ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM THE AERIAL PARTS OF *INDIGOFERA PULCHRA* (WILLD) AND EVALUATION OF POSSIBLE SYNERGISTIC ANTIMICROBIAL POTENTIAL WHEN COMBINED WITH CIPROFLOXACIN AND FLUCONAZOLE

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JANUARY, 2017

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P13PHMC8016

A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA

IN PARTIAL FULFILLMENT OF THE AWARD OF A MASTER DEGREE IN PHARMACEEUTICAL AND MEDICINAL CHEMISTRY

DEPARTMENT OF PHARMACEUTICAL AND MEDICINAL CHEMISTRY

FACULTY OF PHARMACEUTICAL SCIENCES

AHMADU BELLO UNIVERSITY,

ZARIA, NIGERIA.

JANUARY, 2017.

DECLARATION

I declare that the work in this thesis entitled **“**ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM THE AERIAL PARTS OF *INDIGOFERA PULCHRA* (WILLD) AND EVALUATION OF POSSIBLE SYNERGISTIC ANTIMICROBIAL POTENTIAL WHEN COMBINED WITH CIPROFLOXACIN AND FLUCONAZOLE” has been performed by me in the Department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University, Zaria under the supervision of Prof. A. M. Musa and Prof. M. Ilyas. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at any University.

Mu’ammar Dauda Lawal ____________________________

Name of Student Sign & Date

CERTIFICATION

This thesis entitled **“**ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM THE AERIAL PARTS OF *INDIGOFERA PULCHRA* (WILLD) AND EVALUATION OF POSSIBLE SYNERGISTIC ANTIMICROBIAL POTENTIAL WHEN COMBINED WITH CIPROFLOXACIN AND FLUCONAZOLE” by Mu’ammar Dauda Lawal meets the regulations governing the award of the degree of Master of Science in Pharmaceutical and Medicinal Chemistry of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

____________________________ _______________
Prof. A. M. Musa Date
Chairman, Supervisory Committee

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Prof. K. Bala Date
Dean, School of Postgraduate Studies, A.B.U. Zaria

DEDICATION

This research work is dedicated to my parent, late brothers (Rafiq, Kabir and Sulaiman), wife (Fatima) and daughter (Aminatu).

ACKNOWLEDGEMENTS

All praise is due to Allah (S.W.A), the Lord of the worlds, the beneficent the merciful, sovereign of the day of recompense, Whom alone we worship and Whom we ask for help.

My sincere gratitude goes to my supervisors, Prof. A. M. Musa and Prof. M. Ilyas for their patience, constructive criticism, immense guidance and dedication of their time and resources for the success of my research, whom without their efforts, this work wouldn’t have been possible.

My profound gratitude goes to my parents who supported me financially and morally throughout my educational career and worked to unlimited extent in helping me to build a bright future and to my entire family for their patience, love and support.

I would like to extend my appreciation to Dr. Y. M. Sani for all the sacrifices and contributions he made of his time and ideas and other staff members of the department for their encouragement and contributions to the success of this work.

My heartfelt thanks goes to Dr. Yusuf Hassan and M. Magaji Ilu Barde who helped me with specral analysis from Rhodes University, Johannesburg, South Africa; Dr. Aliyu Babando and Mal. Abubakar Falaki for their support with relevant literatures and not forgetiting M. S. Abdullahi of NILEST, Zaria for his full hands in the antimicrobial studies of this work.

I would finally like to thank any other person who had directly or indirectly contributed to the successful completion of this study. I am indeed very grateful.

ABSTRACT

Indigofera pulchra (Willd) is an annual non climbing erect herb or shrub that can grow upto 1m tall. It belongs to the family PAPILIONACEAE. It is widely distributed throughout tropical and subtropical regions of Nigeria, Niger, Togo, Benin, Ghana and Chad. It is also found in Senegal and over eastern and central Africa from Ethiopia to Angola. In ethnomedicine, it is used for the treatment of infected wound, itching skin, as prophylactic against snake bite, and for the treatment of malaria and dysentery. The aerial parts of the plant was subjected to cold maceration to afford the crude ethyl alcohol extract which was further fractionated using hexane, chloroform, ethylactate and butanol fractions. Phytochemical screening was carried out on the crude ethyl alcohol extract and the fractions using standard procedures and the results revealed the presence of flavonoids, tannins, steroids, carbohydrates and saponins. Extensive phytochemical investigations using silica gel column chromatography followed by preparative thin layer chromatography led to the isolation of 2,4-dihydroxy-4’-prenyloxychalcone (coded L1) and 3,5-dimethoxy-4’-O-(2’’-3’’-dihydroxy-3’’-methylbutyl)-dihydrostilbene (coded L2). The structures of the compounds were determined using IR and NMR. Antimicrobial activity was studied using agar well diffusion method and the organisms used are hospital isolates of Methiciline resistant *Staphylococcus aureus, Staphylococcus aureus, Proteus mirabilis, Eschericia coli, Candida albicans* and *Candida tropicalis*. The isolated compounds and the various fractions of the ethyl alcohol extract of *Indigofera pulchra* (Willd) aerial parts were investigated for their effects on the antimicrobial activity of ciprofloxacin and fluconazole. The results of the antimicrobial study obtained indicated that the various fractions of the ethyl alcohol extract of *Indigofera pulchra* (Willd) inhibited the growth of the test microorganisms with zone of inhibition range of 20-30mm in diameter while the isolated compounds inhibited the growth of the test microorganisms with zone of inhibition range of 29-37mm in diameter and was found to be greater than those observed for ciprofloxacin and fluconazole range of 25-33mm in diameter. Further evaluation of the antimicrobial effects of the two isolated compounds combined with the standard drugs (ciprofloxacin and fluconazole) led to increase in zones of inhibition diameter (25-40mm). The result of this study has justified the use of *Indigofera pulchra* (Willd) in ethno-medicine for the treatment of bacterial and fungal infections and that the isolated compounds might be potential lead compounds with antibacterial and antifungal activities.

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ABBREVIATIONS AND ACRONYMS

^{13}C-NMR:	Carbon-13 Nuclear Magnetic Resonance
CDC:	Centre for Disease Control and Prevention
DNA:	Deoxyribonucleic acid
F.D.A:	Food and Drug Administration
^{1}H-NMR:	Proton Nuclear Magnetic Resonance
IR:	Infra-red
MDR:	Multi-drug Resistance
MRSA :	Methicillin Resistant *Staphylococcus aureus*
NCB:	Nature Chemical Biology
NCCIH:	National Center for Complementary and Integrative Health
NPF:	Natural Products Foundation
PABA:	Para-aminobenzoic acid
PBPs:	Penicillin Binding Proteins
TLC:	Thin Layer Chromatography
U.S.A:	United States of America
WHO:	World Health Organisation

CHAPTER ONE

1.0 INTRODUCTION

1.1 Natural Product

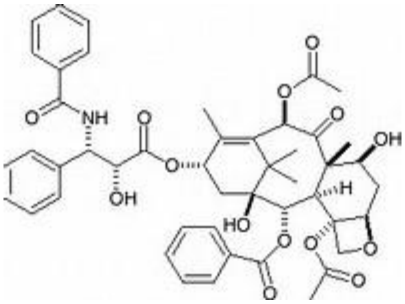
A natural product according to National Center for Complementary and Integrative Health (NCCIH) is defined as a chemical compound or substance produced by a living organism that is found in nature. In the broadest sense, natural products include any substance produced by life. Natural products have also played a central role in the development of the field of organic chemistry by providing challenging synthetic targets. The term natural product has also been extended for commercial purposes to refer to cosmetics, dietary supplements, foods produced from natural sources without added artificial ingredients (NCCIH, 2013).

Within the field of organic chemistry, the definition of natural products is usually restricted to mean purified organic compounds isolated from natural sources that are produced by the pathways of primary or secondary metabolism (Hanson, 2003). Within the field of medicinal chemistry, the definition is often further restricted to secondary metabolites (Willams and Lemke, 2002).

Natural products sometimes have pharmacological or biological activity that can be of therapeutic benefit in treating diseases. As such, natural products are the active components not only of most traditional medicines but also many modern medicines.

Plants are a major source of complex and highly structurally-diverse chemical compounds (phytochemicals); this structural diversity is attributed in part to the natural selection of organisms producing potent compounds to deter herbivory (Dang and van Damme, 2015).

Though the number of plants that have been extensively studied is relatively small, many pharmacologically active natural products have already been identified. Clinically useful examples include the anticancer agents paclitaxel (I) from *Taxus brevifolia* and omacetaxine mepesuccinate (II) from *Cephalotaxus harringtonia* (Kittakoop *et al*., 2014), the antimalarial agent artemisinin (III) from *Artemisia annua* (Kano, 2014) and the acetylcholinesterase inhibitor galantamin (IV) used to treat Alzheimer's disease from *Galanthus* spp. (Russo *et al*., 2013).



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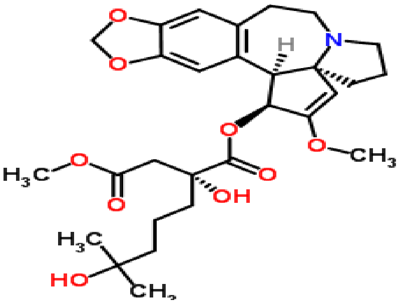

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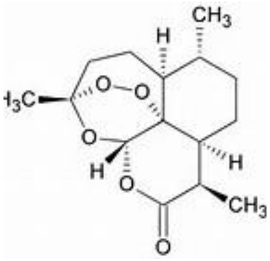

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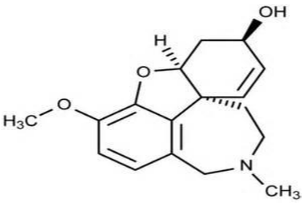

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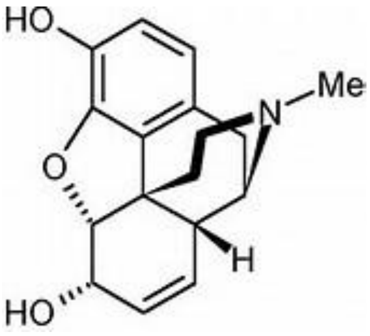
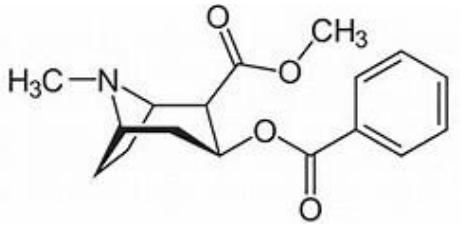
Other plant-derived drugs, used medicinally and/or recreationally include morphine (V), cocaine (VI), quinine(VII), tubocurarine (VIII), muscarine (IX), and nicotine (X) (Dewick, 2009).





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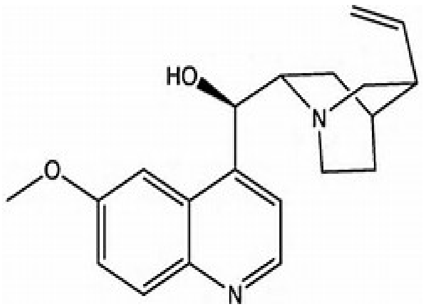

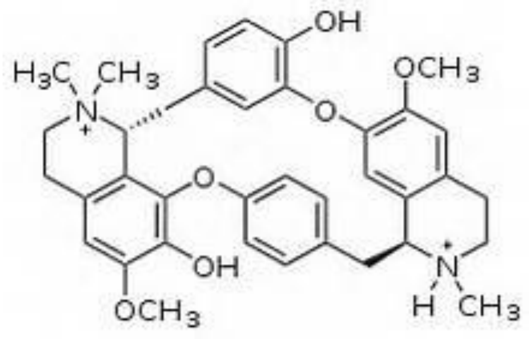

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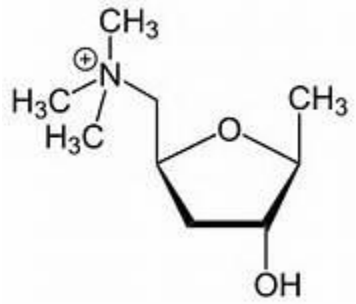

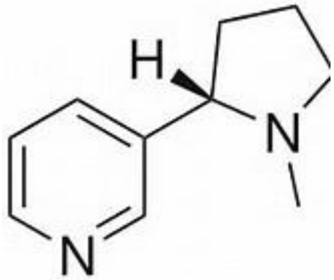

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Following Albrecht Kossel's original proposal in 1891, natural products are often divided into two major classes, the primary and secondary metabolites. Primary metabolites have an intrinsic function that is essential to the survival of the organism that produces them. Primary metabolites as defined by Kossel are components of basic metabolic pathways that are required for life. They are associated with essential cellular functions such as nutrient assimilation, energy production, and growth/development.

Secondary metabolites in contrast have an extrinsic function that mainly affects other organisms (Karlovsky, 2008). Secondary metabolites are dispensable and not absolutely required for survival. Furthermore secondary metabolites typically have a narrow species distribution and a broad range of functions. These include pheromones that act as social signaling molecules with other individuals of the same species, communication molecules that attract and activate symbiotic organisms, agents that solubilize and transport nutrients (siderophores etc.), and competitive weapons (repellants, venoms, toxins etc.) that are used against competitors, prey, and predators (Demain and Fang, 2000).

Animals also represent a source of bioactive natural products. In particular, venomous animals such as snakes, spiders, scorpions, caterpillars, bees, wasps, centipedes, ants, toads, and frogs have attracted much attention. This is because venom constituents such as peptides, enzymes, nucleotides, lipids, biogenic amines etc. often have very specific interactions with a macromolecular target in the body e.g. α-bungarotoxin from cobras (Dossey, 2010).

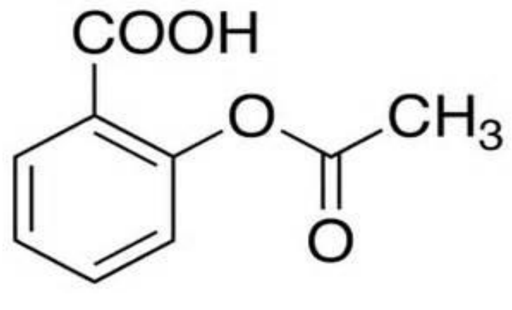
In addition to the terrestrial animals and amphibians, many marine animals have been examined for pharmacologically active natural products, with corals, sponges, tunicates, sea snails, and bryozoans yielding chemicals with interesting analgesic, antiviral, and anticancer activities (Mayer *et al.,* 2010). Two examples developed for clinical use include ω-conotoxin from the marine snail *Conus magus* used to relieve chronic pain (Prommer, 2006), and ecteinascidin 743 from the tunicate *Ecteinascidia turbinata* is used to treat metastatic soft tissue sarcoma (Petek *et al.,* 2015).

1.2 Medicinal Plants

A large amount of archaeological evidence exists which indicates that humans were using medicinal plants during the Paleolithic period, approximately 60,000 years ago. Furthermore, animals such as non-human primates, monarch butterflies and sheep are also known to ingest medicinal plants to treat illness (Judith, 2000).

Angiosperms (flowering plants) were the original source of most plant medicines. Many of the common weeds that populate human settlements, such as nettle, dandelion and chickweed, have medicinal properties (John, 2004).

The therapeutic properties of medicinal plants are conditioned by the presence in their organs of active substances, such as alkaloids, flavonoids, glycosides, vitamins, tannins, and coumarin compounds, which physiologically affect the bodies of humans and animals or which are biologically active in relation to the causative agents of various diseases. The use of herbs to treat disease is almost universal among non-industrialized societies. Many of the pharmaceuticals currently available to physicians have a long history of use as herbal remedies, examples include opium, acetylsalicylic acid (XI), digitalis, and quinine (XII).



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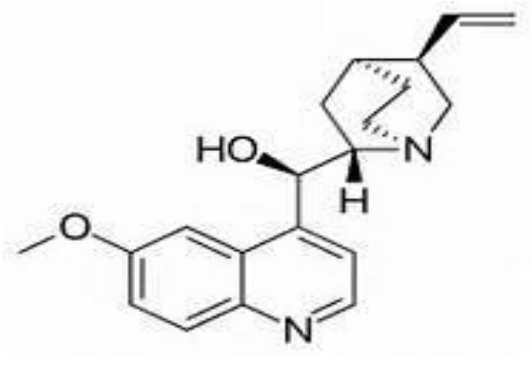

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The World Health Organization (WHO) estimates that 80 percent of the populations of some Asian and African countries presently use herbal medicine for some aspects of primary health care. This may be due to the fact that pharmaceuticals are prohibitively expensive for most of the world's population, half of which lives on less than $2 U.S. per day. In comparison, herbal medicines can be grown from seed or gathered from nature for little or no cost (Edgar *et al.*, 2002).

The use of, and search for drugs and dietary supplements derived from plants have accelerated in recent years. Pharmacologists, microbiologists, botanists, and natural-product chemists are combing the earth for phytochemicals and leads that could be developed for treatment of various diseases (Edgar *et al.,* 2002).

1.3 Bacterial Infection

Bacteria are a type of microbe which can cause different types of diseases. Most bacteria are harmless, and some are even helpful in digestion of food. But harmful types of bacteria can make one feel extremely unwell. Harmful bacteria multiply at lightning speed, and release toxins that can damage tissues considerably.

