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CENTRE DE RECHERCHE ET FORMATION DOCTORALE EN SCIENCES, TECHNOLOGIE ET GEOSCIENCES
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REPUBLIC OF CAMEROON
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THE UNIVERSITY OF YAOUNDE I
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POST GRADUATE SCHOOL FOR SCIENCE, TECHNOLOGY AND GEOSCIENCE
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PHYTOCHEMICAL AND PHARMACOLOGICAL STUDIES OF CLUSIACEAE (*Garcinia brevipedicellata Oliv.*)

THESIS

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DEDICATION

This work is dedicated to:

To God the father almighty

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LIST OFABBREVIATIONS, SYMBOLS AND ACRONYMS

EtOAc :	Ethyl acetate
EtOH :	Ethanol
ATP :	Adenosine TriPhosphate
brs :	Broad singlet
°C :	Degree Celsius
CC :	Column Chromatography
COSY :	COrrelation SpectroscopY
CH_2Cl_2 :	Methylene chloride
CSH :	Chalcone Synthase
d :	Doublet
dd :	Doublet of doublet
ddd :	Doublet of doublet of doublet
DEPT :	Distortionless Enhancement by Polarization Transfer
DMSO :	DiMethyl SulphOxide
G :	*Garcinia*
g :	Gramm
GB :	*Garcinia buchananii*
GBr :	*Garcinia brevipedicellata*
Hex :	Hexane
HIV :	Human Immuno Virus
IR :	Infra-Red
HMBC :	Heteronuclear Multiple Bond Connectivity
HRMS :	High Resolution mass spectrum
$[M+H]^+$:	Molecular ion peak
HSQC :	Heteronuclear Single Quantum Coherence
IC_{50}:	50% Inhibition Concentration
***J*:**	Coupling constant
HSCoA :	AcetylCoenzyme-A
Hz :	Hertz
MHz :	Mega Hertz
***m/z*:**	Mass-to-charge ratio

MS	**:**	Mass Spectrometry
ppm	**:**	Part per million
NMR	**:**	Nuclear Magnetic Resonance
NOESY	**:**	Nuclear Overhauser Enhancement SpectroscopY
OAU	**:**	Organization of African Union
***δ*:**		Chemical shift
s	**:**	Singlet
TOF-MS	**:**	Time Of Flight Mass Spectrum
t	**:**	Triplet
UV	**:**	Ultra Violet
TLC	**:**	Thin Layer Chromatography
TMS	**:**	TetraMethylSilane
$CDCl_3$	**:**	Deuterated chloroform
NTFP	**:**	Non-Timber Forest Products

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMES

ABSTRACT

Within the framework of building a scientific data base of Cameroonian medicinal plants, phytochemical investigations have been carried out on a plant currently used by traditional healers to solve daily health problems of the population. The main aim of this work is to complete the results already obtained from biological and biochemical tests carried out on the extracts of 100 selected plants currently used in folk medicine. The specific objective is to elaborate a good extraction protocol of the leaves of *Garcinia brevipedicellata (Clusiaceae),* and then followed by the purification of the extracts so as to know and compare the different classes of secondary metabolites found in this extract. It is also important to carry out other related tests to identify the biological activities of these constituents.

From the ethyl acetate extract of the leaves of *Gacinia brevipedicellata*, seventeen compounds were isolated which were given the abbreviations GBr-1-GBr-17 and the first nine (GBr-1-GBr-9) were characterized using routine spectroscopic methods including 1D and 2D NMR as well as by comparing obtained data with literature. Among these, two ether biflavnoids trivialy named brevipedicelone D and brevipedicelone E were new. The other compounds were identified as robustaflavone, 4-*O*-Me-robustaflavone, brevipedifloside A, apigenine, 2'-hydroxy -4'- *O*-methylgenistein, amentoflavone and luteoline respectively.

The two new compounds were evaluated for anti-onchocercal activities. It was found that among the two compounds tested only brevipedicelone D showed moderate inhibition of the adult worm motility of *Onchocerca ochengi* by 60 % at the highest concentration (20 µg/ml) and inhibited motility of both the juvenile worms of *O. ochengi* and *Loa loa* 90 % at this same concentration.

Key words: *Garcinia brevipedicellata.* Brevipedicelone. *Anti*- Onchocercal activity

RESUME

Dans le cadre d'une contribution phytochimique à l'élaboration d'une banque de données scientifiques sur les plantes médicinales du Cameroun, l'investigation d'une plante couramment utilisée par les tradipraticiens pour resoudre divers problèmes quotidiens de santé de la population a été faite. Ces travaux ont pour but principal de compléter avec les données phytochmiques, les résultats des tests biologiques et biochimiques effectuées sur les extraits de 100 plantes sélectionnées, régulièrement utilisées en medecine traditionnelle. L'objectif specifique est d'élaborer un bon protocole d'extraction des feuilles de *Garcinia brevipedicellata* (Clusiaceae), puis d'effectuer la purification de l'extrait pour identifier et comparer les différentes classes de métabolites secondaires que cet extrait renferme.

A partir de l'extrait à l'acétate d'éthyle des feuilles de *Garcinia brevipedicellata*, dix sept composés ont été isolés, indexés de GBr-1 à GBr-17, mais seuls les neufs premiers (GBr-1 à GBr-9) ont été caractérisés à l'aide des méthodes spectroscopiques telles que l'UV, IR, et la RMN à une et à deux dimensions. Parmi ces composés, deux éthers biflavonoyles, nommés trivialement brevipedicelone D et brevipedicelone E ont été décrits pour la première fois dans le cadre de ce travail. Les autres composés ont été idenifiés respectivement à la robustaflavone, 4'-*O*-methylrobustaflavone, brevipedifloside A, apigenine, 2'-hydroxy -4'- *O*-methylgenistein, amentoflavone et luteoline.

Du criblage anti-onchocercique de ces derivés nouveaux, seul le brevipedicelone D à presenté une inhibition modérée de la motilité des vers adultes d'*Onchocerca ochengi* de 60% à la concentration la plus élevée (20 µg / ml) et une inhibition de la motilité des deux vers juvéniles d'*O. Ochengi et de Loa loa* de 90% à cette même concentration.

Mots clez : *Garcinia Brevipedicellata*, Brevipedicelone, criblage anti-onchocercique

TABLE OF CONTENTS

INTRODUCTION

Phytochemistry is the study of secondary metabolites present in plants, animals and fungi **(Newman and Cragg, 2007).** The use of plants and plant products in traditional medicine is as old as man himself. Humans have always used medicinal plants to solve their health problems throughout their life, **(Petrovska, 2012, Kirtikar and Basu, 1918, Tang and Eisenbrand, 1992).**

Recently, a lot of effort is being made to identify the natural products responsible for these curative properties from plants in the program drug discovery and development. **(Okada et al., 2010).** Unfortunately, these plant products have always been used as mixtures without the real knowledge of their chemical natures.

The use of modern analytical techniques like chromatography, electrophoresis, isotope techniques and enzymology has contributed significantly to the knowledge of many of these compounds as well as the important biosynthetic pathways explaining the presence of these products in these plants **(Harborne, 1998, Balunas and Kinghorn, 2005).**

For the past fifteen years, an elaborate project is being carried in my host laboratory dealing with the creation of a scientific data base for medicinal plants used in Cameroonian traditional medicine. These plants were chosen from an OAU report containing a scientific data base on medicinal plants**,** biological and biochemical tests were carried out on the crude extracts of different parts of the selected plants. The important results obtained shown inTables **I, II, III and IV**. **(Tih et *al.*, 2000)** led to the decision to carry out their phytochemical investigation

These results constituted the preliminary phase of the scientific data base of these plants and accounted for two PhD theses defended in the Faculty of Science, University of Yaounde I: one in biochemistry and the other in animal biology. These results also inspired us to continue our research in order to know more about the composition and the chemical nature of these plant extracts.

To achieve this goal, the phytochemical laboratory of the Department of Organic Chemistry of the University of Yaoundé I, carried out research in this area in collaboration with other home laboratories as well as others abroad. In general, the phytochemical research aiming at the identification of the secondary metabolites present in these plant extracts is given as research topics to Doctorat/PhD research students. It is in this perspective that I was proposed the theme ‘Phytochemical study of *G. brevipedicellata*.

General objective

To complete the phytochemical information on *G. brevipedicellata* thus contributing to the scientific data base of 100 selected plants mentioned earlier in this program and to carry out biological tests on the secondary metabolites isolated from the leaves of this plant.

Specific objectives:

- To harvest of the plant material (leaves, wood, roots, stem barks, branches etc.) of G*arcinia brevipedicellata*,
- To purify and the isolate of the secondary metabolites from the different crude extracts
- To determine their structures using physico-chemical methods
- To screen on these obtained pure constituents and to compare the obtained information with those obtained previously for their crude extracts.

CHAPTER I

LITERATURE REVIEW

I: BOTANY OF THE CLUSIACEAE FAMILY

I.1. Generalities on Clusiaceae (Guttiferaceae*)*

The *Clusiaceae* family long known by the name (*Guttiferaceae*), can be found in four of the five continents which are Asia, Australia, Africa and south America. The Clusiaceae family has about 1350 species accounting for 47 genera. The most represented are: *Vismia, Garcinia, Clusia, Cratoxylum, Harungana, Mesua, Hypericum, and Kielmeyera*, among others **(Piccinelli et al., 2005)**. They are largely spread in dense humid tropical forest regions. This tropical family is a rich source of isoprenylated xanthones and bioflavonoids (**Xu et al., 2001, Bennett. and Lee., 1989, Goh et al., 1991, Ampofo and Waterman., 1986, Aumond., 2004, Crichton and Waterman., 1979).**

The family Clusiaceae has two sub-families: *Kielmeyeroideae* Engl. and *Clusioideae* Engl. **(Stevens, 2007)** The first includes the *Mammea*, *Endodesmia*, *Lebrunia* and *Calophyllum*, while the second regroups *Allanblackia*, *Garcinia*, *Pentadesma* and *Symphonia*.

The members of this family which can be trees, shrubs or herbs but rarely lignans, are easily recognized by their yellow or orange resinous latex which usually flows slowly when the stems, flowers and fruits are wounded. The leaves hardly produce the latex. **(Ampofo and Waterman, 1986).** The wood of plants of the Clusiaceae family is hard and firm **(Letouzey, 1982)**, the stems of the trees have their branches spread out in such a way that the longer branches are below while the shorter ones are above, giving a conical apprearance; **(Aumond, 2004).**

The leaves have a simple morphology with an opposite disposition and rarely alternate. They possess oil glands. The flowers are polygamous, hardly hermaphrodites and are usually functionally unisexual having cyclic or spiral petals and sepals. The perianth consists of a calyx of 2-10 imbricated sepals and 4-12 petals. They are generally numerous distinct or variously united stamens. The gynoecium consists of a single pistil with 3-5 or more carpels, an equal number of stigmas, and a superior ovary of 3-5 or more locules, each containing many stamens.

The OAU report containing the scientific data base on medicinal plants studied in our laboratory years ago throws more light on the different families and their genera used in this program and the different biological and biochemical test made on them as shown on tables **I, II, III** and **IV** below.

Table 1 : OAU data base information on medicinal plants (Tih and col., 2000).

Plant Identification	Biological and biochemical tests	Phytochemistry
(vernacular name) -Economic uses -Medicinal uses -Scientific name	**Biological tests** Antihypertensive activity - Vasomotive activities - Analgesic effects - Anti-inflammatory effect **Biochemical tests** - Antifungi - Anti--bacteria - Anti-oxydant - Anti-hepatotoxic	**Extraction and purification of extracts** - spectroscopic analysis of pure compounds isolated: - MS, IR, UV, NMR (^{1}H and ^{13}C) - optical rotation - melting point

Table 2: 100 Plants chosen for studies in the program (Tih and al., 2000).

Family	Genus	No. of species chosen	Total No. Of species
Ochnaceae	*Lophira*	2	32
	Campylospermum	8	
	Rhapdophyllum	2	
	Ochna	14	
	Ouratea	6	
Clusiaceae	*Garcinia*	15	17
	Hypericum	2	
Asteraceae	*Vernonia*	9	9
Combretaceae	*Terminalia*	3	3
Hypericaceae	*Harugana*	4	4
Chrysebalanaceae	*Parinari*	6	6
Fabaceae	*Eriosema*	4	12
	Desmodium	8	
Rubiaceae	*Nauclea*	6	12
	Mitragyna	6	
Anonaceae	*Polyalthia*	3	5
	Anona	2	

Table 3: Results of biological tests (Tih and al., 2000)

Family	No. of species tested	Anti-hypertensive activity	Analgesic effect	Vasomotive activities	Anti-inflammatory effect
Ochnaceae	32	18	12	6	10

Clusiaceae	17	13	9	4	8
Asteraceae	9	8	8	3	4
Hypericaceae	4	2	3	1	4
Combretaceae	3	2	3	0	2
Chrysebalanaceae	6	3	2	2	4
Fabiaceae	12	6	8	4	8
Rubiaceae	12	6	4	3	9
Anonaceae	5	5	4	3	2

Table 4: Results of biochemical tests (Tih and al., 2000)

Family	No. of species tested	Anti-fungi activity	Anti-bacteria activity	Anti-oxidant effect	Anti hepato toxic effect
Ochnaceae	32	19	28	13	8
Clusiaceae	17	13	16	10	6
Asteraceae	9	4	6	8	3
Hypericaceae	4	2	4	2	2
Chrysebalanaceae	6	4	5	4	3
Fabiaceae	12	6	8	6	4
Rubiaceae	12	9	6	6	8
Anonaceae	5	2	3	2	3

I.2. Botany of the genus *Garcinia*

I.2.1. Generalities on the genus *Garcinia*

The genus *Garcinia* is the most represented genus of this family and is composed of approximately 400-600 species. Members include dioecious, evergreen trees, distributed in the tropical parts of the world **(Steentoft, 1988; Richards, 1990)**, About 200 of these species have been identified in tropical Africa **(Hutchinson, 1973, Lemee, 1931)**. Commonly, the plants in this genus are called saptrees, mangosteens (which may also refer specifically to the purple mangosteen, *G. mangostana*), garcinias or, ambiguously, **"monkey fruit"**.

I.2.2. Geographical distribution of the genus *garcinia*

Wherever they are found, plants of the genus garcinia grow in forest galleries, river banks or in ombrophilitic sub-woods **(Bamps, 1970).** In Africa, this zone extends from Senegal in the west part of the continent towards Tanzania in the east passing through Ghana, Nigeria, Cameroon and continues towards Angola and the two Congo republics in the South. The 21 species identified in Cameroon show that this genus is represented in all the ten regions of the country (**Guedje, 2002).** Table V.

Table 5 : Geographical distribution of the genus Garcinia in Cameroon (Guedje, 2002).

Species	REGION									
	East	**Adamawa**	**Far North**	**North**	**Centre**	**Western**	**North West**	**South West**	**Littoral**	**South**
G. afzelii			x		x					
G. barteri		x								
G. brevipedicillata					x			x	x	
G. chromocarpa								x		
G. ovalifilia	x	x		x					x	
G. mannii		x			x				x	
G. epunctata,		x						x	x	x
G. polyantha,	x				x		x	x		
G. gnetoids				x	x				x	
G. elliotii,								x		
G. nobilis								x		x
G. mangostana,						x				
G. tinctorial						x				
G. conranana					x	x				
G. preussii	x				x			x		
G. lucida					x			x	x	x
G. kola	x							x		
G. staudtii									x	
G. smeathmannii					x	x	x			
G. letestii									x	
G. punctate	x				x			x	x	x

I.3. Botany of *Garcinia brevipedicellata* (Bak.f.)

G. brevipedicellata is an evergreen tree that can measure about 5-9 meters high and about 30 cm in diameter and very abundant in forest regions. In certain localities some exceptions were noticed where it could grow up to 30 m high. The name of *Garcinia brevipedicellata* suggests it has short pedicels, the flowers are yellow with a red centre and the lateral vein is less dense **(Hutchinson et al. 1954).** The stem is always erect; the stem bark is yellowish in colour and not very thick. The twigs are always dressed but at times they are found creeping with many branches. This plant is easily recognized by the yellow or orange resinous latex which flows slowly out of the stem back when wounded.

Figure 1: *Garcinia brevipedicellata* (photo AKONGWI, 2013)

In the general classification of the Clusiaceae, *Garcinia brevipedicellata* is classified as follows:

Table 6 : Classification of Garcinia brevipedicillata (Cromquist, 1998)

Kingdom	Plantae
Order	Spermatophytes
Family	Guttifereae
Sub- family	Clusoidae
Tribe	Garciniacae
Genus	*Garcinia*
Species	*Brevipedicellata*

I.3.1 Ethnobotanical uses of the species of the genus *Garcinia*

Various parts of plants of the genus *Garcinia* (the stem bark, the grains, roots and the leaves) are diversely used in many parts of the world for nutritional, economic purposes as well as in traditional medicine. Actually, many species are threatened by extinction due to extensive use by indigenes. For example, *G. cadelliana* found on the South Andaman Island is almost completely extinct.

I.3.2. Importance of *Garcinia* species in traditional pharmacopeae

Plants of this genus are used in medicinal preparations destined for the treatments of abdominal pain, dysentery, diarrhea, suppuration, infected wound, leucorrhoea, chronic ulcer and gonorrhea **(Jayaprakasha G.K., P.S. Negi and B.S. Jena, Innov. Food Sci. Emerg.Technol., 7, 250** 2006). For example, the grains of *G. kola* and *G. mangostana* are used in the traditional preparation of many potions in traditional medicine destined for the treatment of gastro-intestinal and lung infections in Cameroon. They have important astringent properties as described in many African countries and Asia. (**Bouquet et Debray 1974).** In Thailand, leaves and grains of *G. dulcis* are used to treat goiter (**Deachatai et al., 2006)**

Most species of *Garcinia* are known for their brownish-yellow gum resin, due to the presence of xanthones. This resin is not only used as purgative or cathartic, but most frequently it serves as a pigment in decorations.

Fruit extracts from Bitter Kola *(G. kola)* have been claimed in stopping the replication of the Ebola virus. When its seeds are consumed with palm wine, they cleanse the stomach. The aqueous extract of its stem bark is used for the treatment of hypertension while that of the leaves generally serve for the treatment of gastro-intestinal and lungs infections. In Asia different parts of *G. mangostana, G. cambogia and G. kola* enter preparations used to treat chronic intestinal infections, catarrh, diabetes and urino-genital infections **(Awaad et al., 2013)**. These extracts are also very effective in the elimination of free radicals and in the fight against eczema and other skin diseases. The antibacterial and antiviral properties have been reported as they inhibit the growth of *Staphylococcus aureus* and suppress the growth of the Ebola virus. The leaves of *G. semseii, G. brevipedicellata and G. livingstoneii* show a moderate activity against HIV virus. **(Yamaguchi et al., 2000, Gustatson et al., 1992).**

The roots of *Garcinia huillensis* are used for the treatment of child diarrhea and parasitic diseases. The stem bark extracts are also used to treat sterility, sexual asthene, urinogenital infections **(Keay, 1954, Bouquet, 1969)**.

I.3.3. Nutritional importance

The ripe fruits of *G. cambogia* and *G. mangostana* have hydroxycitric acid which reduces appetite and for this reason they are consumed to fight against obesity (**Ambasta, 1986).** The fruit of *G. mangostana* contains vitamin C and is used as a spice **(Joy et al 1977).** The grains of *G. mangostana* are very oily and can be used to produce vegetable oil. Those of *G. kola, G. lucida and G. polyantha* readily replace kola **(Burkill, 1994).** Lastly, the grains of *G. Lucida, G. polyantha, G. punctata, G. brevipedicellata* and *G. manni* are consumed as antidote against poison and snake venum **(Nkongmeneck, 2000, Twtrakul et al., 2009)**.

The roots of *G. afzelii* and the grains of *G. kola* are consumed raw as kola while the young flowers of *G. parvifolia* are eaten as vegetables **(Xu et al., 2001).** The grains of *G. brevipedicellata* mixed with curry are used as spice in India and for conservation **(Normand, 1955)**.

I.3.4. Economic importance

The grains and stem barks of *G.* kola are classified among the non-timber forest products (NTFP) with respect to their commercialization importance both nationally and internationally. The commercialization of these NTFP takes place between Occidental Africa and Central African countries, Europe (France, England, Spain etc.) **(Walter and Mbala 2006, Bonannee, Ze et al., 2007).**

In Cameroon for example, the fruits, nuts and the stem bark of *G. kola* are a source of revenue to small and big business people. The price of one grain of bitter cola (commercial name of G. *cola*) is evaluated to be 25 to 50 FCFA in Yaounde, meanwhile in Bertoua, 8-grains are sold for 100 FCFA depending on the sizes. If we take a look at local markets of small cities like Buea, Kumba, Ambam or Abong-bang, there is a significant increase of the total sale of these grains per semester of NTFP (among which we have the grains and the stem bark of *G. cola* at first position) of about 10 million FCFA. These localities sell a limited number of these available products at the spot. The principal NTFP commercialized in 25 humid forest zone markets in Cameroon are shown on **(table 7).**

Table 7 : Principal NTFP commercialized in 25 markets of humid forest zones of Cameroon (average of two questionnaires)

Specie/product	Local name	Sales per semester (value estimated in thousand FCFA)	% of total sales value
Garcinia kola (stem barks)	Onie	2975	31
Garcinia cola (fruits)	Bitter kola, Onie	9944	28
Garcinia lucida (stem barks)	Essok	10994	31
Elaeis guineensis	Palm nut	11089	31
Kola spp	Kola nut Abel	135376	27

Table 8 below shows a summary of main ethnobotanical uses of some species of *Garcinia* in Cameroon.

Table 8 : Ethnobotanical uses of some species of Garcinia in Cameroon (Gustafson et *al*., 1992, Ambasta, 1986, Burkhill, 1994, Xu et *al*., 2001)

Concerned species	Uses	Parts used
Garcinia kola, G. afzeli *G. epunctata, G. mannii,* *G. polyantha, G. brevipedicellata*	Comestible fruits	peelings
G. kola, G. lucida, G. Mannii	Buccal hygiene	grains
G. kola, G. lucida, G.mannii, *G.lucida, G. Kola*	additives for palm wine	Grains and barks of the roots
G. kola, G. Mannii	ORL infections	
G. kola, G. aftelii, G. lucida,G. kola, *G. Mannii G. brevipedicellata*	Buccal and gastro infections	Branches, stem barks and Grains
G. lucida, G. kola, *G. brevipedicellata*	Gynaecological infections and STD's	Stem barks
G. mannii	Occular infections	Stem barks
G. kola, G. staudtii G. lucida, *G. kola, G. mannii, G. polyantha*	Stimulant	Grains, stem barks of the roots
G.lucida ,G.brevipedicellata	Anti poison and venum	Grains, stem barks of the roots
G. kola, G. brevipedicillata	Wound healing	Grains
G. kola, G. Lucida, G.manni, G. staudtii	Hunting	Roots, leaves Grains, wood
G. Lucida, G.manni, G. brevipedicellata	Construction	Wood and branches
G. kola, G. staudtii G. lucida,G. parvifolia	Against evil spirits vegetables and as insectifuge	Leaves
G.mangostana G.gambogia	Against obesity	Fruits

II- PREVIOUS PHYTOCHEMICAL STUDIES ON THE GENUS *GARCINIA*

II.1. Secondary metabolites characterized from the genus G*arcinia*

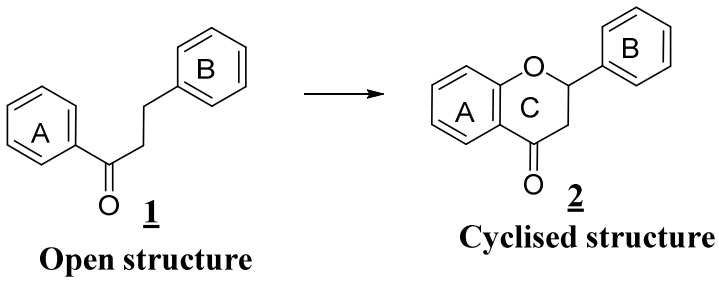
Phytochemical studies carried out on several species of the genus *Garcinia* show structural classes of secondary metabolites which include: flavonoids, biflavonoids, xanthones, steroids, acetogenins, cinamates, phloroglucinols, benzophenones, depsidones, tocotrienols and biphenyls.

II.1.1. Generalities on flavonoids

Flavonoids form the great majority of the phytochemical constituents of the genus *Garcinia*. The word '*flavonoid'* is derived from the Latin word "Flavus" which means yellow. Flavonoids are generally yellow or orange coloured pigments responsible for the coloration of diverse plant tissues like vacuoles where they are found as heterosides.

There are polyphenolic compounds, present in all parts of higher plants: (roots, leaves, and flowers, stem bark, wood, branches) etc. Their principal function is the coloration of plant tissues responsible for the attraction of pollinator insects thus playing a very important role in plant reproduction. **(Bahm et al., 1986).**

The structure of flavonoids show a basic carbon skeleton made of 15 carbon atoms incorporated in two benzene rings A and B linked by a short chain of three carbon atoms.



Scheme 2: The basic structure of flavonoids

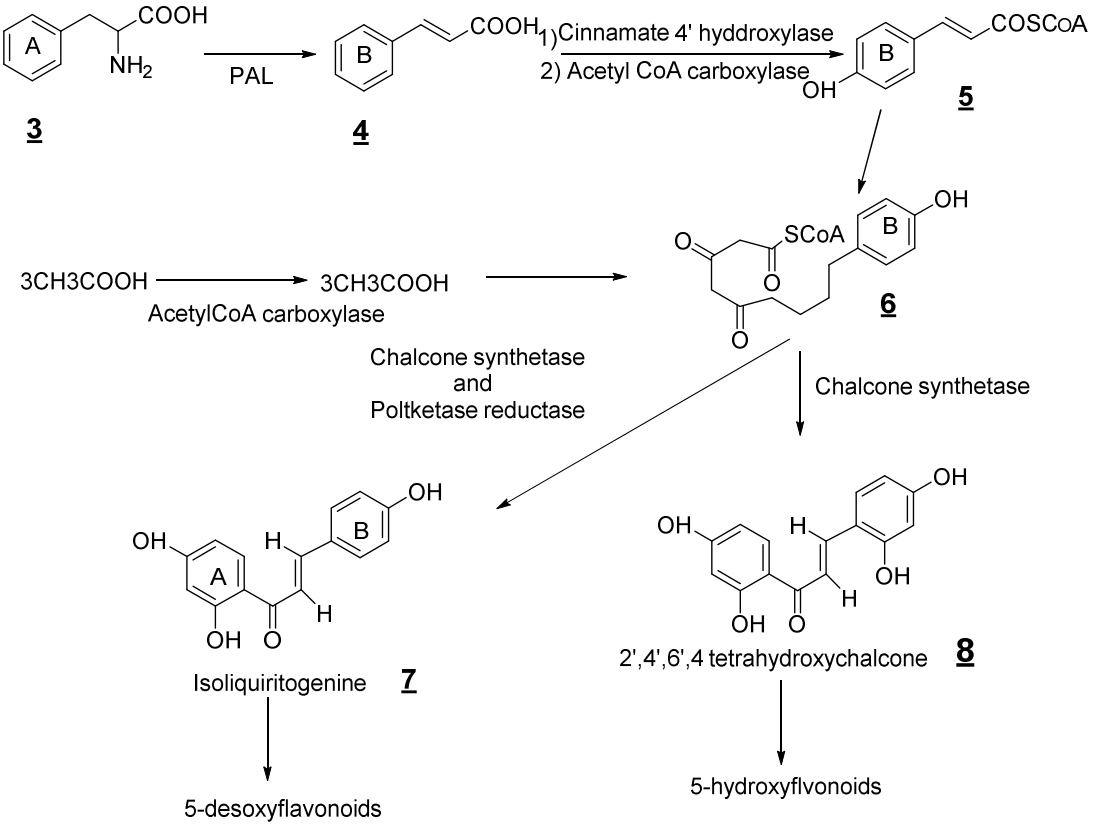
From the biosynthetic point of view, ring A results from the condensation of three acetate units while ring B is derived from phenylalanine transformed to cinnamic acid. The benzene rings can in many cases have oxygenated substituents some of which undergo cyclisation to give a benzo-pyranone ring (cyclic structure).

This basic cyclic structure can be modified by further oxidation and hydroxylation to give a variety of compounds which explain the large structural diversity of flavonoids. From the structural point of view of flavonoids, two major classes are distinguished: simple flavonoids and polyflavonoids

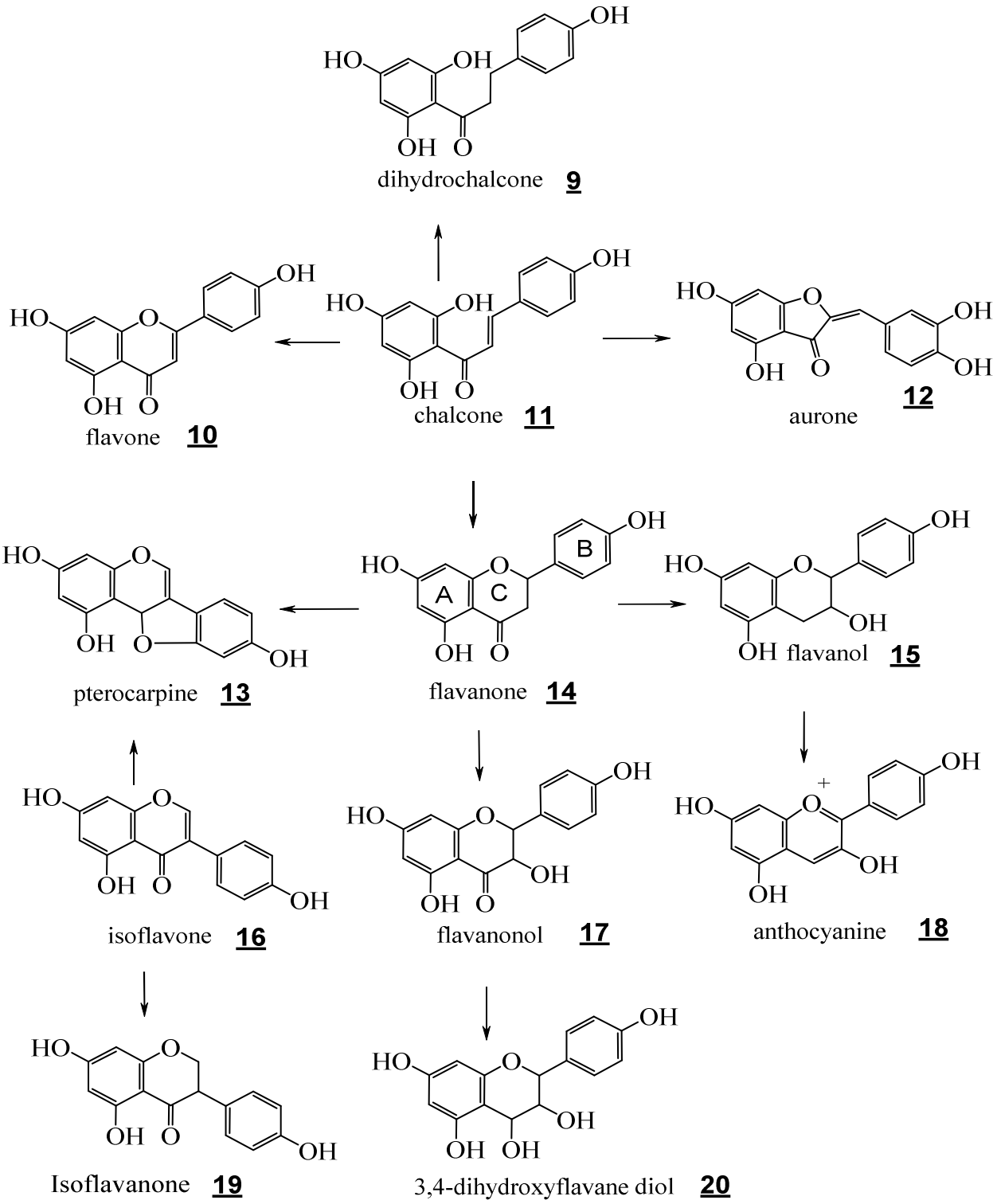
II.1.2. Simple flavonoids

Simple flavonoids have the basic carbon skeleton with 15 carbons atoms. These are the first compounds formed in the flavonoid biosynthetic chain. Members include chalcones which have the open structure of flavonoids, flavanones and flavones. In some cases, we have a 1, 2 shift of the aromatic ring B to give compounds of the isoflavonoid group. The reactions involved in these series of transformations are catalyzed by specific enzymes.

To better understand the biosynthetic pathway of these simple flavonoids, the transformations have been divided into two phases. Phase 1 which shows the formation of the basic chalcone motif **(Scheme 2).** While phase 3 deals with the conversion of this chalcone motif to other cyclized groups of flavonoids. **(Scheme 3).**

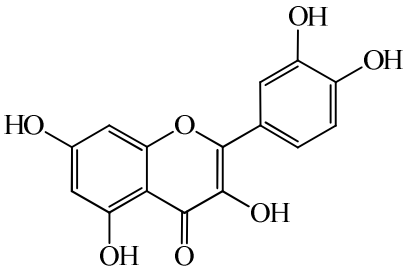


Scheme 3: Biosynthesis of the chalcone basic structure (Forkman, 1992)

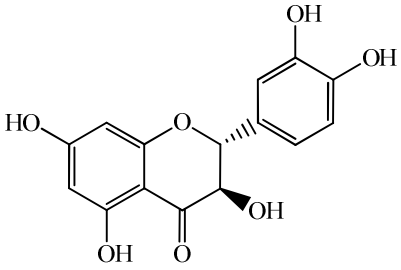


Scheme 4: Formation of different classes of simple flavonoids (Harborne, 1988)

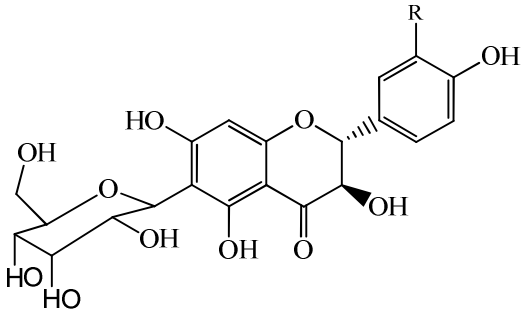
Some examples of simple flavonoids isolated from the genus *Garcinia*.arecompounds **21**-**27**.



Quercetine 21**(Charles et *al*., 2009)**



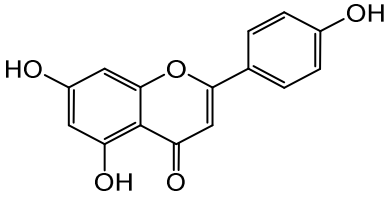
(2R, 3R)-taxifoline **22** **(Tanyi et Tanee 2004)**



R = OH: 2R, 3R)-taxifoline-6-C-ß-D-glucopyranoside **23**

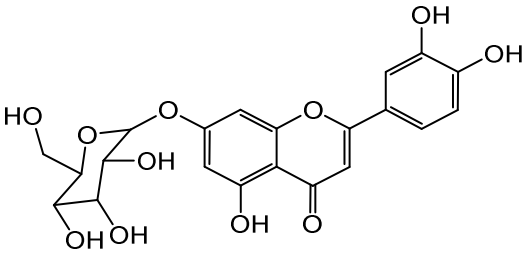
R = H: (2R, 3R)-aromadendrin-6-C-ß-D-glucopyranoside **24**

(Tanyi and Tanee., 2004)



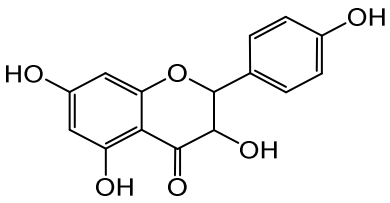
Naringenine **25**

(Hurabielle et al., 1982)



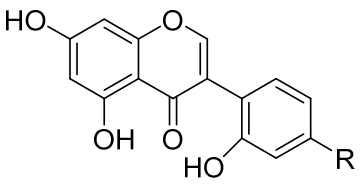
Luteoline-7-O-glucoside **26**

(Lie-Chwen et al., 2015)



Kaempferol **27**

(Fathy et al., 2006)

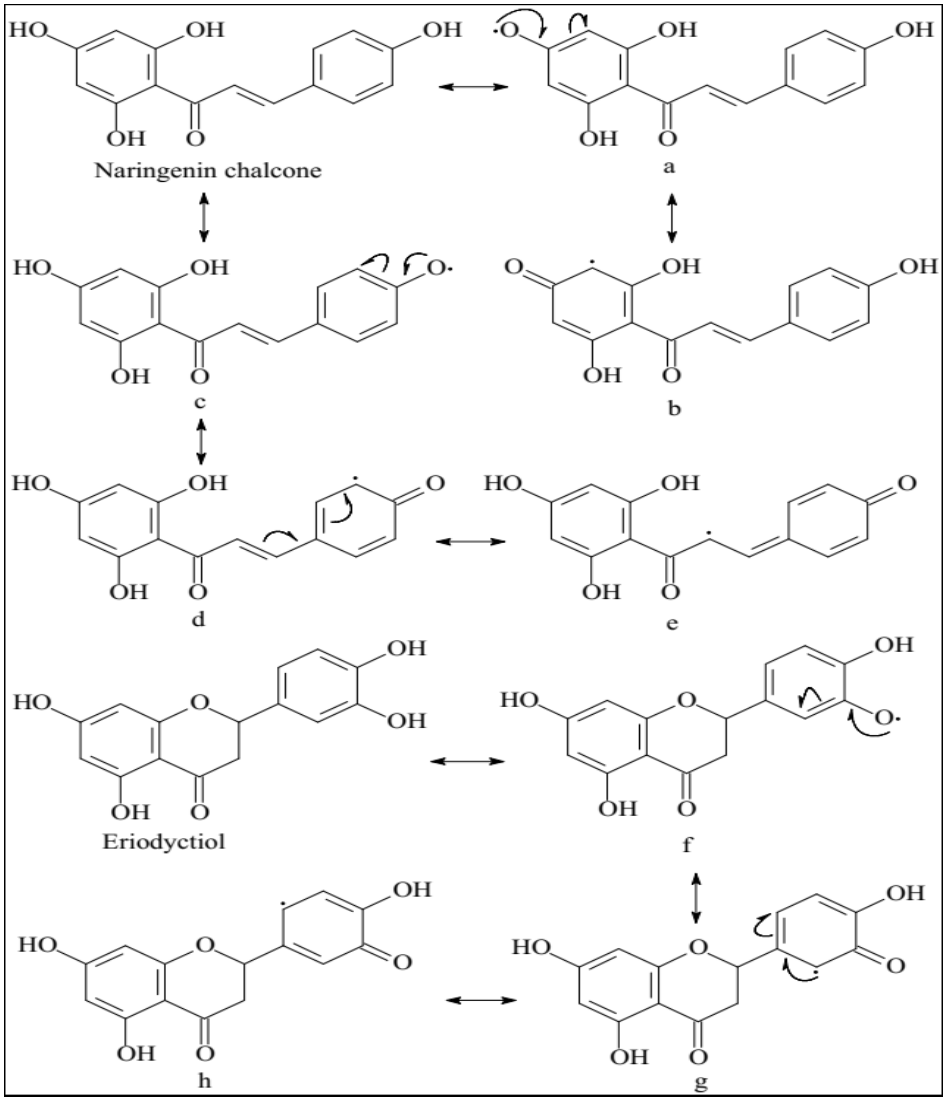


R= OH: 2'-hydroxygenistein **25'**

R= OMe: 2'- hydroxy- 4'- O- methylgenistein **25''**

II.1.3. Polyflavonoids

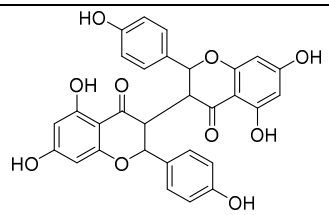
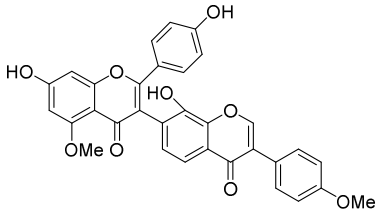
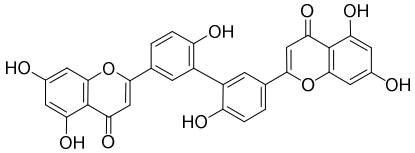
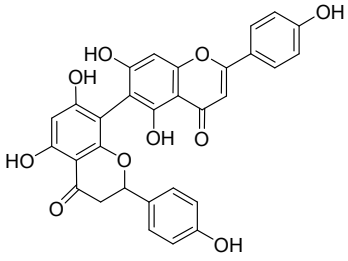
Biflavonoids were proposed to be formed from the radical dimerization of monomeric flavonoids. Because flavonoids are phenolic compounds, they are susceptible to one-electron oxidation to generate radicals. Theoretically, one-electron oxidation can occur in any type of flavonoid. Geiger and Quinn suggested that chalcones, the precursors of flavonoids, undergo one-electron oxidation to afford a series of appropriate radicals which may couple to form biflavonoids as illustrated in **Scheme 4 (Mercader and Pomilio, 2012)**. The removal of a phenol proton brings about an oxygen free radical, which can be stabilized by delocalization. This key radical in turn generates radicals (a-e). In principle, these radicals can freely couple to produce biflavonoids. However not all dimers have been found to exist in nature so far. There are around twenty-four types of simple biflavonoids identified till date, see **Table 9.**

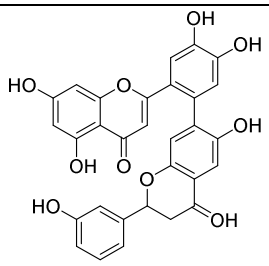
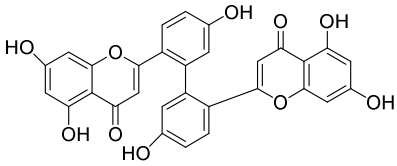
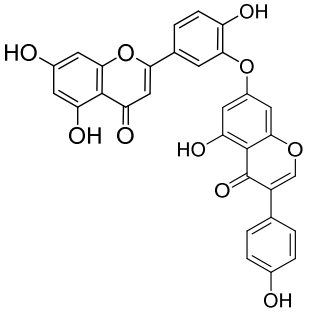
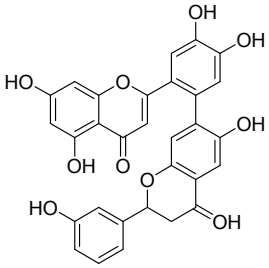


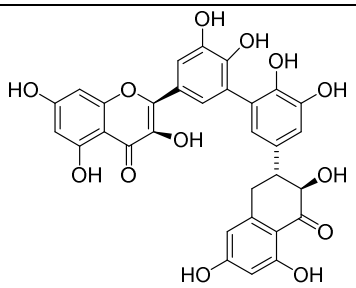
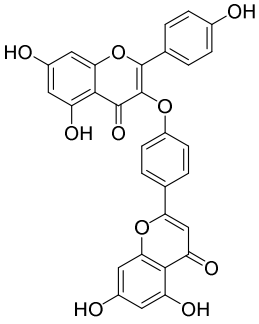
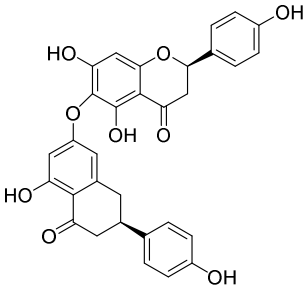
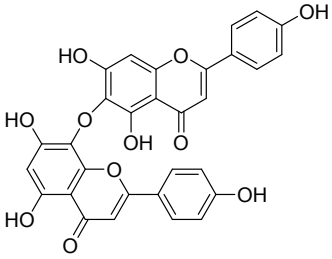
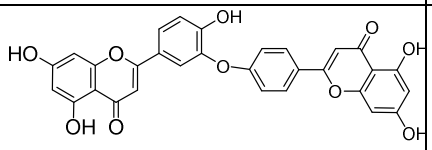
Scheme 5: Mechanism of formation of free radicals (Jackson et al., 1971)

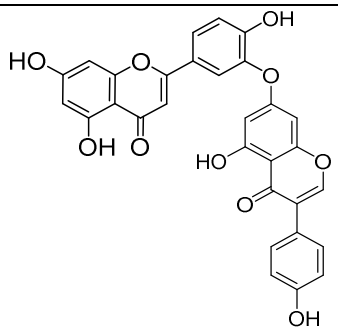
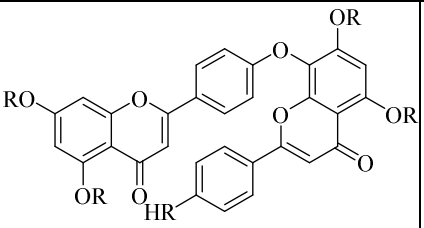
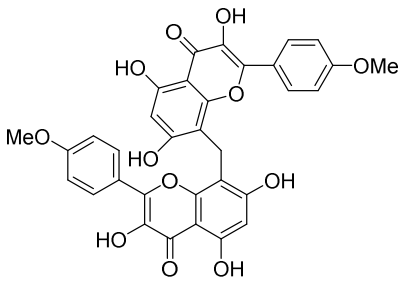
We notice that the free electrons can also be on a carbon atom as well as on an oxygen atom. The coupling of two radicals having free electrons on carbon will lead to a carbon-carbon bond between the flavonoid units. In the case where one of the free electrons is on a carbon atom and the other on an oxygen atom, coupling gives another biflavonoid. Biflavonoids can be formed from any two radicals issued by any known class of flavonoids. In the case where the coupling concerns two different monomers, we talk of hetero-flavonoids. Meanwhile when two monomers of the same sub class are used to form the dimmer, we talk of homo-flavonoids.

Table 9: Some examples of classes of biflavonoids

Combination of radicals	Position of bond	Example	REFERENCE
e+e	I-3, II-33″	 Chamaejasmin	Roots of tellera chamaejasme (Yang et al, 2005)
e + b	I-3, II-8"	 Stephaflavones	From *Stephania tetrandra* and Ridiculaflavones from *Aristolochia ridicula* (Si et al., 2001; Machado and Lopes, 2005)
e + d	I-3, II-3‴	 Taiwaniaflavones	leaves of *Taiwania cryptomeriodides* (Gadek and Quinn, 1985)
b + b	I-6, II-8"		Genus *Agathis* (Ofman et al., 1995)

		Agathisflavones	
g+b	I-2′, II-8″	 Philonostiflavones	From the gametophytes of *Philonotis fontana* (Geiger and Bokel, 1989)
h + h (g + g)	I-2', II-2"	 (2'→2")-Biapigenin	*Garcinia nervosa* (Parveen et al., 2004)
d + b	I-3', II-8"	 Amentoflavones	from Campylospermum flavum **(N**dongo et al., 2010)
h + b	I-6', II-6"	 Hegoflavones	fronds of *Alsophila spinulosa* (Wada et al., 1985)

h + h	I-5', II-5'''	 Bisdihydroquercetin	bark of *Pseudotsuga menziesii* (Lai et al., 1992)
e + c	I-3 ,*O*, II-4''	 Delicaflavone	aerial parts of the *Selaginella delicatula* (Lin and Chou, 2000)
b + a	I-6, *O*, II-7' I-6 ,*O*, II-8'	 Masazinoflavanone  *Biapigenin*	leaves of *Rhus tripartitum* (Mahjoub et al., 2005) *leaves of Viburnum cotinifolium (Muhaisen et al., 2002)*
d + c	I-3', *O*, II-4'	 Ochnaflavones	Genus *Ochna* (Reddy et al., 2008)

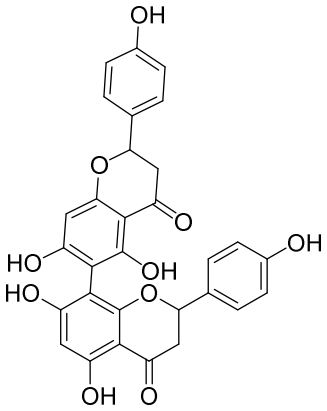
d + a	I-3', *O*, II-7"	 Lophirone L	leaves of *Lophira alata* (Tih et al., 2006)
c + b	I-4', *O*, II-8" I-8, II-8″	 Lanaroflavone  Pentagrametin	From *Campnosperma panamense* (Weniger et al., 2004) Pentagramma triangularis (Roitman et al., 1993)

II.1.4. Biflavonoids of the genus Garcinia

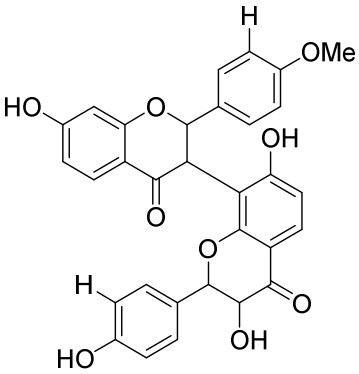
The genus *Garcinia* is also a source of polyflavonoids among which biflavanoids form the greatest sub-class involving more than one hundred biflavonoids identified so far. Two sub- classes: are distinguished:

Those known as biflavonoids with a C-C bond between two simple flavonoid units linking carbons C-3 and C-8´´ or C-3´ and C-8´´.

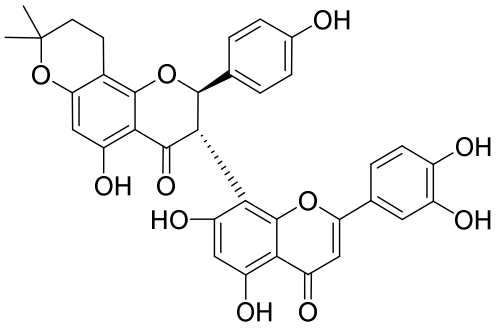
Those having the C-O-C bond as interflavonoid linkage known as biflavonoid ethers. Some examples of biflavonoids having C-C linkage are compounds **28**- **40**:



Lateriflavone **28**

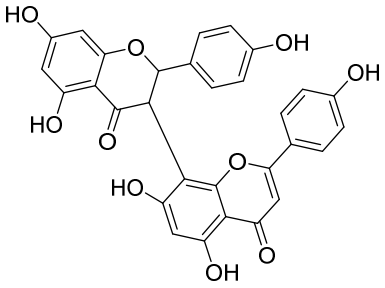


29

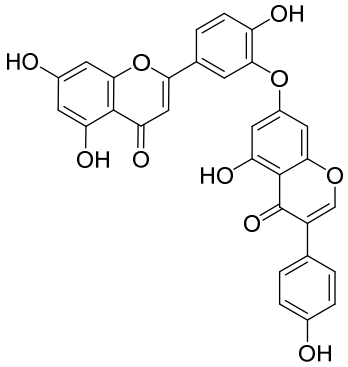


30

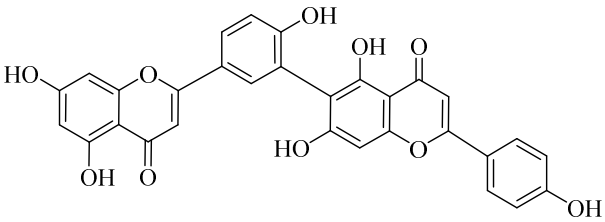
Kolaflavone **(Iwu et al., 1982)** Garciniaflavone F **30 (Tetsuro et *al*., 2013)**



R1=H: Volkensiflavone **31**
(Osorio et al., 2013)

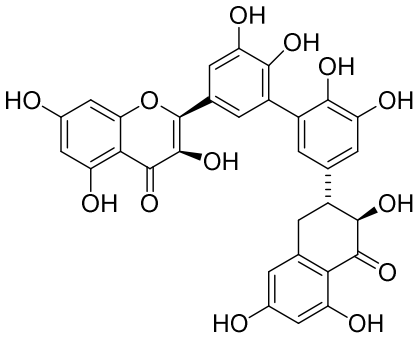


Lophirone L **(Tih et al., 2006)**



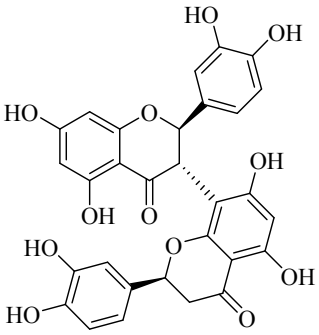
Robustaflavone **33**

(Junxia et al., 2007)

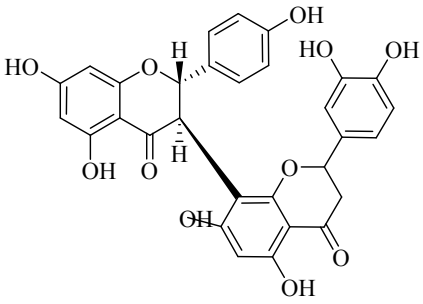


Bisdihydroquercetin **34**

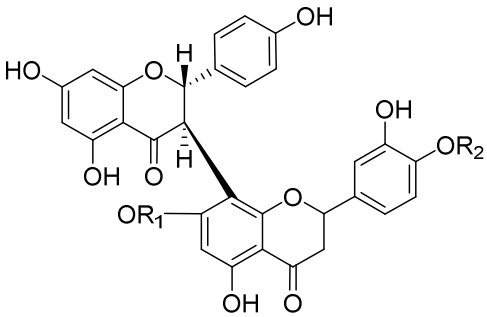
(Lai et al., 1992)



Manniflavanone **35**
(Tetsuro et al., 2013)



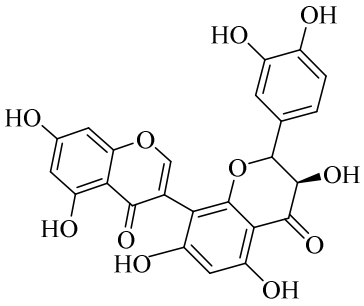
Garciniaflavone E **36**
(Timo et al., 2012)



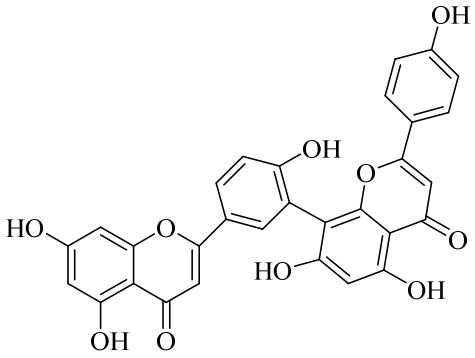
R_1=Glycoside, R_2=H: morelloflavone-7-*O*-ß-D-glucoside **37**

R_1= H, R_2= Glycoside: morelloflavone-4’’’-*O*-ß-D-glucoside **37'**

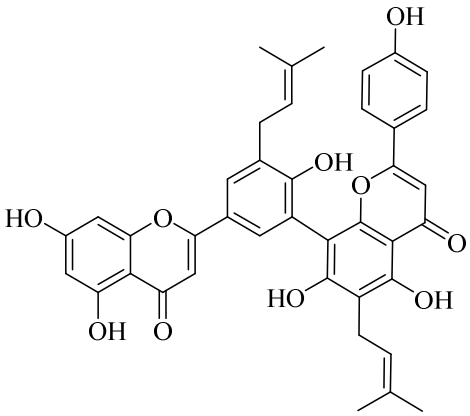
(Vanessa et al., 2012).



