KWAME NKRUMAH UNIVERSITY OF SCIENCE AND

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BIOACTIVE ANTI-LEISHMANIAL AGENTS: PROSPECTS OF THE GHANAIAN BIODIVERSITY

BY

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BIOACTIVE ANTI-LEISHMANIAL AGENTS: PROSPECTS OF THE GHANAIAN BIODIVERSITY

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By

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DECLARATION

The experimental work described in this thesis was carried out at the Department of Pharmacognosy, KNUST and the Department of Biomedical and Forensic Science, University of Cape Coast. This work has not been submitted for any other degree.

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DEDICATION

This work is dedicated to my late grandparents Nana Asafo IV alias Mr Thomas Ekeyeboe Cudjoe and Madam Hannah Aya Ekeyeboe Cudjoe.

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My sincere thanks go to my supervisors Profs. Kofi Annan and Rita Akosua Dickson, for supervising this research. Their continual advice and encouragement have been very helpful. May the Almighty God bless you. I am greatly indebted to Prof. Abraham Yeboah Mensah for his support as Head of the Pharmacognosy Department. I am most grateful to Dr Isaac Kingsley Amponsah for his advice and encouragement throughout this research. I express my sincere appreciations to the teaching staff and my colleague graduate students at the Department of Pharmacognosy, KNUST, for creating the much needed and pleasant working atmosphere during my stay. I thank you all for your warm companionship. I also express my gratitude to the technicians of the Departments of Pharmacognosy, Pharmacology and Pharmaceutical microbiology for their technical assistance. Many thanks to Dr. Solomon Habtemariam, Pharmacognosy Research Laboratories, Medway School of Science, University of Greenwich, for generously running and providing the NMR spectra of the isolates. The technical assistance of the staff at the Greenwich NMR Laboratory and their willingness to allow access to their state-of-the-art, Energy and Physical Science Research Council (EPSRC) National Mass Spectrometry Facilities at Swansea University (Singleton Park, Swansea, UK) are greatly appreciated. I would like to say a big thank you to my dear and lovely wife Anita for her understanding and never failing support through out this work. I am also very grateful to God for His mercies and protection. Finally, my deepest gratitude goes to my entire family; my mother, Mrs. Monique Assuah Cophie and my father, Mr. Alfred

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ABSTRACT

Leishmaniasis, one of the neglected tropical diseases, is currently a significant health problem in North Africa with a rising concern in West Africa because of co-infection with the Human Immunodeficiency Virus (HIV). The aim of this research is to evaluate the anti-leishmanial, anti-inflammatory and antioxidant activities of methanol extracts of four (4) indigenous plants namely root bark of *Erythrophleum ivorense* and the stem barks of *Coelocaryon oxycarpum, Omphalocarpum ahia* and *Anthostema aubryanum* and determine the chemical constituents which confer on the species their purported activities in folklore medicine. These plants are traditionally used in Ghana to treat parasitic infections as well as pain and inflammation. *In vitro* anti-leishmanial potential of the selected plants was evaluated using direct counting assay of promastigotes of *Leishmania donovani*. Amphotericin B was used as positive control. Anti-inflammatory activity of the extracts and compounds were evaluated using the chick carrageenan model with diclofenac and dexamethasone as standards. The antioxidant potential of the extracts and compounds were also evaluated using the DPPH scavenging assay. *E. ivorense* showed the highest anti-leishmanial activity (IC_{50} = 14.10 μg/mL), followed by *A. aubryanum* (IC_{50} = 23.90 μg/mL), *C. oxycarpum* (IC_{50} = 31.31 μg/mL) and *O. ahia* (IC_{50} = 124.04 μg/mL) respectively. *E. ivorense* thus showed considerable activity compared to amphotericin B (IC_{50} = 2.4 μg/mL). Fractionation of *E. ivorense* afforded a moderately active ethyl acetate fraction and a significantly methanol fraction active (IC_{50} = 2.97 μg/mL). *E. ivorense* also showing the highest anti-inflammatory (ED_{50} = 16.91 mg/kg

body weight) and antioxidant (IC_{50} = 1.11 μg/mL) activities, followed by *C. oxycarpum*, *O. ahia* and *A. aubryanum* respectively. Investigation of the pet-ether, ethyl acetate and methanol fractions of *E. ivorense* revealed the ethyl acetate fraction to be the most active. Further fractionation of the ethyl acetate fraction led to an isolation of the novel cassane diterpene erythroivorensin as well as the flavanone eriodictyol and the triterpene betulinic acid. The rank order of leishmanicidal potency of the compounds was erythroivorensin (IC_{50} = 29.10 μg/mL) > eriodictyol (IC_{50} = 103.80 μg/mL) > betulinic acid (IC_{50} = 1000 μg/mL). These compcounds may be working synergistically to produce the observed anti-leishmanial activity of the ethyl acetate fraction. The anti-inflammatory activity of betulinic acid (ED_{50} = 4.367±1.333 mg/kg body weight), the most active anti-inflammatory compound, was comparable to that of diclofenac (ED_{50}= 3.74±0.333 mg/kg body weight). This was followed by erythroivorensin (ED_{50} = 18.66±0.667 mg/kg body weight) and eriodictyol (ED_{50} = 56.25±0.133 mg/kg body weight). With respect to the antioxidant activity of the compounds, eriodictyol was the most active (IC_{50} = 21.06 μg/mL), followed by betulinic acid (IC_{50}= 33.07 μg/mL) and erythroivorensin (IC_{50} = 35.78 μg/mL) respectively. The present studies have shown that extracts of *E. ivorense* have considerable anti-leishmanial activity and could be used for the treatment of leishmaniasis after the establishment of its toxicity profile. The novel compound erythroivorensin possesses anti-leishmanial effects and has considerable anti-inflammatory properties. To the best of my knowledge, eriodictyol and betulinic acid are isolated for the first time in *Erythrophleum ivorense.*

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ABBREVIATIONS

^{13}CNMR	Carbon nuclear magnetic resonance
^{1}HNMR	Proton nuclear magnetic resonance
2D NMR	Two dimensional nuclear magnetic resonance
AAE	Ascorbic acid equivalent
ANOVA	Analysis of variance
ASTM	American Society for Testing and Materials
AUC	Area under curve
BDH	British Drug Houses
BHA	Butylhydroxyanisole
BHT	Butyl hydroxy toluene
CAA	Cellular antioxidant activity
CCDC	Cambridge Crystographic Data Centre
$CDCl_3$	Deuterated chloroform
CI	Chemical ionisation
COSY	Correlation spectroscopy
COX-2	Cyclooxygenase-2
CP	Cross-polarisation
D_2O/CD_3COOD	Deuterated acetic acid
$D_2O/CDCOOD$	Deuterated fomic acid
D_2O/DCl	Deuterium chloride solution

DFQ –COSY	Double quatum filtered Correlation spectroscopy
DMPD	N, N-dimethyl-p-phenyldiamine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2, 2-Diphenyl-1-picrylhydrazyl
DRS	Diffuse reflectance spectroscopy
EAA	Extract of *Anthostema aubryanum*
EC50	Effective concentratin at 50%
ECL	Enhanced chemilluminescence
ECO	Extract of *Coelocaryon oxycarpum*
EEI	Extract of *Erythrophleum ivorense*
EI	Electron impact
ELISA	Enzyme-linked immunosorbent assay
EOA	Extract of *Omphalocarpum ahia*
EPSRC	Engineering and Physical Research council
ERK1/2	Extracellular signing-regulated kinase 1and 2
ESI	Electrospray ionisation
ET	Electron transfer method
EtEI	Ethyl acetate fraction of *Erythrophleum ivorense*
FBS	Fetal bovine serum
FRAP	Ferric decourisation antioxidant power
FTIR	Fourier transform Infra red

GAFCO	Ghana Agro Food Co. Ltd
H_2O_2	Hydrogen peroxide
HAT	Hydrogen Atom Transfer methods
HIV	Human immunodeficiency syndrome
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple-Quantum Correlation
HNE	Hydroxynonrnal
HO	Hydrogen superoxide
HSQC	Heteronuclear single quatum coherence
IC_{50}	Inhibition concentration at 50%
IL	Interleukins
iNOS	Inducible nitric oxide synthase
IOC	Inhibited oxygen uptake
IR	Infra red
JNK	Jun Nterminal kinase
KNUST	Kwame Nkrumah University of Science and Technology
LPIC	Lipid peroxidation inhibition capacity
LPS	Lipopolysaccharide
LTB4	Leukotries B4
MALDI	Matrix assisted laser decomposition ionizsation
MAPK	Mitogen actvated protein kinase
MAS	Magicangle spinning

MBC	Minimum bacterial concentration
MDA	Malondialdehyde
MEI	Methanol fraction of *Erythrophleum ivorense*
MIC	Minimum inhibitory concentory
MS	Mass spectroscopy
N_2O_3	Dinitrogen trioxide
NF-Kb	Nuclear transcription factor-kB
NMR	Nuclear magnetic resonance
NO	Nitrous oxide
NO_2	Nitrogen dioxide
NOESY	Nuclear overhauser effect spectroscopy
O_2^-	Peroxide
ORAC	Oxygen radical absorbance capacity
PAF	Platelet activation factor
PCD	Programme cell death
PCD	Programme cell death
PEI	Petroleum ether fraction of *Erythrophleum ivorense*
pH	Hydrogen ion concentration
***p*-NDA**p-	Butrisidunethyl aniline
RNS	Reactive nitrogen species
RO	Alkoxyl
ROO	Peroxyl

ROS	Reactive oxygen species
SIM	Selective ion monitoring
SIR	Single ion recording
T $[SH]_2$	Trypanothione-bis-(glutathionyl) spermidine
TAC	Total antioxidant capacity
TEAC	Trolex equivalent antioxidant capacity
TLC	Thin layer chromatography
TNF-α	Tumor necrosis factor TNF-alpha
TOSC	Total oxidant scavenging capacity
TRAP	Trapping antioxidant parameter
TryS	Trypanothione synthetase
UK	United Kingdom
USA	United State of America
UV-VIS	Ultra violet-visible
VOA	Voice of America
WHO	World Health Organisation
XAS	X-ray absorption spectroscopy
XES	X-ray emission spectroscopy
XPS	X-ray photoelectron spectroscopy

Chapter 1
INTRODUCTION

1.1 GENERAL INTRODUCTION

There are about 21,000 medicinal plants in the world forest biodiversity. These are concentrated in the global biodiversity hot spots' such as the Amazon rain forest of South America, the Eastern Himalayas and Western Ghats/Sahyadri in Southern India and East Africa. In tropical Africa, for example, more than 4,000 plants species are used for medicinal purposes and 50,000 tons of medicinal plants are consumed annually (Esenam *et al*., 2007). These medicinal plants are used to help meet some of the primary health care needs of people as majority of the world's population, in developing countries, rely on medicines derived from plants for the management of diseases (Ziblim *et al*., 2013). However, in recent times, the ascending trend in the acceptance and use of herbal medicines has seen them being embraced in many developed countries.

In most African countries, the first line of treatment for about 60% of children with fever resulting from malaria is the use of herbal medicines (WHO, 2013). This is particularly true in West Africa where about 50% of the population in the region lack access to essential medicines (Kirigia and Wambebe, 2006). Modern health services are not available and in some instances distant away from the communities. The road network system linking some of these communities to the health facilities may be inaccessible, especially during rainy seasons. These and other factors make it difficult to access quality health care and undoubtedly make traditional medicine, especially herbal medicine, an obvious choice for the rural people in West Africa.

In Ghana, about 80% of rural dwellers rely on medicinal plants from the wild, as their main source of medicine (Falconer, 1994). Primary healthcare is more accessible in the urban centres than in rural communities. The number of patients per public doctor varies between 6200 in Accra to 42,200 in remote rural areas (Van den Boom *et al*., 2008) with majority relying on herbal medicines (WHO, 2003). Reasons assigned to the high patronage of herbal medicines include their availability, affordability and belief of their superior efficacy and safety compared to allopathic medicine (Ziblim *et al*., 2013).

Despite the surge in the use of herbal medicines in Ghana, majority of these medicinal plants remain untested for their efficacy and safety. Modern allopathic medicine had its roots in herbal medicine and with Ghana's rich biodiversity; the development of medicines from leads provided by folklore is a very viable research strategy. The potential of these plants can be harnessed and integrated safely into mainstream primary health care (Gurib-Fakim, 2006).

Erythrophleum ivorense (A. Chev, Fabaceae), *Anthostema aubryanum (*Baill, Euphorbiaceae) *Coelocaryon oxycarpum* (Stapf, Myristicaceae) and *Omphalocarpum ahia* (A. Chev, Sapotaceae) are large evergreen trees widely distributed in tropical regions of West Africa ranging from Gambia to the Central African Republic and Gabon. They have the potential to grow up to 40 m high and are the source of hard-heavy wood; these plants are among the most exploited timber trees in West Africa. They are widely traded for their medicinal uses (Burkill, 1997). The stem barks and roots are particularly employed to treat convulsive disorders, emesis, pain, edema, smallpox, laxative and as anti-helminthic (Oliver-Bever, 1986). Although these plants are used extensively in folklore medicine,

they remain largely untested for their pharmacological activity and chemical principles, with the exception of *E. ivorense*. Ethanol extract of *E. ivorense* has been shown to display anti-convulsant, sedative and anti-microbial effects (Adu-Amoah *et al*., 2013: Wakeel *et al*., 2014).

1.2 JUSTIFICATION OF RESEARCH

Leishmaniasis is one of the 17 neglected tropical disease caused by a protozoan of the genus *Leishmania* transmitted by sandflies. According to a report by the World Health Organization (WHO, 2009), the disease is distributed across 88 countries causing serious health problems especially in developing countries. About 350 million people are at risk of contracting the disease and approximately 2 million new cases being reported each year (Vijay *et al*., 2011). Known treatments against human *Leishmania* include pentavalent antimony (sodium stiboghconate and meglumineantimoniate), Amphotericin B, pentamidine and paramomycin (Chappuis *et al*., 2007: Berman, 2015). These drugs have disadvantages of high cost, lack of oral formulation (Amphotericin B) can be used only "intravenously" or serious side effects. Common untoward side effects of Amphotericin B include injection site reactions such as pain, swelling, irritation, fever, shaking, chills, flushing, loss of appetite, weight loss, dizziness, vomiting, stomach upset, headache, shortness of breath, muscle or joint aches, and tingly feeling that require close monitoring of the patient (Berman, 2015). Also, rapid developments of resistance by the parasite have been reported (Ephros *et al*., 1997: Lira *et al*., 1999: Boelaert *et al*., 2002). Therefore, new treatments are needed to augment or replace currently available therapies. More recently,

emergence of co-infection of leishmaniasis with HIV has made the treatment even more challenging (Laguna, 2003).

Investigation of medicinal plants used in folklore medicine for the treatment of diseases can provide lead compounds, allowing the design and rational planning of new drugs, biomimetic synthesis development and the discovery of new therapeutic properties not yet attributed to known compounds (Hamburger *et al*., 1991). Different cultures in Africa, South America and Asia depend on the use of native plants for the treatment of leishmaniasis. In Africa folklore medicine, the treatment usually consists of the oral administration of the crude plant extracts for visceral leishmaniasis and as topical preparations of the corresponding extracts for the cutaneous form of the disease (Iwu *et al*, 1994). A number of plant-derived natural products have been reported to have leishmanicidal activity. These include diospyrin, a bis-naphthoquinone derivative isolated from the bark of *Diospyros montana* (Ebenaceae). It was reported to be active against promastigotes of *L. donovani* with a minimum inhibitory concentration (MIC) of 1 µg/mL (Hazra *et al.,* 1987). Similarly, Plumbagin, a naphthoquinone isolated from *Plumbago zeylanicum* (Annan *et al*., 2009), is reported to have an activity with effective concentration (EC_{50}) of 0.42 and 1.1 µg/mL against amastigotes of *L. donovani* and *L. amazonensis* respectively (Croft *et al.,* 1985). Berberine, an isoquinoline alkaloid, has been used clinically for the treatment of leishmaniasis for over 50 years and has demonstrated significant activity both *in vitro* and *in vivo* against several species of *Leishmania* parasite. Other natural products with leishmanicidal activities include the alkaloid isoguattouregidine, the diterpenoid phorbol ester 12-O-tetradecanoyl phorbol-13-

acetate and the saponins α-hederin, β-hederin and hedaragenin from the leaves of *Hedera helix* (Chan-Bacab *et al.*, 2001). Undoubtedly, medicinal plants represent a repository of potential leishmanicidal agents.

Immunological response to *Leishmania* parasite infection includes the release of pro-inflammatory mediators and reactive oxygen species such as nitric oxide (NO). Anti-inflammatory agents provide a balance between pro-inflammatory and anti-inflammatory cytokines which determines the outcome of the infection (López *et al.*, 2009). Thus plants with antioxidant and anti-inflammatory activities may be beneficial in the management of leishmaniasis.

It is therefore important to investigate the efficacy of these plants used in folklore medicine for the treatment of parasitic diseases particularly leishmaniasis to avert the menace it poses to the healthcare machinery of Ghana. To this end, the study seeks to evaluate the anti-leishmanial, anti-inflammatory and antioxidant activities of *Erythropleum ivorense, Anthostema aubryanum, Coelocaryon oxycarpum and Omphalocarpum ahia.*

1.3 AIMS AND OBJECTIVES

The aim of this project is to evaluate the anti-leishmanial, anti-inflammatory and antioxidant activities of methanol extracts of the root bark of *Erythrophleum ivorense* and the stem barks of *Anthostema aubryanum, Coelocaryon oxycarpum* and *Omphalocarpum ahia*.

The specific objectives of the research are:

i) To investigate the selected plants for their anti-leishmanial, anti-inflammatory and antioxidant activities.

ii) To fractionate and isolate the compounds responsible for the various biological activities using chromatographic methods.

iii) To elucidate the structures of the isolated compounds using spectroscopic methods.

iv) To evaluate the anti-leishmanial, anti-inflammatory and antioxidant effects of the isolated compounds.

Chapter 2

LITERATURE REVIEW

2.1 LEISHMANIASIS

Leishmaniasis is a parasitic disease caused by a protozoan of the genus *Leishmania* (Schuster and Sullivan, 2002). *Leishmania* belongs to the family of hemoflagellates known as Trypanosomatidae. Trypanosomatidae includes the genera *Leishmania* and *Trypanosoma* which are pathogens of humans and animals. The genus *Leishmania* is divided into the subgenera *Leishmania* and *Viannia* (Attar, 1997), which are further divided into seven main complexes, which can affect human health (Cheesbrough, 1997: Peters and Pasvol, 2002). Complexes of the subgenus *Leishmania* include *L. donovani*, *L. tropica*, *L. major*, *L. aethiopica* and *L. maxicana* where as *L. braziliensis* and *L. guyanesis* belong to the subgenus *Viannia* (figure 2.1).

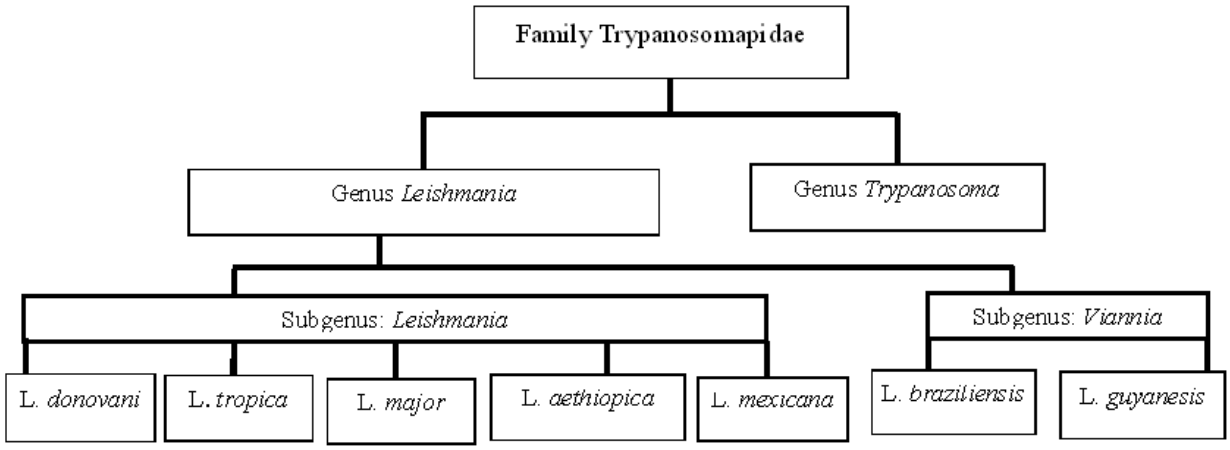


Figure 2.1 Classification of genus *Leishmania* complexes

2.1.1 Historical background of leishmaniasis

Leishmaniasis was reported as far back as the First Century. Natives of Ecuador and Peru had skin lesions and facial deformities that were characteristic of cutaneous and mucocutaneous leishmaniasis (Herwaldt, 1999). These ulcers bore a resemblance to leprous lesions and thus were named “white leprosy” or “valley sickness”. In Africa and India, cases of the disease were reported in the 1750s (Dedet and Pratlong, 2003). It was described as “kal-azar” or “black fever” now known as visceral leishmaniasis. Around 1756, Alexander Russell made vital development in unearthing leishmaniasis after observing a Turkish patient. Russell termed the disease, "Aleppo boil" (Bray, 1987). The disease came to be known as leishmaniasis after William Leishman, a Glasgwegian doctor helping the British Army in India who developed one of the first stains of *Leishmania* in 1901 (Bray, 1987). In Dum Dum, a town near Calcutta, Leishman identified ovoid bodies in the spleen of a British soldier who was suffering from fever, anaemia, muscular atrophy and swelling of the spleen. Leishman called this illness “dumdum fever” and put out his outcomes in 1903. Charles Donovan also came across these symptoms in other patients and published his findings a few weeks after Leishman (Bray, 1987: Cox, 2002: Murray, 2005). After examining the parasite using Leishman's stain, these amastigotes were named as Leishman-Donovan bodies and these species became officially known as, *Leishmania donovani*. By linking this protozoan with kal-azar, Leishman and Donovan named the genus *Leishmania* (Bari, 2006).

2.1.2 Geographical distribution of leishmaniasis

The geographical distribution of leishmaniasis is restricted to tropical and temperate regions, the living area of the sandfly. The disease is known from 88 countries in the tropics and sub-

tropics (16 developed countries and 72 developing countries) where 350 million are at risk of infection with over 12 million people affected and 2 million fresh cases every year. Leishmaniasis is widespread in African countries commonly in the North, Central, East and the Horn of Africa (Figure 2.2). The disease is also widespread in West Africa (Sheik-Mohammed and Velema, 1999). The initial available work representing the occurrence of leishmaniasis was recorded in Niger in 1911. New cases of leishmaniasis have been recorded in West Africa from Mali (Lefrou, 1948), Nigeria (Dyee-Shar, 1924), Senegal (Riou, 1933) and Cameroon (Rageu, 1951). Other countries in West Africa that also recorded cases in the past included, Mauritania, Gambia and Guinea. From the above data, cutaneous leishmaniasis is projected to be widespread from Mauritania, Gambia and Senegal in the west to Nigeria and Cameroon in the east (Boakye *et al.,* 2005).

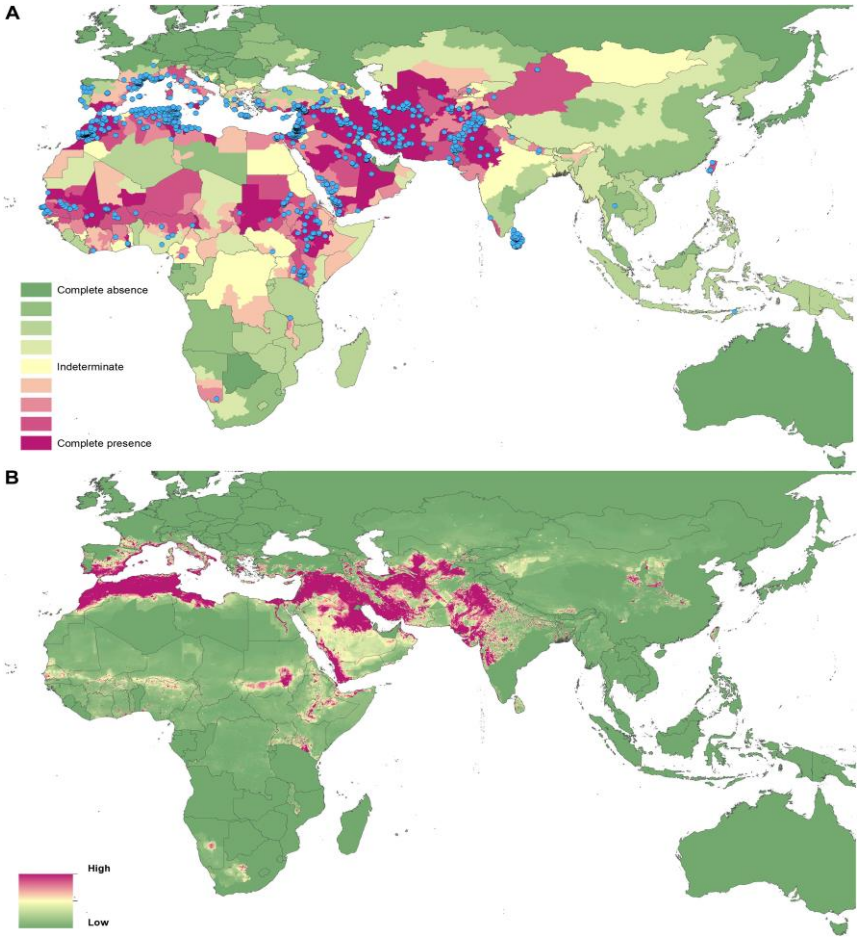


Figure 2.2 Reported and predicted distribution of leishmaniasis

(A) Evidence consensus for presence of the disease (B) Predicted risk of leishmaniasis. The blue spots indicate occurrence points or centroids of occurrences within small polygons (Pigott *et al.,* 2014*)*

2.1.3 Leishmaniasis in West Africa

The disease is reported to affect all age groups. Dedet *et al.,* (1982) reported that cutaneous leishmaniasis infected age groups between 10–40 years. There are small cases of leishmaniasis recorded from people below the above age group in Senegal. The highest number of cases occured during the rainy season. For instance in Burkina Faso, 76.5% of the lesions appear

between July and the end of November each year (Traore *et al*., 1998). Similar reports were made in Niger (Develoox *et al*., 1991) and Senegal (Dedet *et al*., 1982).

2.1.4 Leishmaniasis in Ghana

The first cases of cutaneous leishmaniasis were recorded in the Ho Traditional Area of the Volta Region of Ghana, in 1999 (Ghana Ministry of Health, Annual Report 2004). This led to active research of the disease in Kpando, Ho, and Hohoe Traditional Areas in 2003 (Figure 2.3). The study revealed that about 8,876 cases of leishmaniasis occurred between 2002 and 2003 and were recorded in three municipalities/districts in the Volta Region with 8,533 cases coming from Ho area alone (Table 2.1) (Kweku *et al*., 2011).

Table 2.1 Incidence of leishmaniasis in the Volta Region between 2002 and 2003

Municipality/District	Cases before 2003	Cases in 2003	Total
Ho	2348	6185	8533
Kpondo	76	91	167
Hohoe	2	174	176
Total	2426	6450	8876

(Kweku *et al.*, 2011).

Figure 2.3 Outbreak of cutaneous leishmaniasis in the Volta Region of Ghana
(Kweku *et al.*, 2011).

The results of Ho municipality were then sub-divided into sub-districts (Table 2.2). This indicated that out of the 2,348 cases that occurred in 2002 in the Ho traditional area, Ho-Shia sub-district, recorded the highest numbers followed by the nearby sub-district Kpedze and Tsito, which shares border with Kpedze-vane. The pattern was the same for the three sub-districts in 2002 and 2003.

Table 2.2 Incidence of leishmaniasis in the Ho Municipality

Sub-district	Communities	Households with cases	Cases before 2003	Cases in 2003	Toatal number of cases by November2003
Abutia	5	15	0	0	15
Kpetoe/Ziope	6	16	0	0	17
Adaklu	8	18	0	0	18
Tsito	17	340	42	556	598
Kpedze/Vane	31	1493	770	2725	3495
Ho/Shia	49	2240	1536	2854	4390
Total	116	4107	2348	6185	8533

(Kweku *et al*, 2011).

2.1.5 Classification of human leishmaniasis

There are four major type of leishmaniasis. These are cutaneous, diffused, mocus and visceral leishmaniasis.

2.1.5.1 Cutaneous leishmaniasis

This is the commonest form of the disease which causes mainly skin lesions that may heal by itself within few months but can leave many unpleasant scars. However, in some patients, difficulties may occur as the parasite metastasises through the lymphatic vessels to the lymph nodes, leading to the development of subcutaneous nodules or expansion of the regional lymph nodes (Bryceson, 1987; Kubba and Al-Gindan, 1989). Ulceration of the infection may be as a result of the host immune response (Figure 2.4 -2.5) (Boakye *et al.*, 2005).

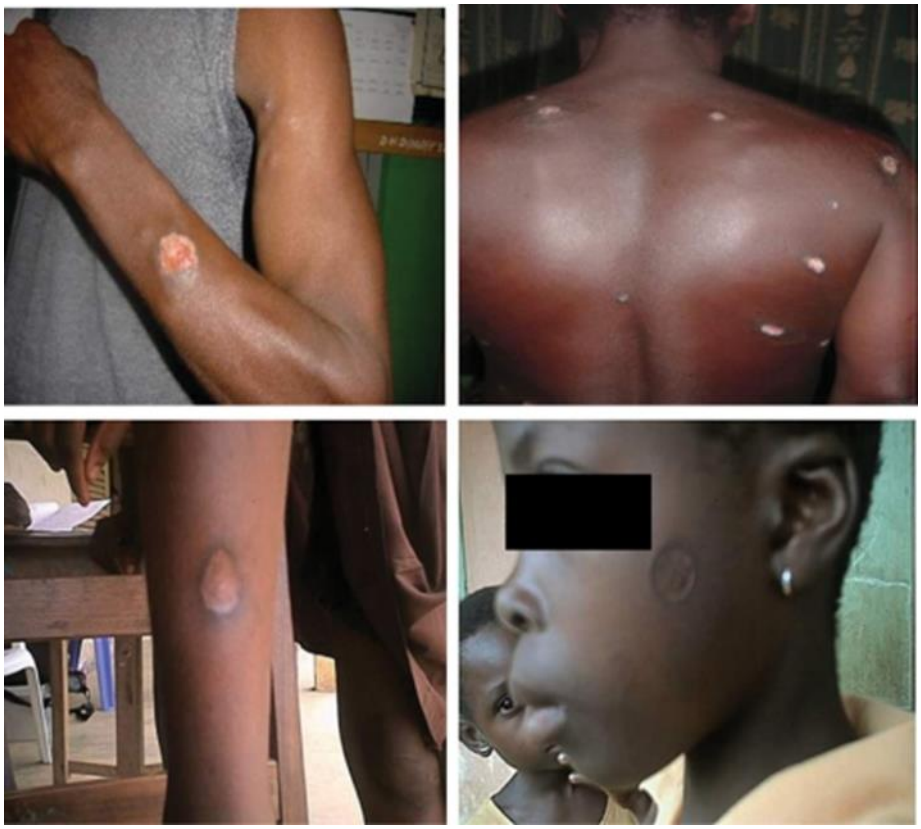


Figure 2.4 Scars and ulcers caused by the leishmania parasite (Kweku *et al*, 2011).

Figure 2.5 Stages of active cutaneous leishmaniasis (Kweku *et al*, 2011

Cutaneous leishmaniasis is classified as 'Old World' and 'New World' based on the geographical location. Old World leishmaniasis (caused by *Leishmania* species found in Africa, Asia, the Middle East, the Mediterranean, and India), which produces cutaneous or visceral disease, and New World leishmaniasis (caused by *Leishmania* species found in Central and South America), which produces cutaneous, mucocutaneous, and visceral disease. Some of the *Leishmania* parasites that cause cutaneous leishmaniasis in the Old World are *Leishmania major*, *Leishmania tropica* and *Leishmania aetiopica* (Alrajhi, 2003: Murray, 2005). *Leishmania major* is characterized by a boil of 5-10 mm in diameter which changes into a large rough ulcer or moist type lesion associated with reddish raised edge (Neva and Brown, 1994; Cheesbrough, 1998). It has numerous lesions that differ in size (Al-Jaser, 2005). *L. tropica* causes dry-type lesion 25-70 mm in diameter. Lesions made by *Leishmania major* infection

requires 3-6 months minimum for self-healing, while the lesion made by *L. tropica* requires 1-2 years minimum for self-healing (Neva and Brown, 1994: Cheesbrough, 1998: Alrajhi, 2003). It used to be thought that long lasting immunity against cutaneous leishmaniasis is acquired after curing the infection (Cheesbrough, 1998), but recent study reported that no life lasting immunity existed and re-infection may occur (Al-Jaser, 2005). The incubation period of *Leishmania major* and *Leishmania tropica* varied from 1-2 weeks to several months (Neva and Brown, 1994).

The primary species which cause 'New World' cutaneous leishmaniasis are *Leishmania mexicana* and *Leishmania vianna*. The lesions caused by *Leishmania mexicana* can be self-healing; but if the ears are infected, it may last for 30 years and destroy the pina of the ear. In the case of *Leishmania vianna* infection, ulcers formed have capacity to heal by itself (Cheesbrough, 1998).

2.1.5.2 Diffused leishmaniasis

This creates long time skin lesions that do not heal by itself and have a tendency to revert when poorly managed. It happens both in 'new world' and 'old world' and associated with wide, firm and smooth skin lesions which peel off and leave a rough surface later (Figure 2.6). Diffuse leishmaniasis in the New world, caused *by L. amazonensis*, is difficult to treat, while Old world diffused leishmaniasis caused by *L. aethiopica* revert after treatment (Cheesbrough, 1998; Alrajhi, 2003).

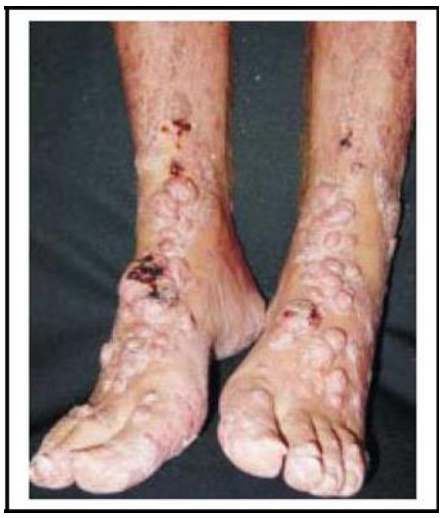


Figure 2.6 Diffused leishmaniasis at an advanced stage (Al-Amer, 2008).

2.1.5.3 Mucocutaneous leishmaniasis

Mucocutaneous leishmaniasis starts as lesions that ulcerate and involves the human mucosal system (Neva and Brown, 1994: Peake *et al.*, 1996). The parasite attacks the nasopharynx or the buccal cavity and slowly destroys the cartilaginous and soft tissues of the nasal septum, lips and larynx (Figure 2.7) (Peake *et al.*, 1996: Cheesbrough, 1998: Cunningham, 2002: Boakye *et.al.*, 2005). This starts with sores on the skin and continues to lesions that cause huge tissue damage of the mouth, nose and throat cavities and serious mutilation. They are usually caused by *L. panamensis* and *L. guyanensis* (Peake *et al.*, 1996). Immune compromised patients also can show mucocutaneous leishmaniasis symptoms by other *Leishmania* species.

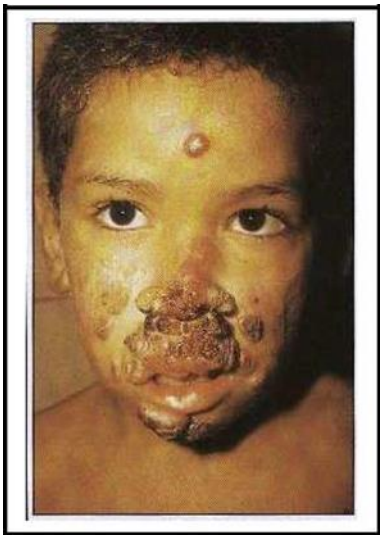


Figure 2.7 Mucocutaneous leishmaniasis showing the destruction of the nose tissue (Al-Amer, 2008).

2.1.5.4 Visceral leishmaniasis

Visceral leishmaniasis is the most severe type and is caused by *L. donovani* and *L. infantum*. It presents with momentous enlargement of the spleen and liver, unstable fever episodes; significant weight loss, anaemia (Figure 2.8) (Peake *et.al*, 1996; Cheesbrough, 1998: Guerin *et al.*, 2002) and death if patient remain untreated (Cheesbrough, 1998; Guerin *et al.*, 2002: Boakye *et al.*, 2005). The development of the disease varies tremendously. The usual duration is between 3-4 months, but some cases can last for one to more than 5 months. The disease begins as small skin boils on the face, progressively widens and extends to cover the entire body. The lesions might finally enlarge and can cause blindness if allowed to reach the eyes (Cheesbrough, 1998).

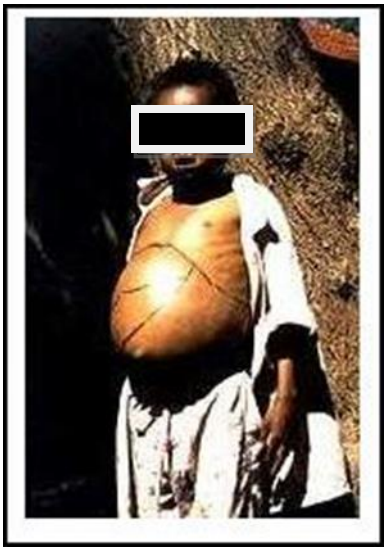


Figure 2.8 Boy suffering from visceral leishmaniasis (Al-Amer, 2008).

2.1.6 Treatment of leishmaniasis

In most circumstances cutaneous leishmaniasis heal without any management. Visceral leishmaniasis are really hard to treat, and may require longer time treatment with pentavelant antimony drugs, though, resistance to these drugs is high in certain parts of the world, mostly in India (Sundar *et al*., 2000). This may require treatment with more toxic and costly drugs such as amphotericin B, a potent antifungal agent with some severe side effects that may cause kidney damage and mild to severe allergic reactions. Miltefosine and fluconazole have displayed potential for management of leishmaniasis with others still in clinical trials. Currently there is no accepted vaccine for the disease, but a number of leishmaniasis vaccines are in different levels of development. These include LEISH-F 1, LEISH-F2, LEISH-F3, Various Lutzomyia sandfly antigens and LdNH36 all are at various stages of trials. Leishmune, Leishtec, and CaniLeish have been licensed for canine visceral leishmaniasis (Jain and jain, 2015).

2.1.7 Life cycle of the *Leishmania* parasite

Leishmaniasis is spread by the bite of infected female phlebotomine sandflies. In sandflies, amastigotes converted into promastigotes (Figure 2.9), grow in the gut and migrate to the proboscis. The sandflies introduce the promastigotes (infectious stage) from their proboscis through blood meals. Promastigotes that reach the punctured wound are phagocytosised by macrophages and other types of mononuclear phagocytic cells (Figure 2.10). Promastigotes change in these cells into the amastigotes (tissue stage of the parasite), which increase in number by cell division and go on to attack other mononuclear phagocytic cells.

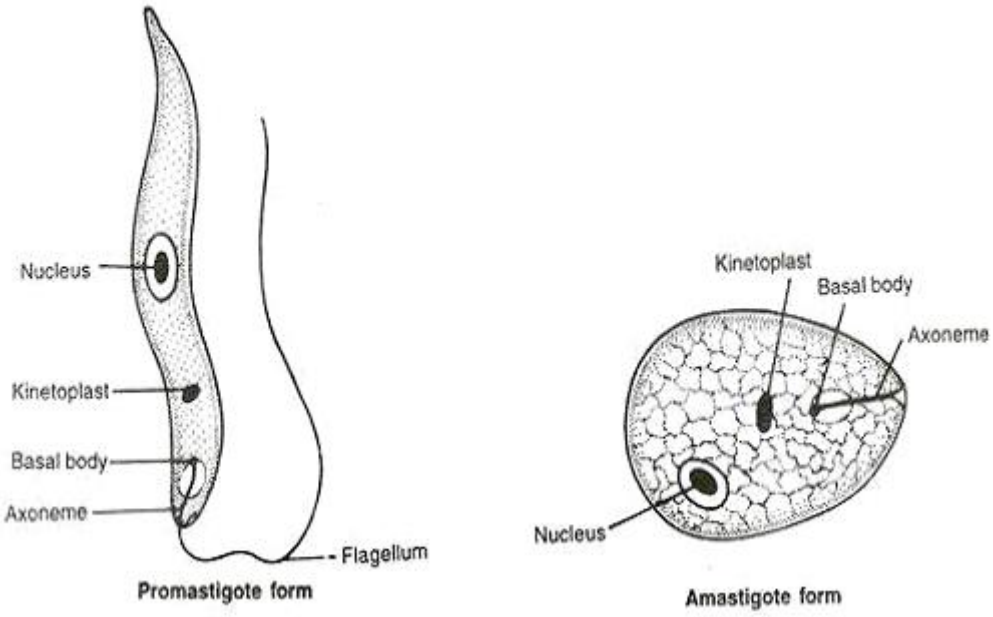


Figure 2.9 Morphological forms of *Leishmania donovani* (Prasath, 2015)

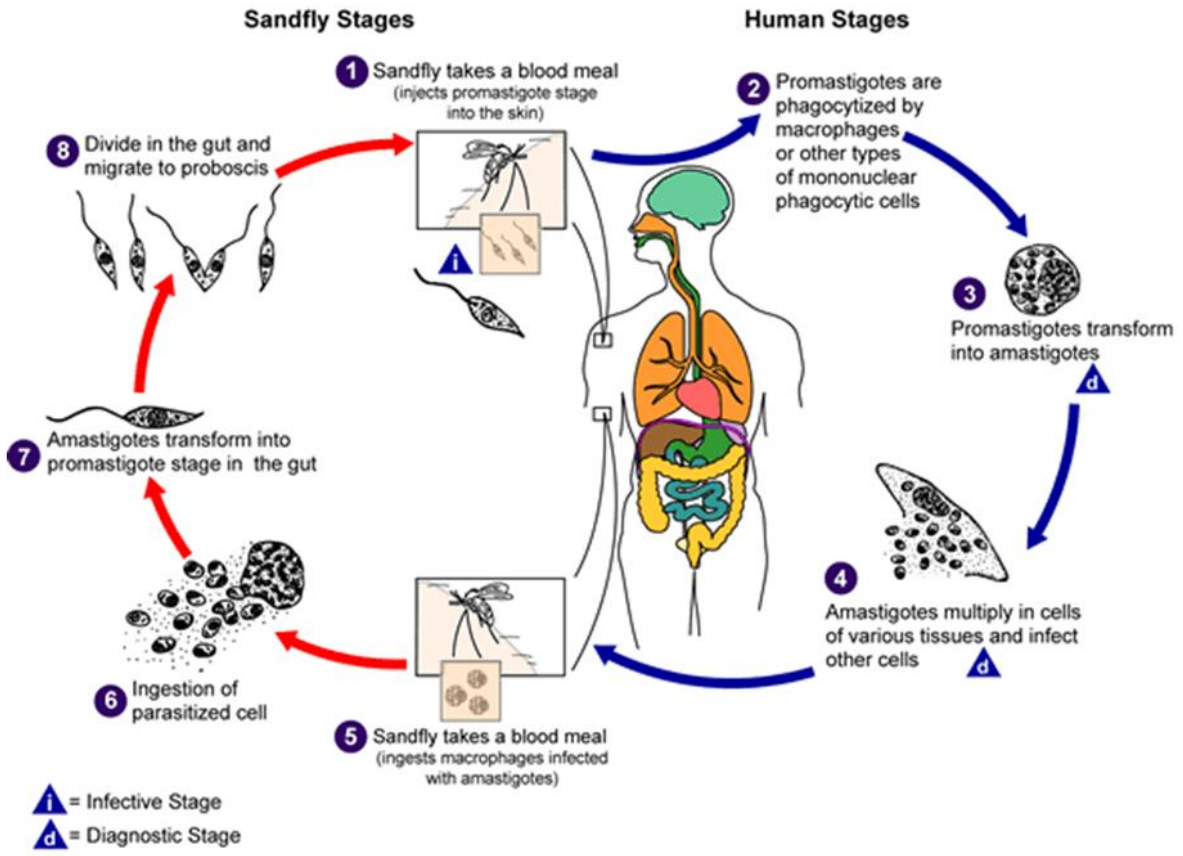


Figure 2.10 Life cycle of *leishmania* parasite
http://www.cdc.gov/parasites/leishmaniasis/biology.html

2.1.8 *Leishmania* infection and the human immune system

Immunity is the body's capacity to repel microbes and other foreign materials or abnormal cells. Immune response may be either nonspecific (innate) or specific (adaptive) (Sherwood, 2004).

When there is an entry of a pathogen, macrophages produced by circulating peripheral blood mononuclear cells, engulf microbes by phagocytosis into phagosomes. The phagosomes bind

with lysosomes to form digestive organelles (phagolysosomes), which damages the pathogen and makes it available to the immune system (Sherwood, 2004). When the micro-organism is engulfed by macrophages, an activation of numerous cellular genes occurs, several of which encode cytokines that stimulate an inflammatory response and resistance to pathogens such as Interleukin 12 (IL-12) and tumor necrosis factor (TNF-α) (Be Souza, 1995: Belkaid, 1998: Chaussabel *et al.*, 2003). The complement system is a group of inactive plasma proteins produced by the liver and circulate in the blood. When activated, a sequential cascade of complement components activation reactions will form the membrane attack complex that destroys plasma membrane of foreign cells (Abbas *et al.*, 1994).

Macrophages form an essential part in *Leishmania* infection. When phagocytosis occurs, *Leishmania* promastigotes enter the vacuole, the parasite changes from promastigotes to amastigotes and multiply in a naive host (Alexander and Russell, 1992). However, *Leishmania* is able to escape from the damage activity of the macrophages by preventing the macrophage capability to present the parasite antigen to other components of immune system (Oliver *et al.*, 2005). As a consequence, *Leishmania* is able to live, change into amastigotes, and increase in number by binary fission within those cells (Chang, 1990).

2.1.9 Intracellular survival of *Leishmania* parasite in the macrophages

Leishmania defends itself from the severe environment inside the phagolysosomes by numerous mechanisms (Cunningham, 2002). It has functional molecular surface and a number of enzyme systems, which help to achieve its survival (Chang, 1990). It achieves this by preventing host phagosome-lysosome fusion, hydrolytic enzymes, cytokines production, nitric oxide production and influences cell signaling pathways (Cunningham, 2002). *Leishmania*

parasite succeeds to impede and influence macrophage apoptosis in order to divide in an aggressive environment before shattering the cell and finding another host cell (Moore and Matlashewski, 1994: Heussler *et al.*, 2001).

2.1.10 Natural products with anti-leishmanial activity

A number of diterpenes and triterpenes have been found to show leishmanicidal activity. The diterpenoid phorbol ester, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) **(1)**, is able to cause a variety of structural changes in the parasites of *L. amazonensis*. This phorbol ester was found to activate protein kinase C, an important enzyme in the development of several cellular functions (Wright and Phillipson, 1990: Vannier-Santos *et al.*, 1988)**.** Other diterpenoids with leishmanicidal activity isolated from Euphorbiaceae species include jatrogrossidione **(2)** and jatrophone **(3)**. These metabolites possess toxic activity against the promastigote forms of *L. braziliensis, L. amazonensis* and *L. chagasi* (Schmeda-Hirschmann *et al.*, 1996)

Triterpenes reported to have anti-leishmanial effects include ursolic acid **(4)** and betulinaldehyde **(5)**, obtained from the bark of *Jacaranda copaia* and the stem of *Doliocarpus dentatus* (Dilleniaceae), respectively. Both metabolites were active against the amastigotes of *L. amazonensis* (Sauvain *et al.*, 1993: Sauvain *et al*., 1996). Two triterpenes obtained from the leaves of *Celaenododendron mexicanum* (Euphorbiaceae), (24*Z*)-3-oxotirucalla-7, 24-dien- 26-oic acid **(6)** and *epi*-oleanolic acid **(7)**, also exhibited leishmanicidal activity on promastigotes of *L. donovani* (Camacho *et al.*, 2000).

