methanolic extract of *P. amarus* from Salem region was screened for the presence of several bioactive compounds. It was evaluated using gc-ms analysis. The methanolic extract revealed the presence of 17 medicinally important bioactive compounds among those 9,12,15- Octadecatrienoic acid showed highest peak of 9.10 %, followed by gamma tocopherol - 6.04 %, oxozalone - 3.59 %, n hexadecanoic acid - 2.59 %, Octadecanoic acid - 1.92 %, 9,17- Octadecadienal (Z) - 0.43 % and Phytol - 0.61 % identified during analysis (Saradhai *et al*., 2015).

Andrew and Keelan (2013) studied the antibacterial activity of long-chain polyunsaturated fatty acids against *Propionibacterium acnes* and *Staphylococcus aureus*. The study aimed to investigate the antimicrobial effects of six LC-PUFAs against *P. acnes* and *S. aureus*. Minimum inhibitory concentrations were determined against *P. acnes* and *S. aureus*, and the LC PUFAs were found to inhibit bacterial growth at 32–1024 mg/L. Generally, *P. acnes* was more susceptible to the growth inhibitory actions of LC-PUFAs, but these compounds were bactericidal only for *S. aureus*. This is the first report of antibacterial activity attributed to 15 hydroxyeicosapentaenoic acid (15-OHEPA) and 15-hydroxyeicosatrienoic acid (HETrE), while the anti-*P. acnes* effects of the six LC-PUFAs used herein are novel observations. During exposure to the LC-PUFAs, *S. aureus* cells were killed within 15–30 min. Checkerboard assays demonstrated that, the LC-PUFAs did not antagonise the antimicrobial potency of clinical agents used presently against *P. acnes* and *S. aureus* (Andrew and Keelan, 2013).

Shettima *et al*. (2013) investigated the Gas Chromatography–Mass Spectrometry (GC-MS) analysis of bioactive components of ethyl acetate root extract of *Guiera senegalensis*. The methanol and ethyl acetate root extracts of *Guiera senegalensis* have been shown to be effective against diarrhea and also have antibacterial activity. The plant was therefore investigated for its bioactive components. The ethyl acetate root extract was investigated using Gas Chromatography-Mass Spectrometry (GC-MS) analysis. Nine components were identified, N-Hexadecanoic acid (46.6 %) as the major component followed by 9-Hexadecenoic acid (20.93 %), methyl ester (7.75 %), 7- Octadecenoic acid- methyl ester, 1, 2-benzene dicarboxylic acid – diisoctyl ester having (6.97 %) respectively; 2–pentanone - 4–hydroxy–4–methyl acid diethyl phthalate (2.32 %), Decane–6–ethyl–2–methyl and nonane, 3–7–dimethyl with (1.55 %) compositions (Shettima *et al*., 2013).

Sermakkani and Thangapandian, (2012) investigated the Gc-ms analysis of *Cassia italica* leaf methanol extract. The methanol leaf extract of *Cassia italica* was analyzed using Gas Chromatography–Mass Spectrometry, while the mass spectra of the compounds found in the extract was matched with the National Institute of Standards and Technology (NIST) library. Gas chromatography mass spectrometry (GC-MS) analysis revealed the presence of 17 compounds. In GC-MS analysis, some of the phytocomponents screened were Phytol, Squalene and n-Hexadecanoic acid. The compounds were identified by comparing their retention time and peak area with that of literature and by interpretation of mass spectra. Many of them are used in industry for various applications like flavor, antioxidant, anti-inflammatory, antimicrobial, pesticide and cancer preventive (Sermakkani and Thangapandian, 2012).

Kitahara *et al*. (2004) studied the antimicrobial activity of saturated fatty acids and fatty amines against methicillin-resistant *Staphylococcus aureus*. The antimicrobial activity of saturated fatty acids and fatty amines was determined by oxygen meters with multi-channels and disposable oxygen electrode sensors (DOX-96). Lauric acid, the most effective among the saturated fatty acids, showed antimicrobial activity at 400 microg/ml against methicillin-susceptible *Staphylococcus aureus* (MSSA) and MRSA. The minimal inhibitory concentration (MIC) of fatty amines depended on each hydrophobic chain length. The MIC of myristylamine was 1.56 microg/ml; most effective of the fatty amines. In time-kill curves, lauric acid and myristyl amine produced a bactericidal effect and a bacteriostatic effect at 4-fold the MIC (Kitahara *et al*., 2004).

Fischer *et al*. (2012) studied the antibacterial activity of sphingoid bases and fatty acids against gram-positive and gram-negative bacteria. They investigated the antibacterial activity, determined MICs and minimum bactericidal concentrations (MBCs) of lipids common to the

skin and oral cavity. The sphingoid bases, D-sphingosine, phytosphingosine, and dihydrosphingosine and the fatty acids sapienic acid and lauric acid--against four gram-negative bacteria and seven gram-positive bacteria. Exact Kruskal-Wallis tests of these values showed differences among lipid treatments ($P < 0.0001$) for each bacterial species except *Serratia marcescens* and *Pseudomonas aeruginosa*. D-sphingosine (MBC range, 0.3 to 19.6 μg/ml), dihydrosphingosine (MBC range, 0.6 to 39.1 μg/ml), and phytosphingosine (MBC range, 3.3 to 62.5 μg/ml) were active against all bacteria except *S. marcescens and P. aeruginosa* (MBC > 500 μg/ml). Sapienic acid (MBC range, 31.3 to 375.0 μg/ml) was active against *Streptococcus sanguinis, Streptococcus mitis,* and *Fusobacterium nucleatum* but not active against *Escherichia coli, Staphylococcus aureus, S. marcescens, P. aeruginosa, Corynebacterium bovis, Corynebacterium striatum,* and *Corynebacterium jeikeium* (MBC > 500 μg/ml). Lauric acid (MBC range, 6.8 to 375.0 μg/ml) was active against all bacteria except *E. coli, S. marcescens, and P. aeruginosa* (MBC > 500 μg/ml). Complete killing was achieved as early as 0.5 h for some lipids but took as long as 24 h for others. Hence, sphingoid bases and fatty acids have different antibacterial activities and may have potential for prophylactic or therapeutic intervention in infection (Fischer *et al*., 2012).

McGaw *et al*. (2002) carried out isolation of antibacterial fatty acids from *Schotia brachypetala* . Activity-directed fractionation of the ethanol extract of the dried leaves yielded 9,12,15-octadecatrienoic (linolenic) acid and methyl-5,11,14,17-eicosatetraenoate. These fatty acids displayed antibacterial activity against the gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* and to a lesser extent, against the gram-negative *Escherichia coli* and *Klebsiella pneumoniae (*McGaw *et al*., 2002).

Muthezilan *et al.* (2012) studied the fatty acid composition and antimicrobial activity of *Solanum torvum*. The chloroform-methanol (2:1, v/v) extracts of stem, leaf, pericarp and root of *Solanum torvum*, which were collected from Medicinal Plant Garden, Ragav Endra Medical Institute of Electropathy, Erode, Tamil Nadu, India. Extraction was by Soxhlet apparatus. The fatty acids obtained from plant extracts were esterified and subjected to detailed GC-MS analysis. The plant extracts from the stem, leaf, pericarp and root contained unsaturated fatty acids such as linoleic acid, oleic acid and saturated fatty acids such as palmitic acid, stearic acid, richinoleic acid, ligniceric acid, lauric acid, myristic acid and behanic acid. The antimicrobial activities of the ethanolic extracts of those samples were determined against ten bacteria and nine fungi. Among the tested bacterial strains, *Bacillus subtilis, Klebsiella pneumoniae, Staphylococcus aureus* and *Micrococcus sp* showed maximum antibacterial activity to all the tested extracts of *Solanum torvum* (Muthezilan *et al.*, 2012).

David *et al*. (2015) studied the toxicological evaluation of aqueous and acetone extracts of *Combretum molle* twigs in Wistar rats. In acute toxicity test, female rats were divided into two groups and treated orally with distilled water (10 mL/kg) and single dose of aqueous extract (2000 mg/kg). Animals were observed for 14 days, during which toxic signs, mortality and body weight were evaluated. Results showed that, the acute toxicity study did not produce any grossly toxic signs, mortality and body weight changes. In conclusion, the aqueous extract did not exhibit acute toxicity, it appears to be relatively non-toxic one.

Nishaa *et al*. (2013) investigated the ethanolic extract of rhizomes of *Maranta arundinacea*. The rhizomes were extracted using maceration method based on the polarity viz., petroleum ether, chloroform, ethyl acetate and ethanol. The ethanolic extract showed the presence of phytochemicals. The GC-MS analysis of the ethanolic extract revealed the presence of 49

compounds. This study forms a basis for the biological characterization and importance of the compounds identified.

Similarly, Dodehe *et al*. (2012) studied the acute and sub acute toxic study of aqueous leaf extract of *Combretum molle* in Wistar rats. The animals were orally administered with doses ranging from 2000 to 8000 mg/kg and observed continuously for the first 4 h, then hourly for the next 24 h, and finally, 6-hourly for 72 h. Control animals received orally normal saline. The rats were observed carefully for mortality, pain as well as respiratory movements. At the end of the treatment period, the animals were sacrificed and their organs (liver, heart and kidney) removed for macroscopic examination. For the acute toxicity test, no death and signs of poisoning were observed in the treated groups. Histological examination of vital organs showed normal architecture suggesting no morphological abnormalities in the heart, kidney and liver (Dodehe *et al*., 2012).

2.3 TEST MICROBES

2.3.1 *Dickeya didantii*

2.3.1.1 Classification

Dickeya didantii formerly known as *Erwinia chrysanthemi* is of the Kingdom: Bacteria, Phylum: Proteobacteria, Class: Gammaproteobacteria, Order: Enterobacteriales, Family: Enterobacteriaceae, Genus: *Dickeya*, Species: *D. didantii*.

2.2.1.2 Biology

Dickeya didantii is a gram-negative bacillus that belongs to the family *Enterobacteriaceae*. It was formerly known as *Erwinia chrysanthemi* but was reassigned as *Dickeya didantii* in 2005 (Samson *et al*., 2005). Members of this family are facultative anaerobes, able to ferment sugars to lactic acid, have nitrate reductase, but lack oxidases. Even though many clinical pathogens are part of the *Enterobacteriaceae* family, most members of this family are plant pathogens. *Dickeya didantii* is a motile, nonsporing, straight rod-shaped cell with rounded ends. Cells range in size from 0.8 to 3.2 μm by 0.5 to 0.8 μm and are surrounded by numerous flagella (peritrichous) (Grenier, 2006).

In the natural plant environment, *Dickeya didantii* causes plant maladies such as necrosis, blight and "soft rot," which is a progressive tissue maceration (Komatsu *et al*., 2012). *Dickeya didantii* contains many pectinases that are able to macerate and break down the plant cell wall material. This exposed part of the plant releases nutrients that can facilitate bacterial growth. Commonly infected plants include potato tubers, bulbs of vegetables, and ornamental crops. *Dickeya didantii* causes disease on several different ornamental and horticultural host plants throughout the world including: tropical, subtropical and temperate climates. The host range of *Dickeya didantii* continues growing as new susceptible species are continuously being documented (Slawiak *et al*., 2009). It has also been found in soils, rivers and irrigation water. Host specificity is not yet fully understood. Originally pathovar groups were documented according to the hosts from which they were isolated. Today 50+ species have been identified and more are possible if another classification system based on biovars were to be used. Disease is most often reported on

bananas, carnations, and chrysanthemums, but the list of host species is quite vast (Barras *et al*., 1994).

Dickeya didantii is phytopathogenic bacterium causing soft rot diseases on many host plants including some which are economically important. *Dickeya didantii*, more commonly known as: soft rot, brown rot or blackleg, causes characteristic symptoms associated with other bacterial wilts, causing final diagnosis to be difficult. The pathogen primarily seeks to attack the plant's xylem vessels located in leaves, stems, blossoms and storage organs of herbaceous plants (Cother and Gilbert, 1990). *D. didantii* is able to infect hosts at any point in its life cycle. In addition to symptoms of wilt, the disease appears as sunken and cracked external lesions also having a brown interior in cross section in subterranean bulbs and tuber. Diseased plants will display a variety of symptoms including: wilting, stunting and vascular discolouration of the stems. Early symptoms include water soaked lesions at the site of infection, gradually expanding chlorotic leaves and loss of turgor in tissues (Robert *et al.*, 2000). The intensity of *Dickeya didantii* colonization relates to the amount of disease and degree of damage. The pathogen is very successful at infiltrating host tissues due to the many pectinases responsible for disassembly of plant cell wall polysaccharides. Once the cell wall is degraded cellular structure collapses and this cell maceration gives a characteristic "water-soaked" or rotted appearance (Komatsu *et al*., 2012). *Dickeya didantii* grow intercellularly, continuing to degrade cells and colonize, until it eventually reaches xylem tissues. Upon reaching the xylem vessels, *Dickeya didantii* possesses the ability to spread to new regions of the host and other areas may begin to display symptoms. Colonization within the xylem restricts flow of water causing loss of turgor pressure and wilting of foliage and stems. Restricted movement of important plant compounds eventually lead to death of the host (Ma *et al*., 2007).

2.3.2. *Klebsiella pneumoniae*

2.3.2.1 Classification

Klebsiella pneumoniae is of the Kingdom: Bacteria, Phylum: Proteobacteria, Class: Gamma Proteobacteria, Order: Enterobacteriales, Family: Enterobacteriaceae, Genus: *Klebsiella* and Species: *K. pneumoniae*.

2.3.2.2 Biology

Klebsiella pneumoniae is a gram-negative, non-motile, lactose fermenting, rod-shape organism. *Klebsiella pneumoniae* is able to grow either with or without free oxygen, deeming it a facultative anerobe. This organism is also surrounded by a capsule, which increases its virulence by acting as a physical barrier to evade the host's immune response (Ryan and Ray, 2004). This capsule also protects the cell from dessication. *Klebsiella pneumoniae* is a home-grown microorganism in that it is resides in the microbiota of humans. It can be found in the mouth, skin, and intestinal tract, where it initially does not cause disease. Although, found in the microbiota, *Klebsiella pneumoniae* can progress into severe bacterial infections leading to pneumonia, bloodstream infections, wound infections, urinary tract infections and meningitis (Podschun and Ullman, 1998).

Pathogenesis

Klebsiella infections is spread through exposure to the bacteria via respiratory tract, which causes pneumonia, or the blood to cause an infection in the bloodstream. *Klebsiella* infections are most well-known in hospitals spread through person-to-person contact by contaminated hands of people within the hospitals, whether it be an employee or a patient. *Klebsiella* is spread

very easily and rapidly, but not through the air. Healthcare settings are most vulnerable to *Klebsiella* infections due to the nature of procedures that allow easy access of bacteria into the body. Patients who are on ventilators, catheters, or surgery wounds are highly prone to catching this deadly infection (Postgate, 1998).

2.3.3 *Xanthomonas axonopodis*

2.3.3.1 Classification

Xanthomonas axonopodis is of the kingdom Bacteria, Phylum: Proteobacteria, Class: Gamma Proteobacteria, Order: Xanthomonales, Genus: *Xanthomonas* and Species: *X. axonopodis*

2.3.3.2 Biology

Xanthomonas axonopodis is a rod-shaped Gram-negative bacterium with polar flagella. The bacterium has a genome length around 5 megabase pairs. A number of types of *Citrus* canker diseases are caused by different pathovars and variants of the bacterium (Gottwald *et al.*,2002). *Xanthomonas axonopodis* are proteobacteria and as with all proteobacteria they have a gram negative wall consisting of an outer membrane made up of lipopolysaccharide (LPS) and a phospholipid inner membrane (Alegria *et al.*,2005).

Ecology

Xanthomonas axonopodis have a parasitic relationship exclusively in plants where they cause *Citrus* cankers on plants. They are most likely originated from southeastern Asia and spread to Japan, South Africa, Australia, the Pacific Islands, South America and the United States (Kim *et al.*,2006). Usually, trees that are infected with *Citrus* canker bacteria, such as the *Xanthomonas*

axonopodis, are burned to prevent the spread of infection because it is difficult to control their rapidly spreading nature. *Xanthomonas* are an agriculturally devastating bacteria. In 1933 they caused more than 6 million dollars damage in Florida alone when they destroyed about 258,000 grove trees and 3 million nursery trees that had become infected. In 1998, Sao Paulo, Brazil suffered from a *Citrus* canker outbreak where they had no choice but to destroy all infected trees as well as trees that were within a 30 meter radius of the infected trees (Lopes *et al.*, 2006).

Pathology

Xanthomonas are exclusively pathogenic to a large group of plants such as *Citrus* trees, rice, cotton, beans and grapes. They cause a highly contagious disease called *Citrus* canker. *Citrus* canker can destroy an entire crop field, but does not pose any danger to humans or animals. The infection has a wide range of effects which include: defoliation, dieback, severely blemished fruit, reduced fruit quality, and premature fruit drop (Graham *et al.*, 2004). *Citrus* canker bacteria can enter through a plant's stomata or through wounds on leaves or other green parts. In most cases, younger leaves are considered to be the most susceptible. Also, damage caused by *Citrus* leaf miner larvae (*Phyllocnistis citrella*) can be sites for infection to occur. Within a controlled laboratory setting, symptoms can appear in 14 days following inoculation into a susceptible host (Silva *et al.*, 2002). In the field environment, the time for symptoms to appear and be clearly discernible from other foliar diseases varies; it may be on the order of several months after infection. Lower temperatures increase the latency of the disease. *Citrus* canker bacteria can stay viable in old lesions and other plant surfaces for several months (Gottwald *et al.*,2002).

2.3.4 *Pseudomonas syringae*

2.3.4.1 Classification

Pseudomonas syringae is of the kingdom: Bacteria, phylum: Proteobacteria, Class: Gamma Proteobacteria, Order: Pseudomonales, Family: Pseudomonadaceae, Genus: *Pseudomonas*, Species: *P. syringae.*

2.3.4.2 Biology

Pseudomonas syringae is a rod-shaped, Gram-negative bacterium with polar flagella. As a plant pathogen, it can infect a wide range of species, and exists as over 50 different pathovars, all of which are available to researchers from international culture collections such as the National Collection of Plant Pathogenic Bacteria (NCPPB), International Collection of Microorganisms from Plants (ICMP), and others (Anzai *et al*., 2000). Whether these pathovars represent a single species is unclear (Anzai *et al*., 2000). *Pseudomonas syringae* is a member of the *Pseudomonas* genus, and based on 16S rRNA analysis, it has been placed in the *P. syringae* group. It is named after the lilac tree (*Syringa vulgaris*), from which it was first isolated (Maki, 1974).

