PHYTOCHEMICAL AND ANTIMICROBIAL STUDIES OF THE LEAVES EXTRACT OF *PILIOSTIGMA THONNINGII* SCHUM Milne - Redh (FABACEAE)

BY

FRIDAY ADIH ECHU

DEPARTMENT OF PHARMACEUTICAL AND MEDICINAL CHEMISTRY,

FACULTY OF PHARMACEUTICAL SCIENCES,

AHMADU BELLO UNIVERSITY, ZARIA

NIGERIA

DECEMBER, 2014

PHYTOCHEMICAL AND ANTIMICROBIAL STUDIES OF THE LEAVES EXTRACT OF *PILIOSTIGMA THONNINGII* SCHUM Milne – Redh (FABACEAE)

BY

Friday Adih ECHU (B. Pharm., A.B.U. 1997)

M. Sc/PHARM. SCI/22426/2012 – 2013

BEING

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

IN PARTIAL FULFILMENT FOR THE AWARD OF MASTER OF SCIENCE IN PHARMACEUTICAL AND MEDICINAL CHEMISTRY

DEPARTMENT OF PHARMACEUTICAL AND MEDICINAL CHEMISTRY,
FACULTY OF PHARMACEUTICAL SCIENCES
AHMADU BELLO UNIVERSITY, ZARIA – NIGERIA.

DECEMBER, 2014

DECLARATION

I declare that the work in this thesis entitled **"PHYTOCHEMICAL AND ANTIMICROBIAL STUDIES OF THE LEAVES EXTRACT OF *PILIOSTIGMA THONNINGII* SCHUM. Milne – Redh (FABACEAE)** has been performed by me in the Department of Pharmaceutical and Medicinal Chemistry under the supervision of Dr (Mrs.) H.S. Hassan and Prof. M.I. Sule. The information derived from the Literature has been dully acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at any other University.

Friday Adih ECHU …….. ……..

Signature Date

CERTIFICATION

This dissertation entitled" **PHYTOCHEMICAL AND ANTIMICROBIAL STUDIES OF THE LEAVES EXTRACT OF *PILIOSTIGMA THONNINGII* SCHUM. Milne – Redh (FABACEAE) by Friday Adih ECHU** meets the regulations governing the award of Master of Science in Pharmaceutical and Medicinal Chemistry of Ahmadu Bello University, Zaria and is approved for its contributions to knowledge and literary presentation.

Dr. (Mrs.) H.S. Hassan	……..	……..
Chairman Supervisory Committee	Signature	Date
Prof. M.I. Sule	……..	……..
Member Supervisory Committee	Signature	Date
Dr. Aliyu M. Musa	……	……..
HOD Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University, Zaria..	Signature	Date
Prof. A. H. Zoaka	.. ..……	….…….
Dean, School of Postgraduate Studies Ahmadu Bello University, Zaria.	Signature	Date

DEDICATION

This research work is dedicated first to **GOD** Almighty for the opportunity He had given me again to achieve this level of education and to my wife, Mrs. (Dcns) OJONUGWA D. ECHU and Children, OGECHA, OMA-AJUMA, ONENYI, OJIMA and OJONIMI.

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my supervisors Dr. (Mrs.) H.S. Hassan and Prof. M.I. Sule for supervising this work and for their sincere advice, encouragements and corrections in the course of this work. I appreciate all you have both done to see the success of this work.

My heartfelt thanks also to Bashar Bawa, Njinga Stanly, Sani Yahaya, Dr. Aliyu Musa (HOD Pharmaceutical and Medicinal Chemistry A.B.U. Zaria), Musa Yahaya Maikafi, Iliyasu Salisu., Mr Obeta Francis., Mr. Williams A., Joy Biko., Umar Usman Umar, Nafiu Garba, Ms Veronica Odinkemere and all other staff of the Department, all my brothers, sisters and their families lead by Mr. Bernard Onuche.

My profound gratitude goes to Prof. Agunu Abdulkareem, Kamilu Mahmoud and Kabiru Ibrahim, all of Department of Pharmacognosy and Drugs Development A. B. U. Zaria, Dr. Busayo Olayinka, Department of Pharmaceutics and Pharmaceutical Microbiology both of Faculty of Pharmaceutical Sciences, A.B.U. Zaria, Prof. Augustin Ahmadu, Niger Delta University, Yenagoa – Bayelsa State, Prof. Iliya Ibrahim Faculty of Pharmacy, University of Maiduguri, Dr. (Pastor) David Abolude, Department of Biological Sciences, A.B.U. Zaria, Prof. M.D. Alegbejo, Department of Crop Protection, Institute for Agricultural Research, A.B.U. Zaria, Mal. Umar Hanwa, Samaru College of Agriculture, Division of Agricultural Colleges A. B. U. Zaria and my former H.O.D. General Hospital, Birnin Gwari Pharm. Mahmoud Muhammed, for his encouragement and contributions to the success of this work, I will forever be grateful to you.

ABSTRACT

The plant *Piliostigma thonningii* which is used in ethno - medicine for the treatment of wounds, ulcers and gingivitis was investigated for its chemical constituents and antimicrobial activity. The leaf of the plant was subjected to extraction procedures to obtain ethanol and n – hexane extracts. The n – hexane extract which was then subjected to preliminary phytochemical screening to revealed the presence of alkaloids, anthraquinones, flavonoids, reducing sugars, saponins, tannins and steroids/terpenes. The phytochemical technique employed was column chromatography. The antimicrobial activity of the n – hexane and ethanol extracts were studied using hospital isolates of ten pathogenic microorganismmms. Extensive chromatographic separation of the n – hexane fraction using silica gel column chromatography led to the isolation of a steroid. Stigmast-5-en-ol (β – Sitosterol), the structure was determined by spectral analysis including 1D and 2D NMR. In the antimicrobial studies, the extracts exhibit activity against six of the ten microorganisms with zone of inhibition ranging from 13 – 16 mm for n – hexane extract and 20 – 27 mm for ethanol extract while the zone of inhibition for the standard antibacterial drug sparfloxacin ranged from 40 – 46 mm and the zone of inhibition for the standard antifungal drug fluconazole ranged from 34 – 37 mm. The results obtained from this study suggest that the fractions of *Piliostigma thonningii* leaves extracts posses some compounds with antimicrobial activity and validate the ethnomedicinal use of the plant in the treatment of microbial infections.

TABLE OF CONTENTS

Content

Page

Cover page… … … … … … … … … … i

Title page… … … … … … … … … … ii

Declaration… … … … … … … … … … iii

Certification … … … … … … … … … … iv

Dedication… … … … … … … … … … v

Acknowledgement… … … … … … … … … vi

Abstract… … … … … … … … … … vii

Table of Contents… … … … … … … … … viii

List of Tables …… … … … … … … … … xiii

List of Figures…… … … … … … … … … xiv

List of plates …… … … … … … … … … xvi

List of Appendices … … … … … … … … … xvii

List of Abbreviations …… … … … … … … … xviii

CHAPTER 1: Introduction

1.1 General Introduction…. …… … … … … … … 1

1.2 Statement of Research Problems…… … … … … … 14

1.3 Justification … … … … … … … … … … 14

1.4 Aim and Objectives of the Study… … … … … … 15

1.5 Research Hypothesis … … … … … … … … … 15

CHAPTER 2: Literature Review

2.1 Botanical classification …… … … … … … 16

2.1.1 Common names …… … … … … … … … … 16

2.1,2 Morphology of *Piliostigma thonningii* …… … … … … 17

2.2 Ethno - medicinal (Traditional) uses of *Piliostigma thonningii* …… … 18

2.3 Phytochemistry of *Piliostigma thonningii* and other species in the genus… 19

CHAPTER 3: Materials and Methods

3.1 Materials…… … … … … … … … … … 22

3.1.1 Solvents and reagents…… … … … … … … 22

3.1.2 Equipments… … … …… … … … … … 22

3.1.3 Collection, identification and preparation of plant material… …… … 22

3.2 Methods

3.2.1 Extraction…… … … … … … … … … 22

3.2.2 Preliminary phytochemical screening…… … … … … 24

3.2.2.1 Test for alkaloids… … … …… … … … … 24

3.2.2.2 Test for anthraquinones... …… … … … … … 24

3.2.2.3 Test for carbohydrates/reducing sugars ... …... 25

3.2.2.4 Test for flavonoids… … … …… … … … … 25

3.2.2.5 Test for glycosides… … … …… … … … … 26

3.2.2.6 Test for saponins… … … …… … … … … 26

3.2.2.7 Test for steroids/terpenes… … …… … … … … 27

3.2.2.8 Test for tannins… … …… … … … … … 27

3.2.3 Chromatographic Procedure/separation of n - hexane extract…… … 28

3.2.3.1 Column chromatography of n - hexane extract… … …… … 28

3.2.3.2 Solubility of compound B 29

3.2.3.3 Chemical tests on compound B 29

3.2.3.4 Spectral analysis … … … …… … … … … 29

3.2.4 Antimicrobial Screening… … …… … … … … 30

3.2.4.1 Test microorganism... ... …… … … … … 30

3.2.4.2 Preliminary antimicrobial studies... … …… … … … 30

3.2.4.3 Minimum inhibitory concentration (MIC)… … …… … … 31

3.2.4.4 Minimum bactericidal concentration/minimum fungicidal concentration (MBC/MFC) …… … … …… … … … … … 31

CHAPTER 4: Results

4.1 Percentage yields …… … … … … … … … 33

4.2 Results of Preliminary Phytochemical Studies .. … … … 34

4.3 Fractions of n – hexane extract chromatography …… … … … 35

4.4 Fractions of the merged fractions 12 & 13 chromatography…… … 36

4.5 Thin layer chromatography of compound B… … …… … … 37

4.6 Results of chemical tests … … … … …… … … 38

4.7 FTIR spectrum of compound B … … … …… … … 39

4.8 Proton nuclear magnetic resonance of compound B… … …… … 40

4.9 Carbon-13 nuclear magnetic resonance of compound B… …….. … 41

5.10 Distortionless enhancement by polarization transfer (Dept) of compound B…42

5.11 Results of susceptibility tests… … … … … … …… 43

4.8.2 The MIC and the MBC/MFC of the ethanol and n – hexane extracts of the leaves of *P. thonningii* on the test microorganisms … … … … … …… 44

CHAPTER 5: Discussion

5.0 Extraction … …… … … … … … … … … 45

CHAPTER 6: Summary, Conclusion, Recommendations and Contributions.

6.1 Summary… …… … … … … … … … … 49

6.2 Conclusion… …… … … … … … … … … 49

6.3 Recommendations…… … … … … … … … 50

References… … … …… … … … … … … 51

LIST OF TABLES

Table 4.1: Percentage yields of n – hexane and ethanol extracts of P. thonningii…33

Table 4.2: Preliminary screening of ethanol and n – hexane extracts of leaves of *P. thonningii* … … …… … … … … … … … 34

Table 4.3: Column chromatography of the n – hexane extract …… … … 35

Table 4.4: Column chromatography of fractions 12 and 13 … …… … 36

Table 4.5 Chemical tests on compound … … … …… … … 38

Table 4:6 Susceptible tests in mm of the leaves extracts *P. thonningii* and the standard drugs on the ten microorganisms … … … … …… … .. 43

Table 4.7 Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal/Minimum Fungicidal Concentrations (MBC/MFC) in mg/ml the ethanol and n – hexane extracts of the leaves of *P. thonningii* on test microorganisms.… 44

LIST OF FIGURES

Figure 4:5 IR Spectra of compound B…… … … … … … 39

Figure 4:2 ^{1}H – NMR Spectra of compound B… …… … … … 40

Figure 4:3 ^{13}C – NMR Spectra of compound B… … …… … … 41

Figure 4:4 DEPT Spectra of compound B… … … …… … … 42

LIST OF PLATES

Plate 1: *p. thonningii* tree on a farm at Sundimina in Birnin Kudu LGA Jigawa State 17

Plate 2: Leaves and fruits of *P. thonningii* … … … … … 18

Plate 4.1 TLC of compound B … … …… … … … … 37

LIST APPENDICE(S)

Appendix 1 … … … … … … … … … 61

ABREVIATIONS

CNMR …	…	Carbon Nuclear magnetic resonance
DEPT …	…	Distortionless enhancement by polarization transfer
HNMR …	…	Proton Nuclear Magnetic Resonance
IR … …	…	Infra-red
IUCN …	…	International Union for Conservation of Nature and Natural Resources
MBC …	…	Minimum bactericidal concentration
MFC …	…	Minimum fungicidal concentration
MIC …	…	Minimum inhibitory concentration
NIPRD …	…	National Institute for Pharmaceutical Research and Development
NMR …	…	Nuclear magnetic resonance
^{o}C … …	…	Degrees Celsius
PHC …	…	Primary Healthcare
PTEE …	…	Piliostigma thonningii ethanol extracts
PTnHE …	…	Piliostigma thonningii n – hexane extract
R_f … …	…	Retardation factor
RMRDC …	…	Raw Materials Research and Development Council
STI … …	…	Sexually transmitted infection
TLC …	…	Thin layer chromatography
US …	…	United States
WHO …	…	World Health Organisation

CHAPTER ONE

INTRODUCTION

1.1 General Introduction

Man have been using medicinal preparations derived from plants and other sources like animals and minerals from time immemorial, the practice being as old as man himself. The use of medicinal plants have passed through several developmental stages from crude and to the present day drugs like tablets, capsules, injections, ointments, creams, etc.

This then make it difficult for the present day man to say exactly when the use of plants in medicines started. However, records of the use of plants in traditional medicines by the ancient civilisation like the Chinese, Greek, Indians and the Babylonians are still in existence. There are still some books or texts that show that the Egyptians were in possession of a good knowledge of plants in traditional medicines, they have recognised the important values of castor oil, dates, garlic and honey (Snowdon and Cliver, *1996*).

Ancient texts of India and China contain exhaustive depictions of the use of a variety of plant-derived medications (Ahmed *et al.,* 2006). The Chinese Emperor Shen Nung published a book on herbs in 370BC. Also an Apothecary by the name Theophrastus born in Athens in the present day Greece around 370BC also published a compendium on the uses of close to 500 medicinal plants. In fact, plants remain the main source of medicines for a large proportion of the world's population, particularly in the third world countries (Barnes, *et al.,* 2008). Man had continued to use traditional medicines from civilisation to civilisation. Recently evidence that proves the uses of plants as drugs were excavated from caves in Texas, United States of America, a fossil remains of *Lephophora williamsii* used by American – Indians were found and dated back to as far as about 7000BC (Plowman,1984). The use of plants remains undisputed despite the advent of the Pharmaceutical Chemistry during the early twentieth century, which brought with it the

ability to synthesize an enormous variety of drug molecules and thereby allowed the treatment of previously incurable and/or life-threatening diseases (Farnsworth *et al., 1984)*.