The biflavonoids amentoflavone **(8)**, podocarpusflavone A **(9)** and B (**10)**, isolated from the leaves of *Celanodendron mexicanum*, showed activity against promastigotes of *L. donovani*.(Camacho *et al*, 2000) The chalcone (*E*)-1-[2,4-hydroxy-3-(3-methylbut-2-enyl)phenyl]-3-[4-

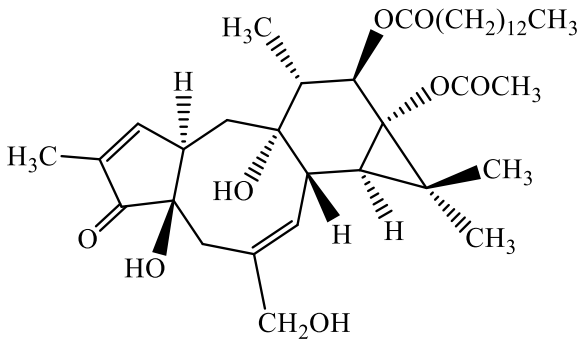
hydroxy-3-(3-methylbut-2-enyl)phenyl]-prop-2-en-1-one (**11)** is reported to be toxic to promastigotes of *L. donovani*, (Christensen *et al.*, 1994**)** while 2,6-dihydroxy-4-methoxychalcone **(12)**, isolated from inflorescences of *Piper aduncum* (Piperaceae), is reported to show a significant activity *in vitro* against promastigotes and amastigotes of *L. amazonensis* (Torres-Santos *et al.*, 1999).

The oxygenated chalcone, licochalcone A **(13)**, obtained from roots of the Chinese licorice plant (*Glycyrrhiza* spp., Fabaceae), inhibited the *in vitro* growth of promastigotes of *L. major* and *L. donovani*. The aurones, a group of metabolites related biosynthetically to the chalcones, have demonstrated anti-leishmanial activity against the promastigote forms of *L. major, L. donovani, L. infantum* and *L. enrietti* (Chen *et al.*, 1994; Chen *et al.*, 1993; Zhai *et al.*, 1995).

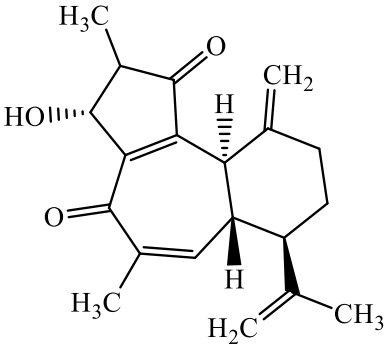
A number of alkaloids have shown leishmanicidal activity according to various reports. Among some of the indole alkaloids reported to possess antiprotozoal activity is harmaline isolated from *Peganum harmala* and *Passiflora incarnata*. The possible mechanism of its antiprotozoal action has been postulated as its ability to intercalate DNA by interfering with the metabolism of aromatic amino acids (Goodwin, 1989: Fournet *et al.*, 1994).

Sarachine **(14)**, a steroidal alkaloid isolated from leaves of *Saracha punctata* (Solanaceae), completely inhibits the growth of the promastigote forms of *L. brazilienis, L. donovani* and *L. amazonensis.* Similarly, eight steroidal alkaloids, including holamine **(15)**, 15-α-hydroxyholamine **(16)**, holacurtine **(17)**, and *N*-desmethylholacurtine (**18),** obtained from the leaves of *Holarrhena curtisii* (Apocynaceae) exhibited leishmanicidal activity against promastigotes of *L. donovani* (Kam *et al.*, 1998). Five indole alkaloids obtained from the bark of *Corynanthe pachyceras* (Rubiaceae) showed marked activity against promastigotes of

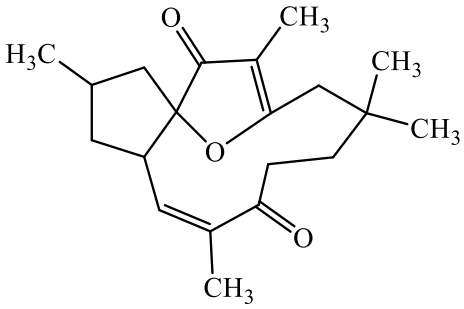
Leishmania major. Among these alkaloids were dihydrocorynantheine **(19)**, corynantheine (**20)** and corynantheidine **(21)**. Anonaine **(22)** and liriodenine **(23)**, obtained from the trunk bark and roots of *Annona spinescens* (Annonaceae), have been reported to show activity against promastigotes of *L. braziliensis*, *L. amazonensis* and *L. donovani* (Queiroz et al, 1996).



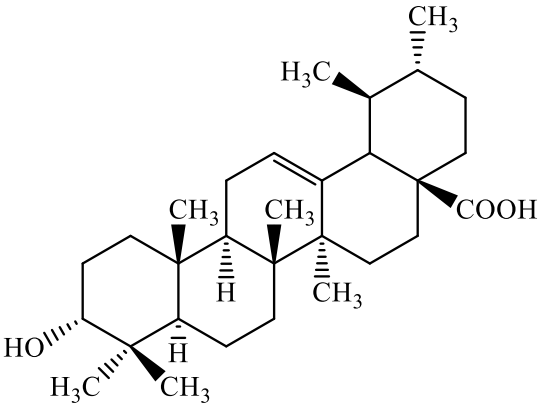
12-O-tetradecanoyl phorbol-13-acetate **(1)**



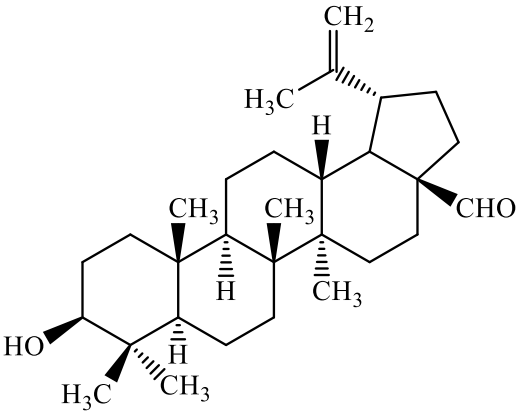
Jatrogrossidione**(2)**



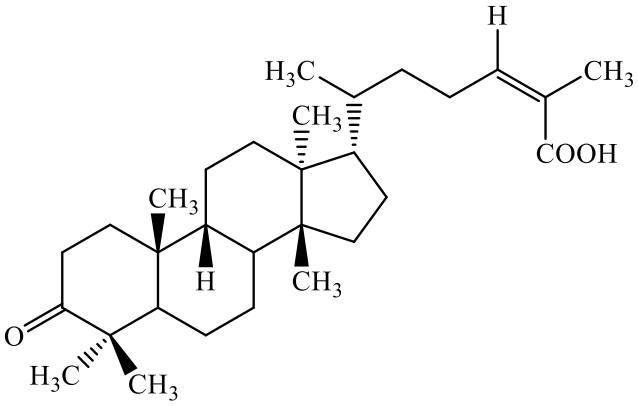
Jatrophone **(3)**



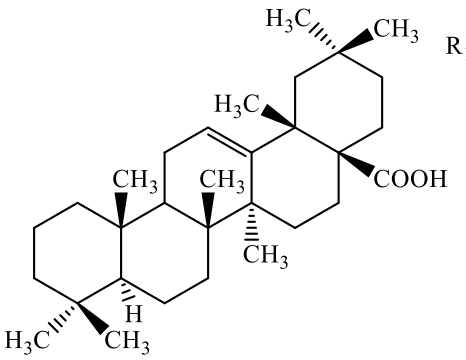
Ursolic acid **(4)**



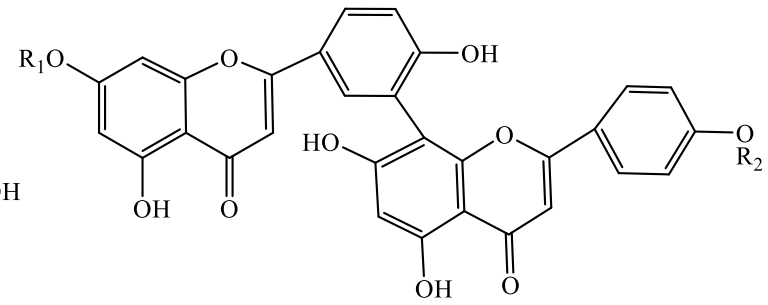
Betulinaldehyde **(5)**



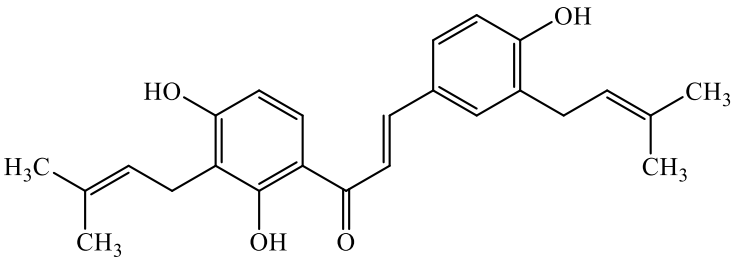
(24Z)-3-oxotirucalla-7,24-dien- 26-oic acid **(6)**



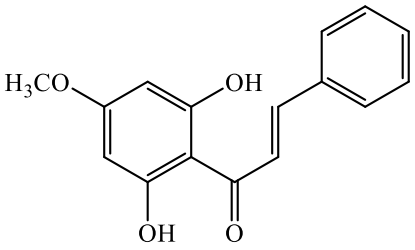
Epi-oleanolic acid **(7)**



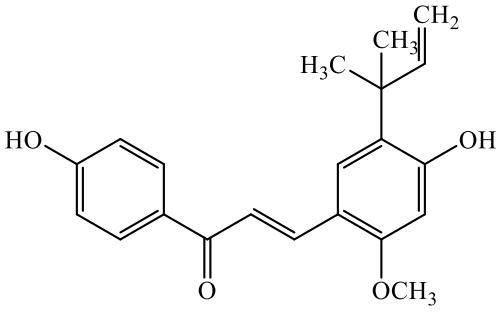
AmentoflavoneR$_1$ = R$_2$ = H **(8)**
Podocarpusflavone A R$_1$=H, R= OCH$_3$ **(9)**
Podocarpusflavone B R$_1$ = OCH$_3$, R$_2$= OCH$_3$ **(10)**



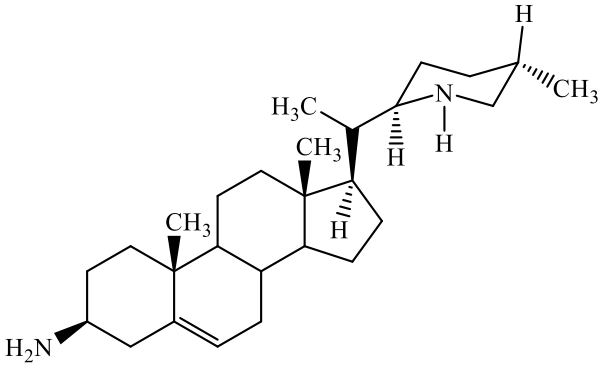
(E)-1-[2,4-hydroxy-3-(3-methylbut-2-enyl)phenyl]-3-[4-hydroxy-3-(3-methylbut-2-enyl)phenyl]-prop-2-en-1-one **(11)**



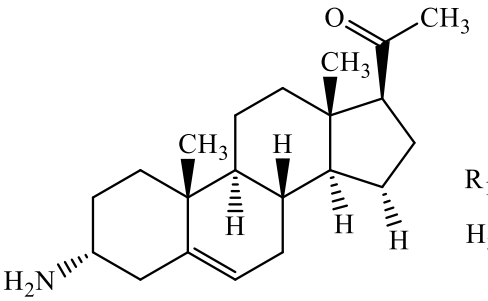
2,6-dihydroxy-4-methoxychalcone**(12)**



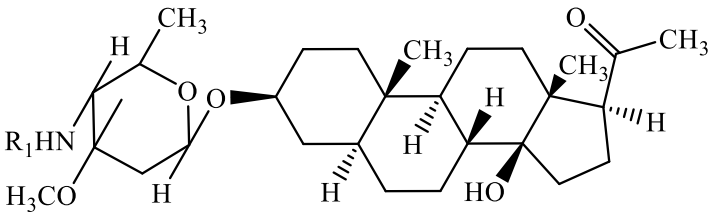
Licochalcone A (**13**)



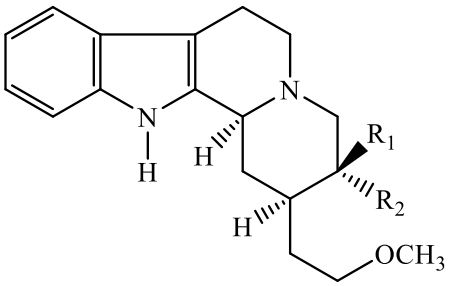
Sarachine **(14)**



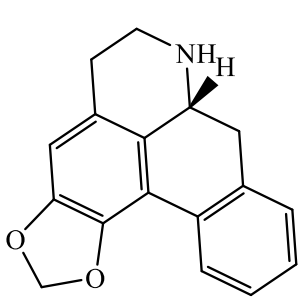
Holamine **(15)** R=H
15-a- hydroxyholamine **(16)** H=OH



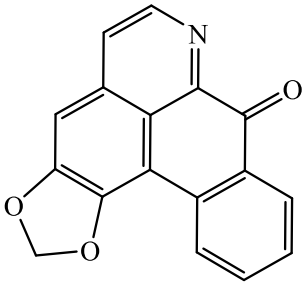
Holacurtine **(17)** $R_1 = CH_3$
N-desmethylholacurtine **(18)** $R_1 = H$



Dihydrocorynantheine **(19)** R_1 =H, $R_2 = C_2CH_5$
Corynantheine **(20)** $R_1 = H$, $R_2 = C_2H_3$
Corynantheidine **(21)** $R_1 = C_2H_5$, $R_2 = H$



Anonaine **(22)**



Liriodenine **(23)**

2.1.11 Molecular mechanism of anti-leishmanial activity

The best drug target is one with high selectivity for the parasite and is vital for its death. Such parasite-specific targets can be known by discovering essential differences in metabolic and/or biochemical targets between host and parasite. A noticeable metabolic difference between *Leishmania* and its mammalian hosts is found in the trypanosomatids redox metabolism that functions to lessen cellular oxidative stress (Lillig *et al.*, 2007: Krauth-Siegel and Leroux, 2012). The important metabolite in the redox system of the *Leishmania* is trypanothione-bis-(glutathionyl) spermidine, $[T(SH)_2]$, which keeps the cellular redox homeostasis (Krauth-Siegel

and Comini, 2008). The functionally similar redox metabolite in the mammalian host is glutathione. The biological synthesis of $T(SH)_2$ is performed by trypanothione synthetase (TryS), one of the main enzymes of the parasite's redox metabolism. T $(SH)_2$ is responsible for various cellular development, such as synthesis of deoxynucleotides, and parasite resistance to chemical stress and anti-leishmanials (Krauth-Siegel and Comini, 2008: Dormeyer *et al*., 2001: Krauth-Siegel and Lüdemann, 1996). The TryS has been confirmed as a drug target by conducting knockout research in *Trypanosome brucei* (Comini, 2004: Ariyanayagam *et al*., 2005). The special nature of TryS in synthesizing $T(SH)_2$ in parasites and its lacking in the host make it a striking target (Saudagar and Dubey, 2011). Betulin **(5)** has been reported as an inhibitor of the TryS with potent anti-leishmanial activity (Saudagar and Dubey, 2011). Betulin **(5)** is an abundant naturally occurring triterpene present in many plants especially in the bark of white birch trees. There are reported works on betulin eliciting anti-inflammatory, anti-human immunodeficiency virus (HIV), (Reutrakul *et al*., 2010) anti-malarial, (Steele *et al*., 1999) and natural medicine anticancer properties (Laszczyk *et al*., 2009: Li *et al*., 2010). Recent studies of betulin and its derivatives have been reported to prevent DNA topoisomerase of *L. donovani* (Chowdhury *et al*., 2011). Antiparasitics, having more than one metabolic target, are normally more active and less probable to bring rapid parasite drug resistance, than drugs having only one mechanism of antiparasite action. Multidrug-resistant leishmaniasis is growing worldwide making the unearthing of novel and effective anti-leishmanials drugs vital. Anti-leishmanial drugs that originate from natural sources often have advantages of low toxicity and fewer or no side effects (Saudagar and Dubey 2011).

2.1.12 Programmed cell death

Programmed cell death (PCD) is a process of cell biology and is believed to have developed to control growth development as well as to protect against viral infections and the rise of cancer (Shaha, 2006) in multicellular organisms. Modern researches have proven the existence of PCD in unicellular organisms, and have hypothesized a functional role of PCD in the biology of unicellular organisms (Lee *et al*., 2007). There is a suggestion that in order to promote and preserve clonality within the population, the Trypanosomatids must have developed an altruistic mechanism to control growth (Shaha, 2006). Recently, PCD has been shown to be involved in the control of cell proliferation of *T. cruzi in vitro* (Lee, 2002) and in the insect vector mid gut. All these observations point towards the existence of a PCD pathway in Trypanosomatids. Whether the type of PCD that exists in Trypanosomatids is the same as in multicellular organism remains to be seen (Marinho *et al.*, 2011).

2.1.13 PCD in stationary phase of cultures of promastigotes

When promastigotes of *L. donovani* is cultured *in vitro*, there is an increase exponentially in cell number in the first 3- 4 days, followed by a constant growth (Figure 2.9A) (Lee, 2002). The constant growth curve is described as stationary phase since there is no significant increase in cell number (Lee *et al.*, 2007). Another study reported the nature of cell death occurring in stationary phase culture, which could be the result of either necrosis or PCD. A typical feature of PCD in multicellular organisms is the formation of a DNA ladder (Figure 2.9B). In stationary phase promastigotes, they reported a DNA fragmentation pattern in multiples of oligonucleosomal length units (Lee, 2002). The intensity of the DNA ladder was more pronounced in the latter stages of the stationary phase (Shaha, 2006).

A reduction in mitochondrial membrane potential has been observed in a number of models of apoptosis (Marinho *et al*., 2011). However, it is not clear whether it is the primary events in PCD (Marinho *et al*., 2011). Current reports suggest that, upon ageing, *Leishmania* promastigotes loose mitochondrial membrane potential (Lee *et al*., 2002). *L. donovani* promastigotes were cultured *in vitro* at an initial concentration of 1 x 10^6 cells/ml. The cells were kept in culture for 10 days and counted as indicated indicated in Figure. DNA was isolated at each time point, run through an agarose gel, and visualized by ethidium bromide (B). DNA size markers are shown in base pairs (Figure 2.9B)

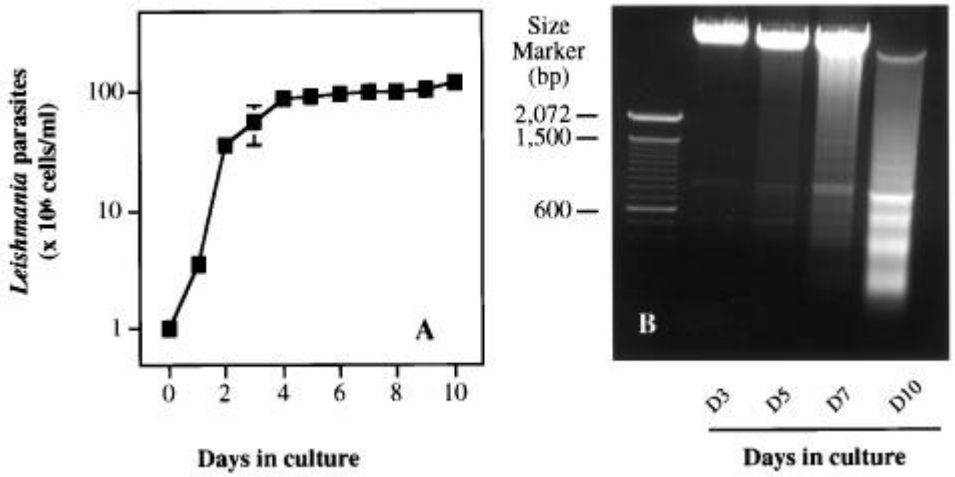


Figure 2.9 Cell growths in culture and the appearance of DNA ladder formation (Lee *et al*., 2002)

2.2 INFLAMMATION

Inflammation is the local physiological response to tissue injury. It is not a disease, but is usually a manifestation of disease. Inflammation is initially protective and acts to remove and repair damaged tissue or to neutralize harmful agents (Fritz *et al*., 2006; Maslinska and Gajewski 1998); destroy invading micro-organisms and ward off infection by walling of the

abscess cavity to prevent spread of infection. This initial phase is the immune systems response to entry of pathogens and tissue injury. It is a rapid self-limiting process, mediated by eicosanoids and vasoactive amines which increase the movement of plasma and leukocytes into infected site (Dinarello, 2010). Inflammation is usually classified according to its time course as acute inflammation and chronic inflammation.

2.2.1 Acute inflammation

It is a transient response to injury or irritant. Important cardinal signs of inflammation include heat and redness that comes from vasodilation of blood vessels, swelling that emerges from edema, pain that results from local release of prostaglandins and kinins, and loss of function. Inflammation targets at removal of the harmful agent and repair of tissue intergrity, but occasionally one has to bear for this benefit might be in the form of scaring or additional forms of inflammatory implications (Jato, 2015). The principal causes of acute inflammation are microbial infections like pyogenic bacteria, viruses, hypersensitivity reactions and physical agents such as trauma, ionising irradiation, heat, cold and chemicals. However, the commonest cause of inflammation is microbial infection. Viruses lead to death of individual cells by intracellular multiplication. Bacteria release specific exotoxins (chemicals synthesised by them which specifically initiate inflammation) or endotoxins (which are associated with their cell walls). Early in the acute inflammatory process, pro-inflammatory mediators such as prostaglandins and leukotrienes play an important role (Samuelsson *et al*., 1987). The progression from acute inflammation to chronic inflammation as in many widely occurring human diseases such as rheumatoid, arthritics, atherosclerosis, cancer and Alzeihmer's

(Medzhitov, 2008; Noah *et al*., 2012), is widely viewed to be due to excess of pro-inflammatory mediators (Serhan *et al*., 2009).

2.2.2 Chronic inflammation

In chronic inflammation, various cytokines and growth factors are released resulting in the recruitment of higher order immune cells such as leukocytes, lymphocytes and fibroblasts. This may lead to persistent tissue damage by these cells (Aggarwal *et al*., 2009; Lin and Karin, 2007).

Chronic inflammation has been widely reported as the leading cause of a number of diseases such as hay fever, periodontitis, rheumatoid arthritis, arteriosclerosis, cardiovascular diseases, diabetes, obesity, pulmonary diseases, neurologic diseases and cancer (Aggarwal *et al*., 2006). Chronic inflammation has been linked to various steps involved in carcinogenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis (Mantovani *et al*, 2005: Coussens and Werb, 2002). Inflammatory response and tissue damage are induced by inflammatory mediators generated through up-regulation of inducible pro-inflammatory genes cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). During the inflammatory process, large amounts of the pro-inflammatory mediator's like nitric oxide and prostaglandins are produced by these pro-inflammatory genes (Akira and Hajime, 2007). Continuous production of these molecules in chronic inflammation has been linked to the development of cancer (Israf *et al*., 2007).

2.2.3 Pathways of inflammation

The acute inflammatory response takes place in three different stages. The first phase is an augmented vascular permeability causing exudation of fluids from the blood into the interstitial

space, the second stage comprises the infiltration of leukocytes from the blood into the tissue whereas the third phase involves granuloma formation and tissue repair (Medzhitov, 2008: Noah *et al*., 2012). Mediators of inflammation come from either plasma (e.g. complement proteins kinins) or from cells (Amponsah, 2012). The release of vigorous mediators is caused by microbial products or by host proteins (kinins) and coagulation structures that are themselves triggered by microbes and destroy tissues. Usually the mediators of inflammation are histamine, prostaglandins (PGs), leukotrienes (LTB4), nitric oxide (NO), platelet-activation factor (PAF), bradykinin, serotonin, lipoxins, cytokines and growth factors (Medzhitov, 2008: Noah *et al*., 2012).

2.2.4 Models of inflammation

Paw oedema, sponge implantation and air pouch granulomas are among the models that are used in inflammation studies. These models employ a variety of agents like formalin, Freunds adjuvant, carrageenan, monosodium crystals and zymosan (Singh and Newman, 2011). Others include vasoactive agents, weakened bacteria such as *E. coli*, chemotactic factors, injection of polymorphonuclear leucocyte, leucotriene B4 and arachidonic acid in acetone (Issekutz and Issekutz, 1989). Inducing these agents into different parts of the body may bring about an acute inflammatory response (Jato, 2015).

2.2.4.1 Models of acute inflammation

Acute inflammatory response can be measured by checking reactions such as foot volume increase formed by oedema (an increase in vascular permeability), monocyte infiltration, polymorphonuclear leucocyte and lymphocyte accumulation (Issekutz and Issekutz, 1989).

Hyperaemia and the migration of leucocytes are the primary indices of the acute inflammatory reaction (Issekutz, 1981). The best suitable initial screening test for anti-rheumatic activity is the carrageenan - induced acute footpad oedema in laboratory animals. This model has been widely used to screen new anti-inflammatory drugs (Singh, 2000) and has been used in this present study with very outstanding result. This model is centered on discharge of several inflammatory mediators by carragenan. Oedema development owing to carrageenan in the rat paw is a biphasic event (Jato, 2015). The first stage is credited to the discharge of histamine and serotonin. The second phase of oedema is owed to the release of prostaglandins, protease and lysosomes (Crunkhon and Meacock, 1971). Subcutaneous injection of carrageenan into the rat paw yields inflammation which result from plasma extravasation, improved tissue water and plasma protein exudation beside neutrophil extravasation, all due to the metabolism of arachidonic acid (Chatpaliwar *et al*., 2002). The initial stage starts directly after injection of carrageenan and reduces in two hours. The second stage starts at the end of the initial stage and rests via the third hour up to five hours.

2.2.5 Anti-inflammatory medicinal plants

The use of plants for pain and inflammation is as old as ancestry. Anti-inflammatory and analgesic drugs produced by leads from folklore medicine include colchicine from *Colchicum autumnale* and morphine from the opium puppy *Papaver somniferum*. A number of plants are still used in traditional medicine to manage and treat serious inflammatory disease with results that is comparable to that of allopathic medicines but without undesirable effect such as gastrointestinal bleeding, renal impairment and dependence (Burke *et al*., 2005). Some of these medicinal plants and the parts used are shown in Table 2.3.

Table 2.3 Plants used in ethnomedicine for pain and inflammation

Scientific name	Family	Part(s) used for the study	Traditional use(s)
Persicaria stagnina	Polygonaceae	Whole plant	Diuretic, analgesic
Albizia lebbeck	Fabaceae	Bark	Toothache, gum diseases
Acalypha indica	Euphorbiaceae	Whole plan	Bronchitis, asthma, arthritis
Azadirachta indica	Meliaceae	Leaves	Rheumatic disorders, antiallergic
Hibiscus sabdariffa	Malvaceae	Calyx	Rheumatic fever, ulcer
Phyllanthus reticulatus	Euphorbiaceae	Aerial parts	Antidiabetic
Zingiber officinale	Zingiberaceae	Rhizome	Pain, inflammation

(Burke *et al*., 2005)

2.3 ANTIOXIDANTS

An antioxidant is a substance which averts the oxidation of different substances (Badarinath *et al.*, 2010). They guard the key cell components through the neutralization of the harmful free radicals that are normal end substances of cell breakdown. Free radicals are produced by oxygen breakdown in the body and they have a single electron in their valence orbital (Winrow, *et al*., 1993). Closest stable molecules are attacked by these free radicals, withdrawing their electron. The particle becomes a free radical and starts a chain reaction (Bae *et al*., 1999). Free radicals could be oxygen derivatives (reactive oxygen species, ROS) or nitrogen derivative (reactive nitrogen species, RNS). The oxygen radicals include O^{-}_{2} (oxygen superoxide), HO (hydroxyl), HO_2 (hydroperoxyl), ROO (peroxyl), RO (alkoxyl) as free radical and H_2O_2 (hydrogen peroxide) oxygen as non-radical. Reactive nitrogen species are mostly nitric oxide (NO), peroxy nitrate (ONOO), nitrogen dioxide (NO_2) and dinitrogen trioxide

(N_2O_3) (Colbert and Decker, 1991). In normal cells, there are enzymes and molecules that counter the actions of free radicals known as antioxidants. However, when free radical production exceeds the defence capacity of the antioxidant system, the results is oxidative stress. As results of oxidative stress, biopolymers including nucleic acids, proteins, polyunsaturated fatty acids and carbohydrates become destroyed.

Lipid peroxidation is oxidative worsening of polyunsaturated lipids and it consists of reactive oxygen species and transition metal ions. It is a mechanism of cell destruction that produces an extensive array of cytotoxic results, greatest of them is aldehydes, such as malondialdehyde (MDA), and 4-hydroxynonenal (HNE). Oxidative stress brings about serious cell destruction resulting in a number of human illnesses (Martinez and Whitaker, 1995), such as Parkinson's disease, cancer, atheroscleorosis, Alzheimer's disease, arthritis, neurodegenerative disorders and immunological incompetence. Insufficient antioxidants in diet also results in oxidative stress. This means the natural antioxidative agents present in food consumed by human population is of great importance (Lindley, 1998).

Antioxidants have been classified into two, which are primary (chain-breaking antioxidants) and secondary (preventative antioxidants). Astley *et al.*, (2003) postulated that secondary antioxidants are substances that delay the speed of oxidation. This might be done in a various ways comprising elimination of substrate or singlet oxygen quenching (Otto, 2006). Primary antioxidants, if exist in small amounts, might one way or the other interrupt or hinder the initiation step by reacting with a lipid radical or inhibit the propagation step by reacting with peroxyl or alkoxyl radicals (Astley *et al.*, 2003). The antioxidant free radical may further

hamper chain-propagation reactions by creating peroxy antioxidant substances (Antolovich *et al*., 2002).

Primary antioxidants might occur naturally or be manufactured synthetically as in Butylated hydroxytoluene (BHT), Butylated hydroxyanisole (BHA), tert-Butylhydroquinone (TBHQ) and the gallates. In the food industry, the synthetic antioxidants are widely used (Cory *et al*., 2003) and are included in the human food (Rice-Evans *et al.,* 1995). These synthetic antioxidants have been reported to cause cancer; therefore, their use in food is being discouraged. The use of naturally occurring antioxidants is therefore being promoted (Taga *et al*., 1984).

2.3.1 Antioxidant determination methods

Antioxidant methods are classified into two main groups; hydrogen atom transfer method and electron transfer method (Badarinath *et al*., 2010). Hydrogen Atom Transfer methods (HAT) include total radical trapping antioxidant parameter (TRAP), oxygen radical absorbance capacity (ORAC) method, lipid peroxidation inhibition capacity (LPIC) assay, crocin bleaching, nitric oxide radical inhibition activity, inhibited oxygen uptake (IOC), scavenging of H_2O_2 radicals, hydroxyl radical scavenging activity by p-butrisidunethyl aniline (p-NDA), scavenging of super oxide radical formation by alkaline (SASA) and ABTS radical scavenging method (Sindhi *et al,* 2013). Electron transfer methods (ET) of determining antioxidants include copper (II) reduction capacity, trolox equivalent antioxidant capacity (TEAC), ferric decolourization reducing antioxidant power (FRAP), DPPH free radical scavenging assay, N, N-dimethyl-p-Phenylenediamine (DMPD) assay and total phenols by Folin-Ciocalteu

(Badarinath *et al*., 2010). Other methods of assaying antioxidants include total oxidant scavenging capacity (TOSC), inhibition of Briggs-Rauscher oscillation reaction, chemilluminescence, electrochemilluminescence, fluorometric analysis, enhanced chemilluminescence (ECL), thin layaer chromatograph (TLC) bioautography, cellular antioxidant activity (CAA) assay and dye-substrate oxidation method (Carocho and Ferreira, 2013).

2.3.2 *In vitro* determination of antioxidant capacity

Antioxidants work by different mechanisms and no one method can apprehend the different means of their actions (Badarinath *et al*., 2010). Therefore, a number of methods are employed in *in vitro* antioxidant assessment. Normal cuvette method of radical scavenging activity using a UV-Visible spectrophotometer to measure the absorbance has been substituted by 96-well titre plate method (Carocho and Ferreira, 2013). The 96-well plate method uses ELISA plate reader for absorbance. The cuvette method is very tiresome, require a lot of time, works with one sample at a time and requires a lot of reagent whereas the latter is not time consuming and can read more than 90 samples at a time, with very small amount of reagent (Badarinath *et al*., 2010).

2.3.3 Quantitative antioxidant analysis

2, 2-diphenyl-1-picrylhydrazyl (DPPH) is one of the methods for measuring antioxidant activity. This technique is based on the reduction of DPPH. This is a free radical, very stable at room temperature, which yields a violet solution in methanol. Once it reacts with an antioxidant, its colour turns to light yellow owing to chain breakage (Badarinath *et al*., 2010).

In the DPPH free radical scavenging capacity method by TLC, the extracts that yield yellow or white spots against the purple background are well thought-out to be antioxidants. The extract is spotted on TLC plates and run it in appropriate solvent systems. The plate is sprayed with a methanol solution of DPPH (2 mg/mL). Antioxidants show yellow bands on a light purple background. The same technique could be used to identify total phenolic and total flavonoid content by altering solvent system and visualizing agent. Vanillin /H_2SO_4 reagent is sprayed on the plate and heated at 110 ^oC for 5 minutes, different functional groups of compounds could be identified (Badarinath *et al*., 2010). Orange-yellow spots indicate polyphenolic compounds. The plate if sprayed with natural substance-polyethylene glycol reagent and observed at UV-365 nm, flavonoids appear as yellow-orange fluorescent spots (Carocho and Ferreira, 2013).

2.4 SPECTROSCOPIC METHODS OF ANALYSING THE COMPOUNDS

2.4.1 Infrared spectroscopy

Infrared (IR) spectroscopy is one of the best and extensively used analytical methods for the characterization of organic compounds. It is centered on atoms vibrations in a molecule. This is determined by passing infrared electromagnetic radiation via a sample that has a permanent or induced dipole moment and determining what fraction of the incident radiation is absorbed at a specific energy (Stuart, 2004). The energy of each peak in an absorption spectrum matches with the frequency of the vibration of a molecule part, thus allowing qualitative identification of certain bond types in the sample (Kumirska *et al*., 2010). An IR spectrometer generally registers the energy of the electromagnetic radiation that is transmitted via a sample as a function of the wavenumber or frequency. Currently, the overall spectrum is studied by an interference procedure and changed into the frequency or wavenumber by means of a

mathematical process known as the Fourier transform. Fourier-transform infrared (FTIR) spectroscopy has tremendously enhanced the quality of infrared spectra and has reduced the time required to get data (Stuart, 2004: Smith, 1996; Günzler and Gremlich, 2002: Griffiths and De Haseth, 2007).

2.4.2 Ultraviolet/visible spectroscopy

Ultraviolet/visible (UV-Vis) spectroscopy is an important analytical technique for basically two motives. It can be used to detect some functional groups in molecule as well as for quantitative analysis. Unlike IR spectroscopy, UV-Vis spectroscopy consists of the absorption of electromagnetic radiation between 200–800 nm range and the successive excitation of electrons to higher energy states. Organic molecules that have chromophores (functional groups) absorb ultraviolet/visible light that contain valence electrons of low excitation energy (Kumirska *et al*., 2010). The UV-Vis spectrum is complex and seems as a continuous absorption band since the superimposition of rotational and vibrational transitions on the electronic transitions provides a combination of overlapping lines. At the moment, the individual detection of electron transfers minus superimposition by neighboring vibrational bands can also be recorded (Hunger and Weitkamp, 2001). With UV-Vis spectroscopy, it is possible to study electron transfers between orbitals or bands of atoms, ions and molecules existing in the gaseous, liquid and solid phase (Kumirska *et al*., 2010). Investigation of solutions and crystals usually takes place in transmission, while powdered samples are often measured in diffuse reflection mode (Diffuse Reflectance Spectroscopy– DRS). Unlike IR spectroscopy, where Fourier transform method dominates, dispersive spectrometers are more or less entirely used in UV-Vis spectroscopy. (Hunger and Weitkamp, 2001: Hollas, 1996: Ojeda and Rojas, 2004: Förster, 2004).

2.4.3 Mass spectrometry

Mass spectrometry (MS) is an analytical method used for determining the features of individual molecules. The basic information gained from mass spectrometric analysis is the molecular mass of a compound, which is determined by assessing the mass to charge ratio (m/z) of its ions. With the ionization method, full particulars about a molecule's elemental composition can be found. MS can analyse chemicals with a wide mass range–from small molecules to complex biomolecules such as carbohydrates, proteins, peptides or nucleic acids. The mass spectrometer is made up of three basic parts: an ion source, an analyser and a detector. The sample must be introduced to the ion source of the mass spectrometer– (Kumirska *et al*., 2010). In the ion source the sample molecules are ionized by one of a variety of methods (electron impact–EI, chemical ionization–CI, electrospray ionization–ESI, matrix assisted laser desorption ionization–MALDI). Next, the stream of ions is transferred to the analyser, where they are sorted and separated according to m/z. The best used analyzers are quadrupoles, ion trap and time of flight. In tandem mass analysis (MS/MS, MSn) a combination of two or more analysers are used. The MS can be operated in three modes: full scan (scanning of a selected mass range), more sensitive SIM (selected ion monitoring is equal to SIR - single ion recording), and the most sensitive MRM (multiple reaction monitoring). Finally, the ions reach the detector when their energy is changed to electrical signals that the computer can read. In the end, mass spectra are attained, and proof of identity of compounds is possible (Smith *et al.*, 2006: De Hoffmann and Stroobant, 2007).

2.4.4 Nuclear magnetic resonance spectroscopy

It is a very important analytical technique used in the structural determination of organic compounds. The most vital factor is to find a right solvent, which should have good solubility properties towards the target compound. When the sample is only incompletely soluble, no quantitative analysis will be accurate and reproducible (Heux *et al*., 2000: Rinaudo *et al*., 1992 Vårum *et al*., 1991). Furthermore, the residual signal of the solvent should not overlap the signals of the sample. The best solvents for liquid-state NMR spectroscopy are deuterium chloride solution (D_2O/DCl), deuterated acetic acid (D_2O/CD_3COOD), and deuterated formic acid (D_2O/DCOOD) (Desbrières *et al*., 1996: Lebouc *et al*., 2005: Weinhold *et al*., 2009: Yang and Montgomery, 2000).

Two type of ^{13}C NMR spectroscopy are available: liquid-state and solid-state. The liquid state has the same disadvantages as ^{1}H NMR spectroscopy, which are, the inadequate solubility of most analyzed materials. Furthermore, ^{13}C NMR spectroscopy is much less sensitive than ^{1}H NMR spectroscopy due to the properties of the carbon nucleus and the only 1% abundance of the ^{13}C isotope in nature. Liquid-state ^{13}C NMR spectra are typically recorded from the same kinds of solutions as are used for producing ^{1}H NMR spectra, but requires several scans from several dozen to hundreds of thousands. Solid-state ^{13}C NMR spectroscopy is a much more potent method. In this technique no solubilisation of samples is required. This means that quite a large amount of sample can be used, which solves the difficulty of the low sensitivity of ^{13}C NMR spectroscopy (Kumirska *et al*., 2010). The ^{13}C NMR spectra of solid samples are normally recorded with magicangle spinning (MAS) and cross-polarization (CP). MAS averages out dipolar interactions and chemical shift anisotropy, generating extremely resolved

spectra. CP significantly upsurges the sensitivity of the method by reducing the relaxation delay due to the magnetization transfer from the ^{1}H to the ^{13}C spins. The amounts of the ^{13}C NMR signals are influenced by the kinetics of the CP process: different contact times affect the intensities of ^{13}C NMR resonances. Hence, it is very important to use the proper contact and relaxation delay times (Duarte *et al*., 2001: Tolaimate *et al*., 2000: Guinesi *et al*., 2006).

2.4.5 Two dimensional NMR spectroscopy

The two dimensional NMR spectroscopy offers much more data about the molecule than 1D NMR and is very beneficial for characterising complex molecules. 2D NMR spectroscopic techniques that are frequently employed are correlation spectroscopy (COSY), nuclear overhauser effect spectroscopy (NOESY) and heteronuclear single quantum coherence (HSQC). COSY (correlation spectroscopy) and double quantum filtered correlation spectroscopy (DQF-COSY) allow one to determine which protons are spin-spin coupled (Kumirska *et al*., 2010). The proton signals that are two or three bonds apart are visible. NOESY displays correlations of all protons which are close enough for dipolar interaction by coupling through space (<5 Å). ^{1}H-^{13}C HSQC allows one to determine which protons are directly bonded with particular carbon atoms (Kumirska *et al*., 2010).

2.4.6 X-ray spectroscopy

Unarguably, X-ray spectroscopy is the most useful and extensively used means of identifying substances of all kinds (Guo, 2009). Generally, there are two forms of structural information that can be obtained by X-ray spectroscopy. These two are electronic structure, which focuses on outermost and innermost electrons that control the chemical and physical properties and

geometric structure that provides evidence about the positions of all or a set of atoms in a molecule at an atomic resolution. This technique covers several spectroscopic methods for determining the electronic and geometric structures of materials using X-ray excitation (Kumirska *et al*., 2010). These include X-ray emission spectroscopy (XES), X-ray absorption spectroscopy (XAS), X-ray photoelectron spectroscopy (XPS) and X-ray Auger spectroscopy. The kind of X-ray spectroscopy which is used rest on whether the target information is electronic, geometric or refers to oxidation states. For instance, XAS (first developed by de Broglie) is used to probe empty states and the shapes of molecules or local structures (Broglie, 1970), and XPS (first developed by Siegbahn) is used to investigate occupied electronic states (Siegbahn, 1970). X-ray spectroscopy is a powerful and flexible tool and a tremendous match to many structural analysis methods such as UV-Vis, IR, NMR or Raman (Kumirska *et al*., 2010).

Chapter 3

SELECTED PLANTS FORTHE STUDY

3.1 INTRODUCTION

Coelocaryon oxycarpum, Omphalocarpum ahia, Athostema aubryanum and Erythrophleum ivorense are well known for their ethnobotanical uses especially in western part of Ghana. These medicinal plants have been used in the management of parasitic infections, pain and inflammation among others. There is however not much scientific data support the purported claims.

3.2 THE FAMILY MYRISTICACEAE

Myristicaceae is a flowering plant family distributed in Africa, Asia, Europe, North and South America. It is occasionally known as the "nutmeg family", after its most renowned member, *Myristica fragrans*, the source of the spices nutmeg and mace. The most recognized genera are Myristica and Virola. The family comprises 20 genera, with 440 species of trees and shrubs found in tropical areas across the world. Species in this family are large trees that are patronised in the timber industry (Burkill, 1997). These species have coloured sap which are typically red and contain essential oils that are used as defense mechanisms that repel or poison many herbivorous organisms. The wood is pink to reddish as the sap exudes a red or yellow resin when cut. The leaves are usually aromatic and are glossy, dark green, simple, evergreen, and leathery. The flowers are normally small and bear either only three petals or no petals at all. The flowers are in groups and discharge a powerful scent. They are a greenish, whitish, or yellowish. The fruits are quite large and, in almost all the species, the fruits break naturally when mature (dehiscent fruits). It contains a single seed (Hawthorne and Jongkind, 2006).

3.2.1 The genus *Coelocaryon*

The genus *Coelocaryon* consists of four species, *botryoides*, *oxycarpum*, *preussii* and *sphaerocarpum*. Most of these species contain edible fat seed. The stem barks of these species are widely used in traditional medicine to manage schistosomiasis, dysmenorrhoea, dysentery and haemorrhoids. Bark decoctions or macerations are taken as expectorant, emetic and anodyne, and to treat cough, lung diseases, tachycardia, rheumatism and oedema. Bark decoctions are used as an enema to treat colic and diarrhoea, and the powder is applied to wounds as haemostatic (Hawthorne and Jongkind, 2006: Irvine, 1961).

3.3 COELOCARYON OXYCARPUM

3.3.1 Botanical description

Coelocaryon oxycarpum belongs to the family Myristicaceae and is a fairly large forest tree about 30 m tall, occurring from Senegal to Ghana. The wood is white on first cutting but a copious sap exudes from the vessels which immediately redden in the air imparting on the wood a clear reddish colour (Burkill, 1997: Irvine, 1961).

Figure 3.1 Whole plant and stem bark of *Coelocaryon oxycarpum* (Armah, 2015)

Ethnomedicinal uses

The wood bark is used to treat chronic pain, manage wounds, boil and piles. It is also used as a laxative and antihelmintic. Despite the widespread traditional uses, the plant remains untested for its pharmacological properties and phytochemistry principles (Irvine, 1961).

3.4 THE FAMILY SAPOTACEAE

Sapotaceae is a family of flowering plants belonging to the order Ericales. The family consists of 65 genera which includes 800 species of evergreen trees and shrubs. They are distributed in the tropical regions. Fruits from most of these species are edible. Species with edible fruits include *Chrysophyllum cainito* (star-apple or golden leaf tree) and *Planchonia careya* (Australian native plum). *Vitellaria paradoxa* produces edible shea butter; which is a major source of lipid for many West African ethnic groups (Burkill, 1997: Irvine, 1961). It is also used in production of cosmetics and ointment for skin medications. Seeds of *Argania spinosa* (L.) yields edible oil harvested in Morocco for traditional uses. The family name is obtained from zapote, a Mexican vernacular name for one of the plants and latinised by Linnaeus as sapota, a name now treated as a synonym of Manilkara. Previous phytochemical studies of the Sapotaceae family have shown the presence of alkaloids, phenolic compounds, glycerol, fatty acids, flavonoids, saponins and triterpenes (Baliga *et al*., 2011: Charrouf and Guillaume, 1999; Charrouf and Guillaume, 2007: Akihisa *et al*., 2010: Akihisa *et al*., 2011).

3.4.1 The genus *Omphalocarpum*

Omphalocarpum is a genus of plants belonging to the family Sapotaceae. The genus is common to tropical Africa. It consists of about twenty-seven species. These rainforest trees are

20-30 m tall and are mostly famous for their woody-walled, pumpkin-sized, and trunk-borne timber (Burkill, 1997; Irvine, 1961; Hawthorne and Jongkind, 2006). Their fruits are mostly eaten and dispersed by elephants (Normand *et al*., 1976: Nchanji *et al*., 2003).

3.4.2 Ethnomedicinal uses

In Africa, decoctions, powders and macerations of plants of the genus *Omphalocarpum* are used for years in traditional medicine to manage headaches; wounds, skin diseases, constipation, elephantiasis, fever, cough, and rheumatism (Irvine, 1961: Bouquet, 1974: Aubréville, 1964: Betti, 2004). In Nigeria, bark decoctions of the plant is used to treat constipation. In Cameroon, bark decoctions together with fruits of *Capsicum annuum* L (Betti, 2004) and *Solanum anguivi* Lam. is used to manage malaria and lactation problems. Decoction of the young leaves is used for the management of cough. Mixture of the seeds and crushed bark with palm wine is used as a laxative in case of poisoning and to manage hernia in Côte d'Ivoire. In Sierra Leone the seeds are used in the management of yaws. In Ghana the bark is used to treat stomach-ache, rheumatism, pain and piles.

3.4.3 Pharmacological activities

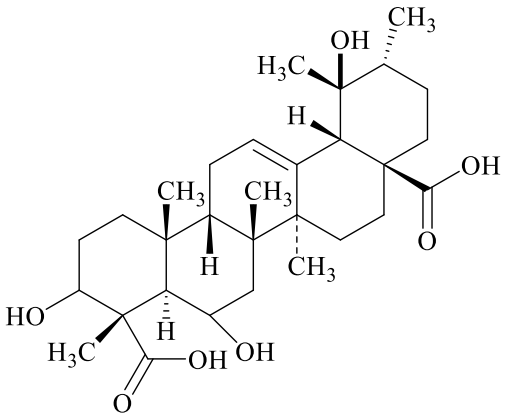
There is scarce information on the biological activity of this genus. Stem bark extract of *Omphalocarpum elatum* Miers (Sapotaceae) has been reported to have weak antibacterial activity (Sandjo *et al.,* 2014). The dichloromethane-methanol (1:1) extract of the fruit pericarp of *Omphalocarpum procerum* exhibited antiplasmodial, anti-leishmania and antitrypanosomal activity (Ngamgwe *et al*., 2014).

3.4.4 Phytochemistry of *Omphalocarpum* species

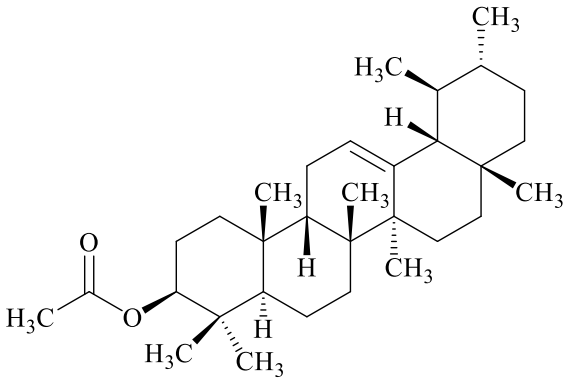
Few compounds, mainly terpenoids and steroids, have been reported from this genus.

