SCREENING FOR BIOACTIVE COMPOUNDS IN FIVE SELECTED NIGERIAN MEDICINAL PLANTS AGAINST SOME PATHOGENIC MICROORGANISMS

By

ISMAIL BABATUNDE ONAJOBI

B.Sc. (Hons.) Microbiology (Ogun), M.Sc. Microbiology (Ibadan)

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CERTIFICATION

I certify that this work was carried out by Ismail Babatunde ONAJOBI in the Department of Microbiology, University of Ibadan, Ibadan, Nigeria.

……

Supervisor
O. E. Fagade
B.Sc., (Ife); M.Sc., Ph.D. (Ibadan).
Professor of Microbiology, Department of Microbiology,
University of Ibadan, Ibadan, Nigeria

List of Abbreviations

MDR	Multi Drug Resistant
TLC	Thin Layer Chromatography
DMSO	Dimethyl Sulphide
TMS	Tetramethyl Saline
PCA	Plate Count Agar
SDA	Sabouraud Dextrose Agar
PDA	Potato Dextrose Agar
NMR	Nuclear Magnetic Resonance
COSY	Correlation Spectroscopy
EXSY	J-Spectroscopy, Exchange Spectroscopy
NOESY	Nuclear Overhauser Effect Spectroscopy
TOCSY	Total Correlation Spectroscopy
2D-COSY	Two Dimensional Correlation Spectroscopy
2D NMR	Two-Dimensional Nuclear Magnetic Resonance
DEPT	Distortionless Enhancement Polarization Transfer
APT	Attached Proton Test
HOHAHA	Homonuclear Hartmann–Hahn Spectroscopy
HSQC	Heteronuclear Single-Quantum Correlation Spectroscopy
HMBC	Heteronuclear Multiple-Bond Correlation Spectroscopy

Abstract

The search for new drugs to combat infectious diseases has stimulated the interest of scientists globally due to emergence of Multi Drug-Resistant (MDR) microorganisms. Indigenous medicinal plants are potential reservoir of bioactive compounds from which new active drugs could be obtained, but are yet to be adequately exploited. This study was therefore designed to screen for new bioactive compounds from some indigenous medicinal plants against selected MDR microorganisms.

Harungana madagascariensis Lam. Ex Poir and *Enantia chlorantha* Oliv. barks, *Senna alata* Linn., *Gossypium hirsutum* Linn. and *Alstonia bonnie* De Wild leaves were collected from a farmland in Idi-Ayunre, Ibadan and authenticated at Forest Research Institute of Nigeria. Ethanol extracts from the leaves and barks were tested against reference strains of *Escherichia coli, Bacillus subtilis, Salmonella typhi, Shigella flexneri, Pseudomonas aeruginosa* and *Staphylococcus aureus, Microsporum canis, Candida albicans, Candida glabrata*, and *Aspergillus flavus* using Agar well diffusion method. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the extracts were determined using tube dilution method. Extract purification was carried out using column chromatographic techniques. Chemical structures of compounds obtained were determined using Mass spectroscopy, Ultraviolet spectroscopy, Infra-Red spectroscopy and (1D and 2D) Nuclear Magnetic Resonance. Purified compounds which showed the best antimicrobial activities were tested against MDR clinical strains of *P. aeruginosa* and *S. aureus* compared to standard antibiotics. The compounds' antioxidant Radical Scavenging Activity (RSA), and *in vitro* alpha-glucoxidase enzyme inhibition activity was measured at 400 nm using Ultraviolet-Visible absorption spectrophotometer with arcabose as positive control. Data were analysed using ANOVA at $p = 0.05$.

Harungana madagascariensis, E. chlorantha and *S. alata* crude extracts showed broad spectrum antibacterial activity, inhibiting all the tested bacterial species with 24.3±0.3, 25.7±0.3 and 27.7±0.6 (mm) diameter zones of inhibition respectively. All the extracts except *A. bonnie* leave extract exhibited more than 90% inhibition against *M. canis. Enantia chlorantha* and *H. madagascariensis* bark extracts showed highest inhibition

against *C. glabrata* (80%) and *C. albicans* (70%) respectively. The MIC of the extracts ranged from 5.0 to 20.0 mg/mL while the MBC ranged from 20.0 to 30.0 mg/mL. Minimum fungicidal concentration of 50.0 mg/mL of the extracts inhibited all the tested fungi. Five new prenylated anthranoids (Harundigin anthrone, kenganthranol D, E, F and G) from *H. madagascariensis* were identified. These purified compounds at 100 µg/mL inhibited all the tested microorganisms. The MDR *S. aureus* was susceptible to isolated kenganthranol G with MIC of 25 µg/mL, but was resistant to standard antibiotics used (oxacillin, amikacin, chloramphenicol, erythromycin, sulfamethozale and ciprofloxacin). *Harungana madagascariensis* exhibited most significant antioxidant activities against 1,1-Diphenyl-2-picrylhydrazyl radical with 92% RSA, IC_{50} (33.3±1.8) at 0.5 µg/mL. Harundigin anthrone and kenganthranol E were good inhibitors (97%) of alpha-glucooxidase at IC_{50} of 69.9±4.2 and 122.4±1.1 respectively as against arcabose with 59.1%.

The prenylated anthranoids obtained from the plants exhibited antimicrobial and anti-enzymatic activities against multidrug-resistant *S. aureus.* They could serve as alternative source of bioactive compounds against resistant strains of *S. aureus*.

Keywords: Antimicrobial, Multidrug-resistant *Staphylococcus aureus*, Alpha-glucooxidase, Prenylated anthranoids.

Word count: 484

DEDICATION

This work is dedicated to my parents, Alhaji and Alhaja R. A. Onajobi, my Brother, Mr. M. Onajobi, My sisters: Mrs. A. A. Ogunbanjo, Mrs. F. Akinwande, Mrs S. A. Orekoya and Mrs B. Okunade, my one and only Mrs A. A. Onajobi, and to my daughter, Miss Bushra and son, Abdul Jawwad Onajobi.

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Ismail Babatunde, ONAJOBI

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CHAPTER ONE

INTRODUCTION

The survival of man has been dependent on his innate curiosity to examine by trial and error all aspects of his environment (Saeed *et al*., 2004). An attempt to promote rural development that reconciles improvement in the quality of life and conservation of natural resources have had more success when based on the local knowledge and current patterns of resource use within the involved communities (IES, 1995). Studies on indigenous uses of plants in several parts of the world have been documented (Idu and Omoruyi, 2003; Chunlin *et al*., 2009). Plants are used for medicinal purposes and they play important part in the culture and tradition of Africa. In Nigeria, traditional plants play an important role in medical system and plant materials remain an important resource to combat serious diseases world wide. The use of plant and its products has a long history that began with folk medicine and through the years has been incorporated into traditional and allopathic medicine (Dubey *et al*., 2011). Scientific exploration of traditional knowledge on the use of herbs in treatment of various ailments is one of the thrust areas of research, thus, up to 80% of the population depends directly on the traditional medicine for primary health care (WHO, 2008b).

Since antiquity, many plants species have been reported to produce and contain a diverse range of bioactive constituents such as alkaloids, steroids, tannins, terpenes, flavonoids, cardiac glycoside and phenolics, which purportedly provide excellent leads for new drug developments (Newman *et al*., 2000) and therefore, should be utilized to combat the disease-causing pathogens (Kamali and Amir, 2010). Secondary metabolites produced by plants constitute a source of bioactive substances and scientific interest in these plants-derived substances has increased, due to identification of many new drugs of plant origin. The major advantage of these new generation drugs is their efficacy with very little or no side effects and their low cost. Medicinal plants have been used in traditional treatments

of numerous human diseases for thousands of years and in many parts of the world and indigenous people have adapted different modes of application and uses of traditional medicine (Adnan *et al*., 2014). Hence, researchers have paid attention to safer phytomedicines and biologically active compounds isolated from plant species used in herbal medicines with acceptable therapeutic index for the development of novel drugs (Warrier *et al*., 1995; Pavithra *et al*., 2010).

1.1 Medicinal plants

The plant kingdom represents a rich storehouse for organic compounds, many of which have been used for medicinal purposes. Natural products are the fascinating varieties among the innumerable gifts of nature and have being inseparable parts of human history. Use of herbal medicines in Nigeria represents a long history of human interactions with the environment. Since mostly, they fulfil our basic requirements. Man has used plants in a variety of ways from the beginning of his existence throughout the ages. It is interesting to note that plants selected and used medicinally in different continents, in the region, as well as in neighbouring countries were similar or even closely related (Reddi *et al*., 2009).

Medicinal plants may be defined as those plants that are commonly used in treating and preventing specific ailments and diseases (Anselem, 2004). The important role of medicinal plants in health care delivery cannot be over emphasized as they are of great importance to the health of individuals and communities. The medicinal value of these plants lies in some chemical substances that produce a definite physiological action on the human body (Edeoga *et al*., 2005). Many of these indigenous medicinal plants are used as spices and food plants. They are also sometimes added to foods meant for pregnant and nursing mothers for medicinal purposes (Okwu, 2001). Utilization of plants as traditional remedies still occupies a central place in developing countries especially among rural communities and many plants have been shown to be highly effective for treating diverse ailments (Omojasola and Awe, 2004; Ali *et al*., 2007).

The rediscovery of the connection between plants and health is responsible for the launching of new generations of multi-component botanical drugs, dietary supplements

and plant-produced recombinant proteins (Raskin *et al*., 2002). Approximately 700 mono and poly-herbal preparations in the form of decoction, tincture, tablets and capsules from more than 100 plants are in clinical use (Chakraborthy, 2008). With the advancement in Science and Technology, remarkable progress has been made in the field of medicine with the discoveries of many natural and synthetic drugs (Preethi *et al*., 2010).

1.2 Antibiotic resistance

Antibiotics are undeniably one of the most important therapeutic discoveries of the 20th century that had effectiveness against serious bacterial infections. However, only one third of the infectious diseases known have been treated from these synthetic products (Sharma, 2011). This is because of the emergence of resistant pathogens is beyond doubt the consequence of years of widespread indiscriminate use, incessant and misuse of antibiotics (Enne *et al*., 2001; Westh *et al*., 2004). Antibiotic resistance has increased substantially in the recent years and is posing an ever increasing therapeutic problem. Serious infections caused by bacteria that have become resistant to commonly used antibiotics have become a major global healthcare problem in the 21st century (Idu *et al.*, 2013).

In developing countries like Nigeria, bacterial infections are still the main cause of deaths (Iwu *et al.*, 1999). Increase in failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents led to the screening of several medicinal plants for their potential antimicrobial activity (Colombo and Bosisio, 1996). One of the methods to reduce the resistance to antibiotics is by using antibiotic resistance inhibitors from plants (Kim *et al*., 1995; Alagesaboopathi, 2011). Plants are known to produce a variety of compounds to protect themselves against a variety of pathogens. It is interesting to note that phytomedicines have shown great potential in the treatment of intractable infectious diseases (Idu *et al.*, 2007). It is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug resistant pathogens (Ahmad and Beg, 2001).

Resistance of both human and animal pathogenic microorganisms to drugs have been reported in recent years from all over the world, particularly in developing countries, due to indiscriminate use of commercial antibiotics in the treatment of infectious diseases (Akinjogunla *et al*., 20011). Though, the resistance development by microbes cannot be stopped, appropriate action will reduce the mortality and health care costs by using antibiotic resistant inhibitors of plant origin (Ahmad and Beg, 2001). The increase in resistance of microorganisms due to multi various use of commercial antimicrobial drugs has encouraged scientists all over the world to search for new antimicrobial substances from various antecedents comprising medicinal plants (Akinjogunla *et al*., 2009).

1.3 Statement of problem and Justification

Infectious diseases account for approximately one-half of all deaths in tropical countries, the worst affected are the developing countries, which account for nearly 99 percent of deaths. In Nigeria, mostly children and young adults die each year because of the same reason, causing a total of 7 million deaths. The infectious diseases most prevalent in the developing world are diarrhoeal, tuberculosis, malaria and measles. Diseases once believed to be under control have re-emerged as major global threats. The emergence of drug-resistant strains of bacteria, viruses and other parasites pose new challenges in controlling infectious diseases. This situation has forced the biomedical scientists to look for new antimicrobial substances from alternate source, such as medicinal plants. The search for new drugs to combat infectious diseases has stimulated the interest of scientists globally due to emergence of Multi Drug-Resistant (MDR) microorganisms. Indigenous medicinal plants are potential reservoir of bioactive compounds from which new active drugs could be obtained, but are yet to be adequately exploited. Therefore, it is important to screen for new bioactive compounds from some indigenous medicinal plants against selected MDR microorganisms in order to develop more effective drugs.

1.4 Research objectives

The objectives of the study, therefore, were to:

a. Extract each plant material using different solvents
b. Phytochemically screen each of the plant extracts
c. Evaluate *in vitro* toxicity of each plant extracts
d. Determine antimicrobial activities of the plant extracts
e. Purify crude plant extracts through chromatographic analysis
f. Structurally elucidate purified bioactive compounds.

CHAPTER TWO

LITERATURE REVIEW

2.1 History of medicinal plants

Plants serve as sources of food and medicinal herbs for Man and other animals in their environment. Thousands of years ago, Man has depended on plants in order to provide solutions to the myriad of health problems plaguing him (Oliver, 1960). Through trial and error it was discovered that some plants are good for food, that some are poisonous, and that some produce bodily changes such as increased perspiration, bowel movement, urination, relief of pain, hallucination, and healing (Saeed *et al*., 2004). Over the millennia these observations were passed orally from generation to generation, with each generation adding to and refining the body of knowledge. Every culture in the world has in this manner developed a body of herbal knowledge as part of its tradition.

The first written record of medicinal plants was created on clay tablets over 5000 years ago by the Sumerians**,** in ancient Mesopotamia (present day Iraq). Around 1500 BC the Ancient Egyptians wrote the Ebers Papyrus which listed over 850 herbal medicines (Foley, 2006). This compilation includes many herbs that are recognized and used today. Bottcher (1965), as cited by Wiart (2006), reported that the Chinese book on roots and grasses "Pen T'Sao," written by Emperor Shen Nung circa 2500 BC, treated 365 drugs (dried parts of medicinal plants), many of which are used even nowadays.

The Ebers Papyrus, written circa 1550 BC, represents a collection of 800 prescriptions referring to 700 plant species and drugs used for therapy such as pomegranate, castor oil plant, aloe, senna, garlic, onion, willow, coriander, juniper, common centaury, etc. (Tucakov, 1964). According to data from the Bible and the holy Jewish book the Talmud, during various rituals accompanying a treatment, aromatic plants were utilized such as myrtle and incense (Dimitrova, 1999).

In Homer's epics the Iliad and the Odysseys, created circa 800 BC, 63 plant species from the Minoan, Mycenaean, and Egyptian Assyrian pharmacotherapy were referred to. Some of them were given the names after mythological characters from these epics; for instance, Elecampane (*Inula helenium* L. *Asteraceae*) was named in honour of Elena, which was the centre of the Trojan War. As regards the plants from the genus *Artemisia*, which were believed to restore strength and protect health, their name was derived from the Greek word *artemis*, meaning "healthy" (Toplak, 2005).

Hippocrates (460-377 BC) the famous Greek physician who believed that the body naturally became diseased and that disease were not caused by superstitions or by gods. He wrote many medical theories that hold true today (Herbal Academy, 2014). He is often referred to as the "Father of Modern Medicine." Doctors today still take the Hippocratic Oath upon completion of their medical degrees. With his writings he preserved the medical practices of the Greeks and Romans. He incidentally wrote about the medicinal properties of willow bark and how it could be used for fevers and pain. Today, this very bark is used to make aspirin (Herbal Academy, 2014).

Theophrast (371-287 BC) founded botanical science with his books "De Causis Plantarium" Plant Etiology and "De Historia Plantarium"—Plant History. He generated a classification of more than 500 medicinal plants known at the time (Pelagic, 1970). Theophrast underscored the important feature for humans to become accustomed to them by a gradual increase of the doses. Due to this, he gained the epithet of "the father of botany," being that he has great merits for the classification and description of medicinal plants (Bazala, 1943; Nikolovski, 1995).

2.2 Medicinal plants, an alternative means of treatment

Use of medicinal plants plays a vital role in alleviating the basic health needs in developing countries like Nigeria, and many plants may offer a new source of antibacterial, antifungal and antiviral agents with significant activity against infective microorganisms (Mingarro *et al*., 2003). Most plant parts e.g. (bark, root, seeds, fruit, leaf) serve as major sources of active ingredients and products of secondary metabolites used in

curing diseases, production of drugs as well as in maintaining good health by both the traditional and orthodox medical practitioners (Cowley, 2002).

Medicinal plants can be used to cure ailments such as malaria, diabetes, gonorrhoea, diarrhoea, measles, dysentery, fever, filariasis, chicken pox, hepatitis, hypertension and many others. Agishi (2004), reported the use of *Mangifera indica* (mango) and *Piliostigma thonningii* in the cure for malaria and diabetes respectively while Tor-Anyiin *et al*. (2003) reported the use of *Lawsonia inermis* (ilele) to treat gonorrhoea, the leaves of *Irvingia gabonensis* to cure diarrhea, *Nauclea latifolia* (uche) to cure measles and *Annona senegalensis* to cure dysentery. Nwachukwu *et al.* (2010) reported the use of *Nauclea latifolia* and *Piliostigma thonningii* to treat *filariasis*, *Azadirachta indica* (dogoyaro) to cure chicken pox, *Zingiber officinale* (ginger) to treat hepatitis and *Acanthus montanus* to cure hypertension while Tringali (1995) reported the use of *Scorparia dulcis* to treat fever. The active ingredients in these plants provide an important natural safeguard which gave rise to the resultant effect of each plant. Rather than using a whole plant, researchers identify, isolate, and extract the individual plant components, thus capturing the active metabolite (Umeobi, 1994).

2.3 Plants' Metabolites

Plants have been classified as an essential source of secondary metabolites used as medicinal agents and a huge number of novel drug components have been derived from natural plant sources and their extracts used in traditional medicine (Obeidat *et al*., 2012). Demain (1980) described metabolites as the intermediate products of metabolism synthesized by plants for both essential functions (primary metabolites) and specific functions (secondary metabolites). Metabolites have various functions; primary metabolites are directly involved in normal growth, development, and reproduction, while secondary metabolites often play an important role in plant defence and other interspecies defences (Stamp, 2003). Examples of secondary metabolites include antibiotics and pigment such as resins and terpenes. In addition to active ingredients, plants contain minerals, vitamins, volatile oils, glycosides, alkaloids, bioflavanoids, and other substances that confer medicinal properties on them (Alaribe, 2008).

2.3.1 Types of Plants' Metabolites

Saponins are secondary metabolites that have similar chemical character to glycosides. They are believed to be useful in the human diet (controlling cholesterol level). According to Ong (2004), saponins can be used as an anti-inflammatory agent and also in the treatment of tuberculosis (Amoros *et al*., 1987).

Cowan (1999), described alkaloids as organic chemical compounds which contain heterocyclic nitrogen. Morphine is the first alkaloid used in medicine as an analgesic drug. Alkaloids can increase nutrient absorption and blood circulation, reduce pain and stimulate nerve system because of its narcotic effect (Ong, 2004).

Steroids have four carbon rings called 'steroid backbone' and classified as cardiac glycoside because of its specific response to the heart (Mohamad *et al*., 2001). Steroids have been used in allergy, arthritis and coronary failure therapy; control of pains during menstrual cycle and increasing women fertility (Ong, 2004).

Triterpenoids have been proven to inhibit human immunodeficiency virus but the mechanism is still unclear and there was a suggestion that the mechanism was related to membrane damage through lipophilic component on the alkaloid involved (Cowan, 1999). These naturally occurring triterpenoids include the lupine, ursane oleanane, lanostane, dammarane (Dang, *et al.*, 2009).

Phenols are some of the simplest bioactive phytochemicals consisting of single substituted phenol ring. Cinnamic and caffeic acids are common representatives of a wide group of phenylpropane-derived compounds which were in the highest oxidation state. The common herbs tarragon and thyme both contain caffeic acid, which is effective against viruses (Wild, 1994), bacteria (Thomson, 1978), and fungi (Duke, 1985).

Quinones are aromatic rings with two ketone substitutions, ubiquitous in nature and characteristically highly reactive. Quinone has potential range of antimicrobial effects leading to inactivation of the protein and loss of functions (Stern *et al.,* 1996). Quinones may render substrates unavailable to the microorganism; hence hindering the growth of the organism (Cowan, 1999).

Flavones are phenolic structures, containing one carbonyl group (as opposed to the two carbonyls in quinones) (Fessenden and Fessenden, 1982). Flavones are known to be synthesized by plants in response to microbial infection (Dixon *et al*., 1883). It should not

be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms.

Tripathi *et al*. (2003) reported that natural product derivatives represent over 50% of all drugs in clinical use, in which natural products derived from higher plants represent about 25% of the total. People in developing countries rely on traditional remedies such as herbs for their daily needs and about 855 traditional medicines include crude plant extracts. This means that about 3.5 to 4 billion of the global population rely on plants resources for drugs (Farnsworth, 1989).

2.3.2 Metabolites Determination Methods

Nuclear magnetic resonance (NMR) is a physical phenomenon in which magnetic nuclei in a magnetic field absorb and re-emit electromagnetic radiation. NMR allows the observation of specific quantum mechanical magnetic properties of the atomic nucleus (Keeler, 2010). Spectroscopy is the study of the interaction of electromagnetic radiation with matter. Nuclear magnetic resonance spectroscopy is the use of the NMR phenomenon to study physical, chemical, and biological properties of matters. As a consequence, NMR spectroscopy finds applications in several areas of science. NMR spectroscopy is routinely used by researchers to study chemical structure using simple one-dimensional techniques. Two-dimensional techniques are used to determine the structure of more complicated molecules. These techniques are replacing x-ray crystallography for the determination of protein structure. Time domain NMR spectroscopic techniques are used to probe molecular dynamics in solutions. Solid state NMR spectroscopy is used to determine the molecular structure of solids. Other scientists have developed NMR methods of measuring diffusion coefficients.

In other words, NMR spectroscopy is a powerful and theoretically complex analytical tool. It is important to remember that, with NMR, experiments are performed on the nuclei of atoms, not the electrons. The chemical environment of specific nuclei is deduced from information obtained about the nuclei (Keeler, 2010). Many scientific techniques exploit NMR phenomena to study molecular physics, crystals, and non-crystalline materials through NMR spectroscopy. NMR is also routinely used in advanced medical imaging

techniques, such as in magnetic resonance imaging (MRI). All isotopes that contain an odd number of protons or neutrons have an intrinsic magnetic moment and angular momentum, in other words a nonzero spin, while all nuclides with even numbers of both have a total spin of zero. The most commonly studied nuclei are ^{1}H and ^{13}C, although nuclei from isotopes of many other elements have been studied by high-field NMR spectroscopy as well.

2.4 Toxicity of Plant Extracts

Toxicity is the degree to which a substance can damage an organism (Wikipedia, 2014). Toxicity can refer to the effect on a whole organism, such as an animal, bacterium, or plant, as well as the effect on a substructure of the organism, such as a cell (cytotoxicity) or an organ such as the liver (hepatotoxicity).

Biological Assay is an experiment carried out on the test plant extracts to determine the level of potency or toxicity of their secondary metabolites. Bioactive compounds could be toxic to *Artemia salina* larvae. The eggs of the brine shrimp *Artemia salina* are readily available as fish food in pet shops. When placed in artificial seawater, the eggs hatch within 48 hours, providing large numbers of larvae. It is a typical experiment to investigate a dose-response relationship and one indicator of the toxicity of plant material is LD_{50} or LC_{50}, which refers to the amount of plant material that kills half of the test organisms. Brine shrimp lethality assay is considered a useful tool for preliminary assessment of toxicity and it has been used for detection of plant extract toxicity (Martinez *et al*., 1998).

The method is attractive because it is a simple, rapid, inexpensive and small amount of plant extracts are required to perform the test. Brine shrimps are small crustaceans related to shrimps and crabs that live in very salty areas that have a salinity of 20 -30 mg/L. This organism makes good test subject for toxicity since it is easy to acquire in large numbers, they survive well in small volumes of water and they do not have advanced nervous systems. All stages of their life cycle have been used, and hatching rate of the eggs after

exposure to contaminant has been used as a criterion for toxicity (Mongelli *et al*., 1996). The commonest stage used is the one of 24 -48 hours after hatching.

2.4.1 Importance of Determining Toxicity

The maximum therapeutic and minimum side effects of herbal remedies have been verified in numerous scientific investigations. Although, isolated or synthesized active compounds can become toxic in relatively small doses; it usually takes a much greater amount of a whole herb, with all of its components, to reach a toxic level. Saponins including those produced by the soapberry are very poisonous if swallowed, and able to lyse red blood cells and cause urticaria or skin rash in many people (Mohamad *et al*., 2001). Alkaloid compounds are sometimes toxic and do not have smell or taste. Plants use steroids to avoid being eaten by vertebrate animals. As with all plant-derived antimicrobials, the possible toxic effects of quinones must be thoroughly examined (Cowan, 1999). Herbs as medicines, however, can have powerful effects which should not be taken lightly (Anselem, 2004).

The concise information on various medicinal plants indicating Botanical name, Family name, Common name, Plant part used and their traditional usage, are presented in Table 2.1.

