SECONDARY METABOLITES FROM *NEWTONIA PAUCIJUGA* AND *ERYTHRINA SACLEUXII* AND BIOACTIVITIES OF THEIR EXTRACTS

By

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A Dissertation Submitted in Partial Fulfilment of the Requirements for the Degree of Master of Science with Education of the University of Dar es Salaam

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September, 2018

CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance by the University of Dar es Salaam a dissertation titled: "***Secondary Metabolites from Newtonia paucijuga and Erythrina sacleuxii and Bioactivities of their Extracts***" in partial fulfilment of the requirements for the degree of Master of Science with Education of the University of Dar es Salaam.

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Dr. Joan J.E. Munissi

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Date. 21/09/2018

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(Supervisor)

Date. 19/09/2018

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AND

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DEDICATION

To

My Late Father George Mayeka

LIST OF ABBREVIATIONS

^{13}C	-	Carbon-13
1D	-	One dimensional
^{1}H	-	Proton
2D	-	Two dimensional
COSY	-	Correlation spectroscopy
d	-	Doublet
dd	-	Doublet of doublet
ddd	-	Doublet of doublet of doublets
DMSO	-	Dimethylsulfoxide
GC-MS	-	Gass chromatography-mass spectrometry
HMBC	-	Heteronuclear multiple bond correlation
HSQC	-	Heteronuclear single quantum coherence
IR	-	Infrared
LC-MS	-	Liquid chromatography-mass spectrometry
m	-	Multiplet
NMR	-	Nuclear magnetic resonance
s	-	Singlet
t	-	Triplet
TOCSY	-	Total correlation spectroscopy

ABSTRACT

The phytochemical investigations reported in this dissertation aimed at establishing the chemical constituents, antiplasmodial and antimicrobial activities of the two plants from the family Fabaceae, *viz. Newtonia paucijuga* and *Erythrina sacleuxii*. The crude extracts were subjected to chromatographic separation to afford the pure compounds. The antiplasmodial activities were evaluated using malaria imaging assay method, while the antimicrobial activities were carried out by disc diffusion and broth microdilution methods. Mearnsetin-3-*O*-α-*L*-rhamnopyranoside (**2.26**), benzyl-3,4,5-trihydroxylbenzoate (**2.28**) and, the mixture of stigmasterol (**2.37a**) and β-sitosterol (**2.37b**) were isolated from the ethanolic extracts of leaves, root and stem barks of *N. paucijuga,* respectively. Chromatographic isolation of ethanolic extracts of the *E. sacleuxii* leaves yielded a new *nor*-sesquiterpenoid namely, sacleuxenone (**2.38**). The structure elucidation of the compounds was based on the 1D and 2D NMR, IR and Mass spectral data. The extracts from the leaves, stem and root barks of *N. paucijuga* and leaves of *E. sacleuxii* exhibited moderate antibacterial activities with activity index value ranging from 0.3 - 0.5 against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia.* The most potent were the leaves and stem bark extracts of *N. paucijuga* (AI = 0.5). The minimum inhibitory concentrations (MIC) for the active extracts ranged from 0.3 - 5.0 mg/mL against *S. aureus*, *P. aeruginosa*, *K. pneumonia* and *B. subtilis*. All the extracts were active against *B. subtilis* with MIC value of 0.3 mg/mL. No any activities were observed against all the fungal species tested. On the other hand, all extracts exhibited antiplasmodial activity against *Plasmodium falciparum* (3D7 strain) with an IC_{50} value ranging from 2.6 – 24.6 μg/mL with selectivity index (SI) against human embryonic kidney 293 cell lines ranging from 6.5 – 21.0. The most potent antiplasmodial was *Newtonia paucijuga* root barks extract (NPRE) (IC_{50} = 2.6; SI = 6.5). Most extracts were safe against HEK 293 cell lines at the concentration of 40 μg/mL. Pure compound were not assayed for their antiplasmodial and antimicrobial activities due to insufficient amount. The study indicated that ethanolic crude extracts from *N. paucijuga* and *E. sacleuxii* have antibacterial and antiplasmodial activities and are recommended for further investigations.

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CHAPTER ONE

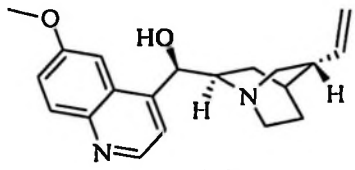
INTRODUCTION

1.1 General Introduction

Diseases are main drawbacks to the economic development of any country all over the world. They affect the health of people leading to poor performance of the manpower, hence low production inculcating to undevelopment and poverty in most countries especially in Africa.[1] Malaria is among the diseases which have continued to be a prominent health problem causing more sufferings and leading to the highest morbidity and mortality in tropical and sub-tropical areas.[2,3,4] The disease is caused by *Plasmodium* species in which, human malaria is currently caused by either *Plasmodium falciparum*; *P. vivax, P. ovale, P. malariae* or *P. Knowlesi* being transmitted from one person to another by an infected female *Anopheles* mosquito.[5,6] *P. falciparum* has high global prevalence, therefore commonly mentioned to be the most dangerous species causing the most lethal kind of malaria.[6,7]

The National Institute of Allergy and Infectious Diseases of the United States of America (NIAID) estimates that 350 to 500 million malaria cases occur annually worldwide.[8] In 2015, 214 million malaria cases and 438,000 deaths were reported, of which 88 and 90%, respectively occurred in Africa across all age groups on a global scale.[9,10] In Tanzania, the disease is still claiming more than one third of all deaths in children aged below five.[11] The persistence of malaria and its massive death toll is due to the failure of current treatment caused by the resistance of malaria pathogens towards antimalarial drugs.[12] Thus, the resistance of *P. falciparum, P. vivax, P. ovale* and *P. malariae* against quinine (**1.1**), chloroquine (**1.2**), amodiaquine (**1.3**),

mefloquine (**1.4**), sulfadoxine (**1.5**), pyrimethamine (**1.6**), proguanil (**1.7**), artemisinin (**1.8**) and artemisinin derivatives has been reported in malaria endemic areas at varying rates.[10,13]



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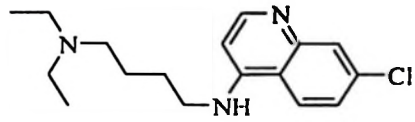

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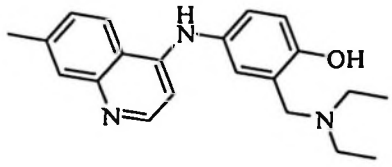

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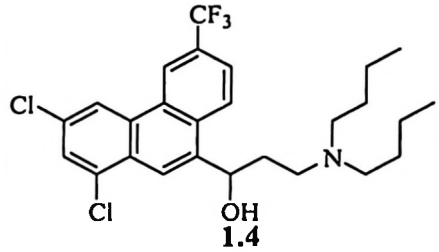

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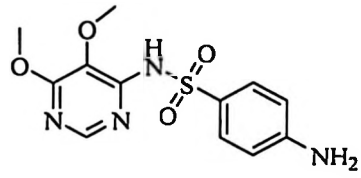

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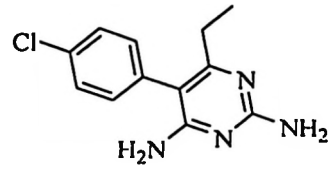

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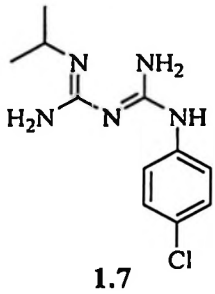

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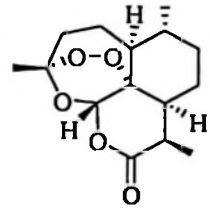

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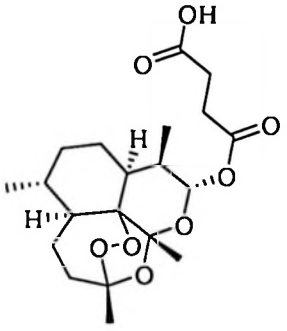
The evolution of malaria parasite resistance towards antimalarial drugs poses the significant challenge in combating the disease.[12] However, the use of combination therapy has been advocated as one of the strategies to improve the efficacy and limit the spread of resistance.[14] Thus, artemisinin-based combinations (ACTs) such as

artesunate (**1.9**) and lumefantrine (**1.10**), lumefantrine (**1.10**) and artemether (**1.11**), and chlorproguanil (**1.12**) and dapsone (**1.13**) have been devised as remedy for uncomplicated malaria and a control of resistance development.[15,16] In areas such as Western Cambodia and Thailand, the use of ACTs has been reported to have low efficacy because their partner drugs have already developed resistance.[17] Thus, the treatment of malaria demands more efforts on searching for alternative antimalarial agents of which plant species are considered such potential sources.



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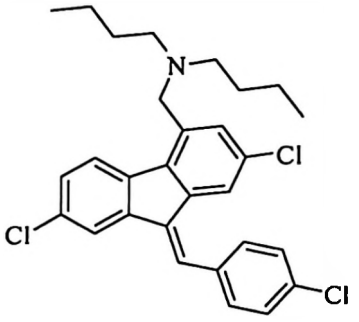

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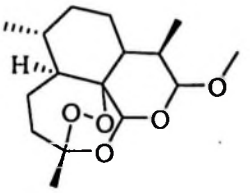

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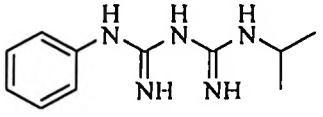

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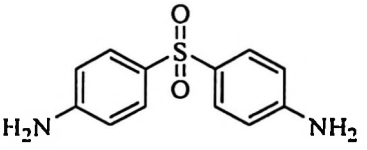

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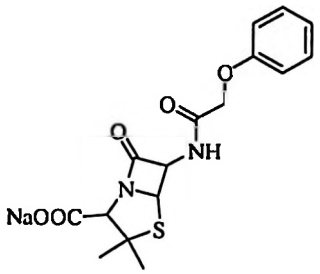
Furthermore, infectious diseases caused by bacteria and fungi have continued to threaten human life posing great burden on many societies worldwide and consequently leading to the high morbidity and mortality.[18] Statistics showed that 1.2 million people lost their lives due to tuberculosis worldwide in 2010, of which 90% of the total impact occurred in Sub-Saharan Africa signifying the persistence of the problem in the region.[19,20] The treatment of microbial diseases has been attempted by

the developed antibiotics such as penicillin (**1.14**), streptomycin (**1.15**), tetracycline (**1.16**), chloramphenicol (**1.17**), clotrimazole (**1.18**) and erythromycin (**1.19**).[18,21]



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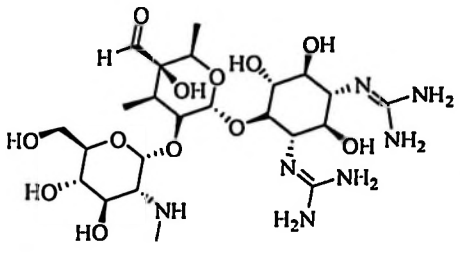

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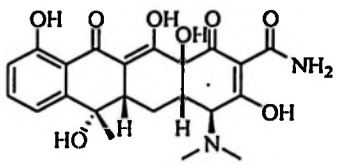

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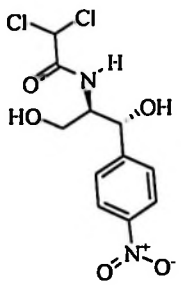

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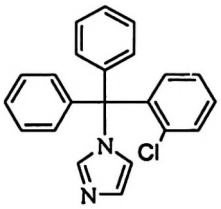

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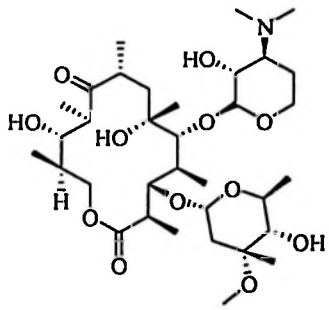

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Over recent years, 50% of the effectiveness of the antibiotics has been challenged by the emergence of drug resistant bacteria and fungi.[18,22] The evolutions of various forms of resistance worldwide have been documented. For example, drug resistant *Neisseria gonorrhoeae*, vancomycin-resistant enterococci, methicillin-resistant *Staphylococcus aureus* and multi drug-resistant *mycobacterium tuberculosis* have emerged and became more threat to the lives of people worldwide.[18,23] Pathogens resistance of any form endangers the efficacy of antibiotic which have transformed medicine and saved millions of lives.[23] The occurrence and spread of drug resistance results from a myriad of behavioural, ecological and evolutionary interacting factors such as genetic adaptations, poor use and abuse of antibiotics, availability of

substandard products, increased global travel, decline in research and development of new drugs, poor application of infection control measures and use of antibiotics in livestocks.[24] Therefore, in order to enhance effective treatment of these infectious diseases, intensive search for alternative bioactive drug agents is required.

The use of plants or extracts from microorganisms as medicine for remedying diseases is as old as human kind.[25,26,27] Plants are acknowledged to offer natural products as alternative source of medicine by providing promising foundation for drug development.[28] Therefore, natural products derived from plants or microorganisms have contributed to the development of most drugs in clinical use today and they account for about 25% of the total drugs.[27] The developed drugs targeted diseases such as cancer, alzheimers, malarial, bacterial, fungal and neurological diseases.[29,30] For example, quinine (**1.1**) and artemisinin (**1.8**) and their derivatives were obtained from *Cinchona* species (Rubiaceae) and *Artemisia annua* (Asteraceae), respectively have been used for treatment of malaria for a long period of time regardless of the noted parasite resistance. On the other hand, the antibiotics penicillin (**1.14**), streptomycin (**1.15**), tetracycline (**1.16**), chloramphenicol (**1.17**), clotrimazole (**1.18**) and erythromycin (**1.19**) which are currently used for combating various forms of bacterial infections were derived from various species of microorganisms such as *Penicillium notatum*, *Streptomyces griseus*, *Streptomyces rimosus*, *Streptomyces venezuelae* and *Saccharopolyspora erythraea*, respectively.[31-35]

Therefore, from the above account, secondary metabolites from extracts of plants or microorganisms and their derivatives have provided remarkable alternatives and potential sources of drugs currently in use due to their efficiency and efficacy on combating malaria and microbial diseases. Thus, due to their usefulness, plants will continue to be a source of lead compounds which are yet to be explored for application in the field of medicine. Hence intensive search for phytochemical constituents from them is highly required in order to unravel their potentials.

The current study investigated the secondary metabolites of *Newtonia paucijuga* (Harms) and *Erythrina sacleuxii* (Hub) which belongs to family Fabaceae to unveil their potentials as sources of bioactive agents for possible control of malaria and microbial infections. The selection of the species for their inclusion in the current study was due to their endemicity and fact that the family and genera level taxa to which they belong are acknowledged for medicinal values from which remarkable natural products with promising treatment of various infections have been discovered. Furthermore, *N. paucijuga* and *E. sacleuxii* have been used traditionally for inducing labour [36] and treatment of malaria,[37] respectively, hence the need for their investigation in order to disclose their phytochemical constituents and biological activities. Thus, the unraveled antimalarial and other anti-microbial activities reported in this work would provide scientific information on new sources of the bioactive principles.

1.2 Literature Review

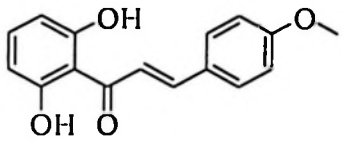
1.2.1 The Family Fabaceae

Family Fabaceae or Leguminosae is commonly recognized as bean, pea or legume family. It occupies the third position among the flowering plants with 730 genera and 19,400 species which include trees, shrubs and herbaceous plants that are well distributed in tropical rainforests of the world.[38,39] Ethnobotanical studies show that the family is of great importance in treatment of various diseases. Thus, diseases such as insomnia, neuritis, malarial, stomatitis, fever, ulcer, worms, cancer, inflammation and oxidative problems have been reported to be controlled by the family members.[39-42] Consequently, their phytochemical studies have revealed the presence of tannins, flavonoids, alkaloids, anthraquinones and terpenoids as the major secondary metabolites some having potent biological activities.[40,43] The specific review of the antimalarial, antimicrobial and other bioactive compounds produced by the family members are briefly given in the following subsections.

1.2.1.1 Antimalarial Compounds from the Family Fabaceae

The use of plant species belonging to the family Fabaceae in traditional medicine is well documented all over the world. For example, *Cajanus cajan,* is used in western Nigeria as food and remedy for various ailments including malaria related fever. Its phytochemical analysis of leaves led to the isolation of cajachalcone (**1.20**) as the active compound possessing antimalarial activities with an IC_{50} 2.0 μg/mL.[44] Extracts from seeds of *Bowdichia nitida* yielded four cassane-type diterpenes namely sucutinirane A (**1.21**), sucutinirane B (**1.22**), 6α-acetoxyvouacapane (**1.23**) and 6α,7β-diacetoxyvouacapane (**1.24**), the latter compound having *in vitro* antimalarial

activities with an IC_{50} 0.39 μg/mL against *P. falciparum* 3D7 and good selective index with regard to cytoxicity with an IC_{50} > 100 μg/mL.[45]



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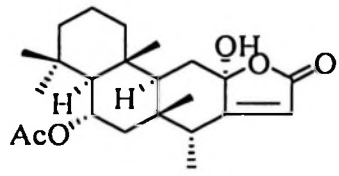

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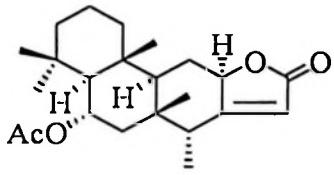

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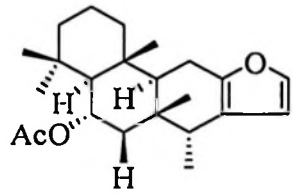

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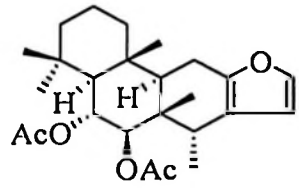

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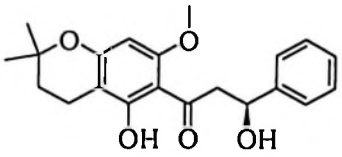
From *Tephrosia elata* seedpods β-hydroxydihydrochalcone (**1.25**), dequelin (**1.26**) and obovatin (**1.27**) were afforded while phytochemical analysis on *Cassia siamea* leaves led to the isolation of chrobisiamone A (**1.28**) and cassiarin B (**1.29**). Compounds **1.25 - 1.29** exhibited antimalarial activities against *P. falciparum* at IC_{50} 8.2, 12.4, 27.6 μM, 5.6 and 22 μg/mL, respectively.[46-50] Furthermore, the stem barks of *C. siamea* gave emodin (**1.30**) and lupeol (**1.31**) which were reported to be active principles for antimalarial property at an IC_{50} 18.5 μM and 11.7 μM against multi-resistant strain of *P. falciparum* (K1), respectively.[48]



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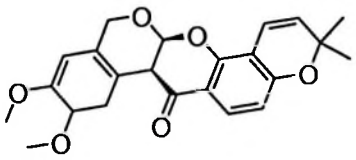

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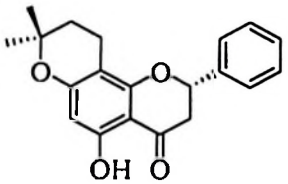

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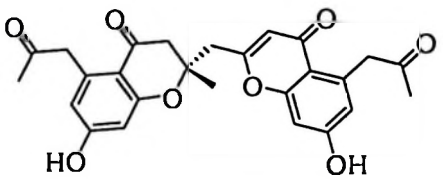

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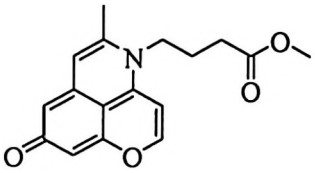

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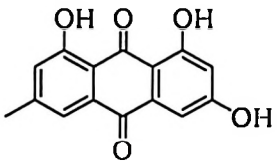

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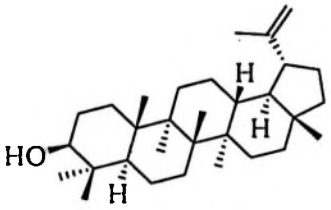

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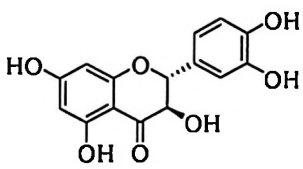
Piptadenia pervillei which is found in Madagascar is reported to have antimalarial activities. Thus, its phytochemical analysis led to the isolation of (+)-catechin (**1.32**), (+)-catechin-5-gallate (**1.33**) and (+)-catechin-3-gallate (**1.34**). Among these, compounds **1.33** and **1.34** showed *in vitro* activity of an IC_{50} 1.2 μM and 1.0 μM, respectively against the chloroquine–resistant strain of *P. falciparum*.[51] The presence of the gallate ester on the compounds was ascribed to the inhibition of the malarial causative agent.[48]



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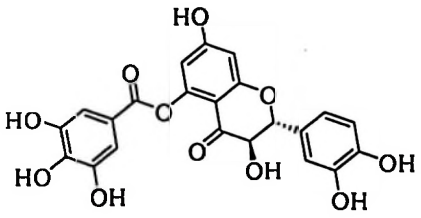

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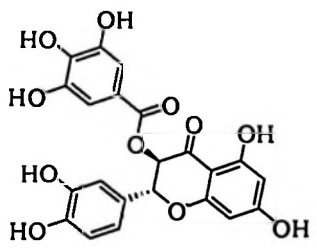
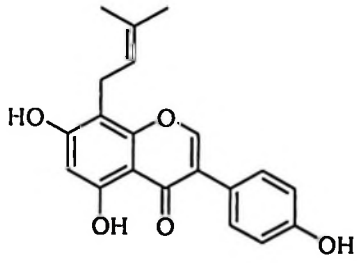


1.34

1.2.1.2 Compounds from the Family Fabaceae

In vitro experiments have proved that plants produces a great number of secondary metabolites in the form of phenols, phenolic acids, quinines, flavones, flavonoids, flavonols, tannins, terpenoids and coumarins that have antimicrobial activitie.[52] Thus, phytochemical analysis of the leaves of *Vatairea guianensis*, an Amazonian species is used for treatment of skin diseases in Brazil, Venezuela, Colombia and French Guiana led to the isolation of lupiwighteone (**1.35**), 5,7,4′-trihydroxy-3′-methoxy-8-prenylisoflavone (**1.36**) and 5,7,3′-trihdroxy-4′-methoxy-8-prenylisoflavone (**1.37**). Compound **1.35** exhibited antifungal activity against *Candida dubliniensis* at the MIC of 32 µg/mL while compound **1.37** showed activity against *Candida parapsilosis* and *candida dubliniensis* at the MIC of 32 and 8 µg/mL, respectively.[53,54] The study conducted on the stem barks of *Swartzia polyphylla* led to isolation of biochanin A (**1.38**) and dihydrobiochanin A (**1.39**) both of which exhibited significant antifungal activities against *Trichophyton mentagnophytes* and *Microsporum gypseum* with the zone of inhibition of 31, 33 and 41,47 mm, respectively.[55] From the root barks of *Vatairea maerocarpa*, vatacarpan (**1.40**) an active principle against *Candida albicans* at the MIC of 0.98 µg/mL was isolated.[56] The isolation of *N*-1,*N*-2,*N*-3-triisopentenylguanidine (**1.41**), nitensidine A (**1.42**), nitensidine B (**1.43**) and nitensidine C (**1.44**) have been reported from the branches of *Pterogyne nitens*. The compounds were active against *Candida albicans*, *C. krusei*, *C. parapsilosis* and *Crytococcus neoformans* at varying efficacy.[57]



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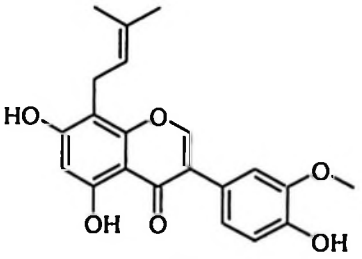

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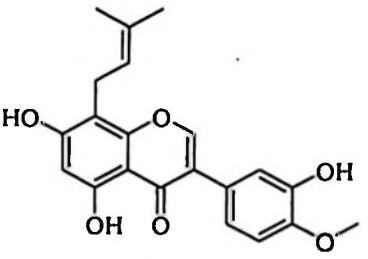

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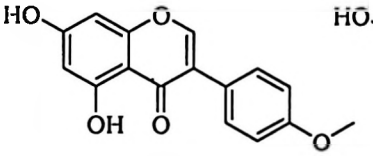

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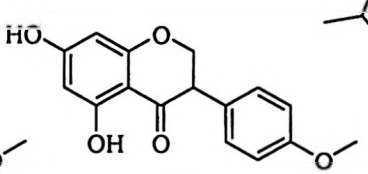

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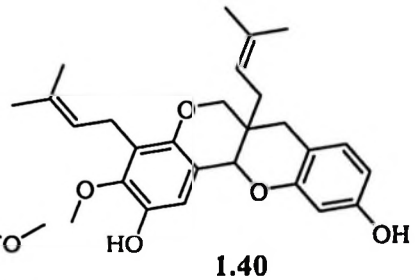

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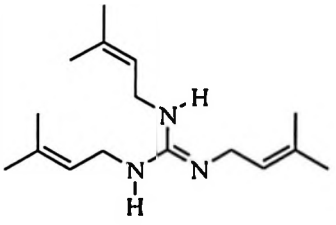

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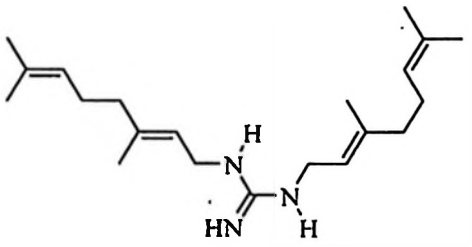

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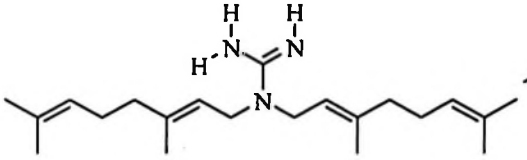

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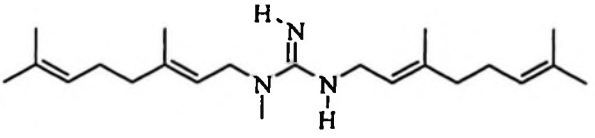
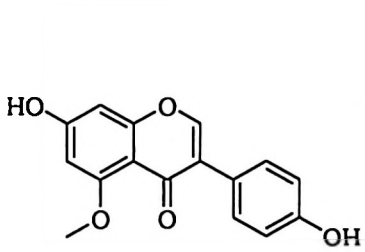


1.44

The investigation of the leaves of *Adenocarpus mannii* led to the isolation of isoprunetin (**1.45**) having activity against *Enterococcus faecalis* at the MIC of 16 μg/mL.[58] Other studies on the leaves of *Senna alata* (previously known as *Cassia alata*) afforded the isolation of cannabinoid dronabinol alkaloid (**1.46**) which inhibited the growth of *Klebsiella pneumonia*, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* with the zone of inhibition of 9±0.1, 7±0.1,

10±0.1 and 14±0.2 mm, respectively, the compound being more active on *P. aeruginosa*.[59] Furthermore, the use of the members of the genus *Sophora* in chinese traditional medicine have been documented. Studies have shown that sophoraflavanone G (**1.47**), a compound isolated from the *Sophora exigua* possess strong antimicrobial activity against methicillin resistant *S. aureus* with MIC 3.13 μg/mL. From the root of *S. flavescens*, quinolizidine alkaloids anagyrine (**1.48**), oxymatrine (**1.49**) and sophoranol (**1.50**) were isolated. Compounds **1.48 – 1.50** showed potent antiviral activities against respiratory syncital virus with an IC_{50} 24.0, 12.0 and 24.0 μg/mL, respectively.[60]



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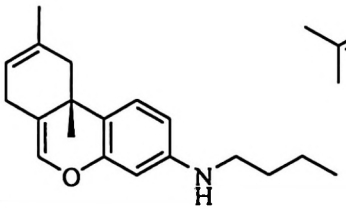

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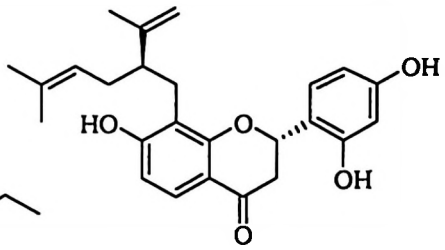

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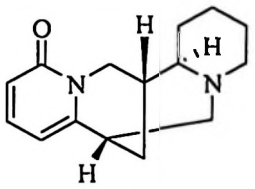

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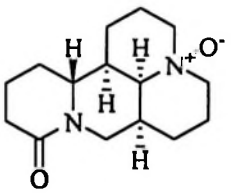

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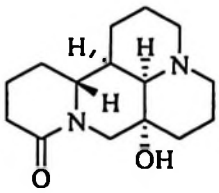

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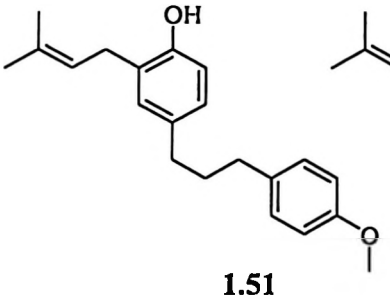
In addition, studies on the genus *Eriosema* species revealed the presence of flavonoids with potent pharmacological activities. Investigation on the whole plant of *E. glomerata* afforded the isolation of two dihydrochalcones, erioschalcone A (**1.51**) and erioschalcone B (**1.52**) of which compound **1.51** had antibacterial activity against *E. coli* with the zone of inhibition of 10 mm while compound **1.52** showed antifungal activity against *Microbotryum riolaceum* with the zone of inhibition of 13 mm.[61]

Eriosemaone A (**1.53**), eriosemaone B (**1.54**) and eriosemaone C (**1.55**) were isolated from the root barks of *E. tuberosum*. Compound **1.53** had significant antimycobacteria activity against *Mycobacterium tuberculosis* at the MIC of 12.5 μg/mL.[61] Therefore; due to their potentiality in combating various forms of microbial diseases as highlighted above, intensive search is required in order to reveal more ingredients from the Fabaceae family members.



