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PHYTOCHEMISTRY AND QUORUM SENSING INHIBITORY STUDIES OF FOUR *VERNONIA* SPECIES GROWING IN NIGERIA

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by

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Supervisors: Dr Brenda Moodley and Professor Neil A. Koorbanally

PREFACE

The research contained in this thesis titled "**Phytochemistry and quorum sensing inhibitory studies of four Vernonia species growing in Nigeria**" was completed by the candidate while based in the Discipline of Chemistry, School of Chemistry and Physics of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville, South Africa. The research was financially supported by the University of KwaZulu-Natal, Durban, South Africa and the Tertiary Education Fund (TETFUND) Abuja, Nigeria.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

Abubakar Babando Aliyu

As supervisors of the candidate, Mr Abubakar Babando Aliyu, we approve the final thesis for submission:

Dr Brenda Moodley, PhD (UKZN)

Professor Neil A. Koorbanally, PhD (Natal)

Date: 30th October 2017

DECLARATION 1: PLAGIARISM

I, Abubakar Babando Aliyu, declare that:

(i) the research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work;

(ii) this dissertation has not been submitted in full or in part for any degree or examination to any other university;

(iii) this dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons;

(iv) this dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a) their words have been re-written but the general information attributed to them referenced;

b) where their exact words have been used, their writing has been placed inside quotation marks, and referenced;

(v) where I have used material for which publications followed, I have indicated in detail my role in the work;

(vi) this dissertation is primarily a collection of material, prepared by myself, published as journal articles or presented as a poster and oral presentations at conferences. In some cases, additional material has been included;

(vii) this dissertation does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the References sections.

Signed: Abubakar Babando Aliyu

Date: 30th October 2017

DECLARATION 2: PUBLICATIONS

I declare that I carried out all the experimental work in the manuscripts below and submitted drafts of all manuscripts to the other authors involved, whose primarily role was supervision of the scientific studies, ensuring that proper scientific protocols were adhered to and that the results presented were as accurate as possible.
The corresponding author is indicated by an * and indicates the author responsible for submitting the manuscript and corresponding with the editors of these journals.

Chapter 2
Aliyu, A.B., Moodley, B., Koorbanally N.A.* 2016. Sesquiterpene lactones from the genus *Vernonia* (Asteraceae): A review. Manuscript prepared for *Chemistry and Biodiversity*.

Chapter 3
Aliyu, A.B., Moodley, B., Chenia, H., Koorbanally, N.A.* 2015. Sesquiterpene lactones from the aerial parts of *Vernonia blumeoides* growing in Nigeria. *Phytochemistry*, 111, 163-168.

Chapter 4

i. Aliyu, A.B., Koorbanally, N.A., Moodley, B., Singh, P., Chenia, H.* 2016. Quorum sensing inhibitory potentials and molecular docking studies of sesquiterpene lactones from *Vernonia blumeoides*. *Phytochemistry*, 126, 23-33.

ii. Aliyu, A.B*., Koorbanally, N.A., Moodley, B., Chenia, H. 2016. Quorum sensing inhibitory potentials of sesquiterpene lactones isolated from *Vernonia blumeoides* (Asteraceae). A poster presentation to the Nigerian Society of Pharmacognosy (NSP) Scientific Conference 2016, 24th to 27th February, 2016, Abuja, Nigeria.

Chapter 5
Aliyu, A.B., Moodley, B., Chenia, H., Koorbanally, N.A.* 2016. Sesquiterpene lactones from *Vernonia perrottetii* and their quorum sensing inhibitory activity. Manuscript prepared for *Phytochemistry Letters*.

Chapter 6
Aliyu, A.B., Moodley, B., Chenia, H., Koorbanally, N.A.* 2016. Bioactive compounds from *Vernonia ambigua* and *Vernonia glaberrima* as quorum sensing inhibitors. Manuscript prepared for *International Journal of Molecular Sciences*.

Signed: Abubakar Babando Aliyu

Date: 30th October 2017

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Lastly, to my parents for their prayers and goodwill, my family for their patience and understanding and to my elder brothers: Alhaji Yunusa Aliyu Babando and Mallam Yakubu Aliyu for their unrelenting efforts at supporting my studies from my high school days to date.

List of Abbreviations

^{1}H NMR	proton nuclear magnetic resonance spectroscopy
^{13}C NMR	C-13 nuclear magnetic resonance spectroscopy
2D-NMR	two-dimensional nuclear magnetic resonance spectroscopy
AHL	acyl homoserine lactone
AMP10	ampicillin (10 micrograms)
ANOVA	analysis of variance
br	broad resonance
c	concentration
$CDCl_3$	deuterated chloroform
cm	centimeter
COSY	correlated spectroscopy
CV	chromobacterium violaceum
d	doublet
dd	doublet of doublets
DCM	dichloromethane
DEPT	distortionless enhancement by polarization transfer
DMSO-d_6	deuterated dimethyl sulfoxide
EtOAc	ethyl acetate
GC-MS	gass chromatography-mass spectrometry
Hex	hexane
HMBC	heteronuclear multiple bond coherence
HPLC	high performance liquid chromatography
HREIMS	high resolution electron impact mass spectrometry
HRMS	high resolution mass spectrometry
HSL	homoserine lactone
HSQC	heteronuclear single quantum coherence
Hz	hertz
IR	infrared
LB	Luria Bertani media
m	multiplet
MeOH	methanol

MDR	multi-drug resistant
MH	Mueller Hinton agar
MHz	megahertz
MIC	minimum inhibitory concentration
Mp	melting point
MRSA	methicillin resistant *Staphylococcus aureus*
NOESY	nuclear overhauser effect spectroscopy
OD	optical density
PAO2	*Pseudomonas aeruginosa*
QS	quorum sensing
QSI	quorum sensing inhibition
s	singlet
SLs	sesquiterpene lactones
TET30	tetracycline (30 micrograms)
t	triplet
TLC	thin layer chromatography
TOF-MS	time-of-flight mass spectrometry
TSA	tryptic soy agar
UV	ultraviolet
UV-vis	ultraviolet-visible spectroscopy
VA	*Vernonia ambigua*
VBL	*Vernonia blumeoides*
VG	*Vernonia glaberrima*
VP	*Vernonia perrottetii*
WHO	World Health Organization

Abstract

This thesis contains the phytochemical analysis of four medicinal *Vernonia* species growing in Nigeria and used in ethnomedicine to treat a variety of medical conditions. The four *Vernonia* species studied were *Vernonia blumeoides*, *Vernonia perrottetii*, *Vernonia ambigua* and *Vernonia glaberrima*. The thesis also contains a comprehensive review of the sesquiterpene lactones from the genus *Vernonia*, their structural diversity and biosynthetic considerations. For each of the plants, the crude extracts and selected compounds were tested for their antibacterial activity using the traditional disc diffusion and broth microdilution as well as anti-quorum sensing assays.

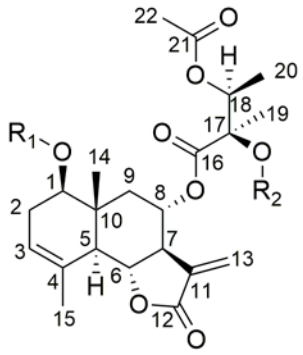
Three of the four plants studied yielded sesquiterpene lactones, *Vernonia blumeoides* yielded four novel eudesmanolide sesquiterpene lactones (blumeoidolides A-D), *Vernonia perrottettii* yielded a novel keto-hirsutinolide 13-acetoxy-1(4*β*),5(6)*β*-diepoxy-8*α*-(senecioyloxy)-3-oxo-1,7(11)-germacradiene-12,6-olide **B1** and the known keto-hirsutinolide 13-acetoxy-1,4*β*-epoxy-8*α*-(senecioyloxy)-3-oxo-1,5,7(11)-germacratriene-12,6-olide **B2** and *Vernonia ambigua* yielded a novel glaucolide sesquiterpene, 5,6-dehydrobrachycalyxolide. The structures of the sesquiterpenes were determined from their ^{1}H, ^{13}C and 2D NMR spectra along with mass spectra. The crystal structure of one of the eudesmanolide sesquiterpenes allowed the configuration of the stereocentres in the molecule to be determined.

In addition to the sesquiterpene lactones, some common sterols and flavonoids were isolated from the plants: stigmasterol was isolated from *V. blumeoides*, lupeol was isolated from *V. blumeoides*, *V. ambigua* and *V. perrottettii* and lupeol acetate from *V. ambigua* and *V. perrottetti*. The flavonoid apigenin was found in *V. blumeoides*, *V. perrottetti* and *V. glaberrima*, luteolin in *V. blumeoides* and *V. perrottetti*, velutin in *V. perrottetti* and *V. glaberrima* and chrysoeriol in *V. ambigua* and *V. glaberrima*. Chrysin was found only in *V. blumeoides* and luteolin 3',4'-dimethyl ether in *V. glaberrima*.

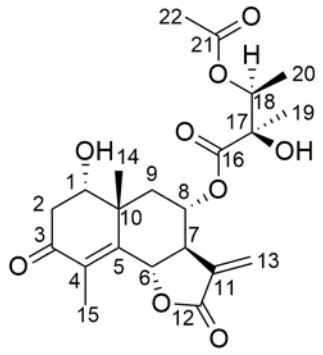
Several of the isolated sesquiterpene lactones showed good anti-quorum sensing inhibition (QSI). QSI ≥80% was obtained for blumeoidolide A at a concentration ≥ 0.071 mg mL^{-1}, blumeoidolide B (≥ 3.6 mg mL^{-1}) and **B1** (1.31 mg mL^{-1}), QSI ≥75% for **B2** (0.33 mg mL^{-1}) and QSI ≥84% for 5,6-dehydrobrachycalyxolide (2.6 mg mL^{-1}). The sterols, lupeol and lupeol acetate, were also found to have QSI ≥84% at 2.6 mg mL^{-1}. Molecular docking studies carried out on blumeodolides A-D in the binding sites of CviR and CviR' (transcription activator proteins) suggested that these molecules are able to bind to certain domains in the target protein, thus eliciting an effect.

The current work adds to the library of sesquiterpene lactones from the genus *Vernonia* and provides some lead compounds to antibacterial activity via quorum sensing inhibition.

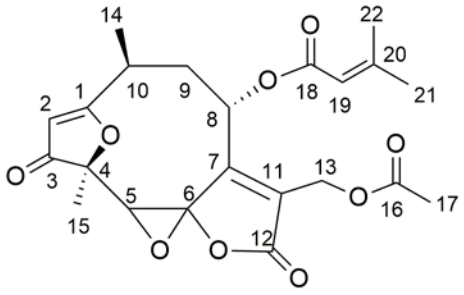
Structures of sesquiterpenoids isolated from *Vernonia* species in this work:



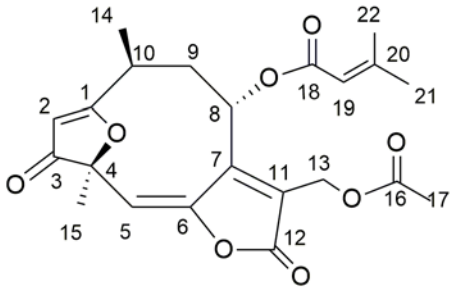
blumeoidolide A, R_1 = C(O)CH_3, R_2 = H
blumeoidolide B, R_1 = H, R_2 = C(O)CH_3
blumeoidolide C, R_1 = H, R_2 = H



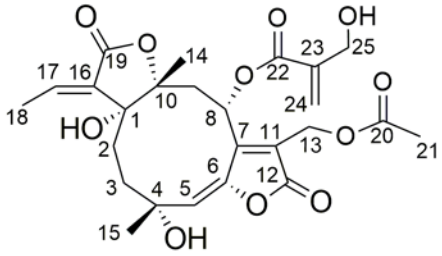
blumeoidolide D



B1



B2

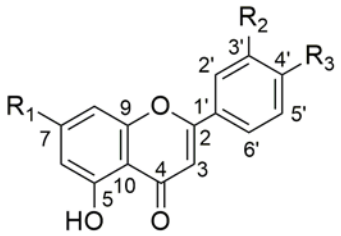


5,6-dehydrobrachycalyxolide

B1 = 13-acetoxy-1(4*β*),5(6)*β*-diepoxy-8*α*-(senecioyloxy)-3-oxo-1,7(11)-germacradiene-12,6-olide

B2 = 13-acetoxy-1,4*β*-epoxy-8*α*-(senecioyloxy)-3-oxo-1,5,7(11)-germacratriene-12,6-olide

Structures of flavonoids isolated from *Vernonia* species in this work:



chrysin R_1= OH, R_2 = H, R_3 = H

apigenin R_1 = OH, R_2 = H, R_3 = OH

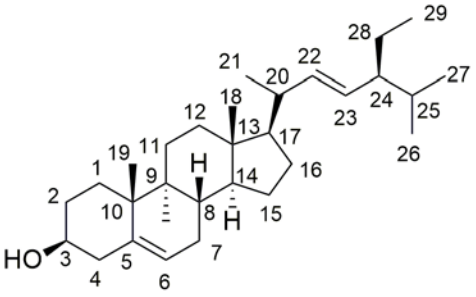
luteolin R_1 = OH, R_2 = OH, R_3 = OH

chrysoeriol R_1 = OH, R_2 = OCH_3, R_3 = OH

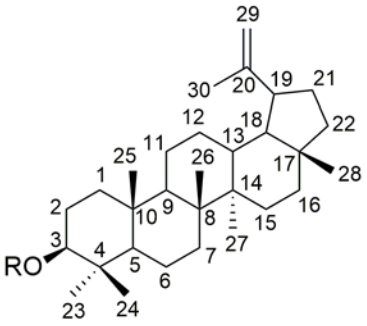
luteolin 3',4'-dimethyl ether R_1 = OH, R_2 = OCH_3, R_3 = OCH_3

velutin R_1 = OCH_3, R_2 = OCH_3, R_3 = OH

Structures of sterols isolated from *Vernonia* species in this work:



stigmasterol



lupeol R = H

lupeol acetate R = $OC(O)CH_3$

A note on numbering used in the thesis

In each chapter, compounds are numbered using arabic numerals, **1**, **2**, **3** etc. In the supporting information, the numbers for the compounds in Chapter 3 are preceded by an **A**, Chapter 5 a **B** and Chapter 6 a **C**. Hence, the spectra for a particular compound may appear in duplicate in the appendices. These however are different samples from different plants and provide proof of isolation of the compound from the plant.

In the abstract and conclusion, common names are used for compounds where available. Where compounds do not have a common name and when the name is too long or complicated, a code equivalent to the numbering **A1**, **A2**, **B1**, **B2** etc. according to the numbering for the appendices is used.

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CHAPTER 1 INTRODUCTION

1.1 The Genus *Vernonia* Schreb. (Asteraceae)

Vernonia is the largest of the ninety-eight (98) genera belonging to the Vernonieae tribe - one of the thirteen (13) tribes that constitute the Asteraceae family, representing one of the largest families of flowering plants (Keeley and Turner, 1990; Jones, 1977). According to Bremer (1994), *Vernonia* comprises approximately 500 species distributed in tropical regions of the world especially Africa and South America. There are approximately 20 species in the USA and about 200 species growing in Brazil (Lopes, 1991, Jones, 1977). In West Africa, 60 species have been reported, of which, 37 were recorded in Nigeria (Hutchinson and Dalziel, 1963). *Vernonia* can therefore be described as a pan tropical genus found throughout the Americas, Africa and Asia (Craig, 2003). It was speculated that *Vernonia* had only two centers of origin; one in Africa and the other in South America. Similar phytoconstituents such as sesquiterpene lactones and flavonoids within species from the two centers support their geographical origin (Harborne and Williams, 1977).

Vernonia species are generally perennial trees, shrubs or annual herbs grown across diverse ecological environments (Ayodele, 1999). Several of them are vegetables and used as medicinal herbs for the treatment of various human and animal diseases (Yeap *et al*., 2010). Approximately 109 *Vernonia* species are reportedly used in herbal medicine across the world. The use of *Vernonia* species as potential sources of modern medicine, their biological activities (*in vitro* and *in vivo*) and toxicity profiling have been reviewed in great detail (Toyang and Verpoorte, 2013).

Vernonia ambigua Kotschy & Peyr.

This plant is described as a herb, approximately 18 inches high with mauve coloured flowers (**Figure 1.1**). It is distributed across North tropical Africa and Angola (Hutchinson, 1921). In Northern Nigeria, *Vernonia ambigua* is used as a remedy for cough, fever and malaria (Kunle and Egharevba, 2009).

Figure 1.1 *V. ambigua* in natural habitat
Photograph by Umar Gallah: Department of Biological Sciences,
Ahmadu Bello University, Zaria, Nigeria

Vernonia blumeoides: Hook f.

V. blumeoides is a perennial herb found in abandoned fields across Northern Nigeria. It is 2-4 feet high with leaves shortly attached to the stems, with purple flower heads (**Figure 1.2**). It is widely used as a remedy for stomach-pain and malaria (Aliyu *et al.*, 2011).

Figure 1.2 *V. blumeoides* in natural habitat
Photograph by Umar Gallah: Department of Biological Sciences,
Ahmadu Bello University, Zaria, Nigeria

Vernonia glaberrima Welw. ex O. Hoffm.

V. glaberrima is an erect shrub with a height of 3-4 feet and containing white flower heads. Its distribution ranges across grasslands in Guinea to Northern Nigeria, Western Cameroon and Central Africa to Angola (Hutchinson, 1921). It is used as an anti-malarial, analgesic, anti-inflammatory, and anti-microbial herb (Abdullahi *et al*., 2015).

Vernonia perrottetii Sch. Bip. ex Walp.

This plant is an annual herb, 60 cm in height with leaves 1-3 cm long and purple flower heads. It is distributed from North to South in tropical Africa. In Northern Nigeria, it is commonly found in abandoned fields and widely used as purgative agents for gastrointestinal problems (Hutchinson *et al*., 1963; Burkill, 1985).

1.2 The phytochemistry of *Vernonia*

The phytochemistry of the genus *Vernonia* was probably reported for the first time in 1967 when vernolide was isolated from *Vernonia colorata* (Toubiana and Gaudemer, 1967). Subsequently, the report of vernolepin and vernomenin from *Vernonia hymenolepsis* (Kupchan *et al*., 1968) was followed by cytotoxic vernodalin and vernomygdin from *Vernonia amygdalina* (Kupchan *et al.*, 1969). Since then, phytochemical studies on *Vernonia* species increased. Several species across the world were investigated, from which triterpenoids, flavonoids, coumarins, steroidal or saponin glycosides, polyacetylenes and sesquiterpene lactones (SLs) were reported (Igile *et al*., 1995; Malafronte *et al*., 2009; Toyang and Verpoote, 2013). Triterpenoids, flavonoids and sesquiterpene lactones are the major compounds frequently reported from *Vernonia* with considerable structural diversity. Of the different types of triterpenoids isolated from *Vernonia*, the oleanane, ursane, taraxarane, friedelane and friedoursane types have similar structures differing only in the

position of methyl groups and double bonds (**Figure 1.3**). Lupane type triterpenoids are somewhat different from these, having a five-membered ring attached to an isopropyl group. Fasciculatol, a triterpenoid containing a dihydrofuran ring, is thus far the only triterpenoid of this class isolated from *Vernonia* (**Figure 1.3**). However, the most commonly isolated triterpenoids from the *Vernonia* species are lupeol, *α*-amyrin and *β*-amyrin (Kiplimo, 2012).

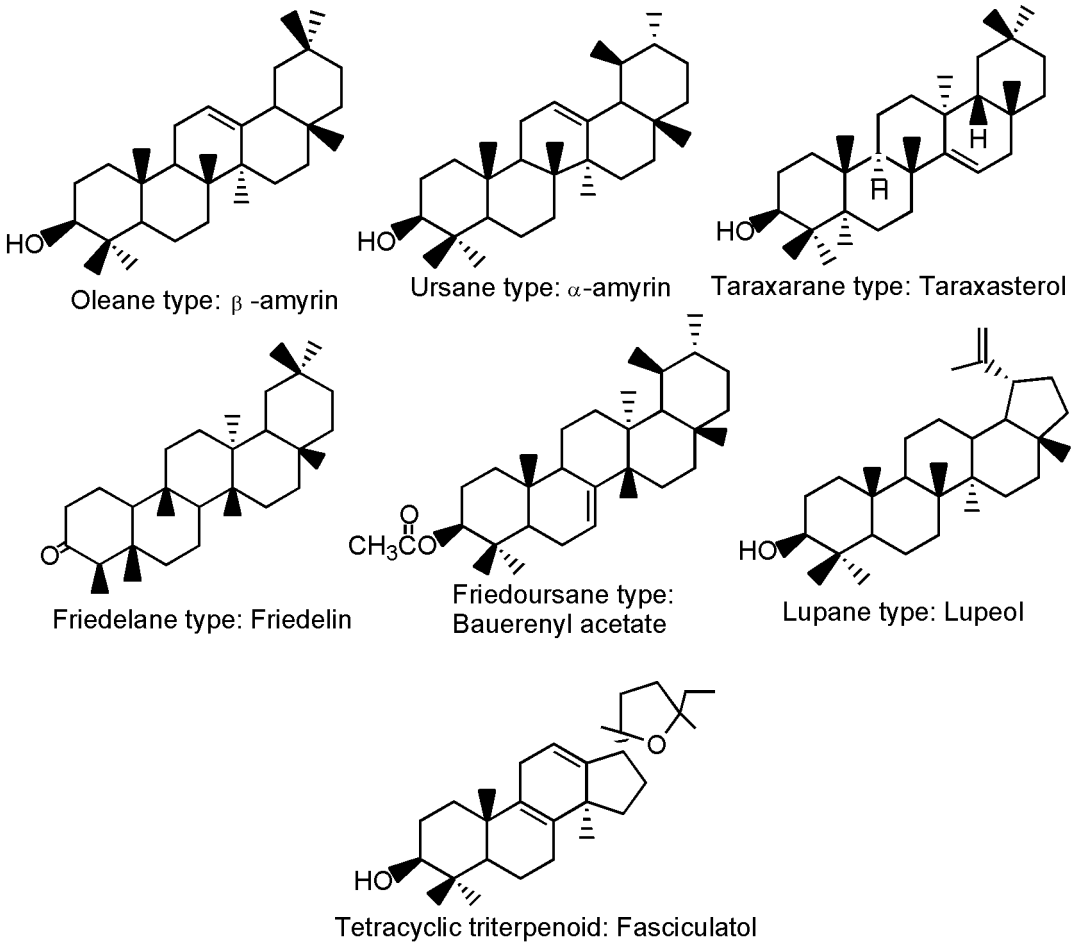

Figure 1.3 Triterpenoids of the various skeletal-types isolated from *Vernonia* species

The flavonoids are another class of compounds commonly found in *Vernonia* and in the Asteraceae, in general. *Vernonia* was found to contain mainly glycosides of luteolin, kaempferol, apigenin and quercetin (**Figure 1.4**). Several *O*-methylated flavones and flavonols are also commonly found in many *Vernonia* species (Bohm and Stuessy, 2001).

African *Vernonia* species produced mainly flavones while South American species contained flavonols or flavones or both (Harborne and Williams, 1977). Flavanones were also reported in several species including *Vernonia brevifolia, Vernonia hindei* and *Vernonia syringifolia* (Bohm and Stuessy, 2001). However, only a few *Vernonia* species contain alkylcoumarin derivatives (Bohlmann and Jakupovic, 1990) (**Figure 1.4**).

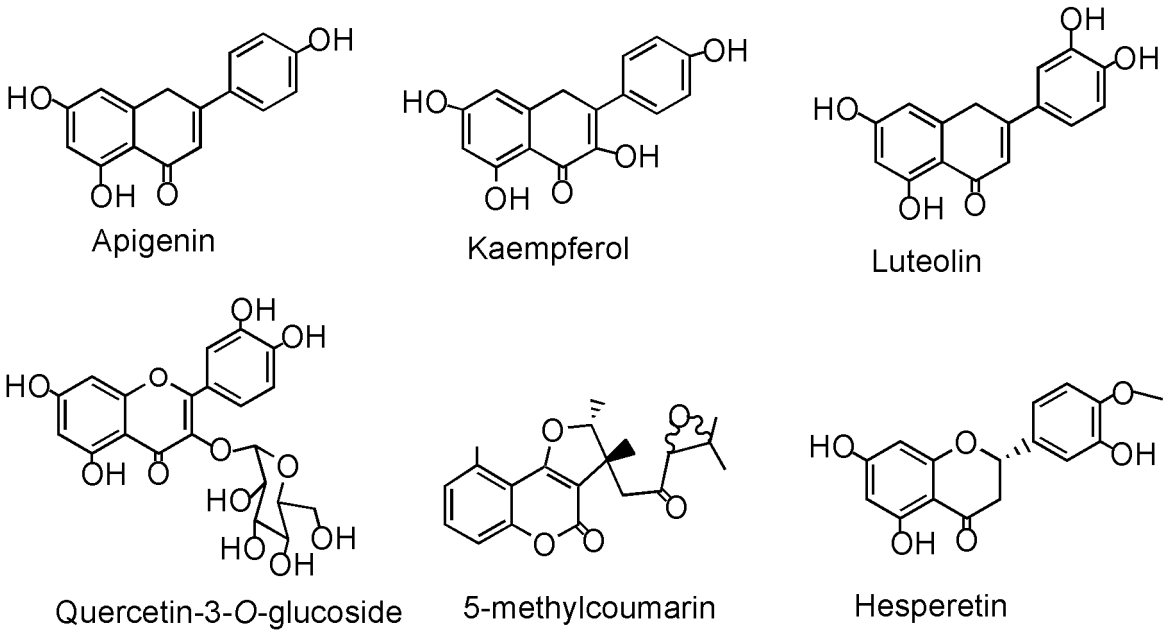

Figure 1.4 Flavonoids of the different skeletal types and a coumarin from *Vernonia*

Sesquiterpene lactones are ubiquitous in most species within *Vernonia*. As such, they are useful as chemotaxonomic markers in the Asteraceae family and an important class of secondary metabolites due to their widespread biological activities. Due to differences in biosynthetic pathways, there are four major skeletal types of sesquiterpene lactones; germacranolides, guiainolides, eudesmanolides and elemanolides (**Figure 1.5**). The germacranolides are the largest and, structurally, most diverse having sub-classes as glaucolides, non-glaucolides, hirsutinolides and cardinanolides. Miscellaneous sesquiterpene lactones in *Vernonia* are derived from germacranolides via transformations into

bourbonenolides (Bohlmann *et al*., 1981), vernonallenolides (Jakupovic *et al*., 1986) and potamopholides (Bila *et al*., 2003) (**Figure 1.6**).

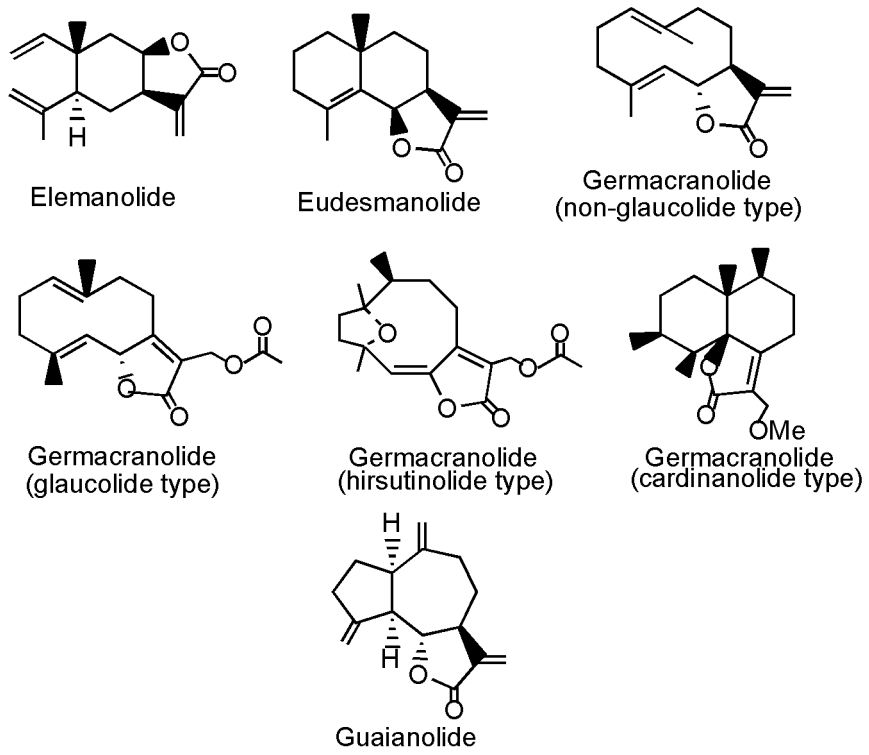

Figure 1.5 Sesquiterpene lactones skeletal types from *Vernonia*

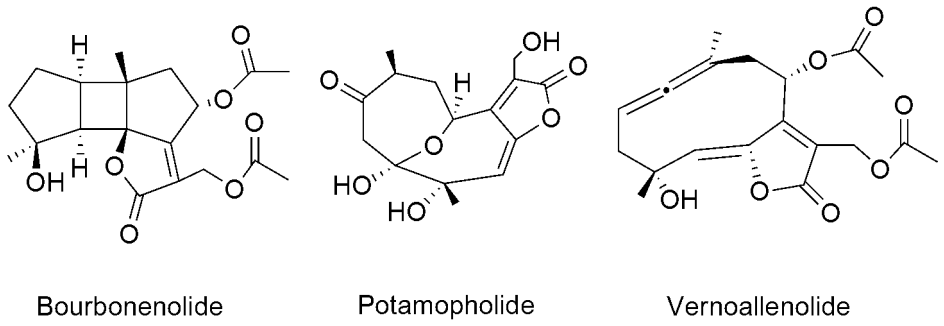

Figure 1.6 Miscellaneous sesquiterpene lactones from *Vernonia*

The phytochemistry of *Vernonia* is diverse; however, very little information is known or understood about the therapeutic potential of several hundreds of chemical compounds isolated from the genus. Of the 103 bioactive compounds from *Vernonia*, as much as 70% are sesquiterpene lactones (Toyang and Verpoote, 2013), emphasising the need to explore the

structural diversity of sesquiterpene lactones from *Vernonia* in the search for new and effective lead compounds with potential pharmacological or medicinal value.

1.3 Antimicrobial drug resistance

Drug resistant infectious diseases are a public health problem due to increasing incidences of multi drug resistance (MDR) pathogens. This situation continues to threaten antimicrobial chemotherapy especially in developing countries (WHO, 2014). The search for new antibiotics to combat the problem of MDR has therefore intensified in recent years. An example of this can be seen in the search for natural compounds effective as inhibitors of bacterial efflux pumps (Stavri *et al*., 2007). Inhibition of bacterial efflux pumps are known to inhibit the proliferation of MDR bacteria (Marquez, 2005). Drug combination strategies involving plant constituents in combination with antibiotics have demonstrated good activity against drug resistant pathogens. Several pure compounds from plant sources have exhibited potentiating effects on antibiotics against drug resistant microorganisms (Gibbons, 2005). Recent research efforts toward the suppression of bacterial virulence using secondary metabolites from plants or microorganisms have proven successful (Teasdale *et al.*, 2009; Martín-Rodríguez *et al*., 2014; Kim Ta and Arnason, 2016). The discovery of antibacterial agents with new targets or novel mechanisms, distinct from currently used antibacterial drugs, can lead to novel active pharmaceutical ingredients to combat drug resistant pathogens (Zhang and Dong, 2004). One such mechanism is quorum sensing (QS), a technique which has attracted much interest.

1.4 Quorum sensing

Quorum sensing (QS) is a bacterial communication system where signal molecules are involved in the co-ordination of group behaviour of intercellular bacteria to achieve biological functions. In Gram negative bacteria, signal molecules such as acyl homoserine

lactones (AHLs) are constantly produced, diffused and accumulated out of the cell. Upon a critical threshold concentration or population density, these molecules bind to specific receptor proteins of neighbouring bacterial cell walls activating transcription of gene expressions used in biofilm formation, resistance production, virulence expressions, bioluminescence, motility, swarming and pigment production, among others (Cámara *et al*., 2002). Gram positive bacteria such as *Staphylococcus aureus* have been found to use oligopeptide signal molecules to achieve QS (Lyon and Novick, 2004). However, the AHL mediated QS systems in Gram -ve bacteria have received greater attention (Geske *et al*., 2008) probably since 70 species of Gram -ve bacteria produce AHLs (Surrette and Bassler, 1998; Atkinson *et al*., 1999; Smith and Iglewski, 2003).

The AHLs- based QS consist of a four-component circuit: (**1**) LuxI-type signal synthase, (**2**) AHLs signal molecule, (**3**) LuxR-type signal receptor and (**4**) the target genes. The signal synthase is an enzyme responsible for the synthesis of AHL-signal molecules at low basal concentration; the AHL molecules are fatty acid acyl chains linked through an amide bond to homoserine lactones (HSLs), differing in the length and oxidation of the acyl side chain (Nisha *et al*., 2013) (**Figure 1.7**). The LuxR-type receptor functions as mediator of signal molecules to transcriptional regulator, LasR, for activation of gene expression (Pearson *et al*., 1997). However, the target genes are intricately related to genes encoding signal synthase, a situation that allows for a continuous response system in which signal production and dissemination become rapidly enhanced (Hentzer and Givskov, 2003). QS is therefore crucial for bacterial cellular functions and survival.

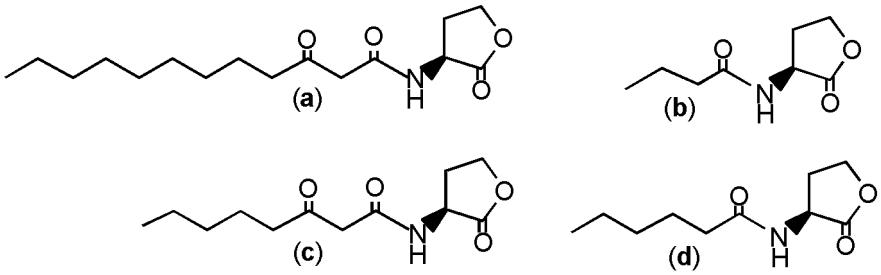

Figure 1.7 AHL-QS signal molecules: (**a**) *Pseudomonas aeruginosa*: 3-oxo-C12-HSL (PAO1), (**b**) *Psedomonas aeruginosa*: C4-HSL (PAO2), (**c**) *Agrobacterium tumefaciens*: 3-oxo-C8-HSL and (**d**) *Chromobacterium violaceum* C6-HSL**.**

1.5 Biological screening

Discovery of potential QS inhibitors depend largely on screening methods. Several techniques have been developed based on the behaviour of bacterial QS including bioluminescence, swarming, motility and pigment production. The commonly used strategy involves biosensor strains containing a reporter gene/operon under QS-controlled LuxR-type promoter. *Chromobacterium violaceum* (CV ATCC 12472) is an opportunistic human pathogen known to produce purple pigment (due to operon-violacein) under QS-control. A mutant of *C. violaceum* CV026 cannot produce its own AHL signals but can respond to exogenous active signal molecules, which interact with CviR to produce purple pigmentation (McLean *et al*., 2004). However, in the presence of QS inhibitors such as plant extracts; expression of reporter gene/operon decreases or is completely eliminated. This can be read quantitatively as absorbance using spectrophotometry. This inhibition of violacein production resulting from loss of purple pigmentation has identified CV organisms as important tools for screening QS inhibitors (Adonizio *et al*., 2006; Rasmussen and Givskov, 2006). CV biosensors are however only able to detect AHLs with acyl chains of four to eight carbons in length and cannot respond to C4-3-oxo-AHL or AHLs with C10 and longer acyl chains (Steindler and Venturi, 2007). This limitation is overcome by using a biosensor with a broader range of AHL detection.

The AHLs biosensors based on the TraI/R QS system have a broad range of detection with significant sensitivity. An example of such a strain is *Agrobacterium tumefaciens* (Steindler and Venturi, 2007). *A. tumefaciens* is a plant pathogen with AHLs mediated QS system. The strain of *A. tumefaciens* A136 expresses *β*-galactosidase under QS control and in response to AHL molecules secreted by the AHL over producer KYC6. In the presence of QS inhibitory compounds, a decrease in the formation of *β*-galactosidase resulting from decreased X-gal hydrolysis is indicated by blue pigmentation, qualitatively observed in a double ring assay (Chenia, 2013). In AHLs mediated QS systems, the signal vulnerability arising from AHL synthesis, dissemination or reception has provided the opportunity to manipulate QS as a novel anti-infective strategy.

1.6 Quorum sensing inhibitors from plants

The search for QS inhibitors from plant sources is attractive due to the numerous plant extracts, dietary phytochemicals and essential oils exhibiting QS inhibition (QSI) with significant activity (Vattem *et al*., 2007; Al-Hussaini and Mahasneh, 2009; Szabó *et al*., 2010; Koh *et al*., 2013; Tolmacheva *et al*., 2014). Since phytochemicals have diverse chemical and biological effects on disease therapy, they could also be viable sources of QS inhibitors. Presently, the most effective natural QS inhibitors are brominated furanones (**Figure 1.8**) isolated from the marine macro alga *Delisea pulchra*. Unfortunately, these furanones are not suitable as antipathogenic agents due to their toxicity (Manefield *et al*., 1999; Bottomley *et al*., 2007).

Higher plants are a natural reservoir of bioactive compounds and chemical compounds isolated from plants are increasingly being identified as potential QS inhibitors. Sesquiterpene lactones of the goyazensolide-type isolated from the Argentine *Centratherum*

punctatum demonstrated significant QS inhibitory activity on *P. aeruginosa*, *in vitro* (Amaya *et al*., 2012). In addition, the reports indicate that drimane sesquiterpenoids from the Chilean *Drymys winteri* (Paz *et al*., 2013; Cárcamo *et al*., 2014), acyl phenol (malabaricone C) from the Malaysian *Myristica cinnamomea* (Chong *et al*., 2011), iberin (isothiocyanate) from the Brassicaceae family (Jakobsen *et al*., 2012a), ellagic acid derivatives from *Terminalia chebula* (Sarabhai *et al*., 2013) and ajoene from garlic (Jakobsen *et al*., 2012b) have demonstrated varying degrees of QSI (Dobretsov *et al*., 2011).

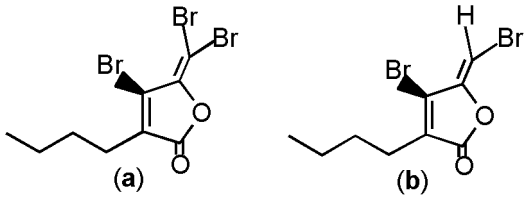

Figure 1.8 (a) 4-Bromo-3-butyl-5-dibromomethylene-2(5*H*)-furanone, (b) 4-Bromo-5-bromomethylene-3-butyl-2(5*H*)-furanone

Quorum sensing inhibitors ideally are low molecular weight, stable compounds capable of preventing synthesis, accumulation and or signal recognition between QS signals and the receptor proteins, in a manner that is target-specific and devoid of toxic side effects on both bacteria and the host (Zhang and Dong, 2004; Rasmussen and Givskov, 2006). Because QS inhibitory mechanisms involve targeting bacterial signalling to attenuate gene expression required by the 'bacterial quorum' to establish infections and resistance, it is a radical departure from well known mechanisms of targeting cell-walls by antibacterial drugs. In addition, QS inhibitors cannot inhibit or kill bacterial cells, unlike antibacterial drugs. Thus, the emergence of bacterial resistance to these drugs is not an issue (Hentzer and Givskov, 2003; Bottomley *et al*., 2007). Therefore, QSI is an anti-infective strategy with potential to provide treatment against bacterial resistance. It is anticipated that chemical compounds from

indigenous medicinal plants will provide effective, stable and viable leads for the discovery and development of antipathogenic drugs.

1.7 Justification for the research

Plant species used in traditional medicine continue to be reliable sources of new compounds for drug discovery. The widely used *Vernonia* species for treatment of various infectious diseases, is a possible source of novel compounds which could be lead compounds in the drug discovery programme.

1.8 Aim

The aim of the present study was to identify bioactive chemical constituents from various plant extracts of *Vernonia* species used in traditional medicine.

1.9 Objectives

i. To isolate and purify secondary metabolites from four *Vernonia* species (*Vernonia ambigua, Vernonia blumeoides, Vernonia glaberrima* and *Vernonia perrottetii*).

ii. To identify and characterize the structures of the isolated compounds.

iii. To evaluate the plant extracts, fractions and pure compounds for potential antibacterial activity.

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CHAPTER 2 SESQUITERPENE LACTONES FROM THE GENUS *VERNONIA* (ASTERACEAE): A REVIEW

Abstract

Sesquiterpene lactones (SLs) have attracted attention in recent years because of their potential as lead compounds for the pharmaceutical industry. They have been isolated from the genus *Vernonia* in the last four decades where a variety of skeletal types have been discovered. The diversity of the sesquiterpene lactone structure was attributed to plant genetics. There are 309 sesquiterpene lactones reported to have been isolated from the genus *Vernonia.* These are largely of the germacranolide, guaianolide, elemanolide and eudesmanolide skeletal types. The largest of these is the germacranolide type representing 71%, followed by the guaianolides (17%), elemanolides (6%), eudesmanolides (3%) and miscellaneous (3%). This review provides an analysis of the chemical and biogenetic relationships of the SLs from *Vernonia* as reported in the literature from 1967 to 2016.

Keywords: *Vernonia,* sesquiterpene lactones, germacranolides, elemanolides, eudesmanolides, guaianolides, hirsutinolides, glaucolides, cardinanolides, bourbonenolides, vernoallenes.

2.1 Introduction

Vernonia belongs to the tribe Vernonieae of the Asteraceae family. It comprises five hundred species distributed in tropical regions of the world especially in Africa and South America (Bremer, 1994). There are approximately twenty species in the USA and approximately two hundred growing in Brazil (Lopes, 1991; Jones, 1992). In West Africa, sixty species have been reported (Hutchinson and Dalziel, 1963). *Vernonia* species are known to synthesise terpenoid secondary metabolites such as the sesquiterpene lactones (SLs). These molecules have been isolated from plants over the last four decades. The structural elucidations of these molecules have revealed several classes with enormous chemical and structural diversity. This diversity has been linked to plant genetic transformation which suggests that all SLs are products of a common biosynthetic origin (Schmidt, 2006).

Phytochemical studies have revealed that the SLs are chemosystematic markers within the Asteraceae (Seaman, 1982). Currently, sesquiterpene lactones isolated from approximately eighty one *Vernonia* species distributed across Africa, the Americas and Asia are documented in the literature. They are largely based on the four major skeletal types: elemanolides, eudesmanolides, germacranolides and guaianolides. The germacranolides are the largest skeletal types consisting of four different subtypes: i. non-glaucolides ii. glaucolides iii. hirsutinolides and iv. cardinanolides. However, the eudesmanolides are rarely found and thus represent the smallest number in the genus. Miscellaneous SLs with unique ring systems or substitutional patterns that are rarely found or less frequently reported also occur. These miscellaneous classes are the vernonallenes, bourbonenolides and potamopholide.

This review provides an analysis of the chemical and biogenetic relationships of the SLs from *Vernonia* as reported from 1967 to 2016 and contains structures that were elucidated using

NMR spectroscopy. SLs identified by HPLC or mass spectrometry without NMR data were not considered in this review. A total of 309 SLs were reported in 81 *Vernonia* species across 53 countries (**Table 2.1**). **Table 2.1** is arranged with the species in alphabetical order and the structures of the compounds can be found in the pages that follow.