2.5 Herbal Medicine Today

Herbal medicine is a major component in all indigenous peoples' traditional medicine and a common element in Ayurvedic, homeopathic, naturopathic, traditional oriental, and Native American Indian medicine (Halberstein, 2005). WHO (2008b), reported that about 74% of One hundred and nineteen (119) plant-derived pharmaceutical medicines are used in modern medicine in ways that correlated directly with their traditional uses of plant medicines by native cultures. Major pharmaceutical companies are currently conducting extensive research on plant materials gathered from the rain forests and other places for their potential medicinal values (Halberstein, 2005). Today, the U.S. *Pharmacopoeia*, with its reliance on herbal compounds, has been all but forgotten. Most modern physicians rely on their desk reference, an extensive listing of chemically manufactured drugs. It is

TABLE 2.1: The scientific names of some plants, their common names and their uses

Scientific Name	Family	English/ Local name	Part used	Common Uses	Reference
Alstonia boonei	*Apocynaceae*	Egbu	Bark	As analgesic for rheumatic pains	Asuzu and Anaga (1991).
Azadirachta indica	*Meliaceae*	Neem/ Dogoyaro	Leaves	Anti malaria	Nwachukwu *et al*. (2010)
Ageratum conyzoides	*Asteraceae*	Goat weed /Imi eshu,	Leaves	As purgative and Eye Problems	Shekhar and Anju (2012)
Telfairea occidentalis	*Cucurbitaceae*	Ugwu	Leaves	Anaemia	Igoli *et al.* (2002)
Solanum torvum Swartz.	*Solanaceae*	Anyihi nyije	Leaves	Antidote	Yamashita *et al.* (1996)
Acanthus montanus	*Acanthaceae*	Elele-nyijuo	Root	Boils	Burkhill *et al.* (1985)
Zanthoxylum zanthoxyloides	*Rutaceae*	Ufu-otachacha	Stem bark	Contraceptive	Ogonwolu *et al.* (1998)
Lawsonia inermis	*Lythraceae*	Ilele	Leaves	Cosmetic	Adjanahoun *et al.* (1991)
Telfairea occidentalis	*Cucurbitaceae*	Ugwu	Leaves	Anaemia	Igoli *et al.* (2002)
Fuirena ciliaris	*Cyperaceae*	Ijan nyohe	Beads	Cosmetic	Adjanahoun *et al.* (1991)
Cassia obtusifolia	*Caesalpiniaceae*	Ufu ochiri	Leaves	Cough	Guo *et al.* (1998)
Phyllanthus muellerianus	*Euphorbiaceae*	Ohunte	Leaves	Cough	Igoli *et al.* (2003)

Scientific Name	Family	English/ Local name	Part used	Common Uses	Reference
Piliostigma thonningii	*Caesalpiniaceae*	Omepa	Leaves	Cough	Burkhill *et al.* (1985)
Ceiba pentandra	*Bombacaceae*	Ufu enwu	Leaves	Cuisine	Igoli *et al.* (2002)
Irvingia gabonensis	*Irvingiaceae*	Ono	Leaves, fruits and stem bark	Diarrhea	Okolo *et al.* (1995)
Acanthus montanus	*Acanthaceae*	False thistle/ Ogwu	Leaves	Chesty coughs and boils	Adjanahoun *et al.* (1991)
Afromomum melegueta	*Zingiberaceae*	Alligator pepper/ Ose oji	Seeds	Stimulant/ Diuretic	Githens (1949)
Anarcadium occidentale	*Anarcadiaceae*	Cashew / kanshuu	Bark, leaves	Ringworm infection, Diuretic	Gbile (1988)
Carica papaya	*Caricaceae*	Papaya, pawpaw/ Okpurukwa	Fruit, seeds	Diuretic, Antimalarial, and Eczema	Ketiku (1976).
Citrus aurantifolia	*Rutaceae*	Lime / oroma nkirisi	Fruit	Catarrh and Stomach aches	Tor-Anyiin *et al.* (2003)
Elaeis guineeensis	*Arecaceae*	Oil Palm tree/ nkwu, akwu	Seeds	Antidote for poisons	Aiyeloja and Bello (2006)
Mangifera indica	*Anarcadiaceae*	Mango / mangoro	Leaves, bark	Malaria	Tor-Anyiin *et al.* (2003)

Scientific Name	Family	English/ Local name	Part used	Common Uses	Reference
Piliostigma thonningii	*Caesalpiniaceae*	Omepa	Roots	Diabetes	Agishi (2004)
Sorghum guinensis	*Poacea*	Igbi	Leaves and seed	Diabetes	Agishi (2004)
Nauclea latifolia	*Rubiaceae*	Uche	Leaves	Dysentery	Adjanahoun *et al.* (1991)
Annona senegalensis	*Annonaceae*	Unwu	Leaves	Dysentery	Adjanahoun *et al.* (1991)
Bryophyllum pinnatum	*Crassulaceae*	Ufu ivo	Leaves and stem	Ear ache	Burkhill (1985)
Euphorbia hirta	*Euphorbiaceae*	Ufu idire	Stem		Burkhill (1985)
Scorparia dulcis	*Scrophulariaceae*	Ufu ija	Leaves	Fever	Tringali (1995)
Erythrina senegalensis	*Papilionoidae*	Eruana	Bark	Fever	Tringali (1995)
Carica papaya	*Caricaceae*	Ubgoja	Latex	Arrow Poison	Adjanahoun *et al.* (1991)
Alchornea cordifolia	*Euphorbiaceae*	Upia	Stem	Poison (arrow)	Adjanahoun *et al.* (1991)
Lawsonia inermis	*Lythraceae*	Ilele	Root	Syphilis/ Gonorrhoea	Okorie (1976)
Ocimum gratissimum	*Labiateae*	Ujuju-okpevu	Leaves	Typhoid fever	Tor-Anyiin *et al.* (2003)
Scoparia dulcis	*Scrophulaceae*	Ufu ija	Leaves	Tooth ache	Agishi (2004)
Alchornea cordifolia	*Euphorbiaceae*	Upia	Stem	Arrow Poison	Adjanahoun *et al.* (1991)

important to note that each entry, in addition to specifying the chemical compound and actions of a particular drug, also includes an extensive list of contraindications and possible side effects (Summer, 2008).

Metabolites derived from the plants remain the basis for a large proportion of the commercial medications used today for the treatment of heart disease, high blood pressure, pain, asthma, and other problems (Cowley, 2002). For instance, ephedra is an herb used in Traditional Chinese Medicine for more than two thousand years to treat asthma and other respiratory problems. Most of the research done on plants recently continues to focus on identifying and isolating active metabolites, rather than studying the medicinal properties of whole plants. However, herbalists believe that the power of a plant lies in the interaction of all its ingredients. Plants used as medicines offer synergistic interactions between ingredients both known and unknown (Oliver, 1960). The efficacy of many medicinal plants has been validated by scientists all over the world. With modern technology, the specific properties and interactions of plant constituents can now be identified and monitored. Though, substantial research is being carried out in other developed countries, drug producing companies and laboratories in the United States so far have not chosen to put resources into botanical research. This implies that herbal medicine does not have the same place of importance or level of acceptance in United States (Nunn, 1996).

2.6 Different Plants and Their Uses

Medicinal plants in the tropics are integral to healthcare and constitute one of the richest forms of tropical forest biodiversity. There is a need for scientific information on utilization, conservation, safety, efficacy and quality control to match the rapid growing demand in this field (Rao, 2009). The World Health Organization (WHO) compiled an inventory of more than 20,000 species of plants and their products used to control diverse diseases such as catarrh, bronchitis, pneumonia, ulcers and diarrhea with greater attention being paid to plants looking for new leads to develop better drugs against bacterial infections (Srinivasan *et al*., 2001).

2.6.1 *Senna alata*

Senna alata Linn (Fabaceae) is a perennial shrub widely distributed in tropical regions of the world, and commonly called "Ringworm bush" or candle stick. It grows well in forest areas of West Africa. *Senna alata* which is an official drug in the Nigerian Herbal Pharmacopoiea (NHP, 2008), is locally used in Nigeria in the treatment of wound infections, bronchitis and asthma as well as several infections, which include ringworm, parasitic skin diseases (Palanichamy *et al*., 1990). The leaves are reported to be useful in treating convulsion, gonorrhoea, heart failure, abdominal pains and oedema is also used as a purgative (Ogunti *et al*., 1993). Ibrahim and Osman 1995, reported that ethanol extract of it plants showed high activity against fungi: *Trichophyton mentagrophytes var interdigitale, T. mentagrophytes var. mentographytes, T. rubrum* and *Microsporium gypseum* (MIC: 125 mg/mL) and *Microsporium canis* (M1C: 25 mg/mL). Several studies (Akinsinde *et al*., 1995; Akinyemi *et al*., 2000) have been conducted to provide scientific basis for the efficacy of it plants used in herbal medicine.

2.6.2. *Gossypium hirsutum*

Gossypium hirsutum, local name Owu (Yoruba) is the principal cultivated cotton throughout the world (Vijayakumar, 2002). Wild cotton is mainly a plant of the coastal strand and lower coastal plains. *Gossypium hirsutum* grows in disturbed places, particularly along roads and on river overflow areas, well inland. Wild cotton can grow in almost all types of well drained soils. Optimum pH is said to be between 5.2 to 7.0 (Center for New Crops and Plants Products, 2002). The pigment of *G. hirsutum*, localized in lysigenous glands distributed in the epidermal layer of most tissues, confers insect resistance and serves as a deterrent to other herbivores. High levels of gossypol are also localized in the epidermal and cortex tissues of cotton roots (Smith, 1961) from which gossypol secreted extracellularly (Hunter *et al.*, 1978). The medicinal properties of gossypol are widely recognized in Africa, China and India and in folk medicine practices of the southern USA.

Several research groups have reported the inhibitory effects of gossypol and related compounds on in-vitro growth of cancer cell lines (Le Blanc *et al.*, 2002). In addition,

gossypol has been used in clinical trials for human gliomas (Bushunow *et al.*, 1999) and metastatic breast cancer (Van Poznak *et al.*, 2001). Gossypol was reported to have antiviral activity against enveloped viruses, including HIV (Vander Jagt *et al.*, 2000), and inhibited the growth of numerous parasitic organisms (Montamat *et al.*, 1982), fungi (Puckhaber *et al.*, 2002), microbes (Yildirim-Aksoy *et al.*, 2004), and insects (Stipanovic *et al.*, 2006).

2.6.3. *Alstonia boonei*

Alstonia boonei De Wild belongs to the family called *Apocynaceaee* which consists of about 50 species widely distributed in the continents of Africa, Asia and America (Iwu, 1993). *Alstonia boonei*, known as *Ahun* in Yoruba, *Egbu-ora* in Igbo, *Ukhu* in Edo and *Ukpukunu* in Urhobo, a widely distributed plant in the lowlands and rain-forest areas of Nigeria. *A. boonei*, a medicinal plant that is widely used across Africa for various ailments. The stem bark of *A. boonei* has been reported to possess anti-inflammatory, analgesic and antipyretic activities (Olajide *et al*., 2000). The stem bark is commonly used in malaria treatment, and it is listed in the African Pharmacopoeia as an antimalaria drug. An infusion of the bark is used as antivenom for snake bites. It is also used in treating painful micturation and rheumatic conditions (Asuzu and Anaga, 1991) with an infusion of the root and stem bark is taken as a remedy for asthma.

A liquid made from the stem bark and leaves (concoction) is drunk to treat impotence. In Nigeria, it is used for the treatment of ulcers, fever, painful micturition, insomnia, chronic diarrhea, rheumatic pains, as anti-venom for snake bites (Adomi, 2008) and in Cameroon and Liberia as remedy for snake bite and arrow poisoning (Kweifo-Okai *et al*., 1995). The chemical constituents include alkaloids, triterpenoids and steroids. Over 90% of the isolated chemical constituents are alkaloids many of which are the indole types. The major alkaloids are echitamine and echitamidine. *In vitro* antiplasmodial activity of the alkaloids against both drug sensitive and resistant strains of *P. falciparum* and *in vivo* activity against *P. berghei* in mice (Olajide *et al.*, 2000), have been reported. In another trial, the anti-inflammatory properties of the alcohol extract *A. boonei* in rat hind paw edema has

been used to justify its use in herbal medicine for the treatment of rheumatic and muscular pains (Osadebe, 2002).

2.6.4. *Enantia chlorantha*

Enantia chlorantha Oliv., commonly known as the African yellow wood, which is a tropical rainforest tree and widespread in central Africa, belongs to the family *Annonaceae*. *Enantia chlorantha* is an ornamental tree of up to 30 m high, with dense foliage and spreading crown. The stem is fluted, the bark fissured geometrically and the outer bark is thin and dark brown; the inner bark is light brown above and pale cream beneath. Its root barks are locally used for the treatment of malaria, hepatic disorders, tuberculosis, (Wafo *et al*., 1999). Phytochemical studies showed that several protoberberine-type quaternary alkaloids (Nyasse *et al*., 2002), of which palmatines a major metabolite (Wafo *et al*., 1999), were isolated from the stem bark of *E. chlorantha*.

The stem bark extracts of this species and related protoberberine alkaloids displayed a great variety of biological and pharmacological activities such as anti-human immunodeficiency virus (HIV) activity, antitrypanosomal and antiplasmodial effects (Kimbi and Fagbero-Beyioku, 1996), hepatoprotection, anticandidal and antibacterial activities (Moody *et al*., 1995), anti-ulcer action (Tan *et al*., 2002), cytotoxicity (Iwasa *et al*., 2001), anti-monoamine oxidase activity (Kong *et al*., 2001), inhibition of biosynthesis of catecholamine and dopamine (Shin *et al*., 2000), anti-acetylcholinesterase activity, nerve growth factor-potentiating activity, inhibition of reverse transcriptase of tumor, antiradical and antioxidant effects (Rackova and Majekova, 2004), lipoxygenase inhibition and rat lens aldose reductase inhibition (Lee, 2002), as well as anti-inflammatory, antinociceptive and antipyretic effects (Kupeli *et al*., 2002).

In addition to palmatine, it is used as a natural pharmaceutical drug for treatment of viral hepatitis B, the reduced form *dl*-tetrahydropalmatine (*dl*-THP) is also one of major bioactive components in *Corydalis yanhusuo* that is well-known as a traditional Chinese herbal medicine. Studies have shown that *dl*-THP exerts remarkable analgesic without any addiction, sedative-tranquilizing, hypnotic anxiolytic (Leung *et al*., 2003), hypotensive

and inhibiting the aggregation of thrombocyles, hypo-locomotion (Lin *et al*., 2001), and neuroprotective actions. Moreover, *dl*-THP has been shown to deplete the levels of dopamine, noradrenaline, serotonin, and monoamine in brain (Chang and Lin, 2001). This compound also is a very effective antiepileptogenic and anticonvulsant agent (Lin *et al*., 2002).

2.6.5. *Harungana madagascariensis*

Harungana madagascariensis Lam. (*Hypericaceae*) is a native of tropical Africa. It is a small- to medium-sized shrub (up to 1.65 m high) with fine stellate hairs and ovate lateral leaves (Irvine, 1961), otherwise called dragon's blood tree. Among Yorubas, the local names are *Amuje, Aroje,* and *Arunje* while called *Uturu* among the Igbos of southeastern Nigeria. It is widespread and locally abundant in areas where annual rainfall exceeds 1300mm (Csurhes and Edwards, 1998).

It pharmacological models allowed proof of their effectiveness in the treatment of a variety of ailments including jaundice, diarrhea, dysentery, typhoid fever, and constipation (Atindehou *et al*., 2002). The stem bark is used for nephrosis, malaria, gastro-intestinal disorders and fever while the leaves and stem bark are used for the treatment of anaemia. Okoli *et al*. (2002), demonstrated anti-microbial activity of aqueous leaf extract on different strains of bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Eschericia coli* and *Salmonella typhi* which support its usage for the treatment of gastro-intestinal disorders. Tona *et al*., 2000, showed the inhibition of *Entamoeba hystolytica* growth by the stem back extract at a concentration less than 10 gm/L.

Previously isolated classes of compounds like flavonoids, alkaloids, saponins, glycosides, and tannins from *H. madagascariensis* constituents were reported by Moulari *et al*. (2006a, b). Kouam *et al.* (2006a,b, 2007) reported three prenylated anthranoids: harunmadagascarins C and D and kenganthranol D, with three prenylated 1, 4-anthraquinone: harunmadagascarins A and B, harunganol B and harungin anthrone. Ndjakou *et al*. (2007) reported a new anthrone derivative, Bazouanthrone, together with other known compounds, feruginin A, harunganin, harunganol A, harunganol B, friedelan-

3-one and betulinic acid from it root bark. Some of these compounds have been reported to exhibit antihypoglycemic (Gunatilaka *et al*., 1984) antioxidant (Minami *et al*., 1995), cytotoxic and platelet aggregation inhibitory (Lin *et al*., 1993) activity as cited by Oboh *et al*., 2010.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Plant Collection, Identification and Authentification

Barks of *Harungana madagascariensis* Lam. Ex Poir and *Enantia chlorantha* Oliv. and leaves of *Senna alata* Linn., *Gossypium hirsutum* Linn. and *Alstonia boonei* De Wild were collected from a farmland in Idi-Ayunre, Ibadan, Oyo State in March, 2010. The plants were selected based on literature review and their traditional uses in the treatment of infectious diseases. They were identified at the Herbarium of the Department of Botany, University of Ibadan, Ibadan, Nigeria, and authenticated at Forest Research Institute of Nigeria (FRIN) with specimen vouchers issued. Identification numbers of FRI-109553, FRI-109554, FRI-109555, FRI-109556 and FRI-109557, were assigned to *Enantia chlorantha*, *Harungana madagascariensis*, *Alstonia boonei*, *Gossypium hirsutum* and *Senna alata* respectively.

3.1.1. Plant Processing

Freshly collected plant materials were air dried under the shade to avoid heat and moisture by spreading and constantly re-spreading to allow air circulation. This process was continued until the plant materials were sufficiently dried. The resulting materials changed colour from green to brown. These were ground into powder using an electric blender (Miller III, Model MS-223, Taiwan, China) and later stored in air-tight plastic container until required.

3.1.2. Extraction of Plant Materials

Cold extraction method was carried out with ethanol. The pulverized plant materials (5.0 kg) poured into big conical flask (5 L) and were submerged with distilled 5 L ethanol for the period of 72 hours. These were filtered with Watmann No. 1 Filter paper and were further soaked repeatedly until it turned colourless. The filtrate was evaporated under

reduced pressure at 25^{o}C. The resulting 500 g crude extracts for each sample were collected and later stored in a refrigerator.

3.2. Phytochemical Investigation of plant extracts

Phytochemical screening of the five medicinal plant extracts obtained was carried out to determine the presence of different classes of secondary metabolites according to standards methods of Odebiyi and Sofowora, (1978); Trease and Evans, (1989) and Banso and Ngbede, (2006) as described below:

3.2.1 Test for Carbohydrates (Reducing Sugar)

Two drops of Molish reagent (a solution of α- naphtol in 95% ethanol) was added to 2 mL of each plant extract in a test tube. A 1 mL volume of concentrated H_2SO_4 was allowed to flow down the side of test tube so that the acid forms a layer beneath the aqueous solution without mixing with it. A reddish brown solution indicates the presence of carbohydrate which serves as the positive test.

3.2.2 Test for Alkaloids

About 0.2 g of plant extract was acidified with 1% hydrochloric acid (HCl) for 2 minutes and was then treated with few drops of Dragendorff's reagent in a test tube. The formation of white precipitate indicated the presence of alkaloids (Odebiyi and Sofowora, 1978; Banso and Ngbede, 2006).

3.2.3 Test for Glycosides

Five milliliter of 1% H_2SO_4 was added to 0.2 g of plant extract in separate test tubes, the mixture was heated in boiling water for 15 minutes. Two drops of fehling solution was then added and the resulting mixture was heated to boiling. A brick-red precipitate indicated the presence of glycosides.

3.2.4 Test for Saponins

Sterile distilled water was used to dissolve 0.2 g plant extract. Two milliliter of the resultant solutions were taken into different test tubes and shaken vigorously for a few

minutes. Frothing which persisted on warming was taken as an evidence of the presence of saponins (Odebiyi and Sofowora, 1978).

3.2.5 Test for Steroid (Salkowski Test for steroidal ring)

About 0.5 g of plant extract was dissolved in 2 mL of chloroform. A 0.2 mL of concentrated H_2SO_4 was carefully added to form a lower layer. A reddish-brown colour ring at the interface between the layers indicated the deoxy-sugar characteristic of cadenolides which indicated the presence of steroids.

3.2.6 Test for Flavonoids (Shinoda's Test)

Plant extracts were dissolved separately in 2 mL dilute NaOH. A yellow solution that turned faint or colourless on addition of few drops of hydrochloric acid and a change in colour while standing indicated the presence of flavonoids.

3.2.7 Test for Resins

About 0.2 g of plant extract was mixed with 5 mL sterile distilled water and filtered. A 1 mL of copper acetate solution was added to 1 mL of the filterate. The resultant solution was shaken vigorously and allowed to separate. A green-colour solution showed the presence of resins.

3.2.8 Test for Anthraquinones (Born-Trager's Test)

Born-Trager's test was used for the detection of anthraquinones. A 0.2 g of plant extract was mixed and shaken with 4 mL of benzene. The resulting mixture was filtered and 2 mL of 10% ammonia solution was added to the filtrate. The mixture was shaken and the presence of red colour in the ammonical solution (lower phase) indicated the presence of free Anthraquinones hydroxyl - anthraquinones.

3.2.9 Test for Cardiac Glycosides (Liberman's Test)

Liberman's test was used to determine the presence of cardiac glycosides. A 0.5 g of plant extract was dissolved in 2 mL of acetic anhydride and cooled with ice. Concentrated H_2SO_4 was then carefully added. A colour change from violet to blue and then to green

indicated the presence of a steroidal nucleus (i.e. a glycone portion of the cardiac glycoside) (Sofowora, 1993).

3.3 Toxicity of the Extracts

Biological Assays were carried out on the plant extracts to determine the toxicity of biologically active substance in their secondary metabolites. The biological assays carried out included: phytotoxicity test, insecticidal activity test and brine shrimp lethality test.

3.3.1 Phytotoxicity Assay

Phytotoxicity assay was carried out according to the method described by Atta-ur-Rahman, (1991). *Lemna minor* was collected from H.E.J. Research Institute, International Centre for Chemical and Biological Sciences, Karachi, Pakistan. *Lemina minor* was washed in sterile distilled water and transferred on to nutrient solution (E- medium) (appendix). Hundred milliliter of E-medium stock solution was added to 900mL of distilled water. The pH of the E-medium stock solution was adjusted to 6.0 by dissolving KOH pellets in 1000 mL sterile distilled water. Thirty miligrame of plant extracts were dissolved in 1.5 mL of ethanol solvent. Three different volumes 10, 100 and 1000 µL of plant extract stock solution were pipetted in to three different flasks to make the concentrations of 10, 100 and 1000 µg/mL of plant extracts. Solvent was allowed to evaporate overnight.

A 20 mL volume of working E. medium was added to ten *Lemna minor* plants, each containing a rosette of two fronds (total of 20 fronds) in each flask. Other flasks were supplemented with E-medium and reference standard drug (Etoposide), serving as negative and positive controls, respectively. The flaks containing the *Lemna minor* plants were incubated in the growth cabinet with $56\underline{+}10^{o}$rh (relative humidity), 9000 lux light intensity at 30^{o}C, for seven days with plants examined daily. Number of fronds retaining their chlorophyll per flasks on day 7 was counted and recorded. Results were analysed as plant growth inhibitor in percentage, calculated with reference to the negative control.

The criterium for interpretation of results was as follows; 0-39% inhibition - Low activity, 40-59% inhibition - Moderate activity, 60-69% inhibition - Good activity, Above 70% - significant activity (Source: Atta-ur-Rahman, 1991).

3.3.2. Insecticidal Assay

Insecticidal activity was determined using impregnated filter paper method as described by Tabassum *et al*. (1997). *Tribolium castaneum, Rhyzopertha dominica* and *Callosobruchus analis* were obtained as laboratory grown insects from H.E.J. Research Institute, International Centre for Chemical and Biological Sciences, Karachi, Pakistan.

Forty milligram (40 mg) of each plant extract was added to 2 mL of the methanol volatile solvent). Filter papers were cut according to the size of Petri plate (9cm or 90mm) and inserted into the plate. Different volumes 10, 100 and 1000 µL of plant extracts were transferred onto the filter paper placed in Petri plate using micropipette. The plates were left for 24 hours to allow the methanol to evaporate completely. Ten active and fully grown insects of each species were added to each plate with the aid of a clean long artistic brush. Petri plates were incubated at 27°C for 24 hours at 50% relative humidity. Standard insecticide, permethrin, was used as control. The Petri plates were observed and numbers of survivors for each insect species were counted. Percentage mortality was calculated according to the following formula:

$$\text{Percentage Mortality} = 100 - \left\{ \frac{\text{No. of insects alive in test sample}}{\text{No. of insects alive in control}} \right\} \text{X } 100$$

3.3.3. Brine Shrimp Lethality Assay

Artemia salina larvae were used to examine the toxicity of the plant extracts. Fifty milligram of *A. salina* egg (*Artemia* Incorporated, USA), was transferred unto filtered 250 mL of artificial seawater, prepared by mixing 38g of sea salt to a liter of distilled water with pH of 7.4. These were transferred to hatching tray with perforated partitions. The mixture was incubated at 37ºC and eggs hatched within 48 hours resulting to large number of larvae. The method was carried out as described by Carballo *et al.* (2002).

Twenty milligram of plant extract was dissolved in 2 mL of methanol. From this solution 5, 50 and 500 µL was respectively transferred into vials in triplicates (i.e. 3 vials per

concentration). Nine vials per plant extract were arranged in a vial tray. The resultant concentrations were 10, 100 and 1000 µg/mL respectively. The solvent was allowed to evaporate overnight. After 48 hours of hatching and maturation as *nauplii*, brine shrimp larvae were collected using dropping pipette. These were introduced into each vial with the aid of Pasteur pipette. The mixture was adjusted to make 5 mL volume with seawater and incubated at 27ºC for 24 hours under illumination. Other vials were supplemented with solvent, and reference cytotoxic drug which served as negative and positive controls, respectively. Data were analyzed with Finny Probit Analysis, 1971 to determine LD_{50} values with 95% confidence interval.

3.4. Antioxidant Activities Assay

3.4.1 DPPH Radical-Scavenging Activity Assay

Antioxidant activities of the plant extracts were determined using 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) (Sigma Aldrich), free–radical scavenging activity as described by Farzana *et al*. (2005)**.** Plant extract was allowed to react with stable free radical, DPPH at a concentration of 300 µM for half an hour at 37ºC. Plant extracts were dissolved in dimethyl sulfoxide (DMSO) while the DPPH solution was prepared with ethanol.

Five different concentrations of plant extract were used which was achieved by means of serial dilutions in a 96 well microplate. After incubation, decrease in absorption was measured at 515 nm using multiplate reader (Spectra MAX-340). Percentage radical scavenging activity by plant extract was determined in comparison with a DMSO treated control group. Percentage radical scavenging activity was calculated using the following formula:

$$\% \text{ RSA} = 100 - \{(\text{OD test compound} / \text{OD control}) \times 100\}$$

All tests and analysis were carried in triplicates with average mean calculated for the results obtained. Propyl gallate a known antioxidant was used as the standard positive control.

3.4.2 Superoxide Scavenging Assay

Antioxidant activities of the plant extracts were further evaluated through superoxide scavenging effect according to the method described by Ferda (2003). In an aerobic reaction mixture containing NADH, phenazine methosulphate (PMS) and Nitroblue tetrazolium (NBT), PMS was reduced by NADH and then gave rise to free O_2^-, which in turn, reduced NBT. On the basis of this PMS has frequently been used to mediate free O_2. The reaction mixture comprised of 40µL of 280µM β-nicotinamide adenine dinucleotide, a reduced form of NADH, 40 µL of 80µM NBT, 20 µL of 8µM PMS, 10 µL of 1mM plant extract and 90 µL of 0.1M phosphate buffer (pH 7.4). The reagents were prepared with buffer and plant extract in DMSO.