Bacterial diseases continue to present a major threat to human health. Tuberculosis, for instance, ranks among the world's leading causes of death. *Streptococcus* (Group B *Streptococcus*), another bacterium, continues to be a frequent cause of life-threatening infection during the first two months of life. Food borne and water borne bacteria such as *Salmonella* and *Campylobacter* are responsible for a recent troubling increase in diarrheal disease. Meanwhile, during the last decade, scientists discovered many new organisms and new strains of many familiar bacteria, such as *Escherichia coli*. Such emerging bacterial diseases present a clear challenge to biomedical researchers (Hooper and Gordon, 2001).

1.4 Antimicrobial Resistance

The WHO defines antimicrobial resistance as a microorganism's resistance to an antimicrobial drug that was once able to treat an infection by that microorganism. There are three main ways by which resistance can occur: by natural resistance in certain types of bacteria, by genetic mutation, or by one specie acquiring resistance from another (WHO, 2014).

Resistance can happen spontaneously owing to random mutations, to a build-up of resistance over time, or to misuse of antibiotics or antimicrobials, although the latter two pathways are the most important. Resistant microbes become increasingly difficult to treat, requiring alternative medications or higher doses, both of which may be more costly or more toxic. According to Centre for Disease Control and Prevention (CDC, 2015), microbes which are resistant to multiple antimicrobials are called multidrug resistant (MDR); these organisms are often referred to as superbugs.

Antimicrobial resistance is an increasingly problematic issue that leads to millions of deaths every year. A few infections become completely untreatable due to resistance. All classes of microbes develop resistance (fungi, antifungal resistance; viruses, antiviral resistance; protozoa, antiprotozoal resistance; bacteria, antibiotic resistance). Of those categories, bacterial antibiotic resistance poses the largest threat to public health (WHO, 2014).

The drug penicillin was introduced in the mid-1940s, thereafter, a few individual *Staphylococcus aureus* bacteria acquired genes that made penicillin ineffective against them. The strains that possessed these special genes had a survival advantage when penicillin was commonly used to treat infections. Strains of *Staphylococcus aureus* that lacked these new genes were killed by penicillin, allowing the remaining penicillin-resistant bacteria to reproduce and over time become more common. Chemists then altered the penicillin molecule, making a different but similar drug, methicillin, which could kill the penicillin-resistant bacteria. Soon after methicillin was introduced, strains of *Staphylococcus aureus* developed genes that made them resistant to methicillin and related drugs. These strains are called methicillin-resistant *Staphylococcus aureus, mRSA* (WHO, 2014).

Resistant bacteria can spread from person to person. Because international travel is so common, resistant bacteria can spread to many parts of the world in a short time. Spread of these bacteria in hospitals is a particular concern. Resistant bacteria are common in hospitals because antibiotics are so often necessary and hospital personnel and visitors may spread the bacteria if they do not strictly follow appropriate sanitary procedures. Also, many hospitalized patients have a weakened immune system, making them more prone to infection (WHO, 2014).

1.4.1 Mechanisms of Antimicrobial Resistance

The main mechanisms by which microorganisms exhibit resistance to antimicrobials are:

1. Drug inactivation or modification: for example, enzymatic deactivation of penicillin G in some penicillin-resistant bacteria through the production of β-lactamases. Most commonly, the protective enzymes produced by the bacterial cell will add an acetyl or phosphate group to a specific site on the antibiotic, which will reduce its ability to bind to the bacterial ribosomes and disrupt protein synthesis (Mukesh *et al.,* 2008).

2. Alteration of target site: for example, alteration of PBPs (Penicillin Binding Proteins) in MRSA and other penicillin-resistant bacteria. Another protective mechanism found among bacterial species is ribosomal protection proteins. These proteins protect the bacterial cell from antibiotics that target the cell's ribosomes to inhibit protein synthesis. The mechanism involves the binding of the ribosomal protection proteins to the ribosomes of the bacterial cell, which in turn changes its conformational shape. This allows the ribosomes to continue synthesizing proteins essential to the cell while preventing antibiotics from binding to the ribosome to inhibit protein synthesis (Mukesh *et al.,* 2008).
3. Alteration of metabolic pathway: for example, some sulfonamide-resistant bacteria do not require para-aminobenzoic acid (PABA), an important precursor for the synthesis of folic acid and nucleic acids in bacteria inhibited by sulfonamides, instead, like mammalian cells, they turn to using preformed folic acid (Mukesh *et al.,* 2008).
4. Reduced drug accumulation: by decreasing drug permeability or increasing active efflux (pumping out) of the drugs across the cell surface. These specialized pumps can be found within the cellular membrane of certain bacterial species and are used to pump antibiotics out of the cell before they are able to do any damage. These efflux pumps are often activated by a specific substrate associated with an antibiotic (Aminov and Mackie, 2007).
5. Horizontal gene transfer: Antibiotic resistance can also be introduced artificially into a microorganism through laboratory protocols, sometimes used as a selectable marker to examine the mechanisms of gene transfer or to identify individuals that absorbed a piece of DNA that included the resistance gene and another gene of interest. A recent study

demonstrated that the extent of horizontal gene transfer among *Staphylococcus* is much greater than previously expected and encompasses genes with functions beyond antibiotic resistance and virulence, and beyond genes residing within the mobile genetic elements (Chan *et al.*, 2011).

6. Unlinked point mutations in the pathogen genome at a rate of about 1 in 10^8 per chromosomal replication. Mutations are rare but the fact that bacteria reproduce at such a high rate allows for the effect to be significant (Cirz *et al.*, 2005).

For a long time, it has been thought that, for a microorganism to become resistant to an antibiotic, it must be in a large population. However, recent findings show that there is no necessity of large populations of bacteria for the appearance of antibiotic resistance. It is known now that small populations of *E.coli* in an antibiotic gradient can become resistant. Any heterogeneous environment with respect to nutrient and antibiotic gradients may facilitate the development of antibiotic resistance in small bacterial populations and this is also true for the human body. Researchers hypothesize that the mechanism of resistance development is based on four SNP mutations in the genome of *E.coli* produced by the gradient of antibiotic. These mutations confer the bacterial emergence of antibiotic resistance (Chan *et al.*, 2011).

1.5 Synergy

The term synergy is derived from the Greek syn-ergos, "working together". Synergies have been described in many settings and situations of life, including mechanics, technical systems, human social life, and many more. In all cases, synergy describes the fact that a system, i.e. the combination and interaction of two or more agents or forces is such that the combined effect is greater than the sum of their individual effects. This definition implies that there are three possible ways of such an “interaction of agents or forces”: these forces could simply add up, not affecting each other (no interaction; A+B = A+A or B+B), their combination could produce a greater than expected result (synergy; A+B > A+A or B+B), or the combination could lead to a result that is less than the sum of the individual effects; antagonism: A+B < A+A or B+B (Hewitt and Vincent, 2003).

Interactions of biologically active agents are an important aspect of pharmacology and biomedicine. In this context, interaction describes the biological activity that results from the presence of several drugs at the same time. Such situations occur in numerous clinical situations; combinations of antibiotics likewise produce better efficacy with fewer side effects and reduced development of resistance; many serious clinical situations require administration of several drugs simply because of multiple therapeutic indications. Although in such a case drug combinations are not formulated to look for synergies, the interactions of these drugs need to be assessed; so also, the effect of one drug may be augmented by another drug that does not produce such an effect on its own. In all these cases, multiple drugs are administered, and will show some form of interaction; synergistic, antagonistic, or none (Hewitt and Vincent, 2003).

Historically, extracts from plants, animals, or even soils were the first classified pharmaceuticals; these were complex mixtures rather than single agents, and some ingredients may have interacted with others (Van Vuuren, 2007).. Over the years of development of pharmacy, isolation, synthesis and marketing of single drugs became the accepted standard. Whether a complex mixture or a combination of drugs is used, the biological interaction of all active substances should be known. Synergy may be observed in simple systems where two drugs only act on one target protein. In such a case we can study the interaction of the drugs mechanistically and determine why and how several drugs can reinforce each other (or why they do not). Synergy may also be observed in complex settings, such as patients receiving multiple medications. Usually, more than one biological target (protein, pathway, or even organ) are involved in such cases, and single mechanistic descriptions are not appropriate (Van Vuuren, 2007).

Additional parameters that justify the concept of synergism are absorption, tissue distribution, and clearance. It may be expected that many drugs interfere with metabolism of other drugs. Thus, a substance B that slows down clearance of an active drug A, say by blocking metabolizing enzymes or excretion, may lead to a higher effective concentration of A that remains in the body for a longer time. As a result, one would notice a greater effect of drug A when given together with B, although the two drugs have completely different modes of action. While certainly the combination of these two drugs would have a “combined effect greater than the sum of their individual effects”, their combination is synergistic in practical application, but not by the strict definition.

1.6 Statement of Research Problem

Antimicrobial resistance is an increasingly problematic issue that leads to millions of death every year (WHO, 2014). In recent years, some bacteria and fungi have developed resistance to antibiotics that used to kill them. This is a big problem in controlling infectious diseases and hence, poses a dire need to search for substances, especially from natural sources to solve the problem. Therefore, this research aims toward finding new compounds from *Indigofera pulchra* (Willd) that can either be irresistible by the microorganisms or potentiate the efficacy of the drugs when combined together and retard the problem of antimicrobial resistance.

1.7 Justification of the Study

Previous studies reported the isolation of 2’,4’-dihydroxy-4-prenyloxychalcone (Musa *et al.,* 2011), a mixture of 3-methoxy-4,5-methylenedioxy-4’-O-(2’’-3”-dihydroxy-3”-methylbutyl)-dihydrostilbene & 3,5-dimethoxy-4’-O-(2”,3”-dihydroxy-3”-methylbutyl)-dihydrostilbene (Musa *et al.,* 2008) from *Indigofera pulchra* (Willd) but, to the best of our literature search, there is no documented report on the isolation of either of the two stilbenes in their pure form and the possible synergistic effect between the fractions, isolated compounds and the standard drugs.

It has been proven that, in addition to the production of intrinsic antimicrobial compounds, plants also produce multi-drug resistant (MDR) inhibitors which enhance the activity of the antimicrobial compounds (Stermitz *et al.*, 2000). The activity of presumed plant antimicrobials against Gram-positive and Gram-negative organisms was significantly enhanced by synthetic MDR inhibitors of associated efflux proteins. The findings provided a basis that plants can be prospective sources of natural MDR inhibitors that can modulate the performance of antibiotics against resistant strains (Tegos *et al.*, 2002).

Therefore, isolation of these compounds in pure form to test for their antimicrobial effects and possible synergistic effects in combination with standard antibiotics will be of great help in designing new antibiotics to combat the problem of antimicrobial resistance.

1.8 Aim of the Study

The aim of this study is to isolate and characterize some bioactive compounds from *Indigofera pulchra* (Willd) and investigate their possible synergistic antimicrobial effect on ciprofloxacin and fluconazole.

1.9 Objectives of the Study

The objectives of the study are as follows:

1. To isolate and characterize some bioactive compounds from *Indigofera pulchra* (Willd).
2. To conduct antimicrobial activity study on the fractions and isolated compounds using agar well diffusion technique.
3. To investigate the effect of the fractions and the isolated compounds on antimicrobial activity of ciprofloxacin and fluconazole.

1.10 Research Hypothesis

The ethyl alcohol extract of *Indigofera pulchra* (Willd) aerial parts contain bioactive compounds with synergistic antimicrobial potential when combined with ciprofloxacin and fluconazole.

CHAPTER TWO

2.0 Literature Review

2.1 *Indigofera*

Indigofera is a large genus of over 750 species of flowering plants belonging to the family Fabaceae. They are widely distributed throughout the tropical and subtropical regions of the wold. Species of *Indigofera* are mostly shrubs, though some are small trees, annual or perennial herbs, most have pinnate leaves, recemes of flowers grow in the leaf axils and most species have flowers in shades of red, but there are few white and yellow flowered species. The fruit is a legume pod of varying size and shape (Burkill, 1995).

Fabaceae is characterized by leaves simple to compound, unifoliate, trifoliate, sometimes phyllodic, or reduced to a tendril, spirally arranged, with stipules present that are sometimes large and leaf-like or developed into spines.

Flowers are usually regular or irregular (i.e., actinomorphic to zygomorphic in symmetry, respectively), bisexual, with a single superior carpel (hypogynous to perigynous), pentamerous, arranged singly or in racemes, spikes, or heads. Ranging in habit from large trees to annual herbs, the family is cosmopolitan in distribution and well represented throughout temperate and tropical regions of the world (Rundel, 1989).

Taxonomically, Fabaceae has been traditionally divided into three subfamilies, the Caesalpinioideae, Mimosoideae, and Papilionoideae (although sometimes these have been ranked as separate families, as in Caesalpiniaceae, Mimosaceae, and Papilionaceae), and considered most closely related to the Connaraceae and Sapindaceae on the basis of anatomy, morphology, and biogeographic distributions (Polhill and Raven, 1981).

The recognition of three subfamilies is based on characteristics particularly of the flower, including size, symmetry, aestivation of petals, sepals (united or free), stamen number and heteromorphy, pollen (single or polyads), but also presence of a pleurogram, embryo radicle shape, leaf complexity, and presence of root nodules (Lewis *et al.*, 2005).

Differences in these characteristics led to the view that the Mimosoideae and Papilionoideae are unique and distinct lineages in the family which arose independently within a paraphyletic "basal" caesalpinioid assemblage. The *Dimorphandra* group of tribe Caesalpinieae and papilionoid tribe Swartzieae were considered likely transitional groups between them, respectively (Polhill, 1994).

The last formal classification by Polhill (1994), published prior to the advent of family-wide molecular phylogenetic studies, recognized 39 tribes and some 670 genera. The recent update of the tribal and generic classification of the family, having the benefit from more than 10 years of intensive molecular phylogenetic studies, recognizes 36 tribes, 727 genera and 19,327 species (Lewis *et al.*, 2005).

2.2 *Indigofera pulchra* (willd)

Plate I – *Indigofera pulchra* (Willd) in its local habitat

The botanical classification of *I. pulchra* is as follows:

Kingdom: Plantae

Division: Angiosperm

Family: Fabaceae

Subfamily: Papillionoideae

Genus: *Indigofera*

Species: *pulchra*

Botanical name: *Indigofera pulchra*

Local names: *bakin buunuu* (Hausa, Nigeria), *ejaomode* (Yoruba, Nigeria).

2.2.1 Morphology (Description)

Indigofera pulchra Willd (Papilionaceae) is an erect stiff, grey-pubescent softly wooded under-shrub that grows up to 1–1.5 m high. It is widely distributed from Senegal to Nigeria and over Eastern and central Africa from Ethiopia to Angola (Burkill, 1995).

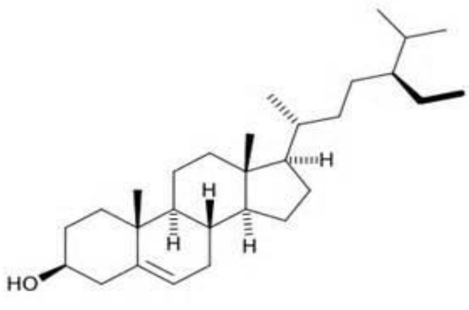
2.2.2 Ethnomedicinal uses of *Indigofera pulchra* (Willd).

In ethnomedicine, the leaves are used to treat infected wound, itching skin and as snake antidote (Burkill, 1995); as prophylactic against snake bite (Sule *et al.*, 2003) and for the treatment of malaria and dysentery (Adamu *et al.*, 2005; Asase *et al.*, 2005).

2.2.3 Phytochemistry of *Indigofera pulchra* (Willd) and other species.

Recent phytochemical investigations reported on *Indigofera pulchra* (Willd) revealed the presence of tannins, saponins, steroids and flavonoids (Musa *et al.*, 2011).

A Beta-sitosterol (XIII), lupeol (XIV), 2’,4’-dihydroxy-4-prenyloxychalcone (XV), and mixture of two dihydrostilbenes; 3-methoxy-4,5-methylendioxy-4’-*O*-(2,3-dihydroxy-3-methylbutyl)-dihydrostilbene (XVI) and 3,5-dimethoxy-4’-*O*-(2,3-dihydroxy-3-methylbutyl)-dihydrostilbene (XVII) were isolated from the methyl alcohol extract of *Indigofera pulchra* (Willd) aerial parts (Musa *et al.*, 2008).



