

UNIVERSITY OF KWAZULU-NATAL

**THE PHYTOCHEMISTRY AND BIOLOGICAL
ACTIVITY OF SECONDARY METABOLITES FROM
KENYAN *VERNONIA* AND *VEPRIS* SPECIES**

2011

JOYCE JEPKORIR KIPLIMO

**THE PHYTOCHEMISTRY AND BIOLOGICAL
ACTIVITY OF SECONDARY METABOLITES FROM
KENYAN *VERNONIA* AND *VEPRIS* SPECIES**

JOYCE JEPKORIR KIPLIMO

2011

A thesis submitted to the school of Chemistry, Faculty of Science and Agriculture, University of KwaZulu-Natal, Westville, for the degree of Doctor of Philosophy.

This Thesis has been prepared according to **Format 4** as outlined in the guidelines from the Faculty of Science and Agriculture which states:

This is a thesis in which chapters are written as a set of discrete research papers, with an Overall Introduction and Final Discussion. Where one (or all) of the chapters has already been published. Typically these chapters will have been published in internationally-recognized, peer- reviewed journals.

As the candidate's supervisor, I have approved this thesis for submission.

Supervisor:

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ABSTRACT

This work is an account of the phytochemical analysis of two genera, *Vernonia* and *Vepris* which are used as remedies for illness by the Kalenjin community of Kenya. Species of *Vernonia* are known to yield sesquiterpene lactones, which typify the genus whereas *Vepris* is rich in alkaloids and limonoids which have a wide range of biological activities. The species studied in this work were *Vernonia auriculifera*, *Vernonia urticifolia*, *Vepris glomerata* and *Vepris uguenensis*.

Phytochemical studies revealed a range of compounds being present in the four species. From *Vernonia*, triterpenoids, a sesquiterpene amine, a carotenoid and a polyene were isolated. This was the first account of a sesquiterpene amine from a plant species and the first account of the novel polyene. The triterpenoids showed moderate antibacterial activity, with β -amyirin acetate and oleanolic acid being effective at decreasing adhesion of selected gram-negative and gram-positive bacteria. Lutein and urticifolene showed good antibacterial activity against *Enterococcus faecium* and *Pseudomonas aeruginosa*.

In *Vepris*, a range of compounds were isolated, belonging to the furoquinoline alkaloids, coumarins, flavonoids, cinnamic acid derivatives, lignins, cinnamaldehydes, triterpenoids and limonoids. Five new compounds; a cinnamaldehyde derivative (glomeral), two flavonoids (veprisinol, uguenenprenol) and two A, D-*seco*-limonoids (uguenensene and uguenensone) were amongst the compounds isolated. Antibacterial studies showed that glomerol inhibited the growth of *Staphylococcus aureus* and *Shigella dysenteriae* at low concentrations (MIC of $2 \mu\text{g mL}^{-1}$ and $0.4 \mu\text{g mL}^{-1}$ respectively). Antioxidant assays of several compounds revealed that, veprisinol, haplopine-3,3'-dimethylallyl ether, uguenenprenol and 7-*O*-methylaromadendrin are good antioxidant agents. The limonoids isolated from *Vepris uguenensis* also make up an interesting biogenetic relationship.

Structural elucidation was carried out by 1D and 2D NMR spectroscopy in conjunction with mass spectrometry, infrared, ultraviolet and circular dichroism analysis where applicable. Biological assays were carried out using standard methods at laboratories in the University of KwaZulu-Natal and Kenya Medical Research Institute (KEMRI-Nairobi).

ABBREVIATIONS

ANOVA	analysis of variance
¹ H NMR	proton nuclear magnetic resonance spectroscopy
¹³ C NMR	C-13 nuclear magnetic resonance spectroscopy
COSY	correlated spectroscopy
DEPT	distortionless enhancement by polarization transfer
DPPH	2,2-diphenyl-1-picrylhydrazyl
EIMS	electron impact mass spectroscopy
FRAP	ferric reducing antioxidant potential
HMBC	heteronuclear multiple bond coherence
HSQC	heteronuclear single quantum coherence
HRMS	high resolution mass spectroscopy
MS	mass spectroscopy
NOESY	nuclear overhauser effect spectroscopy
RSA	radical scavenging activity
UV	ultraviolet
Ac	acetate
br	broad resonance
m	multiplet
c	concentration
cc	column chromatography

d	doublet
dd	double doublet
Hz	hertz
Me	methyl
s	singlet
t	triplet
IR	infrared
Mp	melting point
tlc	thin layer chromatography

DECLARATIONS

DECLARATION 1 - PLAGIARISM

I, Joyce Jepkorir Kiplimo declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
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Signed

DECLARATION 2-PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication)

Publication 1

Kiplimo, J. J., Chenia, H., Koorbanally N. A. **2011**. Triterpenoids from *Vernonia auriculifera* Hiern exhibit antimicrobial activity, *African Journal of Pharmacy and Pharmacology*, 5 (8), 1150 – 1156.

Publication 2

Kiplimo, J. J., Everia, C. A. and Koorbanally, N. A. **2011**. A Novel polyene from *Vernonia Urticifolia* (Asteraceae), *Journal of Medicinal Plants Research*, 5 (17), 4202-4211.

Publication 3

Kiplimo, J. J., Islam, Md. S., Koorbanally, N. A. Novel flavonoid and furoquinoline alkaloids from *Vepris glomerata* and their antioxidant activity, *Natural Products Communication* accepted for publication on 4th October, **2011**.

Publication 4

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Publication 5

Kiplimo, J. J., Islam, Md. S., Koorbanally, N. A. Ring A, D-seco Limonoids and Flavonoid from the Kenyan *Vepris uguenensis* Engl. and their antioxidant activity, manuscript submitted to *Journal of Natural Products* in November 2011.

From all the above publications, my role included carrying out all the experimental work and writing of the publications. The co-authors contribution was that of an editorial nature and checking on the scientific content and my correct interpretation.

Signed:

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CHAPTER ONE

INTRODUCTION

1.1 Introduction to the genus *Vernonia*

1.1.1 Phylogeny

Vernonia is one of the largest genera of flowering plants in the Asteraceae family, which includes more than 1500 species distributed widely in the tropical and sub-tropical region of Africa, Asia and America. It has two major centres of origin, South America and tropical Africa, with approximately five-hundred species found in Africa and Asia, three-hundred in Mexico, Central and South America and sixteen in the USA. Of the five-hundred species found in Africa, thirty are endemic to Kenya (Beentje, 1994; Oketch-Rabah *et al.*, 1997).

It has been difficult to establish relationships within the *Vernonia* species due to their overlapping characteristics. While the original family members (Barnadesioideae) are found in South America, the tribe Vernonieae is believed to have originated in Madagascar and its sister tribe, the Liabeae is found in America. Due to this difficulty in classifying the species it gave rise to the nickname, the 'evil tribe' (Funk *et al.*, 2005). As such there has been no phylogeny proposed for the tribe Vernonieae and only a few relationships had been suggested even among the best known species (Jones, 1977). The tribe Vernonieae has been traditionally placed in the subfamily Cichorioideae (Funk *et al.*, 2005). In a recent study it was found that the species in America were derived from those of Europe, Asia and Africa (Keeley *et al.*, 2007).

The leaves of *Vernonia* are bright green, with a serrated edge and a light yellow central vein. They are alternate with each other along the stems, getting smaller towards the tip. The

stems are upright and stiff and branch sparingly to about 1.2 m high. The flowers are light to dark purple and are grouped in dense clusters at the tip of the stems. The seeds are very light in weight and hairy (Olorode, 1984). The majority of these plants are used as ornaments and vegetables, while others are considered as weeds in agriculture. The vegetables have a bitter taste, hence the name “the bitter genus *Vernonia*” (Aliyu *et al.*, 2011).

1.1.2 Ethnobotanical information of *Vernonia* species

An ethnomedical survey of *Vernonia* species (Table 1) revealed widespread and diverse medical usage. Generally these species are used in the treatment of infectious and parasitic diseases. The infectious diseases ranged from those affecting the skin to those of the stomach (gastrointestinal infections). Other major applications include treatment of bacterial infections, gynaecological diseases and complications, respiratory diseases, diabetes, urinary tract infections and venereal diseases. Parasitic diseases include malaria, worm infection, amoebiasis and schistosomiasis. Some species are also used as antivenom against snakebites and insect bites.

Of the *Vernonia* species, *V. amygdalina* appears to be the most widely used. The leaves are the most commonly used plant part and is prepared as a decoction. In some cases a decoction of the whole plant is prepared. The method of preparation depends on the application, for example, a mixture of the root and leaf decoction is drunk to treat gastrointestinal diseases and as an antipyretic and the leaves soaked in alcohol is drunk to treat diabetes mellitus. The traditional medicinal applications of members of the genus are illustrated in Table 1.

Table 1 Species of *Vernonia* used in traditional medicine

Plant species	Plant part	Traditional use	References
<i>V. ambigua</i>	Whole plant	antimalarial	Builders <i>et al.</i> , 2011
<i>V. amygdalina</i>	leaves	antimalarial, antidiabetic, antipyretic, gastrointestinal diseases, appetite stimulant, dermatological infections, anthelmintic, respiratory tract infections, gynaecological diseases and complications, infertility, antibacterial, antifungal and antivenom (snake bite)	Akah and Okafor 1992; Akah and Ekekwe 1995; Yeap <i>et al.</i> , 2010; Huffman <i>et al.</i> , 1993
	stems	anti-HIV, antiviral and anti-amoebic	Yeap <i>et al.</i> , 2010
	leaves and fruits	antimalarial, anthelmintic, antibacterial and antiviral	Yeap <i>et al.</i> , 2010; Erasto <i>et al.</i> , 2006
	roots	venereal diseases and gynaecological complications	Yeap <i>et al.</i> , 2010; Geissler <i>et al.</i> , 2002
<i>V. anthelmintica</i>	seeds	anthelmintic, respiratory diseases, gastrointestinal diseases and complications, diuretic, anti-inflammatory, kidney protection and anti-ulcer	Otari <i>et al.</i> , 2010
<i>V. branchycalyx</i>	roots	gastrointestinal complication	Oketch-Rabah <i>et al.</i> , 1997
<i>V. cinerea</i>	leaves and roots	anthelmintic, astringent, conjunctivitis, dermatological diseases, diuretic, antipyretic, gastrointestinal diseases, gynaecological diseases, respiratory diseases, antidote and urinary tract diseases	Deborah <i>et al.</i> , 1992; Khare, 2007; Marita <i>et al.</i> , 1999; Misra <i>et al.</i> , 1993
<i>V. colorata</i>	leaves	dermatological infections, respiratory diseases, antidiabetic, gastrointestinal diseases, antipyretic, antiviral (hepatitis) and venereal diseases	Cioffi <i>et al.</i> , 2004; Rabe <i>et al.</i> , 2002; Sy <i>et al.</i> , 2004
<i>V. condensata</i>	leaves	analgesic, anti-ulcer, anti-diarrhoea, gastrointestinal	Frutuoso <i>et al.</i> , 1994; Pereira <i>et al.</i> , 1994

		diseases, liver protection and antivenom (snakebites)	
<i>V. conferta</i>	bark	anti-diarrhoea (bloody)	Aliyu <i>et al.</i> , 2011
<i>V. cumingiana</i>	ns	antirheumatic arthritis, antiviral, bone and muscular injury, respiratory diseases, antimalarial, and dental diseases	Lin <i>et al.</i> , 1985; Zhonghuabencao, 1999
<i>V. ferruginea</i>	ns	anti-inflammatory remedies	Malafrente <i>et al.</i> , 2009
<i>V. galamensis</i>	leaves	antidiabetic (mellitus) and gastrointestinal diseases	Chhabra <i>et al.</i> , 1989
<i>V. guineensis</i>	ns	antidote to poison, aphrodisiac, jaundice, anti malarial and prostatitis	Tchinda <i>et al.</i> , 2002
<i>V. jugalis</i>	bark	anti-diarrhoea (bloody)	Aliyu <i>et al.</i> , 2011
<i>V. kotschyana</i>	roots	abdominal pains, respiratory diseases including TB, antibacterial, dermatological infections, gastrointestinal disorder, analgesic, antiparasitic, antiprotozoa and anti-ulcers	Sanogo <i>et al.</i> , 1998; Nergard <i>et al.</i> , 2004
<i>V. mapirensis</i>	ns	anti-inflammatory	Morales-Escobar <i>et al.</i> , 2007
<i>V. nigritiana</i>	root	diuretic, gastrointestinal infections, emetic, antipyretic and anthelmintic	Aliyu <i>et al.</i> , 2011
<i>V. nudicaulis</i>	whole plant	venereal diseases	Aliyu <i>et al.</i> , 2011
<i>V. pachyclada</i>	ns	dermatological injury	Williams <i>et al.</i> , 2005
<i>V. paltula</i>	whole plant	antimalarial, antirheumatic and gastrointestinal infections	Chiu and Chang, 1987
	leaves	antiamoebic, anthelmintic, antiviral, respiratory tract infection, gastrointestinal infections and dermatitis	Gani, 1998
	roots	gastrointestinal infections, respiratory tract infection and colic	Gani, 1998
	flowers	eye problem, antipyretic and antirheumatic	Mollik <i>et al.</i> , 2010 Gani, 1998
	seeds	anthelmintic, gastrointestinal disorder, urinary tract infection and dermatological infection	Gani, 1998
<i>V. polytricholepis</i>	ns	antipyretic and respiratory infections	Aliyu <i>et al.</i> , 2011

<i>V. potamophila</i>	leaves	anticancer and skin infection	Babady-bila <i>et al.</i> , 2003
<i>V. saligna</i>	ns	respiratory tract infections and gynaecological complications	Huang <i>et al.</i> , 2003
<i>V. scorpioides</i>	leaves	dermatological infection (diabetic lesions) and anti-ulcer	Pagno <i>et al.</i> , 2006; Buskuhl <i>et al.</i> , 2010
<i>V. trichoclada</i>	ns	anti-inflammatory	Morales-Escobar <i>et al.</i> , 2007
<i>V. tweediana</i>	ns	respiratory tract diseases	Zanon <i>et al.</i> , 2008

Key: ns-not specified

1.1.3 Biological activity of extracts from *Vernonia* species

Biological activities of *Vernonia* extracts have been extensively studied as far back as 1969. The variety of secondary metabolites extracted from *Vernonia* species, explains the diversity of their biological activities. Among the diverse biological activities, antibacterial studies are the most reported. Antibacterial activity was found to be common to all species in all extracts followed by conditions associated with pain, fever and inflammation and antiparasitic activity, including malaria, schistomiasis and leishmania. Antibacterial compounds are mainly lipophilic and will partition from an aqueous phase into bacterial membrane structures, causing expansion of the membranes, increased fluidity, disordering of the membrane structure and inhibition of membrane embedded enzymes (Sikkema *et al.*, 1995). Antifungal activity is also reported in five of the *Vernonia* species, while antiviral is not commonly reported in the *Vernonia* with only one report in the ethanol extract of the fruit of *V. amagdalina*.

Seven species have been associated with being active in assays against pain, fever or inflammation, which includes conditions such as arthritis and gastritis. In rural areas where access to healthcare is not readily available, people rely on these extracts for common pain relief, inflammation and fever. Four of the nineteen species have also been reported to have some form of antiparasitic activity, either antiplasmodial, antileishmanial, antischistomatic,

anti-amoebic or antihelminthic. These are mainly reported for the polar extracts such as the aqueous and methanol extracts with few reports being in the chloroform or hexane extract.

Beside the antibacterial, antiparasitic and conditions associated with pain, inflammation and fever, extracts of these plants have also shown other activities such as antidiabetic, antioxidant, anticancer, antiulcer, immunomodulatory, pesticidal and insecticidal activity.

The most extensively studied plant among all the *Vernonia* species is *V. amygdalina*, reported to possess several pharmacological activities such as antidiabetic, antibacterial, antimalarial, antifungal, antioxidant, liver protection and cytotoxic effects (Table 2). The biological activities of *Vernonia* species that have been studied and documented are listed in table 2.

Table 2: Biological activities of extracts from *Vernonia*

Plant species	Biological activity	Extract	Reference (s)
<i>V. ambigua</i>	antibacterial	ethanol ^a and chloroform ^a	Aliyu <i>et al.</i> , 2011
	antiparasitic (antiplasmodial) and antioxidant	water ^{wp}	Builders <i>et al.</i> , 2011
<i>V. amygdalina</i>	antibacterial	water ^r and ethanolic ^l	Ogbulie <i>et al.</i> , 2007
	bactericidal (oral bacterial)	cold water ^{s, b, p}	Rotimi and Mosadomi, 1987
	antifungal	water ^{sb,r} and methanol ^{sb,r}	Nduagu <i>et al.</i> , 2008
	antiviral	ethanol ^{lr}	Vlietinck <i>et al.</i> , 1995
	antiparasitic (antiamoebic)	methanol ^l and water ^l	Moundipa <i>et al.</i> , 2005
	(antileishamianal)	chloroform ^l and ethanol ^l	Carvalho and Ferreira 2001
	(antischistomiasis)	petroleum ether ^l and ethanolic ^l	Adedapo <i>et al.</i> , 2007
	(anthelmintic)	water ^{l,s,r}	Adedapo <i>et al.</i> , 2007
	(antiplasmodial)	ethanolic ^{l,r}	Abosi and Raseroka, 2003
	analgesic and antipyretic	water ^l and ethanol ^r	Tekobo <i>et al.</i> , 2002
	anti-inflammatory	water ^l	Iroanya <i>et al.</i> , 2010
antioxidant	ethanol ^r and methanol ^{ns}	Yeap <i>et al.</i> , 2010	

	cytotoxic	cold water ^{WP}	Vlietinck <i>et al.</i> , 1995
	anticancer	cold water ^I	Izevbigie <i>et al.</i> , 2003
	antidiabetic	water ^{WP} and isopropanol ^{WP}	Erasto <i>et al.</i> , 2006 Taiwo <i>et al.</i> , 2009
	liver protective	methanol ^I and water ^I	Adesanoye and Farombi, 2009
	pesticidal and insecticidal	methanol ^I	Ohigashi <i>et al.</i> , 1991
<i>V. anthelmintica</i>	antibacterial	water ^{NS} -ethanolic ^{NS}	Parekh and Chanda 2008
	anti-arthritic	ethanolic ^{SD}	Otari <i>et al.</i> , 2010
	anti-inflammatory	water ^{NS} -ethanolic ^{NS}	Parekh and Chanda 2008
	antidiabetic and antihyperglycemic	EtOAc:isopropanol (1:1) ^{SD}	Fatima <i>et al.</i> , 2010
<i>V. blumeoides</i>	antibacterial	ethanol ^A and chloroform ^A	Aliyu <i>et al.</i> , 2011
<i>V. branchycalyx</i>	antiparasitic (antileishmanial) (antiplasmodial)	CHCl ₃ :EtOAc(1:1) ^I	Oketch-Rabah <i>et al.</i> , 1998
	(antimalarial)	water ^I	Oketch-Rabah <i>et al.</i> , 1997
<i>V. brasiliiana</i>	(antiplasmodial)	hexane ^I	Alves <i>et al.</i> , 1997
<i>V. cinerea</i>	antibacterial	benzene ^I	Gupta <i>et al.</i> , 2003a; Latha <i>et al.</i> , 2009
	antifungal	methanol ^A	Latha <i>et al.</i> , 2009
	antioxidant	methanol ^A	Kumar <i>et al.</i> , 2009; Latha <i>et al.</i> , 2009
	anti-inflammatory	alcoholic ^{II}	Latha <i>et al.</i> , 1998
	antipyretic	methanol ^{WP}	Gupta <i>et al.</i> , 2003b
	analgesic and antipyretic	chloroform ^I , methanol ^I and ether ^I	Mazumder <i>et al.</i> , 2003
	immunomodulatory	methanolic ^{NS}	Pratheeshkumar and Kuttan, 2010
<i>V. colorota</i>	antibacterial, antiparasitic (antiplasmodial), antidiabetic and anti-inflammatory	CHCl ₃ -MeOH (9:1) ^I	Chukwujekwu <i>et al.</i> , 2009; Cioffi <i>et al.</i> , 2004
	antidiabetic	aqueous ^I	Sy <i>et al.</i> , 2004
<i>V. condensata</i>	analgesic, antigastitis, antiulcer and antinociceptive	acetone-EtOH-EtOAc ^I	Frutuoso <i>et al.</i> , 1994; Sanogo <i>et al.</i> , 1996; Risso <i>et al.</i> , 2010
<i>V. glabra</i>	antifungal	ns	Gundidza, 1986
<i>V. hymenolepis</i>	tumor inhibitory activity	alcoholic ^I	Kupchan <i>et al.</i> , 1969

<i>V. karaguensis</i>	antibacterial and antileukaemic	ns	Mungarulire, 1993; Taiwo <i>et al.</i> , 1999
<i>V. kotchyana</i>	antibacterial, analgesic, anti-gastritis and anti-ulcer	aqueous/ <i>n</i> -butanol ^F extract and acidic ^F	Frutuoso <i>et al.</i> , 1994; Germano <i>et al.</i> , 1996; Sanogo <i>et al.</i> , 1996, 1998
<i>V. oocephala</i>	antibacterial	ethanol/chloroform ^a	Aliyu <i>et al.</i> , 2011
<i>V. paltula</i>	antibacterial, antifungal, anti-inflammatory and antipyretic	ethanolic ^{wp, fl, fr, t, l}	Chiu and Chang 1987
<i>V. polyanthes</i>	antiulcerogenic	methanolic ^a and chloroform ^a	Barbastefano <i>et al.</i> , 2007
<i>V. pogosperma</i>	antibacterial	ns	Tripathi <i>et al.</i> , 1981
<i>V. scorpioides</i>	cytotoxic effects (anti-tumor)	dichloromethane ^l	Pagno <i>et al.</i> , 2006
	bactericidal and fungicidal	chloroform ^l and hexane ^l	Buskuhl <i>et al.</i> , 2010; Freire <i>et al.</i> , 1996
<i>V. thomsoniana</i>	antibacterial and antileukaemic	ns	Mungarulire, 1993; Taiwo <i>et al.</i> , 1999

Key: superscripts, a = aerial parts, fr = fruits, fl = flowers l = leaves, ns = not specified, r = roots, s = stems, sb = stem bark, sd = seeds, wp = whole plant

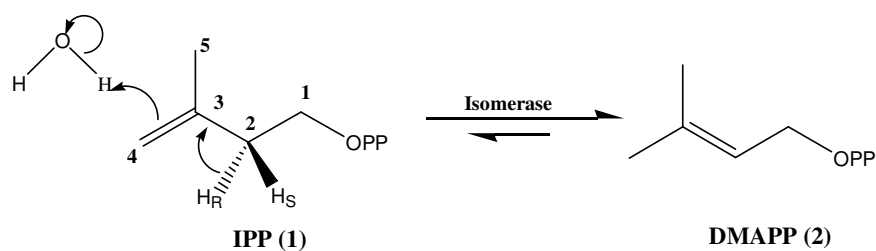
1.1.4 A phytochemical review of the triterpenoids from *Vernonia* species

Phytochemical compounds previously isolated in *Vernonia* include sesquiterpene lactones, triterpenoids, flavonoids, coumarins, steroidal glycosides and carotenoids. Although sesquiterpene lactones are the main chemotaxonomic markers of this genus, none were isolated in this work. To date, there are approximately twenty-six publications on triterpenoids in *Vernonia* species (Scifinder, 2009). The isolation of triterpenoids from this genus is recorded to have begun as early as 1979, where the tetracyclic triterpenoid, fasciculatol was isolated from *V. fasciculata*. This remains to be the only tetracyclic triterpenoid isolated from *Vernonia* and all other phytochemical studies have reported pentacyclic triterpenoids. In this study, triterpenoids were the main compounds isolated from the *Vernonia* species investigated and as a result the literature review which follows focuses on the triterpenoids in *Vernonia*.

1.1.4.1 A Brief Introduction to Triterpenoids

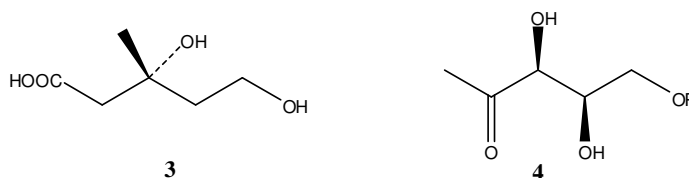
Triterpenoids are compounds based on a 30-carbon skeleton consisting of five six-membered rings or four six-membered rings and a five-membered ring. They are a widespread group of natural terpenoids and are found in most plants. In *Vernonia*, seven classes of triterpenoids have been isolated and reported, of which five are closely related and possess five six-membered rings in their basic skeleton (oleanane, ursane, taraxarene, friedelane and friedoursane), the other two classes being lupane (four six membered rings and a five-membered ring) and a tetracyclic triterpenoid.

Triterpenoids are produced by rearrangement of squalene epoxide (**10**) which is believed to have been biosynthesized by the mevalonate pathway, but recently the route via the deoxyxylulose phosphate pathway (DXP (**4**)) is reported to be more widely accepted (Dewick, 2006). The main precursor of terpenoids is the isoprene unit, which is formed *via* isopentyl diphosphate (IPP) (**1**) and dimethylallyl diphosphate (DMAPP) (**2**), the latter being formed from IPP (**1**) with the isomerase enzyme (Scheme 1).



Scheme 1: Isomerisation of IPP to DMAPP (Dewick, 2006)

These units may be derived from mevalonic acid (MVA) (**3**) or deoxyxylulose phosphate (DXP) (**4**).

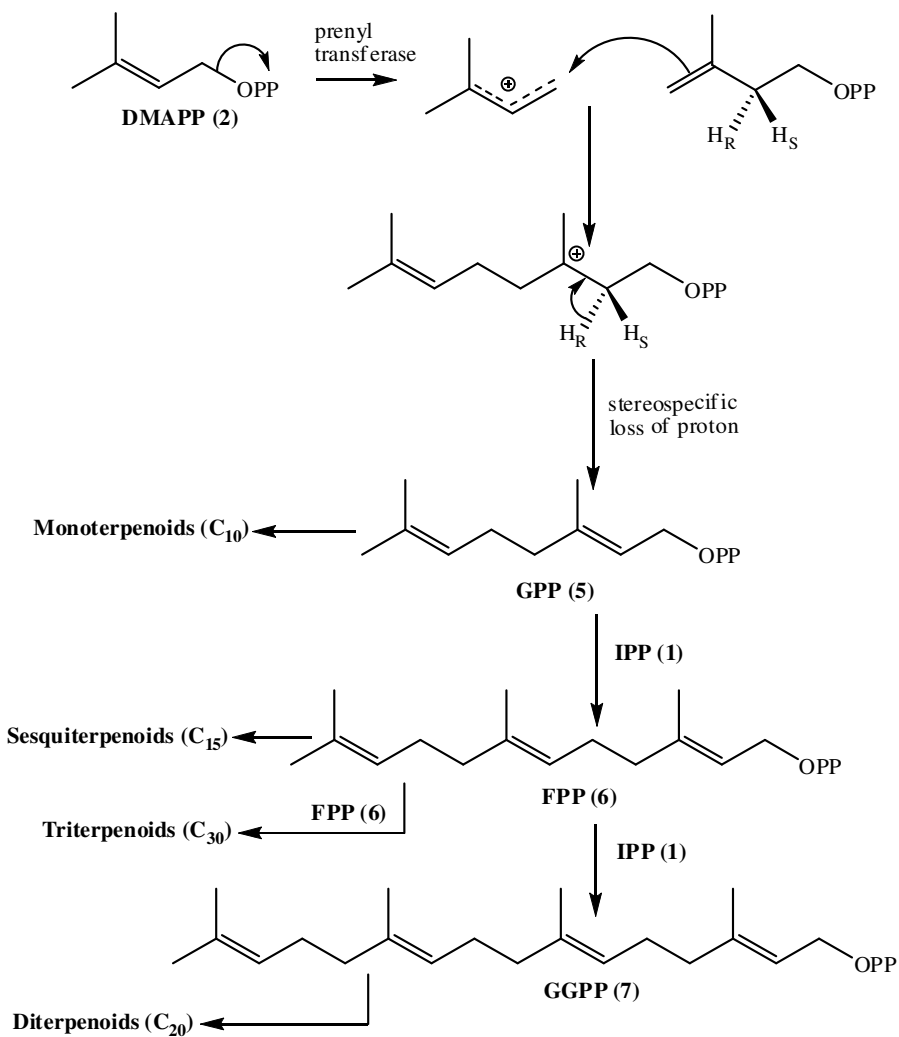


In plants, the enzymes from the mevalonate pathway are found in the cytosol, therefore triterpenoids and steroids (cytosolic products) are thought to be formed through this pathway, whereas in the deoxyxylulose phosphate (4), the enzymes are found mainly in the chloroplasts where the other terpenoids are derived (Dewick, 2006). IPP (1) isomerises to DMAPP (2) via the isomerase enzyme which stereospecifically removes the *pro-R* proton from C-2 and incorporates a proton from water onto C-4. Although the isomerisation reaction of IPP (1) to DMAPP (2) is reversible, the equilibrium favours the formation of DMAPP (2) (Scheme 1).

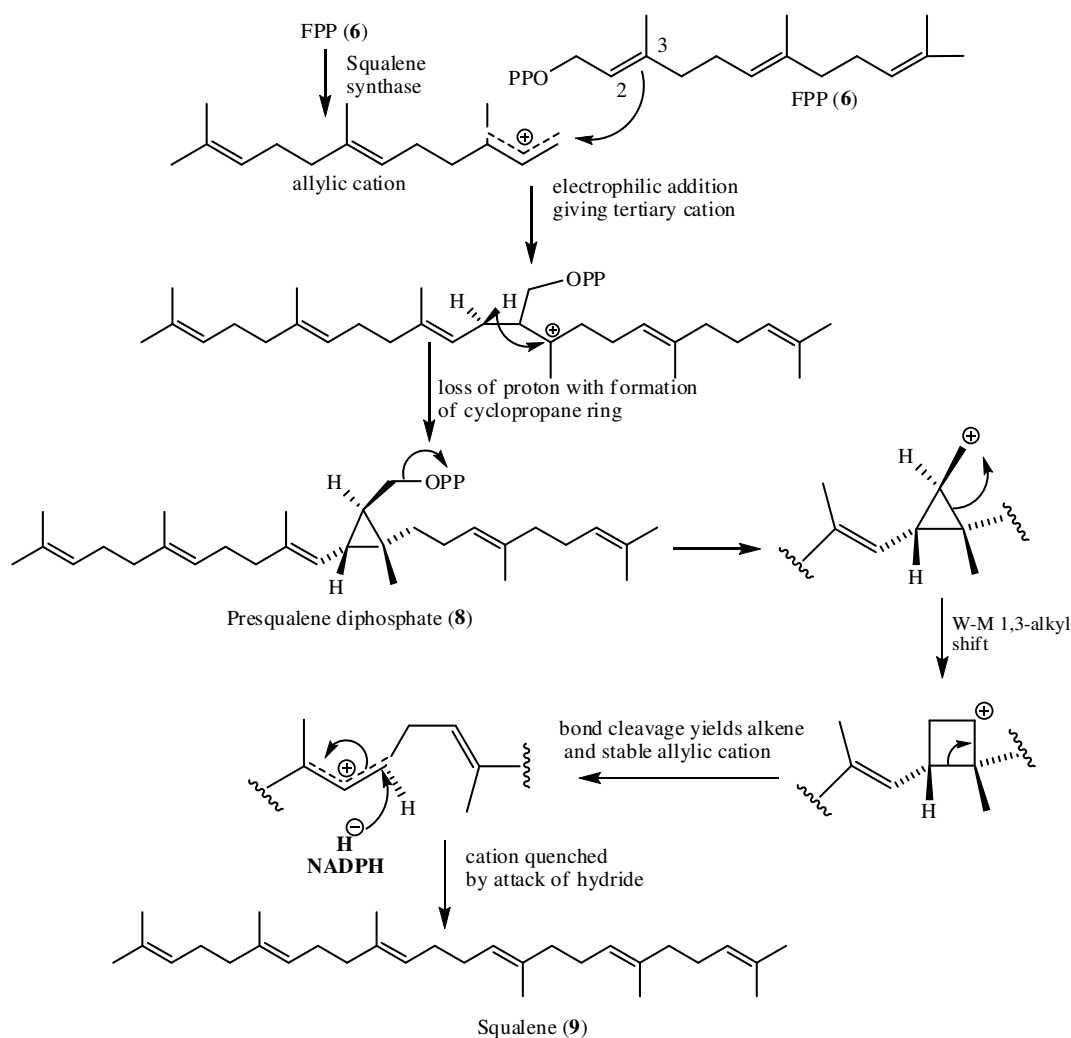
When the phosphate (a good leaving group) of DMAPP (2) leaves, it yields an allylic carbocation that is stabilised by charge delocalisation, therefore making it electrophilic. IPP (1) on the other hand is a strong nucleophile due to the terminal double bond. This differing reactivity of DMAPP (2) and IPP (1) forms the basis of terpenoid biosynthesis in the linkage of isoprene units in a head-to-tail manner (Dewick, 2006; Zulak and Bohlmann, 2010).

The combination of DMAPP (2) and IPP (1) through prenyl transferase mediation, results in the formation of geranyl diphosphate (GPP) (5), the fundamental precursor for monoterpenoid (C₁₀) compounds. This involves ionisation of DMAPP (2) to the allylic cation to which the double bond of IPP (1) adds, followed by the stereochemical loss of the *pro-R* proton. GPP (5) possesses the reactive allyl diphosphate group and the reaction mediated by prenyl transferase continues by addition of an IPP (1) unit to the geranyl cation which results in the formation of farnesyl diphosphate (FPP) (6), the sesquiterpenoid (C₁₅) and geranyl geranyl diphosphate (GGPP) (7), the diterpenoid precursor (Scheme 2).

Triterpenoids are formed by joining two FPP (**6**) units in a tail-to-tail manner to yield the hydrocarbon squalene (**9**), formed from the precursor presqualene diphosphate (**8**) by a reaction that follows a different path from the previous condensation reactions. It was difficult to formulate the mechanism for the formation of squalene (**9**) however it was resolved when presqualene diphosphate (**8**) was isolated from rat liver (Dewick, 2006). The formation of presqualene diphosphate (**8**) (Scheme 3) is by an attack of the Δ^2 double bond of one FPP (**6**) molecule on the farnesyl cation (formed by enzymatic ionisation of the second FPP (**6**) molecule). The resulting tertiary cation is discharged by loss of a proton with the formation of a cyclopropane ring to yield presqualene diphosphate (**8**). This is followed by loss of diphosphate to form an unstable primary cation that undergoes rearrangement to generate a more favourable secondary carbocation and less strained cyclobutane ring. Bond cleavage produces an allylic cation, stabilised by charge delocalisation, which is quenched by attack of a hydride ion from NADPH to form squalene (**9**), an important precursor of cyclic triterpenoids (Scheme 3).



Scheme 2: Formation of triterpenoid precursors GPP, FPP and GGPP (Dewick, 2006, Zulak and Bohlmann, 2010).

