Terpenoid Plant Metabolites - Structural Characterization and Biological Importance

Maldonado, Eliana

2014

Terpenoid plant metabolites

Structural characterization and biological importance

Eliana Maldonado

LUND
UNIVERSITY

Doctoral Dissertation which, by permission of the Faculty of Engineering at Lund University, Sweden, will be publicly defended on Wednesday 1st October 2014 at 9:30 a.m. in Lecture Hall B, at the Center of Chemistry and Chemical Engineering Getingevägen 60, Lund, for the degree of Doctor of Philosophy in Engineering.

Faculty opponent

Dr. Luis Manuel Peña-Rodríguez

Organization	Document name
LUND UNIVERSITY	DOCTORAL DISSERTATION
Centre of Analysis and Synthesis, Department of Chemistry, Kemicentrum, P.O.Box 124, SE-221 00 Lund, Sweden	**Date of issue** 2014-09-05
Author(s)	**Sponsoring organization**
Eliana Maldonado	Swedish International Development Cooperation Agency (SIDA)

Title and subtitle:Terpenoid plant metabolites. Structural characterization and biological importance.

Abstract

In response to the challenges of their local environments, organisms produce of a large number of chemical diverse compounds with complex stereochemistry and reactive functional groups. These characteristics enable them to interact and bind specifically to biological target molecules and exert various biological activities, and have assured that Natural products continues to be an important source of bioactive compounds, which, for example, facilitate the search for new lead structures that can be developed into new pharmaceuticals.

This thesis describes the isolation by different chromatographic techniques, structure elucidation by NMR spectroscopy and high-resolution mass spectrometry and bioactivity of secondary metabolites of four medicinal plants.

Kaunia lasiophthalma G. was found to be particularly rich in sesquiterpene lactones and produced two new eudesmane derivatives (**2.1** and **2.2**) as well as a novel triterpene with a new carbon skeleton (**2.3**). The anticancer activity of several of the isolated metabolites was studied towards a panel of five breast-cancer cell lines, and compared with their cytotoxicity in normal-like breast epithelial MCF-10A cells. In addition, demonstrating the importance of the α-methylene-γ-lactone moiety present in most assayed compounds, a methyl cysteine adduct prepared from **2.10** was also evaluated. These compounds tested were found to exhibit different levels of anticancer and cytotoxicity.

The isolation and identification of the active constituents from extracts of *Trixis antimenorrhoea* and *Lantana balansae* possessing antileishmanial activity was carried out. Two new metabolites, the nortrixane **3.1** and the trixanolide **3.2**, and eleven known compounds, terpenoids and flavonoids, were isolated from *Trixis antimenorrhoea*. *Lantana balansae* yielded eleven previously discovered metabolites, however, a potent cyclopentenone fatty acid derivative (**3.16**) and flavanones (**3.17** and **3.18**) are new classes of secondary metabolites for the genus *Lantana.* The structures of the compounds and the associated leishmanicidal activity towards *L. amazonensis* and *L. braziliensis* are discussed.

Three macrocyclic monoterpene *O*-glycosides (**4.1**-**4.3**) isolated from the leaves and small branches of *Parkinsonia aculeate* L. are reported. The structures were established using NMR and MS techniques. Particularly, 2D NMR experiments, HMQC, HMBC, COSY, TOCSY and ROESY, were crucial for the assembling of the structures.

Key words: Terpenoid, sesquiterpene lactones, breast-cancer cell lines, cytotoxicity, *antileishmanicial*, structural characterization

Classification system and/or index terms (if any)

Supplementary bibliographical information		Language: English
ISSN and key title		ISBN 978-91-7422362-0
Recipient's notes	Number of pages: 75	Price
	Security classification	

Distribution by (name and address)

Signature  Date 2014-09-05

Terpenoid plant metabolites

Structural characterization and biological importance

Eliana Maldonado

LUND
UNIVERSITY

A doctoral thesis at a university in Sweden is produced as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These have either already been published or are manuscripts at various stages (*in press*, *submitted* or *manuscript*).

Centre for Analysis and Synthesis
Department of Chemistry
Lund University
P.O. Box 124
SE-221 00 Lund, Sweden
ISBN 978-91-7422362-0

Printed in Sweden by Media-Tryck, Lund University
Lund 2014

The only limit to our realization of tomorrow will be our doubts of today. Let us move forward with strong and active faith.

Franklin D. Roosevelt.

Abstract

Organisms are constantly challenged by their local environments, and in responce produce of a large number of incredibly diverse compounds with complex stereochemistry and reactive functional groups. Their chemical characteristics give them the ability to bind specifically to biological target molecules, which in turn makes them biologically active. Nature is still our most important source for biologically active compounds, and natural products, or secondary metabolites, provide us with potentially useful new compounds that, for example, can be used for the search of new lead structures that can be developed into new pharmaceuticals.

This thesis describes the isolation by different chromatographic techniques, structure elucidation by high field NMR spectroscopy and high resolution mass spectrometry, and the bioactivity of selected secondary metabolites from four medicinal plants in the search of bioactive natural products.

Kaunia lasiophthalma G. collected in Bolivia was found to be particularly rich in sesquiterpene lactones and produced two new eudesmane derivatives (**2.1** and **2.2**) and a novel triterpene with a new carbon skeleton (**2.3**). The anticancer activity of several of the isolated sesquiterpenes was studied towards a panel of five breast-cancer cell lines, and compared with their cytotoxicity in normal-like breast epithelial MCF-10A cells. In addition, demonstrating the importance of the α-methylene-γ-lactone moiety present in almost all compounds, a methyl cysteine adduct prepared from **2.10** was also evaluated and found to be considerably less active. Several of the terpene lactones having an α-methylene-γ-lactone moiety were found to exhibit potent anticancer activity, but also cytotoxicity.

The isolation and identification of several antileishmanial constituents from extracts of the Bolivian plants *Trixis antimenorrhoea* and *Lantana balansae* was carried out. Two new metabolites, the nortrixane **3.1** and the trixanolide **3.2**, and eleven known compounds, were isolated from *T. antimenorrhoea*. *Lantana balansae* yielded eleven previously reported metabolites, however, a potent cyclopentenone fatty acid derivative (**3.16**) and flavanones (**3.17** and **3.18**) are new classes of secondary metabolites for the genus *Lantana.* The structures of the

isolated compounds and the associated antileishmanial activity towards *L. amazonensis* and *L. braziliensis* are discussed.

In addition, the structures of three macrocyclic monoterpene *O*-glycosides (**4.1**-**4.3**) isolated from the leaves and small branches of *Parkinsonia aculeata* L. is reported. The structures were established using NMR and MS techniques and a combination of the 2D NMR experiments, HMQC, HMBC, COSY, TOCSY, and ROESY, were crucial for the assembling of the structures. The low yields of these compounds unfortunately made further studies to determine the absolute configuration of the individual monoterpenes and monosacharides impossible, and did not make it possible to study any biological activity.

Popular summary

The secondary metabolites found in plants represent an extremely rich and limitless source of novel chemical diversity for drug discovery. In fact, some of the most important drugs currently in use today are derived from plants and they were discovered as a result of the study of medicinal plants used in traditional medicine. In this context, Bolivia is one of the most biological diverse countries of the world maintaining vast intact humid and dry forest ecosystems, which are the habitat of more than 20,000 species of higher plants. Due to the biodiversity and individuality of each geographical region, the medicinal plant knowledge is unique to the ethnical group living and working in these regions and it is therefore essential to preserve them. The current renewed interest in natural products prompted us to study medicinal plants as source of bioactive compounds with anticancer or leishmanial activities.

In this thesis, medicinal plants growing at high altitudes were collected in Cochabamba, Bolivia, and their ethanol crude extracts screened for leishmanicidal activity. *Kaunia lasiophthalma*, *Trixis antimenorrhoea* and *Lantana balansae* showed high or moderate activity and were therefore chosen for detailed phytochemical and biological studies.

Kaunia lasiophthalma yielded two new eudesmane derivatives, a novel terpenoid, and several previously discovered sesquiterpenes and flavonoids. The anticancer activities of the isolated sesquiterpene lactones assayed in five brest-cancer cell lines was compared with the cytotoxicity in the normal-like epithelial cell line, MCF-10A. These biological results demostrate that all assayed compounds showed different levels of cytotoxicity and that the normal-like MCF-10A was the least sensitive to all compounds.

Trixis antimenorrhoea was found to be rich in terpenoids and flavonoids, which are interesting groups of natural products that have shown to exhibit several biological properties including leishmanicidal activity. *T. antimenorrohea* produced two new natural products, a novel nortrixane and a trixanolide derivative. The low yield of the novel nortrixane prevented further studies of this compound, while the new trixanolide exhibited high activity towards the two

strains of *Leishmania* parasite used in this study. The lupane-type triterpernes and the flavonoids displayed different levels of activity.

From the bioactive extract of *Lantana balansae* several known secondary metabolites were isolated, structurally analyzed and their biological activity evaluated. As a result, the cyclopentenone fatty derivative and the two flavanones are new classes of secondary metabolites for the genus *Lantana* and flavonoids were found to be the major metabolites. The potent antileishmanial activity of 12-oxo-phytodienoic and flavonoids explain the significant activity of the crude extract of *L. balansae*.

In addition, structure elucidation of three novel macrocyclic monoterpene *O*-glycosides isolated from the medicinal plant *Parkinsonia aculeata* was established by NMR and MS techniques.

In summary, this research project contributes to the chemical knowledge of these plant species and confirmes that medicinal plants are an important source of bioactive metabolites with anticancer or antileishmanial activities. Further studies to understand the molecular mechanisms governing their beneficial activity should be performed.

List of papers

I. Cytotoxic Sesquiterpene lactones from *Kaunia lasiophthalma* Griseb

Maldonado, Eliana M.; Svensson, Daniel; Oredsson, Stina M.; Sterner, Olov
Scientia Pharmaceutica (2014), 82(1), 147-16

II. A novel cytotoxic terpenoid from the flowers of *Kaunia lasiophthalma* Griseb

Maldonado, Eliana M.; Svensson, Daniel; Oredsson, Stina M.; Sterner, Olov
Phytochemistry Letters (2014), 8, 105-108

III. Antileishmanial metabolites from *Trixis antimenorrhoea*

Maldonado, Eliana M.; Salamanca, Efrain; Giménez, Alberto; Saavedra, Gloria; Sterner, Olov
Submitted to Phytochemistry Letters

IV. Secondary metabolites from *Lantana balansae*

Maldonado, Eliana M.; Salamanca, Efrain; Giménez, Alberto; Sterner, Olov
In manuscript

V. Macrocyclic monoterpene glycosides from *Parkinsonia aculeat*a L.

Maldonado, Eliana M.; Marzouk, Mohamed S.; Bergquist, Karl-Erik; Sterner, Olov
In manuscript

The author's contribution to the papers

I. The author performed the experimental work in collaboration with D. Svensson, the structural characterization of the isolated compounds and wrote most of the paper. Anticancer assays were carried out by Prof. Stina Oredsson.

II. The author performed the experimental work in collaboration with D. Svensson, the structural characterization of the isolated compounds and wrote the paper. Anticancer assays were carried out by Prof. Stina Oredsson.

III. The author performed the experimental work, structural characterization of the isolated compounds and wrote the manuscript. Antileishmanial assays were carried out by E. Salamanca.

IV. The author performed the experimental work, structural characterization of the isolated compounds and wrote the manuscript. Antileishmanial assays were carried out by E. Salamanca.

V. The author contributed to the structural characterization of the isolated compounds and participated in the writing of the manuscript together with the co-authors.

Abbreviations

ACE	Angiotensin converting enzyme
$CDCl_3$	Deuterated Chloroform
C_6D_6	Deuterated Benzene
CC	Column chromatrography
COSY	Correlation Spectroscopy
DMAP	N,N-Dimethylpyridin-4-amine
DNA	Deoxyribonucleic acid
GSH	Glutathione
HER2	Human epidermal growth factor receptor 2
HRESIMS	High resolution electrospray ionization mass spectrum
IC_{50}	Half-effective inhibitory concentration
MTPA	Methoxy(trifluoromethyl)phenylacetic acid
NOESY	Nuclear Overhauser Effect Spectroscopy
ROESY	Rotating-frame Overhauser Spectroscopy
SAR	Structural-Activity Relationship
SLs	Sesquiterpene lactones
TOCSY	Total Correlation Spectroscopy
VLC	Vacuum liquid chromatography

Contents

Abstract v

Popular summary vii

List of papers ix

The author's contribution to the papers x

Abbreviations xi

1. Natural products 1

1.1 Introduction 1

1.2 Natural products (Secondary metabolites) 3

1.3 Sources of natural products 3

1.3.1 Natural products from microorganisms 4

1.3.2 Natural products from marine organisms 4

1.3.3 Natural products from animal sources 5

1.3.4 Natural products from plants 6

1.4 The aim and outline of the thesis 9

2. Cytotoxic sesquiterpenoids 11

2.1 Introduction 11

2.1.1 The genus *Kaunia* (Asteraceae: Eupatorieae) 12

2.2 Chemical investigation of *Kaunia lasiophthalma* (Paper I and II) 13

2.3 Results and discussion 14

2.3.1 Isolation of compounds 14

2.3.2 Structure elucidation of 2.1 14

2.2.3 Structure elucidation of 2.2 15

2.2.4 Structure elucidation of 2.3 17

2.2.5 Identification of known compounds 19

2.2.6 Preparation of compound 2.22 21

2.2.7 Evaluation of the cytotoxic activity 21

3. Antileishmanial metabolites 23
3.1 Introduction 23
3.1.1 The genus *Trixis* (Asteraceae) 24
3.2 Chemical investigation of *Trixis antimenorrhoea* (Paper III) 25
3.3 Results and discussion 25
3.3.1 Isolation of compounds 25
3.2.2 Structure elucidation of 3.1 26
3.2.3 Structure elucidation of 3.2 27
3.2.4 Identification of known compounds from *T. antimenorrhoea* 30
3.2.5 Preparation of the Mosher ester derivatives 3.14 and 3.15 31
3.2.6 Evaluation of the antileishmanial activity (*T. antimenorrhoea)* 31
3.3 The genus *Lantana* (Verbenaceae) 32
3.4 Chemical investigation of *L. balansae* (Paper IV) 33
3.5 Results and discussion 33
3.5.1 Isolation of compounds from *L. balansae* 33
3.5.2 Structure elucidation of 3.16 34
3.5.3 Structure elucidation of 3.17 and 3.18 35
3.5.4 Identification of known compounds from *L. balansae* 37
3.5.5 Evaluation of the antileishmanial activity (*L. balansae)* 37
4. Macrocyclic monoterpene *O*-glycosides 39
4.1 Introduction 39
4.1.1 The genus *Parkinsonia* (Fabaceae) 40
4.2 Chemical investigation of *Parkinsonia aculeata* L. 40
4.3 Results and discussion 41
4.3.1 Structure elucidation of 4.1 42
4.3.2 Structure elucidation of 4.2 44
4.3.3 Structure elucidation of 4.3 44
Conclusions and perspectives 47
Acknowledgments 49
References 51

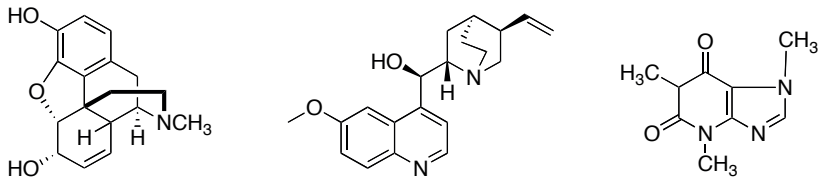
1. Natural products

1.1 Introduction

As long as man has been living on Earth they have utilized plants not only as a source of food and shelter but also as medicines to alleviate a number of diseases and prevent others. The knowledge of healing or poisonous properties of plants was acquired during the development of ancient civilizations, and was passed on through the generations to eventually form the basis of sophisticated traditional medicinal systems. The earliest written information, dating from 2600 BCE records the use of 100 plant-derived substances in Mesopotamia. Examples are *Cedrus* species (cedar), *Glycyrrhiza glabra* (licorice), and *Commiphora* species (myrrh), which still are used today. Egyptians developed the technique of embalming based on their extensive knowledge of plants, but it is the "Ebers Papyrus" (1500 BCE) that documents the use of a large number of drugs, mostly of plant origin. The Chinese Materia Medica, Shennong Herbal and the Tang herbal are evidence of the importance of medicinal plants in ancient China. Likewise, Indian Ayurvedic system has made a great contribution [1]. The development of civilizations also initiated a rapid progress in the understanding of how to use medicinal plants, as powders, simple extracts or tinctures.

It was not until the early nineteenth century that scientists began to isolate the active components from plants. This constitutes the beginning of the natural product chemistry. Morphine, isolated from opium (*Papaver somniferum*) by Friedrich Sertürner became the first purified compound from a plant source, and this discovery opened a new era in the history of medicine portrayed by the isolation and characterization of the active principles from natural sources. The medicinal antimalarial property of the bark of *Cinchona* tree, first discovered by the Quechua Indians of Peru and Bolivia and brought to Europe by the Jesuits, led the isolation of quinine which was the first effective treatment of malaria, and initiated an interest in plants from the New World [2]. Examples of other important natural products isolated are strychnine, caffeine, nicotine, atropine and the mixture of cardioactive glycosides, digitaline (Figure 1.1) [3]. However, to date

approximately 80 % of the world's population in developing countries depend on the plant-derived traditional medicine as their primary healthcare [4].



morphine **quinine** **caffeine**

Figure 1.1 Examples of the first compounds isolated from plants.

Although, plants have been the major source of medicinal products used as leads for pharmaceutical development, fungi, marine organisms and microorganisms have proven to be valuable sources of novel chemical diversity and biologically active compounds. In the past couple of decades, advances in high throughput screening focusing on various molecular disease targets combined with combinatorial chemistry has resulted in a decline of the interest in natural products research by pharmaceutical firms. However, in recent years the discovery of for example Taxol, etoposide and artemisinin together with the apparent failure of combinatorial chemistry to generate useful lead compounds has renewed the interest in drugs of natural origin. In fact, a recent review of the new-drug approvals over the past three decades (1981-2010) reveals that natural products continue to be a significant source of drugs and leads. Approximately half of the drugs used in clinical trials today are from natural origin, in one way or the other. The influence is even greater for anti-infective and anticancer agents [1, 5].

Two main features have contributed to the success of natural products in drug discovery: their structural complexity and diversity, as well as the fact that they often possess highly selective and specific biological activities. A good example of the usefulness of natural product as lead structure are the four new medicinal plant-derived drugs recently introduced on the U.S. market: Arteether (potent antimalarial), Galantamine (for Alzheimer's disease), Nitisinone (for treatment of tyrosinaemia), and Tiotropium (chronic obstructive pulmonary disease, COPD) (Figure 1.2) [6].

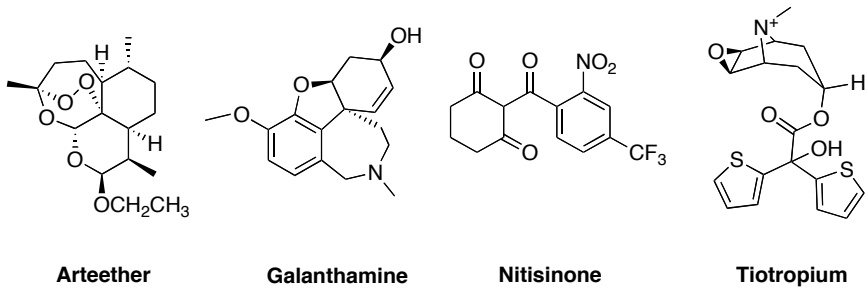


Figure 1.2 Examples of new medicinal plant dugs recently introduced to market.

1.2 Natural products (Secondary metabolites)

Secondary metabolites are, by definition, low molecular weight compounds (less than 2000 amu) that are not necessarily essential for the daily life functions of a cell or an organism. In contrast, the primary metabolites such as carbohydrates, proteins, fats, and nucleic acids are essential for energy production and thereby for the growth, development, reproduction and function of an organism. Improvements of the biochemical techniques and the development of molecular biology has shown that secondary metabolites play important roles in the adaptation of organisms to their environment, in chemical defenses against predators and pathogens, for mediation of spatial competition, to facilitate reproduction, to protect against UV radiation, and for chemical signaling to ensure survival [7].

Secondary metabolites are more characteristic for the particular biological group, such as family or genus, and apparently the biosynthetic pathway involved is related to the evolution of the species [8]. In plants, they are usually classified according to their biosynthetic pathways. Three large molecule families are generally considered: phenolics, terpenes (including steroids), and alkaloids [7].

1.3 Sources of natural products

Natural products are produced by conversions of primary metabolites, and are broadly divided into four categories depending on their biological source.

1.3.1 Natural products from microorganisms

Since the discovery of penicillin and its broad therapeutic usefulness, microorganisms were extensively investigated as sources of novel bioactive compounds. Microorganisms have yielded the most important antibacterial, immunosuppressive, and cholesterol lowering agents, and especially antitumor antibiotics [1, 9]. Some of remarkable examples are shown in Figure 1.3.

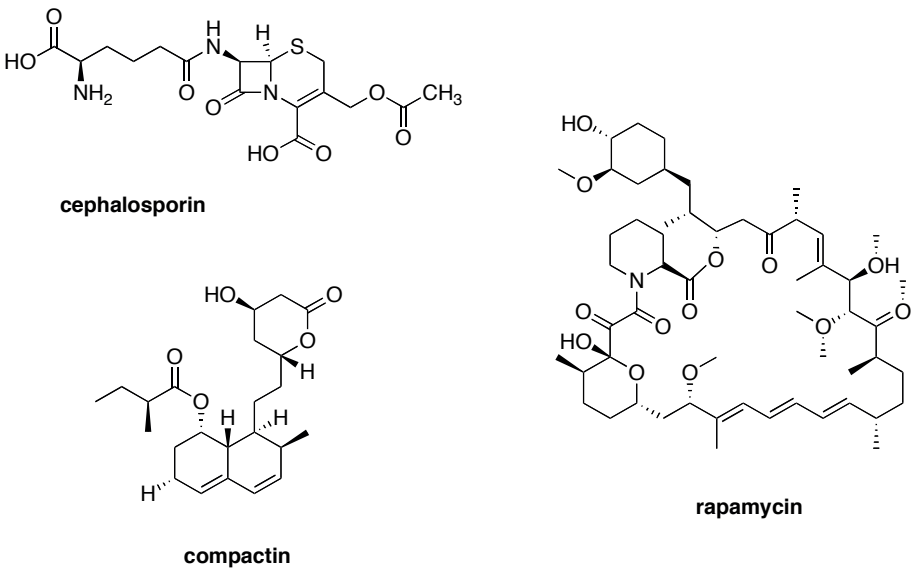


Figure 1.3 Examples of natural products isolated from microorganisms.

1.3.2 Natural products from marine organisms

The marine environment represents a great biodiversity exposed to high salinity, extreme pressure, lack of light and large difference in temperature in comparison with terrestrial organism. Marine organisms are characterized by the production of complex and potent natural products in response to the ecological requirements [3]. The investigations of marine organisms have initially focused on the discovery of new anticancer agents. In Figure 1.4, the first two products that have been approved as drugs are shown: Ziconotide (Prialt®) a non-narcotic analgesic and ecteinascidin (Yondelis®) for the treatment of soft tissue sarcomas [10].

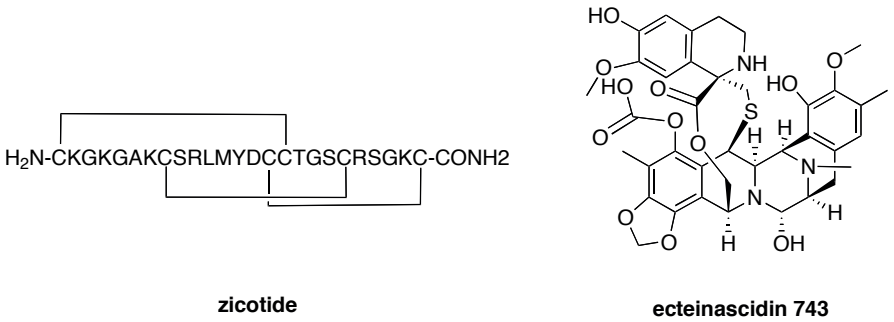


Figure 1.4 First two natural products isolated from marine sources.

1.3.3 Natural products from animal sources

Animals, insects, and arthropods represent another remarkable source of unique natural products. The wide array of defensive chemicals released by the different insect species include: cytotoxins that are potential anticancer compounds, neurotoxins that could be useful for the study and even treatment of central nervous system disorders, as well as antibiotic and antiviral peptides. Venoms and toxins from animals have also inspired the design and synthesis of drugs used in several diseases. Teprotide, for example, isolated from the venom of the pit viper *Bothrops jaracaca*, has led to the development of the ACE inhibitors captopril and enalapril, while epibatidine, isolated from the skin of the poisonous frog *Epipedobates tricolor*, has led to the development of a novel class of potential painkillers (structures are illustrated in Figure 1.5) [1, 11].

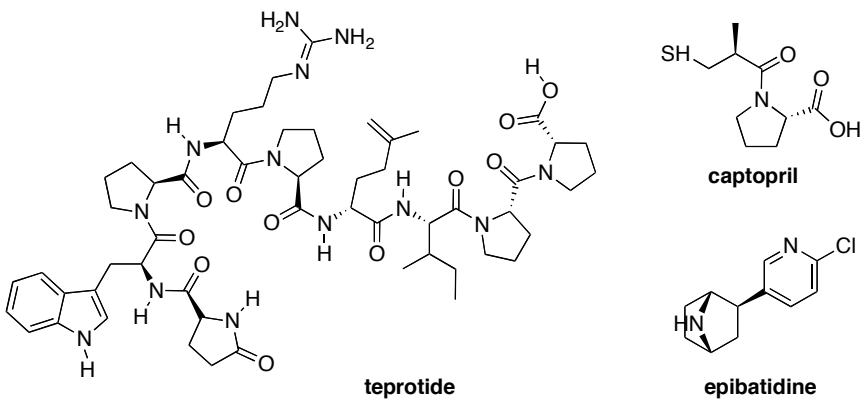


Figure 1.5 Examples of products obtained from animal sources.

1.3.4 Natural products from plants

As discussed above, natural products are produced by all organisms; however, the kingdom of plants has always been the most important provider of clinically used medicines. Ethnobotanical knowledge, the relative easy access to plants, and our skills to identify plant species explain the considerable attention on plant-based natural products. In plants, complex chemical defense systems have evolved resulting in the production of numerous chemically diverse compounds. Remarkably, more than 100,000 low-molecular natural products have been reported from plants [12].

Due to the nature of the scientific investigations conducted in this project, the discussions that follow in the remainder of this thesis will focus on natural products derived from plant species. The work includes the isolation and structure elucidation of the products, as well as the evaluation of their cytotoxic or leishmanicidal activities in collaboration with biologists.

1.3.4.1 Plant-based anticancer agents

Cancer is a leading cause of death in economically developed countries and the second leading cause of death in developing countries. Worldwide, the number of new cases of cancer continues to increase, the reasons may be the aging and growth of the world population as well as the adoption of life styles (e.g. smoking) that are known to cause cancer in the economically developing countries [13].

Medicinal plants have a long history in the treatment of cancer; in fact over the last century most of new clinical applications of plant secondary metabolites and their derivatives were directed towards combating cancer. Anticancer agents from plants currently in clinical use can be categorized into four main classes of compounds (Figure 1.6):

- Vinca alkaloids: Vinblastine and vincristine, isolated from *Catharanthus roseus*. They block cell division by irreversibly binding to tubulin resulting in its depolymerization.

- Podophyllotoxin: Podophyllotoxin was isolated from the resin of *Podophyllum peltatum*. Modified analogs bind to tubulin and cause DNA strand breaks during the G2 phase of the cell cycle, by inhibition of topoisomerase II.

- Paclitaxel: Paclitaxel was isolated from *Taxus brevifolia* and is considered to be the most important plant-derived anticancer drug discovered in recent years. Paclitaxel and derivatives act by binding to tubulin and stabilizing microtubules that can not depolymerize, or by interfering with tubulin assembly.

- Campothecin: Campothecin was isolated from *Campotheca acuminate* and has a unique mechanism of action. It was found to act by selective inhibition of topoisomerase I, causing cell death by DNA damage [6].

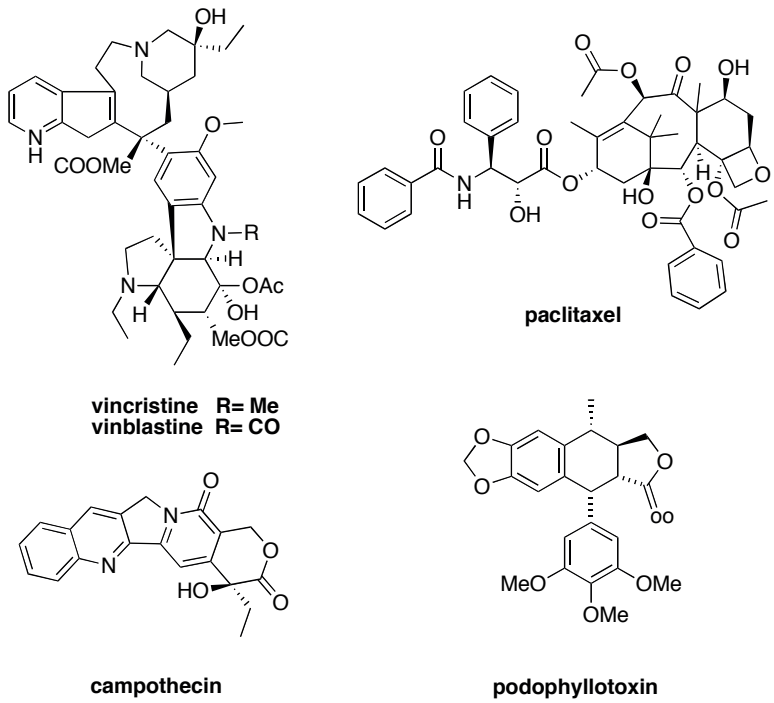


Figure 1.6 Anticancer agents derived from plants and its derivatives.

1.3.4.2 Plant-based antileishmanial agents

Despite the fact that infectious diseases have been identified as the third major cause of death in the world, many fall into the category of "neglected diseases". Among them, leishmaniasis, which is an ancient protozoan disease affecting about 12 million people with 2 million new cases every year, constitutes a serious public

health problem. In the search for better leishmanicidal plant-derived products a great number of crude plant extracts, semi-purified fractions and pure compounds have been evaluated. As a result, several important classes of natural compounds with promising antileishmanial activities have been identified, and include Aurones, Lignans, Chalcones, Flavonoids, Isoflavonoids, Saponins, Quinones, Alkaloids, Tannins, Terpenoids, Iridiods, Terpenes and Oxylipins. Among these, the alkaloids have been found to be the most effective [14, 15].

Some representative examples of natural compounds that have shown potent antileishmanial activity are: Luteolin and quercitin, flavonoids widely distributed in the plant kingdom. Mechanistic studies suggest that these compounds inhibit the synthesis of parasite DNA via inhibition of topoisomerase II. Plumbagin, isolated from *Pera benensis*, exherts activity by generating free radicals in the parasites and induce mammalian topoisomerase II mediated DNA cleavage [14]. The quinolone alkaloids 2-*n*-propylquinoline, chimanine-D and chimanine-B, isolated from *Galipea longiflora*, display antileishmanial activity against the promastigote forms of *Leishmania*, and oral administration of chimanine-D in mice model resulted in 99.9 % reduction of liver parasites [16]. The two species mentioned above, *P. benensis* and *G. longiflora*, are used in Bolivia for the treatment of cutaneos leishmaniasis.

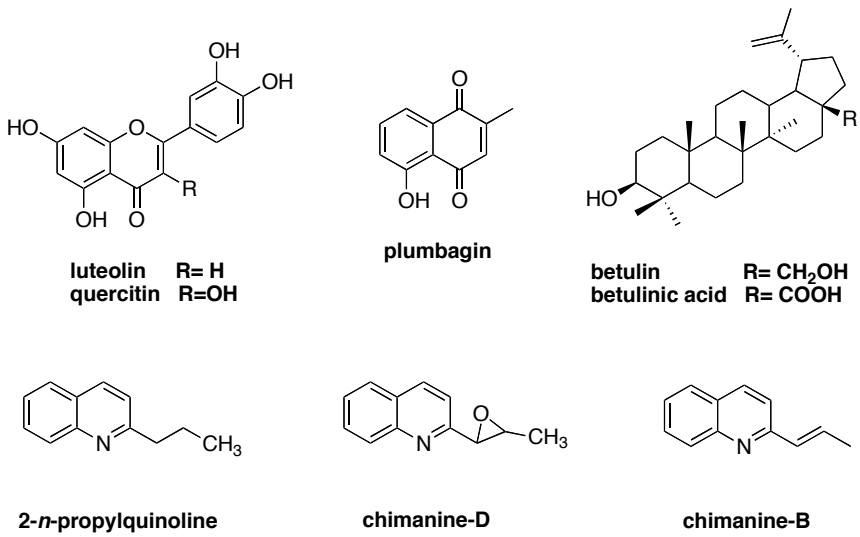


Figure 1.7 Examples of antileishmanial compounds from plants.

Recently, pharmacological studies have point out the importance of testing known anticancer agents against *Leishmania* due to the potential to induce parasite death. Based on this criteria, betulin and betulinic acid derivatives, found predominantly in the bark of birch trees of *Betula spp.*, exhibited activity against promastigotes of *L. amazonensis*[17] and *L. infantum*[18] as well as on amastigotes of *L. donovani*[19]. Betulin derivatives such as dihydrobetulinic acid induces apoptosis by primarily targeting DNA topoisomerase I and II in *L. donovani* [20, 21]. Artemisinin, the active principle of *Artemisia annua*, showed antileishmanial activity against several species of *Leishmania* [22, 23]. The mechanism of action of artemisinin in promastigotes of *L. donovani* is mediated by an iron-dependent generation of reactive intermediates, terminating in a caspase-independent, apoptotic mode of cell death [24].

These discoveries provide strong support that Natural products are and will continue to be used as new chemical entities, but also inspire the design of new semi-synthetic and synthetic analogues and derivatives with improved properties. The structural diversity, often with several chiral centers, concatenated rings and diverse functional groups make them ideal for interacting specifically with biological target molecules. An additional advantage with natural products is that they always, when applicable, are produced as one enantiomer. It is therefore essential that new natural products continuously are sought for and evaluated in both chemical and biological media.

The number of plant species has been estimated to be more than 300,000, but only 5-10 % of them have ever been investigated. Not all of these have been screened for bioactivity, and the reinvestigation of previously evaluated sources may provide even more leads. Development of techniques for the chemical characterization of smalls amounts of bioactive compounds together with advances in genetics and microbiology will facilitate the screening of natural compounds in the future.

1.4 The aim and outline of the thesis

The aim of the research presented in this thesis was to characterize novel and/or bioactive secondary metabolites from selected medicinal plants possessing *in vitro* anticancer and leishmanicidal activities. For these studies pure secondary metabolites were isolated and their structures elucidated.

The chapters outlined in this thesis have unifying themes. Following the general introduction of natural products, the second chapter describes the structure elucidation of the new metabolites isolated from *K. lasiophthalma*. The anticancer activities *in vitro* of the new and previously isolated metabolites are also presented, and a SAR discussion is provided.

In a similar manner, in chapter three the bioassay-guided fractionation of extracts of *T. antimenorrhoea* led to the isolation of two new trixianolides, while 12-oxo-phytodienoic and two flavanones new for the genus *Lantana* were isolated from *L. balansae*. The *in vitro* antileishmanial activity on promastigote form of two strains of *Leishmania* parasite is discussed.

In chapter four, the challenging structure elucidation of three novel macrocylic monoterpene glycosides isolated from *P. aculeata* is described in detail. The structures of these compounds were elucidated by NMR spectroscopy and mass spectrometry.

The last chapter includes the conclusions of this research project and perspectives.

2. Cytotoxic sesquiterpenoids

2.1 Introduction

Breast cancer is, by far, the second most common cancer diagnosed among women worldwide with an estimated 1.67 million new cases reported in 2012. It is also the second leading cause of death after lung cancer in developed countries [25]. Breast cancer is a heterogeneous disease that can be subdivided into different groups, based on gene expression profiles or clinic pathological characteristics such as estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) expression. As a result, all breast cancer subtypes have different tumor biology, treatment options and outcomes [26]. Despite advances in breast cancer treatment and chemopreventive strategies, the development of resistance to therapeutic agents is one of the major obstacles in the successful treatment of breast cancer and one of the main causes of its recurrence. It is therefore of great importance to seek new drugs, more effective treatments or combinations of treatments [27-29].

The search for effective plant-derived anticancer agents has continued to be of interest in drug development. Among others, sesquiterpene lactones have been studied extensively due to their wide range of biological activities including anti-inflammatory, cytotoxic and antitumor activities [30, 31].

Structurally, most of the active sesquiterpene lactones are characterized by an α-methylene-γ-lactone ring that has proved to be essential for the biological activities. They are known to react with biological nucleophiles, especially the thiols of cysteine residues in proteins as well as the free intracellular glutathione (GSH), by a Michael addition to their α-β-unsaturated lactone funcionality. The alkylation of cellular thiols results in the reduction of enzyme activity or causes disruption of GSH metabolism which would seriously affect the cell function. However, recent studies have demonstrated that the formation of the Michael addition adducts is a reversible reaction [32-34].

Interestingly, SLs that lack this reactive structural element exert potent cytotoxic activities in cancer cell lines, although the mechanism of action of this group of SLs is still a matter of debate.

SLs in clinical trials have shown properties that enable them to selectively trigger cell death in cancer cells while leaving normal cells unharmed. Representative examples are parthenolide, artemisinin and thapsigargin (Figure 2.1) [29, 35].

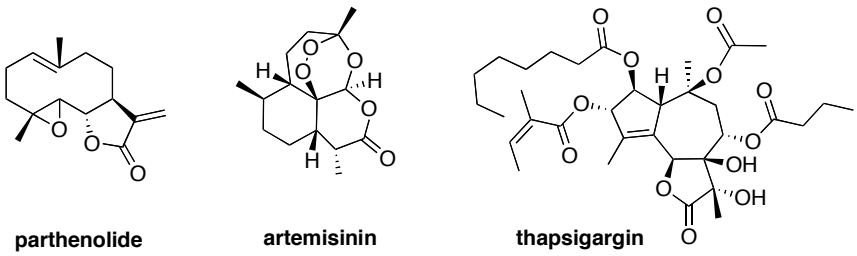


Figure 2.1 Sesquiterpene lactones in clinical trials.

2.1.1 The genus *Kaunia* (Asteraceae: Eupatorieae)

Eupatorieae (Asteraceae) comprises 180 genera with approximately 2,000 species[36]. Most of the plants are herbs, shrubs, climbers or small trees largely distributed through the American continent and mainly concentrated in Mexico, Central and South America, with relatively few species in the Old world. A number of medicinal plants belonging to the Eupatorieae tribe are known, in particular in the genus *Eupatorium*, and used traditionally as antimalarial [37, 38], anti-inflammatory, antibacterial and antifungal agents [39, 40].

The small genus *Kaunia* is represented by 14 Andean species of which 10 grow in Bolivia, but they are also found in Argentina, South of Brazil, Peru and Ecuador. Initially, this genus was placed in the concept of *Eupatorium* but later changed to *Kaunia* by R. M. King and H. Robinson [41].

Previous investigations of the chemical components and the biological activities of *Kaunia* species have revealed that they are characterized by the production of guianolides [42-44] with antitumor and allergenic activities [45, 46], germacranolides and thymol derivatives [43].

2.2 Chemical investigation of *Kaunia lasiophthalma* (Paper I and II)

Kaunia lasiophthalma Griseb (sym. *Eupatorium lasiophthalmun* G.) is an aromatic herbaceous plant bearing white-purple flowers. It grows in the Andean mountains of Cochabamba, Bolivia where is known by the common name of "T'uwi" and used by local people to treat inflammation and headache. As part of a project directed towards searching for biologically active natural products in the Bolivian flora, we prepared ethanol extracts of the leaves and flowers of *K. lasiophthalma*. The fractionation of each individual extract resulted in two new eudesmane derivatives (**2.1** and **2.2**) from the extract of the leaves and a novel terpenoid (**2.3**) from the extract of the flowers along with eighteen known compounds (**2.4**-**2.21**) (see Figure 2.2 and 2.6 for structures). The structure analysis of **2.1**-**2.3** was carried out by analysis of the one- and two-dimensional NMR spectra and results of HR mass spectrometric experiments. The structure elucidation of the new compounds as well as the anticancer activity of sesquiterpenes **2.1**-**2.4**, **2.8**-**2.10**, **2.12**, **2.13**, **2.15** and **2**.**17** and the methyl cysteine adduct **2.22** evaluated *in vitro* with the breast cancer cell lines HCC1937, JIMT-1, L56Br-C1, MCF-7 and SK-BR-3 and compared with the cytotoxicity in the noncancerous breast epithelial cell line MCF-10A will be discussed.