The use of herbal medicines in India dated back to about 5000 years, this was recorded in Ayurveda which contained over 8,000 herbal recipes. The same ancient method of treatment is still in use in over 14,000 dispensaries, traditional medicine is still widely acceptable despite the fact that it is limited by lack of precise diagnosis, hygiene, standardisation, ethics in dosing and sometimes claims or exaggerated claims which cannot be proven scientifically (Elujoba, 1999, Shok, 1999). Although chemically synthesized drug have also gained popularity and became the basis of Pharmaceutical industries. Over the years, however, synthetic drugs have been hijacked by unwanted side - effects, toxicity, high cost and inefficiency, among other problems. Also the search for new drugs against a variety of illnesses through chemical synthesis and other modern approaches has not been too successful. These factors, as well as the emergence of new infectious diseases, the proliferation of disorders such as cancer and growing multidrug resistance in pathogenic microorganisms, have prompted renewed interest in the research and discovery of potential drug molecules from medicinal plants (Arjun Patra, *et. al*, 2010). These information and many others coming out daily make the uses of medicinal plants a continuous one, because drugs from natural sources are less toxic and many are very effective. About half of the world medicinal compounds are derived from plants. Medicinal products from plants are generally more important in developing countries than in developed and industrialized world as 75 – 90 % of the world's rural populations rely comfortably on herbal medicines (Hamann*, 1988)*. This high dependence on herbal medicines and the use of medicinal plants as sources of semi synthetic drugs lead to the World Health Organisation (WHO*)* to carry out a study of medicinal plants in 1978, this

work lead to the identification of about 20,000 new species of medicinal plants, (Zamora-Martinez, *1992)*.

Traditional medicines are recognised and accepted more readily in developing nations because they are more accessible, sometime more effective for some diseases and cheaper than modern drugs (Shok, 1999). In these nations the number of modern health professionals like Pharmacists, Doctors, Nurses, etc are far too low compared to the population they are to serve. For example at the end of 1972, there was only one Doctor (Physician) per 28,000 people (Djukanovic and Mach*, 1975*). Also traditional medicines evolve from and blend with the socio cultural life of the people that make use of them. In developing countries, traditional medicine is indisputably integral part of the Primary Health Care (PHC), as such an attempt should be made to confirm the efficacy of the traditional herbs and also standardize them. This would help tremendously when the economic conditions of the nations are considered. It is obvious that the drug bill for many countries still represent a sizeable proportion of their total expenditure especially in developing countries. These countries have to purchase the drugs at exorbitant and often inflated costs from multinational companies, which spend a disproportionately large amount of money in advertising in developing countries (Sofowora, *1982*). However, the use of phytomedicines is not only limited to the developing nations. Countries like the People Republic of China, the main type of drug therapy is still in the form of plants extract (Anonymous, *1998*). It is therefore disheartening to note that Africans that are situated within the tropical regions and blessed with abundant plant species are making little efforts in standardizing the use of their traditional herbal drugs. It is estimated that over 200,000 out of the 300,000 plant species so far identified in the whole of our planet are in the tropical countries of Africa and elsewhere. Among the potential users of these plants, traditional medicine and are on the top (Adjanahoun *et al., 1991*).

Herbal medicine is now globally accepted as a valid alternative system of therapy in the form of pharmaceuticals, food supplements, nutraceuticals, complementary and alternative medicines, etc., a trend recognised by the World Health Organization (WHO). Africa is a rich ground for many very useful plants from time immemorial but they are largely undocumented, the knowledge are always kept secret within a family or clan and passed from one person to the other leading to a large information being lost from one generation to the other through inappropriate folklore passage of information or sudden deaths of the people or person(s) in the current custody of the knowledge (Arjun Patra, *et. al*, 2010).

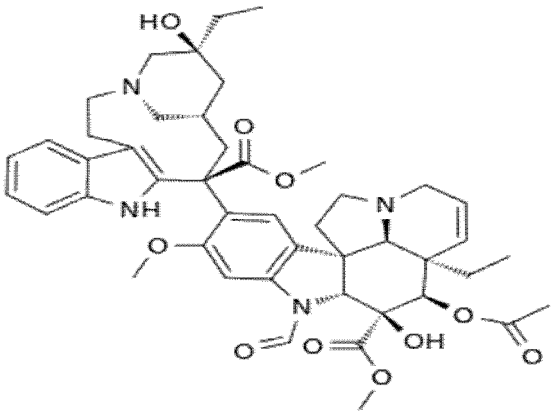
Apart from Chinese, Africans and others mentioned above, Arabs, South American Indians have records that indicate their use of herbal drugs in the ancient times.

Approximately 119 pure chemical substances extracted from higher plants are used in medicine throughout the world (Farnsworth *et al.,* 1984). In United State about 25% of all prescription dispensed from community Pharmacies from 1959 - 1980 contain plant extract or active principle prepared from higher plants (Farnsworth*, 1985*). A survey carried out showed that 60% of population in Netherlands and Belgium and 74% in United Kingdom are in favour of complementary system of medicine being available within the frame work of their national health care system (WHO*, 1996*). Although traditional medicine has been practiced for several thousands of years, it only found a place in World Health Organization (WHO) programs in 1977 (Akerele*, 1991*). The long historical use of medicinal plants in many traditional medical practices, including experience passed from generation to generation has demonstrated the importance, the safety and efficacy of traditional medicine (WHO*, 2000*). World Health Organization (WHO) encourages the inclusion of herbal medicines of proven safety and efficacy in the

healthcare programs of developing countries because of the great potential they hold in combating various diseases (Amos *et al., 2001*).

WHO defined herbal medicine as finished, labelled medicinal products that contain as active ingredient aerial or underground part of a plant or other plant material or combination thereof whether in crude state or as plant preparation. Among the 45 plant drugs of known structure, derived from tropical rain forest species which include those that are of major importance in therapy none is currently produced through synthetic route (Akerele, *1991*). This may be due to the complex nature of their chemical structure and the fact that the natural sources are cheaper. Natural products can contribute to the search for new drugs in three different major ways:

a) By serving as new drugs that can be used in an unmodified state (e.g., vincristine from *Catharanthus roseus*).



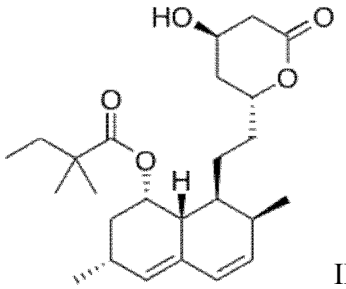
I

b) By providing chemical ‘‘building blocks’’ used to synthesize more complex molecules (e.g., diosgenin from *Dioscorea floribunda* for the synthesis of oral contraceptives).

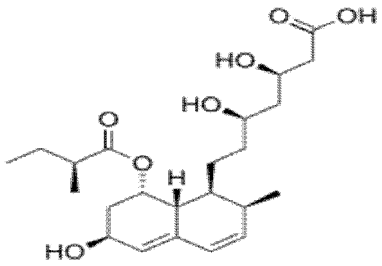
c) By indicating new modes of pharmacological action that allow complete synthesis of novel analogs (e.g., synthetic analogs of penicillin from *Penicillium notatum)*.

Drugs from natural sources may fall into one of these three categories of compounds: those that were isolated from biological microorganisms, those that are modified version

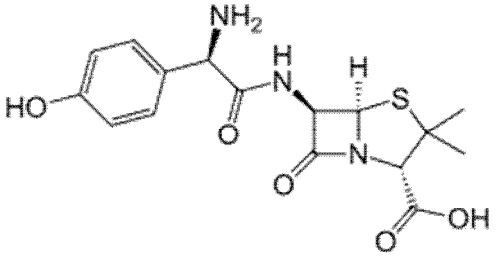
of the natural products and those that are completely synthetic, yet based on models of natural origin (Cragg *et al., 1997*). A recent survey revealed that 61% (*about 535)* of the 877 drugs introduced worldwide can be traced to natural products (Rouhi*, 2003*). It is noteworthy that some of the most important drugs for the past 50 years or so which has revolutionised the modern medical practices have almost all first been isolated from plants, and often from plants which for one purpose or another have been employed or used by the primitive or ancient societies (Schultes, *1986*). By year 2000, approximately 60% of all drugs in clinical trials for the multiplicity of cancers had natural origins. In 2001, eight ((II) simvastatin, (III) pravastatin, (IV) amoxicillin, (V) clavulanic acid, (VI) azithromycin, (VII) ceftriaxone, (VIII) cyclosporin and (IX) paclitaxel) of the 30 top-selling medicines were natural products or their derivatives, and these eight drugs together totalled US *$*16 billion in sales.



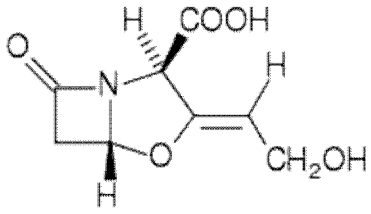
II



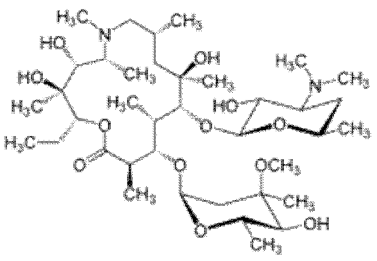
III



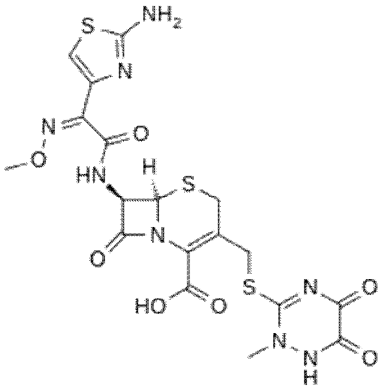
IV



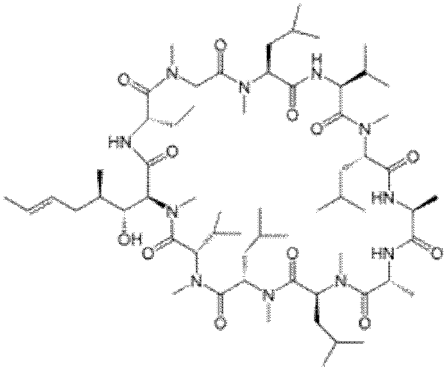
V



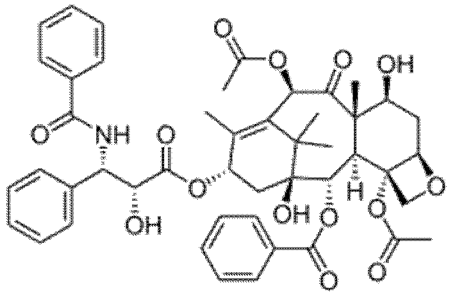
VI



VII



VIII



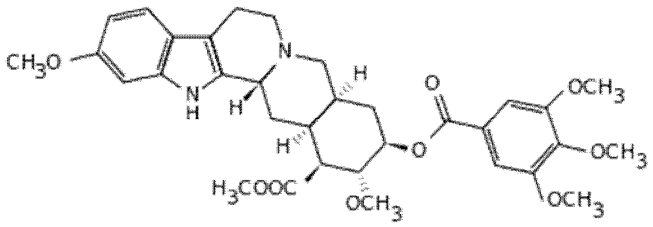
IX

Most or all of these wonder drugs such as curare alkaloids, penicillins and other antibiotics, cortisone, reserpine, vincoleucoblastine, the veratrum alkaloids, podophyllotoxin, strophantine and other new therapeutic agents came from ancient use (Schultes, *1986*). Because of the importance of traditional or herbal medicines it is now being incorporated into the Primary Health Care (PHC) systems of Mexico, People Republic of China, Nigeria and other developing countries of the world. The use of natural or traditional base products as leads are growing in " Natural Pharmaceutical Industries" in Europe and North America and in Nigeria through the Nigeria Institute for

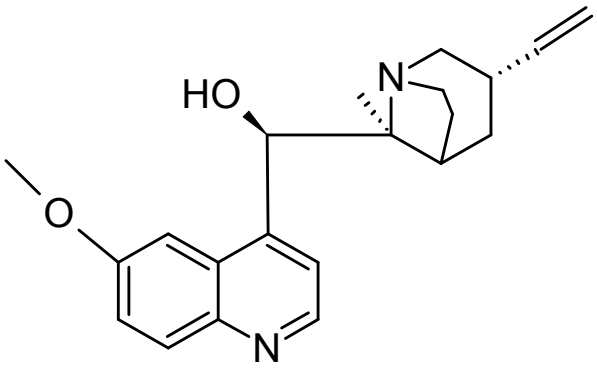
Pharmaceutical Research and Development (NIPRD) and the Raw Materials Research and Development Council (RMRDC) (Wambebe, 1988).

The Nigeria Institute for Pharmaceutical Research and Development (NIPRD) had developed three drugs from plants: (1) Niprisan® (Capsules and syrups) for the managements of sickle cell anaemia, (2) Nipripan® for management of ulcer disease and (3) Niprifan® a highly effective topical antifungal agent from the plant *Mitracarpus scaber* Zucc (Rubiaceae), these drugs are known to have passed clinical trials successfully and are waiting for drugs companies to legally take them for commercial productions. While the Raw Materials Research and Development Council (RMRDC) had sponsored some research areas in the Universities and other research based government institutions (Wambebe, 1988).

Plants are used in herbal medicine because they contain active principles or compounds that bring about the healing or curing of sicknesses and diseases. Plants in the tropics are often used as direct source of drugs, e.g. the alkaloid d-tubocurarine ($C_{37}H_{41}N_2O_6$) isolated from *Chondrodendron tomentosum* is used as muscle relaxant in surgery, and till date chemists are unable to produce it synthetically, although its analogue atracurium besylate was synthesized. It was found to have negligible cardiovascular effects when compared with d-tubocurarine. Other examples are the anticancer vinblastine ($C_{46}H_{60}N_4O_{13}S$) and vincristine ($C_{46}H_{58}N_4O_{14}S$) from *Catharantus roseus*; the tranquilisers rescinamine and reserpine (X) ($C_{33}H_{40}N_2O_9$) from *Rauwolfia serpentina* and the antimalarial quinine (XI) from Cinchona species.

