ISOLATION OF CHEMICAL CONSTITUENTS, ANTIBACTERIAL STUDIES OF THE LEAVES OF *Globimetulaoreophila* (Oliv.) van Tiegh AND POTENTIAL COMBINATION EFFECT WITH CIPROFLOXACIN

BY

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ZARIA-NIGERIA

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DECLARATION

I declare that the work in this dissertationentitled 'ISOLATION OF CHEMICAL CONSTITUENTS, ANTIBACTERIAL STUDIES OF THE LEAVES OF *Globimetula oreophila*(Oliv.) van Tiegh.AND POTENTIAL COMBINATION EFFECT WITH CIPROFLOXACIN' has been carried out by me in the Department of Chemistry. The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other Institution.

Hadiza Sada _____ _____

Signature Date

CERTIFICATION

This dissertationentitled 'ISOLATION OF CHEMICAL CONSTITUENTS, ANTIBACTERIAL STUDIES OF THE LEAVES OF *Globimetula oreophila*(Oliv.) van TieghAND POTENTIAL COMBINATION EFFECT WITH CIPROFLOXACIN' by Hadiza SADA meets the regulations governing the award of the degree of Science in Organic Chemistry of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

Globimetula oreophila(Loranthaceae) growing on *Citrus aurantifolia* is a mistletoe used widely in various cultures for the treatment of cancer, hypertension, diabetes, arthritis, heart problems, insomnia, infertility and pneumonia.Shade-dried powdered plant materialwas extracted using three methods; cold maceration, Microwave assisted extraction (MAE) and Polarity based extraction (PBE). The extractions afforded nine extracts: Crude ethanol (CEE=21.40g) from maceration; hexane (HEX=5.0g), Chloroform (CHL= 5.6 g), ethyl acetate (EA=6.9g) and ethanol (EE=26g) from MAE; and ethyl acetate (EA2=7.0g), chloroform (CHL2=7.5g), chloroform-ethanol (CE=1.14g) and ethanol (EE2=15.6g) from PBE..Repeated gel filtration of extract CE on sephadex LH-20 column eluting with methanol resulted in the isolation of two flavonoids;7-methoxyquercetin and Quercetin. The structures of these compounds were elucidated on the basis ofspectroscopic techniques (UV, FTIR, 1D and 2D NMR) and comparison with literature data.

Antimicrobial Studies was done using the agar well diffusion method which shows that CEE and EE1 had strong antimicrobial activity against all the tested organisms with zones of inhibition ranging from 10-27mmbut has no activity against*E. coli*.All the extracts showed strong activity against *K. pneumoniae* except extract HC which has no activity against all the isolates. The isolated compounds showed activity against all the bacterial strains except *E. coli* and *VRSA*.The extracts and isolated compounds showed potent synergistic activity with ciprofloxacin against the tested organisms.The findings of the study suggests that all the extracts tested have strong antimicrobial activity which might be due to the isolated phytochemical constituents.

Table of Contents

Page

Title Page ----------i

Declaration----------ii

Certification---------- iii

Acknowledgement---------- iv

Abstract---------- v

Table of Contents---------- vi

List of Figures---------- x

List of Tables---------- xii

List of Plates---------- xiii

Abbreviations---------- xiv

CHAPTER ONE ---------- 1

1.0 Introduction ---------- 1

1.1 Infectious Diseases ---------- 1

1.2 Combination Therapy ---------- 3

1.3 Statement of Research Problem 4

1.4 Justification 4

1.5 Aim 5

1.6 Objectives of the Study 5

CHAPTER TWO 6

2.0LITERATURE REVIEW 6

2.1 Loranthaceae 6

2.1.1 Ethnomedical uses of mistletoes (Loranthaceae Family) 7

2.2 *Globimetula oreophila* 7

2.2.1 Taxonomy 8

2.2.2 Ethno-medicinal uses of *Globimetula oreophila* and other species in the Genus 8

2.2.3 Biological activities found in the genus *Globimetula* 8

2.2.4 Phytochemistry of *Globimetula oreophila* and the genus *Globimetula* 9

2.2.5 Flavonoids 9

2.2.6 Compounds isolated from species of *Globimetula* 11

2.3 The host plant-*Citrus aurantifolia* (Rutaceae) **..... 12**

2.3.1 *Citrus aurantifolia* 12

2.3.2 Taxonomy 13

2.3.3Ethnomedical Uses of *Citrus aurantifolia* 14

2.3.4 Pharmacological Activities of *Citrus aurantifolia* 15

2.3.5 Phytochemical Constituent of *Citrus aurantifolia* 18

CHAPTER THREE 23

3.0 MATERIALS AND METHODS 23

3.1 Materials 23

3.1.1 Solvents/Reagents and Chromatographic Materials 23

3.1.2 Test Organisms----------

23

3.2Collection of Plant Material. 23

3.3Extraction 23

3.3.1 Cold Maceration..... **24**

3.3.2 Microwave assisted extraction (MAE) 24

3.3.3 Polarity Based Extraction (PBE) 24

3.4Preliminary Phytochemical Investigation..... 26

3.4.1 Test for Steroids/Terpenes 26

3.4.2Test for flavonoids 26

3.4.3Test for Alkaloids 27

3.4.4Test for tannins 28

3.4.5 Test for Anthraquinones---------- 28

3.4.6Test for saponins 28

3.5Chromatographic Procedure 29

3.5.1Thin Layer Chromatography (TLC) 29

3.5.2 Gel Filtration Chromatography..... 29

3.6Spectroscopic techniques: 30

3.7Antimicrobial assays..... 30

3.7.1 Determination of antimicrobial activity..... 30

3.7.2 Combination Effect..... 31

CHAPTER FOUR..... 32

4.0 RESULTS 32

4.1 Extraction---------- 32

4.2 Preliminary Phytochemical Investigation..... 33

4.3 Thin Layer Chromatography 34

4.4 Gel Filtration chromatography of Chloroform- ethanol extract (CE) 38

4.5 Isolation of SD1 and SD2..... 40

4.6 Analysis of SD1..... 41

4.6.1 TLC analysis of SD1..... 42

4.6.2Chemical test on compound SD1..... 42

4.6.3Melting Point of SD1..... 42

4.6.4Spectroscopic analysis of SD1..... 42

4.7 Analysis of compound SD2..... 57

4.7.1 TLC analysis of SD2..... 57

4.7.2Chemical test on compound SD2..... 58

4.7.3Melting Point of SD2..... 58

4.7.4Spectroscopic analysis of SD2..... 58

4.8 Antimicrobial Assays..... 74

4.8.1 Antimicrobial Activity---------- 74

4.8.2 Combination Effect----------

78

CHAPTER FIVE 80

5.0 Discussions..... 80

CHAPTER SIX 88

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS 88

6.1 Summary..... 88

6.2 Conclusion 88

6.3 Recommendations..... 89

REFERENCES..... 90

APPENDIX ---------- 101

LIST OF FIGURES

Figure Page

3.1: General Procedure for extracting plant tissues and fractionating into different classes according to polarity (Harborne, 1983) ---------- 25

4.1: UV spectrum of SD1 ---------- 43

4.2:FTIR spectrum of SD1 ---------- 44

4.3: Proton NMR spectrum of SD1 ---------- 45

4.4: Proton NMR spectrum of SD1 (expanded 6.0-8.0 ppm) ---------- 46

4.5: Carbon 13 NMR spectrum of SD1 ---------- 47

4.6: DEPT experiment of SD1 ---------- 48

4.7: ^{1}H-^{1}H COSY correlation spectrum of SD1 ---------- 49

4.8: HSQC spectrum of SD1 ---------- 50

4.9: HMBC Spectrum of SD1 ---------- 52

4.10: HMBC Spectrum of SD1 (expanded 3.9- 8.0 ppm) ---------- 53

4.11: NOESY spectrum of SD1 ---------- 54

4.12: UV spectrum of SD2 ----------
59

4.13:FTIR spectrum of SD2 ----------60

4.14: Proton NMR spectrum of SD2 ----------
61

4.15: Proton NMR spectrum of SD2 (expanded 6.0-8.0ppm) ----------62

4.16: Carbon 13 NMR spectrum of SD2 ---------- 63

4.17: DEPT experiment of SD2 ---------- 64

4.18: ^{1}H-^{1}H COSY correlation spectrum of SD2 ---------- 65

4.19: ^{1}H-^{1}H COSY correlation spectrum of SD2 (expanded 5.5- 8.5 ppm) ---------- 66

4.20: HSQC Spectrum of SD2 ---------- 67

4.21: HMBC Spectrum of SD2 ---------- 69

4.22: HMBC Spectrum of SD2 (expanded 6.0-8.0) ---------- 70

4.23: NOESY spectrum of SD2 ---------- 71

5.1: Proposed structure of SD1 ---------- 83

5.2: Proposed structure of SD2 ----------85

List of Tables

Table

Page4.1: Extracts obtained and their yields----------
32

4.2: Preliminary Phytochemical Investigation ----------
33

4.3: Summary of TLC profiles of Extract ----------

374.4: Fractions from Gel filtration Chromatography of extract CE ----------
39

4.5: TLC profile of SD1 using different solvent systems ---------- 41

4.6: Summary of 1D and 2D spectral data for compound SD1 ---------- 55

4.7: Comparison of 1D NMR data of SD1 with literature data---------- 56

4.8: TLC profile of SD2 using two different solvent systems ---------- 57

4.9: Summary of 1D and 2D spectral data for compound SD2 ---------- 72

4.10: Comparison of 1D NMR data of SD2 with literature data---------- 73

4.11: Antimicrobial Activity of Extracts CEE, HC and EE1 of *Globimetula Oreophila* and Ciprofloxacin ---------- 75

4.12: Antimicrobial Activity of Extracts CHL2, CE and EE2 of *Globimetula Oreophila* and Ciprofloxacin ---------- 76

4.13: Result of Antimicrobial Activity of compounds SD1, SD2 and Ciprofloxacin ---------- 77

4.14: Zones of inhibition of the combination effect of CHL2, CE, CEE, EE1 and EE2 with Ciprofloxacin ---------- 78

4.15: Zones of inhibition of the combination effect of the compounds SD1 and SD2 with Ciprofloxacin---------- 79

List of Plates

Plate **Page**

I: The plant *Globimetula oreophila*----------7

II: The host plant (*Citrus aurantifolia*) ---------- 13

III: TLC profiles of HEX, CHL, EA and EE extracts using HEX: EtOAc (10:1) [a] and that of HEX, CHL, EA, EE, and CEE using HEX: EtOAc (5: 2) [b] ---------- 34

IV: TLC profiles of CE using $CHCl_3$: EtOAc (3:1) [a], CHL2 and EA2 using HEX: EtOAc (9:1) [b] and EA and EE using EtOAc: $CHCl_3$: MeOH: H_2O (15: 8: 4: 1) ---------- 35

V: TLC profile of CE, CEE and EE2 using EtOAc: $CHCl_3$: MeOH: H_2O (15: 8: 4: 1) ------- 36

VI:TLC profile of CE collections Chloroform: methanol (20: 1) ---------- 38

VII: TLC profiles of compounds SD1 (a) and SD2 (b) using $CHCl_3$: MeOH (20:1) ---------- 40

List of Abbreviations

TLC	Thin Layer Chromatography
C.	*Citrus*
CE	Chloroform- ethanol extract
CEE	Crude ethanol extract
CHL	Chloroform extract
CHL2	Chloroform extract 2
EA	Ethyl acetate extract
EA2	Ethyl acetate extract 2
EE	Ethanol extract
EE1	Ethyl acetate + ethanol
EE2	Ethanol extract 2
EtOAc	Ethyl acetate
HC	Hexane + chloroform
HEX	Hexane extract
$CHCl_3$	Chloroform
MeOH	Methanol
EtOH	Ethanol
DMSO	Dimethyl Sulfoxide
R_f	Retention Factor
COSY	Correlation Spectroscopy
	Distortionless Enhancement by Polarizability

DEPT	Transfer
^{1}H NMR	Proton NMR
^{13}C NMR	Carbon 13 NMR
HMBC	Hetero nuclear Multiple Bond Correlation
HSQC	Hetero nuclear Spin Quantum Correlation

CHAPTER ONE

1.0 Introduction

Plant kingdom has served as an inexhaustible source of pharmaceuticals from time immemorial (Parikh *et a*l., 2005). The occurrence of natural products with medicinal properties has led to the widespread use of herbal remedies across the world. This is as a result of the development of several drugs and chemotherapeutics from medicinal plants (Gyawali, 2010). More studies have been conducted on the components and the pharmacological properties of these plants (Pieter and Vlietinck, 2005; Soares *et al*., 2005; Jung *et al*., 2006). There are over 10 dozens of drugs which are all single entities extracted from higher plants or modified further synthetically that are currently in use globally (Newman *et al*., 2000).

Interest in medicinal plants as a re-emerging health aid has been fuelled by the rising costs of prescription drugs in the maintenance of personal health and well-being, and the bio-prospecting of new plant-derived drugs (Lucy and DaSilva, 1999). The primary benefits of using plant-derived medicines are that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits at affordable costs (Iwu, *et al*., 1999).

1.1 Infectious Diseases

Infectious diseases account for approximately one-half of all deaths in tropical countries (Iwu, *et al*., 1999); the World Health Organization estimates that nearly 50,000 people die each day throughout the world from infectious diseases (WHO, 2004). Different antibiotics exercise their inhibitory activity on different pathogenic organisms. However, the development and spread of resistance to currently available antibiotics is a worldwide concern.

Antibiotics are defined as natural compounds, produced by microorganisms, with selective antibacterial activity that does not have any strong side effects on human (Chanda and Rakholiya, 2011). Their mechanism of action is either through killing the bacteria (bactericidal effect) or by inhibiting bacterial growth (bacteriostatic effect). The discovery of antibiotics had eradicated the infections that once ravaged humankind. But their indiscriminate use has led to the development of multidrug-resistant pathogens. For example, around 90–95% of *Staphylococcus aureus* strains worldwide are resistant to penicillin (Casal *et al*., 2005) and in most of the Asian countries 70–80% of the same strains are methicillin resistant (Chambers, 2001).

The increasing phenomenon of acquisition of resistance among microorganisms to antimicrobial drugs is attributed to the indiscriminate and improper use of current antimicrobial drugs (Usha *et al*., 2010). Clinically important bacteria are characterized not only by single drug resistance, but also by multiple antibiotic resistance (Levy, 2002). Drug resistance presents an ever increasing global health threat that involves all major microbial pathogens and antimicrobial drugs (Stuart and Bonnie, 2004; Olayinka et al., 2009); these are difficult to treat and are responsible for a variety of infectious diseases. The rate of emergence of antibiotic resistant bacteria is not matched by the rate of development of new antibiotics to combat them (Prescott and Klein, 2002). Antibiotics that work today may not work tomorrow. It is essential to investigate newer drugs to which there is lesser resistance (Sarkar *et al*, 2003). As resistance to old antibiotics spreads, the development of new antimicrobial agents has to be expedited if the problem is to be solved. However, record of rapid, widespread emergence of resistance to newly introduced

antimicrobial agents indicates that even new families of antimicrobial agents will have a short life expectancy (Coates *et al*., 2002).

The steadily increasing bacterial resistance to existing drugs is a serious problem, and therefore there is a dire need to search for new classes of antibacterial substances, especially from natural sources. Sometimes the use of single antibiotic does not produce the desired effective inhibitory effects and to overcome this, a combination of drugs often exercises their synergistic effect which surpasses their individual performance.

An alternative therapy to treat antibiotic resistant microorganisms is the use of plant extracts. There are several reports on the antimicrobial activity of different plant extracts that were effective antimicrobics(Vaghasiya *et al*., 2008; Fisgin *et al*., 2009; Jeong *et al*., 2009; Darwish and Aburjai, 2010; Yoon *et al*., 2011). However, the problem of drug resistance is on the increase. The need of the hour is to develop still newer, useful and important antimicrobial agents (Sharma and Kumar, 2006; Negi and Dave, 2010); or new ways to treat the resistant microorganisms. An alternative approach is the use of combination therapy i.e. synergism between known antibiotics and bioactive plant extracts.

1.2 Combination Therapy

Combination therapy can be used to expand the antimicrobial spectrum, to prevent the emergence of resistant mutants, to minimize toxicity and to obtain synergistic antimicrobial activity, it could be an alternative to monotherapy for patients with invasive infections that are difficult to treat, such as those due to multi-resistant species and for those who fail to respond to standard treatment (Kamatou *et al*., 2006).

As high level acquired resistance to conventional antibiotics is frequent, it is reasonable to use combination therapy in order to achieve bactericidal synergism. The combination can

be of different plant extracts or plant extracts with standard antibiotics or antibiotics with some chemicals. Such combinations against resistant bacteria will have different mechanisms of action and it may lead to new choices for the treatment of infectious diseases.

Synergism is defined as a positive interaction created when two agents are combined and together they exert an inhibitory effect (on the targeted organisms) that is greater than the sum of their individual effects. Plant antimicrobials have been found to be synergistic enhancers in that though they may not have any antimicrobial properties alone, but when they are taken concurrently with standard drugs they enhance the effect of that Drug (Kamatou*et al*., 2006).

1.3 Statement of Research Problem

Infectious diseases account for approximately one-half of all deaths in tropical countries (Iwu, *et al.*, 1999). This may be due to poverty, increasing incidence of multiple drug resistance and side effects associated with currently available antimicrobials. The increasing phenomenon of acquisition of resistance among microorganisms to antimicrobial drugs is attributed to the indiscriminate and improper use of current antimicrobial drugs (Usha *et al*., 2010).

1.4 Justification

This work was conducted because of the dire need to search for newer and more potent antimicrobial agents to which there is lesser resistance in a world where there is an alarming increase in multidrug resistance by microorganisms.There are many advantages of using antimicrobial compounds from medicinal plants, such as fewer side effects, better patient tolerance, less expensive, acceptance due to long history of use, and being

renewable in nature (Gur *et al*., 2006) and also higher plants represent a potential source of novel antibiotic prototypes (Parekh and Chanda, 2007). To the best of our knowledge, there is no reported work on the pharmacological activity of *Globimetula Oreophila* growing on *Citrus aurantifolia.* Also there is no report on the establishment of the phytochemical constituents of the plant as well as isolation and characterization of some bioactive compounds from the plant.

1.5Aim

The aim is to isolate and characterize some of the bioactive compounds in the plant*Globimetula oreophila*and investigate their combination effect with Ciprofloxacin.

1.6 Objectives of the Study

The aim will be achieved through the following objectives:

i. Preliminary Phytochemical screening of the extracts.

ii. Separation, purification and isolation of the bioactive constituents using chromatographic techniques.

iii. Characterization and structural elucidation of the isolated compound(s) using spectroscopic techniques (IR, UV ^{1}H and ^{13}C NMR and MS).

iv. Determination of the antimicrobial activity of the plant extracts and isolated compounds.

v. Investigation of the effect of extracts and isolated compound(s) on antibacterial activity of ciprofloxacin.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Loranthaceae

Mistletoe, with the common name(s) – bird lime and all heal – is a general term for woody shoot parasites in several plant families especially Loranthaceae and Viscaceae (Burkill, 1985, Parker and Riches, 1993, Polhill and Wiens, 1998) and most genera of African mistletoes belong to the family Loranthaceae (Polhill and Wiens, 1998). The Loranthaceae family has seven genera*Helixanthera, Berhautia, Englerina, Globimetula, Agelanthus*, *Tapinanthus* and *Phragmanthera* with about 60 or more species recognized in West Africa (Burkill, 1995) and the group term mistletoe is used for all these species. Besides, the host plant is considered as important as the mistletoe since distinction is made between hosts and not between mistletoes, implying that the biology of the Loranthaceae species is not clearly understood (Adesina *et al*,. 2013). This has implications on the medicinal use of the plant species in the Loranthaceae.

In West Africa, mistletoes are found on many tree crops of economic importance including the shea butter tree (*Vitellaria paradoxa* Gaertn. f.), the neem tree (*Azadirachta indica* L.), citrus species, especially sweet orange (*Citrus sinensis* L.), Lime (*Citrus aurantifolia*. L) and grape (*Citrus paradisi* L.), cocoa (*Theobroma cacao* L.) and rubber (*Hevea brasiliensis* Muell Arg) (Bright and Okusanya, 1998; Overfield *et al*., 1998; Gill and Onyibe, 1990; Begho *et al*., 2007).

2.1.1 Ethnomedical uses of Loranthaceae Family (mistletoes)

Herbal practitioners claimed a general use to counter sorcery and magical powers, to treat mental conditions, insomnia, sterility and health problems associated with urino-genital system, rheumatism and pain. Mistletoes of the Loranthaceae family are widely used in various cultures to treat various diseases including hypertension, cancer, and diabetes, or used as a diuretic agent (Burkill, 1985; Adodo, 2004; and Jadhav *et al*., 2010).

2.2 *Globimetula oreophila*

The plant *Globimetula oreophila* which belongs to the loranthaceae family of parasitic mistletoes grow on trees and shrubs forming a bush to about 1m diameter (Burkill, 1995).It is called afomo in Yoruba speaking area of Nigeria, Kauci in Hausa and Children's matches in Eastern Cameroon.



Plate I: The plant *Globimetula oreophila*

2.2.1 Taxonomy

Kingdom: *Plantae*

Phylum: *Magnoliophyta*

Class: *Magnoliopsida*

Order: *Santales*

Family: *Loranthaceae*

Genus: *Globimetula*

Species: *Oreophila*

2.2.2**Ethno-medicinal uses of *Globimetula oreophila* and other species in the Genus**

Globimetula oreophila have been reportedly used for the treatment of cancer, hypertension, diabetes, arthritis, heart problems, insomnia, infertility and pneumonia (Burkill, 1995; Ogbole *et al.*, 2013). The leaves of *Globimetula cupulata*is used in the management of *diabete*s *mellitus* and high blood pressure(Ojewole and Adewole, 2007).

Globimetula braunii is used by traditional healers in Nigeria for the treatment of cardiovascular diseases, diabetes, headache, rheumatic pains, sickle cell aneamia and pulmonary troubles, (Burkill, 1985).

2.2.3 Biological activities found in the genus *Globimetula*

Biological activities found in *Globimetula* species from various studies include antimicrobial and antimalarial activity in *G. oreophila* growing on *Cola nitida* (Ogbole *et al*, 2013) and *Azadirachta indica*(Dauda, 2016) respectively. *Globimetula braunii* has been

reported to have oxytocic (Le and Zam, 2008), laxative (Fred-Jaiyesimi *et al*., 2008), anti-oxidative(Okpuzor *et al*., 2009a), lipid lowering (Erukainure *et al*., 2011) and anticonvulsant activities (Musa *et al.*, 2014).Significant antioxidant and free radical scavenging activities (Akinmoladun *et al*., 2010), hypoglycaemic and hypotensive (Ojewole and Adewole, 2007) activities were found in Globimetula cupulata.

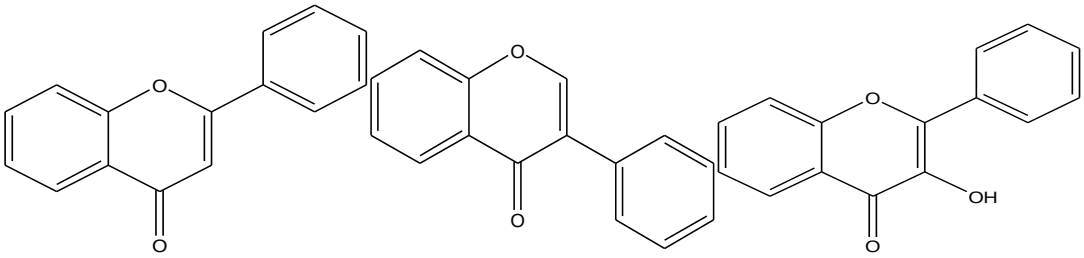
2.2.4 Phytochemistry of *Globimetula oreophila* and the genus *Globimetula*

To the best of our knowledge, no previous phytochemical investigations have been reported on *Globimetula oreophila* growing on *Citrus aurantifolia*but there are few reports on chemical investigations of *Globimetula oreophila* (growing on *Azadirachta indica and cola nitida)* and *Globimetula brauniii*resulting in the isolation of some phenolic compounds,particularly the flavonols. Previous phytochemical studies on *Globimetula oreophila*(growing on *Cola nitida*) revealed the presence of alkaloids, anthraquinones, flavonoids, saponins and tannins (Ogbole *et al.*, 2013).