Pseudomonas syringae also produces ice nucleation active (INA) proteins which cause water (in plants) to freeze at fairly high temperatures (-4 to -2°C), resulting in injury. Since the 1970s, *P. syringae* has been implicated as an atmospheric "biological ice nucleator", with airborne bacteria serving as cloud condensation nuclei (Hirano and Upper, 2000). Recent evidence has suggested that, the species plays a larger role than previously thought in producing rain and snow. They have also been found in the cores of hailstones, aiding in bioprecipitation (Hirano and Upper, 2000). *Pseudomonas syringae* pathogenesis is dependent on effector proteins

secreted into the plant cell by the bacterial type III secretion system. Nearly 60 different type III effector families encoded by *hop* genes have been identified in *Pseudomonas syringae* (Cody and Gross, 1987). Type III effectors contribute to pathogenesis chiefly through their role in suppressing plant defense. Owing to early availability of the genome sequence for three *P. syringae* strains and the ability of selected strains to cause disease on well-characterized host plants, including *Arabidopsis thaliana*, *Nicotiana benthamiana* and tomato (Kreig and Holt, 1987).

Ability to invade plants

Planktonic *P. syringae* is able to enter plants using its flagella and pilli to swim towards a target host. It enters the plant via wounds or natural opening sites, as it is not able to breach the plant cell wall. The role of taxis in *Pseudomonas syringae* has not been well-studied, but the bacteria are thought to use chemical signals released by the plant to find their host and cause infection (Ichinose *et al*., 2013). The pathogens also produce phytotoxins which injure the plant and can suppress the host immune system. One such phytotoxin is coronatine, found in pathovars *Pto* and *Pgl* (Ichinose *et al*., 2013).

Pseudomonas syringae produces polysaccharides which allow it to adhere to the surface of plant cells. It also releases quorum sensing molecules, which allows it to sense the presence of other bacterial cells nearby. If these molecules pass a threshold level, the bacteria change their pattern of gene expression to form a biofilm and begin expression virulence-related genes. The bacteria secrete highly viscous compounds such as polysaccharides and DNA to create a protective environment in which to grow (Fall and Paul, 1995).

2.3.5 *Salmonella enterica* Typhi

2.3.5.1 Classification

Salmonella enterica Typhi is of the Kingdom Eubacteria, Phylum: Proteobacteria, Class: Gamma Proteobacteria, Order: Enterobacteriales, Family: Enterobacteriaceae, Genus: *Salmonella*, Species: *S .enterica*

2.3.5.2 Biology

Salmonella enterica (formerly *Salmonella choleraesuis*), a rod shaped, flagellated, aerobic, Gram-negative bacterium, and a member of the genus *Salmonella* (Gianella, 1996). *Salmonella enterica* has an extraordinarily large number of serovars or strains—over 2000 have been described (Parkhill, 2001). The biomedically most relevant sub-species is called *Salmonella enterica*, whose following Serovars have special clinical significance in human disease: *Salmonella enterica Serovar* Typhi (historically elevated to species status as *S*. Typhi) is the causative of typhoid fever. The genome sequence of *Serovar* Typhi has already been established (Parkhill, 2001). *Salmonella enterica serovar* Typhimurium (also known as *S*. Typhimurium) can lead to a form of human gastroenteritis sometimes referred to as salmonellosis. *Salmonella enterica serovar para* Typhi A has been identified (Adkins, 2006). It is associated with paratyphoid fever, and sometimes known as *Salmonella* Typhi (McClelland, 2002).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection and preparation of plant materials

The *Combretum molle* plant leaves and roots were collected from neigbhouring communities in Samaru, Zaria (latitude 11.07^{o} N, longitude 7.73^{o} E and altitude 613 meters), Nigeria. These were brought and confirmed by a taxonomist with voucher number 900191 at the Herbarium unit of the Department of Biological Sciences, Ahmadu Bello University Zaria. The plant roots and leaves were air-dried for two weeks at room temperature 25^{o} C in the laboratory and then ground into powder.

3.1.1 Preparation of concentration of extracts

Approximately 1 g of each extract was dissolved in 5 mls of distilled water to yield 200 mg/ml. Then 1 ml of the 200 mg/ml was taken and added to 1 ml of distilled water to give a concentration of 100 mg/ml. After this 1 ml of the 100 mg/ml extract concentration was also taken and added to 1ml of distilled water to get a concentration of 50 mg/ml. The procedure was repeated twice to give concentrations of 25 mg/ml and 12.5 mg/ml. For better understanding each fraction was given a code so that identification will be easier during the antibacterial susceptibility testing: minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

3.1.2 Collection and preparation of test microorganisms

The stock cultures of the test microorganisms were obtained from the Department of Microbiology, Ahmadu Bello University, Zaria. Their validity was determined by sub culturing

onto nutrient agar and confirmed by standard cultural, morphological and biochemical techniques as described by Cowan and Steel (2004). The inocula of the test organisms were standardized by the method of Barry and Thormsberry (1991). This was done by suspending each test organism in 5 ml of nutrient broth and the turbidity was compared with that of 0.5 McFarland standard. McFarland standard was prepared by adding 0.6 ml of 1 % barium chloride ($BaCl_2$) to 99.4 ml of 1 % sulphuric acid (H_2S0_4) solution. The turbidity of the 0.5 McFarland standards was used for estimation of the number of bacteria in broth culture (culture for 24 hours at 37^0 C) to pour into 5 ml of distilled water in order to obtain a standard bacterial suspension of 1 x 10^5 cfu/ml (Bauer *et al.*, 2003).

3.2. Extraction procedures

The ground plant roots and leaves were extracted at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, following the methods of Sofowora (2006).

3.2.1 Preparation of Ethanol Extraction of *C. molle*

Approximately 800 g of the dried and powdered leaves and roots of *C. molle* were extracted with 10 liters of 80 % concentration ethanol by maceration at 25^o C for 3 days. The mixture was then strained and filtered. The filtrate was poured into a crucible on a water bath at 80^o C so as to obtain the dry extract after which was stored at -20º C for further studies.

3.2.2 Differential Fractionation of the Ethanol Extract of *C. molle* in Different Solvents

The dried ethanol extract obtained from both the roots and leaves of *C. molle* (50 g) were each suspended in 1 litre of distilled water and partitioned in sequence with ethyl acetate (1 litre), and

n-butanol (1 litre). The different solvent fractions were poured into a crucible on a water bath at 100^{o} C so as to obtain the dry extract after which was stored at -20^{o} C.

3.3 Phytochemical analysis of *Combretum molle* fractions

The method of Sofowora, (2006) was employed for the qualitative test for the presence of the phytochemical properties.

3.3.1 Test for glycosides

Five (5) mls of concentrated sulphuric acid was added to 0.5 g of the fraction and boiled for 15minutes. This was then cooled and neutralized with 20 % KOH and was divided into two portions. Another part of the fraction (0.5 g) was dissolved in distilled water; this was used as control (Sofowora, 2006). Fehling solutions A and B were added to this portion and boiled for five minutes. A brick red precipitate indicates the presence of glycone portion as a result of the glycoside hydrolysis (Sofowora, 2006).

3.3.2 Test for anthraquinones

3.3.2.1 *Borntrager's test*

Approximately 0.5 g of the fraction was mixed with 10 ml of benzene and filtered. 5 ml of 10 % ammonia solution was added to the filtrate and stirred. The production of a pink red or violet colour indicates the presence of free anthraquinones (Sofowora, 2006).

3.3.3 Test for saponins

3.3.3.1 *Frothing test*

Approximately 0.5 g of the fraction was dissolved in 10 mls of distilled water. This was then shaken vigorously for 30 seconds and was allowed to stand for 30 minutes. A honey comb formed for more than 30 minutes indicate saponin (Sofowora, 2006).

3.3.4 Test for flavonoids

3.3.4.1 *Shinoda test*

Approximately 0.5 g of the fraction was dissolved in 1-2 mls of 50 % methanol. The mixture was then heated to 60^{o} C for two minutes after which two tea-spoons metallic magnesium and 4 drops of concentrated hydrochloric acid were added. A red or orange colour indicates the presence of flavonoic aglycones (Sofowora, 2006).

3.3.4.2 *Sodium hydroxide test*

Few drops of aqueous sodium hydroxide were added to 5ml of the fraction, a yellow colouration shows the presence of flavonoids (Sofowora, 2006).

3.3.5 Test for tannins

3.3.5.1 *Ferric chloride test*

Approximately 0.5 g of the fraction was added to 10 ml of distilled water, and then filtered. Few drops of ferric chloride solution were added to the filtrate. Formation of blue-black precipitate indicates hydrolysable tannins and green precipitate indicates the presence of condensed tannins (Sofowora, 2006).

3.3.6 Test for alkaloids

3.3.6.1 *Dragendoff's test*

Four drops of this reagent were added to the fraction (0.5 g). A rose red precipitate indicates the presence of alkaloids (Sofowora, 2006).

3.3.7 Test for steroids and triterpenes

3.3.7.1 *Lieberman- Burchards test*

Approximately 1 ml of acetic anhydride was added to the fraction (0.5 g). Then 1 ml of concentrated sulphuric acid was also added through the side of the tube. The colour change was observed immediately and also after 10 minutes. If a red-pink or purple colour comes up immediately, that indicates the presence of triterpenes while, a change in color after 10 minutes to blue-green indicates steroids (Sofowora, 2006).

3.3.8 Thin layer chromatography screening

Qualitative screening of the constituents in each of the fraction of *C. molle* was conducted using Thin Layer Chromatography (TLC) analysis. 0.10 mls of each sample was loaded on the pre-coated TLC plate via the capillary tubes. The TLC chromatogram was developed from the mixture of different solvent systems namely: Chloroform/ethyl acetate/formic acid (CEF) in the ratio 5:4:1. The solvent was placed in the TLC tank after which the pre-coated plate was inserted for about an hour. For detection of chemical compounds in the extracts vanillin in 10 % sulphuric acid was used to spray the TLC plate. This revealed the different color pigments on the TLC plate which indicated the presence of phytochemical compounds.

3.3.9. Gas chromatography –mass spectrometry (GC-MS)

N-butanol fraction, ethyl acetate, and aqueous fractions from the stem and roots of *C. molle* was subjected to gas chromatography – mass spectrometry analysis, (GCMS-QP2010 PLUS SHIMADZI, JAPAN) Column of 0.25 diameter and 30 mm length was used at column oven temperature of 60^{o} C and an injection temperature of 250^{o} C and a pressure of 100.2 kPa, a column flow of 1.61 mL/min and a total flow of 6.2 ml/ min. Content identified was compared with the database of the NIST library. Compounds with close similarity index were identified and recorded.

3.4 Antibacterial susceptibility testing

The methods of Cowan and Steel (2004) were used. Standard aseptic microbiological methods were followed throughout this antibacterial study. McFarland standard was prepared by adding 0.5 ml of 1 % barium chloride ($BaCl_2$) to 99.5 ml of 1 % sulphuric acid (H_2S0_4) solution. The turbidity (density) of the 0.5 McFarland standards was used for estimation of the amount of bacteria in broth culture (culture for 24 hours at 37^{0} C) to pour into 5ml of distilled water in order to obtain a standard bacterial suspension of 1 x 10^{5} cfu/ml (Bauer *et al.,* 2003).

3.4.1 Well method for antibacterial activity

The Well method was employed to assay the plant fractions for antibacterial activity. Petri dishes were poured with nutrient agar and allowed for 30 minutes to solidify. This was done in duplicate for each fraction and test organism. The test organisms were then inoculated by spreading on the inocula on the surface of the medium using a sterile swab stick. A sterile Cork borer (size 3) was used to bore 4 wells in the medium. The different concentration of the plant

fractions were placed in the wells using a sterile syringe and needle (different for each sample and test organism). These were then allowed a diffusion time of 1 hour after which the plates were incubated at 37^{o} C for 24 hours. The positive control was Ciprofloxacin (100 µg/ml). The potency of the crude extracts was determined by the clear zones of inhibition around the wells and was respectively measured as the diameter zones of inhibition.

3.5 Minimum inhibitory concentration (MIC)

Nutrient broth was used as the medium of cultivation of the test organisms. Seven test tubes were selected. Each test tube contained 2 ml of double strength broth. About 2 ml of the fractions in the various concentrations was introduced into tube 1 and mixed thoroughly. approximately 2 ml of the content was then transferred into tube 2. The procedure was repeated for the remaining tubes up to tube 7. From tube 7 about 2 ml was discarded, with tube 8 containing no fraction (control). To all test tubes a drop (0.02 ml) of the inoculums (18 hr broth culture of the microorganism) was added. The tubes were then incubated overnight at 37 ^{0}C after which they were examined for turbidity (evidence of microbial growth). The growth of the organisms was observed as a change in turbidity. The minimum inhibitory concentration of the extracts is the lowest concentration that is capable of inhibiting the growth of a test organism. The test tube that shows no growth and had a clear solution represents the MIC. This procedure was carried out for all the organisms.

3.6 Minimum bactericidal concentration (MBC)

The minimum bactericidal concentration (MBC) was determined by the method of Rotimi *et al.* (1988). All the tubes from the MIC showing no growth were subsequently sub cultured on freshly prepared nutrient agar and incubated at 37^{0} C for 24 hrs. The MBC is regarded as the

lowest concentration of the extract that inhibited bacterial growth on the nutrient agar after incubation at 37^0 C for 24 hrs.

3.7 Toxicity studies

3.7.1 *Acute Oral Toxicological Evaluation of the fractions of C. molle*

This study was carried out according to the Organisation for Economic Cooperation and Development (OECD) guidelines (OECD, 2000). Acute oral toxicity refers to those adverse effects occurring following oral administration of a single dose of a substance, or multiple doses given within 24 hours. This is a measure of the interaction of induced substance with biomolecules after a single administration within fourteen days

3.7.2 *Limit Test at 5000 mg/kg for the Experimental Rats study*

The limit test is primarily used in situations where the researcher has information indicating that the test plant is likely to be non-toxic. Since there was prior information on the use of the test plant (Wickens, 2000) , therefore the limit test was used.

3.7.3 *Animals* **(**Wistar rats)

A total of 21 female albino rats of Wistar strain weighing about 230 – 280 g were obtained from the Animal house, Department of Pharmacology and Therapeutic, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, Nigeria. They were fed a standard rat pellet diet and water was provided and maintained under standard laboratory conditions (Temperature 21-24 °C, relative humidity 40 - 60 %).

3.7.4 *Grouping and Administration of plant fractions*

A total of 21 female albino Wistar rats were used for the acute toxicity study. The test substance was administered orally, in a single dose by gavage intubation cannula. The animals

were divided into 7 groups of 3 each. Group I rats were given ethyl-acetate root fraction. Group II rats were given ethyl-acetate leaf fraction. Group III rats were given n-butanol root fraction. Group IV rats were given n-butanol leaf fraction. Group V rats were given aqueous root fraction. Group VI rats were given aqueous leaf fraction. Group 7 served as normal healthy control The weight of experimental rats were measured and recorded on Days 1, 7 and 14 after the administration of the fraction. The histological parameters of the experimental animals were evaluated after 14 days. The Wistar rats were subjected to fasting (for food but not water over-night) prior to dosing. Following the period of fasting, they were weighed and the test substance administered. After the substance was administered, food (vital feed grower) was not given to the rats until after 3-4 hours. The plant fractions at fixed doses of 5000 mg/kg body weight were administered to 6 groups, each containing 3 rats which was done in two stages. A single rat each from the first 6 groups were administered the plant fractions (5000 mg/kg) and observed for 24 hours, after which all of them survived. The 2 remaining rats were also administered the dose and were observed during the first 30 minutes, periodically for 4 hours, then hourly the first 24 hours and daily thereafter, for a total of 14 days. All observations were systematically recorded, with individual records being maintained for each animal.

3.8 Histological studies of the kidneys and livers of Wistar rats

The experimental animals were sacrificed (chloroform as anesthesia) and the kidneys and livers were excised and taken to the Department of Human Anatomy, Faculty of Medical Sciences, Ahmadu Bello University, Zaria, Nigeria, for histological studies. The various organs were sliced and placed in embedded tissue baskets. Thereafter, they were fixed with 10 % formalin for 48 hours and afterwards dehydrated with methanol (70, 90 and 100 %) at different concentration in ascending concentration and different time in order to remove water from the tissues. Thereafter,

clearing with toluene was done to remove alcohol and prepare the tissue for waxing. Embedding was done using paraffin wax by impregnating cassettes with molten wax at 60^{o} C for 3 h. Slicing was done at 5 microns using a leica microtome (model no: RM2125RTS). The slide was dried for 20 min on hot plate. Afterwards, dewaxing and hydration were done using xylene and methanol (70, 90 and 100 %) at different concentration in ascending concentration and different time in order to remove water from the tissues. Thereafter, staining was done by placing the slide in cole's hematoxylin for 10 minutes, to stain the nucleus after which eosin was used to stain the cytoplasm for 3 min. Dehydration was once again carried out in alcohol and alcohol cleared with xylene. A mounting medium, dibutylphthalate xylene (DPX) was placed on the tissue section and they were viewed under the microscope.

3.9 Data analysis

One way Analysis of Variance (ANOVA) of was used to assess the efficacy of the plant parts in terms of the antibacterial activity as was shown by the zones of inhibition.

CHAPTER FOUR

RESULTS

4.1 Yield of extracts

The yield was 46 % of the root ethanol extract while that of the leaf ethanol extract was 51 %. Ethyl acetate root fraction yielded 9.2 %, *n*-butanol root fraction yielded 8.1 %, and from distilled water root fraction 28.6 % was obtained. Ethyl acetate leaf fraction yielded 6.7 %, *n*-butanol leaf fraction yielded 10.6 % and from distilled water leaf fraction 27.6 % was obtained (Table 4.1).

4.1.2 Phytochemical screening

The preliminary phytochemical screening of aqueous, n-butanol and ethyl-acetate fractions of the leaves and root *Combretum molle* are presented in Table 4.2. All the root fractions contained Tannins, Flavonoids, Steroids Triterpenes and Saponins. Glycosides were lacking in the aqueous and n-butanol fractions while alkaloids were lacking in n-butanol and ethyl-acetate fractions. All the leaf fractions contained Tannins, Flavonoids, Steroids Triterpenes and Saponins. Anthraquinones were not detected only in the n-butanol and ethyl- acetate fractions of the leaf.