The reaction was performed in a 96-well microtitre plate at room temperature and absorbance measured at 560 nm. The formation of superoxide was monitored by measuring the formation of water soluble blue formazan dye. A lower absorbance in reaction mixture indicated a higher scavenging activity of plant extract. Percent radical scavenging activity (%RSA) by plant extracts was determined in comparison with standard control. n- Propyl gallate with 90.31%RSA and IC_{50} = 106.23± 1.56 was used as standard control. The analysis was carried out for fractions of the test plant extracts with varying concentrations from 500 µM to 15.625 µM. All tests and analysis were performed in triplicates and the results obtained were averaged.

% RSA= 100 – {(OD test compound / OD control) X 100}

3.5. Antibacterial Activity

The test organisms used which included *Staphylococcus aureus (*ATCC 25923**),** *Escherichia coli (*ATCC 25922), *Pseudomonas aeruginosa* (POA 286), *Salmonella typhi* (ATCC 14028), *Bacillus subtilis* (ATCC 6633) and *Shigella flexneri* (ATCC 12022) were obtained from H.E.J. Research Institute, International Centre for Chemical and Biological Sciences, Karachi, Pakistan.

3.5.1. Standardisation of Inoculum

McFarland standard was prepared for adjusting the turbidity of the inoculum by mixing 0.5 mL of 1.75% barium chloride di-hydrate solution with 95.5 mL of 1% sulfuric acid McFarland, (1907). The accurate density of the McFarland standard was measured spectrometrically at 625 nm, with optical density between 0.08 and 0.1. The turbidity adjusted suspension provided an average count of X-colony forming units per mL. Bacterial culture was incubated overnight at 37^{o}C and then compared with the 0.5 McFarland standards**.**

3.5.2. Antibacterial Susceptibility Test

The susceptibility of test organisms to plant extract was examined using the modified agar well diffusion method as described by Alves *et al*. (2000). Twenty eight grams of Nutrient agar (Oxoid) was dissolved in 1L distilled water. Homogenised using microwave and later autoclaved at 121^{o}C for 15min. After cooloing to about 45^{o}C, 50 mL of the medium was poured in sterile 14 cm diameter Petri plate. This was then allowed for proper solidification of the medium and kept at room temperature for 24 hours to check the sterility of the prepared medium. Nutrient Broth and Soft Agar were equally prepared in the same manner with 0.8gm dissolved in 100mL of distilled water with approximately 2 mL and 7 mL respectively was dispense in screw capped test tubes before autoclaved at 121^{o}C for 15min and later kept at room temperature.

Test bacteria were grown on Muller-Hinton II agar (Oxoid) and incubated at 35^{o}C, while slants were prepared and stored at 4^{o}C for further studies. Single colony of the test organism was incubated into nutrient broth for 18 hours and diluted up to 10^5 folds to obtain approximately 10^6 colony forming units (cfu) per mL of bacterial culture. Hundred microlitre of bacterial culture was transferred into sterile soft agar tube and mixed properly. This was aseptically transferred into sterile solidified nutrient agar plate (oxoid) to form lawn and gently swirl to ensure even distribution of the test organism and allowed to properly solidify. Sterile cork borer was used to make wells on solidified medium and was properly labeled. One milligram per one millilitre of test compound was dissolved in DMSO with 100 µL of test compound in respective well plate, according to bacterial

culture in triplicates. Reference antibiotic drug and DMSO were added to separate wells as positive and negative controls. The Petri plates kept for 30min for proper diffusion prior to incubation at 37^{o}C for 24 hours. The zones of inhibition were measured in mm with transparent ruler and the average was calculated as the mean of the triplicate. The criterium for the interpretation result of results is as follows: - = No activity, 9-12 mm = Non significant, 13-15 mm = Low activity, 16-18 mm = Good activity, Above 18 mm = Significant (Source: Alves *et al*., 2000).

3.5.3. Determination of Minimum Inhibitory Concentration (MIC)

Lowest concentration of plant extracts that inhibited the growth of test microorganisms was taken as the minimum inhibitory concentration (MIC). The determination of MIC of the plant extracts at different concentrations in mg/mL was carried out using broth dilution method as described by Clinical Laboratory Standard Institute (2009). Briefly 5, 10, 15and 25 mg of plant extracts was dissolved in 1 mL DMSO to form appropriate stock solutions in mg/mL. From each concentration, 0.1 mL of plant extract stock solutions prepared was introduced into the test tubes containing 9 mL of reference organism (10^{6}cfu/mL) in nutrient broth. The test tubes were incubated at 37^{o}C for 24 hours. Controls were set up with the test organisms in sterile distilled water instead of plant extracts and plant extracts without test organisms. The MIC was taken as the tube with the least concentration of the extracts with no visible growth after incubation for 48 hours.

3.5.4. Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration of the plant extracts on the test organisms was achieved according to the method of National Committee for Clinical Laboratory Standard (1990). From the mixture obtained during determination of MIC, 1mL was pipetted out and was streaked out on nutrient agar plates for 24 hours accordingly. The least concentration of the extract with no visible growth was taken as the minimum bactericidal concentration.

3.6. Antifungal Activity

Agar tube dilution method was determined according to the method described Choudhary *et al*. (1995) for antifungal activity of plant extracts. Test fungal strains used were *Candida albicans, Candida glabarata, Fusarium solani, Microsporum canis and Aspergillus flavus.* All fungal strains were gotten from bioassay laboratory, H.E.J. Research Institute, International Centre for Chemical and Biological Sciences, Karachi, Pakistan. The plant extract (24 mg) was dissolved in 1 mL sterile DMSO which served as stock solution. Sabouraud dextrose agar (SDA) was prepared by mixing 32.5 gm with 500 mL sterile distilled water and then steamed in microwave for proper homogeneity and 4 mL SDA was dispensed into screw caps tubes before autoclave at 121°C for 15 minutes. Tubes were allowed to cool to 45-50°C and non-solidified SDA was loaded with 66.6 µL of plant extract using micropipette from the stock solution. Tubes were then allowed to properly solidify in slanting position at room temperature.

Each tube was inoculated with 4 mm diameter piece of fungus removed from a seven-day-old fungal culture. An agar surface streak was employed for non-mycelial growth, other media supplemented with DMSO and amphotericin B for *Aspergillus flavus* and miconazole for other fungal strains (antifungal drugs) to serve as negative and positive control respectively. The tubes were incubated at 27°C for 3-7 days with cultures examined daily. Growth in the plant extracts adjusted media was determined by measuring linear growth (mm) and growth inhibition was calculated with reference to the negative control.

$$\% \text{ Inhibition} = 100 - \left\{ \frac{\text{linear growth in test sample (mm)}}{\text{linear growth in control (mm)}} \right\} \text{X } 100$$

3.6.1. Determination of Minimum Fungicidal Concentration (MFC)

Agar tube dilution method was carried out according to the method described by Choudhary *et al*. (1995) for antifungal activity of plant extracts. Various concentrations 40, 50, 60, 70, 80, 90 and 100 mg of plant extract was dissolved in 1mL sterile DMSO respectively which served as stock solution. From this stock solution, 0.1 mL was transferred into tube containing 9 mL non-solidified SDA. Tubes were allowed to solidify

properly in slanting position at 25^{o}C. Each tube was inoculated with 4mm diameter piece of fungus removed from a seven-day-old fungal culture. The tubes were incubated at 27°C for 3-7 days with cultures examined daily. Lowest concentration of plant extracts that inhibited test fungal growth was taken as minimum fungicidal concentration (MFC).

3.7. Fractionation and Purification of Secondary Metabolite from Plant Extract

3.7.1. Fractionation of *Harungana madagascariensis* Extract

Previously obtained ethanol extract from bark of *H. madagascariensis*, was further partitioned by solvent - solvent fractionation into different fractions using n-hexane (HEX), dichloromethane (DCM), ethyl acetate (EtOAc) and n-butane (BtOH) successively. The *H. madagascariensis* extract was further fractionated because of it richness in phenol and polyphenol compounds (flavonoids, anthraquinones). Highly oxidized phenol compounds were reported to be inhibitorier (Scalbert, 1991). The fractionation yielded four different fractions which were HEX-fraction, DCM-fraction, EtOAc-fraction and BtOH-fraction.

3.7.2. Purification of Secondary Metabolites

The resulting DCM-fraction was subjected to fractionation using column chromatography (silica gel 200 - 400 µm mesh) and eluted with n-hexane: acetone (95:5) in increasing polarity until absolute acetone polarity was achieved. The fractions were collected in 500 mL volume flasks, concentrated with rotary evaporator, transferred into small vials which resulted into 135 different fractions. These resultant fractions were further purified using column chromatography by eluting with n-hexane:ethyl acetate (97:3) in increasing polarity until absolute ethyl acetate was achieved. Other columns were eluted with n-hexane : dichloromethane : methanol (20:70:10) in increasing polarity. The choice of solvents was based on the observations recorded on Thin Layer Chromatography (TLC) technique experiments previously carried out. Sepadex LH-20 and Reverse Phase Pressure Column Chromatography (RP-18) were used to purify difficult fractions to separate polar fractions. High Pressure Liquid Chromatography (HPLC), Recycling High Performance Liquid Chromatography (RHPLC) and Preparative Plate Thin Layer Chromatography (PPTLC) were also used to further purify the resultant compounds.

3.7.3. Thin Layer Chromatography (TLC) Technique

TLC was performed on pre-coated silica gel plates (DC-Alugram 60 UV254 of E. Merck) and the spots were observed first under UV light (254 nm) and then stained with ceric (iv) sulfate spraying reagent and heated until the appearance of colour. Drangendoff's reagent was also used for the detection of alkaloids. Ceric sulfate used as spraying reagent was prepared by weighing 1 g and dissolved in 5 mL of concentrated hydrogen sulphide in a conical flask. The flask bottom was submerged in ice block for cooling the reaction and continuously shaked for proper homogeneity. Followed by the addition of 95% distilled water and later poured in the spraying flask. This was used for the development of TLC plates /cards after its removal from solvent tank and dried immediately with hot gun for the appearance of colour.

3.8 Structural Elucidation of Isolated Secondary Metabolites Using Spectroscopy

The structures of purified secondary metabolites were elucidated and identified through different spectroscopic techniques. Spectroscopic techniques employed for this purpose were: Ultraviolet, Infra red, Nuclear Magnetic Resonance (^{1}HNMR and ^{13}CNMR), DEPT-90, DEPT-135, APT (HSQC and HMBC) and Mass spectroscopy (EIMS, HREIMS, FABMS-POS, FABMS-NEG, HRFABMS, LCMS and GCMS).

3.9. Bioactivity of Fractions and Isolated Secondary Metabolites

Harungana madagascariensis fractions obtained from partial fractionation of the plant extract were further tested for bioactivity to further confirm if the fractions truly contained active metabolites. The test carried out on plant extract fractions included: antioxidant Radical Scavenging Activity (RSA), *In vitro* alpha-glucoxidase enzyme inhibition activity, and antibacterial activity with multiple antimicrobial resistance activity.

3.9.1. Antioxidant Activity

Antioxidant free radical, 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Scavenging Activity (RSA) was carried out for fraction A, fraction B, fraction C and fraction D obtained from the fractionation of *H. madagascariensis*. The experiment was carried out as described in

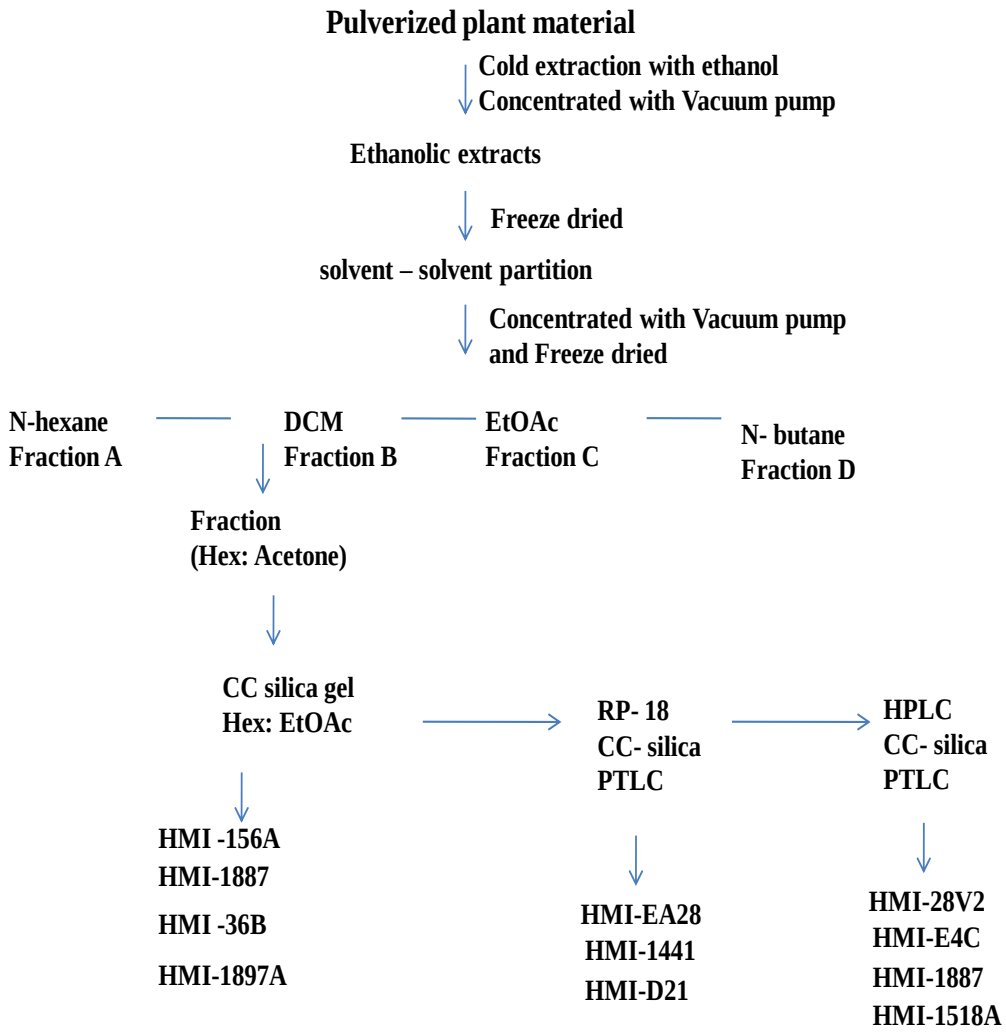

Fig.3.4: Schematic Steps taken to Purify Secondary Metabolites from *H. madagascariensis* Fractions

section 3.4.1. The concentrations were varied from 500 µM to 15.625 µM. Propyl gallate, a known antioxidant was used as the standard positive control.

3.9.2. *In vitro* Assay for Alpha-glucoxidase Enzyme Inhibition

Alpha -glucosidase inhibition assay was performed according to the method of Oki *et al.* (1999). The inhibition was measured spectrophotometrically at pH 6.9 and at 37°C using 0.5 mM *p*-nitrophenyl α-D- glucopyranoside (PNP-G) as a substrate and 250 m units/mL of enzyme, in 50 mM sodium phosphate buffer containing 100 mM NaCl. 1-Deoxynojirimycin (0.425 mM) and acarbose (0.78 mM) were used as positive controls. The increment in absorption at 400 nm, due to the hydrolysis of PNP-G by α-glucosidase, was monitored continuously with a spectrophotometer (Spectra Max, Molecular Devices CA, USA).

3.9.3. Antibacterial Activity of Purified Secondary Metabolites

Fractions obtained from the fractionation of *H. madagascariensis* were examined for antibacterial activity using agar well diffusion method as described in section 3.5.2 above. Varying concentrations of the fractions tested included 30 µg/mL and 100 µg/mL while the concentration of standard antibiotic used (Imipenem) was 10 µg concentration (CT0455B, Oxoid).

3.9.4. Antibacterial activity of selected metabolites and standard antibiotic against MDR *Staphlococcus aureus*

Multiple drug antibacterial activity for the purified secondary metabolites and standard antibiotics was carried out using multi well Micro-plate Alamar Blue Assay against MDR clinical isolate *Staphylococcus aureus*. Alamar blue assay is a simple one step procedure quite amenable to high throughput where metabolic activity resulted in the chemical reduction of alamar blue. One milligram (1 mg) of purified secondary metabolites was dissolved in 1 mL DMSO. One hundred microlitre of nutrient broth was incorporated with alamar blue dye in the micro-plate wells using micro-pipette. Various concentrations of metabolites 25, 50 and 100 µg/mL were introduced into separate wells in triplicates. These

were properly mixed together with the use of micro-pipette. Wells were then inoculated with 0.1 µL of the test bacteria. Micro-plates were incubated at 37°C for 24 hours.

Microtitre plates were observed for colour changed from blue to pink or purple. Alamar blue fluoresces and change colour in response to chemical reduction, and the extent of the conversion is a reflection of cell viability. The concentration at which no visible colour change was observed was taken as the minimum inhibitory concentration. Standard antibiotic used against MDR *S. aureus* included, oxacillin, penicillin, gentamycin, amikacin, chloramphenicol, bacitracin, erythromycin, clindamycin, sulfamethoxazole, ciprofloxacin and trimethoprim. The same method as described above was employed.

3.9.5. Statistical analysis:

Data are mean of three replicates ± SEM and were subjected to Duncan's Multiple Range test using Statistical Package for the Social Sciences, (SPSS 15.0).

CHAPTER FOUR

RESULTS

The five medicinal plants employed for this study were *Harungana madagascariensis* (HMB), *Enantia chlorantha* (ECL), *Senna alata* (SAL), *Gossypium hirsutum* (GHR) and *Alstonia boonei* (ABN). Figure 4.1 below shows the appearance of the plant materials after processing. Crude ethanol extracts obtained were concentrated to form paste. These were examined phytochemically to ascertain the presence of secondary metabolites that could possibly be effective against selected pathogenic microorganisms.

4.1. Phytochemical Investigation

The results of the phytochemical screening (Table 4.1) indicated the presence and absence of alkaloids and reducing sugar respectively in all the extracts. Alkaloid was abundant in *E. chlorantha*, and moderately present in *S. alata*, and *G. hirsutum*, while it was slightly detected in both *H. madagascariensis*, and *A. boonei*. Glycosides were moderately present in *H. madagascariensis*, slightly present in *E. chlorantha* and *S. alata*. Saponins were abundantly present in *H. madagascariensis* but absent in *E. chlorantha* and *S. alata*. Flavonoids were detected abundantly in *H. madagascariensis*, moderately present in *E. chlorantha*, *A. boonei* and *G. hirsutum*, while it was not detected only in *S. alata*. Resins were moderately present in *S. alata*, slightly present in *H. madagascariensis*, *E. chlorantha* and *A. boonei*, while it was not detected in *G. hirsutum* only. Antraquinones were present abundantly in *H. madagascariensis* and was not detected in *E. chlorantha*, *S. alata*, *G. hirsutum* and *A. bonnie*. Cardiac glycosides were moderately present in *E. chlorantha* and *G. hirsutum*, slightly detected in *S. alata* and *A. boonei*, while it was not detected in *H. madagascariensis*. All the plant extracts contained alkaloids while steroids were absent in them and only *G. hirsutum* was positive for Salkowski test. *H. madagascariensis* showed highest abundance in composition of metabolites with

antraquinones, flavonoids, saponins and glycosides while alkaloids were abundant in *E. chlorantha.*

4.2. Toxicity of Selected Plant Extracts

4.2.1. *In vitro* Phytotoxicity Assay

Lemna minor (*Lemnaceae*) was used to determine whether the selected plant extracts has weedicidal or growth stimulating effect. *Lemna minor's* growth was inhibited by *E. chlorantha* at a concentration of 1000 µg/mL 70% (Figure 4.2). *H. madagascariensis, G. hirsutum,* and *A. boonei* exhibited 40%, 25% and 20% *L. minor* growth inhibition at 1000 µg/mL concentration respectively while *S. alata* showed least inhibition (10%) at same concentration. Among the extracts of selected plants tested, only that of *E. chlorantha* exhibited significant growth inhibition. It showed 20% *L. minor* growth inhibition at a lowest concentration of 10 µg/mL tested while *H. madagascariensis* showed moderate inhibitory activity at 1000 µg/mL concentration and *S. alata* had least inhibitory activity at same concentration.

4.2.2. *In vitro* Insecticidal Activity

Mortality rate of plant extracts was examined on *Callosobruchus analis, Tribolium castaneum* and *Rhyzopertha dominica,* insect pest. The percentage growth survival of *C. analis, T. castaneum* and *R. dominica* after treatment with plant extracts is presented in Figure 4.3. Extracts from *H. madagascariensis* and *E. chlorantha* showed 2.0% mortality rate on *C. analis* and *R. dominica* respectively. The *S. alata, A. boonei,* and *G. hirsutum* exhibited no mortality against the selected test insects.

Fig. 4.1: Appearance of Plant Materials after Processing

Table 4.1: Phytochemical Investigation of Selected Plant Extracts

Constituents	HMB	ECL	SAL	ABN	GHR
Reducing sugar	-	-	-	-	-
Alkaloids	+	+++	++	+	++
Glycosides	++	+	+	**N**	**N**
Saponins	+++	-	-	**N**	**N**
Steroids	-	-	-	-	-
Flavonoids	+++	++	-	++	++
Resins	+	+	++	+	-
Anthraquinones	+++	-	-	-	-
Cardiac glycosides	-	++	+	+	++

Keys: +++ = Abundant, ++ = Moderately present , + = Slightly present, - = Absent, N= Not determined, HMB- *Harungana madagascariensis*, ECL- *Enantia chlorantha*, SAL- *Senna alata*, GHR- *Gossypium hirsutum*, ABN- *Alstonia boonei.*

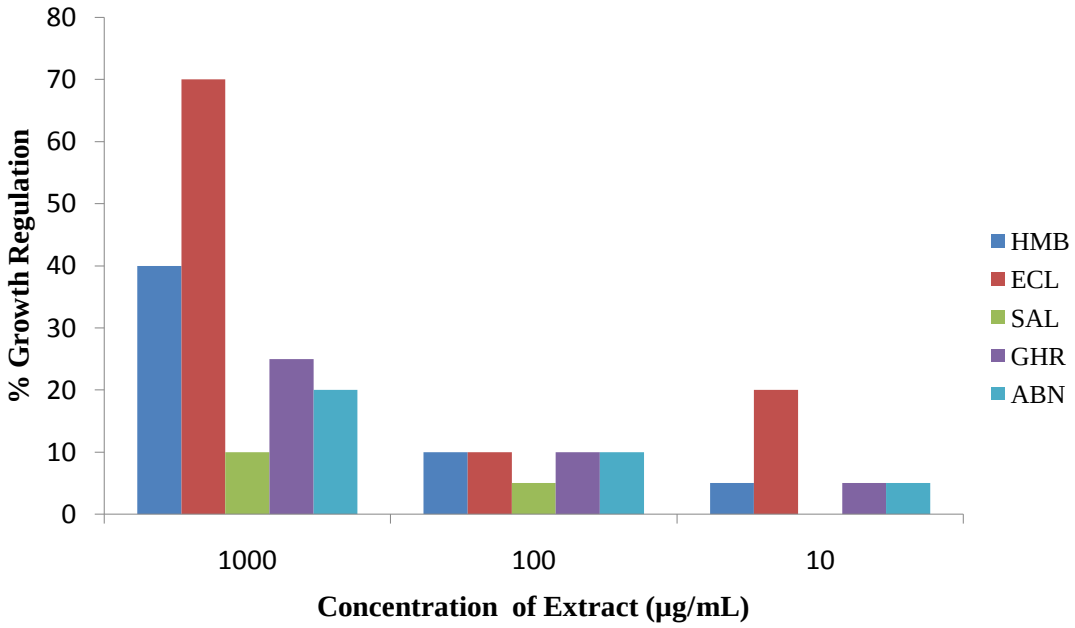

Fig 4.2: *In vitro* Phytotoxicity of selected Plant Extracts

Keys:
HMB- *Harungana madagascariensis*, ECL- *Enantia chlorantha*, SAL- *Senna alata*, GHR- *Gossypium hirsutum*, ABN- *Alstonia boonei*

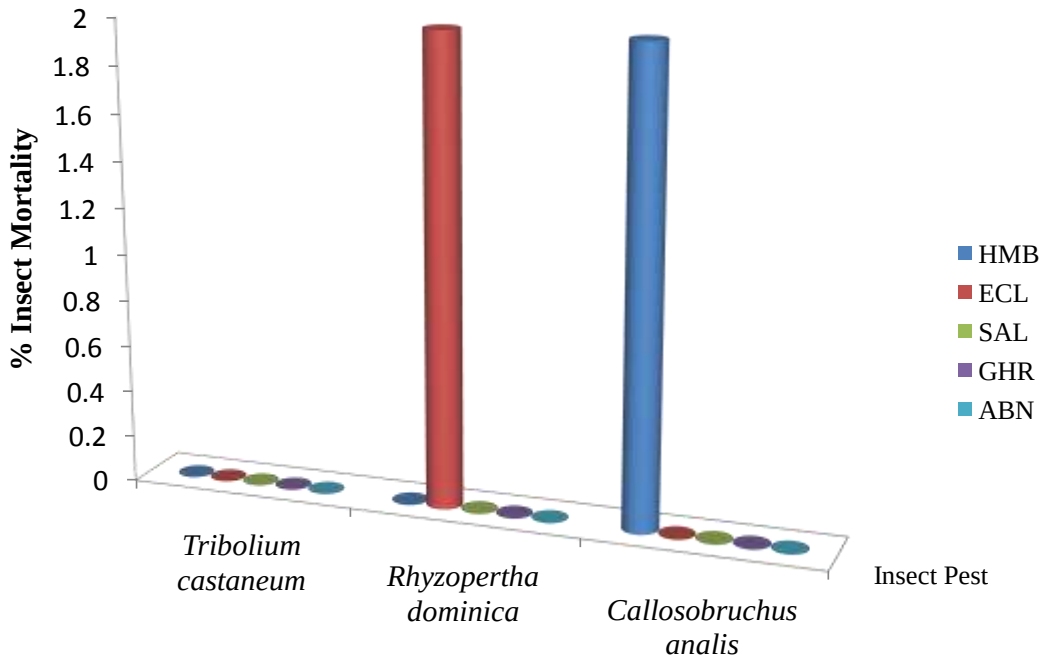

Fig 4.3: *In vitro* Insecticidal Activity of selected Plant Extracts

Keys:
HMB- *Harungana madagascariensis*, ECL- *Enantia chlorantha*, SAL- *Senna alata*, GHR- *Gossypium hirsutum*, ABN- *Alstonia boonei*

4.2.3. Brine Shrimp (*Artemia salina*) Lethality Bioassay

A lethality dosage of 76.747 mg/mL was obtained with *H. madagascariensis* extract while *E. chlorantha* extract lethality dosage (1.9159 mg/mL) was the lowest. This implies that *E. chlorantha* extract was the most toxic to *A. salina* larvae among the selected plant extracts tested while *H. madagascariensis* was the least toxic. None of the extracts of the five selected medicinal plant tested showed significant lethal effect against *A. salina* at 28^{o}C ± 1^{o}C high dosage at 13.785 mg/mL when compared to Etoposide, the standard drug which showed lethal effect at 0.007461 mg/mL concentration as shown in Tables 4.2.