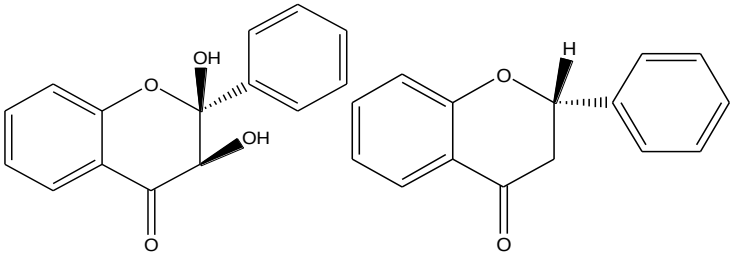
2.2.5 Flavonoids

Flavonoids continue to capture the interest of scientists from different disciplines because of their structural diversity, biological and ecological significance (Williams and Grayer, 2004). Their diverse biological activities include interesting areas such as anti-oxidant, anti-aging, anti-cancer, anti-bacterial and anti-viral (Chen *et al.*, 2002). The flavonoids are structurally related plants' secondary metabolites possessing aC6-C3-C6 skeleton where two aromatic rings are linked through a propanyl chain. They are sub-divided into sub-classes of flavones (I), Isoflavones (II), flavonols (III), flavanonols (IV), flavanones (V), flavans (VI), aurones (VII),chalcones (VIII), catechins (IX) and anthocyanins (X) according to the substitution on the C3 chain (Cushnie and Lamb, 2005; Vukics and

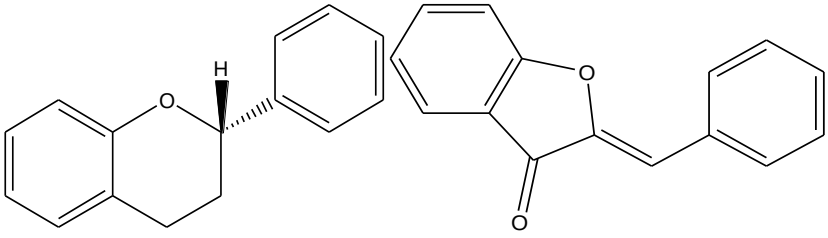
Guttman, 2008). Flavonoids are often hydroxylated in positions 3, 5, 7, 3' 4' and 5'and frequently, one or more of these hydroxyl groups are methylated or prenylated. They also combine with sugar molecules, (directly through the C-bond or through O-bond) to form glycosides, often with distinct biological activities (Reutrakul, *et al.* 2007).In some plant species, flavonoids form complexes of 2 or more molecules with profound chemical and pharmacological properties (Juliana-Feijo *et al*.,2007).



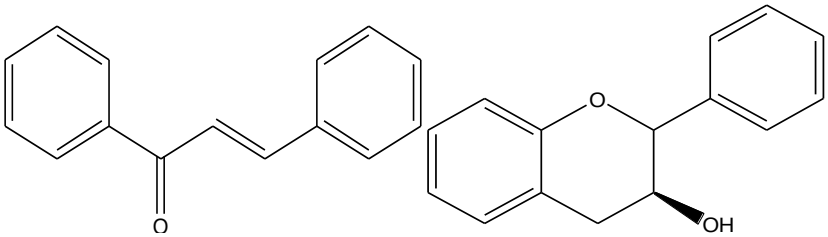
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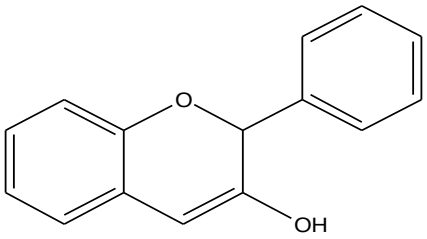
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(VI) (VII)



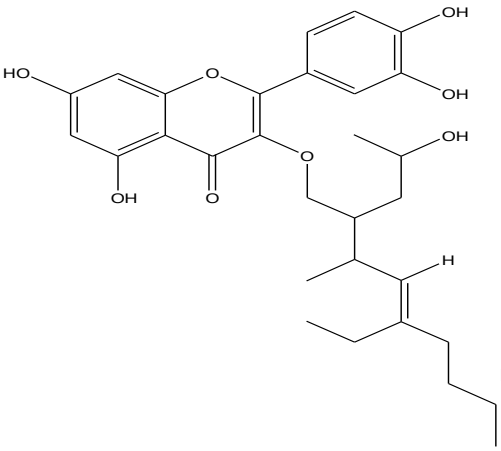
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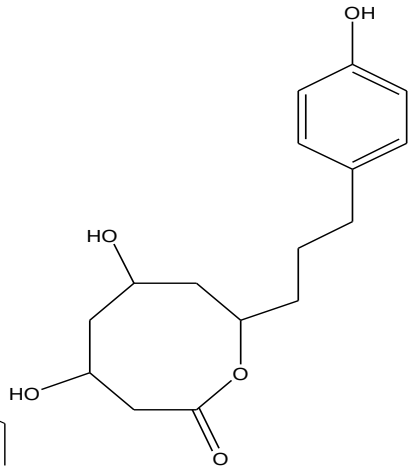
(X)

2.2.6 Compounds isolated from species of *Globimetula*

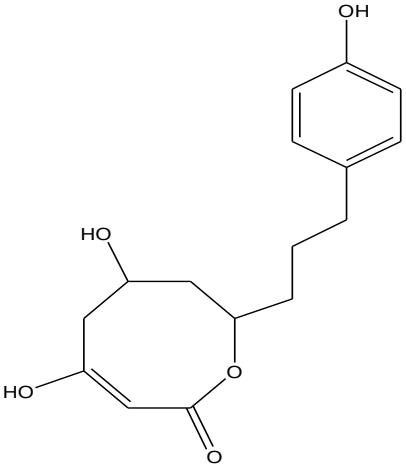
Isolation of a prenylated flavonol – [(E)-2-(3',4'-dihroxyphenyl)-3-(5''-ethyl-2''-(2'''-hydroxypropyl)-3''-methylnon-4''-en-1-yl)oxy-5,7-dihdroxy-4''H-chromene-4-one] (XI) was reported from the leaves of *Globimetula oreophila*growing on *Azadirachta indica*(Dauda, G. unpublished Msc dissertation, 2016). Two octalactones namely 4, 6-Dihydroxy-8-[2-(4-hydroxy-phenyl)-ethyl]-oxocan-2-one (XII) and 6-hydroxy-8-[2-(4-hydroxy-phenyl)-ethyl]-5, 6, 7, 8-tetrahydro-oxocin-2-one (XIII) were isolated from *Globimetula braunii* growing on *Pilostigma thonningii* (Abdullahi *et al*., 2014).



(XI)



(XII)



(XIII)

2.3 The host plant *Citrus aurantifolia* (Rutaceae)

2.3.1 *Citrus aurantifolia*

Citrus aurantifolia (Christm) Swingle is a small shrubby polyembryonic tree which is about 5 m tall. It is an evergreen and ever bearing tree cultivated in many countries all over the world and grows in hot subtropical or tropical regions such as Southern Florida, India, Mexico, Egypt, Nigeria and the West Indies (Patil, 2009).*Citrus aurantifolia* is commonly called Lime (Nigeria), Key lime, Mexican lime, Sour lime, Dayap, bilolo, Indian lime, Egyptian lime (USDA, 2013).



Plate II: The host plant (*Citrus aurantifolia*)

2.3.2 Taxonomy

Kingdom: *Plantae*

Phylum*: Magnoliophyta*

Class: *Magnoliopsida*

Order: *Sapindales*

Family: *Rutaceae*

Genus*: Citrus*

Species: *Citrus aurantifolia* (Sethpakdee, 1992).

2.3.3**Ethnomedical Uses of *Citrus aurantifolia***

C. aurantifolia in its natural state is widely used in West Africa, particularly in Nigeria where it is employed in herbal medicine to treat several illnesses. It forms an essential ingredient in the preparation of most herbal concoctions (Aibinu*et al*., 2007). The decoction of powdered leaves is drunk for stomach ache, used as eye wash and to bath feverish patient. Poultice of leaves are applied to ulcer wounds, used for skin disease and also applied to abdomen after child birth. Crushed leaves are applied to forehead to treat headache and it is squeezed near the nostril for irritant inhalation to treat nausea and resuscitate fainting individuals (Khan and Abourashed, 2010). Infusion of *C. aurantifolia* leaves have been given to treat fever with jaundice, sore throat and oral thrush (Kunow, 2003). A decoction of the flower is believed to help induce sleep for those with insomnia (Khan and Abourashed, 2010). In southwest Nigeria, the roots, bark, stem twigs, leaves and fruits are used in the treatment of malaria (Khare, 2007). In Nigeria, *C. aurantifolia* fruit juice is added to sugar and palm oil or honey to relief cough. It is also used as facial wash to rejuvenate the skin and given in pure form as remedy for diabetes. The diluted juice is used for mouth wash to treat sore mouth and sore throat (Aibinu*et al*., 2007; Khare, 2007). The juice has been used to treat irritation, diarrhoea and swelling due to mosquito bites. It is sometimes incorporated into weight management diet (Akhtar, 2013).

Decoction of roots is used to treat dysentery, diarrhea, gonorrhea and fever (Kunow, 2003). The oil extracted by steam distillation of the fruit rinds is used for cold, sore throat, bronchitis, asthma,arthritis and obesity (Kunow, 2003). It is also used as an astringent and toning action to clear oily skin acneand treat cuts (Kunow, 2003).

2.3.4 Pharmacological Activities of *Citrus aurantifolia*

Pharmacological activities of the extract of different parts of *C. aurantifolia* have been studied and it has been shown that the plant possesses the following pharmacological activities:

2.3.4.1 Antibacterial activity

Studies revealed that the extracts of root of *C. aurantifolia* have been found to be effective in inhibiting the growth of *Staphylococcus aureus, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Beta-haemolytic streptococci, Escherichia coli* and *Neisseria gonorrhoeae* (Aibinu*et al*., 2007), the fruit was found to inhibit anaerobicfacultative bacteria (Rahman*et al*., 2011) and the planthas been shownto have significant antimycobacterial activity especiallyagainst the isoniazid-resistant strain of Mycobacteria (Camacho-Corona*et al*., 2008).

2.3.4.2 Antifungal and antiaflatoxigenic activity

Studies on the essential oil of *C. aurantifolia* shows that the plant has inhibitory action against *Phaeoramularia angolensis*(Dongmo *et al*., 2009), *Aspergillus niger, Aspergillus parasiticus* and its aflatoxins and *Candida albicans* (Aibinu*et al*., 2007).

2.3.4.3 Anticancer/ cytotoxic activity

C. aurantifolia has been shown to inhibit colon cancer (Jayaprakasha*et al*., 2008), breast cancer, neuroblastoma (Poulose*et al*, 2005), pancreas cancer (Patil, 2009) and prostate cancer cells (Gao*et al*, 2006). Its essential oilhas 78% inhibition of human colon cancer cells (Poulose*et al*, 2005).

2.3.4.4 Antioxidant activity

Studies of the juice and fruit peels (Boshtam*et al*., 2011) as well as leaves (Loizzo*et al*, 2012) of *C. aurantifolia* revealed that the plant has a concentration dependent effect on low density lipoprotein (LDL) oxidation.

2.3.4.5 Immuno-modulatory activity

C. aurantifolia juice has been shown to possess immune modulatory activity when it was tested in mitogen activated culture mononuclear cells. The result revealed that the juice could significantly inhibit the proliferation of phytohaemaglutinin activated mononuclear cells at 250 and 500 microgram per litre of the juice extract (Gharagozloo and Ghaderi, 2001).

2.3.4.6 Antiobesity activity

Studies on the effect of essential oil of *C. aurantifolia* on weight gain in mice showed that mice treated with theextract displayed a reduction in both the amount of food intakeand body weight compared with the control group (Asnaashari*et al*., 2010). Anotherinteresting finding was that co-administration of *C.aurantifolia* essential oil and ketotifen caused a significant suppression of weight gain and also decreased weight gain in mice (Asnaashari*et al*., 2010).

2.3.4.7 Antifertility activity

Citrus aurantifolia juice has been shown to reduce the number of ova shed in Sprague-Dawley rats and also caused irregularities of the estrous cycle, partially blocked ovulation and also caused irregularities in the histology of the ovaries and uterus and thus may

possibly compromise fertility (Bakare*et al*., 2012). Its juice showed abortificient effect, although it does not possess teratogenic effect on foetus (Bakare*et al*., 2012). In Nigeria, a survey conducted by Imade *et al*., 2005 and Mairiga *et al*., 2010 revealed that it is a common practice for women to use juice solutions of the plant as a post coital douche with the believe that it prevents pregnancy.

2.3.4.8 Anticholinesterase and antiplatelet activity

The extracts of the fruit peels, leaves and the essential oil of the leaves of *C. aurantifolia* showed anticholinesterase and butyrylcholinesterase activity (Loizzo et al, 2012; Chaiyana and Okonogi, 2012). Similarly, its hydroalcohol extract in the form of tincture has been found to have anti-platelet aggregation activity. It was found to have significantly inhibited ADP and epinephrine-induced platelet aggregation in a concentration dependent manner (Piccinelli*et al*., 2008).

2.3.4.9 Cardiovascular activity

Akhtar,2013 evaluated the cardiovascular effects of *Citrus aurantifolia* fruit on three hypertensive models and found that the methanol extract of the fruit produces hypotensive andantihypertensive effects. Mechanisms responsible for these hadbeen proposed by Souza *et al*., 2011 to be related tocardiodepression and vaso-relaxation.

2.3.4.10 Effects on the bone

The efficacy of *Citrus aurantifolia* against osteoporosis was evaluated in an ovariectomized rat model, and the result revealed that the administration of citrus extracts

increased the trabecular bone mineral content, and bone mineral density of tibia, improved the levels of phosphorus and calcium. The result demonstrates that *the plant* possesses the ability to reduce bone loss (Shalaby*et al*., 2011).

2.3.4.10 Antihelmintic activity

Citrus aurantifolia fruit juice and peels have been shownto possess a remarkable anthelmintic activities against*Heligmosomoides bakeri*. The *in vitro* and *in vivo* anthelminticstudy revealed that *Citrus aurantifolia* fruit juice and peelssignificantly inhibited the hatching of *H. bakeri* eggs andkilled the larvae of the helminth as well as reduced the wormburdens in mice in a concentration and dose-dependent manner(Enejoh*et al*., 2014; Enejoh *et al*., 2015).

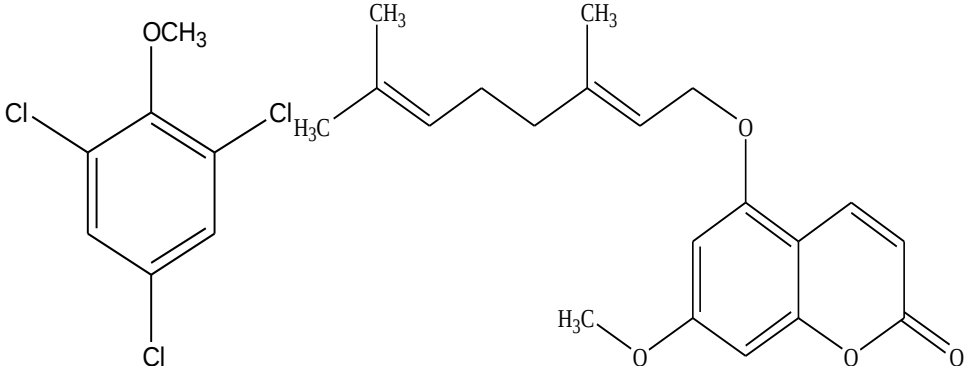
2.3.4.12 Other effects

Citrus fruits are highly recommended for persons suffering from kidney stones, gout and arthritis. Thejuice contains potassium citrate which prevents the formation of kidney stones and eases their dissolution (Roger, 2002). Due to the high content of vitamin C, citrus fruits are used in the treatment ofscurvy. The anti-scurvy effect of citrus fruits is very strong because of the balanced composition of organic acids and minerals (Okwu, 2008).

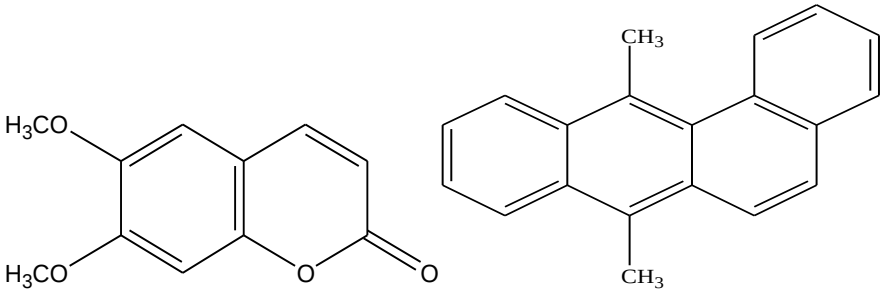
2.3.5 Phytochemical Constituents of *Citrus aurantifolia*

Preliminary phytochemical screening of *Citrusaurantifolia* fruit and other parts showed the presence of alkaloids, flavanoids, tannins, saponins, steroids, cardiacglycosides, carbohydrates, phenols and reducing sugars (Akhtar,2013;. Dongmo*et al*., 2009; Enejoh*et al*., 2015; Wang *et al*., 2007).Detailed analysis of *C. aurantifolia* have led to the

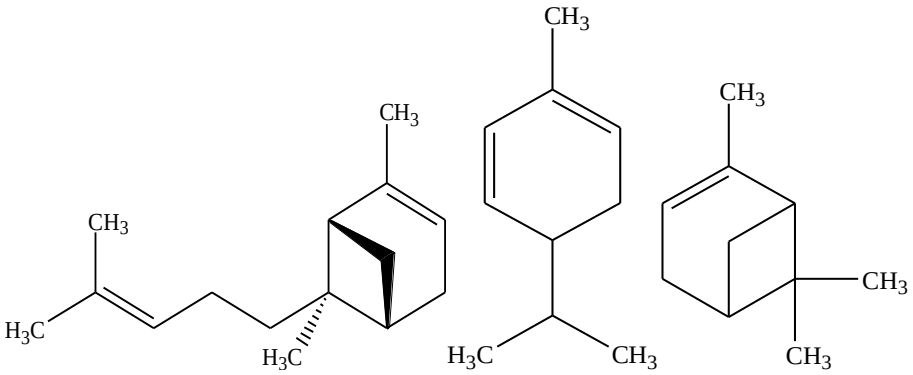
identification of phytochemicals from the plant, which include: 2,4,6-trichloroanisole (XIV); 5-geranoxy-7-methoxy-coumarin (XV); 6,7-dimethoxycoumarin (XVI); 9,10-dimethyl-1,2-benxanthracene (XVII); a-bergamotene (XVIII); a-phellandrene (XIX); a-pinene (XX); aterpineol (XXI); b-pinene (XXII), d-limonene (XXIII);camphene (XXIV); p-cymene (XXV); apigenin (XXVI); citronellol (XXVII); fenchol (XXVIII); imperatorin(XXIX);kaempferol (XXX);nobiletin (XXXI); o-cymene (XXXII); phellopterin (XXXIII);quercetin (XXXIV); rutin (XXXV); sabinene (XXXVI) and terpinolene (XXXVII)(Khan and Abourashed, 2010; Loizzo*et al*., 2012; Chaiyana and Okonogi, 2012; . Berhow*et al*., 1994).



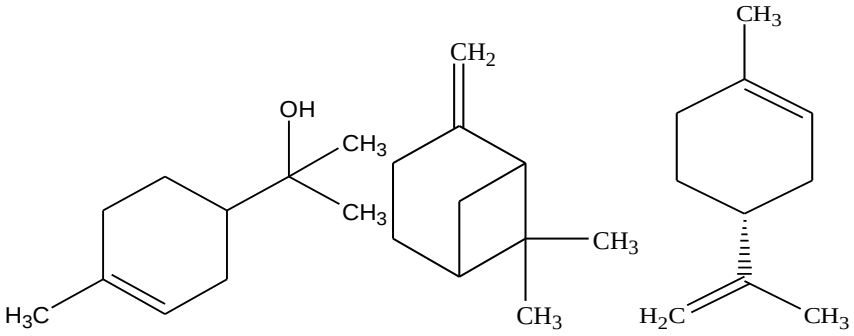
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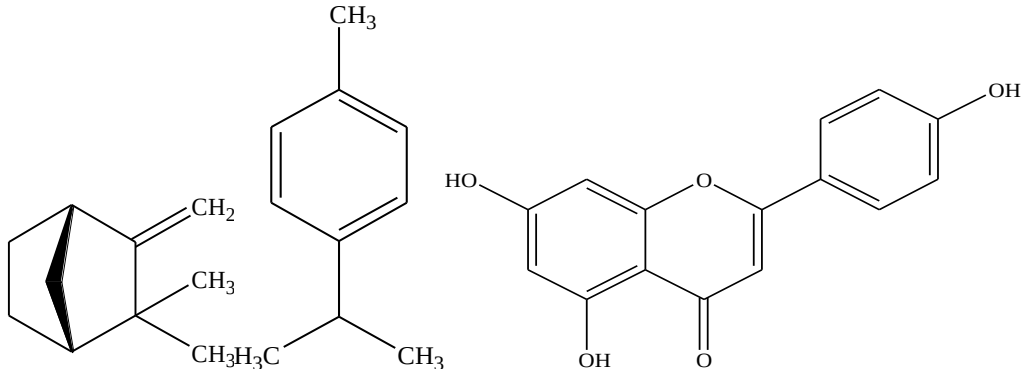
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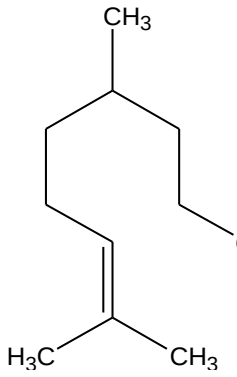
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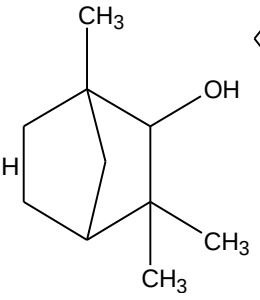
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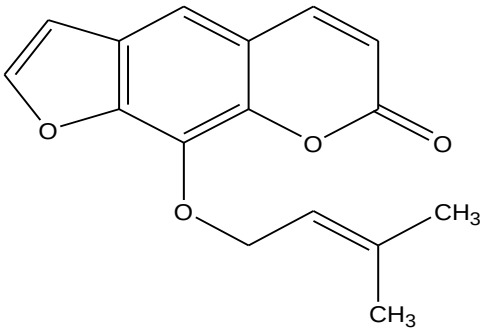
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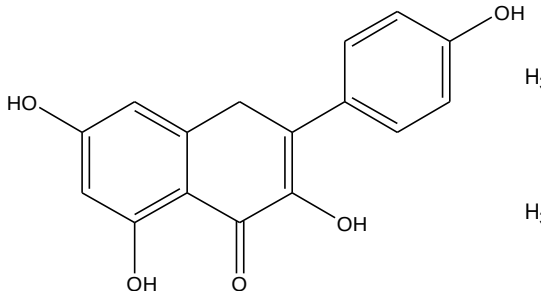
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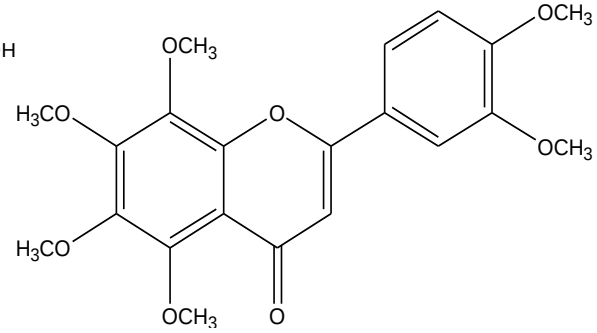
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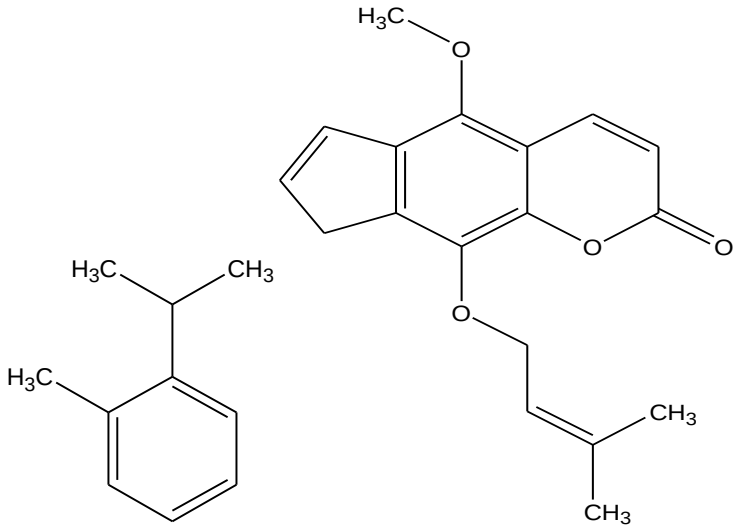
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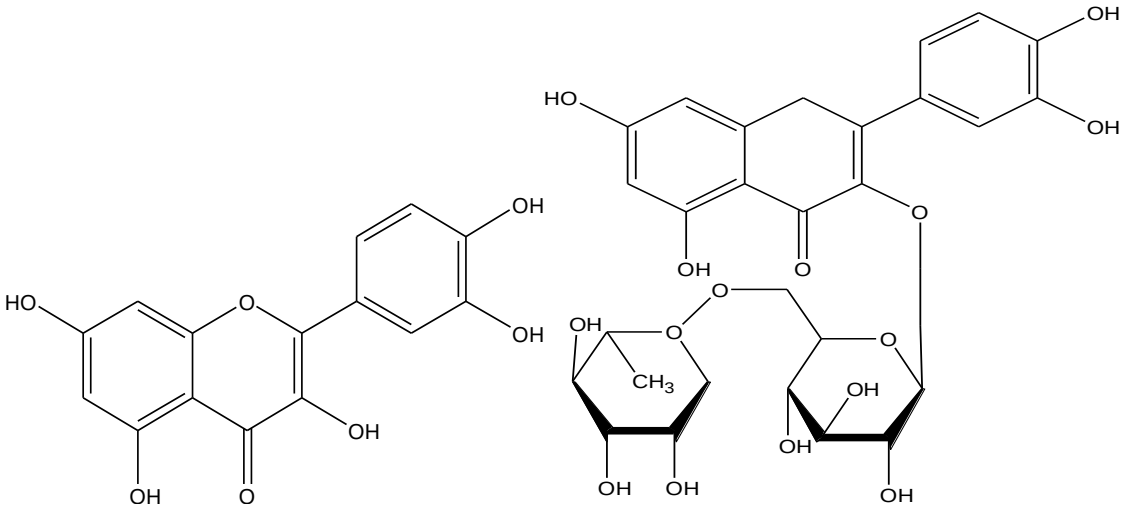
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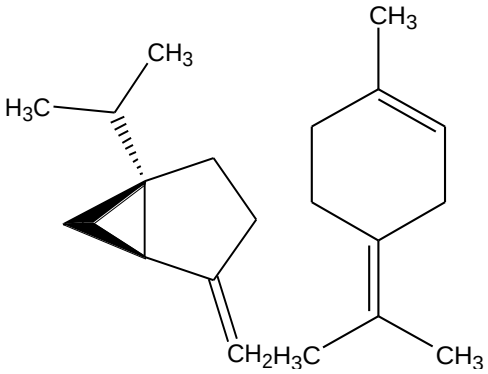
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CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Solvents/Reagents and Chromatographic Materials