Table 4.1. The yield of the fractions of *Combretum molle* after extraction

Plant fractions	Yield in percentage (%)
Root ethanol extract	26.7
Root n-butanol fraction	8.1
Root aqueous fraction	28.1
Root ethyl-acetate fraction	9.2
Leaf ethanol extract	31
Leaf n-butanol fraction	10.6
Leaf aqueous fraction	27.6
Leaf ethyl-acetate fraction	6.7

Table 4.2. Qualitative Phytochemical components of *Combretum molle* in different solvents fractions

Phytochemical	Root Aqueous	Root N-butanol	Root Ethyl-acetate	Leaf Aqueous	Leaf N-butanol	Leaf Ethyl-acetate
Tannins	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+
Glycoside	-	-	+	+	+	+
Alkaloids	+	-	-	+	+	+
Anthraquinones	+	+	-	+	-	-
Steroids	+	+	+	+	+	+
Triterpenes	+	+	+	+	+	+
Saponins	+	+	+	+	+	+

Key: + = Present, - = Absent.

4.2 Thin layer chromatography (TLC)

The chromatograms in Appendix 7, indicated different pigment of colours showing the presence of secondary metabolites. From the TLC chromatogram it was clear that all the fractions of *C. molle* tested contained secondary metabolites. More secondary compounds in the ethyl-acetate root (R2) and leaf (L2) fractions were observed, as seen from the colour pigments on the chromatograms (Appendix 7).

4.3 Antibacterial activities of *C. molle* fractions

The Ethyl-acetate root fraction showed high activity against *E. chrysanthemi, S.* Typhi and *X. axonopodis* at all concentrations, whereas *P. syringae* had zones of inhibition at 100 ml/ml and 50 mg/ml only. The positive control (Ciprofloxacin) performed better than the extracts in terms of zones of inhibition. The Ethyl-acetate root fraction however, did not inhibit the growth of *K. pneumoniae*. Results also showed that, the activities of the Ethyl-acetate root fraction against *X. axonopodis* and *E. chrysanthemi* was better than that of the other microorganisms tested (Table 4.3).

Table 4.3 Antibacterial activity of Ethyl-acetate root fraction of *Combretum molle* against selected test organisms.

Microorganism	Fraction concentration (mg/ml). Zone of inhibition (mm) (Mean±SE)					
	100	50	25	12.5	6.125	control
E. chrysanthemi	24±1.0	22±1.0	20±1.0	16±1.0	15±1.0	21±1.0
S. Typhi	18±0.5	16±1.0	14±1.0	12±1.0	11±1.0	31±1.0
K. pneumoniae	-	-	-	-	-	28±1.0
P. syringae	19±1.0	17±1.0	-	-	-	29±1.0
X. axonopodis	26±1.0	24±1.0	20±1.0	18±1.0	16±1.0	26±1.0

- = No activity, positive Control is Ciprofloxacin (100 µg/ml) for bacteria. Values of inhibition zones above 19 are susceptible, 15-19 is intermediate, while values less than 15 are resistant (CLSI,2014)

The Aqueous root fraction had high activity against *X. axonopodis* at concentrations of100 mg/ml and 50 mg/ml, whereas *E. chrysanthemi, S.* Typhi had zones of inhibition at concentrations of 100 mg/ml, 50mg/ml and 25 mg/ml. *Pseudomonas syringae* had zones of inhibition at concentrations of 100 mg/ml and 50 mg/ml only. Results also showed that the activities of the aqueous root fraction against *X. axonopodis* was the best with a high zone of inhibition of 27 mm. *Klebsiella pneumoniae* was not susceptible to the aqueous root fraction (Table 4.4).

The Ethyl-acetate leaf fraction showed high activity against *E. chrysanthemi, S.* Typhi, *P. syringae* and *X. axonopodis* at all concentrations, whereas *K. pneumoniae* had zones of inhibition at 100 mg/ml and 50 mg/ml only. The positive control performed better than the fractions. The Ethyl-acetate leaf fraction exhibited the highest activity against *X. axonopodis* with a zone of inhibition of 26 mm (Table 4.5).

The N-butanol root fraction showed high activity against *E. chrysanthemi, S.* Typhi and *P. syringae* at all concentrations, whereas *X. axonopodis* and *K. pneumoniae* had zones of inhibition at concentrations of 100 mg/ml and 50 mg/ml. The positive control performed better than the fractions. Results also showed that the activities of the N-butanol root fraction on *E. chrysanthemi, S.* Typhi was the same, but the performance was better than that observed on the other microorganisms (Table 4.6).

Table 4.4 Antibacterial activity of Aqueous root fraction of *Combretum molle* against selected test organisms.

Microorganism	Fraction concentration (mg/ml). Zone of inhibition (mm) (Mean±SE)					
	100	50	25	12.5	6.125	control
E. chrysanthemi	15±1.0	13±1.0	10±1.0	-	-	21±1.0
S. Typhi	20±0.5	17±1.0	10±1.0	-	-	31±1.0
K. pneumoniae	10±1.0	9±1.0	-	-	-	28±1.0
P. syringae	19±1.0	17±1.0	-	-	-	29±1.0
X. axonopodis	27±1.0	24±1.0	20±1.0	18±1.0	15±1.0	26±1.0

- = No activity, positive Control is Ciprofloxacin (100mg/ml) for bacteria. Values of inhibition zones above 19 are susceptible, 15-19 is intermediate, while values less than 15 are resistant (CLSI,2014)

Table 4.5 Antibacterial activity of ethyl-acetate leaf fraction of *Combretum molle* against selected test organisms.

Microorganism	Fraction concentration (mg/ml). Zone of inhibition (mm) (Mean±SE)					
	100	50	25	12.5	6.125	control
E. chrysanthemi	17±1.0	16±1.0	14±1.0	12±1.0	13±1.0	21±1.0
S. Typhi	20±0.5	18±1.0	16±1.0	14±1.0	12±1.0	31±1.0
K. pneumoniae	12±1.0	10±1.0	-	-	-	28±1.0
P. syringae	16±1.0	14±1.0	13±1.0	11±1.0	10±1.0	29±1.0
X. axonopodis	26±1.0	23±1.0	20±1.0	18±1.0	16±1.0	26±1.0

- = No activity, positive Control is Ciprofloxacin (100µg/ml) for bacteria. Values of inhibition zones above 19 are susceptible, 15-19 is intermediate, while values less than 15 are resistant (CLSI,2014)

Table 4.6 Antibacterial activity of n-butanol root fraction of *Combretum molle* against selected test organisms.

Microorganism	Fraction concentration (mg/ml). Zone of inhibition (mm) (Mean±SE)					
	100	50	25	12.5	6.125	control
E. chrysanthemi	18±1.0	16±1.0	14±1.0	12±1.0	-	21±1.0
S. Typhi	18±0.5	16±1.0	14±1.0	13±1.0	10±1.0	31±1.0
K. pneumoniae	14±1.0	12±1.0	-	-	-	28±1.0
P. syringae	18±1.0	16±1.0	13±1.0	11±1.0	10±1.0	29±1.0
X. axonopodis	19±1.0	17±1.0	-	-	-	26±1.0

- = No activity, positive Control is Ciprofloxacin (100mg/ml) for bacteria. Values of inhibition zones above 19 are susceptible, 15-19 is intermediate, while values less than 15 are resistant (CLSI,2014)

The N-butanol leaf fraction showed high activity against *E. chrysanthemi* and *S.* Typhi at all concentrations, and intermediate activity against *P. syringae* and *X. axonopodis* at concentrations of 100 mg/ml, 50 mg/ml and 25 mg/ml. The positive control performed better than the fractions in terms of zones of inhibition. The N-butanol leaf fraction however, did not inhibit the growth of *K. pneumoniae* Results also showed that the activities of the N-butanol leaf fraction against *E. chrysanthemi* was the highest, with high zone of inhibition of 28 mm (Table 4.7).

The Aqueous leaf fraction showed high activity against *E. chrysanthemi, S.* Typhi and *X. axonopodis* at all concentrations of 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml and 6.125mg/ml, whereas *K. pneumoniae* was resistant to the Aqueous leaf fraction. The positive control had higher activity than the fractions. The Aqueous leaf fraction however, had no activity against *K. pneumoniae* as seen in table 4.8. Results also showed that the activities of the Aqueous leaf fraction on *E. chrysanthemi, S.* Typhi were the same, but the highest activity was given by the action of the fraction against *X. axonopodis* with a zone of inhibition of 24 mm (Table 4.8).

4.4 ANALYSIS OF VARIANCE FOR ZONES OF INHIBITION BY EXTRACTS

Analysis of variance carried out to determine the significant effects in the performance of the extracts for the six different fractions indicated that for the treatment, there was no significant difference Similarly, there was no significant difference for interactions for all the extracts used in the study (Table 4.9).

Table 4.7 Antibacterial activity of n-butanol leaf fraction of *Combretum molle* against selected test organisms.

Microorganism	Fraction concentration (mg/ml). Zone of inhibition (mm) (Mean±SE)					
	100	50	25	12.5	6.125	control
E. chrysanthemi	28±1.0	25±1.0	24±1.0	23±1.0	19±1.0	21±1.0
S. Typhi	19±0.5	17±1.0	16±1.0	13±1.0	12±1.0	31±1.0
K. pneumoniae	12±1.0	10±1.0	-	-	-	28±1.0
P. syringae	16±1.0	14±1.0	11±1.0	-	-	29±1.0
X. axonopodis	20±1.0	17±1.0	13±1.0	-	-	26±1.0

- = No activity, positive Control is Ciprofloxacin (100mg/ml) for bacteria. Values of inhibition zones above 19 are susceptible, 15-19 is intermediate, while values less than 15 are resistant (CLSI,2014)

Table 4.8 Antibacterial activity of Aqueous leaf fraction of *Combretum molle* against selected test organisms.

Microorganism	Fraction concentration (mg/ml). Zone of inhibition (mm) (Mean±SE)					
	100	50	25	12.5	6.125	control
E. chrysanthemi	20±1.0	20±1.0	14±1.0	15±1.0	14±1.0	21±1.0
S. Typhi	22±0.5	19±1.0	18±1.0	13±1.0	13±1.0	31±1.0
K. pneumoniae	12±1.0	-	-	-	-	28±1.0
P. syringae	-	-	-	-	-	29±1.0
X. axonopodis	24±1.0	22±1.0	18±1.0	14±1.0	13±1.0	26±1.0

- = No activity, positive Control is Ciprofloxacin (100mg/ml) for bacteria. Values of inhibition zones above 19 are susceptible, 15-19 is intermediate, while values less than 15 are resistant (CLSI,2014)

Table 4.9 Analysis of variance for inhibition zones by Extracts

Source	Df	Leaf Aqueous	Leaf ethyl-acetate	Leaf n-butanol	Root aqueous	Root ethyl-acetate	Root n-butanol
Treatment	5	107.19	64.45	6.42	146.45	144.69	145.41
Error	24	40.18	52.65	15.15	60.40	37.06	64.95
Total	**29**	**147.37**	**117.10**	**2157**	**206.85**	**182.75**	**211.3**

No Significant difference at 95% ($p \geq 0.05$)

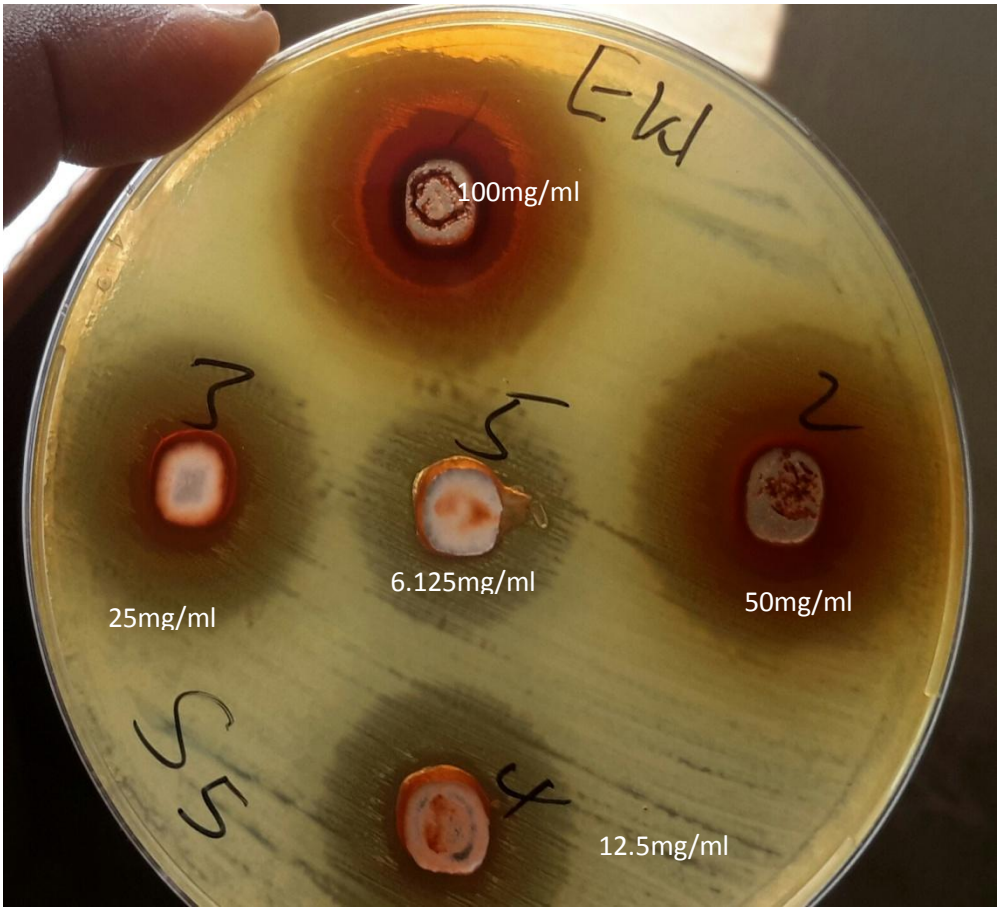


Plate I. The zones of inhibition of the extracts at different concentrations against *Erwinia chrysanthemi*.



Plate II. The zones of inhibition of the extracts at different concentrations against *Salmonella typhi*.

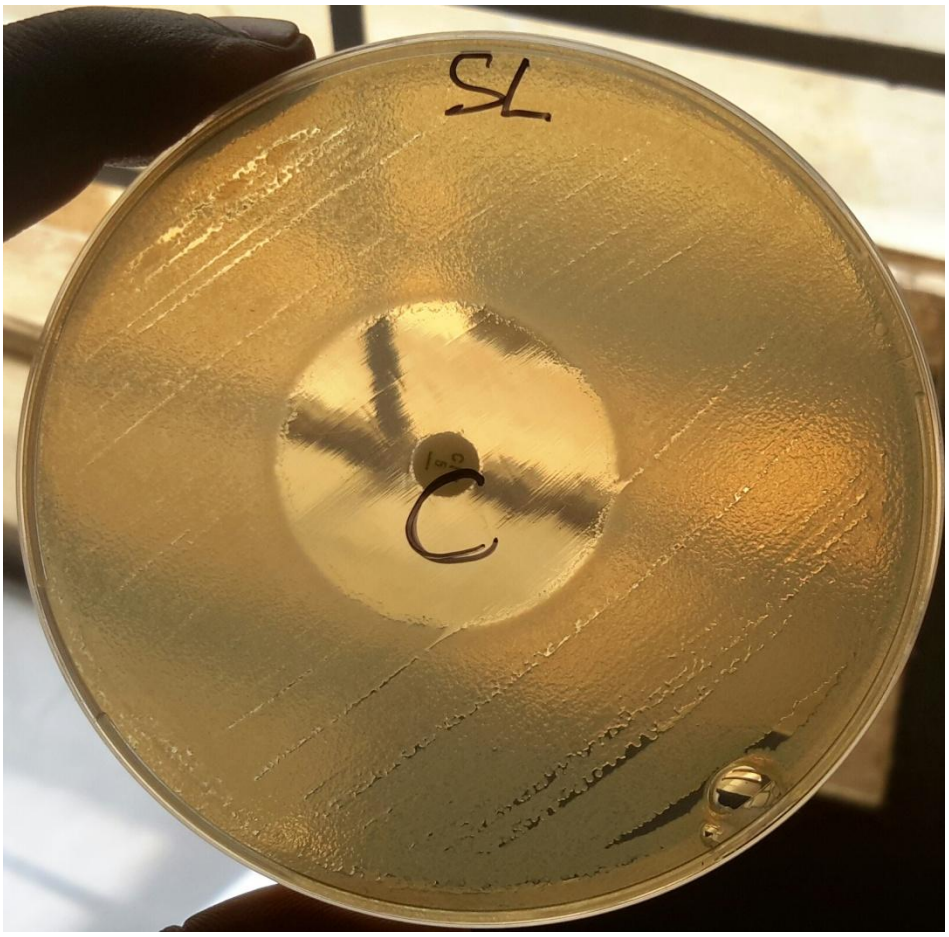


Plate III. Zone of inhibition of the control (ciprofloxacin) tested against *Salmonella typhi*.

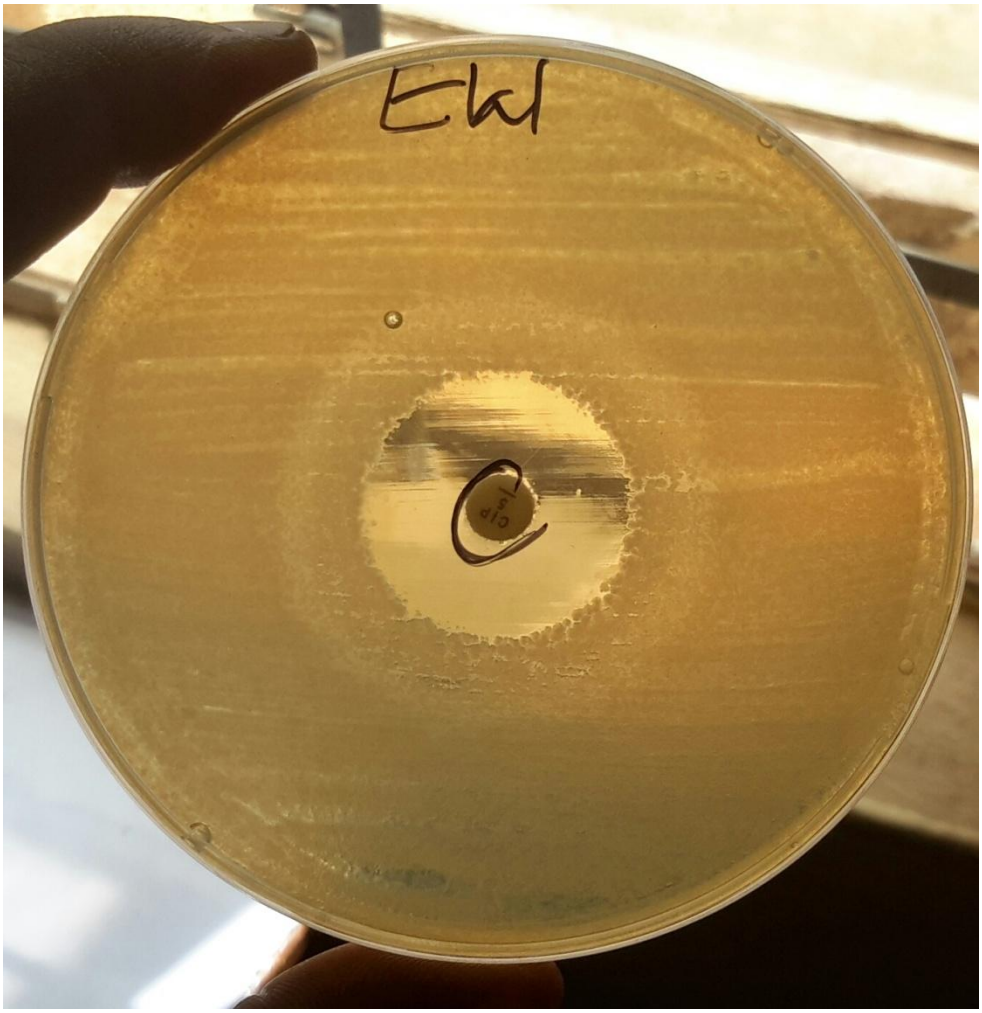


Plate IV. Zone of inhibition of the control (ciprofloxacin) tested against *Erwinia chrysanthemi*.