3.4.5 Terpenoids from *Omphalocarpum* species

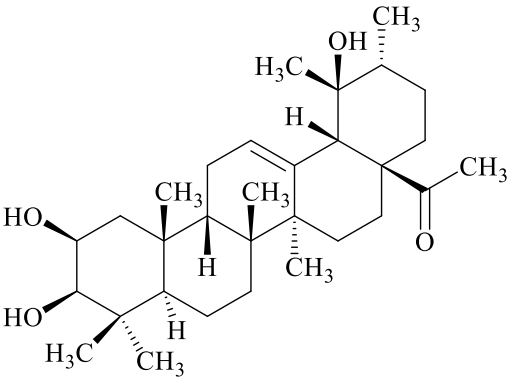
Terpenoids reported from this genus include the new triterpene diastereomer, elatumic acid **(24)**, which was isolated from the stem bark of *Omphalocarpum elatum* Miers (Sapotaceae) along with α-amyrin acetate **(25)** and tormentic acid **(26)** (Sandjo *et al*., 2014). Phytochemical investigation of a dichloromethane-methanol (1:1) extract of the fruit pericarp of *Omphalocarpum procerum* afforded the new fatty ester triterpenoid 3β-hexadecanoyloxy-28-hydroxyolean-12-en-11-one (procerenone) **(27)**, together with, betulin **(28)**, β-amyrin **(29)**, and lupeol acetate **(30)** (Ngamgwe *et al*., 2014).



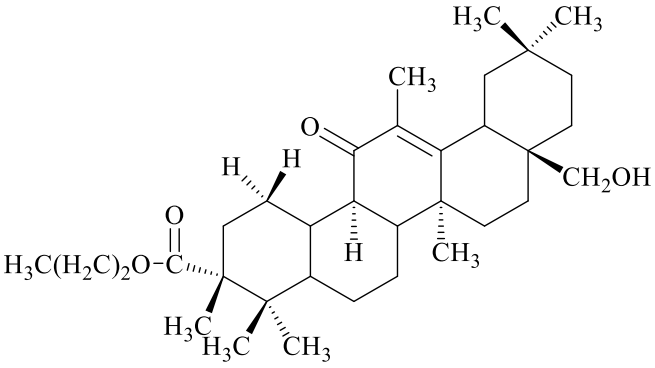
Elatumic acid**(24)**



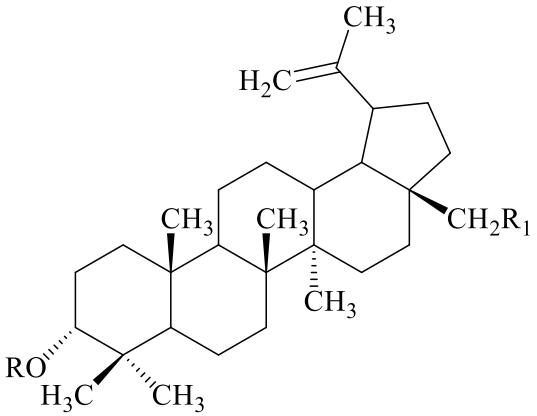
α-amyrin acetate **(25)**



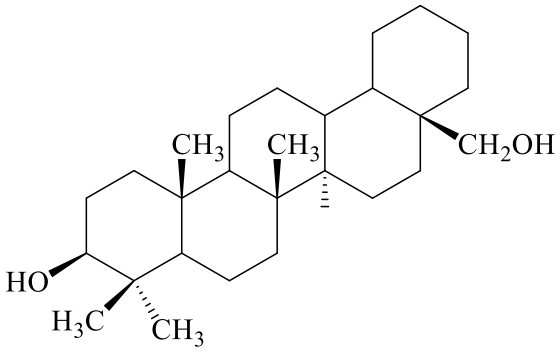
Tormentic acid **(26)**



Procerenone **(27)**



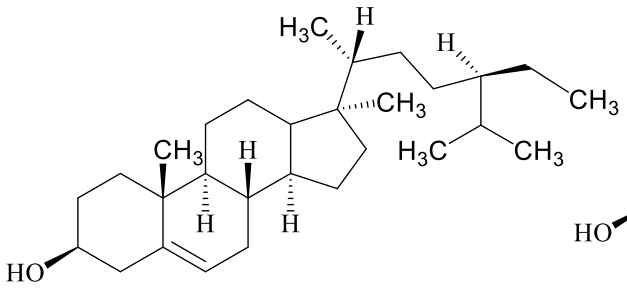
Betulin **(28)** R =H R1 =OH
Lupeol acetate **(30)** R =Ac R1 =H



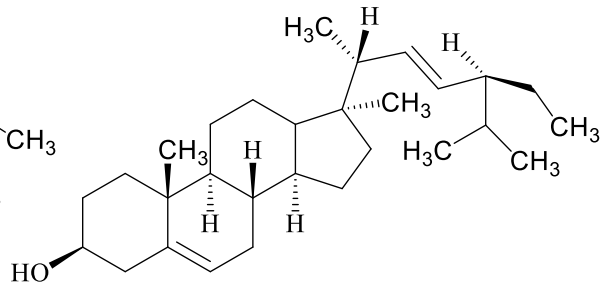
α- amyrin

3.4.6 Steroids from *Omphalocarpum* species

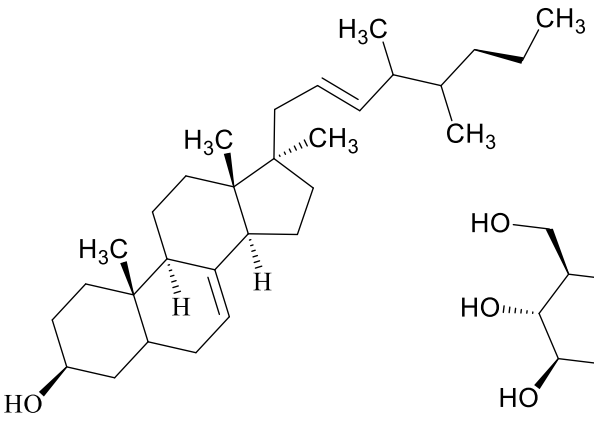
Stigmasterol **(31)** and β-sistosterol **(32)** have been reported from a dichloromethane-methanol (1:1) extract of the fruit pericarp of *Omphalocarpum procerum* (Ngamgwe *et al.*, 2014). Spinasterol **(33)** and spinasterol 3-O-β-D-glucopyranoside **(34)** were obtained from stem bark of *Omphalocarpum elatum* (Sandjo *et al*., 2014).



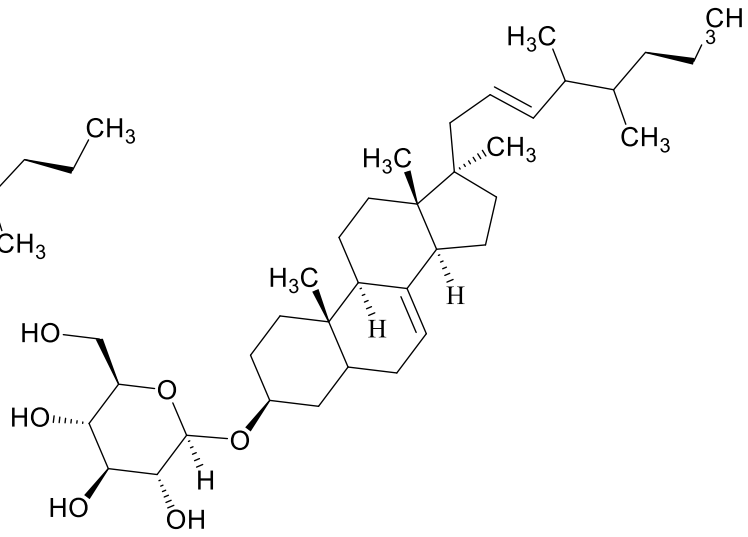
Stigmasterol (31)



β-sistosterol **(32)**



Spinasterol **(33)**



Spinasterol 3-O-β-Dglucopyranoside **(34)**

3.5 OMPHALOCARPUM AHIA

Omphalocarpum ahia occurs mostly in West Africa but majority of them are found in Sierra Leone and Ghana. In Ivory Coast it is called ‘Abe aguia’ or ‘akye ahia’. In Liberia it called ‘mano zẽia’. In Ghana the Asantes called it ‘osonodokono’ while the Nzemas called it ‘asoro’. In Ghana, it is mostly found in the forest regions (Irvine, 1961).

3.5.1 Botanical description

It is an evergreen medium-sized tree, about 30 m tall, and exudes a whitish fluid from the incised bark. The trunk is straight and cylindrical (Figure 3.1) but often slightly furrowed up to

80–180 cm in diammeter, which lacks buttresses; bark is rough and brown with lenticels in longitudinal rows. The inner bark is reddish brown and leathery (Burkill, 1997).

Figure 3. 2Whole plant, stem bark, fruit and leaves of *Omphalocarpum ahia* (Armah, 2015)

3.5.2 Non-medicinal uses

In Ghana the wood is used for house construction and joinery works. The wood is used for timbers implements such as mortars, bowls, handles, seats and drums. It is used in canoes construction. Moreover, the seeds have ornamental value for traditional dancers during festival for example kundum and abisa in Nzema.

3.5.3 Pharmacology and phytochemistry of *O. ahia*

There is no scientific credence for the ethnomedicinal uses of this plant. Phytochemical investigation of this plant is non existent.

3.6 THE FAMILY EUPHORBIACEAE

Most herbs, shrubs, and trees belong to the Euphorbiaceae which are mainly monoecious. It is one of the biggest families of plants with about 300 genera and 7,500 species (Hawthorne and Jongkind, 2006). The species in this family produce a milky sap. The leaves are simple or compound with its arrangement mostly alternate but may be opposite or whorled or sometimes highly reduced. Stipules are sometimes present but may be reduced to hairs, glands or spines. The flowers are unisexual and normally actinomorphic. Cyathium could be found in about 1,500 species including the genera Euphorbia and Chamaesyce. The cyathium is made of a single naked pistillate flower enclosed by cymes of naked staminate flowers, each made up of a single stamen (Brown *et al*., 1913).

3.6.1 The genus *Anthostema*

Anthostema is a flowering plant genus in the Family Euphorbiaceae (Spurge family). It is native to Africa and Madagascar and comprises of three species; *Anthostema aubryanum* Baill, found in West and Central Africa from Ivory Coast to Cabinda (Province of Angonla), *Anthostema madagascariense* Baill., found in Madagascar, and *Anthostema senegalense* A. Juss found mainly in West Africa from Senegal to Benin (Govaerts *et al*., 2000).

3.6.2 *Pharmacology and phytochemistry*

The crude aqueous extract of the stem bark of *Anthostema senegalense* has been reported to show strong anthelmintic activity against the larvae of *Haemonchus contortus*. A crude stem bark extract exhibited significant activity against *Leishmania donovani* with IC_{50} of 9.1 µg/mL, as well as moderate antibacterial and antifungal activities (Abreu *et al*., 1999).

3.7 ANTHOSTEMA AUBRYANUM

Anthostema aubryanum is known in the Ivory Coast as 'mauli'(ABE), 'sese' (Anyi) while Kyama people called it 'Anaya'. In Ghana, among the Akans, it is called 'kyirikisa' which means 'hates talking'. In Nigeria, the Yoruba people call it 'òdògbo' (Hawthorne and Jongkind, 2006).

3.7.1 Botanical description

Anthostema aubryanum belongs to the family Euphorbiaceae. It is a medium size tree, grows up to about 26 m tall with a straight trunk to about 16 m long by 1.60 m in girth (Figure 3.2). It is present in swamp-forests and on river banks in Cote d'lvoire to Nigeria. It is used as a purgative and for the management of edema in Cote d'Ivoire. It's also a good source of fuel to local people in Gabon. It contains latex and is used as a fish poison in Gabon (Hawthorne and Jongkind, 2006).

Figure 3. 3 Whole plant, stem bark and leaves of *Anthostema aubryanum* (Armah, 2015)

3.7.2 Ethnomedicinal and non-medical uses

The latex is toxic, acrid and vesicant, and can cause blindness. In spite of its toxicity, it is used, with suitable precautions, as a strong purgative. In Ghana, it is used to treat leprosy, menstrual pains and help with the expulsion of the placenta (Burkill, 1997). For these purposes, small amounts of latex or pulped roots are added to food. The latex is also used as an antidote for food poisoning; it is diluted with water and drunk till vomiting occurs. Bark macerations are drunk to treat intestinal parasites, kidney problems, oedema, impotence and mental illness (Koné *et al*., 2005). In Sierra Leone, young leaves are ground with flour and the dried paste is taken as a laxative. In Senegal, the plant is chopped into pieces and thrown into pools as fish poison to catch small fishes. The wood is white and lightweight and is easy to work with. It is used for local building purposes, poles for temporary fences, light carpentry work and boxes. In Sierra Leone, the sticky latex is used as bird lime (Neuwinger *et al*., 2000).

3.7.3 *Pharmacological activity*

The plant has been reported to have antimicrobial, antihelmintic anti-leishmanial activity and contains phorbol esters (Abreu *et al*., 1999).

3.8 THE FAMILY FABACEAE

The Fabaceae or Leguminoseae, usually well-known as the legume, pea, or bean family, are a large and economically significant family of flowering plants. It comprises trees, shrubs, and herbaceous plants, perennials or annuals, which are easily recognized by their fruit (legume) compound, and their stipulated leaves. The group usually spread and is the third-largest land plant family in terms of number of species, behind only the Orchidaceae and Asteraceae, with

630 genera and over 18,860 species. (Judd *et al*., 2002: Stevens, 2008). The five largest of the 630 legume genera are Astragalus (over 2,000 species), Acacia (over 1000 species), Indigofera (around 700 species), Crotalaria (around 700 species), and Mimosa (around 500 species), which constitute about a quarter of all legume species. About 18,000 legume species are known, amounting to about 7% of flowering plant species (Judd *et al*., 2002: Magallón *et al*, 2001). Fabaceae is the most common family found in tropical rainforests and dry forests in America and Africa (Burnham and Johnson, 2004).

3.8.1 The genus Erythrophleum

The genus *Erythrophleum* consists of 10 species. These include *Erythrophleum africanum* (Benth) Hharms, *Erythrophleum chlorostachys* (F. Muell.) Baill., *Erythrophleum couminga* Baill, *Erythrophleum fordii* Oliv., *Erythrophleum ivorense* A. Chev, *Erythrophleum lasianthum* Corbishley, *Erythrophleum letestui* A Chev., *Erythrophleum suaveolens* (Guill. & Perr.) Brenan, *Erythrophleum succirubrum* Gagnep and *Erythrophleum teysmannii* (Kurz) Craib (Burkill, 1997: Irvine, 1961; Hawthorne and Jongkind, 2006).

3.8.2 Ethnomedicinal uses of *Erythrophleum* species

The stem barks of Erythrophluem species are traded as ‘sassy-bark’, ‘mancona bark’ or ‘casca bark’. They are used as emetic and purgative. The crushed bark is applied to swellings caused by filaria. In Democratic Republic of Congo, the dried powdered bark is taken as a snuff to cure headache. In Kenya, a diluted decoction of the roots is used as an anthelminthic, especially against tapeworm. In Malawi, a decoction of the roots and bark is applied to soothe general body pain. Pieces of root or bark are a protective and love charm. The bark has been used in

arrow poisons and as ordeal poison and the bark and leaves as fish poison. The use as an anaesthetic for fish in aquaculture is tricky as small differences in dosage will kill, rather than stupefy fish. In the Western world, bark extracts were used in the late 19th century to treat heart failure. Side effects and better results with digitoxine ended this practice (Burkill, 1997: Irvine, 1961: Hawthorne and Jongkind, 2006). Bouquet, (1969) stated that, some of the *Erythrophleum* species are traditionally used to manage inflammation and pain.

3.8.3 Pharmacological activity of *Erythrophleum* species

Very few biological activities have been reported from this genus. Listed among them are their anti- inflammatory and anti-microbial activities.

3.8.4 Anti-inflammatory and analgesic activity

Dongmo *et al.,* (2001) reported the anti-inflammatory and analgesic properties of the stem bark of different fractions from *E. Suaveolens*. The effect of the fractions on carageenan-indeced paw oedema showed a maximum antio-edematous effect of about 47.06 and 38.2 % respectively for 1 and 2 hours after caregeenan administration.

3.8.5 Antimicrobial activity

The ethanolic extract of *E. africanum* exhibited diverse activities against different microbes with zone of inhibition between 12 – 36 mm, MIC ranging from 3.25 60 mg/mL to 60 mg/mL. (Mohammed *et al.*, 2014). Several other *Erythrophleum* species have been reported to have anti-bacterial, anti-termicidal and anti-fungal properties (Antwi-Boasiako and Damoah, 2010).

3.8.6 Antioxidant activity

The antioxidant activities, total phenolic contents and chemical compositions of extracts from 4 Cameroonian woods, showed the toluene /ethanol extracts of *Erythrophleum suaveolens* to exhibit the highest antioxidant activity with $1C_{50}$ of 3.1 mg/L (Saha *et al*, 2013).

3.8.7 Phytochemistry of *Erythrophleum* species

Cassaine type diterpenes are the main chemical constituent of the *Erythrophleum* genus, although other chemical constituents such as triterpene and steroids have been reported.

3.8.7.1 Cassane and norcassane diterpenes

The crystalline diterpene alkaloid cassaine was isolated as the main compound in *Erythrophleum guineense* (Dalma *et al*, 1939). The cassane **(35)** skeleton is basically considered as a tricyclic diterpene with a replacement of ethyl group at C-13 and one methyl group at C-14 position whereas norcassanes **(36)** have one carbon less from cassane each from C-17 or C-16 positions. In some works, it was reported that the cassane skeleton might be obtained from pimarane by the methyl migration from C-13 to C-14 while 17-norcassane diterpenes may be biosynthesized via decarboxylation of cassane-type diterpenes and 16-norcassane-type diterpenes, probably derived from oxidative cleavage of the C-15 and C-16 double bond (Figure 3.3) (Arjun, 2003: Overtone, 1974). These groups of compounds displayed extensive array of pharmacological actions including anti-inflammatory, anti-tumor, antimicrobial, antiviral, antitrypanosomal and antimalarial.

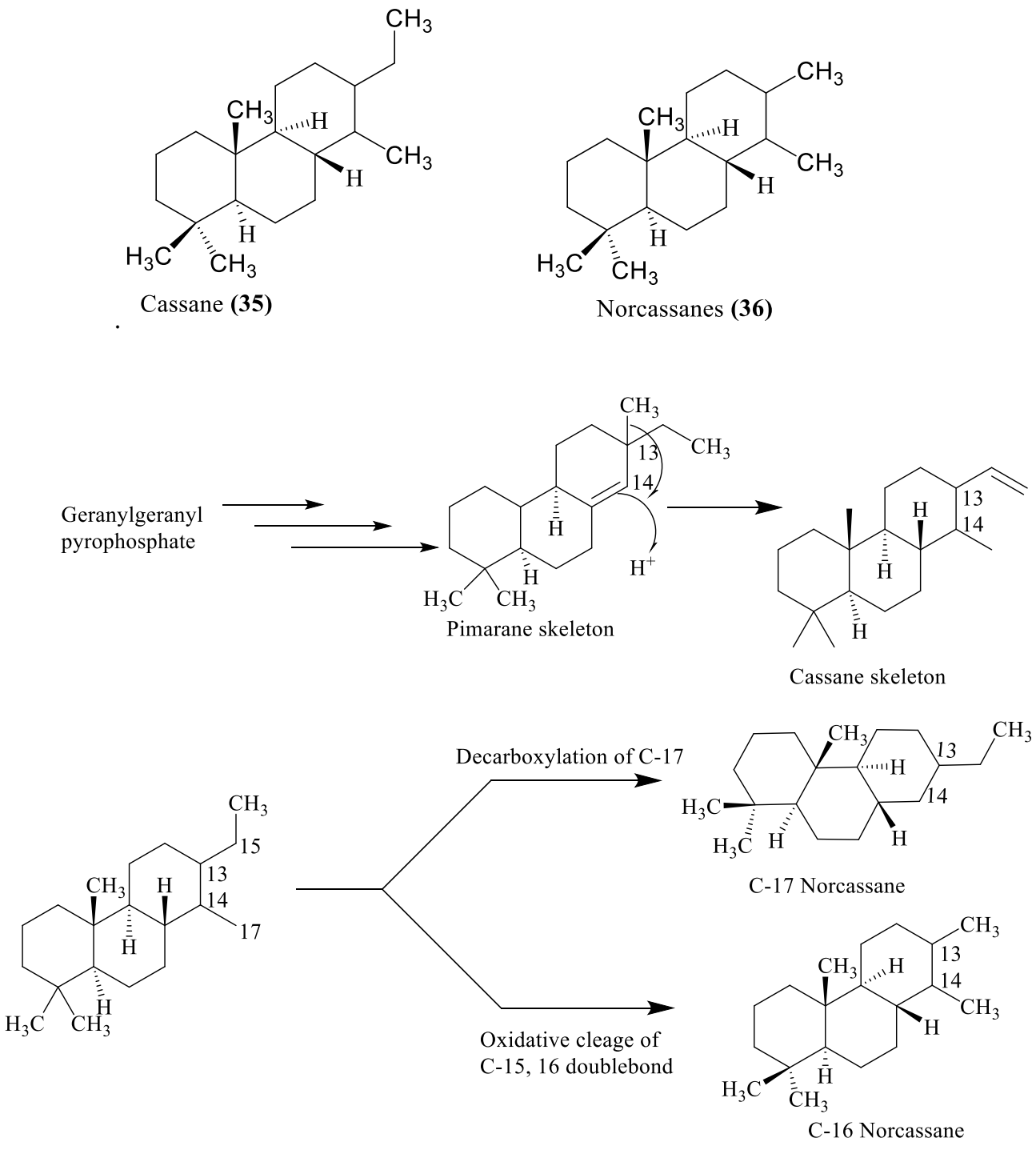


Figure 3. 1 Biosynthetic pathway of cassane and norcassane (Maurya *et al.*, 2012)

Fabaceae has few genera containing cassane diterpenes. However, that of norcassane diterpenes is limited to the genus *Caesalpinia* (*C. crista* or *C. bonduc*) with the exception of one compound isolated from *Chamaecrista* genus. Cassane diterpenes have been reported from *Acacia jacquemontii, Acacia nilotica, Bobgunnia madagascariensis, Bowdichi aniti, Caesalpinia benthamiana, Caesalpinia bunduc, Caesalpinia bonducella, Caesalpinia crista, Caesalpinia decapetala, Caesalpinia magnifoliolata, Caesalpinia major, Caesalpinia minax, Osteospermum muricatum, Swartzia arborescens, Vouacapoua americana*, and one species of the Poaceae family *Oryza sativa*. A cassane diterpene has been reported from the fungi genus *Sarcodon*.

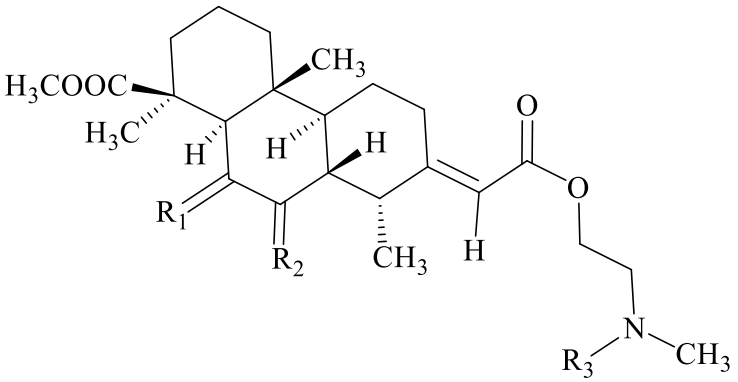
3.8.8 Cassane diterpenoid amines and amides

According to Ruzicka *et al.*, (1944), Cassane diterpenes with nitrogen containing side chain at C-13 position is the principal chemical compound of the *Erythrophleum* genus, called Erythrophleum alkaloids. Basically, they are divided into two sets. They are diterpenoid ester amines and diterpenoid amides.

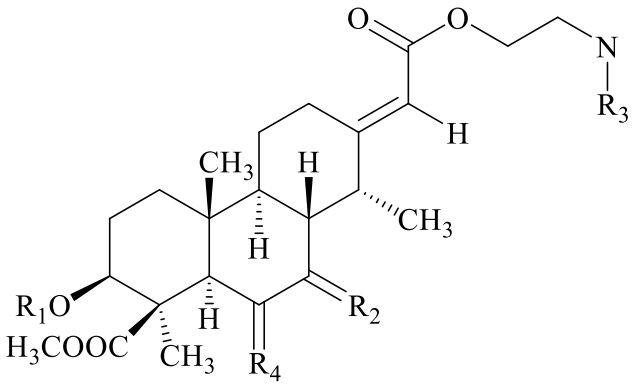
3.8.8.1 Cassane diterpenoid amines

According to Cronlund, 1973 and Hauth *et al.*, 1965, cassane ester amines are the esters of secondary or tertiary N-methylaminoethanol with cassane type diterpenic acid. Cassaine **(37),** coumidine **(38)**, coumingidine **(39),** cassamine **(40)** erythrophleguine **(41)** and erythrosuamine **(42)** have been reported from *Erythrophleum guineense* (Dalma *et al.*, 1939: Clarke, 1997; Tsutsui and Tsutsup, 1959; Thorell *et al.*, 1968). Nor-erythrostachamine **(43),** cassaidine **(44)**, cassamidine **(45)** and norcassamidine **(46)** have also been reported from the bark of

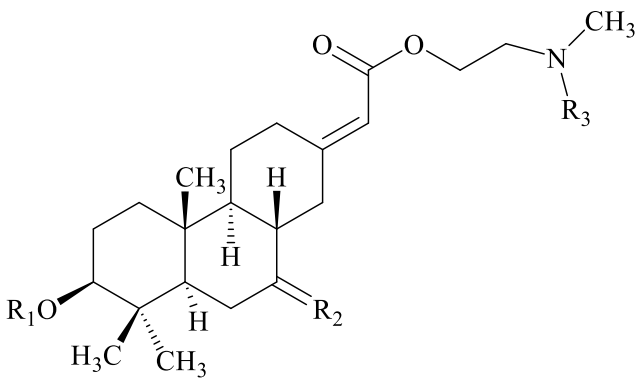
Erythrophleum chlorostachys (Thorell *et al.*, 1968: Blount *et al.*, 1940: Loder *et al*, 1972, 1974). Coumingine **(47)** was isolated from from *Erythrophleum couminga* (Ruzicka *et al*, 1941; Ruzicka *et al*, 1941). Erythrosuavine **(48)** was obtained from the stem bark of *Erythrophleum suaveolens* (Manfouo *et al*, 2005). Erythrophlamine **(49)** was reported from the bark of *Erythrophleum guineense* and *Erythrophleum couminga* and 3β-hydroxy-nor-erythrosuamine and 3β-hydroxynor-erythrosuamine-3-O-β–Gluco-pyranoside **(50–51)** were obtained from the seeds of *E. lasianthum* (Verotta *et al*, 1995).



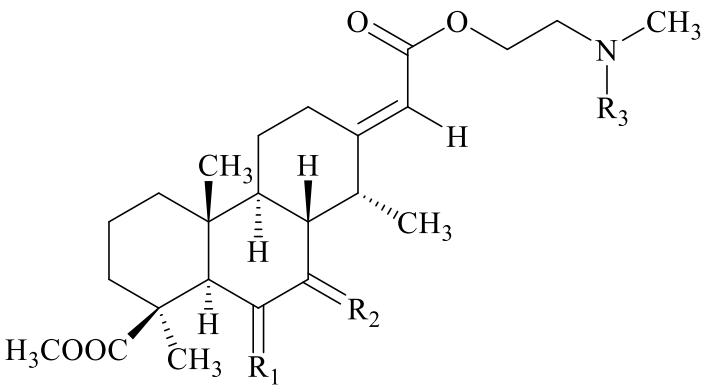
	R_1	R_2	R_3
Cassaine **(37)**	β-H, α-H	O	CH_3
Coumidine **(38)**	α-OH, β-H	O	CH_3
Coumingidine **(39)**	O	β-OH, α-H	CH_3



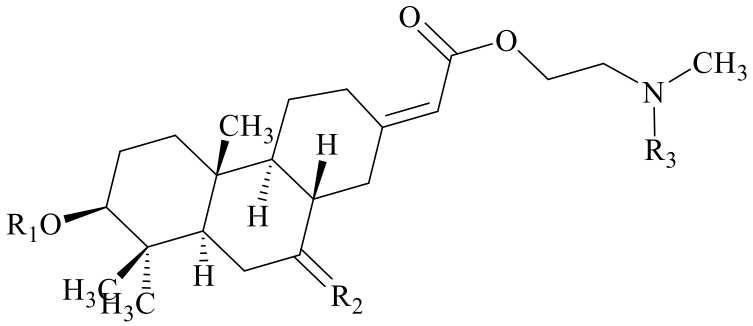
	R_1	R_2	R_3	R_4
Cassamine **(40)**	H	H, OH	H	β-H, α-H



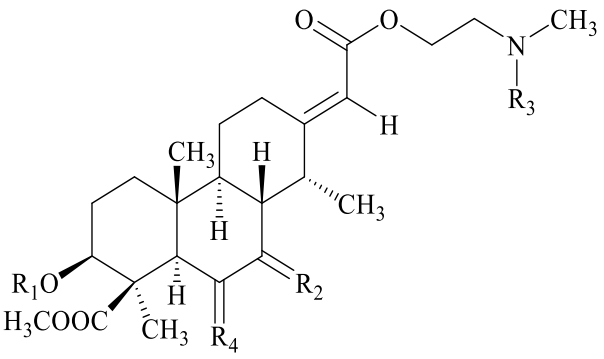
	R_1	R_2	R_3
Erythrophleguine **(41)**	$COCH_2C(OH)(CH_3)_2$	β–OH, α–H	CH_3



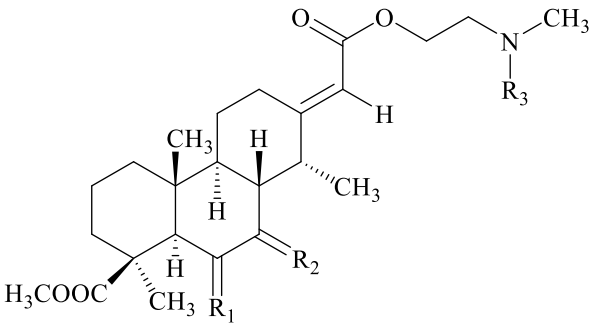
	R_1	R_2	R_3
Erythrosuamine **(42)**	O	β–OH, α–H	CH_3



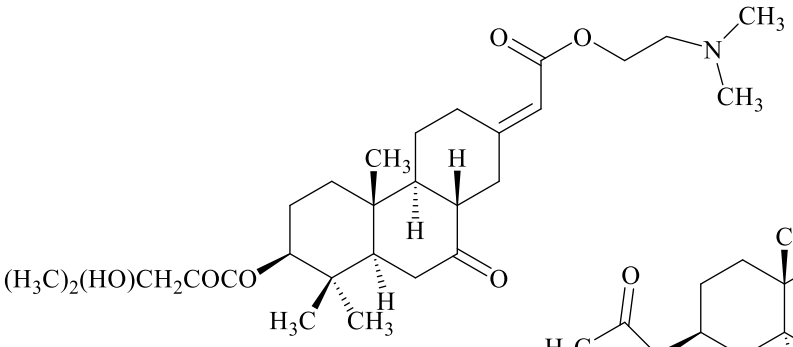
	R_1	R_2	R_3
Nor-erythrostachamine **(43)**	H	β–OH, α–H	CH_3



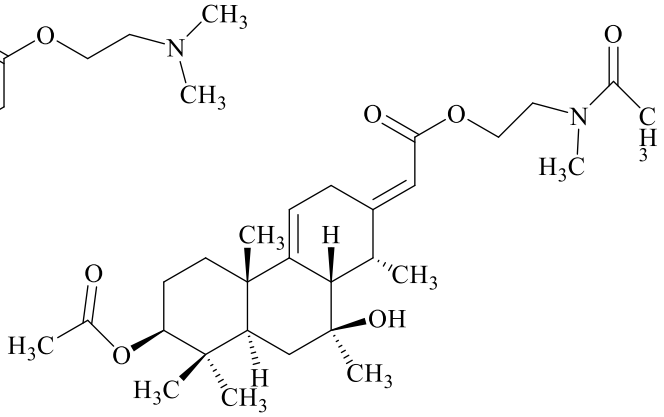
	R_1	R_2	R_3	R_4
Cassaidine **(44)**	H	H, OH	H	β–H, α–H



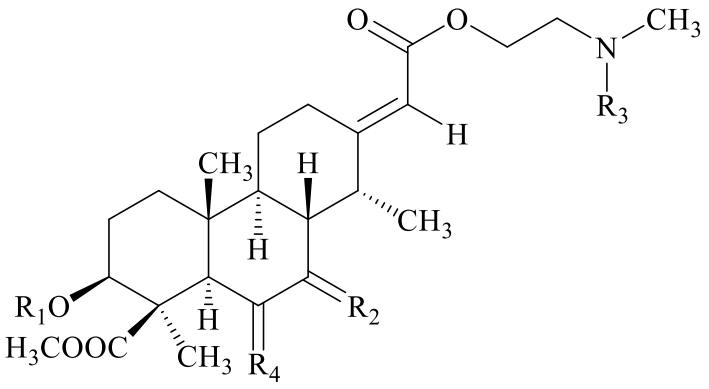
	R_1	R_2	R_3
Cassamidine **(45)**	β–H, α–H	β–OH, α–H	CH_3
Norcassamidine **(46)**	beta-H, alpha-H	β–OH, α–H	H



Coumingine **(47)**



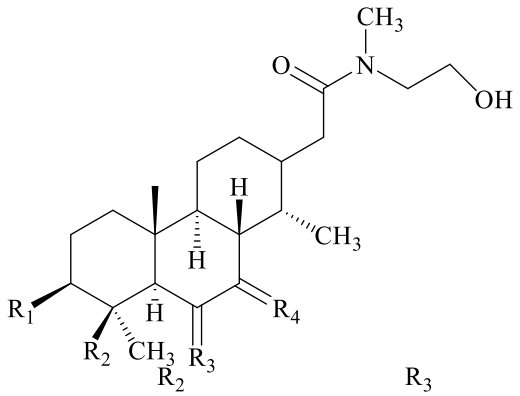
Erythrosuavine **(48)**



	R_1	R_2	R_3	R_4
Erythrophlamine **(49)**	H	O	CH_3	b-H.
3β-hydroxy-nor-erythrosuamine **(50)**	H	OH,	H	O
3β-hydroxynor-erythrosuamine-3-O-β-Gluco-pyranoside **(51)**	β-D-Glucopyranosyl ,	OH	H	O

3.8.8.2 Cassane diterpenoid amides

They are made up of cassane type diterpenoic acid, which is amide linked to N-methylaminoethanol. Nor-cassaidide **(52),** nor-erythrophlamide **(53)** (Loder *et al*, 1972) and nor erythrostachamide **(54)** were reported from *E. chlorostachys* (Cronlund, 1973; Loder *et al*, 1971). 3-acetyl-nor-cassaidide **(55)** (Cronlund, 1973) and nor-cassamide **(56)** were isolated from the bark of *E. fordii, E. ivorense, E. lasianthum* and *E. guineense* (Cronlund, 1973: Loder *et al*, 1972: Sandberg *et al.*, 1971: Qu *et al.*, 2006). 3β-hydroxy-nor-erythrosuamide and 3β-hydroxy-nor-erythrosuamide-3-O-β-D-glucopyranoside **(57–58)** were reported from seeds of *E. lasianthum* (Verotta *et al.*, 1995).

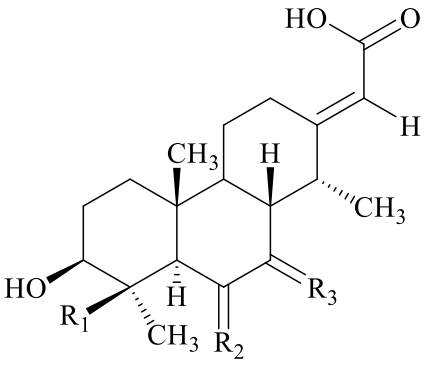


	R_1	R_2	R_3	R_4
52	OH	CH_3	β–H,α–H	β–OH,α–H
53	OH	$COOCH_3$	β–H, α–H	O
54	H	$COOCH_3$	β–H,α–H	O
55	OAc	CH_3	β–H,α–H	β–OH,α–H
56	H	$COOCH_3$	β–H,α–H	O
57	OH	$COOCH_3$	O	β–OH,α–H
58	O-Glucopyranosyl	$COOCH_3$	O	β–OH,α–H
59	O-Tigloyl	$COOCH_3$	β–H,α–H	O
60	OAc	$COOCH_3$	β–H,α–H	O
61	H	$COOCH_3$	O	β–OH,α–H
62	H	$COOCH_3$	α–OH, β–H	O
63	H	$COOCH_3$	β–OH,α–H	O
64	H	$COOCH_3$	O	β–OH,α–H

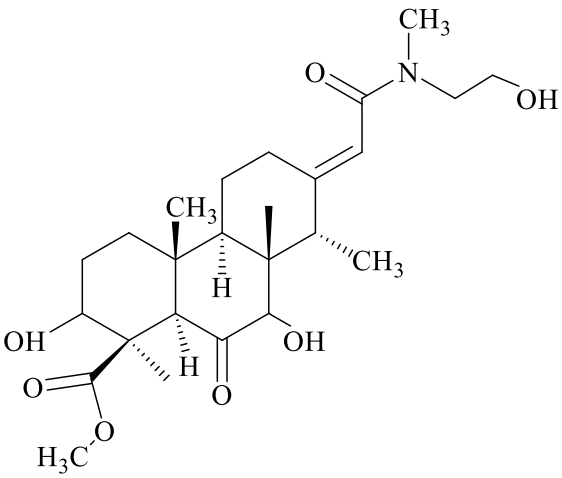
Nor-cassaidide **(52)**, nor-erythrophlamide **(53),** nor erythrostachamide **(54),** 3-acetyl-nor-cassaidide **(55)** nor-cassamide **(56),** 3b-hydroxy-nor-erythrosuamide **(57)**, 3b-hydroxy-nor-erythrosuamide-3-O-b-D-glucopyranoside **(58),** 3b-tigloyl-nor-erythrophlamide **(59)**, 3b-acetyl-nor-erythrophlamide **(60)**, 7-dehydro-nor-erythrosuamide **(61)**, 6a-hydroxy-nor-cassamide **(62)**, 6a-hydroxy-nor-cassamide 22-O-b-D-galactopyranoside **(63)** and nor-erythrosuamide-22-O-b-D-galactopyranoside **(64)**

From the bark of *E. fordii* have been isolated 3β-tigloyl-nor-erythrophlamide, 3β-acetyl-nor-erythrophlamide, 7-dehydro-nor-erythrosuamide, 6α-hydroxy-nor-cassamide, 6α-hydroxy-nor-cassamide 22-O-β-D-galactopyranoside and nor-erythrosuamide-22-O-β-D-galactopyranoside **(59–64)** (Qu *et al.*, 2006).

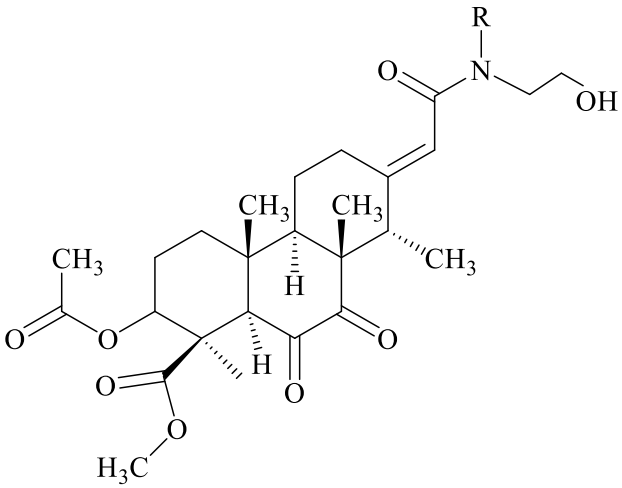
Tsao *et al.*, (2008) also isolated three novel cassaine-type diterpenoids, christened erythrofordin A-C **(65–67)** and norerythrofordin A **(68)** from *E. fordii***.** Two new diterpenoid alkaloids erythroformide and 3β-acetooxynorcassamide **(69- 70)** have been reported from the same plant (Manh *et al.*, 2013). Fractionation and isolation of the extracts of *E succirubrum* produced cassaine diterpenoid dimers erythrophlesins A-D **(71–74)** which have tumor necrosis factor-related apotosis inducing ligand (TRAIL) resistance-overcoming capacity (Miyagawa *et al.*, 2009). Phytochemical investigation of *Erythrophleum fordii* leaves produced cassaine diterpenoid–diterpenoid amide dimmers erythrophlesin E-G **(75–77)** and seven other cassaine diterpenoid amides **(78–84)** (Du *et al.*, 2011). Four new diterpenoid amides 22-acetoxy-nor-cassamide, 22-acetoxy-6α-hydroxy-nor-cassamide, 8-dehyro-nor-cassamide and 6α-hydroxy-nor-erythrophlamide **(85-88)** have been reported from stem bark of *Erythrophleum suaveolens* (Kablan *et al.*, 2014). Four new cassane diterpenoids namely 6α-hydroxy-cassamic acid **(89)**, methyl ester, 4β-carbomethoxy-14-methyltotarol **(90)**, 6α hydroxy-nor-cassamine **(91)**, and 8, 9-dehydro-nor-cassamine **(92)** have been isolated from the same plant (Dade *et al.*, 2015).



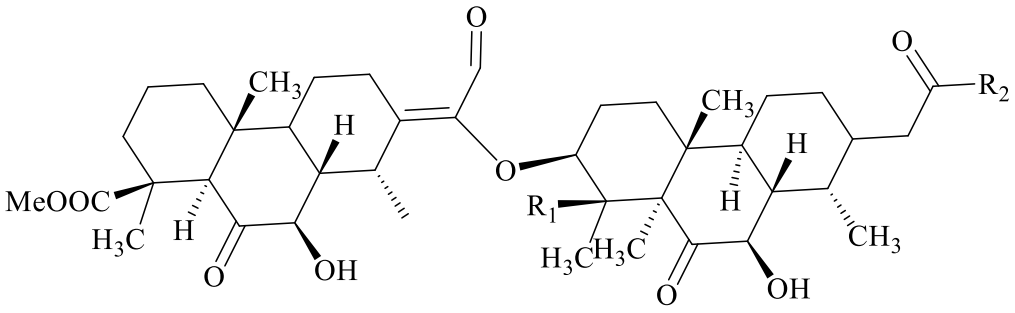
	R_1	R_2	R_3
Erythrofordin A (**65**)	$COOCH_3$	O	β–OH, –αH
Erythrofordin B (**66**)	$COOCH_3$	O	β–OAc, α–H
Erythrofordin C (**67**)	$COOCH_3$	β–OH, α–H	O
Norerythrofordin A (**68**)	H	O	β–OH, α–H



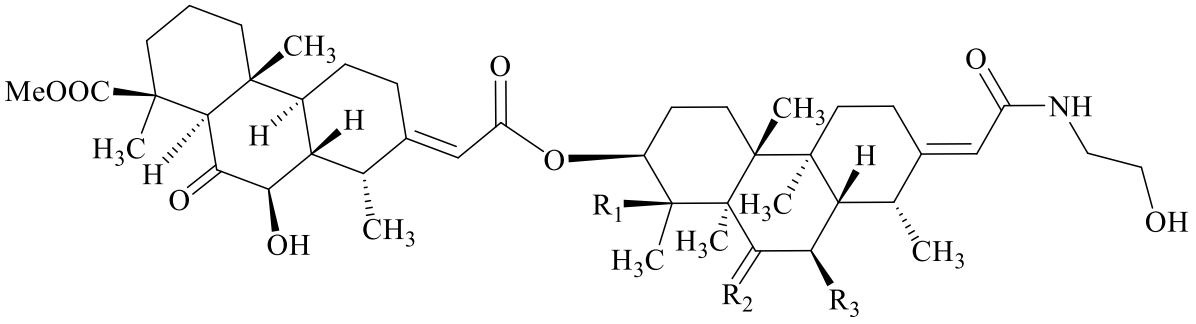
Erythroformide **(69)**



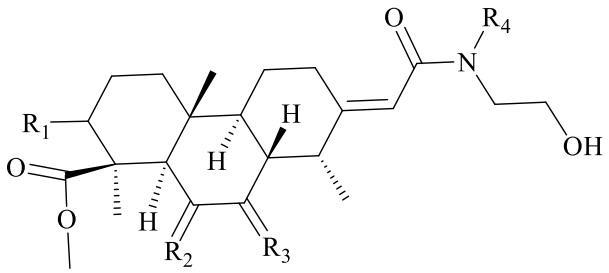
3β-acetooxynorcassamide **(70)**



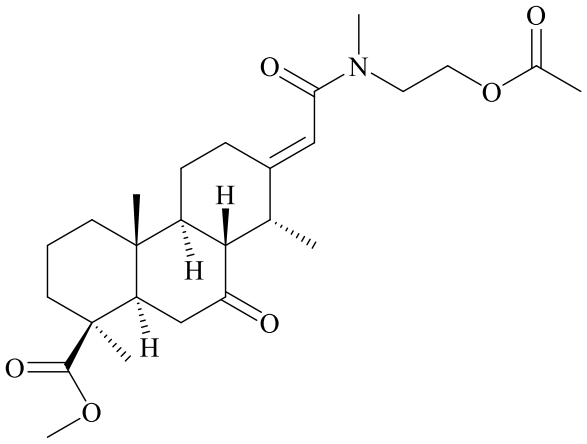
	R_1	R_2
Erythrophlesin A (**71**)	H	OCH_3
Erythrophlesin B (**72**)	CO_2CH_3	OCH_3
Erythrophlesin C (**73**)	H	$N(CH_3)CH_2CH_2OH$
Erythrophlesin D (**74**)	CO_2CH_3	$N(CH_3)CH_2CH_2OH$



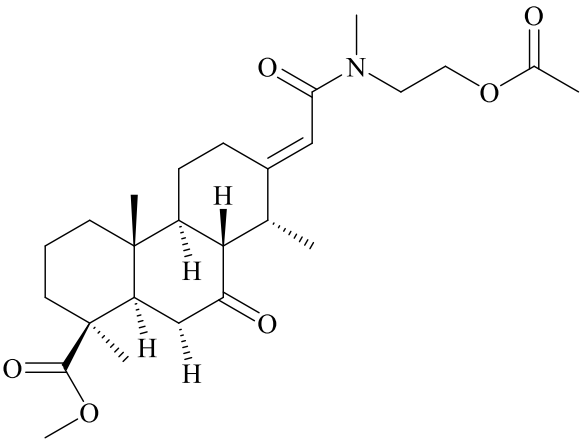
	R_1	R_2	R_3
Erythrophlesin E (**75**)	$COOCH_3$	O	β–OH, α–H
Erythrophlesin F (**76**)	H	O	β–OH, α–H
Erythrophlesin G (**77**)	$COOCH_3$	α–OH, β–H	O



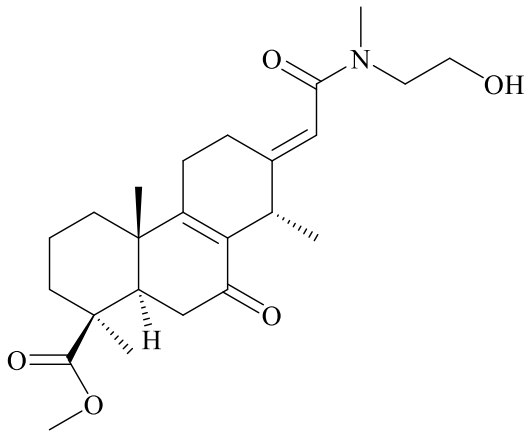
	R_1	R_2	R_3	R_4
3β-hydroxydinorerythrosuamide (**78)**	β– OH	O	β– OH, α–H	H
3β-acetoxydinorerythrosuamide (**79)**	β– $COOCH_3$	O	β– OH, α–H	H
3β-acetoxynorerythrosuamide (**80)**	β– $COOCH_3$	O	β– OH, α–H	CH_3
3β-tigloyloxydinorerythrosuamide (**81)**	β– $COOC(CH_3)=CHCH_3$	O	β– OH, α–H	H
3β-tigloyloxynorerythrosuamide (**82)**	β– $COOC(CH_3)=CHCH_3$	O	β– OH, α–H	CH_3
6α-hydroxydinorerythrophlamide (**83)**	β– OH	α–OH, β– H	O	H
6α- hydroxydinorcassmide 22-acetoxy-nor-cassamide(**84)**	H	α–OH, β– H	O	H



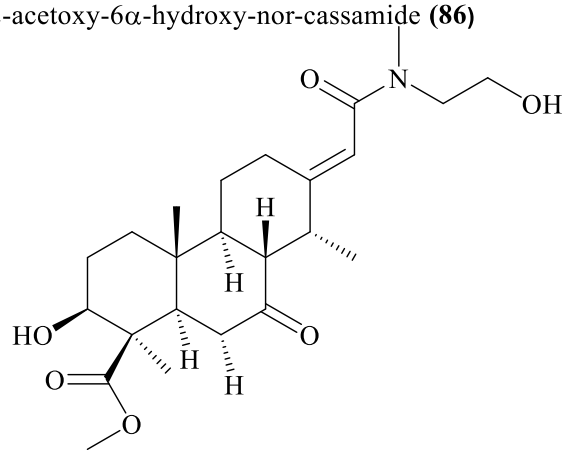
22-acetoxy-nor-cassamide **(85)**



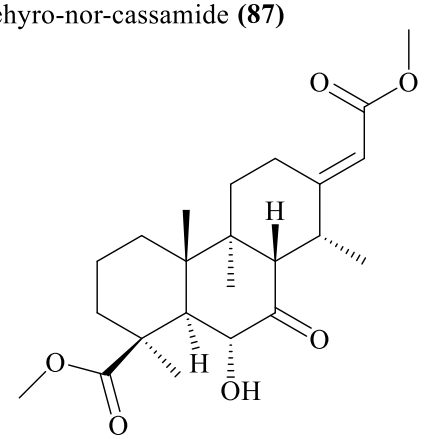
22-acetoxy-6α-hydroxy-nor-cassamide **(86)**



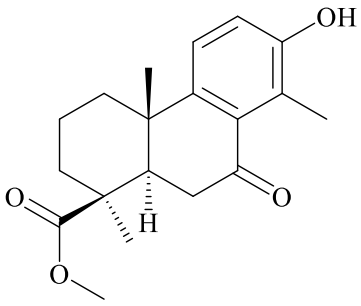
8-dehyro-nor-cassamide **(87)**



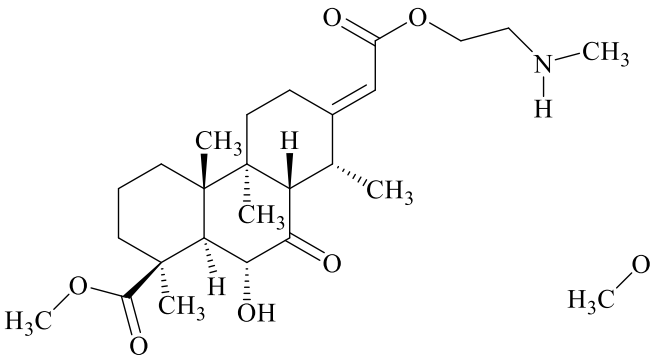
6α-hydroxy-nor-erythrophlamide **(88)**



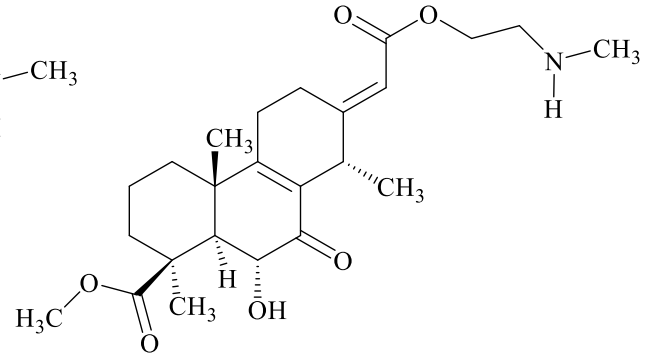
6α-hydroxy-cassamic acid **(89)**



4α-carbomethoxy-14-methyltotarol **(90)**



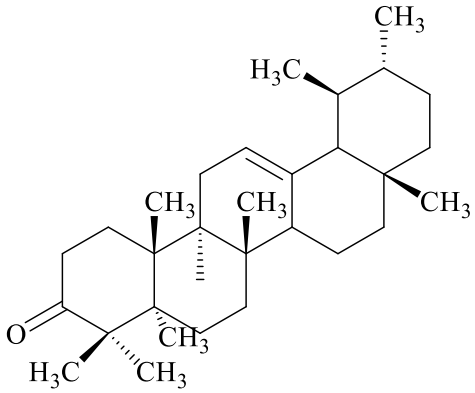
6α– hydroxy-nor-cassamine **(91)**



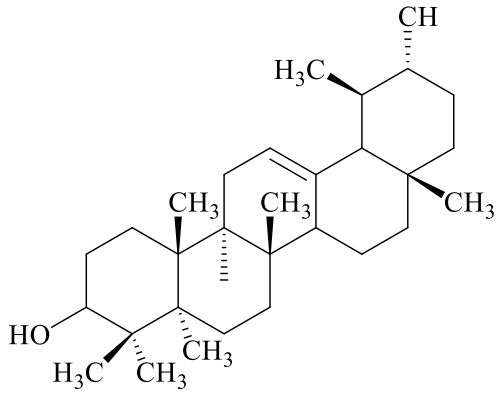
8, 9-dehydro-nor-cassamine **(92)**

3.8.9 Terpenoids from *Erythrophleum* species

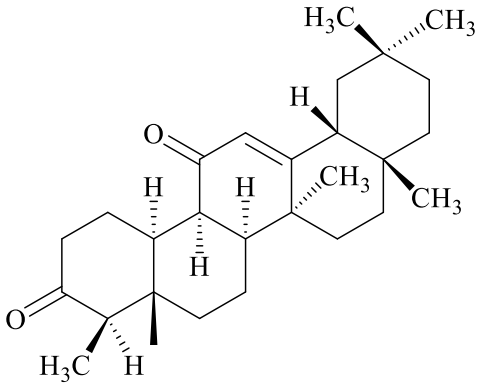
Tsao *et al*, (2008), reported the isolation of anti-inflammatory and antioxidant terpenoids β-amyrinone **(93)**, β– amyrin acetate **(94)**, Cycloartanol **(95)**, 3, 11-dioxoolean-12-ene-30-oic acid **(96)**, friedelin **(97)**, glutin-5-ene-3-β-O-acetate (**98**), glutinol **(99)**, glutinone **(100)**, Lupenone **(101)**, teraxerol **(102)** and taraxerone **(103)** from the leaves of *Erythrophleum fordii* with their anti-inflammatory and antioxidant activity accessed. Betulinic acid **(104)** and Morolic acid **(105)** have also been isolated from the stem bark of *Erythropleum fordii* (Qu *et al.*, 2007).