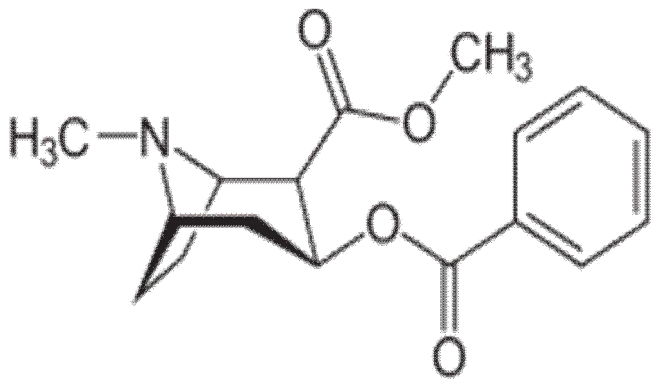


(X)

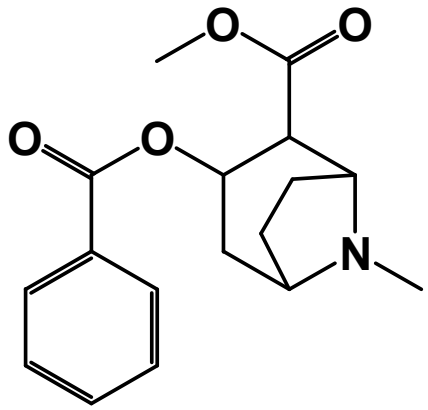


(XI)

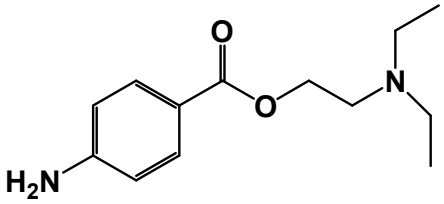
Plants from the tropics have produced compounds that served as lead for the synthesis of new drugs e.g. cocaine $C_{17}H_{21}NO_4$ (XII) from *Erythroxylum coca* has served as lead compound for the synthesis of a number of local anaesthetics such as procaine (XIII) and lignocaine (XIV).



(XII)

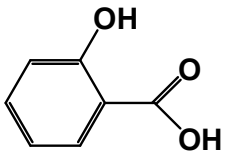


(XIII)

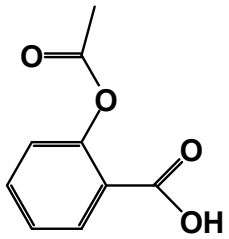


(XIV)

Another example is salicylic acid (XV) which was isolated from the bark of willow tree. It has potent analgesic, anticoagulant and antipyretic agent with severe gastrointestinal toxicity. To overcome this toxicity, it was converted to acetylsalicylic acid (XVI). Acetylsalicylic acid is still the most widely marketed analgesic agent world-wide (Newman *et al., 2000*).

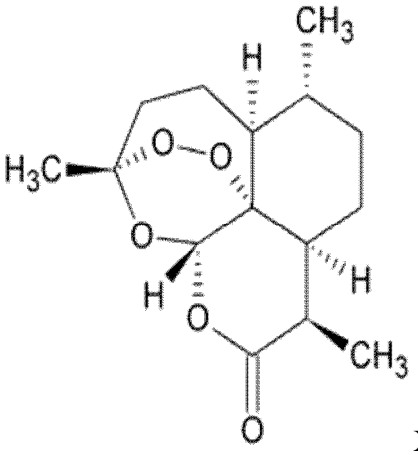


(XV)

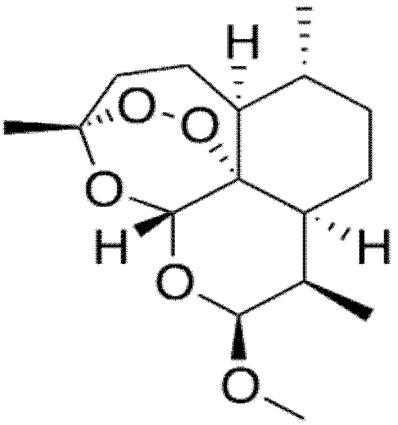


(XVI)

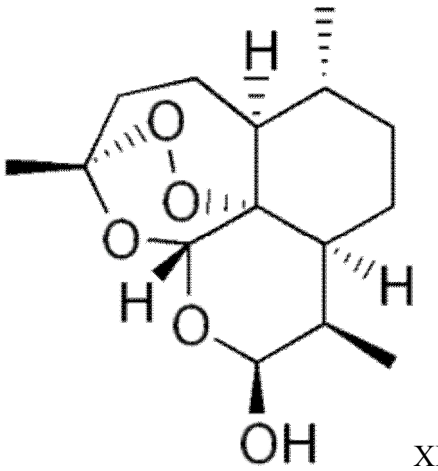
Plants can also serve as sources of starting material for the synthesis of a more complex semisynthetic compounds (drugs) e.g. sapogenins isolated from saponin extract of genus *Dioscorea* is used as starting material for the synthesis of steroids (Oldfield and Linn, 2012*)*. Also a potent antimalarial drug, a sesqueterpenoid endoperoxide named artemisinin (XVII) was isolated from *Artemesia annua* as a remedy against multi-drug resistance strain of *Plasmodium*. Based on the basic structure of artemisinin semisynthetic compounds have been produced. Such compounds include artemether (XVIII) and dihydroartemisinin (XIX) which are potent antimalarial drugs used world - wide (Newman *et al., 2000*).



XVII

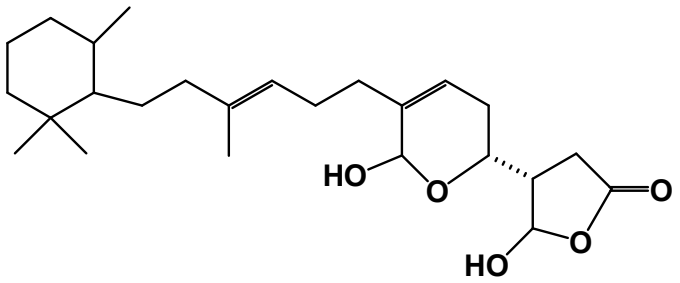


XVIII



XIX

The search for biologically active molecules from marine microorganisms is a relatively new but rapidly expanding branch of natural products chemistry. It was estimated that about 3,000 novel natural products were isolated in the last 30 years (De-Rosa, *2002*). These were isolated from sponges, molluscs, corals, and sea-dwelling microorganisms. An example of bioactive compound isolated from marine source is manoalide, ($C_{21}H_{31}O_5$) (XX) from a sponge, *Luffariella variabilis.* It is a 25-carbon marine natural product with anti-inflammatory activity which acts by selectively inhibiting cyclo-oxygenase (COX)-enzyme (De-Rosa, *2002*).



(XX)

Plant kingdom has been haphazardly investigated; some families have been relatively well studied while others were almost completely overlooked. In general, of the estimated 250,000 higher plant species discovered, only 15% (37,500) have been evaluated

phytochemically while about 6% (1,500) have been screened for biological activity (Fabricant and Farnsworth, *2001*). A major challenge to herbal medicine and development of drugs from herbal medicine is the rapid rate of extinction of plants among the global flora; the world genetic flora is rapidly diminishing. Tropical rain forest plants, which are found exclusively in developing countries, are being destroyed at the rate that many scientists believe to be unjustifiable from both economic and ecological perspectives (Principe, *1989*). According to International Union for Conservation of Nature and Natural Resources (IUCN) and the world wide fund for nature, 60,000 higher plant species could become extinct or near extinct by the middle of this century if the present trend continues (Principe, *1989*). The main causes of deforestation and subsequent extinction of plant species are: outgrowth of population and rural poverty shifting cultivation, agricultural conservation, fuel wood gathering and impact of large development projects (Allen and Bernes, *1985*).

Higher plants have been described as chemical factories that are capable of synthesising unlimited numbers of highly complex and unusual chemical substances whose structure could escape the imagination of synthetic chemist forever (Farnsworth, *1988*).; considering that many of these unique gene sources may be lost forever through extinction and that plants have a great potential for producing new drugs of great benefit to mankind, some action need to be taken to reverse the current apathy (Farnsworth, *1988*).

The validation of the folkloric claims of these medicinal plants will provide scientific basis for the conservation of tropical medicinal resources, the deployment of the beneficial ones as phytomedicine in the primary health care and the development of potential bioactive constituents. These could provide novel lead compounds or precursors in drug development and utilization of isolated compounds in the management of

infections as antimicrobial agents or drugs for treating bacterial, fungal, parasitic or viral infections. Examples of antibiotics in use are tetracycline, oseltamivir, terbinafine, lamivudie, etc, but their usefulness is being threatened or limited by the development of resistance by microorganisms (WHO, 2014). Therefore the need for continuous search for antimicrobial agents, and plants offer a ready one.

1.2 Statement of Research Problems

Infectious diseases continue to be the major causes of death across all age groups (WHO, 2014). With the rise of multidrug - resistant strains of bacteria and a lack of new antibiotic classes in the drug development, alternative strategies are needed to manage bacterial infections (DiGiandomenico, *et al.,* 2014). Antimicrobial resistance (AMR) threatens the effective prevention and treatment of an ever-increasing range of infections caused by bacteria, fungi, parasites and virus. Globally, 6% of new TB cases and 20% of previously treated TB cases are estimated to have Multi Drug Resistance-TB (WHO, 2014).

Hence the need to search for newer agents that are efficacious and are with less side effects.

1.3 Justification

The emergence of antimicrobial resistance (AMR) had lead to high cost, longer duration and failure of treatment leading to death of patients and threatens the effective prevention and treatment of an ever increasing range of infection caused by bacteria, fungi, parasites and virus (WHO, 2014). Antimicrobial resistance is presence in all parts of the world, new resistance mechanisms emerge and spread globally making infections that were hitherto treatable non treatable and now threaten to take the world back to pre antibiotic era (WHO, 2014).

It has been estimated that about 80% of the third world population is almost entirely dependent on traditional medicines for maintaining general health and combating many diseases (Srinivasan, 2005). Plants like *Piliostigma thonningii* among other uses are known to treat infectious diseases like conjunctivitis, cholera (Burkhill, 1995) and among the Igala people of North Central Nigeria the leaf infusion among other uses is used in making *ekamu* for treating loose stool or diarrhoea especially in teething children (Personal information) are available sources of alternative medicine to manage the emerging global antimicrobial challenges.

1.4 Aim and Objective(s) of the Study

Aim

To carry out phytochemical study and to establish the scientific bases for the traditional uses of the plant *Piliostigma thonningii* for treating microbial infections.

Objective(s)

(1) To identify the phytochemical constituents present in the plant.

(2) To isolate some of the compounds present in the plant.

(3) To elucidate the structure of the isolated compound(s).

(4) To establish the antimicrobial properties of the plant.

1.5 Research Hypothesis

Piliostigma thonningii contains phytochemical constituents with antimicrobial activities.

CHAPTER TWO

LITERATURE REVIEW

Piliostigma thonningii

2.1 Botanical classification.

The botanical classification of *P. thonningii* is as follows:

Kingdom:	Plantae
Division:	Angiosperms
Class:	Eudicots
Authority:	Schum. Milne – Redh.
(Unranked):	Resids
Order:	Fabales
Family:	Fabaceae
Subfamily:	Caesalpinioideae
Tribe:	Cercideae
Genus:	Piliostigma
Species:	*thonningii*
Botanical name:	*Piliostigma thonningii*

2.1.1 Common names: Camel's foot, monkey bread, Kalgo (Hausa), Omukpakpa ajalu (Igala), Mchekeche (Swahili), Kharub (Arabs)



Plate 1: *Piliostigma thonningii* tree on a farm at Sundimina in Birnin Kudu LGA of Jigawa State, Nigeria.

2.1.2 Morphology (description)

The plant *Piliostigma thonningii (Leguminocaea, Faba*ceae) is woody plant or tree of 4 – 15m in height with a rounded crown and a short but often crooked bole. The twigs are hairy. The bark is rough and longitudinally fissured, being creamy brown later. Leathery leaves up to 15 x 17 cm, bi - lobed one eight to one third the way down with a small bristle notch, glossy above and heavily veined and somewhat rusty hair below. Flowers with five white to pink petals, pendulous, unisexual with male and female usually on separate trees, ovary topped by a thick flattened globose stigma, pods are indehiscent, up to 26 x 7 cm with rusty brown hairs which wear off as the pods mature, becoming somewhat concorted as they age. The pods persist on the tree but finally fall and decay on the ground to pea sized seeds. An edible pulp surrounds these seeds. This species roots deeply this help them to resist or survive strong winds and also to get water in time of draught. The generic epithet piliostigma have cap like stigma. *Piliostigma thonningii* is

found commonly in open woodland and wooded grasslands of sub humid Africa at medium to low altitudes (Akerele, 1991).



Plate 2: The leaves and fruits of *Piliostigma thonningii.*

Morphologically *Piliostigma thonningii* is a woody plant found in open woodland and wooded grasslands of sub humid Africa. It is found throughout Africa except in Somalia and is always associated with *Annona senegalensis*, *Grewia mollis* and *Combretum spp* (Farnsworth, *1988*).

2.2 Ethno-medicinal (Traditional) uses of *Piliostigma thonningii* and other species in the genus

Piliostigma and other species in the genus have been reported to have a wide range of uses to mankind ranging from food for man and animals and also a wide range of medicinal uses (Ibewuike *et al., 1996*). The medicinal uses include treating loose stool in teething children, wound dressing, ulcers treatment, worms' infestation, arrest bleeding, inflammations, bacterial infections, gonorrhoea, stomach ache, headache, etc (Burkhil, *1995,* Ozolua *et al.,* 2009).