Table 2.1 Sesquiterpene lactones isolated from the genus *Vernonia*

No.	Vernonia species	Sesquiterpene lactones	Reference
1	*V. acunnae*	**279-282**	Budesinky *et al.* 1993
2	*V. adoensis*	**44, 63-67**	Bohlmann *et al.* 1984
3	*V. amygdalina*	**10-11, 13-14, 219-221, 224, 229-231**	Kupchan *et al*. 1969; Jisaka *et al*. 1993; Sinisi *et al*. 2015; Asaka *et al*. 1977; Erasto *et al*. 2006; Luo *et al*. 2011; Toubiana *et al*. 1975; Laekeman *et al*. 1983
4	*V. angusticeps*	**239, 251, 262, 263**	Budesinky *et al*. 1993
5	*V. angulifolia*	**112-116, 131, 133-134**	Bohlmann *et al*. 1978
6	*V. anisochaetoides*	**252, 264**	Bohlmann *et al*. 1978, 1981b
7	*V. anthelmintica*	**228, 232, 234-235, 237**	Zhang *et al*. 2014; Ito *et al*. 2016; Liu *et al*. 2010
8	*V. arborea*	**265**	Kumari *et al*. 2003
9	*V. arkasana*	**1-2, 247, 249, 250, 252, 271, 294-295, 304-308**	Bohlmann *et al*. 1978; 1981a
10	*V. baldawinii*	**27**	Padolina *et al*. 1974
11	*V. blumeoides*	**242-245**	Aliyu *et al*. 2015
12	*V. bockiana*	**20, 151, 157, 163-164, 290-292**	Huo *et al*. 2008
13	*V. brachycalyx*	**104-105**	Oketch-Rabah *et al*. 1998
14	*V. chinensis*	**117-119, 125-129**	Chen *et al*. 2005
15	*V. cinerascens*	**151, 155, 177-179, 182, 187**	Abdel-Saltar *et al*. 2000
16	*V. cinerea*	**42-43, 101, 110-113, 117-126, 128-132, 134-135, 155, 158-160, 289**	Jakupovic *et al*. 1986b; Kou *et al*. 2003; Youn *et al*. 2012, 2014; Chen *et al*. 2006; Chea *et al*. 2006; Zdero *et al*. 1991
17	*V. cognata*	**121, 151-152**	Bardon *et al*. 1988
18	*V. colorata*	**10, 229**	Rabe *et al*. 2002; Chukwujekwu *et al*. 2009
19	*V. compactiflora*	**199-201**	Bohlmann *et al*. 1982
20	*V. conferta*	**38**	Toubiana *et al*. 1972

No.	Vernonia species	Sesquiterpene lactones	Reference
21	*V. cotoneaster*	**299-303**	Bohlmann *et al*. 1982; Jakupovic *et al*. 1986b
22	*V. diffusa*	**282**	Jakupovic *et al*. 1987
23	*V. echitifolia*	**246**	Bohlmann *et al*. 1978, 1981b
24	*V. erdverbengii*	**25, 151, 156, 161**	Dominguez *et al*. 1986
25	*V. eremophila*	**212-216**	Alarcon *et al*. 1990
26	*V. erinaceae*	**83-84**	Tully *et al*. 1987
27	*V. fasticulata*	**21, 85**	Narain, 1977; 1978
28	*V. fastigiata*	**96-100**	Roos *et al*. 1998
29	*V. filigera*	**229**	Abegaz *et al*. 1994
30	*V. fruticulosa*	**27, 205-207, 211**	Bazon *et al*. 1997
31	*V. fultan*	**36-37**	Bohlmann *et al*. 1978
32	*V. galamensis*	**68-76, 94-98, 225**	Perdue *et al*. 1993; Zdero *et al*. 1990; Jakupovic *et al*. 1985
33	*V. gigantea*	**273-275**	Rojas, 2000
34	*V. glabra*	**7, 18, 219, 224-227, 233, 236, 295**	Jakupovic *et al*. 1985; Bohlmann *et al*. 1981b; 1983a
35	*V. greggii*	**151**	Bohlmann *et al*. 1978
36	*V. guineensis*	**4, 229, 238**	Toyang *et al*. 2013; Toubiana *et al*. 1975
37	*V. hirsuta*	**1-2, 8-9, 130, 132, 134, 252**	Bohlmann *et al*. 1978; 1981b
38	*V. holstii*	**99-100**	Zdero *et al*. 1991
39	*V. hymenopsis*	**224, 236**	Kupchan *et al*. 1968
40	*V. incana*	**23-24, 60**	Bardon *et al*. 1990
41	*V. jalcana*	**151, 166-168, 171-172, 174, 206-210, 293, 286-287**	Jakupovic *et al*. 1986b
42	*V. jonesii*	**15-16**	Gershenzon *et al*. 1984
43	*V. jugalis*	**148-150**	Tsichritzis *et al*. 1991
44	*V. karuguensis*	**88**	Mungarulire, 1993
45	*V. lanuginosa*	**29-30**	Bohlmann *et al*.1981a
46	*V. lasiopus*	**222-223**	Koul *et al*. 2003
47	*V. leopoldii*	**137, 265, 272, 297**	Abegaz *et al*. 1994; Marzouk *et al*. 2016
48	*V. marginata*	**52-57, 61-62, 91, 203, 217-218, 283, 288**	Padolina *et al*. 1974a; Jakupovic *et al*. 1986b; Catalan *et al*. 1986
49	*V. menthaefolia*	**209**	Budesinky *et al*. 1993
50	*V. moaensis*	**1, 162**	Budesinky *et al*. 1993
51	*V. mollissima*	**157-158, 162**	Catalan *et al*. 1986

No.	Vernonia species	Sesquiterpene lactones	Reference
52	*V. natalensis*	**26, 42, 45, 58, 86-87**	Zdero *et al*. 1991; Bohlmann and Zdero, 1982; Bardon and Zdero, 1982
53	*V. neocorymbosa*	**6**	Bohlmann *et al*. 1983a
54	*V. nitidula*	**248, 253-261, 266-270, 298**	Bardon *et al*. 1988
55	*V. noveboracensis*	**22, 190, 247, 276**	Padolina *et al*. 1974b; Bohlmann *et al*. 1978, 1981b
56	*V. nudiflora*	**28, 33-35, 47-49, 123, 137-140, 180-181, 183-188, 210**	Bardon *et al*. 1992
57	*V. oligocephala*	**31-32, 44, 59, 136, 277-278, 296**	Bohlmann *et al*. 1978; 1984
58	*V. pachyclada*	**39-40, 46**	Williams *et al*. 2005
59	*V. patens*	**121, 196, 283-285**	Jakupovic *et al*. 1986a
60	*V. patula*	**19**	Qiao-Li *et al*. 2010
61	*V. pectoralis*	**5, 10, 241**	McPhail *et al*. 1975
62	*V. pedunculata*	**81-82**	Lopes, 1991
63	*V. pinguis*	**123-124, 127, 129-131, 197-198, 202-204, 240**	Borkosky *et al*. 1997, Catalan *et al*. 1986
64	*V. polyanthes*	**151, 175-176, 181-184**	Bohlmann *et al*. 1983b
65	*V. poole*	**27**	Gershenzou *et al*. 1984
66	*V. poskeana*	**102-103, 141-146**	Bohlmann *et al*. 1983a; Jakupovic *et al*. 1986a
67	*V. potamophila*	**309**	Bila-Babady *et al*. 2003
68	*V. profuga*	**1-2, 247, 271, 295**	Bohlmann *et al*. 1978; 1981a
69	*V. saltens*	**169-170**	Bohlmann *et al*. 1979
70	*V. saltensis*	**191-192, 194-195**	Bohlmann *et al*. 1979
71	*V. scorpioides*	**17, 22-23, 153-154, 162, 169-170**	Drew *et al*. 1980; Jakupovic *et al*. 1985; Buskuhl *et al*. 2010, Bohlmann *et al*. 1979
72	*V. squamulosa*	**151**	Catalan *et al*. 1986
73	*V. staehelionoides*	**92-93, 142, 146**	Bohlmann *et al*. 1982; Pillay *et al*. 2007
74	*V. steetziana*	**89-90, 141-142, 147**	Zdero *et al*. 1991
75	*V. sutherlandii*	**58**	Bohlmann *et al*. 1984
76	*V. syringifolia*	**58, 77-80, 155**	Bohlmann *et al*. 1984; Abegaz *et al*. 1994; Zdero *et al*. 1991
77	*V. thomsoniana*	**3**	Mungarulire, 1993
78	*V. triflosculosa*	**165-166, 173**	Kos *et al*. 2006
79	*V. uniflora*	**41**	Taylor and Watson, 1976

No.	Vernonia species	Sesquiterpene lactones	Reference
80	*V. vernosissima*	**152, 185, 191**	Bohlmann *et al*. 1981b
81	*V. zanzibarensis*	**106-109**	Zdero *et al*. 1991

2.2 Sesquiterpene lactones

Sesquiterpene lactones consist of C-15 compounds which have at least one lactone (cyclic ester) ring in its structure. For example in costunolide (**Figure 2.1**), a lactone ring occurs usually at C6 and C7 of the cyclodecadiene ring. In addition, three double bonds are present at C-1(10), Δ^4 and Δ^{12} and two methyl groups occur at C-4 and C-10 (**Figure 2.1**). Biogenetic studies of sesquiterpene lactones have indicated that they are derived from the mevalonic acid pathway (Craig 2003). Cyclization of (*E*,*E*)-farnesyl pyrophosphate (FPP) mediated by germacrane A synthase results in germacrane A, which undergoes several oxidation steps and lactonisation to form costunolide, which is regarded as the common precursor to all germacranolide derived sesquiterpene lactones (**Figure 2.1**) (De Kraker *et al*., 2002).

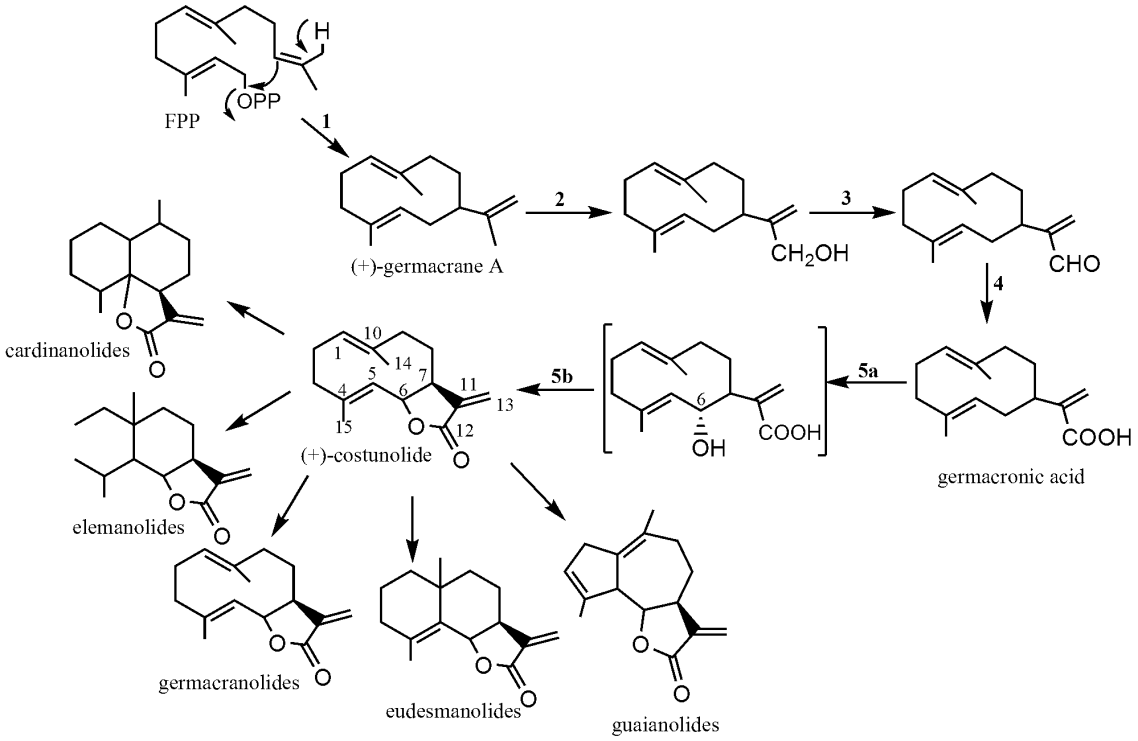


Figure 2.1 Plausible biosynthetic pathway of sesquiterpene lactones. 1 germacrene A synthase, 2-5a oxidations, 5b lactone formation (Majdi *et al*., 2011).

2.3 Germacranolides

Germacranolides are derived from the parent germacrane (**Figure 2.1**) and characterised by a cyclodecadiene skeleton with a C6/C12 γ-lactonized system. They possess an α-methylene-γ-lactone moiety with β-oriented stereochemistry (Milosavljevi *et al*., 1999). Since they contain two double bonds within the cyclodecadiene ring (Kasymov, 1982), they display structural flexibility and can exist as (*E*,*E*)-, (*Z*,*E*)-, (*E*,*Z*)- and (*Z*,*Z*)- conformations (**Figure 2.2**) (Minnaard *et al*., 1999). The predominant conformations are however the (*E*,*E*)- and (*Z*,*Z*)-germacranes in sesquiterpene lactones from *Vernonia*. The germacranolides constitute the largest group of sesquiterpene lactones in *Vernonia*. Their biogenetic formation may involve modifications of the basic skeleton by epoxidation, hydroxylation and esterification reactions at different positions (Picman, 1981). The germacranolides are further classified into four structural sub-types: non-glaucolides, glaucolides, hirsutinolides and cardinanolides based on their structural variations.

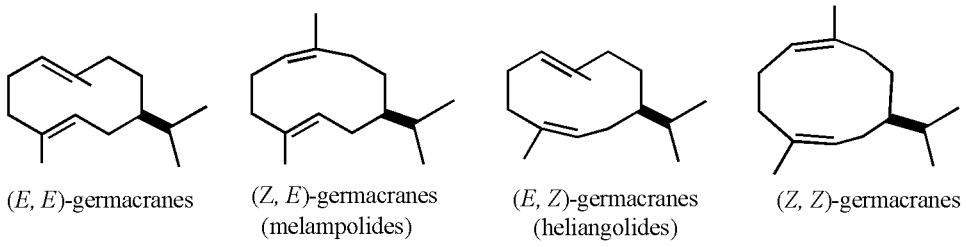


Figure 2.2 Conformational structures of germacrane skeletal types

2.3.1 Non-glaucolides

These are germacranolides characterized by C6/C12 γ-lactonized systems (**Figure 2.3**), with **19** as the only compound bearing a C8/C12 γ-lactone moiety. There are 21 non-glaucolides reported from *Vernonia*, identified by an olefinic methylene group (C-13) and 1(10) and/or Δ^4

olefinic groups as in costunolide (**1**). They occur largely in the (*E*,*E*)-conformation, except artemorin (**18**) having an exocyclic double bond at C-10. The modifications of costunolide involving oxidations and hydroxylation at various positions especially C-8, C-4 and C-10, give rise to variety of costunolide derivatives (**2-21**). The C-8-side chain ester in non-glaucolides contain a methacryloyl moiety or its derivatives with a few exceptions. The occurrence of sesquiterpene lactone non-glaucolides is poor in the *Vernonia* genus. However, the most representatives are costunolides (**1-9**) and vernolides (**10-14**) (**Figure 2.4** and **Figure 2.5**).

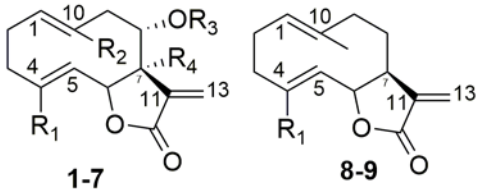
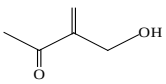
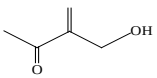
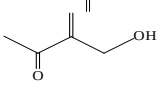
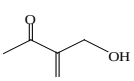
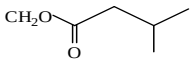
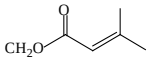
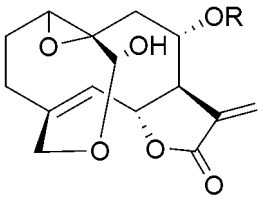


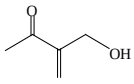
Figure 2.3 Costunolide type germacranolides

No.	Common name	R_1	R_2	R_3	R_4	Mol.-formula	Mol.-mass (g/mol)
1	Costunolide	CH_3	CH_3	H	H	$C_{15}H_{20}O_3$	248
2	Tulipinolide	CH_3	CH_3		H	$C_{17}H_{22}O_4$	290
3	Costunolide-8-*O*-hydroxymethacrylate	CH_3	CH_3		OH	$C_{19}H_{24}O_6$	348
4	Vernopicrin	CHO	CH_3		H	$C_{19}H_{22}O_6$	346
5	Pectorolide	CH_2OH	CH_2OH		H	$C_{19}H_{24}O_6$	348
6	Onopordopicrin	CH_2OH	CH_3		H	$C_{19}H_{24}O_6$	348
7	4'-hydroxy pectorolide	CH_2OH	CH_2OH		H	$C_{19}H_{24}O_7$	364
8	Isovaleryloxy costunolide		-	-	-	$C_{20}H_{28}O_5$	348
9	Senecioyloxy costunolide		-	-	-	$C_{20}H_{26}O_5$	346



(**10-14**)

Figure 2.4 Vernolide type germacranolides

No.	Common name	R_1	R_2	R_3	Mol.-formula	Mol.-mass (g/mol)
10	Vernolide	OH		CH_2	$C_{19}H_{22}O_7$	362
11	Vernomygdin	OH		CH_2	$C_{19}H_{24}O_7$	364
12	11β,13 dihydrovernolide	OH		αCH_3, βH	$C_{19}H_{24}O_7$	364
13	Hydroxyvernolide	OH		CH_2	$C_{19}H_{24}O_8$	380
14	14-*O*-methylvernolide	OCH_3		CH_2	$C_{20}H_{24}O_7$	376

Biogenetic relations have shown that costunolide (**1**) is the common precursor to the synthesis of the vernolides (**10-14**). Plausible biosynthetic pathways showed that C14/C15 oxidative reactions in (**1**) results in pectrolide (**5**) which undergoes epoxidation and heterocyclic ring formation reactions to form the vernolides (Toubiana *et al*., 1975) (**Figure 2.5**).

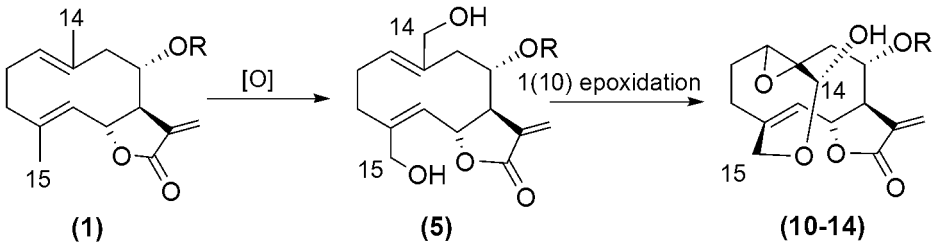
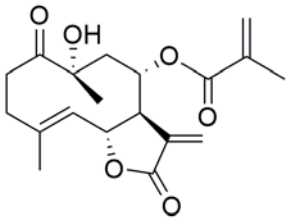


Figure 2.5 Plausible biosynthesis of vernolides (**10-14**)

In other non-glaucolide sesquiterpene lactones, oxidations have occurred on the ring and hence these compounds contain hydroxyl groups, ketones, epoxides, esters and even sugar moieties (**15-21**) (**Figure 2.6a-c**).



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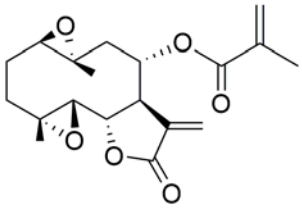
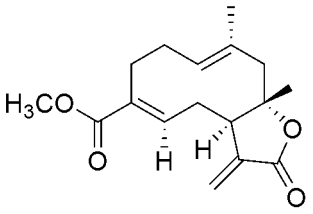


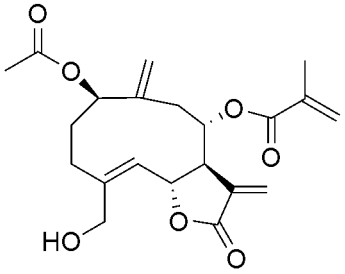
16

Figure 2.6a Non-glaucolide germacranolides

No.	Common name	Mol.-formula	Mol. mass (g/mol)
15	Keto costunolide	$C_{19}H_{24}O_6$	364
16	Diepoxy costunolide	$C_{19}H_{24}O_6$	364



17



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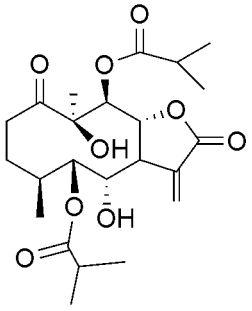
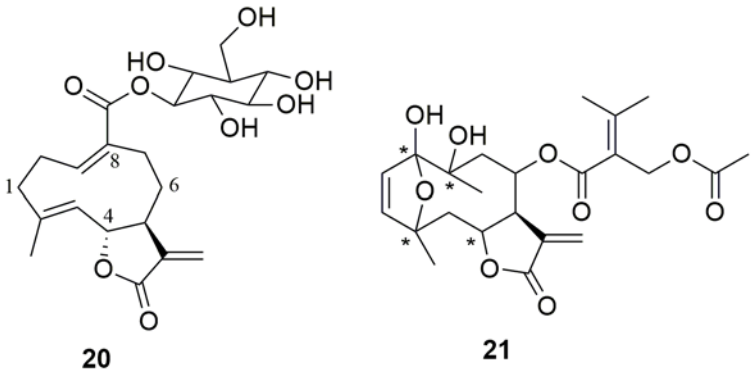


19

Figure 2.6b Non-glaucolide germacranolides (continued)

No.	Common name	Mol. formula	Mol. Mass (g/mol)
17	Scorpiodine	$C_{17}H_{22}O_4$	290
18	Artemorin	$C_{21}H_{26}O_7$	390
19	Incaspitolide D	$C_{23}H_{34}O_9$	545



* stereochemistry not indicated in the literature

Figure 2.6c Non-glaucolide germacranolides (continued)

No.	Common name	Mol. formula	Mol. mass (g/mol)
20	Taraxinic ester	$C_{22}H_{30}O_9$	438
21	Fasciculide-A	$C_{23}H_{30}O_9$	450

2.3.2 Glaucolides

These are a class of sesquiterpene lactones characteristic of the germacranolides. A characteristic feature of this class is that they have an oxygen functionality at C13. They were first isolated from the South American *V. glauca* (Padolina *et al*., 1974), from where they get their name, and are ubiquitous in *Vernonia* species.

The glaucolide constitutes the second largest number (87) of sesquiterpenoids reported from *Vernonia* and are made up of five skeletal types: glaucolides, prevernocisifolides, melampolides, stilpnomentolides and vernozanzibarolide (**Figure 2.7** to **Figure 2.24**). The C8 ester substitution includes the acetate tiglate, methacrylate, seneciolylate, angilate, epoxy angilate and isobutanoylate among others. The occurrence of epoxy and methylene groups in the ring results in reactive sites, and as such, glaucolides can undergo stereospecific transformations leading to many derivatives by *in vivo* reactions involving oxidation, hydrogenation, hydration, dehydrogenation and dehydration, among others (Fischer, 1990).

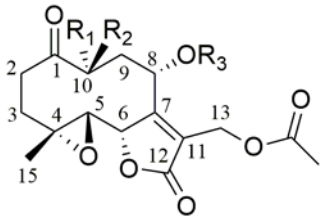
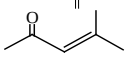
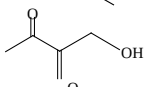
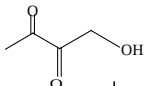
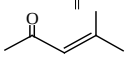


Figure 2.7 Glaucolide A type sesquiterpenoids

No.	Common name	R_1	R_2	R_3	Mol.-formula	Mol.-mass (g/mol)
22	Glaucolide A	$OCOCH_3$	CH_3		$C_{23}H_{28}O_{10}$	464
23	8-Desacylglaucolide A-senecioate	$OCOCH_3$	CH_3		$C_{24}H_{30}O_{10}$	478
24	8-Desacylglaucolide A-angelate	$OCOCH_3$	CH_3		$C_{24}H_{30}O_{10}$	478
25	8-Desacylglaucolide A-tiglate	$OCOCH_3$	CH_3		$C_{24}H_{30}O_{10}$	478
26	19-hydroxy glaucolide A	$OCOCH_3$	CH_3		$C_{23}H_{28}O_{11}$	480
27	Glaucolide B	$OCOCH_3$	CH_3		$C_{21}H_{26}O_{10}$	438
28	Glaucolide B-8-*O*-tiglate	$OCOCH_3$	CH_3		$C_{24}H_{30}O_{10}$	478
29	Glaucolide B-propionate	$OCOCH_3$	CH_3	- $COCH_2CH_3$	$C_{22}H_{28}O_{10}$	452
30	Glaucolide J	$OCOCH_3$	CH_3		$C_{23}H_{30}O_{10}$	464
31	Stilpnotomentolide	H	CH_3		$C_{21}H_{26}O_9$	422
32	Stilpnotomentolide-8-*O*-angelate	H	CH_3		$C_{22}H_{28}O_8$	420
33	Glaucolide -8-*O*- tiglate	CH_3	H		$C_{22}H_{28}O_8$	420
34	Glaucolide-8-*O*-methacrylate	CH_3	H		$C_{22}H_{26}O_8$	418
35	Glaucolide -8-*O*- senecioate	CH_3	H		$C_{22}H_{28}O_8$	420
36	Vernofultan A	CH_3	H		$C_{19}H_{24}O_8$	380
37	Vernofultan B	H	CH_3		$C_{19}H_{24}O_8$	380

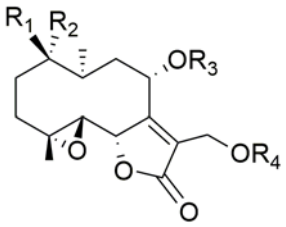


Figure 2.8 Confertolide type sesquiterpenoids

No.	Common name	R_1	R_2	R_3	R_4	Mol.-formula	Mol.-mass (g/mol)
38	Confertolide	$OCOCH_3$	H			$C_{21}H_{28}O_9$	424
39	Glaucolide K	H	$OCOCH_3$			$C_{23}H_{30}O_9$	450
40	Glaucolide L	H	$OCOCH_3$		H	$C_{21}H_{28}O_8$	424

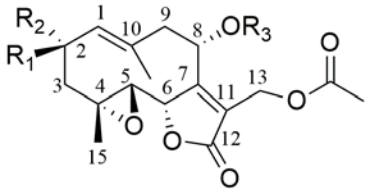
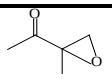
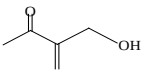
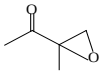


Figure 2.9 Glaucolide-D type sesquiterpenoids

No.	Common name	R_1	R_2	R_3	Mol. formula	Mol. Mass (g/mol)
41	Glaucolide D	H	$OCOCH_3$		$C_{23}H_{28}O_{10}$	464
42	Glaucolide E	H	$OCOCH_3$		$C_{23}H_{28}O_9$	448
43	19-hydroxy glaucolide E	H	$OCOCH_3$		$C_{23}H_{28}O_{10}$	464
44	2-oxo-2-desacetoxy glaucolide D	=O	-		$C_{21}H_{24}O_9$	420
45	2-oxo-2-desacetoxy glaucolide E	=O	-		$C_{21}H_{24}O_8$	404

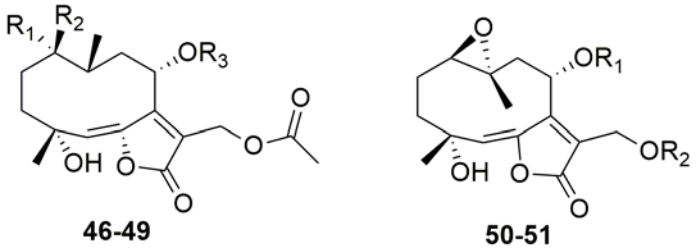
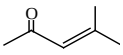


Figure 2.10 Glaucolide M type sesquiterpenoids

No.	Common name	R_1	R_2	R_3	Mol.-formula	Mol. mass (g/mol)
46	Glaucolide M	$OCOCH_3$	H		$C_{23}H_{30}O_9$	450
47	1-oxo-glaucolide M-8-*O*-tiglate	=O	-		$C_{22}H_{29}O_8$	421
48	1-oxo-glaucolide M	=O	-		$C_{21}H_{27}O_8$	407
49	1-oxo-glaucolide M-8-*O*-senecioate	=O	-		$C_{22}H_{29}O_8$	421
50	1(10)-epoxy glaucolide M			-	$C_{21}H_{26}O_8$	406
51	1(10)-epoxy glaucolide M-8-*O*-tiglate			-	$C_{22}H_{28}O_8$	420

The formation of marginatin methyl ester (**57**) was proposed from biogenetic relations, in which acid catalyzed dehydration of 13-deacetyldehydromarginatin (**55**) results in C6/C7 double bond and a free C13 methylene group. The methanol mediated ring opening of the γ-lactone, allows for ketonization of C6 and subsequent elimination of the C8 side ester, thus forming the methyl ester (**57**) (**Figure 2.11**) (Jakupovic *et al*., 1986).

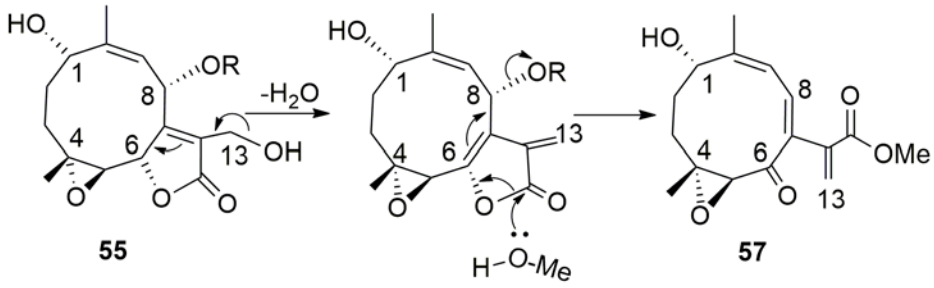


Figure 2.11 Biosynthetic transformation of 13-deacetyldehydromarginatin (55) to marginatin methyl ester (57)

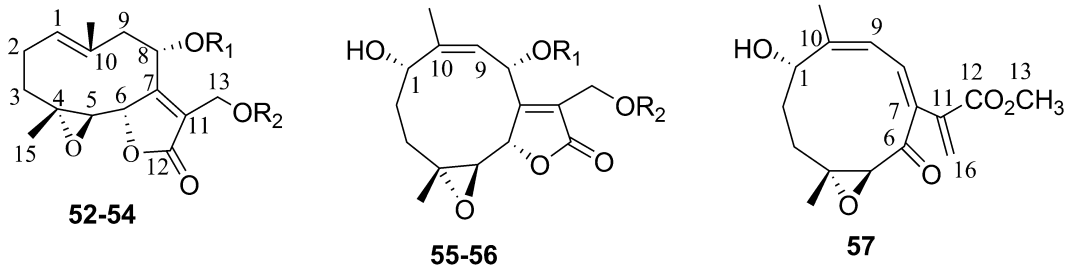
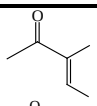
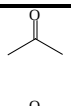
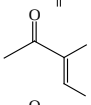
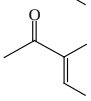
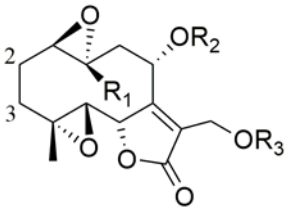
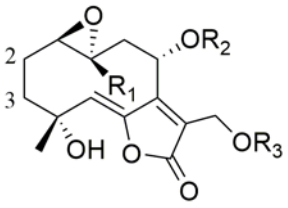


Figure 2.12 Marginatin type sesquiterpenoids

No.	Common name	R_1	R_2	Mol.-formula	Mol.-mass (g/mol)
52	marginatin			$C_{22}H_{28}O_7$	404
53	8-desacylmarginatin methacrylate			$C_{21}H_{26}O_7$	390
54	13-deacetylmarginatin		H	$C_{20}H_{26}O_6$	362
55	13-deacetyldehydromarginatin		H	$C_{20}H_{26}O_7$	378
56	9,10-dehydromarginatin			$C_{22}H_{28}O_8$	420.18
57	marginatin methyl ester	-	-	$C_{16}H_{20}O_5$	292.13

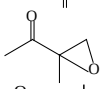
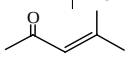
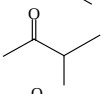
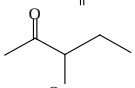


58-65



66-67

Figure 2.13 Vernonataloide-type

No.	Common name	R_1	R_2	R_3	Mol.-formula	Mol.-mass (g/mol)
58	Vernonataloide	βCH_3			$C_{21}H_{26}O_8$	406
59	17, 18-epoxyvernonataloide	βCH_3			$C_{21}H_{26}O_9$	422
60	8-desacylvernonataloide senecioate	βCH_3			$C_{22}H_{28}O_8$	420
61	8,13-bis-desacylvernonataloide tiglate	βCH_3		H	$C_{20}H_{26}O_7$	378
62	8-desacylvernonataloide tiglate	αCH_3			$C_{22}H_{28}O_8$	420
63	8-desacylvernonataloide isobutyrate	αCH_3		CH_3*	$C_{20}H_{28}O_6$	364
64	Δ^2, Vernonataloide methacrylate	CH_2OH			$C_{21}H_{24}O_9$	420
65	Δ^2,Vernonataloide methyl butyrate	CH_2OH			$C_{22}H_{28}O_9$	436
66	4, 13-dihydroxy vernonataloide	βCH_3		H	$C_{19}H_{22}O_8$	378
67	4-hydroxy vernonataloide	βCH_3			$C_{21}H_{24}O_9$	420

***OR_3 = CH_3** (this compound is not oxygenated at this position)

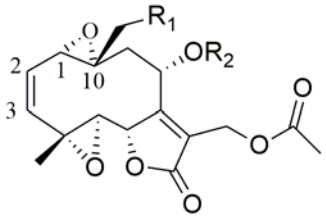
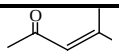
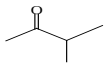
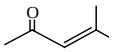
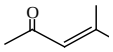
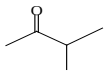


Figure 2.14 Prevernocistifolide type sesquiterpenoids

No.	Common name	R_1	R_2	Mol.-formula	Mol. mass (g/mol)
68	prevernocistifolide A	OH		$C_{22}H_{26}O_9$	434
69	prevernocistifolide B	OH		$C_{20}H_{23}O_8$	391
70	2α,3α-epoxy prevernocistifolide A	OH		$C_{22}H_{26}O_{10}$	450
71	prevernocistifolide C	$OCOCH_3$		$C_{22}H_{25}O_9$	476
72	prevernocistifolide D	$OCOCH_3$		$C_{20}H_{21}O_8$	433
73	prevernocistifolide E	OH		$C_{21}H_{23}O_8$	389
74	prevernocistifolide F	OH		$C_{22}H_{23}O_8$	403
75	prevernocistifolide G	$OCOCH_3$		$C_{22}H_{23}O_9$	431
76	2α,3α-epoxy prevernocistifolide E	OH		$C_{21}H_{25}O_{10}$	437

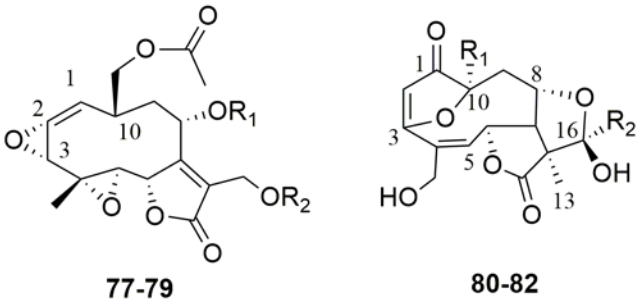
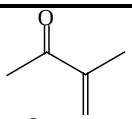
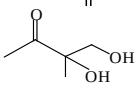
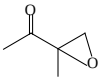
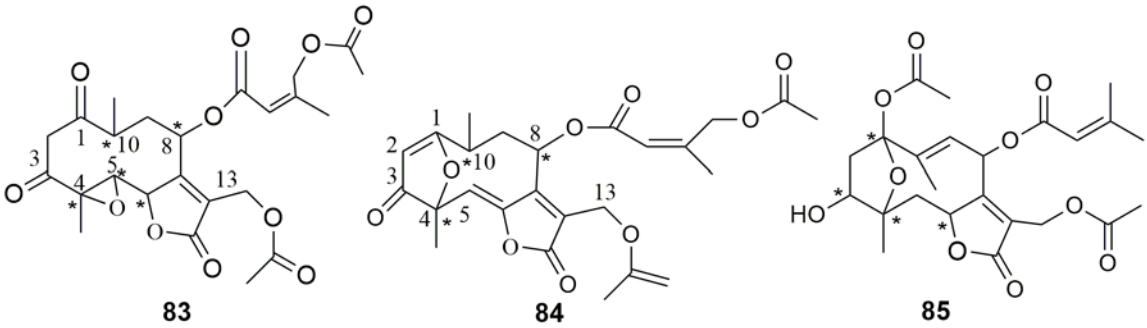


Figure 2.15 Melampolides-type

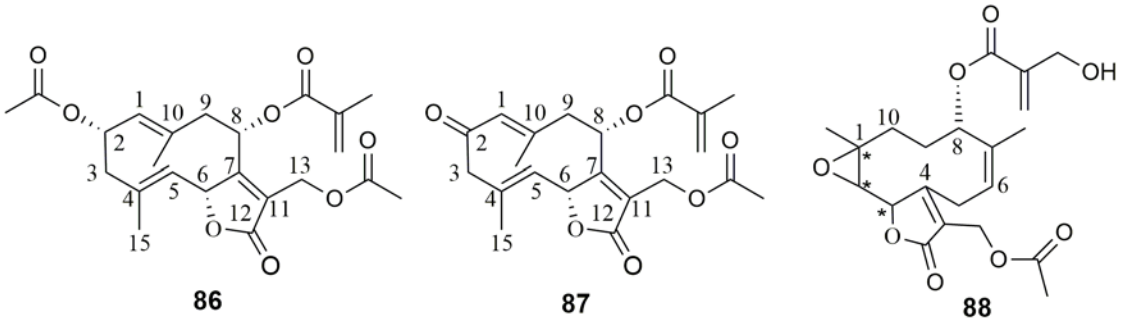
No.	Common name	R_1	R_2	Mol.-formula	Mol.-mass (g/mol)
77	9 desacetoxyvernocistifolide methacrylate			$C_{23}H_{26}O_{10}$	462
78	dihydroxypropanoyloxy malampolide			$C_{23}H_{28}O_{12}$	496
79	propanoyloxy malampolide			$C_{23}H_{26}O_{11}$	478
80	Eremantholide A	CH_3	$-C(CH_3)=CH-CH_3$	$C_{20}H_{25}O_7$	377
81	Eremantholide B	CH_3	$-CH(CH_3)CH_2CH_3$	$C_{20}H_{27}O7$	379
82	Eremantholide C	CH_3	$-C(CH_3)=CH_2$	$C_{19}H_{23}O_7$	363



* stereochemistry not indicated in the literature

Figure 2.16 Keto-glaucolides

No.	Common name	Mol. formula	Mol. mass (g/mol)
83	Glaucolide-1,3-diketone	$C_{24}H_{28}O_{11}$	492
84	Glaucolide erinacolide	$C_{24}H_{26}O_{10}$	474
85	Fasciculide-B	$C_{24}H_{30}O_{10}$	478



* stereochemistry not indicated in the literature

Figure 2.17 Natalensolide type

No.	Common name	Mol.-formula	Mol. mass (g/mol)
86	1,10-desoxidoglaucolide E	$C_{23}H_{28}O_8$	432
87	Natalensolide	$C_{21}H_{25}O_7$	389
88	Unusual glaucolide	$C_{21}H_{25}O_8$	405

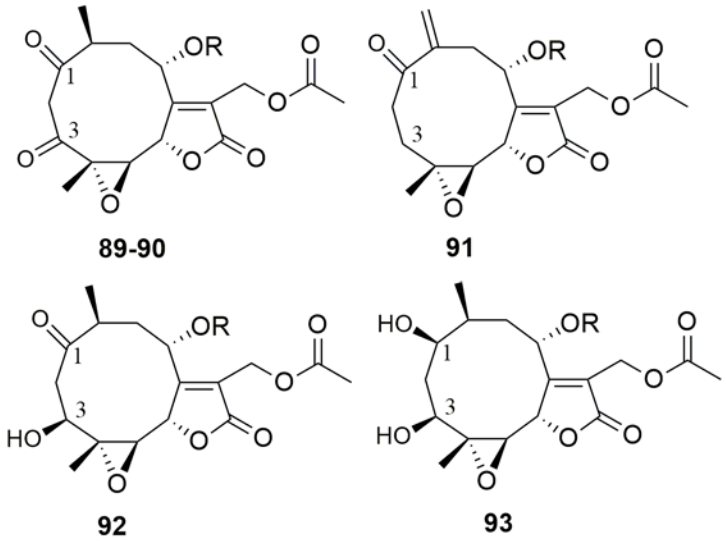
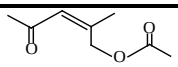
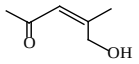
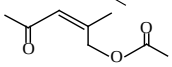
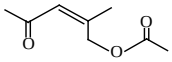


Figure 2.18 Stilpnotomentolide skeletal type

No.	Common name	R	Mol.-formula	Mol.-mass (g/mol)
89	glaucolide-8-*O*-acetoxy senecioate		$C_{22}H_{24}O_8$	416
90	3-oxo-stilpnotomentolide-8-*O*-hydroxy senecioate		$C_{20}H_{22}O_8$	390
91	10,14 dehydrostilpnotomentolide-8-*O*-tiglate		$C_{22}H_{26}O_8$	418
92	3β-hydroxystilpnotomentolide-8-*O*-acetoxy senecioate		$C_{24}H_{30}O_{11}$	494
93	1-epoxy-Δ^5-Stilpnotomentolide-8-*O* -acetoxy senecioate		$C_{25}H_{31}O_{10}$	491

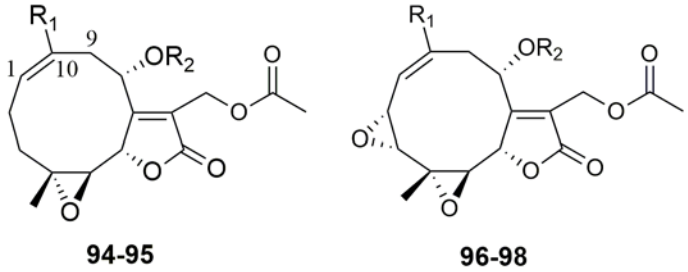
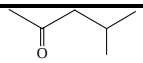


Figure 2.19 Glaucogalamensolide type I sesquiterpenoids

No.	Common name	R_1	R_2	Mol. formula	Mol. mass (g/mol)
94	glaucogalamensolide isovalerate	CHO		$C_{26}H_{24}O_6$	360
95	glaucogalamensolide isobutyrate	CHO		$C_{19}H_{22}O_6$	346
96	bis epoxygermacranolide-8-*O*-angelate	CH_2OH		$C_{22}H_{25}O_9$	433
97	bis epoxygermacranolide-8-*O*-mathacrylate	CH_2OH		$C_{21}H_{23}O_9$	419
98	bis epoxygermacranolide	CHO		$C_{22}H_{23}O_9$	431

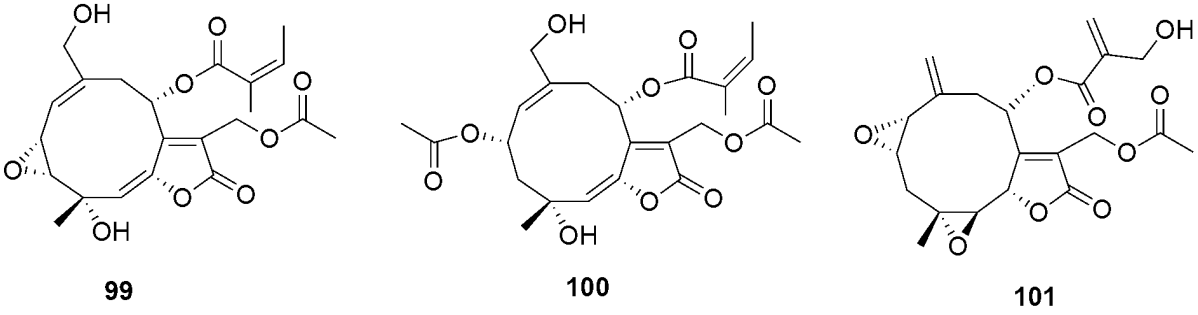
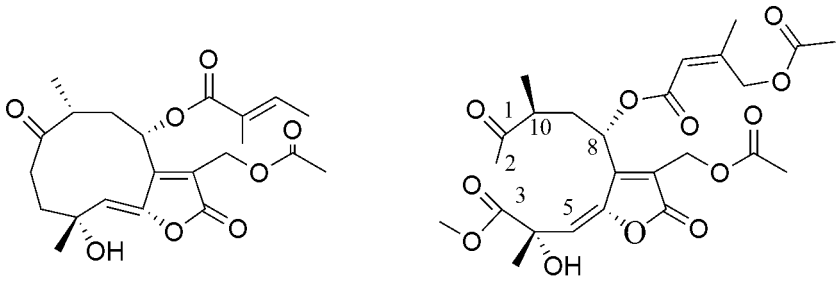


Figure 2.20 Glaucogalamensolide type II sesquiterpenoids

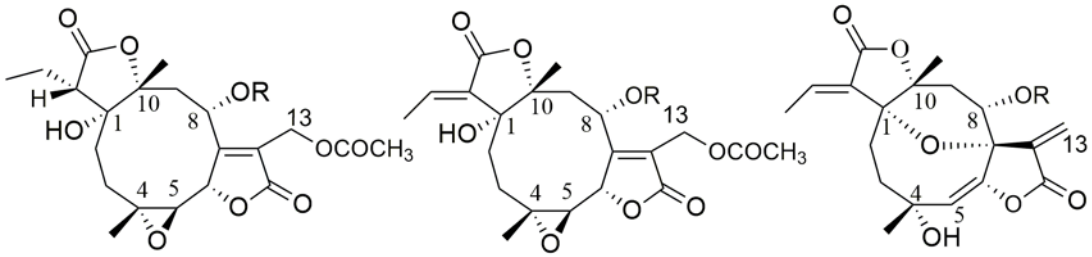
No.	Common name	Mol.-formula	Mol. mass (g/mol)
99	2, 3-epoxy-glaucogalamensolide	$C_{22}H_{26}O_9$	434
100	2-acetoxy- glaucogalamensolide	$C_{24}H_{30}O_{10}$	478
101	vernocinerolide	$C_{21}H_{24}O_9$	420



102 103

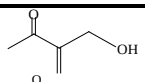
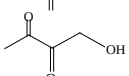
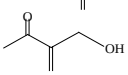
Figure 2.21 Ketovernocinerolide and poskeanolide

No.	Common name	Mol. formula	Mol. mass (g/mol)
102	Ketovernocinerolide	$C_{21}H_{28}O_8$	408
103	Poskeanolide	$C_{25}H_{32}O_{12}$	476



104 105-106 107

Figure 2.22 Germacranolides dilactone

No.	Common name	R	Mol.-formula	Mol.-mass (g/mol)
104	16,17-dihydrobrachycalyxolide		$C_{25}H_{32}O_{11}$	508
105	brachycalyxolide		$C_{25}H_{30}O_{11}$	506
106	16*E*,23-desoxybrachycalyxolide		$C_{25}H_{30}O_{10}$	490
107	16*E*-isobrachycalyxolide		$C_{23}H_{26}O_9$	446

The transformation of brachycalyxolide (**105**) to the corresponding 16*E*-isobrachycalyxolide (**107**) is thought to proceed through an acid catalyzed reaction (**Figure 23**). The ring opening of the epoxide in (**105**) is followed by deprotonation at C6 and subsequent attack of the

hydroxyl group at C1 on C7, leading to the formation of a 1,7-oxygen bridge. This concerted reaction results in the elimination of the acetate group at C13 forming a free methylene system (Zdero *et al*., 1991).