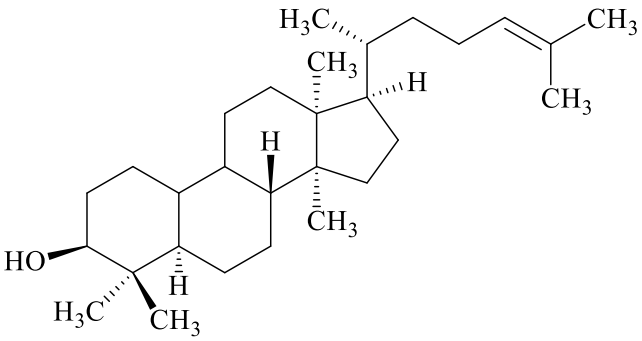
β-amyrinone **(93)**



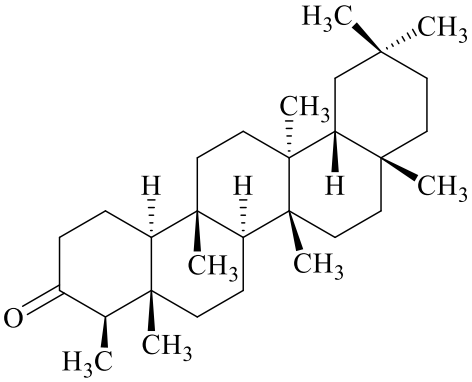
β- amyrin acetate **(94)**



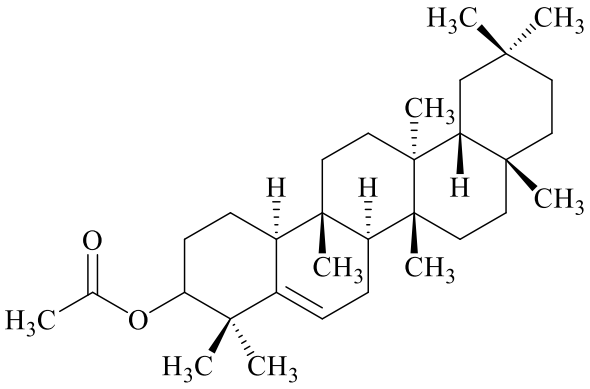
Cycloartanol **(95)**



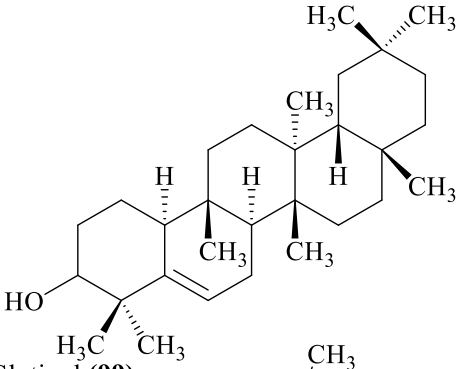
3, 11-dioxoolean-12-ene-30-oic acid **(96)**



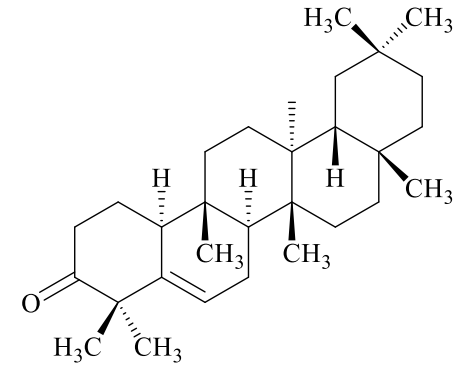
friedelin **(97)**



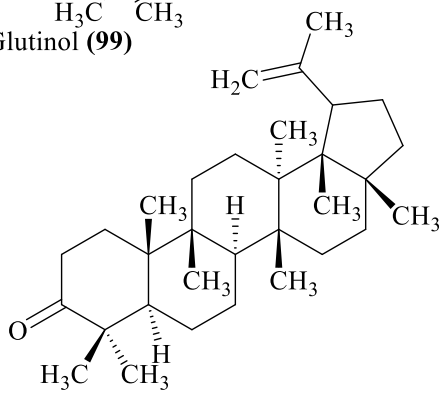
Glutin-5-ene-3-β-O-acetate **(98)**



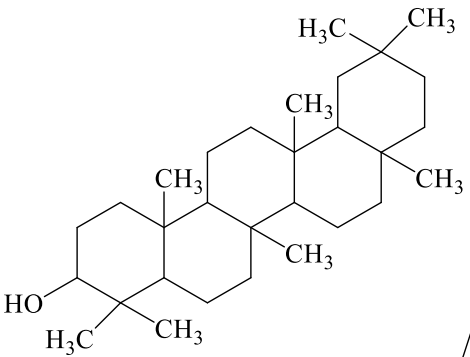
Glutinol **(99)**



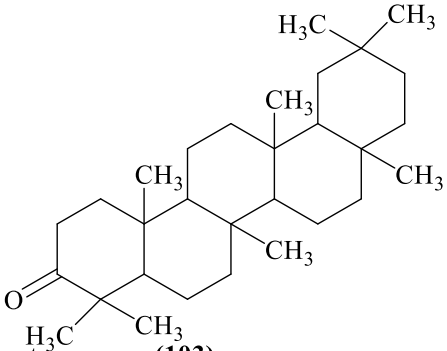
Glutinone **(100)**



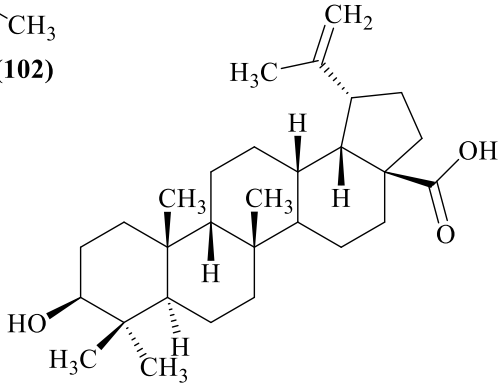
Lupenone **(101)**



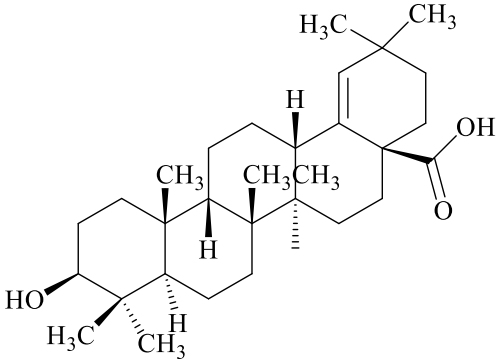
Teraxerol **(102)**



taraxerone **(103)**



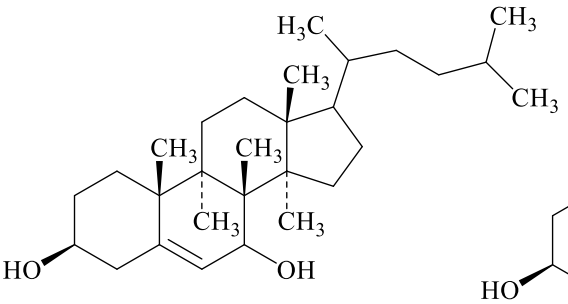
Betulinic acid **(104)**



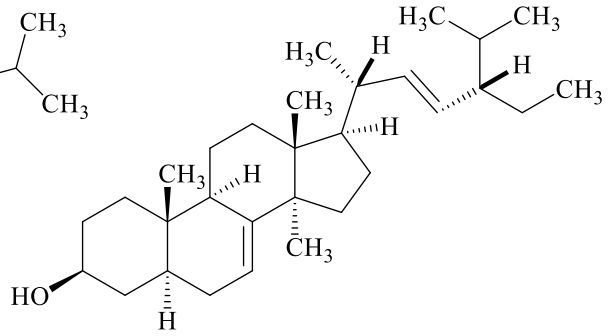
Morolic acid **(105)**

3.8.10 Phytosterols from *Erythrophleum* species

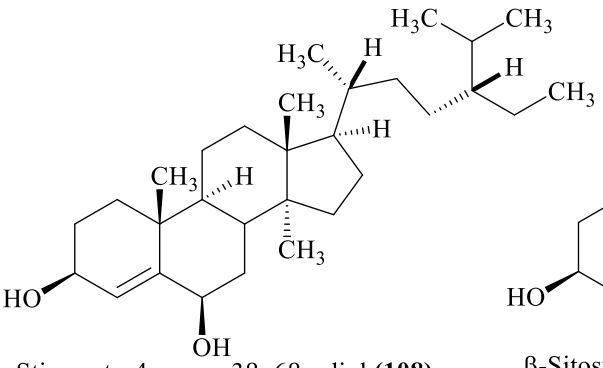
A handful of phytosterols have also been reported from the genus *Erythrophleum.* 5-Stigmasten – 3β, 7β – diol **(106)**, 5α - stigmasta, 7, 22 – diene – 3β – ol **(107)**, Stigmast – 4 – ene – 3β, 6β – diol **(108)**, β-Sitosterol **(109)** and Stigmasterol **(110)** have been reported from *Erythrophleum fordii* (Tsao *et al.*, 2008).



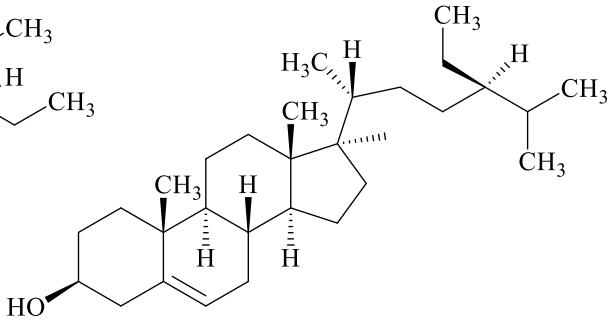
5- Stigmasten - 3β, 7β - diol **(106)**



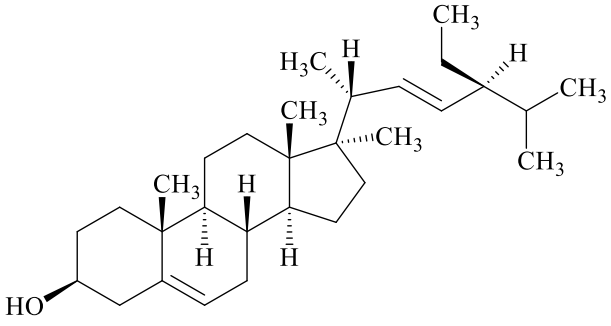
5α - stigmasta, 7, 22 - diene - 3β - ol **(107)**



Stigmast - 4 - ene - 3β, 6β - diol **(108)**



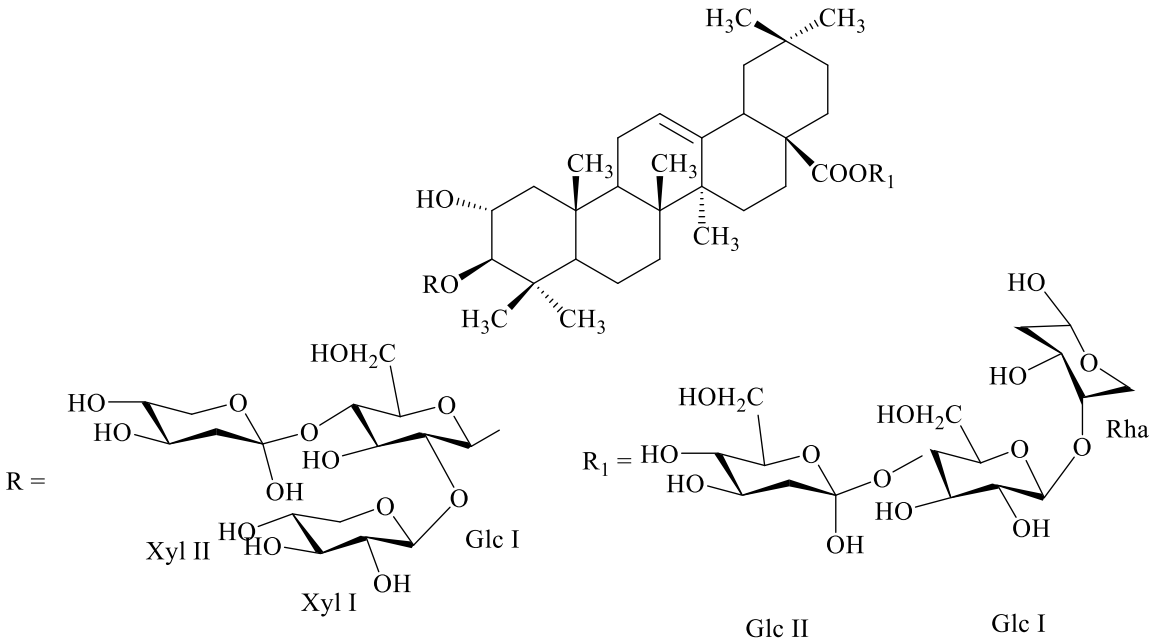
β-Sitosterol **(109)**



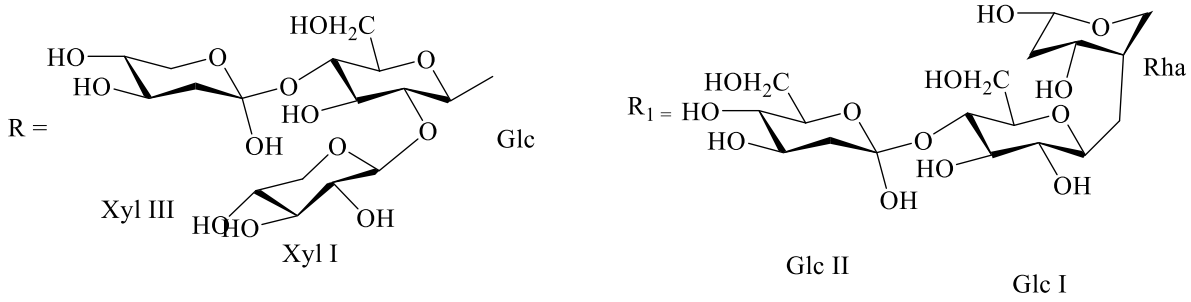
Stigmasterol **(110)**

3.8.11 Saponins from *Erythrophleum* species

Phytochemical studies of the crude ethanol extract of the leaves of *Erythrophleum fordii* led to the isolation of two oleanane-type triterpene saponins; 3β-O-{β-D-xylopyranosyl-(1 → 4)-[β-D-xylopyranosyl-(1→2)]-β-Dglucopyranosyl}-2α-hydroxyolean-12-en-28-O-[β-D-glucopyranosyl-(1 →6)-β-D-glucopyranosyl-(1 → 2)-α-L-rhamnopyranosyl] ester **(111)** and 3β-O-{β-D-xylopyranosyl-(1→4)-[β-D-xylopyranosyl-(1→2)]-β-Dxylopyranosyl}-2α-hydroxyolean-12-en-28-O-[β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1 → 2)-α-L-rhamnopyranosyl] ester **(112)** (Du *et al*., 2011).



3β O-{β-D-xylopyranosyl-(1 → 4)-[β-D-xylopyranosyl-(1→2)]-β-Dglucopyranosyl}-2β-hydroxyolean-12-en-28-O-[β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→ 2)-α-L-rhamnopyranosyl] ester (**111**)



3β O-{β-D-xylopyranosyl-(1→4)-[β-D-xylopyranosyl-(1→2)]-β-Dxylopyranosyl}-2α-hydroxyolean-12-en-28-O-[β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→2)-β-L-rhamnopyranosyl] ester (**112)**

3.9 ERYTHROPHLEUM IVORENSE

3.9.1 Botanical description

Erythrophleum ivorense is a large forest tree which grows to about 40 m tall; the stem is cylindrical, but at times furrowed at the base without buttresses. The bark is scaly with a reddish inner bark. Leaves alternate, and are bipinnately compound with 2–4 pairs of pinnae.

Leaflets alternate, they are elliptical to ovate. The base is asymmetrical and the apex is shortly acuminate (Irvine, 1961: Burkill, 1997).

Figure 3. 2 Whole plant, root bark and leaves of *Erythrophleum ivorense* (Armah, 2015)

3.9.2 Medicinal and non-medicinal uses

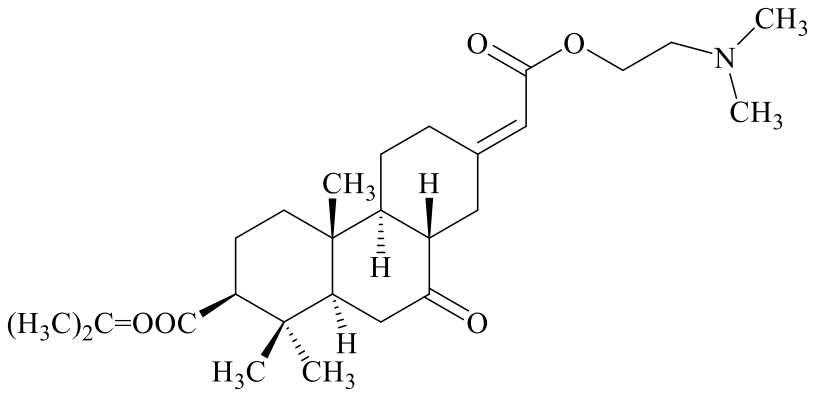
The bark extract is taken orally in Sierra Leone as an emetic and laxative, and is applied as a poultice to relieve pain in Ghana. The bark of young branches of *Erythrophleum ivorense* is used to treat smallpox in Côte d'Ivoire. The bark decoction, added to fermenting palm wine, makes it an excellent powerful drink (Irvine, 1961). The wood is suitable for carpentry, flooring, railway sleepers, harbour and dock work, turnery, construction of bridges. It is also used for boat constructions and wheel hubs. It is used as charcoal and firewood.

3.9.3 Pharmacological activity *of Erythrophleum ivorense*

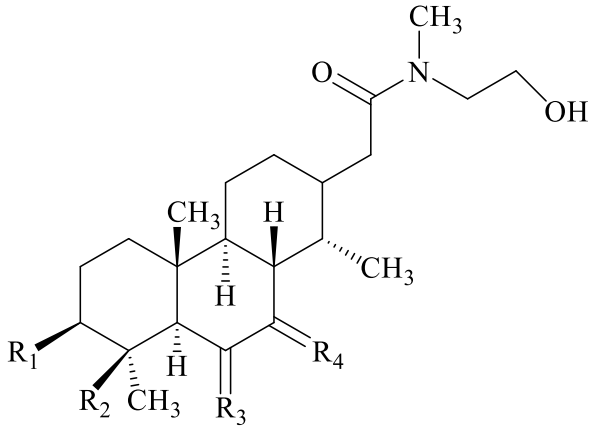
A 70% ethanol extract of the stem bark of *E. ivorense* was reported to show toxicity at a dose of 1000 mg/kg body weight. Again it showed moderate activity against a range or gram positive and gram negative organisms (Adu-Amoah *et al*., 2013). Wakeel *et al.*, (2014) reported the anticonculsant and sedative properties of *E. ivorense* stem bark.

3.9.4 Phytochemistry of *E. ivorense*

The alkaloid composition of *Erythrophleum ivorense* is comparable to that of *Erythrophleum suaveolens.* The distribution of the chief compounds is, however, not the same. First studies revealed the alkaloid erythrophleine. However, this was later recognized as a mixture of different alkaloids with comparable pharmacological action. The alkaloids were identified as esters of tricyclic diterpenic acids and were of two types. These were dimethylaminoethylesters and monomethylaminoethylesters (nor-alkaloids). In addition, other compounds have been identified in which the amine link is substituted by an amide link 3-(3-methylcrotonyl). Cassaine **(113)**, ivorine **(114)**, nor-cassaide **(115),** nor-cassamidide, dehydro-nor-erythrosuamide and norerythrosuamide **(116–118)** have all been isolated from *E. ivorense* (Ottinger *et al.*, 1965; Cronlund, 1973, Loder *et al.*, 1972)



	R_1	R_2	R_3
3-(3-methylcrotonyl)Cassaine **(113)**	$COOCH=C(CH_3)_2$	O	CH_3
Ivorine **(114)**	$COCH=C(CH_3)_2$	O	H



	R_1	R_2	R_3	R_4
Nor-cassaide **(115)**	OH	CH_3	β-H, α-H	O
Nor-cassamidide (**116)**	H	$COOCH_3$	β–H, α- H	β-OH, α-H
Dehydro-nor-erythrosuamide (**117)**	H	$COOCH_3$	O	O
Norerythrosuamide **(118)**	H	$COOCH_3$	O	β-H, α–H

Chapter 4

MATERIALS AND METHODS

4.1 PLANT COLLECTION AND IDENTIFICATION

The root bark of *E. ivorense*, stem barks of *C. oxycarpum, O. ahia* and *A. aubryanum* were harvested from Adukrom, a village in Nzema East Metropolis of Ghana, in October 2014 and were identified by Curators of the University of Cape Coast Herbarium (Ghana). Voucher specimen with numbers (BHM/Eryth/017R/2014, BHM/Coel/019O/2014, BHM/Omph/018A/2014 and BHM/Anth/020A/2014) have been deposited at the Herbarium of the Department of Herbal Medicine, Faculty of Pharmacy, Kwame Nkrumah University of Science and Technology, Ghana.

4.2 MATERIAL PROCESSING AND EXTRACTION

The root bark of *E. ivorense*, stem barks of *C. oxycarpum, O. ahia* and *A. aubryanum* were air dried for 7 days followed by oven drying at 40^{o}C for 48 hours. The dried materials were milled into coarse powder and packed into brown paper bags and kept until needed for extraction. Powdered air-dried root bark of *E. ivorense* (1.2 kg) stem barks of *C. oxycarpum* (600 g), *O. ahia* (900 g) and *A. aubryanum* (500 g) were cold macerated with 70 % methanol for 72 hours. The resulting extracts were then filtered and concentrated under reduced pressure (40 ^{o}C) to give crude extracts with yields of 8.7% $^{w}/_{w,}$ 5.3% $^{w}/_{w,}$ 6.7% ^{w}/w and 4.1% $^{w}/_{w}$ for *E. ivorense*, *C. oxycarpum, O. ahia* and *A. aubryanum* respectively. A portion of *E. ivorense* extract (100 g) was successively partitioned with petroleum ether (5 L), ethyl acetate (5 L) and methanol (5 L) to afford fractions in the yield of 15 g, 36.3 g and 41.2 g respectively.

4.3 PHYTOCHEMICAL ANALYSES

Phytochemical tests were done on the plant materials to determine various secondary metabolites present. Saponins, tannins, flavonoids, triterpenes, glycosides and alkaloids were tested according to the method described by (Harbone, 1975).

4.3.1 Phytosterols

The powdered plant materials were extracted with chloroform. Acetic anhydride (2 mL) was added followed by drops of concentrated H_2SO_4 along the side of the test tube.

4.3.2 Triterpenoid test

The powdered plant materials were extracted with chloroform. Concentrated H_2SO_4 (2 mL) was added in drops to 4 mL of the chloroform extract, along the side of the test tube.

4.3.3 Alkaloids

The powdered plant materials were extracted with ammoniacal alcohol. They were filtered and the filtrate evaporated to dryness. The residues were extracted with 1 % H_2SO_4 .They were filtered and rendered distinctly alkaline with dilute NH_3 solution. They were then shaken with $CHCl_3$ and the chloroformic extracts evaporated to dryness. The residues were dissolved in 1 % H_2SO_4. One drop of Dragendorff's reagent was added to the 1 % H_2SO_4 of the extracts.

4.3.4 Flavonoids

Ethanol extracts of the powdered plant samples were prepared and small pieces of magnesium ribbon were added, followed by drop wise addition of concentrated hydrochloric acid.

4.3.5 Glycosides

200 mg of the powdered plant material was warmed with 5 mL of dil. HCl on a water bath for 2 minutes. It was filtered and made distinctly alkaline by adding 5 drops of 20% NaOH. The pH of the solution was checked with pH paper. 1 mL of Fehling's solution A and B was then added to the filtrate and heated on the water bath for 2 minutes.

4.3.6 Tannins

The powdered plant material was extracted with about 25 mL of water and filtered. 1 mL of the filtrate was then diluted to 10 mLs with water. 1% ferric chloride was then added to the solution.

4.4 ANTI-LEISHMANIA ASSAY OF SELECTED PLANTS

4.4.1 Cultivation of parasite

Promastigote strain of *Leishmania donovani* (LV90) was obtained from the Division of Biomedical and Life Sciences, Faculty of Health and Medicine, Lancaster University, U.K. The promastigotes were cultivated at 25 °C in medium 199 (Gibco, Invitrogen) supplemented with 5 % penicillin/streptomycin, 10 % heat-inactivated fetal bovine serum (FBS), 10 mM adenine (pH 7.5), and 5 Mm L-glutamine Using a pipette, 500 microlitre of parasite (containing $14.4x10^6$ parasites) were picked and added to 15 mL of M199 media in a culturing flask. The mixture was incubated at 25°C for five days. The number of parasites was counted daily using a hemocytometer (Z359629 SIGMA) according to the method described by Umakant *et al.*, (2011).

4.4.2 *In-vitro* anti-leishmanial assay

The anti-leishmanial activity of the selected plants in comparison to Amphotericin B was evaluated *in vitro* against the promastigote forms of *Leishmania donovani* using a heamocytometer counting chamber and a high field microscope with mobility of the parasite as a marker of cell viability. A stock solution of the total crude methanol extract of the plant (1000 µg/mL) was prepared in DMSO. The different concentrations of the extract were prepared by two-fold dilution of the stock as follows. Stock solution (1 mL) was dispensed into the first well of the 24 well microtitre plates. It was topped up with 1 mL of M199 media mixing thoroughly and 1 mL of this solution was again dispensed into the second cell and procedure repeated in subsequent cells. Each cell was then topped with 20 µL of *Leishmania donovani* culture containing 95.5×10^6 cells/mL of the promastigotes. Finally, each well was topped up with a suitable amount of the medium (M199) to make a final volume of 2 mL thereby achieving concentrations of 15.6, 31.2, 125, 250 and 500 µg/mL. The total concentration of DMSO was thus reduced to $0.5\%^{V}/_{V}$, a concentration which has negligible effect on parasite growth rate and morphology (Khan *et al*., 2012). Total solution contained M199, DMSO, plant extract and 20µl promastigotes of *Leishmania* parasite. The same procedure was repeated for all other plant extracts. Similarly, the positive control, amphotericin B and the pet-ether, ethyl acetate and methanol fractions of *E. ivorense* were tested under the same concentrations. The negative control consisted of the medium M199, DMSO and promastigotes of *Leishmania* mixed evenly in a well without the extract. The parasites were incubated up to 72 hours at 25°C. However, the number of viable parasites was counted with a heamocytometer under a

high field microscope after 6, 12, 24, 48 and 72 hours. All the *in vitro* experiments were run in triplicate and the results expressed as a % inhibition in parasite numbers.

4.4.3 Antileishmania activity of isolated compounds

Bioactive ethyl acetate fraction of *E. ivorense* resulted in the isolation of the novel cassane diterpene erythroivorensin, betulinic acid and eriodictyol (section 4.9). These compounds were similarly tested for anti-leishmanial activity as described above using the same concentrations.

4.5 ANTI-INFLAMMATORY ASSAY

4.5.1 Experimental animals

Cockerels (*Gallus gallus*; Akate farms, Kumasi, Ghana) were obtained 1-day post-hatch and were housed in a stainless steel cages ($34 \times 57 \times 40$ cm^3) at a population density of 10 chicks per cage. They were fed with chicks-feed (Chick mash, GAFCO, Tema, Ghana) and clean water was also available. The chicks were routinely maintained for seven days at a room temperature of 30 ^oC. They were tested at 7 days of age.

4.5.2 Anti-inflammatory assay of selected plants

The experimental model used in assaying the anti-inflammatory activity of the extracts was the carrageenan-induced foot edema in chicks (Roach and Sufka, 2003). The chicks were randomly selected and grouped into 5 per cage. Their initial foot volumes were determined by using a digital vernier caliper (Sireeratawong *et al.*, 2013). The anti-inflammatory activities of the extracts were compared to diclofenac and dexamethasone, used as standard drugs.

The chicks were weighed and their doses calculated per their body weight. 10 µL of 2 % carrageenan, freshly prepared in normal saline, was injected sub-plantar into the left foot of the chicks. One hour after the carrageenan challenge, the foot volume was measured again. The 70% methanol extracts of *Omphalocarpum ahia* (EOA), *Coelocaryon oxycarpum* (ECO), *Anthostema aubryanum* (EAA) and *Erythrophleum ivorense* (EEI) were then given orally at 30, 100, and 300 mg/kg body weight. The standard drugs, dexamethasone (0.3, 1, and 3 mg/kg body weight) and diclofenac (10, 30 and 100 mg/kg body weight) were also given orally. The control groups received only the vehicle (normal saline). The foot volumes were measured at hourly intervals for 6 hours. The oedema component of the inflammation was quantified by measuring the difference in foot volume before carrageenan injection and at the various time intervals. The percentage inhibition of edema was calculated based on the following equation:

$$\text{Percentage (\%) inhibition of edema} = \frac{AUC^{O} - AUC}{AUC^{O}} \times 100$$

Where: AUC^{o} is the Area under the curve for the control (non-treated group)

AUC is the Area under the curve for the test samples (treated group).

4.5.3 Anti-inflammatory activity of fractions and isolated compounds

In the anti-inflammatory activity described in section 5.3, the ethanol extract of *E. ivorense* showed the highest activity; hence it was selected and serially fractionated to obtain a pet-ether, ethyl acetate and methanol fraction. These fractions were tested for anti-inflammatory activity using the chick carrageenan model. The fractions were dosed orally at 10, 30 and 100 mg/kg

body weight. Similarly, the isolated compounds from the bioactive ethyl acetate fraction were given orally at 10, 30 and 100 mg/kg body weight.

4.6 *IN VITRO* ANTIOXIDANT ASSAY

The plant extracts were screened for antioxidant activity using the total antioxidant capacity and DPPH radical scavenging assays.

4.6.1 Total antioxidant capacity assay

This experiment is based on the reduction of Mo^{+6} to Mo^{+5}, by the extracts and subsequent formation of a greenish complex (phosphate-molybdate Mo^{+5}) in an acidic medium (Prieto *et al*., 1999). The reagent used in this assay was prepared by mixing freshly prepared solutions of Ammonium molybdate (4 mM), Disodium hydrogen phosphate (28 mM), and Sulphuric acid (0.6 M). To 1ml of the extracts, each of concentrations 500 µg/ml – 62.5 µg/ml, 3 ml of the reagent was added and incubated at 95 ^{o}C for 90 minutes. The process was repeated for Ascorbic acid of concentrations 50-6.25 µg/ml and kept at the same conditions. A blank solution was prepared by mixing 1 ml of methanol with 3 ml of the reagent. After the test sample had cooled to room temperature, the absorbances of the solutions were measured at 695 nm using the UV-visible spectrophotometer. The Ascorbic acid absorbance and concentrations were used to construct a calibration curve. The antioxidant activity was expressed as mg of Ascorbic acid equivalent (AAE) per gram (g) of the extracts.

4.6.2 2, 2-diphenyl-1-picrylhydrazyl radical scavenging assay

In this assay, the method described by Govindarajan *et al.*, (2003) with few modifications by Amponsah *et al.*, 2014 was followed. The extracts (1 mL quantity of each) at concentrations of

500, 250, 125 and 62.5 µg/ml was added to 3 mL methanol solution of DPPH in a test tube and incubated at 25 °C for 30 minutes. The ability of the extract to decolourise the purple colour of DPPH was determined at 517 nm in a spectrophotometer (Cecil CE 7200 spectrophotometer, Cecil instrument limited, Milton Technical Centre, England). The control was prepared by adding 1 ml of methanol and 3 ml of DPPH, incubating at 25 °C for 30 minutes. Vitamin E (100, 50, 25, 12.5 and 6.25 µg/ml) was used as positive control. Results were expressed as percentages of blank. The EC_{50} which is the concentration required to scavenge 50 % of the DPPH molecule was calculated. Each test was carried out using three replicates

$$\% \text{ DPPH Scavenging activity} = \frac{A^o - A}{A^o} \times 100$$

Where: A^o is the absorbance of the blank (negative control)

A is the absorbance of the test sample (positive control).

4.6.3 Antioxidant assay of fractions and isolates

The pet-ether, ethyl acetate and the methanol fractions of *E. ivorense* as well as the isolated compounds were similarly screened for antioxidant activity using the DPPH scavenging assay described in section 4.6.2. The pet-ether, ethyl acetate and methanol fractions were tested in the concentration range of 500-6.125 µg/mL whereas the compounds were tested at concentrations between 3.125-50 µg/mL.

4.7 STATISTICAL ANALYSIS

The raw scores for right foot volumes were individually normalized as percentage of change from their values at time zero then averaged for each treatment group. Total foot volume for each treatment was calculated in arbitrary unit as the area under the curve (AUC). To determine the percentage inhibition for each treatment, the following equation was used.

$$\%\ \text{inhibition of oedema} = \left(\frac{\text{AUC}_{\text{control}} - \text{AUC}_{\text{treatment}}}{\text{AUC}_{\text{control}}} \right) \times 100$$

Differences in AUCs were analyzed by one-way analysis of variance followed by Student-Newman-Keuls' *post hoc t* test. Doses and concentrations responsible for 50 % of the maximal effect (EC_{50}/ IC_{50}) for each drug/extract were determined using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) equation.

$$Y = \frac{a + (b - a)}{(1 + 10^{(Log\,EC50 - X)}}$$

Where, *X* is the logarithm of dose and *Y* is the response. *Y* starts at *a* (the bottom) and goes to *b* (the top) with a sigmoid shape. The fitted midpoints (ED_{50}/IC_{50} values) of the curves were compared statistically using *F* test (Miller, 2003; Motulsky, 2003). Graph Pad Prism for Windows version 5.0 (Graph Pad Software, San Diego, CA, USA) was used for all statistical analyses. $P < 0.05$ was considered statistically significant (Rowe, 2007).

4.8 CHROMATOGRAPHIC TECHNIQUES

The results of the anti-leishmanial (section 5.2), anti-inflammatory (section 5.3) and antioxidant activities (section 5.4) of the four plants revealed *E. ivorense* to be the most active plant. Its anti-leishmanial, anti-inflammatory and antioxidant properties were found to reside in the ethyl acetate fraction. Therefore, the ethyl acetate fraction was fractionated using various chromatographic methods to isolate its bioactive compounds and characterize them by their spectroscopic properties.

4.8.1 Chromatographic materials

Silica gel 60 (70-230 mesh ASTM, Merck Germany) was the main stationary phase material used for all the column chromatographic fractionations. Aluminium precoated silica gel plates 60 F_{254} (0.25 mm thick) was used for the analytical thin layer chromatography.

4.8.2 Solvents and reagents

The solvents petroleum ether, dichloromethane, ethyl acetate, acetone, ethanol and methanol, used for the extraction, column chromatographic fractionation, thin layer chromatography analysis and recrystallisation of isolates, were of analytical grade (BDH Laboratory Supplies, England). Sulphuric acid, glacial acetic acid and p-anisaldehyde were of analar grade and were also purchased from BDH Laboratory Supplies, England.

4.8.3 Detection for analytical thin layer chromatography

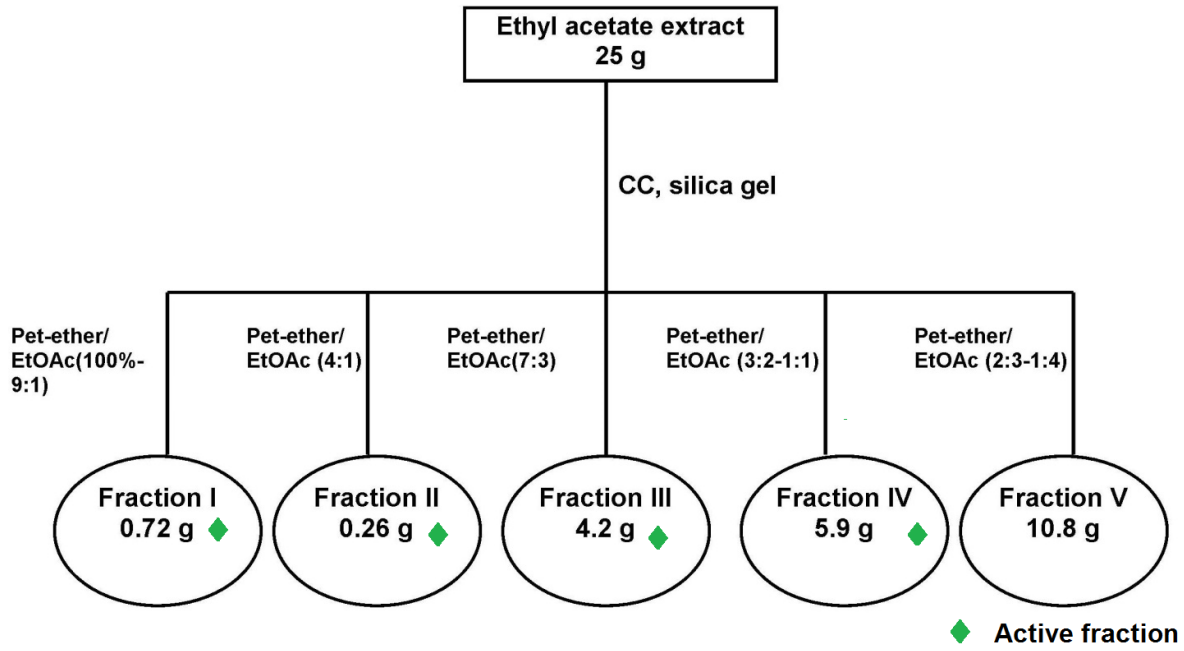
The spray reagents used in this research in detecting and visualizing the separated out compounds on the TLCs included anisaldehyde reagent (prepared by mixing 0.5 mL of p-anisaldehyde, 10 mL of acetic acid, 85 mL of methanol and 5 mL of concentrated sulfuric

acid), Sulphuric acid / MeOH mixture (prepared by mixing 5 mL of concentrated sulfuric acid and 95 mL of methanol) (Stahl, 2003). Application of these reagents was followed by heating at 110 ºC for 10 mins. The TLC chromatograms were also visualized under UV at 365 nm and 254 nm.

4.9 ISOLATION OF COMPOUNDS FROM *E. IVORENSE*

4.9.1 Column chromatographic fractionation of ethyl acetate extract

The glass column (750 mm x 4 mm) was packed with Silica gel 60 (600 g, 70-230 mesh ASTM) using the dry method. The ethyl acetate extract (25 g) was reconstituted with a minimum amount of ethyl acetate 50 mL and mixed with 50 g of silica gel, allowed to dry to attain the same consistency as the silica gel that was used, and spread gently on top of the packed column. A wad of cotton wool was placed on top of the packed column in order not to disturb the surface of the packing. The column was then eluted with a gradient of petroleum ether –ethyl acetate mixtures (100 % 1.5 L pet-ether followed by 10 % 1.5 L, 20 % 1.5 L, 30 % 1.5 L, 50 % 1.5 L, 70 % 1.5 L, 90 % 1.5 L ethyl acetate in pet-ether and 100 % 1.5 L ethyl acetate). Approximately 200 aliquots of 100 mL each were collected. The collected fractions were analysed by thin layer chromatography as described in section 3.9.3 using a mobile phase of pet-ether/ethyl acetate in the ratios 1:4, 3:2 and 2:3. The plates were visualized under UV 254 nm and 360 nm. They were also sprayed with anisaldehyde-sulfuric reagent followed by heating at 110 ºC for 10 minutes. The eluates were then pooled together according to the similarity of their TLC profiles to give five bulked fractions labeled fraction I to V as shown in Figure 4.1.



CC (column chromatograph)
EtOAc (ethyl acetate)

Figure 4. 1 Schematic representation of the isolation of compounds

4.9.2 Isolation of compound 1

Fractions I and II were combined upon analysis of their TLC profiles. Part of the combined fractions (900 mg) was dissolved in ethyl acetate and adsorbed unto silica gel (70-230 mesh ASTM), and spread on top of a column (50 mm × 2 mm) packed dry with silica gel. The column was then eluted with gradient mixtures of pet-ether/ethyl acetate (100 % 500 mL; 90:10 1000 mL; 80:20 1000 mL). 100 fractions of 5 ml aliquots each were collected and bulked into three fractions labeled as FI_A, FI_B and FI_C. Sub-fraction FI_B showed as a single spot on TLC plate developed with pet-ether/ethyl acetate (9:1) as the mobile phase and therefore

labeled as compound 1. Recrystallisation of this compound in acetone yielded colourless needle crystals (400 mg). The scheme for the isolation of compound 1 is shown in Figure 4.2

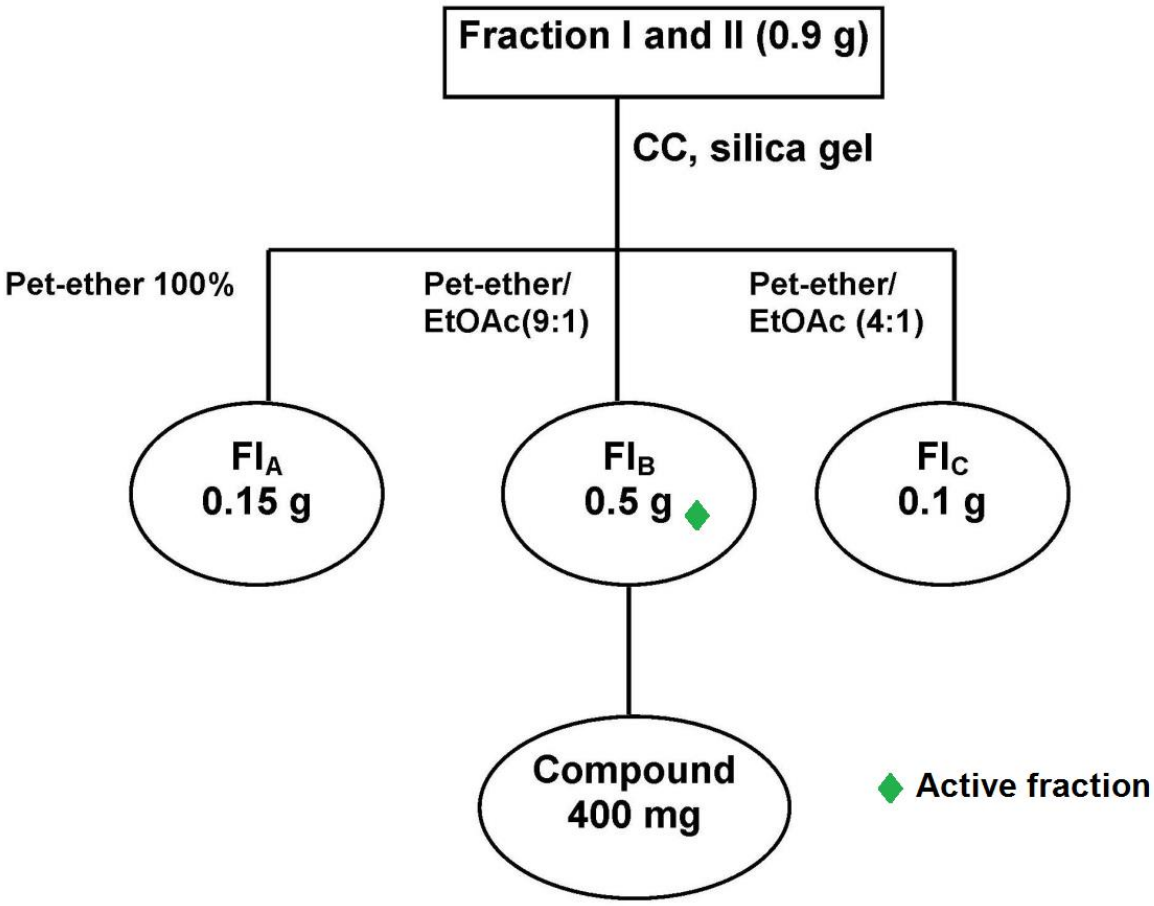


Figure 4. 2 Schematic representation of purification of compound I

4.9.3 Isolation of compound 2

An oily mass of fraction III (4.2 g) was dried in a dessicator after which it was washed several times with petroleum ether (500 mL) to afford a pale yellow amorphous powder. This showed as a single spot on the TLC chromatogram and therefore labeled compound 2 (200 mg).

