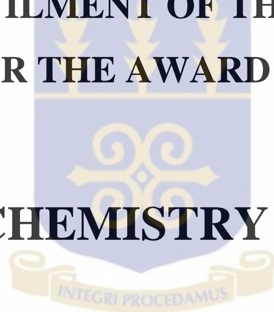
University of Ghana http://ugspace.ug.edu.gh

PHYTOCHEMICAL CONSTITUENTS AND BIOLOGICAL ACTIVITY STUDIES OF THE STEM OF *DICHAPETALUM MADAGASCARIENSE*

THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON

IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF



MPHIL CHEMISTRY DEGREE

BY

ALHAJI MAHAMA APPIAH (BSc.)

(10357509)

DEPARTMENT OF CHEMISTRY
UNIVERSITY OF GHANA, LEGON

JULY, 2013

University of Ghana http://ugspace.ug.edu.gh

DEDICATION

To Almighty ALLAH

University of Ghana http://ugspace.ug.edu.gh

DECLARATION

It is hereby declared that this thesis is my original work produced from research undertaken under supervision and has not been presented for any other degree in this University or another University elsewhere.

……………….……………………..

Alhaji Mahama Appiah (10357509)

(Candidate)

……………………………… ……………….……………

Dr. Mary Anti Chama Dr. (Mrs.) Dorcas Osei-Safo

(Supervisor) (Supervisor)

University of Ghana http://ugspace.ug.edu.gh

ACKNOWLEDGEMENT

All Glory, Honour and Adoration are due to Almighty Allah, for His Guidance and Protection throughout this work.

I acknowledge the effort and assistance of the following:

My Supervisors, Dr. Mary Anti Chama and Dr. (Mrs.) Dorcas Osei-Safo for their constructive criticisms and ideas that made this thesis a reality. Their patience, commitment, insight and encouraging words inspired me and kept me going even when the going was tough. Dr. Mary Anti Chama deserves special commendation in this regard for spending sleepless nights, sacrificing her comfort for the successful completion of this work. Special thanks also go to Prof. I. Addae-Mensah for his immerse contribution to the completion of this project. I can only ask for God‘s blessings for them for influencing my life immensely.

Thanks and appreciation also go to Rev. Dr. W.S.K. Gbewonyo of Department of Biochemistry, Cell and Molecular Biology and Mr. Joseph Otchere of Noguchi Memorial Institute for Medical Research who responded with keen interest to the biological activity studies. They taught and supervised every aspect of the studies. Rev., thanks for all the moral, material and spiritual support. To Mr. Joseph Otchere, I really appreciate the friendship you shared with me.

All the staff in the Department of Chemistry, University of Ghana, especially Mr. J.J. Harrison for his encouragement and pieces of advice. He has actually inculcated the spirit of self-confidence in me. He also read through the thesis and offered helpful suggestions. I thank Prof. W.A. Asomaning, Prof. R.K. Adaboh and Dr. Klakey for their encouragement.

Dr. Reiner Waibel of the University of Erlangen, Germany for running the ^{1}H and ^{13}C NMR. Thanks also to Michael Abbey of Phyto-Riker (GIHOC) for running all the IR spectra. A lot of

University of Ghana http://ugspace.ug.edu.gh

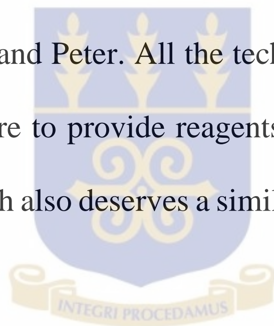
credit goes to Denis Tange of African Regional Postgraduate Programme in Insect Science (ARPPIS) for his assistance especially in the area of statistical analysis.

My family supported me both physically and spiritually. My parents, Mr. N.B. Appiah- Kubi and Madam Hawa Dufie, to whom I owe all my gratitude in life, I pray for prosperity and good health. You are the best parents to have. Ali, Memuna, Iddris, Husein and Hassana, I love you as siblings. Let's keep up the spirit because Allah is with us.

Mr. Braimah Awais of Political Science Department deserves a special recognition for his moral and spiritual support.

Madam Naidatu Hameed for her financial and spiritual support. I am very grateful to you.

Friends, course mates and loved ones, Daniel, Moscoh, Justice, Ibu, Godwin, Banna, Safura, Radia, Mardia, Abiba, Rabia, Letitia and Peter. All the technicians in the department, especially, Mr. Bob Essien who was always there to provide reagents and chemicals that I needed even at short notice. Mr. Samuel Owusu-Atuah also deserves a similar recognition for his prompt technical supports.



University of Ghana http://ugspace.ug.edu.gh

ABSTRACT

This investigation examined petroleum ether, acetone/chloroform, and ethanol extracts of the whole stem of *Dichapetalum madagascariense* Poir. The petroleum ether extract afforded epifriedelanol (friedelan-3β-ol) together with two mixtures- friedelan-3-one and friedelan-3β-ol; and β-sitosterol and stigmasterol. The acetone/chloroform extract (obtained by combining the acetone and chloroform extracts) afforded friedelin (friedelan-3-one) and β-sitosterol but showed dichapetalin A on TLC. The ethanol extract afforded the previously isolated mixture of friedelan-3-one and friedelan-3β-ol. All isolated compounds were identified by comparison of melting points, Retardation factor (R_f) from TLCs, IRs, and ^{1}H- and ^{13}C NMR spectroscopy.

Biological activities involving insecticidal and anthelminthic properties were carried out on the extracts and the isolates. The insecticidal activity investigated the toxicity and grain protectant potential of different extracts and isolates of the plant against *Sitophilus zeamais* in the laboratory using contact toxicity, progeny production and damage assessment assays. The less polar extracts, for example petroleum ether extract, recorded lower LD_{50} value of 0.86 μg/μl after 48hour exposure. Friedelan-3-one, friedelan-3β-ol, and a mixture of friedelan-3-one and friedelan-3β-ol recorded LD_{50} values of 0.48, 0.56 and 0.52 μg/μl respectively following 48hour exposure against *Sitophilus zeamais*. The different extracts caused significant reduction in feeding damage, number of F1 progeny produced, and inhibition of the development of eggs and immature stages of *Sitophilus zeamais*. There was complete protection of grains by the different extracts applied at the highest dosage of 10 g per 100g of grain. The anthelminthic activity using the Egg Hatch Assay (EHA) saw friedelan-3β-ol recording the lowest IC_{50} value of 0.64 μg/μl but a mixture of β-sitosterol and stigmasterol had the largest IC_{50} value of 9.41 μg/μl in inhibiting the hatching of

University of Ghana http://ugspace.ug.edu.gh

Necator americanus eggs. Based on the IC_{50} values, friedelan-3β-ol, friedelan-3-one and β-sitosterol exhibited similar potency. However, no test sample had > 90% inhibition as recommended by WHO but β-sitosterol and friedelan-3β-ol appeared to have significant values > 50%. *D. madagascariense* exhibited some amount of insecticidal and anthelminthic properties and so under practical conditions, relatively higher doses may be required as compared to doses applied in the laboratory experiment.

University of Ghana http://ugspace.ug.edu.gh

TABLE OF CONTENT

DEDICATION i

DECLARATION ii

ACKNOWLEDGEMENT iii

ABSTRACT v

CONTENTS vii

LIST OF TABLES xii

LIST OF REACTION SCHEMES xiv

LIST OF FIGURES xv

CHAPTER ONE 1

1 INTRODUCTION 1

1.1 Characteristics of the family Dichapetalaceae 3

1.2 *Dichapetalum madagascariense* 4

1.2.1 Plant description 4

1.2.2 Geographical location 5

1.2.3 Ethnobotanical uses of *Dichapetalum madagascariense* 5

1.3 Uses of some other Dichapetalaceae species 6

1.4 Bioactivity studies on Medicinal Plants 7

1.4.1 The Maize weevil (*Sitophilus zeamais* Motschulsky) 8

1.4.2 Helminthic infection 10

1.5 Aim of the Project 11

CHAPTER TWO 13

University of Ghana http://ugspace.ug.edu.gh

2 LITERATURE REVIEW 13

2.1 Previous investigation of some species of the family Dichapetalaceae 13

2.1.1 *Dichapetalum cymosum* 13

2.1.2 *Dichapetalum toxicarium* 17

2.1.3 *Dichapetalum braunii* Engl. & K. Krause 19

2.1.4 *Dichapetalum barteri* 19

2.1.5 *Dichapetalum madagascariense* 21

2.1.6 *Dichapetalum gelonioides* Engl 26

2.1.7 Dichapetalins from other *Dichapetalum* species 27

2.1.7.1 Structure activity relationship among the dichapetalins 30

2.1.8 Dichapetalin-type triterpenes from *Phyllantus acutissima* 32

2.2 Previous investigation of grain Insect control 34

2.2.1 Reproductive cycle of the maize weevil (*Sitophilus zeamais* Motschulsky) 39

2.3 Prevalence and importance of Soil-Transmitted Helminths 39

2.3.1 The Biology of hookworms 41

2.3.2 Hookworm Infection 42

2.3.3 Detection and assay of hookworm eggs 44

2.3.4 Previous Investigations of the chemotherapy of Soil-Transmitted Hookworm (STH) and other helminths 45

CHAPTER THREE 49

3 PRESENT INVESTIGATION 49

3.1 Extraction and Phytochemical Screening of stem of *Dichapetalum madagascariense* . 49

3.1.1 Work done on Petroleum ether Extract 51

University of Ghana http://ugspace.ug.edu.gh

3.1.1.1 Isolation of D:A-friedooleanan-3β-ol (Epifriedelanol) SP-a1………………..51

3.1.1.2 Identification of D:A-friedooleanan-3β-ol (Epifriedelanol, [19]) (SP-a1)...... 51

3.1.1.3 Chromatographic Separation of constituents of the oily mother liquor of MP……………... 56

3.1.1.4 Isolation and Identification of a mixture β-Sitosterol and Stigmasterol (MP-G1)………. 58

3.1.2 Work done on chloroform and acetone extracts 63

3.1.2.1 Isolation and Identification of Friedelin (D:A-friedooleanan-3-one) [18] 65

3.1.2.2 Isolation and identification of β-sitosterol [22] (MAC-I1)............................. 69

3.1.3 Work done on ethanol extract 70

3.1.3.1 Isolation and identification of a mixture of friedelin and epifriedelanol (friedelan-3β-ol and friedelan-3-one or friedooleanan-3β-ol and D:A-friedooleanan-3-one) ……………………………………………………………………….71

3.2 Bioactivity studies 73

3.2.1 Insecticidal activities……………………………………………………………...73

3.2.1.1 Toxicity assay……………………………………………………………...74

3.2.1.2 Effects of crude extracts on hidden eggs and immature stages 76

3.2.1.3 Progeny production and damage assessment assays 77

3.2.1.4 Discussion on insecticidal activity studies……………………………………83

3.2.2 Anthelminthic assay 85

3.2.2.1 Discussion on anthelminthic bioassay 88

CONCLUSION 89

RECOMMENDATION……………………………………………………………………90

University of Ghana http://ugspace.ug.edu.gh

CHAPTER FOUR ... 91

4 EXPERIMENTAL ... 91

4.1 General methods ... 91

4.2 Collection and treatment of plant material ... 93

4.3 Reagents ... 93

4.4 Phytochemical screening of the crude extracts of *D. madagascariense* ... 95

4.5 Extractions ... 98

4.5.1 Petroleum ether Extraction ... 98

4.5.2 Chloroform Extraction ... 98

4.5.3 Acetone Extraction ... 99

4.5.4 Ethanol Extraction ... 99

4.5.5 Methanol Extraction ... 100

4.6 Work done on Petroleum ether Extract ... 100

4.6.1 Isolation of SP-a1 ... 100

4.6.2 Identification of SP-a1 ... 101

4.6.3 Isolation and Identification of MP-G ... 101

4.7 Work done on Chloroform and acetone extract (MAC) ... 102

4.7.1 Isolation and Identification of MAC-F1 ... 102

4.8 Isolation and identification of a mixture of friedelin and epifriedelanol (ET-C1) ... 103

4.9 Insecticidal activity ... 103

4.9.1 Culturing of insect ... 103

4.9.2 Preparations of different concentrations of the plant materials ... 104

4.9.3 Contact toxicity by topical assay ... 104

University of Ghana http://ugspace.ug.edu.gh

4.9.4 Effects of extracts on eggs and immature stages....105

4.9.5 Progeny production and damage assessment....105

4.10 Anthelminthic activity.... 106

4.10.1 Sampling....106

4.10.2 Diagnosis of intestinal parasites....106

4.10.2.1 Kato-Katz technique....106

4.10.2.2 Extraction of eggs from faeces....107

4.10.2.3 Egg Hatch Assay (EHA)....108

APPENDIX I 110

TABLES....110

APPENDIX II.... 113

INFRARED SPECTRA (IR) 113

APPENDIX III 117

PROTON (^{1}H) NMR SPECTRA 117

APPENDIX IV 130

CARBON-13 (^{13}C) NMR SPECTRA 130

APPENDIX V 136

DEPT SPECTRA 136

REFERENCE.... 140

University of Ghana http://ugspace.ug.edu.gh

LIST OF TABLES

Table 1: Most significant results on Cytotoxicity of Dichapetalins M and P on brine shrimp and Cancer cell lines……………………………………………………………………….29

Table 2: Cytotoxic activity of some dichapetalins against two human cancer cell lines……….30

Table 3: Comparison of the STH infections with other major diseases in developing Country…………………………………………………………………………….40

Table 4: Infected population estimates of human STH worldwide and in sub-Saharan Africa…………………………………………………………………………….40

Table 5: Relationship between hookworm burden and anaemia and amount of blood loss With different hookworm loads……………………………………………………43

Table 6: Percentage cure rates for the anthelminthics used to control STH……………………45

Table 7: Phytochemical screening of the crude extracts of *D. madagascariense*………………49

Table 8: Identification of D:A-friedooleanan-3β-ol (Epifriedelanol)……………………….....52

Table 9: Infrared values of Epifriedelanol (SP-a1)……………………………………………..52

Table 10: Comparison of ^{13}C- NMR chemical shifts of the epifriedelanol [19] (SP-a1) with literature values…………………………………………………………..54

Table 11: Comparative ^{13}C-NMR chemical shifts of β-sitosterol and stigmasterol from Literature………………………………………………………………………….60

Table 12: Some comparative physical values of MAC-F1 and MAC-F with some authentic samples……………………………………………………………………………64

Table 13: Some IR values of friedelin (MAC-F1)[18]…………………………………………66

Table 14: Comparative ^{13}C-NMR chemical shifts of friedelin (D:A-friedooleanan-3-one, [18])

University of Ghana http://ugspace.ug.edu.gh

from literature…………………………………………………………………...67

Table 15: Infrared values of a mixture of friedelin and epifriedelanol (friedelan-3β-ol and friedelan-3-one or friedooleanan-3β-ol and D:A-friedooleanan-3-one) ET-C1………72

Table 16: Lethal Dosage (LD_{50}) of different extracts and isolates of *D. madagascariense* to *S. zeamais*……………………………………………………………………………….....75

Table 17: Effect of crude extracts of *D. madagascariense* on the number of F1 progeny produced by *S. zeamais*……………………………………………………..…...79

Table 18: Percent weight loss caused *by S. zeamais* in maize treated with the different extracts of *D. madagascariense* …………………………………………………….81

Table 19: Percent seed damage caused *by S. zeamais* in maize treated with the different extracts of *D. madagascariense*…………………………………………………………82

Table 20: Median Inhibitory concentration (IC_{50}) of samples of *D. madagascariense* …………87

University of Ghana http://ugspace.ug.edu.gh

LIST OF REACTION SCHEMES

Scheme 1: The lethal synthetic pathway for the inhibition of Krebs' cycle……………………......15

Scheme 2: Metabolic pathway for the inhibition of Krebs' cycle 15

Scheme 3: Synthesis of ω- fluoro-oleic acid (18-fluoro-*cis*-9-octadecenoic acid)...................... 18

Scheme 4: Flow chart of procedure used in extractions 50

Scheme 5: Separation of constituents of the oily mother liquor of the Petroleum ether 56

Scheme 6: Separation of MAC extract of *Dichapetalum madagascariense* (stem) 63

Scheme 7: Separation of ET extract of *Dichapetalum madagascariense*.................................... 71

University of Ghana http://ugspace.ug.edu.gh

LIST OF FIGURES

Figure 1: Compounds isolated from *Dichapetalum toxicarium* 18

Figure 2: Some compounds isolated from *Dichapetalum barteri* 20

Figure 3: Isolated compounds from *Dichapetalum madagascariense* 24

Figure 4: The structure of Podophyllotoxin... 26

Figure 5: Isolated compounds of *Dichapetalum gelonioides*……………………………………27

Figure 6: Isolated dichapetalin-type triterpenoids from *Phyllantus acutissima* 33

Figure 7: *Sitophilus zeamais* adults produced in maize treated with extracts of *Dichapetalum Madagascariense* and incubated for different periods after oviposition……………....77

Figure 8: Effect of different crude plant extracts on F1 progeny production 80

Figure 9: % seed damage caused by *Sitophilus zeamais* in maize treated with different extracts ....…83

University of Ghana http://ugspace.ug.edu.gh

CHAPTER ONE

1 INTRODUCTION

In the history of civilization, a common practice in many parts of the world, including Africa and for that matter Ghana is the use of plant materials in the treatment of diseases. In the search for more effective remedies to diseases, the plant kingdom continues to remain the major source of phytochemicals for drug discovery[1]. Plants contain active principles that are responsible for their curative action.

Before the advent of the synthetic era, mankind was largely dependent on alternative medicine for prevention and treatment of diseases. But the introduction of scientific procedures enabled man to understand both the therapeutic and toxic principles present in plants[1].

A plant is a biosynthetic laboratory for a multitude of classes of compounds like glycosides, alkaloids, flavonoids, terpenoids, etc. Secondary metabolites are responsible for the medicinal properties of the plant and usually the plant material is subjected to phytochemical screening for the detection of various plant constituents. These classes of compounds have physiological and therapeutic as well as toxic effects[1].

Though, in Ghana, many people are skeptical about the claims of alternative medical practitioners and the efficacy of their preparations, there is an increasing awareness of the potential of these natural products. Currently, plant-based medicine has become an integral part of the primary health care system in many nations[2]. This is why in Ghana, as in other parts of the world, a Centre for Scientific Research into Plant Medicine (CSRPM) has been established, where doctors and clinicians use plant preparations prescribed by herbalists to treat patients. Through physical diagnosis and prognosis, the CSRPM does investigations on how these preparations interact with

University of Ghana http://ugspace.ug.edu.gh

the body and the effects they have on patients. While many plant materials have been evaluated for their putative curative activities by CSRPM and other researchers, several others need to be documented and their efficacy established under controlled experimentation. In this case, in order not to abuse the use of alternative medicine, there is the need to distinguish between tested efficacious and safe products, and the untested ineffective and / or unsafe ones. Medicinal plants also find application in the pharmaceutical, cosmetic, agricultural and food industries[1].

Medicinal plants offer advantages in traditional medicine in the sense that they are cheaper to come by and use. They are also easily accessible to majority of the population in the third world countries where herbal medicine enjoys wider acceptability among the people. It is generally believed that since plants are natural products there is a greater likelihood of their constituents being accepted by the body. Again, as all developing countries are striving to achieve health for all their people, medicinal plants are a potential source of new drugs, as well as a potential source of starting materials or raw materials for the synthesis of known drugs.

It is estimated that only about 20 % of the plant flora has been studied and about 60 % of synthetic medicines owe their origin to plants[1]. Due to this, there is the need to investigate more medicinal plants as potential sources of new medicines, and also strengthen the scientific basis of herbal medicine. It is in this light that the Ghanaian medicinal plant- *Dichapetalum madagascariense* was chosen for the present investigation.

University of Ghana http://ugspace.ug.edu.gh

1.1 Characteristics of the family Dichapetalaceae

The Dichapetalaceae (syn. Chailletiaceae) is one of the major groups of Angiosperms and has three (3) plant genera, namely, *Dichapetalum, Stephanopodium and Tapura* [3, 4, 5] with about 578 species. Of these, 529 are accepted scientific species and 49 infraspecific. All the three genera occur in the tropics and neo-tropics, but only 2 of them occur in Central Amazonia and in the Reserva Ducke. Reserva Ducke is an experimental site of protected primary forest, made up of a large variety of tree species and is 25 km north-east of Manaus (Brazil). About 176 species of the Dichapetalaceae are distributed around the tropics. Five of the 50 neo-tropical species are known to occur in the Reserva Ducke. The family can be most easily recognized by the inflorescence which arises from the petioles in most species and by the bicucullate petals that give it its name.

The Dichapetalaceae are trees or lianas that may be identified by their lenticillate twigs and stipules. The leaves occur as dark greyish-drying blades with secondary veins [6]which alternate with the presence of stipules, often early caduceus which is entirely lobed, partite or fimbriate[7]. Members of this family have a dry or fleshy drupe fruit which are 1−3 (or 4) seeded. The fruit has an exocarp most frequently appressed pubescent and sometimes dehiscent, with its mesocarp thin to thick. The endocarp is hard or parchment-like, indehiscent, glabrous or pubescent within. Plants in this family have seeds that may be pendulous, generally without endosperm but the embryo is large and erect, with plano-convex cotyledons. Germination is hypogeal, the first leaves alternate. Plants of this family are cultivated for poisons in Africa due to their ability to accumulate fluoroacetic acid. Fluoroacetic acid has been found to be one of the major toxic principles, responsible for the observed toxicity to livestock of this family[8, 9].

University of Ghana http://ugspace.ug.edu.gh

1.2 *Dichapetalum madagascariense*

Dichapetalum madagascariense Poir, is a member of Kingdom Magnoliophyta. It is part of the Dichapetalaceae family which belongs to the Magnoliopsida Class. Being part of the order of Malpighiales, it has *Dichapetalum* and *madagascariense* as its genus and species names respectively[3]. The *Dichapetalum*, the largest genus has 418 scientific species. Out of these, 378 are scientific species and 40 are infraspecific [3,4,5].

D. madagascariense Poir has several botanical synonyms: Oliver called it *Chailletia floribunda* whilst Chevalier Bot. referred to it as *D. bocageanum,* also *D. flexuosum* by Engler and *D. guineense* by De Candolle Keay. Thonning named it *D. paniculatum* and Hutchinson and Dalziel called it *D. rowlandii*[10].

D. madagascariense has also been known to many people and as such it has several local names in Ghana. Among the Akans, the Ashantis call it "akwakoraa gyihenim" or "kyekyereantena" whilst it is respectively called by the Twis and Sehwis as "nkorodua" or "ofenwa-biri" and "aunwi (n) dua". The plant is also called "antro" in Ga and the indigenous people of Ewe named it "klesti" or "folie"[9].

1.2.1 Plant description

Dichapetalum madagascariense Poir (Syn. *D. guineense* Keay) is a shrub or small tree which is over 20 m high by 1.70 m girth. The smooth drooping oval-shaped leaves, which are arranged alternatively on the branchlets, grow to about 8-16 cm long and 3-7 cm broad from yellowish branches. The barks of the plant are dry and stringy which normally peel off in scales[10]. The bark exudes a little brownish gum when slashed. The fresh cut wood is white and turns brownish. It is

University of Ghana http://ugspace.ug.edu.gh

hard and contains black veins. The plant bears creamy and fragrant flowers; flowering starts around January. The fruits of the plant are bright orange in colour. Fruiting starts in March.

1.2.2 Geographical location

D. madagascariense is found in West Tropical Africa, especially in the savanna and forest areas from Sierra Leone to Nigeria, and perhaps on to the Congo basin[9]. In Ghana, it is mostly found in the high forest of Ashanti and Eastern Regions; in the savannah areas, it is common in the Accra plains.

1.2.3 Ethnobotanical uses of *Dichapetalum madagascariense*

The fruit-pulp and seeds of *D. madagascariense* are edible and are used as food while the leaves and leaf-sap are generally used in healing and treating liver, gall bladder and spleen (for example jaundice) related diseases. The Baules (Ivory Coast) use the leaf extract of *D. madagascariense* together with the leaf extracts of *Cassia occidentalis* and *Hoslundia opposita* as a nasal instillation in treating jaundice. The stem bark of the plant alone is used in the treatment of jaundice. The whole plant is used as a cure for viral hepatitis, sores and urethritis in some communities. The leaves of the plant are used in soap preparation and as soap-substitutes. Exudations (example gum and resin) are the other products that can be derived from the bark of the plant while the stem is used for carving household, domestic and personal items like bowls and plates due its hardness and toughness[9, 11].

University of Ghana http://ugspace.ug.edu.gh

1.3 Uses of some other Dichapetalaceae species

Plants belonging to the Dichapetalaceae family are believed to contain some active principles which can be curative or toxic. Many of these plant species are used in food or medicine while some are toxic to animals[9].

The leaves of *D. toxicarium* are used for general healing of diseases and for the treatment of chronic sores and urethritis while the fruit-pulp is also edible. Specifically the leaves are for treating heart-related diseases, tremor and palpitations. Its pulverized bark is used to treat dropsy, swellings, oedemas, gout, cough as well as rheumatism[12]. The kernel of the plant is a well-known rodenticide and was used by the indigenous people of Sierra Leone as arrow poisons and adhesives while phytochemicals like alkaloids, glycosides, saponins and steroids are derived from the whole plant[13]. The seeds of *D. pallidum* Engl. are edible and used as food. While the leaves are singly used in the treatment of diarrhea and dysentery, the seeds of *D. pallidum* and *Desmodium adscendens* are used together in treating the same diseases[13]. The leaf of *D. albidum* is a good pain killer and is used for treating menstrual disorders[9]. The whole plant of *D. barteri* is a cheap and potent rodenticide[14]. The leaf juice as well as the tuber decoctions of *D. ruhlandii* Mlundii Engl. is used for the treatment of sore throat. The plant is also poisonous to sheep and goats[15]. The powdered leaves of *D. braunii* Engl. are topically applied to sores[13]. *D. stuhlmanii* is used to poison wild pigs, monkeys, rats in farms and even humans[16, 17]. *D. macrocarpum* is not only poisonous to rodents, head-lice and other animals; it is also used as arrow poisons[16, 17].

University of Ghana http://ugspace.ug.edu.gh

1.4 Bioactivity studies on Medicinal Plants

Over the past several decades, interest in drugs of plant origin has been growing steadily. Previously, chemists were interested mainly in the extraction and structural elucidation of phytochemicals without much testing for bioactivity. Working in collaboration with pharmacologists, microbiologists and pharmacognocists was not common. Many compounds were therefore isolated, characterized and left uninvestigated for their potential medicinal properties and the potential use of these phytochemicals in therapeutics. The situation has however changed considerably in the past fifty years, with teams of scientists working together holistically on plant constituents[18].

Biological testing is now employed by natural product chemists, pharmacologists and biologists to conduct their scientific research and to valorize natural products. Standardization of some of these methods is, therefore, desirable to permit more comprehensive evaluation of plant products, and greater comparability of the results obtained by different investigators. Demonstrating efficacy through bioactivity is, therefore, a critical aspect of product evaluation. Over the years, there have been a lot of reports on the isolation, structure elucidation and antimicrobial activities of novel compounds obtained from plant genera. Several structure-activity relationships exist between microbial and/ or insects and plant products. Examples are the bioactivity of pyrrolizidine alkaloids on *platyphora boucardi* [19] and the effect of alkaloids and other constituents of *Zanthoxylum xanthoxyloides* on *Callosobruchus maculatus* (cowpea beetle) [20].

In this project some of the chemical constituents of *D. madagascariense* are tested for their bioactivity on some insect pests such as *Sitophilus zeamais* and parasites such as hookworm. It is therefore important to understand the nature of these pests and parasites and how they are controlled.

1.4.1 The Maize weevil (*Sitophilus zeamais* Motschulsky)

Sitophilus zeamais Motschulsky is a species of weevil which belongs to the order Coleoptera and family Curculionidae. It is one of the most serious cosmopolitan pests found in field and stored cereal grain, in tropical and sub-tropical regions[21]. The larvae damage maize crops by developing within an individual grain, eat the contents of the maize and remain there until maturity. While reproducing, it releases more crop-damaging larvae. The insect indirectly causes contamination of produce which can result in the accumulation of uric acid[22]. *S. zeamais* is known to cause hypersensitivity pneumonitis in humans (inflammation of the lungs due to the breathing of maize dust from *S. zeamais*), weight loss in the maize, quality loss including reduced nutritional values and seed viability, and fungal growth in the cereals. Fungal growth is known to include *Aspergillus flavus (aflatoxin)* infections, as well as *Fusarium moniliforme, Fusarium semitectum*, *Aspergillus niger, Aspergillus glaucus, Aspergillus candidus, Penicillium islandicum, Penicillium citrinum, Paecilomyces* sp., *Acremonium* sp., *Epicoccum* sp., and yeasts.

About 20-25% out of the 250,000-300,000 tonnes of maize produced in Ghana is destroyed by *S. zeamais* annually[23]. The world's annual stored maize may have heavy infestation of this pest and cause weight losses in the maize as much as 30-40%, although losses[24] are commonly between 4-5% in seed damage. The niggling caused by *S. zeamais* has been recognised as an increasingly important problem in Africa[25] as it can be detrimental to world food security and create economic crisis due to population growth. Known control measures applied against *S. zeamais* infestation are the heavy use of gaseous fumigants and residual chemical insecticides[26] such as pirimiphos methyl (0.5%). Ideally, since the weevils infest foodstuffs, it is not advisable to kill them with insecticides. The safest method for the eradication of the insect should have been freezing or super

University of Ghana http://ugspace.ug.edu.gh

heating grain stores. However these methods are not practicable and may even damage the germ layer required for seeds to be utilized for planting, hence the use of fumigants and insecticides. But the recent development of insect populations resistant to conventional insecticides, together with the high cost of synthetic insecticides, and the growing environmental and human health concerns over the use of many synthetic insecticides[26, 27,28,29] have stimulated interest in the development of alternative control strategies.

Among these methods is the use of powdered plant parts and their extracts and/or isolates. The use of locally available plant materials and traditional botanical pest control agents such as neem seeds and leaves has recently attracted considerable research to establish the scientific basis for their continued use in terms of efficacy, active constituents and appropriate application technology[30]. These alternative control strategies also have advantage of being less toxic to non-target organisms and are biodegradable too.

Devising methods for efficient control of the insects requires a fundamental knowledge of the biology of *S. zeamais*. The biology of *S. zeamais* depends on its response to the effects of environmental and biological factors. The feeding and oviposition of the insects is also crucial. Generally, insect oviposition behaviour is an important contributor to its fitness since that would consequently affect the number and quality of offspring[31-33]. But factors like insect species and strain, population density, environmental conditions, food, age and size of the individual have adverse effects on oviposition behaviour[30].

University of Ghana http://ugspace.ug.edu.gh

1.4.2 Helminthic infection

One of the major health challenges facing people living in remote rural areas in the tropical and subtropical developing countries is helminthic infections. Currently, over half of the world's population is estimated to be infected with intestinal helminths[34,35]. Despite less significant morbidity and mortality rates of helminthic infections in comparison with many other parasitic infections, there are substantial and measureable adverse effects. Helminthic infections often lead to mal-absorption of food nutrients, diarrhea, anaemia and retarded growth in infants and school-age children leading to low cognitive development and even mental retardation[36, 37].

Aside this, millions of livestock are infected with helminthic diseases and as a result, there are significant economic losses in domestic and farm animals. Control measures applied against these parasites are the several synthetic anthelminthic drugs like albendazole and mebendazole. However, the efficacy of single dose treatment in population- based studies is low, at 33% and 15% for albendazole and mebendazole, respectively[38]. Again, there is a major problem of drug resistance in several parasite species, together with the high cost of these modern anthelminthics[39, 40]. All these have stimulated interest in the development of newer and inexpensive drugs and reliance on traditional medicines based largely on medicinal plants.

An anthelminthic is a substance that expels or destroys gastrointestinal worms. It has synonyms like de-wormer, parasiticide, parasitic, antiparasitic, drench, endecticide and nematodicide.

University of Ghana http://ugspace.ug.edu.gh

1.5 Aim of the Project

Some existing orthodox drugs, example anthelminthics and antimicrobial, have become ineffective and expensive in the treatment of diseases. There is therefore the need to identify and unearth more important medicinal plants which usually involves isolating, testing and characterization of known active constituents of some medicinal plants[1].

Literature survey, up to May 2013, revealed that very little work has been carried out on the stem bark of *Dichapetalum madagascariense.* The only work done was that of Darbah who isolated some known triterpenoids from the stem bark[41]. No phenylpyranotriterpenoid compounds (Dichapetalins) were isolated from that research compared with the whole roots of the same plant. Friedelan-3-one, friedelan-3β-ol and β-sitosterol isolated and identified from the stem bark, have also been isolated from the roots of the plant. However, the whole stem of the same plant has not been studied into detail. So far, isolates and some extracts of this plant have been shown to exhibit biological activities such as anti HIV and anticancer[42]. The isolates did not show any antimicrobial[42] (antifungal and antibacterial) activity.

This present project, therefore, involves further chemical and biological investigations on the whole stem of *Dichapetalum madagascariense*. Extraction of the plant material was carried out with different solvents and biological activities such as anthelminthic and insecticidal activities were carried out on the extracts and isolates. Physical, chemical and spectroscopic methods (UV, IR, and NMR) were used to elucidate the structures of isolates.

University of Ghana http://ugspace.ug.edu.gh

Specific objectives

1. Extraction of the plant material with petroleum ether, acetone, chloroform, ethanol and methanol
2. Separation of the different extracts by Column chromatography
3. Structural elucidation of isolated compounds
4. Evaluation of the toxicity, oviposition and damage assessment of extracts and isolates on *Sitophilus zeamais*
5. Evaluation of the inhibition pattern of extracts and isolates on *Necator americanus* and *Ancylostoma duodenale*

University of Ghana http://ugspace.ug.edu.gh

CHAPTER TWO

2 LITERATURE REVIEW

2.1 Previous investigation of some species of the family Dichapetalaceae

The family Dichapetalaceae (syn. Chailletiaceae Engl.) is distributed throughout the tropical and sub-tropical regions of the world. Since the 1900s, there have been scientific investigations of the Dichapetalaceae. Several species of this family, example *Dichapetalum cymosum* and *Dichapetalum toxicarium*, are known to be poisonous to livestock. The research interest then was initially centred on finding the active principle(s) responsible for its poisonous nature. This led to the establishment of fluorinated carboxylic acids as the main active principles responsible for the poisonous nature of the Dichapetalaceae[43]. There was, therefore, little work done on the non-fluorinated constituents of this plant family until the 1990s, when Addae-Mensah *et al* investigated the non-fluorinated constituents of two Dichapetalaceae species- *Dichapetalum barteri* and *Dichapetalum madagascariense*[44].

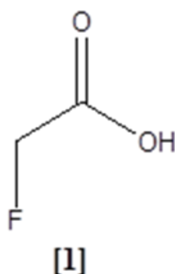
2.1.1 *Dichapetalum cymosum*

Dichapetalum cymosum is known as "gifblaar" (the poison leaf) by the Afrikaans of South Africa[13]. The plant is also found in some localities in Namibia, Zimbabwe, Botswana, Southern Angola and parts of East Africa. *D. cymosum* is known to be poisonous, and ingestion of a quarter to four leaves of this plant has been reported to cause deaths in rabbits, sheep and oxen[45]. The poisonous substance of *D. cymosum* was (at one time) thought by the early researchers, to be a cyanogenic glycoside based on a reported case of isolated hydrocyanic acid from the leaf of the

University of Ghana http://ugspace.ug.edu.gh

plant. However, upon further investigation of plant materials of varying degrees of maturity, two resins were detected and isolated and both were found to be toxic. One of the resins was readily soluble and the other sparing soluble in alcohol[46].

Later in 1906, work carried out on the fruits did not show evidence of the presence of either resin or cyanogenic compounds[46]. Eventually in 1944, work done by Marais established the presence of fluoroacetic acid [1] as the main toxic principle in the leaves of *D. cymosum*[47]. Fluoroacetic acid (15mg/g) was obtained from the dry leaves of the plant. The active principle is soluble in water, thus, its toxicity increases as animals drink water after ingestion of the plant.

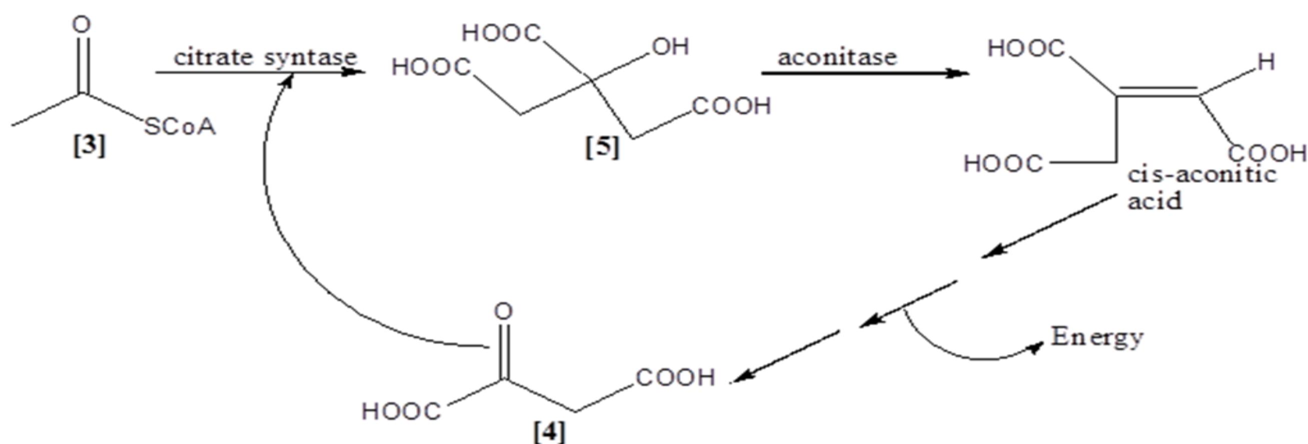


[1]

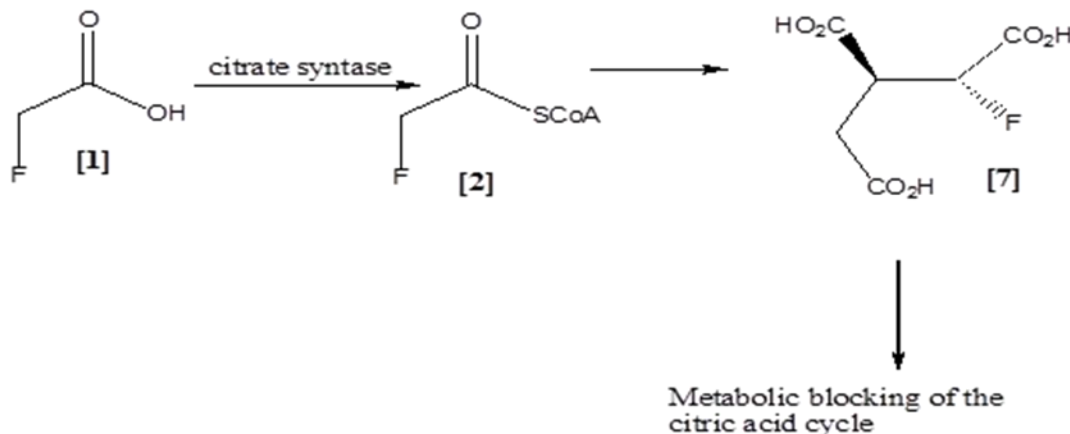
Fluoroacetic acid itself is not toxic; its toxicity results from interference with metabolism of pyruvate particularly at the aconitase stage through beta oxidation of fluoroacetic acid to fluoroacetyl-CoA **[2]** by the pyruvate dehydrogenase complex (scheme 1). In the citric acid cycle, acetyl CoA **[3]** and Oxaloacetic acid **[4]** are converted to citric acid **[5]** by citrate synthase enzyme which further undergoes dehydration to give aconitic acid **[6]**. The aconitic acid is then metabolized through a sequence of reactions with the release of energy for the cell. Oxaloacetic acid is finally regenerated for the cycle to continue. The citric acid cycle then makes fluoroacetate toxic to all cells by being activated to fluoroacetyl CoA. Due to competition between fluoroacetyl CoA and acetyl CoA for citrate synthase, the former is converted to 2R, 3R fluorocitric acid **[7]**. The fluorocitrate stereoisomer, being a potent competitive inhibitor of the subsequent enzyme

aconitase in the cycle halts the regeneration of aconitic acid thus inhibiting respiration. The fluoroacetate deprives the cell of metabolic energy and also binds irreversibly to protein responsible for transporting citrate to the mitochondria. The cell's energy is produced in the mitochondria. Continuous accumulation of the fluorocitrate forms a chelate with calcium ions in the serum of blood and this causes calcium deficiency in mammals. This is as proposed by Peters *et al* [48] in the synthetic pathways in schemes 1 and 2.

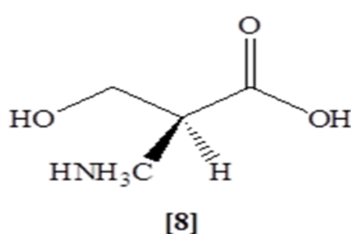
Scheme 1: The lethal synthetic pathway for the inhibition of Krebs' cycle



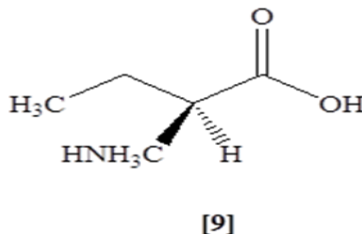
Scheme 2: Metabolic pathway for the inhibition of Krebs' cycle



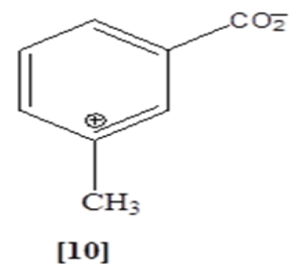
In 1969, Eloff *et al* [49] isolated from the leaves and fruits of the plant two novel amino acids, N-methyl-L-alanine **[8]** and N-methyl-L-serine **[9]** together with trigonelline **[10]** and methyl pentoside **[11]** which were all found to be non-toxic.



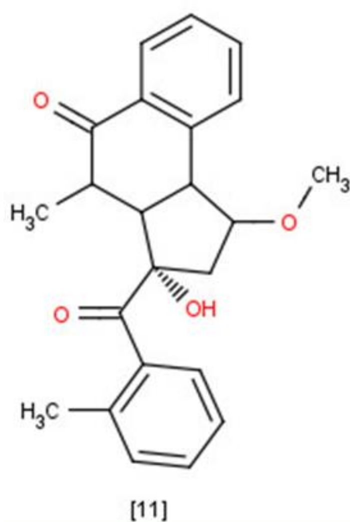
[8]



[9]



[10]



[11]

The roots of this plant are also known to be effective in treating diarrhea and liver-associated problems when administered as a cooled infusion while the whole plant is used in HIV anti-infective therapy[50]. In 1996, it was established that monofluoroacetate occurs in all parts of the plant[51].

University of Ghana http://ugspace.ug.edu.gh

2.1.2 *Dichapetalum toxicarium*

Dichapetalum toxicarium (*Chailletia toxicaria* Don) is a shrub mostly found in Sierra Leone where it is known as "ratsbane" because of the potency of its fruits in killing rats. In Ghana, the Fantes and Twis called the plant ''(e) kum nkura'' as it is used to kill mice. The hardy and woody fruit is extremely toxic to warm-blooded animals. The seeds of the plant have been investigated to contain minute quantities of fluoroacetate that is too low to account for total toxicity. The plant was later found to also contain ω-fluorinated carboxylic acids, ω-fluoro-oleic **[12]** and ω-fluoropalmitic acids **[13]** with strong circumstantial evidence of ω-fluoromyristic acid **[14]**, ω-fluorocapric acids **[15]** and threo-18-9,10-dihydroxystearic acid **[16]**[48,52]. These compounds have a profound effect upon mammalian heart function and the central nervous system and cause ventricular fibrillation and tetanic convulsions in experimental animals[52] hence are also toxic.