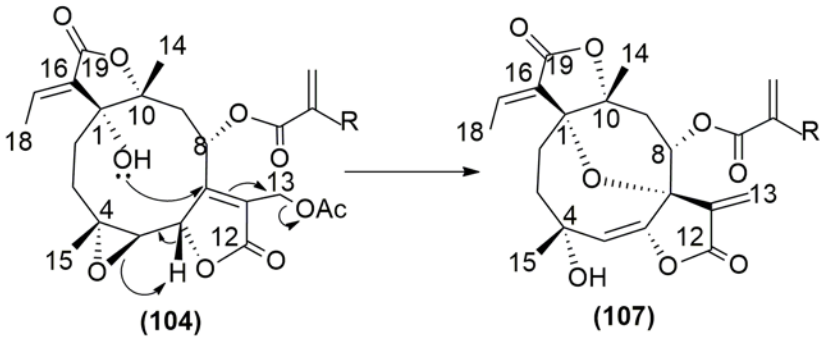


Figure 2.23 Biosynthetic transformation of brachycalyxolide (105) to iso-brachycalyxolide (107)

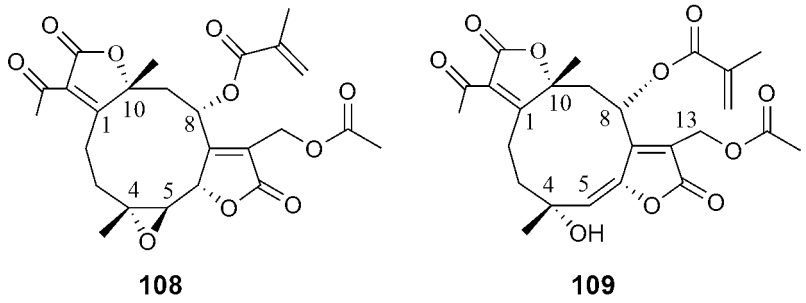


Figure 2.24 Vernozanzibarolide sesquiterpenoids

No.	Common name	Mol. formula	Mol. mass (g/mol)
108	vernozanzibarolide	$C_{24}H_{28}O_{10}$	476
109	isovernozanzibarolide	$C_{25}H_{18}O_{10}$	478

2.3.3 Hirsutinolides

Hirsutinolides are derived from the glaucolides and are represented from **Figure 2.26** to **Figure 2.34**. The trivial name "hirsutinolides" was first given by Bohlmann and co-workers in 1978 when they discovered an unusual sesquiterpene lactone from *Vernonia hirsuta* with an endocyclic double bond and an oxygen at C13 (Bohlmann *et al*., 1978). Hirsutinolides

occur with a (*Z,Z*)-germacrane conformation and a diverse functionalized ester side chain such as the acetate tiglate, methacrylate, seneciolylate, angilate, epoxy angilate and isobutanoylate. They represent the largest number of sesquiterpenoids (91) reported from *Vernonia*.

Hirsutinolides are made up of five skeletal types: Vernolides (**110-140**), keto-hirsutinolides (**141-150**), piptocarphin (**151-186**), iso-hirsutinolides (**187-195**) and 1-desoxy hirsutinolides (**196-201**). Although their structural and substitution patterns are similar, they have a diverse range of compounds. Hirsutinolides are related to the glaucolides by their chemical and conformational characteristics. Biogenetic relationships indicate that germacranolides are the primary precursors to hirsutinolides. The vernolide type hirsutinolides (**110-140**) are probably formed through the diepoxide (**58**) involving oxidation, hydration and dehydration leading to the hirsutinolides (**Figure 2.25**) (Bohlmann *et al*., 1978).

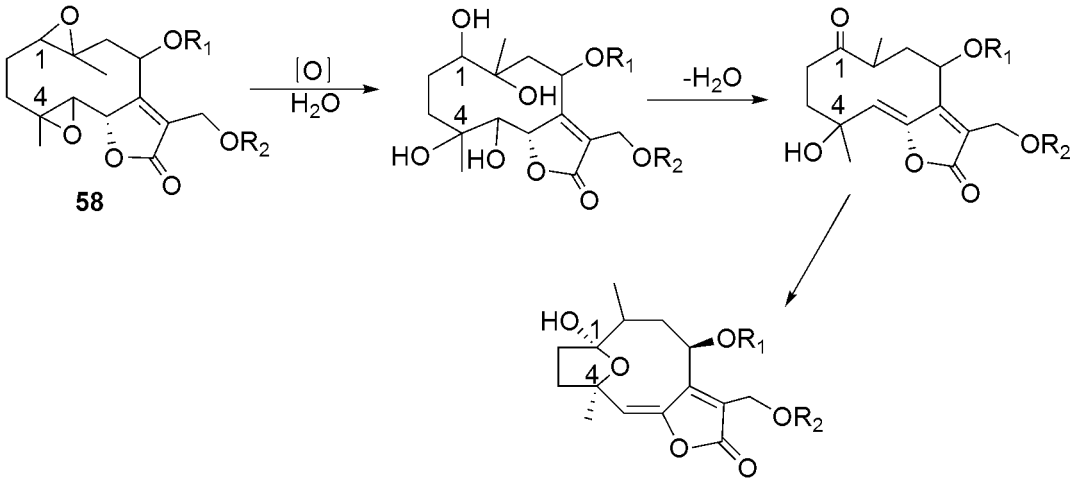


Figure 2.25 Biosynthetic transformation of vernolide type hirsutinolides (Bohlmann *et al*., 1978)

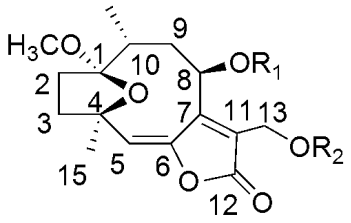
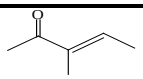
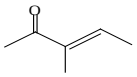
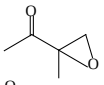
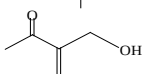


Figure 2.26 Vernolide A and B type sesquiterpenoids

No.	Common name	R_1	R_2	Mol.-formula	Mol.-mass (g/mol)
110	Vernolide A		H	$C_{21}H_{28}O_7$	392
111	Vernolide B			$C_{23}H_{30}O_8$	434
112	Vernolide B-methacrylate			$C_{22}H_{28}O_8$	420
113	Vernolide	H	H	$C_{16}H_{22}O_6$	310
114	Vernolide A-methacrylate		H	$C_{20}H_{26}O_7$	378
115	Vernolide B-epoxy methacrylate			$C_{22}H_{28}O_9$	436
116	Vernolide B-4'-hydroxymethacrylate			$C_{22}H_{28}O_9$	436

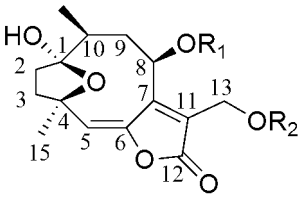
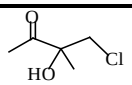
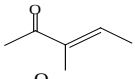
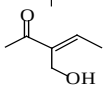
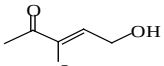
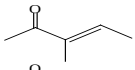
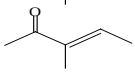
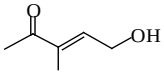
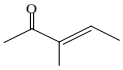
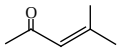
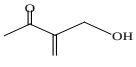
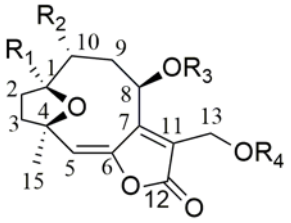
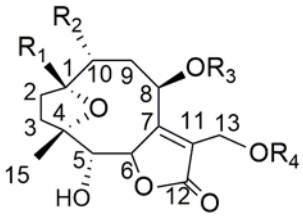


Figure 2.27 Vernolide C and D type sesquiterpenoids

No.	Common name	R_1	R_2	Mol.-formula	Mol. mass (g/mol)
117	Vernolide C			$C_{21}H_{26}O_9Cl$	457
118	Vernolide-8-*O*-tiglate			$C_{22}H_{28}O_8$	420
119	Vernolide-8-*O*-(5'-hydroxy tiglate)			$C_{22}H_{28}O_9$	436
120	Vernolide D			$C_{22}H_{28}O_9$	436
121	Vernolide-8-*O*-angelate			$C_{22}H_{28}O_8$	420
122	13-hydroxy vernolide-8-*O*-angelate		H	$C_{20}H_{26}O_7$	378
123	Vernolide-8-*O*-tiglate			$C_{22}H_{28}O_8$	420
124	13-hydroxy vernolide-8-*O*-tiglate		H	$C_{20}H_{26}O_7$	378
125	13-hydroxy vernolide D		H	$C_{20}H_{26}O_8$	394
126	8-hydroxy vernolide-13-*O*-tiglate	H		$C_{20}H_{26}O_7$	378
127	Vernolide-8-*O*-seneciolate			$C_{22}H_{28}O_8$	420
128	8,13-dihydroxy vernolide	H	H	$C_{15}H_{21}O_6$	297
129	8-hydroxy vernolide-13-*O*-acetate	H		$C_{17}H_{23}O_7$	407
130	Vernolide-8-*O*-methacrylate			$C_{21}H_{27}O_8$	407
131	13-hydroxy vernolide-8-*O*-methacrylate		H	$C_{19}H_{25}O_7$	365
132	Vernolide-8-*O*-(4'-hydroxymethacrylate)			$C_{21}H_{25}O_8$	405

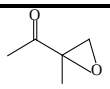
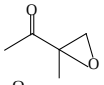
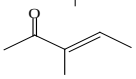


133-138



139-140

Figure 2.28 More vernolide sesquiterpenoids including E and F types

No.	Common name	R_1	R_2	R_3	R_4	Mol.-formula	Mol.-mass (g/mol)
133	13-hydroxy vernolide-8-*O*-epoxymethacrylate	OH	CH_3		H	$C_{19}H_{24}O_8$	380
134	14-*nor* vernolide -8-*O*-epoxymethacrylate	OH	CH_3			$C_{21}H_{26}O_9$	422
135	vernolide-8-*O*-tiglate	OH	CH_3			$C_{22}H_{28}O_8$	420
136	10- hydroxy methyl vernolide E	OH	CH_2OH			$C_{21}H_{25}O_9$	421
137	*β*-methoxy vernolide	OCH_3	CH_3			$C_{22}H_{27}O_8$	419
138	*α*-Acetoxy vernolide E	$OCOCH_3$	CH_3			$C_{23}H_{27}O_9$	447
139	Vernolide E	OH	CH_3			$C_{21}H_{28}O_9$	424
140	Vernolide F	OCH_2CH_3	CH_3			$C_{23}H_{32}O_9$	452

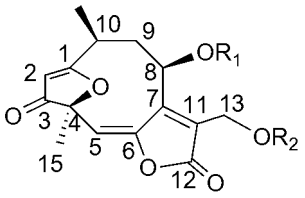
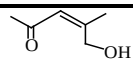
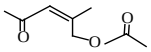
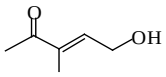
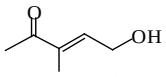
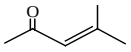
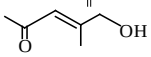
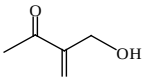
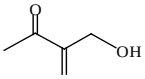
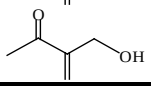
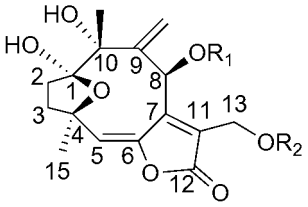
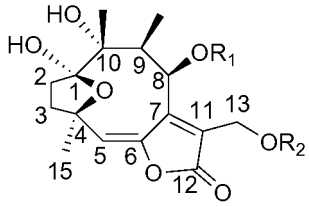


Figure 2.29 Keto hirsutinolides

No.	Common name	R_1	R_2	Mol.-formula	Mol.-mass (g/mol)
141	Keto-hirsutinolides			$C_{22}H_{24}O_9$	432
142	8-*O*-(4',13-diacetoxy senecioyloxy)-keto-hirsutinolide			$C_{24}H_{26}O_{10}$	474
143	8-*O*-(4'-hydroxytigloyoxy) 13-acetoxyketohirsutinolide			$C_{22}H_{24}O_9$	432
144	8-*O*-(4',13-dihydroxytigloyoxy)-ketohirsutinolide		H	$C_{20}H_{22}O_8$	390
145	8-*O*-senecioyloxy-13-acetoxyketohirsutinolide			$C_{22}H_{24}O_8$	416
146	8-*O*-methacryloyoxy-13-acetoxyketohirsutinolide			$C_{21}H_{22}O_8$	402
147	8-*O*-(*E*-4'-hydroxy senecioyloxy)-13-acetoxyketohirsutinolide			$C_{22}H_{24}O_9$	432
148	8-*O*-(4'-hydroxymethacryloyoxy-13-acetoxyketohirsutinolide			$C_{21}H_{22}O_9$	418
149	8-*O*-(4'-hydroxymethacryloyoxy-13-methoxyketohirsutinolide		CH_3	$C_{20}H_{22}O_8$	390
150	8-*O*-(4',13-dihydroxy)-ketohirsutinolide		H	$C_{19}H_{20}O_8$	376

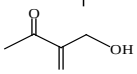
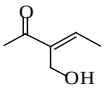
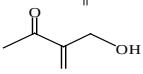
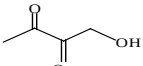


151-168



169-170

Figure 2.30 Piptocarphin A-type

No.	Common name	R_1	R_2	Mol.-formula	Mol. mass (g/mol)
151	Piptocarphin A			$C_{21}H_{26}O_9$	422
152	1,10*α*-dihydroxy piptocarphol ester			$C_{19}H_{24}O_9$	396
153	5,6-*endo*-epoxy- piptocarphol diester			$C_{19}H_{24}O_{10}$	412
154	Piptocarphin isobutanolate			$C_{21}H_{28}O_9$	424
155	Piptocarphin-8-*O*-(4'-hydroxymethacrylate)			$C_{21}H_{26}O_{10}$	438
156	13-hydroxypiptocarphin A		H	$C_{19}H_{24}O_8$	380
157	Piptocarphin C	H	H	$C_{15}H_{20}O_7$	312
158	Piptocarphin D	H		$C_{17}H_{22}O_8$	354
159	Piptocarphin-8-*O*-(5'-hydroxytiglate)			$C_{22}H_{28}O_{10}$	452
160	Hirsutinolide (1F)	H	CH_2CH_3	$C_{17}H_{24}O_7$	340
161	Piptocarphin-8-*O*-angelate			$C_{22}H_{28}O_9$	436
162	13-ethoxypiptocarphin-8-*O*-acetate		CH_2CH_3	$C_{19}H_{26}O_8$	382
163	Piptocarphin F		CH_2CH_3	$C_{21}H_{28}O_8$	408
164	Vernobockolide B		CH_2CH_3	$C_{21}H_{28}O_9$	424
165	13-*O*-methyl hirsutinolide		CH_3	$C_{20}H_{26}O_9$	410
166	13-*O*-methyl Piptocarphin-8-*O*-methacrylate		CH_3	$C_{20}H_{26}O_8$	394
167	13-*O*-methyl Piptocarphin-8-*O*-acetate		CH_3	$C_{18}H_{24}O_8$	368
168	Piptocarphin C-8-*O*-acetylate		H	$C_{17}H_{22}O_8$	354
169	Piptocarphin D-8-*O*-propionylate			$C_{20}H_{26}O_9$	410
170	Piptocarphin-8,13-diester			$C_{19}H_{24}O_9$	396

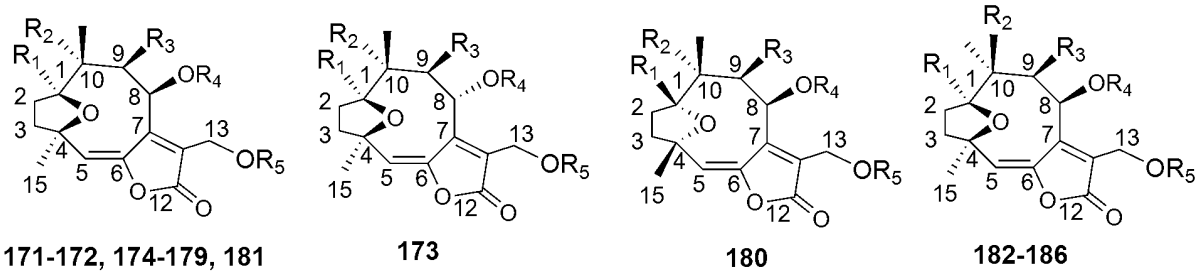
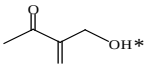
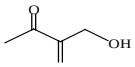
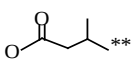


Figure 2.31 Piptocarphin C-type

No.	Common name	R_1	R_2	R_3	R_4	R_5	Mol.-formula	Mol.-mass (g/mol)
171	Piptocarpin C	OCH_3	OH	H		CH_3	$C_{19}H_{26}O_8$	382
172	Piptocarpin C-8-*O*-methacrylate	OCH_3	OH	H		CH_3	$C_{21}H_{28}O_8$	408
173	Piptocarpin C-8-*O*-(4'-hydroxy methacrylate)	OCH_3	OH	H		CH_3	$C_{21}H_{28}O_9$	424
174	10, 13-diacetoxy Piptocarpin C	OCH_3	$OCOCH_3$	H			$C_{22}H_{25}O_{10}$	449
175	10, 13-diacetoxy Piptocarpin C-8-*O*-methacrylate	OCH_3	$OCOCH_3$	H			$C_{24}H_{27}O_{10}$	475
176	13-acetoxy Piptocarpin C	OCH_3	OH	H			$C_{20}H_{26}O_9$	410
177	13-acetoxy Piptocarpin C-8-*O*-(4'-hydroxy methacrylate)	OCH_3	OH	H			$C_{22}H_{28}O_{10}$	452
178	5, 6-dihydro 13-acetoxy Piptocarpin C	OCH_3	CH_3	H			$C_{20}H_{26}O_9$	410
179	10, 13-diacetoxy Piptocarpin C-8-*O*-methacrylate	OCH_3	$OCOCH_3$	H			$C_{22}H_{28}O_9$	436
180	1-isovaleroyl-Pitocarphin C-8-*O*-acetate		OH	H			$C_{24}H_{36}O_{10}$	484
181	1-carboxy-Piptocarphin C-8-*O*-acetate	CHO	OH	H			$C_{20}H_{28}O_9$	412
182	9-methyl-13-acetoxy Piptocarpin C-8-*O*-propionylate	OCH_3	OH	CH_3			$C_{22}H_{30}O_9$	438

No.	Common name	R_1	R_2	R_3	R_4	R_5	Mol.-formula	Mol.-mass (g/mol)
183	9-methyl-13-acetoxy Piptocarpin C-8-*O*-propionylate	OCH_3	OH	CH_3			$C_{22}H_{30}O_9$	438
184	8, 13-dihydroxy,10-dehydroxy Piptocarphin C	OCH_3	H	H	OH	OH	$C_{16}H_{23}O_6$	311
185	1, 13-diacetoxy, 9-methyl Piptocarpin C	$OCOCH_3$	OH	CH_3			$C_{22}H_{29}O_{10}$	453
186	1,13-diacetoxy, 9-methyl Piptocarpin C-8-*O*-propionylate	$OCOCH_3$	OH	CH_3			$C_{23}H_{31}O_{10}$	467

* Note the change in configuration at C-8; ** Note the change in configuration at C-1.

The formation of isohirsutinolides (**187-195**) was also thought to proceed through the costunolide (**1**), which undergoes epoxidation and subsequent isomerization, followed by further oxidation and hydrolysis. This provides the intermediate for a 1,4-*O*-bridge cyclization to the isohirsutinolides (**Figure 2.32**). Other hirsutinolides are presumably formed by a similar biogenetic pathway, often involving oxidation, hydrolysis, acetylation and elimination of water at various stages in the biosynthetic pathway.

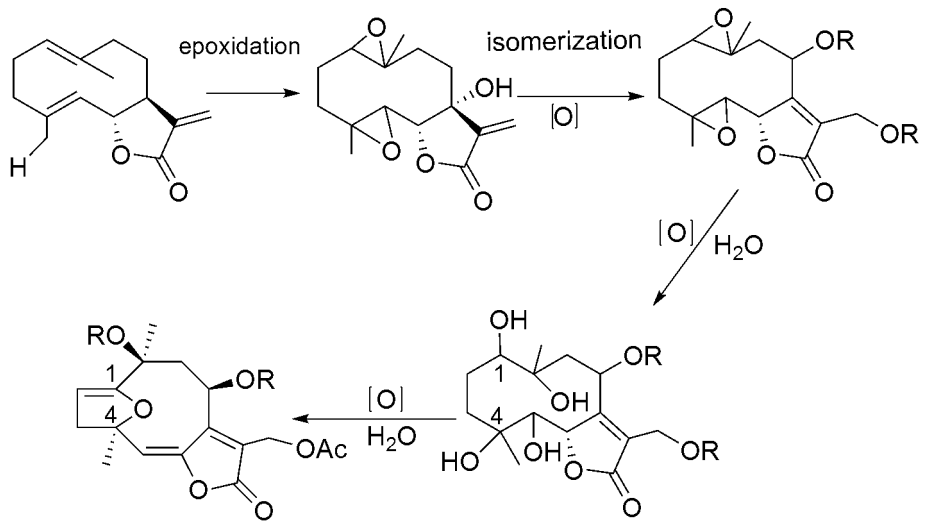


Figure 2.32 Biosynthetic transformation showing the formation of isohirsutinolides (Bohlmann *et al*., 1978)

Although the hirsutinolides are reported as naturally occurring plant compounds, there has been some controversy surrounding this finding, since several authors argue that they are artefacts of isolation during column chromatography. Tully *et al.* (1987) demonstrated that glaucolides undergo transformation under acidic conditions in silica gel resulting in the formation of hirsutinolides. However, Pillay *et al.* (2007) argued that hirsutinolides are natural plant compounds because of their characteristic properties on TLC prior to column chromatography, justifying them as natural products.

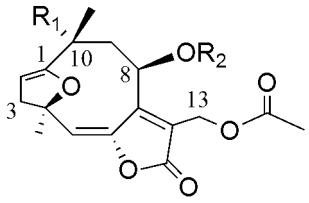
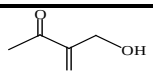
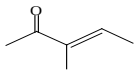


Figure 2.33 Isohirsutinolide sesquitepenoids

No.	Common name	R_1	R_2	Mol.-formula	Mol.-mass (g/mol)
187	8-*O*-(4'-hydroxy methacryloyoxy)-isohirsutinolide	OH		$C_{21}H_{24}O_9$	420
188	8-*O*-methacryloyoxy-isohirsutinolide	OH		$C_{21}H_{24}O_8$	404
189	8-*O*-tigloyoxy-isohirsutinolide	OH		$C_{22}H_{26}O_8$	418
190	8-*O*-methacryloyoxy-isohirsutinolide	OH		$C_{21}H_{24}O_8$	404
191	8-*O*-acetoxy-isohirsutinolide	OH		$C_{19}H_{22}O_8$	378
192	8-*O*-propionoyloxy-isohirsutinolide	OH		$C_{20}H_{24}O_8$	392
193	10-acetoxy-8-*O*-methacryloxy-isohirsutinolide	$OCOCH_3$		$C_{23}H_{26}O_9$	446
194	10-acetoxy-8-*O*-propionoyloxy-isohirsutinolide	$OCOCH_3$		$C_{22}H_{26}O_9$	434
195	8,10-diacetoxy-isohirsutinolide	$OCOCH_3$		$C_{21}H_{24}O_9$	420

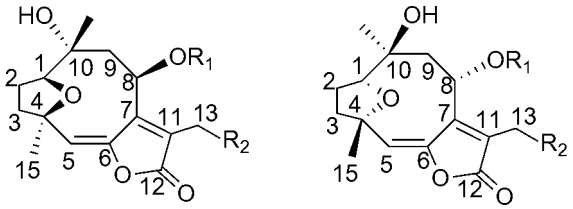
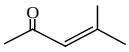
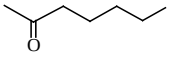
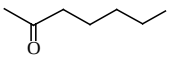
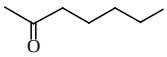


Figure 2.34 1-desoxy hirsutinolide sesquiterpenoids

No.	Common name	R_1	R_2	Mol.-formula	Mol. mass (g/mol)
196	8-*O*-angeloyoxy-1-desoxyhirsutinolides			$C_{22}H_{28}O_8$	420
197	8-*O*-methacryloyoxy-13-methoxy-1-desoxyhirsutinolides		OCH_3	$C_{20}H_{26}O_7$	378
198	8-*O*-senecioyloxy-13-methoxy-1-desoxyhirsutinolides		OCH_3	$C_{21}H_{28}O_7$	392
199	8-*O*-hexanoyloxy-13-methylhydroxy-1-desoxyhirsutinolides		CH_2OH	$C_{22}H_{32}O_7$	408
200	8-*O*-hexanoyloxy-13-methyl-1-desoxyhirsutinolides		CH_3	$C_{22}H_{32}O_8$	424
201	8-*O*-hexanoyloxy-13-carboxy-1-desoxyhirsutinolides		CHO	$C_{22}H_{30}O_9$	438

2.3.4 Cardinanolides

These are sesquiterpene lactones derived from the parent cardinane with a C6/C7 lactone ring and are represented from **Figure 2.38** to **Figure 2.40**. There are seventeen (17) cardinanolides reported in *Vernonia*. Their stereochemistry and position of the lactone ring is unique amongst the sesquiterpenoids. The C8 side chain substitution in all cardinanolides is limited to tiglate and methacrylate esters. Cardinanolides are represented by the vernojalcanolides (**202-211**) and vernomargolides (**212-218**).

The biosynthesis of the cardinane lactones involve cyclization of the (*E,Z*)-farnesyl cation resulting in a *cis*-germacryl cation which subsequently produced the cardinane skeleton after a 1,3-hydride shift (**Figure 2.35**) (Awouafack *et al.*, 2013).

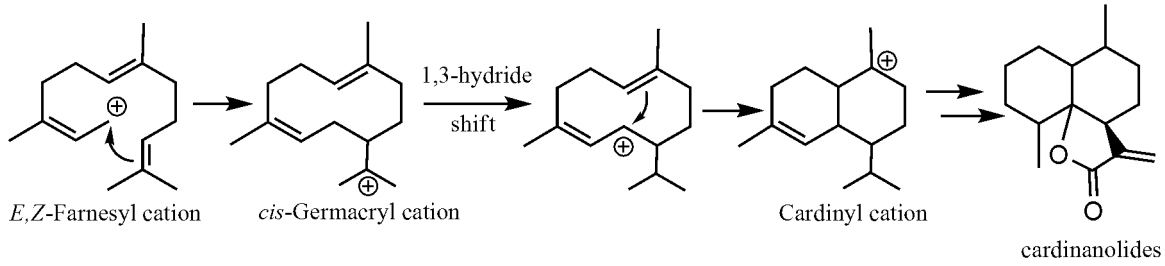


Figure 2.35 Biosynthetic transformation of *E,Z*-farnesyl cation to the cardinolides (Awouafack *et al.*, 2013)

Biogenetic relationships suggest that glaucolide-A is a precursor for cardinanolide transformation. However, Martínez-Vàzquez *et al.* (1986, 1992) demonstrated that glaucolide-A underwent rearrangement to the Vernojalcanolides in the presence of methanol and silica gel (**Figure 2.36**) and *trans* annular cyclization with boron trifluoride (BF_3) to vernomargolides (**Figure 2.37**) through a nucleophillic attack of C2 on C7 with the loss of the C-13 acetoxy group (Rodriguez-Hahn *et al.*, 1988). This led to the belief that cardinanolides are artefacts of isolation and hence should not be regarded as biomarkers.

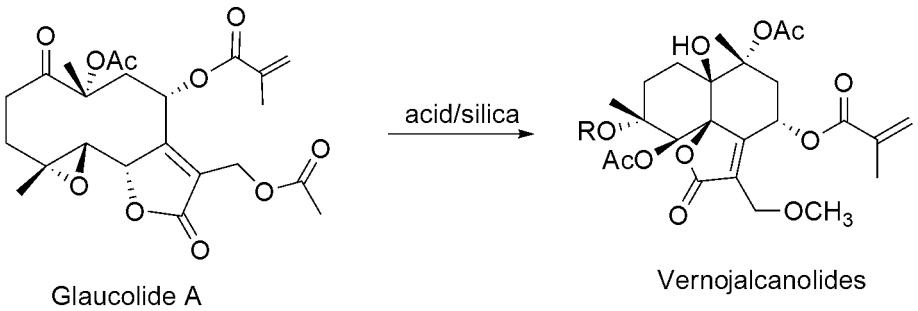


Figure 2.36 Transformation of glaucolide A to the Vernojalcanolide-type sesquiterpene lactones (Martínez-Vàzquez *et al.*, 1986)

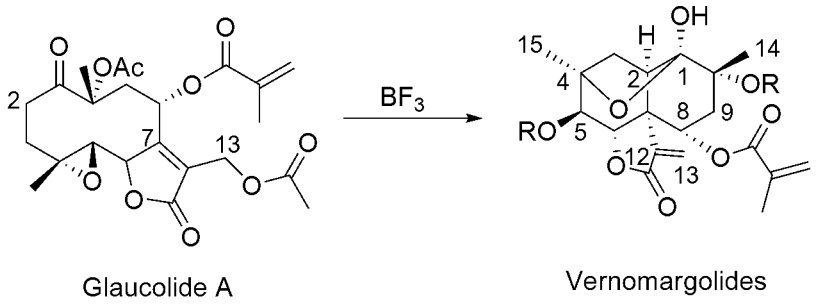


Figure 2.37 *Trans* annular cyclization of glaucolide-A with boron trifluoride (BF_3) resulting in vernomargolides (Rodriguez-Hahn *et al.*, 1988)

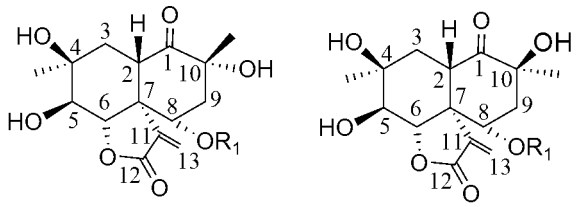
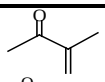
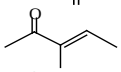
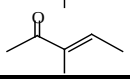


Figure 2.38 Vernopinguisolide type sesquiterpenoids

No.	Common name	R_1	Mol.-formula	Mol. mass (g/mol)
202	Vernopinguisolide A		$C_{19}H_{24}O_8$	380
203	Vernopinguisolide B		$C_{20}H_{26}O_8$	394
204	10β-Vernopinguisolide B		$C_{20}H_{26}O_8$	394

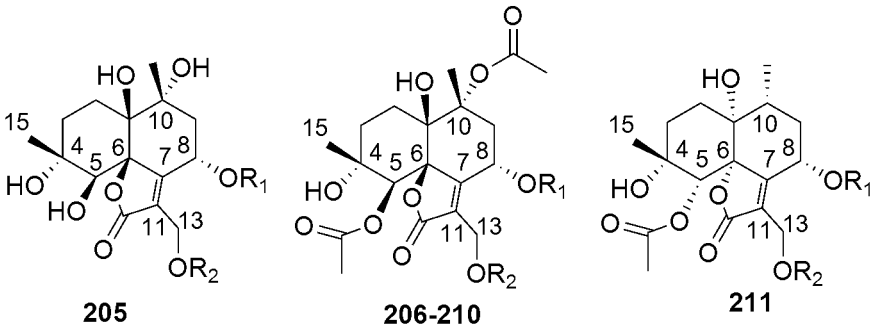
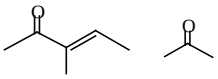
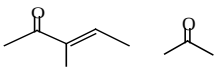


Figure 2.39 Vernojalcanolide-type sesquiterpenoids

No.	Common name	R_1	R_2	Mol.-formula	Mol.-mass (g/mol)
205	13-*O*-methyl-1,4,5,8,10-pentahydroxycardinanolide	H	CH_3	$C_{16}H_{23}O_6$	311
206	5, 8,10-triacetoxy-1,4,13-trihydroxycardinanolide		H	$C_{21}H_{28}O_{11}$	456
207	13-*O*-methyl-5,8,10-triacetoxy-1,4-dihydroxy-cardinanolide		CH_3	$C_{22}H_{30}O_{11}$	470
208	8-*O*-butanoyloxy-5,10-diacetoxy-1,4-dihydroxy-13-methoxycardinanolide		CH_3	$C_{24}H_{34}O_{11}$	498
209	8-*O*-methacryloyloxy-5,10-diacetoxy-1,4-dihydroxy-13-methoxycardinanolide		CH_3	$C_{24}H_{32}O_{11}$	496
210	8-*O*-tigloyloxy-5,10,13-triacetoxy-1,4-dihydroxy cardinanolide			$C_{26}H_{34}O_{12}$	538
211	8-*O*-tigloyloxy-5,13-diacetoxy-1,4-dihydroxy cardinanolide			$C_{24}H_{32}O_{10}$	480

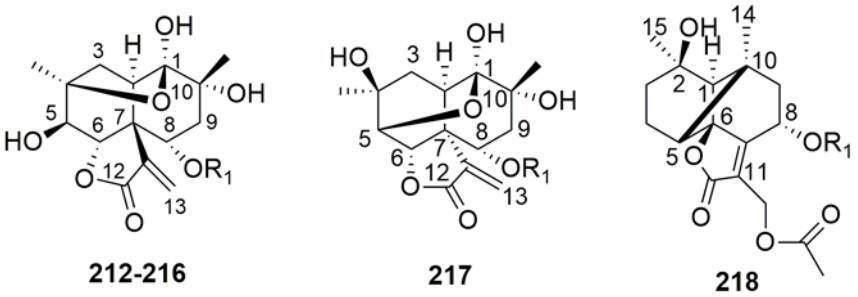
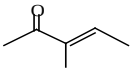
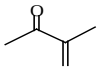
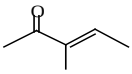
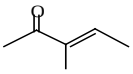
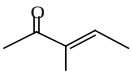


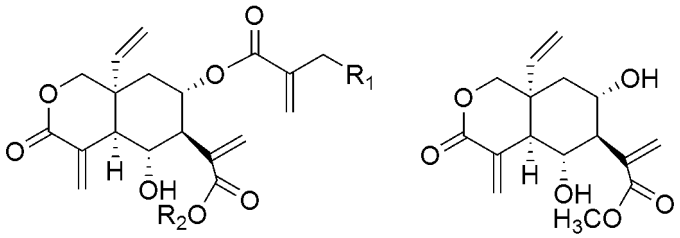
Figure 2.40 Vernomargolide type sesquiterpenoids

No.	Common name	R_1	Mol.-formula	Mol.-mass (g/mol)
212	Vernomargolide-1,4-cyclosemiacetal		$C_{17}H_{20}O_8$	352
213	8-hydroxyvernomargolide-1,4-cyclosemiacetal	H	$C_{15}H_{20}O_7$	312
214	Tigloyloxyvernomargolide-1,4-cyclosemiacetal		$C_{20}H_{26}O_8$	394
215	Methacryloyoxyvernomargolide-1,4-cyclosemiacetal		$C_{19}H_{24}O_8$	380
216	2-epi vernomargolide-1,4-cyclosemiacetal		$C_{20}H_{26}O_8$	394
217	Vernomargolide-1,5-cyclosemiacetal		$C_{20}H_{26}O_8$	394
218	Vernomargolide-5,10-cyclosemiacetal		$C_{20}H_{25}O_7$	377

2.4 Elemanolides

These are derived from the parent elemane and characterised by one or more γ- lactones at either the C6/C7 or C7/C8 positions (**Figure 2.41** to **Figure 2.47**). A total of nineteen (19) elemanolides representing the different but related structural moieties have been reported from *Vernonia* species. The vernodalol skeletal type (**219**-**223**) is a non-lactonized elemane with C6-hydroxy and ester or carboxyl groups attached to C-11. It is presumably a precursor for the synthesis of vernolepin type elemanolides (**224-233**) or products of biogenetic transformation in which the lactone moiety has been oxidized probably by enzyme activity. However, germacranolides such as onopordopicrin (**6**) is a likely precursor of vernolepin

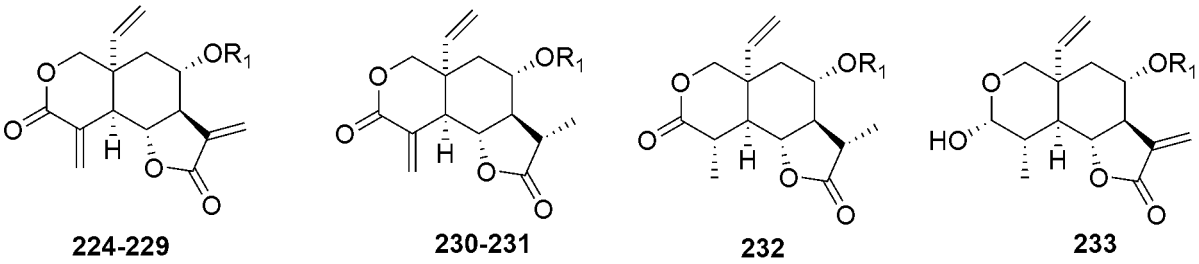
biosynthesis (**Figure 2.43**). This is based on the biogenetic assumptions that germacranolides undergo cope-rearrangement reactions leading to elemanolides (Barrero *et al.*, 1995).



219-222 **233**

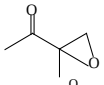
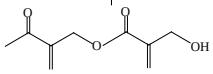
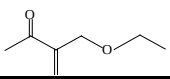
Figure 2.41 Vernodalol type sesquiterpenoids

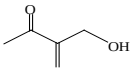
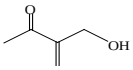
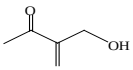
No.	Common name	R_1	R_2	Mol.-formula	Mol. mass (g/mol)
219	Vernodalol	OH	CH_3	$C_{20}H_{25}O_8$	393
220	Vernodalinol	OH	H	$C_{19}H_{23}O_8$	379
221	3'-deoxyvernadalol	H	CH_3	$C_{20}H_{25}O_7$	377
222	Epivernodalol (2β)	OH	CH_3	$C_{20}H_{25}O_8$	393
223	Lasiopulide	H	CH_3	$C_{16}H_{21}O_6$	309



224-229 **230-231** **232** **233**

Figure 2.42 Vernolepin type sesquiterpenoids

No.	Common name	R_1	Mol.-formula	Mol. mass (g/mol)
224	Vernolepin	H	$C_{15}H_{17}O_5$	277
225	Vernolepin-8-*O*-methacrylate		$C_{19}H_{21}O_6$	345
226	Vernolepin-8-*O*-epoxymethacrylate		$C_{19}H_{21}O_7$	361
227	Vernolepin hydroxyl methacryloyl methacrylate		$C_{24}H_{27}O_8$	443
228	Vernonilide B		$C_{21}H_{24}O_7$	

No.	Common name	R_1	Mol.-formula	Mol. mass (g/mol)
229	Vernodalin		$C_{19}H_{21}O_7$	361
230	Vernomygdalin	H	$C_{15}H_{19}O_5$	279
231	11, 13-dihydrovernodalin		$C_{19}H_{23}O_7$	363
232	11, 13-dihydrovernolepin	H	$C_{15}H_{21}O_5$	281
233	Vernolepin-8-*O*-hydroxymethacrylate		$C_{19}H_{23}O_7$	363

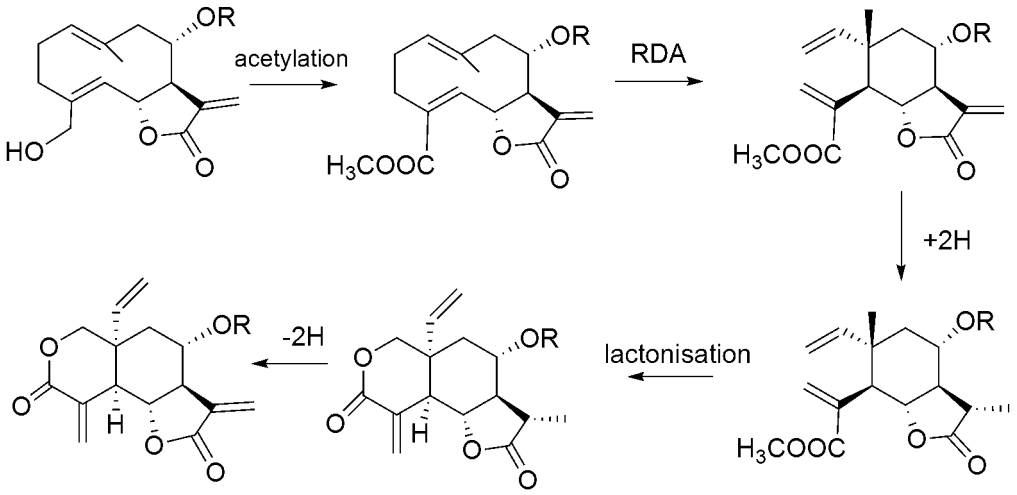


Figure 2.43 Biosynthesis of vernolepin type sesquiterpenoid**s** (**224-233**)

The elemanolide dimmers A (**234**) and B (**235**) were thought to have emerged through Diels-Alder reactions involving vernodalin (**232**) and vernadalol (**219**), respectively (**Figure 2.45**). The synthesis of Vernodalidimer A involved the cycloaddition reaction of the enone moiety of one vernodalin with the methylene group of the other in a region-specific pattern (Liu *et al*., 2010). It was observed that vernodalol **(219-223**) and vernolepin sesquiterpenoids (**224-233**) are the most representative elemanolides.