Preussianone **38** **(Biloa et al., 2012)**



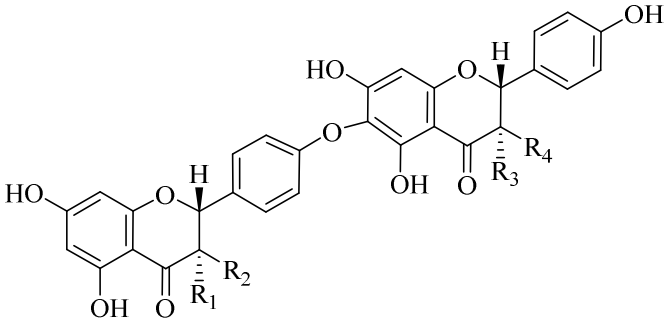
Amentoflavone **39**, **Ndongo et al., 2010**



Dulcisbiflavonoid A **40**, **(Saelee et *al*., 2015)**

Examples of flavonoids having C-C linkage

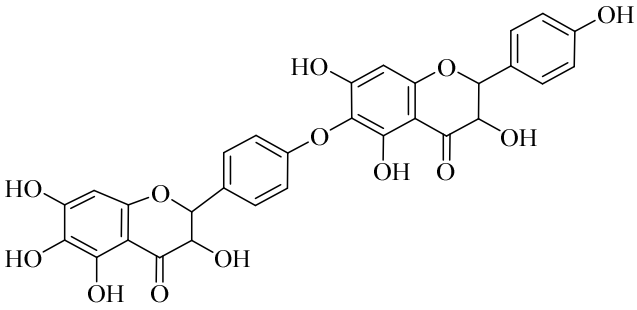
Examples of biflavonoids having C-O-C bond between the two flavonoid units characterized from the genus garcinia are shown below.



R_1=R_2=R_3=R_4=H: Tetrahydrohinokiflavone **41** **(Das et al., 2005)**

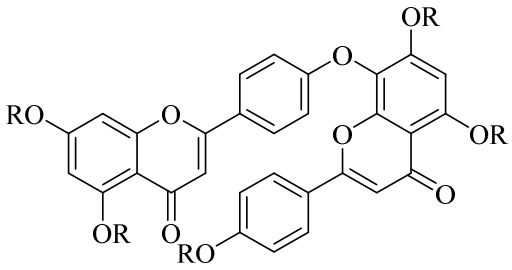
R_1=R_2= R_3=H, R_4=OH: Brevipedicellone A **42** **(Abderaman et al., 2016)**

R_1=R_3=H, R_2=R_4=OH=OH: Brevipedicellone B **43** **(Abderaman et al., 2016)**



Brevipedicelone C **44** **(Abderaman et al., 2016)**

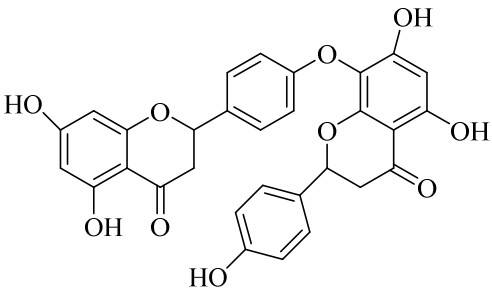
Examples of biflavonoids having C-O-C bond between the two flavonoid units characterized from different plant species.



Lanaroflavone R=H, **45**

Lanaroflavone permethyl ether R=CH_3**,** **46**

(Weniger et al., 2004)

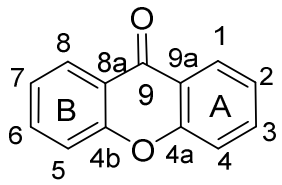


Hynogenol, **47** (**Sievers et al., 1992)**

Examples of flavonoids having C-C linkage

II.2. Xanthones of the genus *Garcinia*

Xanthones are a class of oxygenated heterocyclic compounds which have the basic skeleton of xanthene-9-one or dibenzo-γ-pyrone (**48)**, **Wang et al., (2003**). In general, they have a yellow coloration

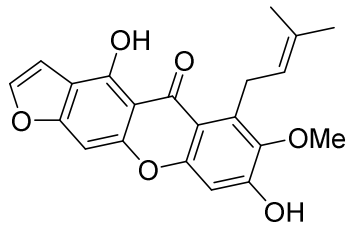


γ- Pyrone 48

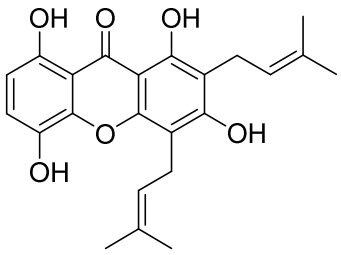
The numbering of the carbon atoms on the basic skeleton follows the biogenetic convention as carbons 1 to 4 are assigned to the acetate derivative of ring A while carbon 5 and 8 are assigned to the shikimic derivative of ring B (**Peres et Nagem., 1997**). Xanthones isolated from the *Garcinia* genus are classified into five subclasses: These include oxygenated xanthones, glycolysed xanthones, prenylated xanthones, xanthonolinoids and furannoxanthones.

Simple oxygenated xanthones can again be divided into subclasses by considering the degree of oxidation of the carbons in the rings to give tri or tetra oxygenated xanthones **(Peres et Nagem., 1997).** Some members of different classes characterized are: Garcimangoxanthone **A**, (**49)** (**Zhang et al., 2010**), Gartanin, (**50)** (**Xuchong et al., 2007**), Bangangxanthone **A** (**51),** Bangangxanthone **B** (**52),** (**Meli et al., 2005**), Garcinone **A, (53**), Cowaxanthone **D** (**54)**, Garcinomangasone-**A** (**55)**, Isocowanol **56**, Garcinomangasone-**B** (**57)** and Garcinomangasone-**C** (**58)**. (**Huang et al., 2001**), Bannaxanthone **I** (**59)**, **(You-Kai Xu, 2010),** β-mangostin **60** (**Ghazali et al; 2010**), 1-hydroxy-3, 6,7-trimethoxy-2,8-bis (3-methylbut-2-enyl) xanthone (**61)**, (**Nilar, Harrison, D.J., 2002**), 9-hydroxycalabaxanthone (**62)** (**Trisuwan, K.)**, tovophyllin **A (63)** (**Antonio, A.A.L. et al ; 1975**) and Garcidepsidone **B** (**64)** (**Bennett, G. et al; 1993**)Afzeliixanthone **(65)** and Afzeliixanthone-B (**66), (Waffo et al., 2006)**, 1,3,7-trihydroxy-4,6-dimethoxyxanthone **(67) (Klaiklay et *al*., 2013)**,5-Farnesyltoxyloxanthone**(Kijjoa et *al*., 2008) (68).**

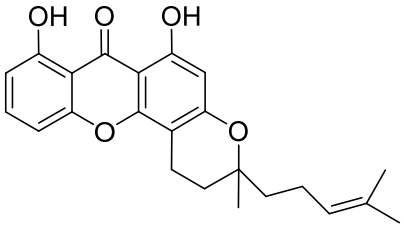
Examples of xanthones from the genus garciniaare compounds **49 - 68**.



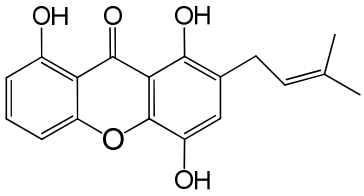
Garcimangosxanthone **A** **49**



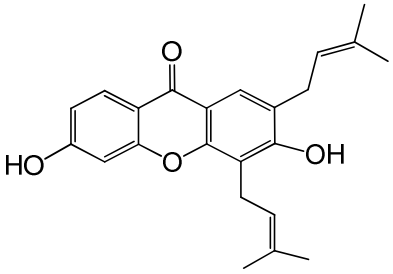
Gartanin **50**



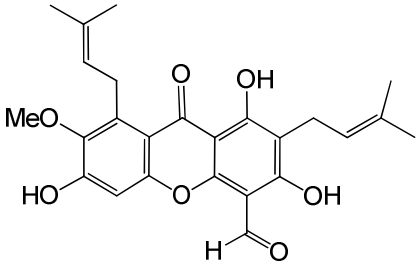
Bangangxanthone **A** **51**



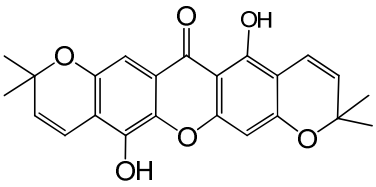
Bangangxanthone **B** **52**



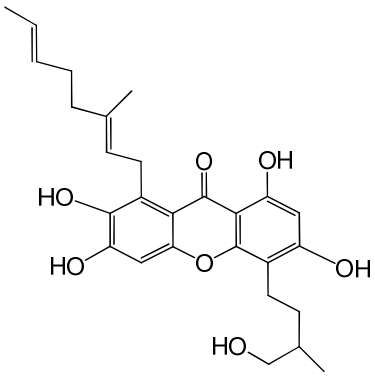
Garcinone **A** **53**



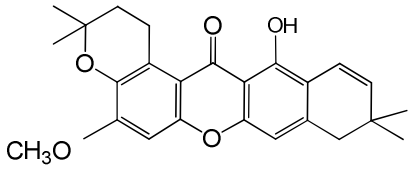
Cowaxanthone D **54**



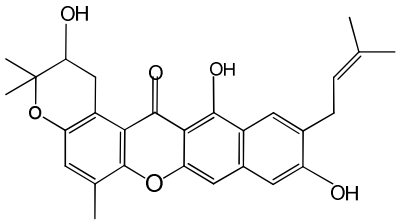
Garcinomangosone **A** **55**



Isocowanol **56**

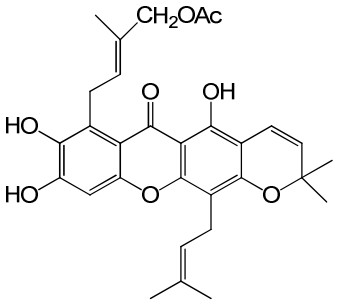


Garcinomangosone **B** **57**

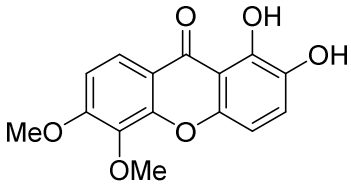


Garcinomangasone **C** **58**

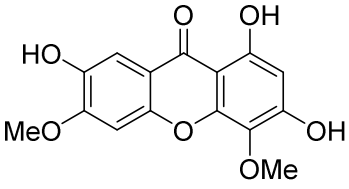
Xanthones isolated from the genus Garcnia



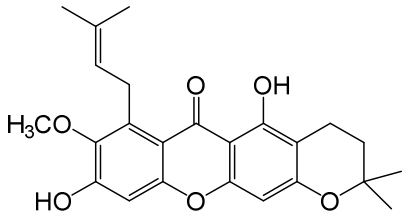
Bannaxanthone I **59**



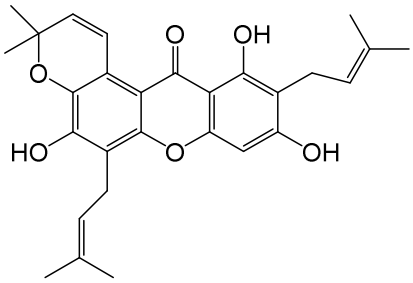
1,2-Dihydroxy-5,6-dimethoxyxanthone **60**



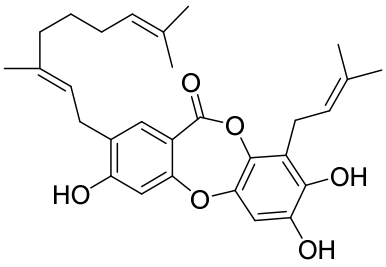
1, 3,7-Trihydroxy-4,6-dimethoxyxanthone **61**



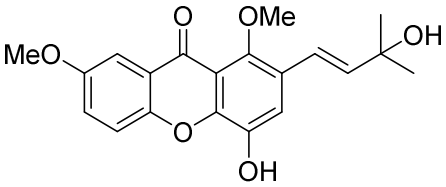
9-hydroxycalabaxanthone 62



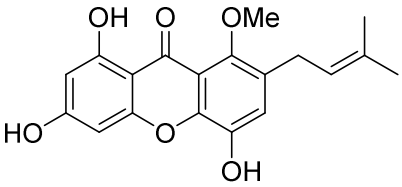
Tovophyllin **63**



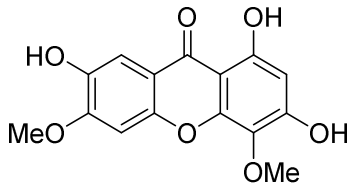
Garcidepsidone B **64**

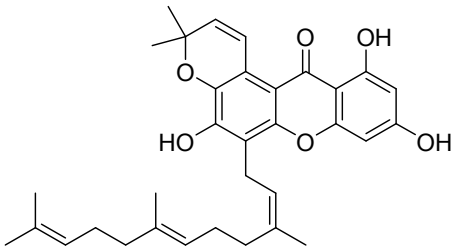


Afzeliixanthone-B 65



Afzeliixanthone (66)





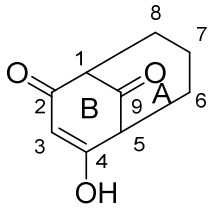
1, 3, 7-trihydroxy-4, 6-dimethoxyxanthone **67**

5-Farnesyltoxyloxanthone **68**

Xanthones isolated from the genus Garcnia

II.3. Benzophenones

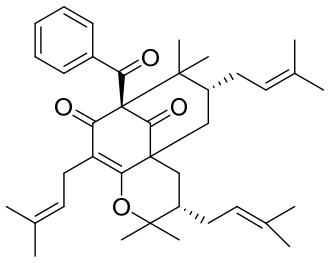
Benzophenones described from *Garcinia* are either simple benzophenones or polyprenylated benzophenones. In both sub-classes the aromatic ring A has undergone oxidation, prenylation and cyclization to form polycyclic benzophenones with a bicyclo [3.3.1] nonane-2, 4, 9-trione (**69**) skeleton **(Zhang et al., 2010)**.



Bicyclo [3.3.1] nonane-2, 4, 9-trione **69**

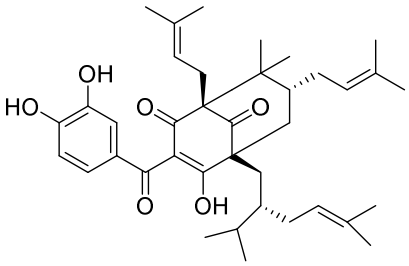
These polycyclic benzophenones are further sub-divided into three structural types depending on the position of the benzoyl group:

- Type A group includes those with the benzoyl group at C-1, such as in garcimultiflorone A (**70**) **(Chen et al., 2009)**.



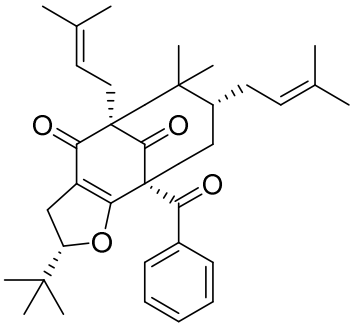
Garcimultiflorone A **70**

- Type B, with the benzoyl group at C-3 such as in pedunculol (**71**) described from *G. pendunculata*, was widely found in the genus **(Sahu et al., 1989)**.



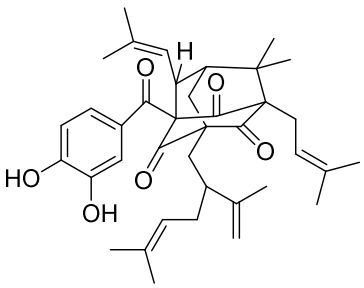
Pedunculol **71**

- Type C with the benzoyl group at C-5 as in Garcinielliptone K (**72)**, described in *G. subelliptica* **(Weng et al., 2004).**



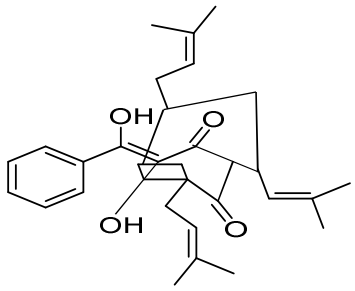
Garcinielliptone K **72**

Cyclization involving the β-diketone and olefinic groups in polyprenylated compounds led to formation of adamantanyl benzophenones such as garciniagifolone A, **(73**) reported from *G. oblongifolia* (**Shan et al., 2012**).



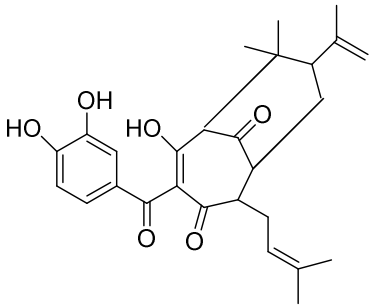
Garciniagifolone A **73**

Doitunggarcinone B **74**, which was isolated from *G. propinqua*, is an unusual transposed benzophenone.



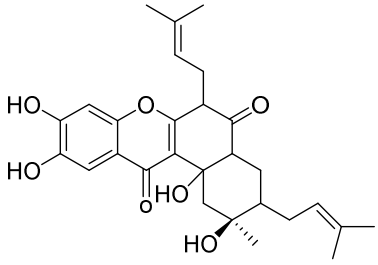
Doitunggarcinone A **74**

In some cases, the ring B can be modified to give a bicyclo [3.3.2] decane-2, 4, 10-trione base structure as found in gambogenone (**75**) described from *G. xanthochymus*.



Gambogenone **75**

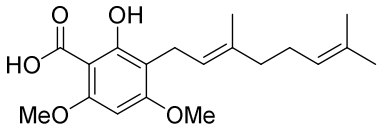
Also, an intramolecular oxidative coupling between the enol and the aromatic ring B is possible forming corresponding polycyclic xanthone derivatives, such as garcinialone **76** **(Chen et al., 2008)**.



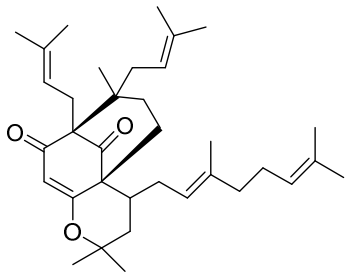
Garcinialone **76**

II.4. Phloroglucinols

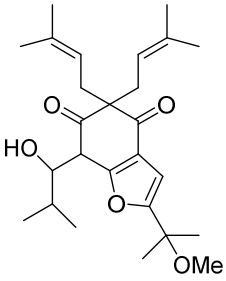
Phloroglucinol derivatives reported in the genus *Garcinia* include simple and complex phloroglucinols derivatives. These are compounds which have a bicyclo [3.3.1] nonane-1, 3, 9-trione base skeleton often oxidized and polyprenylated. Parvifoliol A (**77**) is a simple phloroglucinol isolated from *G. parvifolia* **(Rukachaisirikul et al., 2006),** while Garcinielliptone HB (**78)** is one of seven polyprenylated phloroglucinols identified from *G. subelliptica*, whilst garcicowin A, (**79**) isolated from *G. cowa* is a phloroglucinol derivative with the bicyclo [3.3.1] nonane-1,3,9-trione core **(Lu et al., 2008, Xu et al., 2010)**. Meanwhile, Garcinielliptone HF, (**80**) which was also described from *G. subelliptica*, is a phloroglucinol with an unprecedented skeleton (**Wu et al., 2008)**. Other phloroglucinols include: Guttiférone **A (80a)** isolated from *G. semseii* **(Magadula, 2012)** and Oblongifolin (**80b)** obtained from *G. cowa Roxb* **(Thunwadee et *al*., 2013).**



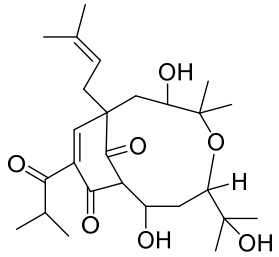
Parvifoliol A **77**



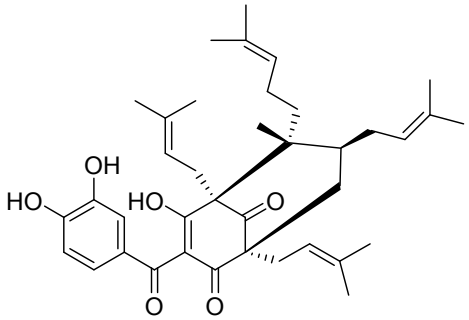
Garcicowin A **79**



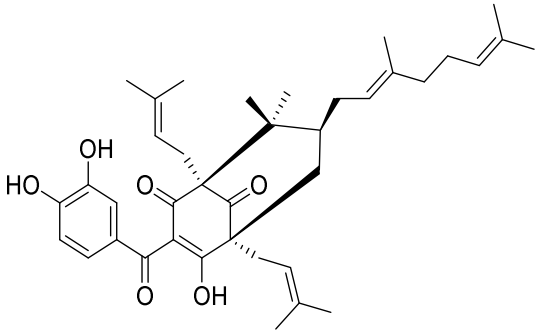
Garcinielliptone HB **78**



Garcinielliptone HF **80**



Guttiférone **A 80a**



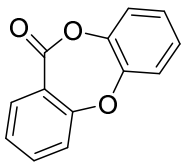
Oblongifolin **80b**

Phloroglucinol derivatives isolated from the genus Garcinia

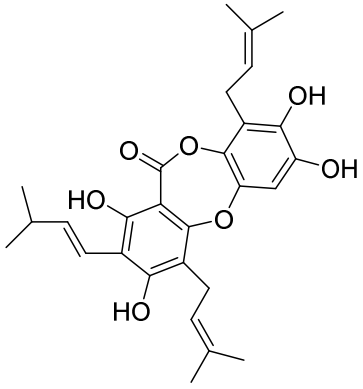
II.5. Depsidones

Depsidones are polyphenolic compounds containing the 11*H*-dibenzo [*b, e*] [1, 4]-dioxepin-11-one (**81)** basic structure. These compounds have been described in lichens, which usually provide polyketide-derived natural products. However, a substantial number of depsidones have now been reported from the genus *Garcinia,* which is well known as a rich source of shikimate-derived aromatic compounds. Depsidones from this genus are commonly have hydroxyl, methoxyl, isoprenyl and geranyl substituent groups. For example, garcidepsidone A (**81a),** one of four prenylated depsidones, was isolated from *G. parvifolia* **(Xu et al., 2000)**.

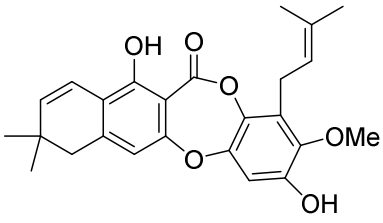
Prenyl side-chains can be cyclized with hydroxyl groups at the *ortho* position to give tetra and penta-cyclic compounds such as garcinisidones B (**81b)** and C (**81c)** respectively, identified from *G. neglecta*, **(Ito et al., 2001)**. Other depsidones are: Cowadepsidone (**82**) **(Cheenpracha et *al*., 2011)**, Parvifolidone B (**82a**) obtained from the leaves of *G. Parvifolia* **(Ruckachaisirikul et *al*., 2006).**



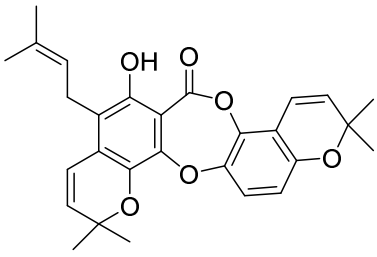
11H-dibenzo[b,e][1,4]-dioxepin-11-one **81**



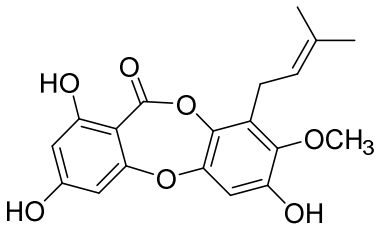
Garcidepsidone **A** **81a**



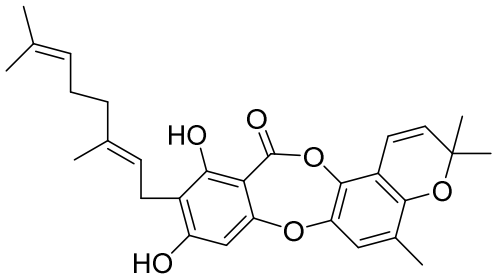
Garcinisidone **B** **81b**



Garcinisidone **C** **81c**



Cowadepsidone **82**

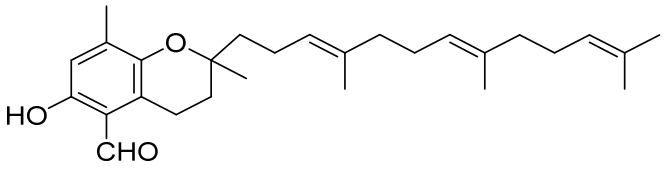


Parvifolidone B **82a**

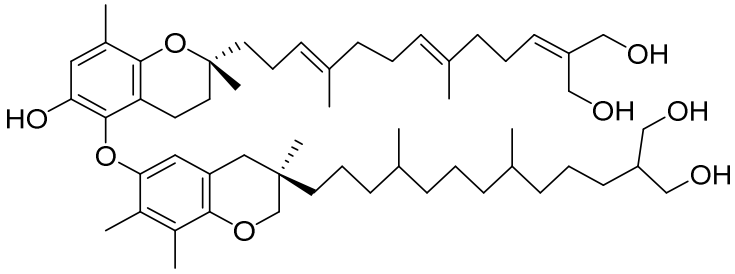
Depsidones isolated from the genus Garcinia

II.6. Tocotrienols

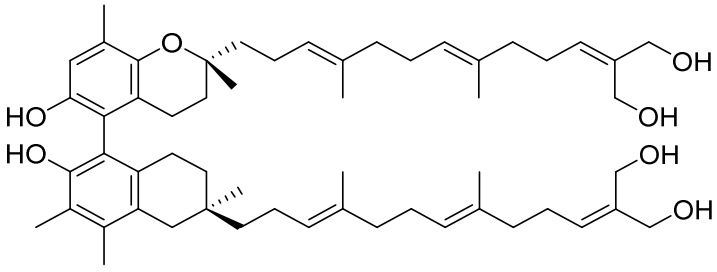
Tocotrienol derivatives have also been isolated also from some *Garcinia* species. They have a 6- chromanol skeleton carried by a farnesyl group at the position C-2. This group can also be oxidized at the level of the two terminal methyl groups in the chain. Mono- or dimeric derivatives have been described. For example, 5-formyl-δ-tocotrienol (**83**) is a mono derivative identified from *G. virgate* **(Merza et al., 2004)**, while δ,γ-Bi-*O*-amplexichromanol, (**84**), δ,γ-biamplexichromanol (**85**) and δ, δ-biamplexichromanoate (**86**) are dimeric tocotrienols reported from *G. amplexicaulis*, **(Lavaud et al., 2015)**.



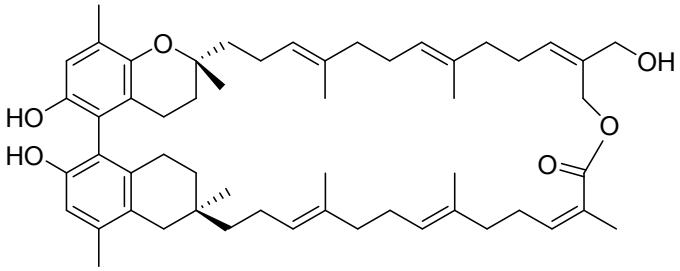
5-formyl- δ-tocotriénol **83**



δ, ɣ-Bi-*O* amplexichromanol **84**



δ, ɣ-Biamplexichromanol **85**

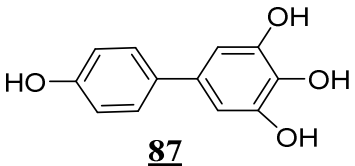


δ, δ-Biamplexichromanolate **86**

Tocotrienol derivative isolated from Garcinia

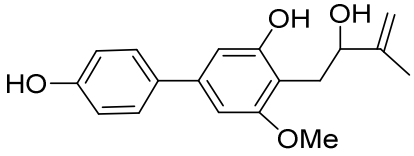
II.7. Biphenyls

A series of biphenyl derivatives have been identified from the genus *Garcinia*. These compounds possess a biphenyl unit in their structure which is usually substituted by hydroxyl, methoxyl and isoprenyl groups. For example, garcibiphenyls B (**87**) and C (**88**) from the root of *G. linii* **(Chen et al., 2006)**. Oblongifoliagarcinines A (**89**) and B (**90**) which were reported from *G. oblongifolia* are respectively tri and tetracyclic biphenyls **(Wu et al., 2008).**



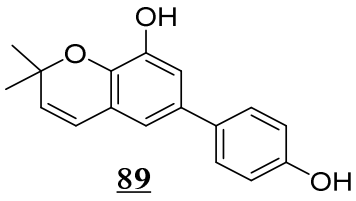
87

Garcibiphenyl B



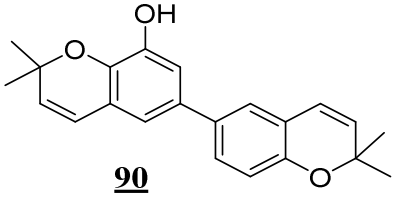
88

Garcibiphenyl C



89

Oblongifoliagarcinine A

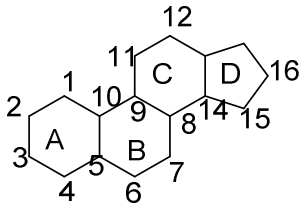


90

Oblongifoliagarcinine B

II.8: Steroids isolated from *Garcinia*

Steroids form part of a large class of natural compounds of terpenic origin having in their structure the basic carbon skeleton of the Perhydrocyclopentenophenanthrene ring (**91).**

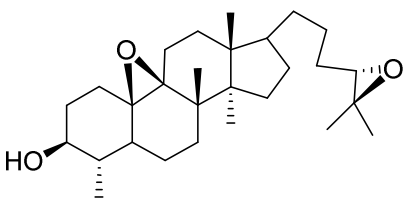


Perhydrocyclopentenophenanthrene ring **91**

This large family of secondary metabolite include: sterols, bile salts, cortic surrenal hormons, sexual hormones, steroidal saponines, lanosterols etc. (**Klyne**. 1966). They are biogenetically derived from triterpenes.

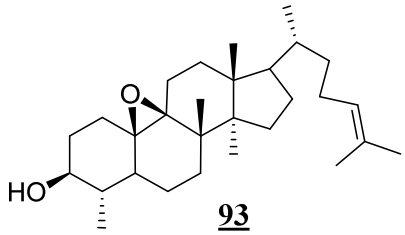
Triterpenes form a large class of about 4000 compounds having a base skeleton of 30-carbon atoms. They are sub divided into over 40 different sub-classes according to the number of rings and the position of the methyl groups substituents on the basic carbon skeleton (**Bruneton, 1993**). Steroids possess four rings designated by A, B, C and D in their base structures

Relatively few steroids have been characterized as constituents of the *Garcinia*. This included a mixture of two epoxides isomers **(92),** 31-norcycloartenol **(93)**, and 30-hydroxycycloartenol **(94)** (**Nyemba et *al.*, 1990**), and six compounds **(95), (96), (97), (98), (99) and (100)** isolated from the plant *G. hombroniana*: (**Rukachaisirikul et *al.*, 2000**).



92

24R and 25S; C-25



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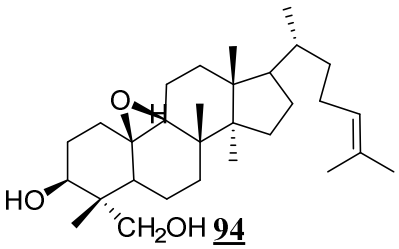
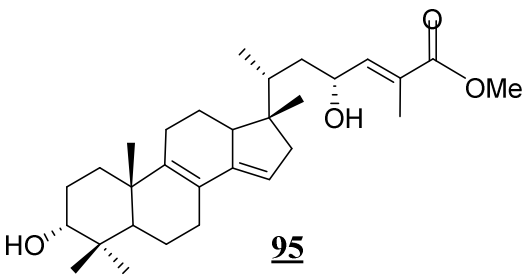


94



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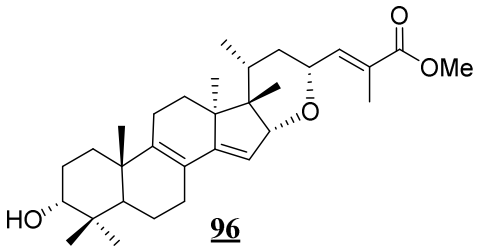

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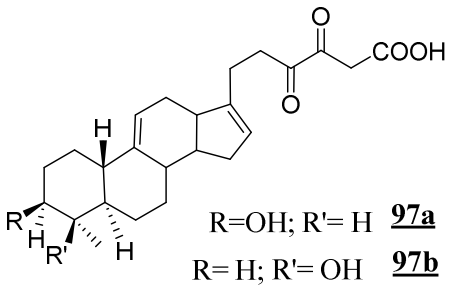
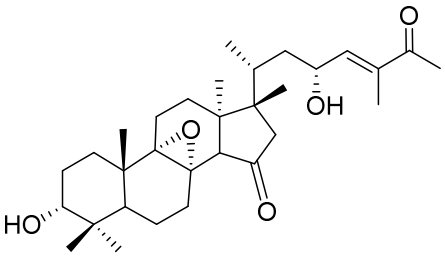
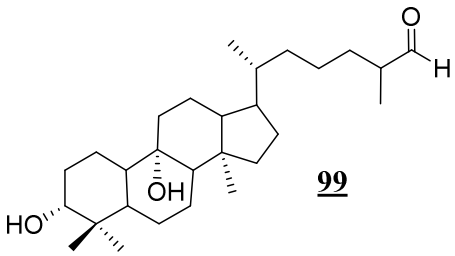


R=OH; R'= H **97a**

R= H; R'= OH **97b**



98



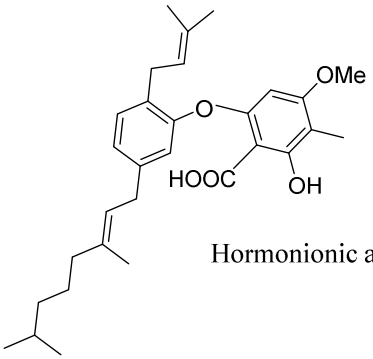
99

Biphenyls isolated from the genus Garcinia

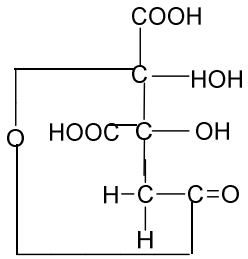
II.9. Acetogenins characterized from the genus *Garcinia*

Acetogenines are long linear chains of compounds obtained biologically from enzymatic condensation of many units of acetic acids activated in the form of Acetyl-CoA. The isolation of hydroxycitric acid (HCA) from certain species of *Garcinia* and mostly biological properties of metabolites has drawn the attention of biochemists and other researchers intervening in the health sector.

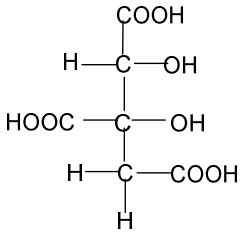
The derivatives of hydroxycitric acid (HCA) are incorporated in many pharmaceutical preparations in addition to other ingredients for their cardio-protective actions, and the correction of certain lipids abnormalities (**Jena et a**l., **2002)**.



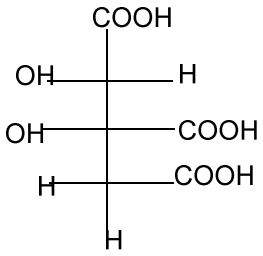
Hormonionic acid **100**



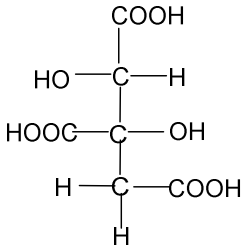
(+)- Allo-hydroxycitric acid lactone **101**



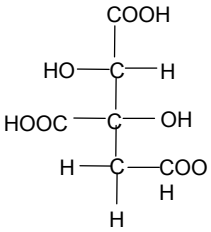
(+)-Hydroxycitric acid **102**



(-) -Hydrocytric acid **102'**



(-)-Allo-hydroxycitric acid **103**

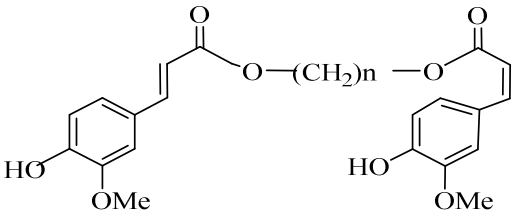


(+)-Allo-hydroxycitric acid **104**

Acetogenins characaterized from the genus Garcinia

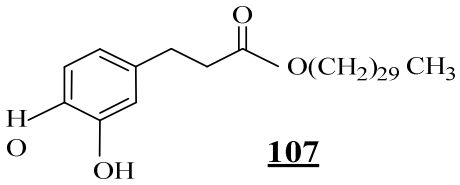
II. 10. Cinnamates characterized from the genus garcinia

Cinnamate are derivatives of cinnamic acid. They are in form of salts or esters with different alcohols. The phytochemical study of the stem barks of *G. multiflora* has permitted the characterization of two cinnamates derivatives (1E, 22Z)-1, 22-diferloyloxyteracosane (**105)** and (1E, 22Z) -1, 24-diferuloyloxteracosane (**106), (Chiang et al., 2003).** The anti-bacteria activity against *staphylococcus aureus* gram+ and *bacillus cereus* was demonstrated for these compounds (**Elseedi et al., 2010).**

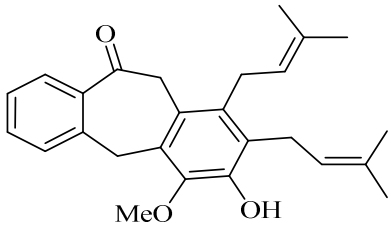


n = 22 **105**

n = 24 **106**



107

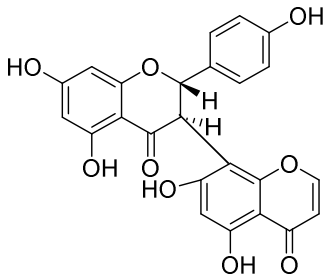


108

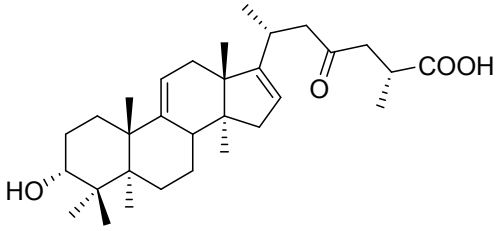
Cinnamates characterized from the genus Garcinia

II. 11. Other compounds

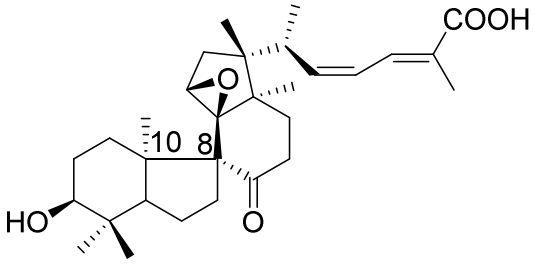
Triterpenes form a vast class of about 4000 compounds distributed in more than 40 different sub-classes according to their carbon skeletons **(Bruneton, 1993).** In addition, the essential oil of some species contains volatile compounds, **(Macleod and Pieris, 1982).**



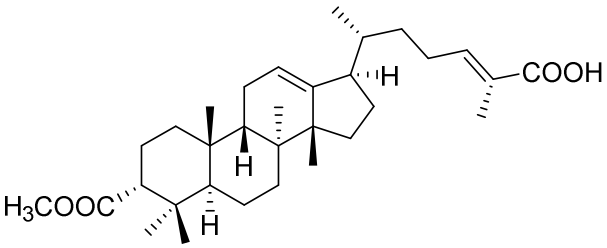
Garcihombronane A **109**



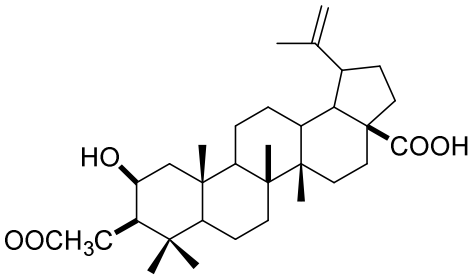
Garcihombronane D **110**



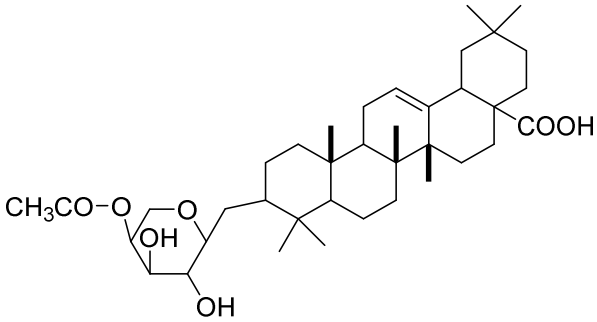
14β-15β-Epoxy-3β-Hydroxy-9-oxo-11[10-8]-abeolanostane-22-*cis*-24-*trans*-dien-28-oic acid (**111)**



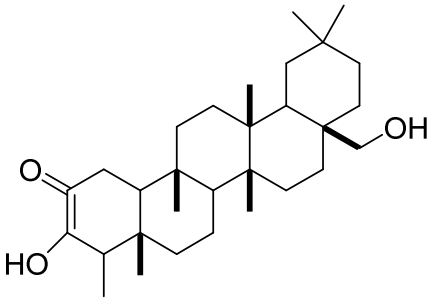
Garciosaterpene A **112**



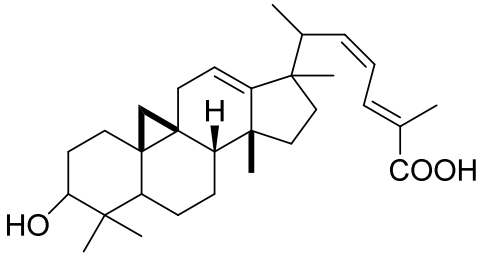
2α-Hydroxy-3β-*O*-acetylllup-20(29)-en-28-oic acid **113**



3-*O*-(4'-*O*-acetyl)-α-L-arabinipyranosyloleanolic acid **114**



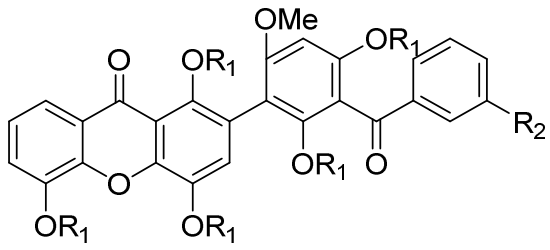
Ovalifolone A **115**

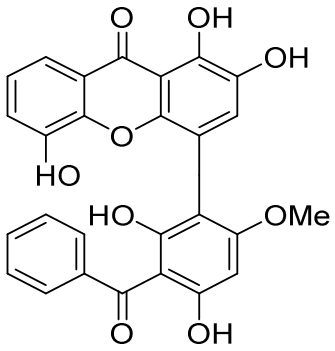


(22*Z*-24*E*)-3α-Hydroxy-17, 13-friedocycloarta-12, 22, 24-trien-26-oic acid **<u>116</u>**

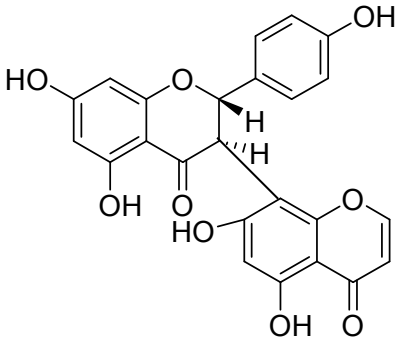
Hydroxycitric acid and its derivatives were isolated from the fruits of three species of *Garcinia* which are: *G. cambogia*, *G. indica* and *G. atroviridis* **(Lewis, 1969)**. (-)-Hydroxycitric **(<u>102'</u>)** acid has drawn all worldwide attention because of its anti-obesity property **(Krishnamurthy and Sapna, 2008).** Some unusual compounds were reported from the genus *Garcinia* such as three benzophenone-xanthone dimers from the root of *G. dulcis*, garciduols A-C (**<u>117</u>**, **<u>118</u>**, **<u>119</u>**) that have been first identified in nature **(Iinuma et al., 1996)**. Two flavanone-chromone dimers, preussianone (**<u>38</u>**) and I-4', I-5, II-5, I-7, II-7-pentahydroxyflavanone [I-3, II-8]-chromone (**<u>120</u>**) were isolated from the leaves of *G. preussii* and *G. dulcis* (**Messi et al., 2012, Ansari and Rahman, 1975)**. The unsubstituted chromone moiety is derived from the elimination of a phenyl ring from a biflavone.

Garcinianins A (**<u>121</u>**) and B **(122**) two new pro-anthocyanidins from the leaves of *G. multiflora*, have been first reported. **(Jiang et al., 2014)**.

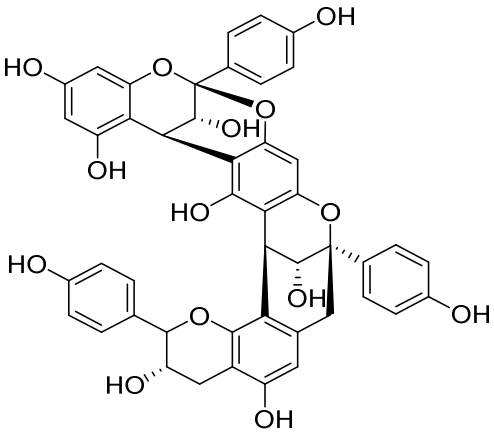




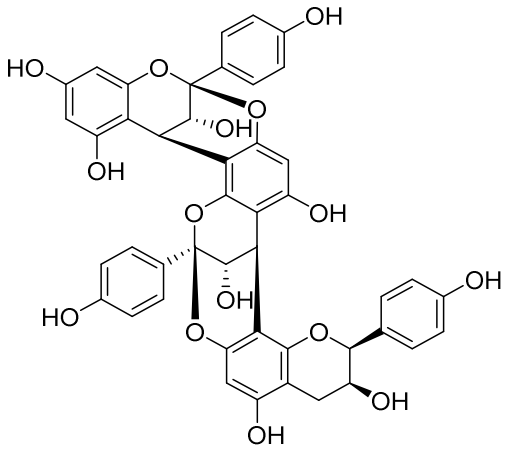
Garciduol A (R_1=R_2=H) **<u>117</u>, Garciduol** B (R_1=H, R_2=OH) **<u>118</u>** and Garciduol C **<u>119</u>**



I-4, I-5, II-5, I-7, II-7-pentahydroxyflavanone [I-3, II-8]-chromone **120**

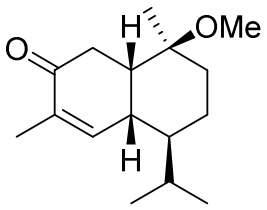


Garcinianin B **122**

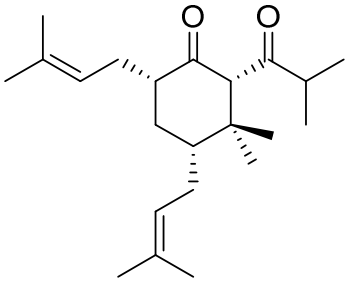


Garcinianin A **121**

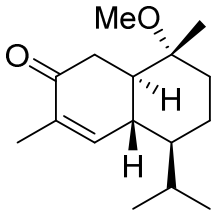
Some sesquiterpenes were identified from the genus, for example, scortechterpenes A (**123**) and B (**124**) from the fruit of *G. scortechinii* **(Sukpondma et al., 2005)**. Garcinielliptones N (**125**) and O (**126**) are two novel terpenoids isolated from the seed of *G. subelliptica* **(Weng et al., 2004)**.



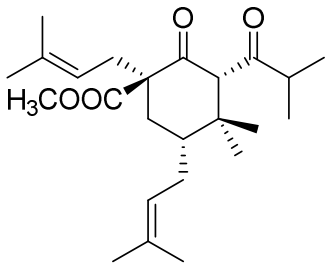
Scortechterpene A **123**



Scortechterpene B **124**



Garcinielliptone N **125**



Garcinielliptone O **126**

Other classes of compounds isolated from the genus Garcinia

II.12: Pharmacological and biological properties of *Garcinia*

Plants in the genus *Garcinia* have numerous therapeutic indications. A lot of research work has been done on different parts of these plants and today we know that they have a lot of important biological activities. Some are used in traditional medicine around the world, particularly in Asia and Africa. The pericarp of *G. mangostana* is used in South East Asia for the treatment of skin infections, wounds, dysentery, diarrhoea, fever, arthritis and inflammation **(Pedraza-Chaverri, 2008)**.

The leaves and seeds of *G. dulcis* are used in Indonesian folk medicine to treat lymphatitis, and parotitis, whereas its stem bark is used in Thailand as an antiseptic and the fruit juice as an anti-scurvy and expectorant. In addition, its root extract is also used as an antipyretic and antitoxin **(Wuttidhammavej, 1997).** The bark of *G. cowa* is used in Thai folk medicine as an antipyretic and antimicrobial agent. Its latex is also used as anti-fever agent **(Na Pattalung et al., 1994).** In India, the fruit of *G. indica* is anthelmintic and useful for piles, dysentery, tumors, pain and heart complaints **(Jena et al., 2002).** *G. cambogia* extract has been used in Indian traditional medicine to treat tumours, ulcers, haemorrhoids, diarrhoea, dysentery, fever, open sores and parasites **(Duke, 2002)**.

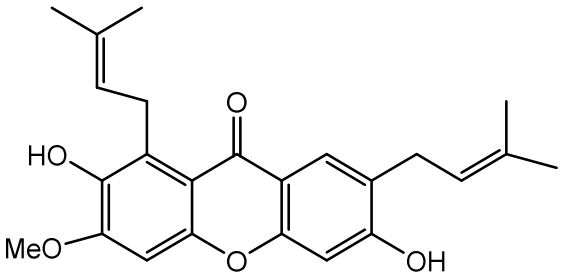
The gum of *G. hanburyii* is used in Thailand as a purgative, vermifuge and for treatment of infected wounds. It is also applied for treatment of chronic dermatitis, haemorrhoids and bedsore. In China, it was developed as an antitumor medicine **(Saralamp et al., 1996; Han et al., 2006)**. *G. xanthochymus* is widely used in Chinese traditional medicine for dispelling worms and removing food toxin **(Lin et al., 2003).** *G. hombroniana*, a seashore mangosteen in Malaysia, is used as protective medicine after child birth and to cure skin allergies **(Jamila, 2014).**

In Africa, *G. preussii* is traditionally used to treat stomach ache and its leaves are prepared as a decoction to relieve toothache **(Bouquet, 1969; Visser, 1975).** *Extracts of G. kola* are used in Nigerian ethnic medicine against laryngitis, cough and liver diseases. Its seeds are used in as an antidote **(Iwu et al., 1985 and 1987).** The leaves and flowers of *G. afzelii* are used in Cameroon and Ghana for antibacterial properties **(Waffo et al., 2006)**.