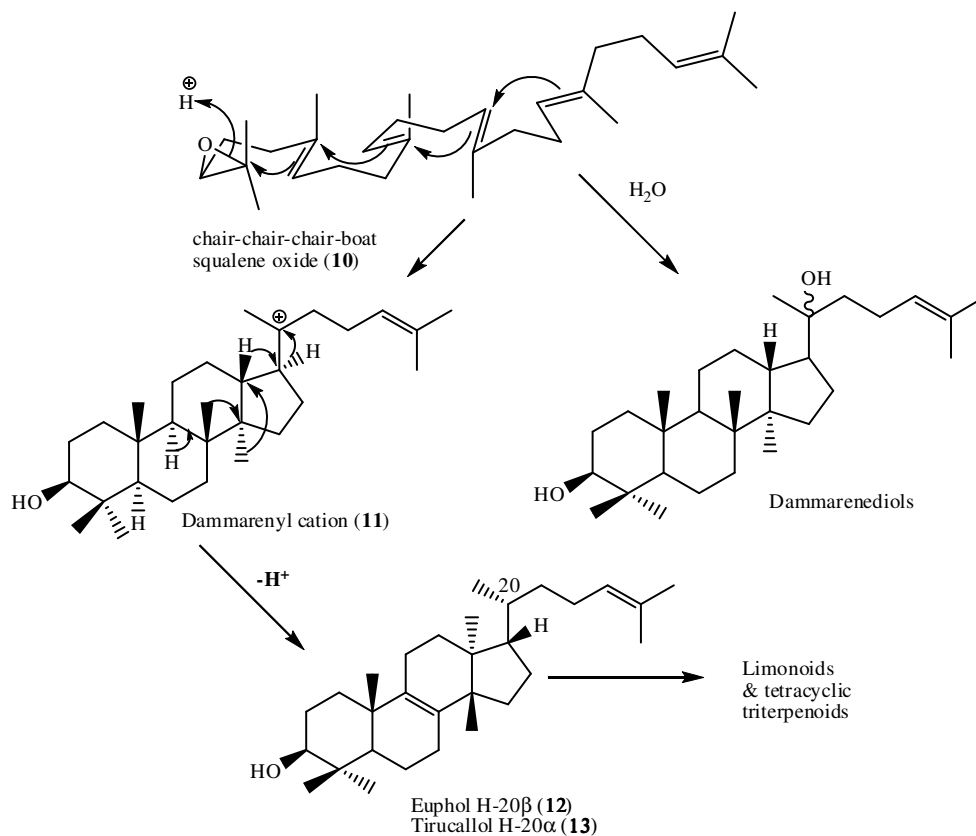


Scheme 3: Formation of squalene (Dewick, 2006)

Cyclisation of squalene (9) is possible through the intermediate squalene-2,3-oxide (10) which is produced in a reaction catalysed by a flavoprotein in the presence of O₂ and NADPH cofactors, resulting in the epoxidation of the terminal double bond in squalene (10).

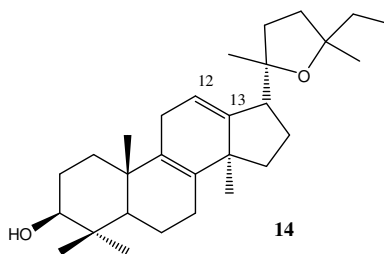
If squalene-2,3-oxide (10) is folded onto a chair-chair-chair-boat conformation on the enzyme surface, the transient dammarenyl cation (11) is produced through a series of cyclisations followed by a sequence of Wagner-Meerwein rearrangements of 1,2-hydride

and 1,2-methyl migrations to form the C-20 epimers, euphol (**12**) and tirucallol (**13**) (Scheme 4) (Dewick, 2006).



Scheme 4: Formation of limonoid and triterpenoid precursors (Dewick, 2006)

1.1.4.2 Tetracyclic triterpenoids

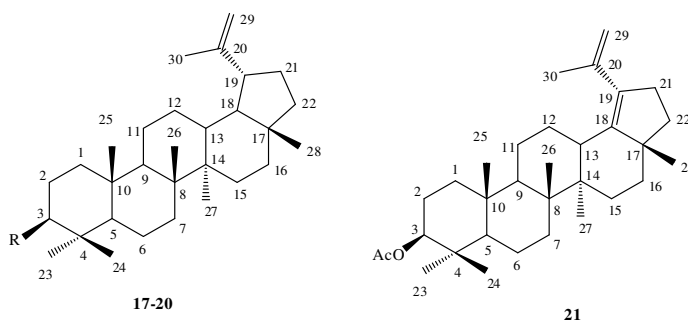


Fasciculatol (**14**) is the only tetracyclic triterpenoid from *Vernonia* being isolated in *V. fasciculata* and is formed by the oxidation and then cyclisation of the side chain of euphol

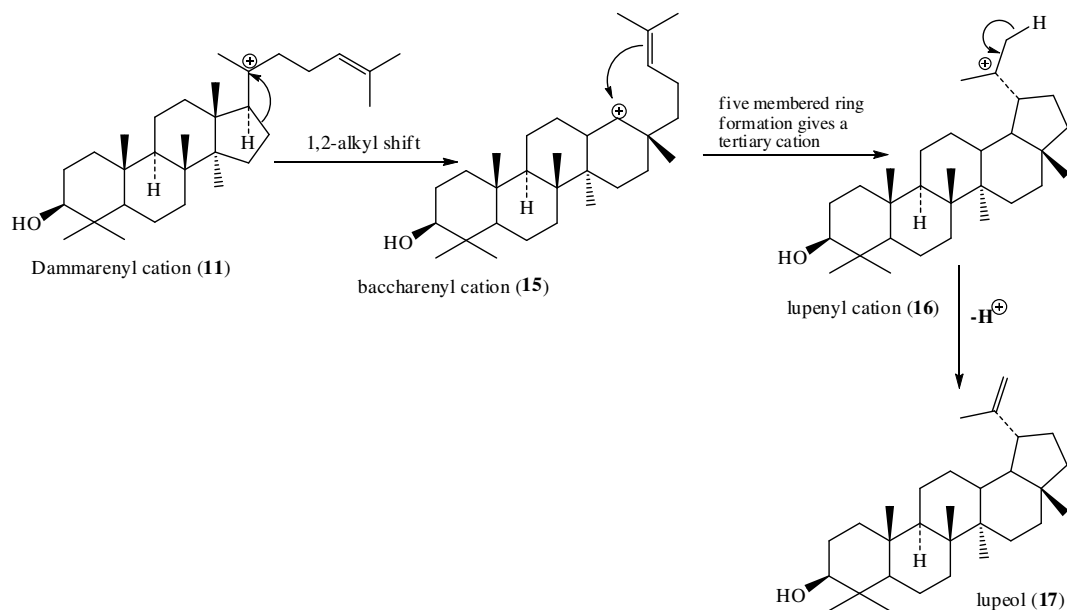
(12)/tirucalol (13). Reduction at position 12 leads to the formation of a double bond with the concomitant loss of the methyl group at position 13.

1.1.4.3 Lupane triterpenoids

The lupane triterpenoids are characterised by four six-membered rings with a fifth five-membered ring to which an isopropyl group is attached. Five lupane triterpenoids have been isolated from *Vernonia* species, all with the same basic skeleton which is formed by a 1,2-alkyl shift in the dammarenyl cation (11) resulting in the bacharenyl cation (15) followed by cyclisation onto ring D leading to a lupenyl cation (16) which ultimately leads to lupeol (17) through the loss of a proton (Scheme 5). All *Vernonia* species except for *V. cinerea*, *V. fasciculata* and *V. saligna* was found to contain lupeol (17). *V. cinerea* and *V. saligna* although not containing lupeol did contain derivatives of lupeol, lupenyl acetate (18) in *V. cinerea* and lupeol palmitate (19), a glycoside (20) and an acetate (21) in *V. saligna*.



		R
17	lupeol	OH
18	lupenyl acetate	OAc
19	lupeol palmitate	OCO(CH ₂) ₁₄ CH ₃
20	lupeol-20(29)en-3β-O-D-glucoside	O-Glu
21	lupeol-18,20(29)-diene-3β-acetate	OAc



Scheme 5: Formation of lupeol (Dewick, 2006)

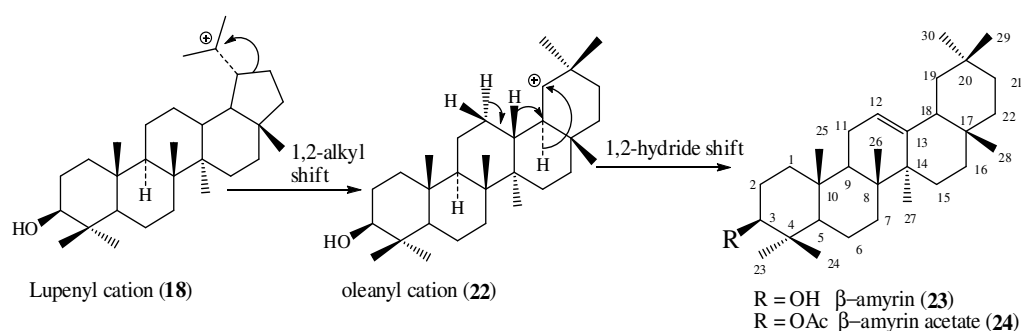
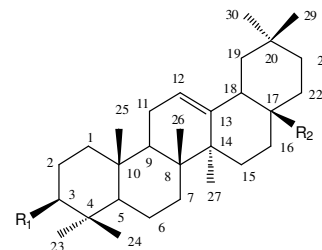
1.1.4.4 Oleanane triterpenoids

Another widely distributed triterpenoid in *Vernonia* is the oleanane triterpenoid, β -amyrin (**23**), being found in all *Vernonia* species except *V. arkansana*, *V. chunii*, *V. fasciculata* and *V. potamophilia*, however *V. chunii* did contain oleanolic acid (**27**). *V. mollissima* and *V. patula* contained β -amyrin acetate (**24**), while *V. saligna* and *V. patula* contained β -amyrin palmitate (**25**) and β -amyrin benzoate (**26**) respectively.

The oleanane triterpenoids are characterised by five six-membered rings with a double bond at Δ^{12} and two methyl groups situated on the same carbon at position 20. They are formed by ring expansion in the lupenyl cation (**16**) by a Wagner-Meerwein rearrangement (a 1,2-alkyl shift) leading to the formation of the oleanyl cation (**22**) (Scheme 6), which is then discharged by hydride migration and loss of a proton to form β -amyrin (**23**). Acetylation, aliphatic esterification and aromatic esterification has led to the other three β -amyrin

derivatives (**24-26**). Oleanolic acid (**27**) is formed through the oxidation of the methyl group at C-17.

		R ₁	R ₂
23	β-amyrin	OH	CH ₃
24	β-amyrin acetate	OAc	CH ₃
25	β-amyrin palmitate	OCO(CH ₂) ₁₄ CH ₃	CH ₃
26	β-amyrin benzoate	OCOPh	CH ₃
27	oleanolic acid	OH	COOH

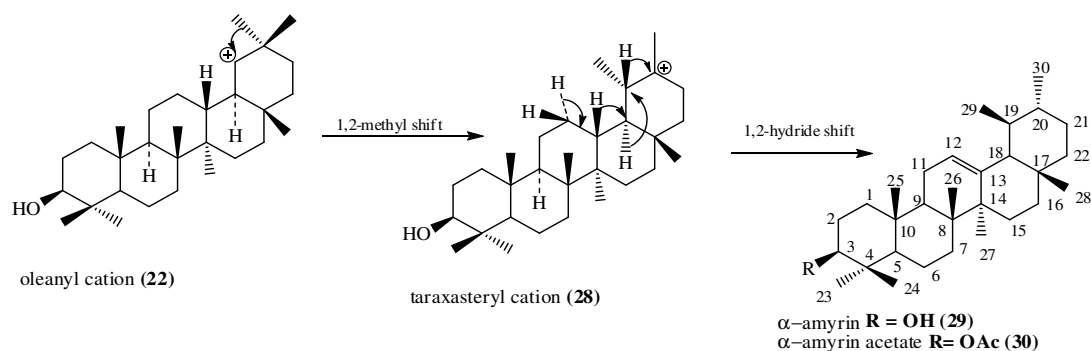


Scheme 6: Formation of oleanyl cation precursor (Dewick, 2006).

1.1.4.5 Taraxarane and Ursane triterpenoids

The difference between the oleanane triterpenoids and the taraxarane and ursane triterpenoids lies in the position of the methyl groups on ring E. In the oleananes there are two methyl groups at C-20 whereas in the taraxaranes and the ursanes, a methyl group migrates from the oleanyl cation (**22**), resulting in the methyl groups being on adjacent carbon atoms, C-19 and C-20. The ursane, α-amyrin (**29**) is the third most common triterpenoid apart from lupeol (**17**) and β-amyrin (**23**), being found in ten of the sixteen *Vernonia* species studied phytochemically. The three compounds, lupeol (**17**), β-amyrin (**23**) and α-amyrin (**29**) form a suite of compounds found in nine of the sixteen species of *Vernonia* and may be used as a chemotaxonomic marker for the genus.

In addition, α -amyrin acetate (**30**) is found in *V. patula* and together with α -amyrin (**29**) in *V. saligna*. Taraxasterol (**32**) is found together with α -amyrin (**29**) in *V. incana* and *V. cognata*, while *V. chalybaea* contains α -amyrin (**29**), pseudotaraxasteryl acetate (**31**) and taraxasteryl acetate (**33**) and *V. cinerea* contains α -amyrin (**29**), α -amyrin acetate (**30**), 24-hydroxytaraxa-14-ene (**34**), 3 β -acetoxyurs-13(18)-ene (**35**) and 3 β -acetoxyurs-19-ene (**36**). Between the taraxaranes and the ursanes, there is a difference in the position of the double bond, which is brought about by the way in which the taraxasteryl cation (**28**) is quenched by the loss of the hydrogen with concomitant hydrogen migrations. In α -amyrin (**29**) and the rest of the ursanes, the H-12 proton is lost and the double bond is formed at Δ^{12} . Acetylation of **29** leads to formation of α -amyrin acetate (**30**) (Scheme 7).

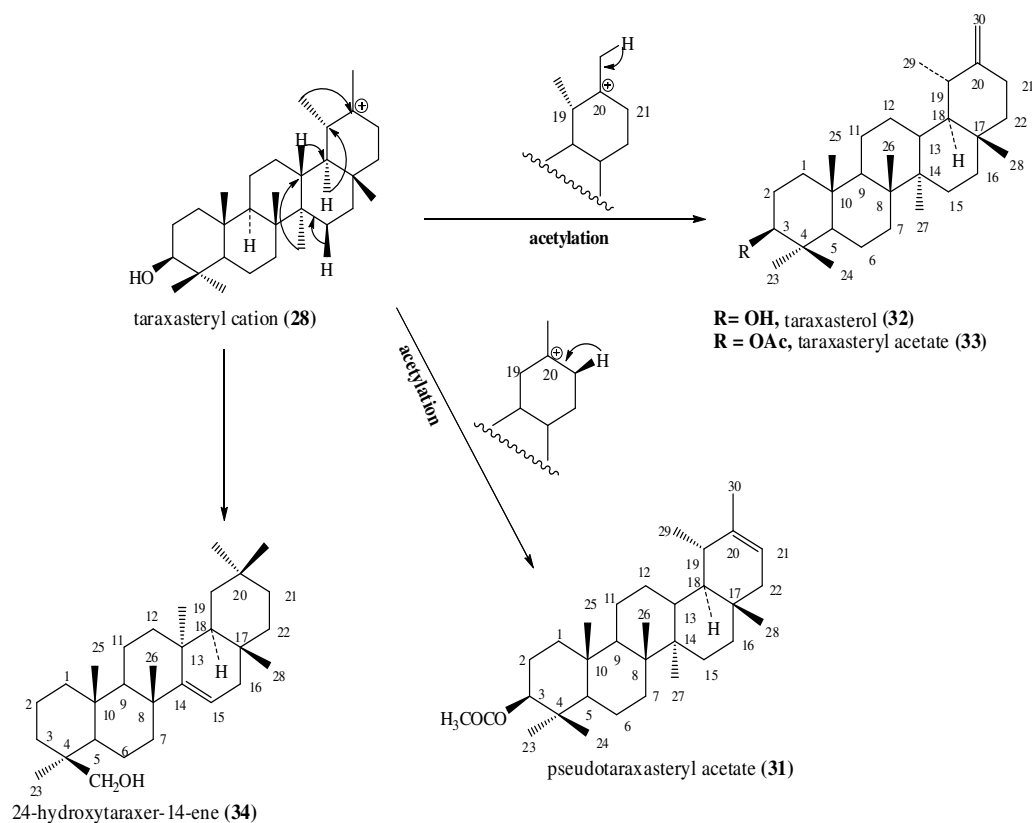


Scheme 7: Formation of taraxasteryl cation and the ursane class triterpenoids

In the taraxaranes, the proton at either C-21 or C-30 is lost, leading to a double bond at either Δ^{20} (**31**) or at 20(30) leading to taraxasterol (**32**) with acetylation of **32** resulting in **33** (Scheme 8).

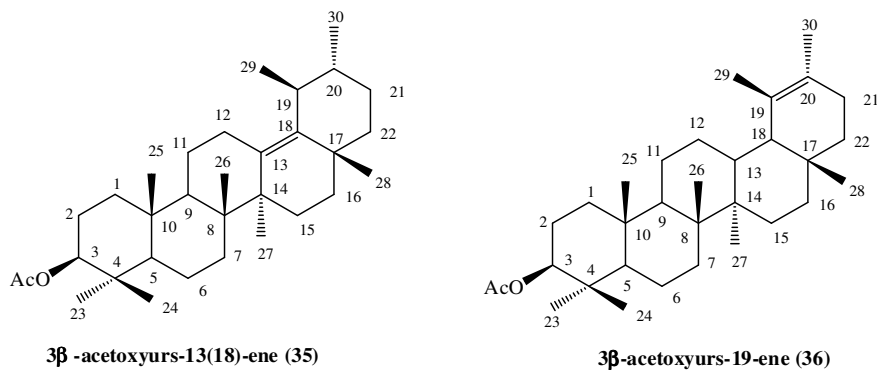
It is highly likely that the compound classified as a taraxarene (**34**) does not belong in this class and should be grouped with other compounds with a double bond at Δ^{14} resulting from

the loss of a proton at C-15 in the oleanyl cation (**22**) with hydride migrations quenching the cation (Scheme 8).



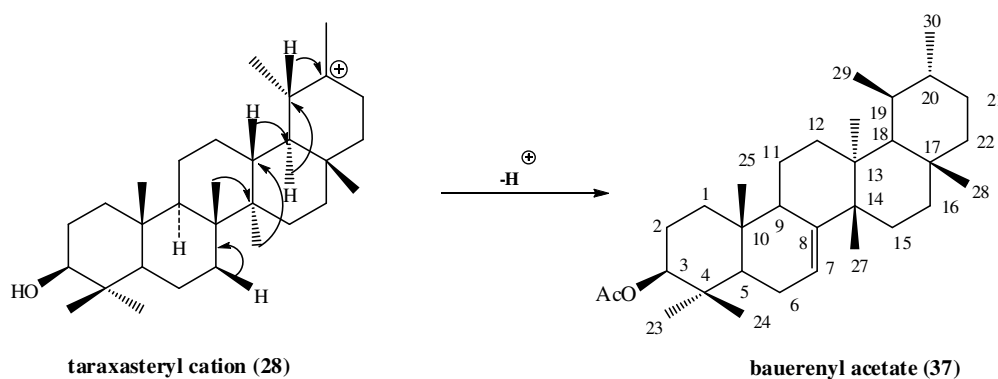
Scheme 8: Formation of taraxastane class triterpenoids from taraxasteryl cation

Compounds **35** and **36** have double bonds in positions different to the ursanes or the taraxaranes, **35** has a double bond between C-13 and C-18 and **36** has a double bond at Δ^{19} and is more likely to be associated with the taraxaranes as the loss of the proton occurs adjacent to the C-20 cation, just as that in **31**.



1.1.4.6 Friedoursane triterpenoids

Unlike the cases where the double bond at Δ^{14} and 13(18) were not given their own classification, those with a double bond at the Δ^7 position are classified as friedoursane triterpenoids as in bauerenyl acetate (**37**) isolated from *V. patula*. They come about by loss of a proton at C-7 with methyl and hydrogen shifts quenching the cation in the taraxasteryl cation (**28**) (Scheme 9).

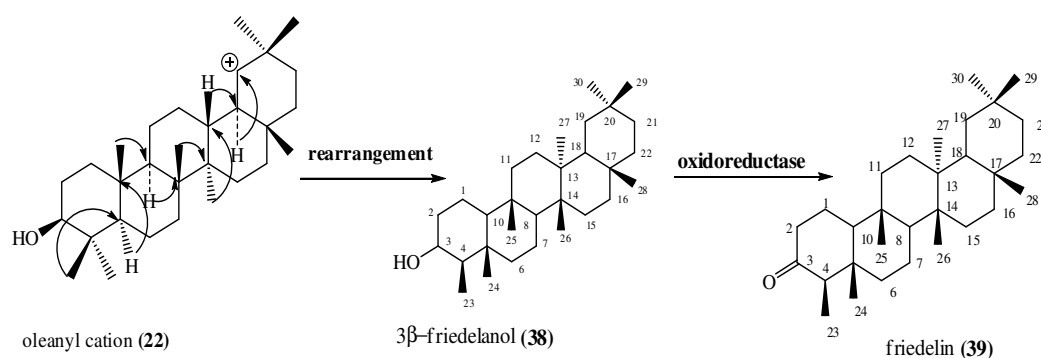


Scheme 9: Formation of bauerenyl acetate from taraxasteryl cation

1.1.4.7 The Friedelane triterpenoids

The Friedelane triterpenoids, 3β-friedelanol (**38**) and friedelin (**39**) are found in *V. patula* while friedelin (**39**) alone is found in *V. chalybaea* and *V. saligna*. The Friedelane triterpenoids are characterised by methyl groups at C-4, C-5, C-9 and C-13, in addition to

those at C-14, C-17 and C-20, which differ to the oleanane triterpenoids which have two methyl groups at C-4 and a methyl group at C-10 instead of C-9 and a proton at C-13 instead of the methyl group. This difference arises from the origin of the methyl migration being from the methyl group at C-4 resulting in a series of methyl and hydrogen migrations to quench the oleanyl cation (**22**) (Scheme 10). Friedelin (**39**) is brought about by the action of an oxidoreductase oxidising the 3 β -hydroxyl group of 3 β -friedelanol (**38**) to a ketone (Corsino *et al.*, 2000) (Scheme 10).



Scheme 10: Formation of friedelane triterpenoids from the oleanyl cation (Corsino *et al.*, 2000)

The species of *Vernonia* from which these compounds have been isolated, including the parts of the plant that contained them are given in Table 3. The table revealed that lupeol (**17**), α -amyrin (**23**) and β -amyrin (**29**) were common to several species. *V. paltula* had the highest number of triterpenoids followed by *V. saligna* and *V. chalybaea*. *V. fasciculata* and *V. potamophilia* each had only one triterpenoid from the tetracyclic and lupane class respectively.

Table 3: Triterpenoids contained in the different *Vernonia* species

Species	Triterpenoids isolated	References
<i>V. arkansana</i>	17 ^r , 18 ^r	Bohlmann <i>et al.</i> , 1981
<i>V. brasiliiana</i>	17 ^l , 23 ^l	Alves <i>et al.</i> , 1997
<i>V. chamaedryis</i>	17 ^{fl,s} , 23 ^{fl,s} , 29 ^{fl,s}	Catalán <i>et al.</i> , 1988
<i>V. chalybaea</i>	17 ^a , 18 ^a , 23 ^a , 29 ^a , 31 ^a , 33 ^a , 39 ^a	da Costa <i>et al.</i> , 2008
<i>V. chunii</i>	17 ^{ns} , 27 ^{ns}	Yuan <i>et al.</i> , 2008
<i>V. cinerea</i>	18 ^r , 23 ^r , 29 ^r , 30 ^r , 34 ^r , 35 ^r , 36 ^r	Misra <i>et al.</i> , 1984a, 1984b, 1993
<i>V. cognata</i>	17 ^{fr,l} , 23 ^{fr,l} , 29 ^{fr,l} , 32 ^{fr,l}	Bardon <i>et al.</i> , 1988b
<i>V. fasciculata</i>	14 ^l	Narain, 1979
<i>V. incana</i>	17 ^{l,fl} , 23 ^{l,fl} , 29 ^{l,fl} , 32 ^{l,fl}	Bardón <i>et al.</i> , 1990
<i>V. mollissima</i>	17 ^a , 23 ^a , 24 ^a , 29 ^a	Catalán and Iglesias, 1986
<i>V. nitidula</i>	17 ^{fl,l} , 23 ^{fl,l} , 29 ^{fl,l}	Bardon <i>et al.</i> , 1988a
<i>V. patula</i>	17 ^{ns} , 18 ^{ns} , 19 ^a , 23 ^{ns} , 24 ^{ns} , 26 ^{ns} , 30 ^a , 37 ^{wp} , 38 ^{wp} , 39 ^{wp}	Liang and Min, 2003; Liang <i>et al.</i> , 2010
<i>V. potamophilia</i>	17 ^l	Babady-Bila <i>et al.</i> , 2003
<i>V. saligna</i>	19 ^{wp} , 20 ^{wp} , 21 ^{wp} , 23 ^{wp} , 25 ^{wp} , 29 ^{wp} , 30 ^{wp} , 39 ^{wp}	Huang and Liu, 2004
<i>V. squamulosa</i>	17 ^a , 23 ^a , 29 ^a	Catalán and Iglesias, 1986
<i>V. tweediana</i>	17 ^l , 23 ^l , 29 ^l	Zanon <i>et al.</i> , 2008

Key: superscripts, a = aerial parts, fl = flowers, fr = fruits, l = leaves, ns = not specified, r = roots, s = stems, wp = whole plant

1.2 Introduction to the genus *Vepris*

1.2.1 Phylogeny

The genus *Vepris* is a member of the Rutaceae family which consists of one hundred and sixty genera. In the scheme proposed by Engler in 1931 and adapted by Schotz in 1964, this genus was put together with the genera *Acronychia*, *Araliopsis*, *Casimiroa*, *Halfordia*, *Hortia*, *Oriciopsis*, *Sargentia*, *Skimmia* and *Toddalia*, as individual sub-tribes of the tribe Toddalioliineae, subfamily Toddaloioideae (Fernandes *et al.*, 1988). A more accepted

taxonomic grouping of the Rutaceae, which included the genera *Acronychia*, *Araliopsis*, *Diphasia*, *Oricia*, *Oriciopsis*, *Vepris* and *Teclea* within the tribe *Aronychia* was proposed in 1983 by Waterman and Grundon (Fernandes *et al.*, 1988). *Vepris* species are widely distributed all over the world and their morphology is diverse. They contain a wide range of secondary metabolites, which include limonoids, flavonoids, coumarins, volatile oils and alkaloids (Groppo *et al.*, 2008).

This genus is comprised of eighty species of trees and shrubs, occurring primarily in tropical Africa, and the Mascarene Islands, and to a lesser extent in tropical Arabia and Southwest India where one species is found (Chaturvedula *et al.*, 2003). African *Vepris* species are found in Cameroon, Democratic Republic of Congo, Ethiopia, Ghana, Kenya, Madagascar, Mauritius, Mozambique, Rwanda, South Africa, Swaziland, Tanzania, Zambia, Zanzibar and Zimbabwe (Waterman, 1986). About sixteen species are endemic to Kenya and thirty are found in Madagascar (Louppe *et al.*, 2008).

Vepris species are plants without prickles and the bark is pale to dark grey and fairly smooth. The leaves are unusually glossy green, large, alternate, fairly smooth and drooping; when crushed they give an aromatic scent. The flowers have short auxiliary heads and are greenish; the fruits are drupaceous, smooth and usually contain 2-4 seeds (Cheek *et al.*, 2009).

1.2.2. Ethnobotanical use of *Vepris* species

Of the eighty *Vepris* species, only a few have documented use in herbal medicine. Species of this genus are mainly used in the treatment of respiratory infections, dermatological infections, as analgesics, antipyretics and for oral health. The two species used for oral health, maintains oral cleanliness by contributing to antimicrobial activity and inhibiting

plaque and also by dislodging cariogenic microorganisms when the plant is chewed. *Vepris* species are also reported to have anti-inflammatory and antimalarial properties (Table 4).

Table 4 Traditional uses of plants belonging to the genus *Vepris*

Plant species	Plant part	Traditional use	Reference (s)
<i>V. ampody</i>	leaves and bark	antimalarial, muscular aches and analgesic	Randrianarivelojosia <i>et al.</i> , 2003
<i>V. ellioti</i>	leaves	aphrodisiac	Poitou <i>et al.</i> , 1995
<i>V. eugenifolia</i>	bark	liver protection, respiratory diseases and kidney disorders	Fratkin, 1996; Hedberg <i>et al.</i> , 1983
<i>V. glomerata</i>	roots	eye infection and antimalarial	Chhabra <i>et al.</i> , 1991
<i>V. heterophylla</i>	leaves	diuretic and antipyretic	Gomes <i>et al.</i> , 1983
<i>V. lanceolata</i>	leaves and stems	gynaecological diseases, heart disease, astringent, burns, eye problem, colic, anti-diarrhoeic, antiviral, antibacterial, antipyretic, analgesic, fortifier, antirheumatic, gout, stimulant, muscular disorder and dermatological infection	Arnold and Gulumian 1984; Gurib-Fakim <i>et al.</i> , 1996; Louppe <i>et al.</i> , 2008; Poullain <i>et al.</i> , 2004; Vera <i>et al.</i> , 1990
	leaves and roots	antimalarial	Gessler <i>et al.</i> , 1994, 1995a, 1995b
	roots	heart diseases, antiviral, analgesic, respiratory infections and gynaecological complications	Arnold and Gulumian 1984; Steenkamp, 2003
<i>V. louisii</i>	stem bark	dermatological diseases and antifungal	Ayafor <i>et al.</i> , 1982a
<i>V. nobilis</i>	leaves	antipyretic	Louppe <i>et al.</i> , 2008
	leaf and root	respiratory diseases antirheumatic and itching	
	root	anthelmintic	
	stem	respiratory disorders	
	bark and leaves	analgesic	
	root and twigs	dental care	
<i>V. paniculata</i>	leaves	respiratory infections	Gurib-Fakim <i>et al.</i> , 1996
<i>V. simplifolia</i>	bark	chest complaint	Louppe <i>et al.</i> , 2008
	root	muscular problems,	

		antibacterial, venereal diseases and dermatological infections	
	leaf	respiratory infection and dermatological infections	
	fruits	dental caries	
	twigs	dental care	

1.2.3. Biological activity of extracts from *Vepris* species

The biological activities of the extracts from various *Vepris* species have not been widely investigated and reported. The few reports are diverse (Table 5) with antimalarial activity being the most studied. Antimalarial activity is reported in the ethanol and chloroform extracts of six species and also in the essential oil of *Vepris elliotii*. Antibacterial activity is reported in two species, *Vepris lanceolata* and *Vepris laendriana*, antifungal activity in *V. laendriana* and *Vepris heterophylla* and antioxidant activity in *V. heterophylla*, and *V. lanceolata*. Cytotoxic activity was reported in *Vepris punctata* to be mild and mainly against human colon and ovarian cancer (Chaturvedula *et al.*, 2003).

Of the *Vepris* species, *V. lanceolata* has been studied the most; its leaf and root bark extracts were found to possess antiplasmodial activity whilst the aqueous leaf and stem extracts were found to have antibacterial and antifungal properties.

Table 5 Biological activities of extracts from *Vepris* species

Plant species	Biological activity	Extract	Reference
<i>V. ampody</i>	antimalarial	ethanol ^s /chloroform ^s	Rasoanaivo <i>et al.</i> , 1999
<i>V. elliotii</i>	antiplasmodial	essential oil ^l	Ratsimbason <i>et al.</i> , 2009
<i>V. fitoravina</i>	antimalarial	ethanol ^s /chloroform ^s	Rasoanaivo <i>et al.</i> , 1999
<i>V. glomerata</i>	antimalarial	aqueous ^l and ethanolic ^l	Innocent <i>et al.</i> , 2009
<i>V. heterophylla</i>	antifungal and antioxidant	methanol ^{lt} and essential oil ^l	Momeni <i>et al.</i> , 2010; Aoudou <i>et al.</i> , 2010
<i>V. lanceolata</i>	antibacterial	hexane ^s , methanol:chloroform(1:1) ^s , methanol:chloroform (1:1) ^l and methanol ^l	Narod <i>et al.</i> , 2004
	antibacterial, antifungal and antimalarial	ns	Gessler <i>et al.</i> , 1994, 1995
	antioxidant	dichloromethane ^s	Poullain <i>et al.</i> , 2004
<i>V. leandriana</i>	antibacterial and antifungal	essential oil ^l	Rakotondraibe <i>et al.</i> , 2001
<i>V. macrophylla</i>	antimalarial	ethanol/chloroform ^s	Rasoanaivo <i>et al.</i> , 1999
<i>V. punctata</i>	cytotoxic	n-hexane/chloroform ^w	Chaturvedula <i>et al.</i> , 2003

Key: ns = not specified, l = leaves, s = stem, w = wood, t = twigs

1.2.4. A phytochemical review of *Vepris* species

Sixteen species of *Vepris* have been studied for their phytochemical properties thus far (Scifinder, 2009). Although furoquinoline alkaloids are the most common isolates, acridone alkaloids, quinol-2-one alkaloids, indoloquinazoline alkaloids, flavonoids, cinnamic acid derivatives, limonoids and terpenoids have also been found.

The species of *Vepris* from which these compounds were isolated, including the parts of the plant that contained them are given in Table 6, with their structures given in the following subchapters.