Solvents used which include methanol, ethanol, ethyl acetate, chloroform, n-hexane and water were of general purpose grade and were distilled once before use. Chromatographic materials include: aluminium pre-coated thin layer chromatography (TLC) plate 60 F_{254}, sephadex LH 20 and chromatographic tank. Drug used was ciprofloxacin.

3.1.2 Test Organisms

The microorganisms tested include Vancomycin resistant *Staphylococcus aureus* (VRSA), methicillin resistant*Staphylococcus aureus* (MRSA*), Bacillus substilis, Salmonella typhi, Klebsiella pneumonia* and *Escherichia coli.* Clinical isolates of the organisms were obtained from the Department of Pharmaceutics and Pharmaceutical Microbiology, Ahmadu Bello University, Zaria, Nigeria.

3.2 Collection of Plant Material.

The plant sample, *Globimetula oreophila* was collected around Area BZ, Ahmadu Bello University, Zaria staff quarters. The plant was authenticated by Namadi Sunusi of the Herbarium unit, Department of Biological Sciences, Ahmadu Bello University Zaria-Nigeria by comparing with an existing specimen with voucher number 2839. The plant material was air-dried and pulverised into powder using pestle and mortar.

3.3 Extraction

The powdered plant material was extracted using three different procedures as follows:

3.3.1 Cold Maceration

The powdered plant material (200 g) was extracted with 2 L of ethanol for 2 days using the cold maceration technique. The extract was concentrated in vacuo at 40^0C to afford a gummy dark green product (21.40 g) and was referred to as crude ethanol extract (CEE).

3.3.2 Microwave Assisted Extraction (MAE)

The pulverized plant material (600 g) was divided into three different portions, a small amount of water (50 mL) was added to each portion and allowed to stand for 30 minutes. Afterward they were placed in a microwave under low heat ($40\ ^0$C) and microwaved at 3 minutes pulses, removed and allowed to cool. The procedure was repeated 5 times for each sample. The microwaved plant material was then extracted exhaustively using n-hexane, chloroform, ethyl acetate and ethanol in increasing order of polarity, the solvent in each case was removed *in-vacuo* to yield 5.0g of n-hexane (HEX), 5.6g of chloroform (CHL), 6.9g of ethyl acetate (EA) and 26.0g of ethanol (EE) extracts respectively. After preliminary phytochemical screening, n-hexaneand chloroform, ethyl acetate and ethanol extracts obtained were merged because they show similarity in their TLC profile to be HC and EE1 respectively for the purpose of antimicrobial assays.

3.3.3 Polarity Based Extraction (PBE)

The method described by Harborne (1983) was adopted with some modification (Figure 3.1)

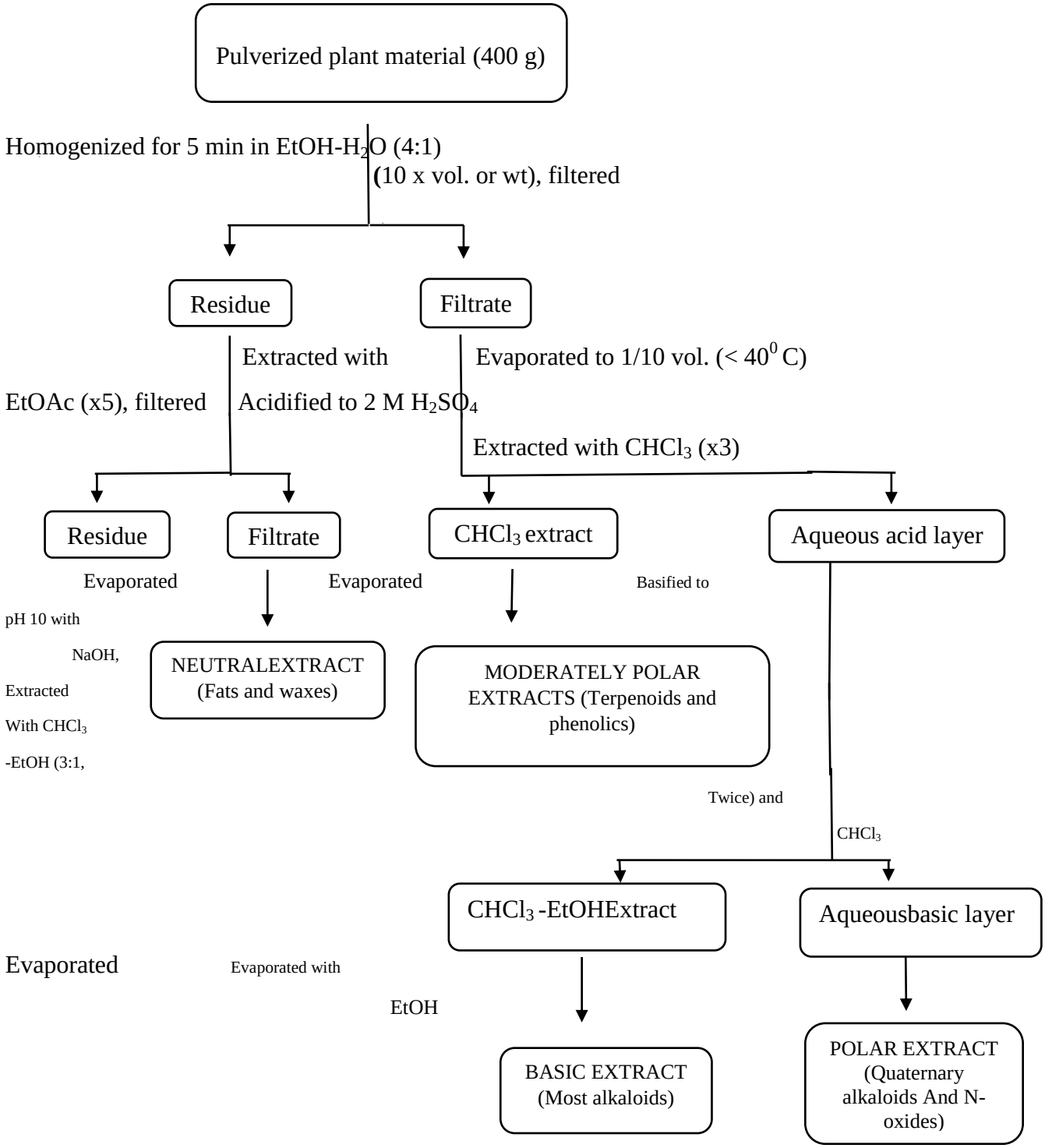


Fig. 3.1 General Procedure for extracting plant tissues and fractionating into different classes according to polarity (Harborne, 1983).

Four extracts were obtained; the ethyl acetate (EA2= 7.0g), Chloroform (CHL2= 7.5g), Chloroform- ethanol (CE=1.14g) and ethanol (EE2=15.6g).

3.4 Preliminary Phytochemical Investigation

The crude ethanol, hexane, chloroform, ethyl acetate, ethanol and Chloroform-Ethanol extracts of *Globimetula oreophila* were subjected to preliminary phytochemical analysis using standard methods (Silva *et al.,* 1998; Trease and Evans, 2002 and Sofowora, 1993).

3.4.1 Test for Steroids/Terpenes

3.4.1.1 Liebermann-Buchard test

The extract (0.2 g) was dissolved in chloroform and 2 drops of acetic anhydride were added followed by 1 ml concentrated sulfuric acid. The mixture was carefully mixed and the resulting solution was observed for blue, green, red or orange color that changes with time (Silva *et al.,* 1998).

3.4.1.2 Salkowski test

The extract (0.1 g) was dissolved in 1ml chloroform and to it 1ml of concentrated sulfuric acid was added down the test tube to form two phases. Formation of red or yellow coloration at the interphase was taken as an indication for the presence of sterols/triterpenes. (Silva *et al.,* 1998).

3.4.2 Test for flavonoids

3.4.2.1 Shinoda test

To 1 ml methanol solution of the extract, three pieces of Magnesium chips were added followed by 3 drops of concentrated hydrochloric acid. Appearance of an orange, pink or red to purple color indicates the presence of flavonoids. (Sofowora, 1993).

3.4.2.2 Sulphuric acid test:

The sample (0.1g) was dissolved in concentrated sulfuric acid. Formation of deep yellow to red solution indicates the presence of flavonoids (Silva *et al.*, 1998).

3.4.2.3 Ferric Chloride test

Extract (0.5 g) was boiled with water and filtered. To 2ml of the filtrate, two drops of freshly prepared ferric chloride solution was added; green, blue or violet colorations indicate the presence of phenolic hydroxyl group (Trease and Evans, 2002).

3.4.2.4 Sodium Hydroxide test

To the extract (0.1g), 10% sodium hydroxide solution was added. This was followed by few drops of concentrated hydrochloric acid (Silva *et al*., 1998).

3.4.3 Test for Alkaloids

Extract (0.5g) was stirred with 5ml of 1% aqueous hydrochloric acid on a water bath and filtered. 3ml of the filtrate was divided into three portions. To the first portion few drops of freshly prepared Dragendoff reagent was added and observed for formation of orange to brownish precipitate. To the second portion 1 drop of Mayer reagent was added and observed for formation of white to yellowish or cream color precipitate. To the third portion 1 drop of Wagner reagent was added to give a brown or reddish or reddish- brown precipitate (Silva *et al.*, 1998).

3.4.4 Test for tannins

The extract (0.2 g) was boiled with water and filtered. Two drops of ferric chloride was added to the filtrate, formation of a blue-black, or green precipitate was taken as evidence for the presence of tannins (Trease and Evans, 2002).

3.4.5 Test for anthraquinones

3.4.5.1 Free anthraquinones

The extract (0.5 g) was shaken with 10 ml of benzene, the content was filtered, and 5ml of 10% ammonia solution was added to the filtrate, the mixture was shaken. Presence of a pink, red or violet color in the ammoniacal layer (Lower phase) indicates the presence of free anthraquinone (Trease and Evans, 2002).

3.4.5.2 Combined anthraquinones

The extract was boiled with 10 ml of aqueous sulphuric acid and filtered hot. The filtrate was shaken with 5ml benzene, the benzene layer was separated and half its own volume, 10% NH_4OH was added. A pink, red or violet colouration in the ammonia phase (lower phase) indicates the presence of combined anthraquinone or anthraquinone derivative (Trease and Evans, 2002).

3.4.6 Test for saponins

The extract (0.5 g) was shaken with water in a test tube. Frothing which persisted for 15 minutes indicates the presence of saponins. (Silva *et al.*, 1998)

3.5 Chromatographic Procedure

3.5.1 Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) was carried out on all the extracts using silica gel 60 PF_{254}pre-coated TLC plates (layer thickness of 0.2mm) by one way ascending technique. Spots were applied manually using capillary tube; plates were dried using air blower and developed at room temperature using an air tight chromatographic tank.Spots on TLC plates were visualized under UV light (254 and 366 nm) and spraying with 10% sulphuric acid, followed by heating at 110˚C for 5-10 min.Various solvent systems were used depending on the material, and they include:

a. Hexane: Ethyl acetate (5:1)

b. Hexane: Ethyl acetate (10:1)

c. Chloroform: Methanol (20:1)

d. Chloroform: Ethyl acetate (3:1)

e. Ethyl acetate: Chloroform: Methanol: Water (15: 8: 4: 1)

3.5.2 Gel Filtration Chromatography

Sephadex LH-20 was used as the stationary phase. The Sephadex was suspended in the eluting solvent (methanol) and allowed to saturate for 24 hours prior to use. It was then transferred into the column and allowed to set, 0.6g of the $CHCl_3$– EtOH extract (CE) was dissolved in a minimum amount of eluting solvent and applied as a thin band on top of the column. It separated into 3 distinct bands in the column which gave 24 fractions. The fractions were further pooled together based on their TLC profile to give four major

fractions coded CE1- CE4. CE2 and CE4 consisting of just one spots were labeled as compounds SD1 and SD2 respectively. Repeated gel filtration of CE3 consisting of 3 spots yielded more of SD1 and SD2.

3.6 Spectroscopic techniques:

a) UV spectroscopy (Hitachi U-3200 spectrophotometer). Methanol was used as the solvent.
b) FTIR spectroscopy (Shimadzu FT-IR8 400S Fourier Transformed Infrared spectrophotometer).
c) 1D and 2D NMR Spectroscopy

NMR spectra of the two compounds isolated (SD1= 5 mg and SD2= 6 mg) were obtained on a Bruker AVANCE spectrometer (600 MHz) using DMSO as solvent. Chemical shift values (δ) were reported in parts per million (ppm) relative to TMS standard.

3.7 Antimicrobial assays

3.7.1 Determination of antimicrobial activity

Antimicrobial activity was measured using agar well diffusion method according to the National Committee for Clinical Laboratory Standard (NCLSI, 2011). Extracts CEE, HC, EE1, CHL2, CE and EE2 of *Globimetula oreophila* (at concentrations of 0.625 mg/ml – 10mg/ml) and the two isolated compounds SD1 and SD2 (at concentration of 0.1 mg/ml) were tested against the organisms. Briefly, Petri plates containing approximately 25-30 ml of Mueller Hinton agar medium were inoculated using a cotton swab with a 4-6 hr old culture of each bacterial strain. Wells of about 10 mm in diameter were punched in the agar and filled with 100µl of the appropriate concentration of the extracts. 100µl of DMSO (2mg/ml) was used as the negative control and no inhibition was observed, Ciprofloxacin

standard (0.03 mg/disc) was placed on the agar plates as positive control and 30 min was allowed for the extracts to diffuse into the agar. The plates were incubated at 37^0C for 20 hrs and the antimicrobial activity was assessed by measuring the inhibition zone diameters around the well.

3.7.2 Combination Effect

Combination effect of the extracts with Ciprofloxacin was determined according to the National Committee for Clinical Laboratory Standard (NCLSI, 2011). A volume of 50µl of CHL2, CE, CEE, EE1, EE2, SD1 and SD2 from a stock solution (10 mg/ml for CHL2, CEE, EE1 and EE2; 2 mg/ml for CE; 0.1 mg/ml for SD1 and SD2) was combined with 50µl of Ciprofloxacin that has already been prepared from stock solution (0.03 mg/ml) and added to each well. The plates were incubated at 37^0C for 20 hrs. Synergistic effect was considered when combinations of the extracts with ciprofloxacin exhibited enlargement of combined inhibition zone diameter size by 0.5mm (Ahmad and Aqil, 2007).

CHAPTER FOUR

4.0 RESULTS

4.1 Extraction

The extractions yielded nine extracts as shown in Table 4.1

Table 4.1: Extracts obtained and their yields

	Extract	Weight (g)
Cold Maceration	Crude ethanol (CEE)	21.40
Microwave Assisted Extraction (MAE)	Hexane (HEX)	5.0
	Chloroform (CHL)	5.6
	Ethyl acetate (EA)	6.9
	Ethanol (EE)	26.0
Polarity Based Extraction (PBE)	Ethyl acetate (EA2)	7.0
	Chloroform (CHL2)	7.5
	Chloroform-ethanol (CE)	1.14
	Ethanol (EE2)	15.6

4.2 Preliminary Phytochemical Investigation

The preliminary phytochemical investigation of the extracts revealed the presence of tannins, saponins, flavonoids and alkaloids in extracts EA, CE, EE and CEE. CHL contain flavonoids and steroids/triterpenes while HEX contains only steroids/ triterpene. (Table 4.2).

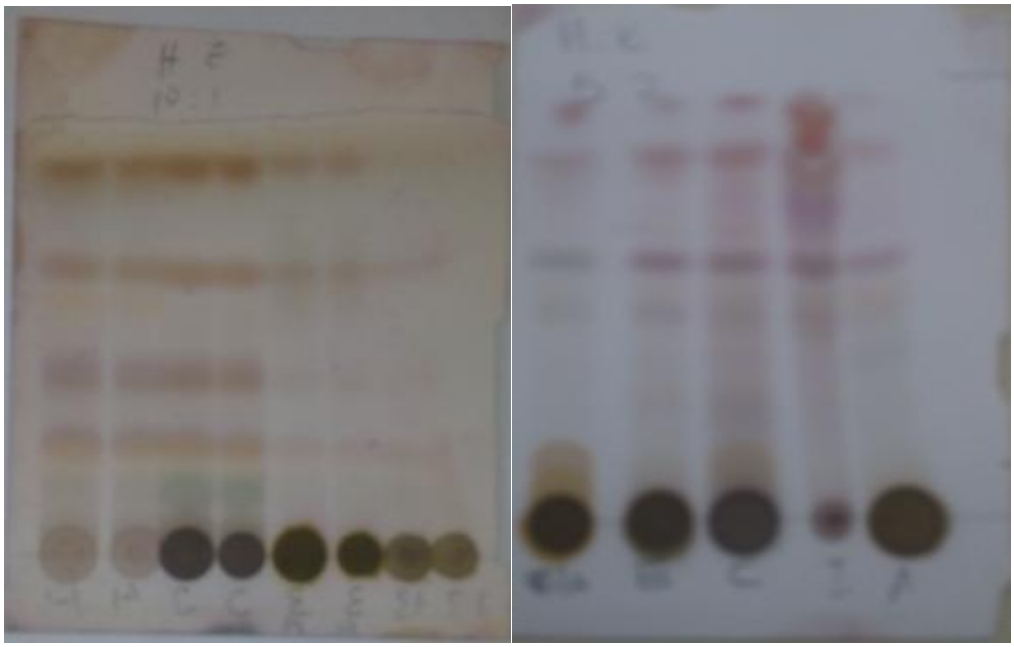
Table 4.2Preliminary Phytochemical Investigation

Constituents	**Test**	**HEX**	**CHL**	**EA**	**EE**	**CE**	**CEE**
Alkaloids	Dragendoff	-	-	+	+	+	+
	Mayer's	-	-	+	+	+	+
	Wagner's	-	-	+	+	+	+
Anthraquinones	Bontrager's	-	-	-	-	-	-
Flavonoids	Shinoda	-	+	+	+	+	+
NaOH		-	+	+	+	+	+
	H_2SO_4-		+	+	+	+	+
	$FeCl_3$-		+	+	+	+	+
Saponins	Frothing	-	-	+	+	+	+
Steroids/Terpenes	Liebermann-		+	+	-	-	-	+
Burchard							
Salkowski+		+	-	-	-	+	
Tannins	Ferric Chloride	-	-	+	+	-	+
Lead Acetate		-	-	+	+	-	+

HEX= hexane, CHL= Chloroform, EA= Ethyl acetate, EE= Ethanol, CE= Chloroform-Ethanol and CEE= Crude ethanol extracts.
Key: + = Present, - = Absent.

4.3 Thin Layer Chromatography

The separation profiles of all the extracts (HEX, CHL, EA, EE, EA2, CHL2, CE, EE2 and CEE) are shown in Plates III- V. Colours observed include green, yellow, black, brown and orange.