In Fiji, an extract of the leaves of *G. pseudoguttifera* is mixed with coconut oil and used to relieve pain in the limbs **(Cambie and Ash, 1994)**. Pharmacological and biological investigations of natural products from the genus *Garcinia* showed that some of them possess a wide range of biological properties such as anti-oxidant, antifungal, antimicrobial, anti-inflammatory, anticancer and antiviral activities **(Hemshekhar et al, 2011).**

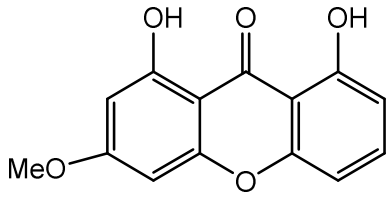
II.12.1. Anti-oxidant activity

1,8-Dihydroxy-6-methoxyxanthone (**128),** a tri-oxygenated xanthone from the wood of *G. subelliptica* exhibited inhibitory activities in three *in vitro* assays viz., anti-lipid peroxidation in rat brain homogenates, DPPH free radical scavenging and superoxide anion scavenging assays at 5 µg/ml **(Minami et al., 1994)**. α-Mangostin (**127**) from the pericarp of *G. mangostana* inhibited 7,12-dimethylbenz[α]anthracene induced pre-neoplastic lesions in a mouse mammary organ culture assay with an IC_{50} of 1.0 µg/ml **(Jung et al., 2006)**. Garcidepsidone B (**129**), a depsidone from the twigs of *G. parvifolia*, gave an IC_{50} of 0.13 µM equal to that of BHT in the DPPH free radical scavenging assay **(Rukachaisirikul et al., 2006).** The antioxidant activity of bigarcinenone A, a bisxanthone (**131**) from the bark of *G. xanthochymus*, is even stronger than that of BHT in a DPPH radical scavenging test. Bigarcinenone A, (**131**) gave an IC_{50} of 9.2 µm, compared to the positive control, BHT with an IC50 of 20 µM, **(Zhong et al., 2008).**



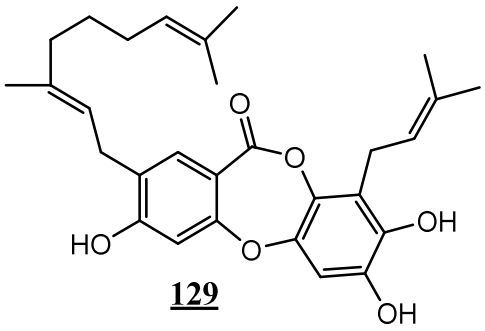
127

α- Mangostin



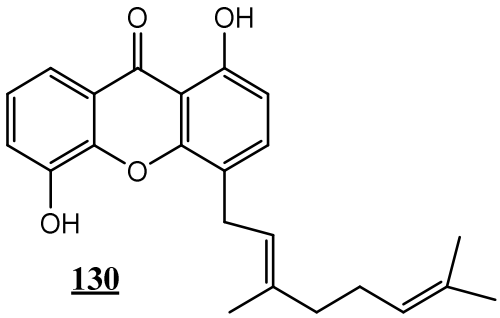
128

1,8-Dihydroxylo-6-methoxyxanthone



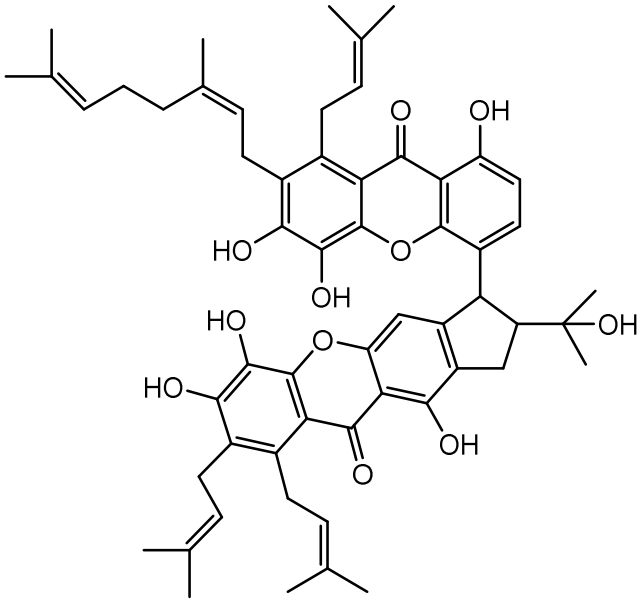
129

Garcidepsidone B



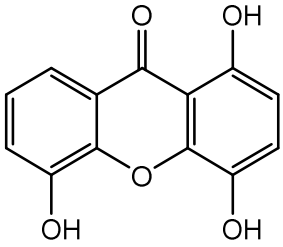
130

4-(3',7'-Dimethylocta-2',6'-dienyl)-1,3,5-trihydroxyxanthone



131

Bigarcinenone



132

1,4,5-Trihydroxy-3-(3-methylbut-2-enyl)-xanthone

Compounds from Garcinia species with anti-oxidant activity

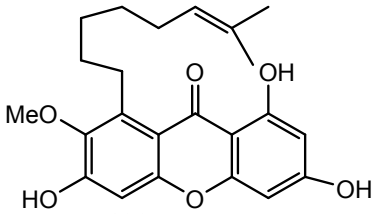
II.12.2. Antifungal activity

Beside the anti-oxidant activity, α-mangostin (**127**) also exhibited the inhibition towards the fungi *Alternaria solani*, *Cunninghamella echinulata* and *Candida albicans* that cause candidiasis with a MIC of 1 mg/ml. It was shown to be more efficient than existing antifungal drugs such as clotrimazole and nystatin **(Sundaram et al., 1983; Kaomonkolgit et al., 2009).** Two isoprenylated tri-oxygenated xanthones, 1,4,5-trihydroxy-3-(3-methylbut-2-enyl) xanthone **(132)** and 4-(3',7'-dimethylocta-2',6'-dienyl)1,3,5-trihydroxyxanthone **(130)** from G. livingstonei showed activity against the plant pathogenic fungus *Cladosporium cucumerinum* at 0.5 and 0.2 µg, respectively, **(Sordat-Diserens et al., 1992).**

II.12.3: Antimicrobial activity

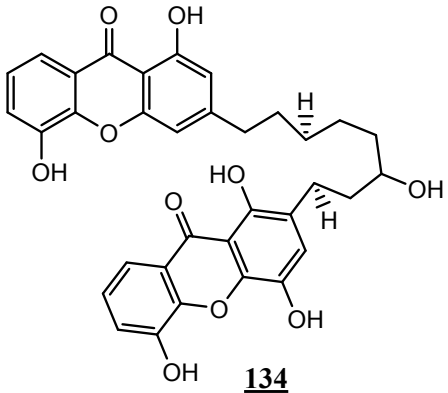
Rubraxanthone (**133)** isolated from G. *dioica* displayed higher activity against Staphylococcal strains (MIC = 0.31-1.25 µg/ml) than the antibiotic, vancomycin with MIC values of 3.13-6.25 µg/ml **(Iinuma et al., 1996)**. Garcivilin A (**134**), a bisxanthone from G. livingstonei, showed a high anti-parasitic activity against two trypanosomes, T. brucei and T. cruzi that cause the fatal human diseases sleeping sickness and chagas disease with IC_{50} values of 0.4 µM and 4.0 µM, respectively.

Moreover, Rubraxanthone (**133)** also exhibited antiplasmodial property against *Plasmodium falciparum* with an IC_{50} of 6.7 µM **(Mbwambo et al., 2006)**. Xanthochymol (**137**), a polyprenylated benzophenone from *G. xanthochymus* and *G. subelliptica*, was evaluated for its antibacterial property against methicillin-resistant *Staphylococcus aureus.* The lowest minimum inhibitory concentration at 3.1-12.5 µg/ml, nearly equal to that of vancomycin **(Iinuma et al., 1995)**. Guttiferone A **(136)**, a polyisoprenylated benzophenone from the fresh fruits of *G. aristata*, showed a potent antiplasmodial effect against *Plasmodium falciparum* with an IC_{50} of 0.5 µM, nearly similar to that of chloroquine (IC_{50} = 0.3 µM), a 4-aminoquinoline drug used in the treatment and prevention of malaria **(Monzote et al., 2011)**. Amentoflavone (**138**), a biflavone from some *Garcinia* species, was reported to be more active against *Mycobacterium smegmatis* than the drug Isoniazid used in the clinical treatment of tuberculosis. Amentoflavone gave a MIC of 0.6 µg/ml compared to isoniazid with a MIC of 1.3 µg/ml **(Kaikabo and Eloff et al., 2011).**



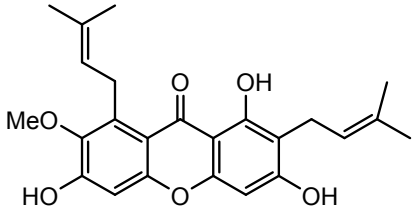
133

Rubraxanthone



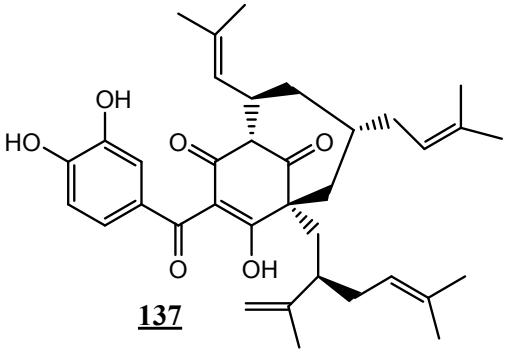
134

Garcivilin A



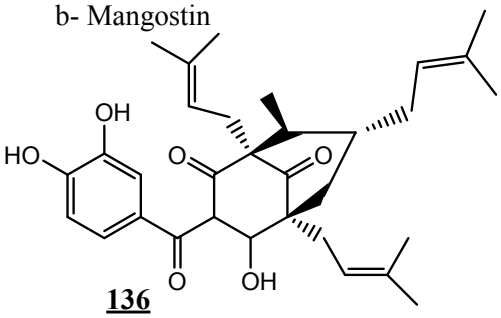
135

b- Mangostin



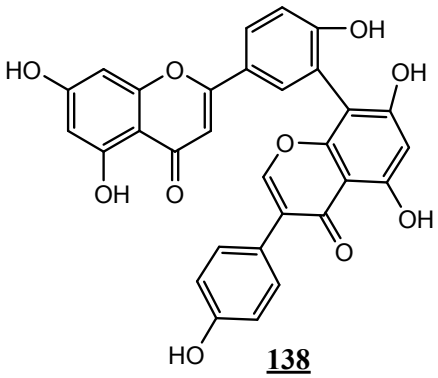
137

Xanthochymol



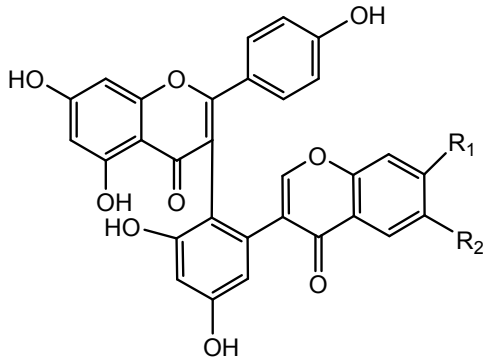
136

Guttiferone A



138

Amentoflavone



GB1(R_1=OH, R_2=H) **139**

GB2 (R1=OH, R2=OH **140**

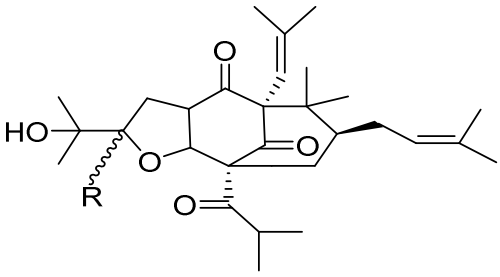
Kolaflavanone(R_1=OMe, R_2= OH **140'**

Compounds from Garcinia species with antimicrobial activity

A biflavonoid complex from the seeds of *G. kola* containing GB-1 (**139**), GB-2 (**140**) and kolaflavanone (**140'**), exhibited potent antiplasmodial activity against *Plasmodium berghei* infection in Swiss albino mice, **(Oluwatosin et al., 2014).**

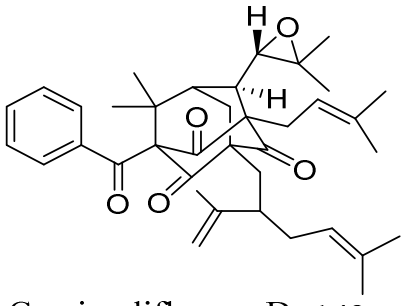
II.12.4: Anti-inflammatory activity

Garcinielliptones L **(141a)** and M (**141b**), two polyisoprenylated phloroglucinols from the seeds of *G. subelliptica*, showed potent inhibitory effects on the release of β-glucuronidase and on histamine from peritoneal mast cell stimulated with p-methoxy-N-methylphenylethylamine in a concentration-dependent manner. They also exhibited potent activities on NO production in culture media of RAW 264.7 cells in response to lipopolysaccharide (LPS) and in culture media of N9 cells in response to LPS/interferon-γ (IFN-γ) **(Weng et al., 2004)**. Two xanthones, α- mangostin **(127)** and γ-mangostin **(143)** from the pericarps of *G. mangostana*, showed significant properties in the expression decrease of TNF-α, IL-1β, IL-6, IL-8, MCP-1 (monocyte chemoattractant protein) and TLR-2 (toll-like receptor). They also potently inhibited the LPS induced NO and PEG2 activity in RAW264.7 macrophages with IC_{50} concentrations of 3.1 and 6.0 µM **(Bumrungpert et al., 2009; Chen et al., 2008)**. In the study of the effects on neutrophil pro-inflammatory responses of benzophenones from *G. multiflora*, garcimultiflorone D **(142)** potently inhibited fMLP/CB-induced superoxide anion generation and elastase release with IC50 values of 7.21 and 6.0 µg/ml, respectively, **(Ting et al., 2012).**

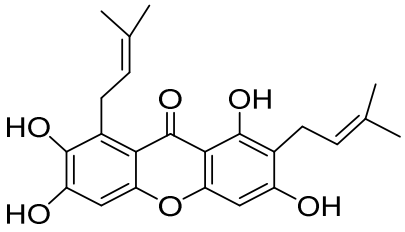


Garcinielliptone L (R= ▬ H) **141a**

Garcinielliptone M (R= ┈┈ H) **141b**



Garcimuliflorone D **142**

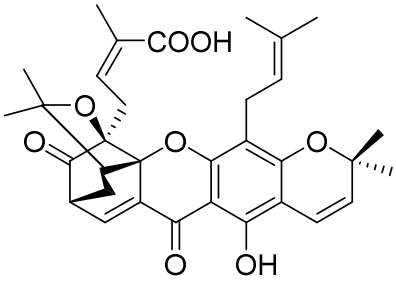


g- mangostin **143**

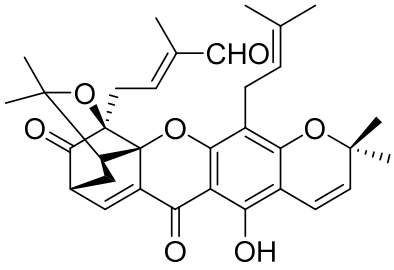
Compounds from Garcinia species with anti-inflamatory activity

II.12.5. Antiviral activity:

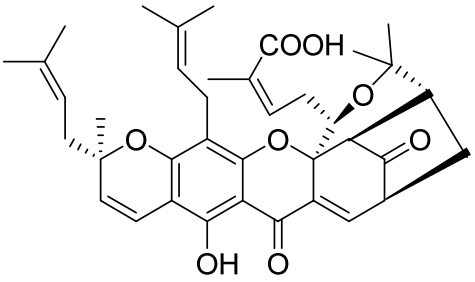
Morelloflavone **(153)** demonstrated potent activity against HIV-1 (strain LAV-1) in phytohemagglutinin-stimulated primary human peripheral blood mononuclear cells at an EC50 value of 6.9 µM and a selectivity index value of approximately 10, while amentoflavone exhibited significant antiviral activity against two strains of influenza A, H1N1 and H3N2 with EC_{50} values of 3.1 and 4.3 µg/ml, respectively (**Lin et al., 1999)**. Morellic acid **(144)**, dihydroisomorellin **(145)** and gambogic acid **(146)**, three caged xanthones from *G. hanburyi*, showed potent HIV-1 RT inhibitory property with IC50 values < 50 µg/ml **(Reutrakul et al., 2007).** Garciosaterpenes A **(147)** and C two pronostanes from the bark of *G. speciosa*, were determined to have strong inhibitory activities against HIV-1 RT with IC_{50} values of 15.5 and 12.2 µg/ml, respectively, **(Rukachaisirikul et al., 2003).**



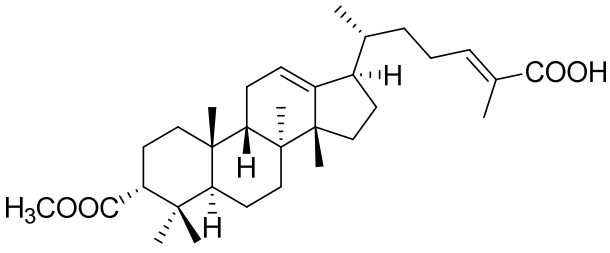
Morellic acid **144**



Dihydroisomorellin **145**



Gambogic acid **146**



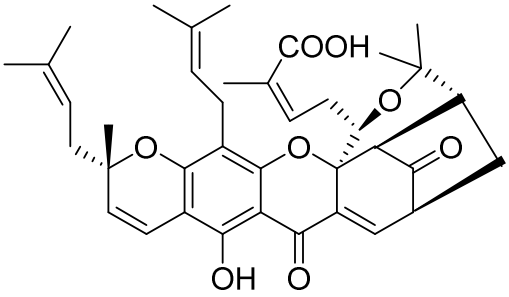
Garciosaterpene A **147**

Compounds from Garcinia species with antiviral activity

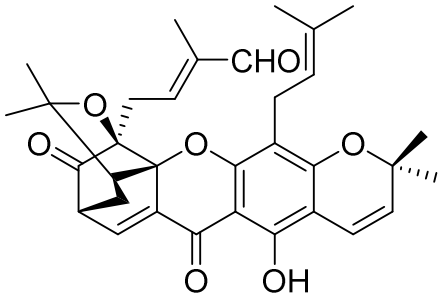
II.12.6: Anticancer activity

Gambogic acid (**146**) and epigambogic acid (**147**), two caged xanthones from the gamboges of *G. hanburyi*, were examined for their cytotoxicity against human leukaemia K562/S and doxorubicin-resistant K562/R cell lines. They were shown to be potent agents

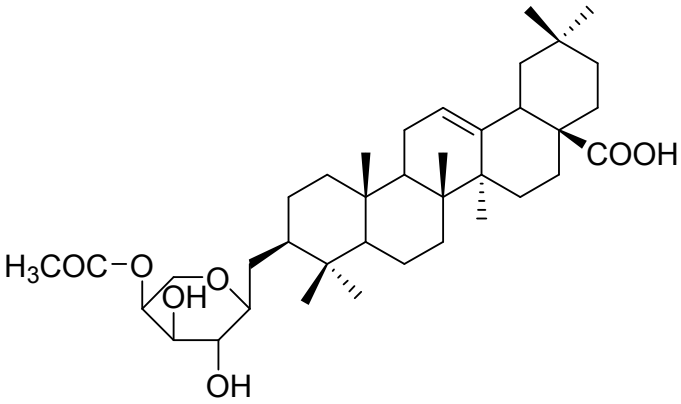
against both cell lines with IC_{50} values of 1.32 and 0.89 µM for gambogic acid (**146**), 1.11 and 0.86 µM for epigambogic acid (**148**), respectively **(Han et al., 2005).** 7-Hydroxyforbesione **(149)**, a caged xanthone from the leaves of *G. cantleyana*, exhibited significant cytotoxicity against MDA-MB-231, CaOV-3, MCF-7 and HeLa cancer cell lines with IC_{50} values ranging from 0.22 - 2.17 µg/ml **(Shadid et al., 2007).** For 3-O(4′-O-acetyl)-α-L-arabinopyranosyloleanolic acid (**151**), a triterpene from the resin of G. hanburyi, the anti-proliferative effects and the apoptosis induction abilities in four human leukaemia cell lines consisting of HL-60, NB4, U937 and K562 were determined with IC_{50} values of 2.45, 2.69, 2.42 and 4.15 µM, respectively (Wang et al., 2008).



Epigambogic acid **148**



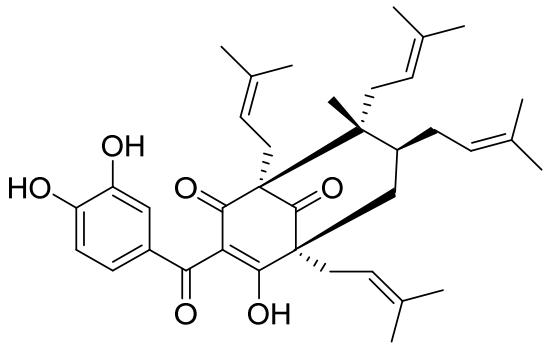
7-Dihydroxyforbesione**149**



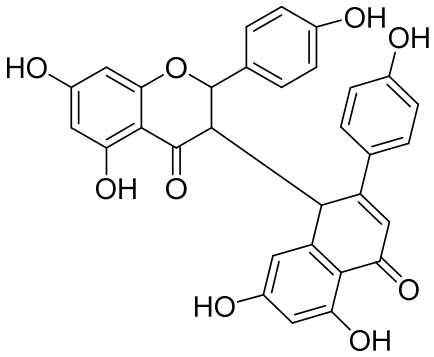
3-*O* (4′-*O*-acetyl)-α-L-arabinopyranosyloleanolic acid **149'**

Compounds from Garcinia species with anticancer activity

Guttiferone A (**150**) an anti-oxidant benzophenone from some Garcinia species, displayed strong activity against HTC-116 and HT29 cell lines with the same IC_{50} values of 5.0 µM **(Yang et al. 2010).** Morelloflavone (**151**), another Garcinia biflavone, was found to inhibit proteasome at an IC_{50} of 1.3 µM, **(Antia et al., 2010; Ren et al., 2010).**



Guttiferone A**150**



Morelloflavone **152**

Compounds from Garcinia species with anticancer activity

II.12.7: Other properties

(-)-Hydroxycitric acid (**102'**), which was found in the fruits of three species *G. cambogia*, *G. indica* and *G. atroviridis,* exhibited the conversion of lactate, acetate and glucose to fatty acids in vitro in bovine and rat adipose tissues **(Hood et al., 1985).** Furthermore, anti-inflammatory, anti-oxidative stress and insulin resistance properties of this acid were evaluated using obese male Zucker rats with type II diabetes associated with inflammation of the IL-6 and plasma C-reactive protein and oxidative stress makers of malondialdehyde, protein carbonyl and protein tyrosine nitration. The results showed that (-)-hydroxycitric acid (**102'**) reduced food-intake, body weight gain as well as decreased the inflammation, oxidative stress and insulin resistance **(Asgar et al., 2007).**

II.12.8: Choice of the theme of research

The natural products laboratory, of the Departement of Organic chemistry of the Faculty of Science of the University of Yaounde I, has put in place a program on establishing a scientific database on medicinal plants on which biological and biochemical tests were carried out uo different parts of their crude extracts. This was done on one hundred plants chosen among our local plants **I, II, III** and **IV**, the different parts of which are used by traditional healers to prepare diverse concoctions destined to solve several health problems. Preliminary anti-microbial tests carried on extracts of these plants have given very prominent results (**Table X).**

Table 10 : Extract of scientific data base of biological activities

Extract tested (solvent MeOH)	**Inhibition Diameter after 24hours**				
	Escherichia coli	***Staphylococcus aureus***	***Klebsiella. pneumoniae***	***Salmonella - typhimurium***	***Candida albicans***
Lophira alata (leaves)	16	11	10	9	12
Lophira alata (wood)	14	12	12	11	10
Garcinia punctata (leaves)	13	14	12	-	10
Garcinia punctata (wood)	12	10	9	-	8
Garcinia brevipedicilata (leaves)	18	16	15	3	9
Garcinia brevipedicilata (wood)	14	9	6	1	9
Garcinia afzeli (leaves)	8	14	10	2	10
*Garcinia afzeli (*wood)	7	5	7	-	8
Gentomycine Reference	18	16	16	15	18

Based on these biological results, my host laboratory, specialized in the chemistry of natural substances is currently carrying out the systematic phytochemistry of these tested extracts in order to know their chemical nature. Students who followed this program before me worked on some parts of these plants and ch

aracterized many compounds with interesting structures.

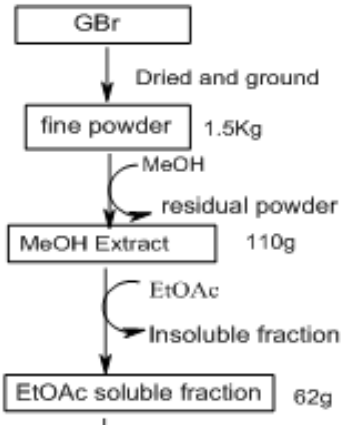
I was then confined with the leaves of *garcinia brevipedicellata* that were not yet investigated to find a good and reproduceable protocol for their purification. These phytochemical investigations led to pure components whose structures are determined. The elaborate extraction protocol will be used later to get more compound to be used for anti-microbial tests shown on table X above. The results obtained shall be compared to those observed for their crude extracts.

CHAPTER III:

RESULTS AND DISCUSSION

III-1: Extraction and isolation of compounds.

Dried leaves of *Garcinia brevipedicellata* after haven been reduced into a fine powder were exhaustively extracted with methanol in a Soxhlet apparatus. The resultant solvent free extract was further washed with hot with ethyl acetate. The removal of the solvent gave crude ethyl acetate extract. **(Scheme 4a)**

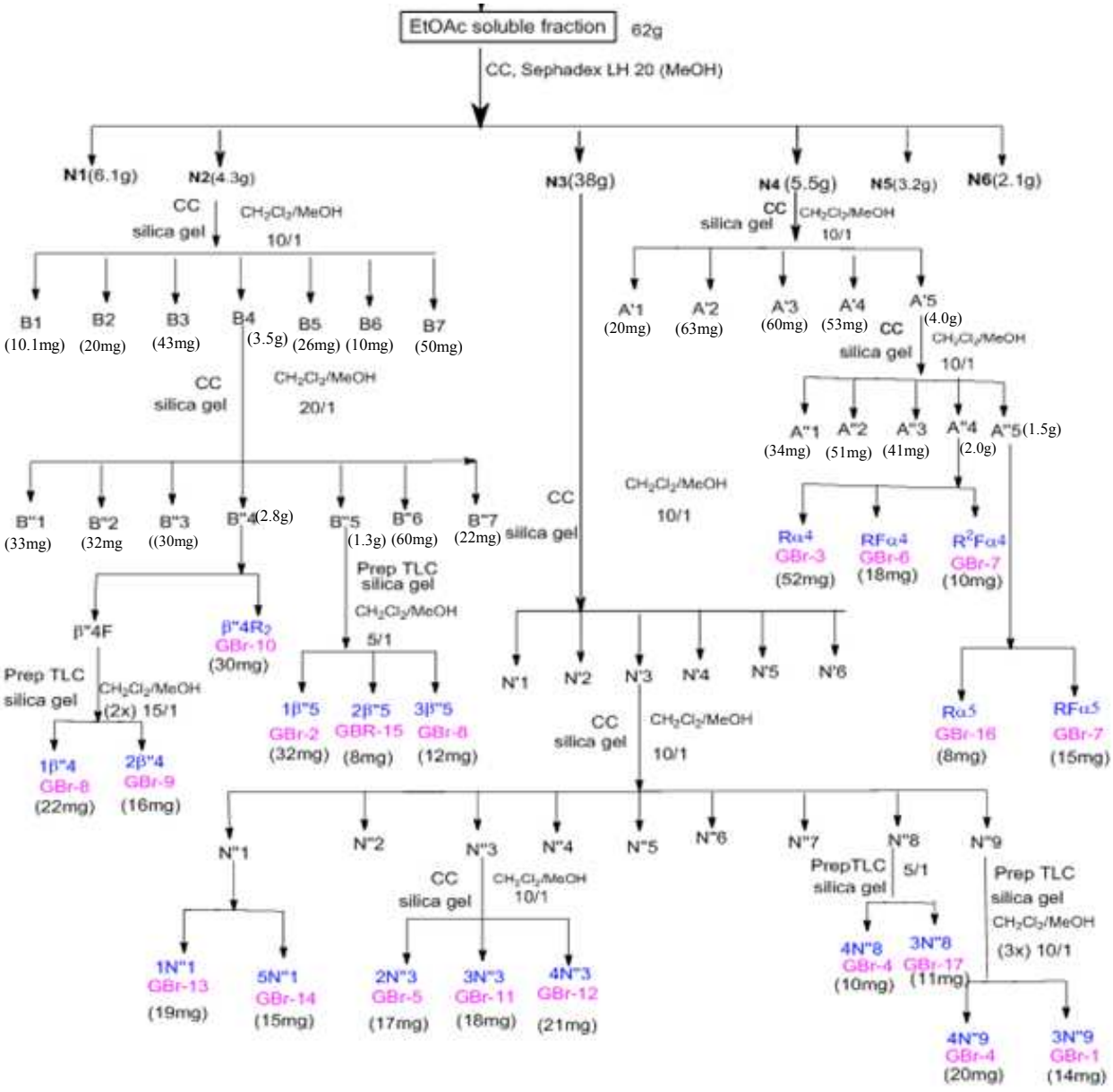


Scheme 6: Protocol of extraction of the leaves of Garcinia brevipedicellata.

The crude ethyl acetate extract was purified using a combination of chromatographic methods, starting with gel filtration on Sephadex LH-20 that gave five sub-fractions. Each of the obtained sub fraction was then purified by a series of column chromatography on silica gel support coupled with preparative thin layer chromatography on silica gel plates. The complete procedure is summarized in scheme **(Scheme 5).**

We obtained seventeen pure compounds, which we gave the etiquettes GBr-1-GBr-17, nine out of which were characterized using 1D and 2D NMR spectroscopic methods. The physical characteristics of these compounds are given in (**table XI)** below. The other eight compounds were also sent for analy

ses but could not be characterized because they were obtained in minut quantities.



Scheme 7: Protocol of purification of the ethyl acetate extract of the leaves of *Garcinia Brevipedicellata*

Table 11: Pure compounds obtained as a result of different purifications.

Etiquette	Physical appearance	Name
GBr-1	Yellow amorphous solid	Robustaflavone
GBr-2	Yellow amorphous solid	4'-*O* -methylrobustaflavone
GBr-3	Yellow amorphous solid	Brevipedicelone D
GBr-4	Cream white powder	Brevipedicelone E
GBr-5	Cream white powder	Brevipedifloside A
GBr-6	Cream white amorphous powder	5, 7,4'-trihydroxy-2-phenylchromen-4-one
GBr-7	White amorphous solid	Tetrahydrohinokiflavone
GBr-8	yellow amorphous powder	Amentoflavone
GBr-9	Pale yellow amorphous powder	Luteoline

The structures of these compounds were elucidated using modern spectrometric and spectroscopic methods including 1D and 2D NMR as well as by comparing obtained data with those reported in literature.

III-2: STRUCTURE ELUCIDATION OF ISOLATED COMPOUNDS

III-2.1: Identification of GBr-1

GBr-1 was obtained as a yellow amorphous solid that responded positively to the test of flavonoids since it gives a brick red coloration when treated with Mg and concentrated hydrochloric acid and a dark blue coloration in the presence of $FeCl_3$ solution. Its (HR-TOF-MS) mass spectrum **(figure 2)** showed the pseudo molecular ion peak $[M+H]^+$ at m/z 539.1090 from which it was deduced that this compound has the molecular formula $C_{30}H_{18}O_{10}$ accounting for 22 unsaturated sites. Gbr-1 is a flavonoid considering its high molecular mass, GBr-1 should be a flavonoid dimer or biflavonoid.

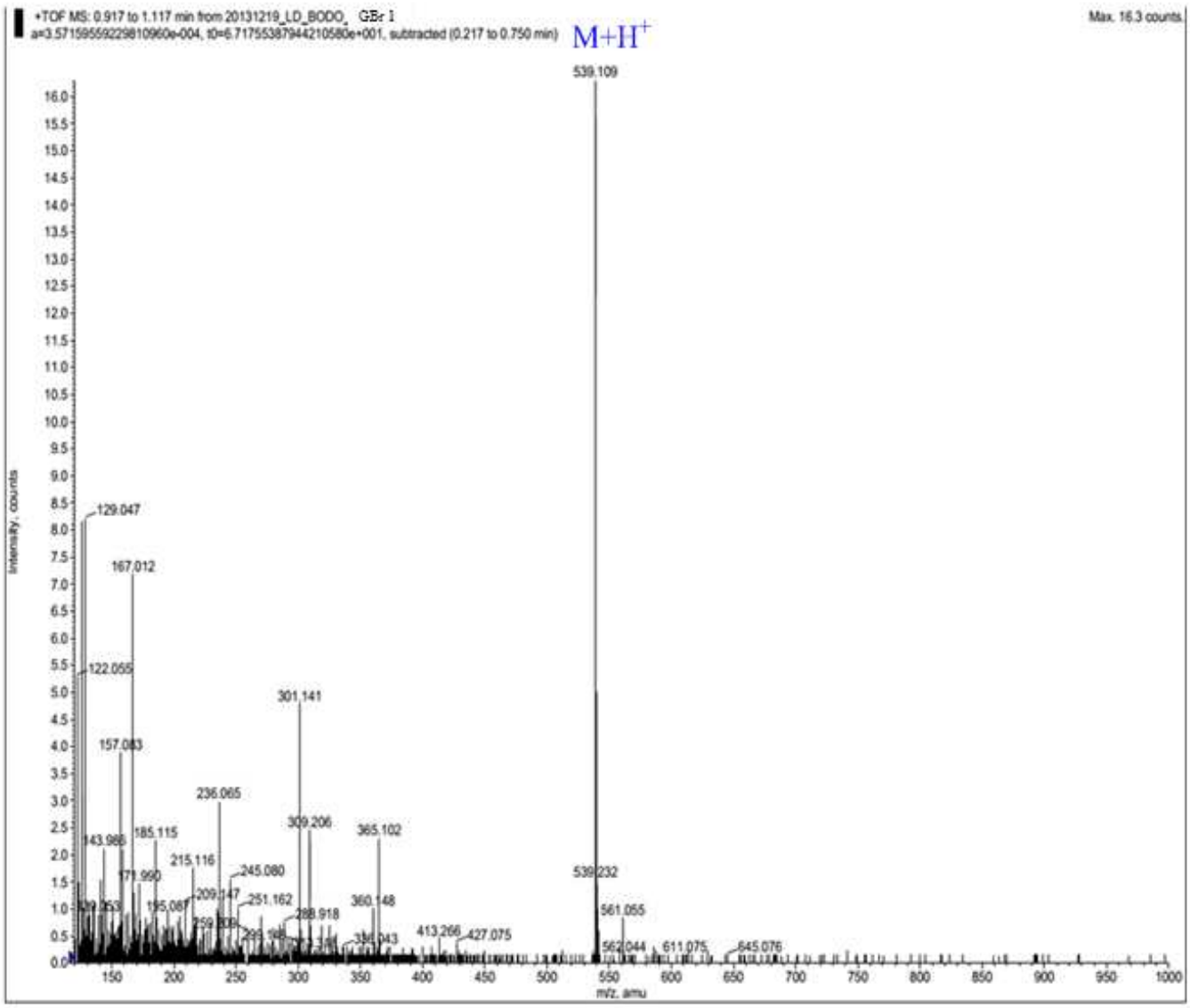


Figure 2: Mass spectrum of GBr-1

Its IR absorption spectrum shows important absorption bands indicating the presence of following functions: phenol (3218 cm^{-1}), a conjugated and chelated carbonyl (1648 cm^{-1}), and aromatic rings (1605, 1503cm^{-1}) and conjugated double bond (1622cm^{-1}) all typical of the flavonoid structure. The complete analysis of its 1D and 2D NMR spectra **(figures 3, 4** and**)** led to the identification of all the different proton systems implicated in the structure of Gbr-1. These included:

- The signal of a highly shielded proton at 5.90 ppm (1H, s, H-8") appearing as a singlet suggesting the presence of a *penta*-substituted benzene ring (ring A') bearing three oxygen atoms.
- Signals of two *Meta* protons at 6.09 ppm (1H, d, *J* = 1.8 Hz, H-6) and 6.19 ppm (1H, d, *J* = 1.8 Hz, H-8) assigned to two residual protons on a tetra substituted benzene ring (ring A) substituted by three oxygen atoms.
- A tri-substituted aromatic ring (ring B) bearing three protons that give signals at 7.81 ppm (1H, d, *J* = 8.46 Hz, H-5'), 8.29 ppm (1H, *J* = 2.21 H, H-2'), 6.81 ppm (1H, dd, *J* = 2.21 and 8.46 Hz, H-6').

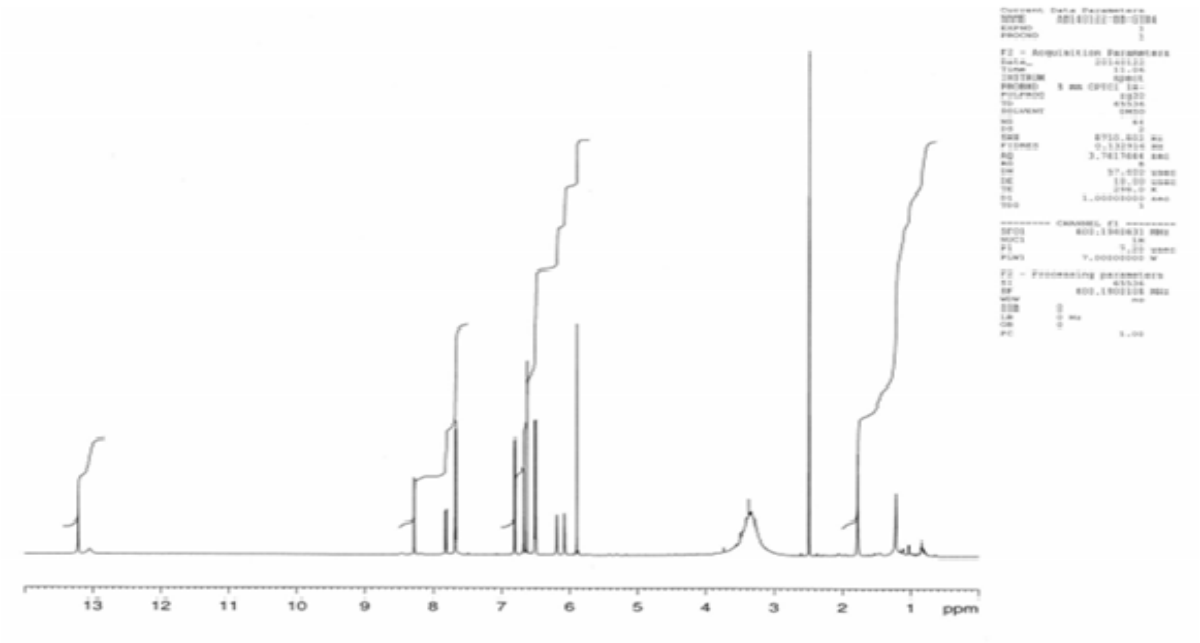


Figure 3: ^{1}H NMR spectrum of GBr-1

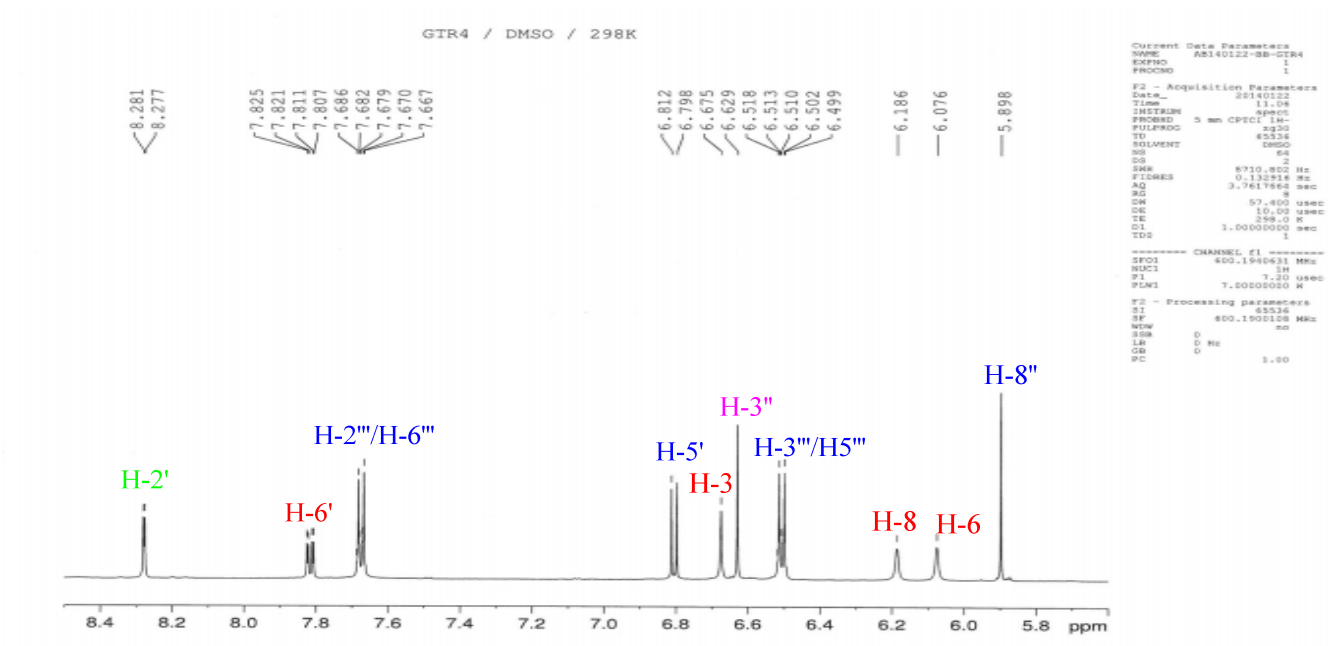


Figure 4: ^{1}H NMR GBr-1 (400MHz, DMSO-d6 (enlarged scale)

- A *para* di-substituted aromatic ring (ring B′) with an AA'BB' spin system of four protons giving signals at 7.68 ppm (2H, d, 9.0Hz, H-2′" and H-6′"), and 6.51ppm (2H, d, 9.0 Hz; H-3'" and H-5'").
- Two separate singlet signals of two protons appearing at 6. 19 ppm (1H, s, H-3) and at 6, 09 ppm (1H, s, H-3"), corresponding to those of the H-3 in the structure flavones. This suggests that GBr-1 is a dimmer with two constituent flavone units.
- Signals of two very deshielded phenolic protons were observed at 13.10 ppm (1H, s, H-5) and 13.22ppm (1H, s, H-5') showing that they are strongly chelated to *peri* phenolic groups

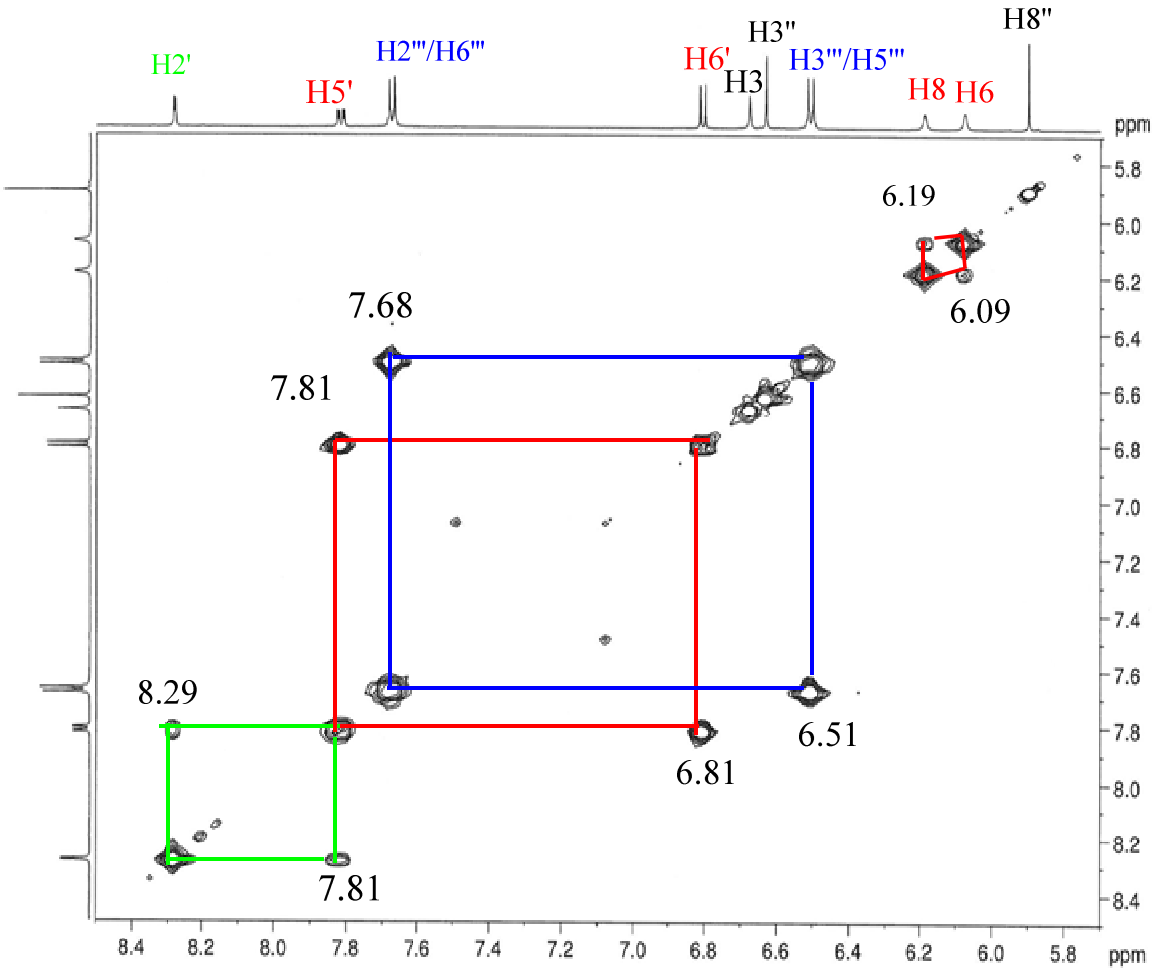


Figure 5: COSY 1H -1H spectrum of GBr-1

All C-H single bonds of the molecule can be determined by the HSQC spectrum and hence it provides information on which protons are bonded to which carbons in the structure of the compound GBr-1**(Figure 6).**

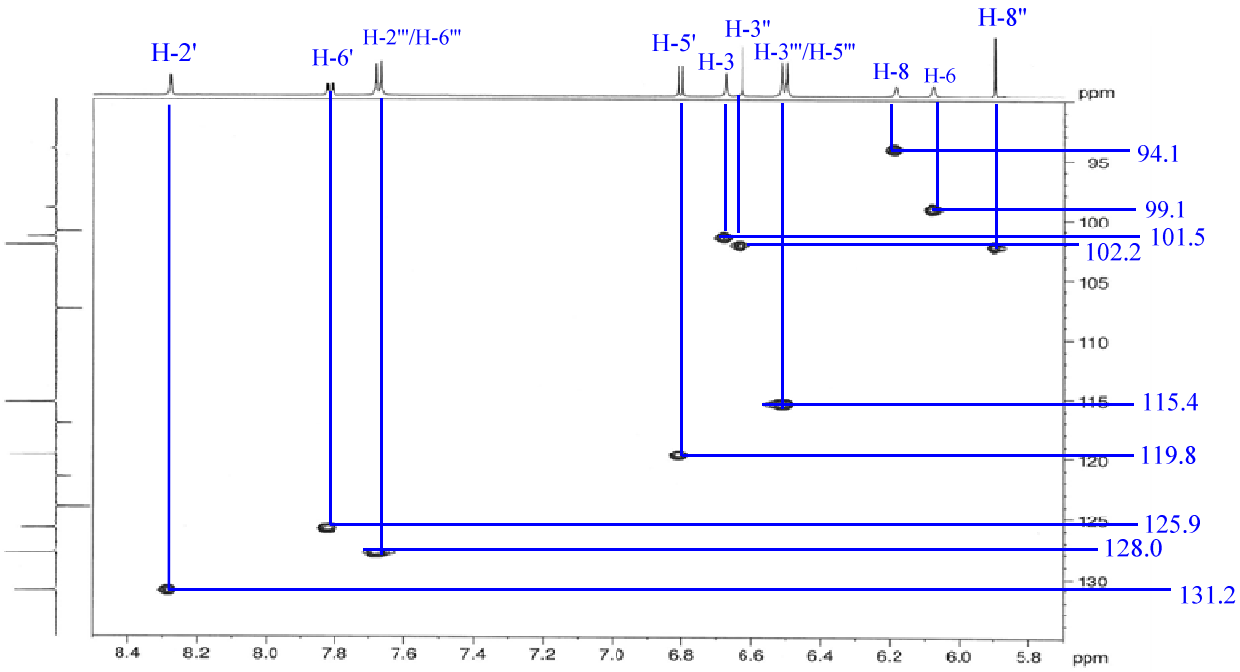


Figure 6: HSQC Spectrum of GBr-1

The obtained results clearly define the three substructures I, IIa and IIb and confirms the implication of two flavone sub units in the structure of GBr-1, obtained from the combination of substructure I with either substructure IIa or IIb **(figure 7).**

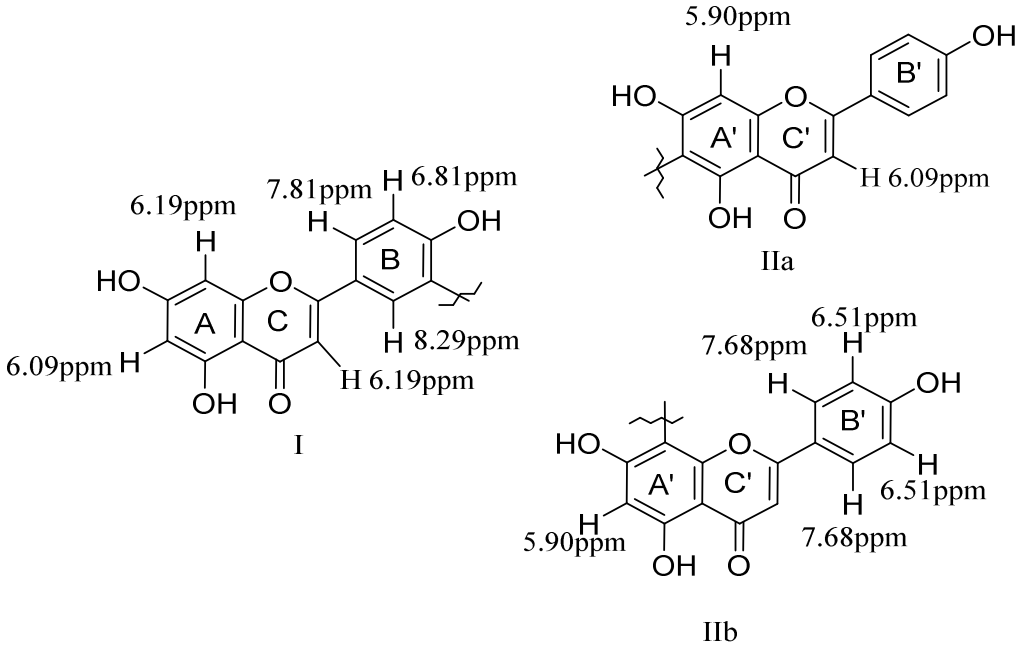


Figure 7: Sub-structures of GBr-1

The DEPTQ spectrum **(figure 8)** of GBr-1 revealed that all the 30 carbon atoms as required in the molecular formula were sp2 hybridized and including two which were of double intensities at 128.0 et 115.4 ppm identified as the carbons C-2'''/C-6''' and C-3'''/5''' respectively of a *para* di-substituted benzene ring (Ring B'). Other signals included those of:

- Two carbonyl carbons atoms at 181.3ppm (x2) C-4 and C-4'',
- 12 methines carbons atoms at 101.2, 98.3, 94.1, 131.2, 119,8, 125.9, 100.8, 103.0 ppm.
- Sixteen quartenary carbon atoms, among which ten were attached to oxygen and give signals at 165.3; 161.4; 159.0; 157.4; 165.5; 162.5; 161.2; 160.4; 154.7 and 160.6 ppm. **(Table XII).**

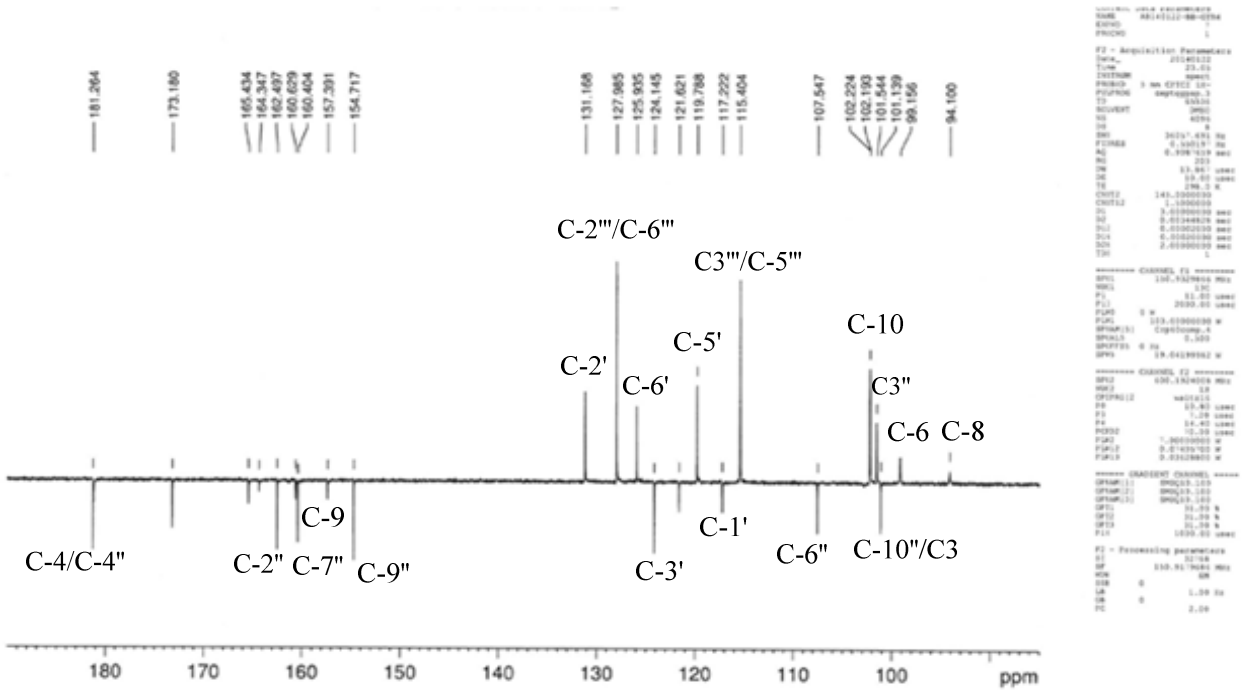


Figure 8: DEPTQ spectrum of GBr-1

An important correlation between proton H-2'of ring B (8, 29 ppm) and carbon C-6"of ring A' (107, 5 ppm) in the HMBC spectrum **(figure 9)** of GBr-1 showed that the interflavonoyl linkage is between carbon C-3' (ring B, sub-structure I) and carbon C-6'' (ring A', sub-structure IIa) and thus leading to the structure 4',4''', 5,5'', 7,7''-hexahydroxy-3'-6''-biflavone or robustaflavone, **152**.

