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PhD Thesis

"Isolation and characterization of plant bioactive compounds and assessment of their use in veterinary science"

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Ogni cosa che puoi immaginare, la natura l'ha già creata

Albert Einstein

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List of abbreviations

µM micromolar
1D-2D mono e bidimensional
ACN acetonitril
anti-Cdc2 (mouse monoclonal, sc-8395)
anti-phospho (Thr161)-Cdc2 p34 (rabbit polyclonal, sc-101654)
AR anthelmintic resistance
ATCC American Type Cell Culture
C^{13}-NMR carbon nuclear magnetic resonance
CA *Candida albigans*
CD_3OD deuterate methanol
CH_2N_2 diazomethane
CH_3COONa sodium acetate
$CHCl_3$ chloroform
COSY correlation spectroscopy
DMEM HeLa
DMSO dimethyl sulfoxide
DQF double-quantum filtered
EHA egg hatch assay
ESI-MS electrospray mass specrtometry
FBS fetal bovine serum
GIBCO Life Technologies, Grand Island, NY, USA
GIN gastrointestinal nematodes
GPP geranil pirophosphate
H^1-NMR proton nuclear magnetic resonance spectroscopy
HCl chloridic acid
HeLa cervical carcinoma
HMBC heteronuclear multiple bond correlation
HPLC high pressure liquid chromatography
HRESIMS high resolution electrosray mass spectrometry
HSQC heteronuclear single quantum correlation
Hz hertz
IC_{50} inhibitory concentration half maxima
IPP isopentenyl pyrophpsphate
J NMR coupling costant
Jurkat T-cell leukemia
LC-MS liquid chromatography coupled with mass spectrometry
m/z mass/ charge
MALDI matrix assisted laser desorbtion ionization
MeOH methanol
mg milligram
Mhz mega Hertz
MS mass spectrometry
MSn massspectroscopy tandem
MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
n-BuOH normal-buthanol
n-HEXANE normal hexane
NHR nuclear hormone receptor
NMR nuclear magnetic resonance
PI propidium iodide
ppm parts per million

List of abbreviations

Q-TOF quadrupole- time of flight
ROESY rotating frame Overhause effect spectroscopy
RP reverse phase
RPMI 1640 Jurkat U937
RU response unit
STA Staphylococcus aureus
TBZ Thiabendazole
TFA Trifluoroacetic acid
TLC thin layar chromatography
TOCSY total correlation spectroscopy
TOF time of flight
t_R retention time
ug microgramm
UPLC Ultra Performance Liquid Chromatography
WAAVP Advancement of Veterinary Parasitology

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Massimiliano

Abstract

L' attività di ricerca del Dottorato di Ricerca in Scienze Veterinarie, XXIX ciclo, si inserisce nell'ambito dello studio chimico-biologico di piante medicinali, rivolto all'isolamento e alla caratterizzazione strutturale dei metaboliti secondari da matrici vegetali e alla valutazione di un loro utilizzo in ambito veterinario ed alimentare. Le specie oggetto di studio sono utilizzate nella medicina popolare o in medicina veterinaria per le loro proprietà biologiche, per la cura di comuni patologie in particolare parassitosi e/o infezioni batteriche.

L'indagine chimica biologica si è concentrata sulle seguenti specie vegetali:

- *Psiadia punctulata* (DC.) Vatke della famiglia delle Asteraceae, diffusa nelle zone aride tropicali, nelle praterie e nei pascoli aridi arbustivi, fino ai margini della foresta, dell'Africa Sud-Orientale, e in Medio Oriente, alcune piante di questa famiglia sono ampiamente usate come antimicrobici.
- *Hypoestes forskaolii* (Vahl) Roem. & Schult, pianta erbacea appartenete alla famiglia delle Acanthaceae, ampiamente distribuita in tutta la regione meridionale dell'Arabia Saudita, alcune piante di questa famiglia sono ampiamente usate come antielmintici.
- *Trichilia maynasiana* C.DC appartenente alla famiglia delle Meliaceae è un albero ampiamente distribuito nelle zone tropicali del Sud-Africa usato in medicina popolare per il trattamento di vari disturbi in particolare antiinfiammatorio, purgante, antiepilettico e antipiretico;
- *Vernonia nigritiana* Oliv. & Hiern. (Asteraceae) è una pianta erbacea o arbusto ampiamente distribuito in Africa occidentale, le foglie e altre parti della pianta sono tradizionalmente utilizzate contro le infiammazioni della pelle, reumatismi, febbre, cefalea e insufficienza. In medicina etnoveterinaria molte piante del genere *Vernonia* sono usate per infezioni batteriche ed elmintiasi.

La purificazione e l'isolamento dei metaboliti secondari è stata realizzata mediante l'utilizzo di tecniche cromatografiche quali: cromatografia su colonna, cromatografia su strato sottile, HPLC (High Pressure Liquid Chromatography). La caratterizzazione strutturale di tutti i composti isolati è stata ottenuta principalmente attraverso tecniche spettroscopiche di

Risonanza Magnetica Nucleare con esperimenti monodimensionli (^{1}H-NMR, ^{13}C-NMR) e bidimensionali (COSY, HSQC, HMBC, ROESY), e tecniche di spettrometria di massa. L'analisi ha condotto all'isolamento di diversi metaboliti secondari per ciascuna delle specie vegetali in esame, alcuni dei quali a struttura non nota in letteratura.

Lo studio fitochimico di *Psiadia punctulata* ha consentito l'isolamento e la caratterizzazione strutturale di 30 composti tra diterpeni kauranici, trachilobanici e flavonoidi, 13 di questi non sono mai stati descritti in letteratura ed appartengono alla classe dei diterpeni ent-kaurani e trachilobani. L'attività antimicrobica dell'estratto diclorometanico di *Psiadia punctulata* è stata valutata contro batteri Gram-positivi (*Staphylococcus aureus*) e Gram-negativi (*Pseudomonas aeruginosa*) ed il fungo dimorfico *Candida albicans*. I risultati hanno mostrato un'attività antibatterica dell'estratto contro *S. aureus* (MIC_{50} = 100 ug/mL), mentre nessuna attività è stata rilevata contro i batteri Gram-negativi. Inoltre, l'estratto ha mostrato una attività antifungina nei confronti di *C. albicans* con MIC_2 = 50 µg / mL e MIC_0 = 130 µg / mL. Le nove frazioni A-I ottenute dopo separazione su colonna di gel di silice sono state testate per la loro attività antimicrobica alle dosi (da 20 a 100 µg/ml), le frazioni A,B e I sono risultate inattive, le frazione C-H hanno mostrato un interessante attività antimicrobica nei confronti di *Staphylococcus aureus* e *Candida albigans* (MIC_2 15 µg/mL ma non nei confronti di *Pseudomonas aeruginosa.* Tutte le molecole isolate dalle frazioni attive sono state investigate per l'attività antimicrobica, solo 4 molecole, 3 flavonoidi e un diterpene hanno mostrato un interessante attività. In particolare Il composto **3,** 3',4',5,7-tetramethoxyflavone, alla concentrazione di 40 µg/mL è stato capace di inibire del 50% la produzione di biofilm di *S. aureus* e del 90% per *Candida albicans.*

Lo studio fitochimico delle foglie di *H. forskaolii* ha permesso di isolare 13 composti appartenenti alla classe dei diterpeni fusicoccanici ed isopimarani, 4 di questi diterpeni fusicoccanici non sono mai stati scoperti precedentemente in natura. Gli estratti *n*-esananico, cloroformico, cloroformio:metanolo 9:1 e metanolico di *Hypoestes forskaolii* sono stati testati nel saggio *in vitro* di attività antielmintica Egg Hacth Assay su diverse specie di nematodi gastrointestinali che naturalmente parassitano i piccoli ruminananti. Gli estratti polari non sono stati in grado di mostrare attività

ovicida significativa (meno del 50% di schiusa delle uova), mentre estratto *n*-esano alla concentrazione di 1 mg/ml ha mostrato una moderata inibizione della schiusa delle uova del 30%. Successivamente i composti isolati sono stati testati per la valutazione del loro potere antiproliferativo *in vitro* su due linee cellulari quali HeLa e Jurkat U973 ma tutti i composti testati non hanno influenzato significativamente la crescita e la vitalità cellulare.

Lo studio di *Trichilia maynasiana* ha condotto all'isolamento di 9 composti di cui 2 molecole mai isolate in precedenza a struttura tirucallanica e secoguaianica.

Lo studio fitochimico condotto su *Vernonia nigritiana* ha portato all'isolamento di 7 composti di cui 2 lattoni sesquiterpenici mai isolati in precedenza.

Abstract

The research activity of the PhD project in Veterinary Science was aimed at the isolation and structural characterization of secondary metabolites from medicinal plants and the evaluation of their use in veterinary science. The selected plants are species used in folk medicine or in the veterinary medicine, particularly plants used as antiparasitic and antimicrobial

The chemical-biological investigation was carried out on the following plants:

- *Psiadia punctulata* (DC.) Vatke of the Asteraceae family it is found in tropical arid areas from the grasslands and shrubs in arid pastures to the edge of the forest, South-Eastern Africa, and Middle East, some plants of this family are widely used as antimicrobial;
- *Hypoestes forskaolii* (Vahl) Roem. & Schult, herbaceous plant belonging to the Acanthaceae family, widely distributed throughout the southern region of Saudi Arabia, some plants of this family are widely used as a natural anthelmintic;
- *Trichilia maynasiana* C.DC belonging to the family of the Meliaceae is a tree widely distributed in tropical areas of South Africa used in folk medicine to treat various anti-inflammatory diseases;
- *Vernonia nigritiana* Oliv. & Hiern. (Asteraceae) is an herbaceous plant or shrub widely distributed in West Africa, the leaves and other parts of the plant are traditionally used against skin inflammations, rheumatism, fever, headache and impairment. In veterinary medicine many plants of *Vernonia* genus are used for bacterial infections and intestinal worms.

The isolation of secondary metabolites was achieved by chromatographic techniques such as column chromatography, thin layer chromatography, HPLC (High Pressure Liquid Chromatography). The structural characterization of all the isolates was performed by spectroscopic techniques, nuclear magnetic resonance experiments monodimensional (^{1}H-NMR, ^{13}C-NMR) and two-dimensional (COSY, HSQC, HMBC, ROESY), and mass spectrometry.

The investigation of *Psiadia punctulata* led to the isolation of 30 compounds including diterpenes and flavones, of which 13 were new natural compounds. The antimicrobial activity of the dicloromethan extract was

tested against Gram-positive bacteria (*Staphylococcus aureus*), Gram-negative (*Pseudomonas aeruginosa*) and dimorphic fungus *Candida albicans*. The results of this study highlighted that the dicloromethan extract showed antibacterial activity against *S. aureus* (MIC_{50} = 100 ug/mL), whereas no activity was detected against Gram-negative bacteria. Moreover, the extract showed antifungal activity against *Candida albicans* (MIC_2 = 50 µg/mL and MIC_0 = 130 µg/mL). The nine fractions A-I obtained after a silica gel column separation were tested for their antimicrobial activity at doses ranged from 20 to 100 µg/ml. The A,B and I fractions were found to be inactive on all species, instead six fractions (C-H) showed a remarkable activity. All the compounds isolated from active fractions were tested for their antimicrobial activity. Compound **3,** 3',4',5,7-tetramethoxyflavone, at a concentration of 40 g/mL was able to inhibit by 50% the production of biofilm of the *S. aureus* and 90% for *Candida albigans*.

The phytochemical investigation of *H. forskaolii* leaves led to the isolation of 13 compounds belonging to the class of diterpenes fusicoccane and isopimarane, among these 4 of fusicoccane diterpenes are new natural compounds. The *n*-hexane, chloroform, chloroform: methanol 9:1 and methanolic extracts of *Hypoestes forskaolii* were tested fot *in vitro* anthelmintic activity via Egg hacth Assay on different species of gastrointestinal netamathodes that naturally parasitize small ruminants. Polar extracts have not been able to show significant ovicidal activity (less than 50% of hatching), while the *n*-hexane extract at a concentration of 1 mg/ml showed moderate inhibition of 30% hatching. All the isolated compounds were also tested *in vitro* for the antiproliferative activity on HeLa and Jurkat U973 cell lines, all compounds did not significantly affect growth and cell viability.

The phytochemical investigation *of Trichilia maynasiana* led to the isolation of nine compounds of which two are new natural compounds. These compounds belonging to the class of tirucallane and secoguaiane terpenes.

The phytochemical study of *V. nigritiana* led to the isolation of seven secondary metabolites including two sesquiterpene lactones, never described before.

Introduction

Plants are the source of an almost uncountable numbers of secondary metabolites, characterized by different chemical structures and a broad range of biological activity. Only in the last decade the role of secondary metabolites has been deeply evaluated and studied. Today, with the word "secondary metabolites" or "specialized metabolites" we indicate compounds nor directly involved in the primary metabolism of the plant, such as growing and reproduction, nor simple catabolic products. In fact, there are experimental evidence that they have an important role in the evolution. Nowadays, the research in the field of biology, chemistry, and medicine, is directed towards the identification and structural characterization of plant secondary metabolites with pharmacological activity with the aim of discover their detailed biosynthesis pathways and as a molecular lead for their industrial production or the synthesis of new drugs. The industrial interest towards active compounds of natural origin is related to the increasing request of natural products (especially in developed countries) instead of synthetic molecules (Gullo, A.M., et al,1994, Newman and Cragg, 2016).

Therefore, plant biodiversity represents the richness and complexity of the earth life and it's the major resource that nature gave to the development of human life. More than 250.000 species of plants have already been identified but we think that more than 50.000 species are still unknown and should be characterized. Moreover, within the 100 cultivated species that produce food and crops, the traditional farmer selected and developed hundreds of thousands different gene varieties. Natural products have provided, and continue to provide food, fats, colouring materials, tissues, and molecules used for the treatment of numerous diseases.

The human activity became so destructive of the earth's resources (the world population is about 6 billion people and their consumption is the 40% of the annual global production) that the biodiversity of the ecosystems has been substantially stressed and threatened with the permanent loss of species, varieties, and groups. The 20th century was the most destructive of the earth's resources and it's estimated that in this century each year about a thousand of species become extinct. This means that we are living a period of continuing destruction and degradation of the environment that will result in the irretrievable loss of many forms of biogenetic diversity. World Conservation Union estimates indicate that one out 8 of the 240.000 plant

species become extinct. The 90% of this species is endemic of a country, thus it means that doesn't grow anywhere (Blunt et al., 2014; Shu, 1998)
The preservation of biodiversity throughout the world is of importance to the human population and to the stability of the entire world. Plant biodiversity plays an important role in providing household food security. There is increasing recognition of the important roles plant biodiversity (including underutilized species) can play in ensuring food security, veterinary medicine and better health (provision of vitamins and other micro-nutrients to combat hidden hunger), supporting income generation and local economy (meeting increasing demand for diversity for food and other products), providing non material benefits (link to cultural traditions and local knowledge) and ecosystem health (mitigation of adverse effects due to environmental changes). The discovery and development of pharmaceutical and other useful agents from natural products research can, under appropriate circumstances, promote economic opportunities and enhanced research capacity in the South of the world. Natural products research should promote the self-sustainability of the research activities, generating income derived from the intellectual property of the scientific discoveries (Jensen, 2016; Kaufman et al., 1999; Sethi, 1998).

I.I Ethnoveterinary-medicine and animal self-medication

The rural and tribal people are not easily accessible to modern veterinary services for their livestock considering that livestock production is the major source of income in rural and semi urban areas of Third World countries. Farmer are less economically healthy to cope with various bovine ailments therefore they depend upon their traditional knowledge of healing animals. The use of plants could be a cheap, safe, biodegradable and easily accessible alternative to the synthetic and modern methods of disease control. Livestock owners use a variety of plants and their products to form traditional medicines for primary health care treatment and maintaining animals productive. Knowledge of the local farmers and tribe have thus laid the foundations take a part in a much bigger concept named ethno-veterinary (SriBalaji and Chakravarthi, 2010).
Ethno-veterinary medicine has evolved through observations, trials and errors, and passes from one generation to the next through verbal communication. Therefore, these practices are hardly documented and unfortunately largely lost, diluted and distorted. In order to understand its

scientific justification, logic and to develop new concepts, it is of utmost need and importance to document the practices involved in EVM. The traditional knowledge of animal healthcare practices requires great attention for pharmaceutical analysis to prospect new drugs in the concerned (Gaur et al., 2010).

When the problem is to discover new chemicals, it would be useful to observe wild animals (and domesticated ones too) as well as carrying out randomized prospection on rainforest plants, or interviewing indigenous peoples about their traditional healing knowledge and practices, since animals can give good indications about new sources of medicines (Huffman, 2003; Huffman and Vitazkova, 2007).

Early in the co-evolution of plant-animal relationships, some arthropod species began to utilize the chemical defences of plants to protect themselves from their own predators and parasites. It is likely, therefore, that the origins of herbal medicine have their roots deep within the animal kingdom. From prehistoric times man has looked to wild and domestic animals for sources of herbal remedies. Both folklore and living examples provide accounts of how medicinal plants were obtained by observing the behaviour of animals. According to primatologist Michael Huffman, "the probability that animals may have something to teach us about the medicinal use of plants is quite high" (Biser, 2006). Field researchers have observed different species of animals seeking and using substances in such a way as to enhance their own health. This behaviour was called as animal self-medication (Confessor et al., 2016).

To date, perhaps the most striking scientific studies of animal self-medication have been made on the African great apes. The great ape diet is often rich in plants containing secondary compounds of non-nutritional, sometimes toxic, value that suggest medicinal benefit from their ingestion. Chimpanzees (*Pan troglodytes*), bonobos (*Pan paniscus*) and gorillas (*Gorilla gorilla*) are known to swallow whole and defecate intact leaves. The habit has been shown to be a physical means of purging intestinal parasites. Chimpanzees and man coexisting in sub-Saharan Africa are also known to ingest the bitter pith of *Vernonia amygdalina* for the control of intestinal nematode infections (Costa-Neto, 2012).

In light of the growing resistance of parasites and pathogens to synthetic drugs, the study of animal self-medication and ethno-medicine offers a novel line of investigation to provide ecologically-sound methods for the treatment of parasites using plant-based medicines in populations and their

livestock living in the tropics. Phytochemical studies have shown a wide range of biologically active properties of medicinal plants also applicable in the field of veterinary medicine.

I.II Natural compounds in veterinary medicine

The recent return to "natural medicine" has emphasized the importance of gathering information about medicinal plants traditionally used to treat animals. Phytotherapy represents one of the most used non- conventional medicines in both human and veterinary medicine. It utilizes plants, parts of them such as flowers, leaves, roots, and seeds, and substances extracted from them to treat many different minor diseases (Abbass et al., 1994; Anon, 1996; Viegi et al., 2003) (*Fig.* 1).

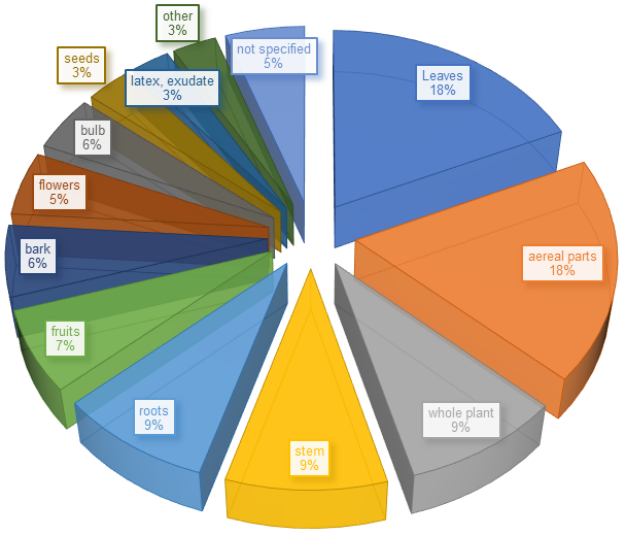

Fig. 1 Organs of the plant most used in veterinary practices

Medicinal properties of the plants are due to the large amount of active compounds that can be found in the vegetable kingdom. Often, active principles extracted from plants are equivalent to synthetic drugs according to their therapeutic efficacy; for this reason they are utilized in veterinary medicine, mainly as antibacterial, antimycotic, antiparasitic, disinfectants and immunostimulants.

Regarding large animals, natural compounds and phytotherapy are mainly utilized in organic farms to reduce the use of allopathic drugs more and

more. For the animal market many of the currently used antimicrobial, feed additive antibacterial, endectocide and anticoccidial drugs are either natural products or synthetics based on natural products (Ruddock, 2000). The majority of these natural products are produced from the fermentation broth of microorganisms, though plants have also been an important source of bioactives. There is an increasing public concern regarding the use of pharmaceuticals in the animal industry. Much of this has been as a result of the emergence of drug resistance. A particular area of criticism has been in the use of antibiotics as growth promoters and the associated risk of developing antibiotic resistance in human pathogens (Barton 2000). In organic farms, not only herbal drugs such as plant extracts and essential oils, but also homeopathic products, nutraceuticals and oligoelements, such as sodium, calcium, phosphorus, magnesium, and sulfur, are considered the main drugs to administer to animals for the treatment of different diseases. Nevertheless, it is possible to use synthetic allopathic drugs only when the previous products are ineffective; in such an eventuality, it is preferable to choose drugs that are metabolized rapidly, with a low environmental impact and less adverse effects on the animals. Herbal drugs used in human practice are often utilized in pets, in particular by owners who use such remedies for themselves. They are given to companion animals to treat respiratory, skin, urinary, digestive, and cardiovascular affections, and to reduce stress; moreover, they are also used to treat some chronic diseases instead of conventional drugs in order to avoid adverse effects that sometimes could occur as a consequence of a prolonged administration of synthetic drugs. Finally, phytotherapy could represent a useful support to conventional therapies in the case of severe illness (Severino and Ambrosio, 2012).

I.II.I Medicinal Plants Used in Veterinary Practice as Antibacterial

Many hundreds of plants worldwide are used in traditional medicine as treatments for bacterial infections. Some of these have also been subjected to *in vitro* screening but the efficacy of such herbal medicines has seldom been rigorously tested in controlled clinical trials. Conventional drugs usually provide effective antibiotic therapy for bacterial infections but there is an increasing problem of antibiotic resistance and a continuing need for new solutions. For these reasons, many researchers have attempted to find natural compounds to replace antibiotics to treat bacterial infections. While few studies have been carried out to evaluate the therapeutic efficacy of

herbal remedies in companion animals, many studies have been reported in the literature relating to the use of plants and plant materials in farm animals.

Several research articles focus on the potential antibacterial activity of medicinal plants used in ethnoveterinary medicine. Luseba et al. (2007) tested dichloromethane extracts for antibacterial and anti-inflammatory activity in a study appraising the efficacy of South African medicinal plants used in the treatment of wounds and retained placenta in livestock. *Cissus quadrangularis* L. (Vitaceae) stem and *Jatropha zeyheri* Sond. (Euphorbiaceae) root extracts showed anti-inflammatory activity against cyclooxygenase-2 enzyme. The extracts tested were not mutagenic in the Ames test against *Salmonella typhimurium* strain TA98.

The antibacterial activity of essential oils derived from plants such as *Salvia* spp. (Lamiaceae) and clove has been demonstrated against many microorganisms such as *Brucella, Salmonella typhimurium*, *E. coli, Bacillus cereus* and *Staphylococcus aureus* in several *in vitro* studies (Bouaziz et al., 2009; Burt, 2004; Horiuchi et al., 2007; Lans et al., 2009; Motamedi et al., 2010).

Intestinal nematodes are extremely important pathogens of domestic livestock, especially sheep, goats and cattle. Collectively, they are responsible for severe losses to livestock agriculture throughout the world. It has been calculated that, in the U.K., intestinal worms constitute the most important disease-related cost of farming sheep, being responsible for an estimated annual loss to the industry of £83 millions (Nieuwhof and Bishop, 2005). In developing countries, intestinal worm infestations are perceived to be the single most important threat to economic success, as was made dramatically clear in a recent review of the attitudes and concerns of small hold farmers in Africa (Perry, 2002).

Intestinal nematodes are also important pathogens of humans, with a range of pathologies and consequences for human health (Bethony et al., 2006); four species dominate: *Ascaris lumbricoides*, *Trichuris trichiura* and two hookworms *Ancylostoma duodenale* and *Necator americanus* (Bethony et al., 2006; Horton, 2003).

The treatment of intestinal nematode infestations in the 21st century is carried out using modern synthetic anthelmintics. Three classes of these anthelmintics dominate the market, each mediating the effect through a different mode of action on the target nematodes:

the benzimidazoles, the nicotinic aceticoline agonist such as pyrantel, levamisole, morantel and the macrocyclic lactones.

Although there are signs that novel synthetic drugs are being developed (e.g., nitazoxanide, cyclic depsipeptides, octadepsipeptides such as emodepside, tribendimidine, diketopiperazines such as paraherquamides, amino-acetonitrile derivatives), no new anthelmintic drugs have become available on the market for the treatment of either livestock or human parasitosis (Cappello et al., 2006; Geary et al., 1999; Harder et al., 2005; Xiao et al., 2005). Different studies have shown that some plant extracts effectively reduce parasite infestation in sheep and are promising alternatives to conventional anthelmintics (Githiori et al., 2006). An alternative to synthetic drugs is to exploit plant-occurring compounds. Medicinal plants and fruits have been used by indigenous people for centuries as sources of extracts used in the treatment of a variety of disorders, including infectious diseases and those caused by parasites, in livestock and humans (Hammond et al., 1997; Mueller and Mechler, 2005; Waller et al., 2001). These are often referred to as ethno-veterinary or ethno-medical remedies, and, in general, they are shunned by traditional, conventional western medicine (Behnke et al., 2008).

The use of ethnoveterinary plant preparations has been documented in different parts of the world (Anon, 1996; Bizimana, 1994; Waller et al., 2001; Wanyama, 1997). In many developing countries, farmers, herders, pastoralists and occasionally veterinary surgeons use plant or plant products to treat cases of parasitism. The related available evidence mainly concerns gastrointestinal helminths, but there is also evidence for effects on blood parasites and external parasites. In traditional societies, a number of plant remedies are described as suitable for each parasitic disease (Githiori et al., 2006). Some of the earliest known medicinal anthelmintic plants include *Carica papaya* L. (Caricaceae), *Ficus* spp. (Moraceae) and *Ananas comosus* (L.) Merr. (Bromeliaceae). Indeed, European doctors used papain and papaya latex for the treatment of worms in the 19th century (Jonxis and Bekins 1953, Stransky and Reyes 1955). The active components of this plant preparations are now known to be cysteines proteinases (CP) that occur naturally in various part of the plants.

Despite evidence of anti-parasitic properties of several plants or plant products, there is still a need to provide validated experimental data of biologically meaningful reductions in infestation levels to support the view that plants may play a direct role in the sustainable control of helminth

infestations in farming situations. Plant products that have shown high activity against nematode parasites *in vitro* need to be evaluated and tested in ruminant hosts. However, plants with moderate anthelmintic activity should still be considered; may be not as a unique alternative to anthelmintic drugs, but as part of an integrated approach specifically designed to achieve sustainable parasite control in a livestock production systems.

I.III Plant molecules for drug discovery

Plant compounds have been the source of inspiration for chemists and physicians for millennia, representing the richest font of novel compound classes and an essential wellspring of drugs and drug leads. According to Newman and Cragg (2016) 62% of the 1142 small-molecule introduced as drugs worldwide during 1981–2014 can be traced to or were inspired by natural products (*Fig.* 2). These include natural products (4%), natural product derivatives (21%), synthetic compounds with natural-product-derived pharmacophores (4%), and synthetic compounds designed on the basis of knowledge gained from a natural product (that is, a natural product mimic; 11%). In some therapeutic areas, the output is higher: 73% of antibacterials and 72% of anticancer compounds are natural products or have been derived from, or inspired by, a natural product.

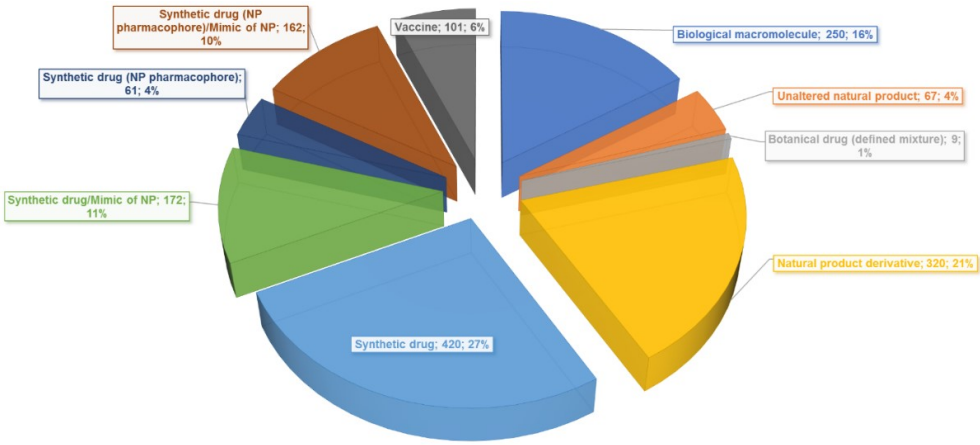

Fig. 2 All new approved drugs 1981−2014; n = 1562. (adopted by Newnan et al 2016)

These numbers are not surprising since it is known that natural products evolved for self-defense. Despite that record of productivity, natural products for drug discovery was de-emphasized in many big pharmaceutical companies in 1990s when combinatorial chemistry had place, and the reasons were primarily practical. But after several years was clear that database of natural products has a major number of unused scaffolds, and couldn't be discarded as starting points for new drugs discovery. The differences between synthetic compounds and natural products are remarkable, especially in their structural properties (Rosén et al., 2009). On average, natural products have higher molecular weights; incorporate fewer nitrogen, halogen, or sulfur atoms, but more oxygen atoms and are sterically more complex, with more bridgehead tetrahedral carbon atoms, rings, and chiral centers. This gives them a high "sterical complexity" due to the fact that the enzymes used for biosynthesis, as well as their molecular targets, are inherently three-dimensional and chiral. Furthermore, nature has a limited palette of building blocks at its disposal, and thus has to generate novelty by branching out common intermediates into different scaffolds (Lahlou, 2013).

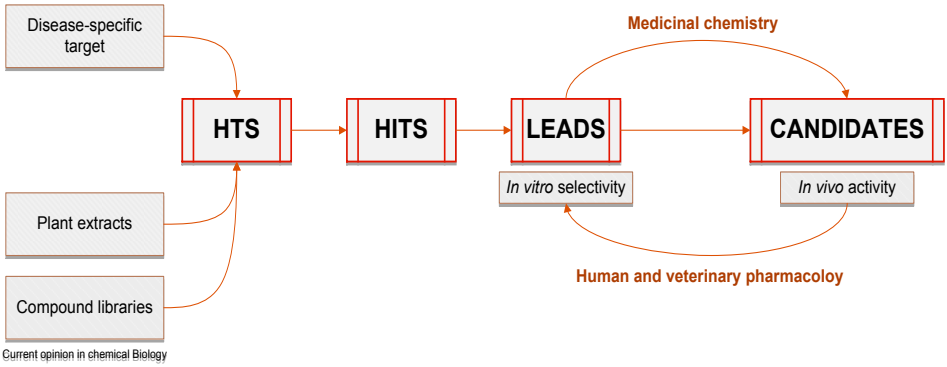

Fig. 3 The current lead discovery process

AIM OF THE PROJECT

The purpose of this project has been the identification of plant derivatives with biological activity mainly for veterinary use.
Accordingly, to this, the research project has been mainly focused on the following objectives:

- to validate the therapeutical properties of plants used in the traditional for both human and veterinary use;
- to isolate the compounds responsible for the biological activity;
- to individuate new natural bioactive compounds as leads for the design of new drugs;
- to search plants secondary metabolites with antiparasitic and antimicrobial activity.

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Chapter 1

Psiadia punctulata (DC.) Vatke

Asteraceae

D'Ambola, M., Malafronte, N., Bader, A., Bisio, A., Severino, L., Porta, A. De Tommasi, N. Antimicrobial activity of costituents of *Psiadia punctulata* (DC.) Vatke (Asteraceae), *manuscript in preparation*

1.1 Asteraceae family

The plant family of Asteraceae is distributed worldwide except for Antarctica. The Asteraceae (Compositae) with its approximately 12 subfamilies, 43 tribes, over 1,620 genera and more than 23,600 species is the largest family of flowering plants (Stevens, 2001)**.** Among these, several plants exhibit economic, medicinal, ornamental and food importance. Moreover plants belonging to this family are important sources of biologically active compounds, and they synthesize different classes of specialized metabolites such as flavonoids, phenylpropanoids, sesquitertenoids, diterpenoids, triterpenoids and a large variety of glycosides. (Yaoita et al., 2012). Particularly, the Asteraceae are rich in sesquiterpene lactones, it is reported that these compounds inhibit tumor growth by selective alkylation of biomolecules which control cell division, thereby inhibiting a variety of cellular functions hence directing the cell into apoptosis. (Chaturvedi, 2011).