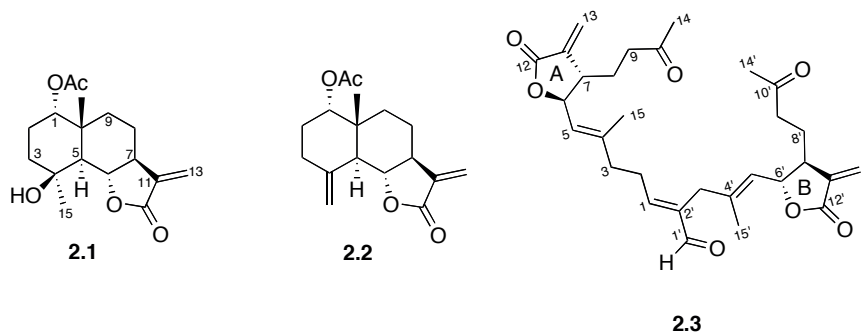


Figure 2.2 New metabolites of *Kaunia lasiophthalma.*

2.3 Results and discussion

2.3.1 Isolation of compounds

The ethanol crude extract of the leaves was suspended in a mixture of 90 % aqueous methanol and partitioned between hexane, CH_2Cl_2, and EtOAc. Further purification of the CH_2Cl_2 fraction, rich in sesquiterpene lactones, by VLC, silica gel column chromatography and Sephadex LH-20 chromatography led to the isolation of the two new eudesmane derivatives **2.1** (1.7 mg) and **2.2** (32.9 mg) as well as four known eudesmanes, **2.8** (10.0 mg), **2.9** (18.9 mg), **2.10** (80.0 mg), and **2.11** (6.3 mg), a germacrane, **2.4** (35.6 mg), an eudesmane unsatured ester, **2.17** (46.1 mg) and three flavonoids, **2.18** (59.0 mg), **2.19** and **2.20** (26.0 mg). Following the same procedure, the CH_2Cl_2 fraction of the flowers was obtained and sequentially purified by VLC, silica gel column chromatography, Sephadex LH-20 and HPLC to give the novel terpenoid, **2.3** (9.8 mg) and fifteen compounds: two germacranes, **2.4** (318.0 mg) and **2.5** (2.9 mg), seven eudesmanes, **2.6** (10.2 mg), **2.7** (9.7 mg), **2.8** (32.0 mg), **2.9** (23.3 mg), **2.10** (96.0 mg), **2.11** (8.0 mg) and **2.12** (10.0 mg), four guianes **2.13** (173.0 mg), **2.14** (2.7 mg), **2.15** (36.0 mg) and **2.16** (1.1 mg), one eudesmane unsaturated ester **2.17** (4.7 mg), and one flavonoid, **2.21** (2.5 mg).

2.3.2 Structure elucidation of 2.1

Compound **2.1** was obtained as a colourless gum. HR-ESI-MS experiments indicated that its elemental composition is $C_{17}H_{25}O_5$, which suggests six degrees of unsaturation. The IR spectrum showed absorption bands corresponding a hydroxyl group (3473 cm^{-1}), an α,β -unsaturated-γ-lactone function (1764 cm^{-1}) and an ester group (1730 cm^{-1}). The NMR spectra (see Table 2.1 for ^1H and ^{13}C NMR data) displayed characteristic signals for an eudesmane lactone. The presence of an exomethylene-γ-lactone ring was established by the 13-H_2 proton signals at δ_H 5.94 and 4.79 and their HMBC correlations to C-7, C-11 and C-12, and the 6-H lactone proton signal at δ_H 3.70 and its strong ^1H-^1H coupling with 7-H and HMBC correlation to C-12. In addition, the NMR data indicated the presence of the acetoxylated tertiary carbon (C-1) at δ_H 4.67 and δ_C 77.2 and HMBC correlations from both 1-H and acetoxy-H_3 to the acetoxy carbonyl carbon (C-1'), and an oxygenated quaternary carbon (C-4) at δ_C 71.0. The complete structural elucidation of **2.1** was achieved by analysis of the HMQC, HMBC and COSY spectra and is fully described in paper I. The relative configuration of **2.1** was suggested by correlations observed in the NOESY spectrum, from 14-H_3 to

1-H, 2β-H, 3β-H, 6-H and 8β-H, as well as from 5-H to 2α-H, 3α-H, 7-H, 8α-H and 15-H_3. The proposed configuration is confirmed by the large coupling constants between 5-H and 6-H as well as 6-H and 7-H, showing that the three protons are axial, while the small coupling constants between 1-H and 2-H_2 show that 1-H is equatorial (Figure 2.3). Consequently, **2.1** was identified as 4-*epi*-1α-acetoxy arbusculin A.

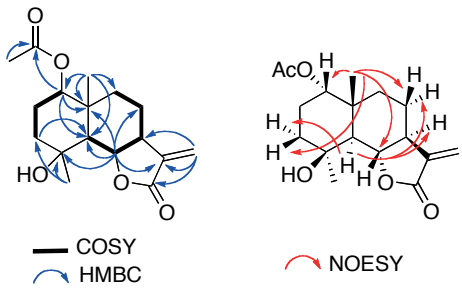


Figure 2.3 Selected 2D NMR correlations of **2.1**.

2.2.3 Structure elucidation of 2.2

Compound **2.2** was isolated as clear oil. The elemental composition was established by HR-ESI-MS experiments to be $C_{17}H_{22}O_4$, indicating seven degrees of unsaturation. The IR spectrum exhibited the presence of an α,β-unsaturated-γ-lactone (1770 cm^{-1}) and an ester group (1726 cm^{-1}). Compound **2.2** exhibited similar ^{1}H and ^{13}C NMR (see Table 2.1) to those of **2.1**, although additional signals and noticeable differences in chemical shift suggested the presence of slightly different functionalities. The COSY and HMBC spectra further confirmed that both compounds shared identical basic skeleton. Like **2.1** compound **2.2** displayed the characteristic signals for an exomethylene-γ-lactone ring that were established by the 13-H_2 proton signals at δ_H 6.09 and 5.40, the 6-H lactone proton signal at δ_H 4.00 and its strong ^{1}H-^{1}H coupling with 7-H and a lactone carbonyl at δ_C 170.5. While the downfield shifts of C-4 (δ_C 143.2) and C-15 (δ_C 110.2) as well as the proton signals at δ_H 5.00 and 4.87 (15-H_2) confirmed an exocyclic methylene group at C-4. An analysis of the NOESY spectrum of **2.2** showed correlations from 14-H_3 to 1-H, 2β-H, 6-H, 8β-H and 9β-H as well as from 5-H to 3α-H, 7-H and 8α-H (see Figure 2.4). Therefore, compound **2.2** was identified as acetyl 1-*epi*-reynosin. Comparison of the ^{1}H NMR data of **2.2** with those of the acetate of reynosin [47] showed that they are

similar and the significant differences are the chemical shifts of 1-H and 5-H as well as the coupling constants between 1-H and 2-H_2 which are small in **2.2** as 1-H is equatorial but large for the acetate of reynosin as 1-H is axial.

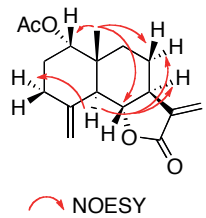


Figure 2.4 Selected 2D NMR correlations of **2.2**.

Table 2.1 ^{1}H and ^{13}C NMR data of compounds **2.1** (C_6D_6) and **2.2** ($CDCl_3$)

Position	**2.1**		**2.2**	
	^{1}H (*J*, Hz)	^{13}C	^{1}H (*J*, Hz)	^{13}C
1	4.67 br dd (2, 2)	77.2	4.68 br dd (2, 3)	76.4
2β	1.99 dddd (13, 13, 4.5, 2.2)	23.0	1.86 dddd (13.9, 13.4, 5.1, 2.7)	27.1
2α	1.53 m		1.81 m	
3β	1.15 m	36.2*	2.20 ddd (13.5, 5.1, 1.9)	30.5
3α	1.53 m		2.32 ddd (13.5, 13.4, 5.4)	
4	-	71.0	-	143.2
5	1.57 d (11.2)	50.5	2.79 d (11.0)	48.7
6	3.70 dd (11, 11)	79.8	4.00 dd (11, 11)	79.8
7	1.76 m	50.7	2.55 ddddd (12, 11, 3, 3, 3.4)	49.3
8β	1.26 m	21.4	1.61 dddd (12, 12, 11, 3.4)	21.2
8α	1.03 m		2.08 m	
9β	0.84 m	36.2*	1.42 ddd (13.0, 2.8, 3.2)	33.1
9α	1.24 br ddd (14, 14, 4)		1.72 ddd (13.0, 13.0, 4.0)	
10	-	40.7	-	41.7
11	-	140.4	-	139.1
12	-	170.0	-	170.5
13	5.94 d (3.2)	115.9	6.09 d (3.2)	117.0
	4.79 d (3.2)		5.40 d (3.2)	
14	0.92 s	20.0	0.92 s	18.0
15	1.29 s	32.9	5.00 br s	110.2
			4.87 br s	
1’	-	169.7	-	170.4
2’	1.74 s	21.1	2.12 s	21.2

δ (ppm) 500 MHz for ^{1}H and 125 MHz for ^{13}C; multiplicities; *J* values (Hz) in parentheses

* May have interchanged

2.2.4 Structure elucidation of 2.3

Compound **2.3**, for which we propose the trivial name kaunial, was isolated as a clear oil. An HR-ESI-MS experiment revealed a molecular ion at *m/z* 533.2531, consistent with the elemental composition $C_{30}H_{38}O_7$ as the exact mass of M + Na^+, $C_{30}H_{38}O_7Na$, is 533.2515. As a confirmation, the ^1H NMR spectrum contained signals integrating for 38 protons while the ^{13}C NMR spectrum displayed signals for 30 carbons (Table 2.2). Kaunial (**2.3**) consequently has 12 unsaturations. The presence of an α,β-unsaturated-γ-lactone, a ketone, and a double bond was indicated by the absorption bands in the IR spectrum at 1758, 1713, and 1681 cm^{-1}, respectively. HMQC experiments suggested that **2.3** has two ketone functions, one aldehyde, two lactone carbonyl, five nonprotonated sp^2 carbons, three monoprotonated sp^2 carbons, two diprotonated sp^2 carbons, two oxygenated methines, two non-oxygenated sp^3 methines, seven sp^3 methylenes, and four methyls. This accounts for all 30 carbons as well as 10 of the 12 degrees of unsaturation, indicating the presence of two rings in **2.3**. As can be seen from the NMR data, kaunial (**2.3**) appears to be composed of two similar subunits, and only the signals for the first three positions (C-1/C-1', C-2/C-2', C-3/C-3') differ substantially. As shall be discussed later, we believe that **2.3** is biosynthesized from two identical C_{15} units obtained from the germacrane lactone costunolide (**2.4**) and therefore the germacrane numbering system has been retained.

Table 2.2 ^1H and ^{13}C NMR data of compound **2.3** in C_6D_6

S-unit A	^{13}C		^1H (*J*, Hz)	**S-unit B**	^{13}C		^1H (*J*, Hz)
1	155.5	CH	6.02 t (7.3)	1'	193.8	CHO	9.26 s
2	27.5	CH_2	2.04 dt (7.8, 7.3)	2'	140.1	qC	-
3	38.0	CH_2	1.79 t (7.8)	3'	33.4	CH_2	2.86 ABq (15.2)
4	142.2	qC	-	4'	141.0	qC	-
5	124.8	CH	5.10 dq (8.8, 1.3)	5'	124.1	CH	4.93 dq (8.9, 1.2)
6	79.2	CH	4.50 dd (8.8, 6.3)	6'	79.4	CH	4.40 dd (8.9, 6.5)
7	45.7	CH	2.52 m	7'	45.6	CH	2.33 m
8	25.8	CH_2	1.62 m 1.53 m	8'	25.7	CH_2	1.37 dt (8.2, 7.2)
9	39.8	CH_2	1.94 t (7.2)	9'	39.7	CH_2	1.87 t (7.2)
10	205.8	qC	-	10'	205.7	qC	-
11	140.3	qC	-	11'	140.1	qC	-
12	169.8	qC	-	12'	169.8	qC	-
13	121.0	CH_2	6.21 d (2.8) 5.09 d (2.1)	13'	121.1	CH_2	6.16 d (2.8) 5.07 d (2.3)
14	29.8	CH_3	1.68 s	14'	29.9	CH_3	1.74 s
15	17.1	CH_3	1.52 d (1.2)	15'	17.5	CH_3	1.50 d (1.2)

The presence of an exomethylene-γ-lactone ring in subunit A was established by the HMBC correlations from 13-H_2 to C-7, C-11 and C-12, the HMBC correlations from 6-H to C-7 and C-12 as well as the COSY correlations from 7-H to 6-H and 13-H_2. The corresponding correlations were observed in subunit B, showing that this also has an exomethylene-γ-lactone ring. With two lactones all unsaturations are accounted for. The connection between the two lactones is demonstrated by COSY and HMBC correlations. 6-H also gives a COSY correlation to 5-H and HMBC correlations to C-5 and C-4, which together with the HMBC correlations from 15-H_3 to C-3, C-4 and C-5 establish the link up to C-3. COSY correlations from 3-H_2 to 2-H_2 and HMBC correlations to C-1, C-2, C-4 and C-5 extend the chain, and HMBC correlations from 1'-H to C-1, C-2' and C-3' as well as from 15'-H_3 to C-3', C-4' and C-5' brings us up to the second lactone. The link between C-5' and C-6' is demonstrated by the COSY correlation between the two protons, as well as by HMBC correlations from 6'-H to C-4' and C-5'. Both C-7 and C-7' are substituted by a 3-oxo-butyl group, as demonstrated by the HMBC correlations from 14-H_3 (14'-H_3) to C-9 and C-10 (C-9' and C-10'), as well as the COSY correlations 9-H_2/8-H_2/7-H (9'-H_2/8'-H_2/7'-H) and the HMBC correlations from 9-H_2 (9'-H_2) to C-7, C-8 and C-10 (C-7', C-8' and C-10') (see Figure 2.5).

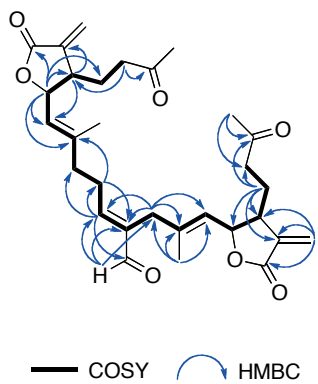
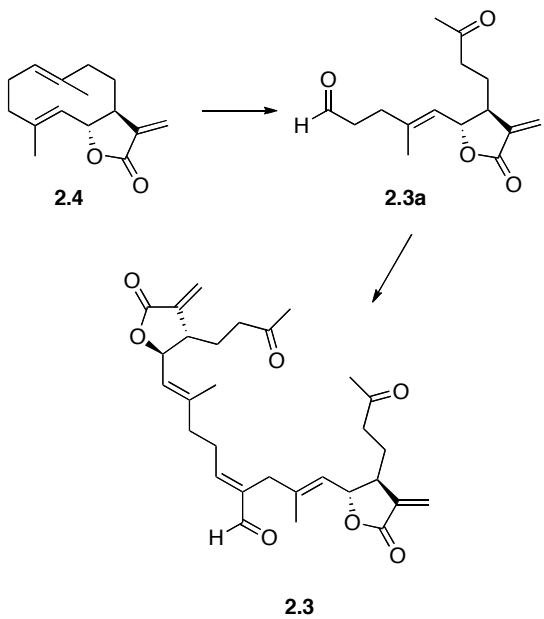


Figure 2.5 Pertinent 2D NMR correlations for **2.3**.

The configurations of the carbon-carbon double bonds of **2.3** were determined by a NOESY experiment, in which important correlations were observed between H-1' and H-1, indicating an *E* configuration for this double bond, and between 3-H_2 and 5-H as well as 3'-H_2 and 5'-H suggesting that both double bonds also are *E*. 6-H correlates with 8-H_2, 9-H_2 and 15-H_3, while 6'-H correlates with 8'-H_2,

9'-H_2 and 15'-H_3, whereas 7-H and 7'-H correlate with 5-H and 5'-H, respectively. This suggests that 6-H/7-H and 6'-H/7'-H are *trans*, which is to be expected if costunolide (**2.4**) is the biosynthetic precursor. This is also supported by the ^{1}H-^{1}H coupling constants between 6-H and 7-H (6.3 Hz) as well as 6'-H and 7'-H (6.5 Hz) which are characteristic in *seco*-lactones with a trans-fused lactone ring [48-51]. Although, these data do not reveal the absolute configuration of kaunial (**2.3**), but assuming that costunolide (**2.4**), whose absolute configuration is known [52], is the starting material, it should be as displayed in Figure 2.2.

The proposed biogenesis of kaunial (**2.3**) involves an oxidative cleavage of the 1,10 bond of the germacrane costunolide (**2.4**), which is a major metabolite produced by this plant, to produce the intermediate aldehyde **2.3a** that subsequently is dimerised by an aldol condensation to yield **2.3** (Scheme 2.1).



Scheme 2.1 Proposed biogenetic route for **2.3**.

2.2.5 Identification of known compounds

The known compounds costunolide (**4**)[53] , haageanolide (**5**)[54], 1β-hydroxyarbusculin A (**6**)[55], 4-epi-1β-hydroxyarbusculin A (**7**)[56], reynosin (**8**)[57], 1-epi-reynosin (**9**)[58], santamarin (**10**)[59], the acetate of santamarin (**11**)[60],

11,13-didehydrovulgarin (**12**)[61], 2β-acetoxy-3α,4α-epoxy-3,4-dihydrokauniolide (**13**)[42], 3α,4α-epoxy-2α-isobutyryloxykau-niolide (**14**)[42], dehydroleucodin (**15**)[62], 3-chlorodehydroleucodin (**16**)[42], baynol C (**17**)[63], hispidulin (**18**)[64], cirsimaritin (**19**)[65], jaceosidin (**20**)[66], eupafolin (**21**)[67] were identified on the basis of their one- and two-dimensional NMR spectra and subsequent comparison to the literature values (Figure 2.6).

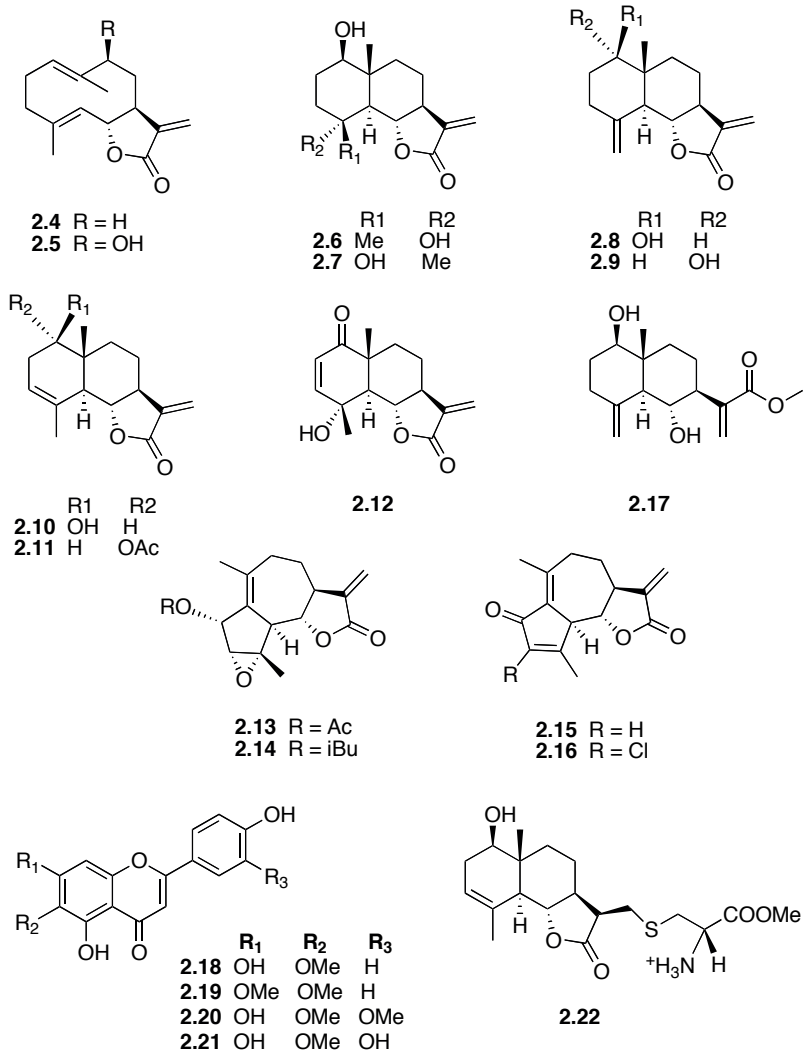


Figure 2.6 Compounds isolated from *K. lasiophthalma* **2.4-2.21** and methyl cysteine adduct **2.22**.

2.2.6 Preparation of compound 2.22

Previous investigations of sesquiterpenoid α-methylene-γ-lactones have indicated that the cytotoxic and antitumor activities are related to their ability to react as Michael acceptors [32]. Therefore, the methyl cysteine adduct of **2.10** was prepared to give **2.22**, and its cytotoxicity was compared with that of the natural products.

10.0 mg (0.04 mmol) of **10** and 8.3 mg (0.048 mmol) methyl-L-cysteine ester hydrochloride were dissolved in methanol (1 mL) and the mixture was heated at 60°C and stirred. After 48 h, 1.6 mg (0.0093 mmol), methyl-L-cysteine ester was added and the reaction mixture left for another 24 h. Then, the solvent was evaporated under reduce pressure and the dry residue dissolved in H_2O (3 mL), washed with $CHCl_3$ (3 x 1.5 mL). The organic phase dried in vacuo, yielded 15.5 mg of a yellow residue, which was purified by Sephadex LH-20 ($CHCl_3$:MeOH 1:1) to give 8.0 mg (0.021 mmol) of **2.22** as yellow oil.

2.2.7 Evaluation of the cytotoxic activity

The anticancer activity of compounds **2.1**-**2.4**, **2.8**-**2.10**, **2.12, 2.13**, **2.15**, **2.17** and the methyl cysteine adduct **2.22** were assayed in the five breast cancer cell lines MCF-7 (luminal A subtype), SK-BR-3 (luminal B subtype), HCC1937 and L56Br-C1 (basal subtypes) and JIMT-1 (HER2 subtype) and compared with the cytotoxicity in the normal-like breast epithelial cell line MCF-10A [68-73]. In breast cancer, each subtype has different prognosis and is subjected to different treatments. The inhibitory concentration 50 values (IC_{50}) were deduced from the obtained dose response curves and are presented in Table 2.3.

Interestingly, the cancer cell lines were more sensitive to all compounds than the normal-like MCF-10A cells. No obvious patterns related to breast cancer cell line subgroup were found. Compounds **2.3** and **2.4** were found to be the most active in all cell lines with IC_{50} values ranging from 0.67 to 7.0 and 2.0 to 6.2 μM, respectively. However, **2.3** is also toxic to the normal cell line MCF-10A, although somewhat less, and the lack of selectivity may depend on the presence of two exomethylene-γ-lactone moieties, while compounds **2.1** and **2.17** exhibited the lowest activity. **2.17** is an unsaturated ester and differs in that respect from the other compounds, but the lower activity of **2.1** compared to the similar compounds was unexpected. In addition, costunolide (**2.4**) together with the two guianes **2.13** and **2.15** shows slightly higher selectivity for the cancer cells compared to the eudesmanes. In **2.12** and **2.14** the presence of a second Michael acceptor function may influence the activity. The difference in activities of **2.8** and **2.9** may depend

on the higher lipophilicity of **2.9**, facilitating its absorption into the cells. The methyl cysteine adducts **2.22** is significantly less potent, however, it is not devoid of activity and its cytotoxicity towards the normal-like MCF-10A cells is similar to that of the α-methylene-γ-lactones. This may depend on the reversibility of Michael additions, by which **2.22** slowly can eliminate methyl cysteine and regenerate **2.10** during the assay condition.

Table 2.3 Cytotoxicity (IC_{50} in μM) of compounds **2.1-2.4, 2.8-2.10, 2.12, 2.13, 2.15, 2.17** and methyl cysteine adduct **2.22**

Comp.	HCC1937 μM	JIMT-1 μM	L56Br-C1 μM	MCF-7 μM	SK-BR-3 μM	MCF-10A μM
2.1	23.0[a]	18.0[a]	9.3[a]	27.0[a]	10.1[a]	38.0[a]
2.2	3.2/7.5[b]	6.3/7.0[b]	8.4/12.0[b]	11.0±1.7[c]	4.7±1.1[c]	24.0[a]
2.3	2.4/2.5[b]	4.3/4.8[b]	0.67/1.3[b]	4.7/7.0[b]	1.1/2.1[b]	4.7/7.0[b]
2.4	2.2/4.2[b]	6.2/6.3[b]	3.7/4.8[b]	5.3±1.4[c]	2.0±1.0[c]	20.0[a]
2.8	10.0[a]	8.5/12.0[b]	11.0[a]	16.4±7.1[c]	5.2±1.6[c]	17.0[a]
2.9	12.0/18.5[b]	12.0/14.0[b]	9.0/10.1[b]	23.0/30.0[b]	7.6/6.4[b]	22.0[a]
2.10	8.1±1.8[c]	6.9/7.2[b]	12.3±5.5[c]	9.7±0.9[c]	3.1±0.6[c]	17.0[a]
2.12	4.8/7.8[b]	10.0/13.0[b]	3.6[a]	10.1/18.0[b]	4.2±2.1[c]	24.0/21.0[b]
2.13	6.0/7.8[b]	7.1/10.1[b]	6.0/10.1[b]	7.1/8.3[b]	4.2±1.1[c]	17.0/38.0[b]
2.15	3.0/5.2[b]	7.5/8.1[b]	5.8/10.0[b]	3.3/4.3	2.5±0.4[c]	20.0/23.0[b]
2.17	24.0[a]	13.0/23.0[b]	16.0[a]	27.4±17.2[c]	9.9±3.0[c]	29.0[a]
2.22	100.0[a]	>100	73.0[a]	>100	51.0/40.0[b]	41.0[a]

Values from: (a) one dose response curve, (b) two dose response curves,
(c) three or more dose response curves.

3. Antileishmanial metabolites

3.1 Introduction

Leishmaniasis is a prevalent protozoan parasitic disease found mainly in subtropical and tropical regions of developing countries, causing significant morbidity or mortality. Although the disease is considered a major health problem due to the high endemicity in more than 90 countries, it has been disregarded by pharmaceutical industries and governments [74].

The parasitic protozoa is transmitted by female sandflies belonging to the genus *Phlebotomus* (The Old Word) and *Lutzomyia* (The New World) [75]. There are approximately 20 different *Leishmania* species and subspecies, and leishmaniasis can exhibit a broad spectrum of clinical manifestations. It is classified in three different clinical forms according to what part of the body is affected the most. Cutaneous leishmaniasis (CL) causes skin lesions that generally self-heal, resulting in life long immunity. In mucocutaneous leishmaniasis (MCL) the parasite affects the mucosal membrane and cause destructive and mutilating skin lesions that are often resistant to treatment and cure. MCL occurs only in The New World and is most common in Bolivia, Brazil, and Peru. Visceral leishmaniasis (VL), also called kala-azar, affects the liver, spleen and bone marrow and can be fatal if left untreated [76, 77].

Leishmania has two main life cycle stages: One as a promastigote form that is present in the sandfly vector, and the other as amastigote that resides in the mammalian host cells. The life cycle of *Leishmania* begins with a bite of the female sandfly to the amastigote-infected host during a blood meal. Once ingested, the amastigotes migrates to the mid gut of the sandfly and rapidly undergoe differentiation to promastigotes. After four or five days promastigotes migrate to the esophagus and reach the salivary glands of the sandfly. The life cycle begins again when the infectious promastigotes are injected into the blood stream of the host vertebrate by the infected sandfly during a subsequent blood meal. In comparison to the short life stage within the sandfly, the amastigote life

stage in the mammalian host may persist for several months or years and even during the entire life of the host [76].

In Bolivia, Leishmaniasis, CL and MCL, are endemic to seven of the nine of the departments. Among them La Paz is the most affected, particularly the municipality of Palos Blancos in the Yungas region that registers the highest incidence of CL. The considerable scale of migration from Andean to tropical areas and uncontrolled logging are two of the main reasons for the emergence of new highly active foci that are continually increasing in scale and extent [74].

Nowadays, due to absence of an effective vaccine, chemotherapy is still one of the most effective treatments. Conventionally, three drugs are used to treat all types of *Leishmania*: pentavalent antimony in the form of sodium stibogluconate (Pentostara ®) or N-methylglucamine antimonate (Glucantime®), Amphotericin B (macrolide polyene anti-fungal antibiotic), and Pentamidine (aromatic diamidines). However, toxicity, high cost in endemic countries, resistance and long-term parental administration have limited their use [78]. Therefore, there is an urgent need to find new antiparasitic agents, for example from natural sources such as plants, to complement the existing drugs.

3.1.1 The genus *Trixis* (Asteraceae)

The neotropical genus *Trixis* P. Browne (Asteraceae: Mutisieae) is represented by nearly 60 species of shrubs, and herbs [79, 80]. The genus has a broad distribution throughout the Southern United States and Mexico to Northern Chile, Northern Argentina and Uruguay. Various members of the genus have been used traditionally to treat skin lesions, bruises and ulcerations, diarrhea, cold, stomach and back aches and as an abortive [81-83].

Phytochemical studies on the chemistry of *Trixis* species have reported that the genus is characterized by sesquiterpene lactones possesing a particular basic skeleton [84-87] named “trixane” by De Riscala and co-workers [88]. Previously, γ-curcume, trixanolides, and a germacrame were isolated from *T. antimenorrhoea*[85], and more recently flavonoids and tannins have been identified to be the major secondary metabolites in a qualitative study [89].

3.2 Chemical investigation of *Trixis antimenorrhoea* (Paper III)

Trixis antimenorrhoea is a shrub with white flowers growing along the river margins or mountainside in the Andean or Tropical regions of Bolivia. The aromatic flowers of this species, locally known as "tian-tian", are used in traditional medicine to treat eye-inflammation and excessive uterine bleeding, known as menorrhagia, from which its name is derived [89, 90]. In our continuing search for novel antiparasitic agents, we prepared the ethanol extract of the whole plant of *T. antimenorrhoea*, which was found to be moderately active towards *L. braziliensis* (IC_{50} 32.8 μg/mL) and therefore chosen for a detailed phytochemical investigation. Bioassayed-guided fractionation led to the isolation of two natural products (**3.1** and **3.2**) as well as eleven known compounds (**3.3**-**3.13**). The isolation and structure elucidation of the novel compounds will be discussed, as well as the antileishmanial activity of compounds isolated in sufficient amount for biological testing.

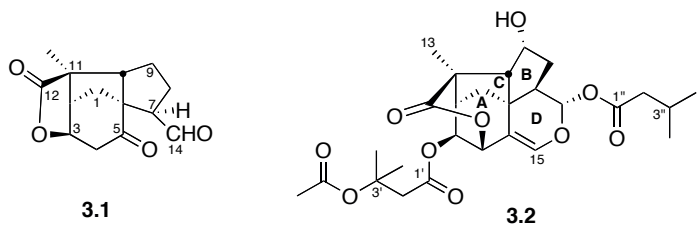


Figure 3.1 New metabolites from *T. antimenorrhoea.*

3.3 Results and discussion

3.3.1 Isolation of compounds

Thirteen compounds were isolated from the aerial parts of *T. antimenorrhoea* by bioassayed-guided fractionation. The crude plant extract was suspended in a mixture of 90 % aqueous methanol and subjected to liquid-liquid partition to produce the hexane, CH_2Cl_2, EtOAc and MeOH fractions. The hexane and CH_2Cl_2 fractions were found to be active towards *Leishmania braziliensis* with IC_{50} 31.6

and 15.7 μg/mL, respectively, and they were therefore investigated in detail. After repeated chromatography on silica gel and Sephadex LH-20, the known compounds **3.3** (1.5 mg), **3.4** (18.0 mg), **3.5** (9.9 mg), **3.6** (10.0 mg), **3.7** (1.8 mg), **3.10** (2.2 mg), and **3.11** (3.6 mg) were isolated from the hexane fraction, while the CH_2Cl_2 fraction yielded the two new natural products **3.1** (0.5 mg), and **3.2** (25.7 mg) as well as compounds **3.8** (4.0 mg), **3.9** (1.5 mg), **3.12** (4.6 mg), and **3.13** (3.5 mg).

3.2.2 Structure elucidation of 3.1

Compound **3.1** was obtained as a colourless oil. The HR-ESI-MS showed a molecular ion at *m/z* 249.1151 $[M + H]^+$, indicating that the elemental composition of **3.1** is $C_{14}H_{16}O_4$ (calcd 249.1127). Absorption bands in the IR spectrum at 1764, 1714 cm^{-1} clearly indicated the presence of aldehyde and ketone functionalities. Indeed, the ^1H NMR of **3.1** showed signals for 16 protons while the ^{13}C NMR spectrum displayed signals for 14 carbons confirming the elemental composition and that **3.1** has seven unsaturations. The ^{13}C and HMQC spectra afforded evidence that **3.1** has one aldehyde function (C-14), one ketone (C-5), one carbonyl (C-12), four sp^3 methylenes (C-1, C-4, C-8, C-9), three sp^3 methines (C-2, C-7, C-10), one oxygenated methine (C-3), two nonprotonated sp^3 carbons (C-6, C-11) and one methyl (C-13). These functionalities account for three of the seven unsaturations, compound **3.1** is consequently tetracyclic. ^1H and ^{13}C NMR data can be found in Table 3.1.

The complete structure of **3.1** was assembled by analysis of the COSY and HMBC spectra. Ring A was established via HMBC correlations from both 1-H_2 and 4-H_2 to C-2, C-3, C-5 and C-6, 4-H_2 also exhibited long range correlation to C-1, and supported by COSY correlations from 3-H to 2-H, and 4-H_2 as well as from 2-H to 1-H_2 and its corresponding HMBC correlations to C-3, C-4 and C-6. The linkage of ring A to C was evidenced by the HMBC correlations from both 1-H_2 and 13-H_3 to C-10 and C-11, together with correlations from 10-H to C-1 and C-5 and from 2-H to C-13. Key HMBC correlations from both 9-H_2 and 8-H_2 to C-6, C-7, and C-10 as well as from 7-H to C-1, C-6, C-8 and C-14 and together with the ^1H-^1H spin system 7-H/8-H_2/9-H_2/10-H close ring B. The presence of an oxygenated carbon at δ_C 73.2 (C-3) and the HMBC correlations from 3-H to the carbonyl ester C-12 (δ_C 180.1) shows that C-3 and C-12 are part of a γ-lactone. Compound **3.1** is suggested to be a norsesquiterpene based on the trixane skeleton with a keto function at C-5, for which we propose the trivial name of nortrixial.

The relative configuration of nortrixial (**3.1)** was determined based on the correlations noted in the NOESY spectrum. The critical correlations are between 13-H_3 and 1α-H, 2-H, 3-H, 4α-H, 7-H, 8α-H, 9α-H, as well as from 8β-H to 9β-H and 10-H and illustrated in Figure 3.2. The systematic name of nortrixial (**3.1**) is consequently (1*R**,3a*S**,3b*R**,6*R**,6a**S*,7a*R**)-3a-methy1-3,8-dioxooctabydro-1*H*-1,6a-ethanopentaleno[1,2-*c*]furan-6-carbaldehyde. Unfortunately, the amounts of **3.1** isolated in this investigation were insufficient for both assaying its biological activity and for the measuring of its optical activity.

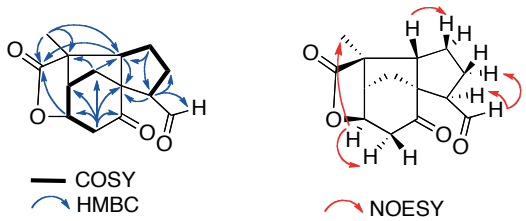


Figure 3.2 Selected 2D NMR correlations of **3.1**.

3.2.3 Structure elucidation of 3.2

Compound **3.2,** obtained as colourless needles, was by HR-ESI-MS experiments suggested to have the elemental composition $C_{27}H_{36}O_{10}$, as the molecular ion was detected at *m/z* 521.2401 $[M + H]^+$ (calcd 521.2387). Its IR spectrum showed bands at 3506, 1732, and 1661 cm^{-1} which were indicative of hydroxyl and carbonyl groups. As a confirmation, the ^1H spectrum displayed signals integrating to 36 protons. The ^{13}C and HMQC NMR spectra indicated that compound **3.2** contained four ester carbonyls (C-12, C-1', C1'', C-4'), one sp^2 quaternary carbon (C-5), one sp^2-oxygenated carbon (C-15), four sp^3-oxygenated carbons (C-3, C-4, C-9, C-14), four sp^3 methylenes (C-1, C-8, C-2', C-2''), four six sp^3 methines (C-2, C-7, C-10, C-3''), three quaternary carbons (C-6, C-11, C-3') and six methyls groups (C-13, C-5', C-6', C-7', C4''/C-5''). These functionalities account for five of the ten unsaturations indicating that **3.2** is pentcyclic.^1H and ^{13}C can be found in Table 3.1. Correlations from HMQC, HMBC and COSY experiments were used to establish the complete structure of **3.2**. In the HMBC spectrum, correlations from both 1-H_2 and 4-H to C-2, C-3, C-5, and C-6 as well as from 2-H to C-1, C-3, C-4, and C-6 describe ring A and confirmed by COSY correlations between 1-H_2 and 2-H, together with the correlation from 3-H via 4-H. The connectivity between ring A and C was suggested through HMBC correlations from 10-H to C-1, C-2, C-5, and C-6 and from 1-H_2 to C-10 and

C-11. The presence of a methyl at C-11 in ring C was demonstrated by the long range correlations of 13-H_3 to C-2, C-10 and C-11. HMBC correlations from both 7-H and 8-H_2 to C-6, C-9 and C-10 as well as between 9-H and C-6, C-7 close ring B which is confirmed by the second COSY spin system 14-H/7-H/8-H_2/9-H/10-H. The only exchangeable proton is a hydroxyl group assigned to C-9 based on the chemical shift of C-9/9-H (73.1/4.41) and the clear HMBC correlations from 9-OH to C-8, C-10 and C-9. The double bond 15-H to C-4, C-5, C-6 and 14-C, while 14-H gives correlation to C-6, C-7, C-8, and C-15 suggesting an oxygen link between C-14 and C-15 to produce ring D. The lactone ring involving also C-4 was demonstrated by the HMBC correlations from 4-H and not 3-H to the carbonyl carbon C-12. In addition, HMBC correlations were critical in the assignment of the ester residues at C-3 and C-14. A 3-acetoxy-3-methylbutanoate residue was defined by correlations from 5'-H_3 to the carbonyl carbon C-4' and C-3', together with correlations from 6'-H_3/7'-H_3 to C-1', C-2' and C-3' extend the chain up to C'-1. In the HMBC spectrum, key correlations between 2'-H_2 and C-1', C-3, and from 3-H to C-1', C1, C-4 and C-11 allowed to place this ester residue at C-3, while the presence of a 3-methylbutanoate linked at C-14 was confirmed by correlations between 2''-H_2 to C-1'', C-3'' and C-4''/C5''. Additionally, 3''-H gives correlations to C-1'', C-2'' and C-4''/C-5'' and 14-H to C-1'', C-6, C-7, C-8 and C-15.

The relative stereochemistry of **3.2** was deduced based on correlations observed in the NOESY spectrum. 9-H correlates with 8β-H, 10-H and 14-H confirming that the four protons are cofacial. Additional correlations were observed between 13-H_3 and 1α-H, 2-H, 3-H, 7-H, and 8α-H, as well as between 4-H and 15-H. Comparing the ^{1}H NMR data of compound **3.2** and 9α-hydroxy-3-β-isovaleryloxy-trixikingolide-14-(3'-acetoxy-isovalerate) [91] show that the trixane core is identical but that the ester residues are linked to different positions.

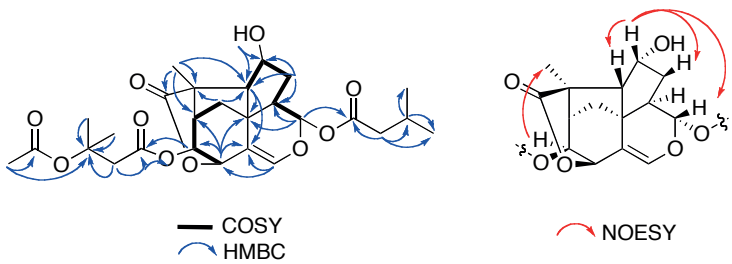


Figure 3.3 Selected 2D NMR correlations of **3.2**.