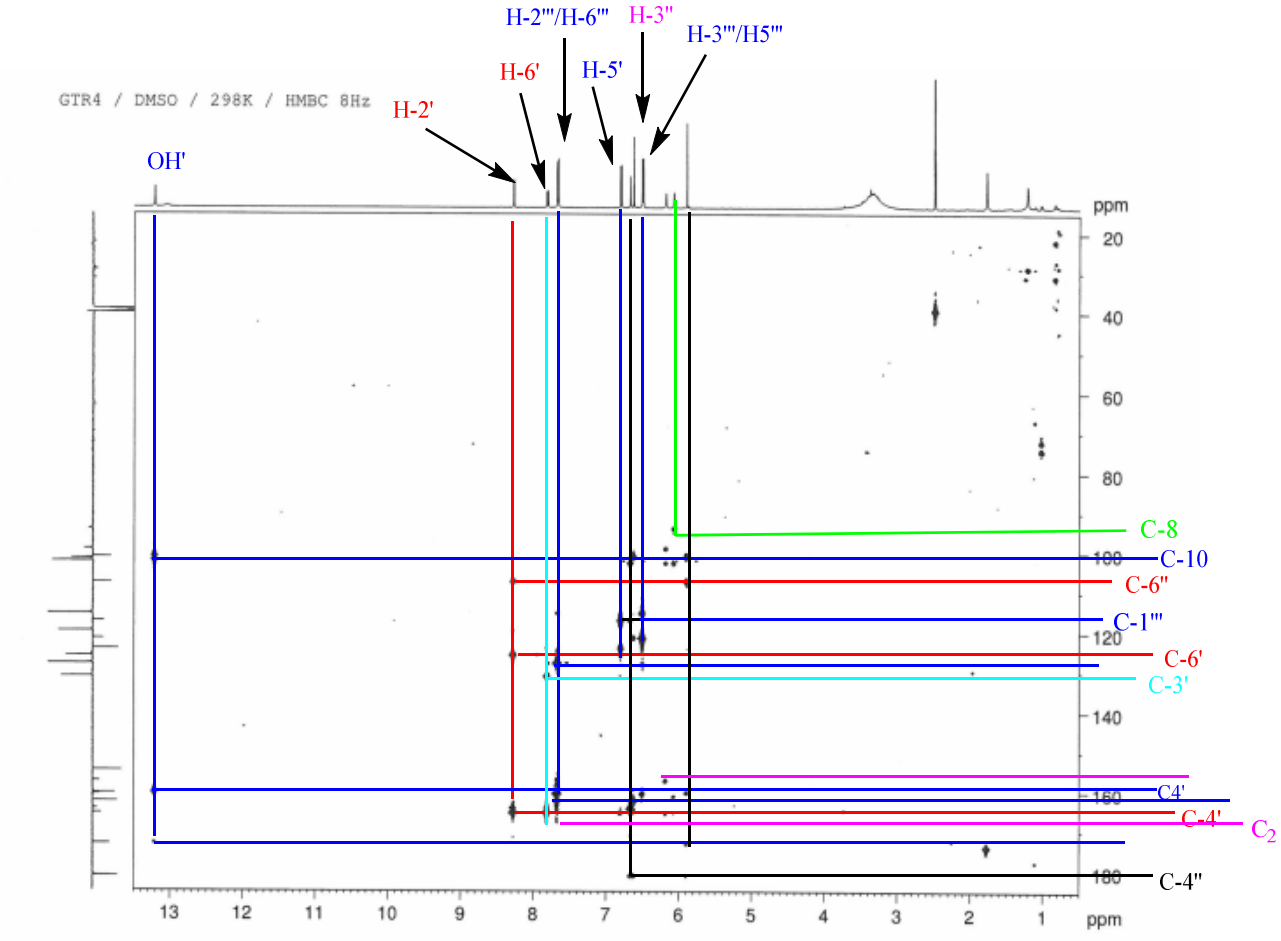
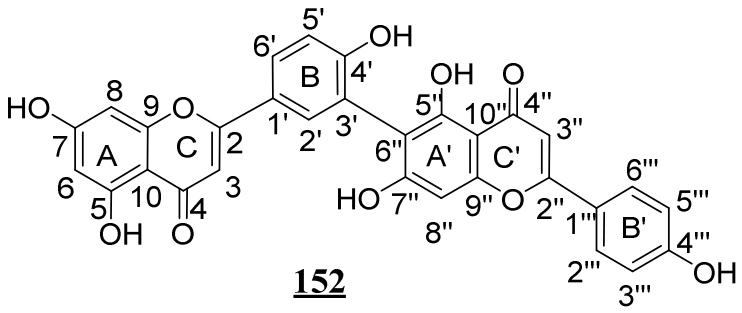


Figure 9: HMBC Spectrum of GBr -1



152

This structure was confirmed by comparing the values obtained for GBr-1 with those of an authentic sample of robustaflavone isolated earlier from Campylospermum *flavum* (**Ndongo et *al*., 2010)**, as shown in table 12.

Table 12 : NMR spectral data of GBr-1 (DMSO-d6):1H (400MHz) and 13C (100 MHz).

N° C	GBr-1			Robustaflavone (acetone) (Ndongo et *al.*, 2010)
	δC ppm	Type of Caron	δH (ppm): *J*(Hz)	δC ppm
2	165.3	C	-	166.3
3	100.8	CH	6.19 (1H, s)	103.2
4	181.3	C	-	181.8
5	161.4	C	13.10 (1H, s)	161.2
6	98.3	CH	6.09 (1H, d, 1.8)	98.6
7	165.5	C	-	163.6
8	94.1	CH	6.19 (1H, d, 1.8)	93.1
9	157.4	C	-	157.3
10	103.0	C	-	102.8
1'	117.2	C	-	123.1
2'	131.2	CH	8.29 (1H, d, 2.2)	134.5
3'	124.1	C	-	119.7
4'	159.2	C	-	159.8
5'	119.8	CH	7.81 (1H, d, 8.4)	119.8
6'	125.9	CH	6.81 (1H, dd, 8.4 and 2.2)	127.5
2"	162.5	C	-	163.9
3"	102.2	CH	6.09 (1H, s)	103.3
4"	181.3	C	-	181.6
5"	161.2	C	13.22 (1H, s)	160.9
6"	107.5	C	-	103.7
7"	160.4	C	-	163.8
8"	94.1	CH	5.90 (1H, s)	93.34
9"	154.7	C	-	156.2
10"	100,8	C	-	103.3
1"	121.6	C	-	120.2
2"'/6"'	128.0	CH	7.68 (2H, d, 9.0)	128.4
3"'/5"'	115.4	CH	6.51 (2H, d, 9.0)	116.4
4"'	160.6	C	-	159.6

III-2.2 Identification of GBr-2

GBr-2 was obtained as a yellow amorphous solid, also gave a positive flavonoids test (Mg/HCl). This was confirmed by it UV spectrum, which displayed two maxima of absorption at λ_{max} 223 nm and 320 nm indicating the conjugated diene and conjugated carbonyl chromophores suggesting the presence of a flavone unit in the structure of GBr-2**.** The high resolution ESI mass spectrum of GBr 2 shows the pseudomolecular ion peak $[M+H]^+$ at m/z 553.1122 with formula $C_{31}H_{21}O_{10}$ from which the molecular formula $C_{31}H_{20}O_{10}$ was deduced with 22 unsaturated sites for GBr-2.

Its infrared spectrum showed intense absorption bands of the following functions: phenol (3354 cm^{-1}), conjugated and chelated carbonyl (1652 cm^{-1}), and aromatic rings (1605 cm^{-1} found in the structure of flavones.

The complete analysis of its ^1H NMR spectrum **(figure 10)** led to the identification of the different proton systems implicated in the structure of GBr-2. These included:

- A *para* disubstituted aromatic ring system with four protons at 6.51 ppm (2H, d, *J* = 8.9Hz, H-3'''/H-5''') and at 7.68 ppm (2H, d, *J* = 8.9Hz, H-2'''/H-6''').

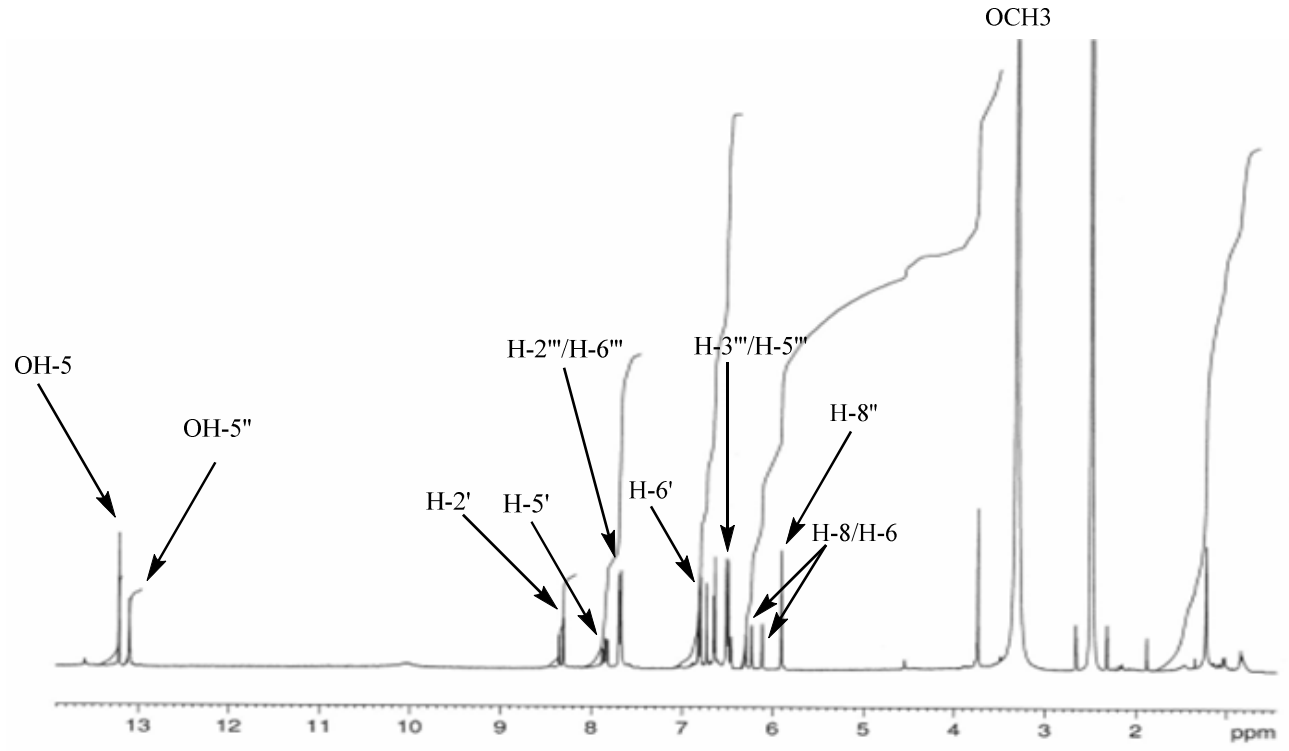


Figure 10: ^1H NMR GBr-2 (400MHz, DMSO-d_6)

- A tri-substituted aromatic ring with three *ortho/meta* protons system that gave signals at 6.81 ppm (1H, dd, *J* = 8.9Hz and 2.4Hz, H-6'), 7.81 ppm (1H, d, *J* = 8.9 Hz, H-5') and at 8.48 ppm (1H, d, *J* = 2.4Hz, H-2').

- Two *meta* coupling protons giving signals at: 6.09 ppm (1H, d, 2.4Hz, H-6) and 6.19 ppm (1H, d, 2.1Hz, H-8).
- The singlet signal of a much-shielded aromatic proton at 5.91 ppm (1H, s, H-8") suggested the presence of a substituted benzene ring (ring A') with three oxygenated substituents.
- Signals of two strongly chelated and much deshielded conjugated phenol protons were observed at 13.10 ppm (1H, s, OH-5") and 13.20 ppm (1H, s, OH-5).
- A three protons singlet signal at 3.45 ppm (3H, s) attributed to that of OCH_3 group which was absent in the proton NMR spectrum of GBr-1 if we take a closer look at both spectra.
- Lastly two remaining singlet signals at 6. 19 ppm (1H, s, H-3) and at 6.09 ppm (1H, s, H-3"), are those of H-3 and H-3″protons found in the structure of flavones and suggest the implication of two flavone units (substructures I and II) in the structure of GBr-2. Sub-structure II presents two possibilities (substructure IIa or IIb) (**figure 11),** thus suggesting two possible positions of the interflavonyl bond.

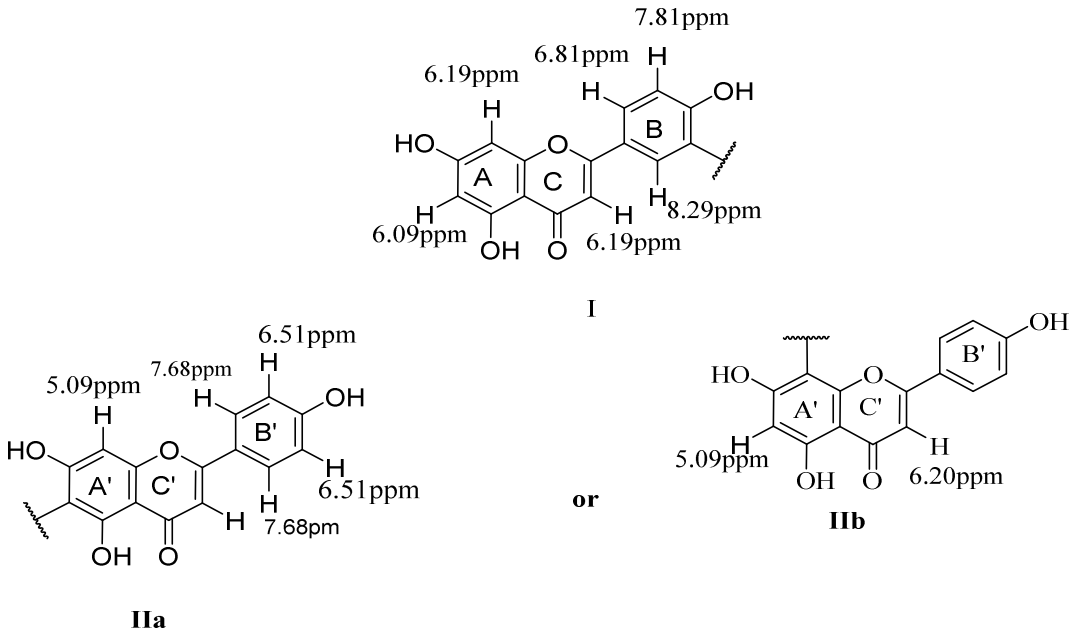


Figure 11: Sub-structures of GBr-2

The ^{13}C NMR spectrum **(figure 12)** of GBr-2, had the signals of all the 31 carbon atoms required by the molecular formula. Apart from the carbon in the CH_3O group which is sp3 hybridised and gave the signal 55.9 ppm, all the other remaining 30 carbons atoms of the molecule are sp^2 hybridized.

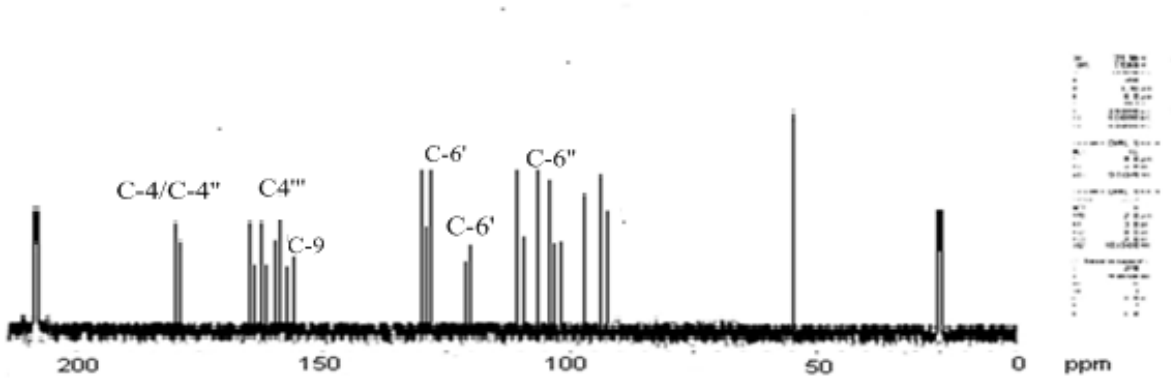


Figure 12: ^{13}C NMR (100 MHz acetone d6) spectrum of GBr-2.

These include signals of:

- Twelve methines sp^2 carbons, [103.4, 96.2, 94.3, 130.8, 120.0, 127.6, 103.1, 93.3, 128.4 (x2), 115.6 (x2)],
- Eighteen quaternary carbon atoms out of which ten were attached to oxygen atoms (163.3, 162.4, 156.8, 160.2, 163.8, 158.4, 161.6, 156.2 and 160.9 ppm) and two of which are carbonyls (181.7 and 181.8 ppm).

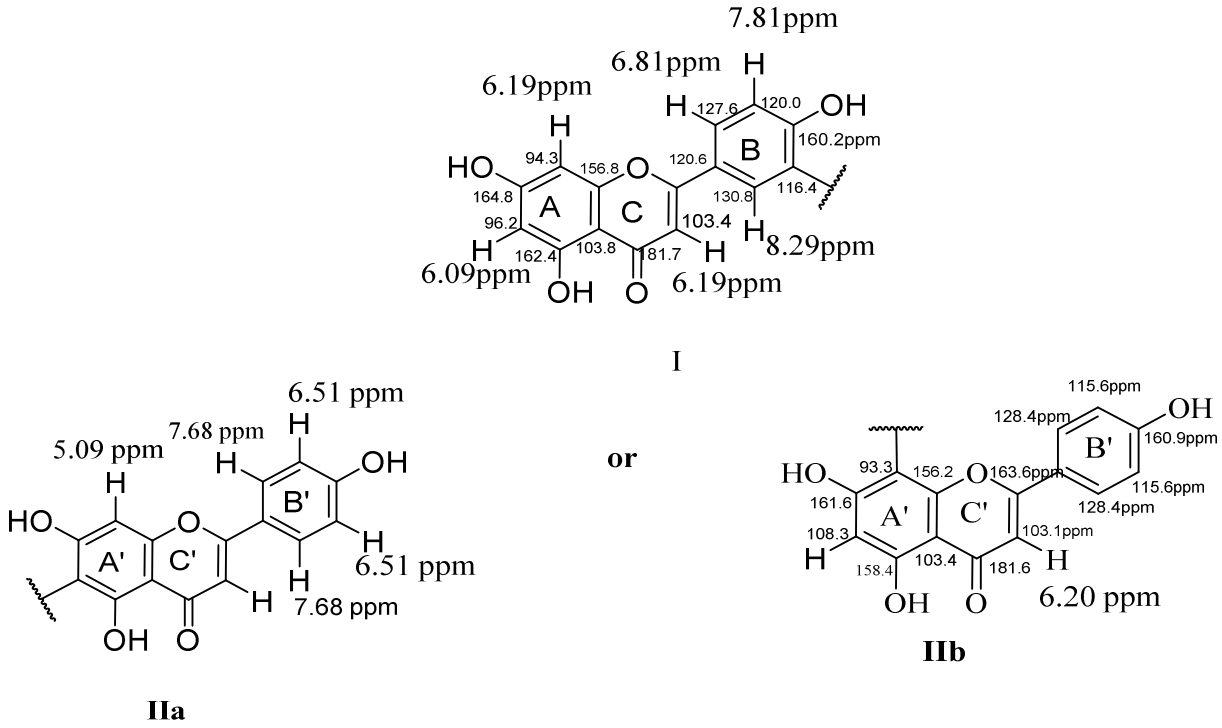


Figure 13: sub-structures of GB-r-2

Now the question that comes up is how did we positioned OCH_3 group on our structure? We positioned the OCH_3 group using the NOESY spectrum **(figure 14)** of GBr-2, which displays the correlations of protons that are close to each other in space. As we can see, it is shown on the spectrum that the OCH_3 group protons at 3.45 ppm are correlated to the chelated hydroxyl proton at 13.10 ppm implying that the interflavonyl bond is between C-3' at 116.4 ppm and C-6" at 108.3 ppm. We could also see other spatial correlations such as that between H-2‴/H-3‴ at 7.68 ppm with that of H-3‴/H-5‴ at 6.51 ppm, that of H-5′ at 7.81 ppm an H-6′ at 6.81 ppm and that between H-2′ at 8.29 ppm and H-6′ at 6.81 ppmrespectively.

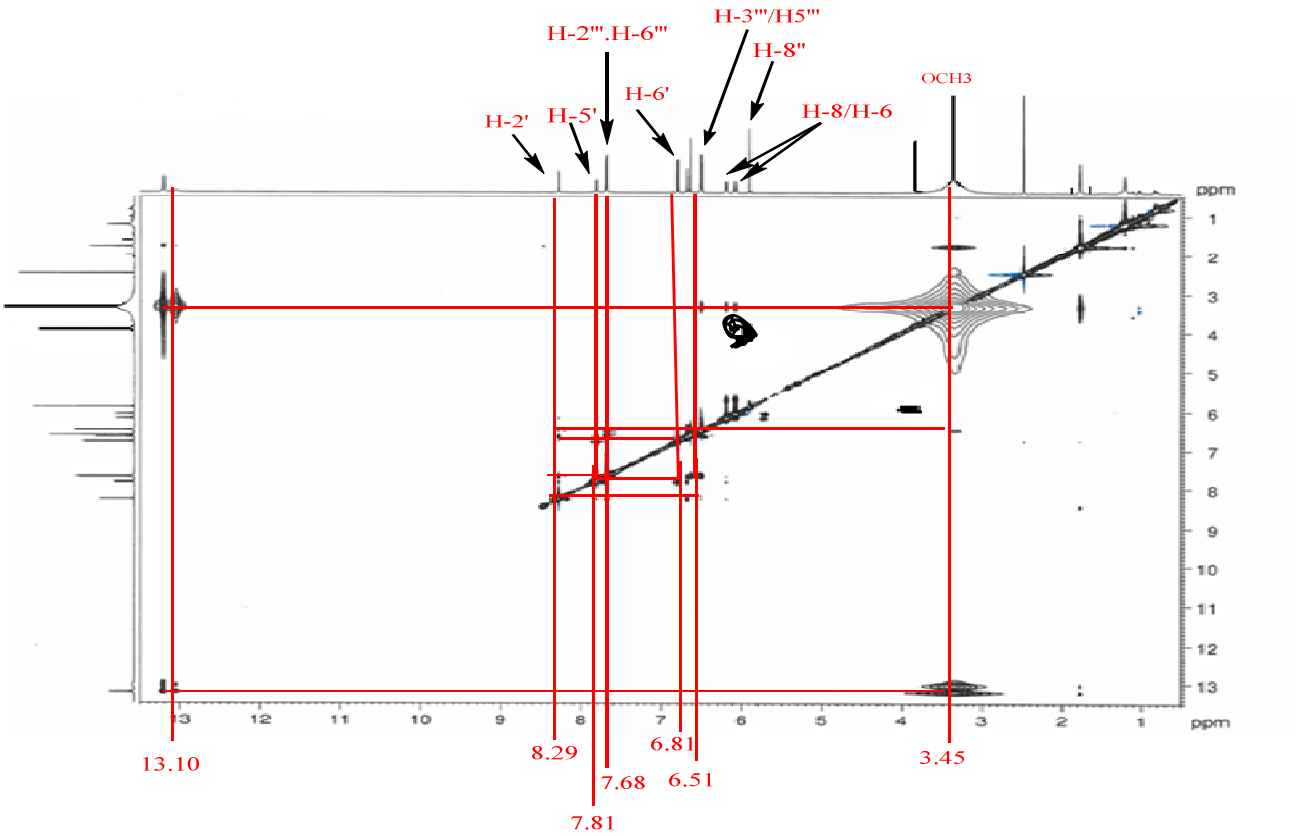
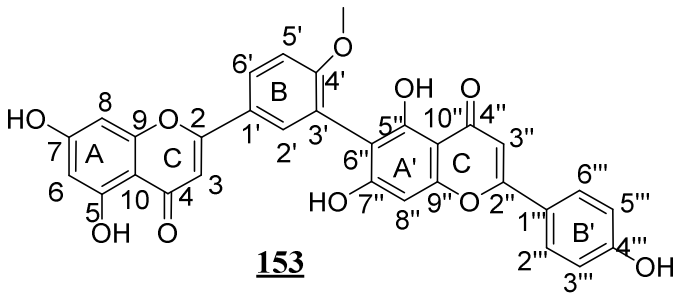


Figure 14: NOESY Spectrum of GBr-2

This leads to the structure 4''',5,5'',7,7''-pentahydroxy-4'-*O*-methoxy-3',6''-biflavone **153** or 4'-*O*–methylrobustaflavone. This structure was confirmed by comparing the values obtained for GBr-2 with those of an authentic sample of robustaflavone isolated in the past from *Campylospermum flavum* (**Ndongo et *al*., 2010)**.



153

Table 13: GBr-2 NMR Data (DMSO-d6): 1H (400MHz) and 13C (100MHz).

Carbon N°	GBr-2			Robustaflavone (acetone) (Ndongo et al., 2010).
	δC	Type of Carbon	δH J (Hz)	δC
2	165.2	C	-	166.3
3	103.4	CH	6.19 (1H, s)	103.2
4	181.7	C	-	181.8
5	162.4	C	13.20 (1H, s, OH)	161,2
6	96.2	CH	6.09 (1H, d, 2.4)	98.6
7	164.8	C	-	163.6
8	94.3	CH	6.19 (1H, d, 2.1)	93.1
9	156.8	C	-	157.3
10	103.8	C	-	102.8
1'	120.6	C	-	123.1
2'	130.8	CH	8.29 (1H, d, 2.4)	134.5
3'	116.4	C	-	119.7
4'	160.2	C	-	159.8
5'	120.0	CH	7.81 (1H, d, 8.9)	119.8
6'	127.6	CH	6.81 (1H, d, 2.4)	127.5
2"	163.8	C	-	163.9
3"	103.1	CH	6,09 (1H, S)	103.3
4"	181.6	C	-	181.6
5"	158.4	C	13.10 (1H, s, OH)	160.9
6"	108.3	C	-	103.7
7"	161.6	C	-	163.8
8"	93.3	CH	5.90 (1H, s)	93.4
9"	156.2	C	-	156.2
10"	103.4	C	-	103.3
1'"	120.8	C	-	120.2
2'"/6'''	128.4	CH	7.68 (2H, d, 8.9)	128.4
3'"/5'''	115.6	CH	6.51 (2H, d, 8.9)	116.4
4'"	160.9	C	-	159.6
OCH_3-4'	55.6	CH_3	3.45 (3H, s)	

III-2.3: Determination of GBr-3

GBr-3, also obtained as a yellow amorphous solid responded positively to the flavonoid test (Mg/HCl). Its UV absorption spectrum had a single absorption maximum at λ_{max} 268 nm and a shoulder at 316 nm thus suggesting the presence of an isoflavone unit in its structure **(Mabry et al, 1970).**

The (HR-TOF-MS) mass spectrum **(Figure 15)** of GBr-3 shows the pseudomolecular ion peak $[M+H]^+$ at *m/z* 479.0588, corresponding to the formula $C_{24}H_{15}O_{11}$ and implying a molecular mass of 478 and the molecular formula $C_{24}H_{14}O_{11}$ accounting for 18 unsaturated sites.

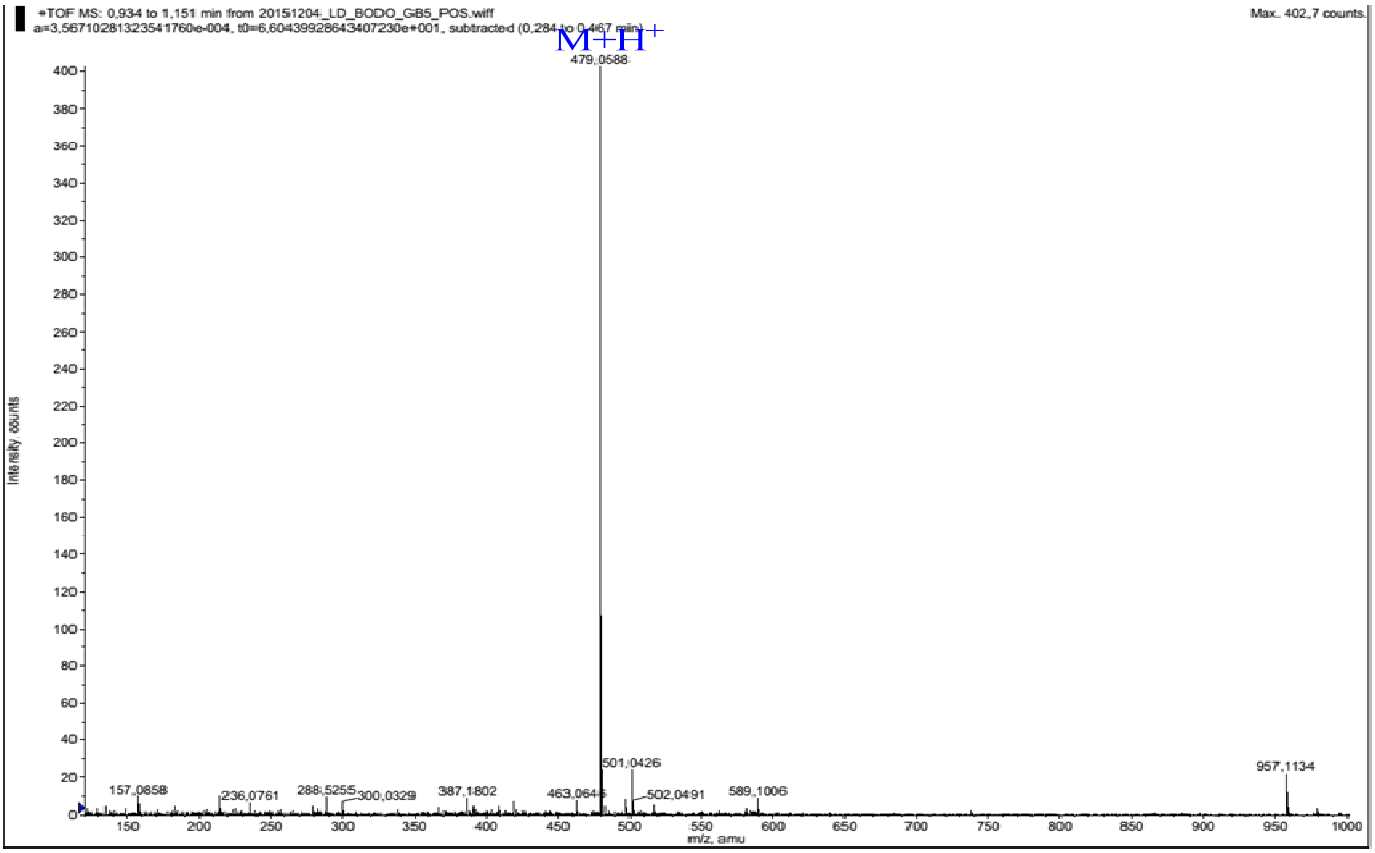


Figure 15: Mass spectrum of GBr-3

Its IR spectrum had intense absorption bands that suggested the presence of the following functional groups: conjugated and chelated carbonyl (1638 cm^{-1}), free phenol groups (3424cm^{-1}) and aromatic rings (1600 and 1498 cm^{-1}).

From the 1D and 2D ^{1}H NMR spectra of GBr-3 **(Figure 16, 17**and **18)** we identified the following proton systems:

- A much deshielded singlet signal of an ethylenic proton at 8.37 ppm (1H, s, H-2) characteristic of H-2 protons of ring C in isoflavones.
- An *ortho-metta* tri-substituted aromatic ring (ring B) with three residual protons giving signals respectively at 7.13 ppm (1H, dd, *J* = 8.46; 2.22 Hz, H-6'), at 6.65 ppm (1H, J = 8.46Hz, H-5'), and at 7.50 ppm (1H, d, *J* = 2.22 Hz, H-2').
- Two separate signals for two *meta* protons on a tetra substituted benzene ring (ring A) substituted by three oxygen atoms appeared at 6.27 ppm (1H, d, *J* = 2.1Hz, H-6) and at 6.46 ppm (1H, d, *J* = 2.1Hz, H-8).

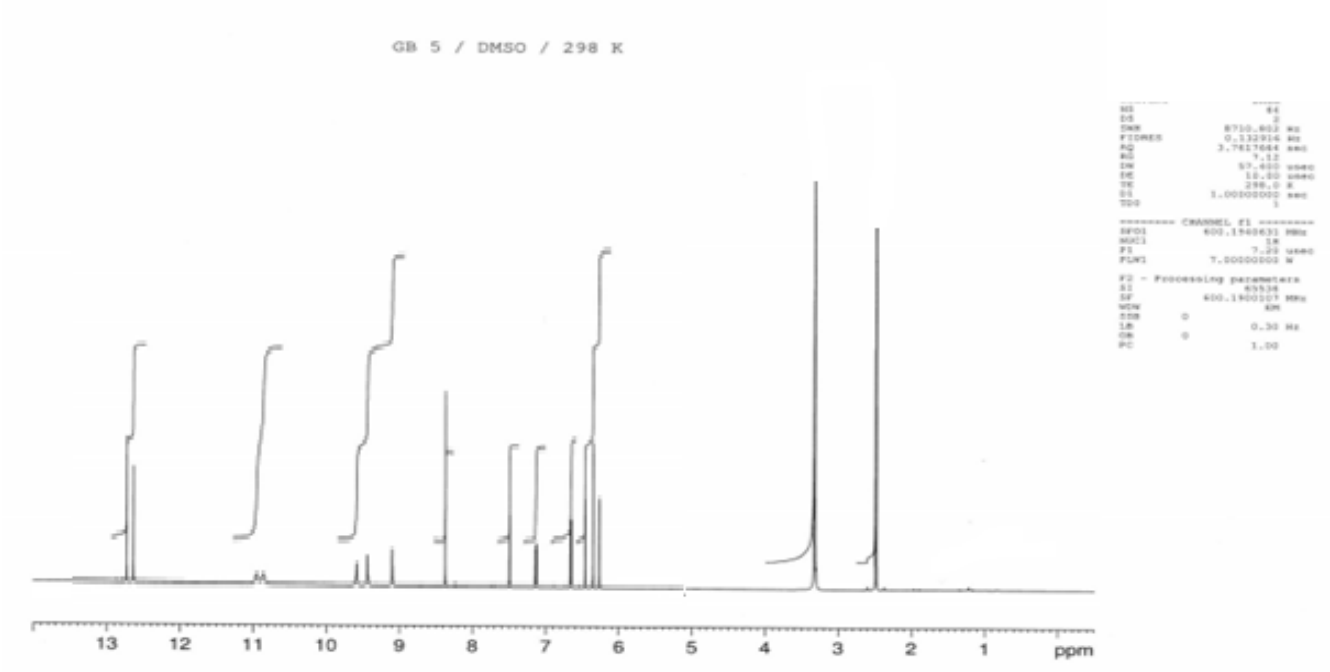


Figure 16: 1H NMR (600MHz DMSO d6) of GBr-3

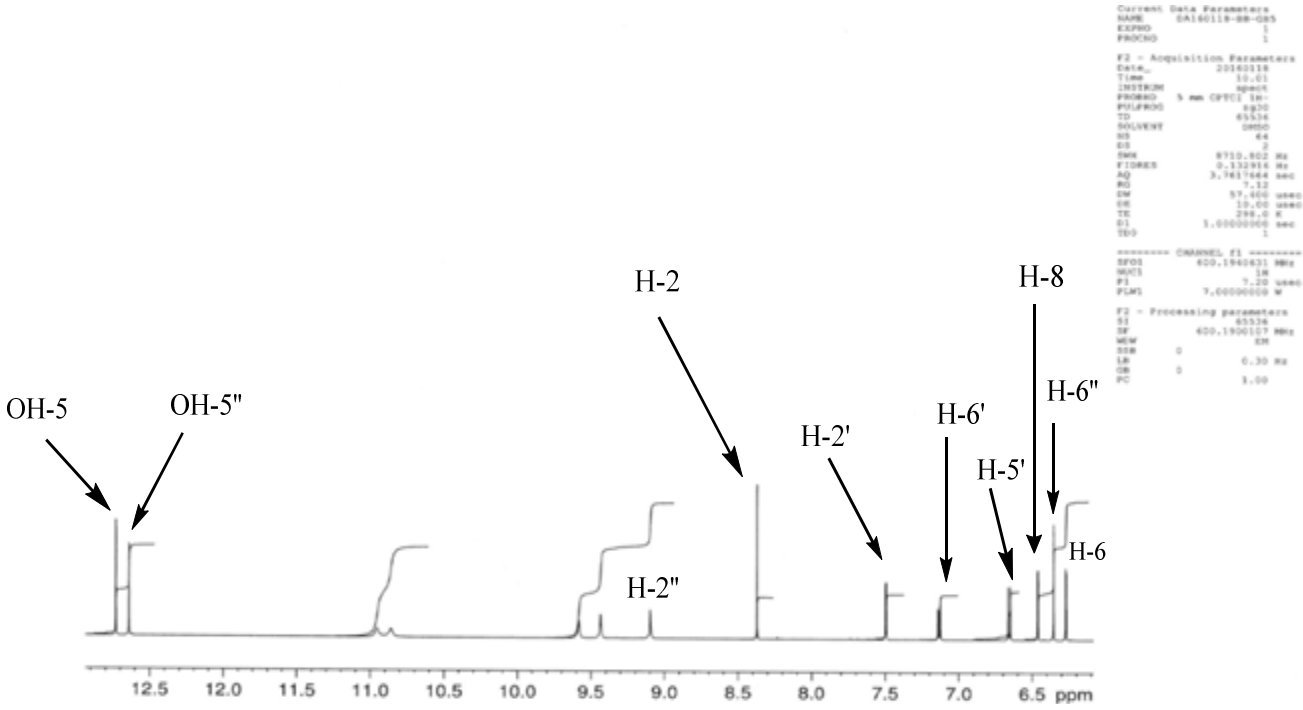


Figure 17: 1H NMR of GBr-3 (600MHz DMSO-d6)

- Also, a singlet proton signal of a proton on a penta-substituted benzene ring (ring A') substituted by four oxygen atoms was observed at δ_H 6.35 (1H, s, H-6"), (sub-structure IIA).
- Another singlet signal observed at δ_H 9.10 (1H, s, H-2") representing that of a more deshielded ethylenic proton characteristic of the H-2 protons on ring C' of isoflavones.
- Two higly deshielded proton singlets at δ_H 12.72 (s, OH-5) and at δ_H 12.63 (s, OH-5") were assigned to the two phenol groups strongly chelated to the two *peri* carbonyl groups.

The COSY spectrum of compound GBr-3 provides information on which proton couples with which one thus indicating also *H-H* connectivities. *Gemical, vicinal* or long range couplings correlations can be observed for all protons except the hydroxylic proton, which is often rapidly exchanged in protonic solvents as shown on **(figure 18).**

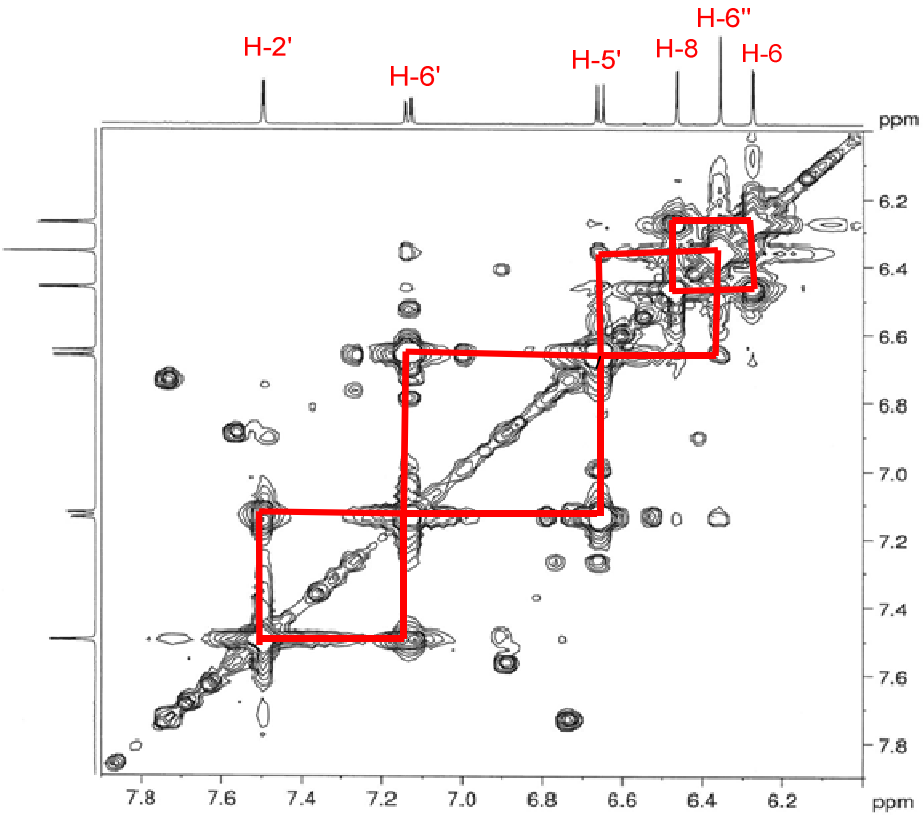


Figure 18: COSY 1H-1H NMR spectrum of GBr-3

The chemical shifts of all the protonated carbon atoms in each of the proton systems in the structure of compound GBr-3 were attributed from the HSQC spectrum **(figure 19).**

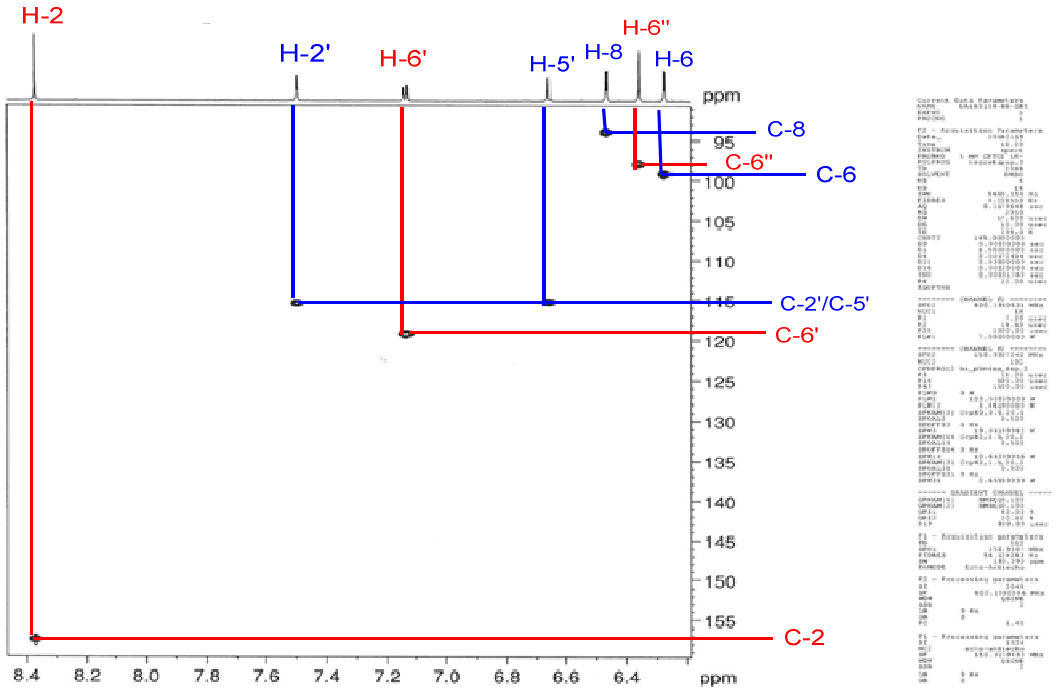
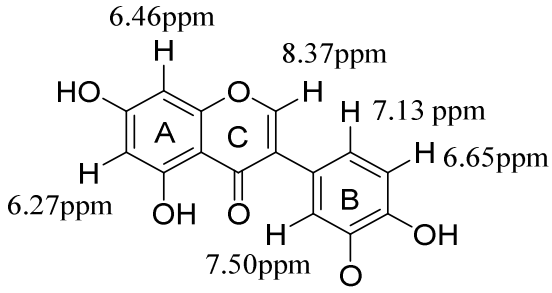
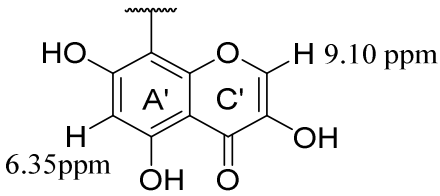


Figure 19: HSQC Spectrum of GBr-3

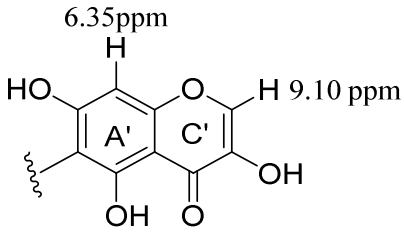
The values obtained were assigned which led to three substructures **I, IIa** and **IIb. (Figure 20).** A detailed analysis of the 1D and 2D ^{1}H NMR spectra **(Figures 16, 17 and 18)** and the HSQC **(Figure 19)** spectrum of GBr-3 revealed the absence of signals of the protons of ring B' and suggested the presence of a chromone motif represented as sub- structures IIa or IIb **(Figure 20).**



Sub-structure I



Sub-structure IIa



Sub-structure IIb

Figure 20: Sub-structures of GBr-3

Analysis of the DEPTQ spectrum **(Figure 21)** of GBr-3 showed that all the signals were those of sp^2 hybridized carbon atoms among which five quartenary carbon atoms (122.0(x2), 120.1, 104.5 and 102.3 ppm), nine are quartenary carbons atoms bearing oxygen substituents at (164.4, 162.0, 161.7,160.9, 157.8 , 157.4, 160.4 147.7 and 146.6) ppm, two are carbonyl carbons at (180.7 and 178.2) ppm and eight are methine carbons atoms (154.0, 144.9, 119.0, 115.3, 115.4, 98.0, 93.9, 99.1) ppm respectively. It is very important to note that the values attributed to the carbon atoms in substructure I are very similar to thoses obtained for the isoflavonoid, genestein.

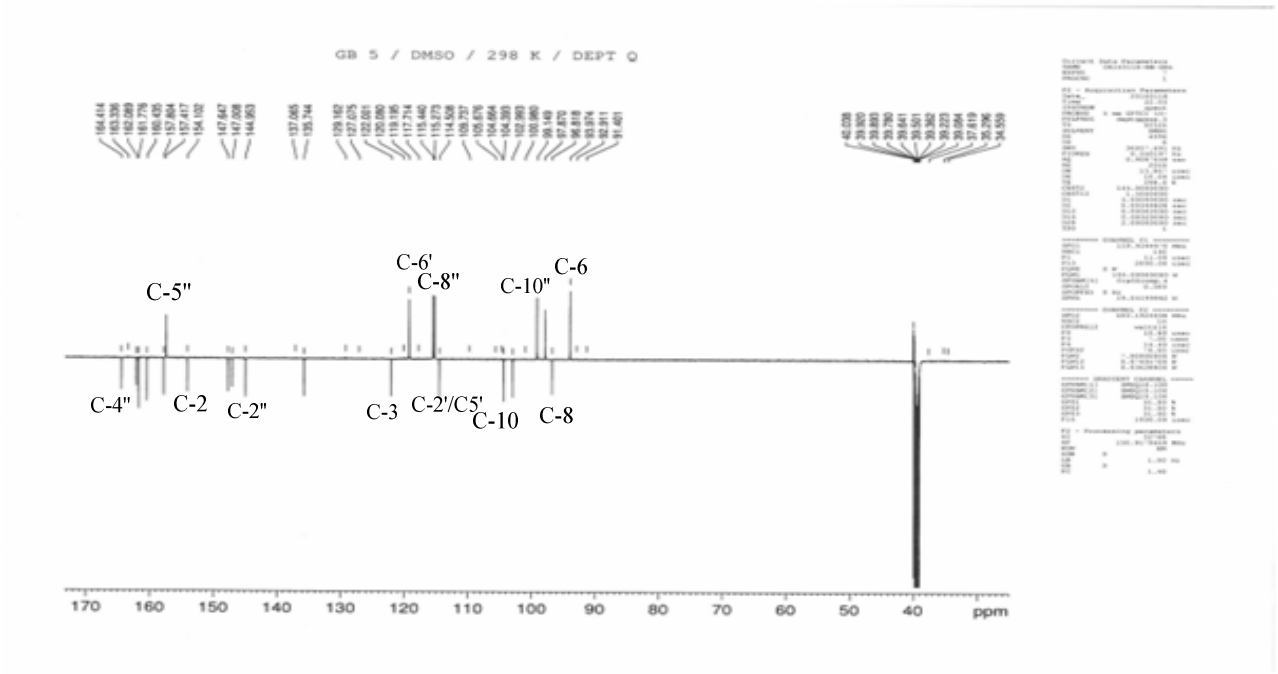


Figure 21: DEPTQ Spectrum of GBr-3

The HMBC spectrum **(figure 22)** showed important correlatios between proton H2' (7.50 ppm, ring B) and the aromatic carbons C-8" (119.0 ppm, ring A'), C-4' at 147.7 ppm and C-3' at 146.6 ppm (ring B) respectively suggesting that the interflavonoyl bond is between the carbon C-3' (146.6 ppm, ring B, sub-structure I) and C-8" (119.0 ppm ring A', sub-structure IIa).