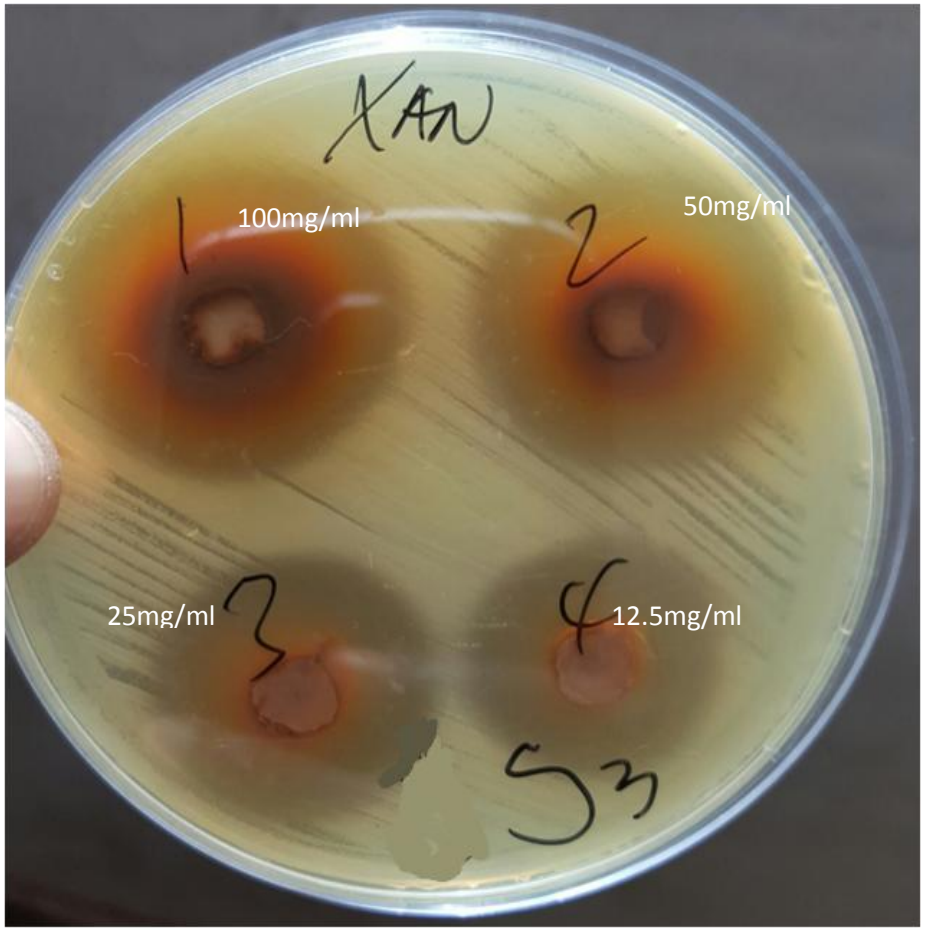


Plate V. Zones of inhibition of the fractions at different concentrations against *Xanthomonas axonopodis.*

4.5 Minimum inhibitory concentration (MIC) tests

The results of the minimum inhibitory concentration (MIC) indicated that, the n-butanol root fraction had the greatest activity against *X. axonopodis* with an MIC value of 1.50 mg/ml, followed by the aqueous leaf fraction against *S.* Typhi with MIC values of 3.063 mg/ml and ethyl-acetate root fraction against *X. axonopodis* respectively. *K. pneumoniae* had its only MIC value of 25 mg/ml against the n-butanol root fraction. *P. syringae* had low MIC values of 12.50 against the n-butanol fractions of both the leaf and root (Table 4.10).

4.6 Minimum bactericidal concentration (MBC)

The results from the minimum bactericidal concentration (MBC) showed that, the n-butanol leaf fraction had the greatest activity against *E. chrysanthemi* with an MBC value of 3.06 mg/ml, followed by the aqueous leaf fraction with MBC values of 6.125 mg/ml respectively. The ethyl-acetate leaf fraction had its greatest activity against *S.* Typhi with a value of 0.76 mg/ml. The least activity was given by the root aqueous and root ethyl-acetate fractions against *P. syringae* with an MBC value of 50 mg/ml. N-butanol root fraction against *K. pneumoniae* had an MBC value of 12.5mg/ml (Table 4.11).

Table 4.10 Minimum inhibitory concentration **(**MIC) for the test microorganism of different extracts of *C. molle in* mg/ml

Test organism	Root ethyl-acetate	leaf Ethyl-acetate	Aqueous root	Aqueous leaf	N-butanol leaf	N-butanol root
E. chrysanthemi	12.5	25	25	6.125	3.063	25
S. Typhi	6.125	12.5	25	3.063	25	25
K. pneumoniae	-	-	-	-	-	25
P. syringae	25	50	25	-	12.50	12.5
X. axonopodis	3.063	12.50	12.50	12.50	12.50	1.50

- = No activity, concentrations below 9 are inhibitory, concentrations above 9-32 is intermediate, while concentrations above 32 is resistant (CLSI, 2014)

Table 4.11 Minimum bactericidal concentration (MBC) for the test microorganism at different extracts of *C. molle in* mg/ml

Test organism	Root ethyl-acetate	Leaf ethyl-acetate	Aqueous root	Aqueous leaf	N-butanol leaf	N-butanol root
E. chrysanthemi	6.125	25	12	6.125	25	6.125
S. Typhi	3.063	0.76	3.063	6.125	1.53	6.125
K. pneumoniae	-	-	-	-	-	12.50
P. syringae	50.00	12.50	50	-	25.00	25
X. axonopodis	12.50	6.125	12.50	12.50	1.53	25

- = No activity, concentrations below 9 are bactericidal, concentrations above 9-32 is intermediate, while concentrations above 32 is resistant (CLSI, 2014)

4.7 Gas chromatography-- mass spectrometry (GC-MS) result

The mass spectrums of the fractions of *C. molle* were obtained from the gas chromatography – mass spectrometry analysis with the following peaks respectively as can be seen in Appendices 1--6. Tributylacetylcitrate had the highest spectrum peak of 99 while 1-hydroxy-2-methylbenzene had the lowest mass spectrum peak of 32 for the n-butanol leaf fraction. 6,9,12,15-docosatetraenoic acid had the highest spectrum peak of 192 while Heptadecanol had the lowest mass spectrum peak of 48 for the n-butanol root fraction. 5-Cholestene had the highest spectrum peak of 136 while 1H-Pyrrole had the lowest spectrum mass peak of 21 for the ethyl-acetate leaf fraction. lupeol had the highest spectrum peak of 214 while 1,6-Methanol (10) annulene had the lowest mass spectrum peak of 44 for the ethyl-acetate root fraction. Octadecanoic acid had the highest spectrum peak of 146 while cyclopropane had the lowest mass spectrum peak of 49 for the aqueous leaf fraction. Oleic acid had the highest spectrum peak of 91 while Undecane had the lowest mass spectrum peak of 13 for the aqueous root fraction.

Table 4.12: Compounds indicated by GC MS in n-butanol fraction of the leaf of *C. molle*.

Line	Compound Name	Formula	Mol. weight	Retention time (Min.)	Similarity index(%)
1.	1-hydroxy-2-methylbenzene	C_7H_8O	108	5.108	90
2.	1-hydroxy-2,3-dimethylbenzene	$C_8H_{10}O$	122	6.400	86
3.	1-dodecene	$C_{12}H_{24}$	168	9.492	95
4.	Tridecanoic acid	$C_{14}H_{28}O_2$	228	11.125	91
5.	Phthalic acid	$C_{14}H_{14}O_4$	246	12.000	76
6.	1-hexadecene	$C_{16}H_{32}$	224	14.425	95
7.	Pentadecanoic acid	$C_{17}H_{34}O_2$	270	16.667	93
8.	1,2benzenedicarboxylic acid	$C_{16}H_{22}O_4$	278	17.433	77
9.	1-pentadecene	$C_{15}H_{30}$	210	18.075	95
10.	11-Octadecanoic acid	$C_{19}H_{36}O_2$	296	19.808	92
11.	Octadecanoic acid	$C_{19}H_{38}O_2$	298	20.167	91
12.	1-propane-1,2,3-tricarboxylic acid	$C_{18}H_{30}O_6$	342	20.617	78
13.	Acetic acid	$C_{20}H_{40}O_2$	312	21.250	93
14.	Tributyl acetylcitrate	$C_{20}H_{34}O_8$	402	21.867	88
15.	Hexadecanoic acid	$C_{37}H_{74}NO_8P$	691	22.275	86
16	4,8,12,16-Tetramethyl heptadecan-4-olide	$C_{21}H_{40}O_2$	324	22.925	87
17.	Hexadecanoic acid	$C_{19}H_{34}O_4$	330	24.392	89

Table 4.13: Compounds indicated by GC MS result in n-butanol fraction of the root of *C. molle*.

Line	Compound Name	Formula	Mol. weight	Retention time (Min.)	Similarity index(%)
1.	Phenol	C_6H_6O	94	3.908	83
2.	1-hydroxy-2-methylbenzene	C_7H_8O	107	5.125	93
3.	Nonanal	$C_9H_{18}O$	142	5.550	87
4.	2,3-dimethylphenol	$C_8H_{10}O$	122	6.417	79
5.	Azulene	$C_{10}H_8$	128	6.817	89
6.	1-methyltridecylacrylate	$C_{17}H_{32}O_2$	268	7.775	83
7.	1-Undecene	$C_{12}H_{24}$	168	8.817	85
8.	1-Dodecene	$C_{12}H_{24}$	168	9.500	94
9.	Phthalic acid	$C_{20}H_{26}O_4$	330	12.017	88
10.	1-Pentadecene	$C_{20}H_{26}O_4$	210	14.425	94
11.	2-Undecanone	$C_{13}H_{26}O$	198	15.225	91
12.	Pentadecanoic acid	$C_{17}H_{34}O_2$	270	16.683	95
13.	Nonadecanoic acid	$C_{19}H_{38}O_2$	298	17.442	76
14.	1-Heptadecanol	$C_{17}H_{36}O$	256	19.550	94
15.	11-Octadecanoic acid	$C_{19}H_{36}O_2$	296	19.808	91
16	Octadecanoic acid	$C_{19}H_{38}O_2$	298	20.175	92
17.	1-propane-1,2,3tricarboxylic acid	$C_{18}H_{30}O_6$	342	20.617	77
18.	Tributyl acetylcitrate	$C_{20}H_{34}O_8$	402	21.883	87
19.	Hexadecanoic acid	$C_{37}H_{74}NO_8P$	691	22.300	85
20.	4,8,12,16Tetramethylheptaden-4-olide	$C_{21}H_{40}O_2$	324	22.993	87
21.	Hexadecanoic acid	$C_{19}H_{38}O_4$	284	24.458	94
22.	6,9,12,15-docosatetraenoic acid	$C_{23}H_{38}O_2$	346	24.608	83
23.	Hexadecanoic acid	$C_{23}H_{42}O_6$	414	25.317	85

Table 4.14: Compounds indicated by GC MS result in ethyl-acetate fraction of the leaf of *C. molle*.

Line	Compound Name	Formula	Mol. weight	Retention time (Min.)	Similarity index(%)
1.	1H-Pyrrole	$C_4H_6N_2O$	98	5.542	74
2.	2-Tridecene	$C_{13}H_{26}$	182	11.983	94
3.	Hexadecene	$C_{16}H_{34}$	226	12.075	96
4.	1-Pentadecene	$C_{15}H_{30}$	210	14.425	95
5.	Pentadecanoic acid	$C_{17}H_{34}O_2$	270	16.675	93
6.	1-Hexadecene	$C_{16}H_{32}$	224	18.083	95
7.	11-Octadecanoic acid	$C_{19}H_{36}O_2$	296	19.817	92
8.	Octadecanoic acid	$C_{19}H_{38}O_2$	298	20.175	91
9.	1-Tetracosanol	$C_{24}H_{50}O$	354	21.100	95
10.	Tridecane	$C_{13}H_{28}$	184	21.175	88
11.	Hexadecanoic acid	$C_{39}H_{34}O_4$	284	22.275	84
12.	1-Iodo-2-methylnonane	$C_{10}H_{21}I$	268	23.117	78
13.	Eicosane	$C_{20}H_{42}$	282	23.392	94
14.	9,12,15-Octadecatrienoic acid	$C_{25}H_{40}O_6$	436	24.342	85
15.	5-cholestene-3-ol	$C_{28}H_{48}O$	400	27.225	80

Table 4.15: Compounds indicated by GC MS result in ethyl-acetate fraction of the root of *C. molle*.

Line	Compound Name	Formula	Mol. weight	Retention time (Min.)	Similarity index(%)
1.	1,6-Methanol(10)annulene	$C_{11}H_{10}$	142	8.342	87
2.	N-(Methyl1-3-isoxazolyl)phthalimide	$C_{12}H_8N_2O_3$	228	11.992	72
3.	3-Heptane,2,2,66-pentamethyl	$C_{12}H_{24}$	168	13.458	82
4.	1-Pentadecene	$C_{15}H_{30}$	210	14.425	94
5.	2-Undecanone	$C_{13}H_{26}O$	198	15.233	84
6.	9-Heptadecanone	$C_{17}H_{34}O$	254	15.717	90
7.	Pentadecanoic acid	$C_{17}H_{34}O_2$	270	16.675	93
8.	Hexadecanoic acid	$C_{18}H_{36}O_2$	284	18.050	91
9.	11-Octadecanoic acid	$C_{19}H_{36}O_2$	296	19.808	93
10.	Octadecanoic acid	$C_{19}H_{38}O_2$	298	20.175	91
11.	9,12-Octadecadien-1-ol	$C_{18}H_{34}O$	266	20.658	91
12.	(E)-9-Octadecanoic acid	$C_{20}H_{38}O_2$	310	20.742	92
13.	Octadecanoic acid	$C_{20}H_{40}O_2$	312	21.067	92
14.	Hexadecanoic acid	$C_{35}H_{68}O_5$	568	22.275	86
15.	Di-n-octyl phthalate	$C_{24}H_{38}O_4$	390	24.767	82
16	Lupeol	$C_{30}H_{50}O$	426	24.925	87
17.	Stigmasta-3,5-dien-7-one	$C_{29}H_{46}O$	410	25.342	81
18.	Cholestane	$C_{27}H_{46}O$	386	26.883	79

Table 4.16: Compounds indicated by GC MS result in aqueous fraction of the leaf of *C. molle*.

Line	Compound Name	Formula	Mol. weight	Retention time (Min.)	Similarity index(%)
1.	Cyclopropane	C_7H_{12}	198	7.725	88
2.	2,4,Decadienol	$C_{10}H_6O$	158	8.133	87
3.	Benzene methanol	$C_{10}H_{14}O$	150	8.383	81
4.	Hexadecanoic acid	$C_{19}H_{30}O_2$	330	17.922	91
5.	9,12,octadecanoic acid	$C_{19}H_{34}O_4$	294	19.925	95
6.	9,12,15,octadecanoic acid	$C_{19}H_{34}O_2$	292	20.033	91
7.	9-Hexadecanoic acid	$C_{16}H_{32}O_2$	254	20.975	92
8.	Octadecanoic acid	$C_{19}H_{38}O_2$	298	21.150	92
9.	Decane	$C_{11}H_{24}$	156	22.483	81
10.	9,12,octadecadienoylchloride	$C_{18}H_{31}ClO$	284	24.292	90
11.	11-Octadecanoic acid	$C_{19}H_{36}O$	280	26.358	88

Table 4.17: Compounds indicated by GC MS result in aqueous fraction of the root of *C. molle*.

Line	Compound Name	Formula	Mol. weight	Retention time (Min.)	Similarity index(%)
1.	1-Decene	$C_{10}H_{20}$	140	3.975	93
2.	Undecene	$C_{11}H_{24}$	156	4.075	89
3.	Carbanic acid	$C_7H_7NO_2$	137	4.250	86
4.	Hexadecanoic acid	$C_{19}H_{34}O_4$	330	15.540	88
5.	n-Hexadecanoic acid	$C_{16}H_{36}O_2$	256	15.958	93
6.	11-octadecanoic acid	$C_{19}H_{36}O_2$	296	17.158	90
7.	Oleoic acid	$C_{18}H_{34}O_2$	282	17.658	91
8.	Octadecanoic acid	$C_{19}H_{38}O_2$	298	17.833	78
9.	13-Docosenoic acid	$C_{22}H_{42}O_2$	338	20.033	84
10.	13-Docosenoic acid	$C_{22}H_{44}O_2$	352	20.575	99

Table 4.18: Compounds common to all the fractions of *C. molle*

S/N	Name of Compound	Formula	Mol. Weight	Retention Time(Min)
1.	11-Octadecanoic acid	$C_{19}H_{36}O_2$	296	19.808
2.	Octadecanoic acid	$C_{19}H_{38}O_2$	298	20.167
3.	Hexadecanoic acid	$C_{19}H_{34}O_4$	330	24.392

4.8 Acute toxicological evaluation

The body weights of all tested groups increased progressively throughout the duration of the experiment (Table 4.19). The effect of the extract in causing drowsiness in all the treated groups was observed for the first 1 hr after administering the fractions of *C. molle*, compared with control which showed no drowsiness. No mortality was recorded for any treated groups throughout the duration of the experiment. Since the treatment did not result in latent toxicity, the LD_{50} was therefore estimated to be above 5000 mg/kg .

