**UNIVERSITÉ DE PARIS**

FACULTÉ DE PHARMACIE DE PARIS
ECOLE DOCTORALE MTCI

Year 2019 N°

DOCTORAT DE L'UNIVERSITÉ DE PARIS

ORTIZ Sergio

# EXPLORATION OF TRADITIONAL MEDICINES AS SOURCE OF BIOACTIVE COMPOUNDS: FROM ETHNOBOTANY TO PHARMACOMODULATION

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Dissertation November 22, 2019

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# LIST OF ABBREVATION

| | | | |
|---|---|---|---|
| **$^{13}$C-NMR** | Carbon-13 nuclear magnetic resonance | **IFN** | Interfeon gamma |
| **1D-2D NMR** | Mono. And bi-dimensional nuclear magnetic resonance | **IKK** | IκB kinase |
| **$^{1}$H-NMR** | Proton nuclear magnetic resonance | **IL-1** | Interleukin 1 |
| **5-LO** | 5-Lipoxygenase | **IL-18** | Interleukin 18 |
| **ABTS** | 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) | **IL-6** | Interleukin 6 |
| **ACN** | Acetonitrile | **IPP** | Isopentenyl pyrophosphate |
| **ACT** | Artemisinin combinatory therapy | **JNK2** | c-Jun N-terminal kinase 2 |
| **AHL** | N-Acyl homoserine lactones | **Kd** | Partition coefficient |
| **AIDS** | Acquired immune deficiency syndrome | **Kobs** | Rate observed |
| **ALP** | Alkaline phosphatase | **LC** | Liquid chromatography |
| **ALT** | Alanine transminase | **LC-MS** | Liquid chromatography coupled to mass spectrometry |
| **AP-1** | Activator protein 1 | **LD50** | Median lethal dose |
| **Arf1** | ADP-ribosylation factor 1 | **L-DOPA** | L-3,4-dihydroxyphenylalanine |
| **ASC** | Apoptosis-associated speck-like protein containing a CARD | **logP** | Logarithm partition coefficient |
| **AST** | Aspartate transaminase | **LPS** | Lipopolysaccharide |
| **ATP** | Adenosine triphosphate | **M199** | Medium 199 |
| **BCE** | Before common era | **MeOD** | Deuterated methanol |
| **Big1** | Brefeldin A-inhibited guanine nucleotide-exchange protein 1 | **MeOH** | Methanol |
| **BLAST** | Basic Local Alignment Search Tool | **MIC** | Minimum inhibitory concentration |
| **$C_6D_6$** | Deuterated benzene | **MPLC** | Medium pressure liquid chromatography |
| **$CDCl_3$** | Deuterated chloroforme | **MS** | Mass spectrometry |
| **CF** | Cystic fibrosis | **MTA** | Material transfer agreement |
| **CHS** | Chalcone synthase | **MTT** | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium |
| **CiTCoM** | Cibles Thérapeutiques et Conception de Médicaments | **MyD88** | Myeloid differentiation primary response 88 |
| **CONC** | Hebario de Botánica Universidad de Concepción | **NADPH** | Dihydronicotinamide-adenine dinucleotide phosphate |
| **COX1-2** | Cytochrome c oxidase I-II | **NF-Kb** | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| **CPC** | Centrifugal partition chromatography | **NLRP3** | NLR family pyrin domain containing 3 |
| **CQ** | Chloroquine | **NOD-like** | Nucleotide-binding oligomerization domain-like receptors |
| **CRP** | C-Reactive protein | **NOS** | Nitric oxide synthase |
| **Cy** | Cyclohexane | **NTD** | Neglected tropical diseases |
| **DCM** | Dichloromethane | **OD** | Optic density |
| **DHFA** | Dihydrofolic acid | **P2X7** | P2X purinoceptor 7 |
| **DHT** | Dihydroartemisinin | **PABA** | 4-Aminobenzoic acid |
| **DMAC** | Dimethylaminocinnamaldehyde | **PAL** | Phenylalanine ammonia lyase |
| **DMEM** | Dulbecco's modified Eagle's medium | **PBS** | Phosphate-buffered saline |
| **DMSO** | Dimethylsulfoxide | **PCA** | Principal component analysis |
| **DNA** | Deoxyribonucleic acid | **PfCPT** | Plasmodium falciparum choloroquine protein transport |

| | | | |
|---|---|---|---|
| **DPPH** | 2,2-Diphenyl-1-picrylhydrazyl | **PGE-2** | Prostaglandin E2 |
| **$EC_{50}$** | Half maximal effective concentration | **PGES-1** | Microsomal prostaglandin E synthase-1 |
| **EIA** | Enzyme immunoassay | ***phz*A-G** | Phenazine biosynthesis proteins |
| **ELISA** | Enzyme-linked immunosorbent assay | **PKD** | Polycystic kidney disease |
| **ESI-MS** | Electrospray ionization mass spectrometry | **PLA2** | Phospholipase A2 |
| **EtOAc** | Ethyl acetate | **PLFE** | Pistacia lentiscus fruit extract |
| **EtOH** | Ethanol | **PLS-DA** | Partial least squares-discriminant analysis |
| **EUCAST** | European Committee on Antimicrobial Susceptibility Testing | **PLS-R** | Partial least squares-regresion |
| **EXANDAS** | EXploitation of Aromatic plaNts' by-proDucts for the development of novel cosmeceuticAls and food Supplements | **PMF** | Polymethoxyflavonoid |
| **FA** | Formic acid | **NPAS** | Produits naturels, analyses et synthèses |
| **FabG** | β-ketoacyl-ACP reductase | **PP2A** | Protein phophatase 2 |
| **FabI** | Enoyl-ACP reductase | **PQS** | Pseudomona quinolone signal |
| **FabZ** | β-hydroxacyl-ACP dehydratase | **PRR** | Pattern recognition receptor |
| **FAS II** | Fatty acid synthases Type II | **PSE** | Pressurized Solvent Extraction |
| **FBS** | Fetal bovine serum | **PYO** | Pyocyanin |
| **FDS** | Farnesyl diphosphate synthase | **qPCR** | Quantitative polymerase chain reaction |
| **FPP** | Farnesyl pyrophosphate | **QS** | Quorum-sensing |
| **FRAP** | Ferric reducing antioxidant power | **RNA** | Ribonucleic acid |
| **GAA** | Germacra-1(10),4,11(13)-trien-12-oic acid | **RNS** | Reactive nitrogen species |
| **GAL** | Germacra-1(10),4,11(13)-trien-12-al | **ROS** | Reactive oxygen species |
| **GAO** | Germacrene A oxidase | **RPMI** | Roswell Park Memorial Institute medium |
| **GAPs** | GTPase-activating proteins | **SAR** | Structure-activity relantionship |
| **GAS** | Germacrene A synthase | **SFE** | Supercritical fluid extraction |
| **GC-FID** | Gas chromatography - flame ionization detector | **spp.** | Species |
| **GC-MS** | Gas chromatography - mass spectrometry | **STOCSY** | Statistical total correlation spectroscopy |
| **GDP** | Guanosine diphosphate | **STPs** | Sesquiterpene synthases |
| **GEF** | Guanine nucleotide exchange factor | **TFE** | Total flavonoid evaluation |
| **GNPS** | Global Natural Products Social Molecular Networking | **THFA** | Tetrahydrofolic acid |
| **GOL** | Germacra-1(10),4,11(13)-trien-12-ol | **TLC** | Thin-layer chromatography |
| **GTP** | Guanosine-5'-triphosphate | **TLR** | Toll-like receptors |
| **HAQ** | 4-Hydroxy-2-alkylquinolines | **TMB** | 3,3′,5,5′-Tetramethylbenzidine |
| **HCN** | Hydrogen cyanide | **TNF** | Tumour Necrosis Factor alpha |
| **HDL** | High-density lipoprotein | **TPC** | Total polyphenol content |
| **HEPES** | 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid | **tPSA** | Topological polar surface area |
| **HetCA** | Hetero-covariance analysis | **TRIF** | TIR-domain-containing adapter-inducing interferon-β |
| **HIV** | Human immunodeficiency virus | **UPLC** | Ultra Performance Liquid Chromatography |
| **HMI-9** | Hirumi's modified Iscove's medium 9 | **UV** | Ultraviolet |
| **HNE** | 4-Hydroxynonenal | **VCAM-1** | Vascular cell adhesion protein 1 |

| | | | |
|---|---|---|---|
| **HPTLC** | High-performance thin layer chromatography | **VEGF** | Vascular endothelial growth factor |
| **$IC_{50}$** | Half maximal inhibitory concentration | **WHO** | World Health Organization |
| **$IC_{90}$** | 90% of maximal inhibitory concentration | **XO** | Xanthine oxidase |
| **ICAM** | Intercellular Adhesion Molecule 1 | | |

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# 0. Preamble

***Plants as source of bioactive compounds***

From the beginning of the humankind, human beings have observed and studied their surrounding in search for new hydric/food resources, tools and new advantages for their survival and development.

Vegetal resources have been essential for the evolution and expansion of humankind. Critical point was the development of agriculture, changing form nomad to sedentary life modes, allowing the construction of cities and the expansion of civilizations. The hours per day, dedicated to the search for food and displacement to new sites, were then available for the development of new technics dedicated to human development as pottery, metallurgy, etc.

Development of medicine was also beneficiated by this new sedentary mode of life. Uses of the environmental resources to treat several health problems are reported from the Antiquity. Vegetal, animal and mineral resources have been used during human evolution as source of elements to heal the physical, psychological and/or spiritual problems. *Diagnosis Handbook* from the Mesopotamia Civilization is the first big compendium of medicinal practice counting diagnosis and therapies, including traditional remedies, for several illness dated from 1050 BCE [1,2]. Edwin Smith papyrus dated from 1600 BCE also collect the medicinal practice of Ancient Egypt, focusing on injuries, fractures, wounds, dislocations and tumors, including details of the types of injury, examination, diagnosis, prognosis and treatment [3].

According to the World Health Organization (WHO) the traditional medicine is defined as “the sum of the knowledge, skills and practices based on the health, beliefs and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as prevention, diagnosis, improvement or treatment of physical and mental illness”. In some regions of Asia and Africa, up to 80% of the population relies on the traditional medicine for their primary health care needs [4].

Until the XIXth century, main remedies were prepared using complex mixtures of extracts or powders of medicinal plants. Development of chemical sciences has led to the isolation of pure active compounds from the vegetal material and to the development of our modern therapeutic. Nowadays, compounds derived from traditional medicins of different cultures still represent drugs of major therapeutic interest such as morphine, an alkaloid isolated from *Papaver somniferum*, or more recently artemisinin, a sesquiterpenie lactone from *Artemisia annua*.

Others drugs also have been discovered from several natural sources such as bacteria and fungi: avermectin (*Streptomyes avermitilis*) and penicillin (*Penicillium notatum*).

During the last decades, several health threat have emerged, coming out as severe worldwide medical problems. Multi-drug resistance of infectious bacteria, the absence of treatment for neglected tropical disease, resistance to artemisinin therapies in the case of malaria, chronic inflammation and aggressive/invasive cancers are some of them.

Traditional medicines and other natural resources are still an inestimable source of bioactive compounds. It is estimated that there are more than 400,000 plant species in the world, amog them near 30,000 have been described and 2,000 new ones are discovered each year [5].

Regarding this large biodiversity, several approaches have been propose in order to select plant species and to study their chemical composition, looking for active products.

- Ethnopharmacological approach: Ethnobotany is the study of plants from a geographical area associated to a specific group of people. This science study the practical uses through the traditional knowledge of a local culture and people [6]. Ethnopharmacology is specifically based on the study of medicinal use of plants. This knowledge is generally compiled in ethnobotanical surveys. In this case, phytochemical analysis enable to identify compounds responsible of the pharmacological activity of the plant (example artemisinin in *Artemisia annua*).
- Chemotaxonomic approach relais on the selection of species regarding their taxonomic classification: clade, family, genus. This approach postulates that if some bioactive compounds are found in a species, similar compounds may be produced by another one belonging to the same genus, or the same family because same biogenetic pathways may be involved. It is the case of some *Streptomyces* species biosynthesizing polyketide antibiotics.

We have divided this dissertation in two parts, depending the approach followed in the natural sources. Classic and modern technics and methodologies have been applied in the different sub-projects carried out in this doctoral thesis. The general workflow is described below:

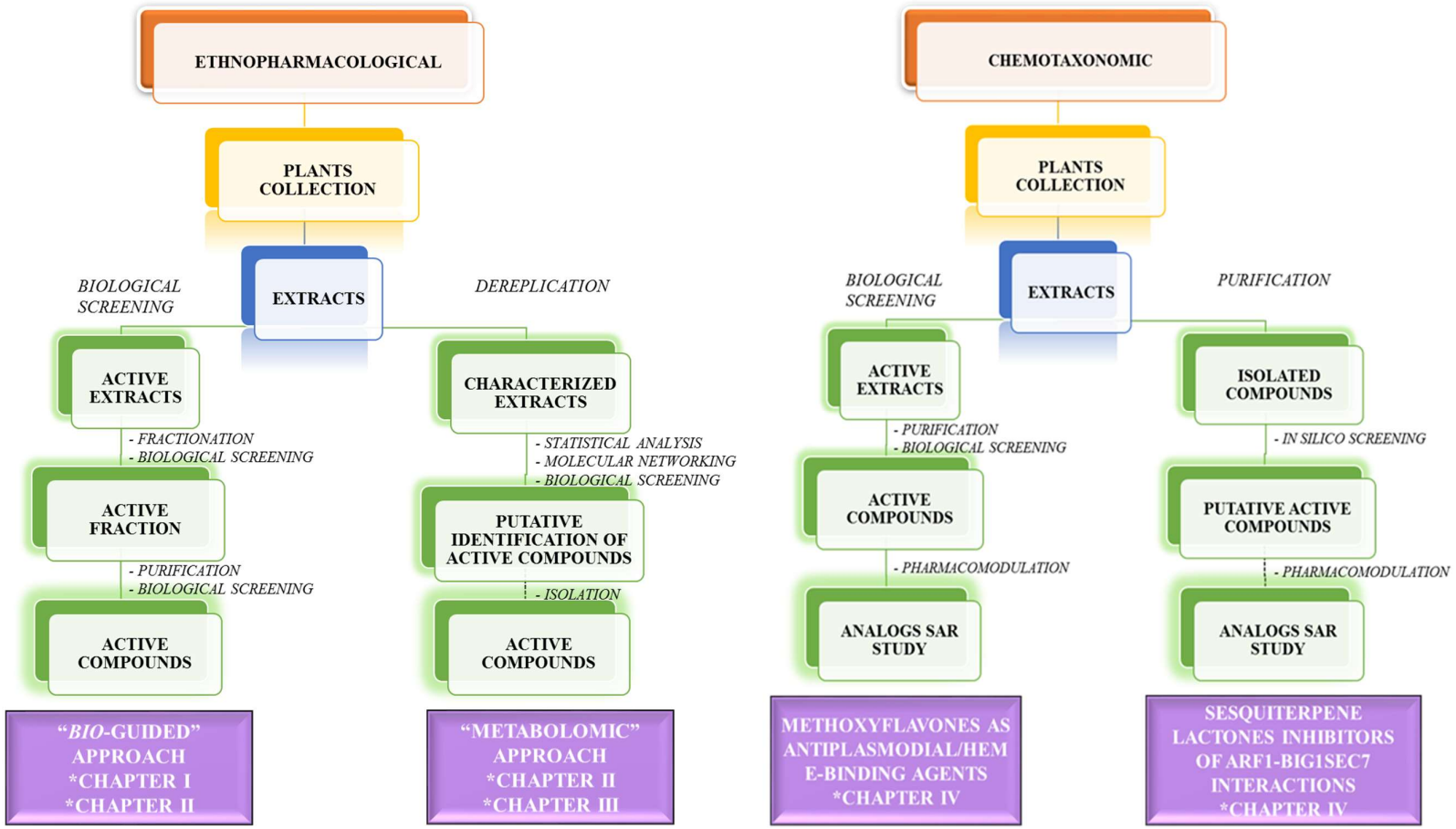



**Figure 1: General workflow followed in this Doctoral Thesis.**

**Part 1**: Ethnopharmacological approach was applied to access to belonging to several traditional medicines, where their therapeutic uses are recorded. In this framework that the traditional medicine of the Atacama People, indigenous culture from north of Chile was explored (**Chapter 1**); *Pistacia lentiscus* fruits in the traditional medicines in Europe and in the North of Africa (**Chapter 2**); and the plants used to fight inflammatory disorders in Europe (**Chapter 3**).

All vegetal material collected were subjected to several extraction procedures. Classic technics such as maceration and hydrodistillation or modern as pressurized solvent extraction (PSE) and supercritical fluid extraction (SFE) were applied according to each project objectives. However, PSE was chosen in first line, due to its low solvent and time consumtion and high extraction yield.

Then, the extracts were evaluated for their in vitro antibacterial, antifungal, antiparasitic, cytotoxic, antioxidant, anti-inflammatory and enzymatic inhibition depending of the respective project.

A bio-guided approach in **Chapter 1** and **2** has consisted in the purification and identification of compounds only from active fractions obtained after a fractionation of active extracts.

In **Chapter 2** and **3**, the modern approach of dereplication analysis by LC-MS/MS methodology was applied in order to identify biomarkers and/or bioactive compounds without previous fractionation or isolation:

- In **Chapter 2**, **molecular networking** approach was performed in order to identify biomarkers of the maturation and geographical origin of *Pistacia lentiscus* fruits.
- **Metabolomic analysis** with LC-MS data was carried out in **Chapter 3** in order to detect bioactive compounds directly from extracts from *Scrophularia* species, with anti-inflammatory effects.

**Part 2**: Chemotaxonomic approach was followed to select several plants from the Asterids clade, known to produce polymethoxyflavonoids (PMFs) and sesquiterpene lactones, as this metabolites posses numerous *in vitro* and *in vivo* biological activities. Several Asterid species were collected in Algeria (Willaya Tamanrasset) and their biological activity were measured, after that ethy acetate extract of *Pentzia monodiana* was chosen for further studies. The antiplasmodial activity by blocking the hemozoin formation by methoxyflavones was evaluated (**Chapter 4**).

In **Chapter 4**, PMFs from *P. monodiana* were identified by a combination of *in vitro* antiplasmodial activity and heme-binding assay affinity by LC-MS. With the blockage of hemozoin formation as plausible antiplasmodial target, pharmacomodulation by semisynthesis of

isolated and commercial flavonoids was performed in order to upgrade the initial effect and to establish structure-activity relationship (SAR) study.

Finally, in **Chapter 5**, of *P. monodiana*, ketopelenolide B, a major compound in the ethyl acetate extracts was found to be structurally similar, after *in silico* evaluation, to brefeldin A, a known inhibitor of the protein-protein interaction of Arf1-Big1Sec7. A library of 13 analogs was built, allowing to improve the bioactivity and to establisj SAR study.

Independently from the selection approach, all vegetal sources were collected respecting the local and French legislation of access to genetic resources: Nagoya Protocol (2010) or Convention on Biological Diversity (1992), depending the local status legislation.

# PART I EXPLORATION OF TRADITIONAL MEDICINES AS SOURCE OF BIOACTIVE COMPOUNDS: ETHNOPHARMACOLOGICAL APPROACH

# Chapter I Exploration of the traditional medicine of the Atacama Community of Taira (Chile)

# I.1. Introduction

In the framework of the exploration of the traditional medicine of Atacama People, it is important to depict the historical, geographical and anthropological context related to this indigenous people and the vegetal biodiversity of the territory.

## I.1.1 Geography and climate

Chile is a South American country delimited as a long and narrow strip of land between the Pacific Ocean with 4,270 km of extention from north to south and an evarages of 177 km from east to west [7,8]. It shares a total of 7,801 km of borders with Argentina (east, 6,691 km), Bolivia (northeast, 942 km) and Peru (north, 168 km). The climatic diversity of Chile ranges of the world's driest desert of Atacama in the north, through a Mediterranean climate in the centre, humid subtropical in Easter Island, to an oceanic climate, including alpine tundra and glaciers in the east and south. Geographers into five regions usually divide the country: the far north, the near north, central Chile, the south and the far south. Each has its own characteristic vegetation, fauna, climate and, despite the omnipresence of both the Andes and the Pacific, its own distinct topography [9].



a



b



c d

**Figure 2: Landscape from four well distinguished geographical zones of Chile. a) Altiplano from northern regions, b) central valleys, c) southern forest from Valdivia to Puerto Montt cities and d) Patagonian landscape from extreme south zone.**

The far north region, which extends from the Peruvian border to about 27 ° south latitude, near the Copiapo River, is extremely arid and contains the Atacama Desert, one of the driest in the World. Four geographic elements can be easily distinguished in the topography of this region from west to east: coastal plains, coastal mountains range, intermediate depression and the Andes mountain range, which includes the Altiplano.

The coastal plains, which extend from the coastal mountain range to the sea, are flat surfaces of only 3 to 10 km of extension. In some cases, e.g. between Iquique and Antofagasta citiesthey are absent: coastal range breaks down abruptly to the sea from elevation of 200-1,000 m altitude (locally knows as “farellones”). The average monthly temperatures fluctuation between 20.5 °C during summer and about 14° C during winter. Inhabitants mostly (68%) lives in the coastal area, where the temperatures are more moderate with high level of humidity and good accessibility the coastal cities of Arica, Iquique and Antofagasta. The coastal mountain range begins in a few kilometres south of Arica until the far south region with elevation of 2,000 m between Iquique and Antofagasta cities [10].

Between the coastal range and the Andes, the intermediate depression, which contains the Atacama Desert and Domeyko Mountains, which covers 128,000 $km^2$. The average rainfall is about 15 mm per year, although some locations, such as Arica and Iquique, receive 1 to 3 mm per year. Moreover, some weather stations in the Atacama have never received rain. Periods up to four years have been registered with no rainfall in the central sector, delimited by the cities of Antofagasta, Calama and Copiapó. Some evidence suggest that during four centuries, the Atacama Desert may not have recieved any

significant rainfall. The cold Humboldt Current, the Pacific anticyclone and the block from moisture on both sides by the Andes mountains and by the Chilean coast range are essential to keep the dry climate of Atacama Desert [11–13].

The eastern parts of this region lie within the higher ranges of the Andes. This arid, bleak land is a continuation southward of the great Altiplano, and is known as the Puna de Atacama. Its average elevation is estimated at 3,300 to 4,000 m of altitude. A line of volcanoes crosses it from those of Licancabur, Láscar, Socompa and Llullaillaco. Large shallow saline lakes are also characteristic features of this area [9].

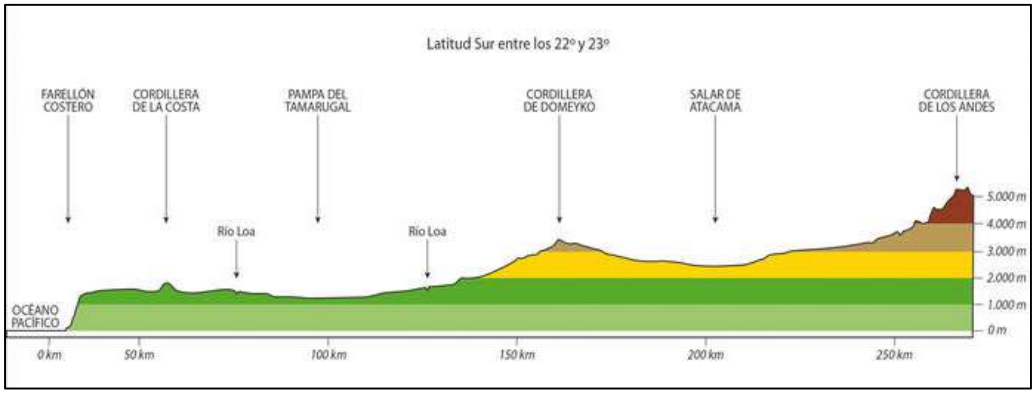



**Figure 3: Topographic profile of northern region of Chile**

## I.1.2 Biodiversity of Chile

The remarkable biodiversity of fauna and flora directly comes from the wide range of latitude and altitude, which definies numerous distinct ecosystems. Chile has hundred protected areas composed of 36 National Parks, 49 National Reserves and 15 National Monuments covering a total of 14.5 million hectares (20 % of the country).

### I.1.2.1 Endemic endangered species in Chile

The country is rich of more than 1,500 native species of plants, animals and fungi. Some species are in critically or endangered conservation status, threatened principally by introduced competitive or depredator species and degrade of their limits habitats, including for example the small shrub yareta (*Azorella compacta* Phil.) from the

Altiplano, used in the traditional medicine for treat diabetes [14], masafuera rayadito (*Aphrastura masafuerae* Phillippi & Landbeck) bird present only in the small island of Isla Más Afuera [15], Darwins's frog (*Rhinoderma darwinii* Duméril & Bibron) from the southern forest [16] or the small marsupial monito del monte (*Dromiciops mondaca* D'Elía, Hurtado & D'Anatro) only present in the forest near Valdivia city [17].

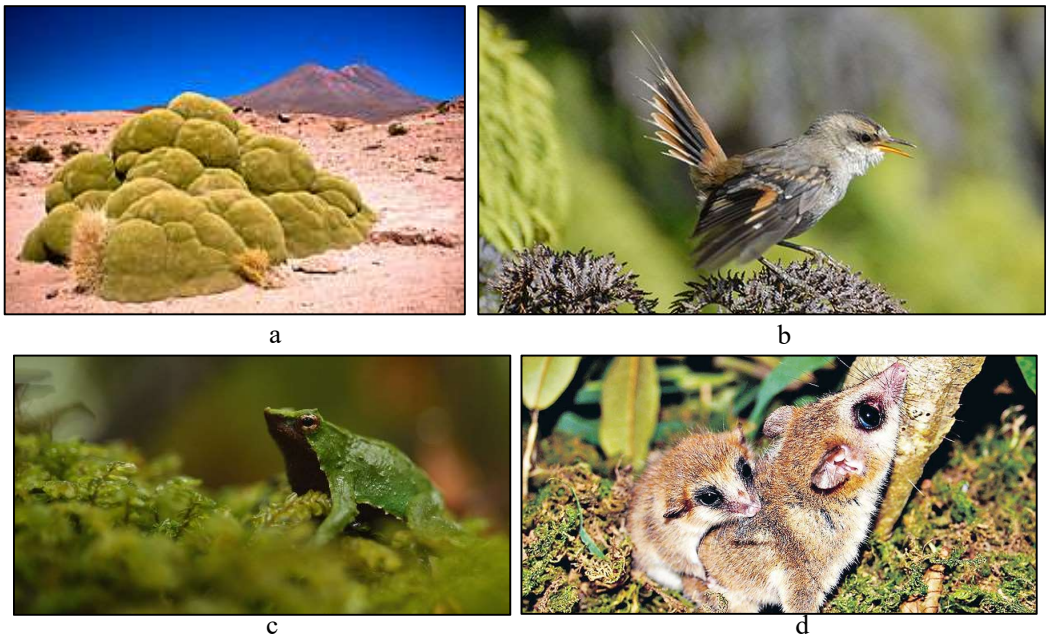



**Figure 4: Some examples of endangered endemic species from Chile. a) *A. compata*; b) *A. masafuerae*, c) *R. darwinii* and c) *D. mondaca*.**

The updated list of endemic species is as follows:

- Plants: 1023 species belonging to 347 genera and 115 families.

- Fishs: 181 species from 124 genera and 74 families.

- Molluscs: 156 species from 48 genera and 20 families.

- Insects: 57 species belonging to 34 genera and 20 families.

- Reptiles: 54 species from 6 genera and 3 families.

- Amphibians: 38 species from 8 genera and 8 families.

- Crustaceans: 21 species from 5 genera and 4 families.

- Fungi: 14 endemic species from several families.

- Mammals: 12 species from 10 genera and 7 families.

- Birds: 10 species belonging to 9 genera and 7 families.

### I.1.2.2 Fauna from North of Chile

The camelids guanaco (*Lama guanicoe* Müller) and vicuña (*Vicugna vicugna* Molina), the southern sea lion (*Otaria flavescens* Shaw), the culpeo fox (*Lycolopex culpaeus* Molina), the Humboldt penguin (*Spheniscus humboldti* Meyen), the grey-hooded sierra finch (*Phygillus gayi* Gervais) or the Guanay cormorant (*Leucocarbo bougainvillii* Lesson) are characteristic of the coastal areas where it is possible to find some vegetation. The Altiplano zone is possible to find also the camelids and wild fox, together with some endangered species such as the mammal long-tailed chinchilla (*Chinchilla lanigera* Bennet), the Andean mountain cat (*Leopardus jacobita* Cornalia) and the bird Andean flamingo (*Phoenicoparrus andinus* Phillipi) may ne encountered in the Altiplano, within restricted areas of national parks.



a) b) c)



d) e)



f)

**Figure 5: Examples of fauna present in the north of Chile. a) *L. jacobita*; b) *L. culpeaus*; c) *L. guanicoe*; d) *P. andinus*; e) *C. lanigera* and f) *S. humboldti***

### I.1.2.3 Flora from North of Chile

A unique vegetation characterizes the north of the country. In accordance with the flora, it is possible to distinguish three sub-regions:

- The coastline rich in cacti like *Copiapoa* spp., *Trichocereus atacamensis* (Phil.) W. T. Marshal & T. M. Bock or *Haageocereus fascicularis* (Meyen) F. Ritter (endemic).
- The central zone with the extremely arid Atacama Desert and consequently almost no vegetation.
- The Altiplano with a rich biodiversity including numerous shrubs (*Baccharis* spp., *Aloysia* spp., *Artemisia* spp, etc.) and trees (*Polylepis* spp. *Prosopis tamarugo* Phil. or *Geoffroea decorticans* (Gilles ex Hook. & Arn) Burkart) resulting from Andes glaciers and summer rain from the Amazonas.



a) b)



c)

**Figure 6: Example of vegetation in north of Chile. a) *Copiapoa* spp. b) *P. tarapacana* and c) shrubs in the Altiplano**

## I.1.3 History of Native Americans in Chile

### I.1.3.1 Pre-Inca and Pre-Hispanic Organization

During the pre-Inca period, Indigenous Peoples belonged to three major groups:

- the northern Peoples, inhabitants of the Altiplano and central valleys (Ayamara, Atacama and Diaguita Peoples), living from agriculture and who developed rich handicrafts and nomads tribes in the coastal areas (Changos and Chinchorro People).
- the Araucanians, who inhabited between the river Choapa (near Coquimbo City) and Chiloe Island, as Picunches, Mapuches, Huilliches, Pehuenches and Cuncos, who have developed a fertile textile and silverwork production and have based their economy on the agriculture in central valleys.
- the Patagonians in the southern fjords composed of various nomadic tribes, living from fishing and hunting (Chonos, Kaweskar, Yaganes and Selknam).




**Figure 7: Distribution of pre-Inka peoples in Chile.**

During the XV$^{th}$ century, the Inca Empire comprised also the north and central regions of Chile. Mapuche People of the central valleys have arrested the Inca expansion further in the south. The same thing was observed when the first Spanish conquerors Diego de Almagro and Pedro de Valdivia in 1535 and 1540 arrived into the Chilean territory. With Pedro de Valdivia started the Arauco War (1550-1772), trying unsuccessfully to colonize and to add the territory between Chiloe and Bio-Bio River to the Spanish domination.



a b

**Figure 8: a) Group of Mapuche photographed around 1860. b) Mapuche Silverwork.**

## I.1.3.2 Independence of Chile and Genocide - Occupation processes.

After the Independence of Chile (1810-1821), the territories between Bio-Bio and Malleco Rivers were not totally under Chilean sovereignty. This occurred after the process called Araucania Occupation (1861-1883), composed of several military campaigns of the Chilean Army into Mapuche territory and leading to the incorporation

of Araucania into Chilean national territory. The main motivation was to profit the valleys soils to the wheat industry, one of the principal exportation of the country in this epoch. The Mapuche population dropped from a total from 500,000 to 25,000 within a generation as results of the occupation and associated diseases and famine. The Chilean government confined the Mapuche to nearly 3,000 camps called “Título de Merced”, losing around 90 % of their ancestral lands. The displaced Mapuches suffered of poverty and racial/social discrimination after the conflict. Despite the fact that all of the Mapuche children were forced to assist public Chilean school, the language (Mapudungun), traditions, cosmovision and culture in general of Mapuche People are quite well preserved. Nowadays, several social programs launched by the State (scholarships, reposition of ancestral lands, several grades of autonomy, parliamentary representation, etc.) are actually in progress aiming at a better integration in the Chilean society and in the preservation of the Mapuche culture [18].

Another process of occupation took place in the extreme Chilean Patagonia. Selknam nomad tribes inhibited the extensive Patagonian plains, precious for lamb and sheep farming. Around 1880, Chilean and Argentinian governments gave concessions to foreign farming societies (principally England companies) to exploit the Patagonian and Isla del Fuego land to rise and commercialize wool, lamb and sheep meat. Selknam tribes, with no concept of private propriety, started to hunt the lamb and sheep, causing problems to the foreign farming companies. These later, under the protection of Argentinian and Chilean governments, payed one pound per Selknam murdered, which was confirmed by presenting the hands, ears and heads of the victims. Hired mercenaries by the companies perpetrated these massacres during 1880 and 1890 decades [19–21].



a b

**Figure 9: a) Selknam men before an initiation ceremony. b) Selknam family.**

Trying to find an alternative to these mass murders, the Chilean government decide to create concentration camps in Dawson Island in the Strait of Magellan with the Selknam survivors and started the procedure of evangelization by Salesians missionaries. The original population of Selknam has been estimated as 4,000 and after the massacres was registered as 2,000. This number was descending through the years in the Dawson Island principally by diseases as tuberculosis and smallpox. At 1905 only 500 Selknam remained, and in 1974 died the last person who met the nomad life style of Selknam People [22]. This process is known in the Chilean historiography as the Selknam Genocide.



a) b)



c)

**Figure 10: a) Mercenaries during the Selknam massacre; b-c) Hail ceremony of men hunting initiation.**

Today is just possible to find some mestizo descendants from Selknam People, but their culture is completely lost. Only some words of their language were preserved and pictures from several ceremonies have been saved. Their social structure, cosmovision, religion, traditions, even their traditional medicine are lost forever.

## I.1.4 Atacama and Aymara cultures in north of Chile

### I.1.4.1 Atacama People or Lican-Antay

The Atacameños, Atacamans, or Lican Antay, which in Kuntza means "the inhabitants of the territory", are descended of the extinct San Pedro culture, which can be traced back at least 1,500 years. At the start of 15$^{th}$ century, Inka Empire conquered their territory, bringing new social and political structures and imposing sun-worship as the new religion. The indigenous population resisted the Spanish conquistadors when they first arrived in 1536; the region needed 20 more years to be pacified through a peace treaty between both sides [23,24].

Towards the 17$^{th}$ century, Spanish evangelization and two customs of conquistadors reached the region: "encomiendas" and "mercedes de tierra". Colonial political and economic structures have maintained the subjugation of indigenous groups as servants under the conquistador lords. After the independence from Spain, the economy of the region grew with the expansion of the mining sector first with saltpeter and later with copper, producing large-scale immigration, proletarianization of the indigenous Peoples of the area and confiscation of water resources by the new industries. Today, 30,369 Chileans (1,04 % of total indigenous population of the country) identify themselves as Atacameños or Lican-Antay [25].




a) b)



c)

**Figure 11: a) Atacama woman; b) actual distribution of Atacama People in north of Chile, northwest of Argentina and Southwest of Bolivia; c) Atacama woman sowing in the farm, Toconce, 2006 [26,27].**

#### I.1.4.1.1 Social organization and economic activities of Atacama People

The Atacama People live in the oases and valleys of the Atacama Salar (salt flat) and Loa river basins in the Antofagasta Region. There are also small populations in northwestern Argentina (Salta and Jujuy) and in the southwestern Altiplano of Bolivia.




a) b)

**Figure 12: a) Atacama exchange fair; b) Atacama woman toasting corn [26,27].**

The Atacama People basic unit is the family, which subsists by occupying an extensive tract of this harsh and arid environment. They often have to walk dozens of kilometers to reach the resources they need. A property may be divided into several pieces of farmland, some often located quite far from the habitations. This social system, based on a traditional structure of family relationships and alliances, is organized to ensure access to and to control the resources spread across the region.

The traditional economy includes agriculture and livestock farming, Cultivation takes place at oases, specifically on terraces built on ravine slopes and irrigated with complex water systems. Subsistence farming implies several kind of crops; a single plot of land might be used to grow potatoes, quinoa, maize, squashes, prickly pears and chili peppers, thereby optimizing the use of water and soil. Animal husbandry was historically based on alpacas and lamas before the arrival of Spanish conquistadors: today, sheep, goats and mules are also bred. Gathering firewood and plants (mostly herbs for medicinal uses) is also common in the region.

More recently, new incomes have been added to Atacama People way of life through sale of local products, eco-tourism within the family communities and as emplyees in urban centres.

#### I.1.4.1.2 Beliefs of Atacama People

In the Atacama cosmogony, certain high mountains are considered as sacred and known as “Providers”. They represent earthly wealth such as pasturelands, minerals, water among other resources. They are associated with farming success, fertility, good weather and health, as well as personal protection and prosperity, and are honored during ceremonies to clean the irrigation canals or to pray for abundant water..



a) b)



c)

**Figure 13: Waki, rite of offering for the Calvary cross; b) Musicians during the carnival; c) lamas during "florement" ritual [26].**

Family rituals are linked to the life cycle: birth, baptism, marriage, roofing new homes and death, along with several related to health and ancestors worship. "*Yerbateros*" are the local experts that are roughly equivalent to shamans or "doctors" [23,24].

#### I.1.4.1.3 Kuntza language

The native tongue of the Atacama People is kuntza, though it has been extincted for at least one century. Only few traces remain, mainly in ceremonial, ritual songs and chants, and to design places and territories. It belongs to the macro-chibcha family and the paezano sub-family, and is not originated from the equatorial Andes, like Quechua and Aymara languages.

Under the Spanish occupation and even after the Independence, several programs were carried aiming at the Hispanicization/castellanization. All children were obliged to learn spanish and kuntza was forbidden in the public sphere from 17$^{th}$ to 19$^{th}$ centuries, resulting that in the 1940's died the last native kuntza speakers [28].

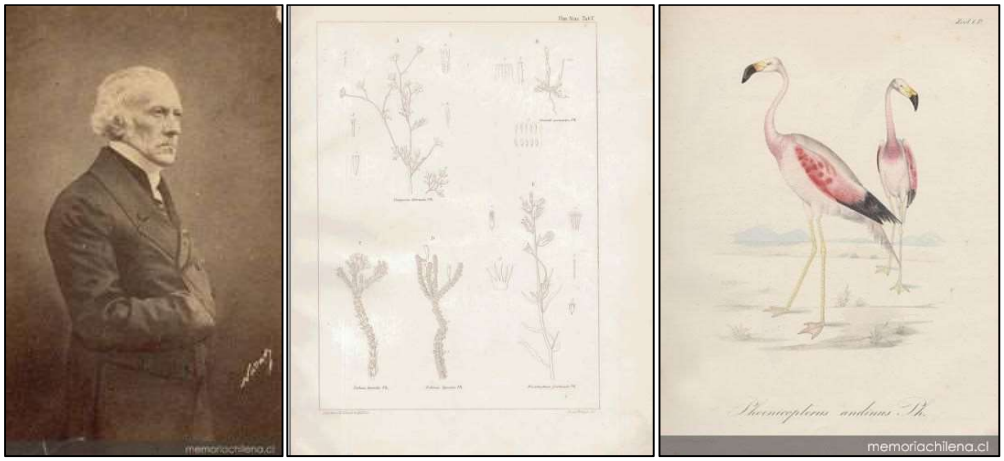
## I.1.5 Traditional Medicine of Atacama People

### I.1.5.1 Previous reports

#### I.1.5.1.1 In the Atacama People communities

The study of the traditional medicine of Atacama and Aymara Peoples started during the first decades after the independence, when the governments contracted European scientists to explore and catalog the natural resources of the new national territory.

During three months in 1853-1854, Rodolfo Philippi (1808 Charlottenburg, Prussia – 1904 Santiago, Chile) has explored oases in the Atacama desert [29]. He has classified around 420 plant species and reported medicinal, fodder and ornamental purposes. Philippi has also collected the amazing total of 3,730 species from all over Chile, having in mind that around 5,000 plant species are growing in the country; 1,317 descriptions are still valid [29,30].




a) c) d)

**Figure 14: a) Portrait of Rodolfo Philippi; b-c) original drawings of R. Philippi during his excursion in the Atacama Desert.**

Munizaga *et al.* (1958) published one of the first reports of the ethnobotanical of Atacama People in the village of Socaire (3,500 m of altitude), with the description of 140 plants species, including 35 with medicinal purposes, principally to treat mainly digestive disorders (stomach ache, diarrheal, etc.), rheumatism and fever (**Table 1**) [31].

| Morbidity | Number of medicinal plants | Examples | Vernacular name |
|---|---|---|---|
| Digestive disorders | 9 | *Artemisia copa* Phil. (Asteraceae) | Copa-copa |
| | | *Ephedra chilensis* C. Presl (Ephedraceae) | Pingo-pingo |
| | | *Junellia origenes* (Phil.) N. O'Leary & P. Peralta (Verbanaceae) | Rica-rica |
| Rheumatism | 5 | *Parastrephia quadrangularis* (Meyen) Cabrera (Asteraceae) | Chacha |
| | | *Haplopappus baylahuen* J. Remy (Asteraceae) | Bailahuén |
| Fever | 5 | *Atriplex retusa* Gay (Chenopodiaceae) | Cachiyuyo |
| | | *Sonchus oleraceus* L. (Asteraceae) | Cerraja |
| Altitude sickness | 4 | *Senecio eriophyton* J. Remy (Asteraceae) | Chachacoma |
| | | *Erythroxylum coca* Lam. (Erythroxylaceae) | Coca |
| Cold and coughs | 4 | *Azorella atacamensis* G.M. Plunkett & A.N. Nicolas (Apiaceae) | Chuquicán |
| Inflammation | 4 | *Xanthium ambrosioides* Hook. & Arn. (Asteraceae) | Cepa caballo |
| Urinary infections | 3 | *Krameria lappacea* (Dombey) Burdet & B.B. Simpson (Krameriaceae) | Tícara |
| Bone fractures | 2 | *Fabiana denudata* Phil. (Solanaceae) | Tolilla |
| Wound healing | 2 | *Rumex patientia* L. (Polygonaceae) | Romasa |
| Heart disorders | 2 | *Loasa* sp. (Loaceae) | Ojman |

**Table 1: Some medicinal plants reported by Munizaga *et al*. (1958).**

In 1983 the ethnobotanical review of the village of Toconce (3,550 m of altitude, 90 km east of the city of Calama), inhabited by Atacama People families was published. One hundred thirty four plant species were identified in the surroundings of the village; the inhabitants have reported medicinal purposes for thirty seven (27.6 %) of them. Only two species in the rich flora of the area were apparently not used, demonstrating that the population had maximized the utilization of the available resources [32].




**Figure 15: Referential geographic position of different ethnobotanical studies previously published in the North of Chile. 1) Taira Community (Atacama people) in Ollagüe Municipality, 2) Socaire Community, 3) City of Iquique, 4) Andes area of Tarapaca-Iquique and 5) Andes area of Antofagasta-Ollagüe.**

Since 1990, ethnobotanical surveys and the relationship between traditional and modern occidental medicines started in the surrounding of Atacama Salar villages (Santiago del Rio Grande, Talabre, Camar y Socaire). Chamorro and Tocornal [33] have described different approaches to cure several health dissorders, classified in three major groups, naturalist, personalist and emotional. Gods or beings connected to the environment, that occupy a place in the Atacama Pantheon, cause the personalist diseases (**Table 2**). For example, *Pachamama*, goddess of hearth and crops, personified as an old woman or with Virgin Mary aspect, is believe to induce the disease "tierra", described as rashes in the body that sting and dry the skin. Illness that are not related to a god or supernatural beings are classified as naturalist. The emotional diseases are linked to post-traumatic states or stress induced by familiar conflicts.




a) b)




b) d)

**Figure 16: Pictures of a) *A. compacta*, b) *E. chilensis*, c) *H. baylahuen* and c) *K. lappacea* [34].**

| God or supernatural being | Specific references in the environment | Diseases related | Symptoms |
|---|---|---|---|
| *Pachamama* | Personified as an old woman or Virgin Mary aspect | *tierra* (ground) | - |
| *Abuelos* (ancestors) | Gentilares (cemeteries of the ancients). Also as strong winds. | *Abuelo* (grandfather) | Body aches, generally in arms and legs. |
| *Souls/deceased ancestors* | Cemeteries are related places for recently deceased. | - | Permanent tiredness and state of predisposition to several diseases. |

**Table 2: Personification of the environment of Atacama People and diseases that cause.**

All kinds of sickness are first treated within the family by medicinal plants. If the troubles remains, a specialist is called, either an Atacama traditional practitioner or a "modern occidental" medical doctor. In case of naturalist diseases, the modern medicine provided by Health Centers (San Pedro de Atacama and/or Calama) are requested.

For emotional and personalist illness, the patient sees a "*yerbatero*", person with several functions, including medical practitioner (based on his knowledge of the magical and curative properties of the plants) and kind of priest, as a mediator between the natural and the divine Worlds. The *yerbatero* use several ways to diagnose the patient such as reading coca leaves, blackjack cards, the Bible or the internal organs of an animal. The therapeutic protocols advised by the *yerbateros* consists mainly in infusions, rubs and sahumerios (kind of inhalation or fumigation) with plants complemented with specific diets. For the personalist diseases are prescribed ritual treatments with spiritual payments, prayers, sacrifices of animals, mesas (altars with coca leaves, alcohol, cigarettes and cereals) and sahumerios.

It happens that the medical prescription of the health centers are not easy to follow due to: 1) the long distance from the villages (60 km), 2) the limited infrastructures and qualified medical staff and 3) insufficient resources of the patients to afford the treatments.

Villagrán and Castro have carried out the biggest ethnobotanical survey in all pre and Altiplano regions in the north of Chile, including Aymara and Atacama Peoples populations. Two hundred and fifty people from around 30 settlements and hamlets were consulted, and almost 500 plant species were listed [35,36].

From all the taxa, 859 uses were described, mainly as forage (37 %) and medicinal (23 %); for 5 % of the species, no uses were reported. In the studied area, 200 medicinal plants linked to diseases related to the respiratory, digestive, bone, circulatory, nervous and reproductive systems including affections associated with fever, allergies, dermic disorders and wounds healing, haemorrhages, head ache, tooth and ear aches, until cancer, diabetes and gonorrhoea were registered. The principal therapeutic procedures are from medicinal baths, ritual sahumerios, infusions and cataplasms.

| Family | Plant Species | Traditional therapeutic uses of some medicinal plants cited by Villagrán and Castro (2003). | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Digestive | Respiratory | Bone | Dermic | Urinary | Circulatory | Reproductive | Nervous | Other |
| Apiaceae | *Azorella compacta* Phil. | * | * | * | | * | | | | |
| Asteraceae | *Artemisia copa* Phil. var. copa | * | | | | | | | | HT, P |
| Asteraceae | *Bidens laevis* (L.) Britton, Stern & Poggenb. | * | * | | | * | | | | |
| Asteraceae | *Chuquiraga spinosa* Less. ssp. rotundifolia (Wedd.) C. Ezcurra | | * | | | * | * | | | G |
| Asteraceae | *Flaveria bidentis* (L.) Kuntze | | | | * | | * | | | LW |
| Asteraceae | *Haplopappus rigidus* Phil. | | * | | | * | * | | | APH |
| Asteraceae | *Parastrephia lepidophylla* (Wedd.) Cabrera | * | * | * | | | | * | | F,S,HT |
| Asteraceae | *Parastrephia quadrangularis* (Meyen) Cabrera | * | | * | * | | | | | S |
| Asteraceae | *Senecio nutans* Sch. Bip. | * | * | | | | | | | F, AD, HT |
| Asteraceae | *Sonchus asper* (L.) Hill | * | * | | | | | | | F |
| Asteraceae | *Werneria glaberrima* Phil. | | | * | | * | | * | | |
| Asteraceae | *Xanthium spinosum* L. var. spinosum | | | | | * | | * | | |
| Asteraceae | *Xenophyllum weddellii* (Phil.) V.A. Funk | * | | | | * | | * | | |
| Cactaceae | *Maihueniopsis camachoi* (Espinosa) F. Ritter | * | * | | * | * | | | | F |
| Calyceraceae | *Moschopsis monocephala* (Phil.) Reiche | | * | * | | * | * | | | |
| Ephedraceae | *Ephedra americana* Humb. & Bonpl. ex Willd. | * | | * | | * | | | | GO |
| Lamiaceae | *Clinopodium gilliesii* (Benth.) Kuntze | * | * | | | | | | | |
| Lamiaceae | *Marrubium vulgare* L. | * | | * | | | * | | | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Loasaceae | *Caiophora chuquitensis* (Meyen) Urb. & Gilg | | * | | * | | | * | | HT, AP |
| Loasaceae | *Caiophora rosulata* (Wedd.) Urb. & Gilg rosulata | * | * | | * | | * | | | |
| Plantaginaceae | *Plantago major* L. | * | | | * | | * | | | |
| Poaceae | *Cortaderia speciosa* (Nees & Meyen) Stapf | * | * | * | | | | * | | F |
| Pteridaceae | *Argyrochosma nivea* (Poir.) Windham | * | * | * | | | | | | |
| Rosaceae | *Polylepis tarapacana* Phil. | | * | | | * | * | | | |
| Solanaceae | *Fabiana densa* J. Remy | * | * | * | | | | | | |
| Verbenaceae | *Aloysia deserticola* (Phil.) Lu-Irving & O'Leary | * | * | | | * | * | | | |
| Verbenaceae | *Junellia seriphioides* (Gillies & Hook. ex Hook.) Moldenke | * | * | | | * | | | | P |
| Verbenaceae | *Lampayo medicinalis* F. Phil. | * | * | * | | | | | * | |

**Table 3: Some examples of medicinal plants cited by Villagrán and Castro 2003 after ethnobotanical surveys in north of Chile. (Modified from Villagrán et al.) AD: altitude sickness; AP: antiparasitic; APH: aphrodisiac; F: fever; G: gentiles (abuelos); GO: gonorrhoea; HT: hair tonic; LW: lose weight; P: pain; S: sahumerio.**

#### I.1.5.1.2 In the urban areas

Medicinal plants based tharapy is not limited to the Atacama communities. Thus, a part of the Chilean population without any family relationship with the indigenous Peoples are using Atacama traditional medicine, mainly in the urban areas of Arica, Iquique, Antofagasta and San Pedro de Atacama [37].

Quantitative surveys have described that 73 % of inhabitants of these areas are concerned and that 195 remedies have been described. Sixty percent of those remedies were based on herbs most frequently to cure digestive affections (16 %). The species rica-rica (*A. deserticola*), llareta (*A. compacta*), bailahuén (*Haplopappus* spp.) are reported both in the urban areas and in the Atacama People communities [38]. The therapeutic properties of those herbs are of common knowledge and they are available in regular markets in Antofagasta, Calama or San Pedro de Atacama.




a) b)




b) d)

**Figure 17: Medicinal plants of the Atacama People traditional medicine in the common markets of a-b) San Pedro de Atacama and c-d) Antofagasta.**

### I.1.5.2 Legal status of the traditional medicine in Chile

In a recent effort to protect and to promote traditional medicines of indigenous cultures, the department of politics and pharmaceutic regulations of health and complementary medicines of the Ministry of Health of Chile, has published a list of 103 herbs, including botanical description, popular uses, recommendations, commercial presentations, instructions of use and habitats [39].

## I.1.6 Phytochemical studies and ethnopharmacology of the Atacama traditional medicine

Scarce phytochemical studies, generally limited to biological evaluations of extracts have been published, exploring the anti-inflammatory, antioxidant, antibacterial and antiparasitic potential of medicinal herbs. Some studies focused on one-plant extracts or on a group of plants with taxonomic relationships. The major results of these investigations are listed and described below, classified by botanical families.

## I.1.6.1 Apiaceae family

### I.1.6.1.1 *Azorella* spp.

Species from the *Azorella* genus as *A. compacta* (*syn. Azorella columnaris* H. Wolff, *Azorella yareta* Hauman, *Azorella prismatoclada* Domin, *Laretia compacta* (Phil.) Reiche) and *A. madreporica* (syn. *Azorella bolacina* Clos, *Azorella piligera* Phil., *Azorella muscoides* Phil., *Azorella obtusiloba* Phil.) are known with the common name "yareta". These species are yellow-green compact resinous cushion shrub growing in the Andes of southern Peru and Bolivia, northeaster and northwester Argentina over the 4000 m of altitude. Therapeutic uses of the roots, flower, seed and resin have been reported [36]. Infusion of these parts are intake to treat kidney and bladder disorders, cough, diabetes, cholesterol lowering agent, general pain and to "purify the blood". They are also utilised as fuelwood. Their current conservation status is declared as vulnerable. Mainly diterpenoids structurally related to mulinoic acid have been isolated from *A. compacta* extracts with several biological activity described.




a) b)

**Figure 18: a) *A. madreporica* and b) *A. compacta*.**

Diterpenoids isolated from *A. compacta* have been assayed for anti-infectious activities by several groups. Compounds **1** and **2** were evaluated by agar diffusion method against methicillin-sensible and resistant strains of *Staphylococcus aureus*, *Enterococcus feacium*, *Escherichia coli* and *Candida albicans*. Two hundred of micrograms of compounds **1** and **2** produced mostly hazy zones of inhibition of 8-12 mm in diameter against the Gram-positive strains (*S. aureus* and *E. feacium*). Not activity was observable in the case of *E. cola* and *C. albicans* [40]. Compound **3** (800 µg per spots) also exhibit

strong antibacterial activity against oral bacteria, as *Actinomyces naeslundii* (DSM 17233), *Porphyromonas gingivalis* (ATCC 33277) and *Streptococcus oralis* (DSM 20697) with zone of inhibition of 10-15 mm [41]. Núñez et al. evaluated compounds **4** and **5** but showed not antibacterial activity by the agar diffusion test against *S. aureus*, *E. coli* and *Mycobacterium smegmatis* (500 µg per spot) [42]. Regarding the antitubercular activity, compound **6** showed MIC value of 20 µg/mL against *M. tuberculosis* $R_{37}Hv$ strain (ATCC 27294).

Compounds **4**, **7-10** were essayed *in vivo* against *Plasmodium berghei* NK65 (protozoan parasite that caused malaria in certain rodents) on infected mice model at an intraperitoneal dose of 10 mg/kg/day. The percentage of inhibition on the parasite erythrocytic life cycle was 25, 29, 60, 42 and 26 respectively. *In vitro* measures were not evaluated [43]. The growth inhibition of *Trichomonas vaginalis* Ant-1 strain (protozoan parasite, pathogen agent of trichomoniasis) *in vitro* by **4**, **11-14**. $IC_{50}$ were recorded as 100, 119 and 40.5 µM for compounds **12-14**. Percentage of inhibition for compounds **4** and **11** were above 50 % even at the lower tested concentration (40 µM)) and $IC_{50}$ were not calculated [44].

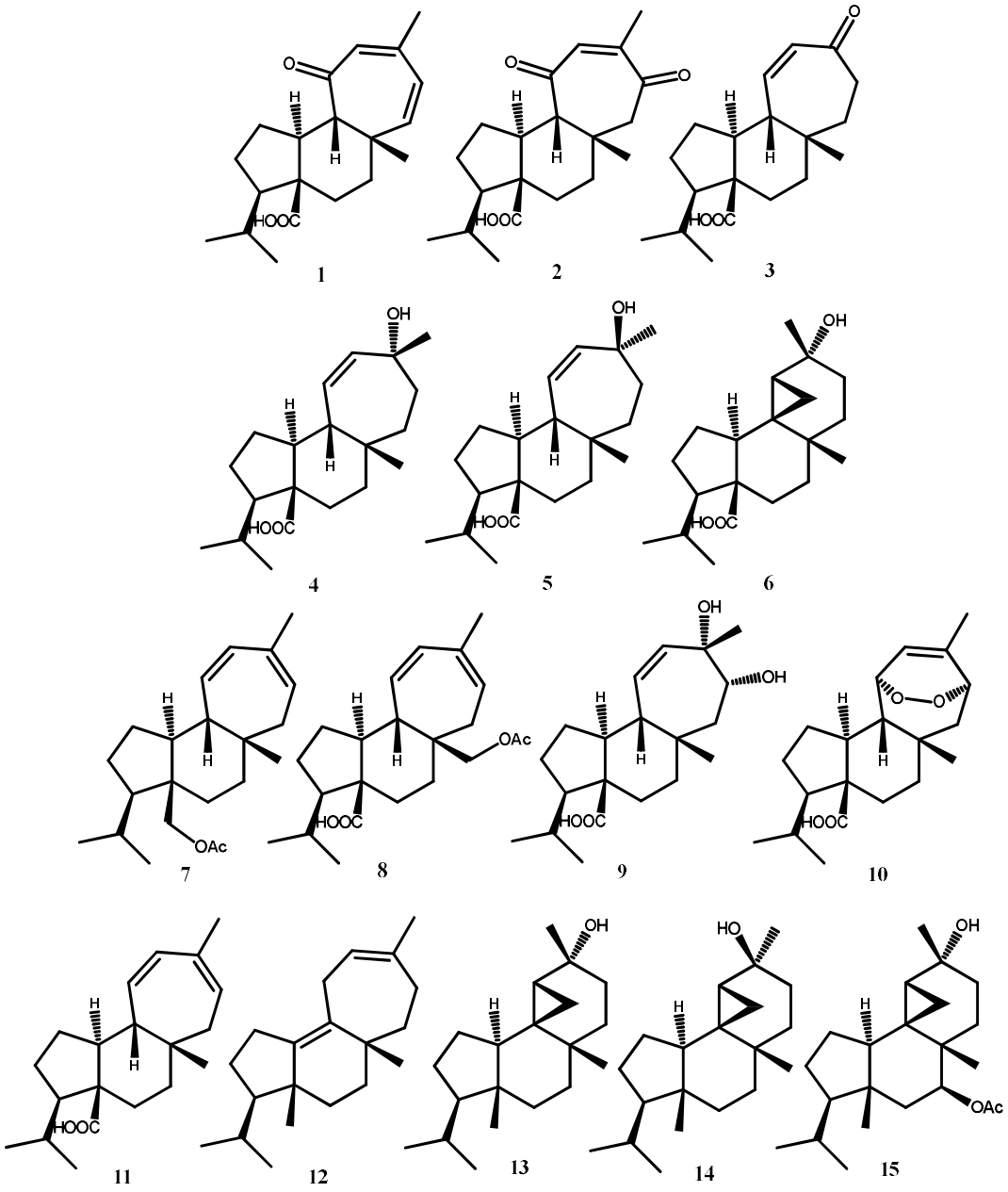



**Figure 19: Diterpenoids from *A. compacta* evaluated dor anti-infectious activities.**

Other kind of evaluations have been performed. The experimental antihyperglycemic effect of compounds **10**, **11** and **14** were explored in the *in vivo* model of streptozotocin diabetic rats. Glycemia of animals treated with **10** and **15** decreased to 243 ± 2 and 247 ± 14 mg/mL respectively, values close to those reached by chlorpropamide injection used in controls. Furthermore, **14** enhanced the production of blood serum insulin from 146 ± 58 pg/mL (controls) to 247 ± 23 pg/mL [45].

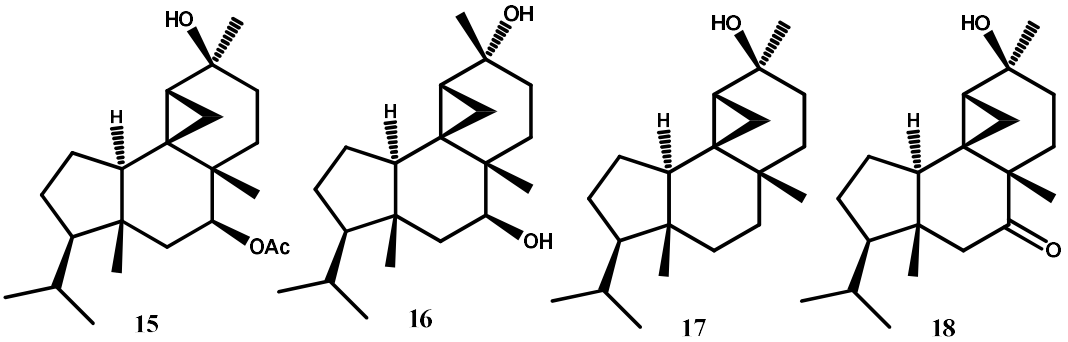



**Figure 20: Diterpenoids from *A. compacta* with anti-inflammatory reports.**

The anti-inflammatory and analgesic properties of compounds **14**-**18** (isolated from *A. compacta*) were essayed in *in vitro* models. Compounds **14**, **16** and **17** were found to inhibit the expression of NF-κB transcription factor in a concentration-dependent manner, inhibited TNFα-induced HIV-1-LTR transactivation. Compound **15** showed not anti-NFκB activity [46]. In addition, the dose-related inhibition of pain and topical anti-inflammatory properties of **14**, **16** and **18** has been reported and **18** was the most potent analgesic but less effective than positive control of sodium naproxen in *in vivo* acetic acid-stimulated mice between 23 to 100 μmol/kg with a reduction of 25, 57 and 60 % of abdominal writhes. Compound **16** showed the higher topical anti-inflammatory potency on arachidonic acid (21 and 23 % reduction at 34 and 66 μmol/kg) and 12-deoxyphorbol-13-tetradeconoate (45, 79 and 66 % reduction at 15, 26 and 40 μmol/kg) induced oedema and its effect was similar to the references drugs (nimesulide and indomethacin) [47]. Additionally, **11** and **18** showed a reduction of the cell viability of MCF-7 cell line at 100 μM, **4** and **16** were not active [48].

### I.1.6.2 Asteraceae family

This botanical families is widely used in the traditional medicine of Atacama People [31,35,36]. , exemplified by the shrubs from *Baccharis* genus, known as “chilkas” (river chilka, mountain chilka, chalkilla, etc.), *Senecio* genus, known commonly as chachakoma (chachakuma, female chachakoma, male chachakoma, people chachakoma, donkey chachakoma, etc.) and *Artemisia copa* variants (copa and trifida) known as copa-copa.

#### I.1.6.2.1 *Artemisia copa*

*Artemisia copa* Phil. var. copa and *Artemisia copa* Phil. trifida Acevedo share the vernacular name "copa-copa" (sometimes as kopa-kopa, kopatola or qopa-qopa). There are small bush, 30-60 cm height, growing in the northwest of Argentina and in the northeast of Chile, above 4,000 m of altitude.




**Figure 21: *A copa* var. copa images[49].**

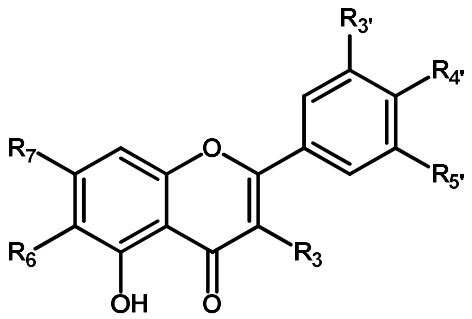
Infusion of aerial parts of *A. copa* is used to treat against digestive disorders, colds, diarrhoea, toothache, bone pain as hair tonic and as sahumerio (kind of inhalation and/or fumigation).

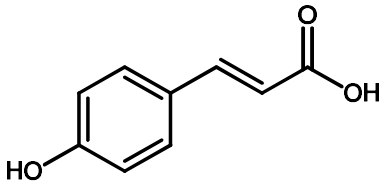
The chemical composition of the essential oil obtained from *A. copa* growing in the high valley region of Bolivia, was determined by GC and GC/MS measurements; the mains constituents were β-thujone (42 %) and chamazulene (6.5 %). No biological evaluation were reported [50].

The vasorelaxant, hypotensive and spasmolytic properties of the aqueous extract of *A. copa* aerial parts have been explored. It has antagonised the rat jejunum contractions induced by acetylcholine and $CaCl_2$ between 3-0.3 and 3-1 mg/mL respectively [51]. At same concentration range, it has induced the relaxation of endothelium-intact aortic rings, pre-contracted with phenylephrine, 5-hydroxytriptamine and KCl with $EC_{50}$ of 1.1, 1.5 and 1.6 mg/mL. Similar behavior was observed in denuded aortic rings contracted by phenylephrine ($EC_{50}$ of 1.8 mg/mL). The relaxation of *A. copa* extract were not blocked by L-NAME, indomethacin, tetraethylammonium or glibenclamide. Same extract in intravenous administration induced a decrease in the mean arterial pressure (10-200 mg/kg between -10 to -30 % of mean arterial pressure) but did not affect the heart rate in hypertensive rats [52].

The vasorelaxant activity of flavonoids **19**, **20**, **22** and **24** isolated from the aqueous extract of *A. copa* were also measured: **19**, **20** and **29** caused dilatation of thoracic aortic rings precontracted

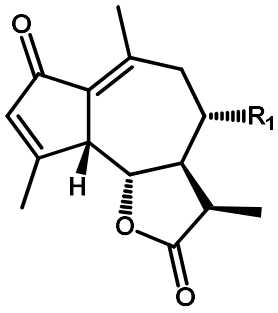
with phenylephrine with $EC_{50}$ of 1.5, 13.2 and 95.2 µg/mL; **19-21** were evaluated at 30 and 15 µg/mL in the reduction of the contraction produced of $CaCl_2$ of rat jejunum, where **20** was the more promising results, showing an inhibition of the maximum contraction of 77 %.




**29**

**19** $R_3 = R_6 = R_{5'} =$ -H; $R_7 = R_{3'} = R_{4'} =$ -OH
**20** $R_3 = R_6 = R_{5'} =$ -H; $R_7 = R_{4'} =$ -OH; $R_{3'} =$ -OMe
**21** $R_3 = R_6 =$ -H; $R_7 = R_{4'} =$ -OH; $R_{3'} = R_{5'} =$ -OMe
**22** $R_3 = R_{3'} = R_{5'} =$ -H; $R_7 = R_{4'} =$ -OH; $R_6 =$ -Glu
**23** $R_3 = R_{5'} =$ -H; $R_7 = R_{4'} =$ -OH; $R_6 = R_{3'} =$ -OMe
**24** $R_{5'} =$ -H; $R_3 = R_7 = R_{4'} =$ -OH; $R_6 = R_{3'} =$ -OMe
**25** $R_{3'} = R_{5'} =$ -H; $R_{4'} =$ -OH; $R_3 = R_6 = R_7 =$ -OMe
**26** $R_{5'} =$ -H; $R_7 = R_{3'} = R_{4'} =$ -OH; $R_3 = R_6 =$ -OMe
**27** $R_{5'} =$ -H; $R_{4'} = R_7 =$ -OH; $R_3 = R_6 = R_{3'} =$ -OMe
**28** $R_{5'} =$ -H; $R_{4'} =$ -OH; $R_3 = R_6 = R_7 = R_{3'} =$ -OMe




**30** $R_1 =$ -H
**31** $R_1 =$ -OH

**Figure 22: Compounds previously isolated from *A. copa*.**

From the dichloromethane extract of aerial parts of *A. copa*, were isolated flavonoids **20**, **21**, **23-26;** their anti-inflammatory activity via production of pro-inflammatory mediators in LPS-stimulated RAW 264.7 mouse macrophage cell line was evaluated. Compounds **23** and **24** weakly inhibited nitric oxide production reduction at 270 and 308 ng/mL in comparison of stimulated control of 422 ng/ml respectively) whereas all flavonoids reduced prostaglandin $E_2$ levels (6.6-11.3 ng/mL in comparison of stimulated control of 17.4 ng/mL) at 10 µM. The most active **23** inhibited cyclooxygenase-2 activity with an $IC_{50}$ of 2.8 µM. All flavonoids partially inhibited synovial phospholipase $A_2$ activity in a range of 13-36 % at 10 µM [53].

In addition, flavonoids **27**, **28** and sesquiterpene lactones **30**, **31** have been isolated from chloroformic extract of aerial part of *A. copa* [54].

#### I.1.6.2.2 *Baccharis* species

*Baccharis* species are resinous shrubs, 30 cm to 2 m of height, known in the region under the vernacular names lejía or léjia (*B. tola* or *B. santelises*) and chilka or pichana (*B. calliprinos*, *B. grisebachii* or *B. scandens*) several therapeutic uses in traditional medicine have been reported [36].

*B. boliviensis* (Wedd.) Cabrera var. boliviensis (syn. *Baccharis boliviensis* (Wedd.) Cuatrec., comb. superfl., *Baccharis boliviensis* (Wedd.) Cabrera var. latifolia, *Baccharis boliviensis* (Wedd.) Cabrera var. latifolia, *Heterothalamus boliviensis* Wedd., *Heterothalamus boliviensis* Wedd. var. latifolius, *Pseudobaccharis boliviensis* (Wedd.) Cabrera, *Pseudobaccharis boliviensis* (Wedd.) Cabrera var. latifolia, *Psila boliviensis* (Wedd.) Cabrera, *Psila boliviensis* (Wedd.) Cabrera var. latifolia, *Baccharis boliviensis* (Wedd.) Malag., comb. superfl.) is an erect ramose shrub of 60 cm max of height present in the northwest of Argentina, north of Chile, very frequent in the Altiplano [55]with the vernacular names of tolilla, tola, or anqo pajariñi. The infusion is used to treat digestive disorders and the bath with this plant are used to heal bone pains [36].




**Figure 23: *B. boliviensis* (Wedd) Cabrera var. boliviensis [34].**

The hydroalcoholic extract of aerial parts of *B. boliviensis* exerted anti-inflammatory properties by the inhibition of COX 1-2 enzymes with $IC_{50}$ of 7.6 and 6.0 µg/mL [56]. The ethanolic extract of *B. boliviensis* has inhibited the growth of Gram-positive and Gram–negative bacterial strains. (MICs values of 80-40 µg/mL for methicillin sensitive or resistant *Staphylococcus aureus* strains, 80 µg/mL against *Enterococcus faecalis* strains) [57].

Flavonoids **32-36** and diterpenes **37-39** were isolated from aerial parts of *B. boliviensis* but not biological activities were reported in those studies.

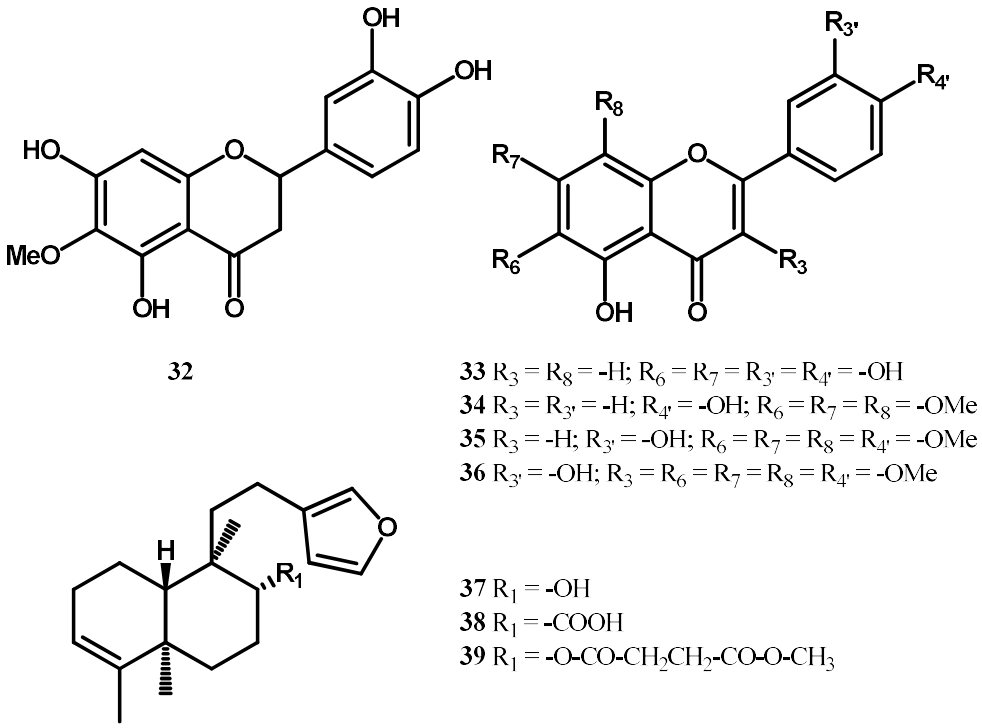



**Figure 24: Flavonoids and diterpenes isolated from *B. boliviensis* extracts.**

The subspecies and variants of *B. tola*, principally *B. tola* Phil. var. incarum (Wedd.) Joch Müll. (syn. *Baccharis magellanica* (Lam.) Pers. var. subviscosa, *Baccharis microphylla* Kunth var. incarum, *Baccharis incarum* (Wedd.) Cuatrec., comb. superfl., *Baccharis incarum* (Wedd.) Heering, comb. superfl., *Baccharis incarum* (Wedd.) Perkins) and *B. tola* Phil. ssp. tola (syn. *Baccharis incarum* (Wedd.) Perkins var. lejía, *Baccharis magellanica* (Lam.) Pers. var. viscosissima, *Baccharis tafiensis* Heering, *Baccharis tola* Phil. var. lejía, *Baccharis lejia* Phil., *Baccharis tola* Phil. var. viscosissima) are shrubs of 5-40 cm tall, larger leaves 4-10 mm long, involucre of female capitula 4.3 – 6.5 mm long present southern Altiplano and north of Uyuni Salar. Vernacular names considered are likia, lejía, léjia, tola lejia, male léjia and ñaka.

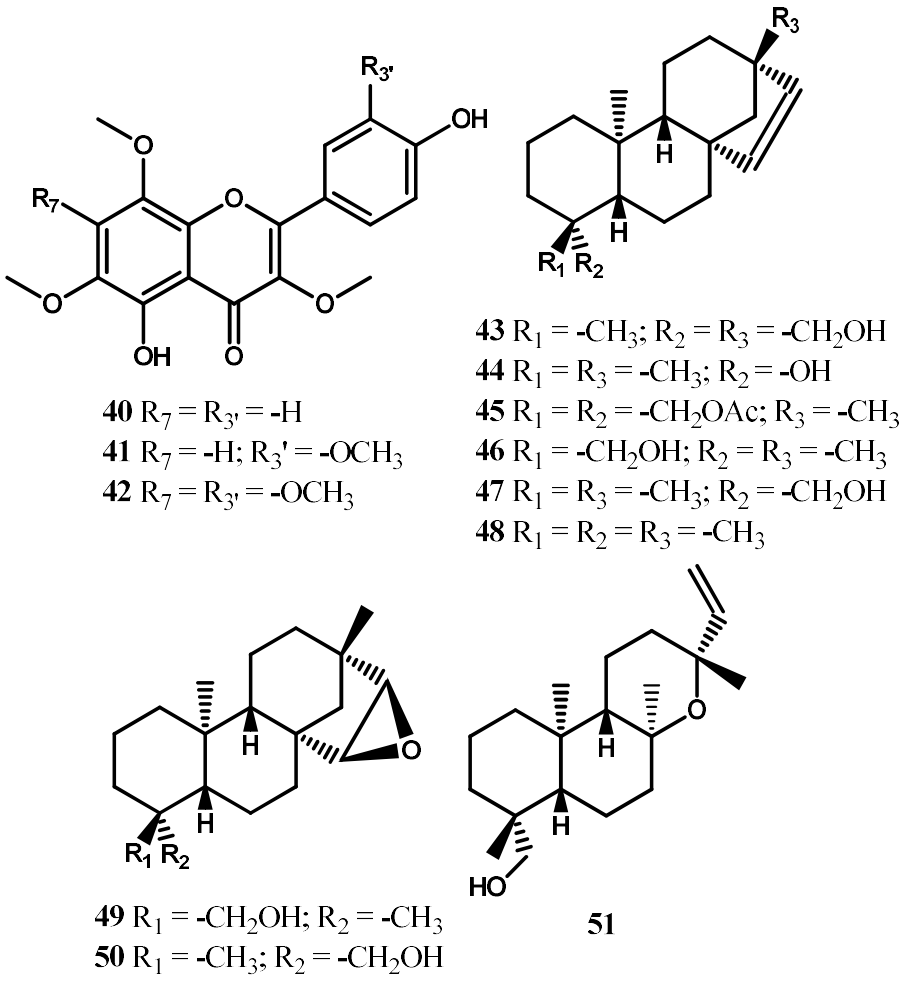


**Figure 25: *Baccharis tola* Phil. spp tola [49].**

In the Atacama people communities, the infusion of *B. tola* species are uses to treat cold, cough, digestive disorders, rheumatism and kidney stones. Cataplasm and bath with this plant is reported to cure bone pains.

The aqueous and methanolic extracts of aerial parts of variants of *B. tola* Phil. var. incarum were evaluated its antimicrobial and antioxidant potential. The methanolic extract showed MICs values of 80-40 µg/mL against methicillin sensitive and resistance *Staphylococcus aureus* and *Enterococcus faecalis*. Isolated flavonoids **19**, **26** and **40-42** from methanolic extract showed MIC values (200-100 µg/mL) higher than original extract [58].

From apolar extract of aerial parts of *B. tola*, a group of diterpenes of *ent*-beyer structure **43-51** were isolated [59,60]. Diterpene **47** showed gastroprotective properties at 100-12.5 mg/kg on the model of HCl/EtOH-induced gastric lesions in mice, reducing the ulcerative index to 10-30 mm respecting the control (40 mm) [61]. In addition, diterpenes **46** and **47** demonstrated antileishmanial activity against intracellular amastigotes of *L. braziliensis* with $IC_{50}$ of 5.3 and 4.6 µg/mL [62].




**40** $R_7 = R_{3'} = -H$
**41** $R_7 = -H$; $R_{3'} = -OCH_3$
**42** $R_7 = R_{3'} = -OCH_3$

**43** $R_1 = -CH_3$; $R_2 = R_3 = -CH_2OH$
**44** $R_1 = R_3 = -CH_3$; $R_2 = -OH$
**45** $R_1 = R_2 = -CH_2OAc$; $R_3 = -CH_3$
**46** $R_1 = -CH_2OH$; $R_2 = R_3 = -CH_3$
**47** $R_1 = R_3 = -CH_3$; $R_2 = -CH_2OH$
**48** $R_1 = R_2 = R_3 = -CH_3$

**49** $R_1 = -CH_2OH$; $R_2 = -CH_3$
**50** $R_1 = -CH_3$; $R_2 = -CH_2OH$

**51**

**Figure 26: Flavonoids and diterpenes isolated from *B. tola* species.**

#### I.1.6.2.3 *Senecio* species

*Senecio* species belonging to the tribe Senecioneae, are small resinous shrubs (20-50 cm) with strong odour, found in Altiplano between 3,600 – 4,800 m of altitude, with vernacular name of "chachakoma" (chachakuma, male or female chachakoma, donkey chachakoma). Several therapeutic purposes are reported for *Senecio* species, for example the treatment of mountain sickness by intake of infusion ade of aerial parts [35,36,63].

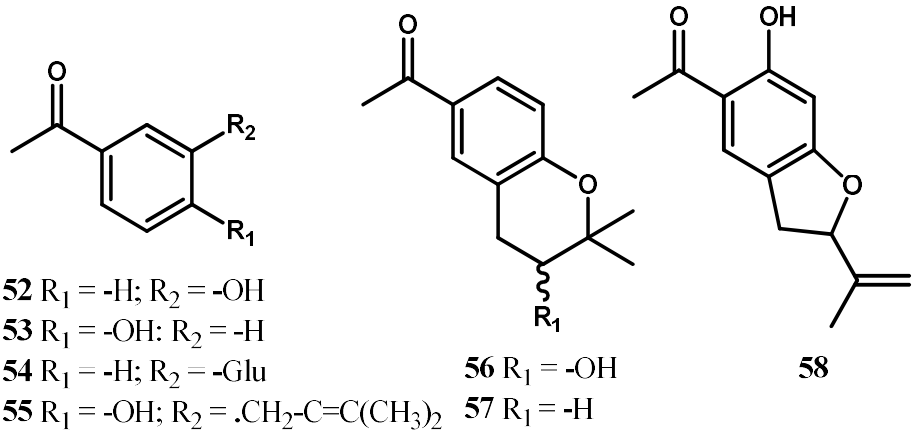


**Figure 27: *Senecio nutans* Sch. Bip. from a) Chile and b) Argentina [34,49].**

*S. nutans* Sch. Bip. (syn. *Senecio graveolens* Wedd., *Senecio graveolens* Wedd. var. psiloachaenius, *Senecio psychrophilus* Wedd.) is the most popular. The infusions are prescribed to treat digestive disorders, urinary infections, mountain sickness, fever and cough. Cataplasm are used to heal muscular pains [36].

Essential oils of aerial part of *S. nutans* were studied, reporting sabanine, α-phellandrene, cymene isomers and α-pinene as main constituents. The essential oil showed weak antifungal activity against toxigenic Fusarium species (*F. graminearum* and *F. verticillioides*) with MICs values of 1.20 mg/mL [64]. The antibacterial activity of *S. nutans* essential oil, collected in Peru showed MICs values of 100-50 µg/mL against Gram-positive strains (*Staphylococcus* spp. *Bacillus subtilis* and *Enterococcus faecalis*) [65]. Specimens collected in Chile (Antofagasta Region) contained methyl cinnamate and *p*-cymenol as main constituents and presented antibacterial activity against *Vibrio cholera* pathogen strain with MIC of 0.4 mg/mL [66].

Hydroalcoholic extract of aerial parts at 40 mg/Kg induced a 30 % and 12 % reduction of the mean arterial pressure in normotensive and hypertensive mice, respectively. It also decreased the sinus rhythm in isolated right atrium of rat [67]. In addition, isolated compounds **52**-**58** were evaluated for their vasodilation properties toward a phenylephrine pre-constricted model. The $EC_{50}$ of relaxation were 3.38 µM, 10.0 µM and 203 µg/mL for acetophenones **55**, **58** and hydroalcoholic extract respectively [68]. Furthermore, **55** showed MICs values of 80-40 µg/mL against *Candida albicans* strains with synergist effects with fluconazole [69]. Finally, antioxidant parameters were also reported in several works of hydroalcoholic and aqueous extracts collected in Chile and Argentina [70,71].




**52** $R_1$ = -H; $R_2$ = -OH
**53** $R_1$ = -OH: $R_2$ = -H
**54** $R_1$ = -H; $R_2$ = -Glu
**55** $R_1$ = -OH; $R_2$ = .$CH_2$-C=C($CH_3$)$_2$
**56** $R_1$ = -OH
**57** $R_1$ = -H
**58**

**Figure 28: Acetophenones from hydroalcoholic extract of *S. nutans*.**

## I.1.6.3 Ephedraceae family

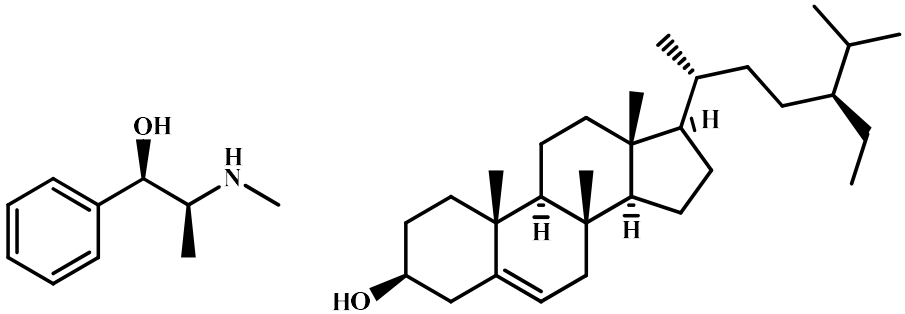
### I.1.6.3.1 *Ephedra* species.

In the Altiplano region is possible to find at least three *Ephedra* species, *E. americana* Humb. & Bonpl. Ex Willd. (syn. *Ephedra breana* Phil., *Ephedra wraithiana* I.M. Johnst., *Ephedra americana* Humb. & Bonpl. ex Willd. var. humboldtii, *Ephedra americana* Humb. & Bonpl. ex Willd. var. humboldtii), *E. chilensis* C. Presl (syn. *Ephedra frustillata* Miers, *Ephedra rupestris* Benth., *Ephedra americana* Humb. & Bonpl. ex Willd. var. andina, *Ephedra americana* Humb. & Bonpl. ex Willd. var. rupestris, *Ephedra andina* Poepp. ex C.A.Mey., *Ephedra andina* Poepp. ex C.A.Mey. f. abbreviata, *Ephedra araucana* Phil., *Ephedra bracteata* Miers, *Ephedra dumosa* Miers, *Ephedra monticola* Miers, *Ephedra nana* Dusén, *Ephedra patagonica* Phil., *Ephedra chilense* Miers, hom. illeg., *Ephedra humilis* Wedd., *Ephedra andina* Poepp. ex C.A.Mey. var. humilis) and *E. multiflora* Phil. ex Stapf (syn. *E. multiflora* Phil.) with the vernacular names "pingo-pingo" (pinko pinko, granada, tumetume), which means "healthy-healthy" in aymara. These shrubs of 20 cm – 1 m of height, presents in all north of Chile between 500 – 4,300 m of altitude [72] are used to treat digestive disorders, bone pains, menstrual pain, kidney dysfunctions, cold, anaemia, urinary infections, rheumatism and gonorrhoea [36].




**Figure 29: *E. americana* [34].**

Hydroalcoholic extract of *E. multiflora* showed inhibition properties of lipoxygenase enzyme with an $IC_{50}$ of 132 µg/mL. LC-$MS^2$ analysis allowed the detection of quercetin glucoside derivatives [73]. In addition, 100 mg of same extract were charged in a micro disk and showed antibacterial properties against Gram-positive strains (*Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus subtilus*, *Acinetobacter baumannii*) in a diffusion disk assay with inhibition halos of 20-6 mm. Ephedrine and β-sitosterol were isolated but not antibacterial activity were observed for these compounds in the same assay with 10 g per disk [74].




**59** **60**

**Figure 30: Isolated compounds from *E. multiflora* collected in Chile.**

## I.1.6.4 Krameriaceae

#### I.1.6.4.1 *Krameria lappacea*

*K. lappacea* (Dombey) Burdet & B. B. Simpson (syn. *Landia lappacea* Dombey, *Krameria triandra* Ruiz & Pav. var. humboldtiana, *Krameria iluca* Phil., *Krameria pentapetala* Ruiz & Pav.) is a shrub, 50-80 cm of height with branchy roots, brown to red thick cortex. It is present between 900-4,000 m of altitude in the Andes of Chile and Peru. The vernacular names are principally chaka-chaka, tikara and chipichapi. The infusion of roots of *K. lappacea* is used to heal urinary infections, gonorrhoea and to treat kidney and bladder dysfunctions.




**Figure 31: *K. lappacea* [34].**

The astringent properties of polar extracts, containing proanthocyanidins derivatives were reported [75]. A dichloromethane extract showed topical antiedematous properties ($ID_{50}$ of 0.31-0.60 µmol/cm$^2$ and 77 µg/cm$^2$) comparable to those of indomethacin (0.29 µmol/cm$^2$) in a mouse ear *in vivo* model. Lignan derivatives **61-75** were then isolated and evaluated for inhibition of enzymes involved in several pro-inflammatory pathways. Compounds **61** and **62** showed the lowest $IC_{50}$ (1-15 µM) against NF-κB, COX1-2, mPGES-1 and 5-LO [76]. In addition, **61**, **63-65**, **69-70** and **74-75** have demonstrated antibacterial activity by disk diffusion method at concentration of 200 µg/per disk, presenting inhibition halos of 10-17 mm against *B. subtilus*, *B. cereus* and *Escherichia coli* [77]. Ethanolic extract of roots showed *in vitro* antiproliferative effect with $IC_{50}$ of 33.5, 33.6 and 89.7 against Hep3B, HepG2 and PLC PFR 5 human hepatocarcinoma cell lines [78].

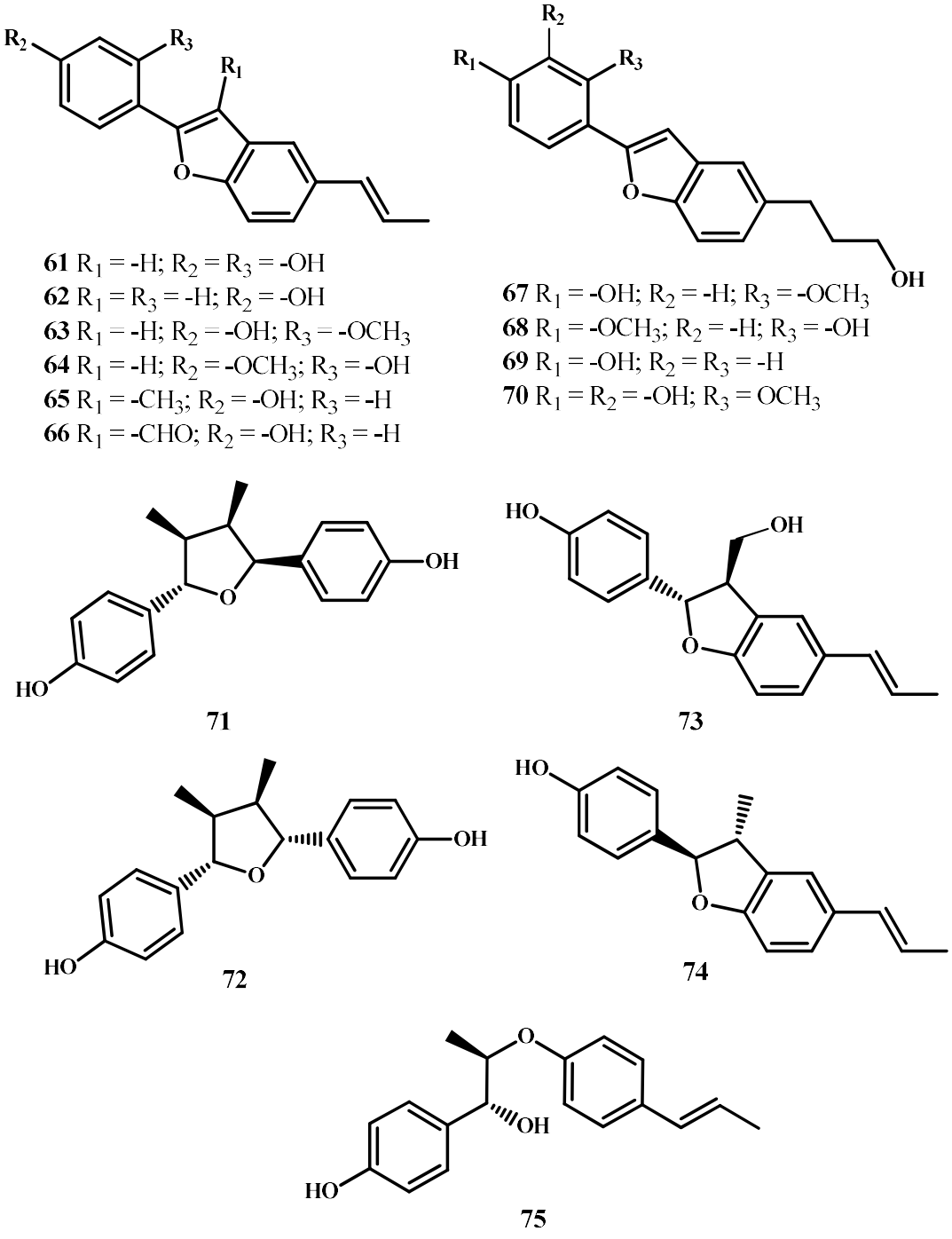



**Figure 32: Isolated compounds from *K. lappacea*.**

## I.1.6.7 Verbenaceae family

The Verbenaceae family, comprising trees, shrubs and herbs, is widely spread over the Altiplano region as in the rest of Chile.

**I.1.6.7.1** ***Aloysia deserticola***

*Aloysia deserticola* (Phil.) Lu-Irving & O'Leary, ex *Acantholippia deserticola* Phil. (syn. *Acantholippia punensis* Botta, nom. superfl., *Lippia deserticola* Phil., *Lippia microphylla* Phil., nom. illeg.) [37,38,79], is a shrub 0.4-1.5 m tall, with spiny branches, small leaves and lilac flowers. It is reported as the medicinal herb "rica-rica" whitin Atacama People communities and the urban areas as Antofagasta, Iquique and Calama. Infusions of leaves and stems are used to treat urinary infections, stomachaches, fever, cold, cough, heart, circulatory and kidney disorders [36]. Infusions of aerial parts of *A. deserticola* and *S. nutans* are prescribed to treat digestive disorders [35]. The leaves are also used as flavouring in dishes and drinks in the traditional Atacama People gastronomy and in some restaurants in Antofagasta, Calama and San Pedro.

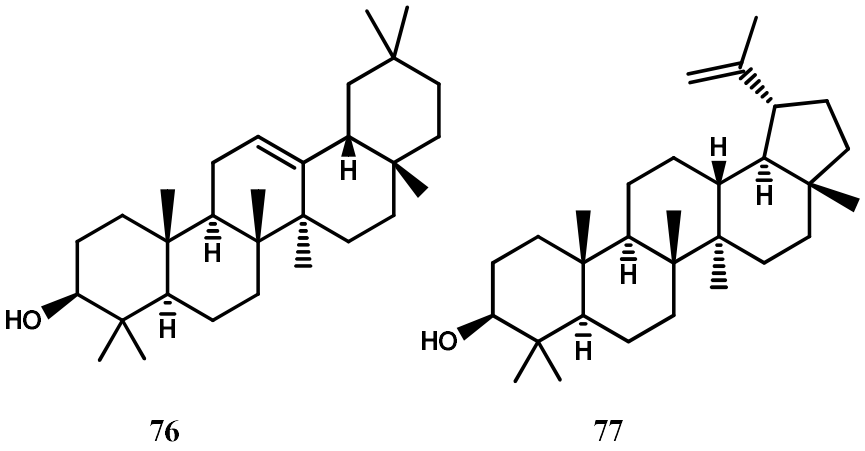



**Figure 33:** ***A. deserticola*** **[34].**

The essential oil of aerial parts mainly contains α- and β-thujone isomers (50-90%) [80,81]. *trans*-Sabinyl acetate and sabinene were also detected (5-10%). No antibacterial or antifungal activity were observable against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* pathogens strains [81]. Nevertheless, mortality of agricultural pests such as *Aleurothrixus floccosus* (97 % after 7 days), *Brevipalpus chilensis* and *Tetranychus urticae* (82 and 89 % after 48 h) was detected [80].

A hydroalcoholic extract showed antioxidant properties *in vivo*, protecting non-enzymatic lipid peroxidation in microsomal membranes of rat, induced by and $Fe^{+2}$-ascorbate system at 100 - 20 mg/mL of concentration [82]. Chloroformic and hydroalcoholic produced inhibition halos of 6 - 10 mm against *Staphylococcus aureus* and *Bacillus subtilus* at 100

mg of extract per disk. β-Amyrin and lupeol were isolated from the extract but did not show any antibacterial activity.




76 77

**Figure 34: Triterpenes from apolar extract of *A. deserticola*.**

#### I.1.6.7.2 *Lampayo medicinalis*

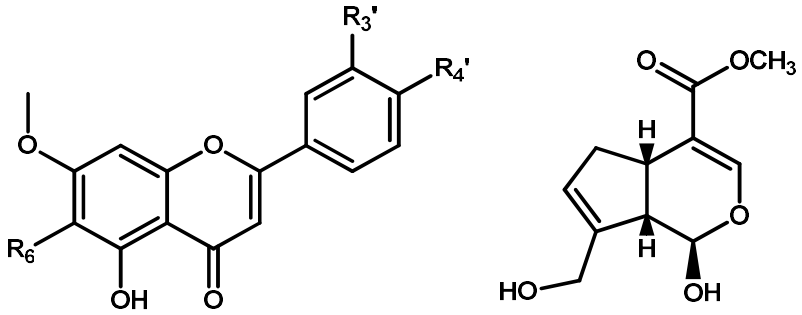
*L. medicinalis* F. Phil. is a dense robust bush, up to 2 m high with thick, coriaceous leaves, flowers of 1.4 – 1.5 cm almost whites in the base and lilac towards limb. The vernacular name in the zone is lampaya or female lampaya. Infusions of aerial parts are prescribed in case of bladder, dysentery, diarrhoea and digestive disorders in general. Baths relieve muscular pain and rheumatism.




**Figure 35: *L. medicinalis* [34].**

Hydroalcoholic extract of *L. medicinalis* showed anti-inflammatory potential in the reduction of oedema paw rat carrageenan-induced model. Reduction of 26-11 % of inhibition at 300-37.5 mg/kg of hydroalcoholic extract [83]. Antioxidant activity was also

measured by different *in vitro* assays (DPPH, ABTS, FRAP, TPC, TFE and reduction potential of phospomolybdate and hexacyanferrate methods) for several extracts. The hydroalcoholic extract showed the more promising results and was subjected to isolation of its main metabolites: flavonoids **78**-**81**, acetophenones **52**, **54** and iridoid **82**. However, their antioxidant activity were not evaluated in this study.




**78** $R_6 = R_{3'} = -H$; $R_{4'} = -OCH_3$
**79** $R_6 = -H$; $R_{3'} = R_{4'} = -OCH_3$
**80** $R_6 = -H$; $R_{3'} = -OCH_3$; $R_{4'} = -OH$
**81** $R_6 = R_{3'} = -OCH_3$; $R_{4'} = -OH$

**82**

**Figure 36: Compounds isolated from hydroalcoholic extract of *L. medicinalis*.**

## I.1.7 Collaborative works between local universities with Atacama People communities

To our point of view, the chemical studies and biological evaluations cited in this document related to the traditional Atacama People medicine, published in national and international journals since 80 years are lacking of interactions between the universities or research institutes with the Atacama communities.

Generally, there are no mentions of ethnobotanical informations nor acknowledgements to the communities, despite the fact that these investigations were based on their culture.

In return, most of the ethnobotanical data available are the results of several anthropologist groups from Universidad Arturo Pratt (Iquique, Chile) and Universidad de Chile (Santiago, Chile), which had performed interviews during the late 1990's in order to classify, categorize and record the plant species growing in the territory with their practical uses (medicinal, foliage, ornamentation,

ceremonial, etc...) [31,32,35,36] but not ethnopharmacological and phytochemical studies are reported from these groups.

In this context, our goal was to bridge the gap between ethnobotanical, phytochemical and biological fields, with establishment of a strong cooperation with the communities.

# I.2 Personal work and collaborations

In the framework of this PhD, a collaborative approach has been established between the Atacama Community of Taira and the team Natural Products, Analysis, Synthesis (ex-Laboratory of Pharmacognosy) from UMR – CNRS 8038 CiTCoM, Paris Descartes University.

## I.2.1 Prospective work and Collection of vegetal material

Since April 2015, a fluent exchange took place via e-mail and telephone with Félix Galleguillos Aymani. Félix and his family are active members of the Atacama Community of Taira, localized near Calama (Antofagasta Region, Chile). Aim of the first discussions was the description of the project and of the prospective preliminary work before collecting ethnobotanical information of currents medicinal plants present and used into the community.

Conversations and interviews with several people have resulted in the organization of two periods of collection of medicinal plants with the assistance and supervision of representatives of Taira Community inside their territory (September 11-13, 2015 and September 9-11, 2016).

Twenty vegetal samples from 18 different species were collected. The parts of plants refered to the indication of the traditional medicine. The community supervised the quantity of collected material, not exceeding 700 g in any case. Voucher specimens of each species were left both in the CONC Herbarium from Universidad de Concepción (Concepción, Chile) and in the Herbarium of NPAS (**Table 5**). Protocols for the vegetal material transfer were signed with the corresponding authority of Chile (**Figure 39**).

The combination of the analysis of previous ethnobotanical reports [32,36,84] with data from the Taira Community before and during the collection periods are listed in **Table 6**.

### I.2.1.1 General objectives

The general and specific objectives and work schema (**Figure 40**) of this collaborative project were delimited in cooperation between NPAS with the Taira Community:

- Investigation of extracts and isolated compounds in basis of their therapeutic uses (antiinfectious, anti-inflammatory, and cytotoxic activities) of collected plants.

- Exploration of extracts and isolated compounds of new therapeutic and cosmetic potential of collected plants.

As a first step, a library of extracts was established. Several extractive procedure were applied such as pressurized solvent extraction (PSE), maceration, supercritical fluid extraction (SFE) and hydrodistillation. Specific protocols are described in each methodology sections.

In order to achieve the objectives, specific task were determined and organized under the different biological activities measured:

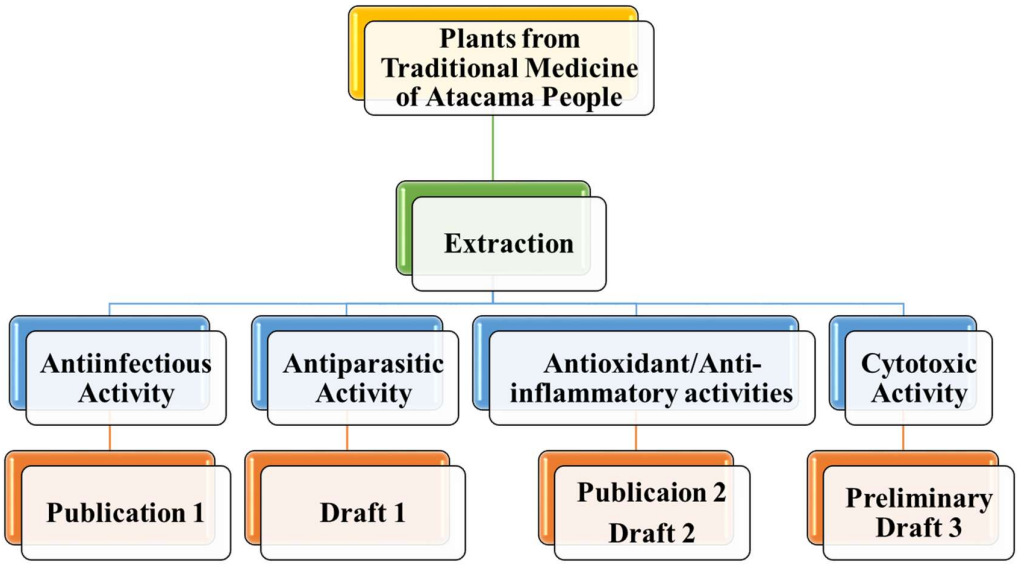



**Figure 37: General organization of the investigation of medicinal plants from North of Chile with the corresponding publication published/proposed.**

### I.2.1.2 Specific objectives – Screening of antiinfectious activities

- *In vitro* antibacterial screening against referential Gram-negative and –positive bacteria of extracts of medicinal plants.
- *In vitro* antifungal screening against referential three fungal pathogen of extracts at 100 µg/mL.
- Determination of the minimal inhibitory concentrations (MICs) of active extracts against reference and clinical isolated bacterial or fungal species.

- Fractionation and *bio*-guided isolation of bioactive compounds from the most active extracts.
- MICs measurement of isolated compounds against referential and clinical isolated bacteria.

#### I.2.1.3 Specific objectives – Screening of anti-inflammatory activity

- Chemical composition analysis of essential oils by GC-FID and GC-MS.
- *In vitro* antioxidant activity of extracts and essential oils measured by free radical scavenging (DPPH), quantification of antioxidant equivalent species (polyphenol, flavonoids, flavon-3-ol) and ferric reducing potential.
- Enzymatic *in vitro* inhibition of ROS-production xanthine oxidase. Measurement of $IC_{50}$ values for the most active samples.
- *In vitro* inhibition by extracts of pro-inflammatory cytokines of LPS-stimulated 264.7 macrophages cells.
- Isolation of metabolites from the most active extracts and biological evaluation of pure compounds.