4.9.4 Isolation of compound 3

Fraction IV (5 g) was adsorbed unto silica gel (70-230 Mesh) and chromatographed over silica gel in a column of dimensions 4 cm x 60 cm. The elution was done stepwise with pet-ether and ethyl acetate mixtures of 7:3 1000 mL, 1:1 1000 mL and 2:3 1000 mL. The fractions collected for each mixture were pooled together to afford three bulked fractions (F4A, F4B, F4C). Fraction F4A (1.5 g) was again column chromatograph over silica eluting with gradient mixtures of pet-ether and ethyl acetate 7:3 (1000 mL) to obtain a yellow amorphous compound (compound 3) (Figure 4.3).

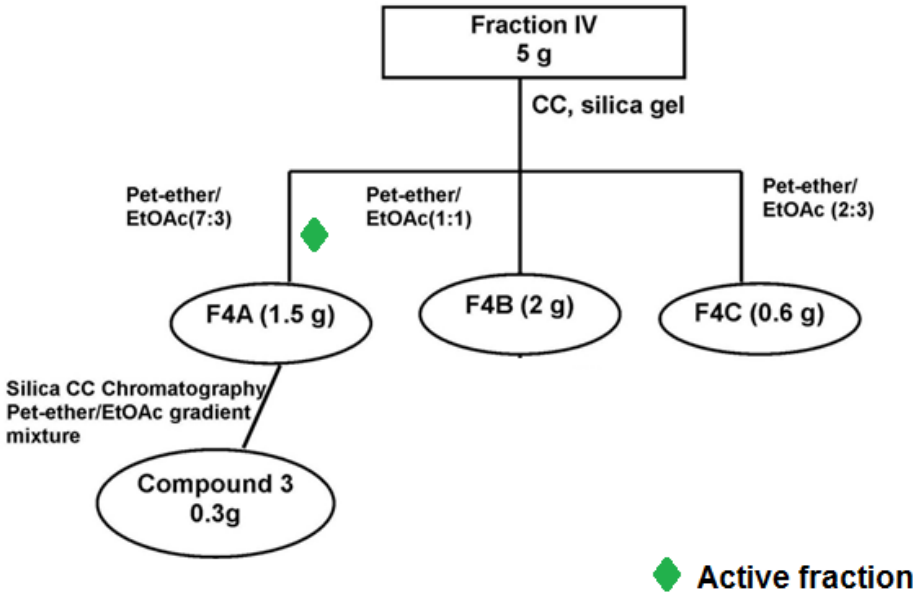


Figure 4. 3 Schematic representation of the isolation of compound IV

4.10 STRUCTURAL ELUCIDATION OF ISOLATED COMPOUNDS 1-3

4.10.1 General experimental procedure

^{1}H NMR, ^{13}C NMR and 2D-NMR (COSY, NOESY, HMQC and HMBC) spectra were obtained on a JEOL 500 MHz instrument. Homonuclear ^{1}H connectivities were determined by using the COSY experiment. One bond ^{1}H–^{13}C connectivities were determined with HMQC while two- and three-bond ^{1}H–^{13}C connectivities were determined by HMBC experiments. Chemical shifts were reported in δ (ppm) using the solvent ($CDCl_3$) standard and coupling constants (*J*) were measured in Hz. Optical activity was recorded using ADP410 Polarimeter (Bellingham and Stanley, Kent, UK) while IR and UV spectra were obtained using Perkin Elmer Spectrum two FTIR (Coventry, UK) and Hitachi U-2900 spectrophotometers respectively. The High resolution mass spectroscopy instrument, Thermofisher LTQ rbitrap XL (Thermofisher Scientific, UK), with an electrospray ionisation probe was used for accurate mass measurement over the full mass range of m/z 50–2000. Nano-electrospray analyses were performed in positive ionisation mode by using nano mate to deliver samples diluted into MeOH + 10% NH_4OAc. The temperature was set at 200 ^{o}C, sheath gas flow of 2 units and capillary (ionising) voltage at 1.4 kV. The accurate mass measurements obtained from this system were far better than 3 ppm.

A single crystal was mounted on a nylon loop and X-ray diffraction data were recorded on an Agilent Super Nova Dual Diffractometer (Agilent Technologies Inc, Santa Clara CA) with Cu-Kα radiation (λ = 1.5418 Å) at 150K. Unit cell determination, data reduction and absorption corrections were carried out using CrysAlisPro[13] The structure was solved by direct methods and refined by full matrix least squares on the basis of F[14] using SHELX 2013[13] within the

OELX2 GUI[15]. The asymmetric unit contains two molecules of $C_{20}H_{30}O_2$. Non-hydrogen atoms were refined anisotropically and hydrogen atoms were included using a riding model. The absolute stereochemistry was confirmed by successful refinement of the Flack parameter (-0.12(4). The details of the structural analysis are described in the supporting information along with the cif file, which has been deposited at the Cambridge Crystallographic Data Centre (CCDC No. 1051612) and can be obtained from www.ccdc.cam.ac.uk/data_request/cif.

Chapter 5

RESULTS

5.1 PRELIMINARY PHYTOCHEMICAL ANALYSIS

The preliminary phytochemical tests of the 70 % methanol extracts of *C. oxycarpum, O. ahia, A. aubryanum* and *E. ivorense* revealed the presence of the major secondary metabolites shown in Table 5.1.

Table 5.1 Secondary metabolites of selected plants

Plant constituents	***O. ahia***	***C. oxycarpum***	***A. aubryanum***	***E. ivorense***
Tannins	+	+	+	+
Flavonoid	-	-	+	+
Terpenoids	+	+	+	+
Alkaloids	-	-	+	+
Glycosides	+	+	+	+
Saponins	+	+	+	+
Phytosterols	+	+	+	+

+ = positive

- = negative

5.2 ANTI-LEISHMANIAL ACTIVITY OF SELECTED PLANTS

The study looked at the anti-leishmanial activity of 70 % methanol extracts of the root bark of *E. ivorense* and the stem barks of *O. ahia, C. oxycarpum* and *A. aubryanum*. *In vitro* anti-leishmanial assay of these plant extracts using promastigotes of *L. donovani* at concentrations of 500 µg/mL, 250 µg/ml, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL and 15.63 µg/mL was done by a direct counting assay based on growth inhibition of the promastigotes. All the extracts

showed varying degrees of anti-leishmanial activities. *E. ivorense* showed the highest leishmanicidal activity (IC_{50}= 14.1±0.17 µg/mL) followed by *A. aubryanum, C. oxycarpum* and *O. ahia* (Table 5.2) (Figure 5.1). The leishmanicidal activity of *E. ivorense* was about ten times less than that of Amphotericin B, used as positive control.

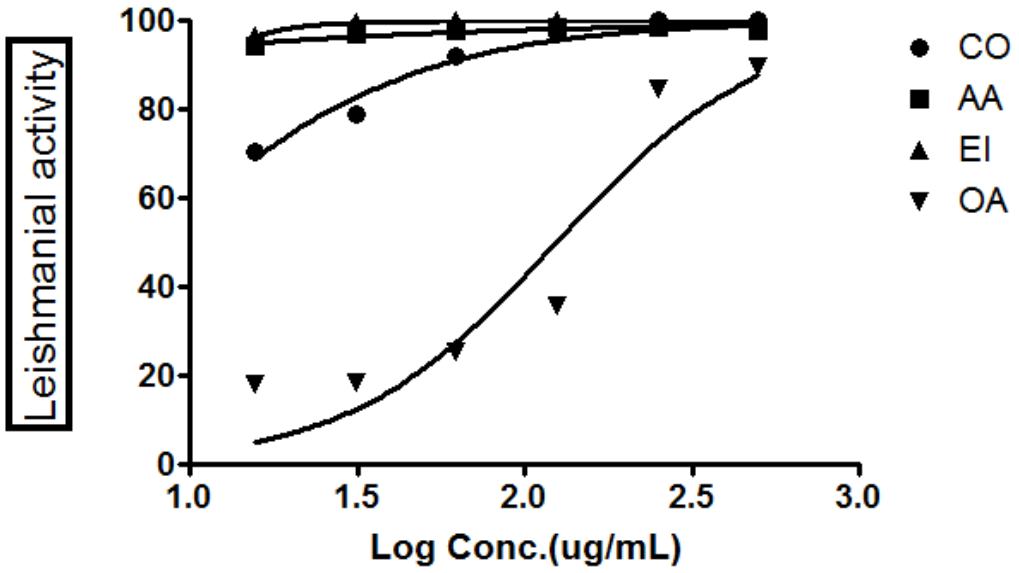


Figure 5.1 Concentration response curves of plant extracts on inhibition of *L. donovani*

Table 5.2 Anti-leishmanial activity of selected plants

Plant materials	**IC_{50} (µg/mL)**
Erythrophleum ivorense	14.1±0.17
Omphalocarpum ahia	124.0±0.67
Anthostema aubryanum	23.9±0.13
Coelocaryon oxycarpum	39.31±1.93
Amphotericin B	2.4± 0.67

5.2.1 Antileishmanial activity of fractions and isolated compounds

The bioactive *E. ivorense* extract was fractionated to give a pet-ether, ethyl acetate and methanol fractions. Investigation of the fractions for leishmanicidal activity showed the methanol and ethyl acetate fractions to be the most active. The activity of the ethyl acetate fraction was very much comparable to that of Amphotericin B (Table 5.3).

The compounds erythroivorensin (compound 1), betulinic acid (compound 2) and eriodictyol (Compound 3) isolated from the active ethyl acetate fraction (Section 4.9) were considerably active as compared to the positive control amphotericin B (Table 5.3). The novel compound erythroivorensin showed moderate anti-leishmanial activity with IC_{50} = 29.10 µg/mL.

Table 5.3 Anti-leishmanial activity of fractions and isolated compounds

Extracts/compounds	IC_{50} (µg/mL)
Petroleum ether	133.6±0.82
Methanol	2.97±0.23
Ethyl acetate	33.07±0.72
Erythroivoresin (1)	29.10±1.11
Betulinic Acid (2)	1000±0.44
Eriodictyol (3)	103.80±0.53
Amphotericin B	2.4± 0.67

5.3 ANTI-INFLAMMATORY ACTIVITY OF SELECTED PLANTS

In this research, the anti-inflammatory potential of the 70 % methanol extracts of *Omphalocarpum ahia* (EOA), *Coelocaryon oxycarpum* (ECO), *Anthostemum aubryanum* (EAA) and *Erythrophleum ivorense* (EEI) using the chick carrageenan induced inflammation model was evaluated. The anti-inflammatory activities of the extracts were compared to diclofenac and dexamethasone, used as standard drugs. Induction of oedema begun one hour

after sub-planter injection of carrageenan (2 %) with the inflammation reaching its peak within 2-3 hours (Figure 5.2 - 5.5). Oral administration of the extracts (30-300 mg/kg body weight) resulted in the reduction of the oedema induced by carrageenan in the chick paw from the second through to the sixth hour. All the extracts showed significant ($p<0.05$) dose-dependent reduction in oedema (Figure 5.2 - 5.5). Similarly, the standard drugs, diclofenac and dexamethasone showed a dose dependent anti-inflammatory activity (Figure 5.6-5.7). The anti-eodematogenic activity was quantified using the ED_{50}. This is the dose required to reduce the inflammation by 50%. The lower the ED_{50}, the higher the anti-inflammatory potency of the extracts. From the results, the rank order of anti-inflammatory potency, as shown by the ED_{50} (Table 5.4) was dexamethasone> diclofenac> *Erythrophleum ivorense*> *Coelocaryon oxycarpum*> *Omphalocarpum ahia*> *Anthostema aubryanum*. Thus *E. ivorense*, again, showed the highest anti-inflammatory activity among the selected medicinal plants.

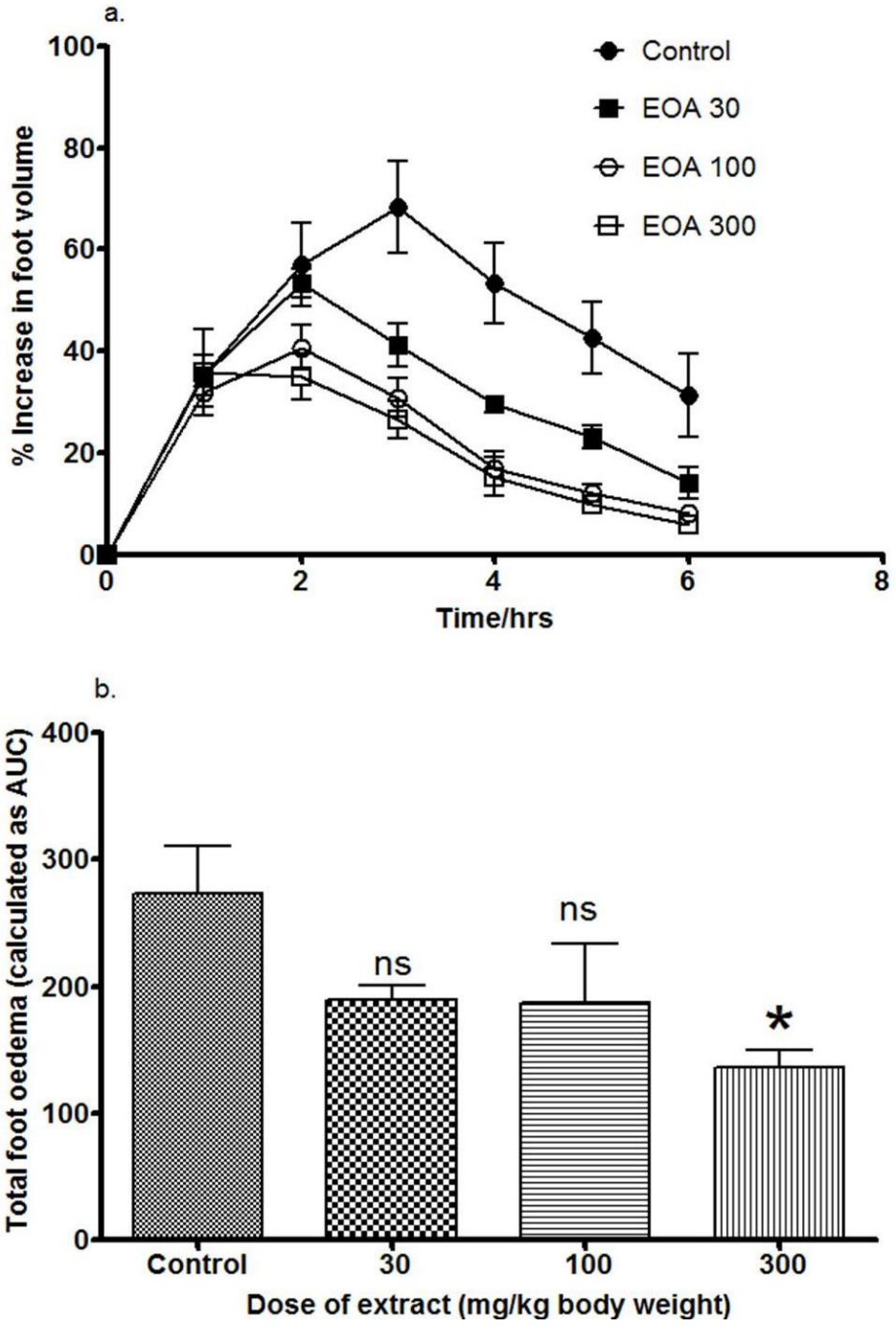


Figure 5.2 Effect of 70 % methanol extract of *Omphalocarpum ahia* (30-300 mg/kg; *p.o*)] on time course curve (a) and the total oedema response, calculated as AUC's, for 6 h, in carrageenan induced paw oedema in chicks (b). Values are means ± S.E.M (n=5), *** p < 0.001, ** p < 0.01, *P < 0.05 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test).

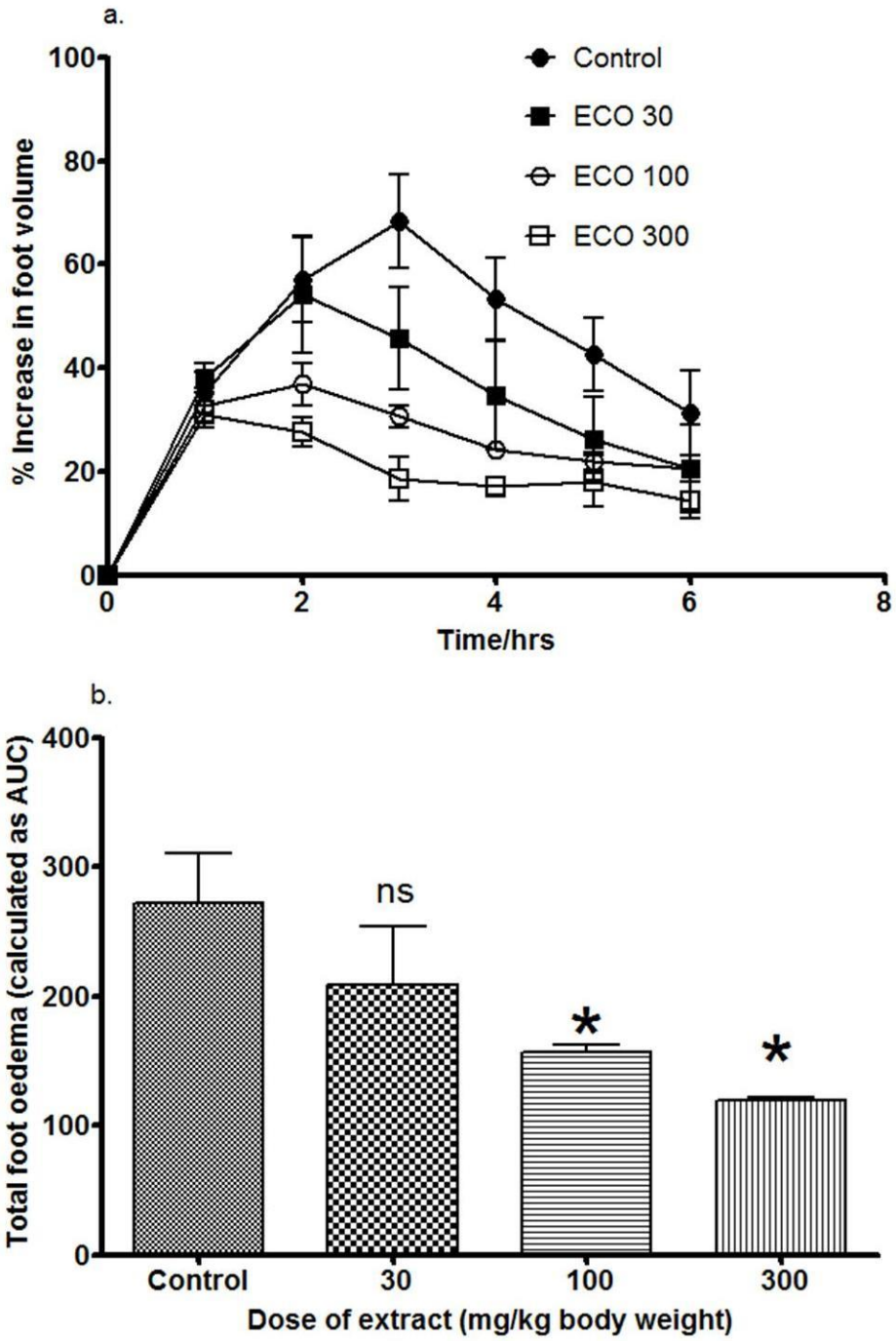


Figure 5.3 Effect of 70 % methanol extract of *Coeryocaryon oxycarpum* (30-300 mg/kg; *p.o*)] on time course curve (a) and the total oedema response, calculated as AUC's, for 6 h, in carrageenan induced paw oedema in chicks (b). Values are means ± S.E.M (n=5), *** p < 0.001, ** p < 0.01, *P < 0.05 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test).

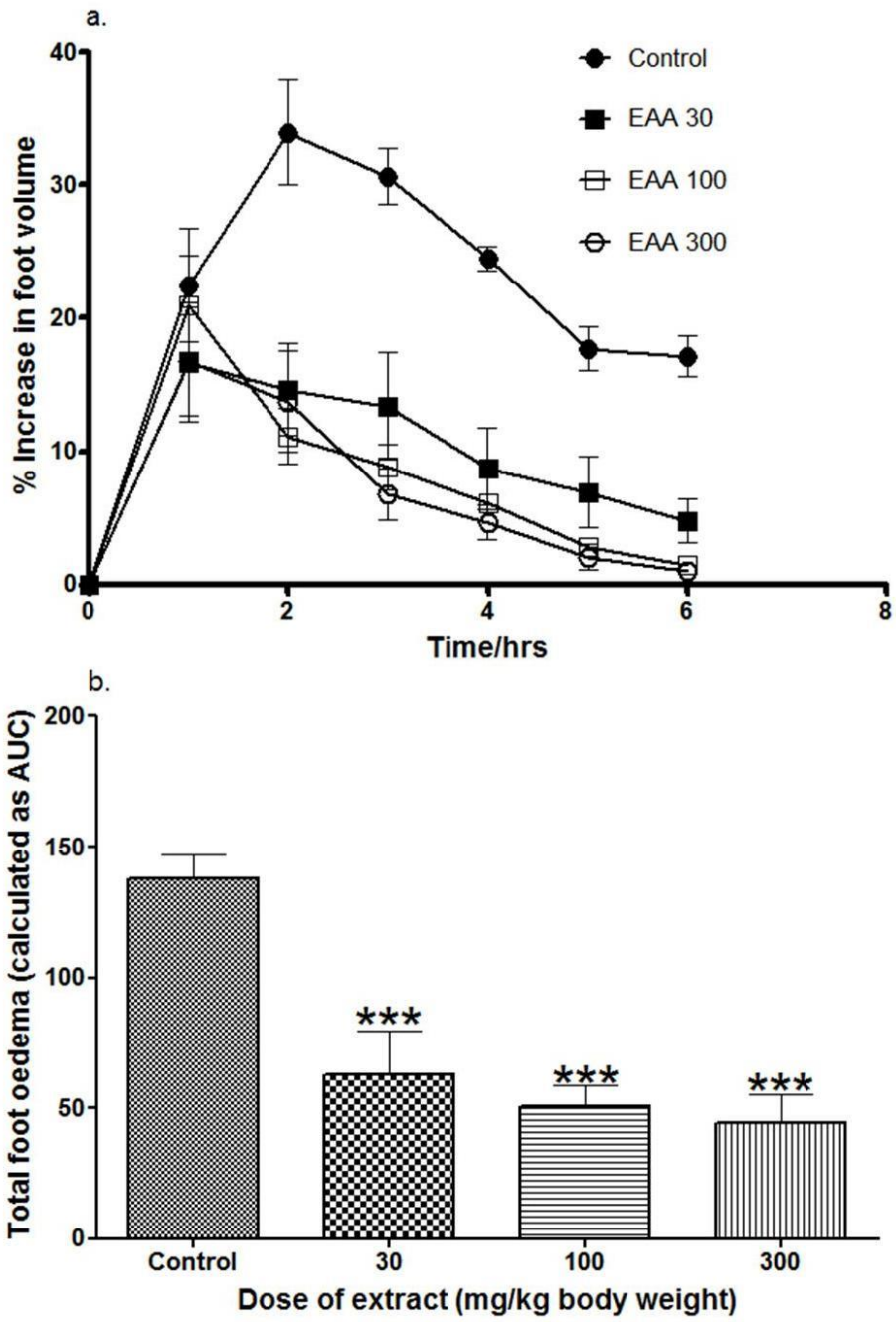


Figure 5.4 Effect of 70 % methanol extract of *Anthostema aubryanum* (EAA: 30-300 mg/kg; *p.o*)] on time course curve (a) and the total oedema response, calculated as AUC's, for 6 h, in carrageenan induced paw oedema in chicks (b). Values are means ± S.E.M (n=5), *** p < 0.001, ** p < 0.01, *P < 0.05 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's post *hoc test*).

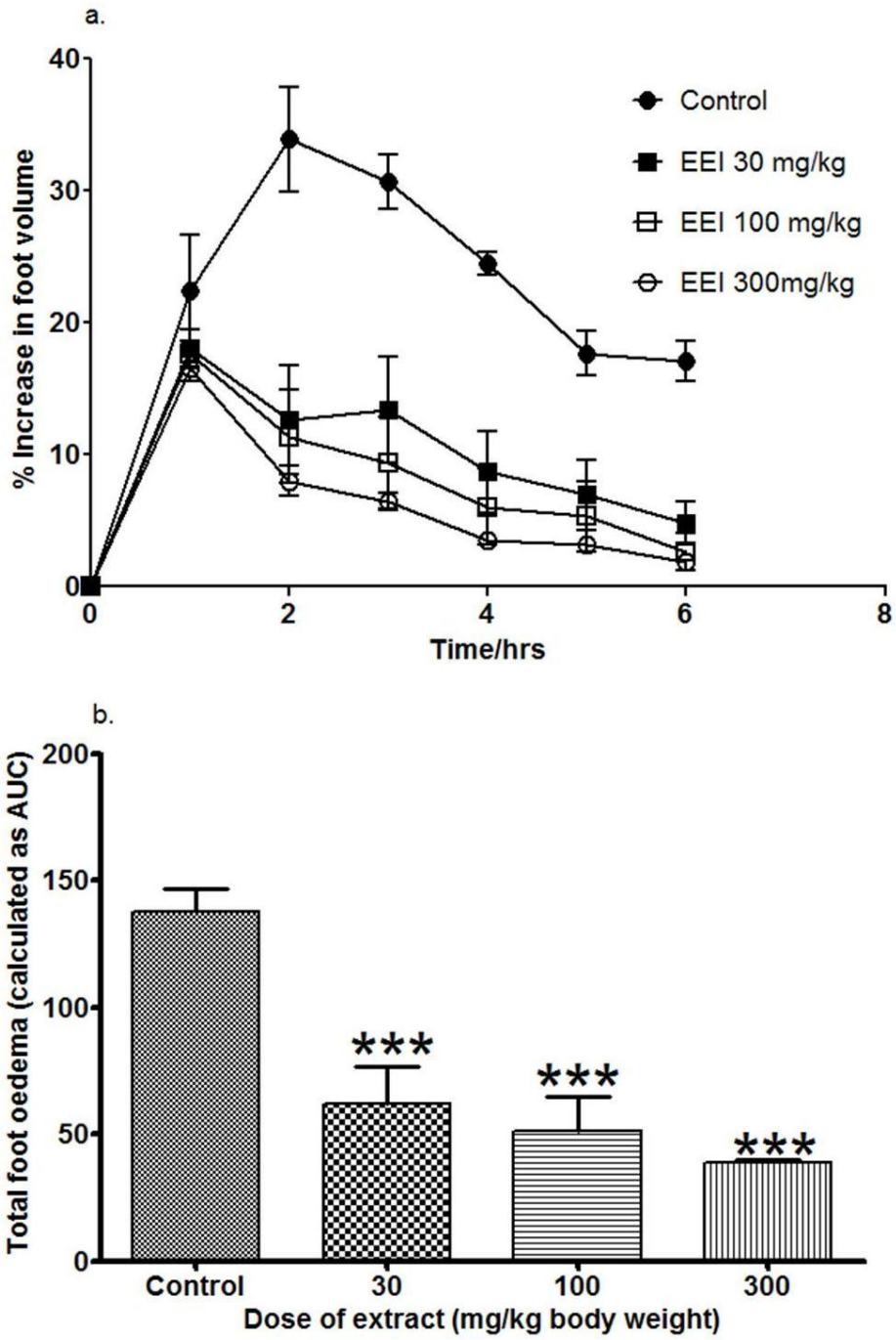


Figure 5.5 Effect of 70% methanol extract of *Erythrophleum ivorense* (EEI: 30-300 mg/kg; *p.o*)] on time course curve (a) and the total oedema response, calculated as AUC's, for 6 h, in carrageenan induced paw oedema in chicks (b). Values are means ± S.E.M (n=5), *** p < 0.001, ** p < 0.01, *P < 0.05 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test).

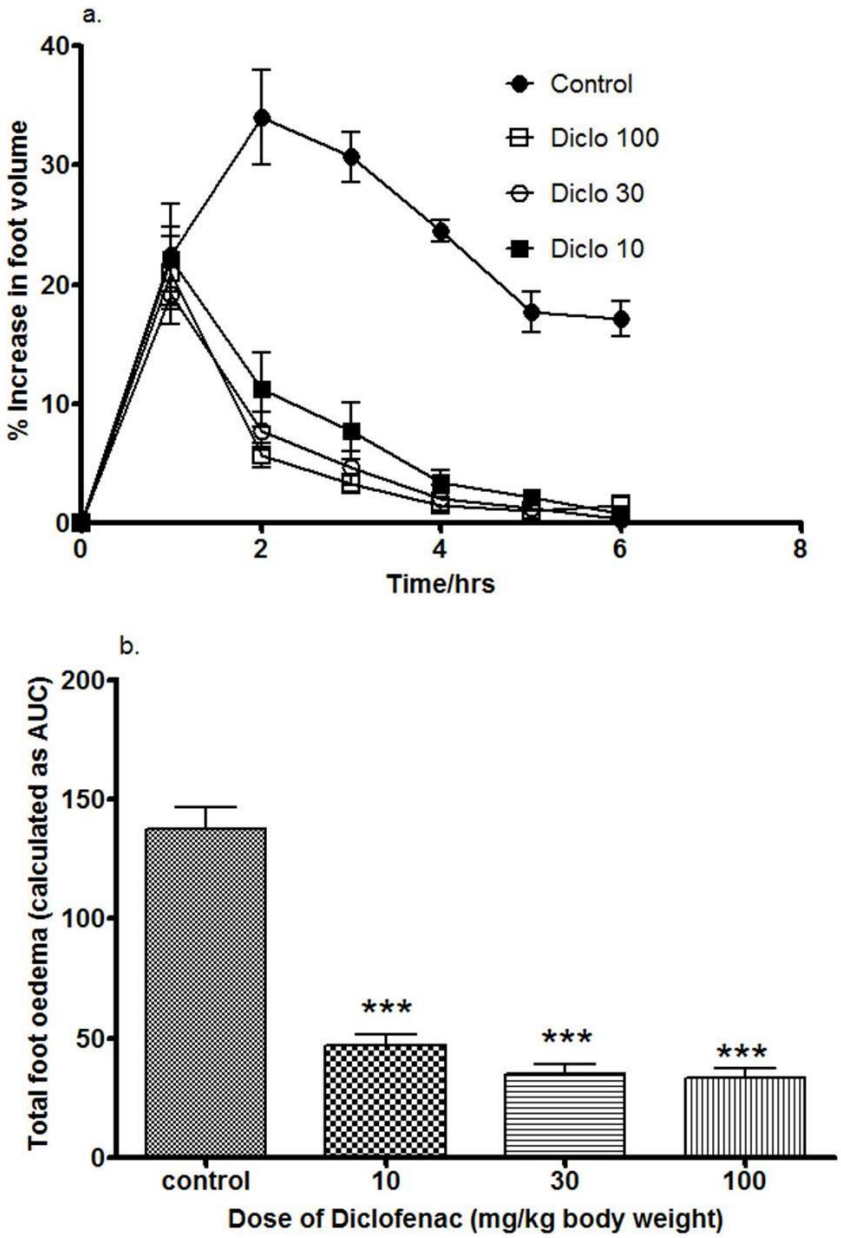


Figure 5.6 Effect of diclofenac (10-100 mg/kg; p.o)] on time course curve (a) and the total oedema response, calculated as AUC's, for 6 h, in carrageenan induced paw oedema in chicks (b). Values are means ± S.E.M (n=5), *** p < 0.001, ** p < 0.01. *P < 0.05 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's post *hoc test*).

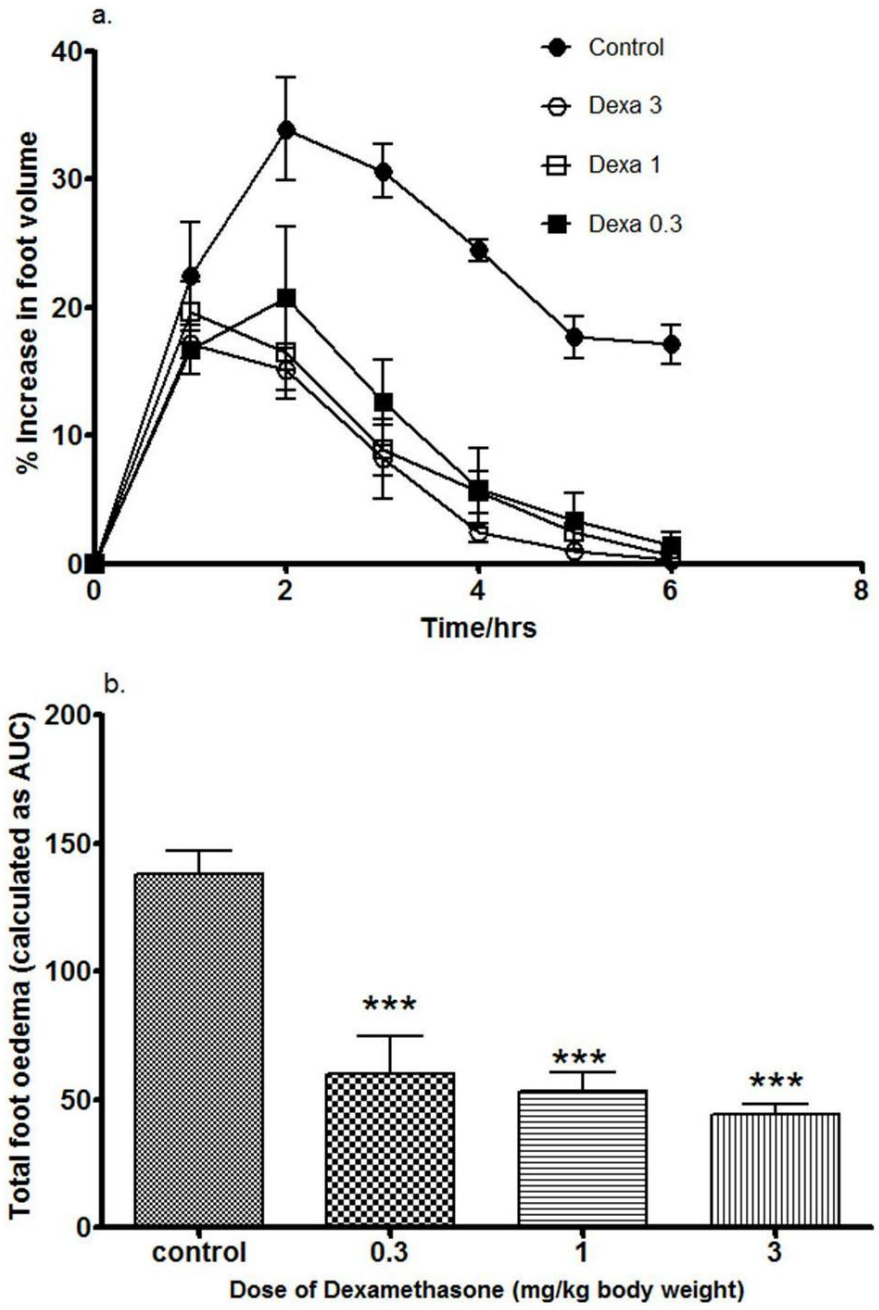


Figure 5.7 Effect of dexamethasonec (0.3 -3 mg/kg; p.o)] on time course curve (a) and the total oedema response, calculated as AUC's, for 6 h, in carrageenan induced paw oedema in chicks (b). Values are means ± S.E.M (n=5), *** p < 0.001, ** p < 0.01. *P < 0.05 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's post *hoc test*).

Table 5.4 Effects of plants extracts and standard drugs on carrageenan-induced oedema

Extracs/Drug	ED_{50}±SEM (mg/kg body weight)
Diclofenac	3.74±0.333
Dexamethasone	1.98±0.133
Omphalocarpum ahia	75.9±0.667
Coelocaryon oxycarpum	20.43±0.332
Anthostema aubryanum	101.3±1.333
Erythrophleum ivorense	16.91±0.667

5.3.1 Anti-inflammatory activity of fractions of *E. ivorense*

The result of the anti-inflammatory activity of the selected plants (section 5.3) revealed *E. ivorense* to be the most potent. It was therefore fractionated into petroleum ether, ethyl acetate and residual methanol fractions (Section 4.2). The fractions were tested for *in vivo* anti-inflammatory activity using the carrageenan induced inflammation model (Section 4.5). All the three fractions exhibited a dose dependent reduction in inflammation (Figure 5.8- 5.10) with the ethyl acetate fraction showing the highest activity. The activity of the ethyl acetate fraction was only 1.4 times less potent than the crude extract but 6 fold less potent than diclofenac used as positive control (Figure 5.11). Thus the ethyl acetate fraction contributed significantly to the anti-inflammatory activity of *E. ivorense* than the pet-ether and methanol fractions which were 7 and 4 times less potent than the total crude extract respectively (Figure 5.11).

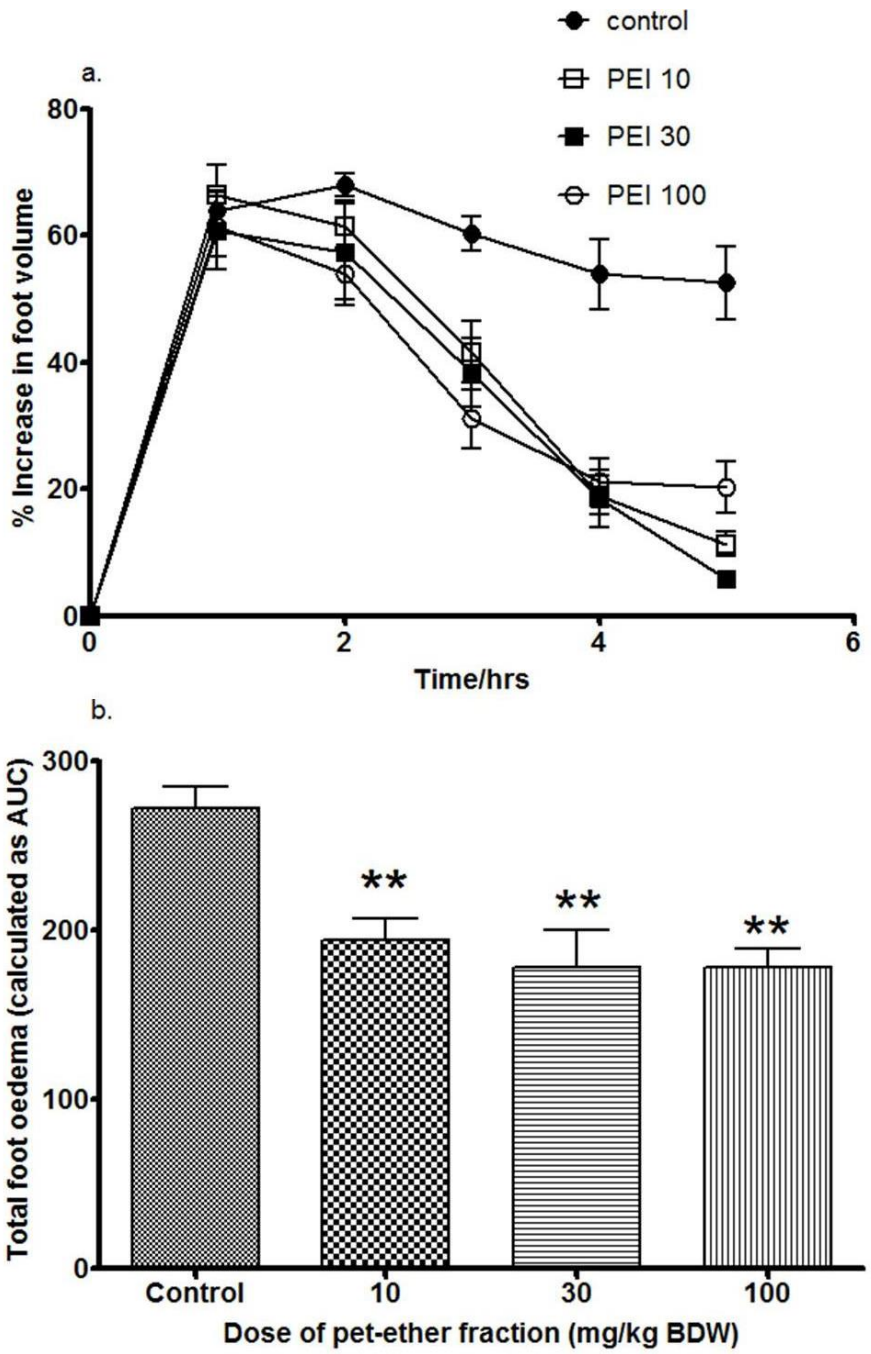


Figure 5.8 Effect of the pet-ether extract of *E. ivorense* (PEI: 10-100 mg/kg; *p.o*)] on time course curve (a) and the total oedema response, calculated as AUC's, for 6 h, in carrageenan induced paw oedema in chicks (b). Values are means ± S.E.M (n=5), *** p < 0.001, ** p < 0.01. *P < 0.05 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test).

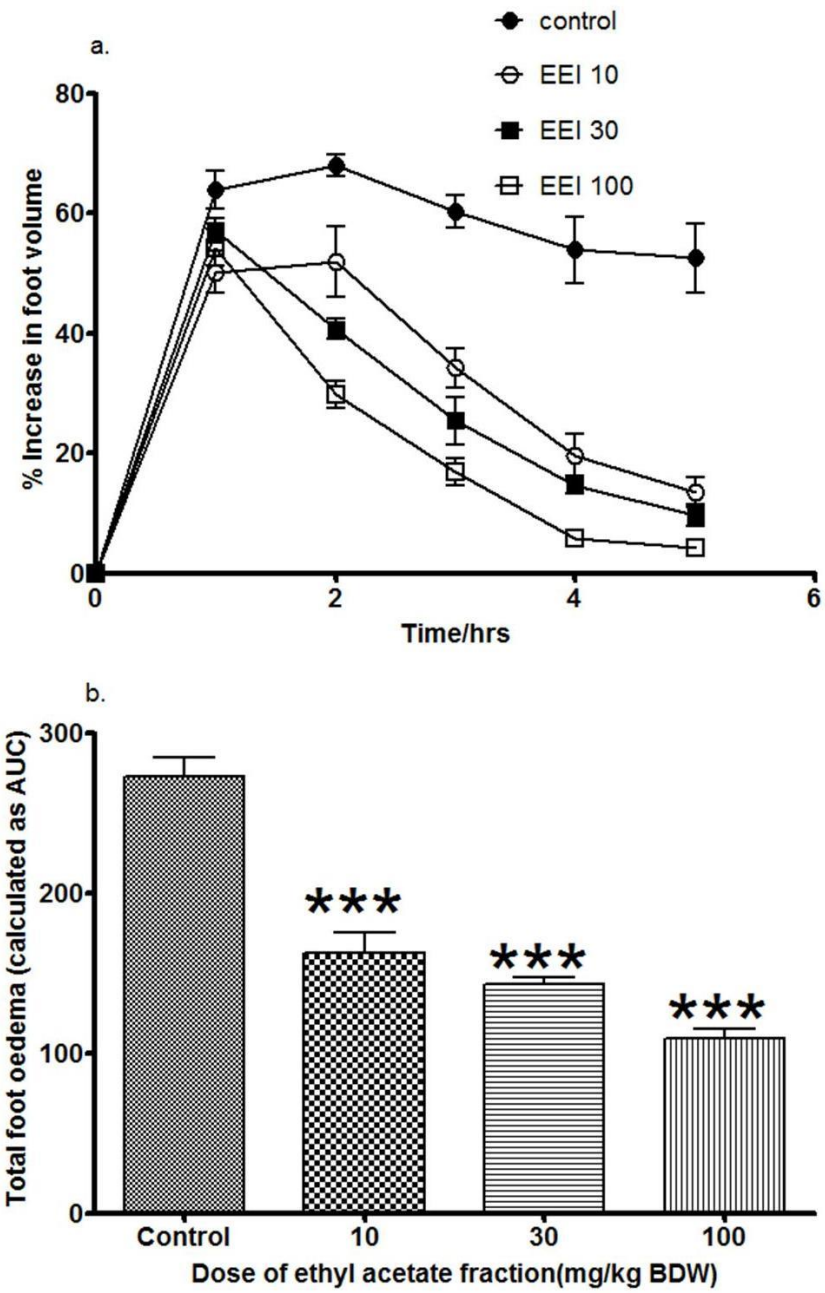


Figure 5.9 Effect of the ethyl acetate extract of *E. ivorense* (EEI: 10-100 mg/kg; *p.o*)] on time course curve (a) and the total oedema response, calculated as AUC's, for 6 h, in carrageenan induced paw oedema in chicks (b). Values are means ± S.E.M (n=5), *** p < 0.001, ** p < 0.01. *P < 0.05 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test).

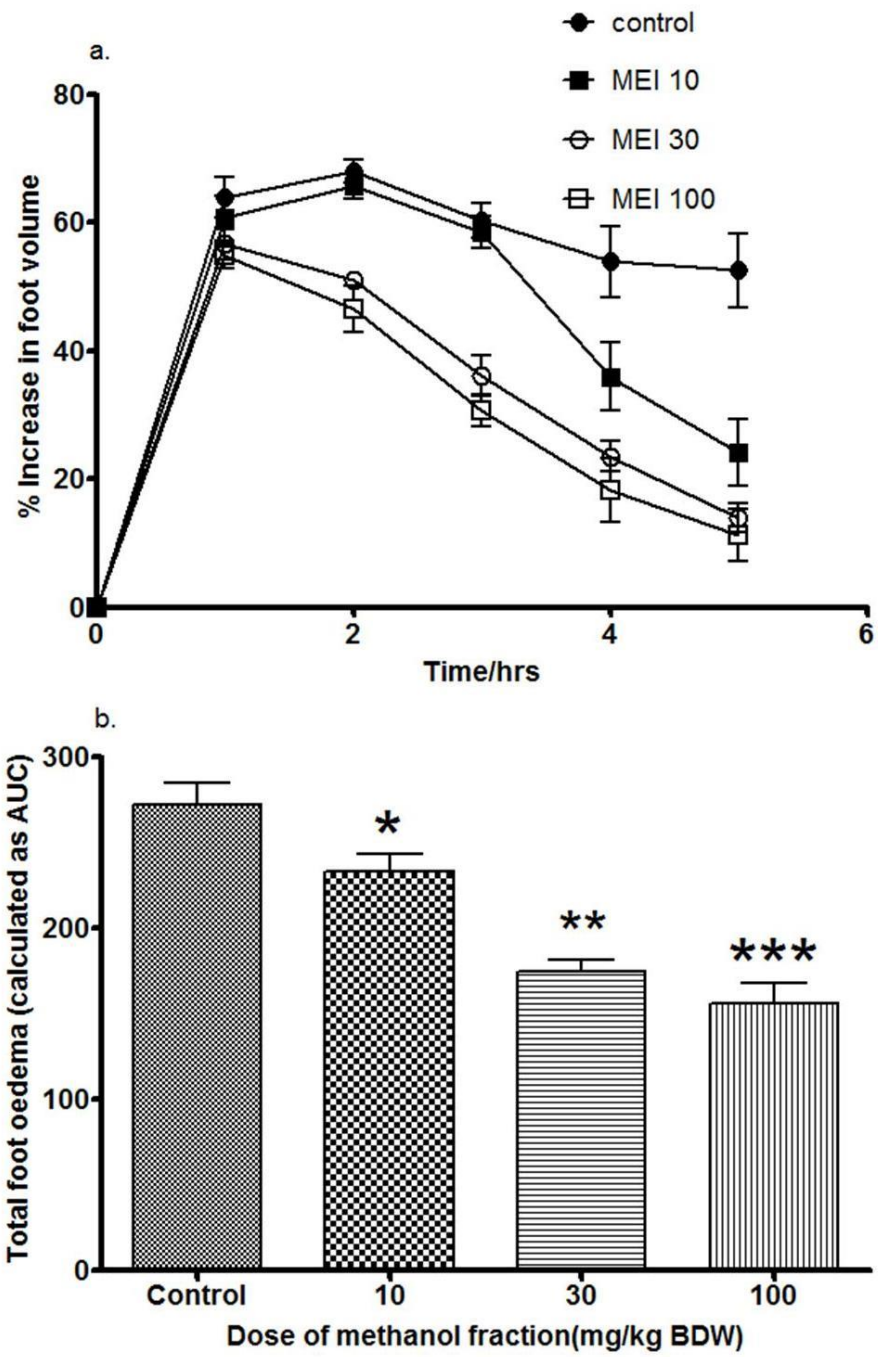


Figure 5.10 Effect of the methanol extract of *E. ivorense* (MEI: 10-100 mg/kg; p.o)] on time course curve (a) and the total oedema response, calculated as AUC's, for 6 h, in carrageenan induced paw oedema in chicks (b). Values are means ± S.E.M (n=5), *** p < 0.001, ** p < 0.01. *P < 0.05 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test).