4.9 Histopathology of the kidneys of the animals

There was no histological alterations or changes at the extract dose of 5000 mg/kg body weight in the kidney organs of rats in the control group, but all the other organs from the fractions tested displayed certain observed alterations. Tubular vacoulation (TVN), Lymphocyte hyperplasia (LH), Glomerular necrosis (GN),Plaques formation (P), Tubular necrosis (TN) and Tubular distortions (TD) were observed in the kidneys of groups I-VI (Plates 4.13---4.18).

4.10 Histopathology of the liver of the animals

There was no histological alterations or changes at the extract dose of 5000 mg/kg body weight in the internal organs of rats in the control group and in the liver of group VI, but all the other organs from the fractions tested displayed certain observed alterations. Vascular congestion (VC), vascular congestion with slight necrosis (VCN) and hepatocellular necrosis (HN) were observed in the livers of groups I--V (Plates 4.19--4.24).

Table 4.19 Average body weight of the rats after oral administration of the fractions of *C. molle.*

	Rat body weight (g)		
GROUPS (fractions of extracts)	Day 0	Day 7	Day 14
I (ethyl-acetate root)	278±1.0	281±1.0	287±1.0
II (ethyl-acetate leaf)	247±1.0	253±1.0	261±1.0
III (n-butanol root)	258±1.0	260±1.0	263±1.0
IV (n-butanol leaf)	235±1.0	237±1.0	241±1.0
V (aqueous root)	278±1.0	283±1.0	288±1.0
VI (aqueous leaf)	256±1.0	260±1.0	263±1.0
VII (control)	229±1.0	234±1.0	236±1.0

Mean body weight ± SE

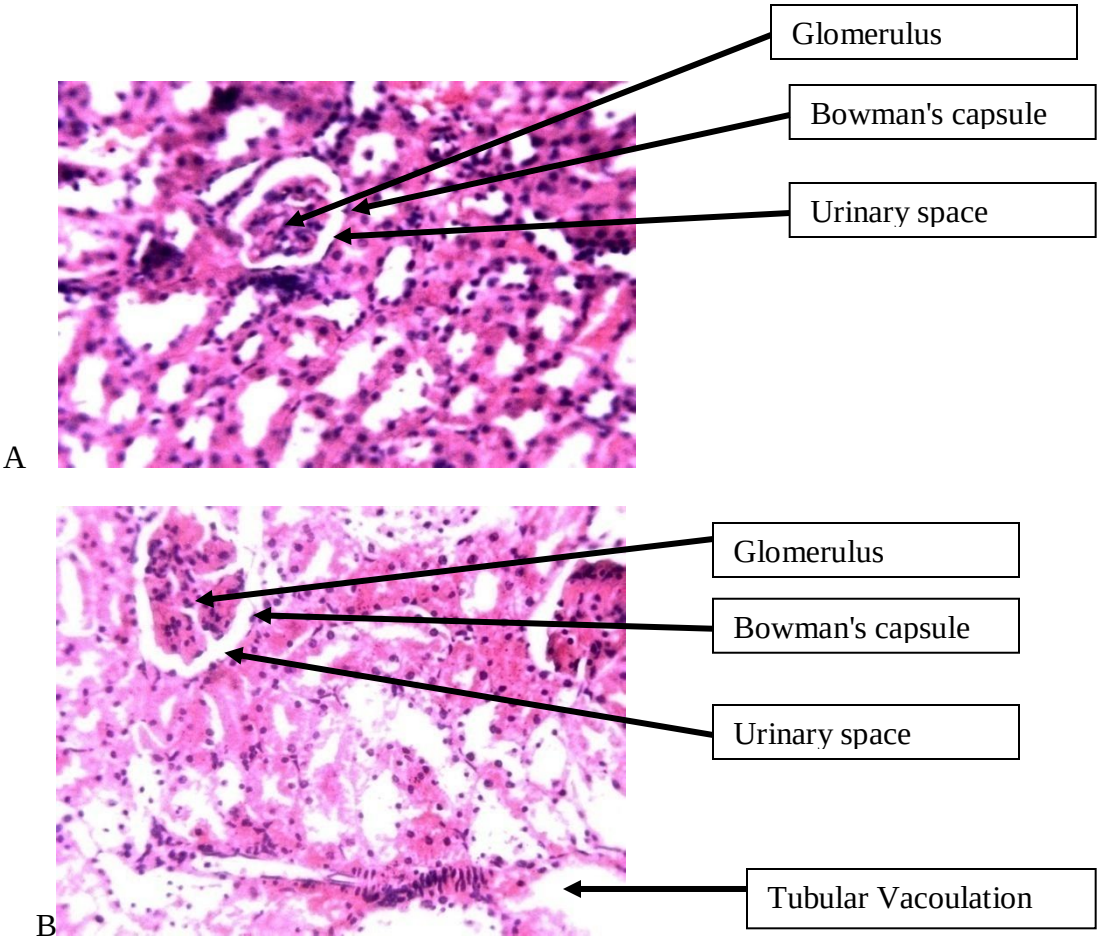
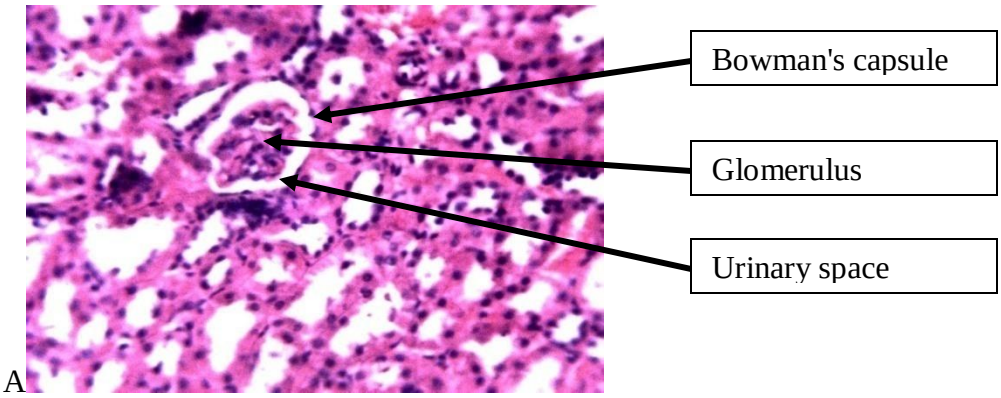
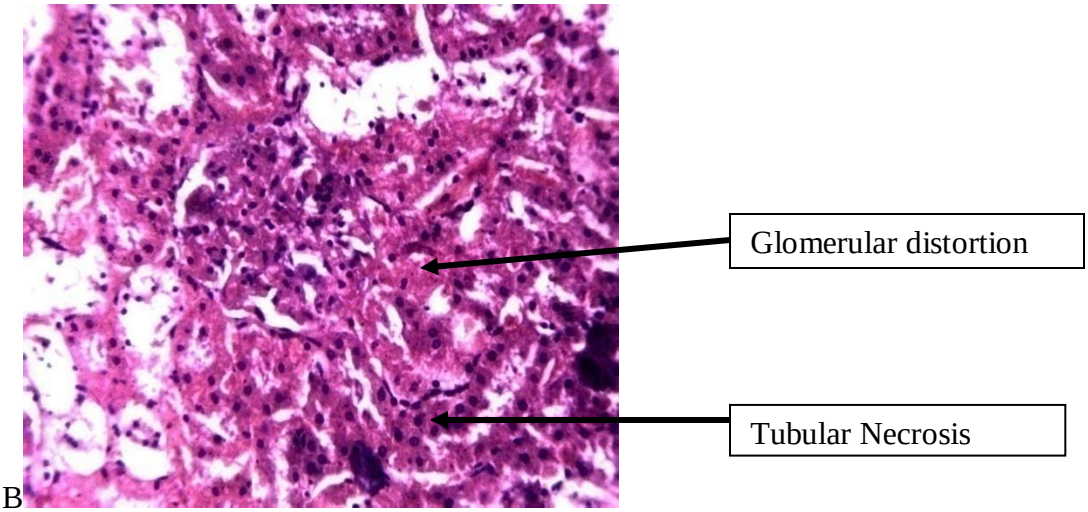


Plate XIII: (A) Photomicrograph of the Rat Kidney under control treatment showing normal tubules and glomerulus, compared with; (B) Orally administered ethyl-acetate root fraction showing tubular vacoulation (TV). (Magnification, × 400).



A



B

Plate XIV: (A) Photomicrograph of the Rat Kidney under control treatment showing normal tubules and glomerulus, compared with; (B) Orally administered ethyl-acetate leaf fraction above showing moderate glomerular distortion (GD) and tubular necrosis (TN). (Magnification, × 400).

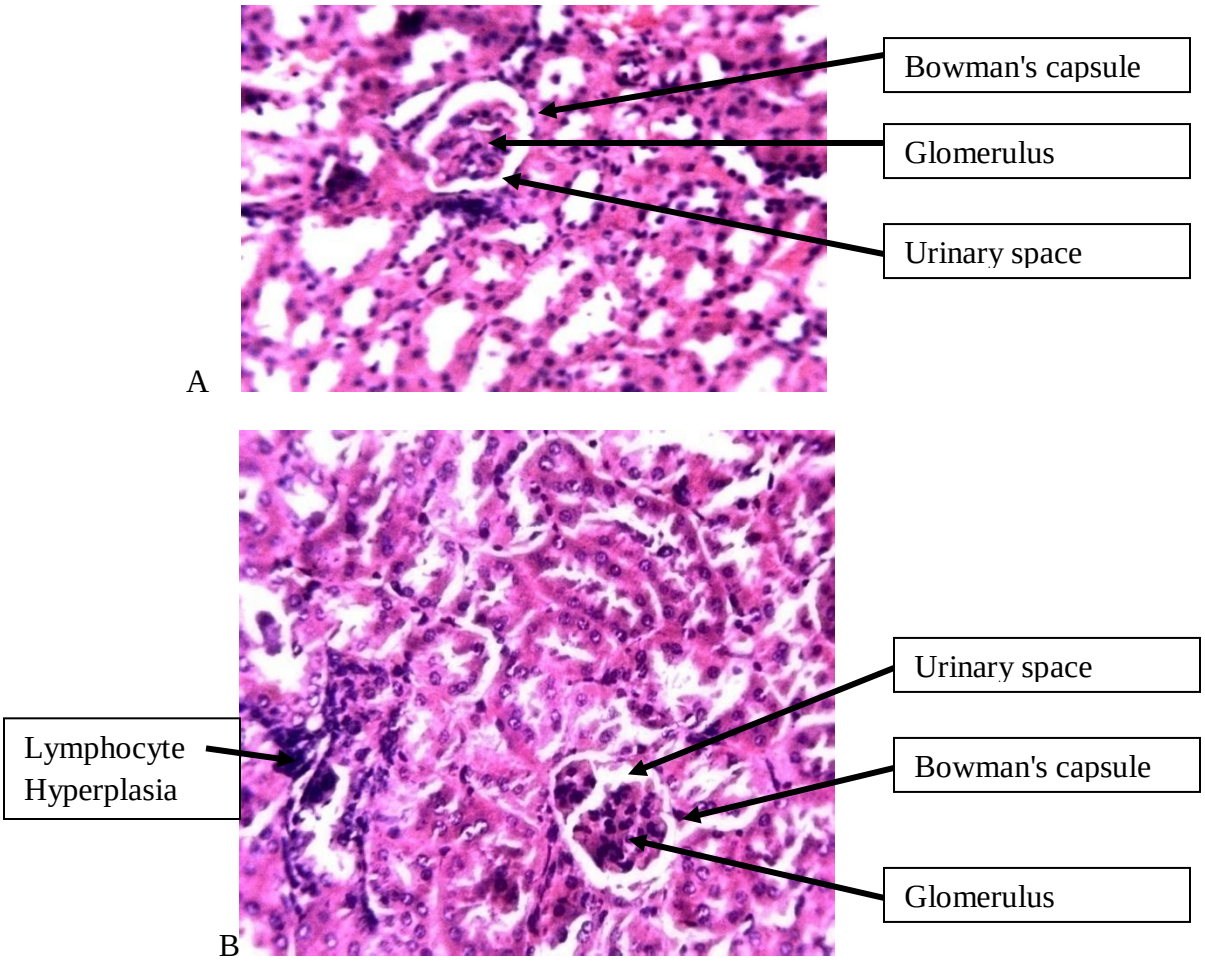


Plate XV: (A) Photomicrograph of the Rat Kidney under control treatment showing normal tubules and glomerulus, compared with; (B) Orally administered ethyl-acetate leaf fraction above showing moderate lymphocyte hyperplasia(LH). (Magnification, × 400).

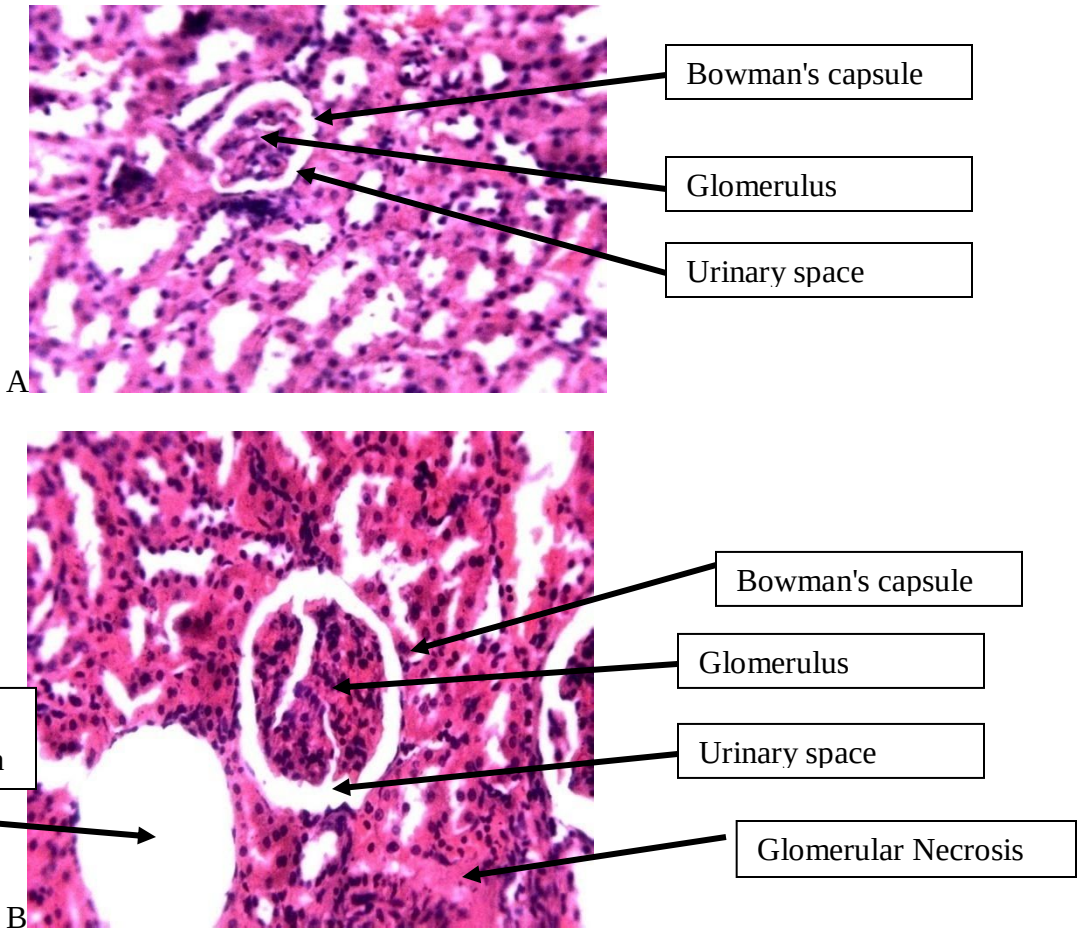


Plate XVI: (A) Photomicrograph of the Rat Kidney under control treatment showing normal tubules and glomerulus, compared with; (B) Orally administered n-butanol root fraction above showing slight glomerular necrosis (GN) and tubular vacoulation (TV). (Magnification, × 400).

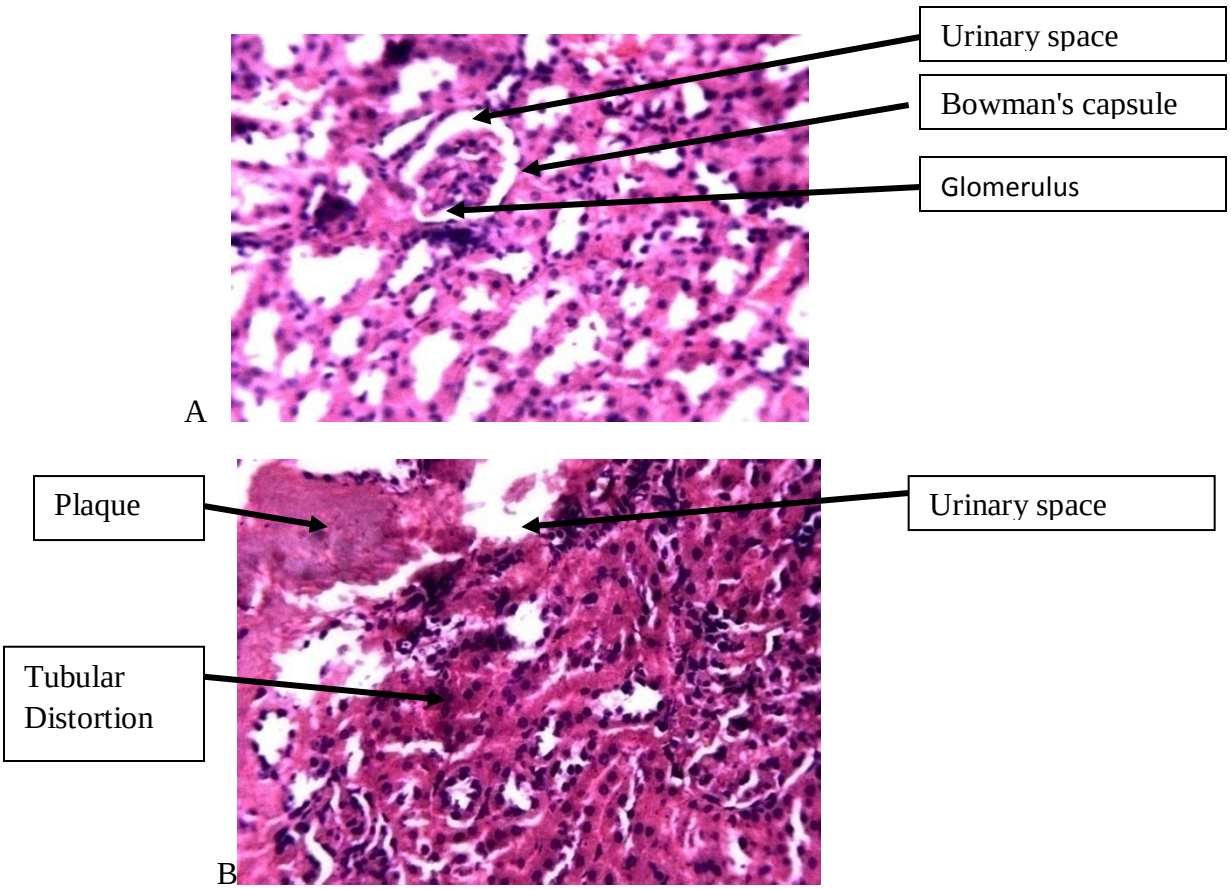


Plate XVII: (A) Photomicrograph of the Rat Kidney under control treatment showing normal tubules and glomerulus, compared with; (B) Orally administered n-butanol leaf fraction above showing plaques formation (P) and slight tubular distortion (TD). (Magnification, × 400).