4.3 Antioxidant Assay

4.3.1: DPPH Antioxidant Activities Assay of Selected Plant Extracts

Gossypium hirsutum and *A. boonei* showed lowest scavenging activity against stable free radical, 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH), while *S. alata* and *E. chlorantha* exhibited moderate level of activity as shown in Table 4.3. The *G. hirsutum* and *A. boonei* exhibited 44 %RSA and 26 %RSA (percent radical scavenging activity) respectively at the concentration of 0.5 µg/mL against DPPH. Moderate scavenging activity of 75 % RSA and 61 %RSA was exhibited by *S. alata* and *E. chlorantha* at the concentration of 0.5 µg/mL with IC_{50} 217.26 and 401.29 respectively against DPPH. Significant level of scavenging activity against free radicals was exhibited by *H. madagascariensis* (92%) RSA, IC_{50} 33.31 with SEM ±1.83 (Standard Error of Mean) at 0.5 µgm/L while the standard used at concentration of 7.13 µg/mL showed only 96% RSA.

Table 4.2 Lethality Bioassay of Selected Plant Extracts on *Artemia salina* Showing Number of Survival

Extracts (µg/mL)	*H. madagascariensis*	*E. chlorantha*	*S. alata*	*G. hirsutum*	*A. boonei*
1000	23	16	23	22	24
100	26	26	25	23	27
10	28	27	29	26	29
Upper limit	0	137.589	0.00763	0.00000015	0
Lower limit	0.237	0.535	1.748	1.857	2.597
LD50(mg/mL)	76.747	1.9159	21.778	28.710	51.704

Keys: Standard Drug used (Etoposide) LD_{50} = 0.007461 mg/mL
Incubation Condition = 28^{o}C ± 1^{o}C
No. of Replicates = 3

Table 4.3: DPPH Antioxidant Activities Assay of Selected Plant Extracts

Sample code	Conc. (µg/mL)	IC_{50} ± SEM (µM)	% RSA
HMB	0.5	33.31±1.83	92
SAL	0.5	217.26±0.28	75
ECL	0.5	401.29±5.9	61
ABN	0.5	-	26
GHR	0.5	-	44
Ascorbic acid	1.0	7.13	96

Keys:

RSA - Radical Scavenging Activity, SEM – Standard Error of Mean, Each value ≥ mean of triplicate, HMB- *Harungana madagascariensis*, ECL- *Enantia chlorantha*, SAL- *Senna alata*, GHR- *Gossypium hirsutum*, ABN- *Alstonia boonei*, BHA - 3-t-butyl-4-hydroxyanisole.

4.4: Superoxide Antioxidant Scavenging Assay of Selected Plant Extracts

Antioxidant activity of selected plant extracts were further examined through superoxide scavenging activity. Extracts of *H. madagascariensis* and *S. alata* showed significant superoxide scavenging effect of 81 %RSA and 70 %RSA respectively. *Enantia chlorantha* and *G. hirsutum* exhibited moderate activity of 42 %RSA and 63 %RSA respectively against free radicals at concentration of 0.5 µg/mL. *Alstonia boonei* exhibited lowest superoxide scavenging activity of 27 %RSA. *H. madagascariensis* extract scavenging activity was significant (81 %RSA) at concentration of 0.5 µg/mL when compared to ascorbic acid (standard) with 96 %RSA at concentration 1.0 µg/mL.

Table 4.4: Superoxide Scavenging Activity

Sample code	Conc. (µg/mL)	%RSA
HMB	0.5	81
SAL	0.5	70
ECL	0.5	42
ABN	0.5	27
GHR	0.5	63
Ascorbic acid	1.0	96
BHA	1.0	95

Keys:

RSA - Radical Scavenging Activity, Each value ≥ mean of triplicate, HMB- *Harungana madagascariensis*, ECL- *Enantia chlorantha*, SAL- *Senna alata*, GHR- *Gossypium hirsutum*, ABN- *Alstonia boonei*, BHA - 3-t-butyl-4-hydroxyanisole.

Data are mean of three replicates ± SEM and were subjected to Duncan's Multiple Range test using Statistical Package for the Social Sciences, (SPSS 15.0).

4.5. *In vitro* Antibacterial Assay

Table 4.5 shows the diameter of zones of inhibition of bacterial growth at varying concentrations of *H. madagascariensis, E. chlorantha, G. hirsutum, A. boonei* and *S. alata* extracts measured in millimeter (mm). For *S. aureus* (ATCC 25923), zones of inhibition increased significantly ($p < 0.05$) from 20.00 ±0.57 to 26.66 ±0.33 and from 14.00 ±0.33 to 19.66 ±0.33 with increasing concentrations from 5 mg/mL to 25 mg/mL of *S. alata* and *G. hirsutum* extracts respectively. Zones of inhibition appeared constant with increasing concentrations of *H. madagascariensis* and *E. chlorantha* extracts from 5 mg/mL to 25 mg/mL. The extracts of *H. madagascariensis, E. chlorantha* and *S. alata* were effective against *S. aureus* ATCC 25923 at lower concentration of 5 mg/mL with zones of inhibition 21.33 ±0.57, 21.33 ±0.57 and 20.33 ±0.57 respectively.

Senna alata, A. boonei and *E. chlorantha* were most effective at 5 mg/mL against *E. coli* ATCC 25922, *P. aeruginosa* POA 286 and *S. flexineri* ATCC12022 with zones of inhibition 20.33 ±0.57, 25.66 ±0.33 and 20.33 ±0.33 respectively. The zone of inhibition significantly increased from 20.33 ±0.33 to 29.00 ±0.57 as the concentration of *E. chlorantha* extracts increased from 5 mg/mL to 25 mg/mL against *S. flexineri* ATCC12022. The *E. chlorantha* extracts exhibited significant zone of inhibition, 20.33 ±0.57 against *S. typhi* ATCC 14028 at concentration of 15 mg/mL. The zones of inhibition 12.33 ±0.33, 16.33 ±0.57 and 20.33 ±0.57 were significant against *S. typhi* ATCC 14028 for *S. alata, H. madagascariensis* and *E. chlorantha* extracts at 5, 10 and 15 mg/mL respectively. The zone of inhibition 29.00 ±1.00 exhibited by *A. boonei* extracts against *P. aeruginosa* POA 286 was greater than 23.00 mm zone of inhibition exhibited by imipinem (standard drug).

Table 4.5: Zones of Bacterial Growth Inhibition at Different Concentrations of Selected Plant Extracts

Conc. (mg/mL)	Extracts	*S. aureus*	*E. coli*	*P. aeruginosa*	*Sh. flexineri*	*Sal. typhi*
5	HMB	21.00 ±0.57a	8.00 ±0.00c	18.33 ±0.33c	7.66 ±0.33c	7.66 ±0.33b
	ECL	21.00 ±0.57a	10.33 ±0.33b	24.33 ±0.33b	20.33 ±0.33a	8.66 ±0.33b
	SAL	20.33 ±0.57a	20.33 ±0.57a	10.33 ±0.33e	13.66 ±0.33b	12.33 ±0.33a
	GHR	14.33 ±0.33b	7.33 ±0.33cd	15.00 ±0.00d	13.66 ±0.33b	5.66 ±0.33c
	ABN	8.33 ±0.33c	6.66 ±0.33d	25.66 ±0.33a	8.66 ±0.33c	5.00 ±0.57c
10	HMB	21.00 ±0.57a	10.33 ±0.00c	20.33 ±0.57b	13.66 ±0.33c	16.33 ±0.57a
	ECL	23.66 ±0.57a	13.66 ±0.33b	20.66 ±0.57b	23.66 ±0.33a	13.00 ±0.33b
	SAL	23.66 ±0.33a	15.33 ±0.33a	13.66 ±0.57d	15.66 ±0.33b	13.66 ±0.33b
	GHR	15.33 ±0.33b	10.66 ±0.33c	15.66 ±0.57c	15.66 ±0.33b	0.00 ±0.33c
	ABN	6.33 ±0.33c	7.66 ±0.00d	29.00 ±1.00a	0.00 ±0.00d	0.00 ±0.33c
15	HMB	19.66 ±0.33c	10.33 ±0.33d	21.00 ±0.00b	14.33 ±0.33d	17.00 ±0.33b
	ECL	22.00 ±0.57a	14.33 ±0.33b	21.33 ±0.33b	25.66 ±0.33a	20.33 ±0.57a
	SAL	25.66 ±0.33a	21.66 ±0.33a	15.33 ±0.33d	15.66 ±0.33bc	15.66 ±0.33c
	GHR	15.66 ±0.33d	12.66 ±0.33c	17.66 ±0.33c	16.66 ±0.33b	0.00 ±0.33e
	ABN	7.33 ±0.33e	7.33 ±0.33e	27.66 ±0.33a	14.66 ±0.33cd	13.66 ±0.33d
25	HMB	20.33 ±0.33b	11.66 ±0.33d	21.00 ±0.33c	15.66 ±0.33c	-
	ECL	20.33 ±0.33b	15.66 ±0.33c	27.66 ± 0.33a	29.00 ±0.57a	-
	SAL	26.66 ±0.33a	23.66 ±0.33a	16.00 ±0.00e	17.00 ±0.00b	-
	GHR	19.66 ±0.33b	15.66 ±0.33c	20.33 ±0.22c	16.66 ±0.33cd	-
	ABN	9.66 ±0.33c	21.33±0.33b	24.66 ±0.33b	13.66 ±0.33d	-
Imipenem	0.010	38.00	25.00	23.00	28.00	28.00

Key: HMB- *Harungana madagascariensis*, ECL- *Enantia chlorantha*, SAL- *Senna alata*, GHR- *Gossypium hirsutum*, ABN- *Alstonia boonei*, - = not determined, Values were mean of three determinations ± S.E.M. Values in each vertical column carrying different letters are significantly different from one another ($p < 0.05$) for each concentration.

4.6. Minimum Inhibitory Concentration (MIC) and Minimum Bacteriocidal Concentrations (MBC) of selected Plant Extracts

In table 4.6, *E. chlorantha, H. madagascariensis* and *Senna alata* exhibited minimal bacteristatic concentrations at 5 mg/mL, 6 mg/mL and 6 mg/mL and minimal bacteriocidal concentrations at 22 mg/mL, 24 mg/mL and 20 mg/mL against *S. aureus* (ATCC 25923) respectively. The MBC: MIC ratios exhibited by *E. chlorantha, H. madagascariensis* and *Senna alata* were 4.4, 4.0 and 3.3 respectively against *S. aureus* ATCC 25923. For *E. coli* ATCC 25922, the minimal bacteristatic concentrations at 8 mg/mL, 9 mg/mL and 10 mg/mL and minimal bacteriocidal concentrations at 25 mg/mL, 20 mg/mL and 25 mg/mL exhibited by *E. chlorantha, Senna alata* and *H. madagascariensis* respectively, while their MBC/MIC ratios were expressed as 3.1, 2.2 and 2.5 respectively against same *E. coli*. *A. boonei* and *E. chlorantha* extracts expressed lowest bacteristatic and bacteriocidal concentrations at 5 mg/mL and 20 mg/mL respectively with lowest MBC/MIC ratio of 4.0 against *P. aeruginosa* (POA 286).

The extracts of *H. madagascariensis* and *Senna alata* had bacteristatic concentrations at 8 mg/mL and 10 mg/mL and bacteriocidal concentrations at 22 mg/mL and 25 mg/mL respectively, while their MBC/MIC ratios were 2.75 and 2.50 respectively against *P. aeruginosa* (POA 286). The bacteristatic activitiy expressed by *E. chlorantha* was significant against *S. flexineri* ATCC 12022 and *S. typhi* ATCC 14028 at concentration of 10 mg/mL each than other plant extracts. The MBC/MIC ratios exhibited by *E. chlorantha* against *S. flexineri* ATCC 12022 and *S. typhi* ATCC 14028 were 25 mg/mL and 2.50 respectively. *G. hirsutum* and *S. alata* extracts expressed their bacteristatic effect at a concentration of 15 mg/mL each while *H. madagascariensis* and *A. boonei* extracts expressed their bacteristatic effect at 20 mg/mL concentration each against *S. flexineri* ATCC 12022. It was observed that *H. madagascariensis, E. chlorantha* and *S. alata* extracts expressed their bacteristatic effect against *S. typhi* ATCC 14028 at 10, 10 and 15 mg/mL respectively.

Table 4.6: Minimum Inhibitory Concentration (MIC) and Minimum Bacteriocidal Concentration (MBC) of selected Plant Extracts in mg/mL

			In vitro Antibacterial Activity				
			Test Isolates				
Fractions	Conc.		*S. aureus*	*E. coli*	*P. aeruginosa*	*Sh. flexineri*	*Sal. typhi*
HMB	MIC		6.0	10	8.0	20	10
	MBC		24	25	22	30	25
	MBC/ ratio	MIC	4.0	2.5	2.8	1.5	2.5
ECL	MIC		5.0	8.0	5.0	10	10
	MBC		22	25	20	25	25
	MBC/ ratio	MIC	4.4	3.1	4.0	2.5	2.5
SAL	MIC		6.0	9.0	10	15	15
	MBC		20	20	25	20	30
	MBC/ ratio	MIC	3.3	2.2	2.5	1.3	2.0
GHR	MIC		10	20	20	15	N
	MBC		25	30	30	25	N
	MBC/ ratio	MIC	2.5	1.5	1.5	1.6	-
ABN	MIC		15	20	5.0	20	20
	MBC		>30	>30	20	>30	30
	MBC/ ratio	MIC	>2	>1.5	4.0	>1.5	1.5

Key: HMB- *Harungana madagascariensis*, ECL- *Enantia chlorantha*, SAL- *Senna alata*, GHR- *Gossypium hirsutum*, ABN- *Alstonia bonnie* , N- not determined *S. a- Staphylococcus aureus; E. coli- Escherichia coli; P. a- Pseudomonas aeruginosa; Sh. f - Shigella flexineri; Sal t- Salmonella typhi;* - no inhibition

4.7 Antifungal Properties of Selected Plant Extracts

4.7. Percentage fungal linear growth inhibition by selected plant extracts is illustrated on table 4.7. *Harungana madagascariensis, E. chlorantha, S. alata* and *G. hirsutum* extracts exhibited significant percentage growth inhibition of 90%, 80%, 85% and 90% respectively against *Microsporum canis* while *A. boonei* extract showed the lowest percentage growth inhibition of 10% against it. The *S. alata, G. hirsutum* and *H. madagascariensis* extracts exhibited significant percentage growth inhibition of 85%, 85%, and 75% against *Aspergillus flavus* respectively. The *E. chlorantha* extract showed moderate growth inhibition of 50% while *A. boonei* extract was ineffective against *Aspergillus flavus.* Against *Candida albicans, A. boonei* and *G. hirsutum* extracts were ineffective while extracts of *H. madagascariensis* and *E. chlorantha* exhibited significant and moderate percentage activity of 70% and 65% respectively. *Candida albicans* was significantly susceptible to *E. chlorantha* extract by 81% while *C. albicans* was moderately susceptible to *H. madagascariensis* extract by 68%.

Minimum fungicidal concentration (MFC) is illustrated on Table 4.8. *Enantia chlorantha* and *S. alata* extracts exhibited minimum fungicidal concentration at 45 mg/mL each while *H. madagascariensis* and *G. hirsutum* extracts showed minimum fungicidal concentration at 50 mg/mL each against *Microsporum canis.* *E. chlorantha* and *S. alata* extracts exhibited minimum fungicidal concentration at 50 mg mL each while *H. madagascariensis* exhibited minimum fungicidal concentration at 60 mg/mL against *C. albicans. Alstonia boonei* extract was ineffective against *A. flavus, C. glabarata* and *C. albicans* while *G. hirsutum* extract was ineffective against *C. albicans* only. The minimum fungicidal concentrations expressed by *E. chlorantha* extract against *M. canis, A. flavus, C. glabarata* and *C. albicans* were 45, 60, 50 and 50 mg/mL respectively as compared to miconazole (control) with minimum fungicidal concentrations of 70, 75, 75 and 65 mg/mL respectively.

Table 4.7: **Percentage Fungal Growth Inhibition by selected Plant Extracts**

Extracts	Percentage Growth Inhibition (%)			
	C. a	*C. g*	*A. f*	*M. c*
H. madagascariensis	70	68	75	90
E. chlorantha	65	81	50	80
S. alata	55	26	85	85
G. hirsutum	N	38	85	90
A. boonei	N	N	N	10

Keys:

C. a - Candida albicans; C. g - Candida glabarata;

M. c - Microsporum canis; A. f - Aspergillus flavus, N= no inhibition,

*Each value was a mean of triplicate

Table 4.8: Minimum Fungicidal Concentrations (MFC) of Selected Plant Extracts

Extracts	Minimum Inhibitory Concentration (mg/mL)		
	C. albicans	*C. glabarata*	*A. flavus*
H. madagascariensis	60	60	50
E. chlorantha	50	50	60
S. alata	50	70	50
G. hirsutum	N	70	70
A. boonei	N	N	N
Control (Miconazole)	65	75	75

Keys: N- No inhibition, *C.* – *Candida*, *A.*- *Aspergillus*, *M.*- *Microsporum*
H- *Harungana madagascariensis*, E- *Enantia chlorantha*, S- *Senna alata*,
G- *Gossypium hirsutum*, A- *Alstonia boonei*

4.8 Fractionation and Purification of *H. madagascariensis* Extract

Among extracts of the plants screened phytochemically, secondary metabolites were abundantly present by 60% in *H. madagascariensis* than others (Table 4.1). Its extracts exhibited promising antioxidant, antibacterial and antifungal activities among other plant extracts tested. Therefore, it was selected for purification in order to further examine the bioactivities of individual secondary metabolites. Partial purification of *H. madagascariensis* extract yielded four different fractions which were coded as fraction-A, fraction-B, fraction-C and fraction-D (Figure 3.4). Column fractionation of the fraction-B resulted into sixteen (16) purified secondary metabolites listed in table 4.9. The purified secondary metabolites were classified into three (3) different groups of metabolites, namely triterpenes, alkaloids and anthraquinone. Their extablished biological functions are also stated (Table 4.9).

Description of Compound HMI-LS

The EI-MS of compound HMI - LS displayed the molecular ion peak (M^+) at *m/z* 342, while the HREI-MS showed the (M^+) at *m/z* 342.1594, corresponding to molecular formula $C_{19}H_{22}N_2O_4$ (calcd. 342.1580). IR spectrum displayed sharp peaks at 3313 (NH), 1709 (C=O) and 1534 (aromatic) cm^{-1}. The UV spectrum showed maximum absorptions at 248, 243 and 209 nm.

The ^{1}H-NMR spectrum showed two signals of four proton each at aromatic region, of δ 7.08 (4H, d, *J* = 8.5 Hz, H-2, 2, 6, 6) and 7.29 (4 H, d, *J*= 8.5 Hz, H-3, 3, 5, 5). The ^{1}H-NMR also displayed two proton containing signal at δ 3.85 s, attributed to H_2-10 and signal for oxygenated methylene protons at δ 4.16 (2H, q, *J* = 7.0 Hz) and one signal for methyl protons at δ 1.28 (3H, t, *J* = 7.0 Hz)

The broad-band decoupled ^{13}C-NMR spectrum showed a total of 19 carbon signals. The DEPT-90 and 135 spectra distinguish as two methyl, three methylene and eight methine carbons. The remaining were found to be quarternary in nature. The 2D-NMR spectra helped to identify the structure of the compound.

Table 4.9: Isolated Secondary Metabolites

Secondary metabolites	Class of compound	Biological Functions with References
HMI-36B (Fig.4.4) HMI-1887 (Fig.4.5) HMI -1414	Triterpenes	Active against tumor promotion (melanoma specific cytotoxicity) Pisha *et al*., 1995. Weak activity against HIV -1 replication (Kashiwada *et al*., 2004).
HMI-EA28 HMI -156A HMI-28V2 HMI-LS (Fig.4.7) HMI-28V2 HMI-1518A	Alkaloids	Palmatine as anti viral hepatitis B agent and dl-THP as a sedative and antihypertensive drug (Gao *et al*., 2008).
HMI-1441 HMI-879A (Fig.4.8) HMI-8A (Fig.4.9) HMI-17 (Fig.4.10) HMI-D21 (Fig.4.11) HMI-79A (Fig.4.12)	Anthraquinine	Antifungal, antibacterial and antiviral activity (Yasodamma *et al*., 2009). Strong antibacterial potential against gram +ve and gram –ve bacterial strains (Pereira *et al*., 2007).

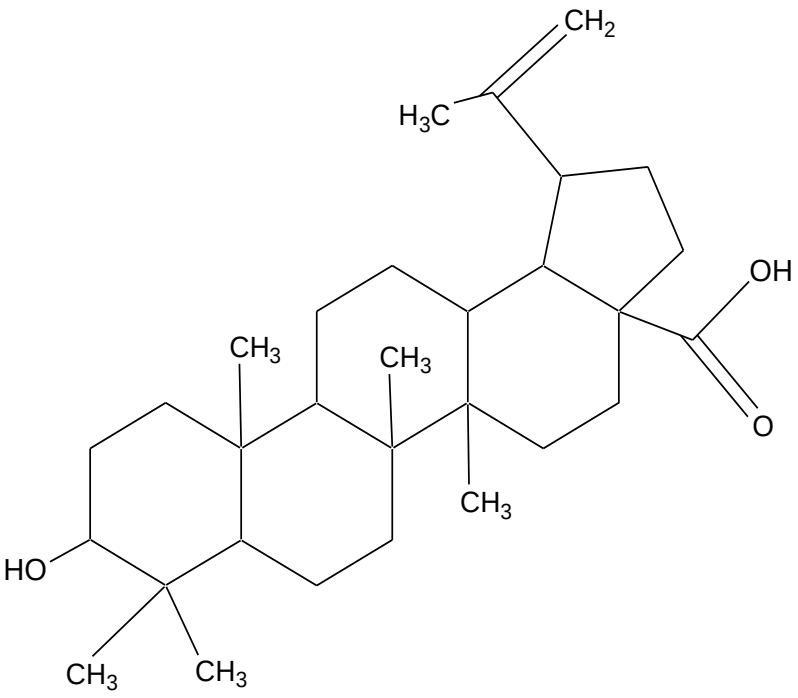

Fig.4.4 : HMI-36B (Triterpene)

IUPAC Name- (1S,5aS,5bR,9S,11aR,11bS)-9-hydroxy-1-isopropenyl-5a,5b,8,8,11a-pentamethylicosahydro-3a*H-cyclopenta[a]chrysene-3a-carboxylic acid*

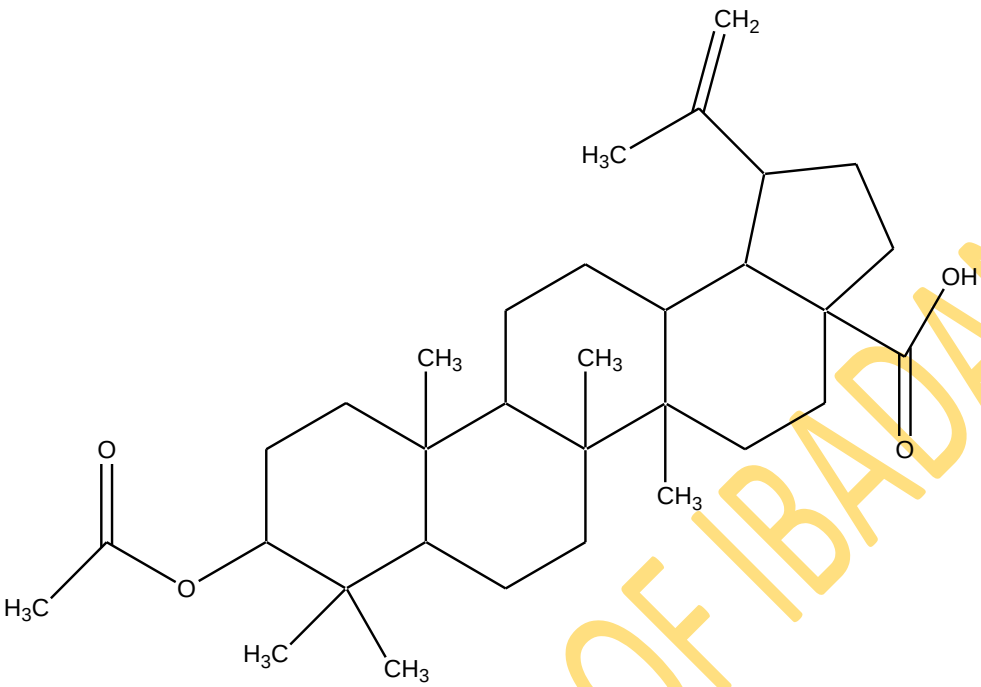

Fig.4.5: HMI- 1887 (Triterpene)

IUPAC Name- (1*S*,5a*S*,5b*R*,9*S*,11a*R*,11b*S*)-9-(formyloxy)-1-isopropenyl-5a,5b,8,8,11a-pentamethylicosahydro-3a*H*-cycopenta(a)chrysene-3a-carboxylic acid

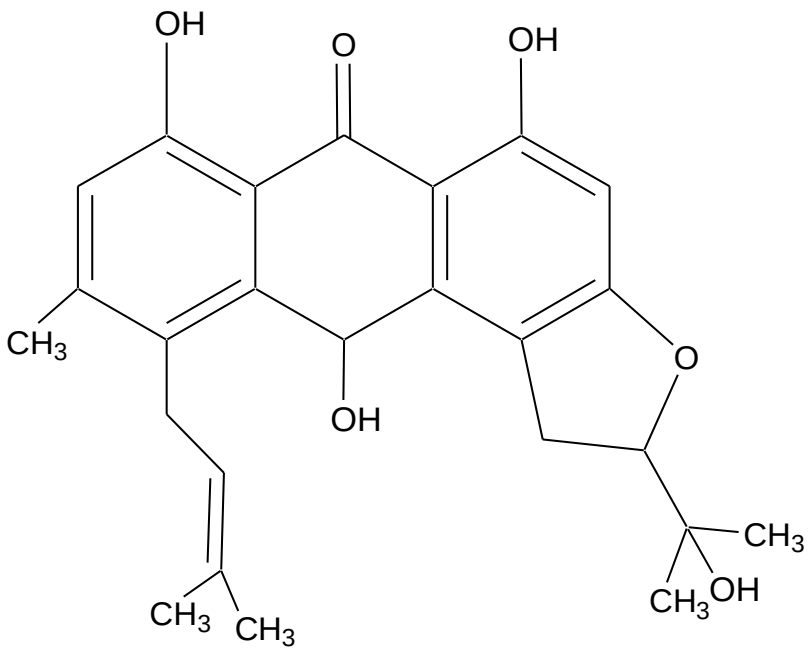

Fig. 4.6: HMI-15H (Anthraquinine)

IUPAC Name- 5,7,11-trihydroxy-2-(1-hydroxy-1-methylethyl)-9-methyl-10-(3-methyl-2-butenyl)-1,11-dihydroanthra[2,1-*b]furan-6(2H)-one*

Fig . 4.7: HMI-LS (Alkaloids)

IUPAC Name - ethyl 4-({[4-(ethoxycarbonyl)anilino]methyl}amino) benzoate

Description of Compound HMI-1879A

Compound HMI-1879A was obtained as brownish powder. The FAB $^{(+)}$ MS showed the *pseudo*-molecular ion peak $(M+H)^+$ at *m/z* 409, whereas FAB $^{(-)}$ MS showed *pseudo*-molecular ion peak $(M-H)^-$ at *m/z* 407. The molecular formula $C_{25}H_{28}O_5$ was deduced from the FAB $^{(+)}$ MS which showed pseudo-molecular ion peak at *m/z* 409.2025 (Calc. for $C_{25}H_{28}O_5$ + H = 409.2015) and ^{13}C-NMR spectra (Broad-band decoupled and DEPT).