#### I.2.1.4 Specific objectives – Screening of cytotoxic activity

- *In vitro* antiproliferative activity of extracts against selected cancer cell lines, related to the therapeutic uses of plants at 25 µg/mL. Measurement of $IC_{50}$ values of the most active extracts.
- Fractionation of the most active extract, *in vitro* biological evaluation and $^{1}$H-NMR analysis of obtained fractions.
- Heterocovariance analysis of fractions in order to identify the bioacives compounds present in the extract.

#### I.2.1.5 Specific objectives – Screening of antiparasitic activity

- *In vitro* antiparasitic activity against *Plasmodium falciparum*, *Trypanosoma brucei brucei* and *Leishmania* spp. (*L. amazonensis*, *L. infantum* and *L. donovani*) of extracts. Measurement of $IC_{50}$ for more active samples were performed.
- Fractionation and *bio*-guided isolation of metabolites from the most active extract against *P. falciparum*. Biological evaluation of pure compounds and $IC_{50}$ determination.

In order to address all these task, several collaboration were set up with laboratories in France, Greece and Germany as described below:

| Task | Responsable | Institution |
|---|---|---|
| Antibacterial activity | Dr Marylin Lecsö-Bornet | Laboratoire Ecosystème Intestinal, Probiotiques, Antibiotiques-EA 4065, Université Paris Descartes, France. |
| Antifungal activity | Dr Sandrine Houze | Laboratoire de Parasitologie-Mycologie, Hôpital Bichat Claude Bernard, France. |
| Antiplasmodial activity | Dr Sandrine Houze | Centre National du Paludisme, Hôpital Bichat Claude Bernard, France. |
| Antitrypanosomal and antileishmanial activities | Dr.Antonia Efstathiou<br>Dr. Despoina Smyrlis | Laboratory of Molecular Parasitology, Hellenic Pasteur Institute, Greece. |
| Anti-inflammatory activity | Dr. Thorsten Rose<br>Dr. Bernd Fiebich | Vivacell Biotechnology, Germany. |
| Cytotoxic activity | Dr. Roxane Tenta | Department of Nutrition & Dietetics, Harokopio University, Greece. |
| Antioxidant activity, Enzymatic inhibition assays, Supercritic fluid extraction, HPTLC analysis, Heterocovariance analysis. | Dr. Nektarios Aligiannis | Departmetn of Pharmacognosy and Natural Prodcuts Chemistry, University of Athens, Greece. |

**Table 4: Collaboration set up for the investigation of medicinal plants from north of Chile.**

| Family | Scientific Name | Vernacular Name | CONC Code | UPD Code | GPS | Altitude (m) |
|---|---|---|---|---|---|---|
| Apiaceae | *Azorella atacamensis* G.M. Plunkett & A.N. Nicolas | chuquicandia | 185885 | MKOSB092015T2A04 | 21°44'43.546" S 68°38'0.451" W | 3254 |
| Asteraceae | *Artemisia copa* Phil. var. copa | copa-copa | 185872-a | MKOSB092015T1B01 | 21°44'35.653" S 68°38'3.476 W | 3255 |
| | | | 185872-b | MKOSB092015T2C03 | 21°41'57.775" S 68°46'23.255" W | 3635 |
| | *Baccharis calliprinos* Griseb. | chilca de cerro | 185873-a | MKOSB092015T2B02 | 21°51'30.785" S 68°38'25.015" W | 3433 |
| | | chilca de río | 185873-b | MKOSB092015T2F01 | 21°56'2.576" S 68°36'40.963" W | 3047 |
| | *Baccharis grisebachii* Hieron. | quinchimaldi | 185874 | MKOSB092015T2C01 | 21°42'33.948" S 68°45'44.013" W | 3570 |
| | *Baccharis tola* Phil. ssp tola | lejía | 185875 | MKOSB092015T2A03 | 21°44'48.768" S 68°38'8.948" W | 3244 |
| | *Chuquiraga atacamensis* Kuntze | candela | 185876 | MKOSB092015T2D01 | 21°40'38.531" S 68°40'39.885" W | 3739 |
| | *Haplopappus rigidus* Phil. | bailahuen | 185881 | MKOSB092015T2C02 | 21°41'53.756" S 68°46'26.963" W | 3650 |
| | *Parastrephia teretiuscula* (Kuntze) Cabrera | coba santiago | 185886 | MKOSB092015T2E02 | 21°38'12.644" S 68°50'37.863" W | 4215 |
| | *Senecio nutans* Sch. Bip. | chachacoma | 185888 | MKOSB092015T2E01 | 21°38'12.644" S 68°50'44.661" W | 4202 |
| Ephedraceae | *Ephedra americana* Humb. & Bonpl. ex Willd. | pingo-pingo | 185878 | MKOSB092015T1C01 | 21°40'28.768" S 68°43'11.063" W | 3495 |
| Krameriaceae | *Krameria lappacea* (Dombey) Burdet & B. B. Simpson | chaca-chaca | 185883 | MKOSB092015T2B01 | 21°51'36.09" S 68°38'16.673" W | 3408 |
| Poaceae | *Cortaderia speciosa* (Nees & Meyen) Stapf | cortadera | 185877 | MKOSB092015T2F02 | 21°56'5.729" S 68°36'43.126" W | 3040 |
| Rosaceae | *Polylepis tarapacana* Phil. | queñua | 185887 | MKOSB092015T2A05 | 21°44'34.074" S 68°37'55.236" W | 3258 |
| Solanaceae | *Fabiana denudata* Miers | tara | 185879 | MKOSB092015T2A09 | 21°44'44.407" S 68°38'55.721" W | 3281 |
| Solanaceae | *Fabiana squamata* Phil. | coba santiago | 185880 | MKOSB092015T2A08 | 21°43'57.193" S 68°37'38.165" W | 3271 |

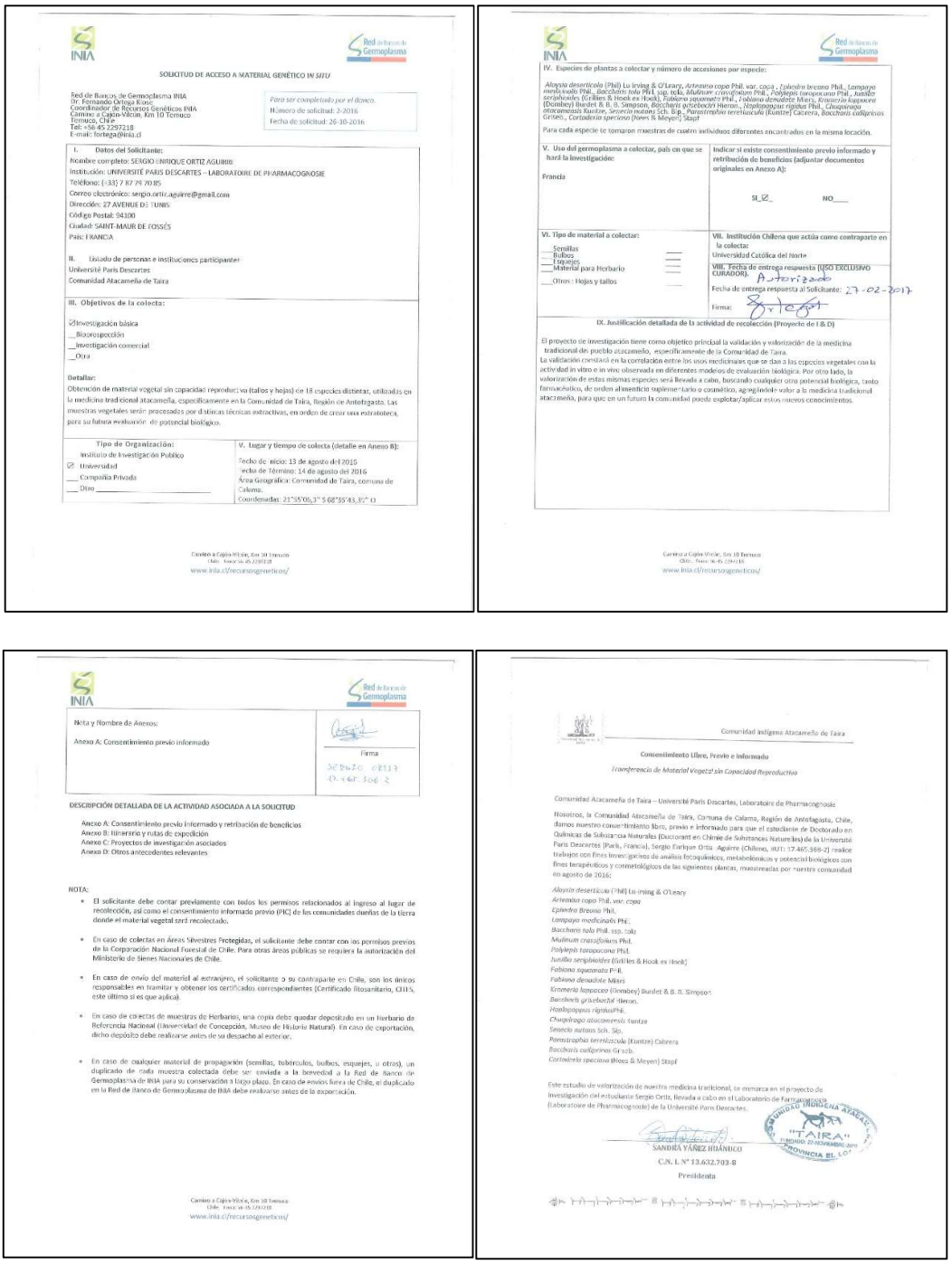
| | | | | | | |
|---|---|---|---|---|---|---|
| Verbenaceae | *Aloysia deserticola* (Phil.) Lu-Irving & O'Leary | rica-rica | 185871-a | MKOSB092015T1A01 | 21°40'57.092" S 68°38'5.163" W | 3248 |
| | | | 185871-b | MKOSB092015T1A02 | 21°40'23.4" S 68°40'02.0" W | 3319 |
| | | | 185871-c | MKOSB092015T1A03 | 21°58'42.9" S 68°37'30.7" W | 3076 |
| | | | 185871-d | MKOSB092015T1A04 | 21°54'22.5" S 68°35'51.1" W | 2946 |
| | *Junellia seriphioides* (Guillies & Hook. ex Hook.) Moldonke | Huira Huira | 185882 | MKOSB092015T2A07 | 21°44'12.692" S 68°37'41.254" W | 3265 |
| | *Lampayo medicinalis* Phil. | lampaya | 185884 | MKOSB092015T2A02 | 21°44'48.281" S 68°38°8.948" W | 3242 |

**Table 5: Collected plant samples inside the Taira community in September 2015 and 2016.**

| Plant Species | Medicinal uses reported in the literature and/or in the Taira Community |
|---|---|
| *Aloysia deserticola* (Phil.) Lu-Irving & O'Leary | Common cold, cough, fever, urinary tract infection, stomach pain, pain, kidney, antidepressant, heart, vomiting, diarrhoea, milk production. |
| *Artemisa copa* Phil. var. copa | Stomach pain, pain, bones, diarrhoea. |
| *Azorella atacamensis* G.M. Plunkett & A.N. Nicolas | Common cold, cough, pain, relaxing, diabetes. |
| *Baccharis calliprinos* Griseb. | Mal de aire, anti-inflammatory, bones |
| *Baccharis grisebachii* Hieron. | Stomach pain, anti-inflammatory, bones |
| *Baccharis tola* Phil. ssp tola | Common cold, cough, stomach pain, pain, bones, rheumatism, dysentery |
| *Chuquiraga atacamensis* Kuntze | Common cold, cough, fever, urinary tract infection, abortifacient, control menstrual cycle, stomach pain, pain, urinary bladder, ovaries pain. |
| *Cortaderia speciosa* (Nees & Meyen) Stapf | Common cold, cough, fever, pain, ovaries pain. |
| *Ephedra americana* Humb. & Bonpl. ex Willd. | Common cold, stomach pain, bonesrheumatism, urinary bladder, kidney, prostate. |
| *Fabiana denudata* Miers | Common cold, cough, stomach pain, anti-inflammatory, bones, dysentery. |
| *Fabiana squamata* Phil. | Stomach pain, anti-inflammatory, bones, dysentery. |

| | |
|---|---|
| *Haplopappus rigidus* Phil. | Common cold, cough, allergy, urinary tract infection, aphrodisiac, stomach pain, rheumatism, urinary bladder, kidney, liver, heart, ovaries pain. |
| *Junellia seriphioides* (Guillies & Hook. ex Hook.) Moldonke | Common cold, cough, fever, urinary tract infection, stomach pain, mal de aire, pain, bones, urinary bladder. |
| *Krameria lappacea* (Dombey) Burdet & B. B. Simpson | Common cold, urinary tract infection, urinary bladder, kidney, gonorrhoea. |
| *Lampayo medicinalis* Phil. | Common cold, cough, stomach pain, pain, bones, rheumatism, urinary bladder, kidney, prostate, dysentery, ovaries pain, diarrhoea. |
| *Parastrephia teretiuscula* (Kuntze) Cabrera | Stomach pain, bones |
| *Polylepis tarapacana* Phil. | Cough, urinary tract infection, kidney, mountain sickness, heart. |
| *Senecio nutans* Sch. Bip. | Common cold, cough, fever, urinary tract infection, stomach pain, pain, mountain sickness. |

**Table 6: Medicinal uses reported in the literature and the current uses in the Taira Community of collected samples.**



SOLICITUD DE ACCESO A MATERIAL GENÉTICO *IN SITU*

Red de Bancos de Germoplasma INIA
Dr. Fernando Ortega Klose
Coordinador de Recursos Genéticos INIA
Camino a Cajón-Vilcún, Km 10 Temuco
Temuco, Chile
Tel: +56 45 2297218
E-mail: fortega@inia.cl

*Para ser completado por el Banco.*
Número de solicitud: 2-2016
Fecha de solicitud: 26-10-2016

I. Datos del Solicitante:
Nombre completo: SERGIO ENRIQUE ORTIZ AGUIRRE
Institución: UNIVERSITÉ PARIS DESCARTES – LABORATOIRE DE PHARMACOGNOSIE
Teléfono: (+33) 7 87 75 70 85
Correo electrónico: sergio.ortiz.aguirre@gmail.com
Dirección: 27 AVENUE DE TUNIS
Código Postal: 94100
Ciudad: SAINT-MAUR DE FOSSÉS
País: FRANCIA

II. Listado de personas e instituciones participantes:
Université Paris Descartes
Comunidad Atacameña de Taira

III. Objetivos de la colecta:
☑Investigación básica
__Bioprospección
__Investigación comercial
__Otra

Detallar:
Obtención de material vegetal sin capacidad reproductiva (tallos y hojas) de 18 especies distintas, utilizadas en la medicina tradicional atacameña, específicamente en la Comunidad de Taira, Región de Antofagasta. Las muestras vegetales serán procesadas por distintas técnicas extractivas, en orden de crear una estratoteca, para su futura evaluación de potencial biológico.

Tipo de Organización:
Instituto de Investigación Público
☑ Universidad
___ Compañía Privada
___ Otro _____

V. Lugar y tiempo de colecta (detalle en Anexo B):
Fecha de inicio: 13 de agosto del 2016
Fecha de Término: 14 de agosto del 2016
Área Geográfica: Comunidad de Taira, comuna de Calama.
Coordenadas: 21°55'06,3" S 68°35'43,3" O

Camino a Cajón-Vilcún, Km 10 Temuco
Chile - Fono: 56-45-2297218
www.inia.cl/recursosgeneticos/

IV. Especies de plantas a colectar y número de accesiones por especie:

*Alonsoa deserticola* (Phil) Lu-Irving & O'Leary, *Artemisia copa* Phil. var. *copa* , *Ephedra breana* Phil., *Lampaya medicinalis* Phil., *Baccharis tola* Phil. ssp. *tola*, *Mulinum crassifolium* Phil., *Polylepis tarapacana* Phil., *Junellia seriphioides* (Gillies & Hook ex Hook), *Fabiana squamata* Phil., *Fabiana denudata* Miers, *Krameria lappacea* (Dombey) Burdet & B. B. Simpson, *Baccharis grisebachii* Hieron., *Haplopappus rigidus* Phil., *Chuquiraga atacamensis* Kuntze, *Senecio nutans* Sch. Bip., *Parastrephia teretiuscula* (Kuntze) Cabrera, *Baccharis calliprinos* Griseb., *Cortaderia speciosa* (Nees & Meyen) Stapf

Para cada especie se tomaron muestras de cuatro individuos diferentes encontrados en la misma locación.

V. Uso del germoplasma a colectar, país en que se hará la investigación:
Francia

Indicar si existe consentimiento previo informado y retribución de beneficios (adjuntar documentos originales en Anexo A):
SI_☑_ NO____

VI. Tipo de material a colectar:
___ Semillas
___ Bulbos
___ Esquejes
___ Material para Herbario
___ Otros: Hojas y tallos

VII. Institución Chilena que actúa como contraparte en la colecta:
Universidad Católica del Norte

VIII. Fecha de entrega respuesta (USO EXCLUSIVO CURADOR):
Autorizado
Fecha de entrega respuesta al Solicitante: 27-02-2017
Firma:

IX. Justificación detallada de la actividad de recolección (Proyecto de I & D)

El proyecto de investigación tiene como objetivo principal la validación y valorización de la medicina tradicional del pueblo atacameño, específicamente de la Comunidad de Taira.
La validación centrará en la correlación entre los usos medicinales que se dan a las especies vegetales con la actividad in vitro e in vivo observada en diferentes modelos de evaluación biológica. Por otro lado, la valorización de estas mismas especies será llevada a cabo, buscando cualquier otro potencial biológico, tanto farmacéutico, de orden alimenticio suplementario o cosmético, agregándole valor a la medicina tradicional atacameña, para que en un futuro la comunidad pueda explotar/aplicar estos nuevos conocimientos.

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Nota y Nombre de Anexos:

Anexo A: Consentimiento previo informado

Firma

DESCRIPCIÓN DETALLADA DE LA ACTIVIDAD ASOCIADA A LA SOLICITUD

Anexo A: Consentimiento previo informado y retribución de beneficios
Anexo B: Itinerario y rutas de expedición
Anexo C: Proyectos de investigación asociados
Anexo D: Otros antecedentes relevantes

NOTA:

- El solicitante debe contar previamente con todos los permisos relacionados al ingreso al lugar de recolección, así como el consentimiento informado previo (PIC) de las comunidades dueñas de la tierra donde el material vegetal será recolectado.
- En caso de colectas en Áreas Silvestres Protegidas, el solicitante debe contar con los permisos previos de la Corporación Nacional Forestal de Chile. Para otras áreas públicas se requiera la autorización del Ministerio de Bienes Nacionales de Chile.
- En caso de envío del material al extranjero, el solicitante o su contraparte en Chile, son los únicos responsables en tramitar y obtener los certificados correspondientes (Certificado fitosanitario, CITES, este último si es que aplica).
- En caso de colectas de muestras de Herbarios, una copia debe quedar depositado en un Herbario de Referencia Nacional (Universidad de Concepción, Museo de Historia Natural). En caso de exportación, dicho depósito debe realizarse antes de su despacho al exterior.
- En caso de cualquier material de propagación (semillas, tubérculos, bulbos, esquejes, u otros), un duplicado de cada muestra colectada debe ser enviada a la bóveda o la Red de Banco de Germoplasma de INIA para su conservación a largo plazo. En caso de envíos fuera de Chile, el duplicado en la Red de Banco de Germoplasma de INIA debe realizarse antes de la exportación.

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Comunidad Indígena Atacameña de Taira

**Consentimiento Libre, Previo e Informado**

*Transferencia de Material Vegetal sin Capacidad Reproductiva*

Comunidad Atacameña de Taira – Université Paris Descartes, Laboratoire de Pharmacognosie

Nosotros, la Comunidad Atacameña de Taira, Comuna de Calama, Región de Antofagasta, Chile, damos nuestro consentimiento libre, previo e informado para que el estudiante de Doctorado en Química de Substancias Naturales (Doctorant en Chimie de Substances Naturelles) de la Université Paris Descartes (Paris, Francia), Sergio Enrique Ortiz Aguirre (Chileno, RUT: 17.465.388-2) realice trabajos con fines investigativos de análisis fitoquímicos, metabolómicos y potencial biológicos con fines terapéuticos y cosmetológicos de las siguientes plantas, muestreadas por nuestra comunidad en agosto de 2016:

*Alonsoa deserticola* (Phil) Lu-Irving & O'Leary
*Artemisia copa* Phil. *var. copa*
*Ephedra Breana* Phil.
*Lampaya medicinalis* Phil.
*Baccharis tola* Phil. ssp. *tola*
*Mulinum crassifolium* Phil.
*Polylepis tarapacana* Phil.
*Junellia seriphioides* (Gillies & Hook ex Hook)
*Fabiana squamata* Phil.
*Fabiana denudata* Miers
*Krameria lappacea* (Dombey) Burdet & B. B. Simpson
*Baccharis grisebachii* Hieron.
*Haplopappus rigidus* Phil.
*Chuquiraga atacamensis* Kuntze
*Senecio nutans* Sch. Bip.
*Parastrephia teretiuscula* (Kuntze) Cabrera
*Baccharis calliprinos* Griseb.
*Cortaderia speciosa* (Nees & Meyen) Stapf

Este estudio de valorización de nuestra medicina tradicional, se enmarca en el proyecto de investigación del estudiante Sergio Ortiz, llevada a cabo en el Laboratorio de Farmacognosia (Laboratoire de Pharmacognosie) de la Université Paris Descartes.

COMUNIDAD INDÍGENA ATACAMEÑA "TAIRA" FUNDADO 22 NOVIEMBRE 1670 PROVINCIA EL LOA

SANDRA YÁÑEZ HUANICO
C.N. I. N° 13.632.703-8
Presidenta

**Figure 38: Material Transfer Agreement, MTA (Solicitud de acceso a material genético *in situ* 2-2016) between the Taira community and the NPAS team.**

# I.3. Antiinfectious activity of medicinal plants from Taira Community

## I.3.1 Multi-resistant bacteria

The struggle of humankind against infectious disease in well known. The discovery of antibiotics like with penicillin in 1928, led to optimism that infections could be controlled and prevented. However, infections are still the leading cause of death in the developing world. From early decades of XX century until nowadays exist several families targeting different bacterial systems as cell wall, nucleic acid and proteins synthesis.

Beta lactams and glycopeptides inhibit cell wall synthesis. Quinolones and fluoroquinolones target bacterial DNA gyrase. Aminoglycosides and tetracycline derivatives act upon the 30S ribosomal subunit, preventing the binding of t-RNA or the translation of mRNA. Macrolide affect the early translocation of protein synthesis, targeting the peptidyl transferase of the 50S ribosomal subunit, resulting in the premature detachment of incomplete peptide chains.

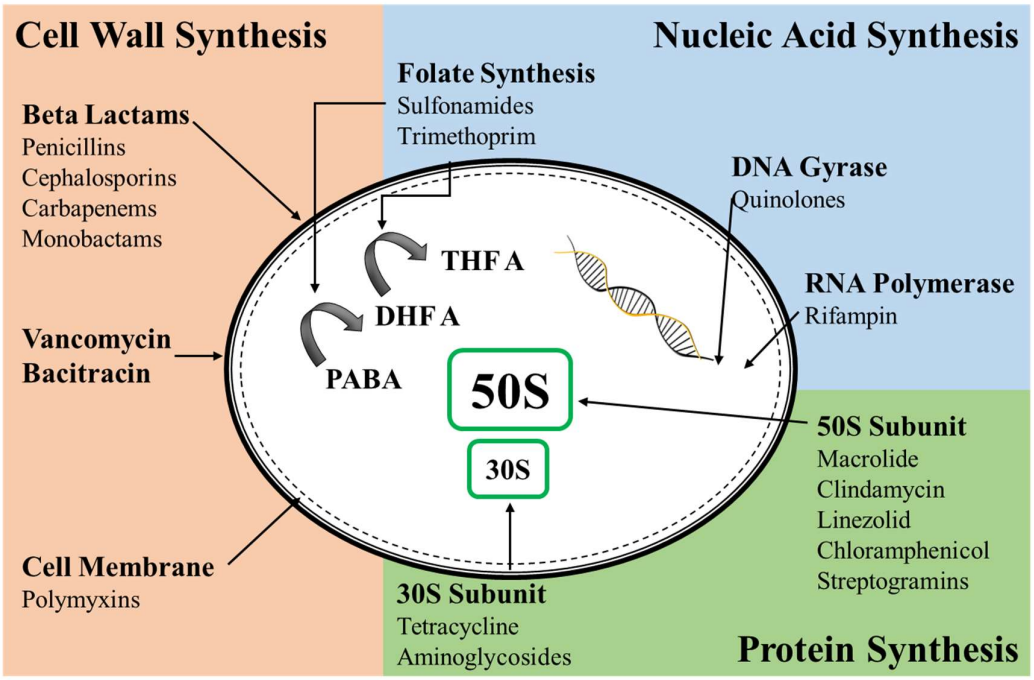


**Figure 39: Mechanisms of action of known antibiotics.**

Increasing demand for antibiotics across many sectors has allowed to decrease their prices. Unfortunately, their irrational use has contributed to increase the natural phenomenon of resistant

strains emergence, occurring via natural gene mutations. The main resistance mechanisms consist in action of efflux pumps able to maintain a low intracellular concentration of antibiotics, alterations of 30S or 50S subunits, structural modifications of cell wall precursors, mutations of DNA gyrase, antibiotic inactivation and variations of antibiotic receptors (like penicillin binding proteins). Thus, there is an urgent need for new antimicrobial agents to fight the high level of multidrug resistance.

Apart from *Baccharis calliprinos*, all of the selected species are used for symptoms which may occur from bacterial or fungal pathogens (**Table 6**, **Figure 49**). Twelve plants are indicated against respiratory system infections (common cold, cough and fever), twelve others to relieve stomachaches or intra-abdominal pain and seven species against urinary infections. Consequently, we have first investigated the antibacterial and antifungal potential of these plants, against pathogens related to respiratory, stomach and/or urinary infections.

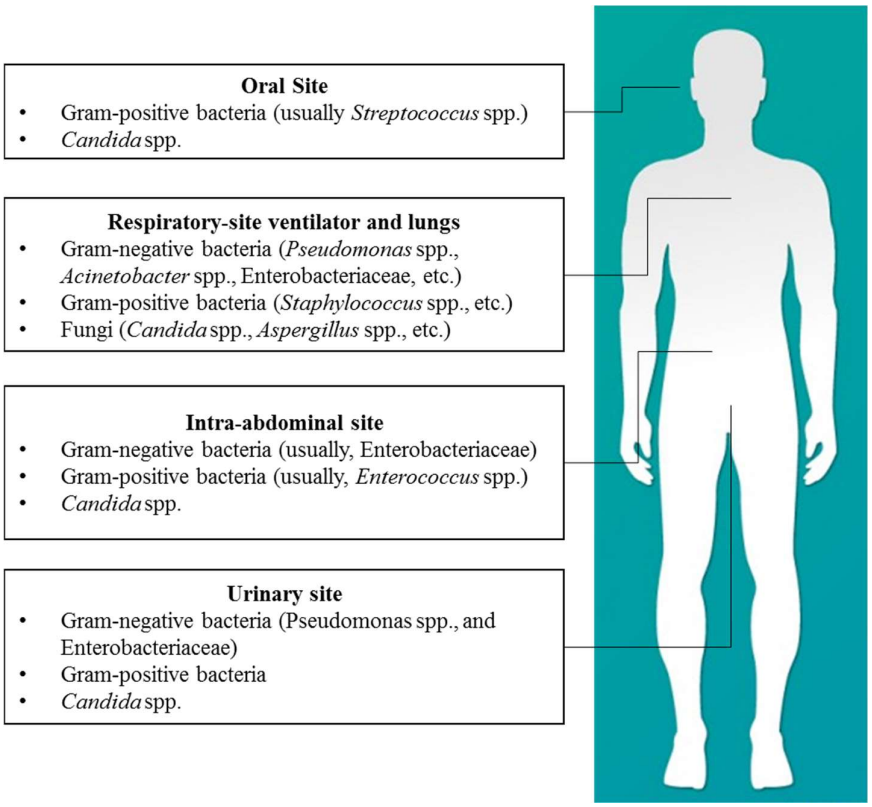


**Figure 40: Types of bacteria or fungi involved in respiratory, oral, intra-abdominal and urinary sites infections.**

## I.3.2 Methodology

The techniques applied for extraction and *in vitro* evaluations of extracts/fractions/isolated compounds are detailed below:

### I.3. 2.1 Extraction (Pressurized Solvent Extraction, PSE)

In order to reduce time and volumes of solvents, Pressurized Solvent Extraction (PSE) using Büchi Speed Extractor E-914, which enables to extract raw material with high pressure and mild to high temperature, was choosen. Ethyl acetate, then methanol, both with a pressure of 100 bar at 50°C were used in order to provide the corresponding extracts.



**Figure 41: Speed Extractor E-914, Büchi, Flawil, Switzerland.**

### I.3.2.2 Antiinfectious activity

#### I.3.2.2.1 Anbacterial activity assay

*In vitro* evaluation was conducted using an agar-dilution method against a panel of forty-one pathogenic reference strains, twenty-five Gram-negative and sixteen Gram-positive (**Figure 42**). Accurate amount of sample (essential oil, extract, fraction or pure compound) and agar medium (Mueller Hinton or Wilkin-Chalgren) were put in a petri dish. After solidification and drying, bacterial suspensions were inoculated with a Steers Inoculator. Plates were then incubated under aerobic conditions for 24 h; afterwards absence of culture growing signifies that the sample possess an antibacterial activity.

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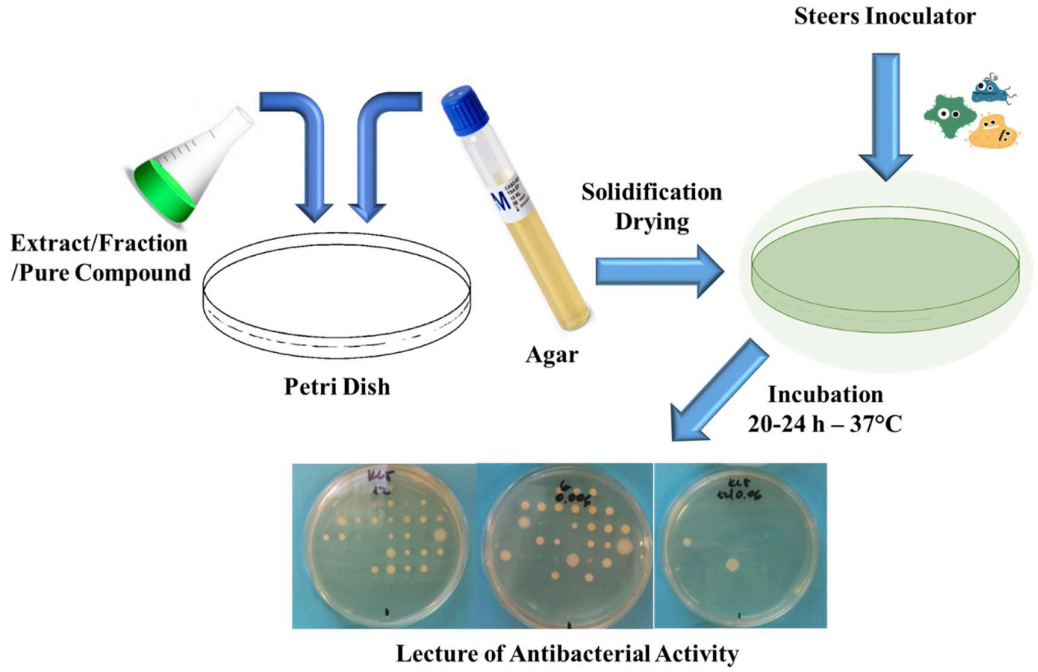


**Figure 42: Workflow of antibacterial agar-dilution method assay.**

#### I.3.2.2.2 Antifungal activity assay

*In vitro* evaluation of the antifungal activity was performed using a micro-dilution method against three fungal reference strains (*Candida albicans*, *Aspergillus fumigatus* and *Trichophyton tonsurans*), following the requiresments of the European Committee of Antimicrobial Susceptibility Testing (EUCAST, European Society of Clinical Microbiology and Infectious Diseases). Briefly, equal volumes of fungal suspension and sample to evaluate, after dilution in RPMI medium, were put in a 96-well plate and incubated in aerobic conditions for 24 h (for *C. albicans*), 48 h (for *A. fumigatus*) and 168 h (for *T. tonsurans*). MICs were recorded for the active extracts.

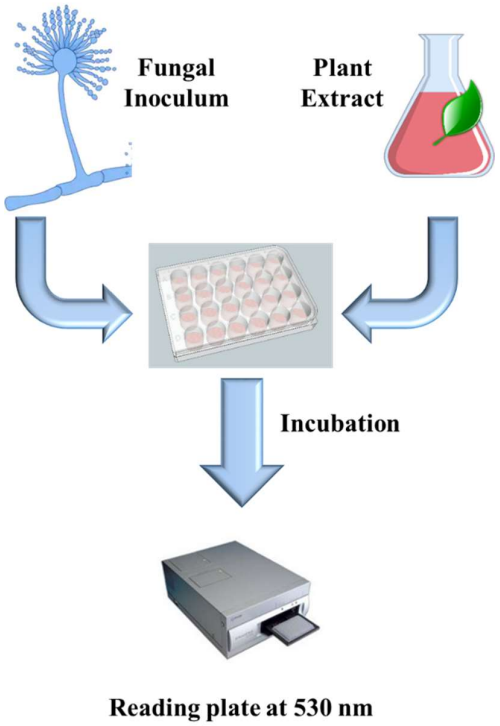


**Figure 43: Workflow on antifungal micro-dilution method assay.**

In addition, discussion of the different ethnobotanical rapport of plant collected was included in this communication.

Gram-positive and –negative representative bacteria were selected for the evaluation due their character pathogenic in pulmonary, digestive and urinary infections. In addition three characteristic fungi pathogen as *Candida albicans*, *Aspergillus fumigatus* and *Trichophyton tonsurans* were also in

Antibacterial and antifungal activities evaluation were established in two steps:

- First screening with a panel of reference bacterial and fungi species was used to evaluate extract samples at elevate concentration.
- Once antibacterial or antifungal activity was observed, minimal inhibitory concentration (MIC) and *bio*-guided isolation of bioactive compounds from more active samples was envisaged against reference and clinical isolated strains species.

The *bio*-guided approach was chosen to investigate the antiinfectious activity of medicina plants and the scheme followed is described below:

- MICs values of active extracts were calculated against a panel of 51 Gram-positive references and clinical isolates strains. MICs values were recorded as a minimal extract concentration where no-growth bacterial colony were observed after incubation.
- Fractionation of more active extracts was performed by silica-gel column chromatography with DCM/MeOH mixture solvents. Collected tubes were resembled by similarity composition (monitored by TLC) in fractions and then evaluated at 50 µg/mL against the same panel of Gram-positive panel.
- Active fractions were purchase to a second silica gel chromatography with gradient of petroleum ether/ethyl acetate or DCM/MeOH mixtures. MICs values of purified compounds from active fractions were evaluated against Gram-positive bacteria strains.
- MICs values against facultative anaerobes *Streptococcus* strains in anaerobic conditions for active extracts and compounds were also performed.

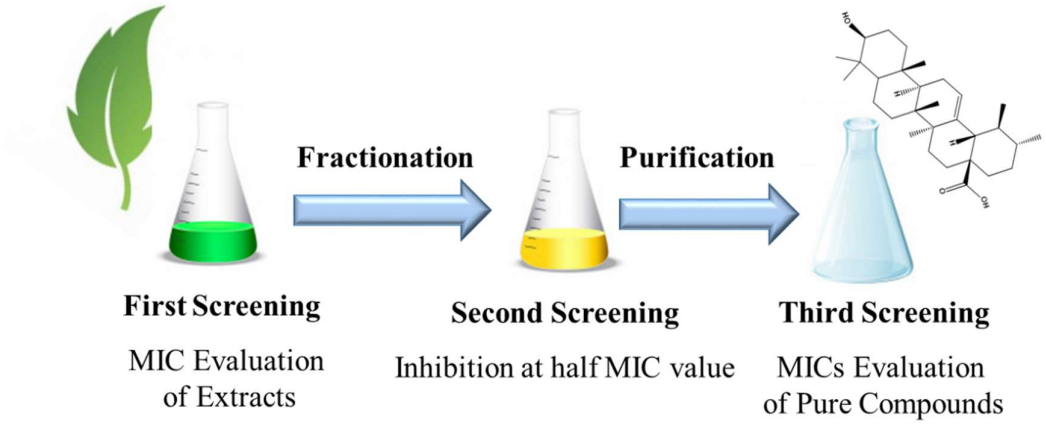


**Figure 44: Workflow of *bio*-guided isolation approach for active antibacterial extracts.**

# I.3.3 Publication 1: *Bio*-guided identification of triterpenoids and neolignans as bioactive compounds from anti-infectious medicinal plants of the Taira Atacama's community (Calama, Chile)

Journal of Ethnopharmacology 231 (2019) 217–229

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

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

## Bioguided identification of triterpenoids and neolignans as bioactive compounds from anti-infectious medicinal plants of the Taira Atacama's community (Calama, Chile)

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[b] Laboratoire Ecosystème Intestinal, Probiotiques, Antibiotiques-EA 4065, Université Paris Descartes Sorbonne Paris Cité, Faculté Sciences Pharmaceutiques et Biologiques, 4 avenue de l'Observatoire, Paris, France
[c] Laboratoire de Parasitologie-Mycologie, Hôpital Bichat Claude Bernard, 46 rue Henri Huchard, Paris 75018, France

A B S T R A C T

*Ethnopharmacological relevance:* Previous ethnobotanical surveys from the north Andean part of Chile, where different ethnic groups are co-existing, with the preeminence of Aymara and Atacama traditions, revealed an extensive domestic use of the local flora. In these communities, traditional medicinal uses are mainly related to the treatment of respiratory, gastro-intestinal and urinary disorders, pain and inflammation, which is closely linked to epidemiological observations.
*Aim of the study:* As these symptoms may be related to infectious diseases, a bioguided evaluation of antibacterial and antifungal activity was conducted on eighteen species selected with the Taira community, in Ollague.
*Materials and methods:* Screening was performed using a large panel of pathogenic germs involved in the main community acquired infectious diseases, represented by Gram positive and Gram negative bacteria of clinical interest and by human pathogenic fungi, using a bioguided approach.
*Results and conclusions:* Gram positive strains of clinical interest were highly sensitive to *Aloysia deserticola* (Verbenaceae) and *Krameria lappacea* (Krameriaceae) extracts. The bioguided approach led us to identify the isolated neolignan from *K. lappacea* conocarpan (1), and triterpenoids form *A. deserticola* (oleanolic acid (6) and ursolic acid (10)), as the main bioactive compounds.

### 1. Introduction

The North Andean part of Chile comprises arid plains, oasis, mountains and valleys located between 2400 and 4000 m of altitude. It is delimited ~~at~~ in the north with the Tarapaca region near Peruvian border, in the east with the Chilean Altiplano nearby the Bolivian-Argentinian border and in the south with the Salar of Atacama (Fig. 1). Despite the presence of the Atacama desert, the most arid desert in the world, the existence of some rivers (El Loa, Lauca, Lluta), summer rains from December to February, and humidity brought by coastal fogs, a rich flora and fauna biodiversity may be found in valleys (Luebert and Pliscoff, 2006).

Colonial and economic mutations have led to co-existence of different ethnic groups, with many intercultural relationships. In the northern Chilean Andes the predominant ethnic group is from Aymara culture, whereas the southern area is occupied by Atacameños with a specific common culture called "Cultura del Desierto" or Likam-Antay (Fig. 1; Villagrán et al., 2003; Tocornal, 2006).

Atacameños represent 0.17% of the total Chilean population with about 30,000 individuals according the last census (Instituto Nacional de estadisticas, 2018). They speak the local Kunza language along with Aymara, Quechua and Spanish. The principal economic areas of Atacameños are agriculture and animal husbandry and these last years, tourism has increased and became an important commercial pole (Villagrán et al., 2003; Tocornal, 2006). Along with other native ethnic groups present in Chile, Atacameños face different forms of racial and social discrimination and encounter the highest rates of unemployment and illiteracy (Merino, 2007; Merino and Quilaqueo, 2003).

Whereas traditional medicine remains extensively employed in all the ethnic groups from North Andean part of Chile, even in the urban

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https://doi.org/10.1016/j.jep.2018.10.029
Received 19 July 2018; Received in revised form 21 October 2018; Accepted 22 October 2018
Available online 06 November 2018
0378-8741/ © 2018 Elsevier B.V. All rights reserved.




**Fig. 1. A-** Collected species in Ollagüe. B- Referential geographic position of different ethnobotanical studies previously published in the North of Chile. **1**- Taira Community (Atacama people) in Ollagüe Municipality, **2**- Socaire Community, **3**- City of Iquique, **4**- Andes area of Tarapaca-Iquique and **5**- Andes area of Antofagasta-Ollagüe.

context of Calama, Antofagasta or Iquique, combined with modern medicine (Madaleno and Delatorre-Herrera, 2013; Tocornal, 2006), few ethnobotanical studies related specifically to Atacameños communities are available. In the oasis of Socaire and towards the Salar of Atacama, among the 200 described species (118 and 173 respectively), about 30% encounter a medicinal use (Baraona et al., 1958; Villagrán et al., 1998). Near Ollagüe, the same proportion is reported; within the 143 collected species between 40 and 60 species are used for medicinal purpose by Atacameños communities (Villagrán et al., 2003; Villagrán and Castro, 2003).

Epidemiological study within the communities living in the Salar of Atacama reports that respiratory troubles such as bronchitis and bronchopneumonia are the predominant diseases, especially during winter where brutal variations of temperature may occur. Secondly, during summer, a high incidence of gastro-intestinal diseases is observed, due to a raise of foodborne infections. At last, osteo-muscular pains represents the third reason for medical consultation (Tocornal, 2006).

It appears that plants properties are generally well-known by inhabitants and a domestic use to treat common disorders such as colds, gastro-intestinal troubles and rheumatisms is widespread (Villagrán and Castro, 2003). Part of the high degree of awareness of the local flora medicinal resources may partly be due to the handover of traditional recipes through folk songs (Araya-Presa et al., 2003).

With the aim to validate traditional medicinal use and to identify bioactive compounds we decided to initiate a collaborative study with the Taira community of Ollagüe.

As a matter of fact, in all previous ethnobotanical surveys in this region, treatment of respiratory, gastro-intestinal and urinary disorders as well as pain and inflammation were among the most cited indications. As these symptoms may be potentially related to infectious diseases, we decided to conduct an ethnopharmacological study based on the evaluation of the anti-infectious activity of medicinal plants used by the community. Collection of eighteen selected plants has been decided together with the Taira community representative. This choice was based on previous ethnobotanical survey, availability of plant species in the collection period (September 2016) and current medicinal use of the species within the community. Collected parts correspond to the part traditionally used for medicinal purpose, mostly aerial parts or roots (Table 1).

A bioguided approach, consisting in studying selectively only active extracts and fractions, was adopted. It was conducted on a large panel of pathogenic germs involved in the main community acquired infectious diseases, represented by Gram positive and Gram negative bacteria of clinical interest and by human pathogenic fungi.

## 2. Material and methods

### 2.1. Chemicals

Müller Hinton agar, trypticase soy broth and Genbox microaer sachet were purchased to BioMérieux. Wilkins Chalgren agar was purchased to Oxoid. TGYH broth (tryptone 30 g/L, glucose 5 g/L, yeast extract 20 g/L and hemin 5 mg/L) was home prepared. RPMI medium 1640 was purchased to Gibco. *Candida* CHROMagar Medium petri dish were purchased to Becton Dickinson and Sabouraud dextrose agar supplemented with gentamicin and chloramphenicol were purchased to Biorad. Voriconazole (powder for solution) was purchased to Accord Healthcare Ltd. Fluconazole (solution for perfusion) was purchased to Fresenius Kobi France Ltd. Silica gel 60 0.025–0.04 mm (Macherey-Nagel GmbH & Co.) was used for column chromatography (CC). Ethyl acetate (AcOEt), methanol (MeOH), dimethylsulfoxide (DMSO), glucose, MOPS (3-(*N*-morpholino)-propanesulfonic acid) and ofloxacin (OFL) were purchased from Sigma-Aldrich. Cyclohexane and dichloromethane (DCM) were purchased from Carlo Erba-SDS. Cyclohexane, ether of petroleum and ethyl acetate were distilled before use. Water was distilled from deionized water.

**Table 1**
Medicinal uses of ethnic Atacama plants.

| Scientific Name | Vernacular name[c] | Part of the plant used[b] | Traditional uses | | | |
|---|---|---|---|---|---|---|
| | | | Socaire Baraona et al., 1958 | Ollague Villagran et al., 2003 | Salar de Atacama Tocornal, 2006 | Iquique Madaleno et Delatorre-Herrera, 2013 |
| **Apiaceae** | | | | | | |
| ***Azorella atacamensis G. M. Plunkett & A. N. Nicolas (ex Mulinum crassifolium)*** | Chuquikandia, Chukikanlla, Chuquican | Leaves and stems (Inf). | *M. crassifolium* cited as Chuquikan, used for bronchitis and cough | *M. crassifolium* cited as Chukikandia used for cough and diabetes | *M. crassifolium* used for colds and cough, grilled and mixed with sugar for infusion | [a]*Azorella compacta* cited as Flor de Yareta |
| ***Asteraceae*** | | | | | | |
| ***Artemisia copa* (Phil.) var. copa** | Kopakopa (Q), Kopatola (Q), Salvia blanca (S). | Leaves and stems (Cat, Inf). | For stomach ache, in infusion | For biliary drainage and stomach ache, abdominal pain | Leaves and stems in infusion for stomach ache, Mountain sickness. Incenses for rituals | |
| ***Baccharis calliprinos* Griseb.** | Chilka de cerro (A), Chilka negra (A). | Leaves and stems (Cat, Inf). | – | | [a] *B. scandens* (Ruiz et Pavón) Pers synonym of *B. callipirinos* Griseb. as Chilca used for digestive troubles, articular pain and rheumatisms. | |
| ***Baccharis grisebachii* Hieron** | Quinchimaldi (A), Quinchamale. | Leaves and stems (Cat, Inf). | – | – | – | – |
| ***Baccharis tola* Phil. ssp tola** | Lejía, Lekia, Léjia (S), Liquia | Leaves and stems (Cat, Inf). | [a]*B. santelices Phil.* cited as Lechia or Litchi | *B. tola* cited as Lejia, nakahembra, used for stomach ache and cough in infusion<br>[a]*B. boliviensis* is cited for tinctorial use | [a]*B. boliviensis* (Wedd.) Cabrera, cited as Tolila used in cataplasms mixed with guano for bone fractures | [a]*B. santelices* Phil. cited as Nakatula for colics |
| ***Chuquiraga atacamensis* Kuntze** | Candela (S), Lengua de gallina (S). | Leaves and stems (Inf). | No scientific name, Azafran cited for the treatments of "blood decomposition" | *C. atacamensis* cited as Chaklampa, Lengua de gallo, used for baths and as abortive. | *C. atacamensis* cited as Candela for prostatic troubles<br>[a]*C. spinosa* Less. subsp. *rotundifolia* (Wedd.) is cited as Azafran used for cough and renal disorders | *C. atacamensis* cited for wound healing and bone fractures |
| ***Haplopappus rigidus* Phil.** | Bailahuén (M). | Leaves and stems (Inf). | Colds in infusion, as men stimulant (aphrodisiac), not for young people | Cough, and colds. Also diuretic and used for menstrual pain. | Cough, colds, renal troubles, heart aches in infusion in milk. | Digestive and carminative and for stomach aches. Such as gastritis. |
| ***Parastrephia teretiuscula*. (Kuntze) Cabrera** | Koba, Tarakoba. | Leaves and stems (Cat, Inf). | | Cited as Kobatola for bones fractures in cataplasms, as well as *P. quadrangularis* (Pulika, Koba). P. lucida cited as febrifuge. | Ritual use as incense. | |
| ***Senecio nutans* Sch. Bip.** | Chachakoma (Q), Chachakuma. | Leaves and stems (Inf). | *Senecio eriphyton* Remy cited as Chachacoma, used for moutain sichkness | *S. nutans* cited as Chachakoma as well as *S. papii* used for cough, colds and influenza.<br>[a]*S. atacamensis* is cited for headaches, vomiting, and digestive troubles | | [a]*S. eriophyton* Remy For high blood pressure and mountain sickness. For cognition. |
| ***Ephedraceae*** | | | | | | |
| ***Ephedra americana* Humb. & Bonpl. ex Willd.** | Pingopingo, pinkopinko, Tume, | Stems (Cat, Inf) | *E. andina* Poepp. cited for stomach ache and colds: cited as Pingopingo and transmontada: cooked stems. | *E. breana* and *E. multiflora* cited as Pingopingo and Granada or Granadilla | *E. breana* cited for urinary troubles: leaves and roots infusion. | E. andina |
| ***Krameriaceae*** | | | | | | |
| ***Krameria lappacea* (Dombey) Burdet & B. B. Simpson** | Chakachaka (K). | Roots (Inf). | *K. iluca* Phil, synonym, cited as Ticara, used for urinary troubles, gonorrhoeae and pulmonary troubles: roots in infusion | | | |
| ***Poaceae*** | | | | | | |
| ***Cortaderia speciosa* (Ness & Meyen) Stapf** | Cortadera [S], cola de zorro [S], Espural | Leaves (Inf). | | | Parto - Diarrea -Firicciones - Infusión | |

(*continued on next page*)

**Table 1** (*continued*)

| Scientific Name | Vernacular name[c] | Part of the plant used[b] | Traditional uses | | | |
|---|---|---|---|---|---|---|
| | | | Socaire Baraona et al., 1958 | Ollague Villagran et al., 2003 | Salar de Atacama Tocornal, 2006 | Iquique Madaleno et Delatorre-Herrera, 2013 |
| | | | | [a]*C. atacamensis* cited as cortadera used for colds, cough and Influenza | | |
| ***Rosaceae*** | | | | | | |
| ***Polylepis tarapacana* Phil.** | Keñoa (Q) or Queñoa | Leaves and stems (Inf). | | | | |
| ***Solanaceae*** | | | | | | |
| ***Fabiana denudata* Miers** | Tara, Taratara (Q). | Leaves and stems (Cat, Inf). | *Fabiana sp.* cited as Monte Derecho, medicinal but no identified use | *F. denudata* cited as Tara tara or Tolila, Tola de Campo: Incenses for rituals | [a]*F. imbricata* cited as Romero (usual name for *Rosmarinus*). Stomach ache, bloating ("aire en el estomago"). | *F. denudata* cited as Kipa hembra used in case of cough. [a]*F. imbricata* cited as Pichi used in case of urinary troubles in infusions and for wound healing and antiseptic in external use. |
| ***Fabiana squamata* Phil.** | Koba[Q], Koba santiago. | Leaves and stems (Cat, Inf). | | *F. squamata* cited as Koba or Tara macho used for ritauls. | | *F. barriosii* Phil. Leaves and tubers used as diuretics, for biliary drainage, in case of gastritis bronchitis and cough and fever. |
| ***Verbenaceae*** | | | | | | |
| ***Aloysia deserticola* (Phil.) Lu-Irving & O'Leary** | Rikarika (S) | Leaves and stems (Inf). | *Verbena origenes* cited as Costilla or Core: infusions in case of stomach ache. | Used in case of vomiting, diarrhoea, including infants diarrhoea. Also used as galactogogue for breastfeeding women, and antidepressant. | Infusion for stomach ache. | In case of colds, stomach ache, menstrual pain and aphrodisiac. |
| ***Junellia seriphioides* (Guilles & Ex Hook.) Moldonke** | Huirohuiro, Kaylla, Rosa del campo | Leaves and stems (Cat, Inf). | | Cited as Rosa del campo, kishka or kaylla: leaves and stems baths or fresh flowers eaten for fever. | Infusion used for stomach ache or digestive troubles. | |
| ***Lampayo medicinalis* Phil.** | Lampaya (Q). | Leaves and stems (Inf, Cat). | | Fresh flowers eaten for fever. In bath for rheumatisms. Infusions for colds and stomach ache | Infusions for colds and stomach ache | Used as diuretic in case of renal troubles (infections, prostatic troubles, stones). Used in infusion in case of colds and articular pain. |

[a] Other cited specie.
[b] Inf: infusion, Cat: cataplasm.
[c] A: Aymara, K: kuntza, M: Mapudungun (Mapuche language), S: Spanish, Q: Quechua.

### 2.2. Plant material

Collect was respectful of Chilean National Biodiversity Strategy and of the Nagoya protocol. Official Material Transfer Agreement was provided from the Taira community and registered at the INIA (INIA, Instituto de Investigaciones Agropecuarias, Ministerio de Agricultura, Chile, application number 2-2016). Eighteen plants were collected within the Taira community (Ollagüe, Chile) in September 2016 (Fig. 1). Plants were selected based on a traditional medicinal use, guided by all previous local surveys (Table 1; Villagrán et al., 1998; Villagrán et al., 2003; Villagrán and Castro, 2003; Baraona et al., 1958; Tocornal, 2006; Madaleno and Delatorre-Herrera, 2013). Botanical identification was ensured by Pr. Alicia Marticorena, responsible of the CONC Herbario, Universidad de Concepción, Chile. Vouchers of each specimen were deposited in the Herbarium of the Laboratory of Pharmacognosy, Paris Descartes University, France, and in CONC Herbario, Universidad de Concepción, Chile.

### 2.3. Samples preparation

All plant materials were air-dried and reduced to powders using an electric grinder Pulverisette 19 (Fritsch GmbH, Mahlen und Messen, Germany). Twenty grams of plant powder were subjected to extraction. For *Aloysia deserticola* and *Artemisia copa* var. *copa*, leaves and stems were separated and extracted separately, due to the different medicinal proposes for each part. Extracts were obtained by pressurized solvent extraction (PSE) using a Speed Extractor E-914 (Büchi, Fawil Switzerland) equipped with four cells (120 mL) and a collector with four flat bottom vials (220 mL) successively with EtOAc and MeOH. Maximum pressure and temperature were adjusted to 100 bar and to 50 °C, respectively. Two extraction cycles with a hold-on time of 15 min were performed in each case. For *Ephedra americana* Humb. & Bonpl. Ex Willd., the PSE methanolic extraction was performed and also maceration of 10 g of vegetal material with 10% $NH_4Cl$ at room temperature was executed. Solvents were removed under reduced pressure. A total of 40 extracts were obtained. Solutions at 40 mg/mL were prepared in DMSO of each extract and stocked at 4 °C until biological evaluations.

### 2.4. General bioguided procedure

The general bioguided procedure consisted in testing at first all the extracts on a large bacterial Gram positive and Gram negative strains panel and against reference pathogen fungal species, at a final concentration of 100 µg/mL. Extracts able to inhibit the total growth of at least one bacterial strain at this concentration were identified as active extracts. They were then tested on a more specific bacterial strains panel with reference and clinical isolated species. At this step, MIC values were defined (MICs evaluation, see Sections 2.5–2.6). Extracts with significant MIC values were submitted to fractionation. In the next step, fractions were tested to identify the most active ones. Active fractions were subjected to an isolation procedure to isolate pure bioactive compounds (see Section 2.9). Finally antibacterial activity of pure isolated compounds was confirmed in the last step of our work.

### 2.5. Bacterial strains

Bacterial strains were reference strains from French (CIP- and CRBI-) or American (ATCC-) national collections or clinical strains (E- and N-) responsible for human respiratory, digestive or urinary infections, such as Gram positive *Bacillus* sp., *Enterococcus* sp., *Listeria* sp., *Staphylococcus* sp., *Streptococcus* sp., or Gram negative *Salmonella sp.*, *Klebsiella* sp., *Enterobacter sp.*, *Serratia sp.*, *Pseudomonas sp.*, *Yersinia sp.*, *Vibrio sp., Citrobacter sp., Achromobacter sp., Aeromonas* sp. …

Bacterial strains, except *Streptococcus pneumoniae*, were cultivated in broth (TGYH broth for *Streptococcus* species, trypticase soy broth for others) for 18–24 h at 37 °C before the assays. Each culture was diluted in sterile distilled water to obtain about $10^7$ UFC/mL and 1 µL of each (about $10^4$ UFC/spot) was applied on test medium using a Steers inoculator. For *S. pneumoniae* strains, the suspension was realized directly from a 24 h culture on blood agar. Two different panels were used for screening of antibacterial activity. The first panel included 28 g positive and 16 g negative strains. The second panel focused on 51 strains of selected genera.

### 2.6. Antibacterial activity assay

#### 2.6.1. Aerobic conditions

The *in vitro* antibacterial assay with the screening panel was conducted using an agar-dilution method with ofloxacin as positive control. Briefly, 250 µL of extract or compound solution in DMSO were poured to 1.75 mL sterile distilled water in a Petri dish and Mueller Hinton agar was added to a final volume of 20 mL and homogenized. After agar solidification, each culture strain solution in sterile distilled water was applied on test medium using a Steers inoculator. Petri dishes were then incubated for 18–24 h at 37 °C in aerobic conditions. The respective DMSO blank was conducted under the same conditions and was determined as non-toxic (1.25% final concentration).

For the first standard screening, extracts were tested at a final concentration of 100 µg/mL. Results were measured visually after incubation. Total absence of colonies was interpreted as growth inhibition by the active extract.

#### 2.6.2. Anaerobic conditions

For the antibacterial assay for anaerobic cultures (*Streptococcus* strains), Wilkins-Chalgren agar was used as medium and the Petri dishes were incubated with a Genbox Microaer sachet to produce anaerobic conditions for 24 h. The other conditions were identical.

#### 2.6.3. MIC determination

MIC was recorded as the lowest concentration at which no bacterial growth was observed. MICs of active extracts were determined using 9 different concentrations (100, 80, 60, 40, 20, 10, 5, 2 and 1 µg/mL). Fractions from active extracts were evaluated at 50 µg/mL. MICs from pure compounds were determined by the evaluation at 8 different concentrations (50, 32, 16, 8, 4, 2, 1 and 0.5 µg/mL). Geometrical dilutions were prepared to obtain final concentrations. The MIC was recorded as the lowest concentration at which no bacterial growth was observed.

### 2.7. Fungal strains

*Candida albicans* ATCC 90028 was cultured in a *Candida* CHROMagar Medium Petri dish and incubated for 24 h at 37 °C. Then, four colonies (≤ 1 mm diameter) were taken and added to 2 mL saline solution until a turbidity between 0,5 and 1 Mc Farland, diluted with 18 mL with RPMI 1640 medium (Gibco) supplemented with glucose (20% w/v) (Sigma Aldritch) and MOPS (0,165 M) (Sigma Aldritch). *Aspergillus fumigatus* ATCC 204305 and *Tricophyton tonsurans* CBS 120133 (Centraalbureau voor Schimmelcultures, Utrecht) were cultured in tubes with 18 mL of Sabouraud-dextrose agar supplemented with gentamicin and chloramphenicol (Biorad) and incubated for 48 and 168 h respectively at 27 °C. Then, 1 mL of medium RPMI was added and gently mixed to obtain a spore suspension. Each spore suspension was diluted with medium RPMI supplemented until a concentration of 200–500 spores/mL. Then 100 µL of inoculum spores solution were added in each well in 96-plates for evaluation.

### 2.8. Antifungal activity assay

The *in vitro* antifungal bioassay was conducted using a microdilution 96-plate method with fluconazole for *C. albicans* and voriconazole for *A. fumigatus* and *T. tonsurans* as positive controls (EUCAST, 2008a, 2008b). Briefly, 100 µL of extract solution was diluted in RPMI 1640 medium modified (as previously described) were poured in the plates and 100 µL of fungal inoculum added and mixed. Then, plates were incubated in aerobic conditions for 24 h for *C. albicans*, 48 h for *A. fumigatus* at 37 °C and 168 h for *T. tonsurans* at 27 °C. The respective DMSO blank was conducted under the same conditions and was determined as non-toxic (0.25% final concentration). Extract were first tested at 100 µg/mL. For active extracts, geometric dilutions were prepared in RPMI modified medium to give final concentrations between 100 and 0.5 µg/mL for the determination of MICs. The MIC was recorded as the lowest concentration at which no bacterial growth was observed.

### 2.9. Bioguided fractionation and isolation of bioactive compounds

A sample of 4 g of ethyl acetate extract of leaves from *Aloysia deserticola* and 2 g of ethyl acetate extract from roots of *Krameria lappacea* were subjected to a silica gel chromatography with a gradient of dichloromethane/methanol mixture solvent (10:0–9:1). For *A. deserticola*, 27 fractions were obtained (FAD1–27) and 23 for *K. lappacea* (FKL1–23). All fractions were evaluated against the panel of Gram-positive bacteria strains. Active fractions were subjected to a second silica gel chromatography with a gradient of increasing polarity using petroleum ether/ethyl acetate (9:1–7:3) for fractions of *A. deserticola* and dichloromethane/methanol (95:5–90-10) for fractions of *K. lappacea*. Structural identification of isolated compounds was checked by mass spectrometry and 1D-, 2D NMR experiments in comparison with published data. Mass spectrometry was performed on a mass spectrometer ZQ 2000 Waters (Saint-Quentin, France). $^{1}H$ and $^{13}C$ NMR experiments were performed in deuterated chloroform ($CDCl_3$). Spectra of pure isolated compounds were recorded on a Bruker AC 300 MHz or a Bruker 400 MHz spectrometer (Wissembourg, France). Rotary values were recorded on a Perkin Elmer Model 341 polarimeter (Perkin Elmer, Courtaboeuf, France) at 20 °C.

## 3. Results

### 3.1. Collected plants and traditional use

The collect at Ollagüe, within the Taira community, provided eighteen medicinal plants, all cited for medicinal use in previous surveys (Table 1). Species from Asteraceae family are the most represented, followed by Verbenaceae species.

Asteraceae were represented by eight species including Kopakopa, identified as *Artemisia copa* var. *copa* Phil., used by both Atacama communities from Ollagüe and Socaire, traditional use of the infusion against digestive disorders. In addition, the cataplasm of the aerial parts is prepared to treat bone fractures in the Taira community in Ollagüe (Villagrán et al., 1998, 2003; Villagrán and Castro, 2003, Baraona et al., 1958). Beside *Artemisia*, three *Baccharis* species were collected corresponding to Chilka (in Aymara, "thorny bush") attributed to several species belonging to the genus *Baccharis* (*B. alnifolia* Meyen & Walp., *B. boliviensis* (Wedd.) Cabrera var. boliviensis, *B. salicifolia* (Ruiz et Pav.) Pers., *B. calliprinos* Grise, and Lejia (or Lekia), identified as *Bacharis santelices* Phil. spp. *santelices* and *B. tola* Phil. spp. *Tola*. This name was also reported for a *Baccharis* in the Socaire community without mention of the species. It is noticeable that in the Bolivian Andes, and in Peru, the Kallawaya community, also attributes the Chilka name to other *Baccharis* species, such as *B. latifolia* (Ruiz et Pav.) Pers, to treat lung diseases (De-la-Cruz et al., 2007; Bastien, 1983). In the Atacama area Chilka and Leija are mainly used to treat rheumatism, bone pain (as cataplasm) and for digestive disorders and as anti-inflammatory in infusion. However, Leija infusion is additionally used against respiratory problems (cough, cold, *etc.)* (Villagrán et al., 1998, 2003; Villagrán and Castro, 2003). Asteraceae were also represented by Bailahuen, the vernacular attributed to *Haplopappus rigidus* Phil. and to *Haplopappus baylahuen* J. Remy), both cited in the Atacama community of Socaire and in the city of Iquique (with Aymara influence) used for rheumatisms, as aphrodisiac and for the treatment of cold and cough (Villagrán et al., 1998; Baraona et al., 1958; Madaleno and Delatorre-Herrera, 2013). The Taira community also reported traditional uses for liver and urinary infections. In Iquique, it is also used for digestive disorders.

Other Asteraceae were "Lengua de gallina", chicken tongue due to the leaves' shape, *Chuquiraga atacamensis* Kuntze, used in Ollagüe, against respiratory disorders, as abortive and for menstrual pain, and Chachacoma, *Senecio. S. nutans* Sch. Bip. or *S. eriophyton* J. Remy, in Iquique, Ollagüe and Socaire. Traditionally used in case of "mountain sickness" in all reports, *S. nutans* may also be used in case of digestive and urinary infections, and as analgesic. It is also used to treat high blood pressure in Ollagüe.

Verbenaceae were represented by the abundant Rikarika, identified as *Aloysia deserticola* (Phil.) Lu-Irving & O'Leary (ex *Acantholippia deserticola* Phil.). This vernacular name is also attributed to *Junellia origenes* (Phil.) N. O'Leary & P. Peralta in Socaire and Iquique (Baraona et al., 1958; Madaleno and Delatorre-Herrera, 2013; O'Leary et al., 2009). Infusion of stems is intensively used by the local communities (Aymara and Atacameños) in the north of Chile, but also in the urban areas (Calama, Antofagasta) for the treatment of digestive problems (Socaire, Ollagüe and Iquique), fever and respiratory troubles (cough) in both Ollagüe and Iquique. In Ollagüe, the infusion of complete aerial part (stems and leaves), is used against urinary infections, digestive pain, emesis, cold, infantile diarrhoea and for milk production for breastfeeding women (Villagrán et al., 2003). Mention of use in case of menstrual pain and as aphrodisiac is documented in Iquique (Madaleno and Delatorre-Herrera, 2013).

Among Verbenaceae, Huirahuira, viravira or vira (in Quechua, "fat butter") was identified as *Junellia serephioides, whereas* in Tarapaca area and Bolivian Andes, it corresponds to *Achyroline* species (*A. ramosissima*, *A. satureioides*) and *Gnaphalium* species (*G. cheiranthifolium*, *G. frigidum*). Its infusion is used against respiratory problems. In Ollagüe it is also used for urinary infections and for bone fracture (cataplasm).

Solanaceae, Ephedraceae, Rosaceae and Krameriaceae are also represented. Among them, the popular Chakachaka ("scratch" in Kunza), *Krameria lappacea* (Dombey) Burdet & B. B. Simpson (syn *Krameria triandria* Ruiz et Pavon), also known as Ticara in Socaire, is used in infusion against urinary infections and respiratory disorders. Aerial parts are used as feed for camelids in the region. Widespread in north Andes, its medicinal use has been exported to Europe since the 18th century, where it is known as Ratanhia, the Peruvian name related to the scientific synonym *Krameria triandra* Ruiz and Pavon. Ratanhia is mainly used as an astringent, due to its high tanins content, to treat haemorrhoids, diarrhoea and stomatitis (Simpson, 1991).

It is also important to notice the use of Solanaceae, *Fabiana* species, against bone fractures, and to treat colds, urinary infection and digestive disorders (Ollague) or cough (Iquique). *Fabiana denudata* Phil., is known as Tolilla in Socaire community, Tara or Taratara in Ollagüe (as well as for other *Fabiana* species) and Kipa hembra in Iquique.

The Ephedraceae Pingopingo commonly used for various species from the genus *Ephedra*, located in the Andes area from Chile and Bolivia should also be mentioned. In Ollagüe community Pingopingo is attributed to *E. americana* Humb. & Bonpl. ex Willd. and its infusion is reported to treat cold and urinary infections. Stems are also used in cataplasm to treat rheumatism. In Iquique Pingopingo is the vernacular name of *E. chilensis* C. Presl used in case of prostate disorders.

### 3.2. Bioguided antibacterial activity

#### 3.2.1. General gram positive and negative panel

At first, the 40 methanolic or ethyl acetate extracts obtained from the eighteen collected species, were tested for their antibacterial activity on a general panel of 44 bacterial strains (28 g negative and 16 g positive), at a concentration of 100 µg/mL. Among these extracts, only eight obtained from 7 different plant species were identified as active extracts, responsible of total growth inhibition at 100 µg/mL at least for one bacterial strain. Interestingly, only Gram positive reference strains from *Bacillus*, *Corynebacterium*, *Enterococcus*, *Listeria* and *Staphylococcus* genus were inhibited by these extracts. No activity was observed on most Gram negative bacteria that may be involved in gastro-intestinal infections, such as *Enterobacter*, *Salmonella*, *Serratia*, *Pseudomonas, Vibrio* or *Yersinia* species (supplementary data).

#### 3.2.2. Specific gram positive panel screening

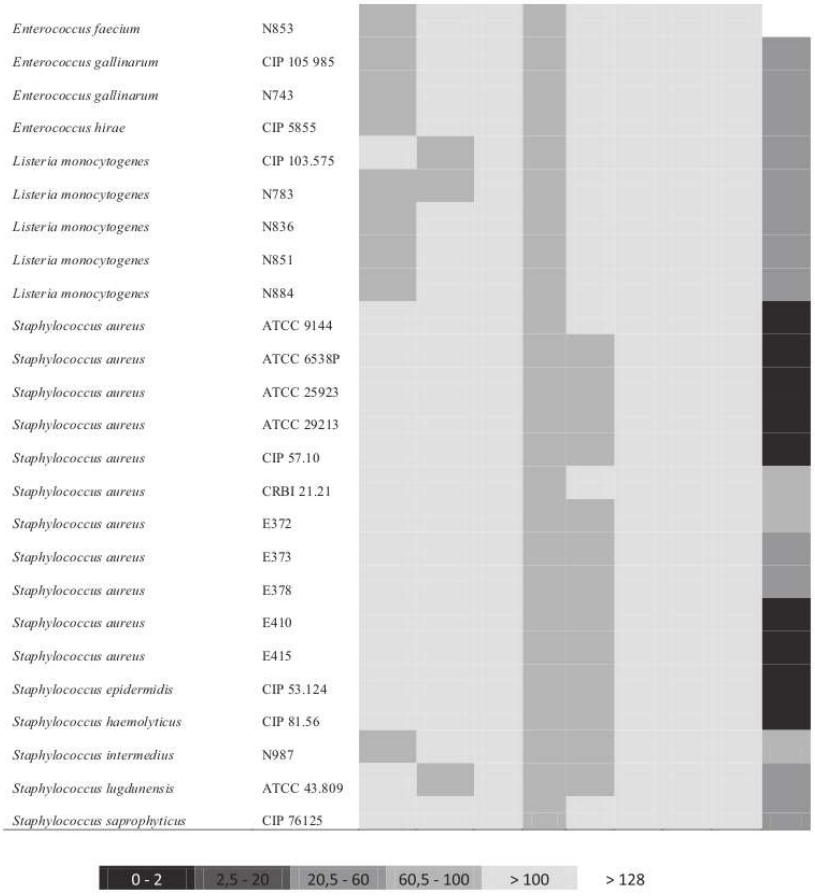
For the next step of the bioguided procedure, a second more specific panel of 51 strains of Gram positive bacteria from these genera was used for screening on active extracts (Table 2). This panel included reference or clinically isolated strains of various bacteria species responsible for digestive infections such as *Enterococcus* sp., *Listeria* sp. and *Staphylococcus aureus*, or respiratory tract infections such as *Streptococcus* sp., *Corynebacterium* sp. At this step, determination of the MIC value of each extract was determined for this second panel. Among the 8 tested extracts, only ethyl acetate extracts of *A. deserticola* (ADA) and *K. lappacea* (KLA), presented promising inhibitory activity against Gram-positive strains with low MIC values. Evaluation of MIC values a third panel of 33 strains of *Streptococcus* species, in anaerobic conditions was performed only for ADA and KLA together with isolated compounds (Table 3).

**Table 2**
MICs (µg/mL) of active extracts against aerobic bacterial strains and reference antibiotic Ofloxacine (OFL). ADA: *Aloysia deserticola* ethyl acetate extract, *Haplopappus rigidus* ethyl acetate extract, JSA: *Junellia seriphioides* ethyl acetate extract, KLA: *Krameria lappacea* ethyl acetate extract, KLM: *Krameria lappacea* methanol extract, AAA: *Azorella atacamensis* ethyl acetate extract, PTeA: *Parastrephia teretiuscula* ethyl acetate extract, PTaA: *Polylepis tarapacana* ethyl acetate extract.

| Bacteria Specie | Reference | MICs valeus of active extracts and reference against Gram positive bacteria (µg/mL) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *ADA* | *HRA* | *JSA* | *KLA* | *KLM* | *AAA* | *PTeA* | *PTaA* | OFL |
| *Bacillus cereus* | CIP 6624 | 200 | 100 | 200 | 50 | 50 | 50 | 200 | 200 | 0.5 |
| *Bacillus cereus* | N551 | 200 | 200 | 200 | 50 | 50 | 50 | 50 | 200 | 0.5 |
| *Bacillus cereus* | N839 | 50 | 50 | 100 | 50 | 200 | 200 | 200 | 200 | 0.5 |
| *Bacillus cereus* | N1060 | 50 | 200 | 200 | 50 | 100 | 200 | 200 | 200 | 0.5 |
| *Bacillus cereus* | N1139 | 50 | 200 | 200 | 50 | 50 | 200 | 200 | 200 | 0.5 |
| *Corynebacterium minutissimum* | CIP 100652T | 200 | 100 | 200 | 50 | 50 | 50 | 50 | 50 | 2 |
| *Corynebacterium striatum* | N1129 | 50 | 200 | 200 | 50 | 100 | 200 | 200 | 200 | 0.5 |
| *Enterococcus avium* | CIP 104 053 | 50 | 200 | 100 | 50 | 200 | 200 | 200 | 200 | 0.5 |
| *Enterococcus avium* | N1061 | 50 | 200 | 200 | 50 | 200 | 200 | 200 | 200 | 2 |
| *Enterococcus avium* | N1064 | 50 | 200 | 100 | 50 | 200 | 200 | 200 | 200 | 1 |
| *Enterococcus casseliflavus* | CIP 103.018T | 50 | 200 | 200 | 50 | 200 | 200 | 200 | 200 | 1 |
| *Enterococcus casseliflavus* | N850 | 50 | 200 | 200 | 50 | 200 | 200 | 200 | 200 | 1 |
| *Enterococcus durans* | CIP 104 999 | 50 | 200 | 100 | 50 | 200 | 200 | 200 | 200 | 1 |
| *Enterococcus durans* | N569 | 50 | 200 | 200 | 50 | 200 | 200 | 200 | 200 | 1 |
| *Enterococcus durans* | N1136 | 50 | 200 | 200 | 50 | 200 | 200 | 200 | 200 | 1 |
| *Enterococcus faecalis* | CIP103.214 | 50 | 200 | 200 | 50 | 200 | 200 | 200 | 200 | 0.5 |
| *Enterococcus faecalis* | CIP 104 676 | 50 | 200 | 200 | 50 | 200 | 200 | 200 | 200 | 0.5 |
| *Enterococcus faecalis* | N491 | 50 | 200 | 100 | 50 | 100 | 200 | 200 | 200 | 1 |
| *Enterococcus faecalis* | N571 | 50 | 200 | 200 | 50 | 200 | 200 | 200 | 200 | 1 |
| *Enterococcus faecalis* | N650 | 50 | 200 | 200 | 50 | 200 | 200 | 200 | 200 | 1 |
| *Enterococcus faecalis* | N755 | 50 | 200 | 200 | 50 | 200 | 200 | 200 | 200 | 4 |
| *Enterococcus faecium* | CIP 103.014 | 50 | 200 | 200 | 50 | 200 | 200 | 200 | 200 | 2 |
| *Enterococcus faecium* | CIP 107.387 | 50 | 200 | 200 | 50 | 200 | 200 | 200 | 200 | 2 |
| *Enterococcus faecium* | N490 | 50 | 200 | 100 | 50 | 200 | 200 | 200 | 200 | 1 |
| *Enterococcus faecium* | N734 | 50 | 200 | 200 | 50 | 200 | 200 | 200 | 200 | 4 |
| *Enterococcus faecium* | N838 | 50 | 200 | 200 | 50 | 200 | 200 | 200 | 200 | 4 |

(*continued on next page*)

**Table 2** (*continued*)




The ethyl acetate extract of roots of *K. lappacea* KLA was highly effective toward all strains of *Staphylococcus* species, *S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. intermedius*, *S. lugdunensis*, and *S. saprophyticus* with MIC values of 20 µg/mL, as well as toward all strains of *Bacillus cereus*, *Listeria monocytogenes* and *Corynebacterium*. KLA was also active toward strains of *Enterococcus* species (MIC 40 µg/mL, and 20 µg/mL against for *E. avium*).

Moderate activity was observed for the methanolic extract of *K. lappacea* (KLM) with MIC comprised between 80 and 100 µg/mL on *Staphylococcus* strains and *Bacillus cereus* strains.

Regarding the *Streptococcus* panel, KLA was found to be highly active on *S. pneumoniae* and *S. pyogenes* growth with MIC values between 6 and 25 µg/mL, and active on main strains of *S. agalactiae* and *S. dysgalactiae* (MIC values between 25 and 50 25 µg/mL).

*Aloysia deserticola* ethyle acetate extract (ADA) showed promising MIC values on all the *Enterococcus* species comprised between 2.5 and 40 µg/mL. ADA was also effective against most *Listeria monocytogenes* strains with a MIC value of 60 µg/mL. Regarding *Bacillus cereus* strains, ADA was not effective against the reference strain whereas it was active on 3 out of 4 of clinical isolated strains (MIC between 20 and 60 µg/mL). ADA seems to be inactive on most *Staphylococcus* species, except *S. intermedius* at 60 µg/mL. Regarding *Streptococcus* genus, ADA was highly effective on main *Streptococcus pneumoniae* clinical strains with MIC values comprised between 3 and 6 µg/mL, and also active on *S. pyogenes* (12–50 µg/mL) and *S. agalactiae* strains. Moderate activity was observed against *S. agalactiae* (CMI between 50 and 100 µg/mL).

Other extracts showed only moderate or low antibacterial activity. The ethyl acetate extract of *Haplopappus rigidus* Phil. (HRA) has demonstrated inhibitory effects against two out of five strains of *B. cereus* and one *C. minutissimum* strains at 40–60 µg/mL. Two out of five *Listeria* strains were also inhibited at 100 µg/mL. The same effect at the same concentration was observed against *S. lugdunensis*. No inhibitory activity was observed against the other *Staphylococcus* or *Enterococcus* species.

*Junellia serephioides* (Guilles & Ex Hook.) Moldonke ethyl acetate extract (JSA) has shown low MIC values between 80 and 100 µg/mL against 5 out of 23 *Enterococcus* strains, but > 100 µg/mL for all other strains.

*Azorella atacamensis* G. M. Plunkett & A. N. Nicolas ethyl acetate extract (AAA) has inhibited the growth of 2 strains of *B. cereus* at 60 µg/mL and *Corynebacterium minutissimum* at 40 µg/mL. *Parastrephia teretiuscula* (Kuntze) Cabrera (PTeA) and *Polylepis tarapacana* Phil. (PTaA) also inhibited *C. minutissimum* strains at 100 µg/mL. No inhibitory effects were observed against the other strains.

**Table 3**
MICs of *Aloysia deserticola* ethyl acetate extract (**ADA)** and *Krameria lappacea* ethyl acetate extract (**KLA)** and of isolated compounds (+)-conocarpan (**1**) and ursolic acid (**10**) against *Streptococcus* species strains, with comparison with reference antibiotic ofloxacine (OFL).

| Bacteria specie | Reference | MICs of extracts and isolated against facultative anaerobic Gram positive bacteria (µg/mL) | | | | |
|---|---|---|---|---|---|---|
| | | **ADA** | **KLA** | **1** | **10** | **OFL** |
| *Streptococcus agalactiae* | CIP 103.227 | | | | | |
| *Streptococcus agalactiae* | SA 1108 4768 UR 1201 | | | | | |
| *Streptococcus agalactiae* | 2651 | | | | | |
| *Streptococcus agalactiae* | N858 | | | | | |
| *Streptococcus agalactiae* | N983 | | | | | |
| *Streptococcus agalactiae* | N1036 | | | | | |
| *Streptococcus agalactiae* | N1311 | | | | | |
| *Streptococcus bovis* | CIP 102302 | | | | | |
| *Streptococcus dysgalactiae* | CIP 107086 | | | | | |
| *Streptococcus dysgalactiae* | N1052 | | | | | |
| *Streptococcus dysgalactiae* | N1328 | | | | | |
| *Streptococcus dysgalactiae* | N1330 | | | | | |
| *Streptococcus dysgalactiae* | N1331 | | | | | |
| *Streptococcus dysgalactiae* | N1332 | | | | | |
| *Streptococcus dysgalactiae* | N1335 | | | | | |
| *Streptococcus dysgalactiae* | N1336 | | | | | |
| *Streptococcus dysgalactiae* | N1337 | | | | | |
| *Streptococcus dysgalactiae* | N1338 | | | | | |
| *Streptococcus pneumoniae* | ATCC 49619 | | | | | |
| *Streptococcus pneumoniae* | N1001 | | | | | |
| *Streptococcus pneumoniae* | N1005 | | | | | |
| *Streptococcus pneumoniae* | N1006 | | | | | |
| *Streptococcus pneumoniae* | N1364 | | | | | |
| *Streptococcus pyogenes* | IP56.1 | | | | | |
| *Streptococcus pyogenes* | SA06115001 | | | | | |
| *Streptococcus pyogenes* | N1259 | | | | | |
| *Streptococcus pyogenes* | N1346 | | | | | |
| *Streptococcus pyogenes* | N1347 | | | | | |
| *Streptococcus pyogenes* | N1356 | | | | | |
| *Streptococcus pyogenes* | N1357 | | | | | |




### 3.3. Isolation of bioactive compounds from active extracts

The two most promising extracts KLA and ADA were selected for fractionation following the procedure described in 2.9. KLA fractionation led to the obtainment of 3 active fractions out of 23 (FKL2 to FKL4) which exhibited inhibitory activity at least against 3 bacterial strains. Isolation procedure by silica gel chromatography using dichloromethane/methanol as eluent afforded five known neolignans represented by 4 benzofurans and one tetrahydrofuran (Fig. 2). Structural identification was checked by 1D-, 2D NMR experiments and mass spectrometry in comparison with published data (Arnone et al., 1988). From FKL2 were isolated conocarpan (**1**, 6.5 mg) and eupomatenoid 6 (**2**, 1.4 mg), from FKL3 conocarpan (2.3 mg), eupomatenoid 6 (4.7 mg) and 2-(2,4-dihydroxyphenyl)-5-(*E*)-propenylbenzofuran (**3**, 2.4 mg) and from FKL4, 2-(2-hydroxy-4-methoxy-phenyl)-5-(3-hydroxypropyl)-benzofuran (**4**, 4.6 mg) and *meso*-3,30-didemethoxynectandrin B (**5**, 2.2 mg) (Fig. 3).

In the case of ADA, 3 out of 27 fractions (FAD14–16) showed bacterial growth inhibition at least toward 3 different bacterial strains. From FAD 14 to 16, seven known pentacyclic triterpenoid derivatives were isolated by silica gel chromatography with petroleum ether /ethyl acetate as eluent (Fig. 4). From FAD14, were isolated oleanolic acid (**6**, 5 mg) (Venditti et al., 2016; Perveen et al., 2014) and betunilic acid (**7**, 3 mg) (Gilbert et al., 2015). From FAD15 lupeol (**8**, 1.2 mg) (Wang et al., 2017) and epilupeol (**9**, 0.8 mg) (Al Musayeib et al., 2013), oleanolic acid (**6** 1.3 mg) and ursolic acid (**10**, 5.6 mg) (Gilbert et al., 2015; Wang et al., 2017) and from FAD16 ursolic acid (**10**, 3.4 mg) and hederagenin (**11** 2.6 mg) (Subba Rao et al., 2008).

#### 3.3.1. Antibacterial activity of isolated compounds

The benzofuran neolignan, conocarpan (**1**) showed a very promising antibacterial profile and could inhibit the growth of all the Gram positive tested strains both in aerobic and anaerobic conditions with MIC values comprised between 1 and 2 µg/mL (Table 4).

Eupomatenoid-6 (**2**) led to total growth inhibition of all *Staphylococcus* and *Corynebacterium* strains at 32 µg/mL. Inhibition of growth of all *Listeria* and *Enterococcus* strains, was observed at 50 µg/mL.

The 2-(2-hydroxy-4-methoxy-phenyl)-5-(3-hydroxypropyl)-benzofuran **4** totally inhibited the growth of *Corynebacterium* strains between 32 and 50 µg/mL. The others neolignans could not be evaluated due to isolation only in minute amount.

From pentacyclic triterpenes, ursolic acid (**10**) was the most active with MIC values comprised between 16 and 50 µg/mL against *Enterococcus* and *Corynebacterium* strains (Table 4). Ursolic acid (**10**) also showed inhibition against *Listeria*, *Bacillus* and *Staphylococcus epidermidis* strains growth. Oleanolic acid (**6**) was slightly less active, with a total growth inhibition of *Enterococcus faecium*, *E. durans*, *E. avium* and *Corynebacterium striatum* at 50 µg/mL. Lupeol (**8**) was only active against *Corynebacterium minutissimum* at the same concentration. Betulinic acid (**7**) and hederagenin (**11**) were not active in this screening. Epilupeol (**9**) was isolated only in minute amounts and could not be evaluated.

### 3.4. Antifungal activity

MIC values of the extracts were measured against representative fungal pathogens strains: *Candida albicans*, *Aspergillus fumigatus* and *Tricophyton tonsurans* (Table 5). Fungal growth inhibition was observed for only four extracts out of forty, obtained from *P. taracana* (PTaA), *K. lappacea* (KLA and KLM) and *Artemisia copa* var. *copa* (ACA), with MIC values comprised between 80 and 100 µg/mL, reflecting a low antifungal activity.

*C. albicans* was inhibited by the ethyl acetate extract of *P. taracana* (PTaA) at 80 µg/mL and by the extracts of *K. lappacea* at 80 (KLA) and 100 µg/mL (KLM).

For *T. tonsurans*, the ethyl acetate extract of *Artemisia copa* var. *copa* (ACA) presented MIC value of 80 µg/mL as well as both ethyl acetate and methanolic extracts of *K. lappacea*. No extract was able to inhibit *A. fumigatus* growth in our conditions.

## 4. Discussion

The bioguided antibacterial screening applied on traditional medicinal plants used within the Taira community led us to corroborate the traditional use for anti-infectious purpose and to identify bioactive compounds for two out of the eighteen studied species: *Aloysia deserticola and Krameria lappacea*.

In our work, *Aloysia deserticola* ethyl acetate extract ADA showed promising activity on large number of strains from different *Enterococcus* species, mainly involved in digestive and urinary infections, and *Listeria* species responsible of food-borne outbreaks. ADA was also active on various strains of *Streptococcus pneumoniae* and *Streptococcus pyogenes* involved in respiratory tract infections. The

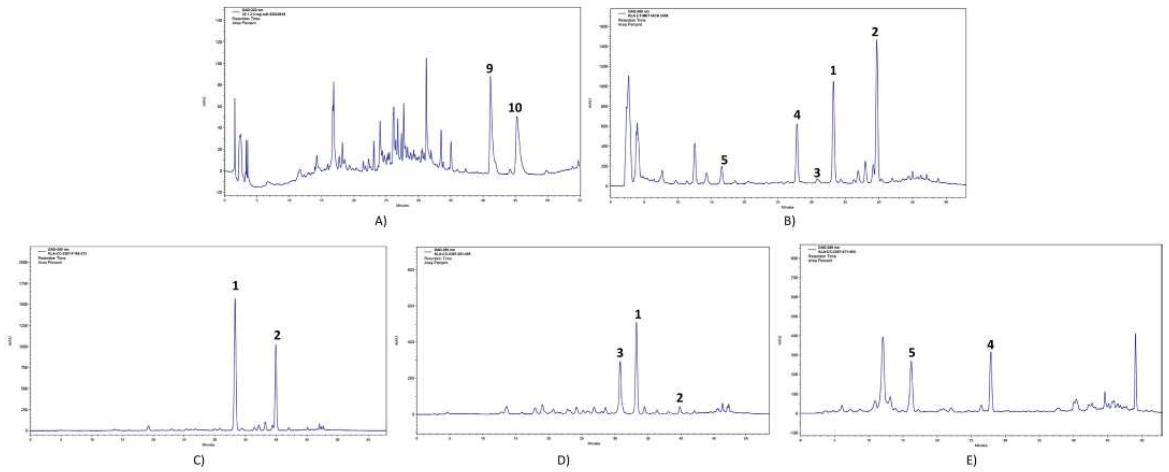



**Fig. 2.** A) Chromatographic profile of **ADA** (*Aloysia deserticola* ethyl acetate extract), **9**: epilupeol, **10**: ursolic acid. B) Chromatographic profile of **KLA** (*Krameria lappacea* roots ethyl acetate extract); C) **KLA** Fraction 2; D) **KLA** Fraction 3 and E) **KLA** Fraction 4. Compounds: **1**: Conocarpan, **2**: Eupomatenoid-6, **3**: 2-(2,4-dihydroxyphenyl)-5-(*E*)-propenylbenzofuran, **4**: 2-(2-hydroxy-4-methoxy-phenyl)-5-(3-hydroxypropyl)-benzofuran and **5**: *meso*-3,3-didemethoxynectandrin B.

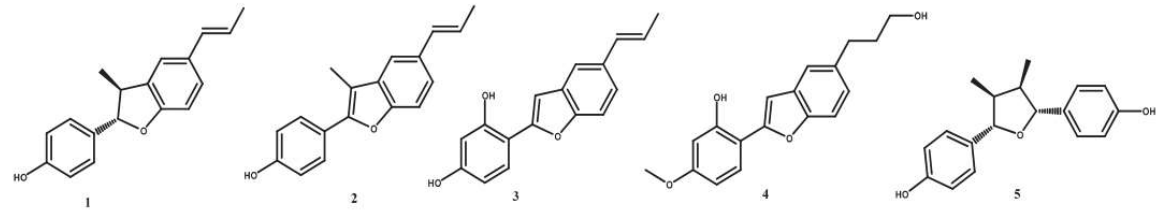


**Fig. 3.** Compounds isolated from *K. lappacea*. **1**: Conocarpan, **2**: Eupomatenoid-6, **3**: 2-(2,4-dihydroxyphenyl)-5-(*E*)-propenylbenzofuran, **4**: 2-(2-hydroxy-4-methoxy-phenyl)-5-(3-hydroxypropyl)-benzofuran and **5**: *meso*-3,3-didemethoxynectandrin B.

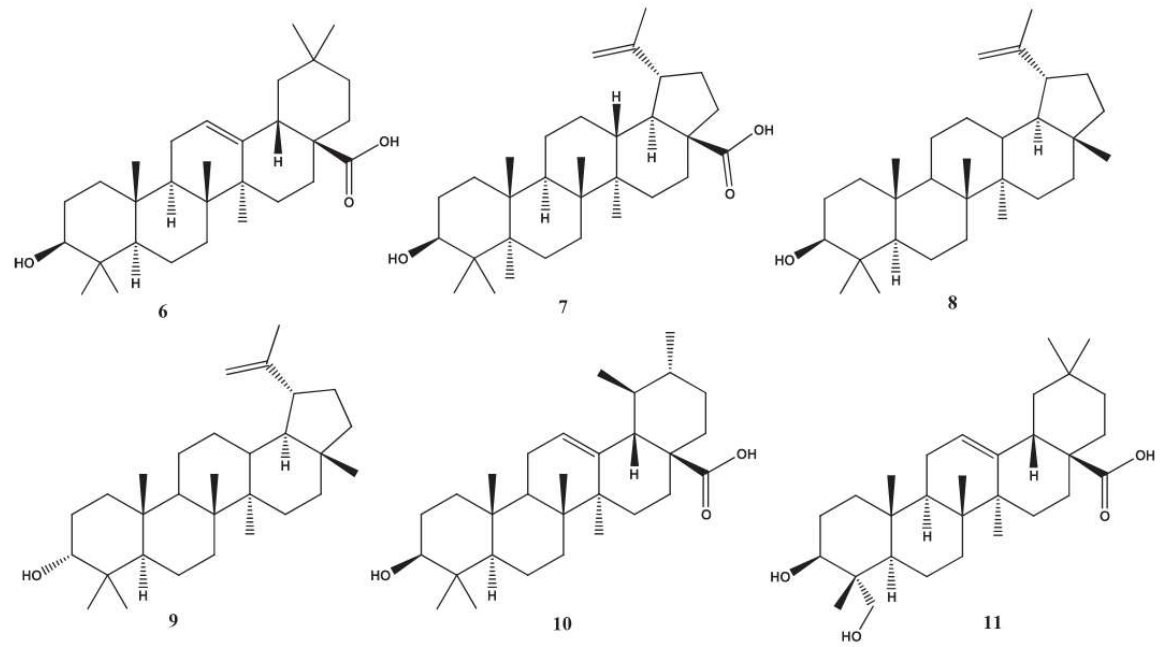


**Fig. 4.** Compounds isolated from *A. deserticola*. **6**: Oleanolic acid, **7**: Betunilic acid, **8**: Lupeol, **9**: Epilupeol, **10**: Ursolic acid and **11**: Hederagenin.

**Table 4**
MICs of pure isolated compounds obtained by bioguided fractionation. Isolated from *Krameria lappacea*: Conocarpan (**1**), Eupomatenoid-6 (**2**), 2-(2-hydroxy-4-methoxy-phenyl)-5-(3-hydroxypropyl)-benzofuran (**4**). Isolated from *Aloysia deserticola*: Oleanolic acid (**6**), Betunilic acid (**7**), Lupeol (**8**), Ursolic acid (**10**) and Hederagenin (**11**).

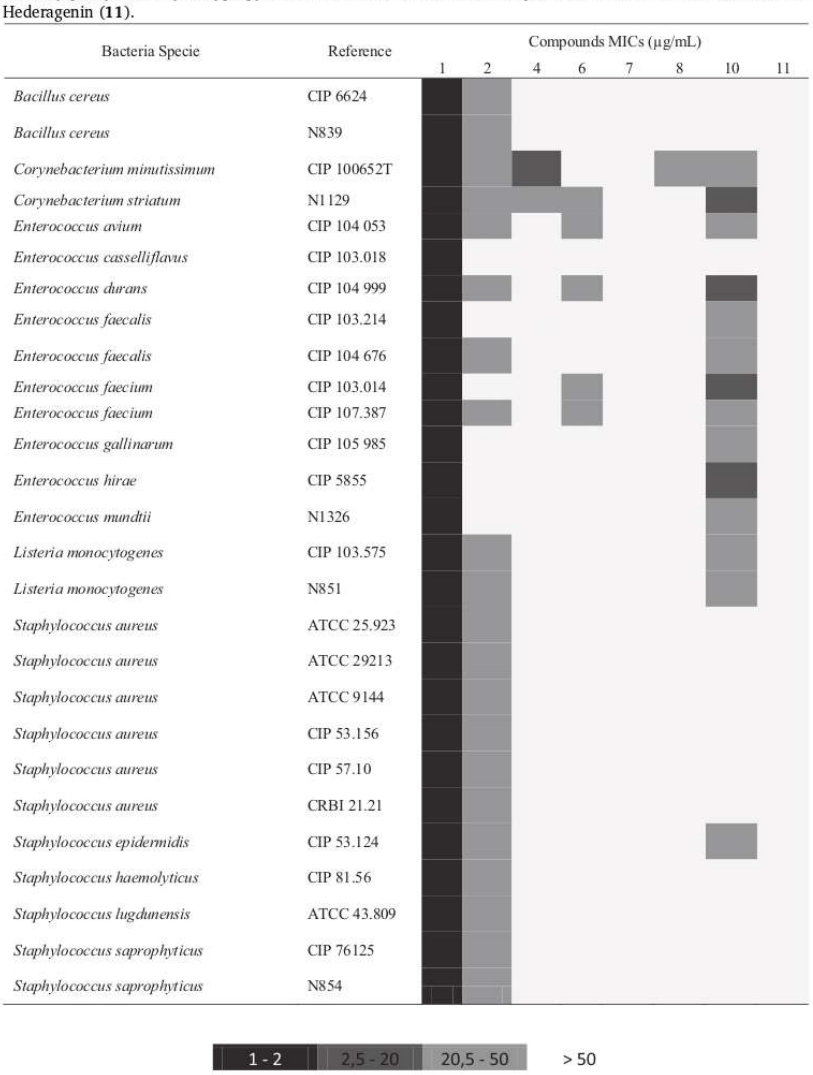
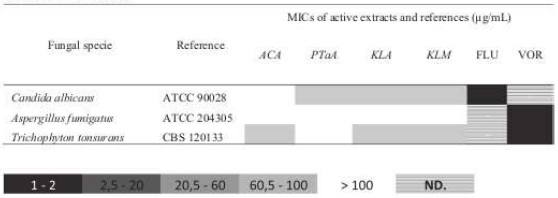



**Table 5**
MIC values of active extracts against fungal reference strains with comparison with fluconazole (FLU) and voriconazole (VOR). ACA: *Artemisia copa* ethyl acetate extract, PTaA: *Polylepis tarapacana* ethyl acetate extract, KLA: *Krameria lappacea* ethyl acetate extract, KLM: *Krameria lappacea* methanol extract, ND: not determinated.




bioguided procedure performed on ADA extract led to the isolation of ursolic acid which exerted the strongest antibacterial activity with a MIC value between 16 and 50 µg/mL against Gram-positive strains *Enterococcus*, *Corynebacterium*, *Listeria*, *Bacillus* and *Staphylococcus epidermidis* strains. These results are in accordance with previous work, describing inhibition of *Staphylococcus aureus* and *Bacillus subtilis* growth by chloroformic extract and hydroalcoholic extracts of *A. deserticola* containing triterpenoids (Morales et al., 2003). Antibacterial screening of triterpenoids isolated from various plant species confirmed that ursolic acid was able to exert a good antibacterial activity limited to Gram positive bacteria. In comparison, other triterpenic analogues, such as oleanolic and betulinic acid showed only weak to moderate activity (Fontanay et al., 2008).

Looking forward the biological targets, ursolic and oleanolic acids were able to affect peptidoglycan synthesis in *Listeria monocytogenes* as

peptidoglycan amount was reduced after the incubation with theses triterpenoids, without inhibition of penicillin-binding proteins (PBPs), essential for the final steps in peptidoglycan synthesis (Kurek et al., 2010). Deeper investigations to elucidate the possible target in the earlier synthesis of peptidoglycan must be carried on to identify the mechanism of action or ursolic and oleanolic acids. Triterpenic compounds are now deeply studied in a large spectrum of other biological activity such as antiviral, anti-parasitic, cytotoxic, anti-angiogenesis, antioxidant and anti-inflammatory activities (Kashyap et al., 2016).

Regarding *Krameria lappacea* (Chakachaka), our results demonstrated that the ethyl acetate extract from roots of *K. lappacea* (KLA) was highly active on mostly all of the Gram positive tested bacterial strains (reference and clinical isolated strains), including also a large number of *Staphylococcus* species.

Among isolated compounds, the neolignan conocarpan (**1**) has been identified as the most promising antibacterial compound as it has shown a strong antibacterial activity with MICs between 1 and 2 μg/mL against all the Gram-positive tested bacteria. Previous evaluation of antibacterial activity of *K. lappacea*, collected in north of Peru, was described for the hydroethanolic extract, inhibiting growth of *Staphylococcus aureus* at 128 μg/mL (Bussmann et al., 2010). Hexane and dichloromethane extracts containing neolignans and norneolignans were active against different bacterial and fungal strains and have also demonstrated anti-inflammatory potential.

Neolignans conocarpan, together with eupomatenoid-5 and -6, isolated from *Piper regnelli* var *pallescens* also exhibited strong activity on *Staphylococcus aureus* and *Bacillus subtilis* strains growth (MIC values 6.25, 1.56 and 3.12 μg/mL respectively) but not on *Escherichia coli* and *Pseudomonas aeruginosa* (Pessini et al., 2003). This selectivity on Gram positive bacteria is in accordance with our results.

Antitrypanosomal, antifungal, anti-biofilm activity have also been described for these neolignans (Luize et al., 2006; Lopes et al., 2017; Brambilla et al., 2017).

In conclusion, bioguided study led us to identify conocarpan **1** and ursolic acid **10** as compounds that may be involved in the antibacterial effect of the plants. Our work pointed out the fact that, in a context of urgent need for antibacterial agents, further studies should be dedicated to the exploration of the antibacterial mechanism and biological targets of both triterpenes and neolignans.

## Acknowledgements

The authors thank Félix Galleguillos and the Taira Community for the collection of plants, to Pr. Alicia Marticorena for the identification of vegetal material and to CONICYT (Comisión Nacional de Investigación Científica y Tecnológica - Chile). Scholarship for PhD studies support of S. Ortiz.

## Author Contribution

Sergio.Ortiz: Investigation, Formal analysis, Writing - original draftMarylin Lecso: Formal analysisChristine Bonnal: Formal analysisSandrine Houze Formal analysisSylvie Michel : SupervisionRaphael Grougnet / Supervision, Formal analysisSabrina Boutefnouchet: Supervision, Project administration, Writing - original draft.

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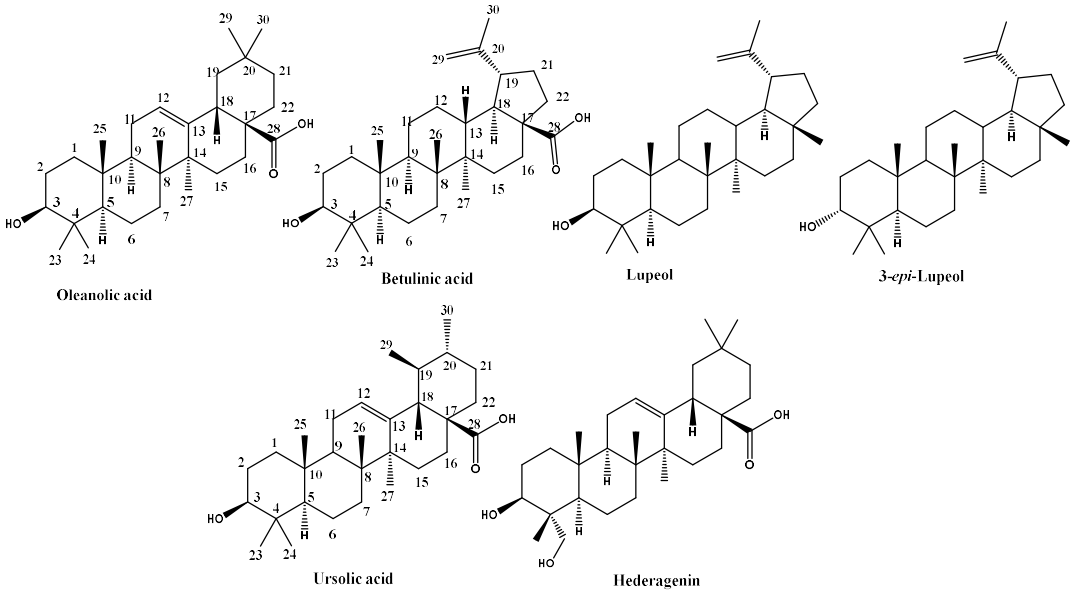
## Supporting Information

**NMR and MS data of isolated compounds.**

Structural elucidation of isolated compounds was achieved by comparison of their experimental spectroscopy ($^1$H, $^{13}$C NMR), specific rotation $[\alpha]_D$ and spectrometry (MS) data with those previously reported in the literature.