1.2 *Psiadia* genus

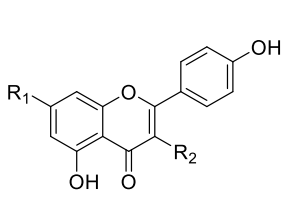
Psiadia genus includes about 60 species around the world (Oliver, 1916). Many plants belonging to the *Psiadia* genus *are* used in folk medicine in many countries. *Psiadia* ssp are used in traditional African medicine as infusion for the treatment of various disorders such as abdominal pain, fever and flu, or as an expectorant for asthma and bronchitis (Kokwaro et al., 1976). In Mauritius Islands, several *Psiadia ssp.* are traditionally used as infusion for the treatment of pulmonary infections, inflammatory diseases, small wounds and burns (Gurib-Fakim & Gueho, 1995 (Li et al., 2013).
During the last years, several plants of the *Psiadia* genus have been investigated for their high chemical diversity, and interesting biological activities. The secondary metabolites most frequently isolated in *Psiadia* ssp. are tetracyclic diterpenes, and highly oxygenated and metoxylated flavonoids. Some of the most characteristic flavonoids and diterpenoids of *Psiadia* genus are briefly reported below.

1.2.1 Flavonoids

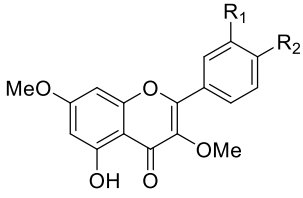
Flavonoids are nearly ubiquitous in plants and are recognized as the pigments responsible for the colours of leaves. Due to their chemical structure, flavonoids are largely known and used as antioxidant although

many biologically activities have been reported for these natural compounds such as anti-inflammatory, antiproliferative, antitumoral, antimicrobial (Middleton et al., 2000).

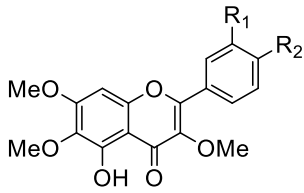
A large number of flavonoids has been discovered in different species of *Psiadia* such as *P.altissima, P. trinervia, P.dentata and P.arabica.* The biological activities reported for *Psiadia* flavonoids are antimicrobial and antioxidant activity. The flavonoids isolated from the genus *Psiadia* are shown in *Fig.* 1.1 (Canonica et al., 1967; El-Feraly et al., 1990; Jakobsen et al., 2001; Mossa et al., 1992a; Wang et al., 1989).



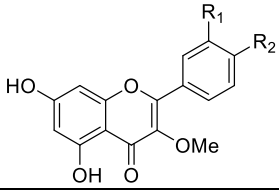
R_1	R_2
OH	H
OH	OH
OCH_3	OH



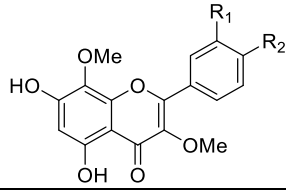
R_1	R_2
H	H
OH	OH
OH	OCH_3



R_1	R_2
H	OCH_3
OCH_3	OCH_3
H	OH



R_1	R_2
OCH_3	OH
H	OH
OH	OH



R_1	R_2
OCH_3	OH
OCH_3	OCH_3

Fig. 1.1. Flavonoids in Psiadia genus

1.2.2 Diterpenoids

Diterpenoids are a vast class of natural products, biosynthesized from mevalonic acid through 2*E*,6*E*,10*E*-geranylgeranyl pyrophosphate (GGPP).

Different biological activities have been described for diterpenes and their derivatives, including plant growth regulating, antimicrobial, antiparasitic, insect antifeedant, cytotoxic, antitumor, anti-HIV, antifertility, hypotensive and antiinflamatory activities, (García et al., 2007). The most important diterpenoids isolated from the genus *Psiadia* particularly *P. altissima* (DC.) Drake and *P. arabica* DC. are shown in *Fig.* 1.2 (Canonica et al.,1967, and El-Domiaty et al. 1993.

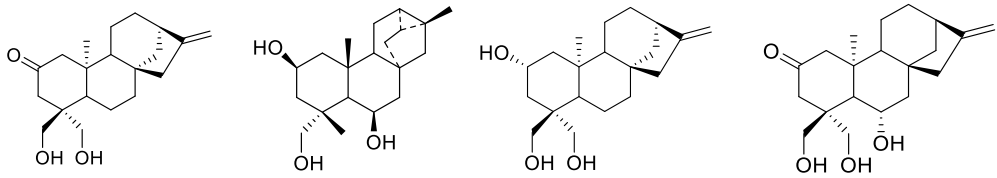


Fig. 1.2 Diterpenoids of Psiadia genus

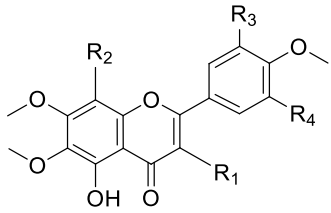
1.3 *Psiadia punctulata* (DC.) Vatke

Psiadia punctulata (DC) Vatke also known as*, Psiadia aparine mosses, Psiadia resiniflua, Baccharis resiniflua* is an African species gradually worldwide diffused. In Saudi Arabia. *P. punctulata* is well known in the antique Arabic medicine, it is called "Tobbag", this plant grows in the mountains surrounding by the Holy Makkah, it produces high amount of leaf exudate. The leaves exudate of *P. punctulata* is produced systematically and it is more abundant during hot seasons (Gushash, 2006). In the Saudi ethnomedicine the leaves rich in exudate are summered in hot water then the oily layer is collected and preserved in a recipient to be used as wounds disinfectant. The leaves are also burned as insect repellant, the warmed fresh leaves are applied locally to accelerate the healing of broken bones in human and animals (Ogweno Midiwo et al., 1997). A leaves decoction is used locally for the treatment of cold and fever (Kokwaro, 1976) and for the removal of ectoparasites from cattle (Beentje, 1994). The shrub is known to be avoided by browsing herbivores like giraffes and goats, even during severe drought.

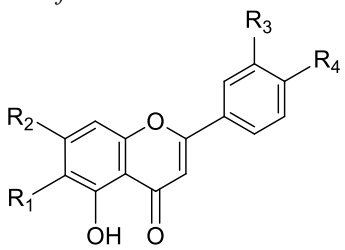
1.3.1 Previous phytochemical studies on Psiadia punctulata (DC) Vatke

Previous phytochemical studies of leaves exudate reveal the presence of different class of metabolites including diterpenes, flavonoids, phenylpropanoids (Juma et al., 2006; Juma et al., 2001; Midiwo et al., 1997),

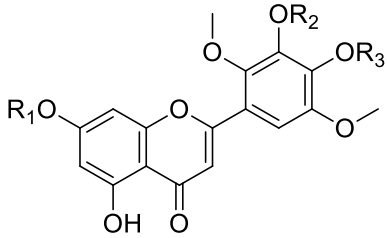
while the propolis obtained from this plant is rich in diterpenes and flavonoids (Almutairi et al., 2014). The main flavonoid isolated in *P.punctulata* exudate are reported in *Fig.* 1.3.



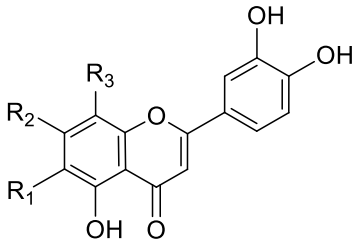
Compounds	**R1**	**R2**	**R3**	**R4**
5 OH-6,7,8,4'-tetrametossiflavon	H	OCH3	H	H
5 OH-3,6,7,4'-tetrametossiflavon	OCH_3	H	H	H
5,3' OH-6,7,4',5'-tetrametossiflavon	H	H	OH	OCH_3
5,3' OH-6,7,8,4',5'-pentametossiflavon	H	OCH_3	OH	OCH_3
5 OH-6,7,3',4',5'-pentametossiflavon	H	H	OCH_3	OCH_3



Compounds	**R1**	**R2**	**R3**	**R4**
Chrysoeriol	H	OH	OCH_3	OH
Cirsilineol	OCH_3	OCH_3	OCH_3	OH
Luteolin	H	OH	OH	OH
Acacetin	H	OH	H	OCH_3
Apigenin	H	OH	H	OH



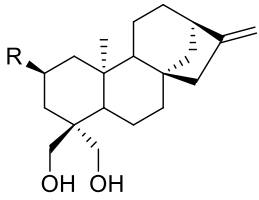
Compounds	**R1**	**R2**	**R3**
5,7- OH-2',3',4',5'-tetrametossiflavon	H	CH_3	CH_3
5- OH-7,2',3',4',5'-pentametossiflavon	CH3	CH3	CH3
5,4'- OH-7,2',3',5'-tetrametossiflavon	CH3	CH3	H
5,7,4'- OH-2',3',5'-trimetossiflavon	H	CH3	H
5,7,3'- OH-2',4',5'-trimetossiflavon	H	H	CH3



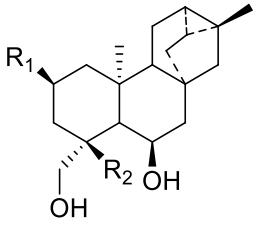
Compounds	**R_1**	**R_2**	**R_3**
Cynaroside	H	O-Glu	H
Orientin	H	OH	O-Glu
Iso orientin	O-Glu	OH	H

Fig. 1.3 Flavonoids of P. punctulata

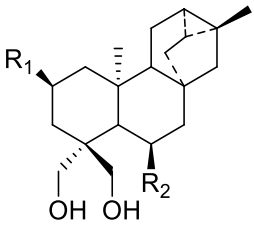
The diterpenoids obtained from the *P. punctulata* exudate are shown in *Fig.* 1.4.



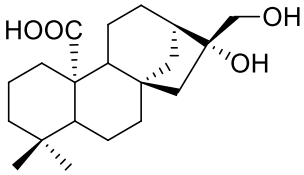
Compounds	**R_1**
(ent)-Kaur-16-en-2α,18,19-triol	OH
(ent)-2-oxokaur-16-en-18,19-diolo	=O



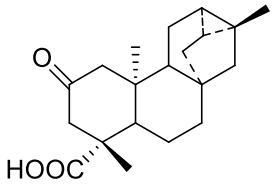
Compounds	**R_1**	**R_2**
Trachyloban-2β,6β, 19-triol	OH	CH_3
2-oxotrachyloban-18,19-diol	=O	CH_3
6α,17,19-ent-Trachylobantriol	H_2	CH_2OH



Compounds	**R_1**	**R_2**
2α,18,19-ent-Trachylobantriol	OH	H
2β,6α,18,19-ent-Trachylobantetraol	OH	OH



(ent)-16β,17-Dihydroxykaur-20-oic acid



ciliaric acid

Fig. 1.4 Kaurane and trachylobane diterpenes of P. punctulata exudate

1.3.2 P. punctulata biological activities

Studies reported the biological properties of *Psiadia punctulata.* Midiwo et al. (2002) highlighted the pesticides effects of the leaves exudate against *Locusta migratoria,* and the antifungal activity against *Colletotricum coffeanum* and *Fusarium ossisporum* respectively fungi that infest coffee and potatoes. Several authors reported the antiprotozoal activity of the bark of *P punctulata* against *Leishmania major* (Mothana et al., 2011).

The methanol extract of *P. punctulata* whole plant was *in vivo* tested in order to investigate Its pharmacological activities. The results showed a blood pressure lowering and the phrenic neuromuscular nerve blocking. The same extract was also test on unstriated muscle of mice trachea showing relaxant properties. Furthermore, methanol extract of *P. punctulata* was demonstrated to induce contraction of mean intestine in rabbit; this pharmacological activity could be compared to effect of Verapamil and may

be due to interference with the depolarization processes linked to the operation of the voltage-gated calcium channels (Achola et al., 1998).

1.3.3 Antimicrobial activity

In recent years, there have been concerns about increased frequencies of antibiotic resistance among bacteria found in food animals and in the environment (Jensen et al., 2001). In fact, the extensive use of antibiotics in animal practice for therapy are considered to be the major reasons for the development of bacterial resistance to antibiotics (Barton, 2000; Sengeløv et al., 2003). Antibiotics are used continuously as feed additives at sub-therapeutic levels to promote growth to increase feed efficiency and to prevent infection and (Jindal et al., 2006; Wegener, 2003).

Presently, there is a growing interest in natural antibacterial derivatives such as extracts of spice and herb for both food preservation and for the development of new antibacterial compounds. Natural compounds could be an alternative source of novel therapeutics, the relatively low frequency of infectious diseases in wild plants suggests that in many cases these natural defence mechanisms can be very effective, than many plants are investigated for their potential and antimicrobial activity (Hemaiswarya et al., 2008; Shan et al., 2007). *Staphylococcus aureus* is a major opportunistic pathogen in humans and one of the most important pathogenic *Staphylococcus* species in veterinary medicine. *S. aureus* is dangerous because of its deleterious effects on animal health and its potential for transmission from animals to humans and vice-versa. It thus has a huge impact on animal health and welfare and causes major economic losses in livestock production (Holmes and Zadoks, 2011). *S. aureus* is a saprophyte of mucous membranes and the skin of man and other mammals. It's able to colonize the udder of milk producing animals (cows, sheep, goats) and also is the most frequently responsible for food poisoning species, is one of the most feared bacteria, because of Its multidrug-resistant strains to antibiotics (Peton and Le Loir, 2014). The study of Gouda et al. (2014) showed that the methanol extract of *P. punctulata* whole plant exhibited a significant antibacterial activity against the Gram-positive *S. aureus* 25913 and antifungal activity against *Candida albicans* ATCC 10231.

Moreover, in the study of Mothana et al. (2011) was investigated the antibacterial activity of the methanolic and water extracts from *P. punctulata* whole plant against *Staphylococcus aureus ATCC 6538; Bacillus subtilis* ATCC 6059; *Micrococcus flavus* SBUG 16; *Escherichia coli* ATCC

11229; *Pseudomonas aeruginosa* ATCC 27853; *Candida maltosa* SBUG; multiresistant *Staphylococcus epidermidis* 847; multiresistant *Staphylococcus haemolyticus* 535; multiresistant Staphylococcus aureus NGR. The result showed that the methanolic extract was more effective than the water extract, as displayed by the respective inhibition diameters against the following microbial strain: *Staphylococcus aureus ATCC 6538* (14mm and 11mm)*; Bacillus subtilis* ATCC 6059 (12mm and 0mm); *Micrococcus flavus* SBUG 16 (22mm and 14 mm); multiresistant *Staphylococcus epidermidis* 847 (20 mm and 15 mm); multiresistant *Staphylococcus haemolyticus* 535 (12 mm and 0 mm); multiresistant Staphylococcus aureus NGR (20 mm and 16 mm). On the other hand, no activity was observed against *Escherichia coli* ATCC 11229; *Pseudomonas aeruginosa* ATCC 27853; *Candida maltosa* SBUG.

AIM

In the present work, the composition of the *Psiadia punctulata* surface secondary metabolites and the antimicrobial activities of the surface extract, crude fractions and pure compounds were investigated.

1.4 Experimental

1.4.1 Plant material

The exudate obtained from the leaves (400g) of *P. punctulata* was collected in Wadi Ghazal, Saudi Arabia, in June 2012. The leaves of the plant were identified by Dr. Ammar Bader. A voucher specimen (SA/IT 2013/1) was deposited in the Laboratory of Pharmacognosy at *Umm Al-Qura University,* Saudi Arabia.

1.4.2 Extraction and isolation

The exudate of *P. punctulata* (10.0 g) was obtained from the leaves (400g) by dipping into dichloromethane for less than 30 seconds and it was dried at 40 °C. Part of exudate (5.0 g) was dissolved in chloroform and separated on silica gel column, eluted with step gradients of $CHCl_3$-MeOH (100:0, 90:10,80:20, 70:30, 50:50 and 0:100). Fractions of 25 mL were collected, pointed and sprayed with a solution of $Ce_2(SO_4)_2/H_2SO_4$, 65% on TLC plates and lastly grouped into nine main fractions (A-I) *Fig.* 1.5.

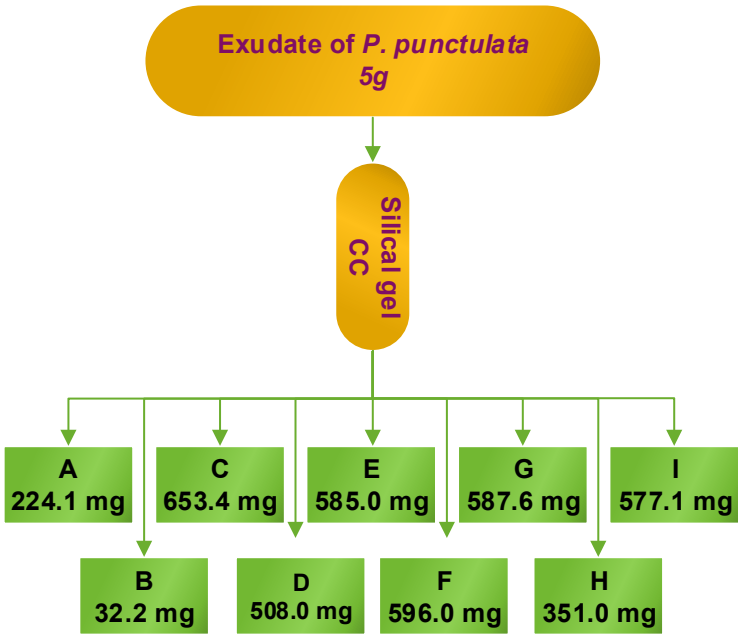


Fig. 1.5 Separation scheme exudate of P. punctulata exudate

Fraction C (653 mg) and D (508.0 mg) were purified by semi-preparative reversed phase HPLC (MeOH-H_2O, 65:35) to yield pure **1** (2.2 mg, t_R 6 min), **2** (4.3 mg, t_R 9.5 min), **3** (7 mg, t_R 14 min), **4** (1.9 mg, t_R 15.5 min), **5** (2.2 mg, t_R 18 min) **6** (1.2 mg, t_R 32 min) and **7** (1.4 mg, t_R 36 min) from fraction C, and **8** (4.3 mg, t_R 8 min), **9** (3.7 mg, t_R 11 min), **10** (4.9 mg, t_R 13 min), **11** (2.7 mg, t_R 15 min) **12** (1.2 mg, t_R 17 min) and **13** (1.2 mg, t_R 20.5 min) from fraction D. Fraction E (585.0 mg) was subjected to RP-HPLC (MeOH-H_2O, 55:45) to give pure compounds **14** (1.0 mg, t_R 14.5 min), **15** (5.3 mg, t_R 22.5 min) **16** (4.1 mg, t_R 32 min) **17** (1.6 mg, t_R 36 min) and **18** (1.1 mg, t_R 40 min). Fraction F (596.3 mg) was subjected to RP-HPLC (MeOH-H_2O, 1:1) to give pure compounds **19** (1.8 mg, t_R 5 min), **20** (1.0 mg, t_R 7.5 min) and **21** (1.5 mg, t_R 41 min). Fraction G (587.0 mg) was separated to RP-HPLC (MeOH-H_2O, 55:45) to yield pure compounds **22** (1.3 mg, t_R 16 min), **23** (1.5 mg, t_R 27.5 min), **24** (1.7 mg, t_R 36.5 min) **25** (1.1 mg, t_R 57.0 min) and **26** (2.3 mg, t_R 62.5 min). Fraction H (351.0 mg) was subjected to RP-HPLC (MeOH-H_2O, 1:1) to give pure compounds **27** (1.0 mg, t_R 3 min), **28** (3.5 mg, t_R 35 min), **23** (0.6 mg, t_R 45 min) **29** (7.5 mg, t_R 60 min) and **24** (1.5 mg, t_R 60 min), **30** (1.7mg, tr 12min).

*1.4.2.1 Compound **10***

White substance; $[\alpha]_D^{25}$: -152.6° (c 0.26, CH_3OH); for ^1H and ^{13}C NMR spectroscopic data, see *Tab.* 1.6; HRESIMS *m/z*: 319,2267 $[M+H]^+$, calcd. for $C_{20}H_{30}O_3$, 318.2195.

*1.4.2.2 Compound **14***

Amorphous white powder; $[\alpha]_D^{25}$: -22.3° (c 0.06, CH_3OH); for ^1H and ^{13}C NMR spectroscopic data, see *Tab.* 1.3; HRESIMS *m/z*: 323.2581 $[M+H]^+$, calcd. for $C_{20}H_{34}O_3$, 322.2508.

*1.4.2.3 Compound **18***

White substance; $[\alpha]_D^{25}$: -67.5° (c 0.04, CH_3OH); for ^1H and ^{13}C NMR spectroscopic data, see *Tab.* 1.6; HRESIMS *m/z*: 321.2422 $[M+H]^+$, calcd for $C_{20}H_{32}O_3$, 320.2351.

*1.4.2.4 Compound **19***

White substance; $[\alpha]_D^{25}$: -109.3° (c 0.15, CH_3OH); for ^1H and ^{13}C NMR spectroscopic data, see *Tab.* 1.7. HRESIMS *m/z*: 335.2215 $[M+H]^+$, calcd for $C_{20}H_{30}O_4$, 334.2144.

*1.4.2.5 Compound **20***

White substance; $[\alpha]_D^{25}$: -65.8° (c 0.08, CH_3OH); for ^1H and ^{13}C NMR spectroscopic data, see *Tab.* 1.7. HRESIMS *m/z*: 335.2216 $[M+H]^+$, calcd. for $C_{20}H_{30}O_4$, 334.2144.

*1.4.2.6 Compound **21***

Amorphous white powder; $[\alpha]_D^{25}$: -85.2° (c 0.14, CH_3OH); for ^1H and ^{13}C NMR spectroscopic data, see *Tab.* 1.4. HRESIMS *m/z*: 321.2422 $[M+H]^+$, calcd for $C_{20}H_{32}O_3$, 320.2351.

*1.4.2.7 Compound **22***

White substance; $[\alpha]_D^{25}$: -20.9° (c 0.07, CH_3OH); for ^1H and ^{13}C NMR spectroscopic data, see *Tab.* 1.8. HRESIMS *m/z*: 337.2371 $[M+H]^+$, calcd for $C_{20}H_{32}O_4$, 336.2301.

*1.4.2.8 Compound **23***

Amorphous white powder; $[\alpha]_D^{25}$: -123.7° (c 0.13, CH_3OH); for ^1H and ^{13}C NMR spectroscopic data, see *Tab.* 1.4. HRESIMS *m/z*: 337.2373 $[M+H]^+$, calcd for $C_{20}H_{32}O_4$, 336.2301.

*1.4.2.9 Compound **24***

White oil-like substance; $[\alpha]_D^{25}$: -75.0° (c 0.14, CH_3OH); for ^1H and ^{13}C NMR spectroscopic data, see *Tab.* 1.8. HRESIMS *m/z*: 335.2218 $[M+H]^+$, calcd. for $C_{20}H_{30}O_4$, 334.2144.

*1.4.2.10 Compound **25***

Amorphous white powder; $[\alpha]_D^{25}$: -18.1° (c 0.06, CH_3OH); for ^1H and ^{13}C NMR spectroscopic data, see *Tab.* 1.5. HRESIMS *m/z*: 323.2580 $[M+H]^+$, calcd. for $C_{20}H_{34}O_3$, 322.2508.

*1.4.2.11 Compound **27***

Amorphous white powder; $[\alpha]_D^{25}$: -77.9° (c 0.04, CH_3OH); for ^1H and ^{13}C NMR spectroscopic data, see *Tab.* 1.5. HRESIMS *m/z*: 353.2331 $[M+H]^+$, calcd for $C_{20}H_{32}O_5$, 352.2250.

*1.4.2.12 Compound **28***

Amorphous white powder; $[\alpha]_D^{25}$: -62.9° (c 0.17, CH_3OH); for ^1H and ^{13}C NMR spectroscopic data, see *Tab.* 1.5. HRESIMS *m/z*: 339.2528 $[M+H]^+$, calcd for $C_{20}H_{34}O_4$, 338.2457.

*1.4.2.13 Compound **30***

Amorphous white powder; $[\alpha]_D^{25}$: -57.0°; for ^1H and ^{13}C NMR spectroscopic data, see *Tab.* 1.3. HRESIMS *m/z*: 339.2531 $[M+H]^+$, calcd. for $C_{20}H_{34}O_4$, 338.2457.

1.4.3 In vitro antimicrobial activity

The *in vitro* minimal inhibitory concentrations (MICs) against *Candida albicans* (ATCC MYA-2876) strains was determined by the micro-broth dilution method in 96-well plates according to the guidelines suggested by the CLSI document M27-A3 (Clinical and Laboratory Standards Institute.

Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts—Third Edition: Approved Standard M27-A3. CLSI, Wayne, PA, USA, 2008.). Colonies from Sabouraud's dextrose agar plate were inoculated into prewarmed RPMI 1640 medium (with l-glutamine, 2% glucose, without bicarbonate, and with phenol red as a pH indicator) buffered to pH 7.0 with 0.165M morpholine propane sulfunic acid (MOPS). Two-fold serial dilutions of extract (concentrations ranging from 10 to 640 ug/ml) or fractions (concentrations ranging from 6.25 to 200 ug/ml) were added to each well containing 2×10^3 yeast/ml. After 24 and 48 h of incubation at 37°C growth was measured by reading the absorbance with a microplate reader (Lab-system Multiskan EX 51118171, Thermo Scientific, Milan, Italy) at 530 nm. MIC_2 were recorded as lowest concentrations of extract or fraction that resulted in 50% decrease in absorbance compared to the control (cells containing only medium and vehicle).
Similarly, the antibacterial activity of extract and fractions were tested against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The MICs were carried out in Mueller-Hinton broth (MHB) by microdilution method using 96-well microtiter plates according to the Clinical and Laboratory Standards Institute (CLSI) guideline (CLSI 2008. Performance standards for antimicrobial susceptibility testing: 18th informational supplement. CLSI document. Wayne, PA: Clinical Laboratories Standards Institute, pp. 46–52). Briefly, bacteria were grown o.n. in MHB medium at 37°C. Cultures were then diluted to approximately 10^6 CFU/mL in fresh MHB medium, and 200 µL was used to inoculate flat-bottom 96-well polystyrene microtiter plate. Different concentration of extract concentrations ranging from 10 to 640 ug/ml) and fractions (concentrations ranging from 6.25 to 200 ug/ml) were added in each well, and after incubation o.n. at 37°C, cells absorbance was measured at 595 nm using a microplate spectrophotometer. Cells treated with only vehicle were used as control.

1.4.3.1 Biofilm formation

In order to determine the capacity of fractions to inhibit the biofilm formation of *Candida*, yeast cells were inoculated into 7ml of Yeast Nitrogen Base (YNB) with 100 mM glucose (pH 7.0) and incubated overnight at 25 °C. Using the McFarland *Tab.*, appropriate dilutions of the o.n. cultures were carried out in order to obtain 10^7 cells/mL. Culture was aliquoted in a 96-wells flat bottom plate (0.2 ml/well). The *Candida* cells

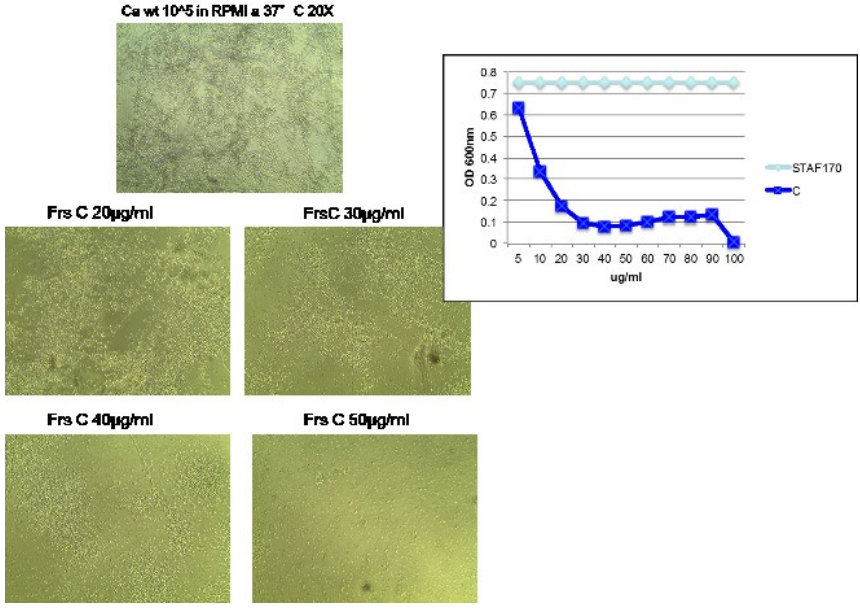
were allowed to adhere for 90 min at 37 °C, afterwards, wells were washed with phosphate buffered saline (PBS, pH 7.2) to remove *Candida* non adherent cells. To allow the adherent yeast cells to germinate and eventually to form biofilms, new YNB medium pH 7.0 with different concentrations of fractions 2, 3, 8 and 27 (40, 60, 80 and 100 ug/ml) were added to each well and incubated at 37 °C. After 48 hours the biofilm mass was washed with PBS, dried and stained with crystal violet (CV, 0.3% (w/v) for 15 min. After rinsing with water, to quantify the biofilm mass, the CV was solubilized with 200 µL of acetic acid (33% v/v), and absorbance was measured at 595nm using a spectrophotometer.
A similar protocol was used to analyse the effect of fractions 2, 3, 8 and 27 (40, 60, 80 and 100 ug/ml) on *Staphylococcus* biofilm formation. Briefly, the culture supernatant was discarded, and the wells were washed twice with PBS to remove loosely adherent cells and then stained for 1 min with 200 µL of CV. The wells were then rinsed with water and dried. The amount of biofilm biomass was quantified by destaining wells with 200 µL of 33% acetic acid and then measuring the absorbance of the CV solution in a microplate spectrophotometer set at 595 nm. Cells treated with only vehicle were used as control.
Each antimicrobial assay was performed in triplicate in separate days.

1.5 Results and Discussions

The antimicrobial activity of the dichloromethane extract was tested against a Gram-positive (*Staphylococcus aureus)*, a Gram-negative bacteria (*Pseudomonas aeruginosa*), and the dimorphic fungi *Candida albicans,* causal agent of opportunistic oral and genital infections in humans. The results showed an antibacterial activity of the extract against *S. aureus* (MIC_{50}= 100 µg/mL) while no activity was detected against the Gram-negative bacteria. Moreover, the extract showed an antifungal activity aganinst *C.* albicans, with MIC_2= 50 µg/mL and MIC_0= 130 µg/mL.
The extract was subsequently separated by column chromatography on silica gel, collecting nine fractions A-I. Fractions were evaluated for their antimicrobial activity at the doses (from 20 to 100 µg/ml). Of the 9 fractions analyzed, fractions A, B and I were inactive on all the species tested while 6 fractions (C-H) were active on the Gram-positive and on *Candida albigans* but not on the Gram-negative strain (*Tab.* 1.1 and *Fig.* 1.6).