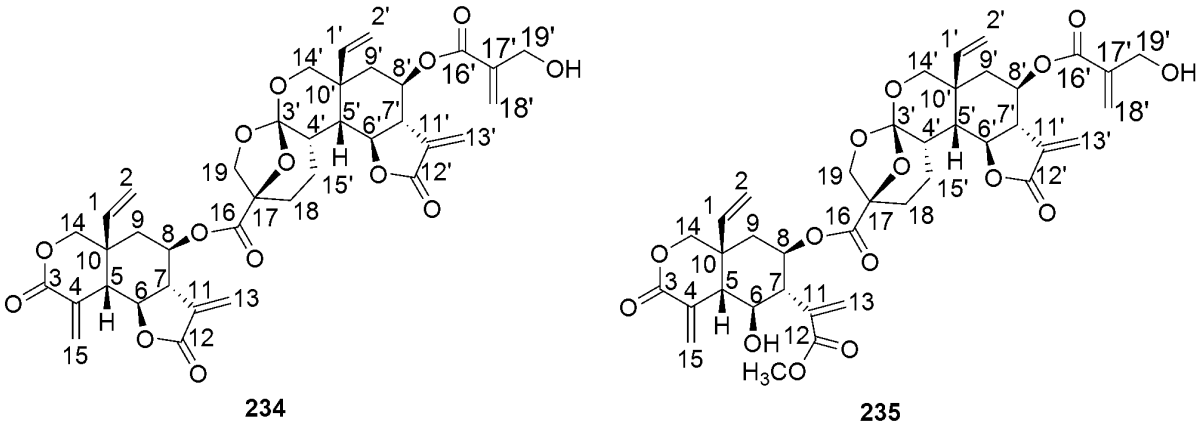


Figure 2.44 Vernodalidimers

No.	Common name	Mol. formula	Mol. mass (g/mol)
234	Vernodalidimers A	$C_{38}H_{40}O_{14}$	720
235	Vernodalidimers B	$C_{39}H_{44}O_{15}$	752

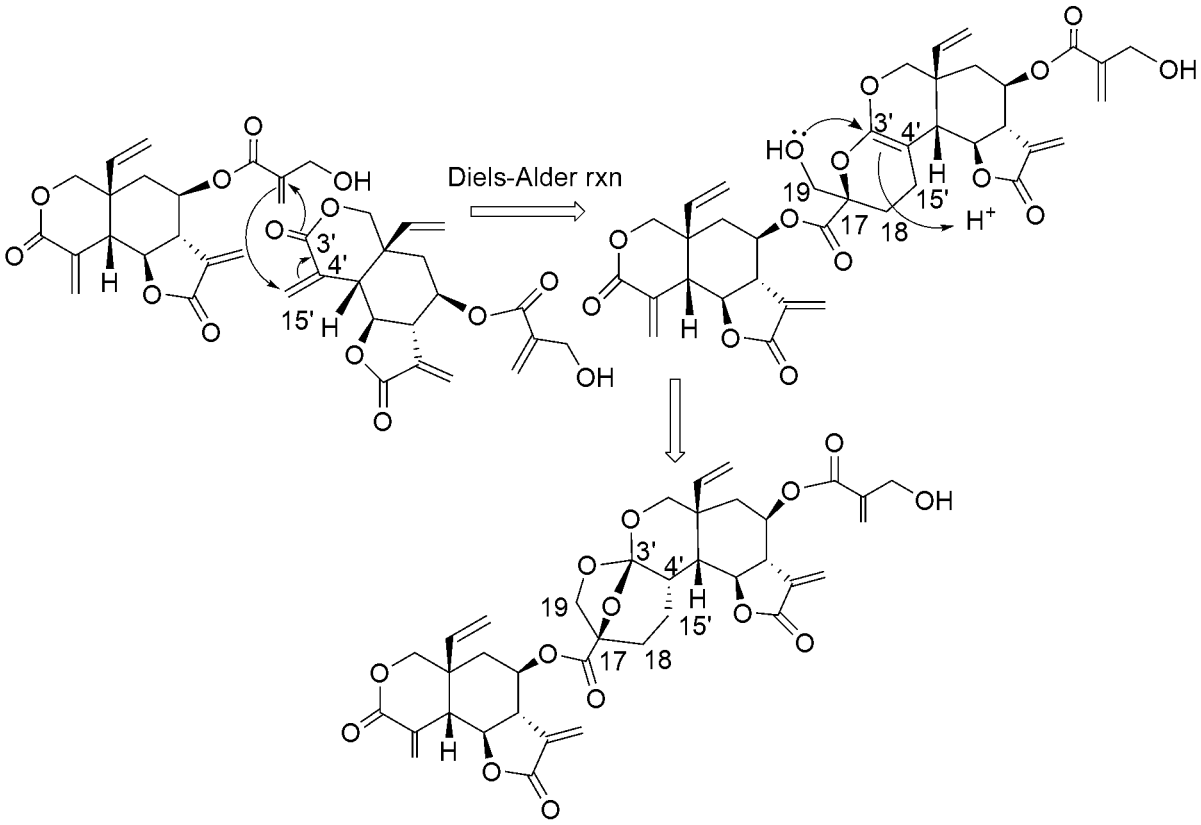


Figure 2.45 Biosynthesis of Vernodalidimer A (**234**)

Vernomenin (**236**) is a rare skeletal type within *Vernonia*, representing the only elemanolides with a C7/C8 lactonized system. Vernomelitensin (**238**) represents an elemanolide with a C2/C3-diene as the only skeletal type in the *Vernonia*. Plausible biogenetic relations (Zhang *et al*., 2014) showed that Vernomelitensin type formation is mediated by an acid catalyzed reaction of 11,13-dihydrovernolepin (**226**) followed by dehydration and reduction (**Figure 2.47**).

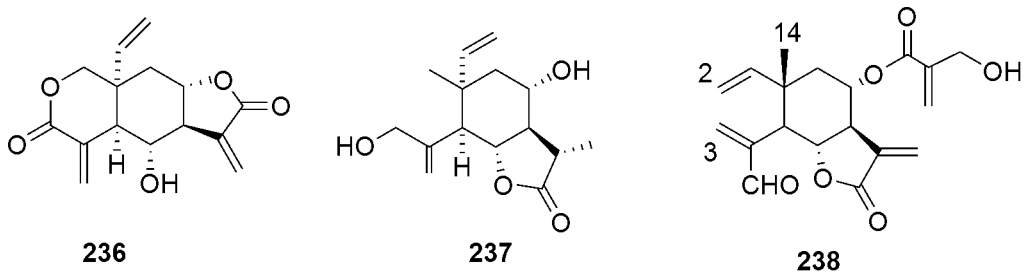


Figure 2.46 Melitensin type sesquiterpenoids

No.	Common name	Mol. formula	Mol. mass (g/mol)
236	Vernomenin	$C_{15}H_{17}O_5$	277
237	Melitensin	$C_{15}H_{22}O_4$	266
238	Vernomelitensin	$C_{19}H_{22}O_6$	346

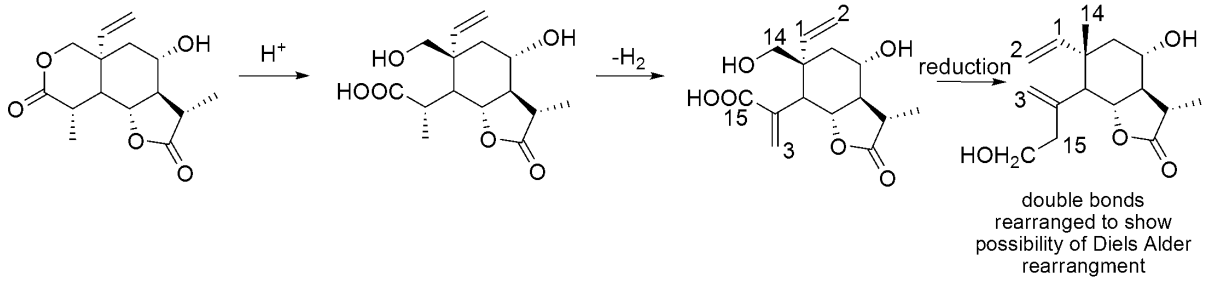


Figure 2.47 Biosynthesis of Vernomelitensin type sesquitepenoids

2.5 Eudesmanolides

These are derived from the parent eudesmanes and characterised by cyclodecane skeleton with either C6/C7 or C7/C8 lactonized systems (**Figure 2.49** and **Figure 2.50**), however only those with a C6/C7 lactone have been reported from *Vernonia*. These compounds are rarely

found in the genus *Vernonia* with only seven that have been previously reported, four of which (blumeoidolides A, B, C and D) were present in *Vernonia blumeoides* growing in Nigeria (Aliyu *et al*., 2015). The blumeoidolides have a characteristic 2-hydroxy-2-methyl butanoyl group at C8. The hydroxy group of this moiety is acetylated in blumeoidolide B. This moiety presumably arises through oxidation of tiglate double bonds, followed by ring opening (**Figure 2.48**) (Aliyu *et al*., 2015).

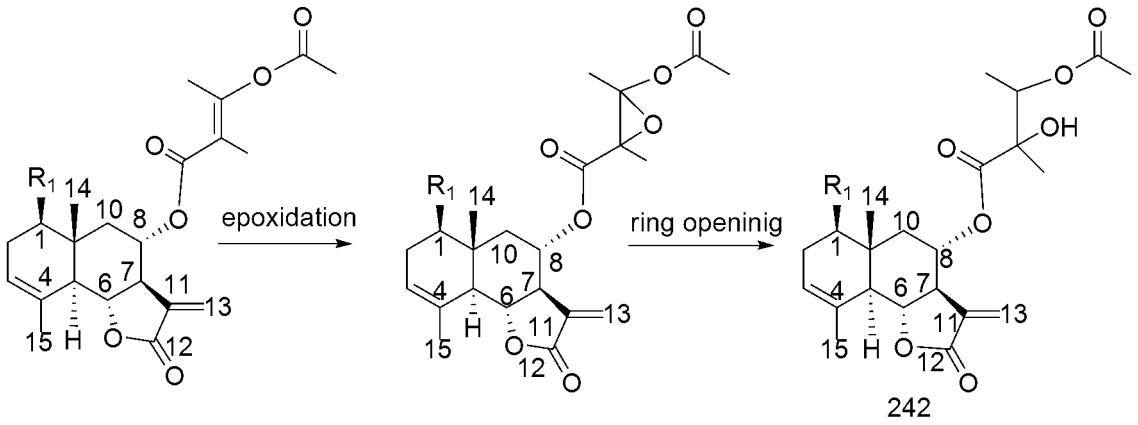


Figure 2.48 Formation of the 2-hydroxy-2-methyl butanoyl group at C8

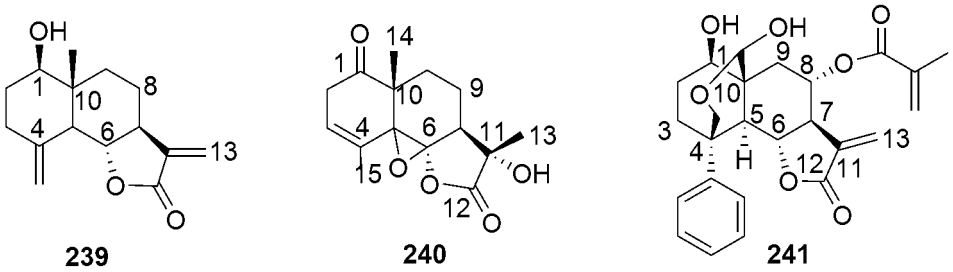


Figure 2.49 Eudesmanolide sesquiterpenes

No.	Common name	Mol. formula	Mol. mass (g/mol)
239	reynosin	$C_{15}H_{20}O_3$	248
240	5,6-epoxyeudesmanolide	$C_{15}H_{18}O_5$	278
241	vernodesmine	$C_{25}H_{28}O_7$	440

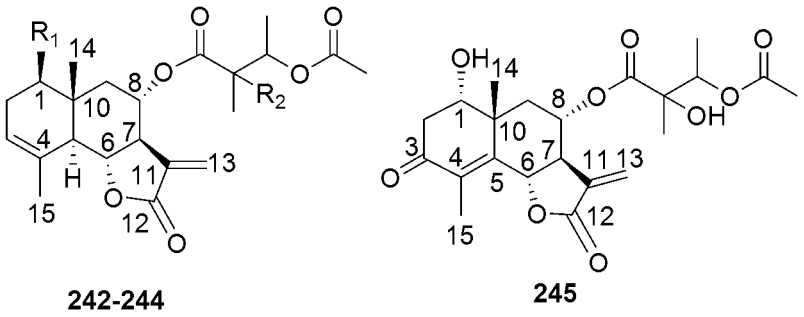


Figure 2.50 Blumeoidolide type

No.	Common name	R_1	R_2	Mol.-formula	Mol.-mass (g/mol)
242	Blumeoidolide A	$OCOCH_3$	OH	$C_{24}H_{32}O_9$	464
243	Blumeoidolide B	OH	$OCOCH_3$	$C_{24}H_{32}O_9$	464
244	Blumeoidolide C	OH	OH	$C_{22}H_{30}O_8$	422
245	Blumeoidolide D	-	-	$C_{22}H_{28}O_9$	436

The four blumeoidolides A (**242**), B (**243**), C (**244**) and D (**245)** have common eudesmane skeletal types and may have similar biogenetic relationships. Biomimetic transformations have indicated germacranolides as primary precursors of eudesmanolides (Fischer, 1990). Costunolide (**1**) is a common precursor in eudesmanolide biosynthesis. The formation of reynosin (**239**) is thought to occur by acid catalyzed epoxidation, followed by opening of the epoxide ring and cyclisation. The olefinic methylene proton arises by loss of the proton at C-15 (**Figure 2.51**).

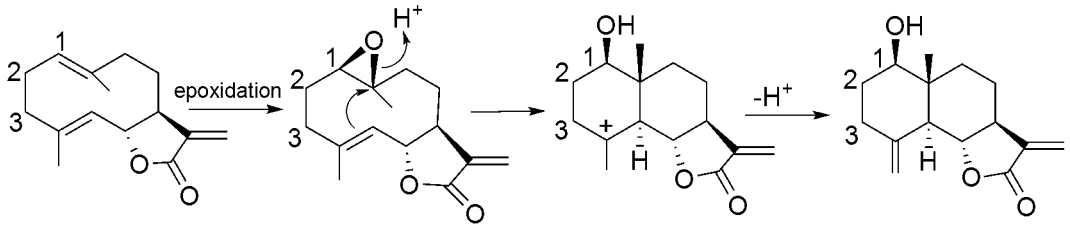


Figure 2.51 Biosynthesis of eudesmanolide: reynosin

2.6 Guaianolides

Guaianolides are sesquiterpenoids of the bicyclic guanine origin. They are chemically bicyclo [5.3.0] decane compounds with a C-5 lactone ring (Miklos, 2012). Guaianolides generally occur as C-6/C-12 or C-8/C-12 lactonized systems (**Figure 2.52** to **Figure 2.61**) predominantly within the Asteraceae, although many have also been isolated from the Apiaceae (Drew *et al*., 2009). There are 51 guaianolides reported to have been isolated from *Vernonia* species. Hydroxy groups, esterified hydroxy groups and epoxy groups are common substituents in guaianolides (Simonsen *et al.*, 2013). The guaianolides in *Vernonia* are broad with interesting chemical diversity. They were largely isolated from South American species with very few reports from species of African and Asian origins.

Zaluzanine guaianolides have a ketone functionality at C-3, with mostly an olefinic methylene at C-4 and C-12, however C-12 is reduced in **250** and **251** and C-4 is reduced in **251** only. Hydroxy or hydroxy derivatives occur at C-8 and C-9.

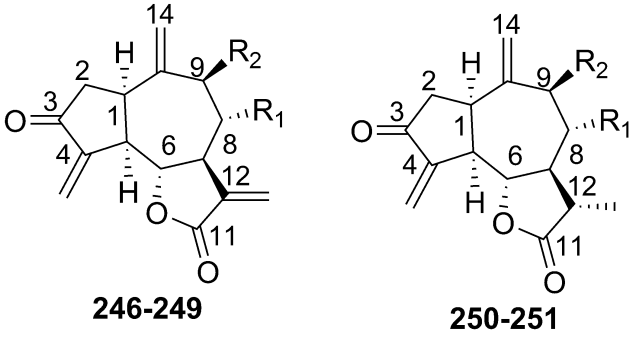
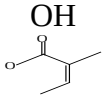


Figure 2.52 Guaianolide type sesquiterpenoids with a ketone at C-3

No.	Common name	R_1	R_2	Mol.-formula	Mol.-mass (g/mol)
246	Hydrozaluzanine	H	OH	$C_{15}H_{16}O_4$	260
247	Dehydrozaluzanin C	OH	OH	$C_{15}H_{16}O_5$	276
248	8-*O*-angeloyloxy Zaluzanin C		OH	$C_{20}H_{22}O_6$	358

No.	Common name	R_1	R_2	Mol.-formula	Mol. mass (g/mol)
249	Zaluzanine	H	H	$C_{15}H_{16}O_3$	244
250	8*α*-Hydroxy-11*β*, 13-dihydro Zaluzanin C	OH	OH	$C_{15}H_{18}O_5$	278
251	3-oxograndolide	H	OH	$C_{15}H_{18}O_4$	262

In **252-264**, the C-3 ketone is reduced to a hydroxy group. The substitution at C-4, C-8, C-9 and C-12 is much like those of **246-251** above.

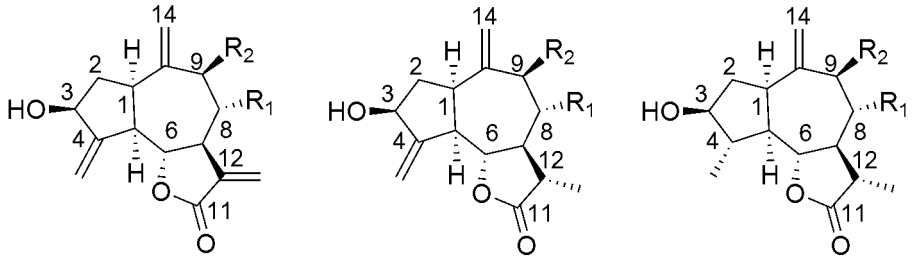
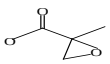
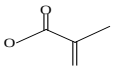
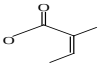
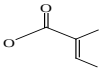
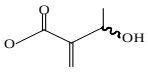
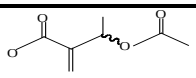
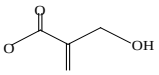
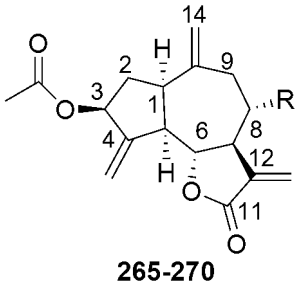


Figure 2.53 Guaianolide type sesquiterpenoids with a hydroxy group at C-3

No.	Common name	R_1	R_2	Mol.-formula	Mol.-mass (g/mol)
252	Zaluzanin C	H	H	$C_{15}H_{18}O_3$	246
253	8-*O*-epoxy-methacryloyoxy Zaluzanin C		H	$C_{19}H_{22}O_6$	346
254	8*α*-hydroxy Zaluzanin C	OH	H	$C_{15}H_{18}O_4$	262
255	8-*O*-acetyloyoxy Zaluzanin C		H	$C_{17}H_{20}O_5$	304
256	8-*O*-methacryloyoxy Zaluzanin C		H	$C_{19}H_{22}O_5$	330
257	8-*O*-angeloyloxy Zaluzanin C		H	$C_{20}H_{24}O_5$	344
258	8-*O*-tigloyloxy Zaluzanin C		H	$C_{20}H_{24}O_5$	344
259	8-*O*-[2-(1-hydroxyethyl) propenoyloxy] Zaluzanin C		H	$C_{20}H_{24}O_6$	360

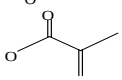
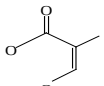
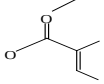
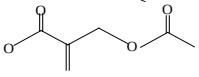
No.	Common name	R_1	R_2	Mol.-formula	Mol.-mass (g/mol)
260	8-*O*-[2-(1-acetoxy ethyl) propenoyloxy] Zaluzanin C		H	$C_{22}H_{26}O_7$	402
261	8-*O*-(4'-hydroxy methacryloyoxy) Zaluzanin C		H	$C_{19}H_{22}O_6$	346
262	3*β*-hydroxy-4, 15 dehydrograndolide	H	OH	$C_{15}H_{20}O_4$	264
263	3*β*-hydroxy grandolide	H	OH	$C_{15}H_{22}O_4$	266
264	Tetrahydrozaluzanin C	OH	H	$C_{15}H_{22}O_4$	266

Compounds **265-270** all have an acetyl group at C-3, with olefinic methylene groups at C-4 and C-12 and no substituent at C-9. C-8 contains esterified groups with the exception of **265**.



265-270

Figure 2.54 Guaianolide type sesquiterpenoids with an *O*-acetyl group at C-3

No.	Common name	R	Mol.-formula	Mol.-mass (g/mol)
265	Zaluzanin D	H	$C_{17}H_{20}O_4$	288
266	8-*O*-acetyloyoxy Zaluzanin D		$C_{19}H_{22}O_6$	346
267	8-*O*-methacryloyoxy Zaluzanin D		$C_{21}H_{24}O_6$	372
268	8-*O*-angeloyloxy Zaluzanin D		$C_{22}H_{26}O_6$	386
269	8-*O*-tigloyloxy Zaluzanin D		$C_{22}H_{26}O_6$	386
270	8-*O*-(4'-acetoxy methacryloyoxy) Zaluzanin D		$C_{23}H_{26}O_8$	430

Compounds **271-275** have either esterified or glycosylated groups at C-3. Compound **274** has a hydroxy group at C-8, while **276** has an epoxy group at C-2 and C-3. All these compounds have olefinic methylene groups at C-4 and C-12 and no substituents at C-9.

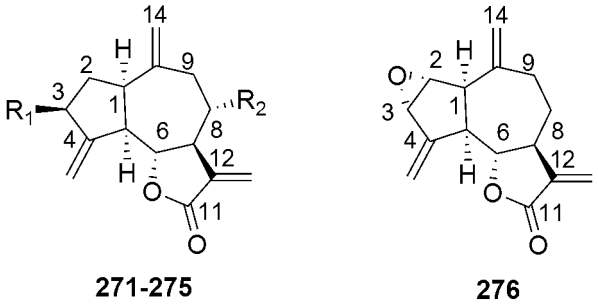
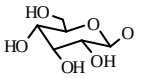
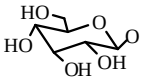
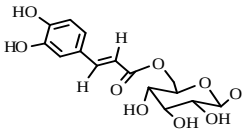


Figure 2.55 Guaianolide type sesquiterpenoids with other oxygenated groups at C-3

No.	Common name	R_1	R_2	Mol.-formula	Mol.-mass (g/mol)
271	Zaluzanin A-3*β*-*O*-senecioate	$OCOCH=C(CH_3)_2$	H	$C_{20}H_{24}O_4$	328
272	Zaluzanin A isobutyrate	$OCOCH(CH_3)_2$	H	$C_{19}H_{24}O_4$	316
273	Glucozaluzanin C		H	$C_{21}H_{28}O_8$	408
274	Dihydro Zaluzanin C-3-*O*-*β*-glucopyranoside		OH	$C_{21}H_{28}O_9$	424
275	6'-*O*-Caffeoyl-glucozaluzanin C		H	$C_{30}H_{34}O_{11}$	570
276	2,3-*α*-epoxy Zaluzanin	-	-	$C_{15}H_{16}O_3$	244

The dehydrocostus lactones (**277-282**) have no functional group at C-3 or C-9. In general, C-4 and C-12 have olefinic methylene groups, however **281-282** has a hydroxymethyl group at C-4 and **282** has a methyl group at C-12.

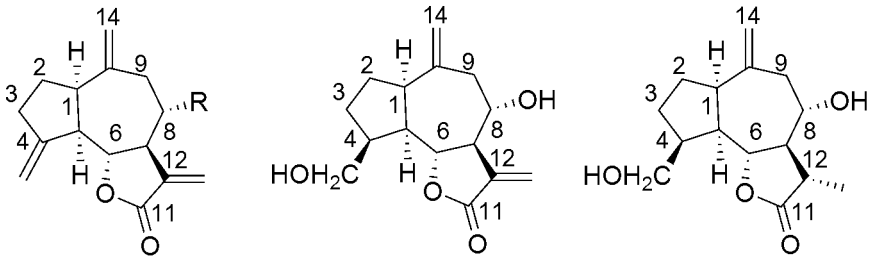
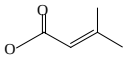


Figure 2.56 Guaianolide type sesquiterpenoids with a fully reduced C-3

No.	Common name	R	Mol.-formula	Mol.-mass (g/mol)
277	Dehydrocostus lactone	H	$C_{15}H_{18}O_2$	230
278	8*α*-*O*-senecioyloxy dehydrocostus lactone		$C_{20}H_{24}O_4$	328
279	8*α*-hydroxy dehydrocostus lactone	OH	$C_{15}H_{18}O_3$	246
280	8*α*-acetoxy dehydrocostus lactone	$OCOCH_3$	$C_{17}H_{20}O_4$	288
281	8*α*,15-dihydroxy dehydrocostus lactone	-	$C_{15}H_{20}O_4$	264
282	13*α*-dehydro-8*α*,15-dihydroxy dehydrocostus lactone	-	$C_{15}H_{22}O_4$	266

Jalcaguaianolide type sesquiterpenes have a guanianolide framework with a fully reduced C-3, an epoxy group at C-4 and C-5 or a hydroxyl at C-4 and oxidized groups at C-8 and C-13. Compounds **283-287** with the epoxide contain hydroxyl and *β*-methyl groups at C-10, whereas in **288** an olefinic methylene group is present at C-10.

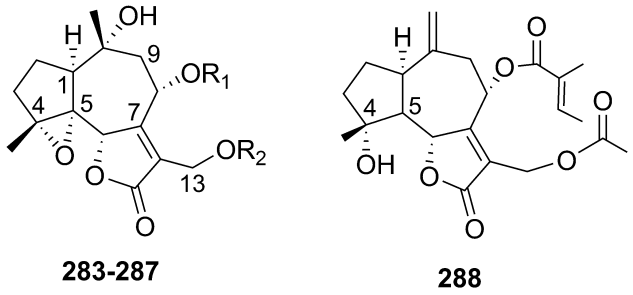


Figure 2.57 Jalcaguaianolide type sesquiterpenes

No.	Common name	R_1	R_2	Mol.-formula	Mol. mass (g/mol)
283	Jalcaguaianolide B		H	$C_{20}H_{26}O_7$	378
284	13-Methoxyjalcaguaianolide B		CH_3	$C_{21}H_{28}O_7$	392
285	13-Acetoxyjalcaguaianolide B			$C_{22}H_{28}O_8$	420
286	13-Methoxy-8-*O*-acetoxyjalcaguaianolide		CH_3	$C_{18}H_{24}O_7$	352
287	8,13-di-*O*-acetoxy jalcaguaianolide			$C_{19}H_{24}O_8$	380
288	Jalcaguaianolide A	-	-	$C_{22}H_{28}O_7$	404

The biosynthesis of jalcaguaianolide type sesquiterpenoids of the guaianolide type involves C-4 epoxidation of costunolide, ring closure forming bicyclo [5.3.0] decane compounds, then loss of a proton, forming the olefinic methylene at C-10 (**Figure 2.58**) (Fischer, 1990).

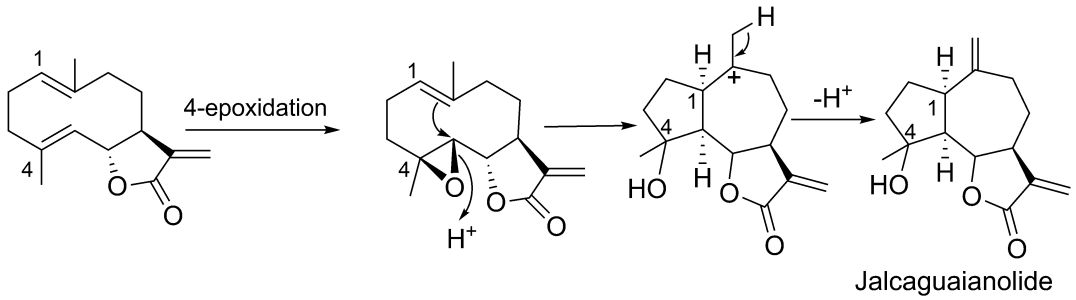


Figure 2.58 Biosynthesis of guaianolides

Guaianolides might also be formed by the Cope rearrangement of elemanolide precursors under UV radiation (Zhang *et al*., 2014). Dehydrogenation at C-10(14), followed by C-11(13) leads to the guaianolides (**Figure 2.59**).

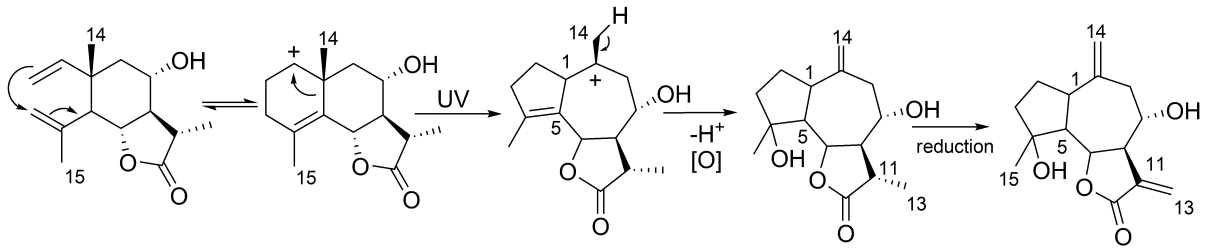


Figure 2.59 Biosynthesis of guaianolides (**283-288**)

In the vernocinolide guaianolide sesquiterpenoids **289-294**, C-4, C-8 and C-10 is oxidised with hydroxy, methoxy or other oxygenated alkyl groups. Vernocinolide and vernobockolide A contain a hydroxy group at C-6 and an oxygenation group at C-13. Compound **292** is the most basic of these sesquiterpenoids, which is not oxygenated at C-6 or C-13. Compounds **293** and **294** contain conjugated double bonds at C-5(6) and C-7(11), and C-6(7) and C-11(13), respectively.

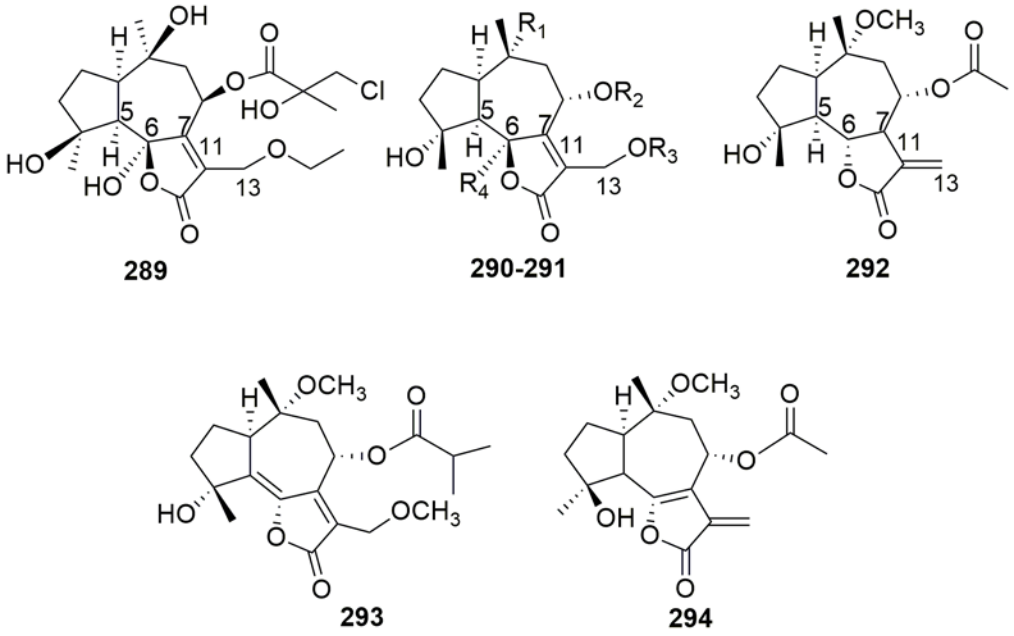


Figure 2.60 Vernocinolide type guaianolide sesquiterpenoids

No.	Common name	R_1	R_2	R_3	R_4	Mol.-formula	Mol.-mass (g/mol)
289	Vernocinolide	-	-	-	-	$C_{21}H_{31}O_9Cl$	462
290	Vernobockolide A	OH		CH_2CH_3	OH	$C_{21}H_{30}O_8$	410
291	13-Methoxyvernocinolide-8-*O*-isobutyrate	OCH_3		CH_3	H	$C_{21}H_{32}O_7$	396
292	4α-hydroxy-8-*O*-acetoxy-10-methoxyvernocinolide	-	-	-	-	$C_{18}H_{26}O_6$	338
293	10,13-Dimethoxy vernocinolide-8-*O*-isobutyrate	-	-	-	-	$C_{21}H_{30}O_7$	394
294	4β-Hydroxy-8-*O*-acetoxy-10-methoxyvernocinolide	-	-	-	-	$C_{18}H_{24}O_6$	336

The eremanthine type sesquiterpenes have olefinic methylene groups at C-4 and C-11 and in some cases a substituent at C-8. In **295-296** a double bond occurs at C-9(10) and in **297**, epoxidation at the same position occurs. In **298**, a double bond is present at C-1(10), with an additional hydroxy group at C-3.

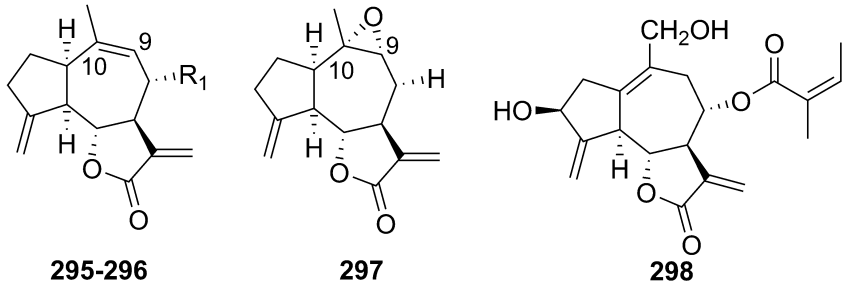
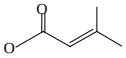


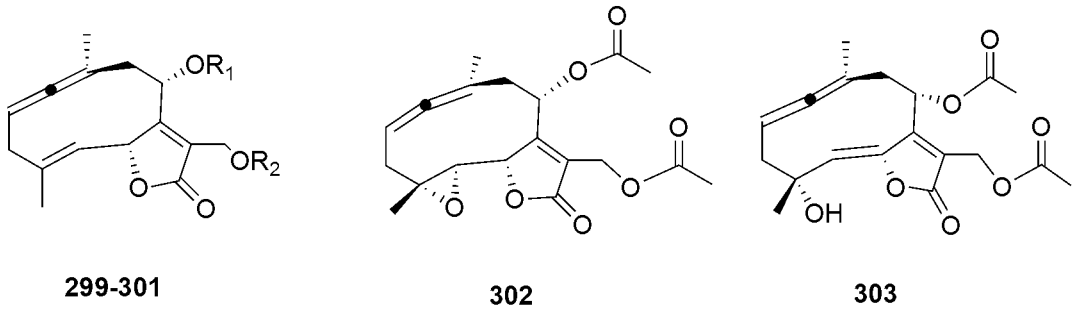
Figure 2.61 Eremanthine type sesquiterpenoids

No.	Common name	R_1	Mol.-formula	Mol. mass (g/mol)
295	Eremanthine	H	$C_{15}H_{18}O_2$	230
296	Eremanthine senecioate		$C_{20}H_{24}O_4$	328
297	9,10-Epoxy eremanthine	H	$C_{15}H_{18}O_3$	246
298	8-*O*-Angeloyloxy 3β,14-dihydroxy-guaian-1(10), 4(15),11(13) trien-6,12-olide		$C_{20}H_{24}O_6$	360

2.7 Miscellaneous types

Miscellaneous sesquiterpene lactones are defined based on unique characteristics and have structural features or unusual substitutional patterns that is rare in the literature. In *Vernonia*, three different classes of compounds are classified as miscellaneous: (i) Vernonallenes (ii) Bourbonenolides and (iii) Potamopholide.

Vernonallenes: These are bicyclic germacranolides bearing endocyclic allenes (**Figure 2.62**) (Bohlmann *et al*. 1981; 1982; Jakupovic *et al*., 1986). Basically, allenes are polyenes having cumulated dienes, characterized by a central carbon (sp-hybridized) sharing a double bond with two adjacent sp^2 hybridized carbons (Luche *et al*., 1980). The vernonallenes (**299-303**) are characterized by a similar skeletal type with varying degrees of substitution such as epoxidation and hydroxylation. No other allenes have been reported from *Vernonia*.

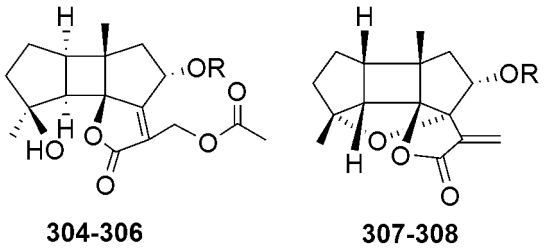


299-301 302 303

Figure 2.62 Vernonallene type sesquiterpenoids

No.	Common name	R_1	R_2	Mol. formula	Mol. mass (g/mol)
299	Vernoallenolide			$C_{19}H_{22}O_6$	346
300	Vernoallenolide diol	H	H	$C_{15}H_{18}O_4$	262
301	Vernoallenolide acetate	H		$C_{17}H_{20}O_5$	304
302	4,5-epoxyvernoallene	-	-	$C_{19}H_{22}O_7$	362
303	4-hydroxyvernoallene	-	-	$C_{19}H_{22}O_7$	362

Bourbonenolides (**Figure 2.63**) are characterised by a unique system with two cyclopentyl rings joined by a central cyclobutane ring in which a lactone moiety at C6/C7 is formed. Biogenetic relationships indicated that the glaucolides such as marginatin (**52**) are the precursor for bourbonenolide biosynthesis (Bohlmann *et al*., 1981). This involves marginatin transformations through acid catalyzed ring opening of the epoxide, intramolecular cyclization of C10/C6, forming the first cyclopentyl ring. Attack on the C4 methylene system by a water molecule results in cyclization through the C1 carbocation to form the second cyclopentyl ring and the central cyclobutane ring (**Figure 2.64**).



304-306 307-308

Figure 2.63 Bourbonenolide type sesquiterpenoids

No.	Common name	R_1	Mol.-formula	Mol. mass (g/mol)
304	Bourbonenolide		$C_{19}H_{24}O_7$	364
305	Bourbonenolide-8-*O*-tiglate		$C_{12}H_{28}O_7$	404
306	Bourbonenolide-8-*O*-methacrylate		$C_{21}H_{26}O_7$	390
307	4,7-oxo-bourbonenolide tiglate		$C_{20}H_{24}O_5$	344
308	4,7-oxo-bourbonenolide methacrylate		$C_{19}H_{22}O_5$	330

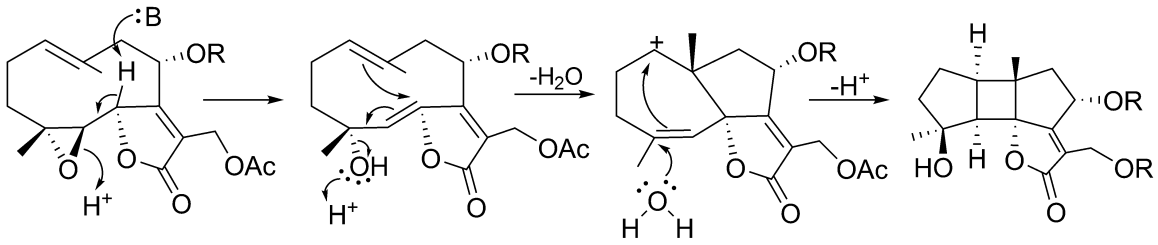


Figure 2.64 Biosynthetic transformation of bourbonenolides (**304**-**308**)

Potamopholide: This is a germacranolide containing 3,8-*O*-bridged 10 membered hemi-acetal skeleton (**309**) (**Figure 2.65**). It is structurally related to the 1,4-*O*-bridged compounds such as the hirsutinolides. The compound was thought to originate from an 8-*O*-acyl precursor such as the *trans* epoxide in the 1,3-diketone glaucolide (**Figure 2.66**). The abstraction of proton from C6 leads to a stable aromatic furan enolate anion. Movement of electrons from the enolate anion, ultimately ending in ring opening of the epoxide with an

attack on the carbonyl group at C-1 results in a 1,4-*O*-bridge similar to the hirsutinolide type. Ring opening of this furan ring and cyclisation of the oxygen at C-8 with the carbonyl group at C-3 leads to the potamopholide (**309**). The compound was isolated from *V. potamophila* found in the Congo DR (Bila *et al*., 2003).

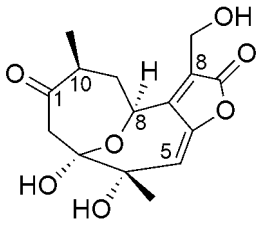


Figure 2.65 The structure of potamopholide

No.	Common name	Mol formula	Mol mass (g/mol)
309	Potamopholide	$C_{15}H_{18}O_7$	310

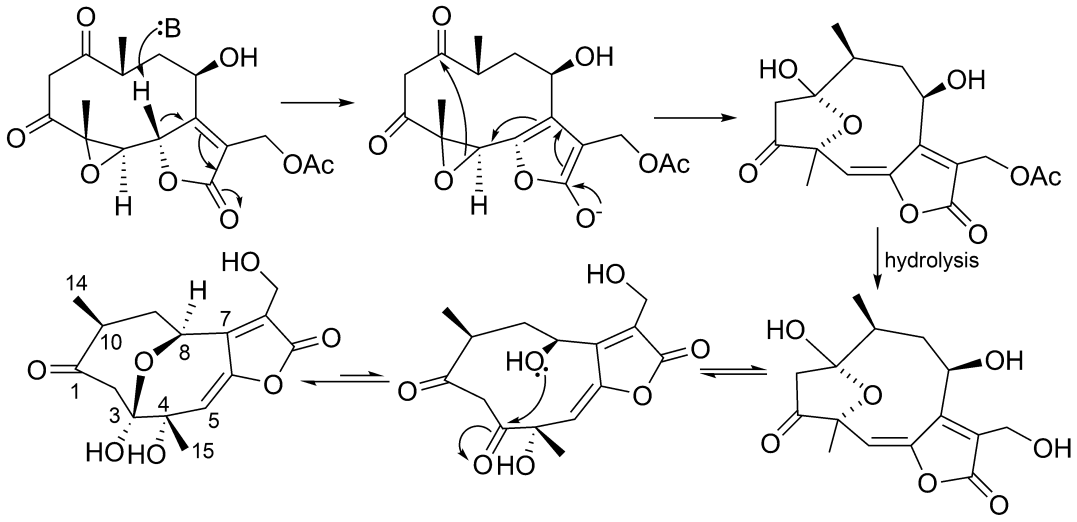


Figure 2.66 Plausible biosynthesis of potamopholide (**309**)

2.8 Conclusion

The sesquiterpene lactones have been studied for four decades with numerous reports on the isolation and structure elucidation of various derivatives. The analysis of phytochemical data on the sesquiterpenoids based on structural variation is intended to provide a holistic picture of these compounds in relation to the number of derivatives that have been isolated from each class. A total of 309 sesquiterpene lactones were reported from 81 *Vernonia* species. The germacranolides represent the largest class with 219 (71%), guaianolides 53 (17%), elemanolides 10 (6%), eudesmanolides 7 (3%) and miscellaneous 10 (3%) as shown in **Figure 2.67**.

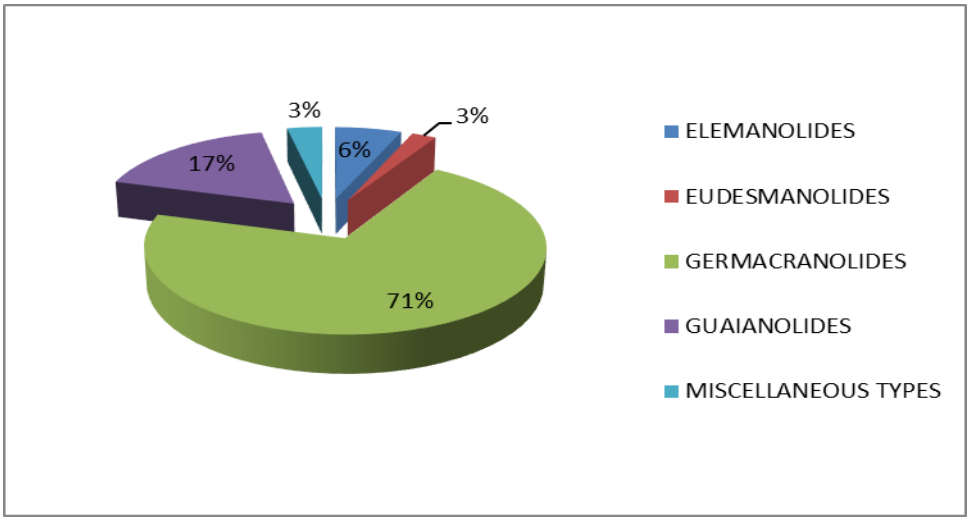


Figure 2.67 Distribution of major sesquiterpene lactones in *Vernonia*

The germacranolides constitutes four subgroups each with structural or chemical features of considerable importance. The non-glaucolides and cardinanolides are poorly represented within the germacranolides with twenty one and five structural motifs representing 7% and 6%, respectively of sesquiterpenoids from *Vernonia*. However, the glaucolides are a highly diverse sub-group of the germacranolides containing thirty one chemical motifs (**Figure 2.7** through to **Figure 2.24**) representing 28% of sesquiterpene lactones of the *Vernonia* genus.

One of the common features in the glaucolides is the consistent substitution pattern at C-1 and C-13.

The hirsutinolides are another class of germacranolides with interesting chemical diversity. They represents 30% of the 309 sesquiterpene lactones from *Vernonia* (**Figure 2.68**), the largest proportion, marginally over the glaucolides at 28%. There are five structural motifs of hirsutinolides based on their different substitution patterns (**Figure 2.26** to **Figure 2.34**). An important re-occuring feature in the hirsutinolides is hydroxylation at C-1. The vernolide C (**Figure 2.27**) and piptocarpin (**Figure 2.30**) skeletal types represent the most hydroxylated hirsutinolides.