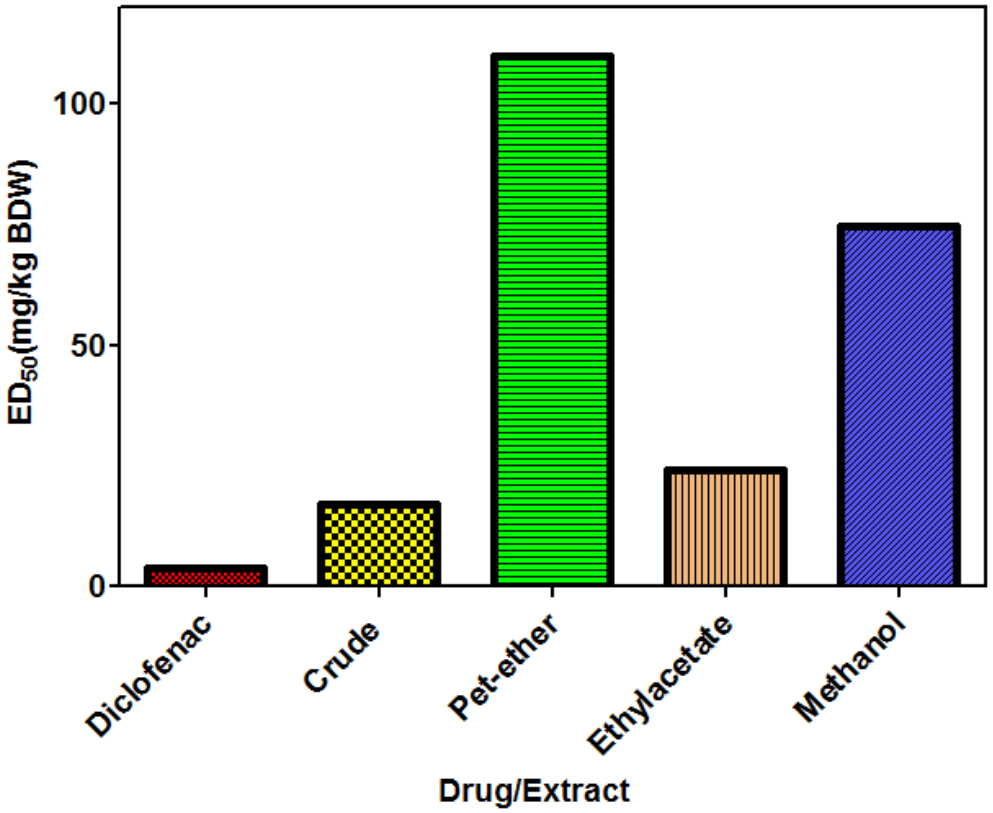


Figure 5.11 Comparison of ED50s of control, crude and fractions of *E ivorense*

5.3.2 Anti-inflammatory activity of compounds isolated from *E. ivorense*

Chromatographic fractionation of the bioactive ethyl acetate fraction afforded the compounds labeled compounds 1, 2 and 3 (Section 4.9) whose structures were elucidated as erythroivorensin, betulinic acid and eriodictyol respectively (Section 5.5). These compounds were evaluated for anti-inflammatory activity (section 4.5.3) to ascertain their contribution to the anti-inflammatory activity of *Erythrophleum ivorense*. All compounds showed significant ($p<0.05$) dose-dependent anti-inflammatory activities. They caused a reduction of the inflammation from the second hour (Figure 5.12 – 5.14). Betulinic acid showed the highest activity (lowest ED_{50}= 4.367±1.333mg/kg body weight) followed by erythroivorensin and eriodictyol respectively (Table 5.5). The activities of betulinic acid, erythroivorensin and

eriodictyol were 1, 5 and 15 times less potent than diclofenac used as standard drug respectively. Thus these compounds worked synergistically to establish the anti-inflammatory activity of the ethyl acatetae fraction of *Erythrophleum ivorense*.

Table 5.5 Effects of isolated compounds and standard drug on carrageenan-induced oedema

Compounds	**ED_{50}±SEM (mg/kg body weight)**
Betulinic acid	4.367±1.333
Erythroivorensin	18.66±0.667
Eriodictyol	56.25±0.133
Diclofenac	3.74±0.333

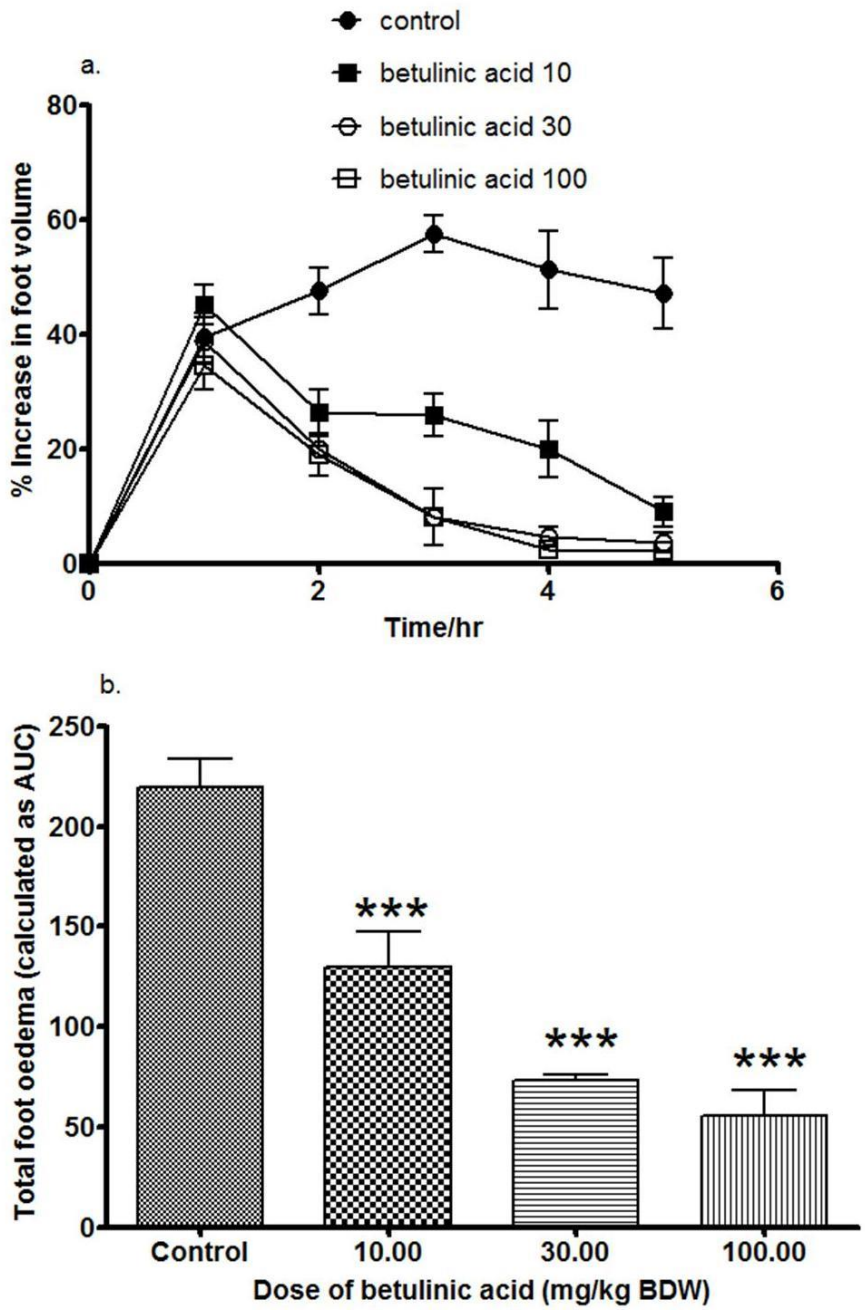


Figure 5.12 Effect of betulinic acid (10-100 mg/kg; *p.o*) on time course curve (a) and the total oedema response, calculated as AUC's, for 6 h, in carrageenan induced paw oedema in chicks (b). Values are means ± S.E.M (n=5), *** p < 0.001, ** p < 0.01. *P < 0.05 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test).

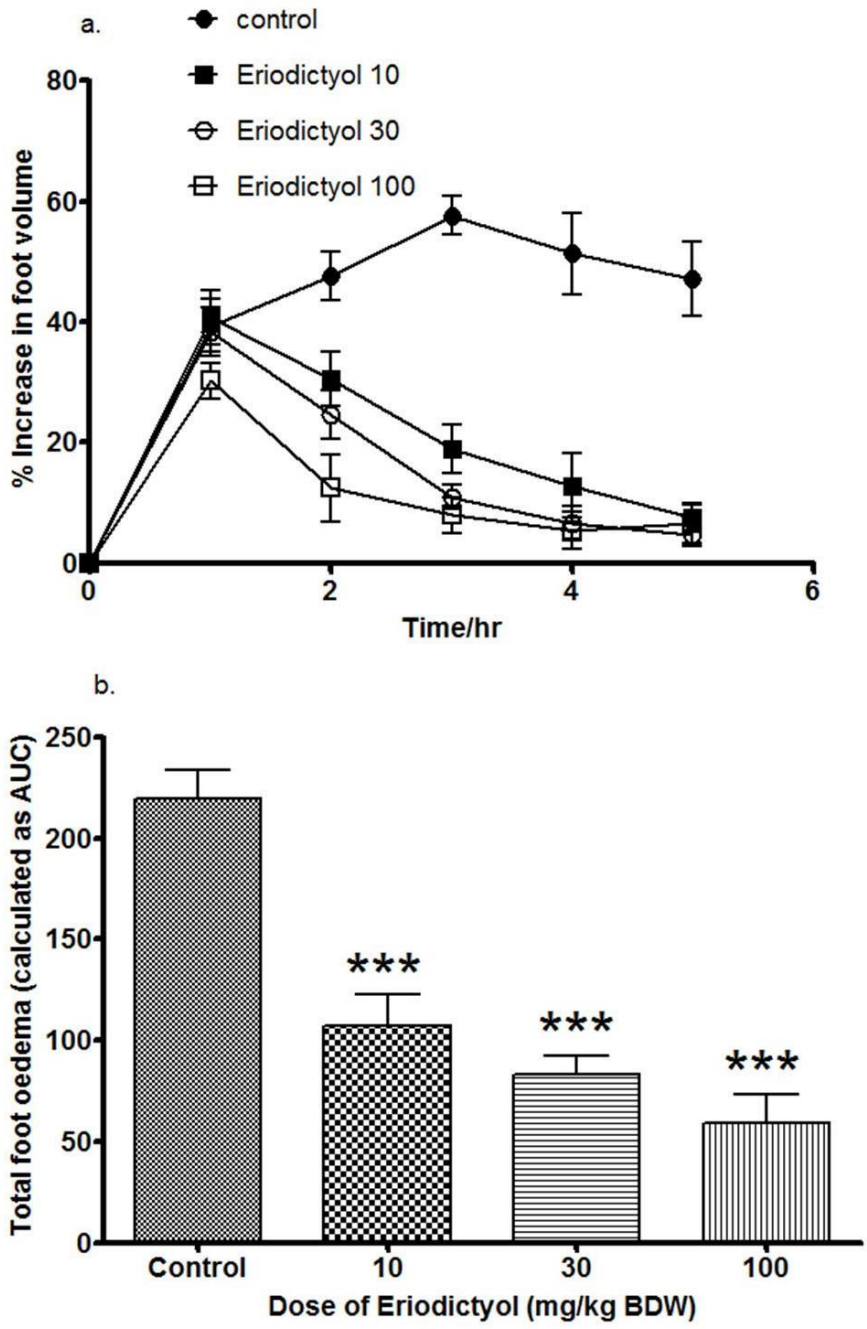


Figure 5.13 Effect of eriodictyol (10-100 mg/kg; *p.o*)] on time course curve (a) and the total oedema response, calculated as AUC's, for 6 h, in carrageenan induced paw oedema in chicks (b). Values are means ± S.E.M (n=5), *** p < 0.001, ** p < 0.01. *P < 0.05 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test).

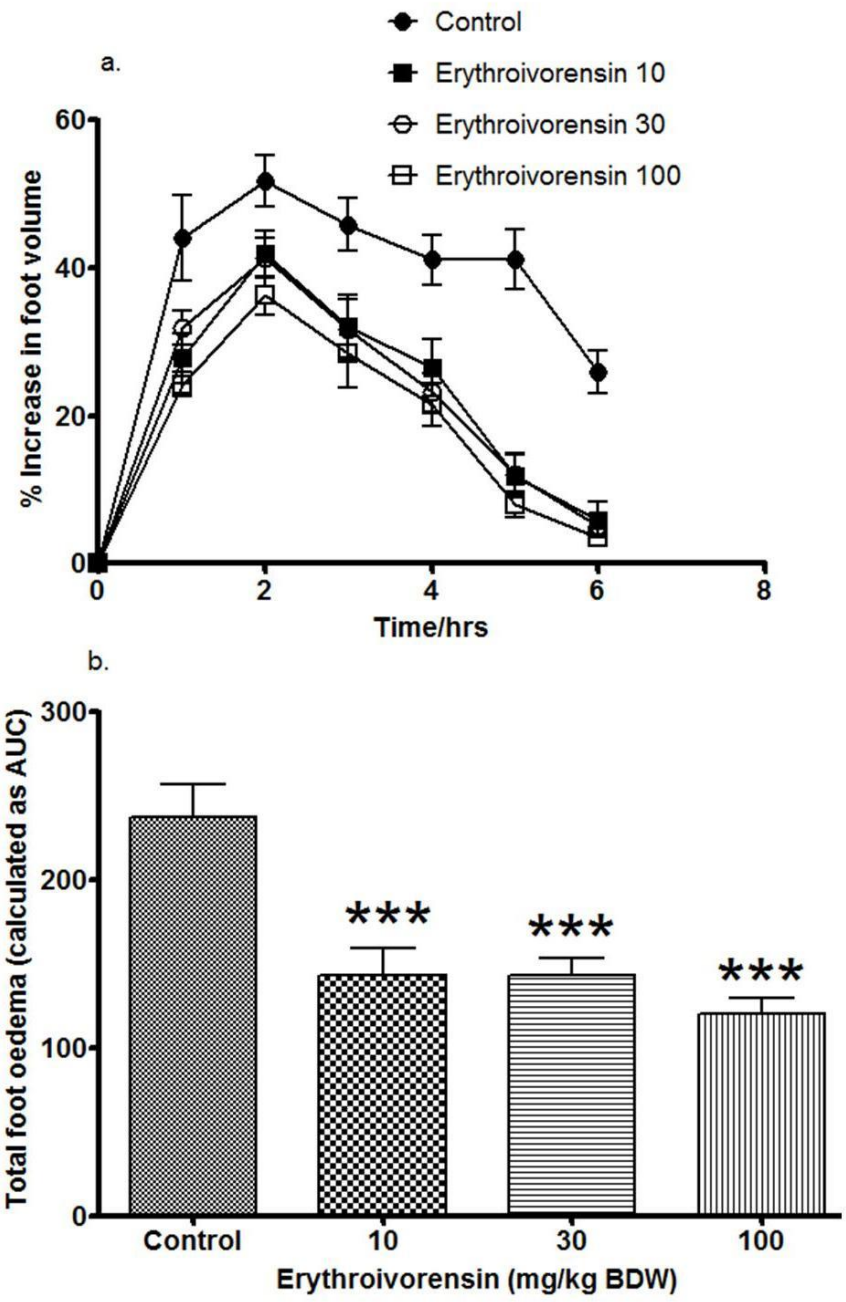


Figure 5.14 Effect of erythroivorensin (10-100 mg/kg; *p.o*)] on time course curve (a) and the total oedema response, calculated as AUC's, for 6 h, in carrageenan induced paw oedema in chicks (b). Values are means ± S.E.M (n=5), *** p < 0.001, ** p < 0.01. *P < 0.05 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test).

5.4 ANTIOXIDANT ACTIVITY OF SELECTED MEDICINAL PLANTS

In the present study, the antioxidant activity of 70 % methanol extracts of *E. ivorense, O. ahia*, *C. oxycarpum* and *A. aubryanum* was evaluated. The scavenging activities of the extracts through the annihilation of the DPPH radical as well as their total antioxidant capacity were investigated.

5.4.1 DPPH scavenging activity of seleceted plants

DPPH is an unstable free radical in methanol or ethanol solutions and accept hydrogen to become a stable diamagnetic molecule. It is used as a substrate to evaluate the antioxidant activity of potential antioxidants. In this research, the DPPH radical scavenging assay was used to evaluate the antioxidant properties of the selected plants. The reduction capability of DPPH radical was determined by a decrease in absorbance at 517 nm. All the four plant extracts showed a concentration dependent DPPH radical scavenging activity (Figure 5.15). The decrease in the absorbance of DPPH was due to phytoconstituents in the plant extracts acting as antioxidants by hydrogen donation. The free radical scavenging activity was compared to vitamin E, a standard antioxidant molecule. The antioxidant activity of the plant extracts, expressed as the EC_{50}, is shown in Table 5.6. The lower the EC_{50}, the higher the activity. The rank order of antioxidant potency, as measured by the EC_{50} was *E. ivorense*> vitamin E> *C. oxycarpum*> *O. ahia*> *A. aubryanum*. Thus the DPPH radical scavenging activity of *E. ivorense* extract was about 3 times higher than that of the standard antioxidant vitamin E. The present results also suggest that all the tested plant extracts have moderate to potent free radical scavenging activity.

Table 5.6 DPPH scavenging activity of the plants extracts and standard drug

Extracts/standard	Free radical scavenging activity EC_{50} ± SEM (µg/ml)
Omphalocarpum ahia	8.70±0.32
Coelocaryon oxycarpum	5.729±0.56
Anthostema aubryanum	23.76±0.61
Erythrophleum ivorense	1.11±0.12
Vitamin E	2.79±0.33

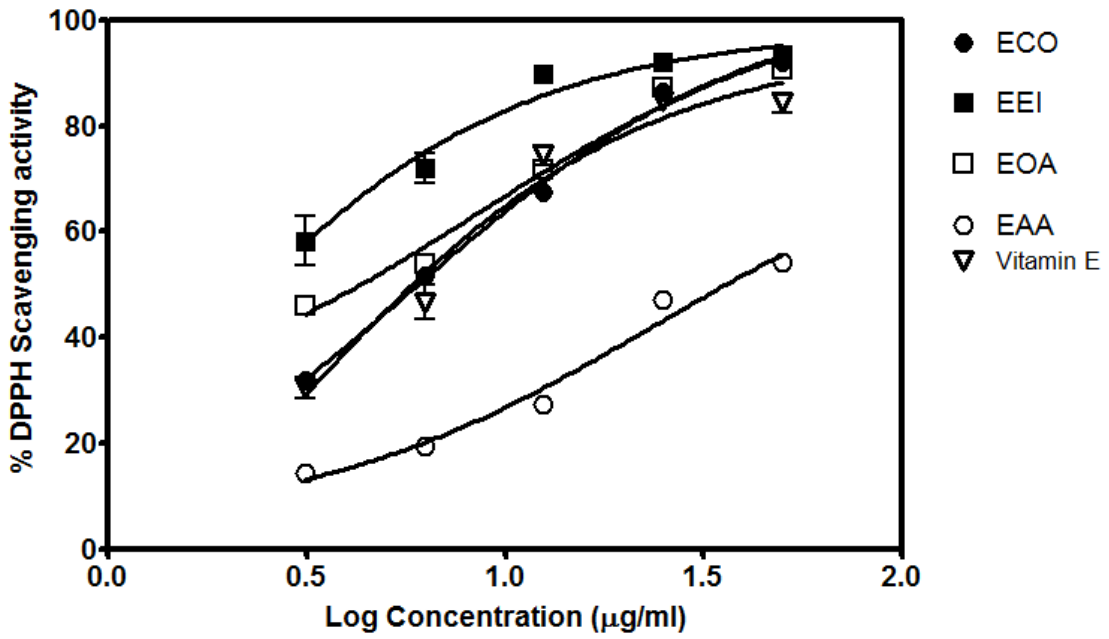


Figure 5.15 Free radical scavenging activity of extracts and standard antioixdant
ECO: *C. oxycarpum*, EEI: *E. ivorense*, EAA: *A. aubryanum*, EOA: *O. ahia* extracts

5.4.2 Total antioxidant capacity of seleceted plants

The measure of total antioxidant capacity considers the cumulative action of all the antioxidants present, thus providing an integrated parameter of all measurable antioxidants

(Ghiselli *et al*., 2000). The capacity of different antioxidants in the plant extracts and their synergistic interaction was therefore assessed in this assay method. It is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of green phosphate/Mo (V) complex at acidic pH (Amponsah *et al*., 2014). In this phosphomolybdate total antioxidant capacity assay (TAC), vitamin C was used as the standard antioxidant. The total antioxidant capacity was calculated in mg of ascorbic acid equivalent per gram of extract (AAE). Vitamin C showed a good linearity in the range 50-3.125 µg/mL with a high positive correlation coefficient (r^2=0.9996) (Figure 5.16). All four plant extracts showed a concentration dependent antioxidant activity. *E. ivorense* gave the highest total antioxidant capacity followed by *C. oxycarpum, O. ahia* and *A. aubryanum* respectively (Figure 5.17). The results indicated that the methanol extracts, of the selected medicinal plants, contain much quantity of antioxidants compounds as equivalents of ascorbic acid to effectively reduce the oxidant (molybdenum) in the reaction matrix.

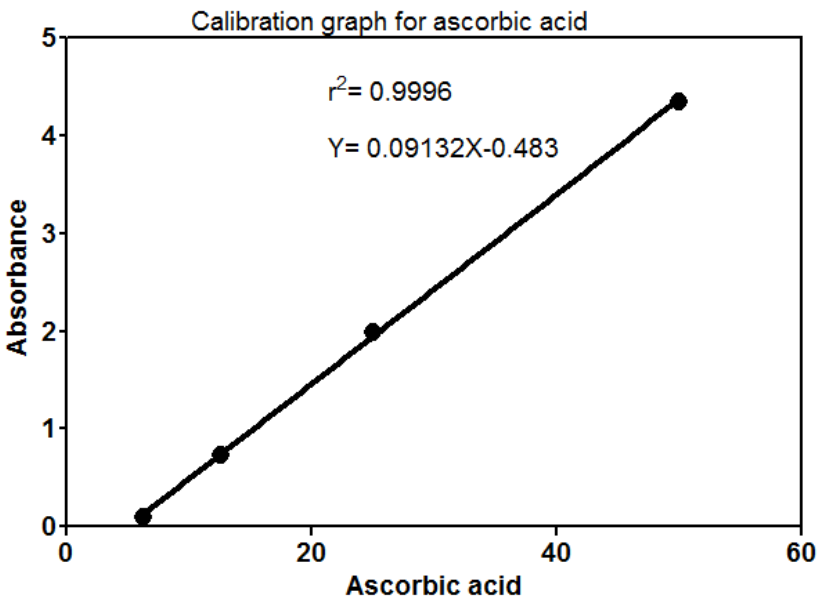
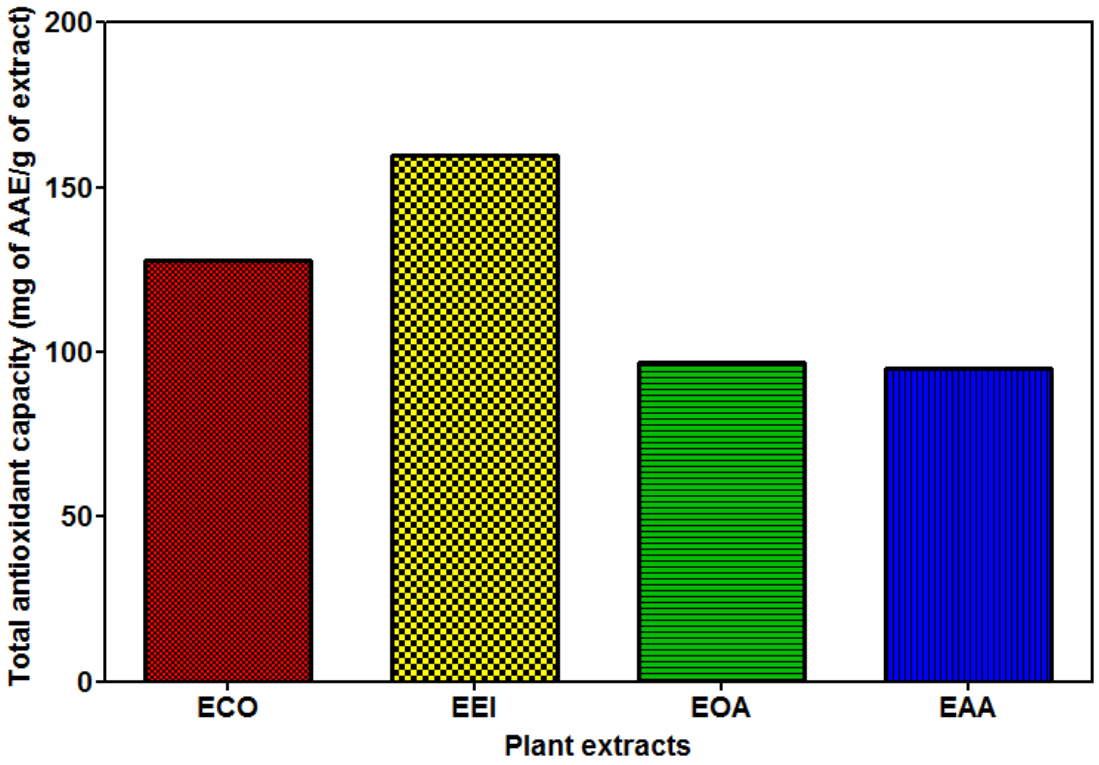


Figure 5.16 Calibration curve for ascorbic acid



ECO: C. oxycarpum, EEI: E. ivorense, EAA: A. aubryanum, EOA: O. ahia extracts

Figure 5.17 Total antioxidant capacity of ECO, EEI, EOA and EAA

5.4.3 Antioxidant activity of fractions of *E. ivorense*

The results of the present study have shown that *E. ivorense* has the highest antioxidant activity. Therefore, in an attempt to track down its antioxidant compounds, the pet-ether, ethyl acetate and methanol fractions of *E. ivorense* were similarly evaluated for their antioxidant activity using the DPPH radical scavenging assay. The ethyl acetate fraction gave the highest EC_{50} followed by the methanol and pet-ether fractions respectively (Table 5.7, Figure 5.18). The antioxidant activity of the ethyl acetate fractions was however about 3 times less potent than the total crude extract and only 1 times lower than the standard antioxidant vitamin E.

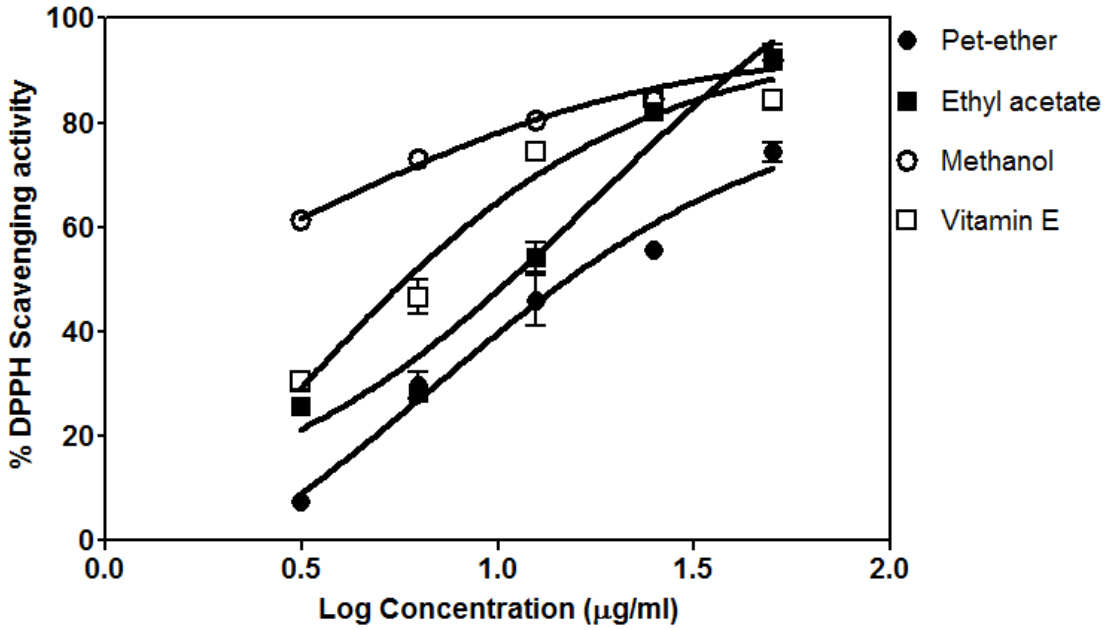


Figure 5.18 Free radical scavenging activity of pet ether, ethyl acetate and methanol (0.25-2mg/mL and standard drug vitamin E (3.75-3.0 µg/mL).

Table 5.7 DPPH scavenging activity of fraction of *E. ivorense* and standard drug

Fractions/standard	Free radical scavenging activity EC_{50} ± SEM (µg/ml)
Pet-ether	17.62±0.0.15
Ethyl acetate	3.70±0.17
Methanol	6.85±0.33
Vitamin E	2.79±0.33

5.4.4 Antioxidant activity of isolated compounds

According to the above results, the ethyl acetate fraction exhibited the strongest DPPH radical scavenging activity. Further fractionation culminated in the isolation of eriodictyol, betulinic acid and erythroivorensin. The antioxidant activities of these compounds were also evaluated via the DPPH radical scavenging assay. The compounds exhibited a concentration dependent radical scavenging activity (Figure 5.19) with the flavonoid eriodictyol showing the highest activity, followed by betulinic acid and erythroivorensin respectively (Table 5.8). The antioxidant activity of the compounds were however lower than the crude ethyl acetate fraction, thus highlighting the synergy of diverse molecules in plants in the establishment of pharmacological activity.

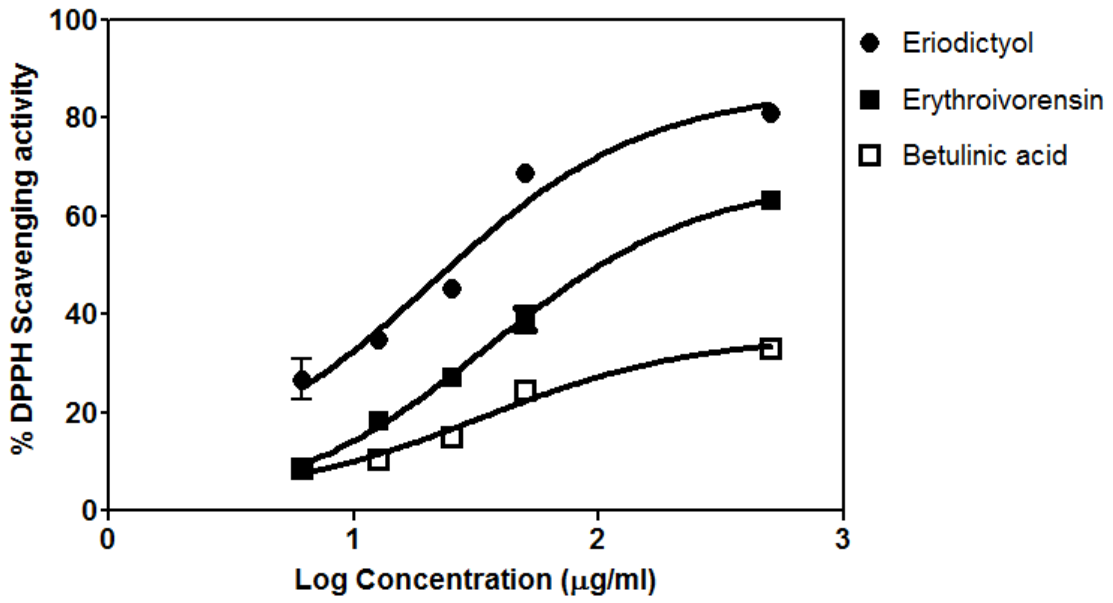


Figure 5.19 DPPH scavenging activity of isolated compounds and standard antioxidant

Table 5.8 DPPH scavenging activity of isolated compounds and and standard drug

Isolates/standard	**EC_{50}±SEM**
Betulinic acid	33.07±0.084
Eriodictyol	21.06±1.011
Erythroivorensin	35.78±1.14
Vitamin E	2.788±0.124

5.5 STRUCTURAL ELUCIDATION OF ISOLATED COMPOUNDS

5.5.1 Identification of compound 1 as Erythroivorensin

Compound 1 was isolated as a colourless needle (Ac_2O); mp 187–189 °C; [α] 21 D = −3.3° (c 0.15, MeOH); UV (MeOH) λmax nm (ε): 231 (999); IR (Universal Attenuated Total Reflectance) νmax; 2919, 2863, 1685, 1293, 1263, 904 cm−1; ^{1}H and ^{13}C NMR (CDCl3). ESI-MS m/z $[M+H]^+$ 303.2321

Accurate mass analysis using high resolution ESI-MS instrument showed the $[M+H]^+$, $C_{20}H_{30}O_2$ plus H, ion at m/z 303.2321 (expected/theoretical 303.2319). The ^{13}C and Dept-135 NMR spectra showed signals indicating the presence of a 20-carbon diterpene skeleton. Of these, one was carbonyl; 4 olefinic of which two quaternary, one methine and one methylene; and in the saturated region, 2 quaternary, 3 methine, 7 methylene and 3 methyl groups (Table 5.9) (Appendix 1a). Further assignment of all NMR signals and the structure came from comprehensive 2-D NMR studies (COSY, HMQC, HMBC and NOESY). Given the molecule 6 double bond equivalents and two double bonds and a carbonyl group (strong IR signal at 1685 cm^{-1} for α, β-unsaturated carbonyl) were evident from the NMR data, the structural assignment was based on a three-ring diterpene skeleton. The classical ABX system in the side chain (C15 methine and C16 methylene olefinic system) was established from the coupling pattern in the ^{1}H NMR spectrum (10.9 Hz as cis- and 17.2 Hz as trans-coupling) (Appendix 1b) and COSY studies. The key 2J and 3J HMBC connectivities shown in Table 5.9 further allowed the assignment of the extended conjugation of this system with the second double bond (C13–C14) and the carboxylic acid at C-17. The C-4 gem dimethyl (C18, C19) and C-20-methyl groups were also good reference points to establish the HMBC-based assignment of the structure as erythroivorensin. Finally, the assignment of ^{1}H NMR signals of the C-16 protons were based on NOESY studies where only H-16B showed an interaction with one of the H-12

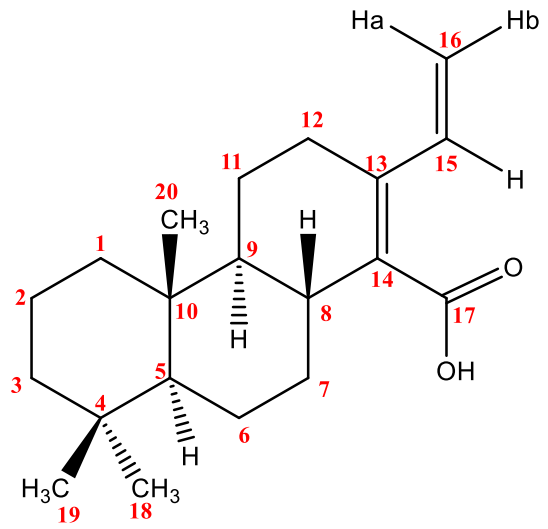
protons at δ 2.46. Although the NOESY studies were helpful in determining the stereochemistry of the compound, unambiguous assignment of the structure as the novel compound named erythroivorensin (compound 1) came from X-ray analysis study (Figure 5.20). Details of the crystal data are presented in Table 5.10.

Table 5.9 NMR spectroscopic data ($CDCl_3$) for erythroivorensin

Position	**$\delta_{C(ppm)}$**	**$\delta_{H(ppm)}$**	**HMBC(major ^2J and ^3Jcorrelation)**
1	38.7 CH_2	1.75 dd (12.6)0.91m	C2, C3
2	31.9CH_2	2.01 dd(12.2, 2.2) 1.12 m	
3	42.2 CH_2	1.43 m 1.38 m	
4	33.2 C		
5	55.4 CH	0.92 m	C4, C9, C20, C18, C19
6	21.8CH_2	1.68 dd (13.3, 3.0) 1.39 m	C8
7	18.9 CH_2	1.61 dt (12.9,3.4) 1.43 m	
8	37.4 CH	2.48 m	C14
9	52.9 CH	0.97 m	C7, C20
10	36.8 C		
11	20.6CH_2	1.86 dd (12.6,5.9) 1.15 m	C8, C9, C12, C13
12	26.1 CH2	2.46 m 2.18 m	C13, C13, C14C13, C13, C14
13	136.5 C		
14	134.5 C		
15	134.9 CH	6.84 dd (17.2,10.9)	C12, C13, C16
16	115.6 CH_2	16a 5.16 (10.9) 16b 5.35 (17.2)	C13, C15
17	174.3 C		
18	33.4 CH_3	0.85 s	C3, C4, C5, C19
19	21.6 CH_3	0.83 s	C3, C4, C5, C18
20	14.2 CH_3	0.87 s	C1, C5, C9, C10

Table 5.10 Crystal data and structure refinement for erythroivorensin

Empirical formula	**$C_{20}H_{30}O_2$**
Formula weight	604.88
Temperature/K	150 (1)
Crystal system	Orthorhombic
Space group	P212121
a/Å	11.99271 (7)
b/Å	14.66394 (7)
c/Å	19.91449 (10)
α/°	90
β/°	90
γ/°	90
Volume/$Å^3$	3502.17 (3)
Z	8
ρcalc g/cm^3	1.147
μ/mm^{-1}	0.554
F(000)	1328.0
Crystal size/mm^3	0.28 × 0.1 × 0.08
Radiation	CuKα (λ = 1.54184)
2Θ range for data collection/°	7.486 to 147.294
Index ranges	$14 \leq h \leq 14$, $-18 \leq k \leq 18$, $-24 \leq l \leq 24$
Reflections collected	59,230
Independent reflections	7031 [Rint = 0.0305, Rsigma = 0.0141]
Data/restraints/parameters	7031/0/405
Goodness-of-fit on F2	1.021
Final R indexes [$I \geq 2\sigma$ (I)]	0.0309, wR2 = 0.0843
Final R indexes [all data]	R_1 = 0.0317, wR_2 = 0.0853
Largest diff. peak/hole/e $Å^{-3}$	0.23/−0.15
Flack parameter	−0.12 (4)



Erythroivorensin

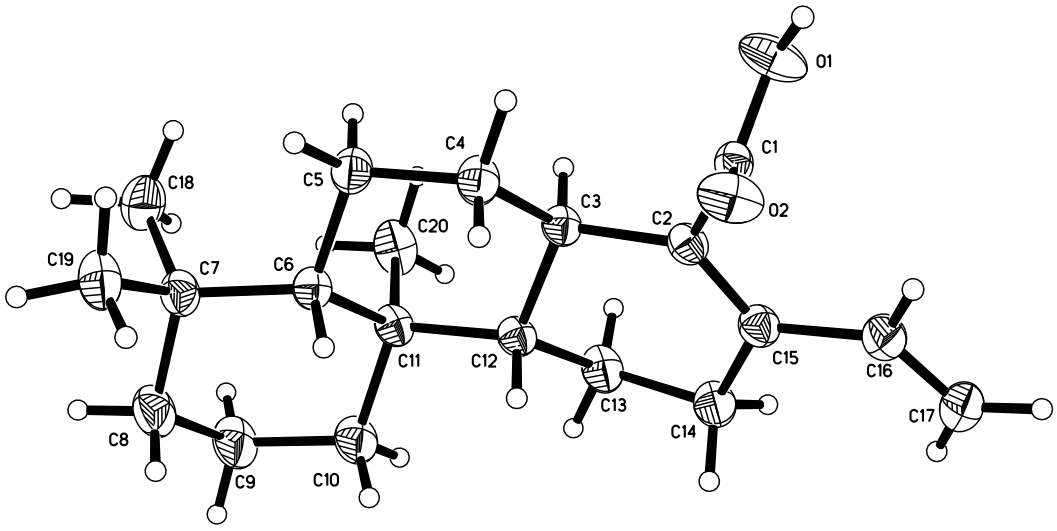


Figure 5.20 ORTEP plot of erythroivorensin showing the atom numbering scheme of one of the two molecules in the asymmetric unit. Thermal ellipsoids are drawn at the 50% probability level (hydrogen atoms are of arbitrary radius).

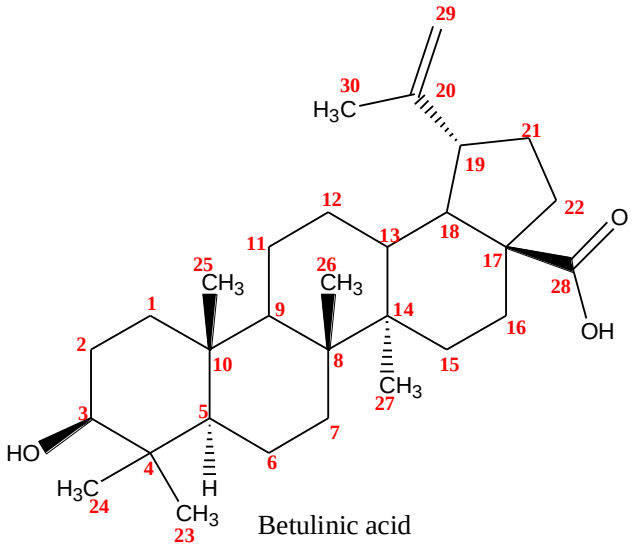
5.5.2 Identification of compound 2

Compound 2 was obtained as a white amorphous powder with melting point of 315-317 ºC. The UV spectrum showed an absorption maximum at 195 nm. The IR spectrum displayed absorption at 3500cm^{-1} for a hydroxyl group, 1700 cm^{-1}for a carboxylic acid moiety and 1625 cm^{-1} for an olefinic group.

The ^{1}H-NMR spectrum of compound 2 (Appendix 2a) displayed diagnostic peaks for five methyl groups at δ 0.75, 0.83, 0.95, 0.95 and 0.98 as well as isopropenyl signals at δ 1.69, 4.72 and 4.59 ppm indicative of a lupane-type skeleton (Saied and Begum, 2004). The signals at δ 4.72 and 4.59 ppm showed as distinct broad doublets arising from terminal olefinic protons assigned H-30. The methylene and methine multiplet envelope occurred between 1.03-3.15 ppm. Confirmation of compound 2 as a triterpene was indicated by the ^{13}C-NMR spectrum which displayed 30 carbon resonances including five methyls, five quartenary carbons, a carbonyl functional group and two olefinic carbons (Appendix 2b). The chemical shifts at δ 179.3, 150.9 and 109.6 were the characteristic peaks for betulinic acid type skeleton assigned to C-28, C-20 and C-29 respectively (Uddin *et al*., 2011). The EIMS displayed a molecular ion peak at M/Z 456 correponding to the molecular formular $C_{30}H_{48}O_3$. The spectral data of compound 2 agreed with that published for betulinic acid (Siddiqui *et al*., 1988) (Table 5.11). Thus the structure of compound 2 was elucidated as betulinic acid. To the best of my knowledge, this is the first report of betulinic acid in *Erythrophleum ivorense*. However, it has been isolated in some species of the the genus *Erythrophleum*.

Table 5.11 ^{13}C-NMR chemical shift of betulinic acid and compound 2

Carbon	Compound 2	Betulinic acid (Siddiqui *et al*., 1988)
C-1	38.5	39.1
C-2	27.1	28.1
C-3	78.9	78.1
C-4	39.0	39.4
C-5	55.6	55.7
C-6	18.5	18.6
C-7	34.5	34.7
C-8	40.9	40.9
C-9	50.8	50.8
C-10	37.3	37.3
C-11	21.1	21.1
C-12	25.7	25.9
C-13	38.4	38.4
C-14	42.6	42.4
C-15	30.8	31.1
C-16	32.5	32.7
C-17	56.4	56.3
C-18	47.2	47.6
C-19	49.5	49.5
C-20	150.9	150.7
C-21	29.9	30.1
C-22	37.5	37.5
C-23	28.0	28.5
C-24	16.2	16.3
C-25	16.0	16.3
C-26	15.5	16.2
C-27	14.8	14.8
C-28	179.3	178.7
C-29	109.6	110.3
C-30	19.4	19.4



Betulinic acid

5.5.3 Identification of compound 3 as Eriodictyol

Compound 3 was isolated as a yellow amorphous powder with a melting point of 263-265ºC. The UV spectrum of compound 3 showed absorption maxima in the range of '310-350 nm' typical of the band A of flavanones with the band B in the range of 250 -290 nm (Bohm, 1998). An absortion maxima appearing as a shoulder at 336 nm for band A and another at 288 for band B is reported to be the flavanone eriodictyol (Bohm, 1998).

The ^{1}H-NMR spectrum of compound 3 (Appendix 3a) showed signals at chemical shifts within the range of δ 6-8 ppm, which was consistent with the aromatic proton envelope. It exhibited two meta coupled doublets at δ 5.89 (d, J = 2.2, 1H) and δ5.87 (d, J = 2.2, 1H), each integrating for one proton, respectively for the aromatic protons H-6 and H-8 on the ring A. Three proton singlets at δ 6.78, 6.78, and 6.91 also depicted the aromatic protons H-5^{1}, H-6^{1} and H-2^{1} respectively on ring B. The chemical shifts of the aromatic protons indicated that compound 3 was a flavanone (Samia, 2010). Again the chemical shift of H-6 and H-8 aromatic protons in the range of δ 5.7-6.9 ppm indicated that ring A of the flavanone nucleus was substituted at positions 5 and 7 by hydroxyl groups (Liu, 2011). Similarly, for flavanones with substituent

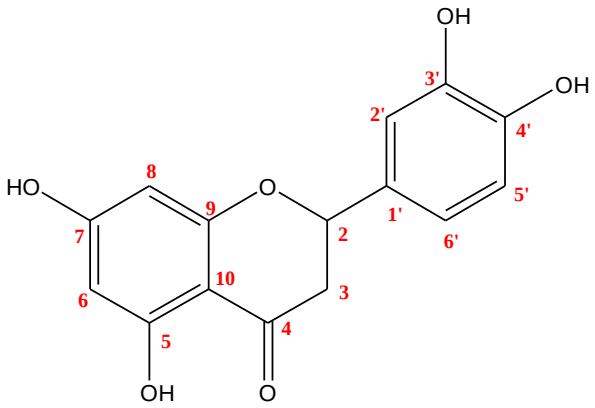
groups at 3^1 and 4^1 on ring B, the chemical shifts occur within the range of δ 6.70-7.10 ppm ((Liu, 2011) and this was consisitent for compound 3 (Table 5.12). The remaining proton signals in the spectrum consisted of a doublet of a doublet at δ 5.26 ppm (1 H, *J*= 3.0, 12.5 Hz) assignable to H-2 and two diastereotropic proton signals at δ 2.88 (dd, *J*= 12.5, 17 Hz) and δ 3.06 ppm (dd, *J*= 12.5, 17 Hz) assigned to H-3a and H-3b on the ring C of the flavanone (Table 5.12) (Appendix 3a). All these proton chemical shifts agreed with that published for Eriodictyol (Samia *et al.*, 2010).