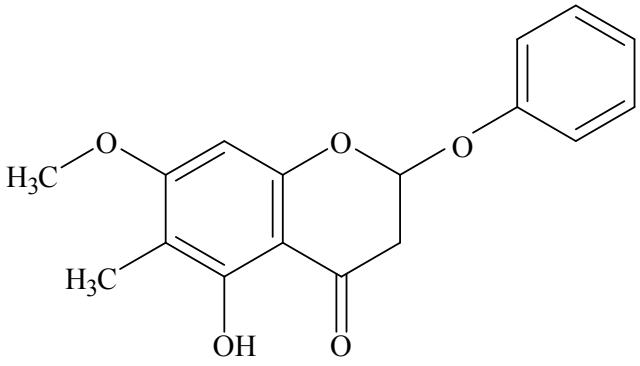
The roots and twigs have been used locally in the treatment of dysentery, fever, respiratory ailments, snake bites, hookworm and skin infections and the leaf extracts has been used for the treatment of malaria all over Eastern Nigeria (Kwaji *et al.,* 2010). The plant is used in ropes making, making of dyestuff or tanning of leather, household utensils, roofing ties, fencing, bridge building and farm implements, because they are deep rooted they are used as erosion control measures, the woods are used as stakes to support plants of weak stems or creepers like yams.

2.3 Phytochemistry of *Piliostigma thonningii* and other species in the genus.

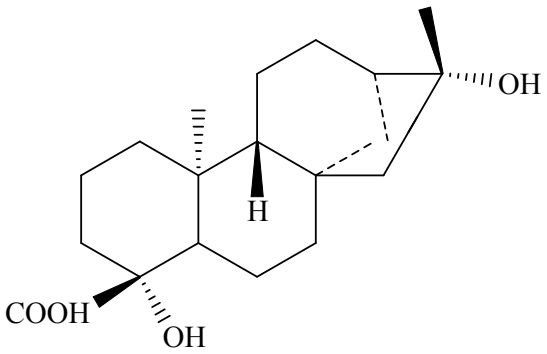
Some phytochemical investigations that have been reported on *Piliostigma thonningii*, showed that the plant contains a wide range of compounds, ranging from alkalloids, anthraquinones, flavonoids, glycosides, saponins, sterols and tannins (Bello *et al.,* 2013*).*

Some compounds have also been isolated from the plant, examples of such compounds include Piliostigmin (XXI), 16α - hydroxy - (-) - kauran-18-oic acid (XXII), (Ibewuike, *et al.,* 1996), other compounds isolated and found to have antibacterial and anti – inflammatory activity reported for the first time by (Ibewuike, *et al.,* 1996) 6, 8 – di – C - methyl quercetin 3 - methyl ether (XXIII), 6 – C - methyl quercetin 3, 7- dimethyl ether (XXIV**)** and 6, 8 – di – C - methyl quercetin3, 7 – dimethyl ether (XXV).

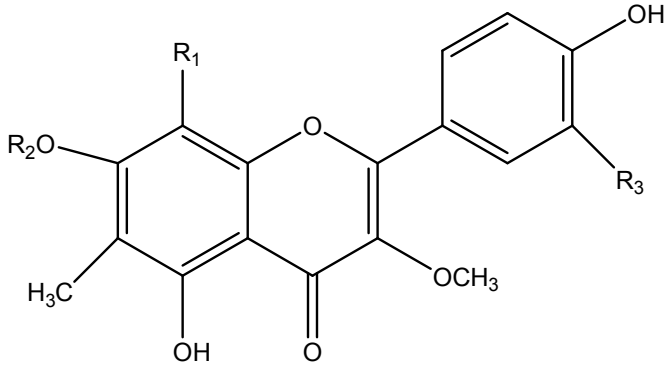
Other isolated flavonoids contributing to activity include quercetin, quercitrin, 6 – C - methyl quercetin 3,3,3 – trimethyl ether (XXVI) and 6, 8 – di – C - methyl kaempferol 3, 7- dimethyl ether (XXVII) (Ibewuike, *et al.,* 1996).



(XXI)



(XXII)



COMPOUND	R_1	R_2	R_3
XXIII	H	CH_3	OCH_3
XXIV	H	H	OH
XXV	CH_3	H	OH

XXVI	H	CH_3	OH
XXVII	CH_3	H	H

Although flavonoids are well known anti - inflammatory agents (Alcaraz and Jimenez*, 1988*), the anti-inflammatory activity of the relatively rare C - methyl flavonoids was reported for the first time by (Ibewuike *et al., 1997*).

A comparative study carried out on the only co – generic sp. *Piliostigma reticulatum* showed similarities both in chemical constituents and biological activity and can be conveniently substituted for each other (Ogundaini*, 2005*)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Solvents and Reagents

Solvents used were of general purpose grade and were distilled twice before used these include n – hexane, chloroform, ethyl acetate, methanol.

3.1.2 Equipments

Infra – red spectroscopy (IR): the IR was carried out on a pye – unicam genesis serried Fourier Transform Infra – red Spectrophotometer (FTIR).

Nuclear Magnetic resonance Spectroscopy was carried out on a Macintosh HD Mac NMR AC 400 MHz.

3.1.3 Collection, Identification and Preparation of Plant Materials.

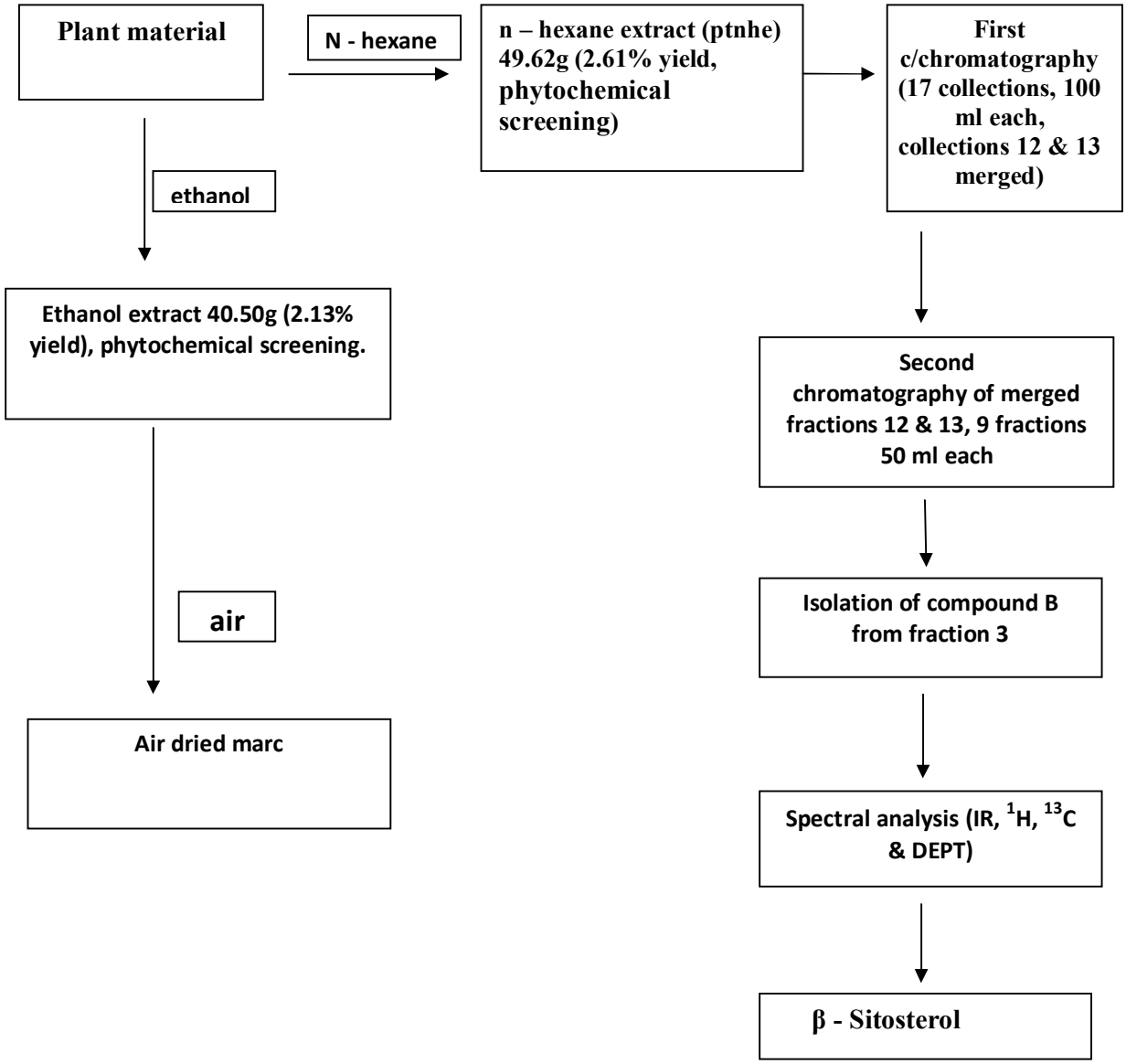
Leaves of the plant growing wild was collected from Sabon Gida in Ikara LGA, Kaduna state, Nigeria on the 15th of June, 2011 and was authenticated by Mr. Galla of the Herbarium unit, Department of Biological Sciences, Ahmadu Bello University, Zaria - Nigeria, where a voucher specimen No.171 was deposited. The leaves were air dried under the shade for 14 days and then powdered using pestle and mortar and hence referred to as the powdered plant material stored for use.

3.2 Methods

3.2.1 Extraction

The powdered plant material (1.9 kg) was macerated with 10 liters n – Hexane (95%) using a 20 litres Pyrex extraction bottle for 14 days, the solvent was recovered using a rotary evaporator. The n – hexane extract yielded a greenish residue (49.62g) and referred to as *Piliostigma thonningii* n - hexane extract (PTnHE). The marc was air – dried and

extracted with 10 litres ethanol (95%) for 7 days to yield a dark greenish residue (40.50g) and referred to as *Piliostigma thonningii* ethanol extract *(*PTEE*)*.



3.2.2 Preliminary Phytochemical Screening

A portion each of the ethanol extract and that of n – hexane extract were subjected to preliminary phytochemical screenings using standard methods as shown below:.

3.2.2.1 *Test for alkaloids*

0.5g of the extract was dissolved in 5ml of 1% aqueous hydrochloric acid on a water bath and filtered. The filtrate was divided into three. To the first portion few drops of freshly prepared Dragendorff reagent was added and observed for formation of orange to brownish precipitate. To the second portion 1 drop of Mayer reagent was added and observed for formation of white to yellowish or cream colour precipitate. To the third portion 1 drop of Wagner reagent was added to give a brown or reddish or reddish- brown precipitate. The presence of precipitate in most or all of the above reagents indicate the presence of alkaloids (Evans, 1989, Silva *et al., 1998*).

3.2.2.2 Tests for anthraquinones

(a) Free anthraquinones

0.5g of the extract was shaken with 10 ml of benzene for 5minutes, the content was filtered and 5ml of 10% ammonia solution was added to the filtrate, the mixture was shaken. Presence of a pink, red or violet colour in the ammonia layer (Lower phase) indicates the presence of free anthraquinones (Cannel, 1998).

(b) Anthraquinones glycosides

0.5g of the extract was boiled with 10 ml of aqueous Sulphuric acid and filtered while hot. The filtrate was shaken with 5ml benzene, the benzene layer was separated and half its own volume, 3ml of 10% NH_4OH solution was added and the mixture shaken. A pink, red or violet colouration in the ammonia phase (lower phase) indicates the presence of anthraquinones glycosides (Sofowora, 1993).

3.2.2.3 Test for carbohydrates/reducing sugar

(a) Molisch's test (general test for carbohydrates)**:**

To 2ml of the extracts dissolved in water was added three drops of Molisch's reagent followed by another three drops of concentrated sulphuric acid down the side of the test tube which was allowed to form a lower layer. A purple to violet coloured interface indicate the presence of carbohydrates. The mixture, when shaken gently and allowed to stand for two minutes, followed by dilution with 5ml of water would produce a dull violet precipitate – another indication of carbohydrates (Evans, 1989).

(b) Fehling's test for free reducing sugar:

5ml of a mixture (1:1) of Fehling solution A and Fehling solution B was added to 2ml of the extract dissolved in water and the mixture boiled on water bath for 5 minutes. A brick – red precipitate indicates the presence of reducing sugars (Trease and Evans, *1996*).

3.2.2.4 Tests for flavonoids

(*a*) *Shinoda test*

0.2g of the extract was diluted with ethanol. Few pieces of magnesium chips were added followed by a few drops of concentrated hydrochloric acid. Appearance of an orange, pink or red to purple colour indicates the presence of flavonoids. (Sofowora, *1993*).

(b) Sodium hydroxide test:

0.2g of the extract was dissolved in 2ml of water and filtered. 2ml of 10% aqueous sodium hydroxide was added. A yellow solution indicates the presence of flavonoids. On addition of dilute hydrochloric acid, the solution becomes colourless, (Brain and Turner, 1975).

(c) Ferric chloride test

0.1g of the extract was boiled with water and filtered. To 2ml of the filtrate, two drops of 10% freshly prepared ferric chloride solution was added; green, blue or violet colourations indicate the presence of phenolic hydroxyl group (Cannel, 1998).

3.2.2.5 Test for glycosides

About 100mg of the extract was dissolved in water. 1ml of strong lead subacetate solution was added and filtered. This was divided into two portions for the following tests:

(a) Kella - killiani's Test

To the first portion the residue was dissolved in 3ml of 3.5% ferric chloride solution in glacial acetic acid. The mixture was allowed to stand for a minute before it was transferred to a test tube. 1.5ml of concentrated sulphuric acid was added down the side of the test tube to form a lower layer on standing, the presence of a brown colour at the junction of the two liquids and a pale green colour in the upper layer indicates the presence of cardiac glycoside (Brain and Turner, 1975).

(b) Kedde's test for Cardenolides:

To the second portion of the filtrate 1ml of 2% solution of 3, 5 – dinitrobenzoic acid in methanol was added followed by 2ml of 5% aqueous sodium hydroxide solution. An immediate violet colour indicates the presence of cardenolides in the vegetable drug (Sofowora, 1993)

3.2.2.6 Test for saponins

(a) Frothing test: 0.5g of the extract was shaken with 5ml of water in a test tube for 30 seconds. A persistent froth for 15 minutes or when warm on water bath indicates the presence of saponins (Sofowora, 1993, Silva *et al., 1998*).