Table 6: Compounds isolated from *Vepris* species

Species	Compounds isolated	References
<i>V. ampody</i>	45 ^{l&br} , 46 ^{l&br} , 47 ^{l&br} , 49 ^{l&br} , 54 ^{l&br} , 75 ^{sb} , 76 ^{sb} , 77 ^{sb} , 82 ^{l&br} , 87 ^{l&br} , 109 ^{l&br} , 110 ^{l&br}	Kan-Fan <i>et al.</i> , 1970; Rasoanaivo <i>et al.</i> , 1999
<i>V. bilocularis</i>	48 ^{sb} , 49 ^{sb} , 54 ^{ns} , 55 ^{sb} , 65 ^l , 63 ^l , 66 ^l , 67 ^l , 78 ^{sb} , 79 ^{sb} , 80 ^l , 81 ^l , 83 ^l , 84 ^{sb} , 89 ^{ns}	Govindachari and Sundararajan, 1961, 1964; Ganguly <i>et al.</i> , 1966; Brader <i>et al.</i> , 1996
<i>V. dainellii</i>	48 ^l , 49 ^l	Dagne <i>et al.</i> , 1988
<i>V. fitoravina</i>	54 ^l , 79 ^l , 83 ^l , 85 ^l , 86 ^l	Koffi <i>et al.</i> , 1987
<i>V. glomerata</i>	48 ^l , 59 ^l	Dagne <i>et al.</i> , 1988
<i>V. heterophylla</i>	49 ^l , 60 ^l , 61 ^l , 93 ^l , 103 ^l -108 ^l	Gomes <i>et al.</i> , 1983, 1994
<i>V. lanceolata</i>	58 ^l , 59 ^l , 61 ^l , 62 ^l , 95 ^{sb,w} , 97 ^{sb,w} , 99 ^{sb,w} , 100 ^{sb,w} , 101 ^{sb,w} , 102 ^{sb,w}	Mbala, 2005
<i>V. louisii</i>	17 ^{sb} , 43 ^{sb} , 44 ^{tb} , 49 ^{tb} , 52 ^{tb} , 64 ^{sb} , 71 ^{ns} , 72 ^{ns} , 73 ^{ns} , 74 ^{ns} , 88 ^{tb} , 113 ^{tb} , 114 ^{tb}	Ayafor <i>et al.</i> , 1980, 1981, 1982a, 1982b, 1982c; Ngadjui <i>et al.</i> , 1982
<i>V. macrophylla</i>	54 ^l , 79 ^l , 83 ^l , 85 ^l , 86 ^l	Koffi <i>et al.</i> , 1987
<i>V. pilosa</i>	49 ^r , 79 ^r , 80 ^r , 88 ^r	Haensel and Cybulski, 1978
<i>V. punctata</i>	17 ^w , 18 ^w , 29 ^w , 32 ^w , 48 ^w , 49 ^w , 53 ^w , 54 ^w , 55 ^w , 56 ^w , 57 ^w , 115 ^w , 116 ^w , 117 ^w	Chaturvedula <i>et al.</i> , 2003, 2004
<i>V. reflexa</i>	17 ^{sb w} , 49 ^{sb&w} , 55 ^{sb&w} , 94 ^{sb,w} , 96 ^{sb,w} , 98 ^{sb,w}	Mbala, 2005
<i>V. stolzii</i>	17 ^{sb} , 48 ^{sb} , 50 ^{sb} , 64 ^{sb} , 68 ^{sb} , 69 ^{sb} , 70 ^{sb}	Khalid and waterman, 1982
<i>V. uguenensis</i>	51 ^r , 55 ^r , 90 ^r , 96 ^r , 111 ^r , 112 ^r	Cheplogoi <i>et al.</i> , 2008

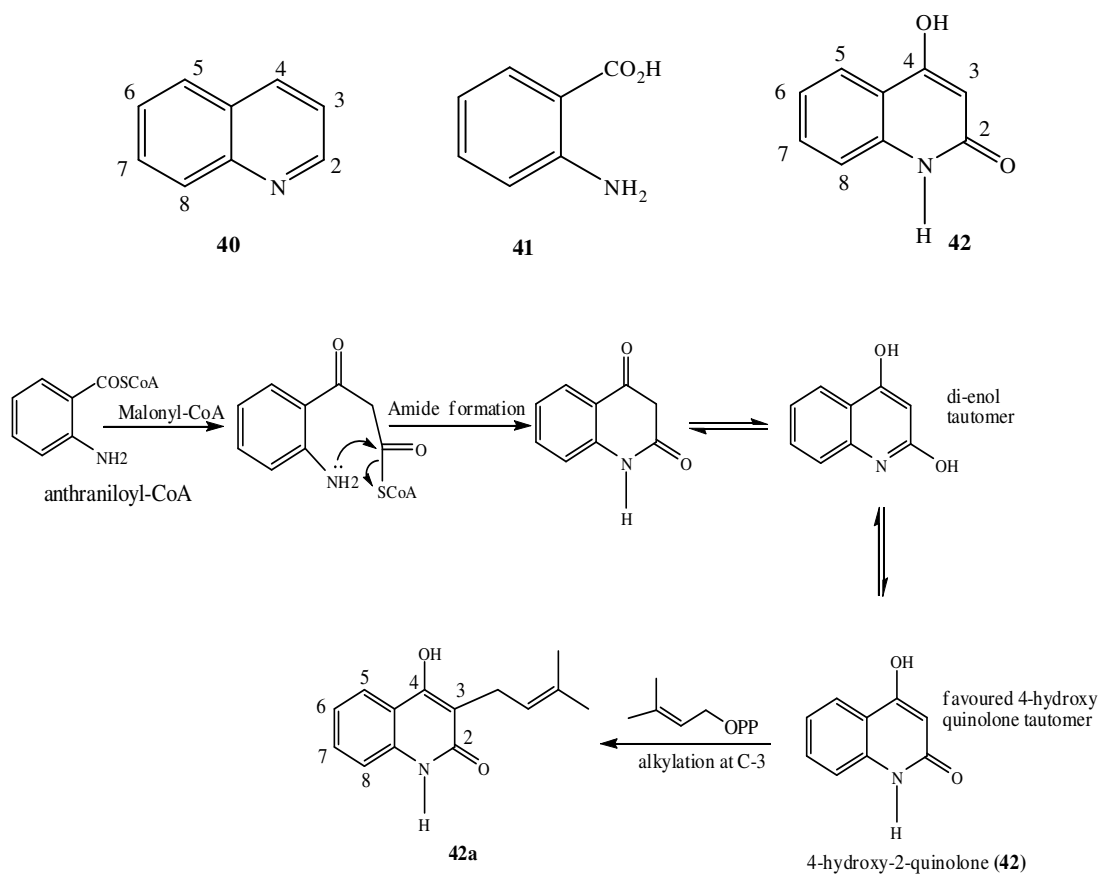
Key: superscripts, a = aerial parts, br = branches, fl = flowers, fr = fruits, l = leaves, r = roots,

s = stems, sb = stem bark, tb = tree bark, w = wood, wp = whole plant,

1.2.4.1. Quinoline alkaloids

The quinoline alkaloids are found in several *Vepris* species (Table 6), with two species, *dainellii* and *glomerata* exclusively containing compounds of this class. Quinoline alkaloids are nitrogenous compounds based on the benzo[b]pyridine or 1-azanaphthalene (40) skeleton. They occur abundantly in the family Rutaceae and have been used in the chemotaxonomic classification of many species in the family. These compounds are biosynthesized from anthranilic acid (41).

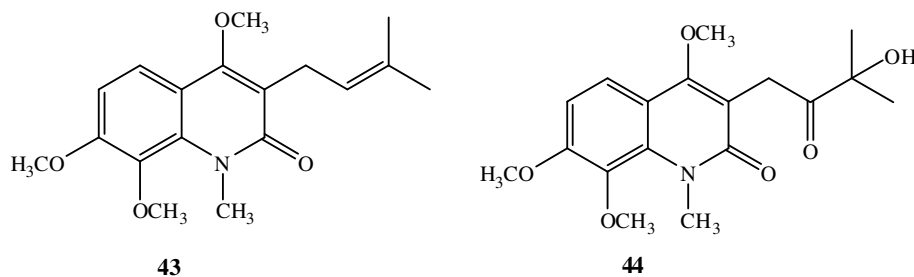
The quinoline ring is formed by the condensation of anthranilic acid (**41**) and malonyl-CoA followed by cyclisation *via* intramolecular amide formation, which results in a heterocyclic system with the more stable 4-hydroxy-2-quinolone (**42**) form. The other form, the di-enol is highly nucleophilic at position 3 and susceptible to alkylation *via* DMAPP (**2**) (Scheme 11).



Scheme 11: Biosynthesis of 2-quinolone alkaloids (Dewick, 2006)

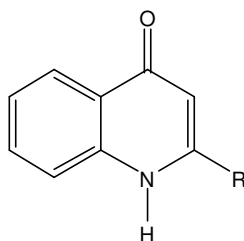
The 2-Quinolone alkaloids

N-methylpreskimmianine (**43**) and veprisilone (**44**) have been found in the stem and trunk bark of *Vepris louisii*. Both these compounds have arisen from the intermediate **42** being methylated both at the secondary amine and the hydroxy group at C-4 as well as being prenylated at C-3. Veprisilone (**44**) is further oxidised in the prenyl ring.

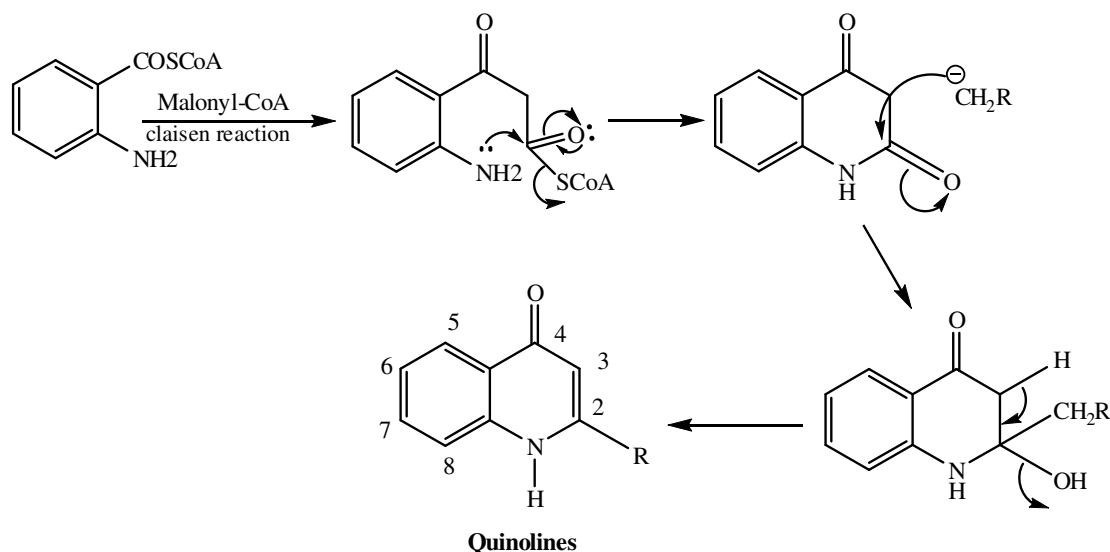


The 4-Quinolone alkaloids

The 4-quinolones **45-47** have been isolated from *Vepris ampody* and differ from the 2-quinolones by the position of the carbonyl which is at C-4. These compounds arise from carbon nucleophiles attacking the C-2 carbonyl group in the quinolodione system, followed by dehydration at C-2 and C-3 resulting in a Δ^2 double bond with alkyl substituents at C-2 (Scheme 12).



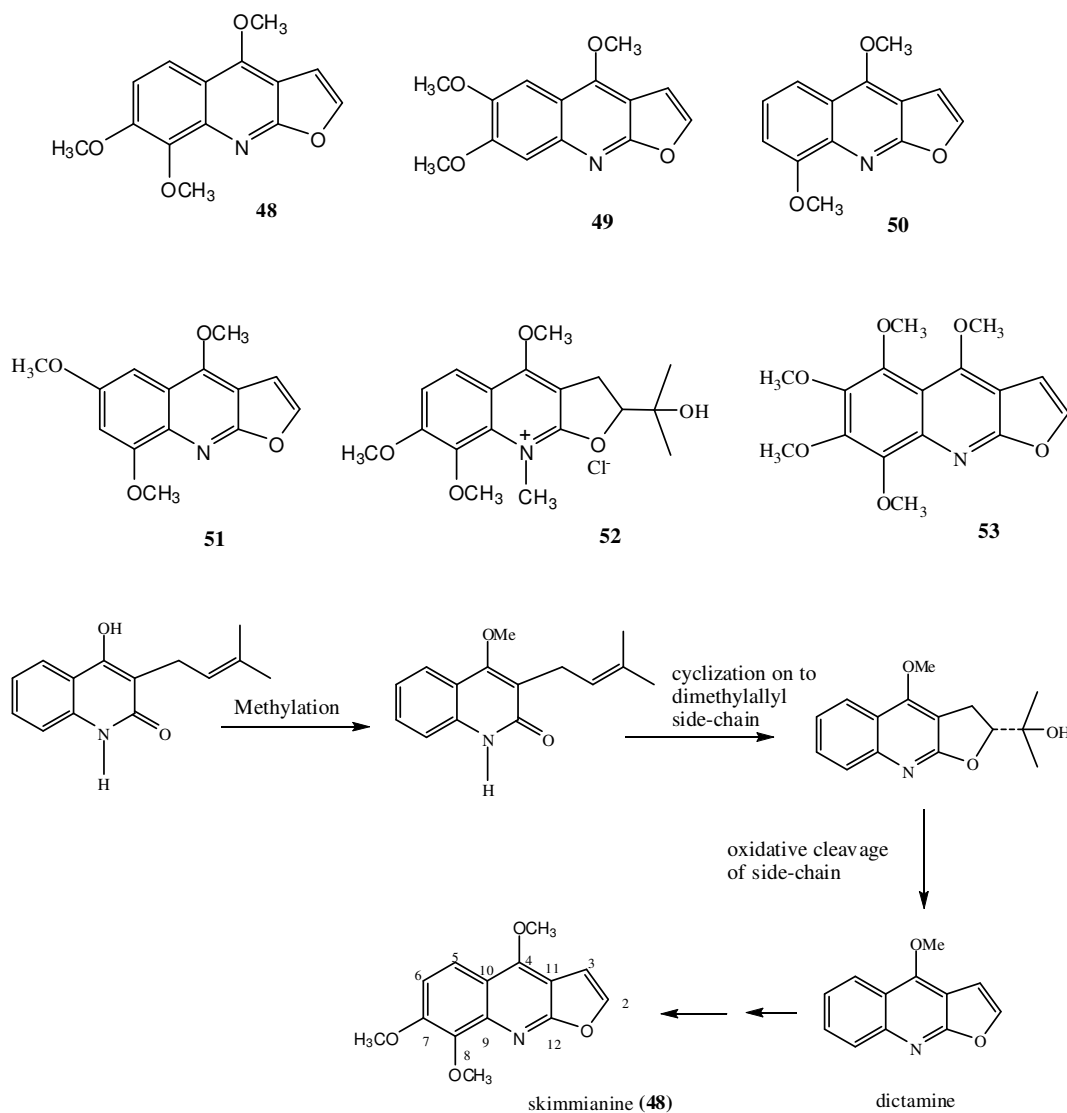
		R
45	2-(Nona-3',6'-diene)-4-quinolone	$(\text{CH}_2)_2\text{CH}=\text{CHCH}_2\text{CH}=\text{CHCH}_2\text{CH}_3$
46	2-(Nona-9'-ol)-4-quinolone	$(\text{CH}_2)_8\text{CH}_2\text{OH}$
47	2-(Undeca-10'-one)-4-quinolone	$(\text{CH}_2)_9\text{COCH}_3$



Scheme 12: Formation of simple 4-quinoline alkaloids (Dewick, 2006).

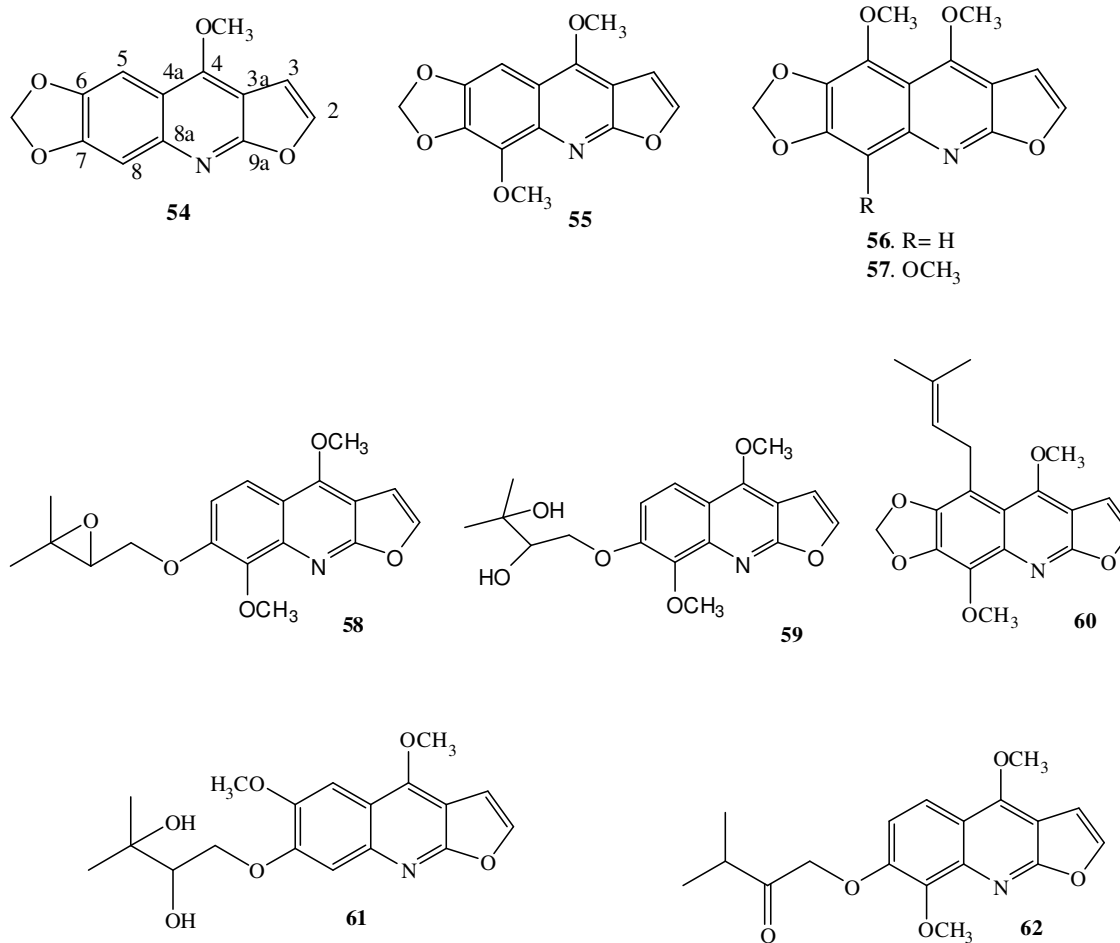
Furanoquinoline alkaloids

This class of alkaloids possesses three rings; a benzopyridine group consisting of two rings to which a furan ring, a third ring, is attached. They are formed from **42** through prenylation, cyclisation and then side chain cleavage of the three carbon fragment (Scheme 13). Various hydroxylations and methylations to dictamine (Scheme 13), the simplest known furoquinoline alkaloid, results in several substituted furoquinoline alkaloids. Aromatic hydroxylation followed by alkylation leads to skimmianine (**48**) found in five *Vepris* species, *bilocularis*, *dainellii*, *glomerata*, *punctata* and *stolzii* (Table 6). Kokusaginine (**49**) is found in the *Vepris* species of *ampody*, *bilocularis*, *dainellii*, *heterophylla*, *louisii*, *pilosa*, *punctata* and *reflexa*, making it the most common furoquinoline alkaloid, being found in eight species. *Vepris stolzii* contains γ -fagarine (**50**) and maculosidine (**51**) was isolated from *Vepris uguenensis*. *V. louisii* contains veprisinium hydrochloride (**52**) and 4,5,6,7,8-pentamethoxymaculine (**53**) is found in *Vepris punctata*.



Scheme 13: Biosynthesis of furanoquinoline alkaloids (Dewick, 2006)

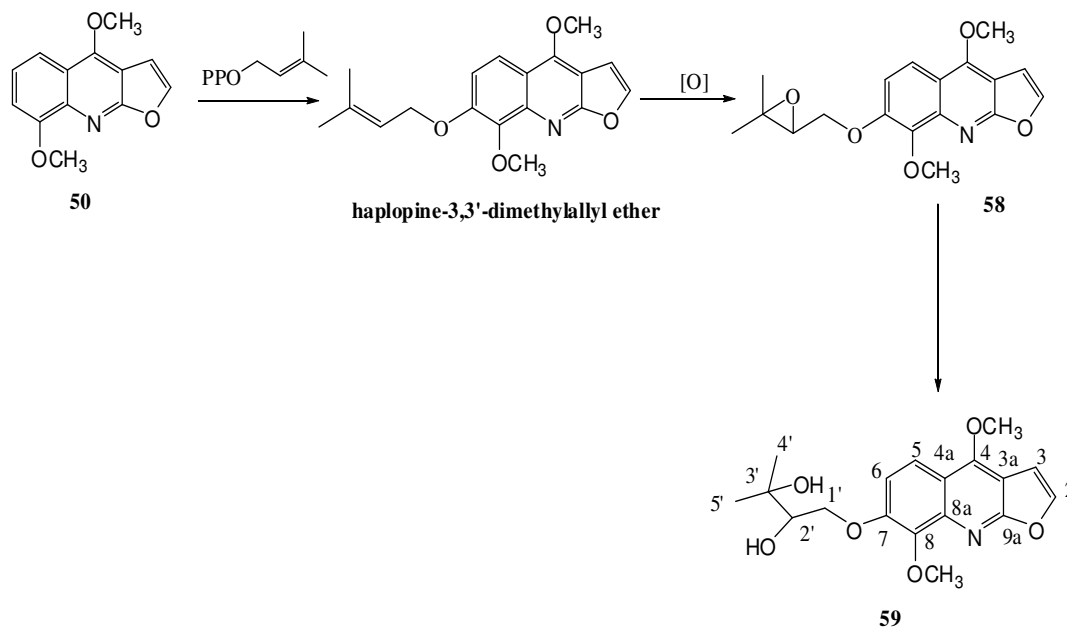
The methoxylated methylenedioxy furanoquinolines, maculine (**54**) and flindersiamine (**55**) are found together in *V. bilocularis* and *V. punctata* with only one of them, **55** being found in *V. reflexa* and *V. uguenensis*. In addition, 5-methoxymaculine (**56**) and 5,8-dimethoxymaculine (**57**), methoxylated at the 5-position as well as the 4- and 8-positions are contained along with **54** and **55** in *V. punctata*. The introduction of a methylenedioxy group at C-6 and C-7 usually arises by cyclisation when a methoxy and hydroxy group are present on adjacent carbon atoms.



The oxidised C-7 *O*-prenylated anhydroevoxine (**58**) and evoxine (**59**) is found in *V. lanceolata* with **59** also being present in *V. glomerata*. These compounds are the epoxidised and dihydroxylated forms of haplopine-3,3'-dimethylallyl ether (not isolated previously from *Vepris* species), which probably arises from the *O*-prenylation at C-7 in γ -fagarine (**50**) (Scheme 14).

V. heterophylla contains a C-5 prenylated furanoquinoline, tecleaverdoornine (**60**), containing a methylenedioxy group at C-6 and C-7. Evolantine (**61**), an isomer of evoxine (**59**), with a methoxy group at C-7 instead of C-5 has been isolated along with **60** in *V.*

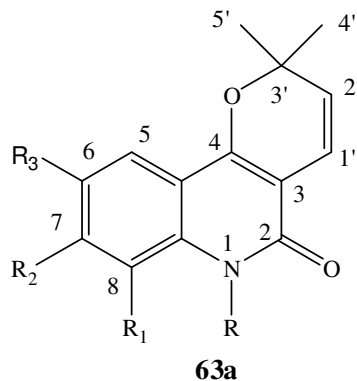
heterophylla. Anhydroevoxine (**58**), evoxine (**59**), evolantine (**61**) and evoxidine (**62**), all isolated in *V. lanceolata*, suggest that all these compounds are related biosynthetically.



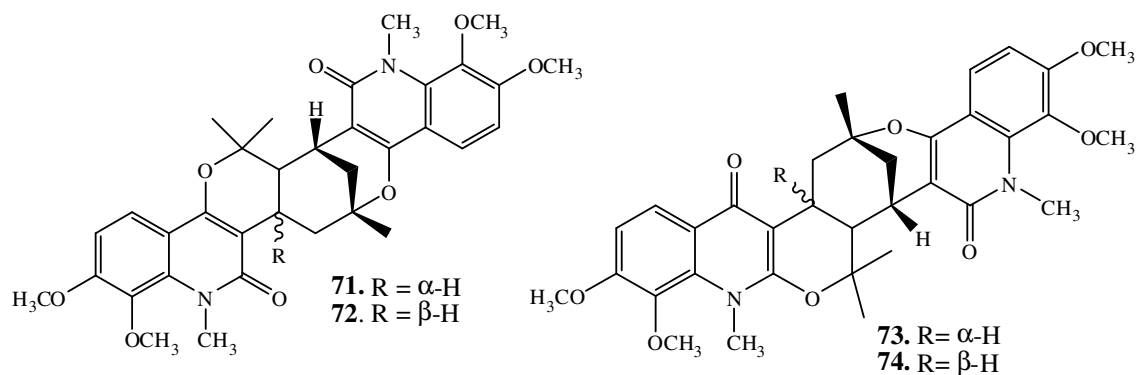
Scheme 14: Biosynthetic pathway to the prenylfuranoquinoline alkaloids (Grundon, 1988)

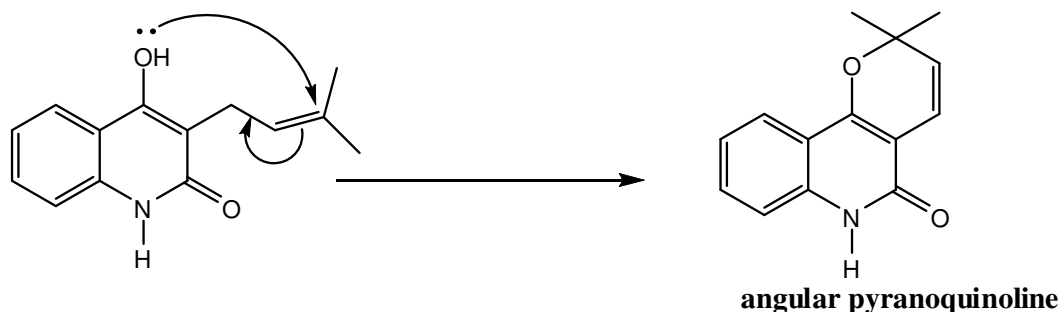
Pyranoquinoline alkaloids

The pyranoquinoline alkaloids **63-74**, all derivatives of flindersine (**63a**) are found in three of the *Vepris* species, *bilocularis*, *louisii* and *stolzii*. They are formed by cyclisation of the prenyl side-chain of 4-hydroxy-2-quinolone (**42a**) (Scheme 15) to form an additional angular pyran ring on the quinoline skeleton. The various pyranoquinoline alkaloid derivatives arise as a result of either methylations or prenylations at the nitrogen, C-7 and C-8. The dimers vepridimerine A-D **71-74** were found only in *V. louisii*.



		R	R₁	R₂	R₃
63	6-methoxyflindersine	H	H	H	OCH ₃
64	veprisine	CH ₃	OCH ₃	OCH ₃	H
65	7-methoxyflindersine	H	H	OCH ₃	H
66	7-prenyloxyflindersine	H	H	OCH ₂ CH=C(CH ₃) ₂	H
67	7-prenyloxy <i>N</i> -methylflindersine	CH ₃	CH ₃	OCH ₂ CH=C(CH ₃) ₂	H
68	<i>N</i> -methyl-8-(3',3'-dimethylallyloxy)flindersine	CH ₃	OCH ₂ CHC(CH ₃) ₂	H	H
69	<i>N</i> -methyl-7-methoxy-8-(3',3'-dimethylallyloxy)flindersine	CH ₃	OCH ₂ CHC(CH ₃) ₂	OCH ₃	H
70	<i>N</i> -methyl-7-methoxy-8-(2',3'-epoxy-3',3'-dimethylallyloxy)flindersine	CH ₃	OCH ₂ CH(O)C(CH ₃) ₂	OCH ₃	H



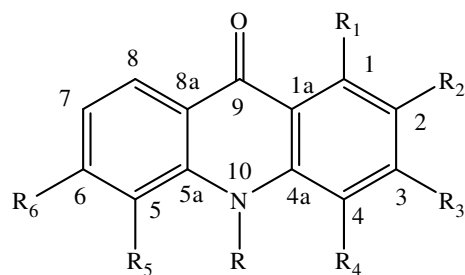


Scheme 15: Formation of angular pyranoquinoline alkaloids (Grundon, 1988)

1.2.4.2. Acridone alkaloids

There are five species of *Vepris* that contain acridone alkaloids, *ampody*, *bilocularis*, *fitoravina*, *macrophylla* and *pilosa* (Table 6), with *fitoravina* and *macrophylla* containing exclusively the same suite of acridone alkaloids, 1,3-dimethoxy-*N*-methylacridone (**79**), arborinine (**83**), 1,3-dimethoxy-10-methylacridan-9-one (**85**) and 1-hydroxy-2,3,4-trimethoxyacridan-9-one (**86**) (Koffi *et al.*, 1987). Together with the furanoquinoline and pyranquinoline alkaloids, they make the quinoline alkaloids one of the taxonomic markers of the genus *Vepris*.

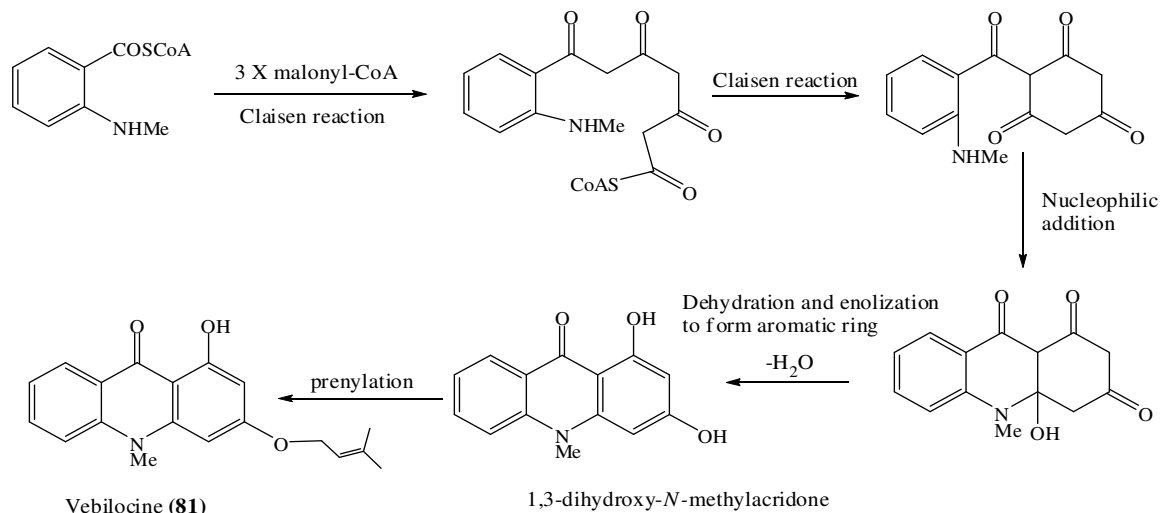
Acridone alkaloids contain a tricyclic ring having nitrogen at position 10 and a carbonyl group at C-9. They are formed by the addition of three malonyl units to anthranilyl-CoA (*N*-methyl derivative of anthranilic acid) and sequential Claisen reactions, followed by heterocyclic ring formation by nucleophilic addition of the secondary amine on the carbonyl group followed by dehydration and enolisation to form the heterocyclic and the second aromatic ring (Scheme 16). They differ from the quinolone alkaloids in that two extra malonyl units are incorporated into the skeletal backbone, resulting in a further aromatic ring. Once again the acetate-derived ring is susceptible to electrophilic attack leading to alkylation (with DMAPP (**1**)) or further aromatic hydroxylation (Scheme 16).



		R	R₁	R₂	R₃	R₄	R₅	R₆
75	melicopicine	CH ₃	OCH ₃	OCH ₃	OCH ₃	OCH ₃	H	H
76	tecleanthine	CH ₃	OCH ₃	OCH ₂ O		H	OCH ₃	H
77	6-methoxytecleanthine	CH ₃	OCH ₃	OCH ₂ O		H	OCH ₃	OCH ₃
78	evoxanthine	CH ₃	OCH ₃	OCH ₂ O		H	H	H
79	1,3-dimethoxy- <i>N</i> -methylacridone	CH ₃	OCH ₃	H	OCH ₃	H	H	H
80	1,2,3- trimethoxy- <i>N</i> -methylacridone	CH ₃	CH ₃	OCH ₃	OCH ₃	H	H	H
82	1-hydroxy-3-methoxy-10-methylacridone	CH ₃	OH	H	H	H	H	H
83	arborinine	CH ₃	OH	OCH ₃	OCH ₃	H	H	H
84	1-hydroxy-2,3, dimethoxyacridan-9-one	H	OH	OCH ₃	OCH ₃	H	H	H
85	1,3-dimethoxy-10-methylacridan-9-one	CH ₃	OCH ₃	H	H	H	H	H
86	1-hydroxy-2,3,4-trimethoxyacridan-9-one	H	OH	OCH ₃	OCH ₃	H	H	H
87	2,4-dimethoxy-10-methylacridan-9-one	CH ₃	H	OCH ₃	OCH ₃	H	H	H

The acridone alkaloids in *Vepris* are mainly hydroxylated and methoxylated at various positions on both the aromatic rings except for C-7 and C-8. Only two of the alkaloids, tecleanthine (**76**) and its 6-methoxy derivative (**77**) were functionalised on the anthranilyl

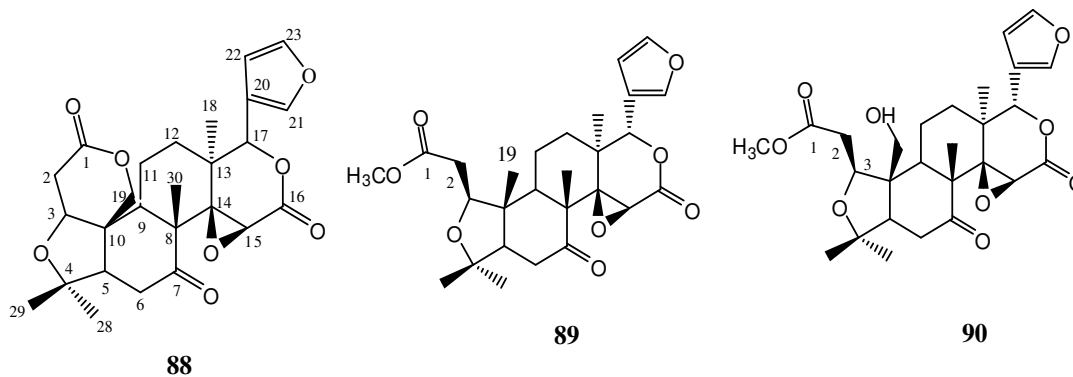
derived ring. The methylenedioxy group between C-2 and C-3 is also a common occurrence and can be seen in **76** and **77** as well as in evoxanthine (**78**).



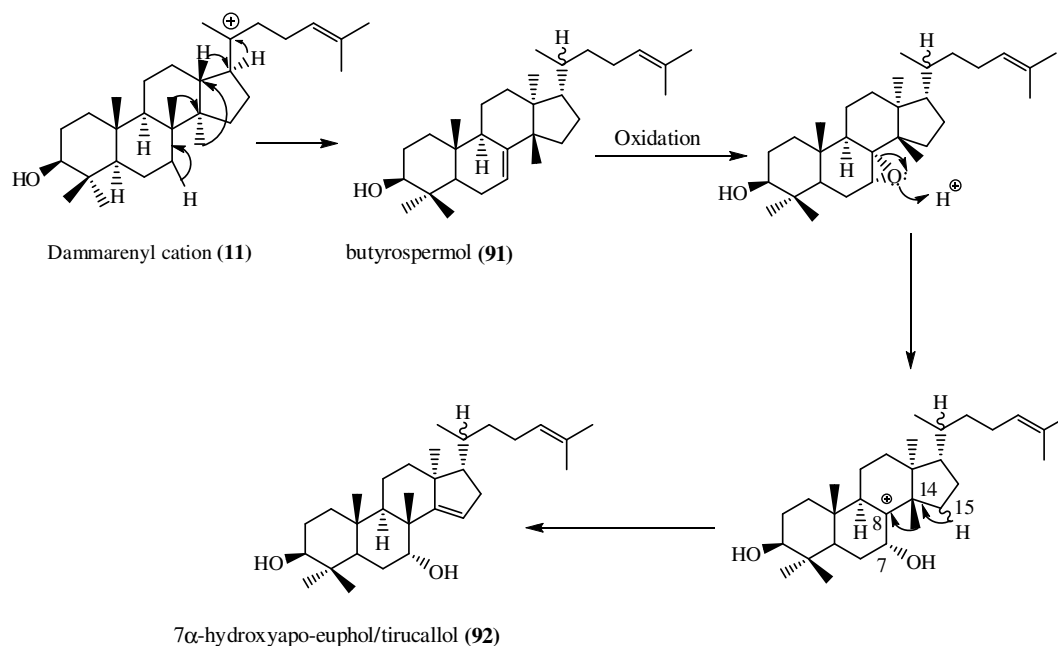
Scheme 16: Formation of acridone alkaloids

1.2.4.3. Limonoids

Three limonoids of the limonin type skeleton, including limonin (**88**) itself have been isolated in three separate *Vepris* species. Limonin (**88**) was isolated from *V. lousii* and *V. pilosa*, veprisone (**89**) from *V. bilocularis* and methyl uguenenoate (**90**) from *V. uguenensis*. This could be the third chemotaxonomic marker for the genus *Vepris*, the quinolone and acridone alkaloids being the first two. Thus, *Vepris* species such as *bilocularis* and *lousii* contain all three classes of compounds. Phytochemists therefore need to keep a careful eye on all three classes of compounds when studying the phytochemical constituents of *Vepris*.