The IR absorption spectrum of HMI-1879A showed absorption of intra molecular hydrogen bonded hydroxyl (2930 cm^{-1}), carbonyl (1685 cm^{-1}), aromatic double bonds (1456 cm^{-1}), and olefinic carbon-carbon bonds (1508 cm^{-1}). The UV absorption bands at λ_{max} 241, 279, 306 and 391 nm indicated extended conjugation and an aromatic chromophoric system which supported anthranoid skeleton (Kouam *et al*., 2007).

The ^{1}H- NMR spectrum of HMI-1879A (Table 4.10) showed two signals for chelated (hydrogen bonded) hydroxyl groups at δ 12.39 (s, H-1/OH) and 12.30 (s, H-8/OH), two aromatic proton signals at δ 6.77 (s, H-5) and 6.32 (s, H-2), and a methyl singlet at δ 2.31 (H_3-21). ^{1}H- and ^{13}C-NMR indicated the presence of two 3,3-dimethylallyl groups by showing resonances at δ_H/δ_C [{4.96 (1H, t, $J_{11,12}$ = 6.1 Hz) /124.2, CH-12}; {5.07 (1H, t, $J_{16,17}$ = 6.2 Hz)/124.5, CH-17}; { 3.50 (2H, d, $J_{11,12}$ = 6.1 Hz)/ 24.6, CH_2-11}; 3.75 (2H, d, $J_{16,17}$ = 6.2 Hz)/27.9, CH_2-16}; {1.82 (3H, s)/18.1, CH_3-14}; {1.70 (3H, s)/26.0, CH_3-15}; {1.85 (3H, s)/17.9, CH_3-19}; {1.75 (3H, s)/25.9, CH_3-20} and two quaternary carbons at δ_C 132.3 (C-13) and 133.1 (C-18). The long-range HMBC correlations of the δ 2.31 (CH_3-21) with C-5 (δ 119.8), C-6 (δ 141.2) and C-7 (δ 132.2) indicated the presence of a methyl group at C-7 position of ring A.

Furthermore, HMBC correlations of protons at δ 12.30 (H-8/OH) and 4.96 (H-12) to C-7 (δ 133.1) indicated the substitution of 3,3-dimethylallyl group at C-7. The proton signal at δ 6.32 (H-2) displayed cross-peaks with two oxygenated aromatic carbons at δ 164.7 (C-1) and 161.7 (C-3), indicating that the other 3,3-dimethylallyl group was located at C-4 of ring A, which was further supported by HMBC correlations between δ 5.07 (H-17) and 122.6 (C-4). In the HMBC spectrum, proton signal at δ 5.84 (H-10) displayed correlations

with the carbon at δ 143.7(C-10a), and 148.6 (C-4a), unambiguously supported the position of hydroxyl group at C-10.

Stereochemistry in compound HMI-1879A was determined with the help of Nuclear Overhauser effect spectroscopy (NOESY) correlations. The H-10 showed NOESY correlation with H-17 which is only possible for the *equatorially* oriented H-10. The NOESY correlation between proton of hydroxyl group of C-8 with H-11 further confirmed the substitution of 3,3-dimethylallyl group at C-7 (Fig. 4.9a). The optical rotation of HMI-1879A was $[\alpha]^{25}_{D} = +18.0$. From above spectral data; the structure of compound HMI-1879A was deduced as 1,6,8,10-tetrahydroxy-3-methyl-2,5-*bis*(3-methyl-2-butenyl)-9(10*H)*-anthracenone.

Table 4.10: Nuclear Magnetic Resonance data (ppm) for Compound HMI-1879A

N	BB	C	CH	CH_2	CH_3	HSQC	HMBC
1	193.3	193.3					
2	164.7	164.7					
3	161.7	161.7					
4	148.6	148.6					
5	143.7	143.7					
6	141.2	141.2					
7	133.1	133.1					
8	132.3	132.3					
9	132.2	132.2					
10	124.5		124.5			4.96	148.6
11	124.2		124.2			5.07	
12	122.6	122.6					
13	119.8		119.8			6.77	161.7, 132.2, 141.2
14	103.0		103.0			6.32	164.7, 122.6, 108.7
15	61.7		61.7			5.84	119.8, 141.2, 122.6, 132.2, 121.5, 143.7
16	**27.9**			27.9		3.38, 3.75	147.5, 141.2, 132.2, 121.5, 143.7
17	26.0				26.0	1.70	132.2, 18.1, 122.6
18	25.9				25.9	1.75	133.1, 132.2, 132.3
19	24.6			24.6		3.50, 3.57	143.7, 131.5, 122.6
20	20.9				20.9	2.31	148.6, 141.2, 119.8, 132.2
21	18.1				18.1	1.82	132.3, 124.2
22	17.9				17.9	1.85	
23	113.7	113.7					
24	121.5	121.5					
25	108	108					

Key: BB- Broad bound; C-C bonds; C - Singlet (s); CH - Doublet (d); CH_2 - Triplet (t); CH_3 – Quartet (q); HSQC - Heteronuclear single-quantum correlation spectroscopy;
HMBC - Heteronuclear multiple-bond correlation spectroscopy

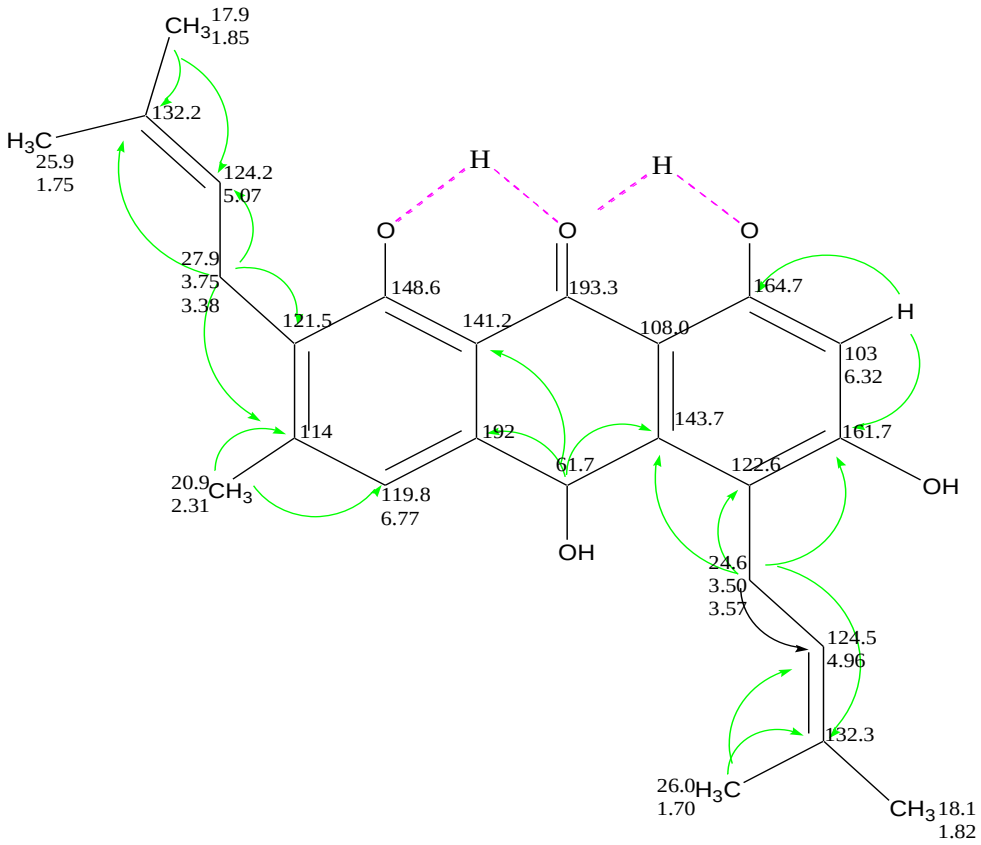

Fig.4.8a: Heteronuclear multiple-bond correlation spectroscopy (HMQC) for compound HMI-1879A

1,6,8,10-tetrahydroxy-3-methyl-2,5-bis(3-methyl-2-butenyl)-9(10*H*)-*anthracenone*

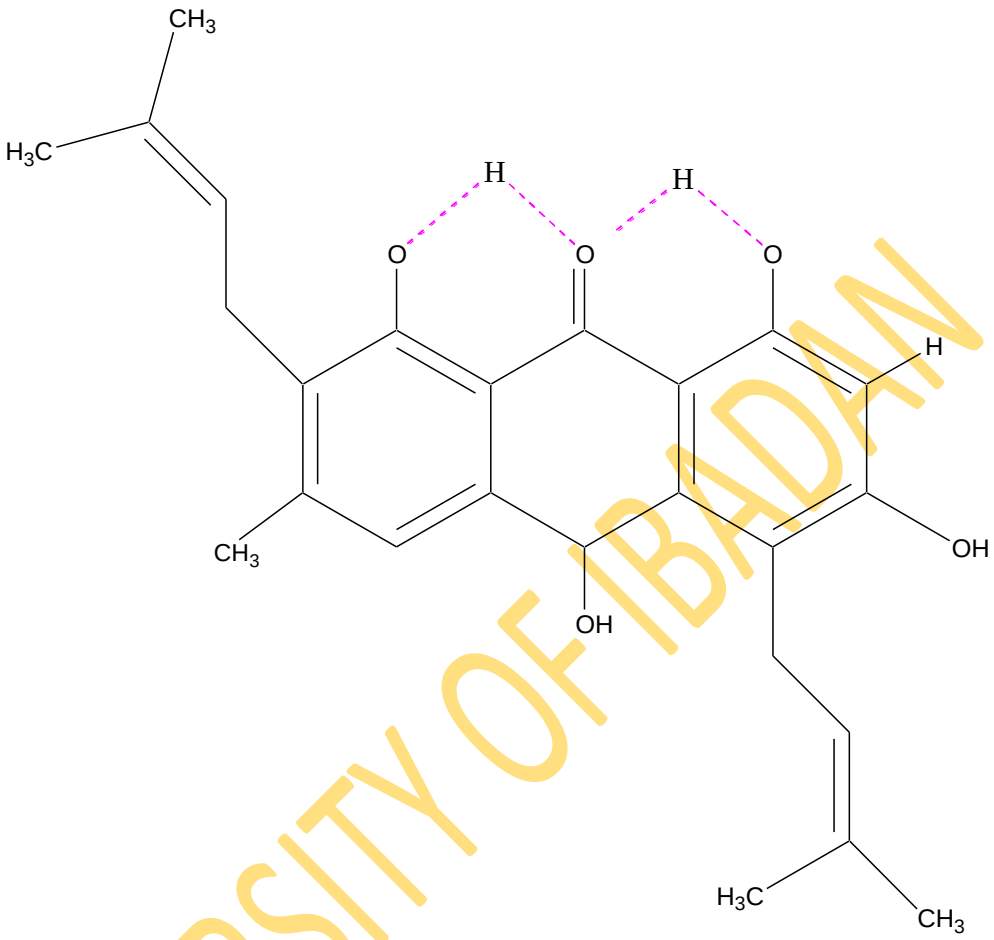

Fig.4.8b: Final Structure for Compound HMI-1879A

IUPAC Name- 1,6,8,10-tetrahydroxy-3-methyl-2,5-bis(3-methyl-2-butenyl)-9(10*H*)-*anthracenone*

Description of Compound HMI-8A

The compound HMI-8A was obtained as yellow crystals, the molecular formular was determined as $C_{30}H_{36}O_6$ by HREIMS $(M)^+$ m/z 492. In conjuction with NMR spectra data, the IR spectrum showed inter and intra- molecularly hydrogen-bonded hydroxyl (2924), a hydrogen bonded carbonyl (1665), olefinic carbons (1598), aromatic double bonds (1440) and carbonyl (1728). The UV bands at *λmax* 242, 285, 307, 395nm indicated an aromatic chromophore system which, in conjuction with the IR spectra data, suggested the anthranoid skeleton (Ritchie and Taylor, 1964a, b).

The ^{1}H NMR spectrum, showed the deshielded hydroxyl group at (δ_H 12.87 and δ_H 12.32). The 1,8,-dihydroxyl anthranoid skeleton was confirmed by the ^{13}C NMR spectrum, it exhibited a resonance of only one carbonyl group at *δc* 191.0, (Delle Monache *et al*., 1980a, b; Kouam, *et al*., 2005 and Kouam et al., 2007). The hydroxyl groups (-OH) were confirmed by resonance at δ_H 12.87 which showed correlations with three carbon *δc* 159.4, 130.1 and 111.5 while the second hydroxyl δ_H 12.32 showed correlations with carbons *δc* 165.6, 107.9 and 98.0 (Table 4.11).

In addition, the ^{1}H NMR spectrum of this compound is similar to that of compound 4 (Kouam, *et al*., 2005) except for the addition of one prynyl group with 1-hydroxyl-1-methyethyldihydrofuran ring in replacement of one prenyl group. The decoupled ^{13}C NMR and DEPT spectra recorded with Cry pro 600Mz, $CDCl_3$ revealed the presence of 30 resonances, including seven methyls, five methines, tree methylenes and fiftheen quaternary carbons.

The long range HMBC correlations of H-2 (δ_H 6.37) were showed with carbonyl carbon (δ_c 191.0) and H-10 (δ_H 5.65) correlate with C-5 (*δc* 130.7). The methylene proton H-16 (δ_H 3.46, 3.68) showed correlation with C-5 (*δc* 130.7), C-6 (*δc* 145.9), C-18 (*δc* 133.0) and H-21 (*δH* 3.44, 1.68) showed correlation with C-7 (*δc* 130.1), C-8 (*δc* 159.4) and C-23 (*δc* 132.3). These suggested the attachement of the prynyl groups at these positions. The methelene proton H-11 (δ_H 1.24, 1.43) showed correlation with methane C-12 (*δc* 91.5), quaternary C-13 (*δc* 71.5) and aromatic carbon C-4 (*δc* 138.2) which suggested the

position of 1-dihyroxyl-1-methylethyl dihydrofuran ring. On the basis of the above spectroscopic data and by comparison with previously reported anthranoids (kouam *et al*., 2005, Kouam *et al*., 2006; Ritchie and Tailor *et al*., 1964a, b and Linuma *et al*., 1995). The structure of this compound (HMI-8A) was fully assigned as 5,7,11-trihydroxy-2-(1-hydroxy-1-methylethyl)-9-methyl-8,10-bis(3-methyl-2-butenyl)-1,11-dihydroanthra [2,1-*b]furan-6(2H)-one*

Table 4.11: Nuclear Magnetic Resonance data (ppm) for Compound HMI-8A

N	BB	C	CH	CH_2	CH_3	HSQC	HMBC
1	191.0	191.0					
2	166.9	166.9					
3	165.6	165.6					
4	159.4	159.4					
5	145.9	145.9					
6	138.2	138.2					
7	135.9	135.9					
8	133.0	133.0					
9	132.3	132.3					
10	130.7	130.7					
11	130.1	130.1					
12	123.3		123.3			5.06	130.1
13	121.5		121.5			5.05	25.7, 18.0
14	119.6	119.6					
15	98.0		98.0			6.37	98.0, 119.6, 165.6
16	91.5		91.5			4.77	71.7
17	71.7	71.7					
18	62.8		62.8			5.65	130.7, 138.2, 135.9, 119.6, 98.0
19	29.6			29.6		1.24, 1.43	91.5, 71.7, 26.2, 138.2
20	27.6			27.6		3.45, 3.68	135.9, 145.9, 123.3, 133, 130.7
21	26.2				26.2	1.37	91.5, 71.7
22	25.7				25.7	1.71	123.3, 18.0
23	25.1			25.1		3.44, 1.68	121.5, 132.3, 159.4, 145.9, 133.0,130.1
24	24.2				24.2	1.47	26.2
25	18.1				18.1	1.85	123.3, 133.0, 26.2
26	18.0				18.0	1.79	123.3, 133.0, 26.2
27	16.5				16.5	2.30	130.7, 145.9, 16.5
28	14.1				14.1		
29	111.5	111.5					
30	107.9	107.9					

Key: BB- Broad; C-C bonds; C - Singlet (s); CH - Doublet (d); CH_2 - Triplet (t); CH_3 - Quartet (q); HSQC - Heteronuclear single-quantum correlation spectroscopy;

HMBC - Heteronuclear multiple-bond correlation spectroscopy

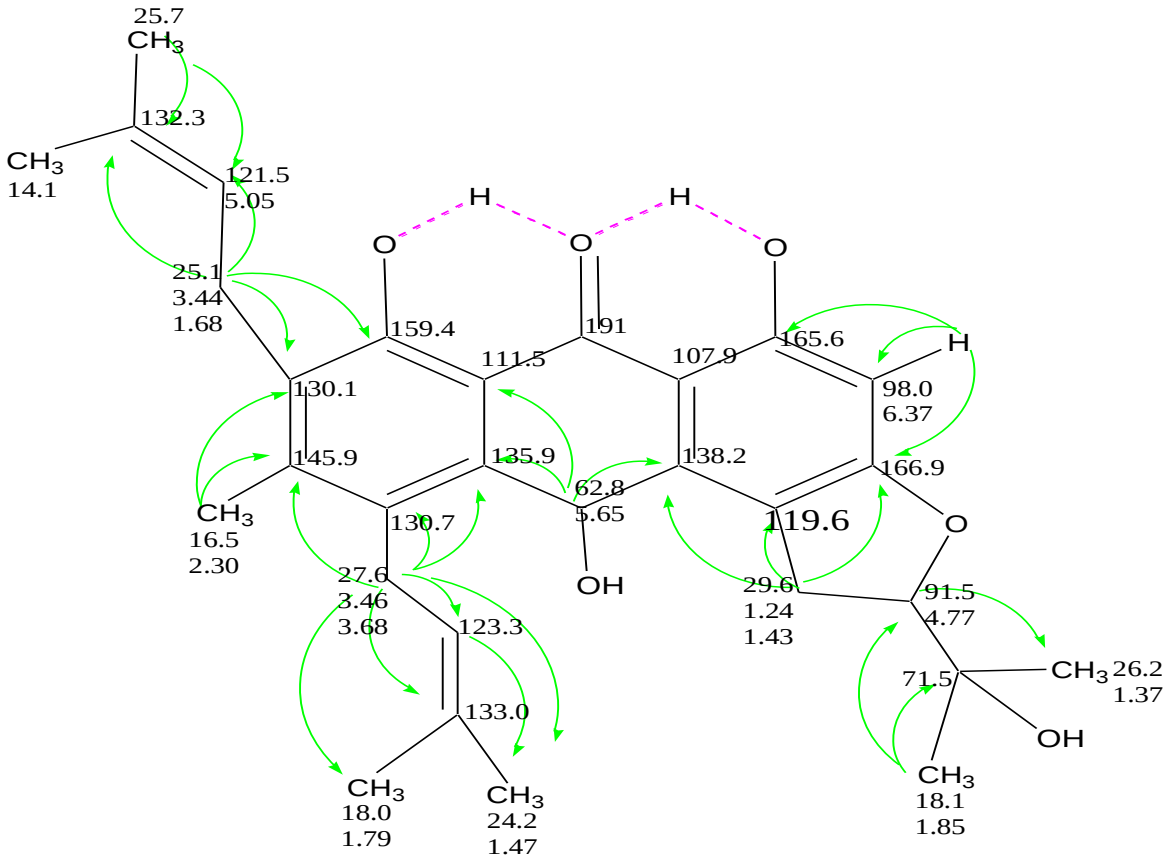

Fig. 4.9a: Heteronuclear multiple-bond correlation spectroscopy (HMQC) for Compound HMI-8A

IUPAC Name- 5,7,11-trihydroxy-2-(1-hydroxy-1-methylethyl)-9-methyl-8,10-bis

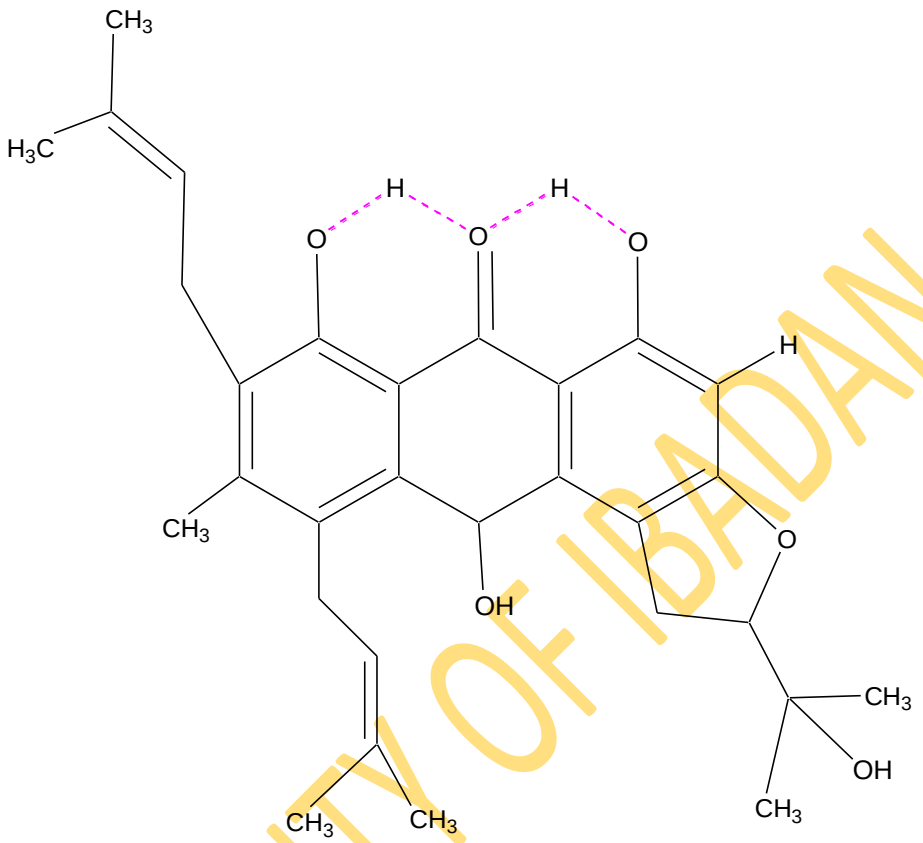

Fig.4.9b: Final Structure of Compound HMI-8A

IUPAC Name- 5,7,11-trihydroxy-2-(1-hydroxy-1-methylethyl)-9-methyl-8,10-bis (3-methyl-2-butenyl)-1,11-dihydroanthra[2,1-*b]furan-6(2H)-one*

Description of Compound HMI-17

The compound HMI-17 obtained as yellow crystals, the molecular formular was determined as $C_{28}H_{34}O_6$ by HREIMS $(M)^+$ m/z 452. In conjuction with NMR spectra data, the IR spectrum showed inter and intra- molecularly hydrogen-bonded hydroxyl (2924), a hydrogen bonded carbonyl (1613), olefinic carbons (1529), aromatic double bonds (1467) and carbonyl (1730). The UV bands at *λmax* 227, 241, 279, 300, 304 and 383nm indicated an aromatic chromophore system which, in conjuction with the IR spectra data, suggested the anthranoid skeleton (Ritchie and Taylor, 1964a, b).

The ^{1}H-NMR and ^{13}C-NMR spectrum are similar to compound HMI-8A above, its deshielded hydroxyl group are at (δ_H 12.90 and δ_H 12.40). The 1,8,-dihydroxyl anthranoid skeleton was confirmed by the ^{13}C NMR spectrum, it exhibited a resonance of only one carbonyl group at *δc* 191.4, (Delle Monache *et al*., 1980a, b; Kouam *et al*., 2005 and Kouam *et al*., 2007). The hydroxyl groups (-OH) were confirmed by resonance at δ_H 12.90 which showed correlations with three carbon *δc* 161.1, 119.9 and 112.7 while the second hydroxyl δ_H 12.40 showed correlations with carbons *δc* 167.3, 108.5 and 98.1 (Table 4.12).

This compound is similar to compound HMI-8A in addition with the ^{1}H NMR spectrum data except for the absence of one prynyl group and addition of a oxygenated methylene C-16 *δc* 64.1 (3.68, 3.79) and a methyl C-17 (*δc* 155.2) to the 1-hydroxyl-1-methyethyldihydrofuran ring in replacement of one prenyl group. The decoupled ^{13}C NMR and DEPT spectra recorded with Cry pro 600Mz, $CDCl_3$ revealed the presence of 28 resonances, including six methyls, five methines, tree methylenes and fourtheen quaternary carbons.

The long range HMBC correlations of H-2 (δ_H 6.42) were showed with carbonyl carbon (δ_c 191.4) and H-10 (δ_H 5.80) correlate with C-5 (*δc* 147.9). The methylene proton H-18 (δ_H 3.35, 3.88) showed correlation with C-5 (*δc* 147.9), C-6 (*δc* 131.6), C-20 (*δc* 129.4). This suggested the attachment of the prynyl group at this position. The methylene proton H-11 (δ_H 1.23, 1.29) showed correlation with methane C-12 (*δc* 94.8), quaternary C-13 (*δc*

71.3) and aromatic carbon C-4 (δc 117.3) which suggested the position of 1-dihyroxyl-1-methylethyl dihydrofuran ring. Furthermore, the hydrogen on the hydroxide group (-OH) of dihydrofuran ring was reduced by addition of methelene proton H-16 (δ_H 3.68, 3.79) showed correlation with methane C-17 (δc 15.2) while the proton H-17 (δ_H 1.34) showed correlation with quaternary C-13 (δc 71.3) and methelene C-16 (δc 64.1).