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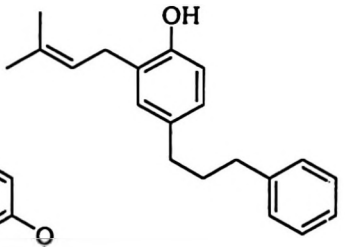

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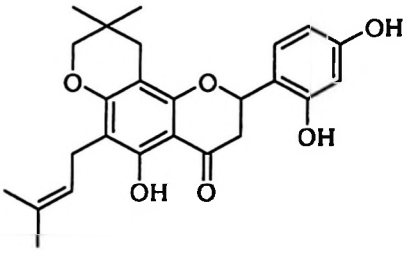

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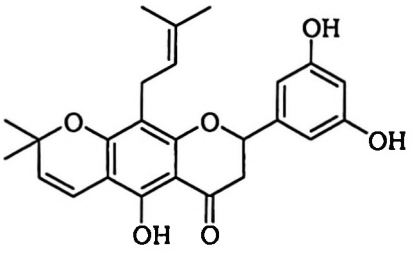

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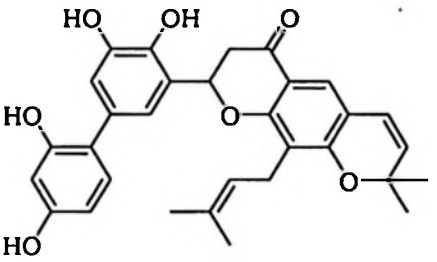
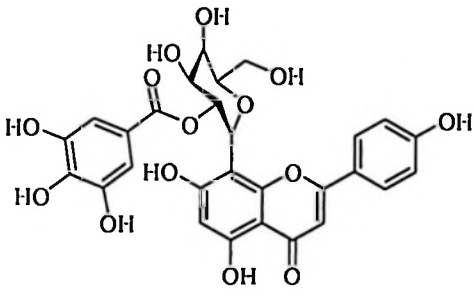


1.55

1.2.1.3 Other Bioactive Compounds from the Family Fabaceae

Members of the family Fabaceae are well known for their promising potential in combating ailments other than malaria, bacterial and fungal diseases. Phytochemical studies on the leaves of *Caesalpinia ferrea* afforded the isolation of 2′′-*O*-galloyl vitexin (**1.56**) which exhibited cytotoxic activity with an IC_{50} 18.5, 22.6, 24.2 and 28.4 μg/mL against the liver HepG-2, HCT-116, Hep-2 and MCF-2, respectively.[62] Indirubin (**1.57**), a nitrogenous compound isolated from *Indigofera tinctoria* is

effective against chronic myelocytic leukemia.[63] Phytochemical investigations on the genus *Eriosema* led to the isolation of flemichin (**1.58**) from the root barks of *E. tuberosum*, khonklonginol A (**1.59**) and lupinifolinol (**1.60**) from the root barks of *E. chinense* and tectorigen (**1.61**) from the twigs of *E. robustum*. The compounds demonstrated potent antioxidant activities at the IC_{50} 0.54, 7.92, 1.77 and 2.55 mM, respectively.[61] On the other hand, cajanol (**1.62**) from the twigs of *E. robustum* inhibited the growth of human breast cancer cells at the IC_{50} 54.0, 58.3 and 83.4 μg/mL after 72, 48 and 24 hours treatments, consecutively.[61] Furthermore, *Astragalus membranaceus,* a plant well-known in Chinese traditional medicine for treating various ailments afforded astragaloside (**1.63**) from its root barks upon phytochemical investigation. The biological assay indicated the compound to possess anti-inflammatory, antioxidant, cardioprotective and immune stimulating activities.[64]



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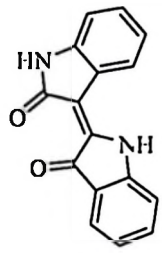

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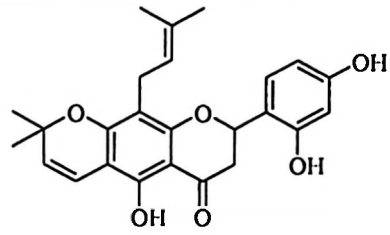

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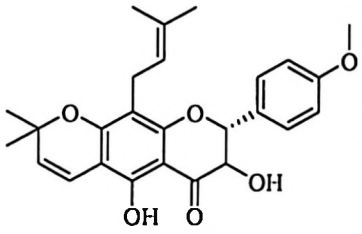

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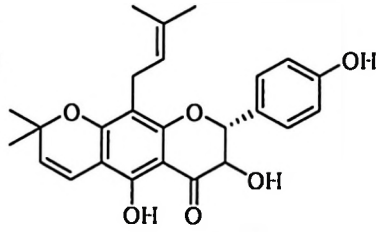

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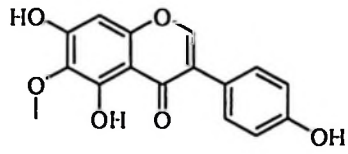

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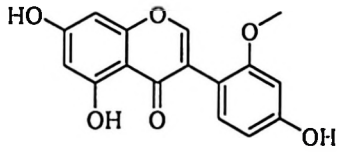

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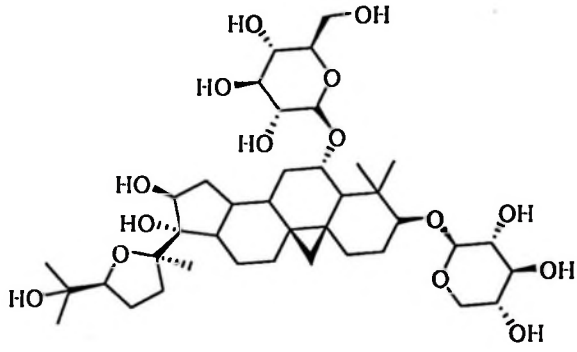


1.63

With the consideration of the phytochemical analyses reviewed from the family Fabaceae, it can generally be concluded that members of this family are rich in bioactive compounds which provide promising alternative for treating various ailments. Therefore, more research on other members of the family is highly essential in order to contribute more chemical scaffolds needed for drug discovery hence, the essence of undertaking the research being reported in this dissertation.

1.3 Statement of the Research Problem

Malaria and microbial diseases have remained to be an enormous burden to numerous societies worldwide due to evolution of pathogens resistance against the existing antimalarial and antibiotics.[22,65] The evolution of pathogens resistance towards the drugs agents pose the need for further finding of alternative and effective agents for treatment of malaria and microbial diseases. Thus, treatment of diseases by using plant extracts or compounds isolated therefrom is currently practiced in many parts of the world.[66] Despite their well known medicinal value in curing diseases such as malaria and microbial infections, only few species in the family Fabaceae have been exploited for their potential as antimalarial and antimicrobial agents.[67] Ethnomedically, *Newtonia paucijuga* is used for labour induction while *Erythrina sacleuxii* is deployed in the treatment for malaria. Previous phytochemical study on the seeds of *N. paucijuga* led to the isolation of a cytotoxic compound namely, 2-piperidinecarboxylic acid (**2.3**). However, the compound was not assayed for its antimalarial and antimicrobial effects. Based on the literature surveyed, the root barks, stem barks and leaves of *N. paucijuga* have not been phytochemically investigated, hence, its inclusion in the investigations reported in this dissertation. On the other hand, only the phytochemical studies of the root and stem bark of *E. sacleuxii* led to the isolation of 5′-deoxy-3′-prenylbiochanin (**2.24**) and 5′-prenylpratensein (**2.25**). Thus, the investigations on the leaves of *E. sacleuxii* have not been reported, hence undertaken in the current study. Furthermore, both *N. paucijuga* and *E. sacleuxii* are endemic in some tropical areas and vulnerable to extinction due to human activities, hence reinforcing the need for their investigation in order to further document their phytochemical constituents.

1.4 Objectives of the Research reported in this Dissertation

The present study aimed at establishing the chemical constituents of *Newtonia paucijuga* and *Erythrina sacleuxii* with the hope of discovering new secondary metabolites with promising antimalarial and antimicrobial activities. Thus, the reported study had the following specific objectives:

i. To determine the in vitro biological activities of the crude extracts from roots, stem barks and leaves of *N. paucijuga* and leaves of *E. sacleuxii* against *Plasmodium falciparum*, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Candida albicans*, *Crytococcus neoformans* and *Aspergillus niger*.

ii. To determine the structures of secondary metabolites isolated from the chosen parts of the two plant species.

CHAPTER TWO

A FLAVONOID, TERPENOID AND OTHER METABOLITES FROM *NEWTONIA PAUCIJUGA* AND *ERYTHRINA SACLEUXII*

Abstract

This chapter reports on the phytochemical investigations of the leaves, stem barks and root barks of *Newtonia paucijuga* and the leaves of *Erythrina sacleuxii*. Chromatographic isolation yielded mearnsetin 3-*O*-α-*L*-rhamnopyranoside (**2.26**), benzyl-3,4,5- trihydroxylbenzoate (**2.28**) and, the mixture of stigmasterol (**2.37a**) and β-sitosterol (**2.37b**) from the ethanolic extracts of leaves, root barks and stem barks of *N. paucijuga*, respectively. Chromatographic separation of the ethanolic extracts of the *E. sacleuxii* leaves led to the isolation of a new *nor*-sesquiterpenoid of the humulyl skeleton namely sacleuxenone (**2.38**). The structural elucidation of the reported compounds in this dissertation was based on the ^{1}H and ^{13}C NMR, IR and mass spectral data.

2.1 Introduction

Newtonia paucijuga Harms (Fig. 2.1)[68] is among the 38 species in the genus *Newtonia* belonging to the family Fabaceae. Most of the members of the genus *Newtonia* are endemic to African forests with a well defined ecological, ethnomedical and economical importance.[69-71] *Newtonia paucijuga* occurs in Kenya and Tanzania, whereas in Tanzania it is found in Zaraninge Forest Reserve in Bagamoyo District, Pugu Forest Reserve and Kazimzumbwi in Kisarawe District, Pwani Region, and Usambara Forest in Tanga region.[72-74] Ethnomedically, *N. paucijuga* has been known for inducing labour upon the use of the root extracts.[36] The species is vulnerable to extinction due to human activities.[75] Thus, the investigations for identification and characterization of it secondary metabolites is highly demanded.

Figure 2.1: ***Newtonia paucijuga*** **Source: Lancetilla (Tela) Botanical Garden**[68]

Erythrina sacleuxii Hua (Fig. 2.2)[76] belongs to the genus which consists of about 130 species distributed in both tropics and subtropics all over the world.[77,78] *E. sacleuxii* is found in the United States of America and well distributed in coastal areas of Tanzania and Kenya.[79] Like other members in the family Fabaceae, members of the genus *Erythrina* are of ethnobotanical importance where their roots, stems, barks, leaves and fruits are used for treating various ailments.[80] For instance, *Erythrina indica* and *E. arborescans* in India are well known for combating malaria, ulcers, helmentic, oxidative, diuretic and osteoporotic health problems.[81] The roots of *E. abyssinica* are used for treating bacterial disease in Kenya while stem barks of *E. addisoniae* are anti-inflammatory in Cameroon.[78] In Tanzania, *E. abyssinica*, *E. schliebenii* and *E. sacleuxii* are reported to be used traditionally in treating malaria and fever diseases.[82] Phytochemical studies of the members of the genus *Erythrina*

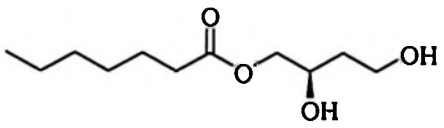
have revealed the presence of terpenes, alkaloids and flavonoids such as isoflavonoids, pterocarpans, flavanones and isoflavanones as the major secondary metabolites just like other members within the family Fabaceae.[83,84]

Figure 2.2: ***Erythrina sacleuxii*** **Source: Koko Crater Botanical Garden**[76]

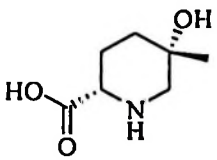
2.2 Phytochemistry of the Genus *Newtonia*

Approximately 38 species belong to of the genus *Newtonia*, but only few out of the described species in the genus have been explored for their phytochemical constituents. The phytochemical study on the stem barks of *N. griffoniana* led to the isolation of newtonoate (**2.1**), an active principle with an anxiolytic properties.[85] Seed extracts from *N. buchananii* and *N. duparquetiana* afforded the isolation of alkaloids 5-hydroxy-2-piperidinecarboxylic acid (**2.2**), 2-piperidinecarboxylic acid (**2.3**) together with djenkolic acid (**2.4**) of which, the latter two possessed cytotoxic activities against the brine shrimp *Artemia salina*.[86,87] *N. paucijuga* in spite of its

traditional use on induction of labour using its root barks extracts, only its seeds have been phytochemically analysed to afford the cytotoxic compound **2.3**.[86]



2.1



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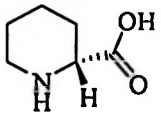

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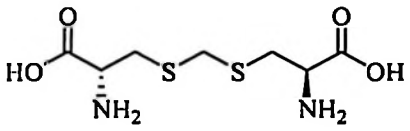
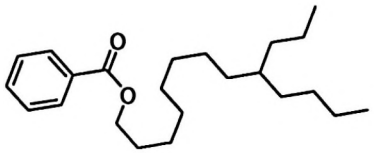


2.4

2.3 Phytochemistry of the Genus *Erythrina*

Previous studies have reported on the phytochemical constituents and biological activities of some members of the genus *Erythrina*. Thus, *Erythrina abyssinica*, a species found in Ethiopia, Kenya, Uganda, Tanzania and Zimbabwe has a pronounced medicinal value like other *Erythrina* species. Phytochemical studies of its root barks led to the isolation of 9-propyltridecylbenzoate (**2.5**) that exhibited antibacterial activities against the Gram-negative bacteria *Shigellae boydi* and Gram-positive bacteria *Staphylococcus aureus* at 23 and 22 mm inhibition zones, respectively.[88] From the stem bark of *E. orientalis* prenylated pterocarpans shinpterocarpin (**2.6**) and phaseollin (**2.7**) were isolated together with flavonoids alpinumisoflavone (**2.8**), 8-prenyl-daizein (**2.9**) and 4′-*O*-methyllisoflavanone (**2.10**).[89] Indonesians use the decocted barks or leaves of *E. orientalis* for treatment of malaria. However, the cytotoxic assay of the isolated compounds showed that compounds **2.6** – **2.9** are active against murine leukemia P-388 cells with the IC_{50}

2.43, 2.55, 4.31 and 5.82 μg/mL, respectively. Compounds **2.7** and **2.9** have also shown good antioxidant properties against DPPH radical scavenging at an IC_{50} 241.9 and 174.2 μM, respectively.[89] Furthermore, the stem bark of *E. fusca* gave loncocarpol A (**2.11**) possessing antimalarial activity with IC_{50} value of 1.6 μg/mL against multi drug resistant strain of *P. falciparum*.[90]



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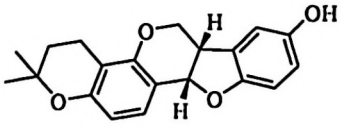

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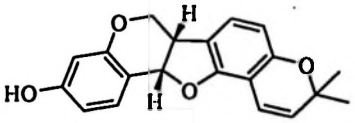

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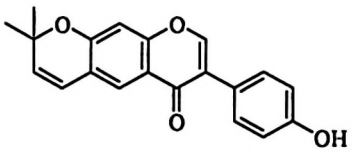

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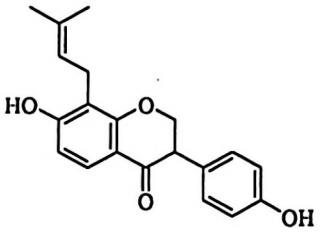

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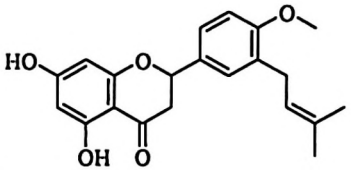

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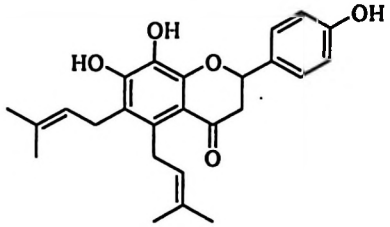
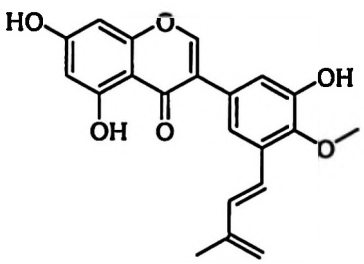


2.11

Phytochemical investigations of *E. schliebenii* carried out by Natural Products Research Group at the Chemistry Department, University of Dar es Salaam (UDSM) yielded schliebenone B (**2.12**), 4′-*O*-methylabyssinone (**2.13**) and parvisoflavone B (**2.14**) and other metabolites. The compounds **2.12** – **2.14** exhibited antimycobacterial activity against *Mycobacterium tuberculosis* with an MIC 87.4, 49.5 and 90.9 μM, respectively.[91] These compounds and other flavonoids isolated from *E. schliebenii* also demonstrated cytotoxicity against the aggressive human breast cancer cell line MDA-MB-231.[91] The plant is used in ethnomedicine for

treatment of stomachache and diarrhea, for prevention of jaundice in newborn babies and as abortive agents.[91]



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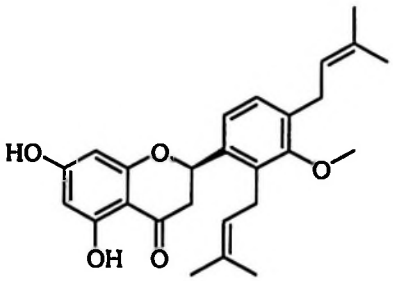

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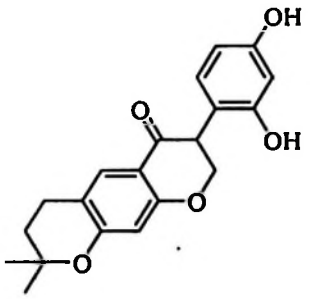
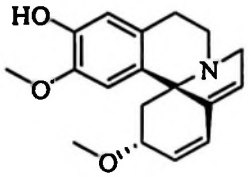


2.14

Although, flavonoids are reported to dominate in the genus *Erythrina*, the existence of alkaloids with potent biological activities has been documented. For example, from the seeds of *E. abyssinica* tetracyclic alkaloids erysodine (**2.15**), erythraline (**2.16**), 11-methoxyerysodine (**2.17**), 8-oxoerythraline (**2.18**) and erysotrine (**2.19**) were isolated.[92] All compounds **2.15 – 2.19** showed cytotoxicity activities with IC_{50} 19.90, 15.90, 11.50, 18.50 and 21.60 μg/mL against HEP-G2 cell lines, respectively and hence provide good indication for their potential use as anticarcinogenic agents.[92] In another study, compounds **2.16** and **2.18** showed lower antileishmanial activity.[93]



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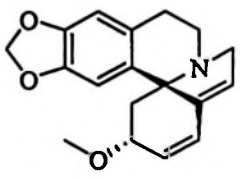

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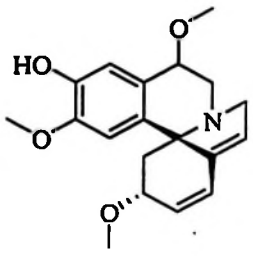

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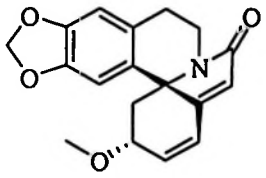

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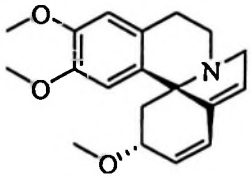
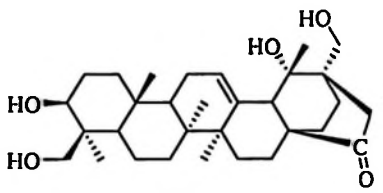


2.19

Triterpenoid gutinosalactone (**2.20**) has been isolated from the stem bark of *E. excelsa* and the compound exhibited antibacterial and antifungal activities showing an inhibition zone of 15 and 11 mm against *A. niger* and *S. aureus*, respectively.[94] Erystagallin (**2.21**), erybraedin (**2.22**) and 5-hydroxysophoranone (**2.23**) were isolated from stem barks of *E. stricta* and *E. subumbrans*. Compounds **2.21 – 2.23** possessed the antimalarial activities with an IC_{50} 9.0, 8.7 and 5.3 μM against multi-drug resistance strain of *P. falciparum*.[95] The investigation on the root bark and stem bark of *E. sacleuxii* growing in Kenya led to the isolation of 5-deoxy-3′-prenylbiochanin A (**2.24**) and 5′-prenylpratensein (**2.25**). Compound **2.24** and **2.25** that exhibited antimalarial activities with an IC_{50} 17.6 and 6.3 μM against D6 strain of *P. falciparum* and 22.5 and 8.7 μM against W2 strain of *P. falciparum*, respectively.[95-97]



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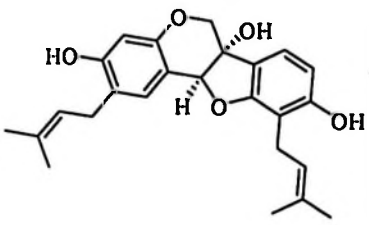

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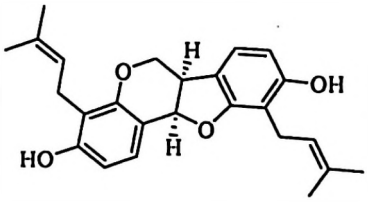

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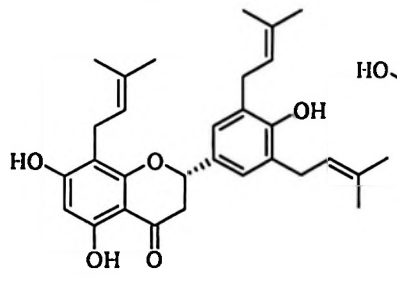

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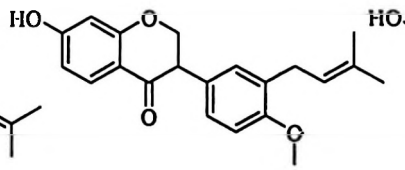

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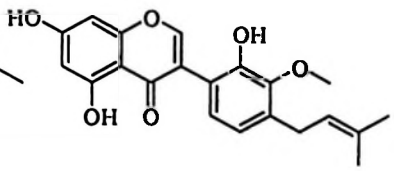
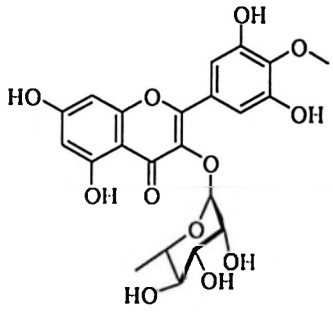


2.25

Previous studies have reported phytochemistry and biological activities for the root barks and stem barks of *Erythrina sacleuxii.* Thus, this investigation embarked on the isolation of compounds from the leaves of *E. sacleuxii* for which no phytochemical studies have been reported.

2.4 Results and Discussion

The chromatographic separation of the ethanolic crude extract of the leaves, root and stem barks of *N. paucijuga* were carried out. The repeated silica gel column chromatography of the fractions obtained from the vacuum liquid chromatography (VLC) of the leaves extracts and further purification using Sephadex® LH-20 afforded the isolation of mearnsetin-3-*O*-α-*L*-rhamnopyranoside (**2.26**). The root barks of *N. paucijuga* afforded the isolation of benzyl-3,4,5-trihydroxybenzoate (**2.28**) upon chromatographic separation. The VLC of the ethanolic extracts from the stem barks of *N. paucijuga* followed by repeated column chromatography using silica gel and Sephadex® LH-20 led to the isolation of the mixture of sterols, stigmasterol (**2.37a**) and β-sitosterol (**2.37b**). On the other hand, the repeated gravitational silica gel and Sephadex® LH-20 column chromatography of the ethanolic extracts of the *E. sacleuxii* leaves led to the isolation of hither to previously unreported *nor*-sesquiterpenoid, sacleuxenone (**2.38**). ^{1}H and ^{13}C NMR, IR and mass spectral data were used to establish of the structures of the isolated compounds.



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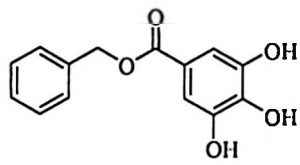

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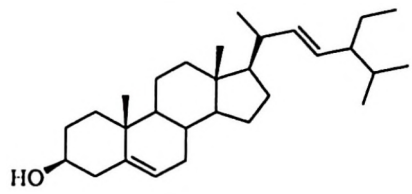
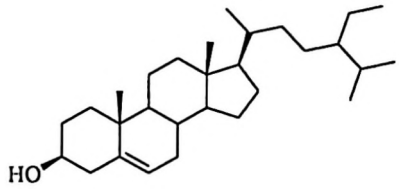
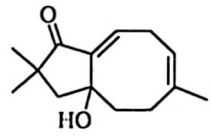


2.37a

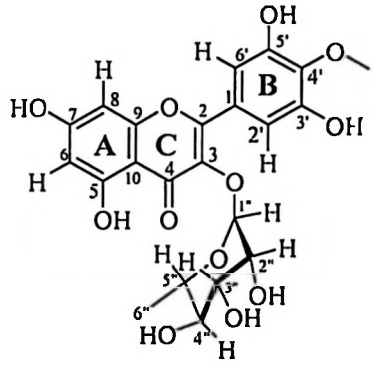


2.37b



2.38

2.4.1 Mearnsetin-3-*O*-α-*L*-rhamnopyranoside (2.26)



2.26

Repeated gravitational column chromatography of the combined VLC fractions 6-7 obtained from the ethanolic extract of the leaves of *N. paucijuga* using of *n*-hexane/ethyl acetate gradient elution yielded a yellow solid whose spectroscopic data were found to be similar to those previously reported for mearnsetin-3-*O*-α-*L*-rhamnopyranoside (**2.26**).[98] The UV observation indicated the presence of a spot of which the anisaldehyde spraying reagent on the TLC and followed with heating displayed dark-brown which indicated the presence of flavonoid molecule with sugar moiety.[98] The mass spectrum (Fig. 2.3) exhibited a $[M+H]^+$ peak at *m/z* 479.6 and its MS fragmentations are shown on scheme 2.1, which together with NMR data

suggested the molecular formula $C_{22}H_{22}O_{12}$. The Optical rotation, $[\alpha]_D$ -28.75° at 20 °C (c 0.0002, $CHCl_3$) of compound **2.26** was observed. The IR spectrum (Fig. 2.4) showed absorptions at 3321.8 cm^{-1} indicating the presence of hydroxyl group, absorptions at 2943.9 and 2836.6 cm^{-1} were typical of the saturated C-H stretches. Absorptions at 1650.45 and 1456.1 were due to C=C. Other absorption due to phenolic C-O appeared at 1030.5 cm^{-1}.[99]

The ^{1}H-NMR spectrum (Fig. 2.5 and Table 2.1) of compound **2.26** revealed two singlet signal integrating to two protons at δ_H 6.88 (s, 2H) that was assigned to two chemically equivalent aromatic protons on ring B of the flavonoid nucleus. Protons at δ_H 6.37 (d, 1H, J = 2.1 Hz) and δ_H 6.21 (d, 1H, J = 2.1 Hz) of which due to their coupling constant, suggested the presence the two protons that are *meta*-coupled, a pattern that is typical of ring A of a flavonoid nucleus.[100] Furthermore, the ^{1}H NMR spectrum suggested the presence of sugar moiety in compound **2.26**. This was characterized by the presence of anomeric proton signal at δ_H 5.31 (d, 1H, J = 1.7 Hz) assigned to H-1'' being in the equatorial position based on the coupling constant. Signals at δ_H 4.23 (dd, 1H, J = 1.7, 3.4 Hz), 3.75 (m, 2H), and 3.33 (d, 1H, J = 8.1 Hz) were consistent with H-2'', H-3''/H-5'' and 4'' of a sugar moiety, respectively. Furthermore, the anomeric H-1'' coupling constant (J = 1.7 Hz) and its chemical shift (δ_H 5.31) suggested that, the sugar moiety of compound **2.26** is of the α-glycoside in nature and linked to the O-C bond at the C-3 of the flavonoids ring C.[101-103] This linkage of the sugar moiety was confirmed by H/C HMBC correlations between signals at δ_H 5.31 to δ_C 136.7 (Fig. 2.7). Methoxy protons (-OCH_3) were observed at δ_H 3.88 (s, 3H) and attached to C-4'of the aromatic ring B as supported

by the $^{3}J_{C\text{-}H}$ HMBC cross-peak between the protons and the carbon at δ_C 139.4. Further analysis of the ^{1}H NMR spectrum indicated the presence of methyl protons at δ_H 0.95 (d, J = 5.7 Hz, 3H) assigned to H-6'' of the sugar moiety. H/H COSY spectrum (Fig. 2.7 and 2.8) depicted some important H/H interactions between (H-6) (δ_H 6.37, d, J = 2.1 Hz, 1H) and H-8 (δ_H 6.21, d, J = 2.1 Hz, 1H), H-1'' (δ_H 5.31,d, J = 1.7 Hz, 1H) and H-2'' (δ_H 4.23, dd, J = 1.7, 3.4 Hz, 1H), H-3'' (δ_H 3.75, m, 1H) and H-4'' (δ_H 3.33, d, J = 8.1 Hz, 1H), H-5'' (δ_H 3.75, m, 1H) and H-6'' (δ_H 0.95, d, J = 6.6 Hz, 3H), H-4'' (δ_H 3.33, d, J = 8.1 Hz, 1H) and H-5'' (δ_H 3.75, m, 1H).

The ^{13}C-NMR spectrum (Fig. 2.6 and Table 2.2) revealed the presence of 20 signals corresponding to 22 carbons that complied with the typical flavonoid monoglycoside nucleus.[100] The close analysis of the spectrum showed that, compound **2.26** was characterized by the presence of carbonyl group indicated by the presence of a downfield signal at δ_C 179.7 which was assigned to C-4. Signals at δ_C 109.8, 100.0 and 94.4 were assigned to aromatic methine carbons C-2′/C-6′, C-6, C-8, respectively. The signal at δ_C 60.9 was due to the methoxy carbon which was deduced from HMBC to be linked to C-4′ of ring B of the flavonoid skeleton. The presence of the methoxy group at C-4′ attributed the further downfield shift of C-3′ and C-5′ which were reported to resonate at δ_C 151.9 each, themselves possessing OH functionality. Chemical shifts at δ_C 73.1, 72.1 and 71.9 represented the oxymethine carbons of the sugar moiety. The signal at δ_C 103.7 was due to the anomeric carbon of the glycoside. Basing on the ^{13}C NMR data, the glycoside present in compound **2.26** was formed by a rhamnosyl group and hence confirming

the compound to be α-rhamnoside. The carbon signal at δ_C 17.1 (C-6′′) was due to the methyl carbon.

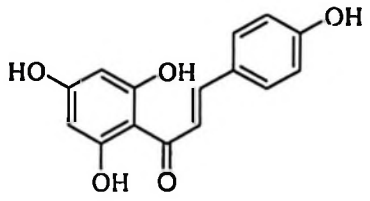
The HSQC spectroscopic data (Fig. 2.11) was used to establish the assignment of all the chemical shifts as given on Table 2.1. The long range multiple bond correlations were deduced from the HMBC spectrum (Figs. 2.9 and 2.10). The spectrum showed that the signal at δ_H 6.21 (H-6) and 6.37 (H-8) correlated with a carbon at δ_C 166.3 (C-7) which was deduced to be an oxygenated sp^2 carbon on ring A. In addition, while H-6 was correlating with carbon signals at δ_C 163.3 (C-5), 105.9 (C-10) and 94.8 (C-8), H-8 (6.37) correlated with carbon signals at δ_C 158.6 (C-9), 105.9 (C-10) and 100.0 (C-6) resulting to the complete assignment of the carbons in ring A of the flavonoid skeleton. Other interactions were observed between the protons signals at δ_H 6.88 (H-2′) with the carbon at δ_C 159.0 (C-2) indicating the linkage of ring B to C at this position. Further correlations in ring B included the interaction of proton signal at δ_H 6.88 with carbon at δ_C 109.8. According to HSQC spectrum, proton (δ_H 6.88, s, 2H) was attached to the carbon at δ_C 109.8. The HMBC spectrum showed that, the proton and its carbon had the long range correlation of ^3J to each other. Therefore, due to the symmetry, the proton at δ_H 6.88 was assigned to H-2′ and H-6′. In addition, H-2′ and H-6′ correlated with the signals at δ_C 151.9 (C-3′ and C-5′). Other correlations in respect to the rhamnoside sugar as a part of the entire molecule were observed between δ_H 5.31 (H-1) and δ_C 72.1 (C-3′′/C-5′′), δ_C 71.9 (C-2′′); δ_H 4.23 (H-2′′) and δ_C 73.1 (C-4′′); δ_H 3.75 (H-3′′) and δ_C 71.9 (C-2′′)/ δ_C 73.1 (C-4′′), δ_H 3.33 (H-4′′) and δ_C 72.1 (C-3′′/C-5′′), δ_H 0.95 (H-6′′) and δ_C 73.1 (C-4′′)/δ_H 72.1 (C-5′′). Therefore the complete assignment of compound **2.26** was achieved by

analyses of the spectroscopic features observed on the COSY, HMBC and HSQC spectra.

Compound **2.26** was previously reported from the leaves, aerial parts and stem barks of *Eugenca jambolana* (Myrtaceae), *Symbolocos racemosa* (Symplocaceae) and *Myrsine Africana* (Primulaceae), respectively.[98,104,105] Thus, its isolation for the first time from the leaves of *Newtonia paucijuga* provides a new source. Compound **2.26** is known to have antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus coagulas* and *Pseudomonas aeruginosa* with an inhibition zone of 15.5, 9.5, 12.5 and 16.7 mm, respectively.[106] The compound also possess antifungal activities against *Aspergilus niger*, *Penicium digitatum* and *Trichoderma viride* with an inhibition zone of 8.4, 7.5 and 9.5 mm, respectively.[106] Based on the antimicrobial activity data reported, it was observed that compound **2.26** was active against both Gram-positive and Gram-negative bacteria and hence recognized to have broad spectrum activity against the microbes. Furthermore, the comparison between antibacterial and antifungal activities indicated that, compound **2.26** is more effective against bacteria than fungi and hence the compound can be a good candidate for antibiotic drug formulations.