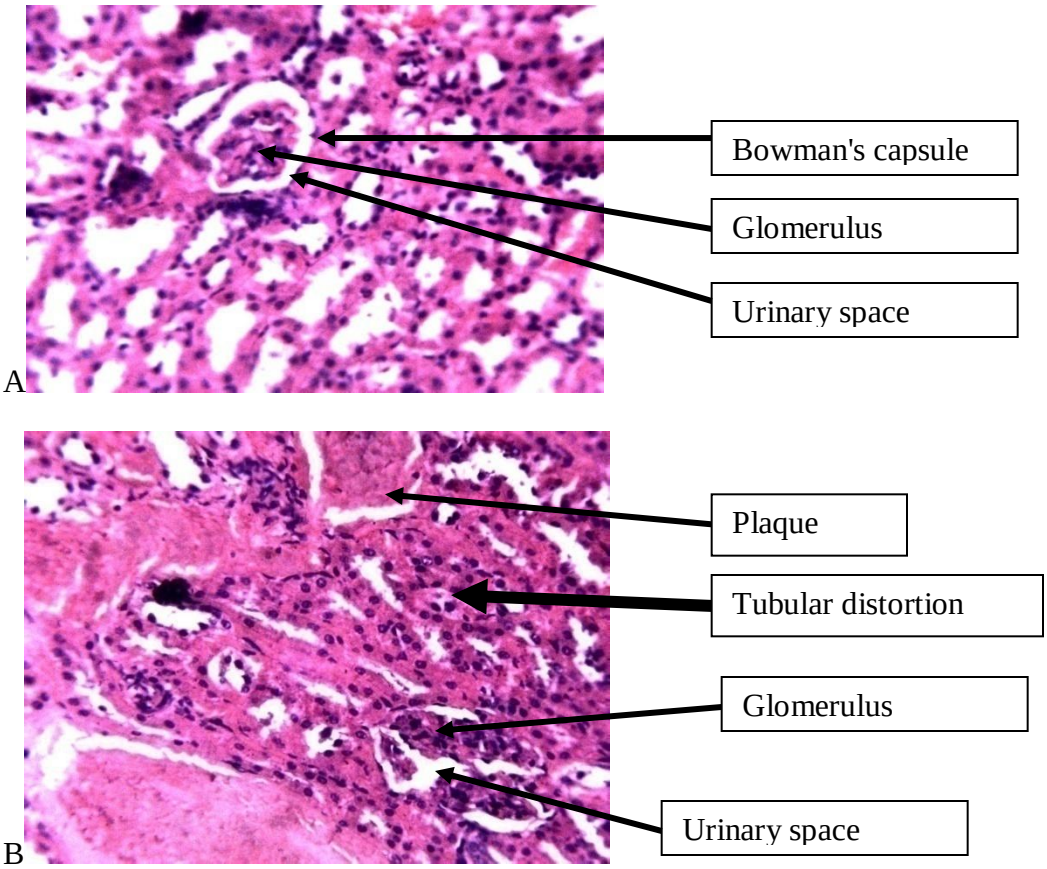
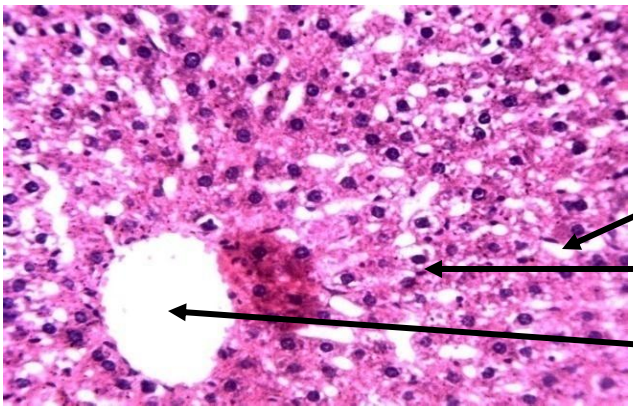


Plate XVIII: (A) Photomicrograph of the Rat Kidney under control treatment showing normal tubules and glomerulus compared with; (B) Orally administered aqueous leaf fraction above showing plaque formation (P), with slight tubular distortion (TD). (Magnification, × 400).

A



B

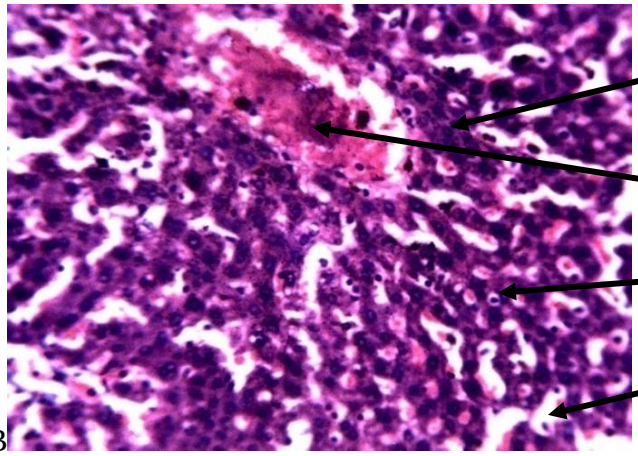
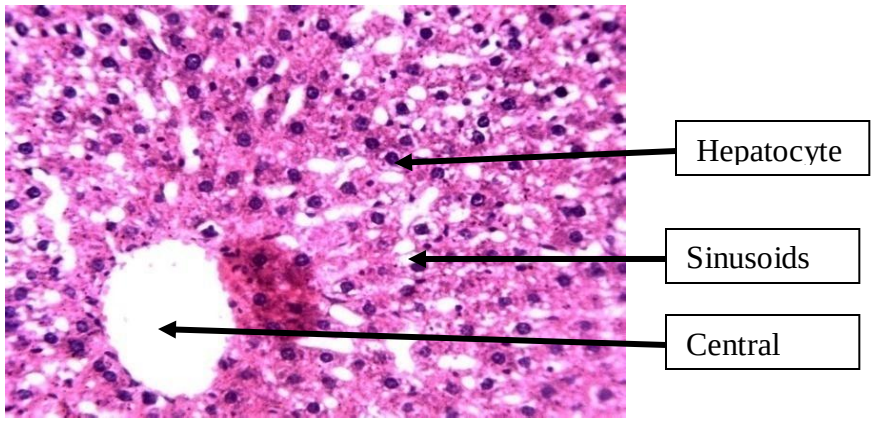


Plate XIX: A) Photomicrograph of the Rat Liver Control showing normal hepatocytes, compared with; (B) Orally administered ethyl-acetate root fraction administered liver which showed vascular congestion (vc). (Magnification, × 400).

A



B

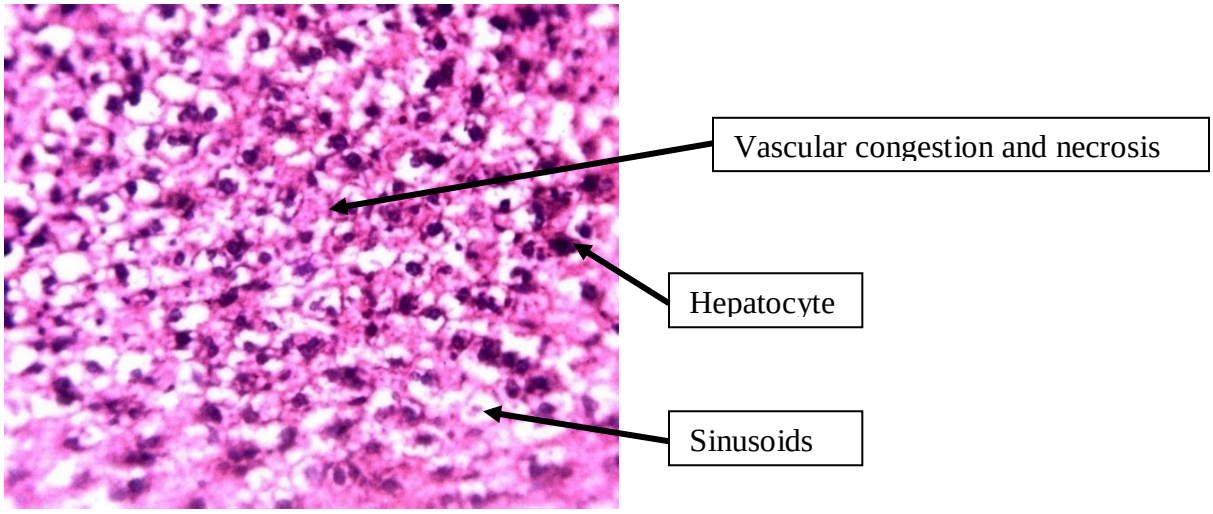


Plate XX: (A) Photomicrograph of the Rat Liver of control showing normal hepatocytes, compared with; (B) Orally administered aqueous root fraction above shows slight vacoulation and necrosis(VCN). (Magnification, × 400).

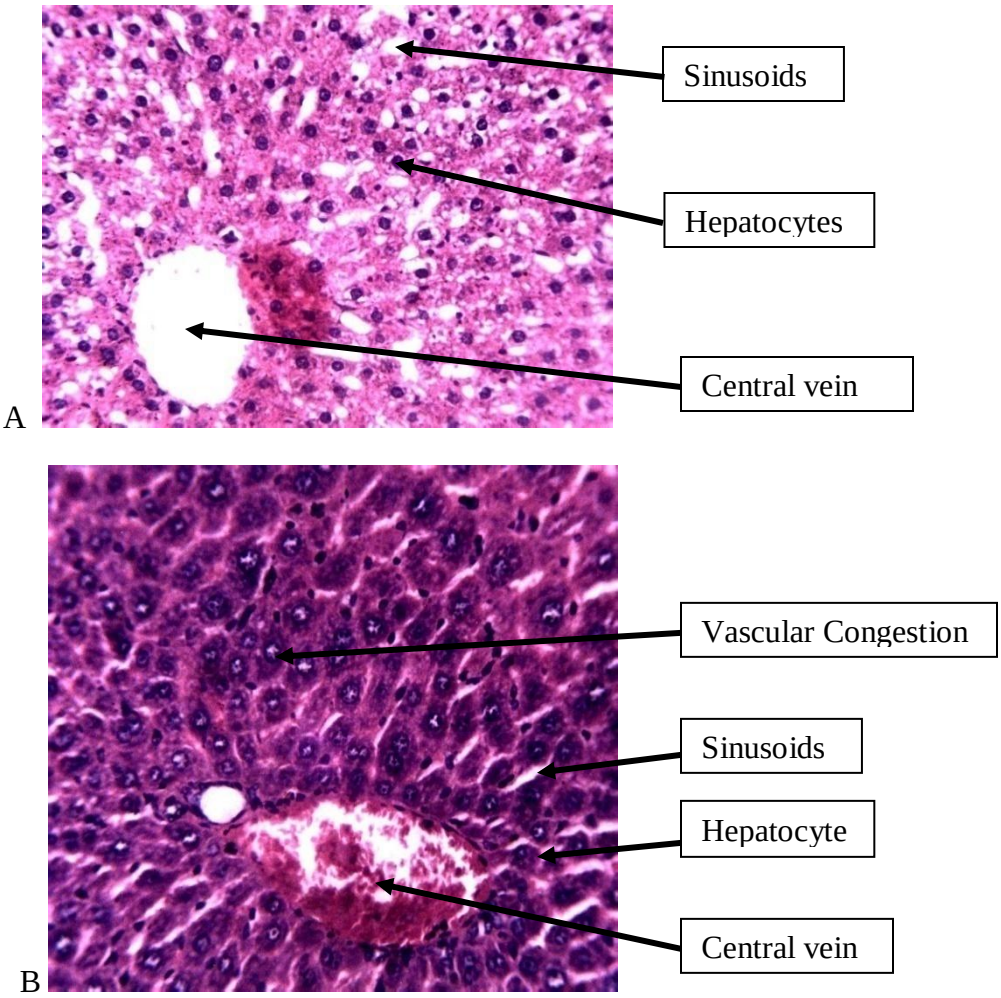
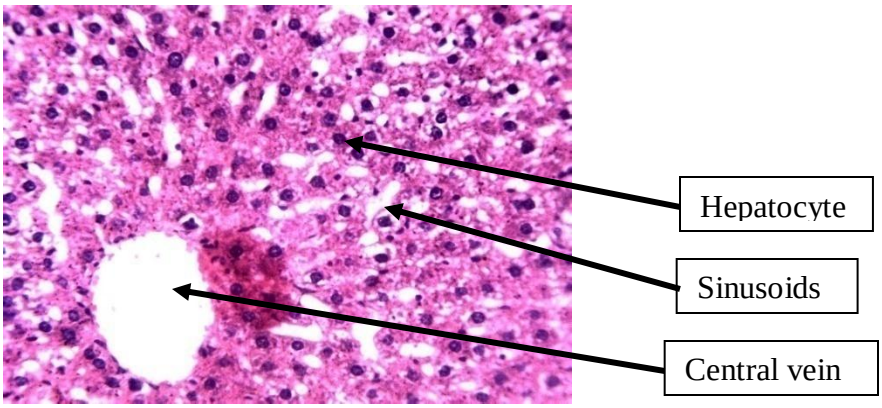


Plate XXI: **(**A) Photomicrograph of the Rat Liver of control showing normal hepatocytes, compared with; (B) Orally administered ethyl-acetate leaf fraction above shows vascular congestion (VC). (Magnification, × 400).

A



B

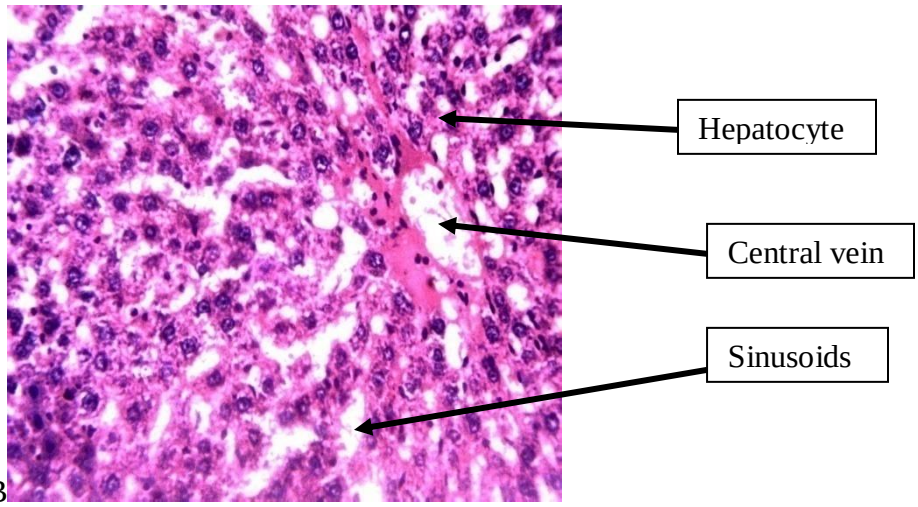
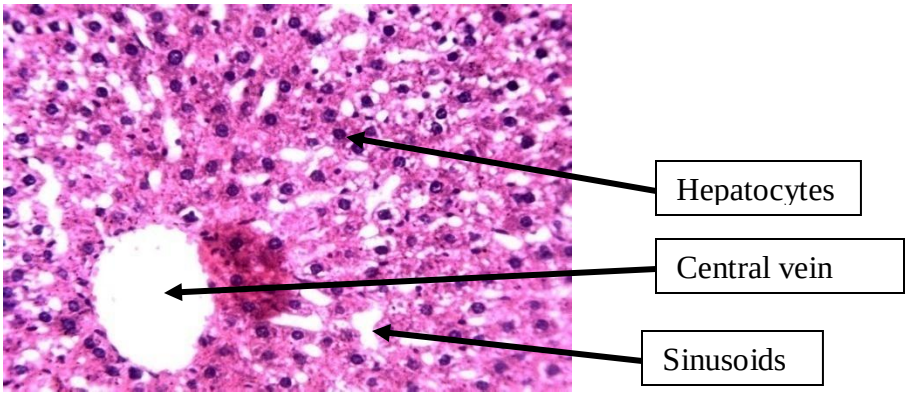


Plate XXII: (A) Photomicrograph of the Rat Liver of control showing normal hepatocytes, compared with; (B) Orally administered N-butanol root fraction above showing normal features. (Magnification, × 400).

