Sudan University of Science and Technology

College of Graduate Studies

Isolation and Characterization of Some Constituents of Ximenia Americana. L Bark

عزل وتشخيص بعض مكونات الحميض الأبيض

A Thesis Submitted for the Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Chemistry

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DIDICATION

I dedicate this work to my beloved family. And my son Mazin

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All thanks premanly and finely for ALLAH, who by his virtue this project is accomplished.

I am greatly indebted to whoever helped me to make this work comes to being.

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ABSTRACT

Ximenia Americana is considered one of medicinal plant which are widely used especially in West of Sudan. This plant has been studied in this research, which is collected from Nuba Mountains and Babanusa.

In part I of the present study, crude extracts of different parts of plant was investigated for their biological activity. Preliminary investigations were carried out to select the plant extracts of the highest activity for further investigation. The extracts were screened for phytochemical constituents: alkaloids, saponins, tannins, flavonoids, triterpenoids/sterols and anthraquinones and assessed for their biological activities against four Gram positive and Gram negative bacteria: *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and one fungi: *Candida albicans* by the agar diffusion method.

The second part of this study dealt with the evaluation of preliminary screening and assessment results of the extracts. The *Ximeniaamericana* bark was selected for further investigation based on its promising biological activity. The bark material of the plant was extracted using solvents with different polarities. The ethyl acetate extract of the bark was selected for further fractionation based on antimicrobial activity results.

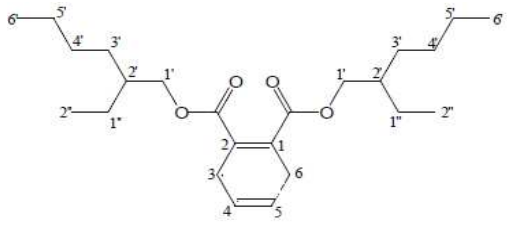
Isolation of active compounds from the different extracts was carried out using bioassay-guided fractionation. Selection of the active fractions for further fractionation was carried out based on antimicrobial activity results. Column chromatography monitored by TLC were used in fractionation and isolation of pure compounds. Various techniques including nuclear magnetic resonance (NMR), mass spectrometry (MS), ultraviolet (UV), infrared (IR) spectrometry and GC/MS were used for structure elucidation of the isolated compounds.

Column Chromatography has been done for ethyl acetate extract which produced (22) semi- pured fractions, then bioactivity of these extracts has been detected against previous bacteria and fungi, also they showed a level of bioactivity especially fraction 16 and has been purified via thin layer chromatography. Many compounds has been isolated and defined via (GCMS) device to give the following compounds:

Phenol, 2, 4-bis (1,1- dimethylethyl) ($C_{14}H_{22}O$), Methylhydroquinone,bis(trimethylsilyl) ($C_{13}H_{24}O_2Si_2$), 8-Octadecanone ($C_{18}H_{26}O$), Benzocyclodecene,tetradecahydro ($C_{14}H_{26}$), n-Heptadecyclocyclohexane ($C_{23}H_{46}$), Nonanoic acid, 9-oxo-,methyl ester ($C_{10}H_{18}O_3$), Phenol,2-4-bis(1,1-dimethylethyl) ($C_{14}H_{22}O$), Dodecanoic acid, dimethyl ester ($C_{13}H_{26}O_2$), Methyl tetradecanoate ($C_{15}H_{30}O_2$), n-Pentadecanol ($C_{15}H_{32}O$), n-Nonadecanol-1 ($C_{19}H_{40}O$), n-Tetracoanol-1 ($C_{24}H_{50}O$), 1-Nonadecene ($C_{19}H_{28}$), Triacontyl heptafluorobutyrate ($C_{34}H_{61}F_7O_2$).

Toxicity of these extracts has been studied via “Brine shrimp lethality” test, whereas study has showed that LD_{50} for Chloroform and ethyl acetate are: 5.1817 µ g/ml and 16.3765 µ g/ml respectively, which confirm toxicity of these extracts. Results show that these extracts have a bioactivity in treatment of cancer.

The name and Structure elucidation of the pure compound zm4 from fraction 16 Di-(2'-ethylhexyl)dihydro phthalate



Structure of Compound Zm4[Di-(2'-ethylhexyl)dihydro phthalate]

المستخلص

يعتبر الحميض الأبيض واحد من النباتات الطبية والتي تستخدم على نطاق واسع لا سيما في غرب السودان. وقد تمت دراسة هذا النبات في البحث، والتي تم جمعها من جبال النوبة وبابنوسة.

في الجزء الأول من هذه الدراسة، تم استخراج المستخلص الخام من أجزاء مختلفة من النبات وفقا لنشاطهالبيولوجي. وأجريت التحقيقات الأولية لتحديد المستخلصات النباتية الاعلى نشاط لمزيد من التحقيق. كما تم عرض المكونات الكيميائيه النبات مثل قلويدات، الصابونين الاسترويد والفلافونيدات، والتريبنويداتالجامدة وانثراكوينونيس وتقييم لأنشطتها البيولوجية ضد البكتيريا سالبة أربعة غرام و الإيجابية غرام مثل العصوية الرقيقة، المكورات العنقودية الذهبية، كولاي، الزائفة الزنجارية والفطريات واحدة : المبيضات البيض من طريقة نشر أجار.

الجزء الثاني من هذه الدراسة تناولت تقييم فحص وتقييم النتائج الأولية لمقتطفات. وقد تم اختيار لحاء الحميض الأبيض لمزيد من التحقيق على أساس النشاط البيولوجي . تم استخراج مواد لحاء النبات باستخدام المذيبات ذات قطبيه مختلفه. وقد تم اختيار مستخلص خلات الإيثيل من الحاء لمزيد من التجزئة بناء على نتائج النشاط المضادة للميكروبات.

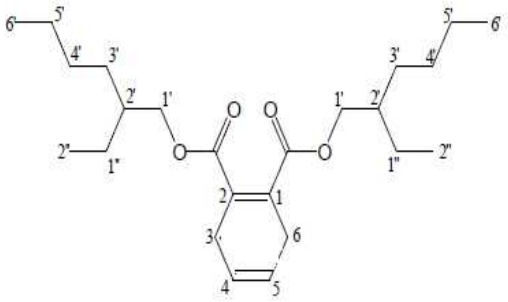
تم عزل بعض المركبات الفعالة من مستخلصات مختلفهباستخدام تجزئة موجهة الأحيائي. وجرى اختيار لمزيد من التجزئة بناء على نتائج النشاط المضادة للميكروبات. واستخدم العمود الكروماتوجرافي اللوني مراقبتها من قبل الطبقه الرقيقهTLC في تجزئة وعزل المركبات النقية. تم استخدام تقنيات مختلفة بما في ذلك الرنين النووي المغناطيسي (NMR)، مطياف الكتلة (MS)، الأشعة فوق البنفسجية (UV) والأشعة تحت الحمراء (IR) الطيف GCMS لتوضيح بنيهالمركبات المعزوله.استخدم العمود الكروماتوجرافي اللوني وانتج(22) نصف نقى من مستخلص خلات الإيثيل ، ثم تم الكشف عن النشاط الحيوي لهذه المستخلصات ضد البكتيريا والفطريات السابقة، كما أنها أظهرت مستوى من النشاط الحيوي وخاصة جزء 16 و تم تنقيته عبر رقيقة اللوني طبقة. تم عزل العديد من المركبات وتعرف عن طريق جهاز (GCMS) لإعطاء المركبات التالية:

Phenol, 2, 4-bis (1,1- dimethylethyl) ($C_{14}H_{22}O$), Methylhydroquinone,bis(trimethylsilyl) ($C_{13}H_{24}O_2Si_2$), 8-Octadecanone ($C_{18}H_{26}O$), Benzocyclodecene,tetradecahydro ($C_{14}H_{26}$), n-Heptadecyclocyclohexane ($C_{23}H_{46}$), Nonanoic acid, 9-oxo-,methyl ester ($C_{10}H_{18}O_3$), Phenol,2-4-bis(1,1-dimethylethyl) ($C_{14}H_{22}O$), Dodecanoic acid, dimethyl ester ($C_{13}H_{26}O_2$), Methyl tetradecanoate ($C_{15}H_{30}O_2$), n-Pentadecanol ($C_{15}H_{32}O$), n-Nonadecanol-1 ($C_{19}H_{40}O$), n-Tetracoanol-1

($C_{24}H_{50}O$), 1-Nonadecene ($C_{19}H_{28}$), Triacontyl heptafluorobutyrate ($C_{34}H_{61}F_7O_2$).

وقد تمت دراسة سمية هذه المقتطفات عبر "فتك الماء المالح الروبيان " ، في حين قد أظهرت الدراسة أن LD_{50} عن الكلوروفورم وخلات الإيثيل هي: 5.1817 µ غ / مل و 16.3765 µ غ / مل على التوالي، التي تؤكد سمية هذه المقتطفات. وتبين النتائج أن هذه المقتطفات لها النشاط الحيوي في علاج السرطان.

الاسم والهيكل استجلاء هذه zm4 مجمع نقية من جزء 16 للعرق (ethylhexyl-'2) ثنائي هيدرو الفثالات



هيكل مجمع Zm4 [عرق (ethylhexyl - '2) ثنائي هيدرو الفثالات]

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CHAPTER ONE

INTRODUCTIONAND LITERATURE REVIEW

1. INTRODUCTION

1.1 Phytochemistry

Phytochemistry, plant chemistry has developed in recent years as a distinct, somewhere in between natural product organic chemistry and plant biochemistry and closely related to both of them. Phytochemistry concerned with the variety of organic substances that are elaborated and accumulated by plants, and deals with the chemical structures of these substances, their biosynthesis, and metabolism, their natural distribution and their biological function (Harborne, 1973). The range and number of discrete molecular structures produced by plants is huge and such that present rate of advance of our knowledge of them that a major problem in phytochemical research is the collection of existing data on each particular class of compound (Harborne, 1973). Phytochemical means plant chemicals that are found in all kind of plants fruit and vegetables.(Harborne, 1973)

Another definition of phytochemical is (phyto) comes from the Greek word plant (phytochemical) could refer to a vary naturally occurring chemical in plants. Phytochemicals refer to naturally occurring compounds that have physiological effect in humans phytochemicals may prevent or delay chronic generative diseases and enhance human health, phrase like (chemo preventive agents) are sometimes used to describe phytochemicals though to reduce risk for captain type cancer. Because most of the components currently of interest for health maintenance and disease prevention are found in plant functional foods are some times used inter changeable with phytochemicals (Hanson, 1972).

Phytochemical progress has been aided enormously by the development of rapid and accurate methods of screening plants for particular chemical techniques (Scheinmann, 1970).

1.2 Natural products

Natural products are, generally, either of prebiotic origin or originate from microbes, plants, or animal sources (Nakanishi, 1999). As chemicals, natural products include such classes of compounds as terpenoids, alkaloids, flavonoids polyketides, amino acids, peptides, proteins, carbohydrates, lipids, nucleic acid bases, ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and so forth (Jarvis, 2000).

Natural products continue to play an important role in the discovery and development of new pharmaceuticals, as clinically useful drugs, as starting materials to produce synthetic drugs, or as lead compounds from which a totally synthetic drug can be designed (Naem *et al*., 2009).

Higher plants have been over time an extremely popular source of natural products, therefore compounds isolated and identified from this source will undoubtedly continue to make strong contributions to modern therapeutics (Connolly, 1997).

Over 100 chemical substances that are considered to be important drugs are either currently in use or have been widely used in one or more countries. In the world they have been derived from a little under 100 different plants (Cragg and Newmun, 2001).

Natural products include any organic substance isolated from living organism (Scheinmann, 1970). Originally, chemists were concerned with the isolation and separation of compounds from natural materials and establishment of their structure. Thus the study of natural products began with the isolation of, identification and classification of

compounds occurring in and associated with living materials (Tedder, *et al*., 1972),natural products are those organic chemical compounds of natural origin produced by living cells, and being those belonging to plants, animals microorganism, etc. They are classified according to their chemical structure and biogenetic organism into alkaloids, antibiotics, terpenoids, flavonoids polyphenols and macrocyclic antibiotic (Culberson, 1969). Many of the natural products are used as therapeutic a gents, food stuffs, tannins, oils and gums (Mann, 1978), the living organism of natural products may be considered as the bio-synthetic laboratories in which many metabolic process take place, not only for chemical compounds (carbohydrates proteins, dates, etc) that are utilized as food for man and animals, but also for multitude of secondary compounds (alkaloids, volatile oils) that exert a physiological effects (Balbaa, *et al*., 1981).

1.2.1 Medicinal Plants

Medicinal plant have a promising future because there are a bout half million plants around the world and most of their medical benefits have not been investigated yet, and their medical activities could be decisive in the treatment of present of future studies (Bassan, 2012).

A half of these have been studied exhaustively for their chemical composition and medicinal value (Cowx and Balick 1994)

A half percent of these have been studied exhaustively for their chemical composition and medicinal value (Cox and Balick, 1994).

The plants are potential source of medicines since ancient times. According to World Health Organization, 80% of the populations in the world depend on traditional system of medical practitioners for their medicinal needs (Elumalai *et, al.* 2009). Traditional systems of medicine

continue to be widely practiced on many accounts, population rise, inadequate supply of drugs, and prohibitive cost of treatments allopathic drugs and development of resistance to currently used drugs for infectious diseases have led to increased emphasis on the use of plant materials as a source of medicines for a wide variety of human ailments (Brindha *et al.,* 2008).

Medicinal plants contain molecular structures and a built-in ability to produce substances (secondary metabolites) to protect itself from injury and disease. Plants are able to produce a wide array of secondary metabolites through intricate metabolic pathways (Robert, 2009). They are rich source of bioactive compounds and thus serve as important raw materials for drug production (Lakshmi *et al*., 2006).

Therefore, medicinal plants are economically important major source for drug production (Tullanithi *et al*., 2010). In this regard, annual expenditures on herbal therapies alone have exceeded of $5 billion in recent years literature indicates that medicinal plants are the backbone of traditional medicine (Fransworth, 1994).

Medicinal plants are known by their required clinical effects on the abnormal living tissues or organs while toxic ones are known by their ability to cause a non required physiological deviations in animals bodies (Clarke *et al*., 1981).

Medicinal plants are used all over the world for different therapeutic action. They are used in traditional and popular medicine often within a conceptual structure with particular taxonomy of ailment and diseases (Hersch, 1993).

1.2.2 History of medicinal plants

The use of plants as medicine is older than recorded history. Probably the earliest evidence for the use of a medicinally active plant is

that for the opium poppy. A number of Linear band keramik sites in the Rhineland and Swiss Neolithic lake villages dated to 5000- 4500 BC have yielded samples of opium poppy seeds (Papaver somniferum) (Merlin, 1984). Early evidence for the use of Ephedra spp. (2000-1500B.C: Sarianidi, 1994), Cannabis saliva (c. 2000 BC; Sherratt, 1991) and Perganum harmela (Syrian rue) (5^{th} millennium BC; Sherratt, 1995).