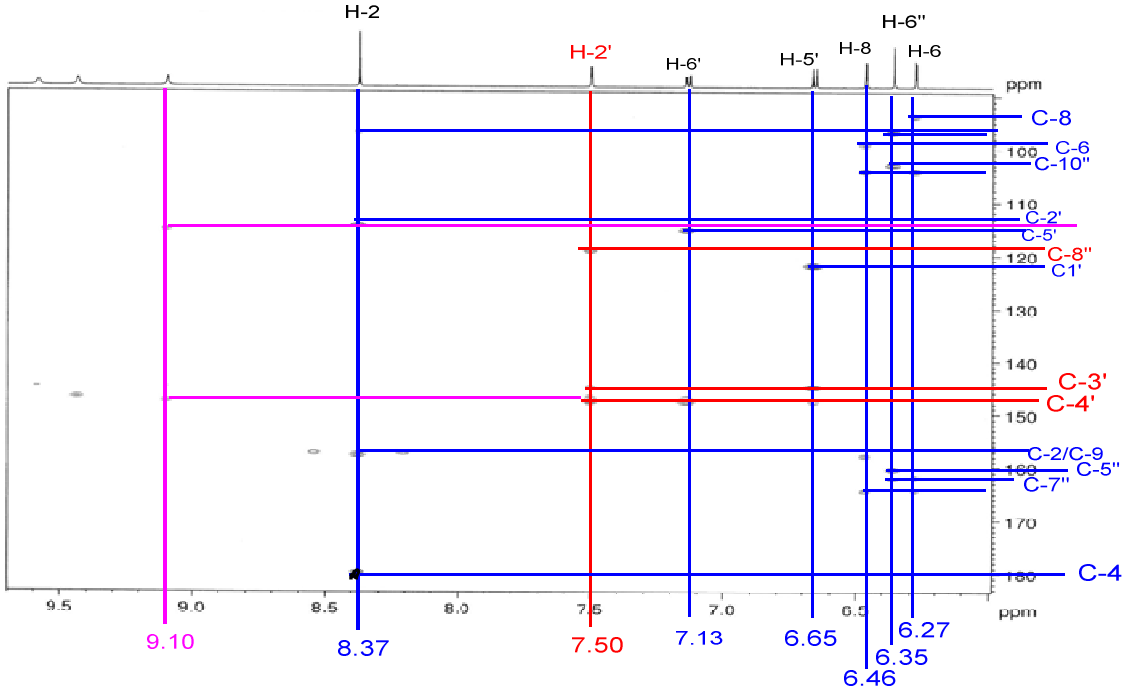
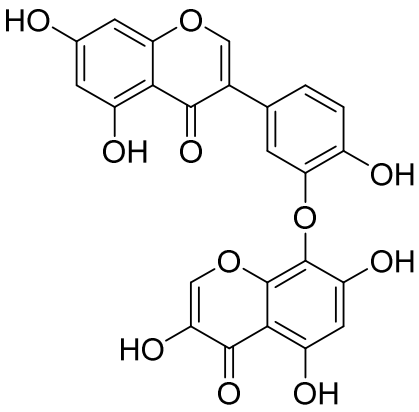


Figure 22: HMBC Spectrum of GBr-3

Table 14: 1H-NMR and 13C-NMR (600 MHz) data of Compound GBr-3 in DMSO-d6

GBr-3				Genistein (GBr-7) (**Madan et *al.*, 2009**).	
Carbon Nº	**δ_C**	**Type of Carbon**	**δ_H, *J*(Hz)**	**δ_C**	**δ_H, J(Hz)**
2	156.1	CH	8.37 (1H, s)	153.9	8.32 (1H, s)
3	122.0	C	-	122.2	
4	180.7	C	-	180.1	
5	161.7	C	12.72 (1H, s, OH-5)	161.9	13.06 (1H, s, OH-5)
6	99.1	CH	6.27 (1H, d, 2.10)	98.9	6.22 (1H, d, 2.0)
7	164.4	C	-	164.2	
8	93.9	CH	6.46 (1H, d, 2.10)	93.6	6.38 (1H, d, 2.0)
9	157.8	C	-	158.1	-
10	104.5	C	-	104.4	-
1'	122.0	C	-	121.1	-
2'	115.3	CH	7.50 (1H, d, 2.22)	130.1	7.37 (2H, d, 8.6)
3'	146.6	C	-	115.1	6.87 (2H, d, 8.6)
4'	147.7	C	-	157.4	-
5'	115.4	CH	6.65 (1H, brd, 8.46)	115.1	6.87 (2H, d, 8.6)
6'	119.0	CH	7.13 (1H, dd, 2.22 and 8.46)	130.1	7.37 (2H, d, 8.6)
2"	144.9	CH	9.10 (1H, s)		-
3"	157.4	C	-		-
4"	178.2	C	-		-
5"	160.9	C	12.63 (1H, s, OH-5")		-
6"	98.0	CH	6.35 (1H, s)		-
7"	162.0	C	-		-
8"	119.0	C	-		-
9"	160.4	C	-		-
10"	102.3	C	-		-

The NOESY Spectrum **(figure 23)** of GBr-3, showed a correlation between the protons H-2" at (δ_H 9.10) and H-2' at (δ_H 7.50) confirming the interflavonyl bond between C-3' carbon (δ_C 156.6, ring B, sub-structure I) and C-8" (δ_C 120.1, ring A', sub-structure IIa) thus leading to the structure **154** which is that of brevipedicellone D.



154

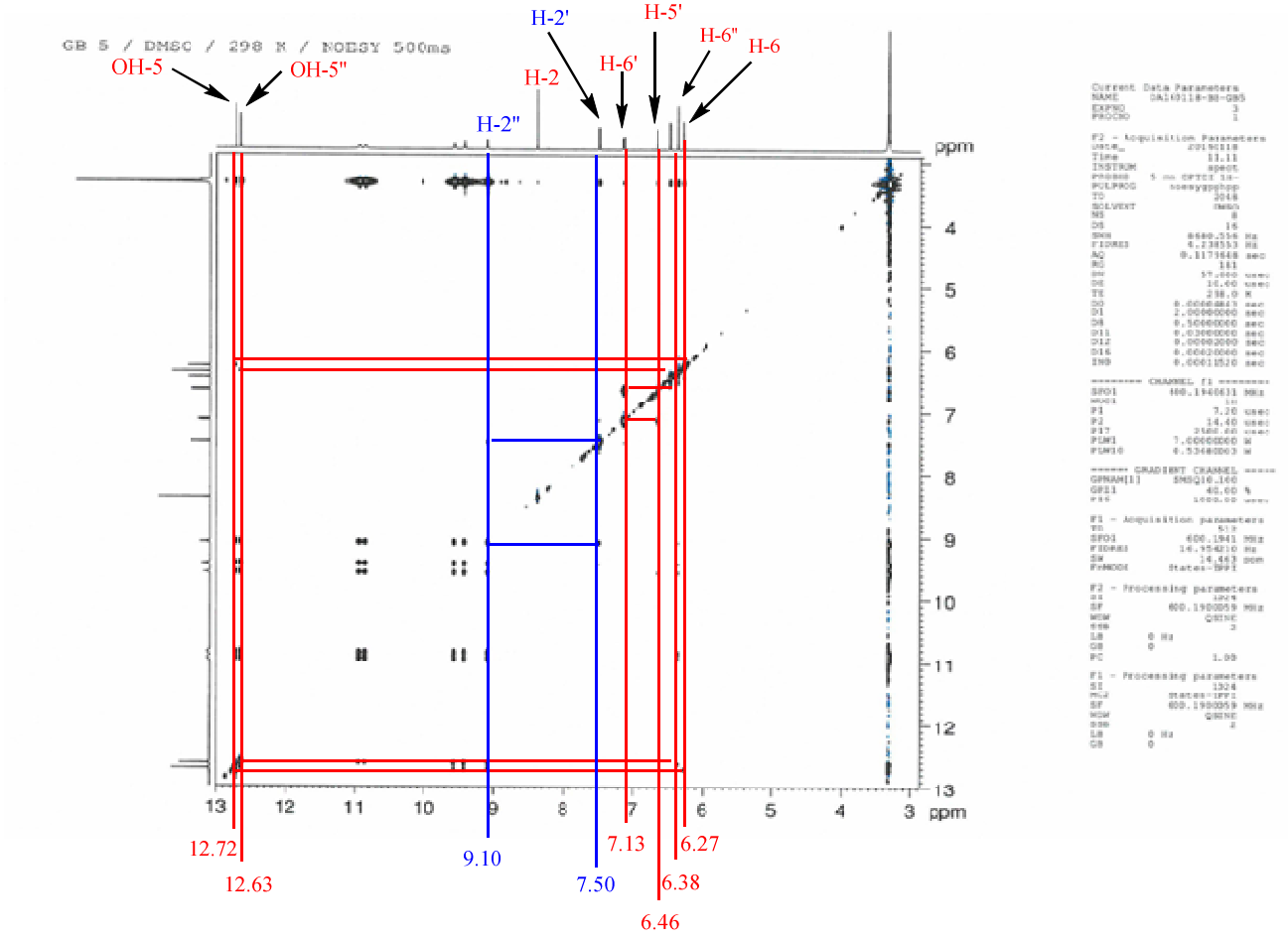


Figure 23: NOESY Spectrum of GBr-3

All given evidence show that the structure of GBr-3 is 8- {-5-(5, 7-dihydroxy-4-oxo-4H-chromen-3-yl)-2-hydroxyphenoxy}-3, 5, 7- trihydroxy-4*H*-chromen-4-one, a new cleaved biflavonoid ether named as **brevipedicellone D**. This structure enriches once more the diversity of biflavonoids, mostly this sub-class of ether biflavonoid which is very less represented.

III.2.4. Determination of GBr -4

GBr-4, isolated as a yellow powder, also gave a positive flavonoid test (Mg/HCl). Its UV spectrum had two absorption bands at λ_{max} 223 and 320 nm associated respectively to the absorption of the chromophores of the conjugated diene and the conjugated carbonyl of the flavone motif**.**

Its HRMS **(figure 24)** shows the pseudomolecular ion peak $[M+H]^+$ at m/z 553.0960 coresponding to the formula $C_{31}H_{21}O_{10}$, thus implying the molecular mass 552 for GBr-4 and the molecular formula $C_{31}H_{20}O_{10}$ which accounts for 22 unsaturated sites.

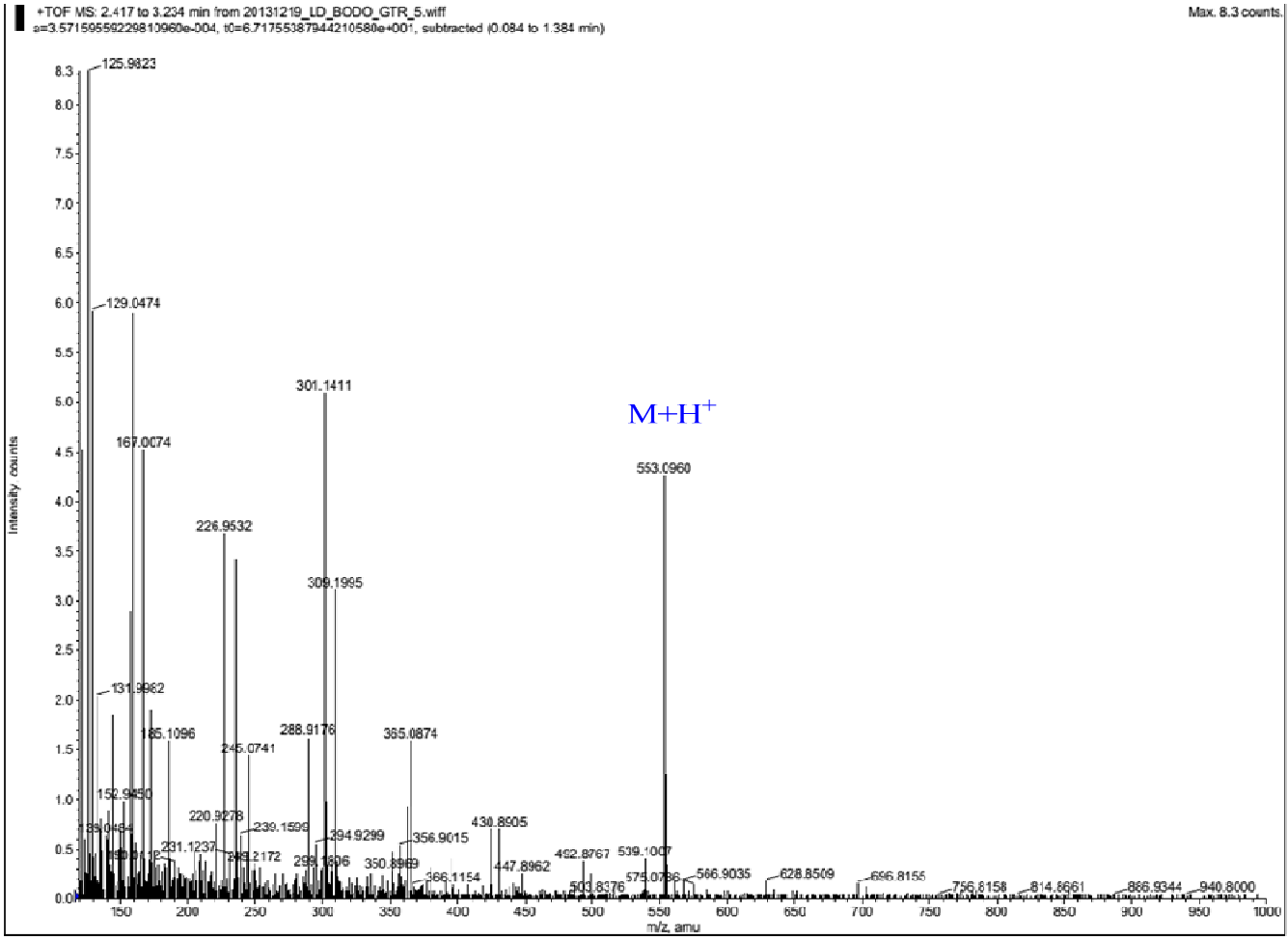


Figure 24: Mass spectrum GBr-4

Its IR absorption spectrum is very similar to that of amentoflavone as it displays absorption bands for the same functional groups: phenol at (3234 cm^{-1}), conjugated and chelated carbonyl (1638 cm^{-1}), conjugated double bond (1628 cm^{-1}) and aromatic rings (1603 and 1508 cm^{-1}). Analysis of the 1D, NMR and 2D ^1H-^1H COSYspectra of GBr-4 **(Figures 25, 26 and 27)** showed that its structure has the following proton systems:

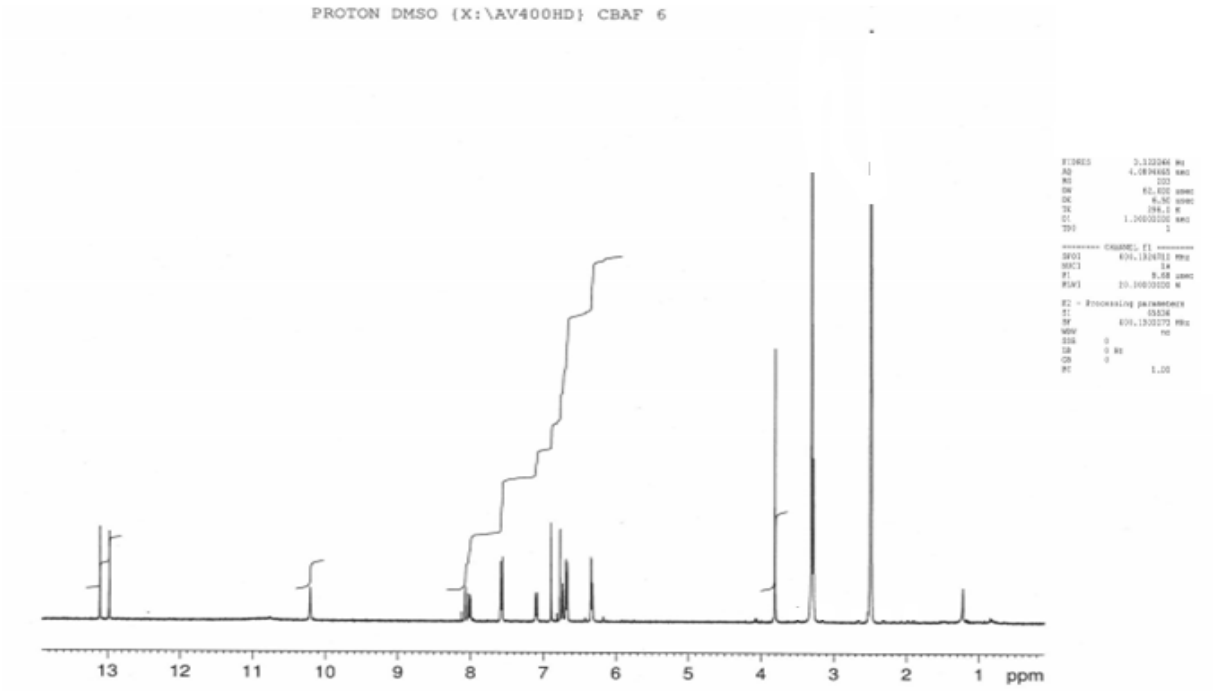


Figure 25: ^{1}H NMR Spectrum of GBr-4 (600 MHz, DMSO-d6)

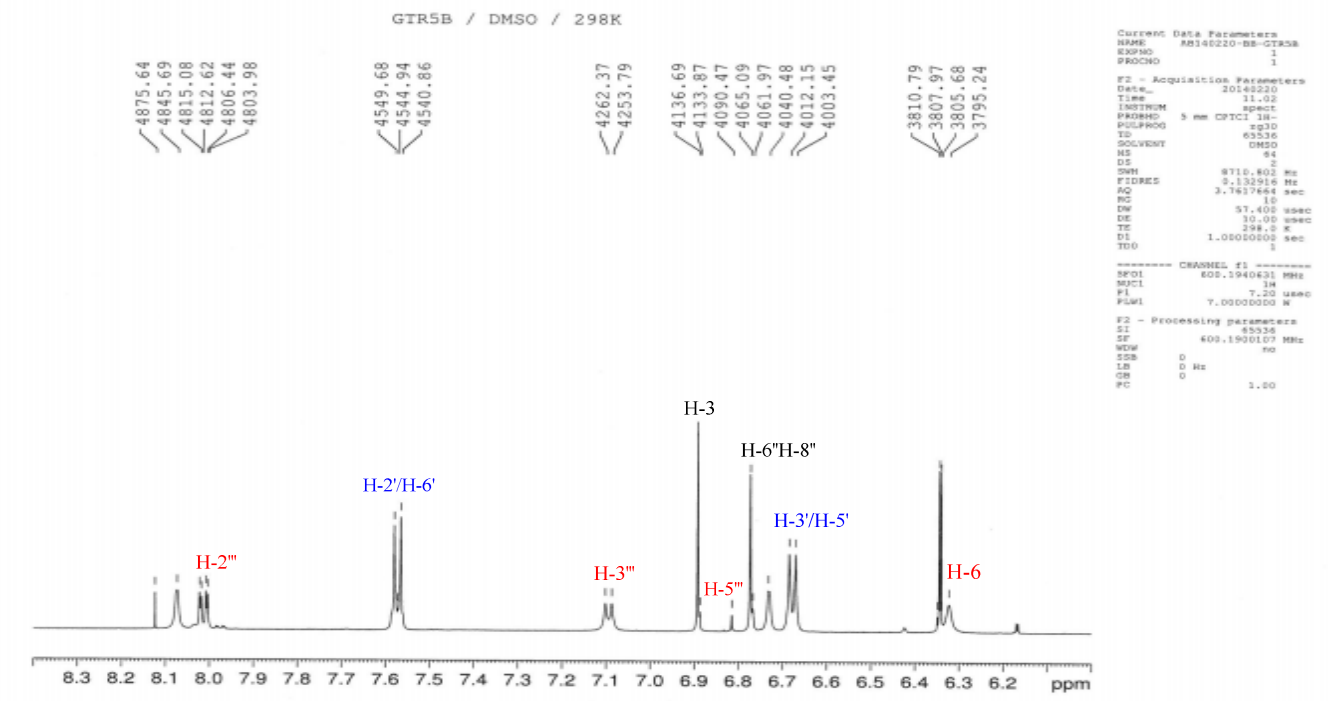


Figure 26: 1H NMR Spectrum of GBr-4 (Enlarged zone) between 6.2 ppm and 8.2 ppm

- Two tetra-substituted aromatic rings with two protons exhibiting *Meta* coupling signals at δ_H 6.35 (1H, d, 2.8 Hz, H-6), δ_H 6.77 (1H, d, 2.8 Hz, H-8) and δ_H 6.81 (1H, d, 2.2 Hz, H-6" and H-8") were attributed to rings A and A'.

- A para di-substituted aromatic ring with signals exhibiting an AA'BB' spin system at δ_H 7.57 (2H, d, 8.8 Hz, H-2' and H-6'), and δ_H 6.71 (2H, d, 8.8 Hz, H-3'and H-5') were attributed to ring B.
- Two distinct singlet signals of two separate protons on two penta-substituted aromatic rings [(δ_H 6.82 (1H, s, H-3) and δ_H 6.89 (1H, s, H-3")] and were attributed to the flavone protons on C-3 and C-3" of rings C and C' respectively.
- A tri-substituted aromatic ring carrying three protons exhibiting an ABX spin system with signals at δ_H 8.04 (1H, d, 8.6 Hz H-2'"), δ_H 7.09 (1H. d, 8.6 Hz, H-3'") and δ_H 6.89 (1H, d, 2.8 Hz, H-5'") was attributed to ring B'.
- Two very deshielded singlets signals at 12.12 ppm (1H, s, OH-5) and at 12.99ppm (1H, s, OH-5") assigned to two phenolic protons, each strongly chelated to a *peri* carbonyl function.
- And finally a sharp singlet signal at 3.85ppm (3H, s) assinged to a CH_3O group.

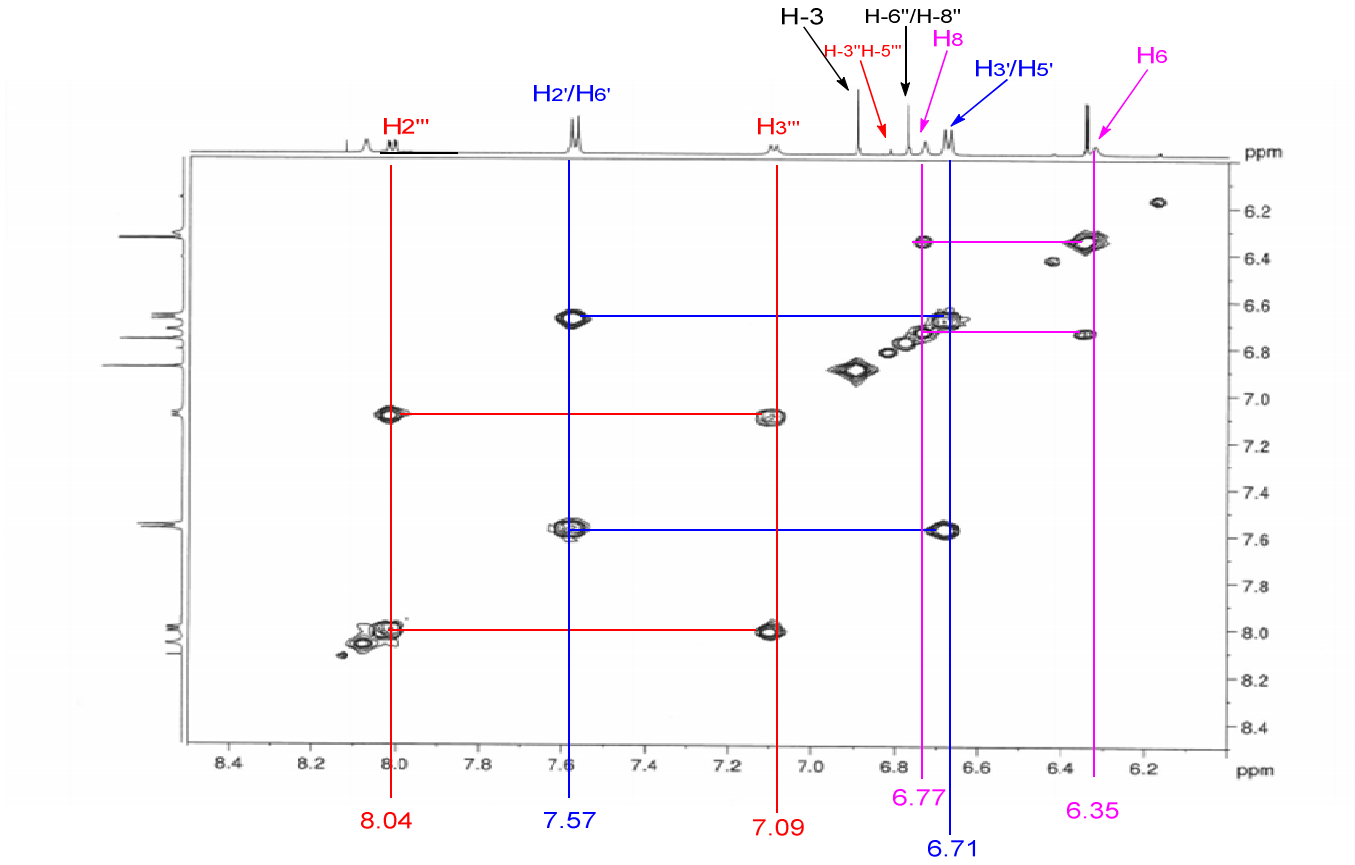


Figure 27: COSY 1H-1Hpectrum of GBr-4

These facts suggest the implication of two flavone units, as accounted for in substructures I and II **(Figure 28).**

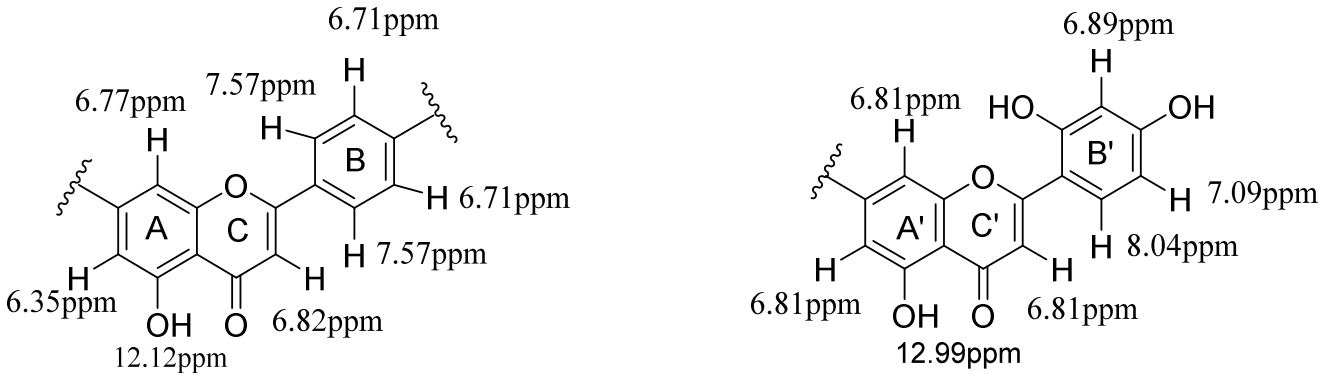


Figure 28: Sub-structures of GBr-4

On the DEPT spectrum **(figure 29)** of **GBr-4**, it was deduced that apart from the C-atom of the CH_3O group which is saturated and whose chemical shift appears at δ_C 56.0, all other remaining 30 carbon atoms are sp^2 hybridized. This gave 28 distinct signals with two of double intensities; 128.1 ppm and115.6 ppm assigned to the carbons C-2'/C6' and C-3'/C-5' of the *para* di-substituted aromatic ring (ring B). Other signals were those of two carbonyls carbons [182.0 (C-4) and at 181.9 ppm (C-4")], eleven methines (CH) [δ_C 101.5, 104.6, 94, 3; 131.4, 121.4, 127.6, 102.8, 92.6, 128.1 (x2), 115.6 (x2)], seventeen quaternary (C) carbon atoms, ten out of which are attached to oxygen atoms (δ_C 154.4, 156.3 (x2), 161.1, 160.5 (x2), 160.9, 163.5, 164.2, 165.0).

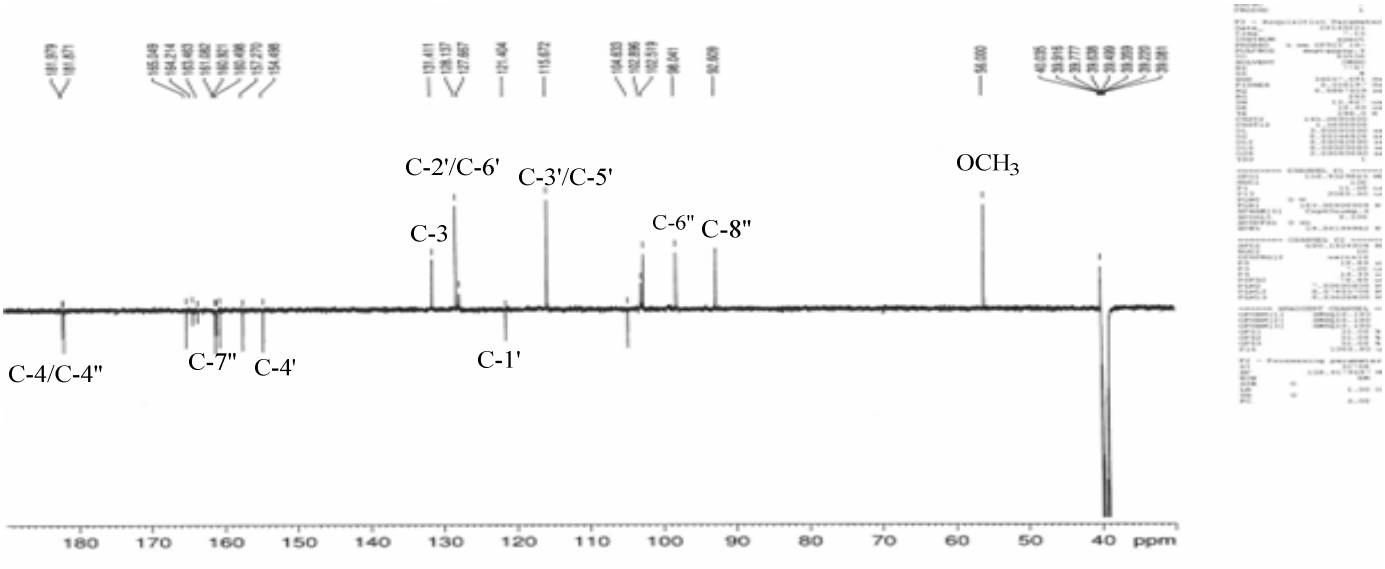


Figure 29: DEPTQ Spectrum of GBr-4

Table 15: 1H and 13C-NMR (600 MHz) data of GBr-4 in DMSO-d6, δ ppm)

Carbon N°	GBr-4		
	δ_C ppm	Type of Carbon	δ_H ppm J(H_Z)
2	165.0	C	-
3	101.5	CH	6.82 (1H, s)
4	182.0	C	-
5	160.5	C	12.12 (1H, s, OH)
6	94.3	CH	6.35 (1H, d, 2.8)
7	167.3	C	-
8	92.6	CH	6.77 (1H, d, 2.8)
9	156.3	C	-
10	104.6	C	-
1´	127.6	C	-
2´/6´	128.5	CH	7.57 (2H, d, 8.8)
3´/5´	115.6	CH	6.71 (2H, d, 8.8)
4´	154.4	C	-
2´´	160.5	C	6.81 (1H, d, 2.2)
3´´	103.5	CH	6.89 (1H, d, 2.2)
4´´	181.9	C	-
5´´	164.2	C	12.99 (1H, s, OH)
6''	96.0	CH	6.81 (2H, d, 8.8)
7´´	160.1	C	-
8"	92.6	CH	6.81 (1H, s, OH)
9´´	156.1	C	-
10´´	104.9	C	-
1´´´	110.4	C	-
2´´'	131.4	CH	8.04 (1H, d, 8.6)
3´´'	121.4	CH	7.09 (1H, d, 8.6)
4´´´	159.1	C	-
5´´'	102.8	CH	6.89 (1H, d, 2.8)
6´´´	157.8	C	-
OCH_3-7	56.0	CH_3	3.85 (3H, s)

The chemical shifts of all the protonated carbon atoms required by the molecular formula of compound GBr-4 were assigned using its HSQC spectrum **(figure 30).**

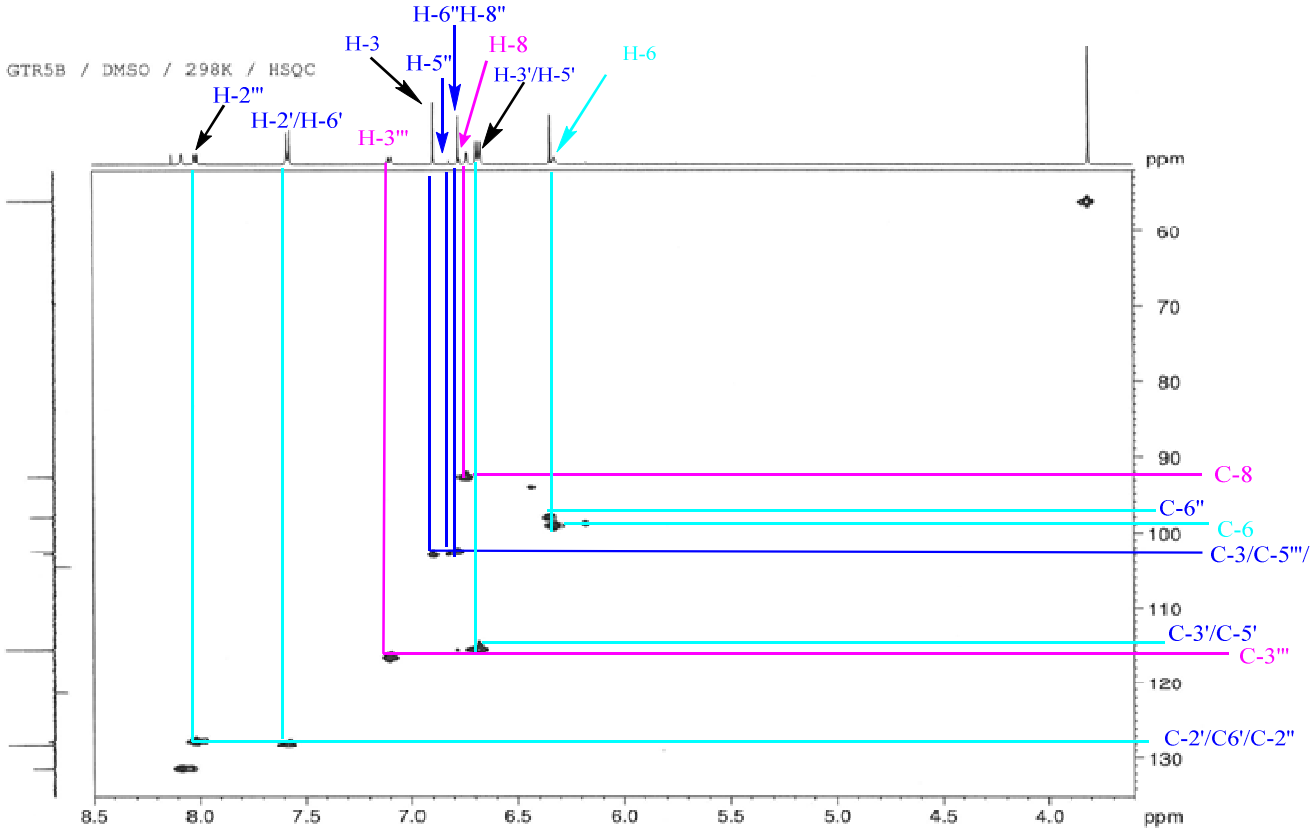
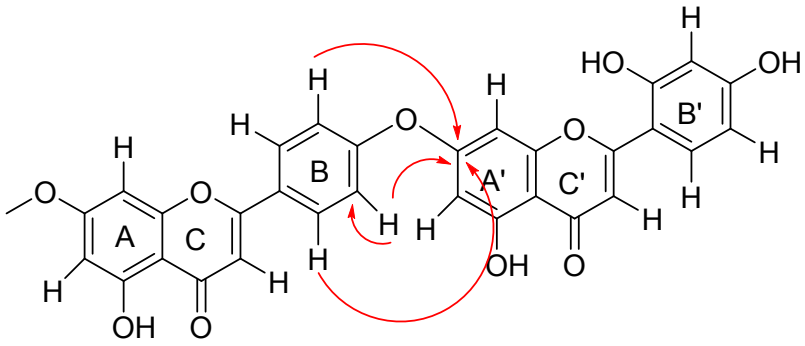
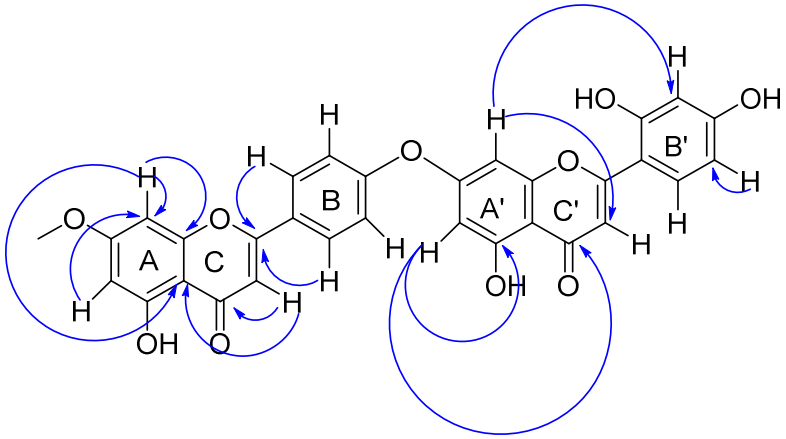


Figure 30: HSQC Spectrum of GBr-4

The HMBC spectrum **(figure 31)** had important correlations which placed the interflavonoyl bond in between carbons C-4' (ring B, sub-structure I) and C-7" (ring A', sub-structure II). This was deduced from the correlations between the protons H-2'/H-6' at 7.57 ppm and the carbon atom C-7" at 160.1 ppm, and that between the protons H-3'/H-5' at 6.71 and the carbon atom C3'/C5' at 115.6 ppm and C-7" at 160.1 ppm respectively as shown below.



Other correlations were observed on the HMBC spectrum of GBr-4 that further confirmed it structure as shown below.



This shows that GBr-4 is 2-(-2,4-dihydroxyphenyl)-5-hydroxy-7-(4,5-hydroxy-7-methoxy-4-oxo-4H-chromen-2-yl) phenoxy)-4H-chromen-4-one **155** which is a new structure, described for the first time which we named **Brevipedicelone E**.

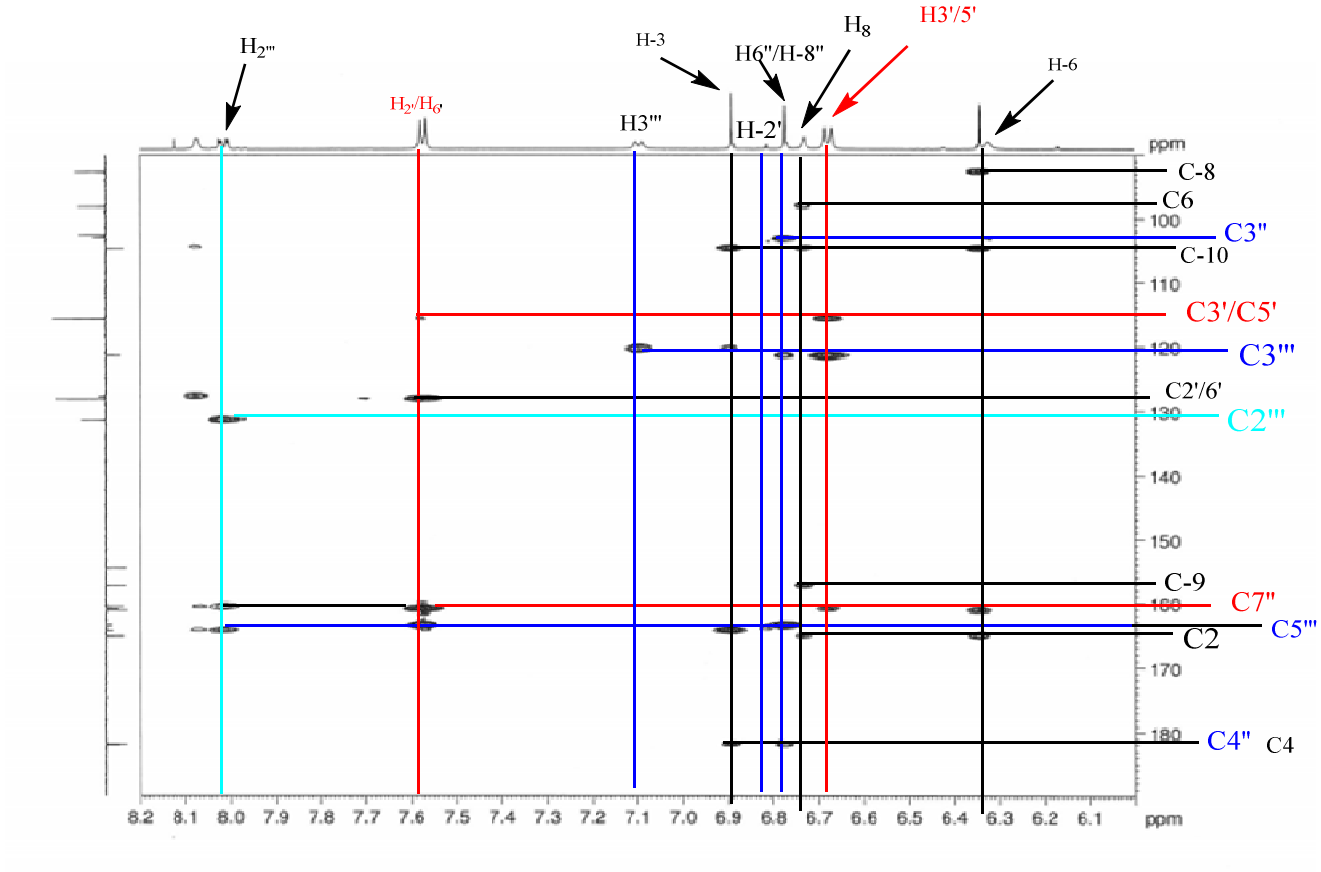
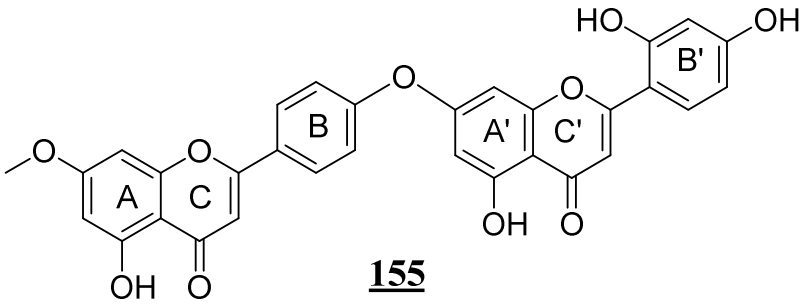


Figure 31: HMBC Spectrum of GBr-4



155

The position of the CH_3O substituent was deduced from connections observed in the NOESY spectrum of GBr-4 **(figure 32)**. These protons that gave the singlet signal at 3.85 ppm are correlated to another proton at 6.75 ppm, (H-8, ring A) implying that the CH_3O group is on carbon C-7 (167.3 ppm, ring A), confirming structure **155** for GBr-4.

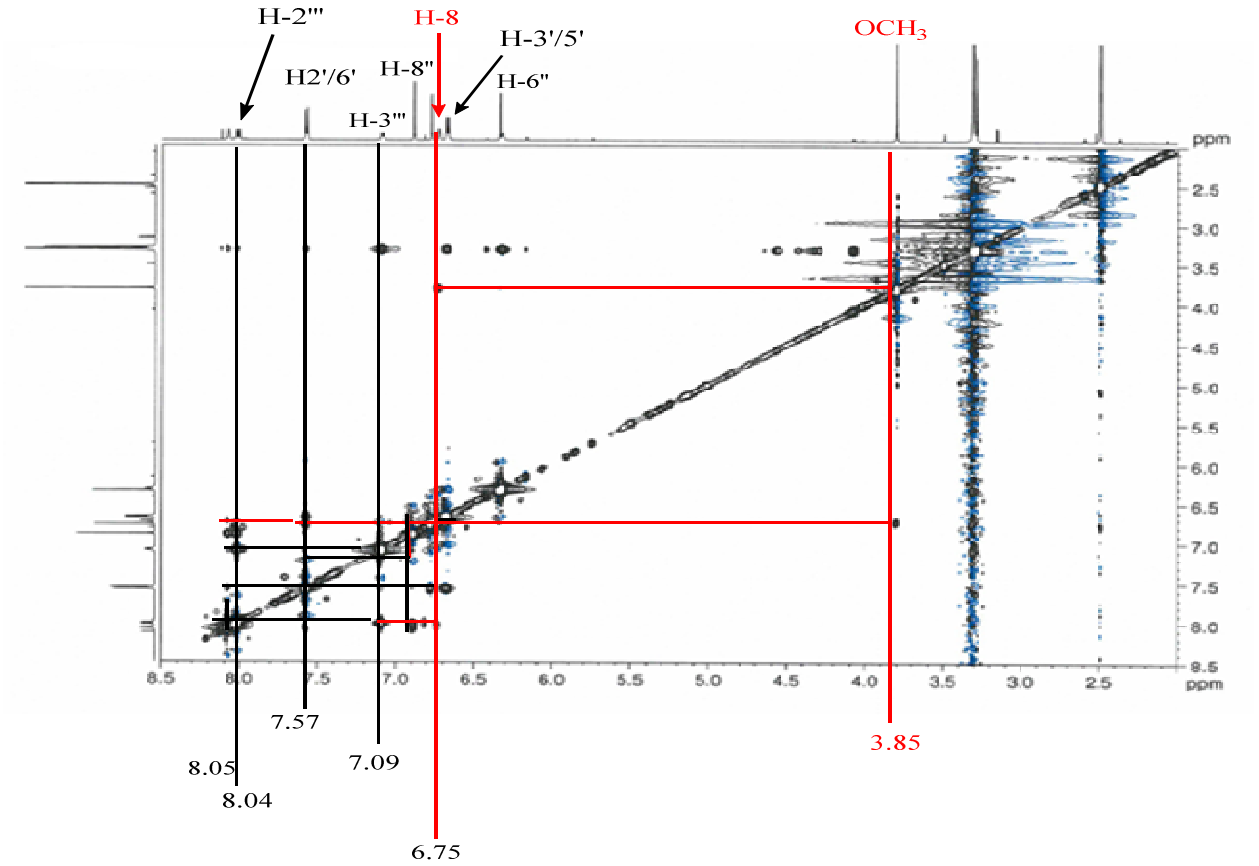


Figure 32: NOESY Spectrum of GBr-4

Comparing the structures of **154** and **155,** it is possible that **154** may have been derived naturally from **155** by cleavage of one of the aromatic ring B'.

III.2.5. Identification of GBr-5

GBr-5 was obtained as a cream white powder soluble in methanol that gave a dark blue coloration with Molish reagent for sugars. Its negative mode HR-TOF-MS mass spectrum showed the quasi molecular ion pic $[M-H]^-$ at *m/z* 496.2701 suggesting the molecular formula $C_{26}H_{38}O_9$ with 8 degrees of insaturations.

Its IR spectrum shows the presence of a hydroxyl group (3396cm-1), of an ester γ-lactone- α, β-saturated with five atoms (1758 and 1729cm-1), of a double bond (1641cm-1) and a furanic ring (1502 and 874cm^{-1}).

The 2D ^1H NMR **(Figure 35)** spectrum helped us to individualize the peaks of this compound. An AB system of two highly deshielded olefinic protons at 5.25 ppm (H-11) and 5.26ppm (H-12) with a coupling constant *J-trans* 15.2 Hz. An AX system of two protons with a *trans* coupling at 4.58 (H-16, d, *J* = 8Hz) and 3.18 (H-15, d, *J* =7.5Hz). An AX_2 system of three protons at δ_H 1.82, 1.91 (H-14) and 1.48 (H-8). Three methyl group at 0.66 (s), 0.77 (s) and the last one which is a secondary methyl at 0.99 (CH_3-18, d, *J*=6.0 Hz). We also noticed the presence of an anomeric protons at 4.25 (H-1, d, *J* = 7.5 Hz). H-6' of glucose appeared at 3.95 (*J* =12.0 and 3.0 Hz) and 3.52 (*J* =12.0 and 5.00 Hz). The remaining sugar protons appeared at δ_H 2.98 (H-2'), 3.12 (H-3') and 3.24 (H-5')

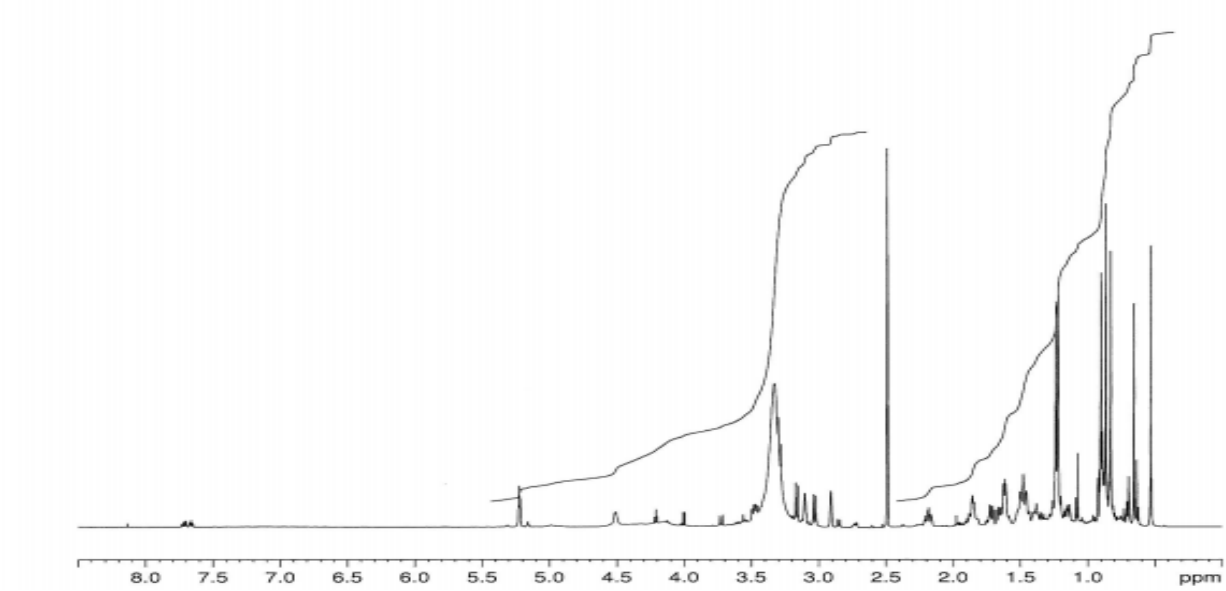


Figure 33: 1H NMR Spectrum of GBr-5 (600MHz, DMSO-d6)

Its DEPT spectrum **(Figure 34)** permitted us to identify a total of 26 carbon signals among which 6 are attributable to a terminal glucopyranoside. Thus compound **156** is a terpenic derivative where by the aglycone possesses 20 carbon atoms which are: three

methyls (δ_C 11.9, 40.1, 12.0 and 20.9), six methylenes (δ_C 34.9, 41.0, 55.5, 75.5, 95.2, 128.6 and 143.5) and four quaternary carbons (δ_C 41.2, 44.7, 63.8 and 179.2) including an ester (δ_C 179.2). This preceeding analysis is confirmed by the ^{1}H NMR spectrum **(Figure 33)** and COSY **(Figure 35)** that permitted us to bring out the structure of GBr-5.