Tab. 1.1 Fractions (A-I) antimicrobial activity

Fractions	MIC (µg/mL)			
	Antibacterial activity		Antifungal activity	
	Staphylococcus aureus	*Pseudomonas aeruginosa*	*Candida albicans*	
	MIC_{50}		MIC_2	MIC_0
A	> 200	> 200	-	> 200
B	87	> 200	20	60
C	8	> 200	15	50
D	18	> 200	50	100
E	73	> 200	80	> 200
F	62	> 200	60	100
G	43	> 200	100	> 200
H	65	> 200	70	90
I	> 200	> 200	80	150



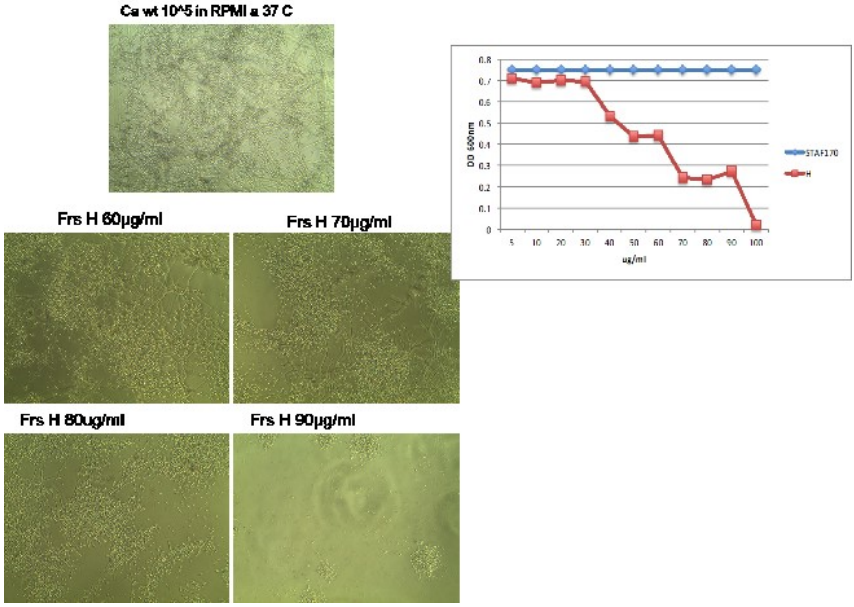


Fig. 1.6 Antibacterial activity of fractions C (Frs C) and H (Frs H) against Staphylococcus aureus (STA) and Candida albigans (CA)

The dichloromethane extract of *P. punctulata* active fractions were then separated by silica gel column chromatography and RP-HPLC to afford thirteen new compounds (**10, 14, 18-25, 27-28, 30**) along with known flavones (**1-8** and **11**) and diterpenes (**9**, **12-13**, **15**, **17**, **26**, **29**) (*Fig.* 1.16). The flavonoids were 3',4',5-trihydroxy-6,7,8-trimethoxyflavone (sideritoflavone) (**1**) (Tomas et al., 1979b), 2',4',5-trihydroxy-6,7,5'-trimethoxyflavone (arcapillin) (**2**) (Kiso et al., 1982), 3',4',5,7-tetramethoxyflavone (**3**) (Joseph-Nathan et al., 1981), 4',5-dihydoxy-6,7,8-trimethoxyflavone (xanthomicrol) (**4**) (Stout and Stout, 1961), 5-hydroxy-3',4',5',6,7,8-hexamethoxyflavone (**5**) (gardenin A) (Rao and Venkataraman, 1968), 4',5-dihydroxy-3',6,7,8-tetramethoxyflavone (**6**) (Pinkas et al., 1968), 3',5-dihydroxy-4',5',6,7,8-pentamethoxyflavon (Gardenin C) (**7**) (Sujatha et al.,2013),5,7-dihydroxy-3′,4'-dimethoxyflavone (Luteolin 3',4' dimethyl ether) (**8**) (Gao and Kawabata., 2004), 4',5-dihydroxy-3',7-dimethoxyflavone (velutin) (**11**) (Das et al., 1970); the diterpenes were *ent*-2-oxo-kaur-16-en-6,19-diol (propsiadin) (**9**) (Almutairi et al., 2014), *ent*-2-oxo-kaur-16-en-18,19-diol (psiadin) (**12**) (Almutairi et al., 2014; El-Domiaty et al., 1993; Mossa et al., 1992b), *ent*-2-oxo-trachyloban-18,19-

diol (**13**) (Midiwo et al., 1997), *ent*-2-oxo-6-hydroxy-kaur-16-en-18,19-diol (**15**) (El-Domiaty et al., 1993), *ent*-trachyloban-2β,6β,19-triol (**16**) (Midiwo et al., 1997), *ent*-trachyloban-6α,18,19- triol (**17**) (Juma et al., 2006), *ent*-kaur-16-en-2α,18,19-triol (**26**) (El-Shafae and Ibrahim, 2003; Midiwo et al., 1997), *ent*-trachyloban-2α,17,18-triol (**29**) (Juma et al., 2006), identified by their physical and spectroscopic data, which were largely consistent with those published in the literature.

Analysis of the NMR data of compounds **14**, **21**, **23**, **25, 27**, **28**, **30** suggested diterpenes featuring an *ent*-kaurane skeleton (García et al., 2007a; Kataev et al., 2011). This observation was confirmed by ^{1}H-^{1}H COSY data that revealed the spin systems H-1—H_2-2—H-3, H-5—H-6—H_2-7and H-9—H_2-11—H-12—H-13—H_2-14 and by HMBC correlations observed from H-5 (δ_H 0.80 - 1.35) to C-4, C-6, C-7, C-10, C-18, C-19, C-20, from H-9 (δ_H 1.07-1.33) to C-7, C-8, C-10, C-11, C-14, C-15, C-20, from H_2-7 (δ_H 1.20-1.69 and 1.37-1.88) to C-5, C-6, C-8, C-14, C-15, from H_2-14 (δ_H 1.27-1.81 and 1.90-2.28) to C-8, C-9, C-13, C-15, C-16 and from H_2-15 (δ_H 1.44-2.18 and 1.31-2.20) to C-7, C-8, C-9, C14, C-16. 1D-ROESY correlations H-9β/H-15β, H-5/H_3-18, H_2-15/H-16, H_3-19/H_3-20, H_2-19 and H_3-20/H_2-14 provided stereochemical information of the *ent*-kaurane skeleton.

Compound **14** (*Fig.* 1.7*)* was obtained as an amorphous white powder. Its molecular formula $C_{20}H_{34}O_3$, equating to four double bond equivalents, was deduced from HRESIMS (*m/z*: 323.2581$[M+H]^+$.

The ^{13}C NMR spectra (*Tab.* 1.2) revealed the presence of four methyls, seven methylenes, five methines of which two oxygenated and four quaternary carbons one oxygenated. The presence of two oxigenated methynes, at C-3 and C-12 (δ_C 79.3 and 67.7, respectively), was confirmed by the ^{1}H-^{1}H COSY and 1D-TOCSY correlations H_2-1—H_2-2, H-3—H_2-2, H-9—H_2-11, H-12—H_2-11, H-12—H-13 and H-13—H-14 and HMBC correlations of H_2-2/C-4, H3/C-18, H3/C-19, H_3-19/C-3, C-18/H-3, H-11/C-12, H-14/C-12. The quaternary oxygenated function was collocated at C-16 (δ_C 79.0), supported by HMBC correlations H-13/C-16, H-14/C-16, H-15/C-16, H-17/C-16. The relative configuration of the basic skeleton of compound **14** was elucidated to be the same with that of *ent*-kaurane-3α,16α-diol (Tori et al., 1993) on the basis of the double doublets resonance of H-3 at δ_H 3.15 and by analysis of the 1D ROESY spectra: selective irradiation of H-3 produced significant enhancement of the proton signal at δ_H 0.79 (H-5) and 0.99 (H_3-18). Furthermore, upon irradiation of H-12 (δ_H

3.90) a ROE response with H-13 (δ_H 1.96) and H-17 (δ_H 1.34) was observed. Thus, **14** was charachterized as *ent*-kaurane-3α,12α,16α-triol.

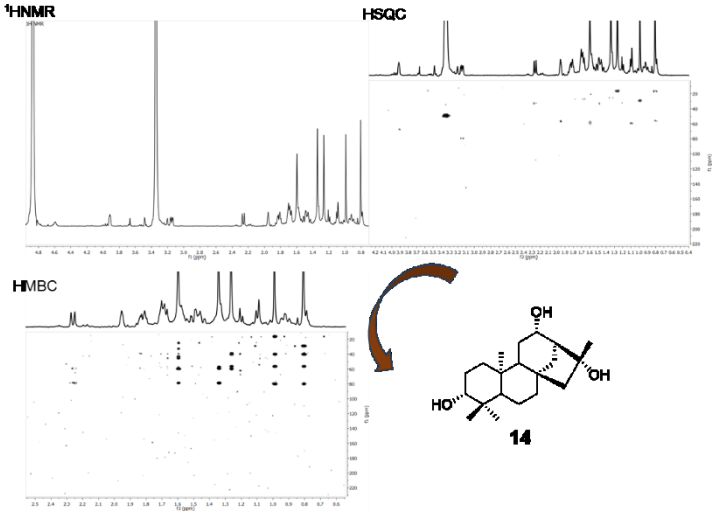
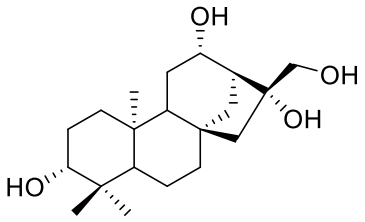


*Fig. 1.7 NMR spectra of compound **14***

Compound **30** was obtained as a colourless powder. The positive HRESIMS gave a quasi-molecular ion peak at *m/z* 339.2531 in accordance with the molecular formula $C_{20}H_{34}O_4$ and four indices of hydrogen deficiency. The NMR data *Tab.* 1.2 of compound **30** showed marked similarities with compound **14**, except for resonances of an additional oxymethylene (δ_H 3.58, 3.65) group and the absence of the signal for one methyl group. The HMBC cross-peaks of signals at ppm 3.58 (1H, d,*J*=13.0 Hz) and 3.65 (1H,d,*J*=13.0 Hz) with C-13, C-15 and C-16 suggested that the hydroxy group was located at C-17.

The 1D-ROE correlations of Me-18 with the H5/H9 indicated a β-orientation of H-5 and H-9 and α-orientation of OH-3. The β orientation of CH_2OH-17 was elucidated comparison of by the ^{13}C NMR data with those of literature (Liug et al.,2003).

Thus, the structure of **30** was defined as *ent*-kaurane-3α,12α,16α,17-tetraol.



30

Compound **21** was obtained as an amorphous white powder and it was assigned the molecular formula $C_{20}H_{32}O_3$ by its HRESIMS *m/z*: 321.2422 $[M+H]^+$, indicating five hydrogens deficiency.

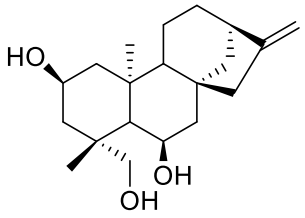
The ^1H NMR spectrum showed the presences of two methyl singlets at δ_H 1.29 (Me-19) and δ_H 1.20 (Me-20); two oxymethines at δ_H 3.82 (H-2) and δ_H 4.07 (H-6), one hydroxymetilene at δ_H 3.96 and 3.50 (H_2-19) and two H-2 olefinic protons at δ_H 4.80 ($1H_1$, s) and 4.85 ($1H_1$, s).

COSY and 1D-TOCSY measurements showed couplings between H1—H3, H5—H7, H9—H14, H15—H17, led to the assignment of the molecule spin systems. The elucidation of the whole skeleton from the above subunits was achieved on the basis of HSQC and HMBC correlations which also allowed the assignment of the resonances of the pertinent carbons

The ^1H and ^{13}C NMR data (*Tab.* 1.3) were quite similar to those reported for *ent*-2-oxo-kaur-16-en-6,19-diol (propsiadin) (Almutairi et al., 2014). The structure of **21** differed from propsiadin in C-2, where the oxo group was replaced by an oxymethine group (δ_C 64.3; δ_H 3.83 m). This was verified by HMBC correlations H1/C2 and H3/C2, H1/C3, H1/C2, H1/C5, H3/C1, H3/C2; H3/C5.

The α-axial orientation of H-2 was inferred by its *J* values (tt, $J_{(H\text{-}3ax/H\text{-}2)}$ = 15.6 Hz, $J_{(H\text{-}3eq/H\text{-}2)}$ = 5.5 Hz, $J_{(H\text{-}1ax/H\text{-}2)}$ = 13.6 Hz. The α-axial orientation of H-6 was inferred by its *J* values (ddd, $J_{(H\text{-}6ax/H\text{-}5)}$ = 13.0 Hz). The relative position of the substituents was confirmed by 1D-Roesy spectra.

From the above information and literature data, the structure of compound **21** was then elucidated as *ent*-kaur-16-en-2β, 6β,19-triol.

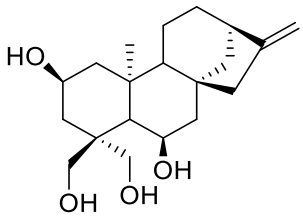


21

Compound **23** was obtained as an amorphous white powder, It showed a molecular formula of $C_{20}H_{32}O_4$, according to an $[M+H]^+$ ion at *m/z* 337.2373 (calcd. for 336.2301) in its HRESIMS, indicative of five indices of hydrogen deficiency.

The ^1H and ^{13}C NMR data of **23** (*Tab.* 1.3) indicated the presence of two hydroxymethylenes and two hydroximethine groups moreover, an exo-methylene group was clearly evident in ^1H NMR spectrum (2 H, br s at δ_H 4.35 and 4.38). The NMR spectral data combined with 1D-TOCSY and COSY suggested the sequences H1—H3, H5—H7, H9—H14, H15—H17.

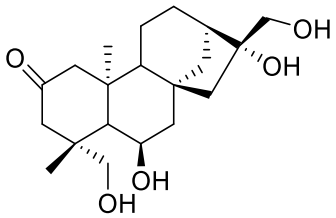
The ^1H and ^{13}C NMR data of **23** showed a high degree of similarity with compound **21**. The signals, in the NMR spectrum of **23**, at δ_H 3.62 d (*J*=11.0 Hz), 4.03 (overlapped signal) and δ_C 65.4 suggested the presence of an additional oxymethyl group, this was located at C-18 on the basis of chemical shift and HMBC correlations H_2-3/C-18, H-5/C-18, H_2-18/C-19, H_2-19/C-18 and H_2-18/C-4 Therefore, the structure of **23** was identified as *ent*-kaur-16(17)-en-2β,6β,18,19-tetraol.



23

Compound **27**, obtained as a white powder, gave a molecular formula of $C_{20}H_{32}O_5$ according to a $[M+H]^+$ ion at *m/z* 353.2331 its HRESIMS requiring five degrees of unsaturation. The ^1H and ^{13}C NMR (*Tab.* 1.4) features of **27** were closely related to those of *ent*-16α-17,19-trihydroxy-kauran-2-one (Chen et al., 2010). The difference between the compounds was that the methilene moiety at position 6 in *ent*-16α-17,19-trihydroxy-kauran-2-one this was replaced by an oxymethine in **27**. This assignment was confirmed by the downfield shifting of C-6 [δ_C 68.7; δ_H 4.04 br d (*J*=3.9 Hz)], C-5 (59.2 ppm) and C-7 (51.3 ppm) and an upfield shift of C4 (42.9 ppm) and by the 1D-TOCSY spectrum showing H-5—H-6—H_2-7 spin system. The β OH-6 was defined by the observation of the H-6 coupling constant [δ_H 4.04 br d (*J* = 3.9)], typical of an axial proton. The 1D ROESY confirmed the relative configuration.

Accordingly, compound **27** was identified as *ent*-kaur-6β,16α,17,19-tetrahydroxy-2-one.



27

Compound **28 (***Fig.* 1.8) was isolated as an amorphous white powder. Its molecular formula was determined as $C_{20}H_{34}O_4$ by the positive HRESIMS at *m/z* 339.2528, and accounted for four degrees of unsaturation.

The ^{1}H and NMR spectrum (*Tab.* 1.4) showed signals for two tertiary methyl groups, two pair doublets centred at δ_H 4.01, 4.03, 3.60, 3.46 for two hydroxymethylenes groups and one hydroxymethine at δ_H 4.01.

The ^{13}C NMR spectra showed the presence of two methyls, 8 methylenes, two hydroxymethylenes, three methynes, one hydrowxymethine and four quaternary carbons, group, one hydroxymethin group; it showed a good agreement with a kaurene derivative (Fraga et al., 2011).

The ^{1}H and ^{13}C NMR spectra of **28** were nearly superimposable with those of **27**, with the exception of the lacking of the signal of the keto group at position 2, replaced by a methylene [δ_C 19.4; δ_H 1.45 and 1.62]. The 1D-TOCSY showed the spin system H-1—H-3. ^{1}H-^{13}C long-range correlations from the HMBC experiment of H_2-1 and of H_2-3 to the signal at ppm 19.4 confirmed the attribution.

Compound **28** was then established as *ent*-kaur-6β,16α,17,19-tetraol.

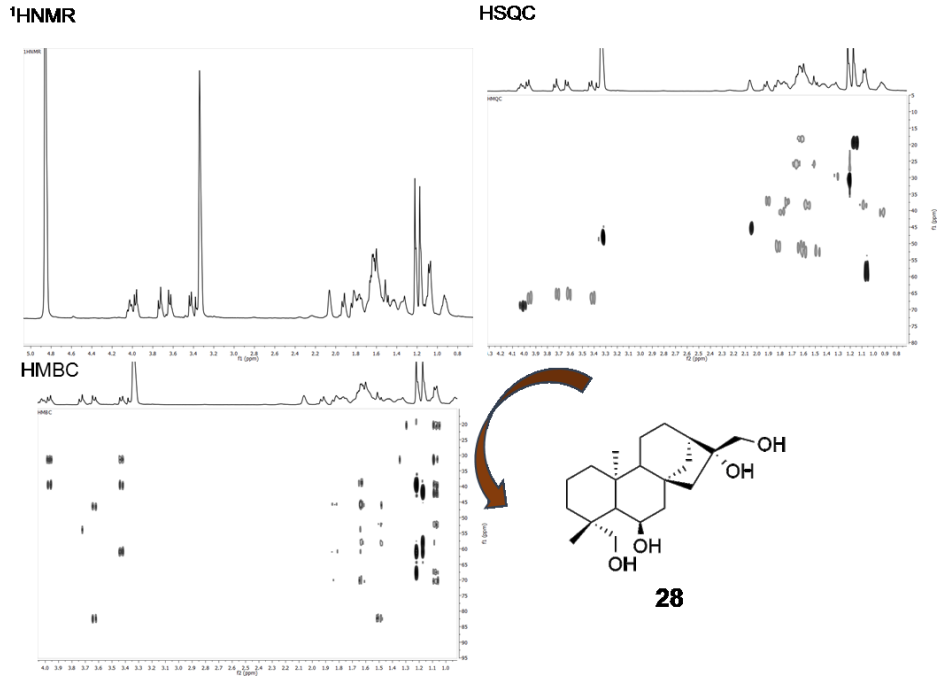
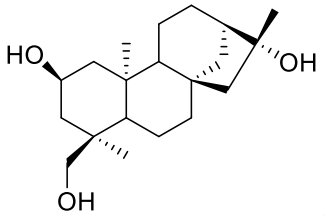


*Fig. 1.8 NMR spectra of compound **28***

Compound **25** was obtained as an amorphous white powder. Its HRESIMS spectrum exhibited a quasimolecular ion $[M+H]^+$ peak at *m/z*: 323.2580 (calcd. for 322.2508) in accordance with the molecular formula $C_{20}H_{34}O_3$, and requiring four degrees of unsaturation. Comparison of the ^1H and ^{13}C NMR data of **25** (*Tab.* 1.4) with those of *ent*-Kaurane-2α,16β,18-triol (Tanaka et al., 1978), indicated that both compounds had similar skeleton and substitution pattern. The only exception was the β orientation of H-2, showed by compound **25**, supported by coupling constants of H-1_{eq} [δ_H 1.38 br dd (J = 13.9, 1.5)] and H-1_{ax} [δ_H 1.67 dd (13.9, 4.7)].
Therefore, compound **25** was determined to be *ent*-kaurane-2β,16,18-triol.



25

Compound **18** (*Fig.* 1.9) was obtained as white substance. Its molecular formula $C_{20}H_{32}O_3$ was established by its HRESIMS data (*m/z*: 321.2422 $[M+H]^+$). This implied five indices of hydrogen deficiency. Compound **18** was assigned a *ent*-trachylobane skeleton by comparison of their spectroscopic and physical data with those reported in the literature (Amone et al., 1979; Fraga, 1994; Juma et al., 2006; Midiwo et al., 1997). Particularly, the two high field resonances at δ_H 0.63 (H-12) and 0.89 (H-13), observed in ^{1}H NMR spectrum of compound **18** that indicated the presence of the cyclopropane ring, characteristic of the trachylobane structures with the methyl group at C-16 (Fraga, 1994). Analysis of ^{1}H NMR spectrum showed the presence of two hydroxymethynes at δ_H 3.94 (1H, m), 3.96 (1H, br dd *J*=11.2 and 3.2 Hz) at δ_H 3.34 and 4.03 (1H, d, *J*=10.9).
The ^{13}C NMR spectrum showed the presence of twenty carbons of which 3 were oxygenated. The analysis of DQF-COSY and 1D-TOCSY let to establish for compound **18** the following spin systems: H-1—H-3, H5—H7, H9—H14. The resonances of all carbons were assigned on the basis of study combination of HSQC, COSY and HMBC spectra.
NMR spectral data (*Tab.* 1.5) indicated that the NMR data of **18** were very similar to trachyloban-2β,6β,19-triol (**16**) (Midiwo et al., 1997). However, differences in the chemical shifts at position 1, [**16**: δ_H 0.73 and 1.85 *vs* **18**: δ_H 1.22 and 1.44], 2 [**16**: δ_H 3.74 *vs* **18**: δ_H 3.95] and 3 [**16**: δ_H 1.87 and 1.02 *vs* **18**: δ_H 1.55 and 1.30] suggested an α orientarion for the hydroxyl group. This fact was also supported by 1D ROESY NMR selective experiments. In these, the selective irradiation of the resonance frequency of Me-18 at δ_H1.28 caused a ROE enhancement in the signals at δ_H 3.92 (H-2, 1.03 CH-5). Consequently, the structure of **18** will be trachyloban-2α,6β,19-triol.

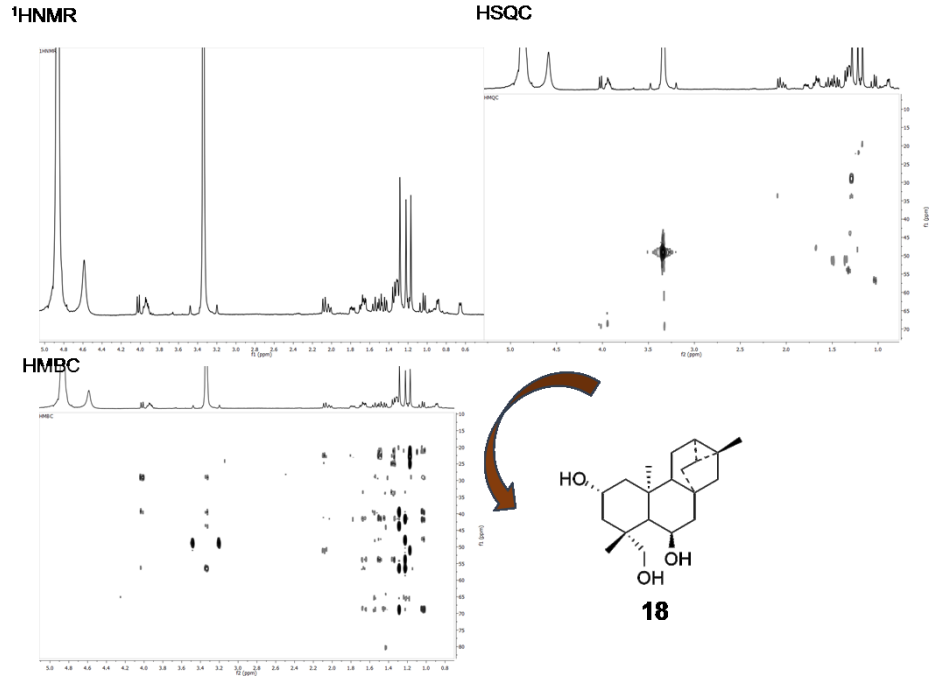


*Fig. 1.9 NMR spectra of compound **18***

Compound **10** (*Fig.* 1.10) was isolated as white substance. **10** showed an HRESIMS spectrum with molecular weight of *m/z*: 319,2267 $[M+H]^+$,which was in agreement with the molecular formula $C_{20}H_{30}O_3$ that indicated six degrees of insaturations. Comparison of the ^1H and ^{13}C NMR data of **10** with those of **18** (*Tab.* 1.5) indicated that both compounds had similar skeleton and substitution pattern. The only difference was that an oxo group in the ^{13}C NMR spectrum showing a low-field signal at 214.8 ppm, was present at position 2 in **10** instead of the hydroxy group present at the same position in compound **18**. This conclusion was verified by the HMBC correlations H_2-1/C-2, C-3 and H_2-3/C2,C5,C3 and H5/C7,C1,C3,C8,C9. Accordingly, compound **10** was identified as trachyloban-6β,19-dihydroxy-2-one.

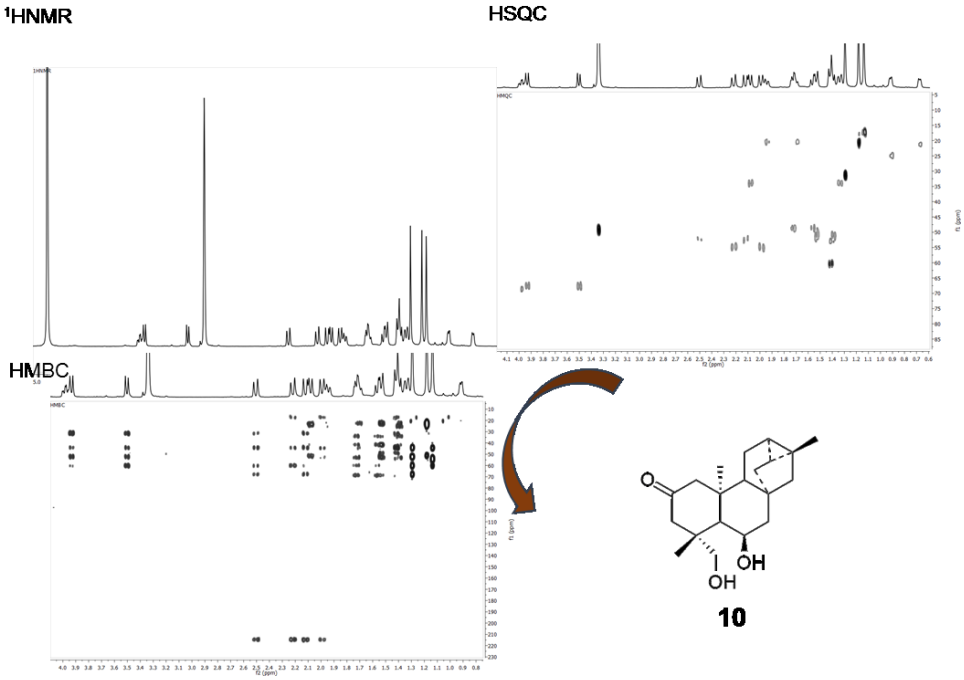


*Fig. 1.10 NMR spectra of compound **10***

Compound **19** (*Fig.* 1.11) was obtained as white substance. Its HRESIMS (*m/z*: 335.2215) suggested a molecular formula of $C_{20}H_{30}O_4$ which was consistent with six degrees of insaturation which were immediately identifiable by NMR spectroscopic data (*Tab.* 1.6). The NMR resonances consist in cyclopropane ring (δ_H 0.89,1.16; δ_C 18.9, 22.3, 30.0), two hydroxymethylen groups at δ_H 3.94, 3.54 and 3.57; δ_C 67.5, 66.7); one hydroxymethine group at δ_H 3.99 and δ_C 68.4; as well as one carbonyl group (δ_C 214.4 attributable to one ketone). In the absence of any other sp, or sp^2 carbons the structure of **19** must be pentacyclic. The ^1H NMR spectrum of **19** exhibited two methyl singlets at δ_H 1.
16 and 1.30. All the spectroscopic data suggest that **19** is a trachyloban derivative (Midiwo et al., 1997). The hydroxymethylenes groups were located at C-19 and C-17 on the basis of the HMBC cross peak of H_2-19 with C-5, C-4 and C-18, Me-18 with C-19, C-4 and C-3, H_2-17 with C-15, C-13 and C-14, H_2-14 with C-9, C-16, C-12, C-7. Long range correlation were also observed between H-6 and C-5, C-9,C-8,C-14; between H15 and C-13,C-8,C-7.

1-D TOCSY and COSY spectra suggest the presence in the molecule of two spin system H5—H7, H9—H14. Direct evidences of the substituent sites were derived from the HSQC and HMBC correlations which also allowed the assignment of all the resonances in the ^{13}C NMR spectrum.
These results were confirmed by the chemical shift of the respective carbons which matched well with related trachylobane (Fraga et al., 2011; Midiwo et al., 1997). The relative configuration of the oxymethylene at 17 was deduced to be β-orientated by 1D ROESY NMR selective experiments that showed correlaation peaks between H-17 and H-12, and H-13
Therefore, compound **19** was found to be trachyloban-6β,17,19-trihydroxy-2-one.

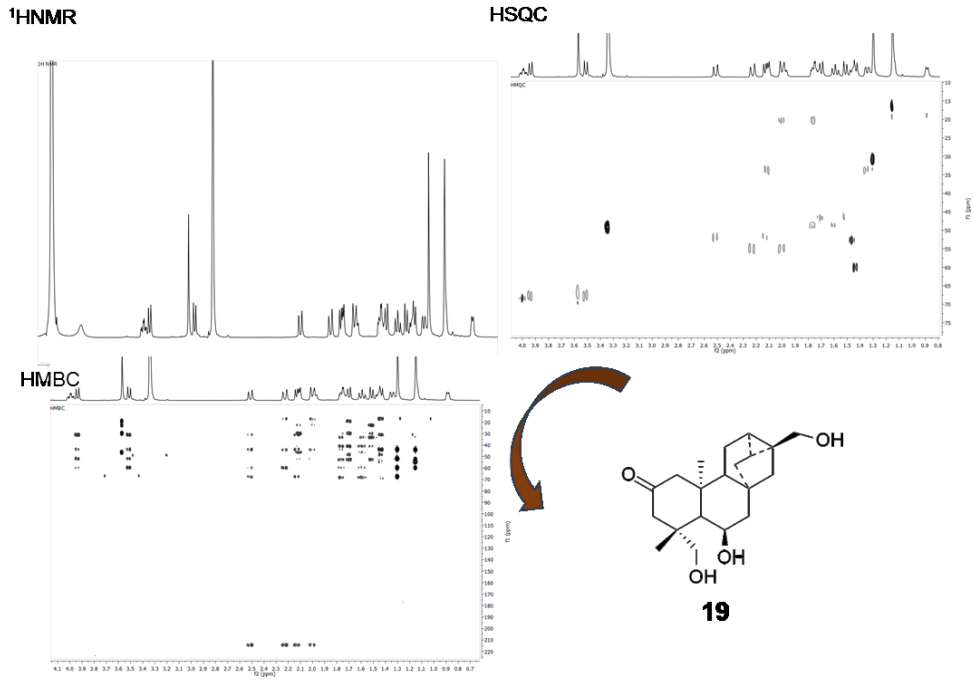


*Fig. 1.11 NMR spectra of compound **19***

Compound **20** (*Fig.* 1.12) was obtained as white substance, had a molecular formula $C_{20}H_{30}O_4$ based on its HRESIMS (*m/z*: 335.2216 $[M+H]^+$ and the ^1H and ^{13}C NMR data (*Tab.* 1.6) suggesting six degrees of unsaturation. Spectroscopic data interpretation indicated that the structure of **20** was similar to **19**. Comparison of the ^{13}C NMR spectrum of compound **20** with that of **19** established a difference in the chemical shift of C-6 [δ_C 21.5 vs

68.7; δ_H 1.76 and 1.77 vs 4.00 (td, J = 11.0, 11.0, 4.0)], implying that the oxymethine was replaced by a methilene, and in the chemical shift of C-18 (δ_C 64.3 vs δ_C 31.4; δ_H 3.48 and 3.59 vs 1.30 s), implying that the methyl was replaced by a hydroxymethylene group. Analysis of HMBC data confirmed the positions of these substituents, showing the cross peaks H-5/C-7, H_2-6/C-5, H_2-6/C-10, H_2-7/C-6 and H_2-3/C-18, H-5/C-18, H_2-19/C-18, H_2-18/C-3, H_2-18/C-4, H_2-18/C-5, H_2-18/C-19, respectively.
The structure of **20** was thus assigned as trachyloban-17,18,19-trihydroxy-2-one.