Egyptian hieroglyphs show physicians of the first and second centuries B.C., treating constipation with sienna pods, and using caraway and peppermint to relieve digestive dyscrasias. The medical papyri reveal substantial use of hyoscyamus, scopolamine, and opium poppy along with ritual and medical procedures. This extensive Egyptian pharmacopoeia later influenced the Greek, Roman, and Hebrew (Frost, 2006).

The oldest written information in the Arabic traditions comes from the Sumerians and Akkadians of Mesopotamia, thus originating from the same areas as the archeological records of Shanidar IV (Heinrich *et al*., 2004).

Traditional use of medicine is recognized as a way to learn about potential future medicines. Researchers have identified number of compounds used in mainstream medicine which were derived from "ethnomedical" plant sources (Fabricant and Farnsworth, 2001)

Ximenia americana

1.2.3.1 Botanical description of *Ximenia americana*

Ximenia Americana is a semi-scandent bush-forming shrub or small trees, 2.7m high from Africa topic (Beentje, (1994). Thrunk diameter seldom greater than 10cm (Booth and wichens, 1988) bark dark brown to pale gray, smooth to scaly (Ambasta, 1986). The lax,

usually divergent branch in forms of a rounded or concial crown (Waston and Dullwitz, 1992). Branchelts purple red with a waxy bloom and tree usually armed with straight slender spines (Storre, 1995). Sometimes semi-parasitic with haustoria on the roots, leaves alternate, lanceolate to elliptic 3-8 to 1.5-4 cm variable thickness; obtuse or emarginated 3-7 pairs veins in conspicuous, petioles short, slender up to 6mm long canaliculated gray, green, hairless and leathery or thin flesh, when crushed, young leaves smell of bitter almonds (Vogt, 1995). The fragrant white yellow – green or pink flowers occur in branched in florescence brown on shortly Pedunculate axially, racemes or ambulates; peduncles 3-7mm long both peduncles or pedicles globrus (Ambasta, 1986).

Fruits globes to ellipsoidal drupes about 3cm long 2.5cm thick, galobrous, greenish when young, becoming yellowish or, rarely orange-red when ripe containing a juicy pulp and one seed.

Seed woody, light yellow up to 1.5cm long, 1.2 cm thick with fatty kernel and a brittle shell (Dale; Greenway, 1961).

1.2.3.2 Distribution

The plant distributed one Rockey Hills and Khor brank in rain full about (800 -1200 mm.) and located in South Elgdarif .and Nuba mountains and wide spread in blue Ninle state(Hamza, 1992).

1.3 Classification of *Ximenia americana*

Current name	: *Ximenia americana*
Kingdom	: Plantae
Unranked	: Angiosperms
Division	: Mongo liophyta
Class	: Mongoliopsida
Subclass	: Rosidae

Order : Sontalates

Family : Olacaceae

Genus : Ximenia

Species : X. americana

1.3. 1 Common names of *Ximenia americana*

Ximenia Americana had different names distributed over the world those are; kleins urpruim (African) inkoy kol (Amhoric): abukhamier, abukhamira, ankwi, humeid abiad, kelto, medica. (Arabic) mulebe, (Bemba); teregi (Bislama); false sondale wood, hogplum, sea side plum, small sourplum, sour plum, tallow nut, tallow wood, wild lime, wild olive, wild plum (England) cerise demer, citron demer, corc, macaby (French) mulutulwa, mungomba, musongwasogwa, mutente (Losi); museka (Luganda); musongwasogwa muvulama (Lunada); nogbe, ntoge, sene, (Mandinka) madarau, madarad, monkey plum (Somali); kamulebe, mtundu, mtunduluwa, ntengel (Nyanja) Almendra de costa, ciruelillo, hiscaco (Spainsh); mpingi, mtumbui, mtundakula , timbui (Swahili); chiru-illantai, kadaranji, struyiland Dai (Tamil); melhtta, mullo (Tigrigan); muchnfwa (Tongan); (Storrs, 1995).

Meal (Bik.), Pangungan (Yak.), Paniungan (Sul.), Sulo-sulo (Bag.), Sour plum (Engl.), Wild olive (Engl.), Wild lime (Engl.), Mountain plume (Engl.), Seaside plume (Engl.), Citron of sea (Engl.), Tallownut (Engl.), Hai tan mu (Chin.)

1.3.2 Ecology and distribution of *Ximenia americana*

1.3.2.1 Natural habitat

A mostly solitary tree dispersed in open country savannah, gallery forest (Eggelling, 1940), long coastal areas, in the under storey, and dry forest in dry woodlands or river banks, *Ximenia americana* is drought resistant (Booth and Wichens, 1988).

1.3.2.2 Geographic distribution

Xaminia americana is wide distributed tree over the world, and it found in the countries (Angola, Argentina, Australia, Benin, Bolivia, Botswana, Brazil, Burkina Faso, Burundi, Cameroon, Chad, Chile,

Colombia, Cotedlvior, Democratic Republic of Congo, Ecuador, Eritrea, Ethiopia, French, Guiana, Gambia, Ghana, GUINEA–Bissau, Guyana, Honduras, India, Kenya, Liberia, Mali, Mexico, Mozambique, Namibia m New Zealand, Nicaragua, Negeria, Panama, Paraguay, Peru, Rwanda, Senegal, Sierra leone, Somalia, South Africa, Sudan, Tanzania, Togo, Uganda, United States of America, Uruguay, Venezuela, Zambia and Zimbabwe) (Burkart, 1976).

In Sudan *Xaminia americana* widely distributed in different regions. It is found in Darfur (Jabal Marra, Radom); Blue Nile (Ingessena Hills) Kordofan (Nuba Mountains, Nuhand); Red sea Hills (Erkwit); Bahar ElGhazal, Upper Nile and Equatoria (Torit) (Hamza, 1992).

1.3.2.3 Soil requirements

Found on many kinds of soil, often poor and dry, including clays, clay loom, loamy sands, sand clay loom and sand (FAO, 1986).

1.3.2.4 Propagation method

Propagation from seed and cutting the tree regenerate naturally from seed and coppice. Fresh seed should be sown for good germinate seed per kg = 1400.

Table (1): *Ximenia americana* requirement for propagation (FAO, 1986).

Rainfall	**Average**
Minimum altitude (m)	20m
Maximum altitude (m)	2000m
Minimum rainfall (mm)	300m
Maximum rainfall (mm)	1250m
Minimum temperature (C̊)	14C̊
Maximum temperature (C̊)	30C̊

1.3.2.5 Growth cycle

On a good site trees produce fruits in after 3years. It flowers and fruits throughout the year, independent of climatic regimes. Seeds are collected in July and august at the coast (Felker, 1981).

1.4 Important Uses of *Ximenia americana*

1.4.1 Food

The fruit is thirst quenching. It is used as a drink and making jams and jellies. Also the fruit is used in cases of habitual constipation and gastric troubles oil from the seed is used to soften human skins and animal (Brown and Massey, 1929). In South Africa the fruit is used for jelly making and north Nigeria it used for making a sour preserve. The young leaf some time cooked and eaten (Watt and Brayer, 1962). The fruits as well as being pleasant to eat raw or cooked can be used to make juice, jams and jellies, or an intoxicating drink (Hines and Eckman, 1993). The pulp of seed and fruit contains hydro-cyanic acid, and it is advisable not to chew the seed (Peltonen, *et al*., 2005). Kernel oil is used as vegetable butter. Young leaves are edible after through cooking (FAO, 1986).

1.4.2 Medicine

It is being investigated by researchers from Nigeria's Ahmadu Bello University. In animal tests, it is showing promise against the parasite that causes sleeping sickness and severe anemia in livestock in many parts of Africa. Leaf extract is active against *Escherischia coli*, *Pseudomonasaeruginosa* and *Candida albicans*. Tannins, flavonoids, alkaloids, saponins, anthraquinones, starch, general glycosides and bitter principles can be found in the extract. Indigenous people of Florida used the bark to treat sore muscles and gums. The

root also contains the fatty acids tariric acid and 10Z, 14E, 16E-octadeca-10, 14, 16-triene-12-ynoic acid (Maikai *et al*., 2009)

1.4.3 Fuel

Firewood and charcoal are the chief uses of the wood, because the trunk is usually too small to make it useful for timber (FAO, 1986).

1.4.4 Tannin or Dyestuff

Bark is used for tanning it contains approximately 17% tannin (Reed, 1995), which gives leather a reddish colour (Deckert *et al*., 2005). Bark is used to strengthen indigo dyes.

Tannins have been traditionally used for protection of inflamed surfaces of the mouth and treatment of catarrh, wounds, hemorrhoids, and diarrhea, and as antidote in heavy metal poisoning.

1.4.5 Lipids

The fruits yield up to 67.4% oil from the seed that has been used as a body and hair oil (Vaughan, 1970). The oil is not edible, and the presence of a rubber like substance excludes is from many industrial uses (Eromose *et al*., 1994).The highest oil obtained was 51% w/v. The physical properties of the oil were found to be reactive index (1.477), density (0.9376 g/ml), boiling point (157°C) and viscosity 42 at 70°C and 227.58 at 25°C. from fixed oil of extracted the seeds of *Ximenia* Americana (Saeed and Bashier, 2010).

1.4.6 Essential Oils

Heartwood of *Ximenia americana* contains an essential oil, which would be used for fumigation, the flowers have an essential oil that could be a good substituent for orange blossom (Biagi, 1994).

1.4.7 Alcohol

In South Africa, The fruits have been used to make a kind of beer (Coastes, 1988).

1.4.8 Traditional Medicine

The use of plant in religious ceremonies as well as for magic and medicinal purposes is very common place and wide spread (Elegami *et al*., 1976), based upon strong primitive roots (Amare, 1970). The art of medicine is still widely practiced while much of this lore is indigenous. Among natives of various countries knowledge of medicine has been passed by word of mouth from one generation next by medicine men (Liao and Shi, 2005). Some of lore is lost at each point of transfer (Benoit, 1996). The plants comprising the remedy are selected from different ecological locations such as high loaned or low land (Ivorra *et al*., 1989).

Ximenia americana bark, fruit and leaves have many uses in local medicine for people and animals (Mwang and malii, 1994). Leaves and twigs are used for fever, cold, as mouth wash for tooth aches, as laxative and an eye lotion (Omer and Ali, 1998), leaves are used for headaches and poison antidote (Frebberger and Vanderjagt, 1998). Roots treat skin problems headachaches, leprosy, hemorrhoids, sexually transmitted diseases, guinea worm, sleeping thickness, oedema and act as an antidote to poison (Teo, 1997).

X. americana seed oil contained oleic, linoleic, linolenic, arachidonic, eicosatrienoic, erucic and nervonic acids (Eromosele and Eromosele, 2002).

The chemical properties of the oil were: iodine value (47.59), acid value (0.2805), peroxide value (30), saponification value (11.43), ester value (9.82), and the ratio value (35.009). The molecular weight of the major component of the oil was 604. The major component of the oil ($C_{40}H_{76}O_3$) was identified as methyl-14,14- dimethyl – 18-hydroxy heptatriacont-27,35-dienoate

$[CH_3OCO(CH_2)_{12}C(CH_3)_2(CH_2)_3CHOH(CH_2)_8CH=CH(CH_2)_6CH=CHC_3]$ (Saeed and Bashier, 2010).

The major con-stituents of the volatile oil of the leaves of *X. americana* were benzaldehyde, hydroxy benzyl cyanide and iso-phorone (Mevy *et al.*, 2006). Mitei *et al*., 2009 reported the contents of α and β tocotrienol, campesterol, β- amyren and lupeol from the seed oil of *X. caffra* in Botswana.

The fruits are eaten in large quantities and act as a vermifuge (Niemi *et al*., 2005), The bark is used dried or powdered and applied to skin ulcers (Kuroki; Conn, 1989).

Fruit is put on the head for headaches, placed in bath water for sick children, and used for kidney and heart complaints (Lason, 2005). The fruits are eaten in large quantities and decoction of the roots or fruits is used to treat dysentery in calves (Katende, 1995). Medicinal plant play a very significant role in health care needs of rural population and other third world countries especially in treatment of diseases (Maikai, 2008); *Ximenia americana* is a plant used in traditional medicine for the treatment of malaria, leproutic ulcers and skin infections of mixed origin in northern parts of Nigeria (Ibrtoye, 2003). Medical plants play a very significant role in health care needs of rural populations in African and other third world countries especially in treatment of diseases.

The plant is used traditional medicine for treatment of malaria, fever, Ieprotic ulcers and skin infections of mixed origin in northern parts of Nigeria (Ogunteye and Ibitoye, 2003). reported the medicinal uses of *X. americana*include treatment of fever, stiffness, onchocerciasis, sore throat asthma, and headaches, (Maikai, *et al*., 2007).

X. americana include treatment of fover stiffness, onchocerciasis, sore threat asthma, and headaches (Maikai *et al*., 2007)

1.5 Chemical constituents of *Ximenia americana*

The nutritional role of wild plants in Nigerian diet was investigated, leaves of seven plants species were analyzed for their mineral, fatty acid and mino acid contents. *X.americana*contain large amounts ofcalcium andsignificant amounts of selenium and phosphorus (Freibbger, *et al.,* 1998).

And also contain tannins, flavonoids, saponins, and starch from the leaves of the plant, but not Alkaloid and Anthra- quinnones. (Trease and Evans 1983),

X. americana pulp, seed and fruit contains hydrocyanic acid. Bark contains approximately 17% tannins materials. Also tannin is found in roots. Seed yield about 67.4% oils, heart wood and flowers contain essential oils (Fatope and Adom, 2005). Fruits, fruits pulp, leaves, twinges and roots contain constituents used in folk medicines (Benoit and Santillana, 2000).

Extraction of fixed oil from the seeds of *X.americana,* the yield percentage, physical and chemical properties of the extracted oil was found, the highest oil obtained was 51% w/v. The physical properties of the oil were found to be reactive index (1.477), density (0.9376 g/ml), boiling point (157°C) and viscosity 42 at 70°C and 227.58 at 25°C. The chemical properties of the oil were: iodine value (47.59), acid value (0.2805), peroxide value (30), saponification value (11.43), ester value (9.82), and the ratio value (35.009). (Saeed and Bashier, 2010).