**Oleanolic acid**

White powder. $[\alpha]_D^{26}$ + 80.1° (c = 0.1, $CHCl_3$). $^1$H-NMR ($CDCl_3$) δ 5.28 [t, *J* = 4.0 Hz, 1H, H-12], 3.22 (dd, *J* = 10.8, 4.2 Hz, 1H, H-3], 2.82 [dd, *J* = 13.5, 4.0 Hz, 1H, H-18], 2.17-2.08 [m, 1H, H-16a], 2.02-1.77 [m, 7H, H-22b, H-16b, H-11, H-2, H-19a], 1.77-1.56 [m, 5H, H-22a, H-9, H-1a, H-6a, H-7a], 1.46-1.30 [m, 4H, H-21a, H-6b, H-7b, H-19b], 1.23-1.20 [m, 3H, H-21b, H-15], 1.13 [s, 3H, H-27], 1.06 [s, 3H, H-23], 1.04-1.00 [m, 1H, H-1b], 0.88-0.86 [m, 1H, H-5], 0.97 [s, 3H, H-26], 0.93 [s, 3H, H-24], 0.90 [s, 3H, H-30], 0.77 [s, 3H, H-29], 0.75 [s, 3H, H-25]. $^{13}$C NMR ($CDCl_3$) δ 183.42 (C-28), 143.56 (C-13), 122.65 (C-12), 79.28 (C-3), 55.12 (C-5), 47.08 (C-9), 46.51 (C-17), 45.75 (C-19), 41.59 (C-14), 39.91 (C-18), 39.05 (C-8), 38.75 (C-4), 38.56 (C-1), 36.98 (C-10), 33.85 (C-21), 33.07 (C-29), 32.63 (C-7), 32.37 (C-22), 30.56 (C-20), 28.31 (C-23), 27.70 (C-15), 27.65 (C-2), 25.28 (C-27), 23.71 (C-30), 23.38 (C-16), 22.98 (C-11), 18.23 (C-6), 17.08 (C-26), 15.38 (C-24), 15.28 (C-25). ESI-MS *m/z* (positive mode) 479.4 $[M+Na]^+$, 935.8 $[2M+Na]^+$ (calculated for $C_{30}H_{48}O_3$: 456.3).

**Betulinic acid**

White powder. $[\alpha]_D^{21}$ + 8.5 (c = 0.06, pyridine). $^1$H NMR ($CDCl_3$) δ 4.73 [br s, 1H, H-29a], 4,61 [br s, 1H, H-29b], 3.20 [dd, *J* = 10.5, 4.2 Hz, 1H, H-3], 3.00 [td, *J* = 10.0, 5.0 Hz, 1H, H-19], 2.24-2.18 [m, 1H, H-13], 1.69 [s, 3H, H-30], 0.98 [s, 3H, H-27], 0.97 [s, 3H, H-22], 0.95 [s, 3H, H-26], 0.82 [s, 3H, H-25], 0.76 [s, 3H, H-24]. $^{13}$C NMR ($CDCl_3$) δ 180.56 (C-28), 151.27 (C-20), 109.86 (C-29), 79.15 (C-3), 56.47 (C-17), 53.24 (C-5), 50.31 (C-9), 49.36 (C-19), 47.08 (C-18), 43.62 (C-14), 40.73 (C-8), 38.23 (C-4), 37.96 (C-13), 37.36 (C-1), 37.18 (C-22), 36.98 (C-10), 33.98 (C-7), 33.00 (C-16), 29.80 (C-15), 29.91 (C-21), 28.13 (C-2), 27.64 (C-23), 24.98 (C-12), 20.71 (C-11), 19.50 (C-30), 18.90 (C-6), 16.29 (C-26), 16.19 (C-25), 15.50 (C-24), 14.85 (C-27). ESI-MS m/z (positive mode) 479.4 $[M+Na]^+$, 935.8 $[2M+Na]^+$ (calculated for $C_{30}H_{48}O_3$: 456.3).

**Lupeol**

White powder. $[\alpha]_D^{22}$ + 25° (c = 0.55, EtOH). $^1$H NMR ($CDCl_3$) δ 4.67 [s, 1H, H-29a], 4.56 [s, 1H, H-29b], 3.18 [ dd, *J* = 10.5, 4.5 Hz, 1H, H-3], 2.32-2.20 [m, 1H, H-19], 1.70 [s, 3H, H-30], 1.02 [s, 3H, H-26], 0.95 [s, 3H, H-23], 0.93 [s, 3H, H-27], 0.82 [s, 3H, H-25], 0.78 [s, 3H, H-28], 0.75 [s, 3H, H-24]. $^{13}$C NMR ($CDCl_3$) δ 150.32 (C-20), 109.48 (C-29), 79.02 (C-3), 55.34 (C-28), 54.21 (C-5), 49.75 (C-9), 48.00 (C-18), 47.65 (C-17), 46.42 (C-19), 41.55 (C-14), 39.98 (C-8), 39.07 (C-1), 38.56 (C-13), 38-05 (C-10), 37.00 (C-4), 33.98 (C-7), 33.52 (C-22), 29.53 (C-16), 29.51 (C-21), 27.03 (C-23), 26.38 (C-15), 25.70 (C-2), 25.08 (C-12), 20.45 (C-11), 19.25 (C-30), 17.98 (C-6), 15.98 (C-25), 15.64 (C-24), 15.32 (C-24), 14.36 (C-27). ESI-MS m/z (positive mode) 449.4 $[M+Na]^+$ (calculated for $C_{30}H_{50}O$: 456.3).

**3-epi-Lupeol**

White powder. $[\alpha]_D^{18}$ + 15.2° (c = 0.01, $CHCl_3$). $^1$H NMR ($CDCl_3$) δ 4.70 [s, 1H, H-29a], 4.64 [s, 1H, H-29b], 3.41 [br s, 1H, H-3], 2.40-2.36 [m, 1H, H-19], 1.97-1.85 [m, 2H, H-21], 1.77-1.64 [m, 2H, H-2], 1.63 [s, 3H, H-30], 1.61-1.40 [m, 5H, H-22, H-16, H-18], 1.40-1-27 [m, 13H, H-15, H-12, H-11, H-7, H-6, H-5, H-1,], 1.25-1.18 [m, 2H, H-13, H-9], 0.99 [s, 3H, H-26], 0.95 [s, 3H, H-27], 0.94 [s, 3H, H-24], 0.86 [s, 3H, H-25], 0.84 [s, 3H, H-23], 0.79 [s, 3H, H-28]. $^{13}$C NMR ($CDCl_3$) δ 152.43 (C-20), 109.11 (C-29), 78.32 (C-3), 51.02 (C-9), 48.98 (C-5), 48.34 (C-18), 48.01 (C-19), 43.18 (C-17), 43.00 (C-14), 42.05 (C-8), 41.31 (C-22), 39.15 (C-13), 37.93 (C-4), 37.26 (C-10), 35.91 (C-16), 34.13 (C-17), 33.21 (C-1), 30.01 (C-21), 28.83 (C-23), 27.52 (C-15), 25.78 (C-2), 25.21 (C-12), 22.12 (C-24), 20.43 (C-11), 19.00 (C-30), 18.51 (C-6), 18.21 (C-28), 16.19 (C-25), 16.13 (26), 13.99 (C-27). ESI-MS m/z (positive mode) 449.4 $[M+Na]^+$ (calculated for $C_{30}H_{50}O$: 426.3).

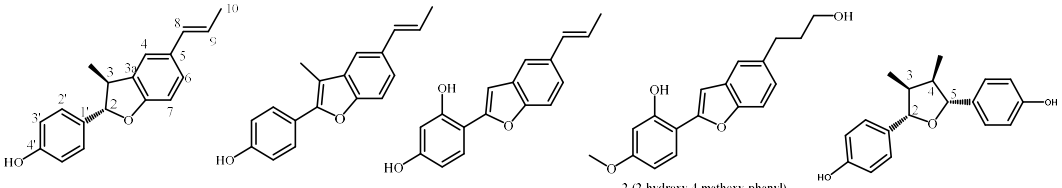
**Ursolic acid**

White powder.$[\alpha]_D^{25}$ +66.7° (c = 1.00, $CHCl_3$). $^1$H NMR ($CDCl_3$) δ 5.25 [ br s, 1H, H-12], 3.22 [dd, *J* = 10.5, 4.3 Hz, 1H, H-3], 2.17 [d, *J* = 11.0 Hz, 1H, H-18], 1.08 [s, 3H, H-27], 0.99 [s, 3H,

H-24], 0.95 [d, *J* = 6.0 Hz, 3H, H-30], 0.93 [s, 3H, H-25], 0.86 [d, *J* = 6.2 Hz, 3H, H-29], 0.78 [s, 3H, H-23]. $^{13}$C NMR ($CDCl_3$) δ 178.27 (C-28), 139.50 (C-13), 123.98 (C-12), 76.78 (C-3), 53.10 (C-5), 52.28 (C-18), 46.78 (C-9), 46.65 (C-17), 42.05 (C-14), 39.01 (C-19), 38.45 (C-20), 38.24 (C-1), 38.12 (C-8), 38.08 (C-4), 36.65 (C-10), 36.12 (C-22), 32.10 (C-7), 30.11 (C-21), 28.29 (C-23), 28.11 (C-2), 27.31 (C-15), 23.90 (C-16), 23.17 (C-27), 22.38 (C-11), 21.21 (C-30), 17.76 (C-6), 16.78 (C-26), 16.53 (C-29), 15.96 (C-25), 15.28 (C-24). ESI-MS m/z (positive mode) 479.4 $[M+Na]^+$, 935.8 $[2M+Na]^+$ (calculated for $C_{30}H_{48}O_3$: 456.3).

**Hedaragin**

White powder. 1H-NMR (CDCl3) δ 5.31 [s, 1H, H-12], 3.69 [d, *J* = 5.0 Hz, 1H, H-24a], 3.55 [d, *J* = 5.0 Hz, 1H, H-24b], 3.31-3.26 [m, 1H, H-3], 2.92 [dd, *J* = 4.5 Hz, 1H, H-18], 1.24 [s, 3H, H-24], 1.04 [s, 3H, H.-27], 1.01 [s, 3H, H-26], 0.98 [s, 3H, H-30], 0.89 [s, 3H, H-29], 0.77 [s, 3H, H-25]. $^{13}$C-NMR ($CDCl_3$) δ 178.9 (C-28), 144.3 (C-13), 122.7 (C-12), 74.1 (C-3), 68.0 (C-23), 48.5 (C-9), 46.8 (C-17), 46.4 (C-19), 43.0 (C-4), 42.5 (C-14), 41.8 (C-18), 40.2 (C-5), 39.5 (C-8), 38.7 (C-1), 36.9 (C-10), 34.6 (C-21), 33.8 (C-22), 33.5 (C-29), 33.0 (C-7), 31.1 (C-20), 28.4 (C-15), 27.5 (C-2), 26.2 (C-27), 24.5 (C-30), 24.0 (C-11), 23.8 (C-16)18.7 (C-6), 17.5 (C-26), 16.0 (C-25), 13.5 (C-24). ESI-MS *m/z* (negative mode) 471.4 $[M-H]^-$, 943.7 $[2M-H]^-$ (calculated for $C_{30}H_{48}O_4$: 472.4).




**Conocarpan**

Colorless gum. $[\alpha]_D^{27}$ = +125 (c=0.5, $CHCl_3$). $^1$H NMR ($CDCl_3$) δ 7.29 [d, *J* = 8.6 Hz, 2H, H-2', H-6'], 7.14 [s, 1H, H-4], 7.13 [d, *J* = 8.6 Hz, 1H, H-6], 6.83 [d, *J* = 8.6 Hz, 2H, H-3', H-5'], 6.77 [d, *J* = 8.6 Hz, 1H, H-7], 6.38 [d, *J* = 15.7 Hz, 1H, H-8], 6.15-6.05 [m, 1H, H-9], 5.20 [br s, 1H, -OH], 5.09 [d, *J* = 9.0 Hz, 1H, H-2], 3.41 [q, *J* = 7.4 Hz, 1H, H-3], 1.87 [ dd, *J* = 6.6, 1.6 Hz, 3H, H-11], 1.40 [d, *J* = 6.8 Hz, 1H, H-10]. $^{13}$C NMR ($CDCl_3$) δ 158.28 (C-7a), 155.71 (C-4'), 132.73 (C-1'), 132.21 (C-3a), 131.29 (C-8), 130.87 (C-5), 127,93 (C-2', C-6'), 126.35 (C-6), 123.11 (C-9), 120.36 (C-4), 115.56 (C.3', C-5'), 109.32 (C-7), 92.70 (C-2), 45.36 (C-3), 18.44 (C-10), 17.91 (C-11). ESI-MS *m/z* (positive mode) 289.3 $[M+Na]^+$ (calculated for $C_{18}H_{18}O_2$: 266.1).

**Eupomatenoid-6**

Colourless gum. $^{1}$H NMR (Acetone-*d6*) δ 7.68 [d, *J* = 7.8 Hz, 2H, H-2', H-6'], 7.53 [d, *J* = 1.8 Hz, 1H, H-4], 7.38 [d, *J* = 8.4 Hz, 1H, H-7], 7.32 [dd, *J* = 8.4, 1.8, 1H, H-6], 7.00 [d, *J* = 7.8 Hz, 2H, H-3', H-5'], 6.52 [d, *J* = 15.6 Hz, 1H, H-8], 6.39-6.01 [m, 1H, H-9], 2.43 [s, 3H, H-11], 1.87 [dd, *J* = 6.5, 1.8 Hz, 3H, H-10]. $^{13}$C NMR (Acetone-*d6*) δ 158.47 (C-4'), 153.72 (C-7a), 152.49 (C-2), 133.79 (C-5), 132.59 (C-8), 132.12 (C-3a), 129.02 (C-2', C-6'), 124.45 (C-9), 123.75 (C-1'), 122.90 (C-6), 117.03 (C-4), 111.25 (C-7), 110.02 (C-3), 18.57 (C-10), 9.43 (C-11). ESI-MS *m/z* (negative mode) 263.2 $[M-H]^-$ (calculated for $C_{18}H_{16}O_2$: 264.3).

2-(2,4-dihydroxyphenyl)-5-(E)-propenylbenzofuran

Yellowish powder. $^{1}$H NMR (Acetone-$d_6$) δ 7.77 [d, *J* = 8.7 Hz, 1H, H-6'], 7.54 [d, *J* = 1.6 Hz, 1H, H-4], 7.41 [d, *J* = 8.7 Hz, 1H, H-7], 7.28 [dd, *J* = 8.7, 1.6 Hz, 1H, H-6], 7.21 [s, 1H, H-3], 6.58 [d, *J* = 1.8 Hz, 1H, H-3'], 6.54-6.47 [m, 2H, H-8, H-5'], 6.29-6.21 [m, 1H, H-9], 1.87 [dd, *J* = 6.6, 1.6 Hz, 3H, H-10]. $^{13}$C NMR (Acetone-$d_6$) δ 159.68 (C-4'), 156.75 (C-2'), 154.80 (C-2), 153.73 (C-7a), 133.89 (C-5), 132.24 (C-8), 131.23 (C-3a), 128.56 (C-6'), 124.37 (C-9), 122.45 (C-6), 118.48 (C-4), 111.04 (C-7), 110.68 (C-1'), 108.53 (C-5'), 104.11 (C-3), 104.01 (C-3'), 18.47 (C-10). ESI-MS *m/z* 265.1 (negative mode) $[M-H]^-$ (calculated for $C_{17}H_{14}O_3$: 266.1)

2-(2-hydroxy-4-methoxy-phenyl)-5-(3-hydroxypropyl)-benzofuran

Yellowish gum. $^{1}$H NMR (Acetone-*d6*) δ 7.86 [d, *J* = 7.5 Hz, 1H, H-6'], 7.40 [d, *J* = 1.8 Hz, 1H, H-4], 7.38 [d, *J* = 7.5 Hz, 1H, H-7], 7.25 [s, 1H, H-3], 7.09 [dd, *J* = 7.5, 1.8 Hz, 1H, H-6], 6.66 [d, *J* = 2.0 Hz, 1H, H-3'], 6.58 [dd, *J* = 7.4, 2.0 Hz, 1H, H-5'], 3.81 [s, 3H, -$OCH_3$], 3.60 [c, *J* = Hz, 2H, H-10], 2.77 [c, *J* = Hz, 2H, H-8], 1.85 [q, *J* = Hz, 3H, H-9]. ESI-MS m/z (negative mode) 297.1 $[M-H]^-$ (calculated for $C_{18}H_{18}O_4$: 298.1).

*meso*-3,3'-didemethoxynectandrin B

White gum. $[\alpha]_D^{25}$ +25.0 (c = 0.05, $CHCl_3$). $^{1}$H NMR ($CDCl_3$) δ 7.27 [d, *J* = 7.5 Hz, 4H, H-2', H6', H-2'', H-6''], 6.75 [d, *J* = 7.5 Hz, 4H, H-3', H-5', H-3'', H-5''], 4.45 [d, *J* = 7.0 Hz, 2H, H-2, H-5], 2.31 [m, 2H, H-3, H-4], 0.97 [d, *J* = 6.8 Hz, 6H, Me-3, Me-4]. ESI-MS *m/z* (negative mode) 283.1 $[M-H]^-$ (calculated for $C_{18}H_{20}O_3$: 284.1).

## Conclusion and perspectives

The most important contribution of the precedent publication are:

- Evidence of the antibacterial activity of *Aloysia deserticola* and *Krameria lappacea*, medicinal plants with vast use against infectious diseases, produced by gram-positive bacteria infection.
- Purification and identification by spectroscopy and spectrometry methodology of active compounds from active extracts by a *bio*-guided approach, resulting in:
    - First phytochemical study of *K. lappacea* roots collected in Chile with the purification and identification of five benzofuran with conocarpan as the more promising compound with MICs of 2-1 µg/mL against Gram-positive reference and clinical isolated bacteria strains.
    - First phytochemical study of *A. deserticola* comprising six compounds with antibacterial properties.
- Conocarpan compound from *K, lappacea* roots exerts antibacterial activity with MICs values at equal range of commercial antibiotic gentamicin.

The main perspectives of this work would be to decipher the mechanisms and target(s) involved in the antibacterial activity of (-)-conocarpan, to isolate other natural benzofurans and to generate a library of semi-synthetic derivatives in order to establish structure-activity relationships.

# I.4 Antiparasitic activity

Aiming to explore new therapeutic potential of medicinal plants from Taira Community, antiparasitic activity were evaluated for extracts and *bio*-guided isolation of bioactive compounds was performed.

The neglected tropical diseases (NTDs) are a group of parasitic disease with a high prevail in tropical and subtropical countries, affecting more than one billion people, and cost developing economies billions of dollars every year. The WHO (World Health Organization) lists fourteen diseases including the African trypanosomiasis, leishmaniasis, echinococcosis, rabies and schistosomiasis. Additionally, 219 million cases of malaria (435000 deaths), caused by Plasmodium parasites and transmitted by the bites of infected female Anopheles mosquitoes, were reported in 2017 (239 millions in 1990, 607000 deaths) [85].

Chile is a country free of endemic malaria, leishmaniasis or trypanosomiasis. The north and central region are the habitat of the vector (*Triatoma infestans*) of the parasite *Trypanosoma cruzi*, which cause the Chagas disease. However, due to campaigns to eradicate the vector, no cases have occurred since 1999 [86].

## I.4.1 Malaria

As mentioned before, in 2017, an estimation of 219 million cases of malaria occurred worldwide, compared with 239 million cases in 2010. Over the 90% of malaria cases are reported in the African region, where Nigeria and Democratic Republic of the Congo concentrate the 35 % of cases worldwide. 435,000 death from malaria were estimated globally in 2017, compared with 607,000 in 2010. It is still estimated that a child dies of malaria every two minutes.

*Plasmodium falciparum* is the most common species not only in this region (accounting for 99.7% of estimated malaria cases in 2017), but also in South-East Asia, Eastern Mediterranean and Western Pacific (62.8, 69 and 71.9% of cases respectively) [87].

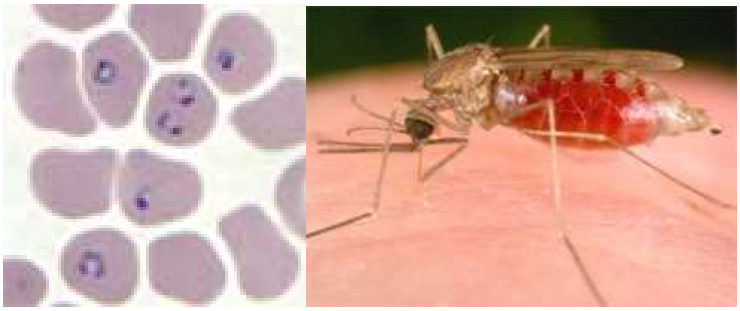


**Figure 45: Ring-form trophozoites of *P. falciparum* in a thin blood smear. *Anopheles* mosquito.**

The lifecycle of malaria involves cyclical infection of human and female *Anopheles* mosquitoes. In the bite, sporozoites of *Plasmodium* parasites are inoculated into the human host. Sporozoites infect liver cells and mature into schizonts, which are lysed and merozoites-parasitic form are released. After this replication liver-stage, the parasites undergo asexual multiplication in the erythrocytes. Merozoites infect red blood cells, which the ring state (trophozoites) mature into schizonts. These schizonts rupture, realising merozoites. Some parasites differentiate into sexual erythrocytes stages, called gametocytes. Female *Anopheles* mosquito ingests these gametocytes during a blood meal. In the mosquito, the multiplication cycle is called sporogonic. The parasite generated zygotes turned in motile and elongated ookinetes, which invade the mosquito gut, where they develop into oocysts. Then, the oocyts grow, rupture and release sporozoites, which arrive to the salivary glands of the mosquito, which by inoculation by bite into a new human host perpetuates the malaria lifecycle [87].

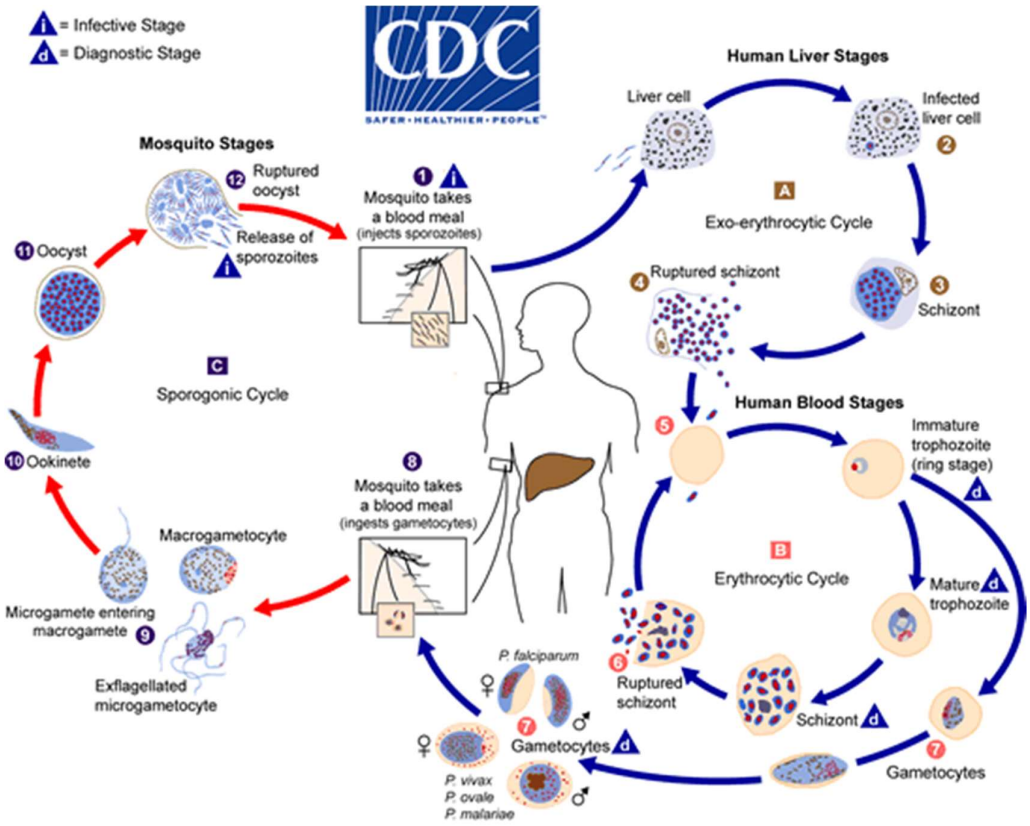



**Figure 46: *Plasmodium falciparum* lifecycle.**

Malaria infections may manifest in a wide variety of symptoms and it can be categorized as uncomplicated or severe infections. More commonly symptoms of uncomplicated infections are fever, chills, sweats, headaches, nausea, vomiting, body aches and general malaise.

Severe malaria occurs when infection are complicated by serious organ failures or abnormalities in the blood and metabolism in the patient. Cerebral malaria present symptoms such as abnormal behaviour, impairment of consciousness, seizures, coma or other neurologic abnormalities. Severe anaemia, haemoglobinuria, blood coagulation disorders, acute respiratory distress syndrome or acute kidney injury are also symptoms of severe malaria infections. Untreated severe malaria could arrive to mortality rates of 10-50%.

Known as artemisinin-combinatory therapy, artemisinin associated with dihydroartemisinin, artemether or artesunate is the most effective treatment to fight *P. falciparum* infection, and contributes to decrease resistance to single drug component. Other drugs are amodiaquine, lumefantrine, mefloquine or sulfadoxine. About 90% of uncomplicated forms are relieved with

this combination. In case of severe malaria, intravenous administration of artemisinins or quinine is often required, together with treatment of the complications.

*Plasmodium falciparum* strain with resistance to chloroquine have been reported from the 1950 decade [88] and in the last years, new strains of *P. falciparum* in South East Asia and Africa have been reported also to be less sensible to artemisinin therapies. Thus, new therapies option against *P. falciparum* infections are still focus of new research.

## I.4.2 Leishmaniasis

Leishmaniasis is caused by protozoan parasites from more than 20 *Leishmania* species, transmitted to humans by the bites of the infected female phlebotomine "sand-fly". There are three main forms of the disease: cutaneous, visceral also known as kala-azar and mucocutaneous [89].




**Figure 47: a) Extracellular form of *Leishmania donovani*, b) sand-fly.**

Promastigote stage are injected by infected bite of phlebotomine sand flies, during their blood meals. After promastigote phagocytised by macrophages, the parasites transform, inside the phagocytic cell, to amastigote form, which multiply by simple division and proceed to infect other phagocytic cells. Then, infection becomes symptomatic and a female phlebotomine sand fly become infected by ingesting infected cells during blood meals from infected host. In sand flies, amastigote are transformed into promastigotes in the gut of the vector and migrate to the proboscis, prepared to infect a new host.

There are 600.000 to 1.000.000 new cases of cutaneous and mucocutaneous are estimated each year worldwide. This form is characterise by severe skin lesions leaving life-long scars and serious disabilities, including the destruction of mucous membranes of the nose, mouth and throat

for the mucocutaneous form. Around 50.000 to 90.000 new cases of visceral leishmaniasis (also known as kala-azar) are estimated each year, worldwide, characterized by fever, enlargement of the spleen and sometimes also the liver.

| Type | Pathogen examples | Location |
|---|---|---|
| Cutaneous leishmaniasis | *L. major*, *L. tropica* and *L. aethiopica* in Old world and *L. mexicana* in America. | Europe, Africa, Asia and America. |
| Mucocutaneous leishmaniasis | *L. braziliensis.* | Latinamerica. |
| Visceral leishmaniasis | *L. donovani* complex (*L. donovani*, *L. infantum*). | Europe, Asia and Africa. |

**Table 7: Pathogen of the different leishmanial diseases form.**

The treatment is determinate by the location where the infection has occured, the specie of *Leishmania* and the type of infection. Visceral form is treated with amphotericin B or a combination of pentavalent antimonial, paromomycin and miltefosine. For cutaneous disease paromomycin, fluconazole or pentamidine could be also effective. Several and serious side effects are associated to these antileishmanial drugs, such as heart inflammation and irregular heartbeat, liver disorders, pancreatitis and/or hearing loss. Despite of these side effects, there were no new drugs on the market since the 1960's, urging the need for research of modern antileishmanial agents.

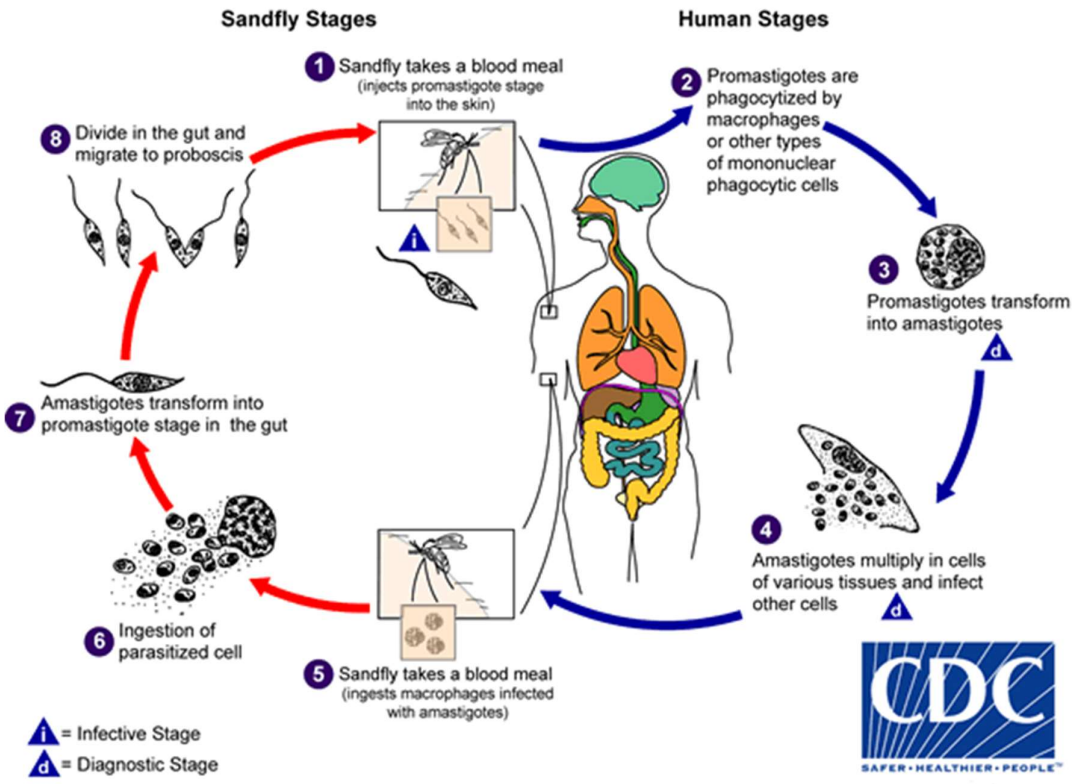



**Figure 48: *Leishmania* lifecycle.**

## I.4.3 Trypanosomiasis

Human African trypanosomiasis, also known as sleeping sickness, is a parasitic disease transmitted by the bite of "Glossina" insect, commonly known as the tsetse fly. The disease mostly affects poor populations living in remote rural areas of Africa. Untreated, it is usually fatal. Generally, new cases are reported in the rural areas of Africa, and in some cases, in suburban areas of big cities in countries where the disease is endemic. Protozoa parasites belonging to the *Trypanosoma* genus are the responsible of African trypanosomiasis. There are two types that infect humans, *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*, which the first causes over 98% of reported cases. *Trypanosoma brucei brucei* causes animal trypanosomiasis, leading weight loss and anaemia, affecting rearing of cattle and agriculture development [90].



**Figure 49: Trypomastigotes of *T brucei* spp. in a blood smear stained with Giemsa. Tsetse fly during a blood meal.**

Trypomastigote are injected into the skin tissue of a new human host, by the bite of an infected tsetse fly (*Glossina* genus). The parasites are transformed into bloodstream trypomastigote in the bloodstream and carry out to other body fluids, continuing the replication by binary fusion. The entire life cycle of African trypanosomes is represented by extracellular stages. Then, the tsetse fly is infected during a blood meal of infected host. The procyclic trypomastigotes continue the binary fission multiplication in the midgut of the fly, transforming into epimastigotes, which then reach the salivary glands of fly and continue the multiplication by binary fission.

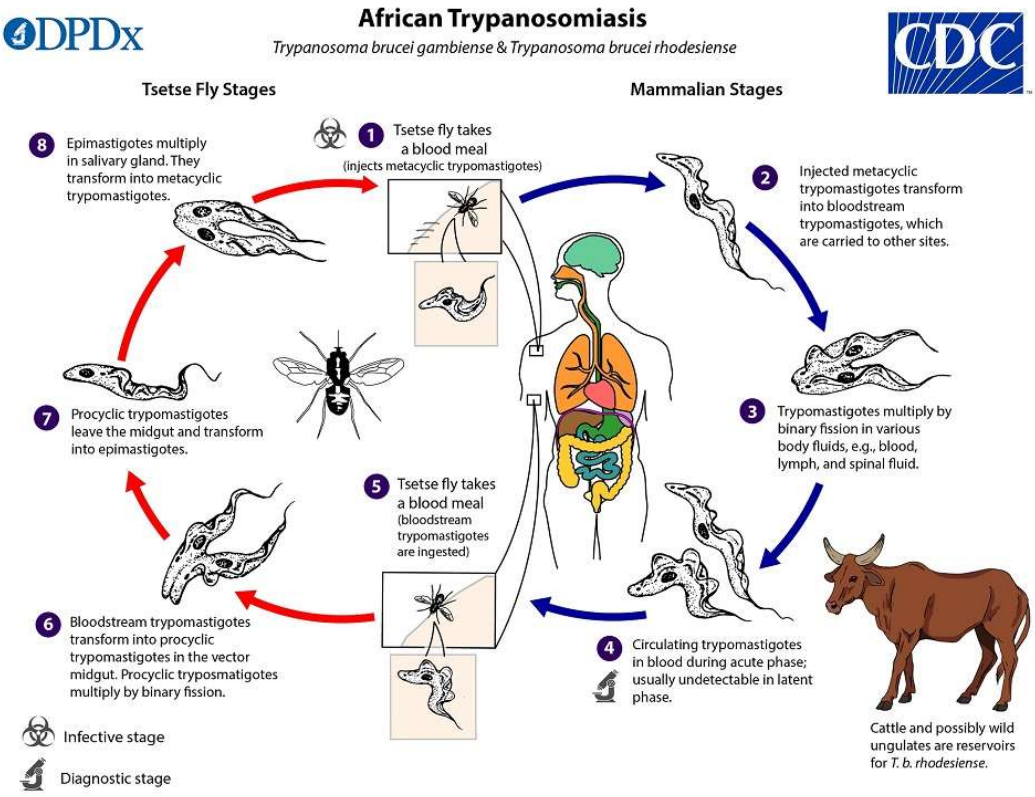



**Figure 50: *Trypanosoma* lifecycle.**

Symptoms are classified in two stages, depending the infection zone in the human body. Fever, headaches, joint pains, itching, characterize the hemolymphatic phase and occasionally red sore is developed at the location of tsetse fly bite. At this stage, if infection is untreated, anaemia, endocrine cardiac and kidney dysfunctions may raise as severe symptoms.

The neurological phase begins when the parasites invade the central nervous system by passing through the blood-brain barrier. Trouble sleeping, confusion and poor coordination characterize this stage. Without treatment, the disease is invariably fatal, with progressive mental deterioration leading to coma, systemic organ failure and death.

In sub-Saharan Africa, 70 million people from 36 countries are at risk of contamination. An estimated 11,000 people are currently infected with 2,800 new cases and around 3,500 deaths reported in 2015.

## I.4.4 Methodology

In a search for new therapeutic options to fight malaria and neglected tropical diseases, a bioguided approach was performed through an *in vitro* screening of medicinal plants from the Atacama People against *Plasmodium falciparum*, *Leishmania* spp. and *Trypanosoma brucei*.

### I.4.4.1 Antileishmanial and antitrypanosomal activities:

*In vitro* fluorescence assays were carried out in order to detect inhibitory activities against *Leishmania* species (*L. donovani*, *L. amazonensis* and *L. infantum*) and *Trypanosoma brucei brucei*. A first evaluation was carried out at several extract concentration allowing us to calculated directly $IC_{50}$ values against promastigote *Leishmania* spp and bloodstream *Trypanosoma* forms. The parasitic viability *in vitro* after incubation with respective extract concentration, was measured after addition of AlamarBlue® reagent. The reduction of this reagent by mitochondrial activity, it is directly proportional to the quantity of parasitic cell viability.

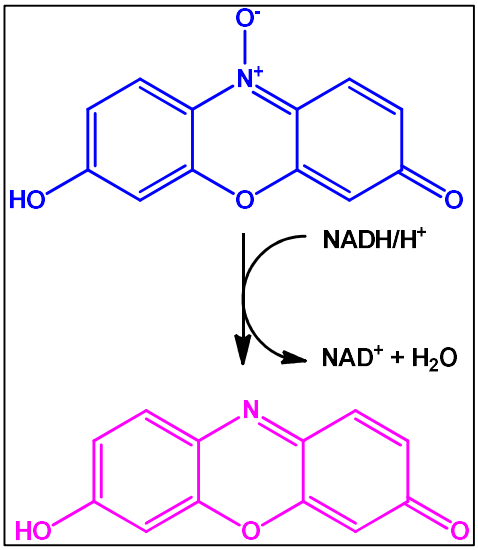



**Figure 51: AlamarBlue® reduction by NADH enzyme.**

### I.4.4.2 Antiplasmodial activity

Hypoxnthine is a natural purine derivative and is required reagent in *Plasmodium in vitro* cultures, since these parasites (as well in certain cell and bacteria) requires a source of nucleic acid synthesis and energy metabolism. The [$^3$H]-hypoxanthine uptake method *in vitro* assay measures

the incorporation of $^{3}$H-hypoxanthine into parasite DNA as a measure of parasite replication in red blood cells.

I*n vitro* [$^{3}$H]-hypoxanthine intake assays was chosen to evaluate the antiplasmodial activity of extracts. A first screening was realized at 100 and 50 µg/mL. The extracts that showed an inhibition higher than 50 % were re-evaluated at several concentration and $IC_{50}$ values were calculated.

Aiming to identify against malaria, trypanosomiasis or leishmaniasis infections, the followed worflow was applied:

- *In vitro* evaluation of plant extracts towards several strains of *Leishmania*, *Trypanosoma* and *Plasmodium*.
- Fractionation of bioactive extracts for compounds purification: apolar extract of *A. atacamensis* was subjected to silica gel chromatography due to low *in vitro* $IC_{50}$ against *P. falciparum* W2 (chloroquine resistant) strain. Mulinic acid (**1**), mulin-11,13-dien-20-oic acid (**2**) were isolated and characterized.
- *In vitro* evaluation of compounds **1**-**2** against chloroquine-sensitive 3D7 strain and chloroquine-resistant W2 strains of *P. falciparum*.

## I.4.5 Draft 1: Exploration of the antiparasitic potential of medicinal plants from North of Chile.

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Introduction

Neglected tropical disease are a group of parasitic diseases with prevalence in the tropical and subtropical regions, such as leishmaniasis, trypanosomiasis and malaria. Because of side effects of drugs on the market and emergence of resistant strains, alternatives for chemotherapy are urgently needed. Ethnopharmacological approach has shown a great interest for discovery of new antiparasitic drugs from plants used in traditional medicines like the well-known artemisinin. Atacama communities (Atacameños), in the north of Chile, report about more than 200 medicinal plants, poorly explored p to date for biological screening.

In this work, eighteen different plants used in the Atacameños traditional medicine were collected. Forty ethyl acetate and methanolic extracts were obtained using Büchi Speed-Extractor and evaluated for their activities against *Leishmania* species (*L. infantum*, *L. donovani* and *L. amazonensis*), *Trypanosoma brucei brucei* and *Plasmodium falciparum* using the alamarBlue® and [$^3$H]-hypoxanthine uptake methodology. In parallel, toxicity of extracts was evaluated on macrophage cells.

2 Experimental

2.1 General

Automatized pressurized extractions were performed on a Speed Extractor E-914 apparatus provided by Büchi (Flawil, Switzerland) equipped with four cells (120 mL) and a collector with four flat bottom vials (220 mL). Maximum pressure and temperature can be adjusted from 50 to 150 bar and from 30 to 200 °C, respectively. Preparative HPLC was performed on an AP-MOD-100 apparatus provided by Armen Instrument (Saint-Avé, France), with C18 PursuitTM Varian (250 mm x 30 mm, 10 µm) column, Büchi UV/Vis Filter-photometer detector, Merck D-2500 integrator and Büchi C-660 collector. Mass spectrometry was performed on a mass spectrometer

ZQ 2000 Waters (Saint-Quentin, France). Silica gel 60 0.025-0.04 mm (Macherey-Nagel GmbH & Co.) was used for column chromatography (CC). $^{1}H$ and $^{13}C$-NMR experiments were performed in deuterated chloroform, benzene and acetone. Spectra were recorded on a Bruker AC 300 MHz or a Bruker 400 MHz spectrometer (Wissembourg, France). $NaHCO_3$, Dimethylsulfoxide (DMSO), methanol (MeOH), ethanol (EtOH), acetonitrile (ACN), dichloromethane (DCM), cyclohexane (Cy) and ethyl acetate (EtOAc) were purchased from Carlo Erba Reagents (Val-de-Reuil, France). HEPES, FBS, RPMI-1640, M199 and HMI-9 mediums were purchased to Gibco (Waltham, Massachusetts). G418 antibiotic was purchased to Sigma (St Louis, Missouri).

2.2 Plant material and extraction

Collection of medicinal 18 plants species was carried out in September 2016, in the Atacama People community of Taira (Calama, Chile) in accordance with the Chilean legislation. Air-dried plant species were subjected to successive extraction by PSE (Büchi E-914, Flawil, Switzerland) with EtOAc and MeOH as previously reported (Ortiz et al., 2018). A total of 40 extracts were obtained. Solutions at 10 mg/mL were prepared in DMSO and stocked at 4°C until *in vitro* evaluations.

2.3 *In vitro* antiparasitic activity of plant extract

2.3.1 AlamarBlue™ method for antileishmanial and antitrypanosomal assays

The alamarBlue™ assays was applied for determining the antileishmanial activity of extracts and amphotericin B was used as a reference drug (Xingi et al., 2009). Stationary-phases of *L. donovani* (MHOM/ET/0000/HUSSEN), *L. infantum* (MHOM/GR/2002/GH12), *L. amazonensis* (MPRO/BR/72/M1845) promastigotes (2.0 x $10^7$ cells/mL) were seeded into 96-well flat bottom plates in 200 µL in M199 medium without phenol red, containing increasing extract concentration in triplicate. The final concentration of DMSO was always <1% and not growth effect of parasite was observed. Following 72 h of incubation at 26 °C, alamarBlue™ (20 µL per well) was added and the plates were incubated at 26 °C for further 12 h. Colorimetric readings were performed at a test wavelength of 550 nm and a reference wavelength of 620 nm. Percentage of growth inhibition was calculated in reference of corresponding DMSO blank. $IC_{50}$ values were determined from dose-response curves via linear interpolation.

*T. brucei brucei* (BSF90-13, Lister 427) bloodstream form parasites (1.0 x $10^6$ cell/mL) were seeded into 96-well flat bottom plates at a density of 5 x $10^4$ cells/mL in 200 µL HMI-9 medium supplemented with 10 % heat inactivated FBS, 10 mM HEPES and 3 µg/mL of G418 antibiotic and containing different extract concentration in triplicate. The final concentration of DMSO was always <1% and not growth effect of parasite was observed. Following 72 h of incubation at 37

°C in 5% $CO_2$, alamarBlue™ (20 µL per well) was added and the plates were incubated at the same precedent conditions for further 24 h. Colorimetric readings were performed at a test wavelength of 550 nm and a reference wavelength of 620 nm. Percentage of growth inhibition was calculated in reference of corresponding DMSO blank. $IC_{50}$ values were determined from dose-response curves via linear interpolation.

2.3.2 [$^3$H]-Hypoxanthine uptake inhibition antiplasmodial assay

*In vitro* activity of plant extracts was determinate following a [$^3$H]-hypoxanthine incorporation assay using an all drug-sensitive strain (3D7) and a chloroquine resistance strain (W2) of *Plasmodium falciparum* (Matile et al., 1990). Dissolved plant extract in DMSO were subsequent diluted in medium of RPMI 1640 in order to have a final concentration of 50 µg/mL in the well. Assays were performed in sterile 96-well flat bottom plate, each well containing 100 µL of parasite culture (0.5 % of parasitaemia, 2.5 % haematocrit) in RPMI 1640 medium (supplemented with HEPES 5.94 g/L, $NaHCO_3$ 2.1 g/L, neomycin (100 U/mL) with [$^3$H]-hypoxanthine) with 5 µL of extract dilution. The plates were incubated for 42 h at 37 °C, 5% $CO_2$, 5% $O_2$ and 90% of $N_2$. After incubation, plates were freeze at -20°C to stop the parasitic growth. Then, plates were defreeze, red blood cells were collected, washed and lysed. [$^3$H]-hypoxanthine incorporated were measured by radioactivity counted in a BetaplateTM counter. Percentages of parasitic growth inhibition were calculated in relation of blanks. For the extracts with a diminution of more than 50 % of parasitic growth and for isolated compounds, $IC_{50}$ were calculated after incubation of a serial of concentration. $IC_{50}$ values were calculated from sigmoidal inhibition curves by algorithmic regression.

2.4 *Bio*-guided isolation of active compounds

Apolar extract of *A. atacamensis*, which had demonstrated the lowest $IC_{50}$ against *P. falciparum* strains, was subjected to silica gel column chromatography fractionation with DCM/MeOH solvent mixtures of increasing polarity (10/0 – 9/1). Fourteen (**AAA1-14**) fractions were obtained and were re-evaluated their antiplasmodial activity at the final concentration at the half of their $IC_{90}$ and $IC_{50}$ values. **AAA1-14** were evaluated at 2.0 and 0.5 µg/mL (final concentration). Percentage of growth inhibition were calculated in basis of respective blanks. The more active fraction **AAA6** was subjected to preparative liquid chromatography on a C18-column with MeOH/$H_2O$ – 90/10 isocratic conditions in order to purify the main metabolites. 1D and 2D NMR spectra of isolated compounds were recorded on a Bruker AC 400 MHz spectrometer (Wissemburg, France) in deuterated chloroform, acetone and benzene.

| Plant Species | Part plant | Solvent Extraction | Code | $IC_{50}$ [µg/mL] of plant extracts against pathogens parasites | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | *Leishmania amazonensis* | *Leishmania donovani* | *Leishmania infantum* | *Trypanosoma brucei brucei* | *Plasmodium falciparum* |
| *Aloysia deserticola* (Phil.) Lu-Irving & O'Leary | L | AcOEt | **ADLA** | > 100 | > 100 | > 100 | 22,8 | > 50 |
| | | MeOH | **ADLM** | > 100 | > 100 | > 100 | > 100 | > 100 |
| | S | AcOEt | **ADSA** | > 100 | > 100 | > 100 | > 100 | > 50 |
| | | MeOH | **ADSM** | > 100 | > 100 | > 100 | 21,95 | > 100 |
| *Artemisia copa* (Phil.) var. copa | L | AcOEt | **ACLA** | 27,6 | 17,7 | <5 | 7,17 | 10,2 |
| | | MeOH | **ACLM** | > 100 | > 100 | > 100 | 70,8 | > 50 |
| | S | AcOEt | **ACSA** | 26 | 11,6 | <5 | 9,7 | 19,8 |
| | | MeOH | **ACSM** | > 100 | > 100 | > 100 | 63,5 | > 50 |
| *Ephedra breana* Phil. | A | MeOH | **EBM** | > 100 | > 100 | > 100 | 17,3 | > 100 |
| | | Basic* | **EBB** | > 100 | > 100 | > 100 | > 100 | > 100 |
| *Lampaya medicinalis* Phil. | A | AcOEt | **LMA** | > 100 | 25,8 | > 100 | 17,1 | > 50 |
| | | MeOH | **LMM** | > 100 | > 100 | > 100 | 67 | >100 |
| *Baccharis tola* Phil. ssp tola | A | AcOEt | **BTA** | > 100 | > 100 | > 100 | 11,25 | > 50 |
| | | MeOH | **BTM** | > 100 | > 100 | > 100 | 62 | 16,9 |
| ***Azorella atacamensis* Phil.** | A | AcOEt | **MCA** | **19,3** | **28,2** | **8** | **11,17** | **1,3** |
| | | MeOH | **MCM** | > 100 | > 100 | > 100 | 29,5 | > 50 |
| *Polylepis tarapacana* Phil. | A | AcOEt | **PTaA** | > 100 | 32,6 | 33,5 | 3,6 | 5,9 |
| | | MeOH | **PTaM** | > 100 | > 100 | > 100 | 83,75 | > 50 |
| *Junellia seriphioides* (Guilles & Ex Hook.) Moldonke | A | AcOEt | **JSA** | > 100 | > 100 | > 100 | 20,75 | 28,8 |
| | | MeOH | **JSM** | > 100 | > 100 | > 100 | >100 | > 100 |
| *Fabiana squamata* Phil. | A | AcOEt | **FSA** | > 100 | > 100 | > 100 | 15 | > 50 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *Fabiana denudata* Miers | A | MeOH | **FSM** | > 100 | > 100 | > 100 | 20,75 | > 100 |
| | | AcOEt | **FDA** | > 100 | 50 | 70,4 | 5,45 | 21,7 |
| | | MeOH | **FDM** | > 100 | > 100 | > 100 | 74,25 | > 100 |
| *Krameria lappacea* (Dombey) Burdet & B. B. Simpson | R | AcOEt | **KLA** | 31 | 35,9 | 6,8 | 18 | 15,5 |
| | | MeOH | **KLM** | > 100 | > 100 | > 100 | > 100 | > 100 |
| *Baccharis calliprinos* Griseb. | A | AcOEt | **BCA** | 30,2 | 19,5 | 8,7 | > 100 | 15,6 |
| | | MeOH | **BCM** | > 100 | > 100 | > 100 | > 100 | > 100 |
| *Baccharis grisebachii* Hieron | A | AcOEt | **BGA** | > 100 | > 100 | > 100 | 9 | > 100 |
| | | MeOH | **BGM** | > 100 | > 100 | > 100 | 71,75 | > 100 |
| *Haplopappus rigidus* Phil. | A | AcOEt | **HRA** | > 100 | > 100 | > 100 | 20,5 | 12,9 |
| | | MeOH | **HRM** | > 100 | > 100 | > 100 | 12 | 11,5 |
| *Chuquiraga atacamensis* Kuntze | A | AcOEt | **CAA** | > 100 | 51,9 | 37,4 | 7,5 | > 50 |
| | | MeOH | **CAM** | > 100 | > 100 | > 100 | 78,4 | > 100 |
| *Senecio nutans* SWch. Bip. | A | AcOEt | **SNA** | > 100 | > 100 | > 100 | 36 | > 100 |
| | | MeOH | **SNM** | > 100 | > 100 | > 100 | > 100 | > 100 |
| *Parastrephia teretiuscula* (Kuntze) Cabrera | A | AcOEt | **PTeA** | > 100 | > 100 | > 100 | 16 | > 50 |
| | | MeOH | **PTeM** | > 100 | > 100 | > 100 | 35 | > 100 |
| *Cortaderia speciosa* (Ness & Meyen) Stapf | A | AcOEt | **CSA** | > 100 | 49,5 | 79,1 | > 100 | 24,1 |
| | | MeOH | **CSM** | > 100 | > 100 | > 100 | > 100 | > 100 |

Table 1: Antiparasitic activity of medicinal plants from the traditional medicine of Atacama People.

3 Results

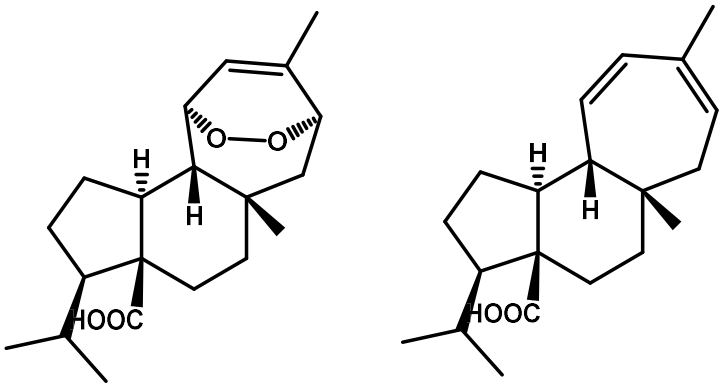
3.1 Antiparasitic activity of plant extracts

In the case of *L. amazonensis*, $IC_{50}$ between 31-19 µg/mL were calculated for active extracts. The apolar extract from *A. copa* and *B. calliprinos* **ACSA**, **ACLA** and **BCA** showed values of 26.0, 27.6 and 30.2 µg/mL respectively. **AAA** from *A. atacamensis* showed the lower value of 19.3 µg/mL. **KLA** from *K. lappacea* also showed antileishmanial activity against promastigotes *L. amazonensis* with an $IC_{50}$ of 31 µg/mL. For *L. donovani*, the same extracts from Asteraceae family showed also $IC_{50}$ values of 11.6, 17.7 and 19.5 µg/mL respectively. In addition, also extract **CAA** of another Asteraceae species (*C. atacamensis*) with $IC_{50}$ value of 51.9 µg/mL. The Apiaceae specie, *A. atacamensis* apolar extract showed an $IC_{50}$ of 28.2 µg/mL. Furthermore, apolar extracts from *P. tarapacana*, *K. lappacea*, *C. speciosa* and *F. denudata* showed $IC_{50}$ values of 32.6, 35.9, 49.5 and 50 µg/mL. For *Leishmania* species, the lower $IC_{50}$ values were recorded against *L. infantum* promastigotes. **ACSA**, **ACLA**, **KLA**, **AAA** and **BCA** extracts showed $IC_{50}$ values of 3.1, 5.5, 6.8, 8.0, 8.7 µg/mL respectively. The higher $IC_{50}$ values were observed for apolar extracts of *F. denudata* and *C. speciosa* of 70.4 and 79.1 µg/mL. Only two plant species were inactive ($IC_{50}$ > µg/mL) against *T. brucei brucei* bloodstream form. The more active extracts were **PTA**, **FDA**, **ACLA**, **CAA**, **BGA** and **ACSA** with $IC_{50}$ values of 3.6, 5.5, 7.2, 7.5, 9.0 and 9.7 µg/mL respectively.

Several Asteraceae species extracts showed interestingly $IC_{50}$ values against *P. falciparum* W2 strain. **ACLA** and **ACSA** extract from *A. copa* showed $IC_{50}$ of 10.2 and 19.8 µg/mL. Both extracts of *H. rigidus* showed $IC_{50}$ values of 12.9 and 11.5 µg/mL. *Baccharis* extracts **BCA** and **BTM** presented $IC_{50}$ values of 15.6 and 16.9 µg/mL. The lower values were observed for the apolar extracts of *A. atacamensis* and *P. tarapacana* of 1.3 and 5.9 µg/mL.

Isolation of bioactive compounds from active fractions.

From **AAA**, fractionation (**AAA1-12**) and subsequently evaluation, **AAA6** was identified as the more active fractions. **AAA6** was subjected to preparative HPLC purification, leading the isolation of two diterpenoids **1-2**. Spectroscopy (1D-, 2D-NMR experiments) and spectrometry (HRMS) data were compared with those published in the literature, allowed us to identify mulinic acid (**1**) and mulin-11,13-dien-20-oic acid (**2**) respectively (Loyola et al., 1997, 1990). Compound **1** showed similar $IC_{50}$ (0.351 and 0.329 µM) values for both resistance and sensible-chloroquine strains. *In vitro* $IC_{50}$ values were also very similar with the drug reference in the case of the W2 strain and just a 10-fold in the case of 3D7 strain. Compound **2** were inactive against the two strains even at the higher concentration level (1 µg/mL).




1 2

Figure 1: Isolated compounds from *A. atacamensis* (**1**-**2**).

| **Antiplasmodial activity of isolated compounds (µM)** | | | |
|---|---|---|---|
| *P. falciparum* strains | Mulinic acid (**1**) | Mulin-11,13-dien-20-oic acid (**2**) | Chloroquine |
| 3D7 Chloroquine-sensible | 0.329 ± 0.008 | n.a. | 0.024 ± 0.002 |
| W2 Chloroquine-resistance | 0.351 ± 0.009 | n.a | 0.284 ± 0.012 |

Table 2: *In vitro* antiplasmodial activity of compounds isolated from the ethyl acetate extract of *A. atacamensis*. n.a not active.

4 Discussion

Azorella species are known to exert several *in vitro* and *in vivo* biological activities. *A. compacta* (yareta), a yellow-green compact resinous cushion shrub growing in the Chilean Andes is used in folk medicine to treat diabetes (Loyola, 1990; Araya, 2003, Bórquez, 2014). From apolar extracts of *A. compacta* have been isolated diterpenes with muline and azorellane skeletal, showing trichomonicidal, antiplasmodial and antimicrobial activities. However, compounds **1**-**2**, previously isolated from *A. compacta*, have been not assayed against these targets (Loyola, 1996).

Derivative 17-acetoxy-mulin acid (which differ with compound **1** with an acetoxy group in position 17), showed not antituberculosis activity against ACTT27294 and CIBIN/UMF15:99 strains. In addition, antiplasmodial *in vivo* activity was essayed for 17-acetoxy-mulin acid. Swiss

male mice infected with *Plasmodium berghei* NK 65 were used as *in vivo* model where the 17-acetoxy derivative of compound **1**, reduced in a 26% the erythrocytic growth, after an intraperitoneal administration dose of 10 mg/kg/day during 3 days (Loyola, 2004). No any *in vitro* or *in vivo* antiplasmodial activity of compounds **1**-**2** were founded in literature.

Regarding the chemical structure of mulinic derivatives, seems that the endoperoxide bond is essential to exert the antiplasmodial activity in comparison with derivative **2**, which not possess this function in its structure. This function is also crucial for the activity of the reference drug artemisinin, a sesquiterpene lactone isolated from *Artemisia annua*. Even if its mechanism of action is still under study, several reports have pointed out the role of the endoperoxide bond. Artemisinin is activated by a reduced iron source (mainly heme, released from haemoglobin parasitic digestion) to provid artemisinin radicals, which quickly react with nucleophile-bearing cellular components as well as with unsaturated membrane lipids and heme itself, blocking the biocrystallization of non-toxic derivative hemozoin. Further studies must be performed in order to clarify if mulinic acid **1** could exert its antiplasmodial activity by similar mechanism of action as artemisinin.

## 5 Conclusion

Mulinic acid, which possess an endoperoxide group, has demonstrated a strong *in vitro* antiplasmodial activity, in the same range to chloroquine. Thus, we have demonstrated that traditional medicines are still a valuable source of bioactive compounds. Even for already described compounds, there is a lack of associated biological evaluations. *Bio*-guided approach could minimize the time consumption in the search for bioactivity.

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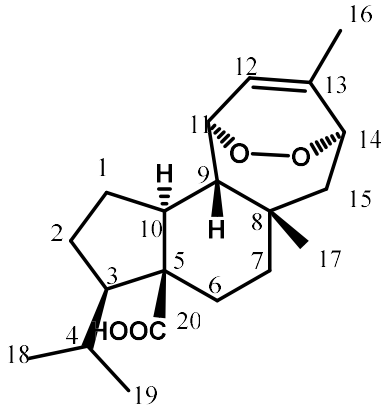
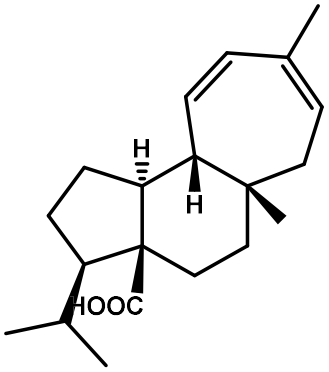
## SUPPORTING INFORMATION

***NMR and MS data of isolated compounds*:**

Structural elucidation of isolated compounds was achieved by comparison of their experimental spectroscopy ($^{1}H$, $^{13}C$ NMR), specific rotation $[\alpha]_D$ and spectrometry (MS) data with those previously reported in the literature.




**1**




**2**

1) Mulinic acid

White crystal. $[\alpha]_D^{23}$ -130.2° (c = 0.3, $CHCl_3$). $^{1}H$ NMR ($CDCl_3$) δ 6.14 [d, *J* = 7.1 Hz, 1H, H-12], 4.59 [t, *J* = 6.6 Hz, 1H, H-11], 4.42 [d, *J* = 4.2 Hz, 1H, H-14], 2.43-2.38 [m, 1H, H-7α], 2.29 [d, *J* = 15.8 Hz, 1H, H-15α], 2.20-2.12 [m, 1H, H-10], 2.05-1.88 [m, 6H, H-16, H-1α, H-2α, H-9], 1.81-1.71 [m, 1H, H-1β], 1.53-1.35 [m, 7H, H-2β, H-3, H-4, H-6, H-7β, H-15β], 1.07 [s, 6H, H-17, H-18], 0.88 [d, *J* = 6.6 Hz, 3H, H-19]. $^{13}C$ NMR ($CDCl_3$) δ 180.8 (C-20), 136.9 (C-13), 124.3 (C-12), 80.5 (C-14), 77.6 (C-11), 57.5 (C-3), 57.0 (C-5), 49.5 (C-9), 49.1 (C-10), 42.5 (C-15), 41.6 (C-6), 33.8 (C-17), 33.6 (C-8), 32.7 (C-7), 31.6 (C-4), 28.4 (C-2), 25.5 (C-1), 22.7 (C-18), 22.5 (C-19), 20.6 (C-16). ESI-MS *m/z* (positive mode) 357.2 $[M+Na]^+$ (calculated for $C_{20}H_{30}O_4$: 334.2).

2) Mulin-11,13-dien-20-oic acid

White gum. $[\alpha]_D^{22}$ -179.1 (c = 0.15, $CHCl_3$). $^{1}H$ NMR ($CDCl_3$) δ 5.71-5.52 [m, 3H, H-11, H-12, H-14], 2.76 [d, *J* = 15.3 Hz, 1H, H-7a], 2.50 [d, *J* = 13.0 Hz, 1H, H-6b], 2.37 [dd, *J* = 5.0, 9.5 Hz, 1H, H-9], 2.13 [dd, *J* = 3.1, 11.0 Hz, 1H, H-1b], 2.00 [m, 1H, H-2b], 1.87 [s, 3H, H-16], 1.77-1.69 [m, 4H, H-1a, H-7b, H-10, H-15b], 1.57-1.42 [m, 5H, H-2a, H-3, H-4, H-6a, H-15a], 1.11 [d, *J* = 5.0 Hz, 3H, H-19], 0.94 [d, *J* = 5.0 Hz, 3H, H-18], 0.94 [s, 3H, H-17]. $^{13}$ C NMR ($CDCl_3$)

δ 182.1 (C-20), 133.3 (C-11), 132.0 (C-13), 128.5 (C-12), 126.0 (C-14), 59.9 (C-5), 58.2 (C-3), 55.6 (C-10), 50.8 (C-9), 41.7 (C-15), 37.0 (C-7), 35.3 (C-8), 33.2 (C-6), 32.0 (C-4), 29.4 (C-2), 27.8 (C-17), 26.2 (C-16), 25.0 (C-1), 23.4 (C-18), 23.0 (C-19). ESI-MS *m*/*z* (positive mode) 325.2 $[M+Na]^+$ (calculated for $C_{20}H_{30}O_2$: 302.2).

## Conclusion and perspectives

The main conclusion of this study is the evidence that bioguided approach still remains efficient to quickly identify promising extracts. By minimizing time, material and solvents consumption, it enables to directly access to active fractions and compounds. This methodology has allowed the characterization of mulinic acid, with *in vitro* $IC_{50}$ values comparable to those of reference drug chloroquine.

In a continuation of this work, *in vivo* evaluation of mulinic acid together with exploration of its toxicity and pharmacokinetic parameters should be performed. Considering the structure similarity with artemisin, especially because of the endoperoxide bridge, the mechanism action has to be deciphered.

# I.5 Anti-inflammatory and antioxidant activity

After infectious diseases, the second most cited traditional therapeutic uses of collected plants was related to inflammatory disorders. In this section, the anti-inflammatory potential of collected species is explored. The antioxidant activity is also evaluated due to its role in the oxidative stress during the inflammation.

## I.5.1 Role of oxidative stress during the inflammatory process

Inflammation represents a fundamental biological process, and occurs in response to any alteration of tissue integrity, in order to restore tissue homeostasis by induction of several repair mechanism. Several evidences have shown that chemical oxidative stress play critical roles in the pathophysiology of inflammation.

Oxidative stress is a state of imbalance between oxidant and antioxidant in favour of the oxidant, leading to damaging effects. Oxidant species, also known reactive oxygen or nitrogen species (ROS and RNS), includes free radicals as O2·-. NO·, ONOO·-, OH· and NO2·. The sources of ROS/RNS are multiple such as sub enzymatic products such as xanthine oxidase, NADPH oxidase (NOX), cyclooxygenases or isoforms of NO synthase or formed during the mitochondrial electron transport chain.

ROS/RNS target proteins, lipids and nucleic acid, producing various molecular modifications, disturbing biological functions. Protein degradation/dysfunction are produced by SH-oxidation/nitrosylation (Cyst), carbonylation (Pro, Thr, Lys, and Arg), and phenolic nitration (Tyr) by action of ROS/RNS. Oxidation of poly-unsaturated fatty acids by ROS lead the lipid peroxidation, producing membrane alteration and cytotoxicity effects. In DNA, ROS can damage guanine nucleobase, resulting in the formation of 8-oxoguanine, with carcinogenic consequences and mutagenic effects.

Inflammation could be organized in five-stage process, including a generating system, a sensor mechanism (danger receptors), transmission of signal with the current production of mediators and the activation of cellular effectors. These stages can be affected in several degrees by ROS/RNS. The presence of danger signals (exogenous or endogenous) alert the immune system through pattern recognition receptors (PRRs) belonging to the Toll-like (TLRs) and NOD-like (NLRs) families, triggering intracellular signaling through the activation of factor NF-κβ and the molecular platform termed the inflammasome, rising the production of multiple inflammatory

mediators. The role of oxidative stress during several inflammatory mechanisms are discussed below.

Amounts of phospholipids, cholesterol and sphingolipids, in the cell membrane, contain the active forms of TLRs, which after cell exposition to external ROS/RNS, results in an increase of this surface-TLRs. The extracellular domain connects through a transmembrane domain to the cytoplasmic Toll/IL-1 receptor (TIR) domain, which interacts with MyD88 and TRIF adaptor molecules, activating the downstream signaling through NF-κβ, activator protein 1 (AP-1) and interferon-regulatory factor 3 (IRF-3), upregulating the expression of inflammatory mediators, comprising oxidant enzyme such as NOX and inducible nitric synthase (iNOS), generating high levels of intracellular ROS. TLR also promotes the generation of ROS in mitochondria and the activation of NOX through enhanced phosphorylation of its $p47^{phox}$ subunit within the cytoplasm. This increase of intracellular ROS levels improve the mobilization and dimerization of cytoplasmic TLR within lipid rafts, creating a cycle of progressive amplification of the TLR response.

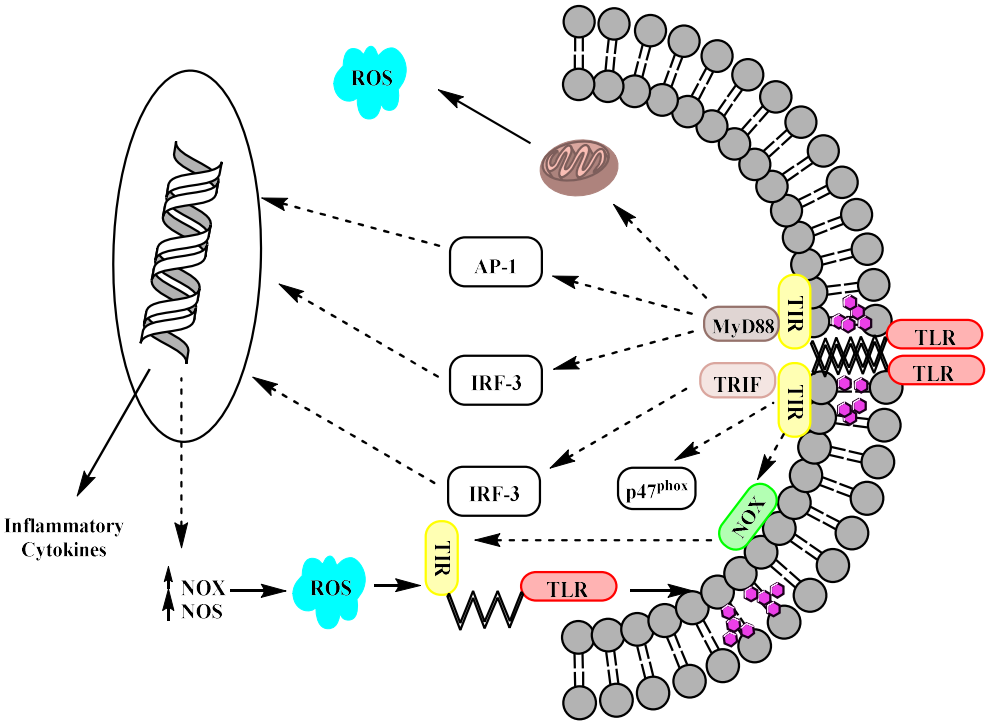



**Figure 52: Interaction between oxidative stress and Toll-like receptors (TLRs).**

After TLR activation, the Iκβ kinase complex (IKK) becomes phosphorylated and active. Active IKK phosphorylates the inhibitor Iκβ on serine 32 and 36, promoting the degradation of Iκβ by

proteasomal action, then free NF-κβ dimers (p50/p65) can translocate to the nucleus and activate genes of pro-inflammatory mediators. Activation of NF-κβ can be ROS promoted by three different pathways: ROS can enhance IKK phosphorylation by activation of protein kinase D (PKD) or inhibition of protein phosphatase 2 (PP2A), which is the responsible of IKK dephosphorylation. ROS can also cause the phosphorylation of tyrosine 42 of Iκβ, producing either its dissociation of NF-κβ or its degradation. ROS also can start several intranuclear changes inducing chromating modelling, which promote DNA binding of the NF-κβ.

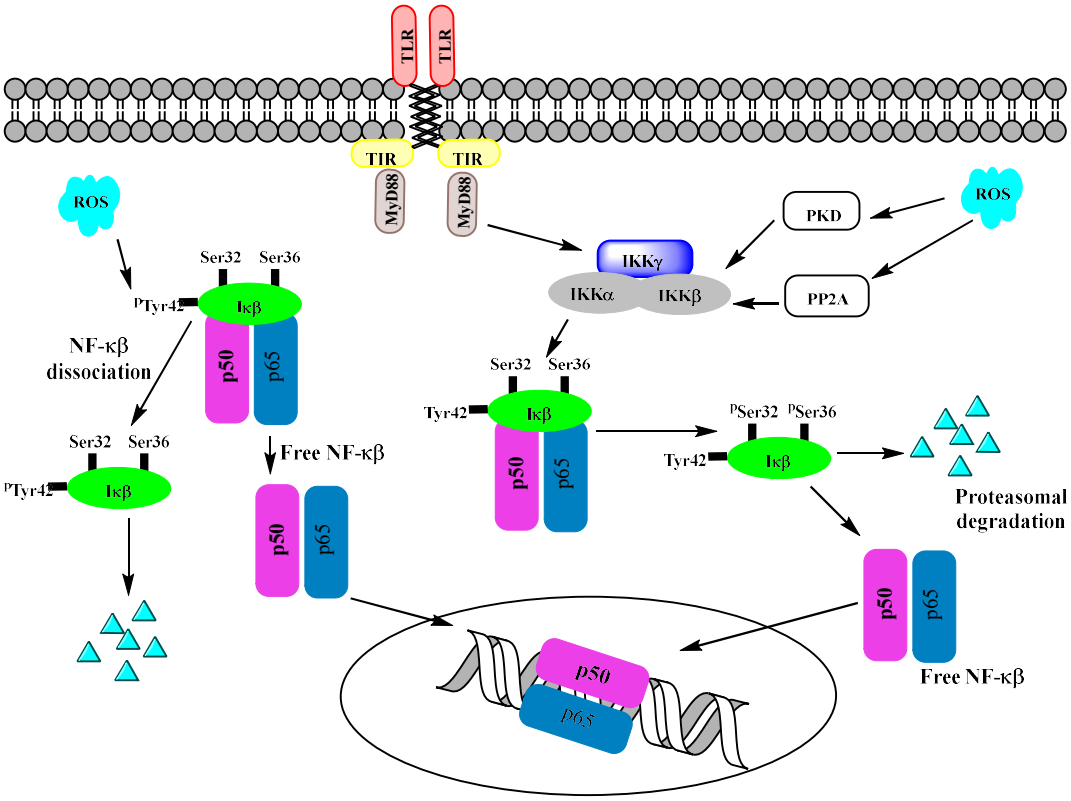



**Figure 53: Positive regulation of NF-κβ by oxidative stress.**

The activation of pathogen or damaged-associated molecular patterns (PAMPs, DAMPs) results in an overexpression of NLRP3 (NOD-like receptor pyrin domain containing 3), pro-IL-1β and pro-IL-18 and the later activation of NLRP3 is then modulated by three distinct mechanisms. First of them by $K^+$ efflux in response of $K^+$ channels, the presence of bacterial pore-forming toxin, or the purigenic receptor channel P2X7 by extracellular ATP. Also by mitochondrial damage and ROS production. ROS can either separate thioredoxin-interacting protein (TXNIP) from its inhibitor thioredoxin (TRX), and TXNIP directly activates NLRP3. Damaged mitochondria also

release oxidized mitochondrial DNA (Ox mtDNA) that activates NLRP3. Lately, activated NLRP3 associates ASC and caspase-1 in a multiprotein platform called inflammasome, which converts pro-Il-1β and pro-IL-18 into mature cytokines.

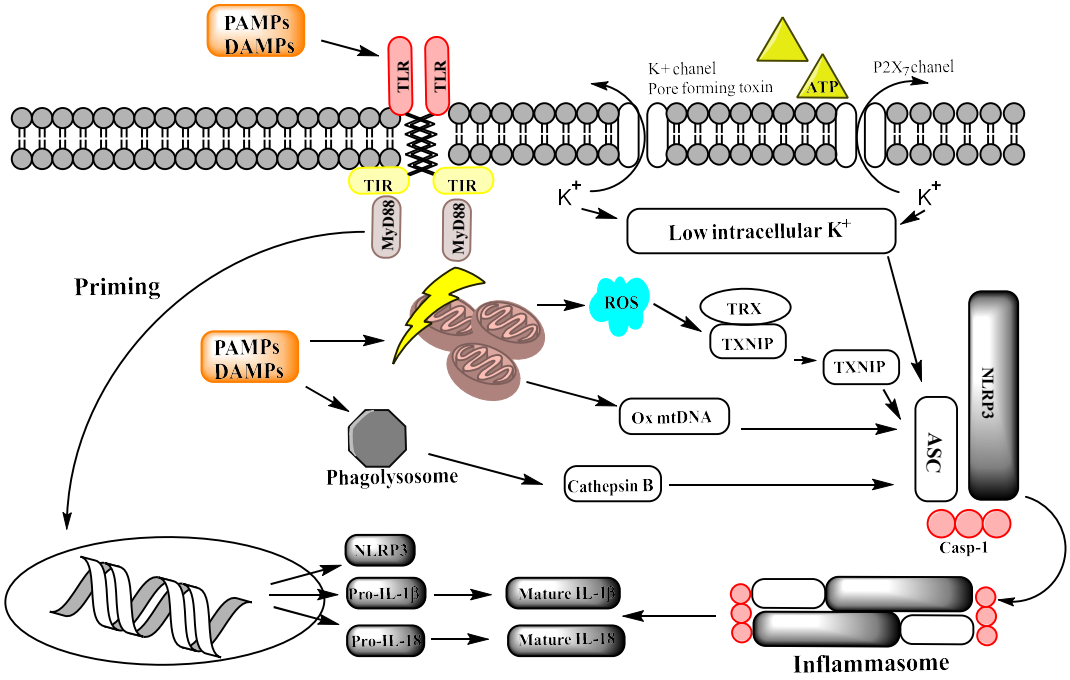



**Figure 54: Role of oxidative stress on the process of NLRP3 activation.**

## I.5.2 Methodology

In order to evaluate the anti-inflammatory and antioxidant potential of collected medicinal plants, we investigate the free radical scavenging activity, inhibition of ROS production enzyme, quantification of antioxidant species and diminution of pro-inflammatory cytokines *in vitro* assays, here below described:

### I.5.2.1 Free radical scavenging activity assays

Evaluation of DPPH and ABTS (**Figure 55**) free radical scavenging capacity was chosen to measure the antioxydant activity of essential oils and extracts. Accurate amounts of sample were put in 96-well plate together with a DPPH solution. Reduction by action of essential oil of DPPH

to its yellowish reduced product is measured after 30 min at 517 nm. Decrease of DPPH absorption underlines the antiradical activity of essential oils.

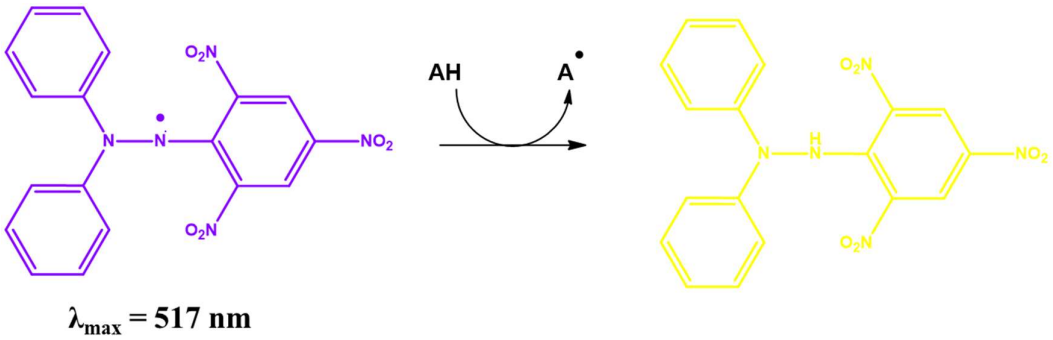



**Figure 55: Reduction of DPPH in presence of antioxidants (AH).**

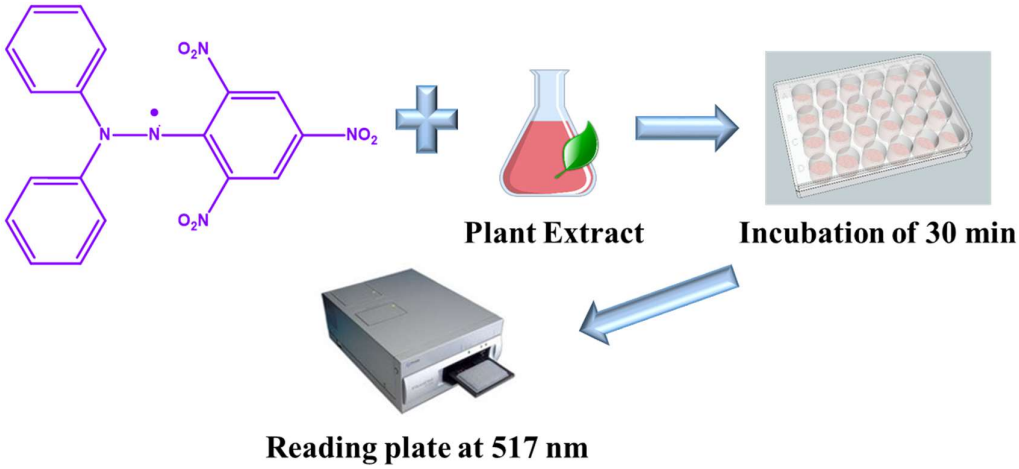



**Figure 56: DPPH scavenging activity assay workflow applied in our investigation.**

## I.5.2.2 Xanthine Oxidase inhibition assay

Xanthine oxidase (XO) is enzyme that generates reactive oxygen species (ROS) in the catalytic conversion of hypoxanthine to xanthine and xanthine to uric acid (**Figure 57**). Evaluation of the xanthine oxidase inhibition capacity is measured by blockage of uric acid production from xanthine. Accurates amount of essential oil are put in well of a 96-well quartz plate with bovine xanthine oxidase enzyme and incubated at room temperature for 5 min. Then xanthine substrate is added and the uric acid production is followed at 295 nm during 30 min. Catalytic reaction rate is measured and percentages of inhibition, based on corresponding blanks, are calculated.

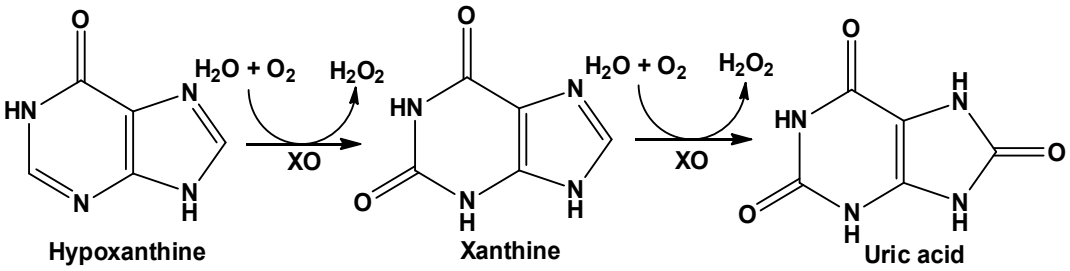



**Figure 57: Biosynthesis of uric acid from hypoxanthine by the catalytic action of xanthine oxidase.**

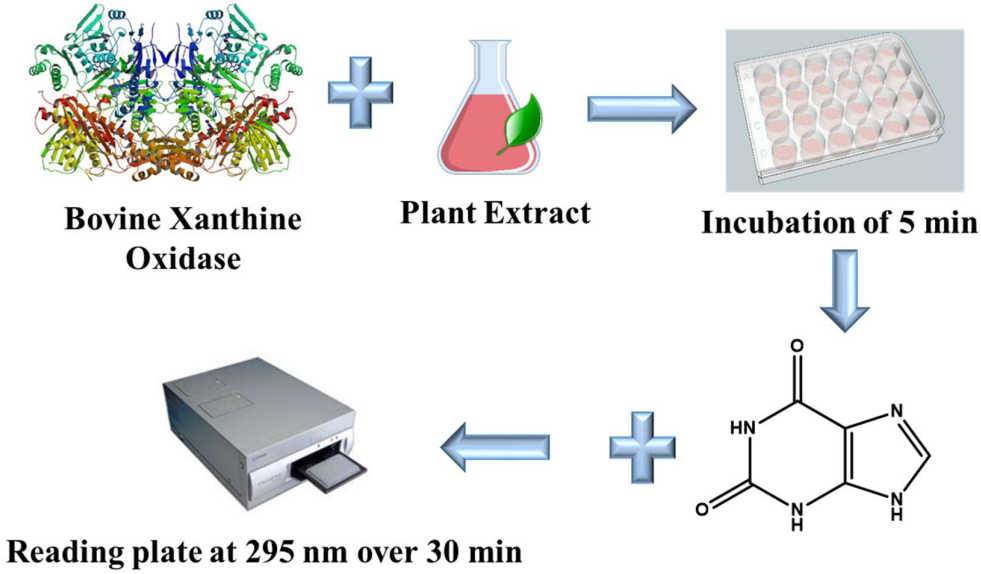



**Figure 58: Xanthine oxidase assay workflow applied in our study.**

## I.5.2.3 Cytokine production assay

Pro-inflammatory cytokine production assay was carried out after incubation of mouse macrophage 264.7 cell line with the corresponding plant extract concentration. After 10 min, macrophages were stimulated with lipopolysaccharide (LPS) in order to create a pro-inflammatory response. To evaluate the effect of plant extract in the inflammatory response, production of IL-6 and TNF-α cytokines (pro-inflammatory mediators) were quantified by enzyme-liked immunosorbent assay (ELISA) tests.

ELISA is a commonly used analytical assay described in 1972. A variant "sandwich ELISA" (**Figure 59**) test was used in our investigation to quantify the pro-inflammatory cytokines. This variant utilise coated plates with a specific capture antibody, which catch the cytokines after

sample addition. A specific antibody is added and binds to the cytokine and then an enzyme-like (horseradish peroxidase in our study) as add binding specifically to the antibody. Finally, 3,3',5,5'-tetramethylbenzidine (TMB) is added and by catalytic conversion by peroxidase, oxidised product can be detected at 450 nm.

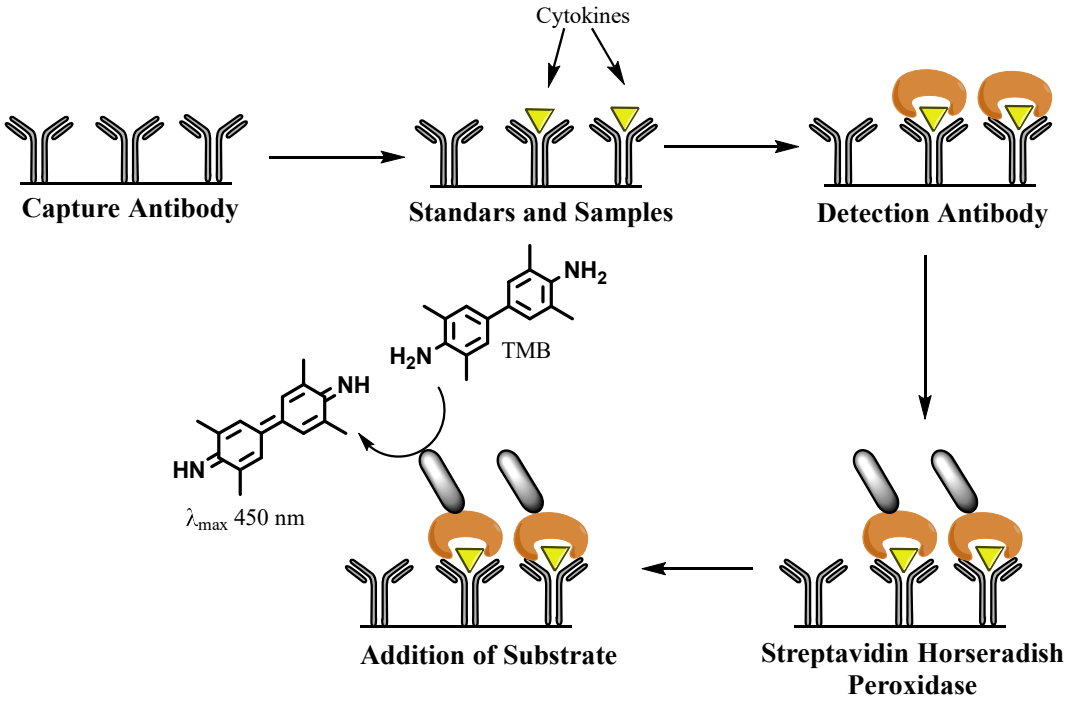



**Figure 59: Workflow of "sandwich ELISA" tests.**

In a second step, we envisaged the isolation of bioactive compounds from most promising extracts, on basis of their *in vitro* antioxidant and anti-inflammatory activities and the evaluation of them, in order to identify the compounds responsible of their activity.

## I.5.3 Antioxidant activity of volatile compounds from *A. copa* and *A. deserticola*

These investigations have started with the analysis of essential oils of *Aloysia deserticola* and *Artemisia copa* var. copa by GC-FID and GC-MS and the evaluation of the antioxidant activity of several samples, collected at different altitudes. These species were selected due to their indications in the Atacama People traditional medicine and in the urban areas by the Chilean population.

Essential oil from *A. copa* var. copa was analysed from samples collected in Cochabamba, Bolivia, but not report of *A. copa* var. copa collected in the Altiplano has been communicated. In other hand, the essential oil of *A. deserticola* from Chile has reported the α-thujone isomer or β-thujone as principal components and its antibacterial activity evaluated only against one strains of *Staphylococcus aureus* and other strain of *Escherichia coli*.

This led us to deeper study the composition as well as antioxidant and antibacterial activities of essential oils of *A. copa* and *A. deserticola* is necessary, in order to corroborate their uses in the traditional medicine of Atacama People.

## I.5.4 Publication 2: Chemical composition and biological activity of essential oils from *Artemisia copa* Phil. var. copa (Asteraceae) and *Aloysia deserticola* (Phil.) Lu-Irving & O'Leary (Verbenaceae), used in the Chilean Atacama's Taira Community (Antofagasta, Chile).

JOURNAL OF ESSENTIAL OIL RESEARCH
https://doi.org/10.1080/10412905.2019.1572549

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# Chemical composition and biological activity of essential oils from *Artemisia copa* Phil. var. copa (Asteraceae) and *Aloysia deserticola* (Phil.) Lu-Irving & O'Leary (Verbenaceae), used in the Chilean Atacama's Taira Community (Antofagasta, Chile)

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**ABSTRACT**

Two aromatic species from the Atacama people traditional medicine, *Aloysia deserticola* (Phil.) Lu-Irving & O'Leary and *Artemisia copa* Phil. var. copa, from Antofagasta area (North of Chile) were investigated for their essential oils (EO) chemical composition, antioxidant and antimicrobial activity. EO of *A deserticola* collected in four different spots unexpectedly contained α-thujone as the major compound instead of the β-isomer described for other Chilean and Bolivian specimens in the literature. For EO of *Artemisia copa* var. copa, chrysanthenone was the main compound whereas β-thujone was identified in a Bolivian specimen. These EO exerted no significant antibacterial activity on a panel of forty-four strains, whereas notable inhibition of xanthine oxidase activity was observed with *A. deserticola* essential oil.

**ARTICLE HISTORY**
Received 23 May 2017
Accepted 4 January 2019

**KEYWORDS**
*Aloysia deserticola*; *Artemisia copa*; thujone; chrysanthenone; xanthine oxidase

## Introduction

The Altiplano (Highland), a region of South America located between North of Chile, North-West of Argentina, West of Bolivia and South of Peru, beneficiates of cool temperatures, semi-arid and arid climate and high level of UV radiations due to the altitude comprised between 2500 and 5000 m. Traditional medicines from different Andean ethnic groups living in the Altiplano (Aymara, Qullahuaya or Quechua) are reported in the literature (1,2). Descriptions of the medicinal use of plants growing in the desert of Atacama, the most arid part of the Chilean altiplano, are more rare (3). *Aloysia deserticola* (Phil.) Lu-Irving & O'Leary (Verbaneceae) (*ex Acanthoippia deserticola* Phil.) and *Artemisia copa* (Phil.) var. copa (Asteraceae) are two aromatic plants used by both Atacama and Aymara communities of the area (4,5). These aromatic species are described in the extreme north of Chile (Antofagasta area), but also in the Argentinian and Bolivian parts of Altiplano.

*A. deserticola*, formerly *Acantholippia deserticola* Phil., locally known as *Rika-rika*, is a medium shrub of 0.4–1.0 m height with cylindrical branches, very reduced opposite yellow-green leaves (1.5 × 1.5–2 mm) and ovate lilacinus flowers. In Atacama people tradition, *A. deserticola* is used for the treatment of common cold, cough, fever, stomach ache, vomiting, diarrhoea, and for urinary tract, kidney and heart problems. In addition, *A. deserticola* is used to stimulate milk production for breastfeeding women (3). Many Verbenaceae species produce essential oils (EO) and are used in traditional medicines all around the world, such as *Lippia alba* and *Aloysia triphylla* (6). Distinction between *Lippia, Aloysia* and *Acantholippia* genus is not really clear. Recently, a study based on chloroplast and nuclear sequence data proposed to expand the definition of *Aloysia* to include various *Acantholippia* species, such as *A. deserticola*.

*Artemisia copa*, locally known as *Kopa-kopa*, is a medium shrub of 0.4–1.0 m height, with small oblong and strongly fragrant leaves, always growing above 3000 m of altitude. It is used by Atacama community for digestive troubles and headaches (3). *Artemisia* species are aromatic species widely used for medicinal purpose, to treat infections, and as febrifuge, emmenagogue or antispasmodic, such as *A. millefolium, A. vulgaris, A. herba-alba* and *A. absinthium*.

We present herein the chemical composition of EO obtained from Chilean specimen of these two species collected in different spots and their biological evaluation for antibacterial and antioxidant activity combining radical scavenging and xanthine oxidase (XO) inhibition.

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## Experimental

### Plant material

Plant material was collected from Antofagasta Region (Chile), within the Taira Atacama Community in September 2015 (7). *A. deserticola* was collected in four different spots located between 2946 and 3319 m and *A. copa* var. copa was collected at 3255 m of altitude (Table 1).

### Plant identification and oil extraction

Plants were identified by Pr Alicia Marticorena from Universidad de Concepción and voucher was deposited

**Table 1.** Chemical composition of the EOs of *A. deserticola* and *A. copa* var. *copa* by GC-FID and GC-MS.

| Compound | | | | | Composition (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample | | | | | 1 | 2 | 3 | 4 | 5 | 6 | |
| EO yield (%) | HP-5 | | DB-WAX | | 1.016 | 0.972 | 1.386 | 0.792 | – | 1.144 | |
| Altitude (m) | $RI^1$ | $RI^2$ | $RI^1$ | $RI^2$ | 2946 | 3076 | 3248 | 3319 | 2946 | 3255 | Identification |
| Tricyclene | 921 | 919 | 1009 | 1005 | 0.5 | 0.2 | 0.3 | 0.1 | – | 1.1 | RI, MS |
| α-Pinene | 939 | 939 | 1020 | 1022 | – | – | – | – | – | 0.2 | RI, MS |
| α-Fenchene | 945 | 942 | 1060 | 1063 | – | – | t | t | – | – | RI, MS |
| **Sabinene** | 977 | 972 | 1119 | 1118 | **16.0** | **9.9** | **10.2** | **4.1** | – | – | RI, MS |
| β-Pinene | 982 | 983 | 1105 | 1103 | – | – | – | – | – | 0.1 | RI, MS |
| Myrcene | 993 | 990 | 1161 | 1168 | 0.3 | 0.3 | 0.3 | 0.2 | – | – | RI, MS, Co-Inj |
| 3-Carene | 1008 | 1008 | 1142 | 1144 | – | – | t | – | – | – | RI, MS |
| α-Terpinene | 1021 | 1016 | 1174 | 1171 | 0.3 | 0.2 | 0.2 | – | – | – | RI, MS |
| 1,2,4-Trimethylbenzene | 1020 | 1021 | 1276 | 1276 | – | – | – | – | – | 0.2 | RI, MS |
| Sylvestrene | 1025 | 1030 | – | – | – | – | – | – | – | 0,3 | RI, MS |
| *p*-Cymene | 1029 | 1022 | 1255 | 1256 | 1.7 | 1.1 | 1.1 | 0.8 | – | 1.2 | RI, MS |
| Limonene | 1033 | 1026 | 1197 | 1194 | 0.1 | 0.1 | 0.1 | – | – | – | RI, MS, Co-Inj |
| 1,8-Cineole | 1037 | 1029 | 1208 | 1209 | 0.2 | 0.1 | 0.6 | 0.1 | – | 3,7 | RI, MS, Co-Inj |
| (*Z*)-β-Ocimene | 1041 | 1040 | 1235 | 1234 | – | 0.2 | 0.2 | t | – | 0.2 | RI, MS |
| (*E*)-β-Ocimene | 1052 | 1053 | 1254 | 1252 | – | – | t | – | – | 0.2 | RI, MS |
| γ-Terpinene | 1063 | 1063 | 1244 | 1246 | 0.9 | 0.5 | 0.6 | 0.4 | – | 0,2 | RI, MS |
| Terpinolene | 1084 | 1090 | 1279 | 1272 | 0.1 | 0.1 | 0.1 | t | – | – | RI, MS |
| cis-Linalool oxide (THF form) | 1076 | 1080 | 1429 | 1430 | – | – | – | – | – | 0.5 | RI, MS |
| Linalool | 1100 | 1100 | 1551 | 1556 | – | – | – | – | – | 4.3 | RI, MS, Co-Inj |
| **α-Thujone** | 1113 | 1118 | 1417 | 1420 | **72.9** | **74.5** | **78.3** | **80.1** | **76.9** | – | RI, MS, Co-Inj |
| **β-Thujone** | 1123 | 1128 | 1436 | 1438 | **0.5** | **7.6** | **4.6** | **11.0** | **12.3** | – | RI, MS |
| Dehydrosabinene ketone | 1120 | 1132 | – | – | 0.1 | 0.1 | 0.1 | t | – | – | RI, MS |
| **Chrysanthenone** | 1124 | 1127 | 1501 | 1508 | – | – | – | – | – | **55.5** | RI, MS |
| trans-*p*-Menth-2-en-1-ol | 1140 | 1138 | 1553 | 1554 | – | – | – | – | – | 0.2 | RI, MS |
| *trans*-Pinocarveol | 1142 | 1140 | 1654 | 1655 | 0.1 | 0.1 | 0.1 | 0.2 | 0.5 | – | RI, MS |
| Neothujol | 1153 | 1157 | – | – | 0.2 | 0.2 | 0.2 | 0.3 | 0.3 | – | RI, MS |
| Nerol oxide | 1164 | 1170 | 1467 | 1471 | – | – | – | – | – | 1.2 | RI, MS |
| Sabina ketone | 1159 | 1162 | 1683 | 1681 | 0.1 | t | t | t | – | – | RI, MS |
| Terpinen-4-ol | 1184 | 1179 | 1603 | 1603 | 1.2 | 0.7 | 0.6 | 0.7 | – | 0.5 | RI, MS |
| α-Terpineol | 1197 | 1190 | 1697 | 1700 | t | t | t | t | – | 1.6 | RI, MS, Co-Inj |
| cis-Piperitol | 1196 | 1190 | 1691 | 1699 | – | – | – | – | – | 0.1 | RI, MS |
| Myrtenol | 1203 | 1194 | 1793 | 1795 | t | t | t | – | – | – | RI, MS |
| Nerol | 1233 | 1237 | 1851 | 1860 | – | – | – | – | – | 0.4 | RI, MS |
| Isobornyl acetate | 1296 | 1297 | 1582 | 1583 | t | 0.1 | 0.1 | 0.2 | – | – | RI, MS |
| Cumin aldehyde | 1249 | 1243 | 1785 | 1785 | t | t | t | t | – | – | RI, MS |
| Carvacryl methyl ether | 1244 | 1248 | 1604 | 1600 | 0.3 | 0.2 | 0.2 | 0.3 | – | – | RI, MS |
| Piperitone | 1252 | 1260 | 1741 | 1750 | – | – | – | – | – | 4.9 | RI, MS |
| Linalyl acetate | 1257 | 1265 | 1568 | 1580 | – | – | – | – | – | 8.2 | RI, MS |
| Carvenone | 1258 | 1259 | 1714 | 1712 | – | t | t | t | – | – | RI, MS |
| Isopiperitone | 1234* | 1248 | 1838 | 1844 | – | – | – | – | – | 0.4 | RI, MS |
| Neo-3-thujanyl acetate | 1276 | 1277 | – | – | 0.1 | 0.1 | 0.1 | t | – | – | RI, MS |
| *p*-cymen-7-ol | 1290 | 1296 | 2108 | 2110 | t | 0.1 | t | t | – | – | RI, MS |
| cis-Pinocarvyl acetate | 1298 | 1295 | 1671 | 1685 | – | – | – | – | – | 0.4 | RI, MS |
| 2-Methyl-undecanal | 1368 | 1363 | – | – | – | – | t | – | – | – | RI, MS |
| Geranyl acetate | 1383 | 1398 | 1766 | 1770 | – | – | – | – | – | 1.1 | RI, MS |
| *cis*-α-Bergamotene | 1412 | 1415 | 1583 | 1587 | – | t | t | – | – | – | RI, MS |
| α-Curcumene | 1498 | 1484 | 1773 | 1774 | t | 0.1 | 0.1 | t | – | 0.1 | RI, MS |
| β-Curcumene | 1515 | 1514 | 1744 | 1746 | t | t | t | t | – | – | RI, MS |
| δ-Cadinene | 1524 | 1522 | 1766 | 1768 | – | – | – | – | – | 0.2 | RI, MS |
| (*E*)-γ-Bisabolene | 1531 | 1533 | 1762 | 1763 | t | t | t | – | – | – | RI, MS |
| α-Calacorene | – | – | 1918 | 1920 | – | – | – | – | – | 0.2 | RI, MS |
| *trans*-Dauca-4 (11),7-diene | 1557 | 1555 | – | – | – | t | t | – | – | – | RI, MS |
| Total compounds identified | | | | | 95.6 | 96.4 | 98.1 | 98.5 | 90.0 | 87.2 | |

Notes: [1]RI in the literature. [2]RI calc.: experimentally determined linear retention indices on an HP-5 or DB-WAX column. Mode of identification: RI, retention index; MS, mass spectrum and Co-Inj, co-injection with pure compound. Content values are means of triplicate analysis; t = trace amounts (<0.1%); – = not detected. Bold values correspond to the percentage of the major compounds identified.

in the Laboratory of Pharmacognosy in the Herbarium of the Laboratory of Pharmacognosy, Paris Descartes University, France, and in CONC Herbario, Universidad de Concepción, Chile, under the references MKOSB092015T1A01–4/CONC185871 (*A. deserticola*) and MKOSB092015T1B01/CONC185872 (*A. copa* var. *copa*). Air-dried aerial parts (10 g) were subjected to hydrodistillation using a Clevenger type apparatus for 3 h. For room-temperature extraction, aerial parts (20 g) were mixed with 200 mL of *n*-pentane and stirred for 4 h and then filtered and the solvent was removed under reduced pressure at room temperature. The obtained oils were stored at 2°C until gas chromatography (GC gas chromatography coupled to mass spectrometry (GC/MS) and *in vitro* assays procedures.

### GC analyses

The GC analyses were carried out with a Hewlett Packard GC 6890/MDS 5972 apparatus equipped with HP-5 (30 m × 0.25 mm × 0.25 µm) and DB-WAX (60 m × 0.25 mm × 0.25 µm) columns for GC-MS and HP-5 (30 m × 0.32 µm × 0.25 µm) for gas chromatography coupled to flame ionization detector (GC-FID) analyses). Carried gas was $H_2$ with a flow of 1.2 mL/min, split 1:20, initial oven temperature was programmed at 60°C for 5 minutes and then increased to 180°C with a rate of 4°C/min. The final oven temperature was maintained for 5 minutes.. The injector and FID temperatures were set at 240°C and 250°C, respectively. The injection volume was 1 µL. The inlet and GC/MS interface temperatures were kept at 200°C. The electronic impact ionization energy was fixed to 70 eV. The ion source temperature was 230°C. MS full scan were registerd from 50 to 600 m/z. . Identification of constituents was achieved by comparing their mass spectra with those of the Mass Spectra Library (NIST 98) compounds, of pure compounds from our chemical database, and also by comparison of their retention indices (RI) calculated relative to the retention times of a series of *n*-alkanes (C8–C20) with linear interpolation and comparison with RI from the literature on both columns (Table 1) (8–23).

### Nuclear magnetic resonance (NMR) analysis

The NMR spectra were recorded at 400 MHz on a Bruker Avance III 400 spectrometer, using Bruker pulse programs. NMR Fourier transform, integration and peak picking were done with Bruker TopSpin software and with MestRenova software version 6. Chemical shifts are reported in ppm, coupling constants (*J*) in Hertz.

### Radical scavenging activity

The free radical scavenging activity was measured using the method described by Clarke et al. (24). Twenty microlitres of solution of EO in methanol (200 µg/mL with 0.1% de DMSO) were mixed with 180 µL of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) in methanol (40 µg/mL) in wells of a 96-well plate. The plate was kept in the dark for 15 min, and then the absorbance was measured at 517 nm in a SPECTROstar nanospectrometer from BMG LabTech. Quercetin was used as positive control. All determinations were performed in triplicate.

### XO inhibition assay

The XO inhibitory activity, measuring the formation of uric acid from xanthine at 295 nm of the EO, was measured using the method previously described (25). The assay mixture consisted of 100 µL of 120 mM phosphate buffer at pH 7.5, 83 µL of 150 µM of xanthine (in buffer pH 7.5), 83 mL of 200 µg/mL EO solutions in water (with 0.1% of DMSO) and 5 µL of enzyme solution 0.5 units/mL (in buffer, pH 7.5). The reaction was initiated by the addition of the enzyme solution and the absorbance was measured at 295 nm at 25°C after 3 min of reaction. Allopurinol was assayed as positive control at concentrations of 50, 25, 12.5, 6.25, 3.13 and 1.56 µM. Inhibition of XO was calculated as $(1 - \Delta Abs_{Blank}/\Delta Abs_{Exp}) \times 100$. All determinations were performed in triplicate.