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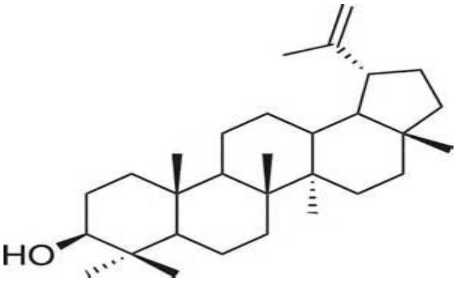

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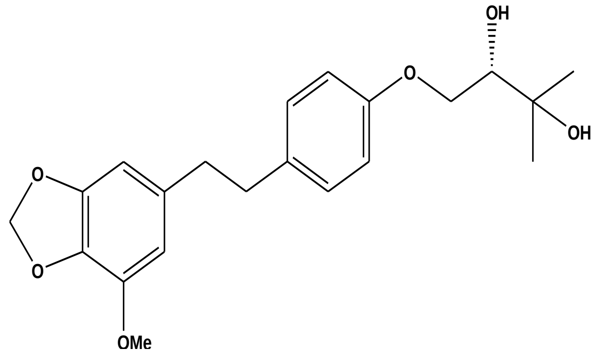

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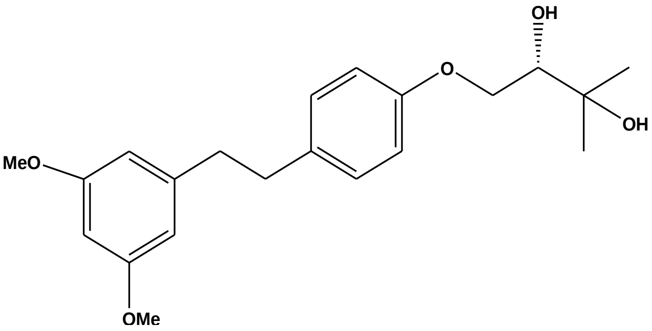
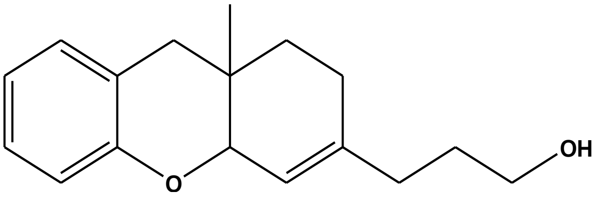


(XVII)

From other species of the genus *Indigofera*, isolation of alkylated xanthenes (XVIII), indigoferic acid (XIX) and a beta-sitosterol (XIII) from the chloroform fraction of the whole plant of *Indigofera oblongifolia* (Sharif *et al.*, 2005).



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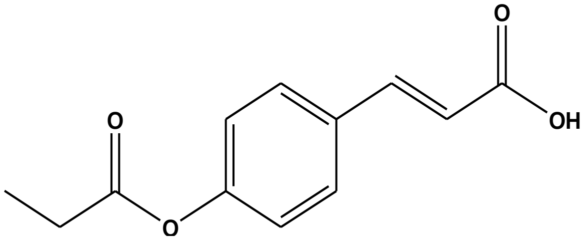
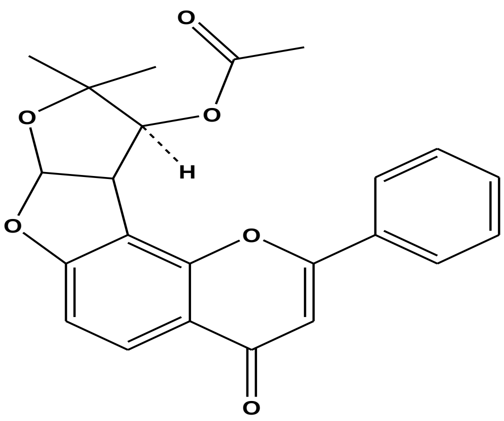


(XIX)

Phytochemical investigation of *Indigofera tinctoria* aerial parts led to the isolation of some flavonoids; pseudosemiglabarin (XX), semiglabarin (XXI) and glabretephrin (XXII) (Rao and Raju, 1984).



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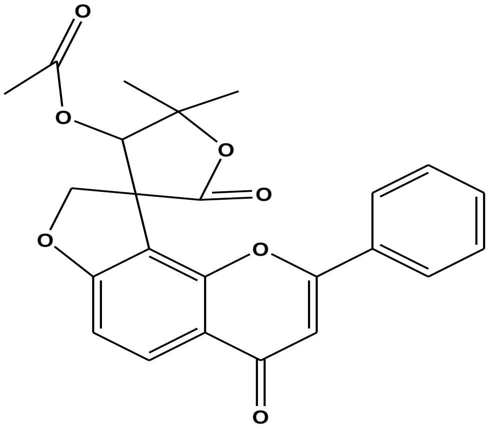

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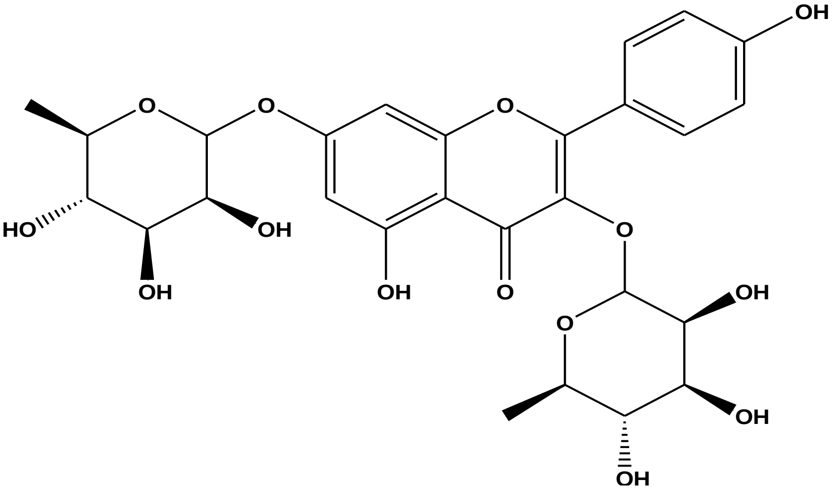
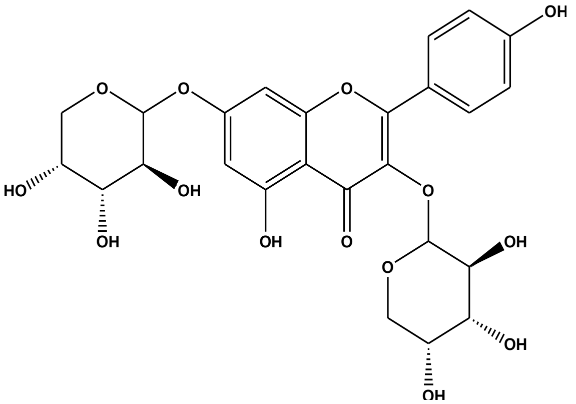


(XXII)

Other reports include Kaemferol-3,7-diarabinoside (XXIII) isolated from the leaves of *Indigofera hebepetala* (Hassan *et al.*, 1994), Kaemferol-3-*O*-α-L-arabinopyranoside-7-*O*-α-L-rhamnopyranoside (XXIV), Kaemferol-3-*O*-α-L-arabinopyranoside-7-*O*-α-L-arabinopyranoside (XXV) and Kaemferol-7-*O*-rhamnoside (XXVI) (Hassan *et al.*, 1996).



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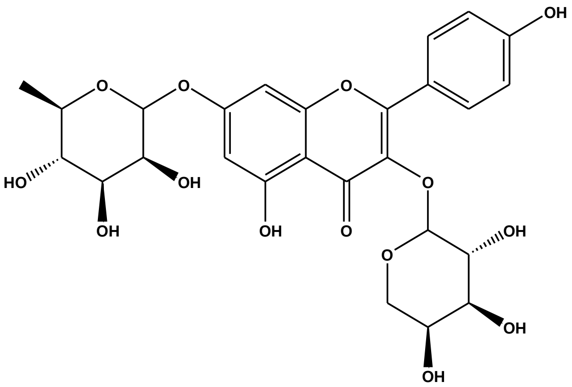

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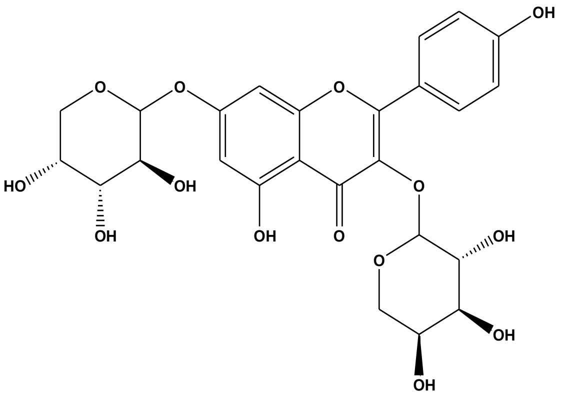

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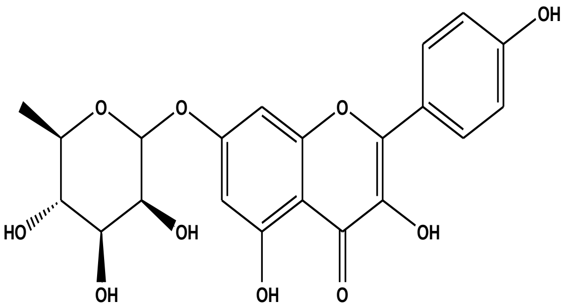


(XXVI)

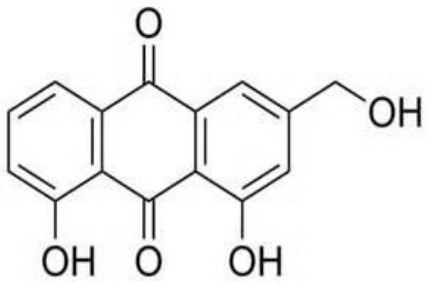
2.2.4 Biological Activities of *Indigofera pulchra* (Willd)

Recent pharmacological studies of *Indigofera pulchra* (Willd) reported the antibacterial activity the methanol leaves extract of *Indigofera pulchra* (Musa *et al.,* 2008). Other pharmacological studies reported include: the analgesic and anti-inflammatory activities of the methanol leaves extract of *Indigofera pulchra* (Willd) (Musa *et al.,* 2010) and anti-*Plasmodium berghei* activities of the methanol leaves extract and its n-butanol soluble fraction (Ibrahim *et al.,* 2011).

2.3 Some plant-derived antimicrobial phytochemicals

Plants have an almost infinite ability to produce aromatic substances, most of which are phenols or their oxygen-substituted derivatives (Kambizi and Afolayan, 2001). Most are secondary metabolites, of which at least 12,000 have been isolated, a number projected to be less than 10% of the total. In many cases, these substances serve as plant defense mechanisms against predation by microorganisms, insects, and herbivores (Van Wyk *et al.,* 1997).

Kambizi and Afolayan (2001) isolated three compounds from Aloe ferox, a plant traditionally used for the treatment of sexually transmitted infections. These compounds include; 1,8 – dihydroxy–3–hydroxymethyl-9,10-anthracenedione (aloe - emodin) (XXVII); 1,8–dihydroxy–3–methyl-9,10-anthracenedione (XXVIII), and 10–C–b–D–glucopyranosyl-1,8–dihydroxy–3–hydroxymethyl-9-anthracenone (aloin A) (XXIX), all the three compounds exhibited antibacterial activities against *Bacillus subtilis, Staphylococcus epidermidis, Shigella sonnei and Escherichia coli.*



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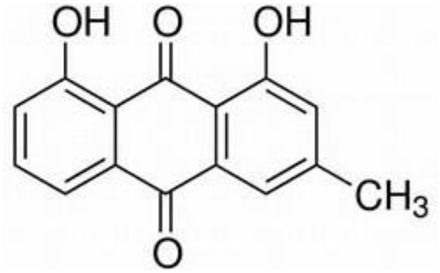

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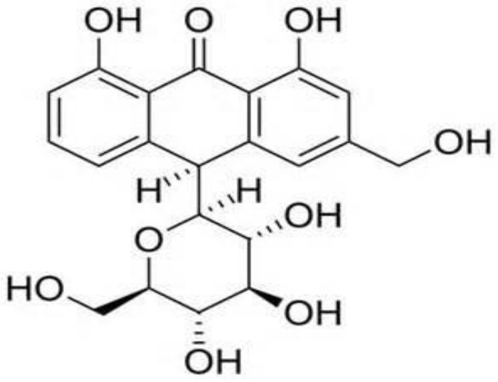
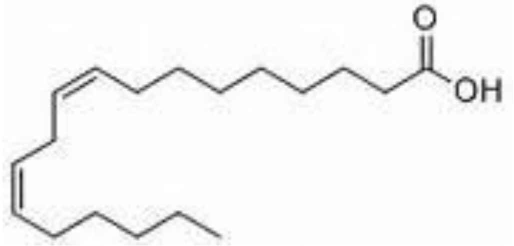


(XXIX)

(Dilika *et al*,. 2000) also reported the antibacterial activities of linoleic (XXX) and oleic (XXXI) acids isolated from the leaf of *Helichrysum pedunculatum*, a plant used to treat wound acquired during male circumcision rites in the Eastern Cape of South Africa, and this study has been corroborated (Aiyegoro *et al.*, 2009). Two lipophilic phytoalexins: α-amyrin (XXXII) and β-amyrin (XXXIII) that have anti-tuberculosis and generally antibacterial activities have also been isolated from *Helichrysum kraussii* (Prinsloo and Meyer, 2006).



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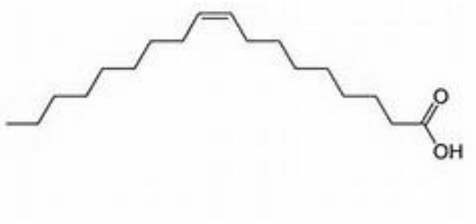

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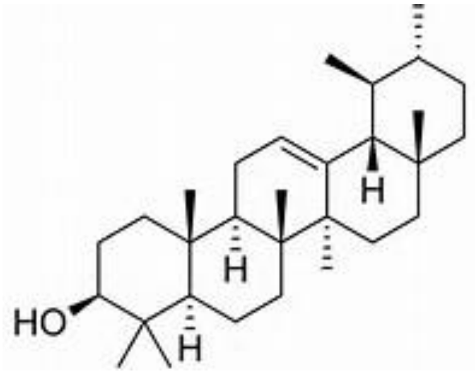

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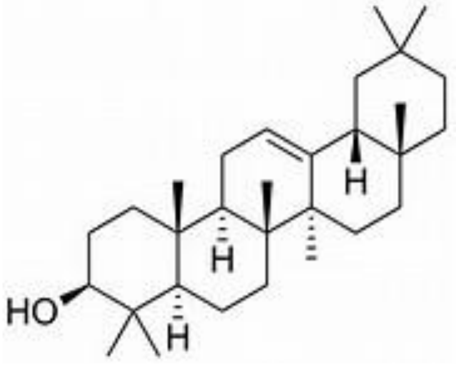


(XXXIII)

Many plant extracts clearly demonstrate antibacterial properties, although the mechanistic processes are poorly understood. Cowan, (1999) describe the mechanism of action for various classes of active components from medicinal plants.

2.4 Antimicrobial synergism and plant products

Many plant phytochemicals have been evaluated potent pharmacological activities which include antimicrobial activities (Cowan, 1999). In many parts of the world, the extracts of medicinal plants are used for their antibacterial, antifungal and antiviral properties (Hassawi and Kharma, 2006). Other studies have also shown improved efficacies of certain natural products when they are combined with clinically useful antibiotics (Lee *et al.,* 2012).

Plants antimicrobials have been found to be synergistic enhancers in that though they may not have any antimicrobial properties alone, but when they are taken concurrently with standard drugs they enhance the effect of that drug (Kamatou *et al.*, 2006).

The synergistic effect from the association of antibiotic and plant extracts against resistant bacteria leads to new choices for the treatment of infectious diseases. This effect enables the use of the respective antibiotic when it is no longer effective by itself during therapeutic treatment (Nascimento *et al.*, 2000).

The application of synergistic principle is evident in commercial preparations for the treatment of various infections (e.g. the antibiotic augmentin). Traditional healers often use combinations of plants to treat or cure diseases (Kamatou *et al.*, 2006). One notable example from the ethnobotanical literature is the concomitant administration of various Salvia species with *Leonotis leonurus* to treat various infections (Masika and Afolayan, 2003).

Kamatou *et al.*, (2006), confirmed the existence of synergism between Salvia *chamela eagnea* and *Leonotis leonurus*, when these two plants extracts were combined together and tested against *Bacillus cereus, Staphylococcus aureus, Escherichia coli* and *Klebsiella pneumoniae*. They also reported synergism when the tincture of *L. leonurus* and various Salvia species were combined together against influenza.

Boik (2001) conducted a large number of combination studies using various natural substances and their results strongly suggested that when used in combination, natural substances can produce synergistic effects. It is thought that phenolic compounds such as flavonoids may increase the biological activity of other compounds by synergistism or other mechanisms.

Experimental evidence of synergistic actions between plants was also shown in a clinical study on the formulation of Chinese herbs used to treat eczema (Williamson, 2001).

2.5 Combinations of bioactive plant products and different classes of antibiotics with specific mechanism of action

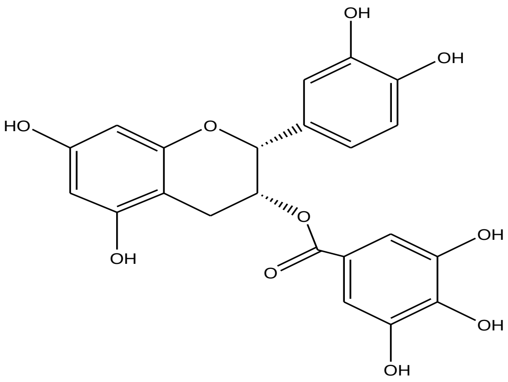
In the treatment of drug resistant infections, combination of antibiotics have often been used as they take advantage of different mechanisms of action. The use of antimicrobial agents displaying synergy is one of the well established justifications for antimicrobial combination therapy (Rybak and McGrath, 1996).