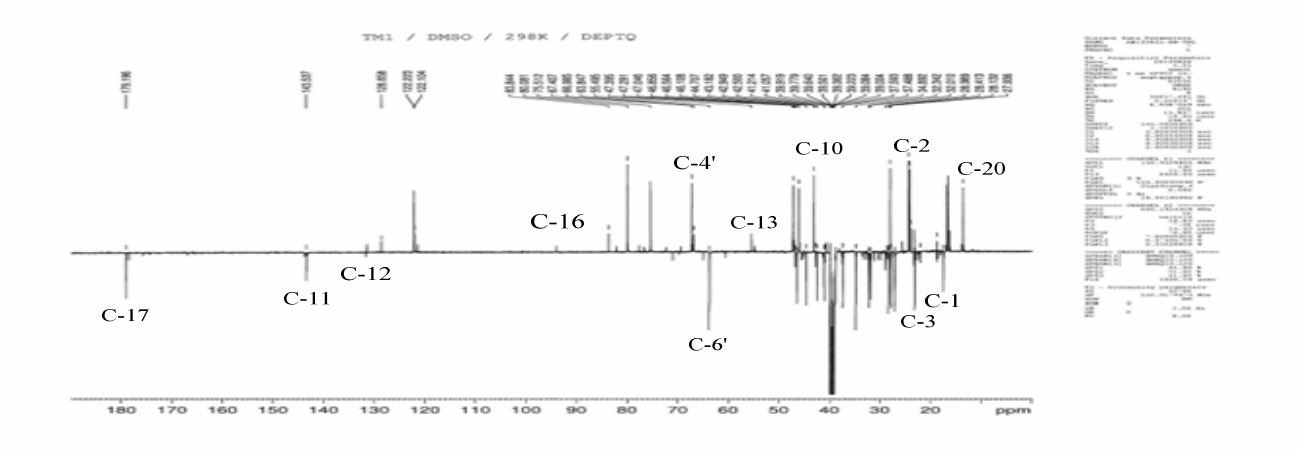


Figure 34: DEPT Spectrum of GBr-5

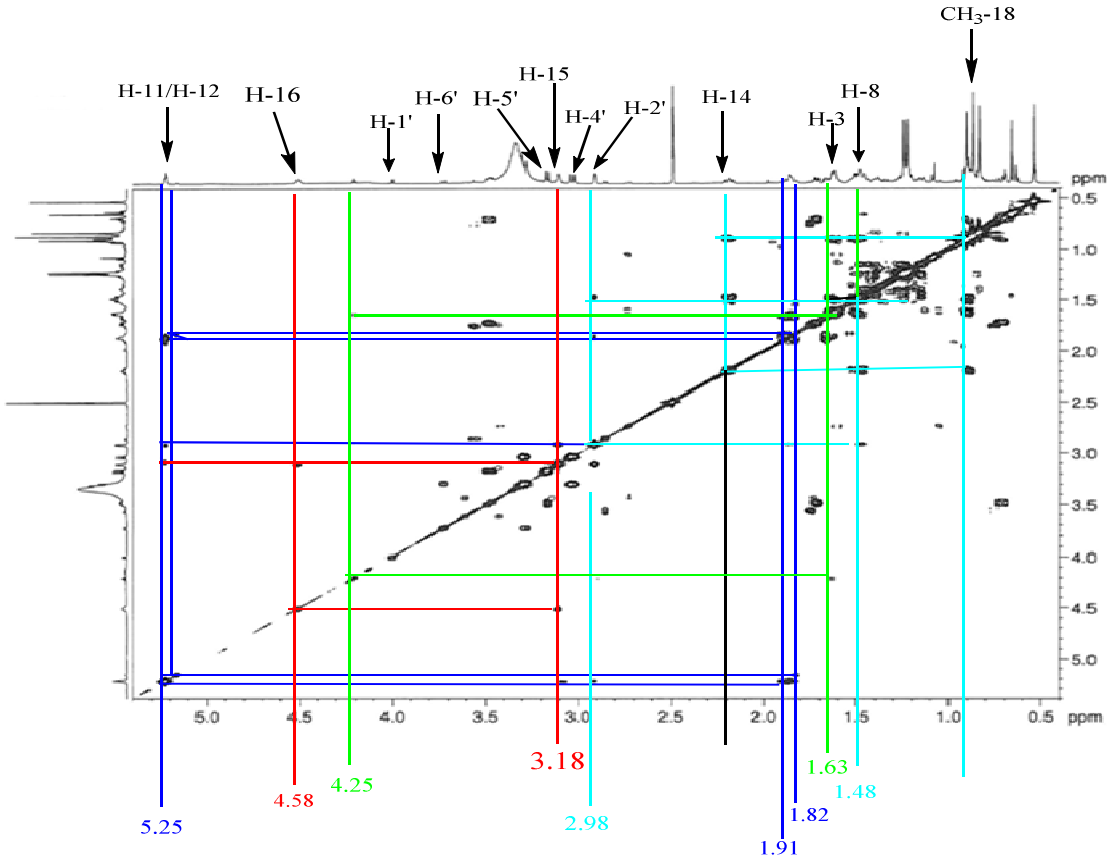


Figure 35: 1H-1H COSY spectrum of GBr-5

Moreover, the HMBC spectrum of GBr-5 **(figure 36)** has permitted us to confirm the structure of GBr-5. In fact, we noticed pro eminent correlations between the anomeric proton

at (4.25 ppm) and the oxygenated carbon C-15 (71.6 ppm) demonstrating that the glucosidic bond is on this carbon (belongs to the furanic ring) **(Agrawal., 1992).**

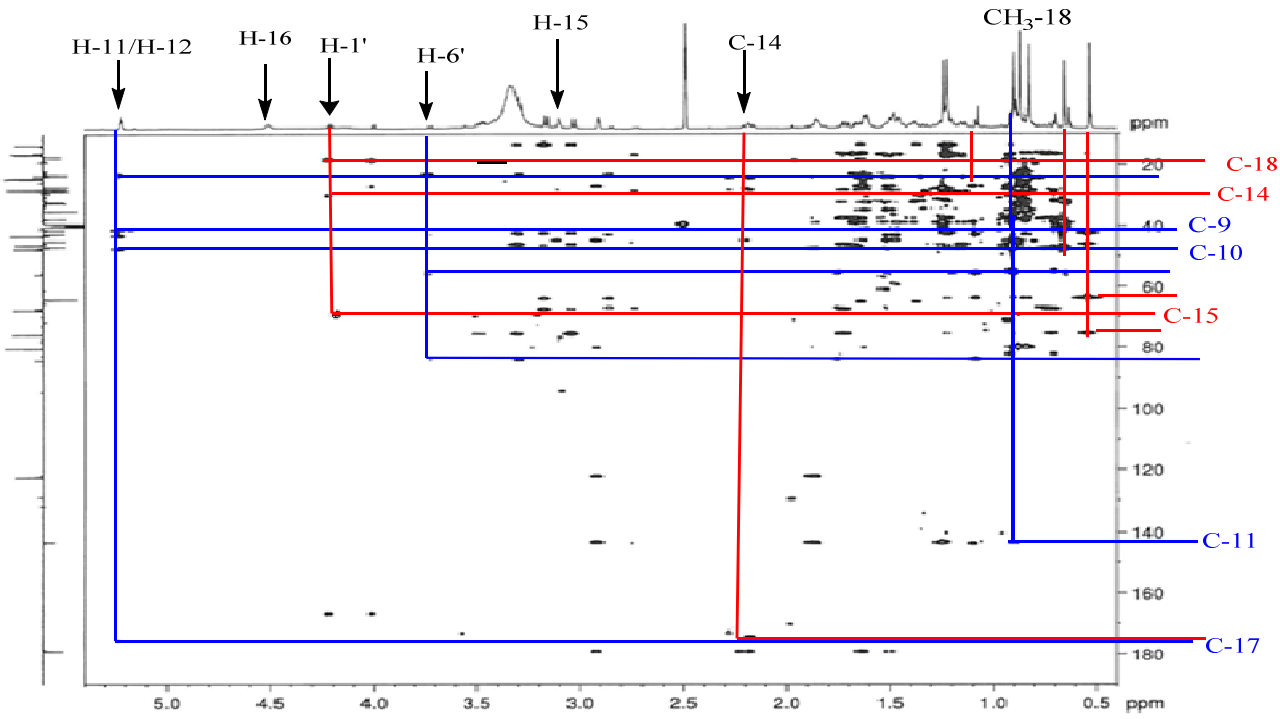
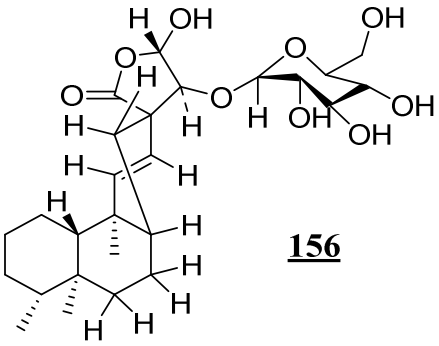


Figure 36: HMBC Spectrum of GBr-5

We equally observed correlations between methylenic protons H-14 (δ_H 2.24) and C-17 (δ_C 179.2) thus demonstrating that the furanic ring is attached to C-13(δc 54.6), between the methyl (δ_H 0.99) and the carbons C-4, (δ_C 40.1), C-5 (δ_C 44.2) and C-11(δ_C 141.6), between the methyl (0.66) and the carbons C-4 (δ_C 40.1), C-5 (δ_C 44.2) and C-10 (δ_C 53.6), the methyl at (δ_H 0.77) and the carbons C-8 (δ_C 34.9), C-9(δ_C 41.9) and C-10 (δ_C 53.6), between the methylenic protons (δ_C 1.24) and the carbons C-6 (δ_C 28.3) and C-14 (δ_C 33.3).

From the analysis of all the spectral data of GBr-5, we can deduce for this compound the formular $C_{26}H_{40}O_9$ corresponding to a calculated mass relatively equal to 495.3064 which is not the molecular ion peak if not it isomer will have an increament of two hydrogens. This compound is elucidated as 15-O-β-D-glucopyranosyl-(13S, 15S, 16S)-16-hydroxy-neo-clerod-11-(E)-en-17, 16-olide, earlier described by **(Ngono Bikobo et al., 2011)** and was named brevipedifloside A **(156).**



156

Table 16: 1H-NMR and 13C-NMR (600 MHz) data of Compound GBr-5 in DMSO-d6 (δ, ppm).

Brevipedifloside A					
Carbon Nº	δ_C (ppm)	m	δ_H ppm	m	J(Hz)
1	21.5	t	1.27	m	
2	23.7	t	1.38	m	
3	24.6	t	1.65	m	
4	40.1	d	1.98	m	
5	44.2	-	-		
6	28.6	t	1.24, 1.75	m	
7	31.2	t	1.26, 1.80	m	
8	34.9	d	1.48	m	
9	41.9	s	-		
10	44.5	d	1.38	m	
11	141.6	d	5.25	d	15.2
12	134.6	d	5.25	d	15.2
13	54.6	s	-		
14	33.3	t	2.24, 1.91	m	
15	71.6	d	3.18	d	7.5
16	98.2	d	4.58	d	8.0
17	172.3	s	-		
18	20.9	q	0.99	d	6.0
19	11.9	q	0.66	s	
20	12.0	q	0.77	s	
1'	100.7	d	4.25	d	7.5
2'	72.6	d	2.98	m	
3'	74.4	d	3.12	m	
4'	71.5	d	3.15	m	
5'	76.6	d	3.20	m	
6'	65.5	t	3.61, 3.43	ench	12.0, 3.0 and 5.0

III.2.6. Identification of GBr-6

GBr-6 was obtained as an amorphous cream white powder and attributed the molecular formula $C_{15}H_{10}O_5$ from the analysis of its high resolution mass spectrum **(Figure 37)** in which the $[M+H]^+$ peak appeared at m/z 271. 0597 (calculated for $C_{15}H_{11}O_5$: 271.2405). This shows that GBr-6 has the molecular mass of 270 and 11 unsaturation sites.

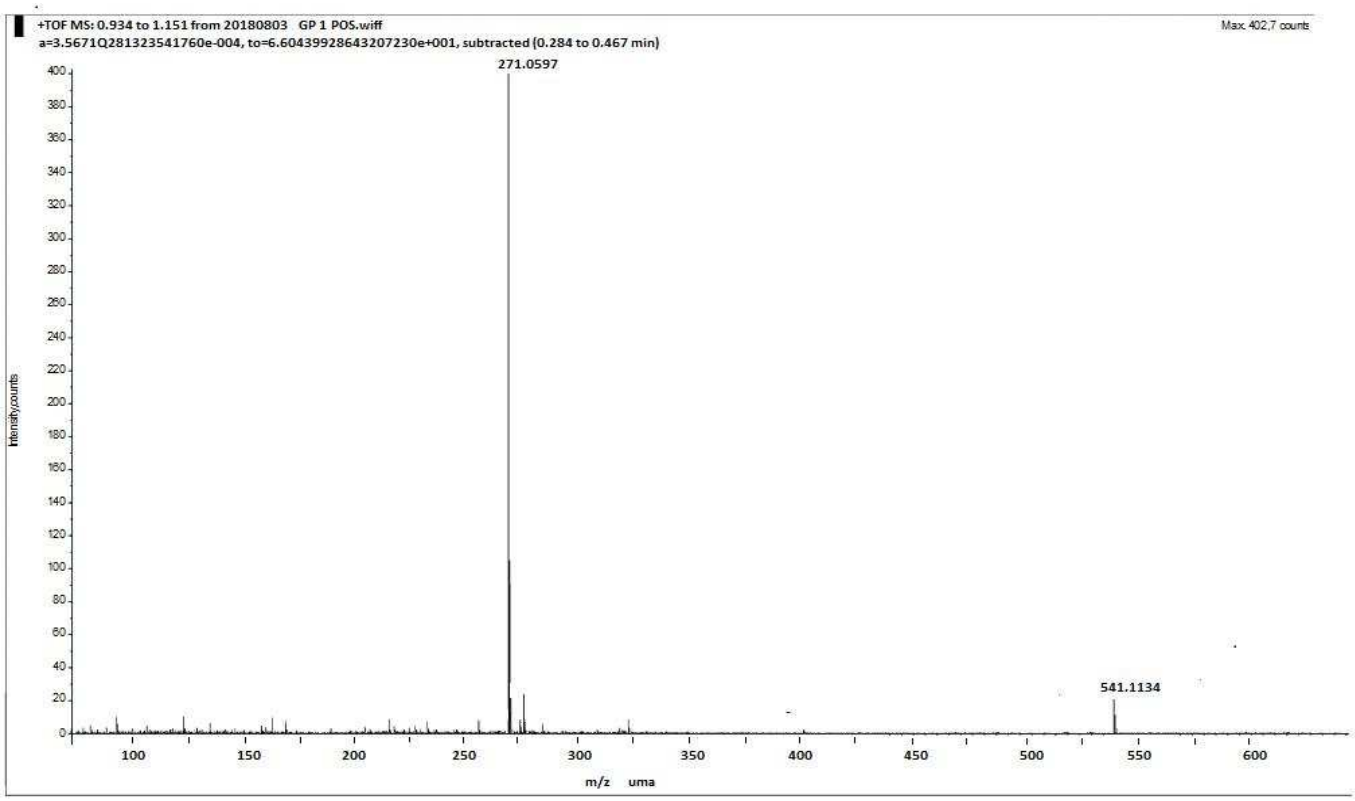


Figure 37: Mass spectrum of GBr- 6

Its IR absorption spectrum shows absorption bands at 3424 cm^{-1} (phenol groups), 1638 cm^{-1} (conjugated and chelated carbonyl) and at 1605 and 1498cm^{-1} (aromatic rings). (**Maciej Heneczkowski et *al.*, 2001).**

The analysis of the ^1H NMR spectrum of GBr-6 **(Figure 38)** led to the identification of the following proton systems:

- A proton singlet signal at 6.78 ppm (1H, s, H-3) characteristic of proton H-3 (ring C) of the chromone moiety in flavones.
- Two separate doublet signals of two protons each at 6.90 ppm (2H, d, *J* = 8.8Hz, H-3' and H-5') and 7.96 ppm (2H, d, *J* = 8.8Hz, H-2' and H-6') assigned to protons of a *para*di-substituted aromatic ring B.

- Two *meta* coupled protons of the tetra substituted aromatic ring A, [6.17 ppm (1H, d, 2.1Hz, H-6) and at 6.42 ppm (1H, d, 2.1Hz, H-8).
- A very deshielded singlet proton signal at 11.78 ppm (1H, s, OH-5), characteristic of a phenol proton strongly chelated to a *peri* carbonyl group in ring C of flavones.

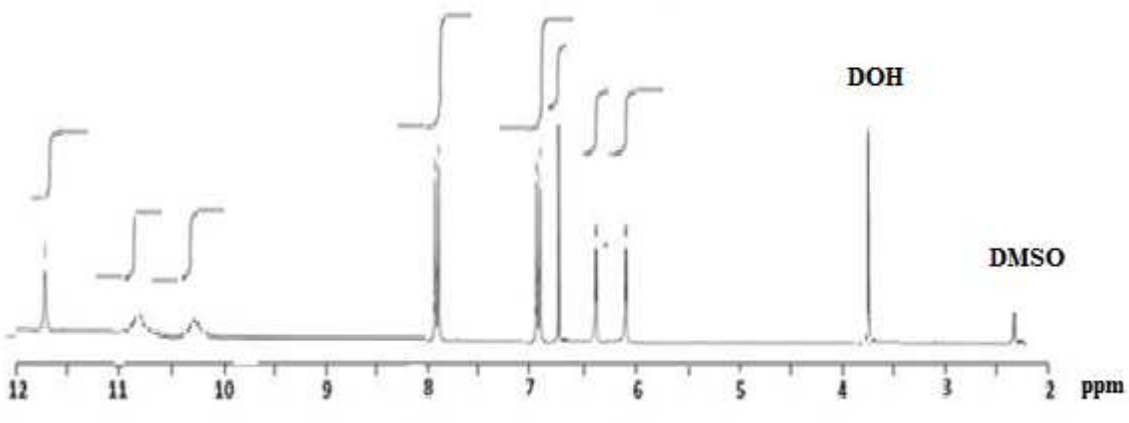
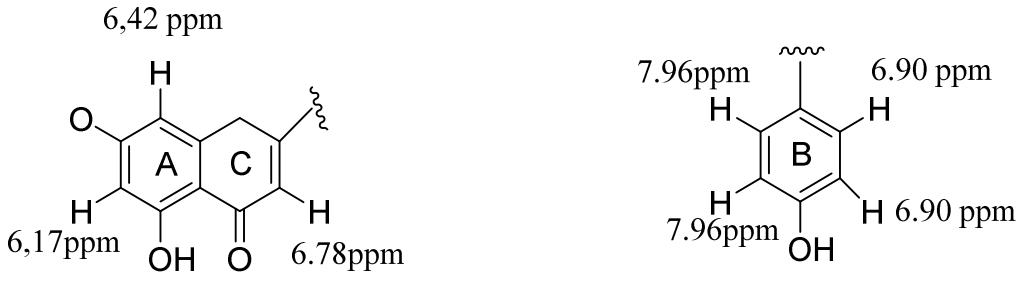


Figure 38: 1H NMR spectrum of GBr-6

The COSY spectrum of GBr-6 **(figure 40)** showed correlations confirming the presence of four protons on the *para* di-substitited aromatic ring B at 6.90 ppm (2H, d, *J* = 8.8 Hz, H-3' and H-5') and at 7.96 ppm (2H, d, *J* = 8.8 Hz, H-2' and H-6'). Also those of the two *meta* protons on the tetra substituted aromatic ring A were observed at 6,17 ppm (1H, d, *J* = 2.1Hz, H-6) and at 6,42 ppm (1H, d, *J* = 2.1Hz, H-8). The above information suggests the implication of two structural units, sub-structures I and II, **(Figure 39)** in the structure of GBr-6.



Sub structure I **Sub structure II**

Figure 39: Sub-structures found in the structure of GBr-6

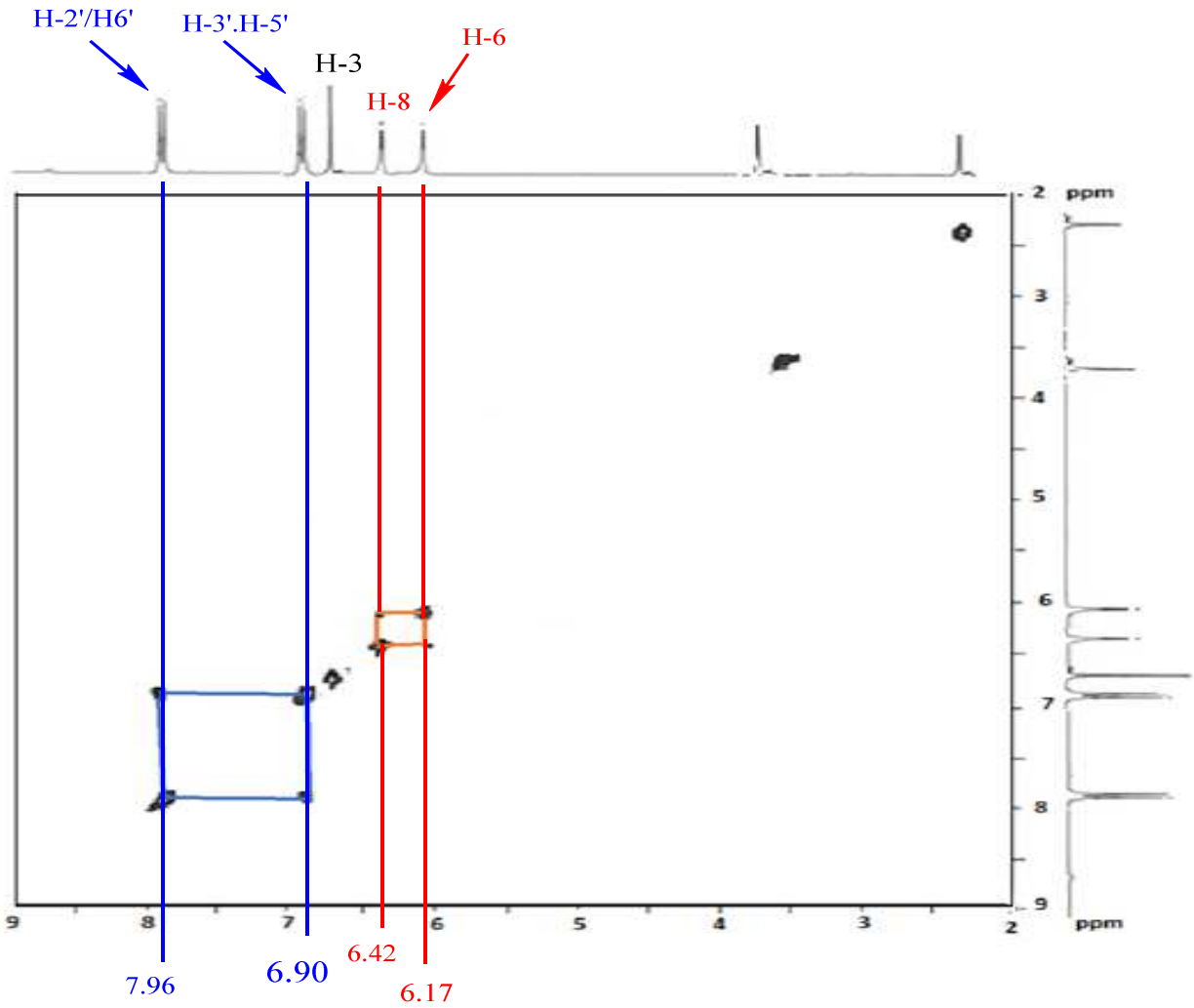


Figure 40: COSY Spectrum of GBr-6

The chemical shifts of all the protonated carbon atoms in the formula of the compound GBr-6 were attributed from it HSQC spectrum **(Figure 41).**

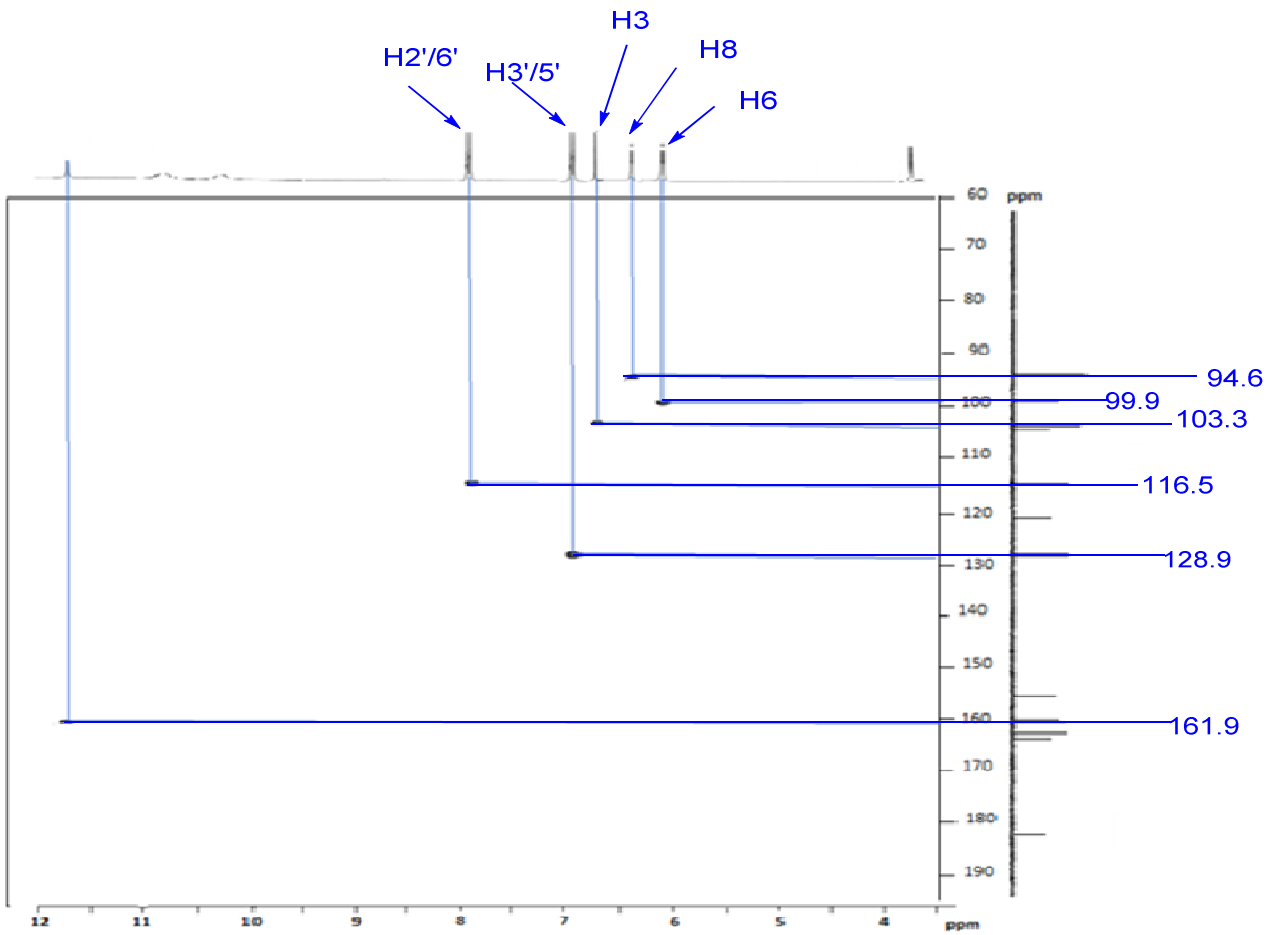


Figure 41: HSQC Spectrum of GBr-6

The analysis of its ^{13}C NMR spectrum **(figure 42)** shows the presence of signals of 13 sp^2 hybridized carbon atom, with two signals having double intensity and assigned to the carbons of the *para* di substituted benzene ring in which is a symmetric structure of this ring.

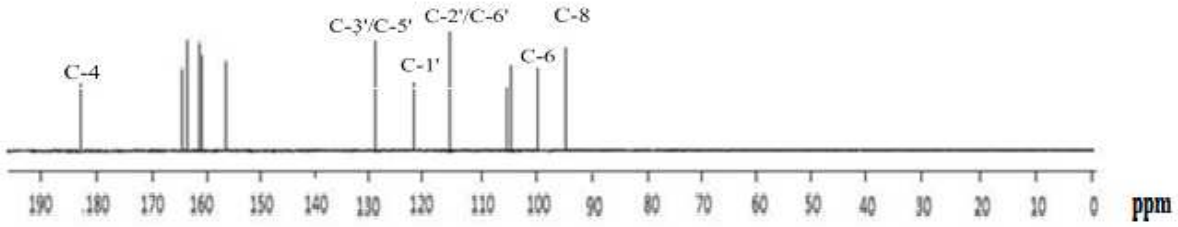


Figure 42: 13C NMRspectrum of GBr-6

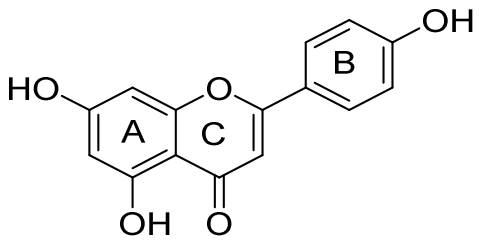
Five signals were identified to be those of methines carbons. They appeared at 128.9 ppm (d, C-3'/C-5'), 116.5 ppm (d, C-2'/C-6'), 103.3 ppm (d, C-3), 99.9 ppm (d, C-6) and 94.6 ppm (d, C-8). Also, signals of twelve quaternary carbons, five of which are

connected to oxygen atoms were displayed at 164.2 ppm (s, C-2), 163.3 ppm (s, C-7), 161.9 ppm (s, C-5), 161.8 ppm (s, C-4') and 157.8 ppm (s, C-9).

Table 17: GBr-6 NMR Data (DMSO-d6): ^{1}H (400MHz) and ^{13}C (100MHz).

Carbon N°	GBr-6			Apigenine (Chaturvedula et al., 2013)	
	δ_C ppm	type	δ_H (ppm), J (Hz)	δ_C ppm	δ_H (ppm)
2	164.2	C		163.9	
3	103.3	CH	6.78(1H, s)	103.1	6.77(1H, s)
4	182.2	C		181.9	
5	161.9	C	11.78 (1H, s, OH)	161.7	12.99 (1H, s; OH)
6	99.9	CH	6.17(1H, d)	99.1	6.22(1H, d,2.1)
7	163.3	C		164.3	
8	94.6	CH	6.42(1H, d)	94.2	6.50(1H, d,2,1)
9	157.8	C		157.5	
10	105.8	C		103.9	
1'	121.6	C		121.4	
2'/6'	116.5	CH	7.96(1H, d, 8.8)	128.6	7.92(1H, d,8.8)
3'/5'	128.9	CH	6.90(1H, d, 8.8)	116.2	6.95(1H, d,8.8)
4'	161.8	C		161.4	

All these analysis data suggest that GBr-6 is a flavonoid. The comparison of the obtained information with those of known flavonoids described led to the identification of GBr-6- as 5,7,4'-trihydroxy-2-phenylchromen-4-one or apigenine **157**.



157

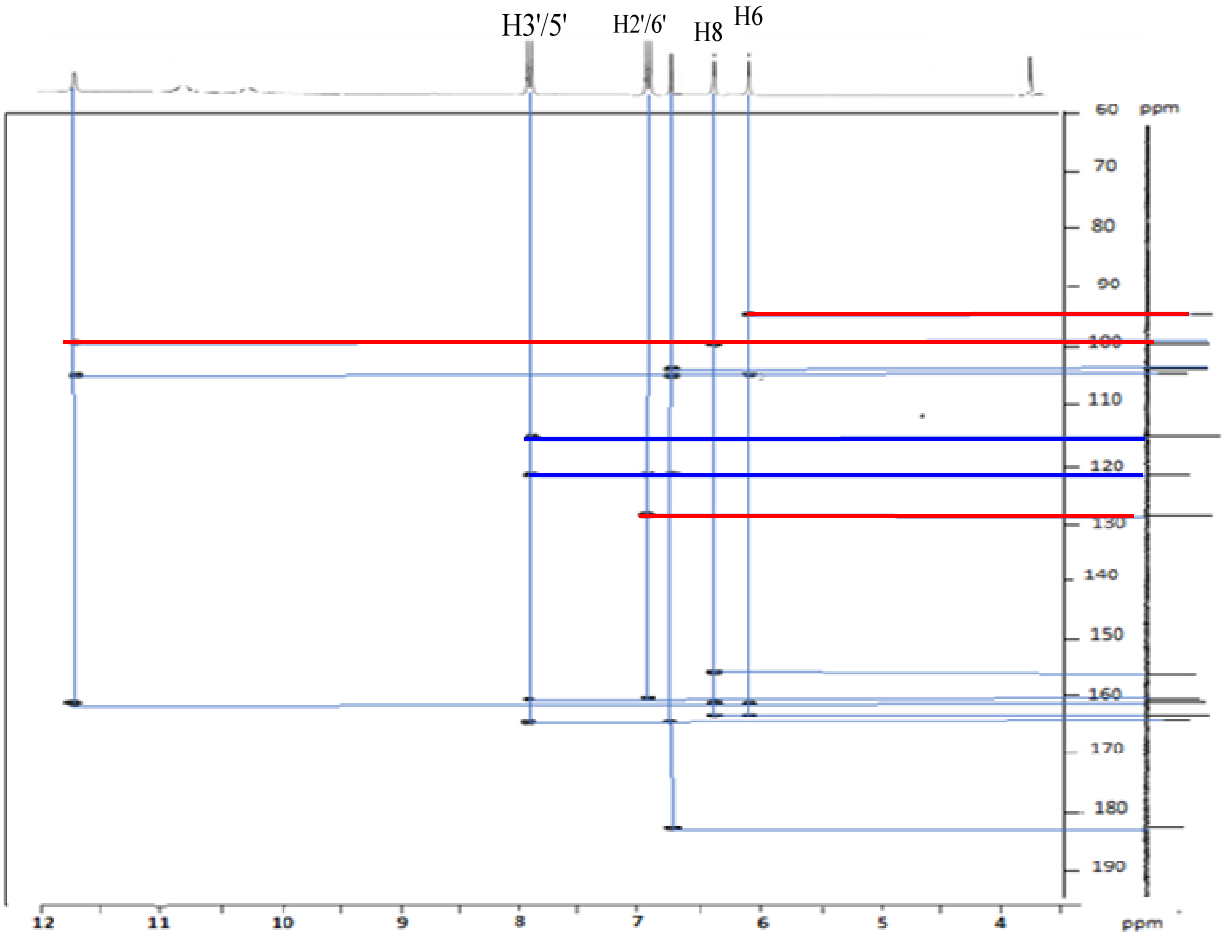


Figure 43: HMBC spectrum of GBr-6

III.2.7. Identification of GBr-7

GBr-7 was obtained as an amorphous berge powder that gives a positive phenol test. It was assigned the molecular formula $C_{16}H_{12}O_6$ from its high resolution mass spectrum **(Figure 44)** as the pseudomolecular ion $[M+H]^+$ was observed at m/z 301.1410 suggesting the molecular formula $C_{16}H_{13}O_6$ for GBr-7.

Its infra-red absorption spectrum was very close to that of 2'-hydroxygenistein **25' (Wantanabe and kinjo, 1993)** as it dsplayed absorption bands at (3424 cm^{-1}) for hydroxyl groups, conjugated and chelated carbonyl (1638 cm^{-1}) and an aromatic ring at (1600 at 1498 cm^{-1}).

Its UV spectrum had two absorption maxima at λ_{max} 263 and 332 nm suggesting an isoflavone structure **(Harborne 1973)**

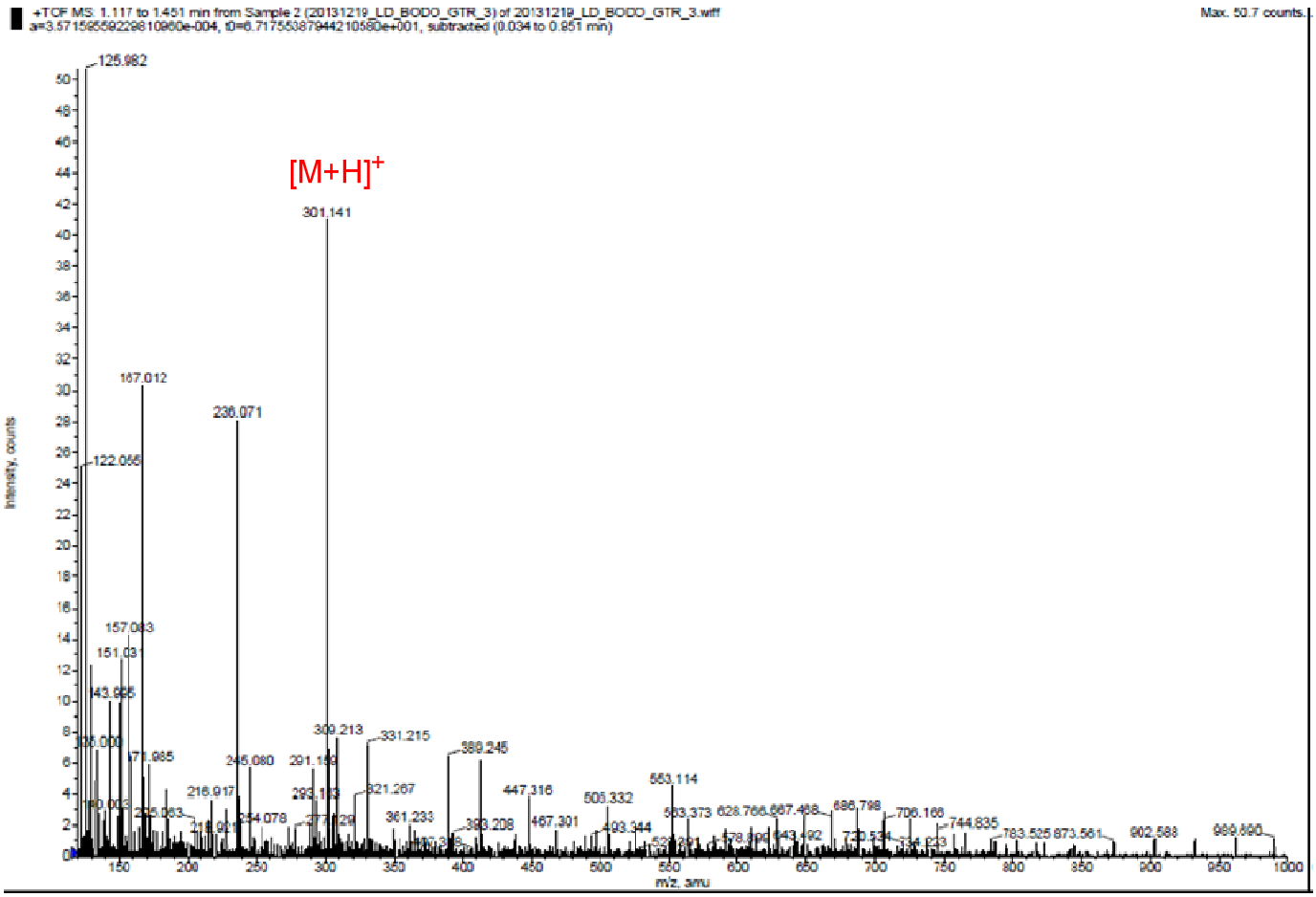


Figure spectrum of GBr-744: Mass

Its ^{1}H NMR spectrum **(figure 43)** had a high deshielded signal at 8.30 ppm (1H, s, H-2) confirms that it is an isoflavone motif. The values of other signals observed are very close to those reported in hydroxy genistein. They include:

- Two doublet signals, each counting for one proton, which are *meta* protons on the tetra substituted benzene ring A [δ_H 5.92 (1H, d, 2.1Hz, H-6) and δ_H 6.62 (1H, d, 2.1Hz, H-8)].
- A singlet signal at 13.23 ppm assigned to a phenol group chelated to *per*i carbonyl group appearing ppm (1H, s, OH-5).
- A three protons system of a trisubstituted benzene ring (Ring B) giving signals respectively at 6.81 ppm (1H, d, 2.2Hz, H-3'), 7.65 ppm (1H, dd, 2.2 and 9.0 Hz H-5') and at 7.85 ppm (1H, d, 9.0Hz, H-6').
- A three proton singlet at 3.69 ppm assigned to methoxy substituent.

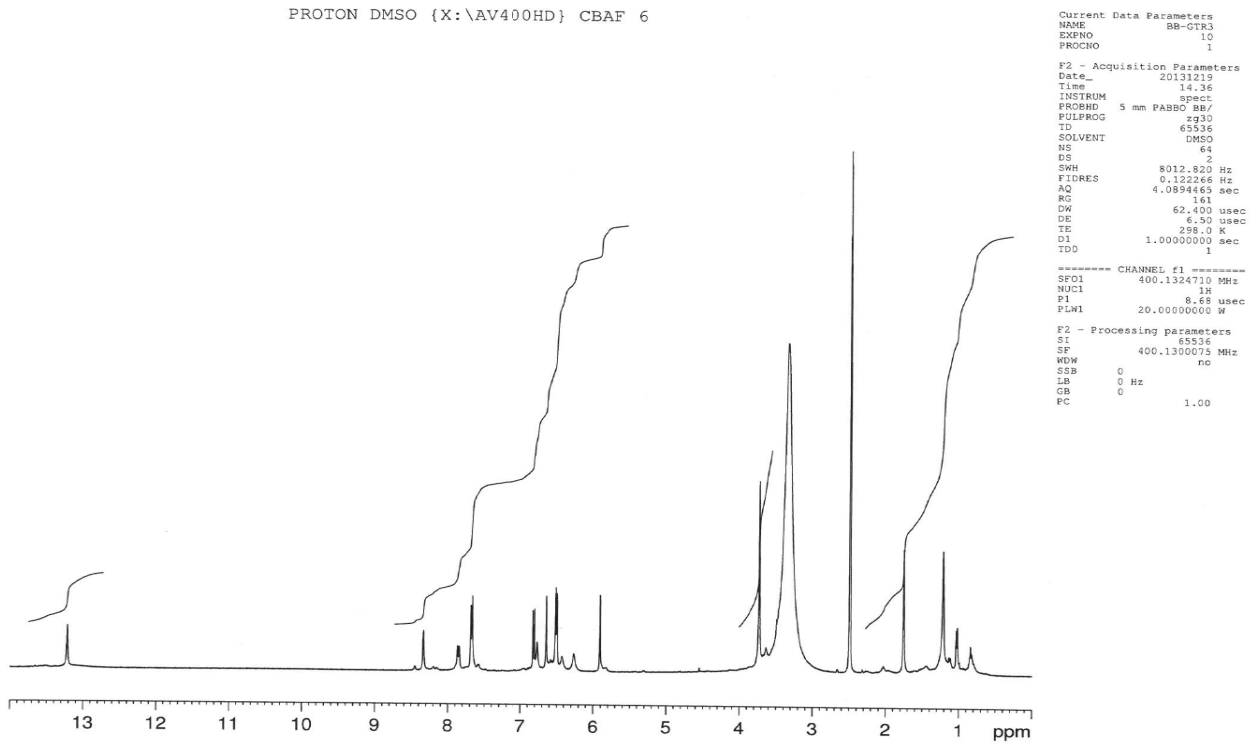


Figure 45: 1H NMR Spectrum of GBr-7

The ^{13}C NMR (**figure 46)** spectrum had signals for all the sixteen carbon atoms in the molecule among which only one was sp3 hybridised (3.69 ppm, CH_3O) and the remaining fifteen are sp^2 hybridised. These include nine quaternary carbons among with five (161.3, 163.6, 158.9, 156.6 (x2) and 158.9 ppm) connected to oxygene atomS and six methine (154.8, 99.3, 94.1, 102.6, 106.4 and 130.6 ppm) carbon atoms and one carbonyl (δ_C 181.2).

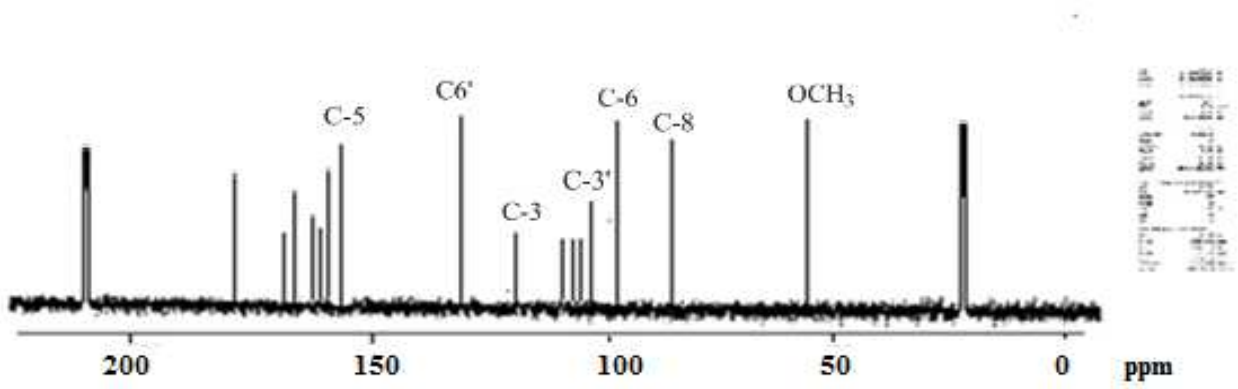


Figure 46: ^{13}C NMR Spectrum of GBr-7

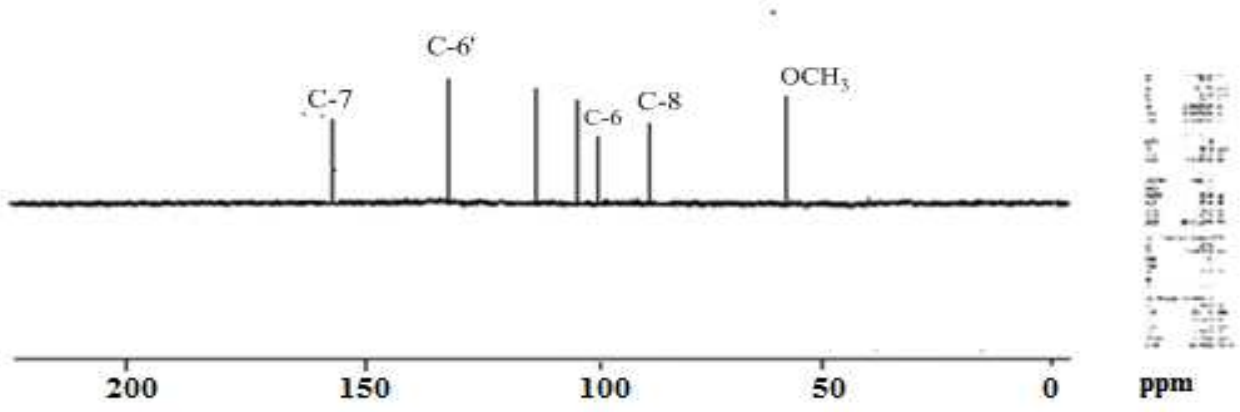


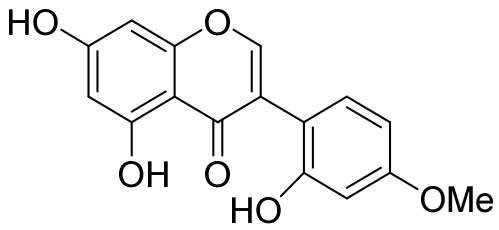
Figure 47: DEPT Spectrum of GBr-7

Table 18: 1H NMR and 13C-NMR (600 MHz) data of Compound GBr-7 in DMSO-d_6 (δ, ppm)

Carbon N°	GBr-7			2´-hydroxygenistein
	δc	Type of C	δ_H	δc
2	154.4	CH	8.30 (1H, s)	155.6
3	121.1	C	-	120.8
4	181.2	C	-	180.9
5	161.3	C	13.23 (1H, s)	162.0
6	99.4	CH	5.92 (1H, d, 2,1)	99.1
7	163.6	C	-	163.8
8	94.2	CH	6.62 (1H, d, 2,1)	93.9
9	156.6	C	-	157.6
10	104.0	C	-	103.9
1´	108.6	C	-	108.6
2´	156.6	C	-	156.1
3´	102.4	CH	6.81 (1H, d, 2,2)	102.8
4´	158.9	C	-	159.4
5´	106.6	CH	7.65 (1H, dd, 9,0 et 2,2)	106.1
6´	130.4	CH	7.85 (1H, d, 9,0)	131.9
OCH_3-4'	56.6	CH_3	3.69 (3H, s)	-

These values are characteristics of the pyron part in the ^{13}C NMR spectrum of isoflavones **(Agrawal, 1990)**. The carbon signals of C-5, C-7, C-2' C-4'which each carry an oxygen atom appeared at 161.4, 163.8, 156.4 and 158.8 ppm respectively. These values are very close to those of 2'-hydroxygenistein and confirm that these two compounds have the same carbon skeleton.

The position of the CH_3O substituent in the molecule was obtained from NOESY spectrum which showed important correlations spots between the CH_3O substituent at 3.69 ppm and the two ring B protons, H-3' (6.81 ppm) and H-5' (7.65 ppm) in the spectrum of GBr-7, These values placed the CH_3O group on C-4' (ring B) and suggested the structure **164** which is that of 2'-hydroxy-4'-*O*- methylgenistein



158

The only difference between the structure of the two compounds is the presence of the CH_3O function in **158** which is absent in 2'-hydroxygenistein. This suggests that one of the phenolic functions in 2'-hydroxygenistein was naturally methylated to have **158**.

III.2.8. Identification of GBr-8

GBr-8 was obtained as a yellow amorphous powder that showed the pseudo molecular ion peak $[M+H]^+$ at m/z 539.0981, which corresponds to the formula $C_{30}H_{19}O_{10}$ accounting for 22 unsaturated sites. Gbr-8 is a flavonoid since it gives a brick red coloration when treated with Mg and concentrated hydrochloric acid and a dark blue coloration in the presence of $FeCl_3$ solution. Considering its high molecular mass, GBr-8 should be a flavonoid dimer or biflavonoid.

In its IR absorption spectrum, we could identify an intense band at (3211 $cm^{-1)}$ (hydroxyl group), a conjugated carbonyl at (1646cm^{-1}), aromatic rings at (1601 and 1493) $cm^{-3}$and conjugated double bonds (1626 cm^{-1}).

Its UV absorption spectrum had two absorption maxima at λ_{max} 223 and 320 nm characteristic of a flavone motif, suggesting the presence of flavone unite in it structure.

It ^{1}H NMR spectra 1D **(figure 48)** and 2D COSY **(figure 49)** permitted us to identify the following proton systems in the structure of GBr-8.

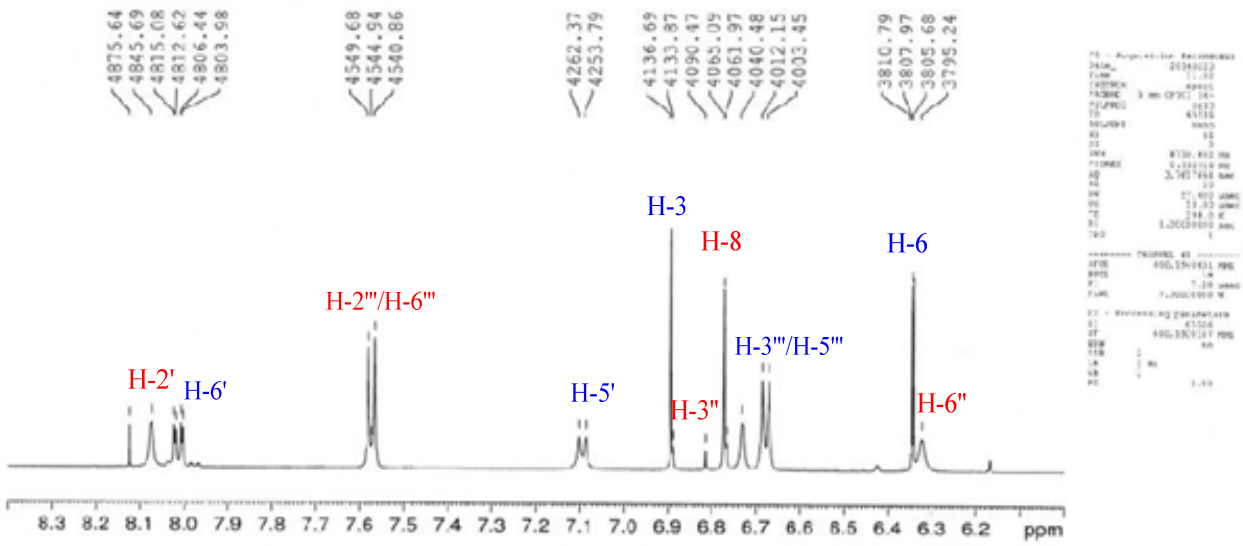


Figure 48: 1H NMR spectrum of GBr-8

Two singlets proton signals found respectively at 6.89 ppm (1H, s, H-3) and at 6.77 ppm (1H, s, H-3'') were assigned to the H-3 proton of two distinct flavone units.