The chemistry of natural products remains a challenge and an important field of research in several science areas (chemistry, biology, medicine, agronomy, botany and pharmacy).The reasons for it’s large

use are the considerable pharmacological potential observed in natural products, in the great development in the process of detection, isolation, purification and, especially, the advances in spectrometric techniques [infrared (IR), mass spectrometry (MS) and nuclear magnetic resonance (NMR ^{1}H and ^{13}C) for structural elucidation of new and complex compounds. (Loganathan *et al.*,1990).

X. americana, commonly called “ameixa do mato”, “ameixa de espinho” and “ameixa daBahia”, is widely distributed in northeast Brazil. A tea obtained from its barks has been used in popular medicine as cicatrizing, astringent and as an agent against excessive menstruation. As a powder, it treats stomach ulcers and the seeds are purgative (Braga, 1976; Pio-Correia, 1984). This specimen has been recently examined (Araújo *et al*., 2008) and the stem ethanolic extract afforded steroids (stigmasterol and sitosterol), triterpenoids (betulinic acid, oleanolic acid , 28-O-(-D-glucopyranosyl) oleanolic acid, 3-oxo-oleanolic acid, 3β–hydroxycicloart-24(E)-ene-26-oic acid and sesquiterpenoids (furanoic and widdrane type). A large number of sesquiterpenes are constituents of essential oils of higher plants and seem to intervene in the pharmacological properties attributed to these volatile fractions (Bruneton, 1999). It has been clarified that the biological activities of the liverworths are due to terpenoids and lipophilic aromatic compounds (Atta, 1988). Steroids and triterpenes with therapeutic interest and manufacturing employment are a group of secondary metabolites of outstanding import ance (Bruneton, 1999). Considerable recent work strongly indicates the great potential of the triterpenoids as source of use medicinal (Mahato *et al*., 1992).

1.6 Biological activity

1.6.1 Antimicrobial activity of *Ximenia americana*

The antimicrobial activity of the extracts of the various parts of the investigated plants such as roots, leaves, seeds, stem barks and fruits, appears to be due to the presence of secondary metabolites such polyphenols, triterpenes, sterols, saponins, tannins, alkaloids, glycosides and polysaccharides (Geyid *et al.*, 2005; James *et al*., 2007; Maikai *et al*.,2009; Ogunleye *et al*., 2003).

X. americana is a plant used in traditional medicine for the treatment of malaria, leproutic ulcers and infectious diseases of mixed origin by natives in Ethiopia, Guinea, Sudan and in the Northern part of Nigeria (Geyid *et al*., 2005; James *et al*., 2007; Magassouba *et al*., 2007; Maikai *et al*., 2009; Ogunleye *et al*., 2003; Omer & Elnima, 2003).

Study of the stem bark of *X.americana* was evaluated for its phytochemical constituents and acute toxicity effect on thirty Swiss albino mice. The extracts were administered intraperitoneally/orally at doses of 10, 100, and 1000 mg/kg body weight. The results revealed no death with doses up to 5000 mg/kg body weight. However, the initial reactions included excitement, restlessness, lack of appetite and later reduced activity during the first 24 h of extract administration. The symptoms were dose dependent with signs noticeable with increasing dosage. Post mortem, hematological and histopathological examination did not show any significant ($P<0.05$) damage as a result of the extract administration. However, there were significant ($P<0.05$) weight changes. Phytochemical screening of the aqueous stem bark extract revealed the presence of cardiac glycosides, flavonoids, saponins, and tannin. The results suggest that the aqueous extract is not acutely toxic to the mice. (Maikai, *et al*., 2008).

Study reported that the antimicrobial properties of the stem bark extracts of *X.americana* were screened against *Escherichia coli, P.aeruginosa, Staphylococcus aureus, P. vulgaris, Candida albicans, B. subtilis* using the disc diffusion method. The result revealed the methanolic and water extract showed significant ($P<0.05$) broad spectrum activity on the growth of many of the test organisms (*E. coli*, and P. *vulgaris, S.aureus*, *P. aeruginosa* and B. *subtilis*), while the butanolic extract had little activity. Phytochemical screening revealed the presence of alkaloids, saponins, flavonoids, cardiac glycosides, terpenoids and tannins. The study supports the traditional usage of this plant by herbalist as remedy in curing microbial infections. (Maikai, *et al.*, 2009).

The ethanolic extract of the bark of X. Americana was subjected to phytochemical and biological activity screening. The extract revealed the presence of Flavonoids, Steroids, Tannins, Alkaloids, Phenolic compounds, Saponin, Terpenoids, and glycosides. Also the extract exhibited significant activity against *Staphylococcus aeruginos* and low activity against *Pseudomonas Aeruginosaorther* investigations led to isolation of two compounds 1 is new : 3-Methyl-1-oxoiso chroman-8-Carboxylic acid, and 2 is known steroid: Ergosta-4, 6, 8, 22-tetraen-3-one, their structures were elucidated by spectroscopic methods. (Abd alfatah. *et al*., 2013).

Physiochemical screening of stem bark solvent extracts of *X. americana* in three solvent extracts revealed the presence of flavonoids, saponins, tannins and terpenoids. However, alkaloids pylobatnins and cardiac glycosides, were absents in the water extracts (Mainkai, 2009). The leaves extract of the *Ximenia Americana*, was found to be active aginst

Escherischia coli, *Pseudomonas aeruginosa* and *Candida albicans* (Ogunleye and Ibitoye, 2003).

The vegetal extract obtained from *X. americana* commonly used in cot, divorces by native healer, for the treatment of malaria were tested on two strain of *Plasmodium falciparum*. The extract had apparent inhibitor effect on *P. falciparum* (Choloroquine. resistant) (Benoitetal, 1996).

1.6.2 Anti canecer activity

The antineoplastic activity of a plant powder used in African traditional medicine for treating cancer was investigated by analyzing the activity of various extracts in *vitro*. the most active , aqueous extract was subsequently subjected to declared investigation in panel of 17 tumor cell lines .Varied in magnitude, from 1.7mg/ml in MCH7 breast cancer cells, 170mg/ml in AR230 chronicmyeloid leukemia cells. Immortalized, non- tumorigenic cell lines showed marginal sensitivity. In addition , kinetic and recovery experiments performed in MCF7 and U87- MG cell and a comparison with the antineoplastic acitivety of miltefosine, gemcitasbine, and cisplatinum in MCF7 and U87- MG , HEP2 and SAOSZ cells revealed on obvious similarity betten the sensitivity profiles of the extract and three standard agents in *vivo* antitumor activity was determined in the (C531 colorectal cancer rat model . significant antitumor activity was found following administration of equitoxic doses of 100 (orally), and 5 mg/kg (intra – peritoneal)

Physicochemical characterization showed that active antineoplastic components of the plant material are proteins with galactose affinity. Moreover, by mass spectrometry, one of these proteins was shown to contain stretch of 11 amino acids identical to a tryptic peptide from the ribsome – in activating protein ricin (Voss, *et al*., 2006)

1.6.2 Cytotoxicity Studyusing Brine Shrimp Lethality Test (BSLT)

Brine Shrimp was used as testing organism to evaluate the cytotoxic effect of the plant extracts.

1.6.2.1 Testing organism

Scientific name: *Artemia salina*

Common name: Brine shrimp

1.6.2.2 Scientific classification

Kingdom: Animalia

Phylum: Arthropoda

Subphylum: Crustacea

Class: Branchiopoda

Order: Anostraca

Family: Artemiidae

Genus: Artemia

Species: *A. salina*

1.6.2.3 Brine Shrimp lethality Description

This assay was developed by Meyer *et al*.,1982 and is widely used as a simple, reliable and cheaper prescreens method to determine the cytotoxicity of crude plant extract and pure natural compounds, especially antitumor compounds from the natural source (Hullatti and Murthy, 2010). Bioactive compounds are often toxic to shrimp larvae (*Artemia salina*); therefore, Brine Shrimp Lethality Assay is in use to monitor different chemicals' in vivo lethality to shrimp larvae (..HarwigandScott, 1971; Solis *et al*., 1993; Lewan *et al*., 1992). The general toxic activity was considered weak when the LD_{50} values of crude extracts and pure substances were between 500 and 1000 µg/ml, moderate when the LD_{50} was between 100and 500 µg/ml, and designated as strong when the LD_{50} ranged from 0 to 100 µg/ml but those with <20

µg/ml were considered to be very active (Padmaja *et al*., 2002). While, (Meyer *et al*., 1982) considered the LD50 values > 1000 µg/ml as non-toxic.

1.7 Aims and Objectives

Sudan has different floristic regions spreading from rich Savannah to poor Sahara. Through these different climate regions, some medicinal plants are distributed over a large area. Therefore Sudan is a rich country plants that are used as folk medicine. *X.americana*. L. the plant subjected for investigation in this study is one of those medicinal plants.

The main objectives of the project are:

1- Phytochemically scanned the bark and leaves of *X. americana* for the different naturally occurring products.

2- To carry out bioassay-directed phytochemical isolation and structure elucidation, identification of the possible active constituents using different spectroscopic methods.

3- Chemical studies of the bark and leaves.

4- To investigate and evaluate the antimicrobial activity of *X.americana*.

5-To assess the cytotoxicity of *X.americana* extracts using brine shrimp lethality test.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant Materials

X. americana brought from the West of Sudan (West Kordafan). The plant collected from the forest (round Babanousa city and Nuba Mountain) in rainy season in July and August in the year 2010. Some of the plants were purchased from the local market. The plant materials were identified by Dr. Wail Elsadig and Dr. Haider Abd AlGadir at the Herbarium of Medicinal and Aromatic Plant Research Institute (MAPRI).

2.1.2 Chemicals and reagents

Acetic acid and Formic acid were obtained from Fluka, Switzerland. Acetic anhydride, Petroleum ether and Aluminum chloride were obtained from Merck. Germany. Acetone technical was obtained from CDH, (P) LTD. England. Ammonium hydroxide was obtained from Bell and Son Co, England. Chloroform AR, Hexene, Benzene, Toluene, Magnesium turuing wire were obtained from S.d. fine. Chem. Limited- India. Ethanol was obtained from (Avondale laboratories, Ltd. Ethyl acetate was obtained from Sharlu, spain. Ferric chloride was obtained from (Modern chemical corporation, Egypt. Hydrochloric acid was obtained from (The British Drug-House.England). Hydrogen peroxide was obtained from Bell, Son and Co, England. Methanol was obtained from loba chem. PVT Ltd. n-butanol was obtained from (Lab tech, India). Potassium hydroxide, Potassium Iodide, Mercuric chloride, Sodium chloride, and Sulphuric acid were obtained from the British Drug House, England. Vanillin was obtained from (BDH chemicals, England).

2.1.3 Equipment and glass ware

Equipments, glass wares and their sources are listed

Beakers, separatory funnel, conical flasks all type of Pyrex were obtained from Iso lab, Germany.

Chromatographic jar, Capillary tubes 75 X 2mm obtained from Marienfeld, Germany. Chromatographic sprayer obtained from Superfit, India. Glass plates 20X20 cm, Silica gel for TLC and Silica gel type GF_{254}-precoated plates were obtained from EMD Chemicals Inc, Germany.

2.1.4 Aapparatus

- Sensitive Balance, electronic obtained from Galenkamp England.
- Column chromatography obtained from Duran, England.
- Soxhelt obtained from karb-kolb, W. Germany.
- TLC spreading device obtained from Heidelberg, W. Germany.
- Rotary evaporator, vaccum Buchi obtained from Switzerland.
- Water bath model 20L obtained from Pittsburgh, England.

2.1.5 Instrument

2.1.5.1 UV Spectrophotometer

Isolated metabolites were dissolved in methanol and were analyzed the spectrum using Ultraviolet-visible spectroscopy (Shimadzu, Japan). Scanning was carried out from 700 to 190nm.

2.1.5.2 IR Spectroscopy

In order to get information about functional groups, infrared (IR) spectrum of isolated samples were recorded separately on a FT-IR spectroscopy (IR affinity - 1) (Shimadzu, Japan) by the KBr pellet technique. 2 mg of isolated each extract separately was mixed with 100 mg of KBr, compressed it into a pellet on an IR hydraulic press and immediately Scanning was done for the wavelength 4000-450 cm-1.

2.1.5.2 Mass Spectrophotometer

Mass Spectroscopic analysis (MS) was carried out at Micro Analytical Center, Cairo university using mass Spectrometric instrument, model QP 1000EX.

2.1.5.3 Gas Chromatography-Mass Spectroscopy

Qualitative and quantitative analysis of total samples are done by using GM/MS technique (GC/MS-QP2010-Ultra) from japans 'Simadzu Company, with capillary column (Rtx-5ms- 30mxO.25 mmxO.25Ilm).All samples are injected by using split mode .The samples are prepared as we disused, helium as the carrier gas passed with flow rate 1.5 ml/min, The temperature program was started with50c with rate 7 c/min to 180c then the rate was changed to 10c/min till 300c . All the samples are analyzed by using scan mode in the range of m1z 50-550 charges to ratio .Identification of components for each sample was achieved by comparing their retention times and mass fragmentation patents with those available in the library ,the National Institute of Standards and Technology(NIST). , results were recorded.

2.1.5.4 ^{1}H NMR

Proton NMR spectra were recorded on a Varian 400 spectrometer.

Proton chemical shifts are reported in ppm (ȝ) relative to internal tetramethylsilane (TMS, ȝ 0.0 ppm), or with the solvent reference relative to TMS employed as an internal standard ($CDCl_3$, ȝ 7.26 ppm). Data are reported as follows: chemical shift (multiplicity [singlet (s), doublet (ȝ), triplet (t), quartet (q), multiplet (m)], coupling constants [Hz], and integration) (Shimadzu Japan).

2.1.5.5 ^{13}C NMR

Carbon NMR spectra were recorded on a Varian 400 (100 MHz) or 500 (125 MHz) spectrometers with complete proton decoupling. Carbon chemical shifts are reported in ppm (ȝ) relative to TMS with the respective solvent resonance as the internal standard ($CDCl_3$, ȝ 77.0 ppm). All NMR spectra were acquired at ambient temperature.

2.1.6 Microorganisms

Five strains of common bacteria and fungi were used in this study from Microbiology laboratory of Medicinal and Aromatic Plant Research Institute as shown in table below:

Species	**Source**
Pseudomonas aeruginosa	ATCC 27853 gram+ve
Candida albicans	ATCC 7596
Bacillus subtilis	NCTC 8236 gram+ve
Escherichia coli	ATCC 25922 gram-ve
Staphylococus aureus	ATCC 25923 gram+ve

2.2 Methods

2.2.1 Preparation of the plant:

The freshly collected bark leaves, roots and stem, were cleaned and dried under shade. The dried barkleaves, roots and stem was grounded to fine powder using grinder and stored in tightly container

2.2.2 Phytochemical screening

Phytochemical screening for major constituents was undertaken using standard qualitative methods as described by (Harborne, 1992), (Fadeyi *et al*., 1987), (Odebiyi and Sofowora, 1990) and (Abulude, 2007). Saponins, tannins, flavonoids, alkaloids, cumarine, anthraquinone, cyanogenic, glycoside, terepenes and sterol were tested in all the fractions.