18-fluoro-*cis*-9-octadecenoic acid (ω-fluoro-oleic acid) $F(CH_2)_8CH=CH\ (CH_2)_7COOH$ has been confirmed as main the toxic principles[13] as illustrated in the synthetic scheme 3 below. A comparison of the spectral and toxicological properties of ω-fluoro-oleic acid isolated from *D. toxicarium* and that synthesized, indicated that the two compounds were the same[48].

University of Ghana http://ugspace.ug.edu.gh

Scheme 3: Synthesis of ω- fluoro-oleic acid (18-fluoro-*cis*-9-octadecenoic acid)

$F(CH_2)_8Br + NaC{\equiv}CH \longrightarrow F(CH_2)_8C{\equiv}CH$

1. $NaNH_2/NH_3$
2. $I(CH_2)_7Cl$

$F(CH_2)_8C{\equiv}C(CH_2)_7Cl \xrightarrow[NaCN]{DMSO} F(CH_2)_8C{\equiv}C(CH_2)_7CN$

1. H_2O_2
2. $OH^{\ominus}$

$F(CH_2)_8C{\equiv}C(CH_2)_7CONH_2 \xrightarrow[EtOH]{KOH} F(CH_2)_8C{\equiv}C(CH_2)_7COOH$

Lindlars catalyst

$F(CH_2)_8$, $(CH_2)_7COOH$, C=C, H, H

F, OH, O

[12]

F, O, OH

[13]

F, O, OH

[14]

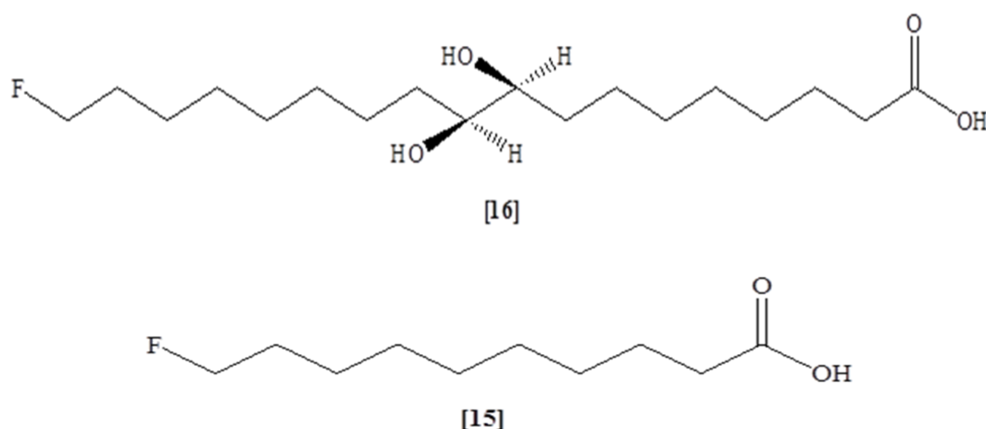


Figure 1: Compounds isolated from *Dichapetalum toxicarium*

2.1.3 *Dichapetalum braunii* Engl. & K. Krause

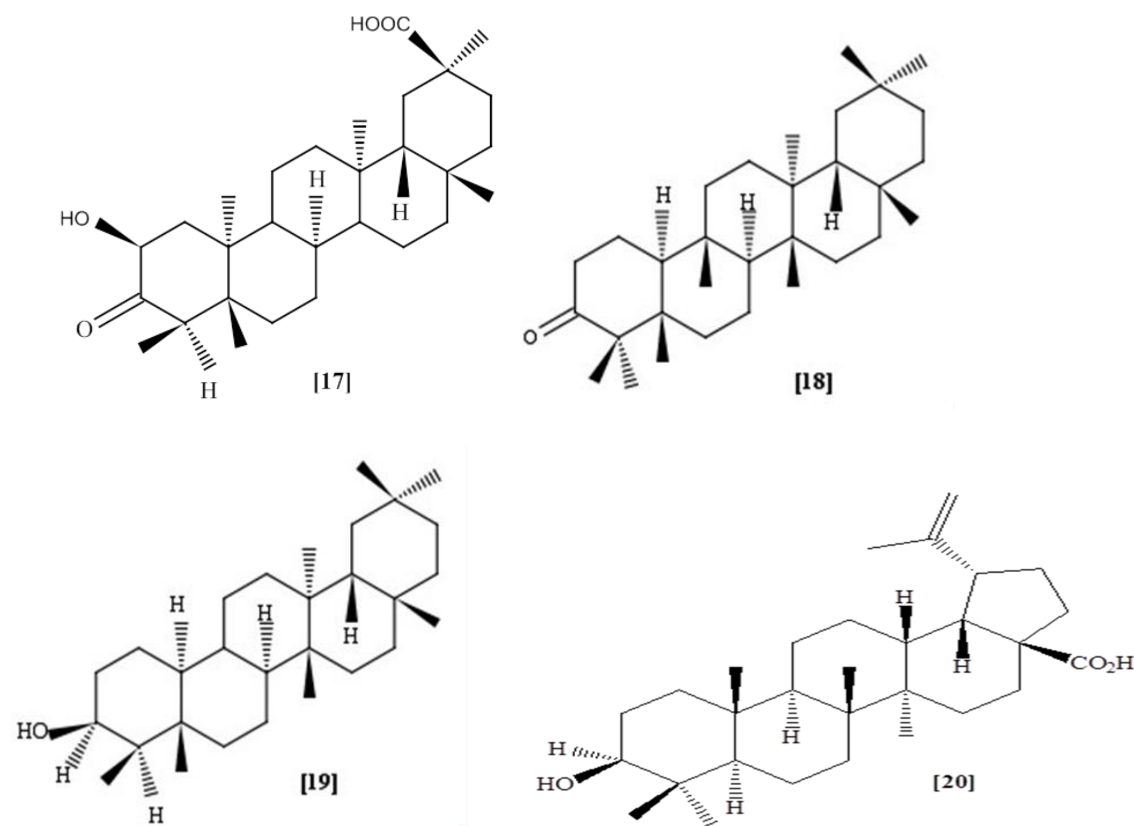
Dichapetalum braunii Engl. & K. Krause can be found in southeast of Tanzania. The plant is known to contain monofluoroacetate, occurring in young leaves and seeds on a dry weight basis at concentrations of 7200 and 8000 ppm respectively, the highest level so far reported from a plant source. However, the fluoride and monofluoroacetate concentrations in this species, especially, in the seeds, do not contain any ω-fluorinated fatty acids when compared with other *Dichapetalum* species. This was confirmed by the use of ion chromatography, ^{19}F- NMR and fluorinated secondary metabolites[53].

2.1.4 *Dichapetalum barteri*

Dichapetalum barteri, mostly found in the southern part of Nigeria is known as "akwuosa" or "ngbuewi" (goat-killer) by the indigenous people of Nigeria. The plant is known to cause deaths in goats and sheep. Nwude *et al* [54] observed that an acute toxicity of leaves and extracts of the plant to mice, rabbits and goats is due to the presence of monofluoroacetate.

University of Ghana http://ugspace.ug.edu.gh

In a study to find out about the relatively non-toxic phytochemicals in the species, the non-fluorinated components of this plant were investigated[55, 56, 57]. The researchers worked on the petroleum ether and acetone extracts and through spectroscopic methods like NMR, MS and IR, several compounds were identified, including the novel D:A-friedoolenanan-29-oic acid **[17]**, friedelan-3-one **[18],** friedelan-3β-ol **[19]** , betulinic acid **[20],** betulonic acid **[21]**, β-sitosterol **[22]**, stigmasterol **[23]**, several long chain esters of ferulic acid ($C_{10}H_{10}O_4$) **[24]**, canophyllol **[25]** and canophyllal **[26]**. Bioactivity studies using the brine shrimp lethality test showed that the crude extracts had strong and selective toxicity as compared to β-sitosterol, betulinic acid, betulonic acid and friedelinol, whereas the rest of the compounds did not show any activity against the brine shrimp.



University of Ghana http://ugspace.ug.edu.gh

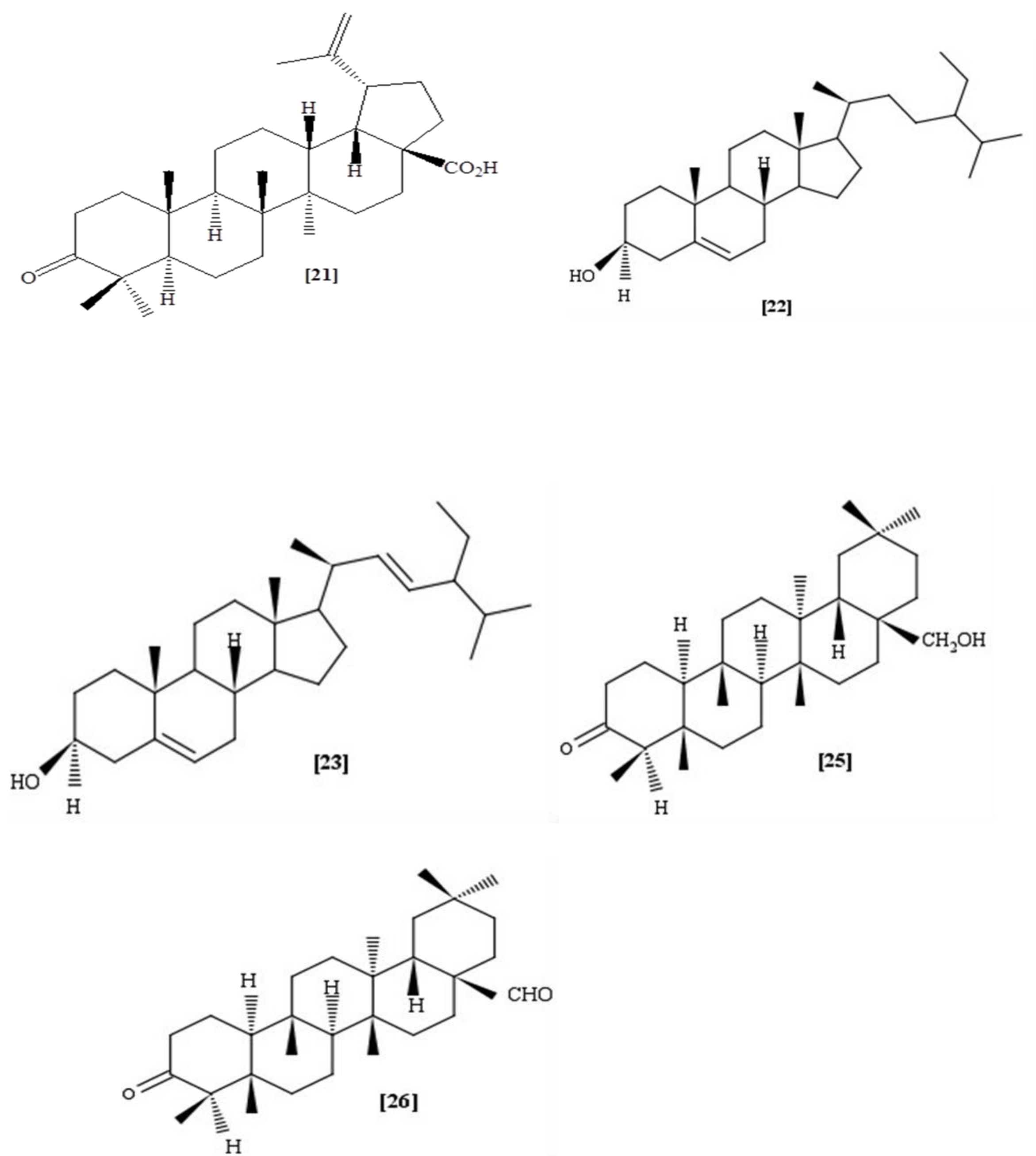


Figure 2: Some compounds isolated from *Dichapetalum barteri*

2.1.5 *Dichapetalum madagascariense*

Work on the non-fluorinated constituents was carried out on the stem bark and whole roots of the plant[41,42,44,,58,59] where some phenylpyranotriterpenoids, ranging from Dichapetalin A to H and M

have been isolated. Altogether, Dichapetalins A **[32**], B **[35]**, C **[33]**, D **[34]**, E **[36]**, F **[37]**, G **[38]**, H **[39]** and M **[40]** have been isolated from the roots of *Dichapetalum madagascariense* Poir[42]. All the dichapetalins have been isolated in acetone extracts except Dichapetalin M which was isolated together with the previously isolated Dichapetalin A from the acetone, diethyl ether and dichloromethane extracts[42]. Octadecanoic acid **[27]**, sucrose **[28]**, mannitol **[29]**, stigmasterol and a mixture of stigmasterol and β-sitosterol from the roots of the plant[58] have also been isolated from petroleum ether extract[42]. A mixture of friedelan-3β-ol and friedelan-3-one[58] was obtained in large quantities from the petroleum ether extract while the dichloromethane and ethyl acetate fractions of ethanol extract yielded pure friedelan-3-one[42].

The effect of friedelan-3-one on the blood sugar levels has been tested. The intraperitoneal administration of a suspension of friedelan-3-one in vegetable oil in concentrations of 8mg/ml and 0.8mg/ml to fasting genetically non-diabetic mice showed significant increase in blood sugar level one hour after administration[41].

The isolations of friedelan-3-one and friedelan-3β-ol were abundantly high in the petroleum ether and acetone extracts from the stem of the plant but the presence of β-sitosterol was seen in both acetone and diethyl ether extracts[41]. Zeylanol **[30]** was also isolated from the stem bark of the plant in small quantities[41].

The absolute configuration of Dichapetalin A was determined by single-crystal X-ray diffraction analysis[60].

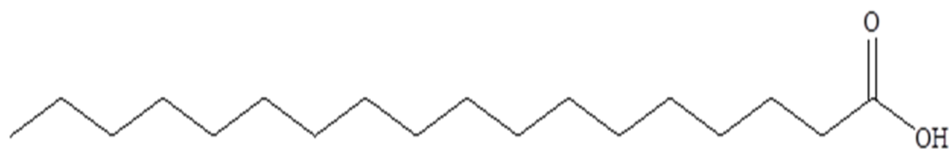
The major Dichapetalin isolated was Dichapetalin A **[32]** and it showed a strong and selective cytotoxicity *in vitro* and *in vivo* but sensitivity reduced drastically *in vivo* whilst Dichapetalin B-H showed less selective cytotoxicity and constitute the minor components[41,44,58,59]. The compound, Dichapetalin A, exhibited pronounced cytotoxicity in the brine shrimp bioassay (LC_{50} 0.31 μg/ml),

exceeding that of Podophyllotoxin **[31]** about seven fold[44,59]. Meanwhile, brine shrimp lethality test of Dichapetalin M exhibited much higher toxicity with LC_{50} value of 0.011µg/ml, exceeding that of Dichapetalin A about twenty eight fold[42].

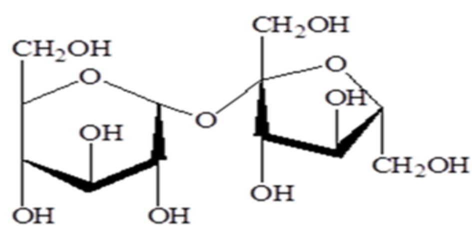
Podophyllotoxin, most times used as a standard for cytotoxicity, is a non-alkaloid toxin lignin extracted from the roots and rhizomes of *Podophyllum* species[61]. Podophyllotoxin and its derivatives are used diversely as remedies for purgative, snake bites, periodontitis, skin disorders, coughs, various intestinal worm diseases, venereal wart *Condyloma acuminatum*, lymphadenopathy and certain tumours. It has shown significant activities in reverse transcriptase inhibition and anti-HIV activity, immunomodulatory, cardiovascular effects, anti-leishmaniasis, 5-lipoxigenase inhibition, antirheumatic, antipsoriasis, insecticidal, phytogrowth inhibitory and ichthyotoxic, and antimalarial and antiasthmatic properties[62].

Dichapetalin C was active to a lesser extent while dichapetalins D & F were almost inactive. Dichapetalin A showed very significant inhibition of cancer cell growth *in vitro* but lost it *in vivo* where there was likelihood of enzymatic hydrolysis of the lactone to an open chain carboxylic acid, the methyl ester side chain. Dichapetalin M has been evaluated for its anti-tumour potential[65]. Dichapetalins A and M were found to be inactive against the HIV-virus; however, the aqueous extract of the plant showed slight activity with an antiviral index of 4.7, indicating the possibility of the presence of anti-HIV principles in the aqueous extract[42].

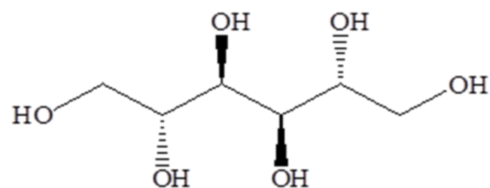
D. madagascariense had also exhibited antimicrobial property. The methanol extract of *D. madagascariense* (leaves) showed significant activity to *Enteroccocus faecalis* at MIC=0.313mg/ml[63].



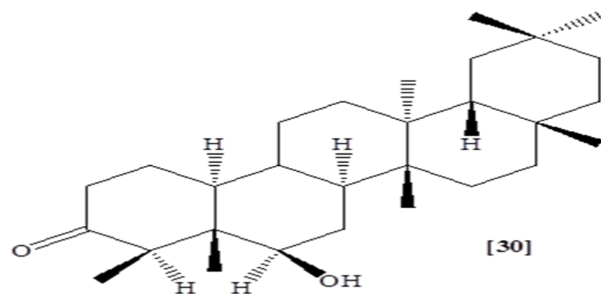
[27]



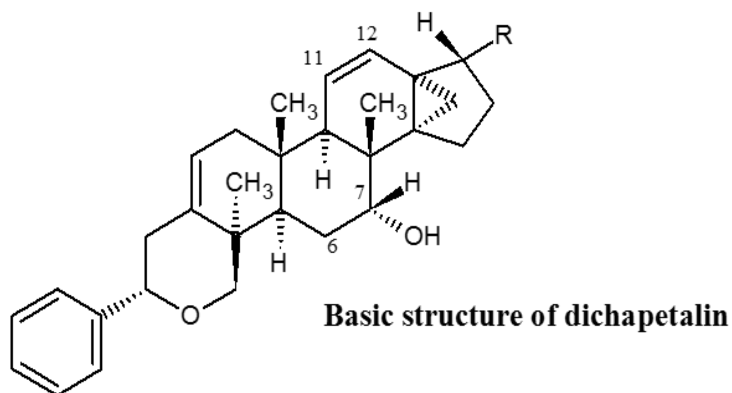
[28]



[29]



[30]



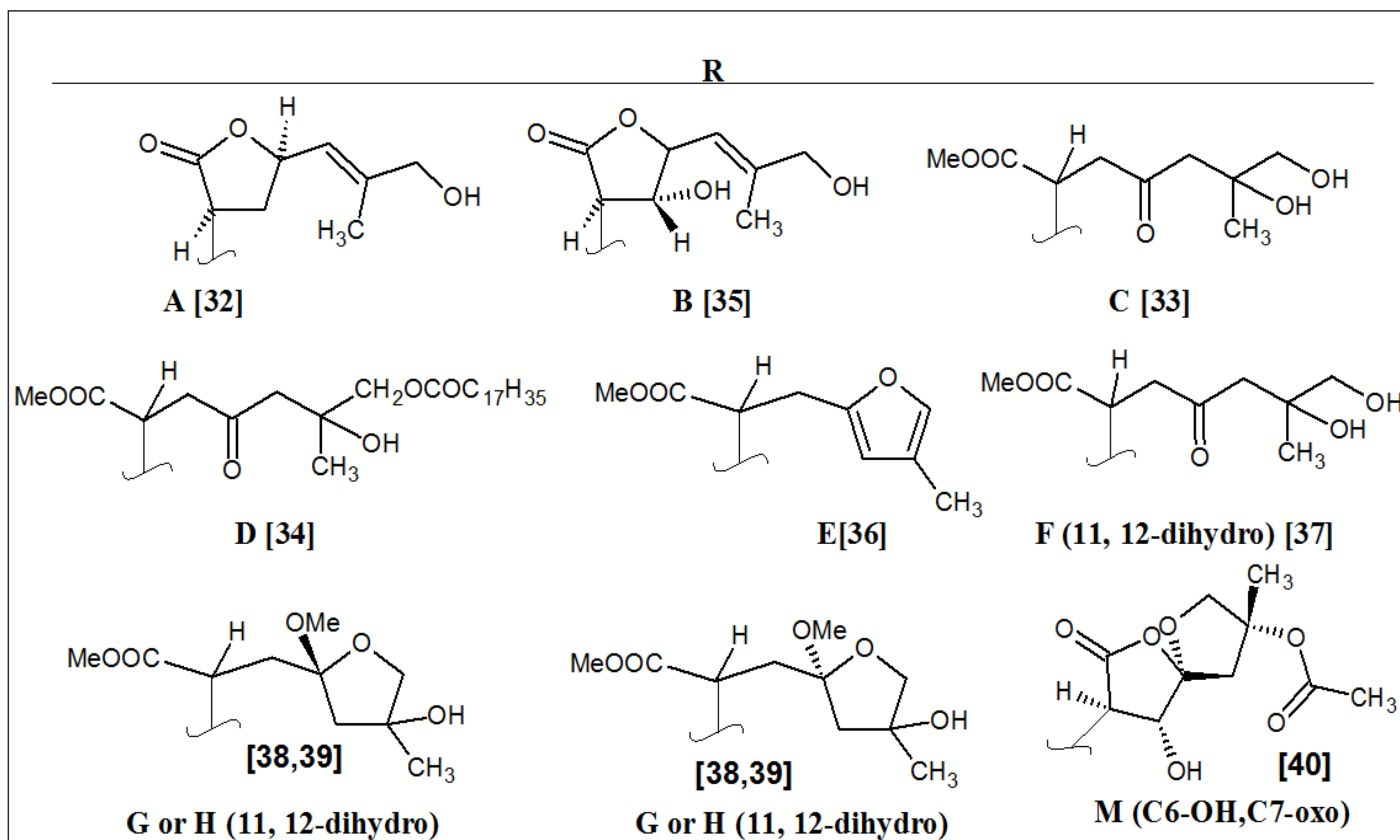


Figure 3: Isolated compounds from *D. madagascariense*

University of Ghana http://ugspace.ug.edu.gh

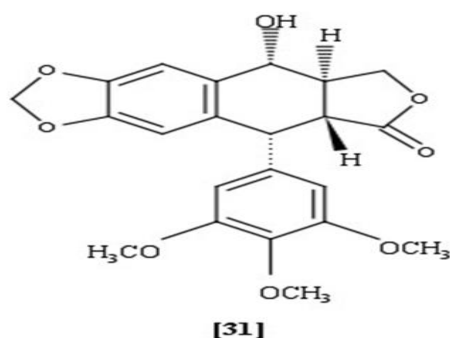
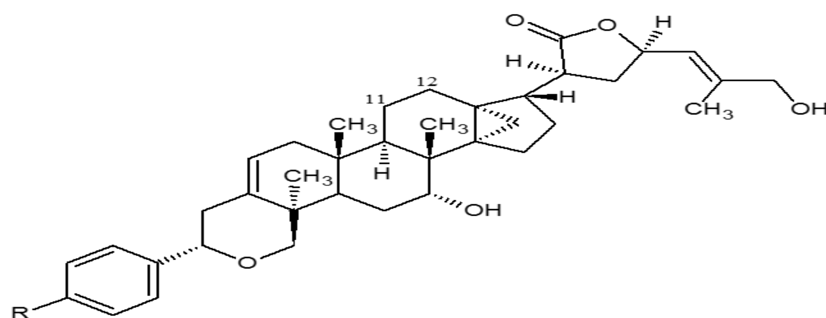


Figure 4: The structure of Podophyllotoxin

2.1.6 *Dichapetalum gelonioides* Engl.

Following the discovery of the dichapetalins in *D. madagascariense*, it has been found that this class of compounds is also present in *D. gelonioides.* This observation has chemotaxonomic significance. Anticancer activity-guided fractionation of the ethyl acetate extract of the plant using eleven (11) human cancer cell lines led to the isolation of eight (8) compounds, five of which were dichapetalins. Four of these dichapetalins were new and were named Dichapetalin I, J, K and L and already known dichapetalin A previously isolated from *D. madagascariense*. Other compounds isolated from the plant were Zeylanol, 28-hydroxyzeylanol and betulinic acid[64]. Zeylanol and betulinic acid have been isolated from *D. barteri*. Dichapetalin K and L exhibited broad cytotoxic activity when tested against a panel of human cancer cell lines. However, dichapetalin A did not show any activity evaluated in an *in vivo* hollow fiber assay in the dose range of 1-6 mg/kg[64].

University of Ghana http://ugspace.ug.edu.gh



Basic structure

Dichapetalin Name	R	Others
A	H	$\Delta^{11,12}$
I	H	12β-OH
J	OMe	12β-OH
K	OMe	$\Delta^{11,12}$
L	H	-

Figure 5: Isolated compounds of *Dichapetalum gelonioides*

2.1.7 Dichapetalins from other *Dichapetalum* species

Following the discovery of the unique cytotoxic properties of the dichapetalins in *D. madagascariense* and *D. gelonioides*, it has been found that this class of compounds is also present in other *Dichapetalum* species. Six more dichapetalins named dichapetalins N-S have been isolated and characterized, together with the known dichapetalins A, B, C, I, L and M from five *Dichapetalum* species[65] as indicated below:

University of Ghana http://ugspace.ug.edu.gh

Dichapetalin A……………………………….....*D. ruhlandii*

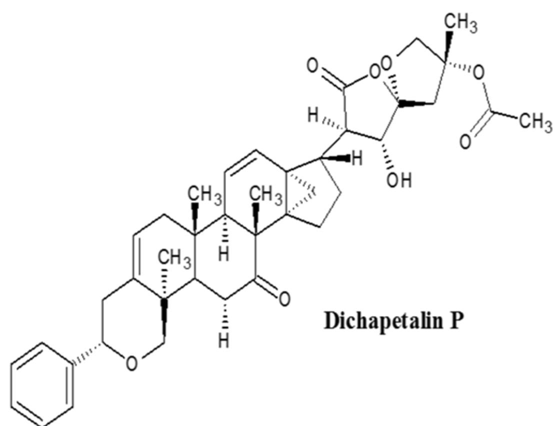
Dichapetalins A, L, N………………………...*D. mombuttense*

Dichapetalins C, I, P, S……………………....*D. leucosia*

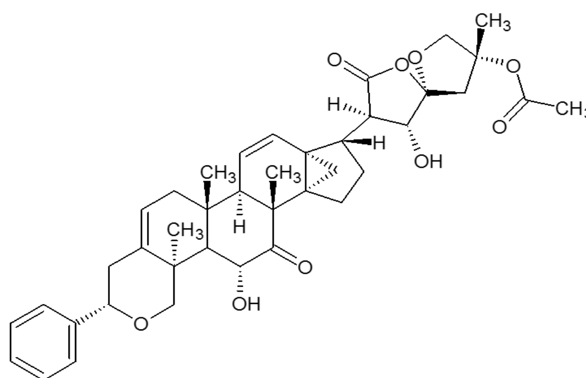
Dichapetalins A, B, L, O, P, Q, R………....*D. zenkiri*

Dichapetalins A, M………………………..*D. eickii*

The researchers worked on the ethyl acetate extracts of the roots of the various *dichapetalum* species and through 1D and 2D-NMR, and MS, the structures of these compounds were elucidated. Dichapetalin P was identified as 6-deoxy dichapetalin M. The compounds were tested against a variety of cancer cell lines and they showed cytotoxic properties and antiproliferative activities in the range of 10^{-6} to 10^{-8} M. The analysis indicated dichapetalins M and P as the most potent[65].



Dichapetalin P



Dichapetalin M

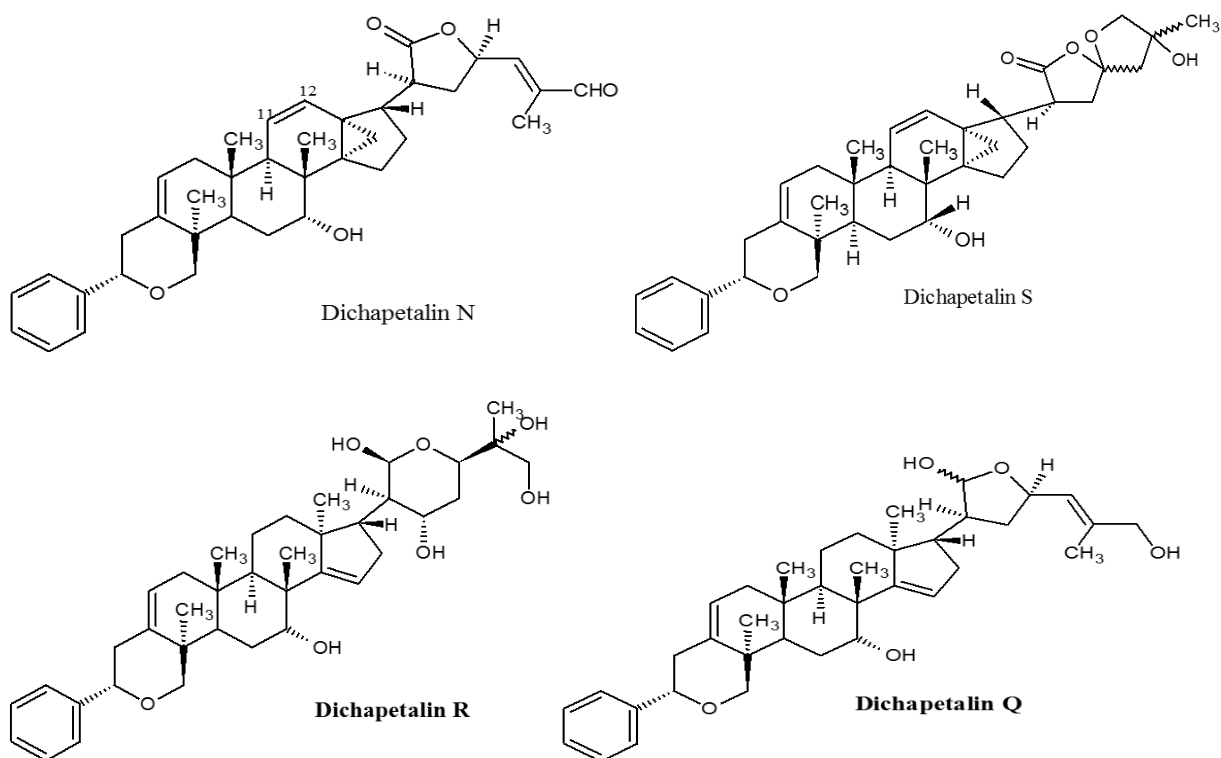


Table 1: Most significant results on Cytotoxicity of Dichapetalins M and P on brine shrimp and cancer cell lines

Bioassay	Activity			
	Type	**Value (M)**	**Outcome**	**Dichapetalin**
Brine shrimp[10]	EC_{50}	2.0×10^{-8}	Active	M
Cytotoxicity against HCT116 human colorectal carcinoma[65]	EC_{50}	9.9×10^{-9}	Active	M
Cytotoxicity against WM 266-4 human melanoma[65]	EC_{50}	7.8×10^{-8}	Active	M
Cytotoxicity against HCT 116 human colorectal carcinoma[65]	EC_{50}	5.8×10^{-8}	Active	P
Cytotoxicity against WM 266-4 human melanoma[65]	EC_{50}	2.3×10^{-7}	Active	P

University of Ghana http://ugspace.ug.edu.gh

Table 2: Cytotoxic activity of some dichapetalins against two human cancer cell lines[65]

Dichapetalin	**Bioassay**	
	Human colorectal carcinoma HCT 116 (EC_{50} /M)	Human melanoma WM 266-4 (EC_{50} /M)
A	2.5×10^{-7}	1.7×10^{-5}
B	8.1×10^{-8}	3.4×10^{-7}
C	5.0×10^{-7}	2.5×10^{-6}
I	2.8×10^{-7}	1.0×10^{-5}
L	6.8×10^{-7}	3.1×10^{-6}
N	9.2×10^{-8}	1.5×10^{-6}
O	8.9×10^{-7}	8.4×10^{-6}
Q	2.5×10^{-6}	2.7×10^{-5}
R	4.2×10^{-6}	3.1×10^{-5}
S	4.5×10^{-7}	1.3×10^{-6}

2.1.7.1 Structure-activity relationship (SAR) existing among the dichapetalins

Comparing the structural differences that exist among the dichapetalins, the toxicities exhibited by brine shrimp and cancer cell lines have so far shown significant activity, especially with dichapetalins possessing a lactone (dichapetalins A, B, I, J, K, L, M, N, P and S) and lactol (dichapetalins O, Q and R) side chain[42,64,65]. It can be inferred that activities with respect to these dichapetalins could be due to the presence of the γ-lactone/lactol in the R group which links to C-17. Dichapetalins M and P have so far exhibited the most significant cytotoxicity results (Table 1).

University of Ghana http://ugspace.ug.edu.gh

Earlier on, hypothetical structure-activity relationship (SAR) studies had revealed the importance of the lactone side chain to cytotoxic activity and the activity of dichapetalin M was envisaged to be significantly higher than that of dichapetalin A due to the stability of the lactone ring provided by the presence of the spiroketal side chain in dichapetalin M[10b]. Analysis by Long *et al*[65] has confirmed the importance of the spiroketal lactone for cytotoxic activity as shown by dichapetalins P and M. However, since dichapetalin S, the only other dichapetalin with a spiroketal side chain, exhibited decreased activity, it is likely that the high activity observed in dichapetalins M and P could be due to the combined effects of the spiroketal lactone ring and carbonyl group in position 7. The presence of an extra hydroxyl group at C-22 possibly increased the hydrophilicity of dichapetalin M. Furthermore, the importance of the 6-hydroxyketone for activity is seen in the reduction in activity of dichapetalin P compared to dichapetalin M towards HCT116. A similar observation can be made in their activities towards WM266-4 (Tables 1 and 2).

Dichapetalins O, Q and R showed a decreased toxicity against cancer cell lines due to the absence of a lactone in the R group[65]. Dichapetalins B, M, N, and P recorded higher selectivity towards human colorectal carcinoma HCT116 than dichapetalin A while human melanoma WM266-4 showed more resistance against dichapetalins Q and R than dichapetalin A. Dichapetalin R was the least sensitive. Dichapetalins B and P had similar activities towards the two cell lines.

Dichapetalins C, D, E, F, G and H possess a methyl ester side chain at C-17. Dichapetalin C was active to a lesser extent while dichapetalins D & F were almost inactive in the brine shrimp test[10b]. So far, only dichapetalin C from the methyl ester side chain group has been evaluated for antiproliferative activity against cancer cell lines. Although it exhibited comparable activities with

University of Ghana http://ugspace.ug.edu.gh

some members of the lactone/lactol side chain group, dichapetalins P and M were far more potent[65].

2.1.8 Dichapetalin-type triterpenes from *Phyllanthus acutissima* (Euphorbiaceae)

A number of dichapetalin-type triterpenes have been obtained from *Phyllanthus acutissima,* which belongs to a totally different plant family, the Euphorbiaceae. This class of dichapetalin-like triterpenes has been named the acutissimatriterpenes. So far, five of these have been isolated, characterized and named acutissimatriterpene A, B, C, D and E. These compounds are similar to the dichapetalins in the possession of a lactone side chain on ring D and the presence of a substituted phenylpyrano group. The spiroketal side chain of acctissimatriterpenes A, B and E resemble that of dichapetalin M from *D. madagascariense* except that the acetoxy group at C-25 in dichapetalin M has been substituted with a methoxy group in the acutissimatriterpenes. These compounds have also shown significant cytotoxic and anti-HIV-1 activities mainly due to the lactone side chain group, a situation similar to the dichapetalins from *D. madagascariense* and *D. gelonioides*. However, the differences are the side chain groups at C-17 and the C-6' substituents and the methylenedioxy units in the phenylpyrano moiety[66].

University of Ghana http://ugspace.ug.edu.gh

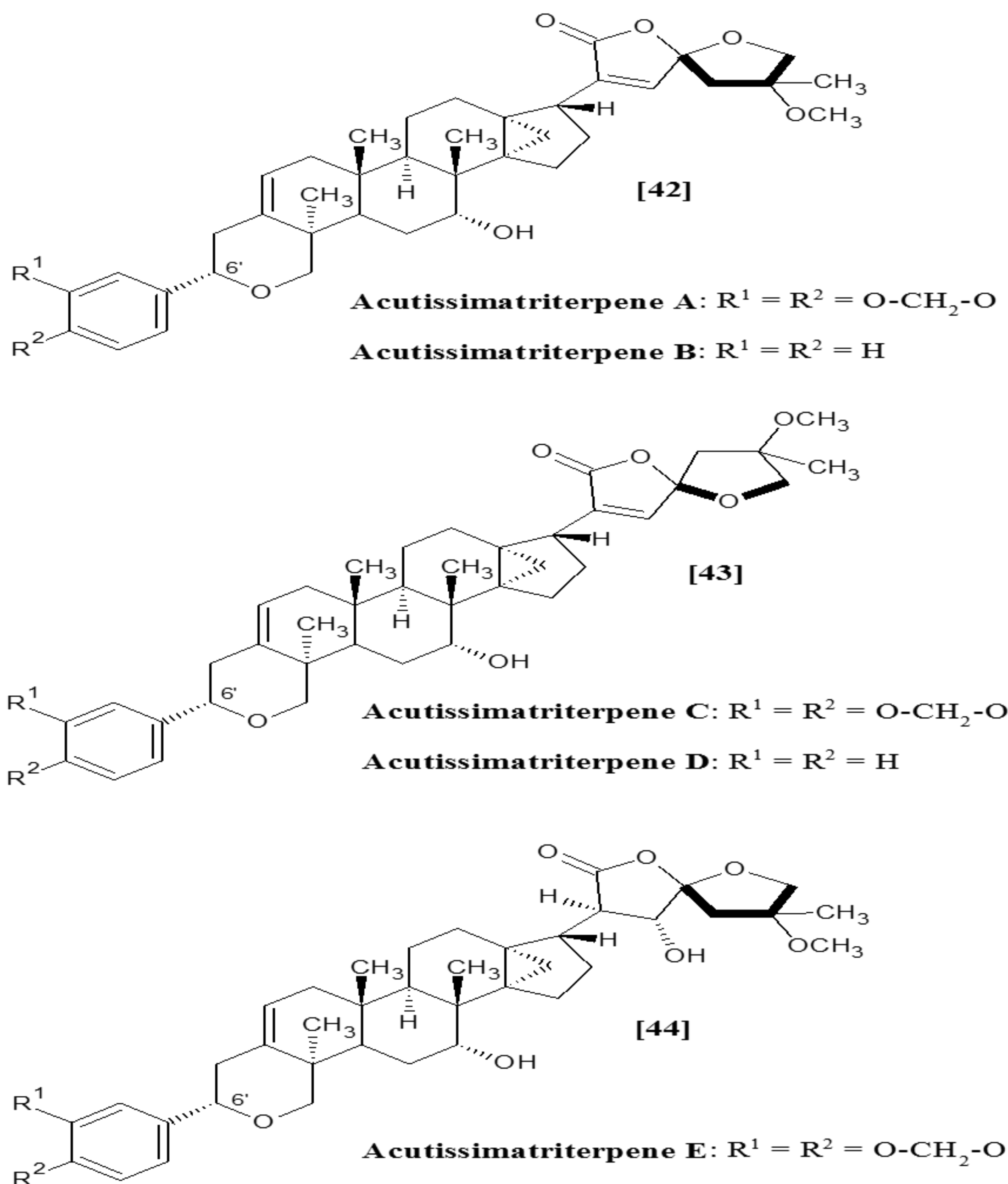


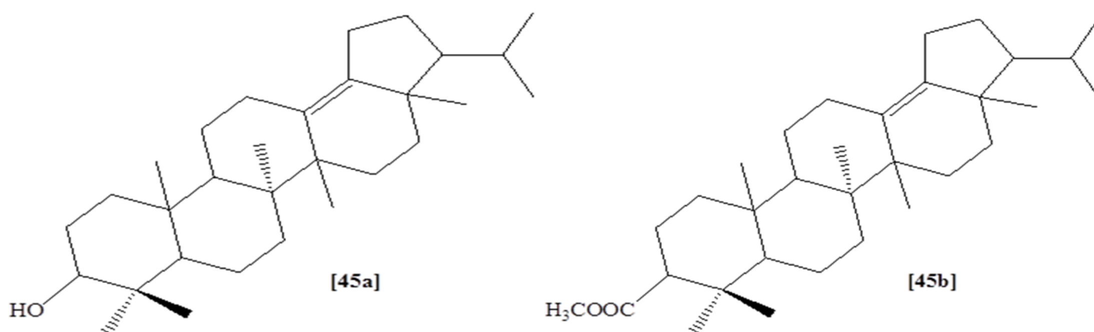
Figure 6: Isolated dichapetalin-type triterpenoids from *Phyllanthus acutissima*

University of Ghana http://ugspace.ug.edu.gh

2.2 Previous Investigations of Grain Insects control

Insects normally oviposit in the punctures created in the storage roots and seeds during insect feeding[67].

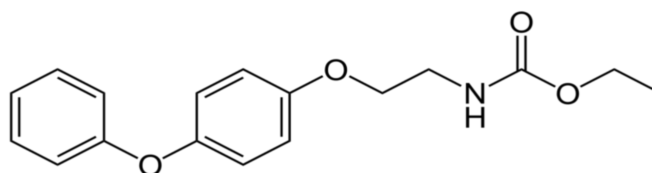
Wilson *et al.*[68] demonstrated that the presence of two triterpenes- boehmeryl acetate **[45b]** and boehmerol **[45a]**, in the periderm of sweet potato stimulate oviposition and feeding. This was because when they removed the periderm, no oviposition or stimulation in feeding occurred. The triterpenes in the periderm of potato caused the stimulated feeding and oviposition in the potato weevil.



However, Darbah's[41] investigation of another two triterpenoids- D:A-friedo-oleanan-3-one [18] and D:A-friedo-oleanan-3β-ol [19] from the stem of the *D. madagascariense* could not deter or stimulate feeding and oviposition in sweet potato weevils because there were no significant differences between the tested compounds and the controls ($p>0.05$).

Letellier *et al* [69] also tested fenoxycarb **[46]** (a carbamate insecticide, $C_{17}H_{19}NO_4$) on *S. zeamais*. After 3 weeks exposure to grain treated at 10 mg/kg, it was observed that 45.5% adult mortality and 95% inhibition of progeny occurred. The fenoxycarb **[46]** reduced oviposition at 10 mg/kg. Hidden immature stages were affected only when treatment took place on grain within 2 weeks of oviposition. Progeny in grain treated with 1 mg/kg fenoxycarb were unaffected at any age.