Combinations of antimicrobials that demonstrate an *In-vitro* synergism against infecting strains are more likely to result in successful therapeutic result. Thus, evidence of *In-vitro* synergism could be useful in selecting most favorable combinations of antimicrobials for the practical therapy of serious bacterial infections (Hooton *et al.*, 1984).

The screening of crude plant extracts for synergistic interaction with antibiotics is expected to provide ways for the isolation of MDR inhibitors. The ability of crude extracts of plants to potentiate the activity of antibiotics has been observed by some researchers (Aiyegoro *et al.*, 2009; Darwish *et al*., 2002; Ahmad and Aqil, 2006) and it is anticipated to form the basis for the bioassay directed fractionation of potential resistance modulators from plants.

Darwish *et al.*, (2002) carried out a study on some Jordanian plants and demonstrated that the efficacy of the antibiotics; gentamycin and chloramphenicol against *S. aureus* was reportedly improved by the use of plant materials. Ahmad and Aqil (2006), also reported that crude extracts of Indian medicinal plants demonstrated synergistic interaction with tetracycline and ciprofloxacin against extended spectrum β-lactamase (ESβL)-producing multidrug-resistant enteric bacteria. Betoni *et al.,* (2006) also observed synergistic interactions between extracts of Brazilian medicinal plants and eight antibiotics on *S. aureus*. The use of *Catha edulis* extracts at sub-inhibitory levels, has been reported to reduce the minimum inhibitory concentration (MIC) values of tetracycline, and penicillin G against resistant oral pathogens, *Streptococcus oralis, Streptococcus sanguis* and *Fusobacterium nucleatum* (Al-hebshi *et al.*, 2006).

A number of compounds with an *in-vitro* activity of reducing the MICs of antibiotics against resistant organisms have also been isolated from plants. Polyphenols - epicatechin gallate (XXXIV) and catechin gallate (XXXV) have been reported to reverse beta-lactam resistance in Methicillin Resistant *Staphylococcus aureus* (MRSA) (Stapleton *et al.,* 2004). Diterpenes, triterpenes, alkyl gallates, flavones and pyridines have also been reported to have resistance modulating abilities on various antibiotics against resistant strains of *S. aureus* (Smith *et al.*, 2007).



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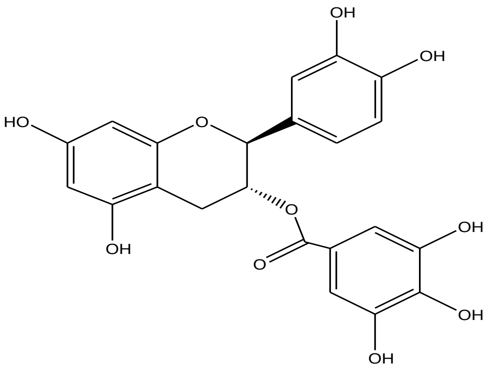


(XXXV)

Plants have been known to contain myriads of antimicrobial compounds such as polyphenols and flavonoids. The antimicrobial and resistance modifying potentials of naturally occurring flavonoids and polyphenolic compounds have been reported in other studies (Cushnie and Lamb, 2005; Sato *et al.*, 2004).

Some of these compounds including polyphenols have been shown to exercise their antibacterial actions/activities through membrane perturbations. This disruption of the cell membrane coupled with the action of beta-lactams on the transpeptidation of the cell membrane could lead to an enhanced antimicrobial effect of the combination (Esimone *et al.,* 2006). It has also been revealed that some plant-derived compounds can improve the *In-vitro* activities of some peptidoglycan inhibiting antibiotics by directly attacking the same site (i.e. peptidoglycan) in the cell wall (Zhao *et al.*, 2001).

While the above explanations may account for the synergy between the extracts and beta-lactam antibiotics that act on the cell wall, it might not apply in the case of the observed synergy with other classes of antibiotics with different targets such as tetracyclines, erythromycin, ciprofloxacin and chloramphenicol. Bacterial efflux pumps are responsible for a considerable level of resistance to antibiotics in pathogenic bacteria (Kumar and Schweizer, 2005). Some plant derived compounds have been observed to augment the activity of antimicrobial compounds by inhibiting MDR efflux systems in bacteria. Such compounds are likely to be broad spectrum efflux inhibitors considering that the synergistic effect of the extract was observed on both Gram-positive and Gram-negative organisms as well as in combination with cell wall inhibiting and protein synthesis inhibiting antibiotics (Tegos *et al.,* 2002)..

Smith *et al*., (2007) reported one efflux inhibitor (ferruginol) from the cones of *Chamaecyparis lawsoniana*, which inhibited the activity of the quinolone resistance pump (NorA), the tetracycline resistance pump (TetK) and the erythromycin resistance pump (MsrA) in *S. aureus.*

CHAPTER THREE

3.0 Materials and Methods

3.1 Materials

All organic solvents used were of general purpose grade (Sigma Chemical Co. St. Louis, U.S.A) and distilled before used. All equipments and capacity are quoted accordingly and operated to standard procedures. The silica gel for column chromatography is of mesh size (60 – 120) and that of thin layer chromatography is 0.25mm thickness. Solvents (ethyl alcohol, n-hexane, chloroform, ethylacetate & n-butanol), TLC plates (Silica gel coated), Column (100 X 4cm), Burette (50cm^3), Foil paper, Distilled water, Cottonwool, Syringes (2ml, 5ml, 10ml & 20ml), Capillary tube, Mueller-Hinton agar medium, Mueller-Hinton broth, DMSO (dimethylsulfoxide), 37°C ambient-air incubator, Incandescent light, Vortex mixer, Weighing balance, Sterile cotton-tipped swabs, Biohazard waste bag, 0.5 McFarland turbidity standard, 13 × 100 mm test tubes with snap caps, Test tube racks, Millimeter ruler, Sterile transfer pipette, Forceps and Inoculating loop or needle.

3.2 Methods

3.2.1 Collection, identification and preparation of plant material

The plant *Indigofera pulchra* (Willd) was collected in the month of September, 2014 from Area BZ, Ahmadu Bello University, Zaria, Samaru Campus. The plant was authenticated by taxonomist Namadi Sanusi at Herbarium Unit, Department of Biological Sciences, Ahmadu Bello University Zaria by comparing with an existing specimen voucher no. 410. The fresh aerial parts of the plant material was carefully cut, air-dried at room temperature, made into powder using mortar and pestle and subsequently referred to as powdered plant material of *Indigofera pulchra* (Willd).

3.2.2 Extraction of plant material

The powdered plant material of *Indigofera pulchra* (Willd) weighed 1.5 Kg was extracted with 7.5 liters of ethyl alcohol using cold maceration method for ten days. Solvent used was removed at reduced pressure and a dark green gummy material (89.25g) was obtained and referred to as the ethyl alcohol extract of *Indigofera pulchra* (Willd). The obtained extract was treated successively with hexane, chloroform, ethylacetate and n-butanol respectively to afford hexane (10.26g), chloroform (25.5g), ethylacetate (7.45g) and n-butanol (4.67g) fractions.

3.3 Preliminary Phytochemical Screening

The ethyl alcohol crude extract, n-hexane, chloroform, ehylacetate, n-butanol fractions labelled as ET, H, C, E and B respectively of the crude ethyl alcohol extract of *I. pulchra* (Willd) were subjected to phytochemical screening using standard procedures (Prashant *et al.*, 2011).

3.3.1 Test for steroids and triterpenes

(a) Salkowski's Test: the extract and the fractions were treated with chloroform and filtered. The filtrates were treated with few drops of conc. sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenes.

(b) Libermann Burchard's test: 1ml of anhydrous acetic acid was added to 1ml of chloroform and cooled to 0˚C, then one drop of concentrated sulphuric acid was added to the cooled mixture followed by the extract/fractions. The solution was observed for blue, green, red or orange colour that changes with time.

3.3.2 Extraction of alkaloids

20g of the powdered material was macerated in 100ml of ethanol and was shaken gently at interval and left over night. The extract was filtered and the residue was placed on a water bath and evaporated to dryness. 20ml of 55% HCl was added to the crude extract and filtered. The filtrate was alkalinified with ammonia and placed in a separating funnel. 3ml of chloroform was added into the separating funnel, mixed and shaken for about five minutes and allowed to separate into two layers. The lower layer of chloform contained the alkaloids and the upper layer the aqueous portion. The upper layer was extracted until the last chloroform extract was collected and concentrated on a water bath. The concentrated extract was labelled and kept for preliminary test for alkaloids (Trease and Evans, 1989).

3.3.2.1 Test for alkaloids

Detection of alkaloids: the extract and the fractions were dissolved individually in dilute Hydrochloric acid and filtered. The filtrate was divided into four portions.

(a) Mayer's Test: first portion was treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow coloured precipitate indicates the presence of alkaloids.

(b) Wagner's Test: second portion was treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.

(c) Dragendroff's Test: third portion was treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Formation of red precipitate indicates the presence of alkaloids.

(d) Hager's Test: fourth portion was treated with Hager's reagent (saturated picric acid solution). Formation of yellow colored precipitate confirmed the presence of alkaloids.

3.3.3 Test for tannins

(a) Gelatin Test: To the extract and the fractions, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

(b) A small quantity of the extract and the fractions extract was boiled with water and filtered. Two drops of ferric chloride was added to the filtrate, formation of a blue-black, or green precipitate was an indication for the presence of tannins.

3.3.4 Test for carbohydrate

The extract and the fractions were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

(a) Molisch's Test: Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube. Formation of violet ring at the junction indicates the presence of carbohydrates.

(b) Benedict's Test: Filtrates were treated with Benedict's reagent and heated gently. Orange red precipitate indicates the presence of reducing sugars.

3.3.5 Test for flavonoids

(a) Alkaline Reagent Test: the extract and the fractions were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

(b) Shinoda Test: About 0.5g of the extract and the fractions was dissolved in ethanol, warmed and filtered. To the filtrate, three pieces of magnesium chips were added followed by few drops of concentrated hydrochloric acid. Appearance of an orange, pink or red to purple colour indicates the presence of flavonoids.

3.3.6 Test for saponins

(a) Froth Test: the extract and the fractions were diluted with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

3.4 Thin Layer Chromatography

Thin layer chromatography was carried out on silica gel Pf_{254} coated aluminum sheet with layer thickness of 0.25mm.

Technique: One dimensional TLC technique was employed

Spotting and development: The spots were applied manually using capillary tubes and the plates developed at room temperature using a shandom chromatotank.

Mobile phase solvent systems: n-hexane : ethylacetate 2:1, 3:1 and 4:1

Detection: Spots on TLC plates were visualized under UV light (254 and 366 nm) and spraying with 10% sulphuric acid or P-anisaldehyde, followed by heating at $110^{O}C$ for 2-3mins.

3.5 Column Chromatography

The following column conditions were employed in running the column chromatography.

(a) Technique - Gradient elution

(b) Column - Glass column (100×4cm) with sintered disc at the bottom of various dimensions

(c) Stationary phase - Silica gel, 60 – 120 mesh size

(d) Column packing - Wet slurry method.

(e) Sample loading - The sample was applied using dry load method (Cannell, 1998), the sample was dissolved in small amount of suitable organic solvent, it was then mixed with a small quantity of silica gel, dried, triturated and then loaded on top of the column.

(f) Solvents for elution – n-hexane, chloroform and ethylacetate were used singly and in combination with varying polarity.

3.5.1 Column chromatographic separation of chloroform fraction (C).

Chloroform fraction (25g) was chromatographed over silica gel (60 – 120 mesh size) packed column of dimension (100 x 4cm), the column was eluted with 100% n-hexane then followed by hexane:ethylacetate in the ratio of 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40 to 30:70. Ninety three fractions (100ml each) were collected and were pooled together based on their TLC profiles to give fifteen major sub-fractions (A1-A15). Sub-fraction A6 using hexane : ethylacetate (2:1) as TLC mobile phase solvent system showed one major spot with R_f value 0.59 and two minor spots and was subjected to purification using preparative thin layer chromatography (PTLC) to afford a pure yellow crystalline solid compound coded L1.

Sub-fraction A11 (1.06g) was packed for repeated silica gel column chromatography using hexane : chloroform of varying polarity (5%) to chloroform 100%. Then with chloroform : ethylacetate mixture in ratio order of 99.5:0.5, 99:1, 98.5:1.5, 98:2, 97.5:2.5, 97:3, 96.5:3.5, 96:4 and 91:9 where one hundred and twenty two sub-fractions were collected. Using hexane : ethylacetate (2:1) as TLC mobile phase solvent system, two major spots with R_f values 0.33 and 0.31 were observed on the TLC profile with one minor spot from pooled sub-fractions C17 and

C18; purified using preparatory thin layer chromatography (PTLC) to afford a white amorphous solid compound coded L2.

3.6 Preparative Thin-layer Chromatography (PTLC)

PTLC was carried out using Fluka silica gel precoated glass plates 20 x 20cm with layer thickness of 0.25mm. A thin line about 1.5cm from the bottom of the plate was drawn with a pencil. The sample to be separated was dissolved in minimum amount of ethylacetate to give an approximate concentration of 20mg/ml. It was then applied uniformly along the thin line using capillary tube. The plate was allowed to dry after which it was developed using n-hexane: ethylacetate (2:1) as elution solvent system. The developed plate was air dried in a fume cupboard and the position of the band of interest found to be visible naked eyes was marked with pencil and scraped off the backing of the plate on to a foil. The scrapped sorbent was transfered into a sintered glass funnel and washed repeatedly with ethylacetate and the solution obtained was evaporated to give the compounds.

3.7 Melting Point (M.P.) Determination

The melting points of the isolated compounds were determined using Gallenkamp melting point apparatus obtained from Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, A.B.U., Zaria.

3.8 Spectral Analysis

Infrared (IR) absorption spectra were recorded using an infrared spectrophotometer Shimadzu 8400S.

NMR spectra were obtained on a Bruker AVANCE (400MHz for ^{1}H and ^{13}C) spectrometer from Rhodes University, Johannesburg, South Africa. Dimethylsulfoxide (DMSO) was used as solvent. Chemical shift values (δ) were recorded in parts per million (ppm) relative to appropriate internal solvent standard.

3.9 Antimicrobial Screening

The antimicrobial activities of Hexane (H), Chloroform (C), Ethylacetate (E) and Butanol (B) fractions; L_1, L_2 and their effects on the antimicrobial activity of ciprofloxacin and fluconazole were determined using some pathogenic microbes (Irobi *et al*., 1996).

The test organisms used for the study are clinical isolates which include two gram positive bacteria (Methicilin Resistant *Staphylococcus aureus* and *Staphylococcus aureus*), two gram negative bacteria (*Proteus mirabilis* and *Escherichia coli*) and two yeasts (*Candida albicans* and *Candida tropicalis*) obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital Zaria.

Agar Well-Diffusion method was used for the screening of the fractions, isolated compounds and the standard antibiotics. Mueller-Hinton agar was used as the growth medium for the test microbes and was prepared according to the manufacturer’s instruction, sterilized at 121ºC for 15 mins, poured into sterile petridish and allowed to cool and solidify.

The sterilized medium was seeded with 0.1ml inoculum of the test microbe, spread evenly over the surface of the medium using a sterile cotton swab and a 6mm well was cut at the center of each inoculated medium using cork borer of 6mm in diameters.

10mg each of the fractions was weighed and dissolved separately in 10ml of DMSO to obtain a concentration of 1mg/ml, 1mg each of the isolated compounds (L_1 and L_2) was weighed and dissolved in 10ml of DMSO to obtain a concentration of 100µg/ml, 1mg each of the drugs was weighed and dissolved separately in 10ml each of DMSO to obtain a concentration of 100µg/ml.

0.2ml solution of the fractions, isolated compounds, ciprofloxacin and fluconazole were separately introduced into the wells of the inoculated media, incubated and the zones of inhibition were observed.

Solutions of the fractions (0.1ml) combined with the standard antibiotics (0.1ml) were introduced into the wells of the inoculated media and that of the isolated compounds (0.1ml) also separately combined with the standard antibiotics (0.1ml) in different inoculated media were incubated and the effects of the fractions and the isolated compounds on the zones of inhibition of the standard antibiotics were observed respectively.

The incubation of the inoculated media was made at 37ºC for 24 hours (bacteria) and 48 hours (fungi) after which each plate of the medium was observed for the zone of inhibition of microbial growth, the zones were measured with a transparent ruler and the results were recorded in millimeters (Irobi *et al*., 1996).

CHAPTER FOUR

4.0 RESULTS

4.1 Percentage Yield of the Extract and Fractions

Extraction (Maceration) of 1500g of *Indigofera pulchra* (Willd) aerial parts in 7.5 litres of ethanol for two weeks yielded 89.25g (5.9%) of crude extract (ethyl alcohol extract) which was further fractionated using n-hexane, chloroform, ethylacetate and n-butanol yielding 10.26g (0.68%), 25.50g (1.7%), 7.5g (0.50%) and 4.67g (0.31%) respectively (Table 4.1).

Table 4.1: Percentage yield of the extract and the fractions from *I. pulchra* (Willd) from 1500g of the prepared plant material.