Like other flavonoids, compound **2.26** is biosynthesized through phenylpropanoids metabolic pathways. The pathways involve the transformation of phenylalanine into 4-coumaroyl-CoA which combines with malonyl-CoA to yield a central C-15 intermidiate chalcone (**2.27**) as a precursor for the formation of all flavonoid.[107,108]

Thus, chalcone (**2.27**) undergoes further reactions as proposed in scheme 2.2 yielding compound **2.26.**



2.27

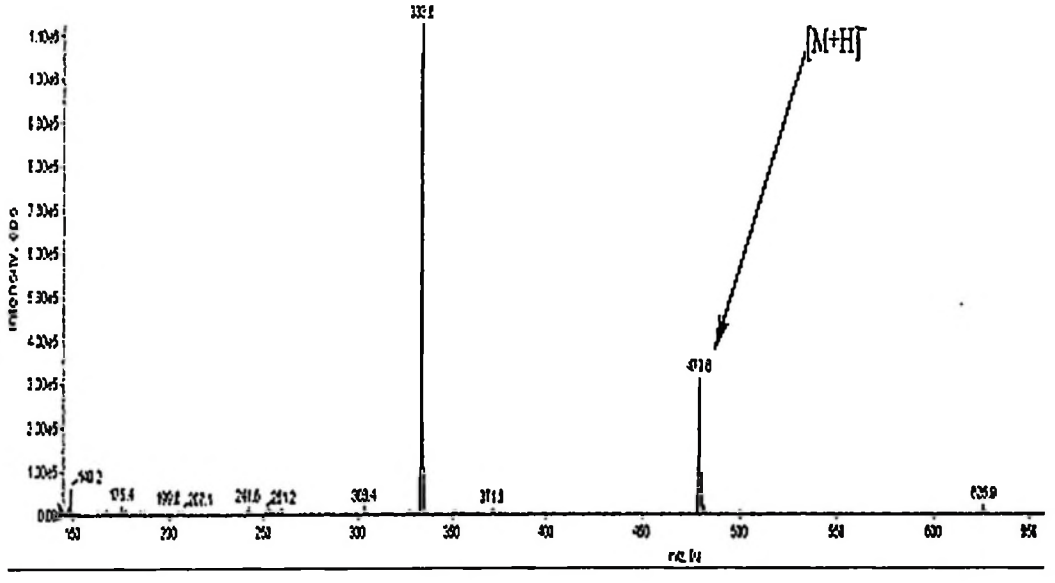
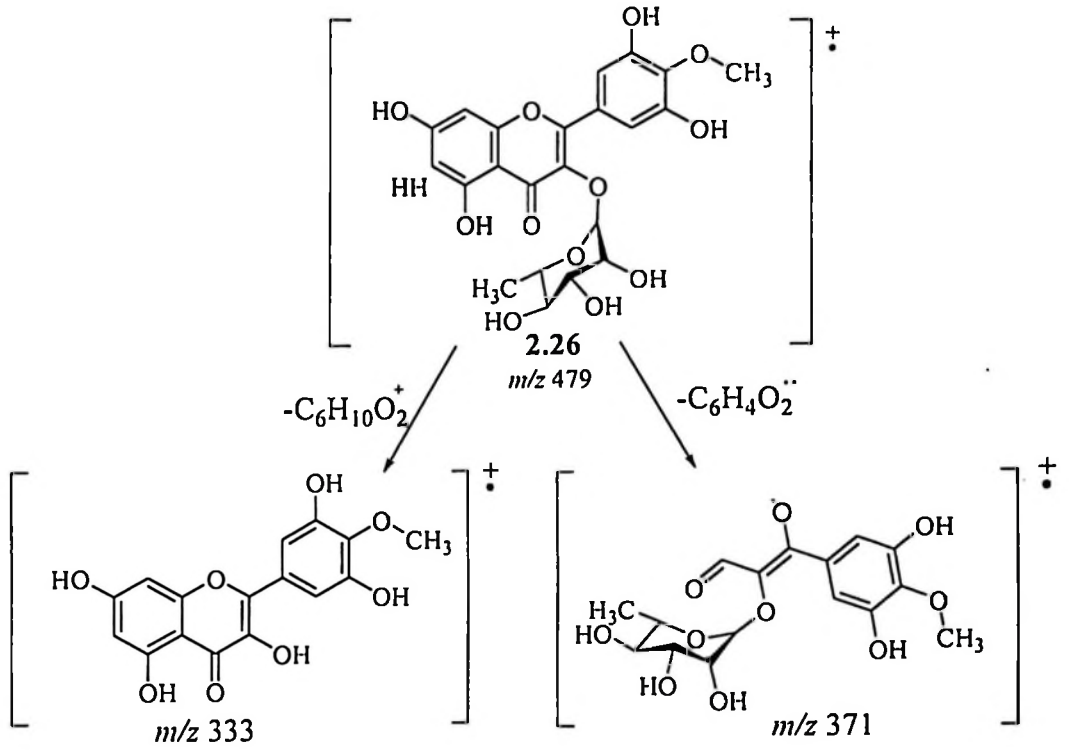


Figure 2.3: The LC-Mass Spectrum for Mearnsetin-3-*O*-α-*L*-Rhamno pyranoside (2.26)



Scheme 2.1: MS Fragmentation Pattern of Mearnsetin-3-*O*-α-*L*-Rhamno pyranoside (2.26)

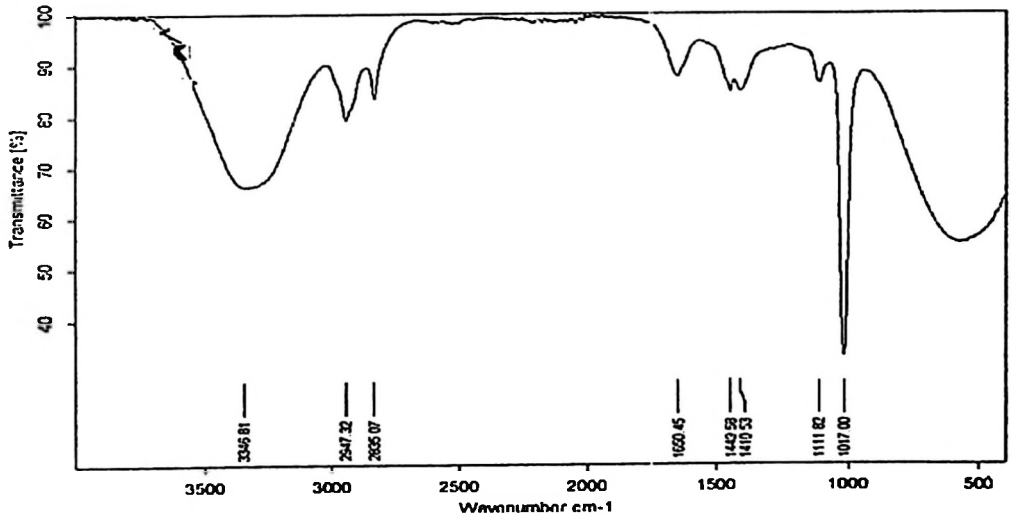


Figure 2.4: IR Spectrum for Mearnsetin 3-*O*-α-*L*-Rhamnopyranoside (2.26)

Table 2.1: **^{1}H NMR Spectroscopic Data for Mearnsetin-3-*O*-α-*L*-Rhamno pyranoside (2.26)**

H	Observed (CD_3D)		Reported [98] (DMSO)	
	δ_H	Mult. *J* (Hz)	δ_H	Mult. *J* (Hz)
6	6.21	d, 2.1	6.21	d, 1.9
8	6.37	d, 2.1	6.38	d, 1.9
2′	6.88	s	6.82	s
6′	6.88	s	6.82	s
1′′	5.31	d, 1.7	5.12	br s
2′′	4.23	dd, 1.7, 3.4	3.97	dd, 1.5, 3.3
3′′	3.75	m	3.51	br s
4′′	3.33	d, 8.1	3.15	m
5′′	3.75	m	3.32	m
6′′	0.95	d, 5.7	0.88	d, 6.1
-OCH_3	3.88	s	3.74	s

Table 2.2: **^{13}C NMR Spectroscopic Data for Mearnsetin-3-*O*-α-*L*-Rhamno pyranoside (2.26)**

C	δ_C		C	δ_C	
	Observed (CD_3D)	Reported[98] (DMSO)		Observed (CD_3D)	Reported[98] (DMSO)
2	159.0	157.28	3′	151.9	150.60
3	136.7	138.80	4′	139.4	137.70
4	179.7	177.80	5′	151.9	150.60
5	163.3	161.30	6′	109.8	108.10
6	100.0	98.75	1′′	103.7	102.12
7	166.3	164.35	2′′	71.9	70.03
8	94.8	93.60	3′′	72.1	70.31
9	158.6	156.50	4′′	73.1	71.13
10	105.9	104.18	5′′	72.1	70.53
1′	127.0	124.80	6′′	17.7	17.45
2′	109.8	108.10	-OCH_3	60.9	59.75

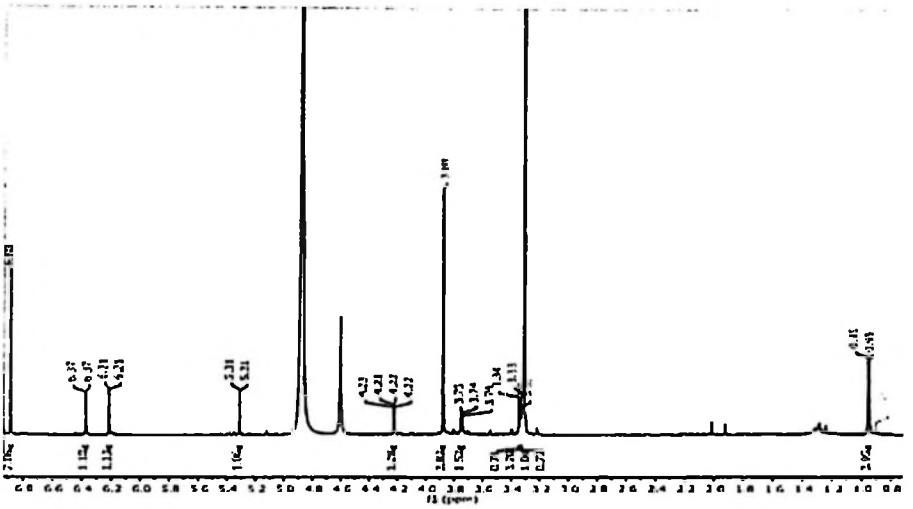


Figure 2.5: The ^{1}H NMR Spectrum of Mearnsetin-3-*O*-α-*L*-Rhamnopyranoside (2.26)

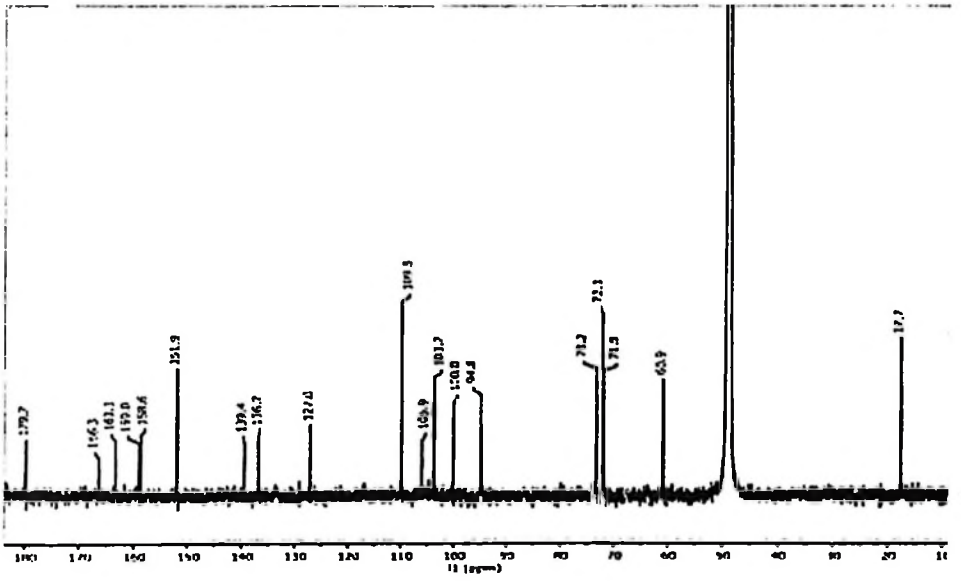


Figure 2.6: The ^{13}C NMR Spectrum of Mearnsetin-3-*O*-α-*L*-Rhamnopyranoside (2.26)

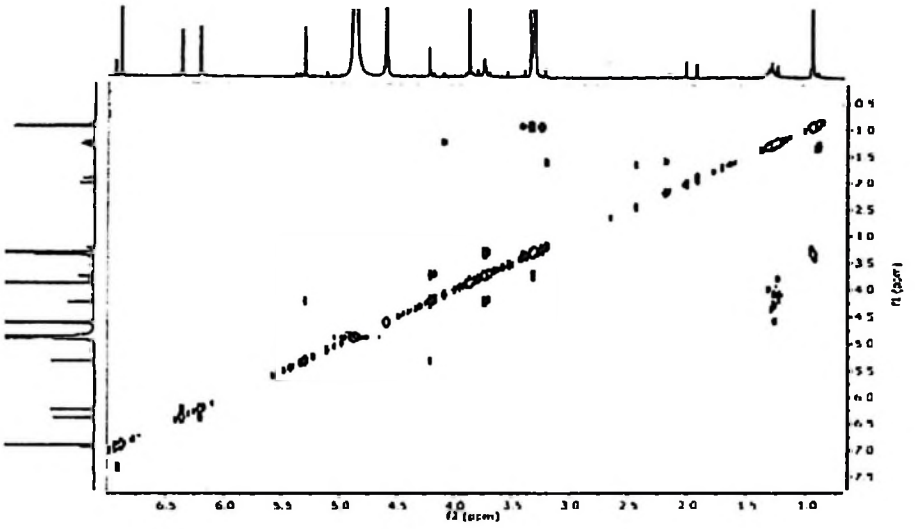


Figure 2.7: **H/H COSY Spectrum of Mearnsetin-3-*O*-α-*L*-Rhamnopyranoside (2.26)**

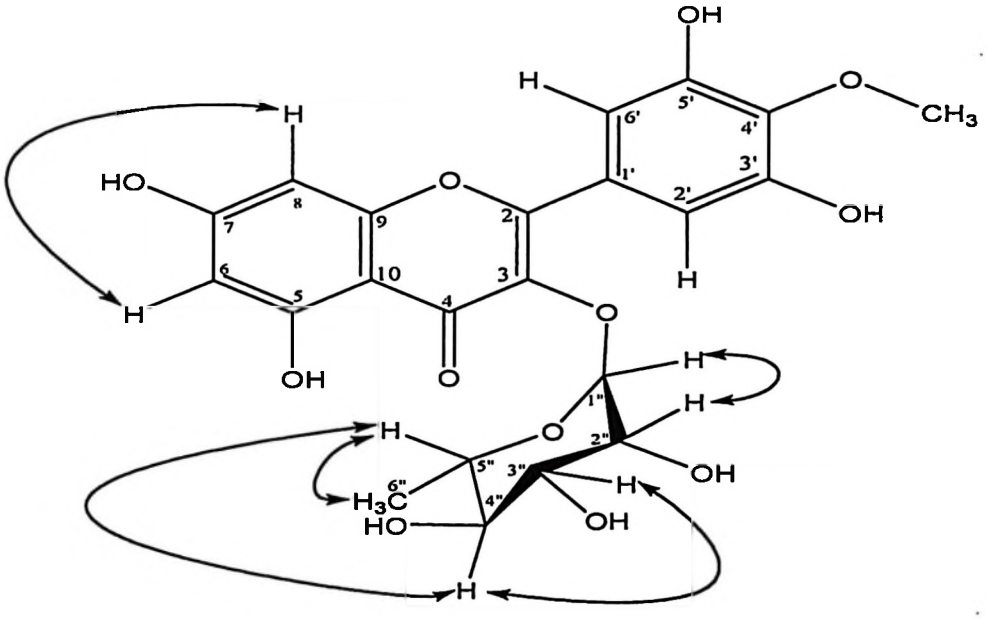


Figure 2.8: **H/H Interactions for Mearnsetin-3-*O*-α-*L*-Rhamnopyranoside (2.26)**

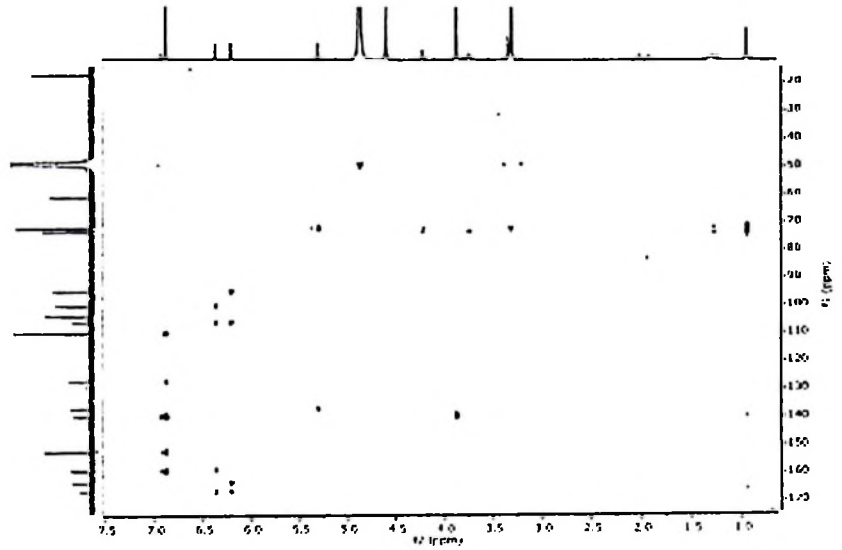


Figure 2.9: The HMBC Spectrum for Mearnsetin-3-*O*-α-*L*-Rhamnopyranoside (2.26)

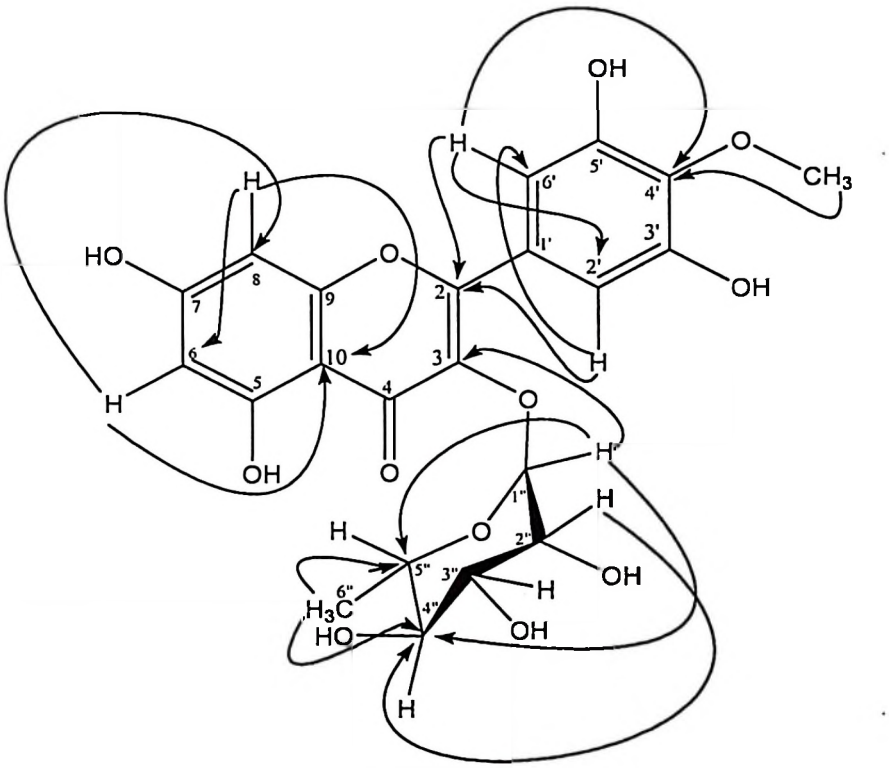


Figure 2.10: Important HMBC for Mearnsetin-3-*O*-α-*L*-Rhamnopyranoside (2.26)

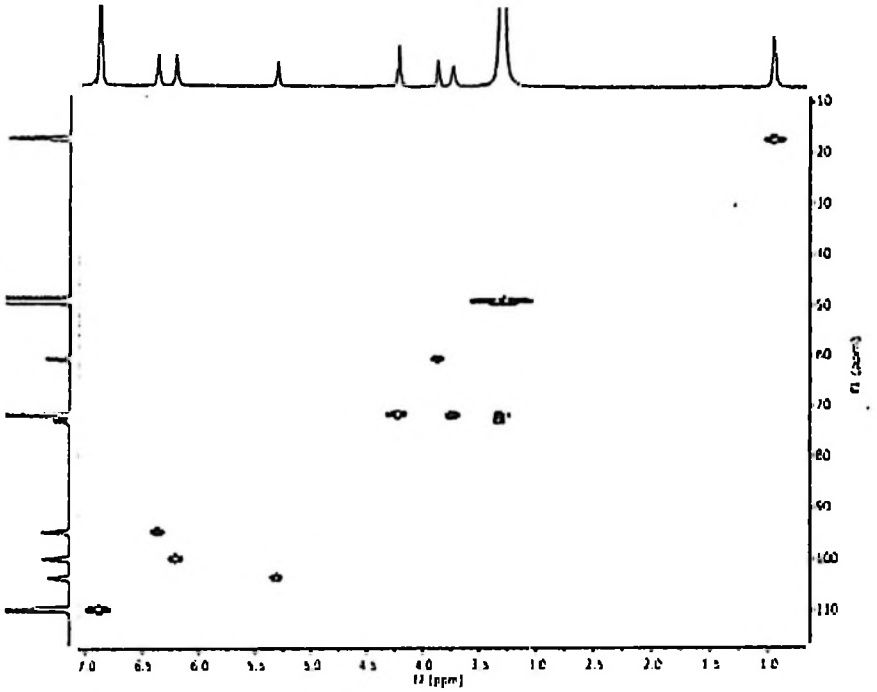
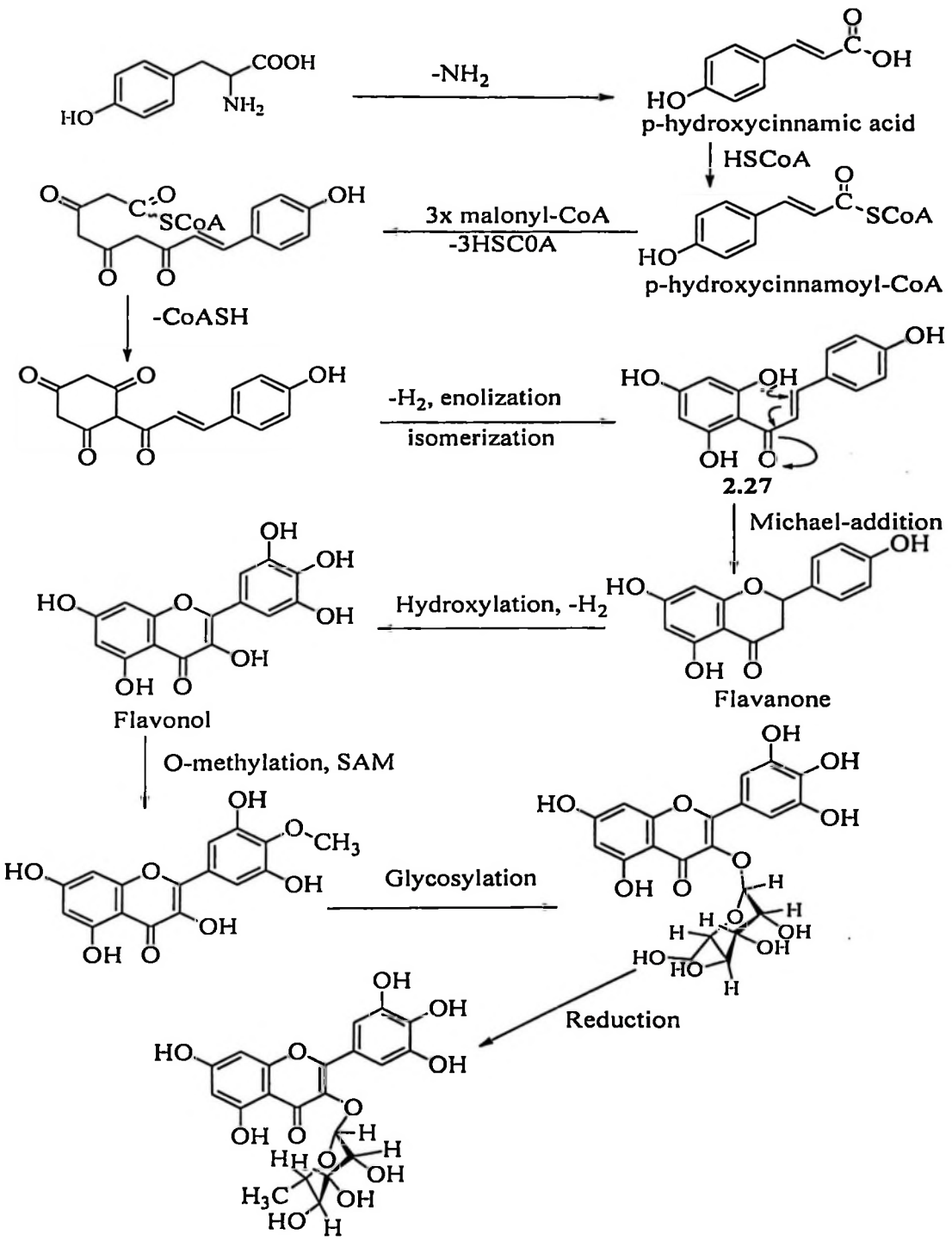
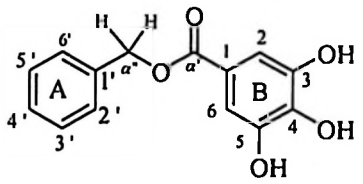


Figure 2.11: HSQC Plot for Mearnsetin-3-*O*-α-*L*-Rhamnopyranoside (2.26)



Scheme 2.2: Proposed Biosynthesis Pathway of Mearnsetin-3-*O*-α-*L* Rhamnopyranoside (2.26)

2.4.2 Benzyl-3,4,5-trihydroxybenzoate (2.28)

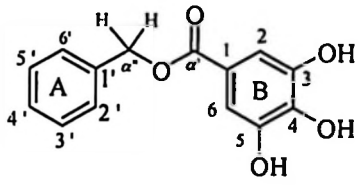


2.28

Repeated column chromatography of the ethanolic extract of the root barks of *Newtonia paucijuga* using silica gel and further purification by gel chromatography on Sephadex® LH-20 led to the isolation of benzyl-3,4,5-trihydroxybenzoate **(2.28)** as a yellow solid. The mass spectrum (Fig. 2.12) showed the presence of a [M+H] peak at *m/z* 261.4 which together with the NMR data were consistent with the molecular formula $C_{14}H_{12}O_5$. The compound was UV positive displaying deep blue spot which changed to red colour upon spraying with anisaldehyde followed by heating. This indicated the presence of aromatics or conjugated systems in the molecule.

The ^{1}H NMR spectrum (Fig. 2.13 and Table 2.3) showed the presence of aromatic proton signals that suggested the presence of two aromatic rings. Three signals in the aromatic region with overall integration of five protons appeared at δ_H 7.43 (2H, d, J = 7.1 Hz), 7.38 (2H, t, J = 7.6 Hz) and 7.32 (1H, t, J = 7.4 Hz) signified the presence of a monosubstituted benzene ring. These protons were assigned to H-2′/H-6′, H-3′/H-5′ and H-4′ positions, respectively. Their coupling constants indicated that, the neighboring protons were *ortho*-coupled to each other enabling their assignments to the respective positions. H/H COSY spectrum (Fig. 2.15 and 2.16) revealed the

2.4.2 Benzyl-3,4,5-trihydroxybenzoate (2.28)



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presence of the H/H interactions between H-2′/H-6′ and H-3′/H-5′ as well as H-4′ and H-3′/H-5′ (Fig. 2.15). Further analysis of the ^{1}H NMR spectrum revealed the presence of more deshielded aromatic protons at δ_H 7.89 (2H, s) which were assigned to H-2 and H-6 constituting part of another aromatic ring. The two protons at δ_H 5.30 that appeared as singlets were assigned to the oxygenated benzylic methylene group (H-α′′) of the compound **2.28**. The downfield shift experienced by the methylene protons was influenced by both the aromatic ring and the electronegative atom oxygen of the ester group. Proton at δ_H 6.77 was identified to be not the part of compound **2.28**.

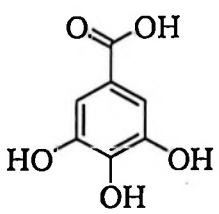
The ^{13}C NMR spectrum (Fig. 2.14 and Table 2.3) together with HSQC spectrum (Fig. 2.19) depicted the presence of five quaternary carbons. A signal at δ_C 168.3 was due to an ester functional group. Signals at δ_C 158.1 (C-3/C-5) and δ_C 154.0 (C-4) were attributed to oxygenated sp^2 carbons of the aromatic ring B. The signals at δ_C 138.0 and δ_C 119.6 were assigned to C-1′ and C-1 carbons. Furthermore, the ^{13}C NMR showed the presence of three signals due to methine carbons at δ_C 129.0, 129.6, and 129.1 which were assigned to the monosubstituted aromatic ring at C-2′/C-6′, C-3′/C-5′ and C-4′, respectively. Other methine carbons were characterized by the presence of a signal at δ_C 132.5 (C-2/C-6). The carbon signal at δ_C 67.2 was due to the methylene carbon at C-α′′, whose downfield shift suggested it was attached to oxygen. The information obtained from the HSQC assisted the complete assignment of the protons on their respective carbons. On the other hand, carbon at δ_C 81.8 did not form part of the idenefied structure of compound **2.28**.

The use of HMBC spectrum (Fig. 2.17) assisted the linking of fragments together through the long range H-C interactions of which the important interactions are depicted in Fig. 2.18. The spectrum indicated that, proton at signal δ_H 7.89 (H-2/H-6) interacts with the carbon at δ_C 168.3 (C=O) linking the aromatic ring B to the ester group of compound **2.28**. Further correlation was observed between the proton at δ_H 5.30 and the carbonyl carbon at δ_C 168.3 joining the methylene group to the ester group within the compound. The monosubstituted aromatic ring A was linked to methylene group of the molecule through the interaction between the methylene protons at δ_H 5.30 with the quaternary carbon at δ_C 138.0 (C-1′) enabling the deduction of the linkage of benzoate group to the benzyl group. Other correlations to complete the assignment of compound **2.28** were observed between δ_H 7.89 (H-2/H-6) and δ_C 132.5 (C-6/C-2), δ_H 7.38 (H-3′/H-5′), δ_H 5.30 (H-α′′) and δ_C 138.0 (C-1′), δ_H 7.38 (3′/H-5′) and δ_C 129.6 (C-5′/C-3′), δ_H 7.43 (H-2′/H-6′) and δ_C 67.2 (C-α′′), δ_H 7.89 (H-2/H-6) and δ_C 154 (C-4), δ_H 7.38 (H-3′/H-5′), δ_H 7.43 (H-2′/H-6′) and δ_C 129.1 (C-4′) and, δ_H 7.43 (H-2′/H-6′), δ_H 7.32 (H-4′) and δ_C 129.0 (C-6′/C-2′).

The isolated compound was identified as benzyl-3,4,5-trihydroxybenzoate (**2.28**) being previously only reported as a synthetic compound.[109,110] Thus, study on the chemoselective esterification of phenolic acids in the presence of sodium bicarbonate led to the synthesis of benzyl-3,4,5-trihydroxybenzoate (**2.28**).[110] Furthermore, compound **2.28** has been synthesized as a derivative of β-resorcylic acids and gallic acids.[109] The synthesized compound **2.28** was evaluated for antioxidant and antiparkinson activities.[109] This is the first report on the isolation of compound **2.28**

from nature, thus its isolation from the roots barks of *N. paucijuga* is hereby reported for the first time.