3.2.2.7 Tests for steroid and terpenoids

(a) ***Liebermann - Buchard test:***

0.5g of the extract was dissolved in 2ml of chloroform and filtered into a clean, dry test tube. 2 ml of acetic anhydride was added to the filtrate and shaken. Few drops of concentrated sulphuric acid were added carefully down the side of the test tube to form a lower layer. A brownish – red or violet ring at the zone of contact of the two liquids and the upper layer turning green denotes the presence of steroids and terpenes (Silva *et al.,* 1998).

(b) ***Salkowski test***

0.5g of the extract was dissolved in 1ml chloroform and to it 1ml of concentrated sulphuric acid was added down the test tube to form two phases. Formation of red or yellow coloration at the interface indicates the presence of steroidal ring (Sofowora, 1993, Silva *et al., 1998*).

3.2.2.8 Test for tannins

(a) Ferric chloride test: 0.5g of the extract was boiled with 5ml of water and filtered. To 2ml of the filtrate in a test tube, two drops of ferric chloride solution were added. A green or greenish black precipitate indicate the presence of condensed tannins, while a blue or bluish black precipitate shows the presence of hydrolysable tannins (Trease and Evans, *1996*).

(b) Lead subacetate test:

To 1ml of the aqueous extract, three drops of lead subacetate solution was added. A coloured precipitate indicates the presence of tannins (Silva *et al.,* 1998).

3.2.3 Chromatographic Procedures/Separation of n - hexane extract.

3.2.3.1 Column Chromatography of n - hexane extract

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

(a) Technique - Gradient eluent

(b) Column - Glass column with sintered disc at the bottom of 3.80 x 50cm 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 (Cannel, 1998), the sample was dissolve 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) Solvent for eluent - Various solvents systems were used depending on the materials. Eluent was carried out using one or mixture of the following solvents:

n- hexane, chloroform, ethyl acetate, acetone and methanol

7.0g of the n - hexane extract was directly applied onto a column of diameter 3.80 x 50cm and eluted gradually with n – hexane 100 %. n – hexane – chloroform mixture, gradually to chloroform 100 %, ethyl acetate 100 % and acetone 100 % The process was monitored using the thin layer chromatography. All collections were in 100 ml aliquots

Fractions were pooled together based on their TLC profile to obtain six fractions. fraction one was discarded because it was small in quantity and oily, fractions two to six were merged because they have TLC profile of five spots, while fraction seven was kept having a major spot and three minor spots but of small in quantity. Fractions eight to eleven were merged because they have three similar spots, fractions twelve and thirteen were merged because they showed two similar spots with same R_f values and fraction

fourteen was also kept, fractions fifteen to seventeen were merged and discarded as the TLC did not show any spot.

Fractions twelve and thirteen were merged and chromatographed on a small column of diameter 0.50 cm and 50 cm in length. The fractions were collected in 50 ml aliquot. Beginning with 100% n – hexane to get fraction number one, n – hexane - chloroform (90:10) to get fraction number two, n – hexane - chloroform (80:20) to get fraction number three and fraction number four, n – hexane - chloroform (70:30) to get fraction number five, n – hexane - chloroform (50:50) to get fraction number six, n – hexane - chloroform (40:60) to get fraction number seven, n – hexane - chloroform (30:70) to get fraction number eight and n – hexane - chloroform (00: 100) to get fraction number nine. The fraction number three above crystallised out and was coded compound B. compound B was re - crystallised with methanol and was found to be soluble in chloroform. On TLC with solvent system of chloroform: ethyl acetate 9:1 it gave an R_f 0.56. The compound B was subjected to melting point, chemical tests and spectral analysis.

3.2.3.2 Solubility of compound B

Compound B was soluble n – hexane and chloroform

3.2.3.3 Chemical tests on compound B

a) **Salkowski test:** Formation of red or yellow coloration at the interface indicates the presence of steroidal ring (Sofowora, 1993, Silva, *et al.,* 1998)

b) **Liebermann Buchard test:** A brownish – red or violet ring at the at the zone of contact of the two liquids and the upper layer turning green denote the presence of steroids and terpenes (Silva, *et al.,* 1998)

3.2.3.4 Spectral analysis of compound B.

The isolated compound B was sent for spectral analysis.

3.2.4 Antimicrobial screening

3.2.4.1 Test microorganisms

The microorganisms for the study used are clinical isolates which include *Staphylococcus aureus, Streptococcus feacalis, Bacillus cereus, Escherichia coli, Shigella dysenterae, Salmonella typhi, Pseudomonas aeruginosa, Proteus vulgaris, Candida albicans* and *Candida krusei.* All the microorganisms were obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Zaria.

3.2.4.2 Preliminary antimicrobial studies

The antimicrobial activities of the ethanol and the n – hexane extracts of *Piliostigma thonningii* were determined using ten hospital isolate microorganisms (mentioned above) obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Zaria.

0.6g of the extracts was weighed each and each dissolved in 10ml of Di-methyl sulfoxide (DMSO) to obtain a concentration of 60mg/ml of the extracts.

Mueller Hinton agar was the growth medium used for the microbes. The medium was prepared according to manufacturer's instructions. The medium was sterilised at 121^{o}C for 15 minutes and was poured into sterilised petri dishes, the plates were allowed to cool and solidify. Diffusion method was used for screening the extracts.

The sterilised and solidified media were seeded with 0.1 ml of the standard inoculums of the test microorganisms. Using standard cork borer of 6 mm in diameter, a well was cut at the center of each inoculated medium. 0.1 ml of the extracts of concentration 60 mg/ml was then inoculated into the cut well on the seeded medium. The inoculated plates were then incubated at 37^{o}C for bacteria and 27^{o}C for 24 hours (standardised) after which the plates were observed for zones of inhibition of growth. The zones were measured with a transparent ruler and the results recorded in mm.

3.2.4.3 Minimum Inhibitory Concentration (MIC)

The Minimum Inhibition Concentration of the extracts were determined using broth dilution method. The Mueller Hinton broth was prepared according to manufacturer's instructions, 10mls of the broth was dispensed into test tubes and the test tubes were sterilised at 121^{o}C for 15minutes and allowed to cool.

Mc Farland's turbidity scale number 0.5 was prepared to give a turbid solution.

Diluent (normal saline) was prepared and the test microorganisms were inoculated and incubated at 37^{o}C for bacteria and 27^{o}C for fungi for 6 hours.

Dilution of the test microorganisms in the normal saline was done until the turbidity marched that of the Mc Farland's scale by visual comparison, at this point the test microorganisms has a concentration of about 1.5 x 10^{8}cfu/ml

Two fold serial dilution of the extracts in the broth was made to obtain the concentrations of 60 mg/ml, 30 mg/ml, 15 mg/ml, 7.5 mg/ml and 3.75 mg/ml. the initial concentrations were obtained by dissolving 0.6 g of the extracts each in 10mls of the broth, 0.1ml of the test microorganisms in the normal saline was then inoculated into the different concentrations. Incubation was made at 37^{o}C for bacteria and 27^{o}C for fungi for 24 hours after which the broth was observed for turbidity. The lowest concentration of the extract in the broth which showed no turbidity was the MIC.

3.2.4.4 Minimum bactericidal concentration/ minimum fungicidal concentration (MBC/MFC)

Minimum Bactericidal Concentration/ Minimum Fungicidal Concentration (MBC/MFC)

Mueller Hinton agar was prepared according to manufacturer's instructions, sterilised at 121^{o}C for 15 minutes and was poured into sterile petri dishes, the plates were allowed to cool and solidify.

The contents of the MIC in the serial dilution were then sub cultured onto the Mueller Hinton agar, incubation was made at 37ºC for bacteria and 27ºC for fungi for 24 hours after which the plates were observed for colony growth. The MBC/MFC was/were the plate(s) with lowest concentration(s) without colony growth.

CHAPTER FOUR

RESULTS

4.1 Extraction

The powdered leaves (1.9 kg) of *Piliostigma thonningii* after maceration using 10 liters each of n – hexane (95%) and ethanol (95%) yielded respectively n – hexane and ethanol extracts in weight in grams (g) and percentage (%) as shown in Table 4.1 below:

Table 4.1: Percentage yields of n – hexane and ethanol extracts of *Piliostigma thonningii*

Extract	Colour	Weight	% yield
N - hexane	Green	49.62g	2.61
Ethanol	Dark green	40.50g	2.13

.

Preliminary phytochemical screening of the powdered leaves of *Piliostigma thonningii* reveals the presence of anthraquinones, carbohydrates, flavonoids and steroids as shown in Table 4.2 below:

Table 4.2: Preliminary screening of ethanol and n - hexane extracts of leaves of *P. thonningii*

Constituents	Test(s)	Observation(s)	Inference	
			Ethanol extract	n-exane extract
Alkaloids	Dragendorff	Precipitate seen	+	-
	Mayer's reagent test	Precipitate seen	+	-
	Wagner's reagent test	Precipitate seen	+	-
Anthraquinones	Free anthrquinones	Pink, red or violet colour produced in the ammonia layer (Lower phase)	+	+
	Anthraquinone glycosides	Pink, red or violet colour produced in the ammonia layer (Lower phase)	+	+
Carbohydrates	Molisch test	Purple colour at interphase	+	+
	Fehlings test	Precipitate seen	+	+
Flavonoids	Shinoda test	Red colouration produced	+	+
	NaOH test	Yellow colouration produced	+	+
	$FeCl_3$ test	Yellow colouration produced	+	+
Glycosides	Kella – Killiani Test	Purple ring at interphase produced	+	-
	Kedde's test	Purple – blue ring produced	+	-
Saponins	Frothing test	Frothing persists for □ 15minutes	+	-
	Haemolysis test	Haemolysis of the RBC	+	-
Sterols/terpenes	Liebermann – Buchard test	Red coloured ringed produced	+	+
	Salkowski's test	A reddish brown colour was formed at the interface	+	+
Tannins	Ferric Chloride test	Black colour formed	+	-
	Lead Sub-acetate Test	White precipitate formed	+	-

Key: + = Present, - = Absent

First column chromatography of n – hexane fraction using column of 3.8 cm x 50 cm a total of 17 fractions of 100 ml were collected as shown in Table 4.3 below:

Table 4: 3 Column chromatography of the n – hexane extract.

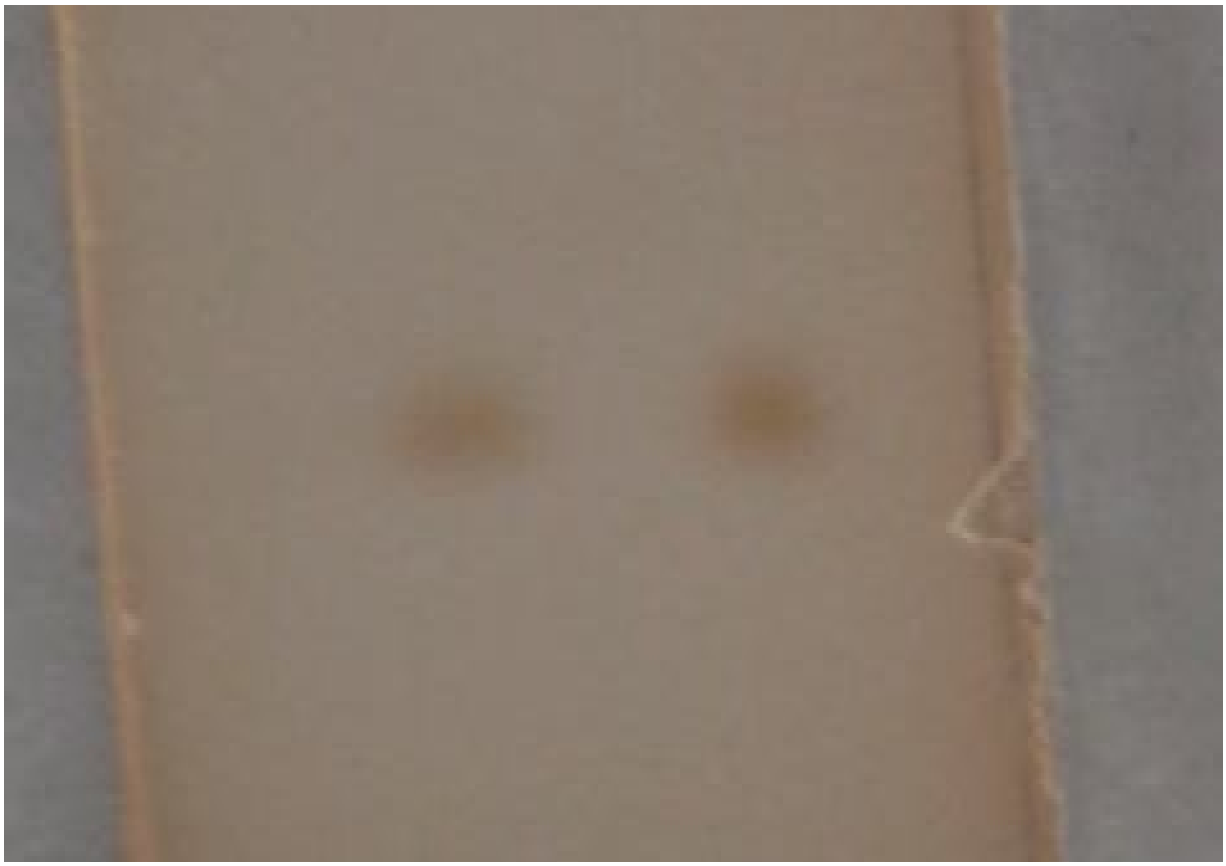
Solvent system	**Fractions**	**No. of fraction(s)**	**TLC Profile**
n – hexane 100%	1	100 ml x 1	oily
n – hexane 100%	2 – 5	100 ml x 4	5 spots
n – hexane: Chloroform 80:20	6	100 ml x 1	5 spots
n – hexane: Chloroform 60:40	7	100 ml x 1	1 major spot + 3 minor spots
n – hexane: Chloroform 60:40	8	100 ml x 1	3 spots
n – hexane: Chloroform 50:50	9	100 ml x 1	3 spots
n – hexane: Chloroform 40:60	10	100 ml x 1	3 spots
n- hexane: chloroform 00: 100	11	100 ml x 1	3 spots
Ethyl acetate 100%	12 & 13	100 ml x 1 each	2 spots Same R_f value merged
Ethyl acetate 100%	14	100 ml x 1	Kept
Acetone 100%	15 - 17	100 ml x 1 each	No spot discarded

Second column chromatography of n – hexane fraction using column of 0.5 cm x 50 cm a total of 9 fractions of 100 ml were collected as shown in Table 4.4 below:

Table 4: 4 Column chromatography of merged fractions number 12 and 13.