2.2.2.1 Preparation of reagent

2.2.2.1.1 Mayers reagent

a. 1.36 g of mercuric chloride dissolved in 60 ml of distilled water.

b. 5 g of potassium iodide dissolved in 10ml of distilled. Solution (a) and (b) mixed and diluted to 100ml with distilled water.

2.2.2.1.2 Valsers reagent

10 g of potassium iodide dissolved was added slowly to red mercuric iodide. The mixture was stirred until a small quantity of mercuric iodide remained and then, the solution was filtered. Other wise, 15g of red mercuric were dissolved in 100ml of 10% potassium iodide.

2.2.2.1.3 1% Aluminum chloride:

1.00 g of aluminum chloride dissolved in 100ml methanol.

2.2.2.1.4 1% Potassium hydroxide:

1.00 g of potassium hydroxide dissolved in 100 ml methanol.

2.2.2.1.5 Saline Solution:

0.90g of sodium chloride dissolved in 100ml of 10g of distilled water.

2.2.2.1.6 Gelatin salt reagent:

10 g of gelatin powder dissolved in 100 ml hot water.

2.2.2.1.7 3% Ferric chloride:

3.00g of ferric chloride dissolved in 100 ml methanol.

2.2.2.1.8 (0.5) N Potassium hydroxide:

2.8g of potassium hydroxide dissolved in 100 ml distilled water.

2.2.2.2 Preparation of the extract:

Extraction was carried out according to the method described by Sukhdev *et al* (2008). 500g of each sample were successively extracted with n-hexane, chloroform, ethyl acetate and methanol using shaker apparatus. Extraction was carried out for about three days for each solvent with daily filtration and evaporation of the solvents under reduced pressure using rotary evaporator apparatus. Extract of each solvent was allowed to air till complete dryness and the yield percents were calculated as follows:

Yield% = Weight of extracts obtained / weight of plant sample ×100

2.2.2.2.1 Test for Alkaloids:

0.2g of extract was stirred with 10 ml of 2M HCl while heating bath for 10 minutes, cooled, filtered and divided into two test tubes. Few drops of one mayer's reagent was added to one tube while valsers reagent was added to the other. Slight turbidity or heavy precipitate in either of the two test tubes was taken as presumptive evidence for the presence of alkaloids.

2.2.2.2.2 Test for Flavonoids:

0.2g of extract was dissolved in 30 ml of 80% ethanol and filtered; the filtrates were used for following test.

1ml of 10% aluminum chloride solution in methanol was added to 3 ml of the filtrate in test tube. Formation of a yellow color indicated the presence of flavonoids, flavones or and chalcone.

2.2.2.2.3 Test for Tannins:

0.2g of each extract was stirred with 10 ml of hot saline, the mixture was cooled, filtered and the volume of the filtrates was adjusted to 10 ml with more saline solution.

5 ml of solution was treated with few drops of gelatin salt reagent. Formation of immediately precipitate was taken as an evidence for the presence of tannins in the plant sample: to another portion of the solution few drops ferric chloride were added. The appearance of blue, black or green color was taken as an evidence for presence of tannins.

2.2.2.2.4 Test for Saponins:

0.2 g of extract was placed in a clean test tube, 10 ml of distilled water was added and the tube was vigorously shaken for about 30 seconds. The tube was then allowed to stand and observed for fifteen minute. The formation of foam was taken as evidence for presence of saponins.

2.2.2.2.5 Test for Anthraquinones glycosides:

0.2g of extract was boiled with 10 ml of 0.5 M KOH containing 1 ml of 3% hydrogen peroxide solution, the mixture was extracted by shaking with 10 ml of benzene. 5 ml of the benzene solution was shaken with 3 ml of 10% ammonium hydroxide solution and the two layers were allowed to separate. The presence of anthraquinones was indicated if the alkaline layer was found to have assumed pink or red color.

2.2.2.2.6 Test of Coumarins:

0.2 g of extract was boiled with 20 ml distilled water in test tube. Spot of the solution was spotted on filter paper saturated with 0.5 N KOH, then the filter paper was inspected under UV light; the presence of coumarins was indicated if the spot was found to be adsorbed the UV light.

2.2.3 Biological activity

0.5 g of the powdered plant material (roots, stem, leaves and bark) was dissolved in 5 ml hexane.

2.2.3.1 Screening for antibacterial activity

A total of five standard microorganisms used, four bacterial cultures (*Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhii* and *Shigella flexenari*) were used in this study. The bacterial strains were grown in Muller-Hinton agar plates at 37^0C and maintained on nutrient agar slants, and the *Candida albicans* fungi.

2.2.3.2 Well-in agar method

Anti-bacterial activity of plant extracts was tested by a modified well-in agar method (Sinclair, 1995). Two ml of the standardized bacterial stock suspension were thoroughly mixed with 250 ml of sterile melted nutrient agar and maintained at 40^0C. 20 ml aliquots of the inoculated media were poured into a sterile Petri dish (9 cm in diameter). The agar was left to set, and in each of these plates, four holes (10 mm in diameter) were made

using a sterile cork borer and agar discs were removed. Alternate holes (wells) were filled with 0.1 ml (100 µ l) of 100 mg /ml sample of each of the extracts and allowed to diffuse at room temperature for two hours. The plates were then incubated, in the upright position, at 37^0C for 18 hours (Sinclair, 1995). Three replicates were carried out for each extract against each of the test organisms simultaneously; controls involving the addition of the respective solvents instead of the extracts were carried out. The results were recorded by measuring the diameter of inhibition zone at the end of 24–48 hours.

2.2.3.3 Nutrient Agar method

Anti-bacterial activity of plant extracts was tested by a modified well-in agar method Two ml of the standardized bacterial stock 28.0 suspensions was thoroughly mixed with 100 ml of sterile melted nutrient agar and maintained at 40^0C.

2.2.3.4 Sabour and Dexrose Agar method

Sixty five g of plant extracts mixed with distilled water for fungi.

2.2.4 Thin layer chromatography

Thin layer chromatography was performed according to the method described by (Stalh, E. 1969)

2.2.4.1 Stationary Phase

The stationary phase used was Silica gel type GF_{254}.

2.2.4.2 Mobile Phase

The following mobile phases were tested:

- Toluene: ethyl acetate: (9.7:0.3)v/v
- Toluene: ethyl acetate: (8.5:1.5)v/v
- Toluene: ethyl acetate: Formic acid (5:4:1)v/v
- Toluene: ethyl acetate: Formic acid (4:5:1)v/v
- Toluene: ethyl acetate: Formic acid (3:6:1)v/v

- Toluene: ethyl acetate: Formic acid (2:7:1)v/v
- Toluene: ethyl acetate: Formic acid (1:8:1)v/v
- Toluene: ethyl acetate: Formic acid (3:6:2.5)v/v
- Toluene: ethyl acetate: Formic acid (2:7:2.5)v/v
- Toluene: ethyl acetate: Formic acid (2:7:1.2)v/v
- Toluene: ethyl acetate: Formic acid (3:6:1.2)v/v
- Butanol: Acetic acid: Water (4: 1: 5) v/v upper layer.
- Butanol: Acetic acid: Water (12.5: 0.5: 1) v/v
- Butanol: Acetic acid: Water (12.5: 0.5: 0.5) v/v
- Methanol: Chloroform (5: 5)v/v
- Methanol: Chloroform (4: 5)v/v
- Methanol: Chloroform (5: 5)v/v
- Methanol: Chloroform (4: 6)v/v
- Methanol: Chloroform (3: 7)v/v
- Methanol: Chloroform (2: 8)v/v
- Methanol: Chloroform (1: 9)v/v
- Methanol: Chloroform (0.2: 9.8)v/v
- Chloroform (10:0) v/v.

2.2.5 Thin layer chromatographic technique (TLC)

Dry clean chromatographic plates (2020 cm) were coated with a slurry of silica gel (GF) $_{254}$. Layers of 0.25 mm thickness were used for analytical separation as well as ready-made plates of different adsorbent were used (Stalh, 1969).

2.2.5.1 Preparation of Plates (TLC)

30g of the silica gel were weighed and shacked well with 60 ml of distilled water (until homogenous slurry was obtained). Five clean glass plates (20x20 cm) were coated with the slurry using Desaga spreader to

get 0.25 cm thickness. The plates were allowed to air dry and then activated by heating in an oven at 105°C.

2.2.5.2 Application of Sample

0.5 g of *X. americana* ethyl acetate extracts were dissolved in 5 ml of ethyl acetate. The sample was spotted at a level about 2 cm from the bottom of the plate, kept to dry by air and placed in a tank containing the selected solvent system. After reaching the height of 15 to 20 cm, the plate was removed from the tank and allowed to air dry until the solvent was completely evaporated. The plate was inspected in day light, then examined under UV and finally sprayed with Vanillin sulphuric acid; RF values of separated spots were calculated as follows.

Distance crossed by spot / distance crossed by solvent front

2.2.6 Column Chromatography

Column was prepared by macerating 363 g of silica gel column grade (60-120) mesh with chloroform for about two hours, poured into 90 cm length and 20 cm wide, clean glass, column and eluted with hexane till complete homogeneity. 11g of extract was mixed with 11g silica gel, and poured at the top of the column and eluted with hexane, chloroform, ethyl acetate and methanol respectively. The graduation of solvent was 5% in each time. The fractions were collected in 50 ml test tube and monitored with thin layer chromatography on silica gel using different solvent systems. The plates were detected under UV and sprayed with vanillin sulphuric acid. Plates containing similar spots were combined, together and allowed to dry and then weighed.

2.2.6.1 Preparative Thin Layer Chromatography

After testing the obtained fractions for their anti-microbial activity, the fractions with potential activity were subjected to preparative thin layer chromatography for more purification.

2.2.6.1.1 Preparation of Preparative Thin Layer Plates

60g of silica gel were weighed and shacked well with 120 ml of distilled water until homogenous slurry was obtained. Five clean glass plates (20x20 cm) were coated with the slurry using desaga spreader to get 0.5 cm thickness. Plates allowed to air dry and activated by heating in an oven at 105°C.

2.2.6.1.2 Application of fractions Isolated from column on TLC plate

The most active fraction was dissolved in 2 ml methanol, spotted as band at a level of about 2 cm from the bottom of the plates, kept to air dry and then inserted in a tank containing the selected saturated solvent system. After attaining the height of 15 to 20 cm, the plate was removed from the tank and allowed to air dry until the solvent was completely evaporated. The plates were examined under UV_{256} and the common spot appeared through all the plates were scratched. Scratched band combined together and re-dissolved in methanol and filtered. Solvent allowed drying and the scratched compound stored till used for spectroscopic analysis.

2.2.6.1.3. Isolation of pure compoundF_{16}(Zm4) from *Ximenia americana:*

Ethylacetate extract from barks was subjected into coloumn chromatography, Tow fractions were obtained (E.A:MeOH; 60:40 and 50:50%) which were collected to give fraction -16 (F_{16}). F_{16} were subjected to preparative thin layer chromatography (PTLC) by usingChloroform: Methanol: formic acid (70:30:2) as solvent system. Zm4 was obtained as colorless mass.

2.2.6.1.4. Isolation of compound $F_{16\text{-}1}$:

Three sub-fractions were obtained from F_{16}, which were re-subjected to preparative TLC again with other solvent system [toluene: ethylacetate:

formic acid (70:30:2)], from which fraction $F_{16\text{-}1}$ and $F_{16\text{-}2}$ were obtained as colorless mass.

2.2.7 Biological assay of Brine-shrimp of Chloroform and ethyl acetate of the bark (Cytotoxicity study)

The brine-shrimp lethality assay was performing following the reported procedure (Meyer, 1982 and Mclaughin, *et al*., 1991).

2.2.7.1 Hatching of Brine shrimp technique:

Brine shrimp (*Artemia salina* leach) eggs were purchased from fish pet shops. The eggs were then hatched in a shallow rectangular plastic dish, filled with artificial seawater prepared by dissolving 38 g of sea salt in 1litre of distilled water. An unequal partition was made in the plastic dish with the help of a perforated device. Approximately 50 mg of eggs were sprinkled into the large compartment that got darkened while the minor compartment was opened to ordinary light. Two days later, nauplii/larvae were collected by a pipette from the lighter side(Mclaughin, *et al*., 1991).

2.2.7.2 Test procedure:

20 mg of crude extract was dissolved in 2 ml of the respective solvent, then 500 μL, 50 μL and 5 μL of this solution was transferred to vials corresponding to 1000, 100 and 10 μg/mL respectively. The solvent was evaporated by leaving the vials overnight. After two days of hatching, 10 Nauplii /larvae were placed into each vial and the volume was adjusted with sea water to 5ml per vial, and incubated at 27 °C for 24 hours under illumination. Then, the number of survivors were counted and recorded. Reference cytotoxic drug (Etoposide) served as positive control.

Table (4): *Artmia salina* test conditions

Test	***Artemia salina***
Colour	Light
Size	up to 0.5 mm
Hatching performance	80%
Number of individuals in 1 dish	10
End point	Mortality
Test conditions	Stable temperature and light (incubator)
Replicates	3
Volume of tested concentration	5 ml in 1 vial
Temperature	25-30
Exposition length	24 hours
Illumination	Continuous light
Chemicals	Initial solutions of tested sample, marine water
Instruments and equipment	Petri dishes, beakers, micropipettes, incubator, hatching tray , vials , Pasteur pipettes

2.2.7.3. Evaluation of the test results

Using the values on died individuals in given concentrations determine the percent of mortality. The data were processed using a Finney computer programme (McLaughin 1991) and LD_{50} values were obtained at 95 % confidence intervals.

CHAPTER THREE

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

3.1 Result of Weight Extracts of *X.americana*

Table (3.1) below shows extraction of 100g of the *X.americana*(roots, stem-bark, bark and leaves) by using 500ml of different solvents (hexane, chloroform, ethyl acetate and methanol).

Table (3.1): Weight Extracts of *X. americana*

Solvent	**Bark**	**Stem**	**Roots**	**Leaves**
Hexane	0.820 %	0.432 %	0.784%	10.1 %
Chloroform	0.502 %	0.265 %	2.681%	2.1 %
Ethyl acetate	0.809 %	0.489 %	0.417%	1.98 %
Methanol	17.918 %	4.181 %	11.51%	3.9 %

Table (3.1) showed different weights yields, in hexane extraction, leaves showed great values comparable with roots stem and bark. In chloroform extraction the root is more than leaves bark and stem respectively. In ethyl acetate extraction leaves is more than bark, stem and roots respectively. In methanol extraction bark showed the greatest value more than the roots, stem and leaves respectively, these different results with different ranges of weights may be due to the difference of nature and the presence of the secondary metabolites of each plant organs tested.