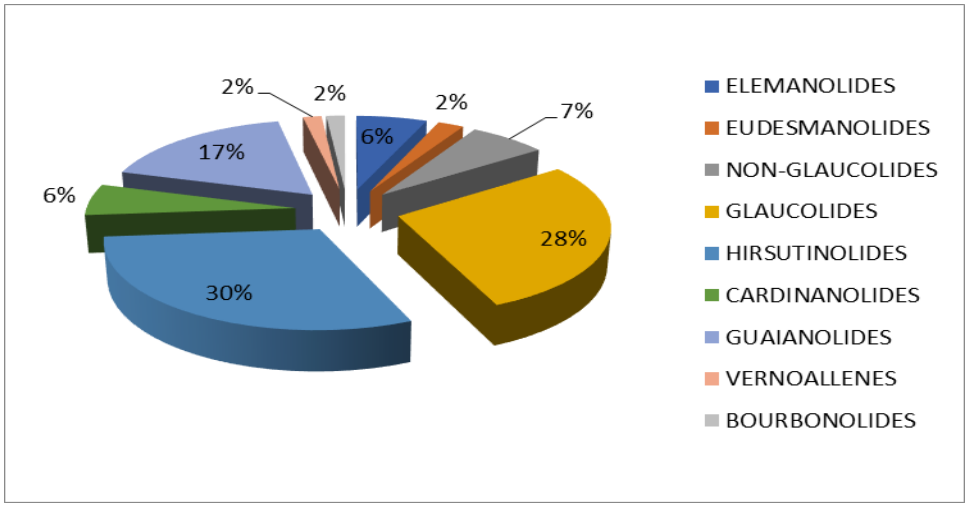


Figure 2.68 Distribution of sesquiterpene lactone skeletal types in *Vernonia*

The guaianolides are represented by only fifty seven compounds from twenty one structural motifs which constitute 17% of sesquiterpene lactones from *Vernonia* (**Figure 2.68**). It was observed that the most representative guaianolide-type sesquiterpenoids are those with a hydroxy group at C-3. The elemanolides (6%), eudesmanolides (2%) and miscellaneous sesquiterpenoids (3%) are represented by poor occurrence in *Vernonia*. However, it is

apparrent that sesquiterpene lactones are diverse in their chemical classification and structural derivatives within *Vernonia*.

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CHAPTER 3 SESQUITERPENE LACTONES FROM THE AERIAL PARTS OF *VERNONIA BLUMEOIDES* GROWING IN NIGERIA

Abstract

Four eudesmanolide sesquiterpene lactones (**1**-**4**) were isolated from the aerial parts of *Vernonia blumeoides* used in Nigerian ethnomedicine for the treatment of diarrhoea and malaria. Compound **1** demonstrated limited but interesting antibacterial activity against *Bacillus, Staphylococcus* and *Streptococcus* species. The crystal structure of compound **1** allowed the absolute configuration of the stereocentres in the molecule to be assigned.

Keywords: eudesmanes, sesquiterpenoids, antibacterial activity, *Vernonia blumeoides*

3.1 Introduction

Vernonia blumeoides Hook f. (Asteraceae) is a perennial herb growing in grasslands or abandoned fields across Northern Nigeria (Hutchinson and Dalziel, 1963). It is an erect shrub of 2-4 feet high with leaves shortly attached to the stems. In full bloom, the flower heads are reddish to purple in colour. The plant is known as "Bagashi" in Hausa language and commonly used in Northern Nigerian traditional medicine for the treatment of diarrhoea and malaria (Aliyu *et al*., 2011; Ibrahim *et al*., 2011). Plants of the genus *Vernonia* are widely used as vegetables and as medicinal herbs in African traditional medicine for the treatment of quite a number of ailments including gastrointestinal disorders, dysentery, malaria, skin diseases, fever, hepatitis, venereal diseases, diabetes and as an anthelmintic, amongst others (Akinpelu, 1999; Cioffi *et al*., 2004; Nergard *et al*., 2004; Tchinda *et al*., 2002). Phytochemical investigations of *Vernonia* species have resulted in the isolation of triterpenoids (Kiplimo *et al*., 2011), steroidal glycosides (Cioffi *et al*., 2004; Liu *et al*., 2009; 2010), flavonoids (Seetharaman and Petrus, 2004) and numerous classes of sesquiterpene lactones with interesting antibacterial (Rabe *et al*., 2002), anticancer (Williams *et al*., 2005; Buskuhl *et al*., 2010; Luo *et al*., 2011; Liao *et al*., 2012), antiplasmodial (Pillay *et al*., 2007) and anti-inflammatory activities (Youn *et al*., 2012). The sesquiterpene lactones are known to serve as chemotaxonomic markers for the genus *Vernonia*.

Although several other *Vernonia* species have been studied, especially from Africa and South America where the genus *Vernonia* was thought to have originated (Harborne and Williams, 1977), no previous phytochemical studies have been carried out on *Vernonia blumeoides*. Herein, we report on the isolation and characterisation of novel sesquiterpene lactones of the eudesmanolide class from the aerial part of the plant and its antibacterial activity.

3.2 Results and Discussion

Chemistry

Eudesmanolides have rarely been found in most African *Vernonia* species. In this study, the aerial parts of *Vernonia blumeoides* yielded four novel eudesmanolides (**1-4**), in addition to the known compounds, chrysin (**5**), apigenin (**6**) (Wawer and Zielinska, 2001), luteolin (**7**) (Li *et al*., 2008), stigmasterol (**8**) (Forgo and Kövér, 2004) and lupeol (**9**) (Laghari *et al*., 2011).

Compound **1** (**Figure 3.1**) was isolated as a white crystalline solid with a molecular formula of $C_{24}H_{32}O_9$ established by the quasi-molecular ion peak at *m/z* 487.1938 $[M+Na]^+$ (calcd. for $C_{24}H_{32}O_9Na$, 487.1944) in the HREIMS. In the IR spectrum, absorption bands of the hydroxyl (3530 cm^{-1}) and the ester carbonyl (1782 and 1736 cm^{-1}) were observed.

The ^1H and ^{13}C NMR data (**Table 3.1**) showed resonances typical of an eudesmane sesquiterpene skeleton similar to 1α,8α-dihydroxy-5αH,10α-eudesma-3,11(13)-dien-6-olide (**10**) (Triana *et al*., 2013), with characteristic oxygenated methine resonances at δ_H 4.79 (1H, dd, *J* = 9.8, 6.8 Hz, H-1), 3.96 (1H, t, *J* = 13.4 Hz, H-6) and 5.22 (1H, td, *J* = 10.8, 4.5 Hz, H-8), a doublet methine resonance at δ_H 2.48 (1H, *J* = 12.5 Hz, H-5), an olefinic resonance at δ_H 5.32 (1H, br s, H-3), two exomethylene protons at δ_H 6.08 (1H, d, *J* = 2.8 Hz, H-13a) and 5.43 (1H, d, *J* = 2.8 Hz, H-13b), an aliphatic methyl resonance at δ_H 0.98 (3H, s, H-14) and an olefinic methyl resonance at δ_H 1.79 (3H, s, H-15).

In addition to the resonances of the eudesmane skeleton, there were two additional methyl resonances at δ_H 1.33 (3H, s, H-19), 1.22 (3H, d, *J* = 6.2 Hz, H-20), an oxygenated methine resonance at δ_H 5.08 (1H, q, *J* = 6.2, H-18), two additional acetyl groups at C-18 and C-1 (δ_C 169.2, 20.7 and 170.1, 20.9) and an additional tertiary carbon at δ_C 76.2 (C-17). An acetyl

group was assigned to C-1 since HMBC correlations from H-1 to C-23 (δ_C 170.1) and H-24 to C-1 (δ_C 76.0) were observed (**Figure 3.2**). An ester group was also assigned to C-8 because of the observed HMBC correlations from H-8 to C-16 (δ_C 174.2). The second acetyl group was assigned to C-18 due to HMBC correlations from H-18 to C-21 (δ_C 169.2). This position was further occupied by the methyl group (C-20), accounting for the splitting pattern of H-18 being a quartet and H-20 being a doublet.

The 2-methylbutanoyl group was established by HMBC correlations from H-18 to C-16 and from H-19 to C-17. These correlations indicated that a 3-acetate-2-hydroxy-2-methyl butanoyl group was present at C-8. This side chain could be considered an oxidized tigloyl group, possibly through epoxidation of the tigloyl double bond followed by ring opening of the epoxide with further acetylation of the hydroxy group. The absolute configuration of **1** was determined by single crystal X-Ray diffraction analysis (**Figure 3.3**). This was supported by selected NOESY interactions (**Figure 3.4**). Compound **1** was thus identified as 1β-acetyl-8α-(3-acetyl-2-hydroxy-2-methyl)butanoyl-5αH,10β-eudesma-3,11(13)-dien-6α,12-olide and given the trivial name blumeoidolide-A.

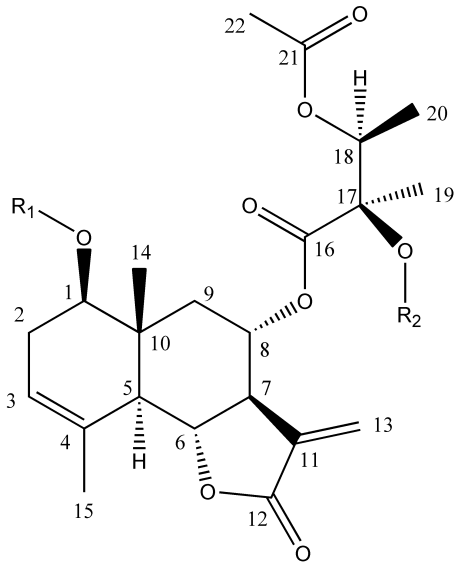
The molecular formula $C_{24}H_{32}O_9$ of compound **2** was deduced from HREIMS, which showed a quasi-molecular ion peak at *m/z* 487.1935 $[M+Na]^+$ (calcd. for $C_{24}H_{32}O_9Na$, 487.1944). Comparison of the ^1H and ^{13}C NMR spectroscopic data with **1** (**Table 3.1**) showed that they were very similar, except for the chemical shift of H-1 at δ_H 3.62 ($\Delta\delta_H$ -1.17) and H-19 δ_H 1.60 ($\Delta\delta_H$ +0.27) as well as C-16 at δ_C 168.4 ($\Delta\delta_C$ -5.8) and C-17 at δ_C 81.6 ($\Delta\delta_C$ +5.4). This prompted us to assign the acetyl group to C-17 instead of C-1 to form a structural isomer of **1**. The NOESY interactions of H-8/H-6 and H-6/H-14 shown as β, and H-18/H-19 shown as α, indicate that the relative configuration of **2** is the same as that of **1**. We have thus

identified compound **2** as 8α-(2,3-diacetyl-2-methyl) butanoyl-1β-hydroxy-5αH,10β-eudesma-3,11(13)-dien-6α,12-olide and given the trivial name blumeoidolide-B.

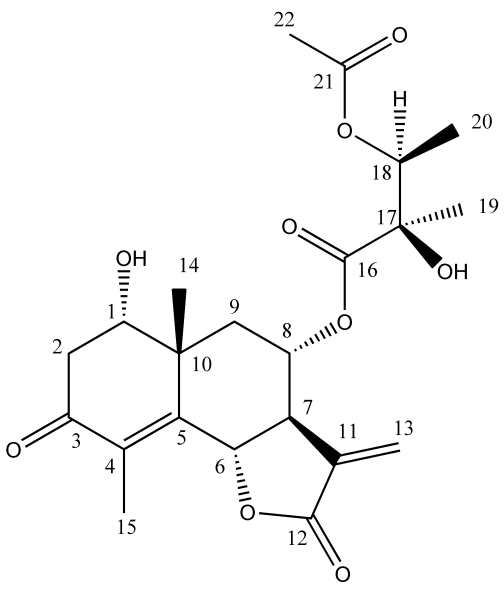
Compound **3** was obtained as a yellow amorphous solid. Unfortunately, HREIMS or elemental analysis data could not be obtained due to insufficient quantity; however, an M^+ - H_2O peak was evident at *m/z* 404 in the EIMS spectrum. The ^1H and ^{13}C NMR data was similar to both **1** and **2**, the difference being the absence of a carbonyl and methyl resonance in the ^{13}C NMR spectrum. This indicated that compound **3** had one less acetyl group than **1** and **2**. Since the NMR data of H-1 and C-1 was the same as that of **1**, and H-16 and C-17 the same as that of **2**, the only acetyl group was assigned to C-18. Both H-6 and H-8 showed NOESY interactions with H-14, indicating the same relative configuration to that of **1** and **2**. Compound **3** was thus identified as the monoacetylated, 8α-(3-acetyl-2-hydroxy-2-methyl) butanoyl-1β-hydroxy-5αH,10β-eudesma-3,11(13)-dien-6α,12-olide, with the trivial name, blumeoidolide-C.

Compound **4** was isolated as a yellowish gummy residue. HREIMS and EIMS data could not be obtained for compound **4** due to insufficient quantity, however the TOF MS indicated an M^+ -CH_3COO^- ion peak at *m/z* 378. In the IR spectrum, absorption bands for hydroxyl (3434 cm^{-1}) and ester carbonyl (1734 cm^{-1}) functional groups were observed. The NMR spectra of compound **4** was acquired in deuterated DMSO and showed a similar pattern to the eudesmane sesquiterpene lactones with the two proton resonances for the methylene group (H-13a and H-13b) at δ_H 6.15 and 5.77 (each 1H, d, J = 2.9 Hz), the oxygenated methine groups, H-6 and H-8 at δ_H 5.28-5.33 (2H, m), H-1 at δ_H 3.69 (1H, dt, J = 13.1, 5.0 Hz) and two methyl groups on the eudesmane skeleton, H-15 at δ_H 1.90 and H-14 at δ_H 1.25. The side chain ^1H NMR resonances for the 3-acetyl-2-hydroxy-2-methyl butanoyl group was the same

as that of **1** and **3**. In comparison to compound **3**, the two methine protons H-5 (δ_H 2.34) and H-3 (δ_H 5.33) were missing. In the ^{13}C NMR spectrum, an additional ketone resonance at δ_C 197.4 was assigned to C-3 and the olefinic resonance at δ_C 152.2 was assigned to C-5. HMBC correlations were seen from both H-2 (δ_H 2.42 and 2.55) and H-15 to C-3 (δ_C 197.4) and from H-15, H-14 and H-9 to C-5 (δ_C 152.2). Thus, the double bond in compound **4** shifted from Δ^3 to the Δ^4 position with a ketone at C-3 resulting in an α,β-unsaturated ketone moiety.



1, $R_1 = C(O)CH_3$, $R_2 = H$
2, $R_1 = H$, $R_2 = C(O)CH_3$
3, $R_1 = H$, $R_2 = H$



4

Figure 3.1 The structures of sesquiterpene lactones isolated from *Vernonia blumeoides*

The relative configuration was determined by the NOESY interactions of H-6/H-1, H-8/H-1, H-6/H-14 and H-8/H-14 (β-orientation) and between H-19 and H-18 (α-orientation). Accordingly, compound **4** was identified as 8α-(3-acetyl-2-hydroxy-2-methyl) butanoyl-1α-hydroxy-3-keto-10β-eudesma-4,11(13)-dien-6α,12-olide and given the trivial name blumeoidolide-D.

Table 3.1 ^{1}H and ^{13}C NMR data for compounds **1-3** (in $CDCl_3$, 400 MHz) and **4** (in DMSO-d_6, 600 MHz) [a, b]

	1		**2**		**3**		**4**	
No	**δ_H**	**δ_C**	**δ_H**	**δ_C**	**δ_H**	**δ_C**	**δ_H**	**δ_C**
1	4.79 (dd, 9.8, 6.8)	76.0	3.62 (dd, 9.9, 6.6)	74.7	3.62 (dd, 10.0, 6.6)	74.5	3.69 (dt,13.1, 5.0)	73.1
2α	*2.45 (m)	29.0	*2.36 (m)	32.5	*2.29 (m)	32.8	2.55 (dd, 16.1, 13.1)	42.7
2β	1.89 (m)		1.92 (m)		1.95 (m)		2.42 (dd,16.1, 5.0)	
3	5.32 (br, s)	121.4	5.34 (br, s)	121.9	**5.33 (br, s)	121.9	-	197.4
4	-	132.0	-	132.4	-	132.4	-	127.9
5	*2.48 (br d, 12.5)	49.7	*2.33 (m)	50.3	2.34 (dd,12.0, 2.2)	50.3	-	152.2
6	3.96 (t,13.4)	78.3	4.00 (t, 11.1)	78.9	4.00 (t, 11.0)	78.8	[#] 5.28-5.33 (m)	78.2
7	2.80 (tt, 10.9, 2.9)	53.1	2.81 (tt, 10.9, 2.8)	53.6	2.81 (tt, 10.9, 3.0)	53.4	3.22-3.27 (m)	50.0
8	5.22 (td, 10.8, 4.5)	70.7	5.27 (td, 10.8, 4.5)	70.9	**5.28 (td, 10.8, 4.6)	71.3	[#] 5.28-5.33 (m)	70.9
9α	1.12 (br t, 9.2)	39.9	1.12 (br t, 9.2)	*40.4	1.11 (br t, 11.9)	40.6	1.25 (m)	43.8
9β	2.12 (dd, 12.6, 4.5)		2.41 (dd, 12.7, 4.5)		*2.28 (dd, 12.2, 4.6)		2.38 (dd, 13.0, 4.5)	
10	-	38.9	-	*40.4	-	40.3	-	43.2
11	-	136.0	-	135.9	-	136.3	-	136.3
12	-	169.0	-	169.8	-	169.4	-	168.8
13a	6.08 (d, 2.8)	119.0	6.15 (d, 2.8)	120.1	6.10 (d, 2.9)	119.0	6.15 (d, 2.9)	121.6
13b	5.43 (d, 2.8)		5.87 (d, 2.8)		5.45 (d, 2.9)		5.77 (d, 2.9)	
14	0.98 (s)	13.3	0.89 (s)	12.1	0.90 (s)	13.2	1.25 (s)	18.4
15	1.79 (br, s)	22.9	1.81 (br, s)	23.2	1.81 (s)	23.1	1.90 (s)	11.0
16	-	174.2	-	168.4	-	174.3	-	173.9
17	-	76.2	-	81.6	-	76.1	-	76.1
18	5.08 (q, 6.2)	73.7	5.17 (q, 6.5)	72.4	5.11 (q, 6.4)	74.3	4.99 (d, 6.2)	73.1
19	1.33 (s)	21.7	1.60 (s)	17.6	1.37 (s)	21.5	1.31 (s)	23.1

	1		2		3		4	
No	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
20	1.22 (d, 6.2)	13.1	1.23 (d, 6.5)	14.5	1.26 (d, 6.4)	12.2	1.18 (d, 6.7)	13.8
21	-	169.2	-	170.0	-	170.4	-	170.0
22	1.91 (s)	20.7	2.05 (s)	21.1	2.01 (s)	21.0	1.96 (s)	21.3
23	-	170.1	-	170.0	-	-	-	-
24	1.98 (s)	20.9	2.07 (s)	21.1	-	-	-	-

[a]Assigned by DEPT, COSY, HMBC and HSQC experiments

[b]Chemical shifts are given in (ppm) relative to internal reference, tetramethylsilane (TMS). Coupling constants (*J*) are given in Hz

[#], * and ** indicate overlapping resonances

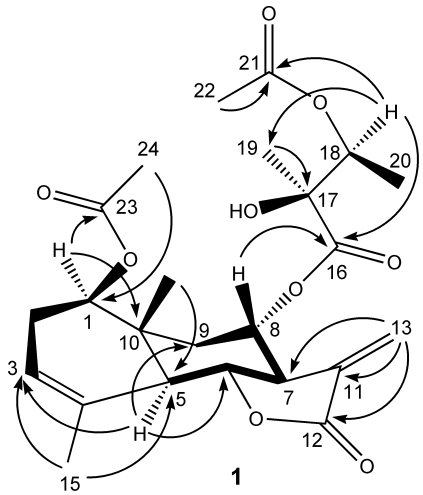


Figure 3.2 Selected HMBC (H→C) correlation for compound **1**

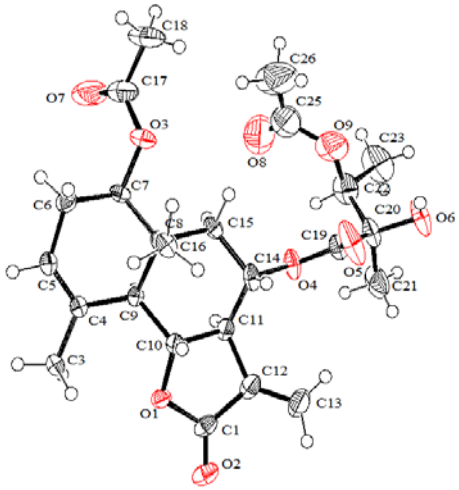


Figure 3.3 Ortep diagram for compound **1**

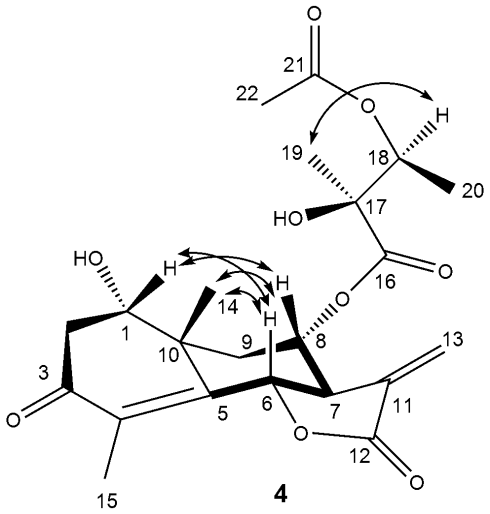


Figure 3.4 Selected NOESY interactions for compound **4**

Antibacterial Activity

In comparison to the standard antibiotics, lactone **1** showed moderate activity against *Bacillus subtilis*, the six *Staphylococcus* spp. strains and *Streptococcus pyogenes* (**Table 3.2**). Similar antibacterial activity was seen with the crude hexane, dichloromethane and ethyl acetate extracts, although the crude extracts were not always active against all of the *Staphylococcus* spp. strains. Lactone **2** showed moderate activity against *Bacillus subtilis* only, with an inhibition zone of 9 mm. The MIC values of lactones **1** and **2** using a 2-fold serial broth microdilution assay were determined to be 2.15, 4.30 and 2.15 mM (lactone **1**) and 4.30, 8.62 and 2.15 mM (lactone **2**) for *S. aureus* ATCC 29213, *S. aureus* ATCC 43300 and *B. subtilis* ATCC 6633, respectively. The antibacterial activity of compound **1** against Gram-positive bacteria was also shown by Erasto *et al*. (2006) who isolated vernolide and vernodalol from the leaves of *Vernonia amygdalina*.

Table 3.2 Antibacterial activity of compounds **1, 2**, Ampicillin, Tetracycline and crude extracts from *V. blumeoides*

	Zones of inhibition (mm)*						
Test organisms	**Ampicillin**	**Tetracycline**	**1**	**2**	**Hex**	**DCM**	**EtOAc**
Bacillus subtilis ATCC 6633	34±0.00	30±0.01	15±0.00	9±0.00	10.5±0.01	10±0.00	12±0.00
Staphylococcus aureus ATCC29213	26±0.01	29±0.00	13±0.01	0	10±0.00	10±0.00	15±0.00
*Staphylococcus aureus*ATCC43 300	30±0.00	25.5±0.01	11±0.12	0	8±0.02	10±0.01	11±0.02
Staphylococcus epidermidis ATCC 14990	32±0.00	23±0.00	11±0.01	0	0	0	0
Staphylococcus saprophyticus ATCC 35552	34±0.01	26±0.01	10±0.00	0	10.5±0.02	10±0.02	12±0.02
Staphylococcus sciuri ATCC29062	35±0.00	25±0.01	10±0.00	0	0	0	9±0.00
Staphylococcus xylosus ATCC 35033	34±0.02	26.5±0.02	10±0.00	0	7±0.01	9±0.03	12.5±0.0 1
Streptococcus pyogenes ATCC 19615	35.5±0.01	27±0.00	14±0.01	0	7±0.01	0	10±0.012

Mass of compounds on disk: **1** (2 mg), **2** (40 μg), Hex: (2 mg), DCM (2 mg), EtOAc (2 mg), Ampicillin (10 μg), Tetracycline (30 μg). Results expressed as mean±SD of three independent determinations. Hex: hexane, DCM: dichloromethane, EtOAc: ethyl acetate

3.3 Experimental

General experimental procedures

All NMR data, ^{1}H, ^{13}C and 2D experiments were recorded on a Bruker Avance^{III} 400 MHz spectrometer. Samples were acquired with deuterated chloroform ($CDCl_3$) or deuterated dimethyl sulfoxide (DMSO-d_6). The spectra were referenced according to the deuteriochloroform signal at δ_H 7.24 (for ^{1}H NMR spectra) and δ_C 77.0 (for ^{13}C NMR

spectra) for $CDCl_3$ and δ_H 2.50 and δ_C 39.51 for DMSO-d_6. The HREIMS spectra were obtained from a Bruker Micro TOF-QII instrument. IR spectra were recorded using a Perkin Elmer Universal ATR spectrometer. Optical rotations were measured at room temperature on a Perkin Elmer™ Model 341 Polarimeter with a 10-cm flow tube. UV spectra were obtained on a Varian Cary UV-VIS Spectrophotometer. The melting points were determined on an Ernst Leitz Wetziar micro-hot stage melting point apparatus. Merck silica gel 60 (0.040–0.063 mm) was used for column chromatography and crude samples were separated on 3 cm diameter columns, while purifications were carried out on 2 cm diameter columns. Merck 20 cm x 20 cm silica gel 60 F_{254} aluminium sheets was used for thin-layer chromatography. The TLC plates were analysed under a UV lamp with wavelength 254 and 366 nm and sprayed with an anisaldehyde- concentrated sulfuric acid-methanol mixture (1:2:97).

Plant material

The plant was collected in August, 2010 along Giwa road in Samaru-Zaria, Kaduna state, Nigeria. It was authenticated by U.S. Gallah at the Herbarium unit of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria where a voucher specimen (1784) was deposited.

Extraction and isolation

The dried aerial part (1700 g) was subjected to sequential extraction with hexane, dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH) and all extracts were concentrated under reduced pressure on a rotary evaporator (Büchi® rotary evaporator R-200) to yield 12.50, 33.89, 19.25 and 28.70 g of extract, respectively. The hexane extract (10.09 g) was separated by column chromatography with a hexane: DCM gradient starting with 100% hexane and gradually increasing the polarity by 10% DCM every 500 mL until 100% DCM

was reached. Fifty five fractions of 100 mL each were collected and combined into six fractions (A- fr. 1-12, B- fr. 13-20, C- fr. 21-29, D- fr 30-39, E- fr. 40-48 and F- fr. 49-55). Fraction C was purified with hexane: DCM (1:1) where fraction 6 yielded an amorphous white compound, stigmasterol (**8**) (53 mg). Purification of fraction D with hexane: DCM (4:6) yielded a light yellowish compound in fractions 22-32. Sub fraction $D_{22\text{-}32}$ was further purified with the same solvent system to yield lupeol (**9**) (88 mg) in fraction 11.

The DCM extract (15.45 g) was separated using a stepwise gradient of hexane: DCM: EtOAc: MeOH and collecting 100 mL fractions, which was further combined into 8 fractions; 100% hexane (A- fr. 1-4), hexane: DCM (7:3) (B- fr. 5-8), 100% DCM (C- fr. 9-13), DCM: EtOAc (9:1) (D- fr. 14-17), DCM: EtOAc (8:2) (E- fr. 18-26), 100% EtOAc (F- fr. 27-31), EtOAc: MeOH (7:3) (G- fr. 32-34) and 100% MeOH (H- fr. 35-38). Fraction E was purified with hexane: EtOAc (7:3) collecting 50 mL fractions, where fractions 27-50 yielded compound **1** (780 mg), which was recrystallised with MeOH. Fractions 55-60 yielded a brown amorphous compound **2**, which was purified with hexane: EtOAc (6:4) to yield the pure compound in fractions 22-24 (10 mg).

The EtOAc extract (8.04 g) was separated on a silica gel column and eluted with a hexane: EtOAc gradient (1:1, 4:6 and 3:7) collecting 100 mL fractions and further combined into 3 fractions; A- fr. 1-12, B- fr. 13-22 and C- fr. 23-30. Fraction A was further purified (with hexane: EtOAc, 1:1) in which fr. 4-9 yielded a mixture of two compounds. This mixture was purified with the same solvent system to yield 18 mg of chrysin **5** in fr. 5-8 and more of compound **1** in fractions 13-18 (32 mg recrystallized with MeOH). Fraction B was purified with hexane: EtOAc (4:6) to give a yellowish residue in fr. 14-16, which was purified with hexane: EtOAc (1:1) to yield compound **3** (6.45 mg) in fr. 10-12. Purification of C with

hexane: EtOAc (3:7) yielded compound **4**, a yellowish gummy residue, which upon recrystallization from MeOH yielded 6.20 mg of the pure compound. The MeOH extract (15.86 g) was dissolved in distilled water (200 mL), filtered and partitioned with an equal amount of butanol. The butanol fraction (3.08 g) was subjected to column chromatography on Sephadex LH-20 (1.5 cm diameter column) using MeOH and collecting 10 mL fractions. Fractions 12-19 was further purified on a 2 cm diameter silica column using hexane: EtOAc (1:1) where fr. 1-4 afforded apigenin (**6**) (10 mg) and fr. 5-8 yielded luteolin (**7**) (15 mg).

1β-acetyl-8α-(3-acetyl-2-hydroxy-2-methyl)butanoyl-5αH,10β-eudesma-3,11(13)-dien-6α,12-olide (blumeoidolide-A) (**1**) white crystalline (780 mg); m.p. 128-129 °C; $[\alpha]_D^{20}$ +29.5° (c. 0.13 $CHCl_3$); IR υ_{max} (cm^{-1}) 3530 (OH), 2990, 2952, 1782 (C=O), 1736 (C=O); ^1H and ^{13}C NMR (see **Table 3.1**); HREIMS *m/z* 487.1938 [M^+ + Na] (calcd. for $C_{24}H_{32}O_9Na$, 487.1944).

8α-(2,3-diacetyl-2-methyl)butanoyl-1β-hydroxy-5αH,10β-eudesma-3,11(13)-dien-6α,12-olide (blumeoidolide-B) (**2**) a brown amorphous solid (10.05 mg); m.p. 84-85 °C; $[\alpha]_D^{20}$ +43.5º (c. 0.1 $CHCl_3$); IR υ_{max} (cm^{-1}) 3483 (OH), 2952, 1744 (C=O), 1449; ^1H and ^{13}C NMR (see **Table 3.1**); HREIMS *m/z* 487.1935 [M^+ + Na] (calcd. for $C_{24}H_{32}O_9Na$, 487.1944).

8α-(3-acetyl-2-hydroxy-2-methyl) butanoyl-1β-hydroxy-5αH,10β-eudesma-3,11(13)-dien-6α,12-olide (blumeoidolide-C) (**3**) a yellowish amorphous solid (6.45 mg); m.p. 124-125 °C; $[\alpha]_D^{20}$ +50.0º (c. 0.1 $CHCl_3$); IR υ_{max} (cm^{-1}) 3448 (OH), 2929, 1736 (C=O), 1458; ^1H and ^{13}C NMR (see **Table 3.1**); EIMS (*m/z*) 404 (45) [M^+ - H_2O], 281 (45), 247 (65), 228 (100), 201 (80), 147 (98).

8α-(3-acetyl-2-hydroxy-2-methyl)butanoyl-1α-hydroxy-3-keto-10β-eudesma-4,11(13)-dien-6α,12-olide (blumeoidolide-D) (**4**) a yellow gummy residue (6.20 mg); m.p. 108-109 ºC;

$[\alpha]_D^{20}$ +35.0º (c. 0.1 CH_3OH); IR υ_{max} (cm^{-1}) 3434 (OH), 2941, 1734 (C=O), 1736 (C=O); ^1H and ^{13}C NMR (see **Table 3.1**); TOF MS ES^+ (*m/z*) 378 (80) [M^+ - CH_3COO], 357 (40), 301 (65), 265 (100).

Antimicrobial susceptibility testing

Antimicrobial testing was done on crude extracts of *Vernonia blumeoides* and the pure lactones **1** and **2** against bacterial isolates (**Table 3.2**) using the disc diffusion method by standard procedures (CLSI, 2007). Compounds **3** and **4** were isolated in small amounts and were not assayed as the samples decomposed before the assays could be carried out. Crude extracts (DCM, hexane, EtOAc and MeOH) and lactone **1** were dissolved in DMSO to a final concentration of 50 mg mL^{-1}. From the stock solutions, 40 µL (0.04 mL) were impregnated onto blank discs (0.04 mL x 50 mg mL^{-1}) resulting in 20 mg of extract concentration absorbed. While for lactone **2**, the final concentration prepared was 1 mg mL^{-1} (1 mg dissolved in 1 mL DMSO), from where 40 µL (0.04 ml) were also impregnated onto blank discs (6 mm; MAST, UK). The concentrations of compound **2** absorbed (0.04 mL x 1 mg mL^{-1}) were 0.04 mg (40 µg). The difference in masses of compounds **1** and **2** was due to quantity of compounds isolated. Compound **1** was isolated up to 100 mg, while compound **2** yielded only 10 mg which depleted after chemical characterization. In all cases, solutions on discs were allowed to dry. Testing was done in duplicate and tetracycline (TE30; 30 µg disc potency) and ampicillin (AMP10, 10 µg disc potency) discs (Oxoid, UK) were used as standard antimicrobial agent controls in addition to DMSO-impregnated discs which served as the negative control. Antibacterial activity was determined by measuring the diameter of the inhibition zone (clear zone) formed around the discs. Criteria for assigning susceptibility or resistance to AMP10 was as follows: Sensitive (S) ≥ 17 mm, Intermediate (I) = 14 – 16

mm, Resistant (R) ≤ 13 mm, while those for TE30 were: (S) ≥ 19 mm, (I) 15 – 18 mm, (R) ≤ 14 mm (CLSI, 2007).

MIC determination using broth microdilution assays

Staphylococcal isolates, *S. aureus* ATCC 29213 and ATCC 43300, and *B. subtilis* ATCC 66333 were cultured overnight on TSA and resuspended in sterile distilled water equivalent to a 0.5 McFarland standard. These were used to determine the minimum inhibitory concentrations using two-fold serial broth microdilution assays (Andrews, 2001). Microtiter plate wells contained 90 µL per well of Mueller Hinton (MH) broth, 10 µL of cell suspension and lactones **1** and **2** at doubling dilution concentrations ranging from 0.5 - 32 mg mL^{-1}. Tetracycline (20 mg mL^{-1}; Sigma, Germany) was used as a reference antimicrobial agent against each bacterium. Wells were inoculated in triplicate for each isolate at each of the specified concentrations and incubated at 37 °C without shaking. The highest dilution (lowest concentration of lactone), showing no visible growth was regarded as the MIC (Andrews, 2001). Negative controls contained MH broth only and growth controls contained MH broth and respective cell suspensions only.

X-Ray crystallography

A crystal of dimension of 0.25 x 0.28 x 0.48 mm^3, was selected and glued on to the tip of a glass fibre. The crystal evaluation and data collection were performed on a Bruker Smart APEXII diffractometer with Mo Kα radiation (λ = 0.71073 Å). The data collection method involved ω scans of width 0.5°. Data reduction was carried out using the program *SAINT+*. The structure was solved by direct methods using SHELXS and refined. Non-H atoms were first refined isotropically and then by anisotropic refinement with full-matrix least-squares calculations based on F^2 using SHELXS. All H atoms were positioned geometrically and

allowed to ride on their respective parent atoms. All H atoms were refined isotropically. The final least-squares refinement of 305 parameters against 4242 data resulted in residuals *R* (based on *F*2 for $I \geq 2\sigma$) and *wR* (based on F^2 for all data) of 0.0519 and 0.1285, respectively. The final difference Fourier map was featureless. Crystal data: $C_{24}H_{32}O_9$, M_W = 464.50, orthorhombic, space group $P2_12_12_1$, crystal cell parameter a = 6.2596(2) Å, b = 10.1164(3) Å, c = 38.2097(9) Å, V = 2419.61(12) Å^3, Z = 4, D_{calc} = 1.275 Mg/m^3. Crystallographic data (excluding structure factors) for the structure in this paper has been deposited with the Cambridge Crystallographic Data Centre, CCDC, 12 Union Road, Cambridge CB21EZ, UK. Copies of the data can be obtained free of charge on quoting the depository number CCDC-909545 (Fax: +44-1223-336-033; E-Mail: deposit@ccdc.cam.ac.uk, http://www.ccdc.cam.ac.uk).

3.4 Conclusion

Four sesquiterpene lactones, all with similar backbones were isolated from the aerial parts of *V. blumeoides* and their structures were elucidated using spectroscopic data and X-Ray crystallography. Compounds **1** and **2** were assayed for their antibacterial activity, of which, **1** showed antibacterial activity against *Staphylococcus* spp. strains and *Bacillus subtilis*. The antibacterial activity displayed by **1** and the crude extracts of the aerial parts of the plant suggest a potential reason for the use of the plant in Northern Nigeria for the treatment of Gram-positive-associated skin infections.

Acknowledgements

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3.5 References

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CHAPTER 4 QUORUM SENSING INHIBITORY POTENTIAL AND MOLECULAR DOCKING STUDIES OF SESQUITERPENE LACTONES FROM *VERNONIA BLUMEOIDES*

Abstract

The increasing incidence of multidrug-resistant Gram-negative bacterial pathogens has focused research on the suppression of bacterial virulence *via* quorum sensing (QS) inhibition strategies, rather than the conventional antimicrobial approach. The anti-virulence potential of sesquiterpene lactones previously isolated from *Vernonia blumeoides* was assessed by inhibition of quorum sensing and *in silico* molecular docking. Inhibition of QS-controlled violacein production in *Chromobacterium violaceum* was quantified using violacein inhibition assays. Qualitative modulation of QS activity and signal synthesis was investigated using agar diffusion double ring assays and *C. violaceum* and *Agrobacterium tumefaciens* biosensor systems. Inhibition of violacein production was concentration-dependent, with ≥90% inhibition being obtained with ≥2.4 mg mL^{-1} of crude extracts. Violacein inhibition was significant for the ethyl acetate extract with decreasing inhibition being observed with dichloromethane, hexane and methanol extracts. Violacein inhibition ≥80% was obtained with 0.071 mg mL^{-1} of blumeoidolide B in comparison with ≥3.6 mg mL^{-1} of blumeoidolide A. Agar diffusion double ring assays indicated that only the activity of the LuxI synthase homologue, CviI, was modulated by blumeoidolides A and B, and *V. blumeoides* crude extracts, suggesting that QS signal synthesis was down-regulated or competitively inhibited. Molecular docking was conducted to explore the binding conformations of sesquiterpene lactones into the binding sites of QS regulator proteins, CviR and CviR'. The computed binding energy data suggested that the blumeoidolides have a tendency to inhibit both CviR and CviR' with varying binding affinities. *Vernonia* eudesmanolide sesquiterpene lactones have the potential to be novel therapeutic agents, which might be important in reducing virulence and pathogenicity of drug-resistant bacteria *in vivo*.

Keywords: *Vernonia blumeoides*; Asteraceae; quorum sensing inhibition; molecular docking; sesquiterpene lactones, blumeoidolides

4.1 Introduction

The increasing incidence of multi-drug resistant bacteria has prompted the search for potent, novel antibacterial drugs or complementary agents against resistant pathogens, with new targets or novel mechanisms, distinct from currently used antibacterial therapies. One such target mechanism which has garnered interest has been quorum sensing (QS), a cell density-dependent chemical signaling process, which is mediated by acyl homoserine lactones (AHL) in Gram-negative bacteria. QS regulates gene expression in bacteria for collective biological functions and significantly influences bioluminescence, plasmid transfer, bacterial virulence, the biosynthesis of secondary metabolites and antibiotics, and biofilm formation (Hirakawa and Tomita, 2013). Therefore, targeting QS mechanisms involving signal production, dissemination or reception could disrupt the QS circuits, curtail bacterial virulence and resistance (Hentzer and Givskov, 2003) and, furthermore, bacteria are unlikely to develop multi-drug resistance since no selection pressure is imposed (Koh *et al*., 2013).

Plants have been used for centuries in traditional medicine due to their diverse secondary metabolites such as alkaloids, flavonoids, saponin glycosides, anthraquinones and sesquiterpenoids, among others. All plants grow in environments with high bacterial densities and have developed an evolutionary co-existence with QS bacteria. Plants have thus developed protective mechanisms against bacterial infections, e.g., being able to produce QS inhibitory compounds or QS mimic compounds, which reduce the pathogenic capability of bacteria (Koh *et al*., 2013; Nazzaro *et al*., 2013). Due to their diverse chemical repertoire, the anti-virulence properties of medicinal plants and their constituent phytochemicals are attracting attention since plants are able to interfere with bacterial communication processes thereby disrupting associated cellular mechanisms or functions (Koh *et al*., 2013; Nazzaro *et al*., 2013). Gram-negative bacteria primarily use the LuxR/I-type QS system. Plant

compounds usually target these Gram-negative bacterial QS systems *via* three different ways, either by inhibiting the signaling molecules from being synthesized by the LuxI synthase, by inhibition of activity of AHL-producing enzymes, by degrading signaling molecules and/or by targeting the LuxR signal receptor (Koh *et al*., 2013; Nazzaro *et al*., 2013). Since QS is crucial to bacterial cellular functions and survival, disrupting the QS signal production or reception, facilitates control of bacterial virulence and resistance (Hentzer and Givskov, 2003).

Plants of the genus *Vernonia* (Asteraceae) represent about 500 species distributed in tropical regions of the world especially in Africa and South America (Bremer, 1994). *Vernonia* species, such as *Vernonia amygdalina*, is widely used in African traditional medicine due to its multiple therapeutic properties for various human and animal diseases (Yeap *et al*., 2010). Phytochemical studies on several *Vernonia* species have resulted in the isolation of flavonoids, triterpenoids and sesquiterpene lactones (SLs) with interesting biological activities (Toyang and Verpoorte, 2013). In the Asteraceae particularly, SLs which are typically localised in leaves and flowering heads, are one of the main contributors to the plant's defense mechanisms. Since plants are constantly under microbial attack, SLs are able to provide defense against fungi, bacteria, and viruses, by disruption of a microbe's cell membrane, due to their polar groups disrupting the phospholipid membrane. Sesquiterpene lactones function as phytoalexins in response to microbial attack, as anti-feedants to deter herbivores, as attractants of pest predators, as hormones, and as allelochemicals (Chadwick *et al*., 2013). Sesquiterpene lactones demonstrate a broad spectrum of biological activity including anti-tumor, anti-inflammatory anti-malarial, anti-viral, anti-bacterial, and anti-fungal activity. The QS and biofilm inhibitory potential of plant SLs has been reported by

Cartegena *et al*. (2007) and Amaya *et al*. (2012), while that of drimane sesquiterpenoids have been reported by Paz *et al*. (2013) and Cárcamo *et al*. (2014).

The binding of the signal molecule to the sensor can be compared to that of a ligand binding to an enzyme active site. According to Goh *et al*. (2005), it is important to analyze the binding of signal antagonists to the receptor protein in order to fully understand the inhibitory effect. In this study, we report on the QS inhibitory potential of previously described eudesmanolide SLs (blumeoidolides) and crude extracts from *Vernonia blumeoides* Hook. f. (Asteraceae) (Aliyu *et al*., 2015) using *Chromobacterium violaceum* and *Agrobacterium tumefaciens* biosensor systems. In addition, *in silico* molecular docking of the SLs (blumeoidolides A, B, C, and D) with LuxR homologues, CviR and CviR' was carried out to confirm and assess the molecular characteristics of the protein-ligand interactions.

4.2 Results and Discussion

Chemical composition of extracts

Table 4.1 indicates the chemical composition of four solvent extracts of *V. blumeoides* as identified by GC-MS analysis and using the NIST library. Fatty acids/esters, terpenoids and steroids constituted the main classes of bioactive compounds. The major components in the hexane (VBL-Hex), dichloromethane (VBL-DCM), ethyl acetate (VBL-EA) and methanol (VBL-MeOH) extracts were 2-(octadeca-9*Z*,12*Z*-dienyloxy) ethanol (**5**) (12.5%), 14,15-epoxy-3,11-dihydroxy-(3β,5β,11α,15β)-bufa-20,22-dienolide (**6**) (33.5%), catechol (**7**) (19.5%) and 3,5-stigmastadien-7-one (**8**) (17.9%), respectively (**Figure 4.1**). Of the main classes of bioactive compounds, terpenoids are ubiquitous components of plant extracts, but chemo-types vary in aggregate composition due to environmental influences or genetic

evolution (Figueiredo *et al*., 2008). This probably determines the potency of biological or pharmacological action on microorganisms.