Solvent	Ratio	Fraction
n - hexane	100%	1
n - hexane - Chloroform	90 : 10	2
n - hexane **- Chloroform**	**80 : 20**	**3**
n - hexane - Chloroform	70 : 30	4
n - hexane - Chloroform	50 : 50	5
n - hexane - Chloroform	40 : 60	6
n - hexane - Chloroform	30 : 70	7
n - hexane - Chloroform	20 : 80	8
n - hexane - Chloroform	00 : 100	9

The TLC of compound B using the solvent system of n – hexane: ethyl acetate 9: 1 is as shown in plate 4.1 below:



Solvent system Hexane: EtOAL 9:1

Plate 4.1 the TLC of compound B.

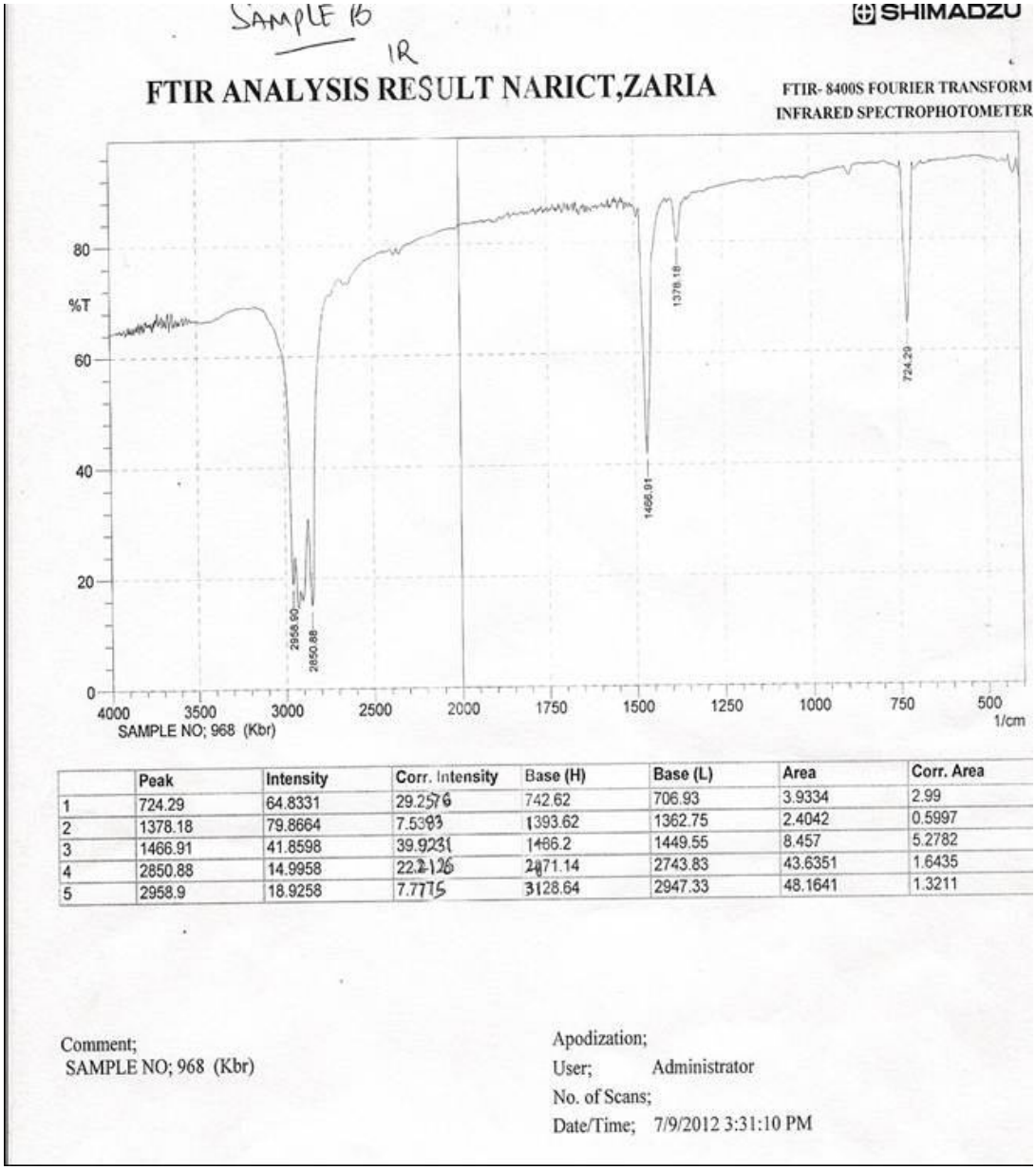
Compound B showed positive tests for steroids as shown in Table 4.5 below:

Table 4: 5 Chemical test on compound B.

Test	Result
Liebermann – Buchard test	**Red coloured ring produced**
Salkowski's test	**A reddish brown colour was formed at the interface**

The IR spectrum of compound B is as shown in Figure 4.1 below:

Figure 4.1 to 4.4 show the IR, ^{1}H, ^{13}C and DEPT spectra of compound B.



	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	724.29	64.8331	29.2576	742.62	706.93	3.9334	2.99
2	1378.18	79.8664	7.5393	1393.62	1362.75	2.4042	0.5997
3	1466.91	41.8598	39.9231	1486.2	1449.55	8.457	5.2782
4	2850.88	14.9958	22.2126	2871.14	2743.83	43.6351	1.6435
5	2958.9	18.9258	7.7775	3128.64	2947.33	48.1641	1.3211

Comment;
SAMPLE NO; 968 (Kbr)

Apodization;
User; Administrator
No. of Scans;
Date/Time; 7/9/2012 3:31:10 PM

Figure 4.1: IR spectrum of Compound B

The proton NMR spectrum of compound B is as shown in Figure 4.2 below:

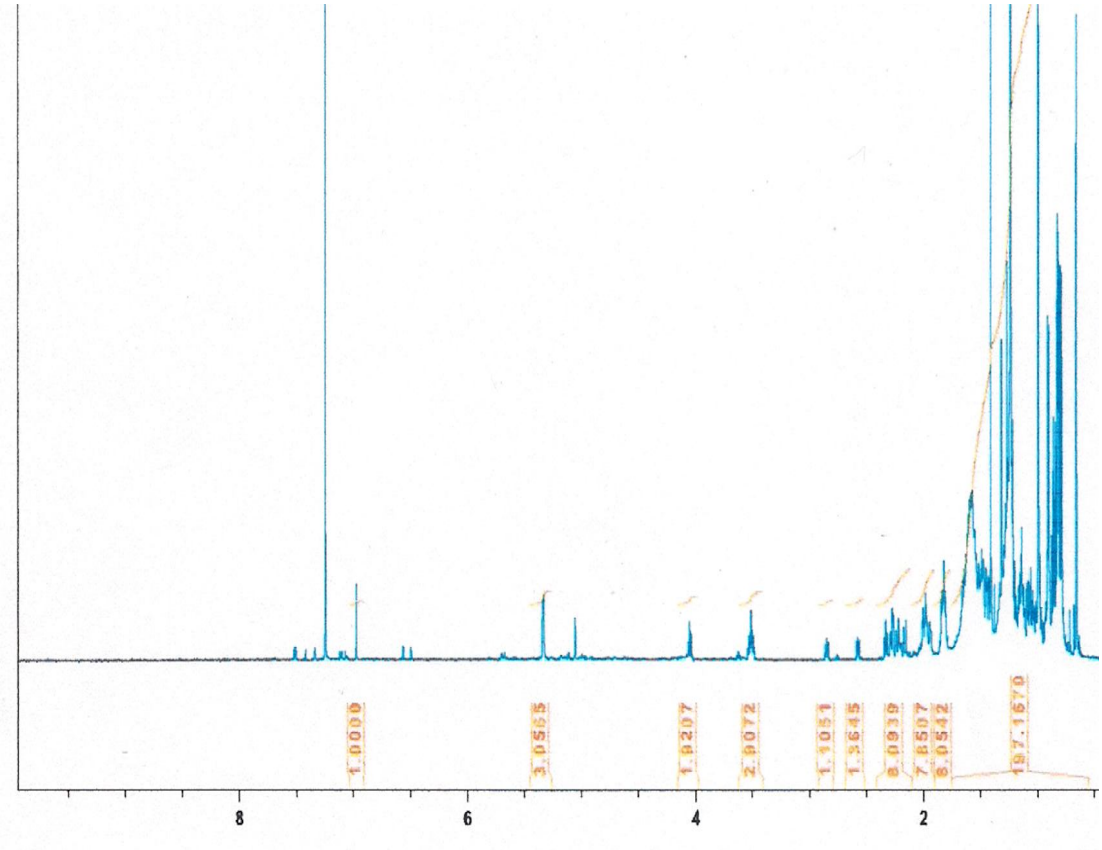


Figure 4.2: ^{1}H - NMR Spectrum of compound B

The ^{13}C – NMR spectrum of compound B is as shown in Figure 4.3 below:

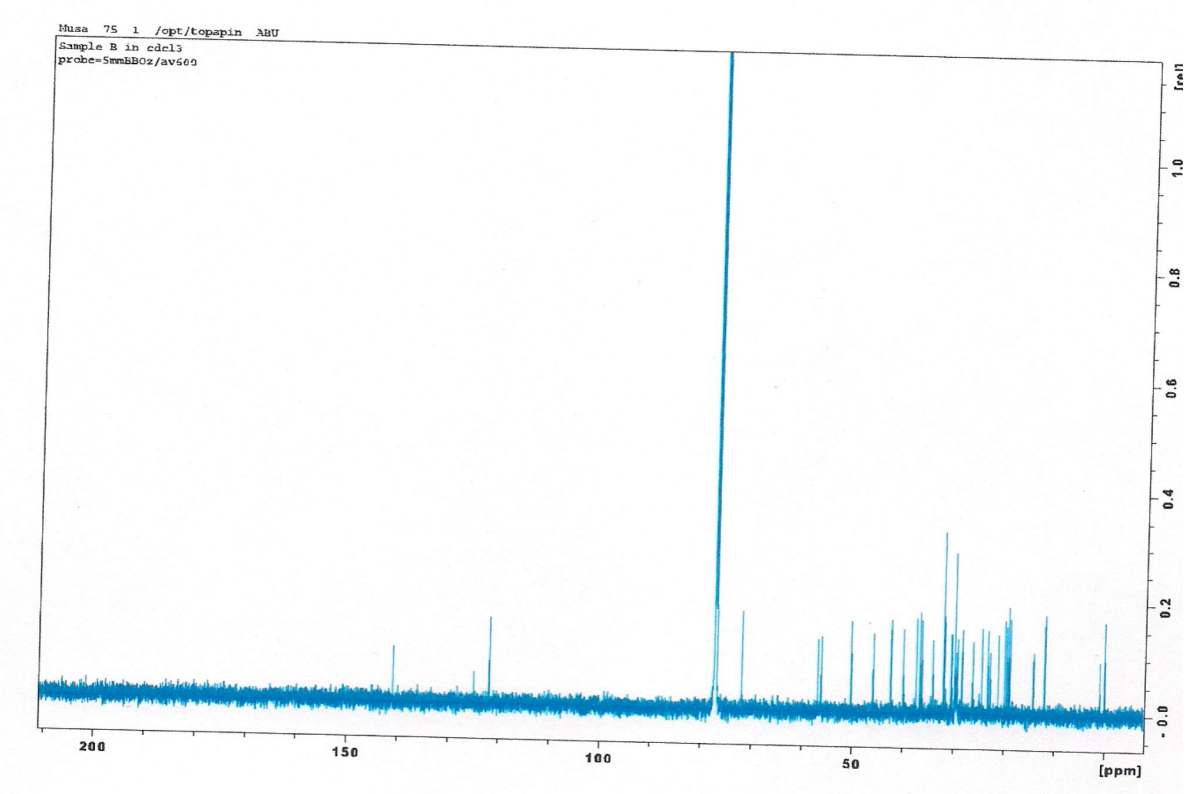


Figure 4.3: ^{13}C - NMR Spectrum of compound B

The DEPT – NMR spectrum of compound B is as shown in Figure 4.4 below:

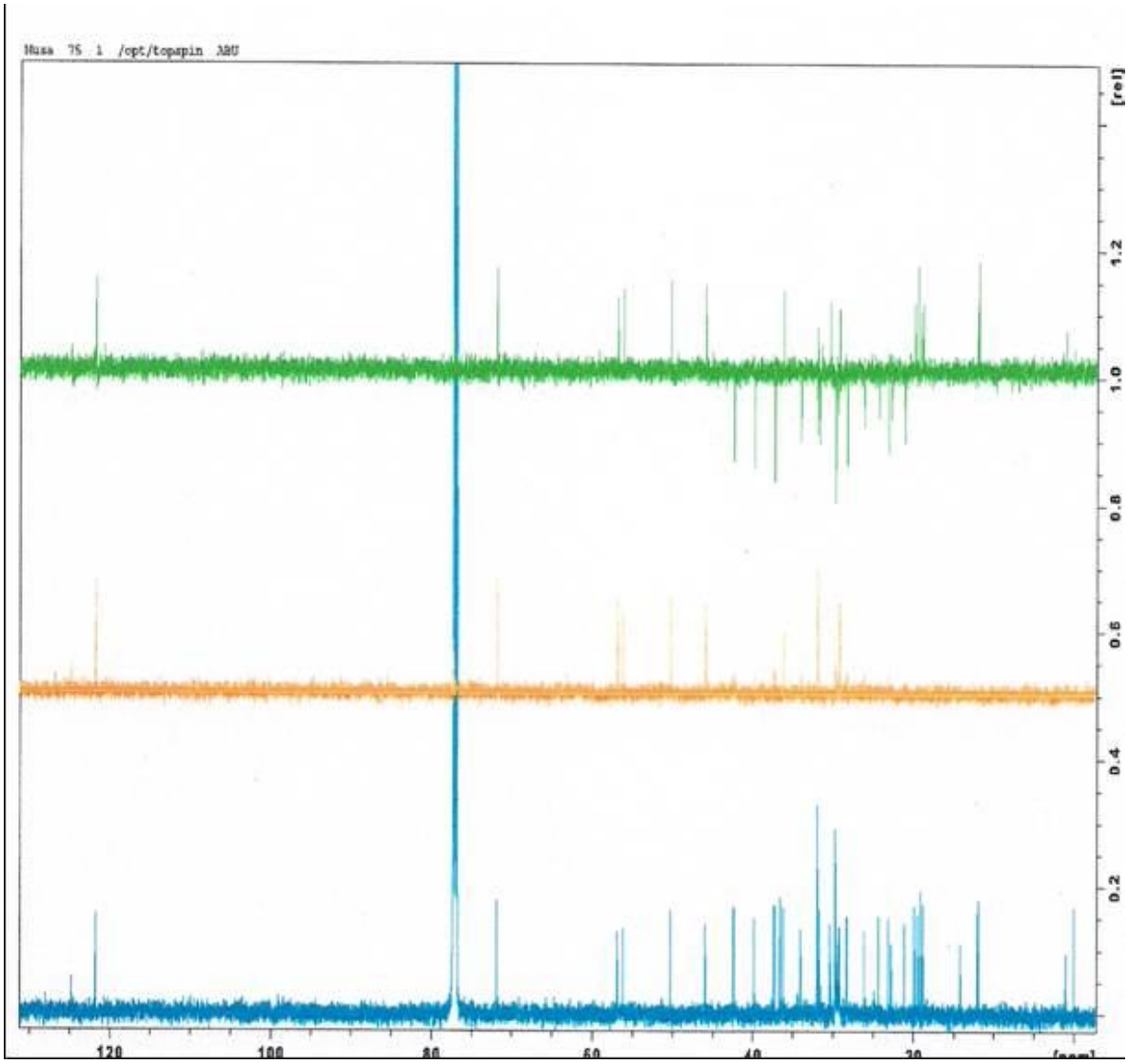


Figure 4.4: Distortionless Enhancement by Polarisation Transfer (DEPT) Spectrum of Compound B.