Extract/Fractions	Yield	% Yield(w/w)
Ethyl alcohol extract	89.25g	5.95%
H	10.26g	0.68%
C	25.50g	1.70%
E	7.45g	0.50%
B	4.67g	0.31%

Keys: H= n-Hexane, C= Chloroform, E= Ethylacetate and B= n-Butanol

4.2 Phytochemical investigations of *Indigofera pulchra* (Willd) extract and fractions

The phytochemical screening of chloroform fraction from *Indigofera pulchra* (Willd) extract revealed the presences of flavonoids, tannins, steroids and carbohydrates while alkaloids and saponins are absent (Table 4.2).

Table 4.2: Preliminary phytochemical screening of *Indigofera pulchra* (Willd) extract and fractions.

Phytochemical Constituents	Test	Extract/Fractions				
		Ethanol Extract	Hexane Fraction	Chloroform Fraction	Ethylacetate Fraction	Butanol Fraction
Alkaloids	Drsgendroff's Test	-	-	-	-	-
	Wagner's Test	-	-	-	-	-
	Mayer's Test	-	-	-	-	-
Carbohydrates	Molisch's Test	+	-	+	+	+
Flavonoids	Shinoda Test	+	+	+	+	+
	Sodium Hydroxide Test	+	+	+	+	+
Saponins	Frothing Test	+	-	-	-	+
Steroids	Salkowski Test	+	+	-	-	-
	Liberman-Burchard Test	+	+	-	-	-
Tannins	Ferric Chloride Test	+	-	-	+	+
	Lead Acetate Test	+	-	-	+	+

Key: - + = Present - = Absent

Table 4.3: Column chromatographic separation of the chloroform fraction of *Indigofera pulchra* (Willd).

Compound L1 was isolated from sub-fraction A6 of the chloroform fraction, it was found to be a yellow chrystalline solid powder and completely soluble in ehtylacetate with a melting point range of 137^{O}C – 141^{O}C. Sub-fraction A11 was thereafter re-chromatographed for the isolation of compound L2.

Eluent	**Sub-fraction**	**Eluenting Solvent**	**Spot**
1–4	A1	Hex 100%	-
5–7	A2	Hex : EtOAc 95:5	3
8–10	A3	Hex : EtOAc 90:10	3
11–14	A4	Hex : EtOAc 85:15	4
15-16	A5	Hex : EtOAc 80:20	5
17-21	**A6**	**Hex : EtOAc 75:25**	**3**
22-29	A7	Hex : EtOAc 70:30	4
30-38	A8	Hex : EtOAc 65:35	3
39-50	A9	Hex : EtOAc 60:40	5
51-56	A10	Hex : EtOAc 55:45	4
57-62	**A11**	**Hex : EtOAc 50:50**	**4**
63-73	A12	Hex : EtOAc 45:55	3
74-80	A13	Hex : EtOAc 40:60	2
81-86	A14	Hex : EtOAc 35:65	2
87-93	A15	Hex : EtOAc 30:70	0

Note: Hex = Hexane, EtOAc = Ethylacetate

Table 4.4: Repeated column chromatographic separation of column fraction A11.

Compound L2 was isolated from sub-fraction A11 of the chloroform fraction, it was found to be a white chrystalline solid powder and completely soluble in ehtylacetate with a melting point range of 144^{O}C – 147^{O}C.

Eluent	Sub-fraction	Eluenting Solvent	Spot
1–124	B1-B103	Hex 100% - 100% $CHCl_3$	-
1-3	C1	$CHCl_3$: EtOAc 99.5:0.5	-
4-6	C2	$CHCl_3$: EtOAc 99:1	2
7-9	C3	$CHCl_3$: EtOAc 98.5:1.5	1
10-13	C4	$CHCl_3$: EtOAc 98:2	3
14-17	C5	$CHCl_3$: EtOAc 97.5:2.5	2
18-19	C6	$CHCl_3$: EtOAc 97:3	2
20-21	C7	$CHCl_3$: EtOAc 96.5:3.5	2
22-25	C8	$CHCl_3$: EtOAc 96:4	1
26-28	C9	$CHCl_3$: EtOAc 95.5:4.5	1
29-32	C10	$CHCl_3$: EtOAc 95:5	2
33-36	C11	$CHCl_3$: EtOAc 94.5:5.5	2
37-40	C12	$CHCl_3$: EtOAc 94:6	3
41-43	C13	$CHCl_3$: EtOAc 93.5:6.5	3
44-47	C14	$CHCl_3$: EtOAc 93:7	2
48-49	C15	$CHCl_3$: EtOAc 92.5:7.5	4
50-53	C16	$CHCl_3$: EtOAc 92:8	4
54-55	**C17**	**$CHCl_3$: EtOAc 91.5:8.5**	**3**
56-57	**C18**	**$CHCl_3$: EtOAc 91:9**	**3**
58-63	C19	$CHCl_3$: EtOAc 90.5:9.5	2

Note: Hex = n-Hexane, EtOAc = Ethylacetate, $CHCl_3$ = Chloroform

4.3 Thin-layer chromatography (TLC) of the isolated compounds

The one dimensional thin layer chromatography of compound L1 showed a yellow colored single spot with R_f value 0.59 while, that of compound L2 showed a red colored single spot with R_f value 0.33 using hexane:ethylacetate in the ratio of 2:1 as the solvent system (mobile phase) and 10% sulphuric acid as spraying agent.

Plate II- TLC of compound L1

Solvent system: Hexane:ethylacetate (2:1)

Spraying Agent: 10% sulphuric acid

Plate III- TLC of compound L2

Solvent system: Hexane:ethylacetate (2:1)

Spraying Agent: 10% sulphuric acid

4.4 Solubility of Compounds L1 and L2

Both the two compounds L1 and L2 are soluble in ethylacetate while insoluble in hexane, chloroform, butanol and methanol.

4.5 Melting Point of Compounds L1 and L2

Compounds L1 and L2 were found to have a melting point range of 137 – 141^{O}C and 144-147^{O}C respectively.

4.6 Infrared Spectral Analysis

The IR spectrum of compound L1 shows absorptions around 3146.83cm^{-1} for aromatic protons stretching, 1737.79cm^{-1} indicating presence of Ketonic C=O stretch, 1200cm^{-1} and 1214cm^{-1} for aromatic hydroxyl groups and at 1143cm^{-1} signifying presence of an ether linkage (Figure 4.1

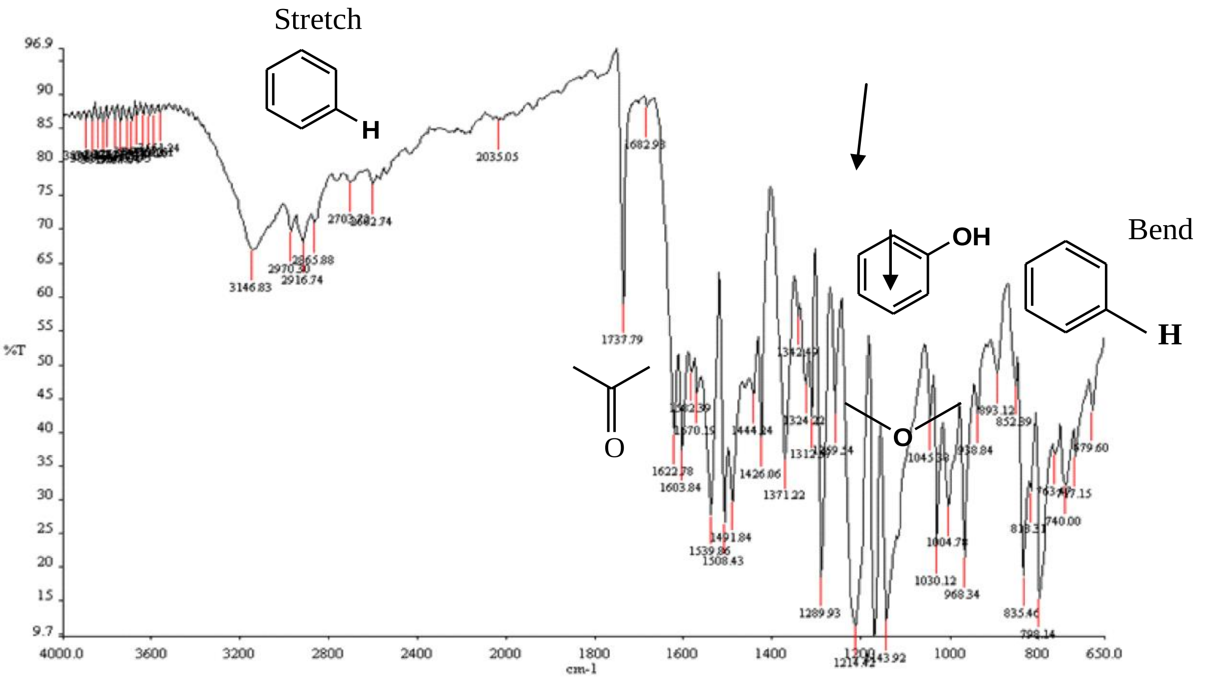


Figure 4.1- IR Spectrum of compound L1

The ^{1}HNMR spectrum of compound L1 shows resonance at δ_H 6.21, δ_H 6.32, δ_H 6.35, δ_H 6.92, δ_H 7.75 and δ_H 8.08,. It also revealed the presence of hydrogen resonances at δ_H 7.78, δ_H 6.95 and at δ_H 4.53, δ_H 5.39, δ_H 1.64, and δ_H 1.9 (Figure 4.2).

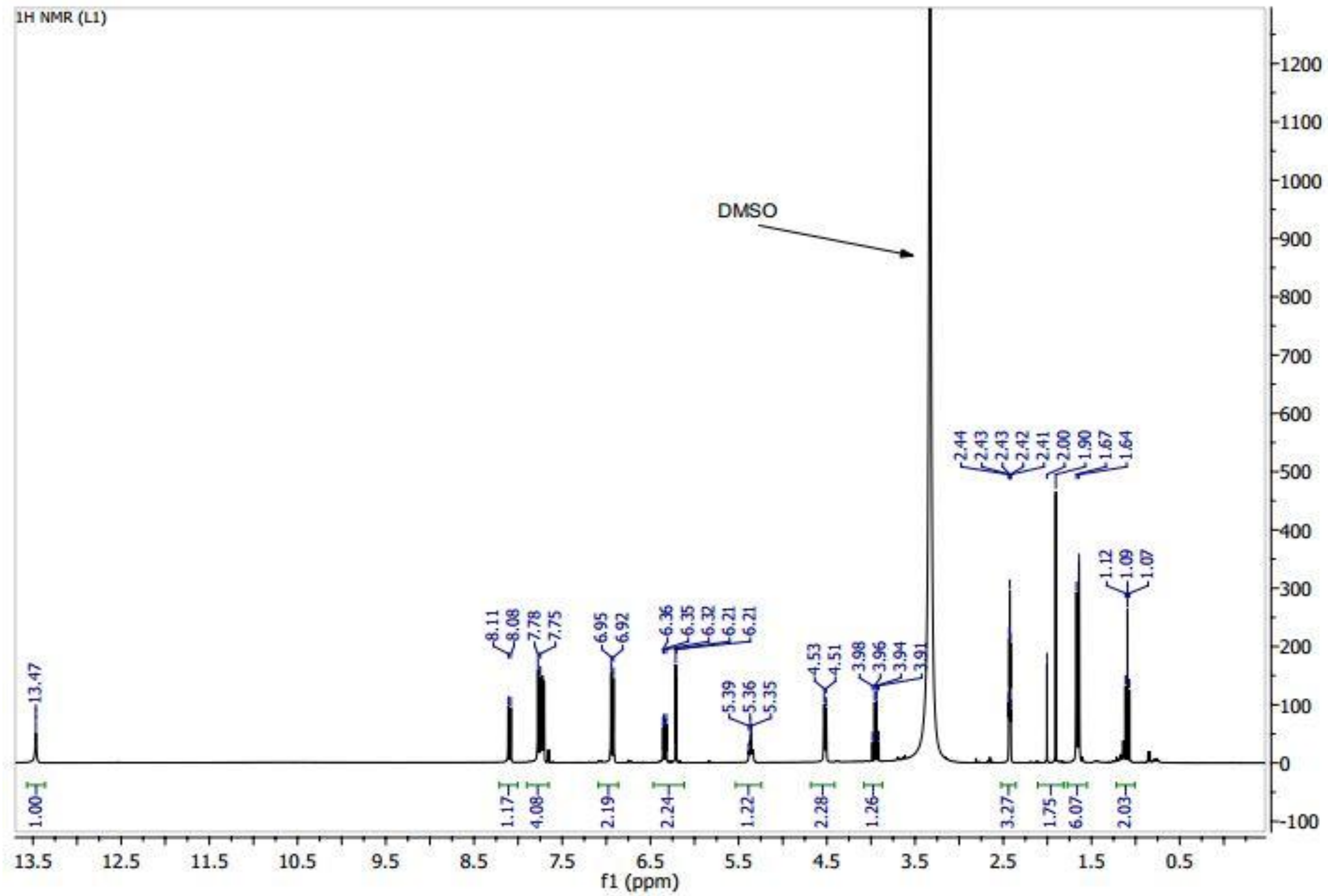


Figure 4.2- ^{1}H-NMR spectrum of compound L1

^{13}C-NMR spectrum of compound L1 revealed a total of 18 carbon resonances of which 10 correspond to carbon atoms in the aromatic region (C2 and C6 resonating within same chemical environment as C3 and C5), 1 for the carbonyl carbon and 7 for aliphatic carbon resonance (Figure 4.3).

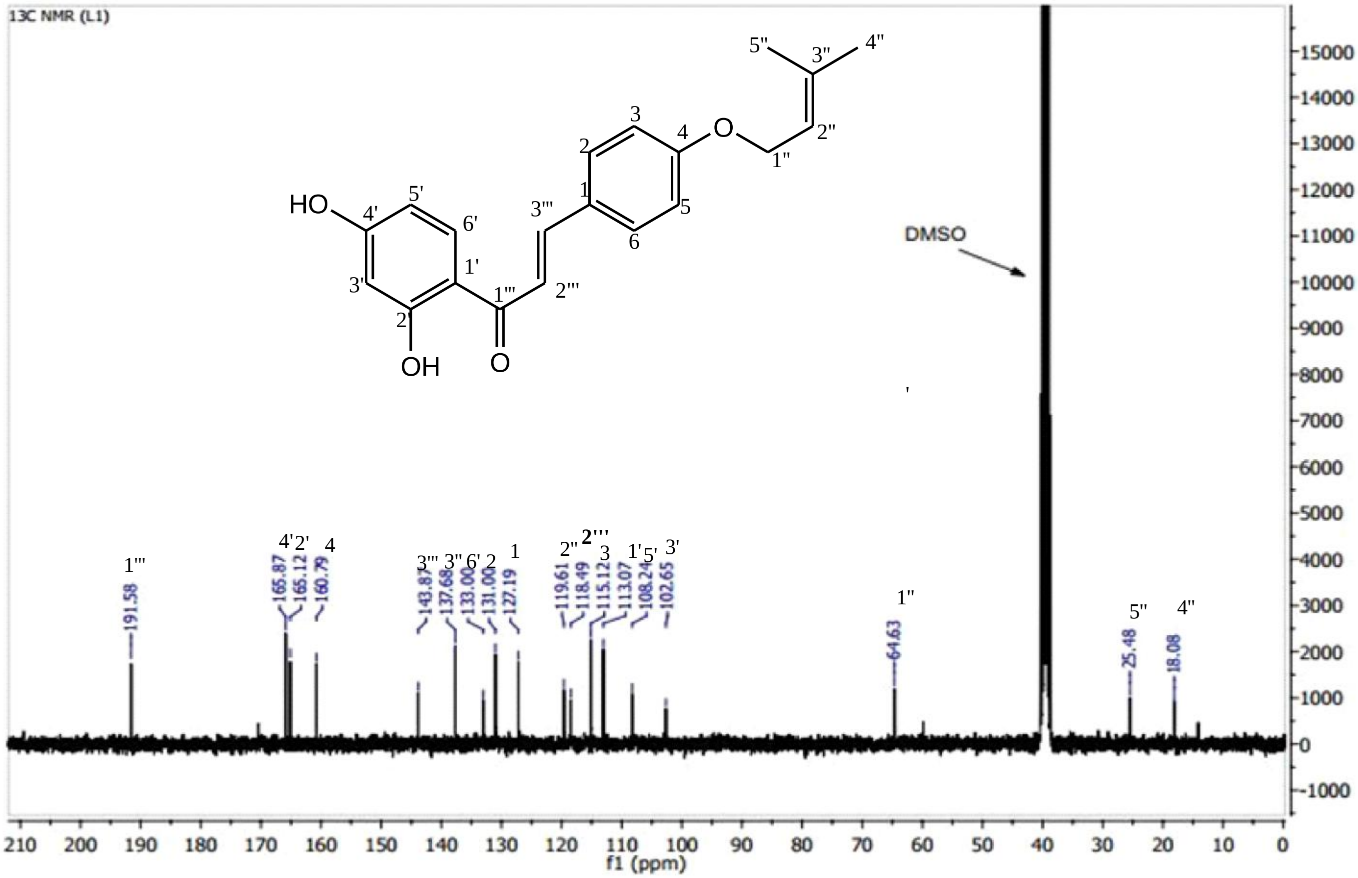


Figure 4.3- ^{13}C-NMR spectrum of compound L1

The ^{13}C - DEPT-NMR spectrum of compound L1 revealed the presence of one methylene only at δ64.63ppm, two methyl at δ18.08 and δ25.48ppm and eight methine resonances (Figure 4.4).