Benzyl-3,4,5-trihydroxybenzoate (**2.28**) is considered as a derivative of gallic acid (**2.29**). Gallic acid derivatives with interesting structures have been reported from various plant species and are acknowledged to have potent biological activitie.[111] Thus, gallic acid derivatives such as methyl stringate (**2.30**), 3,4,5-triomethylgallic acid (**2.31**) and 3,4-dimethoxy-5-hydroxylbenzoic acid (**2.32**) with potent antioxidant activities were obtained from *Tapinanthus bangwensis* (Loranthaceae).[112] 2-methoxybenzyl-2-hydroxybenzoate (**2.33**) was obatained from *Salidago virga-aurea* (Compositae). Compound **2.33** showed good immunostimulatory activity and considered as good candidate for antitumoral therapy.[113] From *Cleistochlamys kirkii* (Annonaceae), cleistophenolide (**2.34**) with potent antiplasmodial activities against *P. falciparum* was isolated.[114] Furthermore, an antiviral agent against infectious bursal disease virus and Newcastle disease virus namely ethyl gallate (**2.35**) was isolated from *Toussaintia patricine* (Annonaceae).[115] Therefore, the isolation from the plant species of compounds with structures related to benzyl-3,4,5-trihydroxybenzoate (**2.28**) ascertain the proof that such compounds are metabolized by the plant species and hence its isolation from *N. paucijuga* was further supported.



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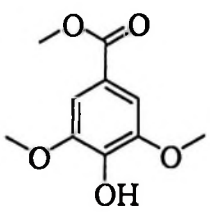

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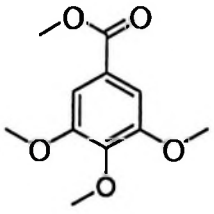

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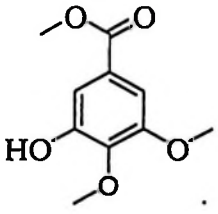

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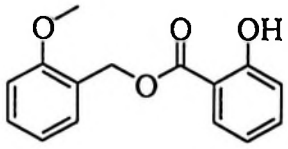

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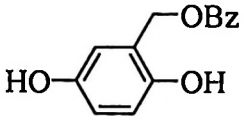
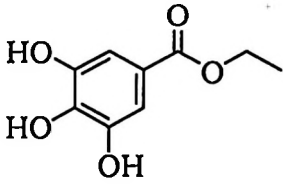


2.34



2.35

Like other gallic acid derivatives, the synthetic benzyl-3,4,5-trihydroxybenzoate (**2.28**) exhibited potent antiparkinson and antioxidant activities.[109] The bioactivities of various gallic acid derivatives are associated by the presence of benzyl moieties and free hydroxyl groups.[109,116] The presence of benzyl moiety and free hydroxyl groups attached to the benzoate ring in the benzyl-3,4,5-trihdroxybenzoate (**2.28**) influences its biological activities and that the compound might be among those candidates with significant biological activities for correcting human ailments in future.

Biosynthetically, benzyl-3,4,5-trihydroxybenzoate (**2.28**) is considered to be synthesized through shikimate pathway, a pathway which serves as the alternative route for formation of aromatic compounds such *L*-phenylalanine, *L*-tyrosine and L-tryptophan and their several biosynthetic products.[117] Thus, the biosynthesis of compound **2.28** utilizes the *L*-phenylalanine which undergoes a series of reaction to obtain a benzoic acid (**2.36**) which is a precursor of various benzyl derivatives.[117] Benzoic acid (**2.36**) is then firstly reduced to benzyladehyde, followed by reduction of the aldehyde to benzyl alcohol,[118] which is subsequently esterified to with gallic acid (**2.29**), another biosynthetic product of shikimate pathway to form benzy-3,4,5-trihydroxybenzoate (**2.28**) as showed in scheme 2.3.

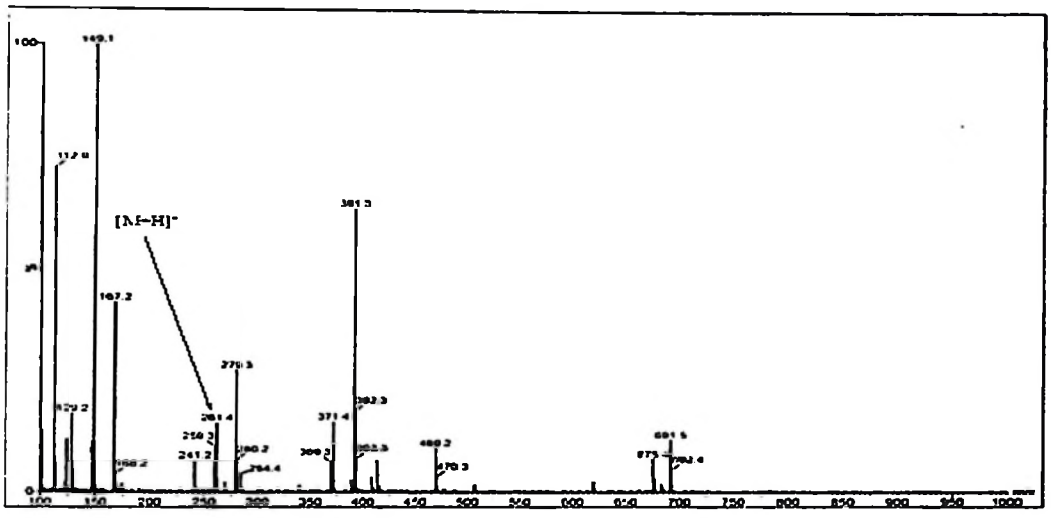


Figure 2.12: LC-Mass Spectrum of Benzyl-3,4,5-Trihydroxybenzoate (2.28)

Table 2.3: ^{1}H,^{13}C NMR Spectroscopic Data, COSY and HMBC for Benzyl-3,4,5-Trihydroxylbenzoate (2.28)

H/C	δ_H	*Mult.*	*J (Hz)*	δ_C	H/H COSY	HMBC H→C
1	--	--	--	119.6	--	--
2	7.89	s	--	132.5	--	α′,4′4,6
3	--	--	--	158.1	--	--
4	--	--	--	154.0	--	--
5	--	--	--	158.1	--	--
6	7.89	s	--	132.5	--	α′,2,4′,4
1′	--	--	--	138.0	--	--
2′	7.43	d	7.1	129.0	2′, 3′	α″,4′,6′
3′	7.38	t	7.6	129.6	3′, 2′/4′	1′,4′,5′
4′	7.32	t	7.4	129.1	4′, 3′/5′	2′,6′
5′	7.38	t	7.6	129.6	5′, 4′/6′	1′,3′,4′
6′	7.43	d	7.1	129.0	6′, 5′	α″,2′,4′
α′	--	--	--	168.3	--	--
α″	5.30	s	--	67.2	--	α′,1′

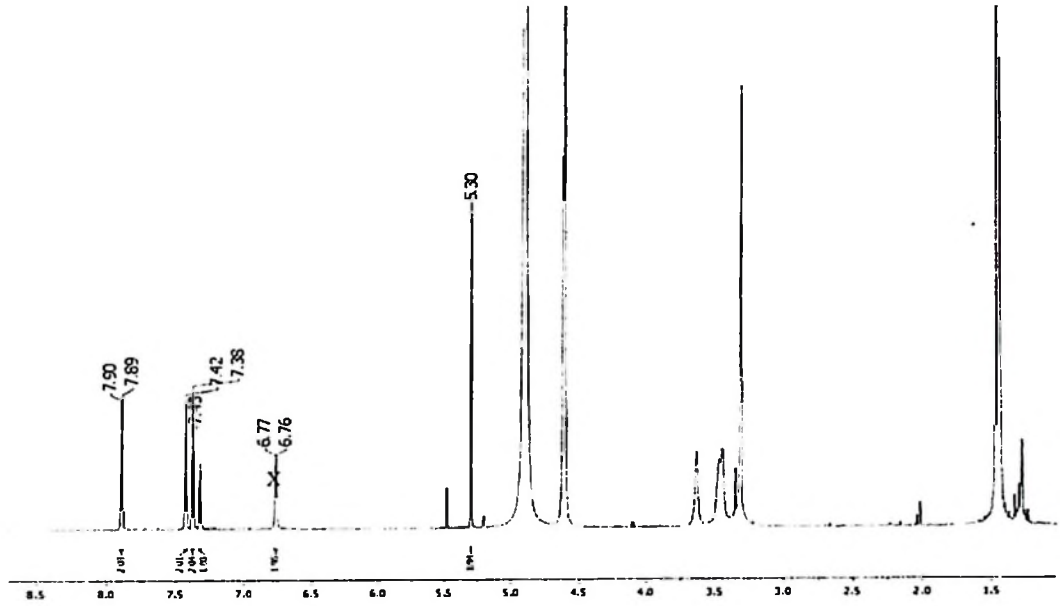


Figure 2.13: ^{1}H NMR Spectrum of Benzyl-3,4,5-Trihydroxybenzoate (2.28)

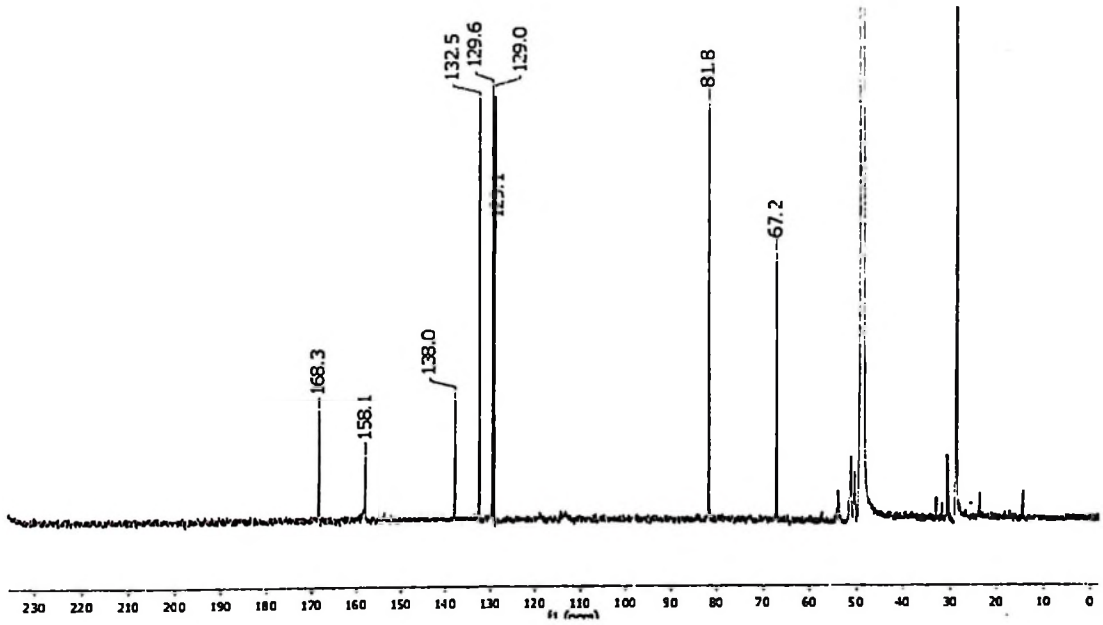


Figure 2.14: ^{13}C NMR Spectrum of Benzyl 3,4,5-Trihydroxybenzoate (2.28)

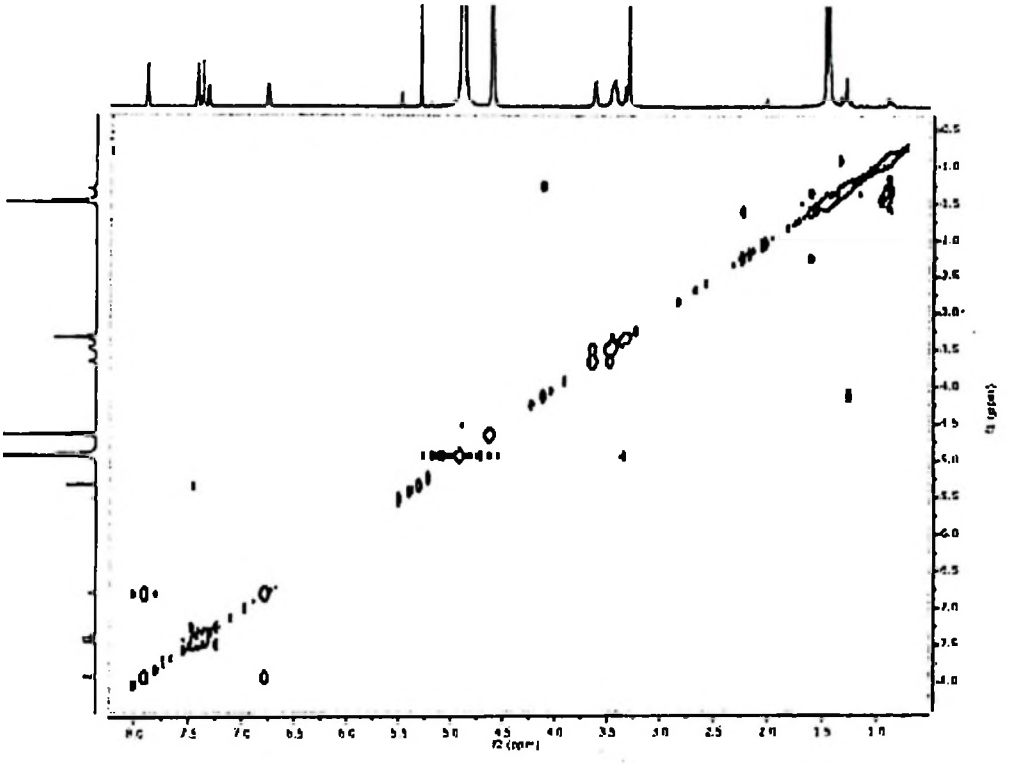


Figure 2.15: H/H COSY Spectrum of Benzyl-3,4,5-Trihydroxybenzoate (2.28)

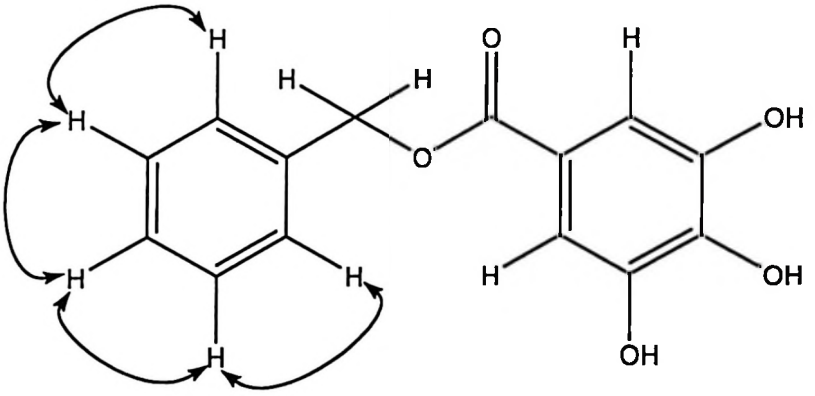


Figure 2.16: H/H Interactions of Benzyl-3,4,5-Trihydroxybenzoate (2.28)

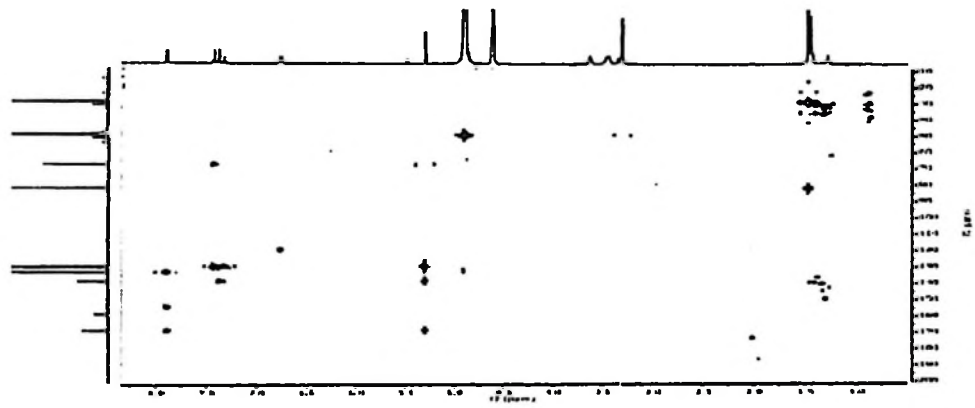


Figure 2.17: The HMBC Spectrum of Benzyl-3,4,5-Trihydroxybenzoate (2.28)

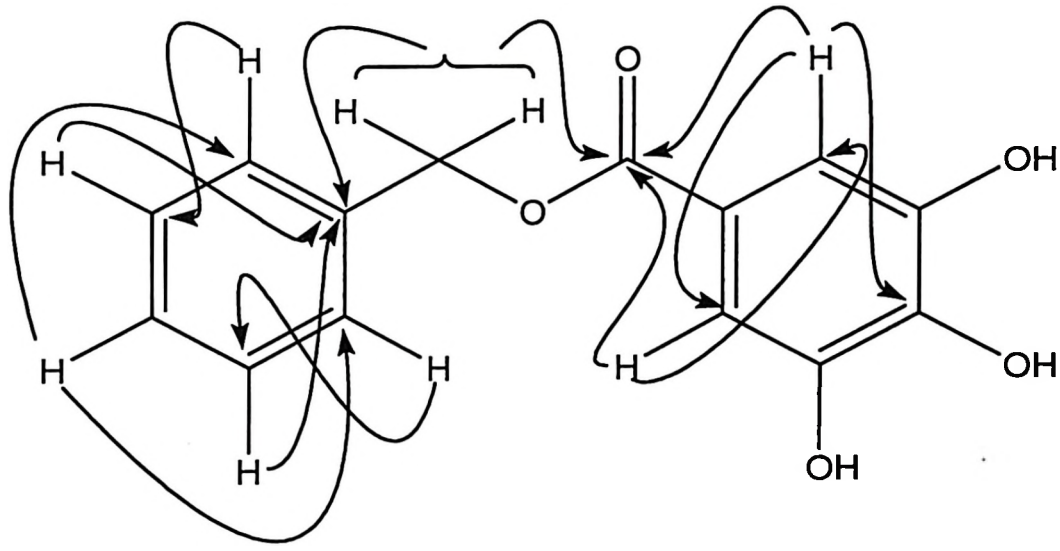


Figure 2.18: Some Important HMBC for Benzyl-3,4,5-Trihydroxy benzoate (2.28)

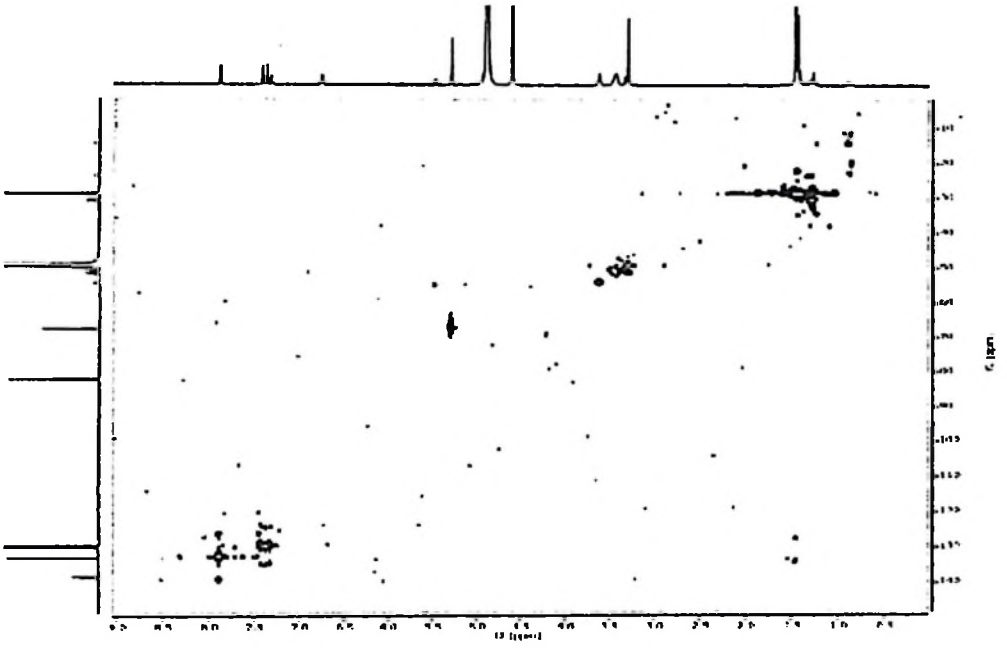
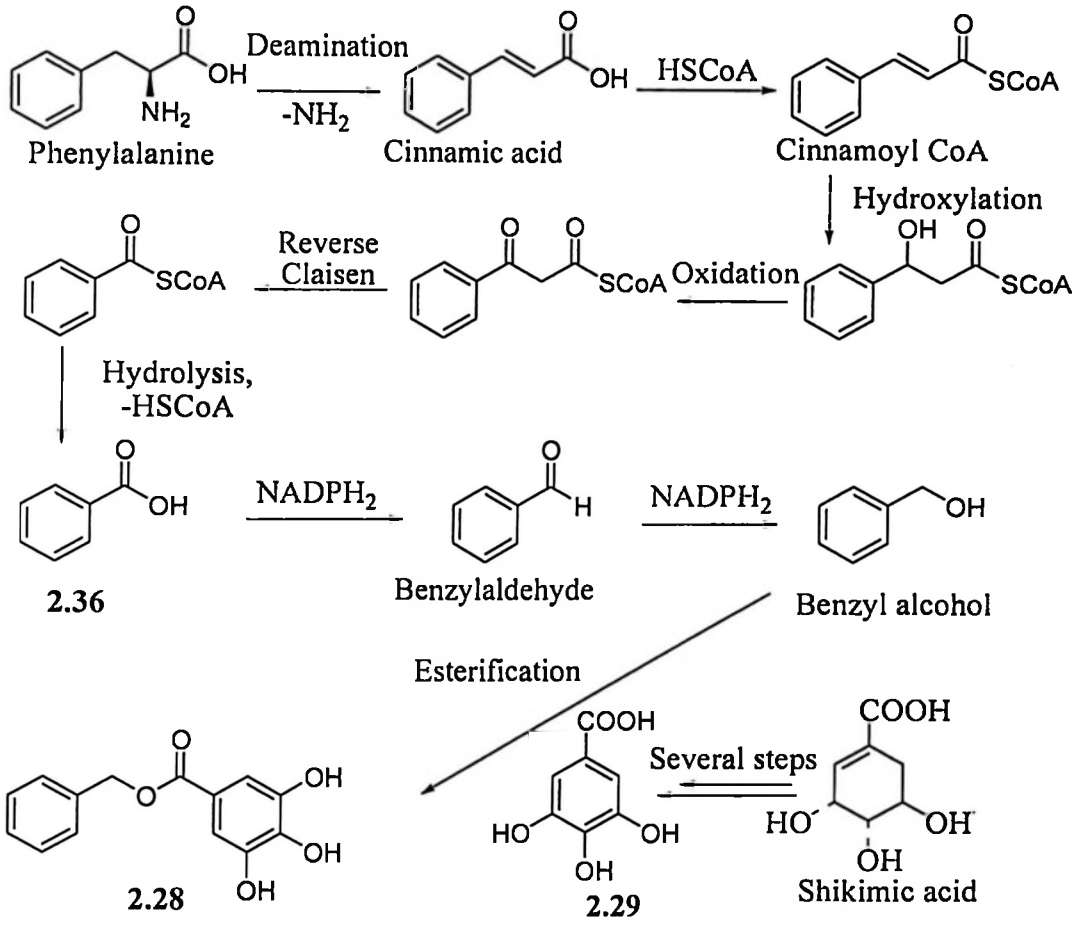
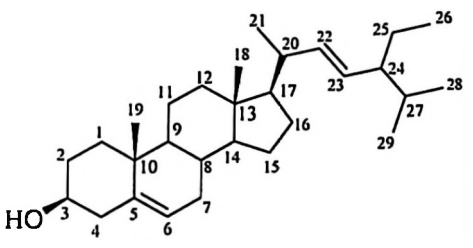


Figure 2.19: The HSQC Spectrum of Benzyl-3,4,5-Trihydroxybenzoate (2.28)

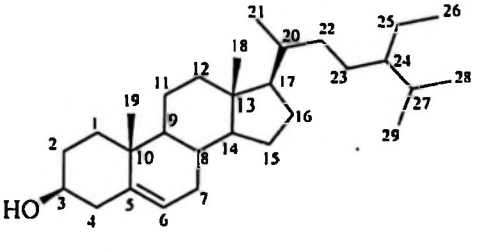


Scheme 2.3: Proposed Biosynthesis Pathway of Benzyl-3,4,5-Trihydroxy benzoate (2.28)

2.4.3 Stigmasterol (2.37a) and β-sitosterol (2.37b)



2.37a



2.37b

Repeated column chromatography of the combined fractions 3-6 obtained from the ethanolic extract of the stem barks of *N. paucijuga* led to the isolation of a mixture of phytosterols namely, stigmasterol (**2.37a**) and β-sitosterol (**2.37b**) as a white solid. Compounds **2.37a** and **2.37b** were established based on the spectroscopic data and with the comparison to the literature.[119,120] The mass spectrum (Fig. 2.20, 2.21a and 2.21b) displayed $[M-H_2O+H]^+$, $[M-H_2O-H]^+$ peaks at *m/z* 393.3, *m/z* 397.1 and the hydrated molecular ion peak at *m/z* 412 and *m/z* 414 which together with the NMR data were consistent with molecular formulae $C_{29}H_{48}O$ and $C_{29}H_{50}O$ for compound **2.37a** and **2.37b**, respectively. The UV visualization indicated the absence of the spot but blue colour was displayed after treatment with anisaldehyde spraying agent suggesting the presence of steroids. The IR spectrum (Fig. 2.22) indicated absorptions due to OH (3346.99 cm^{-1}), aliphatic C-H (2940.87 cm^{-1} and 2834.46 cm^{-1}), C=C (1647.50 and 1449.52 cm^{-1}) and C-O (1020.52 cm^{-1}).[99,121]

The ^1H NMR spectrum (Fig. 2.23 and Table 2.4) of the compounds **2.37a** and **2.37b** showed the presence of a deshielded signal at δ_H 5.35 (1H, m) which was due to an olefinic proton H-6 and signal at δ_H 3.52 (1H, tt, J = 10.8, 4.6 Hz) was assigned to H-3 of both compound **2.37a** and **2.37b**. Basing on the coupling constant (J = 10.8 Hz), H-3 was assigned to an axial position in respect to hydroxyl group attached to C-3, while coupling with protons H-4 and H-2 were suggested to be axial/axial and axial/equatorial couplings, respectively. The spectrum also revealed the presence of a pair of doublet of doublets signals at δ_H 5.15 (J = 15.2, 8.6 Hz, 1H) and δ_H 5.01 (J = 15.2, 8.6 Hz, 1H) which were assigned to unsaturated methine protons as H-22 and H-23 of the compound **2.37a** suggesting the two protons are *trans*-oriented.

Furthermore, six signals due to methyl protons appeared at δ_H 1.01 (3H, s), 0.92 (3H, d, J = 6.6 Hz), 0.84 (3H, m), 0.83 (3H, m) 0.81 (3H, m) and 0.68 (3H, s) and were assigned to H-19, H-21, H-26, H-29, H-28 and H-18, respectively. The proton-proton couplings were also correlated by the COSY spectrum (Figs. 2.25, 2.26 and 2.27). The correlation between δ_H 1.84 (H-1) and δ_H 1.26 (H-2), δ_H 1.84 (H-2) and δ_H 3.52 (H-3), δ_H 3.52 (H-3) and δ_H 2.28 (H-4), δ_H 5.35 (H-6) and δ_H 1.98 (H-7), δ_H 1.16 (H-16) and δ_H 1.15 (H-17), δ_H 2.01 (H-20) and δ_H 5.15 (H-22), δ_H 5.15 (H-22) and δ_H 5.01 (H-23), δ_H 5.01 H-23) and δ_H 1.51 (H-24), δ_H 2.33 facilitated the complete assignment of the protons in the structures of compounds **2.37a** and **2.37b**.

The ^{13}C-NMR spectrum (Fig. 2.24 and Table 2.5) consisted of 31 carbon signals which agreed with the proposed structures that the isolated compound was a mixture of **2.37a** and **2.37b** with 29 carbons each according to the correlation showed by the HSQC spectrum (Fig. 2.28). The ^{13}C-NMR spectrum further revealed the presence of C-C double bonds which were characterized by the presence of deshielded signals at δ_C 140.9 (Cq), 138.5 (CH), 129.4 (CH), and 121.9 (CH).[119,120] The resonance at δ_C 72.0 was due to β-hydroxyl group at C-3.[119,120,122] Furthermore, two angular methyl carbon of the resonance at δ_C 12.0 and 19.6 which were assigned to C-18 and C-19, respectively were observed and, that their values were closely related to other values reported previously.[120] Further close analysis of the spectrum revealed that, the chemical shifts of the carbons of compound **2.37a** and **2.37b** were similar, but differed at carbons 22 and 23. That, the C-C double bond (δ_C 138.5 and 129.4) and C-C single bond (δ_C 34.1 and 29.2) at the C-22 and C-23 are responsible for the differences between **2.37a** and **2.37b**, respectively. Like other phytosterols isolated

from other plants species, compound **2.37a** and **2.37b** each has 29 carbons of which, they are categorized to six methyl carbons, three quaternary carbons, eleven methine carbons and nine methylene carbons for **2.37a**, while six methyl carbons, three quaternary carbons, nine methine carbons and eleven methylene carbons were identified for compound **2.37b**.[119,120,123,124]

The assignment of H/C long-range correlations was depicted by the HMBC spectrum (Fig. 2.29). The spectrum showed the presence of correlations between δ_H 1.84 (H-1) to δ_C 72.0 (C-3) and δ_C 140.9 (C-5), δ_H 2.28 (H-4) to δ_C 28.4 (C-2) and δ_C 121.9 (C-6), δ_H 5.35 (H-6) to δ_C 31.8 (C-8), δ_H 1.98/1.48 (H-7) to δ_C 140.9 (C-5), δ_H 5.15 (H-22) to δ_C 51.4 (C-24) (Fig. 2.30a and Fig. 2.30b).

Compound **2.37a** and **2.37b** are mostly isolated in mixture form with high proportion compound **2.37a** in most cases.[125] It is difficult to obtain the two in their pure form due to similarities in polarity and molecular size. Like other phytosterols, compound **2.37a** and **2.37b** are ubiquitous to most plant species and reported to resemble to cholesterol in both structure and biological activities.[119,120,125,126,127] Stigmasterol (**2.37a**) and β-sitosterol (**2.37b**) isolated from *Indigofera hererantha* (Fabaceae) exhibited good membrane stabilization and antidiabetic activities against human red blood cells (HRBC) and glucose uptake, respectively with minimum stabilization of 62.74% at 10 μg/mL and maximum stabilization of 65.19% at 40 μg/mL, and minimum increase 66.48% at 10 μg/mL and maximum increase 74.46% at 80 μg/mL glucose concentration, respectively.[128] Moreover, the antimicrobial activities of the two compounds were previously reported. Stigmasterol (**2.37a**) and β-sitosterol

(**2.37b**) isolated from *Bulbine natalensis* (Asphodelaceae) were active at 0.16 µg/mL and 0.07 µg/mL, respectively against *Aspergillus flavus*.[129] Other study on the roots of *Malva parviflora* isolated β-sitosterol (**2.37b**) which inhibited the growth of *S. aureus* and *E. coli* with the inhibition zone of 17 ± 0.5 mm and 14.5 ± 1.84 mm, respectively.[130] Furthermore, study on the roots of *Caylusea abyssinica* (Fresen) isolation of stigmasterol (**2.37a**) and β-sitosterol (**2.37b**) which were active against *S. aureus*, *E. coli*, *P. aeruginosa* and *S. typhimurium* with an inhibition zone of 12, 18, 11, 13 mm and 15, 13, 13, 12 mm, respectively.[131] This indicated that, compound **2.37a** and **2.37b** are good candidates as antimicrobial agents and hence can be incorporated in medication formulation.