The result of the susceptibility test of the leaves extract of *Piliostigma thonningii* on the test organisms is as shown in Table 4.6 below:

Table 4: 6 Susceptibility test in mm of the leaves extracts of *P. thonningii* and the standard drugs on the ten microorganisms.

Test Microorganisms	Ethanol Extract	N – Hexane Extract	Sparfloxacin	Fluconazole
Staphylococcus aureus	27	16	45	-
Streptococcus feacalis	0	0	44	-
Bacillus cereus	0	0	46	-
Escherichia coli	22	15	40	-
Shigella dysenterae	25	15	40	-
Proteus vulgaris	0	0	0	-
Salmonella typhi	20	13	42	-
Pseudomonas aeruginosa	0	0	40	-
Candida albicans	22	16	0	34
Candida krusei	20	15	0	37

The results of the MIC and the MBC/MFC tests of the leaves extract of *Piliostigma thonningii* on the test microorganisms are as shown in table 4.7 below:

Table 4: 7 Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal/Fungicidal Concentrations (MBC/MFC) in mg/ml of the ethanol and n – hexane extracts of the leaves of *P. thonningii* on test microorganisms.

Test Microorganisms	**Ethanol extract**		**n – hexane extract**	
	MIC	MBC/MFC	MIC	MBC/MFC
Staphylococcus aureus	15	30	30	60
Streptoccocus feacilis	0	0	0	0
Bacilus cereus	0	0	0	0
Escherichia coli	15	60	30	60
Shigella dysenteriae	15	30	30	60
Proteus vulgaris	0	0	0	0
Salmonella typhi	15	60	30	60
Pseudomonas aeruginosa	0	0	0	0
Candida albicans	15	60	30	60
Candida krusei	15	60	30	60

CHAPTER FIVE

5.0 DISCUSSION

Extraction (Maceration), the results of the maceration showed that the n – hexane extract yielded 49.62 g (2.61%) and ethanol extract yielded 40.50 g (2.13%). The n – hexane extract was more in quantity because the maceration was for two weeks (14 days) thus allowing longer solvent – plant material contact resulting in more quantity of n – hexane soluble material while the ethanol maceration was for one week (7 days) resulting in short solvent – material contact time leading to less quantity of the material macerated even though ethanol is more polar and pick more groups of compounds.

Preliminary Phytochemical Studies, the result of preliminary phytochemical screening carried out on the crude ethanol and n – hexane extracts revealed the presence of flavonoids, tannins, steroids/triterpenes and saponins. These phytochemical constituents are known to possess many biological activities, oleanene, a saponin which occur in the root of umbellifera is used in Chinese medicine in the treatment of hepato-biliary disorders and also possess anti-inflammatory property (*Trease* and *Evans*, 2002). Tannins a major constituent of canberry juice has for long been used to treat bacterial infection of the bladder (Avorn *et al*., 1994). While some flavonoids have anti-tumor, antibacterial or antifungal property, they are used in domestic veterinary medicine, particularly in the form of ointment for treating dermal diseases (Trease and Evans, 1996).

Column Chromatography of n – hexane extract followed by crystallization using methanol lead to the isolation of compound B. The structure of the compound was confirmed by spectral data (IR, ^{1}HNMR, ^{13}CNMR and DEPT) and by compairism with literature (Pateh *et al.,* 2009).

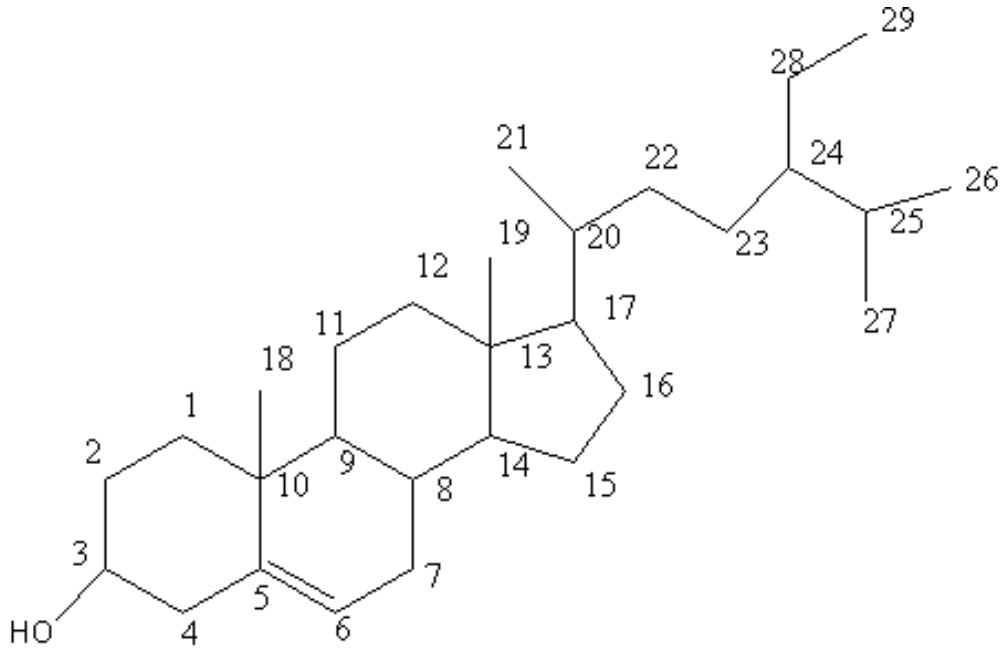
Elucidation of compound B, the IR spectrum of compound B showed five bands at 2958.90 (CH_2), 2850.88 cm^{-1} (CH), 1466.91 cm^{-1} (C = C), 1378.18 (C – H bending), 724.29 cm^{-1} (mono substituted C – H band), (Patra *et al.,* 2010, Khanam and Sultana, 2012).

The ^{1}HNMR show signals at δ 0.70, 0.96, 0.96, 0.96, 1.06 and 1.06 due to methyl protons, signals at δ 1.25, 1.25, 1.29, 1.38, 1.49, 1.52, 1.57, 1.60, 1.60, 2.04 and 2.23 due to methylene protons, signals at δ 1.25, 1.40, 1.44, 1.45, 1.47, 1.47, 1.64, 3.25 and 5.37 for methyne protons. The signals at δ 3.25 H(m) is an indication of a hydroxyl group on carbon 3, signals at δ 1.25, 1.25 and 1.29 are due to methylene groups on aliphatic chain while the signal at δ 5.37 is due to methyne proton on olefinic carbon (C_6). However, lack of signal at C_5, C_{10} and C_{13} shows lack of protons on these carbons, they are quaternary carbons (Patra *et al.,* 2010). The signal at δ1.24 to 1.57 show an indication of methylene proton (Aziz-ur-Rahman *et al.,2*005). The methyl signals observed at δ 0.96 1.24 and 1.26 is atypical of terminal methyl (CH_3) groups (Aziz-ur-Rahman *et al.,* 2005, Rajput and Rajput, 2012)

The ^{13}C – NMR spectra data of compound B (δ ^{1}H and δ ^{13}C) agrees with those reported by Patra *et al,* (2010). The signals at δ 121.7, 140.9 were assigned to olefinic unsaturated carbons (C_5 & C_6) and the signal at 71.7 to alcoholic carbon group (hydroxyl group attached at C_3) suggests strongly that the compound is a sterol. The signals at δ 11.9, 11.9, 18.8, 18.8, 19.4 and 19.8 were assigned to methyl carbons and those signals at δ 21.1, 23.2, 23.2, 24.3, 26.1, 31.7, 31.7, 34.0, 37.2, 39.8 and 41.9 were assigned to methylene carbons, those signals at δ 29.4, 31.4, 36.2, 42.3, 50.2, 56.1, 56.8, 71.7 and 121.7 were assigned to methyne carbons and those signals at δ 36.6, 45.9 and 140.9 were assigned to quaternary carbons.

The ^{13}C – NMR - DEPT spectra data of compound B (δ ^{1}H and δ ^{13}C) agrees with those reported by Patra *et al,* (2010), with signals assigned to 3 quaternary, 6 methyl, 11 methylene and 9 methyne), the nine methyne signals are shown at 90^{O} perpendicular to the base line, the eleven methylene signals are shown at 135^{O} as inverted signals, the six methyl signals are also shown at 135^{O} as perpendicular to the base line, the nine methyne signals are also repeated here. Signals observed at δ $140.9_{(S),}$ $121.9_{(d)}$ were assigned to double bond between C_5 and C_6 **(**olefinic carbons**)**, the signals at δ 26.1, 32.2 and 34.0 were assigned to aliphatic methylene groups. The signals at δ 11.9, 18.8 and 19.8 were assigned terminal methyl (CH_3) carbons and that at δ 21.1 was assigned to methylene carbon attached to a cyclic ring (Aziz-ur-Rahman *et al.,* 2005).

Based on the result of NMR spectra data and with comparison with literature the probable structure of compound B propose is:



Stigmast-5-en-ol (β - Sitosterol (C_{29} H_{50} O)) mp 135 – 137^{o}C

Antimicrobial Studies, the crude ethanol and n – hexane extracts showed strong activity against *Staphylococcus aureus, Escherichia coli, Shigella dysenterea, Salmonella typhi, Candida albicans* and *Candida krusei*. The two extracts have no activity against *Pseudomonas aeruginosa, Bacillus cereus, Streptococcus faecalis* and *Proteus vulgaris*. *Staphylococcus aureus* is known to play a significant role in skin diseases including superficial and deep follicular lesion (Srinivesan *et al.,* 2001), it is also implicated in causing sexually transmitted infections (STIs). So the strong activity of both the ethanol and n - hexane extracts indicates that the plant can be effective against skin and sexually transmitted infections.

It is important to note that the strong activity of both the ethanol and the n – hexane extracts against *Candida albicans* and *Candida krusei* indicates that the plant can be used as a good source of anti - fungal agent, for the treatment of those fungal diseases such as tinea (ringworm) and parasitic disease of dogs and cats which are caused by these microorganisms. The plant extracts can be used for the treatment of skin, hair and nail infections often cause by these microorganisms. It can serve as good agent for the treatment of thrush, vaginitis and other conditions such as pulmonary and generalized infections including endocarditis caused by *Candida albicans* (Thomas, *1979*).

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

Preliminary phytochemical screening of the ethanol and n - hexane extracts of *Piliostigma thonningii,* revealed the presence of alkaloids, anthraquinones, glycosides, flavonoids, saponins, sterols and tannins. Chromatographic investigation led to the isolation of β - Sitosterol. Antimicrobial studies on the ethanol and n - hexane extracts showed that the extracts exhibited antimicrobial activity against *Staphylococcus aureus, Salmonella typhi, Escherichia coli, Candida albicans, Candida krusei, and Shigella dysenterae* but they did not show antimicrobial activities against *Streptococcus feacalis, Pseudomonas aeruginosa, Bacillus cereus and Proteus vulgaris.* The n – hexane extract from which compound B was isolated and the ethanol extract showed antibacterial activity against *Staphylococcus aureus* which is one of the most important economic microorganisms in the research world today because it is resistance to most of the available antibiotics, this further showed the importance of medicinal plants as in the fight against *Staphylococcus aureus*.

6.2 Conclusion

Based on the findings in this work, it can be concluded that the use of *Piliostigma thonningii* in the treatment of diseases caused by sensitive bacterial and fungal infections has scientific basis. This research results are in agreement with some works that had been carried out before by (Ibewuike, *et al.,* 1996). The antimicrobial activity of the ethanol and the n – hexane extracts can be linked to β - Sitosterol isolated and other phenolic compounds present in the extracts.

6.3 Recommendations

We therefore wish to recommend that further work aimed at isolation, characterising and bioassay of compounds to explore other medicinal uses of the plant and in the search for other bioactive agents from nature.

References

Adjanahoun, E., Ahyi, M.R.A., Ake-Assi, L., Elewude, J.A., Dramane, K., Fudoju, S.D., Gbile, Z.O., Goudole, E., Johnson, C.L.A., Keita, A., Morakinyo, O., Ojewole, J.A.O., Olatunji, A.O and Sofowora, E.A. (1991). *Traditional Medicine and Pharmacopoeia Contributio*n to Ethnobotanical floristic studies in Western Nigeria, Pub. Organization of African Unity, Scientific Technical and Research Commission Lagos, Nigeria. 420p.

Ahmad, I., Aqil, F., and Owais, M. (2006). *Modern phytomedicine*. *Turning medicinal plants into drugs.* KGaA, Weinheim: Wieleyvch Verlag GmbH & Co; p. 200.