Table 3.1 ^{1}H and ^{13}C NMR data of compounds **3.1** and **3.2** in $CDCl_3$

	3.1			**3.2**	
	^{13}C	^{1}H (*J*, Hz)		^{13}C	^{1}H (*J*, Hz)
1β	36.1	2.05 dd (13.4, 4.1)	1β	42.0	3.15 dd (11.8, 7.0)
1α		2.36 d (13.4)	1α		1.43 bdd (11.8, 1.7)
2	50.2	3.06 dd (8.9, 3.9)	2	49.1	2.38 ddd (7.0, 1.7, 1.9)
3	73.2	4.98 ddd (1.1, 8.9, 6.2)	3	74.4	4.86 dd (2,8, 1.9)
4β	42.1	2.80 dd (18.5, 1.1)	4	76.6	4.94 dd (2.8, 2.8)
4α		2.72 dd (18.5, 6.2)			
5	207.7	-	5	117.6	-
6	70.3	-	6	53.9	-
7	56.9	2.51 m	7	40.5	2.59 ddd (8.9, 8.6, 6.5)
8β	30.6	2.20 m	8β	40.9	1.86 ddd (15.1, 6.0, 6.0)
8α		2.17 m	8α		2.22 ddd(15.1, 8.6, 8.6)
9β	27.1	2.02 m	9	73.1	4.41 m
9α		1.71 dddd (6.3, 5.9, 5.9, 11.8)			
10	57.8	2.47 m	10	61.9	2,18 d (7)
11	47.5	-	11	49.8	-
12	180.1	-	12	176.8	-
13	17.4	1.32 s	13	20.0	1.56 s
14	201.1	9.8 d (1.8)	14	95.4	5.45 d (8.9)
			15	140.2	6.51 s
			1’	169.3	-
			2’	44.4	2.95 d (14.5)
					2.85 d (14.5)
			3’	79.2	-
			4’	170.5	-
			5’	22.3	2.01 s
			6’	26.6	1.54 s
			7’	26.5	1.54 s
			1’’	171.5	-
			2’’	43.2	2.28 dd (14.8, 7.2)
					2.26 dd (14.8, 7.2)
			3’’	25.6	2.13 sept (6.8)
			4’’	22.3	0.99 d (6.8)
			5’’	22.3	0.99 d (6.8)
			OH		1.68 d (3.2)

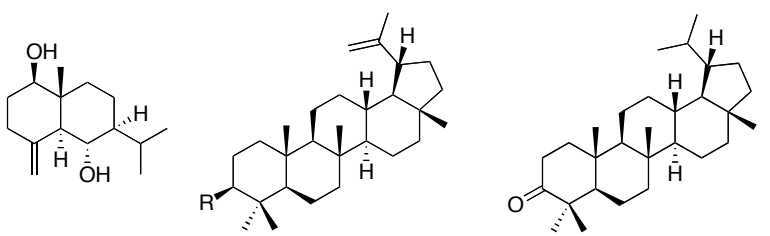
The absolute configuration of **3.2** could determined by application of the Mosher’s ester protocol by acylating the free C-9 hydroxyl group with *S*- and *R*-α-methoxy-α-trifluoromethylphenylacetic acid. The difference in chemical shifts ($\Delta\delta = S\text{-}R$) obtained by comparison of the analogous pairs of protons for the *S*- and *R*-MTPA esters (**3.14** and **3.15**) are presented in Table 3.2 and indicated that the absolute configuration of C-9 to be *R*. Therefore, compound **3.2** was identified as 9α-hydroxy-3-β-acetoxy-3-methylbutanoate trixikingolide-14-(3’-methylbutanoate).

Table 3.2 Selected ^{1}H NMR chemical shift data for diagnostic of the *S*- and *R*-MTPA-methyl Mosher esters **3.14** and **3.15**

^{1}H	3.14 (*S*)	3.15 (*R*)	Δ(*S*-*R*)
	ppm	ppm	ppm
13	0.885	1.140	-0.255
8α	2.344	2.321	+0.023
8β	2.046	2.208	+0.019
7	2.450	2.308	+0.142
14	5.458	5.433	+0.025

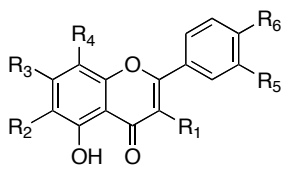
3.2.4 Identification of known compounds from *T. antimenorrhoea*

The structures of the known compounds were elucidated on the basis of their 1D- and 2D-NMR data and after their spectral data compared with those reported in the literature as: voleneol (**3.3**) [92], lupeol-3-acetate (**3.4**) and lupenol (**3.5**) [91], lupan-3-ona (**3.6**) [93], nevadesin (**3.7**) [94], sudachitin (**3.8**) [95], 3,5-dihydroxy-6,7,8,3',4' pentamethoxyflavone (**3.9**) [96], 5-hydroxy-3,3',4',6,7,8-hexamethoxy-flavone (**3.10**) [97], 5-hydroxy-3,4',6,7,8-pentamethoxy-flavone (**3.11**) [98], chrysoeriol (**3.12**) [99] and luteolin (**3.13**) [100].



3.3 **3.4** **3.5** **3.6**

R OAc OH



	R_1	R_2	R_3	R_4	R_5	R_6
3.7	OH	OMe	OH	OMe	H	OMe
3.8	H	OMe	OH	OMe	OMe	OH
3.9	OH	OMe	OMe	OMe	OMe	OMe
3.10	OMe	OMe	OMe	OMe	OMe	OMe
3.11	OMe	OMe	OMe	OMe	H	OMe
3.12	H	H	OH	H	OMe	OH
3.13	H	H	OH	H	OH	OH

Figure 3.4 Secondary metabolites from *T. antimenorrhoea*.

3.2.5 Preparation of the Mosher ester derivatives 3.14 and 3.15

Preparation of the *S*- and *R*-MTPA methyl esters (**3.14** and **3.15):** To a stirred solution of compound **3.2** (5 mg, in 1 ml of $CDCl_3$) under nitrogen atmosphere, Et_3N (4 mg, 5 μL), DMAP (a small crystal, ca 1 mg) and *S*-(+)-MTPA-Cl (3 mg, 5 μL) were added at room temperature. The mixture was stirred for 24 h. The reaction mixture was dissolved in EtOAc (20 ml) and washed with 1 M HCl (10 ml), $NaHCO_3$ (10 ml) and brine (10 ml), filtered through phase separator and concentrated to yellow oil. This crude residue was purified by Silica-gel chromatography eluted with CH_2Cl_2:Et_2O 95:5 to yield 1.2 mg of **3.14**. Treatment with *R*-(-)-MTPA-Cl as described above yielded 0.7 mg of **3.15**.

Table 3.3 *In vitro* leishmanicidal activity of *Trixis antimenorrhoea*

	IC_{50} (μg/mL)[a]	
	L. amazonensis	*L. braziliensis*
EtOH extract	>50	32.8±4.0
Hexane fract.	>50	31.6±3.5
CH_2Cl_2 fract.	44.5±0.75	15.7±1.45
EtOAc fract	>50	>50
MeOH fract.	>100	>100
3.2	0.3±0.01	0.96±0.05
3.4	>100	>100
3.5	78.4±4.2	96.5±0.2
3.6	>100	>100
3.7	42.1±3.15	34.0±5.0
3.8	>100	3.5±1.0
3.10	18.7±0.9	5.8±0.32
3.11	>100	58.7±7.0
3.13	>100	>100
Control[b]	0.21±0.06	0.08±0.04

[a] Data are expressed as mean standard deviation of three determinations.

[b] Amphotericin B was used as positive control.

3.2.6 Evaluation of the antileishmanial activity (*T. antimenorrhoea)*

Compounds **3.2**, **3.4-3.8**, **3.10**, **3.11**, and **3.13** were assesedd *in vitro* for their leishmanial activity. The results are presented in Table 3.3. Compound **3.2** was found to be the most active with IC_{50} values of 0.30 μg/mL for *L. amazonensis* and 0.96 μg/mL for *L. brasiliensis*. This is actually close to the activity of amphotericin B, used as positive control (IC_{50} 0.21 and 0.08 μg/mL, respectively). The potency of **3.2** is noteworthy for a compound with no apparent reactive functionalities. The lupane-type triterpene **3.5,** having a hydroxyl group at C-3, showed an improved activity (IC_{50} of 78.4 μg/mL for *Leishmania amazonensis*

and 96.5 μg/mL for *L. braziliensis.)* when compared with **3.4** (acetylated at C-3) and **3.6** (with a ketone group at C-3). Among flavonoids, **3.10** exhibited significant activity towards the two strains used with IC_{50} of 19 and 5.8 μg/mL. The higher selectivity towards *L. braziliensis* of compounds with the same substitution pattern in ring A but different in ring B, **3.8** vs **3.7** and **3.10** vs **3.11**, appears to be associated with the presence of a second methoxy group at C-5' in ring B. It is important to point out in our investigation, the lack of activity of **3.13** is not in agreement with those reported in the literature [101, 102].

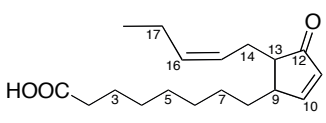
3.3 The genus *Lantana* (Verbenaceae)

The family Verbenacea is represented by 100 genus and approximately 2,600 species distributed in tropical and subtropical regions around the world. An estimated number of 150 species of herbaceous and shrubby plants form the genus *Lantana*, which is native to the tropical and subtropical Americas with a few taxa originally from tropical Asia and Africa. They are considered as garden plants due to the beauty of their flowers or aggressive weeds of disturbed areas. The genus is difficult to classify taxonomically since species are not stable due to a widespread hybridization and change of the shape of inflorescence and flower colours with age and maturity [103-105]. A great number of *Lantana* species are widely used in traditional medicine to treat a wide range of affections, all over the word. *Lantana camara* is the most representative example of the genus. Different parts of the plant are used for bronchitis (whole plant), cuts, rheumatisms, ulcers, malaria, cancer, high blood pressure and others (leaves), as vermifuge (powdered roots), and as antiseptic for wounds or itches (oil) while decoctions apply externally for leprosy and scabies [106-108].

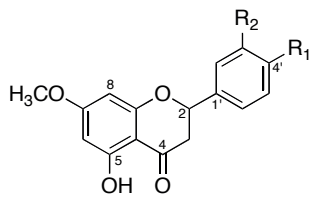
Studies of the chemistry of different *Lantana* species have shown to be characterized mainly by pentacyclic triterpenoids belonging to the oleane series (Lantadenes), flavonoids, phenylethanoid glycosides as well as monoterpenes and sesquiterpenes, steroids, furanonaphthoquinones and iridoid glycosides among others. Biological and pharmacological evaluation of crude extracts, essential oils and isolated compounds have shown that they possess a broad range of biological activities, for example antiprotozoal (antiplasmodial, antimalarial, leishmanicidal), antiviral, antioxidant, antiproliferative and cytotoxic activity [104, 105, 109]. Previous studies of *L. balansae* have reported the antimicrobial activity of the methanol extract [110] and the chemical composition of its essential oil [111, 112].

3.4 Chemical investigation of *L. balansae* (Paper IV)

Lantana balansae (Verbenaceae) is a perennial shrub with very pungent odor and white small flowers that grows in the mountain region of Cochabamba, Bolivia, where it is locally known as "k'ichita". An infusion of fresh leaves of *L. balansae* is used traditionally to alleviate digestive disorders and muscle spasms by people living in the surroundings where the plant is collected. As a part of our collaborative project in the search of bioactive secondary metabolites from the native flora of Bolivia, we prepared an ethanol extract of the aerial parts of *Lantana balansae*, which was assayed for leishmanicidal activity. As this extract displayed significant activity towards *L. amazonensis* (IC_{50} 6.0 µg/mL) and *L. braziliensis* (IC_{50} 4.9 µg/mL), this plant was selected for a detailed study. Bioassayed-guided fractionation of the ethanol extract led the isolation of eleven known compounds (**3.16-3.26**). Interestingly, cyclopentenone fatty acid (**3.16**) and the flavones (**3.17** and **3.18**) are reported as constituents of *Lantana* species for the first time. The structural elucidation of **3.16**, **3.17** and **3.18** are presented, as well as the leishmanicidal activity of compounds isolated in sufficient amounts for biological testing.



3.16



	R_1	R_2
3.17	OMe	OH
3.18	OMe	OMe

Figure 3.5 Important secondary metabolites from *L. balansae.*

3.5 Results and discussion

3.5.1 Isolation of compounds from *L. balansae*

Eleven compounds were isolated from the aerial parts of *L. balansae* by bioassay-guided fractionation. The crude plant extract was suspended in a mixture of 90 % aqueous methanol and partitioned between hexane and EtOAc to yield

three separate fractions. The hexane and EtOAc fractions were found to be active towards *L. amazonensis* and *L. braziliensis* and were therefore investigated in detail. After repeated chromatography on silica gel and Sephadex LH-20, compounds **3.16** (26.1 mg), **3.17** (9.0 mg), **3.18** (7.7 mg), **3.19** (39.3 mg), **3.20** (43.0 mg), and **3.25** (3.3 mg), were obtained from the hexane-soluble fraction, while the EtOAc-soluble fraction yielded **3.21** (5.2 mg), **3.22** (6.4 mg), **3.23** (3.3 mg), **3.24** (4.1 mg), and **3.26** (4.6 mg).

3.5.2 Structure elucidation of 3.16

Compound **3.16** was isolated as a colourless oil. The HR-ESI-MS showed a protonated molecular ion peak at *m/z* 293.2148, corresponding to the elemental composition $C_{18}H_{28}O_3$ for **3.16**. The ^{1}H NMR spectra showed two 1,2-disubstituted carbon-carbon double bonds at δ_H 6.95 (10-H, dd, 5.8, 2.6 Hz) and 6.00 (11-H, dd, 5.8, 2.0 Hz) suggesting an α,β-unsaturated carbonyl system, and at δ_H 5.33 (15-H, dtt, 11, 7, 1 Hz) and 5.42 (16-H, dtt, 11, 7, 1 Hz). Both double bonds are by the coupling constants suggested to have a *cis* configuration. In addition, nine methylenes at δ_H 2.14 (2-H_2, t, 7.4 Hz), 1.50 (3-H_2, quintet, 7.4 Hz), 1.10 (4-H_2, m), three at 1.06 (5-H_2, m; 6-H_2, m; 8-H_2, m), 1.13 (7-H_2, m), 2.50 and 2.34 (14-H_2, m), and 1.97 (17-H_2, dq, 7.4, 1 Hz), two methines at 2.26 (9-H, m) and 1.85 (13-H, ddd, 7.8, 4.5, 2.4 Hz) as well as a methyl at 0.89 (18-H_3, t, 7.5 Hz) were observed (see Table 3.4 for 1D NMR data). The presence of ketone and carboxylic acid functionalities were confirmed in the ^{13}C spectrum, by the signals at δ_C 209.8 and 180.0, respectively, and allowed to identify the positions of the three oxygen atoms in the structure. Since two double bonds and two carbonyls accounts for four of the five degrees of unsaturation, compound **3.16** must have a ring in its structure. The presence of an α,β-unsaturated carbonyl ring was established by HMBC correlations from 10-H to C-9, C-11, C-12, and C-13, and 13-H showed correlations to C-9 and C-12 and further confirmed by the key COSY correlations displayed between 11-H and 9-H via H-10 as well as 8-H with 13-H. The side chain at C-13 was constructed on the basis of the HMBC correlations displayed from 18-H_3 to C-16 and C-17 and from both 15-H and 16-H to C-14, C-17 and C-18 and the ^{1}H-^{1}H spin system 14-H_2/15-H/16-H/17-H. The connectivity at C-13 was demonstrated by key HMBC correlations from 13-H to C-14 and C-15. To complete the structure, HMBC correlations from 9-H to C-8 and C-10 link the eight-carbon saturated fatty acid, octanoic acid, to C-9. These data inferred that **3.16** was 12-oxo-phytodienoic, previously isolated from *Schistostephium* species [58], and the spectroscopic data reported are in agreement with those obtained here. The

relative configuration of **3.16** was confirmed by 2D NMR spectroscopy to be *cis* between C-9 and C-13.

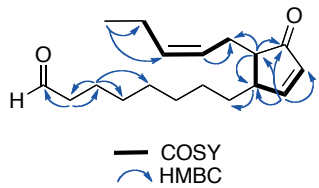


Figure 3.6 Selected 2D NMR correlations for **3.16**.

Table 3.4 ^{1}H and ^{13}C NMR data of compounds **3.16** in C_6D_6

	3.16	
	^{13}C	^{1}H (*J*, Hz)
1	179.9	
2	34.1	2.14 t (7.4)
3	24.9	1.50 quintet (7.4)
4	29.2	1.10 m
5	29.7	1.06 m
6	27.5	1.06 m
7	29.3	1.13 m
8	34.5	1.06 m
9	46.9	2.26 m
10	166.1	6.95 dd (5.8, 2.6)
11	133.1	6.00 dd (5.8, 2.0)
12	209.8	-
13	51.5	1.85 ddd (7.8, 4.5, 2.4)
14	28.5	2.50 m
		2.34 m
15	125.9	5.33 dtt (11, 7, 1)
16	133.8	5.42 dtt (11, 7, 1)
17	20.9	1.97 dq (7.4, 1)
18	14.4	0.89 t (7.5)

3.5.3 Structure elucidation of 3.17 and 3.18

Compound **3.17** was obtained as white fine needles. Positive-ion HR-ESI-MS analysis gave a pseudomolecular ion at *m/z* 317.1046, which suggested a formula of $C_{17}H_{16}O_6$. The ^{1}H NMR spectrum of **3.17** showed the presence of downfield shifted hydroxyl group at δ_H 12.4 (5-OH), three doublet of doublets, one corresponding to an oxymethine at δ_H 4.70 (2-H, dd, 12.8, 3.1 Hz) and two methylene α to the carbonyl at δ_H 2.50 (3-H_2, dd, 17.1, 2.8 Hz) and δ_H 2.30 (3-H_2, dd, 17.1, 3.1 Hz) (see Table 3.5 for NMR data). These data suggested that **3.17**

possess a basic flavanone skeleton. The observation of one doublet at δ_H 6.35 (3'-H, d, 8.4 Hz), one doublet of doublets at δ_H 6.62 (2'-H, dd, 8.4, 2.1 Hz), and one doublet at δ_H 7.03 (6'-H, d, 2.1 Hz) show the presence of 1,3,4-trisubstituted benzene ring, which by the HMBC correlations from 2-H to C-2' and C-6'was shown to be the ring B of a flavanone. In addition, a pair of doublets at δ_H 6.08 (8-H, d, 2.3) and at δ_H 6.21 (6-H, d, 2.3) characteristic of a meta substitution at ring-A. The aforementioned data correspond to persicogenin, previously isolated from other plant species [113].

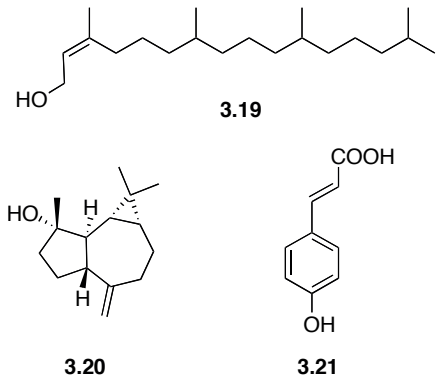
Compound **3.18** was isolated as a white fine neddles. Positive-ion HR-ESI-MS analysis gave a pseudomolecular ion at *m/z* 331.1207, which suggested a formula of $C_{18}H_{18}O_6$. Compound **3.18** exhibited nearly identical ^{1}H and ^{13}C NMR spectra of those of **3.17** indicating that both compounds share the same basic skeleton. The distinction between the two sets of spectra, however, is demonstrated by the presence of an additional singlet at δ_H 3.40 integrating to three protons (OMe-5') in the ^{1}H NMR spectrum of **3.18**, consistent with the additional fourteen mass units found by mass spectrometry. Therefore, compound **3.18** was identified as eriodictyol 3',4',7-trimethyl ether [114].

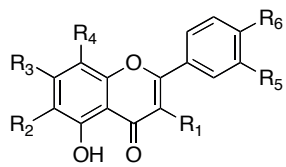
Table 3.5 ^{1}H and ^{13}C NMR data of compounds **3.17** and **3.18** in C_6D_6

	3.17		**3.18**	
	^{13}C	^{1}H (*J*, Hz)	^{13}C	^{1}H (*J*, Hz)
2	79.0	4.70 dd (12.8, 3.1)	79.7	4.76 dd (13.1, 2.9)
3	43.4	2.50 dd (17.1, 12.8)	43.9	2.62 dd (17.1, 13.1)
		2.30 dd (17.1, 3.1)		2.39 dd (17.1, 2.9)
4	196.2	-	196.6	-
5	165.2	-	165.7	-
6	95.3	6.21 d (2.3)	95.6	6.25d (2.2)
7	168.3	-	168.6	-
8	94.5	6.08 d (2.3)	94.9	6.16 d (2.2)
9	163.3	-	163.7	-
10	103.8	-	104.2	-
1´	132.5	-	131.8	-
2´	118.0	6.62 dd (8.4, 2.1)	119.4	6.69 dd (8, 2)
3´	110.1	6.35 d (8.4)	112.2	6.52 d (8.8)
4´	147.0	-	150.6	-
5´	146.5	-	150.8	-
6´	113.3	7.03 d (2.1)	110.95	6.68 d (2)
OMe-7	55.1	3.09 s	5.4	3.07 s
OMe-4'	55.3	3.08 s	55.9	3.36 s
OMe-5'	-	-	55.9	3.40 s
OH-5	-	12.84 br s		12.91 br s
OH-5'		5.47 s		

3.5.4 Identification of known compounds from *L. balansae*

The structures of the known compounds were elucidated by 1D- and 2D-NMR data and comparison with the published data identified as: phythol (**3.19**) [115], spathulenol (**3.20**) [116], 4-hydroxycinnamic acid (**3.21**) [117], onopordin (**3.22**) [118], 5,8-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy (**3.23**) [119], quercetin (**3.24**) [120] , jaceosidin (**3.25**) [66], and 8-hydroxyluteolin (**3.26**) [121].





	R_1	R_2	R_3	R_4	R_5	R_6
3.22	H	H	OH	OMe	OH	OH
3.23	H	H	OMe	OH	OMe	OH
3.24	OH	H	OH	H	OH	OH
3.25	H	OMe	OH	H	OMe	OH
3.26	H	H	OH	OH	OH	H

Figure 3.6 Isolated compounds from *L. balansae*.

3.5.5 Evaluation of the antileishmanial activity (*L. balansae)*

In order to identify the bioactive constituents of *L. balansae*, compounds **3.16**-**3.18** and **3.22**-**3.24** were investigated for their antiprotozoal activity. The results are presented in Table 3.6. Compound **3.16** was slightly less potent than amphotericin, used as a reference control, with values of 0.6 and 0.2 μg/mL for *L. amazonensis* and *L. braziliensis*, respectively, but still possessing an interesting activity. All three evaluated flavonoids showed antileishmanial activity, however, compound **3.23** exhibited the highest degree of activity and selectivity against both *L. amazonensis* (IC_{50} 13.6 μg/mL) and *L. braziliensis* (IC_{50} 2.7 μg/mL). The biological activity seems to be related with the presence of methoxyl groups in ring A and B. Compounds **3.23** methoxylated at C-7 and C-5′ and **3.22** at C-8 showed improved activity than **3.24** which is nonmethoxylated**.** This is possibly due to a lipophilicty effect. The antileishmanial activity of quercitin (**3.24**) is in agreement with those previously reported in the literature [102]. In addition, compounds **3.17** and **3.18** having a flavanone backbone were inactive.

Table 3.6 Antileishmanial activity of *Lantana balansae*

	IC_{50} (μg/mL)[a]	
	L. amazonensis	*L. braziliensis*
EtOH extract	6.0±0.2	4.9±1.2
Hexane fract.	6.1±1.3	1.3±0.3
EtOAc fract.	9.9±0.5	5.3±1.0
3.16	0.6±0.1	0.2±0.01
3.17	>100	90±4.2
3.18	>100	>100
3.22	21.7±4.2	16.4±0.46
3.23	13.6±1.81	2.7±0.2
3.24	19.8±0.77	40.9±2.7
Control[b]	0.21±0.06	0.08±0.04

[a] Data are expressed as mean standard deviation of three determinations.

[b] Amphotericin B was used as positive control.

4. Macrocyclic monoterpene *O*-glycosides

4.1 Introduction

This chapter describes the structural elucidation of two new and one previously discovered macrocylic monoterpene glycosides and therefore will be focus on the biological activity and functional role in plants of glucose esters that contain menthiafolic acid.

Important secondary metabolites in plants are formed by glycosylation of small lipophilic molecules to form glycosides that may play important roles in plant functionality. For example they can act as antiherbivores and in antimicrobial plant defenses, and as free radical scavengers to increase the oxidative stress tolerance. They occur commonly as *O*-glycosides when a glucosyltransferase (GT) links the hydroxyl group of an aglycone to the anomeric center of a glucopyranose in glucosides or di-, tri-, and higher carbohydrate moieties in glycosides. In a similar manner, glucose esters are formed by esterification of aromatic or aliphatic acid aglycones to a carbohydrate at the anomeric or primary hydroxyl position. The neutralization of the acid group by esterification in plants, which affects the lipophilicity and facilitates the transport of an acid, particularly in phloem sap and during partitioning into various other plant tissues and cell compartments [122].

The natural bioactive monoterpene glycosides are widely distributed in nature; on the contrary, only a small number of monoterpene acid glucose esters have been isolated from plants, particularly from *Eucalyptus* species [122, 123]. It has also been observed that the aglycone part is based on two aliphatic acids, oleuropeic acid or menthiafolic acid or both. The biological activity of these secondary metabolites is associated with the presence of an α-β-unsaturated carbonyl group that acts as a Michael acceptor, and they may therefore possess a number of important biological activities.

While many of the oleuropeic acid glucose esters have been assessed for their antitumor, antinflammatory, antioxidant, antiviral, antimicrobial, cytotoxic and repellent properties and found to exhibit different levels of activity, only a few menthiafolic acid glucose esters have been tested, and the interest in them has concentrated on their antioxidant activity [122].

4.1.1 The genus *Parkinsonia* (Fabaceae)

Fabaceae is an important family of flowering plants, comprising about 730 genera and 19,400 species [124]. Many are of considerable ecological, agricultural and scientific importance [125], while others have found various uses in the traditional medicine [126, 127]. The genus *Parkinsonia* contains about 12 species that are native to tropical America, extending from Mexico to South America. It has been naturalized in Florida, Israel, Africa, Australia, Uganda and India [128, 129] where it has been given trivial names such as Mexican paloverde and Jerusalem thorn. Lately, it has attracted interest due to the increased use of the aqueous extract of the leaves in India and Nigeria to treat a number of conditions [130] and many pharmacological studies have been carried out to validate the traditional uses [131]. Previous phytochemical investigations of *P. aculeata* have focused on the isolation and identification of its contents of flavonoids, mainly C-glycosylflavones, and a few rotenoids [131-133], however, a recent study has reported the presence of monoterpene glycosides [134].

4.2 Chemical investigation of *Parkinsonia aculeata* L.

Parkinsonia aculeata is a spiny shrub or a small tree with yellow-orange and fragrant flowers. It has a high tolerance to drought and can grow on most types of soils including sand dunes, clay, alkaline and chalky and even mildly salty soil[135]. As a part of a collaborative project, the bioactive methanol extract of *P. aculeata* (leaves and twigs) was purified and yielded three macrocyclic *O*-glycosides **4.1**-**4.3.** The structure elucidation of these compounds was carried out by the analyses of one- and two-dimensional NMR spectra and HR-mass spectrometry experiments.

4.3 Results and discussion

This investigation reports the structure elucidation of three macrocyclic monoterpene *O*-glycosides, parkinsene F (**4.1**), parkinsene G (**4.2**) and parkinsene E (**4.3**) (see Figure 4.1 for chemical structures). The structure of **4.3** has been reported [134], but as the spectroscopic data were incomplete, they are included herein as well as the structural elucidation of **4.3**.

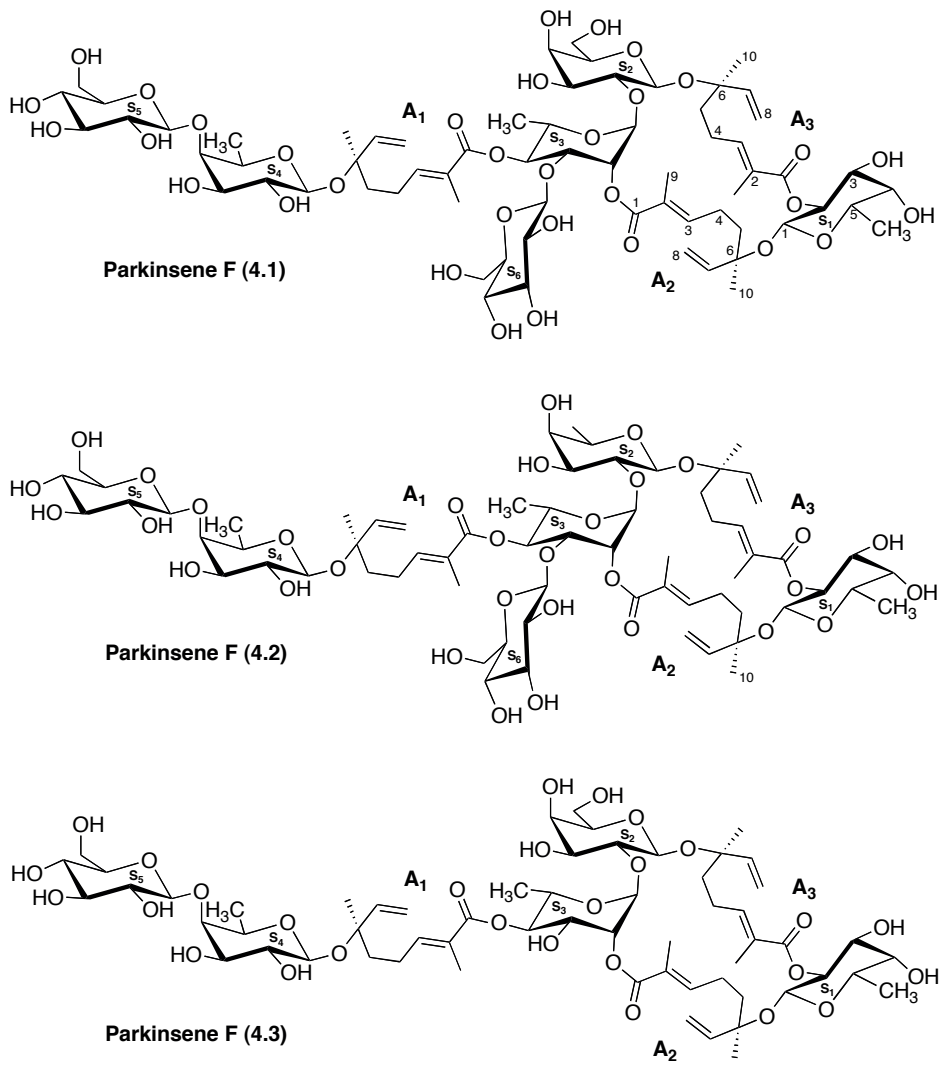


Figure 4.1 Macrocyclic monoterpenoids from *P. aculeate* **(4.1-4.3)**

4.3.1 Structure elucidation of 4.1

Compound **4.1** was obtained as a colourless gum. An LC-MS analysis showed that **4.1** produces the pair of positive ions 1423.7 and 1445.7, indicating that the elemental composition of **4.1** is $C_{66}H_{102}O_{33}$ and that the ions observed are $M+H^+$ and $M+Na^+$. This elemental composition was confirmed by a HRESIMS experiment to be 1423.6404 $[M+H]^+$ (calcd. for $C_{66}H_{103}O_{33}$ 1423.6382) as well as by the 1D NMR data (see Table 4.1), especially the ^{13}C spectrum which indicate the presence of 66 carbon signals. The number of unsaturations is consequently 16. An inspection of the NMR data suggested the presence of three monosubstituted carbon-carbon double bonds, three trisubstituted carbon-carbon double bonds, three carbonyl groups, and six monosaccharide units, accounting for 15 unsaturations. Compound **4.1** therefore has one additional ring.

The NMR data indicate that **4.1** has three identical monoterpene units, 6-hydroxy-2,6-dimethyl-2,7-octadienoic acid, designed A1, A2 and A3 in Figure 4.1. These were identified by the COSY correlations between 3-H and 4-H_2, 4-H_2 and 5-H_2, as well as 7-H and 8-H_2, and the HMBC correlations between 2-CH_3 and C-1, C-2 and C-3, as well as between 6-CH_3 and C-5, C-6 and C-7. The configuration of the trisubstituted double bond was determined to be 2*E* from the ROESY correlations between 2-CH_3 and 4-H_2. The absolute configuration of the monoterpenes could not be determined with the limited amounts available, but assumed to be 6*R*. The monoterpenes are all connected at C-6 with an ether link to the acetal carbon of the monosaccharides S1, S2 and S4 (A1 to S4, A2 to S1 and A3 to S2, see Figure 4.1 for the naming of the monosaccharides), and the expected HMBC correlation from the corresponding acetal protons to C-6 in the monoterpene (S4/1-H to A1/C-6, S1/1-H to A2/C-6 and S2/1-H to A3/C-6) were observed.

The monosaccharide S1 was characterised by the COSY spin systems 1-H/2-H/3-H and 5-H/6-H_3, by the ROESY correlations between 1-H and 3-H as well as 5-H, and between 6-H_3 and 4-H, as well as by the HMBC correlations between 3-H and C-4, between 5-H and C-1, and between 6-H_3 and C-5 as well as C-4. 2-H is a dd in the ^1H NMR spectrum with two large coupling constants (8.0 and 9.7 Hz), showing that 1-H, 2-H and 3-H all are axial. S1 was consequently determined to be a fucose. The signal for 2-H in the ^1H NMR spectrum is downshifted, indicated that C-2 in S1 is acylated, and a HMBC correlation between S1/2-H and A3/C-1 shows that A3 is connected to S1 with an ester bond.

The other end of A3 is as was mentioned above connected to C-1 of S2. S2 is characterised by the spin system 1-H/2-H/3-H/4-H/5-H/6-H_2, by the ROESY correlations between 1-H and 3-H as well as 5-H, and between 6-H_2 and 4-H, as

well as by the HMBC correlations between 3-H and C-4, between 5-H and C-1, and between 6-H_2 and C-5 as well as C-4. 2-H is a dd in the ^{1}H NMR spectrum with two large coupling constants (7.9 and 9.7 Hz), showing that 1-H, 2-H and 3-H all are axial. S2 was consequently determined to be a galactose. Besides to S2/C-1, S2/2-H also gives a HMBC correlation to S3/C-1, while S3/1-H gives a HMBC correlation to S2/C-2. 1-H in S3 is according to the direct ^{1}H-^{13}C coupling constant 170.8 Hz (extracted from the self-correlation in the HMBC spectrum) equatorial. So is S3/2-H, which is demonstrated by the small coupling constants between 1-H and 2-H (1.8 Hz) and 2-H and 3-H (3.3 Hz). On the other hand, 3-H, 4-H and 5-H are all axial which is demonstrated by the large coupling constants of 4-H (9 and 9 Hz). This is supported by the ROESY correlations between 3-H and 5-H, as well as between 4-H and 6-H_3. The suggested structure of S3 is supported by the expected COSY and HMBC correlations, and S3 is therefore a rhamnose. S3/2-H is as S1/2-H downshifted, and a HMBC correlation from S3/2-H to A2/C-1 establishes the connection between S3 and A2 through an ester bond. As A2 is connected to S1 in the other end (*vide supra*) the missing unsaturation is identified as a macrocyclic ring consisting of S1-A3-S2-S3-A2-S1 with 23 atoms involved. Additional HMBC correlations are observed from S3/3-H to S6/C-1 and from S6/1-H to S3/C-3, showing that S2-S3-S6 actually forms a trisaccharide. In S6 all ring protons are axial according to the ^{1}H-^{1}H coupling constants, and this is confirmed by ROESY correlations. S6 is consequently a glucose.

A fourth connection to S3 is to C-4, indicated by the downshift of S3/4-H and the HMBC correlation from this proton to A1/C-1. The final monoterpene unit (A1) is therefore connected to S3 by an ester bond to C-4, while A1/C-6 is connected to an additional monosaccharide (S4) by an acetal link (*vide supra*). In S4, the protons 1-H, 2-H and 3-H are axial according to their ^{1}H-^{1}H coupling constants, while 4-H is equatorial. COSY and HMBC correlations determine the structure of S4, while ROESY correlations between 1-H and 3-H as well as 5-H determine that S4 is a second fucose. HMBC correlations from S4/4-H to S5/C-1 and from S5/1-H to S4/C-4 show that S5 is connected to S4 at C-4, and that S4/S5 is a disaccharide. COSY and HMBC correlations in S5 determine the structure while the ROESY correlations between S5/1-H and S5/3-H as well as S5/5-H show that S5 is a second glucose (See Fig.ure 4.2).

As indicated above, the small amounts of the compounds obtained in this investigation does not permit hydrolysis and characterisation of the individual monoterpenes and monosaccharides. We have therefore assumed that the glucoses are D-glucoses, galactose is D-galactose, while the rhamnose is L-rhamnose and the fucoses are L-fucoses, as expected and as shown in Figure 4.1.

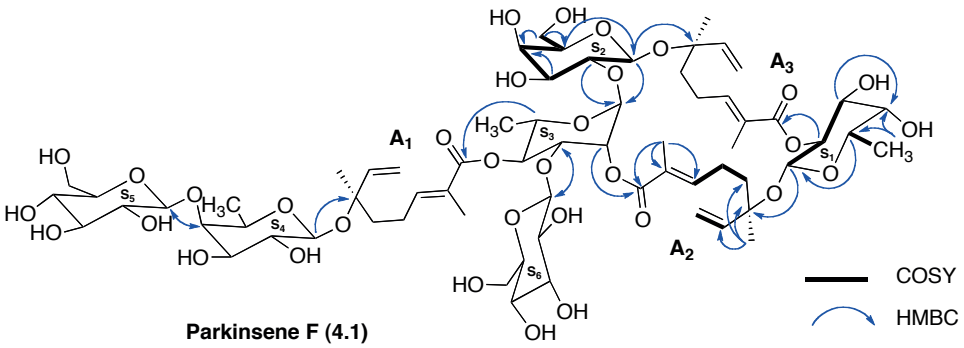


Figure 4.2 Pertinent HMBC and COSY correlations of **4.1**.

4.3.2 Structure elucidation of 4.2

Compound **4.2** was obtained as a colourless gum. The LC-MS analysis showed that **4.2** produces the pair of positive ions 1407.7 and 1429.7, indicating that the elemental composition of **4.2** is $C_{66}H_{102}O_{32}$ and that the ions observed are $M+H^+$ and $M+Na^+$. This was confirmed by 1D NMR data (see Table 4.1), especially the ^{13}C spectrum which indicate the presence of 66 carbon signals and that the number of unsaturations in **4.2** is 16, and the corresponding 15 unsaturations as in compound **4.1** are suggested by an inspection of the NMR data (three monosubstituted carbon-carbon double bonds, three trisubstituted carbon-carbon double bonds, three carbonyl groups, and six monosaccharide units). Compound **4.2** as well has one additional ring. The NMR data of **4.1** and **4.2** are overall very similar, indicating that **4.2** is in fact desoxy-**4.1**. The only significant differences are observed for the signals of S2, in which C-6 obviously is reduced to a methyl group. The corresponding correlations as discussed above for **4.1** were observed for **4.2**, also for S2, which consequently is a third fucose.

4.3.3 Structure elucidation of 4.3

^1H and ^{13}C NMR data of **4.3** are given in Table 4.1. The LC-MS analysis showed that **4.3** produces the pair of positive ions 1261.7 and 1283.7, indicating that the elemental composition of **4.3** is $C_{60}H_{92}O_{28}$ and that the ions observed are $M+H^+$ and $M+Na^+$. This elemental composition was confirmed by a HRESIMS experiment (*m/z* 1423.6404 $[M+H]^+$; calcd. for $C_{66}H_{103}O_{33}$ 1423.6382) as well as by the 1D NMR data (see Table 4.1), especially the ^{13}C spectrum which indicate the presence of 60 carbon signals. Compared to **4.1** and **4.2** it can be assumed that **4.3** is lacking a sugar unit, and it is obvious that only 5 acetal protons can be

observed in the ^{1}H NMR spectrum of **4.3**. Careful analysis of the correlations observed in the 2D NMR spectra reveal that **4.2** has the identical macrocyclic ring as **4.1** with S2 being a galactose.