3.2 Phytochemical screening

Table (3.2) below showed a photochemical screeningof the *X. americana* extracts (bark, leaves stem and root) by using solventsmethanol and water.

Table (3. 2): Phytochemical screening of *X. americana*extracts

Part used	**Bark**	**Leaves**	**Stem**	**Root**	**Observation**
Tannin	+++	+++	+++	+++	Greencolor / turbidity
Flavonoid	++	++	++	++	Yellow color
Saponin	+++	+++	++	+++	Foam
Alkaloid	-	+	-	-	Turbidity
Sterol	+	+	+	+	Observation
Triterpenes	+	++	+++	++	Purple color
Cumarin	+	+	+	+	UV adsorption

+++ = highly present, ++ = moderately present, + = faintly present, -= absent

Phytochemical screening presented to detect its major constituents, the standard method of chemical screening was used. The phytochemical screening was performed for the major constituents of the crude extract ofbark, leaves, roots and stem of *X. Americana L*., the standard method of chemical screening was used shown in table (3.2). The results showed a high presence of tannin and saponin (+++),in all extracts, alkaloids are moderately present (+) in leaf extract, and absent in the rest of others extract, Sterol is present (+) in all sample extract, triterpene is a high present (+++) in stems and is moderately presents in other parts. Flavonoid is (++) moderately present in all extracts. The results showed highly present of tannin and saponin (+++) in the methanol and water extracts of *X. americana L.* bark and leaves which are more comparable with ethanolic extractof *X. americana L.* bark by (Abd alfatah, *et al.*, 2013), tannin and saponin were highly present (++), Flavonoid(++) showed similarity comparable with ethanolic and butanolic extractof *X. americana L.* bark by (Abd alfatah, *et al*., 2013), alkaloids is moderately

present (+) in methanolic extract which showed similarity comparable with ethanolic and butanolic extractof *X. americana L.* bark by (Abd alfatah, *et al*., 2013) and alkaloids is absent in water extract which showed similarity comparable with ethyl acetate extractof *X. Americana L.* bark by (Abd alfatah, *et al*., 2013). Sterol is highly present (++) leaves in the methanol extract of *X. americana L.* comparable *to* bark, which showed moderately present (+), but in water extracts of *X. americana L.* bark and leaves Sterol is absent (-). Terpene is highly present (+++) leaves in the methanol extract of *X. americana L. bark* comparable *to* bark which is absent (-), but ethanolic extractof *X. americana L.* bark by (Abd alfatah, *et al*., 2013) is highly present (++) comparable with the methanol extract of *X. americana L. bark* which terpene is absent (-). A large number of sesquiterpenes are constituents of essential oils of higher plants and seem to intervene in the pharmacological properties attributed to these volatile fractions (Bruneton, 1999). It has been clarified that the biological activities of the liverworths are due to terpenoids and lipophilic aromatic compounds (Atta.1988). Steroids and triterpenes with therapeutic interest and manufacturing employment are a group of secondary metabolites of outstanding importance (Bruneton, 1999). Considerable recent work strongly indicates the great potential of the triterpenoids as source of medicineuse (Mahato *et al*., 1992).

3.3 Antimicrobial activity

X.americana plant organs (leaves, stem, bark and roots) were screened to show their antimicrobial activity against four standard bacteria, two Gram-positive bacterial strains (*Bacillus cereus* and *Staphylococcus aureus*), two Gram-negative bacterial strains (*Escherichia coli* and *Pseudomonas aeruginosa*) and fungal strain (*Candida albicans*) using

the cup plate agar diffusion method. Inhibition zone diameters were measured as shown in table (3.3).

Table (3.3): Antimicrobial activity of different organ extracts of *X. americana*against Standard bacteria and fungi using (100 mg/ml):

Samples	**Solvents**	**Standard tested organisms* /M.D.I.Z (mm) ****				
		S. a	B. s	E. st	Ps. A	Ca. a
Bark	Hexane	-	20	-	11	-
Stem		-	18	-	-	-
Roots		-	18	-	-	-
Leaves		14	22	15	15	-
Bark	Methanol	16	32	14	15	46
Stem		15	25	12	11	11
Roots		15	24	14	14	46
Leaves		14	22	15	15	46
Bark	Chloroform	20	19	18	19	-
Stem		15	14	-	15	-
Roots		20	19	19	20	-
Leaves		14	20	14	14	-
Bark	Ethyl acetate	22	23	22	20	-
Stem		21	24	23	20	-
Roots		22	23	20	20	-
Leaves		20	20	20	20	20

(-) no activity.Values greater than 9mm indicate activity.

*Standard organisms tested: *B.S. = Bacillus subtilis, S.a. =Staphylococcus aureus, E.c. = Escherichia coli, Ps.a. = Pseudomonas aeruginosa, C.a= Candida albicans* . M. D. I. Z=: Mean diameter of growth inhibition zone in

(mm). Result: >18 mm: Sensitive, 14 to 18 mm: Intermediate: <14 mm: Resistant. (-): No inhibitionzone.

The antimicrobial activity of the extracts of the various parts of the investigated plants such as roots, leaves, seeds, stems barks and fruits, appears to be due to the presence of secondary metabolites such polyphenols, triterpenes, sterols, saponins, tannins, alkaloids, glycosides and polysaccharides.

X. americana antimicrobial activities of (hexane, methanol, chloroform and ethyl acetate) extracts of the (roots, stem-bark, bark and leaves) were evaluated against four common bacteria isolates *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus* and fungi *Candida albicans* and was active against all of them. The highest degree of activity in Methanol extracted of the bark was for *B. subtilis* (inhibition zone: 35-30 mm), followed by *S. aureus* (inhibition zone: 17-16 mm), *P. aeruginosa* (inhibition zone: 16-15 mm), *E. coli* (inhibition zone: 15-14 mm),and*C. albicans* (inhibition zone: 12-11 mm). The (roots, stem-bark, bark and leaves) hexane extracts of *X. americana* were tested against five bacteria the growth of *Staphylococcus aureus* and *Escherichia coli w*ere inhibited by only hexane bark aqueous bark, stem aqueous and root aqueous extracts. *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Candida albicans* were not affected by these extracts. Several other studies to determine the presence of antimicrobial activity in crude extracts of *Ximenia Americana* were performed (Magassouba *et al.*, 2007; Maikai *et al.*, 2009). In all, the various extracts were found to have a broad spectrum effect against standard organisms (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Candidaalbicans* and *Bacillus*

subtilis, and supports the traditional usage of this plant as a remedy in treatment of microbial infections.

In general, the antimicrobial activity of extracts of the various parts of the plants appears to be due to the presence of secondary metabolites. In some experiments, was remarked that the plants which accumulate polyphenols, tannins and unsaturated sterols/terpenes showed to inhibit or significantly retard growth of eight of the ten test organisms; the species, which constitute polyphenols and unsaturated sterols/terpenes; and polyphenols, tannins, unsaturated sterols/terpenes, saponins and glycosides inhibited six organisms each while, those with polyphenols, tannins, unsaturated sterols/terpenes, saponins and alkaloids and unsaturated sterols/terpenes inhibited growth of five bacterial strains each (Geyid *et al.*, 2005). Cyanogenetic glycosides are reported to possess antimicrobial activity (Finnermore *etal.*, 1988). Tannins have been traditionally used for protection of inflamed surfaces of the mouth and treatment of catarrh, wounds, haemorrhoids and diarrhea and as antidote in heavy metal poisoning. They have the ability to inactivate microbial adhesions, enzymes, cell envelope transport proteins and also complex with polysaccharide (Maikai *et al.*, 2009; Scalbert, 1991; Ya *et al.*, 1988). Flavonoids are naturally occurring phenols, which posses numerous biological activities including anti-inflammatory, antiallegic, antibacterial, antifungal and vasoprotective effects and, also have been reported to complex with extracellular and soluble proteins and to complex with bacterial cell walls (Dixon *et al.*, 1983; Geyid *et al.*, 2005; James *et al.*, 2007; Maikai *et al.*, 2009; Ogunleye *etal.*, 2003).

Terpenoids have also been reported to be active against bacteria, the mechanism of action involves membrane disruption by the lipophilic compounds (Geyid *et al.*, 2005; James *et al.*, 2007; Maikai *et al.*, 2009; Ogunleye *et al.*, 2003). Although it is difficult to speculate on the mechanism of action of the constituents of the extracts on the basis of studies conducted to date, the antimicrobial activity of these extracts is due, no doubt, the presence of these secondary metabolites. In the case of extracts of *Ximenia Americana,* probably, due the presence of tannins, flavonoids, triterpenes/steroids, saponins or cyanogenetic glycosides.In summary, the results justified the use of *X. americana*as having antibacterial properties and support its use as agent in new drugs for therapy of infectious diseases caused by pathogens.

3.4 Fraction of column

Table (3.4): Weight of the fraction column of the bark of ethyl acetate of *Ximenia Americana*

Fr. No	**Limit**	**Solvent Eluent**	**Weight of Fraction**	**Yield %**	**Solvent system**
1	1-36	Hexane 100% to Hexane 20% + $CHCl_3$ 80%	0.103	0.936	$CHCl_3$: P.E 50:50
2	37-43	Hexane 20%+ $CHCl_3$ 80% to Hexane 5% + $CHCl_3$ 95%	0.023	0.023	$CHCl_3$: P.E 50:50
3	44-58	Chloroform 100%	0.683	6.209	$CHCl_3$: P.E 50:50
4	59-76	Chloroform 100%	0.038	0.345	$CHCl_3$: P.E 50:50
5	77-91	$CHCl_3$ 95%+ E.A 5% to $CHCl_3$ 70% + E.A 30%	0.003	0.290	$CHCl_3$: P.E 80:20
6	92	$CHCl_3$ 65%+ E.A	0.110	1.00	$CHCl_3$: P.E

		35%			80:20
7	93-99	$CHCl_3$ 65%+ E.A 35% to $CHCl_3$ 60% + E.A 40%	0.084	0.763	$CHCl_3$: P.E 80:20
8	100-103	$CHCl_3$ 60%+ E.A 40% to $CHCl_3$ 55% + E.A 45%	0.052	0.472	$CHCl_3$:E.A 93:7
9	104-109	$CHCl_3$ 55%+ E.A 45% to $CHCl_3$ 35% + E.A 65%	0.880	8.00	$CHCl_3$:E.A 93:7
10	110-118	$CHCl_3$ 40%+ E.A 60% to $CHCl_3$ 35% + E.A 65%	0.906	8.236	$CHCl_3$:E.A 93:7
11	119-125	$CHCl_3$ 30%+ E.A 70% to $CHCl_3$ 25% + E.A 75%	0.074	0.672	$CHCl_3$:E.A: F.A 80:20 2drops
12	126-139	$CHCl_3$ 20%+ E.A 80% to $CHCl_3$ 5% + E.A 95%	0.499	4.536	$CHCl_3$:E.A: F.A 80:20 2drops
13	140	$CHCl_3$ 5%+ E.A 95%	0.038	0.345	$CHCl_3$: E.A: F.A.80:20 2drops
14	144-155	E.A 100%	0.951	8.645	$CHCl_3$: E.A: F.A 50:50:2
15	156-182	E.A 100% to E.A 65% + MeOH 35%	0.708	6.436	$CHCl_3$:MeOH: F.A 70:30:2
16	183-188	E.A 60% + MeOH 40%to E.A 50% + MeOH 50%	2.369	21.54	$CHCl_3$:MeOH:F.A 70:30:2
17	189-195	E.A50% + MeOH 50% to E.A30%+ MeOH 70%	2.566	23.32	$CHCl_3$:MeOH: F.A 70:30:2
18	196-199	E.A 25% + MeOH 75%	0.079	0.718	$CHCl_3$:MeOH: F.A 70:30:2
19	200-212	E.A25% + MeOH 75% to E.A5%+ MeOH 75%	0.276	2.509	$CHCl_3$:MeOH: F.A 70:30:2
20	213-231	MeOH100%	0.318	2.890	$CHCl_3$:MeOH: F.A 70:30:2
21	232-239	MeOH100%	0.055	5.0	$CHCl_3$:MeOH: F.A 70:30:2
22	240-249	MeOH100%	0.042	0.381	$CHCl_3$:MeOH: F.A 70:30:2

* MeOH: methanol, CH_3Cl: chloroform, F.A: formic acid, P.E: petroleum ether, E. A: ethyl acetate, Fr. No: Fraction number and Wt: weight

Fraction of column of the *Ximenia Americana* of ethyl acetate extracted of barkby using different solvent system as showed in table (3.4).

Column chromatography is a method used to purify individual chemical compounds. It is often used for preparative applications on scales from micrograms up to kilograms. The fraction done by solvent eluent using Chloroform and Methanol showed good weight and good percentage more than which used other solvent eluent as ethyl acetate. Solvent eluent mixture Methanol and ethyl acetate mixture gave high yield and percentage (2.566, 23.32%) and (2.369, 21.54%) respectively.

3.5 Antimicrobial activity of fraction column

Antimicrobial activity of *X. americana* ethyl acetate fraction obtained from column by using four standard micro-organisms as showed in Table (3.5)

Table (3.5): Antimicrobial activity of a*X.americana* ethyl acetate fraction column

Fraction Number	**Types of Bacetria**				
	S.a	**B.s**	**E.st**	**Ps.a**	**Ca.a**
1	12	10	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-
5	-	-	-	-	-
6	-	-	-	-	-
7	-	-	-	-	-
8	-	-	-	14	-
9	-	-	-	-	-
10	-	20	-	-	-
11	-	20	17	20	-
12	28	30	32	27	-
13	17	22	28	23	-
14	27	31	33	31	-
15	26	29	35	30	-
16	28	30	37	30	-

17	21	33	25	24	-
18	16	25	27	22	-
19	21	25	25	26	-
20	14	16	25	17	-
21	14	15	-	14	-
22	14	-	-	-	-

*(-) no activity, values greater than (9 mm) indicates activity

**B.a: Bacillius subtilis, S.a: Staphylococus aureus, E.st: Escherichia coli, Ps.a: Pseudomonas aeruginosa, and Ca.a: Candida albicans*

Fraction number (1,8,10,11,12,13,14,15,16,17,18,19,20,21and 22 showed activity, fraction 1 showed moderate against *Bacillius subtilis*and *Staphylococus aureus*, fraction 8 showed moderate activity against *Pseudomonas aeruginosa*, fraction 10 showed moderate activity against *Bacillius subtilis* , fraction 22 showed activity only against *Staphylococus aureus* and fractions from 12 to 20 showed very good activity against all types of bacteria, and all extracts gave negative activity aganist *Candida albicans* showed table (3.5).