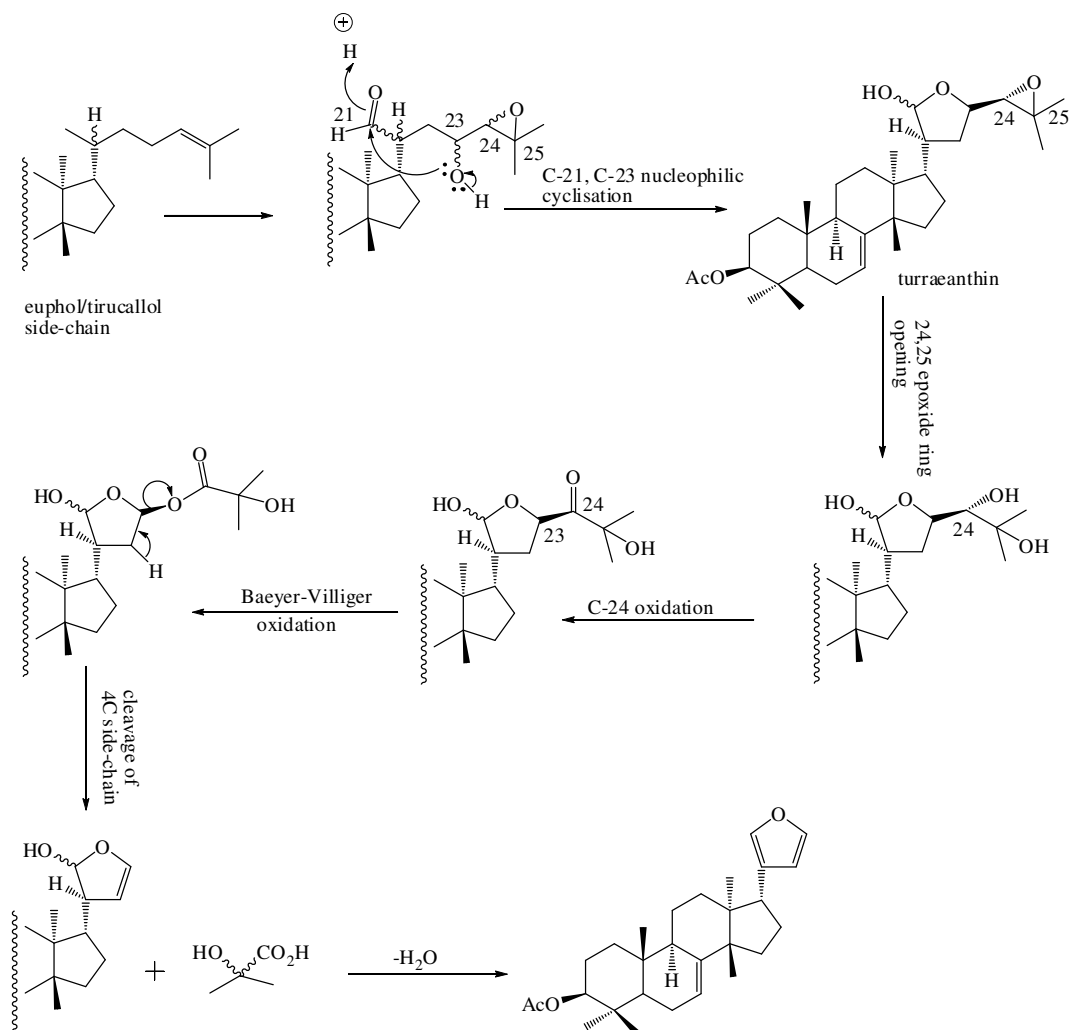


The three *Vepris* limonoids possess the limonin skeleton, which from the 7α -hydroxyapoeuphol precursor have modified rings A and D as well as side chain modification to a furan ring. The triterpene precursor, butyrospermol (**91**) (Scheme 17) is derived directly from the dammarenyl cation (**11**) (from Scheme 4) by the loss of a proton from C-7, catalysing a series of methyl and hydrogen migrations. Since all three compounds isolated previously have a ketone at C-7, the key step in their biosynthesis is the epoxidation of the Δ^7 double bond followed by ring opening with subsequent loss of the H-15 proton and concurrent Wagner-Meerwein shift of the methyl group to C-8, retaining the methyl group in the β -position. Subsequent oxidation to the ketone at C-7 and to the epoxide at Δ^{14} results in the two functional groups present in **88-90**.



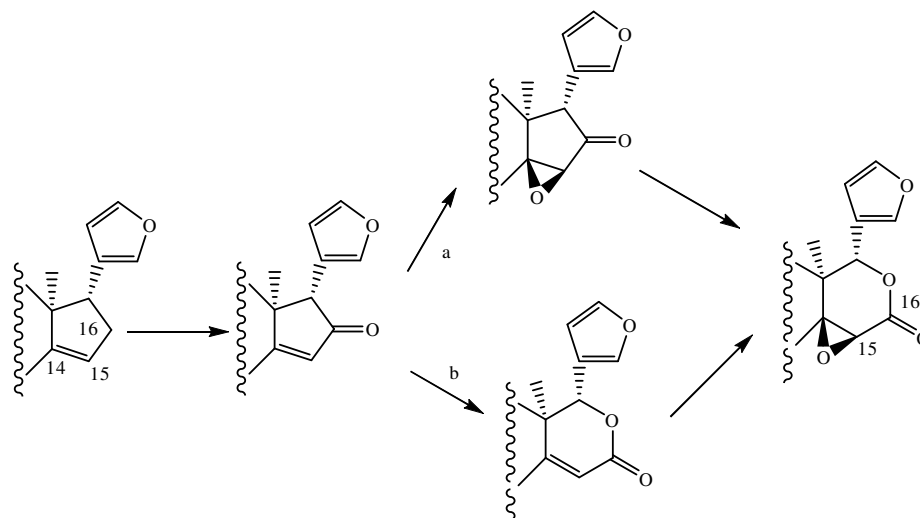
Scheme 17: Formation of 7 α -hydroxy-euphol/tirucallol *via* apo rearrangement

The furan ring is biosynthesized through stepwise oxidation of the protolimonoid's side-chain to produce a hydroxyl group at C-23, a 24,25-epoxy group, and an aldehyde at C-21, followed by nucleophilic cyclisations from the oxygen at C-23 to the carbonyl group at C-21 to form the hemiacetal ring. Opening of the C-24,25 epoxide ring followed by oxidation produces a ketone at C-24, which then undergoes Baeyer-Villiger oxidative cleavage of the C-23,24 bond to give a dihydrofuran ring, which finally forms the furan ring, with the loss of four carbon atoms (Scheme 18).



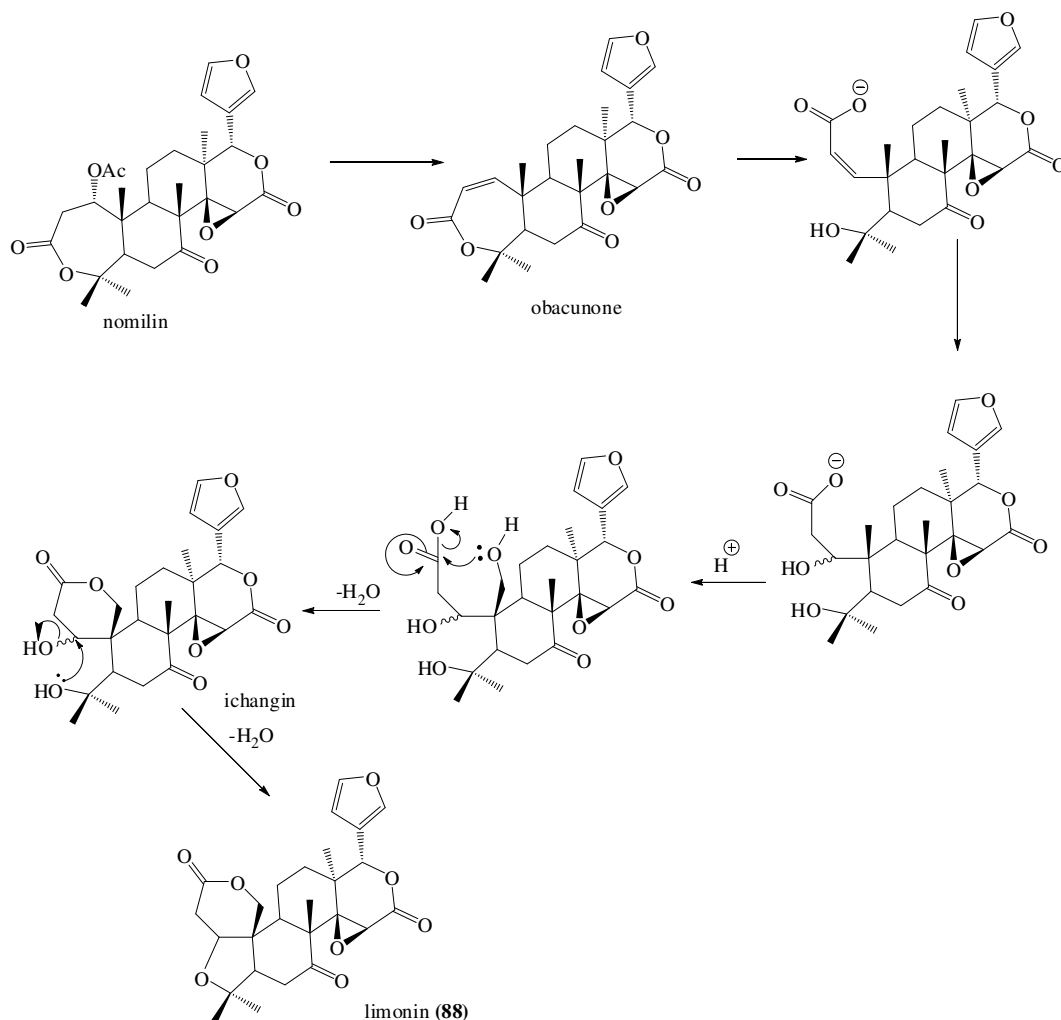
Scheme 18: Furan ring formation via side-chain oxidation (Bevan *et al.*, 1967)

In the expansion of the D ring, the key step is the allylic oxidation at C-16 of the D ring containing the Δ^{14} -double bond to form an α, β unsaturated ketone. This is followed by Baeyer-Villiger ring expansion to the lactone, which can occur either before or after the epoxidation of the Δ^{14} double bond (Taylor, 1984) (Scheme 19).



Scheme 19: Ring D lactone formation (Taylor, 1984).

Ring A is contracted to a five membered ring followed by lactonization which involves the methyl group (CH₃-19) and the carbonyl group at C-3 leading to the formation of A and A' rings (A-seco) in a sequence of events starting from the deacetylation of nomilin (Scheme 20). Methyl uguenenoate (**90**) is a derivative of limonin (**88**) in which the A' lactone ring has undergone cleavage resulting in the hydroxyl-acid, which is followed by methylation.

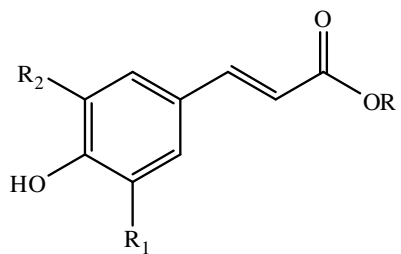


Scheme 20: Biosynthesis of limonin (Adesogan and Taylor 1969)

1.2.4.5. Cinnamic acid derivatives and simple aromatics

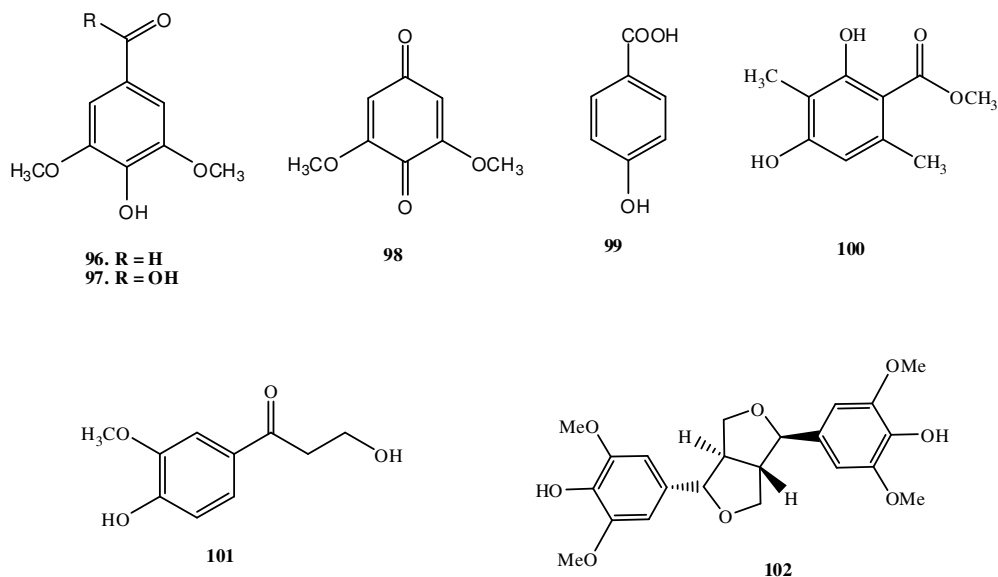
Three cinnamic acid derivatives (**93-95**) and six simple aromatic compounds (**96-102**) were isolated from two species, *V. lanceolata* and *V. reflexa*. Cinnamic acids are plant derived fragrances and flavourings having a common cinnamoyl functionality, which is present in a variety of secondary metabolites of phenyl propanoid biosynthetic origin (De *et al.*, 2011). Chemically, cinnamic acids are aromatic fatty acids composed of an aromatic ring substituted with an acrylic acid group commonly in the more favoured *trans* geometry. The acrylic acid group is a α, β -unsaturated carbonyl group which can be considered as a

Michael acceptor; an active moiety which is often used in the design of anticancer drugs (Ahn *et al.*, 1996). The main precursor in their biosynthesis is *L*-Phenylalanine which undergoes deamination to form *trans*-cinnamic acid. Further hydroxylation and methylation reactions result in the large variety of known cinnamic acids from plant sources. The cinnamic acids also undergo esterification leading to cinnamic acid esters such as **94** and **95**.



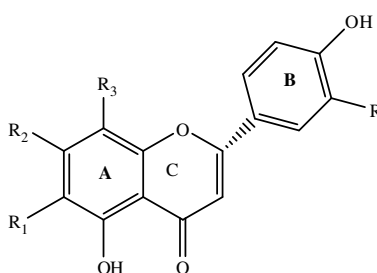
		R	R₁	R₂
93	<i>trans</i> -sinapic acid methyl ester	CH ₃	CH ₃	CH ₃
94	alkyl <i>trans</i> -4-hydroxycinnamate	OCH ₂ (CH ₂) _n CH ₃	H	H
95	alkyl <i>trans</i> -4-hydroxy-3-methoxycinnamate	OCH ₂ (CH ₂) _n CH ₃	H	OCH ₃

Apart from the cinnamic acids, several simple aromatic compounds, including syringaldehyde (**96**) and syringic acid (**97**), the precursors to lignins as well as the aromatic acid, para hydroxybenzoic acid (**99**), a preservative, an ester, methyl 2,4-dihydroxy-3,6-dimethylbenzoate (**100**) and ketone, 3-hydroxy-1-(4-hydroxyphenyl)-1-propanone (**101**) as well as the quinone, 2,6-dimethoxy-1,4-benzoquinone (**98**) were isolated. The isolation of syringaldehyde (**96**) from *V. reflexa* and syringic acid (**97**) from *V. lanceolata* is consistent with the isolation of *meso*-syringaresinol (**102**) from *V. lanceolata*, since both the syringaldehyde and syringic acid are precursors to the lignins.

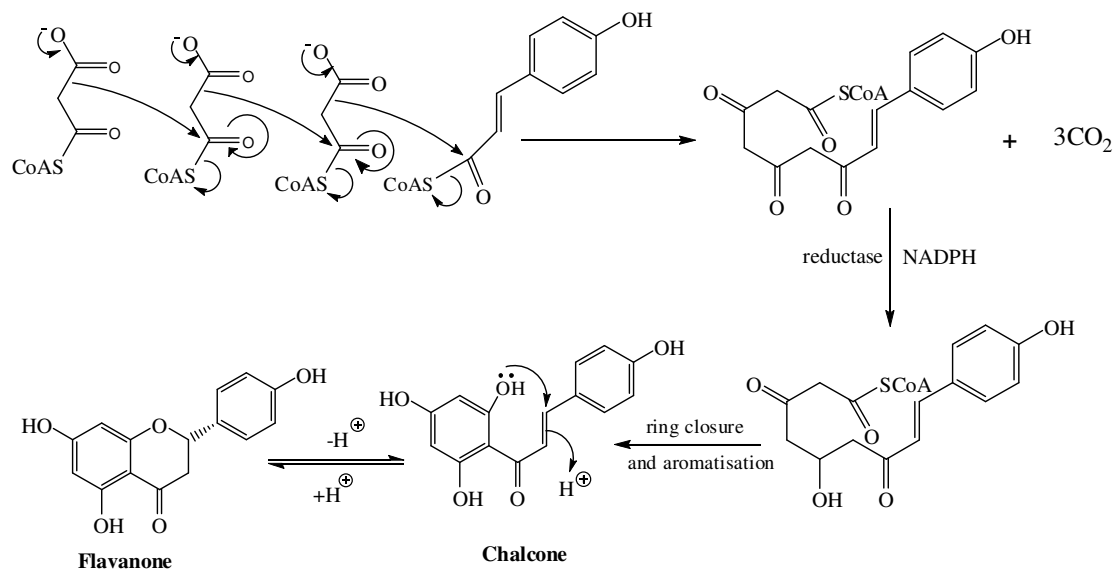


1.2.4.6. Flavonoids

Surprisingly, only *V. heterophylla* was reported to contain flavonoids, which were all glycosides with the sugar moiety on the A ring, **103-108** (Gomes *et al.*, 1983). Flavonoids are derived from three malonyl CoA units which condense with each other and the cinnamoyl CoA unit derived from cinnamic acid which is reduced and ring closed forming the chalcone, the precursor to the flavonoids (Scheme 21).



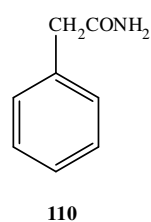
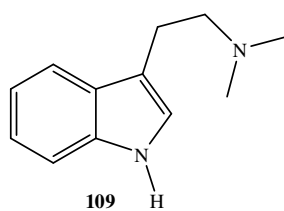
		R	R₁	R₂	R₃
103	vitexin	H	H	H	C-Glu
104	7-O-acylscoparin	H	H	CH ₃ CO	C-Glu
105	Chrysoeriol 7-glucoside	CH ₃ O	H	C-Glu	H
106	2''-O-glucosylvitexin	H	H	H	C-Glu(1→2)]
107	2''-O-glucosylisovitexin	H	C-[Glu(1→2)]	H	H
108	Chrysoeriol 7-rhamnoside	CH ₃	H	C-Rha(1→2)Gal]	H

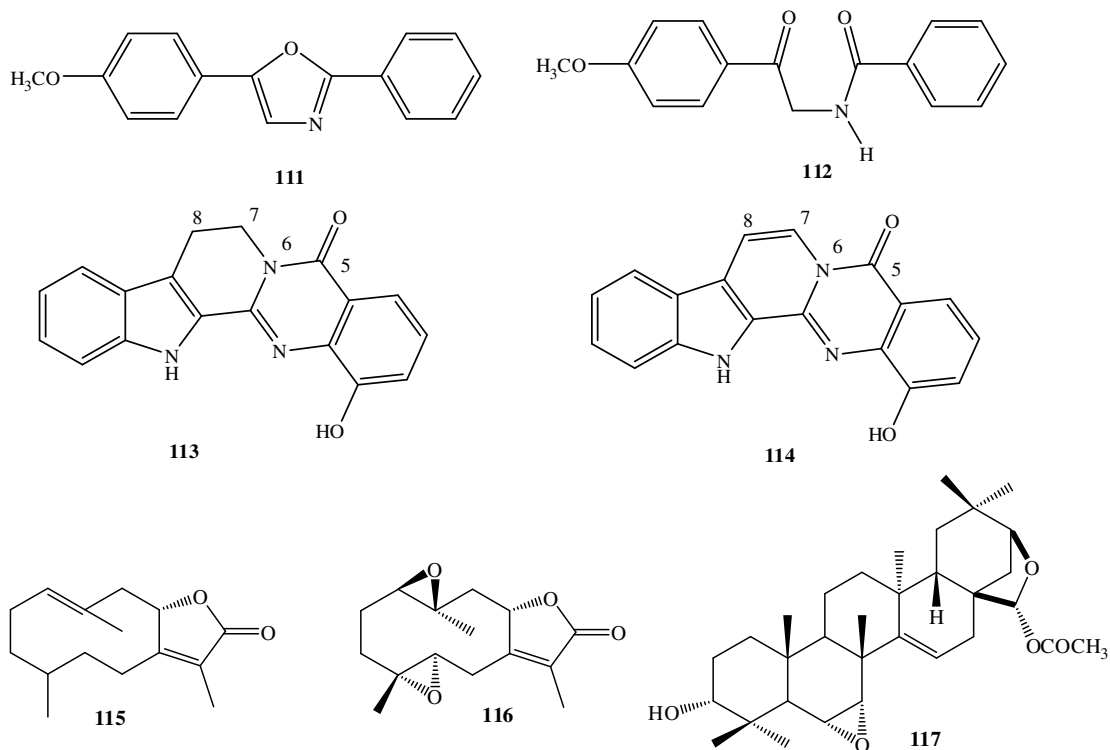


Scheme 21: Condensation of three molecules of malonyl CoA with activated cinnamic acid (Hahlbrock and Grisebach, 1975).

1.2.4.7. Miscellaneous compounds

Apart from the classes mentioned above, *N,N*-dimethyltryptamine (**109**) and phenylacetamide (**110**) have been isolated in *V. ampody* and an azole, unguenenazole (**111**), and amide, unguenenamide (**112**) were isolated from *V. uguenensis*. The indoloquinazoline alkaloids 1-hydroxyrutaecarpine (**113**) and 7,8-dehydro-1-hydroxyrutaecarpine (**114**) were found in *V. lousii*. The terpenoids, glechomanolide (**115**), $1\beta,10\beta:4\alpha,5\alpha$ -diepoxy-7(11)-enegermacr-8 $\alpha,12$ -olide (**116**) and 28-acetyloxy-6 $\alpha,7\alpha:21\beta,28$ -diepoxytaraxer-3 α -ol (**117**) were isolated in *V. punctata*.





1.3 Aim of the study

The main aim of the study was to investigate the species of *Vernonia* and *Vepris* phytochemically in order to determine what secondary metabolites were contained in the various extracts of the plant in order to validate the use of the plants in traditional medicine in Kenya and to provide suggestions for further use of the plant ethnomedicinally by testing the compounds isolated in various identified assays, determined by the class of compound isolated.

1.3.1 Objectives

The research objectives were;

1. To extract and isolate the phytochemicals from *Vernonia auriculifera*, *Vernonia urticifolia*, *Vepris glomerata* and *Vepris uguenensis* leaves, stems and roots.

2. To identify and characterise the isolated compounds using spectroscopic techniques (NMR, IR, UV, CD, and MS).
3. Based on the isolates contained in each plant, identify suitable bioassays to test the compounds and thereby provide information on the further use of the plant or validate its existing use.

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CHAPTER TWO

TRITERPENOIDS FROM *VERNONIA AURICULIFERA*

HIERN EXHIBIT ANTIMICROBIAL ACTIVITY

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ABSTRACT

Phytochemical investigation of *Vernonia auriculifera* afforded farnesylamine, a sesquiterpene amine that has not been found previously in plant species, together with lupenyl acetate, oleanolic acid, β -amyirin acetate, β -amyirin, friedelanone, friedelin acetate, α -amyirin and β -sitosterol. The compounds were characterized using NMR spectroscopy and by comparison with literature values. The isolated triterpenoids exhibited moderate antibacterial activity, α and β -amyirin had MIC values of 0.25 mg/mL against *S. aureus*, *B. subtilis*, *E. faecium* and *S. saprophyticus* while lupenyl acetate and oleanolic acid exhibited MIC values of 0.25 mg/mL against *S. maltophilia*. Sub-MIC exposure of β -amyirin acetate was effective in decreasing adhesion of *S. aureus*, *K. pneumonia* and *E. faecium* while oleanolic acid decreased adhesion of *K. pneumonia* and *P. aeruginosa* significantly at sub-MIC concentrations. These compounds show potential for synergistic coupling with antimicrobial agents to improve therapeutic efficiency in the face of rising bacterial resistance.

Keywords: *Vernonia auriculifera*; triterpenoids; farnesylamine; antibacterial activity

INTRODUCTION

The genus *Vernonia* (Asteraceae family) has more than 1000 species growing all over the world with more than 30 species growing in Kenya (Beentje, 1994; Oketch-Rabah et al., 1997). *Vernonia auriculifera* is a small tree or woody herb that grows between 1-7.5 m high and is easily recognizable by its deep purple flowers. *V. auriculifera* has a wide variety of applications in traditional medicine. A drop of the juice squeezed from the crushed stem bark, inserted into the nostrils, is known to relieve headache (Kusamba, 2001). The Kikuyu people of central Kenya use the leaves of this plant as a wrap for pounded material used as a poultice (Kokwaro, 1976). Heated crushed leaves of *Aspilia mossambicensis*, are tied in the leaf of *V. auriculifera*, and then applied over the eyes to treat conjunctivitis (Muthaura et al., 2007). A cold water infusion of *V. auriculifera* is administered orally in Uganda and Kenya to treat fever associated with viral and bacterial infections (Muthaura et al., 2007; Freiburghaus et al., 1996). In Ethiopia, the roots are used to treat toothache (Mirutse et al., 2009) and snake poison (Mesfin et al., 2009).

Hydroperoxides of unsaturated fatty acid methyl esters previously isolated from *V. auriculifera* were found to have lethal toxicity (Keriko et al., 1995a). Plant growth stimulators have also been identified from this plant (Keriko et al., 1995b). Other *Vernonia* species that have received extensive phytochemical and pharmacological research include: *V. galamensis* (Miserez et al., 1996), *V. brachycalyx* (Oketch-Rabah et al., 1997), *V. colorata* (Rabe et al., 2002), *V. amagdalina* (Erasto et al., 2006), *V. cinerea* (Chen et al., 2006), *V. mapirensis* (Morales-Escobar et al., 2007), *V. cumingiana* (Mao et al., 2008), *V. ferruginea* (Malafrente et al., 2009) and *V. scorpioides* (Buskuhl et al., 2010). Members of

the genus *Vernonia* are an excellent source of sesquiterpene lactones which include vernolide, vernolepin, vernodalin and hydroxyvernolide (Kupchan et al., 1969; Jisaka et al., 1993; Koshimizu et al., 1994). Other compounds have also been isolated from this genus such as triterpenoid glycosides, flavonoids, coumarins and benzofuranones (Miserez et al., 1996; Oketch-Rabah et al., 1997; Mao et al., 2008).

The current study was undertaken primarily to investigate the phytochemistry of *V. auriculifera* from which only fatty acids were previously isolated and to test the isolated compounds for antimicrobial activity since extracts of *Vernonia* species have been cited as antimicrobials in traditional medicine (Kokwaro, 1976).

MATERIALS AND METHODS

General experimental procedure

NMR spectra were recorded using a Bruker Avance^{III} 400 MHz spectrometer. All the spectra were recorded at room temperature with all chemical shifts (δ) recorded against the internal standard, tetramethylsilane (TMS). IR spectra were recorded on a Perkin Elmer Spectrum 100 FT-IR spectrometer with universal ATR sampling accessory. For GC-MS analyses, the samples were analysed on an Agilent GC-MSD apparatus equipped with DB-5SIL MS (30 m x 0.25 mm i.d., 0.25 μ m film thickness) fused-silica capillary column. Helium (at 2 ml/min) was used as a carrier gas. The MS was operated in the EI mode at 70 eV. Optical rotation was recorded using a PerkinElmerTM, Model 341 Polarimeter. Melting points were recorded on an Ernst Leitz Wetzlar micro-hot stage melting point apparatus.

Plant Material

The leaves, stem bark and root bark of *V. auriculifera* were collected in August, 2009 from Egerton University Botanical Garden, Rift Valley Province in Kenya. The plant was identified by taxonomist, Dr S. T. Kariuki, of the Botany Department, Egerton University, Kenya and a voucher specimen (Kiplimo, 02) was deposited in the Ward Herbarium, University of KwaZulu-Natal Westville, Durban, South Africa.

Extraction and isolation

The air-dried and ground plant material of *V. auriculifera* (823 g leaves, 710 g roots, 600 g stems) was sequentially extracted with organic solvents in order of increasing polarity viz; hexane, dichloromethane, ethyl acetate and methanol using a soxhlet apparatus for 24 h in each case. The yields obtained for each solvent were, hexane 66.68 g (leaves), 9.10 g (roots), 16.73 g (stems); dichloromethane 16.77 g (leaves), 5.13 g (roots), 9.25 g (stems); ethyl acetate 8.28 g (leaves), 0.93 g (roots), 5.02 g (stems) and methanol, 27.01 g (leaves), 20.28 g (roots), 15.09 g (stems).

Isolation and purification of compounds 1, 3, 4, 5, 8 and 9

The hexane extract from the leaves (30 g) was separated by column chromatography using a step gradient of hexane: dichloromethane: ethyl acetate gradient, starting with 100% hexane stepped to 10%, 20%, 30%, 50%, 80% and 100% dichloromethane, followed by 20% and 30% ethyl acetate in dichloromethane. Twenty fractions of 100 mL each were collected in each step. Fractions 5-12 were combined and purified using 100% hexane, to produce farnesylamine (**9**) (12 mg). Fractions 21-25 were recrystallised in methanol to yield sitosterol (**8**) (78 mg). Fractions 41-67 were combined and separated with 20% and 30%

dichloromethane in hexane. Lupenyl acetate (**1**) (52 mg) was obtained in fractions 8-12 while fraction 18-35 was further purified using 20% ethyl acetate in hexane where fractions 5-9 afforded β -amyrin acetate (**4**) (150 mg) and fractions 11-18 afforded a mixture of α -amyrin (**5**) and β -amyrin (**3**) (89 mg).

Isolation and purification of compound 2

The ethyl acetate extract (0.93 g) from the roots was dissolved in dichloromethane and separated with a mobile phase consisting of a hexane: ethyl acetate step gradient 1:0 (fractions 1-10), 9:1 (fractions 11-20), 7:3 (fractions 21-38), 6:4 (fractions 52-64) and 3:7 (fractions 65-70). Fractions 22-27 were further purified with 20% ethyl acetate in hexane. Oleanolic acid (**2**) (25 mg) was obtained in fractions 9-13.

Isolation and purification of compounds 6 & 7

The hexane extract from the stems (16.73 g) was subjected to column chromatography. The mobile phase consisted of a hexane: dichloromethane step gradient; 1:0 (fractions 1-45), 9:1 (fractions 46-66), 8:2 (fractions 67-80), 7:3 (fractions 81-98) and 1:1 (fractions 99-121). Friedelin acetate (**7**) (31 mg) was eluted in fraction 24-32 and the pure compound was obtained by recrystallisation in methanol. Friedelanone (**6**) (120 mg) was obtained by purification of fractions 55-80 using 10% dichloromethane in hexane as the mobile phase where the compound was eluted in fraction 7-15, followed by recrystallisation in methanol.

Farnesylamine (9)

White crystals, m/z (rel %): 221 $[M]^+$, 206 (1), 189 (3), 179 (3), 161 (3); IR spectra (V_{\max} cm^{-1}): 3413, 2919, 1357, 1053; ^1H NMR spectral data (400 MHz, CDCl_3) δ_{H} 5.11(H-2, 6, 10), 2.07 (2H-1), 2.06 (2H-4), 2.05 (2H-9), 2.03 (2H-5), 1.69 (3H-12), 1.62 (3H-13, 3H-14),

3H-15), 1.28 (2H-8); ^{13}C NMR spectral data (400 MHz) 134.82 (C-3), 134.16 (C-7), 130.97 (C-11), 124.12 (C-6), 124.02 (C-10), 123.98 (C-2), 39.45 (C-1, 4), 29.42 (C-8), 27.99 (C-5), 26.48 (C-9), 25.41 (C-12), 17.39 (C-13), 15.75 (C-14), 15.71 (C-15).

BIOLOGICAL STUDIES

Minimum inhibitory concentration (MIC)

Four strains of Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 35032, *Klebsiella pneumoniae* ATCC 700603 and *Stenotrophomonas maltophilia* ATCC 13637) and five Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6051, *Enterococcus faecium* ATCC 19434, *Staphylococcus epidermidis* ATCC 14990 and *Staphylococcus saprophyticus* ATCC 35552) were selected for the determination of antimicrobial activity.

The antibacterial activities of the compounds were determined using the broth microdilution method as described by Andrews (2001). Bacterial strains were cultured for 18 h at 37 °C in Tryptone Soy Broth (TSB) and standardized to a final cell density of 1.5×10^8 cfu/mL equivalent to 0.5 McFarland Standard. The 96-well plates were prepared by dispensing into each well, 90 μL Muller-Hinton (MH) broth and 10 μL of the bacterial inoculum. Test compounds were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mg/mL while tetracycline (a broad-spectrum antimicrobial agent) the positive control was dissolved in ethanol. Serial two-fold dilutions were made in a concentration range of 0.002 to 2 mg/mL. Wells containing MH broth only were used as a medium control and wells containing medium and cultures without the test compound were used as the growth control. Plates were covered to avoid contamination and evaporation and incubated for 24 h at 37 °C. The minimum inhibitory concentration (MIC) was described as the lowest concentration of

the test compounds that completely inhibited the growth of microorganisms. The tests were done in triplicate on two separate occasions and the results are as shown in table 2.

Anti-biofilm activity evaluation

To determine the anti-biofilm activity of β -amyrin acetate and oleanolic acid, three strains of Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 35032 and *Klebsiella pneumoniae* ATCC 700603) and four Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 43300, *Enterococcus faecium* ATCC 19434, and *Staphylococcus saprophyticus* ATCC 35552) were used. Bacterial isolates were cultured overnight in TSB to determine the effect of MIC, sub-MIC (0.5×MIC) and supra-MIC (2×MIC) exposures on biofilm formation. Cells were washed and resuspended in distilled water to a turbidity equivalent to a 0.5 McFarland standard. Wells of sterile, 96-well U-bottomed microtiter plates were each filled with 90 μ L Luria Bertani broth (LB) and 10 μ L of cell suspension, in triplicate. Based on individual MICs for each isolate the effect of MIC, sub-MIC and supra-MIC of β -amyrin acetate and oleanolic acid on bacterial adhesion was investigated. Plates were incubated aerobically at 37 °C for 24 h with shaking on an Orbit P4 microtitre plate shaker (Labnet).

The contents of each well were aspirated and then washed three times with 250 μ L of sterile distilled water. To remove all the non-adherent bacteria, the plates were vigorously shaken and the remaining attached cells were fixed with 200 μ L of 99% methanol per well. After 15 min, plates were left to dry and then stained for 5 min with 150 μ L of 2% Hucker crystal violet. Excess stain was washed with running tap water and plates were left to air dry (Basson et al., 2008). The bound stain was resolubilised with 150 μ L of 33% (v/v) glacial

acetic acid per well. The Optical Density (OD) of the contents of each well was obtained at 595 nm using the Fluoroskan Ascent F1 (Thermolabsystems).

Tests were done in triplicate on two separate occasions and the results were averaged (Stepanović et al., 2000). The negative control for both assays was un-inoculated LB, while the positive control was tetracycline, with respective cell suspensions without β -amyirin acetate or oleanolic acid. OD_{595nm} values of treated cells were compared with untreated cells to investigate the increase/decrease of biofilm formation as a result of antimicrobial agent exposure. Treated and untreated samples were compared statistically using paired t-tests and Wilcoxon signed rank tests if normality failed using a SigmaStat V3.5, Systat Software.