University of Ghana http://ugspace.ug.edu.gh



Fenoxycarb

[46]

Arannilewa and Odeyemi[70] also researched on the efficacy of the ethanolic extract of four plant materials: *Zanthoxylum xanthoxyloides* (Lam.) Waterm, *Aristolochia ringens* (Vahl), *Colocasia esculenta* (L) Schott and *Morinda lucida* (L.) on the maize weevil. These researchers assessed adult toxicity, adult emergence, and percentage reduction of grain weight and damage effect of weevils to the grains at concentrations of 0.0, 1.0, 1.5 and 2.0% (W/V). *Z. xanthoxyloides* evoked 100% adult mortality within 24h after treatment while *A. ringens* caused 100% mortality within 48h. *A. ringens* at all concentrations provided good protection to the grains stored for 49 days and also gave 100% reduction value as well as 0.00% damage and 0.00% weevil perforation index.

In 2008, Bisseleua *et al* [71] demonstrated that the crude extracts of *Zanthoxylum xanthoxyloides* (leaves, stems and roots) could be used as a botanical insecticide in alternative control strategies against *Callosobruchus maculatus*. It was observed that the root extract of the plant was highly toxic to the insect (LD_{50} in 24 hours for topical application= 4.98 µg and LD_{50} in 96 hours grain treatment= 0.18 g of root extract/100g of grain). Development of eggs and larvae within grain kernels, as well as progeny emergence were significantly inhibited in the treated grains with dosage $\geq$ 0.4 g per 100 g of grain. There was indication of the plant extracts being repellent to the insect but with considerable variation in their repellency ($P \leq 0.001$). From the investigation, all the parameters were dose-dependent with the exception of repellency.

The biological activity of *Zanthoxylum xanthoxyloides* against *S. zeamais* (Coleoptera: Curculionidae) and *Callosobruchus maculatus* (Coleoptera: Bruchidae) has also been investigated[72] in the laboratory using admixtures of leaf, root and bark powders of *Z. xanthoxyloides.* Methanol extracts of the plant material were applied at 2.0 ml per 50 g of grains for contact mortality, damage assessment, progeny development and repellent action. The results indicated that the root and bark powders caused 100% mortality of both *S. zeamais* and *C. maculatus* while complete protection of grains and progeny inhibition were achieved. Topical application of the extracts caused significant insect mortality ($P<0.01$) with fresh bark extract offering complete protection of grains and complete inhibition of progeny production by both *S. zeamais* and *C. maculatus*. The extracts also evoked moderate repellent effect against the two insect pests.

Udo *et al.* [73] in 2009 investigated the efficacy of powders from plant parts of *Ricinodendron heudelotii* against *S. zeamais* and *C. maculatus* on stored maize and cowpea, respectively. Leaf, bark and root powders were added as admixtures to 100 g of grains to assess contact toxicity, damage assessment, progeny production and grain germination. The results indicated that the plant materials were toxic to the two insect species with over 30% and 75% mortality for *S. zeamais* and *C. maculatus,* respectively. Observable damage level was significantly lower ($p \leq 0.05$) in the treated grains while progeny production by both insect species was significantly reduced ($p \leq 0.05$). Grain germination of both crops was not affected by the powders.

Nukenine *et al.* [74] also investigated Calneem oil from Ghana and local neem oils from two localities in northern Cameroon (Garoua and Maroua). The phytochemicals were tested at 0 (untreated control), 2, 4, 6, 8 and 12 ml/kg of grain, on the adult and immature stages of *S. zeamais*, for

University of Ghana http://ugspace.ug.edu.gh

mortality and reproduction inhibition. Neem oils from Cameroon were extracted using the traditional kneading method and a hydraulic press in the laboratory (refined). Maize grains were coated with the five neem seed oils (Calneem, Garoua traditional and refined, and Maroua traditional and refined, respectively) and adult mortality was recorded at 1, 3, 7 and 14 days after exposure. Within 1 day of exposure, the highest tested concentration (12 ml/kg) of Calneem, Garoua traditional, Garoua refined, Maroua traditional and Maroua refined oils caused similar weevil mortality of 86.3, 93.8, 93.8, 97.5 and 97.5%, respectively. The LC_{50} (24-hour) values for the oils in the same order were 7.0, 6.0, 5.0, 5.0 and 4.8 ml/kg, respectively. It was observed that the lowest (2 ml/kg) and highest (12 ml/kg) concentrations of the oils suppressed progeny production by over 80 and 98%, respectively. The results revealed that all the oils in a dose-dependent manner inhibited the development of the hidden eggs and immature stages of *S. zeamais* in the maize grains in the range of 86.9 –100%. These results agreed with earlier findings by Lale and Abdulrahman[75], that two formulations of neem seed oil (traditional and soxhlet extracted) significantly reduced the development of eggs and larvae to adults in *C. maculatus* since the two insects showed similar physiological effects, irrespective of the method of extraction used.

Roxas *et al.*[76] also evaluated the insecticidal action of five locally available plants namely: *Azadirachta indica* (Neem), *Cymbopogon citratus* (Lemon Grass), *Lantana camara* (Lantana), *Ocimum basilicum* (Basil) and *Tagetes erecta* (African marigold) against maize weevil, *Sitophilus zeamais* Motsch. The investigation was based on the repellency, adult mortality, and antioviposition and growth inhibition tests. Results revealed that all test materials exhibited repellency action against maize weevil. Powdered leaves of Neem and Lantana were noted to be highly repellant while powdered leaves of lemon grass, basil and the African marigold were

University of Ghana http://ugspace.ug.edu.gh

observed to be moderately repellant against maize weevil within 96 hours of exposure. Grains treated with powdered leaves of lemon grass and basil exhibited a low mortality of 5.33% and 0.66%, respectively, at 24 days after insect introduction. Other test plants revealed zero adult mortality. There was no anti-ovipositional and growth inhibitory action against the maize weevil. All examined grains showed larval tunnel. The total development period within which maize weevils emerged from both treated and untreated grains was the same (39 days) and 100% insect survival was noted indicating zero percent of insects that reached larval or pupal stages only.

Using contact toxicity, progeny production, damage assessment and repellency assays, the biological effects of chloroform extract of the leaves of the Siam weed *Chromolaena odorata* (L.) known in Ghana as "Acheampong''and mahogany tree *Khaya senegalensis* (Ders.) A.Juss (Meliaceae) against *Sitophilus oryzae* (L.) in stored maize were assessed in the laboratory[77]. The extract of *K. senegalensis* showed 100% mortality in maize grains treated with the highest concentration of 30 ml/kg after 72 hours exposure while *C. odorata* extract was moderately toxic to the insects. There was complete inhibition in the number of progeny produced by the *S. oryzae* at higher dosages (20 and 30 ml/kg) with grains treated by the extracts of the two plant materials. As the repellency was dose-dependent, both extracts were highly repellent to *S. oryzae.*

It can be inferred from the above that the use of plant extracts and/ or isolates to protect grains from pests is very promising because of several distinct advantages like their ability to adversely affect insect growth and oviposition.

University of Ghana http://ugspace.ug.edu.gh

2.2.1 Reproductive cycle of the maize weevil (*Sitophilus zeamais* Motschulsky)

The adult female *S. zeamais* lays her eggs within the actual grain kernels of the maize at a rate of 25 per day spread over 100 days[78]. It bores into the grain, deposits her ovipositor and lays a single egg. Most eggs are deposited in the endosperm, but 28% are in or around the germ. Maximum daily rate of fecundity is 6.7 eggs per female in 24 hours. Depending on the humidity and moisture content of the grain, the eggs hatch in approximately 3 days. The larvae begin to eat the internal contents of the maize while developing, which takes approximately 18 to 23 days. At this point, the larvae become pupae, and they begin the transformation into the adult weevil form which takes approximately 6 days at 25 ^{o}C[79].

Within the 6 days, the pupae do not eat or move. The duration of development and number of progeny produced are optimal at 30°C and 75% relative humidity (RH)[21]. The lowest temperature for development from egg to adult weevils is 15.6^{o}C and the upper limit is 32.5^{o}C at 75% RH[21]. The weevil then emerges by cutting a small circular hole in the grain, as an adult and begins the process all over again. The entire process takes about 30 to 45 days to complete (averaged 36 days) at 27±1°C, and 69±3% RH[78]. The lifespan of adult maize weevil is approximately 5 to 8 months[78].

2.3 Prevalence and importance of Soil-Transmitted Helminths

Ascaris lumbricoides (roundworm), *Necator americanus/Ancylostoma duodenale* (hookworms) and *Trichuris trichiura* (whipworm) are the three most prevalent Soil-Transmitted Helminths (STH's) worldwide. By estimation, there are more than one billion cases worldwide, of which 500 million people suffer significant morbidity attributable to their infection, the majority of the

University of Ghana http://ugspace.ug.edu.gh

infected being children. The mortality rate of hookworm or roundworm infections is approximately 135,000 deaths per year [80]. Using the disability-adjusted life years (DALYs) lost as a means of measurement, STH infections are as important as malaria or tuberculosis[81]. DALYs means the number of healthy years lost to premature death or disability.

Table 3: Comparison of the STH infections with other major diseases in developing Countries [80,81]

Disease	DALYs Lost annually (million)	Deaths per year
Hookworm	22.1	65,000
Ascariasis	10.5	60,000
Trichuriasis	6.4	10,000
TOTAL	**39.0**	**135,000**
Tuberculosis	34.7	2-3 million
Malaria	46.5	112 million

Table 4: Infected population estimates of human STH worldwide and in sub-Saharan Africa[82,83]

STH	Infected population worldwide (million)	Infected population in sub-Saharan Africa (million)
Hookworm	576-740	198
Ascariasis	807-1221	173
Trichuriasis	604-794	162
Total	**1987-2756**	**533**

University of Ghana http://ugspace.ug.edu.gh

In all the three STHs, the highest prevalence is observed in areas of rural poverty and morbidity and this is attributable to the chronic consequences of infection. The level and intensity of infection determines its morbidity and transmission. The key determinants of epidemiology are inadequate sanitary conditions affecting the world's poorest people. The three STH have low mortality but high morbidity rates and so the affected populations frequently suffer poly-parasitism[82-84]. Notwithstanding the high morbidity and mortality rates of the cases mentioned in Table 4, major pharmaceutical industries are generally not interested in development of drugs to combat them, because of the low profitability of such drugs. This is why these diseases are normally referred to as neglected tropical diseases.

2.3.1 The Biology Hookworms

Necator and *Ancylostoma* are the two different genera of the hookworm. *Necator americanus,* compared to *Ancylostoma duodenale* is the most predominant species of hookworm within the sub-Saharan region of Africa due to the favourable climatic conditions[34, 82]. There are pathobiological differences between the two genera of the hookworm.

Ancylostoma duodenale [82]

This species has the ability to survive in more extreme environmental conditions and cause infection via oral route. It has high fecundity due to the fact that the female can produce 25,000-35,000 eggs per day, and between 18 million and 54 million during its life time. Maximum egg output is reached within 15 and 18 months after infection. Its buccal capsule contains two pairs of sharp teeth. The female is slightly longer compared with its male counterpart: 1.2×0.6 cm against 1×0.5 cm. The adult worm lives for 1-3 years.

Necator americanus [82]

The buccal capsule is smaller compared with *A. duodenale* but this species has blunt cutting plates instead of teeth for attachment to the small intestinal mucosa. It has slightly larger eggs of 70×40 μm but the adult worm is shorter and more slender of measurement 1×0.4 cm. The female produces about 20,000 eggs daily with its life span of 3-5 years but its male counterpart can live up to 10 years.

Life cycle of *N. americanus* [85]

The eggs are passed in the stool. Under favourable conditions like moisture, warmth and shade, the larvae hatch in one to two days. The released *rhabditiform* larvae grow in the faeces and/or the soil. After 5 to 10 days, they become *filariform* (third stage) larvae that are infective. On contact with the human host, the larvae penetrate the skin. They are then carried through the veins to the heart and to the lungs. They penetrate into the pulmonary alveoli, ascend the bronchial tree to the pharynx, and are swallowed. The larvae reach the small intestine, where they reside and mature into adults. The adult worms live in the lumen of the small intestine attached to the intestinal wall and cause resultant blood loss to the host.

2.3.2 Hookworm infection

Hookworm is an important human pathogen, with developing countries recording highest rates of infections with a range of 20-80%[34,36]. *Necator americanus* and *Ancylostoma duodenale* are the two most important hookworm species within the sub-Saharan region due to their widespread prevalence, distribution and ability to contribute to hundreds of millions of infections[34].

University of Ghana http://ugspace.ug.edu.gh

Hookworm infection rate in Ghana is about 3.2% [86]. Based on WHO classification guidelines, this rate of infection is classified as light intensity [1- 1999 eggs per gram (epg)]. The predicted intensity of infection is ≥1 epg[87]. What makes the problem significant and worthy of attention is that about 80% of anaemia and iron-deficiency related diseases in pregnant women in Ghana are estimated to be attributed to hookworm infections[86]. Some of the end results of anaemia are malaise, digestive disturbance and inability of patients to excrete waste products[87]. Hookworm infection is also co-endemic with malaria, tuberculosis, HIV, malnutrition as well as cognitive and educational deficits in both children and adults[88,89]. Morbidity associated with hookworm infection varies from mild, transient clinical signs and symptoms to severe clinical diseases[85].

The presence of only one human hookworm can consume 0.03-0.6 ml of blood per day[90] while 40-160 human hookworms may cause severe anaemia[90]. In fact, the presence of iron supplementation cannot even do away with anaemia if there are 1200 hookworms. The persistent anaemia in children as a result of severe hookworm infection may have severe long-term consequences[87].

Table 5: Relationship between hookworm burden and anaemia and amount of blood loss with different hookworm loads[82].

Hookworm infection intensity	Egg per gram (epg) of faeces	Mean blood loss (ml/day)	Standard deviation
Negative	0	1.24	1.85
Light	1-999	1.46	1.07
Moderate	1000-4999	2.96	3.03
Heavy	>5000	8.79	1.10

University of Ghana http://ugspace.ug.edu.gh

Hookworm eggs found in faeces are characteristically oval-shaped with thin hyaline shell; they measure 60-75 µm. They are usually in the 4- or 8- cell stage in fresh faeces or in a more advanced stage of cleavage in feces that have been kept at room temperature for even a few hours[91].

2.3.3 Detection and assay of hookworm eggs

Kato-Katz technique[91]

The Kato-Katz technique is a (semi) quantitative method that provides information on the intensity of Soil-Transmitted Helminths infection. This technique is employed to detect the presence of hookworm eggs. It is based on the clearing of a thick faecal smear with glycerine in the presence of a background stain, usually malachite green so that the eggs appear unstained. The mesh filters the specimen so that faecal fibres are kept off the slide. The technique is feasible for mass screening as the collected specimen can be examined at leisure in the laboratory.

Egg Hatch Assay / Test (EHA/T)

The EHA is an ovicidal method used to investigate anthelminthic activity of a set of compounds against both laboratory and field isolates of hookworm. It is one of the standardized tests to detect drug resistance recommended by the World Association for the Advancement of Veterinary Parasitology (WAAVP)[92]. The EHA assesses the ability of a drug at given concentrations to inhibit the embryonation and hatching of freshly collected nematode eggs expressed as the dose required to inhibit 50% hatching of the eggs (ED_{50})[91,92]. The EHA is the most appropriate test for use with human hookworm since human hookworm eggs hatch rapidly. It gives accurate and reliable results. In view of this, WHO (1999) has promoted the evaluation and standardization of the EHA in human helminthology.

2.3.4 Previous Investigations of the chemotherapy of Soil-Transmitted Hookworm (STH) and other helminths

Reported cases of Anthelmintic Resistance (AR) in hookworms are limited in published literature based on efficacy level of some synthetic anthelminthics with many of them providing inconclusive results.

In 1992, Coles *et al* [92] reported ED_{50} values of thiabendazole in nematodes to be in excess of 0.1 mg/ml. This was considered as indicative of benzimidazole resistance but the value is too high since the discriminating dose (LD_{99}) ought to be 0.1 mg/ml. Janssens and Stepek *et al* [93] reported some of the percentage cure rate (% CR) of some anthelmintics as indicated in table 6.

Table 6: Percentage cure rates for the anthelminthics used to control STH [93]

Anthelminthic	GI Nematodes (% CR)		
	Hookworms	***A. lumbricoides***	***T. trichiura***
Mebendazole	9-60*	95-100	45-100
Albendazole	33-95	67-100	10-77
Pyrantel	37-88	81-100	0-56
Levamisole	66-100	86-100	16-18
Ivermectin	0-20	50-75	11-80

[* In areas of high transmission (prevalence >50%)]

A study by De Clercq *et al* [94] in 1997 in Mali on *N. americanus* was suggestive that anthelmintic resistance against mebendazole had developed in the region with recorded cure rate of 22.9%, Egg Reduction Rate (ERR) of 6.5% and ED_{50} value of 0.117 instead of 0.069. However, when Sacko

University of Ghana http://ugspace.ug.edu.gh

et al [95] compared the efficacy of pyrantel, albendazole and mebendazole in the same localities they fell short of providing conclusive evidence of anthelmintic resistance against mebendazole.

Reynoldson *et al* [96] suggested that the Australian *A. duodenale* strain was resistant to pyrantel. The results were based on only 15 individuals. On Pemba Island, Zanzibar, the efficacy of mebendazole against hookworms in school children appeared to have fallen over a period of 5 years, during which time the children were regularly treated with mebendazole (ERR fell from 82.4% to 52.1%). This suggested the possibility of emergence of mebendazole -resistant hookworms on Pemba Island[97].

A series of studies were carried out between 1998-2000 on Pemba Island, Zanzibar, to evaluate, adapt and refine the egg hatch assay as a test to detect benzimidazole resistance in human hookworm[98]. A single dose of mebendazole was found to have disappointing efficacy (ERR of 31%) against hookworms infections in Vietnam[99], although repeated dosing and single dose albendazole treatment achieved higher efficacy (75% and 88%, respectively)[93].

A meta-analysis conducted by Nilanthi de Silva from the University of Kelanyia, Zanzibar for the efficacy of mebendazole against the three major STH showed high rates of drug failure (low efficacy) against hookworms, particularly in Pemba[97]. While the biological basis for this has not been extensively investigated there is the possibility of anthelminthic resistance (AR). Similar studies carried out in Papua- New Guinea by Kotze *et al.* [100] showed that the egg hatch assay is a reproducible test to perform in the field and that it proved effective in quantifying drug sensitivity in drug-susceptible hookworms.

University of Ghana http://ugspace.ug.edu.gh

Case studies have showed that due to complexities (anthelminthic resistant and efficacy) and cost in controlling the helminth diseases, treatment from plant sources has been an alternative approach in several societies [101]. It is against this background that the WHO in its Tropical Diseases Control Programme has laid a special emphasis on the use of traditional medicines to combat the menace of parasitic diseases globally[102]. However, there are few reported and published research cases on helminths.

Githiori *et al.*[103] evaluated the anthelmintic efficacy of some ethnoveterinary medicine (EVM) preparations used by pastoralists and Small Holder Farmers (SHF) in Kenya. The research was on the following plant species: *Aframomum sanguineum, Albizia anthelmintica, Ananas comosus, Annona squamosa, Azadirachta indica, Dodonaea angustifolia, Hagenia abyssinica, Hildebrandtia sepalosa, Myrsine africana, Olea europaea* var. *africana, and Rapanea melanophloeos*. Evaluation was carried out in two *in vivo* infection models, *Haemonchus contortusin* in (sheep) and *Heligmosomoides polygyrusin* in (mice). The anthelmintic efficacy of the EVM preparations was monitored through faecal egg count (FEC) reduction at regular intervals for a period of 2 – 3 weeks after treatment in sheep. However, the monitoring in mice was done through FEC and total worm counts (TWC) one week after treatment. For a useful anthelmintic efficacy, an *a priori* cut-off value of 70% reduction of FEC and TWC was used for both sheep and mice. Of all the plant species tested in sheep, the largest decrease of 34% in FEC was measured from a bark preparation of *A. anthelmintica*. None of the other plant species had a significant effect on FEC. Similarly, the seven plant species, apart from *A. sanguineum, D. angustifolia, and H. sepalosa* evaluated in mice did not significantly reduce FEC or remove parasites. Preparations of *A. anthelmintica* at doses above 0.5 g per mouse were toxic. Hence, no reduction of FEC or TWC

University of Ghana http://ugspace.ug.edu.gh

greater than the *a priori* value of 70% was observed in sheep or in mice. Therefore, the plants evaluated were ineffective as anthelmintics in the preparations and forms that were used.

However, an *in vivo* test conducted by Salifou *et al* [104] in evaluating the efficacy of an aqueous extract of leaves of *Chenopodium ambrosioïdes* against nematode parasites in fifteen naturally infected West African Long Legged goats (*Capra hircus*) from Northern Benin, reported a significant (P <0.001) decrease of faecal *strongyl*e eggs in experimental groups. Three levels (1, 2 and 4 ml/kg body weight/3 days treatment) of concentration of the herbal drug were administered to three experimental groups. This was compared with two control groups (one without treatment and the other treated with levamisole as a reference drug). After three days of treatment, the reduction in the rate of egg shedding was over 70% in animals treated with plant extracts, whereas egg shedding rates remained the same in the non-treated group. The rates of reduction in faecal eggs reached almost 100% in a few days (5 to 6 days) among treated animals without a significant dose-dependent effect. Moreover, helminthological autopsy performed on the study animals after treatment showed an almost total absence of worms. Thus *C. ambrosioïdes* aqueous extract exerted a kind of parasite clearing effect in the abomasum and in the small intestine of the animals, an indication of the effectiveness of the extract against gastrointestinal parasitic worms. The lowest dosage of 1ml/kg body weight for three days treatment was, therefore, found to be appropriate and prescriptive. There was no acute toxicity recorded as the autopsy of internal organs revealed only rare cases of congestion and petechiae on the liver and lungs in animals treated with 4ml/kg body weight.

University of Ghana http://ugspace.ug.edu.gh

CHAPTER THREE

3 PRESENT INVESTIGATION

3.1 Extraction and Phytochemical Screening of the stem *Dichapetalum madagascariense*

The air-dried pulverized sample of the stem of *D. madagascariense* (5.0 Kg) was first exhaustively extracted with petroleum ether using a soxhlet extractor. After the petroleum ether extraction, the plant material was air dried and cold extraction by chloroform was carried out, then followed by acetone. Another fresh plant material of the whole stem of *D. madagascariense* (5.0 Kg) was also exhaustively extracted with ethanol followed by extraction with methanol using a soxhlet extractor. The extracts were concentrated and dried under reduced pressure using a rotary vacuum evaporator.

The petroleum ether, chloroform, ethanol, acetone and the methanol crude extracts were then subjected to a series of phytochemical screening and the table 7 below is a summary of the results obtained:

Table 7: Phytochemical screening of the crude extracts of *D. madagascariense*

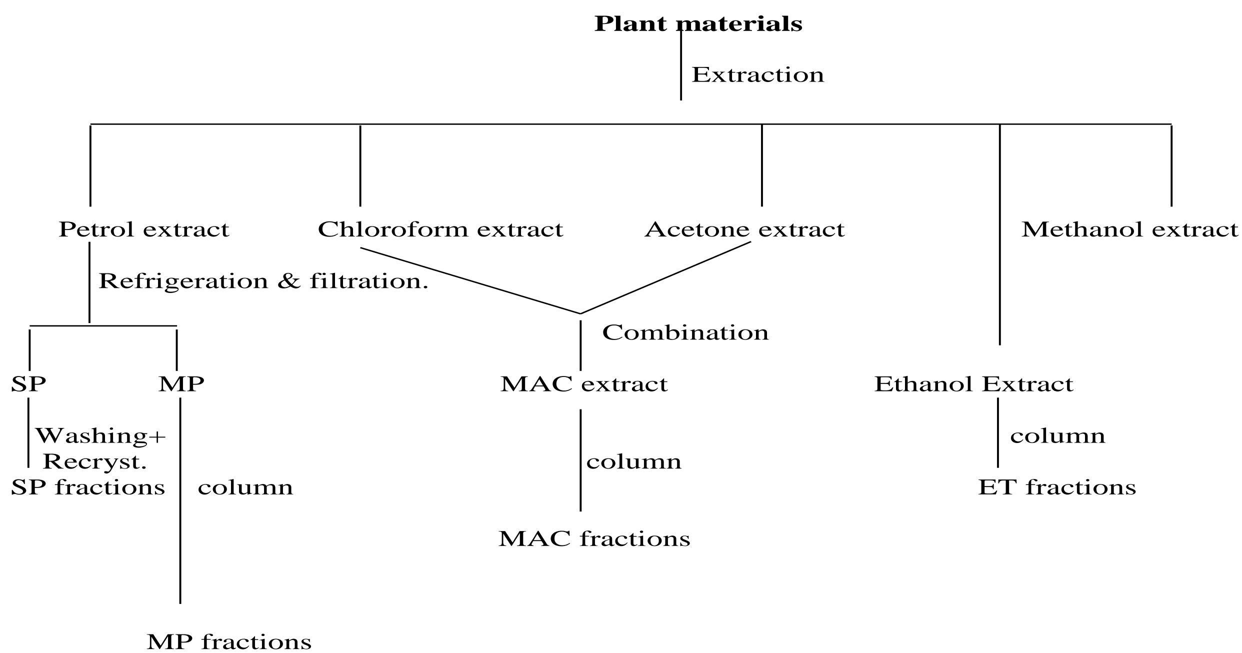
CLASS OF COMPOUND	PETROLEUM ETHER EXTRACT (MPC)	ACETONE EXTRACT (MA)	ETHANOL EXTRACT (ET)	CHLOROFORM EXTRACT (MC)	METHANOL EXTRACT (ME)
Alkaloids	-	-	-	-	-
Anthraquinones & Anthracene	+	+	-	-	-

Flavonoid & Leucoanthocyanin	-	-	+	-	+
Cardiac Glycosides	+	+	+	+	+
Saponins	-	+	+	+	+
Tannins	-	-	+	+	+
Terpenoids	+	+	+	+	+

Key: + means present, - means absent

From the phytochemical screening of the crude extracts of *D. madagascariense* stem, cardiac glycosides and terpenoids were present in all the above extracts but flavonoids and leucoanthocyanins were present only in the ethanol extract; saponins were absent only in the petroleum extract while tannins were present in the ethanol and chloroform extracts. anthraquinones and anthracene were present in both petroleum and acetone extracts.

Scheme 4: Flow chart of procedure used in extractions



University of Ghana http://ugspace.ug.edu.gh

3.1.1 Work done on Petroleum ether Extract

The petroleum ether extract, MPC, upon refrigeration precipitated some solids which were filtered to give a solid coded SP yield and the filtrate coded MP. MP was concentrated to give a mass of 6.50 g.

3.1.1.1 Isolation of D:A-friedooleanan-3β (Epifriedelanol or friedelan-3β-ol) (SP-a1)

SP was obtained as whitish brown grains insoluble in petroleum ether, acetone, ethanol and methanol but partially soluble in chloroform and a mixture of petroleum ether/chloroform. Repeated recrystallization of SP in chloroform and petroleum ether/chloroform mixture gave crystals **SP-a** to **SP-d**. Apart from SP-b which dissolved completely in cold chloroform, the rest dissolved upon warming in chloroform. TLC analysis of all the crystals showed two spots which stained orange and purple when sprayed with anisaldehyde reagent. None of these spots fluoresced under UV light. Attempts to also separate the components by repeated recrystallization proved futile, with the exception of SP-a, which precipitated pure solid coded SP-a1 (150 mg). Comparative TLC with authentic samples of friedelan-3β-ol and friedelan-3-one indicated that SP-a1 was probably D:A-friedooleanan-3β-ol (Epifriedelanol or friedelan-3β-ol).

3.1.1.2 Identification of D:A-friedooleanan-3β-ol (Friedelan-3β-ol) (SP-a1)

Friedelan-3β-ol was isolated as white crystals with a melting point of 270-271°C. The melting point from literature is 274-276 °C[105]. The molecular formula is $C_{30}H_{52}O$[105] and the molecular mass is 428. From the molecular formula of the compound, the double bond equivalent (DBE) is five.

University of Ghana http://ugspace.ug.edu.gh

Table 8: Identification of D:A-friedooleanan-3β-ol (Epifriedelanol or friedelan-3β-ol)

Sample	Mass (mg)	MP (℃)	Solvent system	R_f
SP-a1	150	270-271	Pet ether: EtOAc 3:1	0.83
			Pet ether: EtOAc 20:1	0.29
			100% $CHCl_3$	0.75

The IR spectrum signal for OH group was observed at 3620 and 3477 cm^{-1} and the absorption peak at 3006 cm^{-1} was due to C-H stretching. The C-O stretching bond had an absorption peak of 1089 cm^{-1}. The geminal dimethyl group showed peaks at 1385 and 1362 cm^{-1}.

Table 9: Infrared values of Epifriedelanol (Friedelan-3β-ol) (SP-a1)

IR (KBr) ν_{max} cm^{-1}	Functional group	Literature value
3620 and 3477	OH stretching	3475[105]
3006	C-H stretching	2933[105]
1089	C-O stretching	1080[41]
1385 and 1362	geminal dimethyl	1380 and 1340[41]

The ^{13}C-NMR spectrum, appendix IV, showed the presence of 30 carbons. From DEPT analysis at 135°, the spectrum showed that there were six quaternary, five methine, eleven methylene and eight methyl carbons. The presence of a hydroxyl group is evident in ^{13}C-NMR spectrum by the presence of a signal at δ_C 72.8. Previously isolated friedelan-3β-ol[41] from the same plant part had showed similar spectrum at δ_C 72.75 on C-3.

University of Ghana http://ugspace.ug.edu.gh

The ^{1}H NMR spectrum, appendix III, showed characteristic triterpenoid protons signal at δ_H 0.88–1.55 and also showed a broad signal at δ_H 3.73 ppm on C-3. The presence of double triplet peaks at δ 1.90 (d t, J_1=10.1, J_2 =3.0 Hz) and 1.73 (d t, J_1=12.8, J_2 =3.0 Hz) were due to one of the protons, H-2a on C-2 and a similar one, H-6a on C-6 respectively, while multiple signals at δ_H 1.57 ppm was attributed to the other proton, H-2b on C-2. These multiple signals are caused by a proton each on C-10 and C-8 because of methyl groups around their environments. The presence of eight singlet peaks at upfield was as result of the eight methyl protons. The signals at δ_H 0.73, 0.86, 0.93, 0.95, 0.97, 1.01, 1.05 and 1.17 ppm are attributable to the protons of the methyl groups attached to C-5, C-4, C-9, C-13, C-14, the geminal dimethyl group attached to C-20 and the methyl group on C-17 respectively. The methylene protons also gave signal between δ_H 1.20 and 1.60 ppm. Previous investigation[56] had obtained a broad signal at δ_H 3.35 on C-3 and the rest of signals appearing at δ_H 0.80, 0.85, 0.90, 0.92, 0.96 ppm for the methyl protons attached to C-5, C-4, C-9, C-13, C-14; the geminal dimethyl group attached to C-20 and the methyl group on C-17 showed signals at δ_H 1.02, 1.04 and 1.19 ppm respectively while the methylene protons gave signal between δ_H 1.20 and 1.65 ppm.

The ^{1}H and ^{13}C NMR spectral data for this compound indicate that it belongs to the friedelane group and is identified as friedelan-3β-ol **[19]** based on its identical ^{1}H and ^{13}C NMR data and physical constants reported in literature.

University of Ghana http://ugspace.ug.edu.gh

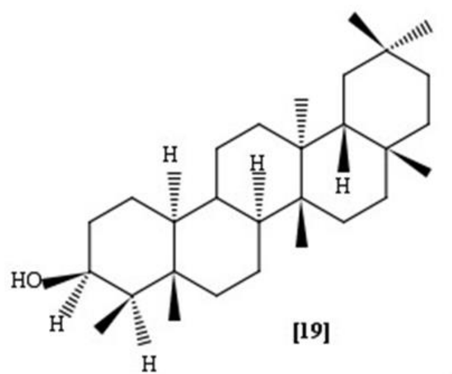


Table 10: Comparison of ^{13}C- NMR chemical shifts of the epifriedelanol (friedelan-3β-ol) [19] (SP-a1) with literature values

POSITION	GROUP	SP-a1	Zj wu[106]	Kundu[107]	Chau[108]	Rahayu[105]
		δ_C	δ_C	δ_C	δ_C	δ_C
1	CH_2	**15.8**	15.8	15.8	16.2	15.8
2	CH_2	**35.4**	35.4	35.2	35.7	35.3
3	CH	**72.8**	72.6	72.8	73.1	72.8
4	CH	**49.2**	48.7	49.2	49.6	49.2
5	C	**37.9**	37.8	37.1	37.5	37.1
6	CH_2	**41.8**	41.7	41.7	42.1	41.7
7	CH_2	**17.6**	17.1	17.6	17.9	17.5
8	CH	**53.2**	52.4	53.2	53.6	53.2
9	C	**37.1**	37.8	38.4	38.2	37.8
10	CH	**61.4**	61.3	61.4	61.8	61.3
11	CH_2	**36.1**	35.5	35.3	35.6	35.2

University of Ghana http://ugspace.ug.edu.gh

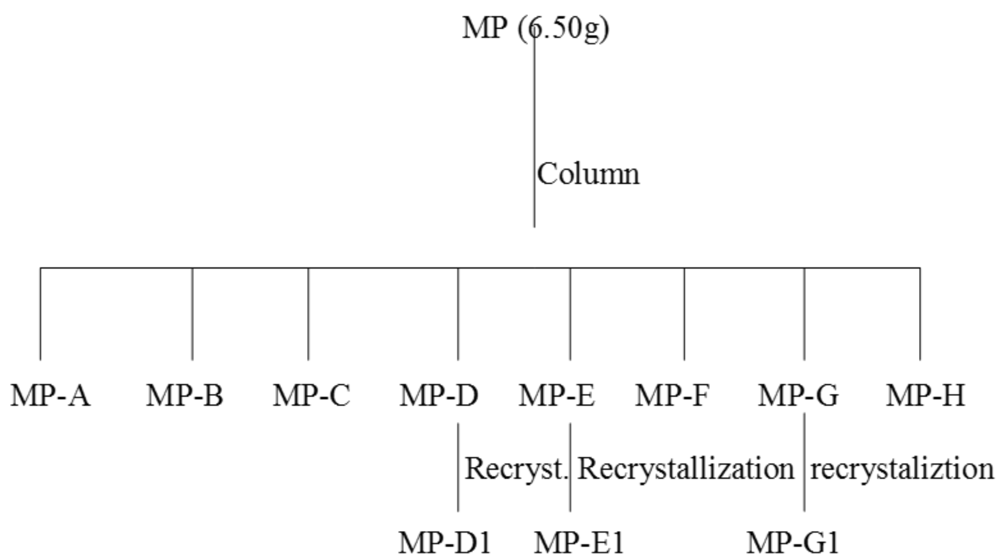
12	CH_2	**30.6**	30.9	30.6	31.0	30.6
13	C	**38.4**	38.4	37.8	38.7	38.4
14	C	**39.3**	38.9	39.7	40.0	39.3
15	CH_2	**32.3**	32.3	32.2	33.2	32.8
16	CH_2	**35.2**	35.4	36.1	36.5	36.1
17	C	**30.0**	30.3	30.0	30.0	30.0
18	CH	**42.9**	42.8	42.8	43.2	42.1
19	CH_2	**35.0**	35.3	35.6	35.9	35.6
20	C	**28.2**	28.0	28.2	28.7	28.2
21	CH_2	**32.8**	32.5	32.8	32.7	32.1
22	CH_2	**39.7**	39.1	39.3	39.7	39.3
23	CH_3	**11.6**	11.1	11.6	12.0	11.6
24	CH_3	**16.4**	16.9	16.4	16.8	16.4
25	CH_3	**18.2**	18.2	18.2	18.6	18.6
26	CH_3	**18.6**	18.7	18.6	19.0	18.6
27	CH_3	**20.1**	20.0	20.1	20.5	20.1
28	CH_3	**31.8**	31.8	31.8	32.5	32.3
29	CH_3	**35.7**	35.5	35.0	35.4	35.0
30	CH_3	**32.1**	32.3	32.1	32.2	31.8

University of Ghana http://ugspace.ug.edu.gh

3.1.1.3 Chromatographic Separation of constituents of the oily mother liquor of the Petroleum ether extract of *Dichapetalum madagascariense* (MP)

Chromatographic separation of MP (6.50 g) was performed using 230 g of silica gel slurry in petroleum ether. Elution of the column was first started with 100% petroleum ether followed by petroleum ether/ ethyl acetate (20:1). The polarity of the eluent was increased gradually until 100 % of ethyl acetate was used. 5 cm^3 portions of the eluate were collected and monitored by TLC and combined into 8 main fractions labeled MP-A to MP-H. Work done on the eight (8) fractions is shown in the scheme 5 below.

Scheme 5: Separation of constituents of the oily mother liquor of the Petroleum ether extract of *D. madagascariense*



Fraction MP-A was collected as a light yellow oil and is UV active (light blue). When the fraction was concentrated to dryness, the oil was left behind. TLC analysis showed a single purple spot

when sprayed with anisaldehyde spray reagent in the solvent systems below. Iodine vapour also stained it at the same R_f as the anisaldehyde spray reagent.

Solvent system	R_f
Petrol: EtOAc 3:1	0.85
Petrol: EtOAc 20:1	0.75
$CHCl_3$: MeOH 98:2	0.85

Fraction MP-B was collected as a deep yellow oil. When it was concentrated to dryness, oil was left behind. TLC analysis showed a single purple spot when sprayed with anisaldehyde reagent in the solvent systems below and the spot fluoresced under UV light (light blue). Iodine vapour also stained it at the same R_f as anisaldehyde.

Solvent system	R_f
Petrol: EtOAc 3:1	0.85
Petrol: EtOAc 20:1	0.75
$CHCl_3$: MeOH 98:2	0.85

Fraction MP-C was collected as a light green oil and was UV active (light blue). When it was concentrated to dryness, oil was left behind. TLC analysis of the oil gave a single purple spot when sprayed with anisaldehyde reagent in the solvent systems below. It fluoresced under the UV light (light blue). The spot was stained with iodine vapour.

Solvent system	R_f
Petrol: EtOAc 3:1	0.85
Petrol: EtOAc 20:1	0.75
$CHCl_3$: MeOH 98:2	0.85

University of Ghana http://ugspace.ug.edu.gh

MP-D as a fraction was collected as a wine-coloured liquid. The iodine vapour gave 5 spots on TLC with 100% chloroform as solvent system, with none of the spots fluorescing under UV light.

Fraction MP-E was collected as a yellow liquid. Based on TLC analysis in chloroform, 4 spots were obtained on staining with iodine vapour. None of the spots fluoresced under UV light.

Fraction MP-F was collected as a yellowish green liquid. Three (3) spots were obtained on TLC with chloroform, and were stained by iodine vapour. None of them fluoresced under UV light.

Fraction MP-G was collected as a light green liquid. TLC in chloroform indicated 1 spot which was stained by the iodine vapour, the spot fluorescing under UV light.

MP-H as a fraction was a dark green liquid. One of the 3 spots fluoresced under UV light when TLC analysis was carried out in chloroform. The spots were stained with iodine vapour.

3.1.1.4 Isolation and Identification of a mixture of β–Sitosterol and Stigmasterol (MP-G1)

The column chromatographic separation of the petroleum ether extract on silica gel afforded MP-G. Recrystallization of MP-G gave the solid MP-G1. It was obtained as a white crystalline substance. TLC with 100% chloroform indicated 1 spot which was stained by the iodine vapour, the spot fluorescing under UV light. Comparative TLC with authentic sample of the β-sitosterol indicated that MP-G1 and the authentic sample had the same $R_{f.}$ MP-G1 gave a positive test to Liebermann Buchard reagent for steroidal or terpenoidal nucleus. The melting point of MP-G1 was determined as 139 - 140 °C.

When MP-G1 (17 mg) was subjected to IR Spectroscopic analysis, there was an absorption band of 3430 cm^{-1} which is characteristic of O-H (stretching). Absorption at 2868 cm^{-1} was assigned to C-H (stretching). 1667 cm^{-1} occurred as a result of weak C=C absorption while 1465 cm^{-1} is a bending frequency for cyclic $(CH_2)_n$ and 1382 cm^{-1} for $–CH_2\ (CH_3)_2\gamma$ (geminal dimethyl group). The absorption frequency at 1242 or 1133 cm^{-1} can be assigned to C-O stretching while absorption at 1052 or 1022 cm^{-1} signifies cycloalkane.

The ^{13}C-NMR, appendix IV, showed recognizable signals 140.76 and 121.71 ppm, which were assigned to C-5 and C-6 doubly bonded carbon respectively. The δ_C value at 71.81 ppm was due to C-3 the β-hydroxyl group. The signals around δ_C 19.4 and 11.9 ppm corresponded to the angular carbon atoms (C-19 and C-18 respectively). The value for C-18 is lower due to γ-gauche interaction that increases the screening of the C-18 hence lower chemical shift. However, the loss of H in C-6 resulted in decrease in screening of the C-19 leading to increase in ^{13}C chemicals shift to higher frequency. This was also tenable as in chemical shift of 21.2 and 11 ppm for C-19 and C-18 respectively.

From the ^{1}H NMR there is existence of signals at δ_H 5.40 – 5.00 ppm (for methine protons) but the proton at C-3 showed a multiple signal around 3.50 ppm due to the coupling and splitting by protons on C-2 and C-4. The signals around δ_H 2.0 ppm is attributed to the methylene protons especially signal 2.30 - 1.90 is attributable to protons on C-2 and C-4. The chemical shift of δ_H 1.70 – 0.70 ppm are for methyl protons like those on C-18 and C-19.