- Three protons on the trisubstituted benzene ring B, gave signals at 7.98 ppm (1H, dd, 7.4 and1, 6 Hz, H-6'), 8.07 ppm (1H, d, 1.6 Hz, H-2') and at 7.10 ppm (1H, d, 7.4 Hz, H-5').
- A proton singlet at 6.32 ppm was that of the residual proton on the penta substituted aromatic ring A'.
- Two *meta* coupling protons on the aromatic ring A gave signals at 6.73 ppm (1H, d, 2.1 Hz, H-8) and at 6.34 ppm (1H, d. 2,1 Hz, H-6),
- Four protons on the *para* di-sustituted aromatic ring (ring B') gave signals at 7.57 ppm (2H, d, 8.2 Hz, H-2'''/6''') and 6.68 ppm (2H, d, 8.2 Hz, H-3'''/5''').
- Lastly, two highly deshielded proton singlets signals were observed at 12.98 ppm (1H, s, OH-5, ring A) and at 13.10 ppm (1H, s, OH-5'', ring A''), characteristic of phenolic protons chelated to *peri* carbonyl groups.

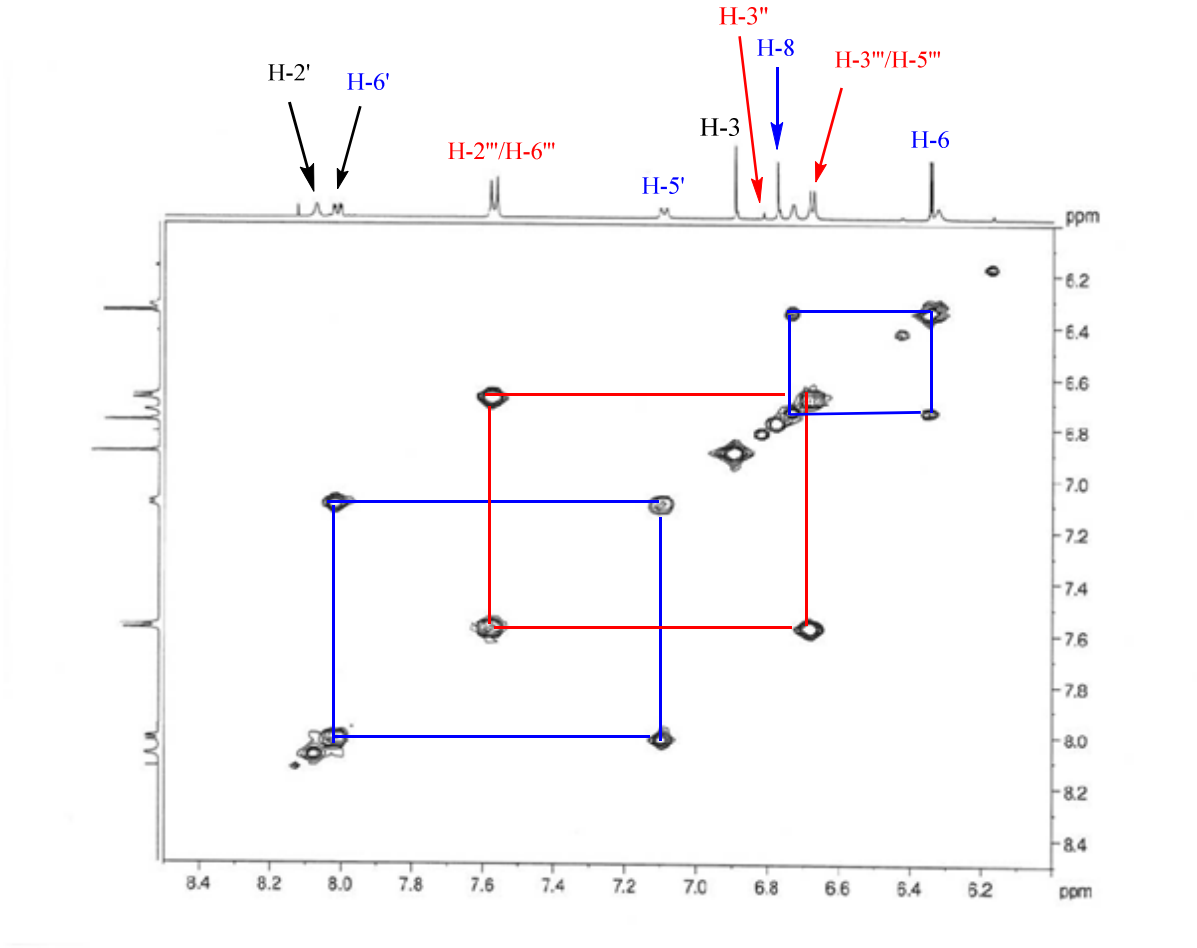


Figure 49: COSY spectrum of GBr-8

This information leads to the definition of two structural flavonoid units: sub-structures I and sub-structure II. In the latter, the ambiguity in assigning the chemical shift to either the proton H-6 or H-8 gives us two structural possibilities sub structure IIa and sub structure IIb **(figure 50).** These details suggest the implication of two flavone sub units obtained from the combination of substructure I with either substructure IIa or IIb to get the structure of GBr-8.

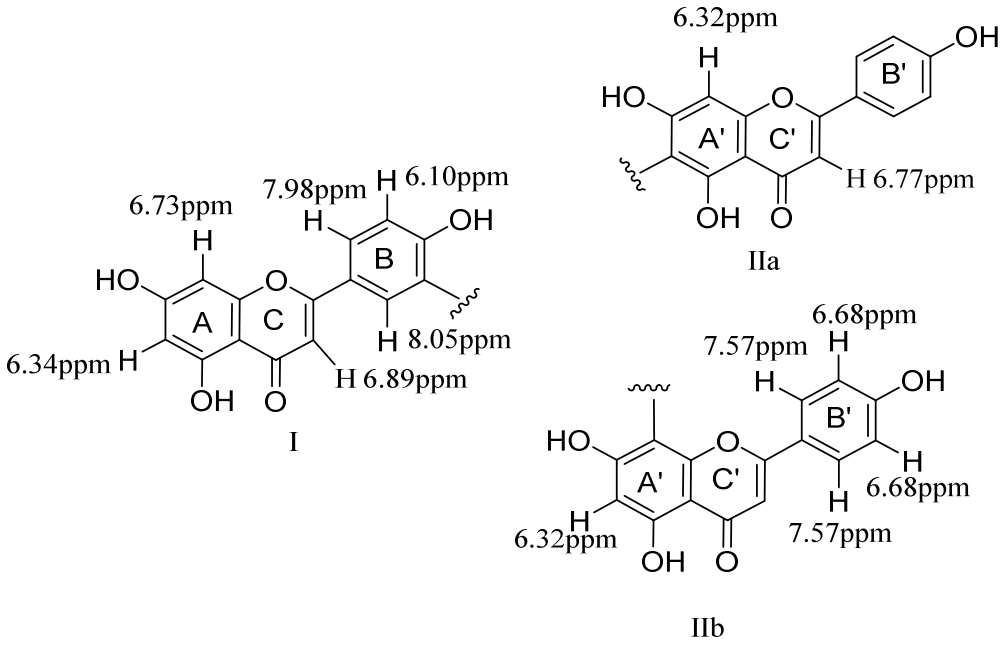


Figure 50: Sub-structures of GBr-8

The DEPTQ spectrum **(figure 51)** of GBr-8 showed that all the 30 carbon atoms found in the molecular formula were sp2 hybridized among which two conjugated and chelated carbonyl carbons at 182.2 and 181.7 ppm. Four other aromatic carbon atoms giving two signals with double intensities at 128.1 and 115.7 ppm were assigned respectively to the carbon atoms C-2'''/C-6''' and C-3'''/5''' of the *para* di-substituted ring B'. Also eight methines carbons gave signals at102.9, 98.0, 92.6, 131.4,116.0 ,127.7,102.5, 97.0 ppm while those of sixteen quartenary carbon atoms, among which ten were substituted by oxygen and appeared at 164.2, 165.1, 160.9, 157.3, 163.5,160.5, 161.6, 160.5, 154.5 and 161.1 ppm.

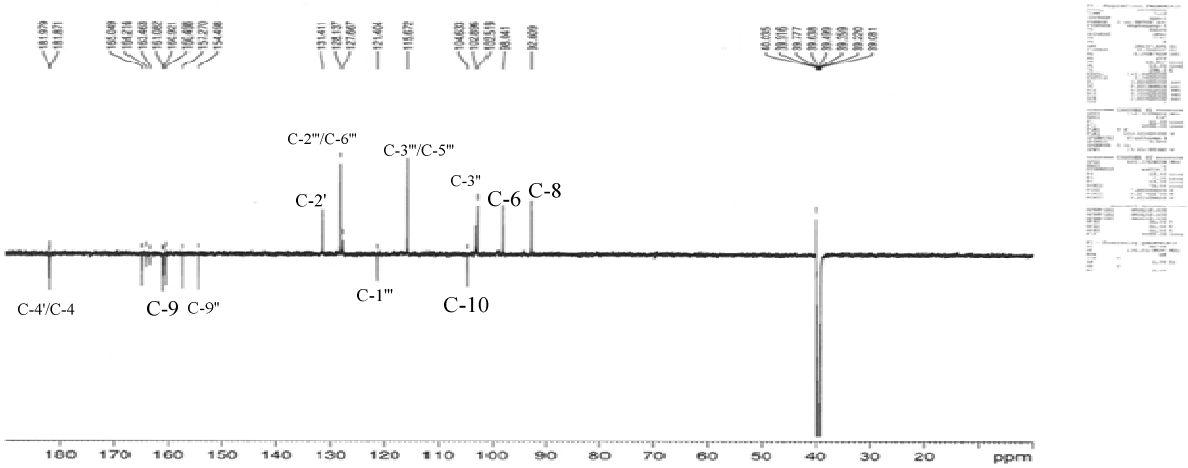


Figure 51: DEPTQ spectrum (100 MHz, DMSO-d6) of GBr-8

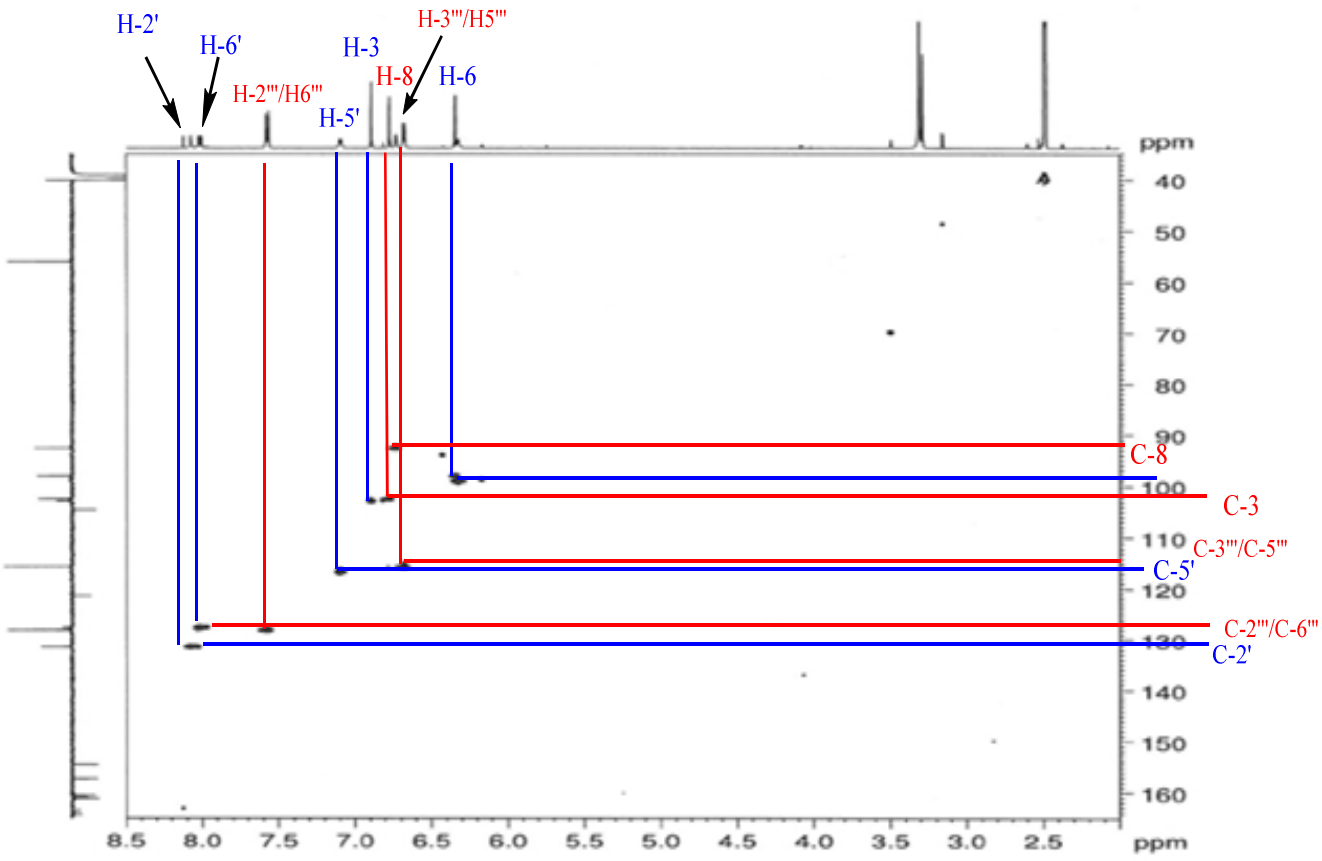


Figure 52: HSQC spectrum of GBr-8

From the connections established on examination of the HMBC spectrum of GBr-8 **(figure 53)** precision was obtained on the location of the interflavonyl bond. The connection between proton H-2' (8.07 ppm, ring B) and carbon atom C-8" (104.4 ppm, ring A') places the interflavonoyl linkage between the carbon atoms C-3' and C-8" thus leading us to structure 4',4''', 5,5'', 7,7''-hexahydroxy-2,2''-bis(4-hydroxyphényl)-3',8''-bis(4H-1-benzopyran)-4,4'-dione **159** or amentoflavone.

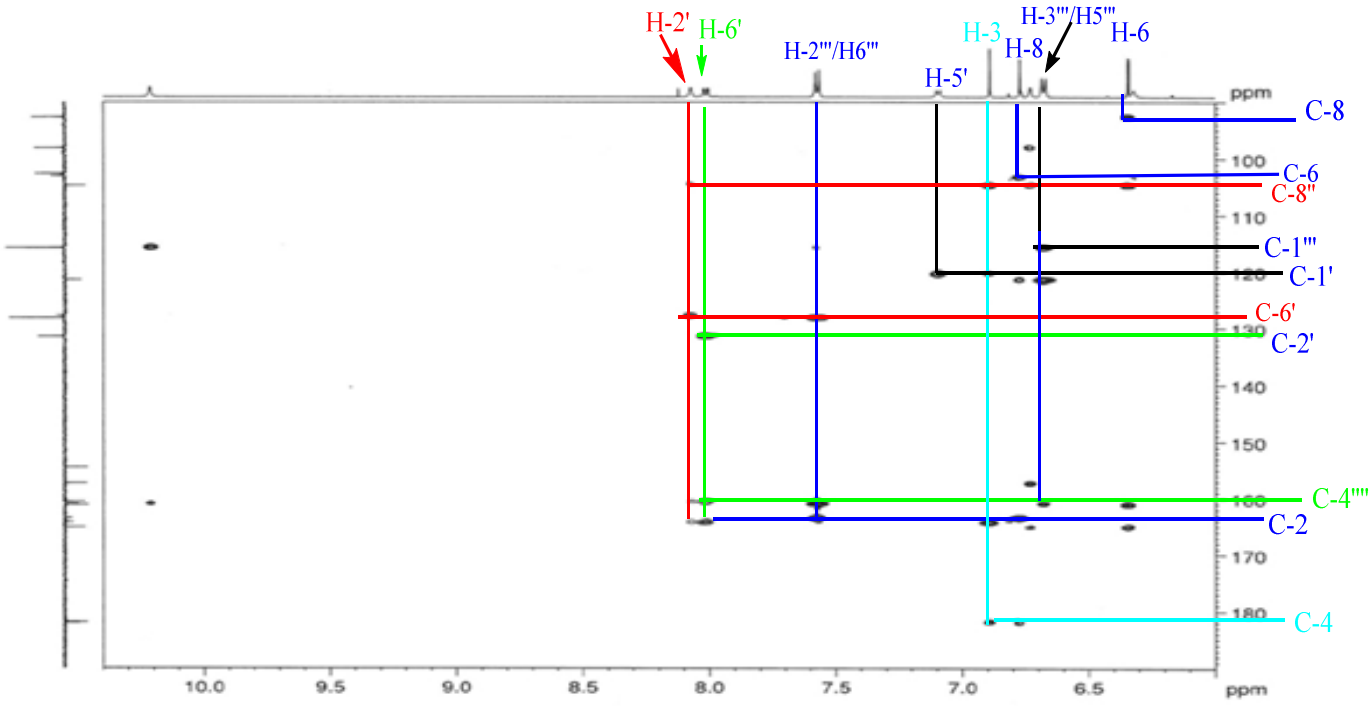
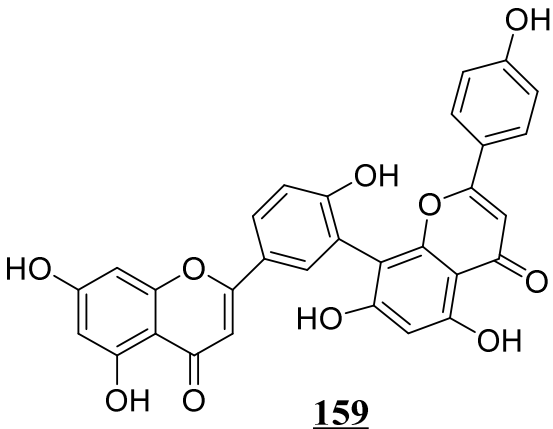


Figure 53: HMBCspectrum of GBr-8

These results were confirmed by comparing the spectroscopic values obtained for GBr-8 with those of an authentic sample of amentoflavone reported from *Campylospermum flavum* (**Ndongo et *al*., 2010)**.



159

Table 19: NMR data[1]H (600 MHz) and [13]C 100 MHz) of Compound GBr-8 (DMSO d6).

Carbon N°	GBr-8			Amentoflavone δC ppm **(Ndongo et *al.*, 2010)**
	δC (ppm)	Type of C	δH (ppm) J (Hz)	
2	164,2	C	-	164,1
3	102,9	CH	6,89 (1H, s)	102,0
4	182,0	C	-	181,7
5	160,5	C	12,98 (1H, s, OH)	161,1
6	98,0	CH	6,34 (1H, d; 2,1)	98,6
7	165,1	C	-	164,1
8	92,6	CH	6,73 (1H, d; 2,1)	93,7
9	157,3	C	-	157,1
10	104,6	C	-	103,4
1’	120,0	C	-	118,3
2’	131,4	CH	8,07 (1H, d ; 1,6)	131,1
3’	121,1	C	-	122,0
4’	160,9	C	-	162,7
5’	116 ,0	CH	7,10 (1H, d ; 7,4)	117,8
6’	127,7	CH	7,98 (1H, dd ; 7,4 et 1,6)	126,6
2’’	163,5	C	-	162,8
3’’	102,5	CH	6,77 (1H, s)	102,2
4’’	181,7	C	-	181,2
5’’	160,5	C	13,10 (1H, s, OH)	160,2
6’’	97,0	CH	6,32 (1H, s)	100,3
7’’	161,6	C	-	161,4
8’’	103,4	C	-	103,6
9’’	154,5	C	-	154,4
10’’	103,2	C	-	102,3
1’’’	121,4	C	-	121,5
2’’’/6’’’	128,1	CH	7,57 (2H, d; 8,2)	127,9
3’’’/5’’’	115,7	CH	6,68 (2H, d; 8,2)	115,3
4’’’	161,1	C	-	160,4

III.2.9. Identification of GBr-9

GBr-9 was obtained as a pale yellow amorphous powder that also gave a positive test with ferric chloride solution for the phenol fonction and with Mg/ HCl suggesting that it is a flavonoid structure. It has the molecular formula $C_{15}H_{10}O_6$, since the pseudo molecular ion peak $[M+H]^+$ appeared at m/z 287.0549 in its high resolution TOF mass spectrum **(figure 54)**. This molecular formula of GBr-9 which has the molecular mass of 286 has one oxygen atom more than that of apigenin and accounts for 11 unsaturation sites.

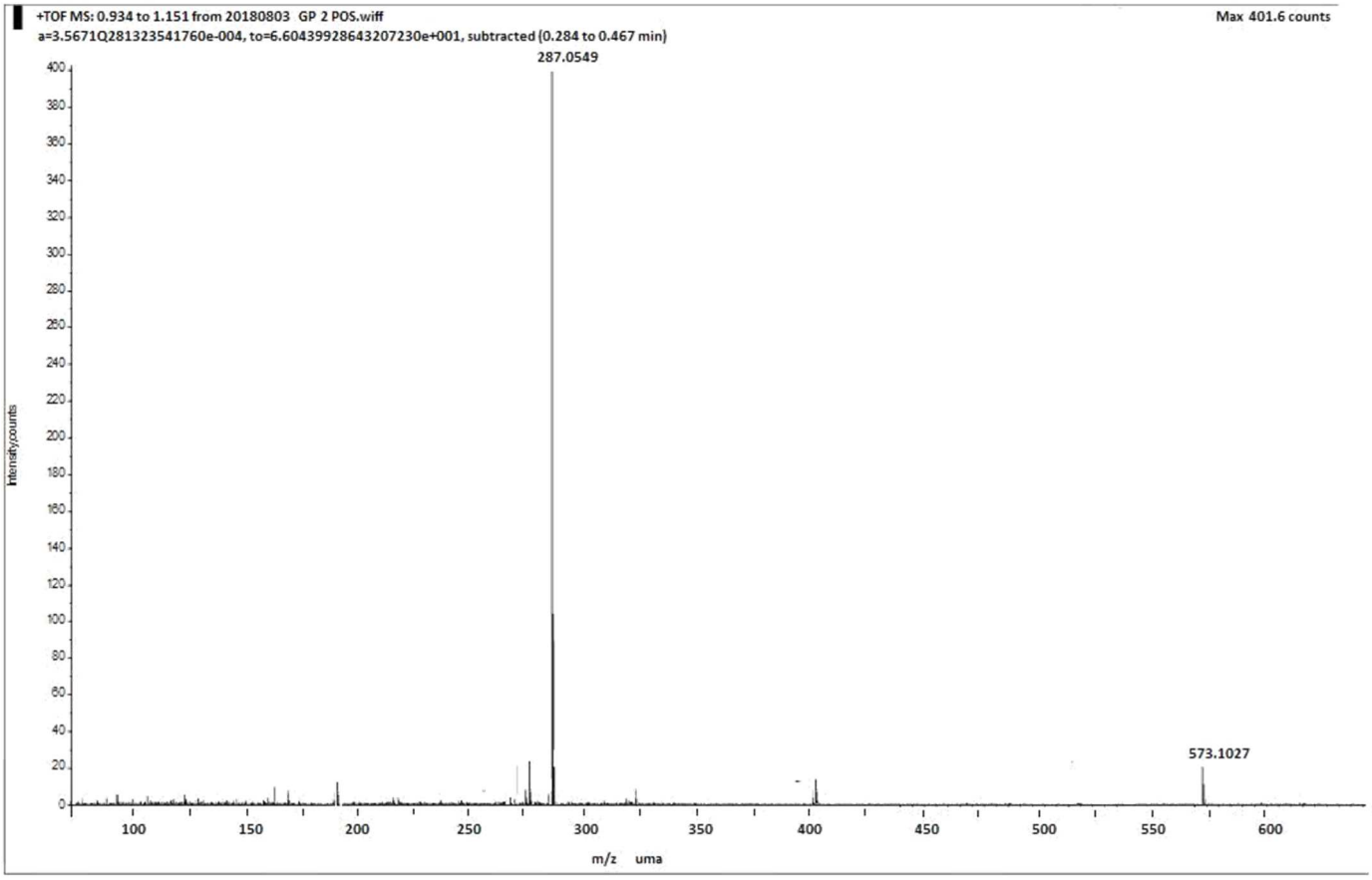


Figure 54: Mass spectrum of GBr-9

Its infrared absorption spectrum shows the following absorption bands: characteristic of flavones: 3186 cm^{-1} for phenol groups, 1638 cm^{-1} for conjugated and chelated carbonyl (1638 cm^{-1}), and 1592 cm^{-1} for aromatic rings .

The analysis of its 1D and 2D ^1H-^1H NMR spectra **(figures 55 and 56)** led to the identification of the following proton systems implicated in the structure of GBr-9:

- Two *meta* coupling protons of a tetra substituted aromatic ring A [(6.26 ppm (1H, d, 2.1Hz, H-6) and at 6.44 ppm (1H, d, 2.1Hz, H-8)].

- Three protons of the trisubstituted aromatic ring B [7.78 ppm, 1H, d, 2.3 Hz, H-2'; 6.88 ppm (1H, d, 2.1 Hz, H-5') and 7.63 ppm (1H, dd, 2.3 Hz and 8.4 Hz, H-6')].
- A singlet signal accounting for one proton at 6.78 ppm (1H, s, H-3) was assigned to the H-3 proton of ring C of the flavones motif.
- A very deshielded proton singlet proton at 12.90 ppm (1H, s, OH-5, ring A) characteristic of phenolic protons that are one proton chelated to a *peri* carbonyl.

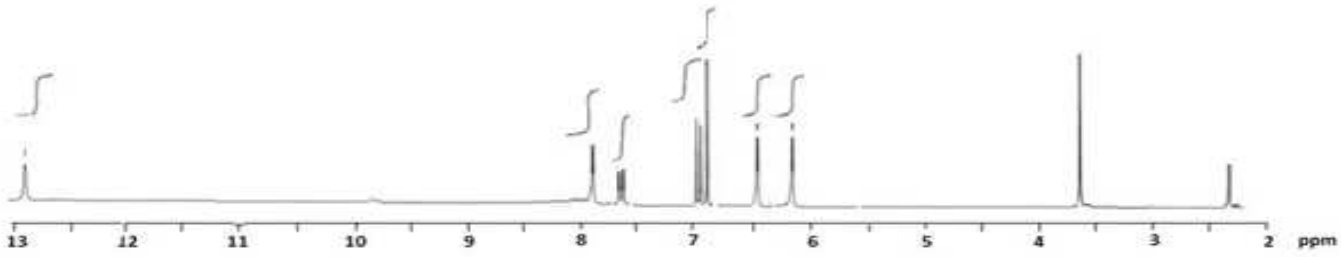


Figure 55: 1H NMR Spectrum (600 MHzDMSO-d6) of GBr-9

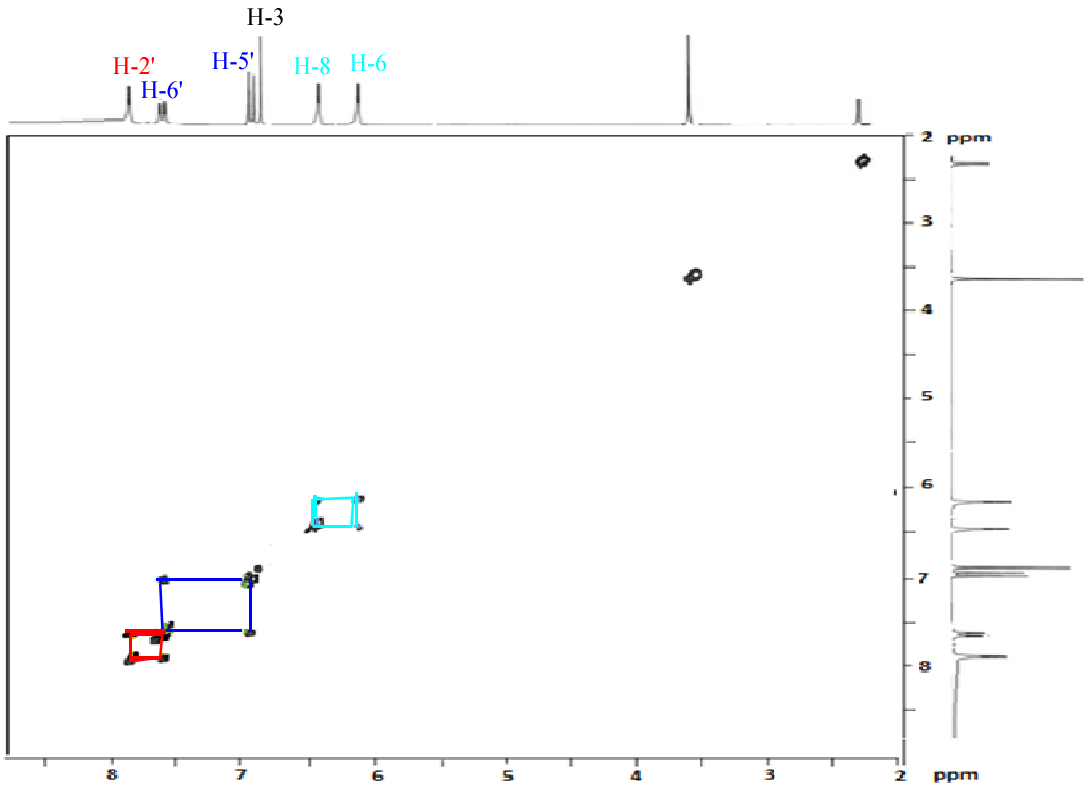
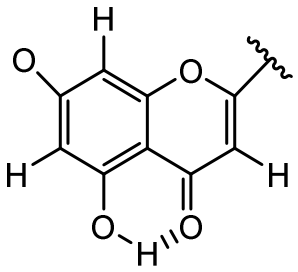
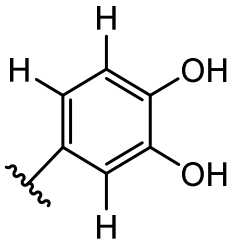


Figure 56: COSY Spectrum of GBr-9

The above information led to two structural units: substructure I and substructure II.



Sub-structure I



Sub-structure II

Figure 57: Sub-structures of GBr-9

The HSQC spectrum **(figure 58)** of GBr-9 had the chemical shifts of all the protonated carbons in the structure of **GBr-9.**

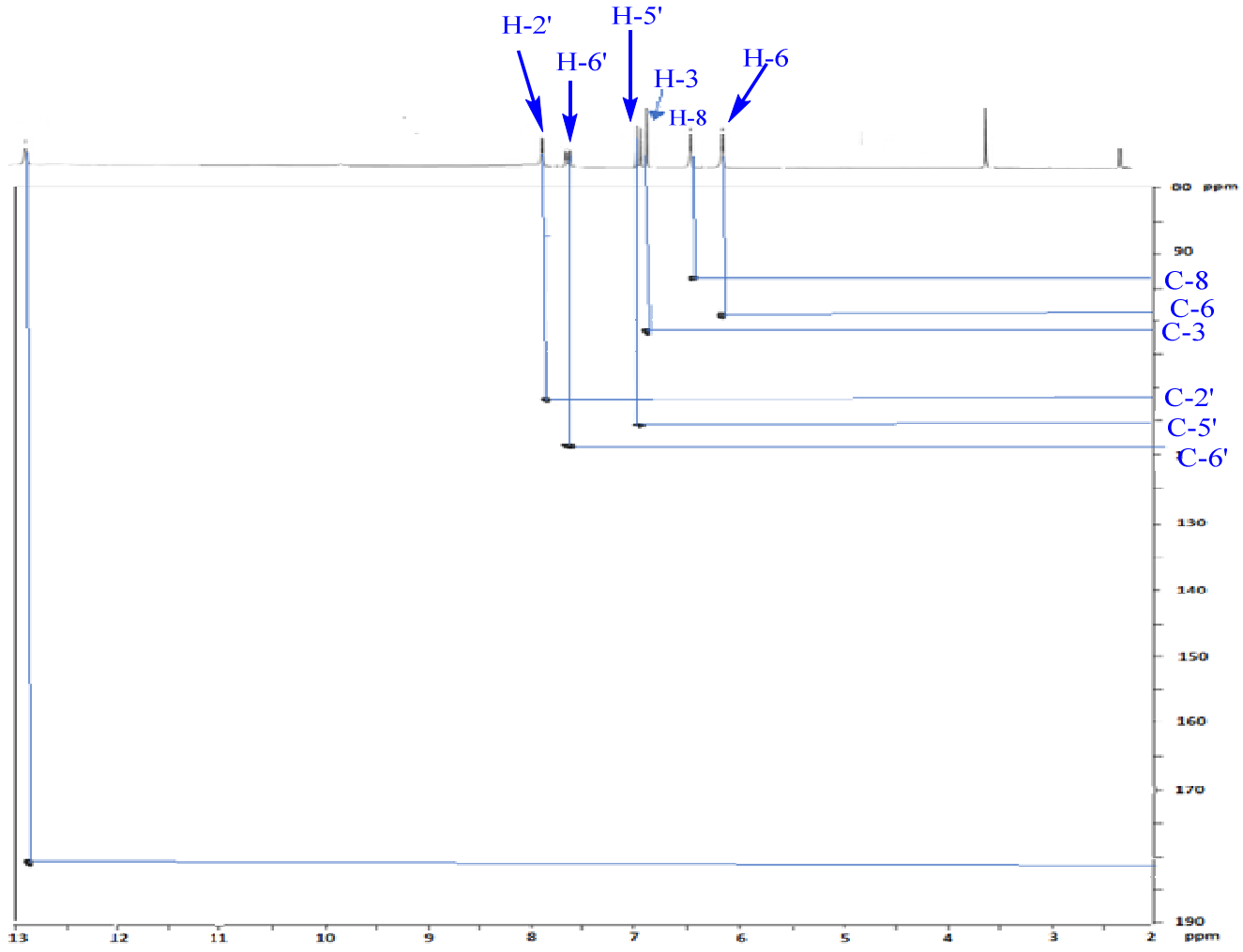


Figure 58: HSQC spectrum of GBr-9

All the 15-carbon atoms in the molecule are sp^2 hybridised. A conjugated chelated carbonyl carbon at 182.4 ppm (s, C-4) and the carbon atoms of aromatic ring A at 161.1 ppm, 99.8 ppm, 163.2 ppm, 94.4 ppm, 157.2 ppm and 103.2 ppm.

Signals at 120 ppm, 113.1 ppm, 145.9 ppm, 152.2 ppm, 116.4 ppm and 118.6 ppm were identified as the six carbon atoms of the aromatic ring B. Two methine carbon atoms in which one was attached to an oxygen atom gave signals at 102.6 ppm and at 157.9 ppm

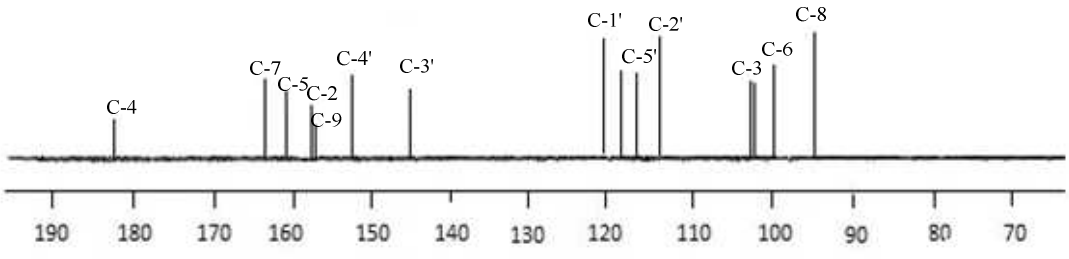


Figure 59: 13C NMR Spectrum of GBr-9

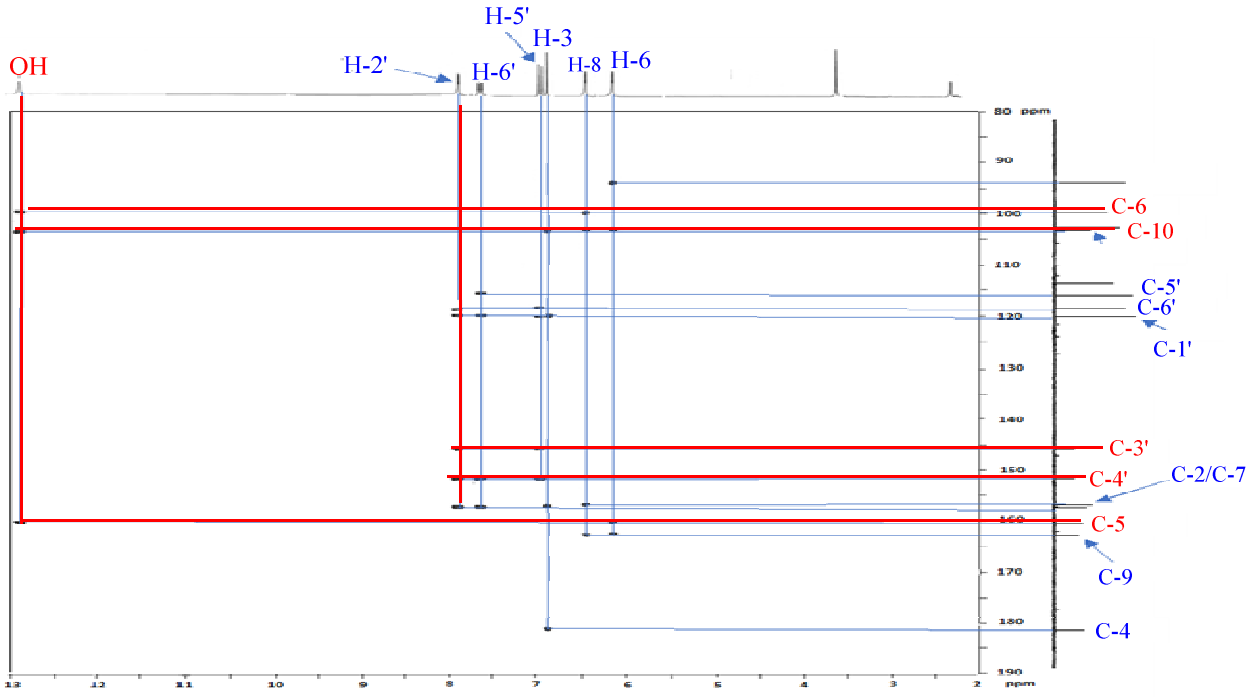
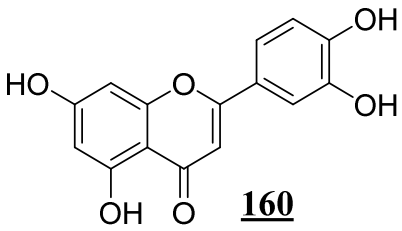


Figure 60: HMBC Spectrum of GBr-9

All the above information shows that GBr-9 has the structure **160**, which is that of earlier reported luteoline or 3, 5, 7, 4'-tetrahydroxy-2-phenylchromen-4-one **(Chaturvedula et al., 2013).**



160

Table 20:1H-NMR and 13C-NMR data of Compound GBr-9

N° C	GBr-9			Luteoline (DMSO), **(Agrawal et al., 1989)**	
	δC pm	Type of C	δH (ppm), J (Hz)	δC ppm	δH (ppm), (J Hz)
2	157.9	C		164 0	
3	102.6	CH	6.78(1H, s)	102.9	6.75(1H, s)
4	182.4	C		181.8	
5	161.1	C	12.90 (1H, s, OH)	157.6	12.97 (1H, s, OH)
6	99.8	CH	6.26(1H, d, 2.1)	99.2	6.28 (1H, d, 2.1)
7	163.2	C		164.3	
8	94.4	CH	6,44 (1H, d, 2.1)	94.7	6.46 (1H, d, 2.1)
9	157.2	C		162.1	
10	103.2	C		103.8	
1’	120.1	C		119.0	
2’	113.1	CH	7.78 (1H, d, 2.3)	113.2	7.36 (1H, d, 2.3)
3’	145.9	C		146.0	
4’	152.2	C		149.7	
5’	116.4	CH	6.88(1H, d,8,4)	116.8	6.85 (H, d, 8.9)
6’	118.6	CH	7.63 (1H, dd ,2.3 and 8,4)	120.8	7.56 (1H, dd, 2.3 and 8.9)

III.2.10: Results of Onchocercal screening of biological activities

GBr-3 was tested to see if it suppressive effect on three parasites: the adult worms of *O. Ochengi* and microfilariae of *O. ochengi* and *L. loa*. The cultures lasted 120 hours after the addition of the test compound.

Concerning the adult worms, the compound showed moderate activity. At the concentration of 20 µg/ml,the motility by 90% of the juvenile form of the parasite were noticed. When screened on *L. loa* mfs, there was no activity at the highest concentration **(Table XXII)**. There was a dose-dependent response for the *O. ochengi* parasite that succumbed to the compound.

Table 21: Effect of GBr-3 on O. ochengi and L. loa

Concentration (µg/mL)	% inhibition of formazan formation by *O. ochengi* adult worm	% inhibition of *O. ochengi* microfilariae motility	% inhibition of *L. loa* microfilariae motility
20	60	90	0
10	20	50	0
5	0	25	0
2.5	0	10	0
1.25	0	0	0

IV: GENERAL CONCLUSION AND PERSPECTIVES

The phytochemical investigation of the ethyl acetate extract (GBr) of the leaves of *Garcinia brevipedicellata* were purified using a combination of chromatographic procedures (CC, TLC, GPC and Prep TLC as well as Sephadex LH-20) led to the isolation of seventeen natural secondary metabolites. The structures of nine of these compounds were determined by studing their complete spectral data (UV, 1D and 2D NMR and MS) as well as by comparing the obtained information with literature. These compounds were characterized as seven earlier described compounds (robustaflavone, 4'''-*O*-methylrobustaflavone, brevipedifloside A, genistein, 2-hydroxygenistein, amentoflavone, and luteoline), and two new compounds (Brevipedicelones D and E). The latter are biflavonoid ethers derivatives that appear to be obtained naturally from the coupling of two flavone units. Most of the compounds were obtained in very small quantities.

Two of these compounds obtained in reasonable quantities were evaluated for anti-onchocercal activities. It was found that among two compounds tested only brevipedicelone D showed moderate inhibition of the adult worm motility of *Onchocerca ochengi* by 60 % at the highest concentration (20 µg/ml) and inhibited motility of both the juvenile worms of *O. ochengi* and *Loa loa* 90 % at this same concentration. These results can be exploited as a new source of anti-onchocercal lead compounds.

The aim of this research was to establish a reproduceable extraction and purification protocol to get pure compounds from the ethyl acetate extract of the selected medicinal plant *G. brevipedicillata*. This protocol shall next be used to get a greater quantity of the isolated compounds which shall be evaluated for their respective biological and antimicrobial properties and results compared with those obtained for the crude extract.

Finally, these results will enrich the information that my host laboratory is gathering on the scientific data base (chemical, biochemical and biological) which the laboratory is establishing on 100 selected plants used in traditional medicine. This will be used to advise traditional healers to adapt scientific notions in their everyday practice to heal patients.

CHAPTER IV:

MATERIAL AND METHODS

(EXPERIMENTAL PROCEDURE)

IV.1. General Experimental Procedures:

UV spectra were recorded on Kronton-Uvikon 930, IR spectra were obtained using a JASCO FITIR-3000E apparatus with specimens presented as transparent KBr discs while Mass spectra were obtained using Time of Flight Mass Spectrometer (TOF-MS). ^{1}H NMR spectra (600 MHz) and RMN ^{13}C (150 MHz) were ran on Bruker WM 600 spectrometer, with compounds dissolved in acetone-d_6 and DMSO-d_6 and using TMS as standard reference. By DEPT experiments, we distinguished methyl, methylene and methane carbons. From COSY ^{1}H-^{1}H spectra, we deduced Homonuclear ^{1}H connectivities. One-bond ^{1}H–^{13}C connectivities were determined with HSQC gradient pulse factor selection. From HMBC experiments, we determined two and three bonds ^{1}H-^{13}C connectivities. Chemical shifts (δ) reported are in parts per million (ppm) and coupling constants (J) were measured in hertz (Hz). For column chromatography, sephadex LH-20 and silica gel 60 were used as stationary phases. Fluorescent silica gel spread on aluminum plates were used for thin layer chromatography. The development of the plates was done with the mixture of CH_2Cl_2 / MeOH (10/: 1, v/v) as eluent. The spots on the plates were revealed by spraying with a solution of H_2SO_4 (3%) followed by heating in an oven at 60˚ for 10 minutes

Preparative silica gel glass plates were used to obtain more compounds in difficult mixtures. The separated compounds were obtained as bands which after visualisation with a UV lamp (λ_{254nm}) were scraped off and washed with methanol to obtain the pure compounds.

IV.2. Extraction, Fractionation and Isolation

IV.2.1. Vegetable material: Harvesting of *Garcinia brevipedicellata*

The leaves of *Garcinia brevipedicellata* were harvested in August 2013 in Malande, a village situated 3 km from DIBANG sub-division in the Nyong et Kelle Division of the Centre Region, Cameroon and identified by Mr. Victor Nana, botanist of the Cameroon National Herbarium, Yaounde, Cameroon where a voucher specimen (No.VN2634) was deposited.

IV.2.2. Extraction of plant material

The leaves of *Garcinia brevipedicellata* (1.5 kg) were dried, reduced to fine powder and then exhaustively extracted with methanol (2L) in a soxhlet extractor, until the extracting solvent was colourless. Next, the solvent was removed using a rotary evaporator to give a residual powder, (the methanol extract). This was further washed with hot ethyl acetate and after removal of the solvent, the ethyl acetate extract (GBr) was obtained.

IV.2.3. Fractionation of crude extracts

The ethyl acetate extract (GBr, 62g) was first divided into four parts (A-D) after which each part was successively subjected to an open CC using a glass column 50cm long and 10 cm in diameter. 600 g of SephadexLH-20 was used as stationary phase and elution was carried out with methanol as mobile phase giving 37 fractions of 30 ml each. After TLC analysis, identical fractions were recombined to give finally six main fractions (N1- N6).

IV.2.4. Purification of fractions

The crude ethyl acetate extract was subjected to purification by a combination of chromatographic methods, starting with gel filtration on Sephadex LH20 that gave five sub-fractions. Each of the obtained sub fraction was then purified by a series of column chromatography on silica gel support coupled with preparative thin layer chromatography on silica gel plates.

The ethyl acetate extract was subjected to an open CC using a column of 10 x 50cm dimension, in which 600 g of Sephadex gel LH-20 as stationary phase was placed and eluted with methanol as mobile phase to give 37 fractions of 30 ml. On the basis of TLC, identical fractions were combined to afford 6 main subfraction fractions N1 (6.1 g), N2 (4.3 g), N3 (34 g), N4 (5.5 g) N5 (3.2 g) and N6 (2.1g). The acetone extract (GBr', 80.5 g) was subjected to the same procedure to give 34 fractions 30 ml of each. Similar fractions were further combined based on TLC chromatogram to give 5 main subfractions: P1 (30 g), P2 (3.3 g), P3 (25 g), P4 (6.6 g) P5 (4.0 g).

Fraction N2 underwent column chromatography on silica gel support using CH_2Cl_2/MeOH: 10/1 (v/v) as solvent mixture to give 25 fractions of 20 ml each, which were reunited to obtain seven sub-fractions (B1, B2, B3, B4, B5, B6 and B7) according to the chromatogram: Sub-fraction B4 was further subjected to chromatography on a silica gel column with CH_2Cl_2/MeOH: 20/1 (v/v) as eluent to obtain 20 fractions of 15ml each which were combined to give five sub-fractions (B"1, B"2, B"3, B"4, and B"5) according to the chromatogram: Sub-fraction B"4 abandoned, gavea whit deposite which was filtered and washed twice with two portions of MeOH to obtain a pure yellow powder (GBr-10 , 30 mg). The filtrate after concentration and subjected to preparative thin layer chromatography (prep TLC) and developed twice using CH_2Cl_2/MeOH: 15/1 as solvent mixture gave two bands. These were scraped off, washed with MeOH and found pure by TLC (GBr-8, 22 mg) and GBr-9 (18 mg). Sub-fraction B"5 under went prep TLC developed with CH_2Cl_2/MeOH: 5/1 as solvent mixture following the multiple migration technique. Three bands which we

scraped and washed with MeOH were found pure (GBr-2 (32 mg), GBr-15 (2 mg) and GBr-8 (12 mg)).

Fraction N3 was first of all subjected to column chromatography eluted with CH_2Cl_2/MeOH: 10/1 (v/v) as solvent mixture to obtain 30 fractions of 15ml each which were pooled together in to six sub-fractions according to the chromatogram: N'1, N'2, N'3, N'4, N'5, N'6. Fraction N'3 under went a similar purification procedure as N3 and nine sub-fractions were again obtained as shown by the chromatogram: N"1, N"2, N"3, N"4, N"5, N"6, N"7, N"8, and N"9. A similar purification procedure as that of N3 was done for fractions N"1 and N"3 and five pure spots altogether were obtained. N"1 gave two pure spots [GBr-13 (19 mg), and GBr-14 (15 mg)] while N"2 gave three [GBr-5 (17 mg), GBr-11 (18mg) ang GBr-12 (21 mg)]. Also, N"8 and N"9 were subjected to prep TLC with the following solvent mixtures: N"8 (CH_2Cl_2/MeOH: 5/1) and N"9 (CH_2Cl_2/MeOH: 10/1) respectively and migrated twice each. N"8 showed two bands which were scraped and then washed with MeOH. They were verified and confirmed pure by TLC [GBr-14 (10 mg) and GBr-17 (11 mg)]. N"9 after treatment following the same procedure gave three bands which were scraped and washed with MeOH (GBr-4 (20 mg), GBr-1 (14 mg).

Fraction N4 was also subjected to CC on silica gel support eluted with the solvent mixture CH_2Cl_2/MeOH (10/1: v/v) to obtain 15 fractions of 15ml each which were pooled together in to five sub-fractions according to the chromatogram: A'1, A'2, A'3, A'4 and A'5 respectively. A’5 was again subjected to the same purification procedure to obtain five other sub-fractions: A"1, A"2, A"3, A"4 and A"5 respectively. By repeating the same procedure again on these subfractions, A"4 gave four compounds [GBr-3 (52 mg), GBr-6 (52 mg) and GBr-7 (16 mg) and GBr-7 (10 mg)] while subfraction A"5 gave two compounds [GBr-16 (8mg) and GBr-7 (15 mg)] respectively.

IV.3. Assays for biological screen (Carried out at the Pan-African ANDI Centre of excellence)

IV.3.1. Extraction of *Onchocerca ochengi* adult worms

O. ochengi adult worm masses were extracted from cattle skin using the method employed by Cho-Ngwa *et al* (2010). Briefly, fresh pieces of umbilical cattle skin containing palpable nodules were obtained from local slaughterhouse in Buea, Cameroon. The piece of skin was immediately transported to laboratory, thoroughly washed with soap and distilled

water, drained, dried by blotting with a piece of cloth and then transferred to a sterile laminar flow hood. It was then entirely covered with 70% ethanol and allowed to evaporate completely on its own. The nodules were carefully dissected using a sterile razor blade and the pale orange-yellow worms (in appearance) were immediately submerged in sterile 12-well culture plates (NUNC, USA) containing 2 ml of complete culture medium [RPMI-1640 supplemented with 25 mM HEPES, 2 g/L sodium bicarbonate, 20 mM L-glutamine, 10% new born calf serum (SIGMA, USA), 2x antibiotic-antimycotic (Sigma, USA)], pH 7.4]. After overnight culture, 1 mL of medium was added before addition of drug making a total volume of 3 mL. Adult worm cultures were carried out at 37ºC under an atmosphere of 5% CO_2 in humidified air in an incubator (HERACell 150, Haraeus, Germany).

IV.5.2. Extraction of *O. ochengi* microfilariae

The microfilariae of *O. ochengi* were extracted by the method of Samje *et al*. (2014), with some slight modifications. Cattle skin got from the slaughterhouse was thoroughly cleaned and sterilized as above. The skin was then firmly attached onto a sterilized flat wooden board using autoclaved thumbnails. The outer surface was carefully shaved with a sterile razor blade, and then rinsed twice with distilled water. A clean dry adsorbent cloth was used to remove excess moisture from the skin. The entire skin was covered with 70% ethanol and allowed to evaporate in a laminar flow hood. This sterilization process was done twice. Once the alcohol had completely evaporated from the skin, skin snips were obtained from different locations of the skin. These sleeves were carefully scrapped and the snips submerged in 15 ml of complete culture medium. The assemblage was incubated at room temperature for 2 hours to allow for emergence of microfilariae. The highly motile microfilariae that emerged were concentrated by centrifugation at 400 x g for 10 minutes. The supernatant was decanted, and the pelleted mfs were re-suspended in fresh complete culture medium. The highly motile microfilariae were quantified using an inverted microscope (Euromex, Holland). One hundred microlitres of culture medium containing microfilariae were distributed into 96 well culture plate containing LLC-MK2 cell layers to obtain an average of 12-15 mfs per well. Culture conditions were the same as that of adult worms above.