RESULTS AND DISCUSSION

The phytochemical investigation of *V. auriculifera* led to the isolation of eight triterpenoids **1-8** and a sesquiterpene amine (**9**) (Figure 1). Extracts from the leaves were found to contain the sesquiterpene amine along with one lupane-type triterpenoid (lupenyl acetate **1**), one ursane-type triterpenoid (α -amyirin **5**), two oleanane-type triterpenoids (β -amyirin **3** and β -amyirin acetate **4**) and a common steroid (sitosterol **8**) (Figure 1). The stem bark afforded friedelanone (**6**) and friedelin acetate (**7**) belonging to the friedelane class. From the roots, oleanolic acid (**2**), the parent oleanane type triterpene, was isolated. Compounds **1-8** were identified using 2D NMR spectral data and by comparison with literature values, which supported the structures as lupenyl acetate (Jamal et al., 2008), oleanolic acid (Seebacher et al., 2003), β -amyirin, β -amyirin acetate, friedelin acetate and α -amyirin (Mahato and Kundu, 1994), friedelanone (Igoli and Gray, 2008) and sitosterol (Kamboj and Saluja, 2011). Although farnesylamine (**9**) was previously reported (Jones et al., 2003), here we are reporting the complete data for the first time.

Compound **9** was isolated as a colourless oily liquid; its molecular formula was assigned as $C_{15}H_{27}N$. The IR spectrum showed the presence of a primary amine (3413 cm^{-1}) and (1375 cm^{-1}). The ^{13}C NMR spectrum showed the presence of six olefinic carbon resonances, 3-protonated carbon resonances at δ_C 124.12 (C-6), 124.02 (C-10) and 123.98 (C-2) and 3-non-protonated carbons at δ_C 130-135. The olefinic methine resonances could also be seen at δ_H 5.11 in the 1H NMR spectrum. The methylene carbon resonances were observed between δ_C 26.48 and δ_C 29.42 except for the methylene bonded to the amine group which was observed downfield at δ_C 39.47. All the methylene proton resonances, including 2H-1 were present at δ_H 2.05 except for one methylene resonance which appeared upfield at δ_H 1.27. Three of the four methyl proton resonances overlap at δ_H 1.62 (3H-13, 14, 15) and one is in a different chemical environment at δ_H 1.69 (3H-12). The methyl carbon resonances can be seen in the ^{13}C NMR spectrum between δ_C 25.41 and δ_C 15.71. This compound has been detected in an extract of the ant *Monomorium fieldi* Forel from Australia (Jones et al., 2003) and has only now been found in a plant species.

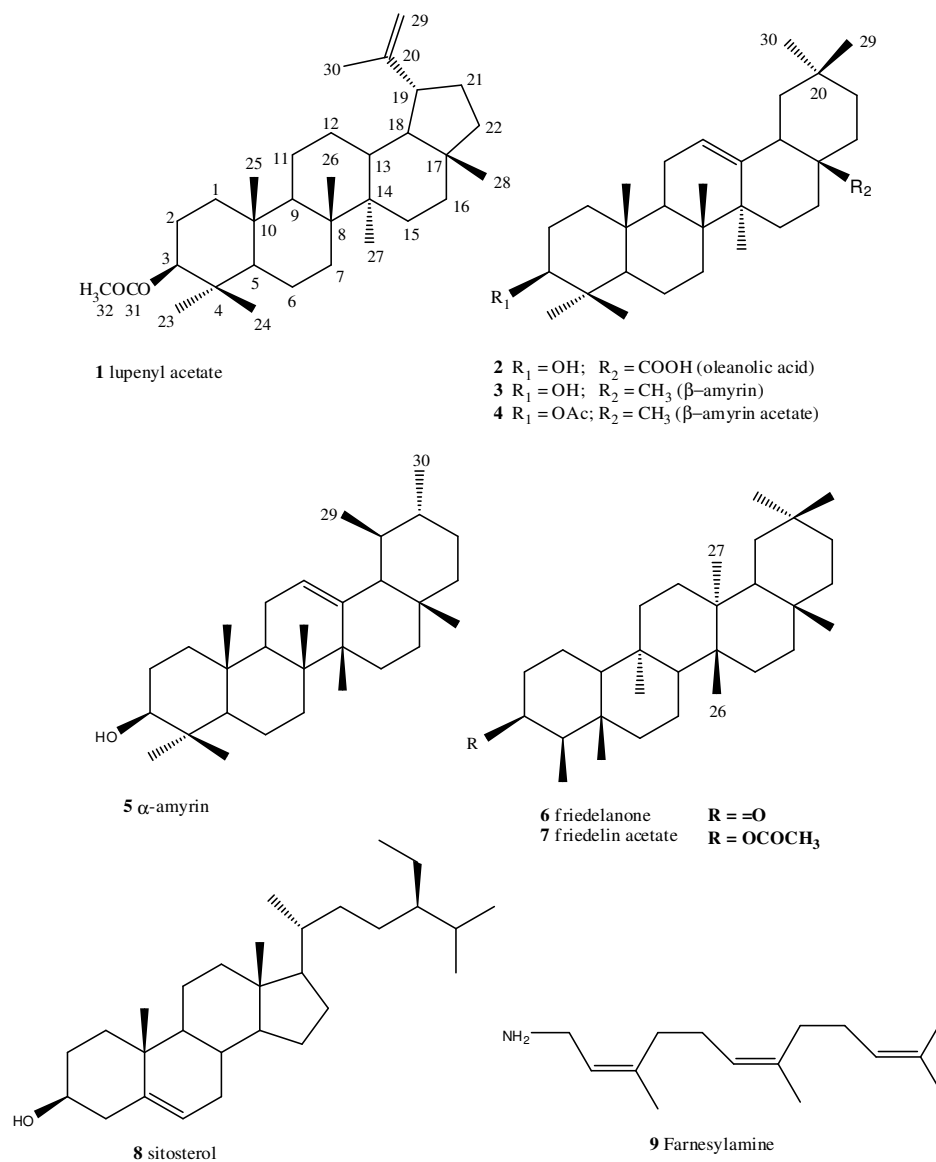


Figure 1: Structures of compounds (1-9) isolated from *Vernonia auriculifera*.

The triterpene family of compounds to which all the isolated compounds belong are reported to possess antibacterial activity (Collins and Charles, 1987). The sesquiterpene, farnesylamine, could not be screened for antibacterial activity due to sample decomposition. MIC values recorded for all tested compounds (Table 2) suggested moderate antibacterial activity. The most active compounds were amyrins (mixture of α - and β -), with MICs of 0.12 mg/mL against *E. coli*, 0.25 mg/mL against *S. aureus*, *B. subtilis*, *E. faecalis*, *S.*

saprophyticus, and 0.5mg/mL against *S. epidermis*, *K. pneumonia*, and *S. maltophilia*. The other compounds **3-7** had a MIC of 0.5 mg/mL against *S. maltophilia*. The least active compounds were **6** and **7** with a MIC of 1.0 mg/mL against six microorganisms. All tested compounds had MICs of 1.0 mg/mL for *P. aeruginosae* and 0.5 mg/mL for *S. maltophilia*. The oleanane triterpenoids (**2-4**) displayed better antibacterial activity than the friedelane triterpenoids (**6-7**). It is reported that the 28-COOH and ester functionality at C-3 contributes to pharmacological activities of pentacyclic triterpenes (Mallavadhi et al., 2004) like lupenyl which has greater antimutagenic activity than lupenyl acetate (Guevara et al., 1996). These effects are observed for friedelanone and friedelin acetate where the ketone has higher activity against *Bacillus subtilis* than the ester.

Table 1: Minimum inhibitory concentrations (MIC in mg mL⁻¹) of compounds isolated from *V. auriculifera*

Microorganism	MIC (mg mL ⁻¹) of the test organisms					
	1	2	3 & 5	4	6	7
<i>S. aureus</i>	1.0	0.5	0.25	1.0	1.0	1.0
<i>B. subtilis</i>	1.0	0.5	0.25	1.0	0.25	1.0
<i>E. faecium</i>	0.5	1.0	0.25	0.5	1.0	1.0
<i>S. epidermidis</i>	1.0	0.25	0.5	1.0	0.5	0.5
<i>S. saprophyticus</i>	0.25	1.0	0.25	1.0	1.0	1.0
<i>E. coli</i>	0.12	1.0	0.12	0.5	1.0	0.5
<i>K. pneumoniae</i>	1.0	0.5	0.5	1.0	1.0	1.0
<i>P. aeruginosa</i>	1.0	1.0	1.0	1.0	1.0	1.0
<i>St. maltophilia</i>	0.25	0.25	0.5	0.5	0.5	0.5

Key: 1= lupenyl acetate, 2 = oleanolic acid, 3 = β -amyryn, 4 = β -amyryn acetate, 5 = α -amyryn, 6 = friedlanone, 7 = friedlin acetate

Biofilm is a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other and are embedded in a matrix of extracellular polymeric substances they have produced. When planktonic bacteria adhere to surfaces, they initiate biofilm formation. The nature of biofilm structure and

physiological attributes of biofilm organisms confer an inherent resistance to antimicrobial agents such as antibiotics, disinfectants or germicides (Donlan et al., 2002).

β -amyrin acetate and oleanolic acid were tested for antibiofilm activity against seven strains of bacteria. β -amyrin acetate decreased adhesion of *S. aureus* (ATCC 43300), *K. pneumonia* and *E. faecium* significantly at sub-MIC concentrations (figure 2). For *K. pneumonia*, this decreased adhesion was also seen at MIC concentrations and in *S. saprophyticus* a marked decrease in adhesion was seen at MIC and supra MIC concentrations. Sub-MIC oleanolic acid exposure also decreased adhesion of *K. pneumonia* and *P. aeruginosa* significantly (figure 3), but MIC and supra-MIC exposures of oleanolic acid increased adhesion of all tested bacterial strains. These results suggest that oleanolic acid and β -amyrin acetate that are relatively abundant, can be used at low concentrations to decrease adhesion of certain bacterial strains to abiotic surfaces. Since bacterial resistance to antibiotics and their survival are associated with their ability to form biofilms (Donlan et al., 2002), compounds which decrease biofilm formation would be useful in being used in conjunction with other antibiotics to decrease bacterial resistance. Agents that decrease adhesion of bacteria may also be useful in improving the efficacy of antibiotics and hygiene in hospitals that have devices such as incubation tubes, catheters, artificial heart valves, water lines and cleaning instruments on which bacterial biofilm have been found (Donlan et al., 2002).

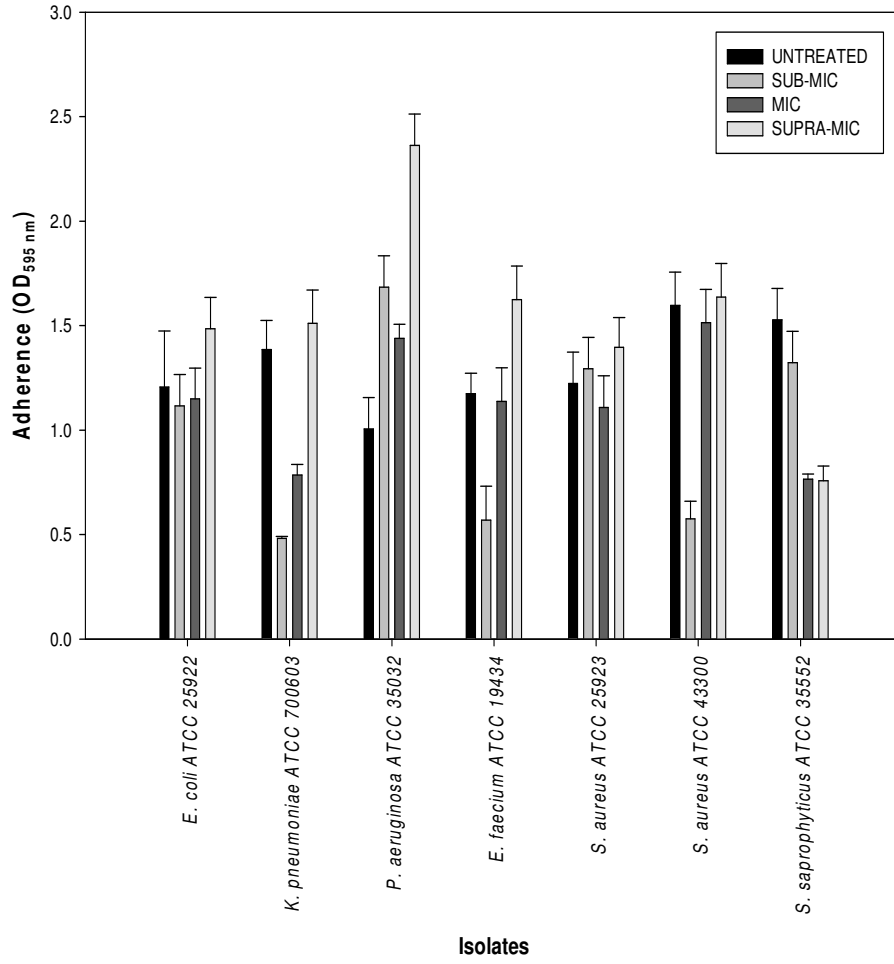


Figure 2: Antibiofilm results for β -amyrin acetate (4)

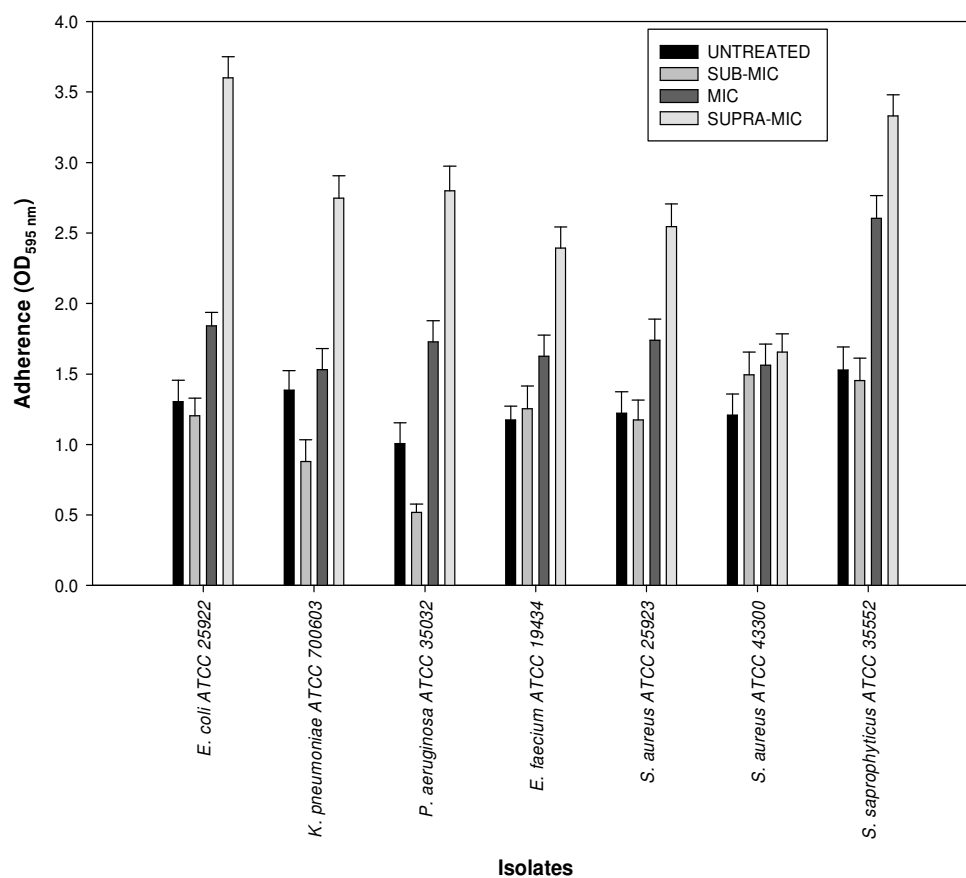


Figure 3: Antibiofilm results for oleanolic acid (2)

CONCLUSION

This is the first report of a phytochemical investigation of *V. auriculifera*. The finding of a sesquiterpene amine in *V. auriculifera* is unique as it has not been isolated from a plant species before. Although the genus *Vernonia* is known to be a rich source of sesquiterpene lactones, none were isolated from *V. auriculifera*. However, eight pentacyclic compounds with moderate antibacterial activity were isolated. Oleanolic acid and β -amyrin acetate exhibited moderate anti-adhesion properties. These compounds show potential for synergistic coupling with antimicrobial agents to improve therapeutic efficiency, in the face of rising bacterial resistance, however this needs further investigation.

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CHAPTER THREE

A NOVEL POLYENE FROM *VERNONIA URTICIFOLIA* (ASTERACEAE)

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ABSTRACT

A new polyene metabolite, urticifolene together with a known carotenoid, lutein and sitosterol were isolated from the leaves of *Vernonia urticifolia* and fully characterized. Lutein (**2**) inhibited the growth of *Enterococcus faecium* at a low concentration (MIC of 8 µg/mL), while urticifolene (**1**) inhibited the growth of *Enterococcus faecium* and *Pseudomonas aeruginosa* at low concentrations (MIC of 16 and 32 µg/mL respectively). In contrast to the related polyenes, nystatin and amphotericin B, which exhibit no activity against bacteria, urticifolene exhibited inhibitory property against all the bacteria investigated.

Keywords: Urticifolene, lutein, *Vernonia urticifolia*, Asteraceae, antibacterial.

INTRODUCTION

Ethnomedicinally, *Vernonia* species are employed in the treatment of a diverse range of ailments, including measles, skin rashes, backache, malaria (Anoka et al., 2008), asthma, bronchitis, dysentery and worms (guinea, round and thread) (Misra et al., 1984). The root of *V. cinerea* is used as an anthelmintic and diuretic (Misra et al., 1984) and to treat, coughs

intestinal colics and chronic skin diseases (Dastur, 1977). *V. calvoana*, a leafy vegetable is found to be a rich source of provitamin A, particularly *cis*- β -carotene (Ejoh et al., 2010).

Several *Vernonia* species have previously been investigated, of which *V. amagdalena* is extensively investigated for its pharmacological properties (Kupchan et al., 1963, Ohigashi et al., 1994). *V. brasiliana* and *V. brachycalyx* have shown potential antiprotozoal activity (Almeida Alves de et al., 1997; Oketch-Rabah et al., 1997). *V. urticifolia*, the subject of the present study is known as Motoiyokwo by the Kalenjin tribe of Kenya, who use it to treat sinuses, allergy and skin rashes (Kokwaro, 1976).

There are no previous phytochemical or pharmacological studies carried out on this plant. The current study was undertaken to investigate the phytochemistry of *V. urticifolia* and to test the isolated compounds for antibacterial activity since extracts of *Vernonia* species have been reported to possess antimicrobial activities and are used in traditional medicine (Kokwaro, 1976). We present herein a detailed isolation, characterization and antibacterial activity of the isolated compounds.

MATERIALS AND METHODS

Plant material

The leaves of *Vernonia urticifolia* (762.87 g) were collected from Nakuru District, Kenya in June 2010. The plant was identified by Mr Ezekiel Cheboi of the Department of Natural Resources, Egerton University, Kenya. A voucher specimen (Kiplimo 03) was retained at the Ward Herbarium University of KwaZulu-Natal Westville, Durban.

Extraction and Isolation of Compounds

The air-dried and ground leaves were extracted sequentially using a soxhlet apparatus with solvents of increasing polarity viz: hexane, dichloromethane, ethyl acetate and methanol.

The dichloromethane extract was concentrated to yield a crude extract (6.94 g) which was separated using column chromatography over silica gel (Merck 9385) and eluted with a hexane/ethyl acetate step gradient, to yield three subfractions D₁-D₃. Compound **1** (32 mg) was obtained in fraction D₂ and compound **2** (43 mg) was obtained in fraction D₃.

GENERAL EXPERIMENTAL PROCEDURE

Melting points were determined on an Ernst Leitz Wetzlar micro-hot stage melting point apparatus. NMR spectra were recorded at room temperature on a 400MHz Varian UNITY-INOVA spectrometer. ¹H NMR spectra were referenced against the CHCl₃ signal at δ_{H} 7.24 and ¹³C NMR spectra against the corresponding signal at δ_{C} 77.0. Coupling constants are given in Hz. For GC-MS analyses, samples were analysed on an Agilent GC-MSD apparatus equipped with a DB-5SIL MS (30 m x 0.25 mm i.d., 0.25 μ m film thickness) fused-silica capillary column. The MS was operated in the EI mode at 70 eV, in *m/z* range 42–350. IR spectra were recorded on a Nicolet Impact 400D Fourier Transform Infrared (FT-IR) spectrometer, using NaCl windows with CHCl₃ as solvent against an air background. UV spectra were obtained on a Varian DMS 300 UV-visible spectrophotometer.

BIOLOGICAL STUDIES

Test organisms

Three strains of Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 35032, *Klebsiella pneumoniae* ATCC 700603), and three Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecium* ATCC 19434, *Staphylococcus saprophyticus* ATCC 35552) were selected to determine the antibacterial activity of the isolated compounds.

Determination of minimum inhibitory concentration (MIC)

The antibacterial activities of the compounds were determined using the broth microdilution method as described by Andrews, 2001; Bacterial strains were cultured for 18 h at 37 °C in Tryptone Soy Broth (TSB) and standardized to a final cell density of 1.5×10^8 cfu/ml equivalent to 0.5 McFarland standard. The 96-well plates were prepared by dispensing into each well, 90 μ l Muller-Hinton (MH) broth and 10 μ l of the bacterial inoculum. Test compounds were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mg/ml while tetracycline (a positive control) was dissolved in ethanol. Serial two-fold dilutions were made in a concentration range of 0.002 to 1 mg/ml. Wells containing MH broth only were used as medium control and wells containing medium and cultures without the test compound were used as the growth control. Plates were covered to avoid contamination and evaporation and incubated for 24 h at 37 °C. The minimum inhibitory concentration (MIC) was described as the lowest concentration of the test compounds completely inhibiting the growth of microorganisms. The tests were done in triplicate on two separate occasions (Table 2).

RESULTS AND DISCUSSION

The dichloromethane extract of the powdered leaves of *V. urticifolia* afforded a new polyene, (urticifolene, **1**), known carotenoid (lutein, **2**) and the known triterpenoid, (sitosterol) that is ubiquitous in the *Vernonia* family. This is the first report of compound **1**; a yellow oily solid which has been assigned the trivial name urticifolene. The molecular formula was established to be $C_{31}H_{52}O_4$, as indicated by the M^+ ion at m/z 488 in the mass spectrum, which implied six degrees of unsaturation. The IR spectrum revealed the presence of hydroxyl (3396 cm^{-1}) and carbonyl (1713 cm^{-1}) functional groups. The UV spectrum of urticifolene, **1** showed maxima with three shoulders at 237, 245 and 322 nm

consistent with the conjugated double bonds present in the compound. The ^{13}C NMR spectrum of urticifolene, **1** (Table 1) showed ten carbon resonances in the olefinic region (Range 123-135 ppm) indicating five double bonds, a carbonyl at δ_{C} 179.23 (accounting for the six degrees of unsaturation) and two oxygenated methine carbon resonances at δ_{C} 72.95 and 72.18. ^1H NMR data displayed two methyl groups bound to aliphatic carbons at δ_{H} 0.97 (3H, s, H-31) and δ_{H} 0.89 (3H, s, H-16) and overlapping olefinic resonances which appeared between 5 and 7 ppm, characteristic of polyenes. Also present in the ^1H NMR spectrum were two overlapping signals at δ_{H} 4.17 (d, $J = 6.60$ Hz) and δ_{H} 4.22 (d, $J = 6.32$ Hz) confirming the presence of two hydroxyl groups at C-3 and C-14.

The COSY spectrum revealed a correlation between H-3 and H-2, and H-2 and H-5 in the olefinic region showed correlations with C-3 (δ_{C} 72.95) in the HMBC spectrum. The geometry of the double bond at C₄, C₆, C₈, C₁₀ and C₁₂ were determined from the proton coupling constants of $J_{6,7} = 15.0$ Hz, and $J_{10,11} = 15.5$ Hz to be *trans*. The other three double bonds were established to be *cis* double bonds because their coupling constants were below 8 Hz. The triplet at δ_{H} 5.97 ($J = 11.0$ Hz, H-8), supported a double bond at Δ^8 with a *cis* geometry. The H-12 and H-13 protons also displayed downfield chemical shift values δ_{H} 5.57 and δ_{H} 5.66 that are close to those observed for the olefinic protons at H-8 and H-9, suggesting the presence of a *cis* double bond at Δ^{12} as well. Urticifolene (**1**) displayed high structural similarity to laetiporic acid isolated from *Laetiporus sulphureus* with the exception of the aliphatic methylene chain containing 16 carbons in urticifolene (**1**) that is absent in laetiporic acid (Davoli et al., 2005).

Compound **2** was identified as Lutein due to characteristic peaks observed in the spectroscopic data. The UV spectrum showed absorption maxima at 454 nm, 480 nm and

430 nm. This was consistent with the UV data of (9Z, 9'Z, 3R, 3'R, 6'R)-lutein isolated from *Brassica napus* (Rape) (Kull and Pfander, 1997).

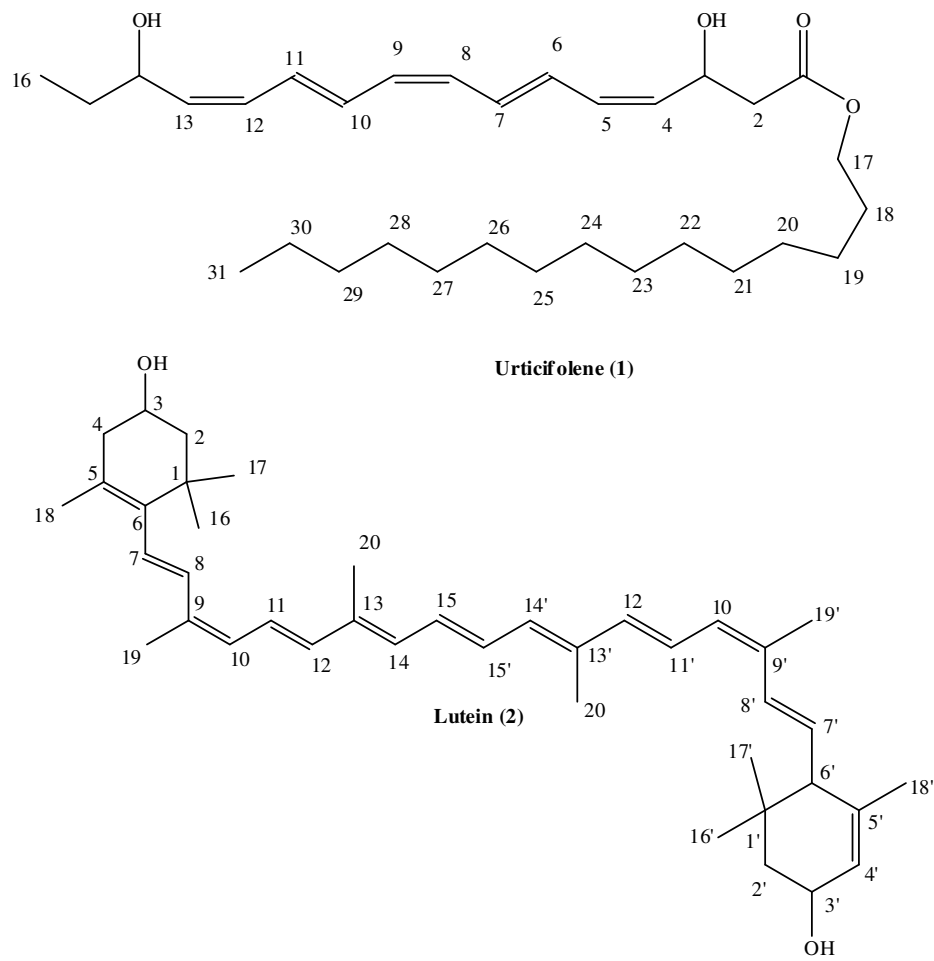


Figure 1. Structures of compounds (1 & 2) isolated from *Vernonia urticifolia*

Table 1. ^1H and ^{13}C NMR spectral data of compound 1 and 2 (400 or 100 MHz)

urticifolene (1)			lutein (2)		
position	δ_{C}	δ_{H}	position	δ_{C}	δ_{H}
1	179.23	-	1	37.13	-
2	35.22	2.33	2	48.45	178, 150
3	72.95	4.22	3	65.10	4.03
4	123.72	5.36	4	42.56	2.05, 2.42
5	125.82	6.50	5	126.17	-
6	125.91	6.50	6	138.00	-
7	127.84	5.97	7	128.73	6.12
8	127.78	5.97	8	130.81	6.65
9	132.81	5.42	9	135.07	-
10	132.90	5.43	10	130.04	6.07
11	134.87	5.65	11	124.49	6.73
12	135.20	5.57	12	137.57	6.28
13	135.71	5.66	13	136.42	-
14	72.18	4.17	14	132.58	6.23
15	33.93	2.33	15	130.09	6.63
16	14.19	0.89	16	30.26	1.08
17	37.25	1.60	17	28.73	1.09
18	31.76	1.28	18	21.62	1.78
19	29.69	1.28	19	29.70	1.97
20	29.69	1.29	20	12.81	1.97
21	29.36	1.33	1'	34.04	-
22	29.34	1.33	2'	44.64	1.37, 1.85
23	28.89	1.33	3'	65.93	4.25
24	28.87	1.33	4'	128.81	5.50
25	27.61	2.19	5'	137.77	-
26	27.58	2.06	6'	54.97	2.47
27	25.09	1.60	7'	131.30	5.47
28	24.61	1.34	8'	130.09	6.65
29	22.59	1.29	9'	135.70	-
30	20.74	2.06	10'	125.60	6.05
31	14.04	0.97	11'	124.94	6.74
			12'	138.50	6.28
			13'	136.49	-
			14'	137.73	6.23
			15'	130.81	6.63
			16'	29.50	0.85
			17'	24.29	1.03
			18'	22.86	1.64
			19'	12.76	1.90
			20'	13.11	1.95

The antibacterial activity results (Table 2) shows that urticifolene, **1** and lutein, **2** had broad spectrum antibacterial activity. The minimum inhibitory concentration (MIC) determination showed that lutein at low concentration of 8 µg/mL completely inhibited the growth of *Enterococcus faecium* (ATCC 19434) and at 32 µg/mL it inhibited the growth of *Staphylococcus aureus* (ATCC 29212), *Escherichia coli* (ATCC 25922) and *Klebsiella pneumonia* (ATCC 700603). Urticifolene inhibited the growth of *Pseudomonas aeruginosa* (ATCC 35032) and *Enterococcus faecium* (ATCC 19434) at low concentrations of 32 µg/mL and 16 µg/mL respectively, but the MIC for *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (ATCC 700603) and *Staphylococcus aureus* (ATCC 29212,) were recorded as 256 µg/mL. These findings indicated that urticifolene (**1**) and lutein (**2**) possessed inhibitory activity against bacteria. Other investigations showed that the carotenoid methanol extract of the citrus the peel of *Shatian pummel* exhibited inhibitory properties within a range of 18.75-140.00 µg/mL against *E. coli*, *S. aureus* and *B. subtilis* (Tao et al., 2010).

The constituents of citrus carotenoids cover a wide range of compounds such as β-cryptoxanthin, violaxanthin isomers, lycopene and β-carotene (Tao et al., 2010) therefore the synergistic effect of these compounds could have possibly enhanced the activity of the methanol carotenoid extract. It has also been reported that some polyenes (of which urticifolene, **1** belongs) such as amphotericin B and nystatin have no antibacterial activity whilst others such as faeriefungin inhibit the growth of a variety of bacterial isolates. The mode of action of carotenoids is not clearly understood but Cucco et al., 2007) suggest that β-carotene could lead to the accumulation of lysozyme (a bacterial immune enzyme that digests bacterial cell walls). But the mode of action of polyenes against bacteria is unknown, though the fungicidal activity involves interaction of the lipophilic polyene

structure with the cytoplasmic membrane sterol found in fungi and mammalian cells resulting in alteration of the cell membrane, leakage of cell constituents and cell death (Mulks et al., 1990).

Table 2 Minimum inhibitory concentration (MIC) of urticifolene and lutein

Test organism	Concentration ($\mu\text{g/ml}$)	
	Urticifolene	lutein
<i>E. faecium</i> , (ATCC 19434)	16	8
<i>S. aureus</i> , (ATCC 29212)	256	32
<i>S. saprophyticus</i> , (ATCC 35552)	128	256
<i>E. coli</i> , (ATCC 25922)	256	32
<i>K. pneumonia</i> , (ATCC 700603)	256	32
<i>P. aeruginosa</i> , (ATCC 35032)	32	256

CONCLUSION

The phytochemical study of this plant resulted in the isolation of two compounds, a polyene and a carotenoid. These compounds exhibited moderate antibacterial activity. In contrast to the related polyene antibiotics, nyastin and amphotericin B, which are inactive against certain bacteria, urticifolene exhibited inhibitory activity against all the bacteria investigated. These results provide scientific validity and credence to the ethnomedicinal use of this plant in the treatment of ailments caused by some of the bacteria used in this study and highlights the usefulness of *V. urticifolia* in the treatment of bacterial infections.

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CHAPTER FOUR
A NOVEL FLAVONOID AND FUROQUINOLINE
ALKALOIDS FROM *VEPRIS GLOMERATA* AND THEIR
ANTIOXIDANT ACTIVITY

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Abstract

The dichloromethane extract of the aerial part of the plant *Vepris glomerata* (Rutaceae) yielded a new flavonoid, which was accorded the trivial name veprisinol (**1**), together with four known furoquinoline alkaloids: haplopine-3,3'-dimethylallyl ether (**2**), anhydroevoxine (**3**), evoxine (**4**) and skimmianine (**5**). The structures of the compounds were established by 1D and 2D NMR spectroscopy, as well as HREIMS. Compounds **1** and **2** have strong antioxidant potential, similar to and in some instances better than ascorbic acid and can be used as beneficial additives to antioxidant supplements.

Keywords: *Vepris glomerata*, veprisinol, furoquinoline alkaloids, antioxidant activity.

Introduction

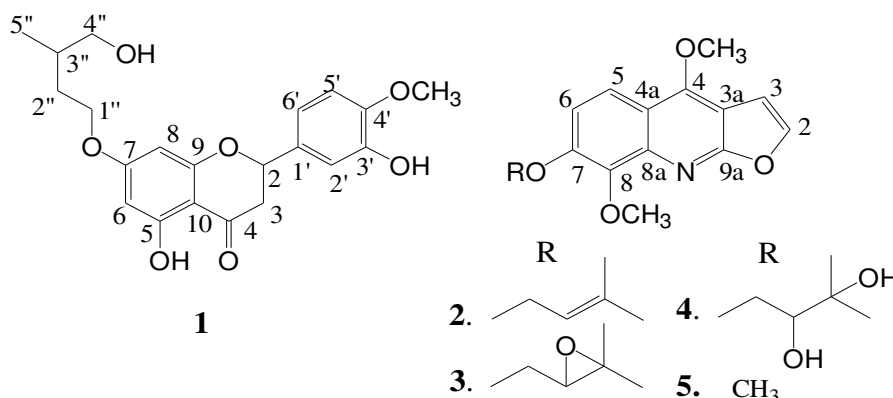
The African *Vepris* species have proved to be a good source of furoquinoline and acridone alkaloids that typify the genus as a whole. *V. bilocularis* has been found to have both

furoquinoline as well as acridone alkaloids [1, 2], while furoquinoline alkaloids alone have been found in *V. ampody* [3], *V. heterophylla* [4], *V. punctata* [5] and *V. stolzii* [6], and acridone alkaloids alone in *V. fitoravina* and *V. macrophylla* [7]. The alkaloids are reported to possess broad spectrum antimicrobial [8], antiradical [9], antioxidant [10], antiplasmodial [11], anticancer [12] and antimutagenic [13] activities. *V. glomerata* is used in African traditional medicine, where its aqueous root extract is used to treat malaria, epilepsy, psychosis and stroke, when mixed with tea [14]. Earlier pharmacological studies on this plant reported antiplasmodial activities of the ethanol extract [15].