(a) (b)

Plate III: TLC profiles of HEX, CHL, EA and EE extracts usingHEX: EtOAc (10:1) [a] and that of HEX, CHL, EA, EE, and CEE using HEX: EtOAc (5: 2) [b]



(a)(b)(c)

Plate IV: TLC profiles of CE using $CHCl_3$: EtOAc (3:1) [a], CHL2 and EA2 using HEX: EtOAc (9:1) [b] and EA and EE using EtOAc: $CHCl_3$:MeOH: H_2O(15: 8: 4: 1)



Plate V: TLC profile of CE, CEE and EE2 usingEtOAc: $CHCl_3$: MeOH: H_2O (15: 8: 4: 1)

The numbers and R_f of observed spots of all extracts are given in the table below

Table 4.3: Summary of TLC profiles of Extracts

Extract	Solvent System	No. of spot	R_f values
HEX	Hexane: ethyl acetate (10:1)	6	0.16, 0.27, 0.42,0.59, 0.68,0.92
	Hexane: ethyl acetate (5: 2)	6	0.43, 0.47, 0.57, 0.67, 0.82,0.91
CHL	Hexane: ethyl acetate (10:1)	5	0.16, 0.27, 0.42,0.68,0.92
	Hexane: ethyl acetate (5: 2)	6	0.11, 0.23, 0.47, 0.57, 0.82,0.91
EA	Hexane: ethyl acetate (10:1)	4	0.27, 0.42,0.68,0.92
	Hexane: ethyl acetate (5: 2)	5	0.09, 0.15, 0.57, 0.82, 0.91
	EtOAc: $CHCl_3$: MeOH: H_2O (15:8:4:1)	2	0.67, 0.71
EE	Hexane: ethyl acetate (10:1)	2	0.27, 068
	Hexane: ethyl acetate (5: 2)	4	0.47, 0.57, 0.82, 0.91
	EtOAc: $CHCl_3$: MeOH: H_2O (15:8:4:1)	2	0.67, 0.71, 0.79
EA2	Hexane: ethyl acetate (10:1)	6	0.16, 0.27, 0.42,0.59,0.68,0.92
CHL2	Hexane: ethyl acetate (10:1)	2	0.30, 0.73
CE	Chloroform: ethyl acetate (3: 1)	2	0.54, 0.76
	EtOAc: $CHCl_3$: MeOH: H_2O (15:8:4:1)	3	0.51, 0.56, 0.89
EE2	EtOAc: $CHCl_3$: MeOH: H_2O (15:8:4:1)	1	0.89
CEE	Hexane: ethyl acetate (10:1)	4	0.47, 0.57, 0.82, 0.91
	EtOAc: $CHCl_3$: MeOH: H_2O (15:8:4:1)	2	0.51, 0.56

4.4 Gel Filtration chromatography of Chloroform- ethanol extract (CE)

Extract CE when subjected to gel filtration chromatography separated into 3 distinct bands (Appendix I) in the column which gave24 fractions. The fractions were further pooled together based on their TLC profile (Plate VI) to give four major fractions coded CE1-CE4 (Table 4.4).

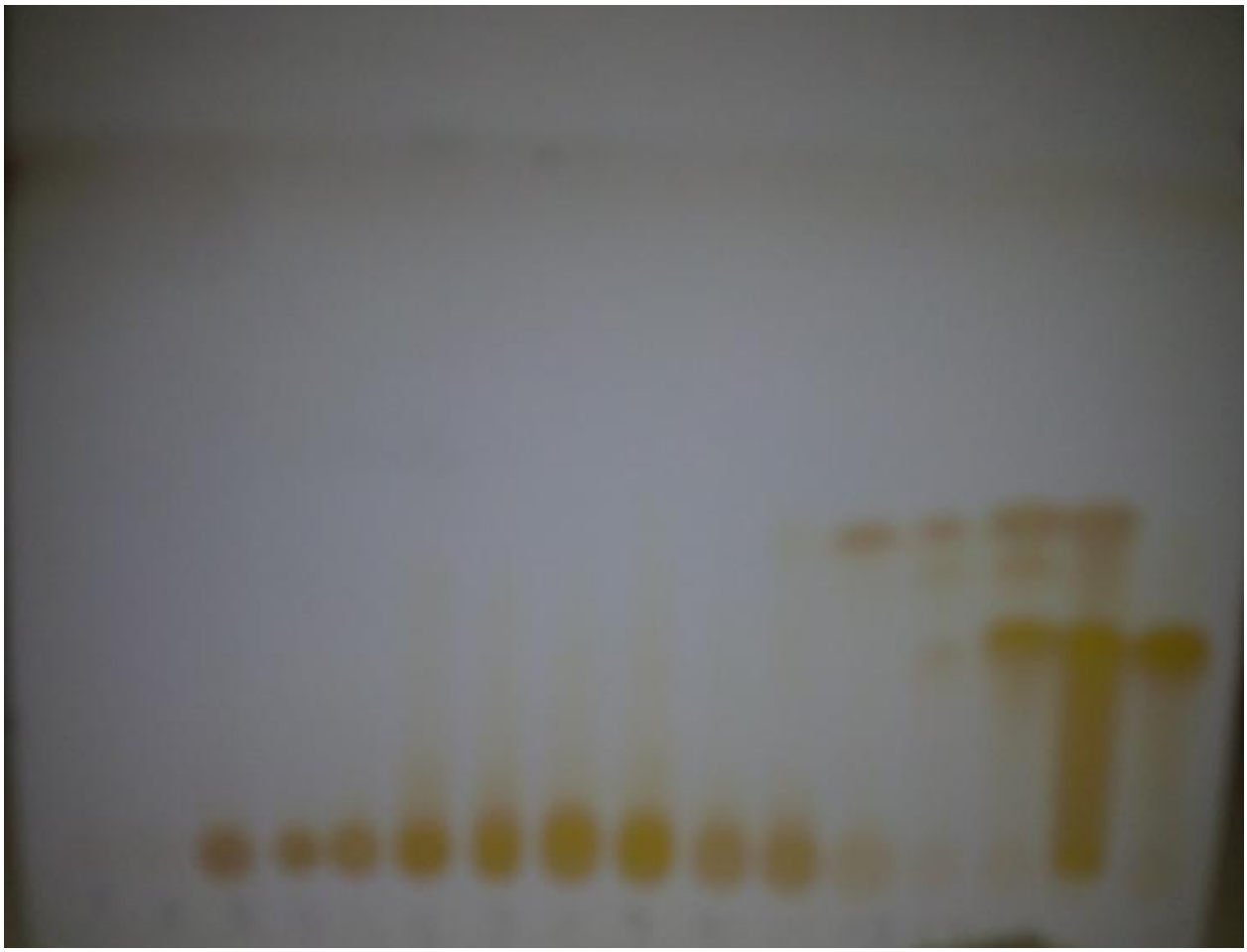


Plate VI: TLC profile of CE collections

Chloroform: methanol (20: 1)

Table 4.4: Fractions from Gel filtration Chromatography of extract CE.

Fraction	Code	No. of spots
1-15	CE1	None
16-18	CE2	1
19-22	CE3	3
23-24	CE4	1

4.5 **Isolation of SD1 and SD2**

CE2 and CE4 consisting of just one spot were labeled as compound SD1 and SD2 respectively. Repeated gel filtration of CE3 consisting of 3 spots yielded more of SD1 and SD2. SD1 (10mg) and SD2 (12mg) were isolated as light yellow and yellow crystals respectively. Chromatograms of SD1 and SD2 are shown in plates VII. The two compounds were subjected to spectroscopic analysis for structural elucidation.



(a) (b)

Plate VII: TLC profiles of compounds SD1 (a)and SD2(b) using $CHCl_3$: MeOH (20:1)

4.6 Analysis of SD1

4.6.1 TLC analysis of SD1

TLC analysis of SD1 using two solvent systems chloroform: ethyl acetate (3:1) and chloroform: methanol (20:1) gave single homogenous orange-yellow spot with R_f values of 0.57 and 0.56 respectively. Spraying with 10% H_2SO_4 and heating gave an orange-yellow colored spot (Table 4.4).

Table 4.5: TLC profile of SD1 using different solvent systems

Solvent system	Number of spot(s)	Color of spot after heating	R_f value
Chloroform: ethyl acetate (3:1)	1	Orange-yellow	0.57
Chloroform: methanol (20:1)	1	Orange-yellow	0.56

4.6.2 Chemical test on compound SD1

Compound SD1 gave a green color in the Ferric chloride test for phenolic compounds.

4.6.3 Melting Point of SD1

The melting point of SD1 was determined to be 294-297^{0}C

4.6.4 Spectroscopic analysis of SD1

4.6.4.1 UV Analysis of SD1

SD1 showed absorbance at wavelengths of 209nm, 256nm and 371nm (Figure 4.1)

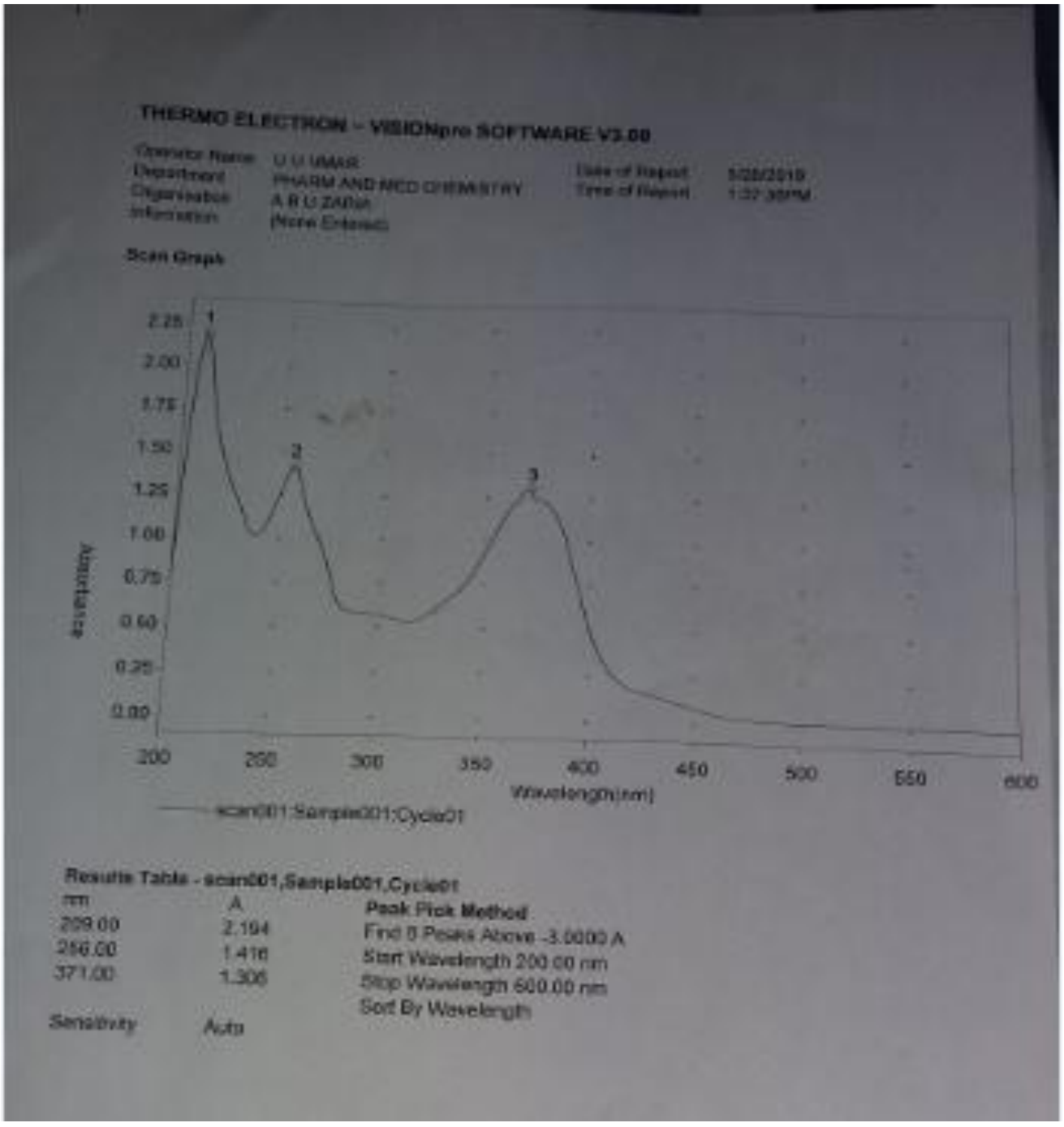


Figure 4.1: UV spectrum of SD1

4.6.4.2 FTIR Analysis of SD1

The IR spectrum of SD1 (figure 4.2) showed bands at 3306 cm^{-1}, 2948 cm^{-1}, 1658 $cm^{-1}$and bands in the finger print region.

Sample ID:SD1
Sample Scans:16
Background Scans:16
Resolution:8
System Status:Good
Method Name:Transmittance Method
User:Admin
Date/Time:2016-08-04T10:15:31.178+01:00
Range:4000 - 650
Apodization:Happ-Genzel
File Location:C:\Program Files\Agilent\MicroLab PC\Results\SD1_2016-08-04T10-15-31.a2r

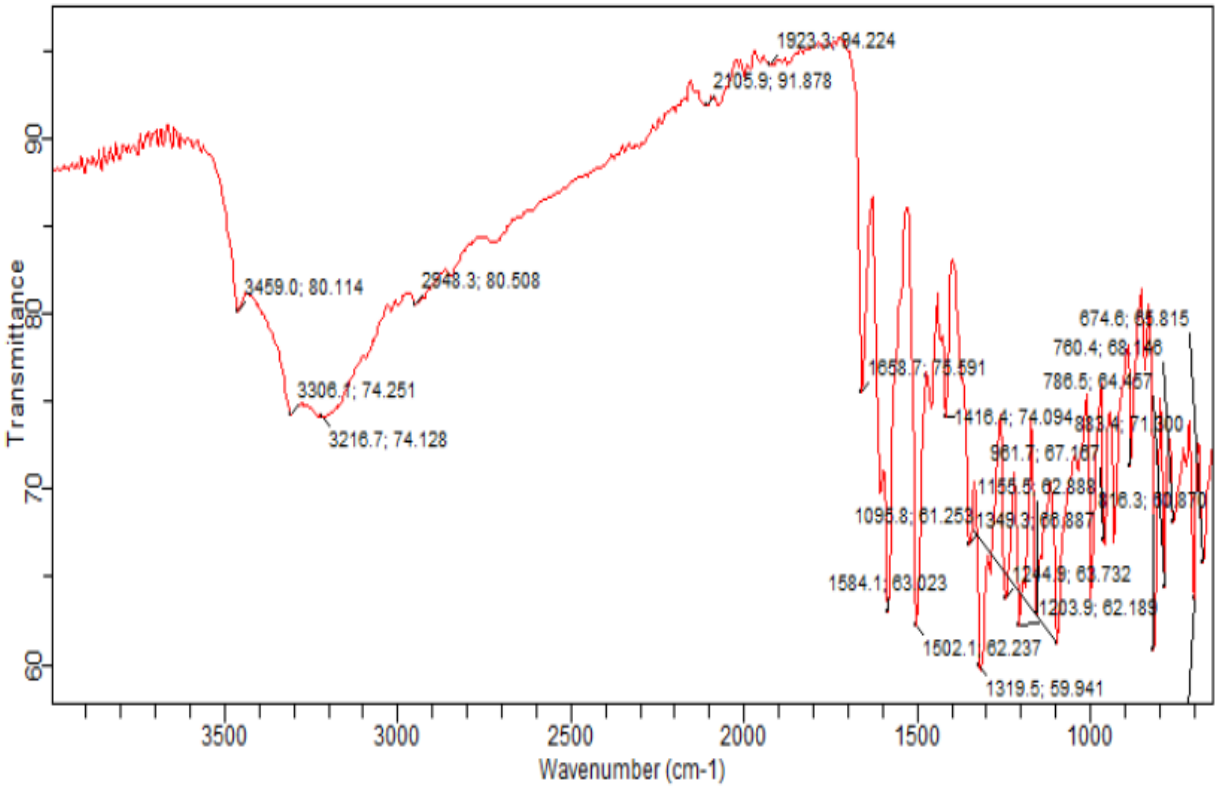


Figure 4.2: FTIR spectrum of SD1

*4.6.4.3 ^{1}H NMR (δppm, 600 MHz, DMSO)*of SD1

The ^{1}H NMR of SD1 revealed resonances at δ: 7.74(d, 1H, J=2.04Hz), 7.56(dd, 1H, J=2.04, 8.58Hz), 6.91(d, 1H, J=8.46Hz), 6.71(d, 1H, J=1.98Hz), 6.36(d, 1H, J=1.98Hz) in the aromatic region and at δ: 3.90ppm integrating for 3 protons (Figures 4.3 and 4.4)

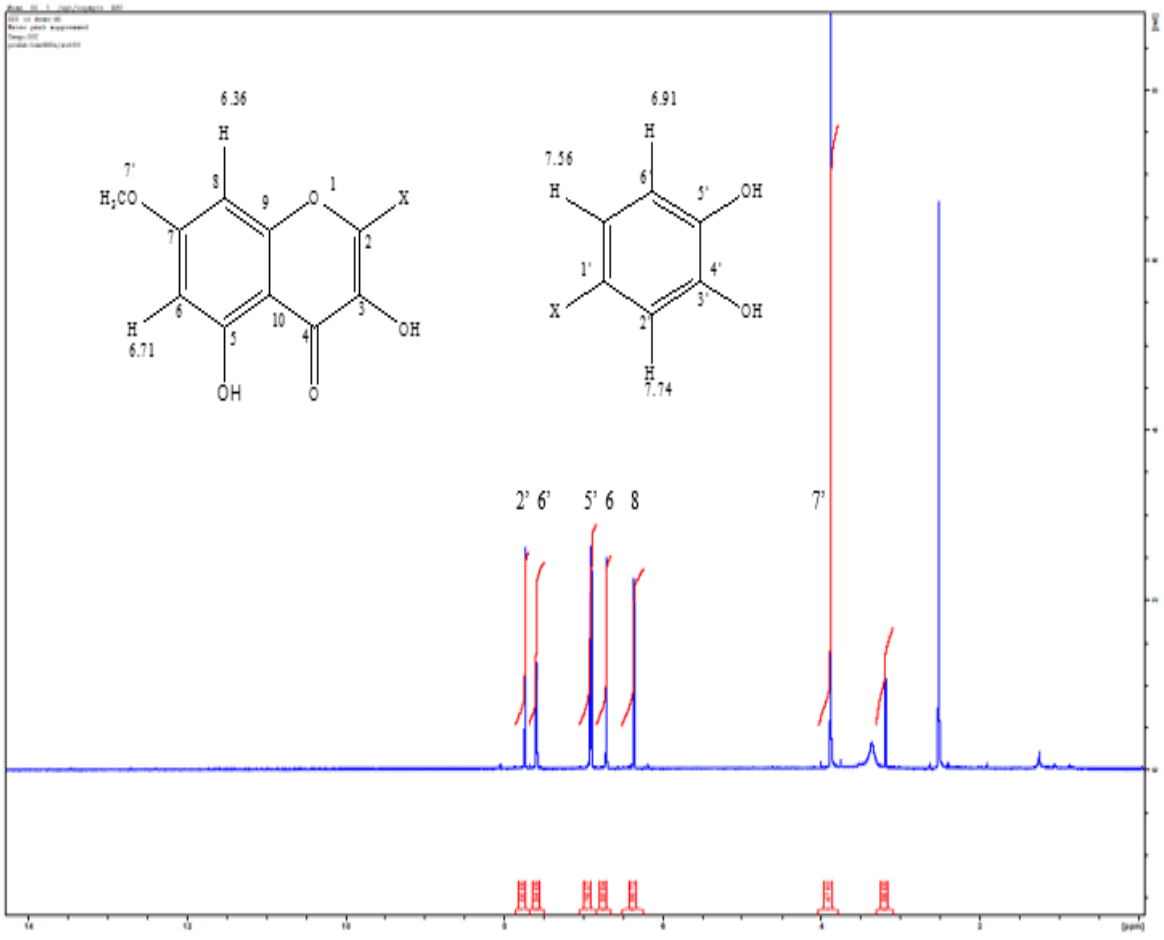


Figure 4.3: proton NMR spectrum of SD1

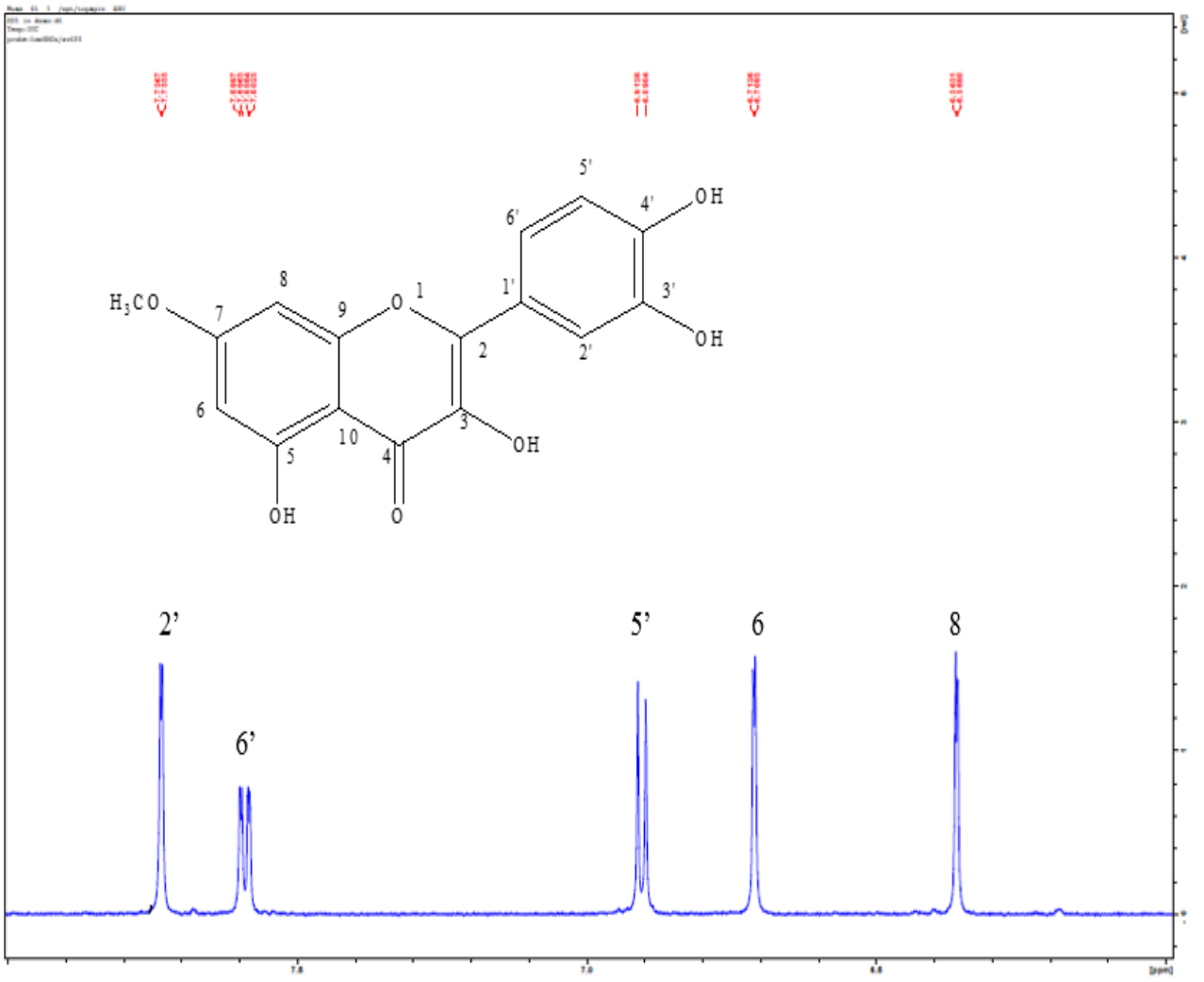


Figure 4.4: Proton NMR spectrum of SD1 (expanded 6.0-8.0 ppm)

4.6.4.4 ^{13}C NMR (δppm, 600MHz, DMSO) and DEPT NMR spectral analysis of SD1

The ^{13}C-NMR and DEPT experiment (figure 4.5 and 4.6) of SD1 showed the presence of 16 carbon atoms at δ:176.45 (C), 165.39 (C), 160.87 (C), 156.56 (C), 148.38 (C), 147.81 (C), 145.59(C), 136.51 (C), 122.33 (C), 120.53(-CH), 116.06 (-CH), 115.72(-CH), 104.49(C), 97.92 (-CH), 92.38 (-CH) and 56.49 ($-CH_3$) indicating that SD1 contains 10 quaternary carbon atom signals, five methine carbon atom signals (-CH-) and one methyl carbon atom signal.