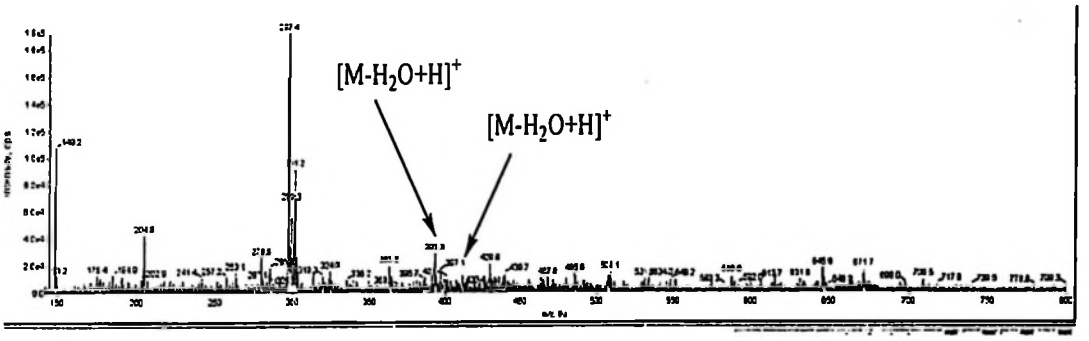


Figure 2.20: The LC-Mass Spectrum of Stigmasterol (2.37a) and β-sitosterol (2.37b)

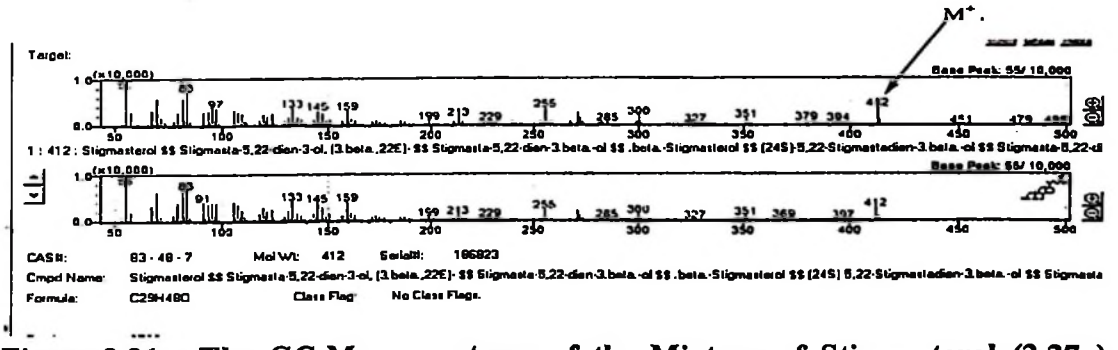


Figure 2.21a: The GC-Mass spectrum of the Mixture of Stigmasterol (2.37a) and β-sitosterol (2.37b)

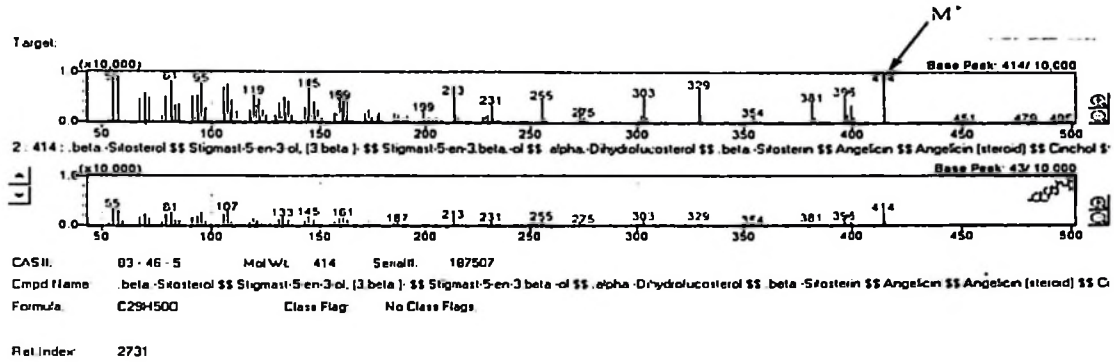


Figure 2.21b: The GC-Mass spectrum of the Mixture of Stigmasterol (2.37a) and β-sitosterol (2.37b)

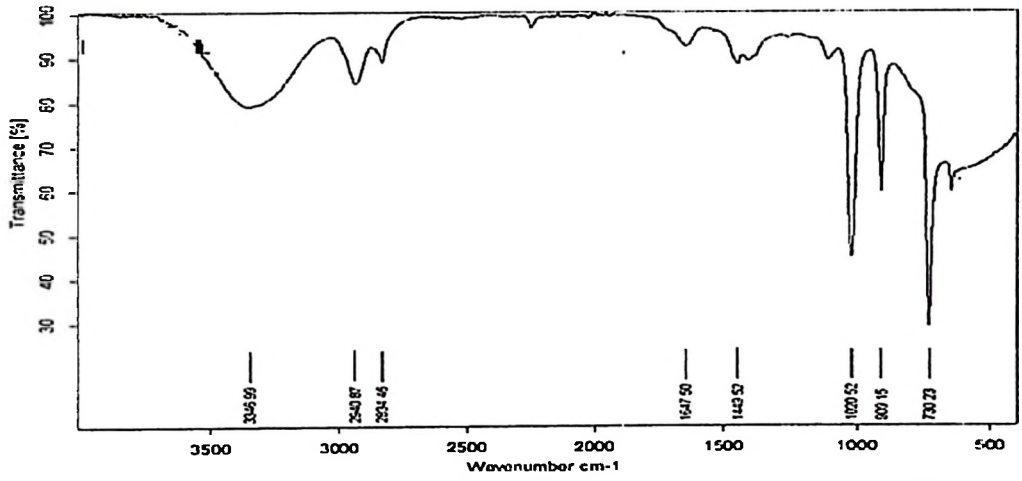


Figure 2.22: IR Spectrum for the Mixture of Stigmasterol (2.37a) and β-sitosterol (2.37b)

Table 2.4: ^{1}H **NMR Spectroscopic Data for Stigmasterol (2.37a) and β-sitosterol (2.37b)**

H	Stigmasterol				β-Sitosterol			
	Observed		Reported[119]		Observed		Reported[119]	
	δ_H	Mult. *J* (Hz)	δ_H	Mult.	δ_H	Mult. *J* (Hz)	δ_H	Mult.
1	1.84 1.08	m m	1.84 1.30	m m	1.84 1.08	m m	1.84 1.30	m m
2	1.84 1.26	m	1.50	m	1.84 1.26	m	1.50	m
3	3.52	tt, 10.8,4.6	3.50	m	3.52	tt, 10.8, 4.6	3.50	m
4	2.28	m	2.27	m	2.28	m	2.27	m
5	---	---	---	---	---	---	---	---
6	5.35	m	5.34	dd	5.35	m	5.34	dd
7	1.98 1.48	m m	1.98	m	1.98 1.48	m m	1.98	m
8	1.84	m	1.70	m	1.84	m	1.70	m
9	0.92	d, 6.6	0.92	m	0.92	d, 6.6	0.92	m
10	---	---	---	---	---	---	---	---
11	1.48		1.50	m	1.48		1.50	m
12	2.01 1.16	m m	2.01	m	2.01 1.16	m m	2.01	m
13	---	---	---	---	---	---	---	---
14	1.01	m	1.10	m	1.01	m	1.10	m
15	1.26	m	1.09 1.70	m m	1.26	m	1.09 1.70	m m
16	1.16	m	1.70	m	1.16	m	1.70	m
17	1.15	m	1.20	m	1.15	m	1.20	m
18	0.68	s	0.68	s	0.68	s	0.68	s
19	1.01	s	1.01	s	1.01	s	1.01	s
20	2.01	m	1.28	m	2.01	m	1.28	m
21	0.92	d, 6.6	0.92	s	0.92	d, 6.6	0.92	s
22	5.15	dd, 15.2, 8.6	5.01	dd	2.33 1.01	m m	1.20	m
23	5.01	dd, 15.2, 8.6	5.16	dd	1.66 1.26	m m	1.70	m
24	1.51	m	1.50	m	1.51	m	1.50	m
25	1.58	m	1.20	m	1.58	m	1.20	m
26	0.84	m	0.83	d	0.84	m	0.83	d
27	1.25	m	1.90	m	1.25	m	1.90	m
28	0.81	m	0.83	d	0.81	m	0.83	d
29	0.83	m	0.83	d	0.83	m	0.83	d

Table 2.5: **^{13}C NMR Spectroscopic Data for Stigmasterol (2.37a) and β-sitosterol (2.37b)**

C	Stigmasterol		β-Sitosterol	
	δ_C		δ_C	
	Observed	Reported[119]	Observed	Reported[119]
1	37.4	37.4	37.4	37.4
2	28.4	28.4	28.4	28.4
3	72.0	71.9	72.0	71.9
4	42.4	42.5	42.4	42.5
5	140.9	140.9	140.9	140.9
6	121.9	121.9	121.9	121.9
7	32.0	32.0	32.0	32.0
8	31.8	32.1	31.8	32.1
9	50.3	50.3	50.3	50.3
10	36.7	36.7	36.7	36.7
11	21.2	20.0	21.2	20.0
12	39.9	39.9	39.9	39.9
13	46.0	46.0	46.0	46.0
14	56.9	56.9	56.9	56.9
15	23.1	23.1	23.1	23.1
16	26.2	26.3	26.2	26.3
17	56.2	56.2	56.2	56.2
18	12.0	12.0	12.0	12.0
19	19.6	19.6	19.6	19.6
20	39.9	40.6	39.9	40.6
21	18.9	19.9	18.9	19.9
22	138.5	138.5	34.1	33.9
23	129.4	129.4	29.2	28.3
24	51.4	51.4	51.4	51.4
25	24.5	24.5	24.5	24.5
26	12.1	12.3	12.1	12.3
27	29.9	29.3	29.9	29.3
28	19.2	19.8	19.2	19.8
29	20.0	21.2	20.0	21.2

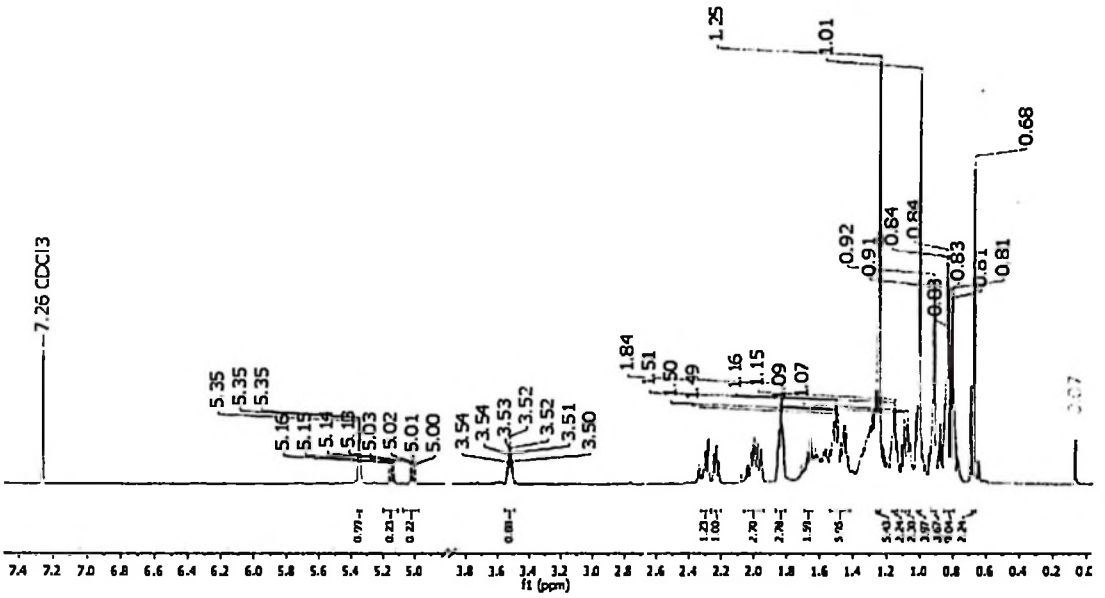


Figure 2.23: ^{1}H NMR Spectrum of the Mixture of Stigmasterol (2.37a) and β-sitosterol (2.37b)

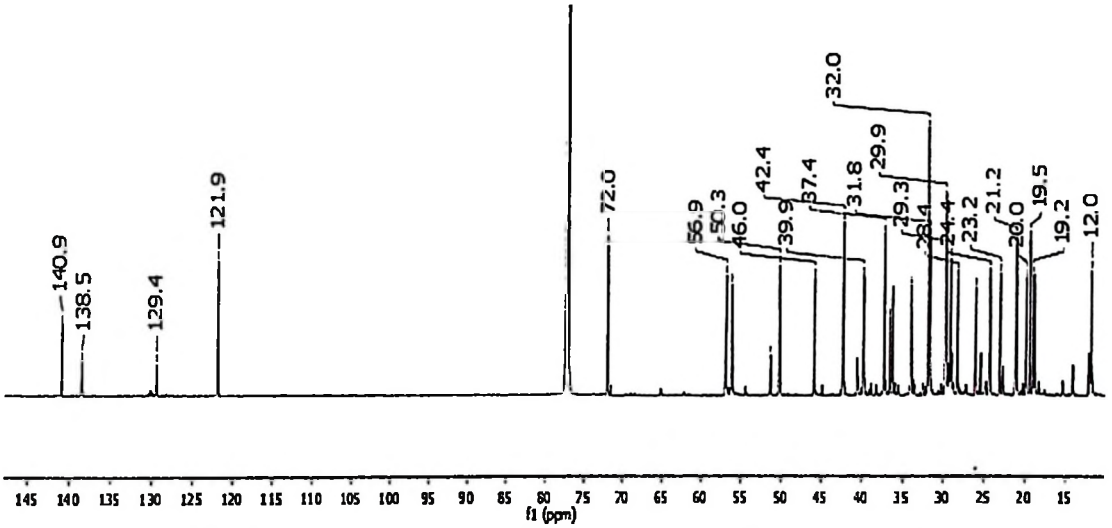


Figure 2.24: ^{13}C NMR Spectrum of the Mixture of Stigmasterol (2.37a) and β-sitosterol (2.37b)

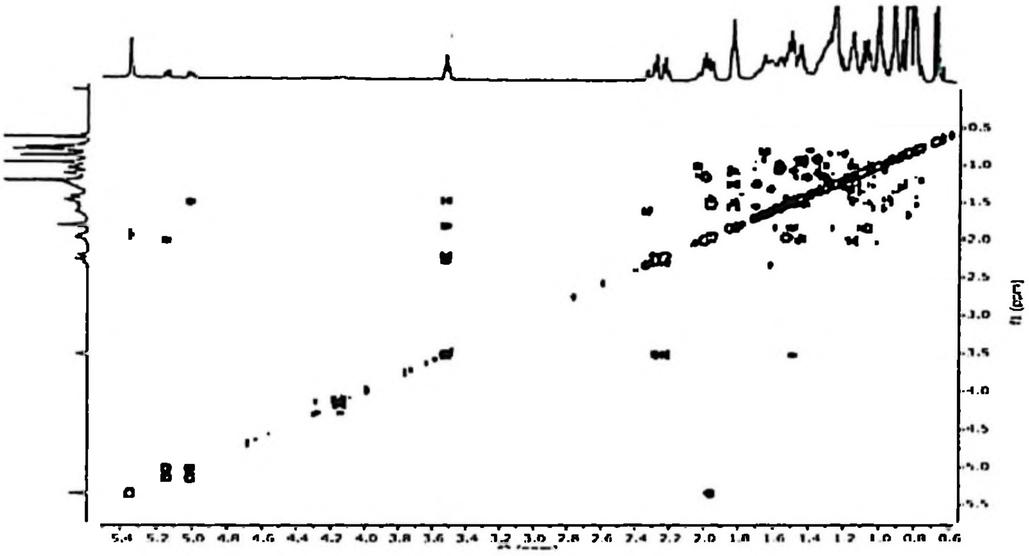


Figure 2.25: H/H COSY Plot for Stigmasterol (2.37a) and β-sitosterol (2.37b)

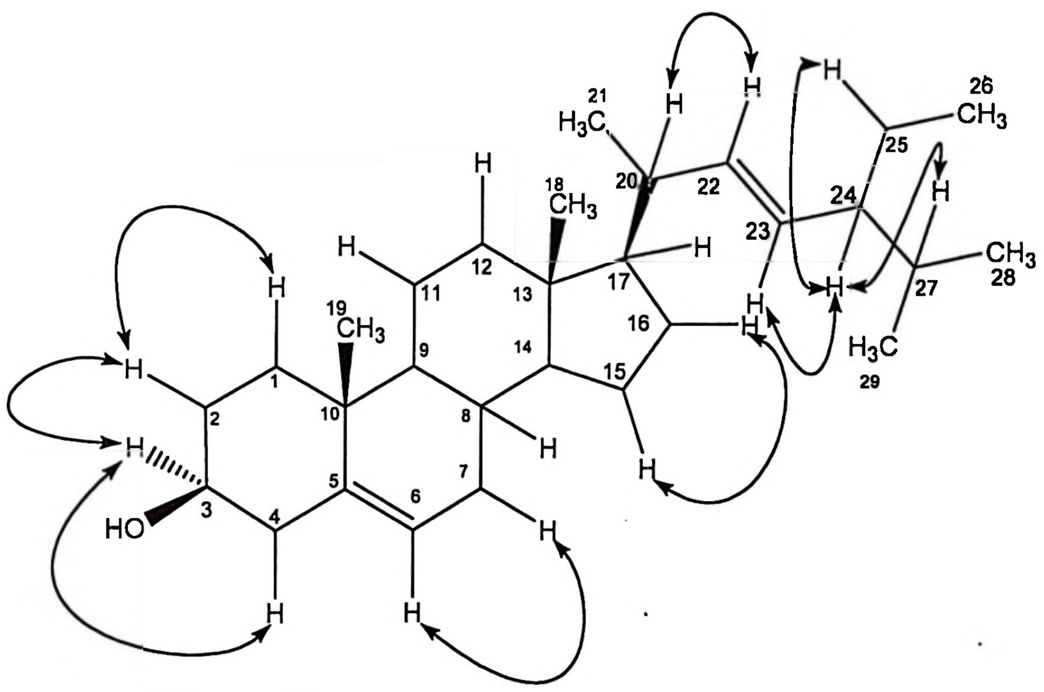


Figure 2.26: Some H/H Interactions for Stigmasterol (2.37a)

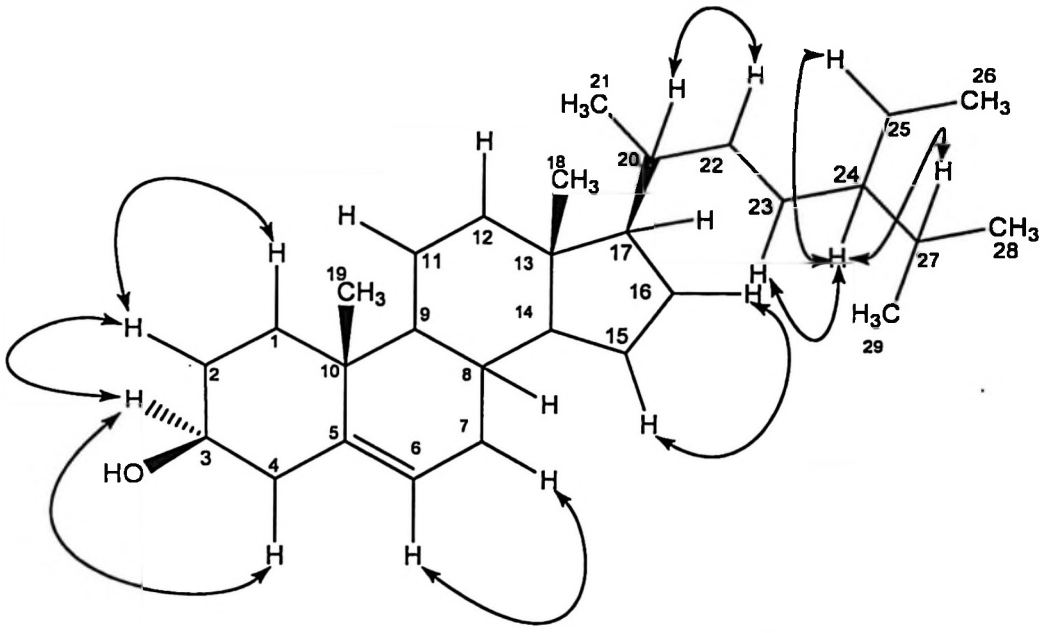


Figure 2.27: Some H/H Interactions for β-sitosterol (2.37b)

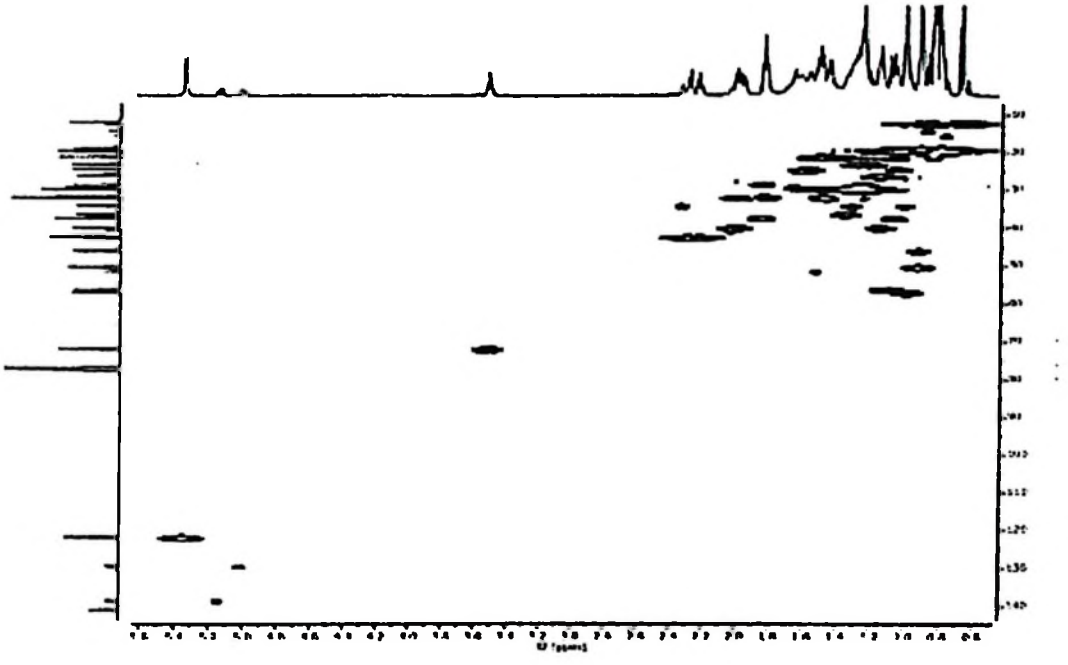


Figure 2.28: HSQC Spectrum for the Mixture of Stigmasterol (2.37a) and β-sitosterol (2.37b)

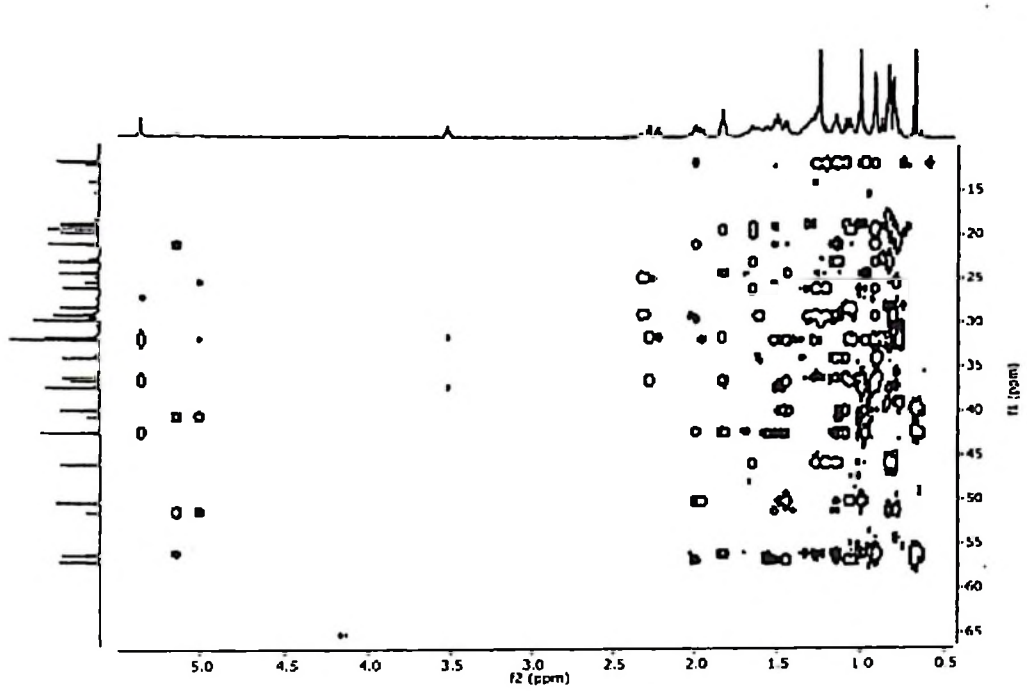


Figure 2.29: HMBC Plot for the Mixture of Stigmasterol (2.37a) and β-sitosterol (2.37b)

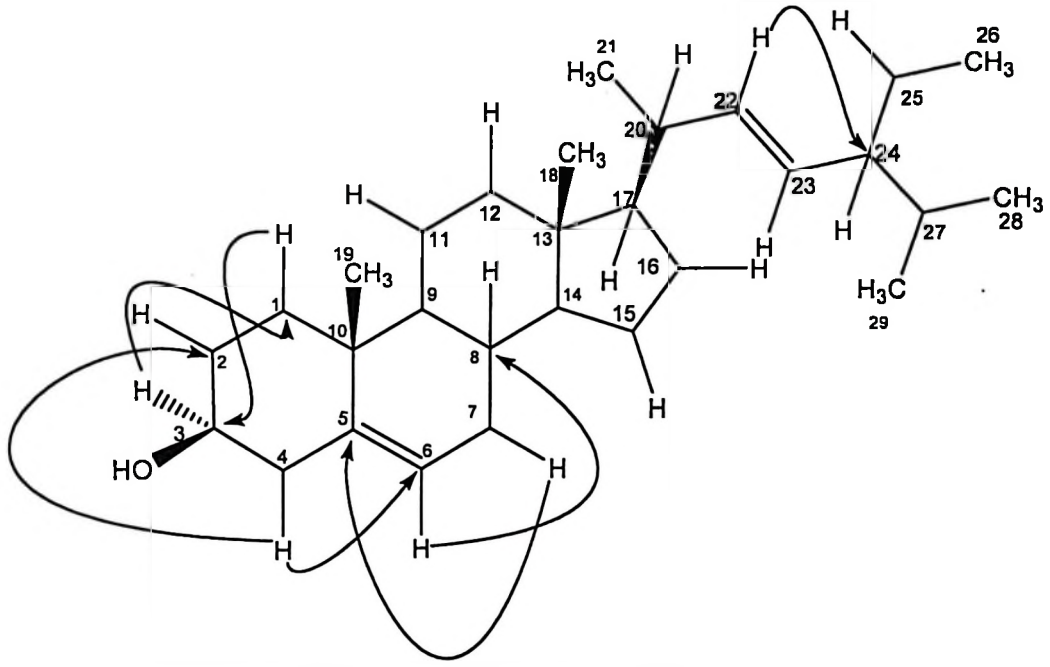


Figure 2.30a: Some HMBC for Stigmasterol (2.37a)

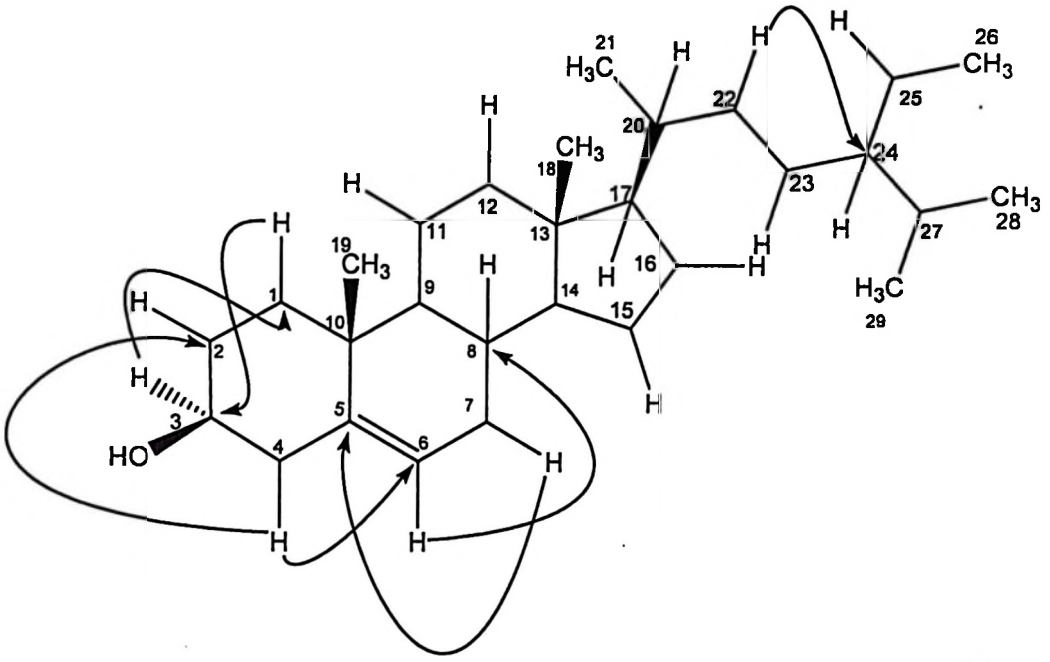
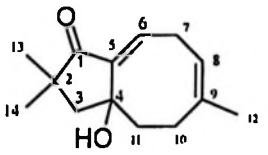


Figure 2.30b: Some HMBC for β-sitosterol (2.37b)

2.4.4 Sacleuxenone (2.38)



2.38

The repeated silica gel column chromatography of the ethanol extracts of the leaves of the *Erythrina sacleuxii* followed by purification using Sephadex® LH-20 afforded compound **2.38** as a white solid. The mass spectrum (Fig. 2.31) displayed the molecular ion peak $[M+H]^+$ at *m/z* 221.2 which together with the NMR data corresponded to the molecular formula $C_{14}H_{20}O_2$. The optical rotation, $[\alpha]_D$ at 20 °C of compound **2.38** was -57.5° (c 0.0013,$CHCl_3$). The IR spectrum (Fig. 2.32) of compound **2.38** consisted of broad band at 3346.81 cm^{-1} attributed to OH absorption. In addition, the spectrum showed the absorption at 2947.32 and 2835.07 cm^{-1} for

aliphatic C-H stretches. A weak absorption at 1650.46 cm^{-1} was due to both C=O and C=C α,β - conjugated functional groups. Furthermore, the absorption at 1017.00 cm^{-1} was ascribed to C-O functional group. Other absorptions were observed at 1449.58 cm^{-1} indicating the presence of the C=C group in the compound **2.38**.[99]

The ^1H NMR spectrum (Fig. 2.33 and Table 2.6) of compound **2.38** revealed the presence of pairs of signals due to diastereotopic protons of methylene groups at δ_H 1.96 (ddd, J = 13.6, 4.9, 1.8 Hz, 1H) and 1.73 (ddd, J= 13.7, 11.9, 5.2 Hz, 1H), 1.73 (ddd, J = 13.7, 11.9, 5.2 Hz, 1H) and 2.16 (m, 1H), 2.17 (m, 1H) and 2.47 (m, 1H), 2.08 (dd, J = 16.6, 1.1 Hz, 1H) and 2.89 (dd, J = 16.6, 1.2 Hz, 1H). These were assigned to H-11α and H-11β, H-7α and 7β, H-10α and H-10β; and H-3α and H-3β, in compound **2.38**, respectively. Signals at δ_H 6.01 (t, J = 1.8, 1H) and 5.66 (s, 1H) and were due to the unsaturated methine protons which were assigned to H-8 and H-6 in compound **2.38,** respectively. The spectrum revealed also the presence of three methyl protons characterized by the signals at δ_H 1.00 (s, H-13), 1.12 (s, H-14) and 1.93 (s, H-12). The proton-proton correlations for compound **2.38** were observed in the COSY spectrum (Figs. 2.35 and 2.36) and TOCSY spectrum (Fig. 2.37) whereby correlations between proton at δ_H 2.89 (H-3α) and 2.08 (H-3β), 2.47 (H-10α) and 2.17 (H-10β), 1.96(H-11α) and 1.73 (H-11β); and 2.17 (H-7α) and 1.73 (H-7β) were observed. The TOCSY spectrum further showed the presence of interactions between protons at δ_H 6.01 (H-8) and 1.93 (H-14), 2.47 (H-10α) and 1.93 (H-12), 2.17 (H-10β) and 1.93 (H-12), 2.89 (H-3α), and 2.08 (H-3β) 1.96 (H-11α) and 1.73 (H-11β) of the compound **2.38**

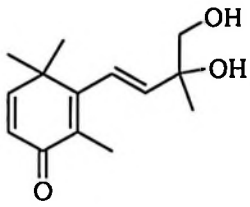
The ^{13}C NMR spectrum (Fig 2.34 and Table 2.6) together with the HSQC spectrum (Fig. 2.38) revealed the presence of five quaternary carbons, four methylene carbons, two unsaturated methine carbons and three methyl carbons. A further downfield signal at δ_C 200.1 was due to a ketonic carbonyl group assigned to (C-1). Other carbons were observed at δ_C 154.4 (C-5) and 150.3 (C-9). Two unsaturated methine carbons appeared at δ_C 123.7 and 122.3 and were assigned to C-8 and C-6, respectively. A signal at δ_C 71.6 assigned to C-4 was deduced to be due to a hydroxylated carbon. The quaternary carbon at δ_C 40.1 was assigned to C-2 in the proposed structure for compound **2.38.** The four methylene carbons were observed at δ_C 49.3 (C-3), 27.9 (C-7, C-10) and 27.7 (C-11). In addition, the three methyl carbons were characterized by the presence of signal at δ_C 24.7 (C-12), 24.6 (C-13) and 23.2 (C-14).