University of Ghana http://ugspace.ug.edu.gh

Table 11: Comparative ^{13}C-NMR chemical shifts of β-sitosterol and stigmasterol from Literature

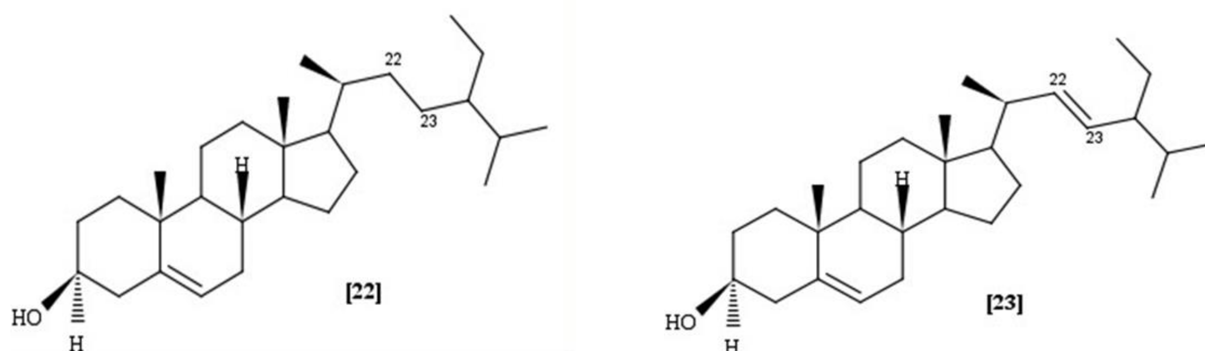
Carbon position	Carbon type	Literature values (β-sitosterol)		Literature values (stigmasterol)		MP-G1
		δ_C[109]	δ_C[110]	δ_C[109]	δ_C[111]	δ_C
1	CH_2	37.3	37.28	37.3	35.5	37.25
2	CH_2	31.6	31.69	31.6	28.4	31.67, 30.27
3	CH	71.8	71.82	71.8	71.8	71.81
4	CH_2	42.2	42.33	42.3	35.4	42.31
5	C	140.8	140.70	140.8	142.0	140.76
6	CH	121.7	121.72	121.7	122.0	121.71
7	CH_2	31.9	31.69	31.9	29.1	32.41
8	CH	31.9	31.93	31.9	30.5	31.90
9	CH	51.2	50.17	51.2	49.6	50.16
10	C	36.5	36.52	36.5	35.9	36.14, 35.88
11	CH_2	21.1	21.10	21.1	17.9	21.08
12	CH_2	39.8	39.80	39.7	46.2	39.78, 39.68
13	C	42.3	42.33	42.3	44.7	42.21
14	CH	56.8	56.79	56.9	35.3	56.77, 56.86

University of Ghana http://ugspace.ug.edu.gh

15	CH_2	24.3	24.37	24.4	24.8	24.30, 24.36
16	CH_2	28.3	28..25	28.4	28.0	28.24
17	CH	56.0	56.09	56.1	34.3	55.96, 56.06
18	CH_3	11.9	11.86	11.0	15.6	11.85
19	CH_3	19.4	19.40	21.2	18.9	19.39
20	CH	36.2	36.52	40.5	41.1	36.50, 40.48
21	CH_3	18.8	18.79	21.2	25.4	18.77, 18.69
22	CH_2, CH	33.9	33.98	138.3	131.1	33.95, 138.30
23	CH_2, CH	26.1	26.14	129.3	25.9	26.08, 129.28
24	CH	45.9	45.88	51.2	50.0	45.84, 51.23
25	CH	29.2	28.91	31.9	47.6	28.91, 29.16
26	CH_3	19.8	19.80	21.2	19.1	19.81
27	CH_3	19.3	18.79	19.0	19.2	18.97, 19.03

28	CH_2	23.1	23.10	25.4	25.7	23.07, 25.40
29	CH_3	12.2	11.99	12.1	15.7	12.24, 12.04

The above IR, ^{13}C-NMR and ^{1}H-NMR spectral data and a comparison of the ^{13}C-NMR signal with those described in the literature showed the structure of MP-G1 to be a mixture of β-sitosterol and stigmasterol. The presence of a double bond on C-22 and C-23 in Stigmasterol and single bond on C-22 and C-23 in Sitosterol is only difference between the two compounds. Pure β-sitosterol has been isolated in petroleum ether extract from the stem bark of *D. madagascariense*[56]; it is difficult to be obtained in its pure state when there is presence of stigmasterol. However, it is best to be separated by HPLC technique. This is the first time of obtaining the mixture from the stem of the plant after isolating it from the roots.



Plant sterols are well known biogenetic precursors of many hormones and oviposition stimulants of some insects[112]. β-sitosterol possesses antihyperlipoproteinaemic, antibacterial and antimicotic activities and has been shown to act as inhibitor of tumor promotion *in vivo*[113] and also inhibited carcinogenesis[114]. Stigmasterol is known to markedly inhibit tumor promotion in two-stage

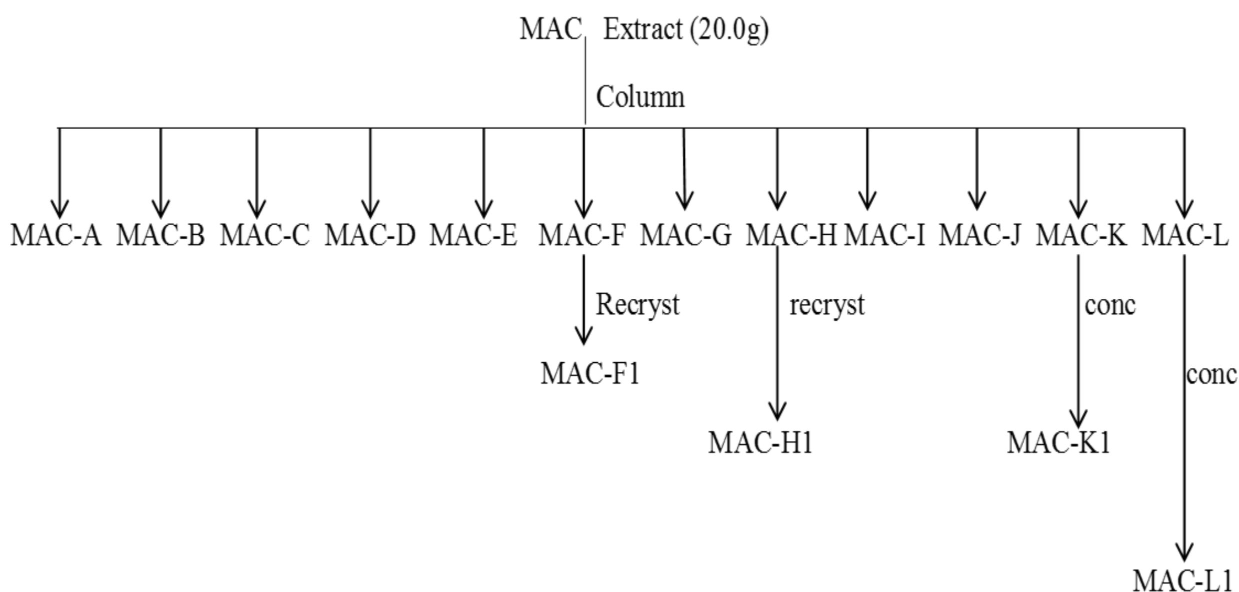
University of Ghana http://ugspace.ug.edu.gh

carcinogenesis in mice[113, 115] and exhibited significant inhibitory effect on HIV reverse transcriptase[116]. However, a mixture of β-sitosterol and stigmasterol is known to possess anti-inflammatory activity[117]. Hence, MP-G1 was subjected to some biological assays to ascertain its activities.

3.1.2 Work done on chloroform and acetone extracts

The TLC profiles of the chloroform and acetone crudes were similar, hence the two extracts were combined and coded MAC. Chromatographic separation of 20.0 g of this extract on silica gel (600 g) with petroleum ether/ ethyl acetate mixtures (the polarity of the eluent was increased gradually until 100 % of ethyl acetate and finally washed with methanol) yielded 12 fractions labeled MAC-A to MAC-L.

Scheme 6: Separation of MAC extract of *Dichapetalum madagascariense* (stem)



University of Ghana http://ugspace.ug.edu.gh

Each of the fractions namely, MAC-A, MAC-B, MAC-C and MAC-D when concentrated left behind oils. Each gave a single purple spot when sprayed with anisaldehyde spray reagent. The spot fluoresced under UV light and was also stained by iodine vapour at the same place as the spot with anisaldehyde spray reagent.

MAC-F precipitated a solid which was filtered and coded MAC-F1. TLC analysis of MAC-F1 showed one purple spot when sprayed with anisaldehyde reagent. The spot did not fluoresce under UV light. Comparative TLC with authentic sample of friedelan-3-one (PAD E_{II}) indicated that they are the same. However, attempts to isolate dichapetalin A (which was shown on TLC with reference standard of dichapetalin A, ADK-1) from the supernatant MAC-F proved futile. This solution was coded MAC-F.

Table 12: Some comparative physical values of MAC-F1 and MAC-F with some authentic samples

Sample	Mass (mg)	MP (°C)	Solvent system	R_f
MAC-F1	190	245-246	PE:EA=3:1	0.90
			PE:EA=20:1	0.46
			CH=100	0.71
MAC-F	n.d	n.d	PE:EA=1:1	0.25
			PE:EA=1:2	0.40
			PE:EA=3:2	0.13
PAD E_{II}	n.d	n.d	PE:EA=3:1	0.90
			PE:EA=20:1	0.46
			CH=100	0.56
ADK-1	n.d	n.d	PE:EA=1:1	0.25
			PE:EA=1:2	0.40
			PE:EA=3:2	0.13

Key: n.d= not determined

University of Ghana http://ugspace.ug.edu.gh

MAC-H precipitated a solid. It was filtered and coded MAC-H1. TLC analysis of the solid gave two purple spots when sprayed with anisaldehyde spray reagent in the following solvent systems: Pet ether: EtOAc 3:1 (0.90); Pet ether: EtOAc 20:1 (0.46). None of the spots of MAC-H1 fluoresced under UV light. Comparative TLC with authentic samples of friedelan-3-one and friedelan-3β-ol (PAD E11 and ADB 1C) indicated that MAC-H1 and the standards had the same R_f. The melting point of MAC-H1 was found to 273- 275 °C.

Fraction MAC-K was collected as a light green liquid. When the fraction was concentrated to dryness, an oil was left behind. It was carefully decanted and coded MAC-K1. TLC analysis of MAC-K1 gave a single orange spot when sprayed with anisaldehyde reagent in the solvent systems below. However, it was not UV active.

Solvent system	R_f
Cyclohexane: acetone (10: 1)	0.13
Cyclohexane: acetone (20:1)	0.13
Petroleum ether (100)	0.10

3.1.2.1 Isolation and Identification of Friedelin (D:A-friedooleanan-3-one) [18]

Friedelin (friedelan-3-one) [18], also known as D:A-friedooleanan-3-one, was isolated as white needles, with melting point of 245-246 °C. The literature value is found to be 249-251 °C[105].

The IR spectrum showed an intense band at 1715 cm^{-1} consistent with a six membered ring ketone. The geminal dimethyl group at C-20 showed absorption peaks at 1390 and 1363 cm^{-1}.

University of Ghana http://ugspace.ug.edu.gh

Table 13: Some IR values of friedelin (MAC-F1)[18]

IR (KBr) ν_{max} cm^{-1}	Functional group	Literature value [41]
1715	C=O	1720
1390	CH_3	1390
1363	CH_3	1375

The ^{13}C- NMR spectrum showed the presence of thirty carbon resonances. The DEPT spectrum showed the presence of a ketone carbonyl at δ_C 213.21 ppm. The rest of the 29 carbon atoms appeared between δ_C 0 and δ_C 60 ppm consisting of eight methyl (CH_3), eleven sp^3 methylene (CH_2), four sp^3 methine (CH) and six quarternary sp^3 carbon (C) groups.

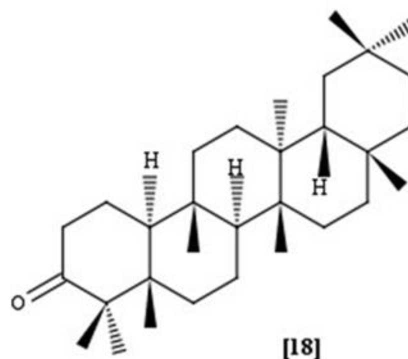
The presence of signals due to one secondary and seven singlet methyls in the ^1H- NMR spectrum suggested the friedelane skeleton. In the ^1H-NMR spectrum the quartet signal integrating for one proton at δ_H 2.25 was assigned to H-4. The neighbouring methyl protons on C-23 are splitting H-4 into a quartet. Meanwhile, the resonances of the eight methyl protons showed signals at δ_H 0.73 (s, 3H-28) on C-17; δ_H 0.88 (s, 6H-29&30) on C-20, indicating geminal dimethyl groups; δ_H 0.95 (s, 3H-25) on C-9; δ_H 1.01 (s, 6H-26 & 27) on C-13 and C-14, these are two methyl groups with the same environment; δ_H 1.05 (s, 3H-23) on C-4; δ_H 1.18 (s, 3H-24) on C-5. The protons on C-2 and C-4 gave multiple signals between 2.42 and 1.64 ppm. Previous investigation[56] on the same plant had obtained resonances of the eight methyl protons showed signals at δ_H 0.75 on C-17; δ_H 0.88 on C-20, indicating geminal dimethyl groups; δ_H 0.95 on C-9; δ_H 1.00 on C-13 and C-14, these are two methyl groups with the same environment; δ_H 1.05 on C-4; δ_H 1.17 on C-5. The protons on C-2 and C-4 gave multiple signals between 2.50 and 1.65 ppm.

The ^{1}H and ^{13}C NMR spectral data for the isolation compound indicated that it belongs to the friedelane group and identified as friedelin (D:A-friedooleanan-3-one, **[18])** based on its identical ^{1}H and ^{13}C NMR data and physical constants reported in literatures.

Table 14: Comparative ^{13}C-NMR chemical shifts of friedelin (friedelan-3-one or D:A-friedooleanan-3-one, [18]) from literature

POSITION	GROUP	MAC-F1	Esobedo[118]	Hisham[119]	Patra[110]	Rahayu[105]
		δ_C	δ_C	δ_C	δ_C	δ_C
1	CH_2	**22.27**	22.30	22.3	22.3	22.3
2	CH_2	**41.53**	41.54	41.5	41.5	41.5
3	C	**213.21**	213.33	213.2	213.2	213.2
4	CH	**58.24**	58.24	58.2	58.2	58.2
5	C	**42.14**	42.16	42.1	42.1	42.1
6	CH_2	**41.29**	41.31	41.3	41.2	41.3
7	CH_2	**18.23**	18.25	18.2	18.2	18.2
8	CH	**53.10**	53.12	53.1	53.1	53.1
9	C	**37.44**	37.46	37.4	37.4	37.4
10	CH	**59.48**	59.50	59.5	59.5	59.4
11	CH_2	**35.62**	35.64	35.6	35.6	35.6
12	CH_2	**30.50**	30.52	30.5	30.5	30.5
13	C	**38.29**	38.31	39.7	39.7	39.7
14	C	**39.70**	39.72	38.3	38.3	38.3
15	CH_2	**32.42**	32.44	32.8	32.4	32.4
16	CH_2	**36.01**	36.03	36.0	36.0	35.9
17	C	**29.99**	30.10	30.0	30.0	29.9
18	CH	**42.79**	42.81	42.8	42.8	42.8
19	CH_2	**35.34**	35.36	35.4	35.5	35.3

20	C	**28.16**	28.18	28.2	28.2	28.1
21	CH_2	**32.77**	32.79	32.4	32.8	32.7
22	CH_2	**39.25**	39.27	39.2	39.2	39.2
23	CH_3	**6.81**	6.83	6.8	6.8	6.8
24	CH_3	**14.65**	14.67	14.6	14.6	14.6
25	CH_3	**17.94**	17.96	17.9	17.9	17.9
26	CH_3	**20.25**	20.27	20.3	20.2	20.2
27	CH_3	**18.65**	18.67	18.7	18.6	18.6
28	CH_3	**32.09**	32.10	32.1	32.1	32.1
29	CH_3	**35.01**	35.04	35.0	35.0	34.9
30	CH_3	**31.77**	31.79	31.8	31.8	31.8



[18]

The molecular formula is $C_{30}H_{50}O$[110] with molecular mass of 426 g/mol

The quantities of friedelan-3-one were very high in the petroleum ether and acetone extracts from the stem and roots of the plant[41,42] and relatively high in the ethanol extract from the roots of the plant[42]. Friedelan-3-one has so far not shown any appreciable cyctotoxity against brine shrimps bioassay, hence, the need to subject the compound to other biological assay.

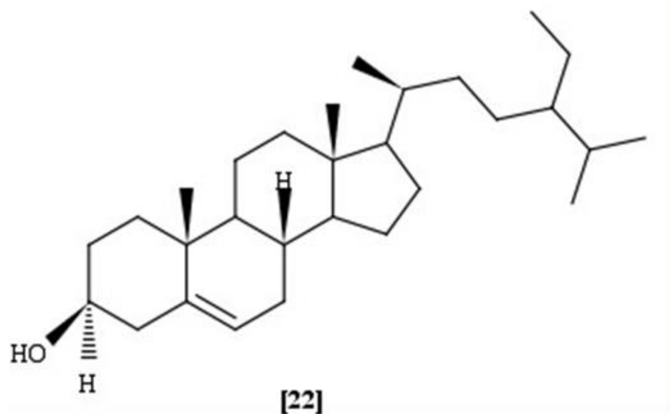
University of Ghana http://ugspace.ug.edu.gh

3.1.2.2 Isolation and identification of β-sitosterol [22] (MAC-I1)

Fraction MAC-I was collected as a light green liquid. TLC profile in chloroform indicated 2 spots which were stained by the iodine vapour, none of the spots fluorescing under UV light MAC-I precipitated a white flaky solid. It was filtered, recrystallized and coded MAC-I1 (26 mg). TLC analysis of the solid gave one purple spot when sprayed with anisaldehyde spray reagent; the spot did not fluoresce under UV light. Comparative TLC with authentic sample of β-sitosterol indicated that MAC-I1 and the standard have the same R_f. The melting point of MAC-I1 was 126-128 °C (the literature value 142-144 °C[120]). The wide difference between the reported melting point and the literature value could be attributed to possible presence of impurities. The R_f values of MAC-I1 in petrol- ethyl acetate 1:1 and 100% chloroform were 0.80 and 0.30 respectively. The compound gave a positive test with Liebermann Buchard reagent for steroidal or terpenoidal nucleus. MAC-I1 was also compared with MP-G1. The only difference between them was that MP-G1 fluoresced under UV light but MAC-I1 did not. The authentic sample, β-sitosterol also did not fluoresce under UV light. Apart from IR spectroscopy, MAC-I1 was not subjected to any other spectral analysis compared to MP-G1 in the present investigation.

The IR spectrum of MAC-I1 showed a peak at 3550 cm^{-1} attributable to the hydrogen-bonded hydroxyl (OH) group. The peak at 2936 cm^{-1} is attributed to the methylene ($-CH_2$) group while 2867 cm^{-1} signal could be assigned to methine (-CH) group. 1638 cm^{-1} signal has C=C stretch while the peak at 1377 cm^{-1} may be assigned to geminal dimethyl group. The peak at 1063 cm^{-1} was assigned to C-O stretch in secondary alcohol. Based on the IR spectrum and other physical constants like melting point of the isolated compound with the authentic sample, MAC-I1 is probably β-sitosterol.

University of Ghana http://ugspace.ug.edu.gh



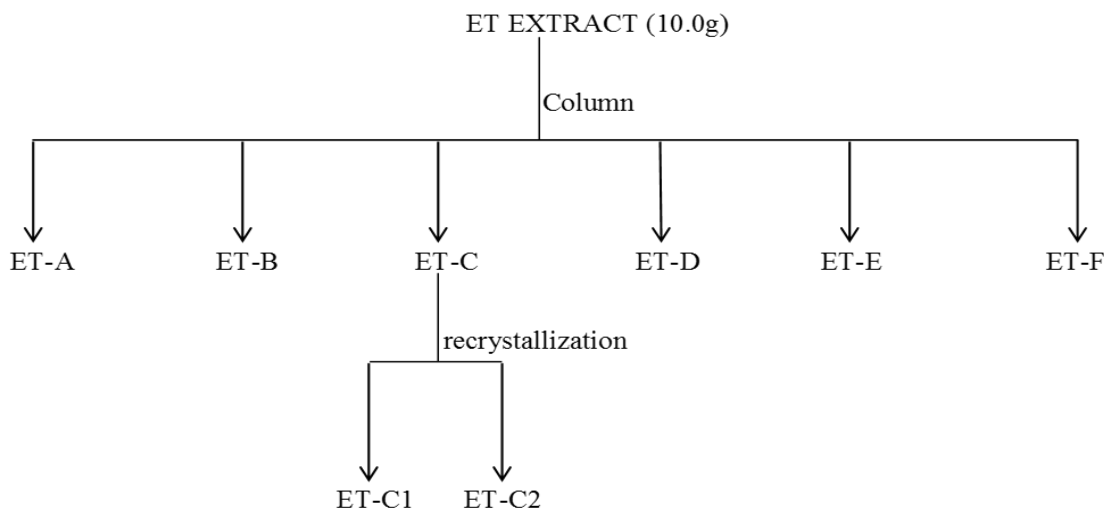
β-sitosterol has also been isolated from the petroleum ether extract of the stem bark of *D. madagascariense*[56].

3.1.3 Work done on ethanol extract

Chromatographic separation of ethanol extract (10.0 g) was performed using 300 g of silica gel slurry in ethanol. Elution of the column was first started with 100% petroleum ether followed by petroleum ether/ ethyl acetate (20:1). The polarity of the eluent was increased gradually until 100% of ethyl acetate was used finally and washed with methanol. 5 cm^3 portions of the eluate were collected and monitored by TLC and combined into 6 main fractions labeled ET-A to ET-F. Work done on the six (6) fractions is shown in the scheme 8 below.

University of Ghana http://ugspace.ug.edu.gh

Scheme 7: Separation of ethanol (ET) extract of *D. madagascariense*



3.1.3.1 Isolation and identification of a mixture of friedelin and epifriedelanol (friedelan-3β-ol and friedelan-3-one or friedooleanan-3β-ol and D:A-friedooleanan-3-one)

ET-C precipitated a solid which was filtered, recrystallized and coded ET-C1 (253.5mg). TLC analysis of the crystal showed two purple spots when sprayed with anisaldehyde reagent. None of the spots fluoresced under UV light. Comparative TLC with reference standards of friedelan-3β-ol and friedelan-3-one (PAD E11 and ADB 1C) indicated that they have the same R_f. ET-C1 was also compared with the already isolated friedelan-3β-ol and friedelan-3-one and was found to be a combination of the two compounds. Attempts to separate the two spots proved futile.

ET-C1 was isolated as white grain with melting point of 257-259 ℃**.**

The IR spectrum signal for an OH group was observed at 3624 cm^{-1} and the absorption peak at 3483 cm^{-1} was due to the C-H stretching bond of alkane functionality. The C-O stretching bond had an absorption peak at 1174 cm^{-1}. The presence of a carbonyl group with a sharp signal was

University of Ghana http://ugspace.ug.edu.gh

found at 1709 cm^{-1}. The presence of a geminal dimethyl group was also evident by the peak at 1452 and 1387 cm^{-1}.

Table 15: Infrared values of a mixture of friedelin and epifriedelanol (friedelan-3β-ol and friedelan-3-one or friedooleanan-3β-ol and D:A-friedooleanan-3-one) ET-C1

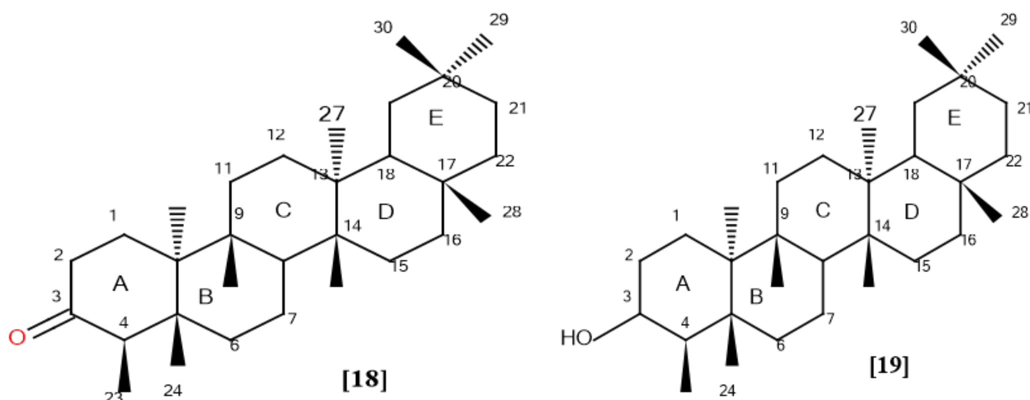
IR (KBr) ν_{max} cm^{-1}	Functional group	Literature[42]
3624, 3483	O-H	3621, 3477
1709	C=O	1710
1452, 1387	Dimethyl group	1449, 1383
1174	C-O	1178

From the ^{13}C-NMR spectrum, the signal at δ_C 213.22 was assigned to the carbonyl carbon at C-3 of the friedelan-3-one while the signal at δ_C 72.74 was assigned to the C-O at C-3 of friedelan-3β-ol as a result of the deshielding effect of the attached OH group in friedelan-3β-ol.

The ^{1}H-NMR spectrum showed a signal at δ_H 3.70 assignable to the proton attached to C-3 of friedelan-3β-ol. Signals resonating at δ_H 2.40 and 2.30 were assigned to the non-equivalent methylene protons (H-2) at C-2 which coupled geminally with each other and further split by neighbouring protons (H-1 and H-3) at C-1 and C-3 to give the observed multiplicity. The methine proton at H-4 expected to couple with the methyl protons of C-23 into a quartet was assigned the signal at δ_H 2.25 (q, J = 7 Hz). The signals between δ_H 2.00 and 1.60 are attributed to methylene protons at C-1 and C-6 (H-1 and H-6). The ^{1}H-NMR spectrum exhibited an intense overlap of signals at high field within the sp^3 alkane region (δ_H 1.60 to 0.70) which led to some difficulty in

University of Ghana http://ugspace.ug.edu.gh

the assignment of chemical shifts to proton (s) as well as their multiplicity and this is due to the saturated nature of both compounds and their presence as a mixture.



While a mixture of friedelan-3β-ol and friedelan-3-one [58] has been obtained in large quantities in the petroleum ether extract, friedelan-3-one has been isolated from both ethyl acetate and dichloromethane fractions of ethanol extract from the roots of the plant[42]. The quantities of friedelan-3-one and friedelan-3β-ol were very large in the petroleum ether and acetone extracts from the stem of the plant[41].

3.2 Bioactivity studies

3.2.1 Insecticidal activities

The degree of insecticidal activities varies in various species of plants due to their chemical composition. Based on the effects of these chemicals on insects, they can be put into two main groups: toxic substances and behaviour-modifying substances. The toxic substances are those considered as ovicidal, larvicidal and adulticidal. The behaviour-modifying compounds are those that act as oviposition deterrents, growth, repellents and development inhibitors and antifeedants.

Thus, the investigation examined these effects (with exception of repellent) of the extracts and/or isolates on *Sitophilus zeamais*.

3.2.1.1 Toxicity assay

Toxicity of a compound is its poisonous potency under a given set of conditions. Toxicity as used in this work for insecticidal activity was defined based on the lethal dosage and concentration of extracts and isolates and these were used to rate the potency and the level of toxicity. The lethal dose (LD_{50}) is the dose that kills 50 percent of the *S. zeamais* to which it is administered and is expressed as microgram or milligram or gram of extracts and isolates per gram (μg/g or mg/g or g/g) of weight of maize grains. This is established by feeding (oral) and contact application (dermal) tests. The dosage that kills 50 percent of the *S. zeamais* administered in microgram of the extracts and isolates per microliter volume of a given solvent (μg/μl) is referred to as lethal concentration and denoted by LC_{50}. It is used mostly in environmental studies.
A hand microapplicator was used to topically apply acetone solutions of the crude extracts and the isolates to the thorax of four- day old adult *S. zeamais* of mixed sexes selected randomly. Ten insects were used for each treatment and treatments were replicated four times. The doses varied geometrically from 0.005 to 5.0 μg/μl and 0.001 to 1.0 μg/μl per insect. The results obtained (Appendix I-table 1 and 2) were analyzed after 48h to ascertain the toxic nature of the plant.

Using probit analysis in *MINITAB* software at 95% confidence interval, LD_{50} values of the dose-response curves at 48h were estimated for all the extracts and isolates (Table 16). From the LD_{50} results obtained, the methanol extract showed the lowest toxicity while the petroleum ether extract demonstrated the highest activity among the extracts. This is because the higher the LD_{50} value,

the lower the toxicity. Thus, among the extracts, the less polar ones generally exhibited high toxicity to the *S. zeamais* than the polar ones. Lower LD_{50} values were obtained for the isolates which were extracted with less polar solvents. Friedelin, epifriedelanol, and a mixture of friedelin and epifriedelanol recorded LD_{50} values of 0.48, 0.56 and 0.52 μg/μl respectively. The various 95% fiducial limits indicate that there is probability of 95% certainty that the true mean, LD_{50}, lies between the lower fiducial limit and upper fiducial limit. Since a result in a biological assay is only approximate and an estimate, there is 95% surety that LD_{50} values were well estimated.

Table 16: Lethal Dosage (LD_{50}) of different extracts and isolates of *D. madagascariense* to *S. zeamais*

Samples	**LD_{50} (μg/ml)**	**95% Fiducial Limits**	
		lower	**upper**
Petrol ether extract	0.86	0.60	1.50
Acetone extract	0.94	0.20	1.67
Chloroform extract	1.23	0.32	2.16
Methanol extract	1.25	0.51	2.06
Ethanol extract	1.14	0.52	1.82
Mix.β-sitosterol &stigmasterol	0.98	0.70	1.64
Friedelan-3-one	0.48	0.33	0.67
Mix.Friedelan-3-one & friedelan-3β-ol	0.52	0.36	0.75

Friedelan-3β-ol	0.56	0.40	0.78

Total number of insects treated= 40. Mortality recorded after 48 h exposure

3.2.1.2 Effects of crude extracts on hidden eggs and immature stages

The effect of *D. madagascariense* crude extracts on the development of eggs and immature stages of *S. zeamais* inside the grains were investigated. Five hundred grams of disinfested maize placed in a 1-liter glass jar were infested with 250 adults of *S. zeamais* to allow for egg laying. The parent adults were removed after seven days. One day after adult removal, 25 g of infested maize were treated with 2 g of the different extracts (dissolved in 2ml of petroleum ether, acetone and methanol). Thereafter these treatments were repeated after one and two weeks of adult removal to determine the effect of the extract on the early and late instars larvae of *S. zeamais*. Each treatment was replicated four times. The controls were each treated with petroleum-ether, acetone and methanol alone. Counts were taken of adults emerging after 2 weeks following the last treatments and the results recorded and analyzed.

The effect of the extracts (methanol, acetone and petroleum ether) on the development of hidden eggs and immature stages of *S. zeamais* inside grains is presented in Figure 7. Maize grains treated with the crude extracts significantly affected the eggs and immature stages of *S. zeamais* ($p<0.05$). Treatments applied 14 days after oviposition recorded higher numbers of emerging *S. zeamais* than in treatments applied earlier than 14 days. Also, none of the extracts completely inhibited the development of *S. zeamais* nevertheless these extracts reduced the progeny emergence during the trial.

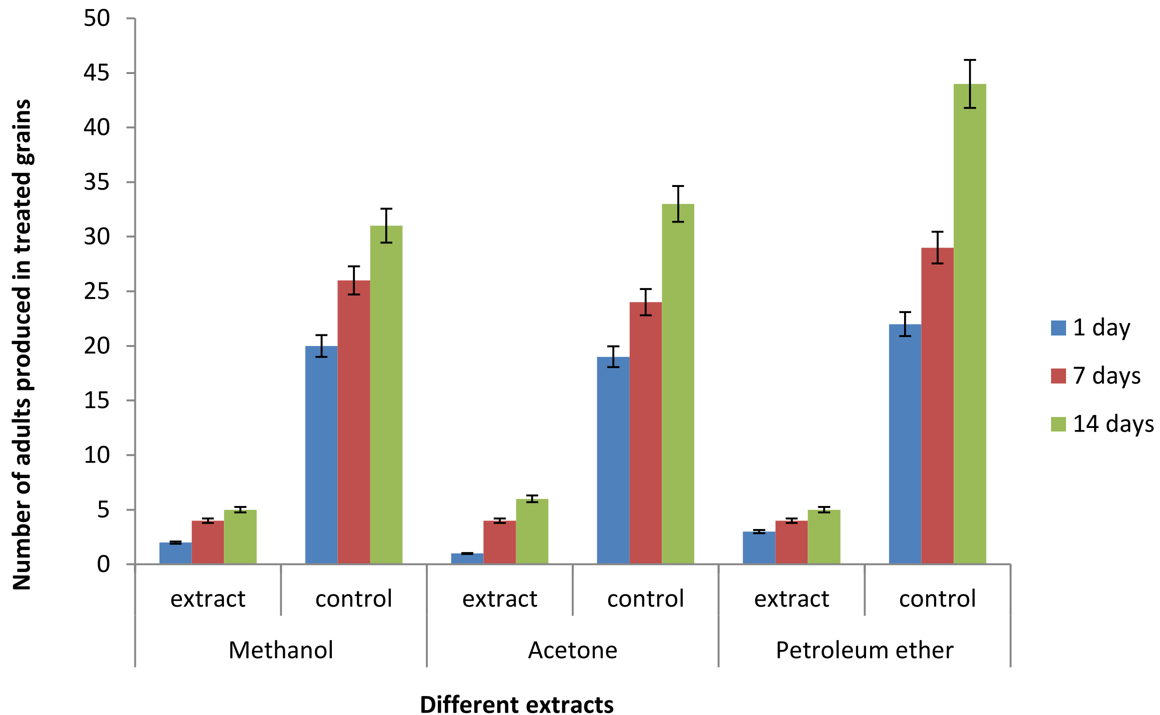


Figure 7: *S. zeamais* adults produced in maize treated with extracts of *D. madagascariense* and incubated for different periods after oviposition

3.2.1.3 Progeny production and damage assessment assays

The effects of crude extracts of *D. madagascariense* on F_1 progeny produced by *S. zeamais* were investigated in maize treated with varying concentrations of 0.0016, 0.08, 0.4, 2, and 10g per 100g of grains. One hundred grams of disinfested maize grains were treated with the above concentrations of different extracts. The different extracts were dissolved in 2ml petroleum-ether, chloroform, acetone, ethanol and methanol and the solvents completely allowed to evaporate within three hours after application before twenty adults of *S. zeamais* were introduced into the grains. The containers were covered with white muslin cloth held in place with rubber bands. Control treatment consisted of grains mixed only with the above solvents used to dissolve the

University of Ghana http://ugspace.ug.edu.gh

extracts. After 21 days oviposition period, the parent adults were removed and insects subsequently emerging were counted to estimate F1 progeny production. Counting began 43 days and stopped after 50 days. Thereafter the damage caused to the grains by *S. zeamais* was assessed. In assessing the level of damage, 100 grains of maize were taken from each jar and the number of damaged grains with characteristic hole(s) and undamaged grains were counted and weighed.

From the observations, extracts applied in grains at dose ≥ 2.0 g per 100g of grains appear to provide the most effective protection to maize against *S. zeamais* attack indicating that for the insecticide to be effective and efficient the dosage must be taken into consideration. The different extracts caused significant reduction in feeding damage and number of F1 progeny produced within the grains.

Progeny production

The number of F1 progeny produced by the *S. zeamais* in treated and untreated maize with the different extracts of the plant is shown in Table 17. The effect of different doses of the extracts on progeny production is also shown in Figure 8. Treatment with the solvents alone (Table 17) gave the highest F1 progeny production. The highest dosage (10g extract/100g grains) completely inhibited the number of progeny produced by the *S. zeamais.* However lower concentrations of the plant extracts were less effective in reducing the number of F1 progeny produced by the *S. zeamais* hence the action of the plant extract was dosage –dependent. Figure 8 indicates the following general trend for inhibition of progeny production: chloroform > acetone > methanol extracts. The effects of ethanol and petroleum ether extracts are fairly similar at higher doses.

University of Ghana http://ugspace.ug.edu.gh

Table 17: Effect of crude extracts of *D. madagascariense* on the number of F1 progeny produced by *S. zeamais*

Dose (g/100g grain)	Total number of F1 progeny produced by *S. zeamais* in different extracts				
	Petroleum ether extract	***Chloroform extract***	***Acetone extract***	***Ethanol extract***	***Methanol extract***
10	0	0	0	0	0
2	3	0	0	4	3
0.4	6	0	3	10	3
0.08	10	1	3	10	5
0.016	20	1	5	11	8
(control) 0.000	72	19	16	22	23

University of Ghana http://ugspace.ug.edu.gh

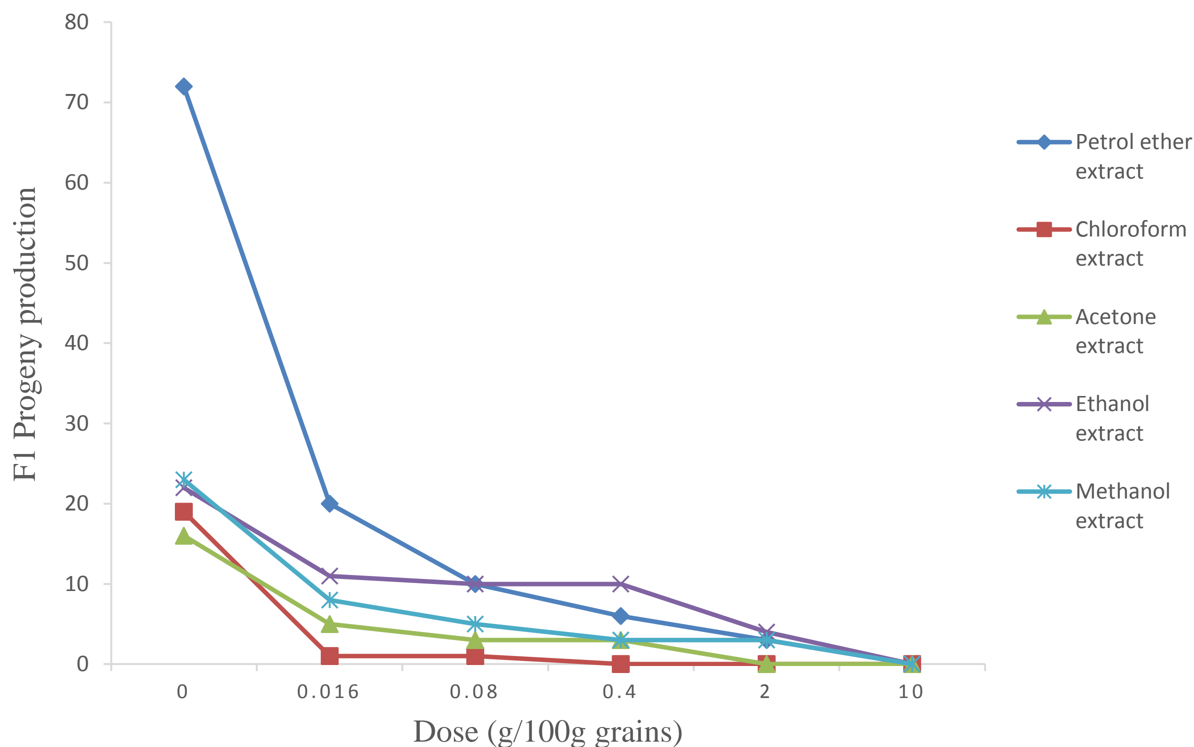


Figure 8: Effect of different crude plant extracts on F1 progeny production

Damage Assessment

Percent weight loss was calculated using the method of Food and Agricultural Organization (FAO) of the United Nations (1985)[121] as follows:

% Weight loss = [(UNd-DNu)/ U (Nd+Nu)]× 100

Where U = weight of undamaged grains; D = weight of damaged grains; Nd = number of damaged grains; Nu = number of undamaged grains.

University of Ghana http://ugspace.ug.edu.gh

Percent seed damaged was also calculated as follows:

% seeds damaged = [Nd/(Nd+ Nu)] × 100

The percent weight loss and the percent seed damage caused by *S. zeamais* to the treated and untreated maize grains are shown in Tables 18 and 19 and the trend of percentage seed damage with treatment with the extracts is shown in Figure 9. The extracts at various dosages applied to the grains offered protection against damage by the *S. zeamais* compared to the untreated grains. Damage caused by the insects to the seeds was more significant ($p<0.05$) in the various controls than maize treated with the other different extracts. More grains were damaged at lower concentrations of the extract. The highest seed damage was recorded for the controls. In case of the grains treated with the extracts at lower concentrations, lower weight loss was observed.

Table 18: Percent weight loss caused *by S. zeamais* in maize treated with the different extracts of *D. madagascariense*

Dose (g/100g grain)	**% weight loss of grain in different extracts**				
	Petroleum ether extract	***Chloroform extract***	***Acetone extract***	***Ethanol extract***	***Methanol extract***
10	0	0	0	0	0
2	0.14	0	0.08	0.07	0.03
0.4	0.33	1.17	0.70	0.62	0.17
0.08	2.43	0.83	0.09	0.47	0.64
0.016	2.44	0.07	0.16	0.87	0.14
0 (control)	4.60	2.52	2.42	4.28	2.38

University of Ghana http://ugspace.ug.edu.gh

Table 19: Percent seed damage caused *by S. zeamais* in maize treated with the different extracts of *D. madagascariense*

Dose (g/100g grain)	% seed damage in different extracts				
	Petroleum ether extract	***Chloroform extract***	***Acetone extract***	***Ethanol extract***	***Methanol extract***
10	0	0	0	0	0
2	3	0	1	1	1
0.4	3	1	2	4	1
0.08	6	2	3	4	2
0.016	6	2	3	4	4
0 (control)	27	13	17	14	14

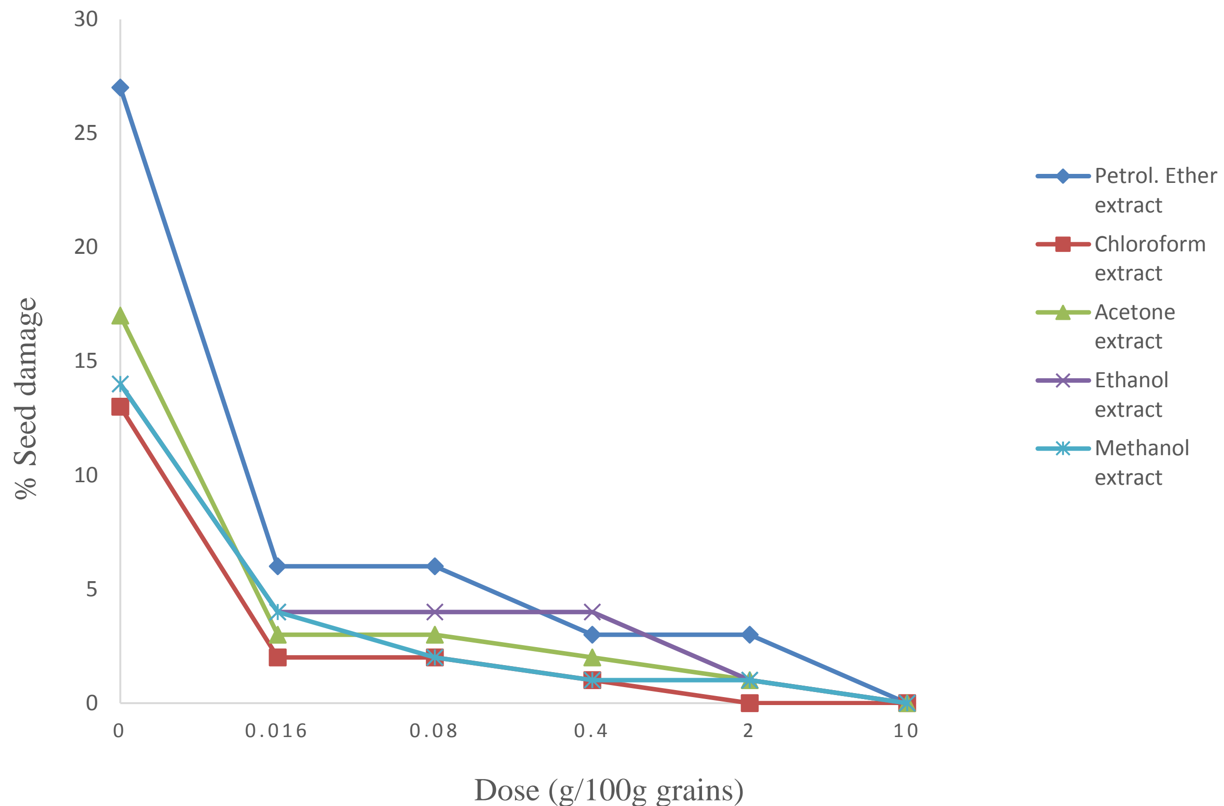


Figure 9: % seed damage caused by *S. zeamais* in maize treated with different extracts

3.2.1.4 Discussion on insecticidal activity studies

Feeding and oviposition by *Cylas puncticollis* (sweet potato weevil) is so far the only reported study on insecticidal activity of *D. madagascariense* where friedelan-3-one and friedelan-3β-ol could not deter or stimulate the feeding and oviposition in *Cylas puncticollis*[41]. The present study on insecticidal activity investigated the toxicity and grain protectant potential of different extracts and isolates of the whole stem of *D. madagascariense* against *S. zeamais* in the laboratory using contact toxicity, progeny production and damage assessment assays. The different extracts and isolates applied topically to insects or on maize grains were toxic to *S. zeamais*, although the activity varied considerably with the different extracts. Lower LD_{50} values were estimated for the

University of Ghana http://ugspace.ug.edu.gh

isolates which were virtually obtained from less polar solvents. The less polar extracts, for example petroleum ether extract, recorded lower LD_{50} value of 0.86 μg after 48h exposure. Friedelan-3-one, friedelan-3β-ol, and a mixture of friedelan-3-one and friedelan-3β-ol recorded LD_{50} values of 0.48, 0.56 and 0.52 μg respectively following 48h exposure against *S. zeamais*. These two compounds, friedelan-3-one and friedelan-3β-ol, have very close LD_{50} values and are analogues ([**18]** & [**19]).** Topically applied insecticides will need to penetrate the insect cuticle which is non-polar. These compounds are terpenes which are relatively non-polar. That friedelan-3-one exhibits slightly higher potency compared to friedelan-3β-ol may be due to the former being able to better penetrate the cuticle. The different extracts applied to maize grains at doses ≥ 2 g per 100 g of grains also provided much better protection to maize against *S. zeamais* attack compared to the lower doses. *S. zeamais* killed in treated grains mostly appeared paralyzed [27], suggesting that toxicity was not only due to ingestion of treated grains but also through contact toxicity. The different extracts caused significant reduction in feeding damage, number of F1 progeny produced, and inhibition of the development of eggs and immature stages of *S. zeamais*. This indicates the higher protectant potential of these extracts against insect damage in storage. It also indicates the presence of growth regulatory and ovicidal substances in the plant[122]. Hence the plant extracts can inhibit eggs hatching and control the growth of *S. zeamais* to some extent.