Table 4.1 Chemical composition of four *Vernonia blumeoides* solvent extracts

Chemical constituents	RT [a]	Percent composition of extracts[b]			
		VBL-DCM	VBL-EA	VBL-Hex	VBL-MeOH
2-methyl-2*Z*-butenoic acid	4.3	-	1.6	-	-
2,3-dihydrobenzofuran	9.1	-	0.9	-	-
Catechol	9.3	-	19.5	-	-
1-Acetyl-2,5-dihydropyrrole-2-carboxylic acid	9.9	-	0.8	-	-
6-hydroxy-4-pyrimidine carboxylic acid	10.5	-	0.8	-	-
8-methoxy-1,3,4,5-tetrahydro-2H-1-benzazepin-2-one	12.6	-	2.3	-	-
4-(1*E*)-3-hydroxy-1-propenyl)-2-methoxyphenol	15.3	-	3.1	-	-
Phytol acetate	16.2	-	0.6	-	-
6,10,14-trimethyl-2-pentadecanone	16.3	-	1.9	6.4	1.3
(Z)-non-3-enyl octyl adipate	17.0	-	0.8	-	8.0
Hexadecanoic acid, 15-methyl, methyl ester-	17.1	-	0.7	-	1.8
(3*β*,13*β*,14*β*)-13,27-Cycloursan-3-ol acetate	17.4	-	-	-	3.4
Ascorbic acid, 2,6-dihexadecanoate	17.6	6.7	-	1.1	-
n-Hexadecanoic acid	17.7	-	6.0	-	7.0
n-Hexadecanoic acid ethyl ester	17.8	-	1.0	-	-
2-hexyldecan-1-ol	17.9	1.0	-	-	-
(10*E*,12*Z*)-methyl octadeca-10,12-dienoate	18.8	0.8	-	-	3.3
Methyl 8-octadecenoate	18.9	0.6	-	-	1.7
Dihydro-5-tetradecyl-2(3H)-furanone	19.0	-	-	1.0	-
10*Z*,12*Z*-Hexadecadien-1-ol acetate	19.3	-	-	-	1.8
9*Z*,12*Z*-octadecadienoic acid	19.4	9.3	8.2	-	-
3-(1-ethoxy-1-oxo-5-phenylpentan-3-yloxy)butanoic acid	20.5	-	1.2	-	-
5-(2-dodecyl-4-methoxyphenoxy)-5-oxopentanoic acid	20.7	-	-	1.4	-
5-methyl-5-(4,8,12-trimethyltridecyl)dihydrofuran-2(3H)-one	21.2	0.6	-	-	-
8,8-dimethyl-2H, 8H-pyrano-(3, 2γ)-chromen-2-one	21.5	0.6	-	-	-
11-Dodecyn-1-ol acetate	22.1	-	-	-	3.4
2-(octadeca-9Z,12Z-dienyloxy)ethanol	22.2	-	-	12.5	-
6-hydroxy-1,4,7-trimethyl-7-vinyl-1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydrophenanthrene-1-carboxylic acid	22.3	-	-	-	9.0
Glycidol stearate	22.4	-	-	5.5	-
(22*E*)-4-methyl stigmasta-4,22-dien-3-ester	22.5	-	-	-	0.9
Lanosterol	22.7	-	-	7.1	-
Digitoxin	23.0	-	-	3.6	-

Chemical constituents	RT[a]	VBL-DCM	VBL-EA	VBL-Hex	VBL-MeOH
Lupan-3-ol acetate	23.5	-	-	6.9	4.6
3,5-stigmastadien-7-one	24.7	-	-	-	17.9
2-(3-acetoxy-4, 4,14-trimethyl andros-8-en-17-yl) propanoate	24.8	-	-	1.6	-
2, 4'-isopropylidene diphenol	25.0	3.0	-	-	-
Tetratetracontane	25.6	-	-	2.4	-
3-acetoxy-4,14-dimethyl-9,19-cycloergost-24(28)-ene	25.9	-	-	-	1.5
β-amyrin acetate	26.2	1.1	4.6	-	-
3β-acetoxylanosta-8,24-diene	28.2	-	-	3.2	-
Lupeol	28.3	4.2	14.2	-	-
3-acetoxy-9,19-cyclolanostane	28.4	-	-	-	1.6
4-hydroxy-4a, 5-dimethyl-3-methylene-3a, 4, 4a, 5, 6, 7, 9, 9a-octahydronaphtho [2,3-b]furan-2(3H)-one	28.7	7.7	-	-	-
3β-acetoxycholest-8-ene	29.0	-	-	-	3.3
14,15-epoxy-3,11-dihydroxy-(3β,5β,11α,15β)-bufa-20,22-dienolide	29.3	33.5	-	-	-
1-(4-isopropylphenyl)-3-(2-furyl) propane	29.6	1.4	-	-	-

[a] Rt = retention time in min.
[b] VBL-DCM = dichloromethane extract, VBL-EA = ethyl acetate extract, VBL-Hex = hexane extract, and VBL-MeOH = methanol extract.

Biosensor antimicrobial susceptibility testing

The antimicrobial activity of crude extracts and eudesmanolide SLs from *V. blumeoides* against Gram-negative and Gram-positive indicator bacteria has already been reported (Aliyu *et al*., 2015). Crude extracts and blumeoidolide A (**1**) (2 and 4 mg mL^{-1}) were initially assessed for their antimicrobial effect against the biosensor and AHL over-producer strains (**Table 4.2**), where 2 mg mL^{-1} was observed to be a sub-inhibitory concentration.

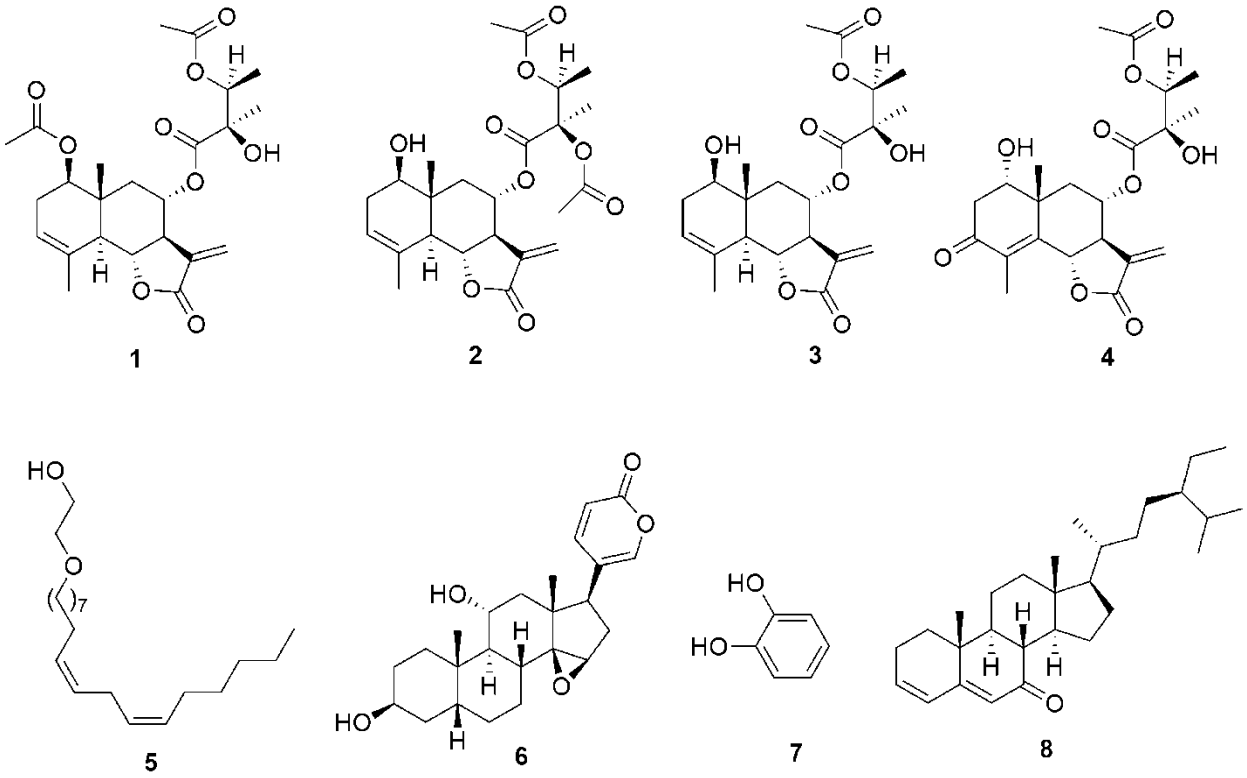

Figure 4.1 Chemical structures of sesquiterpene lactones (1-4) isolated and major constituents identified by GC-MS (5-8) from *Vernonia blumeoides.*

Quantitative anti-quorum sensing activity-violacein inhibition

Inhibition of violacein pigment production by blumeoidolide A (**1**), blumeoidolide B (**2**) and four crude extracts (0.15 – 9.5 mg mL^{-1}), all obtained from our previous study (Aliyu *et al*., 2015), was measured spectrophotometrically and quantified (**Figure 4.2**). Due to a lack of sufficient sample, the inhibition of violacein for blumeoidolides C (**3**) and D (**4**) also isolated in our previous study (Aliyu *et al*., 2015) could not be determined. This range of concentrations was used to identify the lowest concentration at which QS was evident, as well as document any potential growth inhibitory effect. Given the limited yield of blumeoidolide B (**2**), QSI was investigated in a range of 0.003-0.19 mg mL^{-1}. Growth inhibition was observed at concentrations ≥5 mg mL^{-1} and these concentrations were not considered for QSI.

A concentration-dependent inhibition of violacein production by *C. violaceum* ATCC 12472 was observed with ≤4.75 mg mL^{-1} blumeoidolide A (**1**) and four crude extracts (**Figure 4.2**), without inhibition of bacterial growth. The differences in the mean values among the treatment groups was greater than would be expected by chance, thus there was a statistically significant difference ($p < 0.001$). A similar concentration-dependent inhibition of violacein production has been reported with methanol extracts of dried *Capparis spinosa* fruit (Packiavathy *et al*., 2011), *Cuminum cyminum* extract (Packiavathy *et al*., 2012), and aqueous *Moringa oleifera* leaf and fruit extracts (Singh *et al*., 2009), without inhibiting bacterial growth. Inhibition of violacein production ≥90% violacein was observed at concentrations of 2.4 mg mL^{-1} of crude extracts. The four *V. blumeoides* extracts displayed varying levels of QSI potency, with 90% inhibitory activity in the following order: VBL-EA > VBL-DCM > VBL-Hex > VBL-MeOH (**Figure 4.2**). An 88% inhibition in violacein production was obtained with 2 mg mL^{-1} of the *C. spinosa* methanol extract (Packiavathy *et al*., 2011), while 2 mg mL^{-1} of the *C. cyminum* methanol extract resulted in 90% inhibition (Packiavathy *et al*., 2012).

Table 4.2 Antimicrobial susceptibility profiles of biosensors following exposure to 2 mg mL^{-1} (sub-inhibitory concentration) of four crude *Vernonia blumeoides* extracts and blumeoidolide A (**1**)

Bacteria	VBL-DCM[a]	VBL-EA[a]	VBL-Hex[a]	VBL-MeOH[a]	Blumeoidolide A (**1**)
*Ag. tumefaciens A*136	9 (R[b])	0 (R)	9 (R)	0 (R)	10 (R)
Ag. tumefaciens KYC6	10 (R)	0 (R)	10 (R)	10 (R)	10 (R)
C. violaceum VIR07	9 (R)	8 (R)	9 (R)	8 (R)	9 (R)
C. violaceum ATCC 12472	12 (I)	11 (I)	10 (R)	10 (R)	10 (R)
C. violaceum CV026	12 (I)	10 (R)	12 (I)	12 (I)	12 (I)
C. violaceum ATCC 31532	10 (R)	10 (R)	11 (I)	8 (R)	10 (R)

[a] VBL-DCM = dichloromethane extract, VBL-EA = ethyl acetate extract, VBL-Hex = hexane extract, and VBL-MeOH = methanol extract.
[b] (R) and (I) denote resistance and intermediate susceptibility (Chenia, 2013).

Violacein inhibition of ≥85% was obtained with blumeoidolide A (**1**) with ≥3.6 mg mL^{-1} and 22, 81 and 97% with 0.048, 0.071 and 0.095 mg mL^{-1} of blumeoidolide B (**2**), respectively. The IC_{50} for blumeoidolides A (**1**) and B (**2**) were 1.55 and 0.055 mg mL^{-1}, respectively, while those of the crude extracts ranged from 0.45 (VBL-Hex) to 0.77 mg mL^{-1} (VBL-DCM). Blumeoidolide B (**2**) thus had a greater QSI effect than blumeoidolide A (**1**), since it inhibited violacein production at a much lower concentration. The difference in structure between blumeoidolide A (**1**) and B (**2**) is the position of the acetyl group from C-1 on the bicyclic ring to C-17 on the open side chain (**Figure 4.1**). This is indicative that the acetyl group on the flexible side chain was more desirable for violacein inhibition than when it is fixed in a ring system.

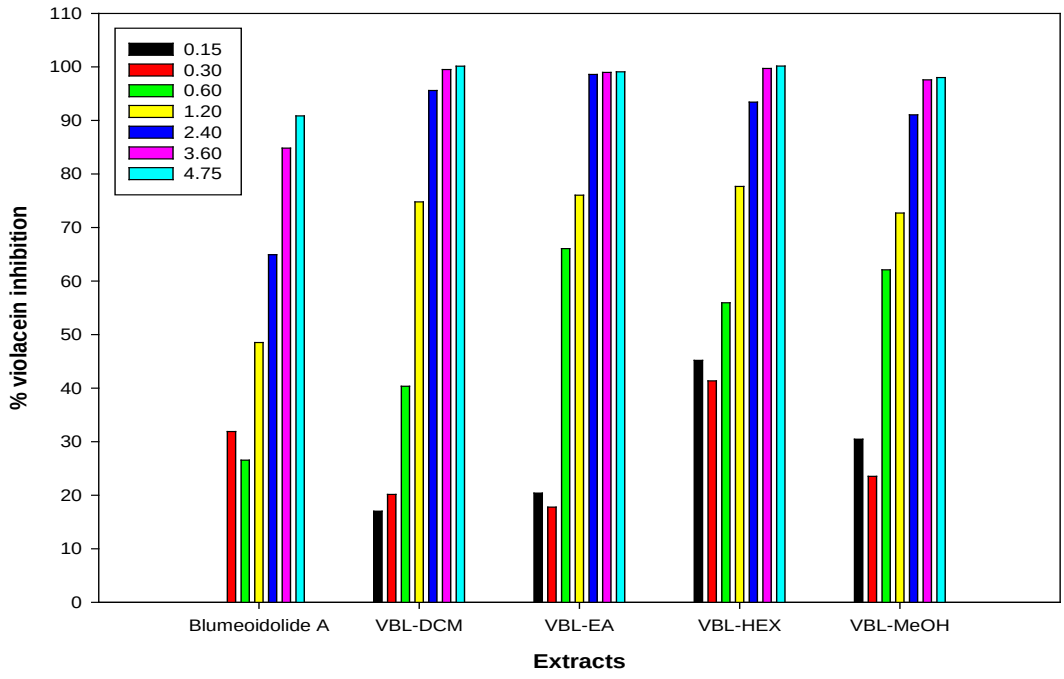

Figure 4.2 Quantitative analysis of the concentration-dependent inhibitory effects of blumeoidolide A and four *V. blumeoides* crude extracts at 0.15-4.75 mg mL^{-1} (VBL-DCM, VBL-EA, VBL-Hex, and VBL-MeOH) on violacein production by *Chromobacterium violaceum* ATCC 12472. Data represents the mean±standard deviation of three independent experiments.

Qualitative modulation of QS activity

In the AHL system, the signal-generating LuxI or its homologues, the *N*-acylhomoserine lactone (AHL) molecule itself, and the signal receptor LuxR or its homologues are potential targets. Many natural extracts inhibit QS by interfering with the AHL activity by competing with them due to their structural similarity and/or accelerating the degradation of the LuxR receptors of AHL molecules (Koh *et al*., 2013; Nazzaro *et al*., 2013). Interference with signal reception may involve competitive and non-competitive molecules which interfere with the binding of AHLs to their cognate LuxR receptor. For competitive molecules to bind to the AHL receptor, they must be structurally similar to AHLs, while for non-competitive binding, these molecules will bind to a site on the receptor other than the AHL binding site. Plants can produce molecules that structurally mimic AHLs, and such competitive binding is effective in blocking activation of QS (Koh *et al*., 2013). A second level of modulation involves modulating the synthesis of AHL molecules by decreasing the expression of the LuxI family of synthases or the ability of phytochemicals to competitively or non-competitively inhibit LuxI activity (Vattem *et al*., 2007). Thus to determine whether potential inhibitors target AHL synthesis (*via* LuxI homologues) or AHL response (*via* LuxR homologues), a double ring bioassay was carried out using the *C. violaceum* and *Ag. tumefaciens* biosensor systems with a sub-inhibitory concentration of SLs, since the goal is not to kill bacterial cells but rather attenuate their virulence abilities by inhibition of QS-regulated processes.

In the CV026/ATCC 31532 system, only CviI (LuxI homologue in *C. violaceum*) inhibition (inhibition of short-chain C4-AHL and C6-AHL signal synthesis) was observed (**Figure 4.3**) to varying degrees: VBL-EA > VBL-MeOH > VBL-Hex > blumeoidolide B (**2**) >

blumeoidolide A (**1**) = VBL-DCM. In the VIR07/ATCC 12472 assay, again only CviI modulation (inhibition of long chain C10-AHL signal synthesis) was observed in the following order: VBL-MeOH > VBL-EA > VBL-DCM > blumeoidolide A (**1**) > VBL-Hex (**Figure 4.4**).

β-galactosidase expression in *Ag. tumefaciens* A136 is under the control of QS and is expressed in response to the presence of AHL molecules secreted by the AHL over-producer KYC6. TraI (LuxI homologue in *Ag. tumefaciens*) inhibition was observed with blumeoidolide A (**1**) and VBL-MeOH and VBL-DCM, with decreased enzyme activity being indicated by decreased X-gal hydrolysis and blue pigment formation. This was indicative of the respective extracts affecting 3-oxo-C8- and 3-oxo-C6-AHL synthesis only. No inhibition was observed with VBL-EA and VBL-Hex. No TraR (LuxR homologue in *Ag. tumefaciens*) inhibition was observed with any of the extracts tested. The CviI modulatory effect was greater when using the VIR07/ATCC 12472 system in comparison to the TraI *Ag. tumefaciens* combination.

Based on the CviI/TraI inhibition observed, it may be suggested that blumeoidolides A (**1**) and B (**2**) potentially modulated the ability of the over-producer bacteria to synthesize AHL molecules. Two scenarios might provide an explanation: the phytochemicals either decrease the expression of the CviI/TraI synthase, which synthesizes the AHL molecules, or decrease AHL synthesis due to their ability to competitively or non-competitively inhibit CviI/TraI activity (Vattem *et al*., 2007; Mihalik *et al.*, 2008). Cartagena *et al*. (2007) evaluated 16 plant SLs, thirteen from the family Asteraceae, and three from the Hepaticaceae family, for their ability to inhibit or stimulate the production of biofilm by *P. aeruginosa*. Six SLs from *Acanthospermum hispidum* and one from *Enydra anagallis* strongly inhibited (69 - 77%) biofilm formation at a concentration of 2.5 mg mL^{-1}. The tested compounds carry a γ -lactone moiety, which is a structural feature similar to

the lactone moiety present in *N*-acyl homoserine lactones, and is a common structural feature of QS inhibitors (Ghantous *et al*., 2010).

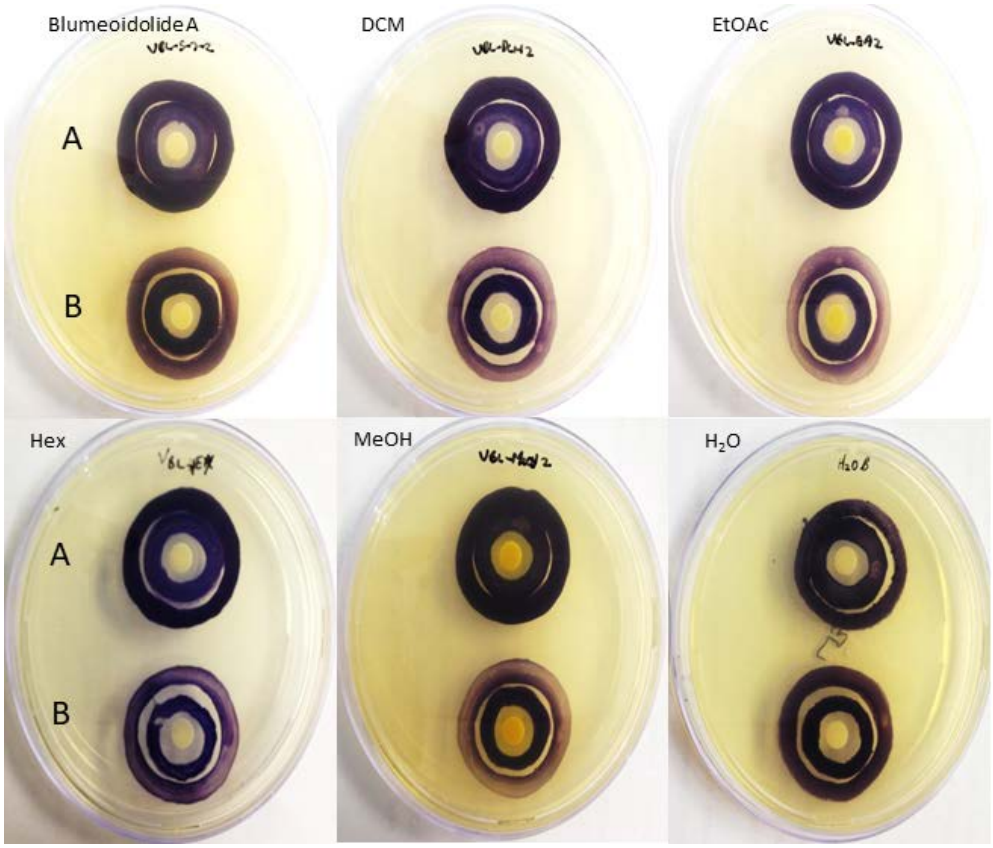

Figure 4.3 Quorum sensing inhibition by sub-inhibitory concentrations (2 mg mL^{-1}) of blumeoidolide A (VBL 5-7), and 4 *V. blumeoides* crude extracts (VBL-DCM, VBL-EA, VBL-Hex and VBL-MeOH) by A] modulation of AHL receptor activity (LuxR) or B] AHL synthesis (LuxI) in the double ring agar diffusion assay with the *Chromobacterium violaceum* VIR07/ATCC 12472 biosensor system. Discs impregnated with 20 µL of sterile distilled water served as a control.

The bioactivity of SLs is mediated by alkylation of nucleophiles through their, *β*- or *α*, *β*, *γ*-unsaturated carbonyl structures, such as *α*-methylene-*γ*-lactones or *α*, *β*-unsaturated cyclopentenones. These structural elements react with nucleophiles, especially the cysteine sulfhydryl groups in proteins by a Michael-type addition. This interaction between SLs and protein thiol groups leads to reduction of enzyme activity (Chaturvedi, 2011). Amaya *et al*. (2012) observed that six SLs of the goyazensolide and isogoyazensolide-type isolated from

Centratherum punctatum were good candidates for the development of new anti-virulence agents instead of being bactericidal. Although these SL compounds were not able to completely inhibit bacterial growth at 100 - 500 µg/mL, they altered biofilm formation, elastase activity, and production of *N*-acyl-homoserine lactones by *Pseudomonas aeruginosa* ATCC 27853 (Amaya *et al*., 2012).

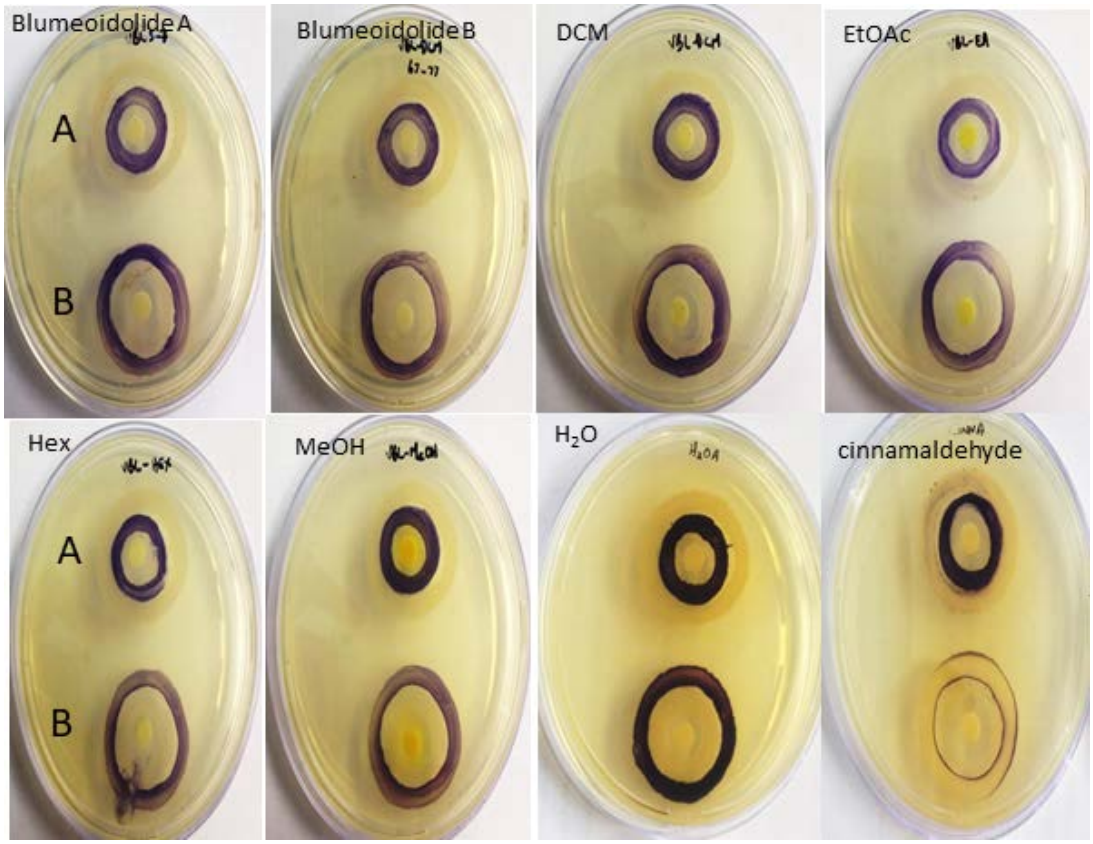

Figure 4.4 Quorum sensing inhibition by sub-inhibitory concentrations (2 mg mL^{-1}) of blumeoidolides A (VBL 5-7) and B (VBL-DCM 67-77), and 4 *V. blumeoides* crude extracts (VBL-DCM, VBL-EA, VBL-Hex and VBL-MeOH) by A] modulation of AHL receptor activity (LuxR) or B] AHL synthesis (LuxI) in the double ring agar diffusion assay with the *Chromobacterium violaceum* CV026/ATCC 31532 biosensor system. Discs impregnated with 20 µL of sterile distilled water and cinnamaldehyde (50 µg/mL) served as controls.

The other documented QS inhibition by sesquiterpenoids involved drimane sesquiterpenoids (polygodial, drimenol and drimendiol) isolated from *Drimys winteri*. Drimendiol (800 µg/mL) decreased violacein production by *C. violaceum* ATCC 12472 by 70% and decreased biofilm formation by *Pseudomonas syringae* (Paz *et al*., 2013). *Drymis winteri, Psoralea*

glandulosa and *Peumus boldus* also displayed inhibitory activity against *C. violaceum* ATCC 12472 (Cárcamo *et al*., 2014). Two α, β unsaturated lactones, cinnamolide and valdiviolide, with the carbonyl on position 12 of the drimane skeleton were found to be inhibitors of QS; while five other drimane lactones were not active. It is highly likely that blumeoidolides A (**1**) and B (**2**) are AHL mimics which competitively inhibit CviI/TraI activity. The secretion of AHL signal mimic molecules by higher plants regulates its associated bacterial populations, either by limiting the activity of pathogens by affecting AHL-regulated behaviors or by activating protection by plant growth-promoting bacteria (Teplitski *et al.*, 2011).

Evaluation of antagonistic activity of blumeoidolides through molecular docking analysis

The availability of the 3D molecular structure of a chosen therapeutic target, particularly the region responsible for its chemical interactions, makes it possible to identify a compound capable of binding to the active site of receptor proteins using computational molecular modeling techniques (Barbosa da Silva *et al.*, 2014). When binding occurs, the properties of the protein change. This technique can be used to identify quorum sensing inhibitors or modulators by examining their structure–activity relationships with AHL receptor protein LuxR and/or its homologues.

The 3D structures of transcriptional regulators involved in QS from *C. violaceum* (Chen *et al*., 2011), *Ag. tumefaciens* (Vannini *et al.*, 2002), and *P. aeruginosa* (Bottomley *et al*., 2007) have been elucidated. *Chromobacterium violaceum* ATCC 31532 synthesizes violacein as a result of cell-to-cell communication using *N*-hexanoyl homoserine lactone (C6-AHL), which is detected via the LuxR-type protein CviR (PDB: 3QP5). Recognition of this native signal

molecule by its receptor CviR is strongly antagonized by C8-AHL and C10-AHL. CviR' (PDB: 3QP1) is the LuxR receptor protein in *C. violaceum* ATCC 12472 which is activated by its cognate ligand, 3-hydroxy-C10-AHL, and responds to C10-AHL, while C6-AHL acts as a partial antagonist. CviR' shares 87% amino acid sequence identity to CviR (Chen *et al*., 2011). In order to substantiate the experimental observations and probe the binding modes of blumeoidolides A-D into the binding site of CviR' (PDB: 3QP1), molecular docking simulations were performed.

All four blumeoidolides A-D were flexibly docked using the Flexible Docking algorithm embedded in the Discovery Studio. First, the native ligands (C10-AHL and C6-AHL) were re-docked into the binding site (BS) of CviR' and CviR, respectively, to check the feasibility of the docking protocol. The computed root mean square deviation of approximately 0.5 Å (for CviR) and 1.2Å (for CviR') between their docked poses and X-ray structures (**Figure 4.5**) indicated a good three dimensional structural correlation between them and validated the predictive efficiency of the docking procedure. Docking of the four SLs was subsequently performed following the same procedure as used for the native ligands.

All compounds docked successfully into the BS of CviR' (3QP1) with good binding affinity as evidenced by the lower computed binding energies ranging between -20 to -44.5 kcal/ mol (**Table 4.3**) with the lowest BE value showing the strongest interaction with the receptor and *vice-versa*. Blumeoidolide B (**2**) exhibited the strongest binding affinity (BE = -44.5 kcal/mol) with CviR', supporting its highest biological activity (inhibition ≥80%, concentration 0.071 mg mL^{-1}) observed under experimental conditions. The binding affinity of the remaining compounds for CviR' (**Table 4.3**) followed the order: blumeoidolide D (**4**)

(BE = -43.3 kcal/mol) > blumeoidolide C (**3**) (BE = -22.1 kcal/mol) > blumeoidolide A (**1**) (BE = -20.0 kcal/mol).

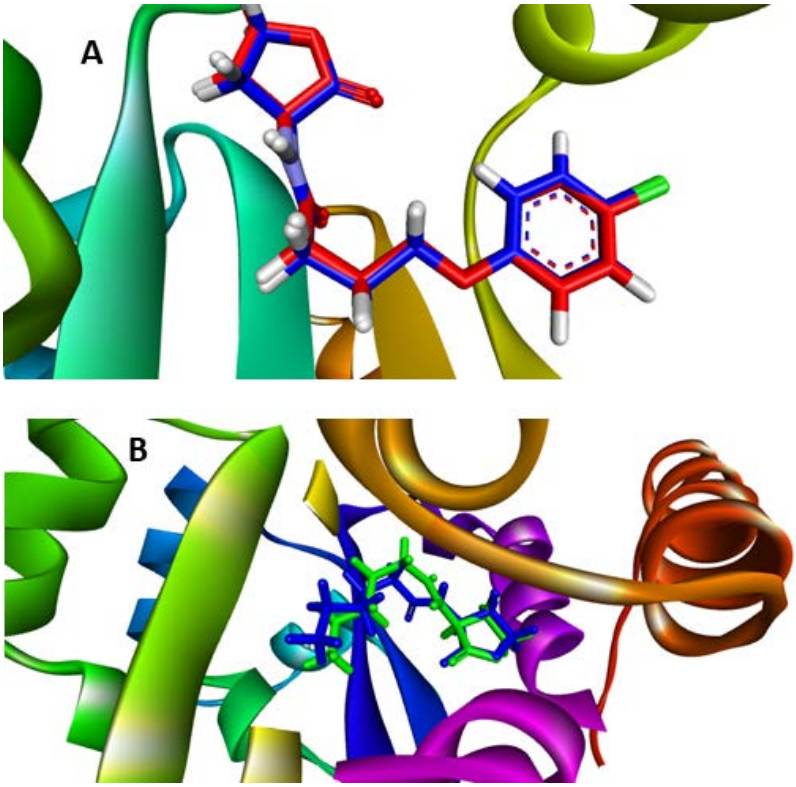

Figure 4.5 [A] Overlay of docked pose of C6-AHL (native ligand; in green sticks) with its X-ray structure (in red sticks) for 3QP5, with RMSD (all atoms)=0.5Å.[B] Overlay of docked pose of C10-AHL (native ligand, in green sticks) with its X-ray structure (in blue sticks) for 3QP1, with RMSD (all atoms)=1.2Å.

Based on computed binding energy data, the order of inhibition of CviR (3PQ5) with SLs (**Table 4.3**) was as follows: blumeoidolide C (**3**) (BE = -101.8 kcal/mol) > blumeoidolide D (**4**) (BE = -33.0 kcal/mol) > blumeoidolide B (**2**) (BE = -18.4 kcal/mol) > blumeoidolide A (**1**) (BE = -14.0 kcal/ mol). Blumeoidolide B (**2**) (BE = -18.4 kcal/mol) also exhibited stronger interaction with the CviR protein (3QP5) compared to blumeoidolide A (**1**) (BE = -14.0 kcal/mol), although blumeoidolide C (**3**) was a stronger inhibitor of CviR rather than blumeoidolide B (**2**).

Table 4.3 Molecular docking energy (kcal/mol) of sesquiterpene lactones, blumeoidolides A-D identified from *Vernonia blumeoides* against CviR/CviR' receptor proteins of *Chromobacterium violaceum*

Compound	Docking score (kcal/mol)	
	CviR	**CviR'**
N-hexanoyl-AHL (natural ligand of CviR)	-55.7	-
N-decanoyl-AHL (natural ligand of CViR')	-	-26.0
Blumeoidolide A (**1**)	-14.0	- 20.0
Blumeoidolide B (**2**)	-18.4	-44.5
Blumeoidolide C (**3**)	-101.8	- 22.1
Blumeoidolide D (**4**)	-33.0	-43.3

AHL antagonists belonging to the lactone, thiolactone and furanone classes of organic compounds are most commonly investigated due to their structural similarities with that of naturally occurring AHL auto-inducers. AHLs or their analogues are characterised by a lactone head which is able to form a H-bond with the nearby Trp84 residue, while the acyl group forms H-bonds with Asp97, Tyr80 and Ser155. The tail part is buried in a hydrophobic pocket made of Val, Leu and Ile residues (Ahmed *et al.*, 2013).

Based on molecular modelling studies, ligands acting as AHL antagonists usually possess a five-membered lactone ring with an acyl group as a spacer and a hydrophobic tail which facilitates binding of these ligands with the active site by H-bonding and hydrophobic interactions (Singh *et al*., 2015). For CviR, the lactone carbonyl forms a direct H-bond with the conserved Trp84 residue, the acyl group –NH forms an H-bond with Asp97 and the carbonyl oxygen forms H-bonds with Tyr80 and Ser155 (Ahmed *et al*., 2013).

In order to determine the mode of action of blumeoidolides, the complexes of blumeoidolides A - D with CviR (**Figure 4.6**) and CviR' (**Figure 4.7**) were visualized to validate whether

these SLs inhibit QS by allosteric inhibition of the receptors or by competitive binding to the receptors. Only interacting amino acids of the protein are shown (in red lines), whereas the lactone pose is depicted in sticks (lemon color) format. Hydrogen bonds are shown as green dotted lines and hydrophobic interactions are depicted with grey dotted lines. Binding sphere is shown as a light-yellow sphere.

The docked complex of blumeoidolide A (**1**) with CviR (3QP5) revealed the presence of a hydrogen bond with Trp84 in addition to hydrophobic interactions with amino acid residues Tyr80, Tyr88, Asp97, Ile99, Trp111, and Met135 (**Figure 4.6A**). Blumeoidolide B (**2**), on the other hand, showed hydrophobic interactions only with Val75, Leu85, Tyr88, and Met89 (**Figure 4.6B**). Chen *et al*. (2011) explained the increased antagonistic activity of C10-AHL against CviR based on the flexible nature of Met89 in accommodating the ligand in the binding site.

Blumeoidolide C (**3**) formed three hydrogen bonds: two with Trp84 and one with Ile99, and hydrophobic interactions with Leu85, Trp111, Phe126, Met135 and Ile153 (**Figure 4.6C**). Blumeoidolide D (**4**) also interacted with CviR through hydrogen bonding with Tyr80, Asp97 and Ile153, and hydrophobic bonding with Leu72, Val75, Leu85, Tyr88, Met89, and Leu100 (**Figure 4.6D**). Ahmed *et al*. (2013) observed that the lactone carbonyl formed a direct hydrogen bond with the conserved Trp84 residue, the acyl group –NH formed a hydrogen bond with Asp97 and the carbonyl oxygen formed hydrogen bonds with Tyr80 and Ser155. Ahmed *et al*. (2013) and Singh *et al*. (2015) have observed similar hydrogen and hydrophobic bonding results with synthesized lactones and thiolactones and *N, N*-disubstituted biguanides, respectively.

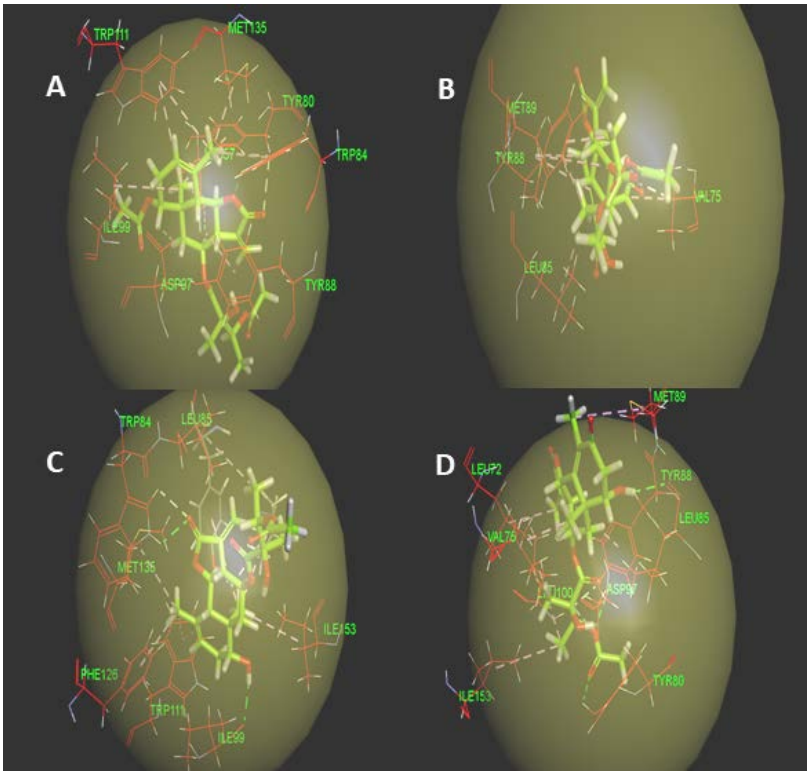

Figure 4.6 Docked complex of blumeoidolide A (A), blumeoidolide B (B), blumeoidolide C (C) and blumeoidolide D (D) with CviR (PDB code: 3QP5).

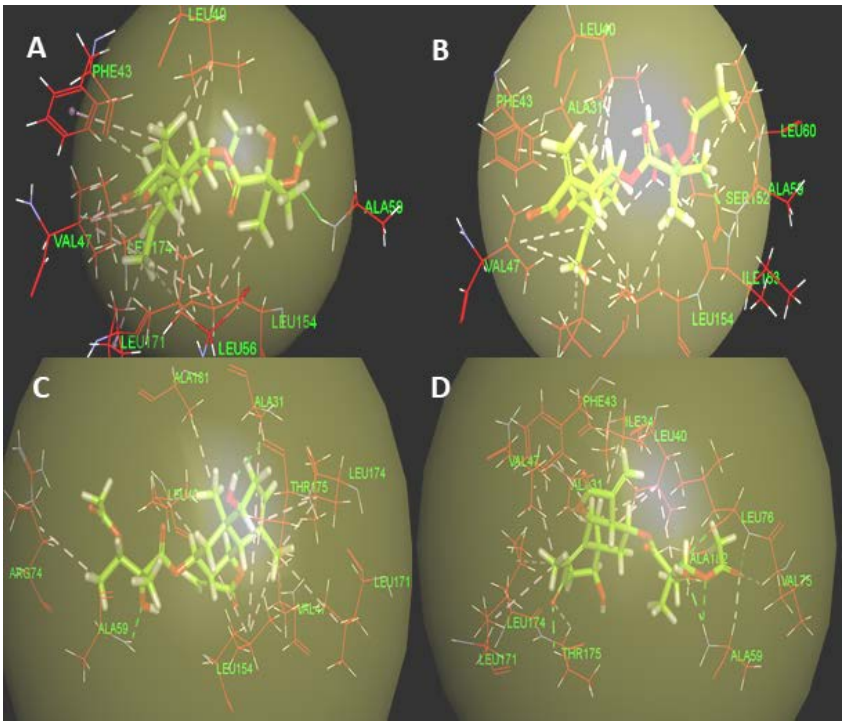

Figure 4.7 Docked complex of blumeoidolide A (A), blumeoidolide B (B), blumeoidolide C (C) and blumeoidolide D (D) with CviR' (PDB code: 3QP1).

CviR' shares 87% amino acid sequence identity to CviR (Chen *et al*., 2011) thus the interactions observed varied. Blumeoidolide A (**1**) exhibited a donor-acceptor interaction between the oxygen (ester functionality) atom of Ala59 of CviR' (3QP1), along with hydrophobic interactions with Phe43, Val47, Leu154, Leu171, and Leu174 (**Figure 4.7A**). Blumeoidolide B (**2**) also showed similar interactions as that of blumeoidolide A (**1**) with CviR', forming a hydrogen bond with Ala59 through its ester oxygen, in addition to hydrophobic interactions with Ala31, Leu40, Phe43, Val47, Leu60, Ser152, Ile153, Leu154, Leu171 and Leu174 (**Figure 4.7B**).

The higher number of hydrophobic interactions exhibited by blumeoidolide B (**2**) suggests its tighter fit into the binding site of CviR', and may account for its stronger interaction (BE = - 44.5 kcal/mol, **Table 4.3**) with the receptor compared to blumeoidolide A (**1**) (BE = - 20.0 kcal/mol, **Table 4.3**), and the higher activity observed under experimental conditions. Blumeoidolide C (**3**) exhibited two hydrogen bonds with Ala59 and Thr175, in addition to hydrophobic interactions with Leu41, Val47, Arg74, Leu154, Leu171, Leu174 and Ala181 of CviR' (**Figure 4.7C**).

Both hydrophobic (Ile34, Leu40, Leu41, Phe43, Val47, Leu171, Leu174, and Ala182) and hydrogen bonding (Ala59, Val75, Leu76 and Thr175) accounted for the binding of blumeoidolide D (**4**) with the CviR' (**Figure 4.7D**). The basic interaction of the four SLs with CviR' appears to involve Phe43, Val47, Ala59, Leu154, Leu171 and Leu174. Overall, the docking results suggest that both the hydrogen bonding and hydrophobic forces play an important role in the stabilization of blumeoidolide- CviR/CviR' -protein complex formation.

4.3 Experimental

General experimental procedures

GC-MS analysis was carried out on an Agilent Technologies (6890 Series) GC coupled with a Mass Selective Detector (5973 Series). It was equipped with an Agilent HP-5MS capillary column (0.25 µm film thickness) with dimensions 30 m (length) × 0.25 micron (I.D). Violacein was quantified using a UV-1800 UV-VIS spectrophotometer (Shimadzu, Japan). The system software was driven by Agilent Chemstation software. All chemical reagents used were of analytical grade and were supplied by Sigma (Germany), or Merck (South Africa). Blank discs for antimicrobial susceptibility testing were obtained from the MAST Group Ltd. (Merseyside, UK).

Test samples

The hexane (VBL-Hex), dichloromethane (VBL-DCM), ethyl acetate (VBL-EA) and methanol extracts (VBL-MeOH) and four SLs [blumeoidolide A (**1**)**,** blumeoidolide B (**2**)**,** blumeoidolide C (**3**) and blumeoidolide D (**4**)] obtained previously from *Vernonia blumeoides* (Asteraceae) (Aliyu *et al*., 2015), were used in the QS inhibitory assays. The structures of compounds **1-4** were elucidated in our previous publication (Aliyu *et al*., 2015).

Gas chromatography-mass spectrometry

The four crude extracts were subjected to GC-MS analysis in order to identify other known secondary metabolites. A sample ionization energy of 70 eV was used for GC-MS detection. Helium was used as the carrier gas at a pressure of 60 kPa, with the oven temperature programmed at 100 °C (for 2 min) to 280 °C (for 30 min) at a ramping rate of 4 °C per min. A DB-5 (Agilent) column was used and a 2 µL sample was manually injected. The injection temperature was at 280 °C with a split ratio of 1:50. The system software was driven by

Agilent Chemstation software. The relative amount of each component as a percentage was calculated by comparing the area of the peak to the total area. The identification of the various compounds was carried out by comparing their fragmentation peaks with those of known compounds in the NIST/NBS 2005 mass spectral database of the GC-MS.