Table 4.1 ^{1}H and ^{13}C NMR data of compounds **4.1**-**4.3** in MeOD

		4.1		**4.2**		**4.3**	
		^{13}C	^{1}H (*J* in Hz)	^{13}C	^{1}H (*J* in Hz)	^{13}C	^{1}H (*J* in Hz)
1	A2	169.1		169.1		169.1	
2		128.2		127.8		128.2	
3		146.1	6.75 m	146.0	6.75 m	145.8	6.79 m
4		24.4	2.39 m	24.0	2.41 m	24.4	2.40 m
			2.10 m		2.10 m		2.13 m
5		41.9	1.83 m	41.5	1.84 m	41.7	1.84 m
			1.65 m		1.65 m		1.65 m
6		81.1		81.1		81.0	
7		143.3	5.68 dd (10.8, 18.0)	143.4	5.68 dd (10.7, 18.2)	143.3	5.72 dd (11.5, 17.2)
8		117.2	5.26 m	116.9	5.26 m	117.1	5.28 m
			5.23 m		5.23 m		5.25 m
9		12.9	1.78 s	12.9	1.78 s	12.8	1.79 s
10		24.1	1.37 s	23.8	1.38 s	24.2	1.37 s
1	S1	97.7	4.46 d (8.0)	97.7	4.45 d (8.0)	97.7	4.47 d (8.0)
2		74.1	5.00 dd (8.0, 9.5)	74.1	5.02 dd (8.0, 9.6)	74.1	5.02 dd (8.0, 9.7)
3		71.8	3.64 m	71.7	3.63 m	71.8	3.63 m
4		73.3	3.61 m	73.3	3.61 m	73.3	3.61 m
5		73.8	3.62 m	73.7	3.61 m	73.8	3.62 m
6		17.0	1.25 d (6.5)	17.0	1.25 d (6.2)	17.0	1.25d (6.5)
1	A3	168.7		168.7		168.7	
2		129.4		129.5		129.4	
3		143.9	6.70 m	143.9	6.70 m	143.9	6.71 m
4		24.3	2.37 m	23.9	2.40 m	24.4	2.38 m
			2.18 m		2.18 m		2.19 m
5		42.9	1.63 m	42.9	1.64 m	42.8	1.66 m
			1.60 m		1.58 m		1.60 m
6		81.4		81.1		81.4	
7		144.3	6.09 dd (11.0, 17.8)	144.6	6.14 dd (11.1, 17.7)	144.2	6.15 dd (11.0, 17.8)
8		117.2	5.31 m	116.1	5.29 m	117.1	5.32 m
			5.28 m		5.26 m		5.29 m
9		12.9	1.82 s	12.8	1.81 s	12.9	1.82 s
10		22.8	1.43 s	22.7	1.40 s	23.0	1.43 s
1	S2	100.1	4.52 d (7.9)	100.4	4.47 d (7.3)	100.2	4.52 d (7.7)
2		81.1	3.23 dd (7.9, 10)	79.0	3.49dd (7, 10)	81.0	3.24 m
3		76.8	3.38 m	76.0	3.52 m	76.6	3.39 m
4		71.7	3.36 m	73.3	3.61 m	71.9	3.35 m
5		71.8	3.17 m	71.4	3.56 m	71.9	3.17 m
6		62.9	3.83 m	17.0	1.24 d (6.3)	62.9	3.83 m
			3.66 m				3.66 m
1	S3	99.3	5.12 d (1.7)	99.9	5.09 d (1.6)	99.5	5.16 d (1.5)
2		72.6	5.56 dd (1.8, 3.4)	72.8	5.55 dd (1.9, 3.2)	72.9	5.28 m
3		76.7	4.51 dd (3, 10)	76.5	4.49 dd (3.4, 10.2)	68.2	4.32 dd (3.8, 10.1)
4		74.2	5.10 dd (9, 9)	74.2	5.13 dd (10.0, 10.0)	75.8	4.98 dd (9.9, 9.9)
5		69.7	4.08 dq (9.9, 6.1)	69.0	4.07 dq (10.1, 6.0)	69.4	4.03 dq (9.9, 6.2)
6		18.6	1.17 d (6.3)	18.6	1.17 d (6.2)	18.6	1.17d (6.2)

Table 4.1 ^{1}H and ^{13}C NMR data of compounds **4.1**-**4.3** in MeOD (continued)

		4.1		**4.2**		**4.3**	
		^{13}C	^{1}H (*J* in Hz)	^{13}C	^{1}H (*J* in Hz)	^{13}C	^{1}H (*J* in Hz)
1	A1	169.6		169.5		169.5	
2		128.8		128.8		128.6	
3		144.9	6.79 m	144.9	6.79 m	144.9	6.82 m
4		24.6	2.32 m	24.4	2.31 m	24.6	2.33 m
5		41.2	1.72 m	41.5	1.70 m	41.3	1.72 m
6		81.0		82.0		81.0	
7		144.3	5.95 dd (11.0, 17.7)	144.3	5.95 dd (11.0, 17.7)	144.3	5.95 dd (11.0, 17.0)
8		116.1	5.28 m	116.1	5.27 m	116.0	5.28 m
			5.22 dd (11, 2)		5.21 m		5.22 m
9		12.8	1.85 s	12.7	1.85 s	12.7	1.85 s
10		23.7	1.38 s	23.9	1.38 s	23.8	1.38 s
1	S4	99.9	4.33 d (7.3)	99.9	4.33 d (7.3)	99.9	4.33 d (7.5)
2		73.2	3.50 dd (7.2, 9.8)	73.3	3.51 dd (7.1, 9.8)	73.2	3.49 dd (7.2, 9.7)
3		75.9	3.52 dd (3.0, 9.7)	75.9	3.52 m	75.9	3.52 dd (3.0, 9.8)
4		82.5	3.83 dd (3.4, 0.6)	82.5	3.82 dd (3.0, 0.6)	82.6	3.81 dd (3.0, 0.5)
5		71.3	3.60 qd (6.6, 0.6)	71.3	3.60 qd (6.1, 0.6)	71.3	3.59 qd (6.4, 0.5)
6		17.7	1.29 d (6.5)	17.5	1.29 d (6.5)	17.6	1.29 d (6.4)
1	S5	106.4	4.55 d (7.8)	106.1	4.55 d (7.8)	106.1	4.55 d (7.8)
2		71.8	3.28 m	71.8	3.28 m	71.8	3.29 m
3		78.5	3.36 m	78.5	3.36 dd (8.8, 8.8)	78.5	3.36 m
4		76.1	3.28 m	76.1	3.28 m	76.1	3.28 m
5		78.3	3.25 m	78.3	3.25 m	78.3	3.25 m
6		63.1	3.85 dd (2.2, 11.9)	63.1	3.85 dd (2.2, 11.9)	63.1	3.85 dd (2.1, 11.8)
			3.66 dd (6, 12)		3.66 dd (5.7, 11.9)		3.65 dd (5.7,11.8)
1	S6	106.3	4.37 d (7.8)	106.3	4.38 d (7.8)		
2		74.8	3.08 dd (7.8, 9.2)	74.9	3.08 dd (7.9, 9.2)		
3		78.3	3.26 m	78.3	3.25 m		
4		71.8	3.17 dd (9.2, 9.5)	71.9	3.16 dd (9.3, 9.3)		
5		78.3	3.27 m	78.3	3.27 m		
6		63.3	3.90 dd (2.0, 11.8)	63.1	3.89 dd (2.1, 12.1)		
			3.61 m		3.60 m		

Also the disaccharide S4-S5 is present in **4.3**, the difference compared to compound **4.1** is that **4.3** is lacking the glucose S6 and there is consequently no HMBC correlation from S3/3-H to another sugar unit. Compound **4.3** was analysed in the same way as compounds **4.1** and **4.2** by COSY, HMQC, HMBC, TOCSY and ROESY NMR experiments, and all corresponding 2D correlations discussed above were also observed for compound **4.3**.

Conclusions and perspectives

Natural products have for a long time been the primary source of commercial medicines and drug leads. In particular, plants have provided us with thousands of novel compounds, many of which have led to the development of some of the most important drugs used today. However, since around 1980 the interest in natural product discovery has experience a decline. Despite of this trend, a recent survey revealed that more half of the NCE-drugs introduced world-wide were either natural products or inspired in natural products, and the numbers are higher for anticancer and anti-infection agents. This has led to a restored interest in natural products that hopefully will be beneficial for the development of novel drugs.

The present thesis summarizes the investigation of bioactive secondary metabolites from three medicinal plants from the native flora of Bolivia, as well as the structure elucidation of novel macrocyclic monoterpene glycosides.

Of the twentyone compounds isolated from *Kaunia lasipohthalma* G., eighteen were known substances while two eudesmane derivatives (**2.1** and **2.2**) and one novel terpenoid with a new carbon skeleton (**2.3)** are reported for the first time. All tested compounds showed different levels of cytotoxicity towards five breast cancer cell line (MCF-7, SK-BR-3, HCC1937, L56Br-C1 and JIMT-1), which in general was higher that the cytotoxicity towards a normal-like breast epithelial cell line (MCF-10A). Differences in activity were also observed for compounds with different carbon skeletons, functional groups or an additional α-β-unsaturated carbonyl group. As the cell lines were more sensitive to all compounds than the normal-like MCF-10A cell, the molecular causes for the lower cytotoxicity of the compounds (specially **2.4**) need to be further explored.

A phytochemical study of bioactive extract of *Trixis antimenorrhoea* led to the isolation of two new metabolites, the nortrixane (**3.1**) and the trixanolide (**3.2**) together with eleven compounds previously reported. The absolute configuration of **3.2** was determined by application of the Mosher's ester protocol, exploiting the free C-9 hydroxyl group in **3.2**. Among the compounds evaluated for antileishmanial activity **3.2** was found to be the most active towards *L.*

amazonensis and *L. brasiliensis* and it may be worthwhile to investigate this class of compounds in more detail.

From the bioactive ethanol extract of *Lantana balansae* were isolated eleven know compounds. The cyclopentenone fatty acid (**3.16**) and the flavones (**3.17** and **3.18**) are new for the genus *Lantana* and therefore contribute to the chemotaxonomy knowledge of this genus. The antileishmanial activity of some of the isolates was measured and **3.16** was found to be particularly more potent towards the two strains tested.

The bioactive methanol extract of *Parkinsonia aculeata* yielded three macrocyclic monterpene glycosides (**4.1**-**4.3**), two of them being new (**4.1** and **4.2**). Their structures were determined using mass spectrometry, 1D and 2D NMR (HMQC, HMBC, COSY, TOCSY and ROESY).

The phytochemical profile and yield of secondary metabolite produced by plants can be highly regulated by environmental and biotic stresses. The influence of these factor were observed in *K. lasiophthalma* collected in Bolvia that produced germacrane, eudesmanolides, guianes, the novel terpenoid (**2.3)** and flavonoids, while guianes were the major metabolites isolated from *K. lasiophthalma* growing in Argentina.

Lately, great advances in separation techniques, screening strategies, new powerful NMR spectrometers, advanced organic syntheses and methodology, and constant and increasing genomic knowledge, have enhanced the discover and screen the biological activities of natural products. It is comforting to conclude that this research area will continue to provide endless opportunities for drug discovery in the future.

Bioassay-guided studies of medicinal plants as source of bioactive compounds may contribute to keep the traditional knowledge of plant uses and conserve the biodiversity.

Considering the wide array of metabolites isolated and their associated cytotoxic and antileishmanial activities, the phytochemical investigation of these medicinal plants and related species should continue and complement with futher biological studies that could help to undertand the mechanism (s) of action of test compounds.

Acknowledgments

The success completion of this thesis would have not been possible without the contribution of many different people, in their different ways. I would like to express my sincere gratitud specially to the following:

To my supervisor, Prof. Olov Sterner, for his timely guidance and exceptional support on structure elucidation.Your experience and knowledge in the field of Natural Products were the key motivation during my PhD.

My special thanks to Dr. Karl-Erik Bergquist, for his guidance through difficult structure elucidation problems, constructive criticism on the manuscripts, and keen interest at all stages of my research. One could not wish for a better co-supervisor.

My appreciation also goes to Prof. Stina Oredesson for conducting the anticancer assays, her valuable contribution to the papers and useful comments on the thesis.

I am deeply grateful to Maria Levin for her kind support and assistance whenever I needed it. Many thanks for my shorts trips to Bjärred and wonderful Summer Concerts!!.

I would also like to thank Bodil Eliasson for her great assitance with chemicals and kindness, Anders Sundin for his valuable support and help with my computer problems and Einar Nilsson for his aid in running HR-ESI-MS.

I wish to specially thank to my colleagues and friends Eira, Gilda, Paula, Rita, Zilma, Dan, Marcelo, Rikard, and Rodrigo for a friendly working environment and interesting conversations outside of the lab. Many thanks to Daniel for his enthusiastic work during his master project.

I would like to thank Ing. Ingrid Trigo, Ing. José L. Balderrama, Dr. Antonio Vilaseca, and Ing. Nelson Hinojosa for their collaboration during my research work at Centro de Tecnologia Agroindustrial. Many thanks to Gloria Saavedra (CTA-UMSS), Alberto Giménez (IIFB-UMSA), and Efrain Salamanca (IIFB-UMSA) for their valuable help with the antileishmanial assays.

A huge thanks to my amazing friends Carola, Gloria, Seda, Vicky, Daniel, and Joseph for their support, care, and encouragement at all times. A special thanks to Tamara for her friendship and for giving me the strenght that I needed throughout my time in Lund, and to Marta for sharing with me her enthusiasm and optimism.

Words are not enough to express my gratitude to my family. Thanks to *my father*, Jorge, for his unconditional help and support in my career, and *my mother*, Norah, for her love, care, and constant encouragement to achieve my goals. I would like to thank *my brothers,* Ivan and Gonzalo, for always believe in me and support my ideas. *My sister*, Linette, who has always been by my side in every stage of my life. Thanks for all your help, your advices and for your patience. This thesis is dedicated to all of you and my wonderful newphews Mauricio, Wadi, Danny, Adrian and Aarón.

Las palabras no son suficientes para expresar mi gratitud a mi familia. *Gracias a mi padre, Jorge*, por su *incondicional* ayuda *y apoyo en mi carrera y a mi madre*, *Norah*, *por su amor*, cuidado *y constante* motivación para *lograr mis metas*. *Quiero agraceder a mis hermanos*, *Iván y Gonzalo*, por confiar en mi y apoyar mis decisiones. *A mi hermana*, *Linette*, quien ha *estado siempre a mi lado en cada etapa de mi vida*, *gracias por tu ayuda*, *consejos y paciencia*. Esta tesis esta dedicada a ustedes y a mis maravillosos sobrinos Mauricio, Wadi, Danny, Adrian y Aarón.

My sincere gratitude to The Swedish International Development Agency (SIDA) for providing the financial support for this research project.

References

1. Cragg, G.M. and D.J. Newman, *Natural products: A continuing source of novel drug leads.* Biochimica et Biophysica Acta (BBA) - General Subjects, 2013. **1830**(6): p. 3670-3695.
2. Yun, U.W., et al., *Plant natural products: history, limitations and the potential of cambial meristematic cells.* Biotechnol Genet Eng Rev, 2012. **28**: p. 47-59.
3. Samuelsson, G. and L. Bohlin, *Drugs of Natural Origin: A Treatise of Pharmacognosy*2010, Stockholm, Sweden: Swedish Pharmaceutical Press. 776.
4. Organization, W.H. *WHO traditional medicine strategy: 2014-2023*. 2013; Available from: http://www.who.int/medicines/publications/traditional/trm_strategy14_23/en/.
5. Newman, D.J. and G.M. Cragg, *Natural Products As Sources of New Drugs over the 30 Years from 1981 to 2010.* Journal of Natural Products, 2012. **75**(3): p. 311-335.
6. Balunas, M.J. and A.D. Kinghorn, *Drug discovery from medicinal plants.* Life Sciences, 2005. **78**(5): p. 431-441.
7. Bourgaud, F., et al., *Production of plant secondary metabolites: a historical perspective.* Plant Science, 2001. **161**(5): p. 839-851.
8. Torssell, K., *Natural Product Chemistry: A mechanistic, biosynthetic and ecological approach*, 1997, Swedish Pharmaceutical Press: Stockholm, Sweden. p. 480.
9. Peláez, F., *The historical delivery of antibiotics from microbial natural products—Can history repeat?* Biochemical Pharmacology, 2006. **71**(7): p. 981-990.
10. Molinski, T.F., et al., *Drug development from marine natural products.* Nat Rev Drug Discov, 2009. **8**(1): p. 69-85.
11. Daly, J.W., T.F. Spande, and H.M. Garraffo, *Alkaloids from amphibian skin: a tabulation of over eight-hundred compounds.* J Nat Prod, 2005. **68**(10): p. 1556-75.
12. Dixon, R.A., *Natural products and plant disease resistance.* Nature, 2001. **411**(6839): p. 843-7.
13. Jemal, A., et al., *Global cancer statistics.* CA Cancer J Clin, 2011. **61**(Copyright (C) 2014 U.S. National Library of Medicine.): p. 69-90.
14. Sen, R. and M. Chatterjee, *Plant derived therapeutics for the treatment of Leishmaniasis.* Phytomedicine, 2011. **18**(12): p. 1056-69.

15. Chan-Bacab, M.J. and L.M. Pena-Rodriguez, *Plant natural products with leishmanicidal activity.* Natural Product Reports, 2001. **18**(6): p. 674-688.
16. Mishra, B.B., et al., *Alkaloids: Future prospective to combat leishmaniasis.* Fitoterapia, 2009. **80**(2): p. 81-90.
17. Dominguez-Carmona, D.B., et al., *Antiprotozoal activity of Betulinic acid derivatives.* Phytomedicine, 2010. **17**(Copyright (C) 2014 American Chemical Society (ACS). All Rights Reserved.): p. 379-382.
18. Sousa, M.C., et al., *Antileishmanial activity of semisynthetic lupane triterpenoids betulin and betulinic acid derivatives: synergistic effects with miltefosine.* PLoS ONE, 2014. **9**(Copyright (C) 2014 U.S. National Library of Medicine.): p. e89939.
19. Alakurtti, S., et al., *Anti-leishmanial activity of betulin derivatives.* J Antibiot, 2010. **63**(3): p. 123-126.
20. Chowdhury, A.R., et al., *Dihydrobetulinic acid induces apoptosis in Leishmania donovani by targeting DNA topoisomerase I and II: implications in antileishmanial therapy.* Mol. Med. (Manhasset, NY, U. S.), 2003. **9**(Copyright (C) 2014 American Chemical Society (ACS). All Rights Reserved.): p. 26-36.
21. Chowdhury, S., et al., *Novel betulin derivatives as antileishmanial agents with mode of action targeting type IB DNA topoisomerase.* Mol Pharmacol, 2011. **80**(Copyright (C) 2014 U.S. National Library of Medicine.): p. 694-703.
22. Sen, R., et al., *Artemisinin triggers induction of cell-cycle arrest and apoptosis in Leishmania donovani promastigotes.* J. Med. Microbiol., 2007. **56**(Copyright (C) 2014 American Chemical Society (ACS). All Rights Reserved.): p. 1213-1218.
23. Sen, R., et al., *Efficacy of artemisinin in experimental visceral leishmaniasis.* International Journal of Antimicrobial Agents, 2010. **36**(1): p. 43-49.
24. Sen, R., et al., *Iron enhances generation of free radicals by Artemisinin causing a caspase-independent, apoptotic death in Leishmania donovani promastigotes.* Free Radic Res, 2010. **44**(11): p. 1289-95.
25. (WHO), I.A.f.R.o.C.I.a.W.H.O., *GLOBOCAN 2012: Estimated cancer incidence, mortality and prevalence worldwide in 2012*, 2014.
26. Heskamp, S., et al., *Tumor-Receptor Imaging in Breast Cancer: A Tool for Patient Selection and Response Monitoring.* Curr. Mol. Med., 2013. **13**(Copyright (C) 2014 American Chemical Society (ACS). All Rights Reserved.): p. 1506-1522.
27. Hortobagyi, G.N. and A.U. Buzdar, *Current status of adjuvant systemic therapy for primary breast cancer: Progress and controversy.* CA: A Cancer Journal for Clinicians, 1995. **45**(4): p. 199-226.
28. Siegel, R., et al., *Cancer statistics, 2014.* CA: A Cancer Journal for Clinicians, 2014. **64**(1): p. 9-29.
29. Wyrebska, A., K. Gach, and A. Janecka, *Combined Effect of Parthenolide and Various Anti-cancer Drugs or Anticancer Candidate Substances on Malignant Cells in vitro and in vivo.* Mini Reviews in Medicinal Chemistry, 2014. **14**(3): p. 222-228.
30. Ghantous, A., et al., *What made sesquiterpene lactones reach cancer clinical trials?* Drug Discovery Today, 2010. **15**(15): p. 668-678.

31. Merfort, I., *Perspectives on sesquiterpene lactones in inflammation and cancer.* Curr. Drug Targets, 2011. **12**(Copyright (C) 2012 American Chemical Society (ACS). All Rights Reserved.): p. 1560-1573.
32. Johansson, M.H., *Reversible Michael additions: covalent inhibitors and prodrugs.* Mini - Reviews in Medicinal Chemistry, 2012. **12**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 1330-1344.
33. Schmidt, T.J., *Helenanolide-type sesquiterpene lactones—III. Rates and stereochemistry in the reaction of helenalin and related helenanolides with sulfhydryl containing biomolecules.* Bioorganic & Medicinal Chemistry, 1997. **5**(4): p. 645-653.
34. Schmidt, T.J., et al., *Helenanolide type sesquiterpene lactones. Part 5: The role of glutathione addition under physiological conditions.* Bioorganic & Medicinal Chemistry, 1999. **7**(12): p. 2849-2855.
35. Denmeade, S.R. and J.T. Isaacs, *The SERCA pump as a therapeutic target: making a "smart bomb" for prostate cancer.* Cancer Biol. Ther., 2005. **4**(Copyright (C) 2014 American Chemical Society (ACS). All Rights Reserved.): p. 14-22.
36. King, R.M. and H. Robinson, *The genera of the Eupatorieae (Asteraceae)*. Monographs in systematic botany from the Missouri Botanical Garden. Vol. 22. 1987, Kansas, USA: Missouri Botanical Garden
37. Lang, G., et al., *Antiplasmodial activities of sesquiterpene lactones from Eupatorium semialatum.* Zeitschrift fur Naturforschung. C, Journal of biosciences, 2002. **57**(3-4): p. 282-286.
38. Maas, M., et al., *An unusual dimeric guaianolide with antiprotozoal activity and further sesquiterpene lactones from Eupatorium perfoliatum.* Phytochemistry (Elsevier), 2011. **72**(Copyright (C) 2012 American Chemical Society (ACS). All Rights Reserved.): p. 635-644.
39. Sánchez-Mendoza, M.E., et al., *Bioassay-guided isolation of an anti-ulcer chromene from Eupatorium aschenbornianum: Role of nitric oxide, prostaglandins and sulfydryls.* Fitoterapia, 2010. **81**(1): p. 66-71.
40. Shen, Y.-C., et al., *Cytotoxic sesquiterpene lactones from Eupatorium kiirunense, a coastal plant of Taiwan.* J Nat Prod, 2005. **68**(Copyright (C) 2012 U.S. National Library of Medicine.): p. 745-50.
41. King, R.M. and H. Robinson, *Studies in the Eupatorieae (Asteraceae). CCII. A new genus Kaunia.* PHYTOLOGIA, 1980. **47**: p. 257-260.
42. De Gutierrez, A.N., et al., *Guaianolides from Kaunia lasiophthalma.* Phytochemistry, 1990. **29**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 1219-25.
43. Bohlmann, F., et al., *Four guaianolides and other constituents from three Kaunia Species.* Phytochemistry, 1981. **20**(10): p. 2375-2378.
44. Bohlmann, F., et al., *A further guaianolide from Kaunia arbuscularis.* Planta Medica, 1984. **50**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 284.
45. Rucker, G., K. Heiden, and E. Schenkel, *Antitumor-active lactones from Kaunia rufescens and Eupatorium cannabinum.* J. Indian Inst. Sci., 2001. **81**(Copyright (C) 2014 American Chemical Society (ACS). All Rights Reserved.): p. 333-334.

46. Rücker, G., et al., *Allergenic sesquiterpene lactones from Eupatorium cannabinum L. and Kaunia rufescens (Lund ex de Candolle)†.* Natural Toxins, 1997. **5**(6): p. 223-227.
47. Jakupovic, J., et al., *Further glaucolides and other sesquiterpene lactones from Brocchia cinerea.* Phytochemistry, 1988. **27**(7): p. 2219-2224.
48. Bohlmann, F. and C. Zdero, *Sesquiterpene lactones and other constituents from Tanacetum parthenium.* Phytochemistry, 1982. **21**(10): p. 2543-2549.
49. Huneck, S., C. Zdero, and F. Bohlmann, *Seco-guaianolides and other constituents from Artemisia species.* Phytochemistry, 1986. **25**(4): p. 883-889.
50. Herz, W. and R.P. Sharma, *Seco-germacradienolide from Liatris pycnostachya.* Journal of Organic Chemistry, 1975. **40**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 392.
51. Herz, W. and R.P. Sharma, *Constituents of Liatris species. XI. Pycnolide, a seco-germacradienolide from Liatris pycnostachya, and other antitumor constituents of Liatris species.* Journal of Organic Chemistry, 1976. **41**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 1248-53.
52. Bovill, M.J., et al., *Sesquiterpenoids. XXV. X-ray crystal structure analysis of costunolide.* Acta Crystallogr., Sect. B, 1976. **B32**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 3203-9.
53. El-Feraly, F.S. and D.A. Benigni, *Sesquiterpene lactones of Laurus nobilis leaves.* Journal of Natural Products, 1980. **43**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 527-31.
54. Kisiel, W., *A new germacranolide from Zinnia haageana.* Phytochemistry, 1978. **17**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 1059-60.
55. Sanz, J.F. and J.A. Marco, *NMR studies of tatridin A and some related sesquiterpene lactones from Tanacetum vulgare.* Journal of Natural Products, 1991. **54**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 591-6.
56. Julianti, E., et al., *Sesquiterpenes from the leaves of Laurus nobilis L.* Phytochemistry, 2012. **80**: p. 70-76.
57. Appendino, G., et al., *Sesquiterpene lactones from Artemisia genipi Weber: isolation and determination in plant material and in liqueurs.* J. Agric. Food Chem., 1982. **30**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 518-21.
58. Bohlmann, F., et al., *Sesquiterpene lactones and other constituents from Schistostephium species.* Phytochemistry, 1983. **22**(7): p. 1623-1636.
59. Romo De Vivar, A. and H. Jimenez, *Structure of santamarine, a new sesquiterpene lactone.* Tetrahedron, 1965. **21**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 1741-5.
60. Romo, J., et al., *Constituents of Artemisia and Chrysanthemum species. Structures of chrysartemins A and B.* Phytochemistry, 1970. **9**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 1615-21.
61. Mata, R., G. Delgado, and A.R. de Vivar, *Sesquiterpene lactones of Artemisia mexicana var. Angustifolia.* Phytochemistry, 1984. **23**(8): p. 1665-1668.

62. Bhadane, N.R., R.G. Kelsey, and F. Shafizadeh, *Sesquiterpene lactones of Artemisia tridentata ssp. Vaseyana.* Phytochemistry, 1975. **14**(9): p. 2084-2085.
63. Matsuda, H., et al., *Anti-alcoholism active constituents from Laurel and Linden.* Tennen Yuki Kagobutsu Toronkai Koen Yoshishu, 2000. **42nd**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 469-474.
64. Herz, W. and Y. Sumi, *Constituents of Ambrosia and related species. III. Constituents of Ambrosia hispida.* Journal of Organic Chemistry, 1964. **29**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 3438-9.
65. Rao, M.M., D.G.I. Kingston, and T.D. Spittler, *Flavonoids from Flourensia cernua.* Phytochemistry, 1970. **9**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 227-8.
66. Ulubelen, A., et al., *Flavonoids of Salvia tomentosa (Labiatae).* Journal of Natural Products, 1979. **42**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 261-3.
67. Kupchan, S.M., et al., *Tumor inhibitors—XXXIII : Cytotoxic flavones from eupatorium species.* Tetrahedron, 1969. **25**(8): p. 1603-1615.
68. Sorlie, T., et al., *Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications.* Proc. Natl. Acad. Sci. U. S. A., 2001. **98**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 10869-10874.
69. Sorlie, T., et al., *Repeated observation of breast tumor subtypes in independent gene expression data sets.* Proc Natl Acad Sci U S A, 2003. **100**(Copyright (C) 2013 U.S. National Library of Medicine.): p. 8418-23.
70. Perou, C.M., et al., *Distinctive gene expression patterns in human mammary epithelial cells and breast cancers.* Proc. Natl. Acad. Sci. U. S. A., 1999. **96**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 9212-9217.
71. Joensson, G., et al., *High-resolution genomic profiles of breast cancer cell lines assessed by tiling BAC array comparative genomic hybridization.* Genes, Chromosomes Cancer, 2007. **46**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 543-558.
72. Perou, C.M., et al., *Molecular portraits of human breast tumors.* Nature (London), 2000. **406**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 747-752.
73. Hu, Z., et al., *The molecular portraits of breast tumors are conserved across microarray platforms.* BMC Genomics, 2006. **7**(Copyright (C) 2013 U.S. National Library of Medicine.): p. 96.
74. Alvar, J., et al., *Leishmaniasis Worldwide and Global Estimates of Its Incidence.* PLoS ONE, 2012. **7**(5): p. e35671.
75. Sen, R. and M. Chatterjee, *Plant derived therapeutics for the treatment of Leishmaniasis.* Phytomedicine, 2011. **18**(12): p. 1056-1069.
76. Patil, R.S., et al., *Synthetic and Natural products against leishmaniasis: A Review.* World Journal of Public Health Sciences, 2012. **1**: p. 7-22.
77. Polonio, T. and T. Efferth, *Leishmaniasis: Drug resistance and natural products (Review).* International Journal of Molecular Medicine, 2008. **22**(3): p. 277-286.

78. Le Pape, P., *Development of new antileishmanial drugs – current knowledge and future prospects.* Journal of Enzyme Inhibition and Medicinal Chemistry, 2008. **23**(5): p. 708-718.
79. Hind, D.J.N., *A New Species and a Commentary on the Genus Trixis (Compositae: Mutisieae) in Bahia, Brazil.* Kew Bulletin, 2000. **55**(2): p. 381-386.
80. Borges, R.A.X. and M. Machado Saavedra, *Trixis forzzae (Compositae: Mutisieae), a new species from Minas Gerais, Brazil.* Kew Bulletin, 2010. **65**: p. 65-68.
81. Thomas, E., et al., *Cultural significance of medicinal plant families and species among Quechua farmers in Apillapampa, Bolivia.* Journal of Ethnopharmacology, 2009. **122**: p. 60-67.
82. Trillo, C., et al., *Persistence of the Use of Medicinal Plants in Rural Communities of the Western Arid Chaco [Córdoba, Argentina]* The Open Complementary Medicine Journal, 2010. **2**: p. 80-90.
83. Agra, M.d.F., et al., *Survey of medicinal plants used in the region Northeast of Brazil.* Revista Brasileira de Farmacognosia, 2008. **18**: p. 472-508.
84. Kotowicz, C., et al., *Absolute configuration of trixanolides from Trixis pallida.* Journal of Natural Products, 2001. **64**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 1326-1331.
85. Bohlmann, F., et al., *Naturally occurring terpene derivatives. Part 346. Trixikingolides and germacrene derivatives from Trixis species.* Phytochemistry, 1981. **20**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 1649-55.
86. Bohlmann, F. and C. Zdero, *Naturally occurring terpene derivatives, 156. A new group of sesquiterpene lactones from the genus Trixis.* Chemische Berichte, 1979. **112**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 435-44.
87. De Riscala, E.C., et al., *Trixanolide and germacratriene derivatives from Trixis grisebachii.* Phytochemistry, 1989. **28**(8): p. 2155-2161.
88. De Riscala, E.C., et al., *Trixane derivatives from Trixis praestans.* Phytochemistry, 1988. **27**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 2343-6.
89. Granato, E.M., et al., *Phytochemical screening of the plant species Trixis antimenorrhoea (Schrank) Kuntze.* Rev. Bras. Farm., 2013. **94**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 130-135.
90. Katinas, L., *Revision de las especies Sudamericanas del género Trixis (Asteraceae, Mutisieae).* Darwiniana, 1996. **34(1-4)**: p. 27-108.
91. Bohlmann, F., et al., *Naturally occurring terpene derivatives. 202. Further isocedrene derivatives from Trixis paradoxa.* Phytochemistry, 1979. **18**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 855-7.
92. Bohlmann, F., et al., *Naturally occurring terpene derivatives. Part 479. Two sesquiterpenes from Senecio species.* Phytochemistry, 1983. **22**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 1675-7.

93. Gupta, M.M., R.K. Verma, and L.N. Misra, *Terpenoids from Hemidesmus indicus*. Phytochemistry, 1992. **31**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 4036-7.
94. Farkas, L., et al., *Constituents of Iva species. V. Isolation, structure, and synthesis of nevadensin, a new flavone from Iva nevadensis and Iva acerosa.* Journal of Organic Chemistry, 1966. **31**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 3228-32.
95. Salmenkallio, M., et al., *The flavonoids of Trichophorum cespitosum*. Phytochemistry, 1982. **21**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 2990-1.
96. Lichius, J.J., et al., *Antimitotic and cytotoxic flavonols from Zieridium pseudobtusifolium and Acronychia porteri.* Journal of Natural Products, 1994. **57**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 1012-16.
97. Simonsen, H.T., et al., *Methoxyflavones from Melicope borbonica and M. obscura (Rutaceae)*. Biochemical Systematics and Ecology, 2003. **31**(3): p. 327-330.
98. Gupta, S.R., T.R. Seshadri, and G.R. Sood, *Flavonols and other components of the leaves of Calycopteris floribunda.* Phytochemistry, 1973. **12**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 2593-40.
99. Kyriakopoulou, I., et al., *Samioside, a new phenylethanoid glycoside with free-radical scavenging and antimicrobial activities from Phlomis samia.* J Nat Prod, 2001. **64**(Copyright (C) 2013 U.S. National Library of Medicine.): p. 1095-7.
100. Takeda, Y. and M.O. Fatope, *New phenolic glucosides from Lawsonia inermis.* Journal of Natural Products, 1988. **51**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 725-9.
101. da Silva, E.R., C.C. Maquiaveli, and P.P. Magalhães, *The leishmanicidal flavonols quercetin and quercitrin target Leishmania (Leishmania) amazonensis arginase.* Experimental parasitology, 2012. **130**(3): p. 183-188.
102. Manjolin, L.C., et al., *Dietary flavonoids fisetin, luteolin and their derived compounds inhibit arginase, a central enzyme in Leishmania (Leishmania) amazonensis infection.* Food Chemistry, 2013. **141**(3): p. 2253-2262.
103. Love, A., et al., *Variability in Foliar Essential Oils among Different Morphotypes of Lantana Species Complexes, and Its Taxonomic and Ecological Significance.* Chem. Biodiversity, 2009. **6**(Copyright (C) 2014 American Chemical Society (ACS). All Rights Reserved.): p. 2263-2274.
104. Ghisalberti, E.L., *Lantana camara L. (Verbenaceae)*. Fitoterapia, 2000. **71**: p. 467-486.
105. Sousa, E.O. and J.G.M. Costa, *Genus Lantana: chemical aspects and biological activities.* Revista Brasileira de Farmacognosia, 2012. **22**: p. 1115-1180.
106. Nirmal, S.A., et al., *An overview of Lantana camara: chemistry and pharmacological profile.* Pharmacologyonline, 2009(Copyright (C) 2014 American Chemical Society (ACS). All Rights Reserved.): p. 520-531.
107. Sharma, O.P., et al., *A review of the hepatotoxic plant Lantana camara.* Critical Reviews in Toxicology, 2007. **37**(Copyright (C) 2014 American Chemical Society (ACS). All Rights Reserved.): p. 313-352.

108. Hussain, H., et al., *Chemistry of some species genus Lantana.* Pak. J. Bot., 2011. **43**(Copyright (C) 2014 American Chemical Society (ACS). All Rights Reserved.): p. 51-62.
109. Grace-Lynn, C., et al., *In Vitro Antioxidant Activity Potential of Lantadene A, a Pentacyclic Triterpenoid of Lantana Plants*. Molecules, 2012. **17**(9): p. 11185-11198.
110. Salvat, A., et al., *Antimicrobial activity in methanolic extracts of several plant species from northern Argentina.* Phytomedicine, 2004. **11**(Copyright (C) 2013 U.S. National Library of Medicine.): p. 230-4.
111. De, V.M.E.L., E.C.J. Talenti, and J.A. Retamar, *Essential oils of Lantana balansae.* Essenze Deriv. Agrum., 1973. **43**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 299-306.
112. Guedes, d.S.F.J., et al., *Chemical and molecular characterization of fifteen species from the Lantana (Verbenaceae) genus.* Biochemical Systematics and Ecology, 2012. **45**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 130-137.
113. Pisutthanan, N., et al., *A new flavonoid from Chromolaena odorata.* Nat. Prod. Res., 2006. **20**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 1192-1198.
114. Fernandez, C., et al., *Flavonoid aglycones from some Canary Islands species of Sideritis.* Journal of Natural Products, 1988. **51**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 591-3.
115. Hasan, M., D.K. Burdi, and V.U. Ahmad, *Diterpene fatty acid ester from Leucas nutans.* Journal of Natural Products, 1991. **54**(Copyright (C) 2014 American Chemical Society (ACS). All Rights Reserved.): p. 1444-6.
116. Vieira, I.J.C., et al., *Hirtinone, a novel cycloartane-type triterpene and other compounds from Trichilia hirta L. (Meliaceae)*. Molecules, 2013. **18**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 2589-2597.
117. Pan, H. and L.N. Lundgren, *Phenolic extractives from root bark of Picea abies.* Phytochemistry, 1995. **39**(6): p. 1423-1428.
118. Reynaud, J. and J. Raynaud, *Presence of onopordin in Doronicum grandiflorum Lam. (Compositae)*. Pharmazie, 1984. **39**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 126.
119. Whalen, M.D. and T.J. Mabry, *New 8-hydroxyflavonoids from Solanum section Androceras.* Phytochemistry, 1979. **18**(2): p. 263-265.
120. Slowing, K., et al., *Flavonoid glycosides from Eugenia jambos.* Phytochemistry, 1994. **37**(Copyright (C) 2013 U.S. National Library of Medicine.): p. 255-8.
121. Taskova, R.M., et al., *Flavonoid profiles in the Heliohebe group of New Zealand Veronica (Plantaginaceae).* Biochemical Systematics and Ecology, 2008. **36**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 110-116.
122. Goodger, J.Q.D. and I.E. Woodrow, *Chapter 12 - Oleuropeic and Menthiafolic Acid Glucose Esters from Plants: Shared Structural Relationships and Biological Activities*, in *Studies in Natural Products Chemistry*, R. Atta ur, Editor 2013, Elsevier. p. 427-452.

123. Dembitsky, V., *Astonishing diversity of natural surfactants: 7. Biologically active hemi- and monoterpenoid glycosides.* Lipids, 2006. **41**(1): p. 1-27.
124. Doyle, J.J., *PHYLOGENY OF THE LEGUME FAMILY - AN APPROACH TO UNDERSTANDING THE ORIGINS OF NODULATION.* Annual Review of Ecology and Systematics, 1994. **25**: p. 325-349.
125. Vietmeyer, N.D., *Lesser-known plants of potential use in agriculture and forestry.* Science, 1986. **232**(Copyright (C) 2014 U.S. National Library of Medicine.): p. 1379-84.
126. Pras, N., et al., *Mucuna pruriens: improvement of the biotechnological production of the anti-Parkinson drug L-dopa by plant cell selection.* Pharm. World Sci., 1993. **15**(Copyright (C) 2014 American Chemical Society (ACS). All Rights Reserved.): p. 263-8.
127. McManus, O.B., et al., *An activator of calcium-dependent potassium channels isolated from a medicinal herb.* Biochemistry, 1993. **32**(Copyright (C) 2014 American Chemical Society (ACS). All Rights Reserved.): p. 6128-33.
128. Sharma, S. and A.P. Vig, *Evaluation of In Vitro Antioxidant Properties of Methanol and Aqueous Extracts of Parkinsonia aculeata L. Leaves.* The Scientific World Journal, 2013. **2013**: p. 7.
129. USDA, A. and N.G.R. Program. *Germplasm Resources Information Network-(GRIN) [Online Database]*. National Germplasm Resources Laboratory, Beltsville, Maryland 2014 [cited 2014 25-04-2014]; Available from: http://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl?26783.
130. Hassan, S.W., et al., *Hepatoprotective effect of leaf extracts of P. aculeata L. against CCl_4 intoxication in albino rats.* International journal of Biological Chemistry, 2008. **2**: p. 42-48.
131. Divya, B., K. Mruthunjaya, and S.N. Manjula, *Parkinsonia aculeata: a phytopharmacological review.* Asian J. Plant Sci., 2011. **10**(Copyright (C) 2014 American Chemical Society (ACS). All Rights Reserved.): p. 175-181.
132. El-Sayed, N.H., et al., *Luteolin 7,4'-dimethyl ether 6-C-glucoside from Parkinsonia aculeata.* Phytochemistry, 1991. **30**(Copyright (C) 2014 American Chemical Society (ACS). All Rights Reserved.): p. 2442.
133. Bhatia, V.K., S.R. Gupta, and T.R. Seshadri, *C-Glycosides of the leaves of Parkinsonia aculeata.* Tetrahedron, 1966. **22**(Copyright (C) 2014 American Chemical Society (ACS). All Rights Reserved.): p. 1147-52.
134. Marzouk, M.S., et al., *Novel Macrocyclic Monoterpene Glycosides from Bioactive Extract of Parkinsonia aculeata L.* Cell Biochemistry and Biophysics, 2013. **65**(3): p. 301-313.
135. Service, U.F. *Pacific Island Ecosystems at Risk (PIER)*. [cited 2014 25-04-2014]; Available from: http://www.hear.org/Pier/species/parkinsonia_aculeata.htm.