The potency of the different extracts of the plant might be due to the oily nature of the extracts, which may block the insect spiracles, coat of the seed testae, act as ovicides by plugging the egg micropyle, thus hindering oxygen supply to the embryo. The complete protection of grains by the different extracts applied at the highest dosage of 10 g per 100g of grain, may suggest the presence of antifeedant properties in the plant. There was some amount of feeding by the insect after

University of Ghana http://ugspace.ug.edu.gh

treatment with the different extracts and this was reflected in weight losses. However, the weight losses were minimal. Though the insects were able to bore into the seeds at lower doses, these grains were not necessarily eaten up by the *S. zeamais* hence some of the extracts appeared to deviate from the normal pattern of weight loss of grains. At lower concentrations, the extracts did not adversely inhibit progeny production of *S. zeamais*. However, under practical storage conditions, relatively higher doses may be required as compared to doses applied in the laboratory experiment.

3.2.2 Anthelminthic assay

Ten (10) samples comprising five different crude extracts and five solid isolates from *D. madagascariense* with unknown anthelminthic activity were tested against *Necator americanus* to ascertain their ovicidal properties *in vitro*.

Following the extraction of hookworm eggs, the average number of eggs per µl of egg suspension was calculated from 10 µl x 3 and the proportion of suspension that would contain 50 eggs/ well was estimated. Albendazole, a known anthelminthic drug, was used as control. The total volume of egg suspension and albendazole solution was adjusted to 100 µl per well. They were plated in water in a 96-well microplate (Costar plate) containing a serial dilution of albendazole (400mg) in DMSO at an initial concentration of 5.0 mg / ml in 50 µl per well in duplicate, each well containing approximately 50 eggs, water serving as the negative control. The plate was incubated for 24 hr. at room temperature (approximately 27 ^{o}C) and the reaction was stopped by freezing the plate. The plate was defrosted and the unhatched eggs and hatched larvae were counted in each well under light microscopy where percent egg hatch inhibition values were calculated as:

University of Ghana http://ugspace.ug.edu.gh

$$\% \text{ egg hatch inhibition} = \frac{No.\ unhatched}{no.\ unhatched + no.\ larvae} \times 100$$

The results were then recorded and analyzed.

From the results recorded, no test sample had > 90% inhibition as recommended by WHO. Only β-sitosterol and friedelan-3β-ol at concentration of 5 μg/μl appeared to have significant values > 50%. However, the controls, Eskaben and Tacizol albendazole 400 mg tablet at concentration of 5 μg/μl had > 90% inhibition. Also from Appendix 1 (table 5), the % inhibition was dose-dependent; the higher the dosage, the better the rate of inhibition of the tested samples.

Using probit analysis in *MINITAB* software at 95% confidence interval, IC_{50} values were estimated for all the extracts and isolates. A mixture of β-sitosterol and stigmasterol had the largest IC_{50} against the *N. americanus* eggs while friedelan-3β-ol recorded the lowest value (Table 20). This means that friedelan-3β-ol was more potent than all other extracts or isolates. The lower the IC_{50} value, the better the inhibition of the eggs from hatching. Friedelan-3β-ol, recording the lowest IC_{50} value of 0.64 μg/μl inhibited the hatching of *N. americanus* eggs but a mixture of β-sitosterol and stigmasterol had the largest IC_{50} value of 9.41 μg/μl against the *N. americanus* eggs. The 95% fiducial limits of friedelan-3β-ol and a mixture of β-sitosterol and stigmasterol were 0.58-0.73 and 5.34-13.44 respectively. Although β-sitosterol inhibited the hatching of *N. americanus* eggs, it failed to achieve the lowest IC_{50}value. Eskaben had IC_{50} value of 0.0024 μg/μl against the *N. americanus* eggs. Fiducial limits are the boundaries within which IC_{50} is considered to be located. Here, there is 95% certainty that IC_{50} value(s) lie within the lower and upper limits.

University of Ghana http://ugspace.ug.edu.gh

Table 20: Median Inhibitory concentration (IC_{50}) of samples of *D. madagascariense*

Samples	IC_{50} (µg/µl)	95% Fiducial Limits lower	upper
Petrol ether extract	1.33	1.19	1.51
Acetone extract	1.13	1.00	1.30
Chloroform extract	1.04	0.93	1.20
Ethyl acetate extract	1.27	1.13	1.44
Ethanol extract	1.02	0.89	1.18
Mix.β-sitosterol &stigmasterol	9.41	5.38	13.44
Friedelan-3-one	0.95	0.86	1.05
β-sitosterol	0.75	0.63	0.88
Mix.Friedelan-3-one & friedelan-3β-ol	1.72	1.48	2.02
Friedelan-3β-ol	0.64	0.58	0.73
Eskaben	0.0024	0.0020	0.0031

University of Ghana http://ugspace.ug.edu.gh

3.2.2.1 Discussion on anthelminthic bioassay

So far, there has not been any report on the anthelminthic activity of any extract or compounds isolated from *D. madagascariense*. The anthelminthic test was carried out on the petroleum ether, chloroform, acetone, ethyl acetate and ethanol extracts of the whole stem of the plant as well as the isolates friedelan-3-one, friedelan-3β-ol, β-sitosterol, a mixture of friedelan-3-one and friedelan-3β-ol, and a mixture of β-sitosterol and stigmasterol. IC_{50} values were estimated for all the extracts and isolates. A mixture of β-sitosterol and stigmasterol gave the largest IC_{50} value against *N. americanus* eggs while friedelan-3β-ol recorded the lowest. This suggests that friedelan-3β-ol is more potent than all other extracts or isolates. The IC_{50} value for friedelan-3β-ol is greater than that of friedelan-3-one, the ketone form of friedelan-3β-ol. It is also observed that the potency of β-sitosterol is similar to that of friedelan-3β-ol. Terpenes such as azadirachtin are known to demonstrate ovicidal activity[123]. Thus the activity demonstrated by friedelan-3-one and friedelan-3β-ol, which are both terpenes, against hatching of *N. americanus* eggs is worthy of note. Also, steroids such as those obtained from or contained in chloroform and benzene extracts of *Padina pavonica* are known to possess ovicidal activity[124].

Based on the IC_{50} and fiducial limits (fiducial limits are the boundries within which IC_{50} is considered to be located), friedelan-3-one, friedelan-3β-ol and β-sitosterol exhibit similar potency. However, friedelan-3-one, friedelan-3β-ol and β-sitosterol only slightly inhibit the hatching of hookworm eggs with increased concentrations.

CONCLUSION

The whole stem of *D. madagascariense* has been investigated. Chemical structures of isolated compounds were determined and identified by comparison of melting points, R_f from TLCs, IRs, DEPT, ^{1}H- and ^{13}C NMR data of each compound as friedelan-3-one, friedelan-3β-ol, β-sitosterol and stigmasterol. Biological activities involving insecticidal and anthelminthic properties were carried out on the extracts and the isolates. The insecticidal activity investigated the toxicity and grain protectant potential of different extracts and isolates of the plant against *S. zeamais* in the laboratory using contact toxicity, progeny production and damage assessment assays. The less polar extracts, for example petroleum ether extract, recorded lower LD_{50} value of 0.86 μg/μl after 48h exposure. Friedelan-3-one, friedelan-3β-ol, and a mixture of friedelan-3-one and friedelan-3β-ol recorded LD_{50} values of 0.48, 0.56 and 0.52 μg/μl respectively following 48hour exposure against *S. zeamais*. The different crude extracts caused significant reduction in feeding damage, number of F1 progeny produced, and inhibition of the development of eggs and immature stages of *S. zeamais*. There was complete protection of grains by the different crude extracts applied at the highest dosage of 10 g per 100g of grain.

The anthelminthic activity, using Egg Hatch Assay, showed friedelan-3β-ol recording the lowest IC_{50} value of 0.64 μg/μl while a mixture of β-sitosterol and stigmasterol had the largest IC_{50} value of 9.41 μg/μl in inhibiting the hatching of *N. americanus* eggs. Based on the IC_{50} values, friedelan-3-one, friedelan-3β-ol and β-sitosterol exhibited similar potency. However, no test sample had > 90% inhibition as recommended by WHO but β-sitosterol and friedelan-3β-ol appeared to have significant values > 50%.

In recent times the World Health Organization (WHO) has been concerned about the lack of interest by the major World pharmaceutical companies in developing new efficacious

University of Ghana http://ugspace.ug.edu.gh

anthelminthic drugs. Helminth infections are classified among the so-called neglected tropical diseases. The WHO, through its Tropical Diseases Research (TDR) unit and the African Network for Drugs and Diagnostics Innovation (ANDI) has therefore initiated major screening programmes for anthelminthic agents of both natural and synthetic origins. Although the present investigation did not result in any highly active anthelminthic compounds (%EHI > 90%), the ≥50% activity shown by some of the well-known triterpenoids such as friedelan-3β-ol may be indication of such compounds being good templates for further search or investigation of anthelminthic agents of plant origin.

RECOMMENDATION

The use of HPLC in isolating the various components in the different extracts of the plant material is highly recommended in order to have a good yield of almost all the components identified on the TLC.

Normally, toxic effect coupled with repellent action of tropical plants increases the protection potential of plant materials against insect bites, crop and grain damage; therefore, it is highly recommended that repellency assay be carried out.

All botanical preparations proposed for use in pest and parasite management should be subjected to acute and subacute toxicological evaluation as well as tests for phytotoxicity. This is because natural products can, under certain circumstances pose appreciable health and safe risks.

University of Ghana http://ugspace.ug.edu.gh

CHAPTER FOUR

4 EXPERIMENTAL

4.1 General methods

Extraction of the plant materials with the various solvents was carried out with a soxhlet apparatus and also by cold percolation. The extracts were concentrated and dried under reduced pressure using Büchi Rotavapor Rotary Vacuum Evaporator.

Isolation and purification of the various components of the crude extracts were done by column chromatography and recrystallization. All analytical thin layer chromatography (TLC) were carried out with aluminum foil slides pre-coated with silica gel (thickness 0.2 mm, type Kiesegel 60 $_{F254}$ Merck). Plates of size 5 cm by 10 cm and 20 cm by 20 cm were used for analytical TLC. Column chromatography was on silica gel 60 (Fluka) and about 20-30 g of the absorbent (stationary phase) was used for 1 g sample. Solvents commonly used included petroleum ether (40-60 °C and 60-80 °C), ethyl acetate, chloroform, acetone ethanol and methanol. All the solvents used were of analytical grade and solvent systems were prepared as % v/v, example, petroleum ether / ethyl acetate (70: 30) means 70 parts by % volume of petroleum ether to 30 parts by % volume of ethyl acetate. Visualization of spots on TLC was under UV light, in iodine vapour, and with anisaldehyde spray followed by heating. Melting points of pure isolates (uncorrected) were determined on a Stuart Scientific Melting Point Apparatus. All the IR spectra were recorded in KBr discs on a Shimadzu IR-408 spectrophotometer.

University of Ghana http://ugspace.ug.edu.gh

^{1}H and ^{13}C NMR and DEPT-135 spectra were recorded in deuterated chloroform with tetramethylsilane (TMS) as the internal reference on a Brüker 250 spectrophotometer at a frequency of 600Hz for ^{1}H, 600Hz and 150Hz for DEPT-135 and 150Hz for ^{13}C. Chemical shifts δ, were expressed in parts per million (ppm), and coupling constant, J, in Hz. In interpreting NMR spectra, the abbreviations used were s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and br = broad.

Materials and reagents used in the Kato-Katz technique included Kato set (template with hole, nylon or plastic screen, plastic spatula), Newspaper or glazed tile, Microscope slides, Gloves, Light microscope with 10x and 40x objectives, Tally counter, 0.85% saline and Cellophane as cover slip, soaked in glycerol-malachite green solution. Again, the materials and reagents used in the extraction of eggs from faeces included faeces (at least 20 g), 100 ml of 0.9% NaCl, 35 ml of 0.015% Brij-35, 35 ml of 2.18M $NaNO_3$ (45 g/250 ml water), 1 sheet double folded gauze, 1 plastic funnel pair of forceps, 2x 50ml conical flask, sachet water, 1x 80µm filter & 1x 20µm filter, filtration unit, 200ml water in a flask or beaker, an empty 250ml beaker and aspirator.

Isolated pure compounds were identified by Retardation factor (R_f) values on thin layer chromatogram (TLC), melting point, IR, DEPT, ^{1}H- and ^{13}C NMR data. These were compared with the respective spectral data of reference spectra from literature. Probit analysis for the determination of LD_{50} and IC_{50} of results on biological activities were analyzed using *MINITAB* computer programme. Mortality of *Sitophilus zeamais* were expressed as means ± standard deviation (SD). The significance differences between the means were measured using analysis of variance (ANOVA) at $p<0.05$ level (Tukey's HSD) with *MINITAB* computer programme.

University of Ghana http://ugspace.ug.edu.gh

4.2 Collection and treatment of plant material

The stem bark of *Dichapetalum madagascariense* was collected at the Legon Botanical Garden in November, 2011 and was identified by Mr. J.V. Amponsah of the Herbarium at the Botany Department, University of Ghana where a voucher specimen of the plant has been deposited. The plant material was chopped into pieces, air-dried in the shade for about four weeks and pulverized into fine powder.

4.3 Reagents

1. Reagents for testing for Alkaloids

a. Wagner's reagent

Potassium iodide (2.0 g) and iodine crystals (1.27 g) were together dissolved in a little distilled water in a volumetric flask and the solution made to 100 cm^3 with distilled water. The presence of alkaloids was indicated by the formation of large brown amorphous and flocculent precipitate after treating the hydrochloric acid solutions of the extracts with a few drops of the reagent[125].

b. Meyer's reagent

Mercuric iodide (1.36 g) was dissolved in distilled water (60 cm^3) and the resulting solution added to the one obtained by dissolving 5 g of potassium iodide in distilled water (10 cm^3). The resulting solution was made up to 100 cm^3 with distilled water. The formation of a pale yellow precipitate when a few drops of the reagent were added to the test solution was indicative of the presence of alkaloids[125].

University of Ghana http://ugspace.ug.edu.gh

c. Dragendorff's reagent.

Hydrated bismuth nitrate (8 g) was dissolved in concentrated nitric acid (20 cm^3) and the resulting solution was slowly added to a solution of potassium iodide (27.2 g) in 50 cm^3 of distilled water with stirring. Crystalline potassium nitrate which precipitates out was filtered off and the filtrate made to 100 cm^3 with distilled water before being used as a reagent for testing for alkaloids. In the test, the solution of the extract was made distinctly acidic with sulphuric acid and completely freed of any ethanol in which the alkaloid precipitates are soluble. Two or three drops of the reagent were used since some of the alkaloid precipitates are soluble in excess reagent. Formation of brown precipitate was indication of alkaloids[125].

2. Iron (III) chloride solution for testing for catechol-type tannins

100 cm^3 of Iron (III) chloride solution was prepared by dissolving 10 g iron III chloride in distilled water. Two to three drops of the solution were added to 3 cm^3 of a test solution. The presence of catechol-type tannins was indicated by the formation of a dark greenish-blue colouration[126].

3. Anisaldehyde spray reagent.

About 55 ml of methanol was added to glacial acetic acid (10 ml). To the mixture, 5ml concentrated tetraoxosulphate (VI) acid was added at 25 °C. To another 30ml methanol, anisaldehyde (0.5 ml) was added. The two mixtures were combined and used as anisaldehyde spray. The reagent was always prepared fresh and used as a spray. The sprayed TLC plates were heated to about 110 °C for 10 minutes before visualization in visible light. The terpenoidal phytochemicals stained blue, violet, green, pink, yellow, red, cream and grey on a light pink background[127, 128].

University of Ghana http://ugspace.ug.edu.gh

4. Liebermann-Buchard Reagent for testing terpenoids

Redistilled acetic anhydride (5 cm^3) was cautiously mixed while cooling with 5 cm^3 concentrated tetraoxosulphate (VI) acid. The resulting mixture was slowly added while cooling in ice to 50 cm^3 of absolute ethanol. As a positive indication for the presence of terpenoids in the various extracts, this reagent when sprayed onto TLC plate produced pink- to-red spots after development followed by heating in an oven at a temperature of 110°C for 10 minutes and then examined under the UV lamp[128].

4.4 Phytochemical screening of the crude extracts of *D. madagascariense*

The petroleum ether, chloroform, acetone and the ethanol crude extracts were all subjected to phytochemical screening as follows:

Screening for Alkaloids

To about 0.1 g of the crude extract in test tube was added 5ml of 2M HCl solution. This was stirred, warmed and filtered. The filtrate from the extract was then divided into three test tubes. To one portion of each test solution was added Dragendorff's reagent. To another portion Meyer's reagent and to the remaining portion Wagner's reagent and then observed for any turbidity or colour change. There were no observable colour changes in all the test solutions. Alkaloids were therefore absent from the crude extracts

Screening for anthraquinones and anthracene derivatives

About 0.2 g of the crude extract was dissolved in 30 cm^3 of distilled water and filtered. The filtrate was shaken up with 10 cm^3 of benzene in a separatory funnel. The benzene layer was transferred into a test tube and shaken with 5 cm^3 of 2M ammonia solution. There was partial dissolution of each of the crude extracts in water. The observation of red colour in the ammonia layer indicated the presence of anthraquinones and anthracene derivatives in the petroleum and acetone crude extracts.

Screening for tannins

About 0.1 g of the extract was dissolved in 10 cm^3 of aqueous methanol (80%) and the solution divided into two portions. To one portion was added two to three drops of freshly prepared iron (III) chloride solution and to the other portion nothing was added to serve as a blank. The absence of a dark greenish-blue colouration on addition of the iron (III) chloride solution signified the absence of catechol-type tannins in any extract. There were positive tests for the ethanol, chloroform and methanol crude extracts.

Screening for flavonoids and leucoanthocyanins (Shinoda Test)

The crude extract (0.2 g) was dissolved in 15 cm^3 ethanol (80%). The resulting solution was filtered and the filtrate divided into three portions. The first test tube served as a blank. To one portion was added magnesium turnings followed by concentrated hydrochloric acid (0.5 cm^3) and observed for colour changes within 10 minutes. The addition of the conc HCl showed effervesce and vigorous reactions with both crude extracts but there was absence of pink colour which indicated the absence of flavonoids in the extracts. To the other portion was added concentrated hydrochloric acid (0.5

University of Ghana http://ugspace.ug.edu.gh

cm^3) and then warmed for about 5 minutes. The absence of any development of light pink colour indicated the absence of leucoanthocyanins in the acetone crude extract but Light pink colour developed within 5 minutes on the surface of the ethanol and methanol crude extracts, indicated the presence of leucoanthocyanins in those extracts.

Screening for cardiac glycosides (Salkowski Test)

Each of the crude extracts (0.2 g) was dissolved in 2 cm^3 of appropriate solvent in a test tube. Concentrated sulphuric acid was carefully added down the side of the test tube to form a lower layer. Formation of a reddish-brown colour at the interface indicated the presence of the aglycone of a cardiac glycoside in all the crude extracts.

Screening for Saponins

About 1.0 g of each of the crude extracts was shaken with water. The presence of foam for about three minutes in all the extracts except petroleum extract was an indication of the presence of saponins.

Screening for Liebermann-Buchard Reagent for testing terpenoids

Redistilled acetic anhydride (5 cm^3) was cautiously mixed while cooling with 5 cm^3 concentrated tetraoxosulphate (VI). The resulting mixture was slowly added while cooling in ice to 50 cm^3 of absolute ethanol. This reagent was sprayed onto TLC plate. The presence of pink- to-red spots after development followed by heating in an oven at a temperature of 110°C for 10 minutes and then examined under the UV lamp was an indication of the presence terpenoids in all crude extracts.

University of Ghana http://ugspace.ug.edu.gh

4.5 Extractions

4.5.1 Petroleum ether extraction

The dried, pulverized stem of *D. madagascariense* (5.0 kg) was exhaustively extracted with petroleum ether 60-80 °C for 72 hours using a soxhlet extractor. Evaporation of the solvent using rotary vacuum evaporator afforded 15.80 g of the extract. This extract was coded MPC. TLC was done on the crude extract and in all seven spots were identified when sprayed with anisaldehyde reagent in $CHCl_3$: Petrol 20:1 and Hexane: $CHCl_3$ 2:3 solvent systems. These spots were also stained by iodine vapour. 4 of the spots fluoresced under UV light.

4.5.2 Chloroform Extraction

The rest of the defatted plant material was extracted with 7.5 L of chloroform using cold maceration technique for 72 hours. The extract was concentrated and dried under reduced pressure using Rotary Vacuum Evaporator. A dark yellowish-green crude extract of mass 5.70 g was obtained. The recovered petroleum ether was then poured back into the plant material for the second extraction. The process was repeated three more times and a total mass of 13.0 g of the crude extract was obtained and coded MC. TLC analysis of the crude extract showed eight spots in each of the following solvent systems; $CHCl_3$: MeOH 98:2 and Hexane: $CHCl_3$ 2:3. The spots were stained by anisaldehyde reagent and iodine vapour, and 4 of the spots fluoresced under UV light. The crude extract was kept in the refrigerator for any precipitation.

University of Ghana http://ugspace.ug.edu.gh

4.5.3 Acetone Extraction

The plant material after chloroform extraction was air-dried. It was then extracted with 7.5 L of acetone using cold maceration technique for 72 hours. The extract was concentrated and dried under reduced pressure using rotary vacuum evaporator. A dark yellowish-green crude extract of mass 4.60 g was obtained. The recovered acetone was then poured back into the plant material for the second extraction. The process was repeated three more times and a total mass of 11.0 g of the crude extract was obtained and coded MA. From the TLC analysis of this extract, nine spots were identified when sprayed with anisaldehyde reagent in $CHCl_3$: MeOH 98:2 and Hexane: $CHCl_3$ 2:3 solvent systems. The spots were also stained by iodine vapour, and 5 of these spots fluoresced under UV light. The crude extract was kept in the refrigerator for any precipitation.

4.5.4 Ethanol Extraction

Another 5.0 kg of fresh pulverized whole stem of *D. madagascariense* were exhaustively extracted with ethanol for 72 hours using a soxhlet extractor. Evaporation of the solvents using rotary vacuum evaporator afforded 31.0 g of ethanol extract and was coded Et. TLC analysis of the crude extract showed eight spots in each of the following solvent systems; $CHCl_3$: MeOH 98:2 and Hexane: $CHCl_3$ 2:3 which were stained by the anisaldehyde reagent and iodine vapour. 4 spots fluoresced under the UV light. The crude extract was kept in the refrigerator for any precipitation.

University of Ghana http://ugspace.ug.edu.gh

4.5.5 Methanol Etraction

The plant material after the ethanol extraction was allowed to air-dry and was then exhaustively extracted with methanol for 72 hours using a soxhlet extractor. Evaporation of the solvents using rotary vacuum evaporator afforded 21.70 g of methanol extract with the code name ME. TLC analysis of the crude extract showed eight spots in each of the following solvent systems; $CHCl_3$: MeOH 98:2 and Hexane: $CHCl_3$ 2:3, which were stained by the anisaldehyde reagent and the iodine vapour. 4 spots fluoresced under UV light. The crude extract was kept in the refrigerator for any precipitation

4.6 Work done on Petroleum ether Extract

The petroleum ether extract, MPC, upon refrigeration precipitated some solids which were filtered to give a solid coded SP and the mother liquor coded MP. MP was concentrated to give a mass of 6.50 g.

4.6.1 Isolation of SP-a1

SP was obtained as whitish brown grains insoluble in petroleum ether, acetone, ethanol and methanol but partially soluble in chloroform and a mixture of petroleum ether/chloroform. Recrystallization of SP in petroleum ether/chloroform mixture gave crystals **SP-a** to **SP-d**. Apart from SP-b which dissolved completely in cold chloroform, the rest dissolved upon warming them in chloroform. TLC analysis of each solid showed two spots for all the crystals which stained orange and purple when sprayed with anisaldehyde reagent. None of these spots fluoresced under UV light. Attempts to also purify the components by repeated recrystallization proved futile, with

the exception of SP-a, which precipitated pure solid coded SP-a1 (150 mg). Comparative TLC with authentic sample of friedelan-3β-ol and friedelan-3-one (PAD E11 and ADB 1C respectively) indicated that SP-a1 is probably epifriedelanol (friedelan-3β-ol). The melting point of epifriedelanol (150 mg) was found to be 270-271℃

4.6.2 Identification of SP-a1

IR spectrum of the compound showed signals at 3620, 3477, 3005, 1089, 1385 and 1362 cm^{-1}.The ^{13}C-NMR spectrum showed the presence of 30 carbons. From DEPT analysis at 135°, the spectrum showed that there are six quarternary, eleven methylene and thirteen methyl and methine carbons. The ^{1}H- NMR spectrum showed a broad signal at δ_H 3.73 ppm on C-3. Other peaks were shown at δ 1.90 (d t, J=10.1, 3.0 Hz) and 1.73 (d t, J=12.8, 3.0 Hz) while multiple signals at δ_H 1.57 ppm was attributed to the protons, H-2b on C-2. There were characteristic triterpenoid protons signal at δ_H 1.55-0.88 ppm. The signals at δ_H 0.73, 0.86, 0.93, 0.95, 0.97, 1.01, 1.05 and 1.17 ppm are attributable to the protons on C-5, C-4, C-9, C-13, C-14, geminal dimethyl group at C-20 and C-17 respectively. The methylene protons also gave signal between δ_H 1.20 and 1.60 ppm.

4.6.3 Isolation and Identification of MP-G

The column Chromatographic separation of MP (6.50 g) using 230 g of silica gel slurry in petroleum ether afforded MP-G.

It was obtained as a white crystalline substance with a melting point of 139 - 140 ° and gave a positive test to Liebermann Buchard reagent for steroidal nucleus. When MP-G was subjected to IR Spectroscopic analysis, there were absorption bands at 3430, 2868, 1667, 1465, 1382, 1242, 1133, 1052, 1022 cm^{-1}. The ^{13}C-NMR showed recognizable signals δ_C 140.76, 121.71, 71.81, 19.4

University of Ghana http://ugspace.ug.edu.gh

and 11.9 ppm. From the ^{1}H NMR there was existence of signals at δ_H 5.40 – 5.00, 3.50, 2.30 - 1.90, 1.70 – 0.70 ppm.

4.7 Work done on Chloroform and acetone extract (MAC)

The column Chromatographic separation of 20.0 g of MAC on silica gel (600 g) with petroleum ether/ ethyl acetate mixtures (the polarity of the eluent was increased gradually until 100 % of ethyl acetate and finally washed with methanol) yielded 12 fractions labeled MAC-A to MAC-L.

4.7.1 Isolation and Identification of MAC-F1

MAC-F precipitated solids which upon recrystallization from petroleum/ethyl acetate mixture gave MAC-F1 as white needles, with melting point of 245-246 °C.

The IR spectrum showed peaks at 1715.45, 1389.63 and 1362.89 cm^{-1}. The ^{13}C- NMR spectrum showed the presence of thirty carbon resonances. The DEPT spectrum showed a broad band at δ_C 213.21 ppm. The rest of the 29 carbon atoms appeared between δ_C 0 and δ_C 60 ppm. In the ^{1}H-NMR spectrum the quartet signal at δ_H 2.25. Other signals were showed at δ_H 0.73 (3H, s), δ 0.88 (6H, s), 0.95 (3H, s), 1.01 (6H, s), 1.05 (3H, s), 1.18 (3H, s). There were multiple signals between 2.42 and 1.64 ppm.

University of Ghana http://ugspace.ug.edu.gh

4.8 Isolation and identification of a mixture of friedelin and epifriedelanol (friedelan-3-one and friedelan-3β-ol) (ET-C1)

ET-C precipitated a solid which was filtered, recrystallized and coded ET-C1. Comparative TLC with authentic samples of friedelan-3β-ol and friedelan-3-one (PAD E11 and ADB 1C) indicated that ET-C1 was a mixture of friedelan-3β-ol and friedelan-3-one. ET-C1 was also compared with the already isolated MAC-F1 and SP-a1 and was found to be a mixture of friedelan-3-one and friedelan-3β-ol (MAC-F1 and SP-a1).

ET-C1 was isolated as white grain with melting point of 257-259 ℃. The literature value was found to 257-163 ℃. The IR spectrum showed signals at 3623.94, 3483.13, 1709.15, 1452.48, 1386.64 and 1173.97 cm^{-1}. The ^{13}C-NMR spectrum showed signals at δ_C 213.22 and 72.74. Other peaks were observed at δ_C 61.34 to 22.27 ppm. The ^{1}H-NMR spectrum showed a signal at δ_H 3.70. Cluster of signals appeared between δ_H 2.40 and 0.70 ppm.

4.9 Insecticidal activity

4.9.1 Culturing of insect

Disease-free *Zea mays* without visible insect infestation were obtained from Madina market, a suburb of Accra. Adults of *S. zeamais* were obtained from the Entomology laboratory, Crop Science Department of the College of Agriculture and Consumer Science, University of Ghana, Legon.

The insects were cultured in the insectary of the Department of Biochemistry, Cell and Molecular Biology.

University of Ghana http://ugspace.ug.edu.gh

The parent stocks of *S. zeamais* were reared on *Zea mays* sterilized by heat disinfestation for four hours at 60°C. Sixty to seventy randomly selected unsexed adults of *S. zeamais* were introduced into 400 g of sterilized *Z. mays* in rearing jars and kept in the insectarium. The place was maintained at 27 ± 1°C and 60 – 70% relative humidity. The seeds were sieved to remove the parent adult insects after 14 days. Progeny emergence began after 21 days and the emerging adults were used for the various assays.

4.9.2 Preparation of different concentrations of the plant materials

For topical applications, the preparation of the concentrations of the crude extracts and the isolates were carried out by measuring a given mass of each compound and dissolving in a solvent (methanol or petroleum ether-methanol mixture) into a 20 ml vial, drying with N_2 gas and re-dissolving in a given volume of acetone to make the stock solution. Serial dilutions of the stock solutions were then carried out as follows: all the different crude extracts, except petroleum ether extract, were diluted from 5.0 to 0.005 µg/µl whilst the petroleum ether extract and the isolates had serial dilutions of 1.0 to 0.001 µg/µl.

4.9.3 Contact toxicity by topical assay

A hand microapplicator was used to topically apply acetone solutions of the crude extracts and the isolates to adult weevils.

One microliter of the test solution was applied on the thorax of four- day old adult *S. zeamais* of mixed sexes selected randomly. Ten insects were used for each treatment and treatments were replicated four times. The doses varied geometrically from 0.005 to 5.0 µg/µl and 0.001 to 1.0

University of Ghana http://ugspace.ug.edu.gh

µg/µl per insect. The treatment series included four groups of *S. zeamais* treated with acetone alone to serve as controls. The insects were chilled in a refrigerator for two minutes to temporarily immobilize them before treatment for easy application. Each group of insects was held in a petri dish (8.5 cm diameter) for 48 hours after treatment and the number of dead insects was recorded.

4.9.4 Effects of extracts on eggs and immature stages

The effect of *D. madagascariense* crude extracts on the development of eggs and larvae of *S. zeamais* in grains was investigated. Five hundred grams of equilibrated maize placed in a 1-liter glass jar were infested with 250 adults of *S. zeamais* to allow for egg laying. The parent adults were removed after seven days. One day after adult removal, 25g of infested maize were treated with 2 g of the extracts (in 2ml of petroleum ether, acetone and methanol). Thereafter these treatments were repeated one and two weeks after adult removal to determine the effect of the extracts on the early and late instars larvae of *S. zeamais*. Each treatment was replicated four times. The controls were each treated with petroleum-ether, acetone and methanol alone. Counts were taken of adults emerging after 2 weeks following the last treatments.

4.9.5 Progeny production and damage assessment

The effects of crude extracts of *D. madagascariense* on F_1 progeny produced by *S. zeamais* were investigated in maize treated with concentrations of 0.0016, 0.08, 0.4, 2, and 10g/100g of grains. One hundred grams of pre-equilibrated maize grains treated with the different concentration of the extracts were each dissolved in 2ml petroleum-ether, chloroform, acetone, ethanol and methanol. The solvents were allowed to completely evaporate within three hours after application and twenty adult *S. zeamais* were introduced into the grains. The containers were covered with white muslin

University of Ghana http://ugspace.ug.edu.gh

cloth held in place with rubber bands. Control treatment consisted of grains mixed only with the above solvents used to dissolve the extracts. After 21 days oviposition period, the parent adults were removed and insects subsequently emerging were counted to estimate F1 progeny production. Counting began 43 days and stopped after 50 days. Thereafter the damage caused to the grains by *S. zeamais* was assessed.

4.10 Anthelminthic activity

The diagnosis of hookworm infestation was carried out at the laboratory of the Kintampo Municipal Hospital in the Kintampo North Municipality of Ghana

.

4.10.1 Sampling

Two main communities selected for this work due to the high prevalence of hookworm infection were Bawa Akura and Jato Akura, all in Kintampo North Municipality. In all, 300 stool samples from school-aged children through to adults were sampled for the analysis.

4.10.2 Diagnosis of intestinal parasites

4.10.2.1 Kato-Katz technique

The Kato-Katz technique is based on the clearing of a thick faecal smear with glycerine in the presence of a background stain, usually malachite green so that the eggs appear unstained. The mesh filters the specimen so that faecal fibres are kept off the slide. The technique is feasible for mass screening as the collected specimen can be examined at leisure in the laboratory. It is a

University of Ghana http://ugspace.ug.edu.gh

quantitative method and provides information on the intensity of Soil-Transmitted Helminths infection.

Procedure

Pre -cleaned microscope slide was labeled and the layer of glazed tile or newspaper prepared. The template with a hole was placed in the centre of a microscope slide. By using gloves, a small amount of faecal material was placed on the newspaper or the glazed tile. The screen was pressed on top so that some of the faeces filtered through, which was then scraped with the flat spatula across the upper surface to collect the filtered faeces. The collected faeces were then put in the hole on the template so that it was completely filled and leveled. The template was carefully removed so that the cylinder of faeces was left on the slide. The faecal material was then covered with the pre-soaked cellophane strip. The microscope slide was inverted and the faecal sample firmly pressed against the cellophane strip on a smooth hard surface such as a tile to spread evenly. The slide was carefully removed by gently sliding it sideways to avoid separating the cellophane strip. The slide with the cellophane was then placed upwards. The prepared slide was left for some time to clear. The smear was examined in a systematic manner and the eggs of each species reported. Based on the template size, a multiplication factor is used to calculate the intensity of infection in eggs per gram of faeces.

4.10.2.2 Extraction of eggs from faeces

This technique is based on the fact that some salts with high specific gravity are able to separate protozoan cysts and certain helminth eggs from faecal debris. The parasitic elements are recovered in the surface film, whereas the debris is found in the bottom of the tube. Sodium nitrate has the property of making parasite eggs float.

University of Ghana http://ugspace.ug.edu.gh

Procedure

For this field isolates, only samples containing 1200 and more eggs were pooled and extracted. This was determined by the equation:

Total egg count = weight of sample × Egg per gram, where Egg per gram was calculated after egg count.

About 20 g faecal material was suspended in 100ml of 0.85% saline and well shaken. It was then filtered through the gauze and centrifuged at 1300 rpm for 6 minutes. The supernatant was discarded. The pellet was washed in 35 ml of detergent (0.015% Brij-35 in distilled water) further centrifuged at 1300 rpm for 6 minutes and the supernatant discarded**.** A second wash was carried out in 35 ml sodium nitrate at 1300 rpm for 6 minutes and about 10 ml of supernatant containing hookworm eggs was decanted into 200 ml sachet water. The egg suspension was clarified by passing through 80 µm and 20 µm nylon mesh membranes respectively. The 20 µm mesh was then washed in 40 ml sachet water which was then spun down and aspirated leaving 5 ml. Egg count was determined in 30 µl of solution on a crayon and the volume adjusted to the desired concentration. This solution was incubated at 27°C or preserved at -20°C.