A



B

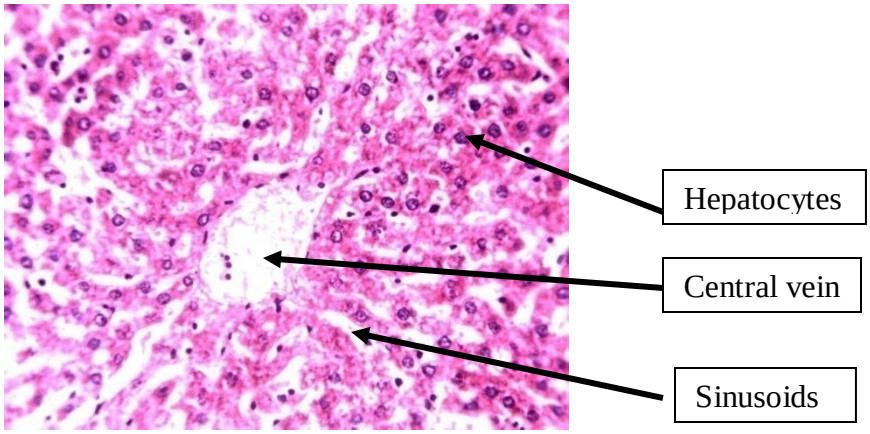
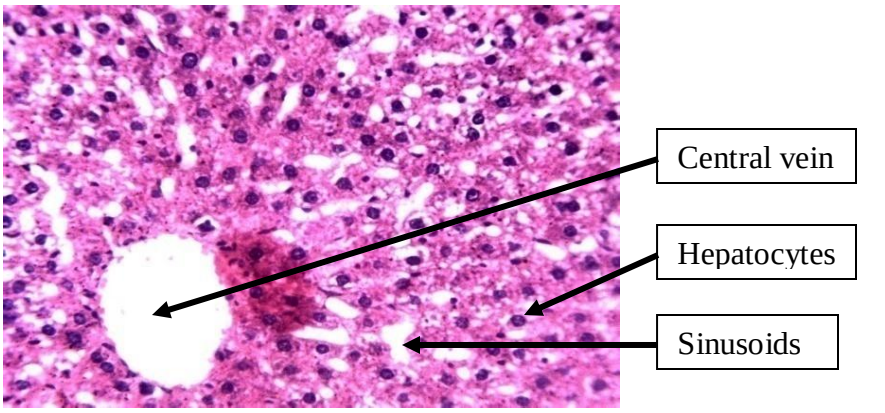


Plate XXIII: (A) Photomicrograph of the Rat Liver of control showing normal hepatocytes compared with; (B) Orally administered N-butanol leaf fraction above also showing normal feature. (Magnification, × 400).

A



B

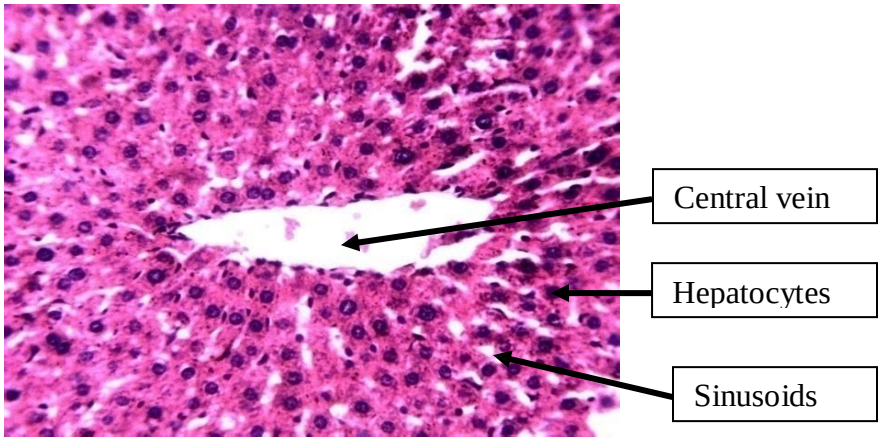


Plate XXIV: (A) Photomicrograph of the Rat Liver of control showing normal hepatocytes compared with; (B) Orally Administered 5000 mg/kg of aqueous leaf fraction also showing normal feature. (Magnification, × 400).

CHAPTER FIVE

5.0 Discussion

The Phytochemical screening of the fractions of *C. molle* revealed the presence of tannins, flavonoids, saponins, triterpenes and steroids. Research work similar is that of Adebayo and Ishola (2009) who investigated the activity of root, stem-bark and leaves extracts of *Terminalia glaucescens'* against some pathogenic organisms. Phytochemical screening of the fractions of *Terminalia glaucescens'* also revealed the presence of alkaloids, tannins, saponins, steroids, flavonoids, anthraquinones and phlobatannins (mostly in root and stem-bark). Also, Igbinosa *et al*. (2009) in the screening of the methanolic and water extracts of the stem bark of *Jatropha curcas* revealed the presence of saponin, steroids, tannin, glycosides, alkaloids and flavonoids in the extracts.

Glycosides were found in all the fractions except the root aqueous and root n-butanol fractions. This confirms the report by many researchers that, *C. molle* contains phytochemical compounds. This also agrees with the work of Fyhrquist *et al.* (2002) who reported the presence of tannins, flavonoids and saponins in the roots and leaves extracts of *C. molle*. Harborne (1999) found tannins and anthraquinones (the largest group of quinones) to posses antibacterial effects by inhibiting nucleic acid synthesis. Alkaloids and anthraquinones were absent in the ethyl-acetate root fraction. This is similar to the work of Nishaa *et al*. (2013) who investigated the ethanol extract of *Maranta arundinacea* rhizomes and found alkaloids to be present in the ethanol extract, but absent in the ethyl-acetate fraction. Similarly, Arun *et al*. (2014) in the screening of constituents and anti-bacterial activity of *Aerva lanata* leaves using similar method found alkaloids to be present in the acetone and ethanol fractions, but absent in the ethyl-acetate

fraction. Also, Cock and Kalt (2012) carried out the Gas chromatography mass spectrometry (GC-MS) analysis of *Xanthorrhoea johnsonii* leaf extract displaying apparent anesthetic effect. They also found alkaloids and anthraquinones to be absent in the ethyl-acetate fraction. The above could possibly imply that ethyl-acetate is a not suitable for the extraction of alkaloids.

Bajaj (1988) reported that, tannins have been used for immediate relief of sore throats, diarrhea, dysentery, haemorrhage, fatigue, skin ulcers and as a cicatrizant on gangrenous wounds. Schuier *et al*. (2005) reported that, epicatechin, quercetin and luteolin (all flavonoids) can inhibit the development of fluids that result in diarrhoea by targeting the intestinal cystic fibrosis transmembrane conductance regulator Cl– transport inhibiting cAMP-stimulated Cl– secretion in the intestine. Tannins have been found to form irreversible complexes with prolinerich protein (Shimada, 2006) resulting in the inhibition of cell protein synthesis. Parekh and Chanda (2007) reported that, tannins are known to react with proteins to provide the typical tannin effect which is important for the treatment of inflamed or ulcerated tissues. Herbs that have tannins as their main components are astringent in nature and are used for treating intestinal disorders such as diarrhea and dysentery (Dharmananda, 2003). Another secondary metabolite compound observed in the root and leaf fractions of *C. molle* was alkaloid. One of the most common biological properties of alkaloids is their toxicity against cells of foreign organisms. These activities have been widely studied for their potential use in the elimination and reduction of human cancer cell lines (Nobori *et al*., 1994). Alkaloids which are one of the largest groups of phytochemicals in plants have amazing effects on humans and this has led to the development of powerful pain killer medications (Kam and Liew, 2002). Just *et al*. (1998) revealed the inhibitory effect of saponins on inflamed cells. Saponin was found to be present in *J. curcas* extracts and has supported the usefulness of this plant in managing inflammation. Steroidal compounds were

also present in the root and leaf fractions of *C. molle*. Quinlan *et al*. (2000) worked on steroidal extracts from some medicinal plants which exhibited antibacterial activities on some bacterial isolates. Neumann *et al*. (2004) also confirmed the antiviral property of steroids. Flavonoids, another constituent of the root and leaf fractions of *C. molle* exhibited a wide range of biological activities like antimicrobial, anti-inflammatory, anti-angionic, analgesic, anti-allergic, cytostatic and antioxidant properties (Hodek *et al*., 2002). Hence, the presence of these compounds in the root and leaf fractions of *C. molle* corroborates the antimicrobial activities observed.

The result of the TLC also showed the presence of phytochemical compounds as was seen from the different array of colours. The result also indicated more secondary compounds in the ethyl-acetate root fraction. This could have been as a result of the environmental conditions or stage of the development of the plant or the method of extraction which are possible sources of variation for the chemical composition, toxicity and bioactivity of extracts (Felix, 1982).

In a the antibacterial study, five microorganisms were tested (*Erwinia chrysanthemi*, *Salmonella* Typhi, *Xanthomonas axonopodis*, *Pseudomonas syringae* and *Klebsiella pneumoniae*) and they were all susceptible to the fractions of *C. molle* at concentrations of 100 mg/ml. This is similar with the investigation of Ernest *et al*. (2012) who evaluated crude extracts from 84 plant species *in vitro* and *in planta* assays for antibacterial activity against bacterial leaf spot of tomato. About 20.2 % of the tested plant extracts totally inhibited growth of bacteria when seed washings from treated seeds were plated on nutrient agar medium. This agrees to the investigation of Misrak *et al*. (2013) who studied the potency of mustard and ginger rhizome extracts, lemon juice, Atella (residue of traditional Ethiopian beer) and cow urine against tomato seed borne pathogen, *Xanthomonas campestris* pv. *vesicatoria* of which Ginger and mustard extracts showed 0 cfu/ml on Xcv with 24 h soaking, 97 to 81% germination and 2303 to 2270 vigour indexes of tomato

seed. Also, it is in line with the findings of Kokwaro (2000) and Chhabra *et al*. (1999) on its use to treat typhoid fever. This also confirms Fyhrquist *et al*. (2002) report that, the extracts of *C. molle* gave good antibacterial effects. *Klebsiella pneumoniae* had a single MIC value of 25mg/ml. This agrees with Adebayo and Ishola (2009) who had minimum inhibitory concentration (MIC) of 40 mg/ml on *K. pneumoniae* after investigating the root, stem-bark and leaves extracts of *Terminalia glaucescens*.

Of the six fractions of *C. molle* screened for their antibacterial effects, the most outstanding effects were obtained with the activity of the n-butanol fraction against *E. chrysanthemi* with zones of inhibition as high as 28 mm at concentration of 100 mg/ml and a zone of inhibition of 19 mm at concentration of 6.125 mg/ml. All the fractions investigated showed excellent antibacterial activities against *E. chrysanthemi*, *X. axonopodis* and *S.* Typhi, whereas their activities against *K. pneumoniae* was generally poor.

In the Minimum inhibitory concentration studies MIC, *E. chrysanthemi*, *X. axonopodis* and *S.* Typhi was to be the most sensitive bacterial species with MIC values of as low as 1.5mg/ml, 3.125mg/ml and 6.25mg/ml. This agrees with the report of Kokwaro (2000) that, *Combretum molle* is used in treating fever. *Klebsiella pneumoniae* had only one MIC value of 25mg/ml against the n-butanol fraction. Similarly, the minimum bactericidal concentration, (MBC) also had low values, such as 0.76mg/ml of the n-butanol leaf fraction against *S.* Typhi, and 1.5mg/ml of the n-butanol leaf fraction against *X. axonopodis*. This agrees with the report of Arses *et al.* (2001) that, the roots of *C. molle* give good antibacterial effects. There was no activity completely by the aqueous leaf fraction against *P. syringae* and *K. pneumoniae* which had only one MBC value of 12.5mg/ml against the n-butanol fraction.

The analysis of variance at $p < 0.05$ showed that there was no significant difference in the activities of the fractions (leaf aqueous, root aqueous, leaf n-butanol, root n-butanol, leaf ethyl-acetate and root ethyl-acetate) on the microorganisms (*Erwinia chrysanthemi*, *Salmonella* Typhi, *Xanthomonas axonopodis*, *Pseudomonas syringae* and *Klebsiella pneumoniae*). The study also shows that the n-butanol leaf fraction had higher activities against the microorganisms than the other fractions tested, even though it was not significantly different as shown by the analysis of variance at $p < 0.05$.

Gas Chromatography – mass spectrometry analysis of the fractions of *C. molle* showed a total of 73 compounds comprising of hydrocarbons, saturated and unsaturated fatty acids. Tributylacetylcitrate, 1-hydroxy-2-methylbenzene, 6,9,12,15-docosatetraenoic acid and Heptadecanol have been reported by Saradhai *et al*. (2015) to be medicinally bioactive compounds. Also, Nandagopalan *et al*. (2015) reported that, Hexadecanoic acid, Octadecanoic acid, Oleic acid, Undecane, Pentadecanoic acid, N-Methoxy-N-methylacetamide and 2-Methyl-l methylmannopyranoside to posses medicinal properties that can be exploited for the treatment of various diseases. The fractions were bactericidal against the test pathogens which agrees with the work of Muthezilan *et al.* (2012), who using Fatty Acid Composition of palmitic acid, stearic acid, richinoleic acid, ligniceric acid, lauric acid, myristic acid and behanic acid found them to be bactericidal against *Bacillus subtilis, Klebsiella pneumoniae, Staphylococcus aureus*. Also, McGaw *et al*. (2002) carried out isolation of antibacterial fatty acids (9,12,15-octadecatrienoic (linolenic) acid and methyl-5,11,14,17-eicosatetraenoate) from *Schotia brachypetala* and found them to be bactericidal against *Bacillus subtilis, Klebsiella pneumoniae* and *Staphylococcus aureus.* Sermakkani and Thangapandian (2012) reported that, Phytol, Squalene and n-Hexadecanoic acid are used in industry for various applications like flavour, antioxidant, anti-

inflammatory, antimicrobial, pesticide and cancer preventive which supports the use of *Combretum molle* in African traditional medicine (since it contains the above fatty acids). Phytol is one among the seventeen compounds of the present study. Phytol was observed to have antibacterial activities against *Staphylococcous aureus* by causing damage to cell membranes (Inoue *et al*., 2005).

Phytol is a key acyclic diterpene alcohol that is a precursor for vitamins E and K . It is used along with simple sugar or corn syrup as a hardener in candies. Muthezilan *et al.* (2012) reported that, the main components of 9, 12 octadecadienoic acid has potential antioxidant and anticancer activities. Linolenic acid is known for its potential antibacterial, antifungal anti arthritic and anti-inflammatory activities. Homo-γ-linolenic acid has gained importance due to its anti-inflammatory and anti-cancer action, and also it has been used in the treatment of rheumatoid arthritis (Inoue *et al*., 2005). Oyewo *et al*. (2012) reported that, Palmitic acid possess antibacterial and cholesterolaemic effects. In addition, palmitic acid also possessed significant cytotoxicity against the MCF-7, WRL- 68, CaCo2, Colo-320 DM cancer cell lines and hepatoprotection against galactosamine.

In the acute toxicological investigation, there was no mortality in the experimental animals at all treatment doses of 5000 mg/kg body weight. The acute toxicity did not result in any mortality therefore the mean lethal dose (LD_{50}) cannot be calculated, rather it can only be estimated that it is above 5000 mg/kg. There were no signs of toxicity effects such as change in skin colour, behavioural pattern nor diarrhea. This finding suggests that, the fractions at the limit dose tested are non toxic and safe in oral formation. According to the chemical labeling and classification of acute systemic toxicity recommended by OECD, (2000) the fractions of *Combretum molle* were assigned class 5 status (LD_{50} > 5000 mg/kg) which is the lowest toxicity class. This is in line

with the investigation of David *et al*. (2015) in the study of the toxicological evaluation of aqueous and acetone extracts of *Combretum molle* leaves in Wistar Rats. They observed that, the limit dose of 2000 mg/kg did not cause any mortality or signs of acute toxicity in the rats tested during the observation period. Similarly, Kamo *et al*. (2015) studied the acute and subacute toxicity effects of hydro - alcoholic extract of *Terminalia mantaly*. They also observed that, the limit dose of 5000 mg/kg did not cause any mortality or signs of acute toxicity in the mice tested during the observation period. Similarly, Dodehe *et al*. (2012) investigated the acute and sub acute toxic study of aqueous leaf extract of *Combretum Molle* in Wistar rats. They observed that the limit dose of 8000 mg/kg did not cause any mortality or signs of acute toxicity in the mice tested during the observation period. Also, Oyewo *et al*. (2012) investigated the effects of aqueous extract of *Citrullus lanatus* on the histology of the kidney of adult Wistar rats. The aqueous extract of *Citrullus lanatus* was given once a day to the animals for 27 days. There was no mortality or signs of acute toxicity in the rats tested during the observation period.

There was a progressive increase in the body weight of all animals throughout the duration of the experiment and when compared to the control, there was no significant difference. The behaviours of all the tested animals revealed that, the animals were alert and responded to pain and touch. The animals showed no signs of depression or restlessness. Monitoring the body weight during treatment provides an index of the general health status of the animals; such information is important for gauging their health (Sharma *et al.*, 2009). According to Rhiouani *et al*. (2008) high doses of plant extracts induce stress in rats, thereby reducing their food and water intake which leads to a loss in weight. Thus, the increase in weight were considered normal. Similar researches are that of Dodehe *et al*. (2012) who carried out the acute and sub acute toxic study of the aqueous leaf of *C. molle* and at dose of 5000mg/kg observed gradual

increase in body weight. In addition, Zaza *et al*. (2016) investigated the oral toxicity of the X_{42} fractions of *Terminalia ivorensis* observed gradual increase in body weight.

There were some histopathological changes in the cellular anatomy of the liver and kidney. This manifested as alterations in the biomarkers of toxicity and cellular damage to the liver and kidney. The kidneys administered ethyl-acetate, aqueous and n-butanol leaf fractions showed slight tubular distortions and plaque formations. Plaque is an interstitial medullary and papillary deposit of calcium phosphate in the form of apatite that begins in the basement membranes of the thin loops of Henle and finds its way into the interstitium and eventually down to the sub epithelial space (Evan *et al*., 2007). They are significant because renal stones are known to grow attached to renal papillae, and specifically to regions of papillae that contain Randall's plaque (interstitial apatite deposits), the mechanisms of stone overgrowth on plaque are not known (Evan *et al*., 2007). From the kidneys administered ethyl-acetate and n-butanol root fraction, slight tubular vacoulation was observed. Tubular vacoulation is said to occur when the vacuoles of the kidneys that were administered the fractions are larger than that of the control (Sharma *et al.*, 2009). The kidney administered leaf ethyl-acetate had slight hyperplasia, which is similar to the work of David *et al*. (2015) who studied the toxicological effects of aqueous and acetone extracts of *Combretum molle* twigs in Wistar rats. They observed hepatotoxicity and mesengial hyperplasia in the livers and kidneys. This is different from the result of Oyewo *et al*. (2012) who did not record any histological distortion of the kidney after the administration of the extracts of *Citrullus lanatus*. This could also be as a result of different concentrations used for the test.