The ^{13}C-NMR spectrum (Appendix 3b) of compound 3 displayed 15 carbon resonances (Table 5.12) comprising of one methylene, six methines and and eight quartenary carbons. The chemical shifts of the olefinic carbons C-5, C-7, C-6 and C-8 occurred at δ 165.4, 168.4, 97.0 and 96.2 ppm respectively with the former two occurring more downfield due to oxygenation (Table 5.12). Similarly, the six olefinic carbons on ring B gave chemical shifts in the range δ 114.7-146.9 ppm with C-3^1 and C-4^1 occurring more downfield due to the presence of hydroxyl substituents. The heterocyclic ring C of the flavanone was identified by the characteristic carbonyl signal at C-4 (δ 197.8 ppm), the methine signal C-2 (δ 80.5 ppm) and the methylene signal C-3 (δ 44.1 ppm). This again agreed with that published for eriodictyol (Samia *et al.*, 2010).

The unequivocal identification was by the EIMS (Appendix 3c), which showed a molecular ion peak at $[M+H]^+$ 289 corresponding to the molecular formular $C_{15}H_{12}O_6$ for eriodictyol. The results of the UV spectrum, 13-carbon and proton NMR as well as the mass spectra analysis agreed with that published for Eriodictyol (Samia *et al.*, 2010). Thus compound 3 was identified as the flavanone eriodictyol. This is the first report of the flavanone in the genus *Erythrophleum.*

Table 5.12 ^{13}C-NMR and ^{1}H-NMR chemical shift of compound 3 and eriodictyol

^{13}C-NMR	^{13}C-NMR of C-3(δ ppm)	^{13}C-NMR of Eriodictyol (Samia *et al* ., 2010)	^{1}H-NMR of C-3(δ ppm)	^{1}H-NMR of C-3(δ ppm)	^{13}H-NMR of Eriodictyol (Samia *et al.* , 2010)
C-2	80.5	80.5	H-2		5.26
C-3	44.2	44.1	H-3a		2.68
			H-3b		3.06
C-4	197.8	197.8			
C-5	165.3	165.4			
C-6	97.1	97.0	H-6		5.89
C-7	168.4	168.4	H-8		5.87
C-8	96.2	96.2	-		
C-9	165.0	164.9	-		
C-10	103.3	103.3	-		
C-1^{1}	131.8	131.8	-		
C-2^{1}	114.7	114.7	H-2^{1}		6.91
C-3^{1}	146.4	146.5			
C-4^{1}	146.8	146.9			
C-5^{1}	116.2	116.2	H-5^{1}		6.78
C-6^{1}	119.3	119.3	H-6^{1}		6.78



Chapter 6
DISCUSSION

6.1 INTRODUCTION

Leishmaniasis is a major public health problem especially in the developing countries. According to the World Health Organization (WHO), about 88 countries are threatened by leishmaniasis and about 350 million people are at risk for the disease (Sadeghi-Nejad *et al*., 2011). Kwakye-Nuako *et al.,* (2015) reported the outbreak of human cutaneous leishmaniasis in the Volta Region of Ghana. DNA sequencing of three isolates revealed them to be *Leishmania*, identical to each other but different from all other forms of *Leishmania* species. Phylogenetic analysis showed the parasites to be new members of the *Leishmania enriettii* complex, a new subgenus of *Leishmania* parasites. This development is worrying at a time where the healthcare delivery system of Ghana is at breaking point with respect to resources to man the sector.

The anti-leishmanial drugs in current use, the antimonials, are toxic and reports of resistance are widespread (Ouakad *et al*., 2007). Other drugs like pentamidine and amphotericin B which are also used to treat leishmaniasis have been limited due to their high toxicity and cost (Borborema *et al*., 2005). Due to the adverse side effects of these treatment regimens, considerable attention has been given to the discovery and development of new, less toxic agents (Pitzer, *et al*., 1998). In an ongoing search for less toxic and cheaper leishmanicidal agents, plant-derived products present a viable option. These plants and their products are readily available and accessible in the communities and majority of the populace rely on herbal medicines. A research of this nature, evaluating the anti-leishmanial, anti-inflammatory and antioxidant potential of indigenous four Ghanaian medicinal plants is therefore timely.

6.2 ANTI-LEISHMANIAL ACTIVITY

The anti-leishmanial activity of *E. ivorense, C oxycarpum, O ahia and Aaubryanum* in comparison to Amphotericin B was evaluated *in vitro* against the promastigote forms of *Leishmania donovani.* To prove that *Leishmania* promastigotes cells functionally respond to pharmacological alteration, *Leishmania donovani* promastigotes were treated with six different concentrations (15.6, 31.2, 62.5, 125, 250 and 500 µg/mL) of the selected plants. Aqueous methanolic extracts (70 % methanol) of the four plants showed variable leishmanicidal activities. A very low IC_{50}, corresponding to high leishmanicidal activity, was recorded for the methanol extract of *E. ivorense* followed by *A. aubryanum, C. oxycarpum and O. ahia* respectively. The activity of *E. ivorense* was comparable to amphotericin B which was used as the positive control (Table 5.2).

Drugs with potential leishmanicidal activity have been found to inhibit the activity of trypanothione synthetase (TryS) which synthesizes trypanothione-bis -(glutathionyl) spermidine; ($T[SH]_2$), a metabolite that keeps the cellular redox homeostasis of the parasite (Krauth-Siegel and Comini, 2008). It maintains redox homeostasis, which is responsible for various cellular developments, such as synthesis of deoxynucleotides, and parasite resistance to stress (Krauth-Siegel and Comini, 2008: Dormeyer *et al*., 2001). Thus inhibition of the activity of TryS can stop the synthesis of $T[SH]_2$ and consequently destroy the mitochondrial membrane of the parasite. The anti-leishmanial activity of the plants may thus be related to this molecular mechanism. This is the first report of the leishmanicidal activity of *E. ivorense, A. aubryanum, C. oxycarpum* and *O. ahia*.

Leishmania donovani is the main cause of the deadly visceral leishmaniasis, the most severe type of the disease that can cause death in almost 100 % of the cases accounting for nearly 50000 deaths per year; a death toll surpassed only by malaria (WHO, 2002). The high cost and

nephrotoxicity of Amphotericin B has limited it use as the main agent for the treatment of this type of leishmaniasis. Thus from the observed activity, medicinal plants, if properly harnessed can be integrated into the treatment of leishmaniasis caused by *L. donovani* as they may present a cheaper and safe alternative to the toxic synthetic analogues.

Fractionation of the active *E. ivorense* extract afforded methanol fraction whose activity (IC_{50}= 2.97 µg/mL) was very much comparable to that of amphotericin B (IC_{50} = 2.4 µg/mL) (Table 5.3). This was followed by the methanol (IC_{50} = 33.07 µg/mL) and pet ether fractions (IC_{50}= 133.6 µg/mL) respectively. Thus purification of *E. ivorense* yielded more active polar and medium polar fractions. Thus it could be speculated that some compounds in the root bark worked antagonistically to reduce the full effect in the total crude methanol extract. Investigation of the bioactive ethyl acetate fraction led to the isolation of the novel cassane diterpene erythroivorensin as well as the flavanone eriodictyol and the triterpene betulinic acid. Although *E. ivorense* elaborates a large number of chemical principles, the isolation of these compounds for the first time in the plant highlights its endless potential. The rank order of leishmanicidal potency of the compounds was erythroivorensin > eriodictyol > betulinic acid. The activity of the novel diterpene was relatively higher than that of the ethyl acetate extract suggesting a weak effect of eriodictyol and betulinic acid in the establishment of the leishmanicidal activity of the extract.

A number of diterpenes have been reported to show marked leishmanicidal activity. Fokialakis *et al*., (2006), reported the anti-leishmanial activity of eleven clerodane and seven labdane type diterpenes from *Cistus monspeliensis* and *Cistus creticus* (Cistaceae) with IC_{50} values of 3.3 - 3.5 µg/mL for the potent compounds. Habtemariam, (2003) also reported the anti-leishmanial activity of diterpene acids with an even greater activity. In a similar report, cassane diterpene acids from *Caesalpina echinata*, with structures similar to erythroivorensin, were found to

show relatively high leishmanicidal activities (Cota *et al*., 2011). They were also non-toxic to human peripheral blood mononuclear cells *in vitro*. Thus in agreement with the results of the present study, diterpenes present a plethora of compounds, whose potential as anti-leishmanial drugs can be harnessed for the treatment of leishmaniasis.

6.3 ANTI-INFLAMMATORY AND ANTIOXIDANT ACTIVITIES

Immunological response to *Leishmania* parasite infection includes inflammation with cytokines and leukotriene B4 (LTB4) playing active roles in the process. Many of the molecules that promote inflammation also activate phagocytes leading to the production of the reactive oxygen species nitric oxide (NO), which acts directly to kill the parasite (Igor *et al*., 2015). However, an exacerbated production of these molecules may also lead to tissue damage. These molecules seem to have a beneficial role at early infection but worsen the disease outcome in established infections (Kostka *et al*., 2006). Studies on Leishmaniasis showed that higher frequency of proinflammatory cytokine production leads to larger lesions. Anti-inflammatory agents provide a balance between proinflammatory and anti-inflammatory cytokines and that determines the outcome of the infection (Lopez *et al*., 2009). Therefore, in this research the anti-inflammatory and antioxidant activity of the plants and their compounds were also investigated. *E. ivorense* again showed the highest anti-inflammatory and antioxidant activities followed by *C. oxycarpum*, *O. ahia* and *A. aubryanum* (Table 5.4-5.8, Figure 5.11). Further fractionation and testing again established the ethyl acetate fraction as the most active fraction with erythroivorensin, eriodictyol and betulinic acid, the main compounds responsible for these activities. The anti-inflammatory activity of betulinic acid (ED_{50} = 4.367±1.333 μg/mL) was comparable to that of diclofenac (ED_{50} = 3.74±0.333 μg/mL) (Table 5.5). Thus purification led to increased anti-inflammatory activity. The stem- and root-barks of *E. ivorense* are routinely

employed in traditional medicine to treat a variety of conditions including inflammatory pain and oedema. This result gives scientific credence to it use in folklore medicine and may help create the balance between proinflammatory and anti-inflammatory cytokines that leads to better prognosis of Leishmaniasis.

Although the major source of the pentacyclic lupane-type triterpenoid betulinic acid is the bark of the common birch tree (*Betula spp.*, Betulaceae), the compound is widely reported from various plant sources. For example, the bark of many tree species that are utilized for timber production are known to contain up to 2.5 % of compound 2 (Maurya *et al*., 1989). Among its reported pharmacological activities are potent anti-inflammatory activities both *in vitro* and *in vivo*. For example, *in vivo* studies on bacterial lipopolysaccharide-induced lung damage demonstrated it anti-inflammatory and antioxidant properties at oral doses as small as 25 mg/kg (Nader and Baraka, 2012). It therapeutic potential in oxidative damage has also been established as protection from ischemia/reperfusion-induced renal damage (Ekşioğlu-Demiralp *et al*., 2010). Similarly, the induction of NF-κB activation by carcinogens has been shown to be suppressed by betulinic acid through inhibition of IκBα kinase and p65 phosphorylation as well as abrogation of cyclooxygenase-2 and matrix metalloprotease-9 (Takada and Aggarwal, 2003). It suppressive effect against pro-inflammatory prostaglandin E2 production *in vitro* (Viji *et al*., 2011) and neutrophil recruitment and inflammatory mediator expression in lipopolysaccharide-induced lung inflammation (Nader and Baraka, 2012: Lingaraju *et al*., 2015) were also demonstrated. Interestingly, the expression of the cytokines mediated (e.g. TNF-δ) adhesion molecules on endothelial cell surface has been shown to be down regulated by betulinic acid (Yoon *et al*., 2010). Other pharmacological effects of betulinic acid include anti-bacterial (Chandramu *et al*., 2003: Fontanay *et al*., 2008), anti-HIV (Fujioka *et al*., 1994), anti-HSV-1 (Ryu *et al*., 1993: Ryu *et al*., 1992), antihelmintic (Enwerem *et al*., 2001) and anti-

cancer effects (Fulda, and. Debatin, 2000: Fulda *et al*., 1999: Pisha *et al*., 1995: Zuco *et al.*, 2002).

The other known compound isolated from the root-bark of *E. ivorense* was the common flavonoid eriodictyol that is also known to possess anti-inflammatory properties in a variety of test models. For example, it has been demonstrated to suppress nitric oxide (NO) production, expression of pro-inflammatory cytokines, inducible nitric oxide synthase and macrophage inflammatory protein in LPS-stimulated activated monocytes (Raw 264.7 cells) and B cells (Lee, 2011: Lee *et al*., 2013). These activities were also shown to be associated with suppression of NF-κB activation and phosphorylation of p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases 1 and 2 (ERK1/2), COX-2 and c-Jun Nterminal kinase (JNK) (Lee, 2011: Lee *et al*., 2013). Eriodictyol is also a known antioxidant polyphenolic compound that possesses numerous pharmacological activities in a variety of assay models (Habtemariam, 1997: Habtemariam and Dagne, 2010). The biochemical mechanism of action of the novel compound, erythroivorensin, remains to be elucidated but given its structural similarities with the steroidal skeleton and/or that of the triterpene betulinic acid, a similar mechanism of action is anticipated.

6.4 CONCLUSION AND RECOMMENDATION

6.4.1 CONCLUSION

This work has established that the selected medicinal plants (*Erythrophleum ivorense*, *Anthostema aubryanum*, *Coelocaryon oxycarpum* and *Omphalocarpum ahia*) exhibit significant anti-leishmanial, anti-inflammatory and antioxidant activities with *E. ivorense* demonstrating the highest activities (14.10 µg/mL, 16.91 mg/kg body weight and 1.11 µg/mL respectively). The bioactivities of fractions (petroleum ether, ethyl acetate and methanol) of *E. ivorense* root bark was highest in the methanol (2.97 µg/mL, 23.98 mg/kg body weight 3.703 µg/mL respectively for the above bioactivities). Phytochemical analysis of the root bark resulted in the identification of a novel compound **erythroivorensin** as well as betulinic acid and eriodictyol. To the best of my knowledge this is the first report of the isolation of these compounds in *E. ivorense*. Eriodictyol is being reported for the first in the genus *Erythrophleum* while betulinic acid is reported for the first time from the specie *Erythrophleum ivorense.* They showed considerable anti-leishmanial, antioxidant and anti-inflammatory activities. Thus this study has provided some scientific justification for the ethnomedical uses of the root bark of *Erythrophleum ivorense* and stem barks of *Anthostema aubryanum*, *Coelocaryon oxycarpum* and *Omphalocarpum ahia* for the treatment of leishmaniasis, inflammatory conditions, wounds and infections.

6.4.2 RECOMMENDATION

1. Toxicity studies of *E. ivorense* extract and compounds should be considered in future work to help in drug formulation.

2. Also future research should consider *in vivo* anti-leishmanial activity of these extracts and compounds since drug formulated from these compounds would be used by humans.

3. In addition, structural modification of erythroivorensin to obtain a possibly more potent anti-leishmanial compound should be considered in future research.

4. Related structures of erythroivorensin should be screened for their anti-leishmanial activity.

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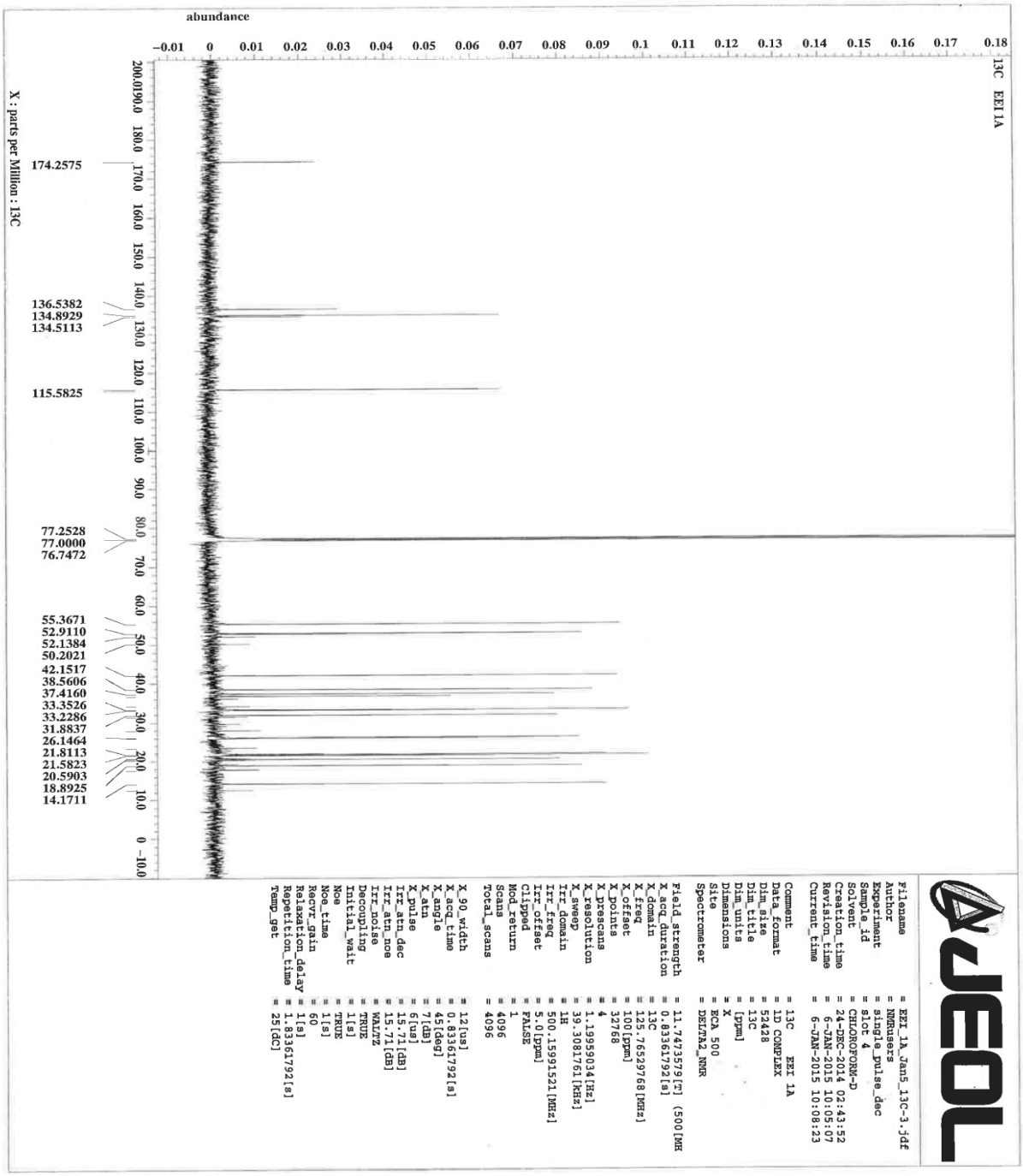
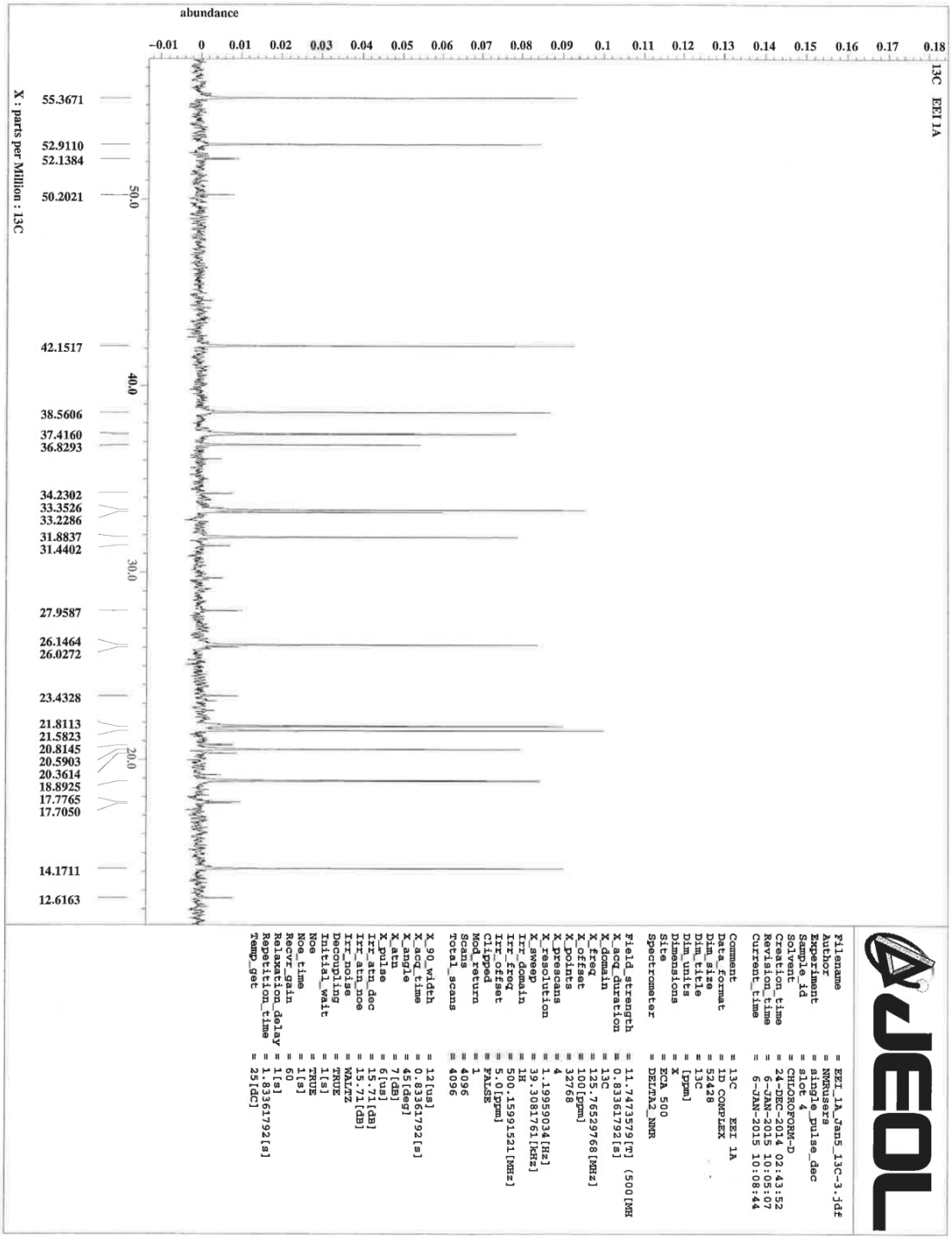
APPENDICES

APPENDIX 1: SPECTRA DATA OF ERYTHROIVORENSIN

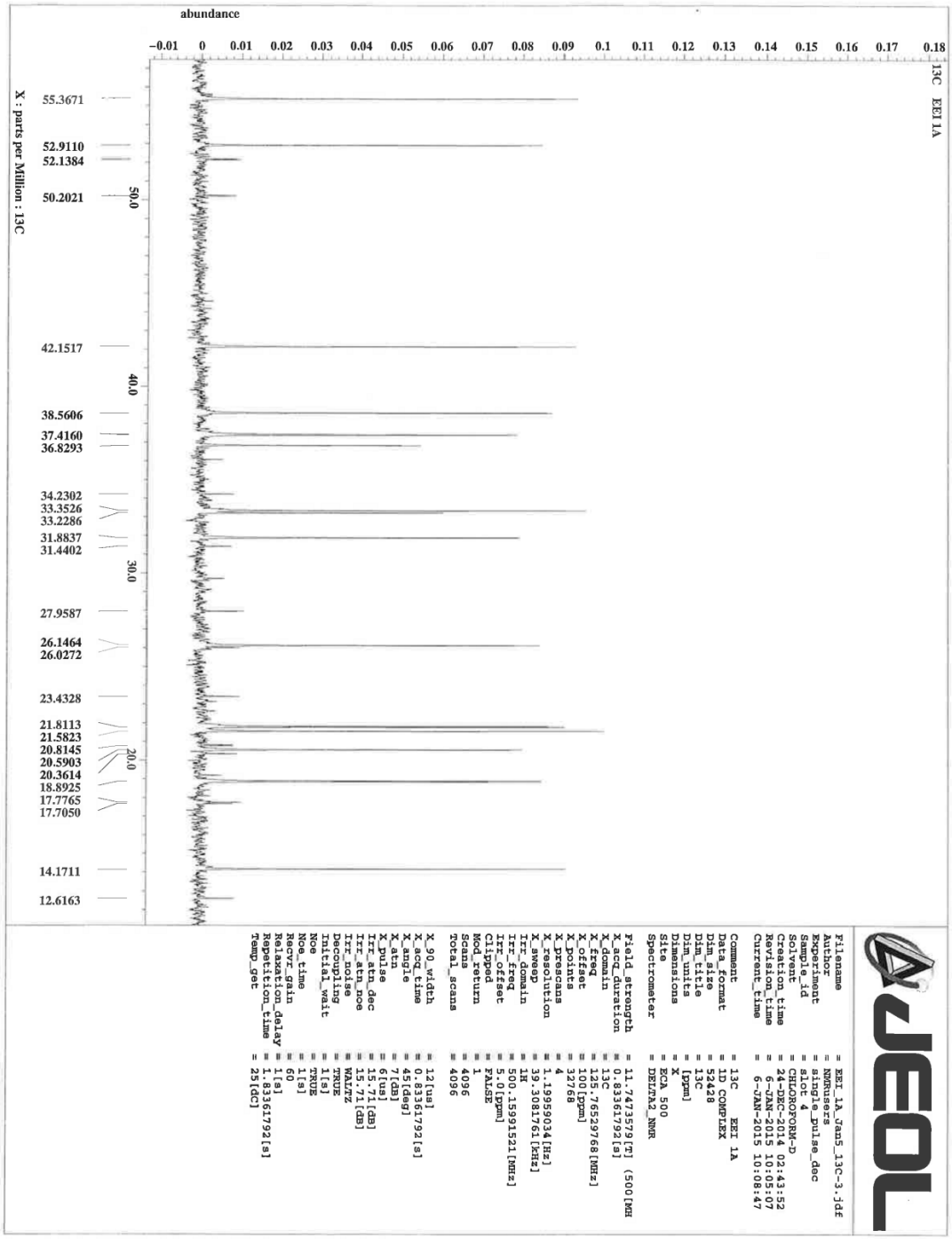
Appendix 1a(i): ^{13}C-NMR spectra of Erythroivorensin



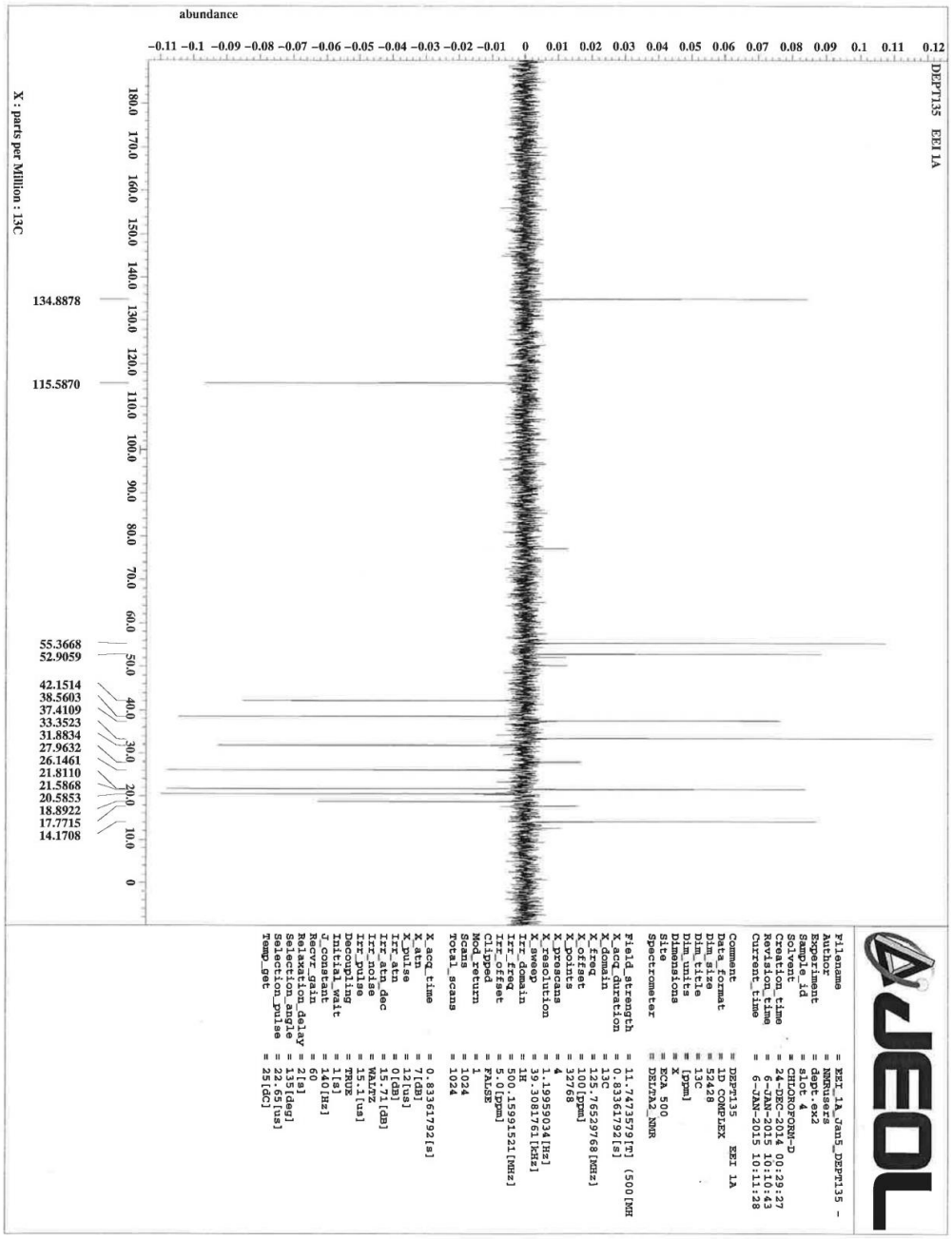
Appendix 1a(ii): ^{13}C-NMR spectra of Erythroivorensin



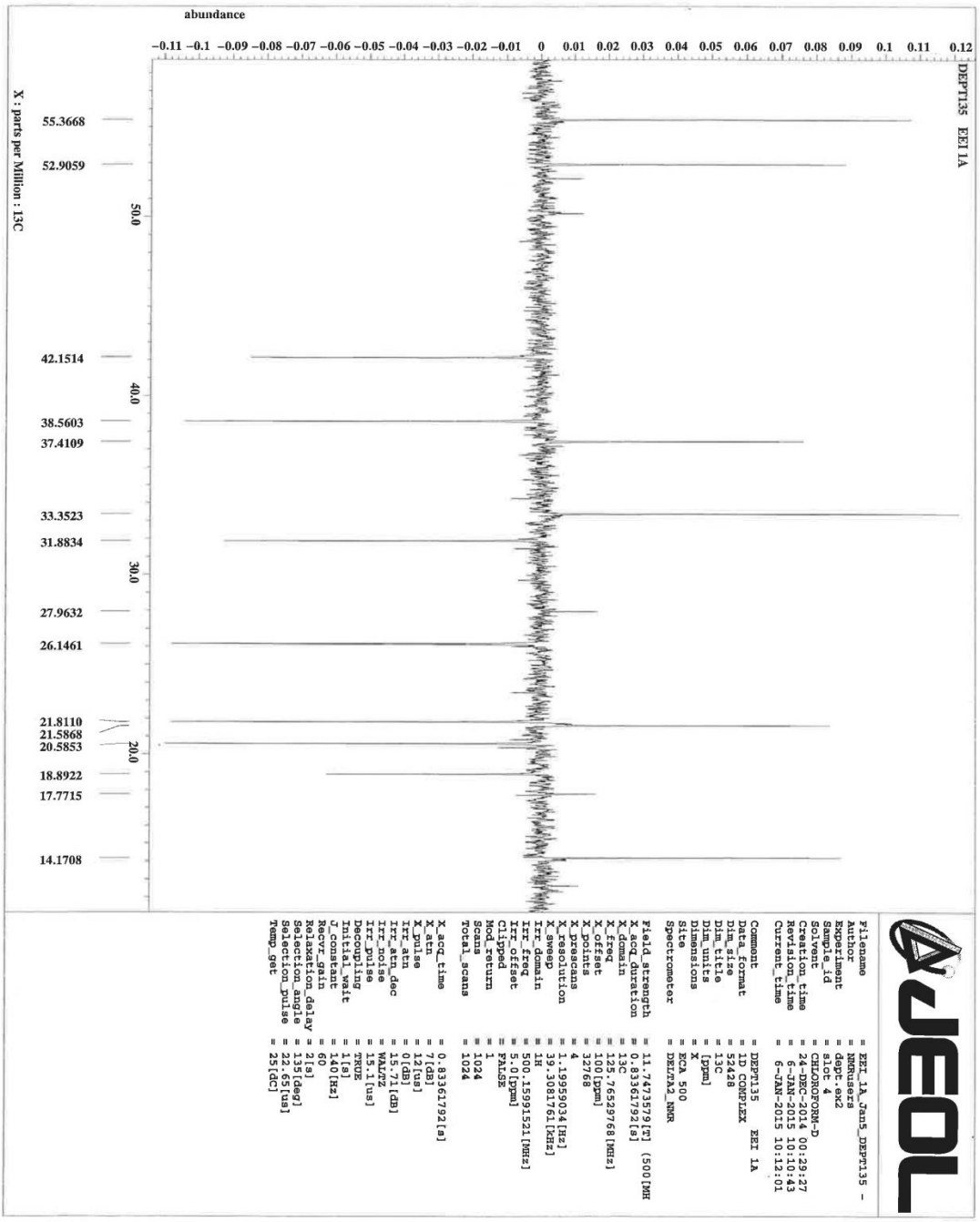
Appendix 1a(iii): ^{13}C-NMR spectra of Erythroivorensin



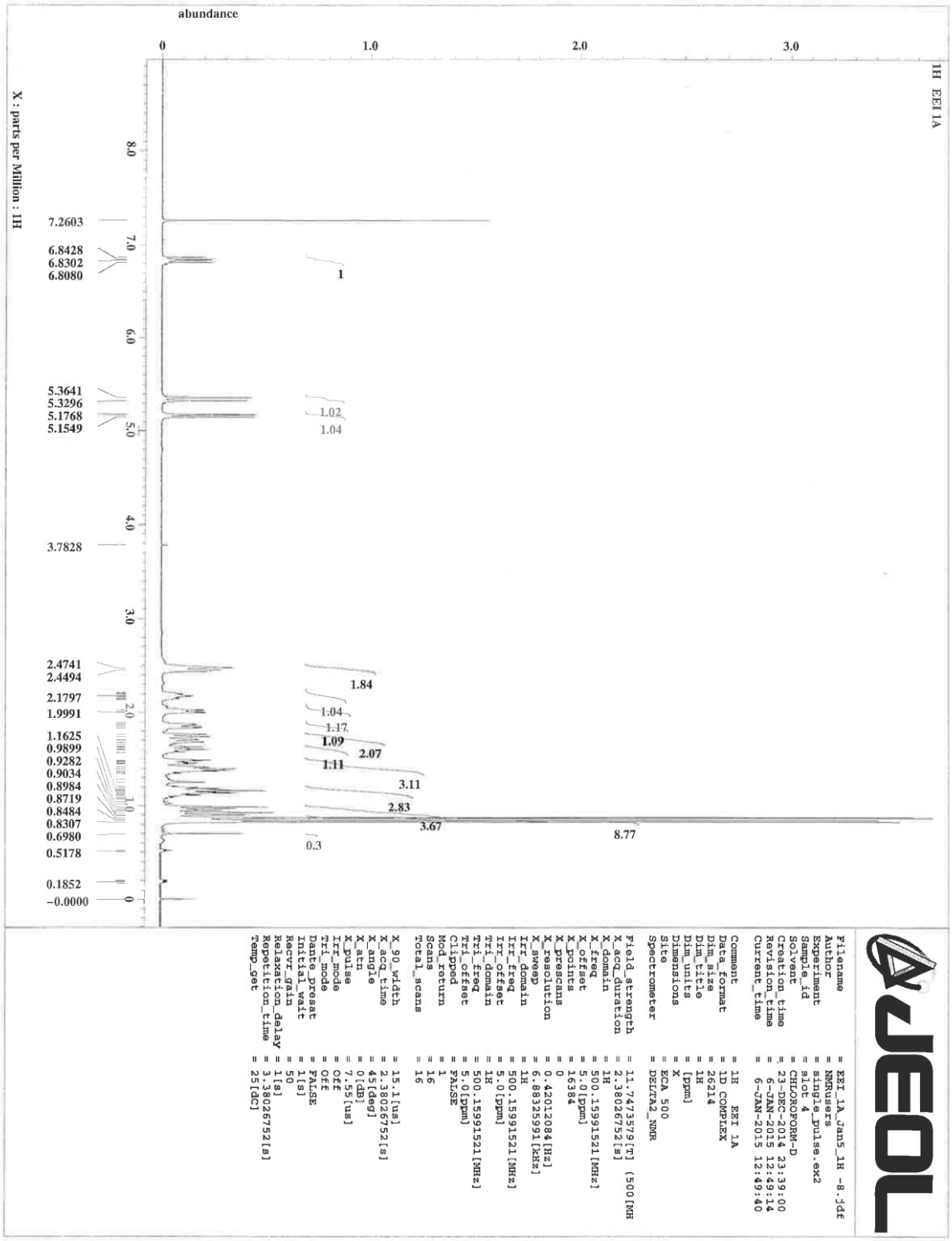
Appendix 1a(iv): ^{13}C-NMR spectra of Erythroivorensin



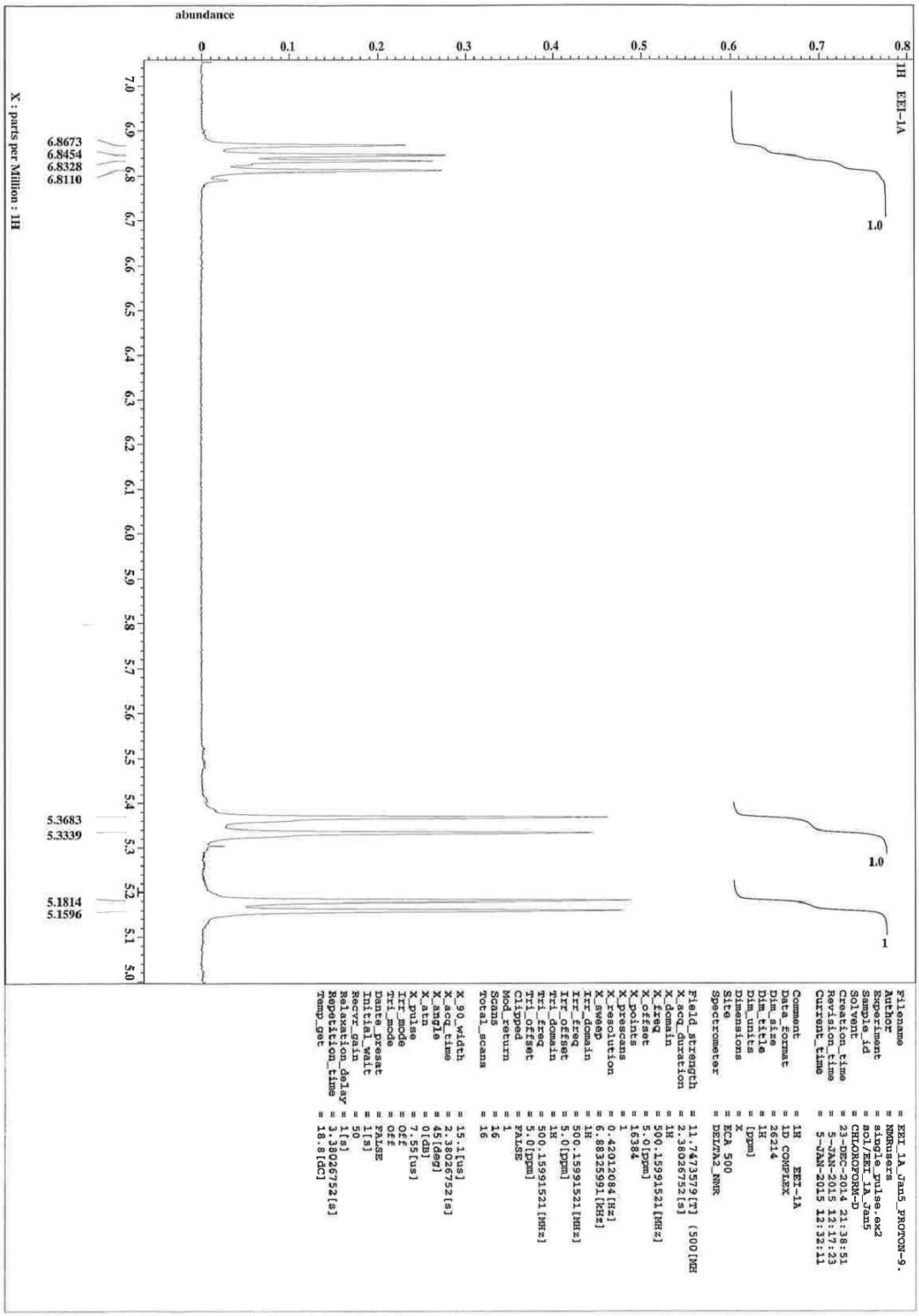
Appendix 1a(v): ^{13}C-NMR spectra of Erythroivorensin



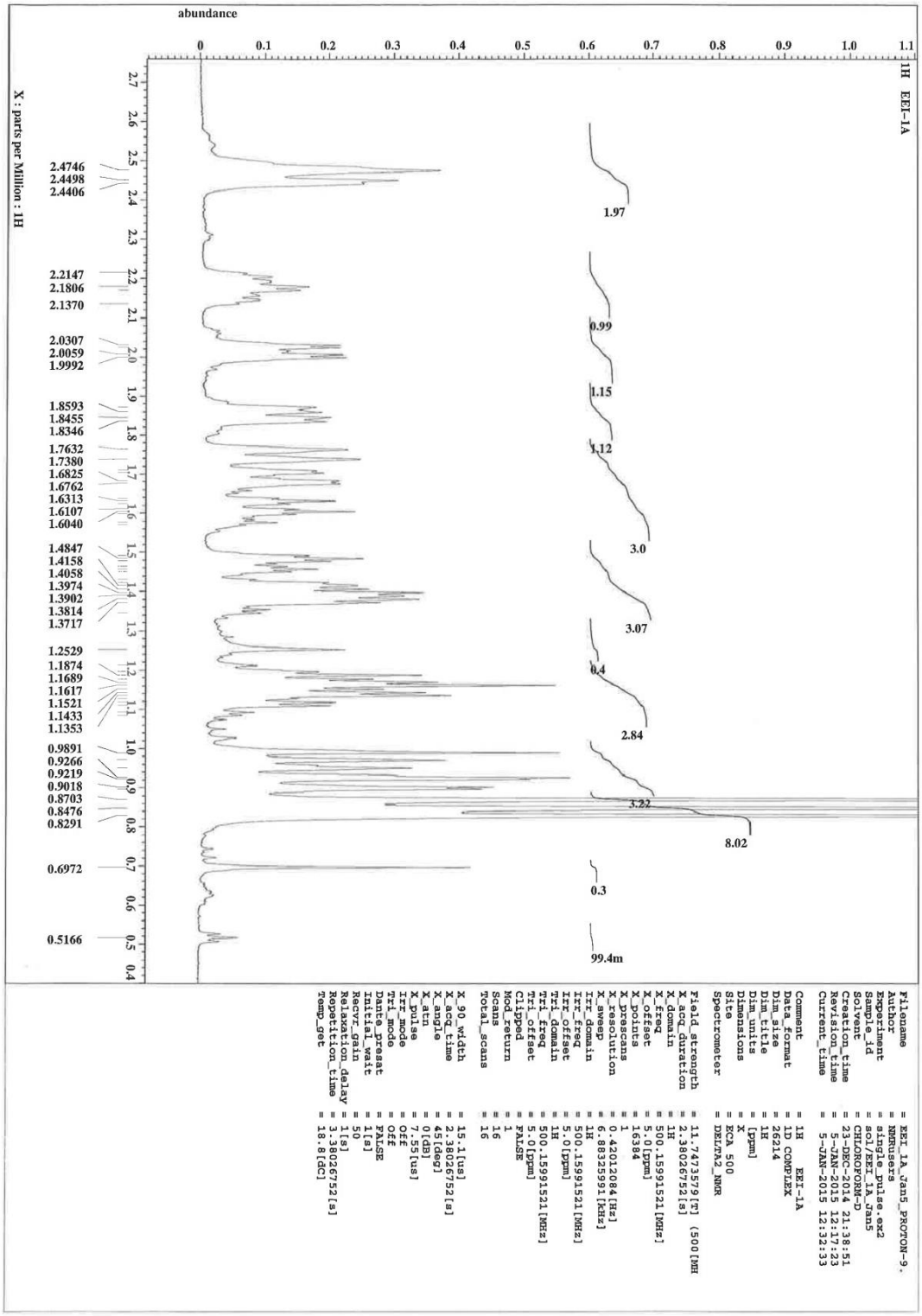
Appendix 1b(i): ^{1}H-NMR spectra of Erythroivorensin



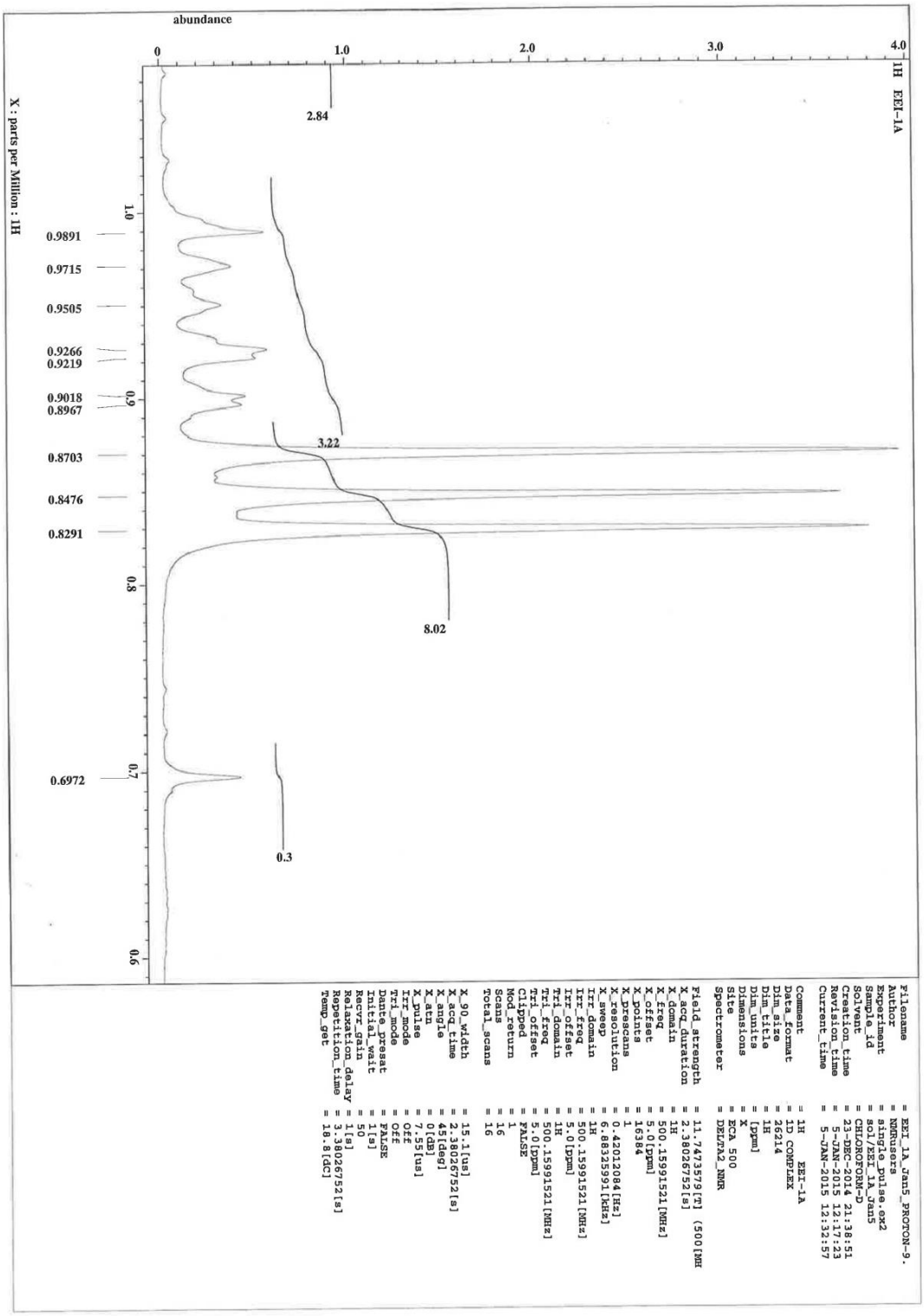
Appendix 1b(ii): ^{1}H-NMR spectra of Erythroivorensin