4.10.2.3 Egg Hatch Assay (EHA)

Following the extraction of hookworm eggs, the average of eggs per µl of egg suspension was calculated from 10 µl x 3 and the proportion of suspension that would contain 50 eggs/ well was estimated. The total volume of egg suspension and albendazole solution was adjusted to 100 µl per well. They were plated in water in a 96-well microplate (Costar plate) containing a serial dilution of albendazole (400mg) in DMSO at an initial concentration of 5.0 mg / ml in 50 µl per well in duplicate, each well containing approximately 50 eggs, water serving as the negative

University of Ghana http://ugspace.ug.edu.gh

control. The plate was incubated for 24 hr. at room temperature (approximately 27 ^{o}C) and the reaction was stopped by freezing the plate. The plate was defrosted and the unhatched eggs and hatched larvae were counted in each well under the light microscope where percent egg hatch inhibition values were calculated as:

$$\% \text{ egg hatch inhibition} = \frac{No.unhatched}{no.unhatched + no.larvae} \times 100$$

University of Ghana http://ugspace.ug.edu.gh

APPENDIX I

TABLES

1. % Mortality of adult S. zeamais using different extracts and isolates of *D. madagascariense*

Sample	% Mortality of adult insects				
	Dosage (µg)				
	0.000	**0.005**	**0.05**	**0.5**	**5.0**
Ethanol extract	0	23	35	55	90
Acetone extract	0	25	33	68	88
Chloroform extract	0	25	30	68	80
Methanol extract	0	23	38	55	85
	Dosage (µg)				
	0.000	**0.001**	**0.01**	**0.1**	**1.0**
Petroleum ether extract	0	20	20	25	55
friedelan-3β-ol	0	15	15	40	73
Mixture of β-sitosterol and stigmasterol	0	13	13	28	50
Friedelan-3-one	0	18	18	43	78
Mixture of friedelan-3β-ol and friedelan-3-one	0	15	20	45	73

University of Ghana http://ugspace.ug.edu.gh

Table 2. Toxicity of samples of *D. madagascariense* against *S. zeamais* with varying doses

Plant extract	Mean (± SD) Mortality of insects at different concentrations (μg/μL) of plant extracts and isolates				
	0.000	**0.005**	**0.05**	**0.5**	**5.0**
Ethanol	0.00	2.25 ± 0.96[a]	3.50 ± 1.29[a]	5.50 ± 0.58[b]	9.00 ± 0.00[b]
Acetone	0.00	2.25 ± 0.50[a]	3.25 ± 0.50[a]	4.75 ± 0.96[b]	8.75 ± 0.50[b]
Chloroform	0.00	2.00 ± 0.82[a]	3.00 ± 0.00[a]	4.75 ± 0.96[b]	8.00 ± 0.00[b]
Methanol	0.00	2.25 ± 0.50[a]	3.75 ± 0.96[a]	7.25 ± 0.50[b]	9.00 ± 0.00[b]
Plant sample	**0.000**	**0.001**	**0.01**	**0.1**	**1.0**
Friedelan-3β-ol +friedelan-3-one	0.00	1.50 ± 0.58[a]	2.00 ± 0.00[a]	4.50 ± 0.58[a]	7.25± 0.96[ab]
Friedelan-3-one	0.00	1.75 ± 0.50[a]	1.75 ± 0.50[a]	4.25± 0.50[ab]	7.75 ± 0.96[b]
Petrol extract	0.00	2.00 ± 0.00[a]	2.00 ± 0.00[a]	2.50 ± 1.00[c]	5.50± 0.58[bc]
β-sitosterol+ stigmasterol	0.00	1.25 ± 0.50[a]	1.25 ± 0.50[a]	2.75 ± 0.50[bc]	5.00 ± 0.82[c]
Friedelan-3β-ol	0.00	1.50 ± 0.58[a]	1.50 ± 0.58[a]	4.00 ± 0.82[abc]	7.25 ± 0.96[ab]

Mean of four replicates of 10 insects each. Mortality recorded after 48 h exposure. Mean values followed by different letter(s) are significantly different at 0.05 level, (Tukey's HSD)

University of Ghana http://ugspace.ug.edu.gh

3. Total number of *S. zeamais* adults produced in maize treated with extracts of *D. madagascariense* at different times after oviposition period

Sample	Number of insects at different times of treatments		
	1 day	7 days	14 days
Methanol extract	2	4	5
control	20	26	31
Acetone extract	1	4	6
control	19	24	33
Petroleum ether extract	3	4	5
control	22	29	44

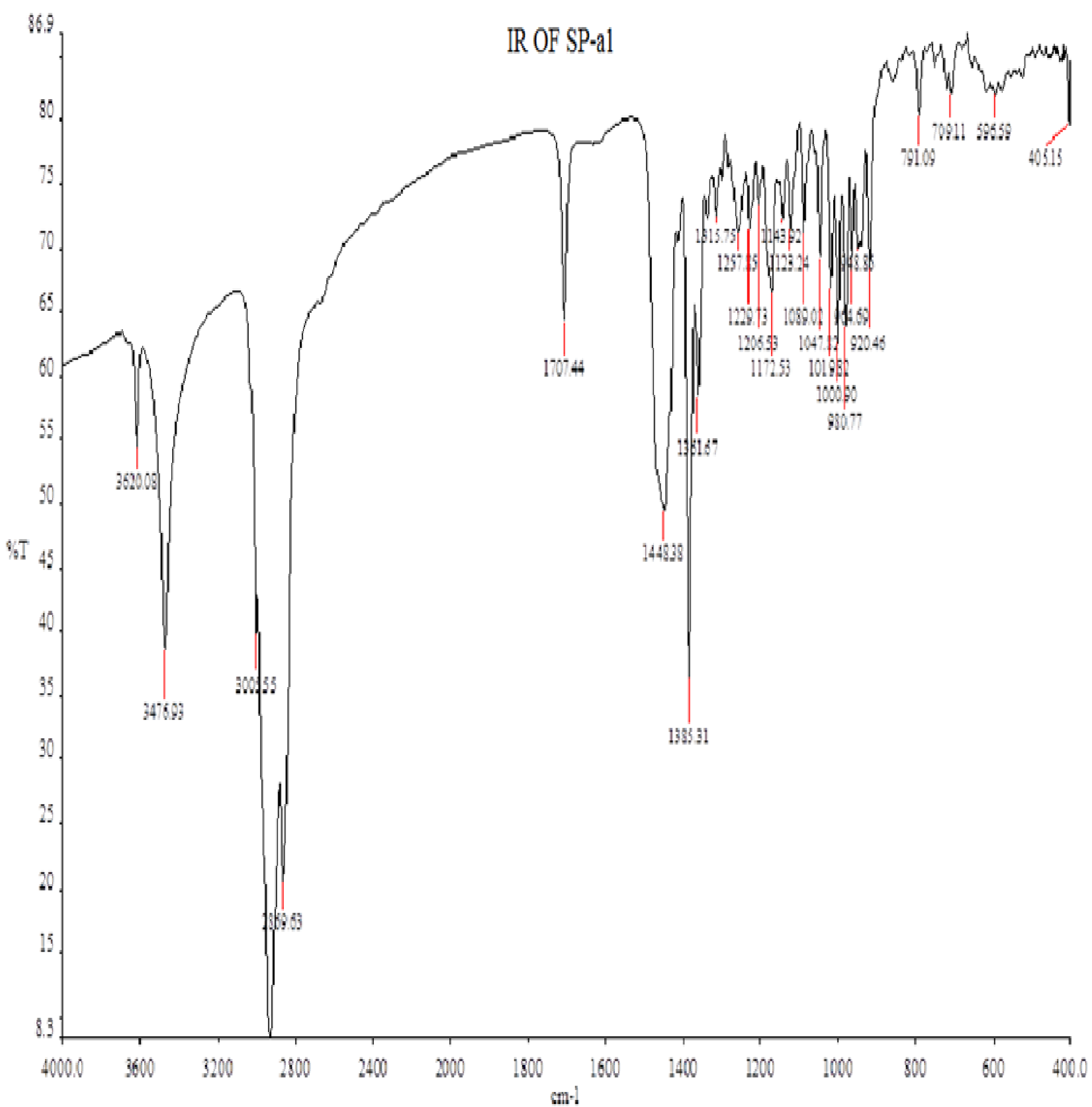
4. *In vitro* average % EHI values for different concentrations of test samples from *D. madagascariense*

Average % EHI							
Test Sample	Concentration (µg/µl)						
	5.000	2.500	1.250	0.625	0.313	0.156	0.078
Petroleum ether extract	100	100	33.6	17.5	2.7	1.5	0.0
Acetone extract	100	100	56.8	13.4	8.2	5.4	4.5
Chloroform extract	100	100	57.1	34.5	7.9	2.8	1.9
Methanol extract	100	100	45.2	10.7	3.1	2.8	2.4
Ethanol extract	100	100	63.6	18.0	17.6	9.1	6.1
β-sitosterol	100	100	79.6	39.5	19.6	19.5	15.5
Mixture of β-sitosterol and stigmasterol	16.7	8.2	7.0	4.0	2.4	1.2	0.0
Friedelan-3β-ol	100	100	100	46.3	6.5	2.5	1.5
Mixture of friedelan-3β-ol and friedelan-3-one	100	60.9	52.0	32.8	14.4	6.2	3.8
Friedelan-3-one	100	100	83.3	10.8	5.3	1.0	0.0
Eskaben (control)	100	100	100	90	86.4	82	80

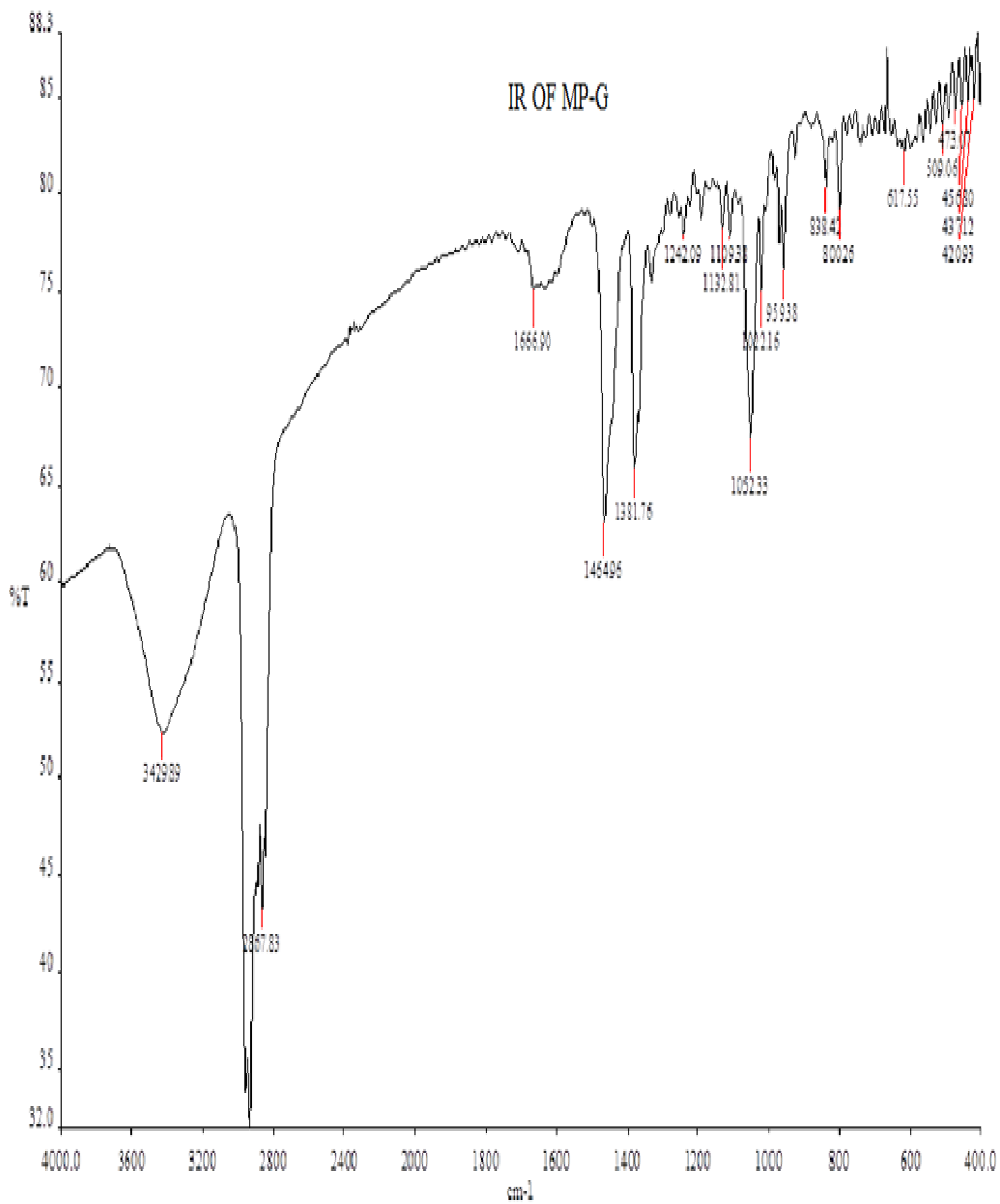
University of Ghana http://ugspace.ug.edu.gh

APPENDIX II

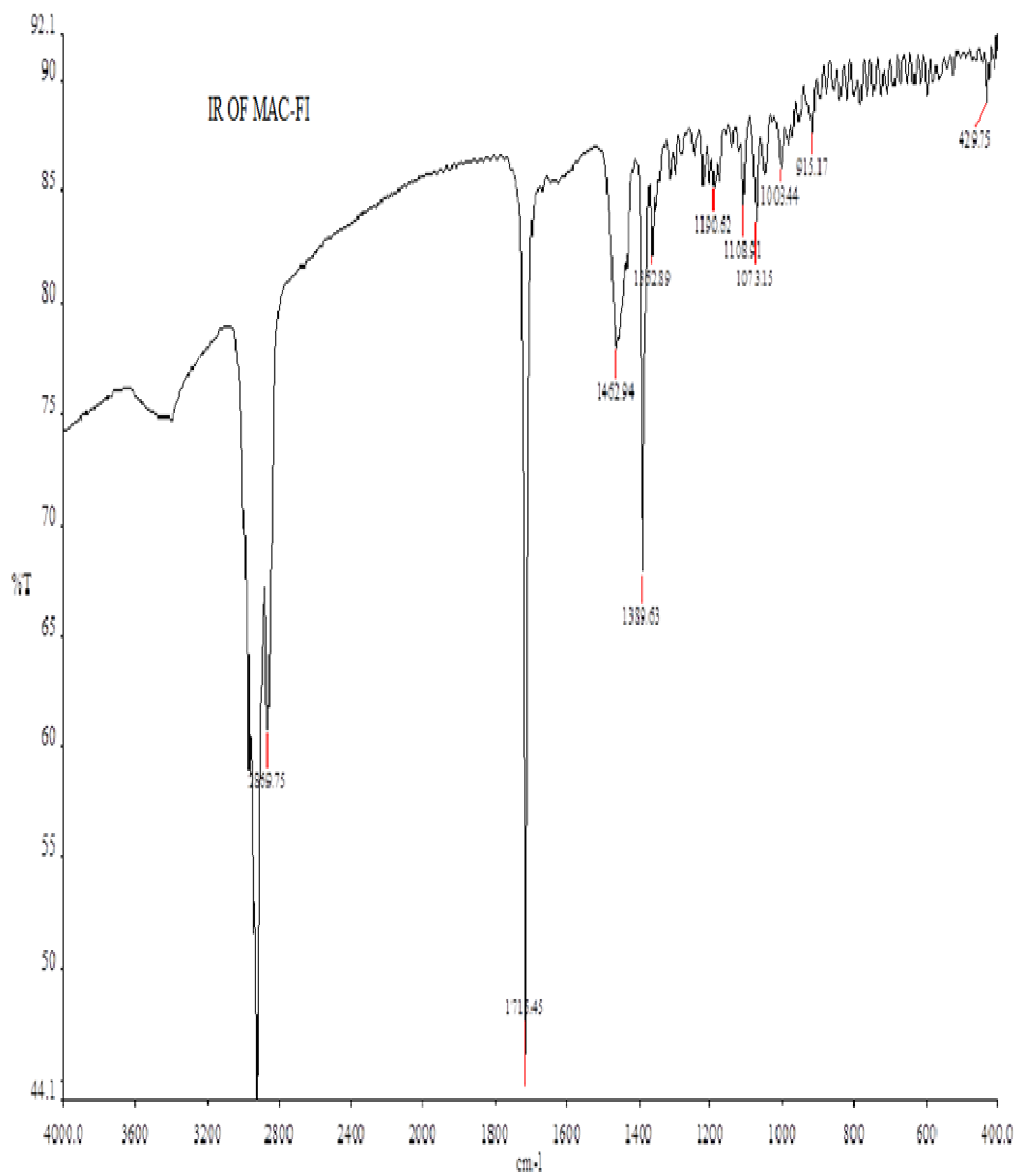
INFRARED SPECTRA (IR)



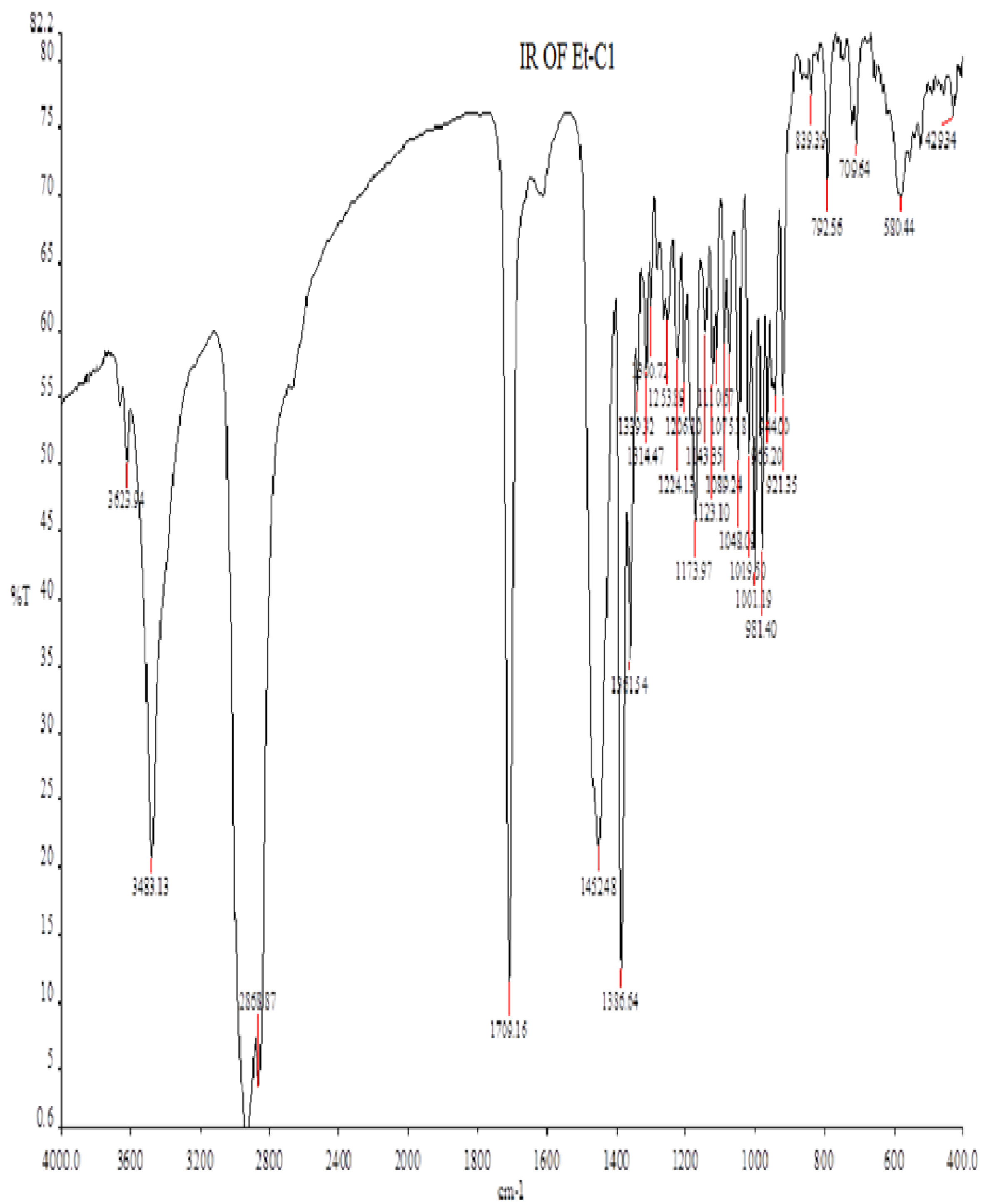
University of Ghana http://ugspace.ug.edu.gh



University of Ghana http://ugspace.ug.edu.gh



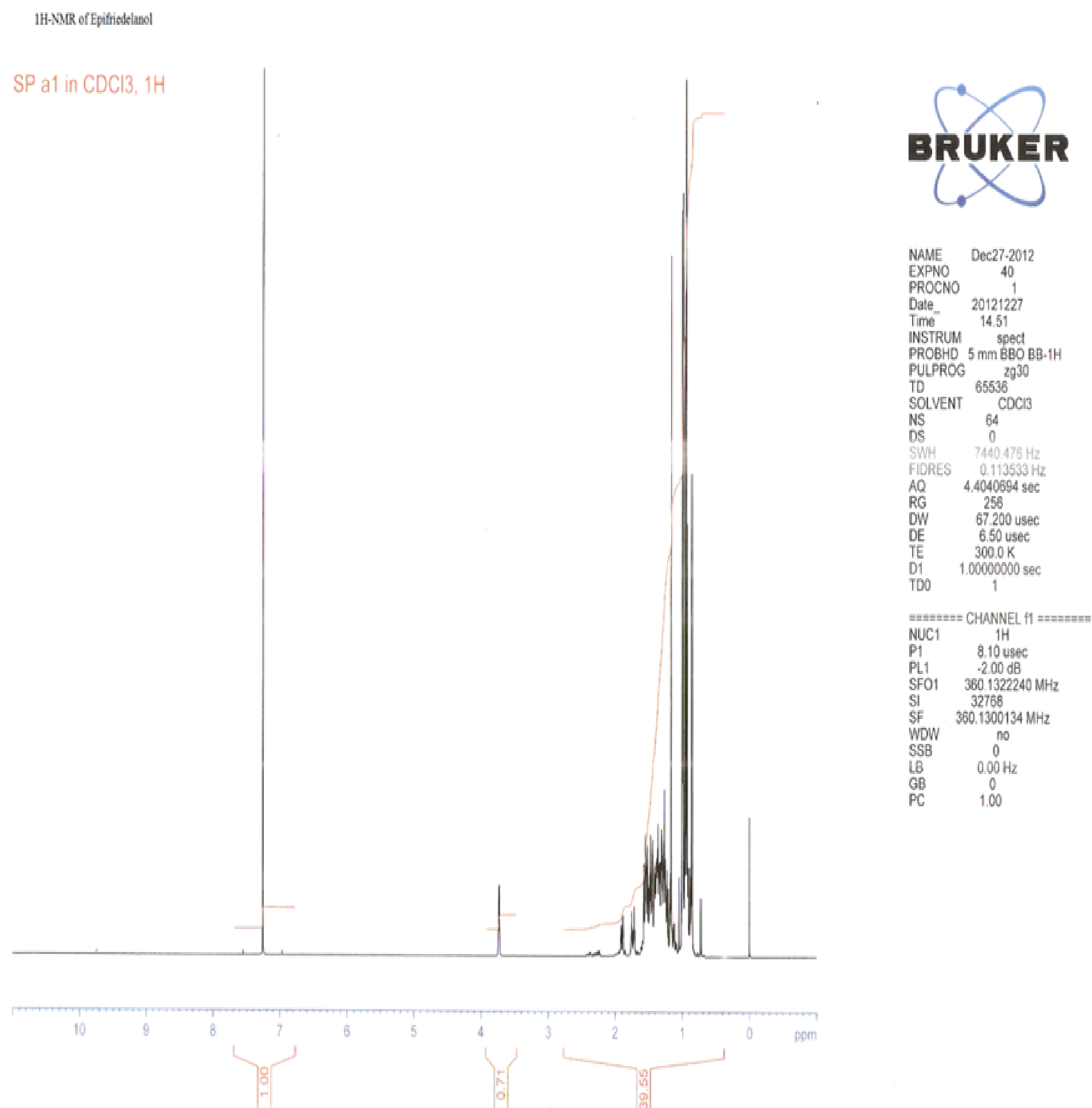
University of Ghana http://ugspace.ug.edu.gh



University of Ghana http://ugspace.ug.edu.gh

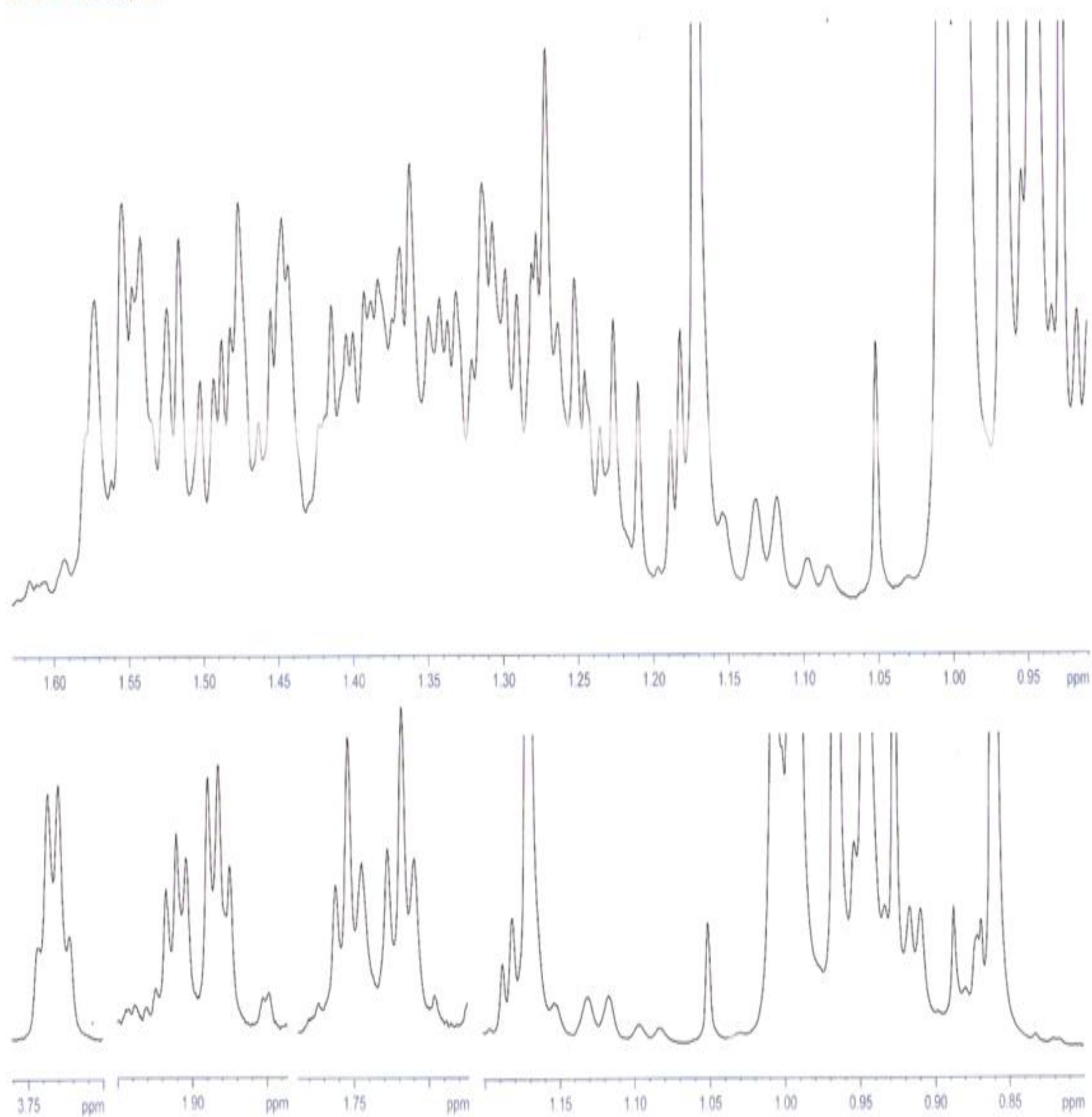
APPENDIX III

PROTON (^{1}H) NMR SPECTRA

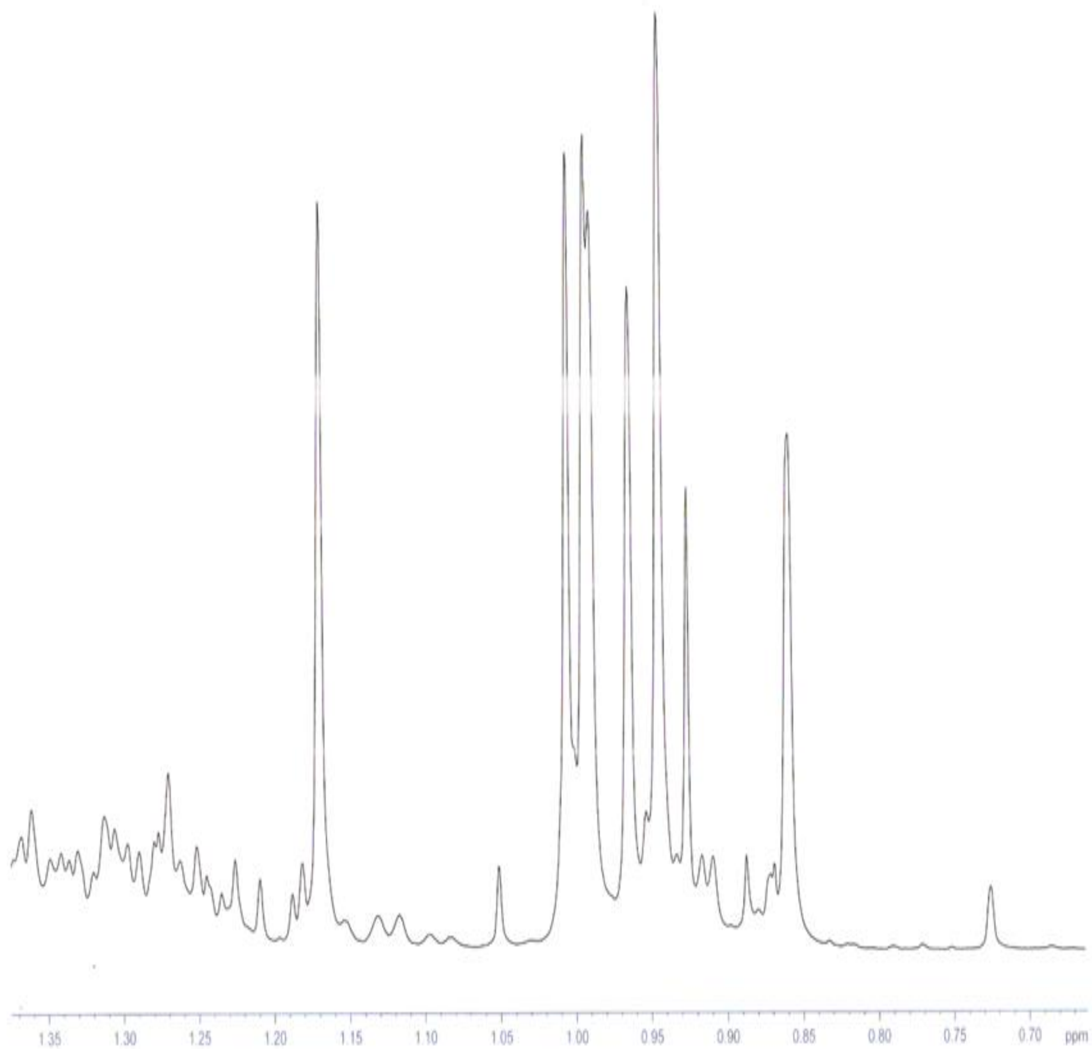


University of Ghana http://ugspace.ug.edu.gh

Expanded 1H-NMR of epifriedelanol

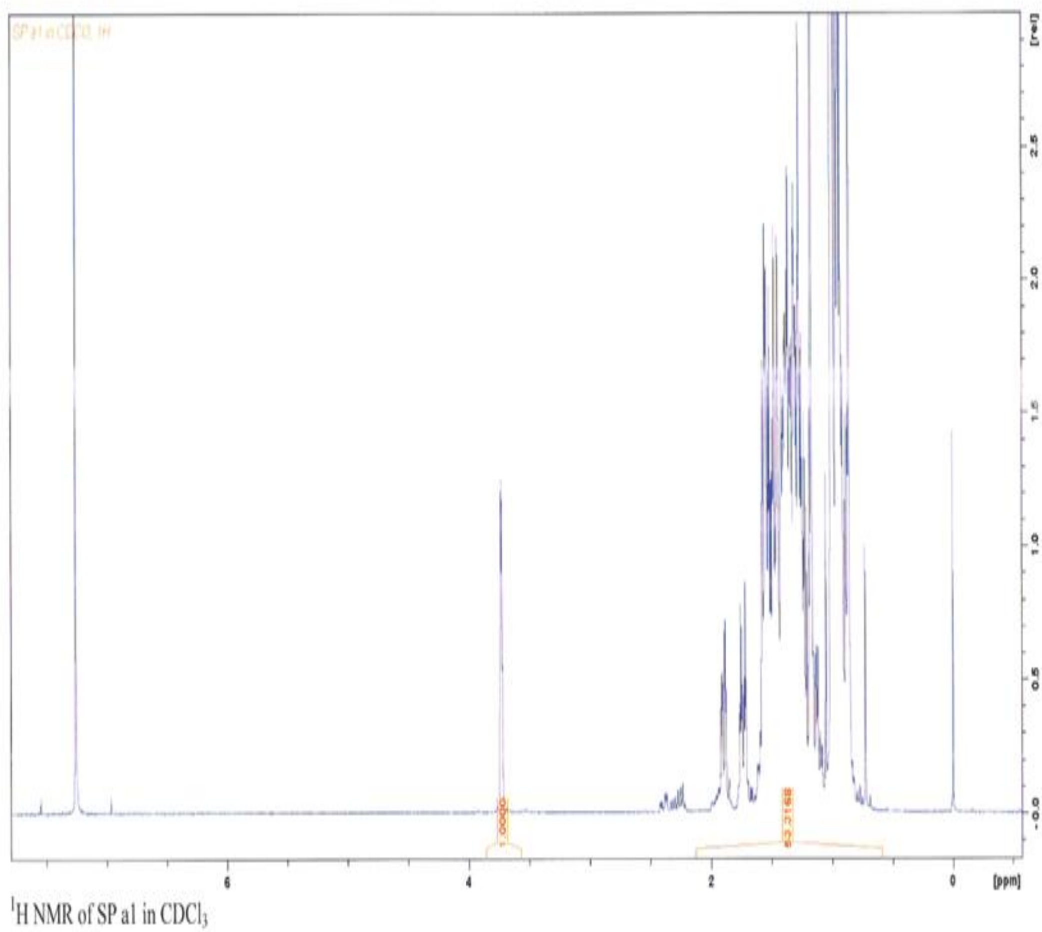


University of Ghana http://ugspace.ug.edu.gh



University of Ghana http://ugspace.ug.edu.gh

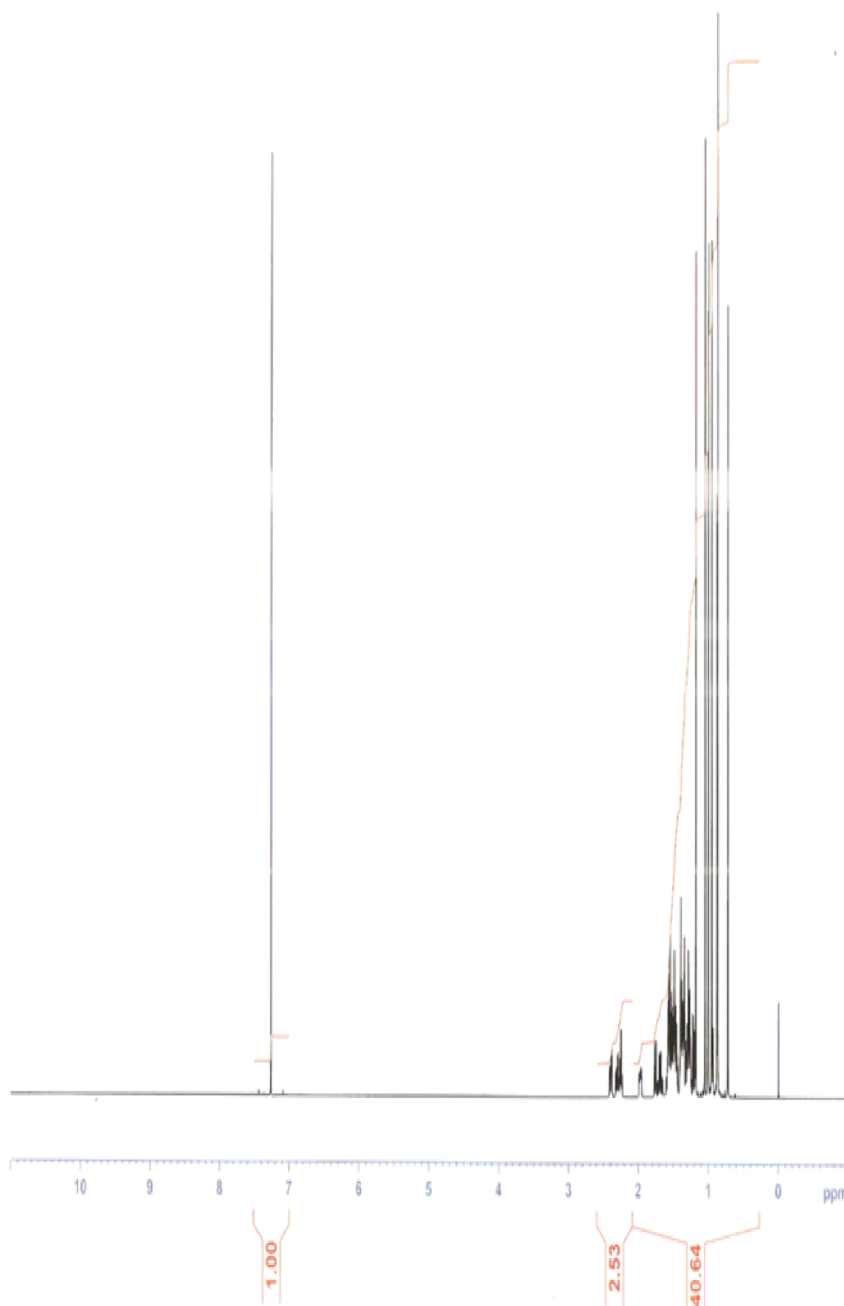
1H-NMR of epifriedelanol



^{1}H NMR of SP a1 in $CDCl_3$

University of Ghana http://ugspace.ug.edu.gh

1H-NMR of Friedelin

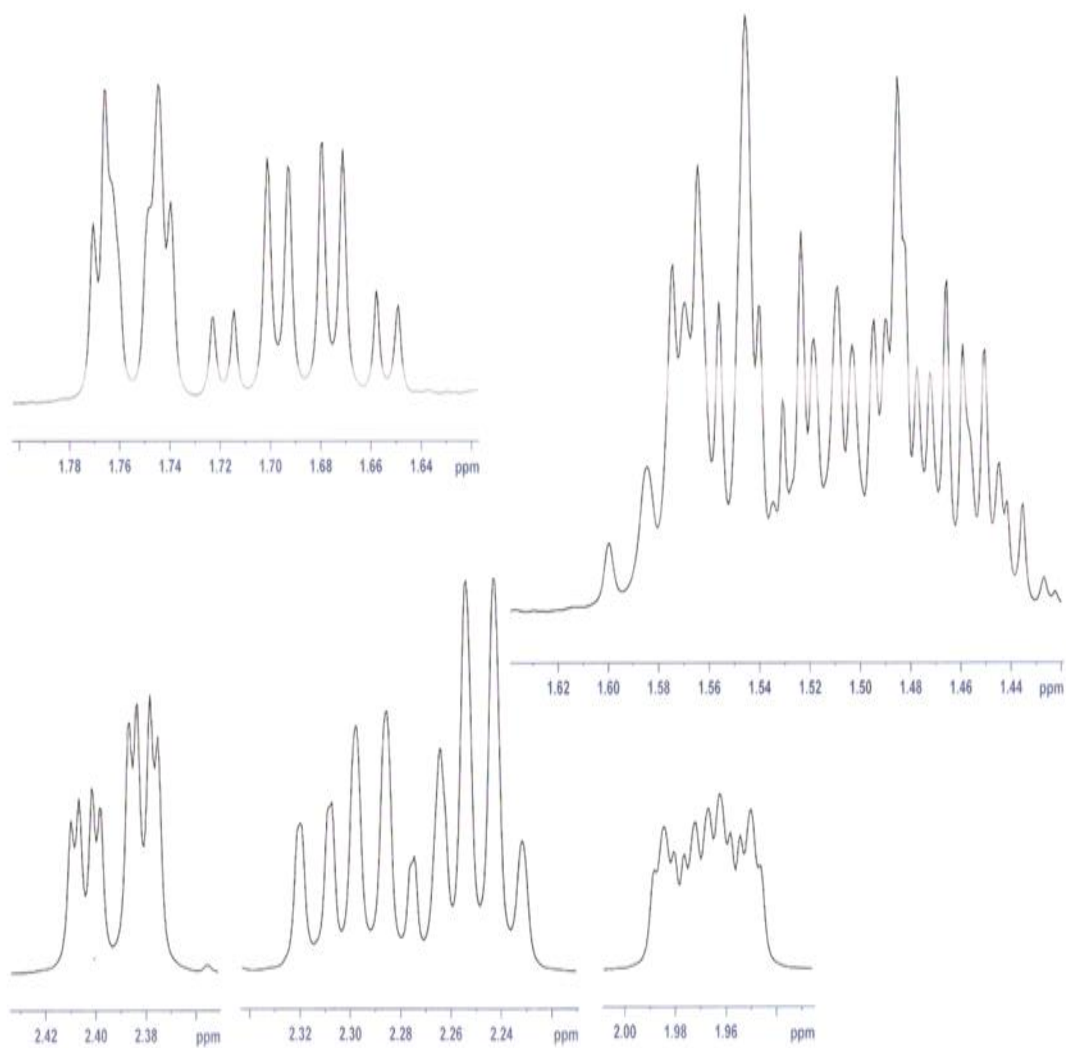


NAME Dec27-2012
EXPNO 40
PROCNO 1
Date_ 20121229
Time 10.20
INSTRUM spect
PROBHD 5 mm PATXI 1H/
PULPROG zg30
TD 65536
SOLVENT CDCl3
NS 64
DS 0
SWH 12376.237 Hz
FIDRES 0.188846 Hz
AQ 2.6477449 sec
RG 80.6
DW 40.400 usec
DE 6.50 usec
TE 300.0 K
D1 1.00000000 sec
TD0 1

======== CHANNEL f1 ========
NUC1 1H
P1 7.70 usec
PL1 4.00 dB
SFO1 600.1337060 MHz
SI 32768
SF 600.1300101 MHz
WDW no
SSB 0
LB 0.00 Hz
GB 0
PC 1.00

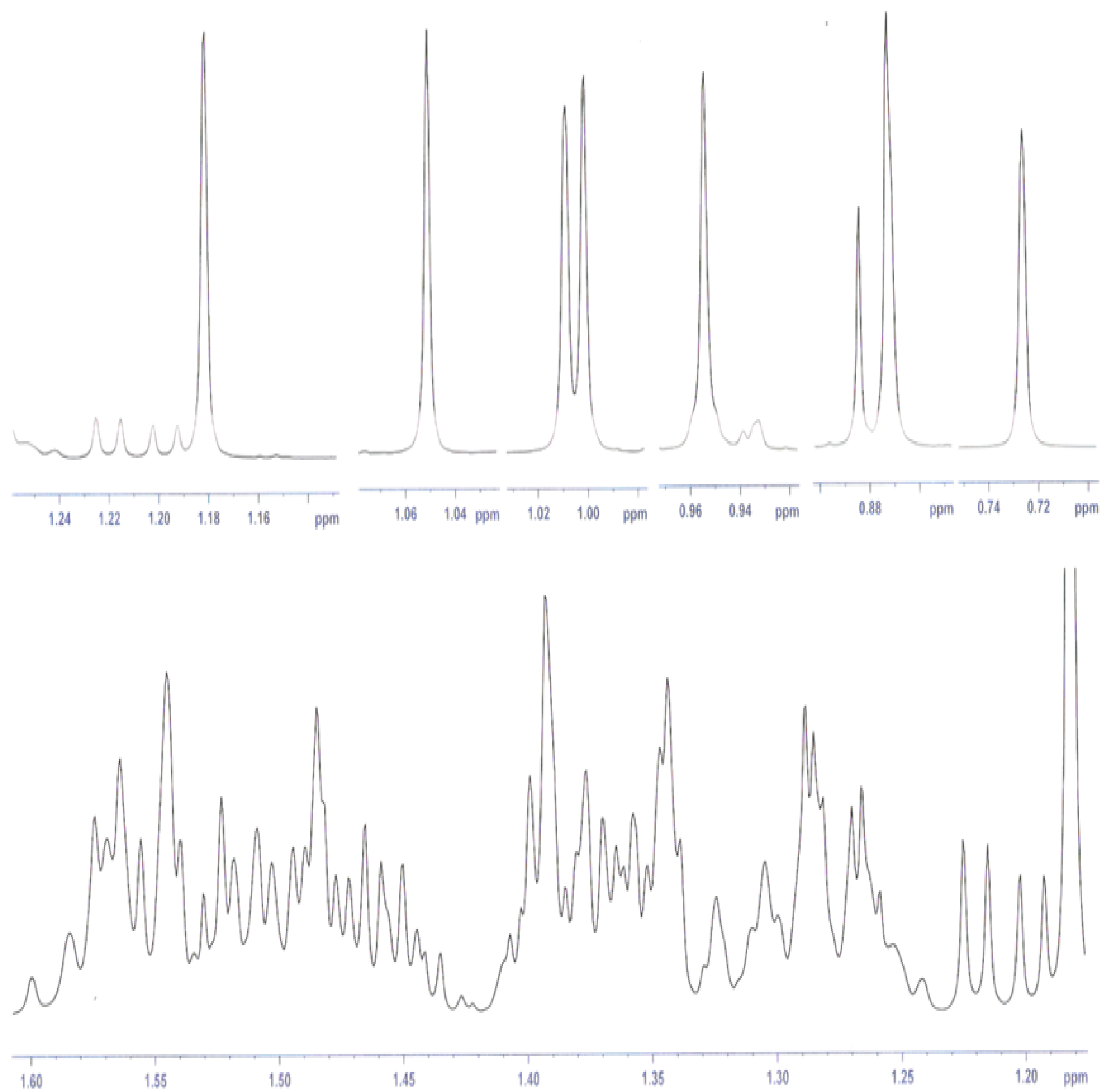
University of Ghana http://ugspace.ug.edu.gh