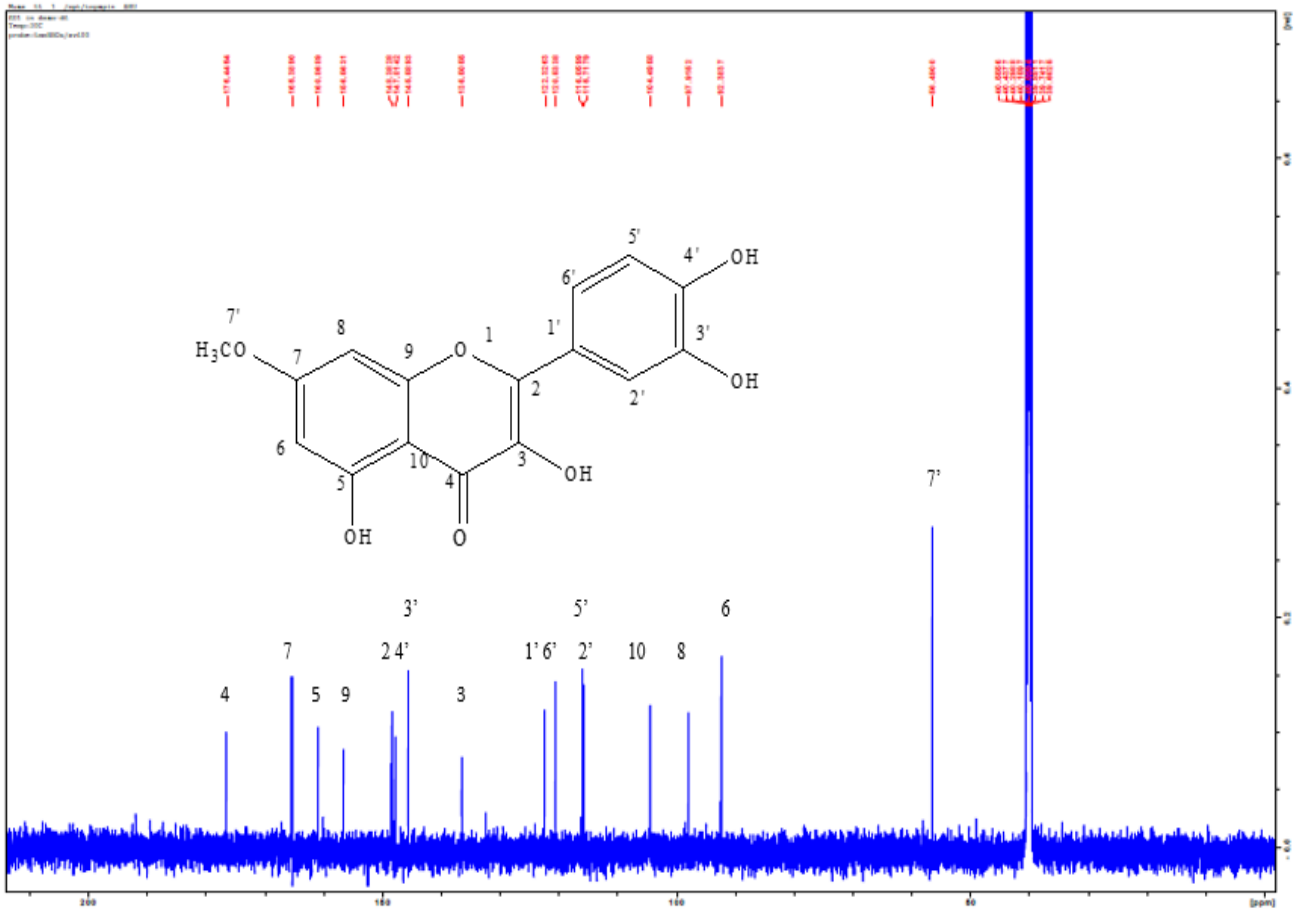


Figure 4.5: Carbon 13 NMR spectrum of SD1

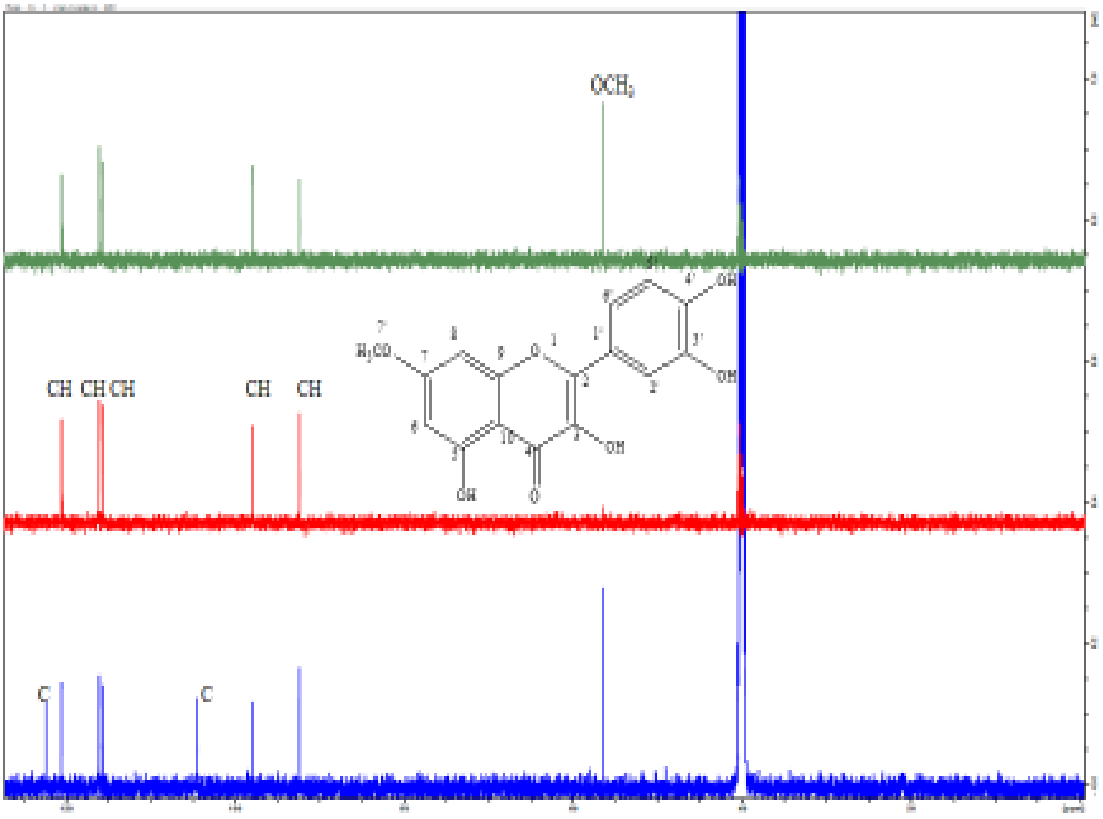


Figure 4.6: DEPT experiment of SD1

4.6.4.5 ^{1}H-^{1}H COSY Spectral Analysis of SD1

The 2D ^{1}H-^{1}H COSY correlation spectrum of SD1 (figure 4.7) show correlation between protons at δ_H; 6.36, 6.71, 6.91 and 7.56.

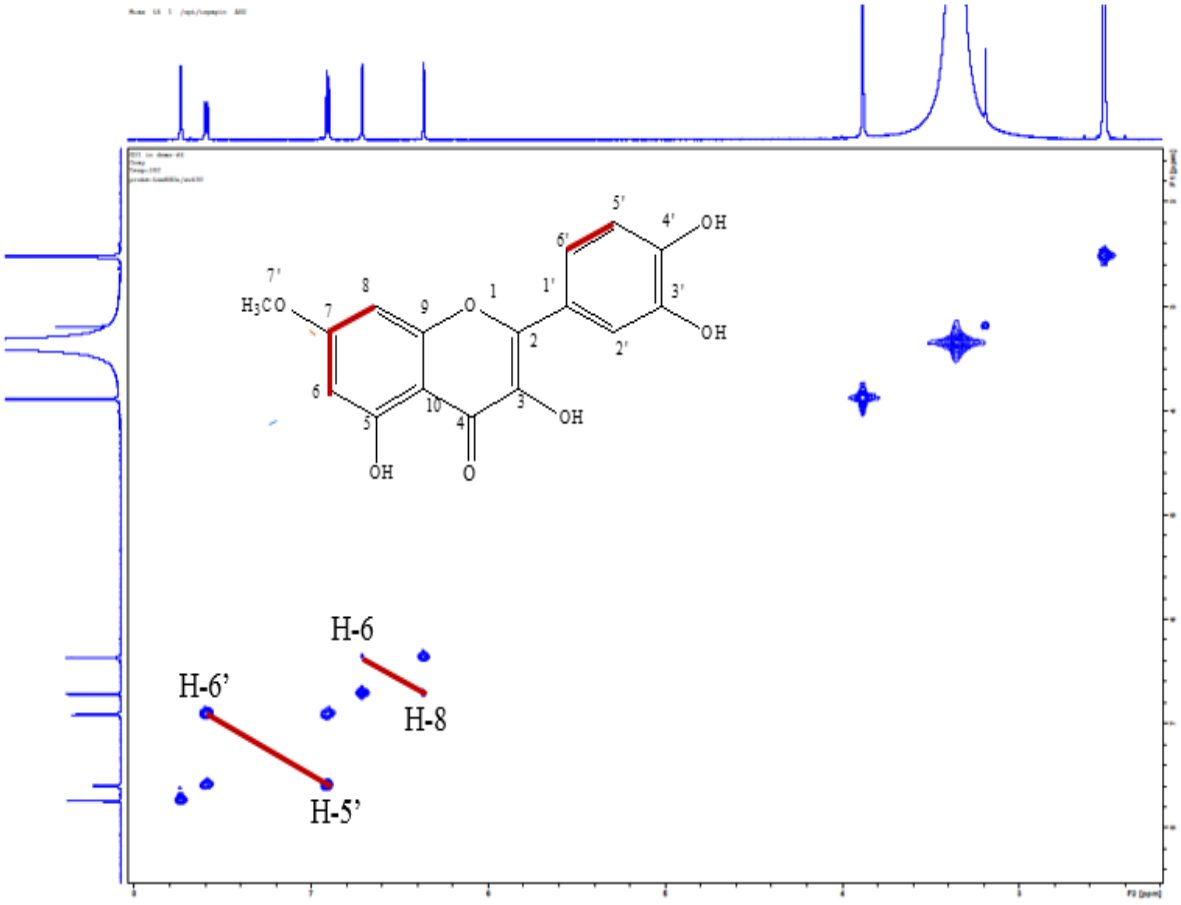


Figure 4.7: ^{1}H-^{1}H COSY correlation spectrum of SD1

4.6.4.6 HSQC Analysis of SD1

The attachment of protons to their respective carbons was established from the HSQC spectrum of SD1 (figure 4.8). The following cross peaks were observed. 7.74(H) → 115.72

(C), 7.56 (H) → 120.53 (C), 6.91 (H) → 116.06 (C), 6.71 (H) → 92.38 (C), 6.36 (H) → 97.92 (C) and 3.90 (H)→56.49 (C).

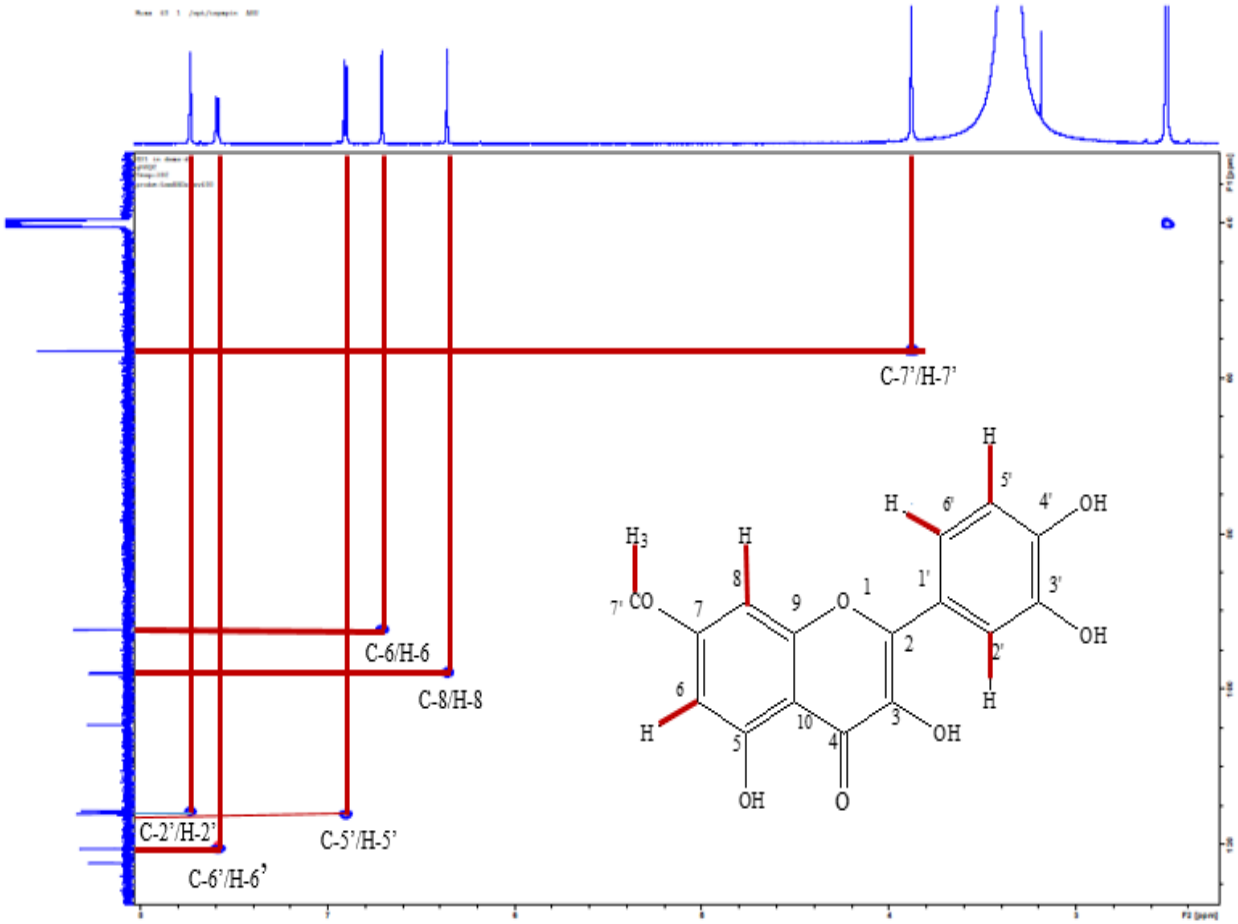


Figure 4.8: HSQC spectrum of SD1

4.6.4.7 HMBC Analysis of SD1

The hetero nuclear multiple bond correlation (HMBC) experiment shows connectivity between protons and carbon atoms up to three (J_3) and sometimes four (J_4) bonds away. The correlation facilitates the connection of the various fragments from the ^{1}H, ^{13}C, COSY

and HSQC experiments. From the HMBC experiment (4.9 and 4.10), the following cross peaks were observed.

(H) (C)

3.90 → 165.39

6.36→ 92.38, 104.49, 160.87, 165.39

6.71→ 97.92, 104.49, 156.53, 165.39

6.91 → 122.33, 145.59, 147.81

7.56 → 115.72, 147.81

7.74→ 120.53, 145.59, 148.38

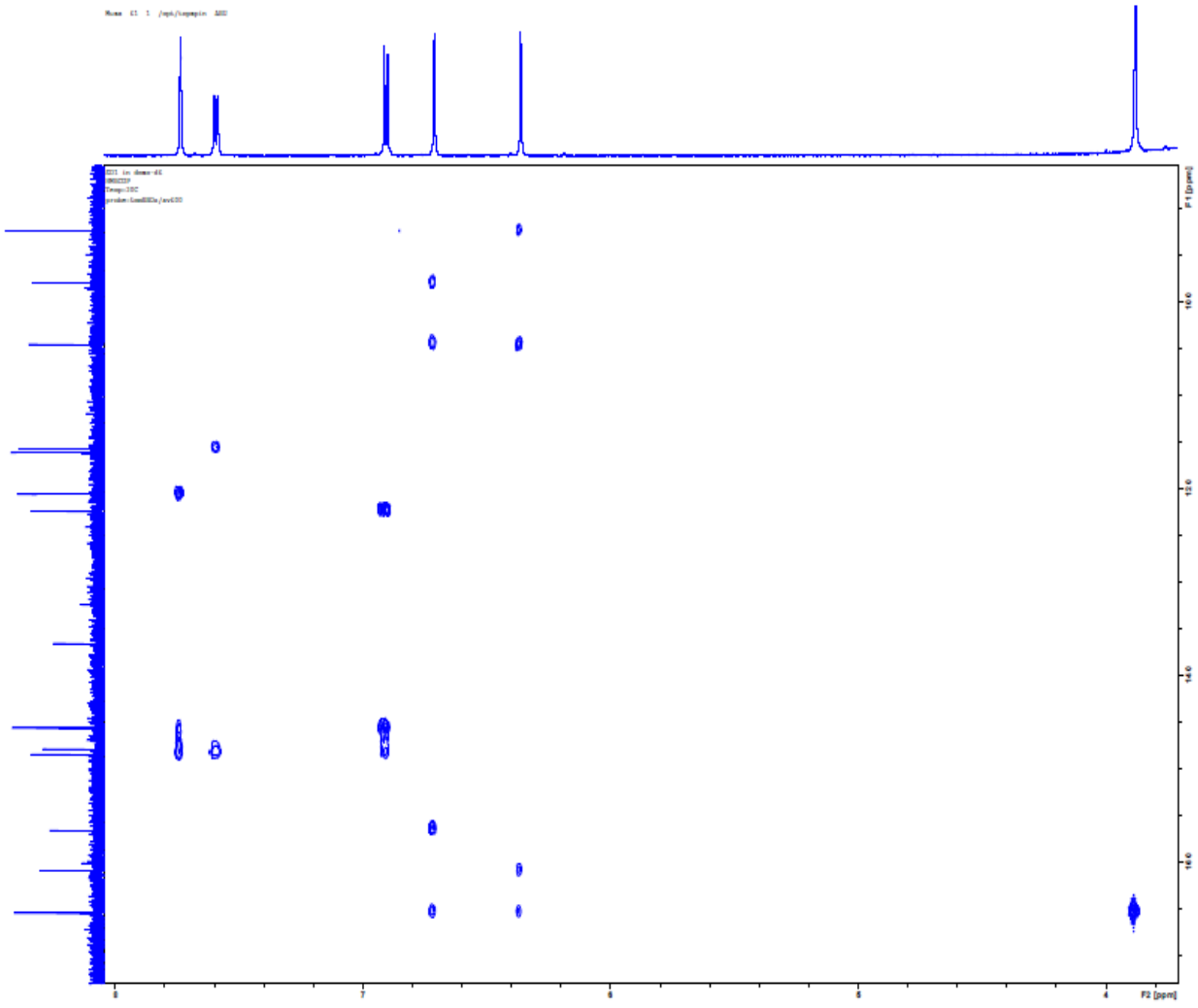


Figure 4.9: HMBC Spectrum of SD1

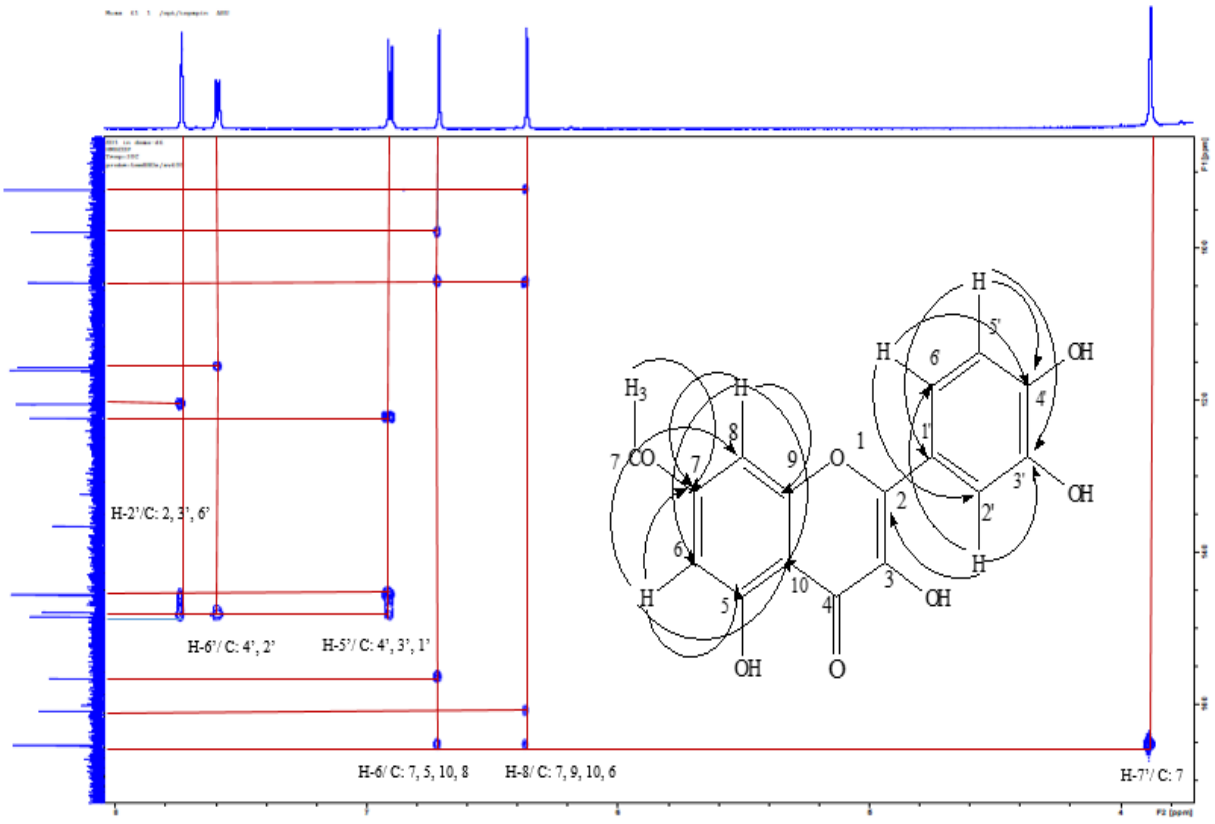


Figure 4.10: HMBC Spectrum of SD1 (expanded 3.9- 8.0 ppm)

4.6.4.8 NOESY Analysis of SD1

The NOESY result of SD1 showed correlations between the protons at $\delta_{H:}$ 3.90, 6.36 and 6.71 (Figure 4.11).