### Antibacterial screening

The *in vitro* antibacterial bioassay was conducted using an agar dilution method with ofloxacin as positive control. The EO were dissolved in DMSO and evaluated at the final concentration of 200 µg/mL. Each strain was incubated for 18 h at 37°C in trypticase soy broth (Bio Mérieux). Cultures were diluted in sterile distilled $H_2O$ and applied on test medium using a Steers inoculator; inoculum was about 1000 UFC/spot. Test media were incubated for 18 h at 37°C. The blank controls of microbial cultures were incubated with 250 µL of DMSO under the same conditions. DMSO was determined to be non-toxic under these conditions. A panel of forty-one strains, twenty-five Gram-negative (*Achromobacter denitrificans* CIP 71.32, *A. xylosoxidans* CIP 77.15, *Acinetobacter baumannii* CIP 70.34, *Aeromonas caviae* CIP 76.16, *A. hydrophila* CIP 76.14 and C74.30T, *Alcaligenes faecalis* CIP 67.23, *Brevundimonas diminuta* CIP 103020, *Burkholderia cepacia* CIP 80.24; *Citrobacter freundii* CIP 57.32, *C. koseri* CIP 82.87, *Comamonas acidovorans* N331, *C. testosteroni* N70, *Enterobacter aerogenes* CIP 6086T, *E. cloacae* CIP 60.85, *Escherichia coli* ATCC

25922, *Klebsiella oxytoca* E282, *K. pneumoniae* CIP 52.145, *Morganella morganii* E324, *Ochrobactrum anthopi* N16, *Pseudomonas aeruginosa* CIP 103.467, *P. putida* CIP 55.191, *Providencia stuartii* CIP 107.808, *Salmonella enterica* CIP 58.58, *Stenotrophomonas maltophilia* CIP 54.90, *Serratia marcescens* CIP 60.93, *Vibrio parahaemolyticus* CIP 75.2 and *Yersinia ebterocolitica* CIP 80.27) and sixteen Gram-positive species (*Bacillus cereus* CIP 6624, *Corynebacterium minutissimum* CIP 100652T, *Enterococcus avium* CIP 104.053, *E. casselliflavus* CIP 103.018, *E. durans* CIP 104.999, *E. gallinarum* CIP 105.985, *E. faecalis* CIP 103.214, *E. faecium* CIP 103.014, *E. hirae* CIP 5855, *Listeria monocytogenes* CIP 103.575, *Staphylococcus aureus* ATCC 25.923, *S. epidermidis* CIP 53.124, *S. haemolyticus* CIP 81.51, *S. lugdunensis* ATCC 43.809, *S. saprophyticus* CIP 76125, *Streptococcus agalactiae* CIP 103.227) were used.

## Results and discussion

### *Composition of the essential oil from* A. deserticola

EO obtained from aerial parts of *A. deserticola* from four different spots around 3000 m revealed slight differences of composition (Table 1). GC-MS and GC-FID analysis of the four samples resulted in the detection of thirty-five compounds (twenty-seven, thirty-one, thirty-five and twenty-six compounds for samples 1–4 representing 95.6%, 96.4%, 98.1% and 98.5% of the total oil, respectively). The main constituent in all the cases was the bicyclic monoterpene ketone, α-thujone (from 72.9% to 80.1%), associated to β-thujone and sabinene.

The occurrence of the α-thujone isomer as the major compound of the EO is intriguing, especially since the two available descriptions of EO obtained from *A. deserticola* specimen from Argentina and from extreme north of Chile (Tarapaca, at 500 km north from Antofagasta) report β-thujone as major compound, with 66.5% and 77.9% respectively (26,27).

Thujone isomers are present in high levels in other Verbenaceae species, accounting for more than 80% of the EOs of some *Aloysia citriodora* and *Lantana trifolia*, collected in North Argentina (28). Thujones are also common in some Asteraceae such as *Artemisia* species (*A. absinthium* or *A. verlotorum*), or *Tanacetum vulgaris* (87% of the EO) (29).

In fact, the α/β ratio in EO is related to the biosynthetic pathway of thujones, starting from oxidation of (+)-sabinene to (+)-cis- or (+)-trans-sabinol, and then to (+)-sabinone. The subsequent reduction of (+)-sabinone may provide either α-thujone or β-thujone (30). This last step is presumed to be under the control of a Nicotinamide Adenine Dinucleotide Phosphate-H NADPH reductase, but to date, these proteins are not yet deeply studied and no data allow us to explain if this reduction step may be stereochemically controlled by the enzyme. In conclusion, this ratio may be in favour of the α-isomer or of the β-isomer.

A recent study of *Aloysia citriodora* Palau collected in north Argentina confirms that the α/β ratio is highly variable. In fact, for wild specimens of *A. citriodora*, a 'thuyone' chemotype was identified besides the most common 'citrals' chemotype. GC analysis of these chemotypes revealed that the major compound of the EO is the β-thujone isomer for three out of five locations (62.6–86.5%), whereas the α-thujone isomer is the main compound (86.7%) in one case (Catamarca area) (31).

The $^{1}$H-NMR spectrum of the crude EO confirmed without ambiguity the stereochemistry of the GC-identified thujone. In fact, chemical shifts of the H-4 proton of each isomer differ significantly at $\delta_H$ 2.22 ppm for the α-isomer and at $\delta_H$ 2.70 for the β-isomer (32). In our case, both signals were observed, but the α-isomer signal, registered at 2.22 ppm, is the major one, in accordance with the GC results.

Finally, we also wanted to exclude the possibility of an inversion of the configuration of thujones due to hydrodistillation. This kind of conversion between α-isomers and β-isomers has already been mentioned (33). Thus, an apolar extract (**5**) obtained by maceration in *n*-pentane at room temperature of sample **1** was subjected to GC-MS analysis. The obtained extract confirmed that the α-thujone is naturally produced as the major isomer by *A. deserticola* (Table 1).

α-Thujone was found to inhibit currents of $GABA_A$ receptor and to reduce 5-HT3 receptor activity, and the β-isomer is reported to be two- or three-fold less active. Thujones containing EO are described as anxiolytic and antidepressant. However, consumption of high doses of thujones may lead to seizures. Thus, various regulations worldwide set maximal levels for thujones in food, beverages and medicines (26,34–36).

The other constituents observed in the EO were *p*-cymene, 4-terpineol, α-fenchene, iso-3-thujanol acetate, turmerone, myrtenol and (*E*)-γ-bisabolene. α-Fenchene, *iso*-3-thujanol acetate and turmerone were only identified in samples **1** and **2**, whereas myrtenol and (*E*)-γ-bisabolene were identified only in samples **3** and **4**. (1*S*)-(+)-3-carene, 2-heptyl acetate, cis-β-ocimene and 2-methylundecanal were identified only in sample **1**.

### *EO from* Artemisia copa *Phil. var. copa*

In the case of *A. copa* EO, GC-MS and GC-FID analyses of sample **6** resulted in the detection of 27 compounds (Table 1), representing 87.2% of the total oil. The major

detected constituent was chrysanthenone (55.5%). The other main constituents were linalool acetate (8.2%), piperitone (4.9%) and linalool (4.3%).

As mentioned above, many *Artemisia* species produce thujones which are thujane-type monoterpens (*A. absinthium, A. verlotorum*). Chrysanthenone is an oxidized pinane type monoterpene. It was reported in high proportions (>30%) in EO from *A. herba-alba* from various origins: North Africa, South of Europe, Middle East, and in another Asteraceae species, *Laggera tomentosa* (37–39). In this latter species, Asfaw et al. showed a fraction of chrysanthenone could isomerize into filifolone, and also transform into (Z)-isogeranic after thermal cleavage due to hydrodistillation (39). These two compounds were not detected by GC-MS in our case.

However, these results strongly differ from a previous study of the EO of *A. copa* from Bolivia, (40). Any of our major constituents (chrysanthenone, linalool or piperitone) was detected in the EO obtained from a Bolivian specimen. In this case, it was composed mainly of β-thujone (42%), chamazulene (6.3%) and 9-(15, 16-dihydro-15-methylene-geranyl)-*p*-cymene (6.1%).

Chrysanthenone, which is also produced in low amounts in some *Chrysanthemum* species, has been studied as behaviour modulator for cabbage moth *Mamestra brassicae* (L.), eliciting upwind flight of mated females (41).

### *Radical scavenger activity and XO inhibition*

The antioxidant activity of EO is of great interest for food preservation purpose and because oxidative stress is involved in many human chronic diseases (chronic inflammatory diseases, diabetes, neurodegenerative disorder). Some EO constituents such as thymol, carvacrol, eugenol and *p*-cymene have demonstrated a high antioxidant capacity in various models (42). In our case, the monoterpene ketones thujones and crysanthenone were the main identified compounds. Literature data reports that thujones are weak antioxidants (43). To date, there is no report on the antioxidant capacity of chrysanthenone.

DPPH inhibition assay is a good indicator of antioxidant activity in a screening approach. It was completed with XO assay because XO is also involved in oxidative stress as it uses $O_2$ to produce superoxide anion $O_2^-$ and uric acid; thus, inhibition of this enzyme may lower the oxidative stress. Moreover, hypouricemiant activity can also be highlighted with this assay.

Table 2 shows that all our EO are weak radical scavengers compared to quercetin in the DPPH assay. However, XO assay revealed high inhibitory activity for *A. deserticola* EO 1–4 containing thujones, the most active one being sample 3 with 27.0 ± 1.6% of inhibition. *A. copa* EO (6) showed no activity in both assays.

**Table 2.** Antioxidant activity and xanthine oxidase inhibitory activity of essential oils of *A. deserticola* at different altitudes and of *A. copa* var. copa.

| | Percentage of activity of EOs at 200 µg/mL (%) | | | | | Reference |
|---|---|---|---|---|---|---|
| Sample | 4 | 3 | 2 | 1 | 6 | EC $_{50}$values |
| 2,2-diphényl-1-picrylhydrazyl radical (DPPH) | n.a. | 7.8 ± 0.1 | 6.1 ± 1.0 | 5.0 ± 0.5 | n.a. | 62 ± 1.3[1] |
| xanthine oxidase (XO) | n.a. | 27.0 ± 1.6 | 6.5 ± 1.8 | 2.8 ± 0.7 | n.a. | 4.2 ± 0.2[2] |

Notes: n.a.: no activity observable; 1) $EC_{50}$ of quercetin; 2) $IC_{50}$ of allopurinol.

### *Antibacterial screening*

Among the forty-four tested strains (twenty-five Gram-negative and sixteen Gram-positive), we did not observe any growth inhibition by EO of *A. deserticola* and *A. copa* var. *copa* in the condition of experiments. For *A. deserticola*, these results are in accordance with the lack of antimicrobial effect by the Tarapaca specimen EO (26).

## Acknowledgments

The authors thank Félix Galleguillos and the Taira Community (Antofagasta, Chile) for helping during the collection of plants and CONICYT Scholarship (Comisión Nacional de Investigación Científica y Tecnológica, Chile) for PhD studies support for S. Ortiz. The authors also thank Pr Pierre Champy for providing access to GC-MS apparatus.

## Disclosure statement

No potential conflict of interest was reported by the authors.

## Funding

This work was supported by the Comisión Nacional de Investigación Científica y Tecnológica, Chile.

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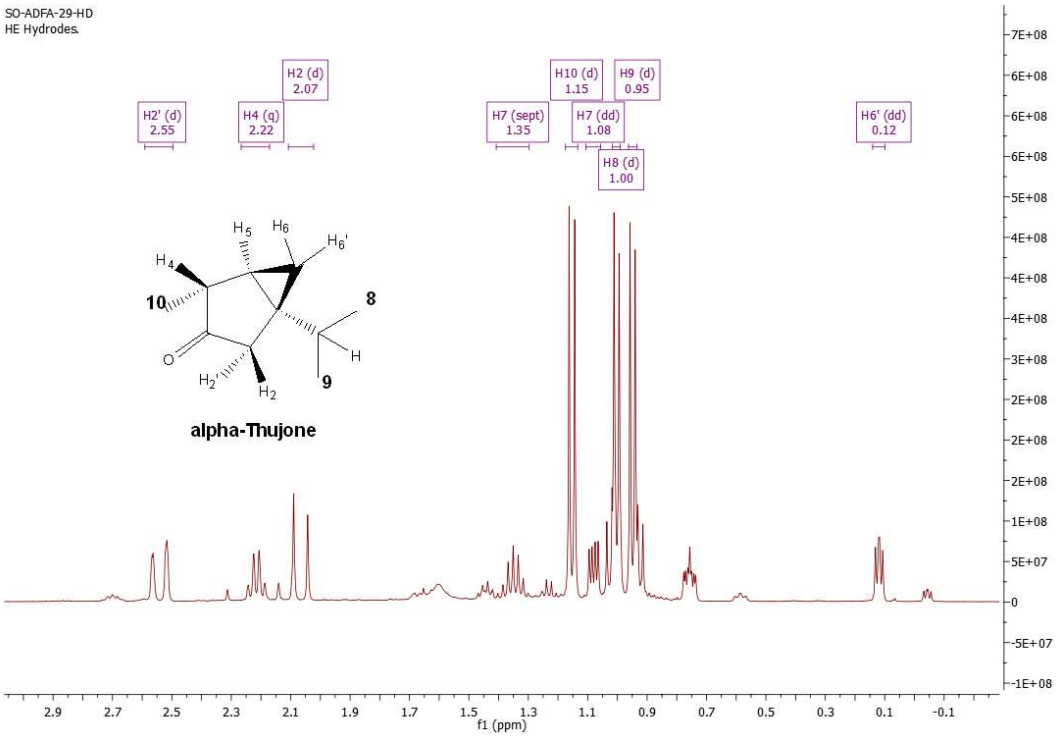
## Supporting Information

$^{1}$H-NMR Spectrum of EO **1** ($CDCl_3$, 400 MHz)



## Conclusion and perspectives

The main conclusions extracted of this communication are:

- The main constituent of *A. deserticola* essential oil was the α-thujone isomer, confirmed by $^{1}$H-NMR analysis. In the case of *A. copa*, chrysanthenone was identified as principal compound present in the essential oil.
- There is a slight correlation between the altitude where *A. deserticola* is collected and the proportion of thujone isomers and sabinene in the essential oils.
- The essential oils obtained by hydrodestillation exerted no significant antibacterial activity.
- Essential oil are weak radical scavenger, but sample **3** from *A. deserticola* showed a percentage of inhibition of xanthine oxidase enzyme 27 % at 200 µg/mL, which also contributes to lower production of radical oxygen species (ROS) *in vivo*.

Many food products available in Chile contain essential oil of medicinal plants such as *A. deserticola*, with no specific regulation from the food security agency. As main perspective, a protocol of detection and quantification of α/β-thujone isomers in essential oil containing commercial food products (beers, cocktails, etc.) derivated from A. deserticola should be set up, due to their neurotoxicity.

## I.5.5 Anti-inflammaory and antioxidant activity of non-volatil extracts and compounds of medicinal plants from Atacama People (Calama, Chile).

After chemical study of essential oil of *A. copa* and *A. deserticola*, we focused on the antioxidant and anti-inflammatory activities of non-volatile compounds from medicinal plants with indications related to inflammatory disordes. In this context, the workflow was as follows:

- Selection of extracts from plants with ethnobotanical reports related to inflammation
- Evaluation of the antioxidant activity by several assays:
    - DPPH and ABTS free radical scavenging activity in 96-well plate colorimetric assays at final concentrations of 200 and 20 µg/mL. Determination of $EC_{50}$ for the most active samples.
    - Xanthine-oxidase inhibition assay by extracts in a 96-well plate UV spectroscopy assay at 100 µg/mL. Determination of $IC_{50}$ for the most active extracs.
    - Polyphenol, flavonoids and flavan-3-ol derivatives total equivalent quantification in 96-well plate colorimetric assay.
- Evaluation of the anti-inflammatory activity of selected extracts and pure compounds by the ability to reduce *in vitro* pro-inflammatory cytokines from LPS-stimulated 264.7 macrophage cell line, as described in point I.3.5.2.3.
- Isolation of compounds from the most promising extracts:
    - Fractionation by silica gel column chromatography with cyclohexane/ethyl acetate of active extracts.
    - Purification of compounds by preparative HPLC with C18 and PFP columns and $H_2O$/MeOH or $H_2O$/ACN solvents mixtures.
    - Structure elucidation by spectroscopic (1D- and 2D-NMR) and spectrometric (MS) techniques.
    - Evaluation of the anti-inflammatory activity of isolated compounds by the reduction of several pro-inflammatory cytokines production as previously described.

## I.5.6 Draft 2: Anti-inflammatory and antioxidant activities of medicinal plants from the traditional medicine of Atacama People (Calama, Chile).

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Abstract

1 Introduction

Chronic inflammatory systemic disease like rheumatoid arthritis, atherosclerosis and many others are a burden to human because of life-long debilitating illness, increased mortality and high costs for therapy and care (Straub et al. 2016). These chronic pathologies together are intrinsically connected with oxidative stress and overproduction of pro-inflammatory mediators (Readnower at al. 2010) ans cytokines such as interleukin 1β, 6 (IL-1β, 6) or tumor necrosis factor α (TNFα), which involve different inflammatory pathways well documented (Svanborg et al. 1999, Delavary et al. 2011).

The Atacama People, an indigenous culture present in the north of Chile and northeast of Argentina, use around 200 medicinal plants for several indications. Six species are still used in the traditional medicine by the community of Taira (Calama, Chile) to fight inflammation symptoms. Anti-inflammatory and antioxidant investigations of these plants and isolation of metabolites could led to the validation of this knowledge and to the discovery of new bioactive compounds against inflammatory disorders.

| Scientific Name | Common Name | Traditional Uses | Part of the plant used | Reference |
|---|---|---|---|---|
| *Artemisia copa* (Phil.) var. copa | Kopa-kopa | Inflamation | Leaves and stems (cataplasm and infusions) | Villagrán and Castro. 2003. |
| *Baccharis calliprinos* Griseb. | Chilka | Rheumatism | Leaves and stems (cataplasm and infusions) | |

| | | | |
|---|---|---|---|
| *Krameria lappacea* (Dombey) Burdet & B. B. Simpson | Chaka-chaka | Inflammation produced by infections | Roots (infusions) |
| *Polylepis tarapacana* Phil. | Keñoa | Inflamation | Leaves and stems (infusions) |
| *Fabiana denudata* Miers | Tara tara | Wound injuries | Leaves and stems (cataplasm and infusions) |
| *Fabiana squamata* Phil. | Koba | Wound injuries | Leaves and stems (cataplasm and infusions) |

Table 1: Medicinal plants with anti-inflammatory reports used in the Taira Atacama People community.

## 2 Experimental

### 2.1 Chemicals

Automatized pressurized extractions were performed on a Speed Extractor E-914 apparatus provided by Büchi (Flawil, Switzerland) equipped with four cells (120 mL) and a collector with four flat bottom vials (220 mL). Maximum pressure and temperature can be adjusted from 50 to 150 bar and from 30 to 200 °C, respectively. Preparative HPLC was performed on an AP-MOD-100 apparatus provided by Armen Instrument (Saint-Avé, France), with C18 PursuitTM Varian (250 mm x 30 mm, 10 µm) or PFP NUCLEODUR® (250 mm x 30 mm x 5 µm) columns, Büchi UV/Vis Filter-photometer detector, Merck D-2500 integrator and Büchi C-660 collector. Mass spectrometry was performed on a mass spectrometer ZQ 2000 Waters (Saint-Quentin, France). SPECTROstar Nano BMG LabTech UV/vis spectrometer was used for colorimetric and enzymatic inhibition assays. Silica gel 60 0.025-0.04 mm (Macherey-Nagel GmbH & Co.) was used for column chromatography (CC). $^{1}H$ and $^{13}C$ NMR experiments were performed in deuterated chloroform. Spectra were recorded on a Bruker AC 300 MHz or a Bruker 400 MHz spectrometer (Wissembourg, France). Dimethylsulfoxide (DMSO), gallic acid, quercetin, Folin-Ciocalteu reagent, 4-(dimethylamino)-cinnamaldehyde (DMAC), hydrochloric acid (HCl), sulfuric acid ($H_2SO_4$), catechin, xanthine oxidase, xanthine, allopurinol, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), lipopolysaccharide (LPS), $FeSO_4$, $FeCl_3 \cdot 6H_2O$, $AlCl_3 \cdot 6H_2O$, $Na_2CO_3$, $CH_3COONa$, $CH_3COOH$, $Na_2HPO_4$, $NaH_2PO_4$, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ABTS were purchased from Sigma Aldrich (St. Lous, USA). Methanol (MeOH), ethanol (EtOH), acetonitrile (ACN), dichloromethane (DCM), cyclohexane (Cy) and ethyl acetate (EtOAc) were purchased from Carlo Erba Reagents (Val-de-Reuil, France). DMEM medium was purchased from Gibco (Thermo Fisher Scientific,

Walthem, MS, U.S.). Quantification of pro-inflammatory cytokines production was conducted by the ELISAs Kits purchased from Immuno Tools (Friesoythe, Germany).

2.2 Plant Material and Pressurized Solvent Extraction (PSE)

Collection of medicinal plants species (*Artemisia copa* var. copa, *Baccharis calliprinos*, *Fabiana denudata*, *Fabiana squamata*, *Krameria lappacea*, *Polylepis tarapacana*) was carried out in September 2016, in the Atacama People community of Taira (Calama, Chile) in accordance with the Chilean legislation. Air-dried collected plant species were subjected to successive extraction by PSE (Büchi E-914, Flawil, Switzerland) with EtOAc and MeOH as previously reported (Ortiz et al., 2018).

2.3 Antioxidant evaluation of extracts

2.3.1 Total phenol content

Total polyphenol content (TPC) was estimated according to Folin-Ciocalteu (F-C) reagent method as previously reported with slight modification (Clarke et al., 2013). Briefly, in a 96-well plate, 25 µL of sample solution at 4 mg/mL were mixed with 125 µL of F-C reagent freshly diluted 1/10 with distilled water. After 5 min, 100 µL the solution was mixed with 100 µL of a solution 7.5 % of $Na_2CO_3$, left for 30 min in the dark and the absorbance was measured at 650 nm. Respective standard curve of gallic acid and blank (replacing F-C reagent with 125 µL of water) were run simultaneously, after that the total phenol content was calculated as mg of gallic acid equivalent per g of extract.

2.3.2 Total flavonoids evaluation

Total flavonoid evaluation (TFE) was performed according to (Bampouli et al., 2015). Briefly, in a 96-well plate, 50 µL of sample solution at 4 mg/mL were mixed with 160 µL of EtOH, 20 µL of 1.8 % of $AlCl_3$ and 20 µL of 0.8 % of $CH_3COONa$. Then, the plates were kept at room temperature for 40 min and the absorbance was measured at 415 nm. Respective standard curve of quercetin and blank (replacing $AlCl_3$ with 20 µL of $H_2O$ distilled) were run simultaneously, then the total flavonoid evaluation was calculated as mg of quercetin equivalent per g of extract.

2.3.3 Total proanthocyanidins equivalent content

The total proanthocyanidins equivalent quantification assay was measured by the 4-(dimethylamino)cinnamaldehyde (DMAC) assay as previous reported (Feliciano et al., 2012). Briefly, an acidified ethanol solution was prepared by adding 12.5 mL of hydrochloric acid (37%) to 12.5 mL of deionized water and 75 mL of ethanol. A 0.1% (w/v) DMAC reagent solution was prepared immediately prior to use. In a 96-well plate, 70 microliters of blanks (80% ethanol), calibration standards, and samples was dispensed into individual wells. A multichannel pipet was

used to dispense 210 µL of DMAC reagent solution into each well, excluding blanks. Immediately after the addition of DMAC reagent, the plate was placed into a plate reader and absorbance readings at 640 nm were taken after 8 min of incubation at room temperature protected by light. The corresponding blank absorbance readings at each time point were subtracted from those from the standard and sample wells. Catechin was used as standard and results are expressed mg of catechin equivalent per g of extract.

2.3.4 Ferric Reducing Antioxidant Power Assay

Ferric reducing antioxidant power was measured according of ferrous tripyridyltriazine complex formation assay previously reported (Clarke et al., 2013). Briefly, 20 µL of extract diluted appropriately in DMSO was mixed with 180 µL FRAP reagent in wells of a 96-well plate, left for 6 minutes, and the absorbance measured at 595 nm. FRAP reagent was prepared freshly by mixing 300 mM acetate buffer pH 3.6, 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3.6H_20$ in the volume ratio 10:1:1. Appropriate blanks of plant extract and of FRAP reagent lacking TPTZ (to correct for colour of the extracts) were run, together with trolox (in DMSO) and $FeSO_4$ as a standard. The results were expressed as mg of trolox equivalent per g extract.

2.3.5 DPPH Scavenging Assay

Radical scavenging capacity was measured using DPPH agent according to previously reported (Clarke et al., 2013). DPPH reagent solution (12.4 mg in 100 mL of EtOH) was prepared freshly just before the assay. Extract were evaluated at a final concentration of 200 µg/mL. In each well were placed 10 µL of sample solution with 190 µL of DPPH solution. Plates were incubated at room temperature by 30 min protected from light. The absorbance was measured at 517 nm. Measured were performed by triplicated. Gallic acid was used as positive control. Results are expressed as percentage of efficacy at 200 µg/mL of extract.

2.3.6 ABTS Scavenging Assay

Radical scavenging capacity was measured using ABTS reagent according to previous reported (Stagos et al., 2012). ABTS reagent was prepared as follows: 10 mL of 7 mM of ABTS solution were mixed with 164 µL of 140 mM of $K_2S_2O_8$ and stored overnight (16 h). The final solution was diluted with distilled $H_2O$ (1:20) to reach an absorbance of 0.70 ± 0.02 before the assay. Extract were evaluated at a final concentration of 200 µg/mL. In each well were placed 100 µL ABTS final solution and 50 µL of sample. Plated were incubated at room temperature by 10 min protected from light. The absorbance were measured at 734 nm. Measured were performed by triplicated. Quercetin was used as positive control. Results are expressed as percentage of efficacy at 20 µg/mL.

2.3.7 Xanthine Oxidase Inhibition Assay

Evaluation of inhibition of xanthine oxidase enzyme (XO) of extracts was determined using the modified protocol from (Havlik et al., 2010). Briefly, in a quartz 96-well plate were added 62.5 µL of extract solution at a concentration of 800 µg/mL with 105 phosphate buffer (0.12 mM, pH 7.5) and 20 µL of xanthine oxidase solution (0.125 units/mL in phosphate buffer) and incubated at room temperature protected from light by 5 min. Then, 62.5 µL of xanthine solution (200 µM in phosphate buffer) were added in each well and the absorbance was measured at 295 nm during 30 min (final DMSO concentration of 0.4 %). Corresponding blanks were carried out in parallel, replacing xanthine oxidase solution by 62.5 µL of phosphate buffer. Initial rate was calculated and the percentage of inhibition was calculated as follows: Percentage of inhibition = (1-((ΔAbs sample – ΔAbs blank sample)/(ΔAbs DMSO – ΔAbs Blank DMSO))) x 100. Allopurinol was used as positive control.

**Antioxidant activity of medicinal plants**

| Plant Specie | TPC[a] | TFE[b] | TPEC[c] | FRAP[d] | DPPH[e] | $EC_{50}$ DPPH | ABTS[f] | $EC_{50}$ ABTS | XO[g] |
|---|---|---|---|---|---|---|---|---|---|
| *Artemisa copa* | 38,6 ± 0,8 | 66,8 ± 1,0 | 2,0 ± 1,1 | 156,2 ± 17 | 21,9 ± 1,3 | | 25,8 ± 3,28 | | **81,0 ± 1,1** |
| | 46,8 ± 1,3 | 88,1 ± 2,7 | 1,9 ± 0,1 | 175,8 ± 3,5 | 47,7 ± 4,1 | | 21,3 ± 13,17 | | 67,8 ± 0,2 |
| *Baccharis calliprinos* | 75,5 ± 3,4 | **111,0 ± 2,4** | 2,0 ± 0,0 | **359,3 ± 5,8** | 59,3 ± 6,0 | | 28,9 ± 0,58 | | **95,1 ± 3,7** |
| | 76,9 ± 0,8 | 75,0 ± 6,1 | 2,0 ± 0,2 | 294,9 ± 9,5 | 78,0 ± 2,0 | | 27,5 ± 1,10 | | 75,0 ± 1,5 |
| *Krameria lappacea* | **201,6 ± 0,3** | 2,3 ± 0,3 | **50,5 ± 0,0** | 252,9 ± 12,3 | **93,1 ± 0,2** | **46,9 ± 5,5** | **85,6 ± 1,71** | **9,1 ± 1,5** | ± |
| | **386,0 ± 1,6** | 17,8 ± 1,1 | **310,3 ± 0,0** | **532,1 ± 0,0** | **94,0 ± 0,0** | **16,2 ± 2,4** | **98,9 ± 0,09** | **6,1 ± 0,7** | **84,2 ± 0,3** |
| *Polylepis tarapacana* | 69,4 ± 1,6 | 14,7 ± 0,99 | 14,5 ± 1,5 | 287,0 ± 13,2 | 67,6 ± 0,7 | | 32,9 ± 0,37 | | ± |
| | **121,2 ± 0,6** | 30,5 ± 0,23 | 17,9 ± 0,8 | **521,2 ± 20,5** | **93,9 ± 0,4** | **38,4 ± 3,0** | **64,5 ± 5,42** | **15,4 ± 0,3** | ± |
| *Fabiana denudata* | 61,6 ± 1,2 | **188,5 ± 3,6** | 1,9 ± 0,0 | 222,8 ± 9,3 | 31,5 ± 2,2 | | 23,0 ± 2,47 | | ± |
| | 62,5 ± 1,0 | 26,1 ± 1,0 | 2,1 ± 0,0 | 183,6 ± 5,7 | 53,1 ± 4,0 | | 27,0 ± 12,64 | | 57,6 ± 0,9 |
| *Fabiana squamata* | 20,0 ± 0,5 | 1,7 ± 0,2 | 2,0 ± 0,0 | 166,6 ± 1,9 | 8,1 ± 2,5 | | 0,0 ± | | 52,8 ± 2,5 |
| | 47,2 ± 0,8 | 14,5 ± 0,3 | 1,9 ± 0,0 | 35,9 ± 2,6 | 28,3 ± 2,7 | | 22,3 ± 10,45 | | ± |
| Gallic acid | | | | | 48,1 ± 2,8 | | | | |
| Trolox | | | | | | | 55 ± 0,2 | | |
| Allopurinol | | | | | | | | | 57 ± 0,4 |

Table 1: a) Total Phenolic Content: mg of gallic acid equivalent per g of extract. b) Total Flavonoid Evaluation: mg of quercetin equivalent per g of extract. c) Total Proanthocyanidins Equivalent Content: mg of catechin equivalent per g of extract. d) Ferric Reducing Activity Power: mg of $Fe^{+2}$ equivalent per g of extract. e) Percentage of DPPH scavenging at 200 µg/mL of extract. f) Percentage of ABTS scavenging at 20 µg/mL. g) Percentage of xanthine oxidase inhibition at 100 µg/mL of extract.

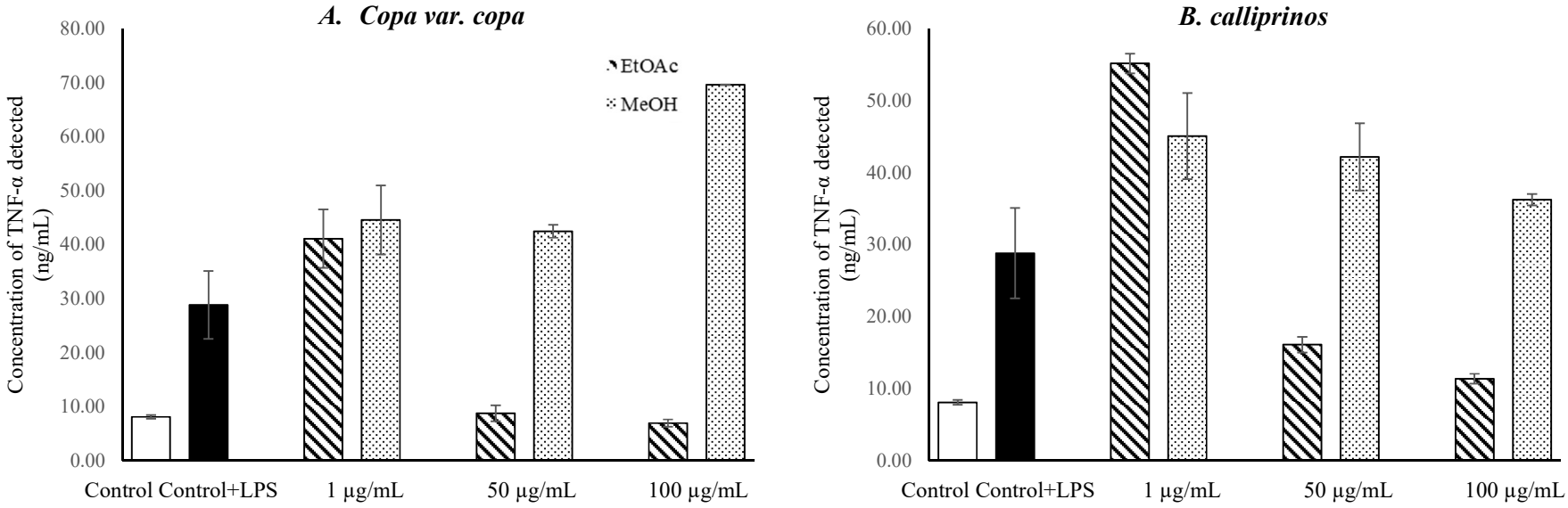


**Fig. X** Effect of Asteraceae extracts on LPS-stimulated cytokine release from RAW 264.7 cell line.

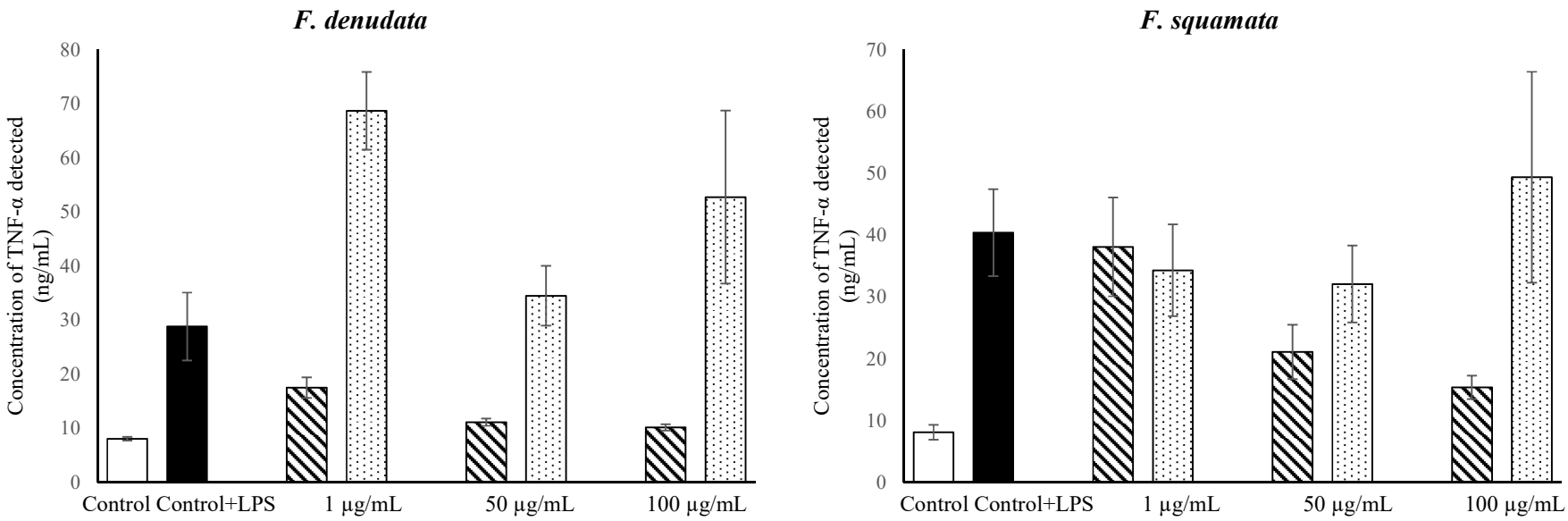


**Fig. X** Effect of *Fabiana* extracts on LPS-stimulated cytokine release from RAW 264.7 cell line.

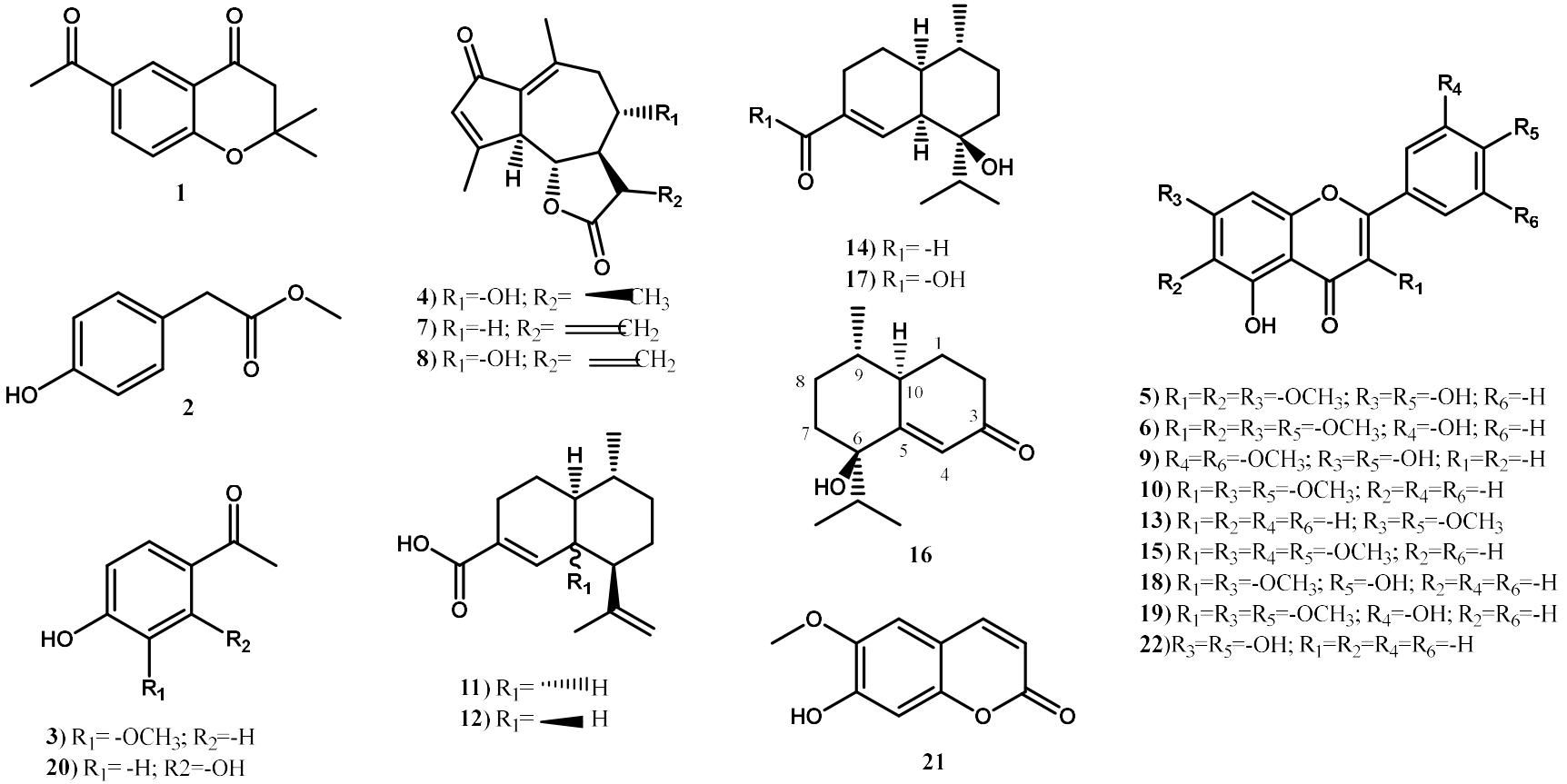


**Figure 2**: Isolated compounds from *A. copa* var. copa, *F. denudata* and *F. squamata*.

2.4 Anti-inflammatory evaluation

2.4.1 Cell culture

RAW 264.7 mouse macrophage cell line was incubated in DMEM supplemented with penicillin and streptomycin at 1%. In a 24-well plate were incubated 1 mL of cell solution (70,000 cell/mL) at 37°C and 5% $CO_2$ atmosphere overnight.

2.4.2 Expression of pro-inflammatory cytokines

Briefly, after incubations, cells were aliquoted in the 24-well plate, 2.5 µL of extract solution in DMSO per duplicate were added in order to have final concentration of 100, 50 and 1 µg/mL (0.25 % DMSO final concentration) and incubated as previously mentioned by 30 min. Then, 10 µL of LPS solution (10 µg/mL) were added in the wells and incubated overnight. Afterward, 1 mL of supernatants were transferred to eppendorfs and frozen them at -20 °C until evaluation.

2.4.3 ELISAs

Quantification of pro-inflammatory cytokines production was conducted using the ELISAs Kits purchased from Immuno Tools (Friesoythe, Germany). Briefly, 96-well plates were previously prepared, transferring 100 µL of anti-cytokine (IL-6 or TNFα) captured antibody previously diluted (1:100) and incubated overnight at room temperature. Then, solution was removed and 300 µL of blocking-buffer mixture (PBS with 2 % PBS and 0.05 % of Tween20) and incubated shaking for 1 h at room temperature. After, solution were removed from plates and 100 µL of samples (diluted in blocking buffer solution in 1:2 for IL-6 and 1:20 for TNFα quantification) and standards (2000-62.5 pg/mL of TNFα and 500-15.6 pg/mL of IL-6 cytokines) were added. The plates were shacked by 2 h at room temperature. Then, plates were washed (PBS with 0.05 % Tween20) 5 times and buffer removed and 100 µL of anti-cytokine detection antibody solution (1:100 diluted) were added to each well. The plates were incubated for 2 h, shaking at room temperatures. Plates were washed 5 times, buffer removed and 100 µL of poly-HRP-streptavidin solution (1:1000 diluted) were added per well and incubated 30 min in room temperature. Plates were washed 5 times, buffer removed and 100 µL of substrate solution (12 mL PBS buffer with 200 µL of TMB at 6 mg/mL and 12 µL of $H_2O_2$ at 3 %) were added and incubated in the dark for 20 min, then 50 µL of 2M of $H_2SO_4$ were added per well to stop the reaction. Absorbance were recorded at 450 nm and results expressed as percentage of cytokine detected in relation to controls.

2.4.4 Cell Viability Assay

Antiproliferative activity of apolar extracts of *A. copa* var. copa, *B. calliprinos*, *F. denudata* and *F. squamata* were evaluated by the alamarBlue™ assay. Briefly, 100 µL of RAW 264.7 cell suspension (50,000 cell/mL) were seeded in a 96-well plate and incubated overnight at 37 °C at 5% of $CO_2$ atmosphere. Then, 100 µL of extract solution at several concentrations (400 – 1 µg/mL) diluted in medium (final DMSO concentration of 0.25%) were added and incubated for 24 h at same conditions. After, 20 µL of alamarBlue™ reagent were added, incubated for 2 h and absorbance recorded at 570 nm. Results are expressed as percentage in relation of respective blanks.

2.5 Isolation of bioactive compounds

2.5.1 *Artemisia copa* var. copa

A sample of 4 g of EtOAc extract of *A. copa* var. copa were subjected to a silica gel chromatography with a gradient of cy/EtOAc/MeOH mixture solvent (10:0:0 to 0:8:2), obtaining 29 fractions (**FAC1-29**). Fractions **FAC11**, **FAC19**, **FAC25** AND **FAC27-28** were purified by preparative-HPLC with a C-18 column and **FAC23-24** with a PFP column using $H_2O$/ACN or $H_2O$/MeOH eluent solvent mixture.

2.5.2 *Fabiana* species

Two grams of EtOAc extract of *F. denudata* and 1 g of EtOAc extract of *F. squamata* were subjected to a silica gel chromatography with a gradient of Cy/EtOAc mixture solvent (10:0 to 0:10), obtaining 22 fractions (**FFD1-22**) and 17 fractions (**FFS1-17**) respectively. For *F. denudata* fractions **FFD9** and **FFD12-15** and for *F. squamata* fractions **FFS8** and **FFS14** were purified by preparative-HPLC (C-18 column) with $H_2O$/ACN mixture solvent.

3 Results

Radical scavenging capacity of extracts

The highest levels of polyphenols detected were founded in both ethyl acetate and methanolic extracts of *K. lappacea* (201 and 386 mg of gallic acid equivalent per g of extract) and in the methanolic extract of *P. tarapacana* (121 mg of gallic acid equivalent), while the lower level was observed in the apolar extract of *F. squamata* (20 mg of gallic acid equivalent). In addition, *K. lappacea* extracts showed hich amounts of proanthocyanidins with 50.5 and 310.3 mg of catechin equivalent per g of extracts. Regarding the flavonoid evaluation, the apolar extract of *B. calliprinos* and *F. denudata* presented the highest flavonoid content with 111 and 188.5 mg per g of extract of quercetin equivalent respectively.

The extracts of *K. lappacea* and methanolic extracts of *P. tarapacana* showed the strongest free radical scavenging capacity with $EC_{50}$ of 46.9, 16.2 and 38.4 against DPPH and 9.1, 6,1 and 15.4

µg/mL against ABTS radicals. Apolar extract of *B. calliprinos* and polar extracts of *K. lappacea* and *P. tarapacana* showed high ferric reducing antioxidant power of 359, 532 and 521 µg of ferrous equivalent per g of extracts.

*In vitro* inhibitin of cytokines production

The apolar extract of *F. denudata* showed the lowest level of TNFα and IL-6 cytokines in all tested concentrations, in comparison the apolar extract of *F. squamata* showed weak inhibition of cytokines production. Polar extracts of *Fabiana* species exerted no diminution of cytokine detected in relation of controls. Regarding the Asteraceae family extracts, the apolar sample of *A. copa* var. copa decreased the TNFα production at same levels that blank controls at 100 and 50 µg/mL of concentration. No anti-inflammatory activity was observed for polar extracts of *A. copa* var. copa and *B. calliprinos*.

Isolated compounds from active extracts

The apolar extract from *A. copa* var. copa, *F. denudata* and *F. squamata* were subjected to fractionation and purification following the procedure described in section 2.5. Structural identification was checked by 1D-, 2D-NMR experiments and mass spectrometry in comparison with published data. For *A. copa* extract, from **FAC11** was isolated 6-acetoxy-2,2-dimethylchroman-4-one (**1**, 38 mg). From **FAC16** were isolated methyl 4-hydroxyphenylacetate (**2**, 6 mg) and acetovanillone (**3**, 5.4 mg). From **FAC19**, **FAC23-25** and **FAC27-28** were isolated hydroxyachillin (**4**, 24 mg), jaceidin (**5**, 32 mg), chrysoplenetin (**6**, 45 mg), dehydrolecodin (**7**, 8 mg), 11,13-dehydrodesacetylmatricarin (**8**, 12 mg) and tricin (**9**, 18 mg).

In the case of *Fabiana* species, ten compounds were obtained from apolar extract of *F. denudata* and three from the same extract of *F. squamata*. Respecting *F. denudata*, from **FFD9** were isolated 3,7,3'-trimethoxykaempferol (**10**, 32.5 mg), pernetic acid B (**11**, 12.3 mg) and pernetic acid C (**12**, 6.8 mg). From **FFD12** were isolated 7,3'-dimethoxyapigenin (**13**, 12.2 mg) and pernatal (**14**, 5.6 mg). From **FFD13** were isolated 3,7,3'-trimethylkaempferol (**10**, 13.8 mg) and 3,3',4',7-tetramethoxyquercetin (**15**, 24.0 mg). From **FFD14** was isolated oxyphyllenone H (**16**, 14.0 mg). From **FFD15** were isolated pernetic acid A (**17**, 8.9 mg), kumatakenin (**18**, 4.6 mg) and ayanin (**19**, 8.9 mg). For fractions of *F. squamata*, from **FFS8** was isolated 2,4-dihydroxyacetophenone (**20**, 7.5 mg) and from **FFS14** were isolated scocoletin (**21**, 4.5 mg) and apigenin (**22**, 2.3 mg).

For compound **16**, $^{1}$H-, $^{13}$C-NMR and HRMS spectra are identical with those of epi-oxyphyllenone reported by Hue et al. (Hue et al 2015). Relative configuration was only reported for C-10 position in comparison of CD spectroscopy of its isomer and determinated as *R*. Spectre $^{2}$D-NOESY spectra were recorded in $CDCl_3$ and $C_6D_6$ in order to clarify the relative configuration

of position C9 and C6. In $CDCl_3$ NOESY spectra, correlations are visualized between H-10 (δ 2.00 ppm) and $CH_3$-14, $CH_3$-13 and H-2α (δ 1.05, 0.75 and 2.26 ppm respectively) and between H-9 (δ 1.48 ppm) with H-2β (δ 2.38 ppm). Same correlations were observed in the NOESY spectra in $C_6D_6$ and in addition an extra correlation between H-11 and H-2α (δ 1.63 and 1.98 ppm respectively). Thus, the relative stereochemistry proposed for **16** is 6*R**, 9*S**, 10*R**.

## 4 Discussion

The antioxidant and anti-inflammatory activities of six medicinal plants from the Atacama People Taira community, together with the isolation and biological activity of main compounds are reported.

Antioxidant activity of extracts.

Regarding the antioxidant potential of extracts, the polar extracts of *K. lappacea* and *P. tarapacana* showed the greatest radical scavenging activity, ferric reducing power and mild xanthine oxidase inhibition. This activity may be related with the polyphenol and flavon-3-ol equivalent content detected in these extracts. In the case of *K. lappacea*, the high amount of catechin equivalents could explain its antioxidant activity. These results are in accordance with previous reports of the presence of flavon-3-ol derivatives (2-14 flavon-3-el oligomers) (Scholz and Rimpler, 1989) with reported antioxidant activity (Škerget et al., 2005).

Some proanthocyanidins oligomers exerted xanthine oxidase inhibition properties, explaining also the inhibition produced by the methanolic extract of *K. lappacea* (Phuwapraisirisan et al., 2006). Poly methoxyflavonoids have been also reported as xanthine oxidase inhibitors, their presence in the apolar extracts of *A. copa* var. copa, and *B. calliprinos*, detected as quercetin equivalents, could explain the inhibitory properties exerted by these extracts (Santi et al., 2018; Nagao et al. 1999).

Anti-inflammatory activity of extracts

The reduction of cytokines TNFα and IL-6 by the apolar extract of *A. copa* are in concordance with previous reports of the anti-inflammatory ability of the dichloromethane extract of this plant in the reduction of ear oedema in rats. Several flavonoids and sesquiterpene lactones isolated from *Artemisia* extracts fight inflammation through several mechanisms as inhibition of production of pro-inflammatory mediators or reduction of radical species.

Antioxidant and anti-nflammatory activity of ethanolic extracts of *Fabiana* species (*F. bryoides*, *F. densa*, *F. patagonica* and *F. punensis*) from the Argentinian Altiplano, have been reported (Cuello, 2011). This activity was directly correlated with the total polyphenolic content. In our

work, the apolar extract of *F. denudata* presents the highest lowering effect on TNFα and IL-6 cytokines production and the highest level of flavonoid content (of quercetin equivalents detected). This large amount of detected flavonoids is in accordance with the quantity of isolated **10**, **13**, **15**, **18** and **19** flavonoids.

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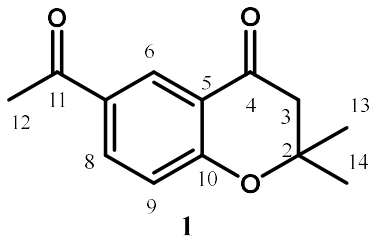
## SUPPORTING INFORMATION

***NMR and MS data of isolated compounds:***

Structural elucidation of isolated compounds was achieved by comparison of their experimental spectroscopy ($^1$H, $^{13}$C NMR), specific rotation $[\alpha]_D$ and spectrometry (MS) data with those previously reported in the literature.

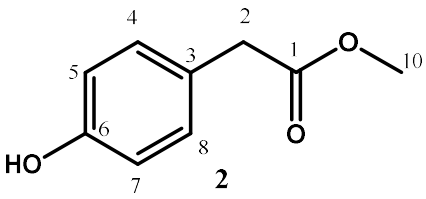
1) 6-Acetyl-2,3-dihydro-2,2-dimethyl-4*H*-1-benzopyran-4-on




**1**

White powder: $^1$H NMR ($CDCl_3$): δ 8.41 [d, *J* = 2.1 Hz, 1H, H-6], 8.10 [ dd, *J* = 7.6, 2.1 Hz, 1H, H-8], 6.96 [d, *J* = 7.6 Hz, 1H, H-9], 2.75 [s, 2H, H-3], 2.57 [s, 3H, H-12], 1.49 [s, 6H, H-13, H-14]. $^{13}$C NMR ($CDCl_3$): δ 196.2 (C-11), 191.0 (C-4), 163.4 (C-10), 135.4 (C-8), 130.2 (C-7), 128.0 (C-6), 119.5 (C-5), 119.0 (C-9), 80.4 (C-2), 48.6 (C-3), 29.6 (C-12), 26.6-26.3 (C-13, C-14). ESI-MS *m*/*z* (positive mode) 219.1 $[M+H]^+$ (calculated for $C_{13}H_{14}O_3$: *m*/*z* 218.1).

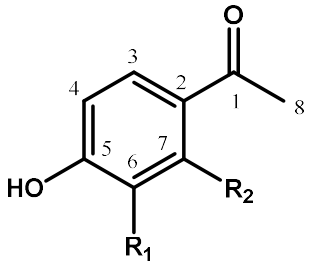
2) Methyl 4-hydroxyphenylacetate




**2**

Oil. $^1$H NMR ($CDCl_3$): δ 7.36 [d, *J* = 7.3 Hz, 2H, H-5, H-7], 7.15 [d, *J* = 7.3 Hz, 2H, H-4, H-8], 3.55 [s, 2H, H-2], 3.50 [s, 3H, H-10]. $^{13}$C NMR ($CDCl_3$): 170.5 (C-1), 157.8 (C-6), 130.9 (C-4, C-8), 126.1 (C-3), 116.3 (C-5, C-7), 52.2 (C-10), 41.0 (C-2). ESI-MS *m*/*z* (positive mode) 167.1 $[M+H]^+$ (calculated for $C_9H_{10}O_3$: *m*/*z* 166.1).

3) Acetovanillone

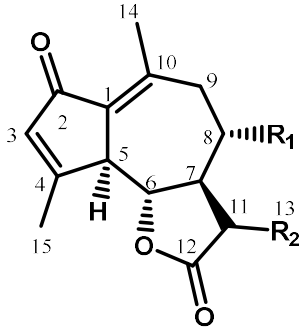



**3**) $R_1$= -$OCH_3$; $R_2$=-H
**20**) $R_1$= -H; R2=-OH

Oil. $^1$H NMR ($CDCl_3$): δ 7.56 [d, *J* = 7.4 Hz, 1H, H-4], 7.52 [dd, *J* = 7.4, 2.1 Hz, 1H, H-3], 6.90 [d, *J* = 2.1 Hz, 1H, H-7], 3.96 [s, 3H, -$OCH_3$], 2.54 [s, 3H, H-8]. $^{13}$C NMR ($CDCl_3$): δ 197.4 (C-1), 150.1 (C-5), 145.4 (C-6), 131.1 (C-2), 121.7 (C-3), 114.6 (C-4), 109.8 (C-7), 56.7 (-OCH3), 26.4 (C-8). ESI-MS *m*/*z* 167.1 $[M+H]^+$ (calculated for $C_9H_{10}O_3$ *m*/*z* 166.1).

20) 2,4-Dihydroxyacetophenone

Oil. 1H NMR (CDCl3): δ 7.70 [d, *J* = 8.2 Hz, 1H, H-3], 6.43 [dd, *J* = 8.2, 2.0 Hz, 1H, H-4], 6.32 [d, *J* = 2.0 Hz, 1H, H-6], 2.53 [s, 3H, H-2]. $^{13}$C NMR ($CDCl_3$): δ 190.0 (C-1), 166.3 (C-5), 164.4 (C-7), 135.0 (C-3), 112.6 (C-2), 107.1 (C-6), 102.1 (C-4), 24.3 (C-8). ESI-MS *m*/*z* 153.1 $[M+H]^+$ (calculated for $C_8H_8O_3$: *m*/*z* 152.0).




**4**) $R_1$=-OH; $R_2$= ◀ $CH_3$
**7**) $R_1$=-H; $R_2$= ═ $CH_2$
**8**) $R_1$=-OH; $R_2$= ═ $CH_2$

4) 8α-hydroxyachillin

White crystal. $[\alpha]_D^{23}$ +42.0° (c = 0.30, $CHCl_3$). $^1$H NMR ($CDCl_3$): δ 6.17 [s, 1H, H-3], 3.86 [d, *J* = 9.8 Hz, 1H, H-6]. 3.77 [dt, *J* = 10.5, 2.3 Hz, 1H, H-8], 3.40 [d, *J* = 9.7 Hz, 1H, H-5], 2.91 [q, *J* = 8.1 Hz, 1H, H-11], 2.76 [m, 1H, H-9a], 2.55 [m, 2H, H-7], 2.43 [m, 1H, H-9b], 2.31 [s, 3H, H-14], 1.27 [d, *J* = 8.1 Hz, 3H, H-13], 2.43 [s, 3H, H-15]. $^{13}$C NMR ($CDCl_3$): δ 196.0 (C-2), 178.5 (C-12), 170.0 (C-4), 146.1 (C-10), 135.6 (C-3), 132.1 (C-1), 80.9 (C-6), 65.0 (C-8), 57.7 (C-7),

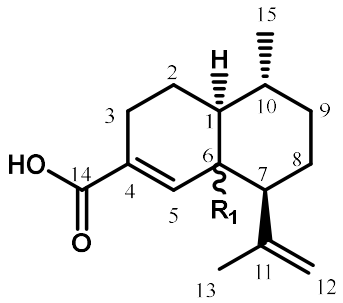
52.9 (C-5), 48.1 (C-9), 38.0 (C-11), 21.5 (C-14), 19.7 (C-15), 9,2 (C-13). ESI-MS *m/z* 285.1 $[M+Na]^+$ (calculated for $C_{15}H_{18}O_4$: *m/z* 262.1).

7) Dehydroleucodine

Colorless crystal. 1H NMR (CDCl3): δ 6.20-6.18 [br s, 2H, H-3, H-13a], 5.46 [d, J = 1.8 Hz, 1H, H-13b]. 3.62 [d, J = 9.8 Hz, 1H, H-6], 3.52 [d, J = 9.8 Hz, 1H, H-5], 2.90 [m, 1H, H-7], 2.54-2.50 [m, 1H, H-9a], 2,45 [s, 3H, H-14], 2.33 [s, 3H, H-15], 2.24-2.22 [m, 1H, H-9b], 1.45 [d, J = 10.3 Hz, 1H, H-8a], 1.26 [d, J = 9.8 Hz, 1H, H-8b]. 13C NMR (CDCl3): δ 195.7 (C-2), 170.0 (C-4), 193.5 (C-12), 153.0 (C-10), 138.1 (C-11), 136.2 (C-3), 132.0 (C-1), 119.3 (C-13), 86.0 (C-6), 53.2 (C-7), 53.1 (C-7), 53.0 (C-5), 37.2 (C-C-9), 25.0 (C-8), 22.2 (C-14), 20.0 (C-15). ESI-MS m/z 245.2 (calculated for C15H16O3: m/z 244.2).

8) 11,13-Dehydrodesacetylmatricarin

Colorless crystals. $^1$H NMR (Acetone-*d6*): δ 6.27 [d, *J* = 1.8 Hz, 1H, H-13a], 6.16-6.14 [br s, 2H, H-3, H-13b], 3.98 [dd, *J* = 9.8, 8.5 Hz, 1H, H-8], 3.78 [d, *J* = 9.8 Hz, 1H, H-6], 3.70 [d, *J* = 8.9 Hz, 1H, H-5], 3.24-3.20 [m, 1H, H-7], 2.92 [dd, *J* = 8.5, 2.1 Hz, 1H, H-9a], 2.45-2.43 [m, 1H, H-9b], 2.40 [s, 3H, H-10], 2.31 [s, 3H, H-4]. $^{13}$C NMR (Acetone-*d6*): δ 195.6 (C-2), 171.1 (C-4), 169.2 (C-12), 147.0 (C-10), 139.2 (C-11), 136.2 (C-3), 134.5 (C-1), 122.2 (C-13), 83.0 (C-6), 68.1 (C-8), 58.3 (C-7), 53.4 (C-5), 49.2 (C-9), 21.3 (C-14), 20.0 (C-15). ESI-MS *m/z* 283.1 (calculated for $C_{15}H_{16}O_4$: *m/z* 260.1).




**11**) $R_1$= ''''''''H
**12**) $R_1$= ◀ H

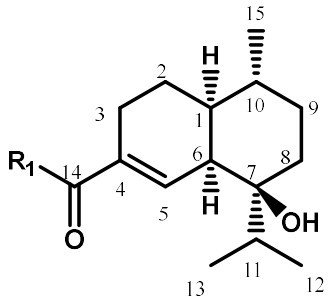
11) Pernetic acid B

White powder. $[\alpha]_D^{25}$ -35.5° (c = 0.5, $CHCl_3$). $^1$H NMR ($CDCl_3$): δ 6.87 [d, *J* = 2.1 Hz, 1H, H-5], 4.96 [d, *J* = 1.5 Hz, 1H, H-12a], 4.74 [d, *J* = 1.5 Hz, 1H, H-12b], 2.69 [dd, *J* = 3.5, 1.8 Hz, 1H, H-6], 2.28 [dd, *J* = 15.6, 5.9 Hz, 1H, H-3a], 2.16 [dd, *J* = 15.1, 6.9 Hz, 1H, H-3b], 2.12-2.07 [m, 2H, H-2b, H-7a], 1.76 [d, *J* = 1.2 Hz, 3H, H-13], 1.70 [d, *J* = 11.5 Hz, 1H, H-9b], 1.60-1.20 [m, 5H, H-10b, H-8a, H-8b, H-2a, H-1a], 1.02-0.99 [m, 1H, H-9a], 0.92 [d, *J* = 6.0 Hz, 3H, H-15]. $^{13}$C NMR ($CDCl_3$): δ 173.0 (C-14), 146.7 (C-11), 142.5 (C-5), 130.1 (C-4), 111.5 (C-12), 47.6

(C-6), 41.7 (C-10), 39.3 (C-7), 35.3 (C-9), 28.9 (C-1), 27.0 (C-8), 25.1 (C-3), 22.6 (C-13), 20.4 (C-2) 19.8 (C-15). ESI-MS *m/z* 257.2 $[M+Na]^+$ (calculated for $C_{15}H_{22}O_2$: m/z 234.2).

12) Pernetic acid C

White powder. $[\alpha]_D^{25}$ +35.5° (c = 0.5, $CHCl_3$). $^1H$ NMR ($CDCl_3$): δ 6.79 [br s, 1H, H-5], 4.86 [br s, 1H, H-12a], 4.75 [br s, 1H, H-12b], 2.45 [dd, *J* = 15.1, 6.5 Hz, 1H, H-3b], 2.21-2-15 [m, 2H, H-2a, H-3a], 1.91-1.67 [m, 4H, H-9b, H-8a, H-7a, H-6b], 1.76 [d, *J* = 1.6 Hz, 3H, H-13], 1.45 [dd, *J* = 12.7, 6.3 Hz, 1H, H-8b], 1.35-1.02 [m, 3H, H-1a, H-2b, H-10], 0.97 [d, *J* = 6.5 Hz, 3H, H-15]. $^{13}C$ NMR ($CDCl_3$): δ 174.1 (C-14), 147.6 (C-11), 144.3 (C-5), 130.0 (C-4), 112.5 (C-12), 50.4 (C-6), 45.6 (C-7), 43.9 (C-10), 36.9 (C-1), 36.3 (C-9), 26.0 (C-2, C-8), 24.8 (C-3), 19.7 (C-15), 19.3 (C-13). ESI-MS *m/z* 257.2 $[M+Na]^+$ (calculated for $C_{15}H_{22}O_2$: *m/z* 234.2).




**14**) $R_1$= -H
**17**) $R_1$= -OH

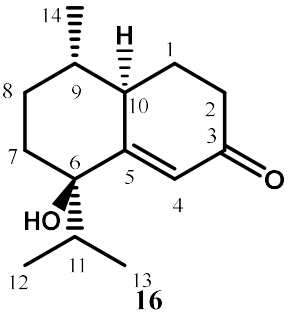
14) Pernatal

Colorless gum. $[\alpha]_D^{21}$ +15.5° (c = 0.5, $CHCl_3$). $^1H$ NMR ($CDCl_3$): δ 9.46 [s, 1H, H-14], 7.16 br s, 1H, H-5], 2.74 [br s, 1H, H-6], 2.24-2.21 [m, 1H, H-3a], 2.17 [q, *J* = 6.2 Hz, 1H, H-11], 2.10-2.01 [m, 2H, H-2a, H-3b], 1.75-1.71 [m, 1H, H-8b], 1.61-1.49 [m, 3H, H-9b, H-2b, H-1a], 1.35-1-25 [m, 2H, H-10b, H-8b], 1.12-1-09 [m, 1H, H-9a], 0.94 [d, *J* = 6.9 Hz, 3H, H-13], 0.94 [d, *J* = 7.2 Hz, 3H, H-15], 0.91 [d, *J* = 6.9 Hz, 3H, H-12]. $^{13}C$ NMR ($CDCl_3$): δ 194.5 (C-14), 153.9 (C-5), 141.2 (C-4), 75.0 (C-7), 44.8 (C-6), 38.0 (C-1), 32.8 (C-8), 30.3 (C-9), 29.5 (C-11), 27.7 (C-10), 24.2 (C-2), 19.1 (C-15), 17.6 (C-3), 15.9 (C-12), 15.7 (C-13). ESI-MS *m/z* 259.2 (calculated for $C_{15}H_{24}O_2$: *m/z* 236.2).

17) Pernetic acid A

White powder. $[\alpha]_D^{21}$ +2.9° (c = 1.2, $CHCl_3$). $^1H$ NMR ($CDCl_3$): δ 7.40 [br s, 1H, H-5], 2.65-2.62 [m, 1H, H-6a], 2.30 [dd, *J* = 11.8, 1.4 Hz, 1H, H-3a], 2.20 [dd, *J* = 11.1, 1.9 Hz, 1H, H-3b], 2.12 [q, *J* = 7.1 Hz, 1H, H-11], 2.07 [d, *J* = 11.4 Hz, 1H, H-2a], 1.70 [dd, 12.8, 3.1 Hz, 1H, H-8a], 1.58-1-49 [m, 3H, H-9a, H-1a, H-2b], 1.45-1.41 [m, 1H, H-10], 1.29 [d, *J* = 12.5 Hz, 1H, H-8b], 1.12-1.09 [m, 1H, H-9b], 0.92 [d, *J* = 7.1 Hz, 6H, H-12, H-13], 0.89 [d, *J* = 7.0 Hz, 3H, H-15].

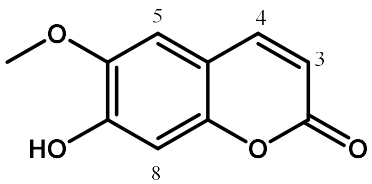
$^{13}$C NMR ($CDCl^3$): δ 173.0 (C-14), 132.9 (C-5), 130.4 (C-4), 75.2 (C-7), 43.8 (C-6), 37.6 (C-10), 32.1 (C-8), 30.5 (C-9), 30.0 (C-11), 28.3 (C-1), 25.0 (C-2), 20.5 (C-3), 19.4 (C-15), 16.0 (C-12, C-13). ESI-MS *m/z* 275.1 $[M+Na]^+$ (calculated for $C_{15}H_{24}O_3$).




**16**

16) Oxyphyllenone H

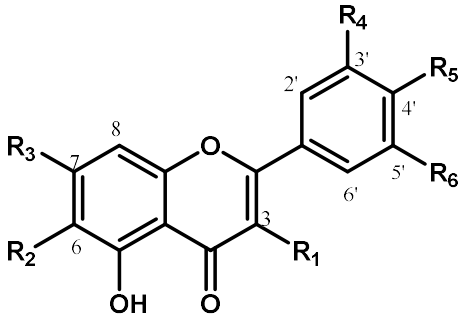
Colorless oil. $[\alpha]_D^{18}$-17.0° (c = 0.015, MeOH). $^1$H NMR ($CDCl_3$): δ 6.23 [d, *J* = 1.4 Hz, 1H, H-4], 2.44-2.35 [m, 1H, H-2b], 2.30-2.14 [m, 3H, H-7a, H-2a, H-1b], 2.08 [sept, *J* = 6.5 Hz, 1H, H-11], 2.05-1.96 [m, 1H, H-10], 1.80-1.67 [m, 3H, H-8b, H-8a, H-1a], 1.51-1.44 [m, 1H, H-9], 1.43-1.31 [m, 1H, H-7b], 1.06 [d, *J* = 6.0 Hz, 3H, H-14], 0.99 [d, *J* = 6.8 Hz, 3H, H-12], 0.75 [d, *J* = 6.8 Hz, 3H, H-13]. $^1$H NMR ($C_6D_6$): δ 6.43 [t, *J* = 1.5 Hz, 1H, H-4], 2.28-2.16 [m 1H, H-2b], 1.98 [td, *J* = 10.7, 4.8 Hz, 1H, H-2a], 1.80 [dd, *J* = 12.5, 2.3 Hz, 1H, H-7a], 1.70-1.64 [m, 1H, H-2b], 1.63-1.56 [m, 1H, H-11], 1.45-1.32 [m, 1H, H-10], 1.26-1.14 [m, 2H, H-2a, H-8b], 1.02-0.86 [m, 3H, H-9, H-8a, H-7b], 0.82 [d, *J* = 6.2 Hz, 3H, H-12], 0.64 [d, *J* = 6.2 Hz, 3H, H-13], 0.63 [d, *J* = 7.0 Hz, 3H, H-14]. $^{13}$C NMR ($CDCl_3$): δ 199.9 (C-3), 170.0 (C-5), 122.4 (C-4), 76.6 (C-6), 41.9 (C-10), 39.0 (C-9), 37.7 (C-7), 35.3 (C-2), 30.9 (C-8), 30.6 (C-11), 26.2 (C-1), 20.0 (C-14), 16.3 (C-13), 15.4 (C-12). $^{13}$C NMR ($C_6D_6$): δ 197.8 (C-3), 168.2 (C-5), 122.9 (C-4), 75.9 (C-6), 41.5 (C-10 ) 38.7 (C-9), 37.6 (C-7), 35.5 (C-2), 30.9 (C-8), 30.4 (C-11), 26.3 (C-1), 19.6 (C-14), 16.4 (C-13), 15.4 (C-12). ESI-MS *m/z* 223.1 $[M+H]^+$ (calculated for $C_{14}H_{22}O_2$: *m/z* 222.19).




**21**

21) Scopeletin

Yellowish powder. $^1$H NMR ($CDCl_3$): δ 7.60 [d, *J* = 9.4 Hz, 1H, H-4], 6.92 [s, 1H, H-5], 6.85 [s, 1H, H-8], 6.27 [d, *J* = 9.4 Hz, 1H, H-3], 3.96 [s, 3H. $-OCH_3$]. ESI-MS *m/z* 193.1 $[M+H]^+$ (calculated for $C_{10}H_8O_4$: *m/z* 192.1].




**5**) $R_1$=$R_2$=$R_4$=-$OCH_3$; $R_3$=$R_5$=-OH; $R_6$=-H
**6**) $R_1$=$R_2$=$R_3$=$R_5$=-$OCH_3$; $R_4$=-OH; $R_6$=-H
**9**) $R_4$=$R_6$=-$OCH_3$; $R_3$=$R_5$=-OH; $R_1$=$R_2$=-H
**10**) $R_1$=$R_3$=$R_5$=-$OCH_3$; $R_2$=$R_4$=$R_6$=-H
**13**) $R_1$=$R_2$=$R_4$=$R_6$=-H; $R_3$=$R_5$=-$OCH_3$
**15**) $R_1$=$R_3$=$R_4$=$R_5$=-$OCH_3$; $R_2$=$R_6$=-H
**18**) $R_1$=$R_3$=-$OCH_3$; $R_5$=-OH; $R_2$=$R_4$=$R_6$=-H
**19**) $R_1$=$R_3$=$R_5$=-$OCH_3$; $R_4$=-OH; $R_2$=$R_6$=-H
**22**)$R_3$=$R_5$=-OH; $R_1$=$R_2$=$R_4$=$R_6$=-H

5) Jaceidin

Yellow powder. $^1$H-NMR (MeOD): 7.60 [dd, *J* = 8.5, 2.0 Hz, 1H, H-6'], 7.57 [d, *J* = 1.9 Hz, 1H, H-2'], 7.02 [d, *J* = 8.5 Hz, 1H, H-5'], 6.47 [s, 1H, H-8], 3.94 [s, 3H, -$OCH_3$-4'], 3.87 [s, 3H, -$OCH_3$-6], 3.76 [s, 3H, -$OCH_3$-3]. $^{13}$C-NMR (MeOD): δ 180.0 (C-4), 157.8 (C-7), 156.6 (C-2), 153.6 (C-5), 153.0 (C-9), 150.9 (C-4'), 147.3 (C-3'), 139.3 (C-3), 131.8 (C-6), 124.3 (C-1'), 121.9 (C-6'), 115.9 (C-2'), 112.2 (C-5'), 106.4 (C-10). 94.6 (C-8), 59.6 (-$OCH_3$-3), 59.4 (-$OCH_3$-6), 55.0 (-$OCH_3$-4'). ESI-MS *m*/*z* 359.1 $[M-H]^-$ (calculated for $C_{18}H_{16}O_8$: *m*/*z* 360.1).

6) Chrysosplenetin

Yellow powder. $^1$H NMR (MeOD): δ 7.68 [dd, *J* = 8.5, 2.0 Hz, 1H, H-6'], 7.64 [d, *J* = 2.0 Hz, 1H, H-2'], 7.07 [d, *J* = 8.5 Hz, 1H, H-5'], 6.76 [s, 1H, H-8], 3.97 [s, 3H, -$OCH_3$-3], 3.95 [s, 3H, -$OCH_3$-3'], 3.84 [s, 3H, -$OCH_3$-6], 3.81 [s, 3H, -$OCH_3$-7]. $^{13}$C NMR (MeOD): δ 178.9 (C-4), 159.2 (C-7), 156.6 (C-2), 152.5 (C-9), 152.0 (C-4'), 150.4 (C-3'), 146.3 (C-5), 138.5 (C-3), 131.9 (C-6), 122.8 (C-6'), 120.8 (C-1'), 114.8 (C-5'), 110.9 (C-2'), 105.9 (C-10), 90.8 (C-8), 59.6 (-$OCH_3$-6), 59.2 (-$OCH_3$-3), 55.6 (-$OCH_3$-7), 55.0 (-$OCH_3$-3'). ESI-MS *m*/*z* 375.2 $[M+H]^+$ (calculated for $C_{19}H_{18}O_8$: *m*/*z* 374.2).

9) Tricin

Yellow powder. $^1$H NMR (MeOD): 7.32 [s, 2H, H-2', H-6'], 6.97 [s, 1H, H-3], 6.55 [d, *J* = 1.8 Hz, 1H, H-8], 6.20 [d, *J* = 1.8 Hz, 1H, H-6], 3.89 [s, 6H, -$OCH_3$-3', -$OCH_3$-5']. ESI-MS *m*/*z* 329.1 $[M-H]^-$ (calculated for $C_{17}H_{14}O_7$: *m*/*z* 330.1).

10) 3,4',7-Trimethoxykaempferol

Yellow powder. $^{1}$H NMR ($CDCl_3$): δ 8.10 [d, *J* = 8.5 Hz, 2H, H-2', H-6'], 7.05 [d, *J* = 8.5 Hz, 2H, H-3', H-5'], 6.47 [d, *J* = 2.0 Hz, 1H, H-8], 6.38 [d, *J* = 2.0 Hz, 1H, H-6], 3.92 [s, 3H, -$OCH_3$-4'], 3.90 [s, 3H, -$OCH_3$-7], 3.88 [s, 3H, -$OCH_3$-3]. $^{13}$C NMR ($CDCl_3$): δ 178.8 (C-4), 165.3 (C-7), 162.0 (C-5), 161.7 (C-4'), 156.7 (C-9), 155.9 (C-2), 138.7 (C-3), 130.1 (C-2', C-6'), 114.1 (C-3', C-5'), 122.8 (C-1'), 106.1 (C-10), 97.2 (C-6), 92.1 (C-8), 60.1 (-$OCH_3$-3), 55.8 (-$OCH_3$-7), 55.4 (-$OCH_3$-4'). ESI-MS *m*/*z* 327.1 $[M-H]^-$ (calculated for $C_{18}H_{16}O_6$: *m*/*z* 328.1).

13) 4',7-Dimethoxyapigenin

Yellow powder. $^{1}$H NMR ($CDCl_3$): δ 7.77 [d, *J* = 8.5 Hz, 2H, H-2', H-6'], 6.95 [d, *J* = 8.5 Hz, 2H, H-3', H-5'], 6.53 [d, *J* = 1.5 Hz, 1H, H-8], 6.51 [d, *J* = 1.5 Hz, H-6], 6.47 [s, 1H, H-3], 3.98 [s, 3H, -$OCH_3$-7], 3.84 [s, 3H, -$OCH_3$-4']. ESI-MS *m*/*z* 299.1 $[M+H]^+$ (calculated for $C_{17}H_{14}O_5$: *m*/*z* 298.1].

15) Retusin

Yellow powder. $^{1}$H NMR ($CDCl_3$): 7.69 [dd, *J* = 8.5, 1.8 Hz, 1H, H-6'], 7.64 [d, *J* = 1.8 Hz, 1H, H-2'], 6.94 [d, *J* = 8.5 Hz, 1H, H-5'], 6.40 [d, *J* = 2.1 Hz, 1H, H-8], 6.30 [d, *J* = 2.0 Hz, 1H, H-6], 3.92 [s, 3H, -$OCH_3$-4'], 3.92 [s, 3H, -$OCH_3$-3'], 3.83 [s, 3H, -$OCH_3$-7], 3.80 [s, 3H, -$OCH_3$-3]. ESI-MS *m*/*z* 381.1 $[M+Na]^+$ (calculated for $C_{19}H_{18}O_7$: *m*/*z* 358.1).

18) Kumatakenin

Yellow powder. $^{1}$H NMR (MeOD): δ 8.09 [d, *J* = 9.0 Hz, 2H, H-2', H-6'], 7.08 [d, *J* = 9.0 Hz, H-3', H-5'], 6.62 [d, *J* = 1.9 Hz, 1H, H-8], 6.34 [d, *J* = 1.9 Hz, 1H, H-6], 3.88 [s, 3H, -$OCH_3$-7], 3.80 [s, 3H, -$OCH_3$-3]. ESI-MS *m*/*z* 315.1 [M+H]+ (calculated for $C_{17}H_{14}O_6$: *m*/*z* 314.1].

19) Ayanin

Yellow powder. $^{1}$H NMR ($CDCl_3$): δ 7.75 (dd, *J* = 8.5, 2.0, 1H, H-6'], 7.72 [d, *J* = 2.0 Hz, 1H, H-2'], 6.99 [d, *J* = 8.5 Hz, 1H, H-5'], 6.47 [d, *J* = 2.2 Hz, 1H, H-8], 6.38 [d, *J* = 2.2 Hz, 1H, H-6], 4.01 [s, 3H, -$OCH_3$-4'], 3.90 [s, 3H, -$OCH_3$-7], 3.89 [s, 3H, -$OCH_3$-3]. ESI-MS *m*/*z* 345.1 $[M+H]^+$ (calculated for $C_{18}H_{16}O_7$: *m*/*z* 344.1).

22) Apigenin

Yellow powder. 1H NMR (MeOD): δ 7.78 [d, *J* = 9.1 Hz, 2H, H-2', H-6'], 6.90 [d, *J* = 9.1 Hz, 2H, H-3', H-5'], 6.51 [s, 1H, H-3], 6.42 [d, *J* = 2.1 Hz, 1H, H-8], 6.22 [d, *J* = 2.1 Hz, 1H, H-6]. ESI-MS *m*/*z* 271.1 $[M+H]^+$ (calculated for $C_{15}H_{10}O_5$: *m*/*z* 272.1).

## Conclusion and perspectives

The most important contribution of the precedent publication are:

- First report of the anti-inflammatory activity of extracts and isolated compounds from medicinal species of the Fabiana, genus that present current uses to treat inflammation disorders.
- First evidence of the antioxidant potential of *P. tarapacana* polar extract.
- First reports of the reduction of pro-inflammatory cytokine production of apolar extract of *F. denudata* and *F. squamata* on macrophage cell line.
- First report of a phytochemical composition of *F. denudata* and *F. squamata*, comprising eleven flavonoids, five sesquiterpene, one coumarin and 1 acetophenone derivatives.

# I.6 Cytotoxic activity.

In the previous communications here described, the antibacterial, antifungal and anti-inflammatory activities of medicinal plant have been explored. Clear evidence of the bioactivity of isolated compounds from *A. deserticola*, *K. lappacea*, *F. denudata* and *A. copa* var. copa has been demonstrated to support the traditional uses of these medicinal plants.

As some reported symptoms may also be related to cancer disease, we have decided to explore the antiproliferative activity of the medicinal plants against several cancer cell lines.

Cancer cell lines were selected on the basis of the traditional medicinal uses of the collected plants:

- Plants to treat cough, chest pain or pulmonary disorder were evaluated against lung cancer cell line.
- Species related to colon pain or digestive disorders; they have been evaluated against colon cancer cell lines.
- Plants to treat bone pain were evaluated against bone cancer cell lines.
- Plants to treat bladder pain or disorders were evaluated against bladder cancer cell lines.

Due the low mass extract of the more active sample found in the antiproliferative screening, a work of dereplication in order to identify the bioactive compounds without need to isolate was programmed.

## I.6.1 HetCA as dereplication tool

The study of natural products has been always challenging due the tedious and long process of fractionation, purification and structural elucidation of metabolites present in extracts. Those, resulting in several cases in the identification of already known compounds with their biological activities already described [91].

Dereplication is the identification of known metabolites in complex mixtures to avoid their re-isolation [91]. Putative compounds are obtained by comparing preliminary spectral data to spectral databases of known metabolites. Recent developments of NMR and MS technics, enhancing their sensitivity, specificity and resolution, are improving the confident degree of dereplication process.

Hetero-covariance is a computer-based NMR spectroscopy methodology, which transfer NMR chemical shift information to other spectroscopic technics. This is visualized as a 2D correlation map, provided a perturbation domain, such as time or concentration change [92].

Heterocovariance analysis (HetCA) approach combines chemical and biological data of fractions obtained after a fractionation process performed on a bioactive extract. The key step of this approach is to have a variation of concentration of constituents over several fractions that correlate with a concentration dependent property, in this case, a biological activity. The resulted 2D correlation map (pseudo-spectra) allows to discriminate between an active-positive region where are the signals that are more present in the active fractions and an inactive-negative region, where is possible to find the signals present in the inactive fractions. This approach allows the early identification of bioactive compounds in the mixture before purification steps [93,94].

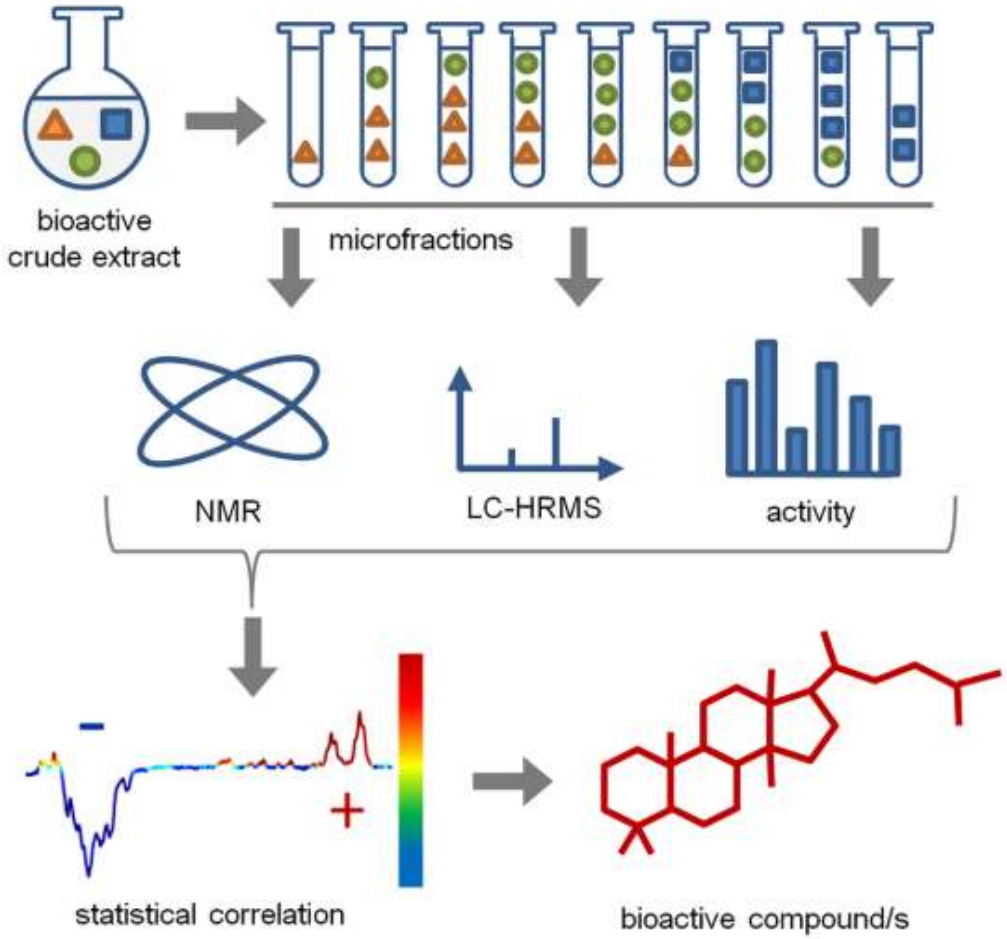



**Figure 60: Schematic overview of Heterocovariance analysis approach** [93,94]**.**

## I.6.2 Centrifugal Partition Chromatography, CPC

Centrifugal partition chromatography is a chromagraphy technic where both mobile and stationary phase are inmiscible liquids and the stationary phase is immobilized by a strong centrifugal force. CPC equipment is composed by series-connected network of extraction cells that operates as elements extractors [95].

Chromatography separation of complex mixture, such as vegetal extracts, by the affinity of constituents in each inmiscible phases (partition coefficient, $K_D$). In this way, compounds with a high affinity for mobile phase ($K_D > 1$) exit in a first place, followed by compounds with an equal affinity for both phases ($K_D \approx 1$) and in a final step, the compounds with an affinity for the stationary phase are expulsed form the cells.

The principal advantages of CPC technic are the no costly solid packing material, no silice uses (cancerigen), no sample loss and almost 100% recovery, no denaturation of fragile molecules, low solvent consumption and the versatility in use with a single column (normal and reverse phase, dual mode, pH-zone refining, ion exchange, etc.).



**Figure 61: SCPC-250+10000 CPC equipment, Armen (France).**

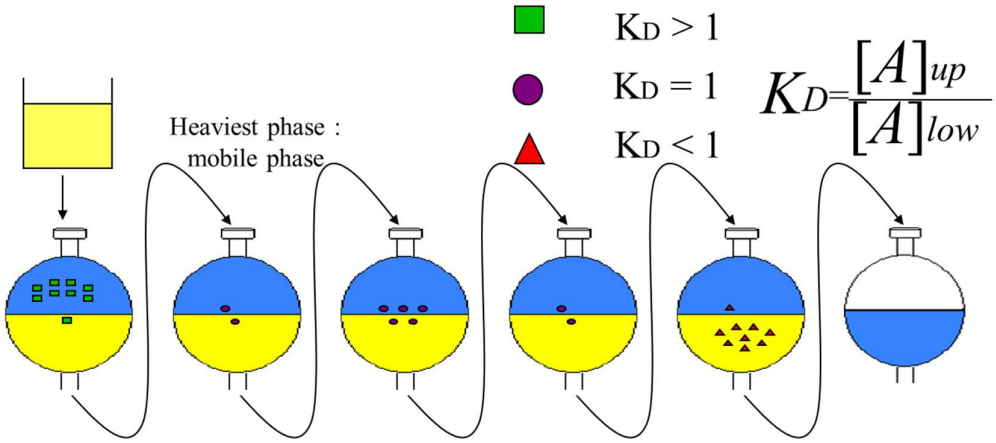



**Figure 62: Partition phenomena of constituents with different $K_D$ values in a byphasic system.**

## I.6.3 Methodology

### I.6.3.1 Supercritical fluid extraction

In this section, supercritical fluid extraction was applied, in addition of PSE procedure in the preparation of extracts. In this methodology, supercritical fluids act as the extracting solvent. $CO_2$ is the most used supercritical fluid, and in this case, the polarity of supercritical fluid is modified by co-solvents such as ethanol. Extraction conditions for supercritical $CO_2$ must be above the critical temperature of 31 °C and the critical pressure of 74 bar. The main advantage of this technic is the fact of use less toxic reagents, as $CO_2$ and ethanol.




**Figure 63: SEPAREX 200 Supercritical fluid extractor**

In our case, 10 g of vegetal material were subjected toa supercritical fluid extraction with $CO_2$ as supercritical fluid and 5, 10 and 20% of ethanol as co-solvent, leading to three consecutive extracts in each case.

### I.6.3.2 Antiproliferative activity

To evaluate the antiproliferative activity of extracts, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyletrazolium bromide) and CellTiterBlue™ (resazurin) colorimetric assays were chosen to assessing cell metabolic activity. In order to minimize the time of screening and $IC_{50}$ evaluation, a first global screening at final extract concentration of 25 µg/mL was executed and only the more active extracts were purchased to the $IC_{50}$ evaluation.

### I.6.3.3 HeteroCovariance analysis

HetCA approach has been used to correlate the biological activity with variation of chemical composition of tested samples. It was chosen due to the expertise developed by our partner laboratories (Department of Pharmacognosy and Natural Products Chemistry, Kapodistrian University of Athens, Athens, Greece).

This approach is segmented in three steps:

- Fractionation of complex matrix: to have a fluctuation of the constituents in the complex matrix, like a plant extract, it is necessary to start with a procedure of fractionation to obtain small part of this matrix (called fractions), with a variation of concentration of the constituents within them. This fractionation procedure could be carried out by different chromatography technics as silica gel column or centrifuge partition chromatography.
- Biological and analytical assessment: analytical and bioactive data must be recover to each fractions obtained on the precedent steps. The analytical data may obtained by $^{1}$D-NMR spectra ($^{1}$H or $^{13}$C-NMR) and/or LC-MS. The bioactive assays of fractions must give quantitative values as MICs, $IC_{50}$, $IC_{90}$, etc. Until now, only anti-enzymatic assays have been applied in the HetCA approach.
- Statistical correlation: data treatment of spectra, previously recovered, by MATLAB® software.

After the large cytotoxic screening, methanolic extract of *Chuquiraga atacamensis* was selected to be subjected to HetCA analysis in order to identifiy the bioactive compounds present in the extract. The next scheme was followed:

Fractionation of more active extract: methanolic extract of *Chuquiraga atacamensis* was selected due its lower antiproliferative $IC_{50}$ values against several cancer cell lines and subjected to fractionation by centrifugal partition chromatography (CPC). This method is a special chromatographic technique where both stationary and mobile phase are liquid, and the stationary phase is immobilized by a strong centrifugal force. This chromatography methodology utilise two phases immiscible solvent mixtures to separate the constituents of a complex mixture based of their affinity to one of the phases defined by their partition coefficient (*Kd*). Several solvents mixture solvents were previously tested in a small scale of 10 mg of extract in 4 mL of final volume. Partition behaviour was monitored by TLC. CPC fractionation was carried out with EtOAc/EtOH/$H_2O$ – 10/1/10 solvent system in an ascendant mode. Twenty tree fractions were obtained. Each of them were dry and analysed by HPTLC-UV and $^1$H-NMR.

Biological and spectroscopy data acquisition: in addition, $IC_{50}$ values of the antiproliferative activity against HCT116 colon cancer cell line. HPTLC-UV was performed with reverse phase silica gel with MeOH/$H_2O$/Formic acid – 50/50/1 as eluent solvent. Plates were dried and observed under 254 and 366 nm UV. $^1$H-NMR spectra were executed in and 600 MHz Avance III Bruker NMR spectrometer in methanol-4d solvent in 1 mg/mL concentration.

HetCA Analysis: $^1$H-NMR spectra and $IC_{50}$ recorded were applied to HetCA scripts in MATLAB® software of all fractions together and in group of four samples as fr1-fr4, fr2-fr5, fr3-fr6, etc. and pseudospectrum were obtained. $^1$H-NMR spectra were bucketed (covered range: $\delta_H$: 0.5 – 8.5 ppm; bucket width: 0.005 ppm, resulting in 1600spectroscopy buckets). This process results in reduced spectra in their complexicity by summation of all the data points per bucket. The intensities of $^1$H-NMR resonances of each bucket were calculated and served as variables for subsequent analyses. Covariance as a measure of the joint variability between two variables (1) 1H-NMR resonance intensity and (2) the biological data was calculated. The normalized version of covariance (correlation coefficient) was calculated and represented by colour coding. Thus, the resulting bucket-specific covariance values were plotted as $^1$H-NMR pseudo-spectrum. In the superior phase of the pseudospectrum are present the proton signal of compounds which are more present in the more active extracts and in the inferior phase, from compounds which are more present in the less active fractions. In that way we can discriminate which compounds contribute more to the biological activity of the extract.

It is important to mention that the next draft presents preliminary results. Deeper NMR studies must be carry out shuch as STOCSY with the aim of elucidate the correct chemical structure of bioactive compounds present in the methanolic extract of *C. atacamensis*.

## I.6.4 Preliminary draft 3: Antiproliferative activity of medicinal plants from the Atacama People traditional medicine.

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Abstract

1 Introduction

The exploration and the valorisation of traditional medicine pass from tedious process of biological evaluation and isolation and re-evaluation of bioactive compounds purified and sometimes finding already known compounds in the literature. Efforts to identified bioactive compounds from already complex matrix as extracts have took place in las decades. Several methodologies have been developed in bases of the $MS^n$ fragmentation or NMR (1D or 2D) spectra analysis of extracts and comparison with databases. Recently, HeteroCovariance Approach (HetCA) have been elaborated with the purpose of identified putative bioactive compounds after a process of fractionation and evaluation of fractions, without the exhausting process of purification and isolation of compounds. HetCA is bases in the variance of concentration of compounds by the process of fractionation. The variance is monitored by NMR experiments as $^1$H-NMR spectra. Recently, HetCA was applied for active fractions of *Paeonia parnassica* from the Greek flora, with anti-enzymatic properties against acetylcholinesterase and hyaluronidase enzymes (Rozalia et al., 2018).

The traditional medicine of Atacama People is largely used in the north of Chile, even in the urban areas of Calama, Antofagasta and Iquique cities. Not previous extensive phytochemical or biological activity studies from medicinal plants are reported. Recently, our group divulged the antibacterial and antifungal activity of extracts and isolated compounds from 18 medicinal plants collected in the Atacama People community of Taira (Ortiz et al., 2018). Besides anti-infectious purpose of medicinal plants, others therapeutic uses as against inflammation, haematuria, cough, chest or bone pain are also reported. As these symptoms may related also to cancer, we have

explored the antiproliferative potential of medicinal plants by HetCA analysis, in order to efficiently identify the active compounds in the extracts.

## 2 Material and methods

### 2.1 Chemicals

Ethyl acetate (EtOAc), methanol (MeOH), dichloromethane (DCM), $NH_4Cl$ (ammonia solution 30%), ethanol (EtOH), dimethylsulfoxide (DMSO), [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide] (MTT), Dulbecco's minimal essential medium (DNEM), Eagle's minimal essential medium (EMEM), fetal calf serum (FCS), fetal bovin serum (FBS) were purchased to SigmaAldrich (St. Louis, USA).

### 2.1 Plant collection

Collection of plants was performed in September 2015 within the Atacama Community of Taira (Calama, Chile). The collect was respectful with the Chilean National Biodiversity Strategy and Nagoya Protocol regulation and the pertinent Official Material Transfer Agreement was provided from the Taira community and registered at the INIA (INIA, Instituto de Investigaciones Agropecuarias, Ministerio de Agricultura, Chile, application number 2-2016). Plants were selected on the basis of their traditional uses against related-symptoms to different types of cancers and by previous ethnobotanics surveys (Baraona et al., 1958; Chamorro P. and Tocornal M., 2005; Madaleno and Delatorre-Herrera, 2013; Villagrán et al., 2003; Villagrán and Castro, 2003). Eighteen different plant species were collected and air-dried. Taxonomic identification was ensured by Pr. Alicia Marticorena, responsible of the CONC Herbario, Universidad de Concepción, Chile. Vouchers of each specimen were deposited in the Herbarium of the Laboratory of Pharmacognosy, Paris Descartes University, France, and in CONC Herbario, Universidad de Concepción Chile.

### 2.2 Extraction

#### 2.2.1 Pressurized Solvent Extraction (PSE)

All plant materials were air-dried and pulverized in an electric grinder Pulverisette 19 (Fritsch GmbH, Mahlen und Messen, Germany). For *Aloysia deserticola* and *Artemisia copa* var. copa, leaves and stems were separated and extracted separately, due to the different medicinal proposes for each part. Twenty grams of aerial parts were subjected to extraction. Extracts were obtained by PSE using a Speed Extractor E-914 (Büchi, Flawil Switzerland) equipped with four cells (120 mL) and a collector with four flat bottom vials (220 mL) successively with EtOAc and MeOH. Maximum pressure and temperature were adjusted to 100 bar and to 50 °C, respectively. Two extraction cycles with a hold-on time of 15 min were performed in each case.

2.2.2 Classic maceration

For *Ephedra americana* Humb. & Bonpl. Ex Willd., the PSE methanolic extraction was performed as well as maceration of 10 g of vegetal material with 10% $NH_4Cl$ (250 mL) at room temperature by 24 h.

2.2.3 Supercritical Solvent Extraction (SFE)

The extraction was conducted using a High Performance Solvent Extraction Lab System SFE 100 ml (Separex Champigneulles, France) system. 220 g of dry powdered herb was placed in the stainless steel extraction cell. Pure carbon dioxide was delivered on extraction basket with the flow of 5.0 kg/h. EtOH at 10 % were used as co-solvent in the experiments. The extraction temperature was set at 50 °C and pressure at 300 bar and kept them by 1 h. Extracts were collected after 1 h.

After the extractions, solvents were removed under reduced pressure. A total of 60 extracts were obtained. Stock solutions (200 mg/mL) were prepared in DMSO of each extract and stocked at 4 °C until biological evaluations.

2.3 CPC Fractionation

2.3.1 Preparation of biphasic system and sample solution

A biphasic mixture EtOAc/EtOH/$H_2O$ – 10/1/10 (v/v/v) was selected after evaluation of five systems. The solvents were thoroughly mixed in a separation funnel at room temperature prior to use and the two phases were collected separately after equilibration of the mixture. Methanolic extract from *C. atacamensis* was diluted in an equal volume of each phase prior the injection.

2.3.2 CPC Apparatus

CPC separations were performed on CPC (centrifugal partition chromatography) preparative 200 Kromaton apparatus (Rousselet Robatel Kromaton, Annonay, France) equipped with a rotor composed of 840 twin cells, with a total column capacity of 266 mL (volume of the cells: 200 mL, of the ducts: 66 mL), a 50 mL injection loop and a UV detector.. Rotation speed was set up between 600 to 1,500 rpm, producing a centrifugal force field in the partition cells about 120 g at 1,000 rpm. Method development, pumping, detection, fraction collection and automated control of FCPC were ensured by the Kromaflash integrated CPC-peripheral system, using Glider software.

2.3.3 CPC Separation procedure

After filling with the stationary phase (lower phase) at slow speed (200 rpm), the rotation was increased to 850 rpm and the mobile phase pumped through the system at a flow-rate of 10

mL/min. After injection of methanolic extract of *C. atacamensis*, 20 fractions of 30 mL were collected. Extrusion was then achieved by pumping the lower phase at 30 mL/min and 600 rpm. All fractions were dried under reduced pressure and weighed.

2.4 HPTLC Profile

HPTLC instrumental set-up was purchased from Camag (Muttenz, Switzerland) and consisted of an ATS4 automated sample applicator, an ADC2 automated development chamber, a TLC Scanner 3 and a Visualiser for the documentation. The system was operating under thewinCats 1.4.9 Planar Chromatography Manager software (Camag). HPTLC reverse phase glass plates (20 cm × 10 cm) pre-coated with silica gel 60 F254were purchased from Merck (Darmstadt, Germany). Extract and dried fractions of *C. atacamensis* derived from CPC as described earlier, were weighted and diluted in methanol at a concentration level of 10 and 1 mg/mL respectively. The sample application volume was set to10µL for samples and the band length was set at 8 mm. The application position was set at 10 mm from the bot-tom of the plate. Plate was loaded with 10 tracks of fractions. Nitrogen flow provided a delivery speed of 150 nL/s through the application syringe. HPTLC plates were developed in a presaturated glass chamber (20 min presaturation) up to 80 mm height. The mobile phase employed was for reverse phase plate (MeOH/$H_2O$/FA – 50/50/1) for extracts and fractions. Plates were subsequently air-dried and pictures obtained under 254 and 366 nm wavelength and at white light after derivatization with vanillin/sulfuric acid reagent.

2.5 Anti-proliferative activity

2.5.1 Cell Cultures

Cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA) and were cultured according to the supplier's instructions. Briefly, human MRC-5 lung normal cells were grown in EMEM containing 4.5 g/L glucose supplemented with 10% FCS and 1% glutamine. Human A549 and H1299 lung carcinomas cells were grown in DMEM containing 4.5 g/L glucose supplemented with 10% FCS and 1% glutamine. Human SJSA-1 osteosarcoma and HCT116 colorectal carcinoma cells were grown in RPMI 1640 containing 10% FCS and 1% glutamine. RT4 transitional cell papilloma from urinary bladder and U-2 OS osteosarcoma cells were grown in McCoy's 5a Medium Modified, FBS 10 % and 1 % of glutamine. All the cell lines were maintained at 37˚C in a humidified atmosphere containing 5% $CO_2$.

2.5.1 MTT Assay

HCT 116 cells were plated at a cell density of 5000 cells/well in 96-well plates 24 h prior to treatment with the appropriate extract solution (diluted in medium) of 200-12.5 µg/mL for 96 h.

The cells were incubated with 10% MTT and added directly in the medium for 4 h at 37°C. The medium was then aspirated and the MTT formazan crystals were solubilized with DMSO. Absorbance was determined in a VersaMax microplate reader (Molecular Devices Corporation) at 540 nm and the results are presented as the percent of optical density (OD) in the treated wells versus the controls.