Expanded 1H-NMR of friedelin

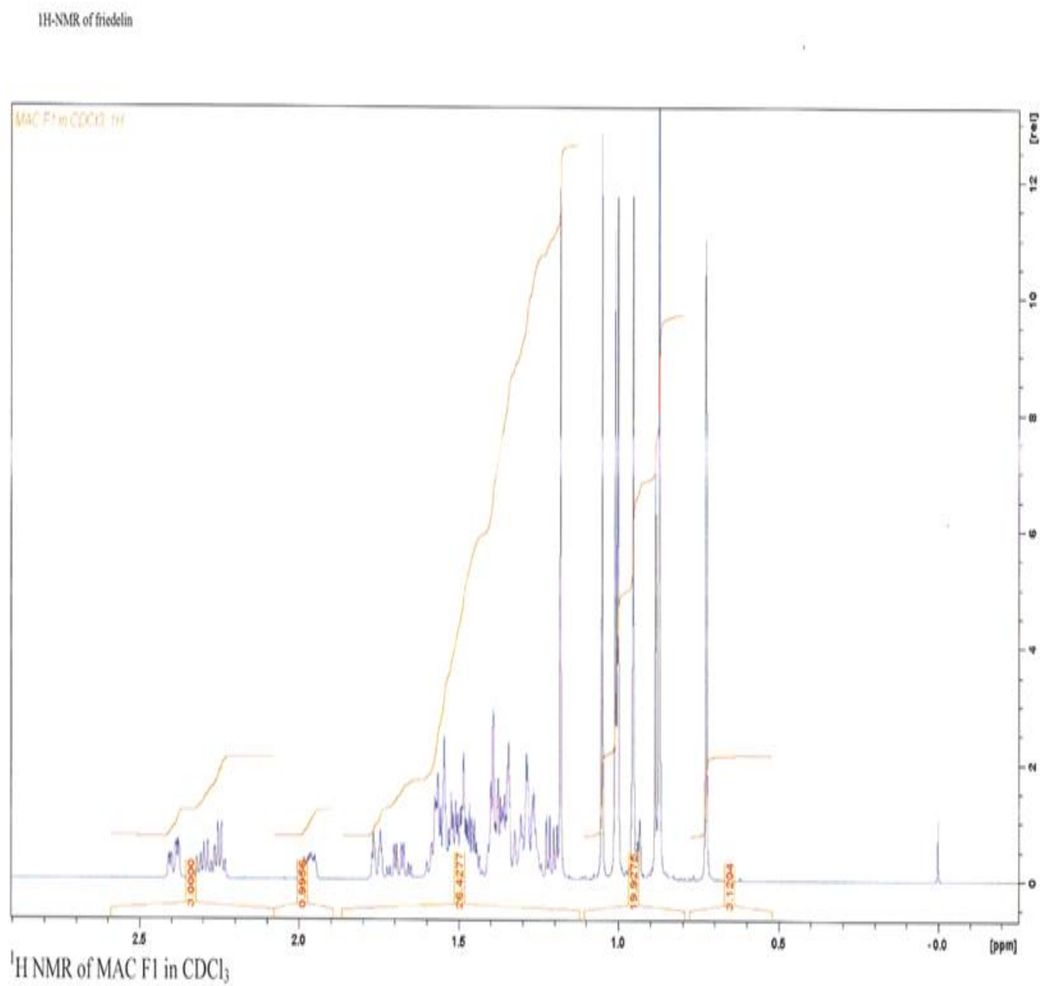


University of Ghana http://ugspace.ug.edu.gh

Expanded 1H-NMR of friedelin

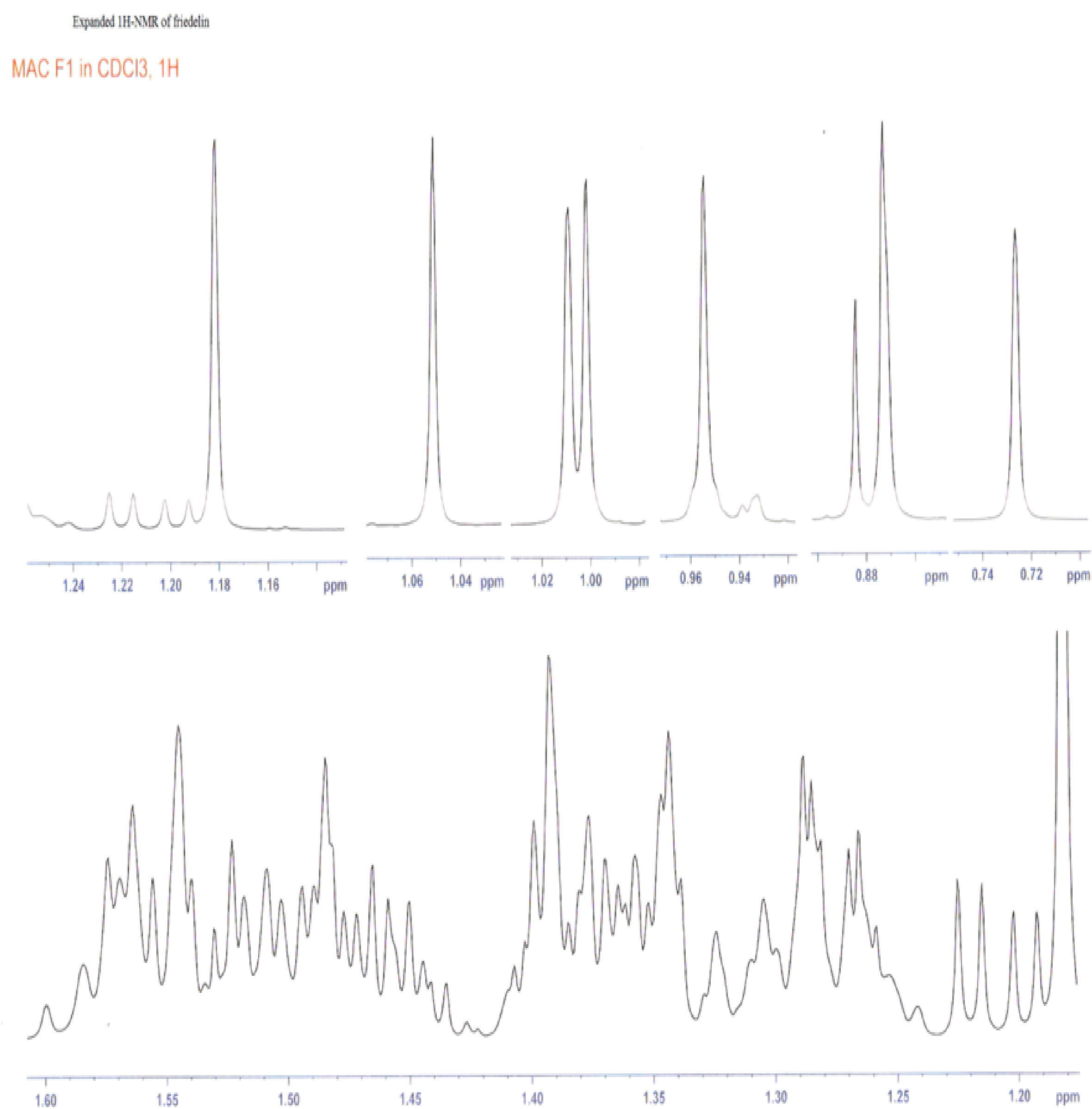


University of Ghana http://ugspace.ug.edu.gh



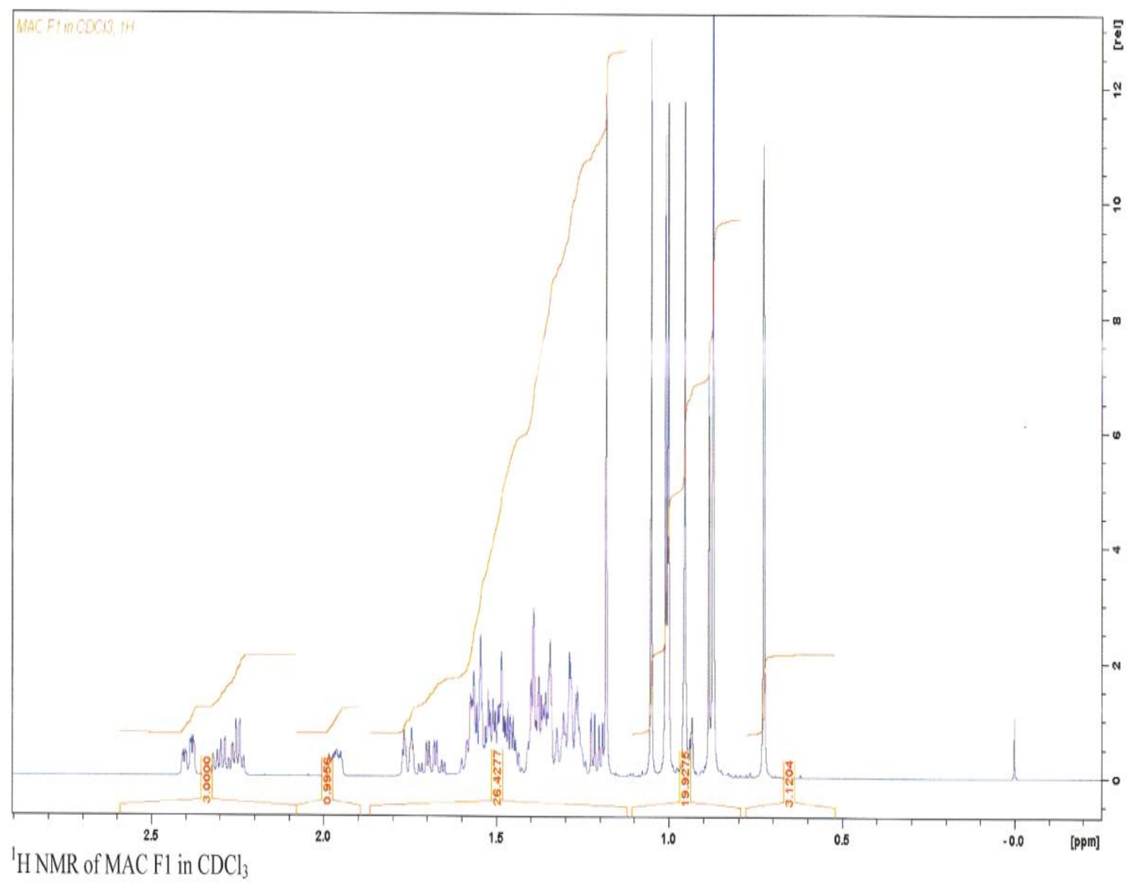
^{1}H NMR of MAC F1 in $CDCl_3$

Expanded 1H-NMR of friedelin



University of Ghana http://ugspace.ug.edu.gh

1H-NMR of friedelin

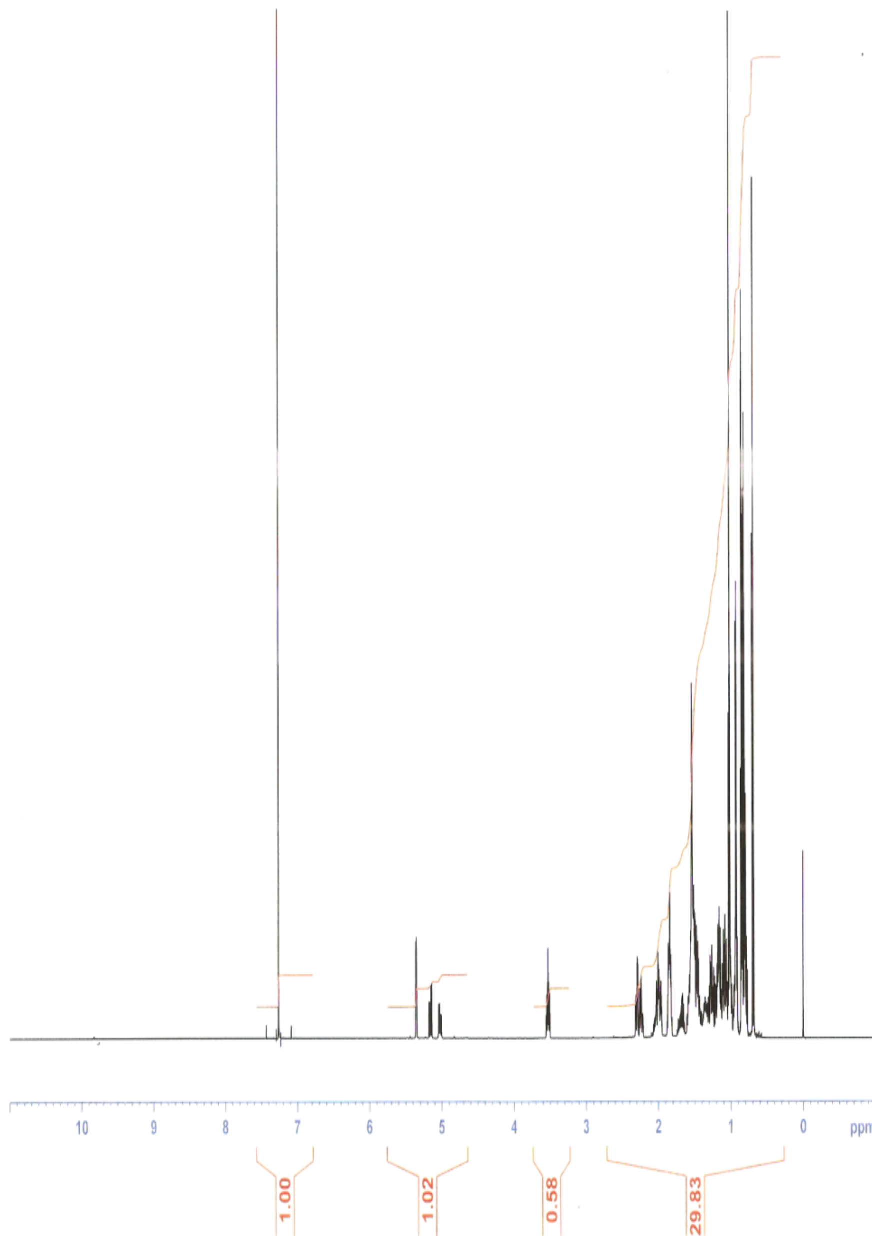


^{1}H NMR of MAC F1 in $CDCl_3$

University of Ghana http://ugspace.ug.edu.gh

1H-NMR of a mixture of beta-sitosterol and stigmasterol

MPG in CDCl3, 1H

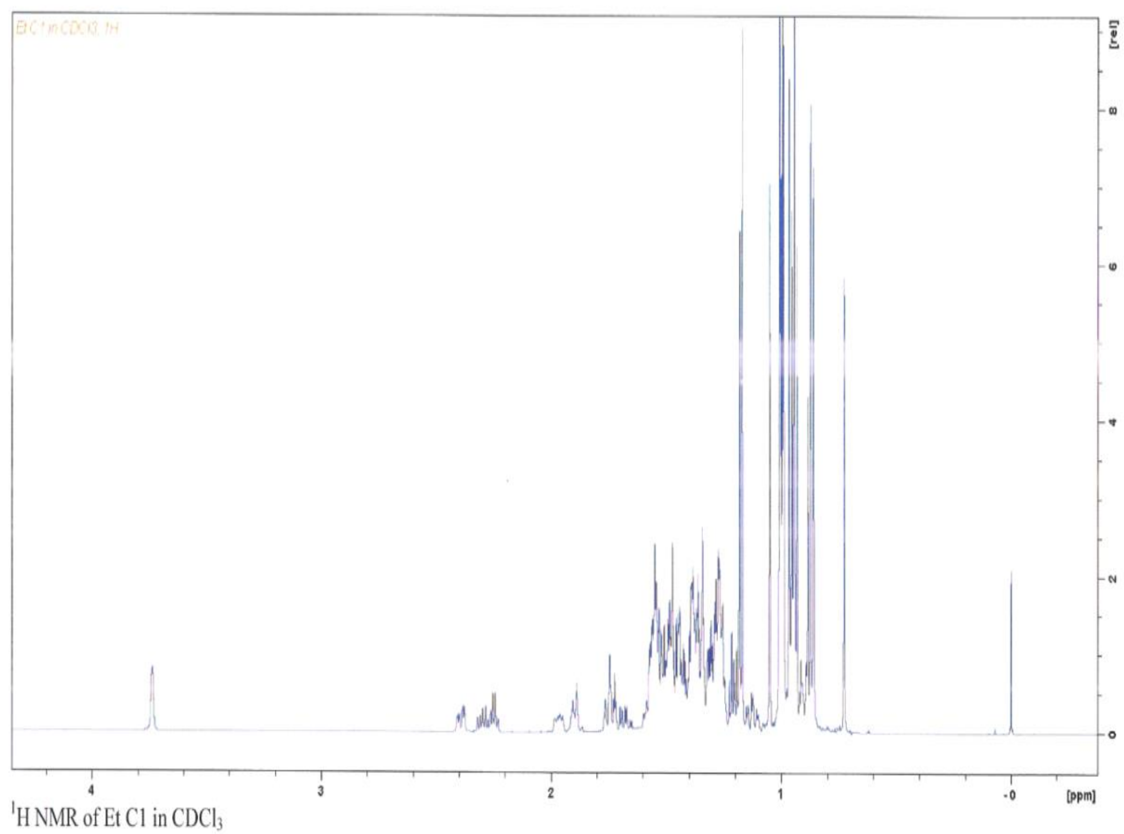


NAME Dec17-2012
EXPNO 80
PROCNO 1
Date_ 20121217
Time 16.41
INSTRUM spect
PROBHD 5 mm PATXI 1H/
PULPROG zg30
TD 65536
SOLVENT CDCl3
NS 64
DS 0
SWH 12376.237 Hz
FIDRES 0.188846 Hz
AQ 2.6477449 sec
RG 144
DW 40.400 usec
DE 6.50 usec
TE 300.0 K
D1 1.00000000 sec
TD0 1

======== CHANNEL f1 ========
NUC1 1H
P1 7.70 usec
PL1 4.00 dB
SFO1 600.1337060 MHz
SI 32768
SF 600.1300105 MHz
WDW no
SSB 0
LB 0.00 Hz
GB 0
PC 1.00

University of Ghana http://ugspace.ug.edu.gh

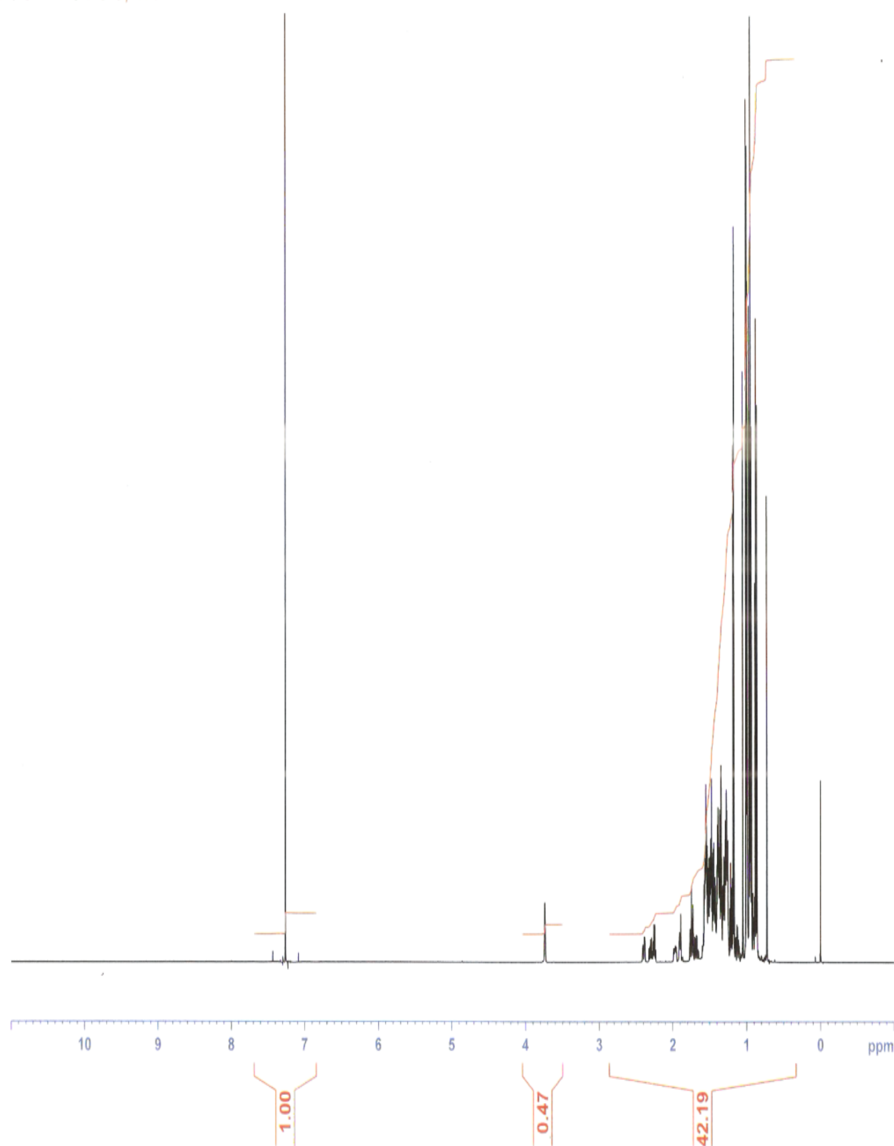
1H-NMR of a mixture of friedelin and epifriedelanol

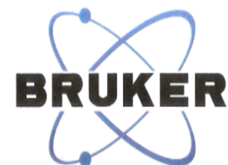


^1H NMR of Et C1 in $CDCl_3$

University of Ghana http://ugspace.ug.edu.gh

1H-NMR of a mixture of friedelanol and epifriedelanol





NAME	Dec27-2012
EXPNO	50
PROCNO	1
Date_	20121230
Time	7.14
INSTRUM	spect
PROBHD	5 mm PATXI 1H/
PULPROG	zg30
TD	65536
SOLVENT	CDCl3
NS	64
DS	0
SWH	12376.237 Hz
FIDRES	0.188846 Hz
AQ	2.6477449 sec
RG	80.6
DW	40.400 usec
DE	6.50 usec
TE	300.0 K
D1	1.00000000 sec
TD0	1

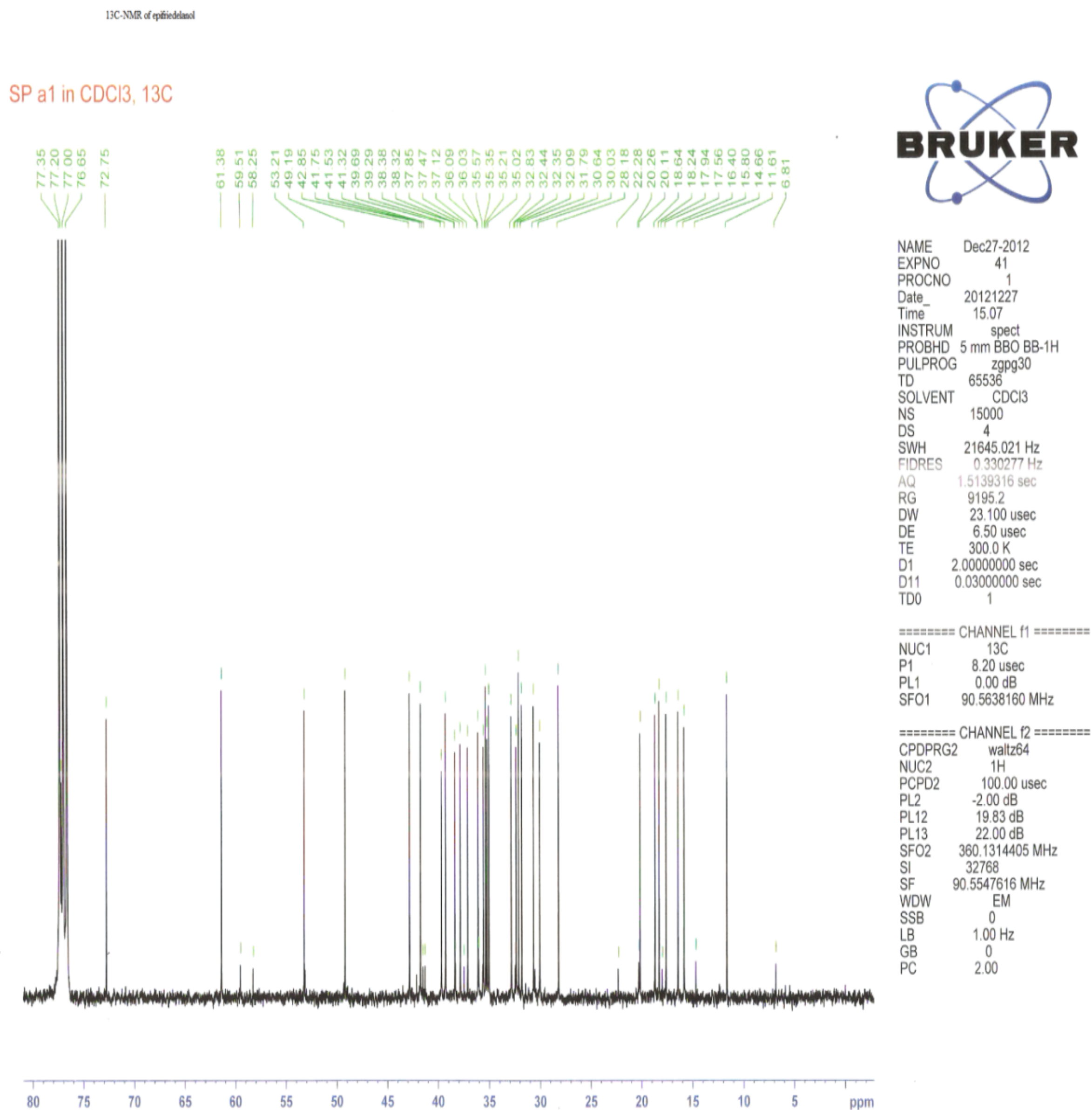
======== CHANNEL f1 ========

NUC1	1H
P1	7.70 usec
PL1	4.00 dB
SFO1	600.1337060 MHz
SI	32768
SF	600.1300105 MHz
WDW	no
SSB	0
LB	0.00 Hz
GB	0
PC	1.00

University of Ghana http://ugspace.ug.edu.gh

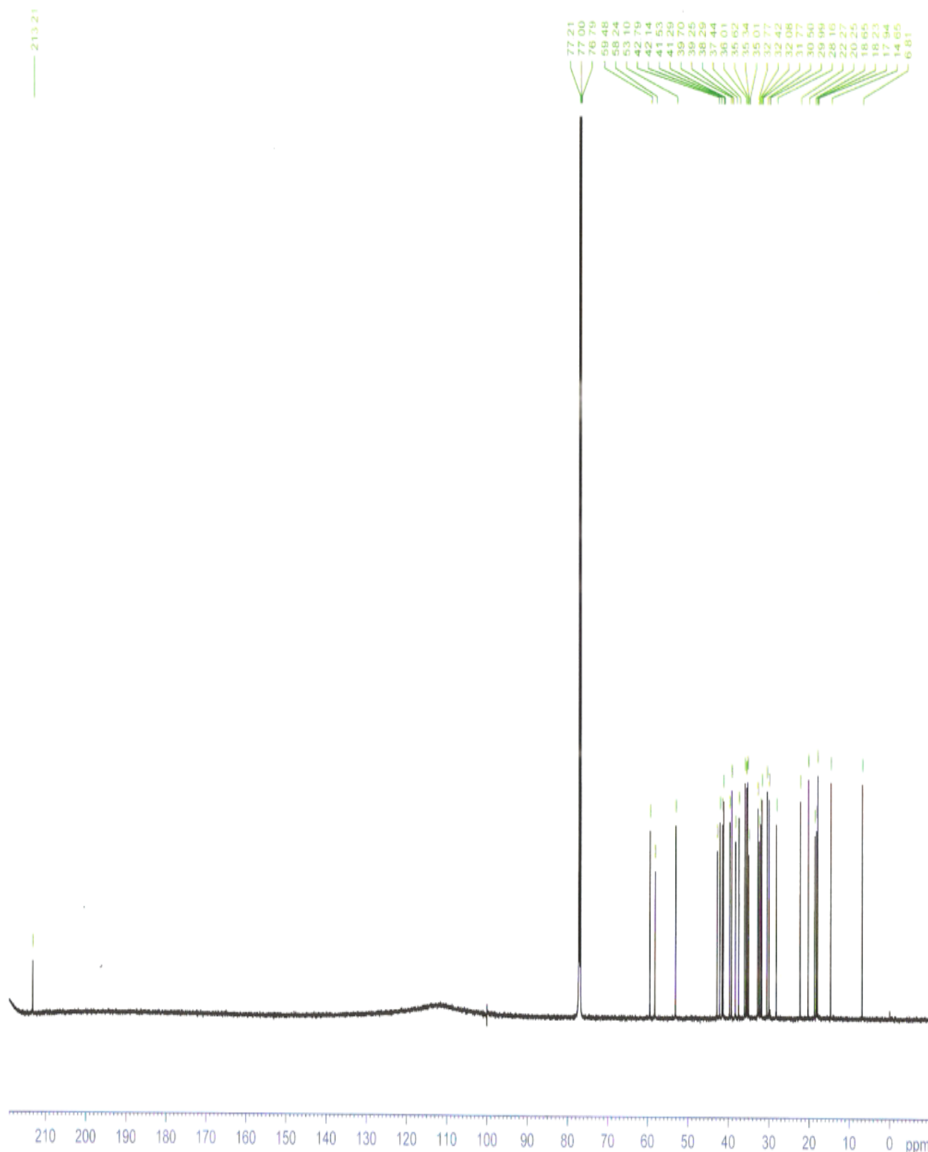
APPENDIX IV

CARBON-13 (^{13}C) NMR SPECTRA



University of Ghana http://ugspace.ug.edu.gh

13C-NMR of Friedelin

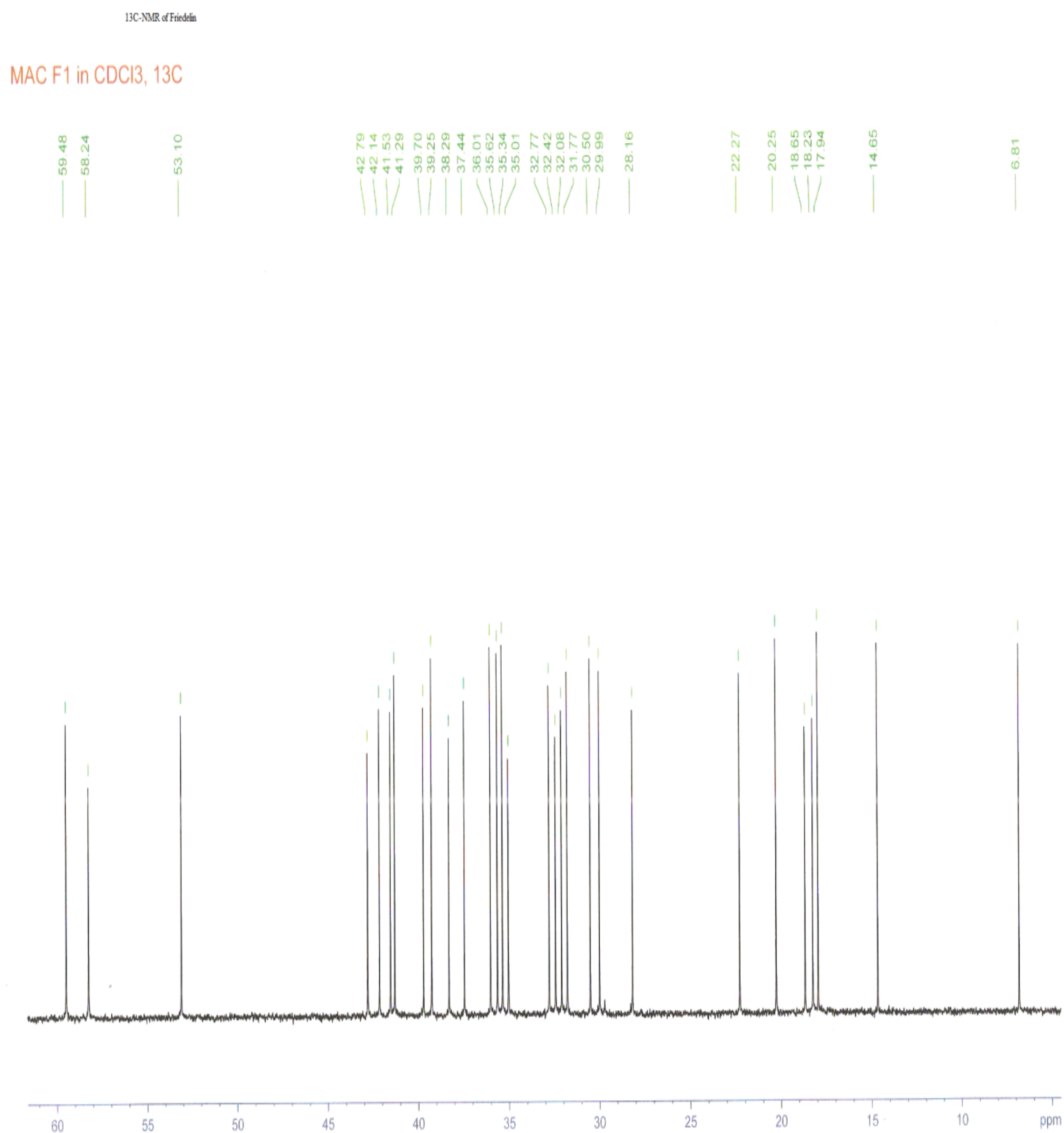


NAME Dec27-2012
EXPNO 41
PROCNO 1
Date_ 20121230
Time 2.53
INSTRUM spect
PROBHD 5 mm PATXI 1H/
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 20000
DS 4
SWH 35971.223 Hz
FIDRES 0.548877 Hz
AQ 0.9110143 sec
RG 362
DW 13.900 usec
DE 6.00 usec
TE 300.0 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1

======== CHANNEL f1 ========
NUC1 13C
P1 10.00 usec
PL1 -3.00 dB
SFO1 150.9178988 MHz

======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
PCPD2 100.00 usec
PL2 4.00 dB
PL12 25.94 dB
PL13 23.00 dB
SFO2 600.1324005 MHz
SI 32768
SF 150.9028098 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 2.00

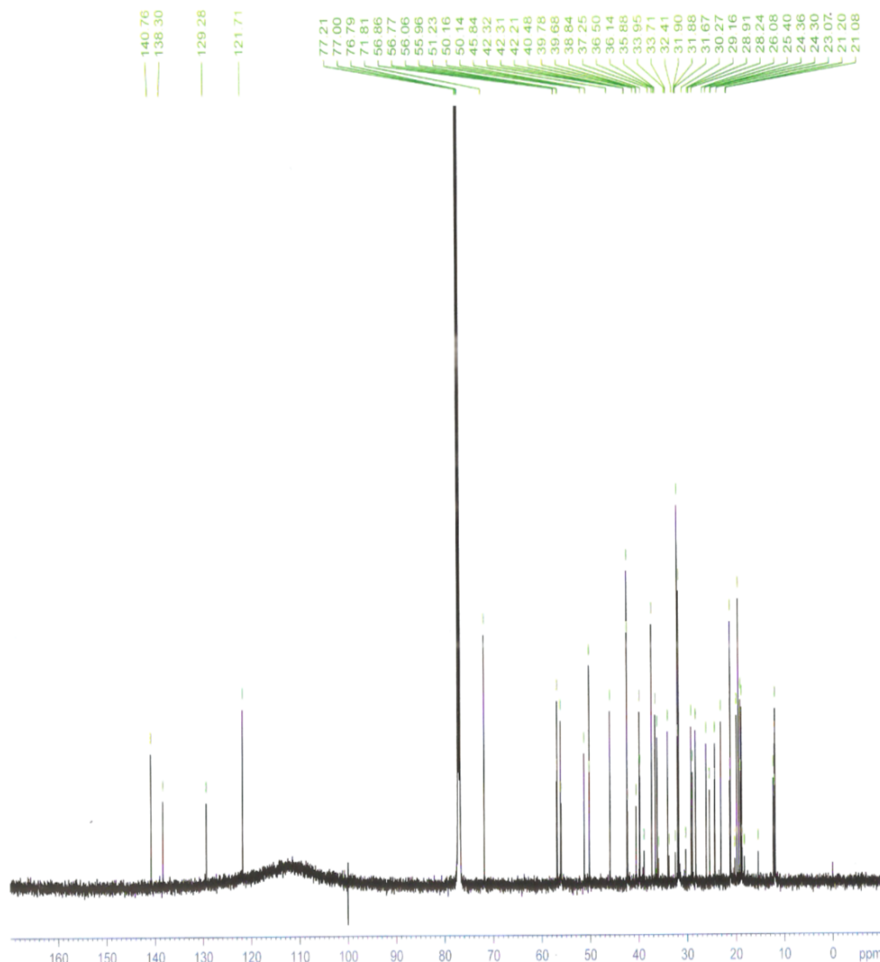
University of Ghana http://ugspace.ug.edu.gh



University of Ghana http://ugspace.ug.edu.gh

13C-NMR of a mixture of beta-sitisterol and stigmasterol

MPG in CDCl3, 13C



NAME Dec17-2012
EXPNO 81
PROCNO 1
Date_ 20121217
Time 16.50
INSTRUM spect
PROBHD 5 mm PATXI 1H/
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 10000
DS 4
SWH 35971.223 Hz
FIDRES 0.548877 Hz
AQ 0.9110143 sec
RG 362
DW 13.900 usec
DE 6.00 usec
TE 300.0 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1

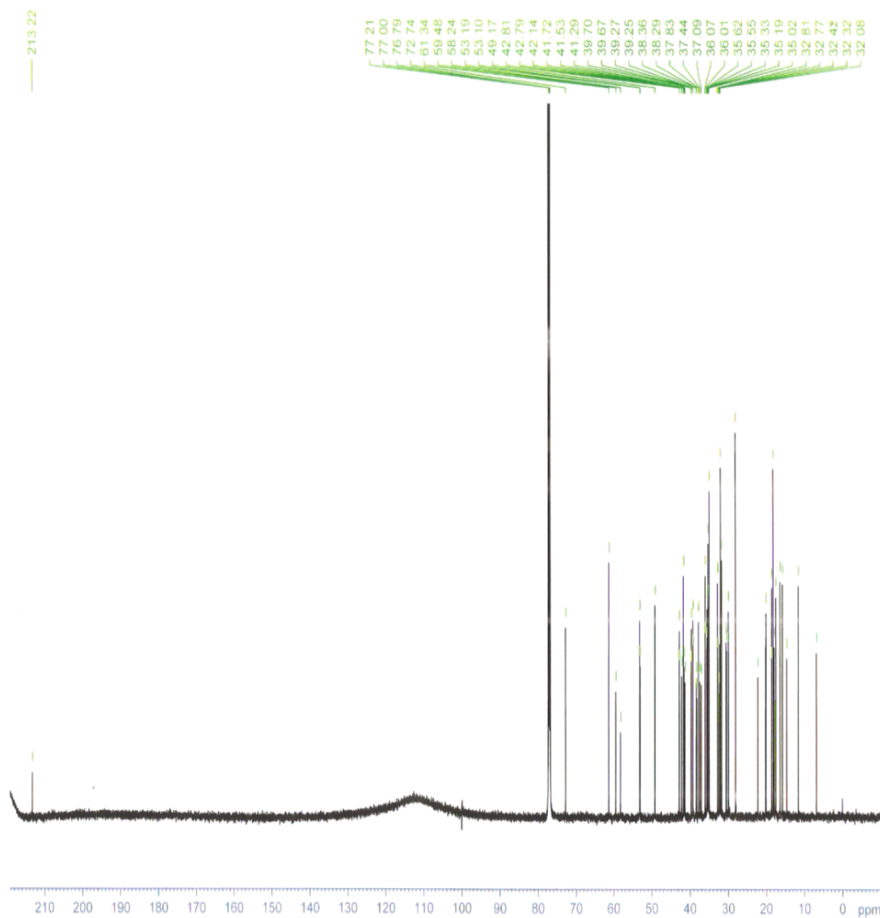
======== CHANNEL f1 ========
NUC1 13C
P1 10.00 usec
PL1 -3.00 dB
SFO1 150.9178988 MHz

======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
PCPD2 100.00 usec
PL2 4.00 dB
PL12 25.94 dB
PL13 23.00 dB
SFO2 600.1324005 MHz
SI 32768
SF 150.9028099 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 2.00

University of Ghana http://ugspace.ug.edu.gh

13C-NMR of a mixture of friedelin and epifriedelanol

Et C1 in CDCl3, 13C



NAME Dec27-2012
EXPNO 51
PROCNO 1
Date_ 20121230
Time 23.48
INSTRUM spect
PROBHD 5 mm PATXI 1H/
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 20000
DS 4
SWH 35971.223 Hz
FIDRES 0.548877 Hz
AQ 0.9110143 sec
RG 362
DW 13.900 usec
DE 6.00 usec
TE 300.0 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1

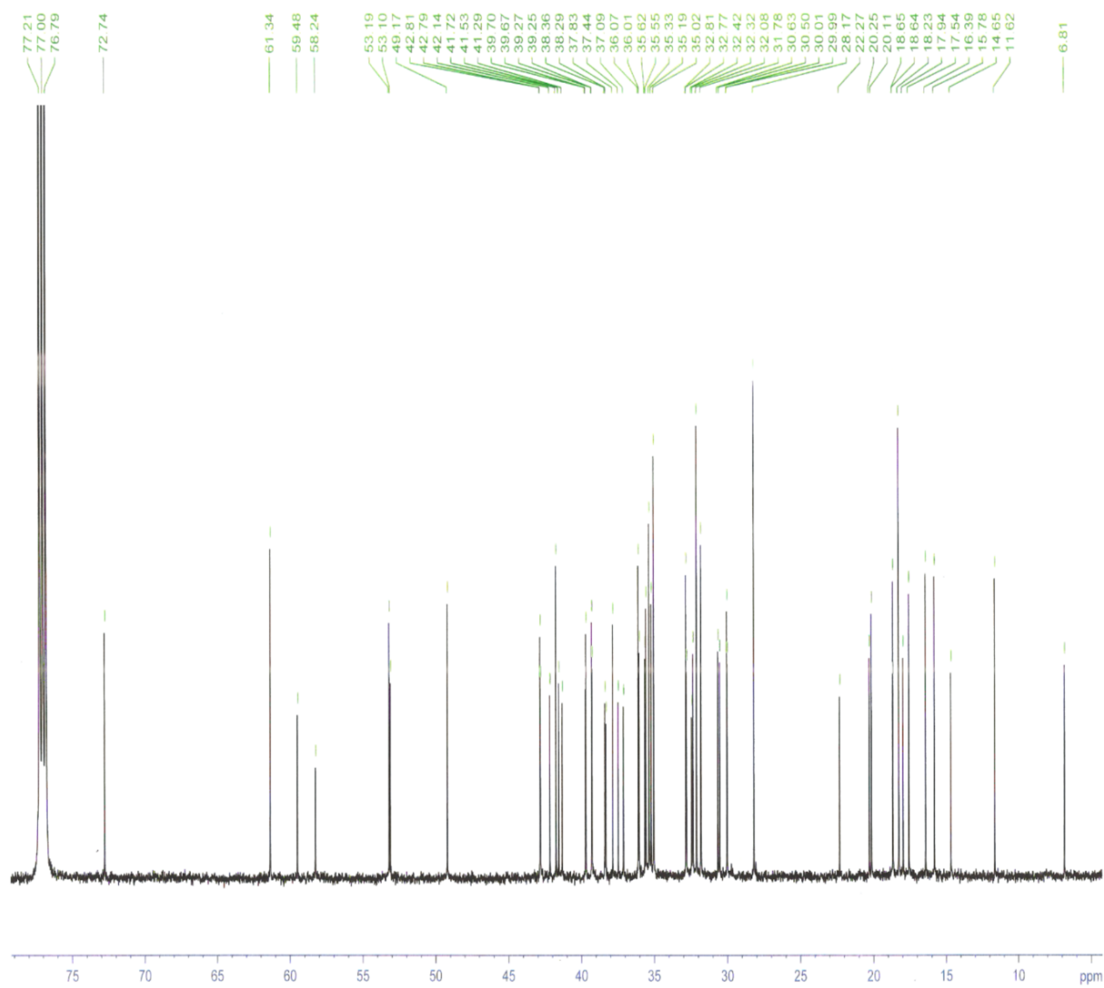
======== CHANNEL f1 ========
NUC1 13C
P1 10.00 usec
PL1 -3.00 dB
SFO1 150.9178988 MHz

======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
PCPD2 100.00 usec
PL2 4.00 dB
PL12 25.94 dB
PL13 23.00 dB
SFO2 600.1324005 MHz
SI 32768
SF 150.9028099 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 2.00