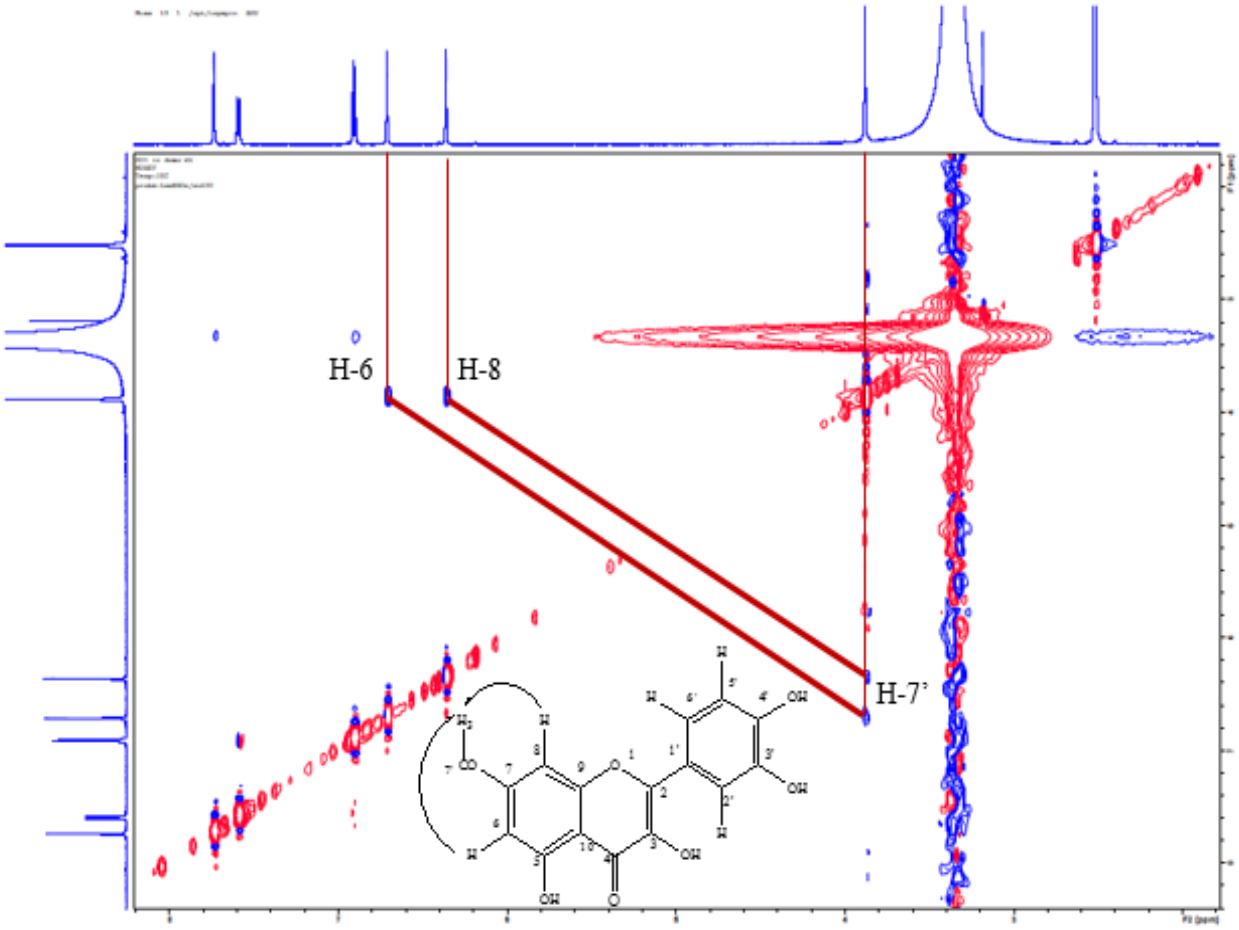


Figure 4.11: NOESY spectrum of SD1

The 1D and 2D NMR data of SD1 are summarized in Table 4.6

Table 4.6: Summary of spectral data for compound SD1

Position	$\delta^{13}C$	$\delta^{1}H$, J (Hz)	DEPT
2	148.38		C
3	136.51		C
4	176.45		C
5	160.87		C
6	92.38	6.71 (d, 1H, J=1.98Hz)	-CH-
7	165.39		C
8	97.92	6.36 (d, 1H, J=1.98Hz)	-CH-
9	156.56		C
10	104.49		C
1`	122.33		C
2`	115.72	7.74 (d,1H,J=2.04Hz)	-CH-
3`	145.49		C
4`	147.81		C
5`	116.06	6.91 (d, 1H, J=8.46Hz)	-CH-
6`	120.53	7.56 (dd,1H, J=2.04,8.58Hz)	-CH-
7’	56.49	3.90	$-CH_3$

Table 4.7: Comparison of 1D NMR data of SD1 with literature data

	Compound SD1 (DMSO)		Pereira et al., 2012 (DMSO-d_6)	
Position	$\delta^{13}C$	$\delta^{1}H$, J (Hz)	$\delta^{13}C$	$\delta^{1}H$, J (Hz)
2	148.38		156.5	
3	136.51		133.9	
4	176.45		177.6	
5	160.87		161.2	
6	92.38	6.71 (d, 1H, J=1.98)	99.1	6.17 (d, 1H, J= 1.7)
7	165.39		165.1	
8	97.92	6.36 (d, 1H, J=1.98)	94.0	6.38 (d, 1H, J= 1.7)
9	156.56		156.8	
10	104.49		103.9	
1`	122.33		121.3	
2`	115.72	7.74 (d,1H,J=2.04)	116.4	7.54 (d, 1H, J= 2.1)
3`	145.49		145.1	
4`	147.81		148.8	
5`	116.06	6.91 (d, 1H, J=8.46)	115.5	6.80 (d, 1H, J= 8.4)
6`	120.53	7.56(dd,1H,J=2.04,8.58)	122.2	7.63(dd, 1H, J= 2.1, 8.5)
7’	56.49	3.90		

4.7 Analysis of compound SD2

4.7.1 TLC analysis of SD2

TLC analysis of SD2 using two different solvent systems chloroform:ethyl acetate (3:1) gave a single homogenous spot with (R_f = 0.43) while the TLC analysis with chloroform: methanol (20:1) gave a single homogenous spot (R_f = 0.44). Spraying with 10% H_2SO_4 and heating gave an orange-yellow colored spot (Table 4.7).

Table 4.8: TLC profile of SD2 using two different solvent systems

Solvent system	Number of spot(s)	Color of spot after heating	R_f value
Chloroform: ethyl acetate (3:1)	1	Orange-yellow	0.43
Chloroform: methanol (20:1)	1	Orange-yellow	0.44

4.7.2 Chemical test on compound SD2

Compound SD2 gave a green color in the ferric chloride test for phenolic compounds.

4.7.3 Melting Point of SD2

The melting point of SD2 was determined to be 315-317^{0}C

4.7.4 Spectroscopic analysis of SD2

4.7.4.1 UV Analysis of SD2

SD2 showed absorbance at wavelengths of 224nm, 256nm and 371nm (Figure 4.12)

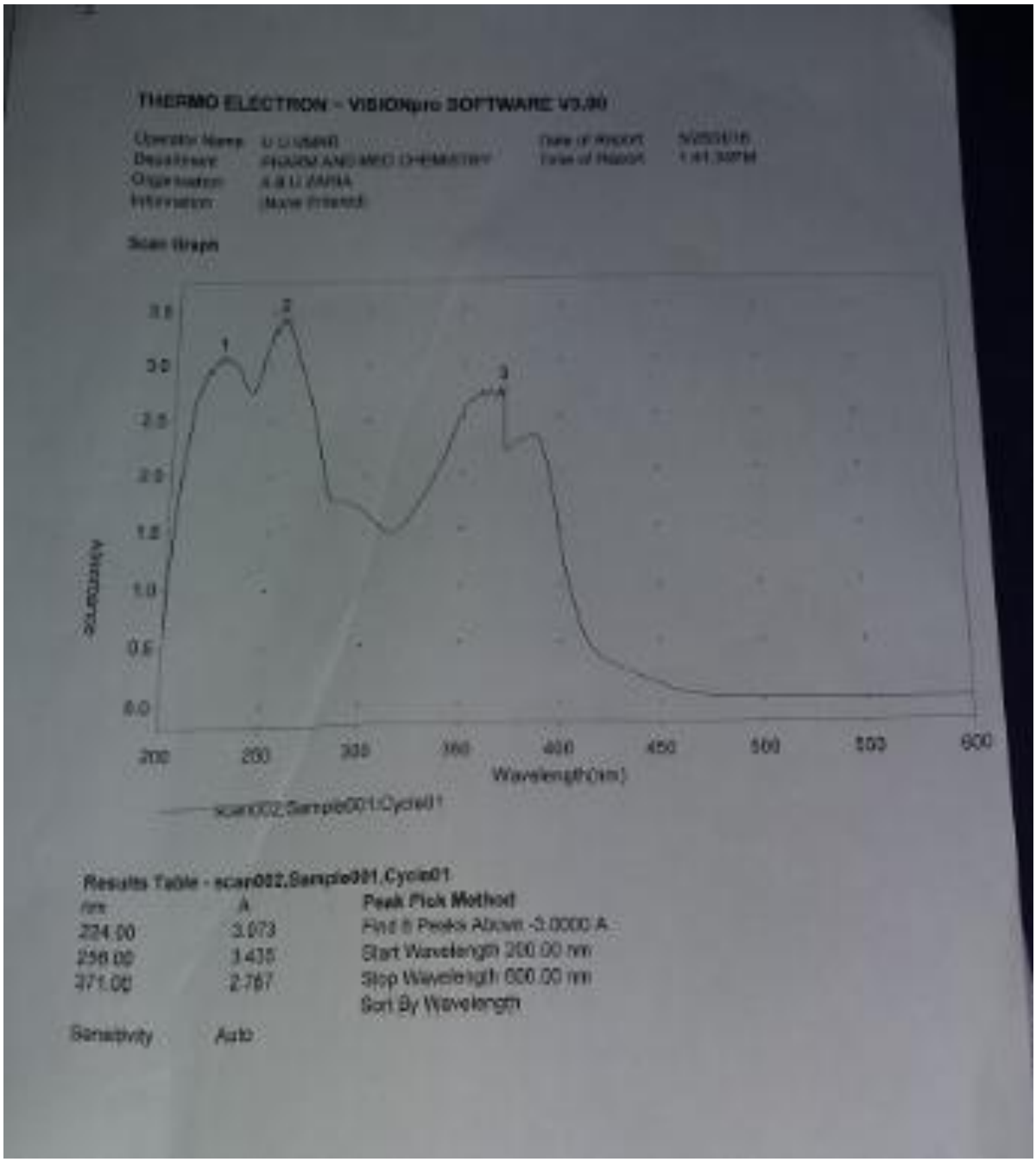


Figure 4.12: UV spectrum of SD2

4.7.4.2 FTIR Analysis of SD2

The IR spectrum of SD2 (Figure 4.13) showed bands at 3291cm^{-1}, 2922 cm^{-1},1654 cm^{-1} and other bands in the finger print region.

Sample ID:SD2
Sample Scans:16
Background Scans:16
Resolution:8
System Status:Good
Method Name:Transmittance Method
User:Admin
Date/Time:2016-08-04T10:19:59.608+01:00
Range:4000 - 650
Apodization:Happ-Genzel
File Location:C:\Program Files\Agilent\MicroLab PC\Results\SD2_2016-08-04T10-19-59.a2r

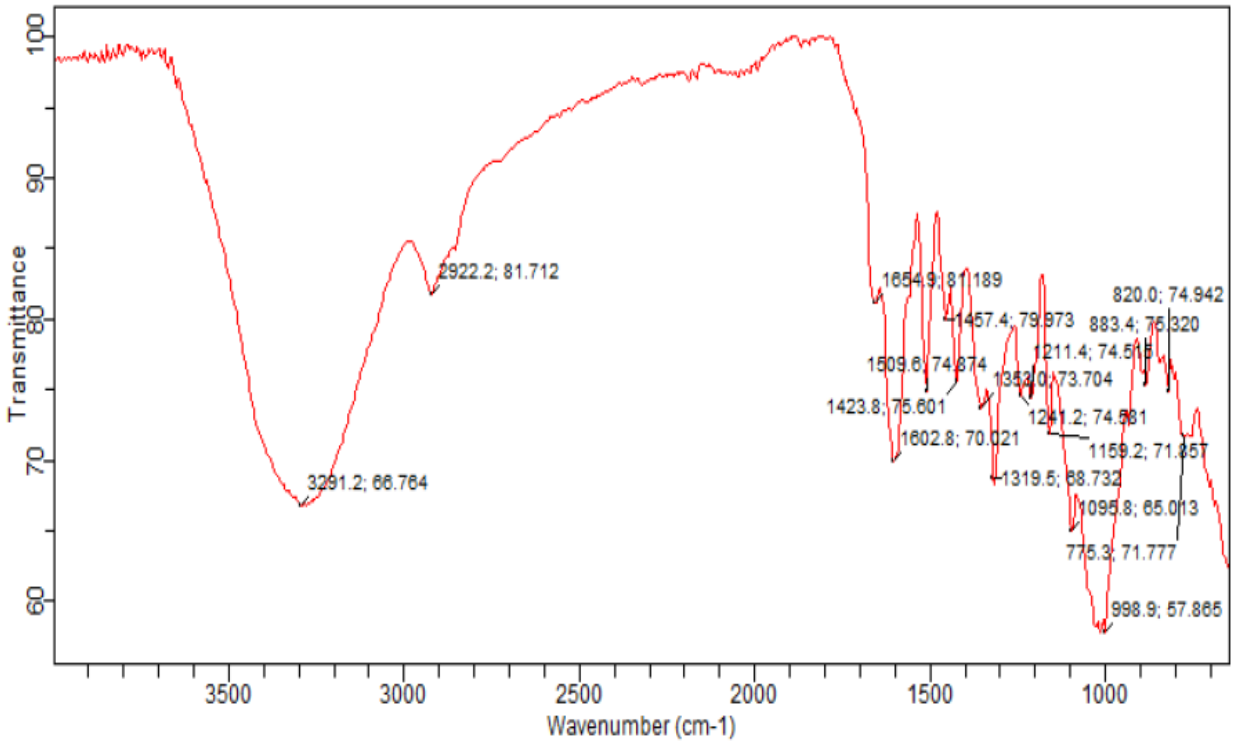


Figure 4.14 FTIR spectrum of SD2

*4.7.4.3 ^{1}H NMR (δppm, 600 MHz, DMSO)*of SD2

Figure 4.14-4.15 show the ^{1}H NMR spectrum of SD2 with resonating signals between 6.20 to 7.69 ppm.

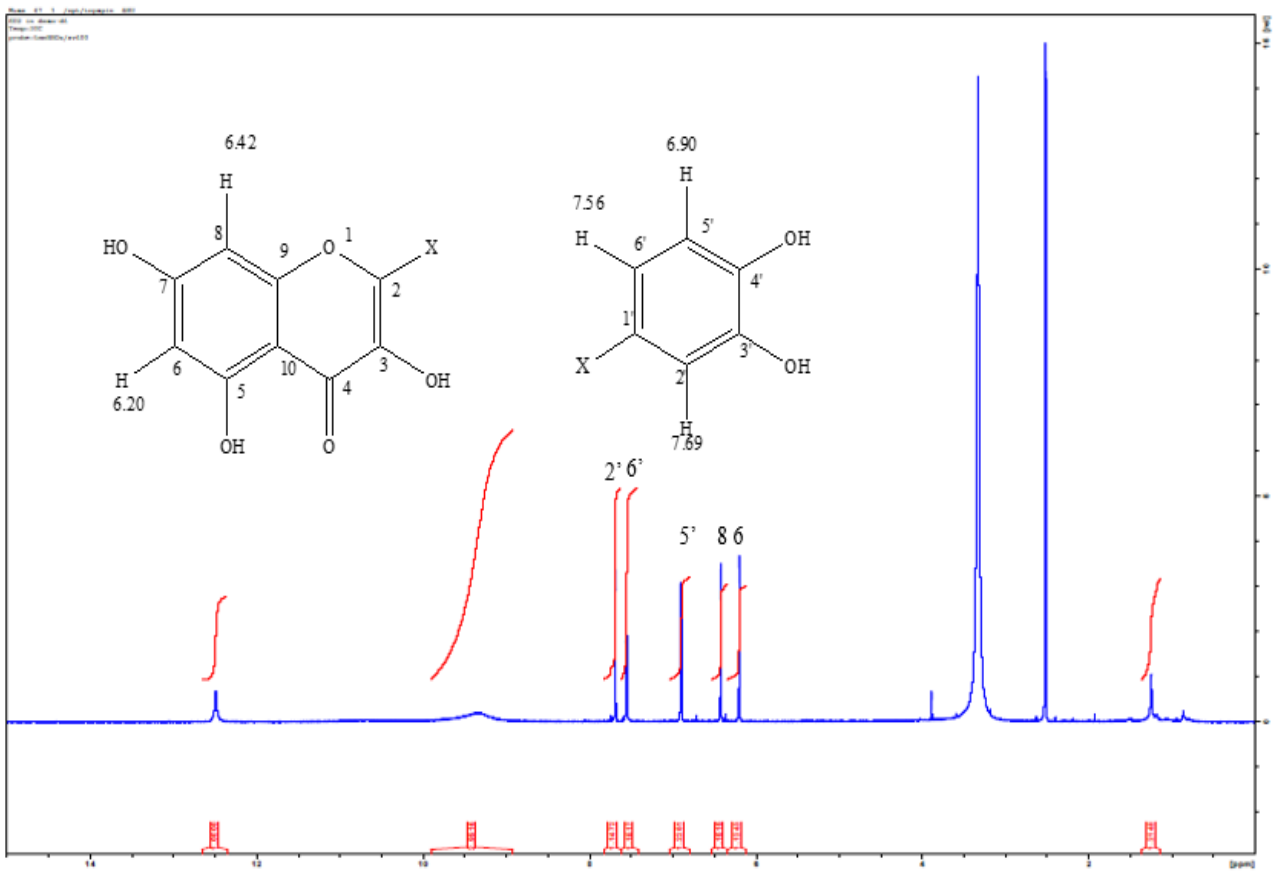


Figure 4.14: Proton NMR spectrum of SD2

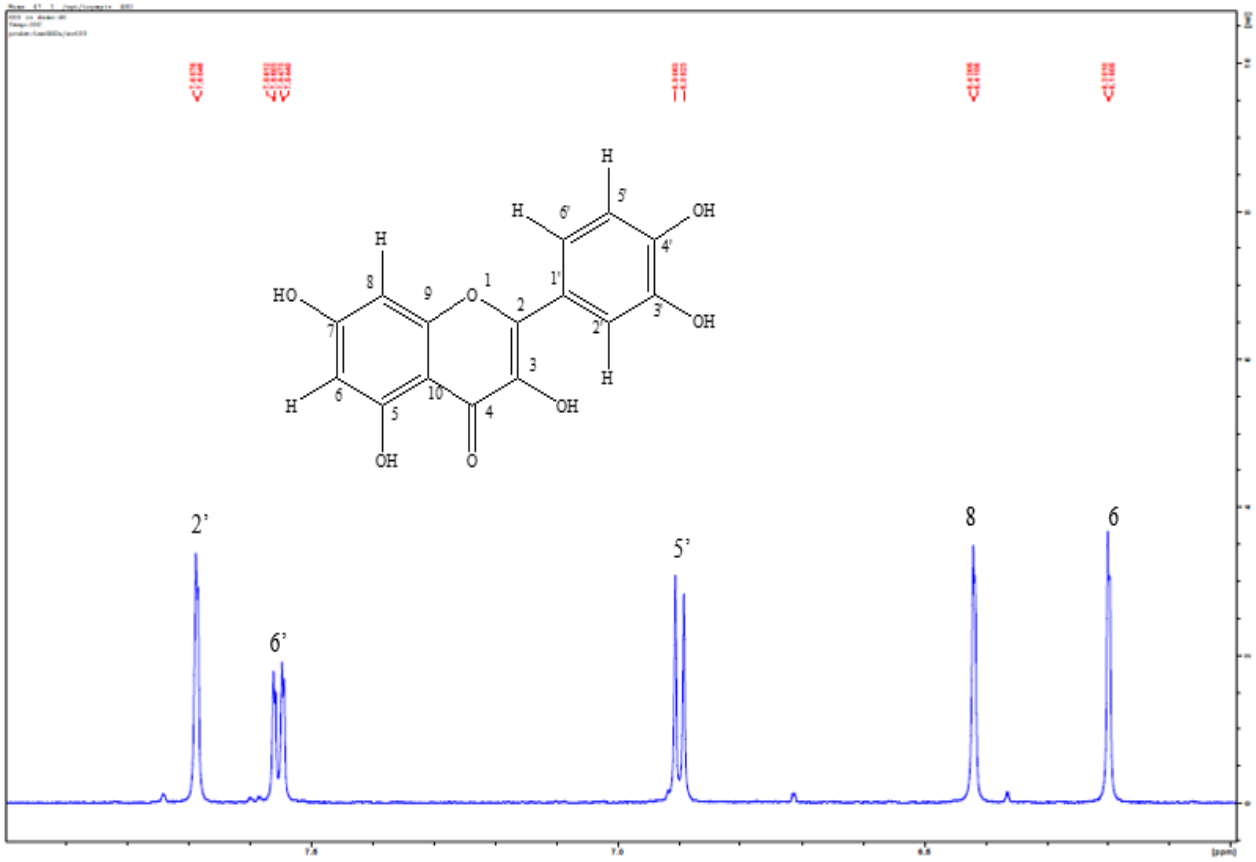


Figure 4.15: Proton NMR spectrum of SD2 (expanded 6.0-8.0ppm)

4.7.4.4 ^{13}C NMR (δppm, 600MHz, DMSO) and DEPT NMR spectral analysis of SD2

The ^{13}C-NMR and DEPT experiment of SD2 showed the presence of 15 carbon atoms; 176.31 (C), 164.43 (C), 161.20 (C), 156.63 (C), 148.19 (C), 147.29 (C), 145.54(C), 136.19 (C), 122.45 (C), 120.45(-CH), 116.08 (-CH), 115.56(-CH), 103.46(C), 98.68 (-CH) and 93.83 (-CH) indicating that SD2 contains 10 quaternary carbon atom signals and five methine carbon atom signals (-CH-) (Figure 4.16- 4.17).

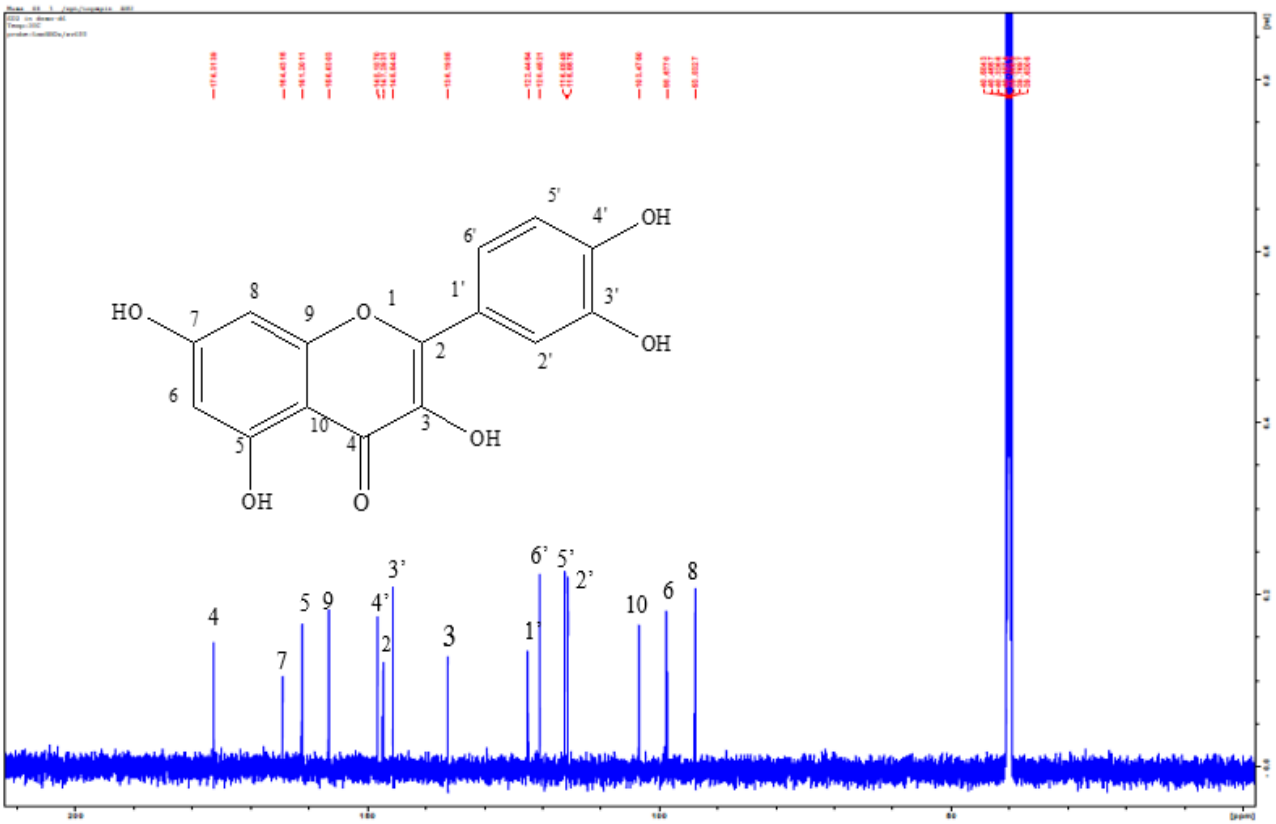


Figure 4.16: Carbon 13 NMR spectrum of SD2

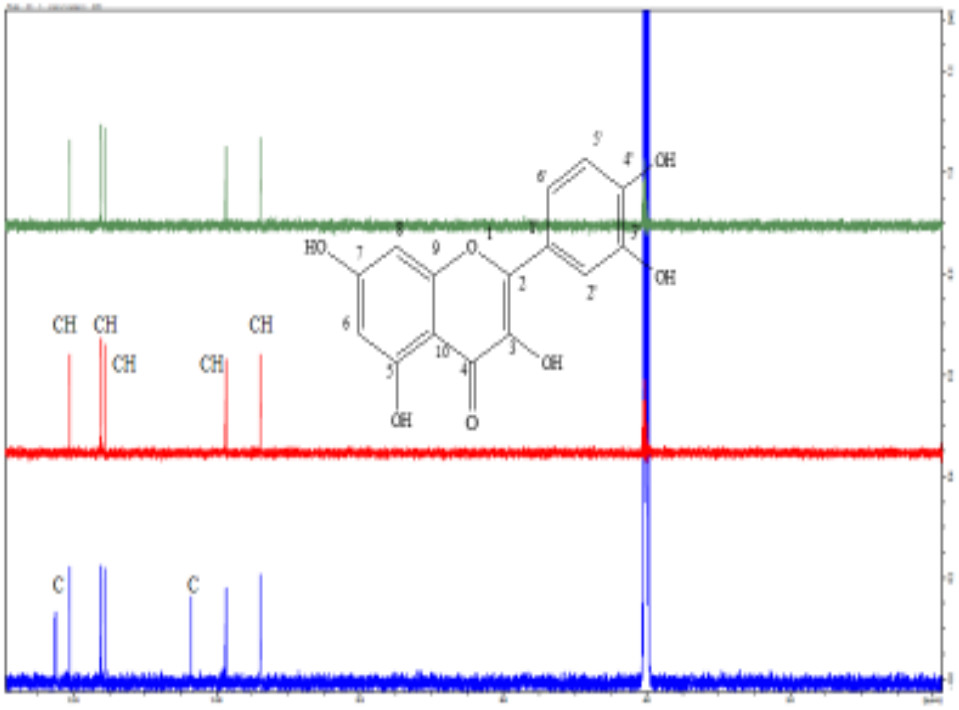


Figure 4.17: DEPT experiment of SD2

4.7.4.5 ^{1}H-^{1}H COSY Spectral Analysis of SD2

The 2D ^{1}H-^{1}H COSY correlation spectrum of SD1 (figure 4.18) show correlation between protons at δ_H; 6.20, 6.42, 6.90 and 7.56.