Paper I

Scientia Pharmaceutica www.scipharm.at

Research article Open Access

Cytotoxic Sesquiterpene Lactones from *Kauna lasiophthalma* Griseb

Eliana M. MALDONADO [1,2], Daniel SVENSSON [1], Stina M. OREDSSON [3], Olov STERNER * [1]

[1] Centre for Analysis and Synthesis, Lund University, Getingevägen 60, 221 00, Lund, Sweden.
[2] Centro de Tecnología Agroindustrial, San Simón University, Cochabamba, Bolivia.
[3] Departament of Biology, Lund University, Sölvegatan 35A, 223 62, Lund, Sweden.

* Corresponding author. E-mail: Olov.Sterner@chem.lu.se (O. Sterner)

Sci Pharm. 2014; 82: 147–160 doi:10.3797/scipharm.1310-18

Published: January 18th 2014 Received: October 21st 2013
Accepted: January 18th 2014

This article is available from: http://dx.doi.org/10.3797/scipharm.1310-18

Abstract

Two new eudesmane derivatives (**3** and **8**) were isolated from the ethanol extract of the aerial parts of *Kaunia lasiophthalma* Griseb, together with 14 known eudesmane, germacrane, and guaiane sesquiterpenes, and four flavones. The structures and relative configurations of all the compounds were established by NMR spectroscopy and high-resolution mass spectrometry. The anticancer activity of sesquiterpenes **1**, **3**, **6**–**9**, **11**, **12**, **14**, and **16** was evaluated *in vitro* with the breast cancer cell lines HCC1937, JIMT-1, L56Br-C1, MCF-7, and SK-BR-3, and compared with the cytotoxicity in the non-cancerous breast epithelial cell line MCF-10A. All compounds were found to possess anticancer activity, and compound **1** was the most potent in all of the investigated cancer cell lines with IC_{50} values ranging between 2.0 and 6.2 μM. In order to demonstrate the importance of the α-methylene-γ-lactone/ester moiety present in all compounds for the effects on the cells, the methyl cysteine adduct **21** was prepared from **9** and found to be inactive or considerably less potent.

Keywords

Kaunia lasiophthalma • Cytotoxicity • Sesquiterpene lactones • Breast cancer cell lines

Introduction

The relatively small genus *Kaunia* (Asteraceae: Eupatorieae) comprises only 14 species of which most grow in Bolivia, but that also are found in Argentina, Brazil, Peru, and Ecuador [1, 2]. Previous chemical investigations of plants classified in this genus have revealed the presence of sesquiterpene lactones, predominately guaianes, and thymol derivatives as the main constituents [3–5]. *Kaunia lasiophthalma* Griseb *(syn. Eupatorium lasiophthalmum* G.) is a shrub bearing white-purple flowers, locally known in Cochabamba, Bolivia by the common name of "Tuwi" and used to treat inflammation and headaches (Lic. Modesto Zárate, personal communication). It has previously been subjected to a phytochemical investigation by Gutierrez and co-workers, who isolated 19 guaiane sesquiterpene lactones [6]. In our search for biologically active compounds in the Bolivian flora, we have isolated and characterized the chemical constituents of the aerial parts of *K. lasiophthalma* G. Two new eudesmanolide derivatives, compounds **3** and **8**, together with 18 known compounds (**1**, **2**, **4**–**7, 9**–**20**) were obtained, and this constitutes the first report of eudesmanolide and germacranolide sesquiterpenes as well as flavones from this species. As most isolated compounds possess an α-methylene-γ-lactone moiety that previously has been associated with various biological activities, we decided to investigate the effects of the sesquiterpenes available in sufficient quantities on cancer cells. Unsaturated lactones are likely to exert biological effects because they react with cell constituents as Michael acceptors, and have consequently been considered to be generally toxic. However, recent studies have shown that Michael acceptors may be selective and several are currently in clinical trials as drug candidates [7].

Human breast cancer can be classified into different molecular subtypes using gene expression profiles [8–13]. The anticancer activity of compounds **1**, **3**, **6–9**, **11**, **12**, **14**, and **16** in the five breast cancer cell lines MCF-7 (luminal A subtype), SK-BR-3 (luminal B), HCC1937, L56Br-C1 (basal subtype), and JIMT-1 (HER2 subtype) was compared with the cytotoxicity in the normal-like breast epithelial cell line MCF-10A [8–13]. In breast cancer, each subtype has a different prognosis and is subjected to different treatment. The luminal A and B subgroups express estrogen receptors and are amenable to hormone therapy, while the HER2 group, expressing the human epidermal growth factor receptor 2, may be subjected to trastuzumab therapy. The basal tumors lack expression of both estrogen receptors and HER2; they are biologically more aggressive and the prognosis is often poorer.

Results and Discussion

The EtOH extract of the leaves and flowers of *K. lasiophthalma* G. were subjected, separately, to sequential liquid-liquid partition with hexane, CH_2Cl_2, and EtOAc. The major constituents were found to be fats, which were not further investigated, and the compounds reported here are minor metabolites of this plant. Vacuum liquid chromatography (VLC) of the CH_2Cl_2 fractions followed by silica gel and Sephadex LH-20 chromatography as well as HPLC fractionation afforded the two new natural products **3** and **8,** along with 18 known compounds, 14 sesquiterpenes and four flavonoids (see Figure 1 for chemical structures).

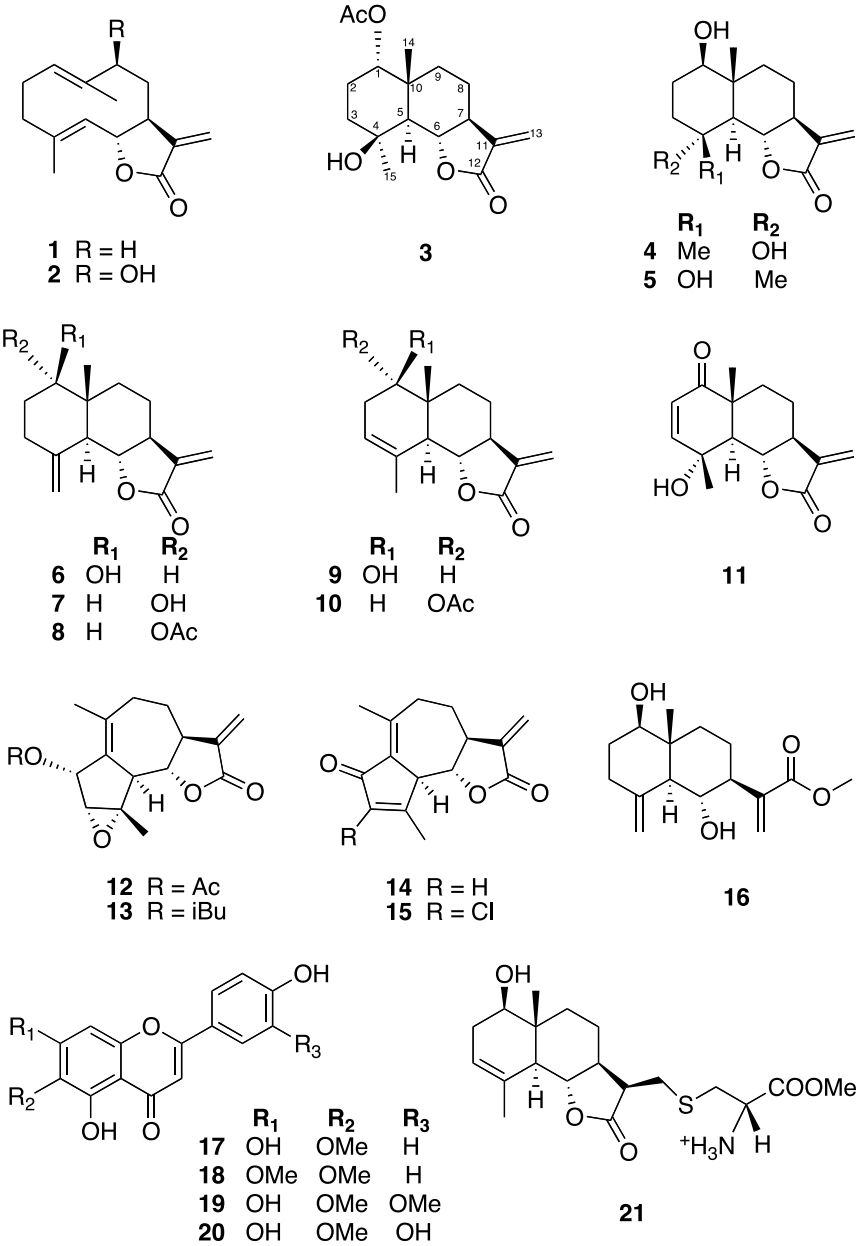


Fig. 1. Structures of the isolated compounds from *K. lasiophthalma* **1–20** and methyl cysteine adduct **21**

The known compounds isolated from leaves (**1**, **6**, **7**, **9**, **10**, **16**–**19**) and flowers (**1**, **2**, **4**–**7**, **9**–**16**, **20**) were identified by combined spectroscopic analyses and comparison with literature data, as costunolide (**1**) [14], haageanolide (**2**) [15], 1β-hydroxyarbusculin A (**4**) [16], 4-*epi*-1β-hydroxyarbusculin A (**5**) [17], reynosin (**6**) [18], 1-*epi*-reynosin (**7**) [19], santamarin (**9**) [20], the acetate of santamarin (**10**) [21], 11,13-didehydrovulgarin (**11**) [22], 2β-acetoxy-3α,4α-epoxy-3,4-dihydrokauniolide (**12**) [6], 3α,4α-epoxy-2α-isobutyryloxykauniolide (**13**) [6], dehydroleucodin (**14**) [23], 3-chlorodehydroleucodin (**15**) [6], baynol C (**16**) [24], hispidulin (**17**) [25], cirsimaritin (**18**) [26], jaceosidin (**19**) [27], and eupafolin (**20**) [28].

Compound **3** was obtained as a colourless gum. The HR-ESI-MS indicated that its elemental composition is $C_{17}H_{25}O_5$, which suggests six degrees of unsaturation. The IR spectrum showed absorption bands corresponding to a hydroxyl group (3473 cm^{-1}), an α,β-unsaturated-γ-lactone function (1764 cm^{-1}), and an ester group (1730 cm^{-1}). The NMR spectra displayed the characteristic signals of an eudesmane lactone, see Table 1 for 1D NMR data and Figure 2 for 2D data. The presence of an exomethylene-γ-lactone ring was established by the 13-H_2 proton signals at δ_H 5.94 and 4.79 and their HMBC correlations to C-7, C-11, and C-12, and the 6-H lactone proton signal at δ_H 3.70 and its strong ^1H-^1H coupling with 7-H and HMBC correlation to C-12. In addition, the NMR data indicated the presence of the acetoxylated tertiary carbon (C-1) at δ_H 4.67 and δ_C 77.2 and HMBC correlations from both 1-H and acetoxy-H_3 to the acetoxy carbonyl carbon (C-1'), and an oxygenated quaternary carbon (C-4) at δ_C 71.0. The complete structural elucidation of **3** was achieved by analysis of the HMQC, HMBC, and COSY spectra. The large ^1H-^1H coupling constant between 5-H and 6-H and the HMBC correlations between 14-H_3 and C-1, C-5, C-9, and C-10 links C-5 and the adjacent γ-lactone to the quaternary C-10. COSY correlations from 1-H via 2-H_2 to 3-H_2, and HMBC correlations from 1-H to C-5 and C-10, from 6-H to C-4, C-5, C-8, and C-10, as well as from 15-H_3 to C-3, C-4, and C-5 close the left cyclohexane ring. The ^1H-^1H spin system 5-H/6-H/7-H/8-H_2/9-H_2 together with HMBC correlations from 6-H to C-8 and from 7-H to C-9 close the second cyclohexane ring and establish the eudesmane skeleton unambiguously. The relative configuration of **3** was suggested by correlations observed in the NOESY spectrum, from 14-H_3 to 1-H, 2β-H, 3β-H, 6-H, and 8β-H, as well as from 5-H to 2α-H, 3α-H, 7-H, 8α-H, and 15-H_3. The proposed configuration is confirmed by the large coupling constants between 5-H and 6-H as well as 6-H and 7-H, showing that the three protons are axial, while the small coupling constants between 1-H and 2-H_2 show that 1-H is equatorial. The comparison of the spectroscopic data of compound **3** with those of 4-*epi*-1β-hydroxyarbusculin (**5**) [17] indicated that they are similar. The difference is that the C-1 hydroxyl group in **5** is acetylated in **3** and that the configuration at C-1 is inversed, which consequently identifies **3** as 4-*epi*-1α-acetoxy arbusculin A.

Compound **8** was isolated as clear oil. The elemental composition was established by HR-ESI-MS to be $C_{17}H_{22}O_4$, indicating seven degrees of unsaturation. The IR spectrum exhibited the presence of an α,β-unsaturated-γ-lactone (1770 cm^{-1}) and an ester group (1726 cm^{-1}). The ^1H NMR data of **8** are similar to those of the acetate of reynosin [29], the significant differences are the chemical shift of 1-H and 5-H as well as the coupling constants between 1-H and 2-H_2 which are small in **8** as 1-H is equatorial, but large for the acetate of reynosin as 1-H is axial. An analysis of the NOESY spectrum of **8** showed correlations from 14-H_3 to 1-H, 2β-H, 6-H, 8β-H, and 9β-H as well as from 5-H to 3α-H, 7-H, and 8α-H. Therefore, compound **8** was identified as acetyl 1-*epi*-reynosin.

The anticancer activities of sesquiterpenes **1**, **3**, **6**–**9**, **11**, **12**, **14,** and **16** were assessed in five breast-cancer cell lines, HCC1937, JIMT-1, L56Br-C1, MCF-7, and SK-BR-3, and compared with the cytotoxicity in the breast-derived non-cancerous cell line MCF-10A using the MTT colorimetric assay. The inhibitory concentration 50 values (IC_{50}) were deduced from the obtained dose-response curves and are presented in Table 2. Interestingly, the cancer cell lines were more sensitive to all of the compounds than the normal-like MCF-10A cells. No obvious patterns related to the breast cancer cell line subgroup (*vide supra*) was found, and all compounds possessed activity. Compound **1** was found to be the most active in all of the cell lines with IC_{50} values ranging from 2.0 to 6.2 μM in the cancer cell lines, while compounds **3** and **16** exhibited the lowest activity. **16** is an unsaturated ester and differs in that respect from the other compounds, but the lower activity of **3** compared to the similar compounds was unexpected. Costunolide (**1**) differs from the eudesmane sesquiterpenes by having the unsaturated lactone fused with a macrocyclic system instead of a cyclohexane ring, and the tension of the lactone ring is likely to be lower in **1**. This would render **1** less reactive and possibly more selective. Indeed, **1** together with the two guaianes **12** and **14**, shows a slightly higher selectivity for the cancer cells compared to the eudesmanes. The difference in the activities of **7** and **8** may depend on the higher lipophilicity of **8**, facilitating its absorption into the cells. In **11** and **14**, the presence of a second Michael acceptor function may influence the activity. It is difficult to speculate from these data, but a trend is that the MCF-7 cells seem slightly less affected than the other cancer cell lines. MCF-7 has a normal wild type *p53* gene, which the MCF-10A cells also have, while the others have a mutated *p53*. Thus, MCF-7 and MCF-10A cells may share a property of being blocked in the G_1 phase of the cell cycle, which has a protective function, while the other cancer cell lines do not.

Previous investigations of sesquiterpenoid α-methylene-γ-lactones have indicated that the cytotoxic and antitumor activities are related to their ability to react as Michael acceptors [7]. We therefore added methyl cysteine to the exocyclic methylene group of **9** to give **21**, and compared its cytotoxicity with the natural products'. As can be seen from the results in Table 2, **21** is significantly less potent, however, it is not devoid of activity and its cytotoxicity towards the normal-like MCF-10A cells is similar to that of the α-methylene-γ-lactones. This may depend on the reversibility of Michael additions, by which **21** slowly can eliminate methyl cysteine and regenerate **9** during the assay conditions [7].

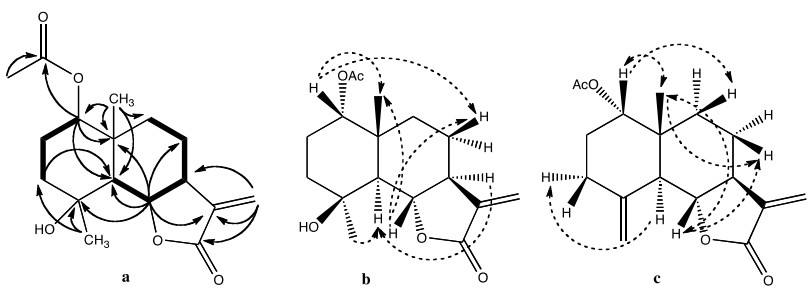


Fig. 2. (a) Important HMBC (arrow) and ^1H-^1H COSY (bold) correlations for **3**
(b) and (c) Key NOESY correlations for **3** and **8**, respectively

In conclusion, the molecular causes for the lower cytotoxicity of the compounds (especially compound **1**) in the normal-like breast epithelial MCF-10A compared to the breast cancer cells lines need to be further exploited and may find clinical use by showing less off-target cytotoxicity.

Tab. 1. ^{1}H and ^{13}C NMR data of compounds **3** (C_6D_6) and **8** ($CDCl_3$)

Position	3		8	
	^{1}H (*J*, Hz)	^{13}C	^{1}H (*J*, Hz)	^{13}C
1	4.67 br dd (2, 2)	77.2	4.68 br dd (2, 3)	76.4
2β	1.99 dddd (13, 13, 4.5, 2.2)	23.0	1.86 dddd (13.9, 13.4, 5.1, 2.7)	27.1
2α	1.53 m		1.81 m	
3β	1.15 m	36.2*	2.20 ddd (13.5, 5.1, 1.9)	30.5
3α	1.53 m		2.32 ddd (13.5, 13.4, 5.4)	
4	–	71.0	–	143.2
5	1.57 d (11.2)	50.5	2.79 d (11.0)	48.7
6	3.70 dd (11, 11)	79.8	4.00 dd (11, 11)	79.8
7	1.76 m	50.7	2.55 ddddd (12, 11, 3, 3, 3.4)	49.3
8β	1.26 m	21.4	1.61 dddd (12, 12, 11, 3.4)	21.2
8α	1.03 m		2.08 m	
9β	0.84 m	36.2*	1.42 ddd (13.0, 2.8, 3.2)	33.1
9α	1.24 br ddd (14, 14, 4)		1.72 ddd (13.0, 13.0, 4.0)	
10	–	40.7	–	41.7
11	–	140.4	–	139.1
12	–	170.0	–	170.5
13	5.94 d (3.2)	115.9	6.09 d (3.2)	117.0
	4.79 d (3.2)		5.40 d (3.2)	
14	0.92 s	20.0	0.92 s	18.0
15	1.29 s	32.9	5.00 br s	110.2
			4.87 br s	
1’	–	169.7	–	170.4
2’	1.74 s	21.1	2.12 s	21.2

δ (ppm) 500 MHz for ^{1}H and 125 MHz for ^{13}C; multiplicities; *J* values (Hz) in parentheses;
* May have interchanged.

Experimental

General

Optical rotations were measured with a Perkin Elmer Model 341 polarimeter. IR spectra were recorded with a Bruker Alpha-P FT-IR instrument in the ATR geometry with a diamond ATR unit. HR-ESI-MS was performed with a Waters Q-TOF Micro system spectrometer (using H_3PO_4 for calibration and as internal standard). 1D and 2D NMR spectra were recorded at room temperature on the Bruker Avance II 400 MHz and Bruker Avance 500 MHz spectrometers, operating at 400 and 500 MHz for ^{1}H and 100 and 125 MHz for ^{13}C, respectively. The chemical shifts (δ) are reported in ppm relative to solvent signals δ_H 7.16 and δ_C 128.39 for C_6D_6, and δ_H 7.26 and δ_C 77.00 for $CDCl_3$, while the coupling constants (*J*) are given in Hz. Vacuum liquid chromatography (VLC) separations

were carried out on the Merck Silica gel 60G (Merck), while column chromatography (CC) was performed using the Silica gel 60 (230-400 mesh, Merck), silver nitrate-impregnated Silica gel 60 [30], and gel permeation on Sephadex LH-20 (GE Healthcare). TLC analyses were carried out using aluminium-backed silica gel 60 F_{254} (0,2 mm thickness, Merck). Chromatograms were visualized under a UV lamp at 254 nm then sprayed with vanillin and $KMnO_4/K_2CO_3$/NaOH solution followed by heating. Preparative TLC (PTLC) was run on 20x20 cm glass-coated plates (1 mm thickness, Analtech) and doped TLC plates in MeCN:$AgNO_3$ solution [31]. HPLC was performed on the Agilent 1260 Infinity Quaternary LC system, equipped with a Standard Autosampler (G1329B), Thermostated Column Compartment (G1316A TCC), a Diode Array Detector VL (G1315D**),** and a semi-preparative column (XTerra RP18, 10x150 mm, 5 µm i.d, Waters).

Tab. 2. Cytotoxicity (IC_{50} in µM) of compounds **1**, **3**, **6–9**, **11**, **12**, **14**, **16**, and **21**

Comp.	HCC1937 µM	JIMT-1 µM	L56Br-C1 µM	MCF-7 µM	SK-BR-3 µM	MCF-10A µM
1	2.2/4.2[b]	6.2/6.3[b]	3.7/4.8[b]	5.3±1.4[c]	2.0±1.0[c]	20.0[a]
3	23.0[a]	18.0[a]	9.3[a]	27.0[a]	10.1[a]	38.0[a]
6	10.0[a]	8.5/12.0[b]	11.0[a]	16.4±7.1[c]	5.2±1.6[c]	17.0[a]
7	12.0/18.5[b]	12.0/14.0[b]	9.0/10.1[b]	23.0/30.0[b]	7.6/6.4[b]	22.0[a]
8	3.2/7.5[b]	6.3/7.0[b]	8.4/12.0[b]	11.0±1.7[c]	4.7±1.1[c]	24.0[a]
9	8.1±1.8[c]	6.9/7.2[b]	12.3±5.5[c]	9.7±0.9[c]	3.1±0.6[c]	17.0[a]
11	4.8/7.8[b]	10.0/13.0[b]	3.6[a]	10.1/18.0[b]	4.2±2.1[c]	24.0/21.0[b]
12	6.0/7.8[b]	7.1/10.1[b]	6.0/10.1[b]	7.1/8.3[b]	4.2±1.1[c]	17.0/38.0[b]
14	3.0/5.2[b]	7.5/8.1[b]	5.8/10.0[b]	3.3/4.3	2.5±0.4[c]	20.0/23.0[b]
16	24.0[a]	13.0/23.0[b]	16.0[a]	27.4±17.2[c]	9.9±3.0[c]	29.0[a]
21	100.0[a]	>100	73.0[a]	>100	51.0/40.0[b]	41.0[a]

Values from: [a] one dose-response curve, [b] two dose-response curves, [c] three or more dose-response curves.

Plant Material

The aerial parts of *Kaunia lasiophthalma* (Griseb) R.M. King and H. Robinson were collected on September 5th, 2009, near Independencia, Cochabamba, Bolivia, at coordinates 17º11.10' S 66º43.58' W and an elevation of 2943 m, during the flowering period. This aromatic herbaceous plant can grow up to 4 m in height and bears white-purple flowers. Lic. Modesto Zárate did the authentication, and voucher specimens have been deposited at Herbario Forestal Martín Cárdenas, Cochabamba (accession number MZ-3948).

Extraction and Isolation

The air-dried and ground leaves (788.5 g) and flowers (1064.0 g) were extracted separately by maceration with 95% EtOH for 24 hours, two times at room temperature. After filtration, the combined extracts were concentrated under reduced pressure and the following crude extracts were obtained: 90.0 g from leaves and 91.0 g from flowers.

Leaves

The crude organic extract (90.0 g) was suspended in a mixture of H_2O:MeOH (9:1, v/v,

500 ml) and partitioned between hexane (four times, 1:1, v/v), CH_2Cl_2 (two times, 1:1, v/v), and EtOAc (one time, 1:1, v/v). After evaporation of the solvent, the extracts weighed 22.68, 33.83, and 7.24 g, respectively. Subjection of the CH_2Cl_2 (18.7 g) to VLC (PE:EtOAc 1:0 to 0:1) gave four major fractions (A-D) based on TLC analyses. Purification of fraction B (10.8 g), using open CC (PE:CH_2Cl_2 1:0 to 0:1; CH_2Cl_2:EtOAc 1:1) yielded nine fractions (B1-B9). Fraction B5 (579.6 mg) was chromatographed by flash CC (heptane:Me_2CO 98:2 to 88:12) and combined into 13 fractions according to its TLC profile (B5.1-B5.13). Stigmasterol (52.4 mg) and **1** (35.6 mg) were obtained pure from B5.1 and B5.3, respectively. Further purification of B5.6 (80.0 mg) by CC (heptane:Et_2O 100:0 to 95:5) and Sephadex LH-20 (MeOH) afforded **10** (6.3 mg) and **8** (32.9 mg). Fraction B7 (2.64 g) was applied on the Sephadex LH-20 CC (MeOH) to give six fractions (B7.1-B7.6). B.7.4 (631.0 mg) and B8 (948.6 mg) were submitted to chromatography on the Sephadex LH-20 CC (MeOH) to give **16** (46.1 mg) after recrystallization in MeOH. Fraction B7.5 (316.0 mg) and B7.7 (153.7 mg) were sequentially purified by the Sephadex LH-20 (MeOH), CC (PE:EtOAc 1:0 to 8:2), and PTLC (PE:EtOAc 6:4) giving **3** (1.7 mg) and **7** (18.9 mg), respectively. Fraction B9 (956.8 mg) was fractionated by CC (heptane:Me_2CO 9:1) affording nine fractions (B9.1-B9.9). Each individual fraction B9.3 (139.4 mg) and B9.4 (470.0 mg) were further purified by the Sephadex LH-20 CC (MeOH) followed by CC (PE:EtOAc 1:0 to 8:2) to obtain **6** (10.0 mg) and **9** (80.0 mg). Compound **17** (59.0 mg) was recrystallized in CH_2Cl_2:MeOH from B9.6 (120.0 mg). Fraction C (589.0 mg) was separated by the Sephadex LH-20 CC (MeOH) to yield seven fractions (C1-C7). A mixture 1:1 of compounds **18** and **19** (26.0 mg) were obtained from C7 (330.0 mg).

Flowers

The flower extract (75.8 g) was partitioned as described above to yield the corresponding hexane (30.19 g), CH_2Cl_2 (26.52 g), and EtOAc (2.13 g) fractions, respectively. The CH_2Cl_2 fraction (24.0 g) was subjected to VLC (PE:EtOAc 1:0 to 0:1) and combined according to its TLC profile into nine fractions (A-H). Fraction B (1.23 g), containing mostly **1,** was dissolved in heptane:MeOH, filtered, concentrated, and recrystallized from heptane:Me_2CO to afford pure **1** (318.0 mg). Fraction C (633.0 mg) was subjected to VLC (PE:EtOAc 1:0 to 0:1) to yield 11 fractions (C1-C11). C3 (360.0 mg) was dissolved in PE and centrifuged to obtain **10** (8.0 mg) as a white powder. The liquid residue was chromatographed by CC (PE:Et_2O 1:0 to 2:3) giving eight fractions (C3.1–C3.8). Compound **8** (4.5 mg) was obtained from C3.5 (29.0 mg) by recrystallization from hexane:CH_2Cl_2 and **13** (2.7 mg) from C3.8 (17.8 mg) by HPLC (MeOH:H_2O 3:7, t_R=13.5 min, 240 nm, 1 ml/min). Subjection of fraction D (1.78 g) to VLC (PE:Et_2O 1:0 to 0:1) afforded six fractions (D1-D6). Compound **12** (173.0 mg) was purified from D4 (390.0 mg) and D5 (386.0 mg) by recrystallization from heptane:Me_2CO. The liquid residue (232.0 mg) was subjected to flash CC (heptane:CH_2Cl_2:Me_2CO 10:10:1) to produce **7** (23.3 mg). D6 (470.0 mg) was applied to the Sephadex LH-20 CC (MeOH) to give **14** (36.0 mg). E (270.0 mg) was fractionated by VLC (heptane:Me_2CO 1:0 to 5:1) to give seven fractions (E1-E7). E3 (151.0 mg) and E4 (642.0 mg) were combined and separated by flash CC (Silica gel doped with 10% $AgNO_3$ w/w, PE:Et_2O:Me_2CO 5:5:1) to yield **9** (96.0 mg) and **6** (32.0 mg) as white needles. Sequential purification of E5 (1.02 g) was achieved by the Sephadex LH-20 ($CHCl_3$:MeOH 1:1) and CC (heptane:CH_2Cl_2:Me_2CO 2:17:1) to yield **15** (1.1 mg), **14** (16.0 mg), **9** (37.0 mg), and **7** (10.0 mg). Fraction F (1.53 g) was applied to VLC (hexane:Et_2O 20:1, 1:3; hexane:Me_2CO 99:1, 80:20) yielding 12 fractions (F1-F12). The CH_2Cl_2 soluble part of F4 (194.0 mg) was purified by VLC (CH_2Cl_2:Me_2CO 1:0 to 4:1)

giving three fractions (F4.1-F4.3). Each fraction was further purified to afford **11** (10.0 mg), **2** (2.9 mg), and **16** (4.7 mg), respectively. Repeated purification of G (950.0 mg) by VLC (CH_2Cl_2:Me_2CO 98:2 to 0:1) and flash CC (CH_2Cl_2:Me_2CO 9:1) afforded **4** (10.1 mg) by recrystallization from heptane:Me_2CO. Fraction I (4.9 mg) was subjected to CC (CH_2Cl_2:Me_2CO 6:4 to 0:1) yielding seven fractions (I1-I7). Sequential purification of I3 (1.06 g) by the Sephadex LH-20 ($CHCl_3$:MeOH 1:1) and CC (CH_2Cl_2:Me_2CO 6:4 to 0:1) produced **5** (9.7 mg) and **20** (2.5 mg).

(+)-(3a*S**,5a*R**,6*S**,9*S**,9a*S**,9b*S**)-9-Hydroxy-5a,9-dimethyl-3-methylidene-2-oxododecahydronaphtho[1,2-*b*]furan-6-yl acetate
[(+)-*rel*-(1α,6α)-1-(Acetyloxy)-4-hydroxy-6,12-epoxyeudesm-11(13)-en-12-one, **3**]

Colourless gum; $[\alpha]_D^{25}$ +70.8 (*c* 0.4, $CHCl_3$); IR ν_{max}(cm^{-1}) 3473, 2933, 2865, 2359, 2342, 1764, 1730, 1375, 1246, 1197, 1134, 1018, 966; ^1H (500 MHz, C_6D_6), and ^{13}C (125 MHz, C_6D_6) see Table 1; HR–ESI-MS m/z 309.1722 $[M + H]^+$ (calcd. for $C_{17}H_{25}O_5$ 309.1702).

(3a*S**,5a*R**,6*S**,9a*S**,9b*S**)-5a-Methyl-3,9-dimethylidene-2-oxododecahydronaphtho[1,2-b]furan-6-yl acetate
[(+)-*rel*-(1α,6α)-1-(Acetyloxy)-6,12-epoxyeudesma-4(14),11(13)-dien-12-one, **8**]

Colourless oil; $[\alpha]_D^{25}$ +170.4 (*c* 0.3, $CHCl_3$); IR ν_{max}(cm^{-1}) 2942, 1769, 1726, 1372, 1240, 1174, 1121, 1039, 1016, 969. ^1H (500 MHz, $CDCl_3$), and ^{13}C (125 MHz, $CDCl_3$) see Table 1; HR–ESI-MS *m/z* 291.1613 $[M + H]^+$ (calcd. for $C_{17}H_{22}O_4$ 291.1596).

Preparation of Compound 21

10.0 mg (0.04 mmol) of **9** and 8.3 mg (0.048 mmol) methyl-L-cysteine ester hydrochloride were dissolved in methanol (1 mL) and the mixture was heated at 60°C and stirred. After 48 h, 1.6 mg (0.0093 mmol) methyl-L-cysteine ester was added and the reaction mixture was left for another 24 h. Then, the solvent was evaporated under reduce pressure and the dry residue dissolved in H_2O (3 mL), and washed with $CHCl_3$ (3 x 1.5 mL). The organic phase dried in vacuo yielded 15.5 mg of a yellow residue, which was purified by the Sephadex LH-20 ($CHCl_3$:MeOH 1:1) to give 8.0 mg (0.021 mmol) of **21** as yellow oil. ^1H NMR (400 MHz, CD_3OD): δ 5.35 (m, 1H), 4.12 (t, *J* = 11.0 Hz, 1H), 3.75 (s, 3H), 3.69 (m, 1H), 3.59 (m, 1H), 3.00 (m, 1H), 2.94 (m, 1H), 2.88 (m, 1H), 2.84 (m, 1H), 2.76 (m, 1H), 2.28 (m, 1H), 1.98 (m, 1H), 1.96 (m, 1H), 1.95 (m, 2H), 1.76 (s, 3H), 1.66 (m, 2H), 1.26 (m, 1H), 0.89 (s, 3H). ^{13}C (100 MHz, CD_3OD): δ 179.5 (C-12), 175.4 (C-3´), 134.7 (C-4), 122.7 (C-3), 82.8 (C-6), 75.9 (C-1), 55.0 (C-2´), 52.7 (C-4´), 52.2 (C-5), 51.8 (C-11), 47.0 (C-7), 41.9 (C-10), 38.6 (C-1´), 35.9 (C-9), 33.5 (C-2), 31.6 (C-13), 24.1 (C-8), 23.6 (C-15), 11.3 (C-14). HR-ESI-MS *m/z* 406.1703 [M + Na]+ (calcd. for $C_{19}H_{29}NO_5SNa$ 406.1664).

Biological Assay

Cell Culture

The L56Br-C1 cell line was established at the Department of Oncology, Clinical Sciences, Lund University, Sweden [32]. The JIMT-1 cell line was purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and the MCF-7, SK-BR-3, HCC1937, and MCF-10A cells were obtained from the American Tissue Type Culture Collection (Manassas, VA, USA). The cell lines were cultured as monolayers at 37°C in a humidified incubator with 5% CO_2 in air. The MCF-10A cells have a population

doubling time of 15 hours, the JIMT-1 cells 24 hours, and the other four cell lines around 35 hours.

The L56Br-C1, MCF-7, SK-BR-3, and HCC1937 cells were cultured as described by Holst et al [33]. JIMT-1 cells were cultured in DMEM/Ham's F12 medium supplemented with 10% fetal calf serum, non-essential amino acids (1 mM), insulin (10 µg/ml), penicillin (100 U/ml), and streptomycin (100 µg/ml). MCF-10A cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, non-essential amino acids (1 mM), insulin (10 µg/ml), epidermal growth factor (20 ng/ml), cholera toxin (50 ng/ml), hydrocortisone (250 ng/ml), penicillin (100 U/ml), and streptomycin (100 µg/ml). The MCF-10A and JIMT-1 cell lines were sub-cultured twice a week, while the L56Br-C1, MCF-7, SK-BR-3, and HCC1937 cells were sub-cultured once a week with an additional change of growth medium once a week.

Dose-Response Assay

Stock solutions (10 or 100 µM) of the compounds were made in 100% DMSO. These were further diluted in PBS to obtain the correct concentrations used for the MTT assay. Appropriate DMSO controls were used. In general, the highest DMSO concentration was 0.1%, however, when treating with 100 µM and starting from a 10 µM stock in 100% DMSO, a final concentration of 1% DMSO was used as the control.

The MTT assay was performed as previously described [34]. Briefly, the cells were trypsinized and counted in a hemocytometer. Aliquots of 180 µl cell suspensions containing 3000 (MCF-10A) and 6000 (MCF-7, SK-BR-3, JIMT-1, L56Br-C1, and HCC1937) cells were seeded in the wells of 96-well plates. Compounds were added 24 hours after seeding to allow the attachment of cells. A concentration range between 0.1 to 100 µM was used in the MTT assays and appropriate DMSO controls. At 72 h of drug treatment, 20 µl of MTT solution (5 mg/ml MTT in PBS) was added to each well and the 96-well plates were returned to the CO_2 incubator for 1 hour. The MTT-containing medium was removed. The blue formazan product formed by the reduction in live attached cells was dissolved by adding 100 µl of 100% DMSO per well after removal of the MTT-containing medium. The plates were swirled gently at room temperature for 10 minutes to dissolve the precipitate. Absorbance was monitored at 540 nm in a Labsystems iEMS Reader MF (Labsystems Oy, Helsinki, Finland) using the DeltaSoft II v.4.14 software (Biometallics Inc., Princeton, NJ, USA). Dose-response curves were drawn based on the % of the control in Excel. The IC_{50} was deduced from the curves.

Acknowledgement

The financial support from The Swedish International Development Agency (SIDA) in a bilateral collaboration between Lund University (Sweden) and San Simon University (Bolivia) is fully acknowledged. We also wish to thank to Dr. Karl-Erik Bergquist for his helpful comments on the manuscript.

Supporting Information

Supporting information containing the ^{1}H-NMR and ^{13}C-NMR spectra of compounds **3** and **8** are available in the online version (Type: PDF, Size: ca. 0.1 MB): http://dx.doi.org/10.3797/scipharm.1310-18.

Authors' Statement

The authors declare no conflict of interest.

References

[1] King RM, Robinson H.
Studies in the Eupatorieae (Asteraceae). CCII. A new genus Kaunia.
Phytologia. 1980; 47: 257–260.

[2] Ulloa C, Jørgensen PM.
Arboles y arbustos de los Andes del Ecuador.
AAU Reports. 1993; 30: 1–264.