Since the species of Rutaceae are often cited as antimalarials or febrifuges in African traditional medicine [14] and the antioxidant activity of alkaloids [10] and flavonoids [16] has previously been demonstrated, all the five compounds isolated were assessed for antioxidant activity using three methods.

Here we report on the isolation and structure elucidation of a new flavonoid, in addition to four known furoquinoline alkaloids: haplopine-3,3'-dimethylallyl ether (**2**), anhydroevoxine (**3**), evoxine (**4**) and skimmianine (**5**) from the dichloromethane extract of *V. glomerata*, together with their antioxidant activities *in vitro*. The structures of the known compounds **2-5** were determined by comparison of their physical and spectroscopic data with those reported in literature; **2** and **3** [17], **4** [18] and **5** [19]. Only skimmianine and evoxine (montrifoline) were previously reported from the leaves of *V. glomerata* endemic to Ethiopia [20]. It is not apparent if the additional compounds found in this study are as a result of either geographical or seasonal differences.

Results and Discussion



Compound **1** was obtained as a yellow solid. Its mass was established to be 388.1573 amu, based on HREIMS data, corresponding to a molecular formula of C₂₁H₂₄O₇, which indicates a double bond equivalence of 10, eight being due to the aromatic rings, one being due to the carbonyl group and one to ring C of the flavanone skeleton. The IR spectrum showed a carbonyl stretching band at 1705 cm⁻¹ and a hydroxyl absorption band at 3364 cm⁻¹. This compound was identified as a flavanone based on its characteristic ¹H NMR spectral pattern. The characteristic ABX coupling system of H-2 β , H-3 α and H-3 β appeared at δ_{H} 5.29 (1H, dd, $J = 12.84, 2.84$ Hz, H-2 β), δ_{H} 3.04 (1H, dd, $J = 17.12, 2.84$ Hz, H-3 α) and δ_{H} 2.75 (1H, dd, $J = 17.12, 12.84$, Hz, H-3 β). These signals also showed COSY and NOESY correlations with each other.

Another characteristic pattern was that of the trisubstituted aromatic B ring. The proton resonances of this ring occurred as a singlet at δ_{H} 7.00 (s, H-2') and doublets at δ_{H} 6.89 and 6.84 (1H each, d, $J = 8.48$ Hz, H-5' and H-6'). The small coupling constant of about 2 Hz for $J_{\text{H}2',\text{H}6'}$ could not be detected for the H-2' resonance. The ¹H NMR spectrum also showed the presence of a methoxy group at δ_{H} 3.88 (s), its position at C-4' being confirmed by both a 1D NOE and a NOESY correlation with the resonances at δ_{H} 6.89 and 6.84 (H-5')

and H-6'). Five aromatic C-O resonances were seen at δ_C 164.0, 167.2, 162.8, 145.0 and 147.0 attributed to oxygenation at C-5, 7, 9, 3' and 4'.

A pair of doublets at δ_H 6.02 (1H, d, $J = 1.76$ Hz, H-6) and δ_H 6.00 (1H, d, $J = 1.76$ Hz, H-8) were attributed to the *meta* coupled, H-6 and H-8 protons on ring A. These two proton resonances showed NOESY correlations to 2H-1'' at δ_H 4.02, confirming the position of the side chain at C-7. Its corresponding carbon resonance showed HMBC correlations to two multiplets at δ_H 1.87 (overlapping resonances of H-2''a and H-3'') and δ_H 1.61 (H-2''b). The H-2'' resonances were diastereotopic and appeared as two separate resonances. COSY correlations were also observed between H-1'' and H-2''a and H-2''b and between H-2''b and H-3''. The H-3'' methine proton was coupled to the methyl proton resonance at δ_H 0.95 (d, $J = 6.52$ Hz) attributed to 3H-5'' and the methylene proton at δ_H 3.50 (2H-4'') in the COSY spectrum. These correlations formed a side chain which was attached to ring A by an ether linkage at C-7. Compound **1** was thus identified as 4H-1-benzopyran-4-one-2,3-dihydro-5-hydroxy-2-(4'-methoxy-3'-hydroxybenzyl)-7-O-(2-methyl butanol) ether, and given the trivial name veprisinol.

The results of the reducing potential (transformation of Fe^{3+} - Fe^{2+}) of the standard (ascorbic acid) and compounds **1-5** are shown in figure 1. The activity of haplopine-3,3'-dimethylallyl ether, **2** and veprisiniol (**1**) was significantly higher than the activity of the other three alkaloids at all concentrations. However, the reducing power of compound **1** was significantly lower than that of compound **2**. The reducing power of the compounds and standard followed the order: ascorbic acid > **2** > **1** > **3** > **4** > **5**.

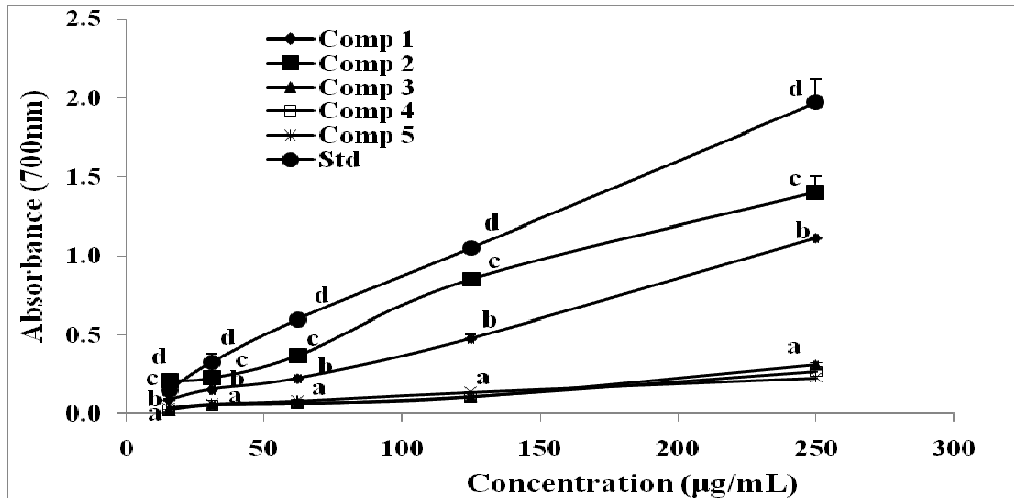


Figure 1: Free radical reducing potential of compounds 1-5 and standard ascorbic acid as evaluated by the spectrophotometric detection of the Fe^{3+} - Fe^{2+} transformation (FRAP method).

The DPPH radical scavenging assay results are shown in figure 2. The results revealed that the scavenging activity of the standard ascorbic acid was significantly higher than all other compounds tested. At concentrations of $62.5 \mu\text{g mL}^{-1}$ and above, the activity decreased in the order ascorbic acid > **1** > **2** > **4** > **3**, whereas at the lower concentrations, 31.25 and $15.625 \mu\text{g mL}^{-1}$, evoxine (**4**) had the highest percentage antioxidant activity of 41%. The activity of compounds **1** and **2** was increased with their concentration and significantly higher than other compounds, particularly at higher concentrations (Figure 2).

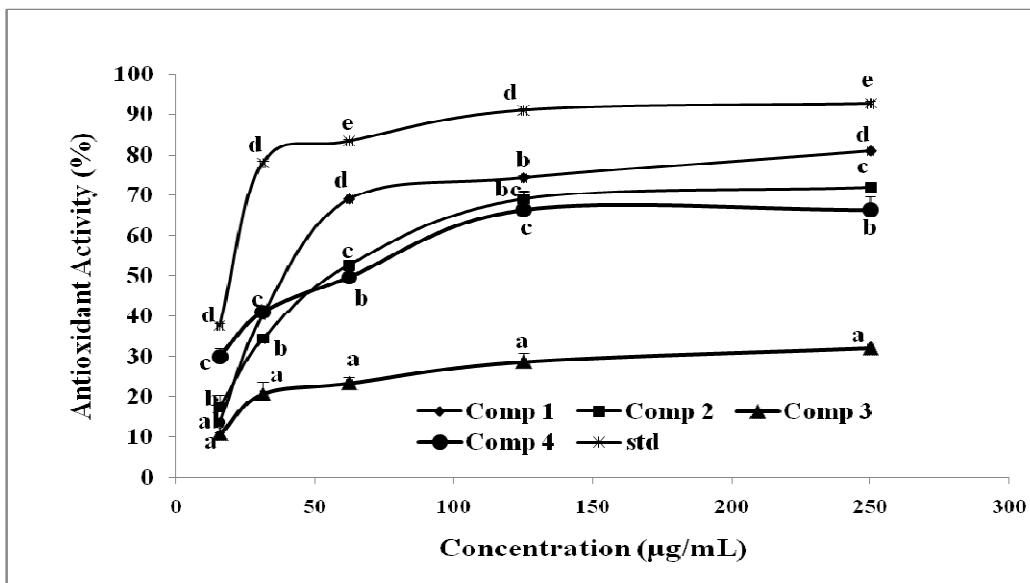


Figure 2: Antioxidant activity of compounds 1-4 and ascorbic acid standard, as measured by the DPPH method.

The hydroxyl radical scavenging activities in the deoxyribose assay are shown in figure 3. The results revealed that compound **1** possessed significantly higher activity than all the other compounds tested, including the standard, ascorbic acid, at most concentrations. Compounds **1**, **2** and **4** had hydroxyl radical scavenging activity comparable with and in the case of **1** and **2**, better than that of ascorbic acid. Skimmianine (**5**) was not tested in either the DPPH or deoxyribose assays due to insufficient amount.

The three assays revealed that compounds **1** and **2** are good antioxidant compounds, while compound **4** shows high activity at a lower concentration in the DPPH assay. Flavonoids are known to be potent antioxidants and their activity is dependent on their molecular structure. The activity of **1** could be attributed to the hydroxyl (OH) groups in the molecule, which donate hydrogen to reduce the DPPH radical to DPPH-H. The alkaloids **2-5** have the same basic skeleton, the only difference being in their side chain.

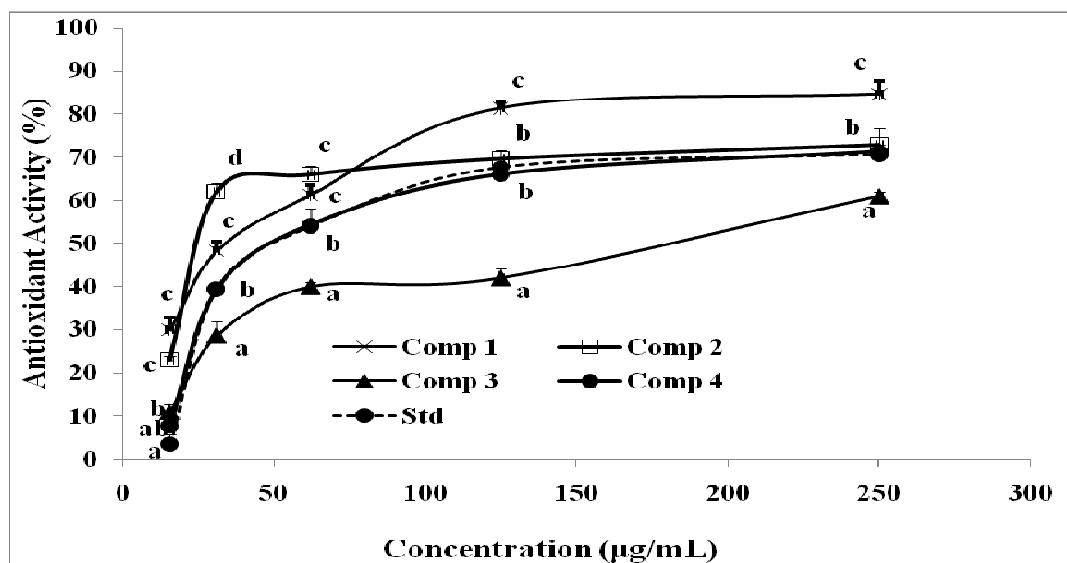


Figure 3: Hydroxyl radical scavenging activity of compounds 1-4 and standard ascorbic acid as measured by the deoxyribose method.

The reductive ability of **2** may be attributed to the double bond of the isoprenyl unit, rich in delocalized pi-electrons, which are easily donated during reduction of Fe^{3+} to Fe^{2+} . Sang *et al.* also reported that the double bond of the isoprenyl group was responsible for the antioxidant activity of garcinol [21]. The antioxidant activity of **2** in the DPPH assay, like **1**, could also be attributed to the hydroxyl groups in the molecule.

In conclusion, five compounds were isolated (a flavonoid and four alkaloids) from the aerial parts of *V. glomerata*. Verification of their antioxidant activities, as well as comparison with known antioxidants, will provide herbalists and traditional healers with scientific evidence for the use of the aerial parts of this plant as natural antioxidants.

Experimental

General experimental procedures: The melting points were recorded on an Ernst Leitz Wetzler micro-hot stage melting point apparatus and are uncorrected. UV spectra were obtained on a Varian Cary UV-VIS Spectrophotometer in chloroform. IR spectra were recorded on a Perkin-Elmer Universal ATR Spectrometer. The 1D and 2D NMR spectra were recorded using a Bruker Avance^{III} 400 MHz NMR spectrometer. All the spectra were recorded at room temperature using deuterated chloroform (CDCl₃) as solvent. The HREIMS was measured on a Bruker Micro TOF-QII instrument. Specific rotations were measured at room temperature in chloroform on a PerkinElmerTM, Model 341 Polarimeter with a 10 mm flow tube. The separation, isolation and purification of compounds were carried out by gravity CC and monitored by TLC. Merck silica gel 60 (0.040-0.063 mm) was used for CC. Merck 20 × 20 cm silica gel 60 F₂₅₄ aluminum sheets were used for TLC. TLC plates were analyzed under UV light (254 and 366 nm) before being sprayed with anisaldehyde: concentrated sulfuric acid: methanol [1:2:97] spray reagent and then heated.

Plant material: *Vepris glomerata* was collected from the Rift Valley province of Kenya and identified by Dr S. T. Kariuki from the Department of Botany, Egerton University, Kenya. A voucher specimen (Kiplimo 01) was deposited at the University of KwaZulu-Natal Ward Herbarium, Westville Campus, Durban, South Africa.

Extraction and isolation: The air-dried aerial parts (980 g) of *V. glomerata* were sequentially extracted with *n*-hexane, followed by dichloromethane in a Soxhlet apparatus for 48 h, yielding crude extracts of 46 and 32 g, respectively. The oily residue of the dichloromethane extract obtained after evaporation under vacuum, was separated by CC on silica gel with *n*-hexane and then increasing the concentration of ethyl acetate from 10 to 80% in *n*-hexane, to give 10 fractions (fr.); fr. 8-16 (1.27 g), fr. 17-19 (0.5 g), fr. 20-26

(2.36 g), fr. 27-32 (2.35 g), fr. 33-39 (1 g), fr. 40-43 (2.1 g), fr. 44-49 (0.5 g), fr. 52-56 (3.9 g), fr. 57-62 (1.75 g) and fr. 63-67 (5.1 g).

Fraction 52-56 was separated by CC with *n*-hexane/EtOAc (7:3) as the solvent to afford sub-fractions A-C. Sub-fraction A was further purified using 100% dichloro-methane to afford compound **2**, a green solid (51 mg). Sub-fraction B yielded compound **3**, a brownish solid (43 mg), which needed no further purification. Sub-fraction C was crystallized in methanol to afford **4** (62 mg). Fraction 44-49 was purified using 100% dichloromethane to afford **5** (60 mg). Fraction 63-67 was separated with *n*-hexane/EtOAc (4:1) to yield 4 sub-fractions A-D. Sub-fraction B was crystallized in methanol to afford yellow crystals of compound **1** (18 mg).

Veprisinol (1); 4H-1-Benzopyran-4-one, 2, 3-dihydro-5-hydroxy-2-(4'-methoxy-3'-hydroxybenzyl)-7-O-(2-methyl butanol) ether.

Yellow solid.

M.p: 78-80°C

$[\alpha]_D^{20}$: +55.30 (*c* 0.056 CHCl₃)

IR: 3364 (O-H), 2928, 1705 (C=O), 1636, 1512, 1162 cm⁻¹

UV λ_{\max} (CHCl₃) nm (log ϵ): 337 (4.45), 285 (5.13), 239 (5.44)

¹H NMR (400 MHz, CDCl₃): 11.97 (H, s, OH), 7.00, (H, s, H-2'), 6.89 (H, d, *J* = 8.28 Hz, H-5'), 6.84 (H, d, *J* = 8.28 Hz, H-6'), 6.02 (H, d, *J* = 1.76 Hz, H-6), 6.00 (H, d, *J* = 1.76 Hz, H-8), 5.29 (H, dd, *J* = 12.84, 2.84 Hz, H-2β), 4.02 (2H, dd, *J* = 12.88, 6.24 Hz, 2H-1''), 3.88 (3H, s, OCH₃), 3.50 (2H, d, *J* = 5.68 Hz, 2H-4''), 3.04 (H, dd, *J* = 17.12, 12.84 Hz, H-3α),

2.75 (H, dd, $J = 17.12, 2.84$, Hz, H-3 β), 1.87 (2H, m, H-2''a and H-3''), 1.61 (H, m, H-2''b), 0.95 (3H, d, $J = 6.52$ Hz, H-5'').

^{13}C NMR: 195.97 (C, C-4), 167.29 (C, C-7), 164.05 (C, C-5), 162.85 (C, C-9), 147.02 (C, C-4'), 145.93 (C, C-3'), 131.52 (C, C-1'), 118.15 (CH, C-5'), 112.71 (CH, C-2'), 110.71 (CH, C-6'), 103.11 (C, C-10), 95.54 (CH, C-6), 94.60 (CH, C-8), 78.92 (CH, C-2), 67.86 (CH₂, C-4''), 66.70 (CH₂, C-1''), 56.06 (OCH₃), 43.15 (CH₂, C-3), 32.94 (CH, C-3''), 32.37 (CH₂, C-2''), 16.60 (CH₃, C-5'').

HREIMS m/z 388.1573 [M]⁺ (calcd. for C₂₁H₂₄O₇, 388.1522)

Antioxidant activity: The total reducing power was determined according to the method described previously [22]. The free radical scavenging activity (antioxidant capacity) of the plant phytochemicals on the stable radical 2, 2-diphenyl- β -picrylhydrazyl (DPPH) was evaluated by the method established by Shirwaikar *et al.* [23], and the deoxyribose assay for hydroxyl radical scavenging activity was performed as described previously by Chung *et al.* [24].

Statistical analysis: The data in figures 1-3 are presented as mean \pm SD of triplicates. ^{a-} ^dValues with different superscript letters for a given concentration are significantly different from each of the other compounds. The data were statistically analyzed using a statistical software program SPSS (SPSS for Windows, version 18, SPSS Science, Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by Tukey's multiple range post-hoc test was employed to find the differences. The data were considered significantly different at $p < 0.05$.

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CHAPTER FIVE

**ANTIBACTERIAL ACTIVITY OF AN EPOXIDISED
PRENYLATED CINNAMALDEHYDE DERIVATIVE FROM
*VEPRIS GLOMERATA***

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Abstract

A prenylated cinnamaldehyde derivative (glomeral), together with the known *p*-hydroxycinnamic acid, caffeic acid, methyl cinnamate, hesperetin, scoparone, skimmianine, syringaresinol and two limonoids (limonin and limonyl acetate) were isolated from the roots and stem bark of *V. glomerata*. The antibacterial assay of the isolated compounds indicated an inhibition zone, ranging from 8 to 16 mm, against standard strains of *Staphylococcus aureus* (ATCC 29213, 25923) and *Shigella dysenteriae*. Glomerol inhibited the growth of *Staphylococcus aureus* and *Shigella dysenteriae* at low concentrations (MIC of 2 µg mL⁻¹ and 0.4 µg mL⁻¹ respectively). Of the other compounds tested, hesperetin displayed good antibacterial activity, the limonoids, scoparone and skimmianine displayed moderate antibacterial activity and the cinnamic acid derivatives were inactive against the test pathogens. This study provides a rationale for the use of *V. glomerata* in its treatment of bacterial infections.

Keywords: *Vepris glomerata*; Rutaceae; glomerol; antibacterial activity.

Introduction

There are about eighty species of the genus *Vepris*, formerly known as *Teclea* distributed in Tropical Africa, Madagascar, the Mascarenes, Tropical Arabia and Southwest India (Chaturvedula *et al.*, 2003). *Vepris glomerata* is a small tree that grows to a height of approximately 7.5 m. In Africa it is distributed in the dry bushland of Sudan, Ethiopia, Somalia, Kenya and Tanzania (Beentje, 1994). A decoction of the roots is used traditionally in the treatment of malaria, whilst the vapour is used to treat eye problems. A decoction of the bark is used in the treatment of cardiac pain while epilepsy, stroke and psychosis is treated using an aqueous root extract of the plant mixed with tea (Innocent *et al.*, 2009; Moshi *et al.*, 2006).

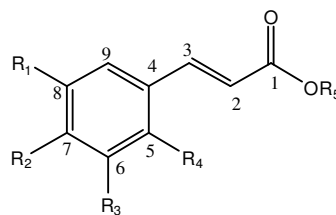
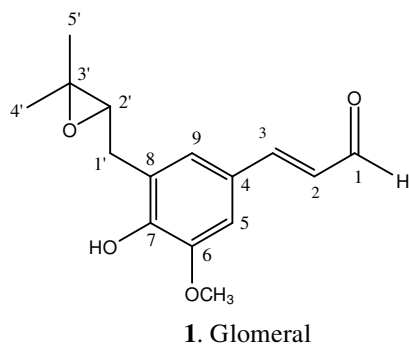
Previous investigations of *Vepris* species indicated that it is rich in furoquinoline alkaloids (Govindachari and Sundararajan, 1961; Ganguly *et al.*, 1966; Rudolf and Eva Maria, 1978; Dagne *et al.*, 1988; Chaturvedula *et al.*, 2003), acridone alkaloids (Rasoanaivo *et al.*, 1999), pyranoquinoline alkaloids (Brader *et al.*, 1996), triterpenoids and sesquiterpenoids (Khalid and Waterman, 1982; Chaturvedula *et al.*, 2004), limonoids (Ngadjui *et al.*, 1982; Cheplogoi *et al.*, 2008), azoles and imides (Cheplogoi *et al.*, 2008) and essential oils (Sidibe *et al.*, 2001; Poitou *et al.*, 1995; Denise and Favre-Bonvin, 1973).

With regard to *V. glomerata*, only one phytochemical study has been undertaken where two furoquinoline alkaloids (skimmianine and monrifoline) have been isolated from the aerial parts of *V. glomerata* indigenous to Ethiopia (Dagne *et al.*, 1988). In terms of pharmacological activity, the ethanolic crude extract of *V. glomerata* collected from Tabora (Tanzania) exhibited antimalarial activity against *Plasmodium berghei* (Innocent *et al.*, 2009). The current study was undertaken primarily to re-investigate the phytochemistry of *V. glomerata* since only two alkaloids were previously found and to test the isolated

compounds for antimicrobial activity since extracts of Rutaceae species have been cited as antimicrobials in traditional medicine (Kokwaro, 1976). Furthermore, limonoids which were isolated in this work are reported to have antifungal activity (Govindachari *et al.*, 1998) and natural phenyl propanoids to which compounds **1-4** are structurally related are well known antibacterial agents (Jimenez and Riguera, 1994). This is the first phytochemical investigation of the roots and stem bark of *V. glomerata*.

Results and discussion

Of the two furoquinoline alkaloids previously isolated, only skimmianine (**7**) (Latip *et al.*, 2005) was isolated in this work. Montrifoline was not detected in the stem bark and roots and may be confined to the aerial parts of the plant. Several other compounds, including a new prenylated cinnamaldehyde derivative (glomeral **1**), known aromatic acids, (*p*-hydroxycinnamic acid **2** and caffeic acid **3** (Lu *et al.*, 1999)), an aromatic ester (methyl cinnamate **4** (Lu *et al.*, 1999)), a flavonoid (hesperetin **5** (Wawer and Zielinska, 2001)), a coumarin (scoparone **6** (Lee *et al.*, 2002)), a lignin (syringaresinol **8** (Ouyang *et al.*, 2007)), and two limonoids (limonin **9** (Khalil *et al.*, 2003) and limonyl acetate **10** (Ruberto *et al.*, 2002)) (Figure 1) were isolated from the stem bark and roots of *V. glomerata*. Compounds **2-10** were identified by their ^1H and ^{13}C NMR spectra and by comparison with spectroscopic data in literature.



- 2.** $R_1 = \text{H}$, $R_2 = \text{OH}$, $R_3 = \text{H}$, $R_4 = \text{H}$, $R_5 = \text{H}$
3. $R_1 = \text{H}$, $R_2 = \text{H}$, $R_3 = \text{OH}$, $R_4 = \text{OH}$, $R_5 = \text{H}$
4. $R_1 = \text{OH}$, $R_2 = \text{OH}$, $R_3 = \text{H}$, $R_4 = \text{H}$, $R_5 = \text{CH}_3$

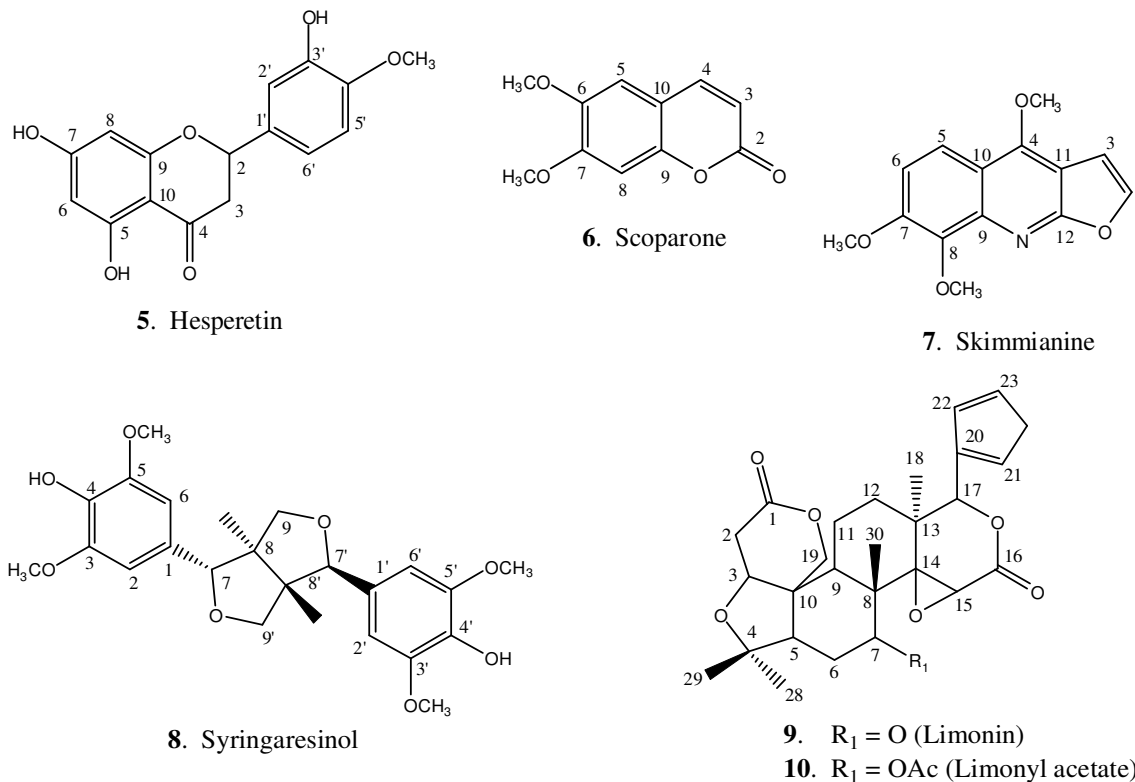


Figure 1: Structures of compounds isolated from *Vepris glomerata*

The HREIMS data of compound (**1**) showed a molecular ion peak at m/z 263.1278 $[M+H]^+$, corresponding to the molecular formula of $C_{15}H_{18}O_4$ and indicating seven double bond equivalents. The IR spectrum of compound **1** revealed the presence of OH and carbonyl stretching frequencies at 3348 cm^{-1} and 1713 cm^{-1} respectively. The ^1H NMR spectrum of compound **1** showed characteristic resonances for prenylated cinnamaldehydes with resonances at δ_{H} 6.57 (1H, dd, $J = 15.80, 7.74$ Hz), δ_{H} 7.37 (1H, d, $J = 15.80$ Hz) and δ_{H} 9.59 (1H, d, $J = 7.74$ Hz), with the large coupling constant of 15.80 Hz indicative of *trans* olefinic protons and were attributed to H-2, H-3 and the aldehyde proton, H-1, respectively. This coupling was also evident in the COSY spectrum.

An ABX coupled system could be seen in the second proton system, which consisted of a triplet at δ_{H} 4.74 (1H, t, $J = 9.15$ Hz, H-2'), which coupled in the COSY spectrum to the two doublet resonances at δ_{H} 3.18 and δ_{H} 3.21, H-1'a and H-1'b, both with $J = 9.15$ Hz. The H-

1'a and H-1'b resonances occurred separately, since these were diastereotopic protons. The two methyl resonances at δ_{H} 1.34 and δ_{H} 1.24 showed HMBC correlations to the C-2' methine carbon resonance at δ_{C} 91.09 and a quaternary carbon resonance at δ_{C} 71.74, C-3'. These COSY and HMBC correlations indicated the presence of a prenyl group with an epoxide ring at C-2' and C-3', also confirmed by the molecular mass of the compound.

Also present in the ^1H NMR spectrum were two aromatic resonances at δ_{H} 6.91 and δ_{H} 7.02 and an aromatic methoxy proton resonance at δ_{H} 3.93. Apart from the C-6 resonance, a further deshielded aromatic C-O resonance was detected at δ_{C} 151.23 indicating the presence of a further aromatic hydroxyl group. The fifteen carbon resonances detected in the ^{13}C NMR spectrum indicated that the substituents on the benzene ring were the cinnamaldehyde group, the prenyl group, the two aromatic hydrogen atoms, the methoxy and hydroxyl group. The prenyl group was placed at C-8 because 2H-1' showed an HMBC correlation to C-9. The remaining hydroxyl group was then placed at C-7.

The absolute configuration of glomerol (**1**) was established by CD exciton chirality method which showed a positive cotton effect at 328 nm ($\Delta\epsilon$ +3.0) due to the $n \rightarrow \pi^*$ transition of the carbonyl group, a negative cotton effect at 292 nm ($\Delta\epsilon$ -0.5) due to the $\pi \rightarrow \pi^*$ transition of the double bond and a positive cotton effect at 218 nm ($\Delta\epsilon$ +6.5) due to the $\pi \rightarrow \pi^*$ transition of the α , β unsaturated aldehydic carbonyl group. The configuration of the chiral carbon C-2' was established to be 2'R in comparison with methyl 12,14-dihomojuvenate which was determined to be (*E,E*)-(10*R*,11*S*)-10,11-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate through Horeau's method (Meyer et al., 1971). Studies indicate that, when the epoxide ring is opened by LiAlH_4 in Et_2O under reflux to yield alcohols, the absolute configuration of the obtained alcohol was established to be *S* and the CD spectrum exhibited a positive exciton

chirality, indicating that the starting material had the R configuration (Solladie-Cavallo et al., 2004). Unfortunately, we did not have sufficient sample to carry out this experiment.

This is the first report of compound **1** and has been given the trivial name glomerol.

Glomerol (**1**) shared high structural resemblance to 3-(3-methyl-2-epoxy-butyl)-*P*-coumaric acid methyl ester isolated from *Psoralea plicata* (leguminosae) except that glomerol (**1**) has an additional methoxy group at C-6 as well as the cinnamaldehyde group at C-4 instead of the ester group (Hamed *et al.*, 1997).

The antibacterial results of the pure compounds (**1**, **5**, **6**, **7**, **9** and **10**) indicate a broad spectrum activity against the test organisms *Staphylococcus aureus* (ATCC 29213 and 25923) and *Shigella dysenteriae* (Table 1). No results were recorded for *Escherichia coli* and the fungal strains investigated since all compounds tested were inactive against these microbes. Compounds **2-4** and **8** were also inactive against all tested strains and therefore the results are not included in the table. The inhibition zone determination shows that the new compound, glomerol (**1**) exhibited good activity with inhibition zones of 16 mm against *S. dysenteriae*, and 15 mm and 14 mm against *S. aureus* (ATCC 29213) and *S. aureus* (ATCC 25923) respectively. Hesperetin (**5**) and scoparone (**6**) also showed good activity with inhibition zones of between 9 and 14 mm against *S. dysenteriae* and the two cultures of *S. aureus*. The observed results for these compounds were comparable with that of chloroamphenicol with an inhibition zone of 20 mm against *S. aureus* and 18 mm against *S. dysenteriae*. The remaining compounds (skimmianine (**7**), limonin (**9**), and limonyl acetate (**10**)) did not show good antibacterial activities.

Table 1: Antibacterial activity of the isolated compounds

Compound	Inhibition zones (mm)			Minimum inhibitory concentration MIC ($\mu\text{g/mL}$)		
	<i>S.a</i>	<i>S.a</i>	<i>S.d</i>	<i>S.a</i>	<i>S.a</i>	<i>S.d</i>
	ATCC 29213	ATCC 25923		ATCC 29213	ATCC 25923	
Glomerol 1	15 \pm 1.0	14 \pm 1.2	16 \pm 1.0	2	2	0.4
Hesperetin 5	12 \pm 1.3	11 \pm 1.0	14 \pm 1.1	0.2	0.2	4
Scoparone 6	10 \pm 1.1	09 \pm 1.5	16 \pm 1.6	16	16	0.2
Skimmianine 7	09 \pm 1.2	06 \pm 1.2	10 \pm 1.3	16	>32	16
Limonin 9	08 \pm 1.6	07 \pm 1.3	08 \pm 1.4	>32	>32	16
Limonin acetate 10	08 \pm 1.4	08 \pm 1.2	10 \pm 1.5	>32	>32	32
Chloroamphenicol	20 \pm 1.0	20 \pm 1.3	18 \pm 1.7	2	2	8

Key: *S.a* – *Staphylococcus aureus*, *S.d* - *Shigella dysenteriae*

The minimum inhibitory concentration (MIC) determination (Table 1) showed that hesperetin (**5**) completely inhibited the growth of *S. aureus* (ATCC 29213 and ATCC 25923) and *S. dysenteriae*, at low concentrations of 0.2 $\mu\text{g/mL}$ and 4.0 $\mu\text{g/mL}$ respectively, even lower than that of chloroamphenicol. The MIC value of glomerol was the same as that of chloroamphenicol for *S. aureus* at 2.0 $\mu\text{g/mL}$ and twenty times lower (0.4 $\mu\text{g/mL}$) than chloroamphenicol against *S. dysenteriae*. Scoparone (**6**) was the most active of all the compounds tested against *S. dysenteriae* at 0.2 $\mu\text{g/mL}$ being forty times lower than that of chloroamphenicol in the same assay. The rest of the compounds (**7**, **9** and **10**) had MIC values of either 16 $\mu\text{g/mL}$ or greater than 32 $\mu\text{g/mL}$.

These results are consistent with previous findings that showed phenyl propanoid derivatives (which are structurally related to glomerol) isolated from *Ballota nigra* (Lamiaceae) to possess moderate activity against *S. aureus* (Didry et al., 1999).