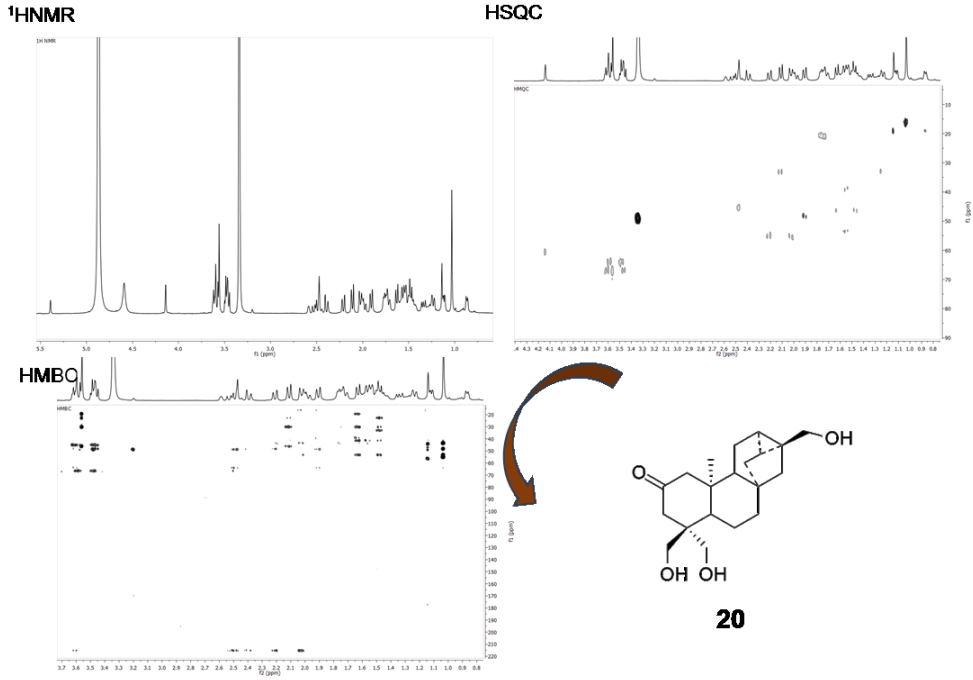
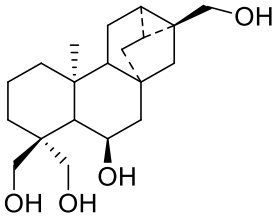


*Fig. 1.12 NMR spectra of compound **20***

Compound **22** was a white oil-like substance, had the molecular formula $C_{20}H_{32}O_4$ as deduced from HRESIMS (*m/z*: 337.2371 $[M+H]^+$, calcd for 336.2301) indicating five degrees of unsaturation. The NMR analysis (*Tab.* 1.7) displayed four oxygenated groups, namely three oxymethilenes [δ_C 63.4, δ_H 3.76 (d, J = 11.1 Hz), 3.90 (d, J = 11.1 Hz); δ_C 72.2, δ_H 3.50 (d, J = 11.2 Hz), 3.59 (d, J = 11.2 Hz); δ_C 67.5, δ_H 3.56 s] and one oxymethine (δ_C 68.9, δ_H 3.91). Two oxymethilenes (δ_C 63.4 and δ_C 72.2) were located at C-4 of the decaline system, C-18 and C-19, respectively, on the basis of the HMBC correlations of their protons to C-3, C-4, C-5. The remaining

oxymethylene with equivalent protons showed HMBC correlations to C-12, C-13, C-15 and C-16 and was thus placed at C-16. The ^{1}H-^{1}H COSY correlations of the oxymethine proton (δ_H 3.91) with H-5 and H_2-7 and the HMBC correlations from this proton to C-4, C-10 and C-8, and from H-5 and H_2-7 to the oxymethine carbon (δ_C 68.9) indicated that this group was located at C-6. The strong cross-peaks H_2-17/ H-12 and H_2-17/H-13, as well as the correlations H_2-19/H_3-20 and H_3-20 /H-6 observed in the 1D- ROESY spectrum, allowed us to conclude that the C-17, C-19 and H-6 were in the β-orientation.

Thus, **22** was elucidated as trachyloban-6β,17, 18,19-tetraol.



22

Compound **24** (*Fig.* 1.13) was obtained as white oil-like substance. The HRESIMS of **24** revelled a molecular ion at *m/z*: 335.2218 $[M+H]^+$ together with a minor ion at *m/z*: 313.2117 corresponding to the loss of a carboxylic group from the previous specie. This information, along with the ^{13} C NMR spectra which sorted 20 carbons into two methyls, seven methilenes, five methines, four quaternary carbons and one carboxylic group, allowed the determination of the molecular formula as $C_{20}H_{30}O_4$ and a trachylobane nucleus for **24**. The molecular formula along with ^{13}C NMR data account for six degrees of insaturation**.** Comparison of the ^{1}H and ^{13}C-NMR data of **24 (***Tab.* 1.7) with those of **19** indicated a close structural similarity of these compounds. The comparative analysis of the ^{13}C NMR data of **24** with those observed for compound **19** revealed a shielded signal attributable to one carboxyl group at δ_C 181.3 (C-17), in place of the signals of the oxymethylene group at δ_C 67.4 observed in **19**. Moreover, in contrast to the observation of keto group at position 2 in compound **19**, the ^{1}H NMR spectrum of **24** indicated the presence of methylene resonances at δ_C 18.8, δ_H 1.37 and 1.51. 1D-ROESY data and coupling constants analysis confirmed that **24** possessed the same relative stereochemistry of **19**. This compound was established as trakyloban-6β,19 dihydroxy,17oic-acid.

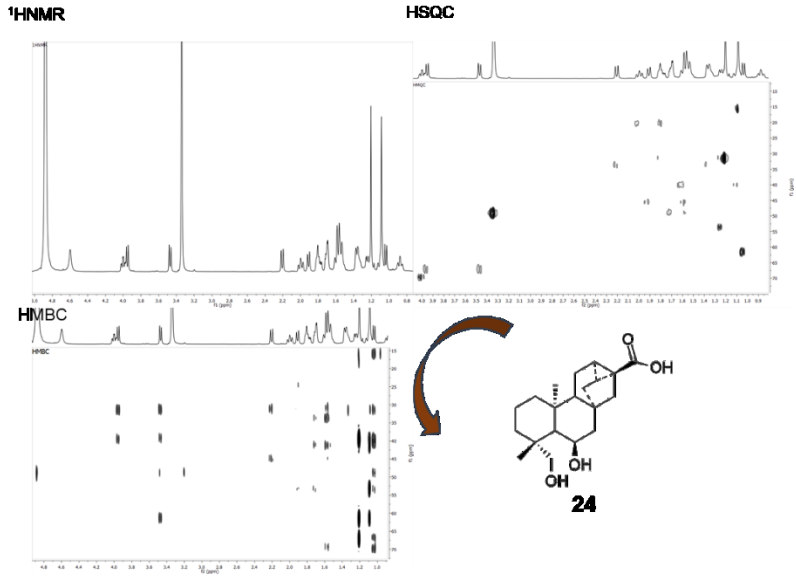


Fig. 1.13 NMR spectra of compound ***24***

Successively, MIC values of all the pure compounds were determined against *S. aureus* and *C. albicans*. Flavonoids, **1**,**3-7** and **11** showed moderate activity (data not shown). Only compound **2**, **3**, **8** and **27** showed interesting antibacterial activity (*Fig.*1.14) (Tab 1.2).

Tab. 1.2 MIC values of the compounds ***2, 3, 8, 27***

	MIC (µg/mL)			
	Antibacterial activity		**Antifungal activity**	
compounds	*Staphylococcus aureus*		*Candida albicans*	
	MIC_{50}	MIC_{100}	MIC_{2}	MIC_{0}
2	40	70	60	140
3	8	25	30	100
8	50	100	100	> 200
27	45	150	100	> 200

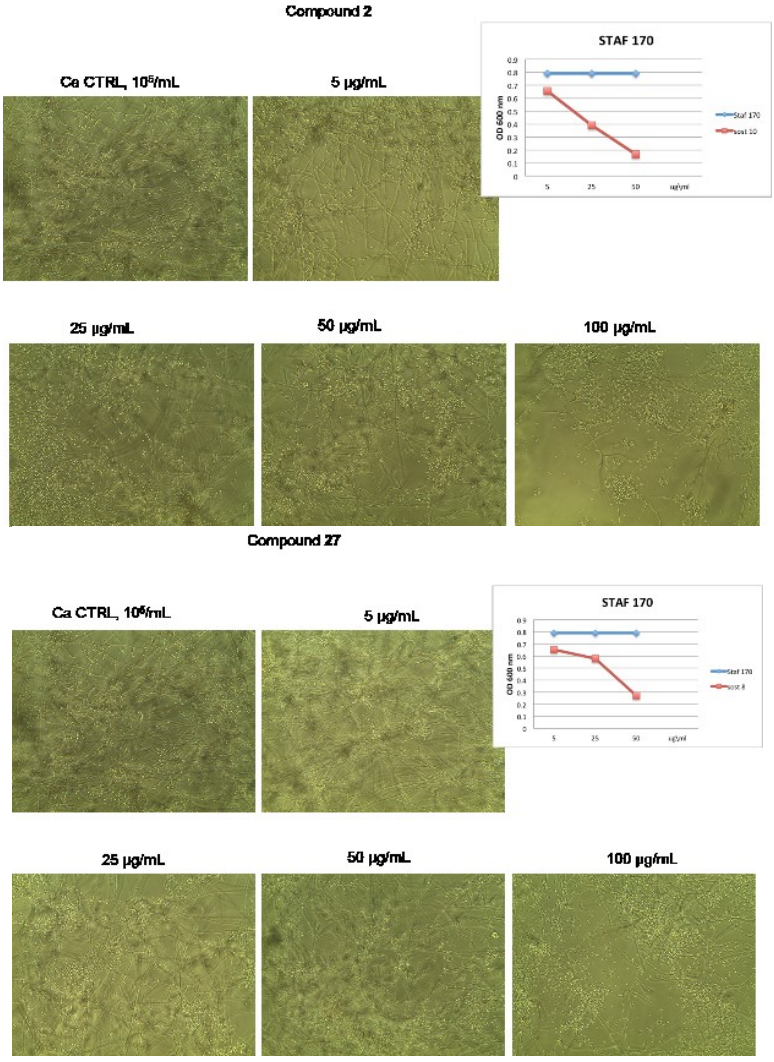


*Fig. 1.14 Antibacterial activity of pure compounds **2** and **27** against S. aureus and C. albicans*

The ability of compounds **2**, **3**, **8** and **27** to inhibit the biofilm formation of *C. albicans* and *S. aureus* was investigated. The blue-violet colour is indicative of biofilm formed on microtiter plates. The biofilm quantification using CV recovered with 200 µl of acetic acid, is reported in *Fig.* 1.15. Compound **3** was the most effective. Compared to the control cells (cells treated with only vehicle), compound **3** at a concentration of 40 µg/mL was

able to inhibit 50% of bacteria biofilm and 90% of *Candida* biofilm formation.

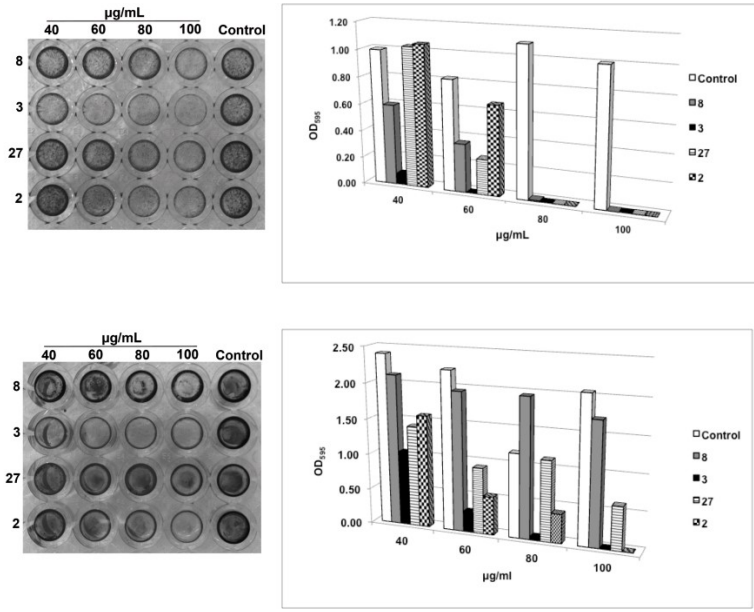


*Fig. 1.15 Ability of compounds **2**, **3**, **8** and **27** to inhibit the biofilm formation of C. albicans and S. aureus*

The antimicrobial activity obtained from *P. punctulata* extracts and compounds was similar to those reported for similar extracts. As expected the flavonoid derivatives manifested a good antibacterial activity on *S. aureus* and *C. albigans*. In conclusion, the dichloromethane extract and six fractions were variously active against gram positive and *Candida albigans*, the potency expressed by the crude extract is of particular interest and could have application as antibacterial preparation.

*Tab. 1.3 ^{1}H and ^{13}C NMR data of compounds **14–30** (CD_3OD, 600 MHz, J in Hz)[a]*

Position	Compounds			
	14		30	
	δ_H	δ_C	δ_H	δ_C
1	0.87; 1.84[a]	39.0	0.93; 1.81	39.7
2	1.58[a]; 1.66[a]	27.7	1.70; 1.84	27.9
3	3.15 dd (4.4, 11.7)	79.3	3.14 dd (12.0. 4.5)	80.1
4	-	39.5	-	39
5	0.80[a]	56.2	0.85	56.0
6	1.57[a] 1.58[a]	19.7	1.61; 1.77	20.1
7	1.55[a]; 1.68[a]	42.6	1.55 1.71	42.9
8	-	41.3	-	46.0
9	1.10 d (8.9)	59.3	1.1	59.0
10	-	40.0	-	38.9
11	1.69[a]; 1.81[a]	27.7	1.62; 1.70	28.0
12	3.92 m	67.7	3.9	67.0
13	1.96[a]	56.8	2.14	54.0
14	1.48[a]; 2.26 d (11.7)	32.7	1.48; 2.28	32.1
15	1.59[a]; 1.60[a]	58.4	1.44 1.57	54.2
16	-	79.0	-	81.0
17	1.34 s	25.2	3.58 (13.0); 3.65 (13.0)	66.0
18	0.99 s	29.3	0.81 s	15.6
19	0.81 s	16.7	0.98 s	28.5
20	1.26 s	16.3	1.25 s	16.4

[a] Data assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments.

*Tab. 1.4 ^{1}H and ^{13}C NMR data of compounds **21**–**23** (CD_3OD, 600 MHz, J in Hz)[a]*

Position	Compounds			
	21		23	
	δ_H	δ_C	δ_H	δ_C
1	0.74 t (13.6, 13.6); 2.19[a]	50.0	1.42[a] 1.77[a]	50.6
2	3.83 br ddd (15.2, 13.6, 5.2)	64.3	3.95[a]	65.8
3	1.03 t (15.6, 15.2); 1.90 dd (15.6, 4.7)	48.7	1.39[a]; 1.89 dd (13.1, 3.9)	36.6
4	-	41.5	-	45.1
5	1.09 d (13.0)	61.1	1.04 d (11.0)	52.5
6	4.08 ddd (14.0, 13.0, 4.6)	69.4	3.99[a]	68.2
7	1.69[a]; 1.74[a]	51.2	1.58[a]; 1.74[a]	50.0
8	-	43.7	-	45.0
9	1.19 d (6.9)	56.8	1.29 br d (8.1)	57.0
10	-	43.3	-	43.3
11	1.72[a]; 1.73[a]	18.9	1.63[a]; 1.80[a]	19.7
12	1.71[a]; 1.55 m	33.6	1.57[a]; 1.73[a]	34.0
13	2.68 br s	44.7	2.69 br s	44.9
14	1.27 dd (13.5, 5.4); 2.04 dd (13.5, 1.3)	41.0	1.25 dd (11.5, 4.6); 1.99 br d (11.5)	40.5
15	2.18[a]; 2.20[a]	48.9	2.14 br s	49.96
16	-	161.7	-	155.8
17	4.81 br s; 4.85[a]	103.4	4.80 s; 4.88[a]	104.1
18	1.30 s	32.3	3.62 d (11.0); 4.03[a]	65.4
19	3.52 d (10.6); 3.97 d (10.6)	67.3	3.47 d (11.1); 4.02[a]	67.8
20	1.21 s	20.0	1.32 s	25.6

[a] Data assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments.

*Tab. 1.5 ^{1}H and ^{13}C NMR data of compounds **25–27–28** (CD_3OD, 600 MHz, J in Hz)[a]*

	Compounds					
	27		**28**		**25**	
Position	**δ_H**	**δ_C**	**δ_H**	**δ_C**	**δ_H**	**δ_C**
1	1.95 d (16.6); 2.57 d (16.6)	56,6	0.94 ddd (12.3, 11.7, 4.9); 1.82[a]	42,0	1.38[a]; 1.67 dd (13.9, 4.7)	46.3
2	-	215,2	1.45[a]; 1.62[a]	19,4	4.14 m	67.9
3	2.07 d (15.0); 2.69 d (15.0)	51,4	1.11[a]; 1.60[a]	39,8	1.49[a]; 1.82 dd (13.9, 3.8)	41.4
4	-	44,7	-	41,6	-	38.5
5	1.34 d (12.2)	59,6	1.08[a]	61,0	1.24 d (8.8)	48.2
6	4.04 ddd (11.2, 10.8, 4.0)	68,7	4.04 ddd (11.0, 10.8, 3.5)	70,1	0.88 m; 1.92 br t (12.4)	28.0
7	1.88 m; 1.67[a]	51,3	1.64[a]; 1.84[a]	52,2	1.19[a]; 1.38[a]	39.7
8	-	45,2	-	45,9	-	51.0
9	1.26 br d (7.9)	56,7	1.07[a]	58,4	1.33[a]	52.7
10	-	43,3	-	39,3	-	39.1
11	1.54[a]; 1.70[a]	19,6	1.44[a]; 1.65[a]	19,1	1.46[a]; 1.48[a]	20.0
12	1.55[a] ; 1.68[a]	26,8	1.54[a]; 1.69[a]	27,2	1.20[a]; 1.37[a]	39.8
13	2.11 br s	46,3	2.07 m	46,3	1.32[a]	38.8
14	1.81 dd (12.0, 4.6); 1.90 dd (12.0, 3.1)	37,9	1.77 dd (12.0, 5.0); 1.93 br d (12.0)	38,8	1.72 d (11.8); 2.11 m	24.8
15	1.53 d (14.7); 1.65[a]	53,6	1.51 d (14.4); 1.62[a]	53,8	1.22[a]; 1.31[a]	58.2
16	-	82,3	-	82,5	-	72.5
17	3.65 d (11.4); 3.75 d (11.4)	66,7	3.64 d (11.3); 3.74 d (11.3)	66,7	1.28 s	30.4
18	1.30 s	31,2	1.23 s	31,4	3.09 d (11.0); 3.37[a]	71.4
19	3.75 d (11.0); 4.02 (11.0)	68,1	3.44 d (10.6); 3.98 d (10.6)	67,6	0.98 s	20.5
20	1.31 s	21,1	1.18 s	20,4	1.34 s	18.1

[a] Data assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments.

Tab. 1.6 ^{1}H and ^{13}C NMR data of compounds ***10-18*** *(CD_3OD, 600 MHz, J in Hz)*[a]

Position	Compounds			
	10		**18**	
	δ_H	**δ_C**	**δ_H**	**δ_C**
1	1.95 m; 2.19 d (15.3)	55.0	1.22[a]; 1.44[a]	48.4
2	-	214.1	3.95[a]	65.8
3	2.08 m; 2.48 d (14.9)	52.3	1.30[a]; 1.52[a]	44.1
4	-	44.5	-	40.0
5	1.39[a]	60.2	1.04 d (10.5)	56.9
6	3.96 br dd (11.0, 3.8)	68.8	3.96[a]	68.0
7	1.55[a]; 1.72[a]	48.5	1.44 m; 1.47[a]	48.1
8	-	41.4	-	41.8
9	1.41[a]	53.0	1.32 dd (5.0, 1.9)	54.2
10	-	44.3	-	42.0
11	1.68[a]; 1.91 m	20.5	1.79 m; 2.06[a]	20.4
12	0.65 br d (7.4)	21.4	0.66 br d (7.8)	21.5
13	0.88 dd (7.7, 2.5)	25.3	0.89 br dd (7.8, 3.1)	25.4
14	1.31 d (12.7); 2.05 d (12.1)	34.1	1.29[a]; 2.08[a]	34.2
15	1.36[a]; 1.50[a]	51.2	1.35 d (11.1); 1.50 d (11.1)	51.4
16	-	23.2	-	23.1
17	1.15 s	20.6	1.17 s	20.6
18	1.27 s	31.4	1.29 s	29.5
19	3.48 d (10.9); 3.90 d (10.9)	68.0	3.34[a]; 4.03 d (10.9)	69.3
20	1.11 s	17.4	1.23 s	21.6

[a] Data assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments.

*Tab. 1.7 ^{1}H and ^{13}C NMR data of compounds **19–20** (CD_3OD, 600 MHz, J in Hz)[a]*

Position	Compounds			
	19		20	
	δ_H	δ_C	δ_H	δ_C
1	2.00[a]; 2.23 d (15.3)	54.9	2.03[a]; 2.21 d (9.2)	55.4
2	-	214.8	-	215.5
3	2.14[a]; 2.52 d (15.0)	52.2	2.39 m; 2.51 d (8.4)	45.5
4	-	44.2	-	49.1
5	1.45[a]	60.0	1.91 d (11.6)	48.5
6	4.00 ddd (11.4, 11.0, 4.0)	68.7	1.76 [a]; 1.77 [a]	21.5
7	1.60 t (12.5, 12.5); 1.77[a]	48.5	1.55[a]; 1.57[a]	39.6
8	-	41.5	-	42.0
9	1.46[a]	52.9	1.56[a]	53.7
10	-	44.5	-	44.0
11	1.76[a]; 2.00[a]	20.5	1.48[a]; 1.99[a]	20.6
12	0.89 dd (7.9, 3.1)	19.6	0.87 br d (8.7)	19.7
13	1.16[a]	23.1	1.12 m	23.1
14	1.36 m; 2.12[a]	33.7	1.23 dd (12.8; 2.8); 2.11 br d (12.0)	33.4
15	1.52 d (11.4); 1.70 d (11.4)	46.7	1.47[a]; 1.63 d (14.5)	46.5
16	-	30.2	-	30.5
17	3.57 br s	67.4	3.56 br s	67.6
18	1.30 s	31.4	3.48[a]; 3.59[a]	64.3
19	3.52 d (11.0); 3.94 d (11.0)	67.8	3.46[a]; 3.61[a]	66.9
20	1.15 s	17.5	1.03 s	16.6

[a] Data assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments.

*Tab. 1.8 ^{1}H and ^{13}C NMR data of compounds **22–24** (CD_3OD, 600 MHz, J in Hz)[a]*

Position	Compounds			
	22		24	
	δ_H	δ_C	δ_H	δ_C
1	0.80; 1.56	39.9	0.87 t (11.8); 1.57[a]	40.0
2	1.38; 1.50	18.1	1.37[a]; 1.51[a]	18.8
3	1.24; 1.74	30.0	1.12[a], 1.59[a]	39.6
4	-	47.5	-	40.5
5	1.15 d (11.0)	58.2	1.03 d (10.8)	61.8
6	3.90 ddd (13.5, 13.0, 4.3)	67.7	3.99 ddd (11.4, 11.2, 3.8)	69.8
7	1.53; 1.97	48.9	1.55[a]; 1.70 dd (12.3, 3.7)	48.7
8	-	42.0	-	41.2
9	1,23	53.3	1.24[a]	53.6
10	-	38.6	-	41.7
11	1.97; 2.00	20.0	1.80[a]; 1.99 br t (12.9)	20.5
12	0,89	18.7	1.23[a]	31.2
13	1,07	22.0	1.81[a]	31.8
14	1.30; 2.14	33.6	1.36[a]; 2.20 d (12.3)	34.0
15	1.46; 1.66	46.7	1.56[a]; 1.90 (11.7)	45.2
16	-	29.0	-	25.2
17	3.56 s	66.7	-	178.0
18	3.71 d (12.8); 3.92 d (12.8)	63.0	1.20 s	31.8
19	3.50 d (10.9); 3.79 d (10.9)	71.0	3.46 d (11.7); 3.96 d (11.7)	67.6
20	1.06 s	15.0	1.08 s	16.3

[a] Data assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments.

Known compounds of *Psiadia punctulata* exudate

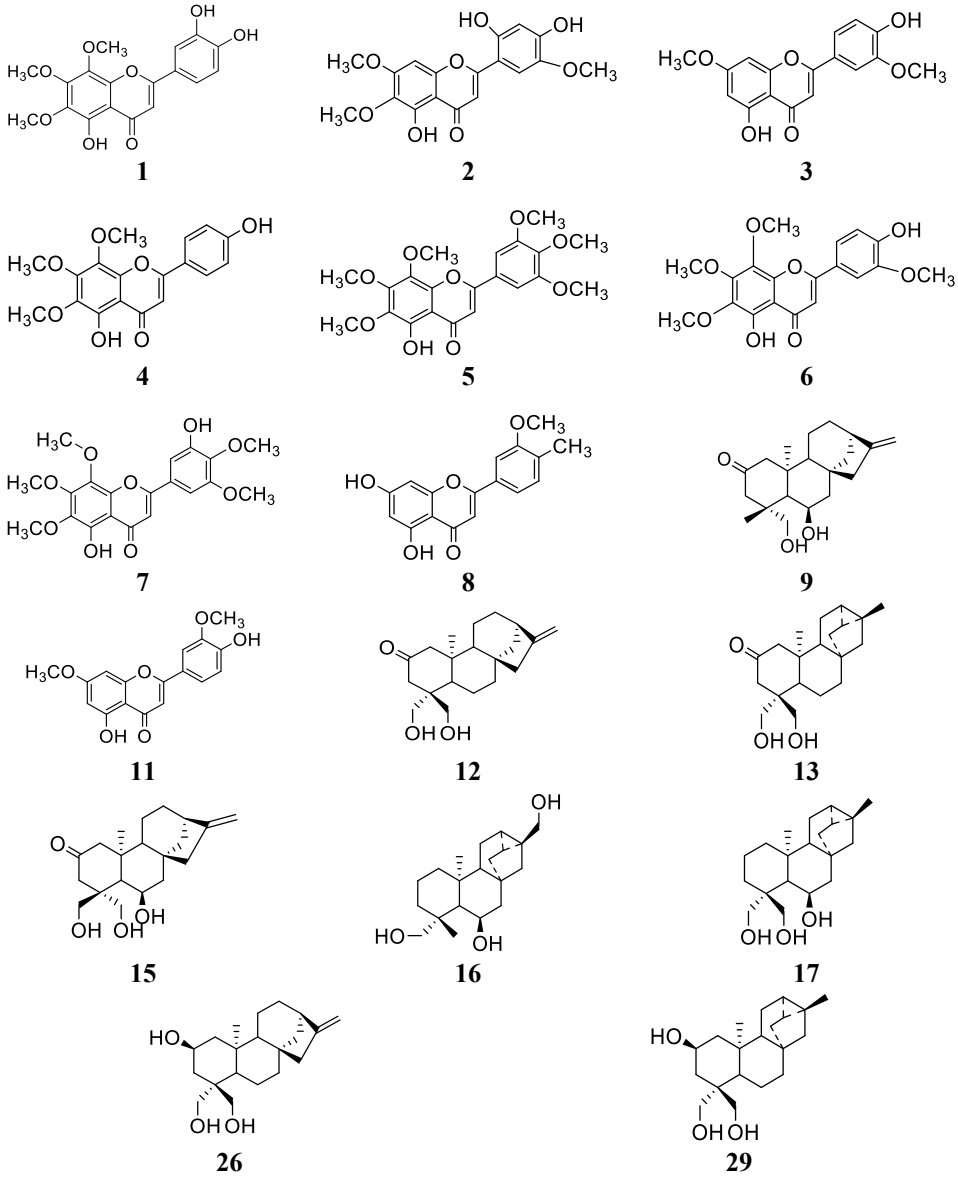


Fig. .1.16 Know compounds of P. punctulata

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Chapter 2

Hypoestes forskaolii (Vahl) Roem. &Schult

Acanthaceae

D'Ambola, M. Malafronte,N. Bosco,A. Amadesi, A. Rinaldi,L. Severino L.
In vitro ovicidal activity of *Hypoestes forskaolii* (Vahl) Roem. & Schult . (Acanthaceae) on sheep gastrointestinal nematodes: preliminary results
XXIV congress SILAE Punta Cana, República Dominicana, 8-12 September 2015

D'Ambola, M. Bosco, A. Amadesi, A. Bader, A. Ariano, A. Rinaldi, L. Cringoli, G. Severino, L.
In vitro anthelminthic efficacy of *Hypoestes forskaolii* (Vahl) R.Br (Acantaceae) extracts on gastrointestinal nematode of sheep.
Research in veterinary science. Submitted

2.1 Acanthaceae family

Acanthaceae is a large pantropical family consisting of approximately 240 genera and 3250 species. In the New World there are approximately 85 genera and 2000 known species. The two largest neotropical genera, *Justicia* (350-600 species) and *Ruellia* (ca. 250 species), are prominently distributed in Bolivia with 48 and 28 species, respectively (Wood, 2004). Plants of the Acanthaceae have found application in African and Asian folk medicine for the treatment of cancer heart disease, gonorrhea, and snake-bite (Pettit et al., 1984). Traditionally, the Acanthaceae leaves are used externally for wounds, but antifungal, cytotoxic, anti-inflammatory, anti-pyretic, anti-oxidant and insecticidal, activities are also reported especially in Middle East areas (Awan et al., 2014; Watt, 1962).

2.2 *Hypoestes genus*

Hypoestes, an important genus belonging to Acanthaceae family, consists of 150 species of woody-based, evergreen perennials, sub-shrubs and shrubs from open woodland in South Africa, Madagascar and S.E. Asia (Ellis 1999). Three species of the genus *Hypoestes* are reported in Southern Africa folk medicine. *Hypoestes aristata* (Vahl) Sol. ex Roem. & Schult native of tropical subSaharan area is used by amaZulu for the treatment of sore eyes (Steenkamp, 2003). *Hypoestes ssp* decoction is used in the treatment of breast disease. Roots are chewed for influenza, cough, colds and sore throats in East Africa (Kokwaro, 1976) (Kokwaro 1976).
The plants belonging to *Hypoestes* genus are the main source of fucosicoccane diterpenes so far identified in higher plants and fungi (Ellis, 2000).

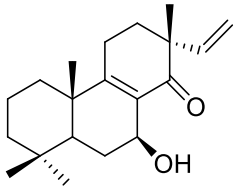
2.3 Previous phytochemical studies of *Hypoestes* genus

Several phytochemical studies have been carried out on *Hypoestes* species such as *H. serpens* L. (Rasoamiaranjanahary et al., 2003; Andriamihaja et al., 2001), *H. verticillaris* (Linn. f.) Soland. (Al-Rehaily et al., 2002), *H. roseae* P.Beauv. (Ojo-Amaize et al., 2001) (Adesomoju et al., 1983; Adesomoju and Okogun, 1984) and *H. purpurea* L. (Shen et al., 2004). Diterpenes belonging to the class of fucossane, isopimaranes and labdane are the main skeleton synthetized by these plant species.

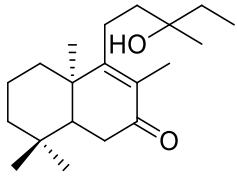
Isopimaranes are common constituents of conifers and are biosynthetically originated from the labdane-derived (+)-copalyl pyrophosphate (Otto and Wilde, 2001). Some isopimaranes exhibit interesting biological properties such as ntitumoral, cytotoxic, antimicrobial, tumour-promoting and anti-inflammatory (Ambrosio et al., 2006; Grace et al., 2006; Phuong et al., 2006).

Labdane skeleton is a natural bicyclic diterpene forming the structural core of a wide variety of natural diterpenes collectively known as labdanes. A variety of biological activities have been determined for labdane diterpenes including antibacterial, antifungal, antiprotozoal, and anti-inflammatory (Cocker and Halsall, 1956; Rahman, 2012).