The long range H/C interactions as observed from the HMBC spectrum (Fig. 2.39 and 2.40) enabled the placement of carbonyl group to carbon signal at C-1 (δ_C 200.1) and carbinol to C-4 (δ_C 71.6). The HMBC spectrum further showed interactions between the methine protons at δ_H 5.66 and 6.01 and methine carbons at δ_C 123.7 and 122.3, respectively. Other important HMBC cross peaks involved the diastereotopic protons at δ_H 2.89/2.08 (H-3α/3β) with carbonyl carbon at δ_C 200.1 (C-1), the quaternary carbon at δ_C 40.1 (C-2) and the carbinol carbon at δ_C 71.6 (C-4) which enabled the assignment of the correlated units in the five-membered ring of the proposed structure **2.38**. Further correlations between δ_H 2.89/2.08 (H-3α/3β) and methyl group at δ_C 24.6 (C-13) and δ_C 23.2 (C-14) suggested a five-membered ring consisting of the carbonyl group and the two methyl groups.

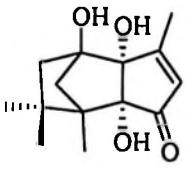
Furthermore, the correlation of δ_H 2.89/2.08 (H-3α/3β) and δ_C 122.3 (C-6) enhanced the linkage of the five–membered ring to the eight-membered ring in the proposed structure **2.38**. Other H/C correlations were observed between δ_H 5.66 (H-6) and δ_C 71.6 (C-4), δ_H 2.16/1.73 (H-7α/7β) and δ_C 150.3 (C-9), 27.9 (C-10), 27.7 (C-11) and 24.7 (C-14). In addition correlations between δ_H 6.01 (H-8) and δ_C 24.7 (C-12) linked the methyl group at δ_H 1.93 (H-12) to the eight-membered ring through δ_C 150.3 (C-9) of structure **2.38**. On the hand, protons signals at δ_H 2.47/2.17 (H-10α/10β) interacted with δ_C 123.7 (C-8), 150.3 (C-9) and 27.7 (C-11) which enabled the linkage of methylene groups at (C-10, C-11) and the methine carbon at δ_C 123.7 (C-8). Furthermore, the attachment of the third methyl group to its respective position was deduced from the presence of correlations between δ_H 1.93 (H-12) and δ_C 150.3 (C-9) and 123.7 C-8), δ_H 1.12 (H-14) and δ_C 49.3 (C-3) and 40.1 (C-2) while δ_H 1.00 (H-13) was correlated with δ_C 200.1 (C-1), 71.6 (C-4), 49.3 (C-3), 40.1 (C-2), and 23.2 (C-14). Therefore, the proposed structure **2.38** belongs to terpenoids class of the secondary metabolites, particularly to *nor*-sesquiterpenoid due to lack of one carbon of the normal sesquiterpenes. The proposed structure **2.38** was identified as hitherto previously unreported compound, hereby having been isolated and characterized for the first time from the leaves of *E. sacleuxii*. It was assigned a trivial name, sacleuxenone.

Nor-sesquiterpenoids are derivatives of sesquiterpenoid upon loss of one carbon atom. The *nor*-sesquiterpenoids with interesting structures have been isolated from various plant and fungi species. Studies on the leaves of *Croton tiglium* (Euphorbiaceae) afforded the isolation of badounoid A (**2.39**) which was inactive

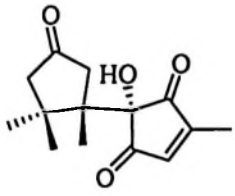
against human cancer cells.[132] From the culture broth of *Coprinopsis cinerea* (Psathyrellaceae), hitoyol A (**2.40**) and hitoyol B (**2.41**) were isolated of which the later showed antimalarial activity with an IC_{50} of 59 μM.[133] Thus, *nor*-sesquiterpenoid regardless of their structure are promising candidates for drug development. Compounds badounoid A (**2.39**), hitoyol A (**2.40**) and hitoyol B (**2.41**) had the molecular formulae of $C_{14}H_{20}O_3$, $C_{14}H_{20}O_4$ and $C_{14}H_{18}O_4$, respectively which related to sacleuxenone (**2.38**) with the molecular formula of $C_{14}H_{20}O_2$. The relationship was accounted by the presence of equal number of carbons to all molecules of which they are lacking one carbon from the normal sesquiterpenoid skeleton. However, the number of hydrogen and oxygen varies from each compound due to various modifications arising during their biosynthesis. Therefore, the sacleuxenone (**2.38**) hereby reported contributes to the pool of new and relatively rare *nor*-sesquiterpenoid class of compounds.



2.39



2.40



2.41

Biosynthetically, sacleuxenone (**2.38**) follow the mevalonic pathway of which one methylene group from farnesyl pyrophosphate (FPP) was lost. The actual biosynthetic pathway involved the subsequent substitution addition of isoprene pyrophosphate (IPP) to geranyl pyrophosphate (GPP) to give a farnesyl pyrophospshate (FPP) which acted as an intermediate during the formation of various forms of terpenoids. Cyclization of the FPP followed by loss of

pyrophosphate group yielded a humulyl cation.[117] Humulyl cation then undergoes a series of reactions including rearrangement, loss of hydrides, oxidation and decarboxylation, hydroxylation, hydrolysis and wagner Meerwein rearrangements to yield sacleuxinoid (**2.38**). The proposed biosynthetic pathway of the proposed compound has been detailed in scheme 2.5.

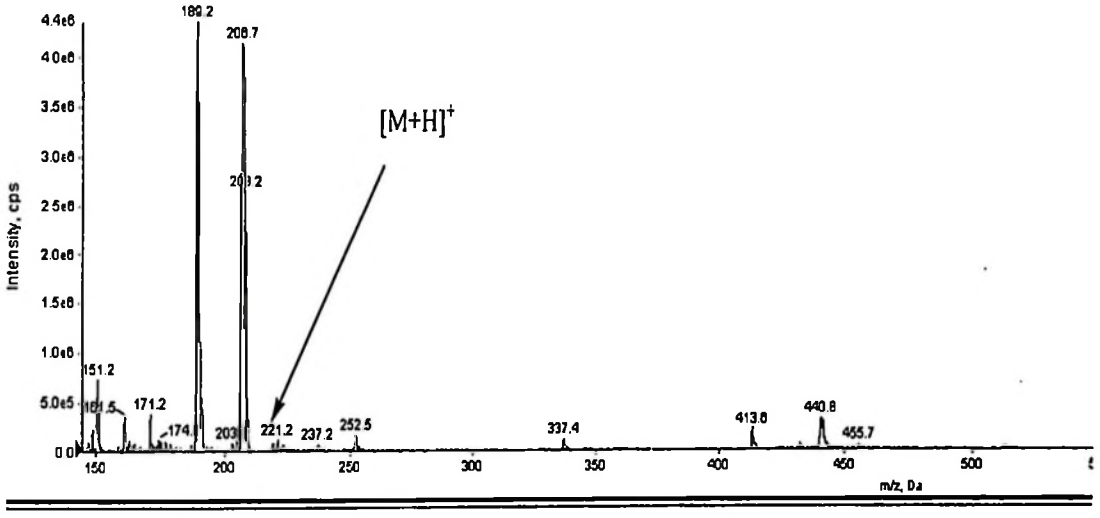
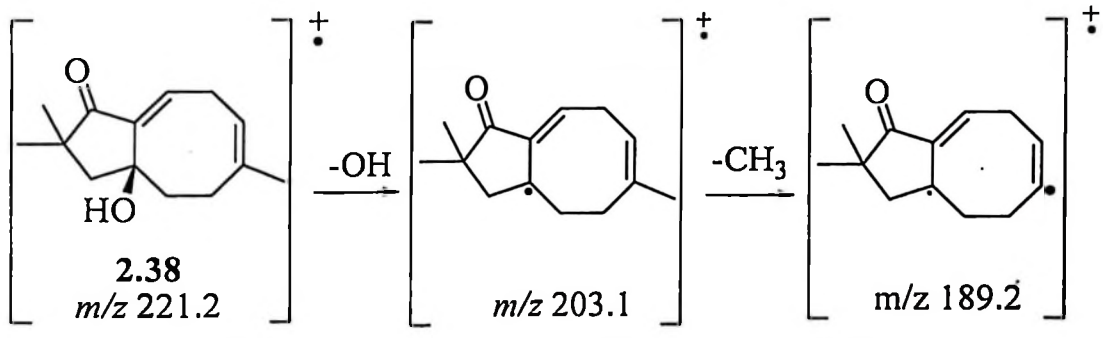


Figure 2.31: LC-Mass Spectrum of Sacleuxenone (2.38)



Scheme 2.4: MS Fragmentation Pattern of Sacleuxenone (2.38)

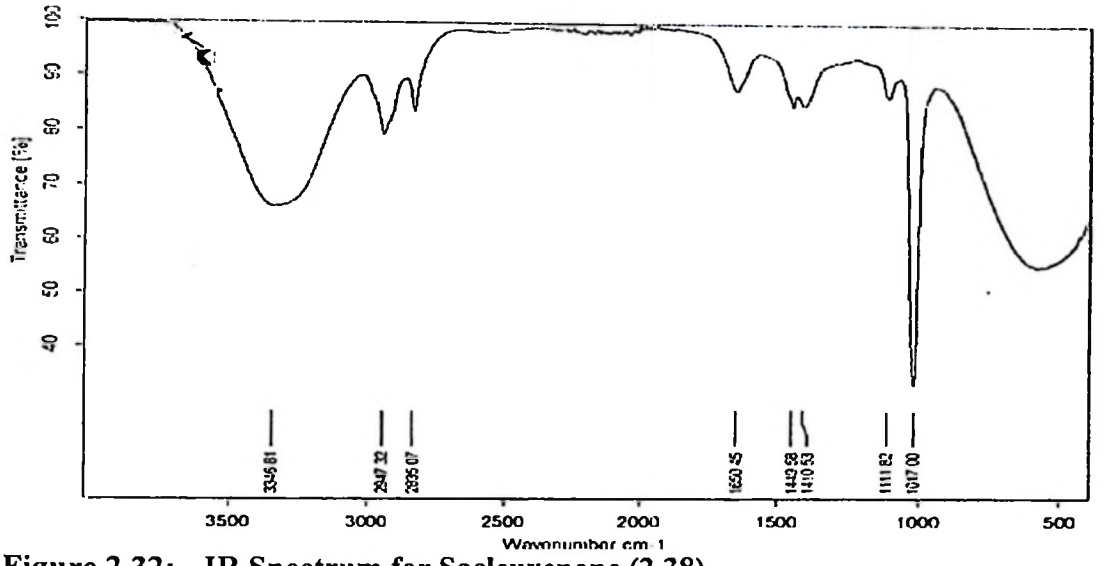


Figure 2.32: IR Spectrum for Sacleuxenone (2.38)

Table 2.6: ^{1}H, ^{13}C NMR Spectroscopic Data and HMBC for Sacleuxenone (2.38)

H/C	δ_H	Mult. *J* (Hz)	δ_C	HMBC H→C
1	---	---	200.1	3α,3β, 13
2	---	---	40.1	3α,3β, 13,14
3α	2.89	dd, 16.6, 1.2	49.3	6,13,14
3β	2.08	dd, 16.6, 1.1		
4	---	---	71.6	8,6,3α/β,11 α/β,13,14
5	---	---	154.4	11
6	5.66	s, br	122.3	8, 3β
7α	2.17	m	27.9	8,11β,12
7β	1.73	ddd, 13.7,11.9, 5.2		
8	6.01	t, 1.8	123.7	6, 10β, 12
9	---	---	150.3	10β,7α, 12
10α	2.47	m	27.9	8,14,11α/β,7α/β
10β	2.17	m		---
11α	1.96	ddd, 13.6,4.9,1.8	27.7	8,12,7β,10β
11β	1.73	ddd, 13.7,11.9,5.2		
12	1.93	s	24.7	8,3α/β,7α,14
13	1.00	s	24.6	8,3α/β, 10β,14
14	1.12	s	23.2	3α,13

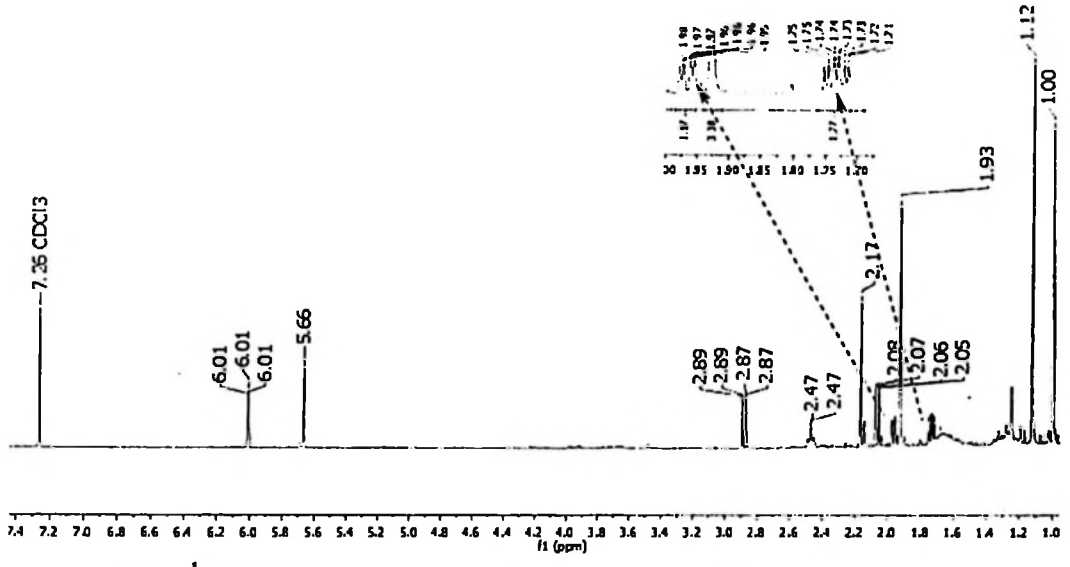


Figure 2.33: ^{1}H NMR Spectrum for Sacleuxenone (2.38)

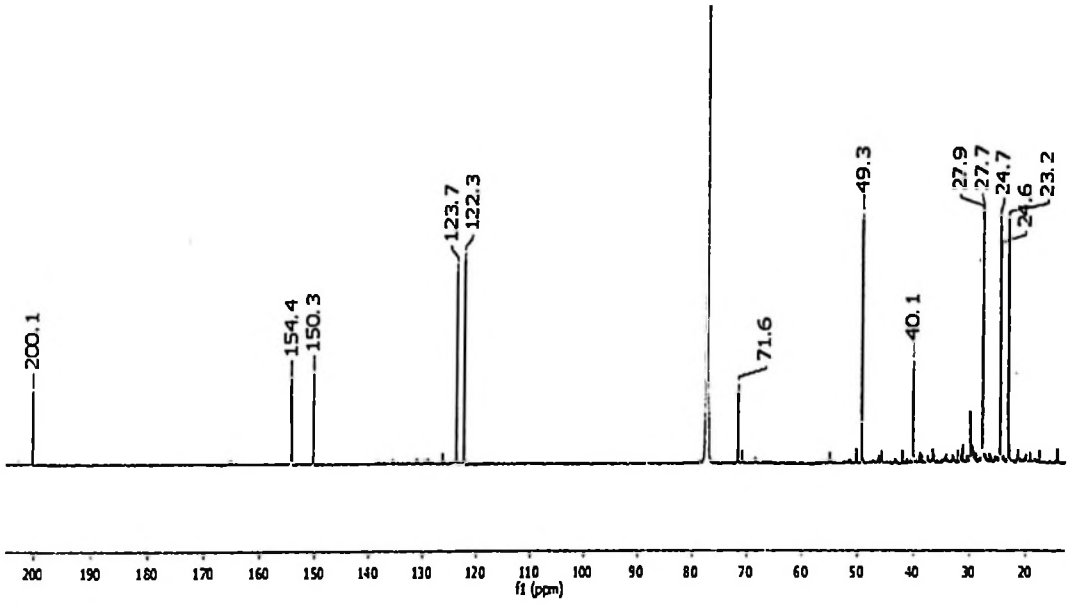


Figure 2.34: ^{13}C NMR Spectrum of Sacleuxenone (2.38)

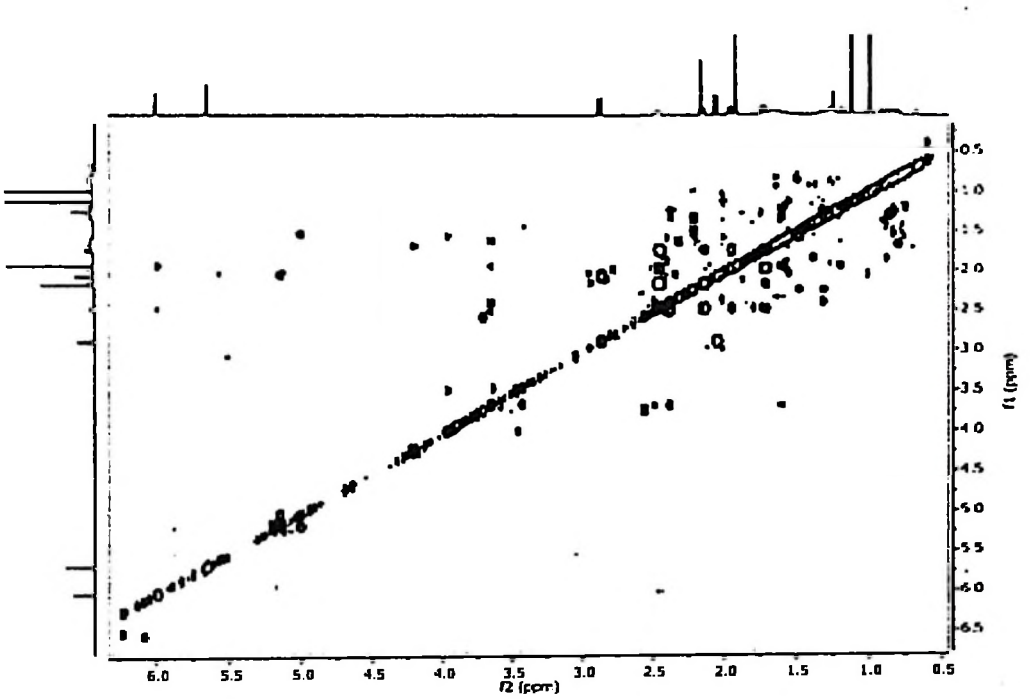


Figure 2.35: The COSY Spectrum of Sacleuxenone (2.38)

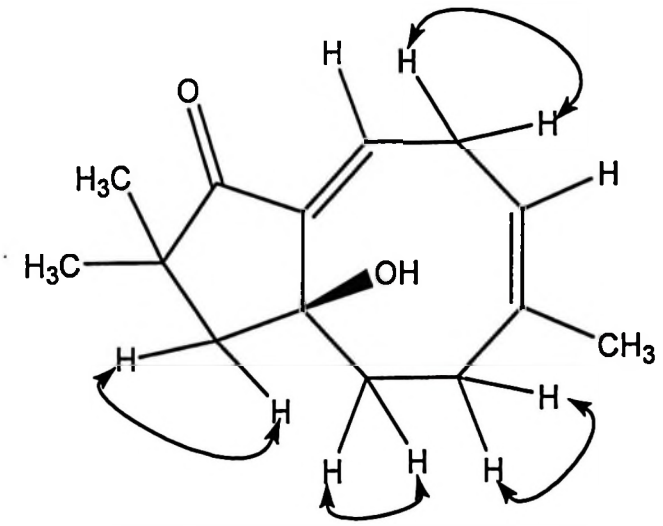


Figure 2.36: The H/H Interactions of Sacleuxenone (2.38)

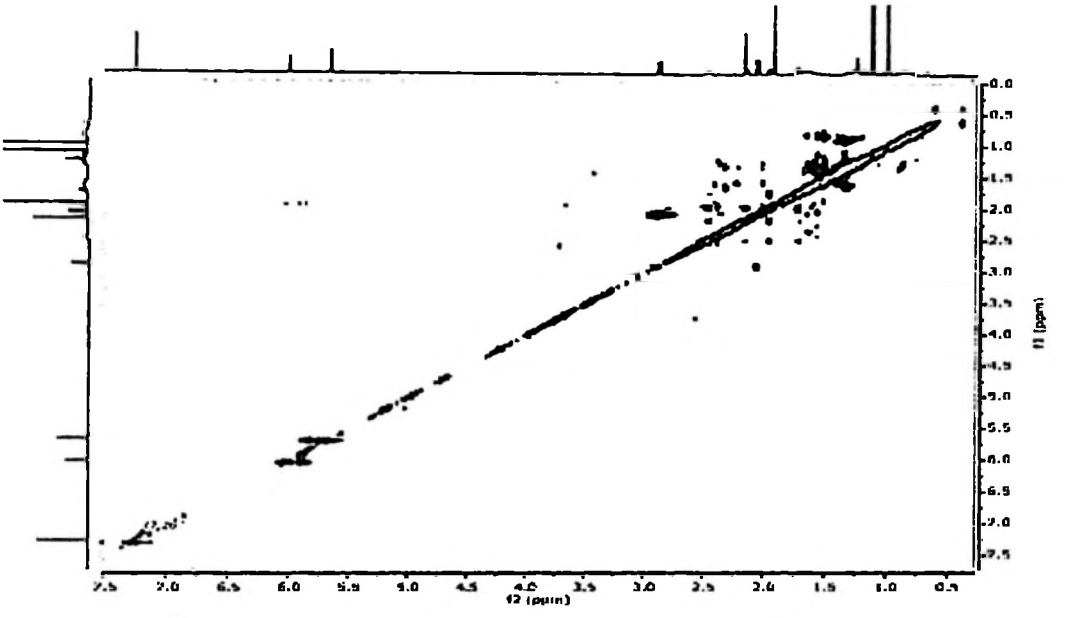


Figure 2.37: The TOCSY Spectrum for Sacleuxenone (2.38)

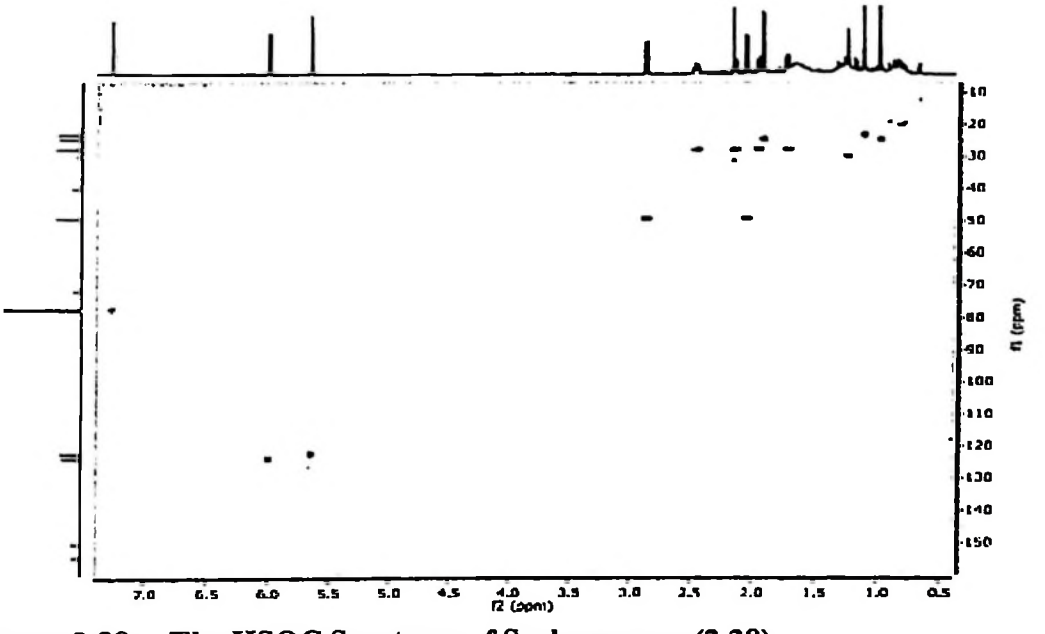


Figure 2.38: The HSQC Spectrum of Sacleuxenone (2.38)

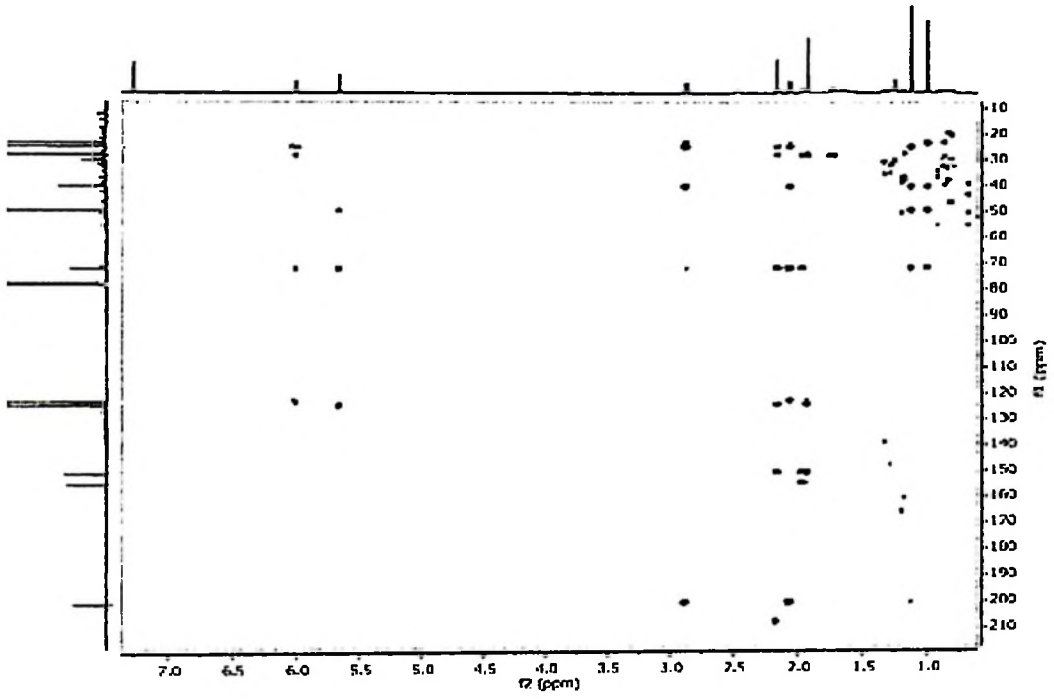


Figure 2.39: The HMBC Spectrum of Sacleuxenone (2.38)

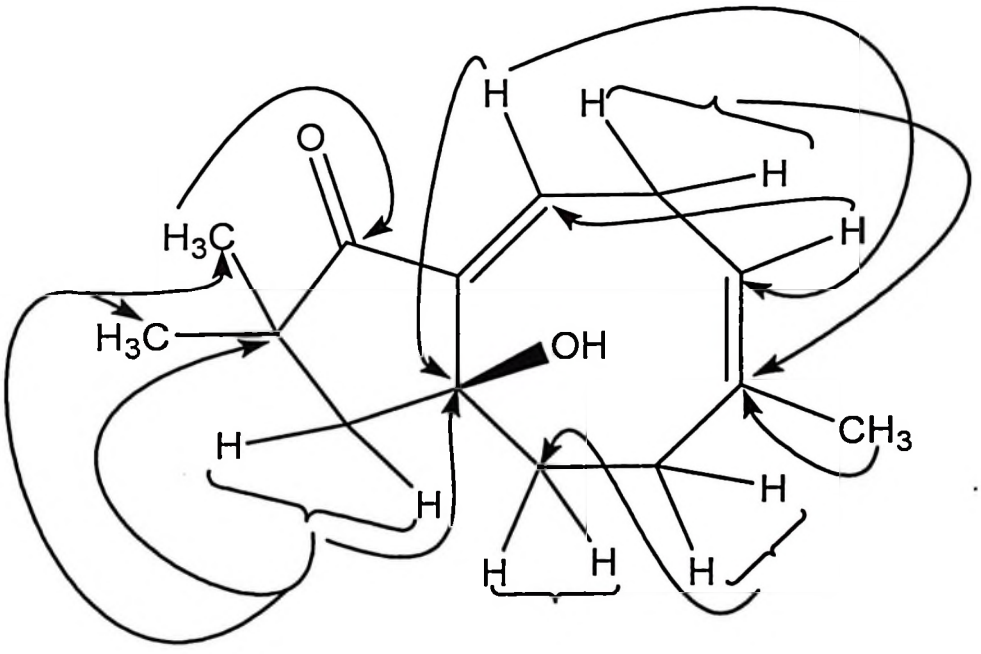
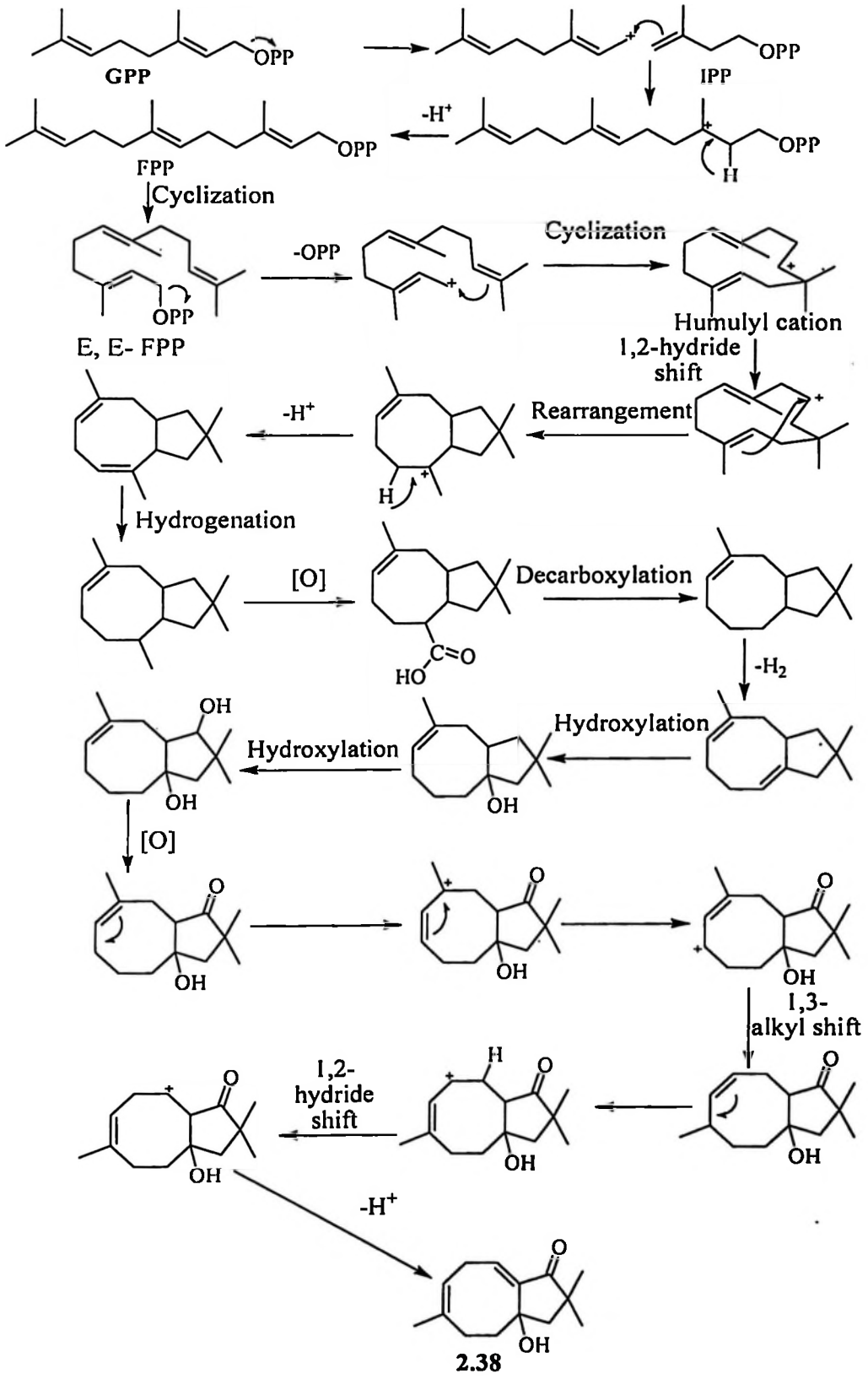


Figure 2.40: Important Interaction of H/C Long Range of Sacleuxenone (2.38)



Scheme 2.5: Proposed Biosynthesis Pathway of Sacleuxenone (2.38)

2.5 Experimental Section

2.5.1 General Experimental Procedures

The isolation of the secondary metabolites was achieved using silica gel 60 (230-400) in the vacuum liquid chromatography (VLC) and gravitational column chromatography (GCC) using analytical grade solvents, namely petroleum ether (b.p 40 – 60 °C), *n*-hexane and ethyl acetate. Gel filtration was carried out over Sephadex® LH-20 eluting with either methanol or mixture of methanol and dichloromethane (1:1 v/v). Analytical thin layer chromatography (TLC) was performed on silica gel 60 F_{254} (Merck) precoated aluminum throughout the TLC analysis. The UV positive spots on TLC plates were evaluated using UV light at wavelength 245 and 366 nm. The plates were then sprayed with anisaldehyde reagent (prepared by mixing 0.5 mL 4-methoxybenzaldehyde, 10 mL gracial acid, 85 mL ethanol and 5mL concentrated sulphuric acid) followed by heating at about 110 °C for identification of UV-negative compounds and detecting of a colour change of the UV-positive spots. 1D (^{1}H and ^{13}C) and 2D (COSY, TOCSY, HSQC and HMBC) NMR spectra were acquired on Avance III HD 201 and 800 MHz and Varian MR 400 MHz spectrometers using deuterated chloroform and were processed using MestReNova-10.0. Mass spectra were determined by the Agilent (Q-TOF-LC-MS) utilizing 1:1 MeCN: H_2O gradient system and Shimadzu (GCMS-QP2010SE) spectrometers. NMR and LC-MS experiments were conducted at the Department of Chemistry and Molecular Biology, University of Gothenburg (UG), and the Swedish NMR Center in Sweden while, GC-MS experiment at the Chemistry Department, University of Dar es Salaam (UDSM).