Experimental

3.1 General experimental procedures. The ^1H , ^{13}C and all 2D NMR spectroscopy were recorded using a Bruker Avance^{III} 400 MHz spectrometer. The spectra were referenced according to the deuteriochloroform signal at δ_{H} 7.24 (for ^1H NMR spectra) and δ_{C} 77.0 (for

¹³C NMR spectra). The HREIMS was measured on Bruker Micro TOF-QII instrument. IR spectra were recorded using a Perkin Elmer Universal ATR spectrometer. Optical rotations were measured at room temperature in a PerkinElmer™, Model 341 Polarimeter with 10 cm flow tube. The Circular Dichroism (CD) spectrum was recorded on chirascan plus spectropolarimeter by applied photophysics at wavelengths 190-400 nm. The melting points were determined on an Ernst Leitz Wetzlar micro-hot stage melting point apparatus. Merck silica gel 60 (0.040-0.063 mm) was used for column chromatography and Merck 20 × 20 cm silica gel 60 F₂₅₄ aluminium sheets were used for thin-layer chromatography. The TLC plates were analysed under UV (254 and 366 nm) before being sprayed and developed with a [1:2:97] anisaldehyde: concentrated sulphuric acid: methanol spray reagent and then heated.

3.2 Plant material. The stem bark and roots of *V. glomerata* were collected in December, 2009 in Rift Valley Province of Kenya. The plant was identified by Dr S. T. Kariuki of the Department of Botany, Egerton University. A voucher specimen (Kiplimo 01) has been retained at the University of KwaZulu-Natal Ward Herbarium, Westville, Durban.

3.3 Serial extraction and isolation. The air-dried and ground plant material of *V. glomerata* (823.3 g stem bark and 719.3 g roots) was sequentially extracted for 24 hours using a soxhlet apparatus with solvents of increasing polarity; hexane, dichloromethane, ethyl acetate and methanol. The collected solution of each extract was individually evaporated using a rotavapor.

Crude extracts were loaded onto a column (4.5 in diameter) packed with a silica gel slurry to a height of 30 cm. For each elution system 2 L volumes were used and 100 mL fractions collected. The collected fractions were analysed using TLC to determine if separation had occurred. Similar fractions were combined and concentrated using a rotavapor.

The dichloromethane extract from the roots was separated with a hexane: ethyl acetate step gradient starting from 100% hexane to 80% ethyl acetate in hexane. The following fractions were combined and purified using the given solvent systems; 19-28 (hexane: ethyl acetate, 80:20) which yielded glomerol (**1**) (56 mg), 52-55 (hexane: ethyl acetate, 70:30) which yielded scoparone (**6**) (19.7 mg), 60-70 (dichloromethane: ethyl acetate, 80:20) yielded skimmianine (**7**) (23 mg), 85-92 (dichloromethane: ethyl acetate, 70:30) yielded *meso*-syringaresinol (**8**) (5.3 mg).

The ethyl acetate extract from the stems yielded compounds **2-5**. The mobile phase in the crude separation consisted of a hexane: ethyl acetate step gradient. Compound **5** (19.3 mg) was obtained from fraction 36-39 and further purified with (70:30) hexane: ethyl acetate. *Trans*-*p*-hydroxy cinnamic acid compound **2** (9.2 mg) was obtained from fraction 27-31 and purified by 100% dichloromethane. Compound **3** (22.3 mg) was obtained from fraction 84-93 and purified with a 1:1 ratio of ethyl acetate and hexane. Fraction 40-48 was purified by 30% ethyl acetate in dichloromethane to yield compound **4** (11.3 mg).

The dichloromethane extract of the stems afforded two limonoids, after the crude extract was separated using a hexane: ethyl acetate step gradient. Both were obtained from fractions 32-49 using hexane: ethyl acetate (70:30). Fraction 32-45 was purified using hexane: ethyl acetate, 80:20 to afford limonin, **9** (21 mg). Fraction 46-49 was purified with 100% dichloromethane which yielded limonyl acetate **10** (33 mg).

3.3.1 Glomerol (1) reddish brownish solid; mp 58-60 °C; $[\alpha]_D^{20} +27.8^\circ$ (*c* 0.016, CHCl₃); UV λ_{max} (CHCl₃) nm (log ϵ) 253 (1.92); IR (cm⁻¹): 3348 (-OH), 2927 (-CHO), 2854, 1713 (>C=O), 1616, 1490; ¹H NMR spectral data (400 MHz, CDCl₃) δ_H 9.59 (1H, d, *J* = 7.80 Hz, H-1), 7.37 (1H, d, *J* = 15.80 Hz, H-3), 7.02 (1H, s*, H-9), 6.91 (1H, s*, H-5), 6.57 (1H, dd, *J* = 15.80, 7.80 Hz, H-2), 4.74 (1H, t, *J* = 9.15, Hz, H-2'), 3.93 (3H, s, OCH₃),

3.18 (1H, d, $J = 9.15$ Hz, H-1'a), 3.21 (1H, d, $J = 9.15$ Hz, H-1'b), 1.35 (3H, s, CH₃-5'), 1.21 (3H, s, CH₃-4'); ¹³C NMR spectral data (100 MHz, CDCl₃) δ_C 193.74 (C-1), 153.38 (C-3), 151.23 (C-7), 144.46 (C-6), 129.37 (C-8), 127.88 (C-4), 126.28 (C-2), 118.69 (C-9), 111.57 (C-5), 91.09 (C-2'), 71.74 (C-3'), 56.05 (OCH₃), 30.64 (C-1'), 26.12 (C-5'), 24.18 (C-4'); EIMS (70 eV) m/z (rel. int): 262 [M⁺] (100), 229 (13), 203 (30), 191 (13), 173 (24), 161 (15), 115 (17); HREIMS (70 eV) 263.1254 [M+1]⁺ (Calc. for C₁₅H₁₉O₄).

*These resonances were not resolved into doublets with $J = 2-3$ Hz as expected for meta coupling, however the resonances were slightly broadened and also coupled in the COSY spectrum, indicating meta coupling.

3.4 Antimicrobial assay. Gram-negative bacteria, *Escherichia coli* and *Shigella dysenteriae*, and Gram-positive bacteria, *Staphylococcus aureus* ATCC 29213 and ATCC 25923 were used for the antibacterial assay and fungal strains, *Candida albicans*, *Candida parapsilosis*, *Cryptococcus neoformans* and *Trichophyton mentagrophytes* were used for the antifungal assay. The bacterial and fungal strains were cultured for 18 hours at 37 °C on Muller Hinton agar (and saboraud dextrose agar for fungi) and standardized to a final cell density of 1.5×10^8 cfu/ml using a barium sulphate standard equivalent to McFarland No. 0.5 standard or its optical equivalent.

Disc diffusion method: Blank discs were sterilized by air drying at 160 °C for 1 h. Test compounds were dissolved in DMSO and 20 µl of the solution (compound and DMSO) impregnated onto the blank sterile discs and placed aseptically onto the inoculated petri dish, which were then incubated at 37 °C for 24 hours for the bacteria while the fungi were incubated for 72 hours at 25 °C. The activity of the compounds was determined by the presence of measureable inhibition zones.

Broth microdilution method (Andrews, 2001): The test compounds and the standard chloroamphenicol (fluconazole for the fungi) were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mg/ml. Serial two fold dilutions were made ranging from 0.002 µg/ml to 32 µg/ml in sterile test tubes. The 96-well plates were prepared by dispensing into each well, 90 µl Muller Hinton and 10 µl of the bacterial inoculums. The first three wells containing Muller Hinton broth were used as a negative control and the next three containing 90 µl Muller Hinton broth and 10 µl of the bacterial inoculums without the test compound were used as growth control. The standard antibiotic tetracycline was used as a positive control. The plates were covered to avoid contamination and evaporation and incubated for 24 hours at 37 °C. All procedures were done in triplicate. The lowest concentration of each compound showing no visible growth was taken as its minimum inhibitory concentration (MIC).

Conclusion

The phytochemical analysis of *V. glomerata* showed the existence of the antimicrobial compounds, glomerol (a new cinnamaldehyde), hesperetin and scoparone. Skimmianine, limonin and limonyl acetate showed low to moderate antibacterial activity. These results provide scientific validity and credence to the ethnomedicinal use of this plant in the treatment of ailments caused by some of the pathogenic microbes used in this study and highlights the efficacy of *V. glomerata* in the treatment of bacterial infections. Further studies on structure-activity relationships and mode of action may be a guide to a better understanding of the relationships between the structures and antimicrobial activities of these compounds.

Acknowledgments

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CHAPTER SIX

**RING A, D-SECO LIMONOIDS AND FLAVONOID FROM
THE KENYAN *VEPRIS UGUENENSIS* ENGL. AND THEIR
ANTIOXIDANT ACTIVITY**

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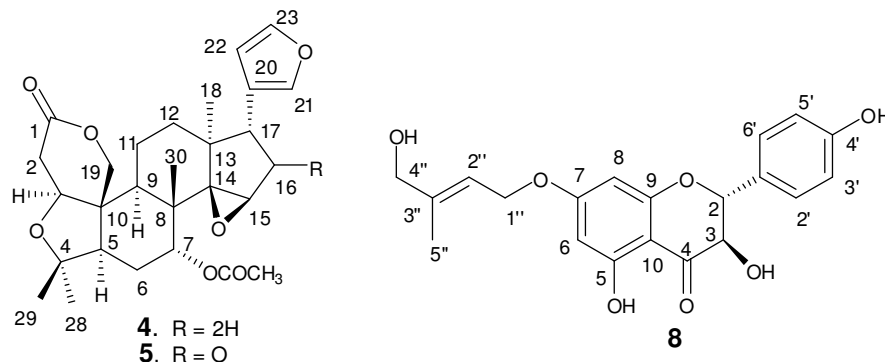
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ABSTRACT

Two new A, D-*seco*-limonoids, accorded the trivial names, uguenensene (**4**) and uguenensone (**5**) and a new C-7 prenylated flavonoid, uguenenprenol (**8**) were isolated from *Vepris uguenensis* (*Rutaceae*). In addition, eleven known compounds, niloticin (**1**), chisocheton A (**2**), kihadalactone A (**3**), limonyl acetate (**6**), methyl uguenenoate (**7**), 7-O-methylaromadenrin (**9**), flindersiamine (**10**), 8 α ,11-elemodiol (**11**), tricoccin S₁₃ acetate, skimmianine, and lupeol were isolated. The structures of the new compounds were elucidated and characterized by spectroscopic analyses (NMR, GC-MS and IR). Antioxidant activity of the isolated compounds using the 2,2-diphenyl- β -picrylhydrazyl (DPPH), Deoxyribose and Ferric Reducing Antioxidant Power (FRAP) assays showed that uguenenprenol (**8**) and 7-O-methylaromadenrin (**9**) are good antioxidant agents. Significantly high antioxidant activity was also exhibited by 8 α ,11-elemodiol (**11**), which was 72% at 250 $\mu\text{g mL}^{-1}$ and 57% at 15.62 $\mu\text{g mL}^{-1}$ when tested with the Deoxyribose

method. The current contribution adds uguenensene (**4**) and uguenensone (**5**) to the class of citrus limonoids common to the Rutaceae which can be used not only for medicinal purposes but also as an antioxidant supplement.



INTRODUCTION

Limonoids are tetranortriterpenoids derived from the acetate-mevalonate pathway with the triterpenoids euphane or tirucallane being the key intermediates^{1,2}. Citrus limonoids have been shown to originate from nomilin which is biosynthesized in the phloem region of stems and then migrate to other tissues such as leaves, fruits and seeds, where other limonoids are biosynthesized³. Limonoids are the main constituents of the Rutaceae and are known to have a wide range of biological activities. The biological activities of limonoids have attracted widespread scientific interest; they are reported to exhibit antifungal⁴, antibacterial⁵, antimalarial⁶, antifeedant⁷, antiprotozoan⁸, antiviral⁹ and anti-inflammatory¹⁰ activities, and recently, the antioxidant capacity of citrus limonoids and limonoid-containing extracts have been evaluated using the racimat experiment, superoxide radical quenching and the DPPH radical scavenging assays¹¹. Limonoids have also been known to inhibit the development of cancer in laboratory animals and in human breast cancer cells¹².

The plant *Vepris uguenensis* is known as ‘Chemchir’ by the Pokot tribe of Kenya, who use it to treat malaria¹³. Previous phytochemical reports indicated that a limonoid (methyl

uguenenoate), an azole (uguenenazole) and an imide (uguenenonamide) were isolated from the roots of *V. uguenensis*¹³. Methyl uguenenoate displayed mild antimalarial activity while the azole and the imide were found to be completely inactive¹³. No phytochemical compounds from the stem bark and leaves of *V. uguenensis* have been reported thus far.

Oxidative stress is a situation where there is an imbalance between the production of reactive oxygen species (ROS) that can damage cell structures and the body's ability to detoxify these molecules or repair the resulting damage. These reactive oxygen species (ROS) have been found to mediate neurological injury in cerebral malaria (CM) which is the most severe neurological complication of infection with *Plasmodium falciparum*¹⁴. During an attack of malaria, the parasite breaches the blood–brain barrier to cause cerebral malaria (CM), resulting in a life-threatening crisis¹⁴. With suitable treatment, patients do recover but often do so with lasting damage to their brain, resulting in a loss of mental function¹⁴. Studies have shown that the lives of many African children have been shattered in this way. It is reported that 21% of children with CM had cognitive deficits six months after discharge from hospital in Uganda¹⁵. This is because cerebral malaria leads to increased production of molecules indicative of oxidative stress in the brain¹⁴. Treatment with a combination of chloroquine and two antioxidant agents, desferoxamine and *N*-acetylcysteine, at the first signs of cerebral malaria prevents both inflammatory and vascular changes in the tissues of the brain, as well as the development of persistent cognitive damage¹⁴. The addition of antioxidants does not weaken the efficacy of chloroquine in eliminating Plasmodia from the blood. Combination therapy with antioxidants has been effective in treating cerebral malaria and preventing subsequent cognitive impairment in mice¹⁴.

Since *V. uguenensis* is used as an antimalarial in African traditional medicine, and limonoids are reported to possess antioxidant activity¹¹, all the isolated compounds were

subjected to antioxidant assays using the ferric reducing antioxidant power (FRAP), the 2,2-diphenyl- β -picrylhydrazyl (DPPH) and the deoxyribose methods. We present herein the details of the isolation and structure elucidation of three new compounds, a C-7 prenylated flavonoid (**8**) and two limonoids (**4** and **5**) as well as the *in vitro* antioxidant activities of the isolated compounds and a brief discussion on the biogenetic relationship of the limonoids (**1-7**).

RESULTS AND DISCUSSION

Repeated column chromatography of the hexane and dichloromethane extract of the leaves, stem bark and roots yielded eleven known compounds; a triterpenoid (lupeol), a sesquiterpene (8 α ,11-elemodiol (**11**)), two furoquinoline alkaloids (skimmianine and flindersiamine (**10**)), a flavonoid (7-O-methylaromadentin (**9**)), two proto-limonoids (niloticin (**1**) and chisocheton A (**2**)) and four limonoids (tricoccin S₁₃ acetate, kihadalactone A (**3**), limonyl acetate (**6**) and methyl uguenenoate (**7**)). A further two novel A, D-seco limonoids and a prenylated flavonoid, which were accorded the trivial names; uguenensene (**4**), uguenensone (**5**) and uguenenprenol (**8**) were isolated. Structures of known compounds were determined using NMR and MS techniques and confirmed by comparison of physical and spectroscopic properties against literature data¹⁶⁻²¹.

The isolated compounds (**1-7**) displayed an interesting limonin biogenetic sequence with structural features expected of intermediates in the limonin biosynthetic pathway (Figure 1). According to the generally accepted scheme, the Δ^7 bond as in niloticin (**1**), is epoxidized to the 7-epoxide and then opened, inducing a Wagner-Meerwein shift of the methyl group at C-14 to C-8 leading to the formation of a double bond at Δ^{14} in **2**, together with modification of the side chain². This is followed by side chain cleavage of the four carbon epoxide group and the expansion of the carbocyclic A-ring in **2** to a seven membered δ -unsaturated lactone

ring as seen in tricoccin S₁₃ acetate. Reduction of the side chain lactone ring to form the furan ring, as well as acetylation at C-1 results in **3**. Contraction of ring A to a five membered ring and lactonization involving the methyl group (CH₃-19) and the carbonyl group at C-3 leading to the formation of A and A' rings (A-seco), occurs with a concurrent epoxidation at Δ^{14} , producing **4**. Ring contraction and lactone formation may occur simultaneously. Oxidation at C-16 results in **5**, which is followed by a Baeyer-villiger oxidation of the D ring to a lactone resulting in **6**. Methanolysis of the A ring lactone results in **7**. Figure 1 shows the postulated biogenetic pathway for the formation of the proto-limonoids and limonoids **1-7**, with **4** and **5** taking its place in the biosynthetic scheme.

Compound **4** was isolated as a reddish brown solid and had a molecular formula of C₂₈H₃₆O₇ as determined by HREIMS, with a molecular ion M⁺ at *m/z* 484.2461 (as expected) indicating 11 degrees of unsaturation. The IR spectrum showed the presence of carbonyl (C=O) stretches at 1733 and 1739 cm⁻¹; epoxide (C-O) stretches at 1231 cm⁻¹ and β -substituted furan ring C-H bending vibrations at 726 cm⁻¹. The ¹H NMR data (Table 1) indicated the presence of a characteristic epoxy group methine proton at δ_{H} 3.39 (s, H-15) and oxygenated protons at δ_{H} 4.01 (d, *J* = 3.96 Hz, H-3), δ_{H} 4.71 (t, *J* = 2.2 Hz, H-7), δ_{H} 4.49 (d, *J* = 13.21 Hz, H-19 *exo*) and δ_{H} 4.34 (d, *J* = 13.21 Hz, H-19 *endo*). The acetyl methyl proton resonance occurred at δ_{H} 2.06. The three furan proton resonances occurred at δ_{H} 7.07 (s, H-21), 6.11 (s, H-22) and 7.33 (s, H-23), with the corresponding carbon resonances of H-21 and H-22 showing HMBC correlations to a methine proton resonance at δ_{H} 2.57 (dd, *J* = 11.25, 6.04 Hz), attributed to H-17. Beside the methyl acetate, there were four other tertiary methyl resonances at δ_{H} 0.88 (3H-30), 0.95 (3H-18), 1.20 (3H-29) and 1.07 (3H-28). The position of the methyl groups were supported by HMBC data; 3H-28 and

3H-29 with C-4 and C-5; 3H-18 with C-14, C-13 and C-12; 3H-30 with C-7, C-8 and C-9. The methine proton at δ_{H} 3.39 (s, H-15) showed HMBC correlations with C-16 and C-17.

Cyclisation between C-19, and C-3 which incorporated C-1 and C-2 was supported by 2D NMR correlations. The H-3 and both H-19 doublets (H-19 *endo* and *exo*) showed HMBC correlations to the carbonyl resonance of C-1 and the formation of the lactone ring was further supported by HMBC correlations between H-3 and both C-19 and C-9. The H-19 doublets also showed HMBC correlations to C-5, C-9 and C-10 as expected and to C-3, only possible with cyclisation between C-19 and C-3.

The selected 1D gradient NOE (GOESY) experiments on compound **4** revealed a twist in the rearranged A-ring structure; this twist is clear in a 3-D model. NOE correlations were observed between H-9 and H-5, H-3 and 3H-18, which were on the backface in the 3-D model. The methyl group 3H-30 showed NOE correlations to H-7, H-6 β , H-11 β , and H-19*exo*; these were on the top face in the 3-D model. This results in H-19 *endo* being situated proximal to the 4 β methyl (3H-29) and H-6 β . The H-17 β resonance exhibited NOE correlations with the furanyl protons H-21 and H-22 and the H-16 β proton resonances.

The absolute configuration was determined by circular dichroism (CD). The CD spectrum of **4** showed a positive cotton effect at 197 nm ($\Delta\epsilon$ +8.5) and a negative cotton effect 210 nm ($\Delta\epsilon$ -22.0). The furan chromophore (λ_{max} 206 nm) was probably masked by the cotton effect of the ester and lactone groups. A positive cotton effect was also observed at 328 nm, ($\Delta\epsilon$ +1.2) due to the $n \rightarrow \pi^*$ transition of the carbonyl groups of **4** and was in accordance with the CD spectra observed for limonoids from *Turraea pubescens*²². The stereochemistry of the epoxide was shown to be β , in accordance with the orientation previously described for limonoids with a Δ^{14} , epoxide^{23,24}. The absolute configuration of

the ten chiral centers in **4** was thus determined as 3*R*, 5*R*, 7*R*, 8*R*, 9*R*, 10*S*, 13*R*, 14*R*, 15*R* and 17*R*.

The spectroscopic data and functionalities established that compound **4** was an A, D-seco limonoid, which are common to the Rutaceae and rare in the Meliaceae²⁵. Ring D resembled evodulone isolated from *Carapa procera*²¹, whereas ring A resembled limonin (A, A' ring system). The other two rings, B and C of compound **4** remained carbocyclic.

Comparison of the ¹H NMR data of compound **5**, a brown solid with that of compound **4**, indicated the notable absence of the methylene protons, H-16 α and H-16 β , which occurred at δ_{H} 2.13 and δ_{H} 1.59 in **4**. This occurred with an additional carbonyl resonance at δ_{H} 208.5, indicating that a ketone functionality was now present at position 16. This was supported by the HREIMS, which showed a molecular ion peak at m/z 498.9120 for C₂₈H₃₄O₈, two hydrogen atoms less and one oxygen atom more than **5**. The presence of the carbonyl functionality at C-16 also affected the proton and carbon chemical shifts at position 17, shifting them downfield to δ_{H} 3.81 from 2.57 and to δ_{C} 50.85 from 39.63. The relative stereochemistry of **5** was deduced to be the same as that of **4** based on their almost superimposable ¹H and ¹³C NMR data and similar NOESY and NOE correlation patterns to those of **4**. The Absolute configuration of **4** and **5** were also identical as indicated by similar CD curves.

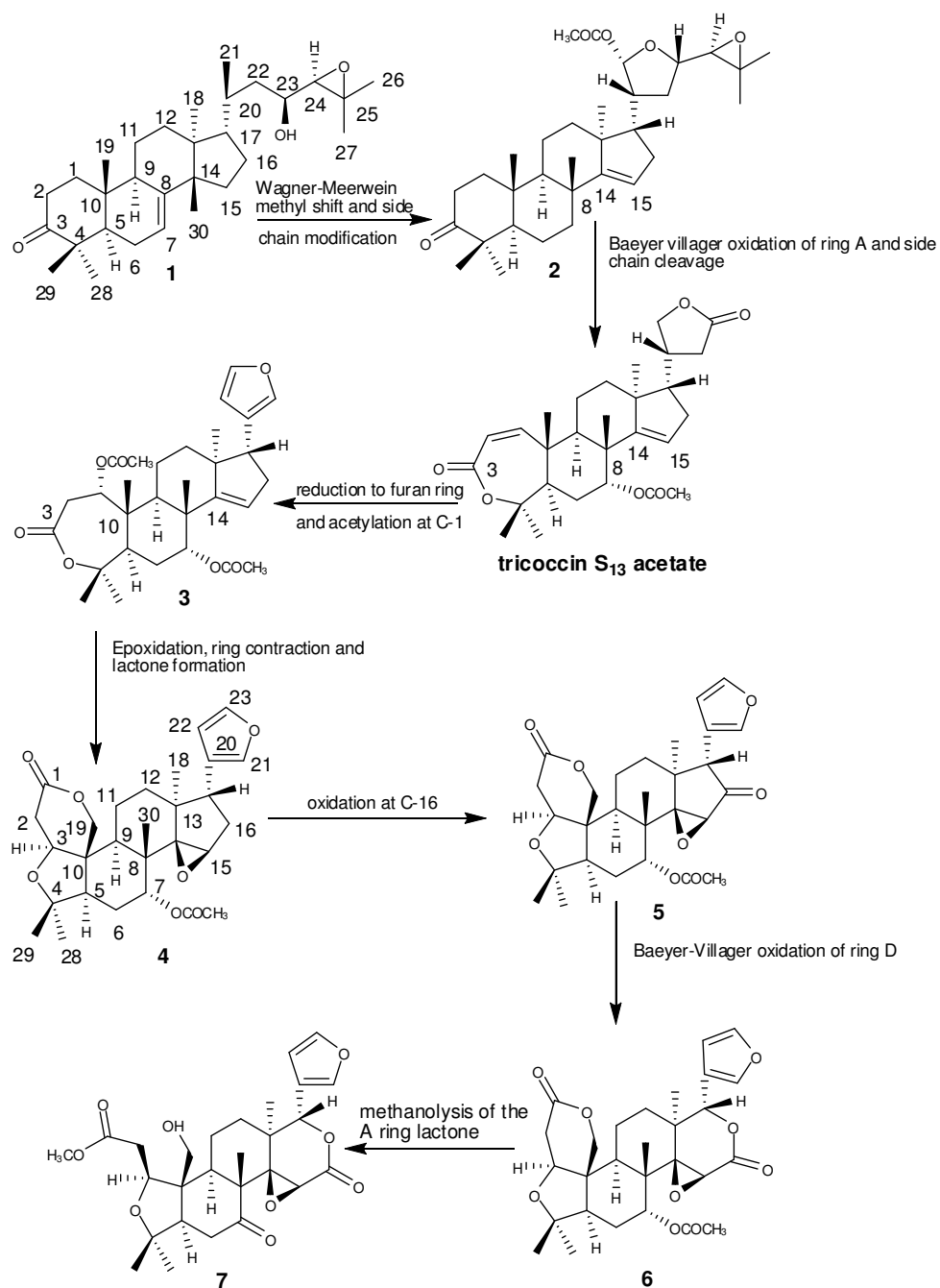


Figure 1. Plausible Biogenetic Pathway for the Formation of Limonoids 1-7 from *Vepris uguenensis*.

Table 1. NMR spectroscopic Data (400 MHz, CDCl₃) for uguenensene (4) and uguenensone (5)

Position	uguenensene (4)			uguenensone (5)	
	δ_C , type	δ_H (J in Hz)	HMBC ^a	δ_C , type	δ_H (J in Hz)
1	169.94, C	-	-	169.80, C	-
2 α	35.95, CH ₂	2.92, dd (16.48, 3.96)	1, 3	35.92, CH ₂	2.93, dd (16.56, 3.96)
2 β	-	2.60, d (16.48)	1, 3	-	2.62, d (16.56)
3	80.26, CH	4.01, d (3.96)	1, 9, 19	80.07, CH	4.01, d (3.96)
4	80.58, C	-	-	80.60, C	-
5	54.19, CH	2.22, m	3, 4, 10, 19, 28, 29	54.16, CH	2.21, dd (13.48, 3.08)
6 α	24.41, CH ₂	1.81, m	5	24.27, CH ₂	1.84, m
6 β	-	1.72, m	-	-	1.78, m
7 β	73.53, CH	4.71, t (2.20)	5, 9, 31	73.35, CH	4.66, t (2.36)
8	42.79, C	-	-	43.10, C	-
9	44.03, CH	2.79, dd (11.8, 4.20)	3, 8, 11, 19	43.31, CH	2.84, dd (13.20, 4.72)
10	45.62, C	-	-	45.58, C	-
11 α	18.55, CH ₂	1.96, m	-	18.27, CH ₂	1.95, m
11 β	-	1.71, m	-	-	1.76, m
12 α	29.64, CH ₂	1.83, m	-	28.30, CH ₂	2.19, m
12 β	-	1.80, m	-	-	1.73, m
13	41.82, C	-	-	42.50, C	-
14	72.40, C	-	-	71.62, C	-
15	57.58, CH	3.39, s	16, 17	57.29, CH	3.34, s
16 α	31.95, CH ₂	2.13, dd (13.35, 6.04)	13, 14, 15, 17	208.05, C	-
16 β	-	1.59, dd (13.35, 11.25)	17	-	-
17	39.63, CH	2.57, dd (11.25, 6.04)	12, 13, 16, 18, 20, 21, 22	50.85, CH	3.81, s
18	21.36, CH ₃	0.95, s	12, 13, 14, 17	24.21, CH ₃	1.02, s
19 <i>exo</i>	66.04, CH ₂	4.49, d (13.12)	1, 3, 5, 10	65.85, CH ₂	4.50, d (13.08)
19 <i>endo</i>	-	4.34, d (13.12)	5, 9	-	4.36, d (13.08)
20	123.46, C	-	-	116.27, C	-
21	139.57, CH	7.07, s	20, 22, 23	141.60, CH	7.50, s
22	110.85, CH	6.11, s	20, 21, 23	110.84, CH	6.17, s
23	143.01, CH	7.33, s	20, 21, 22	142.56, CH	7.35, s
28	21.36, CH ₃	1.07, s	4, 5, 29	21.25, CH ₃	1.06, s
29	30.17, CH ₃	1.20, s	4, 5, 28	30.14, CH ₃	1.19, s
30	19.38, CH ₃	0.88, s	7, 8, 9, 14	19.39, CH ₃	0.96, s
31	169.55, C	-	-	169.50, C	-
OCOCH ₃					
32	21.37, CH ₃	2.06, s	31	21.32, CH ₃	2.01, s
OCOCH ₃					

^aHMBC- correlations are from proton(s) to the indicated carbon (H→C)

Compound **8** was identified as a flavanone by its NMR, UV, IR and mass data. Its molecular formula was established to be C₂₀H₂₀O₇ based on HREIMS data of 372.1158 amu with a double bond equivalence of 11, eight being due to the aromatic rings, one being due

to the carbonyl group, one due to the double bond on the side chain and the remaining one being due to ring C of the flavanone skeleton. The IR data showed the presence of O-H (3330 cm^{-1}), and C=O (1708 cm^{-1}) stretching bands. The ^1H NMR data showed a characteristic AB coupling system of H-2 and H-3 which appeared at $\delta_{\text{H}} 4.58$ (1H, d, $J = 11.65\text{ Hz}$, H-2) and $\delta_{\text{H}} 4.82$ (1H, d, $J = 11.65\text{ Hz}$, H-3), which was supported by COSY correlations between the two resonances. Their coupling constants were characteristic of *trans* protons²⁶, and this *trans* stereochemistry was confirmed by NOESY correlations between H-3 and H-2'/6'.

The second characteristic pattern was that of an AA'BB' system of a 1,4-disubstituted aromatic ring (ring B). These proton resonances occurred as two doublets at $\delta_{\text{H}} 7.36$ (2H, d, $J = 8.52\text{ Hz}$, H-2'/6'), and $\delta_{\text{H}} 6.85$ (2H, d, $J = 8.52\text{ Hz}$, H-3'/5'). COSY coupling between H-2'/6' and H-3'/5' were also observed. Also present in the ^1H NMR data were two aromatic protons at $\delta_{\text{H}} 6.09$ (1H, d, $J = 2.2\text{ Hz}$, H-6) and $\delta_{\text{H}} 6.04$ (1H, d, $J = 2.2\text{ Hz}$, H-8) attributed to ring A. The observed coupling constant of 2.2 Hz was indicative of *meta* coupling.

The third pattern was that of the isoprenol group in ring A. The 2H-1'' and H-2'' resonances were observed at $\delta_{\text{H}} 4.66$ (2H, d, $J = 6.32\text{ Hz}$, H-1'') and $\delta_{\text{H}} 5.70$ (1H, t, $J = 6.32\text{ Hz}$, H-2''). A singlet was also observed at $\delta_{\text{H}} 3.76$ attributed to 2H-4'', which showed HMBC correlations to the quaternary carbon resonance at $\delta_{\text{C}} 142.04$ (C-3''), the methine carbon resonance at $\delta_{\text{C}} 119.65$ (C-2'') and the methyl carbon resonance at $\delta_{\text{C}} 14.03$ (C-5''). These correlations indicated that one of the methyl protons in an isoprenyl unit had been oxidized to a primary alcohol and that the isoprenyl group was attached to C-7 by an ether linkage as the 2H-1'' resonance was seen to be deshielded.

The isoprenol group was placed at C-7 since H-6 and H-8 both showed NOESY correlations with 2H-1''. This would not be possible if the isoprenol group was located at C-5. The hydroxyl groups were thus placed in the remaining C-4', C-3 and C-5-positions, which are also the most common positions to be hydroxylated. The carbon resonances of δ_C 165.02 (C-4'), 85.10 (C-3) and 164.36 (C-5) support these assignments.

The absolute configuration of C-2 and C-3 were determined by CD analysis in comparison with literature values^{26,27}. The CD spectrum showed a positive cotton effect at 205 nm, $\Delta\epsilon$ +8.8, ($\pi \rightarrow \pi^*$ for the double bond), 290 nm, $\Delta\epsilon$ +4.0 ($\pi \rightarrow \pi^*$) and 335 nm, $\Delta\epsilon$ +2.0 ($n \rightarrow \pi^*$ of the carbonyl group). The absolute configuration of the chiral centers was assigned as 2*R*, 3*R*. Compound **8** was thus identified as (2*R*,3*R*)-3,5-dihydroxy-7-((*E*)-4-hydroxy-3-methylbut-2-enyloxy)-2-(4-hydroxyphenyl)chroman-4-one.

Eleven of the fourteen compounds isolated, were evaluated for their antioxidant potentials using three different antioxidant assays; FRAP, DPPH and deoxyribose methods. Two of the compounds, tricoccin S₁₃ acetate and skimmianine were not tested for their antioxidant activity as they were isolated in very small quantities, 23 mg and 13 mg respectively. Only compounds in excess of 30 mg were of sufficient quantities to use in the three assays. Lupeol however did not show any activity.

The results obtained (Table 2) shows that the reducing powers of all of the compounds increased with increasing concentration. The reducing power of the standard ascorbic acid was significantly higher than that of all compounds tested in this assay except for uguenenprenol, (**8**) and 7-*O*-methyларomadenrin (**9**), whose values, although lower than ascorbic acid were found to be significantly higher than the rest of the compounds tested and exhibited good activity even at lower concentrations. The reducing power of the different compounds tested decreased in the following order: uguenenprenol (**8**) > 7-*O*-

methylaromadenrin (**9**) > 8 α , 11-elemodiol (**11**) > chisocheton (**2**) > uguenensone (**5**) = flindersiamine (**10**) > niloticin (**1**) > limonyl acetate (**6**) > kihadalactone A (**3**) > methyl uguenenoate (**7**) > uguenensene (**4**). The high antioxidant activity of the flavonoids in comparison to the other compounds was expected, since flavonoids are known antioxidants. Flavonoids are known to suppress reactive oxygen species formation either by inhibition of enzymes or chelating trace elements involved in free radical production, scavenging of reactive oxygen species and upregulating or protecting antioxidant defences²⁸. The presence of 3-hydroxyl groups as in compounds **8** and **9** in the heterocyclic ring also increases the radical scavenging activity (RSA)²⁸.

Table 2: Reducing power of compounds from *V. uguenensis* as evaluated by the FRAP method.