[3] Bohlmann F, Trinks C, Jakupovic J, King RM, Robinson H.
A further guaianolide from Kaunia arbuscularis.
Planta Med. 1984; 50: 284.
http://dx.doi.org/10.1055/s-2007-969708

[4] Bohlmann F, Kramp W, Gupta RK, King RM, Robinson H.
Four guaianolides and other constituents from three Kaunia Species.
Phytochemistry. 1981; 20: 2375–2378.
http://dx.doi.org/10.1016/S0031-9422(00)82669-2

[5] Rücker G, Schenkel EP, Mayer R, Hausen BM.
Allergenic sesquiterpene lactones from Eupatorium cannabinum L. and Kaunia rufescens (Lund ex de Candolle).
Natural Toxins. 1997; 5: 223–227.
http://dx.doi.org/10.1002/(SICI)1522-7189(1997)5:6<223::AID-NT1>3.0.CO;2-R

[6] De Gutierrez A, Sigstad EE, Catalan CA, Gutierrez AB, Herz W.
Guaianolides from Kaunia lasiophthalma.
Phytochemistry. 1990; 29: 1219–1225.
http://dx.doi.org/10.1016/0031-9422(90)85432-F

[7] Johansson MH.
Reversible Michael additions: covalent inhibitors and prodrugs.
Mini Rev Med Chem. 2012; 12: 1330–1344.
http://dx.doi.org/10.2174/13895575112091330

[8] Sørlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Lønning PE, Børresen-Dale AL.
Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications.
Proc Natl Acad Sci U S A. 2001; 98: 10869–10874.
http://dx.doi.org/10.1073/pnas.191367098

[9] Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, Deng S, Johnsen H, Pesich R, Geisler S, Demeter J, Perou CM, Lønning PE, Brown PO, Børresen-Dale AL, Botstein D.
Repeated observation of breast tumor subtypes in independent gene expression data sets.
Proc Natl Acad Sci U S A. 2003; 100: 8418–8423.
http://dx.doi.org/10.1073/pnas.0932692100

[10] Perou CM, Jeffrey SS, van de Rijn M, Rees CA, Eisen MB, Ross DT, Pergamenschikov A, Williams CF, Zhu SX, Lee JC, Lashkari D, Shalon D, Brown PO, Botstein D.
Distinctive gene expression patterns in human mammary epithelial cells and breast cancers.
Proc Natl Acad Sci U S A. 1999; 96: 9212–9217.
http://dx.doi.org/10.1073/pnas.96.16.9212

[11] Jönsson G, Staaf J, Olsson E, Heidenblad M, Vallon-Christersson J, Osoegawa K, de Jong P, Oredsson S, Ringnér M, Höglund M, Borg A.
High-resolution genomic profiles of breast cancer cell lines assessed by tiling BAC array comparative genomic hybridization.
Genes Chromosomes Cancer. 2007; 46: 543–558.
http://dx.doi.org/10.1002/gcc.20438

[12] Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lønning PE, Børresen-Dale AL, Brown PO, Botstein D.
Molecular portraits of human breast tumours.
Nature. 2000; 406:747–752.
http://dx.doi.org/10.1038/35021093

[13] Hu Z, Fan C, Oh DS, Marron JS, He X, Qaqish BF, Livasy C, Carey LA, Reynolds E, Dressler L, Nobel A, Parker J, Ewend MG, Sawyer LR, Wu J, Liu Y, Nanda R, Tretiakova M, Ruiz Orrico A, Dreher D, Palazzo JP, Perreard L, Nelson E, Mone M, Hansen H, Mullins M, Quackenbush JF, Ellis MJ, Olopade OI, Bernard PS, Perou CM.
The molecular portraits of breast tumors are conserved across microarray platforms.
BMC Genomics, 2006; 7: 96.
http://dx.doi.org/10.1186/1471-2164-7-96

[14] El-Feraly FS, Benigni DA.
Sesquiterpene lactones of Laurus nobilis leaves.
J Nat Prod. 1980; 43: 527–531.
http://dx.doi.org/10.1021/np50010a016

[15] Kisiel W.
A new germacranolide from Zinnia haageana.
Phytochemistry. 1978; 17: 1059–1060.
http://dx.doi.org/10.1016/S0031-9422(00)94281-X

[16] Sanz JF, Marco JA.
NMR studies of tatridin A and some related sesquiterpene lactones from Tanacetum vulgare.
J Nat Prod. 1991; 54: 91–96.
http://dx.doi.org/10.1021/np50074a040

[17] Julianti E, Jang K, Lee S, Lee D, Mar W, Oh K-B, Shin J.
Sesquiterpenes from the leaves of Laurus nobilis L.
Phytochemistry. 2012; 80: 70–76.
http://dx.doi.org/10.1016/j.phytochem.2012.05.013

[18] Appendino G, Belliardo F, Nano GM, Stefenelli S.
Sesquiterpene lactones from Artemisia genipi Weber: isolation and determination in plant material and in liqueurs.
J Agric Food Chem. 1982; 30: 518–521.
http://dx.doi.org/10.1021/jf00111a027

[19] Bohlmann F, Jakupovic J, Ahmed M, Schuster A.
Sesquiterpene lactones and other constituents from Schistostephium species.
Phytochemistry. 1983; 22: 1623–1636.
http://dx.doi.org/10.1016/0031-9422(83)80100-9

[20] Romo de Vivar A, Jimenez H.
Structure of santamarine, a new sesquiterpene lactone.
Tetrahedron. 1965; 21: 1741–1745.
http://dx.doi.org/10.1016/S0040-4020(01)98644-2

[21] Romo J, Romo de Vivar A, Trevino R, Joseph-Nathan P, Diaz E.
Constituents of Artemisia and Chrysanthemum species. Structures of chrysartemins A and B.
Phytochemistry. 1970; 9: 1615–1621.
http://dx.doi.org/10.1016/S0031-9422(00)85285-1

[22] Mata R, Delgado G, Romo de Vivar A.
Sesquiterpene lactones of Artemisia mexicana var. Angustifolia.
Phytochemistry. 1984; 23: 1665–1668.
http://dx.doi.org/10.1016/S0031-9422(00)83464-0

[23] Bhadane NR, Kelsey RG, Shafizadeh F.
Sesquiterpene lactones of Artemisia tridentata ssp. Vaseyana.
Phytochemistry. 1975; 14: 2084–2085.
http://dx.doi.org/10.1016/0031-9422(75)83134-7

[24] Matsuda H, Uemura T, Shimoda H, Ninomiya K, Yoshikawa M, Kawahara Y.
Anti-alcoholism active constituents from Laurel and Linden.
Tennen Yuki Kagobutsu Toronkai Koen Yoshishu, 2000; 42: 469–474.

[25] Herz W, Sumi Y.
Constituents of Ambrosia and related species. III. Constituents of Ambrosia hispida.
J Org Chem. 1964; 29: 3438–3439.
http://dx.doi.org/10.1021/jo01034a526

[26] Rao MM, Kingston DG, Spittler TD.
Flavonoids from Flourensia cernua.
Phytochemistry. 1970; 9: 227–228.
http://dx.doi.org/10.1016/S0031-9422(00)86634-0

[27] Ulubelen A, Miski M, Neuman P, Mabry TJ.
Flavonoids of Salvia tomentosa (Labiatae).
J Nat Prod. 1979; 42: 261–263.
http://dx.doi.org/10.1021/np50003a002

[28] Kupchan SM, Sigel C, Hemingway RJ, Knox JR, Udayamurthy MS.
Tumor inhibitors—XXXIII : Cytotoxic flavones from eupatorium species.
Tetrahedron. 1969; 25: 1603–1615.
http://dx.doi.org/10.1016/S0040-4020(01)82733-2

[29] Jakupovic J, Aal MA, Eid F, El-Dahmy S, Sarg T.
Further glaucolides and other sesquiterpene lactones from Brocchia cinerea.
Phytochemistry. 1988; 27: 2219–2224.
http://dx.doi.org/10.1016/0031-9422(88)80130-4

[30] Li T-S, Li J-T, LiH-Z.
Modified and convenient preparation of silica impregnated with silver nitrate and its application to the separation of steroids and triterpenes.
J Chromatogr. 1995; 715: 372–375.
http://dx.doi.org/10.1016/0021-9673(95)00619-X

[31] Ruprah PK, Cros J-P, Pease JE, Whittingham W, Williams J.
Studies towards the total synthesis of cycloaraneosene and ophiobolin M: a general strategy for the construction of the 5-8 bicyclic ring system.
Eur J Org Chem. 2002; 2002: 3145–3152.
http://dx.doi.org/10.1002/1099-0690(200209)2002:18<3145::AID-EJOC3145>3.0.CO;2-3

[32] Johannsson OT, Staff S, Vallon-Christersson J, Kytöla S, Gudjonsson T, Rennstam K, Hedenfalk IA, Adeyinka A, Kjellén E, Wennerberg J, Baldetorp B, Petersen OW, Olsson H, Oredsson S, Isola J, Borg A.
Characterization of a novel breast carcinoma xenograft and cell line derived from a BRCA1 germ-line mutation carrier.
Lab Invest. 2003; 83: 387–396.
http://www.ncbi.nlm.nih.gov/pubmed/12649339

[33] Holst CM, Staaf J, Joensson G, Hegardt C, Oredsson SM.
Molecular mechanisms underlying N1, N11-diethylnorspermine-induced apoptosis in a human breast cancer cell line.
Anticancer Drugs. 2008; 19: 871–883.
http://dx.doi.org/10.1097/CAD.0b013e32830f902b

[34] Holst CM, Oredsson SM.
Comparison of three cytotoxicity tests in the evaluation of the cytotoxicity of a spermine analogue on human breast cancer cell lines.
Toxicol In Vitro, 2005; 19: 379–387.
http://dx.doi.org/10.1016/j.tiv.2004.10.005

Paper II

Phytochemistry Letters 8 (2014) 105–108

Contents lists available at ScienceDirect

Phytochemistry Letters

journal homepage: www.elsevier.com/locate/phytol

A novel cytotoxic terpenoid from the flowers of *Kaunia lasiophthalma* Griseb

Eliana M. Maldonado [a,b], Daniel Svensson [a], Stina M. Oredsson [c], Olov Sterner [a,*]

[a] Centre for Analysis and Synthesis, Lund University, POB 124, 221 00 Lund, Sweden
[b] Centro de Tecnología Agroindustrial, San Simón University, Cochabamba, Bolivia
[c] Department of Biology, Lund University, Sölvegatan 35A, 223 62 Lund, Sweden

ARTICLE INFO

Article history:
Received 21 January 2014
Received in revised form 18 February 2014
Accepted 21 February 2014
Available online 11 March 2014

Keywords:
Terpenoid
Structure elucidation
In vitro anticancer
Cytotoxicity

ABSTRACT

A phytochemical study of the flowers of *Kaunia lasiophthalma* G. (Asteraceae) yielded a novel triterpene (**1**) together with several known sesquiterpenoids. The structure of the new compound was elucidated by analysis of the spectroscopic data. The biosynthetic origin of **1** is proposed to be a dimerization of an oxidized derivative (**3**) of the germacrane sesquiterpene costunolide (**2**), also present in the flowers. The anticancer activity of **1** in the five breast cancer cell lines HCC1937, JIMT-1, L56Br-C1, MCF-7 and SK-BR-3 was compared with the cytotoxicity in the normal-like breast epithelial cell line MCF-10A. **1** exhibited high cytotoxicity in all investigated cancer cell lines with IC_{50} values ranging from 0.67 to 7.0 μM, although it is lacking selectivity as the MCF-10A cells were almost as sensitive.

1. Introduction

Several species classified in the genus *Kaunia* have been shown to produce biologically active sesquiterpene lactones. *Kaunia lasiophthalma* Griseb (*syn*. *Eupatorium lasiophthalmum* G.) is an aromatic herbaceous plant that can grow up to 4 m in height bearing white-purple flowers, it is found in the Andean Mountains of Cochabamba, Bolivia, where it is locally known by the common name of "Tuwi" and used to treat inflammations and headaches (Lic. Modesto Zárate, personal communication). As has been shown previously, this species is a rich source of sesquiterpenes lactones (De Gutierrez et al., 1990), and in the course of our continuing investigations of medicinal plants from the native flora of Bolivia we were interested of investigating also the metabolites of the flowers. An extract of the flowers unexpectedly yielded a novel triterpene with a new carbon skeleton having two α-methylene-γ-lactone moieties, together with fourteen known compounds. In this report we wish to describe the isolation and structure elucidation of the new metabolite, as well as a comparison of its anticancer and cytotoxic activities when assayed in five breast cancer cell lines and one normal-like breast epithelial cell line.

2. Results and discussion

Compound **1**, for which we propose the trivial name kaunial, was isolated as a clear oil from an ethanol extract of the flowers of *K. lasiophthalma* G., as described in the Experimental part (see Fig. 1 for the structure and numbering of **1**). An HR-ESI-MS experiment revealed a molecular ion at *m/z* 533.2531, consistent with the elemental composition $C_{30}H_{38}O_7$ as the exact mass of $M+Na^+$, $C_{30}H_{38}O_7Na$, is 533.2515. As a confirmation, the ^1H NMR spectrum contained signals integrating for 38 protons while the ^{13}C NMR spectrum (see Table 1 for ^1H and ^{13}C NMR data in C_6D_6) displayed signals for 30 carbons. Kaunial (**1**) consequently has 12 unsaturations. The presence of an α,β-unsaturated-γ-lactone, a ketone, and a double bond was indicated by the absorption bands in the IR spectrum at 1758, 1713, and 1681 cm^{-1}, respectively. HMQC experiments suggested that **1** has two ketone functions (C-10, C-10′), one aldehyde (C-1′), two lactone carbonyl (C-12, C-12′), five nonprotonated sp^2 carbons (C-4, C-11, C-2′, C-4′ and C-11′), three monoprotonated sp^2 carbons (C-1, C-5 and C-5′), two diprotonated sp^2 carbons (C-13 and C-13′), two oxygenated methines (C-6 and C-6′), two non-oxygenated sp^3 methines (C-7 and C-7′), seven sp^3 methylenes (C-2, C-3, C-8, C–9, C-3′, C-8′ and C-9′), and four methyls (C-14, C-15, C-14′ and C-15′). This accounts for all 30 carbons as well as 10 of the 12 degrees of unsaturation, indicating the presence of two rings in **1**. As can be seen from the NMR data (Table 1), kaunial (**1**) appears to be composed of two similar

* Corresponding author. Tel.: +46 705306649.
E-mail address: olov.sterner@chem.lu.se (O. Sterner).

http://dx.doi.org/10.1016/j.phytol.2014.02.012
1874-3900/

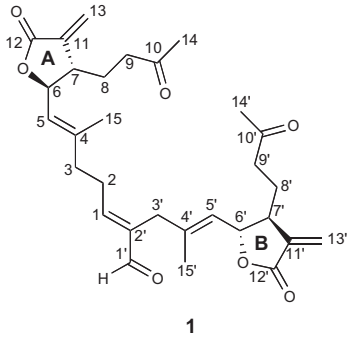


Fig. 1. The structure and numbering of kaunial (**1**).

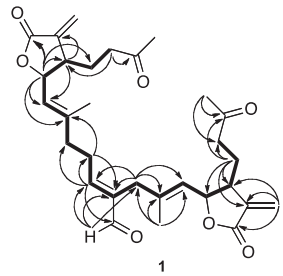


Fig. 2. Pertinent HMBC (arrow) and $^{1}H–^{1}H$ COSY (bold) correlations for **1**.

subunits, and only the signals for the first three positions (C-1/C-1′, C-2/C-2′, C-3/C-3′) differ substantially. As shall be discussed later, we believe that **1** is biosynthesized from two identical C_{15} units obtained from the germacrane lactone costunolide (**2**). All the NMR signals were assigned unambiguously to each of the two subunits A and B, for which the germacrane numbering system has been retained.

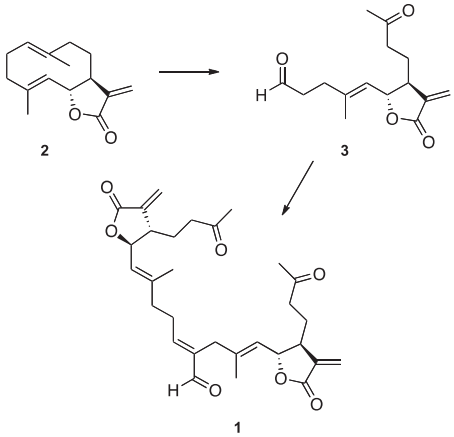
The presence of an exomethylene-γ-lactone ring in subunit A was established by the HMBC correlations from 13-H_2 to C-7, C–11 and C-12, the HMBC correlations from 6-H to C-7 and C-12 as well as the COSY correlations from 7-H to 6-H and 13-H_2 (see Fig. 2 and Table 1 for COSY and HMBC correlations). The corresponding correlations were observed in subunit B, showing that this also has an exomethylene-γ-lactone ring. With two lactones all unsaturations are accounted for. The connection between the two lactones is demonstrated by COSY and HMBC correlations. 6-H also gives a COSY correlation to 5-H and HMBC correlations to C-5 and C-4, which together with the HMBC correlations from 15-H_3 to C-3, C-4 and C-5 establish the link up to C-3. COSY correlations from 3-H_2 to 2-H_2 and HMBC correlations to C-1, C-2, C-4 and C-5 extend the chain, and HMBC correlations from 1′-H to C-1, C-2′ and C-3′ as well as from 15′-H_3 to C-3′, C-4′ and C-5′ brings us up to the second lactone. The link between C-5′ and C-6′ is demonstrated by the COSY correlation between the two protons, as well as by HMBC correlations from 6′-H to C-4′ and C-5′. Both C-7 and C-7′ are substituted by a 3-oxo-butyl group, as demonstrated by the HMBC correlations from 14-H_3 (14′-H_3) to C-9 and C-10 (C-9′ and C-10′), as well as the COSY correlations 9-H_2/8-H_2/7-H (9′-H_2/8′-H_2/7′-H) and the HMBC correlations from 9-H_2 (9′-H_2) to C-7, C-8 and C-10 (C-7′, C-8′ and C-10′).

The configurations of the carbon-carbon double bonds of **1** were determined by an NOESY experiment, in which important correlations were observed between H-1′ and H-1, indicating an *E* configuration for this double bond, and between 3-H_2 and 5-H as well as 3′-H_2 and 5′-H suggesting that both double bonds also have *E* configuration. 6-H correlates with 8-H_2, 9–H_2 and 15-H_3, while 6′-H correlates with 8′-H_2, 9′-H_2 and 15′-H_3, whereas 7-H and 7′-H correlate with 5-H and 5′-H, respectively. This suggests that 6-H/7-H and 6′-H/7′-H are *trans*, which is to be expected if costunolide (**2**) is the biosynthetic precursor. This is also supported by the $^{1}H–^{1}H$ coupling constants between 6-H and 7-H (6.3 Hz) as well as 6′-H and 7′-H (6.5 Hz) which are characteristic in *seco*-lactones with a trans-fused lactone ring (Bohlmann and Zdero, 1982; Herz and Sharma, 1975, 1976; Huneck et al., 1986). Our data do not reveal the absolute configuration of kaunial (**1**), but assuming that costunolide (**2**), whose absolute configuration is known (Bovill et al., 1976), is the starting material, it should be as displayed in Fig. 2.

We suggest that the biogenesis of kaunial (**1**) involves an oxidative cleavage of the 1,10 bond of the germacrane costunolide (**2**), which is a major metabolite produced by this plant, to produce the intermediate aldehyde **3** that subsequently is dimerized by an aldol condensation to yield kaunial (**1**), as shown in Scheme 1. The intermediate **3** was not observed in the extract, nor any other derivatives of **3** except **1**.

Table 1
^{1}H and ^{13}C NMR data of compound **1** in C_6D_6 (400 MHz; δ in ppm).

S-unit A	^{13}C		^{1}H (*J*, Hz)	HMBC (H to C)	S-unit B	^{13}C		^{1}H (*J*, Hz)	HMBC (H to C)
1	155.5	CH	6.02 t (7.3)	2, 3, 1′, 3′	1′	193.8	CHO	9.26 s	1, 2′, 3
2	27.5	CH_2	2.04 dt (7.8, 7.3)	1, 3, 4, 2′	2′	140.1	qC	–	–
3	38.0	CH_2	1.79 t (7.8)	1, 2, 4, 5, 15	3′	33.4	CH_2	2.86 ABq (15.2)	1, 1′, 2′, 4′, 5′, 15′
4	142.2	qC	–	–	4′	141.0	qC	–	–
5	124.8	CH	5.10 dq (8.8, 1.3)	3, 7, 15	5′	124.1	CH	4.93 dq (8.9, 1.2)	3′, 15′
6	79.2	CH	4.50 dd (8.8, 6.3)	4, 5, 7, 8, 12	6′	79.4	CH	4.40 dd (8.9, 6.5)	4′, 5′, 7′, 8′, 12′
7	45.7	CH	2.52 m	5, 11	7′	45.6	CH	2.33 m	5′, 11′
8	25.8	CH_2	1.62 m 1.53 m	6, 7, 9, 10, 11	8′	25.7	CH_2	1.37 dt (8.2, 7.2)	6′, 7′, 9′, 10′, 11′
9	39.8	CH_2	1.94 t (7.2)	7, 8, 10	9′	39.7	CH_2	1.87 t (7.2)	7′, 8′, 10′
10	205.8	qC	–	–	10′	205.7	qC	–	–
11	140.3	qC	–	–	11′	140.1	qC	–	–
12	169.8	qC	–	–	12′	169.8	qC	–	–
13	121.0	CH_2	6.21 d (2.8) 5.09 d (2.1)	7, 11, 12 7, 12	13′	121.1	CH_2	6.16 d (2.8) 5.07 d (2.3)	7′, 11′, 12′ 12′, 7′
14	29.8	CH_3	1.68 s	9, 10	14′	29.9	CH_3	1.74 s	9′, 10′
15	17.1	CH_3	1.52 d (1.2)	3, 4, 5	15′	17.5	CH_3	1.50 d (1.2)	3′, 4′, 5′



Scheme 1. Proposed biogenetic route for **1**.

A number of known compounds were also isolated and identified by comparison of their spectroscopic data with those previously reported in the literature. These were costunolide (El-Feraly and Benigni, 1980), haageanolide (Kisiel, 1978), 1β-hydroxyarbusculin A (Sanz and Marco, 1991), 4-*epi*-1β-hydroxyarbusculin A (Julianti et al., 2012), reynosin (Appendino et al., 1982), 1-*epi*-reynosin (Bohlmann et al., 1983), santamarin (Romo De Vivar and Jimenez, 1965), santamarin acetate (Romo et al., 1970), 11,13-didehydrovulgarin (Mata et al., 1984), 2β-acetoxy-3α,4α-epoxy-3,4-dihydrokauniolide, 3α,4α-epoxy-2α-isobutyryloxykauniolide, 3-chlorodehydroleucodin (De Gutierrez et al., 1990), dehydroleucodin (Bhadane et al., 1975), and baynol C (Matsuda et al., 2000).

Previous studies of α-β-unsaturated-γ-lactones, which react as Michael acceptors, have shown that this group of compounds posses a broad range of biological activities (Ghantous et al., 2010; Picman, 1986), of which the anticancer activity is particularly interesting to us. Therefore, the dose response activity of kaunial (**1**) was assayed in the five breast cancer cell lines HCC1937, JIMT-1, L56Br-C1, MCF-7 and SK-BR-3 as well as in one normal-like breast-derived cell line, MCF-10A, using a MTT colorimetric assay (see Table 2). **1** exhibited high cytotoxicity in all investigated cancer cell lines with IC_{50} values ranging from 0.67 to 7.0 μM. However, it is also toxic to the normal cell line MCF-10A, although somewhat less, and the lack of selectivity may depend on the presence of two exomethylene-γ-lactone. Michael acceptors have, in general, been considered to be toxic and useless as potential drug candidates. However, recent studies have shown that certain Michael acceptors may be selective and several are currently in clinical trials as drug candidates (Johansson, 2012).

Table 2
Cytotoxicity (IC_{50} in μM) of compound **1**.[a]

Cell line	IC_{50} (μM)[b]
HCC1937	2.4/2.5
JIMT-1	4.3/4.8
L56Br-C1	0.67/1.3
MCF-7	4.7/7.0
SK-BR-3	1.1/2.1
MCF-10A	4.7/7.0

[a] The IC_{50} evaluated in dose response curves obtained by an MTT assay.
[b] Two experiments.

3. Experimental

3.1. General

1D and 2D NMR spectra were recorded at room temperature on a Bruker Avance II 400 MHz and a Bruker Avance 500 MHz spectrometers, operating at 400 and 500 MHz for ^1H and 100 and 125 MHz for ^{13}C, respectively. The chemical shifts (δ) are reported in ppm relative to solvent signals δ_H 7.16 and δ_C 128.39 for C_6D_6, while the coupling constants (J) are given in Hz. Vacuum liquid chromatography (VLC) separations were carried out on Merck Silica gel 60G, while column chromatography (CC) were performed using Silica gel 60 (230–400 mesh, Merck), silver nitrate impregnated silica gel 60 (Li et al., 1995) and gel permeation on Sephadex LH-20 (GE Healthcare). Chromatograms were visualized under a UV lamp at 254 nm then spraying with vanillin followed by heating. Preparative thin layer chromatography (PTLC) was run on 20 cm × 20 cm glass-coated plates (1 mm thickness, Analtech). HPLC was carried out on a The Agilent 1260 Infinity Quaternary LC systems, equipped with a Standard Autosampler (G1329B), Thermostated Column Compartment (G1316A TCC), a Diode Array Detector VL (G1315D) and a semi-preparative column (XTerra RP18, 10 mm × 150 mm, 5 μm i.d., Waters). HR-ESI-MS was performed in a Waters Q-TOF Micro system spectrometer (using H_3PO_4 for calibration and as internal standard). Optical rotations were measured with a Perkin Elmer Model 341 polarimeter. IR spectra were determined with a Bruker Alpha-P FT-IR instrument in the ATR geometry with a diamond ATR unit.

3.2. Plant material

The flowers of *K. lasiophthalma* (Griseb) were collected in September of 2009, near Independencia, Cochabamba, Bolivia, during the flowering period. The plant was taxonomically identified by Lic. Modesto Zárate and voucher specimens, MZ-3948, have been deposited at Herbario Forestal Martín Cárdenas, Cochabamba, Bolivia.

3.3. Extraction and isolation

The air-dried and ground flowers (1064.0 g) were extracted by maceration with 95% EtOH, two times at room temperature. After filtration, the solvent was removed under reduced pressure to yield a green-dark residue (91.0 g). 75.8 g of this extract was suspended in a mixture of H_2O:MeOH (8:2, v/v, 500 mL) and partitioned between hexane (three times, 2:1, v/v), CH_2Cl_2 (three times, 2:1, v/v) and EtOAc (one time, 1:1, v/v) to yield three fractions weighing 30.2, 26.5, and 2.1 g, respectively. The CH_2Cl_2 fraction was subjected to vacuum liquid chromatography on silica gel, using step gradient of PE:EtOAc (1:0–0:1) to yield eight fractions (A–I). Fraction B (1.23 g) yielded costunolide (318.0 mg), fraction C (633.0 mg) afforded the reynosin (8.0 mg) and 3α,4α-epoxy-2α-isobutyryloxykauniolide (2.7 mg), fraction D (1.78 g) gave 2β-acetoxy-3α,4α-epoxy-3,4-dihydrokauniolide (173.0 mg), 1-*epi*-reynosin (23.3 mg), and dehydroleucodin (36.0 mg). From E (270.0 mg) santamarin (96.0 mg), reynosin (32.0 mg), 3-clorode-hydroleucodin (1.1 mg), dehydroleucodin (16.0 mg), santamarin acetate (37.0 mg), and 1-*epi*-reynosin (10.0 mg) were obtained. Fraction F (1.53 g) afforded 11,13-didehydrovulgarin (10.0 mg), haageanolide (2.9 mg), and baynol C (4.7 mg). Purification of G (950.0 mg) gave 1β-hydroxyarbusculin A (10.1 mg), while fraction I (4.9 g) produced 4-epi-1β-hydroxyarbusculin A (9.7 mg). Sequential purification of the more polar fraction H by VLC eluted

with a gradient of PE:EtOAc (1:0–0:1) followed by preparative TLC ($C_6H_5CH_3$:Me_2CO 7:3) gave **1** (9.8 mg) as a clear oil.

3.4. Kaunial (*1*)

Clear oil, $[\alpha]_D^{25}$ = +76.1 (*c* = 0.014, $CHCl_3$); IR ν_{max} (cm^{-1}) 1758, 1713, 1618; ^1H (400 MHz, C_6D_6) and ^{13}C (100 MHz, C_6D_6) see Table 1; HRMS-ESI-MS *m/z* 533.2531 [M+Na^+] (calcd for $C_{30}H_{38}O_7Na$, 533.2515).

3.5. Biological assays

One normal-like cell line (MCF-10A) and five breast-cancer cell lines (MCF-7, SK-BR-3, JIMT-1, L56Br-C1 and HCC1937) were used for the dose response assay. The breast cancer cell lines represent breast cancer sub-groups luminal A (MCF-7), luminal B (SK-BR-3), basal-like (L56Br-C1 and HCC1937), and HER2 positive (JIMT-1). The MCF-10A cells have a population doubling time of 15 h, the JIMT-1 cells 24 h and the other four cell lines around 35 h. The MTT assay was performed as previously described (Holst and Oredsson, 2005). Briefly, cells were trypsinized and counted in a hemocytometer. Aliquots of 180 μl cell suspension containing 3000 (MCF-10A), 6000 (MCF-7, SK-BR-3, JIMT-1, L56Br-C1 and HCC1937) cells were seeded in the wells of 96-well plates. Compound **1** was added 24 h after seeding to allow attachment of cells. A concentration range between 0.1 and 100 μM was used in the MTT assays. Appropriate DMSO controls were used. At 72 h of drug treatment, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (5 mg/ml MTT in PBS) was added to each well and the 96 well plates were returned to the CO_2 incubator for 1 h. The MTT containing medium was removed. The blue formazan product formed by reduction in live attached cells was dissolved by adding 100 μl of 100% DMSO per well. The plates were swirled gently at room temperature for 10 min to dissolve the precipitate. The absorbance was monitored at 540 nm in a Labsystems iEMS Reader MF (Labsystems Oy, Helsinki, Finland) using the DeltaSoft II v.4.14 software (Biometallics Inc., Princeton, NJ, USA). Dose response curves were drawn based on % of control in Excel. The inhibitory concentration 50 (IC_{50}) was deduced from the curves.

Acknowledgments

The financial support of the Swedish International Development Agency (SIDA) in a frame of collaboration agreement between Lund University (Sweden) and San Simón University (Bolivia) is gratefully acknowledged. We also thank to Dr. Antonio Vilaseca, Ing. J. Luis Balderrama and Ing. Nelson Hinojosa for useful comments, and Lic. Modesto Zárate for identifying the plant.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2014.02.012.

References

Appendino, G., Belliardo, F., Nano, G.M., Stefenelli, S., 1982. Sesquiterpene lactones from *Artemisia genipi* Weber: isolation and determination in plant material and in liqueurs. J. Agric. Food Chem. 30, 518–521.
Bhadane, N.R., Kelsey, R.G., Shafizadeh, F., 1975. Sesquiterpene lactones of *Artemisia tridentata* ssp. Vaseyana. Phytochemistry 14, 2084–2085.
Bohlmann, F., Jakupovic, J., Ahmed, M., Schuster, A., 1983. Sesquiterpene lactones and other constituents from *Schistostephium* species. Phytochemistry 22, 1623–1636.
Bohlmann, F., Zdero, C., 1982. Sesquiterpene lactones and other constituents from *Tanacetum parthenium*. Phytochemistry 21, 2543–2549.
Bovill, M.J., Cox, P.J., Cradwick, P.D., Guy, M.H.P., Sim, G.A., White, D.N.J., 1976. Sesquiterpenoids. XXV: X-ray crystal structure analysis of costunolide. Acta Crystallogr. B B32, 3203–3209.
De Gutierrez, A.N., Sigstad, E.E., Catalan, C.A.N., Gutierrez, A.B., Herz, W., 1990. Guaianolides from *Kaunia lasiophthalma*. Phytochemistry 29, 1219–1225.
El-Feraly, F.S., Benigni, D.A., 1980. Sesquiterpene lactones of *Laurus nobilis* leaves. J. Nat. Prod. 43, 527–531.
Ghantous, A., Gali-Muhtasib, H., Vuorela, H., Saliba, N.A., Darwiche, N., 2010. What made sesquiterpene lactones reach cancer clinical trials? Drug Discov. Today 15, 668–678.
Herz, W., Sharma, R.P., 1975. Seco-germacradienolide from *Liatris pycnostachya*. J. Org. Chem. 40, 392.
Herz, W., Sharma, R.P., 1976. Constituents of *Liatris* species. XI: Pycnolide, a seco-germacradienolide from *Liatris pycnostachya*, and other antitumor constituents of *Liatris* species. J. Org. Chem. 41, 1248–1253.
Holst, C.M., Oredsson, S.M., 2005. Comparison of three cytotoxicity tests in the evaluation of the cytotoxicity of a spermine analogue on human breast cancer cell lines. Toxicol. In Vitro 19, 379–387.
Huneck, S., Zdero, C., Bohlmann, F., 1986. Seco-guaianolides and other constituents from *Artemisia* species. Phytochemistry 25, 883–889.
Johansson, M.H., 2012. Reversible Michael additions: covalent inhibitors and prodrugs. Mini-Rev. Med. Chem. 12, 1330–1344.
Julianti, E., Jang, K.H., Lee, S., Lee, D., Mar, W., Oh, K.-B., Shin, J., 2012. Sesquiterpenes from the leaves of *Laurus nobilis* L. Phytochemistry 80, 70–76.
Kisiel, W., 1978. A new germacranolide from *Zinnia haageana*. Phytochemistry 17, 1059–1060.
Li, T.-S., Li, J.-T., Li, H.-Z., 1995. Modified and convenient preparation of silica impregnated with silver nitrate and its application to the separation of steroids and triterpenes. J. Chromatogr. A 715, 372–375.
Mata, R., Delgado, G., de Vivar, A.R., 1984. Sesquiterpene lactones of *Artemisia mexicana* var. Angustifolia. Phytochemistry 23, 1665–1668.
Matsuda, H., Uemura, T., Shimoda, H., Ninomiya, K., Yoshikawa, M., Kawahara, Y., 2000. Anti-alcoholism active constituents from Laurel and Linden. Tennen Yuki Kagobutsu Toronkai Koen Yoshishu 42nd, pp. 469–474.
Picman, A.K., 1986. Biological activities of sesquiterpene lactones. Biochem. Syst. Ecol. 14, 255–281.
Romo De Vivar, A., Jimenez, H., 1965. Structure of santamarine, a new sesquiterpene lactone. Tetrahedron 21, 1741–1745.
Romo, J., Romo De Vivar, A., Trevino, R., Joseph-Nathan, P., Diaz, E., 1970. Constituents of *Artemisia* and *Chrysanthemum* species. Structures of chrysartemins A and B. Phytochemistry 9, 1615–1621.
Sanz, J.F., Marco, J.A., 1991. NMR studies of tatridin A and some related sesquiterpene lactones from *Tanacetum vulgare*. J. Nat. Prod. 54, 591–596.

Paper III

Antileshmanial metabolites from *Trixis antimenorrhoea*

Eliana M. Maldonado,[1,2] Efrain Salamanca,[3] Alberto Giménez,[3] Gloria Saavedra,[2] and Olov Sterner[1,*]

[1] Centre for Analysis and Synthesis, Lund University, P.O.Box 124, 221 00, Lund, Sweden
[2] Centro de Tecnología Agroindustrial, San Simón University, Cochabamba, Bolivia
[3] Instituto de Investigaciones Fármaco Bioquímicas (IIFB), San Andrés University, La Paz, Bolivia

ABSTRACT

An ethanol extract of the aerial parts of *Trixis antimenorrhoea* was found to possess potent activity towards *Leishmania brasiliensis*. Two new metabolites, the nortrixane **1** and the trixanolide **2**, and eleven known were isolated and characterized. The antileishmanial activity against the promastigote form of *L. amazonensis* and *L. brasiliensis* was assayed *in vitro*, and **2** exhibited potent activity with IC_{50} values below 1 µg/mL towards both strains. The structures of the isolated compounds were elucidated by NMR spectroscopy and high-resolution mass spectrometry, and the absolute configuration of **2** was determined using Mosher's ester protocol.

Key words: *Trixis antimenorrhoea*, Nortrixane, Trixanolide, Antileishmanial activity, Structural elucidation

1. Introduction

Leishmaniasis is a neglected tropical disease, caused by different species of *Leishmania* protozoa. It is transmitted to humans and animals by the bite of an insect vector, generally females of the *Phlebotomus* sandfly mosquitos. Infections can give a number of different clinical manifestations, from simple cutaneous leishmaniasis (CL) to mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL). Especially the latter can be fatal in untreated cases (Mishra et al., 2009; WHO, 2013). Despite of the efforts to eradicate, or at least to control and prevent the illness, leishmaniasis remains a threat that affects the poorest people living in tropical and subtropical regions, and 1-2 millions new cases are reported every year (WHO, 2013). The drugs available for the treatment of leishmaniasis today have limited usefulness, as they give toxic side effects, are prone to development of drug resistance, require hospitalization during the administration period, or simply are too expensive. There is consequently an urgent need to find new antiparasitic agents, for example from natural sources such as plants, to complement the existing drugs.

Trixis antimenorrhoea belongs to the Asteraceae family, and is a South American shrub with white flowers largely distributed from the Andean to Brazilian regions (Katinas, 1996). The aromatic flowers of this species are used in traditional medicine to treat eye-inflammation and excessive uterine bleeding, known as menorrhagia, from which its name is derived (Granato et al., 2013; Katinas, 1996). Previous phytochemical studies of *T. antimenorrhoea* describe the isolation of trixanolides (Bohlmann et al., 1981) which are the characteristic secondary metabolites of the genus (Bohlmann and Zdero, 1979; De Riscala et al., 1989; De Riscala et al., 1988). Although a great number of compounds with this particular skeleton have been isolated from the *Trixis* species, we have not been able to find any information in regard to their biological activities. In a previous screening for novel antiparasitic agents, the ethanolic extract of *T. antimenorrhoea* showed moderate activity towards *L. braziliensis* and was therefore selected for a detailed investigation. In this paper, we report the isolation and structural elucidation of two new natural products (**1** and **2**), together with eleven known compounds (**3-13**) although they are reported from this plant for the first time. Their structures were elucidated by extensive spectroscopic analysis and the comparison with data previously reported in the literature. With the aim to identify the bioactive agents, compounds isolated in sufficient amounts were assayed for their leishmanicidal activity.

2. Results and discussion

The ethanol extract of the aerial parts of *Trixis antimenorrhoea* was subjected to liquid-liquid partition to produce hexane, CH_2Cl_2, EtOAc and MeOH fractions that were assayed *in vitro* against promastigote forms of two strains of Leishmania (*L. amazonensis* and *L. braziliensis)*. The hexane and CH_2Cl_2 fractions exhibited high activity especially towards *L. braz*iliensis with IC_{50} values of 32 and 16 μg/mL, respectively, and were therefore investigated in detail. After repeated chromatography on silica gel and Sephadex LH-20, the two new natural products **1** and **2** were isolated together with eleven previously reported compounds (**3**-**13)**.

The known compounds were identified by comparison of their spectral data with those reported in the literature as: voleneol (**3**) (Bohlmann et al., 1983a), lupeol-3-acetate (**4**) and lupenol (**5**) (Bohlmann et al., 1979), lupan-3-one (**6**) (Gupta et al., 1992), nevadesin (**7**) (Farkas et al., 1966), sudachitin (**8**) (Salmenkallio et al., 1982), 3,5-dihydroxy-6,7,8,3',4' pentamethoxyflavone (**9**) (Lichius et al., 1994), 5-hydroxy-3,3',4',6,7,8-hexamethoxy-flavone (**10**) (Simonsen et al., 2003), 5-hydroxy-3,4',6,7,8-pentamethoxy-flavone (**11**) (Gupta et al., 1973), chrysoeriol (**12**) (Kyriakopoulou et al., 2001) and luteolin (**13**) (Takeda and Fatope, 1988) (See Fig. 1 for structures)

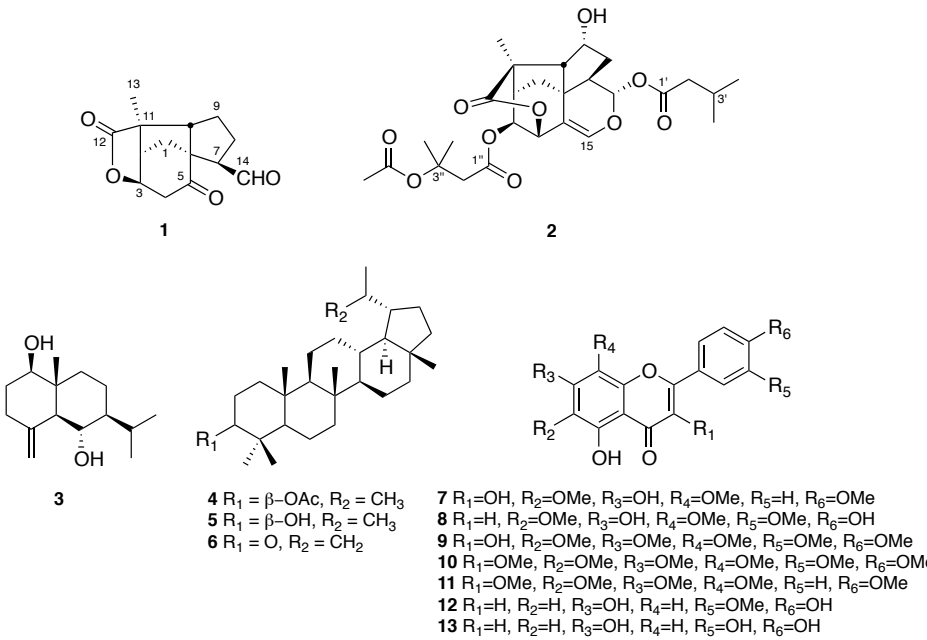


Fig. 1. Structures of the compounds isolated from *T. antimenorrhoea* (**1**-**13**)

1D NMR data for compounds **1** and **2** are presented in Table 1. Compound **1** was obtained as colourless oil. The HR-ESI-MS showed a molecular ion at *m/z* 249.1151 $[M + H]^+$, indicating that the elemental composition of **1** is $C_{14}H_{16}O_4$. Absorption bands in the IR spectrum at 1764, 1714 cm^{-1} clearly indicated the presence of aldehyde and ketone functionalities. Indeed, the ^1H NMR of **1** showed signals for 16 protons while the ^{13}C NMR spectrum displayed signals for 14 carbons confirming the suggested elemental composition and that **1** has seven unsaturations. The ^{13}C and HMQC spectra showed that **1** has an aldehyde (C-14), a ketone (C-5), a carbonyl (C-12), four sp^3 methylenes (C-1, C-4, C-8, C-9), three sp^3 methines (C-2, C-7, C-10), one oxygenated methine (C-3), two quaternary sp^3 carbons (C-6, C-11) and one methyl (C-13). These functionalities account for three of the seven unsaturations, **1** is consequently tetracyclic.