Akerele, O. (1991): The conservation of medicinal plants in: Akerele, O., Heywood, V. and Synge H, (EDs); Conservation of medicinal plants; Cambridge University press (UK) Pp. 3 - 11.

Alcaraz M.J. and Jimenez M.J. (1988). Flavonoids, an anti-inflammatory agents. *Fitoterapia* 59: 25 - 38.

Allen J.C. and Barnes D.F. (1985).The causes of Deforestation in Developing countries. *Annal of the Association of American Geographers* 75(2): 163 - 184.

Amos, S., Kolawale, E., Akah, P., Wambebe, C., and Gamaniel, K. (2001). Behavioral effects of the aqueous extract of *Guira sengalensis* in mice and rats. *Phytomedicine*, 8 (5): 356 - 361.

Anonymousymous (1975). A pictorial history of herbs in medicine and pharmacy, *Herbalgram* 33 – 47.

Avorn, J. Monane, M. Gurwitz, J.H. Glynn, R.J. Choodnovskiy, I. and Lipitz, L.A. (1994). Reduction of bacteriuria and Pyuria after Ingestion of Cranberry Juice. *Journal of the American Medical Association,* 271: 751 - 4.

Aziz-ur-Rahman, N. K. Abbasi, M.A., Khalid, H. Khan, K.M. Ashraf, M. Ahmad, I. and Ijaz, S.A. (2005). Synthesis, spectral characterization and biological activity of S - substituted derivatives of 5-(4-nitrophenyl)-1,3,4- oxadiazole-2-thiol. *Asian J. Pharm. Health Sci*., 2(3): 370 - 376.

Barnes, P. M. Bloom, B. And Nahin, R. (2008). Complementary and alternative medicine use among adults and children: United States. CDC National Health Statistics Report No. 12.

Bello, O. M.. Zack Agbendeh M. and Adikwu J. G. (2013). The comparative studies of Phytochemical Screening of *P. thonningii* root and leaves extracts. *Asian Journal of Plant Science and Research*, 3(6): 74 – 77.

Brain, K. R. and Turner, T.D. (1975). *The Practical Evaluation of Phytopharmaceutical* Wright Scientechnica, Bristol. Pp. 45, 75 – 88, 98 – 107 and 152.

Burkill, H.M. (1995). *The Useful Plants of West Tropical Africa*. Royal botanic garden Kew (UK), 3: 146 - 150.

Cannel R.J.P. (2000). How to approach the Isolatiopn of Natural Product. In: Cannel R.J.P. (ed.) *Natural Product Isolation*., Humana press Totowa, New Jersey (USA). Pp. 1 - 52.

Cragg, G. M., Newman, D. J. and Snader, K. M. (1997). Natural products in drug discovery and development. *Journal of Natural Products*, *60*: 52-60.

De – Rosa, S. (2002) Mediterranean marine microorganisms as source of new potential drugs. In: *Natural Products in the New Millennium:* Prospects and Industrial Applications (Rauter A, Palma FB, Justino J, Araujo ME, Santos SP, eds.) The Netherlands: Kluwer Academic Publishers Pp. 441–461.

DiGiandomenico, A. Keller, A.E. Gao, C. Rainey, G.J. Warrener, P. Camara, M. M. Bonnell, J. Fleming, R. Bezabeh, B. Dimasi, N. Sellman, B. R. Hilliard, J. Guenther, C. M. Datta, V. Zhao, W. Yu, X. Q. Suzich, J. A. And Stover, C. K. (2014). A multifunctional bispecific antibody protects against *Pseudomonas aeruginosa*. Science Translational Medicine **6**, Pp. 262.

Djukanovic V. and Mach, E.P. (1975). *Alternative Approaches to Meeting Basic Health Needs of Populations in Developing Countries: A Joint UNICEF/WHO Study* (Geneva: WHO, 1975), 110.

Elujoba, A. A. (1999). Pharmacognosy for Health and Culture – the PHC Jungle Connection. Inaugural Lecture Series 134OAU Press Ltd, Ile Ife – Nigeria Pp. 9 – 10.

Evans, W.C. (1989). Trease and Evans' *Pharmacognosy,* 13Th edition Bailiere Tindal, London Pp. 15 – 57, 342 – 345, 480 – 517, 546 and 765 – 775.

Fabricant, S.D. and Farnsworth, N.R. (2001). The value of plants used in traditional medicine for drug discovery. *Enviromental Health Perspectives,* 109 (1): 69-75.

Farnsworth N.R. (1984): How can the well be dry when it is filled with water? *Economic Botany*, 38: 4 – 13.

Farnsworth N.R. (1988): Screening plants for new medicines. In: Wilson E.O. (Ed.) *Biodiversity*. Washington D.C. National Academy press (USA). Pp. 83 – 97.

Farnsworth N.R., Akerele, O., Bingel, A.S., Soejarto, D.D. and Guo, Z. G. (1984). Medicinal plants in therapy, *WHO* 63: 965-981.

Gibbons, S. and Gray, A.I. (1998). Isolation by planar Chromatography. In: Cannel R.J.P. (ed.) *Natural products isolation*; Humana press, Totowa New Jersey (USA), Pp.209-246.

Habib, M. R. F. Nikkon, M. Rahman, M.E. Haque and M.R. Karim, (2007). Isolation of stigmasterol and beta sitosterol form methanol extract of root of bark of *Calotropis gigantean* (Linn.). Pak. J. Biol. Sci., 10: 4174-4176.

Hamann, O. (1988). The Joint IUCN – WWF Plants Conservation Programme and its Interest in Medicinal Plants. In: Akerele O., Heywood V. and Synge H. (Eds) Conservation of Medicinal Plants. Cambridge University Press, Cambridge.

Ibewuike, J. C. Ogungbamila, F.O. Ogundaini, A. O. Okeke, I.N. and Bohlin, L. (1996). Antiflammatory and antibacterial activities of C-methyl flavonoids from Pilistigma thonningii. Society of Chemical Industry. John Wiley and Sons, Ltd 61(2): 186 - 190.

Ibewuike, J.C., Ogungbamila, F.O, Ogundaini, A.O., Okeke, I.N. and Bohlin, L. (1997). Antiinflammatory and antibacterial activities of C - methylflavonols from *Piliostigma thonningii*. *Phytotherapy Research* 11(4): 281 – 284.

Khanam S and Sultana, R. (2012) Isolation of β-sitoterol and stigmasterol as active immunomodulatory constituent from the fruits of *Solanum xanthocarpum* (Solanaceae). *Indian Journal of Plant Science and Research*, 3(4): 1057 – 1060

Kwaji, A., Bassi, P.U., Aoill M., Nneji, C.M. and Ademowo, G. (2010). Preliminary studies on *Piliostigma thonningii* Schum leaf extract: Phytochemical screening and *in vitro* antimalarial activity. *African Journal of Microbiology Research*

Vol. 4(9), pp. 735-739, 4 May 2010. Available online http://www.academicjournals.org/ajmr

Li, C., Bu, P.B, Yue, D.K. and Sun, Y.F. (2006). Chemical constituents from roots of *Ficus hirta*. *Zhongguo Zhong. Yao. Za. Zhi*. 31: 131-13

Muhit, M.A., A.M. Tareq, A.S. Apu, D. Basak and M.S. Islam, (2010). Isolation and identification of compounds from the leaf extract of *Dillenia indica* Linn. *Bangladesh Pharmaceutical Journal* 13: 49 – 53

Newman, D. J.,Cragg, G. M. and Snader, K. M. (2000). The influence of natural products upon drug discovery. *Natural Product Reports, 17*: 215 - 234.

Ogundaini, A. O. (2005). *From Greens into Medicine Taking a Lead from Nature.* Inaugural Lecture Series 176. O.A.U. Press Limited, Ile – Ife, Nigeria Pp 12 – 15

Oldfield, E. and Linn, F.Y. (2012). Terpene biosynthesis: Modularity rules. *Angewandle Chemie* 51: 1124 – 1137

Ozolua, R.I., Alonge, P. and Igbe, I., (2009). Effects of leaf extracts of *Piliostigma thonningii* Schum. on aortic ring contractility and bleeding time in rats. *Journal of Herbs, Spices and Medicinal Plants* 15(4): 326 – 333.

Partnership for Maternal, Newborn and Child Health (PMNCH) Summary Report September 16 – 22, 2007.

Patra, A., S. Jha, P.N. Murthy, Manik and A. Sharone, (2010). Isolation and characterization of Stigmast-5-en-3β-ol (β-sitosterol) from the leaves of *Hygrophila spinosa* T. Anders. *International Journal Pharma Sciences and Research*, 1: 95-100.

Plowman, T. (1984). The origin, evolution, and diffusion of coca, Erythroxylum spp., in South and Central America. *Papers PeabodyMuseum Archaeol. Ethnol*., 76: 125 – 163

Principe, P. (1989). The Economic Significance of Plants and their Constituents as Drugs. In Wagner H., Hikino H. and Farnsworth N. *Economic and Medicinal Plant Research,* Vol.3, London: Academic Press, pp.1 - 17.

Rajput, A. P. and Rajput, T.A. (2012). Isolation of Stigmasterol and β-Sitosterol from Chloroform Extract of Leaves of *Corchorus fascicularis* Lam. *International Journal of Biological Chemistry, 6: 130-135*.

Rouhi, A.M. (2003). Rediscovering natural products. *Chemical and Engineering News*, 81: 77 – 91

Schultes, R. E. (1986). Ethno pharmacological Conservation: A key to progress in medicine. *Operabotanica* 92: 217 - 224.

Shok, M. (1999). Allopathic (Othodox) and Alternative (Homeopathic, Traditional, Herbal, etc) Medicines; The Need for Productive co – operation. An Inaugural Lecture, ABU Press Ltd Nigeria. Pp. 9 – 10

Silva, G.L., Lee, I. and Douglas, K.A. (1998). Special problems with extraction of plants .In: Cannel, J.P.R. (Ed.). *Natural Products Isolation.* Humana press publishers, New Jersey (USA). Pp 356 - 358.

Snowdon, J.A. and Cliver, D. O. (1996). Microorganisms in honey, review article. *Internatinal Journal of Food Microbiol*.: 31:1 – 26.

Sofowora, A. (1982). *Medicinal Plants and Traditional Medicine in Africa.* 2nd Edition. John Wiley and Sons Ltd.New York, 214p.

Sofowora, A. (1993). *Medicinal Plants and Traditional Medicine in Africa,* 2nd Edition. John Wiley and Sons Ltd. New York. P. 85, 174, 195 - 198.

Srinivasan, D., Nathan, S., Suresh T. and Perumalsam, P.Z. (2005). Antimicrobial activities of certain Indian medicinal plants used in Folkloric medicine. *Journal of Ethnopharmacology,* 74 (3): 217 - 220.

Srinivasan, K. (2005). Plant Foods in the management of Diabetes Mellitus: species as beneficial antidiabetic food adjuncts. *International Journal of Food Sciences and Nutrition.* 56: 399 – 414.

Thomas, C. G. (1979). *Medicinal Microbiology* 4th ed. Pp 356 - 358 Published in Great Britain.

Trease and Evans, (2002). *Carbohydrates in Pharmacognosy,* 5TH edition, Ed. W.C. Evans, Harcourt Publisher Limted, London,Pp. 193.

Trease, G. E. and Evans, W. C. (1983). *Textbook of pharmacognosy,* 12th (Ed) Balliers Tindall *publisher Limited,* London, United Kingdom. Pp. 343-384.

Trease, K. and Evans, W.C. (1996). *Text book of Pharmacognosy,* 14th edition, Harcourt Publisher Brace and Company, Asia PTE LTD., Pp 251 - 293. Vol.1 (2), 2010, 95-100.

Trivedi, P.C. and N. Choudhrey, (2011). Isolation and characterization of bioactive compound β - Sitosterol from *Withania somnifera* L. *J. Pharmacy Res.,* 4: 4252-4253.

Wambebe, C. (1988). Development of standardised phytomedicines in Africa. *Journal of Pharmaceutical Research and Development* 3: 1 – 11.

WHO (1996). *Facts sheets on traditional Medicine.* Traditional medicine in Europe and America, 3:6

WHO (2000). *General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine* Pp.1 - 20.

WHO (2014) Global Report on Surveillance of Antimicrobial Resistance, with data provided by 114 countries. N^{o} 194. Updated April, 2014

Zamora - Martinez, M. C. (1992). Medicinal plants used in some rural populations of Oaxaca, Puebla and Veracruz, *Mexico J. Ethnopharmacol,* Vol – 37 (3), 179 – 196.

The spectral data of compound B compared with the literature is as shown in Table 4.8 below:

Table 4.8: Spectral data of compound B with the literature reported in ppm.

Position	Group	Compound B ^{13}C (δ)	Reference ^{13}C (δ)	Compound B ^{1}H(δ)	Reference ^{1}H(δ)
1	CH_2	37.2	37.28	1.38	1.47
2	CH_2	31.7	31.69	1.57	1.56
3	CH	71.7	71.82	3.25	3.52
4	CH_2	41.9	42.33	2.23	2.28
5	C	140.9	140.70	-	-
6	CH	121.7	121.72	5.37	5.36
7	CH_2	31.7	31.69	2.04	2.03
8	CH	31.4	31.93	1.45	1.67
9	CH	50.2	50.17	1.44	1.48
10	C	36.6	36.52	-	-
11	CH_2	21.1	21.10	1.52	1.52
12	CH_2	39.8	39.80	1.49	1.49
13	C	45.9	45.88	-	-
14	CH	56.8	56.79	1.40	1.50
15	CH_2	24.3	24.37	1.60	1.60
16	CH_2	23.2	23.25	1.60	1.84
17	CH	56.1	56.09	1.47	1.49
18	CH_3	11.9	11.88	0.70	0.68
19	CH_3	19.4	19.40	1.06	1.02
20	CH	36.2	36.52	1.64	1.64
21	CH_3	18.8	18.79	1.06	0.94
22	CH_2	26.1	26.14	1.25	0.88
23	CH_2	34.0	33.98	1.25	1.04
24	CH	42.3	42.33	1.25	1.50
25	CH	29.4	28.91	1.47	1.65
26	CH_3	19.8	19.80	0.96	0.83
27	CH_3	18.8	18.79	0.96	0.85
28	CH_2	23.2	23.10	1.29	1.04
29	CH_3	11.9	11.99	0.96	0.88