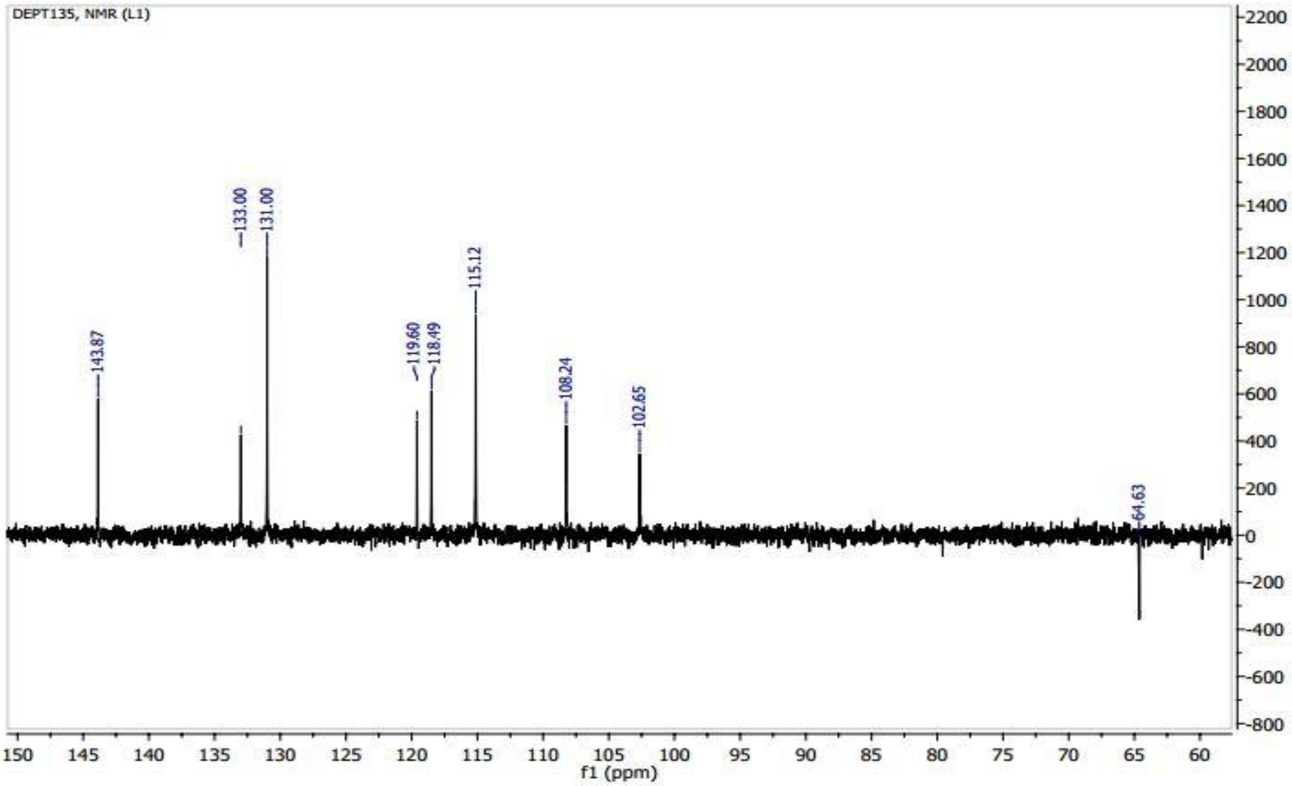


Figure 4.4- ^{13}C - DEPT-NMR spectrum of compound L1

The IR spectrum of compound L2 shows absorptions at 3389.02cm^{-1}, 3568.50cm^{-1}, 1148.44cm^{-1} and at around 2838cm^{-1} – 2993.32cm^{-1} (Figure 4.5).

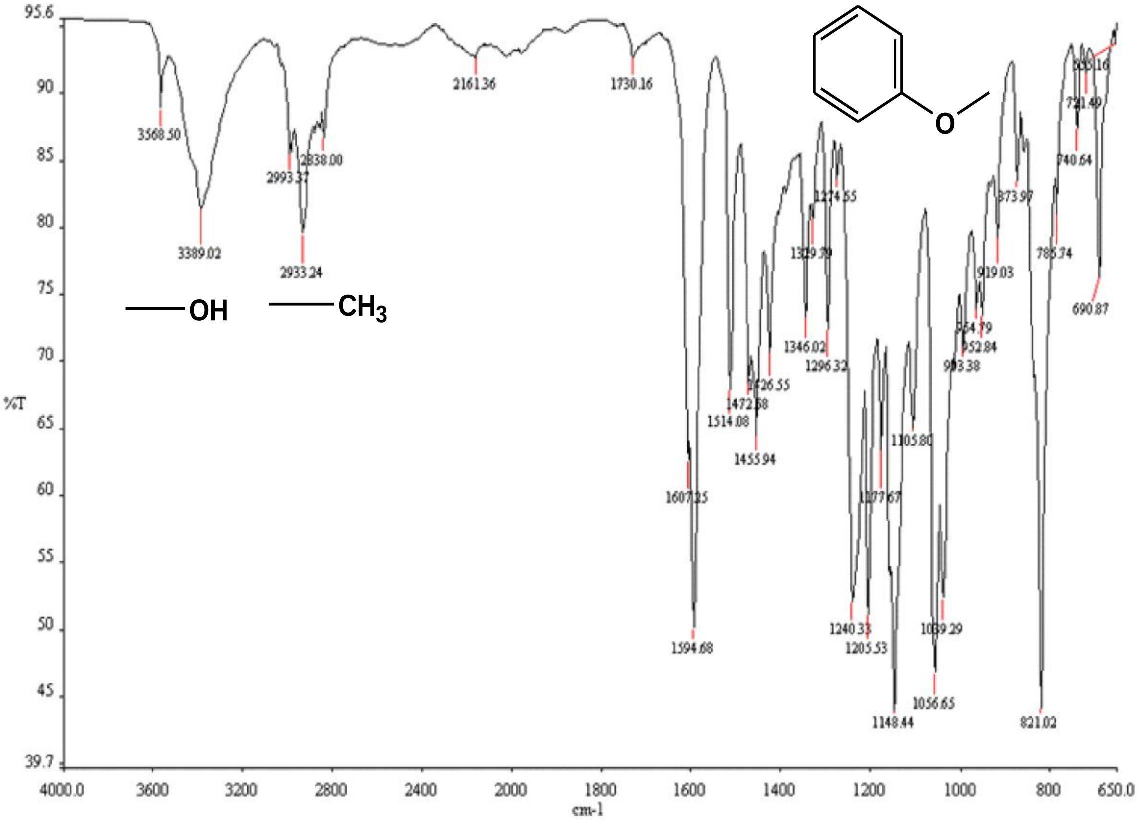


Figure 4.5- IR spectrum of compound L2

^{1}H-NMR spectrum of compound L2 show resonances at δ1.17, δ1.08, δ2.44, δ2.72, δ3.47, δ4.11, δ4.14, δ3.64, δ6.31, δ6.24, δ6.77, δ7.05 and δ8.11 (Figure 4.6).

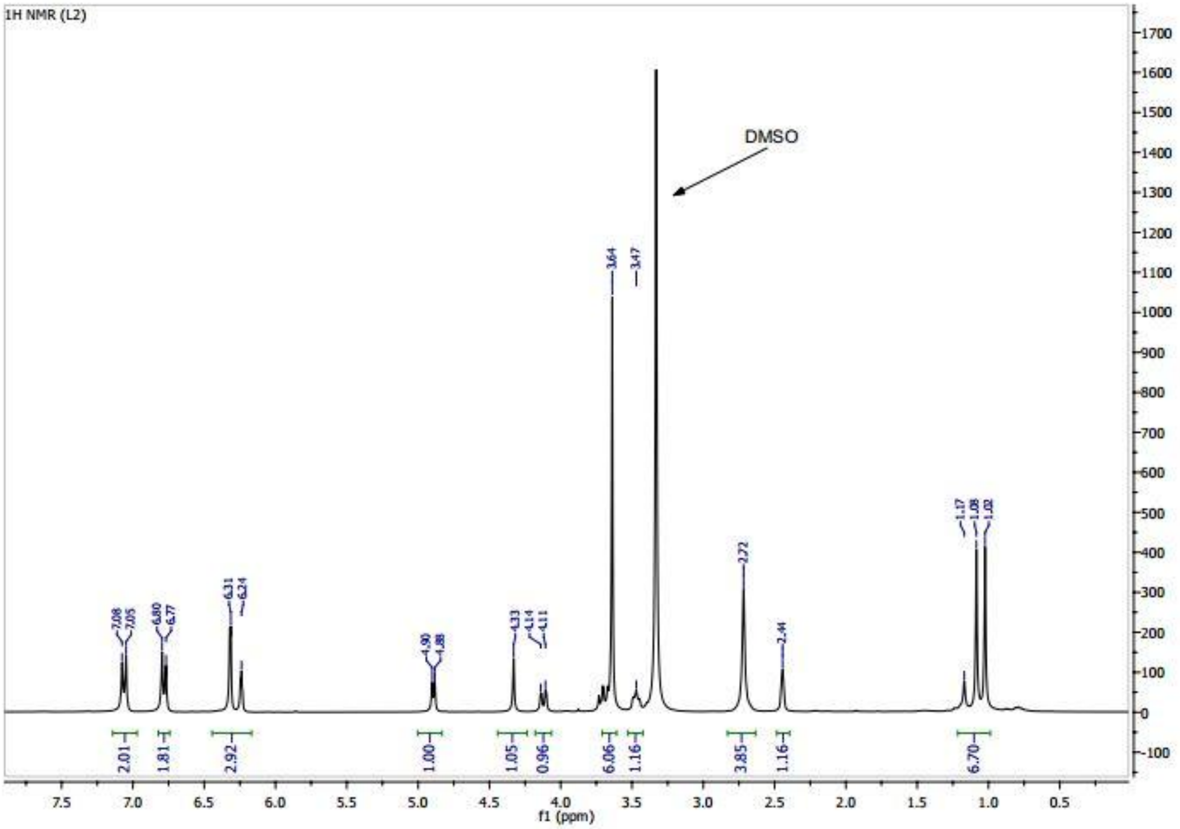


Figure 4.6- ^{1}H-NMR spectrum of compound L2

The ^{13}C-NMR spectrum of compound L2 revealed a total of 16 carbon resonances of which 8 are for aromatic carbons, 3 for methylene carbons and 5 for the aliphatic carbons (Figure 4.7).

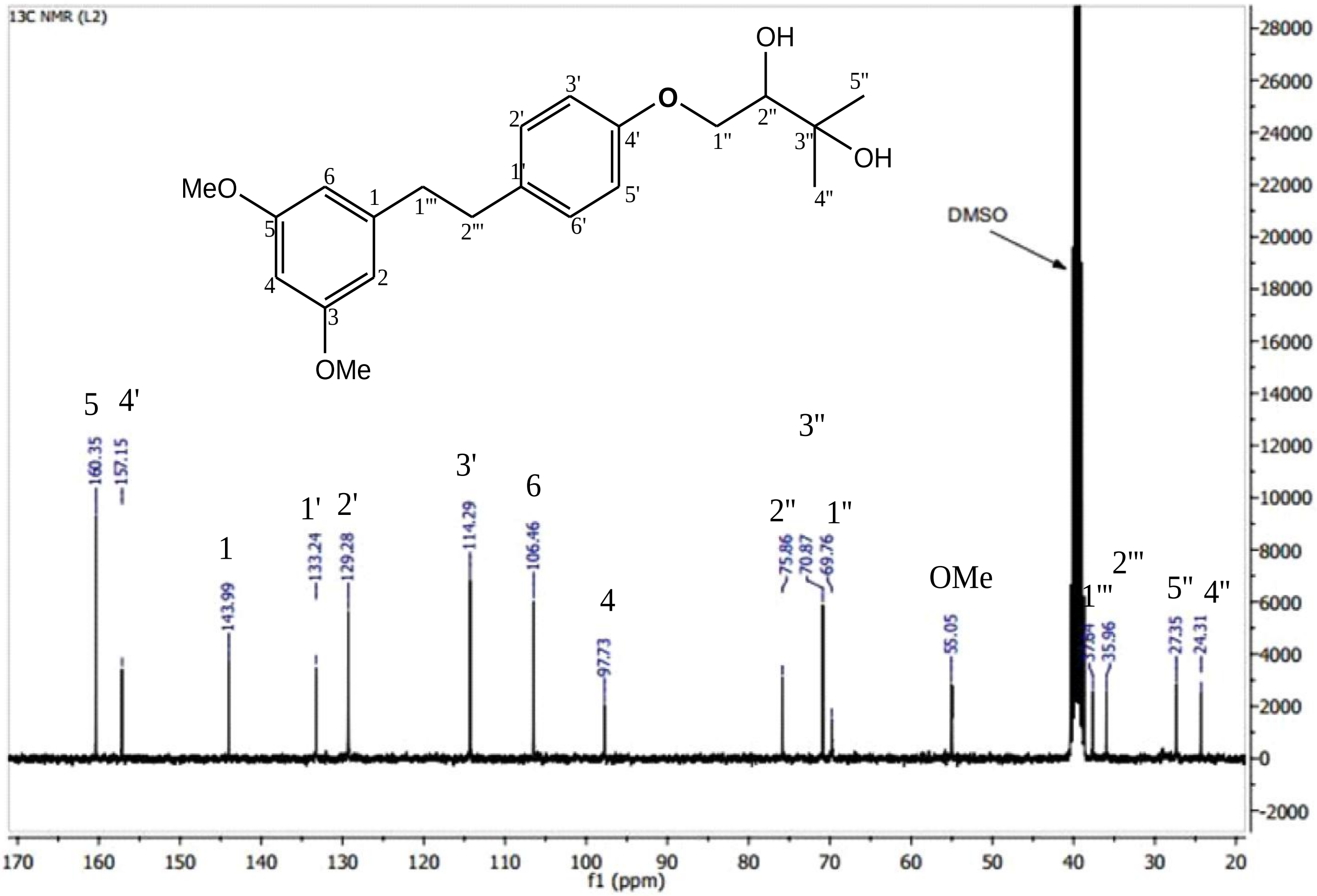


Figure 4.7- ^{13}C-NMR spectrum of compound L2

The ^{13}C-DEPT-NMR spectrum of compound L2 revealed signals for 3 methylene (CH_2), 2 methyl (CH_3), 5 methine (CH) carbon atoms and 1 methoxy group (figure 4.8).

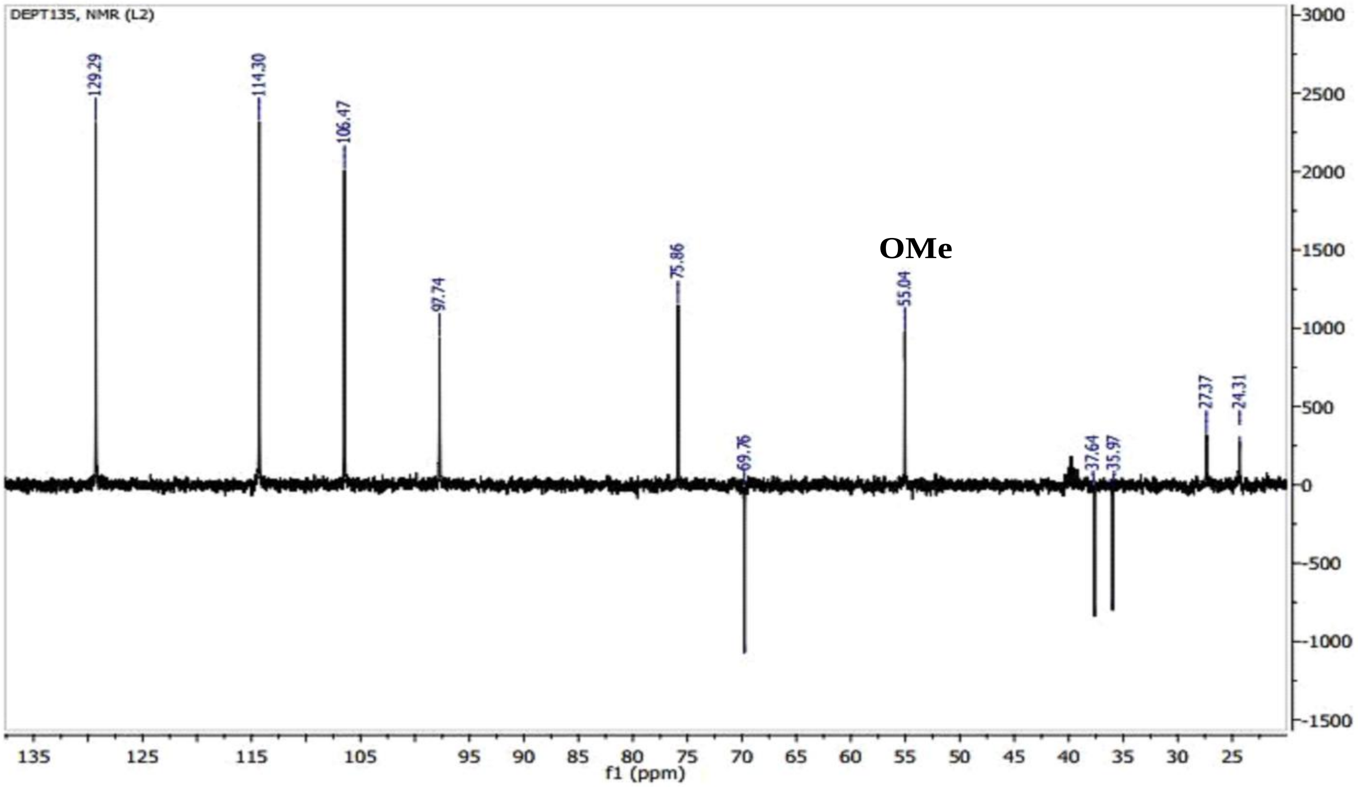


Figure 4.8- DEPT-NMR spectrum of compound L2

4.7 Antimicrobial Studies

Table 4.5: Results of susceptibility test of the fractions of *Indigofera pulchra* (Willd) ethyl alcohol extract and standard antibiotics against selected microorganisms.