Table 1
^{1}H and ^{13}C NMR data of compounds **1** and **2** in $CDCl_3$ (500, MHz; δ in ppm).

	1			**2**	
	^{13}C	^{1}H (*J*, Hz)		^{13}C	^{1}H (*J*, Hz)
1β	36.1	2.05 dd (13.4, 4.0)	1β	42.0	3.15 dd (11.8, 7.0)
1α		2.36 d (13.4)	1α		1.43 bdd (11.8, 1.7)
2	50.2	3.06 dd (8.9, 4.0)	2	49.1	2.38 ddd (7.0, 1.7, 1.9)
3	73.2	4.98 ddd (1.1, 8.9, 6.2)	3	74.4	4.86 dd (2.8, 1.9)
4β	42.1	2.80 dd (18.5, 1.1)	4	76.6	4.94 dd (2.8, 2.8)
4α		2.72 dd (18.5, 6.2)			
5	207.7	-	5	117.6	-
6	70.3	-	6	53.9	-
7	56.9	2.51 m	7	40.5	2.59 ddd (8.9, 8.6, 6.5)
8β	30.6	2.20 m	8β	40.9	1.86 ddd (15.1, 6.0, 6.0)
8α		2.17 m	8α		2.22 ddd (15.1, 8.6, 8.6)
9β	27.1	2.02 m	9	73.1	4.41 m
9α		1.71 dddd (6.3, 5.9, 5.9, 11.8)			
10	57.8	2.47 m	10	61.9	2.18 d (7)
11	47.5	-	11	49.8	-
12	180.1	-	12	176.8	-
13	17.4	1.32 s	13	20.0	1.56 s
14	201.1	9.8 d (1.8)	14	95.4	5.45 d (8.9)
			15	140.2	6.51 s
			1’	171.5	-
			2’	43.2	2.28 dd (14.8, 7.2)
					2.26 dd (14.8, 7.2)
			3’	25.6	2.13 sept (6.8)
			4’	22.3	0.99 d (6.8)
			5’	22.3	0.99 d (6.8)
			1’’	169.3	-
			2’’	44.4	2.95 d (14.5)
					2.85 d (14.5)
			3’’	79.2	-
			4’’	26.6	1.54 s
			5’’	26.5	1.54 s
			1-Ac	170.5	-
			2-Ac	22.3	2.01 s
			OH		1.68 d (3.2)

The complete structure of **1** was assembled by analysis of the COSY and HMBC spectra. Two clear COSY spin systems were observed, from 1-H_2 via 2-H and 3-H to 4-H_2, and from 7-H via 8-H_2 and 9-H_2 to 10-H. The six-membered ring was established by the HMBC correlations from 1-H_2 as well as 4-H_2 to C-2, C-3, C-5 and C-6, and the HMBC correlations from 2-H to C-6 and from 3-H to C-5. 1-H_2 also gives HMBC correlations to C-10 and C-11, and together with the HMBC correlations from 10-H to C-1 and C-5 as well as from 13-H_3 to C-2, C-10, C-11 and C-12 the first five-membered ring is established. There are no exchangeable protons in **1**, and a HMBC correlation from the oxygenated methine proton 3-H to C-12 shows that C-3 and C-12 are part of a γ-lactone. The final ring involves the second spin system, and is established by the HMBC correlations from both 9-H_2 and 8-H_2 to C-6, C-7, and C-10, from 7-H to C-1, C-6, C-8 and C-14, as well as from 14-H to C-7 and C-8. **1** is suggested to be a norsesquiterpene based on the trixane skeleton with a keto function at C-5, for which we propose the name nortrixial. Nortrixanes in which C-14 is lost have been reported (Bohlmann et al., 1983b; Ybarra et al., 1992), but as far as we are aware this is the first nortrixane lacking C-15. The relative configuration of nortrixial (**1**) was determined based on the correlations observed in the NOESY spectrum. The critical correlations are between 13-H_3

and 1α-H, 2-H, 3-H, 4α-H, 7-H, 8α-H and 9α-H, as well as between 8β-H and 9β-H and 10-H. The systematic name of nortrixial (**1**) is consequently (1*R**,3a*S**,3b*R**,6*R**,6a**S*,7a*R**)-3a-methy1-3,8-dioxooctabydro-1*H*-1,6a-ethanopentaleno[1,2-*c*]furan-6-carbaldehyde. Unfortunately, the amounts of **1** isolated in this investigation were insufficient for both assaying its biological activity and for the measuring of its optical activity.

Compound **2**, obtained as colourless needles, was by HR-ESI-MS experiments suggested to have the elemental composition $C_{27}H_{36}O_{10}$, and 10 unsaturations. Its IR spectrum showed bands at 3506, 1732, and 1661 cm^{-1} which were indicative of hydroxyl and carbonyl groups. As a confirmation, the ^{1}H spectrum displayed signals integrating for 36 protons. The ^{13}C and HMQC NMR spectra indicated that compound **2** contained four ester carbonyls (C-12, C-1', C1'', 1-Ac), one sp^2 nonsubstituted carbon (C-5), one sp^2 monosubstituted and oxygenated carbon (C-15), four sp^3-oxygenated carbons (C-3, C-4, C-9, C-14), four sp^3 methylenes (C-1, C-8, C-2', C-2''), four sp^3 methines (C-2, C-7, C-10, C-3'), three quaternary carbons (C-6, C-11, C-3'') and six methyls groups (C-13, C-4', C-5', C-4'', C-5'', 2-Ac). These functionalities account for five of the ten unsaturations showing that **2** is pentacyclic. Correlations from HMQC, HMBC and COSY experiments were used to establish the complete structure of **2**. Starting with the trixane core, the corresponding COSY spin systems from 1-H_2 via 2-H and 3-H to 4-H as well as from 7-H via 8-H_2 and 9-H to 10-H was observed also in **2**, although the latter is extended by the strong coupling between 14-H and 7-H. Again we observe HMBC correlations from both 1-H_2 and 4-H to C-2, C-3, C-5, and C-6 as well as from 2-H to C-6, and from 3-H to C-5, establishing the central six-membered ring. The bond between C-6 and C-10 was demonstrated by the HMBC correlations from 10-H to C-1, C-2, C-5 and C-6, as well as from 1-H_2 to C-10 and C-11 and from 13-H_3 to C-2, C-10, C-11 and C-12 establish the left five-membered ring. HMBC correlations from both 7-H and 8-H_2 to C-6, C-9 and C-10 as well as between 9-H and C-6, C-7 and C-10 close the right five-membered ring. The only exchangeable proton is obviously a hydroxyl group at C-9, based on the chemical shift of C-9/9-H (73.1/4.41) and the HMBC correlations were observed from 9-OH to C-8, C-9 and C-10. The forth ring is a six-membered lactone, involving also C-4 as demonstrated by the HMBC correlations from 4-H and not 3-H to the carbonyl carbon C-12. The double bond proton 15-H gives HMBC correlations to C-4, C-5, C-6, and C-14, while 14-H gives HMBC correlations to C-6, C-7, C-8, and C-15, suggesting an oxygen link between C-14 and C-15 to produce the fifth and final ring of **2**. The nature of C-14 is in fact acetalic, as demonstrated by the chemical shifts of 14-H/C-14 as well as the large direct heteronuclear coupling constant between 14-H and C-14 (160 Hz), and the HMBC correlation from 14-H to C-1' shows that is acylated by 3-methylbutanoic acid. The remaining oxygenated carbon, C-3, is in a similar way acylated with 3-acetoxy-3-methylbutanoic acid, as shown by the HMBC correlation between 3-H and C-1''.

The relative stereochemistry of **2** was deduced based on correlations observed in the NOESY spectrum. 9-H gives NOESYcorrelations to 8β-H, 10-H and 14-H, confirming that the four protons are cofacial. Additional correlations were observed between 13-H_3 and 1α-H, 2-H, 3-H, 7-H, and 8α-H, as well as between 4-H and 15-H. Comparing the ^{1}H NMR data of compound **2** and 9α-hydroxy-3-β-isovaleryloxy-trixikingolide-14-(3'-acetoxy-isovalerate) (Bohlmann et al., 1979) show that the trixane core is identical but that the ester residues are linked to different positions.

The absolute configuration of **2** could be determined by application of the Mosher's ester protocol (Ward and Rhee, 1991) by acylating the free C-9 hydroxyl group with *S*- and *R*-α-methoxy-α-trifluoromethylphenylacetic acid. The difference in chemical shifts (Δδ = *S*-*R*) for the analogous pairs of protons in the *S*- and *R*-MTPA esters are shown in Table 2, and indicate that the absolute configuration of C-9 is *R*. Therefore, compound **2** was identified as 9α-hydroxy-3-β-acetoxy-3-methylbutanoate trixikingolide-14-(3'-methylbutanoate), or (3*S*,3a*R*,3a[1]*S*,5*R*,5a*R*,6*R*,7*R*,8*S*,9*R*)-5-hydroxy-6-methyl-3-((3-methylbutanoyl)oxy)-11-oxo-3a,4,5,5a,6,7,8,9-octahydro-3*H*-9,6-(epoxymethano)-3a[1],7-methanoazuleno[1,8-*cd*]pyran-8-yl 3-acetoxy-3-methylbutanoate.

Table 2
Selected ^{1}H NMR data for diagnostic of the *S*- and *R*-MTPA-methyl Mosher esters **14** and **15**.

^{1}H	**14** (*S*)	**15** (*R*)	Δ(*S*-*R*)
	ppm	ppm	ppm
13	0.885	1.140	-0.255
8α	2.344	2.321	+0.023
8β	2.046	2.208	+0.019
7	2.450	2.308	+0.142
14	5.458	5.433	+0.025

In our search for new antiparasitic agents, the compounds isolated in sufficient quantities (**2**, **4**-**8**, **10**, **11** and **13)** were tested using an *in vitro* antiprotozoal assay (Salamanca et al., 2008), and the results are presented in Table 3. Compound **2** was found to be the most active with IC_{50} values of 0.30 μg/mL for *L. amazonensis* and 0.96 μg/mL for *L. brasiliensis*. This is actually close to the activity of amphotericin B, used as positive control (IC_{50} 0.21 and 0.08 μg/mL, respectively). The potency of **2** is noteworthy for a compound with no apparent reactive functionalities. The triterpenes **4** and **6** were devoid of activity, while **5** having a hydroxyl group at C-3 showed an improved activity with an IC_{50} of 78.4 μg/mL for *L. amazonensis* and 96.5 μg/mL for *L. braziliensis.* Among the flavones, compound **8** exhibited an interesting selectivity towards *L. braziliensis,* with an IC_{50} value of 3.5 μg/mL while it lacked activity towards *L. amazonensis*. In addition, **10** exhibited significant activity towards both strains, and the higher selectivity towards *L. braziliensis* of compounds **8** and **10** compared to **7** and **11** could be associated with the presence of a second methoxy group at C-5' in ring B. The lack of activity of **13** is not in agreement with those reported in the literature (da Silva et al., 2012; Manjolin et al., 2013).

Table 3
In vitro leishmanicidal activity on promastigote form of *Leishmania ssp.*

Compounds	IC_{50} (μg/mL)[a]	
	L. amazonensis	*L. braziliensis*
EtOH extract	>50	33±4.0
Hexane fract.	>50	32 ±3.5
CH_2Cl_2 fract.	44 ±0.75	16 ±1.4
EtOAc fract	>50	>50
MeOH fract.	>100	>100
2	0.30 ±0.01	0.96 ±0.05
4	>100	>100
5	78 ±4.2	96 ±0.2
6	>100	>100
7	42 ±3.15	34 ±5.0
8	>100	3.5 ±1.0
10	19 ±0.9	5.8 ±0.32
11	>100	59 ±7.0
13	>100	>100
Control[b]	0.21 ±0.06	0.08 ±0.04

[a] Data are expressed as mean standard deviation of three determinations.
[b] Amphotericin B was used as positive control.

In conclusion, the ethanol extract of *Trixis antimenorrhoea* is a rich source of terpenoids and flavonoids, which are considered among the most interesting groups of natural products due to their potential as

antileishmanial agents. A new nortrixane (**1**) has been described and accounts are given on the biological activity of a sesquiterpenoid based on a trixane skeleton, compound **2.** The interesting structural and stereo- chemical features together with the potent *in vitro* leishmanicidal activity of **2** should encourage more extensive phytochemical investigations of this and related species to obtain additional trixikingonolide derivatives that could provide an understanding of the structural requirements for their biological activity.

3. Experimental

3.1. General

1D and 2D NMR spectra were recorded at room temperature on a Bruker Avance II 400 MHz and a Bruker Avance 500 MHz spectrometer, operating at 400.13 MHz and 500.20 MHz, respectively, for ^{1}H. The chemical shifts (δ) are reported in ppm relative to solvent signals δ_H 7.26 and δ_C 77.00 for $CDCl_3$, while the coupling constants (*J*) are given in Hz. HR-ESI-MS was performed in a Waters Q-TOF Micro system spectrometer (using H_3PO_4 for calibration and as internal standard). Optical rotations were measured with a Perkin Elmer Model 341 polarimeter. IR spectra were determined with a Bruker Alpha-P FT-IR instrument in the ATR geometry with a diamond ATR unit. Vacuum liquid chromatography (VLC) separations were carried out on Merck silica gel 60G (Merck), column chromatography (CC) was performed using silica gel 60 (230-400 mesh, Merck), and gel permeation on Sephadex LH-20 (GE-Healthcare) and TLC analyses were carried out using aluminum-backed silicagel 60 F254 (0.20 mm thickness plates, Merck). TLC chromatograms were visualized under a UV lamp at 254 nm then spraying with vanillin followed by heating.

3.2 Plant material

The aerial parts of *Trixis antimenorrohoea* (Schrank) Mart. ex Baker in Mart were collec*t*ed in Totora, Carrasco Province, Cochabamba, Bolivia, in March 2011. The plant was identified by Lic. Modesto Zarate. A voucher specimen (MZ 3742) is preserved at Herbario Nacional Forestal Martín Cárdenas, Cochabamba, Bolivia.

3.3 Extraction and isolation

The dried powdered aerial part of *Trixis antimenorrhoea* (1210.0 g) were extracted by maceration with 95 % EtOH for 3 days, two times at room temperature. After filtration, the solvent was removed under reduced pressure to yield a green-dark residue (85.6 g). Of this, 83.4 g was partitioned between hexane and 80 % MeOH, and the aqueous MeOH phase diluted with water to 60 % MeOH and extracted with CH_2Cl_2. Finally, the MeOH layer was further diluted to 50% MeOH and exhaustively extracted with EtOAc. After evaporation of the solvents, the yield of the hexane, CH_2Cl_2, EtOAc and MeOH/water (50%) fractions were 22.2, 15.8, 23.5 and 18.8 g, respectively. The hexane fraction (22.2 g) was fractioned by VLC on silica gel 60G eluted with a step-gradient of hexane:EtOAc (1:0 to 0:1) to give eight major fractions (A-H) based on their TLC profile. Using the same technique and solvent system as describe above, fraction B (9.5 g) was chromatographed and gave fourteen fractions (B1-B14). After repeated purification by flash CC on silica gel (Heptane:CH_2Cl_2 1:0 to 0:1) and recrystallization in MeOH , fraction B6 (4.9 g) yielded compounds **4** (18.0 mg) and **6** (10.0 mg). An aliquot (50 mg) of B12 (798.4 mg) containing mostly **5**, was purified by recrystallization from hexane:MeOH to afford pure **5** (9.9 mg**)** . Fraction D (1.5 g) was chromatographed by Sephadex LH-20 ($CHCl_3$:MeOH 1:1) to produce five fractions (D1-D5). Sequential purification of D5 (303.6 mg) by Sephadex LH-20 ($CHCl_3$:MeOH 1:1) followed by flash CC (heptane:EtOAc 1:0 to 8:2) gave **7** (1.8 mg), **10** (2.2 mg) and **11** (3.6 mg). The CH_2Cl_2 fraction (14.8 g) was subjected to VLC using a gradient of increasing polarity of hexane:EtOAc (1:0 to 0:1) to obtain nine major fractions that were combined according to their TLC profile. Fraction C (805.0 mg) was chromatographed on Sephadex LH-20 ($CHCl_3$:MeOH 1:1) to produce four fractions (C1-C4). Further purification of C4 (338.0 mg) by flash CC (heptane:Et_2O 1:0 to 7:3) gave **1** (0.5 mg). Fraction E (1.2 g) was separated by Sephadex LH-20 CC ($CHCl_3$:MeOH 1:1), which furnished eight fractions (E1-E8). Successive purifications of E3 (418.0 mg) using VLC (PE:Et_2O 3:1, 1% TFA) followed by flash CC (PE:Et_2O 3:1) afforded **3** (1.5 mg). Fraction E4

(213.0 mg) was filtrated over charcoal/silica gel (1:1 w/w) to give E4 (194 mg) free of chlorophylls, which was further purified by Sephadex LH-20 CC ($CHCl_3$:MeOH 1:1) and flash CC (CH_2Cl_2:Et_2O 88:12) to produce **2** (25.7 mg). Compound **9** (1.5 mg) was obtained from E5 (151.0 mg) after flash CC (CH_2Cl_2:Me_2CO 96:4 to 9:1). Fraction F (992.0 mg) and G (1.5 g) were submitted to CC over Sephadex LH-20 ($CHCl_3$:MeOH 1:1) yielding compounds **12** (4.6 mg) and **8** (4.0 mg) and **13** (3.5 mg), respectively.

3.4. (1*R**,3a*S**,3b*R**,6*R**,6a**S*,7a*R**)-3a-methy1-3,8-dioxooctabydro-1*H*-1,6a-ethanopentaleno[1,2-*c*]furan-6-carbaldehyde or nortrixial (**1**)

Colorless oil; IR ν_{max} (cm^{-1}) 2956, 2924, 2853, 1767, 1714, 1459, 1344, 1245, 1083, 1003; ^1H (500 MHz, $CDCl_3$) and ^{13}C (125MHz, $CDCl_3$) see Table 1; HR-ESI-MS *m/z* 249.1151 $[M + H]^+$ (calcd. for $C_{14}H_{17}O_4$ 249.1127).

3.5. 9α-hydroxy-3-β- acetoxy-3-methylbutanoate -trixikingolide-14-(3'-methylbutanoate) (**2**)

Colourless needle crystals; mp 131-134°C; $[\alpha_D^{20}]$ +14.6 (*c* 0.39, $CHCl_3$); *IR* ν_{max} (cm^{-1}) 3506, 2961, 2937, 2873, 1732, 1464, 1368, 1244, 1083, 1006, 918, 733; ^1H (500 MHz, $CDCl_3$) and ^{13}C (125MHz, $CDCl_3$) see Table 1; HR-ESI-MS *m/z* 521.2414 $[M + H]^+$ (calcd. for $C_{27}H_{37}O_{10}$ 521.2387).

3.6. Preparation of the *S*- and *R*-MTPA methyl esters (**14** and **15)**

To a stirred solution of compound **2** (5 mg, in 1 ml of $CDCl_3$) under nitrogen atmosphere, Et_3N (4 mg, 5 μL), DMAP (a small crystal, ca 1 mg) and *S*-(+)-MTPA-Cl (3 mg, 5 μL) were added at room temperature. The mixture was stirred for 24 h. The reaction mixture was dissolved in EtOAc (20 ml) and washed with 1 M HCl (10 ml), $NaHCO_3$ (10 ml) and brine (10 ml), filtered through phase separator and concentrated to yellow oil. This crude residue was purified by Silica-gel chromatography eluted with CH_2Cl_2:Et_2O 95:5 to yield 1.2 mg of **14**. Treatment with *R*-(-)-MTPA-Cl as described above yielded 0.7 mg of **15**.

14: ^1H ($CDCl_3$, 500 MHz) δ 1.21 (1α-H, m), 2.25 (1β-H, brdd, 12.4, 1.8 Hz), 2.08 (2-H, m), 4.77 (3-H, br s), 4.91 (4-H, dd, 2.7, 2.7 Hz), 2.30 (7-H, m), 2.32 (8α-H, m), 2.03 (8β-H, m), 5.27 (9-H, t, 5.6 Hz), 2.38 (10-H, brd, 4.7 Hz), 1.14 (13-H_3, s), 5.43 (14-H, d, 8.8 Hz), 6.51 (15-H, s), 2.92 (2'a-H, d, 14.8 Hz), 2.80 (2'b-H, d, 14.8 Hz), 1.99 (7'-H_3, s), 1.54 (4'-H_3, s and 5'-H_3, s), 2.27 (2''-H_2, dd, 7.2, 3.3 Hz), 2.12 (3''-H, m), 0.99 (4''-H_3, d, 6.7 Hz and 5''-H_3, d, 6.7 Hz), 3.48 (H_3, s, OMe).

15: ^1H ($CDCl_3$, 500 MHz) δ 1.43 (1α-H, brd, 6.7 Hz), 2.18 (1β-H, brd, 12.0 Hz), 2.72 (2-H, dd, 12.1, 7.0 Hz), 4.82 (3-H, brs), 4.93 (4-H, t, 2.7 Hz), 2.45 (7-H, m), 2.34 (8α-H, m), 2.05 (8β-H, m), 5.32 (9-H, t, 5.4 Hz), 2.39 (10-H, brd, 5.1 Hz), 0.88 (13-H_3, s), 5.46 (14-H, d, 9.0 Hz), 6.54 (15-H, s), 2.92 (2'-H, d, 14.6 Hz), 2.80 (2'-H, d, 14.6 Hz), 1.98 (7'-H_3, s), 1.50 (4'-H_3, s), 1.49 (5'-H_3, s), 2.27 (3''-H_2, m), 2.12 (3''-H, m), 0.98 (4''-H_3, d, 6.7 Hz and 5''-H_3, d, 6.7 Hz), 3.58 (H_3, s, OMe).

3.7 Leishmanicidal activity

The colorimetric method-XXT assay was performed as previously described by Salamanca et. al... Briefly, The activity was measured *in vitro* on cultures of the *Leishmania* parasite in the promastigote forms, of complex *L. amazonensis* (clon 1: Lma, MHOM/BR/76/LTB-012) and complex *L. braziliensis* (strand M2904 C192 RJA), established at IIFB, cultivated at 26 °C in Schneider medium (pH 6.8) supplemented with inactivated (by heating to 56 °C for 30 min) bovine calf serum (10 %). Parasites in logarithmic phase of growth, at a concentration of $1x10^6$ parasites/mL, were seeded in the wells of 96-well plates. Solutions of compounds to be assessed at concentration range of 0.09-100 μg/mL were added. DMSO (1%) and Amphotericin B (0,5 mg/mL) were used as negative and positive controls during the evaluations. All assays were performed in triplicate and the micro well plates were incubated for 72 hrs at 26 °C. After incubation, a solution of XTT (1 mg/mL) in PBS (pH 7.0 at 37 °C) with PMS (Sigma-Aldrich, 0.06 mg/mL) was added (50 μL/well), and incubated again for 4 h at 26 °C. Optical density of each well was obtained on a StatFax

(Model 2100 series plate reader) at 450 nm. The IC_{50} values were calculated using Microsoft Excel 2000 program.

Acknowledgments

The financial support of the Swedish International Development Agency (SIDA) through a bilateral collaboration between Lund University (Sweden) and San Simón University (Bolivia) is fully acknowledged. A special thank you is extended to Dr. Rikard Larsson for his guidance in the synthesis of the Mosher esters and helpful comments on the manuscript.

FIGURE CAPTIONS

Fig. 1. Structures of the compounds isolated from *T. antimenorrhoea* (**1**-**13**)

REFERENCES

Bohlmann, F., Ates, N., King, R.M., Robinson, H., 1983a. Naturally occurring terpene derivatives. Part 479. Two sesquiterpenes from Senecio species. Phytochemistry 22, 1675-1677.
Bohlmann, F., Suwita, A., Jakupovic, J., King, R.M., Robinson, H., 1981. Naturally occurring terpene derivatives. Part 346. Trixikingolides and germacrene derivatives from Trixis species. Phytochemistry 20, 1649-1655.
Bohlmann, F., Zdero, C., 1979. Naturally occurring terpene derivatives, 156. A new group of sesquiterpene lactones from the genus Trixis. Chem. Ber. 112, 435-444.
Bohlmann, F., Zdero, C., King, R.M., Robinson, H., 1979. Naturally occurring terpene derivatives. 202. Further isocedrene derivatives from Trixis paradoxa. Phytochemistry 18, 855-857.
Bohlmann, F., Zdero, C., King, R.M., Robinson, H., 1983b. Naturally occurring terpene derivatives. Part 474. A tetracyclic sesquiterpene, further isocedrene, and guaiene derivatives from Jungia stuebelii. Phytochemistry 22, 1201-1206.
da Silva, E.R., Maquiaveli, C.C., Magalhães, P.P., 2012. The leishmanicidal flavonols quercetin and quercitrin target Leishmania (Leishmania) amazonensis arginase. Experimental parasitology 130, 183-188.
De Riscala, E.C., Catalán, C.A.N., Legname, P.R., Gutiérrez, A.B., Herz, W., 1989. Trixanolide and germacratriene derivatives from Trixis grisebachii. Phytochemistry 28, 2155-2161.
De Riscala, E.C., Catalan, C.A.N., Sosa, V.E., Gutierrez, A.B., Herz, W., 1988. Trixane derivatives from Trixis praestans. Phytochemistry 27, 2343-2346.
Farkas, L., Nogradi, M., Sudarsanam, V., Herz, W., 1966. Constituents of Iva species. V. Isolation, structure, and synthesis of nevadensin, a new flavone from Iva nevadensis and Iva acerosa. J. Org. Chem. 31, 3228-3232.
Granato, E.M., Granato, M.M., Gerenutti, M., Silva, M.G., Ferraz, H.O., Vila, M.M.D.C., 2013. Phytochemical screening of the plant species Trixis antimenorrhoea (Schrank) Kuntze. Rev. Bras. Farm. 94, 130-135.
Gupta, M.M., Verma, R.K., Misra, L.N., 1992. Terpenoids from Hemidesmus indicus. Phytochemistry 31, 4036-4037.
Gupta, S.R., Seshadri, T.R., Sood, G.R., 1973. Flavonols and other components of the leaves of Calycopteris floribunda. Phytochemistry 12, 2593-2540.
Katinas, L., 1996. Revision de las especies Sudamericanas del género *Trixis* (Asteraceae, Mutisieae). Darwiniana 34(1-4), 27-108.
Kyriakopoulou, I., Magiatis, P., Skaltsounis, A.L., Aligiannis, N., Harvala, C., 2001. Samioside, a new phenylethanoid glycoside with free-radical scavenging and antimicrobial activities from Phlomis samia. J Nat Prod 64, 1095-1097.

Lichius, J.J., Thoison, O., Montagnac, A., Pais, M., Gueritte-Voegelein, F., Sevenet, T., Cosson, J.-P., Hadi, A.H.A., 1994. Antimitotic and cytotoxic flavonols from Zieridium pseudobtusifolium and Acronychia porteri. J. Nat. Prod. 57, 1012-1016.
Manjolin, L.C., dos Reis, M.B.G., Maquiaveli, C.d.C., Santos-Filho, O.A., da Silva, E.R., 2013. Dietary flavonoids fisetin, luteolin and their derived compounds inhibit arginase, a central enzyme in Leishmania (Leishmania) amazonensis infection. Food Chem. 141, 2253-2262.
Mishra, B.B., Kale, R.R., Singh, R.K., Tiwari, V.K., 2009. Alkaloids: Future prospective to combat leishmaniasis. Fitoterapia 80, 81-90.
Salamanca , E.C., Ruiz , G., Ticona , J.C., Giménez , A., 2008. Método colorimétrico-XXT: como evaluación de alto rendimiento de sustancias con actividad leishmanicida. BIOFARBO 16, 21-27.
Salmenkallio, M., McCormick, S., Mabry, T.J., Dellamonica, G., Chopin, J., 1982. The flavonoids of Trichophorum cespitosum. Phytochemistry 21, 2990-2991.
Simonsen, H.T., Adsersen, A., Smitt, U.W., Strasberg, D., Jaroszewski, J.W., 2003. Methoxyflavones from Melicope borbonica and M. obscura (Rutaceae). Biochem. Syst. Ecol. 31, 327-330.
Takeda, Y., Fatope, M.O., 1988. New phenolic glucosides from Lawsonia inermis. J. Nat. Prod. 51, 725-729.
Ward, D.E., Rhee, C.K., 1991. A simple method for the microscale preparation of Mosher's acid chloride. Tetrahedron Lett. 32, 7165-7166.
WHO, 2013. Leishmaniasis home, http://www.who.int/leishmaniasis/en/.
Ybarra, M.I., Catalan, C.A.N., Diaz, J.G., Herz, W., 1992. A cyperane and trixanes from Jungia polita. Phytochemistry 31, 3627-3629.

Paper IV

Secondary metabolites of *Lantana balansae*

Eliana M. Maldonado [1,2], Efrain Salamanca[3], Alberto Giménez[3], and Olov Sterner[1,*]

[1] Centre for Analysis and Synthesis, Lund University, Getingevägen 60, 221 00, Lund, Sweden
[2] Centro de Tecnología Agroindustrial, San Simón University, Cochabamba, Bolivia
[3] Instituto de Investigaciones Fármaco Bioquímicas (IIFB), San Andrés University, La Paz, Bolivia

ABSTRACT

Eleven compounds, 12-oxo-phytodienoic acid (**1**), persicogenin (**2**), eriodictyol 3',4',7-trimethyl ether (**3**), phytol (**4**), spathulenol (**5**), 4-hydroxycinnamic acid (**6**), onopordin (**7**), 5,8-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy (**8**), quercetin (**9**), jaceosidin (**10**), and 8-hydroxyluteolin (**11**), were isolated from an ethanol extract of *Lantana balansae* that was shown to possess antileishmanial activity. The structures of the compounds were determined by NMR spectroscopy and HR mass spectrometry, and **1**, **2**, **3**, **7**, **8** and **9** were investigated for antiprotozoal activity towards promastigotes of *Leishmania amazonensis* and *L. braziliensis*. Compound **1** was shown to be the most potent with IC_{50} values below 1 μg/mL. All compounds have been reported previously, but this is the first report of the isolation of a cyclopentenone fatty acid (**1**) and flavanones (**2** and **3**) from a *Lantana* species.

Key words: *Lantana balansae*, *Leishmania amazonensis*, *L. braziliensis*, flavanone, 12-oxo-phytodienocic acid.

1. Introduction

The knowledge of the traditional uses of plants to treat different conditions has not only been helpful in the search for biological active constituents but also contributed to preserve the information obtained directly from the people living in isolated rural communities. In this respect, extensive phytochemical studies of different *Lantana* species, particularly *L. camara*, have led to the identification of the characteristic secondary metabolites of this species as lantadenes (pentacyclic triterpenoids), flavonoids and phenylpropanoids. In addition, biological and pharmacological evaluation of crude extracts, essential oils and isolated compounds have shown that they possess a broad range of biological activities, for example antiprotozoal (antiplasmodial, antimalarial, leishmanicidal), antiviral, antioxidant, antiproliferative and cytotoxic activity [1-3].

Lantana balansae (Verbenaceae) is a perennial shrub with a pungent odor that grows in the mountain region of Cochabamba, Bolivia, where it is locally known as "k'ichita". An infusion of fresh leaves of *L. balansae* is used in the traditional medicine to treat digestive disorders and muscle spasms (personal communication with local people where the plant was collected). Previous studies of *L. balansae* have reported the antimicrobial activity of the methanol extract [4] and the chemical composition of its essential oil [5, 6]. As a part of our collaborative project in the search of bioactive secondary metabolites from the native flora of Bolivia, an ethanol extract of *L. balansae* was assayed for leishmanicidal activity towards *Leishmania amazonensis* and *L. braziliensis*. As the extract displayed significant activity towards both strains of the parasite it was selected for a more detailed study. Herein, we wish to report the secondary metabolites isolated from *L. balansae* as well as their leishmanicidal activities.

2. Results and discussion

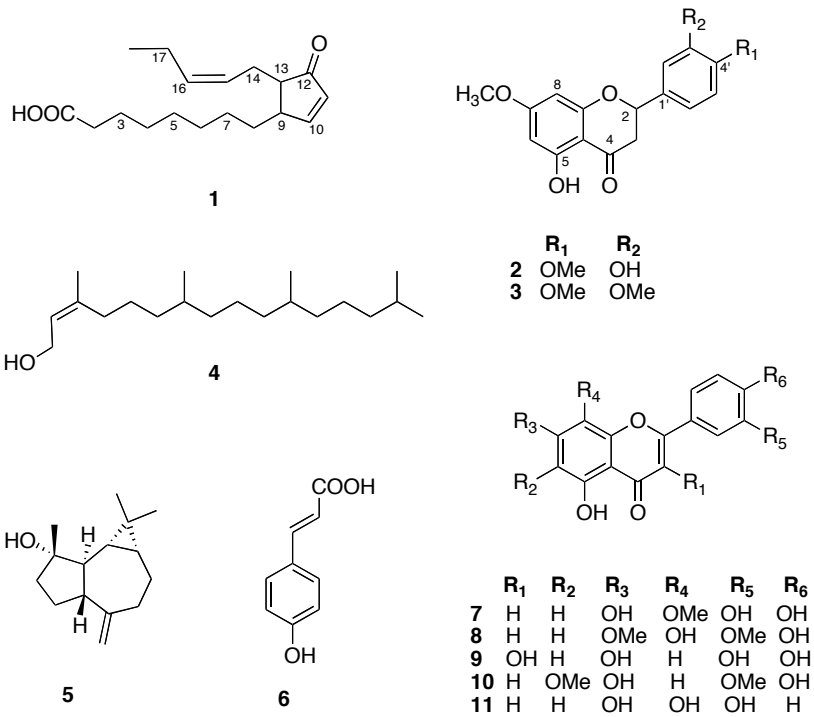
As described in the Experimental part the ethanol extract was purified to a hexane and ethyl acetate fraction from which the 11 compounds were isolated. Compound **1** was obtained as a colourless oil. The HR-ESI-MS showed a protonated molecular ion peak at *m/z* 293.2148 $[M+H]^+$, and as 18 signals could be observed in the ^{13}C NMR spectrum the elemental composition $C_{18}H_{28}O_3$ is suggested for **1.** The ^1H NMR spectrum displayed signals for two 1,2-disubstituted carbon-carbon double bonds, at δ_H 6.95 (10-H, dd, 5.8, 2.6 Hz) and 6.00 (11-H, dd, 5.8, 2.0 Hz) suggesting an α,β-unsaturated carbonyl system, and at δ_H 5.33 (15-H, dtt, 11, 7, 1 Hz) and 5.42 (16-H, dtt, 11, 7, 1 Hz). Both double bonds are by the proton coupling constants suggested to have a *cis* configuration. In addition, nine methylenes at δ_H 2.14 (2-H_2, t, 7.4 Hz), 1.50 (3-H_2, quintet, 7.4 Hz), 1.10 (4-H_2, m), three at 1.06 (5-H_2, m; 6-H_2, m; 8-H_2, m), 1.13 (7-H_2, m), 2.50 and 2.34 (14-H_2, m), and 1.97 (17-H_2, dq, 7.4, 1 Hz), two methines at 2.26 (9-H, m) and 1.85 (13-H, ddd, 7.8, 4.5, 2.4 Hz), as well as a methyl at 0.89 (18-H_3, t, 7.5 Hz) were observed. The presence of a ketone and a carboxylic acid functionalities were confirmed by the signals at δ_C 209.8 and 179.9, respectively, and 2D NMR spectroscopy determined the positions of the two functionalities in the structure. The fifth and final unsaturation of **1** is a ring, which is closed by the COSY correlation between 9-H and 13-H as well as other 2D NMR correlations. A literature search revealed that **1** is identical to the 12-oxo-phytodienoic acid previously isolated from *Schistostephium* species [7], and the spectroscopic data reported are in agreement with those obtained here.

Compound **2** was isolated as a colourless oil. The HR-ESI-MS showed a protonated molecular ion peak at *m/z* 317.1046 $[M+H]^+$, which suggested a molecular formula of $C_{17}H_{16}O_6$. The ^1H NMR spectrum of **2** showed the presence of a hydroxyl group involved in a hydrogen bond at δ_H 12.4 (5-OH), and three doublet of doublets. One corresponds to an oxymethine at δ_H 4.70 (2-H, dd, 2.8, 3.1 Hz) while the remaining two constitute the methylene α to the carbonyl at δ_H 2.50 (3-H_2, dd, 17.1, 2.8 Hz) and δ_H 2.30 (3-H_2, dd, 17.1, 3.1 Hz). These data suggest that **2** possesses a basic flavanone skeleton. The observations of one doublet at δ_H 6.35 (3'-H, d, 8.4 Hz), one doublet of doublets at δ_H 6.62 (2'-H, dd, 8.4, 2.1 Hz), and one doublet at δ_H 7.03 (6'-H, d, 2.1 Hz) show the presence of a 1,3,4-trisubstituted benzene ring, which by the HMBC correlations from 2-H to C-2' and C-6' was shown to be the ring B of a flavanone. In addition, a pair of doublets at δ_H 6.08

(8-H, d, 2.3 Hz) and δ_H 6.21 (6-H, d, 2.3 Hz) characteristic of a meta substitution at ring-A. The aforementioned data correspond to persicogenin, previously isolated from other plant species [8].

Compound **3** was isolated as a colourless oil. The HR-ESI-MS analysis gave a pseudomolecular ion at *m/z* 331.1207 $[M+H]^+$, corresponding to the molecular formula of $C_{18}H_{18}O_6$. The ^1H and ^{13}C NMR spectra of **3** are similar to those of **2**, indicating that both compounds share the same basic skeleton. The distinction between the two compounds is demonstrated by the presence of an additional singlet at δ_H 3.40 integrating to three protons (OMe-5') in the ^1H NMR spectrum of **3**, consistent with the additional fourteen mass units found by mass spectrometry. Identification of **3** as eriodictyol 3',4',7-trimethyl ether was confirmed by comparison of its spectroscopic data with those reported previously in the literature [9].

In addition, phytol (**4**), spathulenol (**5**) [10], 4-hydroxycinnamic acid (**6**) [11], onopordin (**7**) [12], 5,8-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy (**8**) [13], quercetin (**9**) [14], jaceosidin (**10**) [15], and 8-hydroxyluteolin (**11**) [16] were isolated and identified based on their 1D and 2D NMR spectra, and subsequent comparison of their spectroscopic data with those reported in the literature (see Figure 1 for structures).



1

	R_1	R_2
2	OMe	OH
3	OMe	OMe

4

5

6

	R_1	R_2	R_3	R_4	R_5	R_6
7	H	H	OH	OMe	OH	OH
8	H	H	OMe	OH	OMe	OH
9	OH	H	OH	H	OH	OH
10	H	OMe	OH	H	OMe	OH
11	H	H	OH	OH	OH	H

Figure 1. Structures of compounds isolated from *L. balansae*.

Octadecanoid like-compounds or phytooxylipins may possess potent phytohormone activities. They are derived mainly from oxygenized C_{16} and C_{18} fatty acid precursors that are abundant in the cellular membranes of higher plants, by the octadecanoid pathway. Phytooxylipins play an important role in plant response to various environmental stress conditions [17, 18], and recently it was shown that 12-oxo-phytodienoci acid inhibits the proliferation of human breast cancer cells by targeting cyclin D1 [19].

The hexane and ethyl acetate fractions of the ethanol extract of *L. balansae* exhibited potent activity towards promastigotes of *L. amazonensis* and *L. Braziliensis*, with IC_{50} values between 1 and 10 μg/mL (see Table 1 for details). This motivated us to evaluate the antileishmanial activity of the compounds obtained in sufficient quantities, compounds **1**-**3** and **7**-**9**. The results are presented in Table 1. Compound **1** was slightly less potent than amphotericin, used as a positive control, with values of 0.6 and 0.2 μg/mL for *L. amazonensis* and *L. braziliensis*, respectively, but still possessing an interesting activity. All three flavonoids tested showed antileishmanial activity, with compound **8** exhibiting the highest degree of activity and selectivity against both *L. amazonensis* (IC_{50} 13.6 μg/mL) and *L. braziliensis* (IC_{50} 2.7 μg/mL). The biological activity seems to be related with the presence of methoxyl groups in ring A and B. Compounds **8** methoxylated at C-7 and C-5´, while **7**, methoxylated at C-8 is slightly more active compared to **9** which lacks methoxy groups. This is possibly a lipophilicity effect. The antileishmanial activity of quercitin (**9**) is in agreement with those previously reported in the literature [20]. Interestingly, compounds **2** and **3** having a flavanone backbone were practically inactive.

Table 1. *In vitro* leishmanicidal activity on promastigote form of *Leishmania ssp*.

	IC_{50} (μg/mL)[a]	
	L. amazonensis	*L. braziliensis*
EtOH extract	6.0±0.2	4.9±1.2
Hexane fraction	6.1±1.3	1.3±0.3
EtOAc fraction	9.9±0.5	5.3±1.0
1	0.6±0.1	0.2±0.01
2	>100	90±4.2
3	>100	>100
7	21.7±4.2	16.4±0.46
8	13.6±1.81	2.7±0.2
9	19.8±0.77	40.9±2.7
Control [b]	0.21±0.06	0.08±0.04

[a] Data are expressed as mean standard deviation of three determinations.
[b] Amphotericin B was used as positive control.