There were no histopathological changes observed from the liver administered aqueous leaf, root and leaf n-butanol fractions. This is similar to the work of Zaza *et al*. (2016)

investigated the oral toxicity of the X_{42} fractions of *Terminalia ivorensis*, observed no histological distortion of the liver. There were some histopathological changes (Slight vascular congestion) observed from the liver administered root ethyl-acetate, aqueous root and leaf ethyl-acetate fractions. This finding corroborates the finding of Sagar and Vidyasagar (2010) who observed slight changes in cellular architecture of the 202 livers of animals treated with 250 mg/kg body weight of an ethyl acetate extract of *Caesalpinia bonducella.*

5.1. conclusion

From the phytochemical assessment of the fractions of the root and leaf of *C. molle*, secondary metabolites (Tannins, Flavonoids, Glycoside, Alkaloids, Anthraquinones, Triterpenes and saponins) were found present. The screening also shows higher density of compounds in the ethyl-acetate root fraction as shown by the TLC. The leaf and roots of *C. molle* contain several phytochemical compounds. These compounds possess antibacterial effects against *S.* Typhi, *P. syringae, X. axonopodis, K. pneumoniae* and *E. chrysanthemi*. These results validate the ethno botanical use of *C. molle* for ailments and treatment of seeds that may be of bacterial etiology. GC-MS analysis of the fractions of the root and leaf of *C. molle* revealed the presence of a lot of fatty acids and other compounds especially Octadecanoic acid, Hexadecanoic acid and Pentadecanoic acid which together were responsible for the antibacterial activities observed against the test organisms.

Similarly, the fixed dose of *C. molle* at 5000 mg/kg body weight and above is also not toxic to experimental animals. This is because no mortality was observed in all the animals tested. Based on the present assessments and investigations, the daily use of concentrated extracts of the root and leaf of *C. molle* should be carefully considered in view of the observed alterations recorded

in the histological investigation. It is clear from this study that potential toxicity might arise from the continuous intake of plants such as *C. molle* at high concentrations.

5.2. Recommendations.

- Studies should be conducted to evaluate the effect of abiotic factors such as temperature and light intensity on the production of secondary metabolites in *C. molle*.
- Extensive studies should be carried out to assess the impact of *C. molle* on non- target organisms .
- Studies should be conducted with more test organisms so that it can be used in the treatment of infectious diseases as a broad spectrum antibiotic.
- Studies in depth can be further used to find the active compounds present in the fractions of the leaf and root of *C. molle* behind these biological effects.
- Additional research should be taken in order to characterize other toxicological effects which might arise following long term use of these fractions.
- Further toxicity studies are necessary, and more in-depth morphological experiments, to confirm this evidence.

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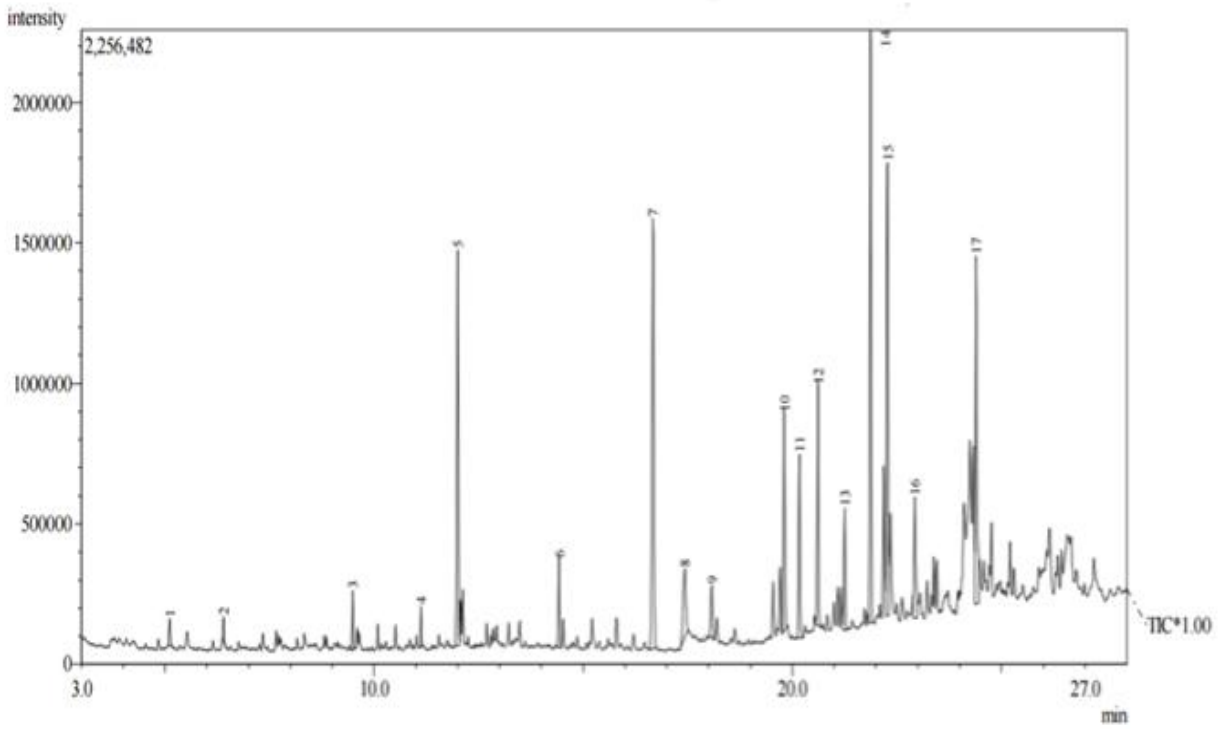
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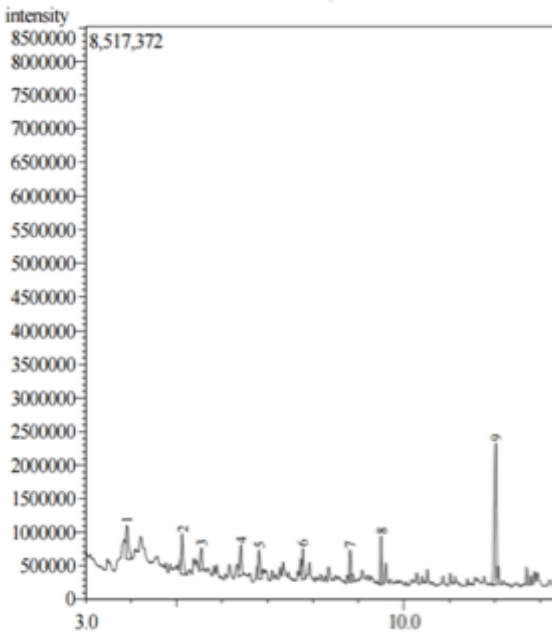
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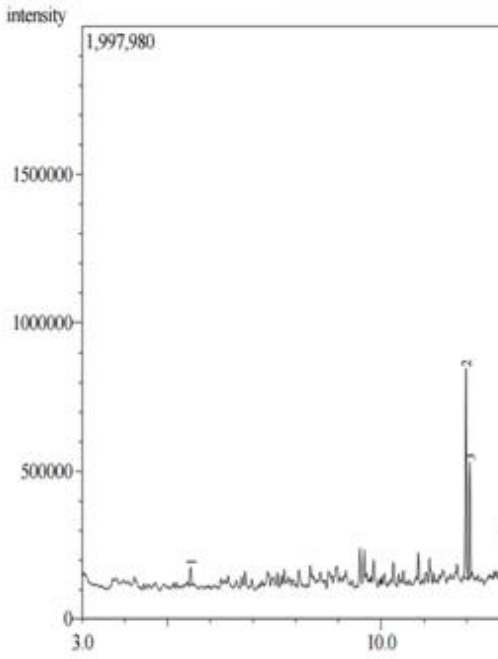
- **APPENDICES**



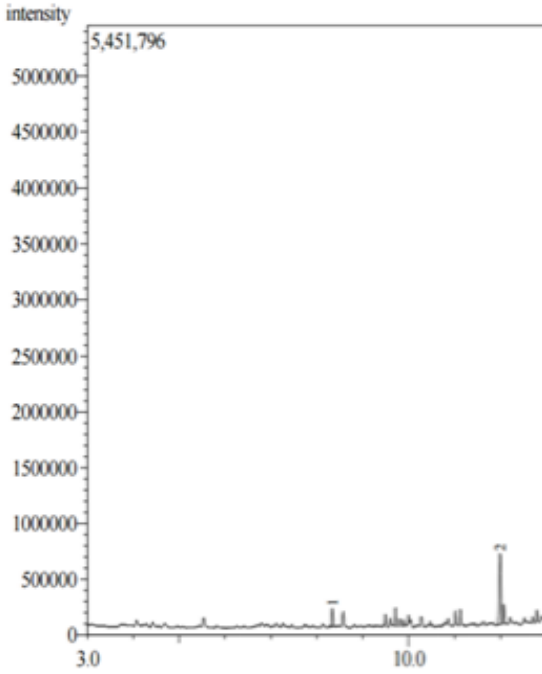
Appendix 1: GC-MS Chromatogram of N-butanol leaf fraction of *Combretum molle*



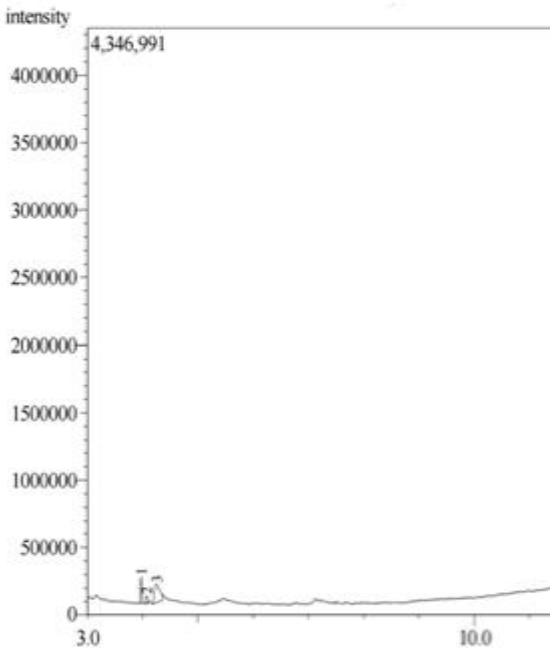
Appendix 2: GC-MS spectrum of N-butanol root fraction of *Combretum molle*



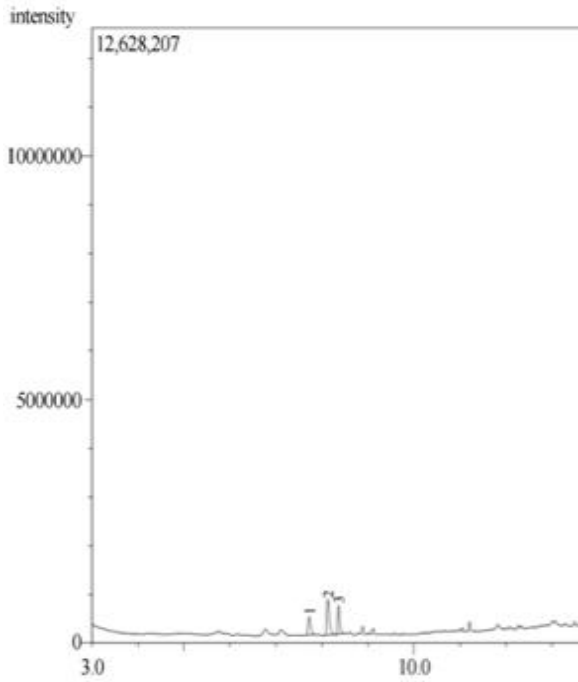
Appendix 3: GC-MS Chromatogram of Ethyl-acetate leaf fraction of *Combretum molle*



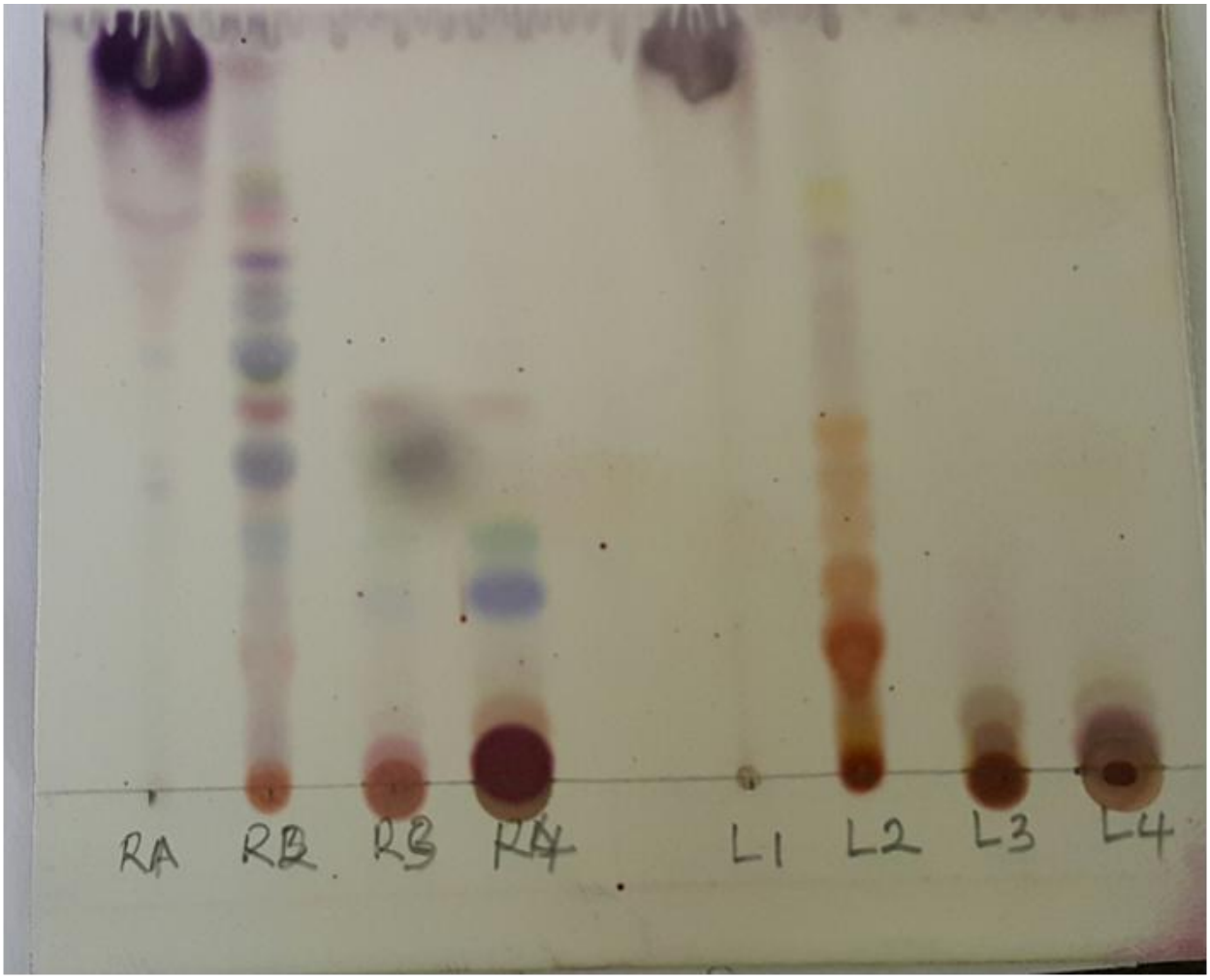
Appendix 4: GC-MS Chromatogram of the Ethyl-acetate root fraction of *Combretum molle*



Appendix 5: GC-MS Chromatogram of the Aqueous leaf fraction of *Combretum molle*



Appendix 6: GC-MS spectrum of the Aqueous root fraction of *Combretum molle*



- **Appendix 7: C**hromatogram of the the n-butanol leaf fraction, n-butanol root fraction, Ethyl acetate root fraction, Ethyl acetate leaf fraction, aqueous leaf fraction and aqueous root fraction of *Combretum molle* using the solvent Chloroform(C), Ethyl acetate (ETA) and Formic acid (F) in the ratio 5:4:1 respectively.

Key: R1= hexane root; R2= ethyl-acetate root; R3= n-butanol root; R4= aqueous root;
L1= hexane leaf; L2= ethyl-acetate leaf; L3= n-butanol leaf; L4= aqueous leaf.

treatment	S1		S2		S3		S4		S5		S6	
	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
100mg/ml	26	26	27	26	26	25	19	19	18	22	24	24
50mg/ml	24	24	24	24	24	21	16	17	14	20	22	21
25mg/ml	21	18	20	20	20	19	0	0	12	13	18	18
12.5mg/ml	18	17	18	18	18	18	0	0	10	0	14	14
6.125mg/ml	16	15	15	16	17	16	0	0	0	0	12	13

- **Appendix 8**: Inhibition zone of the fractions at different concentrations against *Xanthomonas axonopodis*

Treatment	S1		S2		S3		S4		S5		S6	
	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
100mg/ml	19	18	17	18	16	16	18	18	16	15	0	0
50mg/ml	17	17	14	14	14	14	16	16	14	14	0	0
25mg/ml	0	0	12	13	13	12	14	12	10	11	0	0

12.5mg/ml	0	0	10	11	11	11	12	10	0	10	0	0
6.125mg/ml	0	0	9	10	10	9	11	9	0	0	0	0

- **Appendix 9:** Inhibition zone of the fractions at different concentrations against *Pseudomonas syringae*

treatment	S1		S2		S3		S4		S5		S6	
	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
100mg/ml	0	0	10	11	12	13	14	14	12	12	12	12
50mg/ml	0	0	8	9	10	10	12	12	10	10	0	0
25mg/ml	0	0	0	0	0	0	0	0	0	0	0	0
12.5mg/ml	0	0	0	0	0	0	0	0	0	0	0	0
6.125mg/ml	0	0	0	0	0	0	0	0	0	0	0	0

- **Appendix 10:** Inhibition zone of the fractions at different concentrations against *Klebsiella pneumoniae*

treatment	S1		S2		S3		S4		S5		S6	
	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
100mg/ml	24	24	15	15	17	17	18	18	27	28	18	21
50mg/ml	22	22	13	13	16	16	16	16	25	25	22	18
25mg/ml	22	18	10	10	13	14	14	14	24	24	11	17
12.5mg/ml	15	16	0	0	11	12	0	12	24	22	17	14
6.125mg/ml	16	15	0	0	13	13	0	0	18	19	13	15

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- **Appendix 11:** Inhibition zone of the fractions at different concentrations against *Erwinia chrysanthemi*
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treatment	S1		S2		S3		S4		S5		S6	
	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
100mg/ml	18	18	20	20	20	20	19	17	19	19	26	26
50mg/ml	16	15	16	18	18	18	16	16	17	16	20	21
25mg/ml	14	14	11	0	16	16	14	14	16	15	19	21
12.5mg/ml	12	13	0	0	14	14	13	12	13	13	16	18
6.125mg/ml	11	11	0	0	12	12	11	10	12	12	12	13

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- **Appendix 12:** Inhibition zone of the fractions at different concentrations against *Salmonella* Typhi