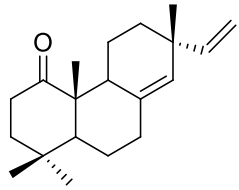
Fusicoccane diterpenes are powerful phytotoxins synthetized by several fungi species (Graniti, 1964) but a large number of these molecules is also synthesized by plants and particularly by *Hypoestes ssp.* Several biological activities are reported for fusicoccanes, mainly antimicrobial, anticancer and hypotensive activity (Gilabert et al., 2011; Mothana et al., 2009; Zapp et al., 1994) All this compounds are characterized by a complex 5-8-5 diciclopenta-cyclooctane nucleus (Muromtsev et al., 1994). Ditepernes isolated from *Hypoestes* ssp are shown below (*Fig.*.2.1)



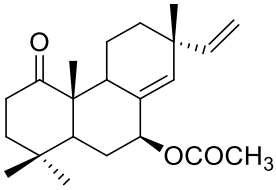
7β-hydroxyisopimara-8,15-dien-14-one



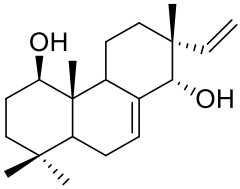
Ketonepoxide



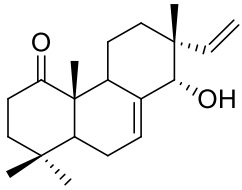
7β-hydroxyisopimara-8(14),15-dien-1-one



7β-acetoxyisopimara-8(14),15-dien-1-one



1 β,14α-dihydroxyisopimara- 7,15-diene



14α-hydroxyisopimara-7,15-dien-1-one

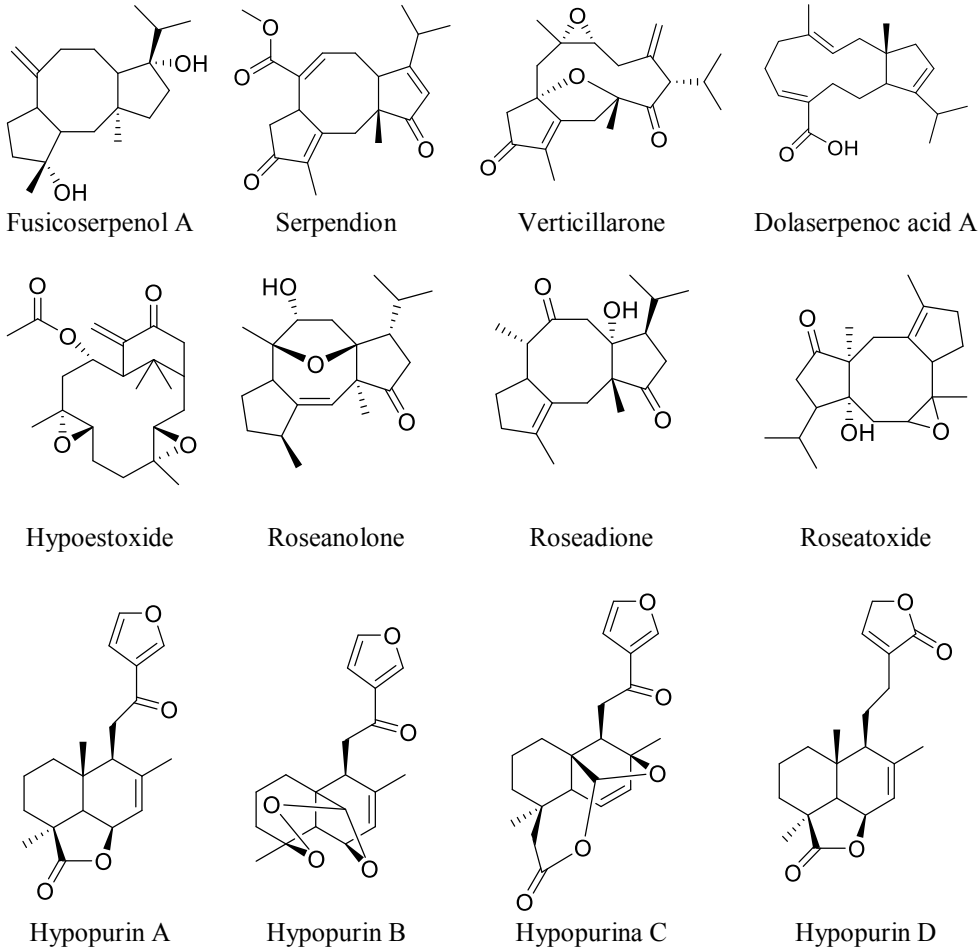


Fig. 2.1 Diterpenes of Hypoestes spp

2.4 *Hypoestes forskaolii* (Vahl) Roem. & Schult

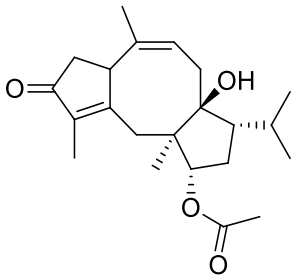
Synonym: *Hypoestes latifolia Hochst. ex Nees; Justicia paniculata Forssk.; Justicia verticillaris L. f. Roem. & Schult.*

The plant is widely distributed in many African countries as well as in high mountains of the Arabian Peninsula. In Saudi Arabia the plant has several popular names, among them "Nadgha", "Majra", "Qumaylah".(Andriamihaja et al., 2001).

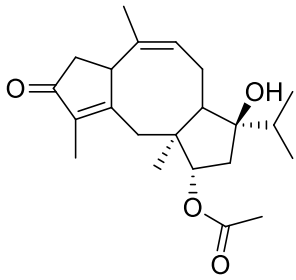
The leaves of this plant have bitter taste so It's not edible plants for animals. *H. forskaolii* is used popularly as natural insecticide, the decoction of this plant is used to wash the goats infested by flea, the fresh leaves are pasted and added to milk to attract and to kill the flies. The fresh leaves are applied to wounds to accelerate the healing. The fresh stems are used to massage the head scalp to kill head lice and to destroy its eggs. Goats with skin diseases specially in the breasts are exposed to vaporise of the burned *H. forskaolii* (Padysakova et al., 2013).

2.4.1 Previous phytochemical studies on Hypoestes forsskaolii

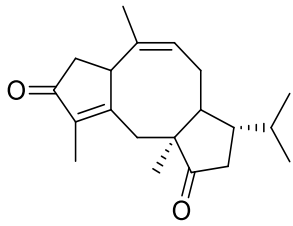
The diterpenes isolated from the leaves of *Hypoestes forsskaolii* are shown below (Al Musayeib et al., 2014)



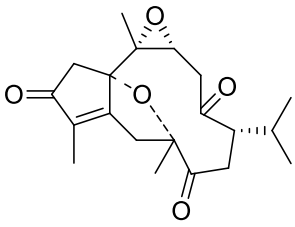
Hypoestenonols A



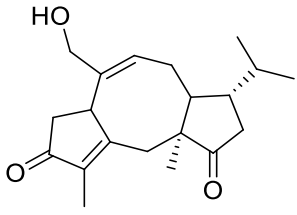
Hypoestenonols B



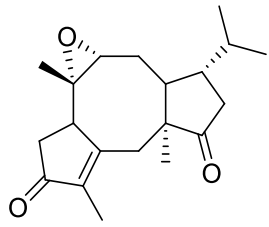
Hypoestenone



Verticillarone



17 – Hydroxyhypoestenone



8(9) α – Epoxyhypoestenone

Fig. 2.2 Diterpenes of H. forskaolii leaves

2.4.2 Biological activities

Several studies have shown that fusicoccanes have multiple biological activities. In particular, serpendione (*Fig.* 2.1) isolated from *H. serpens* was able to reduces of 92.2% the contractile action of noradrenaline at

concentration of 0.05mg / mL. This result supports the use of *H.serpens* for the treatment of hypertension.
Diterpenes isolated from *H. forskalei* as hypoestenone and 8 (9)-α-epoxyhypoestenone, showed antimicrobial activity against the pathogenic fungus *Cryptococcus neoformans*, with a MIC respectively of 20 µg/mL and 50 µg/mL. Hypoestenone showed also a moderate activity against *Pseudomonas aeruginosa* with MIC of 45 µg/mL, and against *Plasmodium falciparium* with IC 50 of 2800 ng/mL.

2.4.3 Anthelmintic activity of plant extracts.

Infections by gastrointestinal nematodes (GIN) remain a major constraint to ruminants' health, welfare and productive performance worldwide (Dipineto et al., 2013; Rinaldi et al., 2015a). These parasites cause direct and indirect losses in different ways such as lowered fertility, reduced work capacity, reduction in food intake, low weight gain and low milk productions (Rinaldi et al., 2015b). The administration of synthetic anthelminthics has long been considered the most effective way of controlling helminth infection to minimize losses caused by these parasites (Charlier et al., 2014) but the use of these drugs has some disadvantages, such as development of anthelmintic resistance (AR), high cost, risk of environmental pollution (Wagil et al., 2015). The availability and affordability of systemic anthelminthics to small-scale sheep farmers is a major problem in many developing countries (Adamu et al., 2010). This problem justifies the need for alternative control methods such as the use of traditional medicinal plants (Githiori, 2004) that are being examined in different parts of the world. The screening and proper evaluation of medicinal plants could offer possible alternative that may both be sustainable and environmentally acceptable (Eguale et al., 2007).
Anthelmintics derived from plants used for the treatment of parasitic infections in human and animals can offer an alternative to minimize some of these problems (Akhtar et al., 2000). One of the novel approaches investigated is the use of indigenous plant preparations commonly used against human parasites. The benefit of using these as possible livestock dewormers is that they are non-toxic and inexpensive, features that are important for farmers in developing countries (Taylor et al., 2001). These possible ethnoveterinary alternatives would be viable for small scale livestock farmers who cannot afford the allopathic drugs and/or for larger,

conventional farmers who cannot rely on the use of conventional veterinary products in their flocks (Gradé et al., 2008). Furthermore, plant-derived anthelminthic products are advantageous as they are less toxic, biodegradable and environmentally friendly (Hammond et al., 1997).
In the last decade, some plants of Acanthaceae family have been studied for their anthelmintic activity (Al-Shaibani et al. 2008, Adamu et al. 2010),

AIM

For all these reasons the aim of this study was to investigate the *in vitro* anthelmintic activity of the leaves extracts (*n*-hexane, chloroform, chloroform: methanol 9:1, methanol) of *H. forskaolii,* plant belonging to Acanthaceae family, on the eggs of GIN infecting sheep in south Italy and to investigate their secondary metabolites.

2.5 Experimental

2.5.1 Plant material

The aerial parts of *H. forsskaolii* were harvested at Wadi Thee Ghazal, Taif in Saudi Arabia, in September 2013. The plant was identified by Dr. Ammar Bader. A sample for aerial parts (N ° SA-EN 2013-2), is deposited in the herbarium of the Faculty of Pharmacy of Pharmacognosy laboratory at Umm Al-Qura University, Saudi Arabia.

2.5.2 Extraction and isolation

The aerial parts (505.0 g) dried and grinded were macerated with increasing polarity solvents: *n*-hexane, chloroform ($CHCl_3$), chloroform / methanol 9: 1 ($CHCl_3$:CH_3OH) and methanol (CH_3OH). The entire extraction process lasted about three weeks, during which the solvent has been renewed continuously (3X2L). The residues obtained were filtered and evaporated at reduced pressure (*Fig.* 2.3).

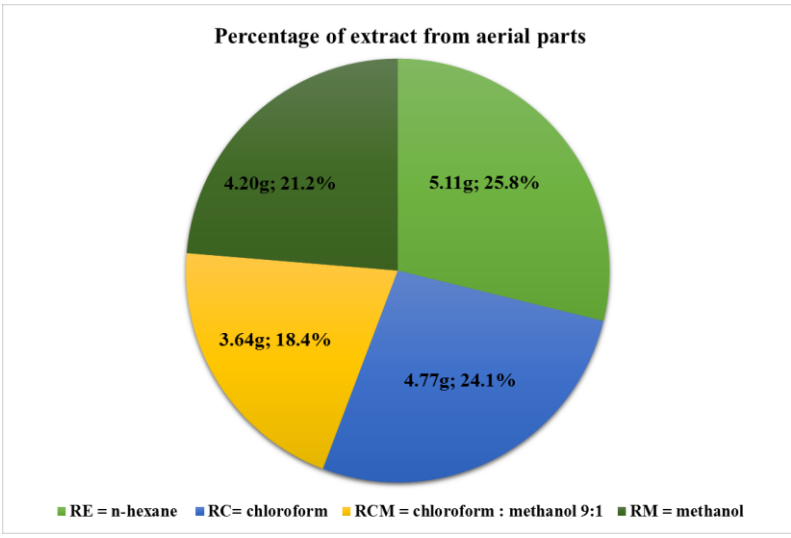


Fig. 2.3 Extracts of H. forskaolii leaves

Part of the *n*- hexane extract (5 g) was dissolved in 15 ml of *n*-hexane and separated on silica gel column, eluted with step gradients of *n*-hexane: $CHCl_3$(100:0, 90:10, 80:20, 70:30, 50:50 and 0:100) and $CHCl_3$:MeOH (100:0, 90:10, 80:20, 70:30, 50:50 and 0:100) Fractions of 25 mL were collected, analysed by TLC and grouped into 9 main fractions based on the similarities revealed on TLC (A-I). Fraction F (2848 mg) was separated by silica gel CC eluting with $CHCl_3$ followed by increasing concentrations of MeOH (between 1% and 100%). Fractions of 15 mL were collected, analysed by TLC pointed and sprayed with a solution of $Ce_2(SO_4)_2/H_2SO_4$, 65% and lastly grouped into TLC and grouped into 6 fractions (F1-F6) (*Fig.* 2.4).

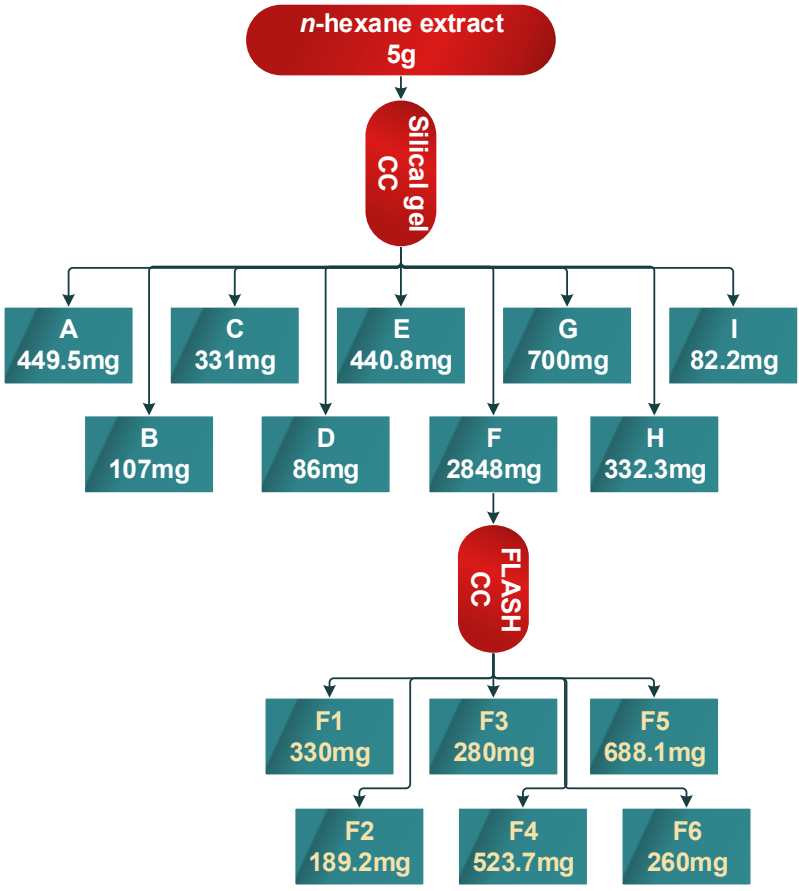


Fig.2.4 Separation scheme of H. forskaolii n-hexane extract

Fraction D (86.0 mg) was subjected to RP-HPLC using a C18 µ-Bondapak column (30 cm x 7.8 mm, 10 µm Waters, flow rate 2.0 mL/min). (MeOH-H_2O, 39:11) to give pure compound **1** (2.5 mg, t_R = 75 m), **2** (1.2 mg, t_R = 90 m). Fraction E (440.8 mg) was subjected to RP-HPLC (MeOH-H_2O, 75:25) to give pure compounds **3** (2.5 mg, t_R = 1h, 12m), **2** (2.5 mg, t_R = 1 h, 20 m) and **5** (2.5 mg, t_R = 1 h,30 m).
Fraction G (700.0 mg) was subjected to RP-HPLC (MeOH-H_2O, 7:3) to give pure compounds **6** (2.5 mg, t_R = 5 m), **7** (2.5 mg, t_R = 7.5 m) **8** (2.5 mg, t_R = 17.5 m), **9** (2.5 mg, t_R = 25 m), **10** (2.5 mg, t_R = 42 m) and **11** (2.5 mg, t_R = 44 m). Fraction F3 (280.0 mg) was subjected to RP-HPLC (MeOH-H_2O, 75:25) to give pure compounds **3** (2.5 mg, t_R = 20 m), **4** (2.5 mg, t_R = 1h, 20

m), **5** (2.5 mg, t_R = 1 h, 32 m) and **12** (2.5 mg, t_R = 1 h, 50 m). Fraction F4 (523.8mg) was subjected to RP-HPLC (MeOH-H_2O, 7:3) to give pure compounds **7** (2.5 mg, t_R = 5 m), **9** (2.5 mg, t_R = 25 m) and **13** (2.5 mg, t_R = 1 h, 22 m). Fraction F5 (688.1mg) was subjected to RP-HPLC (MeOH-H_2O, 65:35) to give pure compounds **7** (2.5 mg, t_R = 10 min), **8** (2.5 mg, t_R = 27.5 m), **9** (2.5 mg, t_R = 48 m) and **11** (2.5 mg, t_R = 75 m).

*2.5.2.1 Compound **1***

Colorless needles $[\alpha]_D^{25}$: +13.0; for ^1H and ^{13}C NMR spectroscopic data, see *Tab.* 2.1; EI-MS m / z 289.2526 $[M + H]^+$ (calcd for $C_{20}H_{32}O_6$; 288.2453).

2.5.2.2 Compound 2

Colorless needles $[\alpha]_D^{25}$:+21.0; for ^1H and ^{13}C NMR spectroscopic data, see *Tab.* 2.1; EI-MS m / z 289.2529 $[M + H]^+$ (calcd for $C_{20}H_{32}O$;288.2453).

*2.5.2.3 Compound **10***

Colorless needles $[\alpha]_D^{25}$: + 88.1 ° (c 0.06 CH_3OH); for ^1H and ^{13}C NMR spectroscopic data, see *Tab.* 2.1; EI-MS m / z 303.2320 $[M + H]^+$ (calcd for $C_{20}H_{30}O9$; 302.2246).

*2.5.2.4 Compound **13***

Oil $[\alpha]_D^{25}$: + 93.0; for ^1H and ^{13}C NMR spectroscopic data, see *Tab.* 2.2; EI-MS m / z 345.2429 $[M + H]^+$ (calcd for $C_{22}H_{32}O_3$; 344.2351).

2.5.3 Reagents and antibodies

Fetal bovine serum (FBS) was from GIBCO (Life Technologies, Grand Island, NY, USA). The antibodies anti-Cdc2 (mouse monoclonal, sc-8395) and anti-phospho (Thr161)-Cdc2 p34 (rabbit polyclonal, sc-101654), were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); appropriate peroxidase-conjugated secondary antibodies were from Jackson Immuno Research (Baltimore, PA, USA).

2.5.4 Cells and treatment

HeLa (cervical carcinoma), and Jurkat (T-cell leukemia), and cell lines were obtained from the American Type Cell Culture (ATCC) (Rockville, MD, USA). Cells were maintained in DMEM (HeLa) or RPMI 1640 (Jurkat and

U937), supplemented with 10% FBS, 100 mg/L streptomycin and penicillin 100 IU/mL at 37 °C in a humidified atmosphere of 5% CO_2. To ensure logarithmic growth, cells were subcultured every two days. Stock solutions (50 mM) of purified compounds in DMSO were stored in the dark at 4 °C. Appropriate dilutions were prepared in culture medium immediately prior to use. In all experiments, the final concentration of DMSO did not exceed 0.15% (v/v).

2.5.5 Cell viability and cell cycle

Cells were seeded in 96-well plates and incubated for the established times in the absence (vehicle only) and in the presence of different concentrations of compounds and Etoposide as positive control. The day before treatments, cells were seeded at a cell density of 1×10^4 cells/well. The number of viable cells was quantified by MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assay. Absorption at 550 nm for each well was assessed using a microplate reader (LabSystems, Vienna, VA, USA). In some experiments cell viability was also checked by Trypan Blue exclusion assay using a Bürker counting chamber. IC_{50} values were calculated from cell viability dose – response curves and defined as the concentration resulting in 50% inhibition in cell survival as compared to controls. Cell cycle was evaluated by propidium iodide (PI) staining of permeabilized cells according to the available protocol and flow cytometry (BD FACSCalibur *flow cytometer,* Becton Dickinson, San Jose, CA, USA). Data from 5000 events per sample were collected. The percentages of the elements in the hypodiploid region and in G_0/G_1, S and G_2/M phases of the cell cycle were calculated using the CellQuest or MODFIT software, respectively.

2.5.6 Statistical analysis

Data reported are the mean values ± SD from at least three experiments, performed in duplicate, showing similar results. Differences between treatment groups were analyzed by Student's t-test. Differences were considered significant when $p < 0.05$.

2.5.7 Recovery GIN eggs

Fresh faecal samples containing GIN eggs were obtained from naturally infected sheep and processed within 4 hours of collection. Specifically, GIN eggs were extracted from the positive samples the mass recovery method,

i.e., a method that employs 5 sieves of different dimension (1 mm, 250 µm, 212 µm, 63 µm and 38 µm) in order to separate the eggs from the faeces. (Coles et al., 2006). The latter filter (38 µm) was washed and eggs were suspended in deionized water and then visualized under a microscope (Leica, 20X) to record if embryonation had not begun. Ten aliquots of 0.1 ml were taken and the number of eggs counted (Godber et al., 2015). The mean of eggs counted in these aliquots was 150 per 0.1 ml of egg suspension.

2.5.8 Faecal cultures

In order to identify the GIN genera involved in this study, larval cultures were needed. An aliquot of faecal positive sample containing GIN eggs was broken up finely, using either a large pestle and mortar or spatula and were placed in a glass jar or petridishes which was closed and incubated at a temperature of about 25 ˚C for 10 days. After incubation, samples were examined for larvae using a binocular microscope (Raza et al., 2013). Third stage larvae were identified using the morphological keys proposed by van Wyk and Mayhew (2013). When a coproculture had 100 or less third stage larvae, all were identified; when a coproculture had more than 100 larvae, only 100 were identified (Cringoli et al., 2003).

2.5.9 Egg hatch assay

The EHA procedure followed that recommended by the World Association for the Advancement of Veterinary Parasitology (WAAVP) (Coles et al., 1992). The protocol to prepare thiabendazole (TBZ, Sigma) solution used deionized water with a neutral pH. Thus, for the preparation of solution A, 50-mg TBZ were dissolved in 5 ml water. Subsequent dilutions were made in deionised water (von Samson-Himmelstjerna et al., 2009). Stock solution B (1 mg TBZ per ml) was obtained by adding 1 ml of stock solution A (10 mg TBZ per ml) to 9 ml water, then 400 µl and 1000 µl of stock solution B were added to 9.6 ml and 9 ml of water, respectively. Therefore, the final concentrations of TBZ in wells were: 0.2 and 0.5 µg per ml. In our study, TBZ was used as positive control.
Five working concentrations were prepared of each *H. forskaolii* extracts: 1 mg/ml, 0.5 mg/ml, 0.1 mg/ml and 0.01 mg/ml. Egg suspension (100 µl with 150 eggs) was placed in plastic wells (24 well tissue culture test plates), 900 µl of water were added and finally1 ml of each extract concentration were

added. Therefore, the final concentrations in wells were: 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.05 mg/ml and 0.005 mg/ml. Each sample was tested in triplicate and at least two negative control samples were used (including the sample in deionized water without any drug/plant extracts). The plates were incubated for 48h at 25-27°C and the assay stopped by adding two drops of Lugol's iodine. The eggs and larvae in each well were carefully washed in a petri dish marked with a grid and counted on a binocular microscope (Coles et al., 2006). Thereafter, the number of larvae present per well was counted, and the percentage hatched was determined as the ratio between the number of larvae to the number of eggs deposited per well. A mean percentage of hatching was calculated for each concentration of each plant extracts.

2.6 Results

2.6.1 EHA test

The most prevalent genera of GIN were *Trichostrongylus* spp.(42.6%), followed by *Chabertia ovina* (22.9%), *Cooperia* spp. (21.3%), *Haemonchus contortus* (8.1%) and *Teladorsagia* spp.(5%).

The results of the EHA for the *n*-hexane extracts of *H. forskaolii*, TBZ and their water controls are shown in *Tab.* 2.1. The results showed a mean of 7.1 % of eggs in negative control (deionized water), a mean of 96.9% of eggs in the first positive control (0.2 µg/ml of TBZ) and a mean of 97.2% of eggs in the second positive control (0.5 µg/ml TBZ). *Tab.* 2.1 show that the *n*-hexane extract has a percentage of inhibition of egg hatching greater than other extracts inhibiting the 30.8% at the concentration of 1 mg/ml showing a dose-dependent effect on GIN eggs hatching. The chloroform:methanol (9:1) extract of *H. forskaolii* showed an high turbidity at 1 mg/ml concentration, therefore it was impossible to examine the sample with a binocular microscope

Tab. 2.1 Percentages of GIN eggs and egg hatch after treatment at various concentrations with thiabendazole, leaves n-hexane extract of H. forskaolii, and distillated water

TBZ (µg/ml)	% egg ± SD	% egg hatch ± SD
0.2 mg/ml	95.00 ± 1.00	5.00± 1.00
0.5 mg/ml	95.88 ± 0.03	4.12± 0.03
*n-h*exane (mg/ml)	% egg ± SD	% egg hatch ± SD
0.005 mg/ml	15.00 ± 0.68	85.00 ± 0.68
0.05 mg/ml	17.82 ± 0.65	82.18 ± 0.65
0.25 mg/ml	18.81 ± 0.24	81.19 ± 0.24
0.5 mg/ml	20.00 ± 0.79	80.00 ± 0.79
1 mg/ml	30.81 ± 0.83	69.19 ± 0.83
DeionisedWater Control	3.67 ± 1.53	93.33 ± 1.53

So, the *n*-hexane extract was studied in order to isolate the secondary metabolites.

2.6.2 Chemical study

The *n*-hexane extract of *H. forskaolii* was separated by silica gel column chromatography and RP-HPLC to afford thirteen compounds (**1-13**) nine of which are already known in literature (**3-9** and **12**).

The known isolated compounds were three labdane diterpenes named Chelodane (**3**) (Rudi and Kashman, 1992), Kolavelool (**4**) (Nagashima et al., 2001), 8,14-labdadien-13-ol (**5**) (Jian and Xiaozhang, 2000) and six fusicoccane diterpenes named 8 (9) α-Epoxy hypoestenone-12 (13) -anhydrohypoestenone (**6**) (El Sayed, 2001), Hypoestenone epoxide (**7**) (Muhammad et al., 1997), Dehydrohypoestenone (**8**) (Muhammad et al., 1998), Hypoestenone (**9**) (Muhammad et al., 1997), 13-Hydroxy-7-oxolabda-8,14-diene (**11**) (Nagashima et al., 2001), Deoxyhypoestenone (**12**) (Muhammad et al., 1998).

Compound **1** (*Fig.* 2.5) was obtained as an amorphous white powder. The molecular formula of compound **1** $C_{20}H_{32}O$ was established by ^{13}C NMR and HRMS (obsd *m/z* 289.2526 for $[M+H]^+$ calcd *m/z* 288.2453), indicating five degree of hydrogen definciences. The ^{13}C NMR of **1** contained

resonances for 20 carbons, while a DEPT experiment revealed the presence of five methyls, six methylenes, five methines, and four quaternary carbons including two sp^2 carbons. The NMR data (*Tab.* 2.2) showed that one of the elements of unsaturation was present as double bond, therefore the molecule was tetracyclic.

The ^1H NMR and ^{13}C NMR spectra showed the presence of epoxy ring resonances (δ_H 2.88, and δ_C 57.4 and 65.0). This group was located at C-8—C-9 on the basis of cross peaks in the ^1H-^1H COSY spectrum between H-9 and H_2-10, and of correlations observed in the HMBC spectrum between H-9 (δ_H 2.28) proton and C -7 (δ_C 52.6), C-17 (δ_C 17.8), C-11 (δ_C 50.8), and Me-17 (δ_H 1.12) and C -7 (δ_C 52.6), C-9 (δ_C 65.0). Furthermore, the analysis of the ^1H NMR data showed signals attributable to the presence of an isopropyl group at δ_H 0.80 (3H, d, J = 6.8 Hz, H-19)/δ_C 19.6 (C-19), δ_H 0.94 (3H,d, J = 6.8 Hz, H-20)/δ_C 23.3 (C-20) e δ_H 1.90 (1H, m, H-18)/δ_C 29.6 (C-18), the presence of this group was confirmed by ^1H-^1H COSY correlations between H-18 and Me-19 and Me-20; this inferred the presence of fusicoccane skeleton (Muhammad et al., 1998), which was supported by correlation of the H-18 resonance in the HMBC spectrum to C-13, C-11, C-20, and of δ_H 0.80 assigned to Me-19 with C-10, C-12. DQF-COSY and 1D-TOCSY measurements showing coupling between H_2-5 (2.33 m) —H_2-6 (1.94 m, 1.84 m), H-7 (2.58 dd, *J*=6.0 and 6.5 Hz) allowed assignment of the H-5—H-7 spin system. Likewise, an HMBC correlation to the two H_2-5 resonances established the $\Delta^{3,4}$ double bond at δ_C 126.0 (C-4), 125.4 (C-3). Extensive analysis of NMR spectra allowed the assignment of most functional groups to the fusicoccane skeleton (Al-Rehaily et al., 2002).

1D-TOCSY and COSY experiments provided evidence for the presence in the molecule of the segments H-9—H-12, H-14—H-11 and H-12—H-20. All protons directly bonded to carbon atoms were assigned on the basis of the cross peaks observed in the HSQC spectrum. The quaternary C—O resonance at δ_H 57.4 was assigned to C-8 by HMBC cross peaks between H-7 and C-8, C-9, C-5, C-3; H-10 and C-8, C-11, and between Me-17 and C-7, C-9.

The relative stereochemistry of compound **1** was obtained on the basis of 1D-ROESY data. The β-orientation of H-9, H-11, H-12 and Me-17 was indicated by ROE cross peaks among proton spatially related particularly H-9 with H-11, and Me-17; and H-12 with H-11 and H-9. This was confirmed by chemical shifts of the respective carbons, which matched well with those

of related fusicoccane-type diterpenes (Muhammad et al., 1998). The 15-methyl proton signal at δ_H 0.67, showed ROE correlations with the signal at δ_H 2.58 (H-7) and 1.90 (H-18), this observation clarified the relative stereochemistry between these proton, the relative stereochemistry of compound **1** was thus found to be identical to that of relate compound roseatoxide (Akindo et al. 1984). On the basis of these data, compound **1** was characterized as a fusicoccane derivative named 8(9)-epoxy, 3(4) fusicoccene

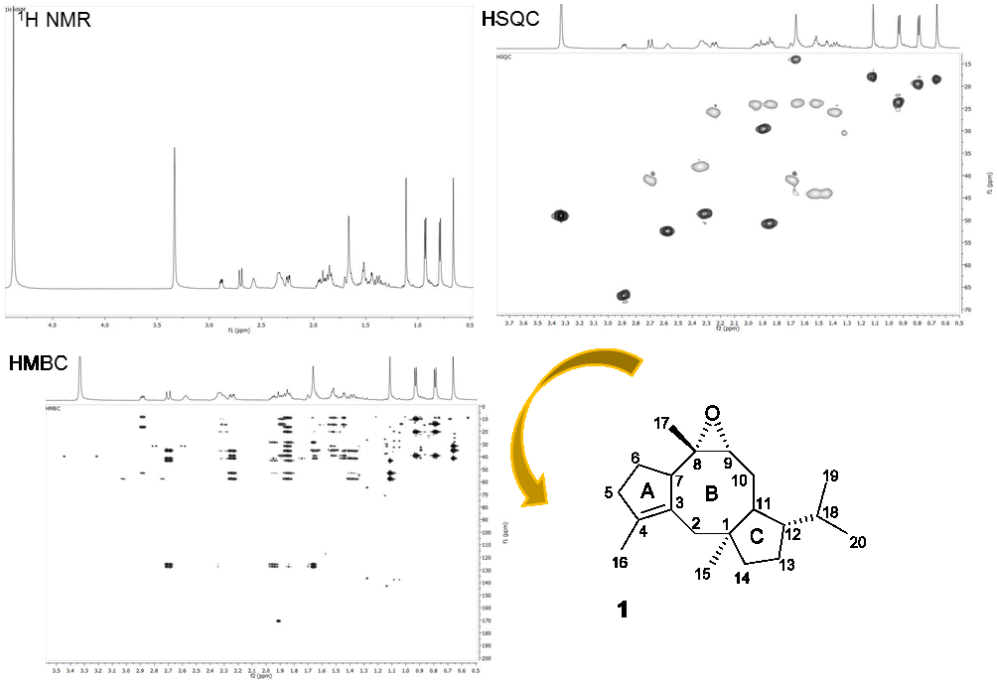


*Fig. 2.5 NMR spectra of compound **1***

Compound **2** (*Fig.* 2.6) was isolated as a colorless, amorphous solid, with molecular formula of $C_{20}H_{32}O$ as determined by HRMS, showing a quasi molecular ion at *m/z* 289.2529 $[M+H]^+$, requiring five degrees of unsaturation. ESI/MS/MS spectrum showed a single fragment ion at *m/z* 271 produced by elimination of a water molecules.