IV.5.3. Isolation and culture of *Loa loa* microfilariae

Blood was collected from *Loa loa* infected individuals at the Edea Health District after confirmation by Giemsa stain. The blood was rapidly transferred to the University laboratory. The microfilariae were isolated by the method of (**Cho-Ngwa *et al*. 2016).**

Freshly collected *L. loa* -infected blood was diluted (1:2) with culture media used above but without sera. The diluted blood was carefully layered on 4 ml of Ficoll-pacque (TM) in a 15 mL centrifuge tube. The tube was spun in a swing bucket centrifuge at 400xg for 15 minutes. The recovered mfs were washed three times with culture media (without sera) and then re-suspended in media containing sera. The mfs were then distributed in wells of a 96-well microtiter plate containing LLC-MK2 cell layers. Each well contained 12-15 mfs in 100 of media.

IV.5.4. Preparation of Compound and assessment of activity

GBr-3 was dissolved in ≥99.9% sterile dimethyl sulfoxide (SIGMA, USA) giving a stock concentration of 5 µg/mL. The compound was prepared at 2X the final concentration and distributed to wells containing parasites. For the microfilariae, 100 µL was added while 1 mL was added to wells containing adult worms to give a final volume of 200 µL and 4 mL

for microfilariae and adult worms respectively. All the cultures were conducted for 120 hours post addition of the compound. Auranofin **(Bulman *et al*., 2015)** served as positive control for adult worm assay while ivermectin and amocarzine was used as positive control drugs for *O. ochengi* mfs and *L. loa* mfs respectively. The diluent (dimethyl sulfoxide) was added to the negative control wells. Inhibition of microfilariae motility was assessed using an inverted **2014**microscope. Effect of compound on adult worm viability was assessed using the MTT-formazan assay following procedures employed by (**Cho-Ngwa *et al*. 2010)** and **(Samje *et al*.,).**

IV.5.5. Ethical issues

Ethical clearance (2013/11/371/L/CNERSH/SP) and administrative authorization (631–06.14) were obtained from the Cameroon National Ethical Committee and the Ministry of Public Health, Cameroon respectively. Local administrative authorization was also obtained from the District Medical Officer of the Edea Health District. Informed consent was obtained freely from individuals who harboured high *L. loa* mf load.

IV.6 DESCRIPTION OF ISOLATED COMPOUNDS

GBr-1: Robustaflavone:**<u>152</u>**

$C_{30}H_{19}O_{10}$

Yellow amorphous powder.

Positive phenol test

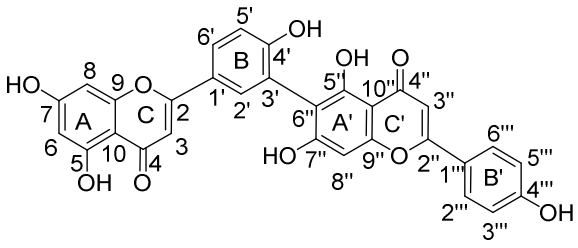
Positive flavonoid test

TOFMS [M+H] $^{+}$ m/z 539.1090

(calculated for $C_{30}H_{18}O_{10}$), 538.0900

IR (KBr) νmaxcm^{-1} 3218; 1648; 1622; 1604

UV λ_{max} (nm) log ε: 223 (4. 3), 320 (3.8)



^{1}H NMR (600MHz, DMSO -d_6) :δ(ppm) : 7.76 (1H, dd, J=8.8 and 2.1 Hz, H-6') , 6.89 (1H, d, J=8.8 Hz, H-5'), 7.84 (1H, d, J=2,1 Hz, H-2'), 6.61(1H, s, H-3), 6.14(1H, d, 1.8 Hz, H-6), 6.24(1H, d, 1.8 Hz, H-8), 6.68(1H, s, H-3"), 6.04(1H, s, H-8"), 7.72 (2H, d, J= 9.0 Hz, H-2''' et H-6'''), 6.64 (2H, d, J= 8.6 Hz, H-3''' and H-5'''), 13.06 (1H, OH-5''). 13.01 (1H, OH-5).

^{13}C NMR (150 MHz, DMSO-d_6): δ(ppm): 165.4 (C, C-2), 103,1 (CH, C-3) ; 180.8 (C, C-4), 160.1 (C, C-5), 99.1 (CH, C-6), 162.6 (C, C-7), 93.8 (CH, C-8), 156.4 (C, C-9), 103.2 (C, C-10), 122.8 (C, C-1'), 134.8 (CH, C-2'), 116.6 (C, C-3'), 157.2 (C, C-4'), 121.1 (CH, C-5'), 128.4 (CH, C-6'), 163.8 (C, C-2"), 103.4 (CH, C-3"), 181.2 (C, C-4") , 161.2 (C, C-5"), 103.8 (CH, C-6"), 164.3 (C, C-7"), 94.1 (C, C-8"), 155.9 (C, C-9"), 104.2 (C, C-10"). 121.6 (C, C-1'"), 128.6 (CH, C-2'"/C-6'"), 116.1 (CH, C-3'"/C-5'") and 158.2 (C, C-4'").

GBr-2: 4'-O-methylrobustaflavone:**<u>153</u>** $C_{31}H_{20}O_{10}$

Yellow amorphous powder.

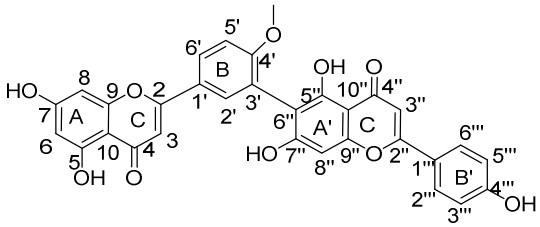
Positive phenol test ;

Positive flavonoid test

TOFMS [M+H]$^{+}$ m/z 553.1122 (calculated for $C_{31}H_{19}O_{10}$), 552,1100

IR (KBr)ν_{max} cm^{-1} 3354, 1652, 1628, 1605, 1508

UV λ_{max} (nm) log ε 223 (3.8), 320 (3.4)

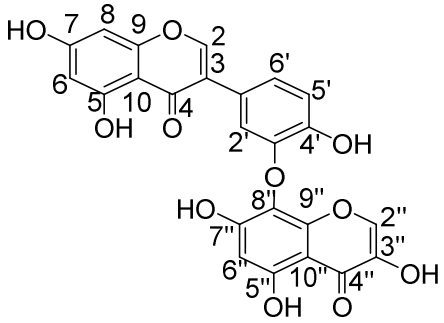


^{1}H NMR(600MHz, DMSO-d_6) :δ (ppm) : 7.94 (1H, dd, J=8.8 et 2.2 Hz, H-6'), 7.28 (1H, d, J=8.8 Hz, H-5'), 8.14 (1H, d, J=2,2 Hz, H-2'), 6.84(1H, s, H-3), 6.24(1H, d, 2.1 Hz, H-6), 6.42(1H, d, 2.1 Hz, H-8), 6.78(1H, s, H-3"); 6.68(1H, s, H-8"), 7.91 (2H, d, J= 8.9 Hz, H-2'" and H-6'"), 6,89 (2H, d, J= 8,9 Hz, H-3'" and H-5'"), 12.96 (1H, OH-5''), 13.03 (1H, OH-5), 3.84 (3H, s, OCH_3-4').

^{13}C NMR (150 MHz, DMSO-d$_6$

): δ (ppm) :163.3 (C, C-2) , 103,4 (CH, C-3) , 181.7 (C, C-4) , 162.4 (C, C-5) : 96.2 (CH, C-6), 164.8 (C, C-7). 94.3 (CH, C-8); 156.8(C, C-9), 103.8 (C, C-10),120.6 (C, C-1'), 130.8 (CH, C-2'), 116.4 (C, C-3'), 160.2 (C, C-4'), 120,1 (CH, C-5'), 127.6 (CH, C-6'), 163.8 (C, C-2"), 103.1 (CH, C-3"), 181.6 (C, C-4") , 158.4 (C, C-5") , 108.3 (CH, C-6"), 161.6 (C, C-7"), 93.3 (C, C-8"),156.2 (C, C-9"), 103.4 (C, C-10"), 120.8 (C, C-1'"), 128.4 (CH, C-2'"/C-6'"), 115.6 (CH, C-3'"/C-5'") and 160.9 (C, C-4'"), 55.9 (H_3CO, C-4').

GBr-3: Brevipedicelone D: <u>154</u> $C_{24}H_{14}O_{11}$,



Yellow powder,

Positive phenol test;

Positive flavonoid test

TOFMS [M+H] $^+$ m/z 479.0588 (calculated for $C_{24}H_{13}O_{10}$), 478.3706

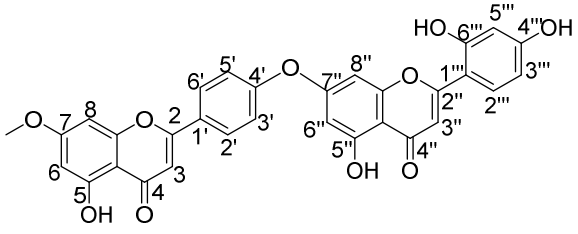
IR (KBr) νmaxcm^{-1}: 3424, 1638, 1600, 1498

UV λ$_{max}$ (nm) log ε 268 (3. 8), 316 (3.4)

^{1}H NMR (600MHz, DMSO-d$_6$) δ (ppm): 8.37 (1H, s,H-2); 7.50 (1H,d,J=2.22Hz, H-2'); 7.13 (1H,dd,J=8.46 & 2.22Hz,H-6'); 6.65 (1H,brd,J=8.46,H-5'); 6.46 (1H,d,J=2.10Hz,H-8) 6.35 (1H, s,H-6''); 6.27 (1H,d,J=2.10Hz,H-6); 12.73 (1H,OH-5); 12.64 (1H, OH-5''); 9.10 (1H, s ,H-2'')

^{13}C NMR (150MHz, DMSO-d$_6$): δ(ppm): 180.7 (C,C-4); 161.7 (C,C-5); 99.1 (CH,C-6); 164.4 (C,C-7); 94.0 (CH,C-8); 160.4(C,C-9); 157.4 (CH,C-2); 122.0(C,C-3); 104.5(C,C-10); 122..0(C,C-1'); 114.5(CH,C-2'); 146.0(C,C-3'); 147.7 (C,C-4'); 115.4 (CH,C-5'); 119.0 (CH,C-6'); 144.9 (CH,C-2''); 158.0 (C,C-3''); 178.2 (C,C-4''); 159.5 (C,C-5''); 98.0 (CH,C-6''); 158.0(C,C-7'');119.0.1 (C,C8''); 159.5(C,C-9''); 102.3 (C,C-10'').

GBr-4: Brevipedicelone E: <u>160</u> $C_{31}H_{20}O_{10}$,



Yellow powder,

Positive phenol test ;

Positive flavonoid test

TOFMS [M+H] $^{+}$ m/z 553.0960 (calculated for $C_{31}H_{19}O_{10}$), 552.1109

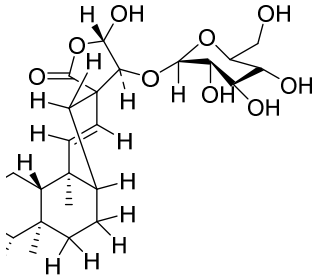
IR (KBr) νmaxcm^{-1}: 3234, 1638, 1628, 1603, 1508

UV λ_{max} (nm) log ε 223 (3. 6), 3320 (3.5)

^{1}H NMR(600MHz, DMSO) : δ (ppm) : 7.57 (2H, d, 8.8, H2′/ 6'), 6.71 (2H, d, 8.8, H3′/H-5'), 6.82 (1H, s, H-3), 6.24(1H, d, 2.1 Hz, H-6),6.77 (1H, d, 2.8, H-8), 6.89 (1H, s, H-3"); 6.68(1H, s, H-8"), 8.04 (1H, d, 8.6, H-2"' and), 7.09 (1H, d, 8.6Hz, H-3"'), 6.81 (1H, d, 2.2 H-2′′), 6.81 (2H, d,8.8, H-6′′/H8′′), 6.89 (1H, d, 2.8Hz H-5′′′), 12.99 (1H, s, OH-5′′), 12.12 (1H, s, OH), 3.85 (3H, s, OCH_3-7).

^{13} C NMR (150 MHz, DMSO): δ (ppm) : 165.0 (C, C-2), 101.5 (CH, C-3), 182.0 (C, C-4), 160.5 (C, C-5), 94.3 (CH, C-6), 167.3 (C, C-7). 92.6 (CH, C-8), 156.3(C, C-9), 104.6 (C, C-10),127.6 (C, C-1'), 154.4 (C, C-4'), 115.6 (CH, C-3'/C-5'), 128.1 (CH, C-2'/C-6'), 160.5 (C, C-2"), 103.5 (CH, C-3"), 181.9 (C, C-4") , 164.2 (C, C-5") , 99.9 (CH, C-6"/C8′′), 166.1 (C, C-7"), 156.1 (C, C-9"), 104.9 (C, C-10"), 110.4 (C, C-1"'), 131.4 (CH, C-2"'), 157.8 (C, C-6′′′), 121.4 (CH, C-3"'), 102.8 (CH, C-5"') and 159.1 (C, C-4"'), 56.0 (H_3CO, C-7).

GBr-5 Brevipedifloside A <u>156</u> $C_{26}H_{38}O_9$



Cream white powder.

Positive sterol test,

Positive Molish test,

TOFMS [M+H]$^{+}$ m/z 496.2701 (calculated for $C_{26}H_{40}O_9$), 538.0978

IR (KBr)ν_{max} cm^{-1} : 3396, 1758, 1729, 1641, 1502, and 847

^{1}H NMR (600MHz, DMSO-d_6) δ (ppm):) δ 3.24 (m, H-5'), 3.12 (m, H-3'), 3.61, 3.43 (ench, H-6'), 6.9 (1H, s, H-1), 1.26, 1.18 (m, H-7), 1.24, 1.75 (m, H-6), 5.25 (d, H-12), 0.99 (d, H-18), 0.66 (s, H-19), 4.58 (d, H-16), 3.18 (d, H15), 0.77 (s,, H-20), 2.24,1.91 (m, H-14), 1.38 (m, H-10) 1.48 (m, H-9).

^{13} C NMR (150 MHz, DMSO-d_6): δ (ppm): 21.5 (t, C-1), 23.7 (t, C-2), 24.6 (t, C-3), 40.1(d, C-4), , 28.6 (t, C-6), 31.2 (t, C-7), 34.9 (d, C-8), 41.9 (s, C-9), 44.5 (d, C-10), 141.6 (d,C-11)

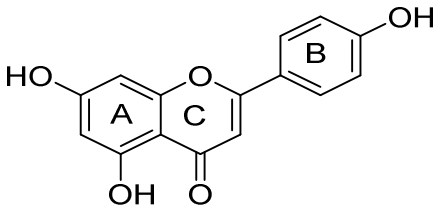
134.6 (d, C-12), 54.6 (s, C-13), 56.22 (CH, C-14), 24.77 (CH_2, C-15), 31.04 (CH_2, C-16),

GBr-6: Apegenine <u>157</u>: $C_{15}H_{10}O_5$

Amorphous white powder

HR- MS [M-H]$^-$ m⁄z 270. 0551(calculated for **$C_{15}H_9O_5$),** m⁄z 271.2423

IR (KBr) ν_{max} cm^{-1}: 3424, 1638, 1605, 1498



^{1}H NMR (600 MHz, DMSO-d$_6$) :δ (ppm) : 8.04 (1H, s, H-2), 6.21(1H, d, J=2,1 Hz, H-6), 6.32 (1H, d, J=2,1 Hz, H-8), 7.34 (1H, d, J=8,9 Hz, H-2' et H-6'), 6.86 (1H, d, J=8,9 Hz, H-3' et H-5'), 13.01(1H, s, OH-5).

^{13}C NMR (150 MHz, DMSO-d$_6$) : δ (ppm) : 154.4(CH, C-2) ;, 123.8 (C, C-3) , 181.2 (C, C-4) , 163.4 (C, C-5) , 99.8 (CH, C-6) , 164.7 (C, C-7) , 94.3 (CH, C-8) , 157.4 (C, C-9) , 103.9 (C, C-10) , 121.2 (C, C-1'), 130.1 (CH, C-2'), 115.3 (CH, C-3'), 157.2 (C, C-4'), 115.3 (CH, C-5'), 130.1 (CH, C-6').

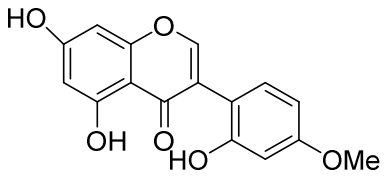
GBr-7: 2'-hydroxy -4'- O-methylgenistein: <u>158</u> $C_{16}H_{13}O_6$

HR-SM: $[M+H]^+$ at m⁄z 301.1410

(calculated for $C_{16}H_{13}O_6$) m/z 300.0712.

IR (KBr) ν_{max} cm^{-1}: 3420, 1650, 1610, 1518, 1240, 1160

UV λ_{max} nm (log ε): 263 (2.3) and 332 (1.6)



^{1}H NMR (400 MHz, DMSO-d$_6$): δ (ppm): 13.02 (1H, s, OH- 5), 11.54 (1H, s , OH - 2'), 8.30 (1H , s, H-2), 6.21 (1H, d, J = 2.1 Hz , H-6), 6.30 (1H, d, J = 2.1 Hz, H-8), 6.48 (1H, d, J = 2.2 Hz, H-3'); 6.64 (1H, dd, J = 2.2 et 9.0 Hz , H-5'), 6.74 (1H, d, J = 9.0 Hz, H-6') ; 3.84 (3H, s, 0CH_3) .

^{13}C NMR (100 MHz, DMSO-d$_6$) : δ (ppm) : 154.8 (CH , C-2) , 121.2 (C , C-3) , 181.1 (C , C-4) ,161.4 (C , C-5), 99.3 (CH , C-6), 163.8 (C , C-7), 94.1 (CH , C-8), 156.8 (C , C-9), 104.1(C, C-10), 108.8 (C , C-1'), 156.4 (C , C-2'), 102.6 (CH, C-3'), 158.8 (C , C-4'), 106.4 (CH, C-5'), 130.6 (CH, C-6'), 56.4(CH_3 , OCH_3-4').

57.67 (CH, C-17), 12.0 (q, C-20), 100.7 (d. C-1'), 72,6 (d, C-2'), 65.5 (t, C-6').

GBr-8: Amentoflavone: <u>159</u> $C_{30}H_{19}O_{10}$

Yellow amorphous solid

Positive phenol test,

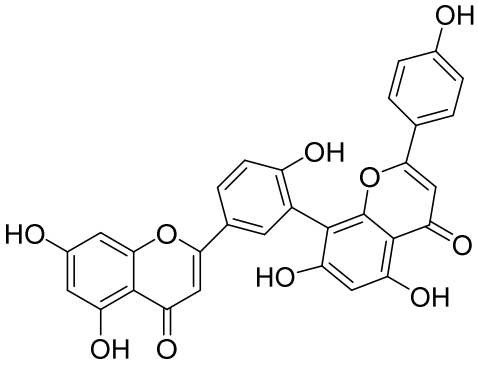
Positive flavonoid test,

TOF-MS: [M+H] $^{+}$543.1194

(calculated for **$C_{23}H_{31}O_7$**)542.1291

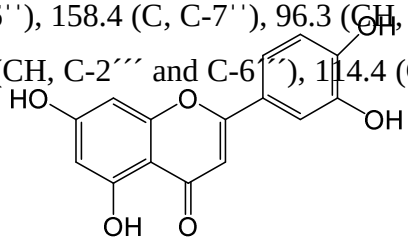
UV(MeOH) : λ_{max} (logε) = 261nm (4.41)

IR (pastilles KBr) λ_{max} cm^{-1}: 3310, 3046, 1712, 1600,1498.



^{1}H NMR (400 DMSO-d_6): (δ ppm) :5.54 (1H, dd, 13.0 and 2.8 Hz, H-2), 3.21 (1H, dd, 13,0 and 17.0 Hz, H-3a), 2.82 (1H, dd, 2.8 and 17.0 Hz, H-3b), 12.86 (1H, s, OH-5), 5.84 (1H, d, 2.0 Hz, H-6), 5.83 (1H, d, 2.0 Hz, H-8), 7.14 (2H, d, 8.0 Hz, H-2' and H-6'), 7.00 (2H, d, 8.0 Hz, H-3' and H-5'), 5.38 (1H, dd, 13.0 and 3.0 Hz, H-2''), 3.18 (1H, dd, 13.0 and17.0 Hz, H-3''a), 2.71 (1H, dd, 3.0 and 17.0 Hz, H-3''b), 12.03 (1H, s, OH-5''), 5.84 (1H, s, H-8''), 7.14 (2H, d, 8.1 Hz, H-2''' and H-6 ''') , 6.65 (2H, d, 8.1 H-3''' and H-5''').

^{13}C NMR (150 MHz, DMSO-d_6): (**δ ppm**): 79.3 (CH, C-2), 43.1 (CH_2, C-3) ,196.2 (C, C-4), 163.1 (C, C-5), 95.8 (CH, C- 6), 165.1(C, C-7), 95.4 (CH, 8), 155.3 (C, C-9), 103.1 (C, C10), 123.1(C, C-1´), 128.4 (CH, C-2' and C-6'), 116.1 (CH , C-3' and C-5'), 158.3 (C, C-4'), 80.1(CH, C-2''), 42.2 (CH_2, C-3''), 196.1(C, C-4''), 155.2 (C, C-5''). 126.1 (C, C-6''), 158.4 (C, C-7''), 96.3 (CH, C-8''), 155.8 (C, C-9''), 103.2 (C, C-10''), 127.4 (C, C-1'''), 128.1 (CH, C-2´´´ and C-6´´´), 114.4 (CH, C-3´´´/C-5´´´), 159.1 (C, C-4´´´).



GBr-9: Luteoline <u>160</u>: C_{15} H_{11} O_6

Pale yellow amorphous powder

Positive phenol test

TOF-MS: [M+H] $^{+}$287.0549

(Calculated for C_{15} $H_{10}O_6$) 286.0524

IR (KBr) ν_{max} cm^{-1} : 3186, 3046, 1638, 1592, 1096

^{1}H NMR (600 MHz, DMSO-d_6):(δppm): 6.78 (1H, s, H-3), 12.90(1H,s, H-5),6.26 (1H, d, 2.1 Hz, H-6), 6.44(1H, d, 2.1 Hz H-8), 7.78(1H, d, 2.3 Hz, H-2'), 6.88(1H, d, 8.4Hz, H-5'), 7.63 (1H, dd, 2.3 and 8.4 Hz, H-6').

^{13}C NMR (150 MHz, DMSO-d_6): (δppm):157.9 (C, C-2), 102.6 (CH, C-3),182.4 (C, C-4), 161,1 (C, C-5), 99.8 (CH, C-6), 163.2 (C, C-7), 94.4 (CH, C-8), 157.2 (C, C-9), 103.2 (C, C-

^{1}H NMR (600 MHz, DMSO-d_6):(δppm): 6.78 (1H, s, H-3), 12.90(1H,s, H-5),6.26 (1H, d, 2.1 Hz, H-6), 6.44(1H, d, 2.1 Hz H-8), 7.78(1H, d, 2.3 Hz, H-2′), 6.88(1H, d, 8.4Hz, H-5′), 7.63 (1H, dd, 2.3 and 8.4 Hz, H-6′).

^{13}C NMR (150 MHz, DMSO-d_6): (δppm):157.9 (C, C-2), 102.6 (CH, C-3),182.4 (C, C-4), 161,1 (C, C-5), 99.8 (CH, C-6), 163.2 (C, C-7), 94.4 (CH, C-8), 157.2 (C, C-9), 103.2 (C, C-10), 120.1 (C, C-1′) 113.1 (CH, C-2′), 145.9 (C, C-3′), 152.2 (C, C-4′), 116.4 (CH, C-5′), 118,6 (CH, C-6′).

10), 120.1 (C, C-1′) 113.1 (CH, C-2′), 145.9 (C, C-3′), 152.2 (C, C-4′), 116.4 (CH, C-5′), 118,6 (CH, C-6′).

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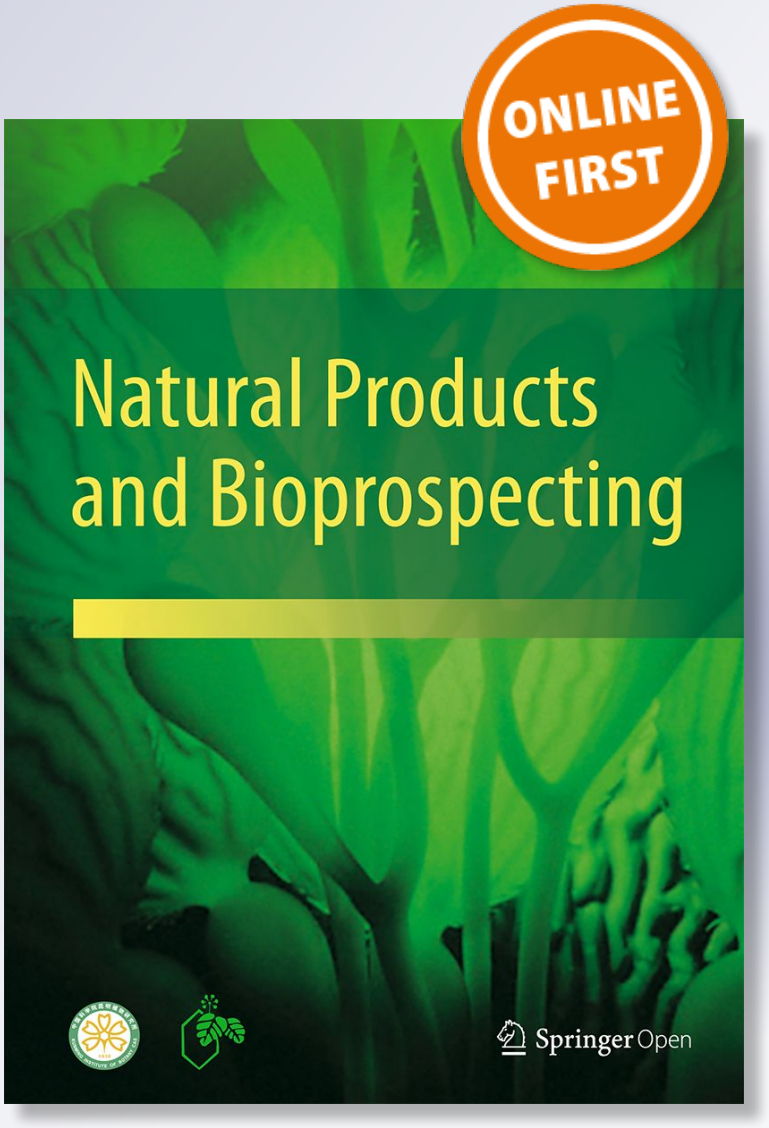

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ORIGINAL ARTICLE

Brevipedicelones D and E, Two C–O–C Flavonoid Dimmers from the Leaves of *Garcinia brevipedicellata* and Anti-onchocercal Activity

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Abstract

A novel isoflavone–chromone flavonoid C–O–C dimmer, brevipedicelone D (**1**), along with one new C–O–C biflavonoid derivative, brevipedicelone E (**2**), were isolated from the ethyl acetate extract of the leaves of *Garcinia brevipedicellata*, a medicinal plant used in folk medicine in parts of Cameroon. Their structures were elucidated by extensive spectroscopic techniques, including 1D- and 2D- NMR, MS experiments, as well as comparing their spectral data with those of known analogues. Anti-onchocercal screening of **1** showed moderate inhibition of adult worm motility of *Onchocerca ochengi* by 60% at the highest concentration (20 µg/mL) and inhibited motility of both the juvenile worms of *O. ochengi* and *Loa loa* by 90% at this same concentration.

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Graphical Abstract

Keywords *Garcinia brevipedicellata* · Brevipedicelone · Anti-onchocercal activity

1 Introduction

Garcinia is the most represented genus of the (Clusiaceae) family. Long known by the name Guttifreae, the Clusiaceae family is the most represented of this family, widely distributed from temperate to tropical regions and are represented by six (06) sub families which are: Kielmeyeroidae, Calophyloidae, Moronoboidae, Lorostermonoidae, Hypericoidae and Clusioidae [1]. The family at times regroups trees, shrubs, herbs and rarely lignans. Members are generally unbeared and the plants are easily recognized by their yellow or orange resinous latex which usually flows slowly when the stems, flowers and fruits are wounded while the leaves hardly produce latex [2]. Largely represented in Africa and Asia this family is made up of about 1350 species regrouped into 47 genera in low altitudes humid dense forests and are composed of six genera in Africa (i.e. *Allanblackia, Calophyllum, Garcinia, Pentadesma, Symphonia* and *Mammea*) [3]. *Garcina brevipedicellata* Oliv. is an African species that is abundant in forest regions of Cameroon and is a deciduous shrub that attains a height of about 5–9 m and about 30 cm in diameter. Previous phytochemical investigation of the leaves and stem bark of *G. brevipedicellata* have shown the presence of sterols, depsidones and tannins [4–6]. In a previous report from our lab., eight biflavonoids were isolated and characterized from the methanol extract of the stem heartwood of this plant, with three of them namely; brevipedicelones A, B, and C, being new flavonoid C–O–C dimmers [7]. This prompted further investigation of the plant.

Further investigation of the ethyl acetate extract of the leaves of this plant resulted in the isolation of a novel isoflavone–chromone flavonoid C–O–C dimmer, brevipedicelone D (**1**) and a new C–O–C biflavonoid derivative, brevipedicelone E (**2**), along with three known biflavonoids, robustaflavone (**3**), *O*-methylrobustaflavone (**4**) brevipediclone C (**5**) and one flavonoid, luteoline (**6**) (Fig. 1). We now report the isolation, structure elucidation of compounds **1** and **2** and the anti-onchocercal activity of **1** on the adult and juvenile worms (microfilariae) of *Onchocerca ochengi* and *Loa loa*, parasites responsible for human onchocerciasis (river blindness).

2 Results and Discussion

The air-dried and powdered leaves of *garcinia brevipedicellata* were extracted with methanol to obtain a gum which was re-extracted with warm ethyl acetate to give a crude extract which was first fractionated by exclusion column chromatography on Sephadex LH-20. The fractions obtained were subjected to a combination of column and preparative thin layer chromatography over silica gel that led to the isolation of compounds: **1** (52 mg), **2** (10 mg), **3** (11 mg), **4** (32 mg), **5** (8 mg) and **6** (12 mg).

Compound **1** was obtained as yellow amorphous solids soluble in methanol. It gave a positive test for flavonoid (Mg/HCl). From the high resolution time of flight mass spectrum (HRTOFMS) we deduced a pseudomolecular ion peak ($M+H^+$) at m/z 479.0588, corresponding to the molecular formula $C_{24}H_{14}O_{11}$. The IR absorption bands indicated the existence of conjugated and chelated

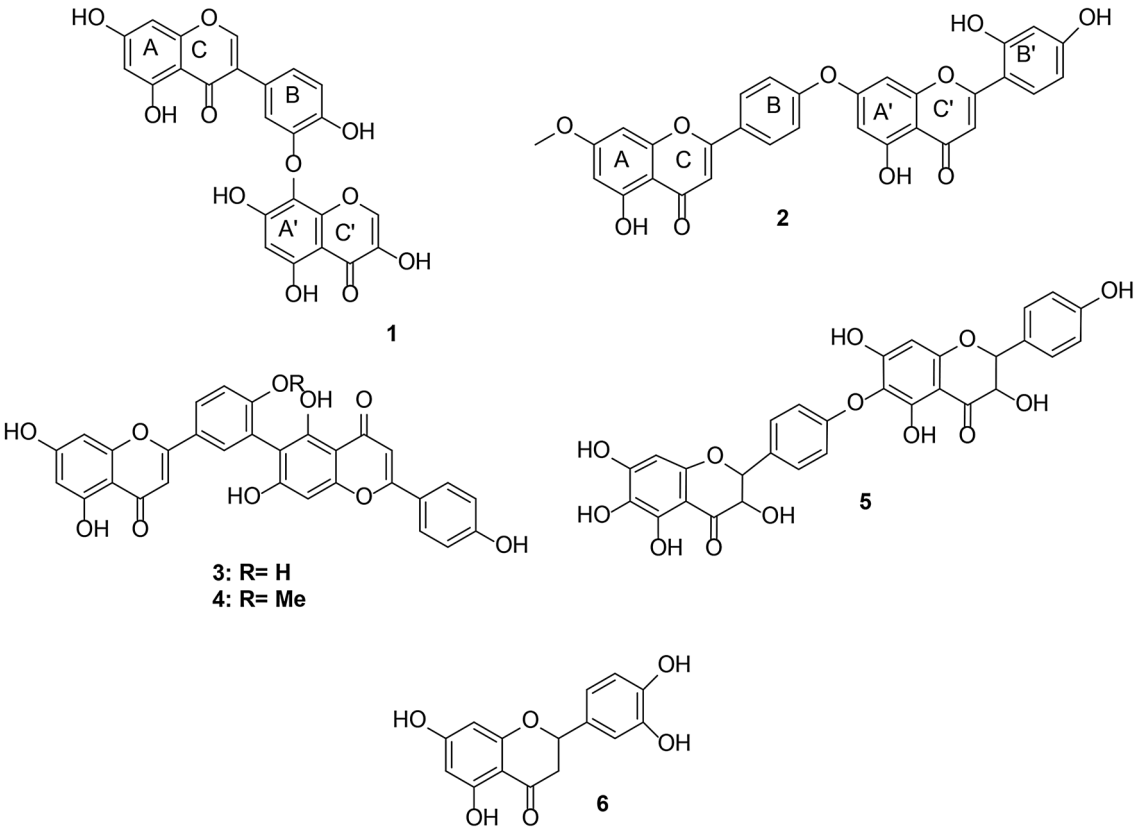

Fig. 1 Structures of compounds **1–6**

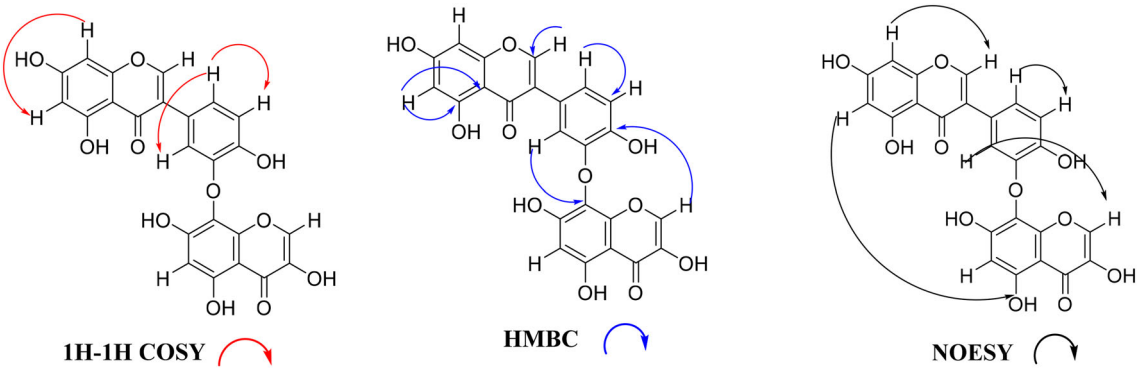

Fig. 2 Selected COSY, HMBC and NOESY correlations of Compound **1**

carbonyls (1638 cm^{-1}), hydroxyl groups (3424 cm^{-1}) and aromatic rings (1600 and 1498 cm^{-1}). The ^{1}H NMR spectrum showed a singlet at δ_H 8.37 (1H, s, H-2) indicating the presence of an isoflavone proton [8]. On the COSY spectrum, aromatic protons on ring A at δ_H 6.27 (1H, d, 2.1 Hz, H-6) and δ_H 6.46 (1H, d, 2.1 Hz, H-8) displayed *meta* coupling and were assigned to carbons C-6 and C-8 respectively. Three aromatic protons observed at δ_H 7.50 (1H, d, 2.22 Hz, H-2′), 7.13 (1H, dd, 8.46 Hz and 2.22 Hz, H-6′) and 6.65 (1H, brd, 8.46, H-5′) were attributed to carbons C-2′, C-6′ and C-5′ respectively of a trisubstituted benzynic ring (ring B). A singlet observed at δ_H 6.35 (1H, s, H-6″) was attributed to the proton on carbon C-6″ of a penta-substituted benzynic ring (ring A′).

In addition, a highly deshielded proton observed at δ_H 9.10 (1H, s, H-2″) suggested a chromone building block. The DEPT spectrum of **1** displayed signals for six hydroxylated carbons, six aromatic carbons and two

ethylenic carbons bearing an oxygen bridge to the pyran ring of the benzopyran moieties. We also noticed in its HMBC spectrum a correlation between H-2′ and C-8″ and the axis was assigned to be located at C-3′/C8″ by a typical downfield shift of C-8″ (119.0 ppm), along with an upfield shift at C-2′ (114.5 ppm) which further confirmed the ether linkage between the two derivatives via C-3′ and C-8″ (Table 1). This ether linkage was confirmed by selected HMBC, COSY and NOESY interactions (Fig. 2). From the above spectroscopic data leading to the structure shown for compound **1**, it is characterized as a novel isoflavone–chromone flavonoid C–O–C dimmer described for the first time and named brevipedicelone D.

Compound **2** was obtained as yellow powder in CH_2Cl_2/MeOH (10:1) mixture. The molecular formula was $C_{31}H_{20}O_{10}$ as deduced from HRTOFMS.

On its ^{1}H NMR spectrum, two tetra-substituted aromatic rings with two protons exhibiting *meta* coupling signals at δ_H 6.35 (1H, d, 2.8 Hz, H-6), δ_H 6.77 (1H, d, 2.8 Hz, H-8) and δ_H 6.81 (1H, d, 2.2 Hz, H-6″ and H-8″) were attributed to rings A and A′. A para di-substituted aromatic ring with signals exhibiting an AA'BB' spin system at δ_H 7.57 (2H, d, 8.8 Hz, H-2′ and H-6′), and δ_H 6.71 (2H, d, 8.8 Hz, H-3′ and H-5′) were attributed to ring B. Two isolated protons on a penta-substituted aromatic rings appeared at δ_H 6.82 (1H, s, H-3) and δ_H 6.89 (1H, s, H-3″) and were attributed to the flavone protons on C-3 and C-3″ of rings C and C′ respectively. A tri-substituted aromatic ring carrying three protons exhibiting an ABX spin system with signals at δ_H 8.04 (1H, d, 8.6 Hz H-2‴), δ_H 7.09 (1H.d, 8.6 Hz, H-3‴) and δ_H 6.89 (1H, d, 2.8 Hz, H-5‴) was attributed to ring B′. A singlet at δ_H 3.85 that integrated for three protons was attributed to the three protons of the CH_3O group.

On the DEPT spectrum, it was deduced that apart from the C-atom of the CH_3O group which is saturated and whose chemical shift appears at δ_C 56.0, all the other 30 carbon atoms of the molecule are sp^2 hybridized, with twelve methines (CH) [δ_C 102.4, 104.6, 94,3; 131.4, 121.4, 127.6, 102.8, 92.6, 128.1 (× 2), 115.6 (× 22)], eighteen quaternary (C) carbon atoms, ten of which carry an oxygen atom each (δ_C 154.4, 156.3 (× 2), 161.1, 160.5 (×2), 160.9, 163.5, 164.2, 165.0) and two carbonyls (δ_C 181.9 and 182.0) (Table 1).

The HMBC spectrum of **2** showed correlations between the two protons carried by ring B (H-2′ and H-6′ at δ_C 7.59) and the carbon atom at δ_C 164.5, (C-3, ring C). A first flavone sub-structure in **2** was deduced from correlations between proton H-3 (δ_H 6.82) and the C-4 (δ_C 181.9) carbonyl. Correlations were also observed on one hand between proton H-2‴ (ring B′) at δ_H 8.04 and H-3″ (ring C′) at δ_H 6.89 and on the other hand between the carbonyl carbon (C-4″ ring C′) at δ_C 182.0 and the proton H-3″ at δ_H 6.89 (ring C′). These led to the suggestion of a second flavone sub-structure in **2**.

On the NOESY spectrum of **2**, it was observed that there exist a correlation between H-3′ and H-5′ (δ_H 6.71, ring B) with H-6″ and H-8″ (δ_H 6.81, ring A′). This suggested that the linkage between the two flavonoid units in **2** is between C-4 (ring C) and C-7″ (ring A′). From the above spectroscopic data leading to the structure shown for compound **2**, it is characterized as a new ether biflavonoid derivative also described for the first time and named brevipedicelone E.

Biflavonoids, which are C–O–C dimmers, up to date still form a very small class of biflavonoids which for the moment have been characterized only in the Ochnaceae, Cycadaceae, Caprifoliaceae, Fabaceae and Lauraceae families. Representatives include three compounds reported from the Ochnaceae, i.e. ochnaflavone and 7″-methylochnaflavone [9], *lophirone* L [10], and *lophirone* O [11], four from the Cycadaceae, i.e. hinokiflavone [12], 7,7″-di-*O*-methyltetrahydrohinokiflavone [13], 2″,3″ dihydrohinokiflavone [14], and 2,3,2″,3″-tetrahydrohinokiflavone [15], two from the Caprifoliaceae, ioniflavone and 3′-methyl ioniflavone [16], two from the Fabaceae, beilschmieflavonoids A and B [17], and one in the Lauraceae, tepicanol A [18].

Even though the genus *Garcinia* is known as a major source of xanthones [19–26], and C–C linked flavonoid dimers [27–31], this is the first report on the characterization of a novel isoflavone–chromone flavonoid C–O–C dimmer, brevipedicelone D **(1)**, along with one new C–O–C biflavonoid derivative, brevipedicelone E **(2)**, in this genus and anti-onchocercal evaluation of compound **1** on the adult worms and microfilariae of *Onchocerca ochengi* and *Loa loa*. These characterized compounds are the first examples of flavonoid dimmers with constituent isoflavone–chromone or flavone–flavone sub-units in their structures, illustrating that the Clusiaceae family is rapidly emerging as a potential source of dimeric C–O–C flavonoids.

Compound **1** was screened on both adult and microfilaria worms of *O. Ochengi* and microfilariae (mfs) of *L. loa*. All cultures lasted for 120 h post addition of the compound. On the adult worms, the compound showed moderate activity at the highest concentration of 20 µg/ml. The compound inhibited *O. ochengi* microfilariae motility by 90% at the 20 µg/ml thus demonstrating activity at this juvenile form of the parasite. When screened on *L. loa* mfs, there was no activity at the highest concentration (Table 2). There was a dose-dependent response for the *O. ochengi* parasite that succumbed to the compound.

Table 1 ^{1}H and ^{13}C-NMR data of Compounds **1** and **2** in (δ in ppm, DMSO)

Compound **1**				Compound **2**			
Position	δ_C	Type of carbon	δ_H, mult., J(Hz)	Position	δ_C	Type of carbon	δ_H, mult., J(Hz)
2	154	CH	8.37 (1H,s)	2	165.0	C	–
3	122	C	–	3	101.5	CH	6.82 (1H, s)
4	180.7	C	–	4	182.0	C	–
5	161.7	C	–	5	160.5	C	12.12 (1H, s, OH)
6	99.1	CH	6.27 (1H,s, 2.10)	6	94.3	CH	6.35 (1H, d, 2.8)
7	164.4	C	–	7	167.3	C	–
8	93.9	CH	6.46 (1H,s, 2.10)	8	92.6	CH	6.77 (1H, d, 2.8)
9	157.8	C	–	9	156.3	C	–
10	104.5	C	–	10	104.6	C	–
1′	122	C	–	1′	127.6	C	–
2′	115.3	CH	7.50 (1H,d, 2.22)	2′/6′	128.1	CH	7.57 (2H, d, 8.8)
3′	146.6	C	–	3′/5′	115.6	CH	6.71 (2H, d, 8.8)
4′	147.7	C	–	4′	154.4	C	–
5′	115.4	CH	6.65 (1H,brd, 8.46)	2″	160.5	C	6.81 (1H, d, 2.2)
6′	119	CH	7.13 (1H, dd, 2.22 & 8.46)	3″	103.5	CH	6.81 (1H, d, 2.2)
2″	144.9	CH	9.12 (1H, s)	4″	181.9	C	–
3′’	135.7	C	–	5″	164.2	C	12.99 (1H, s, OH)
4″	178.2	C	–	2‴	131.4	CH	8.04 (1H, d, 8.6)
5″	160.9	C	–	3‴	121.4	CH	7.09 (1H, d, 8.6)
6″	98	CH	6.35(1H, s)	5‴	102.8	CH	6.89 (1H, d, 2.8)
7″	162	C	–	OCH3-7	56.0	CH_3	3.85 (3H, s)
8″	120.1	C	–				
9″	160.4	C	–				
10″	102.3	C	–				

Table 2 Anti-Onchocercal activity of Compound **1** on *O. ochengi* and *L. loa*

Concentration (μg/ml)	% Inhibition of formazan formation by *O. ochengi* adult worm	% Inhibition of *O. ochengi* microfilariae motility	% Inhibition of *L. loa* microfilariae motility
20	60	90	0
10	20	50	0
5	0	25	0
2.5	0	10	0
1.25	0	0	0
0.625	0	0	0

3 Experimental

3.1 General

The mass spectra (HRTOFMS) were measured in a time of flight mode spectrometers. The ^{1}H NMR spectra were registered on a 600 MHz NMR spectrometer with tetramethylsilane (TMS) as an internal standard; while ^{13}C NMR spectra were recorded on a 150 MHz NMR spectrometer using DMSO as solvent. Methyl, methylene and methine carbons were distinguished by DEPT experiments. Homonuclear ^{1}H connectivities were determined by using the COSY experiment. One-bond ^{1}H–^{13}C connectivities were determined with HMQC gradient pulse factor selection. Two and three bonds ^{1}H-^{13}C connectivities were determined by HMBC experiment. Chemical shifts were reported in δ (ppm) and coupling constants (J) were measured in Hz.

3.2 Plant material

The leaves of *Garcinia brevipedicellata* were harvested in August 2013 in Malande a village situated at 3 km from DIBANG sub-division in the Nyong et Kelle Division of the Centre Region, Cameroon and identified by Mr. Victor Nana, botanist of the Cameroon National Herbarium Yaounde, Cameroon where a voucher specimen (No.VN 2634) was deposited.

3.3 Extraction and Isolation

Air dried and ground leaves of *Garcinia brevipedicellata* (1.5 kg) were extracted using methanol. After evaporating the solvent the methanolic residue obtained was exhaustively fractionated into three, by extraction with three solvents in increasing polarity. First with ethyl acetate, followed with acetone and lastly with methanol. Only the ethyl acetate fraction (62 g) was investigated. This extract was divided into four equal portions A-D and each portion fractionated by size exclusion chromatography on Sephadex gel LH-20 and eluted with methanol. Sub-fractions were pooled together to obtain five main fractions. N_1 (6.1 g), N_2 (4.3 g), N_3 (34 g), N_4 (5.5 g) and N_5 (3.2 g). Fraction N_4 was purified twice by chromatography on a silica gel column with CH_2Cl_2/MeOH (10:1) to afford Compound **1** (52 mg). N_3 and N_2 were repeatedly purified on an open silica gel column with CH_2Cl_2/MeOH (10:1) followed by preparative thin layer chromatography to give Compounds **2** (10 mg), **3** (11 mg) and **4** (32 mg), **5** (8 mg), **6** (12 mg) and more of compound **3** (8 mg) respectively.

3.3.1 Brevipedicelone D (1)

Yellow amorphous powder, UV (MeOH): λmax (log ε) = 268 and 316 nm, IR (KBr): ν_{max}:1638, 3424, 1600 and 1498 cm^{-1} ^1H and ^{13}C NMR spectroscopic data, see Table 1; HRTOFMS (pos.) m/z 479.0588, ($M+H^+$) (calc. for $C_{24}H_{15}O_{11}$, 478.0536).

3.3.2 Brevipedicelone E (2)

Yellow powder, UV (MeOH): λmax (log ε) λmax 223 and 320 nm, IR (KBr): ν_{max}: 3234, 1638 1628 cm^{-1}), 1603 and 1508 cm^{-1}. ^1H and ^{13}C NMR spectroscopic data, see Table 1; HRTOFMS (pos.) m/z, 553.0960 ($M+H^+$) (calc. for $C_{31}H_{21}O_{10}$, 552.1122).

3.4 Anti-onchocercal Screening

3.4.1 Extraction of *Onchocerca ochengi* Adult Worms

Onchocerca ochengi adult worm masses were extracted from cattle skin by the method employed in [32]. Briefly, fresh pieces of umbilical cattle skin containing palpable nodules were obtained from local slaughterhouse in Buea, Cameroon. The piece of skin was immediately transported to laboratory. The skin was thoroughly washed with soap and distilled water, drained, dried by blotting with a piece of cloth and then transferred to a sterile laminar flow hood. It was then entirely covered with 70% ethanol and allowed to evaporate completely on its own. The nodules were carefully dissected using a sterile razor blade and the pale orange-yellow worms (in appearance) were immediately submerged in sterile 12-well culture plates (NUNC, USA) containing 2 ml of complete culture medium [RPMI-1640 supplemented with 25 mM HEPES, 2 g/L sodium bicarbonate, 20 mM L-glutamine, 10% new born calf serum (SIGMA, USA), 2 × antibiotic–antimycotic (Sigma, USA)], pH 7.4]. After overnight culture, 1 mL of medium was added before addition of drug making a total volume of 3 mL. Adult worm cultures were carried out at 37 °C under an atmosphere of 5% CO_2 in humidified air in an incubator (HERACell 150, Haraeus, Germany).

3.4.2 Extraction of *Onchocerca ochengi* Microfilariae

The microfilariae of *O. ochengi* were extracted by the method of [33], with some slight modifications. Cattle skin got from the slaughterhouse was thoroughly cleaned and sterilized as above. The skin was then firmly attached onto a sterilized flat wooden board using autoclaved thumbnails. The outer surface was carefully shaved with a sterile razor blade, and then rinsed twice with distilled water. A clean dry adsorbent cloth was used to remove excess moisture from the skin. The entire skin was covered with 70% ethanol and allowed to evaporate in a laminar flow hood. This sterilization process was done twice. Once the alcohol had completely evaporated from the skin, skin snips were obtained from different locations of the skin. These sleeves were carefully scrapped and the snips submerged in 15 mL of complete culture medium. The assemblage was incubated at room temperature for 2 h to allow for emergence of microfilariae. The highly motile microfilariae that emerged were concentrated by centrifugation at 400×*g* for 10 min. The supernatant was decanted, and the pelleted mfs were re-suspended in fresh complete culture medium. The highly motile microfilariae were quantified using an inverted microscope (Euromex, Holland). One hundred microlitres of culture medium containing microfilariae

were distributed into 96 well culture plate containing LLC-MK2 cell layers to obtain an average of 12–15 mfs per well. Culture conditions were the same as that of adult worms above.

3.4.3 Isolation and Culture of *Loa loa* Microfilariae

Blood was collected from *Loa loa* infected individuals at the Edea Health District after confirmation by Giemsa stain. The blood was rapidly transferred to the University laboratory. The microfilariae were isolated by the method of [32]. Freshly collected *L. loa* -infected blood was diluted (1:2) with culture media used above but without sera. The diluted blood was carefully layered on 4 mL of Ficoll-pacque (TM) in a 15 mL centrifuge tube. The tube was spun in a swing bucket centrifuge at $400\times g$ for 15 min. The recovered mfs were washed three times with culture media (without sera) and then re-suspended in media containing sera. The mfs were then distributed in wells of a 96-well microtiter plate containing LLC-MK2 cell layers. Each well contained 12–15 mfs in 100 of media.

3.4.4 Preparation of Compound and Assessment of Activity

Compound **1** was dissolved in $\geq$ 99.9% sterile dimethyl sulfoxide (SIGMA, USA) giving a stock concentration of 5 µg/ml. The compound was prepared at 2× the final concentration and distributed to wells containing parasites. For the microfilariae, 100 µL was added while 1 mL was added to wells containing adult worms to give a final volume of 200 µL and 4 mL for microfilariae and adult worms respectively. All the cultures were conducted for 120 h post addition of the compound. Auranofin [34], served as positive control for adult worm assay while ivermectin and amocarzine were used as positive control drugs for *O. ochengi* and *L. loa* mfs respectively. The diluent (dimethyl sulfoxide) was added to the negative control wells. Inhibition of microfilariae motility was assessed using an inverted microscope. Effect of compound on adult worm viability was assessed using the MTT-formazan assay following procedures employed by [32, 33].

3.4.5 Ethical Issues

We got ethical clearance (2013/11/371/L/CNERSH/SP) and administrative authorization (631–06.14) from respectively, the Cameroon National Ethical Committee and the Ministry of Public Health, Cameroon. Local administrative authorization was also obtained from the District Medical Officer of the Edea Health District. Informed consent was obtained freely from individuals who harbored high *L. loa* mf load.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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