3. Experimental procedures

3.1. General

1D and 2D NMR spectra were recorded at room temperature on a Bruker Avance II 400 MHz spectrometer, operating at 400.13 MHz for ^{1}H. The chemical shifts (δ) are reported in ppm relative to solvent signals δ_H 7.16 and δ_C 128.0 for C_6D_6, δ_H 2.05 and δ_C 206.0 for acetone-d_6, and δ_H 2.49 and δ_C 39.5 for DMSO-d_6 while the coupling constants (*J*) are given in Hz. HR-ESI-MS experiments were performed with a Waters Q-TOF Micro system spectrometer (using H_3PO_4 for calibration and as internal standard). Vacuum liquid chromatography (VLC) separations were carried out on Merck silica gel 60G (Merck), while column chromatography (CC) was performed using silica gel 60 (230-400 mesh, Merck) and gel permeation on Sephadex LH-20 (GE-Healthcare). Chromatograms were visualized under a UV lamp at 254 nm, and then by spraying with vanillin followed by heating.

3.2. Plant material

The aerial parts of *Lantana balansae* Briq. (Verbenaceae) were collected in September 2009 at the coordinates 17º11.17' S 66º43.58' W and an elevation of 2789 m, near Independencia, Cochabamba, Bolivia. Voucher specimens, taxonomically identified by Lic. Modesto Zárate, are kept at "Herbario Forestal Martín Cárdenas", Cochabamba, under accession number MZ-3946.

3.3. Extraction and isolation

The air-dried and ground leaves and flowers of *L. balansae* (1308.0 g) were extracted by maceration with 95 % EtOH for 48 hours, two times at room temperature. After filtration the combined extracts were concentrated under reduced pressure to yield 136.6 g of a dark residue. The crude organic extract was suspended in a mixture of H_2O:MeOH (9:1, v/v, 500 ml) and extracted with hexane (four times, 500 ml) followed by the extraction with ethyl acetate (four times, 500 ml). After evaporation of the solvents, the two extracts (23.5 and 43.6 g, respectively) were further fractionated. Subjection of the hexane-soluble fraction to VLC (hexane:CH_2Cl_2 1:0 to 0:1) gave ten major fractions (A-J) based on TLC analyses. Fraction E (3.0 g) was submitted to VLC (heptane:EtOAc 1:0 to 8:2) to obtain nine fractions (E1-E9). E4 (582.5 mg) was purified by Sephadex LH-20 ($CHCl_3$:MeOH) to yield **5** (43.0 mg) and **4** (39.3 mg). Fraction F (1.2 g) was chromatographed on Sephadex LH-20 CC ($CHCl_3$:MeOH) yielding six fractions (F1-F6). Compound **10** (3.3 mg) was obtained pure from F5 (53.0 mg). Fractions G (485.0 mg) and H (789.0 mg) were chromatographed on Sephadex LH-20 ($CHCl_3$:MeOH 1:1) to yield eleven (G1-G11) and five fractions (H1-H5), respectively. Compounds **2** (9.0 mg) and **3** (7.7 mg) were obtained pure from G8 and G11, respectively. Sequential purification of H3 (510.0 mg) by Sephadex LH-20 ($CHCl_3$:MeOH 1:1) and VLC (PE:EtOAc 1:0 to 7:3) yielded **1** (26.1 mg). Purification of the EtOAC-soluble fraction (8.0 g) by VLC (CH_2Cl_2:Me_2CO 1:0 to 0:1) yielded seven major fractions (A-G). Fraction C (1.36 g) was subjected to CC on Sephadex LH-20 (MeOH) to give fifteen fractions (C1-C15). Compounds **7** (6.4 mg), **11** (4.6 mg) and **9** (4.1 mg) were obtained pure from C10, C14 and C15, respectively, while

C8 and C11 were purified by PTLC (CH_2Cl_2:Me_2CO 8:2) to give **8** (3.3 mg) and **6** (5.2 mg).

3.4. 12-Oxo-phytodienoic acid (**1**): ^{1}H (400 MHz, C_6D_6) δ_H 2.14 (2-H_2, t, 7.4 Hz), 1.50 (3-H_2, quintet, 7.4 Hz), 1.10 (4-H_2, m), 1.06 (5-H_2, m), 1.06 (6-H_2, m), 1.13 (7-H_2, m), 1.06 (8-H_2, m), 2.26 (9-H, m), 6.65 (10-H, dd, 5.8, 2.6 Hz), 6.00 (11-H, dd, 5.8, 2.0 Hz), 1.85 (13-H, ddd, 7.8, 4.5, 2.4 Hz), 2.50 (14-H, m), 2.34 (14-H, m), 5.33 (15-H, dt, 11, 7. 1 Hz), 5.24 (16-H, dtt, 11, 7.1 Hz), 1.97 (17-H_2, dq, 7.4, 1 Hz), 0.89 (18-H_3, t, 7.5 Hz); ^{13}C (100 MHz, C_6D_6) δ_c 179.9 (C-1), 34.1 (C-2), 24.9 (C-3), 29.2 (C-4), 29.7 (C-5), 27.5 (C-6), 29.3 (C-7), 34.5 (C-8), 46.9, (C-9), 166.1 (C-10), 133.1 (C-11), 209.8 (C-12), 51.5 (C-13), 28.5 (C-14), 125.9 (C-15), 133.8 (C-16), 20.9 (C-17), 14.4 (C-18); HR-ESI-MS *m/z* 293.2148 $[M+H]^+$ (caldc. for $C_{18}H_{29}O_3$ 293.2117).

3.5. Persicogenin (**2**): ^{1}H (400 MHz, C_6D_6) δ_H 4.70 (2-H, dd, 12.8, 3.1 Hz), 2.50 (3-H, dd, 17.1, 12.8 Hz), 2.30 (3-H, dd, 17.1, 3.1 Hz), 6.21 (6-H, d, 2.3 Hz), 6.08 (8-H, d, 2.3 Hz), 6.62 (2'-H, dd, 8.4, 2.1 Hz), 6.35 (3'-H, d, 8.4 Hz), 7.03 (6'-H, d, 2.1 Hz), 3.09 (OMe-7, s), 3.08 (OMe-4', s), 12.84 (OH-5, brs), 5.47 (OH-5', s); ^{13}C (100 MHz, C_6D_6) δ_c 79.0 (C-2), 43.4 (C-3), 196.2 (C-4), 165.2 (C-5), 95.3 (C-6), 168.3 (C-7), 94.5 (C-8), 163.3 (C-9), 103.8 (C-10), 132.5 (C-1'), 118.0 (C-2'), 110.1 (C-3'), 147.0 (C-4'), 146.5 (C-5'), 113.3 (C-6'), 55.1 (OMe-7), 55.3 (OMe-4'); HR-ESI-MS *m/z* 317.1046 $[M+H]^+$ (caldc. for $C_{17}H_{17}O_6$ 317.1025).

3.6. Eriodictyol 3',4',7-trimethyl ether (**3**): ^{1}H (400 MHz, C_6D_6) δ_H 4.76 (2-H, dd, 13.1, 2.9 Hz), 2.62 (3-H, dd, 17.1, 13.1 Hz), 2.39 (3-H, dd, 17.1, 2.9 Hz), 6.25 (6-H, d, 2.2 Hz), 6.16 (8-H, d, 2.2 Hz), 6.69 (2'-H, dd, 8, 2 Hz), 6.52 (3'-H, d, 8.8 Hz), 6.68 (6'-H, d, 2 Hz), 3.07 (OMe-7, s), 3.36 (OMe-4', s), 3.40 (OMe-5', s), 12.91 (OH-5, brs); ^{13}C (100 MHz, C_6D_6) δ_c 79.7 (C-2), 43.9 (C-3), 196.6 (C-4), 165.7 (C-5), 95.6 (C-6), 168.6 (C-7), 94.9 (C-8), 163.7 (C-9), 104.2 (C-10), 131.8 (C-1'), 119.4 (C-2'), 112.2 (C-3'), 150.6 (C-4'), 150.8 (C-5'), 111.0 (C-6'), 55.4 (OMe-7), 55.9 (OMe-4'), 55.9 (OMe-5'); HR-ESI-MS *m/z* 331.1207 $[M+H]^+$ (caldc. for $C_{18}H_{19}O_6$ 331.1182).

3.7. Antileishmanial assay

The colorimetric method-XXT assay was performed as previously described by Salamanca and co-workers [21]. Briefly, the activity was measured *in vitro* on cultures of the *Leishmania* parasite in the promastigote forms, of complex *L. amazonensis* (clon 1: Lma, MHOM/BR/76/LTB-012) and complex *L. braziliensis* (strand M2904 C192 RJA), established at IIFB, cultivated at 26 °C in Schneider medium (pH 6.8) supplemented with inactivated (by heating to 56 °C for 30 min) bovine calf serum (10 %). Parasites in logarithmic phase of growth, at a concentration of $1x10^6$ parasites/mL, were seeded in the wells of 96-well plates. Solutions of compounds to be assessed at concentration range of 0.09-100 µg/mL were added. DMSO (1%) and amphotericin B (0,5 mg/mL) were used as negative and positive controls during the evaluations. All assays were performed in triplicate and the micro well plates were incubated for 72 hrs at 26 °C. After incubation, a solution of XTT (1 mg/mL) in PBS (pH 7.0 at 37 °C) with PMS (Sigma-Aldrich, 0.06 mg/mL), was added (50 µL/well), and incubated again for 4 h at 26 °C. Optical density of

each well was obtained on a StatFax (Model 2100 series plate reader) at 450 nm. The IC_{50} values were calculated using Microsoft Excel 2000 program.

Acknowledgments

This investigation was supported by The Swedish International Development Agency (SIDA) in a bilateral collaboration between Lund University (Sweden) and San Simón University (Bolivia).

REFERENCES

1. Sousa, E.O. and J.G.M. Costa, *Genus Lantana: chemical aspects and biological activities.* Revista Brasileira de Farmacognosia, 2012. **22**: p. 1115-1180.
2. Grace-Lynn, C., et al., *In Vitro Antioxidant Activity Potential of Lantadene A, a Pentacyclic Triterpenoid of Lantana Plants.* Molecules, 2012. **17**(9): p. 11185-11198.
3. Ghisalberti, E.L., *Lantana camara L. (Verbenaceae).* Fitoterapia, 2000. **71**: p. 467-486.
4. Salvat, A., et al., *Antimicrobial activity in methanolic extracts of several plant species from northern Argentina.* Phytomedicine, 2004. **11**(Copyright (C) 2013 U.S. National Library of Medicine.): p. 230-4.
5. De, V.M.E.L., E.C.J. Talenti, and J.A. Retamar, *Essential oils of Lantana balansae.* Essenze Deriv. Agrum., 1973. **43**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 299-306.
6. Guedes, d.S.F.J., et al., *Chemical and molecular characterization of fifteen species from the Lantana (Verbenaceae) genus.* Biochemical Systematics and Ecology, 2012. **45**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 130-137.
7. Bohlmann, F., et al., *Sesquiterpene lactones and other constituents from Schistostephium species.* Phytochemistry, 1983. **22**(7): p. 1623-1636.
8. Pisutthanan, N., et al., *A new flavonoid from Chromolaena odorata.* Nat. Prod. Res., 2006. **20**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 1192-1198.
9. Fernandez, C., et al., *Flavonoid aglycones from some Canary Islands species of Sideritis.* Journal of Natural Products, 1988. **51**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 591-3.
10. Vieira, I.J.C., et al., *Hirtinone, a novel cycloartane-type triterpene and other compounds from Trichilia hirta L. (Meliaceae).* Molecules, 2013. **18**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 2589-2597.
11. Pan, H. and L.N. Lundgren, *Phenolic extractives from root bark of Picea abies.* Phytochemistry, 1995. **39**(6): p. 1423-1428.
12. Reynaud, J. and J. Raynaud, *Presence of onopordin in Doronicum grandiflorum Lam. (Compositae).* Pharmazie, 1984. **39**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 126.
13. Whalen, M.D. and T.J. Mabry, *New 8-hydroxyflavonoids from Solanum section Androceras.* Phytochemistry, 1979. **18**(2): p. 263-265.

14. Slowing, K., et al., *Flavonoid glycosides from Eugenia jambos.* Phytochemistry, 1994. **37**(Copyright (C) 2013 U.S. National Library of Medicine.): p. 255-8.
15. Ulubelen, A., et al., *Flavonoids of Salvia tomentosa (Labiatae).* Journal of Natural Products, 1979. **42**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 261-3.
16. Taskova, R.M., et al., *Flavonoid profiles in the Heliohebe group of New Zealand Veronica (Plantaginaceae).* Biochemical Systematics and Ecology, 2008. **36**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 110-116.
17. Pollmann, S., *Plant oxylipins: the versatile functions of cyclic octadecanoids and jasmonates.* FEBS Journal, 2009. **276**(17): p. 4665-4665.
18. Böttcher, C. and S. Pollmann, *Plant oxylipins: Plant responses to 12-oxo-phytodienoic acid are governed by its specific structural and functional properties.* FEBS Journal, 2009. **276**(17): p. 4693-4704.
19. Altiok, N., et al., *A plant oxylipin, 12-oxo-phytodienoic acid, inhibits proliferation of human breast cancer cells by targeting cyclin D1.* Breast cancer research and treatment, 2008. **109**(2): p. 315-323.
20. Manjolin, L.C., et al., *Dietary flavonoids fisetin, luteolin and their derived compounds inhibit arginase, a central enzyme in Leishmania (Leishmania) amazonensis infection.* Food Chemistry, 2013. **141**(3): p. 2253-2262.
21. Salamanca , E.C., et al., *Método colorimétrico-XXT: como evaluación de alto rendimiento de sustancias con actividad leishmanicida.* BIOFARBO, 2008. **16**: p. 21-27.

Paper V

Macrocyclic monoterpene glycosides from *Parkinsonia aculeata* L.

Eliana M. Maldonado,[1,2] Mohamed S. Marzouk,[3] Karl-Erik Bergquist,[1] and Olov Sterner*[1]

[1] Centre for Analysis and Synthesis, Department of Chemistry, Lund University, P.O.Box 124, 221 00 Lund, Sweden.
[2] Centro de Tecnología Agroindustrial, San Simón University, Cochabamba, Bolivia.
[3] Department of Pharmacognosy, Faculty of Pharmacy, Helwan University, Helwan, Egypt.

ABSTRACT

The structures of three macrocyclic monoterpene *O*-glycosides, parkinsenes E–G (**1**–**3**), isolated from the leaves and small branches of *Parkinsonia aculeata* L. (Fabaceae), were determined by high resolution mass spectrometry and NMR spectroscopy. The elemental compositions of the compounds are $C_{66}H_{102}O_{33}$, $C_{66}H_{102}O_{32}$, and $C_{60}H_{92}O_{28}$, respectively. All three are built around a 23-membered macrocyclic ring consisting of three monosaccharides and two menthiafolic acid (monoterpene) units, and with 2D NMR experiments all proton and carbon signals could be assigned.

Key words: *Parkinsonia aculeata*; Macrocyclic monoterpene glucosides; Structure determination.

1. Introduction

Fabaceae is an important family of flowering plants, comprising about 730 genera and 19,400 species [1]. Many are important plant source for human and animal food [2], while others have found various uses in the traditional medicine [3, 4]. *Parkinsonia aculeata* L., with the common name Jerusalem thorn, has attracted interest due to the increased use of the aqueous extract of the leaves in India and Nigeria to treat a number of conditions [5]. Previous phytochemical investigations of *P. aculeata* have focused on the isolation and identification of its contents of flavonoids [6, 7], however, a recent study has reported the presence of monoterpene glycosides [8]. This investigation reports the structure elucidation of three macrocyclic monoterpene *O*-glycosides, parkinsene F (**1**), parkinsene G (**2**) and parkinsene E (**3**) (see Figure 1 for chemical structures). The structure of **3** has been reported [8], but as the spectroscopic data were incomplete, it is included in this report as well as the structural elucidation of **3**.

2. Results and discussion

Compound **1** was obtained as a colourless gum. An LC-MS analysis showed that **1** produces the pair of positive ions 1423.7 and 1445.7 (see the experimental part for details), indicating that the elemental composition of **1** is $C_{66}H_{102}O_{33}$ and that the ions observed are $M+H^+$ and $M+Na^+$. This elemental composition was confirmed by a HR-ESI-MS experiment (see experimental section) as well as by the 1D NMR data (see Table 1), especially the ^{13}C spectrum which indicate the presence of 66 carbon signals. The number of unsaturations is consequently 16. An inspection of the NMR data suggested the presence of three monosubstituted carbon-carbon double bonds, three trisubstituted carbon-carbon double bonds, three carbonyl groups, and six monosaccharide units, accounting for 15 unsaturations. Compound **1** therefore has one additional ring.

The NMR data indicate that **1** has three identical monoterpene units, 6-hydroxy-2,6-dimethyl-2,7-octadienoic acid, designed A1, A2 and A3 in Figure 1. These were identified by the COSY correlations between 3-H and 4-H_2, 4-H_2 and 5-H_2, as well as 7-H and 8-H_2, and the HMBC correlations between 2-CH_3 and C-1, C-2 and C-3, as well as between 6-CH_3 and C-5, C-6 and C-7. The configuration of the trisubstituted double bond was determined to be 2*E* from the ROESY correlations between 2-CH_3 and 4-H_2. The absolute configuration of the monoterpenes could not be determined with the limited amounts available, but assumed to be 6*R*. The monoterpenes are all connected at C-6 with an ether link to the acetal carbon of the monosaccharides S1, S2 and S4 (A1 to S4, A2 to S1 and A3 to S2, see Figure 1 for the naming of the monosaccharides), and the expected HMBC correlation from the corresponding acetal protons to C-6 in the monoterpene (S4/1-H to A1/C-6, S1/1-H to A2/C-6 and S2/1-H to A3/C-6) were observed.

The monosaccharide S1 was characterized by the COSY spin systems 1-H/2-H/3-H and 5-H/6-H_3, by the ROESY correlations between 1-H and 3-H as well as 5-H, and between 6-H_3 and 4-H, as well as by the HMBC correlations between 3-H and C-4, between 5-H and C-1, and between 6-H_3 and C-5 as well as C-4. 2-H is a dd in the ^1H NMR spectrum with two large coupling constants (8.0 and 9.7 Hz), showing that 1-H, 2-H and 3-H all are axial. S1 was consequently determined to be a fucose. The signal for 2-H in the ^1H NMR spectrum is downshifted, indicated that C-2 in S1 is acylated, and a HMBC correlation between S1/2-H and A3/C-1 shows that A3 is connected to S1 with an ester bond.

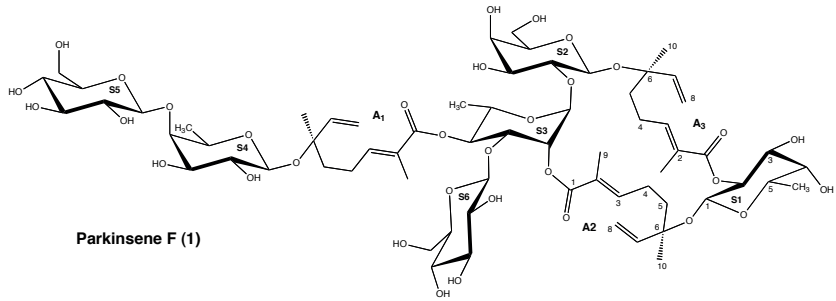
The other end of A3 is as was mentioned above connected to C-1 of S2. S2 is characterized by the spin system 1-H/2-H/3-H/4-H/5-H/6-H_2, by the ROESY correlations between 1-H and 3-H as well as 5-H, and between 6-H_2 and 4-H, as well as by the HMBC correlations between 3-H and C-4, between 5-H and C-1, and between 6-H_2 and C-5 as well as C-4. 2-H is a dd in the ^1H NMR spectrum with two large coupling constants (7.9 and 9.7 Hz), showing that 1-H, 2-H and 3-H all are axial. S2 was consequently determined to be a galactose. Besides to S2/C-1, S2/2-H also gives a HMBC correlation to S3/C-1, while S3/1-H gives a HMBC correlation to S2/C-2. 1-H in S3 is according to the direct ^1H-^{13}C coupling constant 170.8 Hz (extracted from the self-correlation in the HMBC spectrum) equatorial. So is S3/2-H, which is demonstrated by the small coupling constants between 1-H and 2-H (1.8 Hz) and 2-H and 3-H (3.3 Hz). On the other hand, 3-H, 4-H and 5-H are all axial which is demonstrated by the large coupling constants of 4-H

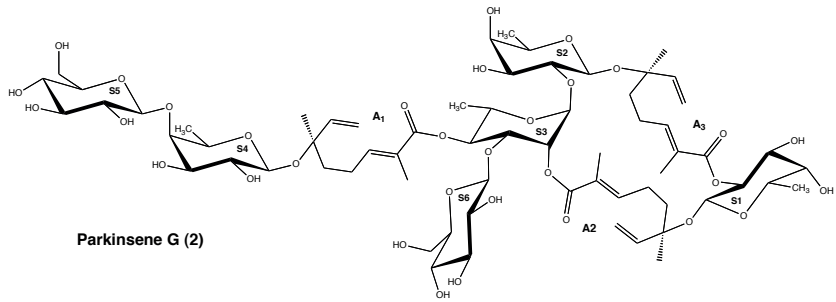
(9 and 9 Hz). This is supported by the ROESY correlations between 3-H and 5-H, as well as between 4-H and 6-H_3. The suggested structure of S3 is supported by the expected COSY and HMBC correlations, and S3 is therefore a rhamnose. S3/2-H is as S1/2-H downshifted, and a HMBC correlation from S3/2-H to A2/C-1 establishes the connection between S3 and A2 through an ester bond. As A2 is connected to S1 in the other end (*vide supra*) the missing unsaturation is identified as a macrocyclic ring consisting of S1-A3-S2-S3-A2-S1 with 23 atoms involved. Additional HMBC correlations are observed from S3/3-H to S6/C-1 and from S6/1-H to S3/C-3, showing that S2-S3-S6 actually forms a trisaccharide. In S6 all ring protons are axial according to the ^{1}H-^{1}H coupling constants, and this is confirmed by ROESY correlations. S6 is consequently a glucose.

A fourth connection to S3 is to C-4, indicated by the downshift of S3/4-H and the HMBC correlation from this proton to A1/C-1. The final monoterpene unit (A1) is therefore connected to S3 by an ester bond to C-4, while A1/C-6 is connected to an additional monosaccharide (S4) by an acetal link (*vide supra*). In S4, the protons 1-H, 2-H and 3-H are axial according to their ^{1}H-^{1}H coupling constants, while 4-H is equatorial. COSY and HMBC correlations determine the structure of S4, while ROESY correlations between 1-H and 3-H as well as 5-H determine that S4 is a second fucose. HMBC correlations from S4/4-H to S5/C-1 and from S5/1-H to S4/C-4 show that S5 is connected to S4 at C-4, and that S4/S5 is a disaccharide. COSY and HMBC correlations in S5 determine the structure while the ROESY correlations between S5/1-H and S5/3-H as well as S5/5-H show that S5 is a second glucose.

As indicated above, the small amounts of the compounds obtained in this investigation (approximately 2 mg of each) does not permit hydrolysis and characterisation of the individual monoterpenes and monosaccharides, especially since we were interested in probing the biological activities of them. We have therefore assumed that the glucoses are D-glucoses, galactose is D-galactose, while the rhamnose is L-rhamnose and the fucoses are L-fucoses, as expected and as shown in Figure 1.

Also compound **2** was obtained as a colourless gum. The LC-MS analysis showed that **2** produces the pair of positive ions 1407.7 and 1429.7, indicating that the elemental composition of **2** is $C_{66}H_{102}O_{32}$ and that the ions observed are $M+H^+$ and $M+Na^+$. This was confirmed by 1D NMR data (see Table 1), especially the ^{13}C spectrum that indicate the presence of 66 carbon signals. Also with **2** the number of unsaturations is 16, and the corresponding 15 unsaturations as in compound **1** are suggested by an inspection of the NMR data (three monosubstituted carbon-carbon double bonds, three trisubstituted carbon-carbon double bonds, three carbonyl groups, and six monosaccharide units). Compound **2** as well has one additional ring. The NMR data of **1** and **2** are overall very similar, indicating that **2** is in fact desoxy-**1**. The only significant differences are observed for the signals of S2, in which C-6 obviously is reduced to a methyl group. The corresponding correlations as discussed above for **1** were observed for **2**, also for S2 which consequently is a third fucose.





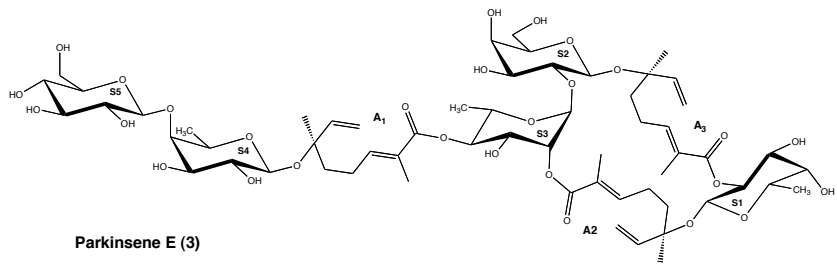


Figure 1. Macrocyclic terpenoids from *P. aculeata* (1-**3**).

The structure of compound **3** was published recently [8], although the NMR data obtained in this investigation differ significantly in several respects and our data are given in Table 1. The LC-MS analysis showed that **3** produces the pair of positive ions 1261.7 and 1283.7, indicating that the elemental composition of **3** is $C_{60}H_{92}O_{28}$ and that the ions observed are $M+H^+$ and $M+Na^+$. This elemental composition was confirmed by a HR-ESI-MS experiment (see experimental section) as well as by the 1D NMR data (see Table 1), especially the ^{13}C spectrum which indicate the presence of 60 carbon signals. Compared to **1** and **2** it can be assumed that **3** is lacking a sugar unit, and it is obvious that only 5 acetal protons can be observed in the ^1H NMR spectrum of **3**. Careful analysis of the correlations observed in the 2D NMR spectra reveals that **3** has the identical macrocyclic ring as **1** with S2 being a galactose. Also the disaccharide S4-S5 is present in **3,** the difference compared to compound **1** is that **3** is lacking the glucose S6 and there is

consequently no HMBC correlation from S3/3-H to another sugar unit. Compound **3** was analysed in the same way as compounds **1** and **2** by COSY, HMQC, HMBC and ROESY NMR experiments, and all corresponding 2D correlations discussed above were also observed for compound **3.**

Table 1. ^{1}H and ^{13}C NMR data of compounds **1-3** in MeOD.

		1		**2**		**3**	
		^{13}C	^{1}H (*J* in Hz)	^{13}C	^{1}H (*J* in Hz)	^{13}C	^{1}H (*J* in Hz)
1	A2	169.1		169.1		169.1	
2		128.2		127.8		128.2	
3		146.1	6.75 m	146.0	6.75 m	145.8	6.79 m
4		24.4	2.39 m	24.0	2.41 m	24.4	2.40 m
			2.10 m		2.10 m		2.13 m
5		41.9	1.83 m	41.5	1.84 m	41.7	1.84 m
			1.65 m		1.65 m		1.65 m
6		81.1		81.1		81.0	
7		143.3	5.68 dd (10.8, 18.0)	143.4	5.68 dd (10.7, 18.2)	143.3	5.72 dd (11.5, 17.2)
8		117.2	5.26 m	116.9	5.26 m	117.1	5.28 m
			5.23 m		5.23 m		5.25 m
9		12.9	1.78 s	12.9	1.78 s	12.8	1.79 s
10		24.1	1.37 s	23.8	1.38 s	24.2	1.37 s
1	S1	97.7	4.46 d (8.0)	97.7	4.45 d (8.0)	97.7	4.47 d (8.0)
2		74.1	5.00 dd (8.0, 9.5)	74.1	5.02 dd (8.0, 9.6)	74.1	5.02 dd (8.0, 9.7)
3		71.8	3.64 m	71.7	3.63 m	71.8	3.63 m
4		73.3	3.61 m	73.3	3.61 m	73.3	3.61 m
5		73.8	3.62 m	73.7	3.61 m	73.8	3.62 m
6		17.0	1.25 d (6.5)	17.0	1.25 d (6.2)	17.0	1.25d (6.5)
1	A3	168.7		168.7		168.7	
2		129.4		129.5		129.4	
3		143.9	6.70 m	143.9	6.70 m	143.9	6.71 m
4		24.3	2.37 m	23.9	2.40 m	24.4	2.38 m
			2.18 m		2.18 m		2.19 m
5		42.9	1.63 m	42.9	1.64 m	42.8	1.66 m
			1.60 m		1.58 m		1.60 m
6		81.4		81.1		81.4	
7		144.3	6.09 dd (11.0, 17.8)	144.6	6.14 dd (11.1, 17.7)	144.2	6.15 dd (11.0, 17.8)
8		117.2	5.31 m	116.1	5.29 m	117.1	5.32 m
			5.28 m		5.26 m		5.29 m
9		12.9	1.82 s	12.8	1.81 s	12.9	1.82 s
10		22.8	1.43 s	22.7	1.40 s	23.0	1.43 s
1	S2	100.1	4.52 d (7.9)	100.4	4.47 d (7.3)	100.2	4.52 d (7.7)
2		81.1	3.23 dd (7.9, 10)	79.0	3.49dd (7, 10)	81.0	3.24 m
3		76.8	3.38 m	76.0	3.52 m	76.6	3.39 m
4		71.7	3.36 m	73.3	3.61 m	71.9	3.35 m
5		71.8	3.17 m	71.4	3.56 m	71.9	3.17 m
6		62.9	3.83 m	17.0	1.24 d (6.3)	62.9	3.83 m
			3.66 m				3.66 m
1	S3	99.3	5.12 d (1.7)	99.9	5.09 d (1.6)	99.5	5.16 d (1.5)
2		72.6	5.56 dd (1.8, 3.4)	72.8	5.55 dd (1.9, 3.2)	72.9	5.28 m
3		76.7	4.51 dd (3, 10)	76.5	4.49 dd (3.4, 10.2)	68.2	4.32 dd (3.8, 10.1)
4		74.2	5.10 dd (9, 9)	74.2	5.13 dd (10.0, 10.0)	75.8	4.98 dd (9.9, 9.9)
5		69.7	4.08 dq (9.9, 6.1)	69.0	4.07 dq (10.1, 6.0)	69.4	4.03 dq (9.9, 6.2)
6		18.6	1.17 d (6.3)	18.6	1.17 d (6.2)	18.6	1.17d (6.2)

Table 1. ^{1}H and ^{13}C NMR data of compounds **1-3** in MeOD (continue).

		1		**2**		**3**	
		^{13}C	^{1}H (*J* in Hz)	^{13}C	^{1}H (*J* in Hz)	^{13}C	^{1}H (*J* in Hz)
1	A1	169.6		169.5		169.5	
2		128.8		128.8		128.6	
3		144.9	6.79 m	144.9	6.79 m	144.9	6.82 m
4		24.6	2.32 m	24.4	2.31 m	24.6	2.33 m
5		41.2	1.72 m	41.5	1.70 m	41.3	1.72 m
6		81.0		82.0		81.0	
7		144.3	5.95 dd (11.0, 17.7)	144.3	5.95 dd (11.0, 17.7)	144.3	5.95 dd (11.0, 17.0)
8		116.1	5.28 m	116.1	5.27 m	116.0	5.28 m
			5.22 dd (11, 2)		5.21 m		5.22 m
9		12.8	1.85 s	12.7	1.85 s	12.7	1.85 s
10		23.7	1.38 s	23.9	1.38 s	23.8	1.38 s
1	S4	99.9	4.33 d (7.3)	99.9	4.33 d (7.3)	99.9	4.33 d (7.5)
2		73.2	3.50 dd (7.2, 9.8)	73.3	3.51 dd (7.1, 9.8)	73.2	3.49 dd (7.2, 9.7)
3		75.9	3.52 dd (3.0, 9.7)	75.9	3.52 m	75.9	3.52 dd (3.0, 9.8)
4		82.5	3.83 dd (3.4, 0.6)	82.5	3.82 dd (3.0, 0.6)	82.6	3.81 dd (3.0, 0.5)
5		71.3	3.60 qd (6.6, 0.6)	71.3	3.60 qd (6.1, 0.6)	71.3	3.59 qd (6.4, 0.5)
6		17.7	1.29 d (6.5)	17.5	1.29 d (6.5)	17.6	1.29 d (6.4)
1	S5	106.4	4.55 d (7.8)	106.1	4.55 d (7.8)	106.1	4.55 d (7.8)
2		71.8	3.28 m	71.8	3.28 m	71.8	3.29 m
3		78.5	3.36 m	78.5	3.36 dd (8.8, 8.8)	78.5	3.36 m
4		76.1	3.28 m	76.1	3.28 m	76.1	3.28 m
5		78.3	3.25 m	78.3	3.25 m	78.3	3.25 m
6		63.1	3.85 dd (2.2, 11.9)	63.1	3.85 dd (2.2, 11.9)	63.1	3.85 dd (2.1, 11.8)
			3.66 dd (6, 12)		3.66 dd (5.7, 11.9)		3.65 dd (5.7,11.8)
1	S6	106.3	4.37 d (7.8)	106.3	4.38 d (7.8)		
2		74.8	3.08 dd (7.8, 9.2)	74.9	3.08 dd (7.9, 9.2)		
3		78.3	3.26 m	78.3	3.25 m		
4		71.8	3.17 dd (9.2, 9.5)	71.9	3.16 dd (9.3, 9.3)		
5		78.3	3.27 m	78.3	3.27 m		
6		63.3	3.90 dd (2.0, 11.8)	63.1	3.89 dd (2.1, 12.1)		
			3.61 m		3.60 m		

3. Experimental

3.1. General

The NMR spectra were recorded at 500 MHz for ^{1}H and 125 MHz for ^{13}C with a Bruker Avance 500 NMR spectrometer, in CD_3OD with the solvent peaks (3.31 ppm for ^{1}H and 49.15 for ^{13}C) as reference. HR-ESI-MS was performed with a Waters Q-TOF Micro system spectrometer (using H_3PO_4 for calibration and as internal standard). Optical rotations were measured with a Perkin Elmer Model 341 polarimeter. For column chromatography, Sephadex LH-20 (Pharmacia, Uppsala, Sweden), microcrystalline cellulose (Merck, Darmstadt, Germany) and polyamide S (Fluka, Steinheim, Switzerland) were used. Whatman No. 1 sheets (Whatman Ltd., Maidstone, England) were used for paper chromatography. The pure compounds were visualized by spraying with Naturstoff reagent (a) 1 % diphenyl boryloxyethanolamine in ethanol, (b) 5 % polyethylene glycol 400 in methanol, then heating the dry chromatogram at 120 °C for 10 min and visualizing under UV light (365 nm)] and $FeCl_3$ (1 % in ethanol). Solvent systems S_1 [*n*-BuOH/HOAc/H_2O (4:1:5, v/v/v top layer)], S_2 (15 % aqueous HOAc), S_3

[*n*-BuOH/2-propanol/H_2O (4:1:5, v/v/v top layer)] and S_4 [$CHCl_3$/MeOH/H_2O (14:6:1, v/v/v)], were used [8].

3.2. Plant material

The leaves and small branches of *P. aculeata* L. were collected from plants growing in Al-Azhar park (Salah Salem St.), Cairo, Egypt, during June-July 2007. The identification of the plants was performed by Dr. Wafaa M. Amer, Professor of Botany, Botany Department, Faculty of Science, Cairo University, Cairo, Egypt. Voucher specimen is deposited in the herbarium of Pharmacognosy Department, Faculty of Pharmacy, Helwan University, Helwan, Egypt.

3.3. Extraction and isolation

The powder of air-dried leaves and small branches of *P. aculeata* (750 g) was subjected to exhaustive extraction with hot 80 % aq. MeOH under reflux (70 °C, 4 x 3 L). The combined extract (**AME**) was then dried in vacuum (45 °C) to give 150 g dry extract that is preliminary purified on dry conditions with hot $CHCl_3$ under reflux (70 °C, 3 x 1 L) to give dry $CHCl_3$-soluble portion of 21 g. The residue was then taken with warm MeOH to produce 100 g of MeOH-soluble portion. Chromatographic examination showed that the MeOH-soluble portion is rich in polyphenols and monoterpene glycosides, while $CHCl_3$-soluble portion was found to be poor in such metabolites. Accordingly, the MeOH-soluble portion was fractionated on polyamide column (300 g) using H_2O and then stepwise gradient H_2O-MeOH to yield seven fractions (I-VII). Fraction II was successively fractionated from H_2O with *n*-BuOH to produce dry 500 mg *n*-BuOH-soluble portion. Thereafter it was fractionated on a silica gel column under positive pressure (flow: 2ml/min) and $CHCl_3$, then MeOH–H_2O gradient with gradual increase of polarity to isolate the macrocyclic monoterpene glycosides **1**–**3**.

3.4. **Parkinsene F (1):** Colourless gum (2.4 mg were obtained for this investigation); $[\alpha]_D^{25}$ +32.66 (*c* 0.4, MeOH). See Table 1 for ^{1}H (500 MHz, MeOH) and ^{13}C (125 MHz, MeOH) NMR data. Positive ESI-MS: *m/z* 1271.9 $[4M+Na+H]^+$, 959.6 $[3M+Na+H]^+$, 360.3 $[M+2Na+2H]^+$; negative ESI-MS 1283.7 $[4M+Cl]^-$, 971.5 $[3M+Cl]^-$, 372.3 $[M+CH_3COO]^-$; HR-ESI-MS *m/z* 1423.6404 $[M+H]^+$ (calcd. for $C_{66}H_{103}O_{33}$ 1423.6382).

3.5. **Parkinsene G (2)**: Colourless gum (aprox. 2 mg were obtained for this investigation); See Table 1 for ^{1}H (500 MHz, MeOH) and ^{13}C (125 MHz, MeOH) NMR data; ESI-MS**:** positive ion mode *m/z* 1271.5 $[4M+Na+H]^+$, 959.4 $[3M+Na+H]^+$, 335.3 $[M+Na]^+$; negative ion mode 971.4 $[3M+Cl]^-$, 935.5 $[3M–H]^-$.

3.6. **Parkinsene E (3**): Colourless gum (2.2 mg were obtained for this investigation); $[\alpha]_D^{25}$ -19.31 (*c* 0.7, MeOH); See Table 1 for ^{1}H (500 MHz, MeOH) and ^{13}C (125 MHz, MeOH) NMR data. Negative ESI-MS: *m/z* 1295.7 $[M+Cl]^-$, 1259.7 $[M–H]^-$; positive ESI-MS: *m/z* 1283.5 $[M+Na]^+$; HR-ESI-MS *m/z* 1423.6404 $[M+H]^+$ (calcd. for $C_{66}H_{103}O_{33}$ 1423.6382).

References

1. Doyle, J.J., *Phylogeny of the legume family-An approach to understanding the origins of nodulation.* Annual Review of Ecology and Systematics, 1994. **25**: p. 325-349.
2. Vietmeyer, N.D., *Lesser-known plants of potential use in agriculture and forestry.* Science, 1986. **232**(Copyright (C) 2014 U.S. National Library of Medicine.): p. 1379-84.
3. Pras, N., et al., *Mucuna pruriens: improvement of the biotechnological production of the anti-Parkinson drug L-dopa by plant cell selection.* Pharm. World Sci., 1993. **15**(Copyright (C) 2014 American Chemical Society (ACS). All Rights Reserved.): p. 263-8.
4. McManus, O.B., et al., *An activator of calcium-dependent potassium channels isolated from a medicinal herb.* Biochemistry, 1993. **32**(Copyright (C) 2014 American Chemical Society (ACS). All Rights Reserved.): p. 6128-33.
5. Hassan, S.W., et al., *Hepatoprotective effect of leaf extracts of P. aculeata L. against CCl_4 intoxication in albino rats.* International journal of Biological Chemistry, 2008. **2**: p. 42-48.
6. El-Sayed, N.H., et al., *Luteolin 7,4'-dimethyl ether 6-C-glucoside from Parkinsonia aculeata.* Phytochemistry, 1991. **30**(Copyright (C) 2014 American Chemical Society (ACS). All Rights Reserved.): p. 2442.
7. Bhatia, V.K., S.R. Gupta, and T.R. Seshadri, *C-Glycosides of the leaves of Parkinsonia aculeata.* Tetrahedron, 1966. **22**(Copyright (C) 2014 American Chemical Society (ACS). All Rights Reserved.): p. 1147-52.
8. Marzouk, M.S., et al., *Novel Macrocyclic Monoterpene Glycosides from Bioactive Extract of Parkinsonia aculeata L.* Cell Biochemistry and Biophysics, 2013. **65**(3): p. 301-313.