2.5.2 CellTiter Assay

Cell viability was assessed using Promega CellTiterBlue™ reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, A549, SJSA-1, U-2 OS, H1299, RT4 and MRC-5 cells were seeded in 96- well plates (5000 cells/well) containing 50 µL of growth medium. After 24 h of culture, the cells were supplemented with 50 µL of appropriate extract solution (diluted in medium) to give a final concentration of 25 µg/mL (final DMSO of 0.025 %). After 72 h of incubation, 20 µl of resazurin was added for 2 h before recording fluorescence (560 nm Ex/590 nm Em) using a Victor microtiter plate fluorimeter (Perkin-Elmer, Waltham, MA, USA). Extracts that showed an anti-proliferative activity above 50% in comparison with control, a serial of different concentration were measured to calculate the corresponding $IC_{50}$ (extract concentration that induced a 50% decrease in fluorescence of treated cells compared with untreated cells). All experiments were performed in triplicate.

2.6 Nuclear magnetic resonance analysis (NMR)

The fraction of *C. atacamensis* aerial parts extracts derived from CPC were diluted in methanol-*d4* at 5 mg/mL. NMR experiments were conducted on a 600 MHz Avance III Bruker NMR spectrometer equipped with a 5 mm PABBI 1H/D-BB inverse detection probe. Temperature was controlled by a BVT-3000 digital controller. A standard operation was followed (temperature calibration, water suppression) and the spectra were recorded using pulse sequences included in the standard pulse program library of Bruker. ICONNMR software v. 4.2.6 build 9 for TopSpin 2.1, controlling a B-ACS 60 position autosampler, was used for full automated NMR operation. For all fractions a $^{1}H$ 1D spectrum (noesygppr1d), with presaturation was recorded.

2.7 NMR-HeteroCovariance Approach (HetCA)

MATLAB software was used for the bucketing of the spectra and the correlation with their biological activities throught HetCA as previously described (Aligiannis et al., 2016). The correlation between NMR resonances and bioactivity appeared as an NMR pseudospectrum. Each point of this function was additionally colour-coded according to the respective correlation values. For the development of the optimum workflow in order to identify potential bioactive compounds via MATLAB, an automated procedure was performed using repeats of four subsequent spectra

each time (e.g. $^{1}$H-NMR of fractions 1-4, Fractions 5-8, etc.), which is the minimum number of spectra for a successful correlation to be achieved.

Antiproliferative activity of plant extracts against different cell lines

| Scientific Name | Part | Solvent Extraction | Code | A549 | H1299 | RT4 | HCT116 | SJSA 1 | U2OS | SW620 | MRC5 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Aloysia deserticola* (Phil.) Lu-Irving & O'Leary | Flower and Leaves | AcOEt | 1 | 91 ± 3 | 32 ± 0,4 | 64 ± 1,8 | 70 ± 1 | | | 52 ± 6 | 12 ± 0,5 |
| | | MeOH | 2 | 86 ± 0,5 | 68 ± 5 | 100 ± 0,1 | 90 ± 1,5 | | | 118 ± 2 | 98 ± 0,6 |
| | Stems | AcOEt | 3 | 102 ± 2 | 58 ± 1,7 | 99 ± 0,7 | 104 ± 9 | | | 75 ± 4 | 56 ± 2 |
| | | MeOH | 4 | 86 ± 1,4 | 84 ± 2 | 99 ± 1,4 | 99 ± 5,5 | | | 113 ± 5 | 98 ± 0,9 |
| *Artemisia copa* (Phil.) var. copa | Flower and Leaves | AcOEt | 5 | | | | | 47 ± 0,8 | 16 ± 2 | 76 ± 4 | 51 ± 0,6 |
| | | MeOH | 6 | | | | | 87 ± 1 | 76 ± 3 | 93 ± 1 | 95 ± 1 |
| | Stems | AcOEt | 7 | | | | | 53 ± 9 | 83 ± 0,3 | 73 ± 10 | 77 ± 1 |
| | | MeOH | 8 | | | | | 52 ± 4 | 80 ± 1 | 100 ± 2 | 103 ± 2 |
| *Ephedra breana* Phil. | Aerial Parts | Basic | 9 | 91 ± 1,8 | 88 ± 4 | 103 ± 1,2 | 76 ± 14 | 91 ± 0,8 | 64 ± 1 | 71 ± 4 | 99 ± 0,8 |
| | | MeOH | 10 | 87 ± 0,4 | 82 ± 0,2 | 92 ± 2 | 73 ± 5 | 92 ± 0,5 | 85 ± 0,3 | 103 ± 1 | 99 ± 0,2 |
| *Lampaya medicinalis* Phil. | Aerial Parts | AcOEt | 11 | 78 ± 0,4 | 71 ± 6 | 72 ± 6 | 62 ± 7,6 | 91 ± 0,8 | 64 ± 1 | 71 ± 4 | 99 ± 5 |
| | | MeOH | 12 | 77 ± 0,3 | 79 ± 2 | 74 ± 0,4 | 83 ± 5 | 92 ± 0,6 | 85 ± 0,3 | 103 ± 0,3 | 92 ± 0,6 |
| *Baccharis tola* Phil. ssp tola | Aerial Parts | AcOEt | 13 | 79 ± 3 | 66 ± 1 | | | 93 ± 2 | 66 ± 1 | 76 ± 0,7 | 91 ± 1 |
| | | MeOH | 14 | 86 ± 1 | 54 ± 1 | | | 81 ± 3 | 54 ± 1 | 85 ± 1 | 99 ± 1 |
| *Azorella atacamensis* Phil. | Areal Parts | AcOEt | 15 | 84 ± 2 | 80 ± 7 | | | | | | 104 ± 6 |
| | | MeOH | 16 | 78 ± 1 | 84 ± 2 | | | | | | 90 ± 2 |
| *Polylepis tarapacana* Phil. | Aerial Parts | AcOEt | 17 | 73 ± 0,5 | 65 ± 2 | 92 ± 0,1 | 39 ± 5 | | | | 83 ± 2 |
| | | MeOH | 18 | 81 ± 0,1 | 90 ± 0,1 | 86 ± 0,4 | 94 ± 8 | | | | 80 ± 2 |
| *Junellia seriphioides* (Guilles & Ex Hook.) Moldonke | Aerial Parts | AcOEt | 19 | 77 ± 0,4 | 77 ± 0,4 | 99 ± 0,3 | 35 ± 2,2 | 103 ± 1 | 71 ± 2 | 60 ± 0,7 | 99 ± 5 |
| | | MeOH | 20 | 93 ± 1 | 93 ± 1 | 108 ± 4 | 83 ± 2 | 94 ± 0,7 | 83 ± 3 | 112 ± 3 | 96 ± 1 |
| *Fabiana squamata* Phil. | Aerial Parts | AcOEt | 21 | | | | | 66 ± 0,7 | 85 ± 0,2 | 89 ± 1 | 92 ± 0,3 |
| | | MeOH | 22 | | | | | 85 ± 1 | 87 ± 1 | 110 ± 2 | 101 ± 0,9 |
| *Fabiana denudata* Miers | Aerial Parts | AcOEt | 23 | 95 ± 2 | 92 ± 2 | | | 80 ± 1 | 58 ± 2 | 89 ± 1 | 63 ± 3 |
| | | MeOH | 24 | 102 ± 0,2 | 97 ± 0,7 | | | 95 ± 1 | 80 ± 2 | 111 ± 5 | 82 ± 0,8 |

| | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Krameria lappacea* (Dombey) Burdet & B. B. Simpson | Roots | AcOEt | 25 | | | | | | | 51 | ± | 3 | 118 | ± | 13 | | | | | | | | | | 60 | ± | 1 |
| | | MeOH | 26 | | | | | | | 108 | ± | 2 | 109 | ± | 10 | | | | | | | | | | 100 | ± | 1 |
| *Baccharis calliprinos* Griseb. | Aerial Parts | AcOEt | 27 | | | | | | | | | | | | | 61 | ± | 0,9 | 60 | ± | 0,1 | | | | 92 | ± | 0,7 |
| | | MeOH | 28 | | | | | | | | | | | | | 74 | ± | 1 | 71 | ± | 0,1 | | | | 99 | ± | 2 |
| *Baccharis grisebachii* Hieron | Aerial Parts | AcOEt | 29 | | | | | | | | | | | | | 61 | ± | 0,9 | 50 | ± | 1 | 76 | ± | 2 | 97 | ± | 0,1 |
| | | MeOH | 30 | | | | | | | | | | | | | 74 | ± | 1 | 77 | ± | 0,5 | 111 | ± | 8 | 91 | ± | 1 |
| *Haplopappus rigidus* Phil. | Aerial Parts | AcOEt | 31 | 88 | ± | 1,6 | 95 | ± | 1,6 | 28 | ± | 0,6 | 39 | ± | 9 | 80 | ± | 2 | 46 | ± | 2 | 84 | ± | 4 | 71 | ± | 2 |
| | | MeOH | 32 | 93 | ± | 3 | 102 | ± | 8 | 64 | ± | 1 | 76 | ± | 2,4 | 99 | ± | 3 | 89 | ± | 4,5 | 98 | ± | 2,5 | 78 | ± | 2,7 |
| *Chuquiraga atacamensis* Kuntze | Aerial Parts | AcOEt | 33 | 79 | ± | 0,9 | 100 | ± | 0,9 | 68 | ± | 2 | 17 | ± | 6 | | | | | | | 79 | ± | 8 | 21 | ± | 2 |
| | | MeOH | 34 | 19 | ± | 0,3 | 30 | ± | 0,3 | 34 | ± | 0,7 | 11 | ± | 0,7 | | | | | | | 62 | ± | 3 | 20 | ± | 2 |
| *Senecio nutans* SW ch. Bip. | Aerial Parts | AcOEt | 35 | 84 | ± | 0,5 | 90 | ± | 0,5 | 35 | ± | 1,2 | 80 | ± | 8 | | | | | | | 67 | ± | 3 | 48 | ± | 1 |
| | | MeOH | 36 | 82 | ± | 0,3 | 89 | ± | 0,3 | 82 | ± | 5 | 99 | ± | 1,3 | | | | | | | 120 | ± | 8 | 78 | ± | 0,5 |
| *Parastrephia teretiuscula* (Kuntze) Cabrera | Aerial Parts | AcOEt | 37 | | | | | | | | | | | | | 55 | ± | 1 | 43 | ± | 1 | 75 | ± | 5 | 59 | ± | 3 |
| | | MeOH | 38 | | | | | | | | | | | | | 38 | ± | 1 | 88 | ± | 0,6 | 117 | ± | 2 | 97 | ± | 3 |
| *Cortaderia speciosa* (Ness & Meyen) Stapf | Aerial Parts | AcOEt | 39 | 89 | ± | 1,5 | 91 | ± | 1,5 | | | | | | | | | | | | | | | | 86 | ± | 1 |
| | | MeOH | 40 | 92 | ± | 0,8 | 85 | ± | 0,8 | | | | | | | | | | | | | | | | 97 | ± | 0,4 |

Table 1: Percentage of cellular viability after incubation of different extracts at 25 µg/mL.

| Antiproliferative activity of more active extracts against selected cancer cell lines | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Scientific Name | Code | A549 | | | H1299 | | | RT4 | | | HCT116 | | | SJSA 1 | | | U2OS | | | MRC5 | | | | | |
| *Aloysia deserticola* | 1 | | | | 46,9 | ± | 0,32 | | | | | | | | | | | | | 28,74 | ± | 5,49 | | | |
| *Artemisia copa* var. copa | 5 | | | | | | | | | | | | | | | | 16,04 | ± | 1,49 | | | | | | |
| *Polylepis tarapacana* | 17 | | | | | | | | | | 22,2 | ± | 0,4 | | | | | | | | | | | | |
| *Junellia seriphioides* | 19 | | | | | | | | | | 12,1 | ± | 2,1 | | | | | | | | | | | | |
| *Haplopappus rigidus* | 31 | | | | | | | 55,1 | ± | 2,63 | 22,2 | ± | 1,2 | | | | | | | | | | | | |
| *Chuquiraga atacamensis* | 33 | | | | | | | | | | 4,6 | ± | 0,1 | | | | | | | 10,73 | ± | 1,58 | | | |
| | 34 | **6,39** | ± | **0,18** | **1,69** | ± | **0,32** | **1,98** | ± | **0,49** | **0,6** | ± | **0,2** | | | | | | | **1,88** | ± | **0,57** | | | |
| *Senecio nutans* | 35 | | | | | | | 55,3 | ± | 3,63 | | | | | | | | | | | | | | | |
| *Parastrephia teretiuscula* | 38 | | | | | | | | | | | | | 104,21 | ± | 0,18 | | | | | | | | | |

Table 2: $IC_{50}$ of Antiproliferative activity of extracts in µg/mL against different cancer cell line and MRC5 fibroblast human line.

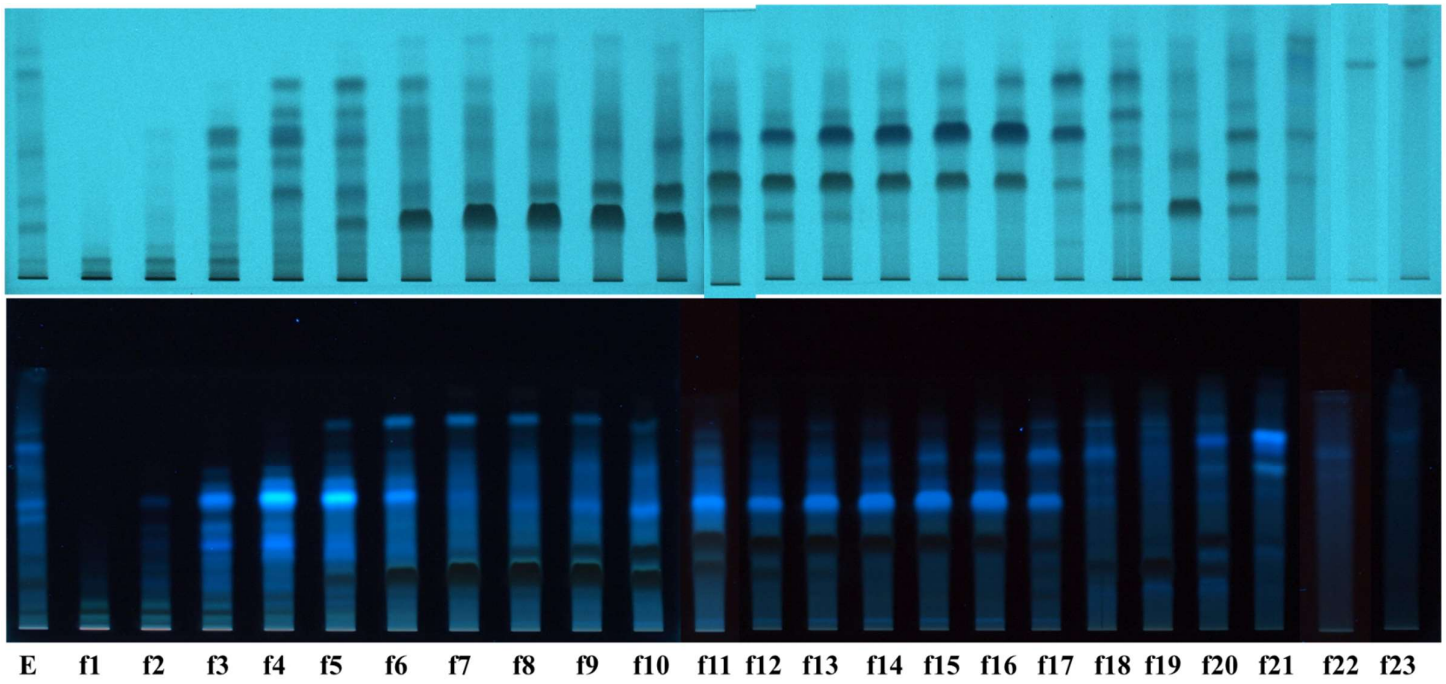


Figure 1: HPTLC profile of extract **34** from *C. atacamensis* and of fractions obtained by CPC. Mobile phase: MeOH/$H_2O$/FA – 50/50/1, pictures recorded under UV light at 254 and 366 nm.

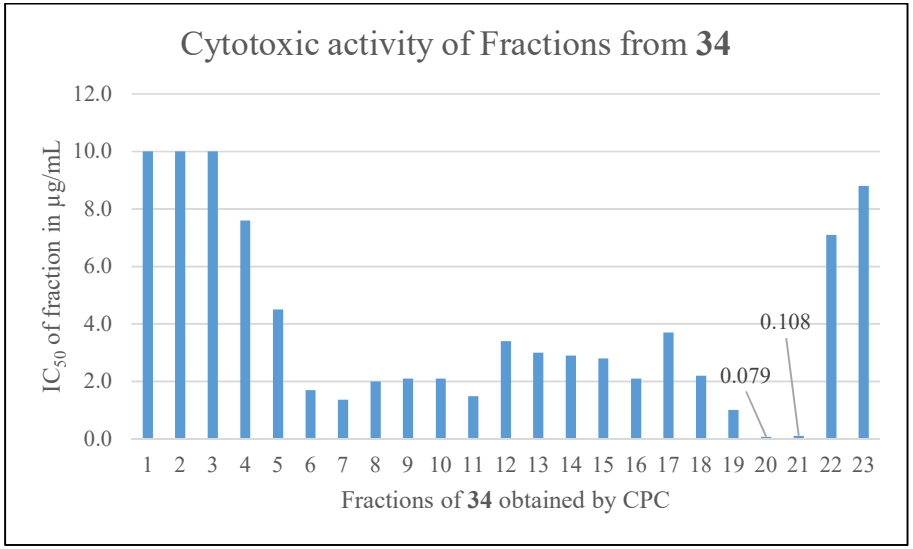


Figure 2: *In vitro* antiproliferative activity against HCT 116 cancer cells of fractions of **34** obtained by CPC.

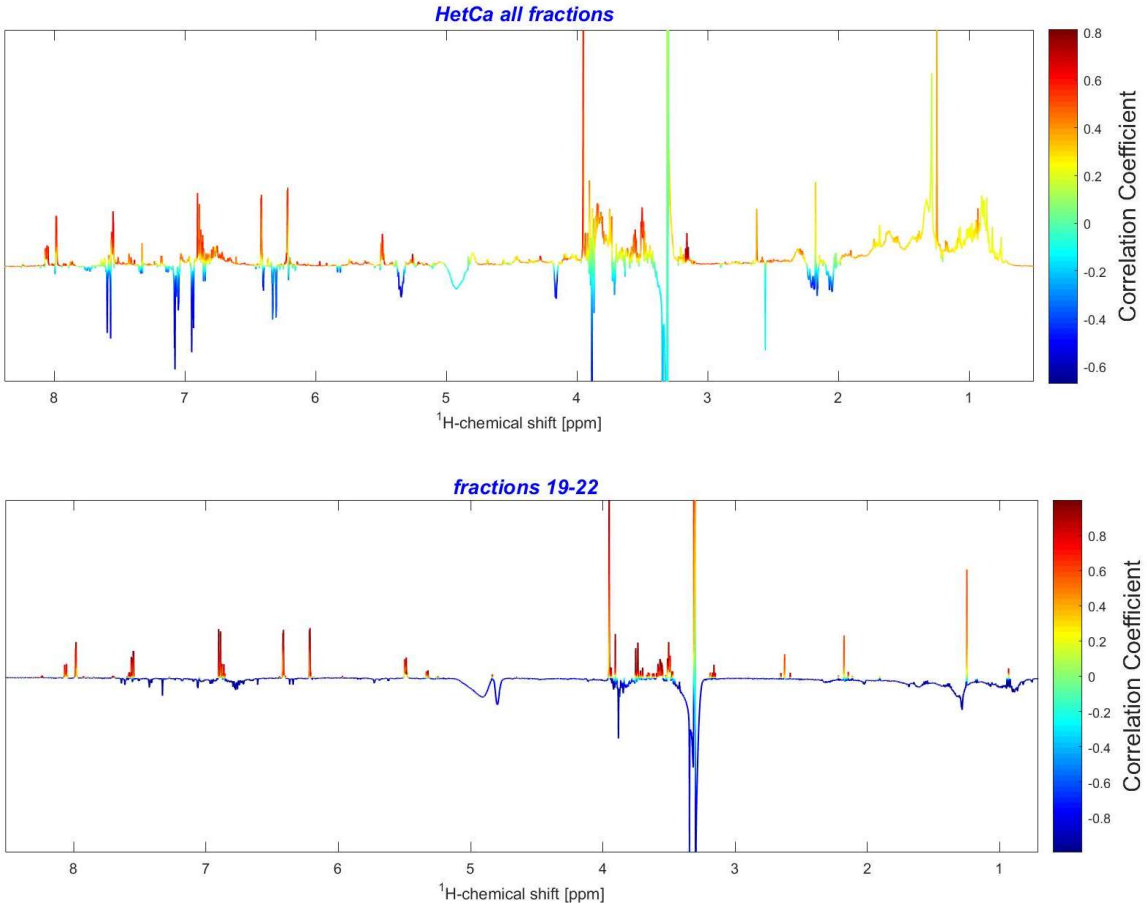


Figure 3: HetCA profiles of a) all fractions of extract **34** and b) HetCA correlation of more active fractions fr19-fr22 against HCT116 cancer cell line.

3 Results

Antiproliferative screening

For Verbaneceae family, *A. deserticola* and *J. seriphioides* exert antiproliferative activity against different cell lines. EtOAc extract of flowers and leaves of *A. deserticola* (**1**) showed a diminution of the cellular proliferation of H1299 cell line of 68 % ($IC_{50}$ = 46.9 µg/mL) but also against the normal cell line MRC5 almost at 90 % ($IC_{50}$ = 28.7 µg/mL). The EtOAc extract of aerial parts of *J. seriphioides* (**19**) against HCT116 with an $IC_{50}$ of 12.1 µg/mL. For Rosaceae family, apolar extract of *P. tarapacana* (**17**), exerted the decrease of cell proliferation of HCT116 colon cancer cell line with an $IC_{50}$ of 22.2 µg/mL.

From Asteraceae family, four medicinal plants showed relevant antiproliferative activity. The apolar extracts of *A. copa* (**5**), *S. nutans* (**35**) and *P. teretiuscula* (**38**) showed $IC_{50}$ level lower than 25 µg/mL, against U2OS (16.04 µg/mL), RT4 (55.3 µg/mL) and SJSA1 (104.21 µg/mL) respectively. *H. rigidus* (**31**) showed also interesting $IC_{50}$ values against RT4 (55.1 µg/mL) and HCT116 (22.2 µg/mL) cancer cell lines. The higher antiproliferative activity was recorded for the methanolic extract of *C. atacamensis* (**34**) against HCT116 cancer cell line with an $IC_{50}$ of 0.6 µg/mL and its apolar extract (**33**) with a value of 4.6 µg/mL. Values between 2-1 µg/mL were also registered against H1299, RT4 and MRC5 cell lines. In the case of *C. atacamensis*, its extract obtained by supercritical fluid extraction was also evaluated for its antiproliferative activity against HCT116 cell line showing an $IC_{50}$ of 14.3 µg/mL.

Fractionation and fraction evaluation

To have an appropriate variance of compounds concentration, CPC was the selected chromatography technic for the fractionation of extract 34, due to its lack of solid stationary phase, high percentage of recovery, wide range of solvent systems and high loading capacity. Several biphasic systems based on the combination of cyclohexane, EtOAc, BuOH, EtOH, MeOH and partition behaviour was evaluated by thin layer chromatography. For extract **34** from *C. atacamensis* the fractionation CPC procedure was performed with the biphasic system of EtOAc/EtOH/$H_2O$ – 10/1/10 given 23 fractions. The fluctuation of the concentration of the different compounds constituents of the extract **34** is possible to visualize in **Figure 1** of HPTLC profiling. Only fractions fr1-3 were not active with $IC_{50}$ values > 10 µg/mL against HCT116 cancer cell line. Fractions fr5-fr19 present $IC_{50}$ values between 4 to 1 µg/mL. The lower $IC_{50}$ were observed to the fractions 20 and 21 with values of 79 and 108 ng/mL.

NMR experiments and HetCA analysis

Twenty-one HetCA plot were obtained for the 23 fractions of *C. atacamensis* extract in correlation with their antiproliferative activity against HCT116 colon cancer cell line *in vitro*. HetCA plot of all fractions correlated with their *in vitro* antiproliferative activity (**Figure 3**) shows the $^{1}$H signals of compounds that are more present in the more active fractions in the positive region of the pseudo-spectra, annoted with red colour. Contrary, the $^{1}$H signals for compounds that are more present in the inactive fractions are annoted in blue. From the first pseudo-spectra we can observe that aromatic $^{1}$H signals (8.10 - 6.00 ppm) in both positive and negative regions. Negative signals are also recorded at 5.3, 4.1 and 2.0 ppm. Between 4.0 and 3.0 ppm are represented signals in the positive region but with weak (yellow) to strong (red) correlation with the antiproliferative activity.

HetCA plot of more active fractions (20-21) allow us to observe in a clearer way the $^{1}$H signals from compounds that could contribuite to the antiproliferative activity. In the positive regions it is still observable the aromatic $^{1}$H signals between 8.0 – 6.0 ppm, in addition $^{1}$H signals between 4.0 – 3.5 that could correspond to glycosides units are strongly correlated with the antiproliferative activity.

## 4 Discussion

*C. atacamensis* has been subject of phytochemical studies in the past. Triterpenes as lupeol, betulin, erythrodiol and taraxas-20(21)-en-3β,6-diol have been isolated and flavonoids quercetin and kaempferol glucosides identified from *C. atacamensis* (Hoeneisen at al. 2000, Juáres and Mendiondo, 2002).

From examination of HetCA plot of more active fractions, aromatic and glycosides $^{1}$H signals may correspond from a glycoside flavonoid structure. In fact, pseudo-spectra of fractions 19-22 showed charesteristic $^{1}$H signals of a kaempferol-like glucoside derivative with two doublets with small couplage constant of 2 Hz (at ~ 6.50 and 6.20 ppm, corresponding to the B ring of flavonoid structure) and two more doublets with *orto* couplage constant (~ 7.5 Hz) representing the 1H signals of the C rings of a kaempferol-like flavonoid. In addition, signal at 5.40 ppm may correspond to an anomeric proton of a glucoside group. Deeper 1D and 2D NMR experiments must be carry out in order to clarify from which flavonoid-like compounds may correspond the $^{1}$H signals in the positive region in the pseudo-spectra obtained by HetCA approach.

## 5 Conclusion

HetCA approach is a powerfool and modern tool that can be applied to understand and characterize an bioactive extract in the early stages of the investigation. In our case, more 1D, 2D spectra (HSQC, HMBC and STOCSY) must be performed to identify the bioactive compounds responsible of the antiproliferative activity of the methanolic extract of *C. atacamensis*.

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## Conclusions and perspectives

The most important contribution of the precedent publication are:

- First communication of the high antiproliferative activity of extracts against several cancer cell lines for several medicinal plant species.
- First report of the high antiproliferative activity of methanolic extract from *C. atacamensis* **34**.

The main perspective of this work is the isolation of identify compounds in the more active, in order to evaluate their antiproliferative activity against HCT116 colon cancer cell line with the principal aim to validate the HetCA approach.

It is important to mention that the precedent draft presents preliminary results. Deeper NMR studies must be carry out shuch as STOCSY with the aim of elucidate the correct chemical structure of bioactive compounds present in the methanolic extract of *C. atacamensis*.

## I.7 Personal contribution

My personal contribution includes both the chemical study i.e. extraction, fractionation, purification and structure elucidation of isolated compounds, and the biological evaluations of samples with the establishment of colorimetric antioxidant assays in our laboratory, as described below:

- Collection of medicinal plants.
- Extractions procedures by PSE with two different solvents, hydrodistillation, SFE extraction (with ethanol as co-solvent) and maceration in the case of *E. americana*.
- Preparation and accomplishment of *in vitro* antibacterial, antifungal, antileishmanial, antitrypanosomal, antiplasmodial, anti-inflammatory, antioxidant and antiproliferative assays of extracts, fractions and/or pure compounds.
- Fractionation of active extracts by silica gel chromatography or centrifugal partition chromatography.
- Isolation of active compounds by silica gel chromatography or preparative HPLC from the most active fractions.
- Structure elucidation of pure compounds by spectroscopic (mainly 1D- and 2D NMR) and spectrometric (MS) techniques.
- Redaction of original manuscripts.

# Chapter II Valorization of *Pistacia lentiscus* L. from Mediterranean Basin.

# II.1 EXANDAS Project

As seen before, plants used in the traditional medicine are a source of bioactive compounds. These proprieties could be extended to cosmeceutical, pharmaceutical or nutraceutical applications.

EXANDAS (Exploitation of aromatic plants' by-products for the development of novel cosmeceuticals and food supplements) program is a research European project, which aims to apply emerging and green technologies in the field of natural products in order to fully and efficiently exploit these by-products of important aromatic/medicinal plants utilized in the Mediterranean Basin neighbouring. The consortium consists of six academic (University Paris Descartes, University of Athens, Bulgarian Academy of Sciences, Centre de Biotechnologies de Sfax, University Ibn Zohr, CRAPC, Algeria) and six non-academic members, under the coordination of Dr. Nektarios Aligiannis from University of Athens, join forces and exchanges expertise through an extended secondment scheme to advance research and innovation [96].

Several by-products (extracts, plant parts without no industrial purpose, etc.…) have been investigated, for example, for *Rosa damascena*, *Geranium macrorrhizum*, *Lavandula angustifolia*, *Sideritis scardica*, *Ocimum basilicum* and *Pistacia lentiscus* industries. NPAS team has been involved in the exploration of the antimicrobial, antivirulence, antioxidant, skin whitening, anti-inflammatory and cytotoxic activities of *Pistacia lentiscus* fruits collected in Algeria and Greece. The main results are described in this chapter.

# II.2 Introduction

## II.2.1 Mediterranean Basin

The Mediterranean basin, spread over three continents, includes the north of Maghreb region and the southern part of Europe comprising the Iberian, Italian and Balkan Peninsulas, and extends into western Asia, including the west of Turkey, the west and south of Syria, Lebanon and Israel. The Mediterranean climate, is characterized by mild humid winters and hot to warm, dry summers [97].




**Figure 64: Map of Mediterranean Basin.**

## II.2.2 Vegetal biodiversity of the Mediterranean Basin

Due to variations of rainfalls, elevations, latitude and soils defining microclimates, the region hosts forests, woodlands of shrubs with rich biodiversity: around 25,000 native species (50% of endemic species). Aleppo pine (*Pinus halepensis* Miller), Mediterranean cypress (*Cupressus sempervirens* L.), Greek strawberry tree (*Arbutus andrachne* L.) and mastic (*Pistacia lentiscus* L.) are among the emblematic endemic species [98].



a) b)



c) d)

**Figure 65: Prominent Mediterranean vegetation. a) *P. halapensis*, b) *C. sempervirens*, c) *A. andrachne* and d) *P. lentiscus*.**

## II.2.3 *Pistacia lentiscus*

*Pistacia lentiscus* L. (syn *Lentiscus massiliensis* Fourr., *Lentiscus vulgaris* Fourr. and *Terebinthus lentiscus* (L.) Moench), from family Anacardiaceae, is a widely distributed in Mediterranean Europe, Morocco and the Iberian Peninsula in the west trough southern France and northern Africa to regions of Turkey, Iraq and Iran. In the Greek island of Chios, it is possible to find the endemic variety *P. lentiscus* var. chia.

### II.2.3.1 Botanical description

Kingdom: Plantae

Clade: Angiosperms

Order: Sapindales

Family: Anacardiaceae

Genus: *Pistacia*

Species: *Pistacia lentiscus*

Binominal name: *Pistacia lentiscus* L.

*P. lentiscus* is a dioic evergreen tree, 1 to 5 m tall. It can growth until 40-50 years. The stems and trunks are reddish in the youth and become grey at older years. The leaves are alternate, leathery and pinnately composed (no terminal leaflet) with winged petioles and five or six pairs of deep-green leaflets. Flowers are small, the male with five stamens, the female is divided into three lobes. The fruit is a drupe, first red and then black, of about 4 mm of diameter. The resin has a strong smell; it is known as mastic gum when coming from *P. lentiscus* var. chia, and appears as transparent yellow-white tears [99,100].



**Figure 66: Fruits and resin produced by *P. lentiscus*.**

## II.2.3.2 Medicinal and industrial uses of *P. lentiscus*

Fruits, resin, galls and leaves of *P. lentiscus* has been used for treatment of various diseases since the time of the Ancient Greeks.

In Algeria and Morocco, the leaves are used in case of digestive disorders, and as appetizer and astringent.

The edible oil is mainly produced in the north of Algeria. Fruits are harvested during the summer, are then air-dried during one week before grinding. After maceration in hot water, they are expressed with a mechanic press. The oil is recovered after a final step of

decantation. The yield depends of the soil and climate conditions. It is consumed in daily diet in salads and pastries and indicated in the treatment of respiratory disorders, allergies, sore throats and locally applied to relieve psoriasis, wounds and burns [99].

The mastic gum is used to treat stomachaches and ulcer, dyspepsia, intestinal disorders, hepatic inflammation, tooth disease, hypercholesterolemia and diuretic in Greece.

Around 20 villages in the Island of Chios, known as mastichochoria, are dedicated to the cultivation, harvest and production of mastic. The process is controled by the Chios Gum Mastic Grower Association, a cooperative founded in 1938, which put on the market resin, mastic chewing gum and mastic oils for culinary recipes [101].




**Figure 67: Commercial products of mastic gum from Chios Island.**

The oil from the fruits of *P. lentiscus* is principally produced in the north of Algeria. The harvest of fruits is comprised between the first months of summer until the start of fall. After harvest, the fruits are air-dry during one week and grinding. The fruits are macerated in hot water and then crushed with the help of mechanic press. The oil is recovered by a final step of decantation. The final yield depend of the soil and climate conditions.

### II.2.3.3 Previously phytochemical and pharmacological studies of *P. lentiscus*

Leaves, fruits, branches and resin from, branches and resin from *P. lentiscus* species, collected in several Mediterranean regions, have been subjected to phytochemical analysis and biological activities evaluation of pharmacological, cosmetological and nutritional relevance. Here below are descripted the more relevant results communicated in the scientific literature.

#### II.2.3.3.1 Resin (mastic gum)

Properties of mastic gum, specifically those related to the digestive system are reported since the Antiquity.

It contains a polymer of myrcene, a wide variety of neutral and acidic tetra and pentacyclic triterpenes (**83**-**93**) and an essential oil with α-pinene (**94**) as main compound.

Oral dose of 500 mg/kg of body weight was administered to mice suffering gastric or duodenal ulcer model where the pathogenesis was caused either mechanically (pyloric constriction), chemically (administration of 50% ethanol) or pharmaceutically (administration of aspirin, phenylbutazone or reserpine). In all *in vivo* models, mastic gum has considerably reduced the degree of gastric mucosa damages with more than 50% of the lesion score in aspirin, phenylbutazone and ethanol-induced models. As *Helicobacter pylori* infections have been correlated with gastric diseases, the anti-*H. pylori* activity of mastic gum against clinical isolated strains was assayed by the microdilution method. Antibactericidal activity was observed against twelve strains, killing 50% of the bacteria at concentration of 125 µg/mL and 90% at 500 µg/mL. An extract of mastic gum without polymer was evaluated and *in vivo* by administration to *H. pylori* (SS1 strain) infected mice over a period of 3 months with a dos of 0.75 mg/day led to a 30-fold reduction colonization (1.5 logCFU/g of tissue). Isomasticadienolic acid **86** was determined as the most active compound with MBC value of 0.202 µg/mL (0.4 mM) [102–104].

Essential oil obtained from mastic gum has demostrated antibacterial and antifungal activity with inhibition zones of 26-18 mm against Gram-positive (*Staphylococcus aureus* and *S. epidermis*) and Gram-negative (*Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Escherichia coli*) strains and of 26 (*Candida albicans*) and 14-10 (*C. tropicalis* and *Torulopsis glabrata*) mm against fungal strains. The neutral (organic layer after the fractionation of the total mastic extract without polymer re-suspended in ether and washed with 5% $Na_2CO_3$) and acid fractions (organic layer of the re-extraction with ether of the aqueous layer previously mentioned acidified with 1N HCl) were less efficient against the Gram-positive strains. α-Pinene accounted for 66% of the composition [105].

In addition, the anti-inflammatory activity was also evaluated. Mastic gum inhibited the NO and $PGE_2$ with $IC_{50}$ values of 29 ± 2.3 and 49 ± 0.2 µg/mL respectively by RAW

264.7 cells LPS-stimulated. At the same conditions, expression of iNOS and COX-2 proteins were also inhibited, even below the detection limit in western blot analysis at the higher concentration tested of 100 µg/mL. Neutral extract of mastic gum also inhibited the expression of adhesion molecules VCAM-1 and ICAM-1 in a TNF-α-induced model with human aortic endothelial cells. Phosphorylation of NF-κB also decreased at concentration range of 200-25 µg/mL of neutral extract. Similar effects were observed with tirucallol **87** isolated from the neutral extract [106].

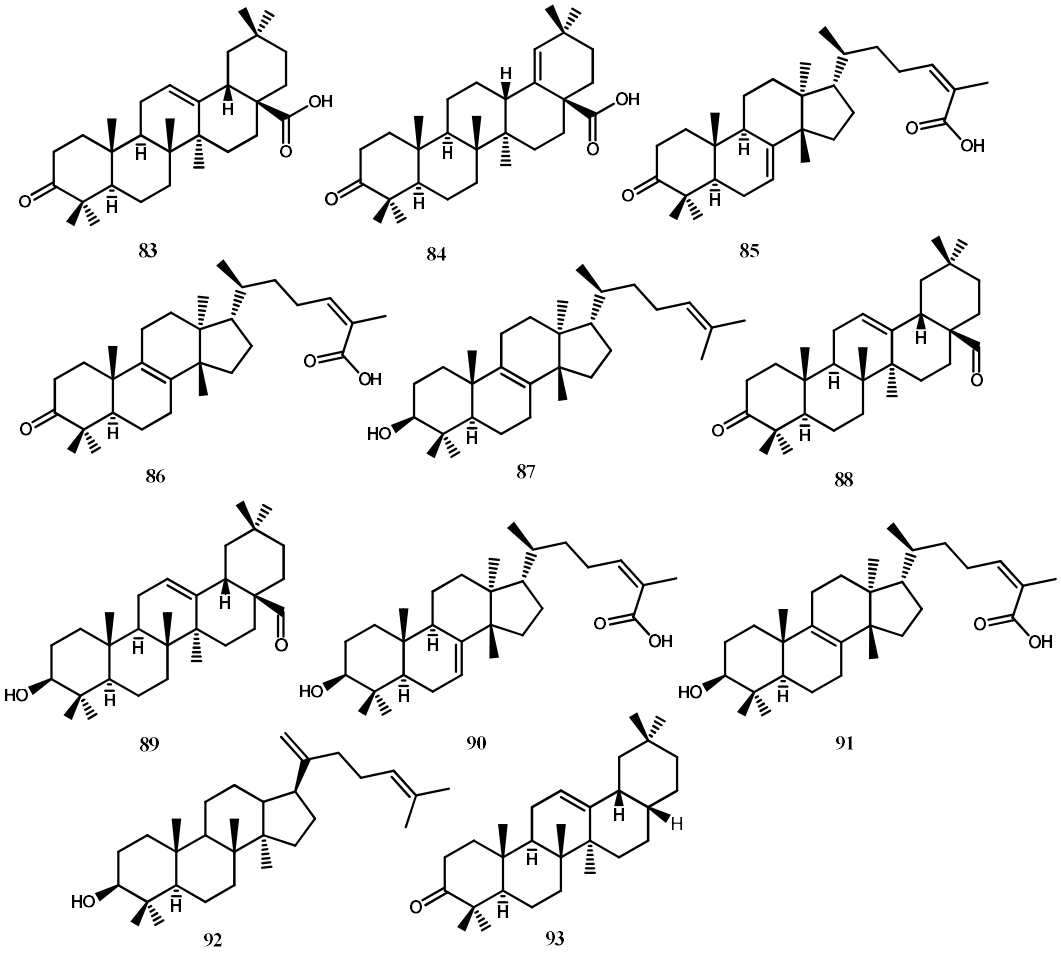
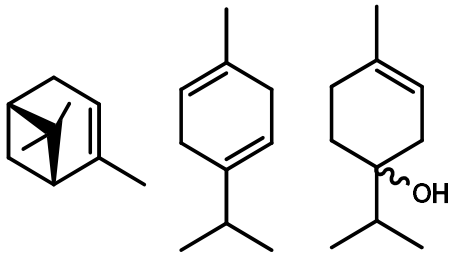



**Figure 68: Some tetra and pentacyclic triterpenes isolated from *P. lentiscus* var. chia mastic gum with antibacterial and anti-inflammatory activities reported.**

**II.2.3.3.2 Leaves**

The antibacterial activity of the essential oils of leaves collected in Tunisia, showed significant inhibitory effects against *Salmonella typhimurium*, *S. enteritidis* and *Staphylococcus aureus* evaluated by a microdilution agar method, with MICs values of 620-30 µg/mL, but no effect on *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Escherichia coli* up to 1 mg/mL were observed. GC-MS analysis allowed to identify as the main, constituents of the essential oil, α-pinene (17%), γ-terpinene (9%) and terpinen-4-ol (12%) [107,108].




94 95 96

**Figure 69: Major compounds detected in essential oils *P. lentiscus* leaves: α-pinene (94), γ-terpinene (95) and terpinen-4-ol (96).**

Ethanolic extracts of leaves exerted antibacterial activity, by disc diffusion method with paper disc charged with 5 or 10 µL of a solution of 0.32 mg/mL, with inhibition halos of 26-11 mm against *Salmonella typhi*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Enterobacter cloacae* strains. Antifungal activity was also reported against *Aspergillus flavus*, *Rhizopus stolonifer* with antifungal index of 13-45% at the same concentration [109].

The hepatoprotective proprieties of boiled and non-boiled aqueous extract of leaves collected in Jordan were measured in a $CCl_4$-induced rat hepatotoxicity *in vivo* model after 4 mL/kg extract administration. The non-boiled extract was the most active toward all essayed parameters (enzymes ALP, ALT, AST and bilirubin levels, which are overproduced after cellular damage), with a reduction of 60-80% of enzyme overproduction. The boiled extract was not so effective [110]. Several phenolic acids, flavonols, flavones glucosides, anthocyans, gallic acid and coumarin acid derivatives have been identified from the polar extracts [99].

In addition, *in vivo* anti-inflammatory activity of $CHCl_3$, ethyl acetate, ethanol and aqueous extracts was evaluated. The aqueous extract showed an inhibition of rat paw

oedema carrageenan-induced test of 60% after 5h at a dose of 100-200 mg/kg. Moreover the same extract inhibited the ulcer lesion formation in an HCl/ethanol induced model at 50-70% at the same dose [111]. In *in vitro* assays, the hydroethanolic extract of leaves showed an inhibition of pro-inflammatory cytokine IL-1β in an ATP-stimulated THP-1 monocytes cell line of 40% in relation with respective blanks at 100 µg/mL. Analysis by UPLC-MS analysis of this extract led to identify gallic acid and glucoside flavones derivatives **97**-**105** [112].

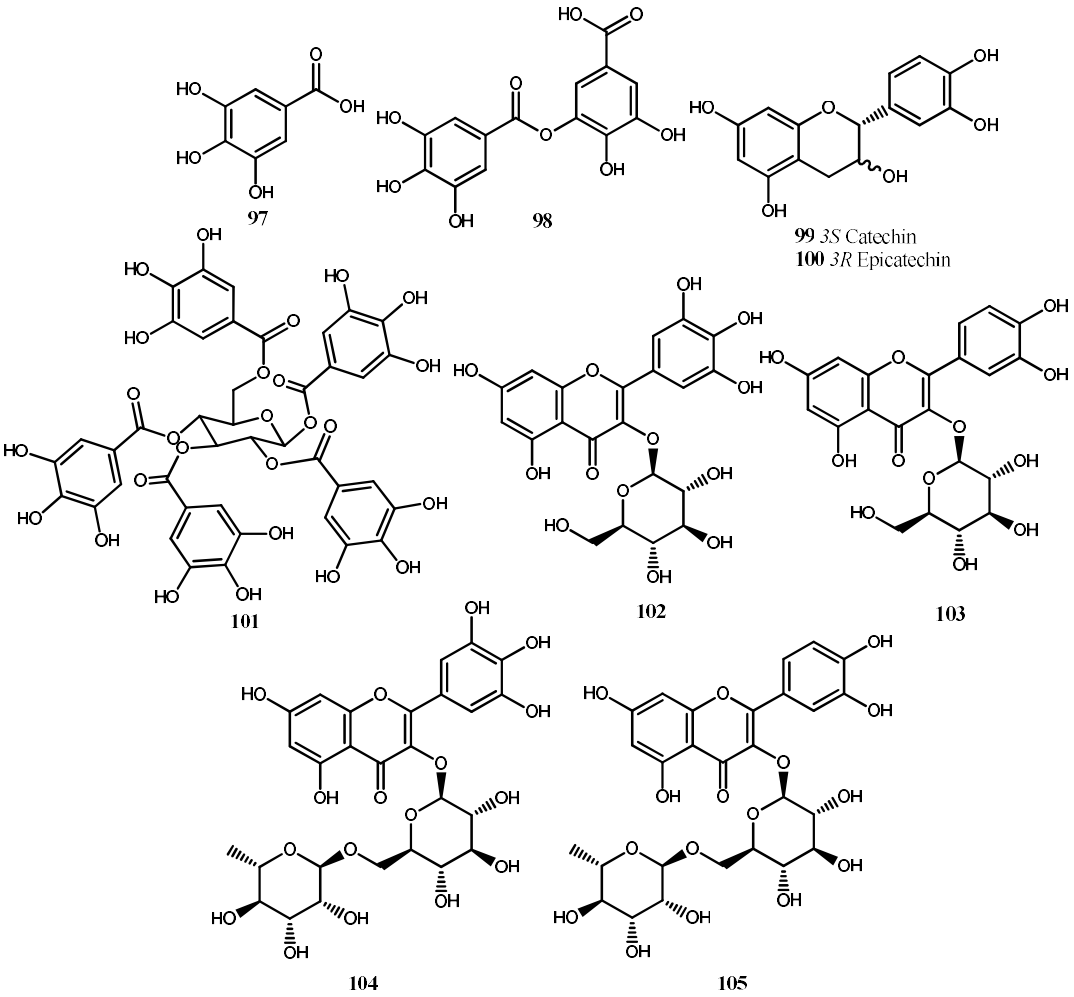



**Figure 70: Compounds isolated or identified from polar extracts of *P. lentiscus*.**

A comparative study of different extraction procedures to obtain the higher antioxidant activity of leaves of *P. lentiscus* var. chia was performed by soxhlet, microwave, ultrasound assisted and supercritical fluid extractions. The aqueous extracts obtained by

microwave and ultrasound assisted procedures showed the higher free radical scavenging activity with $EC_{50}$ values of 37 µg/mL [113].

**II.2.3.3.3 Fruits**

Fruits of *P. lentiscus* have been also subjects of several investigations. The ethanolic extract of fruits collected in Algeria was evaluated for hepatoprotection. Administration of 250 mg/kg before damages induced by paracetamol (165 mg/kg/d for 3 days) showed and inhibition of the overproduction of ALT and ALP transaminases enzymes and total bilirubin detected in serum [114].

Antidiabetic activity was also measured in *in vivo* streptozotocin-induced diabetic rat model. The glucose level significantly decreased after one hour of treatment with 125 mg/kg ethanolic extract. This may be related to the enzymatic inhibition of 60% of α-amylase [114].

In other hand, in *in vitro* model of ATP-induced monocytes inflammation model, hydroethanolic extract of *P lentiscus* fruits showed non-significant effects in the inhibition of pro-inflammatory cytokine IL-1β [112].

In addition, antioxidant activities of isolated compounds from polar extracts of *P. lentiscus* fruits was evaluated in several in vitro models; gallic acid derivatives **97**, **98** and **101** have demonstrated noticeable effect in DPPH and ABTS free radical scavenging assay (**97** and **101** at 2 and 1 µg/mL, respectively). Compound **98** has also inhibited xanthine oxidase (21 % of inhibition at 150 µg/mL) and lipid peroxidation in human K562 cell line pre-treated with $H_2O_2$ (30 µM) [115,116].

The oil of the fruits was subjected to anti-inflammatory, antioxidant and wound healing evaluations. *In vivo* carrageenan-induced paw rat oedema was performed and several inflammatory parameters were measured after dermal application of 2µL per paw. Significant reduction of oedema was observed after treatment with the oil. Some parameters of oxidative stress such as superoxide dismutase level, catalase and glutathione peroxidase activities were measured in the treated paw tissue. Volatile compounds were identified by GC-MS: α-pinene (13.4%), β-phellandrene (10.4%), α-phellandrene (10.1%) and germacrene D (6.9%) [117].

Oil of fruits harvested in Algeria and Tunisia have been also subjected to evaluation of wound healing proprieties. Excision and laser burn models were performed and topical

administration of was applied (dose of 0.56 µl/mm$^2$ in a wound area of 2.2 cm$^2$). Complete healing was observed after 8 days of oil administration. Collagen level was also detected as normal degrees after the same period of time [118]. In an excision model, a dose of 15mg/kg/d of oil and its saponifiable and unsaponifiable fractions were applied and their wound healing effects evaluated during 26 days. Oil and unsoponifiable were the most active, especially from day 6 to 18 after wounding. Moreover, wound inspections confirmed the presence of infections in most of the animals, except for those treated with *P. lentiscus* oil and unsaponifiable fraction [119].

The fatty acid composition of oil obtained from Tunisian fruits showed high proportions of monoinsatured C18 acids, that significantly increased during fruits ripening, while levels of polyinsatured fatty acid decreased. β-Sitosterol was found to be the major sterol (100-400 mg per 100 g of oil). Total sterols amount in oil decreased during fruits ripening [120,121].

## II.3 Personal work and collaboration

In the framework of EXANDAS project, we focused on the pharmacological and cosmetic potential of fruits of Pistacia lentiscus collected at two different stage of maturation and from two geographical origin. Collection were performed during September-October 2016 and 2018 in Chios Island (Greece) and in north of Algeria.

After examination of the literature, we first decided to explore antioxidant, anti-inflammatory and tyrosinase inhibition activities using the modern analytical tool of molecular networking to identify active compounds but also possible geographical and/or ripening biomarkers. As the attenuation of *Pseudomonas aeruginosa* virulence factors was identified in a general screening for the apolar fraction of Algerian fruits extract, a classical *bio*-guided approach was carried out to characterize the bioactive compounds (**Figure 72**).

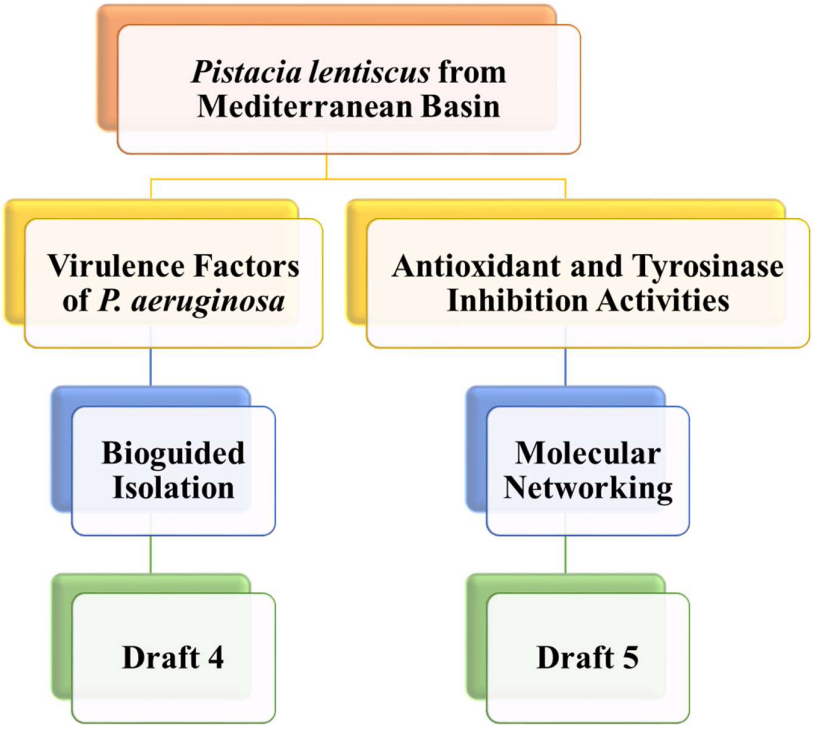



**Figure 71: General workflow of the biological evaluations and chemical studies of P. lentiscus fruits extracts.**

To accomplish this aim, several collaboration were set up with different national and international laboratories:

| Task | Responsible | Institution |
|---|---|---|
| Pyocyanin production, qRT-PCR, membrane fluidity. | Pr. Sylvie Chevalier, Dr. Ali Tahrioui | Laboratory of Microbiology Signals and Microenvironment, Université de Rouen, Évreux, France. |
| Antioxidant, tyrosinase inhibition activities. | Dr. Nektarios Aligiannis | Department of Pharmacognosy and Natural Products Chemistry, University of Athens, Greece. |
| Anti-inflammatory activity | Dr. Thorsten Rose | Vivacell Biotechnology, Germany. |

**Table 8: Collaboration set up during the execution of Chapter II.**

## II.3.1 General Objectives

The study of pharmacological/cosmetic potential of *P. lentiscus* fruits has included:

- Exploration of biological properties with pharmaceutical and/or cosmetic interest (antioxidant, anti-inflammatory and antityrosinase activities) of *P*. *lentiscus* fruits collected in Algeria and Greece.
- Investigation of the antivirulence effects on *Pseudomonas aeruginosa* of *P. lentiscus* fruits harvested in Algeria.

### II.3.2 Specific objectives – Attenuation of *Pseudomonas aeruginosa* virulence factor

- Assessment of influence of *P. lentiscus* fruits samples in the pyocyanin (virulence factor) production of *P. aeruginosa*.
- *Bio*-guided fractionation of more active extract to identify the bioactive compounds.
- *In vitro* and *in vivo* virulence attenuation assessment of active samples.
- Evaluation of the influence in the quorum sensing system and in the membrane fluidity of *P. aeruginosa* by *P. lentiscus* extracts.

### II.3.3 Specific objectives – Comparative studies of *P. lentiscus*

- Dereplication of methanolic extracts of *P. lentiscus* from Greece and Algeria using molecular networking analytical tool.
- Evaluation of antioxidant and antityrosinase activities of samples by *in vitro* colorimetric assays.
- Evaluation of the capacity of extracts to reduce production of pro-inflammatory mediators on LPS-stimulated human monocytes (anti-inflammatory activity).

# II.4 *Pistacia lentiscus* fruit extracts prevent *Pseudomonas aeruginosa* virulence

As discussed in **I.3**, bacterial infections still constitute a serious public health threat. Common antibiotics (methicillin, vancomycin, carbapenems, clarithromycin, etc…) are no longer so effective against pathogen strains including *Enterococcus faecium*, *Staphylococcus aureus*, *Acinobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp., also known as "ESKAPE" pathogens [122]. Resistant bacteria are a major cause of death in intensive care units worldwide. According to the European Centre for Disease Prevention and Control, 33,000 people die every year due to antibiotic-resistant bacteria, similar to influenza, tuberculosis and HIV/AIDS together [123]. This underlines once more the urgent need for antibiotics with original mode(s) of action.

Targeting bacterial virulence is an alternative that can be used to disarm pathogens in the host. The overall strategy is to inhibit specific mechanisms that promote infection and are essential to the persistence of a pathogenic cascade (binding, invasion, subversion of host defences and chemical signaling) and/or that cause symptoms (secretions of toxins) [124].

## II.4.1 Virulence of *Pseudomonas aeruginosa* as potent therapeutic target

*P. aeruginosa* is a ubiquitous, gram-negative bacterium that thrives in diverse habitats and environments. Usually a commensal of the host, *P. aeruginosa* is able to turn into an opportunistic pathogen when there is a breach of host tissue barriers or a suppressed immune system. *P. aeruginosa* is an important nosocomial pathogen, affecting a wide category of hospitalized people, including patients with cystic fibrosis, traumatized cornea, burns, open fractures, long-term intubated, immuno-compromised, lung diseases and elderly. The infections are usually resistant to treatments by multiple antibiotics and may become severe and persistent.

Several researches have been performed to characterized and understand the bacterial cell-cell communication mechanism, known as quorum-sensing system, which plays a key role in the modulation of expression of virulence genes in *P. aeruginosa*. Quorum sensing bacteria produce and release small chemical signals in a high population density, which interact with receptors to induce the transcriptional expression of various target virulence factor-encoding genes [125].

Virulence mechanism of *P. aeruginosa* are summarized in **Figure 73**. Proteins like elastase, rhamnosy-transferase and protease are secreted by the bacteria during infections causing deleterious effect to the host. Rhamnosyl-transferase and alkaline protease degrade the immune

system by degradation of cytokines and causing necrosis of host macrophage, allowing persistent colonization and biofilm development.

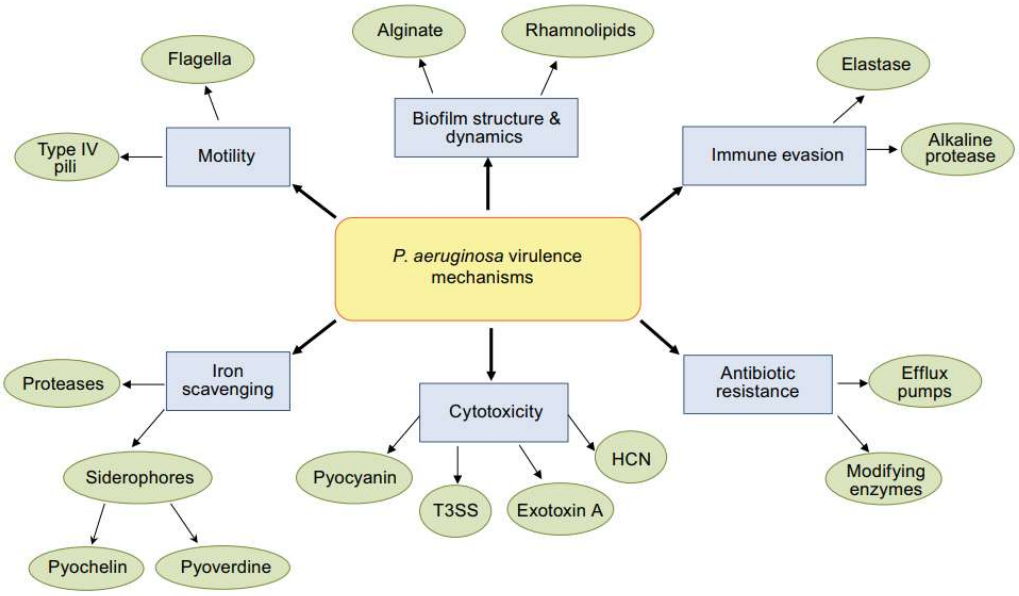



**Figure 72: Virulence mechanisms employed during *P. aeruginosa* infections** [126]**.**

## II.4.2 Pyocyanin virulence factor and other quorum-sensing dependent factors

The transcriptional expression and release of small molecule such as hydrogen cyanide (HCN) or pyocyanin (PYO) is also regulated by the quorum sensing system. These compounds produce cellular host respiration arrest and degrade lung function, enhancing the bacterial colonization.

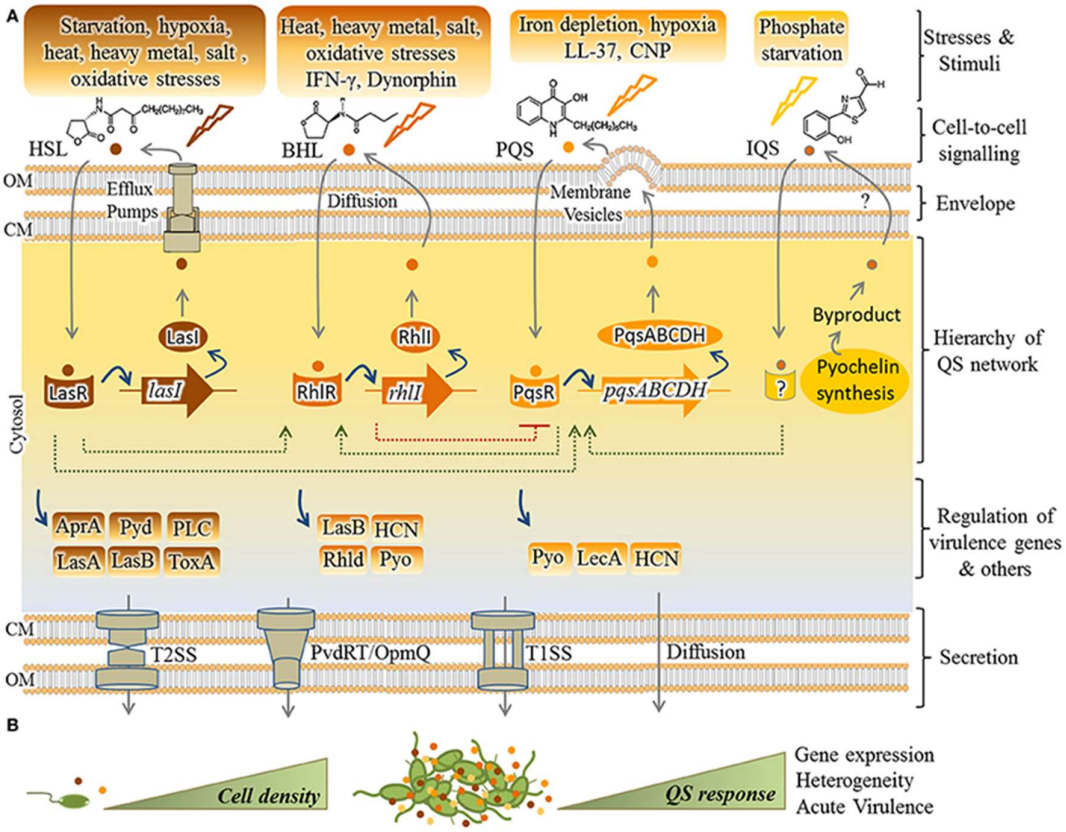
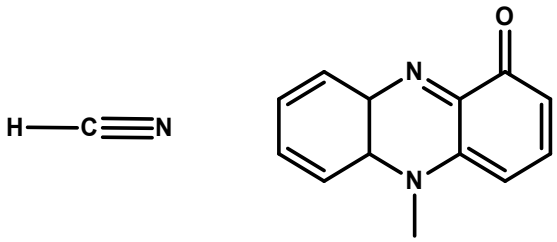



**Figure 73: Quorum-sensing regulation of pyocyanin production by *P. aeruginosa*** [127].




**106** **107**

**Figure 74: Structure of hydrogen cyanide 106 and pyocyanin 107.**

Pyocyanin (**Figure 75**) is a redox active phenazine-derived pigment secreted during *P. aeruginosa* infections. (**Figure 74**). Pyocyanin readily enters to host cells, leading to increased intracellular formation of reactive oxygen species, causing oxidative stress, affecting host cellular respiration, paralysis of airway cilia, delay of inflammatory response through neutrophil damage (by

pyocyanin-induced overproduction of IL-8 host cytokine) [128,129]. These processes benefit the bacteria in the establishment of infection, enhanced colonization and immune evasion.

- Quorum sensing (QS) is a process of cell-cell communication that allows bacteria to share information about cell density and adjust gene expression accordingly. QS circuitry in *P. aeruginosa* controls strongly the biosynthesis of pyocyanin. This process starts with the synthesis of the N-acyl-L-homoserine lactone (AHL) type signal molecules followed by the *Pseudomonas* quinolone signal (PQS) involving 4-hydroxy-2-alkylquinolines (HAQ) [130].

In order to demonstrate the importance of pyocyanin, an *in vivo* CD-1 mice model was infected with *P. aeruginosa* wild types (PA14 and PA01) and mutants with pyocyanin-production deficiency. The viable counts of pyocyanin-deficient mutant strain after infection decreased by 2.83-4.47 logs compared to that of wild types. Co-administration of deficient mutants strains with pyocyanin rescues the virulence, showing the same levels of bacterial counting in comparison with wild types. Competitive mixed-infection assays showed only 18-36% as competitive as their parent wild-type strains [131].

## II.4.3 Methodology

### II.4.3.1 Pyocyanin quantification

The effect on pyocyanin production by co-incubation of *P. aeruginosa* H103 and *P. lentiscus* fruits extracts were carried out by colorimetric assay. After incubation, culture supernatants were recovered and pyocyanin was extracted by liquid-liquid partition with equal volume of chloroform. Then, the chloroformic layer was extracted with equal volume of 0.5 N HCl, where pyocyanin is acidified and consequently soluble in the aqueous layer. Finally, optical density is measured at 580 nm.

In this study, we have evaluated the attenuation of pyocyanin production of *P. aeruginosa* by several extracts of *P. lentiscus* fruits extracts and exploration of the action mechanism of extract and isolated compounds and their implication in the attenuation of *P. aeruginosa in vivo* model. The workflow was as follows:

- Successive extraction in cyclohexane, ethyl acetate, methanol and water of unripe and ripe *P. lentiscus* fruits collected in Algeria.
- Evaluation of the anti-pyocyanin activity of extracts by colorimetric *in vitro* assay in *P. aeruginosa* H103 strain.

- Virulence attenuation by *P. lentiscus* fruits extracts, enriched fractions and isolated compounds *in vitro* on A549 human lung cells and *in vivo C. elegans* infected by *P. aeruginosa* H103 strain.
- Influence of more active extracts on *P. aeruginosa* H103 strain growth and cell viability.
- Influence on the HAQ signalling pathway by evaluation of the expression of involved genes (*phzM*, *phzS*, *phzH*) in the pyocyanin biosynthesis by qPCR assays in *P. aeruginosa* H103 strain.
- Bio-guided isolation of active compounds from more active *P. lentiscus* extract
- Action of more active extract in the bacterial membrane stiffness.

## II.4.4 Draft 4: Membrane-interactive compounds from *Pistacia lentiscus* L. thwart *Pseudomonas aeruginosa* virulence

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### 1. INTRODUCTION

Bacterial infections still constitute a serious public health threat even though their prevention and treatment have improved over the last decades. Common antibiotics are no longer effective against several pathogen strains including *Enterococcus faecium*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and Enterobacter species, also known as "ESKAPE" pathogens (Pendleton et al., 2013). *P. aeruginosa* is predominantly responsible for life-threatening infections affecting the respiratory system, the urinary tract, related to burns and wounds, as well as medical implant devices (Van Delden and Iglewski, 1998). In a recent report published by the World Health Organization (WHO) it was categorized as the second of the "critical priority pathogens" for which there is an urgent call for the discovery of innovative new therapies due to its highly resistance to antimicrobials including carbapenems (WHO, 2017). This notorious multidrug resistant opportunistic Gram-negative bacterium produces a wide variety of virulence factors and host-degrading enzymes as well as multiple secondary metabolites (Lee and Zhang, 2015). Pyocyanin is an important virulence factor produced and secreted abundantly by nearly 95% of *P. aeruginosa* isolates. This blue-green phenazine-derived pigment confers a greenish hue to the sputum of cystic fibrosis (CF) of individuals suffering *P. aeruginosa* chronic lung infection. Moreover, it is a highly diffusible redox-active metabolite which plays an important role in several physiological processes (Lau et al. 2004). Thus, pyocyanin inhibition may have consequences regarding the cytotoxic effects and the full virulence of the bacterium during infections related to airways in CF, making it a good target. The complex quorum sensing

(QS) circuitry in *P. aeruginosa* strongly controls the biosynthesis of pyocyanin. This process starts with the synthesis of the N-acyl-L-homoserine lactone (AHL) type signal molecules followed by the Pseudomonas quinolone signaling (PQS). Next, PQS regulates the expression of phzA-G operons resulting in the production of phenazine-1-carboxylic acid (PCA) which is then predominantly turn into pyocyanin (modification encoded by phzM).

In the search for alternatives to fight the pathogenicity of *P. aeruginosa*, the traditional medicines reported to treat infectious diseases were explored and our attention has focused on *Pistacia lentiscus* L. (Anacardiaceae), a tree growing in the Mediterranean basin. Aerial parts has traditionally been used in case of hypertension, stomachaches, ulcer, kidney stones, cough, sore throat, eczema, burn wounds, as healing agent, stimulant and diuretic (Bozorgi et al., 2013, Ben Khedir et al., 2016). The resin, known as mastic gum, is traditionally used as chewing gum, antiseptic, to relieve stomach pain and to treat respiratory infections (Duru et al., 2003).

## 2. RESULTS

### 2.1. Pyocyanin inhibition by *P. lentiscus* L. fruit extracts

Planktonic cultures of *P. aeruginosa* H103 were exposed to cyclohexane, ethyl acetate, methanol, and aqueous extracts of *P. lentiscus* fruits at concentrations of 100, 50, 25, 12.5, 6.25, 3.12, 1.6 and 0.8 µg $mL^{-1}$ and assayed for interference with pyocyanin production (**Figure 1A**). Untreated H103 strain cultures and cultures treated with 1% v/v DMSO were also measured as negative control and defined as 100% pyocyanin production. **PLFE1** (cyclohexane extract) showed statistically significant inhibition of pyocyanin biosynthesis at all the tested concentrations. The strongest effect was observed at 100 µg mL-1 with 82% ($P<0.0001$) decrease of pyocyanin production in comparison with the untreated control; $IC_{50}$ was determined as 4.9 µg mL-1. In addition, **PLFE1** inhibitory activity showed to be dose-dependent. **PLFE2** (ethyle acetate extract) also inhibited pyocyanin biosynthesis following the same behaviour. However, it was less active, with approximately 50% of inhibition at 100 µg $mL^{-1}$ ($P<0.001$). **PLFE3** (methanol extract) and **PLFE4** (water extract) were not active. Consequently, **PLFE1** was choosen for further set of experiments. The inhibitory activity of **PLFE1** on phenazine biosynthetic pathway was then investigated at the level of genes expression. In the presence of **PLFE1** at 100 µg mL-1, qPCR analyses results showed significant down-regulation of the phzA gene involved in the production of phenazine-1-carboxylic acid (PCA), *phzM*, and *phzS* which converts PCA into pyocyanin (**Figure 1B**). However, the expression levels of the *phzH* gene were not significantly different in H103 strain treated with **PLFE1** compared to untreated H103 (**Figure 1B**). In summary, the repression of the expression levels of genes involved in phenazine biosynthesis correlate with

pyocyanin production inhibition indicating that **PLFE1** have the ability to function as a strong inhibitor of the pyocyanin virulence factor in *P. aeruginosa* H103 strain.

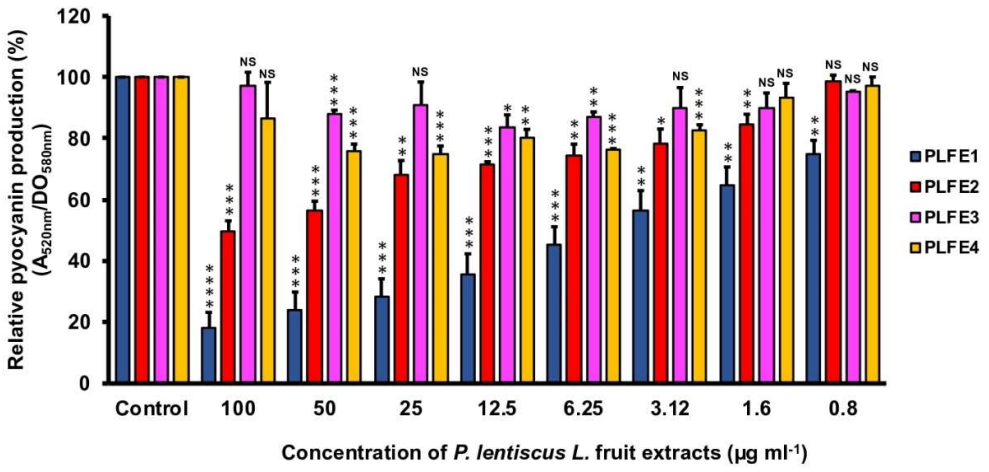



**Figure 1A**: *P. lentiscus* fruits extracts inhibition effect on pyocyanin production at different concentrations.

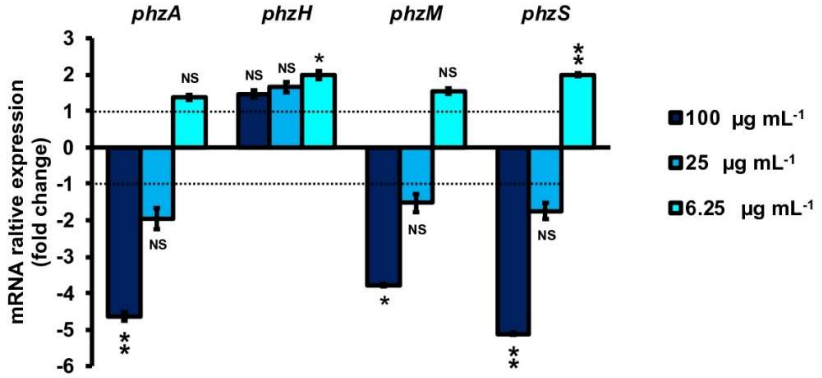
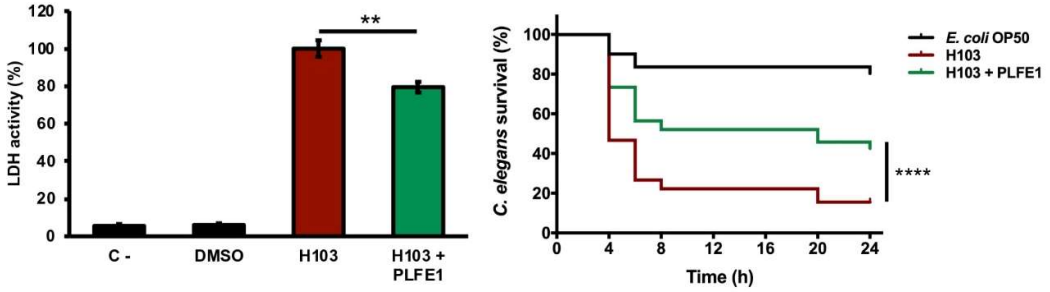



**Figure 1B**: Relative expression levels of representative genes from phenazime biosynthesis pathways compared to relatives mRNA levels in control conditions (H103 untreated).

2.2. *In vivo* virulence attenuation on *P. aeruginosa* by *P. lentiscus* L. fruit extract **PLFE1**

In a next step, the antivirulence effect of **PLFE1** on H103 *P. aeruginosa* strain using human lung A549 cells and *Caenorhabditis elegans* infection models was evaluated. LDH activity of A549 cells after infection with H103 strain treated with **PLFE1** (100 µg $mL^{-1}$) for 20h incubation decreased of 20% ($P \leq 0.05$) in comparison with A549 cells infected with non-treated strain H103 (**Figure 2A**). These result revealed that **PLFE1** exhibited significant *in vivo* antivirulence effect toward *P. aeruginosa* in human lung A549 cells. In addition, *in vivo* anti-virulence activity of **PLFE1** was assessed using a *C. elegans* fast-killing infection assay. When *C. elegans* were placed in a lawn of H103 strain, the percentage of nematodes death drastically decreased (65%;

P<0.0001) after 24h incubation as compared to *C. elegans* fed with *E. coli* OP50 (**Figure 2B**). Interestingly, the presence of **PLFE1** (100 µg mL-1) significantly (27%; P<0.0001) protected *C. elegans* from killing by *P. aeruginosa* as compared to *C. elegans* when applied to lawns of non-treated H103 strain (**Figure 2B**). Altogether, these data clearly indicate that **PLFE1** has attenuated the virulence of *P. aeruginosa* strain H103 in human lung A549 cells and *C. elegans* infection models.




a) b)

Figure 2: a) Antivirulence effect of **PLFE1** in human A549 lung cells infection model after 20 h of infection; b) *P. aeruginosa* H103 virulence attenuation in a *C. elegans* infection model by PLFE1, alive nematodes were scored at 4, 6, 8, 20 and 24 h after the start of the assay. ** P=0.001 to 0.01 (two-tailed t test) and **** P<0.0001 (long-rank [Mantel-Cox] test) versus untreated cells.

2.3. Effect of *P. lentiscus* L. fruit extract **PLFE1** on *P. aeruginosa* growth and cell viability

The impact of **PLFE1** on cell growth of *P. aeruginosa* H103 was monitored at 37 ºC during 24 h. At all the concentrations (100, 50, 25, 12.5, 6.25, 3.12, 1.6 and 0.8 µg $mL^{-1}$) no impact on planktonic cell growth of H103 strain compared to the untreated culture (H103 strain grown in presence of 1 % v/v DMSO) was noticed (Supporting Information). Moreover, the effect of **PFLE1** on the H103 strain cell viability was evaluated by flow cytometry using BacLight live/dead stain. Similarly, all the concentrations tested of **PLFE1** showed no effect on the cell viability according to normalized events counting of live, injured, and dead cells when compared to untreated control (Supporting Information). Thus, the inhibition of pyocyanin production by **PLFE1** was achieved without affecting the growth of the bacteria and cell viability, at least *in vitro*.

2.4. *P. lentiscus* L. fruit extract **PLFE1** modulates the production of HAQ-signaling molecules

To assess whether the inhibition of pyocyanin was directly due the effect of **PLFE1** on PQS signalling system that is known to strongly regulate pyocyanin biosynthesis, *P. aeruginosa* H103 cultures were exposed to several concentrations of **PLFE1** (100, 50, 25, 12.5, 6.25, 3.12, 1.6 and

0.8 µg mL$^{-1}$). Then HAQs molecules were extracted twice with ethyl acetate. The production of HAQs was determined by a PAO1 Δ*pqsA* CTX-*pqsA*::*lux* biosensor strain, which does not produce HAQs molecules and shows response to exogenous HAQs. The H103 cultures treated with 100, 50, and 25 µg mL-1 of PLFE1 showed significant reduced HAQs production (**Figure 3A**). Additionally, the expression levels of *pqs*A, *pqs*H, *pqs*L, and *pqs*R genes representative of PQS signalling system was measured by using RT-qPCR. The assays were performed after 24h of growth of H103 untreated and treated with 100, 25, and 6.25 µg mL$^{-1}$ of **PLFE1** (**Figure 3B**). The expression of *pqs*A, *pqs*H and *pqs*L involved in the biosynthesis of the major molecules from the HAQs family, namely 3,4-dihydroxy-2-heptylquinoline (Pseudomonas quinolone signal PQS), its precursor 4-hydroxy-2-heptylquinoline (HHQ) and 4-hydroxy-2-heptylquinoline N-oxide (HQNO) were all significantly repressed upon exposure to **PLFE1** at 100 and 25 µg mL-1. However, the expression of *pqs*R encoding for the cognate receptor of HHQ and PQS signalling molecules did not change in the presence of 100 and 25 µg mL$^{-1}$ **PLFE1**. Taken together, these data indicate that **PLFE1** repress gene expression levels of the PQS QS-signalling system that correlate with the reduction in HAQs molecules production suggesting that **PLFE1** exhibits anti-pyocyanin production effect possibly through an anti-QS activity.

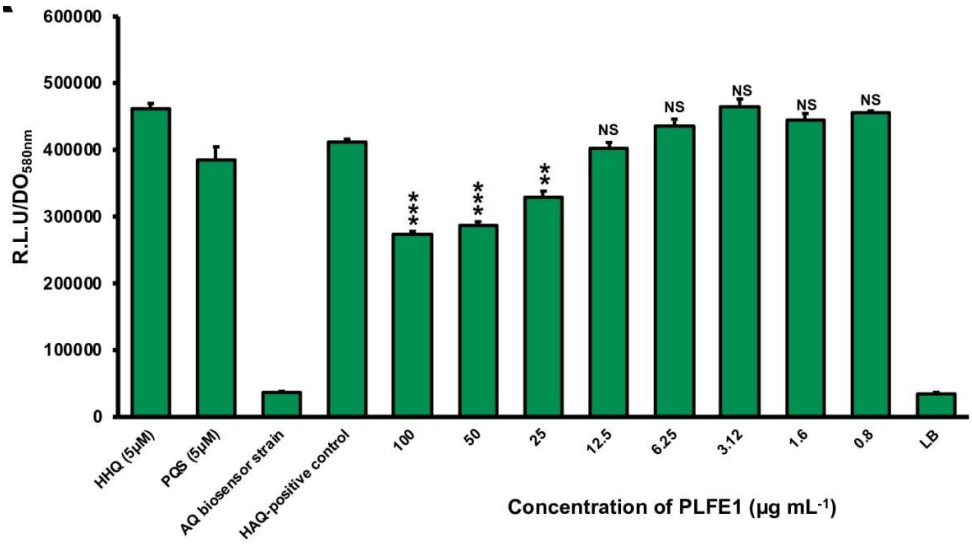



**Figure 3A**: Normalized mean maximal bioluminescence output from HAQ-biosensor strain in the presence of crude ethyl acetate HAQs extracts prepared from cultures of H103 treated with different concentration of **PLFE1**.

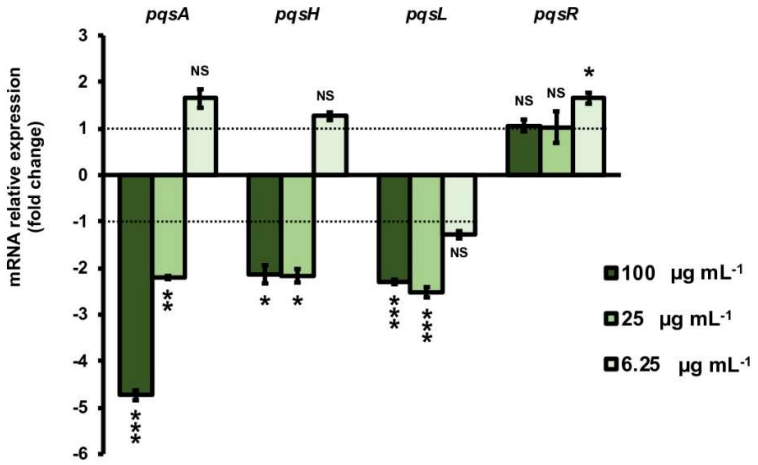



**Figure 3B**: Relative expression levels of representative genes from HAQ signalling system in H103 treated with **PLFE1** at 100, 25 and 6.25 µg $mL^{-1}$ compared to the relative mRNA levels in the control conditions (H103 untreated). *** *P*=0.0001 to 0.001; ** *P*=0.001 to 0.01, * *P*=0.01 to 0.05, NS (not significant) P<0.05.

2.5. *P. lentiscus* L. fruit extract **PLFE1** leads to increased membrane stiffness

Furthermore, the effect of **PLFE1** on *P. aeruginosa* H103 strain membrane fluidity homeostasis was evaluated. Planktonic cell growths of the H103 strain exposed to **PLFE1** at concentrations of 100, 50, 25, 12.5, 6.25, 3.12, 1.6 and 0.8 µg $mL^{-1}$ were assayed for fluorescence anisotropy (FA) using 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescent probe (**Figure 4A**). Anisotropy of H103 cells increased in a concentration dependent-manner to achieve a maximum FA value of 0.223 ± 0.003 (*P*<0.001) at 100 µg $mL^{-1}$ of **PLFE1** in comparison to the FA value of untreated control (0.186 ± 0.002). This result reflects a significant decrease of approximately 20% on membrane fluidity (membrane rigidification) revealing physiological changes and adaptations of the cellular envelope of H103 strain in response to **PLFE1** exposure. Interestingly, mRNA expression levels of *sigX* encoding the extracytoplasmic function sigma factor (ECFσ) *SigX* required to maintain cell envelope stress and its known representative targets *accA*, *accB*, and *fabY* involved in fatty acid biosynthesis significantly decreased upon exposure of H103 strain to **PLFE1** at 100 and 25 µg $mL^{-1}$ when compared to mRNA expression levels in the control conditions (untreated H103) (**Figure 4B**). Moreover, *sigX* expression was monitored during growth in the presence of **PLFE1** at 100, 25 and 6.25 µg $mL^{-1}$ by using a transcriptional fusion construction where *sigX* promoter region was fused to the promoter-less *luxCDABE* cassette in the replicative pAB133 vector (pAB-P*sigX*) (Figure S2, Supporting Information). Remarkably, in the presence of **PLFE1**, the relative bioluminescence output from H103 harbouring pAB-P*sigX* increased in a dose dependent-manner reaching significant maximal activity at 100 µg $mL^{-1}$ during the transition from the late exponential phase to early stationary phase (**Figure 4C**; Figure

S2A, Supporting Information). However, bioluminescence activity of pAB-P*sigX* decreased significantly in a concentration dependent-manner during the late stationary phase achieving minimal activity at 100 µg $mL^{-1}$ of **PLFE1** (**Figure 4C**; Figure S2A, Supporting Information). These results reveal that *P. aeruginosa* exposure to **PLFE1** induced increased membrane stiffness suggesting alterations in envelope homeostasis most likely through the activation of the cell wall stress ECFσ SigX. To further validate this hypothesis, we used a Δ*sigX* deleted mutant strain (Bouffartigueset al. 2012) to determine the FA in the absence or presence of various concentrations of **PLFE1**. The FA values appeared to be unaffected in Δ*sigX* in the response to **PLFE1** exposure compared to the control condition (untreated Δ*sigX*) (Figure S2A, Supporting Information). Altogether, these findings indicate that **PLFE1** contains bioactive membrane-interactive compounds that reduce membrane fluidity where the ECFσ SigX seems to play a key role.

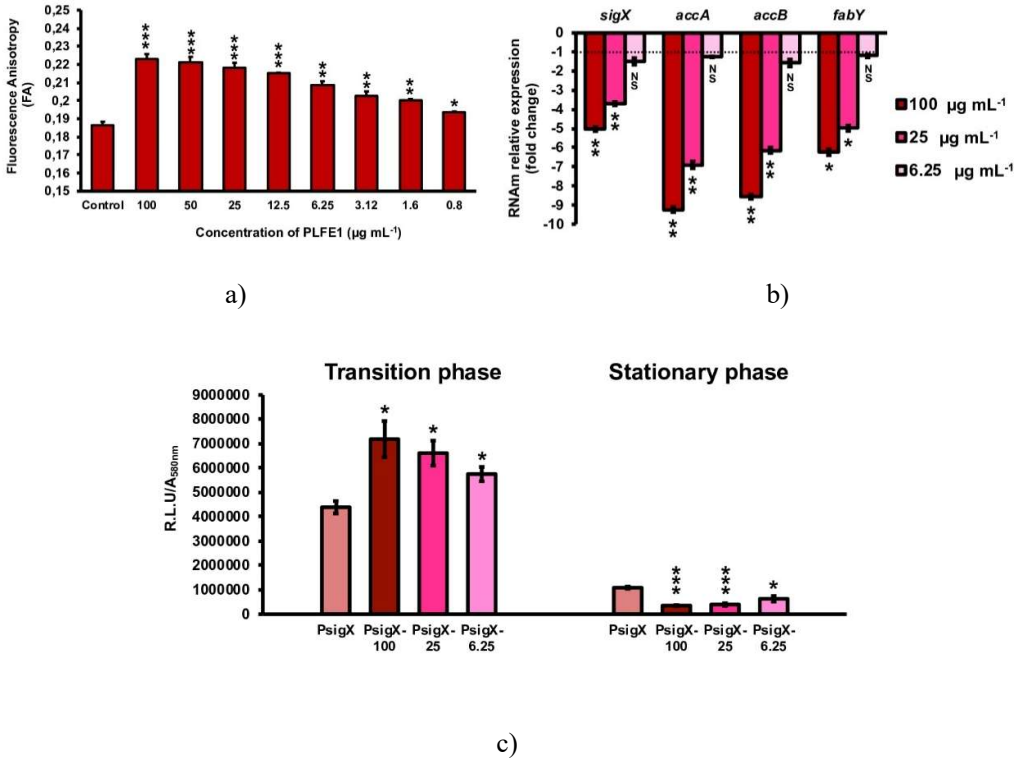



**Figure 4**: a) fluorescence anisotropy (membrane fluidity) measuremts in *P. aeruginosa* H103 strain exposed to various concentrations of **PLFE1** compared to untreated control; b) Relative expression levels of *sigX* encoding the SigX extracytoplasmic function sigma factor (ECFσ) and its known target genes *accA*, *accB* and *fabY* involved in fatty acid biosynthesis in H103 treated with several **PFLE1** concentrations compared to the relative mRNA levels of H103 untreated controls; c) Relative bioluminescence levels of H103 strain harbouring the pAB-Ps*igX* plasmid treated with **PLFE1** at several concentration compared to the relative levels in the control

condition. Statistics were achieved by a two-tailed *t* test: **** *P*<0.0001, *** *P*=0.0001 to 0.001, ** *P*=0.001 to 0.01, * *P*=0.01 to 0.05, NS (Not significant) *P*>0.05.

2.6. Isolation and identification of major bioactive compounds of *P. lentiscus* L. fruit cyclohexane extract **PLFE1**

To isolate and identify the major constituents of **PLFE1**, multiple chemical analyses were performed. The fractionation of **PLFE1** by MPLC allowed to recover 13 fractions labelled **PLFE1**-(**1**-**13**) (Table S2, Supporting Information). Thin layer chromatography (TLC) examination showed that the major compounds of **PLFE1** were present in fractions **PLFE1**-(**2**-**4**) most likely as a result of their important masses. **PLFE1**-(**2**-**4**) were then selected for further analyses. For **PFLE1**-**2**, its $^{1}$H-NMR spectra, proton signals between 7.36 and 6.78 ppm showed the presence of a tri-substituted aromatic ring. The signal at 5.36 ppm [t, *J* = 4.6 Hz, 2H] accounted for an olefinic double bond. The major derivative ginkgolic acid (C17:1) with a molecular formula of $C_{24}H_{38}O_3$ (*m/z* 373.1 $[M-H]^-$) displayed one unsaturation in its aliphatic chain. Ozonolysis reaction of **PLFE1**-**2** allowed to localize the double bound of ginkgolic acid (C17:1, GA) between positions C-8' and C-9' (**Figure 5)**. LC-ESI-MS analyses corroborated the presence of ginkgolic acid (C17:1) in **PLFE1**-**2** and showed the occurence of hydroginkgolic acid (C15:0, HGA) derivative and a mixture of both ginkgolic acids (C17:1/C15:0) in **PLFE1-3** and **PLFE1**-**4**, respectively. (**Figure 5)**. Altogether, these results indicate that ginkgolic acid (C17:1) and hydroginkgolic acid (C15:0) are the main metabolites in **PLFE1** and might be responsible for *P. aeruginosa* virulence attenuation.

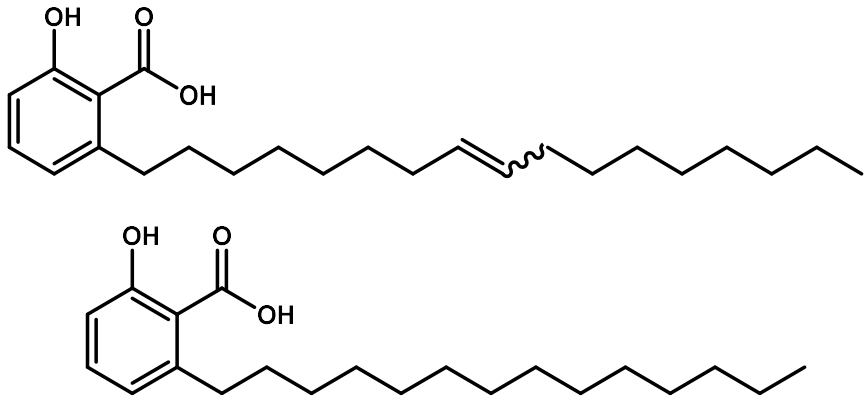



**Figure 5**: Chemical structure of ginkgolic acid derivatives identified in **PLFE1**-**2**-**4**

2.7. Ginkgolic acid-enriched fractions from *P. lentiscus* L. fruit extract **PLFE1** are involved in virulence attenuation of *P. aeruginosa*

To further confirm whether GA (C17:1), HGA (15:0) or a mixture of both (C17:1/C15:0) are the main compounds involved in the anti-virulence activity against *P. aeruginosa*, their effect on

pyocyanin production and *in vivo* virulence attenuation in human lung A549 infection model was evaluated. In accordance with the results of pyocyanin inhibition by the crude extract **PLFE1**, H103 strain treated with GA-enriched fractions showed a dose-dependent reduction of pyocyanin production when compared to untreated H103 (**Figure 6A**). Interestingly, the fractions enriched in GA (C17:1) or GA/HGA (C17:1)/(C15:0) showed approximately 90% pyocyanin inhibition activity at 100 µg $mL^{-1}$. The GA C17:1 and HGA C15:0 displayed an $IC_{50}$ of 6.3 µg $mL^{-1}$ (16.75 µM) and 12.5 µg $mL^{-1}$ (35.75 µM), respectively (Figure S3, Supporting Information). About *in vivo* virulence attenuation, H103 strain exposed to GA-enriched fractions showed significant reduced LDH activity (20%) when compared to the control conditions (H103 untreated) revealing that GA-enriched fractions were able to protect the human lung A549 line cells (**Figure 6B**). These results provide important insights into the involvement of GA-enriched fractions on the antivirulence activity against *P. aeruginosa*.

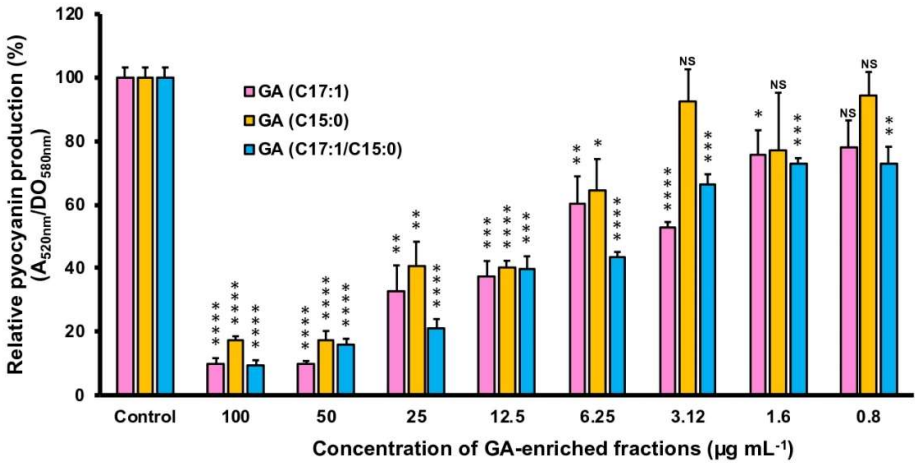



**Figure 6A**: Pyocyanin production upon exposure to different concentrations of GA-enriched fractions.

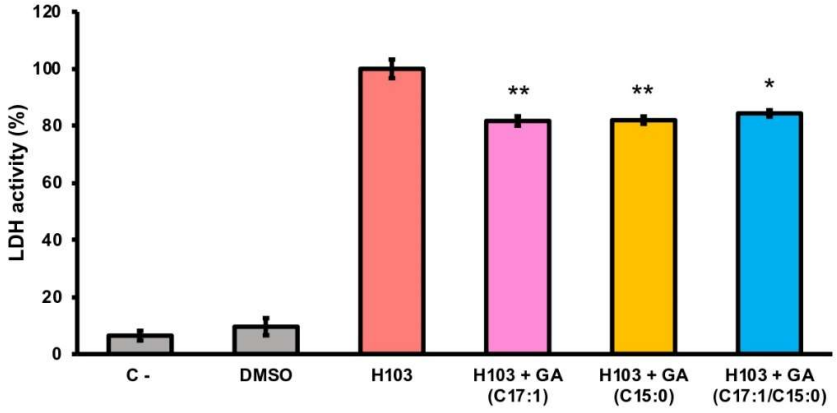


**Figure 6B**: *In vivo* antivirulence of GA-enriched (100 µg $mL^{-1}$) in human A549 lung cell infection after 20 h of infection. Statistic were achieved by a two-tailed *t* test: ** *P*=0.001 to 0.01, * *P*=0.01 to 0.05.

2.8. Cytotoxicity of *P. lentiscus* L. fruit extract (**PLFE1**) and GA-enriched fractions

The last part of this study was to investigate the cytotoxicity of **PLFE1** and its GA/HGA-enriched fractions to human lung A549 cells. As depicted in **Figure 7A**, no significant differences in cytotoxicity levels were found between A549 cells treated with various concentrations of **PLFE1** after 1h incubation in comparison with untreated control. However, a moderate cytotoxic effect was observed when A549 cells were incubated for more than 3h with **PLFE1** at concentrations above 12.5 µg $mL^{-1}$. Remarkably, when A549 cells were treated with **PLFE1** at $IC_{50}$ value of pyocyanin activity inhibition (4.9 µg $mL^{-1}$) there was no difference for the cytotoxicity at the different time points incubation assayed (1h, 3h, 6h, and 24h) (**Figure 7B**). Moreover, similar results were obtained for the fraction enriched with GA/HGA (C17:1/C15:0) when tested at 5.4 µg $mL^{-1}$ ($IC_{50}$ for pyocyanin inhibition, **Figure 7B**). On the other hand, after 3h incubation, the cytotoxicity slightly increased when A549 cells were exposed to enriched fractions of GA (C17:1) and HGA (C15:0) separately at their $IC_{50}$ values (6.3 µg mL-1 and 12.5 µg $mL_{-1}$, respectively) (**Figure 7B**). However, this cytotoxicity increased by about 3-fold after 24h exposure when compared to the control conditions (A549 cells treated with DMSO). In summary, these results indicate that **PLFE1** and its complex GA-enriched fraction (C17:1/C15:0) displayed no significant cytotoxicity in the human lung A549 line cells when tested at their $IC_{50}$ for pyocyanin inhibition values.

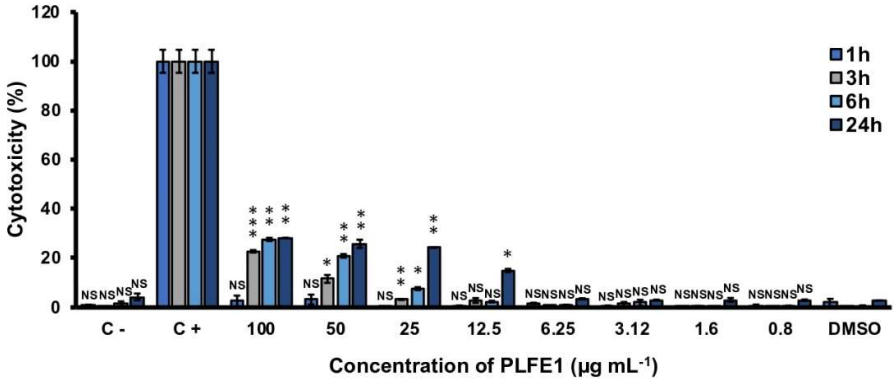



**Figure 7A**: Cytotoxic activity of **PLFE1** extract at different concentration against A549 cell line.

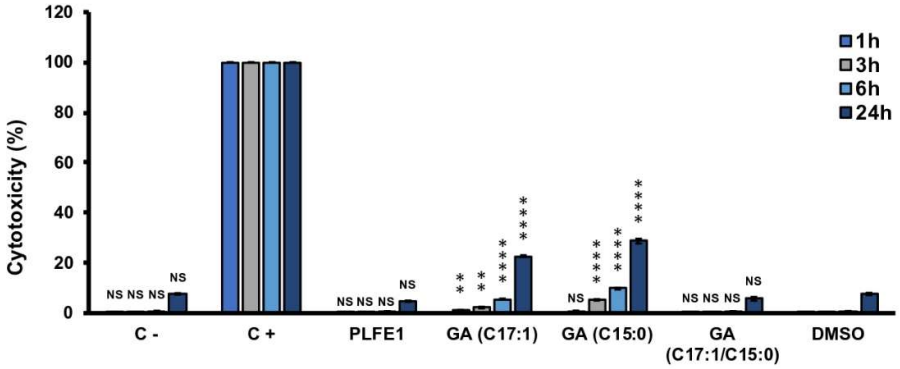



**Figure 7B**: Cytotoxic effect of **PLFE1** and GA-enriched fractions at different concentration at several time interval. Statistics were achieved by a two-tailed *t* test: *** *P*=0.0001 to 0.001, ** *P*=0.001 to =.01, * *P*=0.01 to 0.05, NS (Not significant) *P*>0.05.

## 3. DISCUSSION

Antibiotic-resistant pathogens have become such a major worldwide public health threat that new therapeutic strategies are urgently needed (Wagner et al. 2016). The anti-virulence approaches represent an attracting alternative in the treatment of bacterial infections (Rasko et al. 2010; Dickey et al. 2017). These new therapies mainly target the prevention of virulence factors production by pathogens, rather than their survival; they also aim to thwart the regulatory mechanisms controlling their expression (Martínez et al. 2019). In addition, by targeting essential virulence determinants, the emergence and spread of bacterial resistance would be reduced due to decreased selective pressure and the natural microbiota would be preserved. In this scenario, the current study was designed to explore new anti-virulence agents to fight the multidrug-resistant *P. aeruginosa* responsible for numerous kinds of pathologies. Thus, traditional medicines reported to relieve infectious symptoms were examined. In particular, the fruit of *P. lentiscus* L. is indicated for the treatment of respiratory tract infections and in ointments for articular pain, burn wounds and ulcer among other traditional uses.

Our findings show that *P. lentiscus* L. fruit cyclohexane extract (**PLFE1**) contains potent inhibitor compounds that restrict *P. aeruginosa* virulence by abolishing pyocyanin production, known to facilitate colonization of the host and subsequent infection. This anti-virulence activity does not perturb growth and cell viability which can minimize selective pressures toward the development of resistance compared to conventional antibiotics (Rasko et al. 2010). Nonetheless, additional investigations need to be undertaken to assess whether *P. aeruginosa* can develop resistance toward **PLFE1** upon repeated exposures. Further, by exploring the mechanism of action behind the anti-pyocyanin activity, we have shown that **PLFE1** interferes with HAQs QS-molecules production. In case of *P. aeruginosa* infections, QS is the master regulator involved in the expression of many virulence factors such as phenazines, exoproteases (elastase, alkaline

protease), siderophores and toxins among others, its inhibition appears as a valuable adjuvant therapy able to potentiate the activity of the available antibiotics applied to handle early *P. aeruginosa* infections (Martínez et al. 2019). In the last five years, a huge amount of papers have depicted the potential of natural products in targeting bacterial virulence factors (Silva et al. 2016). Several plant-derived compounds that belong to alkaloids, organosulfurs, coumarins, flavonoids, phenolic acids, phenylpropanoids, terpenoids among other chemical classes have been found to be active against pyocyanin production (Silva et al. 2016). However, the specific underlying molecular mechanisms of action have been poorly deciphered.

A noticeable result of this study was that the membrane stiffness of *P. aeruginosa* has significantly increased upon exposure to **PLFE1**. Furthermore, we have demonstrated that **PLFE1** decreases the expression of the ECFσ SigX, which is involved in the regulation of membrane lipid composition (Boechat et al. 2013; Blanka et al. 2014), and thus, triggers modifications on membrane fluidity and homeostasis. Accordingly, our data show a decreased expression levels of accA and accB encoding subunits of the biotin-dependent enzyme acetyl-CoA carboxylase complex (ACC) that catalyzes the first step of fatty acid biosynthesis in *P. aeruginosa* (Kondakova et al. 2015). In addition, fabY (PA5174) is another gene encoding the β-keto acyl synthase (FabY) involved in fatty acid biosynthesis that showed decreased expression in presence of **PLFE1**. Interestingly, these results are in accordance with those observed in *P. aeruginosa* sigX mutant and over-expressing strains when compared to the wild-type strain (Boechat et al. 2013; Fléchard et al. 2018; Blanka et al. 2014). Moreover, ΔsigX display reduced membrane fluidity (membrane rigidification), HAQs QS-molecules production, pyocyanin production and virulence toward a *C. elegans* model (Fléchard et al., 2018). Overall, these data provide evidences that **PLFE1** most likely contains membrane-interactive compounds, as it is the case for many plant extracts (Kopec et al. 2013; Tsuchiya et al., 2015). **PLFE1** interaction with membrane lipid bilayers seems to be involved in the increased membrane stiffness, which might trigger the modulation of the ECFσ SigX. Thus, we suggest that **PLFE1** exert its anti-virulence activity through a new potential mechanism that at least involves the ECFσ SigX since no impact of **PLFE1** was observed in membrane fluidity of the ΔsigX mutant strain.