2.5.2 Plant Materials

The root, stem barks and leaves of *Newtonia paucijuga* were collected in January, 2016 from Zaraninge Forest Reserve located in Bagamoyo District, Pwani Region, Tanzania. *Erythrina sacleuxii* was collected in March 2014 from the open woodland in north of Maili Kumi village, Korogwe District, Tanga Region in Tanzania. The plant species were identified by Mr. Mbago, a senior taxonomist in the Botany Department, UDSM where by voucher specimen FMM 3717 and FMM 3637 for *N. paucijuga* and *E. sacleuxii,* respectively are deposited. The collected root barks, stem barks and leaves samples were air dried under shade and pulverized before extraction.

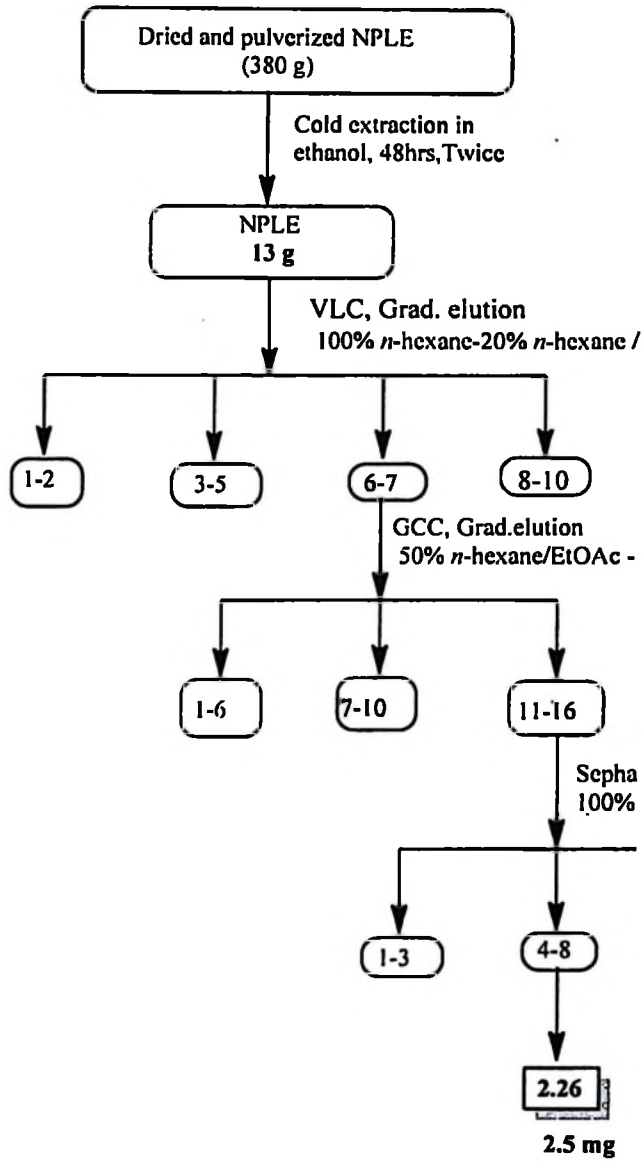
2.5.3 Extraction

From the air dried and pulverized plant samples, 1200 g of root barks, 1000 g of stem barks and 380 g of the leaves of the *Newtonia paucijuga* were soaked twice in 2000 mL, 1500 mL and 1000 mL of ethanol, respectively for 48 hrs at room temperature. The extracts were filtered and concentrated under reduced pressure at 40 °C using rotary evaporator. Crude extract weighing 98 g, 77 g, 13 g were obtained from root barks, stem barks and leaves of *N. paucijuga*, respectively. Similarly, 400 g of the leaves of *E. sacleuxii* were soaked twice in 1000 mL of ethanol for 48 hours, at room temperature. The extract were filtered and concentrated under reduced pressure at 40 °C using rotary evaporator of which 24 g of crude extract was obtained. The crude extracts were then subjected to chromatographic separation, antiplasmodial and antimicrobial activities assays.

2.5.4 Isolation of Mearnsetin-3-*O*-α-*L*-Rhamnopyranoside (2.26)

Ethanolic crude extract of the leaves of *N. paucijuga* (13 g) were adsorbed on silica gel and subjected to vacuum liquid chromatography (VLC) employing gradient elution at 100% *n*-hexane, 80, 60 and 20% *n*-hexane/ethyl acetate proportions of which 10 fractions were obtained. The fractions were analysed and separation monitored using TLC visualized under UV light followed by spraying with anisaldehyde reagent heated. Following TLC analysis fractions were reorganized into four fractions based on their similarities. Fractions 1-2 contained less polar mixtures whose separation was difficult. Fractions 3-5 contained undefined components as there was no promising spot to allow further separations. Fractions 8-10 were not further worked on due to the insufficient amount. Thus, fractions 6-7 were combined and subjected to gravitational column chromatography dry packing employing gradient elution at 50, 30 and 20% *n*-hexane/ethyl acetate from which 16 fractions were obtained. The TLC analysis led to the combinations of fractions 1-6, 7-10 and 11-16. The combined fractions 1-6 and 7-10 due to insufficient amount and uninteresting TLC spots were not separated and analysed further. Combined fractions 11-16 were subjected to Sephadex® LH-20 gel chromatography eluting with 100% MeOH of which 10 fractions of approximately 3 mL each were obtained. The obtained fractions were subjected to the TLC analysis from which sub-fractions 4-8 contained mearnsetin-3-*O*-α-*L*-rhamnopyranoside (**2.26**, 2.5 mg) Summary for the isolation of compound **2.26** from *N. paucijuga* leaves is shown on the chart **2.1**

Chart 2.1: Isolation of Mearnsetin-3-*O*-α-*L*-Rhamnopyranoside (2.26)



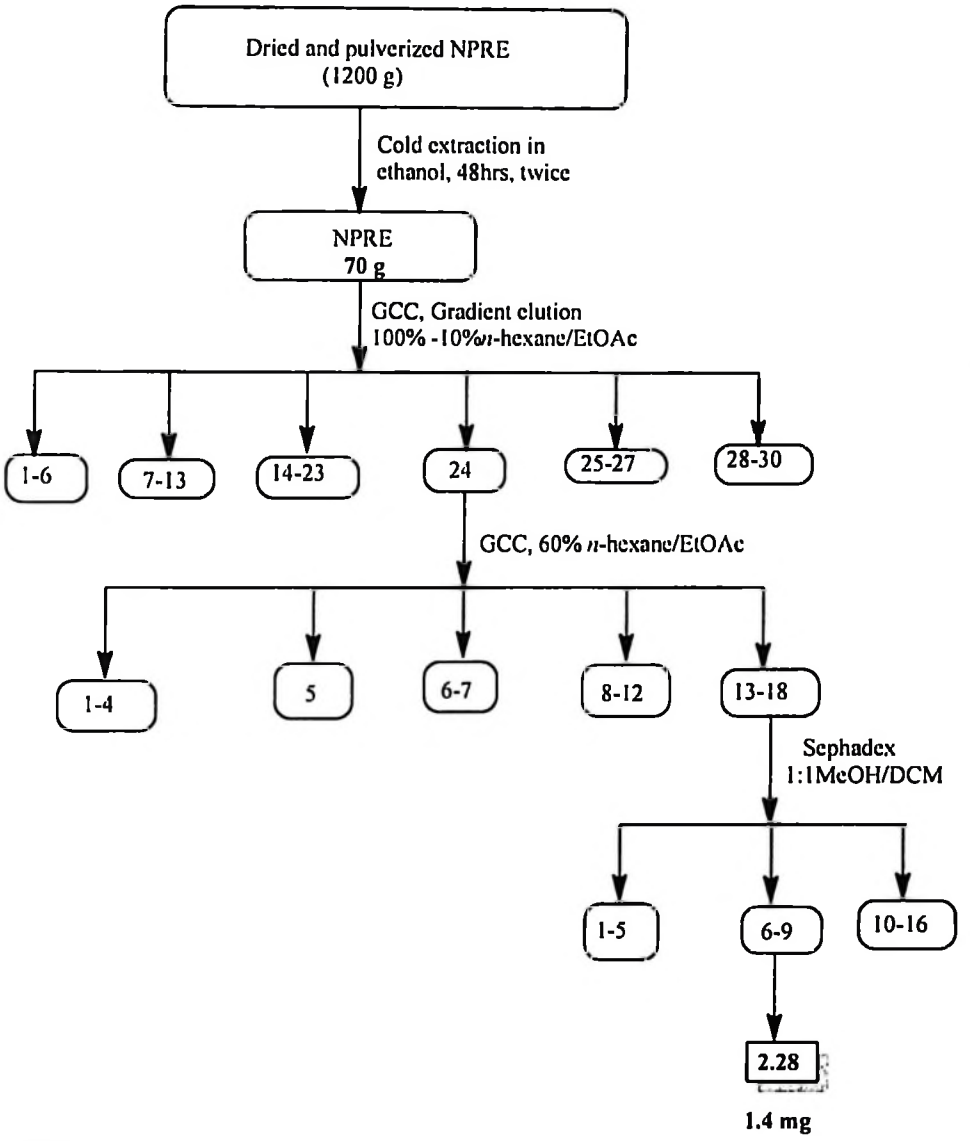
Key:
NPLE = *Newtonia Paucijuga* Leaves Extracts
VLC = Vacuum Liquid Chromatography
GCC = Gradient Column Chromatography

2.5.5 Isolation of Benzyl-3,4,5-trihydroxybenzoate (2.28)

Ethanolic crude extract (70 g) from the root barks of *N. paucijuga* was subjected to gravitational column chromatography dry packing utilizing gradient elution at 100%

n-hexane to 10% n-hexane/ethyl acetate of which 30 fractions of 250 mL each were obtained. Following the TLC analysis on UV light and anisaldehyde spraying reagent, fractions were combined as fraction 1-6, 7-13, 14-23, 24, 25-27 and 28-30 based on similarities of their spot. Attempts to separate and analyze fractions other than fraction 24 were made, but no successful achievements due to the complexity of the mixtures and associated tailing of the components when traced with TLC eluted by different solvent systems. Fraction 24 when subjected to the gravitational column chromatography dry packing eluting at 60% *n*-hexane/ethyl acetate afforded 18 fractions of approximately 50 mL each whereby the similar fractions were combined based on the TLC analysis leading to fractions 1-4, 5, 6-7, 8-12 and 13-18. Fractions 1-4, 5, 6-7, 8-12 contained undefined components and hence their further separation and analysis were not undertaken. Fractions 13-18 were combined and subjected to Sephadex® LH-20 gel chromatography eluting at 1:1 MeOH/CH_2Cl_2 of which 16 fractions of approximately 3 ml each were collected. The combined sub-fractions 6-9 afforded pure compound benzyl-3,4,5-trihydroxybenzoate (**2.28**, 1.4 mg). The isolation of compound (**2.28**) is presented in Chart 2.2.

Chart 2.2: Isolation of Benzyl-3,4,5-Trihydroxybenzoate (2.28)



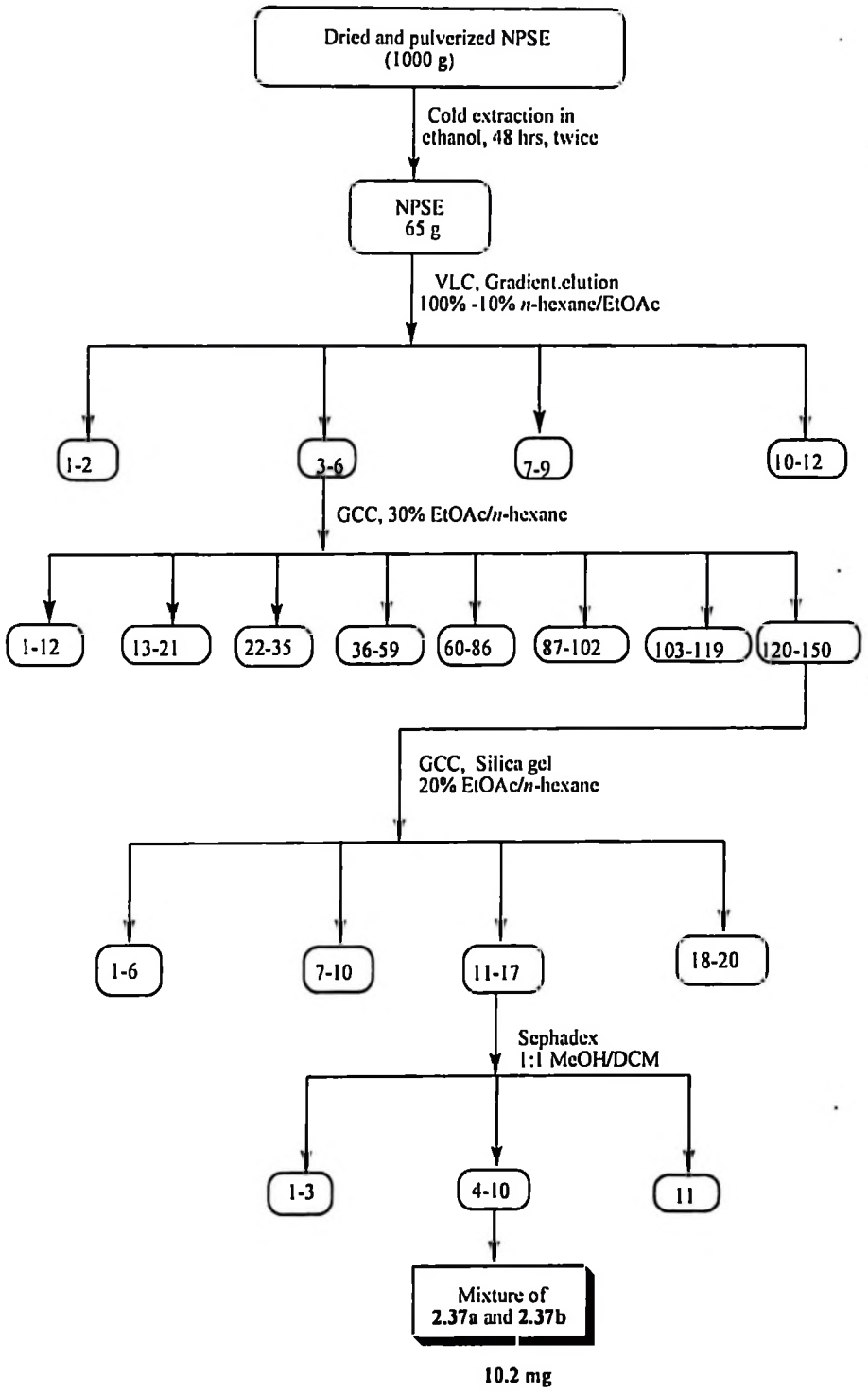
Key:
NPRE = *Newtonia paucijuga* root barks extratcts
GCC = Gradient Column Chromatography
DCM = Dichloromethane

2.5.6 Isolation of the Mixture of Stigmasterol (2.37a) and β-sitosterol (2.37b)

About 65 g of the ethanolic crude extract of *N. paucijuga* stem barks was adsorbed on silica gel and subjected to vacuum liquid chromatography eluting at 100% *n*-hexane to 10% *n*-hexane/ethyl acetate. From the VLC, 12 fractions of approximately

150 mL each were obtained. Similar fractions were visualized on TLC under UV light and upon spraying with anisaldehyde were combined resulting to fractions 1-2, 3-6, 7-9 and 10-12. Fraction 1-2 was not further analysed as it consisted of least polar oily stuff. Fraction 7-9, due to absence of promising spot on TCL were left behind, while combined fraction 10-12 was extremely polar and attempt to separate its components was unsuccessful. The combined fraction 3-6 was subjected to column chromatography eluting with 30% ethyl acetate/*n*-hexane of which fractions of 50 mL each were obtained. TLC analysis was done and similar fractions were combined leading to 1-2, 3-21, 22-35, 36-59, 60-86, 87-102, 103-119 and 120-150 sub-fractions. Fractions, other than 120-150 were not separated and analysed further due to either insufficient amounts or lack of interesting spots. Thus, a combined fraction 120-150 was adsorbed on silica gel and subjected to gravitational column chromatography by dry packing eluting with 20% ethyl acetate/*n*-hexane of which 26 fractions of approximately 20 mL each were obtained and analysed by TLC from which 4 fraction due to combination of similar fractions were obtained. Of the four sub-fractions, fractions 11-17 was subjected to gel chromatography using Sephadex® LH-20 with the mixture of MeOH/DCM (1:1 v/v) as a mobile phase from which 11 sub-fractions were obtained. Upon TCL analysis sub-fractions 4-10 afforded 10.2 mg of stigmasterol **2.37a** and β-sitosterol **2.37b**. Summary of the isolation a mixture of compound **2.37a** and **2.37b** is shown in the Chart 2.3.

Chart 2.3: Isolation of the of Stigmasterol (2.37a) and β-sitosterol (2.37b)

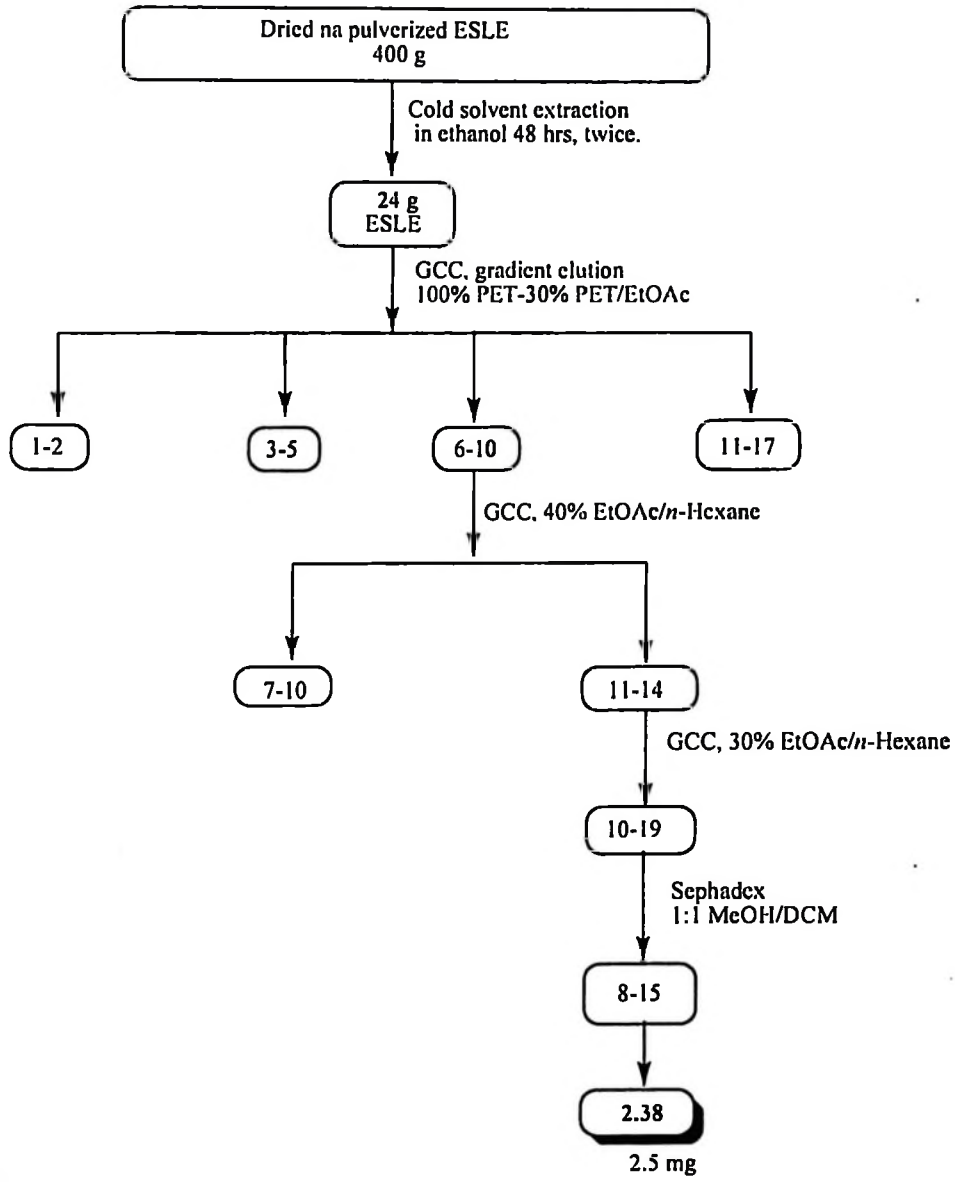


Key:
NPSE = *Newtonia paucijuga* Stem barks Extracts, VCL = Vacuum Liquid charomatography
GCC = Gradient Column Chromatography, DCM = Dichloromethane

2.5.7 Isolation of Sacleuxenone (2.38)

About 24 g of the ethanolic crude extract from the leaves of *E. sacleuxii* was adsorbed on silica gel and subjected to the gravitational column chromatography (GCC) employing gradient elution from 100% petroleum ether to 30% petroleum ether/ethyl acetate to obtain 17 fractions of 100 mL each. TLC was used to monitor the similarity of the fractions of which four fractions were obtained. Fractions 1-2, 3-5 and 11-17 were not further separated and analysed due to their complexity. Fraction 6-10 was adsorbed on silica gel and further subjected to GCC utilizing gradient elution at 40% ethyl acetate/*n*-hexane from which 14 fractions were obtained. Sub-fractions 1-6, 7-10 and 11-14 were combined based on the TLC analysis. Sub-fraction 1-6 displayed undefined components and hence was not further worked on. Fraction 7-10 was subjected to Sephadex® LH-20· filtration eluting with 100% methanol to obtain 25 fractions of approximately 3 mL each. TLC analysis afforded the combination of the sub-fractions 20-25 from which 1.5 mg of unidentified compound was obtained. On the other hand, the combined sub-fraction 11-14 was adsorbed on silica gel and further subjected to GCC eluting at 30% ethyl acetate/*n*-hexane from which 19 fractions were obtained. Upon TLC analysis, sub-fractions 1-9 and 10-19 were grouped. Combined fractions 1-9 did not show any promising component, hence left without further separation. Combined sub-fraction 10-19 was subject to Sephadex® LH-20 separation eluting with 1:1 MeOH/DCM from which 17 fractions were obtained and analysed by TLC from which sub-fractions 8-15 yielded sacleuxenone (**2.38**, 2.5 mg). Summary of the isolation of compound **2.38** is shown in the Chart 2.4.

Chart 2.4: Isolation of Sacleuxenone (2.38)



Key:
ESLE = *Erythrina sacleuxii* Leaves extracts
PET = Petroleum ether
GCC = Gravitational Column Chromatography
DCM = Dichloromethane

2.6 Properties of the Compounds isolated from *Newtonia paucijuga*

Mearnsetin-3-*O*-α-*L*-rhamnopyroside (2.26): Yellow solid, yield 2.5 mg. Optical rotation, $[\alpha]_D$ at 20 °C, -28.75° (c 0.0002, $CHCl_3$). $IR_{\nu max}$ (KBr) 3321.8, 2943.9, 2836.5, 1456.1, 1420.3, 1122, 1030.5 and 660.55 cm^{-1}; MS *m/z* 479.6 $[C_{22}H_{22}O_{12}]^+$;

^{1}H NMR (CD_3OD, 800 MHz) δ_H 6.88 (2H, s), 6.37 (1H, d, J = 2.1 Hz), 6.21 (1H, d, J = 2.1 Hz), 5.31 (1H, d, J = 1.7 Hz), 4.23 (1H, dd, J = 3.4, 1.7 Hz), 3.88 (3H, s), 3.75 (2H, m), 3.33 (1H, m) and 0.95 (3H, d, J = 5.7 Hz); ^{13}C NMR (CD_3OD, 201 MHz) δ_C 179.7, 166.3, 163.3, 159.0, 158.6, 151.9, 139.4, 136.7, 127.0, 109.8, 105.9, 103.7, 100.0, 94.8, 103.7, 72.1, 71.9, 60.9 and 17.7.

Benzyl-3,4,5-trihydroxybenzoate (2.28): Yellow solid, 1.4 mg. MS *m/z* 261.4 [$C_{14}H_{12}O_5$]; ^{1}H NMR (CD_3OD, 800 MHz) δ_H 7.89 (2H, s), 7.43 (2H, d, J = 7.1 Hz), 7.38 (2H, t, J = 7.6 Hz), 7.32 (1H, t, J = 7.4 Hz) and 5.30 (2H, s); ^{13}C NMR (CD3OD, 201 MHz) δ_C 168.3, 158.1, 154.0, 132.5, 129.6, 129.6, 129.0, 119.6 and 67.2.

Mixture of Stigmasterol (2.37a) and β-sitosterol (2.37b): White solid, 10.2 mg. IR_{Vmax} (KBr) 3346.99, 2940.87, 28.46, 1647.50, 1449.52, 1409.75, 1110.42, 1020.52, 909.15, 730.23 and 646.49 cm^{-1}; MS m/z 412 $[C_{29}H_{48}O]^+$ and 414 $[C_{29}H_{50}O]^+$; ^{1}H NMR ($CDCl_3$, 800 MHz) δ_H 5.42 – 5.30 (1H, m), 5.15 (1H, dd, J = 15.2, 8.6 Hz), 5.01 (1H, dd, J = 15.2, 8.6 Hz), 3.52 (1H, tt, J = 10.8, 4.6 Hz), 2.36 – 2.17 (3H, m), 2.08 – 1.93 (3H, m), 1.84 (4H, m), 1.69 – 1.63 (2H, m), 1.56 – 1.43 (7H, m), 1.25 (6H, s), 1.15 (4H, t), 1.11 – 1.05 (3H, m), 1.01 (5H, s), 0.92 (5H, d, J = 6.6 Hz), 0.86 – 0.82 (7H, m), 0.68 (3H, s); ^{13}C NMR ($CDCl_3$, 201 MHz) δ_C 140.9, 138.5, 129.4, 121.9, 72.0, 56.9, 56.2, 50.3, 46.0, 42.4, 39.9, 37.4, 32.0, 31.8, 29.9, 28.4, 24.4, 23.2, 21.2, 20.0, 19.2, 18.9, 12.1 and 12.0.

2.7 Properties of the compound isolated from *Erythrina sacleuxii*

Sacleuxenone (2.38): White solid, 2.5 mg. optical rotation, $[\alpha]_D$ at 20 °C, -57.5° (c 0.0013,$CHCl_3$); IR_{Vmax} (KBr) 3346.81, 2947.32, 2835.07, 1650.45, 1449.58, 1410.53, 1111.82 and 1017.00 cm^{-1}; MS m/z 221.2 $[C_{14}H_{20}O_2]^+$; ^1H NMR ($CDCl_3$, 800 MHz) δ_H 601 (1H, t, J = 1.8 Hz), 5.66 (1H, br s), 2.89 (1H, dd, J = 16.6, 1.2 Hz), 2.47 (1H, m), 2.17 (2H, m), 2.08 (1H, dd, J = 16.6, 1.1 Hz), 1.96 (1H, ddd, J = 13.6, 4.9, 1.8 Hz), 1.93 (3H, s), 1.73 (2H, ddd, J = 13.7, 11.9, 5.2 Hz), 1.12 (3H, s) and 1.00 (3H, s); ^{13}C NMR ($CDCl_3$, 201 MHz) δ_H 200.1, 154.4, 150.3, 123.7, 122.3, 71.6, 49.1, 27.9, 27.7, 24.7, 24.6 and 23.2.

CHAPTER THREE

ANTIPLASMODIAL AND ANTIMICROBIAL ACTIVITIES OF CRUDE EXTRACTS FROM NEWTONIA PAUCIJUGA AND ERYTHRINA SACLEUXII

Abstract

This chapter reports on the antiplasmodial, antibacterial and antifungal activities evaluation of crude extracts from *Newtonia paucijuga* and *Erythrina sacleuxii*. The antiplasmodial activities were assayed using malaria imaging assay method while both antibacterial and antifungal assays were evaluated by disc diffusion method. For antimicrobials, the minimum inhibitory concentrations (MIC) of the extracts with remarkable activities were determined through the use of broth microdilution method. The investigations revealed that extracts from the leaves, stem and root barks of *N. paucijuga* and leaves of *E. sacleuxii* had moderate antibacterial activities with activity index (AI) value ranging from 0.3 to 0.5 against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia*. The highest activity was observed for the leaves and stem bark extracts of *N. paucijuga* (AI = 0.5). Furthermore, the minimum inhibitory concentration (MIC) for the active extracts ranged from 0.3 - 5.0 mg/mL against *S. aureus*, *P. aeruginosa*, *K. pneumonia* and *Bacillus subtilis*. No any activities were observed against all form of the tested fungi at 500 mg/mL. All extracts exhibited antiplasmodial activity against *Plasmodium falciparum* (3D7 strain) at an IC_{50} value ranging from 2.6 – 24.6 µg/mL and selectivity index (SI) ranging from 6.5 – 21.0 against human embryonic kidney (HEK 293) cell lines. The isolated pure compounds were not assayed for their antiplasmodial and antimicrobial activities due to insufficient amount. Antiplasmodial and antimicrobial activities observed for the investigated plant extracts validate some of their use in traditional medicine and further underscore the need for their conservations.