Antibacterial susceptibility testing

Antibacterial efficacy of the SL blumeoidolide A (**1**) and crude extracts against biosensor system strains was determined using the disc diffusion method (CLSI, 2012). Blumeoidolide A (**1**) was dissolved in DMSO to a final concentration of 100 mg mL^{-1}. Blank discs (6 mm; MAST, UK) were impregnated with 2 and 4 mg mL^{-1} of the SL or crude extracts and allowed to dry. Biosensor (*Ag. tumefaciens* A136, *C. violaceum* CV026 and VIR07) and over-producer (*Ag. tumefaciens* KYC6, *C. violaceum* ATCC 12472 and ATCC 31532) strains were grown overnight on Luria-Bertani (LB) agar plates and re-suspended to a turbidity equivalent to that of a 0.5 McFarland standard. Suspensions were used to inoculate Mueller-Hinton (MH) agar plates by streaking swabs over the entire agar surface followed by the application of the respective extract/lactone discs. Plates were then incubated for 24 h at 30 °C. Testing was done in triplicate and DMSO-impregnated discs served as the negative control. Zone diameters were determined and averaged. Antibacterial activity was determined by measuring the diameter of the inhibition zone (clear zone) formed around the well in millimeters and classified as follows: Resistant (R): ≤ 10 mm; Intermediate (I): 11-14 mm; Sensitive (S): ≥ 15 mm (Chenia, 2013).

Quantitative anti-quorum sensing activity-violacein inhibition

Blumeoidolide A (**1**), blumeoidolide B (**2**) and four crude *V. blumeoides* extracts were screened for QS inhibitory properties using the violacein inhibition assay, with inhibition of the *C. violaceum* ATCC 12472 purple pigment, violacein, being indicative of anti-quorum sensing activity (McLean *et al*., 2004). *Chromobacterium violaceum* ATCC 12472 was cultured overnight in 5 mL of LB broth at 30 °C with or without crude extracts and blumeoidolide A (**1**) in a concentration range of 0 - 9.5 mg mL^{-1}. For blumeoidolide B (**2**), concentrations ranged from 0.003 – 0.071 mg mL^{-1} due to insufficient sample being available from our previous isolation. QS inhibition (QSI)-positive controls, cinnamaldehyde and vanillin (Sigma) were tested at concentrations of 0.008 - 2.05 mg mL^{-1}.

A 1 mL aliquot of culture was centrifuged at 13 000 rpm for 10 min. The culture supernatant was discarded and the resulting pellet of precipitated violacein was re-solubilised in 1 mL of DMSO, followed by centrifugation at 13 000 rpm for 10 min to precipitate the cells. The supernatant (1 ml) was aliquoted and violacein was quantified using a UV-1800 UV-VIS spectrophotometer (Shimadzu, Japan) at a wavelength of 585 nm. Tests were done in triplicate determinations (Truchado *et al.*, 2012). The following formula was used to calculate the percentage of violacein inhibition: percentage of violacein inhibition = (control $OD_{585\ nm}$ - test $OD_{585\ nm}$ / control $OD_{585\ nm}$) (Packiavathy *et al*., 2012).

Differences in violacein inhibition with and without the addition of varying concentrations of extract was determined using pair-wise testing based on Student's *t*-tests using SigmaStat 3.5 (Systat Software Inc., San Jose, CA, USA), with $p \leq 0.05$ being considered significant. Differences in violacein inhibition mean values between extracts were determined using one-way repeated measures and ANOVA with $p \leq 0.05$ being considered significant. To isolate

the extract or extracts that differed from the others, the Holm-Sidak multiple pairwise comparison procedure was carried out, with $p \leq 0.05$ being considered significant.

Qualitative modulation of QS activity

The modulation of AHL activity and inhibition of AHL synthesis by *V. blumeoides* crude extracts and sesquiterpene lactones –blumeoidolides A (**1**) and B (**2**) was determined using agar diffusion double ring assays (Vattem *et al*., 2007) at sub-inhibitory concentrations (2 mg mL^{-1}). The effect on short chain AHL inhibition was investigated with the *C. violaceum* biosensor system consisting of biosensor strain CV026 and *C. violaceum* ATCC 31532 as the C6-AHL over-producer (McClean *et al.*, 1997). Two biosensor systems were used to investigate the effect on long chain AHL inhibition, i.e., the *Ag. tumefaciens* biosensor system consisted of the biosensor strain A136 (pCF218) (pCF372) and strain KYC6 as the 3-oxo-C8- and 3-oxo-C6-AHL over-producer (Zhu *et al*., 1998), while the long chain *C. violaceum* biosensor system consisted of biosensor strain VIR07 and *C. violaceum* ATCC 12472 as the C10-AHL over-producer (Morohoshi *et al.*, 2008).

Blumeoidolides A (**1**) and B (**2**) and four crude *V. blumeoides* extracts, at sub-inhibitory concentrations (2 mg mL^{-1}), were impregnated on sterile filter paper disks and the AHL over-producer and biosensor strains inoculated in concentric circles in proximity to the impregnated disks (Chenia, 2013). All biosensor and over-producer strains were first assessed for their resistance to 2 mg mL^{-1} of blumeoidolide A (**1**) and the crude extracts. Potential LuxI homologue inhibition was assessed by placing the AHL over-producer in close proximity to the test substance and the AHL biosensor distally. LuxR homologue inhibition was assessed by reversing the location of the AHL over-producer and biosensor strains. Modulation was inferred by observation of a lower signal from the AHL biosensor than from

the over-producer (Vattem *et al.*, 2007). For the *Ag. tumefaciens* A136 system, 20 µL of 5-bromo-4-chloro-3-indolyl-*β*-D-galactopyranoside (X-gal, 20 mg mL^{-1} in DMSO) was spread evenly on LB agar plates and allowed to dry for 60 min, prior to inoculation. Discs impregnated with cinnamaldehyde (50 µg/mL), vanillin (200 µg/mL) and water were used as controls.

Evaluation of antagonistic activity of blumeoidolides through molecular docking analysis

The 3D structural coordinates of transcription activator proteins CviR and CviR' were retrieved from the Protein Data Bank (PDB code: 3QP5 and 3QP1, respectively). The native ligands and water molecules were removed using the Discovery Studio visualizer. The protonated states of the proteins were determined at physiological pH using the Prepare Protein algorithm in DS. The minimization of proteins was subsequently performed using the conjugate gradient algorithm with the CHARMm force field to remove the bad contacts. The conformational profile of all compounds (blumeoidolides A-D) was explored using the Generate Conformation algorithm, and the lowest energy conformation obtained was further optimized at density functional theory (DFT) level using DS. Prior to docking, a binding sphere covering all the active site residues was generated using the Define and Edit Binding Site module embedded in Discovery Studio. Docking of compounds was subsequently performed using the Flexible algorithm (Koska *et al*., 2008). The best pose selected based on the scoring function (-CDOCKER energy) was subjected to binding energy calculations and further analysis.

4.4 Conclusions

The anti-virulence potential of eudesmanolide SLs from *V. blumeoides* was assessed *via* quantification of QS-controlled violacein production and qualitative modulation of QS activity and signal synthesis using agar diffusion double ring assays using three (*C. violaceum* and *Ag. tumefaciens*) biosensor systems. Blumeoidolide B (**2**) demonstrated greater activity than blumeoidolide A (**1**) and both SLs were able to modulate CviI synthase activity. The docking of blumeoidolides A – D into the binding sites of CviR and CviR' suggested their differential binding affinities for the target proteins. Based on biological and *in silico* investigations of the QS inhibitory potential of eudesmanolide SLs from *V. blumeoides*, it may be suggested that they have the potential to be novel anti-pathogenic agents, with the ability to reduce virulence and pathogenicity of drug-resistant bacteria *in vivo*.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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CHAPTER 5 SESQUITERPENE LACTONES FROM *VERNONIA PERROTTETII* AND THEIR QUORUM SENSING INHIBITORY ACTIVITY

Abstract

A new sesquiterpene lactone of the keto-hirsutinolide type, 13-acetoxy-1(4*β*),5(6)*β*-diepoxy-8*α*-(senecioyloxy)-3-oxo-1,7(11)-germacradiene-12,6-olide **1**, was isolated from the hexane extract of the leaves of *Vernonia perrottetii* in addition to the known sesquiterpene lactone, 13-acetoxy-1,4*β*-epoxy-8*α*-(senecioyloxy)-3-oxo-1,5,7(11)-germacratriene-12,6-olide **2**. Three common flavonoids (apigenin **3**, luteolin **4** and velutin **5)** were also isolated from the ethyl acetate and hexane extracts of the leaves. The structures of the compounds were elucidated using ^{1}H, ^{13}C and 2D-NMR spectroscopy. The antibacterial and quorum sensing inhibitory activities of compounds **1** and **2** and crudes extracts showed limited activity on *Bacillus subtilis* and *Staphylococcus aureus*, with no activity on Gram negative bacteria. However, the quorum sensing inhibitory (QSI) experiments indicated that the keto-hirsutinolides (**1** and **2**) and the four crude extracts had interesting inhibitory activity on the biosensor organism *Chromobacterium violaceum* ATCC 12472 in the range of 0.33-5.25 mg mL^{-1}, with compound **1** being the most effective at 0.33 mg mL^{-1}.

Keywords: *Vernonia perrottetii*, sesquiterpene lactones, keto-hirsutinolides, quorum sensing inhibition

5.1 Introduction

Vernonia perrottetii Sch. Bip. ex Walp. (Asteraceae) is an annual herb that grows up to 60 cm high with leaves of 1-3 cm long. It is commonly found in abandoned fields in Northern Nigeria, where the leaves are widely used as purgative agents for gastrointestinal problems (Burkill, 1985; Hutchinson *et al*., 1963). Species within the genus *Vernonia* have been used in traditional medicine for decades across Africa and South America. Its activity is probably due to secondary metabolites such as sesquiterpene lactones (SLs) found in most *Vernonia* species. These compounds can be considered major biochemical markers within the genus (Seaman, 1982). Sesquiterpene lactones such as guaianolides (Bohlmann *et al*., 1978), glaucolides (Williams *et al*., 2005), hirsutinolides (Pillay *et al*., 2007) and eudesmanolides (Aliyu *et al*., 2015) were isolated from African species of *Vernonia*.

Plant-derived sesquiterpene lactones are promising in modern drug discovery since they contain interesting pharmacophores such as the α, β-unsaturated-γ-lactone group (Ghantous *et al*., 2010). A number of pharmacological activities including cytotoxicity (Buskuhl *et al*., 2010), anti-inflammatory (Walshe-Roussel *et al*., 2013), antibacterial (Duraipandiyan *et al*., 2012) and quorum sensing inhibitory activity (Amaya *et al*., 2012) have been attributed to the lactone moiety.

The problem of antibacterial drug resistance has been a major concern in recent years and the discovery of effective phytochemicals to combat this has been a major research focus of many researchers in the field of drug discovery. Quorum sensing (QS) has received attention in recent years since it is a cell density dependent bacterial communication system using signal molecules to co-ordinate gene expression that activates the transcription of resistance production, biofilm formation and virulence expression, especially in Gram negative bacteria

(Cámara *et al*., 2002). Quorum sensing mechanisms are critical to bacterial virulence and development of antibiotic resistance; hence its inhibition has become a novel strategy to control bacterial resistance.

Secondary metabolites from plants have previously been reported as effective inhibitors of QS (Nazzaro *et al*., 2013). For instance, the sesquiterpene lactones goyazensolides (Amaya *et al*., 2012), 6-gingerol isolated from *Zingiber officinale* (Kim *et al*., 2015) and natural stilbenoids (Sheng *et al*., 2015) have all exhibited potent QS inhibitory activities. In addition, blumeoidolides A and B from *V. blumeoides* demonstrated good QS inhibitory potential (Aliyu *et al*., 2016). In our continued investigation of chemical compounds from *Vernonia* and their QS inhibitory potential, we report on the isolation and structure elucidation of a new sesquiterpene lactone **1** together with a known related sesquiterpenoid **2** and three known flavonoids. The QS inhibitory potential of the two hirsutinolides **1** and **2** and the crude extracts were evaluated.

5.2 Results and Discussion

A novel sesquiterpene lactone **1** of the germacranolide type was isolated from the hexane extract of the leaves of *Vernonia perrottetii*. In addition, a known sesquiterpene lactone 13-acetoxy-1,4*β*-epoxy-8*α*-(senecioyloxy)-3-oxo-1,5,7(11)-germacratriene-12,6-olide **2** (Bohlmann *et al*., 1983) was isolated from the same extract. Three known flavonoids apigenin **3** (Wawer and Zielinska, 2001), luteolin **4** (Li *et al*., 2008), and velutin **5** (Kang *et al*., 2011) were also isolated from the ethyl acetate (**3-4**) and hexane (**5**) extracts of the leaf (**Figure 5.1**). The sesquiterpene lactone **2**, was previously isolated from *V. poskeana* (Bohlmann *et al*., 1983). The structures of the known compounds were determined from their ^{1}H and ^{13}C NMR spectra together with 2D NMR spectra and confirmed by comparing

the NMR data with those contained in literature (Bohlmann *et al*., 1983; Wawer and Zielinska, 2001; Li *et al*., 2008; Kang *et al*., 2011). Some assignments for the ^{1}H NMR data for compound **2** contained in Bohlmann *et al*. (1983) were revised based on HMBC correlations and ^{13}C NMR data provided, which are absent in Bohlmann *et al*. (1983). Germacranolides are the largest class of sesquiterpene lactones reported from *Vernonia* with hirsutinolides being the most frequently reported skeletal type. The two hirsutinolide compounds (**1** and **2**) and the crude extracts were evaluated for their QS inhibitory potential.

Compound **1** was isolated as a brown crystalline solid with a m.p of 95-96 ºC and molecular formula of $C_{22}H_{24}O_9$ as established by the EI-MS showing a molecular ion peak at 455.2 $[M+Na]^+$ calculated for $C_{22}H_{24}O_9Na$. Absorption bands at 1781 and 1713 cm^{-1} for the ester carbonyl and ketone group, respectively were observed. The ^{1}H and ^{13}C NMR spectra (**Table 5.1**) showed resonances typical of the hirsutinolide type sesquiterpenoid similar to 13-acetoxy-1,4*β*-epoxy-8*α*-(senecioyloxy)-3-oxo-1,5,7(11)-germacratriene-12,6-olide **2** (Bohlmann *et al.*, 1983), with characteristic resonances for the olefinic methine protons at δ_H 5.69 (1H, s, H-19) and 5.44 (1H, s, H-2), the methine protons H-8 at δ_H 6.14 (1H, d, *J*=8.3 Hz) and H-10 at δ_H 2.94 (1H, m), the geminal methylene protons at δ_H 4.81 and 4.96 (each 1H, d, *J*=13.4 Hz, H-13a, H-13b) and five methyl resonances at δ_H 1.25 (3H, d, *J* =6.9 Hz, H-14), 1.60 (3H, s, H-15), 1.91 (3H, s, H-21), 2.07 (3H, s, H-17), and 2.14 (3H, s, H-22). The ^{13}C NMR spectrum showed the olefinic oxygenated carbon resonance C-1 at δ_C 195.3, C-2 at 99.2 and the ketone at 201.8. Three other carbonyl resonances at δ_C 165.6 (C-12 lactone), 170.1 (C-16 ester) and 165.4 (C-18 ester) and two other olefinic carbon resonances at δ_C 157.5 (C-7) and 133.6 (C-11) were also observed similar to compound **2**, as was the oxygenated C-4 carbon resonance bearing the methyl group at δ_C 83.1. However, a notable difference in comparison to compound **2** was the absence of the olefinic proton resonance of

H-5 at δ_H 5.87, which was replaced by δ_H 3.64 (an oxygenated methine resonance) and two deshielded oxygenated carbon resonances at δ_C 66.4 and 90.2 instead of the olefinic carbon resonances at δ_C 117.1 and 146.5 seen in **2**. This was indicative of the double bond at C-5 being replaced by an epoxide. The carbon resonance of δ_C 66.4 was typical for epoxides with δ_C 90.2 being more deshielded due to the oxygen from the lactone ring.

The structure was confirmed by HMBC correlations from H-15 to C-5 and H-5 to C-4 and C-6. Other relevant HMBC correlations were H-2 to C-4; H-14 to C-1 and C-10; H-8 to C-18 and H-13a/b to C-7, C-11 and C-16, confirming the positions of the double bond at C-1 and the 14-methyl 8*α*-senecioyloxy and 13-acetoxy groups (**Figure 5.2**). The stereochemistry at the chiral centres of C-4, C-10 and C8, was assigned by comparing with the known related compound **2** since the methyl groups at C-4 and C-10 and the senecioyloxy group at C-8 had very similar proton resonances to that of compound **2**. The H-15 resonance was then used to assign the stereochemistry of H-5 as alpha, since a NOESY correlation was observed between H-5 and H-15. The epoxide at C-5 and C-6 was thus assigned as beta. Compound **1** was thus identified as 13-acetoxy-1(4*β*),5(6)*β*-diepoxy-8*α*-(senecioyloxy)-3-oxo-1,7(11)-germacradiene-12,6-olide. This is the first report of sesquiterpene lactones from *V. perrottetii*.

Keto hirsutinolides with a 3-oxo-1-desoxy-1,2-dehydro hirsutinolide-13-*O*-acetate moiety have previously been isolated from *V. poskeana* (Bohlmann *et al.,* 1983), *V. jugalis* (Tsichritzis *et al*., 1991) and *V. staehelinoides* (Pillay *et al*., 2007).

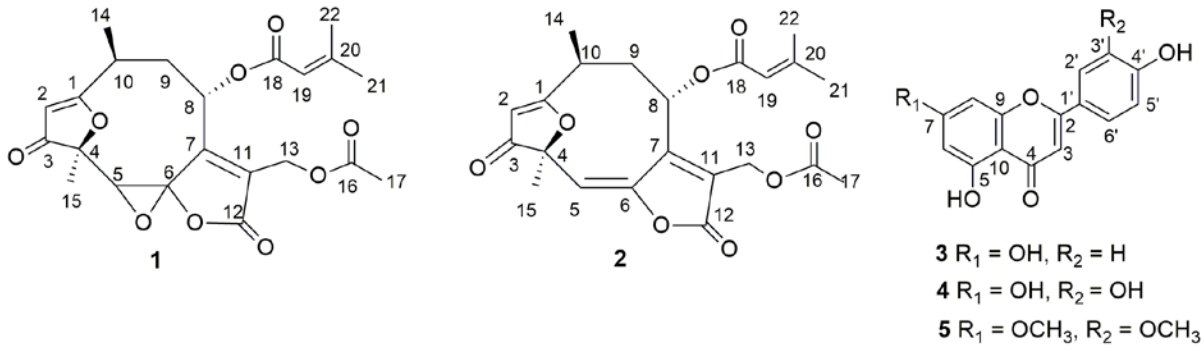

Figure 5.1 Structures of compounds **1-5** isolated from the leaves of *V. perrottetii*

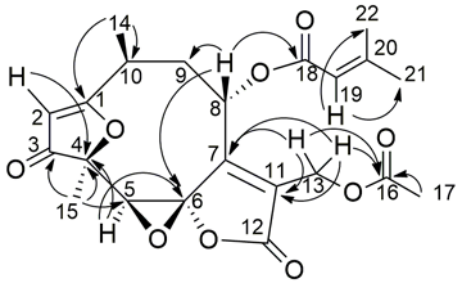

Figure 5.2 HMBC correlations in compound **1**

The hirsutinolides **1-2** are biogenetically related to the glaucolides. However, Tully *et al*. (1987) have argued that the hirsutinolides are artefacts of isolation during column chromatography on silica gel. It was also demonstrated that *trans* annular cyclization of glaucolides on silica under acidic conditions yielded **2** (Tully *et al*., 1987). However, Pillay *et al*. (2007) have shown the hirsutinolides to exhibit characteristic TLC profiles before being subject to purification by column chromatography, supporting their existence as natural products. It is quite possible that due to the conformation of the germacrene skeleton, biosynthetic transformations are responsible for glaucolides to be transformed into the hirsutinolides **1-2** (Minnaard *et al*., 1999).

Table 5.1 ^{1}H and ^{13}C NMR data for compounds **1**and **2** (in $CDCl_3$, 400 MHz)

No.	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C
1	-	195.3	-	195.1
2	5.44 (s)	99.2	5.45 (s)	99.4*
3	-	201.8	-	202.3
4	-	83.1	-	86.9
5	3.64 (s)	66.4	5.87 (s)	117.1
6	-	90.2	-	146.5
7	-	157.5	-	152.1
8	6.14 (d, *J*=8.3)	66.7	6.19 (br s)	65.0
9*α*	2.71 (ddd, *J*= 6.0, 8.3, 14.4)	42.0	2.76 (ddd, *J*= 5.6, 7.7, 14.7)	40.0
9*β*	1.76 (dd, *J*=11.9, 14.4)	-	1.72 (m)	-
10	2.94 (m)[#]	30.7	3.02 (m)[$]	31.5
11	-	133.6	-	131.5
12	-	165.6	-	166.4
13a	4.81 (d, *J*=13.4)	54.9	4.92 (d, *J*=13.4)	55.8
13b	4.96 (d, *J*=13.4)	-	5.00 (d, *J*=13.4)	-
14	1.25 (d, *J*=6.9)	16.4	1.24 (d, *J*=7.0)	15.3
15	1.60 (s)	17.3	1.59 (s)	21.0
16	-	170.1	-	170.2
17	2.07 (s)	20.6	2.02 (s)	20.7
18	-	165.4	-	164.9
19	5.69 (s)	114.7	5.64 (s)	114.6
20	-	159.2	-	160.0
21	1.91 (s)	27.6	1.89 (s)	27.6
22	2.14 (s)	20.5	2.13 (s)	20.5

*indicated by a slight hump in the spectrum; [#,$] resonance is theoretically a ddq but appears as a septet[#] and sestet[$] due to overlapping resonances. Chemical shifts are given in ppm relative to the internal reference, tetramethyl silane (TMS). Coupling constants (*J*) are given in Hz.

Antibacterial activity

Of the four crude extracts tested, only the DCM extract showed good antibacterial activity against *Bacillus subtilis* and the two *S. aureus* strains (ATCC 29213 and methicillin-resistant ATCC 43300) (**Table 5.2**). The lactone **1** also showed good activity against *Bacillus subtilis* with **2** having weaker activity against the same strain.

Table 5.2 Antibacterial activity of crude extracts and keto-hirsutinolides **1** and **2** from *Vernonia perrottetii*

Bacterial isolates	Zone diameter (mm)							
	HEX	**DCM**	**EtOAc**	**MeOH**	**1**	**2**	**TET30**	**AMP10**
Bacillus subtilis ATCC6653	9(R)	20(S)	10(R)	11(I)	18(S)	12(I)	36(S)	40(S)
Enterococcus faecalis ATCC 51299	0(R)	0(R)	0(R)	0(R)	0(R)	0(R)	22(S)	24(S)
Enterococcus faecium ATCC 19431	0(R)	0(R)	0(R)	0(R)	0(R)	0(R)	22(S)	24(S)
Staphylococcus *aureus* ATCC 29213	8(R)	15(S)	0(R)	0(R)	0(R)	14(I)	28(S)	25(S)
Staphylococcus aureus ATCC 43300 (methicillin resistant)	10(R)	16(S)	0(R)	9(R)	9(R)	10(R)	26(S)	11(I)
Staphylococcus saprophyticus ATCC 35552	9(R)	0(R)	0(R)	0(R)	0(R)	0(R)	26(S)	11(R)
Staphylococcus scuiri ATCC 29062	8(R)	14(I)	0(R)	8(R)	8(R)	12(I)	25(S)	34(S)
Staphylococcus xylosus ATCC 35033	10(R)	0(R)	0(R)	8(R)	10(R)	0(R)	36(S)	32(S)
Streptococcus agalactiae ATCC 13813	0(R)	0(R)	0(R)	0(R)	0(R)	0(R)	27(S)	0(R)
Streptococcus pyogenes ATCC 19615	0(R)	0(R)	0(R)	0(R)	0(R)	0(R)	20(S)	34(S)
Escherichia coli ATCC 25922	8(R)	7(R)	0(R)	8(R)	8(R)	0(R)	27(S)	20
Escherichia coli ATCC 35218	9(R)	0(R)	0(R)	0(R)	0(R)	0(R)	23(S)	0(R)
Klebsiella pneumoniae ATCC 700603	0(R)	0(R)	0(R)	0(R)	0(R)	0(R)	12(R)	0(R)
Pseudomonas aeruginosa ATCC 27583	9(R)	0(R)	0(R)	0(R)	0(R)	0(R)	15(I)	0(R)
Pseudomonas aeruginosa ATCC 35032	8(R)	0(R)	0(R)	0(R)	0(R)	0(R)	14(R)	0(R)

*Crude extracts and compounds **1** and **2** were tested at 4 mg mL^{-1}, AMP10=Ampicillin (10 μg) and TET30= Tetracycline (30 μg). HEX=Hexane, DCM= Dichloromethane, EtOAc=Ethyl acetate, MeOH=Methanol Resistant (R): ≤ 10 mm; Intermediate (I): 11-14 mm; Sensitive (S): ≥ 15 mm (Chenia, 2013). The criteria for assigning susceptibility or resistance to AMP10 was as follows: (S) ≥ 17 mm, (I) = 14-16 mm, (R) ≤ 13 mm, while those for TE30 were: (S) ≥ 19 mm, (I) = 15-18 mm, (R) ≤ 14 mm (CLSI, 2012)

Quorum sensing inhibitory activity

All four crude extracts and compounds **1** and **2** demonstrated QSI potential based on the violacein inhibition assay. Anomalously, no growth inhibition was observed with **2** at concentrations ≥ 5.25 mg mL^{-1}. Compound **2** had the most effective QSI, with ≥ 75% at 0.33 mg mL^{-1}. Compound **1** demonstrated QSI ≥ 80% at 1.31 mg mL^{-1}. All tested samples demonstrated QSI ranging from 66-96% at 1.31 mg mL^{-1} (**Figure 5.3**). There was a statistically significant difference ($p \leq 0.001$) in the mean values among the treatment groups

based on concentration. However, no statistically significant differences (p = 0.109) were observed between the crude extracts or compounds **1** and **2** (**Figure 5.3**). The effective QS inhibition of the hirsutinolide **2** surpassed the results of drimane sesquiterpenoids from Chilean flora both in concentration and potency (Cárcamo *et al*., 2014). This is an indication of the potential for **2** to have therapeutic significance.

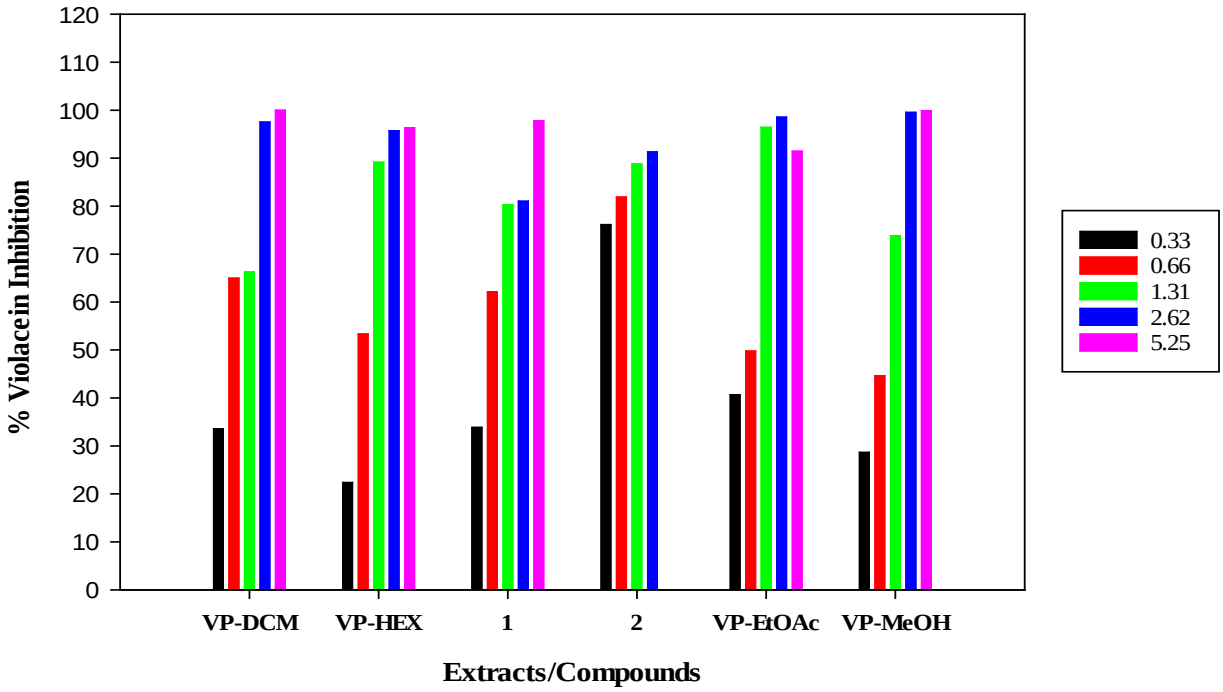

Figure 5.3 Quantitative analysis of the concentration-dependent, violacein production inhibitory effects of keto-hirsutinolides (**1** and **2**) and four *Vernonia perrottetii* crude extracts at 0.33-5.25 mg mL^{-1} (VP-DCM, VP-EtOAc, VP-HEX, and VP-MeOH) against *Chromobacterium violaceum* ATCC 12472. Data represents the mean±standard deviation of three independent experiments.

5.3 Experimental

General experimental procedures

Solvents used for the extraction and purification were reagent grade and distilled prior to being used. ^{1}H, ^{13}C and 2D NMR spectra were recorded on a Bruker Avance^{III} 400 MHz and 600 MHz spectrometers. The spectra were referenced according to the deuteriochloroform

signals at δ_H 7.24 and δ_C 77.0 and dimethyl sulfoxide δ_H 2.50, δc 39.51 (for ^{1}H NMR and ^{13}C NMR spectra, respectively). The HREIMS was measured on a ThermoFinnigan trace 132 GC, coupled with a Polaris Q mass spectrometer. IR spectra were recorded using a Perkin Elmer Universal ATR spectrometer. Optical rotations were measured at room temperature on a Perkin Elmer^{TM} Model 341 Polarimeter with a 10-cm flow tube. UV spectra were obtained on a Varian Cary UV-VIS Spectrophotometer. The melting points were determined on an Ernst Leitz Wetziar micro-hot stage melting point apparatus. Merck silica gel 60 (0.040–0.063 mm) was used for column chromatography and Merck 20 cm x 20 cm silica gel 60 F_{254} aluminium sheets were used for TLC. The TLC plates were analyzed under UV (254 and 366 nm) before being sprayed and developed with anisaldehyde: concentrated sulfuric acid: methanol spray reagent.

Plant material

Vernonia perrottetii was collected in August, 2011 along Giwa Road in Samaru-Zaria, Kaduna State. It was authenticated by U.S. Gallah at the Herbarium unit of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria where a voucher specimen (no. 250) was deposited.

Extraction and isolation

The dried leaves (1 kg) was subjected to sequential extraction with hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH) and all extracts were concentrated under reduced pressure on a rotary evaporator to yield 18.20, 25.50, 15.80 and 22.60 g of extract, respectively.

The hexane extract (15.2 g) was separated by column chromatography with a Hex: DCM gradient starting with 100% hexane and gradually increasing the polarity by 10% DCM every 500 mL until 100% DCM was reached. Fraction 68-79 (120 mg) was purified on a small column (2.5 cm in diameter) using Hex: DCM (2:3), where fractions 23-26 yielded yellowish crystals for compound **2** (40 mg), fractions 32-39 yielded compound **1** (48 mg) as brown crystals and fractions 48-59 yielded compound **5** (15 mg) as a yellow amorphous residue.

The EtOAc extract (10.00 g) was separated on a silica gel column and eluted with a hex: EtOAc gradient (1:1, 4:6 and 3:7) collecting 20 mL fractions. Fractions 67-74 eluted with hex:EtOAc (4:6) yielded a yellowish residue as compound **3** (18 mg). Fractions 145-157 eluted with hex:EtOAc (3:7) were combined and purified with hex:EtOAc (1:1), which yielded a yellowish compound from fractions 16-20 as **4** (25 mg).

Antibacterial susceptibility testing

Antibacterial efficacy of the SLs **1** and **2**, and the four crude extracts was assessed against 10 Gram-positive bacterial strains (*Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 51299, *Enterococcus faecium* ATCC 19434, *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* ATCC 43300, *Staphylococcus saprophyticus* ATCC 35552, *Staphylococcus xylosus* 35033, *Staphylococcus sciuri* ATCC 29062, *Streptococcus agalactiae* ATCC 13813, and *Streptococcus pyogenes* ATCC 19615) and five Gram-negative bacterial strains (*Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 35218, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa* ATCC 35032) using the disc diffusion method (CLSI, 2012).

Crude extracts and compounds **1** and **2** were dissolved in DMSO to a final concentration of 100 mg mL^{-1}. Blank discs (6 mm; MAST, UK) were impregnated with 4 mg mL^{-1} of the SLs or crude extracts and allowed to dry. Indicator bacteria were grown overnight at 37 °C on Tryptic soy agar plates and resuspended to a turbidity equivalent to that of a 0.5 McFarland standard. Suspensions were used to inoculate Mueller-Hinton (MH) agar plates by streaking swabs over the entire agar surface followed by the application of the respective extract/lactone discs. Plates were then incubated for 24 h at 37 °C. Tetracycline (TE30) and ampicillin (AMP10) discs (Oxoid, UK) were used as standard antimicrobial agent controls, while DMSO-impregnated discs were used as negative controls. Zone diameters were determined and averaged. Antibacterial activity was determined by measuring the diameter of the inhibition zone (clear zone) formed around the well in millimeters and classified as follows: Resistant (R): ≤ 10 mm; Intermediate (I): 11-14 mm; Sensitive (S): ≥ 15 mm (Chenia, 2013). The criteria for assigning susceptibility or resistance to AMP10 was as follows: (S) ≥ 17 mm, (I) = 14-16 mm, (R) ≤ 13 mm, while those for TE30 were: (S) ≥ 19 mm, (I) = 15-18 mm, (R) ≤ 14 mm (CLSI, 2012).

Quantitative anti-quorum sensing activity-violacein inhibition

SLs **1** and **2**, and four crude *V. perrottetii* extracts were screened for QS inhibitory properties using the violacein inhibition assay, with inhibition of the *C. violaceum* ATCC 12472 purple pigment, violacein being indicative of anti-quorum sensing activity (McLean *et al*., 2004). *Chromobacterium violaceum* ATCC 12472 was cultured overnight in 3 mL of LB broth at 30 °C with or without crude extracts and SLs **1** and **2** between 0-5.25 mg mL^{-1}. QS inhibition (QSI)-positive controls, cinnamaldehyde and vanillin (Sigma) were tested at concentrations of 0.008-2.05 mg mL^{-1}. One mL of culture was aliquoted and centrifuged at 13 000 rpm for 10 min. The culture supernatant was discarded and the resulting pellet of precipitated

violacein was resolubilised in 1 mL of DMSO, followed by centrifugation at 13000 rpm for 10 min to precipitate the cells. The supernatant was aliquoted (1 mL) and violacein was quantified using a UV-1800 UV-VIS spectrophotometer (Shimadzu, Japan) at a wavelength of 585 nm. Tests were done in three independent experiments (Truchado *et al.*, 2012). The following formula was used to calculate the percentage of violacein inhibition: percentage of violacein inhibition = (control OD_{585} - test OD_{585} / control OD_{585}) (Packiavathy *et al*., 2012).

Differences in violacein inhibition with and without the addition of varying concentrations of extract was determined using pair-wise testing based on Student's *t*-tests using SigmaStat 3.5 (Systat Software Inc., San Jose, CA, USA), with $p \leq 0.05$ being considered significant. Differences in violacein inhibition mean values between extracts were determined using one-way repeated measures and ANOVA with $p \leq 0.05$ being considered significant. To isolate the extract or extracts that differed from the others, the Holm-Sidak multiple pairwise comparison procedure was carried out, with $p \leq 0.05$ being considered significant.

5.4 Conclusion

Keto-hirsutinolides were isolated from the leaves of *V. perrottetii* and their structures elucidated using spectroscopic information including 2D-NMR. This is the first report of 13-acetoxy-1(4*β*),5(6)*β*-diepoxy-8*α*-(senecioyloxy)-3-oxo-1,7(11)-germacradiene-12,6-olide (**1)** and the first report of sesquiterepene lactones from *Vernonia perrottetii*. The crude extracts and 13-acetoxy-1(4*β*),5(6)*β*-diepoxy-8*α*-(senecioyloxy)-3-oxo-1,7(11)-germacradiene-12,6-olide **(1)** and 13-acetoxy-1,4*β*-epoxy-8*α*-(senecioyloxy)-3-oxo-1,5,7(11)-germacratriene-12,6-olide **(2)** have limited antimicrobial activity against selected Gram-positive bacteria belonging to the genera *Bacillus* and *Staphylococcus,* with no antimicrobial activity against the recalcitrant Gram-negative bacteria. Both the keto-hirsutinolides (**1** and **2**) and four *V.*

perrottetii crude extracts demonstrated QSI activity in the range of 0.33-5.25 mg mL^{-1}, with **2** being the most effective at 0.33 mg mL^{-1}, suggesting a potential candidate for the development of antibiotics.

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CHAPTER 6 BIOACTIVE COMPOUNDS FROM *VERNONIA AMBIGUA* AND *VERNONIA GLABERRIMA* AS QUORUM SENSING INHIBITORS

Abstract

The leaf extract of *Vernonia ambigua* was found to contain a novel sesquiterpene lactone, 5,6-dehydrobrachycalyxolide **3**. In addition, the ubiquitous lupeol **1** and lupeol acetate **2** and a flavonoid, chrysoeriol **4** were isolated from the same extract. The leaf extract of *Vernonia glaberrima* was found to contain **1**, **2** and **4** as well as three other flavonoids, velutin **5**, luteolin 3',4'-dimethyl ether **6** and apigenin **7**. The discovery of the sesquiterpene **3** provides a missing link between brachycalyxolide and isobrachycalyxolide. The antibacterial activity of the crude extracts from *V. ambigua* and *V. glaberrima* was poor except for the effect that the hexane and dichloromethane extracts of *V. glaberrima* had on methicillin resistant *Staphylococcus aureus* (MRSA). Quorum sensing (QS) inhibitory activity of the triterpenoids lupeol **1**, lupeol acetate **2** and 5,6-dehydrobrachycalyxolide **3** and all crude extracts showed inhibition ≥84% with **1**, **2** and **3** at 2.6 mg mL^{-1}, while those of the crude extracts ranged from 61-92% at the same concentration. However, the qualitative QS inhibition of **1-3** and crude extracts, using double ring assays, showed predominant activity of the LuxI synthase homologue, indicating that CviI was modulated by all tested compounds and extracts. This suggests that short chain QS signal synthesis was down-regulated or competitively inhibited in the order: methanol extracts > ethyl acetate extracts > brachycalyxolide (**3**) > dichloromethane extracts for *V. ambigua*, and lupeol (**1**) > dichloromethane extracts > lupeol acetate (**2**) > hexane extracts for *V. glaberrima*. The long chain QS signal synthesis was also down-regulated or competitively inhibited, though to a lesser degree.

Keywords: *Vernonia ambigua*, *Vernonia glaberrima*, triterpenoids, sesquiterpene lactone, quorum sensing inhibition.

6.1 Introduction

Plants of the genus *Vernonia* (Schreb) are distributed in the tropical regions especially Africa and South America, where about sixty species have been found in West Africa (Hutchinson and Dalziel, 1963). Several *Vernonia* species are used as vegetable foods and herbal remedies in African traditional medicine (Yeap *et al*., 2010). *Vernonia ambigua* (Kotschy & Peyr) is a plant of approximately 18 inches in height with mauve coloured flowers. The leaves are used as a remedy for cough, fever and malaria (Kunle and Egharevba, 2009). *Vernonia glaberrima* (Welw. ex O. Hoffm) is an erect shrub about 3-4 feet high with a white flower head. In Northern Nigeria, the leaves are used as an analgesic, anti-inflammatory and anti-microbial agent (Abdullahi *et al*., 2015). Since it is widely used in traditional medicine, several *Vernonia* species have been studied for their antibacterial (Aliyu *et al*., 2011), anti-inflammatory (Pandey *et al*., 2014) and antimalarial (Adebayo and Krettli, 2011) activities. These studies have been in many cases accompanied by phytochemical analyses resulting in the isolation of flavonoids (Seetharaman and Petrus, 2004), triterpenoids (Kiplimo, 2016) steroidal glycosides (Ma *et al*., 2016) and sesquiterpene lactones (Aliyu *et al*., 2015), among others.

In recent years, antipathogenic properties of medicinal plants have attracted widespread attention due to the modulating effects of plant extracts against bacterial virulence (Yarmolinsky *et al*., 2015) through quorum sensing (QS) and related properties (Packiavathy *et al*., 2012; Truchado *et al*., 2012). QS is a bacterial communication system involving signal molecules such as the acyl homoserine lactones (AHLs) used to regulate group behavior of bacterial cellular functions in Gram negative bacteria. They bind to specific receptor proteins when secreted out of neighboring bacterial cell walls, activating the transcription of resistance production, biofilm formation, virulence factor expression, bioluminescence and

pigment production (Cámara *et al*., 2002). The mechanisms of QS and related cellular functions are essential to survival of intercellular bacteria. Hence disrupting the QS signals was demonstrated to be a novel strategy for controlling bacterial virulence and resistance (Hentzer and Givskov, 2003).

Previous reports have shown the efficacy of plant chemicals as QS inhibitors (Amaya *et al*., 2012; Nazzaro *et al*., 2013; Yarmolinsky *et al*., 2015; Aliyu, *et al*., 2016). In our continued investigation of chemical compounds from *Vernonia* and their QS inhibitory potential, we report on the isolation and structure elucidation of a novel sesquiterpenoid in addition to known triterpenoids and flavonoids and on their QS inhibitory activity.

6.2 Results and discussion

The leaf extract of *Vernonia ambigua* (Kotschy and Peyr.) yielded the triterpenoids lupeol **1**, lupeol acetate **2**, a novel sesquiterpene, 5,6-dehydrobrachycalyxolide **3** and a flavonoid, chrysoeriol **4.** The leaf extract of *Vernonia glaberrima* (Welw. ex O.Hoffm.) was found to contain the triterpenoids **1** and **2**, the flavonoid **4** and three additional flavonoids, velutin **5**, luteolin 3',4'-dimethyl ether **6** and apigenin **7** (**Figure 6.1**). The known compounds were identified from their ^{1}H and ^{13}C NMR spectra and confirmed by comparison with data from literature (Nakanishi *et al*., 1985; Wawer and Zielinska, 2001; Park *et al*., 2007; Jamal *et al*., 2008; Kang *et al*., 2011).

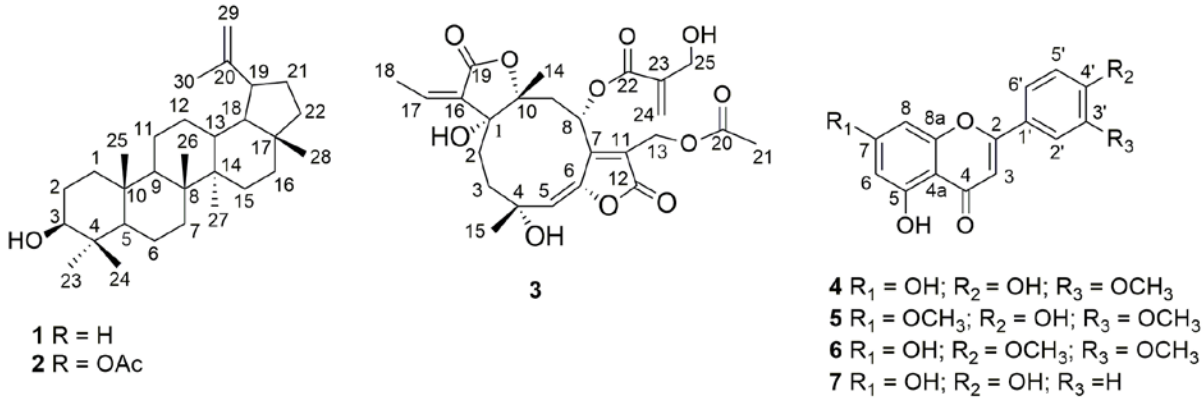

Figure 6.1 Compounds isolated from *V. ambigua* [**1-4**] and *V. glaberrima* [**1-2** and **4-7**]

The dichloromethane leaf extract of *V. ambigua* yielded a glaucolide sesquiterpene **3**, with a ^{1}H NMR spectrum very similar to that of brachycalyxolide, previously isolated from *V. brachycalyx* (Jakupovic *et al*., 1987; Oketch-Rabah *et al*., 1998). It was isolated as a crystalline solid with a melting point of 92-93 ºC and optical rotation of 62º. An ester carbonyl absorption band was evident in the IR spectrum at 1755 cm^{-1}. The ^{1}H NMR spectrum showed the presence of four methyl resonances, three singlets at δ_H 1.34, 1.50 and 2.08, the first two assigned to two methyl groups on the ten carbon cylic ring framework, H-14 and H-15, and the third to an acetyl methyl group, H-21 located on C-11 of the lactone ring. The fourth methyl resonance at δ_H 2.32 (J = 7.2 Hz) was seen coupled to a very deshielded quartet at δ_H 6.70 (J = 7.2 Hz). This was characteristic of the vinylic proton and vinylic methyl group, H-17 and H-18, respectively. H-17 showed a HMBC correlation to the oxygenated singlet carbon resonance at δ_C 89.5. The H-13 methylene group appeared as two coalescing doublets at δ_H 4.95 and 4.91 with J = 12.5 Hz and could be seen correlated the two olefinic resonances C-7 and C-11 and the lactone carbonyl C-12 all in the lactone ring as well as the acetyl carbonyl resonance, C-20.