Herein, the fractionation of **PLFE1** led to the isolation and identification of fractions mainly enriched in C17:1 and C15:0 ginkgolic acids (GA, HGA) or a mixture of both (C17:1/C15:0). To the best of our knowledge, the presence of these chemical constituents in *P. lentiscus* L. fruit has never been previously reported. Nevertheless, according to the literature, GA has already been characterized from *Ginkgo biloba* that had long been used in traditional Chinese medicine (Mahadevan et al. 2008). The GA are a mixture of several 2-hydroxy-6-alkybenzoic acid congeners that differ in carbon alkyl group length and unsaturation and are structurally similar to salicylic acid which is reported to decrease the pathogenicity of *P. aeruginosa* PA14 strain by

repressing pyocyanin, elastase, and protease production (Prithiviraj et al. 2005). Numerous pharmacological effects have been attributed to GA, such as antibacterial, antifungal, insecticidal, anti-tumor, and neuroprotective actions. Moreover, GA have also proven to be effective as anti-biofilm molecules against bacterial pathogens such as *Escherichia coli* O157:H7 strain, *Staphylococcus aureus*, *Streptococcus mutans*, *Salmonella* spp., *Listeria spp*. (Het et al. 2013; Lee et al. 2014; Wu et al., 2016). In line with our results, antibacterial activity studies show that GA did not affect Gram-negative bacteria; however, they exhibit strong anti-microbial activity against Gram-positive strains (Het et al., 2013; Hua et al., 2017; Choi et al., 2009; Yang et al., 2004). This indicates that **PLFE1** anti-virulence properties can be mostly attributed to the identified GA/HGA-enriched fractions (C17:1, C15:0, C17:1/C15:0) since they are able to function as pyocyanin production inhibitors without negative effect on bacterial growth. Moreover, these fractions are capable of inducing *P. aeruginosa* membrane rigidification (data not shown). These results are supported by the fact that the lipid soluble components in the cell wall of Gram-negative bacteria have shown to intercept GA derivatives (Hua et al., 2017). Following demonstration of **PLFE1** and GA/HGA-enriched fractions for *in vitro* anti-virulence properties against *P. aeruginosa*, their *in vivo* efficacy using human lung A549 cells and the nematode *C. elegans* as models host was evaluated. In both infection models, the virulence of *P. aeruginosa* upon exposure to **PLFE1** at a dose of 100 µg/mL is shown to be significantly attenuated. Furthermore, the cytotoxicity of **PLFE1** and GA/HGA molecules on A549 lung human cells was not significative at $IC_{50}$ values. Taken together, these *in vivo* studies provide a promising perspective for the potential future development of **PLFE1** or GA/HGA derivatives as adjuvants to fight *P. aeruginosa*. Nonetheless, the antibiotic-potentiating activity of **PLFE1** and GA/HGA, both *in vitro* and *in vivo*, against the pathogen *P. aeruginosa* remains to be checked.

All of these data strongly suggest that the anti-virulence efficacy of **PLFE1** and GA/HGA-enriched fractions may arise from interactions with the lipid bilayer membranes of *P. aeruginosa* resulting in the modification of membrane fluidity. Therefore, the increased membrane stiffness in presence of **PLFE1** and GA-enriched fractions seems to be mediated through the modulation of the ECFσ SigX which is known to be involved in the regulation of *P. aeruginosa* virulence. These findings need further experimental evidences, in order to identify the specific molecular mechanisms of action leading to impact on the ECFσ SigX as being a potential molecular target to alter the expression of virulence in *P. aeruginosa*.

## 4. EXPERIMENTAL SECTION

### Bacterial strains, media and growth conditions

The *P. aeruginosa* H103, H103-pAB-P*sigX*, and H103-Δ*sigX* (Bouffartigues et al. 2012) used in this study are all derivatives of *P. aeruginosa* wild type PAO1. Planktonic cultures were grown

aerobically at 37 ºC in LB broth on a rotary shaker (180 rpm) from an initial inoculum adjusted to an OD at 580 nm of 0.08. The antibiotics stock solutions used in this study were sterilized by filtration through 0.22-µm filters, aliquoted into daily-use volumes and kept at -20 ºC.

Collection and preparation of *P. lentiscus* L. fruit extracts

Fruits of P. lentiscus L. were collected within the wilaya of Jijel, Algeria, in September 2016, with the collect agreement (48/MC/DGCE/DSPEC/2016) delivered by the Research Centre on Analytical Chemistry (CRAPC). A voucher herbarium specimen is deposited in the herbarium of NPAS laboratory, Université Paris Descartes (France). A sample of 8.98 g of fruits was subjected to a pressurized solvent extraction (PSE) using a Speed Extractor E-914 (Büchi, Flawil Switzerland) equipped with four cells (120 mL) and a collector with four flat bottom vials (220 mL) successively with cyclohexane, ethyl acetate, methanol, and water. Maximum pressure and temperature were adjusted to 100 bar and to 50 °C, respectively. Two extraction cycles with a hold-on time of 15 min were performed in each case. Solvents were removed under reduced pressure. A total of 4 extracts were obtained (Table S3, Supporting Information). Solutions at 10 mg $mL^{-1}$ were prepared in DMSO of each extract and stored at 4 °C until use.

Pyocyanin quantification assay

To perform pyocyanin pigment quantification assay, *P. aeruginosa* H103 cells untreated (grown in presence of DMSO at 1%) and treated to 100, 50, 25, 12.5, 6.25, 3.12, 1.6 and 0.8 µg $mL^{-1}$ of *P. lentiscus* L. fruit extracts (**PLFE1**-**4**) or ginkgolic acid-enriched fractions (**PLFE1**-(**2**-**4**)) were grown on 96-well microtiter plate for 24 h at 37 ºC on a rotary shaker (180 rpm). Then, supernatants samples were collected by centrifugation and extracted with chloroform. The chloroform layer (blue layer) was acidified by adding 0.5 M HCl. The absorbance of the HCl layer (pink layer) was recorded at 520nm using the Spark 20M multimode Microplate Reader controlled by SparkControlTM software Version 2.1 (Tecan Group Ltd., Männedorf, Switzerland) and the data were normalized for bacterial cell density (OD580nm).

Virulence attenuation of *P. aeruginosa* using human lung A549 line cells

The human lung A549 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Lonza, BioWhittaker®) supplemented with 4.5 g $L^{-1}$ glucose, L-Glutamine, 10% heat-inactivated (30 min, 56 ºC) fetal bovine serum (FBS), and 100 Units $mL^{-1}$ each of penicillin and streptomycin antibiotics. Cells were grown at 37 ºC under the atmosphere of 5% $CO_2$ and 95% air with regularly medium change until a confluent monolayer was obtained. The antivirulence effect of **PLFE1** or ginkgolic acid-enriched fractions (**PLFE1**-(**2**-**4**)) on *P. aeruginosa* H103 strain was determined using an enzymatic assay (PierceTM LDH Cytotoxicity Assay Kit, Thermo ScientificTM) which measures lactate dehydrogenase (LDH) released from the cytosol of damaged A549 cells into the

supernatant. After overnight incubation with the *P. aeruginosa* H103 strain (108 CFU $mL^{-1}$) previously treated or untreated (control condition), the supernatants from confluent A549 monolayers grown on 24-well tissue culture plates were collected and the concentration of the LDH release was quantified. A549 cells exposed to 1X Lysis Buffer were used as a positive control of maximal LDH release (100% lysis) as specified by the manufacturer's recommendations. The background level (0% LDH release) was determined with serum free culture medium. The LDH release assays were also used to determine the cytotoxicity of **PLFE1** or ginkgolic acid-enriched fractions (**PLFE1**-(**2**-**4**)) in A549 cells upon direct exposure after 1-, 3-, 6-, and 24-h incubation.

*Caenorhabditis elegans* fast-killing infection assay

The *C. elegans* wild-type Bristol strain N2 worms were grown at 22 °C on nematode growth medium (NGM) agar plates using *Escherichia coli* OP50 as a food source. *The P. aeruginosa*-*C. elegans* fast-kill infection assay was performed as described previously (Blier et al. 2011) with minor modifications. Briefly, cultures of *P. aeruginosa* H103 strain untreated or treated with **PLFE1** at 100 µg $mL^{-1}$ were seeded on a 24-well plate containing each well 1 mL of Peptone-Glucose-Sorbitol (PGS) agar. Control wells were seeded with 25 µL of *E. coli* OP50 from an overnight culture. The plate was then incubated at 37 ºC for 24 h to make bacterial lawns and then shifted to 22 ºC for 4 h. For each assay, 15 to 20 L4-synchronized worms were added to the killing and control lawns and incubated at 22 °C. Worm survival was scored at 4-, 6-, 8-, 20-, and 24-h after the start of the assay, using an Axiovert S100 optical microscope (Zeiss, Oberkochen, Germany).

Bacterial cell viability assays by flow cytometry

Cell viability assays of *P. aeruginosa* H103 cells untreated and treated to different concentrations of *P. lentiscus* L. fruit extract (**PLFE1**) were assessed by using the LIVE/DEAD™ BacLight™ Bacterial Viability and Counting Kit, for flow cytometry (Invitrogen, Molecular Probes, Carlsbad, CA). Briefly, P. aeruginosa H103 untreated and treated suspensions were diluted in filter-sterilized PBS to reach a final density of 1 x 106 CFU $mL^{-1}$, then stained following the manufacturer's instructions. The data were acquired using CytoFlex S flow cytometer (Beckman coulter Life science, Indianapolis, United States). An aliquot of cells was killed with 100% ethanol and used as a control of death cells. The SYTO9 stained cells were detected by an excitation with 22 mW blue laser at 488 and with emission wavelength at 525 nm (green, with band pass filter of 40 nm), while the PI stained cells were detected by 690 nm (red, with band pass filter of 50 nm).

Extraction and quantification of HAQs

Planktonic cultures of *P. aeruginosa* H103 untreated (control DMSO 1%) and treated to 100, 50, 25, 12.5, 6.25, 3.12, 1.6 and 0.8 µg $mL^{-1}$ of **PLFE1** were subjected to HAQ molecules extraction following the technique described in a previous study (Tahrioui et al, 2011). HAQs were quantified by a combined spectrophotometer/luminometer microplate assay using the biosensor strain PAO1 pqsA CTX-lux::pqsA (Fletcher at al., 2007). The HAQ biosensor strain was grown overnight and OD was measured and adjusted with fresh LB medium to achieve OD580nm of 1. For each test well, 5 µL of HAQs crude extracts were diluted in 100 µL of LB and added to 100 µL of 1 in 50 dilution of the HAQ biosensor strain. Further, bioluminescence and OD580nm were monitored in specialized white sided and clear bottom 96-well microtiter plate every 15 min for 24 h at 37 ºC using the Spark 20M multimode Microplate Reader controlled by SparkControlTM software Version 2.1 (Tecan Group Ltd., Männedorf, Switzerland). Both HHQ and PQS synthetic standard (Sigma-Aldrich) at a final concentration of 5 µM, used as positive controls, were added to 1 in 100 dilution of the HAQ biosensor strain, as both activate bioluminescence production. The recorded bioluminescence as relative light units (R.L.U) were normalized to OD580nm of cultures suspensions.

Membrane fluidity measurement by fluorescence anisotropy

Fluorescence anisotropy analysis of *P. aeruginosa* cells were performed as previously described (Fléchard at al., 2018). Briefly, cell pellets of *P. aeruginosa* H103 untreated (control DMSO 1%) and treated to 100, 50, 25, 12.5, 6.25, 3.12, 1.6 and 0.8 µg $mL^{-1}$ of PLFE1 were washed two times (7500 × g, 5 min, 25 °C) in 0.01 M $MgSO_4$ and resuspended in the same wash solution to reach an OD580nm of 0.1. One µL of a 0.004 M of 1,6-diphenyl-1,3,5-hexatriene (DPH) stock solution (Sigma-Aldrich) in tetrahydrofuran was added to 1 mL aliquot of the resuspended cultures and incubated in the dark for 30 min at 37 °C to allow the probe to incorporate into the cytoplasmic membrane. Measurement of the fluorescence polarization was performed using the Spark 20M multimode Microplate Reader, equipped with an active temperature regulation system (Te-CoolTM, Tecan Group Ltd., Männedorf, Switzerland). Excitation and emission wavelengths were set to 365 nm and 425 nm, respectively, and the Fluorescence Anisotropy (FA) was calculated according to Lakowicz. Three measurements were performed for each sample and data were recorded using SparkControlTM software (Version 2.1, Tecan Group Ltd., Männedorf, Switzerland). The relationship between fluorescence polarization and membrane fluidity is an inverse one, where increasing anisotropy values correspond to a more rigid membrane and vice versa. All values are reported as means of triplicate analyses for each experimental variable.

Transcriptional fusion PsigX::*luxCDABE* construction and sigX promoter activity analysis in response to *P. lentiscus* L. fruit extract

The promoter region of the *sigX* gene (P*sigX*) was amplified by PCR with primers PsigX-SacI-F and PsigX-SpeI-R (Table S4, Supporting Information) incorporating SacI and SpeI linkers, respectively. The promoter region of *sigX* gene was fused to the *luxCDABE* cassette in the promoterless pAB133 vector (Bazire et al., 2010). Upon amplification, DNA was digested with the appropriate restriction enzymes, and ligated to pAB133 for the construction of pAB-P*sigX*. The pAB-P*sigX* plasmid was then transformed separately into one shotTM *E. coli* TOP10 competent cells (Invitrogen, Carlsbad, CA). The constructions were confirmed by DNA sequencing. Finally, the plasmids were transferred by electroporation into *P. aeruginosa* H103. Promoter activity was analysed by monitoring bioluminescence during course time.

Reverse transcription-quantitative PCR analysis (RT-qPCR)

Total RNAs from three independent H103 untreated and treated cultures with **PLFE1** at 100, 25, and 6.25 µg $mL^{-1}$ were isolated by the hot acid-phenol method (Bouffartigues et al., 2012) followed by treatment with Turbo DNA-free$^{TM}$ kit (Invitrogen) according to the manufacturer's protocol. Synthesis of cDNAs and RT-qPCR were achieved as previously described (Bouffartigues et al., 2015) using the oligonucleotides listed in Table S4 (Supporting Information). The mRNAs levels were calculated by comparing the threshold cycles (Ct) of target genes with those of control sample groups and the relative quantification was measured using the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001) using DataAssist$^{TM}$ software (Applied Biosystems).

*P. lentiscus* L. fruit extract (**PLFE1**) fractionation

Two hundred mg of **PFLE1** were subjected to fractionation by medium pressure liquid chromatography (MPLC) with 9.6 mg of silice 60 M with cyclohexane/ethyl acetate as mobile phase solvent mixture with increasing polarity. A total of 13 fractions were recovered and designated **PLFE1**-(**1**-**13**) (Table S2, Supporting Information).

Structure determination of compounds present in PLFE1-(2-4) fractions by NMR and ozonolysis

To determine the chemical structure of the main compounds, present in **PLFE1**-(**2**-**4**) fractions, $^{1}D$ and $^{2}D$ NMR experiments were conducted in a Bruker 400 MHz spectrometer apparatus (Wissembourg, France) and recorded in deuterated chloroform ($CDCl_3$). To identify the double bond position of the main compound identified in **PLFE1-2** fraction, an ozonolysis reaction was carried out. Briefly, a solution of 10 mg (0.027 mmol, 1.0 equiv) of **PLFE1-2** was prepared in a round bottom flask containing 20 mL of distilled dichloromethane and a small amount of Sudan III. A stream of ozone ($O_3$) was bubbled into the solution at -78 °C until the pink solution became colourless (10 min). Then, 200 µL of dimethyl sulphide ($(CH_3)_2S$) (2.7 mmol, 100 equiv) were

added to the reaction mixture and stirred for 1 h at 20 °C. From the reaction mixture, 1 µl was directly analysed by GC-MS.

Gas chromatography-mass spectrometry (GC-MS) analyses

The GC-MS analyses were carried out in a Hewlett Packard GC 6890/MSD 5972 apparatus (manufacturer, country) equipped with HP-5 (30 m x 0.25 mm x 0.25 µm). Carried gas was Ar with a flow of 0.9 mL min-1, split 1:20, oven was programmed increasing from 180°C to 270°C at 8°C $min^{-1}$ with an initial and final hold of 1 and 65 min, respectively. The inlet and GC-MS interface temperature were kept at 240 °C. The temperature of EI (electron impact) 70 eV was 220°C with full scan (80-500 *m/z*). The injection volume was 1 µL. Identification of constituents was achieved by comparing their mass spectra with those of the Mass Spectra Library (NIST 98) compounds. The major compound resulted from ozonolysis was nonanal.

Statistical analyses

Statistical significance was evaluated using Prism GraphPad online tool (https://www.graphpad.com/quickcalcs/ttest1/). The data were statistically analysed using two-sample unpaired two-tailed t test to calculate P values. The mean with standard error of the mean (SEM) were calculated and plotted. *C. elegans* survival curves were prepared using GraphPad Prism 8 free trial (GraphPad Software, Inc., San Diego, CA) to perform a statistical log-rank (Mantel-Cox) test.

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**Supporting Information**

| H103 cells | Normalized Live Events/µl | Normalized Injuried Events/µl | Normalized Dead Events/µl |
|---|---|---|---|
| Untreated | 0.963 ± 0.007 | 0.006 ± 0.001 | 0.029 ± 0.007 |
| Treated with **PLFE1** (µg/ml) | | | |
| 100 | 0.965 ± 0.006[a] | 0.005 ± 0.001[a] | 0.029 ± 0.007[a] |
| 50 | 0.955 ± 0.003[a] | 0.008 ± 0.002[a] | 0.037 ± 0.002[a] |
| 25 | 0.958 ± 0.001[a] | 0.006 ± 0.001[a] | 0.035 ± 0.002[a] |
| 12.5 | 0.960 ± 0.002[a] | 0.005 ± 0.000[a] | 0.034 ± 0.002[a] |
| 6.25 | 0.953 ± 0.006[a] | 0.005 ± 0.001[a] | 0.042 ± 0.007[a] |
| 3.12 | 0.952 ± 0.002[a] | 0.005 ± 0.001[a] | 0.043 ± 0.002[a] |
| 1.6 | 0.951 ± 0.002[a] | 0.005 ± 0.001[a] | 0.043 ± 0.002[a] |
| 0.8 | 0.941 ± 0.006[a] | 0.006 ± 0.001[a] | 0.053 ± 0.006[a] |

[a] Not Significant ($P \geq 0.05$)

**Table S1.** Effect of **PLFE1** on cell viability as determined by flow cytometry.

| Code | Mass (mg) | Percentage (m/m, %) |
|---|---|---|
| **PLFE1-1** | 3.0 | 1.5 |
| **PLFE1-2** | 35.5 | 17.8 |
| **PLFE1-3** | 16.5 | 8.2 |
| **PLFE1-4** | 16.2 | 8.1 |
| **PLFE1-5** | 16.8 | 8.4 |
| **PLFE1-6** | 9.6 | 4.8 |
| **PLFE1-7** | 4.2 | 2.1 |
| **PLFE1-8** | 21.3 | 10.6 |
| **PLFE1-9** | 5.3 | 2.6 |
| **PLFE1-10** | 1.2 | 0.6 |
| **PLFE1-11** | 8.7 | 4.4 |
| **PLFE1-12** | 6.1 | 3.0 |
| **PLFE1-13** | 2.9 | 1.4 |

**Table S2**. Fractions of PLFE1 obtained from MPLC

| Plant material | Date of collection | Place | GPS position | Code | Solvent | Percent Yield |
|---|---|---|---|---|---|---|
| *P. lentiscus* L. fruit (PLF) | 20/09/2016 | Wilaya Jijel (Algeria) | 36° 43' 25.248" N 5° 52' 22.4148" E | **PLFE1** | Cyclohexane | 2.7 |
| | | | | **PLFE2** | Ethyl acetate | 0.6 |
| | | | | **PLFE3** | Methanol | 18.2 |
| | | | | **PLFE4** | Water | 8.3 |

**Table S3.** Collection and preparation of *P. lentiscus* L. fruit extracts

| PA number | Gene name | Primer name | Sequence (5' > 3')[a] | Length |
|---|---|---|---|---|
| Construction of the transcriptional fusion pAB-P*sigX* | | | | |
| *PA1775-PA1776* | *cmpX-sigX* | P*sigX*-*SacI*-F | taataa-**GAGCTC-**gagtcgctcggcctgca | 29 |
| | | P*sigX*-*SpeI*-R | taataaa-**CTAGTG-**gtggaacagctccgagtgcg | 33 |
| Quantification of mRNA levels by RT-qPCR | | | | |
| *PA4210* | *phzA* | *phzA-F* | AACCACTACATCCATTCCTTCG | 22 |
| | | *phzA-R* | CGGCTATTCCCAATGCAC | 18 |
| *PA0051* | *phzH* | *phzH-F* | CGCGGGTTGGGTGGAT | 16 |
| | | *phzH-R* | ATGACCGATACGCTCGCC | 18 |
| *PA4209* | *phzM* | *phzM-F* | GCTGCGCGTAATTTGATACAAG | 22 |
| | | *phzM-R* | GATCCCGCTCTCGATCAGATC | 21 |
| *PA4217* | *phzS* | *phzS-F* | CCTGCGCGAATACGAAGAAG | 20 |
| | | *phzS-R* | CGGCCCATTCCTCTTTTTC | 19 |
| *PA0996* | *pqsA* | *pqsA-F* | CGGAGTTGCTGGCATTGC | 18 |
| | | *pqsA-R* | CTGTTGCCCATGCCATAGC | 19 |
| *PA2587* | *pqsH* | *pqsH-F* | CTCCATCGTGCAGATCCT | 18 |
| | | *pqsH-R* | CGGAATGACGCAAGGTC | 17 |
| *PA4190* | *pqsL* | *pqsL-F* | CGGTATCGCCTCCTACGTG | 19 |
| | | *pqsL-R* | GGAAGCTCACCACCAGTCG | 19 |
| *PA1003* | *pqsR* | *pqsR-F* | AACCTGGAAATCGACCTGTG | 20 |
| | | *pqsR-R* | TGAAATCGTCGAGCAGTACG | 20 |
| *PA1776* | *sigX* | *sigX-F* | AATTGATGCGGCGTTACCA | 19 |
| | | *sigX-R* | CCAGGTAGCGGGCACAGA | 18 |
| *PA3639* | *accA* | *accA-F* | TCTTCGGCAATCTGACCAGTT | 21 |
| | | *accA-R* | GTAGCCGATGTAGTCGAGGGTA | 22 |
| *PA4847* | *accB* | *accB-F* | AAGCCATGAAGATGATGAACC | 21 |
| | | *accB-R* | CGTTCTCCACCAGGATCGA | 19 |
| *PA5174* | *fabY* | *fabY-F* | AGGGCGACCTGGAGATCAT | 19 |
| | | *fabY-R* | GCGCGTCCTTCTTGTATACCA | 21 |
| | *16S* | *16S-F* | AACCTGGGAACTGCATCCAA | 20 |
| | | *16S-R* | CTTCGCCACTGGTGTTCCTT | 20 |

**Table S4.** List of primers used in this study

[a]All the primers used in this study were synthesized by Eurogentec and are based on *P. aeruginosa* PAO1 genome sequence (http://www.pseudomonas.com). Bold nucleotides indicate restriction endonuclease sites inserted within primer sequences.

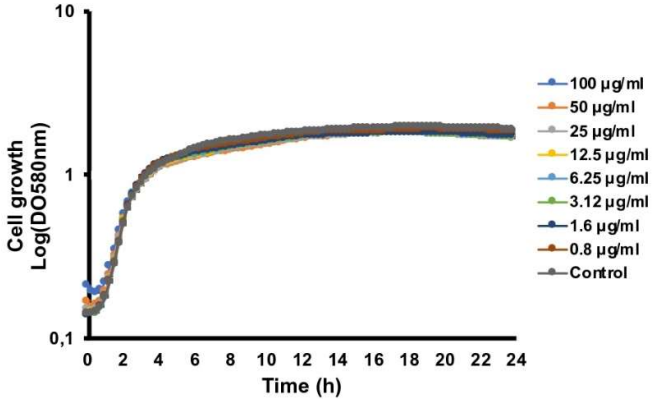


**Figure S1A**: Growth kinetics of *P. aeruginosa* strain H103 treated with *P. lentiscus* L. fruit cyclohexane extract (**PLFE1**) at various concentrations compared to the growth in the control condition (H103 untreated). Each point indicates the mean (± SEM) of OD580nm values.

***NMR and MS data of isolated compounds:***

Structural elucidation of isolated compounds was achieved by comparison of their experimental spectroscopy ($^{1}$H, $^{13}$C NMR) and spectrometry (MS) data with those previously reported in the literature.

1) Ginkgolic acid C17:1

Colorless oil. $^{1}$H NMR ($CDCl_3$): 7.36 [t, *J* = 8.2 Hz, 1H, H-4], 6.88 [d, *J* = 8.3 Hz, 1H, H-5], 6.78 [d, *J* = 8.3 Hz, 1H, H-3], 5.36 [t, *J* = 4.6 Hz, 2H, H-8', H-9'], 2.98 [t, *J* = 7.7 Hz, 1H, H-1'], 2.08-2.00 [m, 4H, H-7', H-10'], 1.67-1.59 [m, 2H, H2'], 1.31-1.25 [m, 20H, H-3'-H6', H-11'-H16'], 0.88 [t, *J* = 6.1 Hz, 3H, H-17']. $^{13}$C NMR ($CDCl_3$): δ 175.5 (C-1''), 163.6 (C6), 147.7 (C-2), 135.4 (C-4), 122.7 (C-5), 115.9 (C-3), 110.4 (C-1), 130.0 (C-8'), 129.8 (C-9'), 36.5 (C-1'), 32.0 (C-2'), 29.0 (C-3'-C-6', C-11'-C-16'), 27.2 (C-7'), 26.9 (C-10'), 14.1 (C-17'). ESI-MS *m/z* 375.5 $[M+H]^+$ (calculated for $C_{24}H_{38}O_3$: *m/z* 374.5).

2) Ginkgolic acid C15:0

Colorless oil. 1H NMR (CDCl3): 7.31 [t, *J* = 8.0 Hz, 1H, H-4], 6.84 [d, *J* = 8.0 Hz, 1H, H-5], 6.75 [d, *J* = 8.0 Hz, 1H, H-3], 2.94 [t, *J* = 7.9 Hz, 2H, H-1'], 1.59-1.49 [m 2H, H-2'], 1-20-1.38 [m, 24H], 0.88 [t, *J* = 6.5 Hz, 3H, H-15´]. $^{13}$C NMR ($CDCl_3$): δ 175.0 (C-1''), 163.0 (C-2), 147.6 (C-6), 135.0 (C-4), 122.7 (C-5), 115.7 (C-3), 111.2 (C-1), 36.3 (C-1'), 32.1 (C-2'), 31.9 (C-3'), 29.4-29.8 (C-4'-C-14'), 14.1 (C-15'). ESI-MS *m/z* 349.5 $[M+H]^+$ (calculated for $C_{22}H_{36}O_3$: *m/z* 348.5).

## Conclusion, contribution and perspectives

This investigation presents the antivirulence proprieties of an apolar extracts of *P. lentiscus* fruits, translated in the virulence attenuation in *in vitro* and *in vivo* models. This activity may be exerted by the presence of ginkgolic acid derivatives present in the cyclohexane extract of *P. lentiscus* fruits. The study demonstrates the antivirulence effects of gingkolics acids derivatives.

Deeper studies must be carried out to understand the elements of structure-activity relationship of ginkgolic acid derivatives. Explore the effect of double position, instauration degree, aliphatic chain length or aromatic substitution pattern on the antivirulence activity must be envisaged together with biological molecular assays to better comprehend the mechanistic action of ginkgolic acids derivatives.

Personal contribution:

- Consecutive extraction by PSE in different solvents (cyclohexane, ethyl acetate, methanol and water) of *P. lentiscus* fruits.
- Fractionation of **PLFE1** by medium performed liquid chromatography (MPLC) with solvent mixture of cyclohexane/ethyl acetate.
- NMR and LC-MS analysis of more important fractions obtained by MPLC.
- Ozonolysis of fraction **PLFE1-2** to determinate the double bond position of ginkgolic acid derivative.

# II.5 Molecular networking analysis and antioxidant, anti-inflammatory and tyrosinase inhibitory proprieties of *P. lentiscus* L collected in Algeria and Greece

## II.5.1 Molecular networking approach as new analytical tool to dereplication and metabolomics

In natural products research, dereplication, i.e. identification of known compounds without prior isolation or purification, is a strategy that allows to shorten time, efforts and costs in the process of complex matrix analysis. Current dereplication strategies includes hyphenated techniques such as HPLC-UV-MS, HPLC-NMR or HPLC-NMR-MS. These approaches are based on the fact that structurally related or identical molecules share similar physical properties including UV-vis profiles, MS, NMR chemical shifts or chromatographic retention times. However, level of confidence remains limited in comparison with assessment of full set of physical data with already published ones.

MS is more sensitive than NMR, however it is difficult to dereplicate a given compound due to the huge number of results corresponding to one exact mass in databases (Dictionary of Natural Products, SciFinder, Reaxys…). MS/MS fragmentation is much more characteristic of a molecule and more discriminatory than parent MS alone. The MS/MS fragmentation pattern depends of the structure and occurrence of functional groups and of their reactivity in collision-induced dissociation in gas phase.

Mass spectrometry-based molecular networking relies on the observation that structurally similar molecules share similar MS/MS fragmentation patterns. Moreover, similar isolated compounds, sharing similar MS/MS fragmentation patterns, molecular families trend to cluster together within the network. Thus, molecular networking not only dereplicate known compounds, from natural products complex matrixes, also is captures related analogues, in cases potential new structures, allowing to identify new natural products.

Mass spectrometry-based molecular networking relies on the observation that structurally similar metabolites share similar MS/MS fragmentation patterns and clusters can be established within a network. Related analogues, even with unreported structures, may also be connected enabling the identification of putative original compounds.

Molecular networking strategy is a complementary approach of dereplication and could be divided in four steps:

- Data acquisition: collection of MS/MS spectra are acquired often in UPLC-MS coupled systems with Orbitrap or qToF (time-of-flight) MS spectrometers.
- MGF file creation: the treatment of data collected is essential to generate the corresponding MGF file, with the MS, MS/MS, charge and retention time information for each peak detected in the LC-MS chromatograms.
- Molecular networking generation: the molecular network is generated in the on-line open free access platform GNPS (Global Natural Products Social Molecular Networking), setting different parameters to generate the respective molecular networking. In the same platform is possible to match the experimental obtained MS/MS spectra with several on-line open access MS/MS fragmentation databases to identify compounds present in the natural product mixture.
- Analysis of molecular network: finally, deeper analysis and customize of originated molecular networking is possible with Cytoscape software.

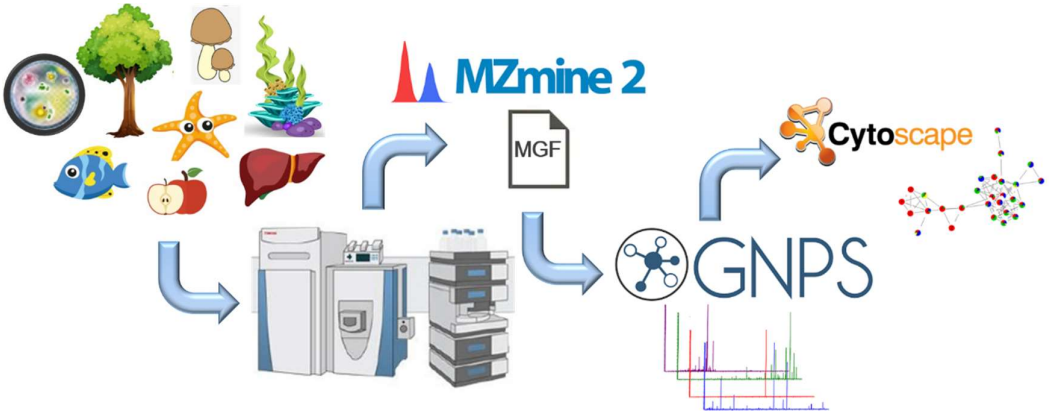


**Figure 75: General workflow in the creation of molecular networking.**

## II.5.2 HPTLC analysis

High performance thin layer chromatography (HPTLC) is an analytical tool, derived from thin layer chromatography, which provides better separation and resolution. The superior efficiency Each step is achieved by automatization (sample deposing, plate elution and scanning, visualization, treatment) and controlled by an integrative software.




**Figure 76: Different elements of HPTLC system.**

The plates are also different than those used for TLC, with lower mean particle size, particle distribution and absorption and fluorescence detection limits. Consequently, HPTLC systems are often quick and efficient tools for quantitative and qualitative analyses.

## II.5.3 Tyrosinase inhibitors as skin whitening agents

Tirosinase is a metal-containing enzyme with monophenolase and diphenolase activities, present in plant, fungi and animal tissues that catalysed the production of melanin and other pigments. Melanin is mainly responsible for the pigmentation of human skin, eyes and hair. Tyrosinase is found inside melanosomes, which are produced in epidermis melanocytes. In response to ultraviolet B (UVB)-irradiation, melanocytes synthesized melanin through the process called melanogenesis. The synthetized melanin in melasomes is transported to neighbouring keratinocytes in epidermis. Under normal physiological conditions, melanin production, has a beneficial effect on the photo-protection of human skin against UV injuries and exerts an important role in evolutionary camouflage and animal mimicry [132].

However, overproduction of melanin causes dermatological disorders as melisma, freckles and solar lentigo as well as post inflammatory melanoderma and cancer.

The melanogenesis process is started with the oxidation of *L*-tyrosine to dopaquinone by the key enzyme tyrosinase in two steps (mono oxidation in *o* position and oxidation of *o*-diphenol to o-quinone). The resulting dopaquinone will serve as substrate for the synthesis of eumelanin and pheomelanin. After dopaquinone formation, intramolecular cyclization is carried out, producing leukodopachrome. The redox exchange between leukodopachrome and dopaquinone generate dopachrome and L-DOPA, which is also a substrate for tyrosinase and oxidized to dopaquinone again by the enzyme. Decarboxylation of produced dopachrome release dihydroxyindole (DHI) and tautomerization by tyrosinase-related protein-2 (TRP-2) produce dihydroxyindole-2-carboxylicacid (DHICA). DHI and leukodopachrome are late re-oxidised to indole-5,6-quinone and indole-5,6-quinone-carboxilic acid. These two quinone with DHICA are oxidized to eumelanin. Tyrosinase-related protein-1 (TRP-1) is believed to catalyse the oxidation of DHICA to eumelanin. The biosynthesis of pheomelanin is initiated by the nucleophilic addition of

glutathione or cysteine to dopaquinone, producing cysteinyldopa or glutothionyldopa. Intramolecular cyclization gives benzothiazine intermediates which after subsequent oxidation produce pheomelanin [132,133].

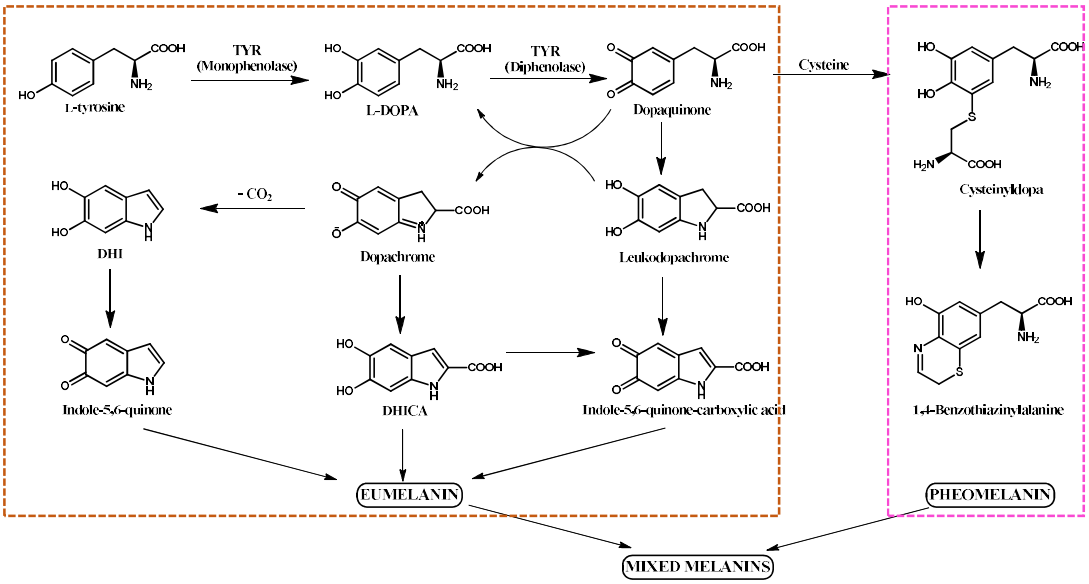


**Figure 77: Biosynthetic pathway of melanin.**

Tyrosinase inhibitors are major skin whitening agents in the cosmetic markets. Estimations predict that the worldwide market for such derivatives will reach US$ 23 billion in 2020 [134].

## II.5.4 Methodology

### II.5.4.1 Antioxidant/anti-inflammatory

The antioxidant and anti-inflammatory activities protocols applied in this section have been previously described in point **I.5.2**.

### II.5.4.2 Tyrosinase inhibition assay

*In vitro* colorimetric assay was performed with tyrosinase from the mushroom *Agaricus bisporus* (common mushroom), due to the commercial availability. The inhibitory activity of investigated substance (plant extract, fraction or pure compound) is measured by conversion of L-DOPA to dopaquinone by mushroom tyrosinase, monitored at 405 nm [135].

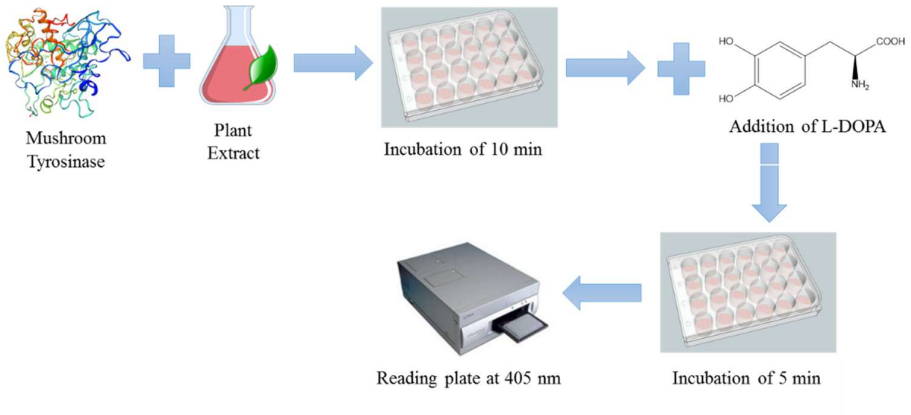


**Figure 78: Workflow of mushroom tyrosinase inhibitory activity assay by measuring the conversion of L-DOPA to dopaquinone at 405 nm.**

## II.4.2.3 Dereplication by molecular networking

Molecular networking allows dereplication and visualization of complex matrixes such as vegetal extracts. After acquisition, LC-MS/MS data are pooled in as network of clusters. Each cluster is made of nodes and each nodes represents a hit in the LC-MS/MS analysis with an associated fragmentation. The interconnection of nodes in each cluster is based on the similarities of each fragmentation pattern. Thus, structurally-related compounds could be linked within the cluster. During the process of molecular networking generation, it is possible to dereplicate the data by comparison of MS/MS spectra with those available in on-line open data bases. Process details are described in section **II.5.1**.

In section **II.5.5**, molecular networking approach was applied to analyse (dereplication and representation) two *P. lentiscus* fruits samples collected in Greece and Algeria, before and after ripening, together with their antioxidant, anti-inflammatory and antityrosinase activities. The workflow was as follows:

- Collection of unripe and ripe fruits of *P. lentiscus* in Greece and Algeria in 2016.
- Successive extraction in cyclohexane, ethyl acetate, methanol and water of dried and ground fruit.
- SPE fractionation of methanolic extracts.
- Analysis of extracts and fractions:
    - HPTLC and UPLC-MS analysis of raw extracts and fractions.

    - Molecular networking analysis by workflow described in section **II.4.2.3** and **II.6.1**
- Antioxidant evaluation of extracts:
    - Radical scavenging activities against DPPH and ABTS by colorimetric assays.
    - Quantification of polyphenol, flavonoids and flavon-3-ol equivalents in extracts and/or fractions by colorimetric assays.
- Anti-inflammatory activity of extracts by reduction of production of pro-inflammatory mediators (PGE2 and IL-6) after incubation of LPS-stimulated monocytes.
- Antityrosinase activity of samples by colorimetric assay, measuring the conversion of L-DOPA to dopaquinone at 405 nm by mushroom tyrosinase.

## II.5.5 Draft 5: Comparison of metabolite profiles and biological activities of two Mediterranean varieties of *Pistacia lentiscus* L. fruits

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### 1. INTRODUCCION

*Pistacia lentiscus* L. (Anacardiaceae) is widespread all around the Mediterranean basin. Medicinal uses are described since Antiquity. However, they may differ either in the therapeutic indications or in the plant part, depending of the geographical area. In Greece, the resin ("mastic gum") of *Pistacia lentiscus* var. chia endemic to Chios island and is mainly used in dentistery and in case of gastrointestinal disorders. In Algeria, Morocco and Tunisia, the fruits and seeds oil is indicated for the treatment of respiratory tract infections and in ointments for articular pain, burns and ulcer. Essential oil from leaves is also used in aromatherapy.

In recent years, molecular networking (MN) has been developed as a complementary technic for the dereplication of extracts, enabling to shorten time and costs of analysis. This approach is based on the comparison of experimental MS/MS fragmentation pattern with those of known metabolites available in online databases.

In this study, MN was applied in order to decipher the composition of two samples of ripe and unripe fruits of *P. lentiscus* harvested in Algeria and in Greece, together with their antioxidant (radical scavenging activity and estimation of flavonoid and polyphenol contents), anti-inflammatory (PGE2 and IL-6 pro-inflammatory mediators production of LPS-stimulated monocytes) and tyrosinase inhibition activities.

## 2. MATERIAL AND METHODS

### 2.1 General

SPECTROstar Nano BMG LabTech UV/vis spectrometer was used for colorimetric and enzymatic inhibition assays. Methanol (MeOH), dimethylsulfoxide (DMSO), ethanol, (EtOH), dichloromethane (DCM), gallic acid, quercetin, Folin-Ciocalteu reagent, $AlCl_3 \cdot 6H_2O$, $Na_2CO_3$, $Na_2HPO_4$, $NaH_2PO_4$, tyrosinase enzyme from mushroom, L-3,4-dihydroxyphenylalanine, kojic acid, $CH_3COONa$, lipopolysaccharides (LPS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ABTS were purchased from Sigma Aldrich (St. Lous, USA). SPE Sep-Pak Vac 3cc C18 cartridges were purchased from Waters (Milford, Massachusetts, USA). HPTLC normal and reverse phase glass plates (20 cm × 10 cm) pre-coated with silica gel60 F254were purchased from Merck (Darmstadt, Germany). Phosphate buffered saline (PBS) was purchased from Invitrogen, Carlsbad, California, USA). Enzyme immunoassay (EIA) kit for PGE2 quantification was purchased from Biotrend (Köln, Germany) and the enzyme-linked immunosorbent assay (ELISA) KITS for IL1-α, IL-β and IL-6 quantification were purchased from HISS (Frieburg, Germany).

### 2.2 Plant collect and preparation of extracts

Fruits from *P. lentiscus* from Algeria and *P. lentiscus* var. chia were collected before and after ripening in 2016 (**Table 1)**. Voucher specimens are available at the herbarium of NPAS. Raw material was freeze-dried for 48 h, then ground.

#### 2.2.1 ASE extractions

Extract were obtained by PSE (Pressurized Solvent Extraction) extractor (E-914, Büchi, Flawil, Switzerland). Extraction cells were filled with the dried mashed fruits and charged in the apparatus. Subsequent extraction program consisted in two distinct cycles of 20 min with cyclohexane, ethyl acetate, methanol and water. Each cycle comprised a pre-heat period of 5 min, pumping of corresponding solvent through the extraction cell unit at a final pressure of 100 bar and at 50 °C, a hold-on time fixed at 5 min and a final flush of extract into the corresponding vial. Subsequent extraction using cyclohexane, ethyl acetate, methanol and water were performed for each fruits sample.

| Collection Places of *Pistacia lentiscus* fruits | | | | |
|---|---|---|---|---|
| Country | Specie | Date | Location | GPS Position |
| Algeria | *P. lentiscus* L. | 20-09-2016 | Willaya Jijel | 36° 43' 25.24" N<br>5° 52' 22.41" E |
| Greece (Chios) | *P. lentiscus* var. chia (L.) | 30-09-2016 | Willaya Jijel | 36° 43' 25.24" N<br>5° 52' 22.41" E |
| | | 06-10-2016 | Katarakis | 38°15'56,31" N<br>26°05'43,15" E |
| | | 10-10-2016 | Sikilia | 38°16'10,27" N<br>26°03'52,66" E |
| | | 10-10-2016 | Cadiz Stone | 38°15'14,60" N<br>26°01'56,89" E |
| | | 10-10-2016 | Kalamoti | 38°14'18,29" N<br>26°02'46,57" E |

**Table 1**: Collection location and dates of *P. lentiscus* fruits.

2.2.2 Polyphenol extraction and SPE fractionation

Five grams of lyophilized fruits were subjected into a polyphenol extraction by maceration with 75 mL of EtOH/$H_2O$/HCl – 50/50/0.01. Three cycles of 30 min were performed. Supernatants were recovered together after each cycle, solvent removed under reduced pressure and lyophilized. The extracts obtained were re-suspended in MeOH/$H_2O$ – 1/1 to achieve a concentration of 50 mg/mL. Then, 3 mL of these solution were subjected to SPE fractionation using C18 cartridges and $H_2O$/HCl – 1/0.01 (20 mL), EtOAc (40 mL), MeOH (20 mL) and MeOH/HCl – 1/0.01 (10 mL) consecutively as eluents. Each fraction was recovered, solvent removed under reduced pressure and lyophilized.

2.2.3 Sample preparation

For biological tests, a solution of 10 mg/mL in DMSO and stocked at -4 °C until evaluation. For HPTLC and LC-MS experiments, solution at the same concentration were prepared in MeOH and stored and -20 °C until evaluation.

2.3 Metabolite Profile

2.3.1 HPTLC

HPTLC instrumental set-up was purchased from Camag (Muttenz, Switzerland).The system was operating under winCats 1.4.9 Planar Chromatography Manager software (Camag). The sample application volume was set to10µL and the band length was set at 8 mm. The application position was set at 10 mm from the bot-tom of the plate. HPTLC plates were developed in a presaturated glass chamber (20 min presaturation) up to 80 mm height. For cyclohexane and ethyl acetate extracts, normal phase plate was used and eluted in two systems of $CH_2Cl_2$/MeOH/HCOOH at 95/05/1 and 90/10/1. For methanolic extracts, for normal phase plate, eluent of AcOEt/MeOH/H2O/Formic Acid – 50/10/7/1 was used and for reverse phase plate, eluent of $H_2O$/MeOH/AcOH – 50/50/1 was used. Plates were subsequently air-dried and viewed in the photo-documentation chamber and the images were captured at 254 nm, 366 nm and at white light after derivatisation with vanillin–sulphuric acid reagent.

2.3.2 UHPLC-MS

The LTQ-Orbitrap XL mass spectrometer (Thermo Scientific (Bremen), Bremen, Germany) was used for the LC-MS study. The analysis was performed in positive and negative ion mode with a mass range of m/z 100–1100. The optimized ESI parameters were set as follows: capillary temperature of 250 ◦C; gas (nitrogen) flow of 30 arb.; auxiliary gas (nitrogen) flow of 10 arb.; source voltage of 4.25 kV; capillary voltage of 25 V; tube lens voltage of 110 V. The resolution of the Orbitrap mass analyser was set at 30,000. The isolation width was 2 amu, and the normalized collision energy (CE) was set to 10, 30 and 50 V. All instruments were controlled by the Xcalibur data system, and the data acquisition was carried out by analyst software Xcalibur (version 2.1) (Waltham, MA, USA) from Thermo Electron Corp. Chromatography separation was carried out in a XSelect ® Phenyl-Hexyl 130Å, 2.5 µm, 4.6 mm x 50 mm column with ACN (0.1% formic acid, A) and $H_2O$ (0.1% formic acid, B) as solvents. After 2 min with 93% of B, gradient to 70 % of B was applied during 10 min, then to 10% of B by 5 min and finally at 93% during 5 min.

2.3.3 Molecular Networking

Molecular networking of metabolites in *P. lentiscus* fruit extracts was created using the online wokflow at GNPS (http://gnps.ucsd.edu). The raw data was pre-treated with MZmine 2.0 software. Noise level was settled as $3.0x10^{+4}$ and the *m/z* tolerance as 0.008 *m/z* (or 8.0 ppm). Files .csv and .mgf were created and uploaded to GNPS platform. The data were then clustered with a parent mass tolerance of 0.02 Da and a MS/MS fragment ion tolerance of 0.02. Minimum matched fragment ion was settled as 2. Costumization of resulted networking was performed in Cytoscape software.

2.4 Antioxidant and anti-inflammatory evaluation

2.4.1 Total phenol content (TPC)

Total polyphenol content were estimated according to Folin-Ciocalteu (F-C) reagent method as previously reported with slight modification (Clarke, Ting, Wiart, & Fry, 2013). Briefly, in a 96 well plate, 25 µL of sample solution at 4 mg/mL were mixed with 125 µL of F-C reagent freshly diluted 1/10 with distilled water. After 5 min, 100 µL the solution was mixed with 100 µL of a solution 7.5 % of $Na_2CO_3$, left for 30 min in the dark and the absorbance was measured at 650 nm. Respective standard curve of gallic acid and blank (replacing F-C reagent with 125 µL of water) were run simultaneously, after which the TPC was calculated as mg of gallic acid equivalent per g of extract.

2.4.2 Total flavonoid evaluation (TFE)

Total flavonoid evaluation were performed according to (Bampouli et al., 2015). Briefly, in a 96-well plate, 50 µL of sample solution at 4 mg/mL were mixed with 160 µL of EtOH, 20 µL of 1.8 % of $AlCl_3$ and 20 µL of 0.8 % of $CH_3COONa$. Then, the plates were kept at room temperature for 40 min and the absorbance was measured at 415 nm. Respective standard curve of quercetin and blank (replacing $AlCl_3$ with 20 µL of $H_2O$ distilled) were run simultaneously, after which the TFE was calculated as mg of quercetin equivalent per g of extract.

2.4.3 Total flavon-3-ol content

The total flavon-3-ol equivalent quantification assay was measured by the 4-(dimethylamino)cinnamaldehyde (DMAC) assay as previous reported (Feliciano et al., 2012). Briefly, an acidified ethanol solution was prepared by adding 12.5 mL of hydrochloric acid (37%) to 12.5 mL of deionized water and 75 mL of ethanol. A 0.1%

(w/v) DMAC reagent solution was prepared immediately prior to use. In a 96-well plate, 70 microliters of blanks (80% ethanol), calibration standards, and samples was dispensed into individual wells. A multichannel pipet was used to dispense 210 µL of DMAC reagent solution into each well, excluding blanks. Immediately after the addition of DMAC reagent, the plate was placed into a plate reader and absorbance readings at 640 nm were taken after 8 min of incubation at room temperature protected by light. The corresponding blank absorbance readings at each time point were subtracted from those from the standard and sample wells. Catechin was used as standard and results are expressed mg of catechin equivalent per g of extract.

2.4.3 DPPH Scavenging Assay

Radical scavenging capacity was measured using DPPH agent according to previously reported (Skotti et al., 2014). DPPH reagent solution (12.4 mg in 100 mL of EtOH) was prepared freshly just before the assay. Extract were evaluated at a final concentration of 200 µg/mL. In each well were placed 10 µL of sample solution with 190 µL of DPPH solution. Plates were incubated at room temperature by 30 min protected from light. The absorbance were measured at 517 nm. Measured were performed by triplicated. Gallic acid was used as positive control. Results are expressed as percentage of efficacy at 200 µg/mL of extract.

2.4.4 ABTS Scavenging Assay

Radical scavenging capacity was measured using ABTS reagent according to previous reported (Skotti et al., 2014). ABTS reagent was prepared as follows: 10 mL of 7 mM of ABTS solution were mixed with 164 µL of 140 mM of $K_2S_2O_8$ and stored overnight (16 h). The final solution was diluted with distilled $H_2O$ (1:20) to reach an absorbance of 0.70 ± 0.2 before the assay. Extract were evaluated at a final concentration of 200 µg/mL. In each well were placed 100 µL ABTS final solution and 50 µL of sample. Plated were incubated at room temperature by 10 min protected from light. The absorbance were measured at 734 nm. Measured were performed by triplicated. Quercetin was used as positive control. Results are expressed as percentage of efficacy at 200 µg/mL.

2.4.5 Tyrosinase inhibition assay

The ability of extract to inhibit the tyrosinase enzyme was performed in 96-well plates as previously reports (Chaita, Gikas, & Aligiannis, 2017). Briefly, phosphate-buffered saline

were prepared in order to dilute enzyme, substrate, samples and positive blanks as follows: 8.26 g of $Na_2HPO_4$ with 4.94 g of $NaH_2PO_4$ diluted in 1 L of $H_2O$, final pH of 6.7. In each plate were placed 40 µL of sample diluted in PBS (initial concentration 1,5 mg/mL, final concentration 300 µg/mL, final DMSO~3%), 80 µL of PBS and 40 µL of mushroom tyrosinase enzyme (initial concentration 92 U/mL in PBS). The plates were incubated at room temperature protected from light for 10 min and 40 µL of L-DOPA (L-3,4-dihydroxyphenylalanine solution at 0.5 mM in PBS) were added and re-incubated for 5 min in the same conditions as before. Negative (replacing the samples with 40 µL of PBS) and positive control were performed in parallel (replacing the sample with 40 µL of kojic acid solution at 10 µg/mL initial concentration, final concentration in the well of 2 µg/mL with 50% of inhibition). Blank of each measured were made. The absorbance were measured at 475 nm. The percentage of inhibition were calculated as follows: [(A - B) - (C - D)]/(A - B) × 100, where A is the Control [without (w/o) sample], B the Blank (w/o sample, w/o tyrosinase), C the Sample, D the Blank Sample (w/o tyrosinase).

2.5 Cytotoxic and anti-inflammatory activity

2.5.1 Isolation of human peripheral monocytes and cell culture

Monocytes from healthy human donors were prepared following a standardized protocol using a completely endotoxin-free cultivation (English & Andersen, 1974; Noble, Cutts, & Carroll, 1968). By using 50 mL tubes, 25 mL Ficoll was loaded with 25 mL blood of buffy coats from healthy blood donors. The gradient was established by centrifugation at 1800 rpm, 20 °C for 40 min by using slow acceleration and brakes. Peripheral blood mononuclear cells in the interface were carefully removed and re-suspended in 50 mL pre-warmed PBS followed by centrifugation for 10 min at 1600 rpm and 20 °C. The supernatant was discarded and the pellet washed in 50 mL PBS and centrifuged as described above. The pellet was then re-suspended in 50 mL RPMI-1640 low endotoxin medium supplemented with 10% human serum (PAA). After counting the amount of cells in a particle counter (Euro Diagnostics, Krefeld), the cells were seeded in 24-well plates and incubated at 37 °C and 5% $CO_2$. The medium and the non-adherent cells (lymphocytes) were removed and fresh RPMI-1640 medium containing 1% human serum was added. The monocytes were ready for use in the experiments.

2.5.2 Pro-inflammatory mediator production

Measurements of the cytokine IL-6, and PGE2 were performed using commercially available EIAs (PGE2) and ELISA (IL-6) kits. The monocytes obtained from isolation step, were seeded in 24-well plates at concentration of 70,000 cell/mL and incubated overnight at 37 °C under 5% $CO_2$. After incubation, 2.5 µL of respective extract solution were added per triplicate to obtained final concentration of 100 and 10 µg/mL per well (final DMSO concentration 0.25%). Then 30 min prior to stimulation with LPS (10 ng/mL) the cells were at 37 °C and 5% $CO_2$ for 24 h and then, the supernatants were removed, centrifuged and investigated for IL-6 and PGE2 concentrations. Data were expressed as a percentage of the LPS control values and the mean SE calculated.

2.5.3 Alamar blue viability Assay

Briefly, 100 µL of monocytes cell suspension (50,000 cell/mL) were seeded in a 96-well plate and incubated overnight at 37 °C at 5% of $CO_2$ atmosphere. Then, 100 µL of extract solution at several concentrations (400 – 1 µg/mL) diluted in medium (final DMSO concentration of 0.25%) were added and incubated for 24 h at same conditions. After, 20 µL of alamarBlue™ reagent were added, incubated for 2 h and absorbance recorded at 570 nm. Results are expressed as percentage in relation of respective blanks.

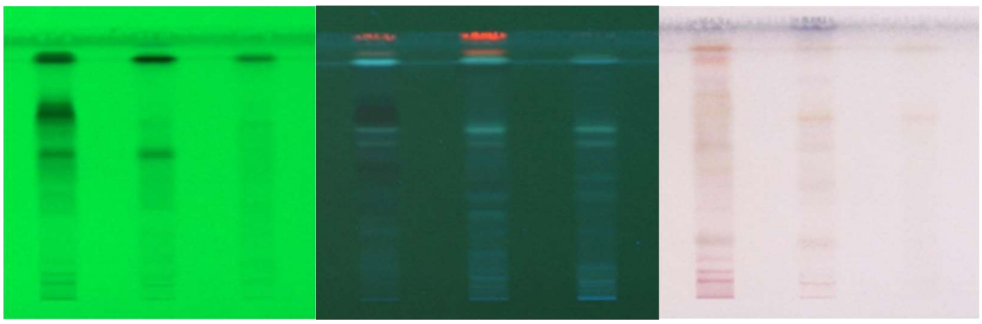

Figure 1: HPTLC profile of **3**, **7** and **11** extracts eluted with system AcOEt/MeOH/$H_2O$/Formic Acid – 50/10/7/1 and revealed at 254 and 366 nm and after vanillin-sulfuric acid derivatization at white light.

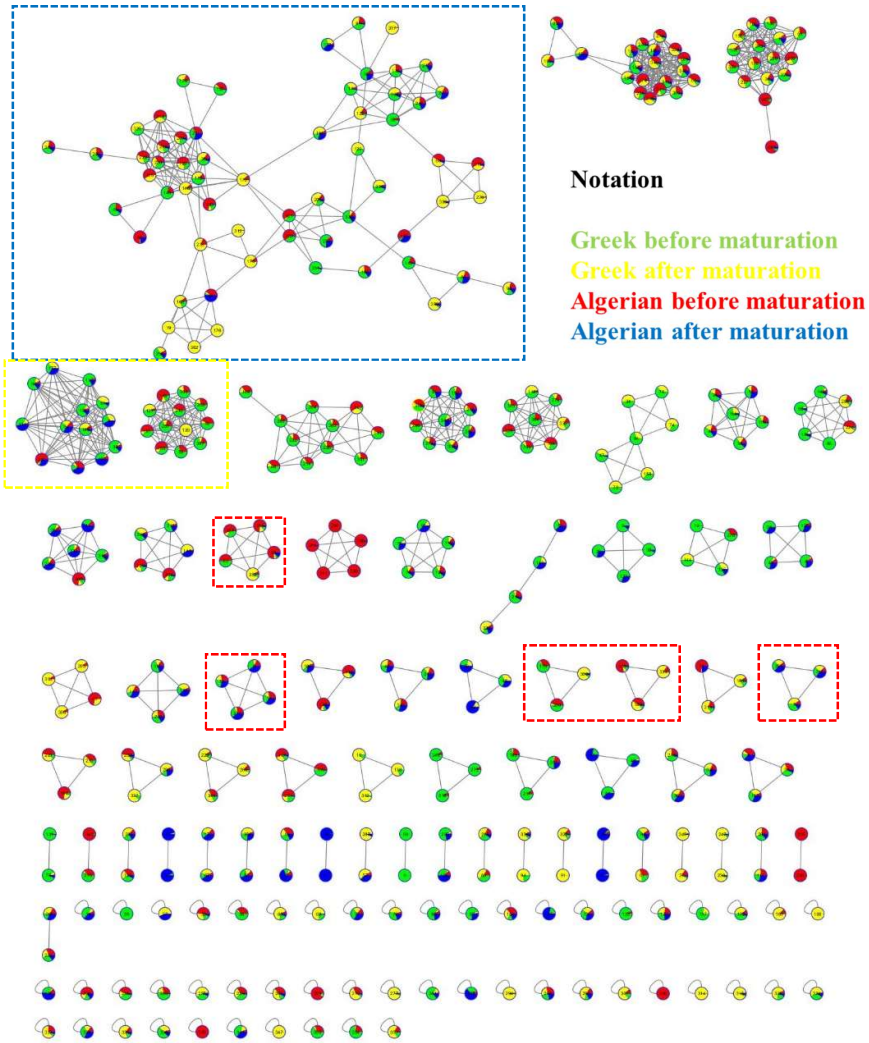


**Figure 2**: Molecular networking of methanolic extracts **3** (green, Greek before maturation), **7** (red, Algerian before maturation), **11** (blue, Algerian after maturation) and Greek after maturation (yellow). In blue box, phenolic acid cluster; yellow box, flavonoids cluster; and red boxes, cluster with gallic acid ester derivatives.

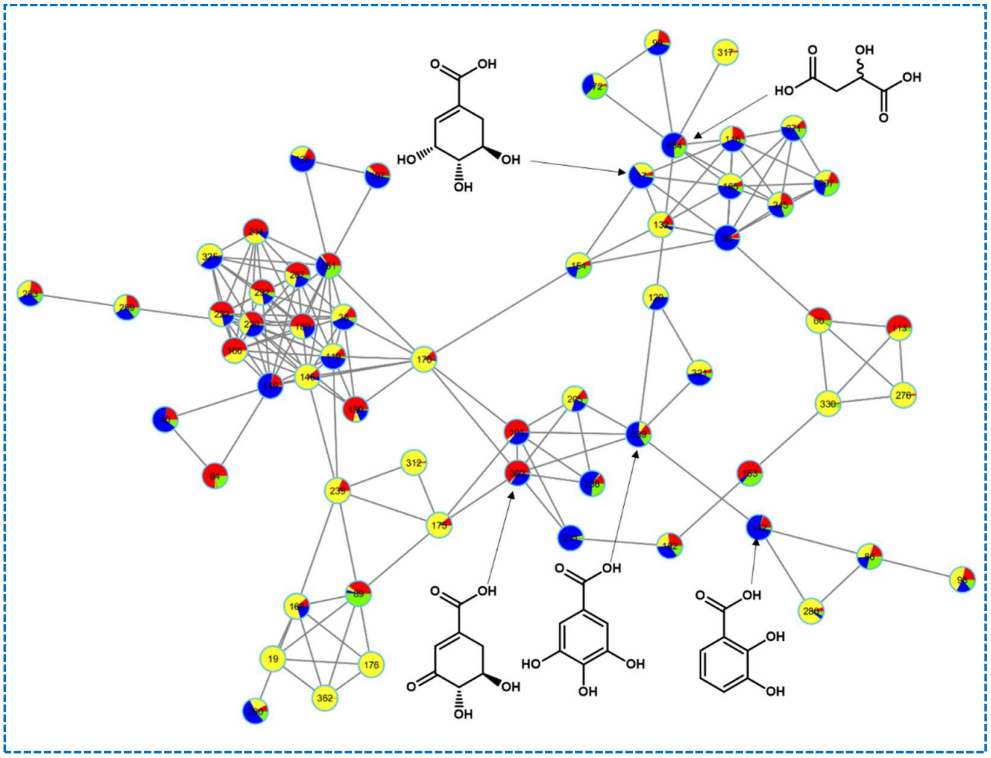

**Figure 3**: Molecular cluster of phenolic acids with identified structure of gallic and quinic acid derivatives.

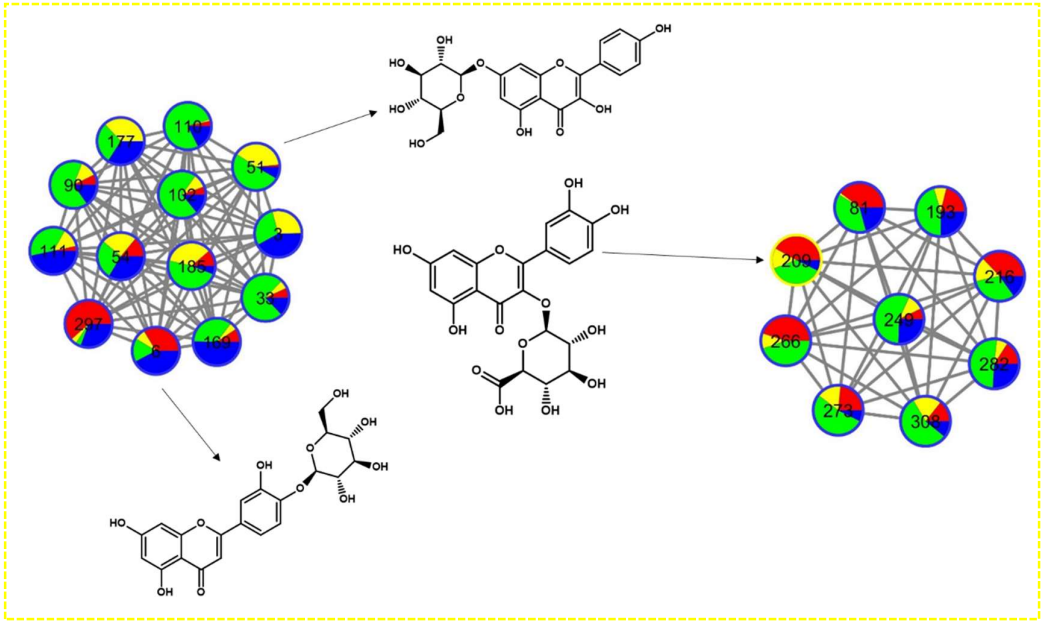

**Figure 4**: Molecular cluster with flavonoids derivatives identified.

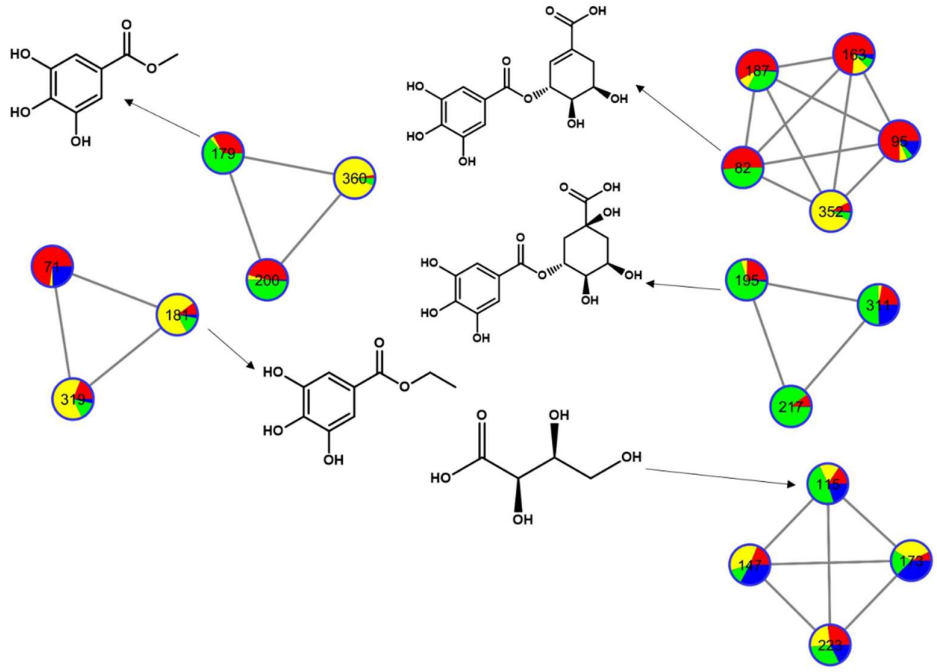


**Figure 5**: Divers molecular cluster with gallic acid ester and threonic acid derivatives identified.

| | | | | Antioxidant and antienzymatic activity of *P. lentiscus* fruits extracts | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *P. lentiscus* samples | | Solvent of extraction | Code | TPC[a] | TFE[b] | PAC[c] | DPPH[d] | ABTS[e] | TA[f] |
| ASE Extracts | Greece before maturation | | Cyclohexane | **1** | 19,6 | 4,8 | | 4,8 | 16,5 | -1,1 |
| | | | EtOAc | **2** | 28,0 | 14,4 | | 32,9 | 95,1 | 16,2 |
| | | | MeOH | **3** | **113,9** | 17,9 | | **95,2** | **99,8** | **80,6** |
| | | | $H_2O$ | **4** | **126,8** | -9,6 | | **91,7** | **99,8** | **90,8** |
| | Algeria before maturation | | Cyclohexane | **5** | 19,5 | 7,3 | | 2,8 | 15,3 | 29,1 |
| | | | EtOAc | **6** | **272,8** | 24,6 | | **95,2** | **100,2** | 46,9 |
| | | | MeOH | **7** | 58,8 | 4,9 | | **94,5** | **100,1** | 26,9 |
| | | | $H_2O$ | **8** | 66,5 | 6,5 | | **91,9** | **99,9** | 32,8 |
| | Algeria after maturation | | Cyclohexane | **9** | 2,9 | -0,1 | | 3,1 | 6,1 | -14,9 |
| | | | EtOAc | **10** | 10,2 | 0,2 | | 22,2 | 55,6 | -7,9 |
| | | | MeOH | **11** | **67,2** | 7,4 | | **74,3** | **99,6** | 39,6 |
| | | | $H_2O$ | **12** | **74,6** | 6,7 | | **92,6** | **99,9** | 52,9 |
| SPE Extracts | Algeria | | $H_2O$/HCl | **13** | | 23,1 | 7,4 | 94,1 | 93,3 | |
| | | | MEOH | **14** | | -0,6 | 1,8 | 46,9 | 16,6 | |
| | | | MEOH/HCl | **15** | | 0,8 | 0,1 | 94,5 | 99,7 | |
| | Greece | | $H_2O$/HCl | **16** | | 4,7 | 2,5 | 92,8 | 82,0 | |
| | | | MEOH | **17** | | 3,2 | 11,6 | 96,8 | 99,4 | |
| | | | MEOH/HCl | **18** | | 2,9 | 49,6 | 92,9 | 99,4 | |

**Table 1**: Antioxidant and antienzymatic activity of *P. lentiscus* (Algeria) and *P. lentiscus* var. chia (Greece) fruits extracts. a) Total Polyphenolic Content (TPC) mg of gallic acid equivalent per g of extract. b) Total Flavonoid Equivalent (TFE) mg of quercetin equivalent per g of extract. c) Proanthocyanidins Equivalent Content: mg of catechin equivalent per g of extract. d) Percentage of DPPH scavenging at 200 µg/mL of extract. e) Percentage of ABTS scavenging at 20 µg/mL of extract. f) Percentage of inhibition of tyrosinase enzyme at 100 µg/mL of extract.

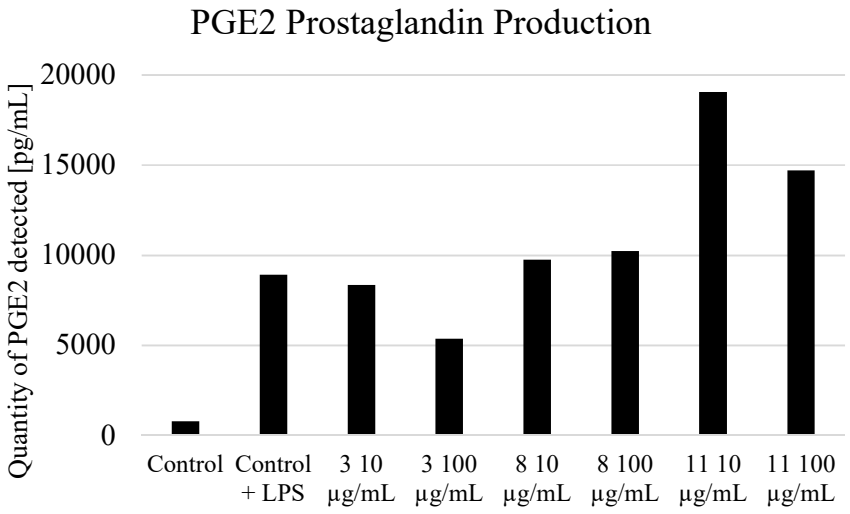


**Figure 6**: PGE2 production by LPS-stimulated monocytes after incubation with extracts **3**, **8** and **11** at 10 and 100 µg/mL.

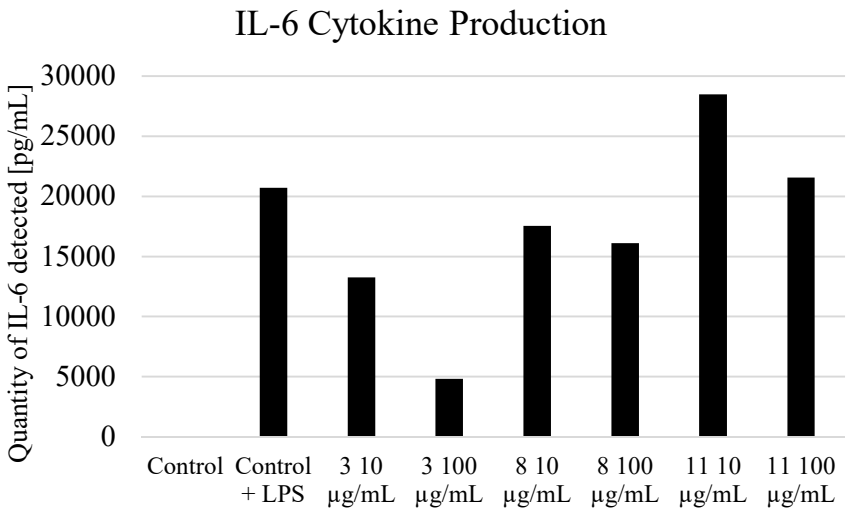


**Figure 7**: IL-6 production by LPS-stimulated monocytes after incubation with extracts **3**, **8** and **11** at 10 and 100 µg/mL.

## 3. RESULTS AND DISCUSSION

### 3.1. Antioxidant activity

Regarding the antioxidant activity, the strongest scavenging effects (DPPH and ABTS) were recorded for the methanolic and aqueous extracts **3**, **4**, **6-8**, **11-13, 15-18** (75-100%). Polyphenols content follows the same trend for extracts **3**, **4**, **6**, **7**, **8**, **11** and **12** from 58.8 to 272.9 mg of gallic acid equivalents per g of extract. Flavonoids equivalents were

detected at noticeable levels only for **13**. These data are in accordance with the radical scavenging activity of polyphenols substances widely reported in the literature (Clarke et al., 2013).

Tyrosinase inhibition activity was very strong for the polar extracts **3** and **4** and moderate for **6**, **11** and **12**. Correlation with the polyphenol content quantification should be underlined (over 75 mg per g of extract). Indeed, polyphenols have previously been reported as tyrosinase inhibitors (Zolghadri et al., 2019). This subject will be further discussed in section 3.3.

3.2. Anti-inflammatory activity

Reduction of pro-inflammatory mediators (PGE2 and IL-6) after LPS stimulation on monocytes cells was recorded for extract **3** mainly considering IL-6 cytokine (75% and 30% at 100 and 10 µg/mL, respectively). A low inhibition of IL-6 production was detected for **8** only at 100 µg/mL. Extract **11** has demonstrated pro-inflammatory effect inducing increased quantities of PGE2 and IL-6 mediators after incubation with LPS-stimulated monocytes.

3.3. Dereplication and Molecular Networking analysis

Molecular networks of methanolic extracts of ripe and unripe fruits collected in Greece and Algeria were established (**Figures 2-5**). Dereplication process has allowed the putative identification of 13 compounds (phenolic acids, flavonoids and gallic acid esters) by MS/MS fragmentation pattern comparison with open databases.

Gallic acid, 3-dehydroshikimic acid, 2,3-dihydroxybenzoic acid, shikimic acid and malic acid have been identified in the phenolic acids cluster (blue). These compounds have been found in larger amount in Greek ripe fruits, except for 3-dehydroshikimic acid, which was found in higher quantity in the Algerian unripe fruits. Only low levels of flavonoids have been detected in all of the extracts, identified as kaempferol and quercetin glycosides. Gallic acid esters (methyl, ethyl, quinic and shikimic acid derivatives) have been also identified in smaller clusters.

The major compounds detected by UPLC-MS analysis were methyl gallate and 5-galloylshikimic acid, in both samples of unripe fruits **3** and **7** while ethyl gallate was the main metabolite in ripe fruits extracts **11**. All of these compounds have been already identified from *Pistacia* species.

The nodes of methyl gallate is included in a network with nodes 200 and 360, both with similar MS value (*m/z* 183.0064) but with different retention times (RT = 6.7 and 9.9 min, respectively). Examination of MS/MS spectra showed also a fragmentation ion at 168.0064 m/z -which may arise from the loss of a methyle group- but with various intensities. These nodes may represent isomers of methyl gallic acid, where the methoxyle group could be located in different positions, inducing changes in polarity (increasing the retention time) and in the intensity of fragmentation.

Methyl and ethyl gallate have demonstrated strong radical scavenging activities (DPPH) with $IC_{50}$ of 2.9 and 3.8 µg/mL, respectively (Kyeong Ko et al., 2011). This could explain the free radical scavenging activity determined for the methanolic extracts. Moreover, methyl gallate has moderately inhibited xanthine oxidase with an $IC_{50}$ of 60 µM contributing to the antioxidant potential of extracts where this compound was detected in high amount.

Furthermore, methyl gallate has also inhibited tyrosinase with $IC_{50}$ values of 40.5 - 64.4 µg/mL (Kyeong Ko et al., 2011, Kubo et al., 2000). However, in deeper studies, ester derivatives of gallic acid with short chains ($C_1$-$C_3$) acted as inhibitors and substrate for tyrosinase enzyme. The oxidised product of this reaction presented yellowish colour, observable in in vitro assays (Kubo et al., 2000).

Additionally, methyl gallate was also able to inhibit cyclooxygenase-2 (COX-2), reducing the generation of the pro-inflammatory mediators prostaglandin $D_2$ (PGD2) and leukotriene $C_4$ ($LTC_4$) in bone marrow-derived mice cells with $IC_{50}$ of 17.0 µM and 5.3 µM. The same compound also reduced the catalytic conversion of arachidonic acid to PGD2 in a free-cell assay ($IC_{50}$ of 19.0 µM). COX-2 protein expression was not reduced by incubation with various concentrations of methyl gallate, indicating a direct enzyme inhibition (Kim et al., 2006). These data may explain the reduction of PGE2 production by action of extract **3**, where methyl gallate was detected as main constituent.

## CONCLUSION

In this work, molecular networking analytical tool was applied for the visualization and dereplication of methanolic extracts of *P. lentiscus* fruits, resulting in the identification of 13 compounds and of the determination of major compounds of each samples. Antioxidant, anti-inflammatory and tyrosinase inhibition activities were determined for

this by-product of mastic gum industry, emphasizing its pharmaceutical and cosmetic potential

ACKNOWLEDGMENT

Authors would like to thank the H-2020 RISE program for funding and mobility of groups researchers involved in EXANDAS program.

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## Conclusion, contribution and perspectives

In the precedent work, Molecular Networking analytical tool was applied for the visualization and dereplication of methanolic extracts of *P. lentiscus* fruits, resulting in the identification of 13 compounds, counting the major compounds of samples. Together, antioxidant, anti-inflammatory and antityrosinase activities are reported of *P. lentiscus* fruits, plant part that are not been exploited in the mastic gum industry, emphasizing the pharmaceutical and cosmetological interest *P. lentiscus* fruits may have.

Personal contribution:

- Collection of *P. lentiscus* var. chia fruits in September 2016 (Chios, Greece).
- Extract preparation of Greek samples.
- HPTLC and Molecular Networking analysis of extracts.
- Antioxidant and tyrosinase inhibition assay.
- Redaction of original draft manuscript.

# Chapter III Exploration of the inhibition of the $P2X_7$ receptor in inflammatory disorders by European medicinal plants.

# III.1 Introduction

As explained in point **I.3.5.1**, inflammation represents a fundamental biological process that stands at the foreground of a large number of acute and chronic pathological conditions. The inflammatory process is closely related to the oxidative stress and can start by pathogen infections or associated toxins. One of these mechanisms involves the negative $K^+$ gradient, produced by $K^+$ channel opening, bacterial pore toxin formation or efflux by $P2X_7$ receptor activation. The activation of $P2X_7$ receptor is stimulated by extracellular adenosine triphosphate (ATP), which is massively released by stressed or dying cells. The subsequent purigenic activated signaling pathway is involved in a variety of pathological conditions, including inflammatory diseases such as lateral and multiple sclerosis, Alzheimer's and Parkinson's diseases and rheumatoid arthritis.

Consequently, therapeutic relevance of $P2X_7$ receptors has increased in the last decade. In this chapter, we have explored the inhibitory activity of $P2X_7$ receptors by European medicinal plants, traditionally used to treat several inflammatory diseases. These investigations were followed by statistical analysis of active extracts in order to identify the active compounds, responsible for the antagonist activity on $P2X_7$ receptors.

## III.1.1 Role of $P2X_7$ receptor in the inflammatory process.

Extracellular ATP (eATP) is known to activate two classes of membrane-bound receptors; the P2Y (purigenic G protein-coupled receptors) with eight identified and cloned receptors in humans ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11-14}$) and seven P2X receptors (ATP-gated receptor cation channel). P2X receptors can form either functional homo- or heterotrimers. When three molecules of eATP bind to P2X in the extracellular domain, the channel open within milliseconds, allowing cations ($K^+$, $Na^+$ or $Ca^{2+}$) to flow. $P2X_7$ receptors, previously known as P2Z ($P2X_7R$), the most extensively studied receptors, differ in that they have extra amino acids in the intracellular C terminus, which appears to facilitate interactions with intracellular proteins. The binding of eATP to $P2X_7R$ within milliseconds induces the opening of a selective efflux of small cations (resulting in changes in the ionic homeostasis of the cell), and within seconds, a large pore opens, which allows permeation by molecules with a mass of up to 900 Da, leading the release of inflammation cytokines and apoptosis. $P2X_7R$ is widely expressed by different immune cells including monocytes, macrophages, neutrophils, lymphocytes, mast cells among others.

As previously described, during innate immune response, damage- or pathogen-associated molecular pattern (DAMPs or PAMPs) activate pattern recognition receptors (PRRs) inducing ATP release, which can activate $P2X_7R$. Furthermore, Toll-like receptors (TLRs) mediated NF-κβ pathway activation act as the first signal promoting the transcription of several genes encoding

pro-inflammatory mediators, including pro-IL-1β and inflammasome components, such as NLRP3 and ASC. $P2X_7R$ stimulation represent the second signal to inflammasome activation by triggering $K^+$ efflux, inflammasome assembly and subsequent caspase-1 activation. Inflammasome processes pro-IL-1β to its mature form and release to extracellular milieu, propagating the inflammatory response to neighbouring cells. In addition, $P2X_7R$ also promotes IL-6 in a $Ca^{2+}$ dependent mechanism and the activation of caspases and phospholipases.

IL-1β is a cytokine with major roles in inflammation, fibrosis and innate immune response. This cytokine induce the production of pro-inflammatory mediators (chemokines and cytokines, such as IL-6 and TNF-α) and proteases (such as matrix metalloproteinases) associated with neutrophil recruitment and proliferation of resident cells.

#### III.1.1.1 Musculoskeletal diseases

In *in vivo* studies, IL-1β levels induced via P2X7R-ATP stimulated were higher, compared with respective controls in blood samples from rheumatoid arthritis patients. Different polymorphic variants of the P2X7R with high or reduced periprosthetic osteolysis in the long-term complication of total hip arthroplasty due to osteoarthritis. Treatment with AZD9056, a selective P2X7R antagonist produced pain-relieving and anti-inflammatory effects in an osteoarthritis rat-model study. Arthritis animal *in vivo* models have provided evidence for the important role of P2X7R in the progression of this inflammatory disease. P2X7R KO mice showed reduced incidence and severity levels of anti-collagen-induced arthritis symptoms and blocking the P2X7R signalization pathways, inhibited the peripheral inflammatory tissue destruction. Thus targeting P2X7R could be a promising therapeutic treatment for rheumatism arthritis.

#### III.1.1.2 P2X7R and diseases of the CNS

P2X7R are expressed on nerve terminals, astrocytes and microglia, and they are upregulated various disease conditions. Exogenous stress signals as mechanical injuries or bacterial/chemical toxins, leading the release of ATP, activating the P2X7R on neighbouring cells. In nerve terminal and astrocytes, following the influx of cations through P2X7R activation, glutamate are released by both exocytotic and non-exocytotic mechanism, which may rise excitotoxicity and enhance of ROS production. In microglia, production of IL-1β and release of pro-inflammatory cytokines IL-6 and TNF-α are promoted after P2X7R activation by eATP. These mechanisms could be

manifested and contribute to disease pathology in Alzheimer, Parkinson, Huntington, lateral and multiple sclerosis, stroke, pain and mood disorders.

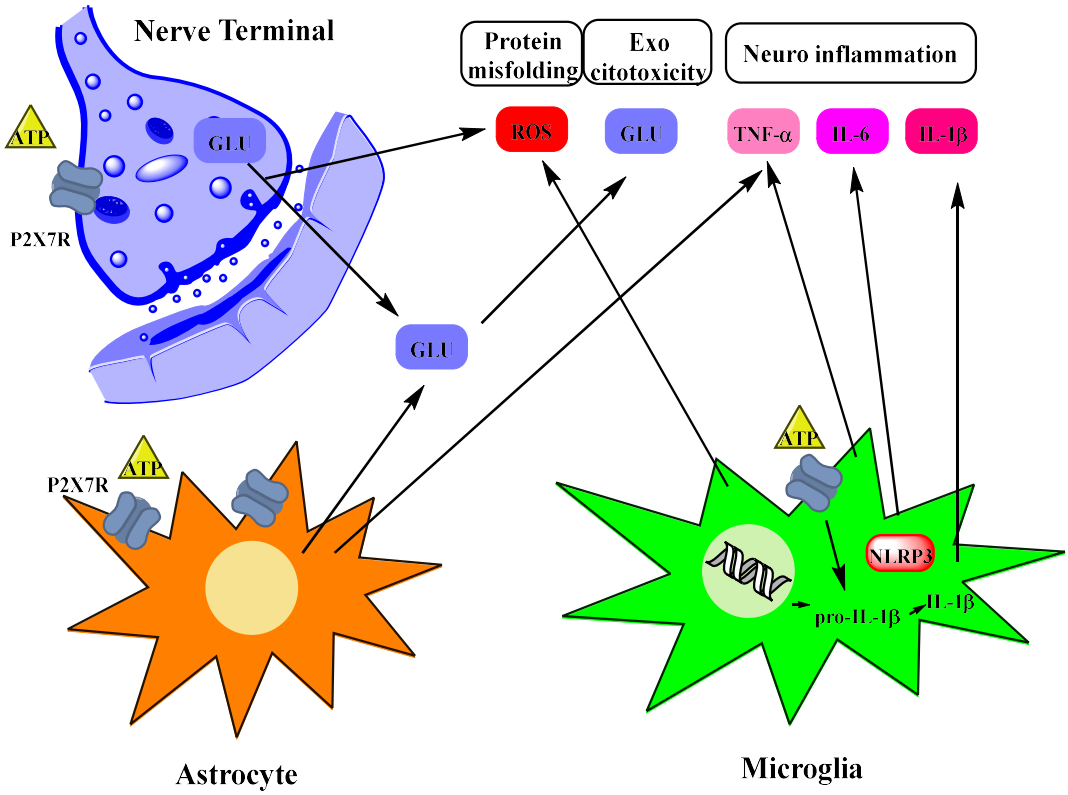


**Figure 79: P2X7R participation in common CNS disease mechanisms.**

## III.1.2 Medicinal plants from Europe with anti-inflammatory uses



**Figure 80: Anti-inflammatory medicinal plants used in the European tradition (*Achillea millefolium* and *Scrophularia nodosa*).**

Traditional uses of medicinal plants to treat several inflammatory disorder in Europe is large and widely documented in several scientific reports and also in local pharmacopeas [136,137]. In the framework of the exploration of the role of $P2X_7$ receptor in the inflammatory pathways, we selectioned medicinal plants with a large usage in the European tradition, such as *Achillea* spp. [138], *Artemisia* spp. [137], *Helichrysum* spp. [139], *Scrophularia* spp. [137], *Gentiana* spp.[140] and *Plantago* spp. [141]. They are known to synthetize metabolites with anti-inflammatory properties, such as sesquiterpene lactones (*Achillea*, *Artemisia* and *Helichrysum* genus) and iridoids (*Scrophularia*, *Gentiana* and *Plantago* genus) [142,143]

Excursions dedicated to plants collection were performed in collaboration with several Institutes in France and in Serbia in 2014-2015. In some cases, the same species was collected in France and in Serbia. Details are given in section **III. 4**.

## III.2 Personal work and collaboration

With the principal aim to explore the role of P2X7 receptor in the anti-inflammatory action of European medicinal plants, the followed shema of work was proposed (**Figure 80**). After collection of plant parts, all samples were subjected to extraction by ultrasound methodology and the resulted extracts were evaluated *in vitro* their antioxidant and anti-inflammatory activities.

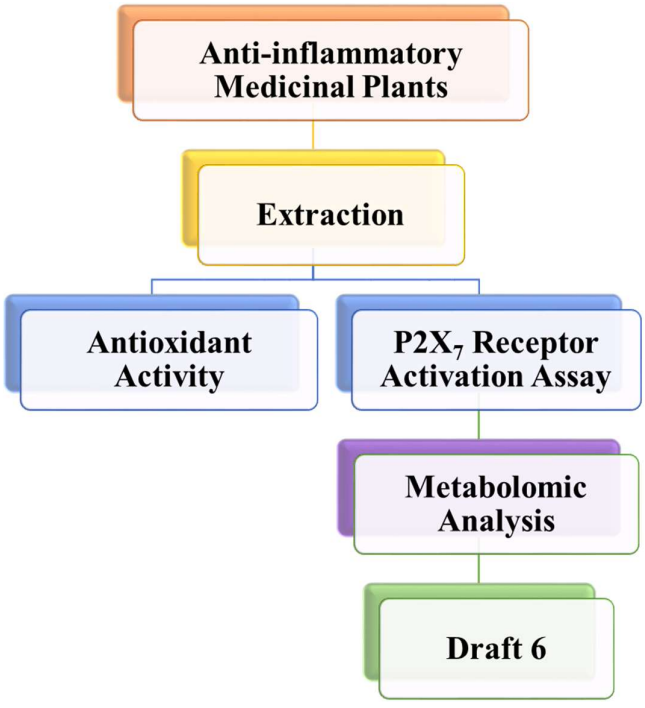


**Figure 81: General workflow of Chapter III.**

Then, *Scrophularia* plants focused our attention as they present active and inactive samples. Metabolomic analysis of their LC-MS data allowed to us to dereplicate the active extracts and identify the putative compounds, responsible of the biological activity.

Aiming at this, a collaboration was set up through the bilateral PHC program PAVLE-SAVIC between NPAS team and JOSIF PANCIC Institute in Serbia:

| Task | Responsible | Institution |
|---|---|---|
| Collection of Balkan samples, extraction, phytochemical analysis. | Dr. Gordana Zdunic | Josif Pancic Institute, Belgrade, Serbia |
| P2X7 receptor fluorescence assay | Pr. Patrice Rat, Dr France Massicot | Laboratoire de Toxicologie, Université de Paris, Paris, France |

**Table 9: Collaboration set up during execution of Chaper III.**

## III.2.1 General Objective

As previously described or principal objective in this project is:

- Evaluation of the role of $P2X_7$ receptor in the anti-inflammatory activity of medicinal plants used in Europe.

## III.2.1.1 Specific Objectives

- Collection of medicinal plants uses to treat inflammatory disorders in France and in the Balkans.
- Ultrasound extraction of collected material.
- Antioxidant and $P2X_7$ receptor antagonist effects *in vitro* assay activities evaluation of extracts.
- LC-MS analysis of all samples obtained.
- Dereplication analysis of more active extracts from Scrophularia genus.
- Metabolomic approach through statistical analysis in order to identify the responsible bioactive compounds.

# III.3 Role of $P2X_7$-receptor in the anti-inflammatory activity of medicinal plants

## III.3.1 Methodology.

### III.3.1.1 *In vitro* YO-PRO-1 fluorescent assay

YO-PRO-1 dye is a DNA probe, which enters specifically through P2X7R of apoptic cells and was utilised to measure the antagonist character of selected medicinal plant extracts. Pre-stimulated with LPS macrophages seeded in a 96-well plate were incubated with several extract concentration and incubated during 22 h at 37°C with 5% of $CO_2$ atmosphere. Then, 100 µL of YO-PRO-1 solution was added per well re-incubated for 10 min and fluorescence was measured ($\lambda_{excitation}$ = 491 nm, $\lambda_{emission}$ = 509 nm). Major fluorescence means major YO-PRO-1 passage and opening of P2X7 receptor, meanwhile minor fluorescence degree means lower YO-PRO-1 passage through P2X7 receptor may be due to an antagonist effect of evaluated extract.

### III.3.1.2 Principal component analysis (PCA)

LC-MS data of extracts was treated in order to create a .csv file including m/z value and intensity of each peak detected. Once the file was prepared, it was subjected into www.metaboanalyst.ca site web for its treatment. Statistical Analysis option was selected and the .csv file was upload as Peak intensity table as data type. Normalization of data set was applied by "pooled sample from group" option and the analysis were submitted.

Several analysis are displayed, such as PCA, PLSDA, sPLSDA, Heatmap, etc. We analyzed PCA in order to elucidate any correlation between the observed biological activities and the chemical consititution of extracts.

## III.3.2 Draft 6: Exploration of the $P2X_7$-receptor pathway involvement in the anti-inflammatory activity of medicinal plants from the European traditional medicine.

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Abstract

Numerous assessments of anti-inflammatory activity of plants used in folk medicine report their ability to decrease proinflammatory cytokines production through different models, or inhibit some key enzymes in cytokines or eicosanoids production. As purinergic receptor P2X7R is reported to be involved in inflammation process, and may be activated in case of tissue injury, we managed to evaluate the protective effect on P2X7R activation by extracts obtained from plants used as anti-inflammatory in European folk medicine. Our results revealed that among our selected plants, species from the Lamiids tribe, producing iridoids, were able to decrease significatively P2X7R activation. A focus on Scrophularia species allowed us to identify harpagoside as potent inhibitor of P2X7R activation. According to our knowledge, no evaluation of anti-inflammatory activity of medicinal plants based on inhibition of P2X7R activation has been reported until now.

Introduction

Use of medicinal plants in folk medicine to treat inflammatory disorders is widespread through Europe, most particularly for articular pain such as chronic arthritis (Barnes et al., 2007; Duke, 2002). From a chemotaxonomic point of view, these medicinal plants are mainly represented by species related to Asteraceae family, or attached to the Lamiales and Gentianales orders such as Plantaginaceae, Scrophulariaceae and Gentianaceae. The traditional use of herbal-based preparations obtained from species belonging to these families is well documented in the long

history of European cultures (Barnes et al., 2007; Duke, 2002). However, in many cases, efficacy is not evidence-based, the identification of bioactive metabolites and the subsequent mechanism of action remains unclear. Nevertheless, some *in vivo* and *in vitro* models are widely used to assess the anti-inflammatory activity of these traditional remedies.

*In vivo* assessment of anti-inflammatory activity of medicinal plant

Assessment of an anti-inflammatory activity is mainly based on a few in vivo assays of chemically induced inflammation. Models of induced oedemas are widely used as they reflect the most visible consequence of inflammation. Animals are exposed to chemical agents such as polysaccharides (carrageenan, dextran, lipopolysaccharide-LPS), phorbol esters derivatives (croton oil, tetradecanoyl phorbol acetate-TPA), physiological mediators of inflammation (arachidonic acid, serotonin, complete Freund's adjuvant CFA) or other irritant compounds such as dithranol, acetic acid. In these models of acute inflammation, the main outcomes of the studies is to evaluate the oedema volume. Anti-inflammatory activity is then related to an anti-oedematous activity (Bouclier et al., 1990; Crunkhorn and Meacock, 1971; Webb, 2014). These kind of assays were used to assess antiinflammatory activity of Asteraceae species used in folk medicine such as *Achillea millefolium*, *Artemisia vulgaris*, and *Helichrysum* species. *A. millefolium* aqueous extract reduced mouse and rat paw oedema, induced by yeast and various inflammatory substances, including histamine, carrageenan and prostaglandin (Goldberg et al., 1969; Shiposhliev et al., 1984). Methanolic extract of *Artemisia vulgaris*, showed anti-inflammatory activity in *in vivo* model of carrageenan-induced rat paw oedema (Ashok and Upadhyaya, 2013) , while apolar extract of *Helichrysum plicatum* was able to reduce carrageenan-induced rat paw oedema (Karaca et al., 2009). Extracts and isolated compounds from *H. italicum* showed antiinflammatory activity *in vitro* and in *in vivo* models. Polar extracts were able to reduce the oedema in mice ears induced by TPA and ethylphenylpropionate induced models. Several acetophenones as 12-hydroxytremetone-12-O-β-D-glucopyranoside, 3-(2-hydroxyethyl) acetophenone-4-O-β-D-glucopyranoside and maltol β-D-Oglucupyranoside, isolated from Helichrysum italicum reduced paw mice oedema induced by phospholipase A2 (PLA2). Flavonoids gnaphaliin, pinocembrin and tiliroside isolated from methanolic extract of *H. italicum* reduced paw mice oedema induced by PLA2 by 50% and serotonin by 40% (Antunes Viegas et al., 2014).

Regarding genera from the Lamiales and Gentianales orders, *Plantago*, *Scrophularia* and *Gentiana*, assessment of anti-inflammatory activity is also reported (Bakuridze et al., 1987; Pasdaran and Hamedi, 2017; Rønsted et al., 2000). *P. major* and *P. lanceolata* extracts showed antiphlogistic activity in carrageenan- and PGE1-induced inflammation in rats (Shipochliev et al., 1981). Acteoside, the main phenylethanoid glucoside from *P. lanceolate* inhibits arachidonic acid induced mouse ear oedema (Murai et al., 1995). Polar and apolar extracts from *G. lutea* rhizomes,

reduced oedema in a carrageenan-induced rat paw, xylol-induced mouse ear oedema and cotton pellet-induced chronic inflammatory models in dose-dependent form (Mathew et al., 2004).

*In vitro* inhibition of production of inflammation mediators

To clarify anti-inflammatory mechanism, *in vitro* inhibition of inflammation mediators may be measured indirectly by evaluation of enzymatic inhibition of inflammation key enzymes such as cyclooxygenases, lipoxygenases or metalloproteases, but also directly measuring cytokines production such as TNFα, IL-1, IL-6 (Koeberle and Werz, 2014). Deeper studies on signalling pathways involved in chronic inflammation are also described, such as inhibition of NF-κB transcription, activated by cytokines and oxygen reactive species, leading to iNOS transcription, NRLP3 inflammasome activation and subsequent IL-1β secretion.

Some extracts obtained from selected Asteraceae including *Artemisia* species, exert dual inhibition on both cyclooxygenase-1 (COX-1) and 5-lipoxygenase (LP-5) (Chagas-Paula et al., 2015; Tan et al., 1998). Sesquiterpene lactones isolated from *Achillea* spp. are able to inhibit serine protease human neutrophil elastase (HNE) and matrix metalloproteases MMP2- and -9, which are known to be involved in the inflammatory process (Benedek and Kopp, 2007). This mechanism is not very specific as it is well known that exomethylene-bearing sesquiterpene lactones (SLs) can act as Michael acceptor, reacting with biological nucleophiles, especially the sulfhydryl group of the cysteine residue of proteins. This pharmacophore is able to inhibit the transcription factor NF-κB by preventing the IκB-α and IκB-β degradration (Hehner et al., 1998). Such SLs were isolated from *A. vulgaris* (verlotorin, anhydroverlotorin and artemorin) and *A. absinthium* (3- hydroxyanabsinthin) (Aberham et al., 2010; Geissman, 1970). In addition, flavonoids from *Artemisia* species can inhibit phosphodiesterases and protein kinases, as well as production of cytokines and histamine release (Emerenciano et al., 2001a). Acetophenone from *Helichrysum italicum*, arzanol, inhibit the 5-LO and COX-1 enzymes ($IC_{50}$=8-2μM) as well as IL- 1β, TNFα, IL-6, IL-8 and PGE2 production in LPS-stimulated human peripheral monocytes and the NF-κB transcription (Antunes Viegas et al., 2014; Mao et al., 2017). Aqueous extract and flavonoids isolated from *H. arenarium* inhibited the expression of inflammatory factors as CRP, VEGF, JNK2 and p38 on A7r5 cell line (Czinner et al., 2000).

For the iridoids biosynthesizing species, the ability to reduce production of cytokines was also assessed. *P. major* polar extracts are able to reduce the generation of pro-inflammatory plasma cytokines (IL-1α, IL-1β and TNFα) levels in an acetaminophen-induced liver injury *in vivo* (Hussan et al., 2015; Samuelsen, 2000). In addition, hydroethanolic extract of *P. lanceolate* inhibited NO production and iNOS mRNA expression by LPS/IFN-γ- stimulated murine macrophages of the J774A.1 cell line (Stevenson et al., 2002). Iridoids and phenylethanol glycosides from *Scrophularia umbrosa* exerted NO inhibitory in mouse macrophages raw 264.7

cell line with $IC_{50}$ of 40-9 µM range (Han et al., 2018). Gentiopicroside from *Gentiana officinalis* and *G. lutea* showed analgesic effects involving the modulation of NR2B receptors in the anterior cingulate cortex (ACC) to persistent inflammatory pain (Chen L. et al., 2008). In addition, *G. lutea* extracts and its flavonoid isovitexin exhibit anti-atherosclerotic activities by blocking leukocyte adhesion and generation of reactive oxygen species in human umbilical vein endothelial cells (HUVECs) and rat aortic smooth muscle cells (RASMCs) and inhibiting TNFα induced endothelial inflammation (Kesavan et al., 2016).

Purinergic receptor $P2X_7R$ and inflammation

Beside these main signaling pathways involved in chronic inflammation, involvement of purinergic receptors P2X in inflammation is discussed. The ionotropic purinergic receptor $P2X_7R$ may be activated in case of excessive extracellular ATP concentrations, generally observed in a case of inflammation linked to local necrosis (Burnstock and Knight, 2018; Khalafalla et al., 2017). This activation leads to the opening of large pores formed by pannexin-1 (PanX1) proteins. The $P2X_7R$-Panx1 complex activation leads to potassium efflux and inflammasome activation associated with subsequent proinflammatory cytokines release (IL-1β and IL-18) (Burnstock and Knight, 2018; Khalafalla et al., 2017; Yue et al., 2017). Targeting $P2X_7R$ may be a potential treatment for rheumatismal arthritis or osteoarthritis, and $P2X_7R$ antagonists may produce pain relieving and reduce the destruction of inflammatory tissue (Gicquel et al., 2017). Until now, no evaluation of anti-inflammatory activity of medicinal plants based on inhibition of $P2X_7R$ activation has been described.