The carbon resonances at δ 124.4 and 131.0, which was assigned to one oleofinic group based on COSY, HSQC, and HMBC data, accounted for one degree of unsaturation and suggested the presence of four ring in **2**. Results

obtained from 1D-TOCSY and COSY experiments established the connectivity of protons H-5—H-7, H-9—H-14, H-12—H-20. The elucidation of the whole skeleton from the above subunits was achieved on the basis of HSQC and HMBC correlations, which also allowed the assignment of all the resonance in the ^{13}C NMR spectrum of the pertinent carbons (*Tab.* 2.2). In the ^{13}C NMR spectrum two signals at δ 69.0 and 64.4 ppm allowed to establish the presence of an epoxy ring which was located at C-3/C-4 on the basis of HMBC experiment. Key correlation peaks between H-2—C-3, H-2—C-7, H-5—C-3, H-7—C-7, H-6—C-3, H-6—C-4, H-6—C-8 were observed.

The spin system of ring B was obtained starting from proton at δ 5.68 (H-9) which correlated with signals at δ 2.20 and 2.14 (H_2-10), 1.69 (H-11), 2.23 (H-12), 1.90 (H-18), 0.84 (H-19), and 0.92 (H-20), 1.59 and 1.51 (H_2-13), and 1.59 and 1.48 (H_2-14). Moreover, ring B was connected with rings A and C by HMBC correlations: H-2/ C-4, C-7, C-11; Me-15/C-3, C-11, C-14; H-9/C-7, C-11, C-17, C-12, H-6/C-4, C-5, C-8; H-18/C-11, and C-20. Therefore, the structure of **2** was defined as 3,4-epoxy-8(9)-fusicoccene.

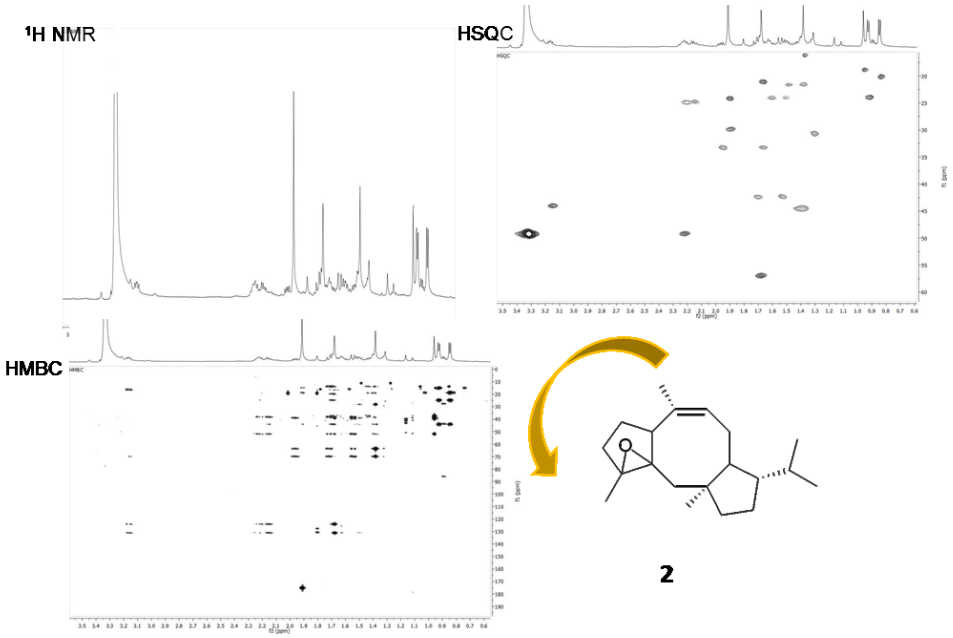


Fig. 2.6 NMR spectra of compound **2**

Compound **10** (*Fig.* 2.7) was obtained as an amorphous white powder. The molecular formula of compound **10** ($C_{20}H_{30}O_2$) was established by ^{13}C NMR and HR-MS (obsd *m/z* 303.2320 for $[M+H]^+$ calcd *m/z* 302.2246) indicating six degrees of unsaturation. The NMR data (*Tab.* 2.3) showed that three of the elements of unsaturation were present as one carbonyl group, and two double bonds, therefore, the molecule was tricyclic.

In the ^1H NMR spectrum of **10**, two methyl singlets linked to sp^2 carbons resonating at δ 1.72 and 1.52 were assigned as C-16 and C-17, respectively; while a methyl singlet at δ 0.83 was assigned as C-15. Two methyl doublets at δ 0.88 (*J* = 7.0 Hz, Me-19) and δ 0.95 (*J* = 7.0 Hz, Me-20) were also present. The hydroxymethine resonance at δ_C 80.0 and δ_H 3.69 (1H, dd *J*=10.5 and 5.5 Hz) was assigned to C-14 by HMBC cross peaks between H-14 and C-1, C-11, C-12, C-15; Me-15 and C-1, C-11, and C-3, and between H-12 and C-11, C-1, C-18, and C-19. Three spin systems were recognized from 1D TOCSY and DQF-COSY spectra: H-9—H-14, H-6—H-7, H-12—H-20. In the ^1H NMR spectrum one proton signal at δ_H 5.73 (m, H-9) was also present showing in HSQC experiment a cross peak with a carbon to δ_C 130.0 indicating the presence of a double bond. The double bond was located at C8-C9 on the basis of the HMBC longe range correlations observed between H-9/C-7, H-9/C-11, and H-9/C-17, and Me-17/C-9, Me-17/C-8, Me-17/C-7, and H-7/C-3, H-7/C-5, H-7/C-9.

The tetra-substituted double bond located between C-3 and C-4 was supported by the HMBC correlations of Me-16 with H-2 and H-6; C-3 with H-7, H-2. Moreover the HMBC spectrum showed correlations between the proton signal at δ_H 4.15 (H-7) and C-3, C-5, C-8; between the signal at δ_H 3.18 (H-2) and C-7, C-3, C-4, C-15: between the signal at δ_H 1.83 (H-11) and C-1, C-2, C-12, C-14 locating the α,β unsaturated carbonyl group at C-5 $\Delta^{3.4}$.

The relative stereochemistry was assigned on the basis of the correlations observed in 1D-ROESY spectrum and by comparison with the coupling constants and the ^1H NMR chemical shifts reported in the literature (Muhammad et al. 1997,1998). In fact, in the 1D- ROESY spectrum correlations between H-7 (δ_H 4.15), Me-15 (δ_H 0.83), H-18 (δ_H 1.95) indicated that they were placed on the same side of the molecule. The 1D-ROESY indicated also that the protons at position 11, 12, and 14 were *syn*, relative to each other (Musayeib, et al 2014).

From these results, the structure of compound **10** was determined as fusicoccane derivative and named Hypoestanonol H 14-hydroxy,3(4),8(9)diene-fusiccon-5-one.

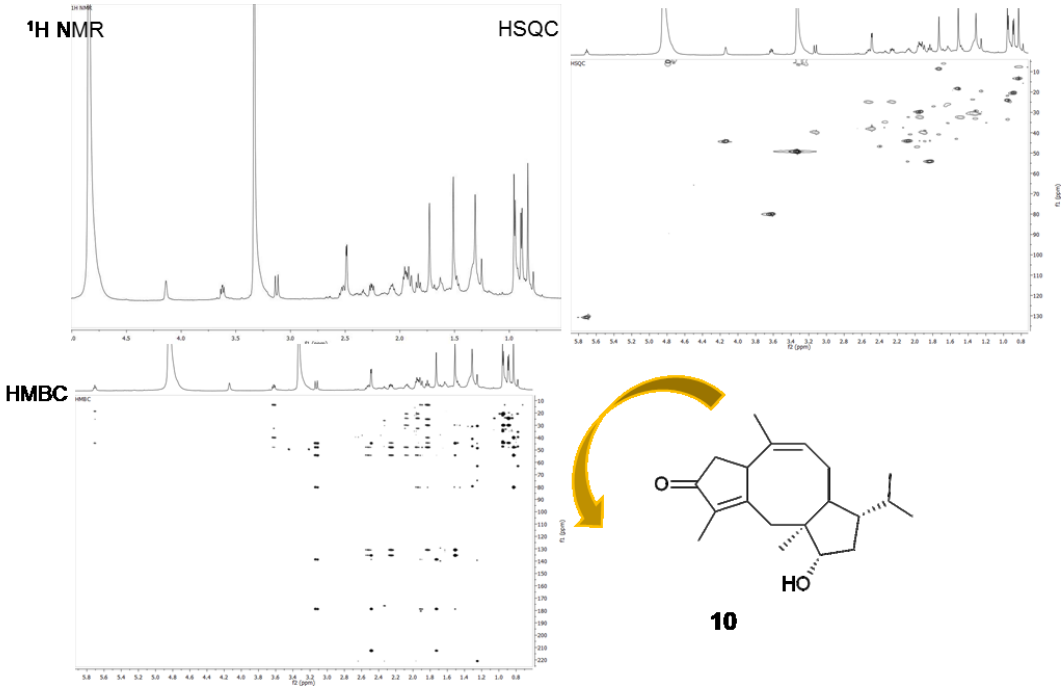


Fig. 2.7 NMR spectra of compound **10**

Compound **13** (*Fig.* 2.8) was obtained as an amorphous white powder. The molecular formula of compound **13** $C_{22}H_{32}O_3$ was established by ^{13}C NMR and HRMS (obsd *m/z* 345.2429 for $[M+H]^+$ calcd *m/z* 344.2351) indicating six degree of unsaturations. The ^{13}C NMR of **13** contained resonances for 22 carbons, while a DEPT experiment revealed the presence of six methyls, four methylenes, six methines, and six quaternary carbons including four sp^2 carbons, and one carbonyl group. The NMR data (*Tab.* 2.3) showed that three of the elements of unsaturation were present as two double bonds and one carbonyl, therefore the molecule was tricyclic.

An α,β unsaturated carbonyl group occurring in ring A ($\Delta^{3,4}$) was deduced from the signal due to a methyl linked at a sp^2 carbon at δ 1.70 and from the sub-spectrum obtained by a 1D-TOCSY experiments. The irradiation of the signal at δ 4.12 (H-7) showed connectivity with chemical shifts at δ 2.48

(H_2-6), 1.51 (Me-17). These observations were substantiated by the HMBC correlations of Me-16 (δ 1.70) and C-5, and C-4; H-7 (δ 4.12) and C-5, C-4, and C-2; H-2 (δ 2.90) and C-1, C-3, C-4 and C-7. The spin system of ring B was obtained starting from proton at δ 5.71 (H-9) which correlated with signals at δ 2.29 and 2.55 (H_2-10), 1.92 (H-11), 2.15 (H-12), 1.97 (H-18), 0.88 (H-19), and 0.95 (H-20). Moreover, ring B was connected with rings A and C by HMBC correlations: H-2/ C-4, C-7, C-11; Me-15/C-3, C-11, C-14; H-9/C-7, C-11, C-17, C-12, H-6/C-4, C-5, C-8; H-18/C-11, and C-20.
Analysis of its NMR data revealed signals which matched very closely those obtained for compound **10**. Comparative analysis of the NMR data of compound **13** with diterpene **10** showed close similarities, a point of difference was the presence of an acetyl group. Since the proton and carbon signals of the –CHOH at C-14 were typically shifted downfield (δ_H 4.72 ; δ_C 82.0) with respect to compound **10** the C-14 position must be esterified by the acetyl group. The position of the acetyl residue was confirmed by the results of the HMBC spectrum of **13** which showed clear long-range correlation peaks between the carbonyl carbon signal of acetyl group (δ171.00) and the proton signal of CHOH at C-14.
The relative stereochemistry was elucidated from the ROESY spectrum to be identical to that of **10**. Therefore, the structure of **13** was defined as 14-acetyl-Hypoestanonol H.

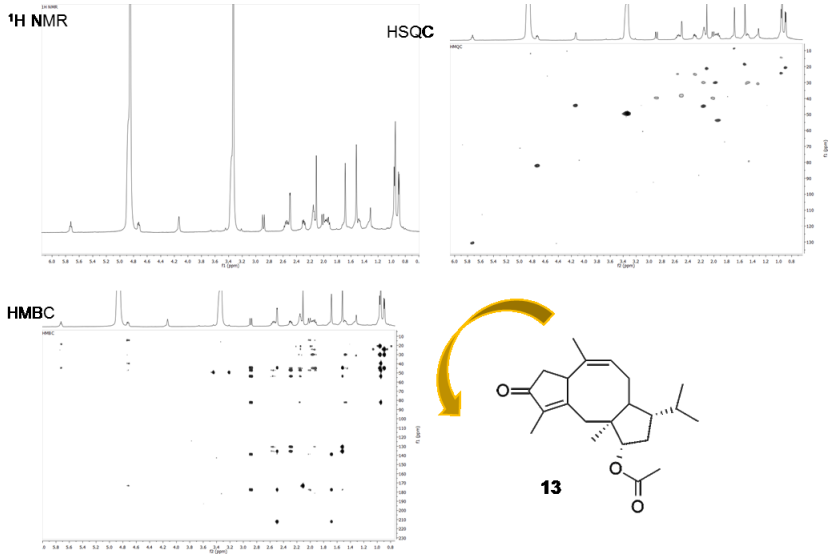


*Fig. 2.8 NMR spectra of compound **13***

Tab. 2.2: ^{1}H and ^{13}C NMR data of compounds **1**–**2** *(CD_3OD, 600 MHz, J in Hz)*[a]

Position	Compounds			
	1		**2**	
	δ_H	**δ_C**	**δ_H**	**δ_C**
1	-	35.0	-	36.0
2 a	2.69 d (9.0)	40.8	1.71 d (14.5)	42.1
2 b	1.68 d (9.0)		1.53 d (14.5)	
3	-	125.4	-	63.4
4	-	126.0	-	69.4
5	2.33 m	37.8	1,98 dd (11, 7.7)	33.0
			1.68 m	
6 a	1.94 m	23.9	1.50 [a]	21.4
6 b	1.84 m		1.38 m	
7	2.58 dd (6.5, 6.0)	52.6	3.18 dd (6.63,10)	43.8
8	-	57.4	-	131.0
9	2.88 m	65.0	5.68 t (8)	124.4
10 a	2.24 ddd (8.0,11.0,14.0)	25.6	2.14 br dd(14, 8)	24.7
10 b	1.39 [o]		2.20 br dd(14, 7)	
11	1.85 br dd (7.4, 11.3)	50.8	1.69dd (12, 7.5)	56.6
12	2.30 m	48.5	2.23m	49.0
13 a	1.65 m	23.7	1.59 [o]	23.7
13 b	1.52 [o]		1.51[o]	
14 a	1.51 [o]	43.9	1.59 [o]	44.3
14 b	1.44 m		1.48 [o]	
15	0.67 s	18.4	0.95 s	18.7
16	1.67 s	13.9	1.36 s	16.0
17	1.12 s	17.8	1.66 s	20.0
18	1.90 m	29.6	1.90 m	29.6
19	0.80 d (6.8)	19.6	0.84 d (6.8)	20.5
20	0.94 d (6.8)	23.3	0.92 d (6.8)	23.8

[a] Data assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments
[o] Overlapped signal

*Tab. 2.3 ^{1}H and ^{13}C NMR data of compounds **10**–**13**(CD_3OD, 600 MHz, J in Hz)*[a]

Position	Compounds			
	10		**13**	
	δ_H	**δ_C**	**δ_H**	**δ_C**
1	-	48.0	-	46.9
2a	3.18 d (8.7)	39.5	2.90 d (9.3)	39.5
2b	1.89 d (8.7)		2.01 d (9.3)	
3	-	178.8	-	177.4
4	-	138.6	-	146.2
5	-	213.0	-	212.2
6	2.49 m	38.0	2.48 [o]	37.9
	1.80 m			
7	4.15 br t (6.5)	44.0	4.12 br d (6.5)	44.1
8	-	135.0	-	135.5
9	5.73 m	130.0	5.71	128.7
10a	2.54 ddd (9.3,14.0,5.0)	24.8	2.55 [o]	24.7
10b	2.26 m		2.29 [o]	
11	1.83 br dd (11.5,7.0)	54.1	1.92 m	53.4
12	2.07 m	43.8	2.15	44.6
13a	1.94 m	32.3	2.18	27.9
13b	1.47 m		1.45	
14	3.69 dd (10.5,5.5)	80.0	4.72 br s	82.0
15	0.83 s	13.3	0.96 s	14.0
16	1.72 s	8.62	1.70 s	6.7
17	1.52 s	18.0	1.51 s	18.3
18	1.95 m	29.4	1.97	28.2
19	0.88 d (7.0)	20.6	0.88 d (6.8)	20.4
20	0.95 d (7.0)	23.8	0.95 d (6.8)	24.6
			$CH_3\underline{CO}$ -	171.0
			CH_3CO 2.10 s	20.9

[a] Data assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments.
[o] Overlapped signal

Known compounds isolated from *H. forskaolii* leaves

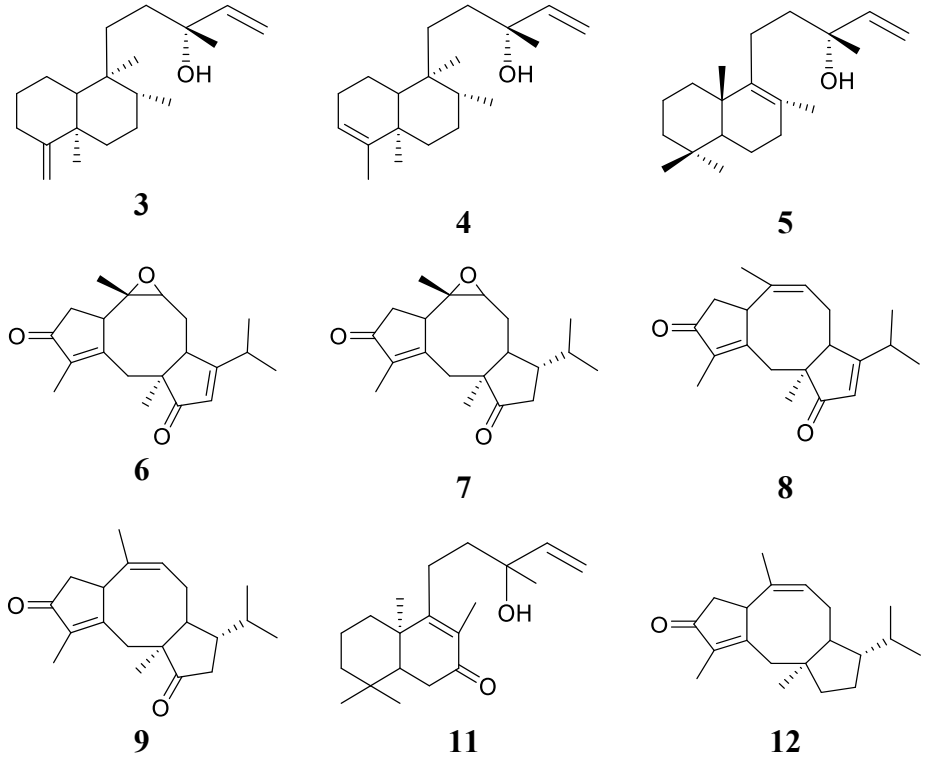


Fig. 2.9 Known compounds of H. forskaolii

2.7 Discussion

GIN are a major cause of disease in grazing sheep. Despite the progress in the development of parasite vaccines, anthelmintic are still indispensable for the for worm treatment and control (Molento et al., 2011). However, the indiscriminate use of anthelmintic to control helminths has led to a widely AR in sheep (Jackson et al., 2012). The development of AR to commercially available drugs as well as the risks that are associated with the presence of these products in the environment and in foods of animal origin have encouraged the search for new active ingredients that are less toxic, able to minimize the presence of drug residues in food of animal origin and more efficient.

In this context, products of plant origin may be an effective alternative for the control of parasites (Nery et al., 2009). The use of plants with anthelmintic properties is considered as one of the most viable alternative methods for the control of GINs even though crude drugs are less efficient with respect to cure of diseases but are relatively free from side effects.
A large number of medicinal plants could possess anthelmintic properties in the traditional system of medicine and are also used by ethnic groups in the world, unfortunately, only a small number of plant species shows an appreciable ovicidal activity (Mali and Mehta, 2008).
The results of this study show that extracts from the leaves of *H. forskaolii* exhibit a weak ovicidal activity against GIN, whereas only two works have succeeded in demonstrating that two plants, belonging to Acanthaceae family, show a considerable *in vitro* ovicidal activity, but only within their polar residues, such as aqueous and ethanolic. Nevertheless, there are no studies which focus on apolar extracts such as the *n*-hexane and the chloroformic. In a study by (Al-shaibani et al., 2008), the aqueous and ethanolic extracts of *Adhatoda vasica* presented ovicidal activity against the GIN eggs. The ethanolic extract was slightly more effective compared to aqueous extract on eggs. In the study of (Adamu et al., 2010) it was instead taken into account only the aqueous extract of *Acanthus montanus* (Nees) T. Anders (Acanthaceae). In this work, in addition to polar methanolic extract, the apolar and medium polar residues, respectively the n-hexane, chloroform and chloroform methanol 9: 1, were analyzed for the first time in a plant belonging to the Acanthaceae family.
In conclusion, in the adopted experimental conditions, the *n*-hexane extract showed a rather mild egg hatch inhibition and a dose-dependent effect on nematode eggs hatching while on the contrary in other studies, conducted in various experimental conditions, the n-hexane extract and other apolar extracts from different plant species showed dissimilar ovicidal activities against nematodes in sheep, such as *H. contortus, Teladorsagia spp., Trichostrongylus spp., Strongyloides papillosus, Oe. columbianum, Chabertia ovina* (Assis et al., 2003; Devi Rajeswari, 2014; Maciel et al., 2006).
According to the adopted experimental approach, it was not possible to test the extracts in concentrations higher than 1mg/ml because of the turbidity of the solutions created in wells during the EHA. Despite using surfactant substances to increase the solubility of the extracts, it was impossible to count the eggs or larvae in those wells where concentrations exceeded the

highest cited. Moreover, limited to the chloroform:methanol 9:1extract, it was impossible to analyse the concentration of 1mg/ml due to the turbidity of the solution that was to already created at that concentration.
The inhibition of egg hatch *in vitro* is an indication of the possible usefulness of various plant as potential anthelminthic and, in Particular, *H. forskaolii* appears to possess a dose-dependent anthelmintic activity with a mild activity shown. For these reasons, under our experimental conditions we have obtained encouraging results and extracts of this plant can be tested on other species of helminths and it could be interesting to evaluate the efficacy of n-hexane extracts of the other plants of Acanthaceae family in order to study their activity, toxicity, mechanism of action, identification of phytochemicals.
Finally, the phytochemical study of *n*-hexane extract led to the isolation of several pure compounds which they have not been tested yet because of the very few amounts. Hence, the antiproliferative potential of the all isolated compounds was tested *in vitro*. Exponentially growing cultures of HeLa (cervical carcinoma) and Jurkat (T-cell leukemia) cells were exposed to increased concentrations of all isolates and cell viability was evaluated at 48 h by MTT assay. All tested compounds did not affect significantly cell growth and viability.

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Chapter 3

Trichilia maynasiana C.DC
Meliaceae

D'Ambola, M., Malafronte, N., Gualtieri, M., Hernández, V., Vassallo, A., Severino, L.
2016 A Novel Tirucallane-type Triterpene and Sesquiterpene from *Trichilia maynasiana* C.DC
Natural Product Communication. (4):447-8.

3.1 Meliaceae family

The Meliaceae family, belonging to the order Rutales, is split in four subfamilies: Swietenioideae, Melioideae, Quivisianthoideae and Capuronianthoideae (Salles, 1995). This family classified 51 genera containing about 1400 species of the tropics and subtropics in both hemispheres (Banerji and Nigam, 1984). Plants of this genus present great economic interest by the wood and essential oils industries (Mosqueta, 1995; Penington, 1981)**.** Meliaceae are a widely distributed in subtropical and tropical angiosperm family occurring in a variety of habitats, from rain forests and mangrove swamps to semi deserts areas (Pérez-Flores et al., 2012). The major specialized metabolites of Meliaceace family are limonoids (tetranortriterpenoids) and terpenoids even though alkaloids, flavonoids and phenolic compounds are also present in remarkable amount. Many biological activities have been reported for the species belonging to Meliaceae family such as antimicrobial activity (Ragasa et al., 2014; Fang et al., 2009; Irungu et al., 2014), cytotoxicity against many tumour cell lines and uterotonic activity. These interesting activities suggest that chemical studies and biological screenings of small molecules of Meliaceae family are needed. (Kaur and Arora, 2009; Rashed, 2014; Yang et al., 2012)

3.2 *Trichilia* Genus

The *Trichilia* genus is composed by 70 species, mainly present in tropical America and Africa, of which 43 species occur in Brazil (del Carmen Ramírez et al., 2000; Pupo et al., 2002). *Trichilia* is a genus which has the largest number of species in Meliaceae. (Patrício and Cervi, 2005; Rocha, 2010). A significant number of reports showed biological and pharmacological activities of crude extracts and pure secondary metabolites isolated from different parts of plants belonging to *Trichilia* genus. The main biological activity is antibacterial activity against plant parasites, and bactericide activity against *Bacillus cereus*, *Eschericha coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*; cytotoxic and antioxidant activities are also reported for this genus (del Carmen Ramírez et al., 2000; Jolad et al., 1981; Kubo and Klocke, 1982; Pizzolatti et al., 2002; Tang et al., 2007). The crude extracts of *T. catigua* (A. Juss.)*, T. connaroides* (Wight & Am.) Bentv.*, T. elegans* a. juss*,* showed antiplasmodium and insecticidal activity (Agripino et al., 2004; Kumar et al., 2011; Matos et al., 2009). The ethyl

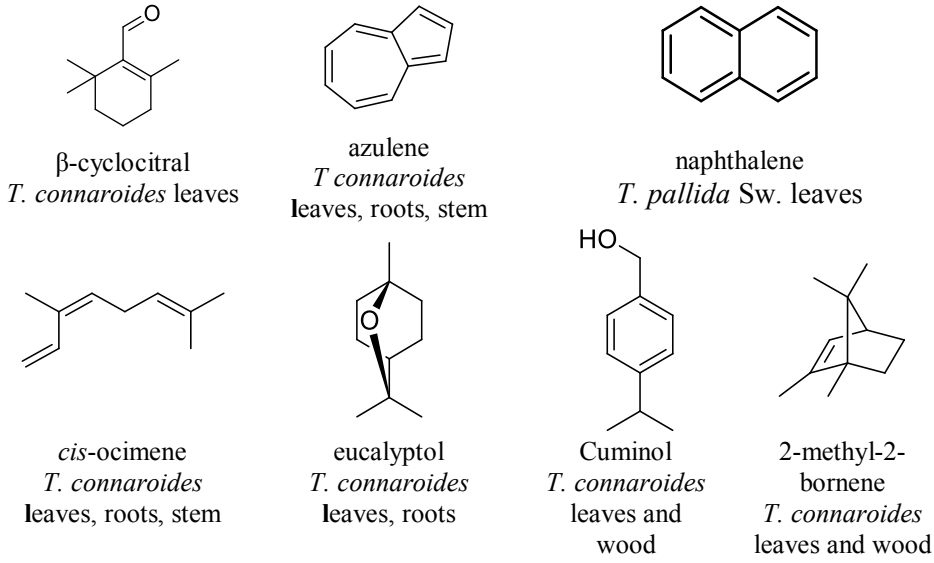
acetate extract of aerial parts of *T. quadrijuga* (Miq.) Kunth showed high antibacterial activity against *S. aureus* and *S. epidermidis* (Rodrigues et al., 2009), while the aqueous leaves extract of *T. glabra* L. was found to possess anti-inflammatory effect (Benencia et al., 2000).

3.2.1 Previous phytochemical studies on Trichilia genus

Chemical composition of *Trichilia* species revealed the presence of secondary metabolites from the metabolic pathway of terpenoids. Particularly limonoids, nor-triterpenes with high oxygenation, appear to be the major chemosystematics markers of the Meliaceae family (Lagos, 2006; Rodrigues et al., 2009). The secondary metabolites isolated from the genus *Trichilia* are shown below.

3.2.1.1 Monoterpenes

6 monoterpenes were identified in the *Trichilia* genus (*Fig.*3.1) (Kumar et al., 2011; Amaro, 2007).



β-cyclocitral
T. connaroides leaves

azulene
T connaroides
leaves, roots, stem

naphthalene
T. pallida Sw. leaves

cis-ocimene
T. connaroides
leaves, roots, stem

eucalyptol
T. connaroides
leaves, roots

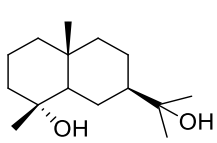
Cuminol
T. connaroides
leaves and wood

2-methyl-2-bornene
T. connaroides
leaves and wood

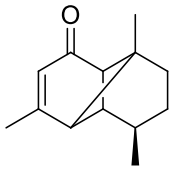
Fig. 3.1 Monoterpenes of Trichilia genus

3.2.1.2 Sesquiterpenes

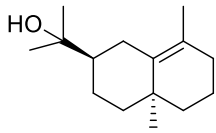
57 sesquiterpenes were identified in the *Trichilia* genus, revealing various skeletons, (Aladesanmi and Odediran, 2000; Amaro, 2007; Kumar et al., 2011) (*Fig.*.3.2).



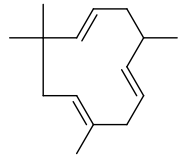
cryptomeridiol
T. connaroides
wood



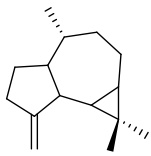
mustacone
T. silvatica
leaves



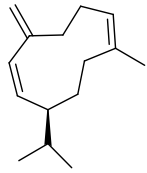
10-epi-γ-eudesmol
T.pallida leaves



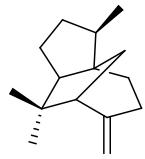
α-humulene
T. pallida
leaves



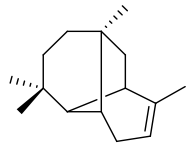
β-gurjunene
T. connaroides
wood



germacrane D
T. connaroides
stem and wood



β-cedrene
T. connaroides
wood

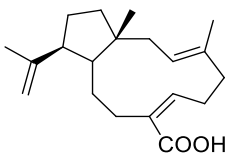


i-longipinene
T. connaroides
roots

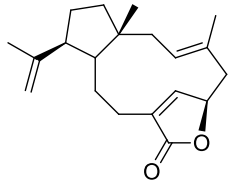
Fig. 3.2 Sesquiterpenes of Trichilia genus

3.2.1.3 Diterpenes

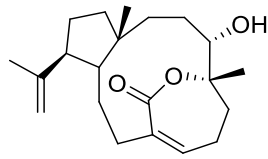
7 diterpenes were identified in the *Trichilia* genus (Aladesanmi and Odediran, 2000; del Carmen Ramírez et al., 2000) (*Fig*.3.3).