3.6: Cytotoxicity assay of *Ximenia americana* extracts

Cytotoxicity 0f *Ximenia americana* of the chloroform and ethyl acetate bark extracts were conducted via brine shrimp lethality.

Table (3.6): Evaluation of Cytotoxicity (Brine shrimp) of Chloroform Bark extract of *Ximenia americana* by Finney probity Analysis (Model):

Concentration chloroform µg/ml)	No. of shrimps	Survived	Dead	St. drug (Etoposide ug/ml)
10	30	11	19	7.4625
100	30	0	30	
1000	30	0	30	

LD_{50} = 5.1817 µg/ml

Table (3.7) Cytotoxicity of the *Ximenia americana* Ethylacetate Bark extract

Concentration ethylacetate µg/ml	No. of shrimps	**Survived**	**Dead**	**St. drug (Etoposide µg/ml)**
10	30	21	9	7.4625
100	30	0	30	
1000	30	0	30	

Key: ≤ 249: highly toxic; 250 – 499: Moderate; 500 - 1000: non-toxic.

LD_{50} = 16.3765 µg/ml

The level of toxicity (LD_{50} Toxicity: <20µg/ml: Very toxic, <100µg/ml: Toxic, >1000µg/ml: No-Toxic).

The results stated that LD_{50}values below 249 µg/ml ware considered as highly toxic, 250 to 499 µg/ml as medium toxicity and 500 to 1000 µg /ml as mild toxicity. Values above 1000µg/ml were regarded as non-toxic (McLaughlin *et al*., 1998). Chloroform extract of *X. americana*, showed LD_{50} =5.1817µg/ml whereas, Ethyl acetate extract showed LD_{50} =16.3765µg/ml, the extracts were very toxics. For uniformity of the result, the tests were repeated three times. The *in vivo* lethality in a simple zoological organism, such as brine shrimp lethality test might be used as a simple tool to guide screening and fractionation of physiological active plant extracts, where one of the simplest biological responses to monitor is lethality, since there is only one criterion; either dead or alive (Solis, *et al*.,1993; Gupta, *et al*., 1996). Therefore, this test has been successfully used by many researchers as a simple biological test in order to detect anti-tumour compounds (Solis, *et al*.,1993) .There is a general toxicity test agreement that LC50 above 100µgmL-1 is non-toxic while that below 100µgmL-1 is indicative of toxicity(Solis, *et*

al.,1993; Gupta, *et al*., 1996). Cytotoxicity of chloroform and ethyl acetate fractions extracts of *X.americana*were conducted via brine shrimp lethality test).The results stated that LD_{50} values below 249 µg /ml were considered as highly toxic, 250 to 499 µg/ml as medium toxicity and 500 to 1000 µg /ml as light toxicity. Values above 1000 µg /ml were regarded as non-toxic (McLaughlin *et al*., 1998). Chloroform extract of *X. americana*, showed LD_{50} =5.1817µg/ml whereas, ethyl acetate extract showed LD_{50} =16.3765µg/ml.

The results indicated that *X. americana*revealed a significant cytotoxic activity against brine shrimps, according to (Padmaja *et al*., 2002). The LD_{50} ranged from 0 to 100µg/ml and those with <20 µg/ml were considered to be very active, therefore, the chloroform extract (LD_{50} =5.1817 µg/ml) and ethyl acetate extract (LD_{50} =16.3765 µg/ml) found to be very active against brine shrimp comparing to the standard drug (Etoposide 7.4625 µg/ml). Cytotoxic activity using normal cell should be conducted for the possibility of anticancer activity.

3.7 Structure Elucidation of $F_{16\text{-}1}$and $F_{16\text{-}2}$:

The TLC chromatogram of **$F_{16\text{-}1}$ and $F_{16\text{-}2}$,** using mobile phase Butanol: Acetic acid: water [97:3] showed one fluorescent spot under U.V (366nm) and pink color with vanillin sulphuric acid.

The GC chromatogram of **$F_{16\text{-}1}$,** exhibited nine major peaks and **$F_{16\text{-}2}$** exhibit (% match with data bank ≥ 95) atdifferent retention times (R_t) indicating presence of nine compounds (fig (3.1)) for **$F_{16\text{-}1}$** and compounds for **$F_{16\text{-}2}$** (fig (3.2), while MS spectrum displayed $[M]^+$ at different m/z values, corresponding to different molecular ions. Suggestion of the separated components was accomplished using computer search by matching spectra with reference spectra in the

computer library. The suggested compounds are shown in Table (3.9) and (3.10).

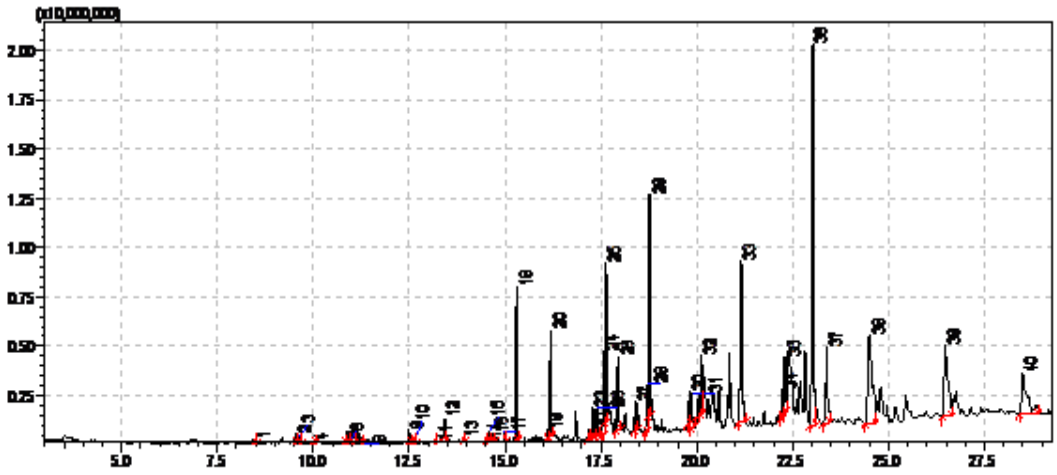


Fig (3.1): GC chromatogram of **F_{16-1}**

Table(3.8): GC-MS Analysis of F_{16-1}:

Compound number	Retention time in min. (R_t)	Compound name	Chemical formula	Area %	Match with data bank
1	8.446	Nonanoic acid, 9-oxo-,methyl ester	$C_{10}H_{18}O_3$	0.16	99
2	9.535	Phenol,2-4-bis(1,1-dimethylethyl)	$C_{14}H_{22}O$	0.30	99
3	9.645	Dodecanoic acid, dimethyl ester	$C_{13}H_{26}O_2$	0.21	99
4	12.489	Methyl tetradecanoate	$C_{15}H_{30}O_2$	0.50	99
5	14.671	n-Pentadecanol	$C_{15}H_{32}O$	0.27	99

6	17.295	n-Nonadecanol-1	$C_{19}H_{40}O$	0.99	99
7	20.125	n-Tetracoanol-1	$C_{24}H_{50}O$	7.7.2	99
8	23.022	1-Nonadecene	$C_{19}H_{28}$	4.26	99
9	28.496	Triacontylheptafluorobutyrate	$C_{34}H_{61}F_7O_2$	6.70	99

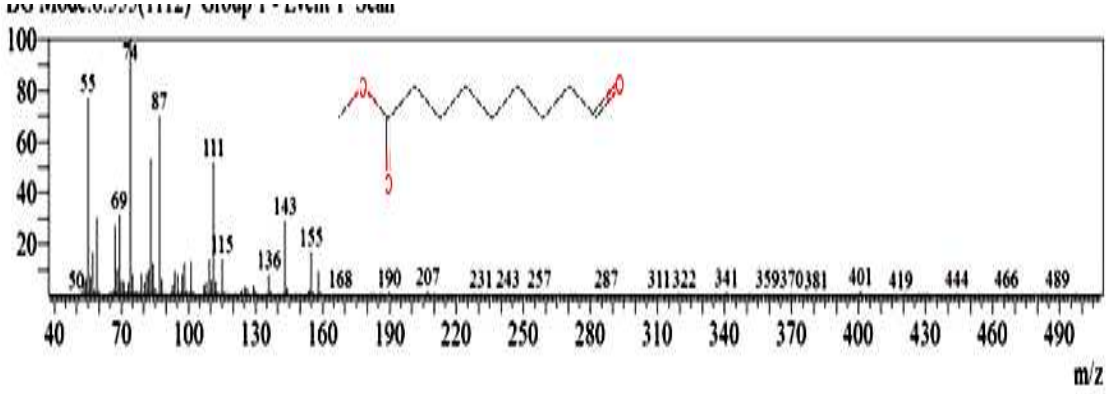


Fig (3.2): MS of Nonanoic acid, 9-oxo-,methyl ester

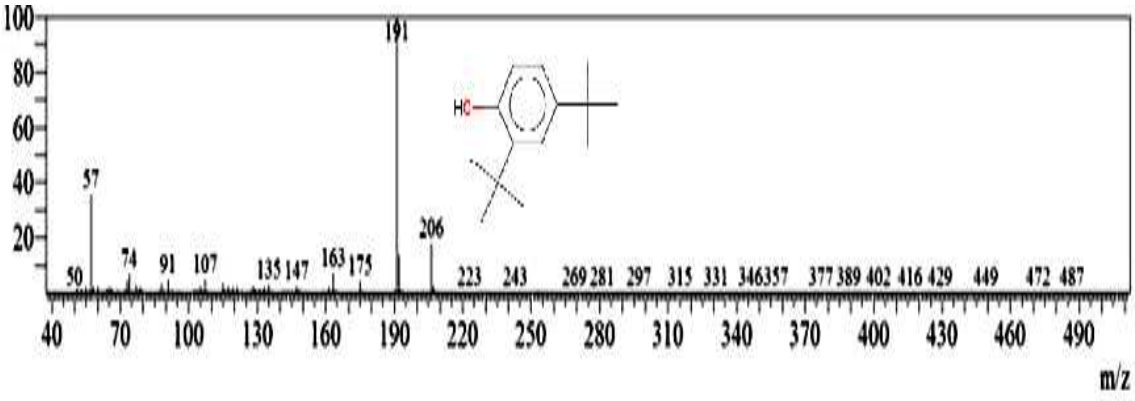


Fig (3.3): MS of Phenol,2-4-bis(1,1-dimethylethyl)