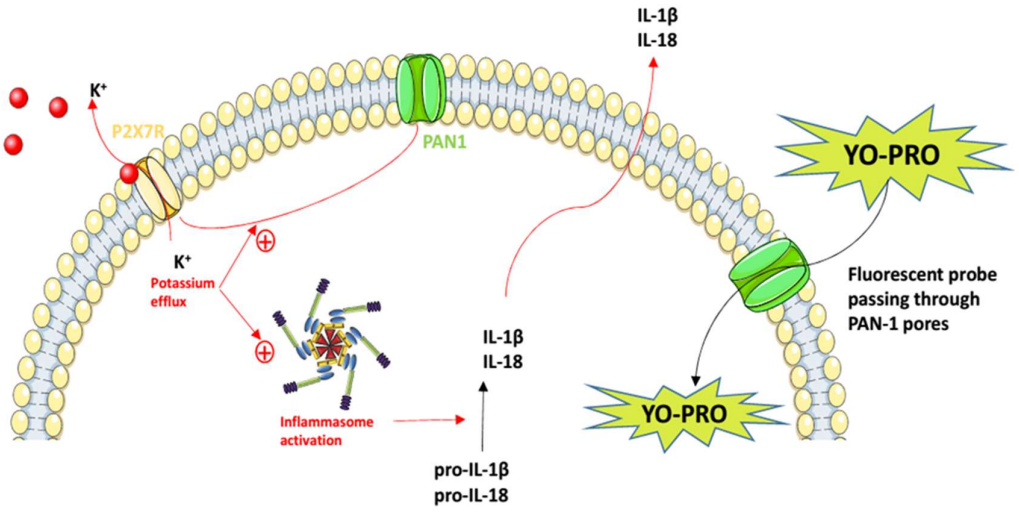


Figure 1: YO-PRO dye permeabilization subsequent to $P2X_7R$ activation.

Plants used in European folk medicine and $P2X_7R$ activation

In our study, we aimed to investigate the involvement of the $P2X_7R$-Panx1 complex in the anti-inflammatory mechanism of some selected medicinal plants used in European folk medicine for treating rheumatismal disorders, through the in vitro $P2X_7R$ activation bioassay using the YOPRO fluorescent dye, based on the permeabilizing effect of $P2X_7R$ activation (Figure 1) (Dekali et al., 2013; Dekali et al., 2013; Dutot et al., 2008; Wakx et al., 2016; Karasawa and Kawate, 2018; Rassendren et al., 1997). As oxidative stress and excessive uric acid production may be involved in the inflammatory process, correlation with antioxidant activity, phenolic content and xanthine oxidase inhibition were also investigated.

The main endpoint of our proposal was to identify bioactive compounds directly from complex mixture through a metabolomics based approach. This attractive methodology is mainly based on using analytical tools usually combining the LC-MS analysis with pertinent statistical analysis allowing to highlight compounds involved in biological activity. Cell viability and membrane integrity were also evaluated. In parallel, LCMS analysis allowed the metabolome dereplication to identify main metabolites presents in the extracts. LC-MS data were then used for statistical analysis, based on principal components analysis (PCA), partial least square regressive (PLS-R) and discriminant analysis (PLS-DA). For the eight Scrophularia species extracts, PCA was used to identify variables responsible of the difference of biological activity.

| Collection Places | | | | |
|---|---|---|---|---|
| **Code** | **Country** | **Region** | **GPS Coordinates** | **Altitude (m)** |
| FR1 | France | Hautes Alpes | 45° 02′ 07″ N 6° 24′ 20″ E | 2000 |
| FR2 | France | Savoie | 45° 13′ 38″ N 6° 44′ 32″ E | 1500 |
| FR3 | France | Loiret | 48° 02′ 23″ N 3° 03′ 22″ E | 130 |
| FR4 | France | Val-de-Marne | 48° 47′ 24″ N 2° 34′ 39″ E | 100 |
| SR1 | Montenegro | Planinica | 42° 38′ 01″ N 19° 37′ 42″ E | 1750 |
| SR2 | Serbia | Šumadija and Western Serbia | 44° 07′ 16″ N 20° 10′ 54″ E | 700 |
| SR3 | North Macedonia | Galičica | 40° 56′ 38″ N 20° 48′ 46″ E | 1800 |
| SR4 | Serbia | North Serbia | 44° 52 ′22″ N 20° 42′ 03″ E | 75 |

Table 1: Collection places. Plant harvested were performed during July and August 2014.

Experimental

General

The LC-MS experiments were performed using an Acquity UPLC (HSS C18 reversed-phase column 1.7 µm, 2.1 mm 100 mm, Waters Corp., MA, USA) system coupled to a Sypnapt G2-HDMS mass spectrometer system (Waters Corp., MA, USA). The ESI source conditions were capillary cone voltage 50 V in negative mode, spray voltage 3.4 kV. SPECTROstar Nano BMG LabTech UV/vis spectrometer was used for colorimetric and enzymatic inhibition assays.

TECAN Safire2 Cytofluorometer (Lyon, France) adapted to microplate was used for fluorescence assay. Methanol (MeOH), dimethylsulfoxide (DMSO), ethanol, (EtOH), gallic acid, quercetin, FolinCiocalteu reagent, $AlCl_3 \cdot 6H_2O$, $Na_2CO_3$, $CH_3COONa$, 2,2-diphenyl-1-picrylhydrazyl (DPPH) xanthine oxidase enzyme from bovine milk, xanthine, allopurinol and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ABTS were purchased from Sigma Aldrich (St. Lous, USA). YO-PRO-1 sonde was purchased from Termo Fisher, Waltham, (MA, USA) and neutral red (NR) from Fluka (Buchs, Switzerland).

Plant material

Plant parts were collected in accordance of their traditional uses in France and the Balkan Peninsula, during July and August 2014. In France, samples were obtained from the Station Alpine Joseph Fourier in Grenoble ("Lautaret"), and from the Conservatoire National des Plantes Médicinales et Aromatiques de Milly-la-Forêt (CNPMAI). In the Balkans, samples were collected from locations in Serbia, Montenegro and North Macedonia (Table 1). A total of 24 species were collected including 7 *Achillea*, 3 *Artemisia*, 4 *Helichrysum*, 3 *Scrophularia*, 5 *Plantago* and 4 *Gentiana* species, either in France or in the Balkans (Table 2). For *Achillea millefolium*, *Scrophularia nodosa*, and *Gentiana lutea* both French and Serbian samples were studied. For *Scrophularia* species, roots and aerial parts were considered separately. Voucher specimen was deposited in the herbarium of the department of Pharmacognosy, Paris-Descartes University, France (voucher specimen MKOSB2014PHCPS01 to MKOSB2014PHCPS31).

Extraction

One gram of dry plant material was submitted to extraction by ultra-sound with 10 mL of $H_2O$:MeOH – 1:1 mixture for 2 x 5 min, at room temperature, then filtered, solvent evaporated under reduced pressure and lyophilized. Extraction yield are reported in Table 2. Aliquots of 10 mg/mL in DMSO:$H_2O$ – 1:1 were prepared for biological assays and kept at -4 °C until evaluation.

UPLC-MS data acquisition

Qualitative on-line UPLC-MS analysis of extract was performed with the following method: water containing 0.1 % of formic acid (solvent A) and acetonitrile containing 0.1 % of formic acid (solvent B) at a flow rate of 0.4 mL/min. Gradient elution started with 95% A and 5% B by 6 min and changed to 100% B in 2 min and maintained for other 2 min. The mass spectral data were acquired in ESI in negative mode. The capillary voltage, source cone voltage, and extraction cone voltage were maintained at 3.25 kV, 30 V, and 4 V, respectively for the positive mode and at -2.5 kV, -40 V, and 4 V, respectively for negative mode. Nitrogen was applied as the desolvation gas at a flow rate of 900 L/h. The source and desolvation temperature were maintained

at 120 °C and 550 °C, respectively. Mass spectra were acquired over the m/z range of 100-1700 at a mass resolution of 22000 FWHM (full width half at maximum).

Total polyphenol content

Total polyphenol content (TPC) was estimated according to Folin-Ciocalteu (F-C) reagent method as previously reported with slight modification (Clarke et al., 2013). Briefly, in a 96 well plate, 25 µL of sample solution at 4 mg/mL were mixed with 125 µL of F-C reagent freshly diluted 1/10 with distilled water. After 5 min, 100 µL the solution was mixed with 100 µL of a solution 7.5 % of $Na_2CO_3$, left for 30 min in the dark and the absorbance was measured at 650 nm. Respective standard curve of gallic acid and blank (replacing F-C reagent with 125 µL of water) were run simultaneously, after which the total phenol content was calculated as mg of gallic acid equivalent per g of extract (Table 3).

Total flavonoid evaluation

Total flavonoid evaluation (TFE) was performed according to (Bampouli et al., 2015). Briefly, in a 96 well plate, 50 µL of sample solution at 4 mg/mL were mixed with 160 µL of EtOH, 20 µL of 1.8 % of $AlCl_3$ and 20 µL of 0.8 % of $CH_3COONa$. Then, the plates were kept at room temperature for 40 min and the absorbance was measured at 415 nm. Respective standard curve of quercetin and blank (replacing $AlCl_3$ with 20 µL of $H_2O$ distilled) were run simultaneously, after which the total flavonoid evaluation was calculated as mg of quercetin equivalent per g of extract (Table 3).

DPPH scavenging assay

Radical scavenging capacity was measured using DPPH agent according to previously reported (Clarke et al., 2013). DPPH reagent solution (12.4 mg in 100 mL of EtOH) was prepared freshly just before the assay. Extract were evaluated at a final concentration of 200 µg/mL. In each well were placed 10 µL of sample solution with 190 µL of DPPH solution. Plates were incubated at room temperature by 30 min protected from light. The absorbance was measured at 517 nm. Measured were performed by triplicated. Gallic acid was used as positive control. Results are expressed as percentage of efficacy at 200 µg/mL of extract (Table 3).

| Family | Collected Samples | | | | | Extraction yield |
|---|---|---|---|---|---|---|
| | Sample | Plant Species | Part Collected | Collection Place | | |
| | | | | France | Balkans | |
| Asteraceae | Genus | *Achillea* | | | | |
| | 1 | *A. millefolium* L. | | FR1 | | 11.1 |
| | 2 | *A. nana* L. | | FR1 | | 13.0 |
| | 3 | *A. fraasii* Sch. Bip. | | | SR1 | 6.7 |
| | 4 | *A. millefolium* L. | Aerial parts | | SR2 | 13.7 |
| | 5 | A. *holosericea* Sm. | | | SR3 | 14.4 |
| | 6 | *A. chrysocoma* Friv. | | | SR3 | 13.4 |
| | 7 | *A. ageratifolia* (Sibth. & Sm.) Benth. & Hook.f. | | | SR3 | 12.5 |
| | | *Artemisia* | | | | |
| | 8 | *A. absinthium* L. | | FR1 | | 17.4 |
| | 9 | *A. vulgaris* L. | Aerial parts | FR1 | | 20.1 |
| | 10 | *A. alba* Turra. | | | SR2 | 25.0 |
| | | *Helichrysum* | | | | |
| | 11 | *H. zivojinii* Černjavski & Soska | Aerial parts | | SR3 | 18.8 |
| | 12 | *H. arenarium* (L.) Moench | Flowers | | SR4 | 16.1 |
| | 13 | *H. plicatum* DC. | Flowers | | SR4 | 9.7 |
| | 14 | *H. italicum* (Roth) G. Don | Aerial parts | | SR4 | 22.5 |
| Scrophulariaceae | | *Scrophularia* | | | | |
| | 15 | *S. umbrosa* Dumort. | Aerial parts | FR3 | | 9.7 |
| | 16 | *S. umbrosa* Dumort. | Roots | FR3 | | 15.2 |
| | 17 | *S. nodosa* L. | Aerial parts | FR4 | | 24.2 |
| | 18 | *S. nodosa* L. | Roots | FR4 | | 19.3 |
| | 19 | *S. nodosa* L. | Aerial parts | | SR2 | 30.4 |
| | 20 | *S. nodosa* L. | Roots | | SR2 | 42.7 |
| | 21 | *S. bosniaca* Beck | Aerial parts | | SR1 | 16.6 |
| | 22 | *S. bosniaca* Beck | Roots | | SR1 | 7.9 |
| Plantaginaceae | | *Plantago* | | | | |
| | 23 | *P. media* L. | Whole plant | FR2 | | 20.2 |
| | 24 | *P. alpina* L. | | FR1 | | 10.4 |
| | 25 | *P. maritima* All. | | FR2 | | 18.6 |
| | 26 | P. major (var P.minima) | | FR2 | | 20.7 |
| | 27 | *P. lanceolata* L. | | FR2 | | 22.7 |
| Gentianaceae | | *Gentiana* | | | | |
| | 28 | *G. lutea* L. | Aerial parts | FR1 | | 35.0 |
| | 29 | *G. nivalis L.* | | FR1 | | 29.8 |
| | 30 | *G. lutea* L. | | | SR2 | 31.0 |
| | 31 | *G. pneumonanthe* L. | | | SR2 | 24.1 |

Table 2: Collected samples and extraction yields. Extract obtained from dry vegetal aterial in MeOH:$H_2O$ 1:1.

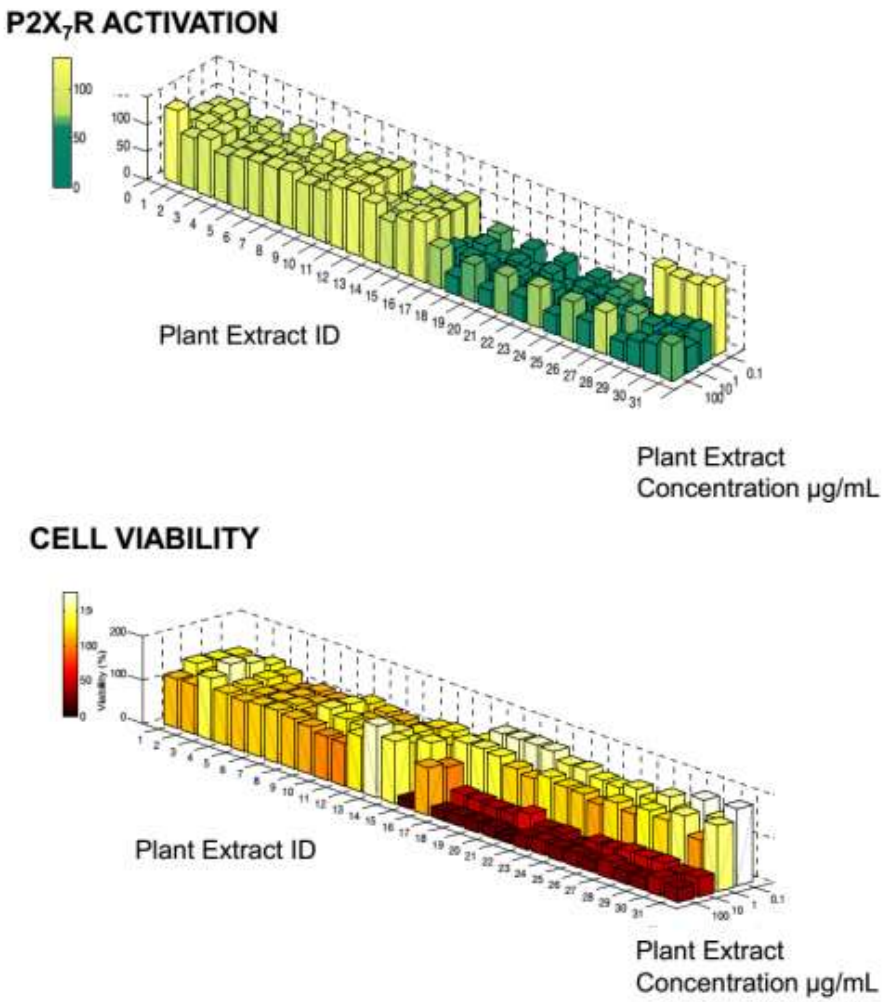


Figure 2: Plant extracts (**1-31**) effects at different concentration (0.1-100 µg/mL) on $P2X_7$ activation and cell viability (U-937, macrophage cells).

ABTS scavenging assay

Radical scavenging capacity was measured using ABTS reagent according to previous reported (Stagos et al., 2012). ABTS reagent was prepared as follows: 10 mL of 7 mM of ABTS solution were mixed with 164 µL of 140 mM of $K_2S_2O_8$ and stored overnight (16 h). The final solution was diluted with distilled $H_2O$ (1:20) to reach an absorbance of 0.70 ± 0.02 before the assay. Extract were evaluated at a final concentration of 200 µg/mL. In each well were placed 100 µL ABTS final solution and 50 µL of sample. Plated were incubated at room temperature by 10 min protected from light. The absorbance were measured at 734 nm. Measured were performed by triplicated. Quercetin was used as positive control. Results are expressed as percentage of efficacy at 200 µg/mL (Table 3).

Xanthine oxidase inhibition assay

Evaluation of inhibition of xanthine oxidase enzyme (XO) of extracts was determined using the modified protocol from (Havlik et al., 2010). Briefly, in a quartz 96 well plate were added 62.5 µL of extract solution at a concentration of 800 µg/mL with 105 phosphate buffer (0.12 mM, pH 7.5) and 20 µL of xanthine oxidase solution (0.125 units/mL in phosphate buffer) and incubated at room temperature protected from light by 5 min. Then, 62.5 µL of xanthine solution (200 µM in phosphate buffer) were added in each well and the absorbance was measured at 295 nm during 30 min (final DMSO concentration of 0.4 %). Corresponding blanks were carried out in parallel, replacing xanthine oxidase solution by 62.5 µL of phosphate buffer. Initial rate was calculated and the percentage of inhibition was calculated as follows: Percentage of inhibition = (1-((ΔAbs sample – ΔAbs blank sample)/(ΔAbs DMSO – ΔAbs Blank DMSO))) x 100. Allopurinol was used as positive control (Table 3).

Cell Culture

U-937 monocytes cell line were incubated in RPMI medium (with 1% of glutamine, penicillin and streptomycin). Monocytes were differenced to macrophages by incubation at 37 °C, 5% $CO_2$ with PMA during 48h. After differentiation, 100 µL of cell suspension at 5 x $10^5$ cell/mL were placed into 96 well plates and incubated for 24h.

YO-PRO-1 assay

Activation of P2X7 receptor was measured using YO-PRO-1 DNA probe as previously reported (Karasawa and Kawate, 2018; Rassendren et al., 1997). Briefly, macrophages were treated with different extract concentration (100, 10, 1 and 0.1 µg/mL final concentration, 1% of DMSO) and incubated during 2h, in triplicate. Then, macrophages were stimulated with 10 µL of LPS (0.5 µg/mL) and incubated at 22h at 37°C with 5% $CO_2$. A 100 µL of the YOPRO-1 solution (2 µM in PBS) was then distributed in each well, protected from light, and the plate was incubated in the dark at room temperature for 10 min and fluorescence were measured ($\lambda_{excitation}$ = 491 nm, $\lambda_{emission}$ = 509 nm, band-pass 5 nm). Fluorescence of the DMSO control was brought to 100% (Figure 2).

MTT viability assay

Macrophages were incubated with the concentration for extracts described above and 10 µL of 10% MTT solution, added directly in the medium for 4 h at 37°C. The medium was then aspirated and the MTT formazan crystals were solubilized with DMSO. Absorbance was measured at 540 nm and the results are presented as the percent of optical density (OD) in the treated wells versus blanks (Figure 2).

Statistical analysis

The matrix of *m/z* values of detected ions obtained from the UPLC-MS analysis for the 31 extracts was submitted to principal component analysis (PCA) and partial least squares-discrimination analysis (PLS-DA) using MetaboAnalyst 4.0 software (Chong and Xia, 2018) and partial least squares regression (PLS-R) were obtained using XLSTAT 2019 add-on for Microsoft Excel. The matrix of m/z data was obtained by directly chromatogram export in CSV format file by Agilent MassHunter Qualitative Analysis B.07 software. Matrix data file was upload to MetaboAnalyst 4.0 web site. Upload parameters were defined as peak intensity table and samples in row format. Interquantile range (IQR) was used for data filtering. Normalization by a pooled sample from group (the less active extracts) was applied before analysis. For PLS-R analysis, same matrix of Scrophularia extract was used in Microsoft Excel 2013 software with XLSTAT add-on installed. Pearson coefficient and a significance level of 5% were applied (Figure 3).

For TPC and TFE evaluation, rich extracts were defined as more than 50 mg of gallic acid or quercetin equivalents per g of extract, medium between 50 – 25 mg of gallic acid or quercetin equivalent per g of extracts and as poor extracts with levels lower than 20 mg per extract. For DPPH and XO inhibition analysis, active extracts were defined with percentage of inhibition more than 50 %, moderate extract between 50-20 % of inhibition, poor extract between 20-5 % and as not active with percentage of inhibition less than 5 %. For P2X7 activation, active extracts were defined if a decrease of fluorescence associated to YO-PRO dye cell penetration was higher than 50%.

UPLC-MS based dereplication of *Scrophularia* extracts

UPLC-MS data obtained from Acquity Sypnapt apparatus were used for putative identification of compounds present in *Scrophularia* extracts **17**-**22**. Exact mass values extrapolated from the *m*/*z* values of most probable adducts ($[M-H]^-$, $[M+FA-H]^-$ or $[2M-H]^-$) were investigated in the Dictionary of Natural Products and through previous phytochemical reports of different *Scrophularia* species (Table 4) (Pasdaran and Hamedi, 2017).

Antioxidant activity of extracts based on phenolics weight and radical scavenging capacities.

| Code Sample | TPC[a] | TFE[b] | DPPH[c] | ABTS[c] | XO[d] |
|---|---|---|---|---|---|
| 1 | 62.8 ± 0.1 | 12.0 ± 0.9 | 33.1 ± 1.4 | 99.1 ± 0.1 | 55.2±1.2 |
| 2 | 23.5 ± 0.1 | 6.7 ± 0.1 | -2.3 ± 1.8 | 37.4 ± 0.3 | 79.6±2.8 |
| 3 | 62.0 ± 0.5 | 6.7 ± 0.9 | 31.8 ± 1.0 | 99.2 ± 0.1 | 66.9±1.2 |
| 4 | 84.4 ± 0.3 | 0.6 ± 0.1 | 58.9 ± 1.7 | 100.0 ± 0.4 | 37.4±0.5 |
| 5 | 72.1 ± 1.0 | 43.5 ± 2.3 | 49.4 ± 3.3 | 99.0 ± 0.5 | 34.2±2.8 |
| 6 | 62.4 ± 0.3 | 18.8 ± 2.8 | 34.5 ± 1.0 | 99.1 ± 0.2 | 50.0±1.3 |
| 7 | 38.2 ± 0.1 | 13.9 ± 0.2 | 9.9 ± 0.1 | 81.7 ± 1.7 | 69.7±2.7 |
| 8 | 43.0 ± 0.1 | 2.9 ± 0.2 | 21.3 ± 0.7 | 83.3 ± 3.8 | 84.0±0.4 |
| 9 | 46.4 ± 0.2 | 8.6 ± 1.0 | 25.6 ± 0.9 | 87.6 ± 1.2 | 9.6±2.4 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 10 | 87.8 | ± | 0.8 | 26.9 | ± | 3.6 | 47.1 | ± | 1.9 | 100.0 | ± | 0.2 | 37.7±1.4 |
| 11 | 74.5 | ± | 0.5 | 102.0 | ± | 5.5 | 34.2 | ± | 1.4 | 98.4 | ± | 0.3 | 6.3±3.6 |
| 12 | 81.2 | ± | 0.2 | 90.6 | ± | 5.2 | 37.2 | ± | 1.2 | 98.5 | ± | 0.3 | n.e. |
| 13 | 65.9 | ± | 0.5 | 56.2 | ± | 2.8 | 30.9 | ± | 0.4 | 98.4 | ± | 1.2 | 26.6±1.4 |
| 14 | 93.5 | ± | 0.3 | 0.4 | ± | 1.5 | 60.8 | ± | 2.7 | 99.4 | ± | 0.3 | n.e. |
| 15 | 99.0 | ± | 0.8 | 6.3 | ± | 1.7 | 58.4 | ± | 2.5 | 99.8 | ± | 0.2 | 46.5±4.3 |
| 16 | 37.2 | ± | 0.2 | 10.2 | ± | 0.9 | 10.0 | ± | 0.2 | 90.6 | ± | 0.8 | 55.9±0.7 |
| 17 | 25.6 | ± | 0.1 | 0.8 | ± | 1.7 | -4.3 | ± | 0.3 | 55.2 | ± | 2.1 | 20.9±2.0 |
| 18 | 14.9 | ± | 0.3 | 0.8 | ± | 2.6 | -4.3 | ± | 0.2 | 40.4 | ± | 1.9 | 58.9±1.6 |
| 19 | 40.0 | ± | 0.1 | 0.2 | ± | 0.2 | 3.8 | ± | 0.4 | 75.8 | ± | 1.0 | 17.4±3.3 |
| 20 | 25.0 | ± | 0.4 | 0.6 | ± | 1.6 | 5.5 | ± | 0.2 | 54.1 | ± | 2.6 | 60.1±1.1 |
| 21 | n.e. | | | n.e. | | | n.e. | | | n.e. | | | 43.6±2.5 |
| 22 | 47.4 | ± | 0.5 | 0.5 | ± | 2.2 | 19.5 | ± | 1.3 | 97.4 | ± | 4.0 | 68.3±0.7 |
| 23 | 19.3 | ± | 0.1 | 2.8 | ± | 0.4 | 1.7 | ± | 0.1 | 49.3 | ± | 0.7 | 63.8±2.9 |
| 24 | 74.9 | ± | 0.5 | 27.7 | ± | 0.3 | 33.4 | ± | 0.9 | 99.4 | ± | 0.2 | 72.4±1.8 |
| 25 | 15.3 | ± | 0.1 | 0.5 | ± | 1.4 | -2.0 | ± | 1.4 | 41.3 | ± | 5.5 | 77.7±1.7 |
| 26 | 19.4 | ± | 0.1 | 2.8 | ± | 1.1 | 0.5 | ± | 0.8 | 51.0 | ± | 6.2 | 67.8±4.6 |
| 27 | 108.2 | ± | 1.7 | 0.6 | ± | 0.5 | 50.6 | ± | 2.5 | 99.5 | ± | 0.1 | 68.1±0.9 |
| 28 | 19.8 | ± | 0.1 | 0.9 | ± | 0.2 | -1.2 | ± | 0.5 | 35.4 | ± | 2.0 | 63.3±8.2 |
| 29 | n.e. | | | n.e. | | | n.e. | | | n.e. | | | 53.6±0.4 |
| 30 | n.e. | | | n.e. | | | n.e. | | | n.e. | | | 62.0±1.3 |
| 31 | 33.8 | ± | 0.2 | 1.6 | ± | 0.4 | 6.4 | ± | 0.1 | 76.1 | ± | 2.3 | 62.3±0.9 |
| Gallic acid[e] | - | | | - | | | 50.3 | ± | 0.5 | - | | | - |
| Trolox[f] | - | | | - | | | - | | | 54.5 | ± | 2.3 | - |
| Allopurinol[g] | - | | | - | | | - | | | - | | | 58.6±0.7 |

Table 3. Antioxidant of obtained extracts. [a] Total polyphenol content expressed in mg of Gallic acid equivalent per g of extract. [b] Total flavonoid evaluation expressed in mg of Quercetin equivalent per g of extract. [c] Percentage of scavenging at 200 µg/mL. [d] Xanthine oxidase inhibition by extract at 200 µg/mL. [e] Percentage of efficacy at 5 µg/mL. [f] Percentage of efficacy at 8 µg/mL. [g] Percentage of inhibition at 3.13 µM. n.e.: not enough quantity for evaluation. -: not evaluated.

Results

Inhibition of $P2X_7R$ activation

Activation of $P2X_7$ receptor was measured using YO-PRO-1 DNA probe. Fluorescence associated to YO-PRO dye cell penetration was reduced by more than 50% when cells were pre-treated with plant extracts **17** to **31**, corresponding to extracts obtained from *Scrophularia*, *Plantago* and *Gentiana* species, but not for *S. umbrosa* extracts (**15**-**16**) (Figure 2). By contrast, *S. nodosa* and *S. bosniaca* (**17**-**22**) decreased significantly $P2X_7$ activation with decrease of more than 50% of fluorescence. Surprisingly, a clear difference was measured for extracts **1** to **14**,

corresponding to the Asteraceae family species, revealing a very weak decrease of fluorescence. The PLS-DA analysis confirmed a clear difference between extracts from Asteraceae family regarding Plantaginaceae, Scrophulariaceae and Gentianaceae families.

TPC, TFE and radical-scavenging activity (DPPH and ABTS radicals) The extract **4**, **14** and **15** showed the highest radical-scavenging activity in DPPH assay with $EC_{50}$ > 50 %. Extracts from *Achillea* and *Helichrysum* genus showed $EC_{50}$ of 30-60 %, except extracts **2** and **7**. DPPH and ABTS scavenging efficacy are directly correlated with polyphenol and flavonoid content in the extracts. Extract from *Scrophularia*, *Plantago* and *Gentiana* genus showed the lowest flavonoid and polyphenol content with the lowest $EC_{50}$ DPPH values (less than 10 %), except extracts **22** and **24** (Table 3). Several PCA analysis for either TPC, TFE and radical-scavenging activity (DPPH and ABTS radicals) were envisaged in order to identify any discrimination based on the level of each parameter. In each case, PCA showed the overlapping for the different level of measured parameters (polyphenol and flavonoids content and radical-scavenging activity) (Figure 3).

Xanthine oxidase inhibition (XO)

The ability to inhibit xanthine oxidase enzyme was also evaluated, due to its implication in chronical inflammatory diseases and reactive oxygen species (ROS) production. The most active extracts were obtained from *Plantago*, *Gentiana* and *Achillea* species with a percentage of inhibition varying from 50 to 84% at 200 µg/mL. Extracts with the lowest XO inhibition (< 10%) were obtained from *Artemisia* species, *A. absinthium* and *A. lobelii* (9, 11). Furthermore, Scrophularia extracts **17** and **19**, which highly inhibit $P2X_7$ activation, presented a poor xanthine oxidase inhibition (17-20 %) (Table 3). PCA analysis showed partial overlapping of the three cluster based on the low, medium or high level of XO inhibition (Figure 3).

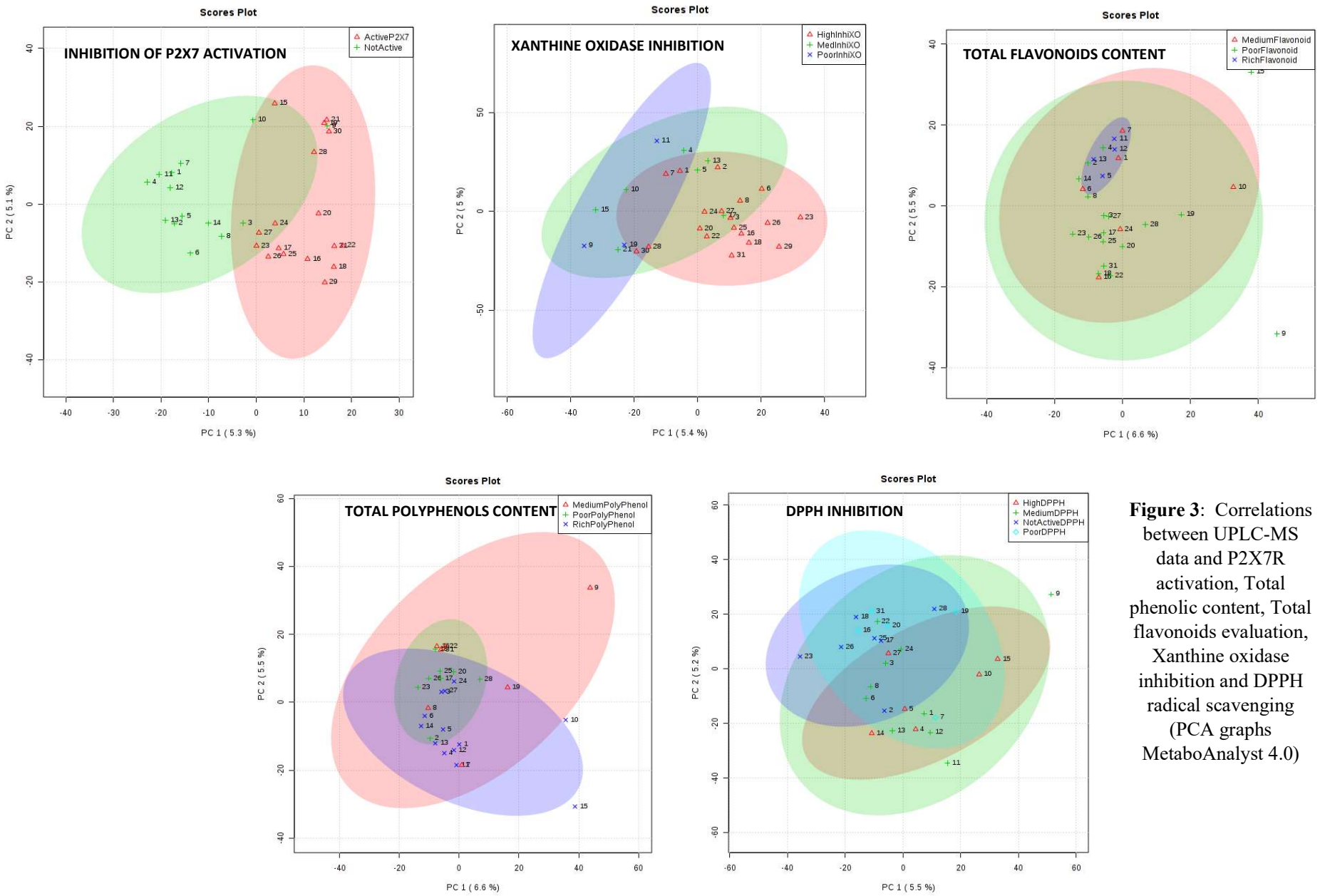


**Figure 3**: Correlations between UPLC-MS data and P2X7R activation, Total phenolic content, Total flavonoids evaluation, Xanthine oxidase inhibition and DPPH radical scavenging (PCA graphs MetaboAnalyst 4.0)

**Putative identified compounds detected by LC-MS[1] from *Scrophularia* extracts**

| Peak | t [min] | [M-H]$^-$ | [M-H+FA]$^-$ | [2M-H]$^-$ | Putative Identification | 17 | 18 | 19 | 20 | 21 | 22 | Previously isolated in | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| #1 | 0,39 | 341,1079 | 387,1136 | 683,2221 | n.d. | | X | X | X | X | X | | |
| **#2** | **0,56** | **345,1125** | **391,1232** | **691,2401** | **Aucubin** | **X** | **X** | **X** | | **X** | **X** | ***S. cryptophila, S. ilwensis, S. koraiensis, S. lepidota, S. ningpoensis, S. trifoliata, S. scorodonia.*** | Caliş et al., 1993a; Li et al., 2009; Pachaly et al., 1994; Ramunno et al., 2006; Santos et al., 2000; Tasdemir et al., 2008, 2005 |
| #3 | 0,57 | 285,0979 | 331,1033 | 571,2027 | n.d. | | | | X | | | | |
| **#4** | **0,94** | **363,125** | **409,1275** | **727,251** | **Harpagide** | **X** | **X** | | **X** | **X** | **X** | ***S. ilwensis, S. crytophila, S. dentata, S. koraiensis, S. scorodonia.*** | Caliş et al., 1993a; Pachaly et al., 1994; Santos et al., 2000; Tasdemir et al., 2008; Zhang et al., 2014 |
| #5 | 1,01 | - | 329,0876 | 567,1716 | 4,5-Dihydroxyanthraquinone-2-carboxylic acid | | | | X | X | | *S. nodosa* | Pasdaran and Hamedi, 2017 |
| #6 | 1,31 | 327,1082 | - | 655,2233 | n.d. | | | | X | | | | |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| #7 | 1,42 | 313,093 | 359,0987 | 627,1952 | Scrodentoid B or Scrophulein | | | | X | | | *S. dentate, S. grisshemii* | Akhmedov and Litvinenko, 1969, Zhang et al., 2015 |
| #8 | 1,68 | 329,1214 | 375,1282 | 659,2485 | Scrodentoid C or Scrodentoid D | | | | | X | | *S. dentata,* | Zhang et al., 2015 |
| **#9** | **1,97** | **405,1331** | **451,1373** | **811,2677** | **8-*O*-Acetyl harpagide** | **X** | **X** | **X** | | **X** | **X** | ***S. cryptophila, S. dentata, S. deserti; S. ilwensis, S. scorodonia*** | Ahmed et al., 2003; Caliş et al., 1993; Santos et al., 2000; Tasdemir et al., 2008; Zhang et al., 2014 |
| #10 | 2,04 | 371,1323 | 417,1396 | 743,2726 | | | | | X | | | | |
| #11 | 2,31 | 653,1939 | - | - | Scrodentoside F | | | X | | X | X | *S. dentata* | Zhang et al., 2016 |
| #12 | 2,39 | 727,2475 | 773,2527 | 1455,4986 | | | | | X | | | | |
| #13 | 2,51 | 683,2174 | - | - | Scropolioside G or Scropolioside H | | | | | X | | *S. dentata.* | Zhu et al. 2015 |
| #14 | 2,60 | 653,2007 | - | - | Scrodentoside F | X | | | | | | *S. dentata* | Zhang et al., 2016 |
| #15 | 2,62 | - | - | - | n.d. | | | | | X | | | |
| #16 | 2,66 | 637,2266 | - | - | Scrophuside or Jionoside D | | | X | | | | *S. ningpoensis, S. nodosa.* | Miyase and Mimatsu, 1999, Hua et al., 2014 |
| #17 | 2,66 | 741,2646 | 787,271 | 1483,5326 | n.d. | | | | X | | | | |
| #18 | 2,68 | 491,2082 | - | 983,4256 | n.d. | | | | | X | | | |
| #19 | 2,73 | 623,2016 | - | 1247,4036 | Verbascoside, Isoverbascoside | | X | | | | X | *S. amplexicaulis, S. nodosa, S. scopolii* | Miyase and Mimatsu, 1999; Calis et al., 1988b; Pasdaran et al., 2016 |
| #20 | 2,79 | - | - | - | n.d. | | X | | | | | | |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| #21 | 2,96 | 711,2485 | 757,254 | - | | | X | X | | | | |
| #22 | 3,01 | 783,2489 | - | 1567,4949 | Angoroside C; Isoangoroside C | | X | | | | *S. cryptophila, S. ilwensis, S. lepidota, S, ningpoensis, S. nodosa, S. scopolii, S. scorodonia* | Miyasi and Mimatsu, 1999; Caliş et al., 1988a; Caliş et al., 1993; Li et al., 2009; Santos et al., 2000; Tasdemir et al., 2008, 2005 |
| #23 | 3,02 | 591,2053 | 637,2079 | - | Osmanthuside B | | | | X | | *S. dentata* | Pasdaran and Hamedi, 2017 |
| #24 | 3,11 | - | - | - | n.d. | | X | | | | | |
| #25 | 3,12 | - | - | - | n.d. | | | | | X | | |
| #26 | 3,15 | 679,2214 | 725,2275 | - | Scrospioside B; Scorodioside | | X | | | | *S. dentata, S. spicata* | Santos Galindes et al. 2001, Zhang et al., 2014 |
| #27 | 3,27 | 421,1997 | 467,205 | - | n.d. | | X | | X | | | |
| #28 | 3,30 | 825,2785 | - | 1265,971 | n.d. | | | X | | | | |
| #29 | 3,32 | - | - | - | n.d. | | | | X | | | |
| #30 | 3,36 | - | - | - | n.d. | X | | X | | | | |
| **#31** | **3,40** | **591,1704** | **637,1756** | **1183,3448** | **Osmanthuside B** | | | | **X** | **X** | ***S. dentata*** | Pasdaran and Hamedi, 2017 |
| #32 | 3,46 | 667,2055 | 713,2116 | 1335,4139 | Scropheanoside I or Verbascoside A | | X | | | | *S. koraiensis or S. nodosa* | Miyasi and Mimatsu, 1999; Pachaly et al., 1994 |
| #33 | 3,49 | 799,2594 | - | - | n.d. | X | | X | | | | |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **#34** | **3,57** | **493,1646** | **539,1697** | **987,3334** | **Harpagoside, 8-cis-cinnamoylharpagide or 6'-cinnamoylharpagide** | **X** | **X** | **X** | **X** | **X** | ***S. buergeriana, S. dentata, S. deserti, S. korainensis, S. scorodonia or S. ningpoensis*** | Ahmed et al., 2003; Jeong et al., 2008; Li et al., 2009; Pachaly et al., 1994; Santos et al., 2000; Zhang et al., 2014 |
| #35 | 3,65 | 523,1713 | 569,1771 | 1047,3485 | 8-O-4-Methoxy-Z-cinnamoylharpagide; 8-O-4-Methoxy-E-cinnamoylharpagide; 6'-O-4-Methoxy-E-cinnamoylharpagide | | | | X | | *S. buergeriana,* | Jeong et al., 2008 |
| #36 | 3,87 | 679,2266 | 725,229 | 1359,4526 | Scrospioside B; Scorodioside | | X | X | | | *S. scorodonia, S. spicata* | de Santos Galindez et al., 2001 |
| #37 | 3,91 | 709,2186 | - | 1419,4372 | Scrophuloside A5; Buddlejoside A5; Buddlejoside A3; Buddlejoside A4 | | X | | | | *S. nodosa* | Miyasi and Mimatsu, 1999 |
| #38 | 3,96 | 767,2204 | - | - | Scrophuloside A8 | | X | | | | *S. nodosa* | Miyasi and Mimatsu, 1999 |
| #39 | 4,02 | 709,2186 | 755,2237 | 1419,4372 | Scrophuloside A5; Buddlejoside A5; Buddlejoside A3; Buddlejoside A4 | | X | | | | *S. nodosa* | Miyasi and Mimatsu, 1999 |
| #40 | 4,68 | 721,2454 | 767,2518 | 1443,4884 | Scropolioside D | X | X | | X | X | *S. ilwensis, S. koraiensis or S. spicata* | Caliş et al., 1993a; de Santos Galindez et al., 2001; Pachaly et al., 1994 |
| #41 | 4,84 | 1103,613 | 1149,6209 | - | Ilwensisaponin C | X | | | | | *S. ilwensis* | Caliş et al., 1993b |
| #42 | 4,95 | - | - | - | n.d. | X | | | | | | |
| #43 | 5,42 | 520,1721 | - | - | n.d. | | X | | | | | |
| #44 | 5,43 | - | - | - | n.d. | X | | X | | X | | |
| #45 | 5,77 | 809,2653 | 855,2754 | - | Koelzioside or Scropolioside B | | X | | | | *S. koelzii or S. scopolii* | Bhandri et al., 1992; |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | Calis et al., 1988b |
| #46 | 6,23 | - | - | - | n.d. | | | | | X | | |
| #47 | 6,57 | 297,1857 | - | 595,375 | Scrodentoid A; Scrodentoid E | | X | | | X | *S. dentata* | Zhang et al., 2015 |
| #48 | 7,22 | - | - | - | n.d. | | | | X | | | |
| #49 | 7,72 | - | - | - | n.d. | X | X | X | | X | | |
| #50 | 7,78 | - | - | - | n.d. | | | | X | | | |
| #51 | 8,26 | - | - | - | n.d. | X | X | X | X | X | | |

Table 4: Putative identification of compounds detected by LC-MS from active extracts 17-22.

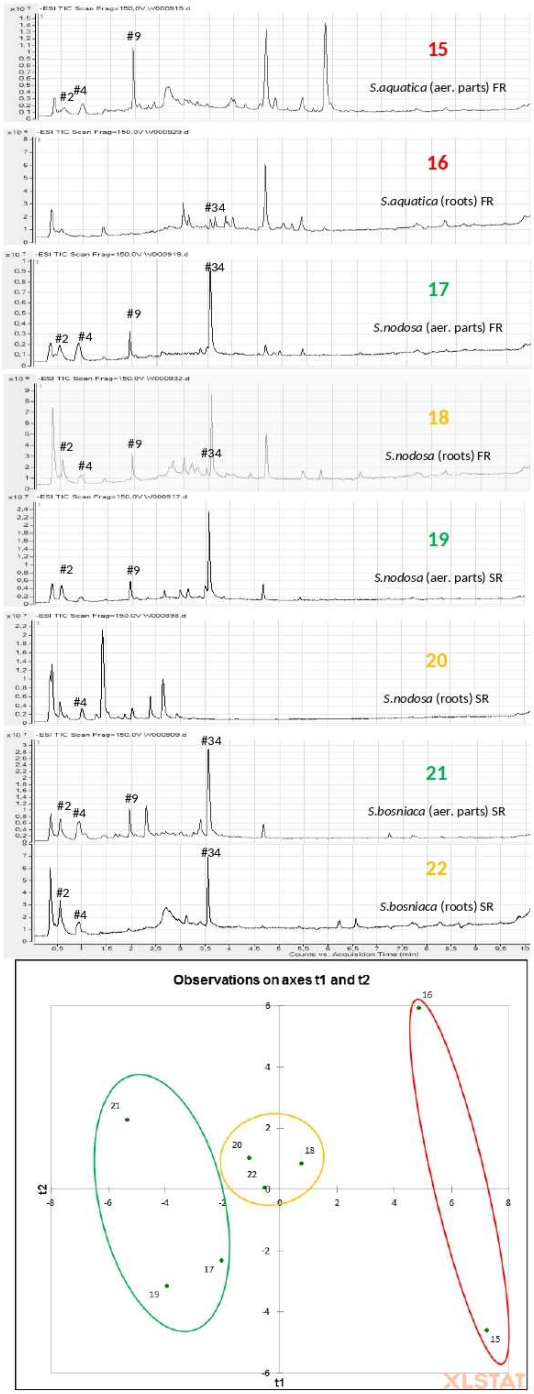


**Figure 4**: UPLC-MS Chromatograms of Scrophularia extracts **15-22** between 0-10 min and XLSTAT 2019 PLS-R graph of UPLC-MS data correlated with $P2X_7$ activation. In red: inactive extracts, yellow: moderately-active extracts and in green: active extracts.

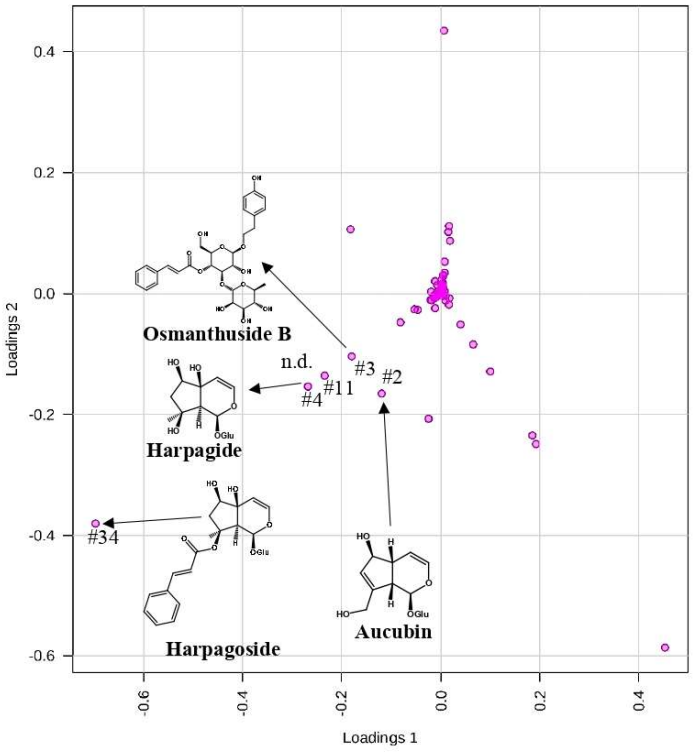


**Figure 5**: Loading scores plot of PCA obtained from *Scrophularia* species

Inhibition of $P2X_7$ Receptor activation by *Scrophularia* extracts

Phytochemical profiles ofScrophularia extracts **15**-**22** obtained from UPLC-MS analysis show a clear difference between active and inactive extracts. An intense peak **#34** is present only in the active extracts whereas minoritary compounds vary between these two groups. Statistical analysis PCA and PLS-R analysis also revealed a significative difference in the metabolome between active and inactive Scrophularia extracts (Figure 4). Loading score plot of PCA analysis graph revealed the m/z peaks which strongly influence the PCA scores. In our case, peak **#34** was identified as the stronger contributor to the biological activity, in accordance with the observation of the UPLC-MS chromatogram, followed by peaks **#4**, **#11**, **#31** and **#2** (Figure 5).

Dereplication of *Scrophularia* extracts

In order to identify putatively compounds related to peaks **#2**, **#4**, **#11**, **#31** and **#34**, dereplication of actives *Scrophularia* extracts was envisaged. Peak **#34**, the main contributor to biological activity, was identified as harpagoside. Other contributors were putatively identified as iridoids: aucubin (**#2**), harpagide (**#4**), osmanthuside B (**#31**). Peak **#11** could not be matched with previously isolated

compounds from *Scrophularia* species. Dereplication of the extracts also allowed us to identify compounds related to other peaks, such as cinnamoylated glycosides iridoids and phenylethanoid glycosides, but also some diterpenoids and triterpenoid glycosides in the more and mild active extracts **17**-**22** (Table 4).

Cell viability

Regarding the cell viability, *Achillea*, *Artemisia* and *Helichrysum* extracts from Asteraceae family, did not alter cell viability significantly and no loss of membrane integrity was observed. For low concentrations (0.1-1 µg/mL) of *Scrophularia*, *Plantago* and *Gentiana* extracts, no alteration of cell viability was observed. However, a loss of viability (50-80 %) was observed at higher concentrations (10-100 µg/mL). Extracts **15** and **16** obtained from *S. umbrosa* responsible of a weak protection from $P2X_7R$ activation, were not responsible of loss of viability or membrane integrity even at 100 µg/mL

Discussion

Anti-inflammatory and antioxidant proprieties of extracts Species from Asteraceae family produce phenolic compounds (flavonoids, phenolic acids) and terpenoids, and specially sesquiterpene lactones, which represent a chemotaxonomic marker of the family (Emerenciano et al., 2001; Seaman, 1982). Lamiales and Gentianales orders, including the Plantaginaceae, Scrophulariaceae and Gentianaceae species produce phenolic acids, flavonoids and many different terpenoid compounds, from monoterpenes to di- and tri-terpenes. Iridoids, which are glycosylated monoterpenes are largely represented in this order, especially in Plantaginaceae, Scrophulariaceae and Gentianaceae families (Bakuridze et al., 1987; Pasdaran and Hamedi, 2017; Rønsted et al., 2000). In our study, total phenolic content of tested extracts is correlated to DPPH and ABTS radical inhibition. However, PCA analysis did not reveal a meaningful cluster separation for TPC and TFE. This suggest that no chemotaxonomic marker strongly influence the radical scavenging activity (DPPH and ABTS) in our extracts. This is obviously related to the ubiquitous nature of phenolic compounds. On the contrary, statistical analysis for the inhibition of $P2X_7$ receptor activation revealed a good segregation of active and not active extracts, revealing two distinct clusters for both groups. Meanwhile the active cluster is represented by the Lamiales and Gentianales orders extracts, whereas the inactive cluster is composed by the Asteraceae family extracts. In consequence, this suggest that chemotaxonomic markers from *Achillea*, *Artemisia* and *Helichrysum* do not block the activation of $P2X_7$ receptors. On the other hand, chemotaxonomic markers of Lamiales and Gentianales species are able to inhibit activation of the $P2X_7$ receptors during the inflammation process.

Interestingly, our extracts have been slightly segregated regarding their XO inhibition, but this discrimination is not based on their phylogenetic classification. This may be explained by the fact that

different groups of natural compounds related to both clades are able to inhibit XO. Polyphenol such as flavonoids are known to inhibit XO with in some cases lower $IC_{50}$ values in comparison of allopurinol (Chang et al., 1993; Cos et al., 1998; Lin et al., 2002). On the other hand, some iridoids and phenylethanoids also exert moderate to high inhibition of XO (Cheng et al., 2015; Sampath Kumar et al., 2005; Vitale et al., 2017). In addition, the phenylethanoid isoacteoside isolated from Brandisia hancei (Scrophulariaceae) has also showed XO inhibitory proprieties at same order as allopurinol (Kong et al., 1999).

Iridoids as potential inhibitors of $P2X_7$ receptor activation

Regarding the $P2X_7$ activation assay, the clear difference between Asteraceae family and Lamiales or Gentianales orders revealed a strong link between their chemotaxonomic markers and $P2X_7$ receptor activation. Our efforts were then focused on eight *Scrophularia* extracts, revealing high levels of inhibition of all extracts except *S. umbrosa* extract, showing only decrease of 30% of $P2X_7$ activation at the lower concentration of 0.1 µg/mL.

Dereplication and statistical analysis of *Scrophularia* samples, lead to the identification of iridoids such as harpagoside, harpagide and in a lower degree aucubin and the phenylethanoid osmanthuside B, which were the highest contributors to the protective effect against $P2X_7R$ activation. PCA analysis showed that polyphenol and flavonoid content is not correlated with this activity. Indeed, anti-inflammatory properties of harpagoside have been largely described in the literature. Harpagoside is the major bioactive compound of *Harpagophytum procumbens*, a very used herb as anti-rheumatic agent in the European traditional medicine. From previous studies, harpagoside showed inhibitory effect on LPS induced-NO synthase in RAW 264.7 cell line and diminution in the expression of COX-2 and iNOS mRNA in HepG2 cell line (Huang et al., 2006). Furthermore, harpagoside exhibited dose-dependent inhibition of the LPS-induced NF-κB transcriptional activity with a $IC_{50}$ of 96,4 µM, inhibiting the production of IL-1β, IL-6 and TNF-α (Huang et al., 2006; Inaba et al., 2010). Also harpagoside inhibits the production of the leukotriene Cys-LT (cysteinyl-leukotriene) and TXB2 (thromboxane B2) *in vitro* (Loew, 2001). In mechanistic studies of anti-inflammatory activity of harpagoside, a cell free assay, looking for the interaction of this iridoid with the COX1-2 enzyme, showed not diminution of the activity of these enzymes (Zhu et al., 2015).

Conclusions

Our results suggest that iridoids producing species from the Lamiales and Gentianales orders such as *Scrophularia*, *Plantago* and *Gentiana* species are able to significantly decrease $P2X_7R$ activation in case of inflammation. Presence of the cinnamoylated iridoid harpagoside in *Scrophularia* extracts was

positively correlated to this activity. Thus deeper research on the effect of cinnamoylated iridoids on $P2X_7R$ activation is needed.

Acknowledgements

Authors would like to thank the French-Serbian bilateral program PAVLESAVIC for funding, Mr Rolland DOUZET from the Station Alpine Joseph FOURIER for providing alpine samples, the CNPMAI for providing other French species, and the Primivege Platform from UMR 8038 for UPLC-MS analysis.

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## Conclusion, contribution and perspectives

The main conclusion we may postulated on basis of this work would be that the anti-inflammatory activity of iridoids as harpagoside could be exerted by blockage of purinic receptors as P2X7, obstructing the activation pathways, which leads to an inflammation response.

Deeper studies must be carry out to explore the antagonist role of iridoids derivatives as harpagoside on the membrane purinic receptors, with the aim to elucidated the anti-inflammatory mechanism of action of these compounds.

Personal contribution:

- Obtention of hydromethanolic extracts by ultrasonic extraction of plant material.
- Antioxidant and andtixanthine oxidase in vitro evaluation of extracts.
- Statiscal analysis of LC-MS data of extracts correlated with their biological activity.
- Dereplication of Scrophularia extract LC-MS data.
- Redaction of original draft manuscript.

# PART II CHEMOTAXONOMIC APPROACH IN THE PHARMACOMODULATION OF FLAVONOIDS AND SESQUITERPENE LACTONES

# Introduction

As seen in **Part I** of this manuscript, medicinal plants from different origenes are a priceless source of bioactive compounds. These metabolites result from the evolutionary process, in which the plants have developed defences against pathogens (herbivores, microbes, viruses or competing plants) or communication (pollinators attraction or seed dispersing animals) systems [144,145].

These mechanisms are generally not exclusive to one species, related species (genus, tribe, family) often produce similar chemical structures.

Chemotaxonomy is defined as the organization of species based on the biosynthesis of similar metabolites. Thus, chemotaxonomic approach is a powerful tool in the discovery of natural products with close chemical structures and consequently similar biological activities.

In that way, sesquiterpene lactones and polymethoxyflavonoids (PMFs) are major chemotaxonomic element of the Asteraceae family. The wide variety of chemical skeletons so far discovered matches with a diversity of biological activities such as cytotoxic, antibacterial, antifungal and antiparasitic [146,147].

In this context, the search for bioactive compounds by a chemotaxonomic approach within the Asteraceae family was performed, exploring antiparasitic and cytotoxic activities. The investigations have started in 2013 with the collection of 13 medicinal plants including asteraceaeous, from Ahaggar Mountains (South Algeria), followed by PSE protocol. *In vitro* antiprotozoal activity and heme-binding affinity were measured. *Pentzia monodiana* Maire (Asteraceae) was, due to its antiplasmodial activity and the affinity of compounds to form adducts with heme, subjected to fractionation and purification of metabolites. Six sesquiterpene lactones and four PMFs were isolated and their biological activities evaluated. Two PMFs have demonstrated moderate antiplasmodial activity and stable heme-adducts (**Publication 9**). These results encouraged us to further explore the blockage of hemozoin by heme-adducts formation.

In a next step, a library of PMFs with several substitution pattern family was semisynthetized from isolated and commercial compounds and subjected to antiplasmodial (chloroquine sensitive and resistant strains), cytotoxic (HUVEC cells) and heme-adduct stability evaluation (**Draft 10**).

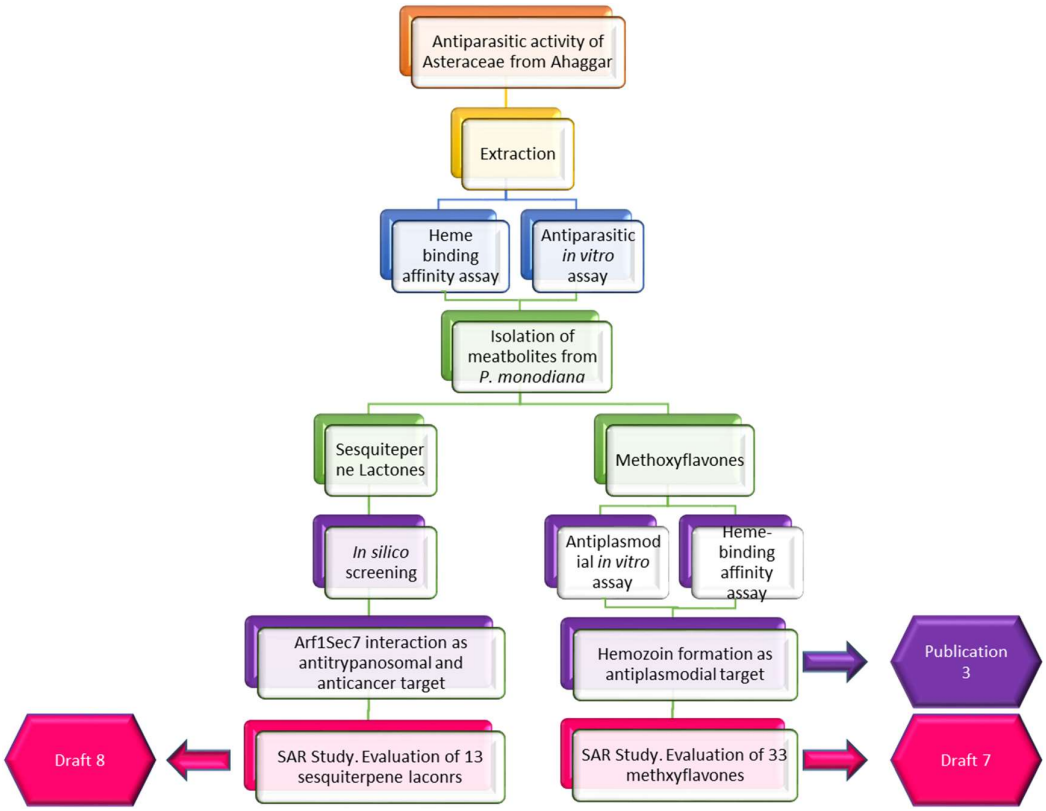


**Figure 82: Organigram of pharmacomodulation studies of compounds isolated from *P. monodiana*.**

Simultaneously, the sesquiterpene lactone ketopelenolide B (**K1**) isolated from *P. monodiana*, has displayed moderate antitrypanosomal activity. *In silico* evaluation of **K1** in an Inverse Virtual Screening with more than 2000 proteins (PDB) structures was performed (7). Among others, **K1** presented in silico affinity against human Arf1-Sec7 protein-protein system, which plays a major role in the cellular vesicle formation and tumor progression. In addition, remarkable structural similarity between **K1** and the Arf1-Sec7 known antagonist, brefeldin A, was observed. Furthermore, a BLAST analysis showed the presence of trypanosomal Arf1 (tArf1) protein expressed by *Trypanosoma brucei brucei*. These preliminary results led us to deeper study the potential of Arf1-Sec7 affinity activity by ketopelenolide derivatives for trypanosome growth inhibition and cytotoxicity.

Thirteen original derivatives were semisynthetized from **K1** and *in vitro* Arf1-Sec7 system inhibition was measured. 3-Ethoxyimin-ketopelenolide B (**K7A**) presented a stronger inhibitory activity in comparison with **K1**. Evaluation towards other Sec7 proteins confirmed the specificity of **K7A** for this system, with similar inhibitory profile as brefeldin A (**Draft 11**). *In vitro*

antiproliferative activity of derivatives against several cancer cell lines is actually in progress, as well as *in vivo* evaluation with tArf1.

In the next two chapters, after some details about chemotaxonomy and biosynthetic pathways, are detailed and discussed the pharmacomodulation studies targeting heme-adducts formation and Arf1-Sec7 protein-protein interaction of PMFs (**Chapter IV**) and sesquiterpene lactones (**Chapter V**) isolated from *Pentzia monodiana* (Maire).

## Classification of Asteraceae family

Last classification of Angiosperm Phylogeny Group (APG IV) published in 2016 (and based on nuclear (mitochondrial and plastid analysis), has classified Asteraceae family within the Asterales order in the asterids clade (superasterids major clade). Cornales and Ericales orders are directly split from Asterids. The remaining orders cluster into two clades, the lamiids and the campanulids.

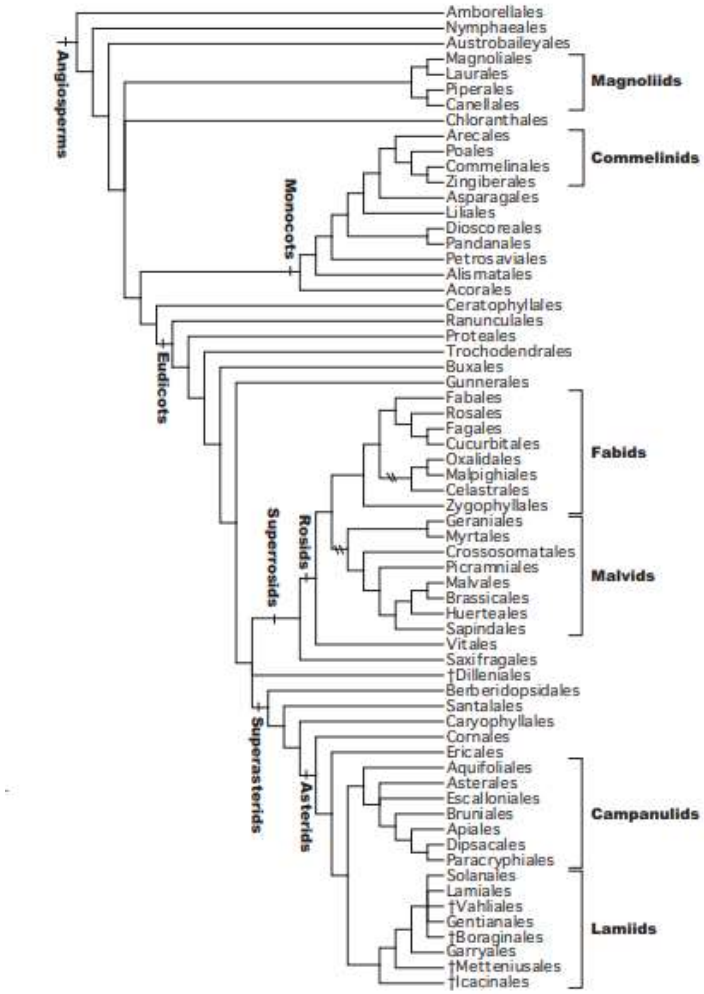


**Figure 83: Interrelationships of the APG IV orders and some families** [148]

Asterales order includes 11 families, the largest are Asteraceae (about 25,000 species) and Campanulaceae (about 2,000 species).

Asteraceae family is divided in 13 subfamilies for 1,911 genera. Almost all of the species bear composite flowers in dense heads (capitula) surrounded by involucral bracts. The Asteraceae name comes from ancient greek ἀστήρ, which means “star”, referring to the star-like form of inflorescences. Most of them are annual or perennial herbs, but a significant number are shrubs, vines or trees with worldwide distribution.

## Elements of chemotaxonomy and biosynthesis of the Asteraceae family

The chemistry of Asteraceae is rich, complex and has evolved along the Evolution. Early-branched clades seem to be less specialized than younger groups. Storage of polysaccharide inulin is a common trait, which is also shared with most other members of Asterales. Sesquiterpene lactones and PMFs are often associated with Asteraceae, such as acetylenes (mainly in the resin ducts), also detected in the Goodeniaceae family (Asterales).

# Sesquiterpene lactones as chemotaxonomic markers of Asteraceae family

Sesquiterpene lactones constitute a major class of metabolites with lipophilic character, generally colourless and bitter, found in the Asteraceae but also occuring in some other botanical families. The backbone is made of fifteen carbons, from condensation of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate catalysed by the action of farnesyl diphosphate synthase (FDS). This enzyme is mainly located in the cytosol and in the mitochondria of the plant cells.

Then, several rearrangements occur. The first step is the cyclisation of FPP by sesquiterpene synthases (STPs). The best studied STPs is germacrene A synthase (GAS), converting FPP into germacrene A, which is the precursor of germacranolide type of sesquiterpene lactones [149]. Then, the isopropenyl chain of germacrene A is oxidized in allylic position by the cytochrome P450 enzyme germacrene A oxidase (GAO) to form GOL, which is further oxidized to GAL and GAA [150,151].

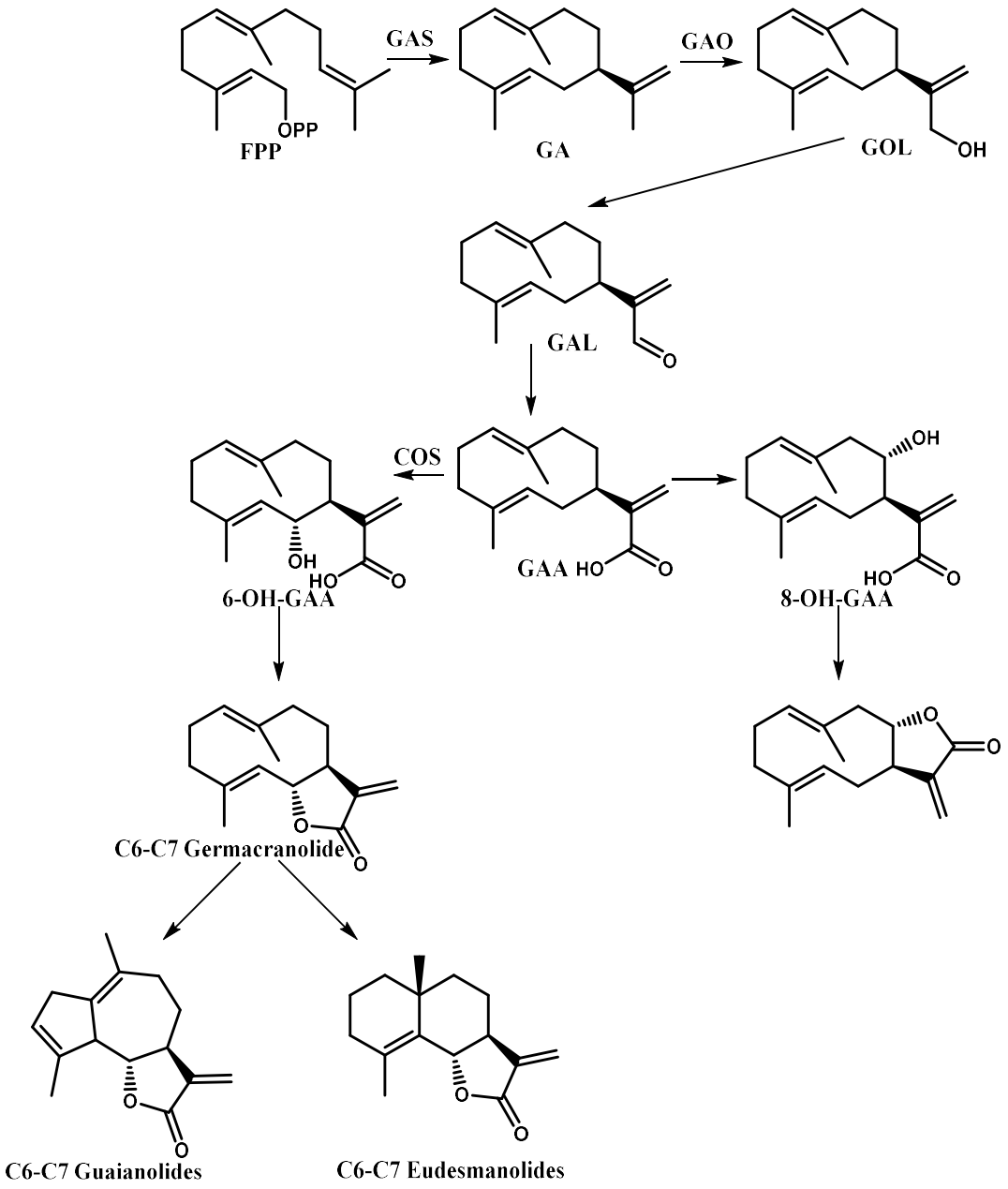


**Figure 84: Biosynthesis of germacranolide, guaianolide and eudesmanolide-types sesquiterpene lactones from FPP.**

GAA is then hydroxylated in allylic position C-6 by costunolide synthase (COS), resulting in an unstable intermediate (6α-OH-GAA), which cyclized by dehydratation into costunolide, the precursor of C6-C7 costunolide-types sesquiterpene lactones. If hydroxylation occurs in position C-8 instead of C-6, further cyclizations will provide eudesmanolide and guaianolide skeletons [150].

There are several types of sesquiterpene synthases. In addition, further modifications such as oxidation, reduction, hydrogenation, carboxylation, glycosylation may happen, and explain the hudge number of identified structures: over 5000 derivatives have still been described [152].

From an ecological point, they act as antifeedants, hormones [153], allelochemicals and UV protection [154]. Toxicity to insects has also been reported: while attracting predators and "warning" other nearby plants, they allow them to rise their defences in anticipation of insect-attacks [155].

## Biological activities of sesquiterpene lactones

Numerous studies dealing with biological evaluations of sesquiterpene lactones have been achieved. Besides those dealing with cytotoxicity and anti-tumor potential [156], prevention of neurodegeneration [157–160], anti-inflammatory [161–165], sedative and antimicrobial [166–169] activities have been investigated. In addition, some derivatives have shown strong potential in the treatment of cardiovascular and parasitic diseases. This last point together with cytotoxicity will be further developed.

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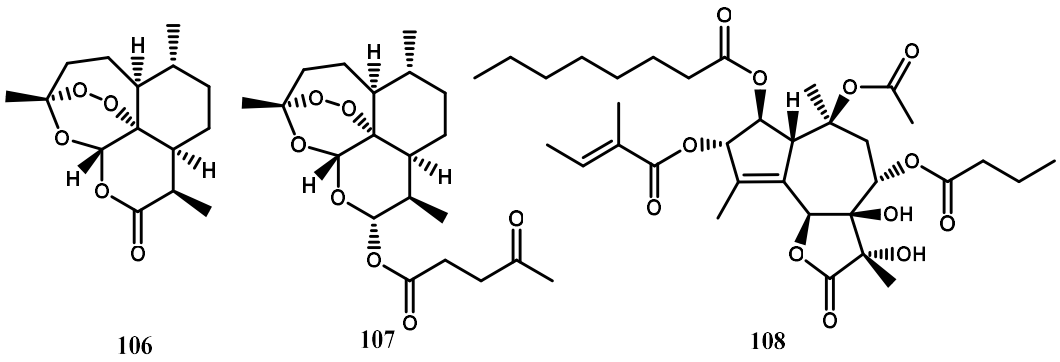


**Figure 85: Chemical structures of artemisinin 106, artesunate 107 and thapsigargin 108.**

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## Antiparasitic activity

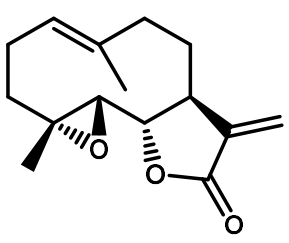
### Antiplasmodial activity

Artemisinin isolated from *Artemisia annua* and its semisynthetic derivatives have become the standard treatment of *P. falciparum* malaria, administrated as artemisinin-combination therapy (ACT). Under this form, it reduce the development of resistance by the parasites.

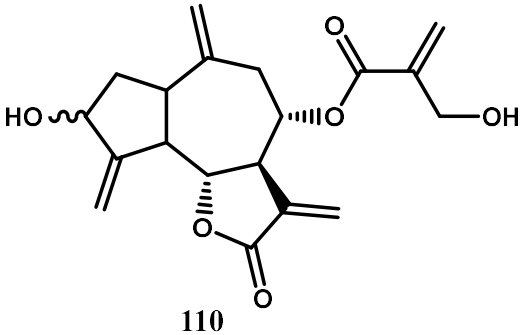
Mechanism of actions of artemisinin is not completely deciphered, due of the complex interactions involved. The drugs undergo physiological chemical modifications, resulting in their active form, dihydroartemisinin (DHT). Activation of DHT is carry out by $Fe^{+2}$ of heme groups (liberated after haemoglobin degradation), which breaks the endoperoxide ring, producing free radicals that damage susceptible proteins, which are involved in the essential biological processes of the parasite [170]. Several proteomic approaches have allowed the identification of artemisinin binding with more than 120 proteins and with heme [171]. Such broad targeting spectrum disrupts the biochemical landscape of the parasite and causes its death. Unfortunately, emergence of parasite resistance to ACT has been recorded and new cases are reported each year since 2000 [172].

### Antitrypanosomal activity

Antitrypanosomal *in vitro* and *in vivo* activities against several *Trypanosoma* species have been reported. Costunolide derivatives have demonstrated low *in vitro* $IC_{50}$ values (between 0.62-0.019 µg/mL) against *Trypanosoma brucei brucei* GUTat 3.1 strain. Parthenolide **109** was 83-112 fold more potent than reference drugs eflornithine and suramin respectively [173].




**109**

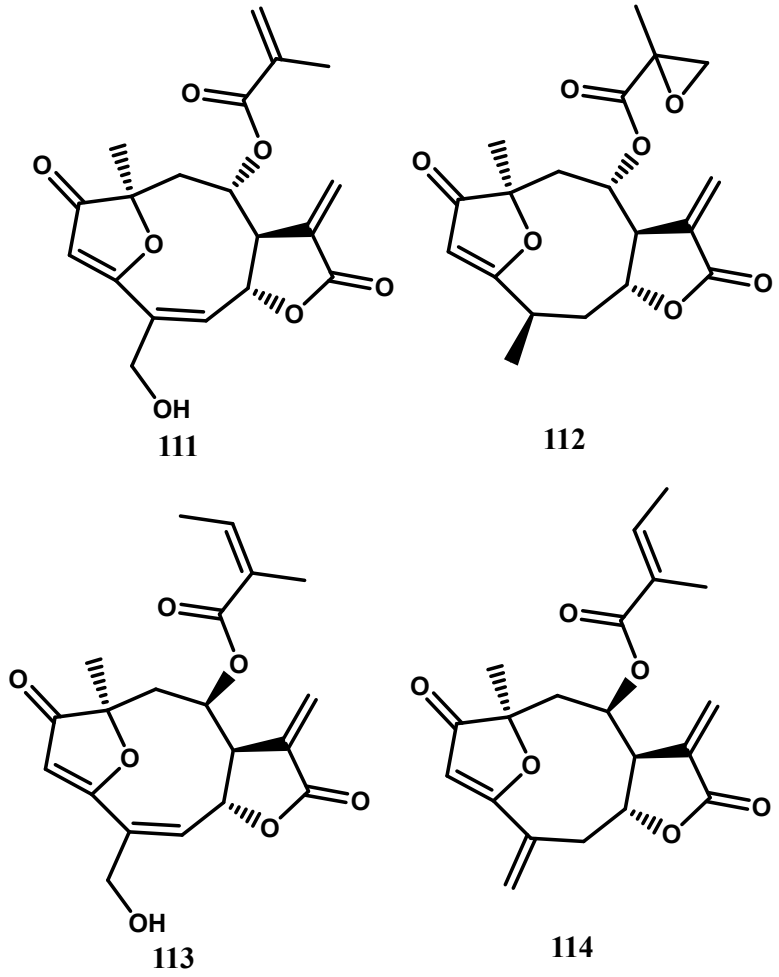



**110**

**Figure 86: Chemical structures of parthenolide 109 and cynaropicrin 110.**

A large screening of 1800 plants and fungi extracts carried has led to the identification of antitrypanosomal activity for an apolar extract of *Centaurea salmantica* L. (Asteraceae). HPLC-based profiling allowed to identify cynaropicrin **110** as a potent *in vitro* inhibitor of *Trypanosoma brucei gambiense* ITMAP 141267, *T. b. rhodesiense* STIB 900 and *T. cruzi* species with $IC_{50}$ values of 0.2, 0.3 and 4.4 µM, respectively. Intraperitoneal administration of 10 mg/kg body weight/day during 2 days in the *T. b. rhodesiense* acute mouse model led to a 92% reduction of parasitemia compared to untreated controls [174].

*In vitro* antitrypanosomal activity of furanoheliangolide-type sesquiterpene lactones, such as goyazensolide **111**, 4,5-dihydro-2',3'-epoxy-15-deoxygoyazensolide **112**, budlein A **113** and 4,15-isotriplicolide tiglate **114,** was reported with $IC_{50}$ values of 0.07, 0.2, 0.07 and 0.15 µM against *T. b. rhodesiense* STIB 900 strain [175].




**111** **112**

**113** **114**

**Figure 87: Chemical structures of furanoheliangolides.**

The antitrypanosomal mechanism of action of sesquiterpene lactones has not been extensively studied. Psilostachyin C, a sesquiterpene lactone isolated from *Ambrosia scabra* (Asteraceae) which induces apoptosis of *Trypanosoma cruzi* epimastigotes, is active *via* inhibition of the biosynthesis of ergosterol, causing an accumulation of squalene and the death of parasites [176].

## Cytotoxic activity

Anti-cancer/cytotoxic activity is one of the most explored biological activity for sesquiterpene lactones. Several subclasses have shown *in vitro* and *in vivo* strong potential against several of cancer cell lines. Some derivatives have been subjected to clinical trials. During phase 1, the safety and tolerance dose in metastatic breast cancer patients of semisynthetic artesunate **107** have been determined [177]; mipsagarin (pro-drug of thapsigargin **108**) has also reached phase 2, where the safety and efficacy against renal carcinoma [178], hepatic cancer [179], prostatic neoplasms [180] and glioblastoma multiform [181] will be deeper assessed.

Two major chemical features have been identified as responsible for the anti-cancer properties of sesquiterpene lactones: Michel-receptor and side chain/lipophilicity.

### Alkylating centre reactivity – Michel receptor

Since the early 70's, the α-methylene-γ-lactone moiety has been identified to be deeply involved in the cytotoxic activity of sesquiterpene lactones. This moiety reacts as a Michel receptor with biological nucleophiles like thiol containing cysteine residues of proteins. This major role was also demonstrated for *in vivo* anti-tumor effect. It is responsible for modifications through Michael reaction, e.g. regulation of gene expression by deactivating/activating transcription factors. Additional carbonyl α-β unsaturated moiety has been suggested to strengthen Michael-receptor affinity [182,183].

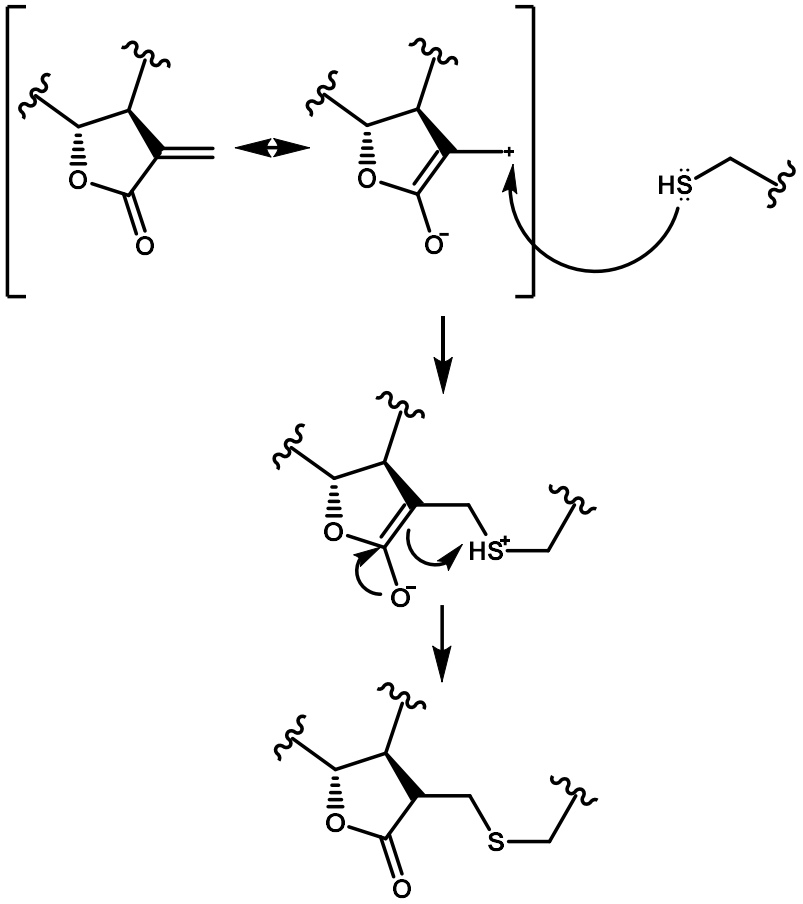


**Figure 88: Michael addition reaction between α-methylene-γ-lactone moiety and cysteine residues of proteins.**

Nuclear factor kappa-light-chain-enhanced of activated B cells (NF-κB) is a protein complex of two subunits (p50 and p65) that mediates immune response in humans by regulating the transcription of DNA, production of cytokines, inflammatory effectors and cell adhesion compounds. It also modulates apoptosis and metastasis pathways and act as transcription factor on several genes when it is released from the NF-κB inhibitors (IκB).

It has been postulated that the α-methylene-γ-lactone moiety may react with the cysteine residues (Cys 38 and Cys 120) of the p65 subunit of NF-κB, forming stable covalent bonds and inhibiting the transcriptional activity of this complex [184].

Parthenolide **109** has shown *in vitro* and *in vivo* anti-cancer activity against several cancer cell lines. It has inhibited proliferation of OUR-10 renal cancer cell line and has triggered *in vitro* apoptosis *via* inhibition of NF-κB. Subcutaneous injection or oral administration of parthenolide has led to significant *in vivo* tumor growth inhibition *via* decreased production of IL-8 cytokine and vascular endothelial growth factor (VEGF). The analysis of the tumors have revealed a

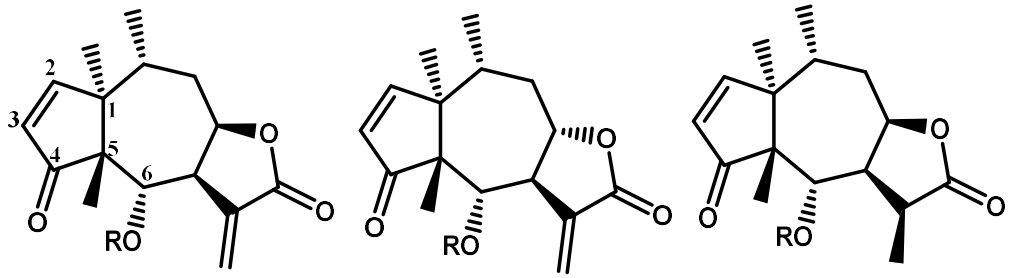
decrease of localization and phosphorylation of NF-κB complex and of the subsequent expression of MMP-9, Bcl-xL and COX-2, in comparison with the untreated control [185].

**Side chain/lipophilicity**

It has been demonstrated that the esterification of hydroxyl groups in the sesquiterpene lactones structures increase the lipophilicity, facilitating the penetration through the cell membrane, increasing the *in vitro* cytotoxic activity. However, this correlation is limited by the chain length, due the decrease of solubility in physiological fluids.

For derivatives with two Michael receptors, there is a positive correlation between the lipophilic conjugated ester added to the structure with the cytotoxic activity. This was observed for helenalin **115** and mexicanin I **116** derivatives bearing esters in position C-6, when evaluated *in vitro* and *in vivo* against Ehrlich ascites carcinoma [186]. On the contrary, the *in vitro* cytotoxic activity of 11α,13-dihydrohelenalin **117**, which has only one Michael receptor, was limited by the lipophilic character of the adjacent esterification moiety. Higher-sized esters have led to an important loss of solubility and of *in vitro* activity [186].

Thapsigargin **108**, a higly esterified derivative isolated from *Thapsia* genus (Apiaceae) diffuses through the intracellular membranes of cancer cells and blocks the SERCA pump through lipophilic interactions, inhibiting the transportation of $Ca^{+2}$ from the cytosol into the endoplasmic reticulum, increasing the $Ca^{+2}$ concentration in the cytosol, stressing the ER and causing subsequent cell death. Toxicity towards normal cells has also been reported; then, prodrug of thapsigargin targeting prostate-specific antigen enzymes of prostate cancer achieved to increase the concentration of the cytotoxic agent selectively into cancer cells.




**115** **116** **117**

**Figure 89: Chemical structures of helenalin 115, mexicanin I 116 and 11α,13-dihydrohelenadin 117.**

# Flavonoids as chemotaxonomic element of Asteraceae family

Flavonoids, a class of polyphenols with as 15-carbons skeleton that consists of two phenyl (A and B) and a heterocyclic ring (C), have been isolated in great scale from Asteraceae species. Due to their wide structural diversity, they were postulated as taxonomic markers at low hierarchical levels. Major diversity was found in the Asteroideae subfamily, mainly flavonols were detected in the Barnadesioideae subfamily. The ratio flavone/flavonol has been proposed as an indicator of evolutionary process, proposed by Harborne *et al*. and later corroborated by emerciano *et al*. [187,188]. In Barnadesioideae species, high levels of flavonols are in accordance with studies using chloroplast DNA (cpDNA), which placed this subfamily at a basal position within the Asteraceae family [187,188].

Biosynthesis of flavonoids occurs through shikimate pathway, which provides phenylalanine. The enzymes of the shikimate biosynthetic pathway are conserved between different species of plants, bacteria and fungi. Then, phenylalanine ammonia lyase (PAL) followed by cinnamate 4-hydroxylase (C4H) and 4-coumarate-CoA ligase (4CL) are acting. These enzymes are involved in the early steps of the phenylpropanoid pathways of a variety of metabolites such as lignins, lignans, coumarins, stilbenoids and flavonoids. The next step is catalyzed by chalcone synthase (CHS), which condensates 4-coumaroyl-CoA with three acetate units from malonyl-CoA to generate naringenin chalcone, precursor of all flavonoids.

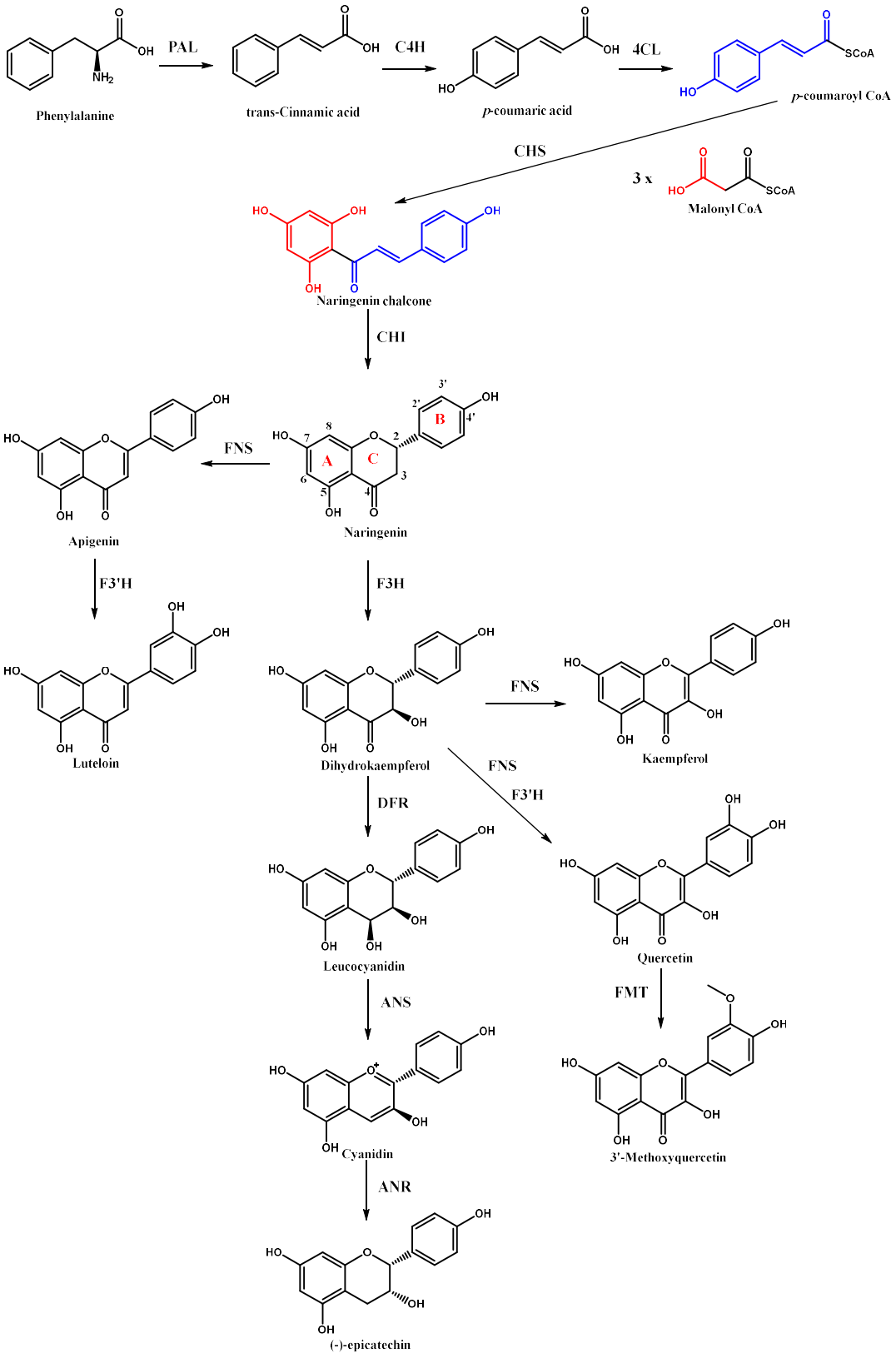



**Figure 90: Biosynthesis pathway of flavonoids in plants.**

Then, chalcone isomerase (CHI) catalyzes the stereospecific cyclization of naringenin chalcone to (2*S*)-naringenin. Flavone synthase (FNS), belonging to the cytochrome P450 family, dehydrogenates the C-2 and C-3 positions on the C-ring. FNS shares its substrate, naringenin, with F3H (flavanone 3β-hydroxylase), which converts (2*S*)-flavanones to dihydroflavonols (dihydrokaempferol). Other cytochrome P450 monooxygenases (under CYP75B group) are the F3'H and F3'5'H that add hydroxyl groups at 3'- and/or 5'-position of the B-ring of flavonoids [189,190].

Flavonoid methyltransferases are involved in the methylation of hydroxyl groups of flavonoids. The transfer of methyl groups is carried out from *S*-adenosyl-L-methionine to hydroxyl group producing *S*-adenosyl-L-homocysteine [191].

Flavonoids are responsible for colour and aroma of flowers and fruits, thus attracting pollinators and contributing to pollen and seeds dissemination. They also protect plants from several kinds of biotic and abiotic stress, acting as UV-filter signal molecules, phytoalexins and antimicrobial defensive compounds. Roles in drought resistance and freezing tolerance have also been attributed to flavonoids [192].

# Biological activities related to flavonoids isolated from Asteraceae family

## Antioxidant activity

The antioxidant activity of flavonoids is deeply connected to the degree and position of hydroxyl groups. Several mechanisms have been described, such as radical scavenging and metal ion chelation ability. Regarding the direct radical scavenging activity, the low redox potential of flavonoids allows thermodynamical reduction of highly oxidized/oxidizing free radicals such as superoxide, peroxyl and hydroxyl radicals by hydrogen atom donation. Due to their capacity to chelate metal ions, flavonoids can also inhibit free radicals formation.

3',4'-Catechol in the B ring, double bond on position C2-C3 and 4-oxo group are fundamental for antioxidant activity. Generally, O-methylation of hydroxyl groups decreases the radical scavenging capacity, also observed in the case of glycosides in comparison with the corresponding aglycones.

In addition, flavonoids indirectly act by inhibition of ROS-producing enzymes, such as microsomal monooxigenase [193], glutathione S-transferase [194,195], mitochondrial succinoxidase [196,197], NADH oxidase [198], xanthine oxidase [199,200], among others.

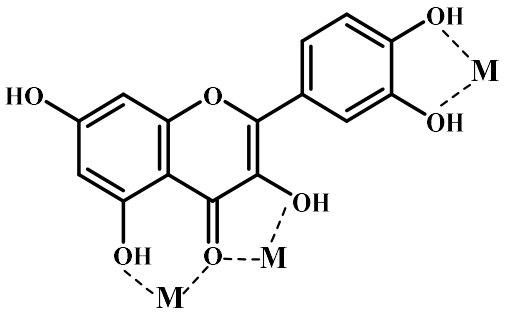



**Figure 91: Chelation position for a quercetin derivative skeleton.**

## Anti-inflammatory activity

As before mentioned, inflammation is a biological response to tissue injuries, microbial pathogen infections and chemical irritations. Relationships between inflammatory process with oxidative stress were discussed in **I.5.1**. Flavonoids may specifically affect the function of enzymes systems critically involved in the generation and progression of inflammation, like tyrosine and serine-threonine protein kinases [201–203].

Apigenin has shown inhibition properties of HIF-1α and VEGF expression by blocking PI3K/Akt signaling and LPS-stimulated pro-inflammatory cytokines expression by blocking NF-κβ through the suppression of p65 phosphorylation [204]. Luteolin has supressed IFNγ-induced CD40 expression and the production of TNF-α and IL-6 cytokines through downregulated phosphorylation of STAT-1 in microglia. In addition, it was found to regulate MAPK and NF-κβ signaling that inhibits TNF-α-induced production of the pro-inflammatory cytokine IL-8 [205].

Tangeretin, a polymethoxyflavones from tangerine and other citrus peels, suppresses IL-1β-induced COX-2 expression through inhibition of MAPK and Akt in human lung carcinoma cells [206]. The polymethoxyflavone nobiletin, isolated from citrus peel as well, inhibits leukocyte infiltration, protein expression of iNOS and COX-2 as tumorigenesis in mouse skin model. Nobiletin derivatives have inhibited 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-induced tumor promotion and skin inflammation by suppressing MAPK and PI3K/Akt pathways [207].

The common flavonols kaempferol and quercetin were reported to decrease excessive pro-inflammatory cytokines. Kaempferol antagonized TNF-α-induced p65 translocation and production of IL-6 and MCP-1 [208]. Quercetin decreased the level of circulating inflammatory markers, including IFNγ, IL-1α and IL-4 in high fat diet animal models [209]. Oral feeding with quercetin significantly reduced the size of atherosclerotic lesion in the aortic sinus and thoracic

aorta through decrease of superoxide generation, improving endothelial NO synthase (eNOS) function in the apolipoprotein E (ApoE)(-/-) gene knockout mouse model [210].

In high cholesterol-fed New Zealand white rabbits, diet supplemented with the flavanone naringenin has reduced gene expression of vascular cell adhesion factors such as VCAM-1, ICAM-1, and MCP-1 in endothelial cell. It has also decreased the plasma lipoprotein levels, total cholesterol, triglyceride and high-density lipoprotein (HDL) [211,212].

## Antibacterial activity

As flavonoids biosynthesis is, among other purposes, a response to microbial infections, it is not surprising that flavonoids-rich plant extracts as well as pure compounds demonstrate antibacterial activity [213].

Recently, the prenylated flavone gancaonin Q and the bisflavone amentoflavone isolated from *Dorstenia* spp. (Moraceae) showed MICs values against *Bacillus* spp. of 2.4 and 3 µg/mL. The formation of complex with the cell wall components and consequently inhibition of further adhesions and of the microbial growth were reported [214,215].

Other mechanism of action involved the downregulation of quorum-sensing regulators: baicalein was able to downregulate the regulators agrA, RNAIII, sarA and gene expression on intracellular adhesion in *Staphylococcus aureus* bio-film production at 64-32 µg/mL [216].

Artonin I was active against *Staphylococcus aureus* by inhibition of bacterial efflux pump, increasing the susceptibility of commercial antibiotics [217].

Quercetin showed also antibacterial activity against *Staphylococcus aureus*, methicillin-resistant *S. aureus* and against oral bacteria with MICs values until 0.0125 µg/mL (*Porphyromonas gingivalis*). Positive synergy with amoxicillin was also observed [218].

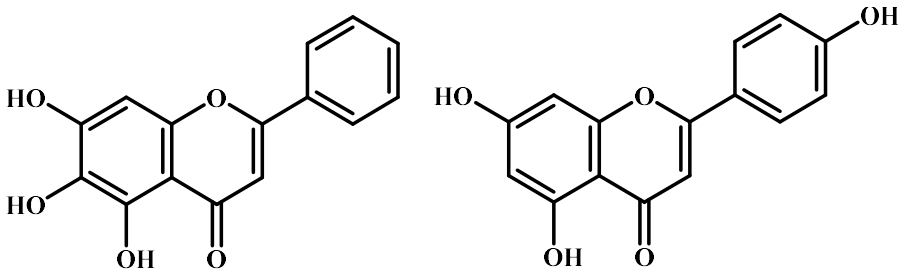
Other mechanisms of action of flavonols (quercetin, kaempferol, myricetrin, morin, galangin,...) have been recently deciphered including the inhibition of β-ketoacyl carrier protein synthase I (related to the elongation of unsaturated fatty acids in bacterial fatty acid synthesis); alteration of bacterial topoisomerase IV activity and inhibition of the formation or destruction of biofilm [219–222].

## Antiparasitic activity of flavonoids

Numerous results dealing with antiparasitic potential of flavonoids against several kinds of protozoa have been published. We will only provide some examples of antitrypanosomal and antiplasmodial activities.

## Antitrypanosomal activity

More than 100 polyphenols including several kinds of flavonoids (flavones, flavonols, flavanones isoflavones and flavon-3-ol) were evaluated *in vitro* against *Trypanosoma cruzi*, *T. brucei brucei* and L6 cell line. Three flavonoids were then selected for *in vivo* assay on *T. brucei brucei* STIB795 mouse model. Intravenous injection of 50 mg/kg/day of 7,8-dihydroxyflavone **106** for 4 days has increased the mean survival time from 6 (untreated control) to 13 days [223].




**118** **119**

**Figure 92: Structure of 7,8-dihydroxyflavone and apigenin.**

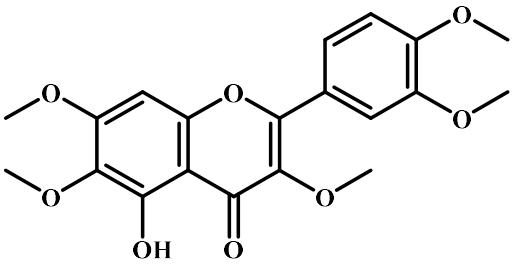
In contrast with humans, *Trypanosoma* are not able to synthetize their own purine bases essential to the parasite life cycle, and must take them from the host and modify them in order to synthetize their own purines (purine salvage pathway). Trypanosomal nucleoside hydrolase is the key enzyme that cleaves the N-glycoside bond of nucleosides to yield the purine bases. *In silico* evaluation showed an affinity for the planar structures of flavonoids in the pocket of active site. Apigenin **107** was the most promising compound and was proposed to *in vitro* evaluation [224].

## Antiplasmodial activity

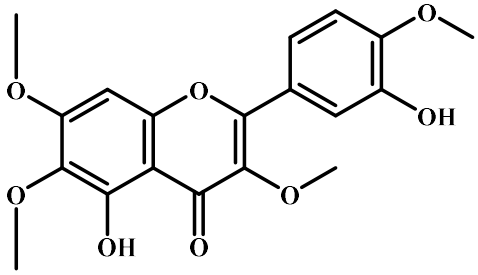
Eleven flavonoids from dietary sources were evaluated *in vitro* for antiplasmodial activity against 3D7 chloroquine-sensitive *P. falciparum* 7G8 chloroquine-resistant strains. Both were more affected by luteolin ($IC_{50}$ = 11 and 12 µM) and quercetin ($IC_{50}$ = 15 and 14 µM). Acacetin was effective against 7G8 strain ($IC_{50}$ = 13 µM) but not against 3D7 ($IC_{50}$ > 100 µM)[225].

The polymethoxyflavones artemetin, isolated from *Artemisia gorgonum*, showed antiplasmodial activity against *P. falciparum* FcB1 strain with an $IC_{50}$ of 3.5 μg/mL [226], while also 5,6,7,3',4',5'-hexamethoxyflavone, 4'-hydroxy-5,6,7,3',5'-pentamethoxyflavone, eupalitin and 5,6,7,5'-tetramethoxy-3',4'-methylenedioxyflavone from *Ageratum conyzoides* (Asteraceae) have demonstrated $IC_{50}$ values of 7.43, 9.25, 10.99 and 11.04 μM against K1 strain [227].

Several flavonoids isolated form *Artemisia annua* (natural source of the sesquiterpene lactone artemisinin) showed moderate *in vitro* antiplasmodial activity. Artemetin **120**, casticin **121**, chrysoplenetin **122**, chrysosplenol D **123**, cirsilineol **124** and eupatorin **125** had $IC_{50}$ values of 26, 24, 23, 32, 36 and 65 μM against *P. falciparum*, much higher than artemisinin (0.03 μM) [228]. Nevertheless, they were able to potentiate the activity of artemisinin when administred in combination [229]. Antiplasmodial activity and synergism artemisinin were also assessed for quercetin [230].