On the basis of the above spectroscopic data and by comparison with previously reported anthranoids (Kouam *et al*., 2005, Kouam *et al*., 2006; Ritchie *et al*., 1964a, b and Linuma *et al*., 1995). The structure of this compound (HMI-17) was fully assigned as 2-(1-ethoxy-1-methylethyl)-5,7,11-trihydroxy-9-methyl-10-(3-methyl-2-butenyl)-1,11-dihydroanthra[2,1-*b*]furan-6(2*H*)-one (Fig. 4.9a, b).

Table 4.12: Nuclear Magnetic Resonance data (ppm) for Compound HMI-17

N	BB	C	CH	CH_2	CH_3	HSQC	HMBC
1	191.4	191.4					
2	167.3	167.3					
3	167.0	167.0					
4	161.1	161.1					
5	147.9	147.9					
6	143.3	143.3					
7	137.1	137.1					
8	131.6	131.6					
9	131.6	131.6					
10	129.4	129.4					
11	122.9		122.9			5.03	78.2, 25.6, 18.1
12	119.9		119.9			6.83	161.5, 20.9, 131.6, 112.7
13	117.3						
14	112.7						
15	108.5						
16	98.1		98.1			6.42	167.3, 117.3, 108.5
17	94.8		94.8			4.57	78.2, 167.0, 26.8, 24.7
18	78.2		78.2			5.22	94.8, 71.3, 64.1, 117.3, 167.0
19	71.3						
20	64.1			64.1		3.68, 3.79	15.2
21	61.8		61.8			5.80	147.9, 137.1, 131.6, 117.3, 112.7, 108.5
22	29.6			29.6		1.23, 1.29	26.3, 71.3, 94.8, 134.5, 117.3
23	27.2			27.2		3.35, 3.88	147.9, 137.1, 131.6, 129.4, 122.9
24	26.3				26.3	1.34	24.7, 94.8, 71.3, 64.1
25	25.6				25.6	1.70	131.6, 122.9, 18.0
26	24.7				24.7	1.23	29.6, 26.3, 71.3
27	20.9				20.9	2.33	147.9, 131.6, 119.9
28	18.1				18.1	1.84	131.6, 122.9, 25.6
29	15.2				15.2	1.34	24.7, 94.8, 71.3, 64.1

Key: BB- Broad; C-C bonds; C - Singlet (s); CH - Doublet (d); CH_2 - Triplet (t); CH_3 - Quartet (q);
HSQC - Heteronuclear single-quantum correlation spectroscopy;

HMBC - Heteronuclear multiple-bond correlation spectroscopy

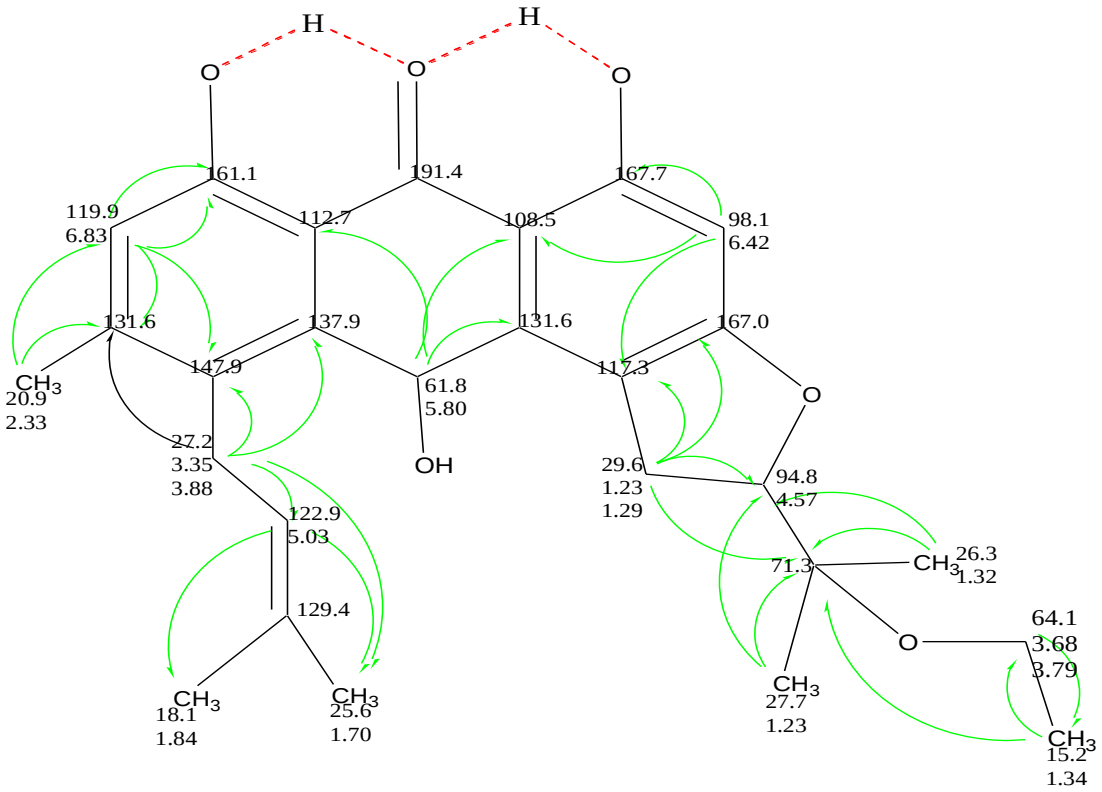

Fig.4.10a Heteronuclear multiple-bond correlation spectroscopy (HMQC) for Compound HMI-17

IUPAC Name- 2-(1-ethoxy-1-methylethyl)-5,7,11-trihydroxy-9-methyl-10-(3-methyl-2-butenyl)-1,11-dihydroanthra[2,1-*b*]furan-6(2*H*)-one

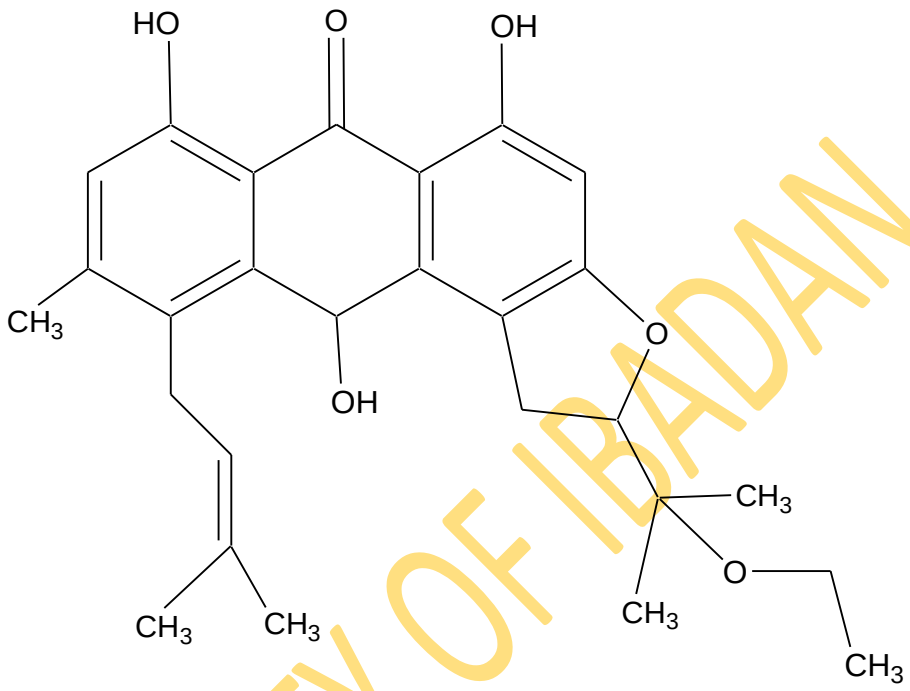

Fig.4.10b: Final Structure of Compound HMI-17

IUPAC Name- 2-(1-ethoxy-1-methylethyl)-5,7,11-trihydroxy-9-methyl-10-(3-methyl-2-butenyl)-1,11-dihydroanthra[2,1-*b]furan-6(2H)-one*

Description of Compound HMI-D21

The HRFABMS and ESI data suggested the molecular mass 438 and calculated 439.1856 with molecular formular {$C_{25}H_{26}O_7$}. The IR spectrum revealed the hydroxyl groups and carbonyl groups**.** The proton NMR showed the deshielded hydroxyl group at (δ_H 12.92 and 11.95). The 1,8- dihydroxyl anthronoid skeleton was confirmed by the ^{13}CNMR spectrum, it exihibited a resonance of only one carbonyl group at δ_C 189.9 (Delle Monache *et al*. 1980a, b; Kouam *et al*. 2005; Kouam *et al*. 2007). The hyroxyl groups (OH) were confirmed by resonance at δ_H 11.95 which showed correlations with three carbons δ_C 160.4, 118.8, and 110.3, while the second hydroxyl δ_H 12.92 showed correlation with δ_C 167.6, 109.2 and 99.3 (Table 4.13).

The ^{1}HNMR specrum showed resonance with aromatic methyl (δ_C 2.69) and a 2,2 – dimethyl pyran moiety (δ_H 1.44 and 1.22), 3-H each and a 1-hydroxy-1-methyethyldihydrofuran ring (δ_H 4.67 (^{1}H, dd, J oxymethine proton); 3.65 (^{1}H, dd, J, methylene proton); 3.72 (^{1}H, dd, J methylene proton; δ_H 1.21, 1.34 each 3H, S gem-dimethyl protons).

The broad band and DEPT specta showed the presence of 25 carbon signals including 14 quaternery carbons, 4 methines, 2 methylenes and five methyls. Among them resonances for methyl ethyl dihydrofuran ring was supported by the following ^{13}CNMR signals; δ_C 29.6 (methylene), δ_C 91.4 (oxomethine), δ_C 71.5 (oxygenated sp^{3}c), δ_C 26.0, δ_C 24.1 for (gem-dimethyl carbon), whereas δ_C 81.5 (quatinery oxygenated carbon, δ_C 82.2 (oxymethine), δ_C 24.1 and δ_C 29.8 (two methyl resonance).

The long range HMBC correlation of H-2 (δ_H 6.42) showed with C-1 (δ_C 167.6), C-3 (δ_C 166.3), C-4 (δ_C 119.5) and C-9a (δ_C 109.2) while H-12 (δ_H 4.67) shown correlation with C-11 (δ_C 29.6). C-15 (δ_C 26.0) and H-11 (δ_H 3.65) showed correlation with C-4 (δ_C 119.5), C-4a (δ_C 133.7) and C-12 (δ_C 91.4) further confirmed the dihydroxyfuran skeleton (Kouam *et al*. 2006).

The HMBC spectrum at two C-18 methyl protons resonance (δ_H 1.22 and 1.42) correlate with the oxymethine C-17 (δ_C 82.2) and oxygenated carbon atom C-18 (δ_C 81.5) and oxmethine H-17 (δ_H 4.68) correlated with C-10 (δ_C 120.1) and C-5 (δ_C 137.9) confirmed the deduction of fragment 13 (Fig. 4.11a,b) of compound HMI-D21.

In COSY spectrum, H-17 (δ_H 4.68) showed cross correlation with two resonance at δ_H 3.13 and δ_H 2.70 which supported the position of methylene proton at C-16. The aromatic methyl proton δ_H 2.69 showed HMBC correlation with C-5 (δ_C 137.9) and C-6 (δ_C 144.1) further confirmed the already biosynthetically proved (Melvyn, *et al*., 1992) at position C-6 (Gill *et al*., 1992) while H-7 (δ_H 6.76) showed resonance with C-8 (δ_C 160.4), C-8a (δ_C 110.5) and C-21 (δ_C 19.5). The molecular model studied which led to the conclusion that the 10, 17 oxygen bridge is *cis* relative configuration. On the basis of the above spectroscopic interpretation and also comparing with the previously reported data (Delle *et al*., 1980a, b; Nagem 1990; Kouam *et al*., 2007) confirmed the structure of compound HMI-D21.

Table 4.13: Nuclear Magnetic Resonance data (ppm) for Compound HMI-D21

N	BB	C	CH	CH_2	CH_3	HSQC	HMBC
1	189.9	189.9					
2	167.6	167.6					
3	165.3	165.3					
4	160.4	160.4					
5	145.9	145.9					
6	137.9	137.9					
7	133.7	133.7					
8	129.4	129.4					
9	120.1	120.1					
10	119.5	119.5					
11	118.8		118.8			6.76	120.1, 110.3, 160.4, 18.5
12	110.3	110.3					
13	109.2	109.2					
14	99.3		99.3			6.42	109.2, 119.5, 167.6, 166.3
15	91.3		91.3			4.67	29.6, 26.0, 24.1
16	82.2		82.2			4.68	29.6, 26.0, 24.1, 120.1
17	81.5	81.5					
18	71.5	71.5					
19	29.9			29.9		3.30, 3.58	91.3, 71.5, 167.6, 120.1, 133.7, 118
20	29.8				29.8	3.58	
21	29.6			29.6		3.36, 3.65	29.8, 29.6, 71.5, 91.3, 167.6, 119.5, 133.9
22	28.0			28.0		2.72, 3.20	28.0, 29.9, 145.9, 137.9, 120.1
23	26.0				26.0	1.34	24.1, 29.9, 91.3
24	24.1				24.1	1.21	26.0, 29.9, 29.8, 91.3, 81.5, 82.2, 71.5
25	24.1				24.1	0.8	
26	19.5				19.5	2.2	145.9, 120.1

Key: BB- Broad; C-C bonds; C - Singlet (s); CH - Doublet (d); CH_2 - Triplet (t); CH_3 - Quartet (q);
HSQC - Heteronuclear single-quantum correlation spectroscopy;
HMBC - Heteronuclear multiple-bond correlation spectroscopy

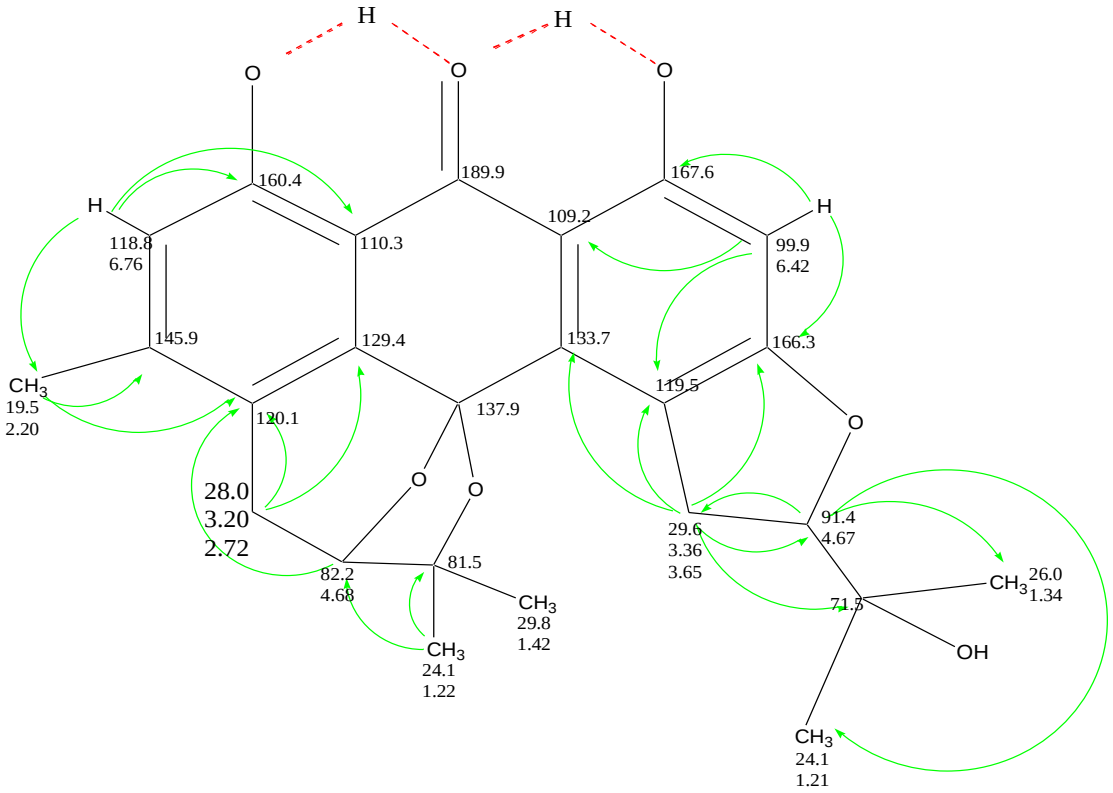

Fig. 4.11a: Heteronuclear multiple-bond correlation spectroscopy (HMQC) for Compound HMI-D21

IUPAC Name- (1*R,18S*)-9,13-dihydroxy-5-(1-hydroxy-1-methylethyl)-15,19,19-trimethyl-6,20,22-trioxahexacyclo[10.8.1.$1^{1,18}$.$0^{2,10}$.$0^{3,7}$.$0^{16,21}$]docosa-2,7,9,12(21),13,15-hexaen-11-one

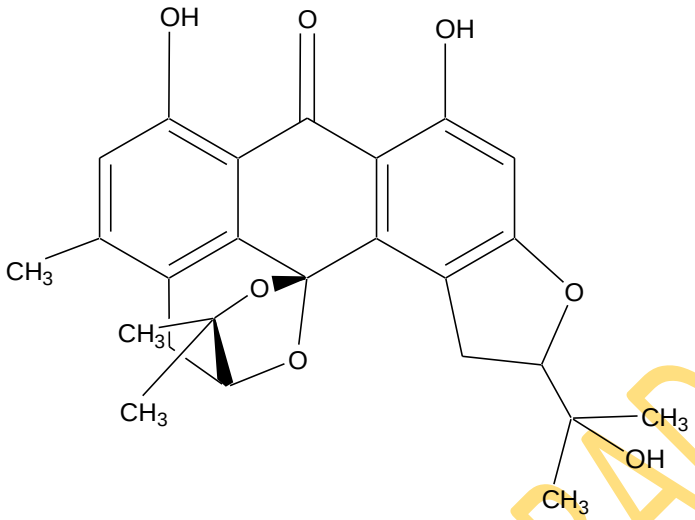

Fig.4.11b: Final Structure of Compound HMI-D21

IUPAC Name- (1*R,18S)-9,13-dihydroxy-5-(1-hydroxy-1-methylethyl)-15,19,19-trimethyl-6,20,22-trioxahexacyclo[10.8.1.1*^{1,18}*.0*^{2,10}*.0*^{3,7}*.0*^{16,21}*]docosa-2,7,9,12(21),13,15-hexaen-11-one*

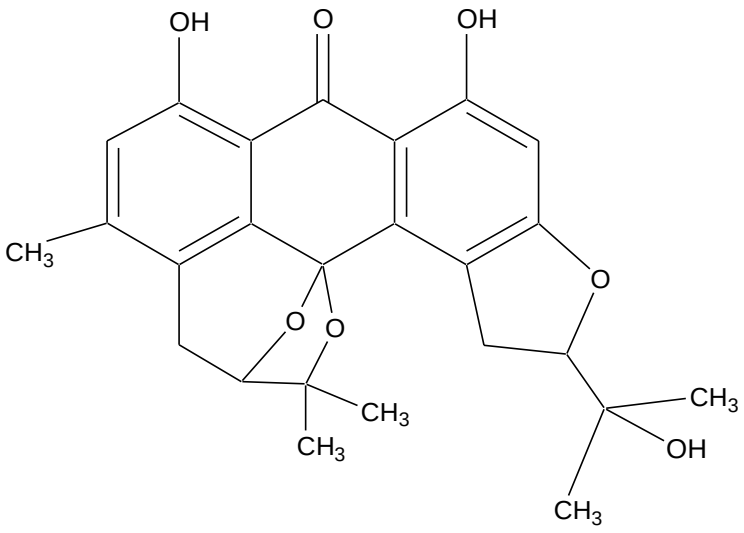

Fig.4.12: Final Structure of Compound HMI-79A

IUPAC Name- 9,13-dihydroxy-5-(1-hydroxy-1-methylethyl)-15,19,19-trimethyl-6,20,22-trioxahexacyclo [10.8.1.1^{1,18}.0^{2,10}.0^{3,7}.0^{16,21}]docosa-2,7,9,12(21),13,15-hexaen-11-one

4.8 Bioactivity of Partial Purified Fractions and Isolated Secondary Metabolites from *H. madagascariensis*

Partially purified fractions obtained from *H. madagascariensis* were examined for antioxidant Radical Scavenging Activity (RSA) and antibacterial activity, while the isolated secondary metabolites were subjected to invitro alpha-glucoxidase enzyme inhibition and antibacterial activity using MDR *Staphylococcus aureus.*

4.8.1. Antioxidant Activity

Partially purified fractions obtained from *H. madagascariensis* namely fraction-A, fraction-B, fraction-C and fraction-D, were further examined for antioxidants due to the fact that *H. madagascariensis* exhibited significant antioxidant activities as described in Table 4.3. Antioxidant activity was investigated using free radical, 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Scavenging Activity (RSA). The EtOAc-fraction at concentration of 0.5 µg/mL exhibited significant 90 %RSA activity with IC_{50} 15.58 µg/mL and SEM 2.03 against DPPH free radical. The EtOH- fraction of *H. madagascariensis* exhibited significant activity against free radicals up to 92 %RSA, IC_{50} 33.31 µg/mL and SEM 1.83 as compared with the standards used (Table 4.10).

Table 4.14: DPPH Antioxidant Activities Assay of HMB Fractions

Sample code	Conc. (µg/mL)	IC_{50} ± SEM (µM)	% RSA
EtOH	0.5	33.31±1.83	92
EtOA	0.5	15.58±2.03	90
DCM	0.25	115.20±2.5	65
HEX	0.5	42.49±1.49	84
Ascorbic acid	1.0	7.13	96
BHA	1.0	8.06	95

Keys:

RSA - Radical Scavenging Activity, SEM – Standard Error of Mean,

Each value ≥ mean of triplicate, HMB- *Harungana madagascariensis*,

EtOH- fraction-D, EtOA- fraction-C, DCM- fraction-B, HEX- fraction-A.

4.8.3. Antibacterial Activity

Table 4.15, shows the diameter of zones of inhibition of bacterial growth at varying concentrations of partially purified *H. madagascariensis* fractions. Fraction-A, Fraction-B, Fraction-C and Fraction-D were further examined for antibacterial effects due to the significant activities exhibited by its crude extract (Table 4.5). All the fractions tested exhibited significant antibacterial activities against *S. aureus* (ATCC 25923) between 25.66 ±0.33 and 28.00 ±0.00. For *E. coli* (ATCC 25922), the zones of inhibition increased significantly ($p < 0.05$) with increasing concentrations of the fractions from 10.33 ±0.33 to 20.33 ±0.33. The same pattern was observed for each of the other isolates.

However, various concentrations of extract Fractions A, B, C, and D produced wider zones of inhibition for *S. aureus* (ATCC 25923) and *P. aeruginosa* (POA 286) which were 28.00 ±0.00 and 27.66 ±0.33 respectively at 5mg/mL. Fraction-B expressed antibacterial activity against all test isolates including Gram negative *P. aeruginosa* (POA 286) at 5 mg/mL except for *S. typhi* (ATCC 14028), which was only susceptible to Fraction-A. Fraction-D, Fraction-C and Fraction-A exhibited 30.33 ±0.33, 20.33 ±0.33 and 34.66 ±0.33 as most significant activity at concentrations of 10 mg/mL, 25 mg/mL and 15 mg/mL against *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922) and *P. aeruginosa* (POA 286) respectively. Antibacterial activity was expressed against *S. flexneri* (ATCC 12022) by all the fractions tested. This result suggested that these fractions contained antibacterial compounds in appreciable quantity.

Table 4.15: Zones of Bacterial Growth Inhibition at Different Concentrations of *H. madagascariensis* Fractions

Conc. (mg/mL)	HMB Fractions	*S. aureus*	*E. coli*	*P. aeruginosa*	*Sh. flexineri*	*Sal. typhi*
5	HEX	28.00 ±0.00a	10.33 ±0.33b	24.00 ±0.00b	10.00 ±0.00d	10.66 ±0.00a
	DCM	21.33 ±0.33b	12.00 ±0.00a	27.66 ±0.33a	14.66 ±0.33a	4.00 ±0,00b
	EtOAc	18.00 ±0.00c	8.33 ±0.33c	15.66 ±0.33d	13.66 ±0.33b	0.00 ±0.00b
	BtOH	8.33 ±0.33d	8.66 ±0.33c	21.66 ±0.33c	11.66 ±0.33c	2.00 ±0.00b
10	HEX	28.33 ±0.00b	12.00 ±0.00b	26.00 ±0.00a	15.66 ±0.33b	10.66 ±0.33a
	DCM	23.00 ±0.00c	14.00 ±0.00a	20.33 ±0.00c	16.00 ±0.00a	6.00 ±0.00b
	EtOAc	16.66 ±0.33d	10.00 ±0.00c	18.00 ±0.33d	16.00 ±0.33ab	0.00 ±0.00c
	BtOH	30.33 ±0.33a	11.66 ±0.57b	22.66 ±0.33b	14.00 ±0.00c	2.00 ±2.00c
15	HEX	27.66 ±0.33a	12.00 ±0.00c	34.66 ±0.33a	-	-
	DCM	24.66 ±0.33b	15.66 ±0.33a	23.00 ±0.00c	-	-
	EtOAc	18.33 ±0.33c	14.33 ±0.33b	22.00 ±0.00c	-	-
	BtOH	18.00 ±0.00c	15.00 ±0.00ab	29.33 ±0.33b	-	-
25	HEX	23.66 ±0.33b	11.00 ±0.00c	22.00 ±0.00b	16.33 ±0.33c	17.33 ±0.33a
	DCM	15.66 ±0.33c	10.33 ±0.33c	18.00 ±0.00c	17.33 ±0.33b	7.00 ±0.00c
	EtOAc	25.66 ±0.33a	20.33 ±0.33a	25.66 ±0.33a	19.33 ±0.33a	9.00 ±0.00b
	BtOH	16.00 ±0.00c	15.66 ±0.33b	26.00 ±0.00a	16.00 ±0.00c	0.00 ±0.00d

Keys: HMB- *Harungana madagascariensis;* EtOH- fraction-D*;* EtOA- fraction-C; DCM- fraction-B*;* HEX- fraction-A*;* -= not determined*;* Values are means of three determinations ± S.E.M.
Values in each vertical column carrying different letters are significantly different from one another ($p < 0.05$).

4.8.4. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration of partially purified *H. madagascariensis* Fractions.