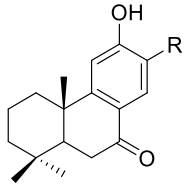
(1R,3E,7Z,11S,12S)-dolabella-3,7,18-trien-17-oic acid
T. heudelotii (Th) leaves



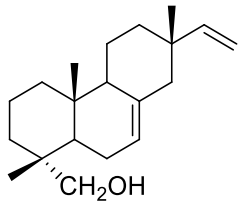
(1*R*,3*E*,6*R*,7*Z*,11*S*,12*S*)-dolabella-3,7,18-trien-6,17-olide
T. trifolia (A.Juss.) stem



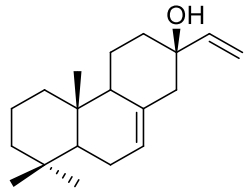
(1*R*,3*S*,4*R*,7*Z*,11*S*,12*S*)-3-hydroxydolabella-7,18-dien-4,17-olide
T. trifolia stem



R=CH_3 nimbiol;R=C_3H_7 7-ketoferruginol
T. heudelotii leaves



Isopimarinol
T. heudellotti leaves

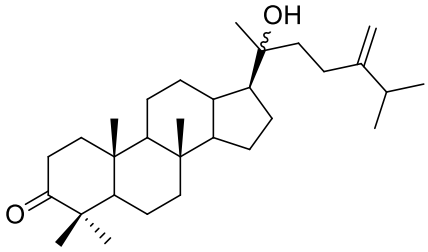


12β-hydroxysandaracopimar-15-ene
T. heudellotti leaves

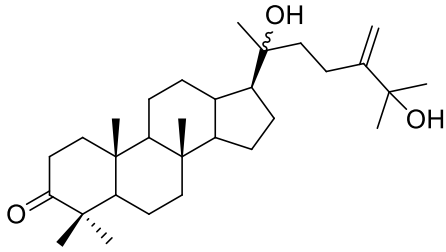
Fig. 3.3 Diterpenes of Trichilia genus

3.2.1.4 Triterpenes

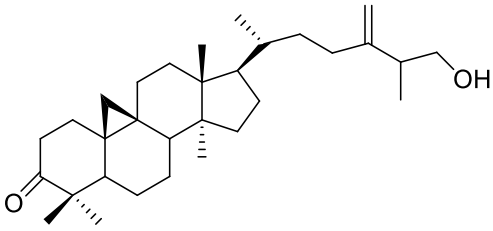
57 triterpenes were identified in *Trichilia* of which 32 tetracyclic triterpenes, fourteen cycloartane-type, four triterpenes with A-*seco*-ring and seven pentacyclic triterpenes (Inada et al., 1994; Cortez et al., 1998; Figueiredo, 2010; Garcez et al., 1996; Rocha, 2010; Ambrozin, 2004) (*Fig*.3.4).



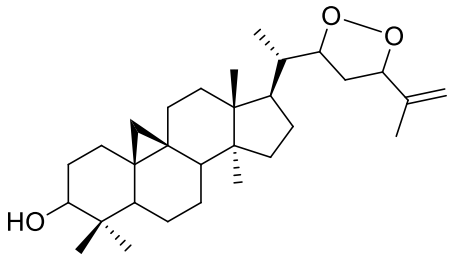
vellozone
T. connaroides leaves



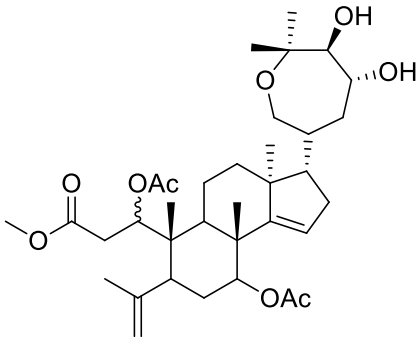
Isofouquerione
T. estipulate (Wight & Arn.) fruits



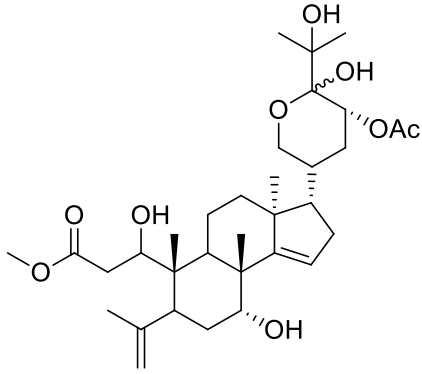
24-methylen-26-hydroxycicloartan-3-one
T. casaretti C.DC. leaves



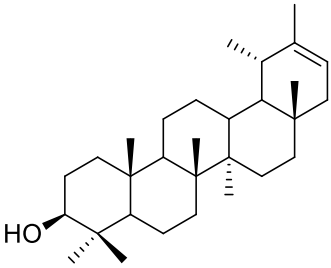
trichiliol
T. casaretti leaves



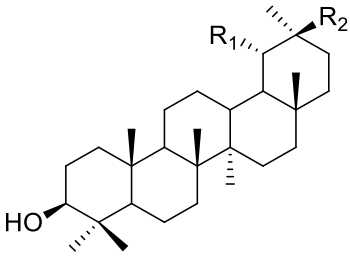
methyl-1ξ,7(R)-diacetoxy-23(R),25(S)-dihydroxy-20(S)-21,25-epoxy-3,4-seco-apotirucall-4(28), 4(15)-dien-3-oate
T. elegans seed



methyl-1(*S*),23(*R*)-diacetoxy-7(*R*),24,25-trihydroxy-20(*S*)-21,24-epoxy-3,4-*seco*-apotirucall-4(28), 14(15)-dien-3-oate
T. elegans seed



pseudotaraxasterol
T. sylvatica Kunth leaves

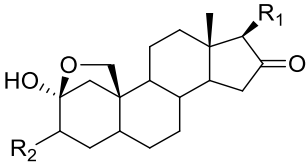


R_1=CH_3; R_2=H α-amirine
R_1=H; R_2=CH_3 β-amirine
T. ramalhoi C.DC. leaves

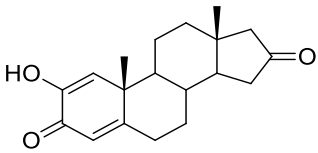
Fig. 3.4 Triterpenes of Trichilia genus

3.2.1.5 Steroids

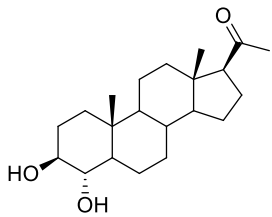
A total of 30 steroids were isolated of *Trichilia ssp*, distributed in the leaves and stem of the species *T. claussenii* and *T. connaroides* (*Fig*.3.5)



R_1=H; R_2=OH
2α,3α-dihydroxyandrostan-16-one-2β,19-hemiketal
T. claussenii C.DC. leaves
(Pupo et al., 2002)



*Trichilia*sterone B
T. americana C. DC. stem
(Hantos et al., 2001)

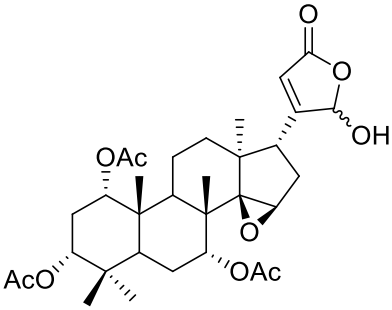


3β,4α-dihydroxypregnan-21-one
T. connaroides stem
(Wang et al., 2008)

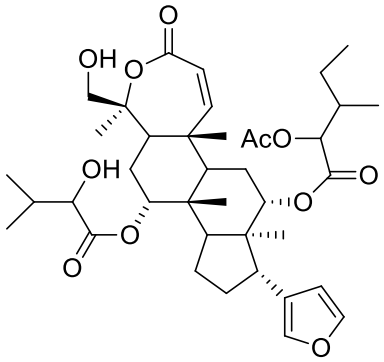
Fig. 3.5 Steroids of *Trichilia* genus

3.2.1.6 Limonoids

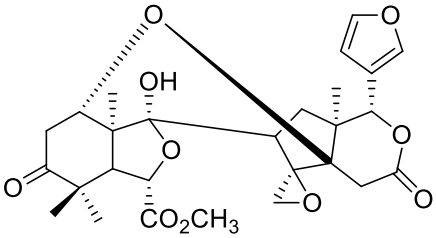
Among all the compounds isolated and identified by phytochemical investigation of species of the *Trichilia* genus, the limonoids represent the largest number. 129 limonoid were isolated and characterized from *T. connaroides*, *T. emetica*, *T. havanensis,* the last specie revealed the higher amount of these compounds (*Fig*..3.6).



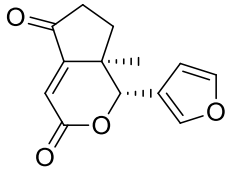
Hydroxybutenolide *T. havanensis* seeds
(Arenas and Rodriguez-Hahn, 1990)



dregeana-5 *T. dregeana SOND* stem
(Connolly et al., 1976)



trichilin A (235)
T. catigua seed
(Hill and Sutherland, 2009)



trichiconnarin A 286
T. connaroides leaves
(Wang et al., 2008)

Fig. 3.6 Limonoids of Trichilia genus

3.3 *Trichilia maynasia* C. DC.

Trichilia maynasiana C. DC. syn. *Trichilia senxanthera,* (Meliaceae), known with the name of "Sulafinzan" in the language of Bambara, is a tree widely distributed in tropical areas and in West-Africa lands. The plant is employed for the treatment of various disorders in Mali as purgative, antiepileptic, antipyretic and antimalarial agent, bronchial and inflammation deseases (Iwu, 1993),while, in South America especially in Venezuela, this plant is used in traditional medicine in the treatment of hemorrhoids, gastrointestinal disorders and as an antimalarial (Malgras, 1992).

AIM

The aim of this work was the isolation and structural characterization of secondary metabolites from *T. maynasiana* and the evaluation of their cytotoxic activity.

3.4 Experimental

3.4.1 Plant material

The leaves of *Trichilia maynasiana* were collected in Mérida-Venezuela and identified by Ing. Juan Carmona. A voucher specimen number (No 011) was deposited at the Herbarium MERF.

3.4.2 Extraction and isolation

The dried leaves of *T. maynasiana* (200 g) were powdered and exhaustively extracted using *n*-hexane, $CHCl_3$ and MeOH in an ASE 2000 extractor to yield 4.2, 6.2, 3.0 g of the respective residue (*Fig.*.3.7).

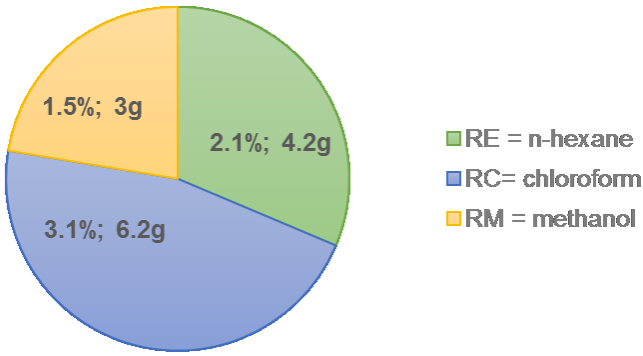


Fig. 3.7 Extracts of T. maynasiana leaves

Part of the *n*-hexane (3.5 g) extract was separated using silica gel, eluting with *n*-hexane followed by increasing concentrations of $CHCl_3$ in *n*-hexane (between 1% and 100%) and MeOH in $CHCl_3$ (between 1% and 100%). Fractions of 25 mL were collected, analysed by TLC and grouped into 11 fractions (A-K) (*Fig.*.3.8).

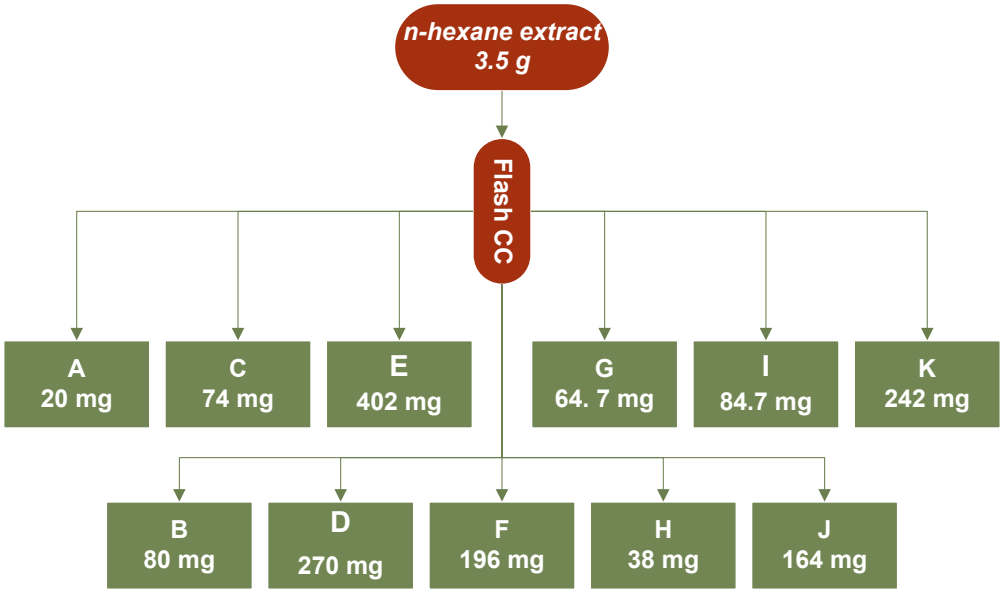


Fig. 3.8 Separation scheme of *n*-hexane extract

Fraction D (270 mg) was subjected to RP-HPLC with $CH_3OH:H_2O$ (95:5) as eluent. The spectroscopic analysis of the resolved peaks, has led to the characterization of pure 3β-25-dihydroxytirucalla-7,23-diene (1.2 mg, t_R 20 min); Fraction E (402 mg) was separated by RP-HPLC with $CH_3OH:H_2O$ (9:1) as eluent to give pure spathulenol (1.5 mg, t_R 8 min), compound **1** (3.0 mg, t_R 28 min) and 3β-25-dihydroxytirucalla- 7,23-diene (2 mg, t_R 32 min); Fraction I (84,7 mg) was subjected to RP-HPLC with $CH_3OH:H_2O$ (17:8) as eluent. The spectroscopic analysis of the resolved peaks, has led to the characterization of pure aromadendrane-4β,10α-diol (1.0 mg, t_R 15 min); Fraction J (164 mg) was was separated by RP-HPLC with $CH_3OH:H_2O$ (65:35). The spectroscopic analysis of the resolved peaks, has led to the characterization of pure 1,7-azulenediol,1,2,3,3α,4,5,6,7-octahydro-1,4-dimethyl-7-(1-methylethyl) (2.9 mg, t_R 50 min).

Part of the $CHCl_3$ extract (5.0 g) was subjected to silica gel column chromatography eluting with $CHCl_3$, followed by increasing concentrations of MeOH in $CHCl_3$ (between 1% and 100%). Fractions of 25 mL were collected, analysed by TLC using as mobile phase a solution of *n*-$BuOH:AcOH:H_2O$ (60:15:25) and $CHCl_3:CH_3OH:H_2O$ (80:18:2) sprayed with a $Ce(SO_4)_2$ (10% H_2SO_4) reagent and grouped into 13 fractions (Aa-Mm) (*Fig.*.3.9).

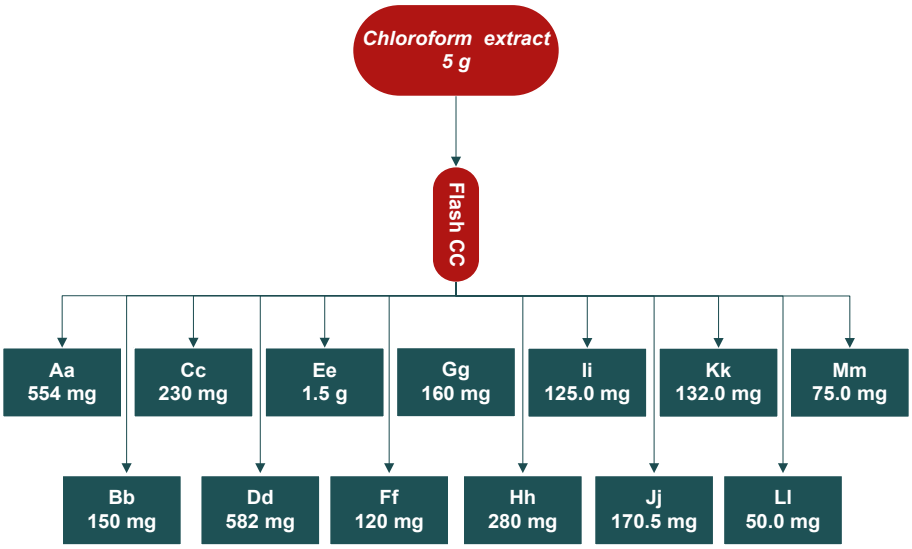


Fig. 3.9 Separation scheme of chloroformic extract

Fraction Dd (610 mg) was chromatographed over RP-HPLC with CH_3OH:H_2O (95:5) as eluent to give pure butyrospermol (8.0 mg, t_R 40 min); Fraction Ee (1800 mg) was subjected to RP-HPLC with CH_3OH:H_2O (85:15) as eluent to give pure compound **1** (1.6 mg, t_R 65 min), 3β-25-dihydroxytirucalla-7,23-diene (8.0 mg, t_R 72 min) and masticadienediol (2.2 mg, t_R 90 min); Fraction Hh (296,2 mg) was chromatographed over RP-HPLC with CH_3OH:H_2O (31:19) as eluent to give pure compound **2** (1.1 mg, t_R 10 min) and aromadendrane-4β,10α- diol (1.6 mg, t_R 18 min); Fraction Ii (124,8 mg) was subjected to RP-HPLC with CH_3OH:H_2O (35:15) as eluent. The spectroscopic analysis of the resolved peaks, has led to the characterization of pure lochmolin f (1.2 mg, t_R 11 min) and 1,7-azulenediol.

3.4.3 Analysis of sulfate group

Briefly, a 1 mg aliquot of compound **1** was refluxed with 10% HCl (4 mL) for 4 h and extracted with Et_2O. An aliquot of the aqueous layer was treated with 70% $BaCl_2$ to give a white precipitate ($BaSO_4$).

*3.4.3.1 Compound **1***

Amorphous white powder; $[\alpha]_D^{25}$: -26.2 (*c* 0.13, MeOH); for ^1H and ^{13}C NMR spectroscopic data see *Tab.* 3.1; ESIMS *m/z*: 523 $[M + H]^+$, 443 [(M +H) –80], HRESIMS *m/z*: 523.3440 $[M+H]^+$ (calcd for $C_{30}H_{50}O_5S$, 522.3379).

*3.4.3.2 Compound **2***

Amorphous white powder; $[\alpha]_D^{25}$: +46.7 (c 0.11, MeOH); for ^1H and ^{13}C NMR spectroscopic data see *Tab.* 3.1; ESIMS *m/z*: 253 $[M + H]^+$ HRESIMS *m/z*: 253.1795 $[M+H]^+$ (calcd for $C_{15}H_{24}O_3$, 252,1725).

3.4.4. Cells and treatment

HeLa (cervical carcinoma), and Jurkat (T-cell leukemia), and cell lines were obtained from the American Type Cell Culture (ATCC) (Rockville, MD, USA). Cells were maintained in DMEM (HeLa) or RPMI 1640 (Jurkat and U937), supplemented with 10% FBS, 100 mg/L streptomycin and penicillin 100 IU/mL at 37 °C in a humidified atmosphere of 5% CO_2. To ensure logarithmic growth, cells were subcultured every two days. Stock solutions (50 mM) of purified compounds in DMSO were stored in the dark at 4 °C. Appropriate dilutions were prepared in culture medium immediately prior to use. In all experiments, the final concentration of DMSO did not exceed 0.15% (v/v).

3.4.5. Cell viability and cell cycle

Cells were seeded in 96-well plates and incubated for the established times in the absence (vehicle only) and in the presence of different concentrations of compounds and Etoposide as positive control. The day before treatments, cells were seeded at a cell density of 1×10^4 cells/well. The number of viable cells was quantified by MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assay. Absorption at 550 nm for each well was assessed using a microplate reader (LabSystems, Vienna, VA, USA). In some experiments cell viability was also checked by Trypan Blue exclusion assay using a Bürker counting chamber. IC_{50} values were calculated from cell viability dose – response curves and defined as the concentration resulting in 50% inhibition in cell survival as compared to controls. Cell

cycle was evaluated by propidium iodide (PI) staining of permeabilized cells according to the available protocol and flow cytometry (BD FACSCalibur *flow cytometer,* Becton Dickinson, San Jose, CA, USA). Data from 5000 events per sample were collected. The percentages of the elements in the hypodiploid region and in G_0/G_1, S and G_2/M phases of the cell cycle were calculated using the CellQuest or MODFIT software, respectively.

3.4.6. Statistical analysis

Data reported are the mean values ± SD from at least three experiments, performed in duplicate, showing similar results. Differences between treatment groups were analyzed by Student's *t*-test. Differences were considered significant when $p < 0.05$.

3.5 Result and discussion

The purification process led to isolation of 9 natural compounds, of which 2 are new molecules. The structural characterization of all the secondary metabolites isolated from the aerial parts of *T. maynasiana* were achieved by using different spectroscopic techniques (NMR, MS, UV).

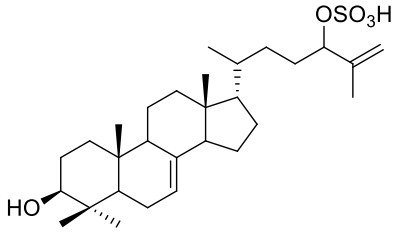
NMR and MS data of compound **1** demonstrated the molecular formula $C_{30}H_{48}O_4S$ (HRESIMS at *m/z* 523, 3440 $[M+H]^+$), which suggested the presence of a sulphate group in the molecule. Acid hydrolysis of compound **1** followed by treatment with $BaCl_2$, confirmed the presence of a sulphate group.

The ESI-MS in positive ion mode showed the $[M+H]^+$ ion peak at *m/z* 523, and fragments by MS^n analysis at *m/z* 443 $[M+H-80]^+$ corresponding to the loss of the sulphate group. This information, along with the ^{13}C NMR spectrum allowed the determination of seven double bond equivalents. Resonances consistent with two double bonds (δ_C 119.0, 146.7, 149.0, and 113.0) were immediately identifiable from the NMR spectroscopic data of compound **1**.

The ^1H NMR spectrum of compound **1** demonstrated the presence of five tertiary methyls, a vinyl methyl, a secondary methyl, an oxymethine proton , and three vinyl protons. 1D TOCSY and COSY spectra suggested the presence in the molecule of four spin systems attributable to C-1—C-3, C-5—C-7, C-9—C-12, and C-15—C-24. Direct evidence of the substituent sites was derived from the HSQC and HMBC correlations, which also

allowed the assignment of all the resonances in the ^{13}C NMR spectrum. Long-range correlations were observed between Me-18 and C-12, C-14, C-17, between H-17 and C-21, C-16, C-14, and between H-7 and C-9, C-8, C-14, Me-21 and C-17, C-21, C-22, H- 24 and C-27, C-26, C-23, Me-27 and C-25, C-24. The α-orientation of H-9, H-5 and Me-30, and the β-orientation of Me-18 and Me-19 were indicated by ROE correlations. These results were confirmed these results were confirmed by the chemical shifts of the carbons, which matched well with those of the related tirucallane (Makino et al., 2004).

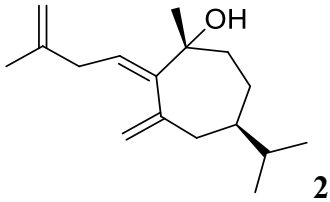
On the basis of these data, compound **1** was characterised as 3β,24-dihydroxy-tirucalla-7,25-diene, 24-sulfate.



1

The ^{13}C NMR spectrum of compound ***2*** showed signals for 15 carbons, including two carbonyl groups, one of them α,β unsaturated (δc 146.3, 152.0, 201.1 and 210.6). The HRESIMS of ***2*** showed a quasi-molecular ion at *m/z* 253.1799 $[M+H]^+$. From this and ^{13}C NMR data a total of eight hydrogen deficiencies were determined, one of which was a ring. The chemical shifts of ***2*** were obtained from the correlations observed in COSY, HSQC, and HMBC experiments. The HMBC spectrum showed correlations between the methyl signal at δ_H 2.10 and C-4, C-2; the methyl signal δ_H 1.31 and C-2, C-9: the signal at δ_H 6.47 and C-5, C-4, C-6, C-10; and the signal at δ_H 2.35 and C-12, C-5, C-8, C-11, which locating the α,β unsaturated carbonyl group at C-1,C-2 and C-5, the carbonyl group at C-4, and the carbinol group at C-10. The relative stereochemistry of ***2*** was determined by 1D ROESY experiments and comparison with literature data (Pereira et al., 2012). Correlations were observed between H-8β at δH 1.90 and Me-14, H-11β, showing that the Me-14 re in a β position.

The structure established for ***2*** was 7-*epi*-10-hydroxychabrol-1(2)-en-dione.



2

*Tab. 3.1 ^{1}H and ^{13}C NMR data of compounds **1** and **2** (CD_3OD, 600 MHz, J in Hz)*[a]

Position	1		2	
	δ_H	δ_C	δ_H	δ_C
1a	1.18 m	37.8	-	146.3
1b	1.73 m			
2a	1.85 m	26.8	6.47	152.0
2b	1.90 m			
3	3.28 dd, (4,8, 11,0)	79.0	2.08	37.8
4	-	40.0	-	210.1
5	1.33 m	52.0	-	201.6
6a	2.16 d (8)	25.3	2.35 dd (6.0, 12.5)	42.3
6b	2.01 m		2.31 d (12.5)	
7	5.31 br s,	119.0	2.38	44.1
8a	-	146.7	1.90 m	25.6
8a	-		1,60 m	
9a	2.25 dd (10.0, 5.0)	50.0	2.47 m	36.3
9b	-		2.60 m	
10	-	36.4	-	69.4
11	1.58 m	19.4	1.31 s	33.0
12a	1.48 m	35.2	0.79 d (6.5)	20.9
12b	1.67 m		-	
13	-	52.2	0.88 d (5.0)	20.5
14	-	45.0	1.43 s	27,6
15a	1.83 br d (14.0)	35.2	2.10 s	29.7
15b	1.56 m		-	
16a	1.91 br d (6.0)	28.00		
	1.95 br d (6.0)			
17	1.53 m	54.0		
18	0.87	22.0		
19	0.80	13.5		
20	1.43 m	37.0		
21	0.92 d (6.0)	18.8		
22a	1.12 m	33.0		
22b	1.45 m			
23	1.33	28.0		
24	4.19 d (6.0)	90.0		
25	-	149.0		
26a	4.90 d (10.5)	113.0		
26b	4.94 m			
27	1.72 br s	17.0		
28	0.88	15.6		
29	0.96	28.0		
30	1.03	27.0		

[a] Data assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments

Known compounds of Trichilia maynasia

Seven known compounds were also identified as *3β-25*-dihydroxytirucalla-*7,23*-diene **(3)** (Su et al., 2010), Butyrospermol (**4**) (Ozen et al., 2008), 1,7-azulenediol (**5**) (Baba et al., 2007), Masticadienediol (**6**) (Luo et al., 2000), Spathulenol (**7**) (Ozen et al., 2008), Lochmolin F (**8**) (Luo et al., 2000), Aromadendrane-4β,10α-diol (**9**) (Ozen et al., 2008) by detailed NMR and MS analyses and compared with literature data as in *Fig.*. 3.10.

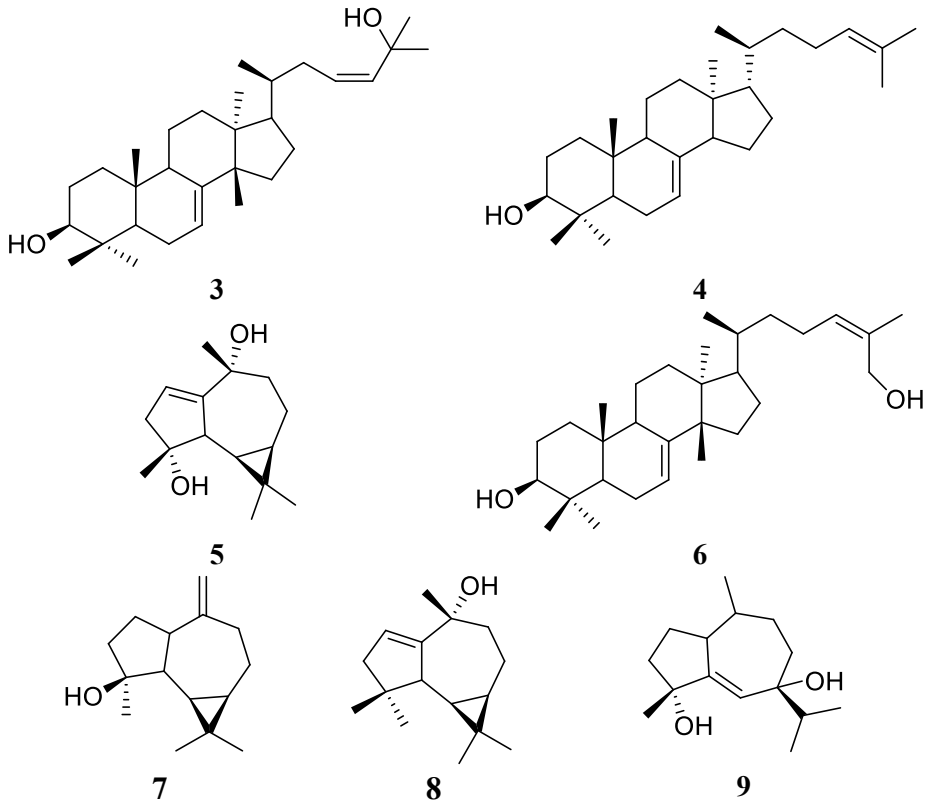


Fig. 3.10 Known compounds of T. maynasiana

All the isolates were tested for the antiproliferative activity towards Hela and Jurkat, no activiy was observed.

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Chapter 4

Vernonia nigritiana Oliv. & Hiern
Asteraceae

Esposito, E., Malafronte, N. Sanogo, R. Vassallo, A. D'Ambola, M. Severino, L.
2016 *Sesquiterpene Lactones from Vernonia nigritiana.*
Natural Product Communications (5) 583 – 584

4.1 Vernonia genus

For further information about Asteraceae family see Chapter 1.
Vernonia (Asteraceae) is the largest genus in the tribe *Vernoniea*e with close to 1000 species (Keeley and Jones Jr, 1979). The genus *Vernonia* is named after William Vernon, an English botanist who collected and identified this genus in Maryland (Quattrocchi, 1999). The genus is distributed both in the New and Old World although it is to be found mostly in the tropical regions. *Vernonia* species grow in a wide range of habitats of broad ecological diversity and climatic conditions including tropical forest, marshes and wet areas, dry plains, tropical savannahs and even frosty regions of eastern North America (Gleason, 1923; Keeley and Jones Jr, 1979). In regard to chemical diversity, plants of the *Vernonia* genus are the source of many terpene type compounds particularly sesquiterpenes, steroids and their glycosides (Django et al., 2007; Huffman and Seifu, 1989; Mølgaard et al., 2001; Yeap et al., 2010). Plants this genus are widely used in ethnomedicine, ethnoveterinary medicine and in zoopharmacognosy particularly by chimpanzees and gorillas. A total of 109 species of *Vernonia* are reported to be used in folk medicine. *Vernonia* genus are used in ethnoveterinary medicine as anthelmintic, hepatoprotective and insecticidal (Burkill, 1985; Yineger and Yewhalaw, 2007; Regassa, 2000; Hussain et al., 2008; Githiori et al., 2006).

4.1.1 Vernonia genus: phytochemistry bioactive compounds

4.1.1.1 Phenols compounds

Flavonoids are among the two major classes of compounds encountered in the *Vernonia* genus (Carvalho et al., 1999). Most of the flavones and phenolic compounds have been isolated from *Vernonia amygdalina* Delile and *Vernonia cinereal* Less. and have exhibited potent antioxidant as well as urease inhibitory activity (Ahmad et al., 2011; Igile et al., 1994; Abeysekera et al., 1999) (*Fig.*.4.1).