3.1 Introduction

Protozoa, bacteria and fungi are responsible for diseases affecting the health of human beings. Efforts to combat the diseases are inevitable, particularly through the use of drugs. However, treatments of malaria, bacterial and fungal diseases for a long period of time have been challenged with the prevalence of resistance of the causative agents against some of existing drugs. Hence, the search for alternative of more potent drug agent is desired. Antimalarial and antimicrobial agents from natural products have been one of the world reliable sources of drug discovery in order to treat diseases such as malaria, bacterial and fungal diseases.[134] According to World Health Organization (WHO) records, about 25% of prescription drugs and 11% of the essential drugs among which are synthetic drugs are obtained from natural products or precursor compounds originating from plant signifying the important role served by plants as a potential source for novel drugs.[25]

N. paucijuga and *E. sacleuxii* species included in the reported investigations are restricted to particular geographical areas and were previously reported to be used traditionally for treatment of diseases. Particularly, the roots of *N. paucijuga* are used for labour induction, while the *E. sacleuxii* leaves for remedy of malaria indicating the presence of bioactive constituents within the species responsible for the alignments. Therefore, with the effort for obtaining antimalarial, antibacterial and antifungal agents, the results of the bioassays of crude extracts obtained from *N. paucijuga* and *E. sacleuxii* are hereby reported in this chapter.

3.2 Materials and Methods

3.2.1 Extraction

The plant materials were air dried, pulverized and extracted as reported in chapter two of this dissertation. The crude of *N. paucijuga* root barks ethanolic extract (NPRE), *N. paucijuga* stem barks ethanolic extract (NPSE), *N. paucijuga* leaves ethanolic extract (NPLE) and *E. sacleuxii* leaves ethanolic extract (ELSE) were stored in the refrigerator at -19 °C until the time of testing.

3.2.2 Antiplasmodial and Cytotoxic Assay

Antiplasmodial activities for the crude extracts from *N. paucijuga* and *E. sacleuxii* were evaluated against *Plasmodium falciparum* (3D7, Chloroquine-sensitive strain) using malaria imaging assay.[135] The crude extracts were diluted in 100% DMSO while the stock solution of the reference drug molecule was prepared at 10 mM in 10 mM in 100% H_2O (Chloroquine). Stock solutions of the extracts were diluted to a final assay concentration of 133 – 400 µg/mL, depending on the stock solution concentration, while the reference antiplasmodials were diluted to 40 µM. The final DMSO concentration for all extracts was 0.4% in the assay. The extracts were tested in 11-points dose-response using three concentrations per log dose.

Plasmodium falciparum parasite (3D7 strains) were grown in RPMI 1640 supplemented with 25 mM HEPES, 5% AB human male serum, 2.5 mg/mL Albumax II and 0.37 mM hypoxanthine. Parasites were subjected to two rounds of sorbitol synchronization before undergoing treatment. Ring stage parasites were exposed to the experimental extracts and reference compounds in 384-wells imaging

micro-plates (PerkinElmer Cell Carrier) as previously described.[135] Plates with the samples were incubated for 72h at 37 °C, 90% N_2, 5% CO_2, 5% O_2, then the parasites were stained with 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI) and imaged using an Opera QEHS micro-plate confocal imaging system (PerkinElmer). The digital images obtained were then analyzed using the Perkin Elmer Acapella spot detection software where spots which accomplish the criteria established for a stained parasite count. The % inhibition of parasite replication was the calculated using DMSO and DHA control data. The experiments were carried out in two independent biological replicates, each consisting of two identical technical replicates. Raw data was normalized using the in-plate positive and negative controls to obtain normalized % inhibition data, which was then used to calculate IC_{50} value, through a four parameter logistic curve fitting in GraphPad Prism v.6.

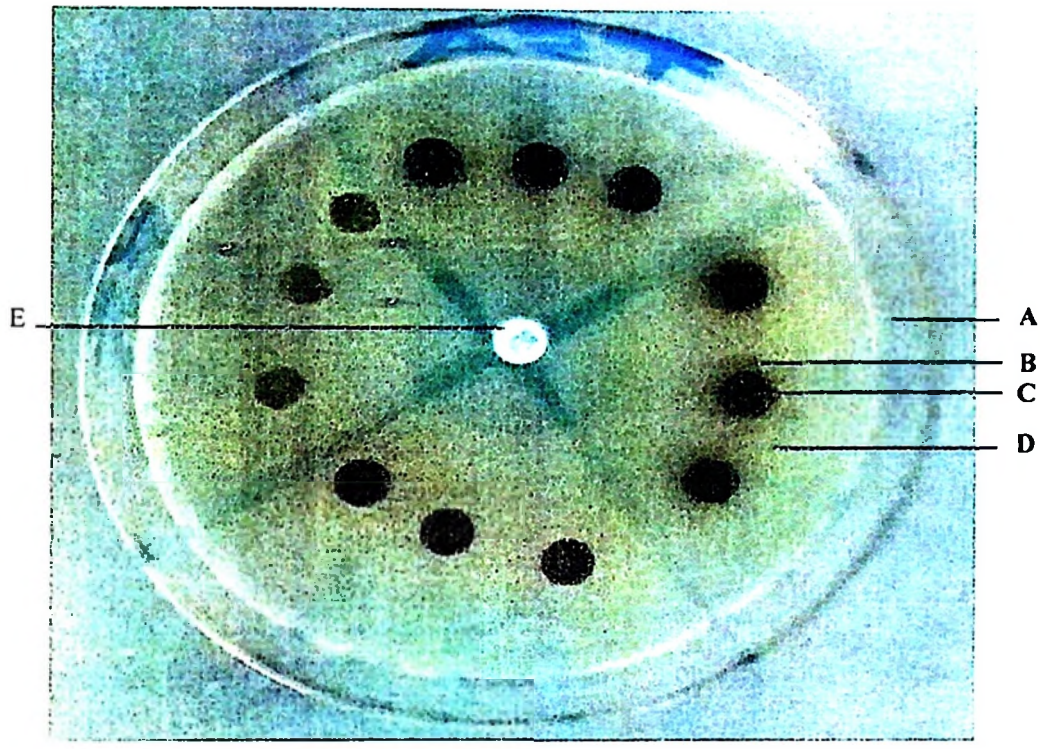
The assessment of cytotoxicity of the crude extracts was determined using human embryonic kidney (HEK 293) cells toxicity assay following already established procedures.[135,136] Briefly, HEK 293 cells were maintained in DMEM medium supplemented with 10% FBS. HEK were exposed to the extracts in TC-treated 384-well plates (Falcon). Plates were incubated for 72h at 37 °C, 5% CO_2 and 95% humidity, the total fluorescence (excitation/emission: 50/595 nm) was measured using an Envision plate reader (PerkinElmer). Puromycin 5 μM and 0.4% DMSO were used as positive and negative controls to obtain normalized % inhibition data, which was then used to calculate IC_{50} value, through a 4 parameter logistic curve fitting in GraphPad Prism v.6. The experiments were carried out in two independent biological replicates, each consisting of two technical replicates. The selectivity

index (SI) was computed with respect to HEK 293/3D7 SI ratio in comparison to puromycin, a standard nonselective compound.[114] All the bioassays were done at Eskitis Institute for Drug Discovery, Griffith University, Australia through existing collaboration involving Natural Products Research Group (NPRG) of Chemistry Department, UDSM and Halogen Bonding Group of the Gothenburg University, Sweden.

3.2.3 Antibacterial Assay

The antibacterial activities of crude extracts from *N. paucijuga* and *E. sacleuxii* were evaluated using five organisms of which *Staphylococcus aureus* (ATCC 25923) and *Bacillus subtilis* represented the Gram-positive, *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniea* (ATCC 708903), *Salmonella typhii* (ATCC 19430), *Pseudomonas aeruginosa* (ATCC 27853) represented Gram-negative bacteria. All the test organisms were obtained from the Department of Microbiology and Immunology, Muhimbili University of Health sciences (MUHAS), except *Bacillus subtilis* whose antibacterial screening was evaluated at the Department of Chemistry and Molecular Biology, University of Gothenburg, Sweden. The discs diffusion method was used to test for activity against the selected bacteria following already established protocol.[137] Discs with the diameter of 5 mm were prepared from a filter paper Whatman No. 1 of which 10 µL of the extracts drawn from the fresh prepared stock made by dissolving 500 mg of the extract in 1 mL of DMSO were placed. All discs were allowed to dry in air at room temperature for 24 hrs. The media were prepared by dissolving 23g of trytoptic soya agar (TSA) in 500 mL of distilled water. The TSA was produced by Liofilchem, Roseto d. Abruzzi, Italy whose chemical

composition consisted of pancreatic digest of casein 15 g/L, papaic digest of soya bean 5 g/L, sodium chloride 5 g/L and agar 15 g/L at pH of 7.2 ± 0.2 and 25 °C. The sterilized trytoptic soya agar was aseptically aliquoted at volumes of 25 mL into petri dishes and left to congeal. The inocolums equivalents to McFarland 0.5 turbidity were prepared into vials containing sterilized distilled water by emulsifying few colonies of the test bacteria from the sub-cultured plates. The agar was then inoculated aseptically with test organisms using streaking method. Thirteen discs (5 mm in diameter) were placed on the agar at the appropriately equidistant intervals using sterile needles, twelve for the extracts, and one for a standard drug. The plates were then incubated for 24 h at 37 °C. The zones of inhibition were measured in millimeters whereby the diameter was taken as a measure of antibacterial activity of the test extract. Each test was done in triplicate while using 10 µg/mL of Gentamicin as a standard drug. The antibacterial assays reported in this dissertation were carried out at the Institute of Traditional medicine (ITM) of the Muhimbili University of Health and Allied Sciences (MUHAS) and the Department of Chemistry and Molecular Biology of the University of Gothenburg.



A = Petri dish B = Zone of inhibition C = Filter paper dipped in a sample solution
D = Bacterial growth E = Disc coated with gentamycin

Figure 3.1: Petri Dish Showing the Disc Diffusion Method

3.2.4 Antifungal Assay

3.2.4.1 Assay against Yeast-like Fungi

Two fungi strain *viz. Candida albicans* (ATCC 13803) and *Cryptococcus neoformans* (ATCC 90112) were used as the test organisms for the determination of antifungal activities of crude extracts from *N. paucijuga* and *E. sacleuxii*. Test organisms were obtained from the Department of Microbiology and Immunology, Muhimbili University of Health sciences (MUHAS). The disc diffusion method was used to test for activity against the selected fungi.[137] The medium was prepared by dissolving 18 g of sabouraud dextrose agar (SDA) in 250 mL of distilled water. The SDA was produced by Liofilchem, Roseto d. Abruzzi, Italy whose chemical composition consisted of dextrose 40.0 g/l, peptic digest of animal tissue 5.0 g/L,

pancreatic digest of casein 5.0 g/L and agar 15 g/L at the pH of 5.6 ± 0.2 and 25 °C. The sterilized SDA was aseptically aliquoted at volumes of 30 mL into petri dishes and left to congeal. The general experiment procedures were conducted as prescribed in section 3.2.3. Each test was done in triplicate while using 20 µg/disc of clotrimazole as a standard drug. The zones of inhibition were measured in millimeters whereby the diameter was taken as a measure of fungal activity of the test extract.

3.2.4.2 Assay against Filamentous Fungus

Aspergillus niger strain (AZN 8240) was used as a representative filamentous fungus for the determination of antifungal activities of crude extracts from *N. paucijuga* and *E. sacleuxii*. The general experimental procedures for this assay were carried out as described in section 3.2.4.1. The antifungal assays reported in this dissertation were carried out at ITM, MUHAS.

3.2.5 Determination of Minimum Inhibitory Concentration (MIC)

Crude extracts which showed antimicrobial activities from the disc diffusion experiments were tested to determine the minimum inhibitory concentrations (MIC). The broth micro dilution method adapted from Yanez *et al.*[138] was used during the conduction of the experimental procedures. The growth medium was made by dissolving 3.8 g of tryptic soya broth (TSB) containing pancreatic digest of casein 17.0 g/L, papaic digest of soya bean 3.0 g/L, sodium chloride 5.0 g/L, dipotassium hydrogen phosphate 2.5 g/L, glucose monohydrate 2.5 g/L at pH of 7.3 and 25 °C obtained from Liofilchem, Roseto d. Abruzzi, Italy in 125 mL of distilled water. The

broth was warmed in a microwave for 2 minutes followed by sterilization at 121 °C, 130 mPa for 30 minutes in an autoclave. 50 µl of TSB were placed in each well of the microtiter plate. A 20 mg portion of the test sample was dissolved in 1 mL of DMSO of which 50 µL of the extract were added to the wells of the microtiter containing 50 µl of TSB followed by serial two fold dilution making the concentration of 5.00, 2.50, 1.25, 0.63, 0.31, 0.16, 0.08 and 0.04 mg/mL. 50 µL of the bacterial suspension equivalent to 0.5 McFarland turbidity was added in the wells of the microtiter plates. The plates were prepared in triplicate and incubated overnight at 37 °C. 30 µl of the 0.2% nitrotetrazoleum indicator were added into each well followed by further incubation for 2 hours. The colour change was assessed virtually and, the lowest concentration at which the colour change observed was considered as the MIC value. The average of the 3 values was calculated providing the MIC value of the tested extract. Gentamycin with the concentrations of 2.5×10^{-2}, 1.3×10^{-2}, 6.3×10^{-3}, 3.1×10^{-3}, 1.6×10^{-3}, 7.8×10^{-4}, and 3.9×10^{-3} and 2.0×10^{-4} mg/mL with sequential two fold dilution was used as a standard drug.

3.2.6 Determination of the Activity Index (AI)

The Activity Index (AI) of the test samples were calculated as the ratio of inhibition zone of the test sample to the inhibition zone of the standard drug, represented by the following formula:

$$AI = IZ_{TS}/IZ_{SD}$$

Whereby IZ_{TS} is the diameter of the inhibition zone of the test sample at a give concentration and IZ_{SD} is the diameter of the zone of inhibition of a standard drug at a known concentration.

3.3 Results and Discussion

3.3.1 Antiplasmodial and Cytotoxic Activity

Antiplasmodial activities of crude extracts from *N. paucijuga* and *E. sacleuxii* were evaluated against drug resistant *Plasmodium falciparum* (3D7 strain), the results of which are summarized in Table 3.1. The NPLE, NPSE, NPRE and ESLE exhibited antiplasmodial activity at IC_{50} value of 11.6, 8.4, 2.6 and 24.6 μg/mL, respectively. Antiplasmodial activity is considered when the IC_{50} value less than or equal100 μg/mL and further classified as high activities (2 – 40 μg/mL), moderate activities (41 – 75 μg/mL) and mild (76 – 100 μg/mL).[139] Thus, based on that classification, the results obtained from the present study revealed that all extracts NPLE, NPSE, NPRE and ESLE showed high activity against *P. falciparum* (3D7). The cytotoxicity evaluations of the extracts against HEK 293 cell lines revealed that ESLE and NPLE were not toxic at 40 μg/mL while NPRE and NPSE showed moderate cytotoxicity with an IC_{50} of 17.2 and 179.0 μg/mL, respectively. Selective index (SI) of the NPRE, NPSE, NPLE and ESLE against *P. falciparum* vs HEK 293 cell lines were 6.5, 21.0, 17.2 and 16.3 respectively. Since, the SI ratio below 16 against HEK 293 mammalian cells are considered to be toxic,[114] the results showed that despite of its potent antiplasmodial activity, NPRE (IC_{50} = 2.6; SI = 6.5) is nonselective against HEK 293 cell lines. On the other hand, NPLE (IC_{50} = 11.6; SI = 17.2) and ESLE (IC_{50} = 24.6; SI = 16.3) are marginally selective while NPSE could be considered safe as compared to puromycin standard drug. Therefore, the results reported in this work validate the use of extracts from the leaves of *Erythrina sacleuxii* as antimalarial plant in East Africa folk medicine. However, precaution should be taken

due to its marginal safety. Moreover, the results provide baseline information for the potentiality of the extracts from *Newtonia paucijuga* for antiplasmodial purposes.

Table 3.1: Antiplasmodial and Cytotoxic Activity of Crude Extracts from *N. paucijuga* and *E. sacleuxii*

Test Sample	Antiplasmodial (3D7), µg/mL	Cytotoxicity (HEK 293), µg/mL	Selectivity Index (SI), (HEK 293/3D7)
NPRE	2.6	17.2	6.5
NPSE	8.4	179	21.0
NPLE	11.6	Inactive	17.2
ESLE	24.6	Inactive	16.3
Chloroquine (Standard)	0.0045	0.06	4000
Puromycin (Standard)	0.023	0.36	15.9

Key:
NPRE = *Newtonia paucijuga* root extracts, NPSE = *Newtonia paucijuga* stem barks extracts
NPLE = *Newtonia paucijuga* leaves extracts, ELSE = *Erythrina sacleuxii* leaves extracts
Inactive = Not toxic at 40 µg/mL

3.3.2 Antibacterial Activity

Antibacterial activities of crude extracts from *N. paucijuga* and *E. sacleuxii* against *S. aureus E. coli*, *K. pneumoniea*, *S. typhii* and *P. aeruginosa* are shown in Table 3.2. The activity of the extracts were categorized as no activity, moderate and highly active if the activity index was 0.0-0.3, > 0.3-7.0 and > 0.7-1.0, respectively.[140] Therefore, close analysis of the data revealed that, *Newtonia paucijuga* leaves extracts (NPLE) and *Newtonia paucijuga* stem barks extracts (NPSE) exhibited antibacterial activity against *S. aureaus* with an activity index (AI) of 0.4 and 0.4 respectively. This indicated that both (NPLE) and (NPSE) were moderately active against *S. aureaus* as Gram-positive representative bacteria. On the other hand,

NPLE and NPSE were moderately active against Gram-negative bacteria *P. aeruginosa* and *K. pneumonia* with an activity index of 0.5, 0.5 and 0.4, 0.4, respectively. Thus, the bioactivities of both NPLE and NPSE against Gram (+) and Gram (-) bacteria suggested that the extracts would be possible source of broad spectrum antibacterial agents. *Newtonia paucijuga* root barks extracts (NPRE) and *Erythrina sacleuxii* leaves extracts (ESLE) had no activity against *S. aureus*. Contrary to lack of activities against Gram (+) bacteria, NPRE had moderate activity against Gram (-) bacteria *P. aeruginosa* with an AI value of 0.4 while ELSE showed less activity against *P. aeruginosa* and *K. pneumonia* at and AI value of 0.3 each. Thus, it was revealed that all the extracts were selectively active against some Gram (-) representative bacteria but remained inactive against other Gram (-) species (*E. coli* and *S. typhii*). Contrary to NPLE and NPSE, the activities of NPRE and ESLE were only manifested on Gram (-) species indicating that, the extracts may be possible narrow spectrum antibacterial agents.

The antibacterial activities of ESLE reported in the current work concur with other studies previously conducted on the *Erythrina* species. Djeussi *et al.* contended that leaves extract from *Erythrina sigmoides* are active against Gram-negative bacteria namely *E. coli*, *K. pneumoniae*, *Providencia stuartii* and *P. aeruginosa*.[141] Study conducted by Lagu and Frederick reported on the antibacterial activities of leaves extracts from *E. abyssinica* collected from Busenyi district in Uganda against gram-negative bacteria *P. aeruginosa* with the zone of inhibition of 8 mm, though the root and stem barks of the species showed antibacterial activities against both Gram-positive bacteria *S. aureus* and gram-negative bacteria *P. aeruginosa* with the zone

of inhibition diameter ranging from 7-15 mm.[142] Leaves extract of *E. abyssinica* collected from Mbarara and Ntungamo districts were inactive against the tested bacteria. Nagaraja *et al.* reported on the antibacterial activities of chloroform and ethanolic extracts of stem barks of *Erythrina mysorensis* at the concentration of 50, 100 and 200 µg/mL. Chloroform extract was active against Gram-negative bacteria *E.coli* and *P. aeruginosa* with the zone of inhibition diameter of 10, 12 and 22 mm; and 10, 14 and 20 mm, with an AI ranging from 0.63-1.28 and 0.42-0.83 respectively.[143] On the other hand, ethanolic extract was active against Gram-positive bacteria *S. aureus* and *Bacillus subtilis* with the zone of inhibition diameter of 10, 14 and 16 mm and 10, 12 and 16 mm, respectively with an AI ranging from 0.45-0.73. The stem barks of *Erythrina senegalensis* inhibited the growth of *S. aureus*, *E. coli*, *P. aeruginosa*, *Streptococcus pyogenes* and *S. typhii* at the concentration of 40 mg/mL with the zone of inhibition ranging from 4-14 mm.[144] Furthermore, the leaves extracts from *E. variegata* showed high activities against *S. aureus*, *P. aeruginosa*, *E. coli* and *P. vulgari*.[145]

The results from the leaves extract of *E. sacleuxii* indicated activity against Gram-negative bacteria which was similar to antibacterial activities of *E. abyssinnica* (Busenyi district), *E. sigmoides* and the chloroform extract of *E. mysorensis*.[141-143] However, the results were contrary to the antibacterial activities of *E. senegalensis* and *E. variegata* of which were reported to be active against both Gram-positive and Gram-negative bacteria with the exception of ethanolic extract of the stem bark of *E. mysorensis* which was reported to be active against Gram-positive bacteria.[143-145] Furthermore, the reported antibacterial activities of different parts of *Erythrina*

species were high compared to that of ESLE in terms of zone of inhibition diameter and activity index. Although, the activities of ESLE were moderate compared to the counterpart members of the genus *Erythrina* previously studied on the same, still the antibacterial activities of the extract were significant and hence *E. sacleuxii* leaves have potential contribution to the availability of other sources of antibacterial agents against Gram-negative bacteria.

On the other hand, the antibacterial activities of the extracts from root, stem barks and leaves of *N. paucijuga* were moderate. Moreover, on the best of the literature review, there was no any study on the antibacterial activity from the species of other members of the genus *Newtonia*. Thus, the reported results from this work suggest the foundation for more investigations on antibacterial agents on the *N. paucijuga* or other species in the genus *Newtonia*.

Table 3. 2: Antibacterial Activity of Crude Extracts from *N. paucijuga* and *E. sacleuxii*

Test Sample	BACTERIA									
	S. aureus		*P. aeruginosa*		*E. coli*		*K. pneumonia*		*S. typhii*	
	IZ_{TS}	AI	IZ_{TS}	AI	IZ_{TS}	AI	IZ_{TS}	AI	IZ_{TS}	AI
NPRE	0.0	0.0	9.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0
NPLE	8.5	0.4	10.0	0.5	0.0	0.0	8.0	0.4	0.0	0.0
NPSE	9.0	0.4	11.0	0.5	0.0	0.0	8.0	0.4	0.0	0.0
ESLE	0.0	0.0	6.0	0.3	0.0	0.0	7.0	0.3	0.0	0.0
Gentamycin (Standard)	21	1.0	22	1.0	21	1.0	23	1.0	18	1.0

Key:
NPRE = *Newtonia paucijuga* root extracts, NPSE = *Newtonia paucijuga* stem barks extracts
NPLE = *Newtonia paucijuga* leaves extracts, ELSE = *Erythrina sacleuxii* leaves extracts

3.3.3 Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentrations (MIC) of extracts NPRE, NPLE, NPSE and ELSE were determined against *S. aureus*, *P. aeruginosa*, *K. pneumonia* and *B. subtilis*. The evaluation for the first three test organisms was carried out at the ITM, MUHAS while the later organism was evaluated at the Department of Chemistry and Molecular Biology, UG, the results of which are summarized in Table 3.3. The NPLE and NPSE exhibited activity against *S. aureus*, *P. aeruginosa*, *K. pneumonia* and *B. subtilis* at MIC value of 2.5, 2.5, 5.0 and 0.3 mg/mL and 2.5, 1.3, 2.5 and 0.3 mg/mL, respectively. NPRE showed the activity against *P. aeruginosa* and *B. subtilis* at MIC value of 2.5 and 0.3 mg/mL, respectively while ESLE was active against *P. aeruginosa* and *K. pneumonia* at MIC value of 2.5 and 2.5 mg/mL, respectively.

Based on surveyed literature, plant extracts with MICs <100 µg/mL (0.1 mg/mL) are considered to be highly active antimicrobial agents, MICs ranging from 100 to 500 µg/mL (0.1 - 0.5 mg/mL) are classified as active, MICs from 500 to 1000 µg/mL (0.5 - 1 mg/mL) are considered as moderately active whereas MICs ranging from 1000 to 2000 µg/mL (1 - 2 mg/mL) are considered to have low activities and MICs > 2000 µg/mL (> 2 mg/mL) are considered as inactive.[146] Thus, based on such classification, the results obtained from the present study revealed that NPRE, NPSE and NPLE were active against *B. subtilis* (MIC = 0.3 mg/mL) while ELSE was not active against *B. subtilis*. NPSE and NPRE were moderately active against *P. aeruginosa* (MIC = 1.25 mg/mL each). On the other hand, NPLE (2.5 mg/mL) against *S. aureus* and *P. aeruginosa*, NPSE (2.5 mg/mL) against *S. aureus* and *K. pneumonia*, ELSE (2.5 mg/mL) against *P. aeruginosa* and *K. pneumonia* and NPLE (5.0 mg/mL)

against *K. pneumonia* were inactive. NPRE was not tested against *S. aureus* and *K. pneumonia* while ELSE was not tested against *S. aureus*. Therefore, this study revealed that, NPLE, NPSE, and NPRE had promising antimicrobial activities to some of the tested organisms.

Table 3.3: Minimum Inhibitory Concentrations (MIC) of some of *Crude* Extracts from *N. paucijuga* and *E. sacleuxii*

Test Sample	Minimum Inhibitory Concentration, MIC (mg/mL)			
	Test Organism			
	S. aureus	*P. aeruginosa*	*K. pneumonia*	*B. subtilis*
NPLE	2.5	2.5	5.0	0.3
NPSE	2.5	1.3	2.5	0.3
NPRE	NT	1.3	NT	0.3
ESLE	NT	2.5	2.5	0.3
Gentamycin (Standard)	$1.6x10^{-3}$	$2.0x10^{-4}$	$2.0x10^{-4}$	$2.0x10^{-4}$

Key:
NPRE = *Newtonia paucijuga* root extracts, NPSE = *Newtonia paucijuga* stem barks extracts
NPLE = *Newtonia paucijuga* leaves extracts, ELSE = *Erythrina sacleuxii* leaves extracts
NT = Not tested

3.3.4 Antifungal Activity

The antifungal activity of extracts NPRE, NPLE, NPSE and ELSE were evaluated against *Candida albicans*, *Cryptococcus neoformans* and *Aspergilus niger*. Results as summarized in Table 3.4 indicated that, all crude extracts were inactive against all tested fungi implying that the extracts are not good candidates for antifungal agents. Other studies on antifungal activity of Fabaceae species revealed some members in the family are active against fungi. For instance, Stem bark extract of *E. excelsa* was found to be active against some forms of bacteria, but was inactive against *C. albicans*, *C. neoformancs*, *Trychophyton mentagrophytes* and *microsporum*

gypsem.[147] The study on biological screening of Brazilian medical plants revealed the absence of antifungal activities for *Erythrina murungu* plant parts.[148] However, the ethanolic extracts of the stem bark of *E. mysorensis* revealed the presence of the antifungal activities against *C. albicans* and *A. niger*.[143] There is no literature reporting on the antifungal activities of members of the genus *Newtonia*.

Table 3.4: Antifungal activity of Crude Extracts from *N. paucijuga* and *E. sacleuxii*

Test Sample	FUNGUS					
	C. albicans		*C. neoformans*		*A. niger*	
	IZ_{TS}	AI	IZ_{TS}	AI	IZ_{TS}	AI
NPRE	0.0	0.0	0.0	0.0	0.0	0.0
NPLE	0.0	0.0	0.0	0.0	0.0	0.0
NPSE	0.0	0.0	0.0	0.0	0.0	0.0
ESLE	0.0	0.0	0.0	0.0	0.0	0.0
Clotrimazole (Standard)	21	1.0	20	1.0	21	1.0

Key:
NPRE = *Newtonia paucijuga* root extracts, NPSE = *Newtonia paucijuga* stem barks extracts
NPLE = *Newtonia paucijuga* leaves extracts, ELSE = *Erythrina sacleuxii* leaves extracts

CHAPTER FOUR

CONCLUSIONS AND RECOMMENDATIONS

4.1 Conclusions

The phytochemical investigations reported in this Dissertation were aimed at establishing antiplasmodial and antimicrobial activities crude extracts and isolated compounds from *Newtonia paucijuga* and *Erythrina sacleuxii* (family Fabaceae) in addition to elucidating the structures of their isolated constituents. Chromatographic separation of extracts from the leaves, stem barks and root barks of *N. paucijuga* yielded mearnstein-3-*O*-α-*L*-rhamnopyranoside (**2.26**), mixture of stigmasterol (**2.37a**) and β-sitosterol (**2.37b**), and benzyl-3,4,5-trihydroxybenzoate (**2.28**), respectively. Compounds **2.26**, **2.37a** and **2.37b** have been previously reported, **2.37a** and **2.37b** being ubiquitous plant metabolites whereas compound **2.28** is previously only known from synthetic source, hence its isolation from the root barks of *N. paucijuga* is hereby reported for the first time. On the other hand, chromatographic separation of extracts from the leaves of *Erythrina sacleuxii* yielded a new *nor*-sesquiterpenoid, sacleuxenone (**2.38**).

The isolated compounds were not assayed for antiplasmodial and antimicrobial activities due to insufficient amount. However, some of the isolated compounds are already reported to possess antimicrobial activities. The antimicrobial activities of the crude extracts of NPLE, NPSE, NPRE and ESLE were investigated and the results revealed that all extracts were active against some of the tested organisms. NPLE and NPSE were found to be moderately active against *P. aeruginosa* and *K. pneumonia* and NPRE had moderate activity against *P. aeruginosa*. ELSE had low

activity against *P. aeruginosa* and moderately active against *K. pneumonia*. Furthermore, the minimum inhibitory concentration, MIC of the extracts revealed activities ranging from 0.3 to 5.0 mg/mL against *S. aureus*, *P. aeruginosa*, *K. pneumonia* and *B. subtilis*. The present study revealed that, *N. paucijuga* and *E. sacleuxii* extracts had broad spectrum activities against the both Gram-positive and gram-negative bacteria. No antifungal activities were observed for the tested fungal strains namely, *C. albicans*, *C. neoformans* and *A. niger*.

Moreover, all extracts exhibited antiplasmodial activity against *P. falciparum* (3D7, Chloroquine-sensitive strain) at an IC_{50} value ranging 2.6 – 24. 6 μg/mL and Selective index (SI) ranging from 6.5 – 21.0 against human embryonic kidney (HEK 293) cell lines. The NPRE (IC50 = 2.6; SI = 6.5) showed potent antiplasmodial activity. Unfortunately, its non-selectivity indicated toxicity. NPLE, NPSE and ESLE were moderated active and marginal selectivity indicating that they are safe. The antiplasmodial activities together with high selective index and no toxicity of the some of the extracts reported in this work validate the traditional use of the extracts from *E. sacleuxii* leaves for treatment of malaria in various communities in East Africa.

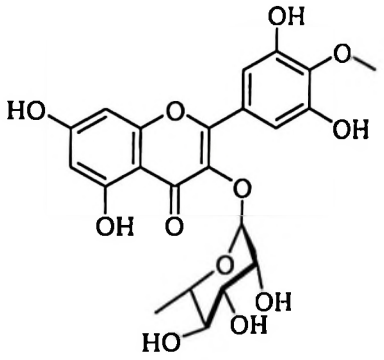
4.2 Recommendations

Sequential extraction procedures are highly recommended in order to overcome the challenge of working with more compounds possessing varying polarities at once. This will ensure isolation and characterization of more ingredients from the plants. Furthermore, since the amounts of the pure compounds obtained were insufficient to

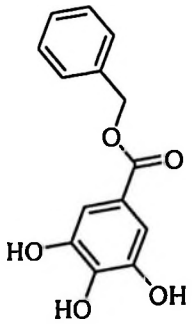
carry out many biological assays. Further studies to obtain more compounds or their laboratory synthesis and biological assays not reported in this work are recommended.

COMPOUNDS ISOLATED IN THIS STUDY

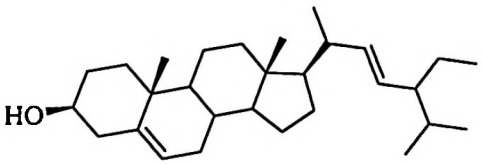
A: Compounds from *Newtonia paucijuga*



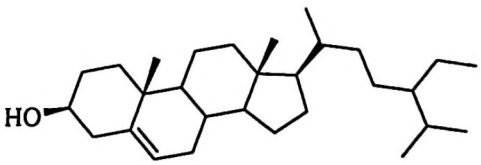
2.26



2.28

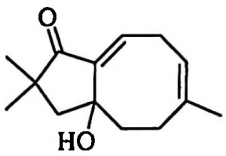


2.37a



2.37b

B: Compound from Erythrina sacleuxii



2.38
NEW

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