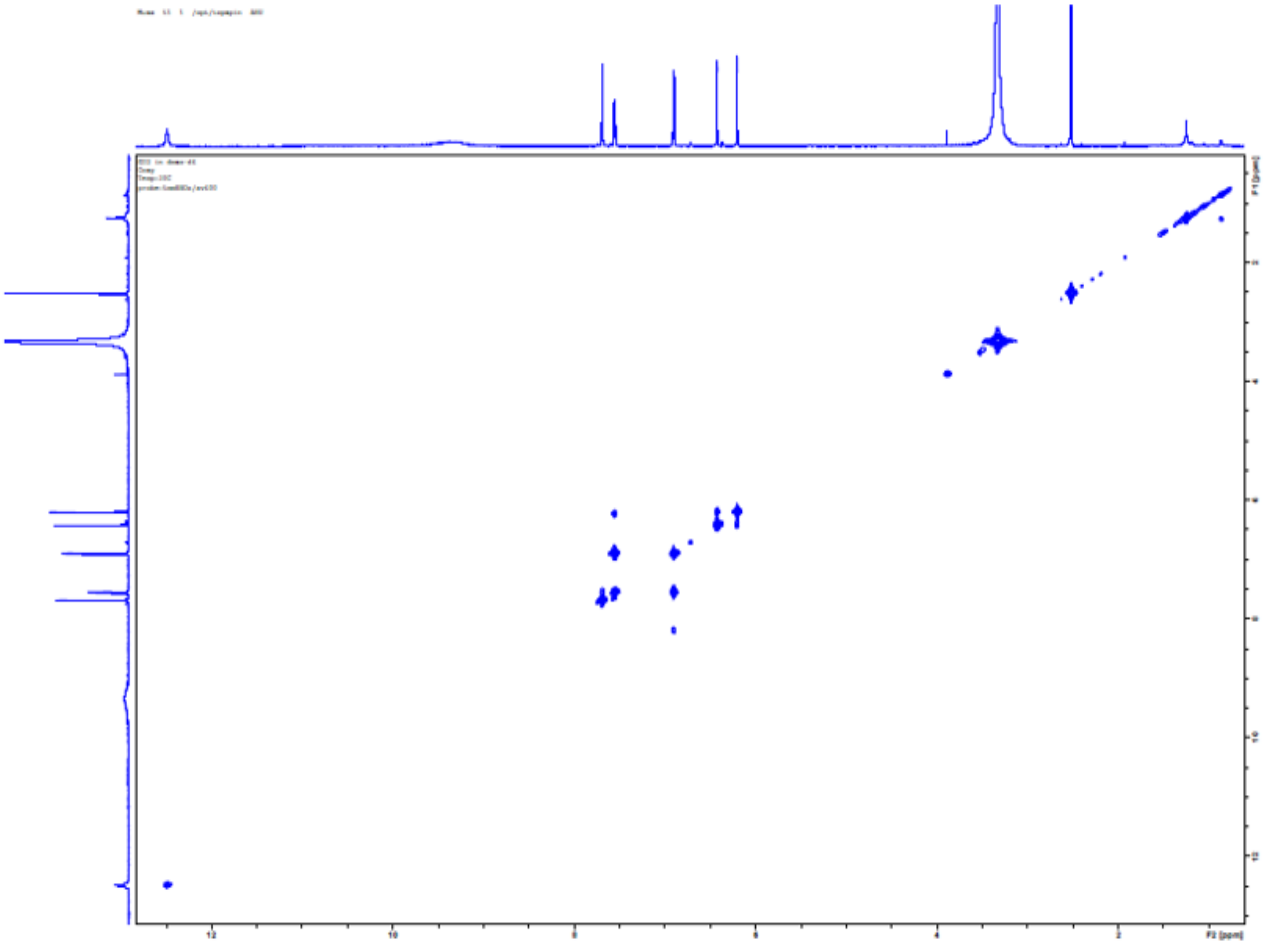


Figure 4.18: ^{1}H-^{1}H COSY correlation spectrum of SD2

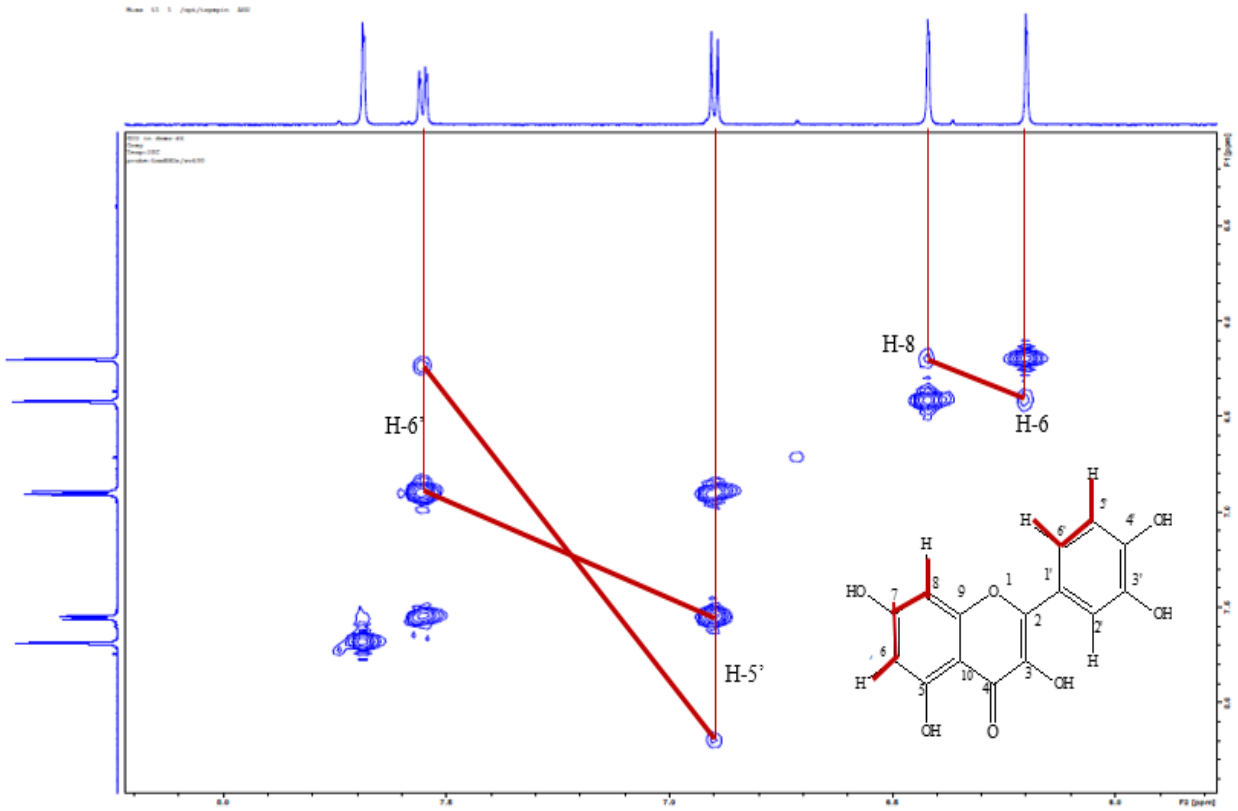


Figure 4.19: ^{1}H-^{1}H COSY correlation spectrum of SD2 (expanded 5.5-8.5)

4.7.4.6 HSQC Analysis of SD2

The attachment of protons to their respective carbons was established from the HSQC spectrum of SD2 (figure 4.20). The following cross peaks were observed: 7.69(H) → 115.56(C), 7.56 (H) → 120.45 (C), 6.90 (H) → 116.08 (C), 6.42 (H) → 93.83 (C), 6.20 (H) → 98.68 (C).

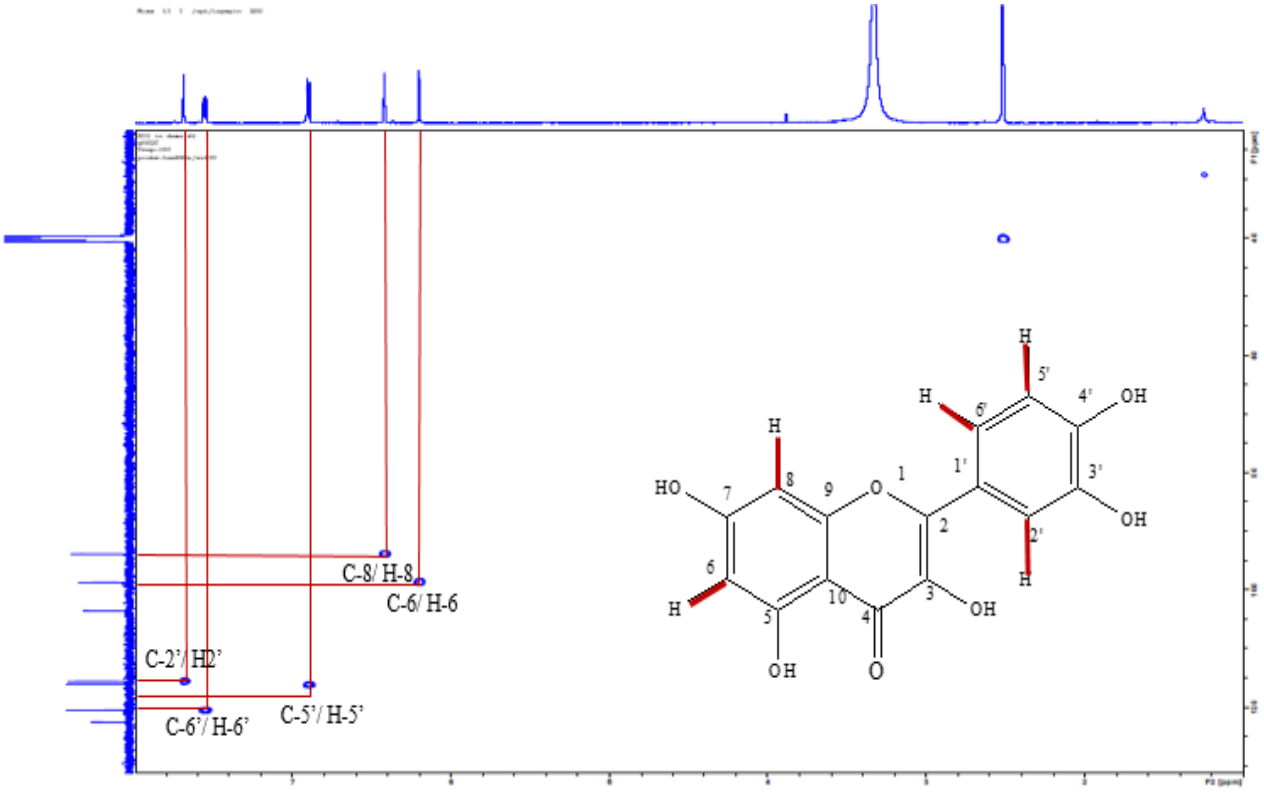


Figure 4.20: HSQC Spectrum of SD2

4.7.4.7 HMBC Analysis of SD2

The HMBC experiment shows connectivity between protons and carbon atoms up to three (J_3) and sometimes four (J_4) bonds away. The correlation facilitates the connection of the various fragments from the ^{1}H, ^{13}C, COSY and HSQC experiments. From the HMBC experiment (4.21 and 4.22) the following cross peaks were observed.

(H) (C)

6.20→ 93.83, 103.48, 161.20, 164.43

6.42→ 98.68, 103.48, 156.63, 164.43

6.90 → 122.45, 145.54, 148.19

7.56 → 115.56, 148.19

7.69→ 120.45, 145.54, 147.29

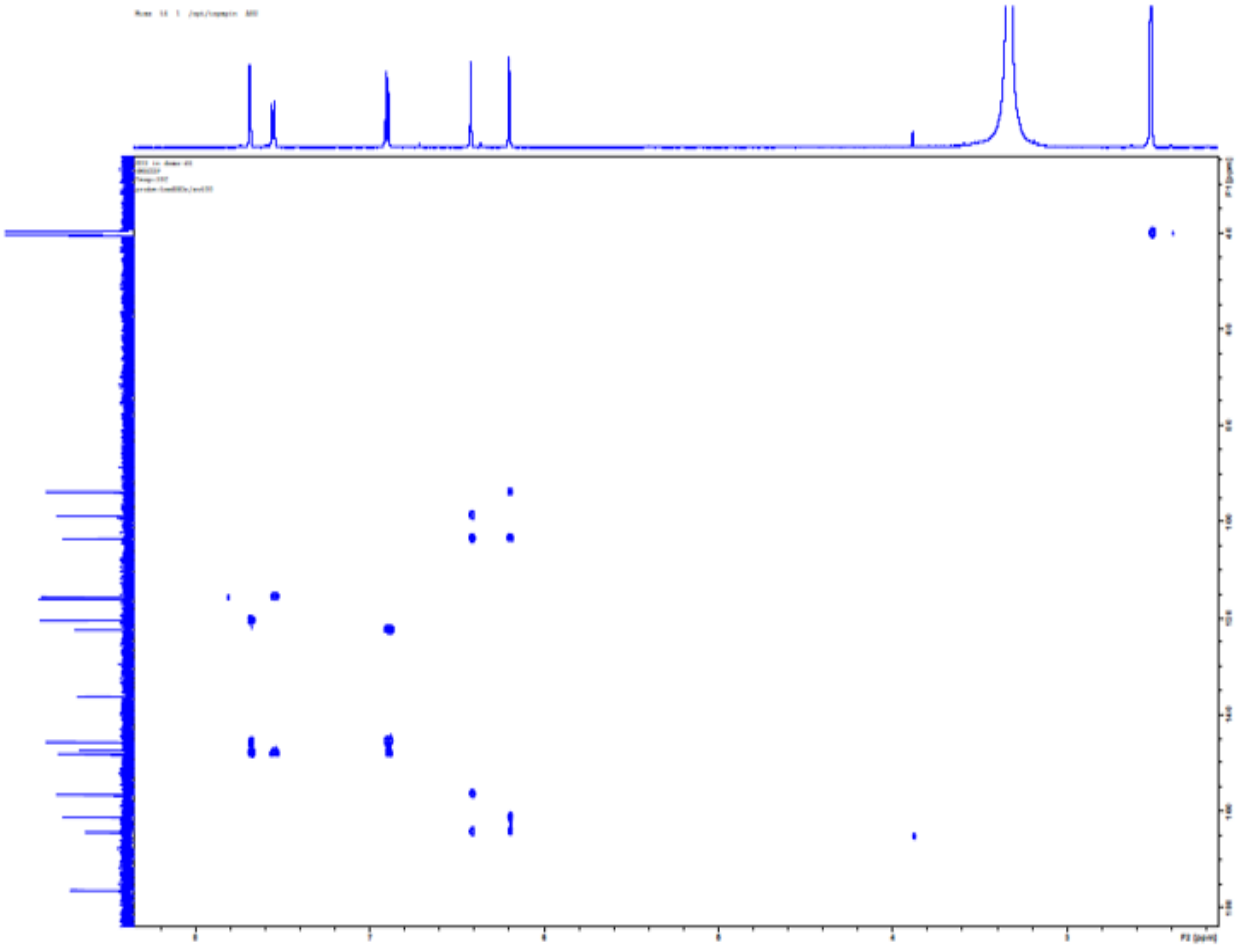


Figure 4.21: HMBC Spectrum of SD2

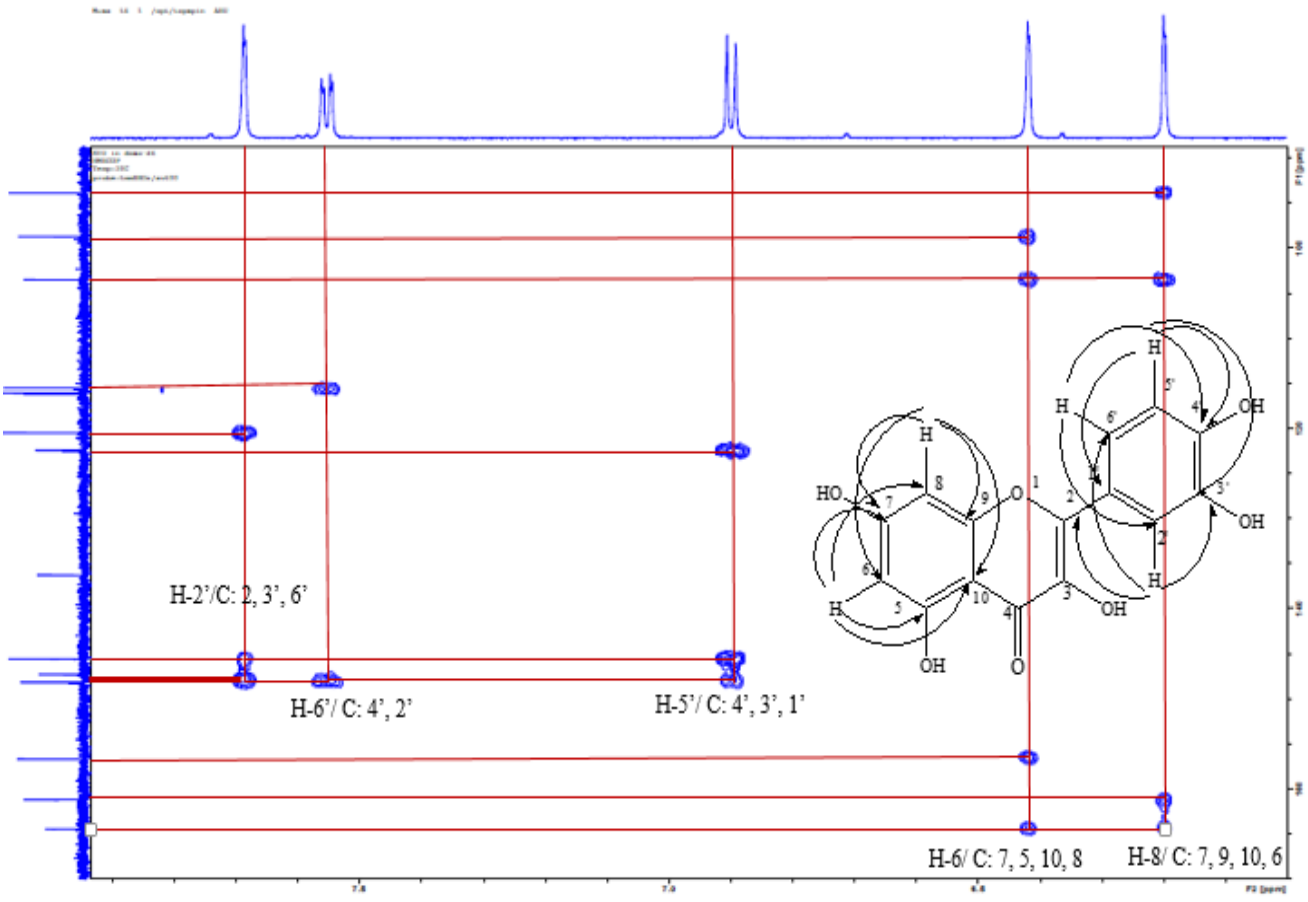


Figure 4.22: HMBC Spectrum of SD2 (expanded 6.0-8.0 ppm)

4.7.4.8 NOESY Analysis of SD2

The NOESY result of SD2 (Figure 4.23) showed correlations between the protons at $\delta_{H:}$ 6.90 and 7.56.