The MIC and Bactericidal activity of the fractions of *H. madagascariensis* extract were presented in Table 4.16. All tested fractions exhibited potent activity against both Gram positive *S. aureus* (ATCC 25923) and Gram negative *P. aeruginosa* (POA 286) with bacteristatic concentration of 5 mg/mL and bacteriocidal concentration of 20 mg/mL with MBC/MIC Ratio of 4.0. Similarly, all fractions exhibited same bacteristatic and bacteriocidal activity against *S. flexneri* (ATCC 12022) at 15 mg/mL and 30 mg/mL respectively with MBC/MIC ratio of 2.0 except Fraction-C which exhibited lower bacteriocidal concentration at 25 mg/mL.

Fraction-A expressed lowest bacteriostatic and bacteriocidal activity against *S. typhi* (ATCC 14028) at 10 mg/mL and 25 mg/mL with MBC/MIC ratio of 2.50. *E. coli* (ATCC 25922) was susceptible to both Fraction-A and Fraction-C at same bacteristatic concentration (15 mg/mL). All the tested fractions exhibited potent activity against one of the tested bacterial pathogens. The fractions of *H. madagascariensis* tested showed significant antibacterial activity more than the main crude extract. Fraction-A and Fraction-C were more significant in their action against all pathogenic strains tested while Fraction-B and Fraction-D were ineffective against *Salmonella typhi* (ATCC 14028).

Table 4.16: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration of *H. madagascariensis* Fractions in mg/mL

Fractions	*In vitro* Antibacterial Activity					
		Test Isolates				
		S. aureus	*E. coli*	*P. aeruginosa*	*Sh. flexineri*	*Sal. Ty*
HEX-fraction	MIC	5	15	5	15	10
	MBC	20	30	20	30	25
	MBC/ MIC ratio	4	2	4	2	2.5
DCM-fraction	MIC	5	20	5	15	N
	MBC	25	>30	20	30	N
	MBC/ MIC ratio	5	>1.5	4	2	-
EtOAc-fraction	MIC	5	15	5	15	15
	MBC	20	25	20	25	30
	MBC/ MIC ratio	4	0.6	4	0.6	2
BtOH-fraction	MIC	5	20	5	15	N
	MBC	25	30	20	30	N
	MBC/ MIC ratio	5	1.5	4	2	-

Keys: *S. aureus- Staphylococcus aureus, E. coli- Escherichia coli, P. aeruginosa- Pseudomonas aeruginosa, S. flexineri – Shigella flexineri, Sal. typhi- Salmonella typhi, N-* not determined, HEX - Fraction-A, DCM - Fraction-B, EToA- Fraction-C and BtOH – fraction-D represent fractions from *Harungana madacascariensis.*

4.8.5. Antimicrobial Activity of Secondary Metabolites against Selected Bacterial Strains

Four new identified secondary metabolites identified as kenganthranol D, E, F and G were examined for antibacterial activity against pathogenic strains of *S. aureus* ATCC 25923**,** *P. aeruginosa* POA 286, *S. typhi* ATCC 14028, *B. subtilis* and *S. flexneri* ATCC 12022 obtained from International Centre for Chemical and Biological Sciences, Karachi University, Pakistan (Table 4.17). *B. subtilis* was susceptible to Kenganthranol E and G, by 15.0 mm and 16.0 mm respectively at concentration of 100 µg/mL. At a concentration of 100 µg/mL Kenganthranol G inhibited the growth of *S. tyhpi*, *S. flexineri*, *P. aeruginosa*, *S. aureus* and *B. subtilis* by 11.0 mm, 12.0 mm, 14.0 mm, 13.0 mm and 16.0 mm respectively. *P. aeruginosa* and *B. subtilis* were susceptible to Kenganthranol D by 10.0mm and 14.0 mm respectively at concentration of 100 µg/mL. Kenganthranol E inhibited *B. subtilis*, *S. aureus* and *P. aeruginosa* by 15.0 mm, 13.0 mm and 12.0 mm at 100 µg/mL concentrations. All the secondary metabolites examined exhibited antibacterial activity against the selected bacterial isolates at concentration of 30 µg/mL.

Table 4.17: Antibacterial Activities of Compound 1-6[A] Against Selected Bacterial Strains

Compounds	*Bacillus subtilis*		*Staphylococcus aureus*		*Pseudomonas aeruginosa*		*Shigella flexineri*		*Salmonella typhi*	
	a	b	a	b	a	b	a	b	a	b
HMI-36B	11	15	7	9	7	12	7	10	9	15
Butuline	14	18	8	10	6	8	10	14	8	14
HMI-D21	10	16	9	13	11	14	8	12	7	11
HMI-1879A	11	14	7	9	8	10	6	9	6	8
HMI-8A	8	14	9	12	7	11	7	10	7	10
HMI-17	9	15	8	13	8	12	7	11	8	11
Imipenem	30		28		23		24		24	

Keys:

[a] Compound with a concentration of 30µg/mL was tested against microorganism

[b] Compound with a concentration of 100µg/mL was tested against microorganism

[A] Results were recorded after 24h of treatment and inhibitory zone diameters were measured in mm.

Imipenem 10µg concentration (CT0455B, Oxoid).

HMB-1879A - kenganthranol D, HMB-17 – kenganthranol E, HMI-36B - Triterpene

HMB-8A – kenganthranol F, HMB-D21 – kenganthranol G, But – Betulinic acid

4.8.6. Antibacterial Activity of Selected Secondary Metabolites and Standard Antibiotics against MDR *Staphylococcus aureus*

The purified secondary metabolites identified as alkaloid, triterpene and Kenganthranol G were screened against MultiDrug-Resistant (MDR) *S. aureus.* Alkaloids, triterpene and Kenganthranol G at 25 µg/mL concentration inhibited the growth of MDR *S. aureus*. The MDR *S. aureus* was susceptible to isolated kenganthranol G, with MIC of 25 µg/mL, but was resistant to standard antibiotics oxacillin, amikacin, chloramphenicol, erythromycin, sulfamethozale and ciprofloxacin used (Table 4.16 and Table 4.17).

In Figure 4.17, gram positive MDR *S. aureus* was susceptible to alkaloid, triterpene, and kenganthranol G by 28.2%, 13.1% and 22.6% respectively at a concentration of 25 µg/mL. Secondary metabolites alkaloid, triterpene, and kenganthranol G, exhibited 28.5%, 13.7% and 23.9% percentage inhibition respectively against MDR *S. aureus* at concentration of 50 µg/mL. Moreover, at a concentration of 100 µg/mL, the metabolites alkaloid, triterpene, and kenganthranol G, exhibited 31.0%, 24.2% and 30.7% percentage inhibition respectively against MDR *S. aureus.* The susceptibility of MDR *S. aureus* to all these secondary metabolites increased as the concentration increases. Metabolites alkaloid and kenganthranol G were more potent than the metabolite triterpene.

HMI-LS is an alkaloid and HMI-D21 is an anthraquinone while HMI-36B is triterpene. Alkaloids and phenol compounds are known to be highly effective against resistant microorganisms. This suggested the reason for their expressed anti-bacterial activity against Gram positive MDR strain of *S. aureus*. On the other hand, this gram positive MDR strain of *S. aureus* was resistant to standard antibiotics used oxacillin 1024 µg/mL, amikacin 1284 µg/mL, chloramphenicol 1284 µg/mL, erythromycin 1024 µg/mL, sulfamethozale 2048 µg/mL and ciprofloxacin 1284 µg/mL (Table 4.18).

Table 4.18: Antibacterial MIC of Different Antibiotic Classes and Purified Metabolites on Multidrug Resistant Strain *Staphylococcus aureus*

	Antibiotic Class	Antibiotics/ Metabolites	Inhibitory conc. (µg/mL)
Standard Antibiotics	Penicillin	Oxacillin	1024
		Penillin	512
	Aminoglycosides	Gentamycin	512
		Amikacin	1284
	Tetracycline	Chloramphenicol	1284
		Bacitracin	Nil
	Marolides	Erythromycin	1024
		Clindamycin	1024
	Sulfonamide	Sulfamethozale	2048
		Trimethoprim	Nil
	Fluoroauinolones	Ciprofloxacin	1284
Secondary Metabolites	Triterpene	HMI-36B	25
	Alkaloid	HMI-LS	25
	kenganthranol G	HMI-D21	25

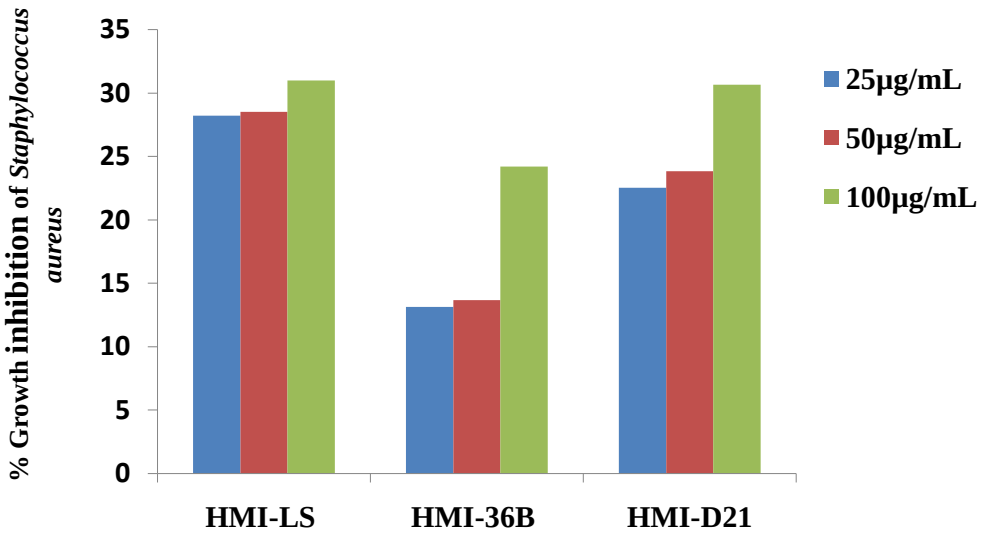

Fig. 4.17: Percentage Growth Inhibition of Clinical MDR *Staphylococcus aureus* by Purified Secondary Metabolites at Different Concentrations

Key: HMI-LS - Alkaloid), HMI-36 B -Triterpene, HMI-D21 - kenganthranol G

4.8.7. *In vitro* Assay for Alpha-glucoxidase Enzyme Inhibition

Secondary metabolites HMB-1879A, HMB-17, HMB-8A and HMB-D21, identified as kenganthranol D, kenganthranol E, kenganthranol F and kenganthranol G were examined for α-glucoxidase enzyme inhibition activity. Kenganthranol D and kenganthranol E exhibited significant enzyme inhibitory activity of 91.7% and 97.9% with IC_{50} of 69.9±4.21 and 122.4±1.13 respectively at 0.5 mM concentration. kenganthranol F was ineffective against α-glucoxidase enzyme while it was not determined for kenganthranol G. Kenganthranol D and kenganthranol E were good inhibitors of alpha-glucooxidase with IC_{50} of 69.9±4.21 and 122.4±1.13 respectively as compared to acarbose (standard) which exhibited 59.1% enzyme inhibition at IC_{50} of 840±1.23.

Table 4.19: Alpha-glucoxidase Activities Assay of Pure Secondary Metabolites

Sample code	Conc. (mM)	IC_{50} ± SEM (µM)	% Inhibition
HMB-1879A	0.5	69.9±4.21	97.1
HMB-17	0.5	122.3±1.13	97.9
HMB-8A	0.5	-	9.7
HMB-D21	0.5	-	NA
Std. (Acarbose)	1.0	840±1.23	59.1

Keys:

SEM – Standard Error of Mean, Each value ≥ mean of triplicates

NA – Not determined,

HMB-1879A - kenganthranol D, HMB-17 – kenganthranol E,

HMB-8A – kenganthranol F, HMB-D21 – kenganthranol G

CHAPTER FIVE

DISCUSSION

The qualitative determination of phytochemicals in extracts of selected five medicinal plant showed that *Harungana madagascariensis*, *Enantia chlorantha*, *Senna alata*, *Gossypium hirsutum* and *Alstonia boonei* contained alkaloids, flavonoids, glycosides, phenols, phlobatanins, saponins and tannins but varied in their qualitative test results. Reducing sugars and streroids were absent in all plant extracts. Some of the phytochemicals such as alkaloids, flavonoids, glycosides, phenols, phlobatanins, saponins, tannins, have previously been reported in *S. alata* extracts by (Ano and Ubochi, 2007) and in *E. chlorantha* by (Okwu, 2001).

Flavonoids and antraquinones, a phenol and polyphenol compounds were abundantly detected in *H. madagascariensis.* Phenol classes of compound has been reported for their effectiveness in the treatment of infectious diseases and possess strong antibacterial potential against both Gram positive and Gram negative bacterial strains (Pereira *et al*., 2007). However, Okoli *et al.* (2002) reported the absence of anthraquinone in *H. madagascariensis* extract which was detected in this work (Fomekong *et al.*, 2007). The descripancy in the result obtained could be due to the fact that extract from bark of *H. madagascariensis* was investigated in this study while Okoli *et al.* (2002) worked on the extract from the leaves of *H. madagascariensis.*

The extracts of *E. chlorantha* qualitatively have more concentration of alkaloids than the extracts of *S. alata, G. hirsutum, A. boonei* and *H. madagascariensis.* Moderate concentrations of flavonoids and cardiac glycosides were qualitatively detected in *E. chlorantha* extracts. The presence of alkaloids and flavonoids, and absence of steroids in *E. chloratha* extracts agreed with earlier report of (Adesokan *et al*., 2007), but

different in the case of glycosides which was qualitatively detected in this study. Tannins are polymeric phenol substances possessing astringent property. These compounds are soluble in water, alcohol and acetone and give precipitates with proteins (Basri and Fan, 2005). Coumarins are phenol substances made of fused benzene and -pyrone rings (O'Kennedy and Thornes, 1997). The presence of these secondary metabolites supported the use of these medicinal plants for infectious treatment means by the traditional healers.

Enantia chlorantha extracts showed seventy percent (70%) of *Lemna minor* growth inhibition while *H. madagascariensis, G. hirsutum, S. alata* extracts exhibited 40%, 25% and 10% of *Lemna minor* inhibition at the concentration of 1000 µg/mL respectively. This implied that *E. chlorantha* extracts had significant phytotoxic effect as compared to *G. hirsutum, A. boonei* and *S. alata* extracts which showed no significant activity at concentration of 1000 µg/mL against *L. minor* growth.

In vitro insecticidal activity of the selected medicinal plant extracts indicated that *H. madagascariensis* and *E. chlorantha* extracts affected the survival of *C. analis* and *R. dominica* respectively at 40 mg/mL while *S. alata*, *A. boonei,* and *G. hirsutum* extracts exhibited no significant toxicity against the survival of test insects as shown in Figure. 4.3. Growth inhibition may result from toxic properties of the plant extracts (El-Lakwah *et al*. 1996; Akhtar and Isman, 2004; Erturk, 2006). The insecticidal activity of the stem bark of *A. boonei* extracts against *Maruca vitrata Fabricius* was reported by (Oigiangbe *et al*., 2007), while Jeong *et al*. (2001) observed the bioactivity of the stem bark of other species in the family *Apocyanaceae* against different insect species. Extracts from the stem bark of *A. boonei* may likely contain more potent compounds than their leave extracts examined in this work.

Lethality bioassay of the selected plant extracts on *Artemia salina* indicated that the LC_{50} at $28^{o}C \pm 1^{o}C$ of extracts from *H. madagascariensis* and *E. chlorantha* were 76.747mg/mL and 1.916mg/mL respectively as against LC_{50} 0.007mg/mL obtained for the first drug Etoposide. The LC_{50} of *G. hirsutum, A. boonei* and *S. alata* extracts was 28.710 mg/mL, 51.704 mg/mL and 21.778 mg/mL respectively which indicated that extracts

tested showed no adverse effect. This implies that their consumption will probably not result in any cytotoxic effects to human when used. The test provided a scientific validation for the continued use of the plants as alternative or supplement to synthetic drugs in treatment of ailments.

In this present investigation, *H. madagascariensis* extract, demonstrated highly significant 1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging activity with 92%RSA value, IC_{50} 33.31±1.83 at 0.5 µg/mL. *Senna alata* and *E. chlorantha* exhibited moderate level of activities with 75 %RSA value and IC_{50} 217.26 ± 0.28 and 61 %RSA, IC_{50} 401.29 ± 5.9 at 0.5 µg/mL respectively as presented in Table 4.3. This indicated their ability to act as radical scavengers. The results obtained implied that extract of *H. madagascariensis, S. alata* and *E. chlorantha* contained appreciable quantity of phenol contents which conferred a very high DPPH scavenging activity on them. Furthermore, *H. madagascariensis* extract significantly showed 81 %RSA at 0.5 µg/mL concentration on superoxide scavenging activity (Table 4.4). This antioxidant result is close to the hypothesis of Momo *et al*. (2009) and Brand-Williams *et al*. (1995) that the DPPH kinetics is proportional to the amount of –OH groups present on the phenol compounds, that were detected in *H. madagascariensis* extracts qualitatively. Phenol compounds are the largest group of phytochemicals and have been said to account for most of the antioxidant activity of plant extracts (Okwu, 2005).

Cancer and cardiovascular as chronic diseases, are the main causes of death in the world (Halliwell, 1999), where oxidative stress induced by Reactive Oxygen Species (ROS) is one of the foci related to these diseases (Hanaa *et al*., 2008). ROS are highly reactive oxidant molecules that were endogenously generated through regular metabolic activity, lifestyle activities and diet, and react with cellular components, causing oxidative damage to such critical cellular bio-molecules such as lipids, proteins and DNA (Halliwell, 1999). Hence, there was strong evidence that this damage may play a significant role in causation of several chronic diseases (Halliwell, 1999). It has been reported that the presence of certain substances as antioxidant or free radicals scavengers may protect the body from the

consequences of oxidative stress (Qi *et al*., 2006). Thus, antioxidants play an important role in the protection of cells against oxidative damage caused by ROS (Qi *et al*., 2006).

The results obtained showed that lower concentrations of plant extracts tested were effective against *S. aureus* (ATCC 25923) and *P. aeruginosa* (POA 286) at 5 mg/mL. The result showed significant ($p < 0.05$) increase in the zones of inhibition when treated with the extracts of *S. alata* and *G. hirsutum* while the zones of inhibition appeared constant with increased concentrations of extracts from *H. madagascariensis* and *E. chlorantha. P. aeruginosa* POA 286, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *S. typhi* ATCC 14028 but not to *S. flexineri* (ATCC12022) were susceptible to extracts of *H. madagascariensis.* The susceptibility of Gram positive *S. aureus* to the extracts from *H. madagascariensis* correlates with the findings of Okoli *et al.* (2002) but different in activity reported for the extracts against gram negative *P. aeruginosa*.

Alstonia boonei and *E. chlorantha* extracts showed significance activity against *P. aeruginosa* (POA 286) when compared to other tested plant extracts. The potency expressed by *A. boonei* extracts against Gram negative *P. aeruginosa* (POA 286), probably due to chemical constituents which include alkaloids, triterpenoids and steroids (Ojewole, 1984). The antibacterial potency exhibited by the extracts of *E. chlorantha* against *S. typhi* (ATCC 14028) in this study was highly significant compared to the result of Adesokan *et al*. (2007), who reported 100 mg/mL and 150 mg/mL as the bacteriostatic and bacteriocidal concentrations respectively as against 10 mg/mL and 25 mg/mL concentrations reported in this study. Wafo *et al*. (1999) reported the antiviral activity of aqueous extract of the dried stem bark of *E. chlorantha* in Cameroon. Stem back extract was used to treat jaundice and urinary tract infections (Adjanohoun *et al*., 1996).

Antifungal activity against *M. canis* by all plant extracts investigated was eighty percent (80%), except *A. boonei* extracts with less than 15% inhibition. *Senna alata*, *G. hirsutum* and *H. madagascariensis* extracts exhibited average percentage growth inhibition of 75% against *A. flavus*. *Alstonia boonei* and *G. hirsutum* extracts were not effective against *C. albicans.* About 90% fungicidal activities were expressed by both *G. hirsutum* and *H.*

madagascariensis extracts against *M. canis. Candida albicans* was susceptible to both *H. madagascariensis* and *E. chlorantha* with fungicidal activity of 70% and 60% respectively. Similarly, 81% and 68% fungicidal activity was exhibited by both *H. madagascariensis* and *E. chlorantha* respectively against *C. glabarata*. Except for *A. boonei*, all plant extracts tested invitro exhibited significant activity. The potency of *S. alata* extracts against *A. flavus* correlates with the findings of Adedayo *et al*. (2002). All plant extracts tested expressed minimum fungicidal concentrations lower than the standard drugs used as control. This suggested that selected plant extracts have many chemical constituents which possess strong antifungal potential alone or in combination amongst them in nature (Table 4.1).

Traditionally used medicinal plants have recently attracted the attention of the biological scientific communities. This prompted the detailed isolation and identification of secondary metabolites. Fraction-B from *H. madagascariensis* was further purified to reveal a total of sixteen (16) secondary metabolites. Twelve (12) out of these secondary metabolites, comprise of known triterpenes and alkaloids, while the remaining four are new anthraquinine compounds that have never been reported as secondary metabolites isolated from the stem, leaf and root of *H. madagascariensis.* The four newly identified secondary metabolites (anthraquinine) belong to the phenol class substituted compounds. Phenol, triterpenes, flavonoids, tannins and saponins detected in the extracts were compounds that have been reported to possess medicinal properties and health-promoting effects (Kameswara *et al*., 1999).

Partially purified fractions of *H. madagascariensis* were further examined for 1,1-Diphenyl-2-picrylhydrazyl (DPPH) antioxidants due to the fact that the crude extract exhibited significant antioxidant activities as previously described. Among these fractions, fraction-A and fraction-C had very significant activity of 90% RSA and 84% RSA at 0.5 µg/mL respectively, similar to crude extract which had its activity of 92% RSA at 0.5 µg/mL. Coupled with this output was the significance of fraction-B with 65% RSA and IC_{50} 115.20±2.5 which was examined at 0.25 µg/mL. These results suggested that the *H. madagascariensis* extracts was very rich in phenol contents and compounds. This further

supports the use of this plant by traditional system of medicine for the treatment of diarrhoea and other gastrointestinal disorders (Tona *et al*., 2000; Atindehou *et al*., 2002). Radical scavengers play active roles in more than sixty different health conditions, including the aging process, cancer and atherosclerosis (Sou *et al*., 2000). Reducing exposure to free radicals and increasing intake of antioxidant nutrients have the potential to reduce the risk of free radical-related health problems.

The partially purified fractions were more significant in antimicrobial activities than the ethanol crude extract of *H. madagascariensis*. These fractions were able to inhibit the growth of Gram positive *S. aureus* (ATCC 25923) and Gram negative *P. aeruginosa (*POA 286) with bacteriostatic and bacteriocidal concentrations of 5 mg/mL and 20 mg/mL respectively as compared with 8 mg/mL and 24 mg/mL exhibited by the crude extracts. All the fractions tested exhibited significant activities against *E. coli* (ATCC 25922), which significantly ($p < 0.05$) increase in the zones of inhibition with increase in concentrations of the fractions. The same pattern was observed for each of the other isolates.

Antibacterial activity of the four new secondary metabolites against *S. aureus* ATCC 25923**,** *P. aeruginosa* POA 286, *S. typhi* ATCC 14028, *B. subtilis* and *S. flexneri* ATCC 12022 showed that zones of inhibition increased significantly ($p < 0.05$) with increasing concentrations of the secondary metabolites against all the test isolates. Though, the zones of inhibitions observed were lower compared to the standard drug (impenem). This suggested that the metabolites could be working in synergy to suppress the growth of pathogenic organisms, similar to Yasodamma *et al.* (2009) assertions.

The purified secondary metabolites HMI-LS, HMI-36B and HMI-D21 were further tested against Multi Drug Resistant (MDR) *S. aureus*. The susceptibility of MDR *S. aureus* to all these metabolites increased from 18% to 33% as the concentration increased from 25 µg/mL to 100 µg/mL. Two metabolites HMI-LS and HMI-D21 exhibited 33% growth inhibition each at 100 µg/mL, which confirm their potency over the metabolite HMI-36B with 23% growth inhibition at 100 µg/mL. The minimum inhibitory concentrations of 25

µg/mL exhibited by these secondary metabolites was lower than the MIC showed by the standard antibiotics, which suggested that these plant derived metabolites were more potent against MDR strain of *S. aureus* than the standard antibiotics. The compounds HMI-LS, HMI-D21 and HMI-36B were identified as alkaloids, anthraquinine and betulinic acid respectively. Alkaloids and phenolic compounds are known to be highly effective against the resistance microorganisms. This suggested the reason for their expressed anti-bacterial activity against the MDR strain of *S. aureus*.

Compound HMI-D21 (1*R,18S)-9,13-dihydroxy-5-(1-hydroxy-1-methylethyl)-15,19,19 -trimethyl-6,20,22-trioxahexacyclo[10.8.1.1*^{1,18}*.0*^{2,10}*.0*^{3,7}*.0*^{16,21}*]docosa-2,7,9,12(21),13, 15-hexaen-11-one*) obtained from *H. madagascariensis* has very similar structure to Artemisinin which is a sesquiterpene lactone obtained from *Artemisia annua* has been found to be highly potent antimalarial drug that is active against chloroquin resistant *Plasmodium* sp. The compound has a complex chemical structure that includes a peroxide bridge within the ring (Bharel *et al*., 1996). Efforts to chemically synthesize artimisinin have met with poor success and the yields have been very low. So, the presence of this compound can open up new sources for antimalaria drug.

Compound HMI-36B, (9-hydroxy-1-isopropenyl-5a, 5b,8,8,11a pentamethylico sahydro-3a*H-cyclopenta[a]chrysene-3a-carboxylic acid*), a known triterpene obtained from dichloromethane main fraction-36 of *H. madacascariensis* which belongs to the class of pentacyclic triterpenes. Pentacyclic triterpenes have been widely investigated for pharmacological activities. Pentacyclic triterpenoids are the dominant constituents of the triterpenoids class and are all based on a 30-carbon skeleton comprising five, six-membered rings and one five-membered ring, these compounds occur commonly and are mostly concentrated in fruits, vegetables, leaves, stem bark and several medicinal plants (Nagaraj *et al*., 2000).

Previous investigations by Habila *et al*. (2010) showed that bioactive pentacyclic triterpenes from plants have the ability not only to inhibit fungi, but also to completely kill them. They are devoid of any prominent toxicity. These naturally occurring triterpenoids

include the lupine, ursane oleanane, lanostane, dammarane, e.t.c. (Dang *et al.*, 2009). This has listed Betulinic acid a pentacyclic triterpene, as one of the compounds with potential that could be used in the treatment of infection caused by *Candida* species and molds, among its many other applications.