The two methylene protons at C-24 appear as singlets at δ_H 6.02 and 6.37 and showed long range coupling to the methylene proton resonance at δ_H 4.35, which was then assigned to H-25. H-8 appeared as a broadened singlet at δ_H 5.95 due to the -O-C-H group and H-5 as a singlet at δ_H 6.06. H-8 showed HMBC correlations to the lactone carbons C-6 and C-7 at δ_C 147.9 and 152.1 and H-5 showed HMBC correlations to C-4, C-3 and C-15 at δ_C 83.4, 39.7 and 31.8, respectively. The methylene protons, H-2, H-3 and H-9 all appear between δ_H 2.38 and 2.79. The four carbonyl resonances are seen at δ_C 165.5 (C-22), 167.16 (C-19), 167.24 (C-12) and 170.4 (C-20) in the ^{13}C NMR spectrum and were assigned based on HMBC correlations; H-24 with C-22, H-21 with C-20, H-13 with C-12 and H-18 with C-19. The stereochemistry at C-1, C-4, C-8 and C-10 was assigned on the basis of brachycalyxolide, since the NMR data was similar. This was supported by NOESY correlations between H-17 and H-15 and between H-14*β* and H-8*β*. Compound **3** was thus identified as 5,6-dehydrobrachycalyxolide and is the intermediate in the biosynthetic sequence from brachycalyxolide to isobrachycalyxolide (Jakupovic *et al*., 1987) (**Figure 6.2**).

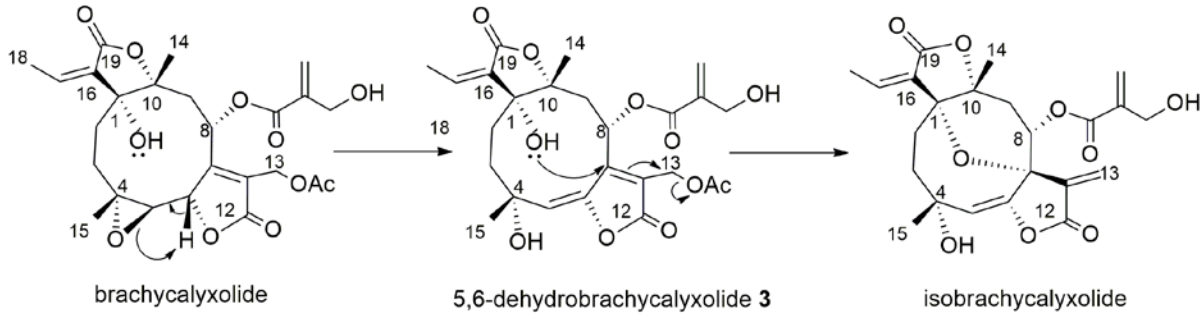

Figure 6.2 Biosynthetic pathway from brachycalyxolide to isobrachycalyxolide incorporating 5,6-dehydrobrachycalyxolide **3**

Antibacterial activity

Both *V. ambigua* and *V. glaberrima* crude extracts demonstrated no antimicrobial activity against the panel of sensitive and resistant bacteria. The only exception was the effect that *V.*

glaberrima hexane and dichloromethane extracts had on methicillin-resistant *S. aureus* (MRSA) (**Table 6.1**). The importance of the *Vernonia* species as remedies for infectious diseases is validated due to the susceptibility of hospital associated infectious pathogens (MRSA) to *V. glaberrima* extracts. This corroborates previous reports on the efficacy of *V. oocephala* chloroform extracts to MRSA (Aliyu *et al*., 2011). However, *Vernonia* species have generally been observed to exhibit activity toward Gram positive bacteria, perhaps due to easy penetration of plant chemicals through the bacterial cell wall.

Table 6.1 Antibacterial susceptibility of *V. ambigua* and *V. glaberrima* extracts

	Zone diameter (mm)							
	E. coli		*P. aeruginosa*		*S. aureus*		*S. sciuri*	*S. xylosus*
Extract	ATCC 25922	ATCC 35218	ATCC 27583	ATCC 35032	ATCC 29213	ATCC 43300	ATCC 29062	ATCC 35033
V. ambigua								
Hex	9 (R)	10 (R)	7 (R)	10 (R)	7 (R)	12 (I)	8 (R)	0 (R)
DCM	8 (R)	10 (R)	10 (R)	8 (R)	0 (R)	12 (I)	10 (R)	11 (I)
EA	10 (R)	7 (R)	9 (R)	10 (R)	0 (R)	12 (I)	7 (R)	11 (I)
MeOH	7 (R)	7 (R)	8 (R)	9 (R)	0 (R)	8 (R)	7 (R)	7 (R)
V. glaberrima								
Hex	10 (R)	9 (R)	10 (R)	11 (I)	0 (R)	16 (S)	10 (R)	12 (I)
DCM	10 (R)	10 (R)	10 (R)	9 (R)	0 (R)	16 (S)	12 (I)	10 (R)
EA	10 (R)	7 (R)	8 (R)	8* (R)	0 (R)	9 (R)	10 (R)	12 (I)
MeOH	8 (R)	7 (R)	8 (R)	10* (R)	0 (R)	10 (R)	9 (R)	10 (R)
AMP10	20 (S)	0 (R)	0 (R)	0 (R)	25 (S)	20 (S)	32 (S)	34 (S)
TET30	27 (S)	23 (S)	14 (I)	15 (I)	28 (S)	36 (S)	30 (S)	25 (S)

* Loss of pyocyanin pigmentation was observed. AMP10 = Ampicillin at 10 µg, TET30= Tetracycline at 30 µg. Resistant (R): ≤ 10 mm; Intermediate (I): 11-14 mm; Sensitive (S): ≥ 15 mm (Chenia, 2013)

Quantitative anti-quorum sensing activity-violacein inhibition

Inhibition of violacein pigment production by *V. ambigua* and *V. glaberrima* crude extracts and compounds **1-3** (0.33-10.5 mg mL^{-1}), was measured spectrophotometrically and quantified (**Figure 6.3** and **Figure 6.4**). This range of concentrations was used to identify the lowest concentration at which QS was evident, as well as document any potential growth inhibitory effect. Growth inhibition was observed at concentrations ≥7.87 mg mL^{-1} and these

concentrations were not considered for QSI. For 5,6-dehyrobrachycalyxolide (**3**), a concentration-dependent inhibition of violacein production by *C. violaceum* ATCC 12472 was observed, up to 86% at 5.25 mg mL^{-1}, while for the four *V. ambigua* crude extracts, inhibition ranged between 92-96% at the same concentration (**Figure 6.3**), with minimal bacterial growth inhibition. The four *V. ambigua* extracts displayed varying levels of QSI potency, with as much as 80% or greater inhibitory activity at 2.62 mg mL^{-1} in the following order: 5,6-dehydrobrachycalyxolide (**3**) > *V. ambigua* dichloromethane (VA-DCM) > *V. ambigua* methanol (VA-MeOH) > *V. ambigua* hexane (VA-Hex) > *V. ambigua* ethyl acetate (VA-EtOAc) (**Figure 6.3**). The IC_{50} for 5,6-dehydrobrachycalyxolide (**3**) and the four *V. ambigua* crude extracts was within the range of 1-1.5 mg mL^{-1}. The differences in the mean values among the treatment groups was not statistically significant ($p = 0.069$).

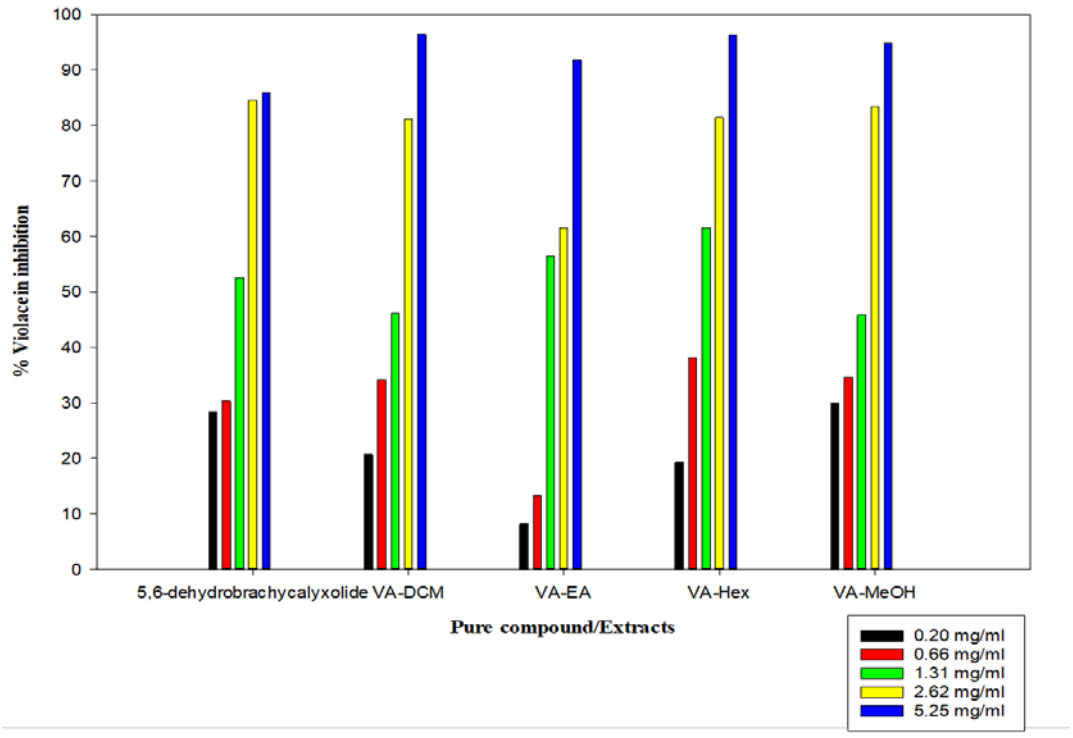

Figure 6.3 Quantitative analysis of the concentration-dependent, violacein production inhibitory effects of 5,6-dehydrobrachycalyxolide (**3**) and four *Vernonia ambigua* crude extracts at 0.33-5.25 mg mL^{-1} (VA-DCM, VA-EA, VA-Hex, and VA-MeOH) on *Chromobacterium violaceum* ATCC 12472. Data represents the mean±standard deviation of three independent experiments.

For lupeol (**1**) and lupeol acetate (**2**) a concentration-dependent inhibition of violacein production by *C. violaceum* ATCC 12472 was observed, up to 94% at 5.25 mg mL^{-1}, while for the four *V. glaberrima* crude extracts inhibition ranged between 90-96% at the same concentration (**Figure 6.4**), with minimal bacterial growth inhibition. The four *V. glaberrima* extracts displayed varying levels of QSI potency, with ≥70% inhibitory activity at 2.62 mg mL^{-1} in the following order: *V. glaberrima* dichloromethane (VG-DCM) > *V. glaberrima* ethyl acetate (VG-EA) > lupeol (**1**) > lupeol acetate (**2**) > *V. glaberrima* hexane (VG-Hex) > *V. glaberrima* methanol (VG-MeOH) (**Figure 6.4**). There was a statistically significant difference (p = 0.007) in the mean values among the treatment groups.

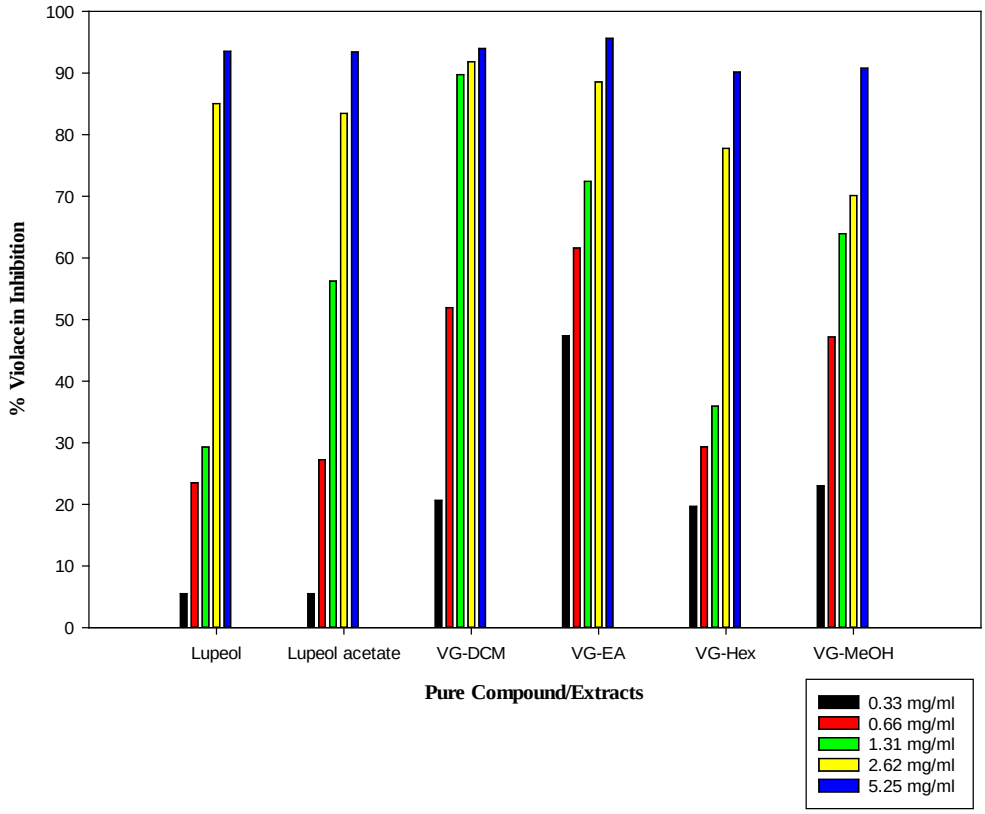

Figure 6.4 Quantitative analysis of the concentration-dependent, violacein production inhibitory effects of lupeol (**1**), lupeol acetate (**2**) and four *Vernonia glaberrima* crude extracts at 0.33-5.25 mg mL^{-1} (VG-DCM, VG-EA, VG-Hex, and VG-MeOH) on *Chromobacterium violaceum* ATCC 12472. Data represents the mean±standard deviation of three independent experiments.

Qualitative modulation of QS activity

Potential targets in the acyl homoserine lactone (AHL) system are the signal-generating LuxI or its homologues, the *N*-acyl homoserine lactone molecule itself, and the signal receptor LuxR or its homologues. Many natural extracts inhibit QS by interfering with the AHL activity by competing with them due to similar structure and/or accelerating the degradation of the LuxR receptors of AHL molecules (Koh *et al*., 2013; Nazzaro *et al*., 2013). Interference with signal reception may involve competitive and non-competitive molecules which interfere with the binding of AHLs to their cognate LuxR receptor. For competitive molecules to bind to the AHL receptor, they must be structurally similar to AHLs, while for non-competitive binding, these molecules will bind to a site on the receptor other than the AHL binding site. Plants can produce molecules similar to the AHLs moieties, and such competitive binding is effective in blocking activation of QS (Koh *et al*., 2013). A second level of modulation involves modulating the synthesis of AHL molecules by decreasing the expression of the LuxI family of synthases or the ability of phytochemicals to competitively or non-competitively inhibit LuxI activity (Vattem *et al*., 2007). Thus, the double ring bioassay was carried out using two *C. violaceum* biosensor systems to determine whether potential inhibitors target AHL synthesis (*via* LuxI homologues) or AHL response (*via* LuxR homologues).

In order to assess the correct concentration to be used for the assay, biosensor strains were subjected to susceptibility tests at 2 and 4 mg mL^{-1} of crude extracts (**Table 6.2**). Based on this data, 2 mg mL^{-1} was selected as the sub-inhibitory concentration to be used. A sub-inhibitory concentration was used since the goal was not to kill bacterial cells but rather attenuate their virulence abilities by inhibition of QS-regulated processes.

Table 6.2 Antimicrobial susceptibility of biosensors to *V. ambigua* and *V. glaberrima* extracts

	Zone diameter (mm)							
	Hex		DCM		EA		MeOH	
Conc. (mg mL^{-1})	**2**	**4**	**2**	**4**	**2**	**4**	**2**	**4**
V. ambigua								
CV026	7 (R)	11 (I)	8 (R)	11 (I)	7 (R)	10 (R)	10 (R)	14 (I)
ATCC 31532	0 (R)	0 (R)	0 (R)	11 (I)	0 (R)	0 (R)	0 (R)	0 (R)
VIR07	7 (R)	11 (I)	8 (R)	10 (R)	7 (R)	10 (R)	10 (R)	14 (I)
ATCC 12472	8 (R)	13 (I)	10 (R)	12 (I)	8 (R)	10 (R)	10 (R)	13 (I)
V. glaberrima								
CV026	0 (R)	10 (R)	9 (R)	12 (I)	10 (R)	14 (I)	7 (R)	12 (I)
ATCC 31532	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	10 (R)	0 (R)	0 (R)
VIR07	0 (R)	12 (I)	9 (R)	12 (I)	7 (R)	10 (R)	10 (R)	14 (I)
ATCC 12472	7 (R)	11 (I)	8 (R)	12 (I)	11 (I)	15 (S)	10 (R)	14 (I)

Hex: hexane, **DCM**: dichloromethane, **EA**: ethyl acetate, **MeOH**: methanol

When assessing the effect of *V. ambigua* crude extracts and 5,6-dehydrobrachycalyxolide **3** on inhibition of short-chain C4-AHL and C6-AHL signal synthesis using the CV026/ATCC 31532 system, only CviI (LuxI homologue in *C. violaceum*) inhibition was observed to varying degrees: VA-MeOH > VA-EA > 5,6-dehydrobrachycalyxolide **3** > VA-DCM (**Figure 6.5A**). In the VIR07/ATCC 12472 assay (**Figure 6.5B**), CviI modulation (inhibition of long chain C10-AHL signal synthesis) was also observed in the following order: VA-DCM > 5,6-dehydrobrachycalyxolide **3** > VA-MeOH. The VA-Hex extract was also able to inhibit CviR' causing decreased pigmentation of the VIR07 sensor **(Figure 6.5B**). The glaucolide 5,6-dehydrobrachycalyxolide **3** potentially modulated the ability of the over-producer bacteria to synthesize both short and long chain AHL molecules, however, the strongest effect was observed for short chain AHL synthesis with VA-MeOH and VA-EA.

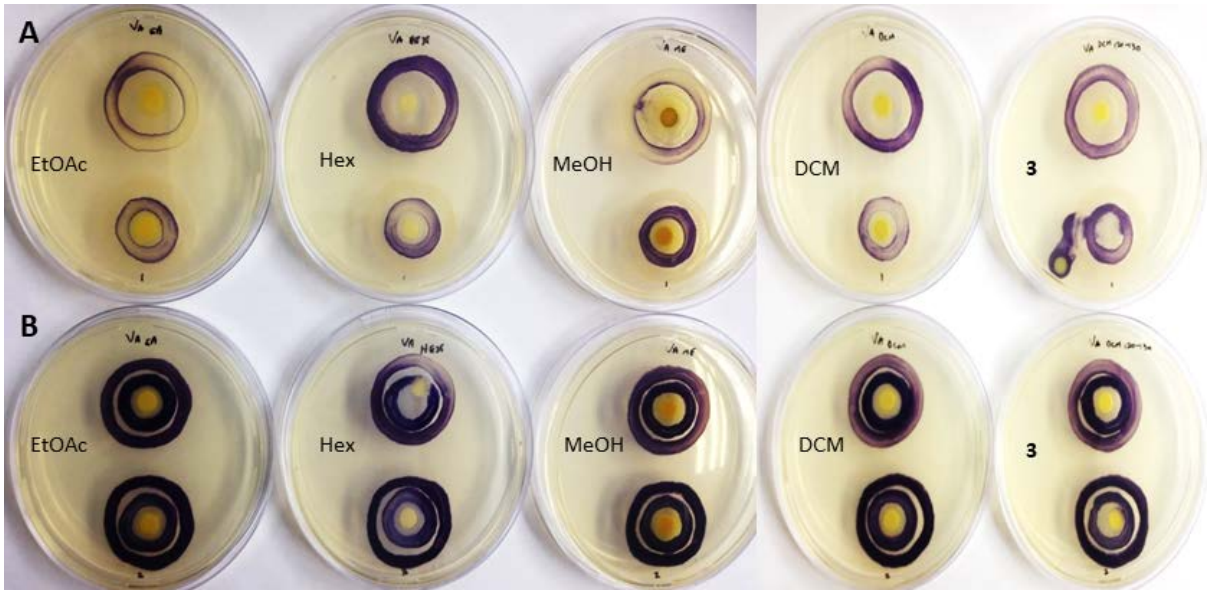

Figure 6.5 Quorum sensing inhibition at sub-inhibitory concentrations (2 mg mL^{-1}) of 5,6-dehydrobrachycalyxolide **3** and four *Vernonia ambigua* crude extracts; (A) modulation of AHL synthesis (LuxI); (B) modulation of AHL receptor activity (LuxR).

When assessing the effect of *V. glaberrima* crude extracts and triterpenoids lupeol **1** and lupeol acetate **2** on inhibition of short-chain C4-AHL and C6-AHL signal synthesis using the CV026/ATCC 31532 system, both CviI (LuxI homologue in *C. violaceum*) and CviR inhibition was observed. CviI inhibition was observed to varying degrees in the following order: lupeol **1** > VG-DCM > lupeol acetate **2** > VG-Hex (**Figure 6.6A**). CviR inhibition was also observed with VG-DCM and VG-Hex, suggesting that compounds in these extracts are able to inhibit both AHL synthesis and reception. In the VIR07/ATCC 12472 assay (**Figure 6.6B**), CviI modulation (inhibition of long chain C10-AHL signal synthesis) was also observed in the following order: lupeol **1** > VG-DCM > VG-Hex > lupeol acetate **2** > VG-EA > VG-MeOH.

These two scenarios might provide an explanation for the CviI inhibitory activity: phytochemicals within the crude extracts or the purified compounds themselves either decrease the expression of the CviI synthase, which synthesizes the AHL molecules, or

decrease AHL synthesis due to their ability to competitively or non-competitively inhibit CviI activity (Vattem *et al*., 2007; Mihalik *et al*., 2008). Secretion of molecules by higher plants that mimic AHL signal molecules allows a plant to regulate its associated bacterial populations, due to the limiting activity of pathogens by affecting AHL-regulated behaviors or by activating protection by plant growth-promoting-bacteria (Teplitski *et al*., 2011). It is highly likely that 5,6-dehydrobrachycalyxolide **3** is an AHL mimic, which competitively inhibits CviI activity.

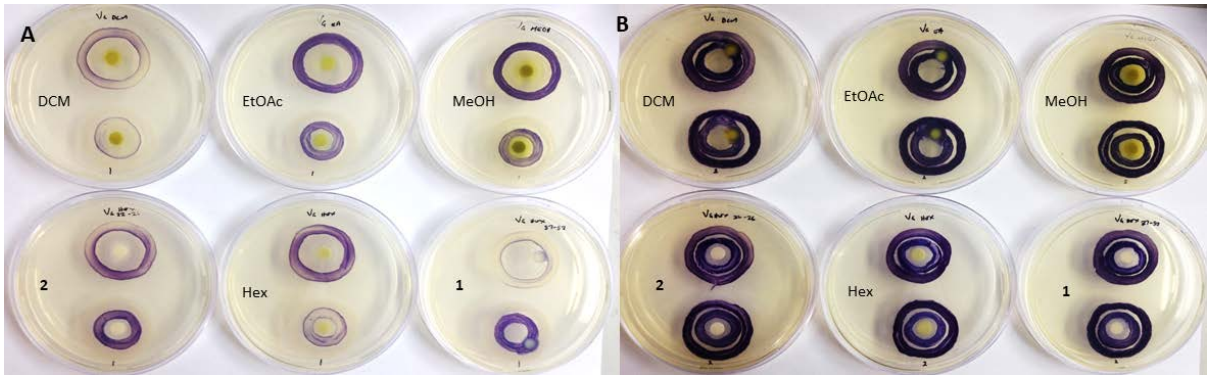

Figure 6.6 Quorum sensing inhibition at sub-inhibitory concentrations (2 mg mL^{-1}) of lupeol **1**, lupeol acetate **2** and the four *Vernonia glaberrima* crude extracts; (A) modulation of AHL synthesis (LuxI); (B) modulation of AHL receptor activity (LuxR)

6.3 Experimental

General experimental procedures

^{1}H, ^{13}C and 2D NMR data were recorded on Bruker Avance^{III} 400 MHz and 600 MHz spectrometers. The spectra were referenced according to the deuteriochloroform signals at δ_H 7.24 and δ_C 77.0 and dimethyl sulfoxide δ_H 2.50 and δc 39.51 (for ^{1}H NMR and ^{13}C NMR spectra, respectively). Samples were acquired with deuterated chloroform ($CDCl_3$) and dimethyl sulfoxide (DMSO-d_6). IR spectra were recorded using a Perkin Elmer Universal ATR spectrometer. Optical rotations were measured at room temperature on a Perkin

Elmer^{TM} Model 341 Polarimeter with a 10-cm flow tube. UV spectra were obtained on a Varian Cary UV-VIS spectrophotometer. Melting points were determined on an Ernst Leitz Wetziar micro-hot stage melting point apparatus. Merck silica gel 60 (0.040–0.063 mm) was used for column chromatography and Merck 20 cm x 20 cm silica gel 60 F_{254} aluminium sheets were used for thin-layer chromatography (TLC). The TLC plates were analysed under UV (254 and 366 nm) before being sprayed and developed with anisaldehyde: concentrated sulfuric acid: methanol spray reagent (1:2:97).

Plant material

The aerial parts of *V. ambigua* (Kotschy and Peyr.) and the leaves of *V. glaberrima* (Welw. ex O.Hoffm.) were collected in March 2011 along Samaru to Giwa in Zaria, Kaduna State, Nigeria. The plants were identified by Umar S. Gallah of the Herbarium, Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria. Voucher specimen numbers 870 and 215, respectively were deposited for future reference.

Extraction and isolation

Dried aerial parts of *V. ambigua* (1.50 kg) and leaves of *V. glaberrima* (1.00 kg) were separately subjected to sequential extraction with hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH) for 12 h on a shaker (Labcon, South Africa). All extracts were filtered and concentrated under reduced pressure on a rotary evaporator (Buchi Rota vapor R-210) at 25 ^{o}C. The following yields for crude solvent extracts were obtained respectively for *V. ambigua*: (Hex: 12.50 g; DCM: 33.89 g; EtOAc: 19.25 g; MeOH: 28.70 g) and *V. glaberrima*: (Hex: 21.20 g; DCM: 18.40 g; EtOAc: 10.50 g and MeOH: 18.80 g).

***V. ambigua*:** The hexane extract of *V. ambigua* (10.20 g) was separated by column chromatography with a Hex: DCM step gradient starting with 100% hexane and gradually increasing the polarity by 10% DCM every 500 mL until 100% DCM was reached. Fraction 52-54 yielded a white amorphous compound **1** (50 mg). The DCM extract (20.00 g) was separated similarly, but with a Hex: DCM: EtOAc step gradient. Compound **2** (32 mg) was eluted with Hex:DCM (8:2) and purified with the same solvent system. Compound **3** (28 mg) eluted with 100% EtOAc and was purified with Hex: EtOAc (1:1). The MeOH extract of *V. ambigua* (20.00 g) was dissolved in distilled water (200 mL), filtered and partitioned with an equal amount of n-butanol. The n-butanol fraction (100 mg) was subjected to column chromatography on Sephadex LH-20 (1.5 cm diameter column) using MeOH and collecting 10 mL fractions. Compound **4** (10 mg) eluted in fractions 18-25 and was purified on a silica column using Hex: EtOAc (1:1).

V. glaberrima: The hexane leaf extract (20.04 g) was separated by column chromatography with a Hex: DCM gradient as above. Fraction 22-26 yielded a white amorphous compound **1** (45 mg). The DCM extract (18.00 g) was separated as above with a Hex: DCM: EtOAc step gradient. Compound **2** (32 mg) eluted with hex:DCM (1:1) and was recrystallized from MeOH. The methanol extract of *V. glaberrima*: (18.00 g) was partitioned with n-butanol as above and subjected to column chromatography on Sephadex LH-20 (1.5 cm diameter column) using MeOH and collecting 10 mL fractions. Fractions 12-42 were combined and further purified on a silica column using Hex: EtOAc (1:1), collecting 10 mL fractions, where fr. 14-28 afforded a yellowish compound **5** (12 mg), fr. 37-41 compound **6** (18 mg) and fr. 44-50 compound **7** (10 mg).

(3E,3aR,6R,7E,11S,12aR)-10-(acetoxymethyl)-3-ethylidene-3a,6-dihydroxy-6,12a-dimethyl-2,9-dioxo-2,3,3a,4,5,6,9,11,12,12a-decahydrocyclodeca[1,2-b:5,6-b']difuran-11-yl 2-(hydroxymethyl)acrylate (5,6-dehydrobrachycalyxolide) (**3**) white solid (28 mg); m.p. 92-93 °C; $[\alpha]_D^{20}$ +62° (c. 0.1 $CHCl_3$); IR υ_{max} (cm^{-1}) 3468 (OH), 1755 (C=O); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.70 (1H, q, *J* = 7.2 Hz, H-17), 6.37 (1H, s, H-24a), 6.06 (1H, s, H-5), 6.02 (1H, bs, H-24b), 5.95 (1H, bs, H-8), 4.95 (1H, d, *J* =12.5 Hz, H-13a), 4.91 (1H, d, *J* =12.5 Hz, H-13b), 4.35 (2H, s, CH_2-25), 2.65-2.80 (2H, m, H-3*α*, H-9*β*), 2.45-2.65 (2H, m, CH_2-2), 2.37-2.45 (2H, m, H-3*β*, H-9*α*), 2.32 (3H, d, *J* = 7.2 Hz, CH_3-18), 2.08 (3H, s, CH_3-21), 1.50 (3H, s, CH_3-15), 1.34 (3H, s, CH_3-14); ^{13}C NMR (100 MHz, CDCl3) *δ* 170.4 (C-20), 167.24 (C-19*), 167.16 (C-12*), 165.5 (C-22), 152.1 (C-7), 147.9 (C-6), 144.0 (C-17), 138.3 (C-23), 131.9 (C-16), 127.9 (C-24), 126.4 (C-11), 124.3 (C-5), 89.5 (C-1), 83.4 (C-4), 77.2 (C-10), 65.9 (C-8), 61.9 (C-25), 55.6 (C-13), 41.8 (C-9), 39.7 (C-3), 32.9 (C-2), 31.8 (C-15), 23.6 (C-14), 20.9 (C-21), 14.6 (C-18).

sample decomposed before elemental analysis and mass spectrometry could be carried out; * assignments may be interchanged

Antibacterial susceptibility testing

Antibacterial efficacy of crude extracts was assessed against four Gram-positive (*Staphylococcus aureus* ATCC 29213, methicillin-resistant *Staphylococcus aureus* ATCC 43300 (MRSA), *Staphylococcus xylosus* ATCC 35033 and *Staphylococcus sciuri* ATCC 29062) and four Gram-negative (*Escherichia coli* ATCC 25922, *β*-lactam resistant *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853 and *Pseudomonas aeruginosa* ATCC 35032) indicator bacteria using the disc diffusion method (CLSI, 2012). Crude extracts were dissolved in DMSO to a final concentration of 100 mg mL^{-1}. Blank discs (6 mm; MAST, UK) were impregnated with 4 mg of the pure compounds or crude

extracts and allowed to dry. Indicator bacteria were grown overnight at 37 °C on Tryptic soy agar plates and re-suspended to a turbidity equivalent to that of a 0.5 McFarland standard. Suspensions were used to inoculate Mueller-Hinton (MH) agar plates by streaking swabs over the entire agar surface followed by the application of the respective extract/lactone discs. Plates were then incubated for 24 h at 37 °C. Tetracycline (TE30 – 30 µg) and ampicillin (AMP10 – 10 µg) discs (Oxoid, UK) were used as standard antimicrobial agent controls, while DMSO-impregnated discs were used as negative controls. Antibacterial activity was determined by measuring the diameter of the inhibition zone (clear zone) formed around the well in mm and classified as follows: Resistant (R): ≤ 10 mm; Intermediate (I): 11-14 mm; Sensitive (S): ≥ 15 mm (Chenia, 2013). The criteria for assigning susceptibility or resistance to AMP10 was as follows: (S) ≥ 17 mm, (I) = 14-16 mm, (R) ≤ 13 mm, while those for TE30 were: (S) ≥ 19 mm, (I) = 15-18 mm, (R) ≤ 14 mm (CLSI, 2012). Experiments were conducted in duplicate and averaged.

Quantitative anti-quorum sensing activity-violacein inhibition

The QS inhibitory properties of compounds **1-3**, along with *V. ambigua* and *V. glaberrima* crude extracts were quantified using the violacein inhibition assay. Inhibition of the *C. violaceum* ATCC 12472 purple pigment, violacein, was indicative of anti-quorum sensing activity (McLean *et al*., 2004). *Chromobacterium violaceum* ATCC 12472 was cultured overnight in 5 mL of Luria-Bertani (LB) broth at 30 °C with or without crude extracts and compounds **1-3** in a concentration range of 0-9.5 mg mL^{-1}. QS inhibition (QSI)-positive control, cinnamaldehyde (Sigma, Germany) was tested at concentrations of 0.008-2 mg mL^{-1}. One mL of culture was aliquoted and centrifuged at 13 000 rpm for 10 min. The culture supernatant was discarded and the resulting pellet of precipitated violacein re-solubilised in 1 mL DMSO, followed by centrifugation at 13 000 rpm for 10 min to precipitate the cells. The

supernatant was aliquoted (1 mL) and violacein quantified using a UV-1800 UV-VIS spectrophotometer (Shimadzu, Japan) at a wavelength of 585 nm. Testing was done in duplicate on two separate occasions. The following formula was used to calculate the percentage of violacein inhibition: percentage of violacein inhibition = (control $OD_{585\ nm}$ - test $OD_{585\ nm}$ / control $OD_{585\ nm}$). Differences in violacein inhibition mean values between extracts were determined using one-way repeated measures and ANOVA (SigmaStat 3.5; Systat Software Inc., San Jose, CA, USA) with $p \leq 0.05$ being considered significant. To isolate the extract or extracts that differed from the others, the Holm-Sidak multiple pairwise comparison procedure was carried out with $p \leq 0.05$ considered significant (Chenia, 2013).

Qualitative modulation of QS activity

The antibacterial efficacy of compounds **1-3** and *V. ambigua* and *V. glaberrima* crude extracts against biosensor system strains (biosensors *C. violaceum* CV026 and VIR07 and over-producers *C. violaceum* ATCC 12472 and ATCC 31532) was determined using the disc diffusion method (CLSI, 2012). This was done to ensure that a sub-inhibitory concentration was used in the modulation assay in order to eliminate bactericidal effects. The modulation of AHL activity and inhibition of AHL synthesis by *V. ambigua* and *V. glaberrima* crude extracts and compounds **1-3** were determined using agar diffusion double ring assays (Vattem *et al*., 2007) at sub-inhibitory concentrations (2 mg mL^{-1}). The effect on short chain AHL inhibition was investigated with the *C. violaceum* biosensor system consisting of biosensor strain CV026 and *C. violaceum* ATCC 31532 as the C6-AHL over-producer (McClean *et al*., 1997). The long chain *C. violaceum* biosensor system consisted of biosensor strain VIR07 and *C. violaceum* ATCC 12472 as the C10-AHL over-producer (Morohoshi *et al*., 2008).

Compounds **1-3**, *V. ambigua* and *V. glaberrima* crude extracts, at sub-inhibitory concentrations (2 mg mL^{-1}), were impregnated onto sterile filter paper disks and the AHL over-producer and biosensor strains were inoculated in concentric circles in proximity to the impregnated disks (Chenia, 2013). Potential LuxI homologue inhibition was assessed by placing the AHL over-producer in close proximity to the test substance and the AHL biosensor distally. LuxR homologue inhibition was assessed by reversing the location of the AHL over-producer and biosensor strains. Modulation was inferred by observation of a lower signal from the AHL biosensor than from the over-producer (Vattem *et al.*, 2007). Discs impregnated with cinnamaldehyde (2 μg) and water were used as positive and negative controls, respectively.

6.4 Conclusion

A new glaucolide sesquiterpenoid (5,6-dehydrobrachycalyxolide **3**) was isolated from the leaf extract of *V. ambigua*. This compound provides evidence of the biosynthetic pathway from brachycalyxolide to 5,6-dehydrobrachycalyxolide. The quorum sensing inhibitory activities of lupeol **1**, lupeol acetate **2** and **3** as well as *V. ambigua* and *V. glaberrima* crude extracts have demonstrated varying degrees of QS inhibition. The quantitative modulation of violaceum inhibition using the CV026 biosensor showed that **1**, **2**, and **3** at 2.6 mg mL^{-1} showed an inhibition of ≥84%, while the crude extracts ranged from 61-92% at the same concentration. QS inhibition of isolated compounds **1-3**, using the qualitative double ring assays showed predominant activity of the LuxI synthase homologue, which indicated that CviI was modulated by **1**, **2** and **3**, as well as *V. ambigua* and *V. glaberrima* crude extracts. The implication is that short chain QS signal synthesis was down-regulated or competitively inhibited. The long chain QS signal synthesis was also down-regulated or competitively

inhibited though to a lesser degree. This study has shown that *V. ambigua*, *V. glaberrima* and their compounds are potential sources of quorum sensing inhibitors.

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CHAPTER 7 CONCLUSIONS AND RECOMMENDATIONS

The four species of *Vernonia* studied in this work, *V. ambigua, V. blumeoides, V. glaberrima* and *V. perrottetii* are herbal remedies for infectious diseases in Nigerian traditional medicine. Species of *Vernonia* are rich sources of sesquiterpene lactones, a class of compounds associated with pharmacological activity. The four *Vernonia* species were therefore subjected to phytochemical analysis in order to search for novel sesquiterpenes and identify lead compounds as antibacterial agents. Novel compounds are often desirable in natural products research as bioactivty of known compounds are often contained in the literature. During the course of this work, our interests also shifted to testing the isolated compounds for their anti-quorum sensing ability, since compounds having the ability to inhibit quorum sensing could potentially be future antibacterial agents.

Phytochemistry

From our phytochemical analysis of these species of *Vernonia*, we isolated sesquiterpene lactones from three of the four plants studied, *V. blumeoides*, *V. perrottettii* and *V. ambigua*. Only *V. glaberrima* did not yield any sesquiterpene lactones. This indicated that species of *Vernonia* are sources of sesquiterpene lactones, which could also serve as a chemotaxonomic marker for this genus. In total, seven sesquiterpene lactones were isolated, six of which were novel. *V. blumeoides* yielded four novel compounds of the eudesmanolide skeleton (blumeoides A-D), *V. perrottetti* yielded one novel keto-hirsutinolide compound (13-acetoxy-1(4*β*),5(6)*β*-diepoxy-8*α*-(senecioyloxy)-3-oxo-1,7(11)-germacradiene-12,6-olide **B1**) in addition to a known keto-hirsutinolide (13-acetoxy-1,4*β*-epoxy-8*α*-(senecioyloxy)-3-oxo-1,5,7(11)-germacratriene-12,6-olide **B2**) and *V. ambigua* yielded a novel glaucolide sesquiterpene (5,6-dehydrobrachycalyxolide).

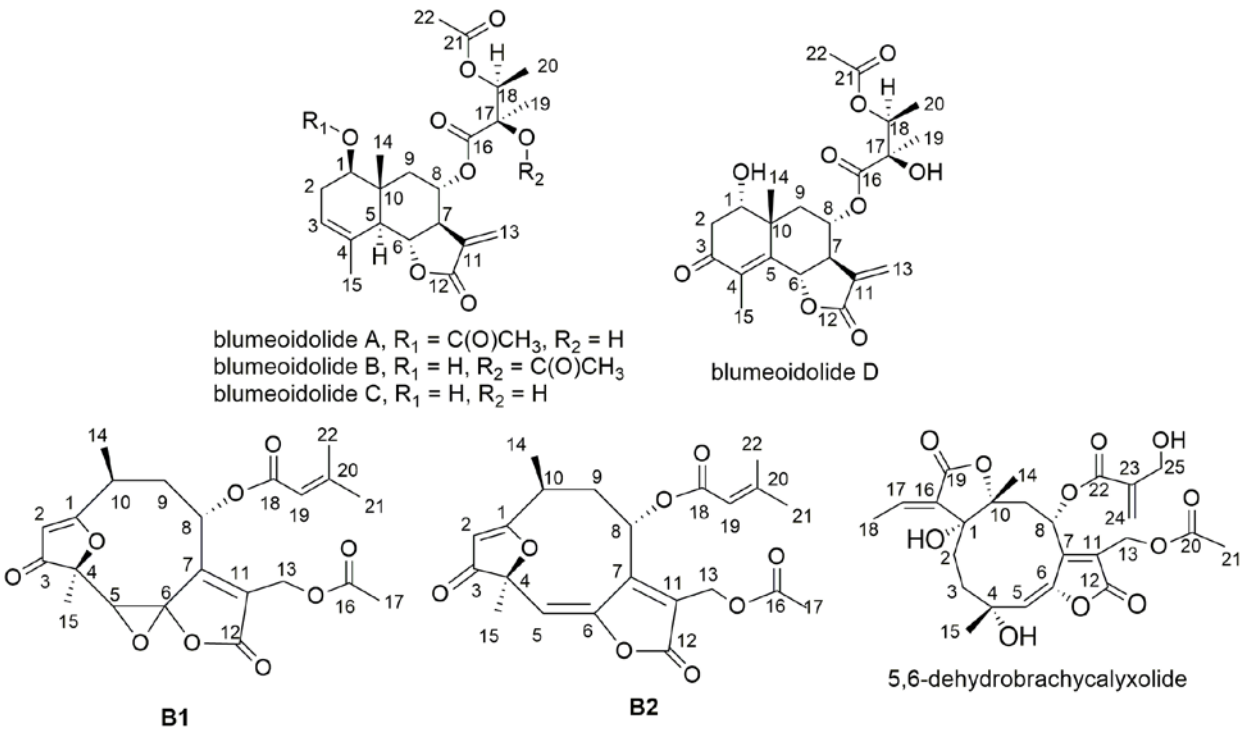

Figure 7.1 Sesquiterpene lactones isolated from *Vernonia* in this work

The four blumeoidolides A-D were all related and make up a good biosynthetic pathway themselves, blumeoidolides A and B being acetyl derivatives of blumeoidolide C and blumeoidolide D being the oxidised biosynthetic product of blumeoidolide C. It may be highly probable that further investigation of the same species at a different time may lead to the isolation of the di-acetylated blumeoidolide C and that acetylated forms of blumeoidolide D may also occur, although, different location and age of plant may be a considered factor. The novel compound **B1** is the epoxidised form of **B2** and 5,6-dehydrobrachycalyoxilde forms a biosynthetic link between brachycalyxolide and isobrachycalyxolide.

Whilst searching for sesquiterpene lactones, several flavonoids were also isolated in the process, all of which were hydroxylated, and it must be noted that this genus is also rich in hydroxylated flavonoids that are well known for their antioxidant properties. In addition, the ubiquitous sterols (lupeol, lupeol acetate and stigmasterol) were isolated from some of the

plants studied. It is well documented that these sterols have immune boosting properties amongst other pharmacological effects.

Antibacterial activity

Selected sesquiterpene lactones (based on availability of sample) were tested for their antibacterial activity, by both the conventional disc diffusion method and quorum sensing inhibition (QSI) assay using the *Chromobacterium violaceum* CV026 and CV-VIR07 biosensors. Several of the sesquiterpene lactones showed good QSI in the assays. QSI at approximately 80% was shown by blumeoidolide A (at ≥ 0.071 mg mL^{-1}), blumeoidolide B (≥ 3.6 mg mL^{-1}), B1 (1.31 mg mL^{-1}), B2 (0.33 mg mL^{-1}) and 5,6-dehydrobrachycalyxolide (2.6 mg mL^{-1}). The sterols, lupeol and lupeol acetate, all had QSI $\geq 84\%$ at 2.6 mg mL^{-1}. *In silico* docking studies with blumeoides A-D in the binding sites of quorum sensing regulator proteins CviR and CviR' indicated that these molecules bind to certain domains of these proteins, thus eliciting a response.

Thus, the sesquiterpene lactones and sterols isolated from the different *Vernonia* plants may be lead compounds, which could be developed further into antibacterial drugs.

Future work

Since the sesquiterpene lactones isolated from *Vernonia* in this work were isolated in small amounts, more of the compounds need to be extracted or a method to synthesise them needs to be developed in order to test them against an array of bioassays, such as anticancer, anti-HIV and anti-TB assays. Cytotoxicity studies also need to be carried out on these compounds to determine whether or not they are viable candidates for the pharmaceutical industry.