The antibacterial and antifungal activities (zones of inhibition) of fractions H, C, E and B of the crude ethyl alcohol extract at 1mg/ml concentration and the reference drugs ciprofloxacin and fluconazole at a concentration of 100µg/ml on the test microorganisms measured in millimeter (mm) are shown in Table 4.5.

Test Organisms	Cp (20µg)	Fz (20µg)	Fractions (0.2mg)			
			H	C	E	B
mRSA	29	-	27	0	0	0
S. aureus	33	-	25	21	26	20
E. coli	28	-	26	21	22	22
P. mirabilis	27	-	0	20	25	0
C. albicans	-	26	27	22	24	0
C.tropicalis	-	25	25	0	23	20

Keys: H= Hexane, C=Chloroform, E=Ethylacetate, B=Butanol, Cp=Ciprofloxacine, Fz=Fluconazole

Table 4.6: Effect of fractions of *Indigofera pulchra* (Willd) ethyl alcohol extract on the antimicrobial activity of the standard antibiotics.

The zones of inhibition for the drug-fraction combinations measured in millimeter (mm) shows the synergistic effect of the fractions of *Indigofera pulchra* (Willd) aerial parts ethyl alcohol extract on the antimicrobial activity of the standard antibiotics except for chloroform fraction which shows antagonism against methicillin resistant *Staphylococcus aureus* .

Test Organisms	Cp (20µg)	Cp (10µg) + Fractions (0.1mg)				Fz (10µg)+ Fractions (0.1mg)				Fz (20µg)
		H	C	E	B	H	C	E	B	
mRSA	29	29	0	27	21	-	-	-	-	-
S. aureus	33	30	27	28	23	-	-	-	-	-
E. coli	28	26	24	25	25	-	-	-	-	-
P. mirabilis	27	21	27	28	26	-	-	-	-	-
C. albicans	-	-	-	-	-	28	20	24	21	26
C.tropicalis	-	-	-	-	-	25	21	26	23	25

Keys: H= Hexane, C=Chloroform, E=Ethylacetate, B=Butanol, Cp=Ciprofloxacine, Fz=Fluconazole

Table 4.7: Results of susceptibility test of the isolated compounds and the standard antibiotics against selected organisms.

The zones of inhibition of the isolated compounds L1 and L2 measured in millimeter (mm) in Table 4.7 shows that compound L1 has greater antibacterial activity than compound L2 and ciprofloxacin agains *P. mirabilis* and *mRSA* whereas L2 is resisted by *mRSA* but with greater activity than L1 and ciprofloxacine against *S. aureus* and *E. coli*.

Organisms	L1 (20μg)	L2 (20μg)	Cp (20μg)	Fz (20μg)
mRSA	29	0	29	-
S. aureus	30	31	33	-
E. coli	31	33	28	-
P. mirabilis	35	34	27	-
C. albicans	32	37	-	26
C. tropicalis	30	32	-	25

Keys: Cp=Ciprofloxacin, Fz=Fluconazole

Table 4.8: Effect of L1 and L2 on the antimicrobial activity of the standard antibiotics.

The antimicrobial actions of the isolated compounds L1 and L2 separately and their effect on the antibacterial activity of ciprofloxacin and the antifungal activity of fluconazole are shown in Table 4.8.

Test Organisms	Cp (20μg)	Fz (20μg)	L1 (10μg) + Cp (10μg)	L2 (10μg) + Cp (10μg)	L1 (10μg) + Fz (10μg)	L2 (10μg) + Fz (10μg)
mRSA	29	-	30	33	-	-
S. aureus	33	-	26	31	-	-
E. coli	28	-	27	29	-	-
P. mirabilis	27	-	25	25	-	-
C. albicans	-	26	-	-	29	40
C.tropicalis	-	25	-	-	27	39

Keys: Cp=Ciprofloxacin, Fz=Fluconazole

CHAPTER FIVE

5.0 Discussion

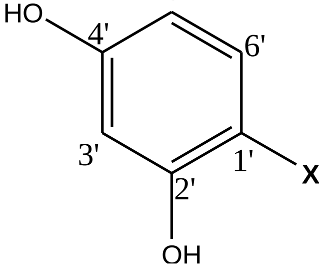
The result of preliminary phytochemical screening carried out on the ethyl alcohol extract of *Indigofera pulchra* (Willd) aerial parts and its fractions revealed the presence of carbohydrates, flavonoids, tannins, steroids/triterpenes and saponins. These phytochemical constituents have been reported to be associated with the pharmacological activities of plants (Cowan, 1999). Flavonoids and triterpenes have been found to contain compounds with good antimicrobial activities (Rojas *et al.*, 1992).

Column chromatographic seperation of the chloroform fraction from ethyl alcohol extract of *Indigofera pulchra* (Willd) aerial parts followed by preparative thin-layer chromatography lead to the isolation of two compounds (L1 and L2); L1 obtained as a yellow powder with a melting point range of 137-141^{O}C while L2 a white crystalline solid with a melting point range of 144-147^{O}C and were both found to be soluble in ethylacetate at room temperature. Thin layer chromatography examination of the isolated compounds using the solvent system n-hexane: ethylacetate (2:1) gave a single yellow and reddish spots with R_f values of 0.59 and 0.33 respectively. The compounds were further confirmed by positive Shinoda test for the presence of flavonoid nucleus.

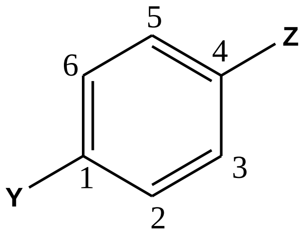
The IR spectrum of L1 shows absorption bands around 3310cm^{-1} for O-H stretching vibration for hydroxyl group, at 3146cm^{-1} indicating aromatic C-H stretching and at 2865.88, 2916.74 & 2970.30 cm^{-1} showing presence of methyl protons. Strong absorption at 1737.39 cm^{-1} signifies a carbonyl vibration of a ketonic functional group, Ar-OH at 1200cm^{-1}, 1143.92cm^{-1} for aromatic C-O- and Ar-H bending vibrations around 835.46cm^{-1} (Kalsi, 2004).

The ^{1}HNMR spectrum of compound L1 shows signals for ring A aromatic protons at δ_H 6.21ppm (1H, dd) proposed to be for C-3' proton while at δ_H 6.35ppm (1H, d) for C-2'and δ_H6.92ppm (1H, d) C-5' protons. The protons of ring B usually appear in the range 6.7-7.9ppm which is downfield from the region where A-ring protons absorb and the signal observed for the B-ring protons is characteristic for substitution pattern of that ring and, in addition, suggests the oxidation level of ring C; if ring B is oxygenated at C-4', a typical four peak pattern of two doublets is observed; the doublets for C-3' and C-5' protons always appears upfield from C-2' and C-6' and generally falls in the range 6.65-7.1ppm for all types of flavonoids but to some extent depends on the oxidation level of ring C; however, it consistently appears at lower field (7.1-8.1ppm) than the C-3', C-5' doublet (Mabry *et al.*, 1970). These therefore, proves the signals observed in the spectrum of compound L1 at the range of 7.1 - 8.1ppm to represent protons at C-2,3,5 and 6. The H-α and H-β protons of chalcones occur as doublets in the ranges 6.7-7.4 ppm (H-α) and 7.3-7.77 ppm (H-β).

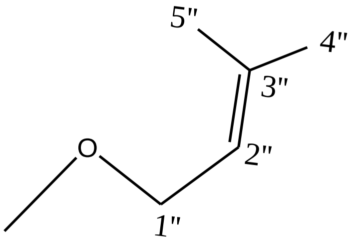
The ^{13}C NMR revealed eighteen carbon chemical shift signals of which δ 118.49, δ 143.87 were assigned to the CH carbons at position 2'' and 3'', signals at δ 165.12 and 165.87 for C2' and C4' of ring 'B' while those at δ 102.65, δ 108.24, δ 133.00 were assigned to aromatic methylene carbon atoms at position 3', 5' and 6' respectively while δ 131.0 for the quartenary carbon at 1' which revealed the trisubstituted nature of the ring.



Signal at δ191.58 was assigned to a carbonyl group attached to ring 'B', signal at δ 115.2 was assigned to the tertiary aromatic carbons at position C3 and C5 while at δ 131.00 represents C2 and C6.



At δ 160.79 shows the chemical shift signal for =C-O group of an oxygenated aromatic ring while signal at δ 64.63 of an O-methylene confirmed the presence of a prenyloxy side chain. Also, at δ 119.60 and 137.68 signified a signal for typical unsaturated carbon atoms and signal δ 18.08 and δ 25.48 for two methyl substituents which confirmed an oxyprenyl side chain.



The ^{13}C - DEPT-NMR spectrum of compound L1 revealed the presence of only one methylene (for O-CH_2) at δ 64.63ppm, eight methine (CH) at δ 102.65, δ 108.24, δ 115.12, δ 118.49, δ 119.60, δ 131.00, δ 133.00, δ 143.87ppm and two methyl (CH_3) at δ 17.5 and δ 25.5ppm. Based on the analysis of the ^{1}H and ^{13}C NMR and comparing with the literature (Appendix 1.0), the compound was proposed to be 2',4'-dihydroxy-4-prenyloxychalcone.

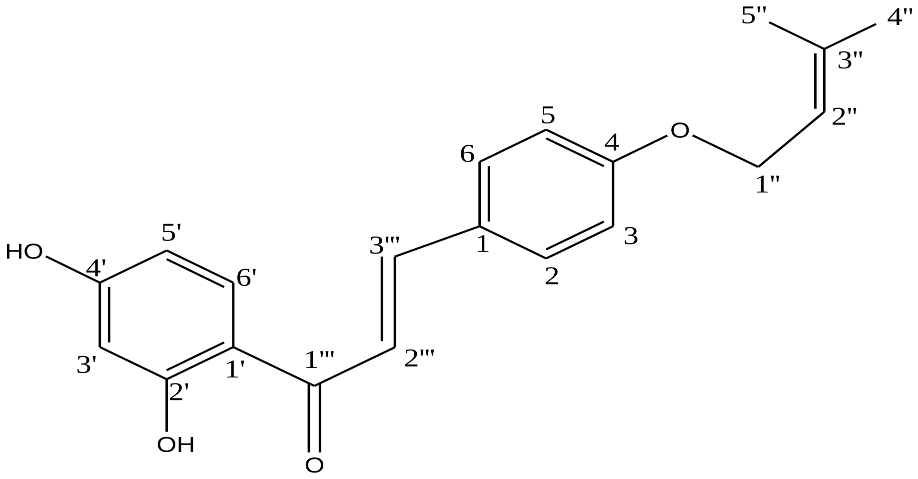
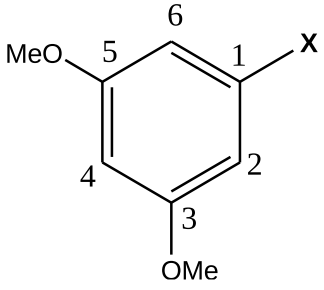


Figure 5.1: Structure of 2’,4’-dihydroxy-4-prenyloxychalcone.

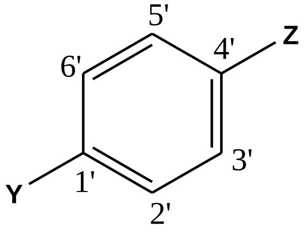
The IR spectrum of L2 shows absorptions at 3568.50 cm^{-1} and 3389.02 cm^{-1} for O-H stretching. Strong absorption band at 1148.44 cm^{-1} signifies presence aromatic C-O- (ether) and Ar-H bending vibrations around 873.97 cm^{-1} (Kalsi, 2004).

^{1}H-NMR spectra of compound L2 shows signals within a range of 6-8 ppm. For ring A, signal at δ 6.31ppm (2H, d) shows absorption for C-2 and C-6 protons which appear at lower field than C-4 proton at δ 6.24ppm (1H, d) due to shielding effect of C-3 and C-5 methoxyl groups. Ring B protons appears downfield to A-ring protons; B-ring proton signals ranges within 6.65-7.1ppm depending on the nature of oxygen attachment to the ring on the ring (Mabry *et al.,* 1970). This therefore, proves that C-3’ and C-5’ protons shows absorption at around δ 6.7 ppm (2H, dd) upfield to where C-2’ and C-6’ protons absorb around δ 7.1 ppm (2H, dd) due to shielding effect of oxygen at C-4’. Other protons include the C-4’’ and C-5’’ methyl protons that appeared around δ1.0- δ1.2ppm, the C-1’’’ and C-2’’’ at δ2.3-2.8ppm, oxymethylene C-1’’ at δ4.88, δ4.9ppm doublet and hydroxyl protons at a range of δ4.0-4.4ppm (Kalsi, 2004).

13C NMR spectra of compound L2 revealed eighteen carbon chemical shift resonances; signal at δ55.05 is assigned to methoxy carbon atoms at C3 and C5 which resonate with chemical shift signal δ 160.35 and δ 97.73 is assigned to C4 resonance at higher field due to C3 and C5 methoxy groups' shielding effect in ring 'A' thus, signifying the tri-substituted nature of the ring.



Recognizable signal at δ 133.24 is due for C1' quartenary carbon atom of ring 'B' and at δ 157.15 for carbon at position 4' while C2' and C6' resonates at same signal of δ 129.28, likewise for C3' and C5' at δ 114.29. This suggested the di-substituted nature of ring 'B'.



Other resonances observed at δ24.31 and 27.35 represents Sp^3 C4'' and C5'' methyl carbons of the methylbutyl side chain, δ35.96 and δ37.64 for C1''' & C2''' ethylene carbons that links the two aromatic rings. Signals at δ69.72 signals for oxy-methylene carbon C1'', δ75.86 for C2'' carbon.

The ^{13}C - DEPT-NMR spectrum of compound L2 revealed the presence of three methylene (for O-CH_2) at δ35.97, δ37.64 and δ69.76ppm, seven methine (CH) at δ24.31, δ27.37, δ75.86, δ97.74, δ106.47, δ114.30, δ129.29 ppm, two methyl carbons (CH_3) at δ24.31 and δ27.37, and two methoxy carbons ($O-CH_3$) at δ55.05ppm and δ25.5ppm. Based on the analysis of the ^1H and ^{13}C NMR and comparing with the literature (Appendix 2.0), the compound was proposed to be 3,5-dimethoxy-4'-O-(2''-3''-dihydroxy-3''-methylbutyl)-dihydrostilbene.

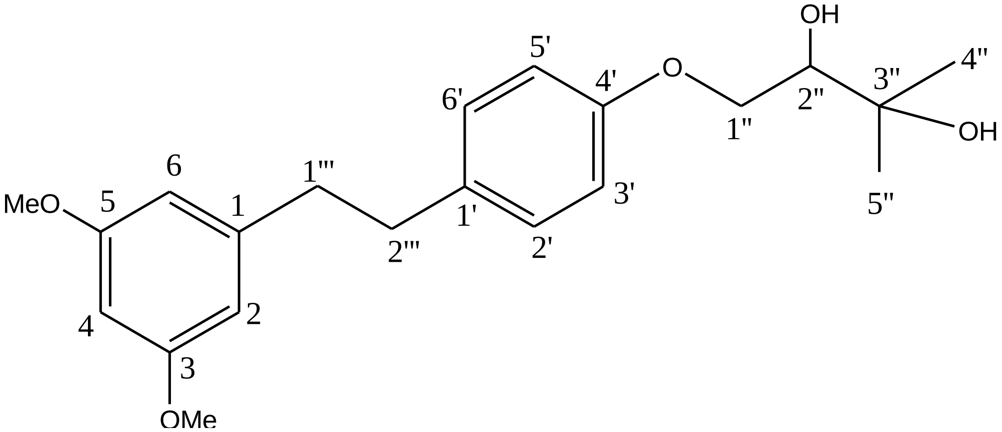


Figure 5.2: Structure of 3,5-dimethoxy-4'-O-(2''-3''-dihydroxy-3''-methylbutyl)-dihydrostilbene.

Antimicrobial studies

The results of this study have shown that fractions of the ethyl alcohol extract of *Indigofera pulchra* (Willd) aerial parts posses a wide range of antimicrobial activity against both Gram positive and Gram negative bacteria and hence, against yeast (*Candida*). This, therefore, shows that the fractions contain substance(s) that can inhibit the growth of some microorganisms. The observed antimicrobial effect of the fractions on the isolates is believed to be due to presence of constituents like flavonoids, saponins and tannins which have been shown to posses' antimicrobial properties (Draughon, 2004).