University of Ghana http://ugspace.ug.edu.gh

13C-NMR of a mixture of friedelin and epifriedelanol

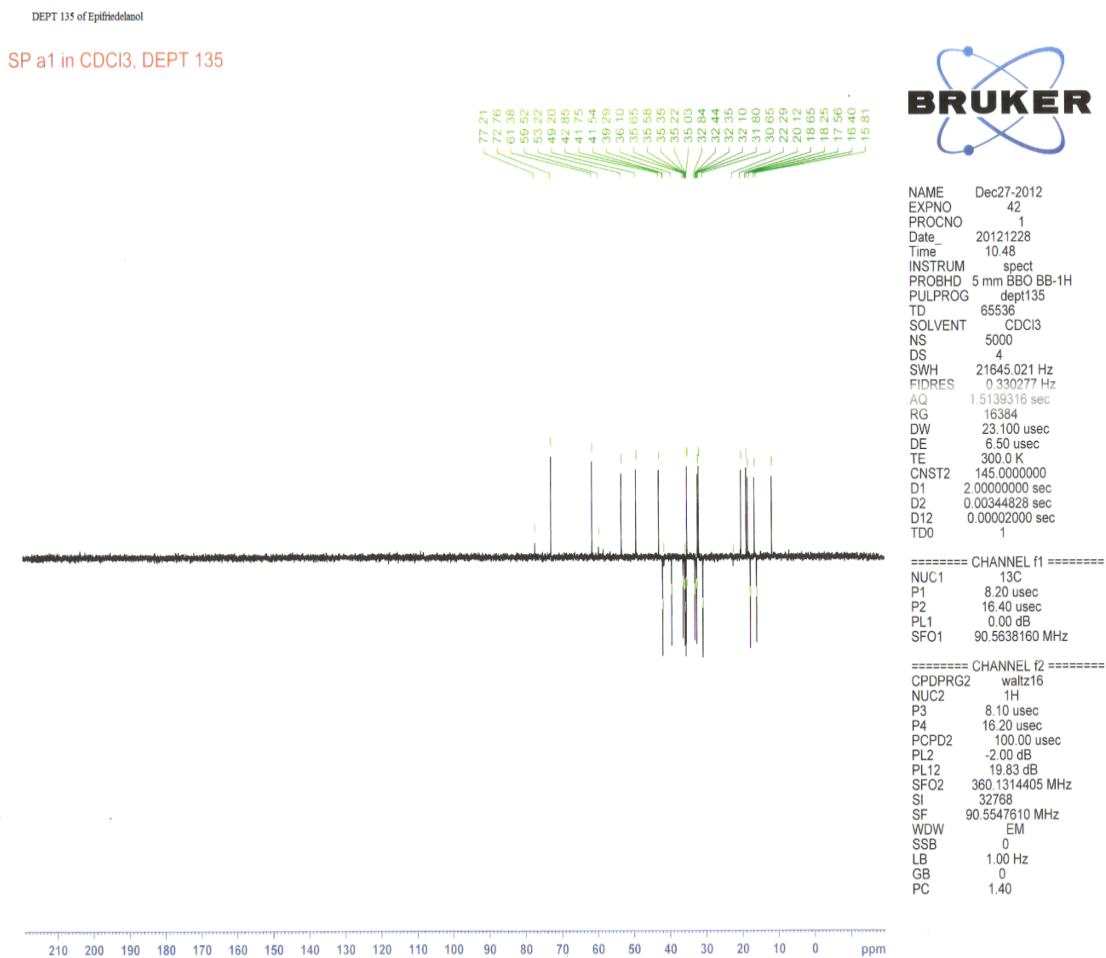
Et C1 in CDCl3, 13C



University of Ghana http://ugspace.ug.edu.gh

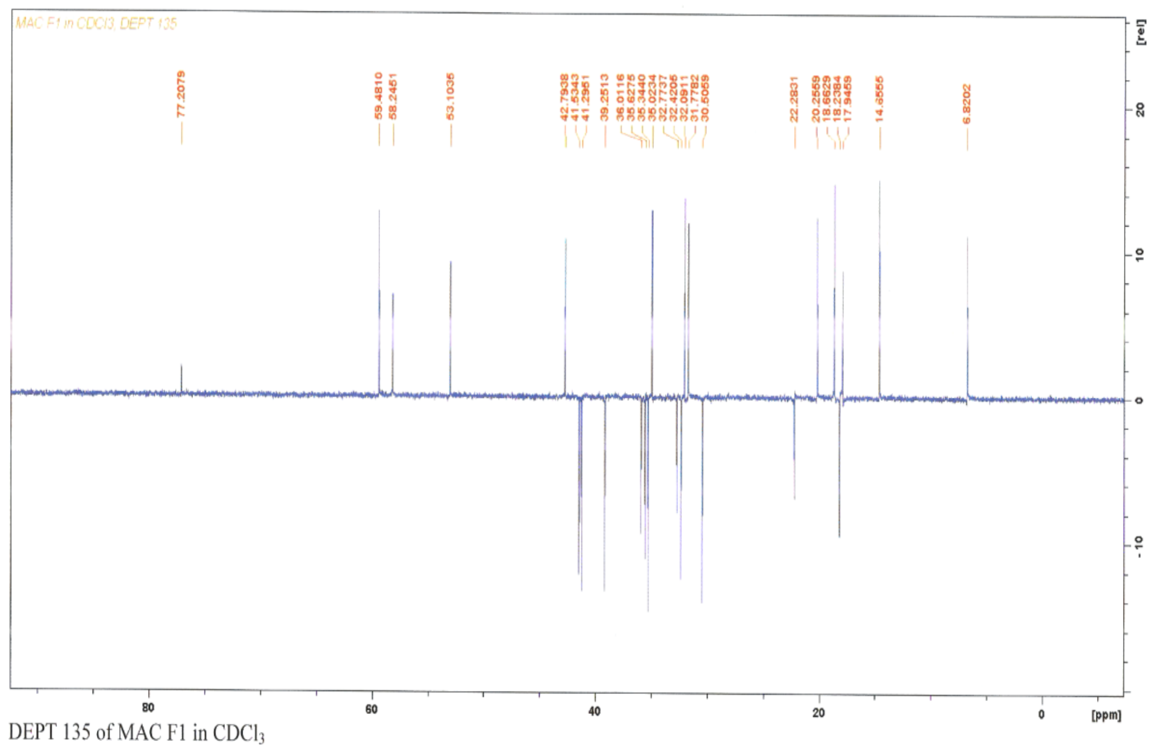
APPENDIX V

DEPT SPECTRA



University of Ghana http://ugspace.ug.edu.gh

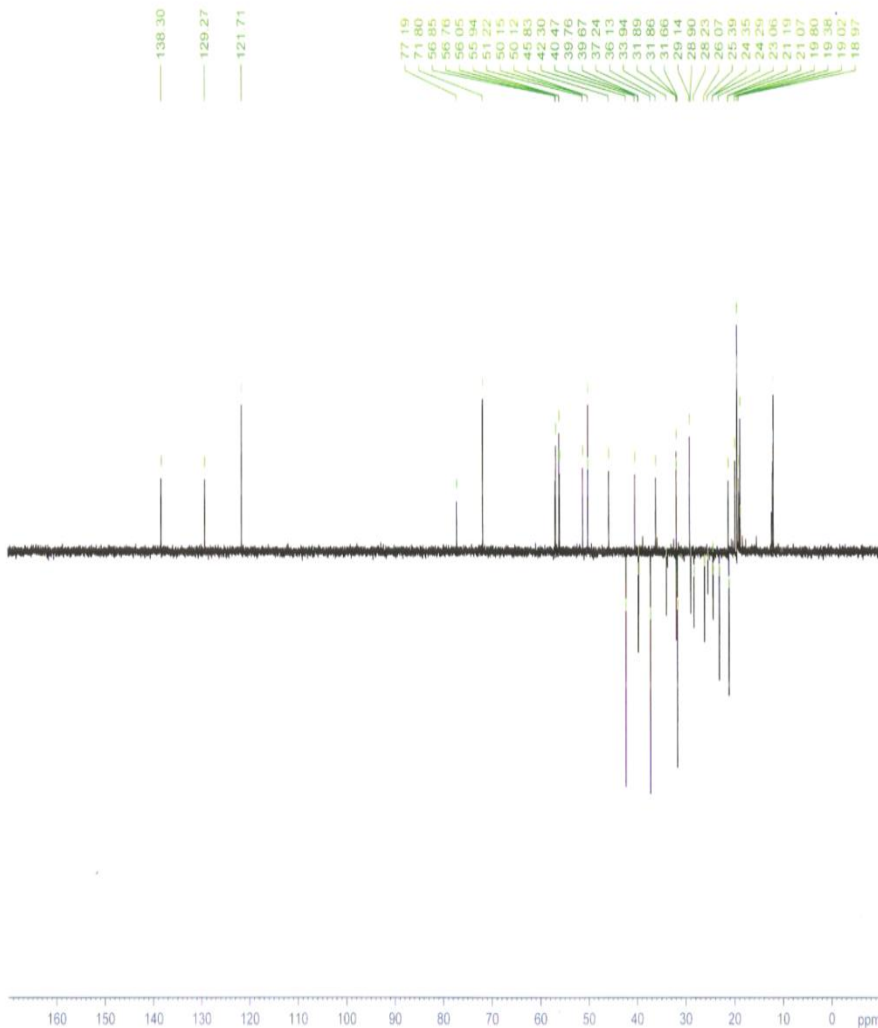
DEPT 135 of Friedelin



DEPT 135 of MAC F1 in $CDCl_3$

University of Ghana http://ugspace.ug.edu.gh

DEPT 135 of a mixture of beta-sitosterol and stigmasterol



NAME Dec17-2012
EXPNO 82
PROCNO 1
Date_ 20121218
Time 5.09
INSTRUM spect
PROBHD 5 mm PATXI 1H/
PULPROG dept135
TD 65536
SOLVENT CDCl3
NS 5000
DS 4
SWH 35971.223 Hz
FIDRES 0.548877 Hz
AQ 0.9110143 sec
RG 16400
DW 13.900 usec
DE 6.50 usec
TE 300.0 K
CNST2 145.0000000
D1 2.00000000 sec
D2 0.00344828 sec
D12 0.00002000 sec
TD0 1

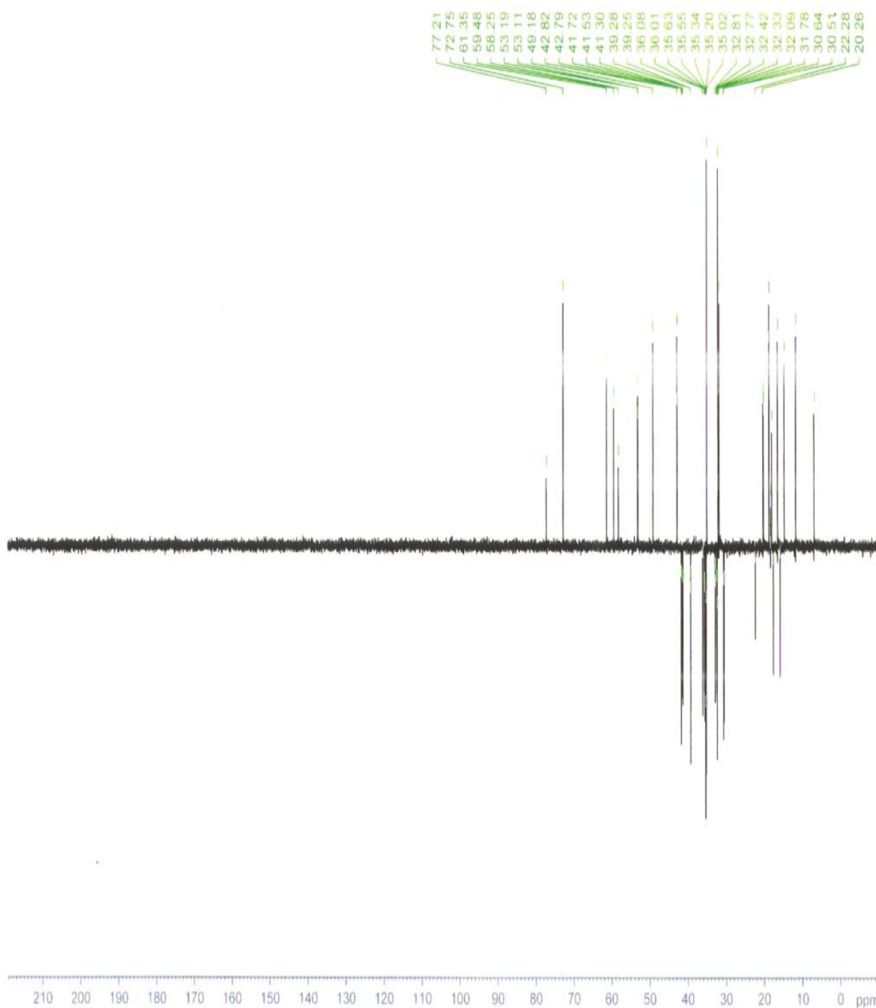
======== CHANNEL f1 ========
NUC1 13C
P1 10.00 usec
P2 20.00 usec
PL1 -3.00 dB
SFO1 150.9178988 MHz

======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
P3 8.00 usec
P4 16.00 usec
PCPD2 100.00 usec
PL2 4.00 dB
PL12 25.94 dB
SFO2 600.1324005 MHz
SI 32768
SF 150.9028114 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 2.00

University of Ghana http://ugspace.ug.edu.gh

DEPT 135 of a mixture of friedelin and epifriedelanol

Et C1 in CDCl3, DEPT 135



NAME Dec27-2012
EXPNO 52
PROCNO 1
Date_ 20121231
Time 3.56
INSTRUM spect
PROBHD 5 mm PATXI 1H/
PULPROG dept135
TD 65536
SOLVENT CDCl3
NS 5000
DS 4
SWH 35971.223 Hz
FIDRES 0.548877 Hz
AQ 0.9110143 sec
RG 16400
DW 13.900 usec
DE 6.50 usec
TE 300.0 K
CNST2 145.0000000
D1 2.00000000 sec
D2 0.00344828 sec
D12 0.00002000 sec
TD0 1

======== CHANNEL f1 ========
NUC1 13C
P1 10.00 usec
P2 20.00 usec
PL1 -3.00 dB
SFO1 150.9178988 MHz

======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
P3 8.00 usec
P4 16.00 usec
PCPD2 100.00 usec
PL2 4.00 dB
PL12 25.94 dB
SFO2 600.1324005 MHz
SI 32768
SF 150.9028090 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 2.00

University of Ghana http://ugspace.ug.edu.gh

REFERENCE

1. Bates S.H, Robert B. J., Clifford J. B., (2000), *Journal of Asian Natural Product Research*, **2**(4), 321-327.
2. WHO, (2002-2005), WHO Traditional medicine strategy. WHO/EDM/TRM/2002.1, 66.
3. The Plant List, (2010), Version 1. Published on the internet; http:// www.theplantlist.org/
4. Chase M.W. and Reveal J.L., (2009), A Phylogenetic classification of the land plants to accompany APG III. *Botanical Journal of the Linnean Society*, **161**, 122- 127.
5. The Angiosperm Phylogenetic Group, (2009), *An update of the Angiosperm Phylogemy Group classification for the orders and families of flowering plants: APG III. Botanical Journal of the Linnean Society*, **161**, 105- 121.
6. William H., Corel J., (2006), Woody plants of Western African Forests: A guide to the forest trees, shrubs and Lianas from Senegal to Ghana, KEW Publishing, Royal Botanic Gardens, KEW, 212- 221.
7. Prance, G.T., (1972), Monograph of Dichapetalaceae, *Flora Neotropica* **10**, 1-84
8. Robert F. R., (1996), Plant Alkaloids: A guide to their Discovery and Distribution, *Food products press, Binghamton, New York*, 74.
9. Burkill H.M., (1985), The useful Plants of West Tropical Africa, 2nd Ed. **1**, Royal Botanical Gardens, KEW, 646- 649; 733- 776.
10. a/ Osei-Safo D., Chama M.A, Addae-Mensah I., Waibel Reiner, Asomaning W. A. and Oppong I.V., (2008), Dichapetalin M from *Dichapetalum madagascariensis, Phytochemistry Letters*, **1**, 147-150.

University of Ghana http://ugspace.ug.edu.gh

b/ Osei-Safo D., Chama M. A., Addae-Mensah I. and Waibel R., (2012). The Dichapetalins -Unique Cytotoxic Constituents of the Dichapetalaceae, Phytochemicals as Nutraceuticals - Global Approaches to Their Role in Nutrition and Health, Dr Venketeshwer Rao (Ed.), ISBN: 978-953-51-0203-8, InTech, Available from: http://www.intechopen.com/books/phytochemicals-as-nutraceuticals-global-approaches-to-their-role-in-nutrition-and-health/the-dichapetalins-unique-cytotoxic-constituents-of-the-Dichapetalaceae

11. Lewis H.W. and Elvin-Lewis M.P.F., (1977), Medical Botany: Plants affecting man's Health, *Wiley Interscience*, 39.

12. Breteler F. J., (1973), The African Dichapetalaceae, XVII, A Taxonomical Revision 4, Wageningen, 11.

13. Dear R. E. A. and Pattison F. L. M., (1962), The Synthesis of Toxic principle of *D. toxicarium, Journal of American Chemical Society*, **85**, 622-626.

14. Hall R.J., (1973), *D. barteri* as Potent Rodenticide, *New Phytologist*, **71**, 855- 860.

15. Kokwaro J. O., (1976), Medicinal Plants of East Africa, *East Africa Literature Bureau,* Kampala, Nairobi, Dar-es-Salaam, 83.

16. Hutchinson J., Dalziel J.M., (1958), Flora of West Tropical Africa, 2nd Ed., **1**(2), *Whitefriars Press*, London, 433.

17. Watt J. M., Breyer- Brandwijk M. G., (1962), Medicinal and Poisonous Plants of Southern and Eastern Africa, *Edinburgh*, 2nd ED., 375- 383.

18. Sam T. W., (1993), Bioactive Natural Products: Detection, Isolation and Structural Determination, *Taylor and Francis Group*, USA, 441- 456.

University of Ghana http://ugspace.ug.edu.gh

19. Pasteels J.M., Theuring C., Wittle L.and Hartmann T., (2003) Sequestration and metabolism of protoxic pyrrolizidine alkaloids by larvae of the leaf beetle *Platyphora boucardi* and their transfer via pupae into defensive secretions of adults. *Journal of Chemical Ecology,* **29**, 337-355.

20. Bisseleua B.D.H., Obeng-Ofori D. and Gbewonyo W.S.K., (2008) Bioactivities of crude extracts of the Candlewood, *Zanthoxylum xanthoxyloides* Lam. (Rutaceae) against the cowpea beetle, *Callosobruchus maculatus* (Walp). *International Journal of Biological and Chemical Sciences,* **2** (3), 316-323.

21. Throne J.E., (1994) Life history of immature maize weevils (Coleoptera: Curculionidae) on corn stored at constant temperature and relative humidities in the laboratory. *Environmental Entomology*, **23**, 1459- 1471.

22. Hill D.S., (2002) Pest of stored foodstuffs and their control. Kluwer Academic Publishers, Dordrecht, The Netherlands, Boston, MA, 476.

23. Mould H.A., (1973) Grain storage in Ghana. *Tropical Stored Products*, **25**, 44.

24. Casey Sclar D., (1994) Neem: Mode of Action of Compounds Present in Extracts and Formulations of *Azadirachta indica* Seeds and their Efficacy to Pests of Ornamental Plants and to Non-target Species. Retrieved from http://www. colostate.edu /Depts/Entomology/Courses/en570/Papers_1994/sclar.html.

25. Markham R.H., Bosque-Perez N.A., Borgemeister C. and Meikle W.G., (1994), Developing pest management strategies for the maize weevil, *Sitophilus zeamais* and the larger grain borer, *Prostephanus truncatus* in the humid and sub-humid tropics. *FAO plant protection Bulletin,* 42.

University of Ghana http://ugspace.ug.edu.gh

26. White N.D.G. and Leesch J.G., (1996), Chemical control, *Integrated management of insects in stored products*, 287-330.
27. Obeng-Ofori D., Reichmuth C.H., Bekele J. and Hassanali A., (1997), Biological activity of 1,8-cineole, a major component of essential oil of *Ocimum kenyense (Ayobangira)* against stored product pests. *Journal of Applied Entomology*, **121**, 237-243.
28. Champ B.R. and Dyte C.E., (1977), FAO Global survey of pesticide susceptibility of stored grain pests. *FAO Plant Protection Bulletin,* **25**, 49-67.
29. Subramanyam B. and Hagstrum D.W., (1996), Resistance measurement and management, *Integrated Management of Insects in Stored Products*, 331-397.
30. Belmain S.R., Neal G.E., Ray D. and Golob P., (2001), Insecticidal and vertebrate toxicity associated with ethnobotanicals used as post-harvest protectants in Ghana. *Food Chemical Toxicology*, **39**, 287-291.
31. Smith R.H., (1986), Oviposition, competition and population dynamics in storage insects. *Proceeding of the 4th International Working Conference on stored-product protection*, Tel Aviv, Israel, September, 427- 433.
32. Honek A., (1993), Intraspecific variation in body size and fecundity of insects: A general relationship. *Oikos,* **67**, 483- 492.
33. Stejskal V. and Kucerowa Z., (1996), The effect of grain size on the biology of *Sitophilus granaries* L. (Coleoptera: Curculionidae). Oviposition, distribution of eggs and adult emergence. *Journal of Applied Entomology,* **120**, 143- 146.
34. Horton J., (2003), Human gastrointestinal helminth infections: Are they now neglected diseases? *Trends in Parasitol,* **19**, 527-531.

University of Ghana http://ugspace.ug.edu.gh

35. De Silva N.R., Brooker S., Hotez P.J., Montresor A., Engels E. and Savioli L., (2003), STH infections: Updating the global picture. *Trends in Parasitol,* **19**, 547-551.

36. Ehrenberg J.P. and Ault S.K., (2005), Neglected diseases of neglected populations: Thinking to reshape the determinants of health in Latin America and the Caribbean. *BMC Public Health,* **5**, 119, doi: 10.1186/1471-2458-5-119.

37. Hotez P.J., Molyneux D.H., Fenwick A., Kumaresan J., Sachs S.E., Sachs J.D. and Savioli L.. (2007). Control of neglected tropical diseases. *New England Journal of Medicine,* **357**, 1018-1027.

38. WHO, (2002-2005), WHO Traditional Medicine Strategy. WHO/EDM/TRM/2002.1, 66

39. Sangster N.C., (2008), Anthelmintic resistance: past, present and future. *International Journal of Parasitol,* **29**, 115-124

40. Alfredo-Skrebsky C.A.S., Toscan G., Camillo G., Sangioni L.A., Ribas H.O. and Vogel F.S.F., (2010), Multiple resistance of gastrointestinal nematodes to nine different drugs in a sheep flock in Southern Brazil, *Veternary Parasitol,* **173**, 157-160.

41. Darbah V.F., (1994), Chemical Constituents and Biological Activity of the Stem Bark of *Dichapetalum madagascariense* Poir, M.Phil (Chemistry), Thesis submitted to the University of Ghana.

42. Chama M.A., (2007), Chemical Investigation of Two Ghanaian Medicinal Plants- *Dichapetalum madagascariense and Scoparia dulcis*, Ph.D. Thesis submitted to the University of Ghana

43. Meyer M., O'Hagan D., (1992), Rare fluorinated natural products, *Chemistry in Britain*, **28**(9), 785-788.

University of Ghana http://ugspace.ug.edu.gh

44. Addae-Mensah I., Achenbach H., Asunka S. A., Waibel R. and Oppong I. V., (1996), The Dichapetalins – A new class of triterpenoids, *Phytochemistry,* Elsevier Science Ltd, **43** (3), 649 – 656.

45. Anon, A., Hydrocyanic Acid and Two Resins from *D. cymosum*, Bulletin Imp. Inst., London, 14 (cited in 7).

46. Power F.B. and Tutin, F., (1906), The Toxic Property of *Chailletia toxicaria*, *Journal* of *American Chemical Society*, **28**, 1170.

47. a/ Marias, J. S. C., (1943), Monofluoroacetic Acid, The Toxic Principle of *D. cymosum* (Hook) Engl. "Gifblaar," *Onderstepoort Journal of Veternary Science Animal* Ind., **18**, 203, b/ Marias, J. S. C., (1944), Monofluoroacetic Acid, The Toxic Principle of *D. cymosum* (Hook) Engl. "Gifblaar," *Onderstepoort Journal of Veternary Science Animal* Ind., **20**, 67

48. Ward P. F. V., Peter R. A and Peter R. A., (1964), Fluoro Fatty Acids in the Seeds of *Dichapetalum toxicarium*, *Nature*, **201**, 611

49. Eloff J.N. and Grobbelaar N., (1969), *Photochemistry*,**8**, 2201

50. Myers A.G., Barbay J.K., Zhong B., (2001), Asymmetric synthesis of chiral organofluorine compounds: Use of nonracemic fluoroiodoacetic acid as a practical electrophile and its application to the synthesis of monofluorohydroxyethylene dipeptide isosteres within a novel series of HIV protease inhibitors, *Journal of American Chemical Society*, **123** (30), 7207-7219.

51. Kellerman T.S., Naude T.W., Fourie N., (1996), The distribution, diagnosis and estimated economic impact of plant poisoning and mycotoxicocis in South Africa, *Onderstepoort Journal of Veterinary Research* **63** (2), 65-90.

University of Ghana http://ugspace.ug.edu.gh

52. Peter, R. A., Hall, R. J., (1960), Fluorine Compounds in some species of *Dichapetalum*, *Nature*, **187**, 573 – 575.

53. O'Hagan D., Harper D.B., Pery R., Lock J. M., Meyer J. J. M., Dasaradhi L. and Hamilton J.T.G., (1993), High levels of Monofluoroacetate in *Dichapetalum braunii*, *Phytochemistry*, **33** (5), 1043-1045

54. Nwude N., Parson L.E and Aduadi A.O., (1977), Acute toxicity of the leaves and extract of *D. barteri* in Mice, *Toxicology*, **7**, 23-29.

55. Addae-Mensah I., Adu-Kumi S., Waibel R., Asunka S. A. and Oppong I. V., (2007), A novel D: A- friedooleanane triterpenoid and other constituents of the stem bark of *D. barteri* Engl. *Archives of Organic Chemistry*, IX, 71 – 79.

56. Adu-Kumi S., (1997), Phytochemical and Bioactivity studies on the chemical constituents of the stem bark of *D. barteri* Engl. MPhil (Chemistry), Thesis submitted to the University of Ghana.

57. Osei-Frimpong A., (1996), Isolation and characterization of the chemical constituents of the roots of *D. barteri* Engl. MPhil (Chemistry), Thesis submitted to the University of Ghana.

58. Asunka S. A., (1994), Phytochemical and Pharmacological Investigations of the roots of *Dichapetalum madagascariense* Poir, MPhil (Chemistry) Thesis submitted to the University of Ghana.

59. Achenbach H., Asunka S. A., Waibel R., Addae-Mensah I. and Oppong I. V., (1995), Dichapetalin A, A novel plant constituent from *Dichapetalum madagascariense*, *Natural Product Letters*, **7**, 93 – 100.

University of Ghana http://ugspace.ug.edu.gh

60. Weckert E., Mattern G., Addae-Mensah I., Waibel R. and Achenbach H., (1996), The Absolute Configuration of Dichapetalin A, *Phytochemistry*, Elsevier Science Ltd, **43** (3), 657 – 660.

61. Xu H., Lv M., Tian X., (2009), A review on hemisynthesis, biosynthesis, biological activities, mode of action, and structure-activity relationship of Podophyllotoxin: 2003-2007, *Current medicinal chemistry* **16** (3), 327-349

62. a/ Maria D.P., Manel R. and Isabel S., (2005), Synthesis and biological activity of new class of deoxygenated anticancer agent. *Anti-cancer agents' medicinal chemistry*, **5**, 215-237.

b/ Gordaliza M., García P. A., Miguel del Corral J. M., Castro M. A., Gómez-Zurita M. A., (2004), Podophyllotoxin: distribution, sources, applications and new cytotoxic derivatives. *Toxicon*, **44**, 441-459.

63. Lagnika L., Anago E., Sanni A., (2011), Screening for antibacterial, antioxidant activity and toxicity of some medicinal plants used in Benin folkloric medicine, *Journal of Medicinal Plants Research* **5** (5), 773-777.

64. Fang, L., Ito, A., Chai, H., Pezzuto, J. M., and Kinghorn, A. D., (2006), Cytotoxic Constituents from the stem bark of *Dichapetalum gelonioides*, *Journal of Natural Products*, **69**, 332-337.

65. Long C., Aussagues Y., Molinier N., Marcourt L., Vendier L., Samson A., Poughon V., Mutiso P.B.C., Ausseil F., Sautel F., Arimondo P.B., Massiot G., (2013), Dichapetalins from *Dichapetalum* species and their cytotoxic properties, *Phytochemistry*. http://dx.doi.org/10.1016/j.phytochem.2013.03.023.

66. Tuchinda P., Kornsakulkarn J., Pohmakotr M., Kongsaeree P. Prabpai S. Yoosook C., Kasisit J., Napaswad C., Sophasan S., Reutrakul V., (2008), Dichapetalin-type triterpenoids and

University of Ghana http://ugspace.ug.edu.gh

lignans from the aerial parts of *Phyllanthus acutissima*, *Journal of Natural products*, **71** (4), 655-663.

67. Nottingham S.F., Wilson D.D., Kays S.J., and Severson R.F., (1987), Feeding and Oviposition preferences of the sweet potato weevil on the outer periderm and exposed inner core of storage roots of selected sweet potato cultivars, *Entomology Experimental Application*, **45** (3), 271-275.
68. Wilson D.D., Severson R.F., Son K.C. and Kays S.J., (1988), Oviposition Stimulant in sweet potato periderm for the sweet potato weevil, *Cylas formicarius elegantulus* (Coleoptera: Curculionidae), *Environmental Entomology*, **17** (4), 691-693.
69. Letellier C., Haubruge E. and Gaspar C., (1995), Biological activity of fenoxycarb against *Sitophilus zeamais* Motsch. (Coleoptera: Curculionidae). *Journal of Stored Product Research,* **31** (1), 37-42.
70. Arannilewa S.T. and Odeyemi O.O., (2007), Insecticidal Evaluation of some plant materials as grain protectants against the maize weevil, *S. zeamais* Mots. (Coleoptera: Curchionidae). *Agricultural Journal*, **2** (1), 155-159.
71. Bisseleua B.D.H., Obeng-Ofori D. and Gbewonyo W.S.K., (2008), Bioactivities of crude extracts of the Candlewood, *Zanthoxylum xanthoxyloides* Lam. (Rutaceae) against the cowpea beetle, *Callosobruchus maculatus* (Walp). *International Journal of Biological and Chemical Sciences*, **2** (3), 316-323.
72. Udo I.O., Epidi T.T. and Osakwe J.A., (2009), Susceptibility of *Sitophilus zeamais* Mots. and *Callosobruchus maculatus* F. to plant parts of *Ricinodendron heudelotii*. *Journal of Plant Protection Research,* **49** (4), 411-415.

University of Ghana http://ugspace.ug.edu.gh

73. Udo I.O., (2011), Potentials of *Zanthoxylum xanthoxyloides* (Lam.) for the control of stored product insect pests. *Journal of Stored Products and Post-harvest Research,* **2** (3), 40-44.

74. Nukenine E.N., Tchiegang C., Mekouo A.A.T., Tofel K.H., Adarkwah C., Obeng-Ofori D. and Adler C., (2011), Efficacy of Calneem derived from Ghanaian neem seed oils from two localities in Cameroom against *Sitophilus zeamais* (Coleoptera: Curculionidae) on maize. *International Journal of Tropical Insect Science*, **31** (4), 225-234.

75. Lale N.E.S. and Abdulrahman H.T., (1999), Evaluation of neem (*Azadirachta indica* A. Juss) seed oil obtained by different methods and neem powder for the management of *Callosobruchus maculatus* (Coleoptera: Bruchidae) in stored cowpea. *Journal of Stored Products Research,* **35**, 135-143.

76. Maribet L. Parugrug and Aurea C. Roxas, (2008), Insecticidal action of five plants against maize weevil, *Sitophilus zeamais* Motsch. (Coleoptera: Curculionidae), *KMITL Science Technology Journal*, **8** (1), 24-38.

77. Obeng-Ofori D. and Akuamoah R.K., (2000), Biological effects of plant extracts against the rice weevil *Sitophilus oryzae* in stored maize. *Journal of the Ghana Science Association,* **2** (2), 62-69

78. Longstaff B.C., (1981), Biology of the grain pest species of the genus *Sitophilus* (Coleoptera: Curculionidae): A critical review. *Protection Ecology,* **2**, 83-130.

79. Hwang J.S., Hsieh F.K. and Kung K.S., (1983), Influence of temperature and relative humidity on the development and reproduction of the *Sitophilus zeamais* Motsch. *Plant Products Bulletin* (Taiwan), **24**, 41-52.

80. Awasthi S. and Bundy D., (2007), Intestinal nematode infection and anaemia in developing countries. *British Medical Journal,* **334**, 1065-1066.

University of Ghana http://ugspace.ug.edu.gh

81. Chan M.S., (1997), The global burden of intestinal nematode infections – fifty years on. *Parasitology of Today,* **13**, 438-443.

82. Hotez P.J. and Kamath A., (2009), Neglected tropical diseases in sub-Saharan Africa: review of their prevalence, distribution, and disease burden. *PLoS neglected tropical diseases,* **3**, e412.

83. Brooker S., Clement A.C. and Bundy D.A., (2006), Global epidemiology, ecology and control of STH infections. *Advance Parasitol,* **62**, 221-261.

84. Hotez P.J., Bethony J., Bottazzi M.E., Brooker S., Loukas A. and Xiao S.H., (2004), Hookworm infection. *New England Journal of Medicine,* **351**, 799-807.

85. Brooker S., Bethony J. and Hotez P.J., (2004), Human hookworm infection in the 21st century, *Advances in parasitology*, 58.

86. Baidoo S.E., Tay S.C.K., Obiri-Danso K. and Abruquah H.H., (2010), Intestinal helminth infection and anaemia during pregnancy: A community based study in Ghana, *Journal of Bacteriology Research,* **2** (2), 9-13

87. Soares Magalhaes R.J. Biritwum N.K. Gyapong J.O., Brooker S. and Zhang Y., (2011), Mapping helminth co-infection and co-intensity: Geostatistical prediction in Ghana. *PLoS Neglected tropical diseases,* **5**(6), e/200. doi:10.1371/journal.pntd.0001200.

88. Hotez P.J., Mistry N., Rubinstein J. and Sachs J.D., (2011), Integrating neglected tropical diseases into AIDS, tuberculosis, and malaria control. *N. England Journal of Medicine,* **364**, 2086-2089.

89. Bethony J., Brooker S., Albonico M., Geiger S.M., Loukas A., Diemert D. and Hotez P.J., (2006), STH infections: ascariasis, trichuriasis and hookworm, *Lancet*, **367**, 1521-1532

90. WHO, (2009), Action against worms. Newletter Issue 13/http//www.who.int/neglected_

University of Ghana http://ugspace.ug.edu.gh

diseases/preventive_chemotherapy/ Newsletter 13_En.pdf

91. WHO, (1994), Bench Aids for the Diagnosis of intestinal parasites.
92. Coles G.C., Bauer C. Borgsteede F.H., Geerts S., Klei T.R., Taylor M.A. and Waller P.J., (1992), World Association for the Advancement of Veterinary Parasitology (WAAVP). Methods for the detection of anthelmintic resistance in nematodes of veterinary importance. *Veterinary Parasitology,* **44**, 35-44
93. Stepek G., Buttle D.J., Duce I.R. and Behnke J.M., (2006), Human gastrointestinal nematode infections: Are new control methods required? *International Journal of Experimental Pathology,* **87**, 325-341.
94. De Clercq D., Sacko M., Behnke J., Gilbert F., Dorny P. and Vercruysse J., (1997), Failure of mebendazole in treatment of human hookworm infections in the southern region of Mali. *American Journal of Tropical medicine and Hygiene,* **57**, 25-30.
95. Sacko M., De Clercq D., Behnke J.M., Gilbert F.S., Dorny P. and Vercruysse J., (1999), Comparison of the efficacy of mebendazole, albendazole and pyrantel in treatment of human hookworm infections in the southern region of Mali, West Africa. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* **93**, 195-203.
96. Reynoldson J.A., Behnke J.M., Pallant L.J., Macnish M.G., Gilbert F., Giles S., Spargo R.J. and Thompson R.C., (1997), Failure of pyrantel in treatment of human hookworm infections (*Ancylostoma duodenale*) in the Kimberley region of northwest Australia. *Acta tropica,* **68**, 301-312.
97. Albonico M., (2003), Methods to sustain drug efficacy in helminth control programmes, *Acta tropica,* **86**, 233-242.

University of Ghana http://ugspace.ug.edu.gh

98. Albonico M., Wright V., Ramsan M., Haji H.J., Taylor M., Savioli L. and Bickle Q., (2005), Development of the Egg Hatch Assay for detection of anthelmintic resistance in human hookworm. *International Journal for Parasitology,* **35**, 803-811.

99. Flohr C., Tuyen L.N., Lewis S., Minh T.T., Campbell J., Britton J., Williams H., Hien T.T., Farrar J. and Quinnell R.J., (2007), Low efficacy of Mebendazole against hookworm in Vietnam: Two randomized controlled trials, *American Journal of Tropical Medicine and Hygiene,* **76**, 732-736.

100. Kotze A.C., Coleman G.T., Mai A. McCarthy J.S., (2005), Field evaluation of anthelmintic drug sensitivity using *in vitro* Egg Hatch and Larval mortality assays with *Necator americanus* recovered from human clinical isolates. *International Journal for Parasitology,* **35**, 445-453.

101. Akerele O., (1990), Medicinal Plants in traditional medicine. In economic and medicinal research. Plants and Traditional Medicine (Eds. Farnsworth N.R. and Wagner H.). Academic Press Ltd., London.

102. Savioli L.D., Bundy D.A.P. and Tompkins A., (1992), Intestinal parasitic infections: a soluble public health problem. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **86**, 353-354.

103. Githiori J.B., Höglund J., Waller P.J. and Baker R.L., (2003), Evaluation of anthelmintic properties of extracts from some plants used as livestock dewormers by pastoralist and smallholder farmers in Kenya against *Heligmosomoides polygyrus* infections in mice. *Veternary Parasitol,* **118** (3-4), 215-226.

104. Salifou S., Daga D.F., Attindehou S., Deguenon R. and Biaou C.F., (2013), Antiparasitic effects on the water extract from *Chenopodium ambrosioides* L. (Chenopodiaceae) against

University of Ghana http://ugspace.ug.edu.gh

some gastrointestinal nematodes in West African long legged goats, *Journal of Parasitology and Vector Biology,* **5** (2), 13-16.

105. Rahayu U., Nurhasniza K., Mohd A.S., Mawardi R., Ahmad B.A., (2013), Phenolic contents, antioxidant and cytotoxic activities of *Elaeocarpus floribundus* Blume. *Pakistan Journal of Pharmaceutical Sciences*, **26** (2), 245-250

106. Zhi-Jun W., Lei Shan, Yun-Heng S., Jian T., Wei-Dong Z., (2010), Chemical constituents from *Inula cappa*, *Chemistry of natural compounds*, **46** (2), 298-300.

107. Kundu J.K., Rauf A.S.S., Nazmul H.M., Hassan C.M., Rashid M.A.,(2000), Antitumor activity of epifriedelanol from *Vitis trifolia, Fitoterapia* **7**, 577-579.

108. Chau V. M., Phan V.K., Hoang T.H., Nguyen T.D., Nguyen H.N., Jung J.L., Young H.K., (2005), Chemical investigations and biological studies of *Mallotus apelta*, *Journal of chemistry* **43** (2), 235-239.

109. Pateh U.U., Haruna A.K., (2009), Isolation of stigmasterol, β-sitosterol and 2-hydroxyhexadecanoic acid methyl ester from rhizome of *Stylochiton lancifolius*, *Nigerian Journal of Pharmaceutical Sciences***, 8** (1), 19-25

110. Patra A. , Murthy P.N., (2010), Isolation and characterization of stigmast-5-en-3β-ol (β-sitosterol) from the leaves of *Hygrophila spinosa* T. Anders, *International Journal of pharmaceutical sciences and Research* **1** (2), 95-100.

111. Shukranul M., Ikram M.S., (2012), Chemical constituents of *Garcinia prainiana, Sains Malaysiana* **41** (5), 585-590.

112. Harborne J.B., (2001), Twenty-five years of chemical ecology, *Natural product reports*, **18**, 361-379.

University of Ghana http://ugspace.ug.edu.gh

113. Yasukawa K., Takido M., Matsumoto T., Takeuchi M., and Nakagawa S., (1991), Sterol and triterpene derivatives from plants inhibit the effects of tumour promoter and sitosterol and betulinic acid inhibit tumour formation in mouse skin two-stage carcinogenesis. *Oncology* **41**, 72-76.

114. Raicht R., Cohen B., and Fazzini E., (1980), Protective effect of plant sterols against chemically induced co- ion tumours in rats, *Cancer Research*, **40**, 403-405.

115. Kasahara Y., Kumaki K., Katagiri S., Yasukawa K., Yamanouchi S., Takido M., Akihisa T., and Tamuta T., (1994), Carthami flos extract and its component, stigmasterol, inhibit tumour promotion in mouse skin two-stage carcinogenesis. *Phytotherapy Res*earch, **68**, 327-331.

116. Akihisa T., Ogihara J., Kato J., Yasukawa K., Ukiya M., Yamanouchi S., and Oishi K.., (2001), Inhibitory effects of triterpenoids and sterols on human immunodeficiency virus-1 reverse transcriptase. *Lipids* **36**, 507-512

117. Gomez M., Saenz M., Garcia M., and Farnandez M., (1999), Study on topical anti-inflammatory activity of *Achillea ageratum* on chronic and acute inflammation, *Z. Naturforsch* **55c**, 937-941.

118. Escobedo-Martinez C., Concepcion L.M., Hernandez-Ortega S., Villarreal M.L., Gnecco D., Enriquez R.G., Reynolds W., (2012), ^{1}H and ^{13}C-NMR Characterization of new cycloartane triterpenes from Mangifera indica, *Magnetic Resonance in Chemistry*, **50**, 52-57.

119. Hisham A., Kumar J. G., Fujimoto Y., Hara N., (1995), Salacianone and salacianol- two triterpenes from *Salacia beddomei, Phytochemistry* **40**, 1227-1231.

University of Ghana http://ugspace.ug.edu.gh

120. Heilbron I., Bunbury H.M., (1953), Dictionary of Organic compounds, London Eyre and Spottiswoode, **2**, 565.

121. FAO, (1985), Prevention of post-harvest food losses, Training series, **10** (122), Rome, *Food and Agricultural Organization of the United Nations*, 120.

122. Ogunwolu O. and Idowu O., (1994), Potential of powdered *Zanthoxylum xanthoxyloides* (Rutaceae) root bark and *Azadirachta indica* (Maliaceae) seed for control of cowpea seed bruchid, *Callosobruchus maculatus* in Nigeria. *Journal of African Zoology*, **108** (6), 521-528.

123. Irigaray F.J., Moreno-Grijaba F., Marco V., Perez-Moreno I., (2010), Acute and reproductive effects of Align, an insecticide containing azadirachtin, on the grape berry moth, Lobesia botrana, *Journal of insect science*, **10** (33), 11.

124. Sahayara K. and Kalida S., (2011), Evaluation of nymphicidal and ovicidal effect of a seaweed *Padina pavonica* (Lim.) on cotton pest, *Dysdercus cingulatus* (Feb.), Indian Journal of Geo-Marine sciences, 40 (1), 125-129.

125. Peach K. and Tracy M. V., (1956), Modern methods of plant analysis, *Springer-Verlag, Berlin*, 40.

126. Mann F.G. and Saunders B.C., (1972), Practical Organic Chemistry, 4th ED., Longman, *Lowe and Brydone Ltd., London*, 523.

127. Kirchner G.J., (1967), Techniques of Organic Chemistry, **XII** (Thin Layer Chromatography), *Interscience Publishers*, *New York*, 153.

128. Stahl E., (1967), Thin Layer Chromatography, a Laboratory Handbook, *Academic Press Inc.*, *New York*, 153.