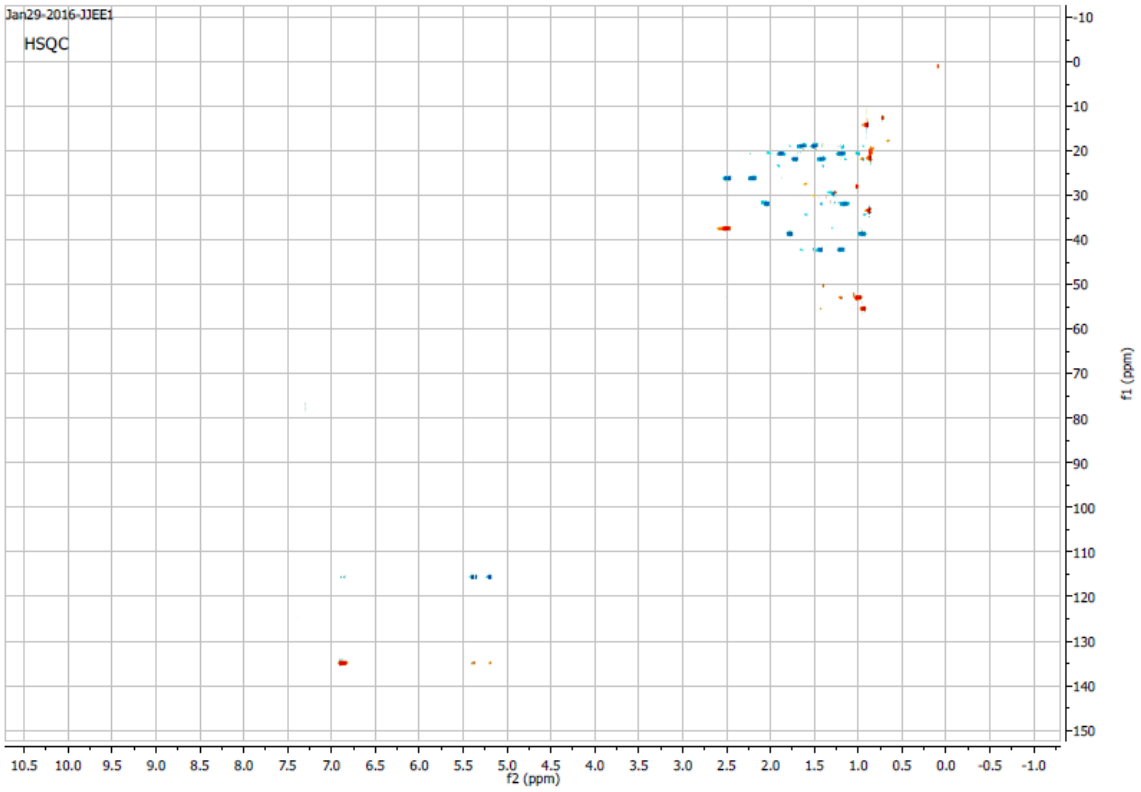
Appendix 1b(iii): ^{1}H-NMR spectra of Erythroivorensin

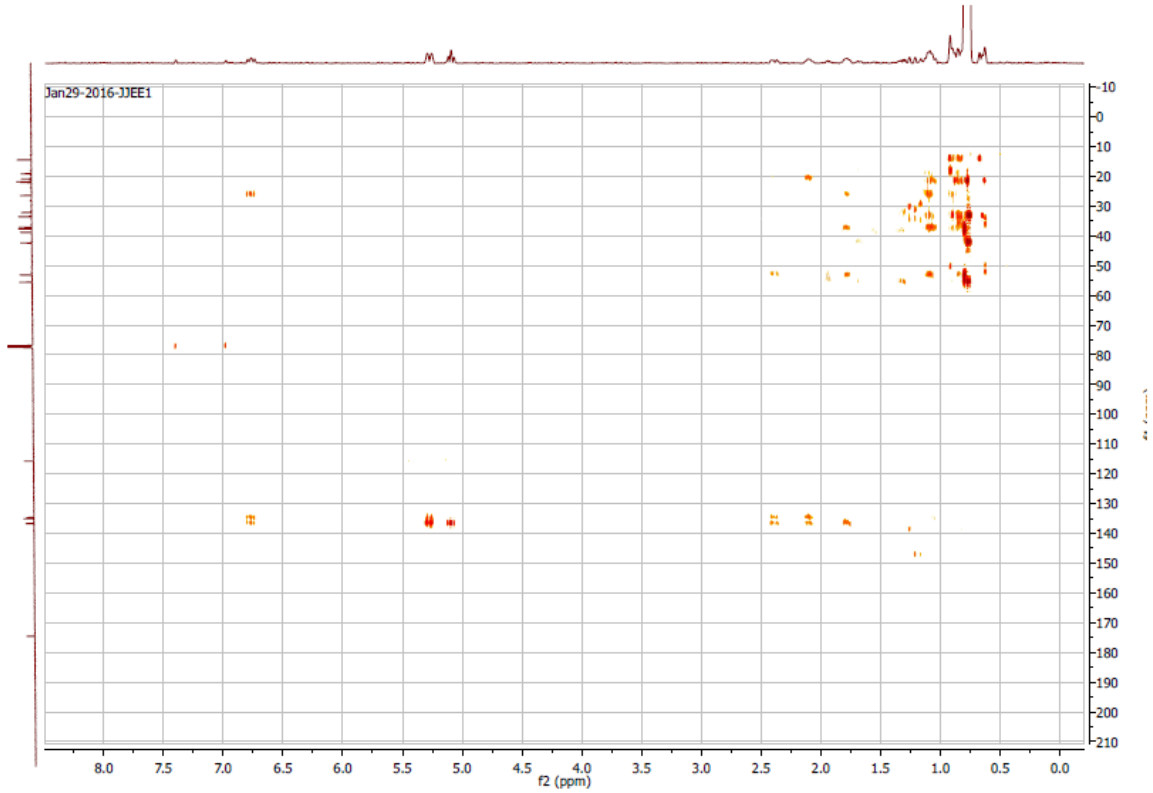


Appendix 1b(iv): ^{1}H-NMR spectra of Erythroivorensin

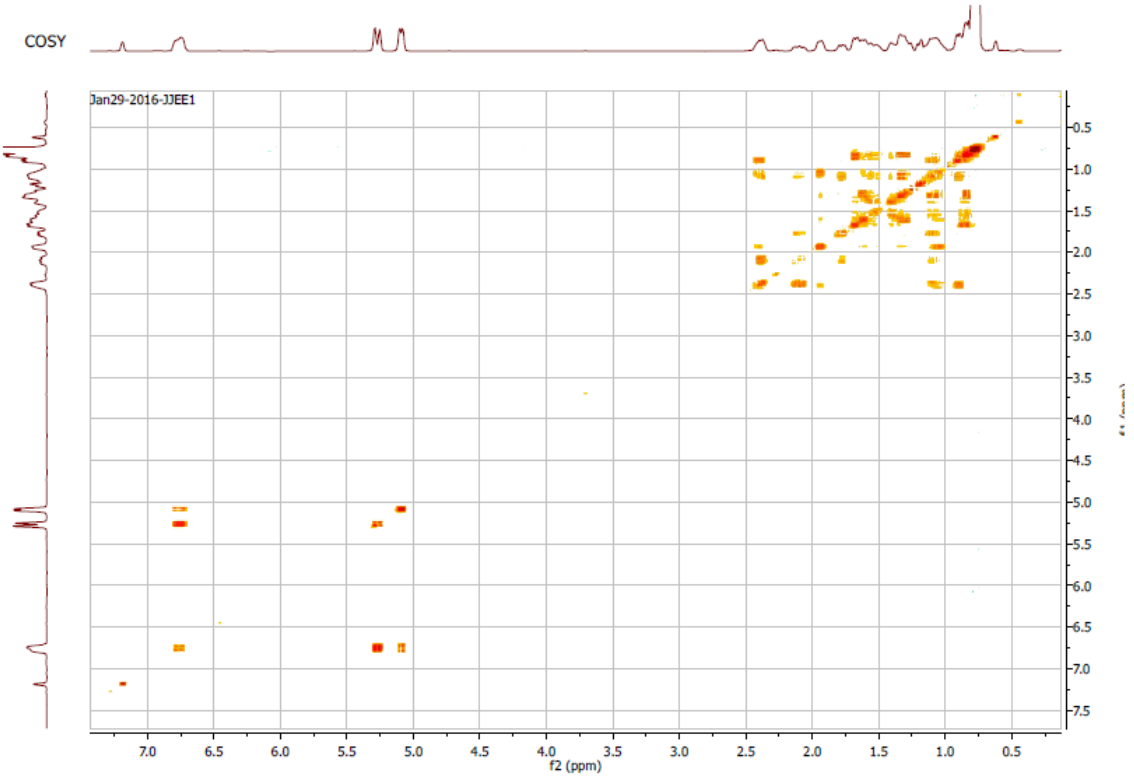


Appendix 1c: HSQC and HMBC spectrum of Erythroivorensin

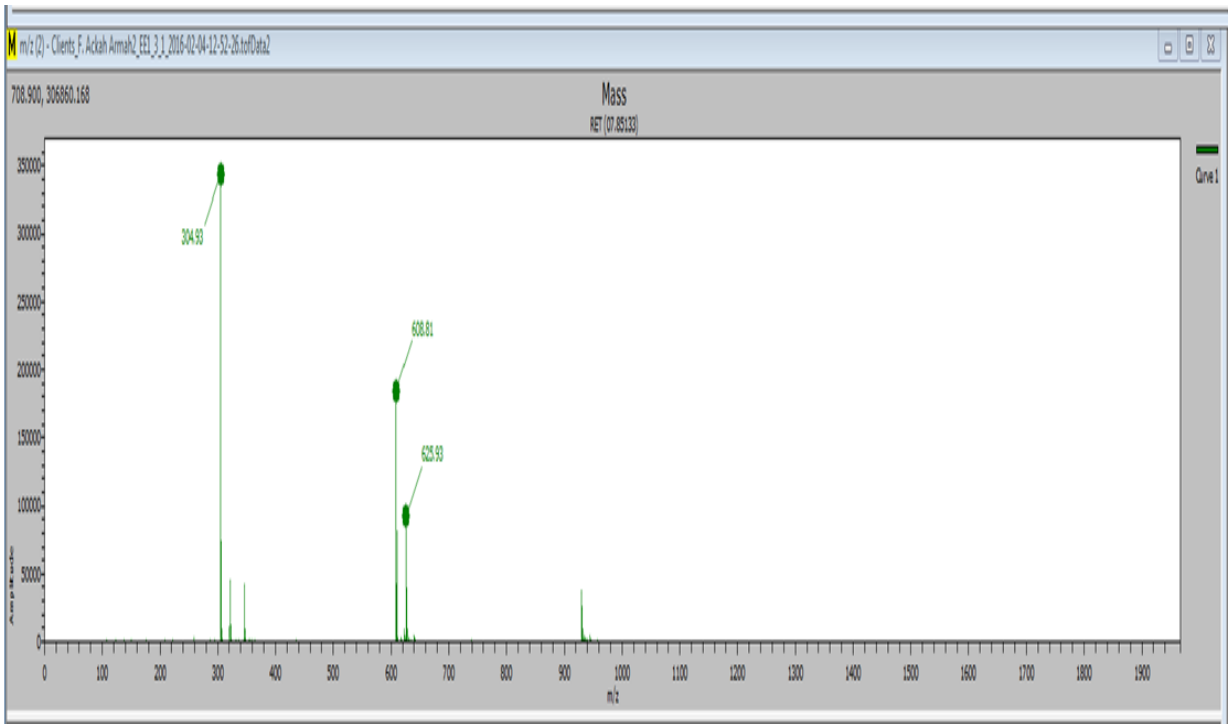




Appendix 1d: COSY spectrum of Erythroivorensin

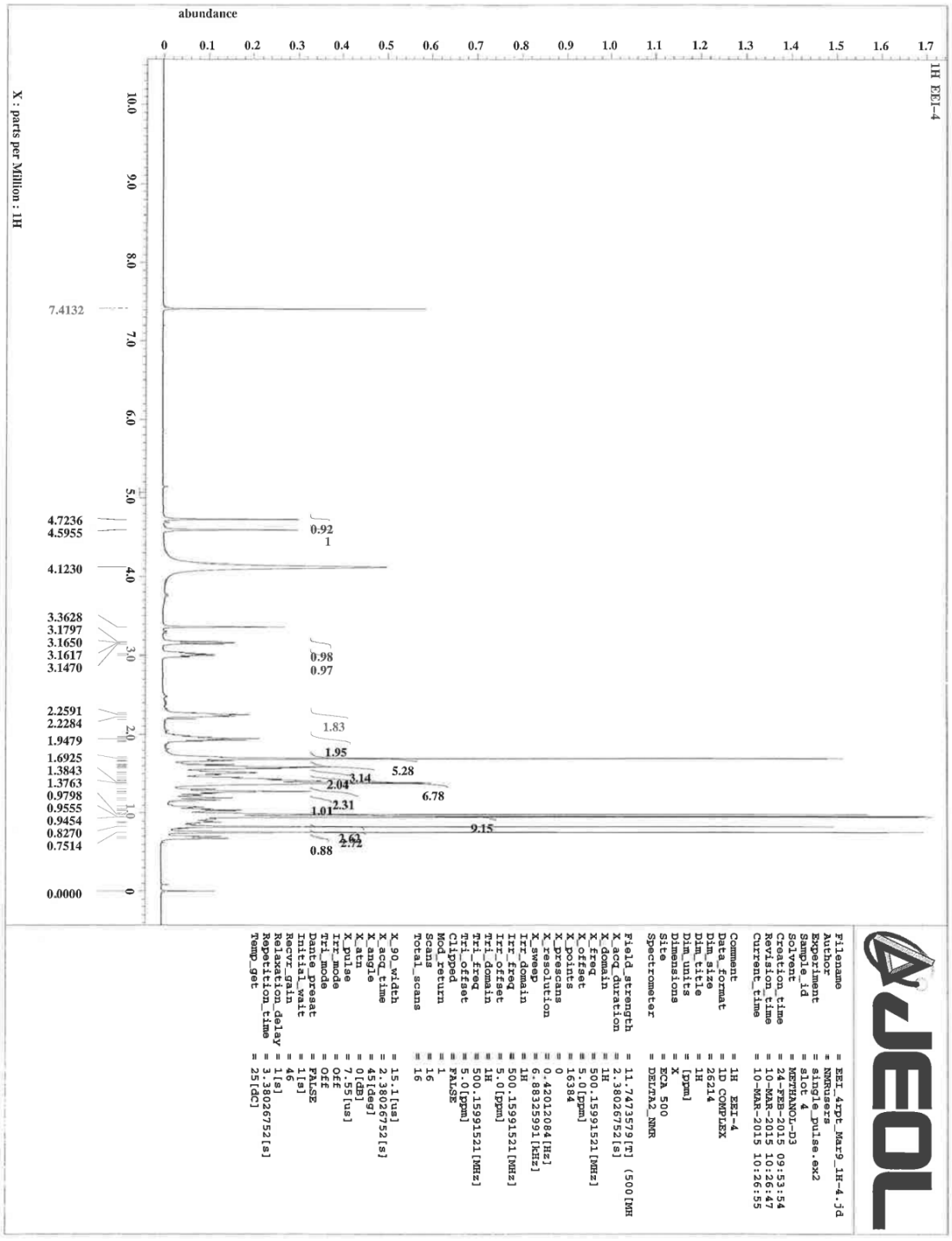


Appendix 1e: Mass spectrum of Erythroivorensin

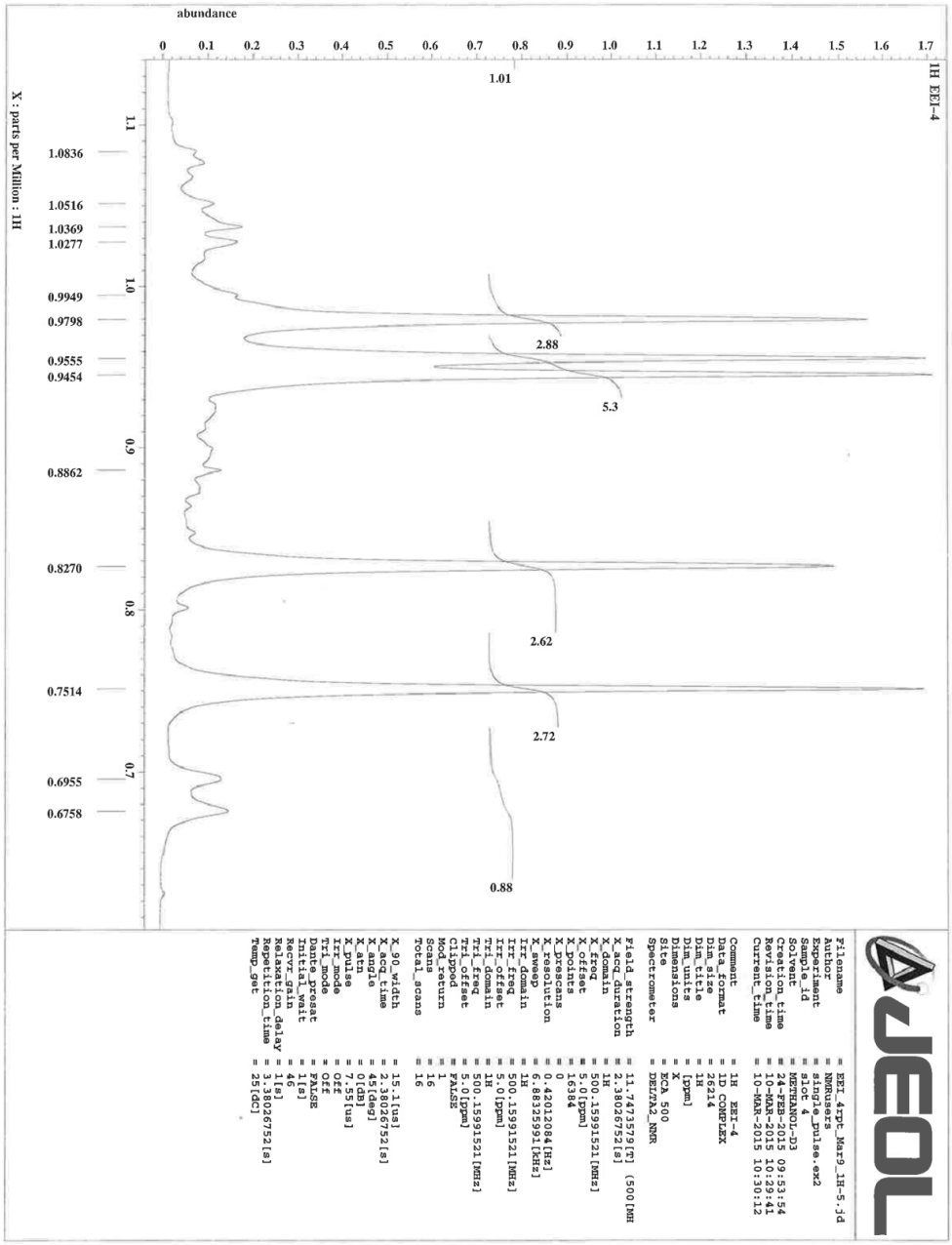


APPENDIX 2: SPECTRA DATA OF BETULINIC ACID

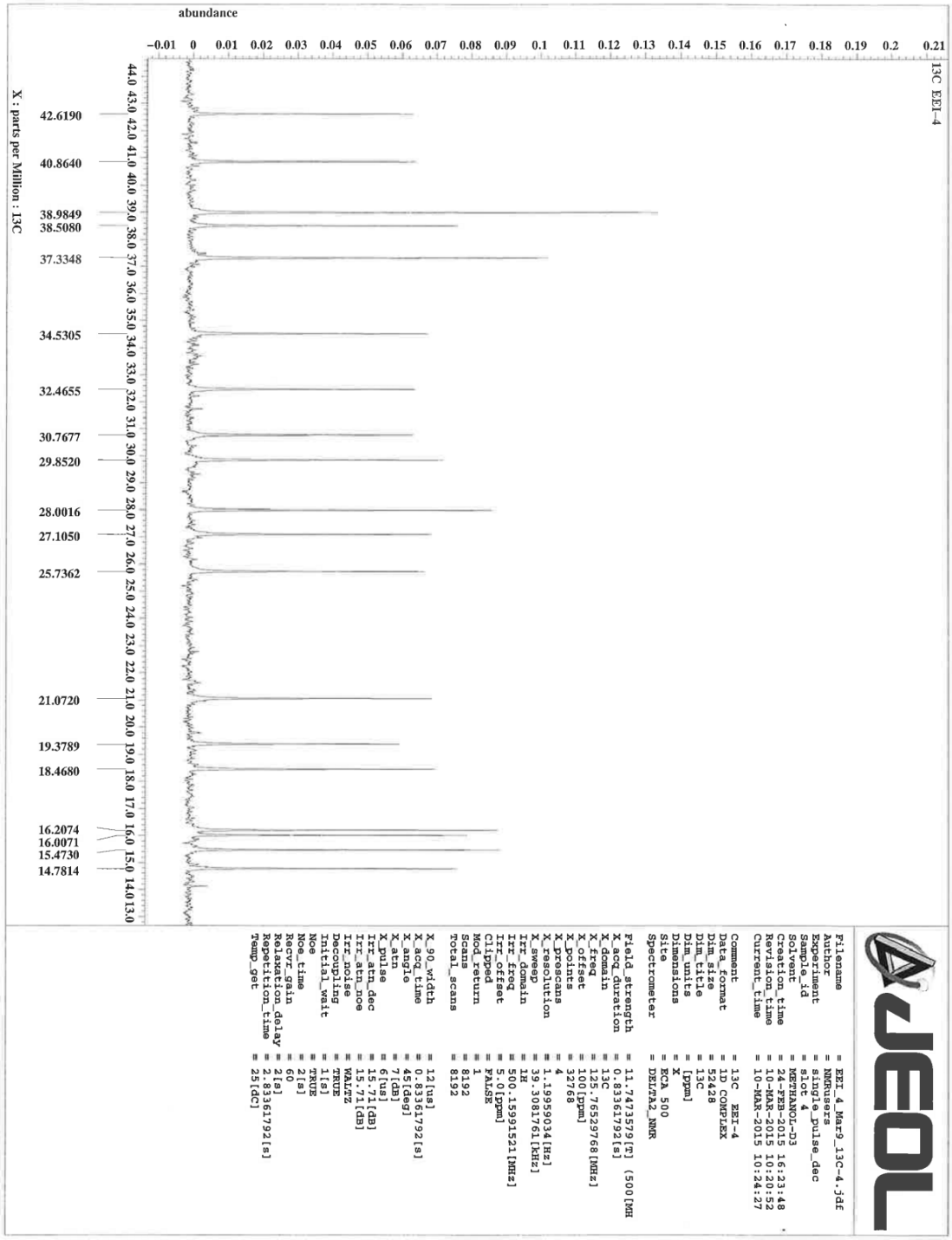
Appendix 2a(i): ^{1}H-NMR spectra of Betulinic acid



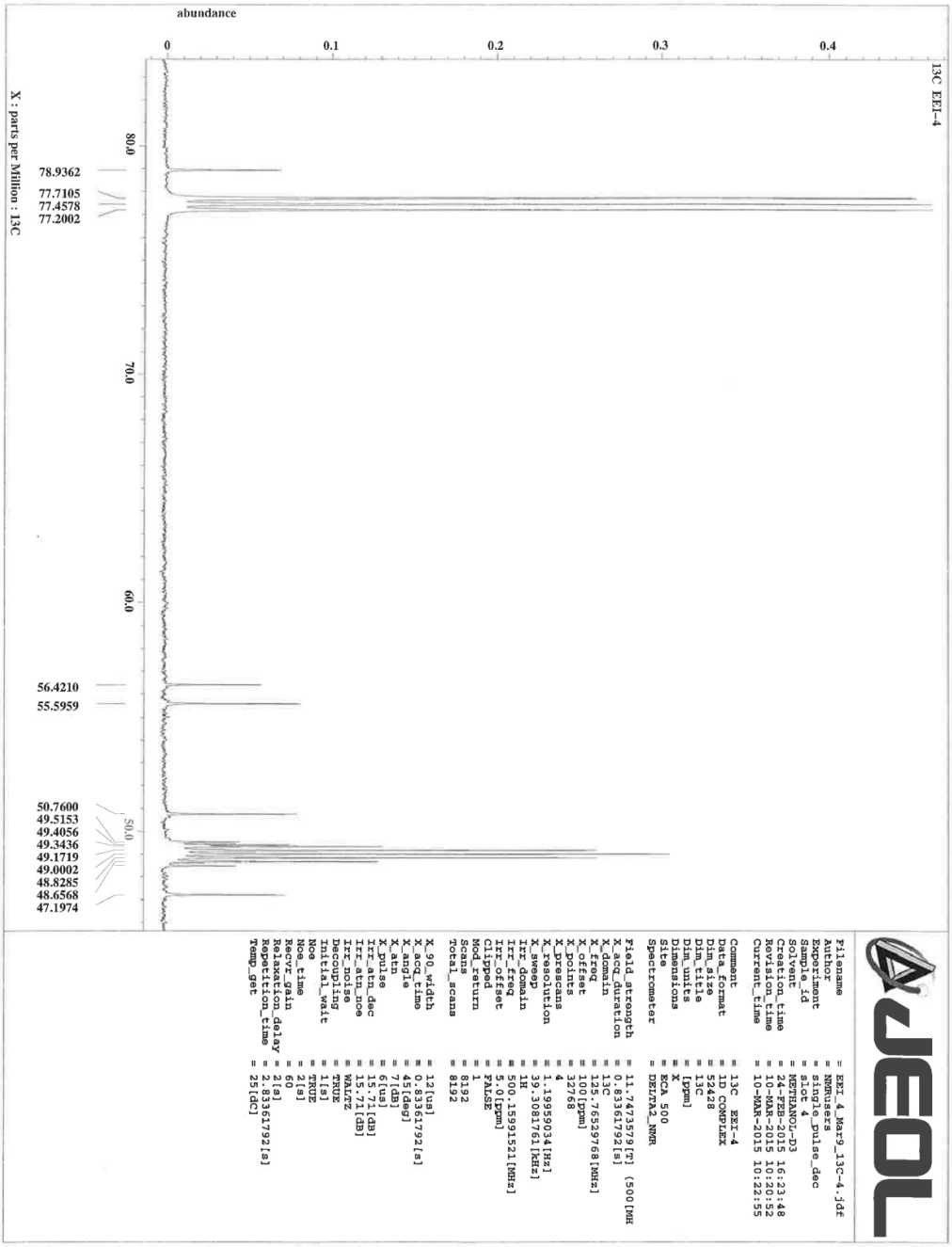
Appendix 2a(ii): ^{1}H-NMR spectra of Betulinic acid



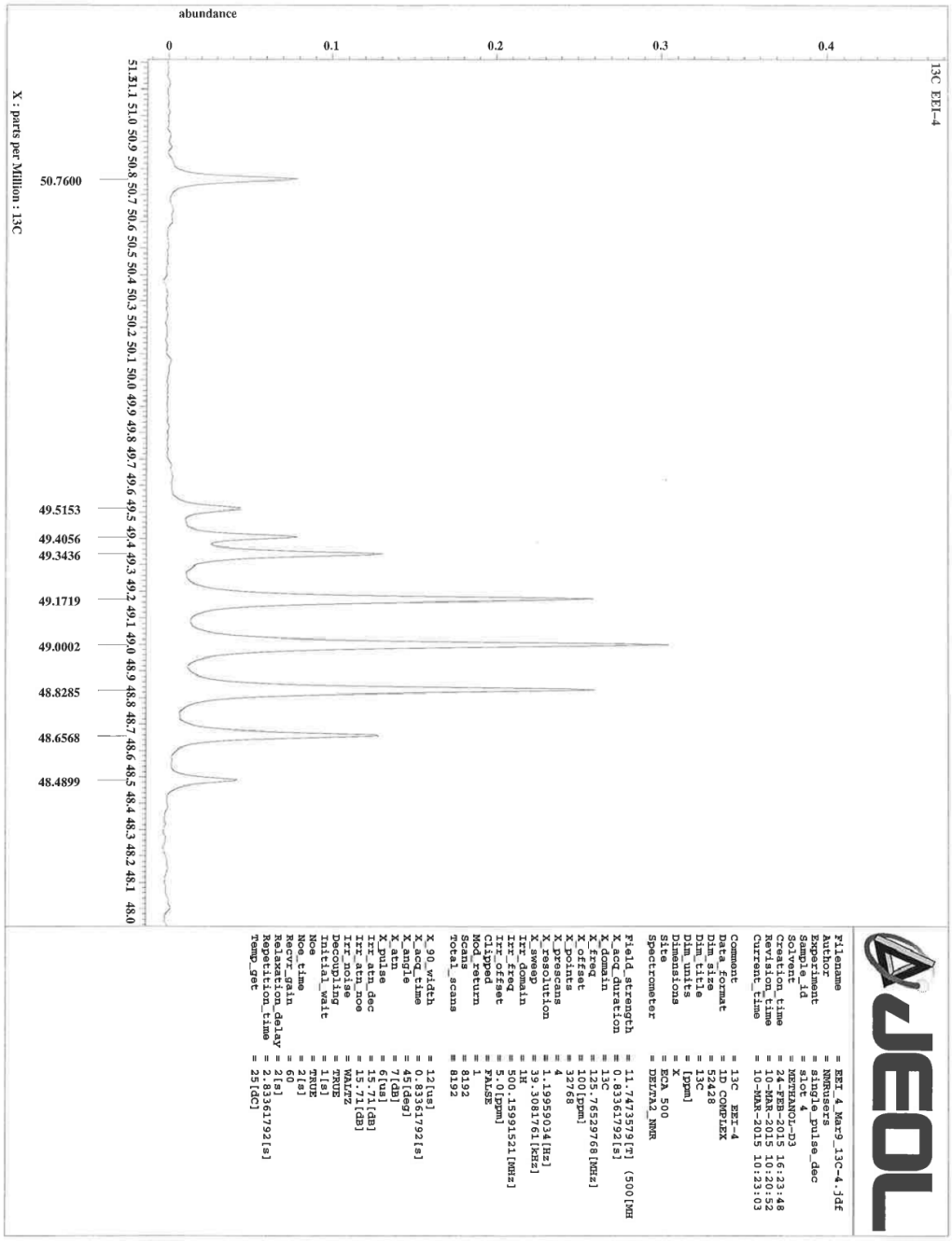
Appendix 2b(i): ^{13}C-NMR spectrum of Betulinic acid



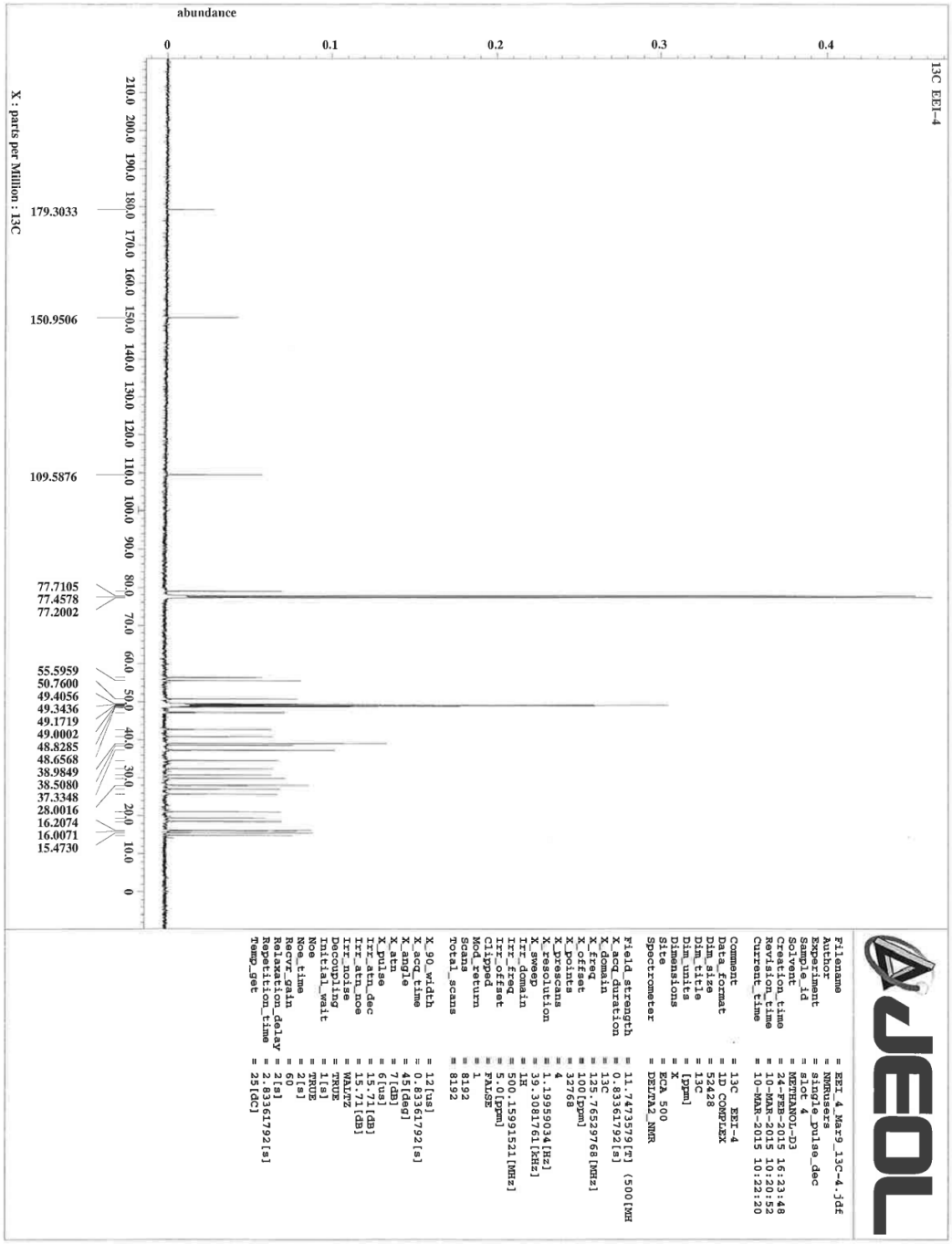
Appendix 2b(ii): ^{13}C-NMR spectrum of Betulinic acid



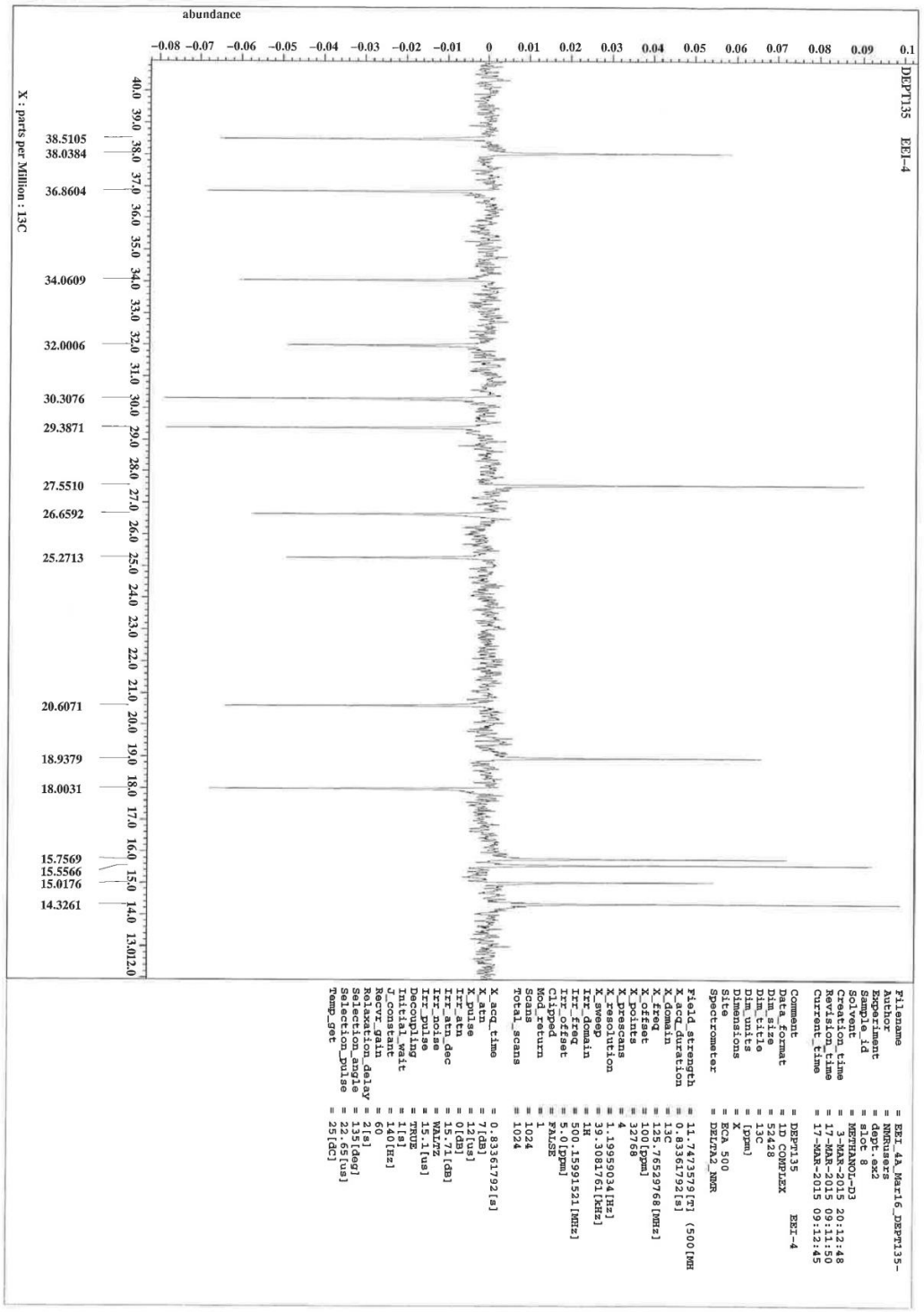
Appendix 2b(iii): ^{13}C-NMR spectrum of Betulinic acid



Appendix 2b(iv): ^{13}C-NMR spectrum of Betulinic acid

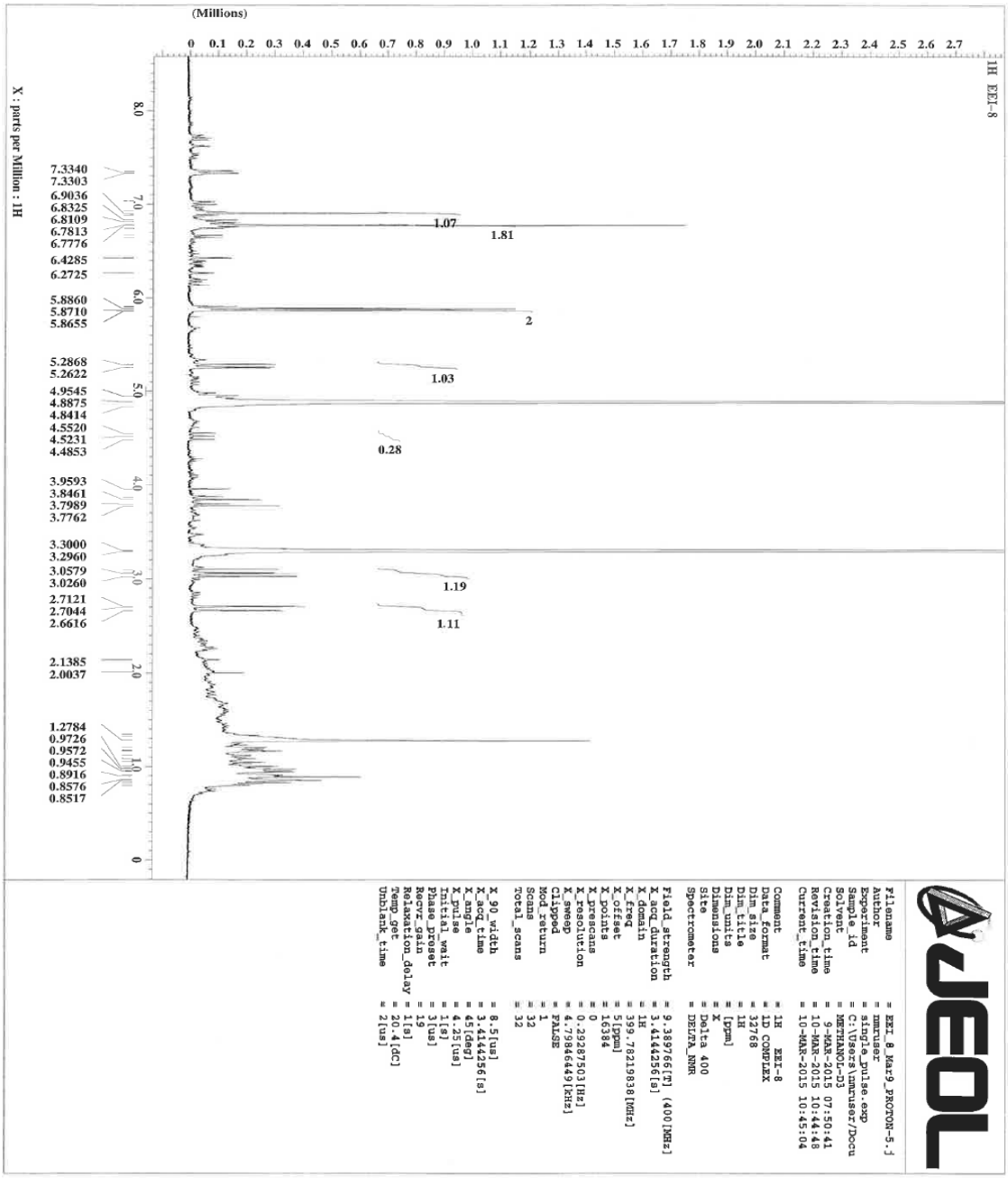


Appendix 2b(v): ^{13}C-NMR spectrum of Betulinic acid

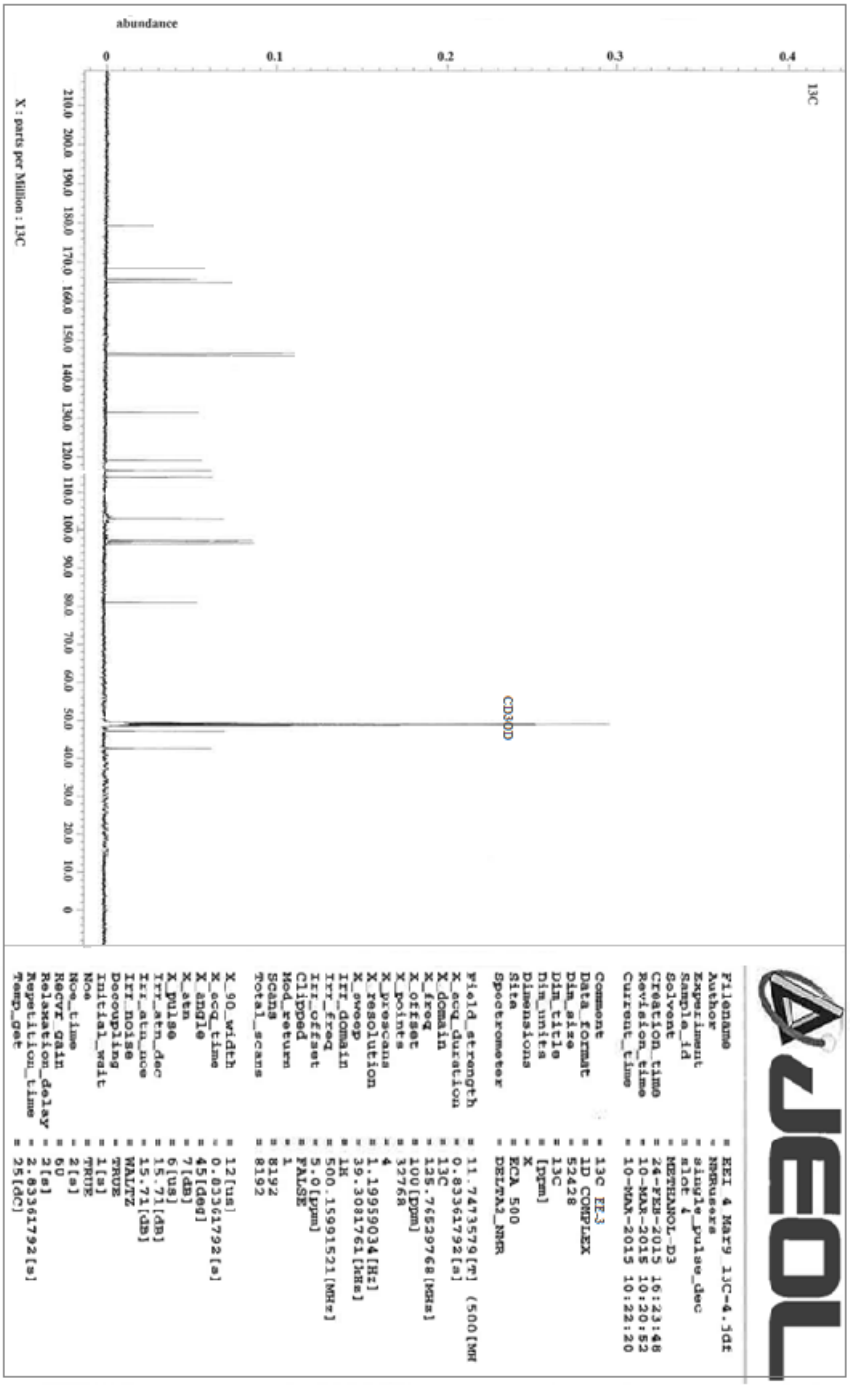


APPENDIX 3: SPECTRA DATA OF ERIODICTYOL

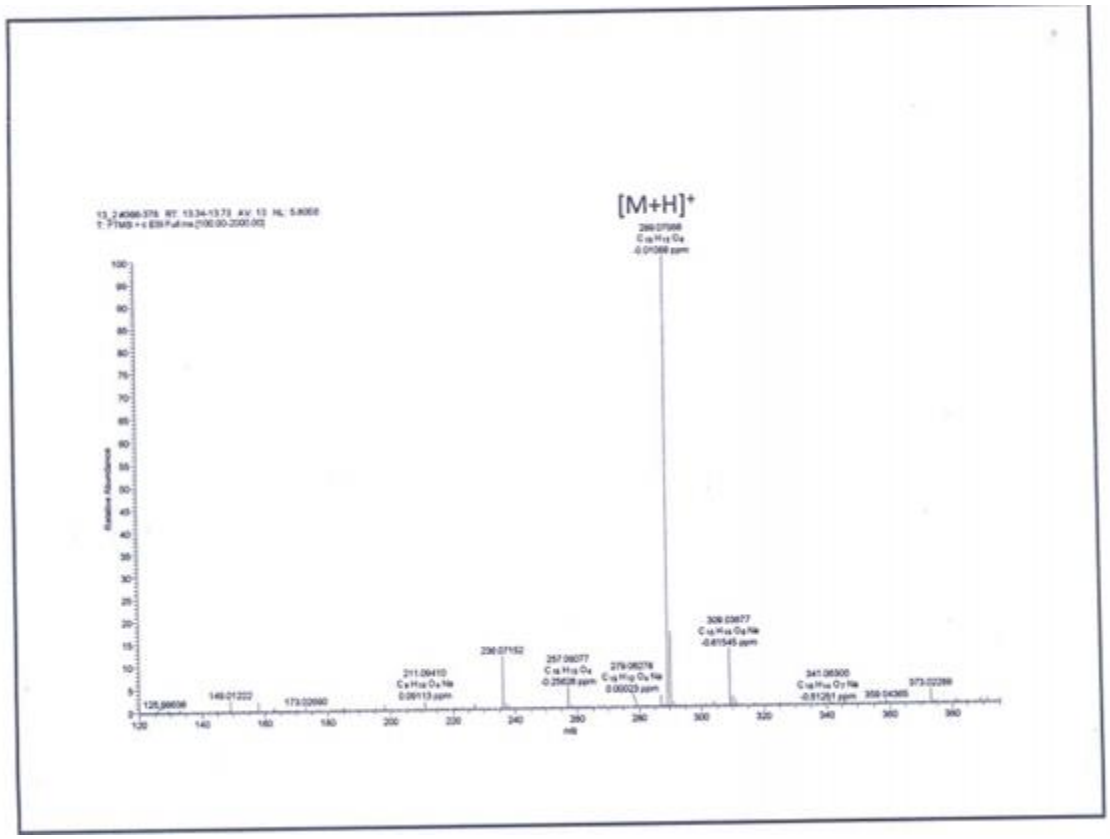
Appendix 3a: ^{1}H-NMR spectrum of Eriodictyol



Appendix 3b: ^{13}C-NMR spectrum of Eriodictyol



Appendix 3c: ESI-MS spectra of Eriodictyol



APPENDIX 4 PUBLICATIONS ARISING FROM THE RESEARCH