CHAPTER SIX

CONCLUSION, CONTRIBUTIONS TO KNOWLEDGE AND RECOMMENDATIONS

The extracts of selected plant possess broad-spectrum antibacterial activities against Gram-positive and Gram negative bacteria investigated. The broad-spectrum antibacterial activities of the selected plant extracts possibly due to the identified alkaloids, quinonones, flavones, phenolic acids, further confirm their uses as antibacterial agent in folklore medicine of Nigeria and may thus, be useful in the treatment of bacterial infections.

Phytochemical investigation revealed the presence of alkaloids, phenols, chalcones, saponins, cardiac glycosides, flavonoids and tannins. The plants extracts contained many constituents that were valuable sources of new and biologically active molecules possessing antimicrobial property. This study clearly points out that the selected plant tested has many chemical constituents which possess strong antibacterial potential alone or in combination amongst them.

Four new compounds were obtained in this study from *H. madagascariensis* which had previously been extensively worked upon for several years since the inception of ethnomedicine. Newer analytical tools and methods employed gave insights into the presence of these new compounds in the plant. Efficient collaborations with microbiologists, pharmacologists, organic chemists and plant pathologists are thus crucial to see complete development of compounds into exploitable products.

Contributions to Knowledge

- Four new compounds were obtained in this study from *H. madagascariensis* which had previously been extensively worked upon for several years since the inception of ethnomedicine.

- The enzymatic potential and good antimicrobial properties of these compounds especially against resistant strains of *S. aureus* paved ways for the development of new effective drugs.
- The findings enhance the scientific research to plants with antimicrobial properties which are abundant in the environment but yet underutilized as natural means of treatment.

RecommendationsIt is thus recommended that the search and research towards the discovery of lead compounds (to benefit human race) against the human pathogens and multi drug resistant pathogens should be intensified. It is recommended that the government should support the scientific research and further utilization of the research outcome for the benefit of human and the nation at large by adequate funding in order to facilitate the discovery of new biologically active plant products with more systematic and interpretation of results.

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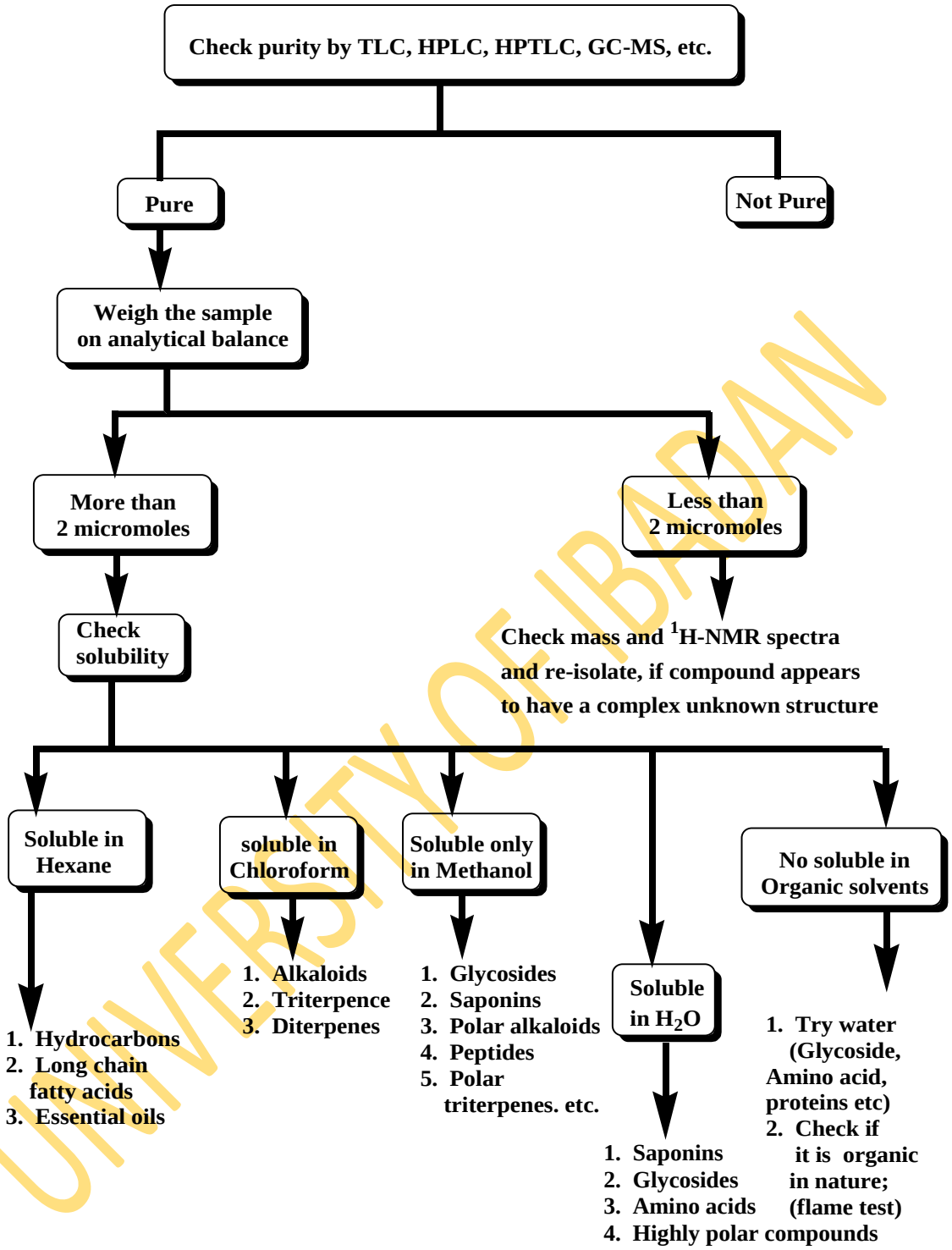
APPENDIX

Table 1: Composition of E-Medium

S/ No.	Chemical Name / Constitutes	g/L
1.	Potassium dihydrogen phosphate (KH_2PO_4)	0.68
2.	Potassium nitrate (KNO_3)	1.515
3.	Calcium nitrate ($Ca(NO_2)_2.4H_2O$)	1.180
4.	Magnesium sulfate ($MgSO_4.7H_2O$)	0.492
5.	Boric acid (H_3BO_3)	0.00286
6.	Manganous chloride ($MnCl_2.4H_2O$)	0.00362
7.	Ferric chloride ($FeCl_3.4H_2O$)	0.00540
8.	Zinc sulfate ($ZnSO_4.5H_2O$)	0.00022
9.	Copper Sulfate ($CuSO_4.5H_2O$)	0.00022
10.	Sodium Molybdate ($Na_2MO_4.2H_2O$)	0.00012
11.	Ethylene diamino tetra acetic acid (EDTA)	0.01120

Table 2: Commonly Stored Grain Pests

1. Red flour beetle	***Tribolium castaneum***
Rearing temperature	30 °C
Relative humidity	50 – 70%
Rearing media	Wheat flour
Life cycle	22-25 days
2. Rice weevil,	***Sitophilus oryzea***
Rearing temperature	25 °C
Relative humidity	50 – 70%
Rearing media	Wheat and rice
Life cycle	26-28 days
3. Lesser grain borer,	***Rhyzopertha dominica***
Rearing temperature	30 °C
Relative humidity	50 – 70%
Rearing media	Wheat and gram seeds
Life cycle	30 days
4. Khapra beetle,	***Trogoderma granarium***
Rearing temperature	30 °C
Relative humidity	50 – 70%
Rearing media	Wheat
Life cycle	30 days
5. Pulse beetle,	***Callosobruchus analis***
Rearing temperature	25-35 °C
Relative humidity	50 – 70%
Rearing media	Mung seeds
Life cycle	25-30 days



Only pure (over 98%), more than 2 micromolar samples should be taken up for further spectral analysis.

Fig. 3.3: PREPARATION OF SAMPLE FOR NMR ANALYSIS

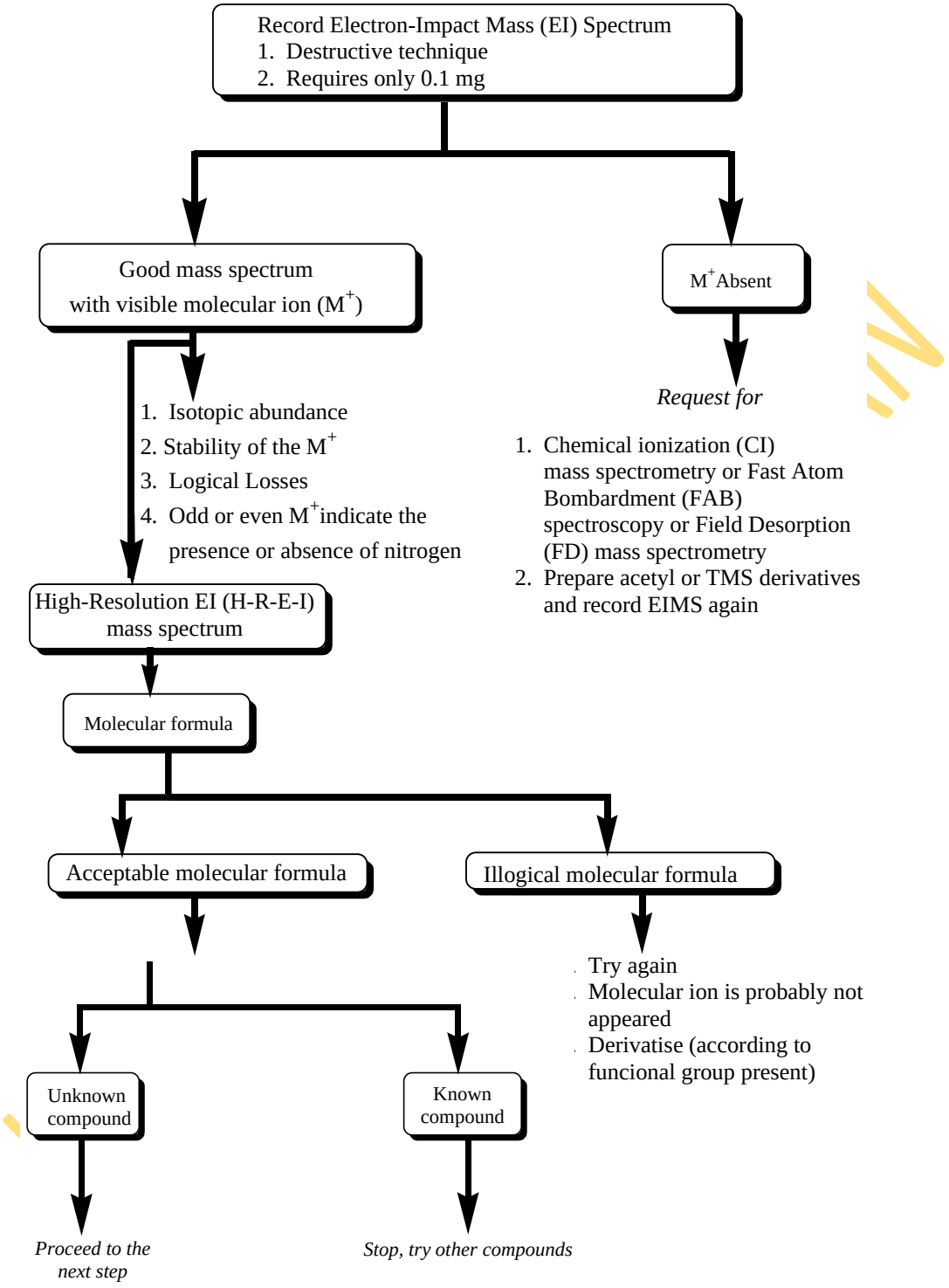


Fig. 3.4: DETERMINATION ISOLATED COMPOUND MOLECULAR FORMULA

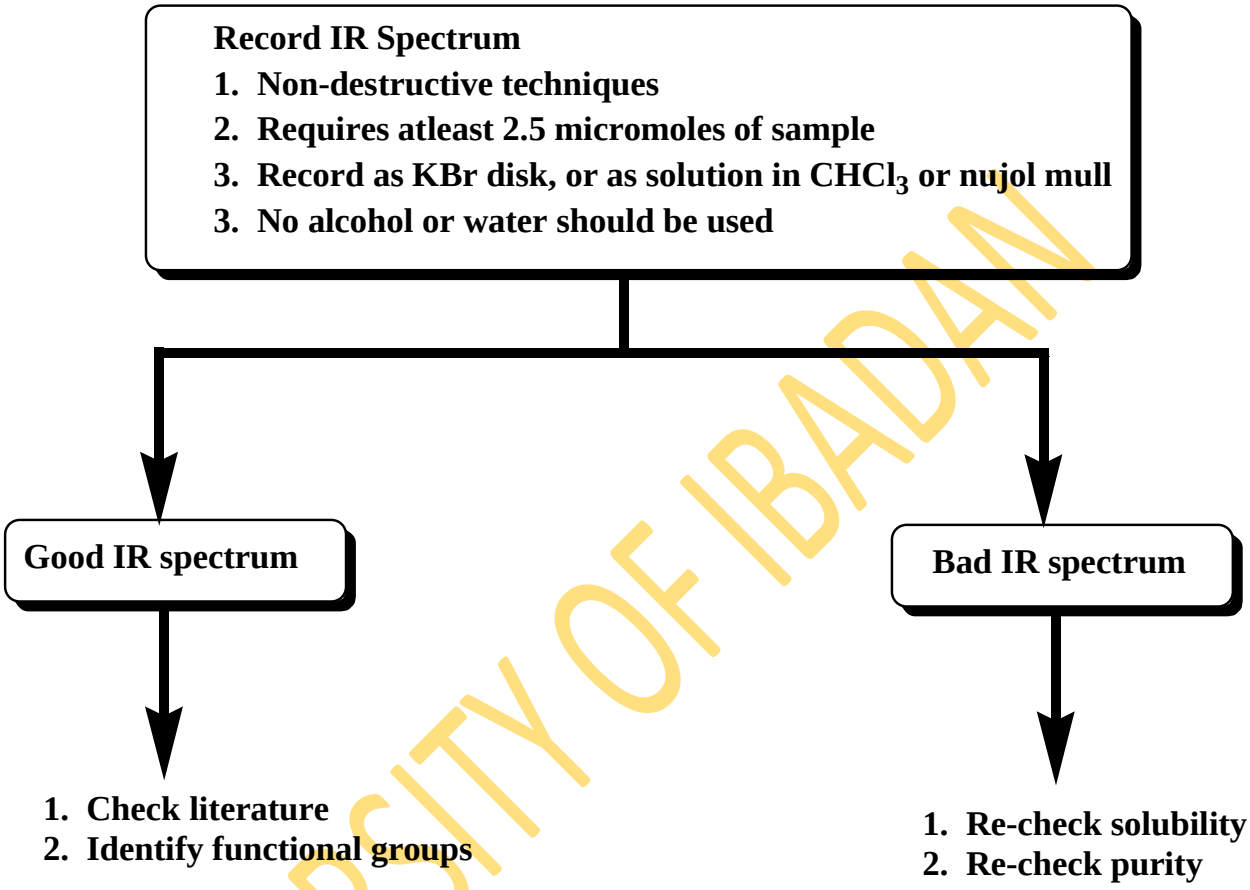


Fig. 3.5: DETERMINATION OF FUNCTIONAL GROUPS OF ISOLATED COMPOUNDS BY IR SPECTROPHOTOMETRY

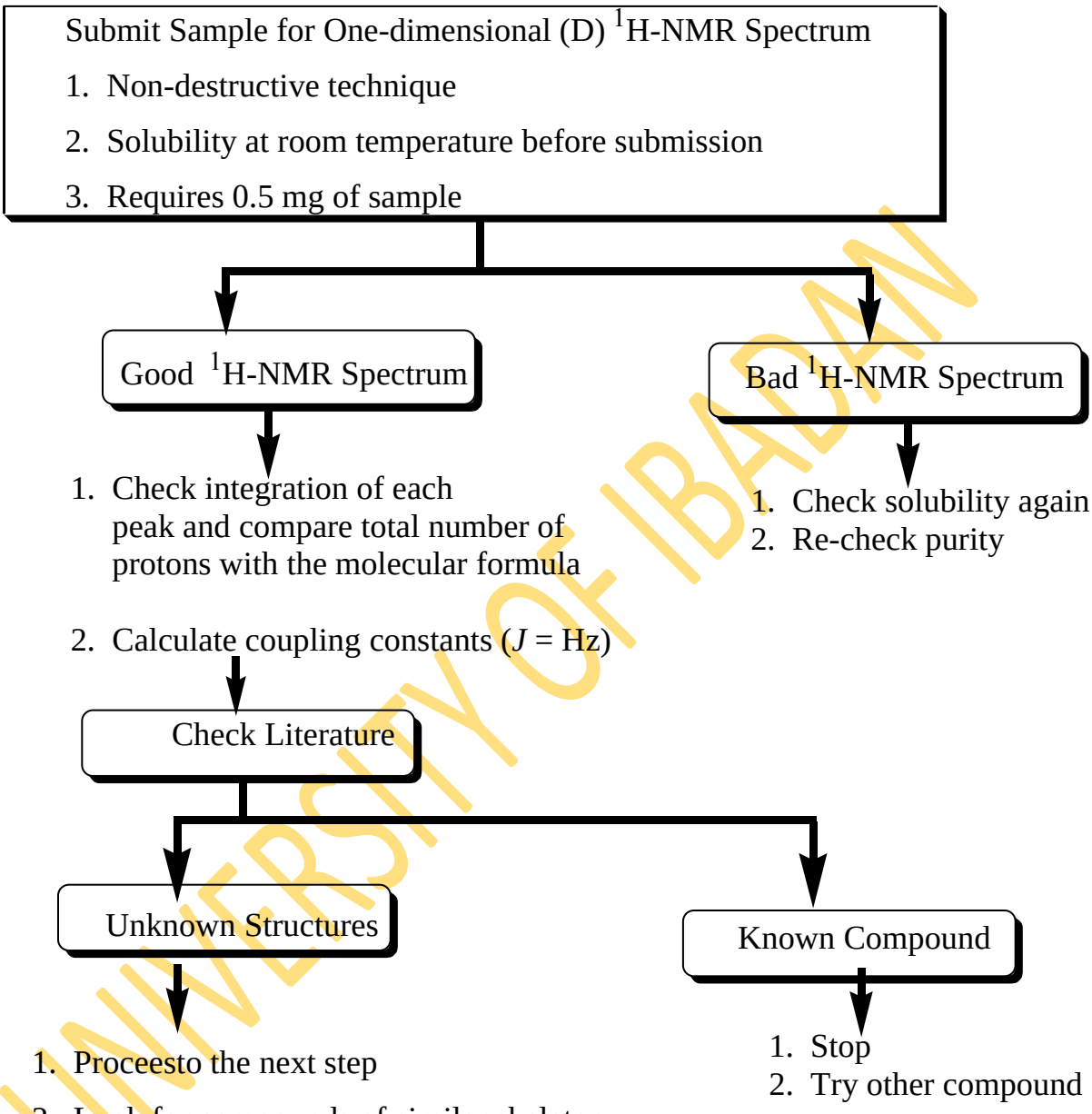


Fig. 3.7: PROTON NMR SPECTRSCOPY

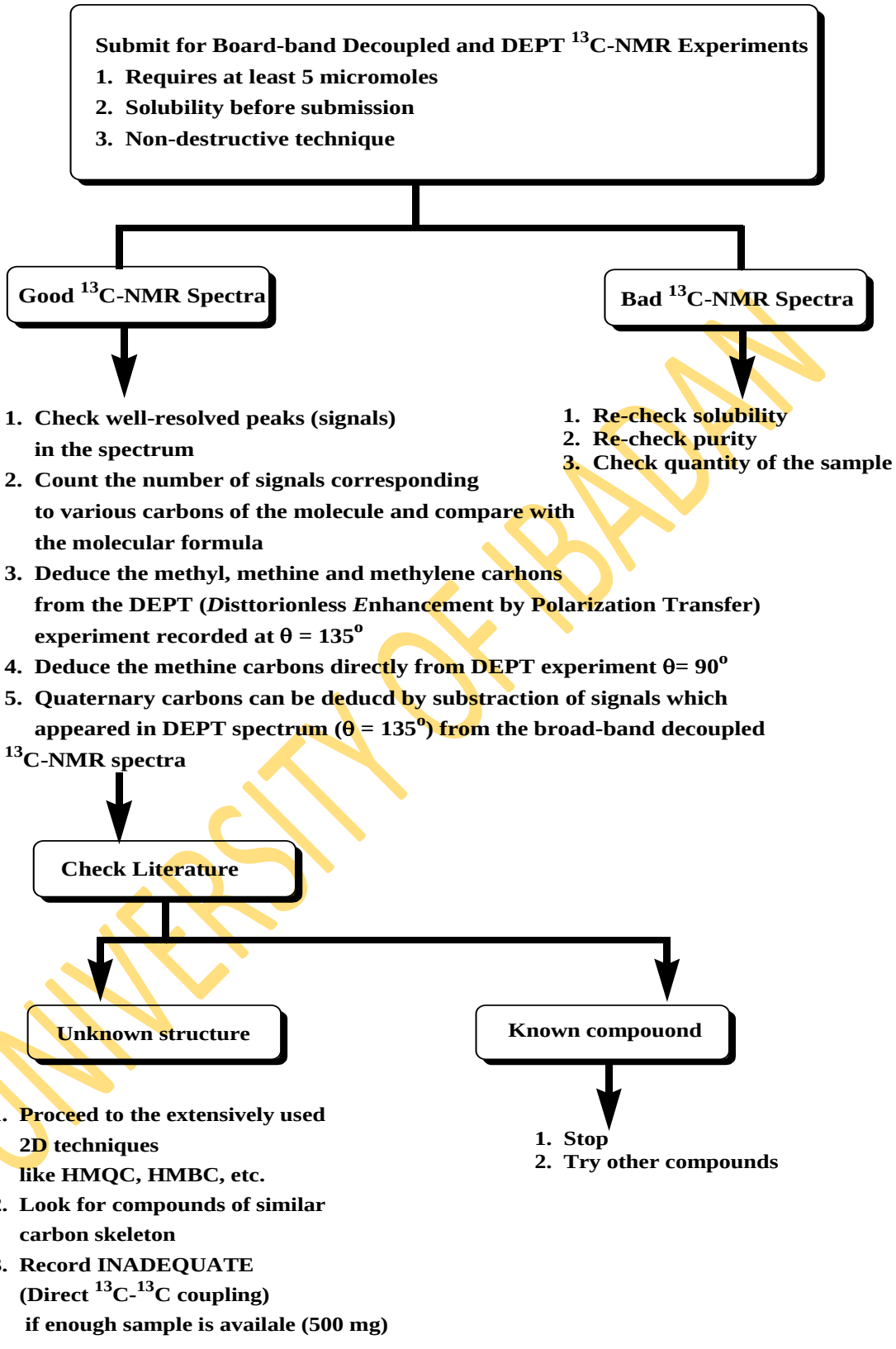


Fig. 3.8: ^{13}C-NMR SPECTROSCOPY

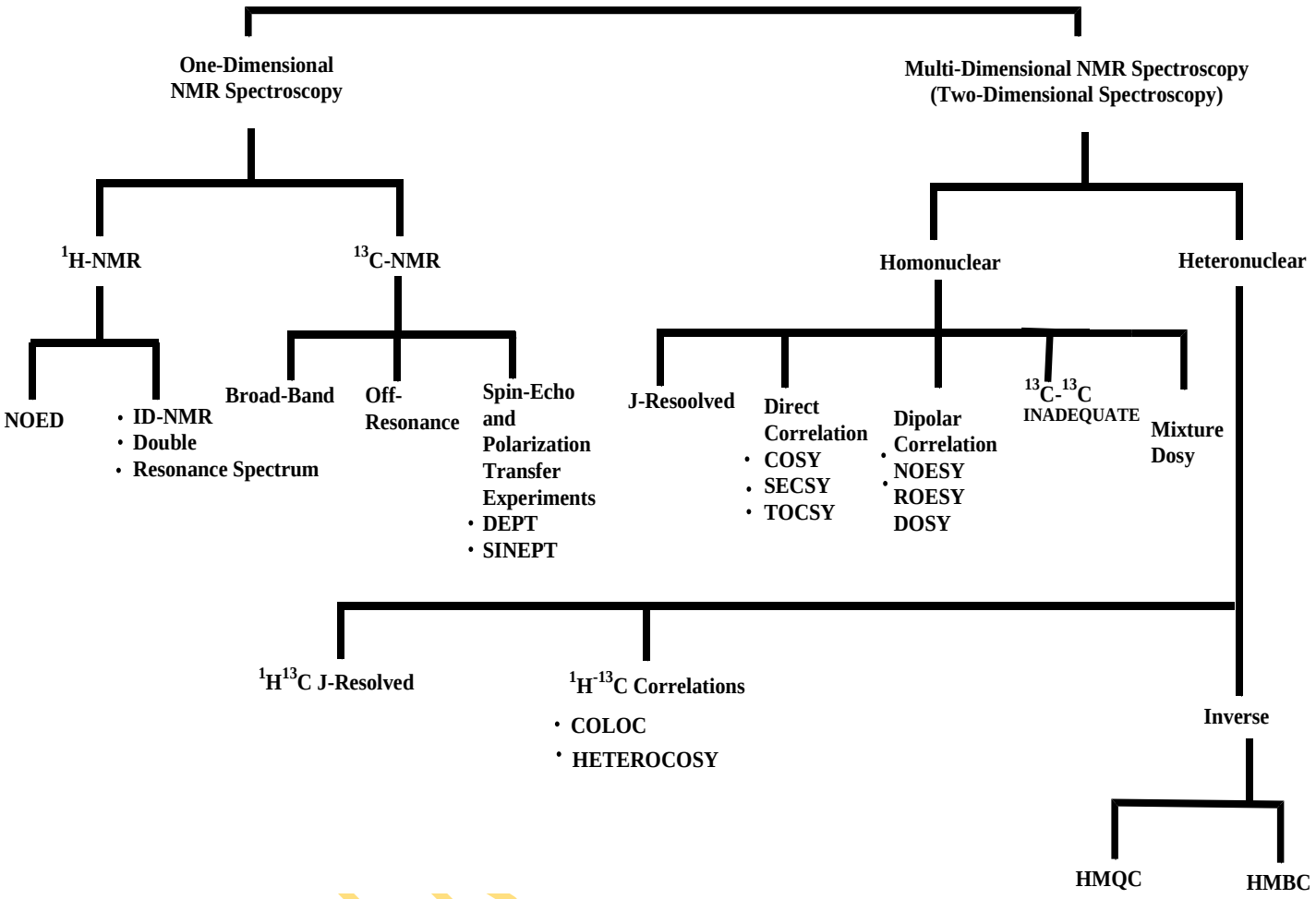


Fig. 3.9: NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY TYPES OF SPECTRA