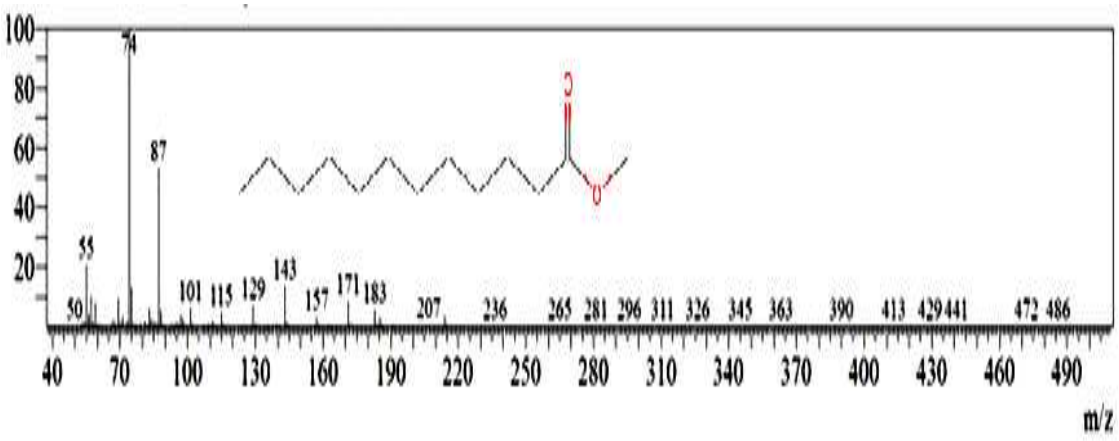


Fig (3.3): MS of Dodecanoic acid, dimethyl ester

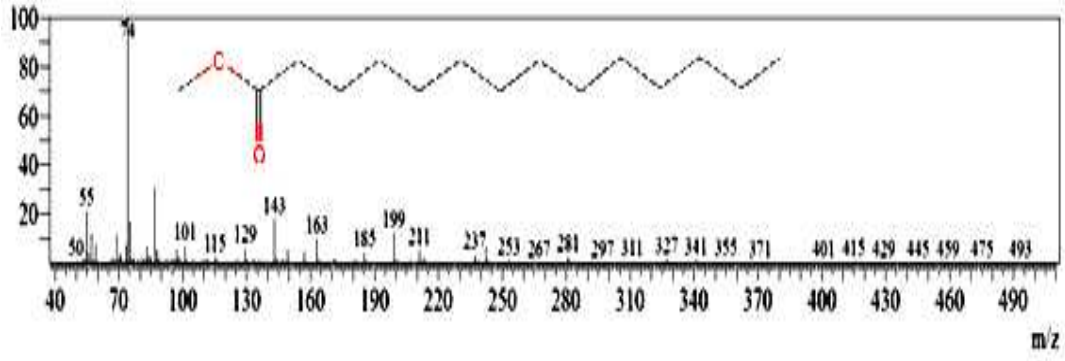


Fig (3.4): MS of Methyl tetradecanoate

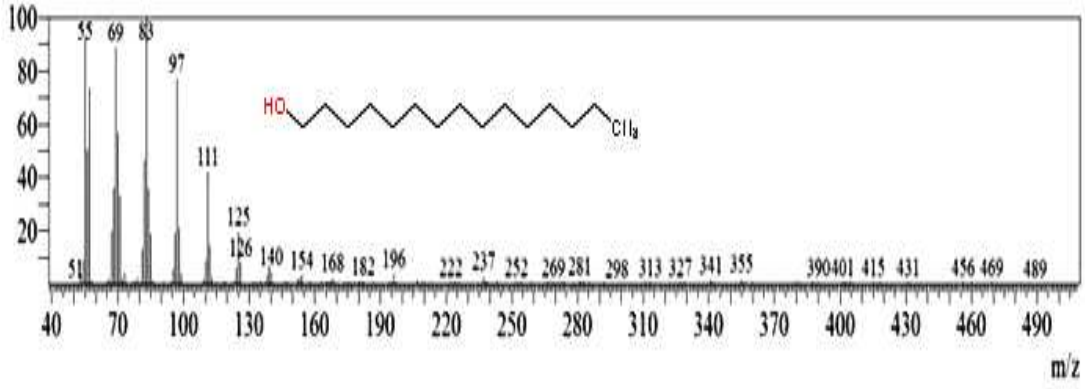


Fig (3.5): MS of n-Pentadecanol

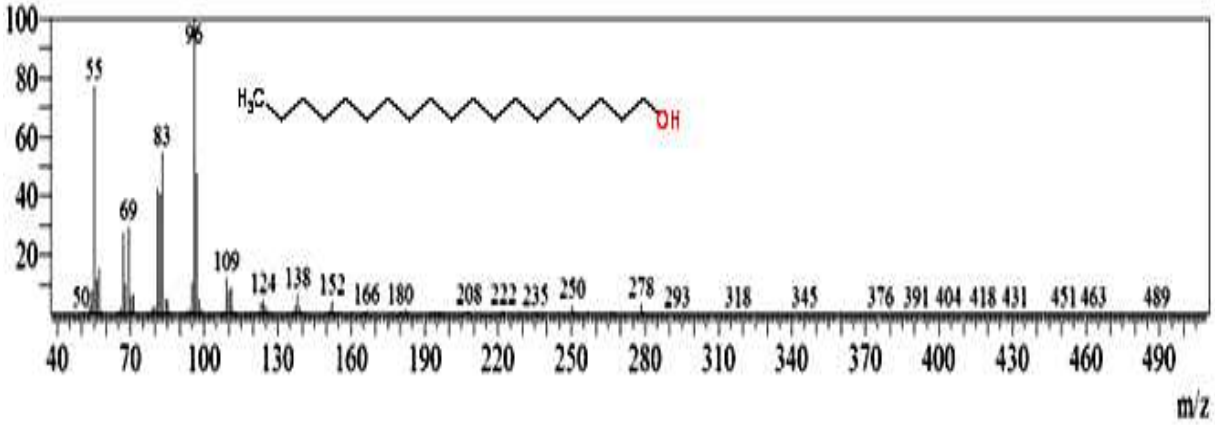


Fig (3.6): MS of n-Nonadecanol-1

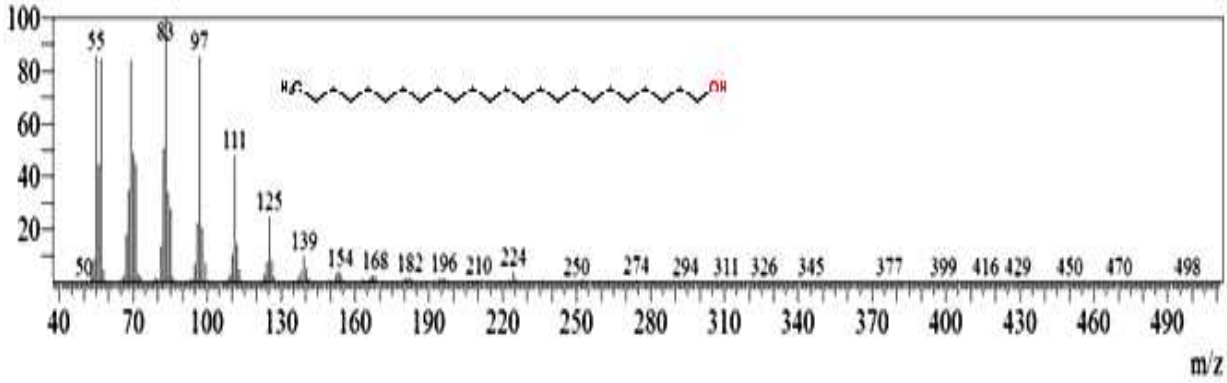


Fig (3.6): MS of n-Tetracoanol-1

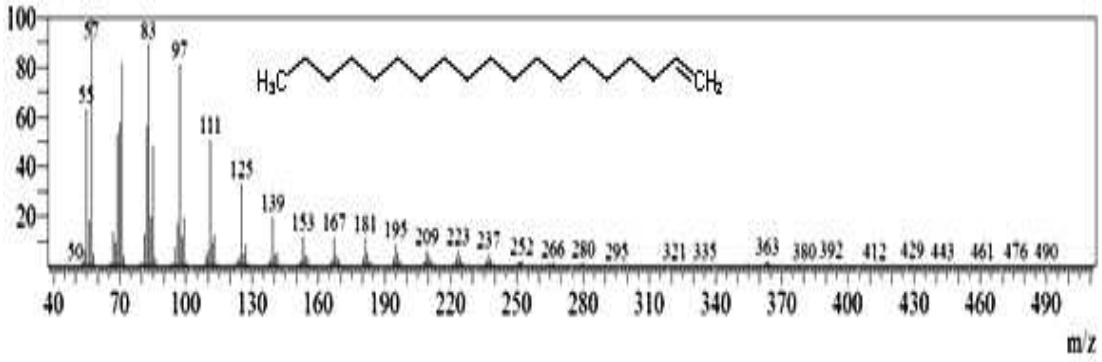


Fig (3.7): MS of 1-Nonadecene

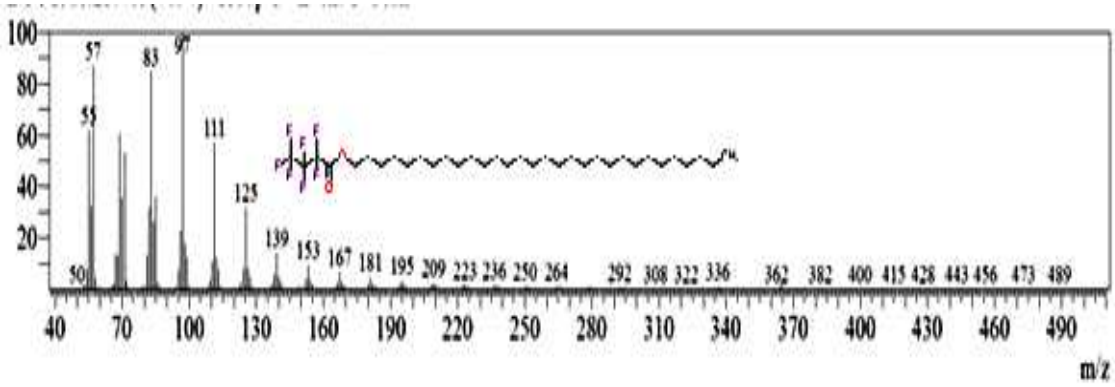


Fig (3.8): MS of Triacontyl heptafluorobutyrate

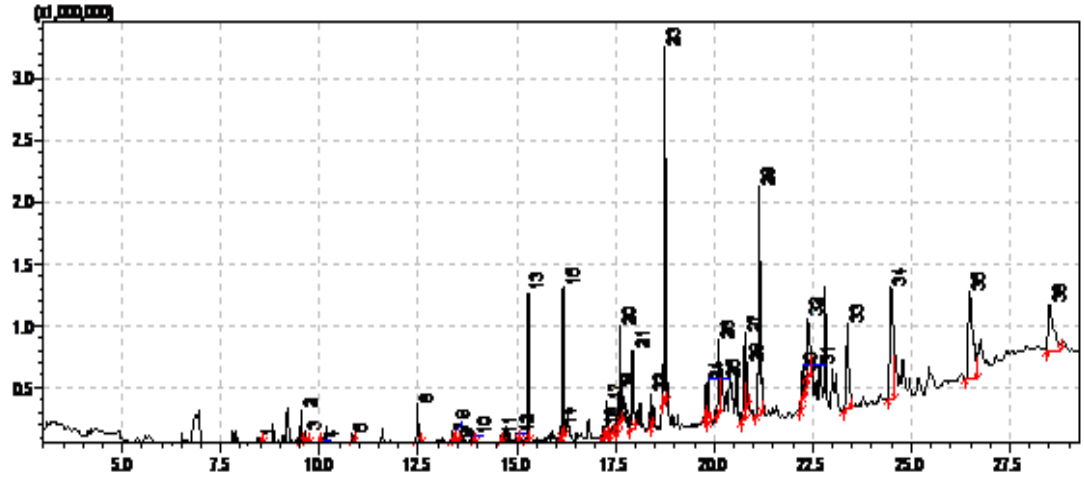


Fig (3.2): GC chromatogram of $\mathbf{F_{16\text{-}2}}$

Table (3.9): GC-MS Analysis of $F_{16\text{-}2}$:

Compound number	Retention time in min.(Rt)	Compound name	Chemical formula	Area %	Match with data bank
1	9.530	Phenol,2,4-bis(1,1-dimethylethyl)	$C_{14}H_{22}O$	1.00	96
2	13.375	Methylhydroquinone,bis(trimethylsilyl)	$C_{13}H_{24}O_2Si_2$	0.66	95

3	14.597	8-Octadecanone	$C_{18}H2_6O$	0.27	98
4	19.766	Benzocyclodecene,tetradecahydro-	$C_{14}H_{26}$	1.02	95
5	19.863	n-Heptadecyclocyclohexane	$C_{23}H_{46}$	1.39	98

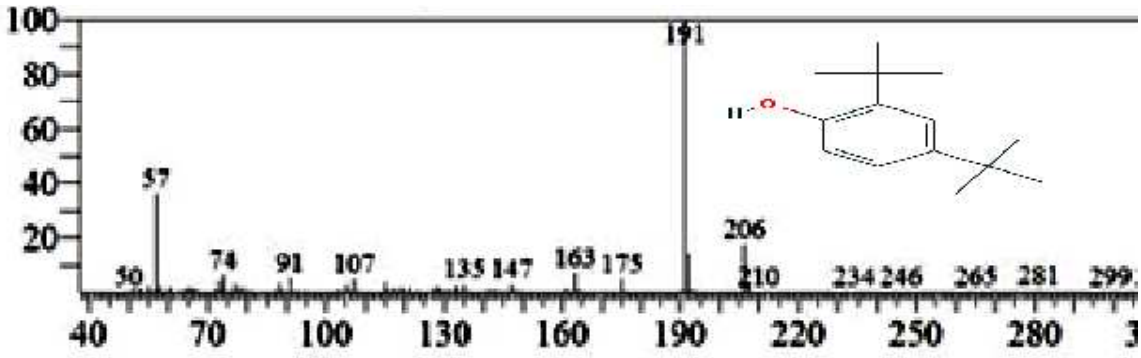


Fig (3.9): MS of Phenol ,2,4-bis(1,1- dimethylethyl)

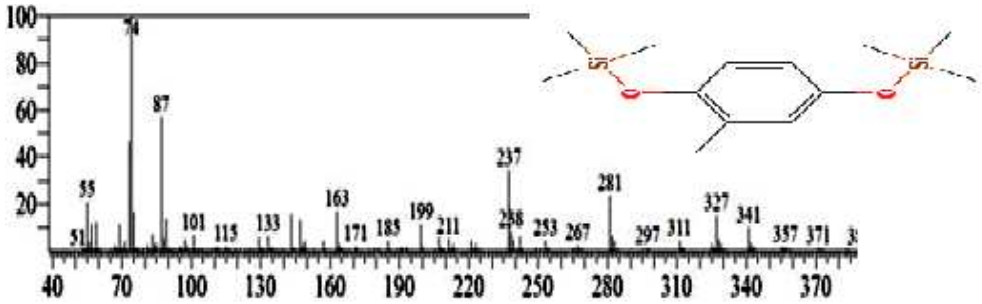


Fig (3.10): MS ofMethylhydroquinone,bis(trimethylsilyl)

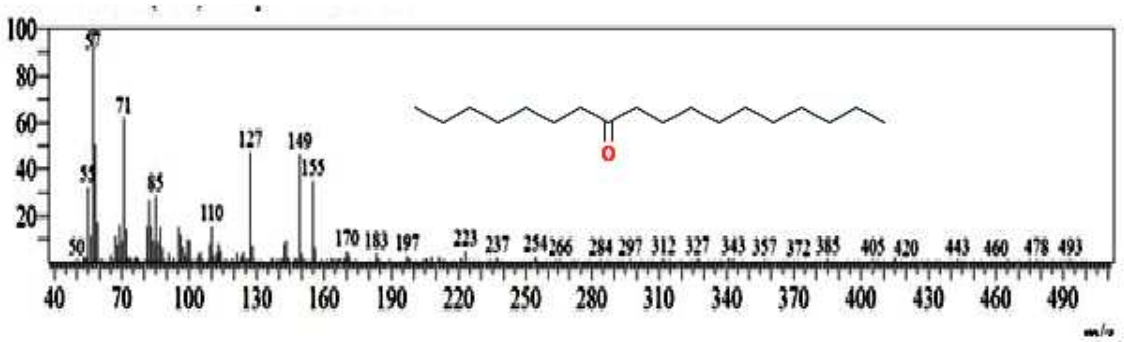


Fig (3.11): MS of 8-Octadecanone

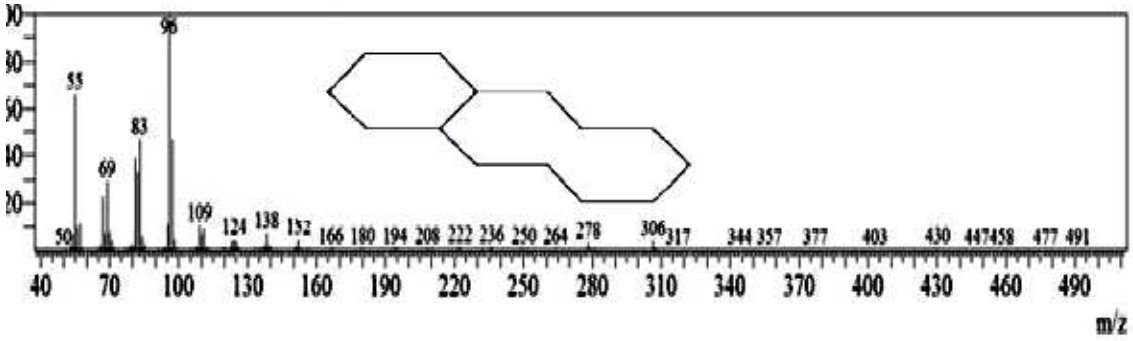


Fig (3.12): MS of Benzocyclodecene,tetradecahydro-

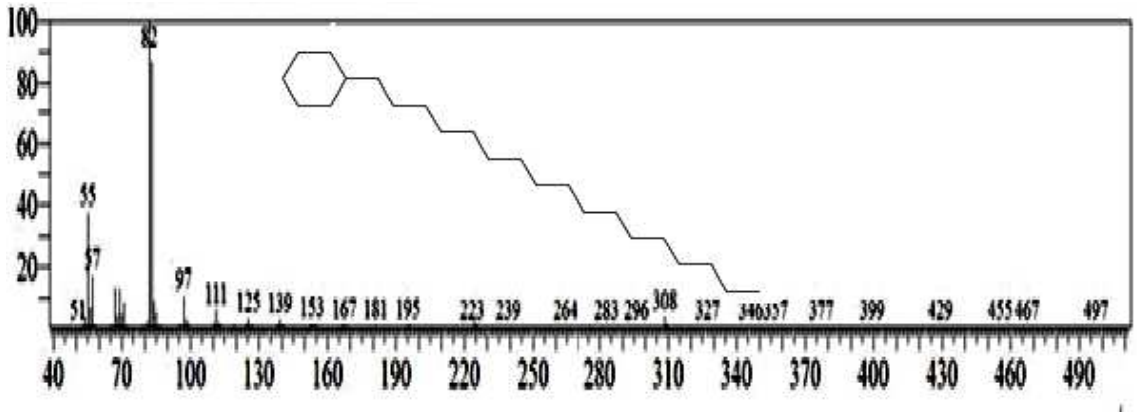


Fig (3.13): MS of n-Heptadecyclocyclohexane

The GC-MS results of the extract of *Ximenia Americana* have revealed some of the chemical components of the plant. The strong antibacterial activities exhibited by the extract are attributed to the presence of glycosides and phenols by way of their synergistic effects. These investigations provide supporting evidence to the use of *Ximenia Americana* in herbal medicine for the treatment of diseases and infections.