The zones of inhibition observed for the susceptibility of the various fractions tested against some microbes revealed that all the fractions are active against the test microorganisms except for *mRSA* which was found to be inhibited by hexane fraction only. *Proteus mirabilis* also shows resistance against hexane and butanol fractions, *Candida albicans* resisted butanol fraction and *Candida tropicalis* resisted chloroform fraction only.

The isolated compounds (L1 and L2) show appreciable antimicrobial activity against all the test microorganisms used except for *mRSA* which was observed to completely resist compound L2 whereas, the standard antibiotics used; ciprofloxacin inhibit all the bacteria isolates used and the same is observed for fluconazole against the *Candida* isolates.

Compound L1 was found to enhance the antibacterial activity of ciprofloxacin against all the test microorganisms but with L1 alone showing better activity against *E. coli* and *P. mirabilis* than ciprofloxacin. Synergistic effect of compound L1 on the antifungal activity of fluconazole was also observed against both *C. albicans* and *C. tropicalis* with L1 showing better activity than fluconazole. Compound L2 was also found to enhance the antibacterial activity of ciprofloxacin against all the test microorganisms with L2 showing better activity against *E. coli* and *P. mirabilis* than ciprofloxacin. Synergistic effect of compound L2 on the antifungal activity of fluconazole was observed against both *C. albicans* and *C. tropicalis* with L2 showing better activity than fluconazole. This can therefore be a basis to attribute the antimicrobial activity of *Indigofera pulchra* to the presence of the isolated compounds.

CHAPTER SIX

6.0- Summary, Conclusion and Recommendations

6.1- Summary

Preliminary phytochemical investigation of chloroform fraction of the ethyl alcohol extract of *Indigofera pulchra* (Willd) aerial parts revealed the presence of sterol/triterpenes, saponins, flavonoids, steroids, tannins, carbohydrates while alkaloids are absent.

Chromatographic investigations using silica gel column chromatography and preparative thin layer chromatography led to the isolation of 2’,4’-dihydroxy-4-prenyloxychalcone coded L1 and 3,5-dimethoxy-4’-O-(2’’,3’’-dihydroxy-3’’-methylbutyl)-dihydrostilbene coded L2.

Studying the antimicrobial potentials of the various fractions and isolated compounds using agar well-diffusion technique revealed their ability to inhibit some bacteria and fungi. The combination of the fractions separately with ciprofloxacin and fluconazole shows synergistic interaction except *mRSA* which resist the combination of ciprofloxacin and chloroform fraction. Larger zones of inhibition were observed even at half concentration of the isolated compounds separately combined with the standard antibiptics against the entire test micerobes used for the study.

6.2 Conclusion

Based on the findings of this work, it can be concluded that the use of *Indigofera pulchra* (Willd) aerial parts in the treatment of infections caused by bacteria and fungi has a scientific basis which can be justified by the activity exhibited by the various fractions and the isolated compounds (L1 and L2) separately and in combination with standard antibiotics.

6.3 Recommendation

There is need for further isolation of more compounds from the plant to study their antimicrobial activitiy aginst other resistant bacteria and fungi. There is also a need to re-isolate these compounds to test for their anti-malarial and snake venom detoxifying activities. Furthermore, considering the compounds’ ability to singly inhibit some infectious bacteria and fungi and in combination synergistically potentiates the antimicrobial activities of ciprofloxacin and fluconazole, it is highly recommended to test for toxicity of these compounds, design, synthesize and extensively study their structure-activity relationship towards utilizing their potentials as antimicrobial lead compounds.

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APPENDICES

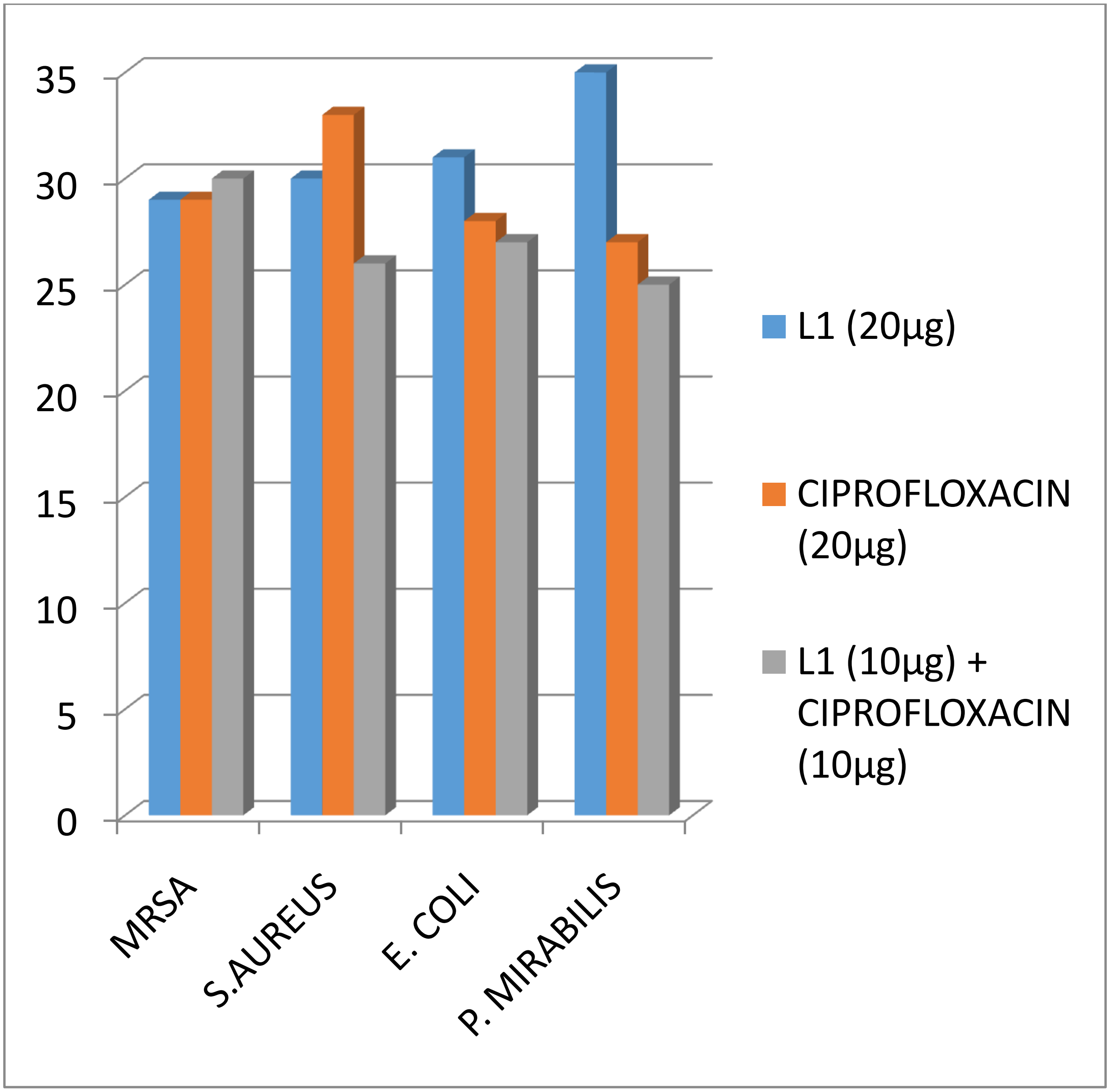
Appendix 1.0: Comparison of ^{13}C-NMR data of compound L1 and 2’,4’-dihydroxy-4-prenyloxychalcone (Musa *et al.*, 2011**).**

Position	δ_c of compound L1	δ_c of reference compound (D5)	DEPT
1	127.19	129.0	C
2,6	131.00	131.5	CH
3,5	115.12	116.2	CH
4	160.79	162.7	C
1’	113.07	114.8	C
2’	165.12	166.5	C
3’	102.65	103.6	CH
4’	165.87	167.5	C
5’	108.24	108.9	CH
6’	133.00	133.4	CH
1’’	64.63	66.1	CH_2
2’’	119.61	120.8	CH
3’’	137.68	139.2	C
4’’	18.08	17.5	CH_3
5’’	25.48	25.5	CH_3
1’’’	191.58	193.5	C=O
2’’’	118.49	119.3	CH
3’’’	143.87	145.2	CH

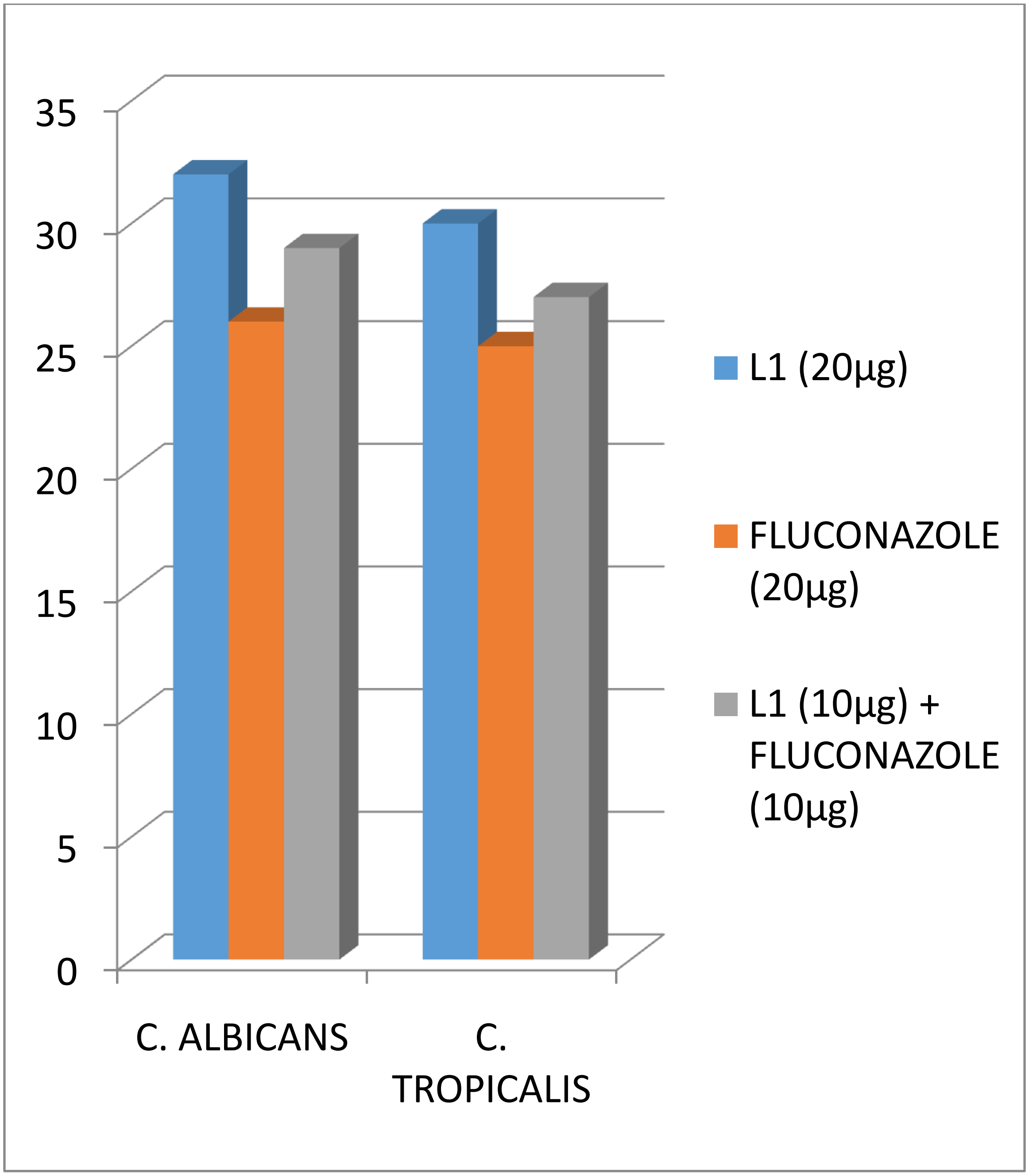
Appendix 2.0: Comparison of ^{13}C-NMR data of compound L2 and 3,5-dimethoxy-4’-O-(2’’,3’’-dihydroxy-3’’-methylnutyl)-dihydrostilbene (Musa *et al.*, 2008**).**

Position	δ_c of compound L2	δ_c of reference compound (2)	DEPT
1	143.49	145.0	C
2	106.46	115.5	CH
3	160.35	162.3	C
4	97.73	99.1	CH
5	160.35	162.3	C
6	106.46	115.5	CH
1’	133.24	135.3	C
2’,6’	129.28	130.5	CH
3’,5’	114.29	115.6	CH
4’	157.15	158.1	C
1’’	69.76	70.2	CH_2
2’’	75.86	76.7	CH
3’’	70.87	71.9	C
1’’’	37.64	39.4	CH_2
2’’’	35.96	38.2	CH_2
4’’	24.31	26.0	CH_3
5’’	27.35	27.1	CH_3
3-OMe	55.05	55.5	CH_3
5-OMe	55.05	55.5	CH_3

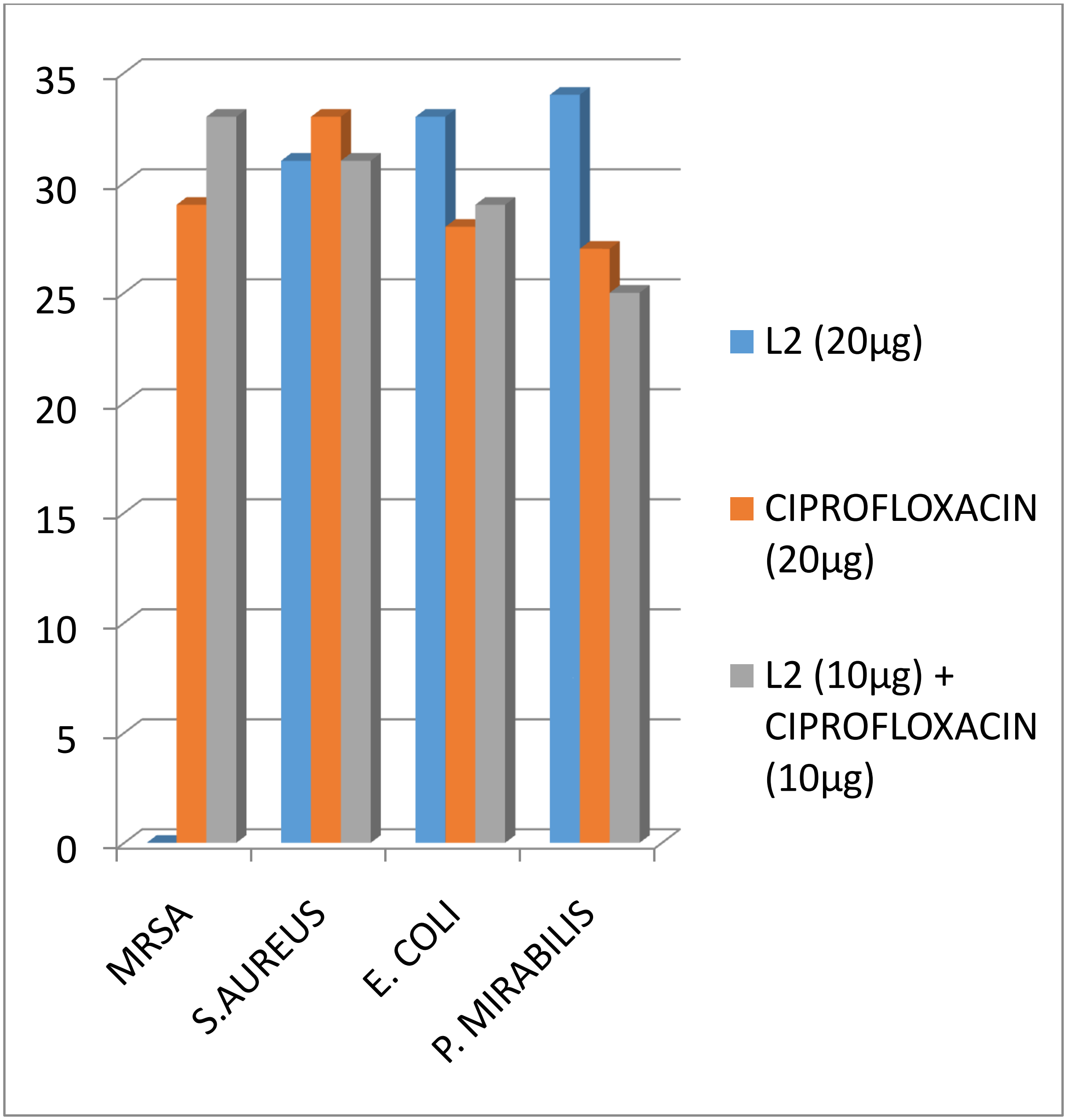
Appendix 3.0: Effect of L1 on antibacterial activity of ciprofloxacin



Appendix 4.0: Effect of L1 on antifungal activity of fluconazole



Appendix 5.0: Effect of L2 on antibacterial activity of ciprofloxacin



Appendix 6.0: Effect of L2 on antifungal activity of fluconazole