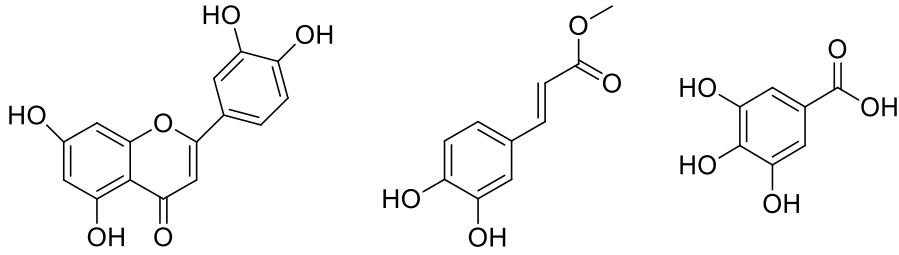
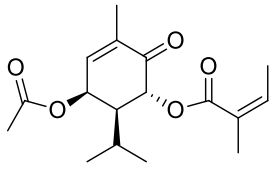


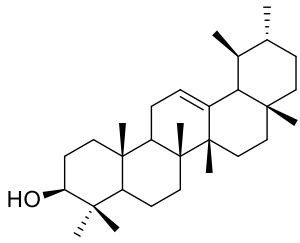
Fig. 4.1 Main phenols of *Vernonia ssp*

4.1.1.2 Terpenoids

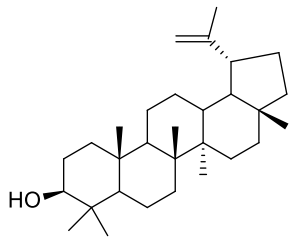
Terpenoids are the largest known class of secondary metabolites in plants (Harborne, 1999). They play a role in interaction of plants with their environment and terpenoids have been shown to have a broad range of biological activities such as antibiotic, cytotoxic, antimalarial, antifeedant, insecticidal, moluscidal and herbicidal (Zhang et al., 2005; Roberts, 2007; Gershenzon and Dudareva, 2007; Kaur et al., 2009). Among plants of the *Vernonia* genus so far studied for biological activity, a number of the reported biological activities have been associated with the presence of terpenoids. In *Fig.*.4.2 some terpenoids isolated from *Vernonia* genus are shown.



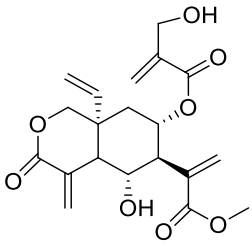
Vernonione
Urease inhibitor
(Ahmad et al., 2012)



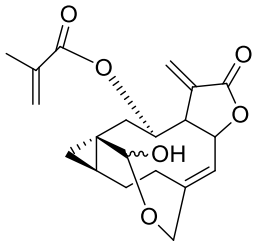
α-amirina
Antibacterial activity
(Kiplimo et al., 2011)



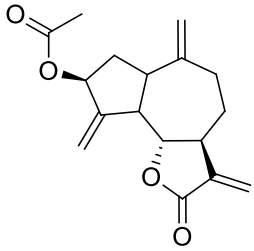
Lupeol
Antiplasmodial
(de Almeida Alves et al., 1997)



Vernodalol
Antifungal
(Erasto et al., 2006),



Vernolide
Antibacterial, Antifungal
(Erasto et al., 2006)



Zaluzanin D
Antifungal
(Kumari et al., 2003)

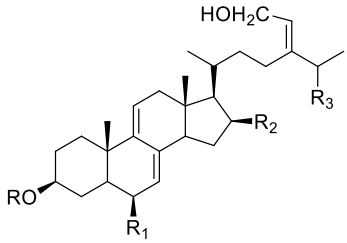
Fig. 4.2 Terpenoids of *Vernonia genus*

4.1.2 Vernonia nigritiana Oliv. & Hiern

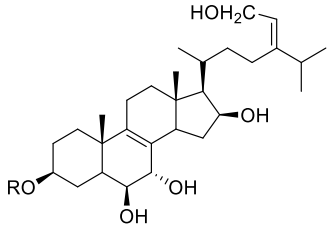
Vernonia nigritiana Oliv. & Hiern. (Asteraceae) *Synonyms: Cacalia nigritana Kuntze; Linzia nigritiana (Oliv. & Hiern) Isawumi* is an annual herb or shrub widely distributed in West Africa, where the leaves and other parts of the plant are traditionally used against skin inflammations and infections, rheumatism, fever, headache and digestive insufficiency (Johri and Singh, 1997; Toyang and Verpoorte, 2013).

Burkill (1985) isolated a bitter monoterpene glycoside (Vernonin) from the roots of *V. nigritiana*. Vernonin showed a digitalis (digoxin) like action on the heart but it is weaker than digoxin.

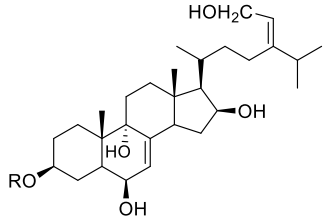
Vassallo et al. (2013) reported the topical anti-inflammatory properties of apolar extracts of *V. nigritiana* leaves, supporting the use in the West African traditional medicine for the treatment of inflammatory diseases. Phytochemical investigation of the *Vernonia nigritiana* leaves extracts led to the isolation of nine new polyhydroxylated stigmasterol glycosides (**1–9**) and six new stigmastanes (**10–15**) with different potency as anti-inflammatory agents (*Fig.*.4.3).



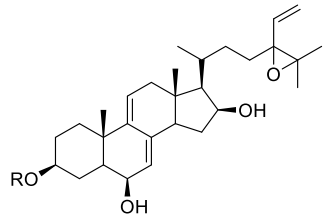
	R	R_1	R_2	R_3
1	β-D-Glc	OH	OH	CH_2OH
2	β -D-Glc	H	OH	CH_3
3	β -D-Glc	OH	OH	CH_3
4	β -D-Glc	=O	OH	CH_2OH
5	β -D-Glc	=O	OH	CH_3
7	β -D-Glc	H	OH	CH_2OH
10	H	=O	OH	CH_2OH
11	H	OH	OH	CH_3
12	H	H	H	CH_2OH
13	H	=O	H	CH_3
14	H	OH	OH	CH_2OH



6 β-D-Glc **15** H



8 β -D-Glc



9 β -D-Glc

*Fig. 4.3 Structures of compounds **1–15** isolated from Vernonia nigritiana*

AIM

In the present study, a phytochemical and pharmacological investigation of *V. nigritiana* leaves extracts was carried out to identify the relevant active principles.

4.2 Experimental

4.2.1 Plant materials

The leaves of *V. nigritiana* were collected in 2012 from the Bougouni, Sikasso region of Mali, near Bandiagara,. The plant material was identified

by Prof. Rokia Sanogo of DMT, where a voucher specimen was deposited (voucher number 1396).

4.2.2 Extraction and isolation

The leaves of *Vernonia nigritiana* (300 g) were dried at 40°C carefully grinded and extracted using increasing polarity solvent light petroleum, chloroform, chloroform/ methanol (9:1 v/v) and methanol by extensive maceration (3 times x 2 L) the solvent was evaporated under vacuum system obtaining the following yields 2.0, 5.7, 2.4, 9.0 respectively (*Fig.*.4.4).

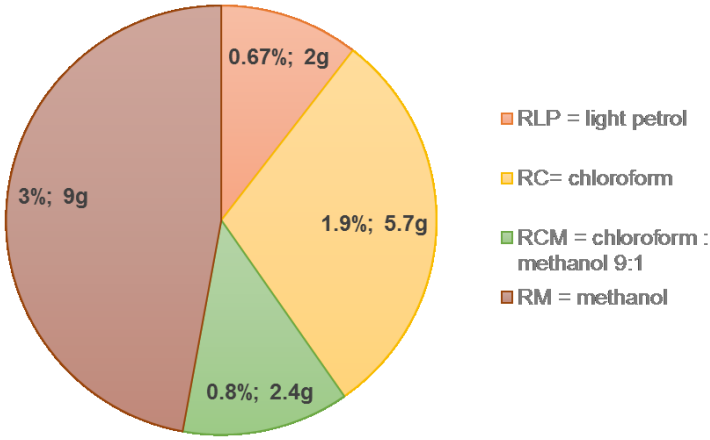


Fig. 4.4 Percentages and grams of extract obtained from *V. nigritiana* leaves

Part of the $CHCl_3$ extract (4.0 g) was separated by silica gel column chromatography (CC) eluting with $CHCl_3$, followed by increasing concentrations of MeOH (between 1% and 100%). Fractions of 25ml were collected, analysed by TLC and grouped into 9 fractions (A-I) (*Fig.*. 4.5).

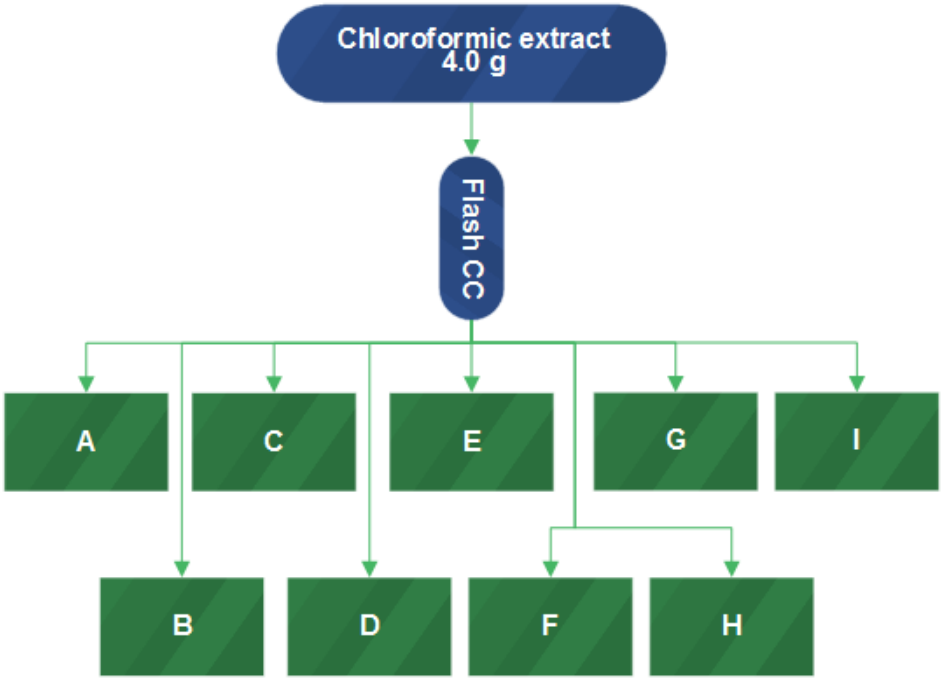


Fig. 4.5 Scheme of silica gel column of chloroformic extract

The fraction G was subjected to RP-HPLC with CH_3OH:H_2O (65:35) as eluent. The spectroscopic analysis of the resolved peaks, has led to the characterization of pure Glaucolide G (5 mg, t_R 37 min) and Glaucolide A (2 mg, t_R 41 min); fraction H was separated by RP-HPLC with CH_3OH: H_2O (7:3) as eluent to give pure 8α-(4-2 hydroxytigloyloxy)-hirsutinolide (2 mg, t_R 41 min).

Part of the $CHCl_3$/MeOH (9:1) extract (2.0 g) was separated by Sephadex LH- 20 with MeOH as eluent. Fractions of 10 mL were collected, analyzed by TLC and grouped into 10 fractions (A–L). Fraction D (500 mg) was separated by silica gel CC eluting with $CHCl_3$ followed by increasing concentrations of MeOH (between 1% and 100%). Fractions of 5 mL were collected, analyzed by TLC and grouped into 14 fractions (*Fig.*.4.6).

The fraction Kk (43mg) was subjected to RP-HPLC with CH_3OH:H_2O (29:16) as eluent to give pure compound **1** (1.4 mg, t_R 5min). Fraction Ll (26mg) was chromatographed over RP-HPLC and eluted with CH_3OH: H_2O

(1:1) to give of pure vernolide-A (0.8 mg, t_R 8 min) and vernolide-C (1 mg, t_R 12 min). Fraction Mm (72mg) was subjected to RP-HPLC with CH_3OH:H_2O (1:1) as eluent to give pure compound **2** (1.8 mg, t_R 15 min).

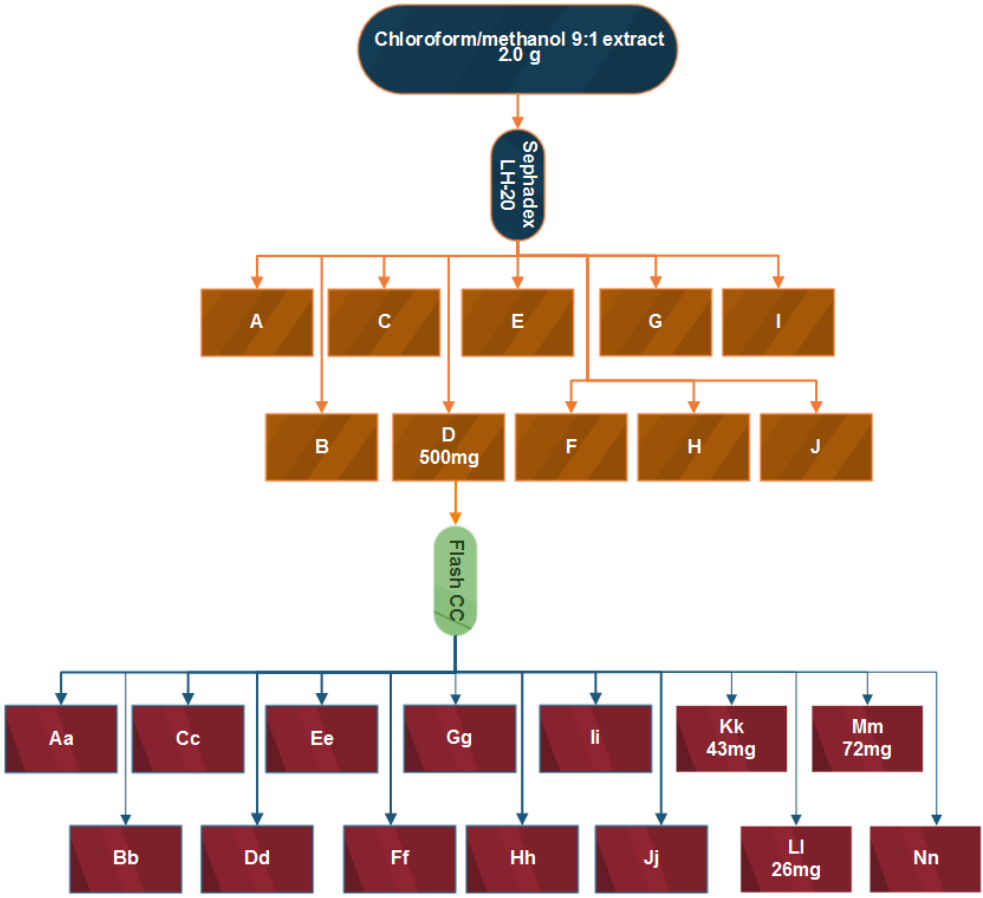


Fig. 4.6 Scheme of silica gel column of chloroform:methanol 9:1 extract

*4.2.2.1 Compound **1***

Amorphous white powder $[\alpha]_D^{25}$: +26.2 (c 0.13, MeOH); for ^1H and ^{13}C NMR spectroscopic data, see *Tab.* 4.1; ESIMS *m/z*: 409 $[M+H]^+$; HRESIMS *m/z*: 409.1854 [M+H] (calcd for $C_{21}H_{28}O_8$: 408.1784).

*4.2.2.2 Compound **2***

Amorphous white powder $[\alpha]_D^{25}$: +33.4 (c 0.11, MeOH); for ^{1}H and ^{13}C NMR spectroscopic data, see *Tab.* 4.1; ESIMS *m/z*: 423 [M + H]$^+$;HRESIMS *m/z*: 423.2011 [M+H]$^+$ (calcd for $C_{22}H_{30}O_8$: 422.1941).

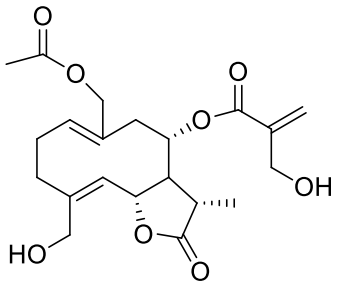
4.3 Results and discussion

The purification process led to isolation of 7 natural compounds, of which 2 are never isolated molecules. Their structural characterization was obtained on the basis of extensive NMR spectroscopic and mass spectrometric studies.

Compound **1** was assigned the molecular formula $C_{21}H_{28}O_8$ from ^{13}C, ^{13}C DEPT NMR, and HRESIMS data. The ESI-MS in positive ion mode showed the $[M+H]^+$ ion peak at *m/z* 409, and fragments at *m/z* 349 $[M+H-60]^+$ corresponding to the loss of an acetyl group, and *m/z* 263 $[M+H-60-86]^+$ corresponding to the subsequent loss of esterified groups. The ^{13}C NMR spectra indicated that compound **1** contained two CH_3, three CH_2, and two CH carbons, as well as three hydroxymethylenes, two hydroxymethines, six sp^2 carbons, and three ester functionalities. The features of the NMR spectra suggested a germacrane skeleton with two tetra-substituted double bonds at $\Delta^{1(10)}$ and $\Delta^{4(5)}$. From two pairs of doublets and one singlet at δ_H 4.73 (1H, br d, J = 13.5 Hz), 4.59 (1H, d, J = 13.5 Hz), 4.13 (1H, br d, J = 14.0 Hz), 4.11 (1H, br d, J = 14.0 Hz), and 4.31 (2H, s) three hydroxymethyl groups were evident. The presence of a 4-hydroxymethacryloyl group at C-8 was deduced from the signals at δ_H 6.30 (s) and 5.95 (s) for H2-3' and δ_H 4.31 (br s) for H-4', while the presence of an acetyl moiety was deduced from the signals at δ_H 2.09 (s). Results obtained from 1D TOCSY and COSY experiments established the correlations of all protons in compound **1**, showing the sequences H-1—H-3, H-5—H-9 and H-5—H-13.

The ^{13}C NMR spectrum was assigned on the basis of a HSQC experiment. The location of the 4-hydroxymethacryloyl group, the acetyl group, the hydroxymethylene moiety, and the lactone ring were confirmed by the key peaks observed in the HMBC spectrum. The signal of H-1 correlated with C-19, C-3, C-14; H-5 with C-15, C-3, C-6, C-7; H-8 with C-10, C-1', C-11; and H-6 with C-11, C-4, and C-8. 1D ROESY measurements supported the

proposed structure and allowed the relative stereochemistry at C-8, C-6, and C-11. Irradiation of H-6 affected H-8, H-11 and H-3β signals, while irradiation of H-7 influenced the H-13 and H-9α signals. Consequently, compound **1** was established as 8α-(4-hydroxy-methacryloyl)-14-acethoxy-salonitenolide.

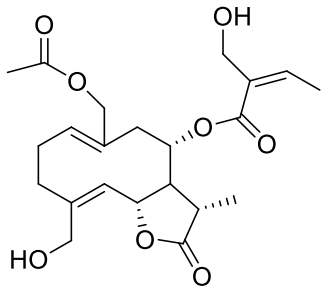


1

The ^{13}C NMR spectrum of compound **2** showed signals for 22 carbons, including three ester groups. The HRESIMS of **2** showed a quasi-molecular ion at *m/z* 423.2011 $[M+H]^+$. This information, along with the ^{13}C NMR spectra, which sorted the 22 carbons into three methyls, six methylenes, seven methines, and six quaternary carbons, allowed the determination of eight double bond equivalents, two of which was a ring. 1D TOCSY and COSY spectra suggested the presence in the molecule of three spin systems attributable to C-1—C-3, C-5—C-9, and C-3'—C-5'. Also, for this compound, the features of the NMR data suggested a germacrane ring similar to that of compound **1** except for the acyl moiety linked at C-8. This was deduced from the position and pattern of the H-8 signal at δ_H 5.56 (br ddd 11.0, 9.4 and 2.0), and was characterized by the presence of a methyl doublet at δ_H 1.92, which was coupled with a quintet of a methine group (δ_H 6.96), and a hydroxymethyl moiety (δ_H 4.25). The chemical shift and the pattern of this signal, according to HSQC results, suggested the presence of a 2-hydroxymethyl 2-butenoyl group. The HMBC spectrum showed correlations between the hydroxymethyl signal at δ_H 4.73 and C-1, C-9 and C-1'; between the signal at δ_H 4.91 and C-3, C-7, C-15: between the signal at δ_H 5.51 and C-10, C-6, C-9, C-1'; and between the signal at δ_H 2.84 and C-12, C-13, C-9, C-5, locating the lactone group at C-6, C-7, the acetyl group at C-14, and the 2- hydroxymethyl 2-butenoyl group at C-8. The relative stereochemistry of ***2*** was determined by 1D ROESY experiments and comparison with literature data (Kuo et al., 2003; Youn et al., 2014). Significant correlations were observed between H-8 and H-6, and H-11

showing that the Me-13 group and 2-hydroxymethyl 2-butenoyl group at C-8 were in were in the α position.

The structure established for compound ***2*** is 8α-(2-hydroxymethyl 2-butenoyl)-14-acetoxy-salonitenolide.



2

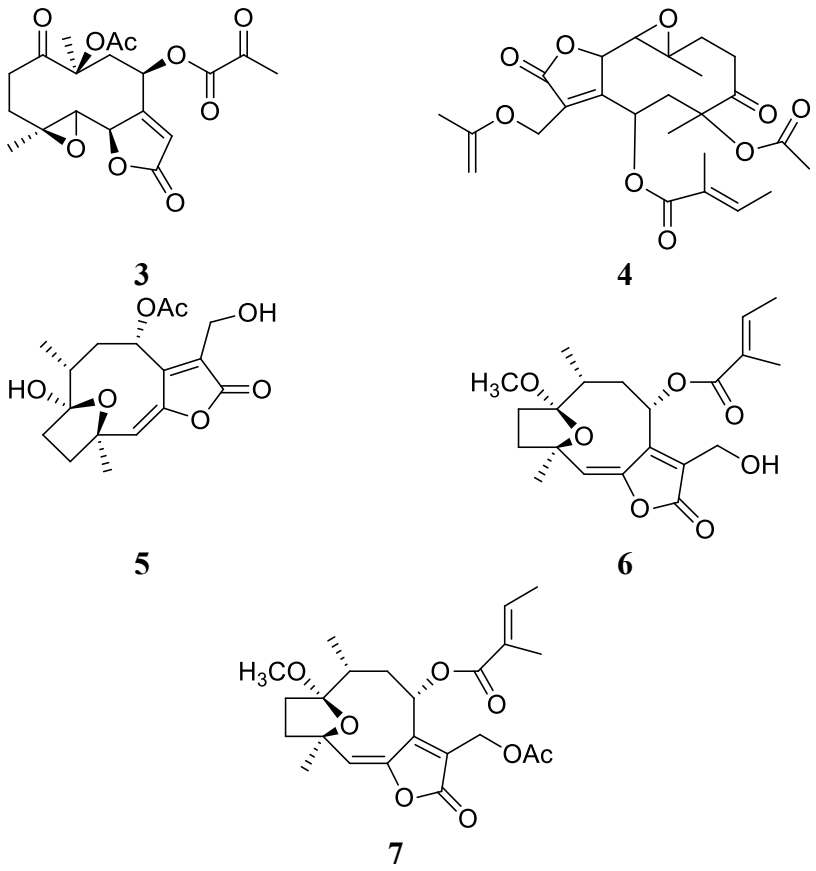
*Tab. 4.1 ^{1}H and ^{13}C NMR data of compounds 1–**2** (CD_3OD, 600 MHz, J in Hz)*[a]

Position	Compounds			
	1		**2**	
	δ_H	**δ_C**	**δ_H**	**δ_C**
1	5.17dd (10.0, 5.0)	135,0	5.17dd (10.0, 5.2)	135,0
1’		165,9		167,7
2	2.27m 2.32m	27,7	2.27m 2.32m	27,7
2’		141,0		133,0
3	2.11ddd (12.0, 11.6 ,6.4) 2,58ddd (11.0, 4.4, 2.4)	35,9	2,11ddd (12.0, 11.6 ,6.4) 2,58ddd (11.0, 4.4 2.4)	35,9
3’	5,95s 6,30s	125,9	6,96q (7.0)	142,2
4		140,0		140,0
4’	4.31 s	61,6	1.92 d (7.0)	15
5	4,91br d (9.0)		4.93br d (9.0)	130,3
5’		130,3	4,25 br s	59,2
6	5.34dd (9.0, 8.7)	77,4	5,34dd (9.0, 8.7)	77,4
7	2.82m	54,0	2.84m	54,0
8	5.51br ddd (11.0 8.4, 2.4)	73,0	5.56 ddd (11.0, 9.4, 2.0)	73,0
9	2.37br dd (12.6 10.5) 2,95br d (10.5)	45,2	2.38br dd (12.6, 10.5) 2.95br d (10.5)	45,2
10		132,7		132,7
11	2.87m	41,0	2.88m	41,0
12		181,0		181,0
13	1.32d (6.2)	10,05	1.34d (6.5)	10,5
14	4.59d (13.5) 4.73d (13.5)	63,0	4.60d (13.5) 4.73d (13.5)	63,0
15	4.11br d (14.0) 4.13br d (14.0)	61,4	4.13br d (14.0) 4.14br d (14.0)	61,4
COMe	2.09	21,0	2.07s	21,0
COMe		172,0		172,0

[a] Data assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments.

Known compounds of *Vernonia nigritiana*

The seven known compounds were identified as Glaucolide A (**3**) (Padolina et al., 1974), Glaucolide G (**4**) (Youn et al., 2014), 8α-(4-hydroxytigloyloxy)-hirsutinolide (**5**) (Youn et al., 2014), Vernolide-A (**6**) (Kuo et al., 2003), Vernolide-B (**7**) (Kuo et al., 2003), by detailed NMR and MS analyses and compared with literature data as in *Fig.*. 4.7.



3 **4** **5** **6** **7**

Fig. 4.7 Compounds of V. nigritiana

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Chapter 5

Materials and Methods

5.1 Chromatographic techniques

5.1.1 Thin Layer Chromatography

TLC (Thin Layer Chromatography) was performed on precoated Kieselgel 60 F254 plates (Merck), 0.25 mm thick, with glass or aluminium as support. The spots were revealed using UV detection with lamp at 254 or 366 nm and successively using specific spray reagents, allowing to the development of typical colouring that can give information about the nature of examined compounds. Ce2S04/H2S04: it is a general reactive used to reveal all compounds contained in mixtures; it can also give specific information, for example triterpenes give typically violet spots. It is a saturated solution of cerium sulphate in 65% sulphuric acid. Heating at 120 °C for 15 min is needed to reveal the spots.
As eluting solvents in the TLC analyses, two mixtures of solvents were mainly used:

- $CHCl_3$-MeOH-H_2O (80:18:2) for apolar mixtures
- *n*-BuOH-AcOH- H_2O (60:15:25) for polar mixtures

5.1.2 Chromatographic columns

Gel filtration

Gel filtration chromatography was performed over Sephadex LH-20 (25-100 µm, Pharmacia Fine Chemicals) using columns 100 cm x 3 cm, for 2-4 g of extract, and 100 cm x 5 cm, for 5-10 g of material, and a peristaltic pump Pharmacia Fine Chemicals PI. The eluent was always methanol, at Constant flow rate (1.5 mL/min).

Flash chromatography

Flash chromatography was performed over Si gel 60 (230-410 pm, Merck), eluting with $CHCl_3$ containing an increasing amount of MeOH.

Diameter (mm)	Length (mm)	Material (mg)
10	160	40-100
20	160	160-400
30	160	400-900
40	160	800-1600
50	160	1000-2500

HPLC

Reversed-phase HPLC (High Performance Liquid Chromatography) separations were conducted on a Shimadzu LC-8A series pumping system equipped with a Waters R401 refractive index detector and a Shimadzu injector, using Waters C-18 μ-Bondapak (30 cm x 7,8 mm) and a mobile phase consisting of MeOH-H2O mixtures at a flow rate of 2.0-2.5 ml/min.

5.2 Chemical-physical techniques

5.2.1 Optical rotations

Optical rotations were measured on a Perkin-Elmer 241 polarimeter equipped with a sodium lamp (589 nm) and a 1 dm microcell.

5.3 Spectroscopic and spectrometric methods

5.2.2 Nuclear Magnetic Resonance

The structural determination of secondary metabolites was performed by one and two-dimensional NMR spectroscopy (^{1}H, ^{13}C, ^{13}C DEPT, 1D-Tolsy, COSY, HSQC, HMBC, 2D-ROESY) and confirmed by mass spectrometry. The ^{1}H and ^{13}C NMR spectra provide information about the number of protons present, their coupling constants and the value as well as information on the number of carbon atoms. With the aid of the technique multi impulse DEPT (distortionless Enhancement by Polarization Transfer) can also be determined by the number of protons attached to each carbon atom. The chemical shifts are reported in δ (ppm) relative to TMS (δ 0) used as a standard; the coupling constants (J) are measured in Hertz.
The experiment 1D-TOCSY (Total Correlation Spectroscopy) is useful in spectroscopic analysis of the spin systems and in all cases in which the signal of a single proton is very complicated reveals in simple ^{1}H NMR spectrum, this experiment allows to build a spin system selectively energizing a proton that, thanks to the magnetization transfer, allows to highlight all the other protons belonging to the same spin system. Of fundamental importance are the two-dimensional spectroscopy experiments. These allow you to get a huge increase in the spectral resolution since the signals are dispersed in two dimensions, providing additional information on the correlation of the same. The experiments used:

- DQF-COSY (Correlated Double Quantum Filtered-Spectroscopy), which allows to correlate the chemical shifts of the protons through the homonuclear scalar couplings, by assigning the various spin systems present in the molecule even when you have small pairs.
  - HSQC (Heteronuclear Single Quantum Coherence), the analogue of the heteronuclear COSY, which, by creating a heteronuclear coherence and employing the proton for signal detection, allows for the assignment of a spectrum 13C by the corresponding proton spectrum and vice versa, through the proton-carbon direct correlation;
  - HMBC (Heteronuclear Multiple Bond Coherence), the long range heteronuclear correlation experiment that allows to reveal the correlations to more than one link (2-4) between a proton and the carbon not adjacent, with the advantage of highlight correlations with quaternary carbons.

A spectrometer Bruker DRX 600 (XWIN-NMR and TopSpin software), operating at 599.19 MHz for ^{1}H and 150.86 MHz for ^{13}C was used to record the NMR spectra. In the ^{1}H-NMR spectra in CD_3OD it was used as a standard the signal relating to CHD_2OD to 3.34 ppm; for measurement in $CDCl_3$ we were used the signal relative to $CHCl_3$ to 7.27 ppm. The ^{1}H-NMR values are reported in δ ppm for, together with the multiplicity, to the values of the constants J and coupling to allocations.

For the $_{13}C$-NMR spectra was used as reference the signal of the solvent, to 49.0 ppm respectively for $CD^{3}OD$ and 77.0 ppm for $CDCl_3$. The DEPT-135 ° experiments were performed using polarization transfer by means of a 90 ° pulse liquid to achieve only -CH groups and at 135 ° to get positive signals for -CH and -CH 3 groups and negative for the $-CH_2$ groups. The delay in the transfer of polarization has been correlated to a mean C-H coupling of 135 Hz. The experiment of direct correlation omonucleare (DQF-COSY, Double Quantum Filtered COSY) was performed using the conventional pulse sequence. The experiment of direct correlation heteronuclear ^{1}H-^{13}C HSQC has been realized generally on a matrix 512 x 2048, using a time constant of 135 Hz C-H coupling and a relaxation delay of 1.5 sec. (Kay, L.E. et al., 1992;). The long range heteronuclear correlation experiment (HMBC) was obtained using the sequence of Bax et al., A constant average long range of 6-8 Hz. And a matrix 512 x 2048 (Bax & Summers, 1986). The 1D-TOCSY experiments were obtained using a XWIN-NMR software; in particular, the 1D-TOCSY was performed using a Gaussian pulse generator using, during the mixing time, the pulse sequence of the type MLEV- 17 (mixing time 80-100 ms).

The NOESY and ROESY experiments were conducted using the commercial microprograms software provided with the instrument. For ROESY experiments it was used a 400 or 500 ms mixing time.

5.2.3 Mass spectrometry (ESI-MS)

Mass spectra were recorded both in positive ion mode and negative ions using one tool Finnigan LCQ Deca ion trap with electrospray source, manufactured by Thermo Finnigan (San Jose, CA, USA), Excalibur software for processing of the acquired data.
The samples (isolated compounds) were analysed by direct infusion at a flow of 10 µL / min., Using the following instrumental parameters: capillary temperature 288.20 ° C; Sheath Gas Flow 45.95 psi; flow of the auxiliary gas 2.68; capillary voltage -25.54 V. Data were acquired either through a scanning MS1 through MSn, making use of the ion trap; for the determination of MSn it was used a collision energy of 30 eV.

5.3 Solvents

Solvents for HPLC and MS, acetonitrile and methanol, were purchased from JT Baker (Mallinckrodt Baker, Phillisburg, NJ), formic acid and trifluoroacetic acid (TFA) were purchased from Merck while the water nanopure (HPLC grade) was prepared by using a purification system, Millipore Milli-Q. For the extraction of the dried plant material they have been used: *n*-hexane, chloroform and methanol supplied from VWR International (Milan, Italy), as well as chloroform and methanol, used for the eluition of the compounds from the silica column