Concentration $\mu\text{g/mL}$	Absorbance				
	15.6	31.1	62.5	125	250
Niloticin (1)	0.01 \pm 0.00 ^d	0.03 \pm 0.00 ^{bc}	0.05 \pm 0.00 ^{bc}	0.10 \pm 0.01 ^b	0.21 \pm 0.00 ^{bg}
Chisocheton (2)	0.02 \pm 0.01 ^{bd}	0.03 \pm 0.01 ^{bc}	0.06 \pm 0.01 ^b	0.11 \pm 0.01 ^b	0.27 \pm 0.02 ^b
Kihadalactone A (3)	0.01 \pm 0.00 ^d	0.02 \pm 0.00 ^e	0.04 \pm 0.01 ^{ge}	0.07 \pm 0.00 ^{be}	0.13 \pm 0.00 ^{eg}
Uguenensene (4)	0.01 \pm 0.01 ^d	0.02 \pm 0.00 ^e	0.03 \pm 0.00 ^g	0.05 \pm 0.00 ^e	0.11 \pm 0.00 ^e
Uguenensone (5)	0.03 \pm 0.00 ^b	0.05 \pm 0.00 ^{bc}	0.07 \pm 0.00 ^b	0.13 \pm 0.00 ^f	0.23 \pm 0.00 ^{bg}
Limonyl acetate (6)	0.06 \pm 0.01 ^c	0.07 \pm 0.00 ^c	0.11 \pm 0.03 ^f	0.11 \pm 0.01 ^f	0.20 \pm 0.03 ^{be}
Methyl uguenenoate (7)	0.01 \pm 0.00 ^d	0.02 \pm 0.00 ^e	0.03 \pm 0.01 ^g	0.05 \pm 0.00 ^e	0.11 \pm 0.00 ^e
Uguenenprenol (8)	0.06 \pm 0.01 ^c	0.12 \pm 0.00 ^d	0.35 \pm 0.02 ^d	0.87 \pm 0.00 ^d	1.27 \pm 0.10 ^d
7- <i>O</i> -methylaromadenrin (9)	0.07 \pm 0.00 ^c	0.07 \pm 0.00 ^c	0.14 \pm 0.00 ^c	0.36 \pm 0.00 ^c	0.86 \pm 0.02 ^c
Flindersiamine (10)	0.02 \pm 0.00 ^{bd}	0.04 \pm 0.00 ^{bc}	0.05 \pm 0.01 ^{bc}	0.08 \pm 0.00 ^{bc}	0.23 \pm 0.02 ^{bg}
8 α ,11-elemodiol (11)	0.03 \pm 0.00 ^b	0.04 \pm 0.01 ^{bc}	0.05 \pm 0.00 ^{bc}	0.10 \pm 0.01 ^b	0.28 \pm 0.01 ^b
Ascorbic Acid	0.15 \pm 0.02 ^a	0.33 \pm 0.06 ^a	0.60 \pm 0.01 ^a	1.05 \pm 0.01 ^a	1.97 \pm 0.15 ^a

Data are presented as means \pm SD of triplicate. ^{a-g}Values with different superscript letters for a given concentration are significantly different from each of the other compounds (One way ANOVA followed by Tukey's multiple range post-hoc test, $p < 0.05$).

The RSA results shown in table 3 revealed that the scavenging ability of the novel flavonoid, uguenenprenol (**8**) and 7-*O*-methylaromadenrin (**9**) was 94% at 250 $\mu\text{g mL}^{-1}$. Their scavenging effects was marginally higher than that of ascorbic acid in almost all concentrations except at the lower concentrations 15.62 $\mu\text{g mL}^{-1}$ and 31.12 $\mu\text{g mL}^{-1}$ where

ascorbic acid was marginally higher. Again, the antioxidant activity of the flavonoids **8** and **9** in this assay were significantly higher than the rest of the compounds. Of the limonoids tested, **2**, **5** and **7** displayed significantly higher activities compared to the rest of the limonoids. The RSA of all compounds in decreasing order was: 7-*O*-methyларomadenrin (**9**) > uguenenprenol (**8**) > uguenensone (**5**) > methyl uguenenoate (**7**) > chisocheton (**2**) > limonyl acetate (**6**) > uguenensene (**4**) > flindersiamine (**10**) > nilotycin (**1**) > 8 α ,11-elemodiol (**11**) > kihadalactone A (**3**).

Table 3: Results of the antioxidant activity of compounds isolated from *Vepris uguenensis* as measured by the DPPH method

Concentrations ($\mu\text{g/mL}$)	Percentage scavenging activity				
	15.6	31.1	62.5	125	250
Nilotycin (1)	2.00 \pm 1.00 ^b	8.33 \pm 0.58 ^c	12.00 \pm 1.00 ^b	14.67 \pm 0.58 ^b	22.00 \pm 3.61 ⁱ
Chisocheton (2)	24.00 \pm 1.00 ⁱ	39.67 \pm 0.58 ^h	43.33 \pm 0.58 ^e	47.67 \pm 1.53 ^d	54.67 \pm 1.53 ^h
Kihadalactone A (3)	1.33 \pm 0.58 ^b	6.00 \pm 1.00 ^g	9.00 \pm 1.00 ^b	11.33 \pm 0.58 ^f	13.67 \pm 0.58 ^g
Uguenensene (4)	4.00 \pm 2.00 ^h	8.33 \pm 1.53 ^c	21.33 \pm 1.53 ^c	32.33 \pm 2.08 ^e	38.67 \pm 4.04 ^f
Uguenensone (5)	16.00 \pm 0.00 ^g	22.00 \pm 1.73 ^f	42.00 \pm 6.93 ^e	51.33 \pm 3.79 ^d	68.00 \pm 1.73 ^d
Limonyl acetate (6)	13.00 \pm 1.00 ^f	22.33 \pm 0.58 ^f	26.00 \pm 1.73 ^f	29.00 \pm 1.00 ^e	49.67 \pm 0.58 ^e
Methyl uguenenoate (7)	29.67 \pm 2.08 ^d	35.67 \pm 1.15 ^e	45.33 \pm 2.52 ^e	50.33 \pm 0.58 ^d	64.67 \pm 0.58 ^d
Uguenenprenol (8)	41.00 \pm 1.00 ^c	72.00 \pm 1.00 ^d	87.00 \pm 1.00 ^d	91.33 \pm 1.53 ^a	93.67 \pm 1.53 ^a
7- <i>O</i> -methyларomadenrin (9)	30.67 \pm 0.58 ^d	75.00 \pm 2.65 ^d	91.00 \pm 1.00 ^d	92.67 \pm 1.15 ^a	94.33 \pm 0.58 ^a
Flindersiamine (10)	7.00 \pm 0.00 ^c	9.00 \pm 1.00 ^c	19.00 \pm 1.00 ^c	20.67 \pm 0.58 ^c	28.67 \pm 2.31 ^c
8 α ,11-elemodiol (11)	0.33 \pm 0.58 ^b	1.67 \pm 0.58 ^b	9.00 \pm 1.00 ^b	14.67 \pm 2.52 ^b	18.00 \pm 1.00 ^b
Asorbic Acid	37.67 \pm 0.58 ^a	78.00 \pm 0.58 ^a	79.67 \pm 0.58 ^a	91.00 \pm 0.58 ^a	92.67 \pm 0.58 ^a

Data are presented as means \pm SD of triplicate. ^{a-i}Values with different superscript letters for a given concentration are significantly different from each of the other compounds (One way ANOVA followed by Tukey's multiple range post-hoc test, $p < 0.05$).

The fact that **2**, **5** and **7** have some antioxidant activity in this assay is worth noting as limonoids are not common antioxidants. Due to the low redox potential of flavonoids they are thermodynamically able to reduce highly oxidizing free radicals such as superoxide, peroxy, alkoxy and hydroxyl radicals by hydrogen atom donation²⁸. Besides scavenging, flavonoids may stabilize free radicals involved in oxidative processes by complexing with

them²⁹. The flavanols containing a catechol group in ring B as in compound **8** and **9** are highly active because of the presence of the 3-hydroxyl group. Additionally, the catechol group present in these compounds has strong electron donating properties and target free radicals²⁹.

The results of the deoxyribose test are presented in table 4. The results revealed that the hydroxyl radical scavenging activities of the isolated compounds increased with increasing concentrations. As in the other two assays, the highest activity was observed for the flavonoids, **8** (77% at 250 $\mu\text{g mL}^{-1}$) and **9** (83% at 250 $\mu\text{g mL}^{-1}$) and showed higher activity than ascorbic acid at all concentrations. The sesquiterpenoid (**11**), and the alkaloid **10**, both had comparable activities to that of ascorbic acid of 72% and 69% at 250 $\mu\text{g mL}^{-1}$. Ascorbic acid had an activity of 71% at 250 $\mu\text{g mL}^{-1}$. The new limonoid uguenensone (**5**) exhibited an activity of 44% at 15.62 $\mu\text{g mL}^{-1}$, 50% at 31.25 $\mu\text{g mL}^{-1}$ and 59% at 62.50 $\mu\text{g mL}^{-1}$, which was significantly higher than that of ascorbic acid at the corresponding concentrations, however it did not increase at the same rate with increasing concentration as ascorbic acid.

Although flindersiamine (**10**) and 8 α ,11-elemodiol (**11**) did not show high radical scavenging activity by the DPPH method, these two compounds showed potency when assayed using the deoxyribose method. Although both of these methods measure hydroxyl radical scavenging activity, a plausible explanation for this finding is that these compounds (alkaloid and sesquiterpenoid) efficiently target different radicals i.e the DPPH method measures the ability to scavenge a phenolic radical whereas the deoxyribose method measures the scavenging activity of aliphatic hydroxyl radicals³⁰. Generally, the scavenging activity of the compounds was found to decrease in the order: 7-*O*-methylaromadentin (**9**) > uguenenprenol (**8**) > 8 α ,11-elemodiol (**11**) > ascorbic acid > flindersiamine (**10**) >

uguenensone (5) > chisocheton (2) > methyl uguenenoate (7) > limonyl acetate (6) > kihadalactone A (3) > niloticin (1) > uguenensene (4). Higher hydroxyl radical scavenging activity in flindersiamine could be attributed to electron rich benzene rings and the double bond at Δ^2 in the structure which is responsible for electron delocalization upon donation of a proton²⁹.

Table 4: Results of the percentage hydroxyl radical scavenging activity of compounds from *V. uguenensis* and ascorbic acid measured by the deoxyribose method.

Concentrations ($\mu\text{g/mL}$)	Percentage scavenging activity				
	15.6	31.1	62.5	125	250
Niloticin (1)	13.33 \pm 1.53 ^f	19.67 \pm 1.53 ^g	27.00 \pm 1.00 ^f	29.00 \pm 2.00 ^f	36.67 \pm 1.53 ^f
Chisocheton (2)	34.33 \pm 2.52 ^d	40.67 \pm 1.53 ^a	43.67 \pm 1.53 ^g	47.67 \pm 1.53 ^h	55.33 \pm 1.53 ^d
Kihadalactone A (3)	21.33 \pm 1.53 ^e	22.67 \pm 1.53 ^f	27.00 \pm 1.00 ^f	30.00 \pm 1.00 ^f	43.33 \pm 3.06 ^e
Uguenensene (4)	5.33 \pm 3.06 ^a	11.00 \pm 1.00 ^{de}	19.33 \pm 1.53 ^e	21.00 \pm 1.00 ^e	34.00 \pm 3.61 ^f
Uguenensone (5)	43.67 \pm 2.08 ^c	50.33 \pm 1.53 ^c	59.33 \pm 1.53 ^c	56.67 \pm 1.53 ^g	59.67 \pm 2.52 ^d
Limonyl acetate (6)	3.00 \pm 1.00 ^a	12.67 \pm 1.15 ^e	20.00 \pm 1.00 ^e	32.00 \pm 2.00 ^f	45.33 \pm 1.53 ^e
Methyl uguenenoate (7)	5.33 \pm 2.08 ^a	10.00 \pm 1.00 ^d	14.00 \pm 1.00 ^d	22.67 \pm 0.58 ^e	53.33 \pm 3.06 ^d
Uguenenprenol (8)	37.33 \pm 1.53 ^d	58.00 \pm 2.00 ^b	63.33 \pm 1.53 ^b	73.00 \pm 1.00 ^d	76.67 \pm 1.15 ^c
7-O-Methylaromadentin (9)	46.00 \pm 1.00 ^c	50.33 \pm 0.58 ^c	54.67 \pm 2.52 ^a	76.33 \pm 3.21 ^c	83.00 \pm 1.73 ^b
Flindersiamine (10)	43.00 \pm 2.00 ^c	49.67 \pm 1.53 ^c	58.67 \pm 3.21 ^c	62.00 \pm 1.73 ^b	69.00 \pm 1.00 ^a
8 α ,11-elemodiol (11)	56.67 \pm 1.53 ^b	59.00 \pm 1.00 ^b	64.67 \pm 1.53 ^b	68.33 \pm 1.53 ^a	71.67 \pm 1.53 ^a
Ascorbic Acid	23.33 \pm 1.53 ^a	39.67 \pm 1.53 ^a	54.00 \pm 1.00 ^a	67.67 \pm 0.58 ^a	70.67 \pm 1.53 ^a

Data are presented as means \pm SD of triplicate. ^{a-h}Values with different superscript letters for a given concentration are significantly different from each of the other compounds (One way ANOVA followed by Tukey's multiple range post-hoc test, $p < 0.05$).

In conclusion, the results of this study show that the classes of compounds and concentrations have significant effects on their antioxidant activity. The flavonoids, as expected, showed high activity in all of the antioxidant assays, and in some cases even higher than ascorbic acid. Unexpectedly, the limonoids **2**, **5** and **7** showed some significant antioxidant activity in the DPPH assay and the alkaloid **10** and sesquiterpene **11** showed significantly higher activity in the deoxyribose assay compared to many other compounds. Whereas the flavonoids are non selective for any particular radical, the limonoids, alkaloids

and sesquiterpene may be more selective to certain radicals, hence their activities were not consistently observed in all three assays used in this study.

These results provide scientific validity and credence to the ethnomedicinal use of this plant in the treatment of malaria as the antioxidants could reduce the ROS linked to cerebral malaria in children in Africa. This study also highlights the efficacy of compounds from *V. uguenensis* as antioxidant additives. Further to this, the current contribution adds uguenensene (**4**) and uguenensone (**5**), both of the limonin-type limonoids to the list of previously identified Rutaceae limonoids.

EXPERIMENTAL SECTION

General Experimental Procedures

The melting points were recorded on an Ernst Leitz Wetzlar micro-hot stage melting point apparatus. Specific rotations were measured at room temperature in chloroform on a PerkinElmer™, Model 341 Polarimeter with a 10 mm flow tube. UV spectra were obtained on a Varian Cary UV-VIS Spectrophotometer. The Circular Dichroism (CD) spectra were recorded on chirascan plus spectropolarimeter by applied photophysics at wavelengths 190-400 nm. IR spectra were recorded on a Perkin-Elmer Universal ATR Spectrometer. The ¹H, ¹³C and all 2D NMR spectra were recorded using a Bruker Avance^{III} 400 MHz spectrometer. All the spectra were recorded at room temperature using deuterated chloroform (CDCl₃) as solvent. High-resolution mass data was obtained using a Bruker microTOF-Q II ESI instrument operating at ambient temperatures, with a sample concentration of approximately 1 ppm.

The separation, isolation and purification of compounds were carried out by gravity column chromatography and monitored by thin layer chromatography (TLC). Merck silica gel 60

(0.040-0.063 mm) was used for column chromatography and Merck 20 × 20 cm silica gel 60 F₂₅₄ aluminum sheets were used for thin-layer chromatography.

Plant material

The leaves, stem bark and roots of *V. uguenensis* were collected from the Rift Valley province of Kenya in December 2010 and were identified by Mr Ezekiel Cheboi of the Department of Natural Resources, Egerton University, Kenya. A voucher specimen (PKC 02 NH) was deposited at the Natal Herbarium, Durban.

Extraction and Isolation

The air dried powder of *V. uguenensis* leaves (400 g), stem bark (980 g) and roots (900 g) were each sequentially extracted for 24 hours using a soxhlet apparatus with solvents of varying polarity; hexane, dichloromethane, ethyl acetate and methanol. Each extract was concentrated using a rotavapor prior to being separated. The crude extracts were separated by column chromatography (CC). The hexane extract from the leaves (13 g) was separated with a hexane: dichloromethane and dichloromethane: ethyl acetate step gradient. For the crude extract, a 4.5 cm diameter column was used and fractions of 100 mL were collected and monitored by thin layer chromatography (TLC). Fractions with similar TLC profiles were combined. From this column separation, eleven fractions (A-K) were obtained. Fraction C was recrystallised in methanol to yield lupeol (118 mg) while fraction G was purified using hexane: dichloromethane (7:3) to afford three subfractions G₁-G₃ of which subfraction G₃ yielded 8 α ,11-elemodiol (**11**) (59 mg). Fraction H was purified using hexane: EtOAc (8:2) to yield subfractions, H₁-H₄ of which subfraction H₄ was washed several times with hexane to yield niloticin (**1**) (583 mg). Fraction J was purified with 100% dichloromethane and then EtOAc: dichloromethane (1:4) to afford five subfractions, J₁-J₅. Subfraction J₃ contained chisocheton (**2**) (83 mg) while subfraction J₅ was purified

further using dichloromethane: EtOAc (9:1) to give limonyl acetate (**6**) (134 mg). The dichloromethane extract of the leaves (9 g) was also separated using a hexane: EtOAc and EtOAc: methanol step gradient from 100% hexane to 1:4 (methanol: EtOAc) to give 9 fractions (A-I). Fraction E was purified with hexane: EtOAc (4:1) to afford 7-*O*-methylaromadennin (**9**) (51 mg), while fraction G was purified using hexane: dichloromethane (1:1) to give four subfractions G₁-G₄ of which fraction G₂ was further purified with 100% dichloromethane to yield uguenensone (**5**) (92 mg). Fraction H was purified with dichloromethane: EtOAc (4:1) to give uguenenprenol (**8**) (38 mg).

The hexane extract (32 g) from the stems yielded the same compounds as the hexane extract of the leaves. The dichloromethane extract of the stems (15 g) was eluted with a hexane: EtOAc and EtOAc: methanol step gradient starting from 100% hexane and stepped up to 100% EtOAc and then to 10% MeOH in EtOAc to give nine fractions A-I. Fraction H was purified with hexane: EtOAc (6:4) to give five subfractions H₁-H₅. Subfraction H₂ was further purified using dichloromethane: EtOAc (4:1) to yield uguenensene (**4**) (241 mg). Subfraction H₁ was purified with dichloromethane: EtOAc (4:1) into four other subfractions H_{1a}-H_{1d}. Fraction H_{1a} yielded tricoccin S₁₃ acetate (23 mg) with 100% dichloromethane. Fraction H_{1c} was purified using dichloromethane: EtOAc (7:3) to afford three fractions after combination, H_{1ca}-H_{1cc}. Kihadalactone (**3**) (117 mg) was contained in fraction H_{1ca} while methyl uguenenoate (**7**) (120 mg) was obtained in fraction H_{1cc}.

The dichloromethane extract from the roots was separated with hexane: EtOAc and then EtOAc: MeOH. More methyl uguenenoate (**7**) was obtained after purification of fraction D with dichloromethane: EtOAc (4:1) and the two alkaloids skimmianine and flindersiamine (**10**) were contained in fraction H. Their separation was achieved after repeated column

chromatography using dichloromethane: EtOAc (1:1), where fraction H_{2c} yielded skimmianine (13 mg) and H_{2d} yielded flindersiamine (**10**) (45 mg).

Compound 4: Reddish brown; mp 105-109 °C; $[\alpha]_D^{20}$ -62.50 (*c* 0.056 CHCl₃); UV (CHCl₃) λ_{\max} (log ϵ) 240 (1.28) nm; CD (CH₃CN) λ_{\max} ($\Delta\epsilon$) 197 (+8.5), 210 (-22.0), 328 (+1.2) nm; IR ν_{\max} 2926, 1733, 1370, 1231, 1031, 729 cm⁻¹; ¹H NMR and ¹³C NMR data: see Table 1; EIMS *m/z* (rel. int.) 484 [M]⁺ (33), 469 (10), 441 (10), 424 (100), 409 (88), 347 (50), 330 (68); HREIMS *m/z* 484.2324 [M]⁺ (calcd. for C₂₈H₃₆O₇, 484.2461).

Compound 5: Dark brown solid; mp 132-134 °C; $[\alpha]_D^{20}$ -14.85 (*c* 0.404, CHCl₃); UV(CHCl₃) λ_{\max} (log ϵ) 246 (0.72) nm; CD (CH₃CN) λ_{\max} ($\Delta\epsilon$) 197 (+8.5), 210 (-22.0), 328 (+1.2) nm ; IR ν_{\max} 2960, 1732, 1369, 1230, 1028, 726 cm⁻¹; ¹H NMR and ¹³C NMR data: see Table 1; EIMS *m/z* (rel. int.) 498 [M]⁺ (100), 482 (25), 467 (25), 438 (38), 423 (50), 384 (38), HREIMS 498.9120 [M]⁺ (calcd. for C₂₈H₃₄O₈, 498.5648).

Compound 8: Light yellow crystals; mp 75-78 °C; $[\alpha]_D^{20}$ +52.30 (*c* 0.056 CHCl₃); UV (CHCl₃) λ_{\max} (log ϵ) 337 (4.45), 285 (5.13), 239 (5.44) nm; CD (CH₃CN) λ_{\max} ($\Delta\epsilon$) 205 (+8.8), 290 (+4.0), 335 (+2.0) nm; IR ν_{\max} 3330, 2924, 1708, 1634 cm⁻¹; ¹H NMR (CD₃OD, 400 MHz) δ 7.36 (2H, d, *J* = 8.56 Hz, H-2'/6'), 6.85 (2H, d, *J* = 8.56 Hz, H-3'/5'), 6.09 (1H, d, *J* = 2.20 Hz, H-6), 6.04 (1H, d, *J* = 2.20 Hz, H-8), 5.70 (1H, t, *J* = 6.32 Hz, H-2''), 4.82 (1H, d, *J* = 11.65 Hz, H-3), 4.66 (2H, d, *J* = 6.32 Hz, 2H-1''), 4.58 (1H, d, *J* = 11.65 Hz, H-2), 3.76 (2H, s, H-4''), 1.54 (3H, s, H-5''); ¹³C NMR (CD₃OD, 100 MHz) δ 198.98 (C, C-4), 168.97 (C, C-7), 165.01 (C, C-5), 164.36 (C, C-4'), 159.28 (C, C-9), 142.04 (C, C-3''), 130.40 (2CH, C-2'/6'), 129.16 (C, C-1'), 119.65 (CH, C-2''), 116.18 (2CH, 3'/5'), 102.61 (C, C-10), 96.71 (CH, C-6), 95.64 (CH, C-8), 85.10 (CH, C-3), 73.72 (CH, C-2), 67.63 (CH₂, C-4''), 66.25 (CH₂, C-1''), 14.03 (CH₃, C-5''); HREIMS *m/z* 372.1158 [M]⁺ (calcd. for C₂₀H₂₀O₇, 372.1209).

ANTIOXIDANT ACTIVITY

Determination of the reducing potential using the Ferric Reducing Antioxidant Power (FRAP) assay

The total reducing power of each compound from *Vepris uguenensis* was determined according to the Ferric Reducing Antioxidant Power (FRAP) method as described³¹. A 2.5 mL volume of different concentrations of the compounds (250, 125, 62.5, 31.25 and 15.625 $\mu\text{g mL}^{-1}$) were mixed with 2.5 mL phosphate buffer solution (0.2 M, pH = 6.6) and 2.5 mL of 1% potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$] in test tubes. The mixture was placed in a water bath of 50 °C, for 20 minutes. A volume of 2.5 mL of 10% trichloroacetic acid (TCA) was added to the mixture and mixed thoroughly. A volume of 2.5 mL of this mixture was then mixed with 2.5 mL distilled water and 0.5 mL FeCl_3 of 0.1% solution and allowed to stand for 10 min. The absorbance of the mixture was measured at 700 nm using a UV-VIS spectrophotometer (UV mini 1240, Shimadzu Corporation, Kyoto, Japan); the higher the absorbance of the reaction mixture, the greater the reducing power. Ascorbic acid was used as a positive control for this assay. All procedures were performed in triplicate.

Measurement of free radical scavenging activity using the DPPH assay

The free radical scavenging activity (antioxidant capacity) of the plant phytochemicals on the stable radical, 2,2-diphenyl- β -picrylhydrazyl (DPPH) was evaluated by the method established by Shirwaikar *et al.*³². In this assay, a volume of 1.5 mL of methanolic solution of the compound at different concentrations was mixed with 0.5 mL of the methanolic solution of DPPH (0.1 mM). An equal amount of methanol and DPPH without sample served as a control. After 30 minutes of reaction at room temperature in the dark, the absorbance was measured at 517 nm against methanol as a blank using a UV

spectrophotometer as mentioned above. The percentage free radical scavenging activity was calculated according to the following equation:

$$\% \text{ Scavenging activity} = [(Ac-As) / Ac] \times 100$$

Where Ac = Absorbance of control and As = Absorbance of sample

Determination of hydroxyl radical scavenging activity using the Deoxyribose assay

The deoxyribose assay for hydroxyl radical scavenging activity was performed as described previously by Chung *et al.*³³. A mixture of 2-deoxyribose (5.6 mM, 500 μ L), Fe³⁺ chloride (0.1 mM, 100 μ L) and EDTA (0.1 mM, 100 μ L), H₂O₂ (1 mM, 100 μ L), 100 μ L of sample (concentrations ranging from 15.625 to 250 μ g/mL), 500 μ L of 50 mM potassium phosphate buffer (pH 7.4) and 100 μ L of vitamin C (1 mM) was made and incubated for 30 min at 50 °C. The Fe³⁺ chloride and EDTA was mixed prior to combining the other reagents. A solution of thiobarbituric acid (TBA) (1 mL, 1% w/v) and trichloroacetic acid (TCA) in aqueous solution (1 mL, 2.8% w/v) was added, and the mixture was heated for 30 minutes in a water bath at 50 °C. The absorbance of the amount of chromogen produced was measured at 532 nm using a UV spectrophotometer as mentioned above. The hydroxyl radical scavenging activity (percentage inhibition rate) was calculated according to the equation as follows:

$$\% \text{ Scavenging activity} = [(Ac-As) / Ac] \times 100$$

Where Ac = Absorbance of control and As = Absorbance of sample

All experiments were performed in triplicate.

Statistical Analysis

The data are presented as mean \pm SD of triplicates. The data were statistically analyzed by using a statistical software program SPSS (SPSS for windows, version 18, SPSS Science,

Chicago, IL, USA). The one-way analysis of variance (ANOVA) followed by the Tukey's multiple range post-hoc test was employed to find the differences. The data were considered significantly different at $p < 0.05$.

ASSOCIATED CONTENT

Supporting Information

1D and 2D NMR spectra of compounds **5**, **6** & **9** are available free of charge *via* the internet at <http://pubs.acs.org>.

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CHAPTER SEVEN

SUMMARY AND CONCLUSION

7.1 Summary

This study focused on the isolation and characterisation of secondary metabolites from four plants belonging to the genera *Vernonia* and *Vepris* which are used in Kenyan traditional medicine as an analgesic, antibacterial, antimalarial, anti-allergy medication, and antivenom (snake bite) and for dermatological infection, dental caries, respiratory tract infections, eye problems, cardiovascular complications, neurological disorders and psychotic disorders. The study was undertaken in order to validate the use of these plants in what they are being traditionally used for as well as to provide the traditional healers with a scientific basis for using the plants to treat additional medical symptoms and diseases.

In order to do this, the plants were investigated for their phytochemical constituents and based on what was present, a literature search on the medicinal properties of the compounds were undertaken to provide a rationale for the use of the plants in different medicinal areas. In addition, bioassays were identified to test the activity of the isolated compounds, thereby providing additional information on the use of the plant in traditional medicine.

7.1.1 Recommendations for *Vernonia auriculifera*

Vernonia auriculifera is used in Kenyan traditional medicine for the treatment of headache, conjunctivitis, toothache, snake bite and bacterial infection (wound infection). Previous phytochemical studies have found hydroperoxides of unsaturated fatty acid methyl esters and plant growth stimulators from *V. auriculifera* leaves. Our studies have revealed the leaves to contain a sesquiterpene amine (farnesylamine), four triterpenoids, (lupenyl acetate, α -amyrin, β -amyrin and β -amyrin acetate) and a steroid (β -sitosterol). The stem bark

afforded two triterpenoids, friedelanone and friedelin acetate. From the roots, one triterpenoid, oleanolic acid was isolated. The triterpenoids have been reported to be used as antibacterials, antifungals, anti-inflammatories, anti-leishmanials and antimalarials. The plant could therefore be used in traditional medicine to treat the symptoms of inflammation and infections by bacteria, fungus and parasites (malaria and leishmania).

In addition we have tested the compounds for antibacterial and antibiofilm activities using the broth microdilution method and found that β -amyrin, α -amyrin and β -amyrin acetate had moderate antibacterial activity. At sub-MIC concentrations, oleanolic acid and β -amyrin acetate exhibited antibiofilm activity. Due to the inavailability of health facilities and expensive pharmaceutical supplies in most of rural Kenya, the plant *V. auriculifera* could replace common commercial antibiotics.

The isolation of pentacyclic triterpenoids in *V. auriculifera* also provides a chemotaxonomic link to the genus *Vernonia* as pentacyclic triterpenoids could be an additional chemotaxonomic marker to the sesquiterpene lactones.

7.1.2 Recommendations for *Vernonia urticifolia*

Vernonia urticifolia is used in Kenyan traditional medicine for the treatment of sinuses, allergy, respiratory tract infection and skin infections. There are no previous phytochemical or pharmacological studies carried out on this plant. Our studies have indicated the leaves to contain, a polyene, urticifolene, a carotenoid and a sterol, β -sitosterol. Polyenes are known to be used as anti-fungal agents and carotenoids are known to have antibacterial, anticancer and antioxidant properties. The plant could therefore be used in traditional medicine to treat the symptoms of bacterial infections, fungal infections and cancer. It can

also be used as an antioxidant supplement due to its carotenoid component which are reported to be antioxidants.

We assessed the compounds for antibacterial activity using the broth microdilution methods and found that lutein and urticifolene possessed antibacterial activity. This plant could substitute/supplement modern antibiotics in most of rural Kenya where modern health facilities are inaccessible. The compounds isolated from *V. urticifolia* add to the existing compounds from the genus *Vernonia*.

7.1.3 Recommendations for *Vepris glomerata*

Vepris glomerata is used in Kenyan traditional medicine for the treatment of malaria, eye problem, cardiac pain, epilepsy, stroke and psychosis. Previous phytochemical studies have found the leaves of the plant to contain two furoquinoline alkaloids, skimmianine and (montrifoline) evoxine.

Our studies have indicated the leaves to contain a flavonoid (veprisinol) and four furoquinoline alkaloids, haplopine-3,3'-dimethylallyl ether, anhydroevoxine, evoxine, and skimmianine. The stem bark was found to contain two cinnamic acid derivatives, (*p*-hydroxycinnamic acid and caffeic acid), an aromatic ester (methyl cinnamate), a flavonoid (hesperetin) and two limonoids (limonin and limonyl acetate). The roots contained a cinnamaldehyde derivative (glomeral), a coumarin (scoparone) and a lignin (syringaresinol). Surprisingly most of these compounds are derived from cinnamic acids, a key intermediate in the shikimate and phenylpropanoid pathways where these classes of compounds are biosynthesized. Cinnamic acids have been known to possess a wide range of biological activities such as anti TB, antidiabetic, antioxidant, antimicrobial, hepatoprotective, CNS depressant, anticholesterolemic, antifungal, antihyperglycemic, antimalarial, antiviral, cytotoxic,

anti-inflammatory as well as to absorb UV rays. Flavonoids have been known to be used as antioxidants while alkaloids are reported to possess antimicrobial, antiradical, antioxidant, antiplasmodial, anticancer, and antimutagenic activities. Limonoids are reported to exhibit antifungal, antibacterial, antimalarial, antiprotozoan, antiviral, anti-inflammatory and antioxidant activities.

The parts of this plant could therefore be used in traditional medicine to treat a wide range of diseases including; the symptoms of malaria, bacterial and fungal infection, cancer, diabetes, TB and inflammation and could also be taken as an antioxidant supplement due to its flavonoid component.

In addition we have tested the flavonoids and alkaloids for antioxidant activity using the DPPH, FRAP and deoxyribose methods and found that two of the compounds were even more active than ascorbic acid as an antioxidant in the deoxyribose test. Compounds isolated from the stem bark and roots were also assessed for antibacterial activity using the disc diffusion and broth microdilution methods. Glomerol and hesperetin were found to be active. Due to the inaccessibility of pharmaceutical supplies in most of rural Kenya, *V. glomerata* could replace common commercial antibiotics and antioxidants such as vitamin C in these communities.

Since veprisinol and haplopine-3, 3'-dimethylallyl ether shows high antioxidant activity whereas glomerol exhibited good antibacterial activity, further work is necessary to first investigate a possible synthetic scheme for the synthesis of these compounds and then determine whether veprisinol and haplopine-3, 3'-dimethylallyl ether can be developed into a commercial antioxidant supplement. Glomerol can be developed into a commercial antibiotic in the face of rising bacterial resistance

The isolation of limonin-type limonoids in *V. glomerata* also provides a chemotaxonomic link to the family Rutaceae as this class of limonoids are exclusive to members of the family *Rutaceae*. The isolation of furoquinoline alkaloids provides a chemotaxonomic link to the genus *Vepris*.

7.1.4 Recommendations for *Vepris uguenensis*

Vepris uguenensis is used in Kenyan traditional medicine for the treatment of malaria. Previous phytochemical studies have found the root bark of this plant to contain methyl uguenenoate, uguenenazole, uguenenonamide, flindersiamine, maculosidine and syringaldehyde. Our studies have indicated the leaves to contain a triterpenoid (lupeol), two protolimonoids (niloticin and chisoche-ton), limonoids (uguenensone and limonyl acetate), flavonoids (uguenenprenol and 7-*O*-methy-laromadenrin) and sesquiterpenoid, 8 α ,11-elemodiol. The stem bark contained the protolimonoid (chisoche-ton) limonoids (kihadalactone A, uguenensene and tricoc-cin S₁₃ acetate) and a furoquinoline alkaloid, (skimmianine) and the roots were found to contain a limonoid (methyl uguenenoate) and furoquinoline alkaloid, flindersiamine. Alkaloids, flavonoids and limonoids have a wide range of biological activities as discussed above (section 7.3).

V. uguenensis could therefore be used in traditional medicine to treat the symptoms of malaria, inflammation, bacterial, viral and fungal infections and could also be taken as an antioxidant supplement due to its flavonoid component.

Since uguenenprenol and 7-*O*-methy-laromadenrin shows high antioxidant activity, further work is necessary to investigate the synthesis of these compounds and then develop these flavonoids into commercial antioxidant supplements.

The isolation of six limonoids of in *V. uguenensis* also provides a chemotaxonomic link to the family Rutaceae as this class of limonoids are common to members of the family Rutaceae and rare in other limonoid producing families such as the Meliaceae family. The limonoids and the protolimonoids isolated, altogether formed an interesting limonin biosynthetic pathway. The isolation of uguenensene and uguenensone provided the missing compounds in this biosynthetic scheme.

7.2 Conclusion

The two *Vernonia* and *Vepris* species studied in this work produced a range of secondary metabolites, which have shown to have pharmaceutical effects, both in the literature and by our own bioassays. This knowledge is important for the ethnomedicinal healers in that they could use this as a rationale for using the plants in African traditional medicine. Furthermore, these plants provide an inexpensive alternative to the pharmaceuticals already on the market, which in many cases is inaccessible to the local communities in Kenya.

Our work has shown, in particular that these plants could be used as antioxidant supplements and antibacterial agents.

SUPPORTING INFORMATION

Supporting information includes 1D NMR, 2D NMR, IR, UV, MS and CD experiments.

Chapter 2: Lupenyl acetate P1-1, oleanolic acid P1-2, β -amyrin & α -amyrin P1-3 & 5, β -amyrin acetate P1-4, friedelanone P1-6, friedelin acetate P1-7, β -sitosterol P1-8 and farnesylamine P1-9.

Chapter 3: Urticifolene P2-1 and lutein P2-2.

Chapter 4: Veprisinol P3-1, haplopin-3,3'-dimethylallyl ether P3-2, anhydroevoxine P3-3, evoxine P3-4 and skimmianine P3-5.

Chapter 5: Glomerol P4-1, *p*-hydroxycinnamic acid P4-2, caffeic acid P4-3, methyl cinnamate P4-4, hesperetin P4-5, scoparone P4-6, syringaresinol P4-7, limonin P4-8 and limonyl acetate P4-9.

Chapter 6: Niloticin P5-1, chisocheon P5-2, kihadalactone A P5-3, uguenensene P5-4, uguenensene P5-5, methyl uguenensate P5-6, uguenenprenol P5-7, 7-*O*-methylaromadennin P5-8, flindersiamine P5-9, 8 α ,11-elemodiol P5-10, tricoccin S₁₃ acetate P5-11 and lupeol P5-12.