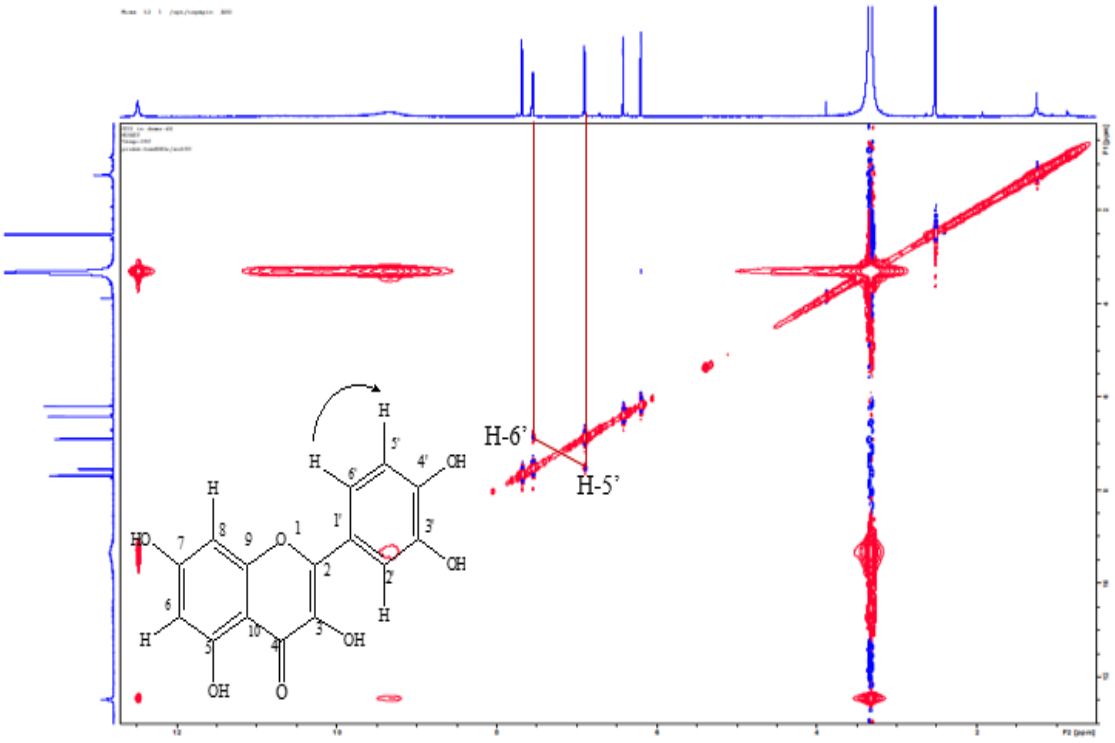


Figure 4.23: NOESY spectrum of SD2

The 1D and 2D NMR data of SD2 are summarized in Table 4.9

Table 4.9: Summary of 1D and 2D spectral data for compound SD2

Position	^{13}C	^{1}H, *J* (Hz)	DEPT
2	147.29		C
3	136.19		C
4	176.31		C
5	161.20		C
6	98.68	6.20 (d, 1H, *J*=1.50)	-CH-
7	164.43		C
8	93.83	6.42 (d, 1H, *J*=1.14)	-CH-
9	156.63		C
10	103.48		C
1`	122.45		C
2`	115.56	7.69 (d,1H, *J*=1.80)	-CH-
3`	145.54		C
4`	148.19		C
5`	116.48	6.90 (d, 1H, *J*=8.40)	-CH-
6`	120.45	7.56 (dd,1H, *J*=8.34,1.74)	-CH-

Table 4.10: Comparison of 1D NMR data of SD2 with literature data

	Compound SD1 (DMSO)		Pereira et al., 2012 (DMSO-d_6)	
Position	$\delta^{13}C$	$\delta^{1}H$, J (Hz)	$\delta^{13}C$	$\delta^{1}H$, J (Hz)
2	147.29		156.5	
3	136.19		133.9	
4	176.31		177.6	
5	161.20		161.2	
6	98.68	6.20 (d, 1H, *J*=1.50)	99.1	6.17 (d, 1H, J= 1.7)
7	164.43		165.1	
8	93.83	6.42 (d, 1H, *J*=1.14)	94.0	6.38 (d, 1H, J= 1.7)
9	156.63		156.8	
10	103.48		103.9	
1`	122.45		121.3	
2`	115.56	7.69 (d,1H, *J*=1.80)	116.4	7.54 (d, 1H, J= 2.1)
3`	145.54		145.1	
4`	148.19		148.8	
5`	116.48	6.90 (d, 1H, *J*=8.40)	115.5	6.80 (d, 1H, J= 8.4)
6`	120.45	7.56 (dd,1H, *J*=8.34,1.74)	122.2	7.63 (dd, 1H, J= 2.1, 8.5)

4.8 Antimicrobial Assays

4.8.1 Antimicrobial Activity

The results of antimicrobial studies of the extracts shows that CEE and EE1 had strong antimicrobial activity against all the tested organisms with zones of inhibition ranging from 10-27mm but has no activity against *E. coli*. All the extracts showed strong activity against *K. pneumoniae* except extract HC which has no activity against all the isolates (Table 4.11 and 4.12).

	Zone of Inhibitions (mm)															
	CEE (mg/ml)					HC (mg/ml)					EE1 (mg/ml)					CP
	10	**5**	**2.5**	**1.25**	**0.625**	**10**	**5**	**2.5**	**1.25**	**0.625**	**10**	**5**	**2.5**	**1.25**	**0.625**	**0.03mg/disc**
B. subtilis	20	15	0	0	0	0	0	0	0	0	0	10	11	12	0	30
E. coli	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	32
K. pneumoniae	25	20	18	0	0	0	0	0	0	0	21	23	26	20	24	32
MRSA	14	0	0	0	0	0	0	0	0	0	16	13	12	11	0	32
VRSA	17	15	0	0	0	0	0	0	0	0	14	13	11	10	0	25
S. typhi	17	14	23	30	28	0	0	0	0	0	15	18	23	0	0	25

Table 4.11: Antimicrobial Activity of Extracts CEE, HC and EE1 of *Globimetula Oreophila*and ciprofloxacin.

CEE= Crude ethanol, HC= Hexane + Chloroform, EE1= Ethyl acetate + ethanol extracts and CP= Ciprofloxacin. MRSA= Methicillin resistant Staphylococcus aureus and VRSA= Vancomycin resistant Staphylococcus aureus.

Organism	Zone of Inhibitions (mm)															
	CHL2 (mg/ml)					CE (mg/ml)					EE2 (mg/ml)					CP
	10	**5**	**2.5**	**1.25**	**0.625**	**10**	**5**	**2.5**	**1.25**	**0.625**	**10**	**5**	**2.5**	**1.25**	**0.625**	**0.03mg/disc**
B. subtilis	18	0	0	0	0	21	18	0	0	0	15	13	12	0	0	30
E. coli	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	32
K. pneumoniae	16	13	0	0	0	27	21	18	11	0	14	15	0	0	0	32
MRSA	0	0	0	0	0	21	16	0	0	0	0	0	0	0	0	32
VRSA	15	13	0	0	0	0	0	0	0	0	0	0	0	0	0	25
S. typhi	15	0	0	0	0	18	13	0	0	0	13	0	0	0	0	25

Table 4.12: Antimicrobial Activity of Extracts CHL2, CE and EE2 of *Globimetula oreophila*and ciprofloxacin.

CHL2= Chloroform 2, CE= Chloroform-Ethanol and EE2= Ethanol 2 extracts. MRSA= Methicillin resistant Staphylococcus aureus and VRSA= Vancomycin resistant Staphylococcus aureus.

The isolated compounds showed activity against all the bacterial strains with inhibition zones of 13-18 mm at far less concentrations than the extractsexcept *E. coli* and *VRSA*(Table 4.13).

Table 4.13: Result of Antimicrobial Activity of compounds SD1, SD2 and Ciprofloxacin

Organism	**Zone of Inhibitions (mm)**		
	C/P (0.03mg/disc)	**SD1 (0.05 mg/ml)**	**SD2 (0.05 mg/ml)**
B. subtilis	30	13	0
E. coli	32	0	0
K. pneumoniae	32	17	18
MRSA	32	0	18
VRSA	25	0	0
S. typhi	25	18	13

4.8.2 Combination Effect

The result of combination therapy of the extracts and isolated compounds with ciprofloxacin exhibited enlargement of combined inhibition zone diameter size by not <0.5mm (Table 4.14 and 4.15)

Table 4.14: Zones of inhibition of the synergistic effect of CHL2, CE, CEE, EE1 and EE2 with Ciprofloxacin.

Organism	**Concentration of extracts (mg/ml) + Ciprofloxacin (mg/ml)**					
	CHL2	**CE**	**CEE**	**EE1**	**EE2**	**CP**
	Extract + **CP**	5 + 0.015	1 + 0.015	5 + 0.015	5 + 0.015	5 + 0.015	0.03
B. subtilis		34	36	36	33	36	30
E. coli		34	44	33	46	37	32
K. pneumoniae		35	30	37	31	34	30
MRSA		33	42	33	36	34	32
VRSA		34	40	33	40	32	30
S. typhi		32	33	35	44	36	30

CP= Ciprofloxacin, CHL2= Chloroform 2, CE= Chloroform-ethanol, CEE= Crude ethanol, EE1= Ethyl acetate + ethanol, EE2= Ethanol 2. MRSA= Methicillin resistant Staphylococcus aureus and VRSA= Vancomycin resistant Staphylococcus aureus.

Table 4.15: Zones of inhibition of the synergistic effect of the compounds SD1 and SD2 with Ciprofloxacin.

Organism	**Concentration of compounds (mg/ml) + Ciprofloxacin (mg/ml)**		
	SD1	**SD2**	**CP**
	Compound 0.05 + **CP** 0.015	0.05 + 0.015	0.03
B. subtilis	32	35	30
E. coli	35	37	32
K. pneumoniae	32	36	30
MRSA	34	35	32
VRSA	35	35	30
S. typhi	35	35	30

CP= Ciprofloxacin**,** SD1 and SD2 are the compounds isolated. MRSA= Methicillin resistant Staphylococcus aureus and VRSA= Vancomycin resistant Staphylococcus aureus.

CHAPTER FIVE

5.0 Discussions

The result of phytochemical investigation (Table 4.2) revealed the presence of tannins, saponins, flavonoids and alkaloids in extracts EA, CE, EE and CEE while CHL contain flavonoids and steroids/triterpenes, HEX contains only steroids/triterpenes. These secondary metabolites have been associated with several pharmacological properties of plants (Cowan, 1999). Sedative activity have also been associated with most of these constituents (Edewor-Kuponiyi, 2013).

Purification of chloroform-ethanol extract (CE) through repeated gel filtration over sephadex LH-20 eluting with methanol led to the isolation of two compounds coded SD1 (light yellow solid) and SD2 (yellow solid). The melting point of SD1 was determined to be 294-297^{0}C. This indicates the purity, pure compounds have sharp melting points.The positive chemical test with ferric chloride and Shinoda test observed with SD1 is an indication of free hydroxyl group(s) (Silva et al., 1998) and flavonoid nucleus (Sofowora, 1993) respectively.

The UV spectrum (Figure 4.1) of SD1 showed absorption at 209nm, 256nm and 371nm wavelengths which is indicative of conjugated π-electron system (Bohm, 1999) while the IR spectrum of SD1 (Figure 4.2) showed absorption at 3302cm^{-1}which is characteristic of

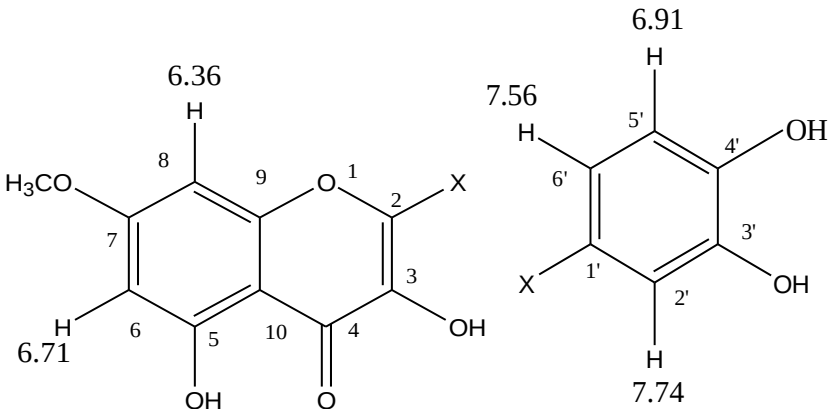
O-H stretching or phenolic hydroxyl groups, the signal at 1658cm^{-1}is indicative of carbonyl carbon stretch (C=O), the signal at 2948cm^{-1}is assignable to an Sp3 C-H and also the bands observed in the finger print region are due to various C-H stretching and bending modes (Wade, 2006). This also suggest a flavonol nucleus (Han *et al*., 2004; Lee *et al*., 2007).

The ^{1}H NMR spectrum of SD1 (Figure 4.3 and 4.4) exhibited a meta-coupled system at δ6.36 (d, J=1.98Hz) and δ6.71 (d, J=1.98Hz) assignable to H-6 and H-8 respectively. A tri-substituted ABX coupling system was portrayed on ring B at δ7.74 (d, J=2.04Hz), δ6.91 (d, J=8.46) and δ7.56 (dd, J=2.04, 8.58Hz) representing H-2', H-5' and H-6' respectively. These coupling system indicates a 3, 5, 7, 3', 4' penta-oxygenated flavonol typical of quercetin nucleus (Mabry *et al*., 1970; Markham *et al*., 1978; Abdullahi *et al*., 2011). The spectrum also showed signal at δ3.90ppm integrating for three protons indicating the presence of a methoxy group.

The ^{13}C NMR Spectrum (Figure 4.5) revealed the presence of 16 carbon atoms; 1 in the carbonyl region, 14 in the aromatic region and 1 in the methoxy region. The down field signal at 176.45 ppm was due to the carbonyl group at C-4. The DEPT experiment (Figure 4.6) confirmed the multiplicity of carbon atoms as 10 quaternary, 5 methine and 1 oxymethyl.

The ^{1}H-^{1}H COSY (Figure 4.7) established the correlations between protons; 6.36 (H-8) and 6.71 (H-6), 6.91 (5') and 7.56 (6').

The presence of two major fragments viz rings A and B was established from the above information.



Ring A **Ring B**

Further 2D NMR correlation experiments (HSQC and HMBC) were used to establish the structure of the compound SD1.

The HSQC experiment (Figure 4.8) established the attachments of various protons to their respective carbons. Among these attachments are: 3.90 (H)→56.49 (C), 6.36 (H-8) → 97.92 (C-8), 6.71 (H-6) → 92.38 (C-6),6.91 (H-5') → 116.06 (C-5'), 7.56 (H-6') → 120.53 (C-6') and 7.74(H-2') → 115.72 (C-2').

HMBC Spectroscopy was used to establish linkages between the two fragments. The result (Figure 4.9 and 4.10) showed a long range correlation between δ_H6.71 (Ring A, H-6) and quaternary carbon δ_C104.49 (C-10) connected to the carbonyl carbon (C-4) and between δ_H 7.74 (Ring B, H-2`) and δ_C 148.38 (C-2) of the chromone ring, other correlations are shown in (Table 4.6, figures 4.9 and 4.10).

In the NOESY spectrum, correlations were observed between methoxy proton at δ_H 3.90 and anomeric protons at δ_H 6.36 and 6.71. This confirms the attachment of OCH_3at position 7 (Figures 4.11)

Based on the analysis of spectroscopic data (UV, IR, 1D and 2D) and after comparing with data in literature , the structure of SD1 was proposed to be a 7-Methoxy-3,5,3',4'-

tetrahydroxyflavone or 7-Methoxyquercetin (Rhamnetin) with the following structure (Figure 5.1).

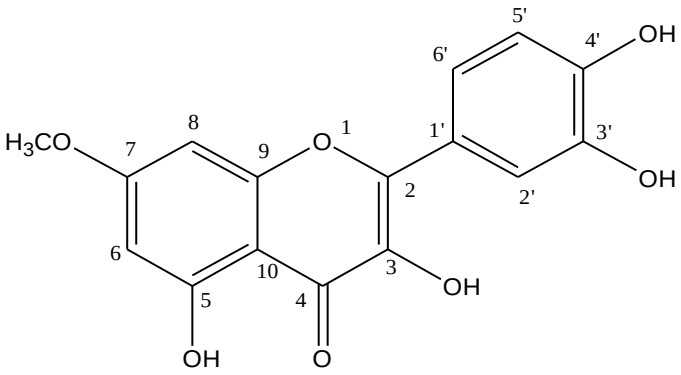


Figure 5.1: Proposed structure of SD1

Melting point of SD2 was determined to be 314-317^{0}C. The sharp melting point displayed the compound is an indication of purity. The positive chemical test with ferric chloride and Shinoda test observed with SD2 is an indication that it is a flavonoid with free hydroxyl group(s) (Silva et al., 1998; Sofowora, 1993).

The existence of three absorbance at 224nm, 256nm and 371nm wavelengths in the U.V spectrum of SD2 is an indication that it might be a flavonoid with conjugated π-electron system (Bohm, 1999).

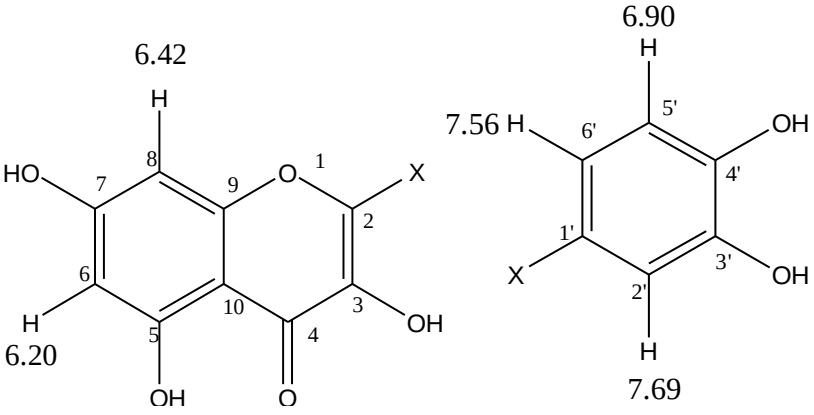
The very broad band at 3291cm^{-1}observed in the IR spectrum of SD2 is characteristic of OH stretching or phenolic hydroxyl groups, band at 1654cm^{-1}is for the carbonyl group, the peak at 2922cm^{-1}is assignable to an Sp3 C-H and also the bands observed in the finger print region are due to various C-H stretching and bending modes (Wade, 2006). This suggest a flavonol nucleus (Han *et al*., 2004; Lee *et al*., 2007).

The ^{1}H NMR spectrum of SD2 (Figure 4.14 and 4.15) exhibited a meta-coupled system at δ6.20 (d, J=1.50Hz) and δ6.42 (d, J=1.14Hz) assignable to H-6 and H-8 respectively. A tri-substituted ABX coupling system was portrayed on ring B at δ7.69 (d, J=1.80Hz), δ6.90 (d, J=8.34) and δ7.56 (dd, J=1.74, 8.40Hz) representing H-2', H-5' and H-6' respectively. These coupling system indicates a 3, 5, 7, 3', 4' penta-oxygenated flavonol typical of quercetin nucleus (Mabry *et al*., 1970; Markham *et al*., 1978; Abdullahi *et al*., 2011).

The ^{13}C NMR Spectrum (Figure 4.16) revealed the presence of 15 carbon atoms; 1 in the carbonyl region and 14 in the aromatic region. The down field signal at 176.31 ppm was due to the carbonyl group at C-4. The DEPT experiment (Figure 4.17) confirmed the multiplicity of carbon atoms as 10 quaternary and 5 methine.

The ^{1}H-^{1}H COSY (Figure 4.18 and 4.19) established the correlations between protons; 6.20 (H-6) and 6.42 (H-8), 6.90 (5') and 7.56 (6').

The presence of two major fragments viz rings A and B was established from the above information.



Ring A Ring B

Further 2D NMR correlation experiments (HSQC and HMBC) were used to establish the structure of the compound SD2.

The HSQC experiment (Figure 4.20) established the attachments of various protons to their respective carbons. Among these attachments are: 6.42 (H-8) → 93.83.92 (C-8), 6.20 (H-6) → 98.68 (C-6),6.90 (H-5') → 116.08 (C-5'), 7.56 (H-6') → 120.45 (C-6') and 7.69(H-2') → 115.56 (C-2').

HMBC Spectroscopy was used to establish linkages between the two fragments. The result (Figure 4.21 and 4.22) showed a long range correlation between δ_H6.20 (Ring A, H-6) and quaternary carbon δ_C103.48 (C-10) connected to the carbonyl carbon (C-4) and between δ_H 7.69 (Ring B, H-2`) and δ_C 147.29 (C-2) of the chromone ring, other correlations are shown in (Table 4.6, figures 4.21 and 4.22).

The NOESY spectrum of SD2 (Figure 4.23) further confirms the correlation between the protons at δ_H6.90 and 7.56.

Based on the analysis of spectroscopic data (UV, IR, 1D and 2D) and after comparing with data in literature (Pereira *et al*, 2012), the structure of SD2 was proposed to be a -3,5,7,3',4'-Pentahydroxyflavone or Quercetin with the following structure (Figure 5.2).

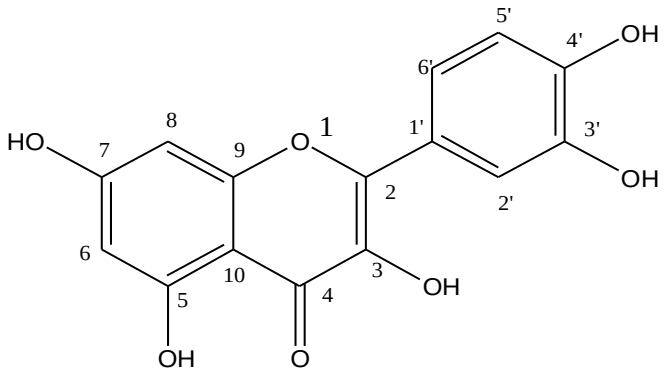


Figure 5.2: Proposed structure of SD2

The results of antimicrobial studies on the extracts and the isolated compounds are quite revealing. Extracts CEE and EE1 showed strong antimicrobial activity against all the organisms tested with zone of inhibitions ranging from 10mm-30mm but has no effect on *E. coli*. The activity observed against MRSA and VRSA (inhibition zones of 10-17 mm) is worth noting. *Staphylococcus aureus* is known to play a significant role in skin diseases including superficial and deep follicular lesion (Srinivesan *et al.,* 2001),so the strong activity of extract CEE and EE1 indicates that the plant can be a source of compound that can be effective against skin infections. The ability of the extracts to inhibit *S. typhi* isolate used in the study with inhibition zones of not <13 mm showed the plant to be promisingin controlling the incidence of typhoid fever especially nowthat there are reports of *S. typhi* acquiring resistance to theexisting antibiotics used in its control (Naxhije, *et al*., 2011). All the extracts showed strong activity against *K. pneumonie*with inhibition zone diameters of 13-27mm even at lower concentrations of about 0.625 mg/ml of the extracts. Infact, an increase in activity against *K. pneumonie* was observed as the concentration of extract EE1 decreases; extract HC has no activity against all the isolates. Flavonoids have strong activity against *K. pneumoniae* with MICs ranging from32-64 µg/ml (Xu and Lee, 2001).

The isolated compounds SD1 and SD2 showed activity against all the bacterial strains with inhibition zones of 13-18 mm at far less concentrations than the extracts except *E. coli* and *VRSA*. The absence of activity against *E. coli* and *VRSA* in any of the 2 isolated compounds suggests the presence of some other bioactive principles in *Globimetula oreophila* plant, yet to be isolated.

The extracts and isolated compounds showed potent synergistic activity with ciprofloxacin against the tested organisms. The combinations of the extracts and compounds with

Ciprofloxacin exhibited enlargement of combined inhibition zone diameter size by not <0.5mm. In fact, the extracts and compounds showed strong synergistic effect with ciprofloxacin even when they do not have any activity against the tested organisms on their own. Plant antimicrobials have been found to be synergistic enhancers in that though they may not have any antimicrobial properties alone, but when they are taken concurrently with standard drugs they enhance the effect of that drug(Kamouto *et al*., 2006).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

Preliminary phytochemical investigation of the hexane, chloroform, ethyl acetate, ethanol, chloroform-ethanol and crude ethanol extracts of *Globimetula oreophila* revealed the presence of steroids/triterpenes, flavonoids, saponins, tannins and alkaloids.

Gel filtration chromatography of chloroform-ethanol extract (CE) led to the isolation of SD1 (7-methoxyquercetin) and SD2 (Quercetin).

The extracts showed moderate to good antimicrobial activity against a wide range of bacterial organisms tested. The isolated compounds also exhibited anti-microbial activity at far less concentrations than the crude extracts. This underscores the possibility of utilizing some of these isolated compounds as lead drugs in the development of more effective anti-bacterial agents.

The extracts tested and the isolated compounds showed great synergistic potentials with ciprofloxacin at far less concentrations than when they are used singly.

6.2 Conclusion

This research into the validation of the use of *Globimetula oreophila* in the management ofinfectious diseases has achieved the stated aim. Two bio-active principles (quercetin and 7-methoxyquercetin) were isolated and tested some of which could serve as potential lead

drugs and sensitized the scientific community into more research in largely uninvestigated *Globimetula* species in Nigeria.

To the best of our knowledge, this is the first report of isolation of Quercetin and 7-methoxyquercetin (Rhamnetin) from *Globimetula Oreophila*.

Based on the findings in this work, it can be stated that the use of*Globimetula Oreophila*in the treatment of infectious diseases is justified.

6.3 Recommendations

1. There is need to carry out bioassay guided evaluation on the constituents of the plant with a view of isolating the bioactive compounds responsible for the observed Central Depressant Activity and combination therapy.

2. Further studies need to be carried out on the isolated compounds.

3. Comparative studies should be carried out between the plant and its host because these mistletoes are hemi-parasitic on their host and has been reported in the literature that there is a strong relationship in terms of bioactive constituents and biological activities between them.

4. There is also need to carry out chronic toxicity studies on the plant to establish its safety upon prolonged usage.

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APPENDIX

Appendix I: Gel filtration chromatography of extract CE