The chemistry of natural products remains a challenge and an important field of research in several science areas (chemistry, biology, medicine, agronomy, botany and pharmacy).The reasons for its large use are the considerable pharmacological potential observed in natural

products, in the great development in the process of detection, isolation, purification and, especially, the advances in spectrometric techniques [infrared (IR), mass spectrometry (MS) and nuclear magnetic resonance (NMR ^{1}H and ^{13}C) for structural elucidation of new and complex compounds. (Loganathan, *et al.* 1990).

3.8. **Structure Elucidation of compound Zm4:**

The TLC chromatogram of compound Zm4 using mobile phase Chloroform:Methanol : formic acid (70:30:2) showed one compound fig (3.9), which gave violet colour with vanillin sulphuric acid.

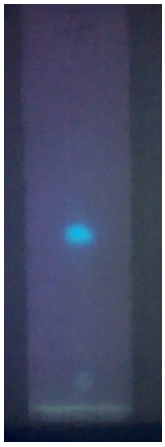


fig (3.14): **Zm4 under UV light (366)**

Table (3.10): TLC Detection of Compound Zm4:

Spot No.	R_f value	Color					
		Day light		UV Light			
				254 nm		366 nm	
1	0.5	Before spray	After spray	Before spray	After spray	Before spray	After spray
		blue	violet	No color	No color	blue	black

The purity of Compound **Zm4** was indicated by TLC, while its structure was elucidated by ^{1}DNMR (^{13}CNMR and ^{1}HNMR), and confirmed by MS.

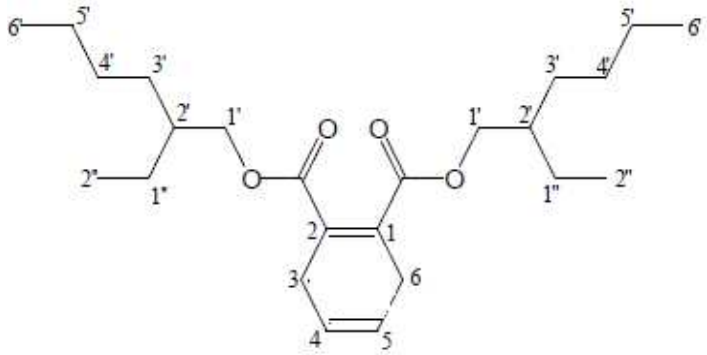
^{1}HNMR. Spectra showed the presence of two terminal methyls at δ 0.97 and 0.91 assigned for H6'and $H_{2''}$, three downfield signals at δ 4.60, 4.22 and 3.66 are assigned for $H_{1'}$, $H_{2'}$and H_3, respectively. In addition to downfield aromatic signals at δ7.7 assigned for H_4 respectively. fig (3.15)

^{13}CNMR data showed intense signals attributed to two quaternary carbons including ester carbonyl group at δ 168.9 and carbon attributed to endocyclic double bond at δ 131.00, two methine signals at δ128.4 (attribute to aromatic ring) and δ 38.8 ($C_{2'}$); six methylenes including an oxygen-bearing at δ 67.6 ($C_{1'}$) and endocyclic at δ30.2 (C_3); and two terminal methyl groups at δ 12.9 and 9.9.fig (3.16)

Data obtained from ^{1}H and^{13}C spectrum showed that this compound has molecular formula of $C_{12}H_{20}O_4$ corresponding to MW equal to 196 which is half of the value obtained from its MS fig (3.17) which indicates the molecular ion peak [M] $^{+}$ion at *m/z* 392.Thus we must have two fold symmetry in the molecule with a sum formula of $C_{24}H_{40}O_4$.

The higher order aromatic signals shows an AA'BB' spin system implicate an orthodi substituted benzene moiety (AA': 0.5, AB: 7.7, AB': 1.3 and BB': 7.5). Oxygentated methylene indicated the direct attach of carbonyl group into benzene ring and two different methyl groups indicated that remaining side chain is branched octyl.

The NMR spectral data of compound Zm4were in good agreement with that reported data for di-(2'-ethylhexyl) dihydro phthalate as shown in Table (3.11).



Structure of Compound Zm4[Di-(2'-ethylhexyl)dihydro phthalate]

Table (3.11): ^{1}H and ^{13}C NMR Assignment of Compound Zm4 (500 MHz, MeOD) and in Comparison with the Reported Data for di-(2'-ethylhexyl)dihydro phthalate (in MeOD)(ref.)

Position	δ ^{1}H (Multiplicity, *J* in Hz)	δH (M, *J* in Hz) of di-(2'-ethylhexyl) phthalate [ref]	δ ^{13}C	δ^{13}C of di-(2'-ethylhexyl) phthalate [ref.]
1 & 2	-	-	131.00	130.9
3 & 6	3.6 (*m*)	3.6 (*m*)	30.21	30.46
4 & 5	7.7 (*m*)	7.70 (*m*)	128.44	128.84
1'	4.6	4.77 (*d*, 13.2)	67.70	68.16
2'	4.2	4.22 (*sept*)	38.76	38.84

3'	2.1 (m)	2.80	29.33	29.01
4'	1.9 (m)	1.23-1.30 **	23.54	23.84
5'	1.3 (m)	1.23-1.30 **	22.62	23.05
6'	0.9 (m)	0.91**	12.99	13.9
1"	1.3	1.23-130 **	28.72	28.84
2"	0.91	0.71	9.99	9.2
C=O	-	-	168.97	167.72

** Overlapped signals

(Ref.D. Lyutskanova, V. I. (2009).Aleksieva and V. Peltekova. Isolation and characterization of a psychrotolerant streptomyces strain from permafrost soil in spitsbergen, producing phthalic acid ester. *24*.

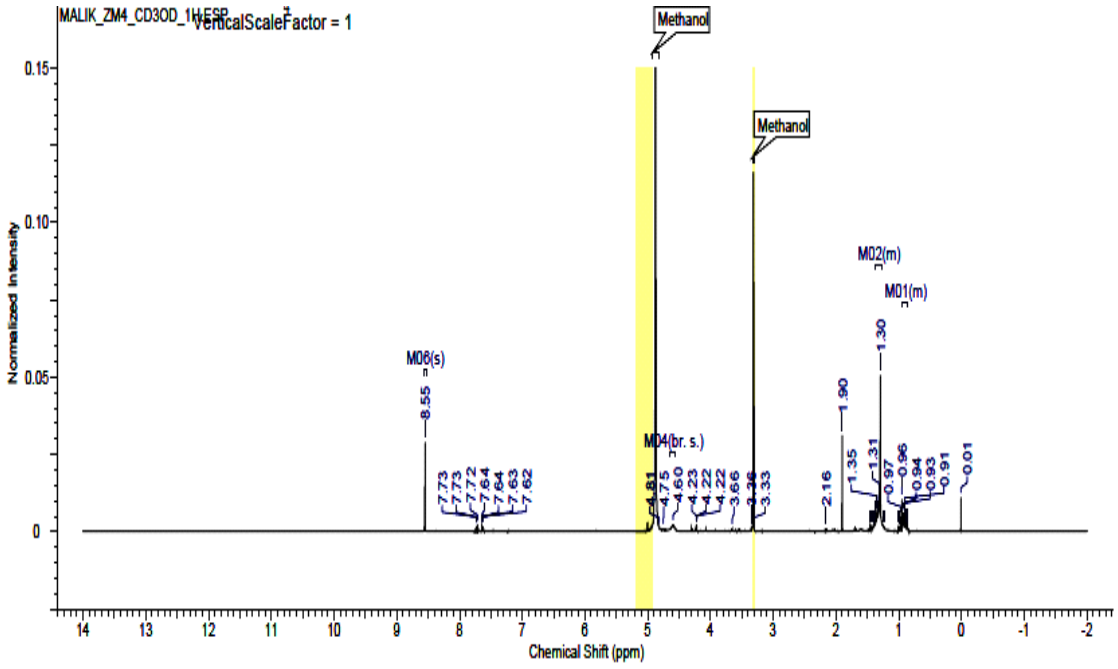


Fig (3.15): ^{1}H-NMR-spectrum of compound **Zm4** (500MHz, MeOD).

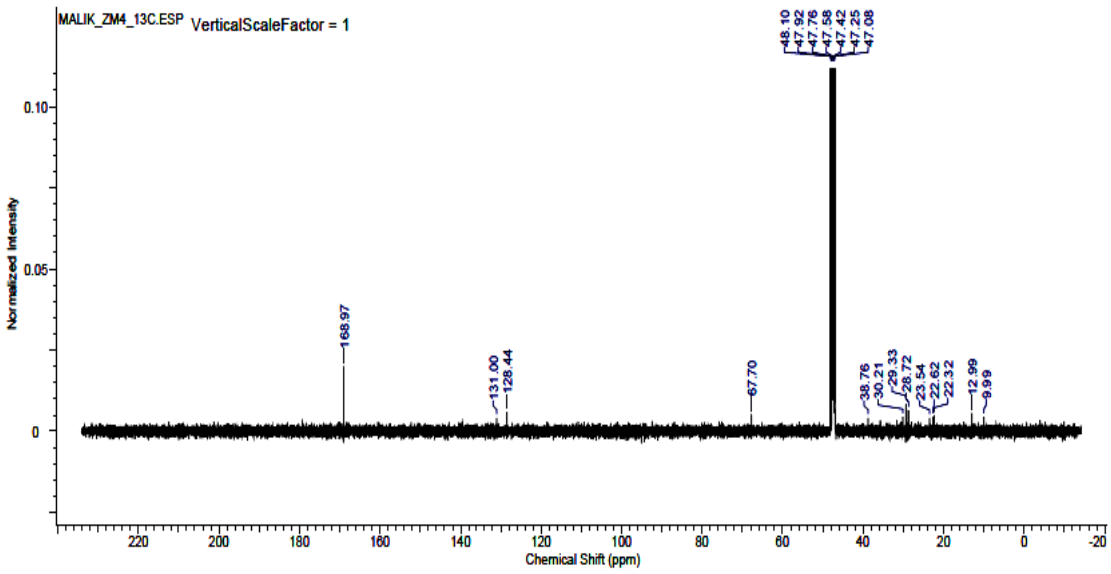


Fig (3.16): ^{13}C- NMR spectrum of compound **Zm4**(500MHz, MeOD).

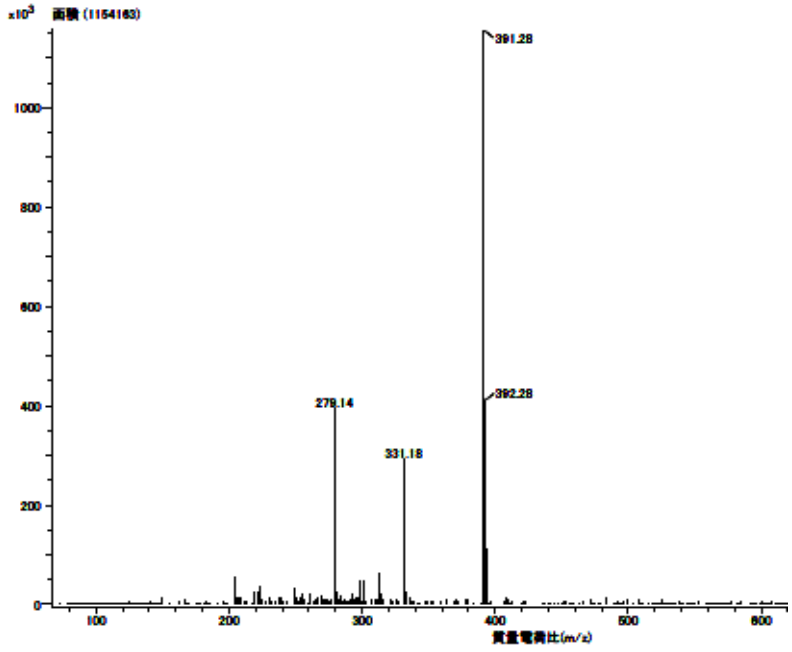


Fig (3.17): **Mass spectrum of compound Zm4**

CHAPTER FOUR

REFERENCES

CONCLUSION AND RECOMMENDATIONS

Conclusion

* The different analysis of *Ximena Americana* showed possible potential activities of these extracts and that validated its traditional uses.

* The phytochemical screening of different parts reveals the presence of steroids, terpinoids, alkaloids, flavonoids, tannins, saponins and glycosides. This result supports the use of this plant in traditional medicine for treatment of different diseases.

* The antimicrobial bioassay tests of the ethyl acetate extract of *X. Americana* bark showed high activity against four common bacteria isolates *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus* and fungi *Candida albicans* and was active against all of them. The highest degree of activity in Methanol extracted of the bark was for *B. subtilis* (inhibition zone: 35-30 mm), and fungi *Candida albicans* were not affected by these extracts The plant could be a veritable and a cheaper substitute for conventional drugs from the plant is easily obtainable and the extract can easily be made via simple processes.

* One of the phthalate derivatives was obtained as pure compound beside other nine semi pure compounds.

* The toxicity of these extracts has been studied via “Brine shrimp lethality” test, whereas, study has shown that LD50 for Chloroform and ethyl acetate are: 5.1817 µ g/ml and 16.3765 µ g/ml respectively, which confirm toxicity of these extracts. Results show that these extracts have a bioactivity in treatment of cancer.

Recommendations:

* Further studies should be conducted for other biological activities.

* Other morphological parts of the plants should be screened.

* The plant could be a veritable and cheaper substitute for conventional drugs since the plant is easily obtainable and the extract can easily be made via simple processes of maceration or infusion.

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