**120**



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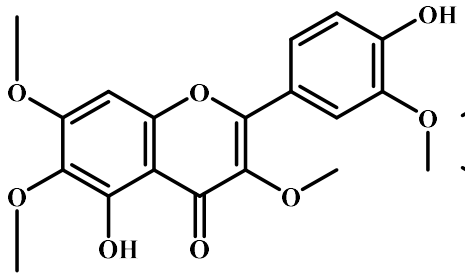

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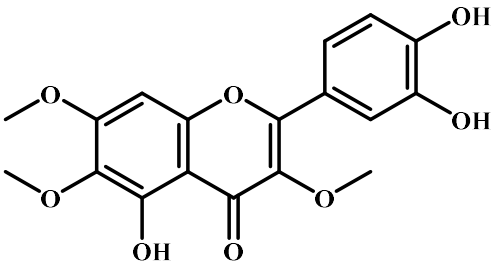

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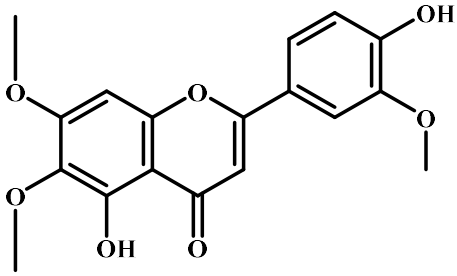

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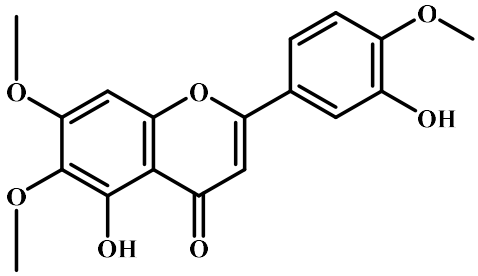

**125**

**Figure 93: PMFs isolated from *Artemisia annua* with synergistic effects with artemisinin.**

This synergistic effect was investigated considering:

- Homology between plasmodial with human serine-threonine kinase.
- Homology between plasmodial with human thioredoxin reductase (HTR). These enzymes are essential to the survival of the erythrocytic stage of *Plasmodium*. Some flavonoids such as quercetin are inhibitors of the human enzyme, and are therefore also expected to inhibit the plasmodial HTR [231,232].
- Heme-binding affinity: the toxic heme is detoxified to a non-toxic hemozoin crystal by the parasite; some flavonoids by binding heme may inhibit this process and kill the parasite.

The synergism between flavonoids with artemisinin could be explained from this latter mechanism. Indeed, artemisinin has a short half-life (2-5 hours) due to the fast metabolism in the human body carried out by cytochromes enzymes. As heme is structurally related to cytochromes, its inhibition by binding with flavonoids allows to upgrade the *in vivo* pharmacokinetics properties of the sesquiterpene lactone [233].

Inhibition role of flavonoids in the plasmodial fatty acid biosynthesis by flavonoids has also been explored; polyhydroxyflavones (luteolin, kaempferol, quercetin, fisetin, morin and myricetin) have exhibited strong activity toward the plasmodial FabG, FabZ and FabI enzymes with $IC_{50}$ values of 0.5-8 µM. Luteolin and (-)-catechin gallate were found as non-competitive inhibitor to FabG, FabI and competitive to FabZ [234].

# Chapter IV Antiplasmodial and heme-affinity activities of polymethoxyflavones

# IV.1 Introduction

## IV.1.1 Role of hemozoin formation in the *Plasmodium* parasites life cycle

Malaria kills around one million people every year. One of the major obstacles in the control and eradication of malaria is the rise of drug resistance worldwide. Current protocols for uncomplicated malaria are based on chloroquine in combination with primaquine for chloroquine-sensitive strains, or artemisinin (**106**) combination therapy (ACT). Quinine and artemisinin derivatives artemether **126** and artesunate **107** are available to treat severe forms of the disease. Resistance to chloroquine, sulfadoxine-pyrimethamine and ACT are rising in Africa and south east Asia. Thus, alternative are needed to find new targets and/or to overcome drug resistance by parasites [235–238].

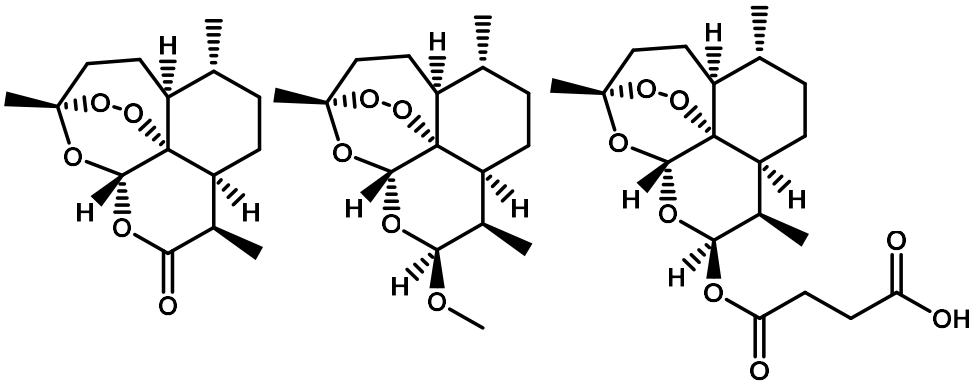


**Figure 94: Structure of artemisinin, artemether and artesunate.**

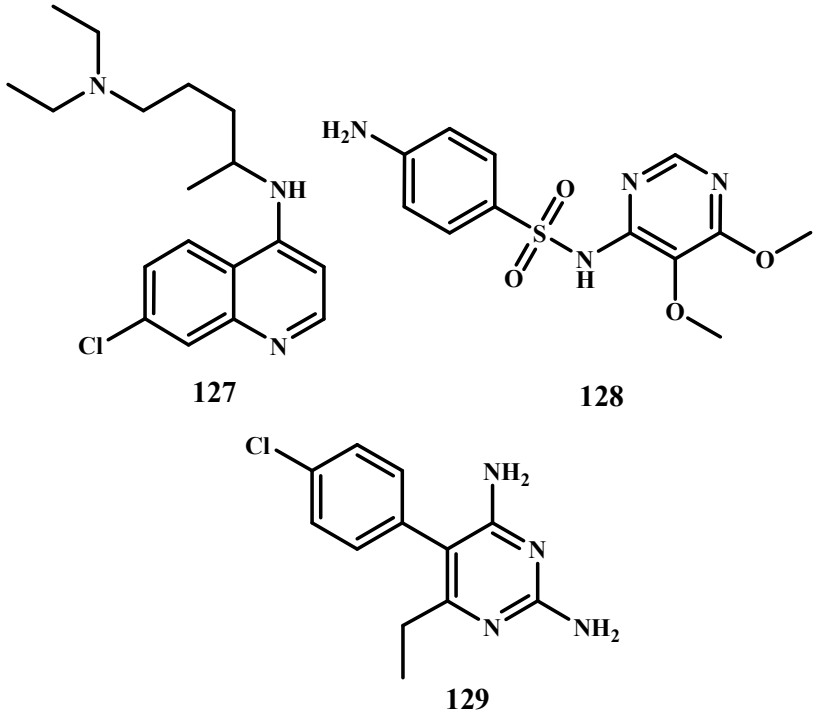


**Figure 95: Structures of chloroquine, sulfadoxine and pyrimethamine.**

Degradation of haemoglobin by the parasites during the red blood cell stage provides nucleoacids necessary to its development. Heme, the porphyrin part of haemoglobin, is not digested and is toxic for the parasite, which will turn it into non-toxic hemozoin [239].

Haemoglobin degradation occurs in the vacuole of the parasite at acidic pH (5.0 – 5.4) [240,241]. under actions of several enzymes, mostly plasmepsin I and II and falcipain [242,243]. In addition, a protein complex of about 200 kDa formed by falcipain, plasmepsin II and IV, histo-aspartic protease and Heme Detoxification Protein (HDP) is needed for this process [244].

Heme, toxic to the parasite, is then transformed in hemozoin (β-hematin) with paramagnetic properties. Hemozoin formation has been investigated as follows:

- Under non-biological conditions: formation was found to be spontaneous in acetate solution at moderately acidic pH [245,246].
- In presence of biological material: HDP converts heme into hemozoin in a very efficient way *in vivo*, with assistance of Histidine rich protein 1 (HRP1), Neutral lipid bend (NLB) and monopalmitoylglycerol (MPG) lipids [247–249].

Nevertheless the global mechanism still remains unclear.

The crystal structure of hemozoin has been studied by different techniques such as X-ray diffraction, infrared and Raman spectroscopy and chemical synthesis. The molecules were paired into dimers by iron-oxygen bonds, linked into dimers through reciprocal iron-carboxylate bonds to the propionic side chains of each porphyrin, and the resulted dimers form chains linked by hydrogen bonds within the crystal [250].

Finally, the spin state of the iron centre of hemozoin was elucidated by multi-frequency high field electron paramagnetic resonance studies. The obtained spectra for hemozoin and synthetic β-hematin could only correspond to a high spin of Fe(III) [251,252].

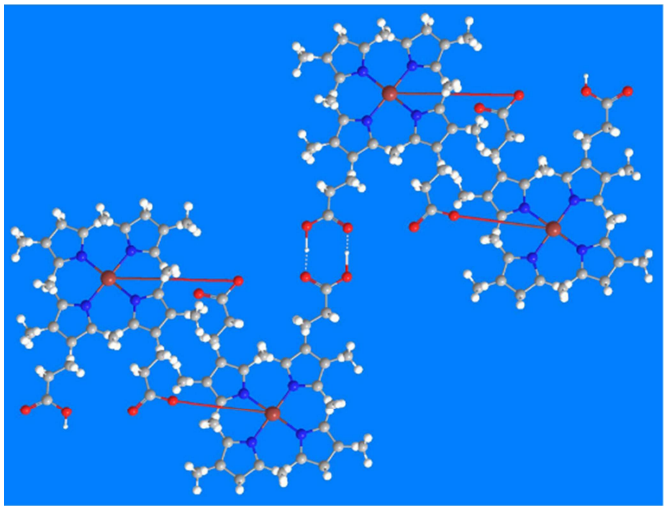

**Figure 96: Crystal structure of hemozoin.**

### IV.1.1.1 Hemozoin as drug target

Heme detoxification pathway is specific to the parasite and is the target of many antimalarial drugs including chloroquine and artemisinin. The mechanism of action of chloroquine by blocking hemozoin biocristallization is well-understood.

Chloroquine enters into the parasitic digestive vacuole through simple diffusion. Due the acidic pH inside, the molecule is protonated and cannot go back through the membrane, remaining trapped inside the vacuole. The protonated “salt” chloroquine interferes with detoxification process by inhibiting heme transformation into hemozoin, leading to the accumulation of heme causing the death of the parasites [253]

Studies have shown that chloroquine prevent hemozoin its assembly *via* non-covalent π-π interactions with adjacent porphyrins. This mechanism of action is now also accepted for other 4-aminoquinolines drugs (amodiaquine and hydroxychloroquine) [254–257].

The resistance of the parasites against chloroquine has emerged in most part of the world due to specific mutations in a chloroquine transport protein (PfCPT). This latter, present in the parasitic vacuole is responsible for the efflux of the protonated chloroquine, avoiding its accumulation in the organelle. In chloroquine sensitive strains, threonine 76 and serine 163 contribute to a positive charge in the active site of PfCPT, decreasing the transport activity. Specific mutations by lysine and arginine in the PfCPT sequence increases its activity, transporting the protonated form of chloroquine outside of the vacuole. However, the exact mechanism by which PfCPT works as transporter of chloroquine is still under progress [258,259].

In the context of increasing resistance to antimalarial drugs, hemozoin appears as one of the major target for the discovery of new effective agents. PMFs, by synergistic actions, could restore and enhance the biological activity of conventional treatments.

## IV.2 Personal work and collaborations

The laboratory of Pharmacognosy, now partner of NPAS team was involved in ChemBioFight European program (Exploring the Chemical Biodiversity with Innovate Approaches to Fight Chagas Disease and Leishmaniasis, PIRSES-GA-2009-269301, 2011-2015). This project aimed to find new antiprotozoal compounds. Asteraceae family was identified as a valuable privilegied source of antiparasitic metabolites. Following a chemotaxonomic approach, the potential of sesquiterpene lactones and PMFs was investigated.

The collaborations with several research units were established.

| Task | Responsible | Institution |
|---|---|---|
| Collection of samples, extraction, phytochemical analysis. | Dr. K. Dali-Yahia | Department of Pharmacy, Tlemcen University (Tlemcen, Algeria), |
| Antiparasitic activity of extracts and/or pure compounds | Dr. Laila Salmen Espindola | Laboratory of Pharmacognosy, Universidade de Brasilia (Brasilia, Brazil) |

| | | |
|---|---|---|
| Antiparasitic activity of extracts and/or pure compounds | Dr. Philippe Grellier | Unité Molécules de Communication et Adaptation des Microorganismes (MCAM, UMR 7245) from Muséum National d'Histoire Naturelle (Pairs, France). |
| Heme-binding affinity and stability by LC-MS methodology | Dr. Alexandre Maciuk and Dr. Pedro Vásquez-Ocmín | Equipe "Chimie des substances naturelles" BioCIS, Université Paris Sud (Châtenay-Malabry, France). |
| Antiplasmodial, hemozoin formation and cytotoxic activities | Dr. Sandrine Cojean | Equipe "Chimiothérapie antiparasitaire" BioCIS Université Paris Sud (Châtenay-Malabry, France). |

**Table 10: Collaboration with different national and international laboratories during the execution of Chapter IV.**

## IV.2.1 Objectives and methodology

### IV.2.1.1 General Objectives

To carry out our project, the general objectives were:

- Identification of metabolites from Asteraceae with antiplasmodial activity due to heme-binding affinity giving stable adducts.
- Generation by semisynthesis of a library of flavonoids for heme-binding affinity assay and *in vitro* evaluation of hemozoin formation.

#### IV.2.1.1.1 Specific objectives – Identification of active secondary metabolite family of Asteraceae species

- Collection of plant from the Asteraceae family or related families in the Algerian Southern Sahara region, in Ahaggar area nearby the Tahat Mountain (Willaya of Tamanrasset).
- Successive extractions by Pressurized Solvent Extraction (PSE) with ethyl acetate and methanol.
- *In vitro* evaluation of antileishmanial, antiplasmodial and heme-binding assay of extracts.
- Purification of major compounds of ethyl acetate extract of *Pentzia monodian*a (due to its antiplasmodial activity and ability of compounds to establish stable adducts with heme).
- Antiplasmodial *in vitro* and heme-binding assay of isolated compounds in order to identify the metabolites responsible for antiplasmodial activity and heme-adducts formation.

#### IV.2.1.1.2 Specific objectives – Evaluation of antiplasmodial, heme-binding assay and hemozoin formation of a Flavonoid Library

- Isolation from several sources (apolar extracts of aerial parts of *Artemisia copa* var. copa, *Fabiana denudata* and exudates of *Gardenia oudiepe* and *G. urvillei*) by flash chromatography and centrifugal partition chromatography of flavonoids.
- Semysynthesis from isolated and from commercial flavonoids (apigenin, chrysin, quercetin and kaempferol) by single or multiple methoxylation.

- *In vitro* antiplasmodial (3D7 and W2 strains) and cytotoxic (HUVEC cell line) evaluations of the flavonoids library.
- Heme-binding assay by LC-MS and adduct stability evaluation of the flavonoid library.
- Hemozoin formation evaluation *in vitro* of selected compounds.

## IV.2.2 Methodology

### IV.2.2.1 Evaluation of antiparasitic activity.

#### IV.2.2.1.1 Antiplasmodial [$^3$H]-hypoxanthine uptake *in vitro* assay.

The *in vitro* procedure to evaluate the antiplasmodial activity in vitro by [$^3$H]-hypoxanthine uptake is already described in section **I.2.2.2.2**.

#### IV.2.2.1.2 Antiplasmodial SYBR® Green I *in vitro* assay

In the second part of our work, SYBR® Green I dye fluorescence binds to the parasite DNA. After *in vitro* incubation of parasites with the molecules to evaluate, the plates are treated with 3 freeze-thaw cycles to achieve a complete hemolysis. Then, the lysis suspension is diluted in buffer and SYBR® Green I dye solution is added per well. Finally, the fluorescence resulting from the incorporation of the dye in the parasite DNA is measured and percentage of parasite growth inhibition is calculated in relation to respective blanks. Chloroquine is used as positive control.

#### IV.2.2.1.3 Antitrypanosomal *in vitro* assay

Antitrypanosomal activity evaluation of *Pentzia monodiana* extracts and isolated compounds was performed *in vitro* by the resazurin assay. After *in vitro* incubation of *Trypanosoma brucei brucei* bloodstream form with the compound/extract to evaluate, 20 µL of resazurin (45 mM final concentration) are added per well and re-incubated for 4 h at 37 °C under 5% CO2 and then at room temperature for 22 h. Resazurin is then reduced by NADPH or NADH dehydrogenase to resorufin. Quantity of formed resorufin is directly proportional with the growth of *T. brucei brucei*. Fluorescence was measured and percentage of inhibition are calculated in relation to respective blanks.

### IV.2.2.2 Evaluation of heme-adducts affinity.

#### IV.2.2.2.1 Heme-binding assay

LC-MS approach was used to identify the ability of compounds to form adducts with heme with some modifications [260]. Heme and compounds are dissolved in DMSO at 5mM in 384 well-plate with 5 µL tween 20 (10 µM in water) and 85 µL of citric buffer saturated octanol buffer at

pH 5.2, in order to mimic the acidity of parasite vacuole. After mixing, 5 µL of mixture are injected on LC-MS instrument (Accurate-Mass Q-ToF). Positive ionization (ESI) is apply in the MS detector: Masses are detected for heme at m/z 616.16 and for adducts of compound of mass X at (616.16 + X). Chloroquine is used as positive control.

To measure the stability of formed heme-adducts, increasing collision energy from 0 to 32 eV in the MS/MS mode under the same parameters previously described. Dissociation voltage at 50% is the collision energy dissociating 50% of the initial adduct.

# IV. 3 Antiplasmodial and heme-binding of aromatic and medicinal plants from Ahaggar, Algeria

Central Sahara and more precisely Ahaggar area are a rich source of biodiversity with very few phytochemical reports. Ethnobotanical surveys have been done on about 55 species used for medicinal purposes; Asteraceae species are the most frequently cited, followed by Zygophyllaceae, Lamiaceae, Fabaceae and Apiaceae families.

Species belonging to the Asterids clade were selected after chemotaxonomic evaluation, due to their potential content of metabolites from taxonomic elements such as flavonoids, sesquiterpene lactones and acetylenes with known biological activities..

Nine Asteraceae, three Apiaceae and one Lamiaceae species were collected near Tahat Mountain (Willaya of Tamanrasset) in accordance with an agreement delivered by the Ahaggar National Park Office. Species were identified on the basis of "Quezel and Santa's new flora of Algeria and desert and meridionals areas and Ozenda's flora of Sahara", with the assistance of Mr. Belghoul, head of the Department of Natural Patrimony Development of the Cultural Park of Ahaggar.

Extraction of collected material was performed with ethyl acetate and methanol. Then antiplasmodial in vitro activity and heme-binding assay were performed on the resulted extracts.

After preliminary results, ethyl acetate extract from *Pentzia monodiana* was selected for fractionation and isolation of major compounds, in order to identify the metabolites responsible of its activity.

# IV.3.1 Publication 3: Heme-binding activity of methoxyflavones from *Pentzia monodiana* Maire (Asteraceae)

Fitoterapia 118 (2017) 1–5

Contents lists available at ScienceDirect

Fitoterapia

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

## Heme-binding activity of methoxyflavones from *Pentzia monodiana* Maire (Asteraceae)

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ARTICLE INFO

*Article history:*
Received 1 December 2016
Received in revised form 20 January 2017
Accepted 29 January 2017
Available online 3 February 2017

ABSTRACT

A heme-binding assay based on mass spectrometry was performed on *P. monodiana* Maire (Asteraceae) extracts to identify metabolites able to form adducts with heminic part of haemoglobin, as potential antimalarial drugs. Main adducts were characterized and their stability was measured. Isolation of main constituents of *P. monodiana* Maire lead to identification of the two methoxyflavones 3′-*O*-methyleupatorin (**7**) and artemetin (**8**) involved in the adducts formation. Four *seco*-tanapartholides (**1**–**4**), a guaianolide (**5**), a germacranolide (**6**) and two other methoxyflavones (**9**, **10**) were also characterized. Evaluation of isolated compounds on *P. falciparum* and *T. brucei brucei* showed a moderate antiprotozoal activity of the two methoxyflavones.

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### 1. Introduction

Activity of the antimalarial drug artemisinin, as well as quinine derivatives, involves an interaction with the prosthetic group of haemoglobin. Formation of covalent or non-covalent adducts with free heme can inhibit its cristallization into insoluble, non-toxic hemozoin, leading to the parasite death. Such adducts can be characterized by mass spectrometry. An efficient LC/MS *in vitro* assay, described by Muñoz-Durango et al. is based on the identification, in complex mixtures such as plant extracts, of small molecules able to form adducts with heme [1].

In the continuation of our previous investigation of heme-binding molecules, we focused on *P. monodiana* Maire, from the Asteraceae family. *Pentzia monodiana*, "Tehatit" in Tamachek, grows in the southern part of Algeria [2]. Traditional uses of *Pentzia* species are reported for infectious and digestive troubles [3]. Phytochemical studies in the *Pentzia* genus reported occurrence of polymethoxyflavones, sesquiterpene lactones (both from germacranolide and guaianolide type). C-13, C-14 and C-17 acetylenes have also been isolated together with C14-spiroacetal enol-ethers [4,5].

### 2. Experimental

#### 2.1. General

Automatized pressurized extractions were performed on a Speed Extractor E-914 apparatus provided by Büchi (Fawil, Switzerland) equipped with four cells (120 mL) and a collector with four flat bottom vials (220 mL). Maximum pressure and temperature can be adjusted from 50 to 150 bar and from 30 to 200 °C, respectively. Mass spectrometry was performed on a mass spectrometer ZQ 2000 Waters (Saint-Quentin, France). Silica gel 60 0.025–0.04 mm (Macherey-Nagel GmbH & Co.) was used for column chromatography (CC). Preparative HPLC was performed on an AP-MOD-100 apparatus provided by Armen Instrument (Saint-Avé, France), with a C18 PursuitTM Varian column (250 mm × 30 mm, 10 μm), Büchi UV/VIS Filter-photometer detector, Merck D-2500 integrator and Büchi C-660 collector. The experiments were conducted at room temperature. CPC was performed on a SCPC-250 + 1000B apparatus provided by Armen Instrument (Sain-Avé, France) fitted with two rotors (total volume: 250 mL or 1 L), containing respectively 1953 or 2016 twin-cells. Rotation speed can be adjusted from 0 to 3000 rpm for the 250 mL rotor and from 0 to 1500 rpm for the 1 L rotor. A valve incorporated in the CPC apparatus allowed operation in ascending or descending mode. The system is

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http://dx.doi.org/10.1016/j.fitote.2017.01.012
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equipped with a gradient pump and a 10 mL or a 50 mL loop injection 6-way valve. $^{1}H$ and $^{13}C$ NMR experiments were performed in deuterated chloroform. Spectra were recorded on a Bruker AC 300 MHz or a Bruker 400 MHz spectrometer (Wissembourg, France). All the reagents and solvents were purchased from Carlo Erba-SDS. Cyclohexane and ethyl acetate were distilled before used. Water was distilled from deionized water.

### 2.2. Plant material

Aerial parts of *P. monodiana* were collected in the Algerian Southern Sahara, in Tassili Ahaggar area nearby the Tahat mountain (Wilaya of Tamanrasset, 23° 8′39.95″, 5°43′46.28″, alt.: 2019 m), in the Ahaggar National Park (Wilaya of Tamanrasset, Algeria), in September 2013, with the collect agreement delivered by the Ahaggar National Park Office, as *P. monodiana* is an Algerian endemic protected species. The collect agreement was delivered by the director of the Ahaggar National Park Office (OPNA), n°965/DPLBCVPC/MC/13 and 111/SDV/MC/13. Identification was under supervision of M. Belghoul, head of Department of Study of Natural Resources from the Ahaggar National Park. A voucher specimen was deposited in the herbarium of the department of Pharmacognosy, University Paris-Descartes University, France (voucher specimen MKOSB2013KDHPM).

### 2.3. Isolation of metabolites from P. monodiana Maire

#### 2.3.1. Extraction

A sample of 557 g of aerial part of *P. monodiana* was mixed with sand (1:1). Extraction cells were filled with the mixture and charged in the apparatus. Extraction program consisted in two distinct cycles of 20 min with ethyl acetate. Each cycle comprised a pre-heat period of 5 min, pumping of ethyl acetate through the extraction cell unit at a final pressure of 100 bar and at 50 °C, a hold-on time fixed at 5 min and a final flush of acetate extract into the corresponding vial. The extracts were concentrated under reduced pressure affording 42.72 g (7.67%) of ethyl acetate extract. The residual vegetal material was successively extracted with methanol, using the same extraction procedure, to afford 46.65 g of methanolic extract.

#### 2.3.2. Silica gel chromatography

A sample of 10 g of ethyl acetate extract was subjected to a silica gel chromatography with a gradient of cyclohexane/ethyl acetate (100:0 to 10:90 v/v) to give 32 fractions. Compound **6** recrystallized in ethyl acetate from fraction 13 and 14 (562 mg). Fraction 25 was identified as a mixture of compounds **9** and **10** with 1:1 a ratio (13 mg). Fractions 17 to 22 (F17–22) (1 g) were pooled and submitted to CPC separation, whereas fractions 26 to 28 (F26–28) (400 mg) were submitted to a preparative chromatography (preparative HPLC). Fraction 32 was subjected to a second silica gel CC eluted with cyclohexane/ethyl acetate (50:50, v/v) to afford compound **1**.

#### 2.3.3. CPC separation

Solvents systems were determined using shake-flask method followed by UV examination at 254 and 366 nm and vanillin spraying. Two CPC procedures were developed, the first one using the quaternary solvent system S1 (cyclohexane/ethyl acetate/MeOH/$H_2O$ (1/4/1/4 v/v/v/v)), applied to the ethyl acetate extract, and the second one, using the ternary solvent system S2 (ethyl acetate/THF/$H_2O$ (4/2/5 v/v/v)), applied to previous silica gel chromatography fraction F17–22. An aliquot of 400 mg of material (ethyl acetate extract or F17–22 fraction) was dissolved in 10 mL of a 1:1 (v/v) mixture of both phases of the biphasic systems S1 and S2 respectively. After injection, separation was performed in ascending mode on the 250 mL rotor, at a flow rate of 5 mL/min and at 1600 rpm. A total volume of 1.5 L of mobile phase was collected in 10 mL fractions. Extrusion step was initiated in ascending mode using the lower phase solvent as the mobile phase to ensure that any residual extract was recovered. Compounds **2** (14 mg), **3** (10 mg) and **4** (23 mg) were isolated from ethyl acetate extract and compound **5** was isolated from F17–22 (17 mg).

#### 2.3.4. Preparative HPLC of F26–28

An aliquot of 10 mg of F26–28 was dissolved in 10 mL of a $H_2O$/MeOH mixture (40:60 v/v), filtered on Acrodisc© Premium 25 mm 0.45 μM nylon filters (Pall Life Science, Port Washington, New York, USA), and injected. Subsequently, a linear elution is started using $H_2O$/MeOH mixture (40:60 v/v), flow rate: 20 mL/min. Compound **7** (3.4 mg) and **8** (4.1 mg) were isolated.

Identification of isolated compounds was confirmed with $^{1}D$ and $^{2}D$ NMR analysis and comparison with literature data: 5-βH-*seco*-tanapartholide (**1**) *seco*-tanapartholide A (**2**), *seco*-tanapartholide B (**3**) [6], iso-*seco*-tanapartholide (**4**) [7], Artecanin (**5**) [8], 3′-*O*-methyleupatorin (**7**) artemetin (**8**) [9], cirsilineol (**9**) [10] and chrysosplenetin (**10**) [11].

### 2.4. Heme binding assay

Hemin (Fe (III) ferriprotoporphyrin IX) was dissolved just before use at 5 mM in aqueous ammonia 25%. Extracts were dissolved in DMSO

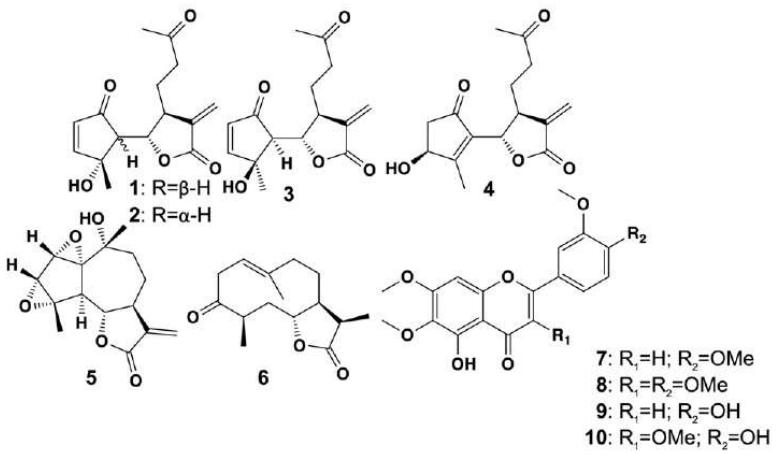


**Fig. 1.** Structures of compounds **1–10**.

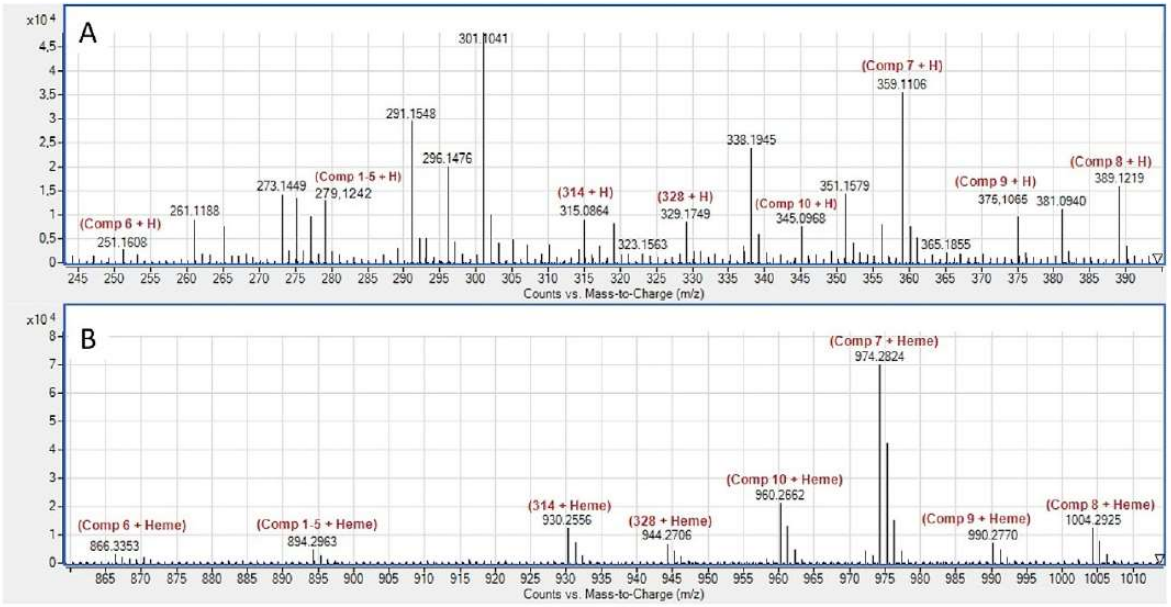


**Fig. 2.** Spectrum of *P. monodiana* ethyl acetate extract before (2.A) and after (2.B) incubation with hemin.

(5 mM), chloroquine diphosphate was dissolved in water (5 mM). Mixtures were prepared so as to obtain 100 μL of final equimolar concentrations of 250 μM for each compound (hemin, drug or extract) in methanol [1].

*2.4.1. Mass spectra acquisition*

Mass spectrometry was performed on a LC/MS Agilent (LC 1260 Infinity, MS 6530 Q-Tof (Agilent, Les Ulis, France). The ionization parameter (ESI positive mode) were: nebulizer pressure 40 psig, capillary voltage 3500 V, drying temperature 320 °C, drying gas flow 10 L/min, fragmentor 150 V. Experiments with a collision-induced dissociation (CID) were performed in FIA mode (flow injection analysis) with an voltage increment ranging from 0 to16 eV with 2 eV increments using MS/MS targeted mode (isolation width = 1.3 *m/z*). Injection was done at a flow rate of 300 μL/min of methanol/water (1:1).

*2.5. Adducts stability*

Data from CID experiments were analyzed by GraphPad Prism software. Relative abundance of heme was plotted *vs* the energy increments and fitted by a sigmoidal dose-response curve.

*2.6. Antiprotozoal activity of isolated compounds*

Antiplasmodial activity was determined on the FcB1 strain of *P. falciparum*. A modified [$^3$H]-hypoxanthine incorporation assay was used. Briefly, infected human red blood cells in culture were exposed to serial drug dilutions (50, 10 and 1 μM) in 96-well microtiter plates. Each compound at each concentration was tested in triplicate. After 48 h of incubation at 37 °C in a reduced oxygen atmosphere, 25 mL of [$^3$H]-hypoxanthine was added to each well. Cultures were incubated for a further 24 h prior to harvesting on glass-fiber filters and washing with distilled water. Radioactivity was counted using a 1450 Microbeta trilux (Wallac) (liquid scintillation and luminescence counter). The results were recorded as counts per minute per well at each drug concentration and expressed as a percentage of the untreated controls. Antitrypanosomal activity was determined by using the bloodstream form of *T. brucei brucei*. Briefly, bloodstream forms ($10^5$ parasites/mL) were exposed to 50 and 1 μM drug concentrations in 96-well microtiter plates. Each compound and each concentration was tested in triplicate. Plates were then incubated for 72 h at 37 °C under an atmosphere of 5% $CO_2$. 20 mL of resazurin (45 mM final concentration) was then added to each well. Incubation was continued for 4 h at 37 °C under 5% $CO_2$, then at room temperature for 22 h. Wells were read at excitation (535 nm), emission (590 nm) on an FL 600 Microplate Fluorescence Reader (Biotek) and results expressed as a percentage of the untreated control.

## 3. Results and discussion

Plant material was extracted using pressurized solvent extraction affording an ethyle acetate and a methanolic extract, which were both evaluated for their antiplasmodial activity on chloroquine-resistant strain FcB1/Colombia of *P. falciparum* ([$^3$H]-hypoxanthine test). Ethyl acetate extract showed a promising activity with an $IC_{50}$ = 3.5 μg/mL, whereas methanolic extract showed no significant activity ($IC_{50}$ > 100 μg/L). This interesting antiplasmodial activity led us to look for heme-binding compounds in the ethyle acetate extract of *P. monodiana* Maire.

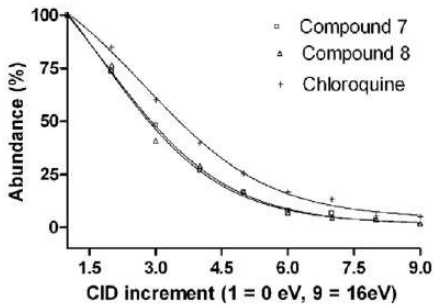


**Fig. 3.** Comparative stability curves of compounds **7**, **8** and chloroquine.

**Table 1**
Antiprotozoal activity of compounds **1–10** against *P. falciparum* and *T. brucei brucei* (inhibition percentage). nt: not tested.

| | Antiplasmodial activity [%] | | | Antitrypanosomal activity[a] [%] | | |
|---|---|---|---|---|---|---|
| | 50 μM | 10 μM | 1 μM | 50 μM | 10 μM | 1 μM |
| *1* | **97,0 ± 4,9** | **69,4 ± 7,6** | 31,7 ± 8,4 | **102,4 ± 2,7** | 46,7 ± 8,4 | 24,0 ± 3,1 |
| *2* | **99,5 ± 0,7** | 45,2 ± 2,1 | – | **50,8 ± 8,1** | 9,4 ± 8,8 | – |
| *3* | **99,8 ± 0,6** | **51,6 ± 4,6** | 1,2 ± 6,2 | **67,0 ± 9,4** | – | – |
| *4* | **99,7 ± 0,8** | 40,6 ± 6,7 | 3,5 ± 4,8 | 38,7 ± 10,8 | 13,8 ± 11,8 | – |
| *5* | nt | nt | nt | nt | nt | nt |
| *6* | 17,0 ± 13,5 | 5,4 ± 10,5 | – | **95,8 ± 3,4** | 39,5 ± 6,8 | – |
| *7* | **62,4 ± 6,4** | 19,7 ± 2,8 | 4,7 ± 5,1 | **96,9 ± 2,9** | **91,8 ± 16,0** | 16,0 ± 6,8 |
| *8* | **88,4 ± 0,8** | **56,1 ± 4,6** | 1,2 ± 6,2 | **98,1 ± 4,9** | **90,5 ± 3,0** | 37,4 ± 1,7 |
| *9* | 48,9 ± 4,4 | 38,8 ± 6,0 | 16,3 ± 15,0 | **88,4 ± 7,9** | **80,6 ± 1,7** | 31,0 ± 15,0 |
| *10* | **85,5 ± 4,9** | **69,4 ± 7,6** | 31,7 ± 8,4 | **95,5 ± 3,9** | **82,4 ± 4,2** | 32,2 ± 8,4 |

[a] The $IC_{50}$ of pentamidine, reference compound on *Trypanosoma brucei* is 5 nM.

### 3.1. Phytochemical study of P. monodiana Maire

The phytochemical study of *P. monodiana* was subsequently initiated and led to isolation and identification of six sesquiterpenes **1–6** and four methoxyflavones **7–10** (Fig. 1). Sesquiterpenes were of seco-guaianolides type (5β-*H* seco-tanapartholide(**1**), seco-tanapartholide A (**2**), seco-tanapartholide B (**3**), *iso-seco*-tanapartholide(**4**)), guaianolide (artecanin **5**), and germacranolide type (ketopelenolide B **6**). Both tri-, tetra- and penta-methoxyflavones have been characterized: 3′-*O*-methyleupatorin (**7**) artemetin (**8**), cirsilineol (**9**) and chrysosplenetin (**10**).

### 3.2. Heme-binding assay

Heme-binding assay protocol consisted in incubation of plant extracts in presence of hemin. Mass spectrometry analysis used an ESI-API ionization in positive mode with Q-TOF detection. Peaks arising only in the incubated extracts are assumed to be adducts. Eight peaks of $m/z > 860$ appear following the incubation of ethyl acetate extract with hemin (Fig. 2A), and every compound suspected to form adducts is detected in the extract (Fig. 2B), corresponding to sesquiterpenic lactones **1–6** and methoxyflavones **7–10** (Fig. 1).

### 3.3. Stability of heme-adducts

Stability of observed adducts was subsequently regarded. Previous study showed that adducts stability can be evaluated using mass spectrometry by measuring abundance of an adduct increasing the fragmentor voltage. Dissociation of adducts with increasing fragmentor voltages involves a decrease of detected ion abundance. This can be illustrated by stability curves measuring adduct ion abundance as a function of voltages. Compounds **1–6** and **9–10** would form adducts of very low stability and no curve could be obtained, whereas compounds **7** and **8** formed more stable adducts. Stability curves of compounds **7** and **8** and chloroquine heme-adducts are presented in Fig. 3. Compounds **7** and **8** showed a lower stability compared to chloroquine, what is in favor of a weak π-π stacking interaction that may be involved in an antimalarial activity.

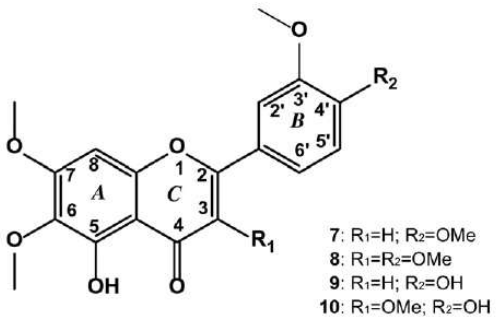


**Fig. 4.** Structures of methoxyflavones **7–10**.

### 3.4. Antiprotozoal activity of isolated compounds

To corroborate these results, we evaluated antiplasmodial and antitrypanosomal activities of isolated compounds *in vitro* against of *Plasmodium falciparum* (FcB1-Colombia strain) and *Trypanosoma brucei brucei* (Table 1). Methoxyflavones, showed low activity with percentage of growth inhibition not superior to 37% at 1 μM. *Seco*-guaianolides (**1–4**) showed low activity with percentage of inhibition not higher than 50% at 1 μM. Thus, no $IC_{50}$ were measured. These results suggest that the heme-binding activity of methoxyflavones **7** and **8** is not correlated with a direct toxic effect on parasites.

## 4. Discussion

Screening of heme-binding compounds in *P. monodiana* Maire showed that two methoxy-flavones 3′-*O*-methyleupatorin (**7**) and artemetin (**8**) present a good affinity for the heminic part of haemoglobin. Such data focused our attention on methoxyflavones as they are known to interact with drug metabolizing enzymes, and by this way, able to enhance drug activity. Several flavonoids have been reported to modulate the drug efflux proteins (P-glycoprotein, breast cancer resistance protein BCRP…), and thus to reverse drug resistance [12]. However, flavonoids can also interact with cytochromes P450, responsible of the phase 1 metabolism of xenobiotics, and consequently, enhance the pharmacokinetic profile of drugs [13]. This undesired effect in case of enhancement of toxicity, may be advantageous to decrease drugs inactivation by cytochromes. For example, the methoxyflavone chrysoplenetin has been reported to inhibit the enzymatic activity of cytochromes CYP1A2 and CYP2C19 *in vitro* and that its co-administration with the antimalarial drug artemisinin is associated to an enhanced antimalarial activity *in vivo*, although the compound showed no antiplasmodial activity [14]. Investigations on the structure-activity relationship of flavonoids regarding inhibition of cytochromes P450 have been reported in several studies [15]. Heterosides are generally poor inhibitors of CYP, as well as 2–3 dihydrogenated flavonoids. An extensive study on the SAR of 33 flavonoids describes the structural requirements for a CYP1A1, 1A2 and 1B1 inhibition. A 5,7-dihydroxy substitution on A ring, associated to a 4′-methoxy group on B ring was observed for the most potent inhibitors [16]. Moreover, the molecular docking studies showed that the B ring of strong inhibitors interacts with the iron center of the heme part of the CYP, and is surrounded by amino-acids residues.

Regarding our results, the two more stables heme-adducts were observed with 3′-*O*-methyleupatorin (**7**) and artemetin (**8**), bearing a 5-hydroxy,-7-methoxy substitution on A ring, and a 3′,4′-dimethoxy

substitution pattern on B ring (Fig. 4). On the other hand, very transient heme-adducts were observed with chrysoplenetin (**9**), and cirsilineol (**10**), where the 4′-methoxy group is lacking on the B ring, which is in accordance with the importance of the 4′-methoxy substitution. Nevertheless, chrysoplenetin has been reported to inhibit CYP1A2 and CYP2C19 at the micromolar range (4.61 and 6.23 μM respectively), with a subsequent significative biological effect *in vivo*, meaning that surrounding amino-acids may contribute to stabilize the compound in the active site [14].

Thus, despite a weak direct antiplasmodial activity, our results are in favor of a good affinity of methoxyflavones for heme, the prosthetic group of hemoproteins such as haemoglobin and cytochromes P450. As a matter of fact, this affinity can lead to two positive effects in case of malaria: the inhibition of haemoglobin crystallization into hemozoin with subsequent plasmodium death, and enhancement of artemisinin activity by inhibition of cytochromes P450. These preliminary data should be investigated more deeply. For a better understanding of a positive effect of methoxyflavones co-administration with antimalarial drugs, further studies should be undertaken to evaluate the adduct formation of methoxyflavones on isolated heme in presence of artemisinin, as artemisinin also binds to the heminic part of haemoglobin. Moreover, evaluation or cytochrome P450 inhibition of methoxyflavones **7**–**10** alone and in combination with artemisinin should be evaluated for their cytochromes P450 inhibitory activity.

## Acknowledgements

Financial support was provided by the ChembioFight European program (269301) (FP7-People-2010-IRSES) and the Algerian PROFAS research funding.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2017.01.012.

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## IV.3.2 Conclusion and perspectives

Chemotaxonomic approach was carried out to identify an antiplasmodial active extract (from *Pentzia monodiana*) and able to form heme-adducts in an LC-MS methodology.

This work has led to the identification of flavonoids from *P. monodiana* as main metabolites underlying the heme-adducts formation, despite a moderate antiplasmodial *in vitro* activity. In addition, 3-*O*'-methyleupatorin and artemetin formed stable adducts but weaker than chloroquine, with $DV_{50}$ near 3.0 eV.

Deeper studies will be carried out for a better understanding of the correlation and influence of methoxyl/hydroxyl substitution pattern of flavonoids in the formation and stability of heme-adducts in the LC-MS assay. Furthermore, in order to corroborate their heme affinity detected in the LC-MS assay, effect of the flavonoids involved in heme-adducts formation must be explored *in vitro* on infected erythrocytes.

## IV.4 Antiplasmodial and heme-binding activities of poly-methoxyflavones

In the continuation of our previous work, we have designed library of 33 flavonoids with different methoxyl/hydroxyl substitution pattern and perform the evaluation of their antiplasmodial, cytotoxic and heme-binding affinity activities, in order to better understand of the antimalarial potential of these compounds *via* inhibition of hemozoin formation.

## IV.4.1 Draft 7: Antiplasmodial activity and heme-binding affinity of methoxyflavonoids.

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Abstract

*Plasmodium falciparum*, responsible for uncomplicated and severe forms of malaria in humans, is considered as one the most dangerous parasite worldwide. Researches in biology and chemistry remains essential to have an extensive understanding of antiplasmodial drug targets. The main antimalarial mechanism consists in the interruption of heme crystallization during erythrocytic phase, perturbating parasite detoxification strategy. It involves interaction of drugs with free heme to form adducts that can be monitored by mass spectrometry. Formation of heme adducts is used as an antiplasmodial screening assay. We propose to determine the nature of the correlation between *in vitro* antiplasmodial activity and heme-binding profile of a set 33 methoxyflavones chosen to represent a wide range of substitutions pattern. Results showed that physicochemical and geometrical properties induced by methoxylation impacts binding of methoxyflavones with heme.

Keywords: methoxyflavone, heme-binbing, antiplasmodial, hemozoin.

## 1. Introduction

Malaria is the most important and dangerous parasitic disease affecting humans [1]. In 2016, an estimated 216 million cases of malaria occurred worldwide and US$ 2.7 billions were invested globally in control and elimination efforts by governments of countries where the disease is endemic and by international institutions [2]. Malaria is transmitted by the bite of female mosquitoes of the *Anopheles* genus infected by *Plasmodium* spp. [3,4]. During the trophozoite stage of the intraerythrocytic life cycle, *P. falciparum* internalizes up to 80% of the host hemoglobin through a phagocytic organelle known as the cytostome [5]. This latter then transports hemoglobin into an acidic digestive vacuole (pH 5.2) [6]. There it is digested by proteolytic enzymes into small peptides used as nutrients by the parasite [7]. For every consumed molecule of hemoglobin, four molecules of heme (ferriprotoporphyrin IX) are released. Free heme-Fe(III) is toxic, and cells usually degrade it by heme oxygenase [8]. In *Plasmodium*, free heme-Fe(III) is crystallized into an insoluble crystal (hemozoin). Hemozoin has been described as being responsible for multiple deleterious *in vitro* and *ex vivo* immunomodulating effects [9]. Methods of quantification of free or crystallized heme-Fe(III) have been investigated, however the knowledge about its accumulation *in vivo* remains limited [10].

Heme detoxification pathway is specific to the parasite and is the target of many antimalarial drugs including 4-aminoquinolines [11,12] and artemisinin [13,14]. Several methodologies have been described to assess compounds for their binding to heme [15–17]. Our previous work has

identified methoxyflavonoids as heme binders. Thus, we hypothesize that the methoxylation pattern could be of some significance for antiplasmodial activity, increasing hydrophobic interaction with heme [18]. We have tested a library of 33 methoxyflavones, both on the heme-binding assay by mass spectrometry and *in vitro* on two strains of *P. falciparum*. In addition, *in vitro* formation of hemozoin of selected derivatives was performed in order to validate their heme-binding affinity observed in LC-MS assay.

## 2. Results and discussion

### 2.1. Preparation of the flavonoids library

Thirty-three methoxyflavonoids were obtained from purification from plants extracts and semisynthesis from isolated compounds and from commercial standards and were subjected to heme-binding assay, *in vitro* antiplasmodial evaluation on 3D7 (chloroquine-sensible), W2 (chloroquine-resistant) strains, and *in vitro* cytotoxic activity against HUVEC cells. These 33 compounds can be pooled in two main groups, flavones (**1**-**5**, **23**-**25**) and flavon-3-ols (**6**-**22**, **26**-**33**). Furthermore, it is possible to identify nine sub-groups depending of the substitution pattern in positions 3, 5, 6, 7, 3', 4' and 5' by hydroxyl or methoxyl groups on the flavonoid core.

In addition to *in vitro* biological measurement, computing estimation of physico-chemical parameters as partition coefficient logP and topological polar surface area to obtain indications about lipophilicity of compounds depending on methoxylation/hydroxylation degree and its relationship with the heme-binding affinity and/or antiplasmodial in vitro activity was carried out.

### 2.2. Correlation of methoxylation and affinity to heme

In each sub-group of derivatives, it is possible to observe a clear correlation between the methoxylation degree of compounds with its logP and tPSA estimated values. Methoxylation substitution in the flavonoid skeleton increase the estimated partition coefficient values. This may due to the loss of the capacity of methoxylation in the hydroxyl groups to form hydrogen bonds, increasing the hydrophobic character of derivatives. Nevertheless, this trend is not followed by the methoxylation in position 5 of flavonoid skeleton, showing a decrease of estimated logP values.

Similar observations was deduced in the stability of formed adducts of heme-flavonoids. Heme is hydrophobic, and hydrophobic interactions are involved in the formation of adducts with heme [19]. As methoxylation increases hydrophobicity of the flavones, an effect of methoxylation can be expected on the ability to bind to heme. Indeed, a higher hydrophobic interaction can easily explain the increasing stability of adduct with increasing cLogP through methoxylation. Nevertheless, the R5 position does not follow this scheme: its methoxylation decreases cLogP, but systematically increases the stability of the binding to heme, by a factor 2. This drop of

hydrophobicity for the methoxylated analogue is at first sight surprising, but it is well known for flavonoids: the 5-OH is engaged in a strong intramolecular hydrogen bound with the carbonyl in position 4 [20]. This bound effectively diminishes the partial negative charge on the carbonyl oxygen atom and partially blocks the carbonyl group from dipolar interactions with other polar molecules. Paradoxically, a methoxy group remains the electron pairs of the oxygen atoms free to form hydrogen bounds. If the 5-OH is substituted by a methoxy group, this effect is cancelled and even if the hydrophobicity is partly increased by the addition of a methoxy group, the total hydrophobicity decreases. This effect is obvious when comparing the following pairs of compounds: **9**/**10**, **13**/**14**, **21**/**22**, **28**/**29** and **32**/**33**. For compounds **18**/**19** show that either hydroxyl on position 3 or 5 can form hydrogen bound with the carbonyl. This effect also has an effect on the binding to heme: unexpectedly, methoxylation of position 5 significantly increases the binding to heme. In each methoxyflavone family, an increased $DV_{50}$ is observed for the 5-OMe derivative **(Figure 2**).

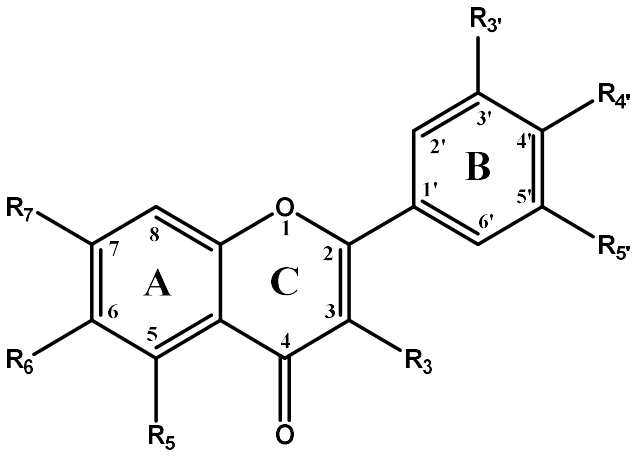


**Figure 1**. Methoxyflavones skeleton

Table 1. *In vitro* biological activity and heme-test of methoxyflavones

| Derivatives methoxyflavones sub-families | Compounds | Substitutions | | | | | | | *P. falciparum* 3D7 sensitive strain, $IC_{50}$ ± SD, µM | *P. falciparum* W2 resistant strain, $IC_{50}$ ± SD, µM | HUVEC cells cytotoxicity, $CC_{50}$ ± SD, µM | $DV_{50}$ (eV) | cLogP (ACD/ChemSketch) | tPSA ($Å^2$) (FAF-Drug4) | Selectivity index, $IC_{50}$/ $CC_{50}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | C ring | A ring | | | B ring | | | | | | | | | |
| | | R3 | R5 | R6 | R7 | R3' | R4' | R5' | | | | | | | |
| **Chrysin derivatives** | 1 | -H | -OH | -H | -OH | -H | -H | -H | >50 | >50 | >50 | 7.79 | 2.88±0.53 | 70,34 | ≤1 |
| | 2 | -H | -OH | -H | $-OCH_3$ | -H | -H | -H | >50 | >50 | 25.45±1.99 | 7.81 | 3.13±0.49 | 59,67 | ≤1 |
| **Apigenin derivatives** | 3 | -H | -OH | -H | -OH | -H | -OH | -H | >50 | >50 | >50 | 7.93 | 2.10±0.56 | 90,57 | ≤1 |
| | 4 | -H | -OH | -H | $-OCH_3$ | -H | $-OCH_3$ | -H | 11.94±2.07 | 30.61 ± 6.3 | 27.89±2.42 | 8.09 | 3.40±0.60 | 68,9 | 2.33 |
| | 5 | -H | $-OCH_3$ | -H | $-OCH_3$ | -H | $-OCH_3$ | -H | >50 | >50 | 30.12±2.85 | 15.94 | 3.53±0.95 | 57,9 | ≤1 |
| **Kaempferol derivatives** | 6 | -OH | -OH | -H | -OH | -H | -OH | -H | 3.78±0.88 | 0.18 ± 0.05 | >50 | NA | 2.05±0.60 | 110,8 | 13.22 |
| | 7 | $-OCH_3$ | -OH | -H | -OH | -H | -OH | -H | >50 | >50 | >50 | NA | 2.22±1.41 | 99,8 | ≤1 |
| | 8 | $-OCH_3$ | -OH | -H | $-OCH_3$ | -H | -OH | -H | 26.46±3.14 | 30.95 ± 3.48 | >50 | 7.95 | 2.79±1.41 | 89,13 | 1.89 |
| | 9 | $-OCH_3$ | -OH | -H | $-OCH_3$ | -H | $-OCH_3$ | -H | >50 | >50 | 30.18±2.20 | 7.95 | 2.97±1.25 | 78,13 | ≤1 |
| | 10 | $-OCH_3$ | $-OCH_3$ | -H | $-OCH_3$ | -H | $-OCH_3$ | -H | >50 | >50 | >50 | 15.77 | 2.49±1.37 | 67,13 | ≤1 |
| **Viscosine derivatives** | 11 | $-OCH_3$ | -OH | $-OCH_3$ | -OH | -H | -OH | -H | 6.06±0.96 | 10.01 ± 1.19 | 23.98±2.07 | 7.85 | 1.80±1.46 | 109,03 | 3.96 |
| | 12 | $-OCH_3$ | -OH | $-OCH_3$ | -OH | -H | $-OCH_3$ | -H | 30.29±4.69 | >50 | 14.60±2.04 | 7.91 | 1.98±1.41 | 98,03 | ≤1 |
| | 13 | $-OCH_3$ | -OH | $-OCH_3$ | $-OCH_3$ | -H | $-OCH_3$ | -H | >50 | >50 | >50 | 8.16 | 2.67±1.41 | 87,36 | ≤1 |
| | 14 | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | -H | $-OCH_3$ | -H | 24.97±3.53 | >50 | >50 | 15.83 | 2.30±1.45 | 76,36 | ≤1 |
| **Quercetin derivatives** | 15 | -OH | -OH | -H | -OH | -OH | -OH | -H | 8.52±2.25 | 10.02 ± 3.53 | >50 | 4.11 | 2.07±0.72 | 131,03 | 2.0 |
| | 16 | $-OCH_3$ | -OH | -H | -OH | -OH | -OH | -H | 3.33±0.63 | 29.66 ± 3.96 | 17.81±0.81 | 7.93 | 2.02±1.44 | 120,03 | 5.35 |
| | 17 | $-OCH_3$ | -OH | -H | $-OCH_3$ | -OH | $-OCH_3$ | -H | 7.99±1.03 | >50 | 18.45±1.22 | 8.05 | 2.43±1.37 | 98,36 | 2.31 |
| | 18 | -OH | $-OCH_3$ | -H | $-OCH_3$ | -OH | $-OCH_3$ | -H | 6.88±0.58 | >50 | >50 | 8.04 | 3.06±1.14 | 98,36 | 7.27 |
| | 19 | $-OCH_3$ | $-OCH_3$ | -H | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | -H | 3.96±1.36 | 9.51 ± 1.51 | >50 | 15.89 | 2.37±1.37 | 76,36 | 12.63 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Chrysoplenetin derivatives** | 20 | $-OCH_3$ | -OH | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | -OH | -H | >50 | >50 | 15.50±2.03 | 11.96 | 2.26±1.46 | 107,59 | ≤1 |
| | 21 | $-OCH_3$ | -OH | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | -H | >50 | >50 | 18.45±2.45 | 11.72 | 2.55±1.41 | 96,59 | ≤1 |
| | 22 | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | -H | 12.7±1.34 | 9.51±1.51 | >50 | 12.25 | 2.18±1.45 | 85,59 | 3.94 |
| **Eupatilin derivatives** | 23 | -H | -OH | $-OCH_3$ | -OH | $-OCH_3$ | $-OCH_3$ | -H | 29.31±3.58 | 14.52 ± 2.36 | 6.25±2.03 | 8.03 | 2.46±1.22 | 98,03 | ≤1 |
| | 24 | -H | -OH | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | -OH | -H | >50 | >50 | 31.25±2.35 | 8.29 | 1.67±1.14 | 98,36 | ≤1 |
| | 25 | -H | -OH | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | -H | >50 | 16.38 ± 4.81 | 25.91±1.02 | 11.98 | 2.83±1.20 | 87,36 | ≤1 |
| **Myricetin derivatives** | 26 | $-OCH_3$ | -OH | -H | -OH | $-OCH_3$ | $-OCH_3$ | -OH | >50 | >50 | 15.02±1.26 | 8.01 | 1.85±1.37 | 118,26 | ≤1 |
| | 27 | $-OCH_3$ | -OH | -H | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | -OH | >50 | >50 | >50 | 8.15 | 2.43±1.38 | 107,59 | ≤1 |
| | 28 | $-OCH_3$ | -OH | -H | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | 28.04±3.76 | 11.08 ± 1.26 | >50 | 8.09 | 2.62±1.26 | 96,59 | 17.83 |
| | 29 | $-OCH_3$ | $-OCH_3$ | -H | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | >50 | >50 | >50 | 16.01 | 2.13±1.38 | 85,59 | ≤1 |
| **5'-Hydroxyquercetagetin derivatives** | 30 | $-OCH_3$ | -OH | $-OCH_3$ | -OH | $-OCH_3$ | $-OCH_3$ | -OH | 30.29±4.63 | >50 | >50 | 8.03 | 1.43±1.45 | 127,49 | ≤1 |
| | 31 | $-OCH_3$ | -OH | $-OCH_3$ | -OH | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | 18.5±3.91 | >50 | >50 | 7.99 | 1.62±1.41 | 116,49 | 2.7 |
| | 32 | $-OCH_3$ | -OH | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | 7.28±3.34 | 9.93±0.68 | >50 | 11.76 | 2.32±1.42 | 105,82 | 6.87 |
| | 33 | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | 12.94±1.68 | >50 | >50 | 15.96 | 1.95±1.45 | 94,82 | 3.86 |
| | Chloroquine | | | | | | | | 0.02± 3.14 | 0.13±32.29 | NT | 15.86 | NT | NT | NT |

NA= not adducts; NT= not tested.

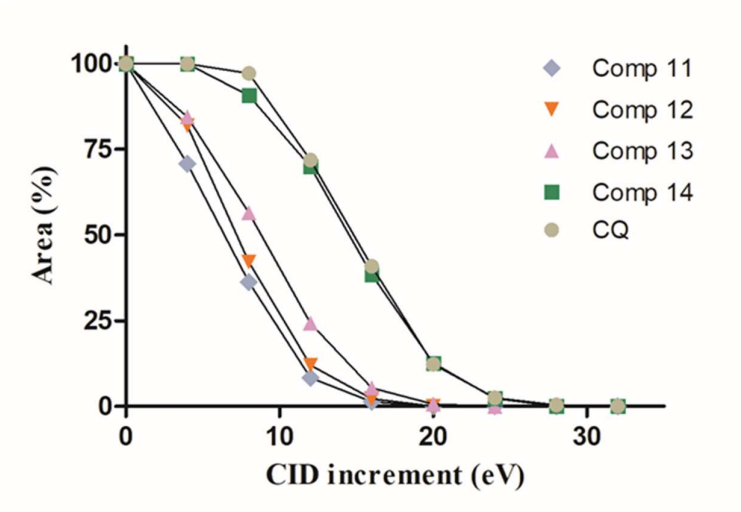


**Figure 2**. $DV_{50}$ of the viscosine derivatives.

2.3 *In vitro* antiplasmodial activity of flavonoid library

In the case of 3D7 strain, compounds **6**, **11**, **15-19** and **32** showed $IC_{50}$ values below the 10 µM, considered as active compounds by the WHO Guidelines. Derivatives **4**, **22** and **33** presented $IC_{50}$ values between 12-13 µM. For the kaempferol sub-group (**6-9** compounds) only kaempferol is active and the methoxylation of any hydroxyl group generate a loss of the antiplasmodial activity against 3D7 strain. On the other hand, all quercetin derivatives (**15-19**) were active against 3D7 strain regardless of their degree of methoxylation. The degree of methoxylation takes importance in the chrysoplenetin and 5'hydroxyquercetagetin, where only the derivatives with high methoxylation degree are actives (**22**, **33**). Eupatolin and myricetin sub-groups were not active against 3D7 strain.

For W2 strain, compounds **6**, **11**, **15**, **19**, **22**, **28** and **32** presented $IC_{50}$ values below the 10 µM. Derivatives **23** and **25** showed also $IC_{50}$ values between 10-15 µM. As in the case of 3D7 strain, kaempferol **6** was the more active compound with an $IC_{50}$ value of 0.18 µM. Similar trend, in the case of 3D7 strain, the rest of kaempferol derivatives were not active against the W2 strain. The rest of active derivatives (**19**, **22**, **28** and **32**) shared a higher methoxylation degree, all of them with methoxyl groups in R3, R7, R3' and R4'.

Six compounds **6**, **11**, **15**, **19**, **22** and **32** were actives against both chloroquine-sensitive and resistant strains. These compounds could present different mechanisms of action in comparison of chloroquine, to explain the comparable *in vitro* antiplasmodial activity of these compounds. Indeed, kaempferol and quercetin (**15**) present inhibitory activity on FabG, FabZ and FabI enzymes, involved in the type II fatty acid biosynthesis (FAS-II) of *P. falciparum*, essential for

the cycle life of parasites [21]. However, if these active flavonoids present similar mechanism of action than chloroquine, they may be not affected by the resistance mechanism of *Plasmodium* parasites. This mechanism, exerted after specific mutation of chloroquine protein transport (PfCPT) may be specific to chloroquine as substrate [22].

## 2.4 Hemozoin *in vitro* formation

To better understand the impact of the heme-binding affinity observed in the LC-MS assay, we performed a quantification of hemozoin after incubation *in vitro* of erythrocytes parasite with the chloroquine-sensible 3D7 *P. falciparum* strain with 3 methoxyflavones derivatives: kaempferol **6**, active against 3D7 but with not adduct with heme detected; tetramethoxykeampferol **10**, not active *in vitro* against 3D7 but with very stable heme-adduct observed with and $DV_{50}$ of 15.77 eV; and pentamethoxyquercetin **19**, with low $IC_{50}$ (3.96 μM) against 3D7 strain and stable heme-adduct formed (15.89 eV).

In accordance with the other test results, kaempferol showed no influence in the quantity of hemozoin formation, reinforcing the idea that this compound may exert its antiplasmodial *in vitro* activity by other pathways such as inhibition of essential fatty acids previously described [21]. In addition, plasmodial serine-threonine kinase and thioredoxin reductase (essential for the growth and survival of *Plasmodium* parasites), present a high homology with their human equivalents, in which flavonoids as kaempferol and/or quercetin present high levels of inhibition proprieties [23–25]. Deeper studies of the antiplasmodial mechanism of action of kaempferol must by carried out taking account these homologies between plasmodial and human enzymes.

Tetramethoxykaempferol **10** do not modify the *in vitro* quantity hemozoin formation after incubation with 3D7 *P. falciparum* strain, but is able to form stable adduct detected in the LC-MS assay. Methoxylated flavonoids have been previously reported with poor membrane permeability in different *in vitro* with biological membrane models [26]. These poor proprieties could hamper the entry of methoxyflavonoids first into erythrocyte membrane and then through the parasite membrane to finally arrive into the parasite vacuole, where hemozoin formation take place. This highlights one of the limitation of the LC-MS assay, which it is a cell-free methodology, giving false positive cases, due other factors present in biological *in vitro* assays (membrane permeability, solubility of compounds, pH stability, degradation of substrate at assay temperature, etc.), not taken into account by this heme-binding affinity assay.

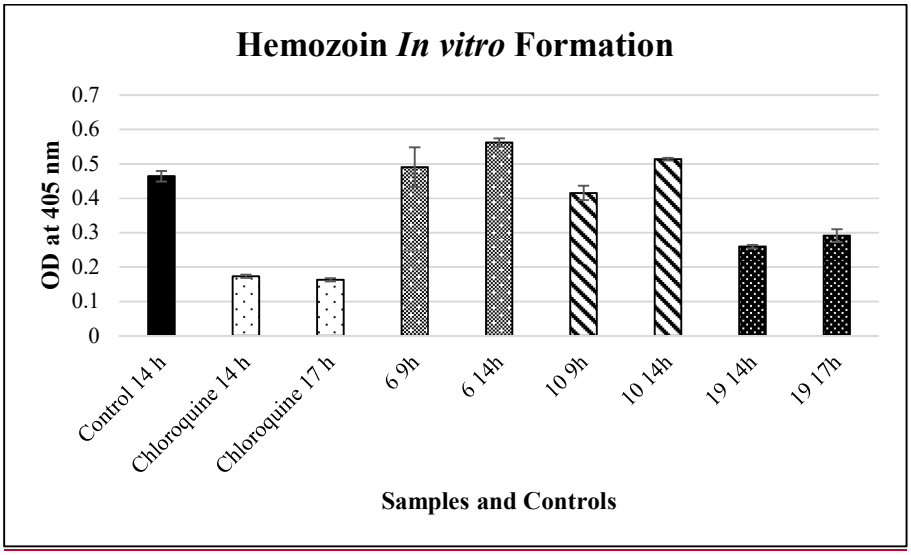


**Figure 3**: Effect of the quantity of hemozoin formation by 3D7 *Plasmodium falciparum* strain after incubation with chloroquine, kaempferol **6**, tetramethoxykaempferol **10** and pentamethoxyquercetin **19** at two time points.

At last, pentamethoxyquercetin **19** showed a spectacular reduction of 44% of the hemozoin formed after 14 h of incubation. This effect was similar to the effect detected for chloroquine, which reduced in a 63% of hemozoin synthesis by 3D7 *Plasmodium* strain. This observation confirms the heme-binding affinity exerted by this compound in the LC-MS assay, by creating adducts with heme, may also be responsible of blocking the hemozoin biocrystallization *in vitro*. Together with the $IC_{50}$ values obtained by this compound against both *P. falciparum* strains, we showed that one of the mechanism of antiplasmodial activity of pentamethoxyquercetin is the obstruction of the main detoxification mechanism of heme, by impeding hemozoin formation.

## 3.Conclusions

Impairment of hemozoin formation is the major mechanism of action of most antimalarial drugs. Their binding of heme is the "Achilles hell" of the parasite. The thorough assessment of a series of methoxyflavones for their in vitro antiplasmodial activities, their affinity for heme and their physicochemical characteristics allowed to establish that the number and location of methoxylated phenols has a significant influence on heme binding, principally methoxylation in position 5. Nevertheless, this set of methoxyflavones would not allow to establish any structure activity relationship. Only compound **19** showed simultaneously a significant biological activity

and a strong interaction with heme. At the contrary, compound **6** (kaempferol) showed a surprising antiplasmodial activity especially on the resistant W2 strain, without binding to heme.

## 4. Material and methods

### 4.1. Chemical and reagents

Mass spectrometry analyses of compounds was performed on a Waters ZQ 2000 mass spectrometer (Saint-Quentin, France). Silica gel 60 25-40 µm (Macherey-Nagel GmbH & Co, Düren, Germany) was used for column chromatography (CC). $^{1}H$ and $^{13}C$ NMR experiments were performed in deuterated chloroform on a Bruker AC 300 MHz or a Bruker 400 MHz spectrometer (Wissembourg, France). All the reagents and solvents were purchased from Carlo Erba-SDS (Chaussée Du Vexin, France). Cyclohexane and ethyl acetate were distilled before us. Deionized water was used for all experiments. Compounds **1** and **3** were purchased from Sarsyntex (Merignac, France); **6** and **15** from Sigma Aldrich (St. Louis, MO, USA).

### 4.2. Flavonoids library

Flavonoids library is composed of either isolated, commercial or semi-synthetic derivatives. Compounds **23**, **30** and **31** were previously isolated from bud exudates of *Gardenia oudiepe* (Rubiaceae); **7**, **11** and **12** from *G. urvillei*; and **20**, **21**, **24** and **25** from *Pentzia monodiana* (Astearceae) [18,27]. Compounds **26** and **27** were isolated from bud exudates of *G. oudiepe* (Rubiaceae). Coumpounds **1**, **3**, **6** and **15** were purchased from Sarsyntex (Merignac, France) or Sigma Aldrich (St. Louis, MO, USA). Compounds **7-8**, **13-14** and **32-33** were previously synthetized [27]. Compound **2** was obtained by methylation from 1; **4** and **5** from **3**; **9** and **10** from **6**; **16-19** from **15**; **22** from **20** and **28-29** from **27** (see section 4.5).

### 4.3. Plant material

*G. oudiepe* budswere collected in October 2008 in Forêt Plate, North Province of New Caledonia. Voucher specimens (POU-0290 respectively) were deposited at the Herbarium of the Botanical and Tropical Ecology Department of the IRD Center, Noumea, New Caledonia.

### 4.4. Extraction and isolation

55 g of *G. oudiepe* exudate were subjected to silica gel chromatographic separation using cyclohexane/DCM, EtOAc/DCM and DCM/MeOH mixtures of increasing polarity as eluent. Fraction obtained with AcOEt/DCM 60/40 (3.28 g) was subjected to another silica gel chromatography with AcOEt/DCM as eluent and allowed the isolation of compound 26 (15 mg) and 25 (2.1 g, previously isolated). 27 g of exudate of *G. urvillei* were also subjected to silica gel chromatography with cyclohexane/DCM and DCM/MeOH as eluent. Fraction obtained with cyclohexane/DCM (65:35) (128 mg) was subjected to another silica gel chromatography with cyclohexane/DCM mixture as eluent, allowing the isolation of compound 27 (20 mg). Identification of isolated compounds was confirmed by NMR analysis and MS spectrometry data and by comparison with literature data: 3',5,7-trihydroxy-3,4',5'-trimethoxyflavone (compound **26**) and 5,5'-dihydroxy-3,7,3',4'-tetramethoxyflavone (compound **27**) [28,29].

### 4.5. Synthesis of the compounds **2**, **4-5**, **9-10**, **16-19**, **22**, **28** and **29**.

Semisynthesis of compounds **8** from **7**; **13** and **14** from **12**; **32** and **33** from **31** were previously reported, using dimethyl sulfoxide ($Me_2SO_4$, 4 equiv.) and 1,8-diazobiciclo[5.4.0]undec-7-en (DBU, 2 equiv.) in dried acetone as solvent at room temperature [27]. Furthermore, methylation of isolated compounds **20** and **27** and synthesis of compounds **2**, **4-5**, **9-10**, **16-19** from the commercial available compounds **1**, **3**, **6** and **15** were performed. Methylation consisted in stirring the substrate with methyl iodide (MeI, 13.5 equiv.) and carbonate of potassium ($K_2CO_3$, 6.6 equiv.) in dried acetone at room temperature for 1 h. The crudes reaction were precipitated and washed with ice water. The resulting residue were solubilized with ethyl acetate (EtOAc, 10 mL) and treated with 1N HCl solution (3 mL). The final products were extracted with EtOAc (3x10 mL) and the organic phases were washed with saturated NaCl solution and dried over $Na_2SO_4$. After filtration, and solvent evaporation, methylated compounds were purified by silica gel chromatography column, using a mixture of DCM/MeOH (99.1/0.1 to 95/5 v/v) as eluent [30].

Identification of resulting pure compounds were confirmed by comparison of $^{1}H$, $^{13}C$-NMR and MS analysis with literature data.

Methylation of 50 mg (0.20 mmol) of 5,7-dihydroxyflavone (chrysin, **1**) afforded **2** (48.5 mg, 92 % of yield) [31].

Methylation of 50 mg (0.19 mmol) of 5,7,4'-trihydroxyflavone (apigenin, **3**) gave **4** (31.6 mg, 60 %) and **5** (8.3 mg, 15 %) with a ratio of 4/1 [31].

Methylation of 100 mg (0.35 mmol) of 5,7,3',4'-tetrahydroxyflavone (kaempferol, **6**) gave 9 (71.1 mg, 62 %) and **10** (14.3 mg, 12 %) with a ratio of 5/1 [32].

Methylation of 150 mg (0.5 mmol) of 3,5,7,3',4'-pentahydroxyflavone (quercetin, **15**) gave **16** (15.6 mg, 10 %), **17** (15.6 mg, 9.5 %), **18** (97.8 mg, 55 %) and **19** (17.0, 9.2 %) with a ratio of 1/1/6/1 [33–36].

Methylation of 6 mg (0.017 mmol) of isolated compound **20** gave **22** (5.5 mg, 85 %) [37].

Methylation of 12 mg (0.032 mmol) of isolated compound **27** gave **28** (7.5 mg, 61 %) and **29** (3.9 mg, 28 %) in ratio of 2/1 [38].

### 4.6. Heme-binding assay by MS

Hemin and compounds were dissolved in DMSO at 5 mM. An auto sampler (G1367E 1260 Hip ALS, Agilent Technologies, Les Ulis, France) was used to realize the automatic mixing in 384 well-plates of: compound 5 µL + heme 5 µL + tween 20 (10 µM in water) 5 µL + CBSO (citric buffer saturated octanol, pH = 5.2) 85 µL. Citric buffer saturated octanol (CBSO) was prepared by mixing 5 mL of citric acid 50 mM pH 5.2 and 15 mL of n-octanol (anhydrous, ≥99%) and letting it to settle at 23 °C for 30 min. Upper phase of CBSO was used. Five microliter of the sample were injected in a 6530 Accurate-Mass Q-ToF LC/MS instrument (Agilent Technologies). Positive ESI (electrospray ionization) was used, in 1700 range / 2 GHz mode. Ionization source conditions were: drying gas temperature 325 °C, drying gas flow 11 L/min, nebulizer 35 psig, fragmentor 175 V, skimmer 65 V. Mass range for MS acquisition was m/z 200-1700. Masses were detected for heme at m/z 616.16 and for adducts of compound of mass "X" at m/z (616.16 + "X")+. Chloroquine diphosphate (5 mM in water) was used as positive control.

### 4.7. Collision induced dissociation of non-covalent adducts

Dissociation voltage at 50% was determined by means of applying increasing collision energy from 0 to 32 eV. MSMS targeted mode, with isolation width of ~1.3 m/z and flow injections analyses (FIA) were used. MSMS parameters in source were the same as described above. Results were plotted and sigmoidal regression curve was calculated using GraphPad Prism software. $DV_{50}$ is the collision energy dissociating 50% of the initial adduct [15].

### 4.8. Parasite culture of *Plasmodium falciparum*

The chloroquine-sensitive 3D7 *P. falciparum* strain and W2 chloroquine-resistant stain were obtained from Malaria French National Reference Center (CNR Paludisme, Hôpital Bichat Claude Bernard, Paris). The strains were maintained in O+ human erythrocytes in albumin supplemented RPMI medium under continuous culture using the candle-jar method. The parasites were synchronized to the ring stage by repeated sorbitol treatment [39,40].

### 4.9. *In vitro* antiplasmodial activity on *Plasmodium falciparum*

A 2.5% (V/V) erythrocytes suspension with 1% parasitemia was incubated with the compounds. Compound concentrations ranged between 48.5 nM and 1 µM, obtained by serial dilution, in duplicates. After 44 h of incubation at 37 °C, the plates were subjected to 3 freeze-thaw cycles to achieve complete hemolysis. The parasite lysis suspension was diluted 1:5 in lysis buffer. Monitoring of in vitro susceptibility uses the concentration that inhibits 50% of the parasite's growth (IC50). Parasite growth was determined by using SYBR® Green I, a dye showing strong fluorescence enhancement upon contact with parasite DNA. Incorporation of SYBR® Green I (Applied Biosystems, Strasbourg, France) in parasite DNA was measured using the Mastercycler epRealplex (Eppendorf, France) according to the following program to increase the SYBR® Green I incorporation: 90 °C (1 min), decrease in temperature from 90 °C to 10 °C (during 5 min), followed by fluorescence reading. Untreated infected and uninfected erythrocytes were used as controls and chloroquine diphosphate (Sigma, France) as reference drug. $IC_{50}$ was calculated by IC-estimator online software (http://www.antimalarial-icestimator.net).

### 4.10. Cytotoxic activity

For determination of cytotoxicity $IC_{50}$, human umbilical vein endothelial cells (HUVECs) were cultured in Dulbecco's Modified Eagle medium/nutrient mixture F-12 (DMEM-F12) medium in the presence of 10% fetal bovine serum (FBS) plus 1% streptomycin and incubated in 5% $CO_2$ at 37 °C. The cytotoxicity of compounds (starting concentration of 10 mM) was evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric method. HUVECs were seeded in a 96-well plate at 15,000 cells per well and incubated for 24 h until cells reached >80% confluence. After discarding the old medium, the cells were incubated in the medium containing test solutions at eight concentrations ranging from 0.78 to 50 µM. After 24 h incubation, 20 µL MTT (5 mg/mL) was added to each well and cells were incubated for another 3 h. Finally, the culture medium containing MTT solution was removed and 100 µL DMSO were added to dissolve formazan crystals. Absorbance was read with an Eppendorf plate reader at 546 nm. $CC_{50}$, defined as extract concentration that reduces the number of viable cells by 50%, was calculated using GraphPad Prism Software (Version 5.0, San Diego, CA, USA).

### 4.11. ADMET Prediction

FAF-Drugs4 [41] and ChemSketch (ACD/Labs Products) software were used to screen the flavonoids library in order to predict their ADMET proprieties. For FAF-Drugs4 on line service, the input structures (previously generated SDF files) were formatted according to FAF-Drugs4's requirements using Bank-Formatter. XLOGP3 was chosen as the clogP computation program to estimate the lipophilicity and the derived ADMET descriptors. For ChemSketsh software, the input file were previously generated as SK2 files and prediction of clogP were estimated for all compounds.

### 4.12. Hemozoin *in vitro* formation

Hemozoin *in vitro* formation was evaluated by Heller and Roepe methodology with slightly modifications [42]. Briefly, 8 mL of synchronized plasmodial culture of 3D7 *P. falciparum* strain at 2.5% (v/v) erythrocytes suspension with 3% of parasitemia were incubated with compounds **6**, **10**, **19** and chloroquine at $IC_{90}$ concentration respectively in duplicates and cultivated as previously described conditions. After 9, 14 and 17 h of incubation, the suspension culture were centrifuged and parasite were released with 0.1% saponin solution (w/v). Samples were then

washed four times with ice-cold PBS buffer to yield purified parasites pellets. Then, pellets were washed once with 150 µL of PBS, 50 µL of $H_2O$ and 50 µL of 1 M HEPES to remove any final traces of hemoglobin. Four hundred microliters of 4% SDS in 0.1 M $NaHCO_3$ were added to the pellets, and the mixture was incubated and shaken at 37°C for 2.5 h and then centrifuged at 1,300 rpm. The pellet containing hemozoin was washed three times with bicarbonate buffer and finally dissolved in 500 µL of 3 N NaOH solution. One hundred microliters were placed into 96-well plate and UV absorption was reading at 405 nm.

Acknowledgments

The authors thank Dr. Guy Lewin for fruitful discussion and north Province of New Caledonia for permission of field investigations.

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| Pos. | **2**[a] | **4**[a] | **5**[a] | **9**[a] | **10**[a] | **16**[b] | **17**[b] | **18**[a] | **19**[a] | **22**[a] | **26**[a] | **27**[a] | **28**[a] | **29**[a] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 3 | 6.65 s | 6.56 s | 6.63 s | | | | | | | | | | | |
| 6 | 6.49 d 2.0 | 6.35 d 2.1 | 6.37 d 2.0 | | 6.34 d 2.2 | 6.25 d 2.2 | 6.23 d 2.0 | 6.35 d 2.1 | 6.35 d 2.5 | | 6.21 s | 6.37 s | 6.34 d 2.2 | 6.35 d 2.5 |
| 7 | | | | | | | | | | | | | | |
| 8 | 6.36 d 2.0 | 6.47 d 2.1 | 6.57 d 2.0 | 6.46 s | 6.50 d 2.2 | 6.49 d 2.2 | 6.69 d 2.0 | 6.44 d 2.1 | 6.44 d 2.5 | 6.75 s | 6.45 s | 6.46 s | 6.43 d 2.2 | 6.50 d 2.5 |
| 2’ | 7.87 dd 7.7, 1.6 | 7.00 d 9.1 | 7.00 d 9.0 | 7.05 d 9.2 | 7.01 d 9.6 | 7.70 d 2.2 | 7.66 d 2.0 | 7.69 d 2.0 | 7.70 d 2.7 | 7.70 dd 7.9, 2.4 | 7.24 s | 7.37 s | 7.35 s | 7.36 s |
| 3’ | | 7.83 d 9.1 | 7.82 d 9.0 | 8.23 d 9.2 | 8.06 d 9.6 | | | | | | | | | |
| 4’ | 7.56-7.48 m | | | | | | | | | | | | | |
| 5’ | | 7.83 d 9.1 | 6.57 d 2.0 | 7.05 d 9.2 | 8.06 d 9.6 | 6.99 d 8.9 | 7.14 d 9.0 | 6.97 d 9.0 | 6.96 d 8.7 | 6.99 d 7.9 | 7.18 s | 7.35 s | 7.35 s | |
| 6’ | 7.87 dd 7.7, 1.6 | 7.00 d 9.1 | 7.00 d 2.0 | 7.05 d 9.2 | 7.01 d 9.6 | 7.58 dd 8.9, 2.2 | 7.70 dd 9.0, 2.0 | 7.71 dd 9.0, 2.0 | 7.69 dd 8.7, 2.7 | 7.69 dd 7.9, 2.4 | | | | 7.36 s |
| OMe-3 | | | | 3.99 s | 3.96 s | 3.86 s | 3.86 s | 3.87 s | 3.89 s | 3.87 s | 3.82 s | 3.90 s | 3.86 s | 3.88 s |
| OMe-5 | | | 3.92 s | | 3.88 s | | | 3.99 s | 3.95 s | 4.01 s | | | | 3.97 s |
| OMe-6 | | | | | | | | | | 3.92 s | | | | |
| OMe-7 | 3.87 s | 3.87 s | 3.88 s | 3.89 s | 3.89 s | | 3.96 s | | 3.95 s | 3.97 s | | 4.01 s | 3.86 s | 3.91 s |
| OMe-8 | | | | | | | | | | | | | | |
| OMe-3’ | | | | | | | | | 3.95 s | 3.97 s | 3.86 s | 3.96 s | 3.93 s | 3.94 s |
| OMe-4’ | | 3.89 s | 3.91 s | 3.89 s | 3.87 s | | 3.93 s | 3.87 s | 3.87 s | 3.98 s | 3.77 s | 3.89 s | 3.94 s | 3.93 s |
| OMe-5’ | | | | | | | | | | | | | 3.93 s | 3.94 s |

**Table 1**: $^{1}$H-NMR Chemical shift of synthetized (**2**, **4-5**, **9-10**, **16-19**, **22** and **28-29**) and isolated (**26-27**) compounds. a) $^{1}$H-NMR (400 MHz, $CDCl_3$), b) $^{1}$H-NMR (400 MHz, $CD_3COCD_3$).

## IV.4.2 Contribution and perspectives.

Even though correlation between the antiplasmodial *in vitro* activity with the methoxylation/hydroxylation pattern remains unclear, pentamethoxyquercetin was identified with antiplasmodial activity against two strains of *P. falciparum* and present heme-adducts formation, blocking 40% of the hemozoin formation in infected erythrocytes.

Other important contribution of this work is the implementation of an *in vitro* protocol to measure the hemozoin formed *in vitro* on infected erythrocytes.

Main perspectives could be drawn in two parts:

- Study of the principal molecular interaction involved in the heme-adduct formation between polymethoxyflavones as pentamethoxyquercetin, with the heme. *In silico* studies have been started in order to better understand these interactions.
- *In vivo* efficacy of pentamethoxyquercetin of the antiplasmodial activity and *in vitro*/*in vivo* synergism between this flavonoid with artemisinin, based on the previous effect reported for this drug with polymethoxyflavones [261].

# Chapter V Arf1-Sec7 Protein complex as pharmacological target of new sesquiterpenes lactones

# V.1 Introduction

As treated in **Chapter IV**, the main objective of the chemotaxonomic approach is discover potential bioactive compounds from identified metabolites families. Species from Asteraceae family biosynthesize sesquiterpene lactones and PMFs. These kind of compounds have been reported in the literature with a wide range of pharmacological, cosmetic and nutraceutical potential, including cytotoxic and antiparasitic activities.

Asteraceae species plant from Algeria were collected and extracted with ethyl acetate and methanol. Then, in addition to experiments dealing with PMFs, the sesquiterpene lactone ketopelenolide B was isolated in high yield from the same extract of *P. monodiana*, and had demonstrated a moderate antitrypanosomal activity.

In **Chapter V** we describe the semisynthesis of a library of sesquiterpene lactones from ketopelenolide B and the *in vitro* evaluation of their cytotoxic and antitrypanosomal activities together with *in silico* modelization, which revealed a high similarity with brefeldin A, a known inhibitor of the protein-protein interaction of Arf1-Sec7 protein system.

# V.2 Personal work and collaboration

In this section are described the main objectives proposed in the study of the antitrypanosomal and cytotoxic activities of ketopelenolide B derivatives, natural and semisynthetic sesquiterpene lactones isolated from *P. monodiana*.

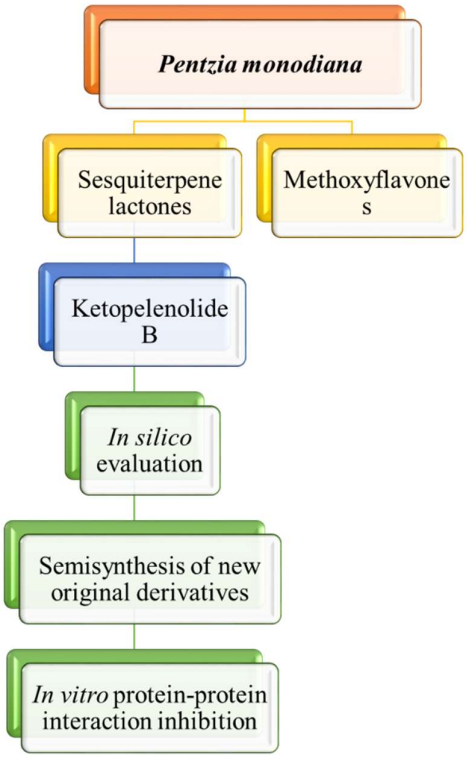


**Figure 97: General workflow applied in Chapter V.**

Three important partnerships were established in order to reach general and specific objectives of this study.

| Task | Responsible | Institution |
|---|---|---|
| In silico screening | Dr. Euzébio Guimarães Barbosa | Department of Pharmacy, Federal University of Rio Grande do Norte, Brazil |
| Antiparasitic activity | Dr.Antonia Efstathiou<br>Dr. Despoina Smyrlis | Laboratory of Molecular Parasitology, Hellenic Pasteur Institute, Greece. |
| Arf1-Sec7 inhibition | Pr. Jacqueline Cherfils | Laboratoire de Biologie et Pharmacologie Appliquée, |

**Table 11: Collaboration stablished during Chapter V.**

## V.2.1 Objectives and methodology

In order to explore the antitrypanosomal and cytotoxic activities of ketopelenolide derivatives, several general and specific objectives were proposed:

### V.2.1.1 General objective

- *In silico* evaluation of ketopelenolide B against a bank of protein structure in order to identify potential molecular targets.
- *In vitro* evaluation of the inhibition of Arf1-Sec7 protein-protein interaction by semisynthetic ketopelenolide B derivatives.

### V.2.1.2 Specific objectives

- Semisynthetic structural modification of ketopelenolide B by reduction/epoxidation of its double bond, ring expansion and condensation with R-hydroxylamines.
- Nucleotide exchange factor inhibition evaluation of Arf1-GDP to Arf1-GTP by action of different ArfGEFs of original ketopelenolide B derivatives.

## V.2.2 Methodology

### V.2.2.1 *In vitro* cell free Arf1-Sec7 inhibition interaction assay

The nucleotide exchange assay of Arf1-Sect systems was carried out as previously. [262]. This methodology is based on the fact that nucleotide exchange of Arf1 from its deactivated

conformation, linked with a GDP group changes after exchange with GTP, its active form. Only its active form Arf1-GTP shows tryptophan fluorescence ($\lambda_{exi}$ 292 and $\lambda_{emi}$ 340 nm).

The fluorescence is followed after solution mixture of Arf1-GDP, GTP, GEF protein (containing the Sec7 domain) with the evaluated compound. Exchange rates ($k_{obs}$) were determined and brefeldin A was used as positive control.

# V.3 Inhibition of Arf1-Big1Sec7 interaction by natural and semisynthetic sesquiterpene lactones

Arf1 (ADP-ribosylation factor 1) belongs to the small GTPases protein family. Hydrolases from this large group bind to the nucleotide guanosine triphosphate (GTP) and hydrolyse it to guanine diphosphate (GDP). The GTPase state bonding with a GDP is considered inactive and is located in the cellular cytosol. The other state, bonding a GTP, is considered as the active form, added to hydrophilic membrane in several organelles or cellular compartment, where the small GTPase activates downstream pathways, exerting fundamental functions in secretion, endocytosis, phagocytosis, cytokinesis, cell adhesion and tumor-cell invasion[263].

The alternation of both GDP/GTP states is complex and well-studied for some GTPase systems only. Due the fact that GDP is in general tightly bound to GTPases and GTP is hydrolysed very slowly, small GTPases require the assistance of guanine nucleotide exchange factor (GEFs) that facilitates GDP dissociation and of GTPase activating proteins (GAPs) that stimulate GTP hydrolyzation [263].

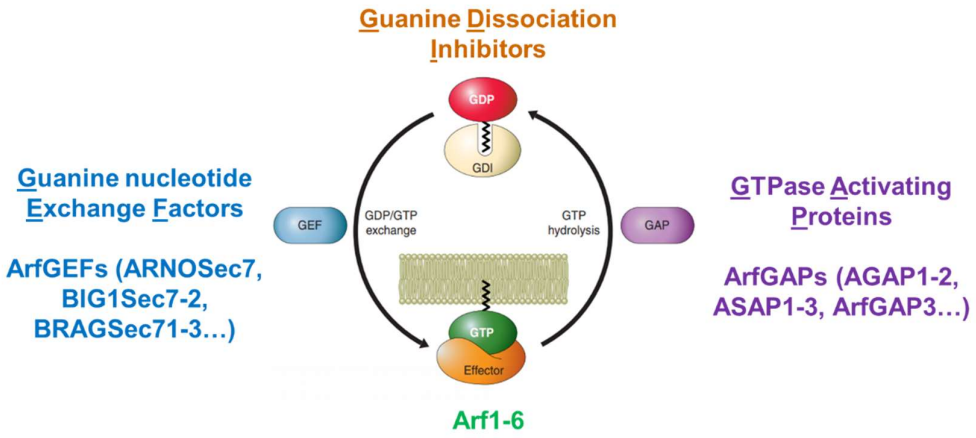


**Figure 98: General scheme of of small GTPase system.**

GTPases that carry a farnesyl or geranylgeranyl group, which are used for their addition to the membranes in their active state (in their C-terminus) are combined with guanine dissociation inhibitors proteins GDIs, which help GTPs to form soluble complex by shielding their lipids in their inactive state. The actual molecular system is then composed of a small GTPase, a GEF, a GAP and sometimes a GDI, rather than a small GTPase alone [263].

One of the subfamilies of GTPase presents in almost all eukaryote cells is the Arf family, comprised by six members, where five of them are found in human cells (Arf1, 3-6), all of which have in common a much larger GDP/GTP conformational change in comparison to other GTPase families.

## V.3.1 Role of Arf1 protein in the tumor development

Arf1 and Arf6 are quite well-studied GTPase, localized in different cell compartments. Arf6 is only found in the plasma membrane, while Arf1 mainly in the Golgi membrane [264]. They are activated by ArfGEFs, which contain a catalytic conserved Sec7 domain and inactivated by GAPs, which contain a conserved ArfGAP domain, all combined forming a system Arf, ArfGEFs and ArfGAPs [265].

The main function of Arf proteins in the cells is the regulation of vesicular traffic and organelle structures by recruiting coat proteins, regulating phospholipids metabolism and modulating the structure of actin at membrane surfaces. Arf1, which under its active form, is mainly located in the Golgi membrane and in the endoplasmic reticulum – Golgi intermediate compartment, recruits numerous coats to different membrane sites, regulating the essential trafficking between Golgi and ER organelles [266,267].

Arf1 is abnormally expressed in different cancer cell types such as breast, gastric, prostate and lung human cancers [268–273]. Arf1 plays an active and essential role in the cancer cell migration. Arf1 was shown to regulate the formation of focal adhesions, which couple the cancer cell membrane to the actin cytoskeleton and the extracellular matrix (FA), in invasive breast cancer cells through the phosphorylation of FA kinase, interacting also with several proteins within the FA complex, like paxillin and talin, regulating their interactions [274,275]. It has shown also that the inactivation of Arf1 protein lead to a decrease in cancer migration via depletion of its ArfGEFs: BIG1 and BIG2 [276,277].

## V.3.2 Role of trypanosomal Arf proteins in the development of Trypanosoma parasites

Numerous efforts have been done to better understand the molecular biology of *Trypanosoma* parasites. From early 2000 decade, several groups have identified trypanosomal small GTPase from *T. brucei* and *T. cruzi* parasites [278–281], including the 3D structural determination of the

trypanosomal Arl6 (ADP-ribosylation factor-like protein 6) protein from *T. brucei gambiense* [282].

*Trypanosoma brucei* Arf1 protein (tArf1), which shared a 75% of its amino acid identity with human Arf1 was characterized. As in humans, tArf1 is localized in the Golgi complex, ERGIC (endoplasmic reticulum-Golgi intermediate compartment) and Golgi-proximal endosomes of the parasites [278].

The disrupting expression of tArf1 using a tetracycline-inducible RNAi caused a rapidly lethal phenotype 24h after the induction. A large vacuole-like structure was observed in 86% of the parasites, which was identified as a grossly enlarged flagellar pocket, due to loss of vesicular transport, accumulating the excess of membrane and raising the internal pressure. This effect was also observed in the disrupt expression of actin, Rab5 and Rab11 proteins [283–285]. The absent of tArf1 allowed to identified the main physiological role of this protein in *T. brucei*, seemed essential for endocytosis, regulation of glycosome proliferation and exocytosis [278]. In addition, overexpression tArf1 produced also a lethal phenotype, quickly stoping cell division and Golgi-to-lysosome transportation.

## V.3.3 Brefeldin A as inhibitor of Arf1-Big1Sec7 system

Brefeldin A, a macrolide-type lactone isolated from *Penicillium* species since 1958 [286,287], has exerted a series of potent biological activities such as antitumor [288–290], antifungal [291]and antiviral [292]. I*n vitro* and *in vivo* cytotoxic activity were reported against a wide panel of cancer cell lines, with $IC_{50}$ values (MTT assay) of 7.1 (Meth A fibrosarcoma) to 0.08 µM (KATOIII gastric carcinoma)[293], and 13.9 nM against bone cells [294]. SAR analysis on the cytotoxic activity against HCT 116 colon cancer cell line showed the importance of the stereochemistry of position 4, where the *S* isomer is 300 fold more active than the *R* isomer ($IC_{50}$ 0.2 µM/60 µM) [295].

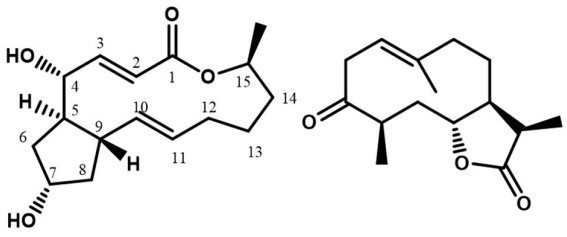


**Figure 99: Brefeldin A (left) and ketopelenolide B (right) structures.**

Mechanistic studies showed that brefeldin A inhibits the exchange reaction through an uncompetitive process with formation of an Arf-GDP-brefeldin A-Sec7 complex, which cannot proceed to nucleotide exchange, blocking the vesicle formation and transport from the Golgi complex [296]. Brefeldin A interacts with Arf1-Sec7 system by hydrogen bonds and hydrophobic contacts with both proteins. Chiral hydroxyls are engaged in hydrogen bonds Trp 78 and Tyr 190* (asterisk indicates a residue of the Sec7 domain) and Asp67. Hydrophobic contacts are formed between the macrolide cycle and Trp66, Tyr81 and Met194* [297].

The toxicity levels of brefeldin A, together with poor physical and pharmacokinetics proprieties hampered further developments as anti-cancer agent: indeed, *in vitro* cytotoxic activities in the low micromolar range against various mammalian normal cell lines (5-0.01 μM) [298] and $LD_{50}$ values of 250 mg/kg in mice (intraperitoneal) and 275 mg/kg in rats (oral administration) have been reported.

Brefeldin A was inactive against *Trypanosoma* parasites, mainly due to the sequence and structure of the trypanosomal GEFs.

Due the structural similarity between brefeldin A with ketopelenolide B, a preliminary *in silico* screening was performed in order to find any affinity between ketopelenolide B with the Arf1-Sec7 system. The observed affinity was corroborated by a cell free *in vitro* assay, where ketopelenolide B weakly inhibited the nucleotide exchange (5%). A library of semisynthetic derivatives was generated in order to upgrade the initial activity. Main results are detailed in Draft 11.

## V.3.4 Draft 11. Synthesis of new sesquiterpene lactones with Arf1-Big1Sec7 inhibitory properties

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Abstract

1. Introduction

Arf (ADP-ribosylation factor proteins) are small GTPase proteins, dedicated to the formation and trafficking of vesicle structures. They are present in all eukaryotes cells [1]. Overproduction of some members of this family such as Arf1, Arf4 and Arf6 in cancer cells has been reported [2]. Arf1 have been identified to play an important role in the cell migration on tumor development and inhibition of its expression and/or activity has decreased *in vitro* and *in vivo* cancer cell proliferation [3,4].

Brefeldin A (BFA), a natural macrolide lactone isolated from *Penicillium* species, has shown to inhibits Arf1 activity by stabilization of the protein complex formed by Arf1 with guanine diphosphate and ArfGEFs proteins. These latter include a well-conserved domain called Sec7 [5,6]. Several ArfGEFs have also been identified but brefeldin is selective to the complex Arf1-Big1Sec7 complex [7]. BFA has shown potent *in vitro* and *in vivo* cytotoxic activity against a wide range of cancer cell lines [8,10]. Unfortunately, toxicity and poor pharmacokinetics proprieties hampered its further development as anticancer drug candidate.

The natural sesquiterpene lactone ketopelenolide B (**1**), isolated in our group [11] displays structural similarities with BFA. After a preliminary in silico docking of **1** with the Arf1-Sec7, the semisynthesis of 11 (**2a**-**8**) original derivatives of **1** was performed and their Arf1 inhibition activity was evaluated.

2. Results

2.1 *In silico* evaluation of ketopelenolide B

A preliminary *in silico* docking of ketopelenolide B **1** was performed by Inverse Virtual Screening approach. Eight conformation of **1** were drawn on basis of their lower energy, and were submitted into a directory of more than 2000 protein structures. Docking evaluation showed a great score function of -10.2 of conformation structure of compound **1** with the structure of Arf1-GDP in complex with a Sec7 domain (Entry 1S9D). Structure similarities between **1** and brefeldin A consist in the presence of a lactone group, a five member ring and a bigger one (10 or 13 atoms). Even though **1** do not establish hydrogen bond, it efficiently fits into the active site of Arf1-Sec7 complex.

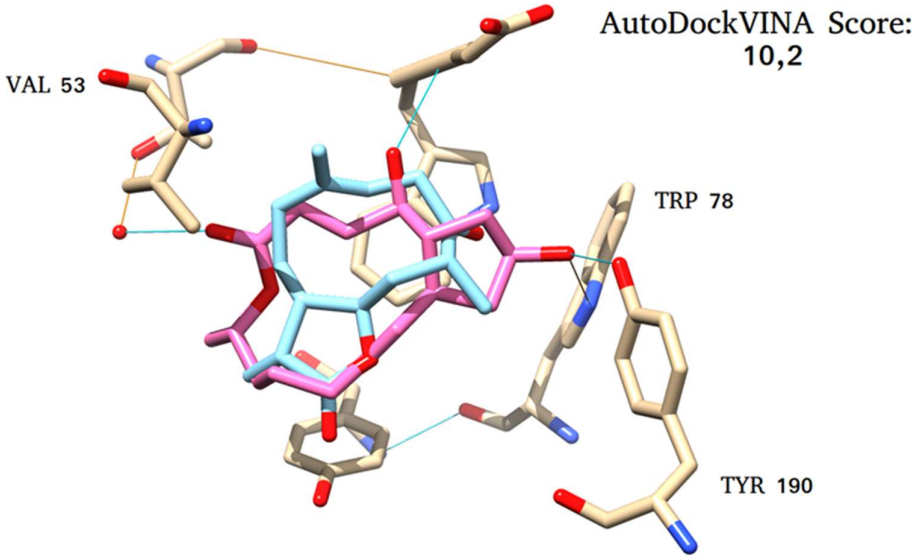


**Figure 1**: Docking with Arf1 (ID 1S9D) illustrate of resemblance between the brefeldin A (red) and ketopelenolide B (blue). The hydrogens bond are symbolized with sticks.

2.2 Chemistry

A series of analogues of **1** was prepared as shown in **Figure 2**. Thus, ring expansion of **1** with $TMSCHN_2$ as methylation agent provided **2a** and the sub-product **2b**. Conventional epoxidation of **1** was performed with *m*-CPBA, giving the epoxy analogue **3a** and the sub-product **3b**. Bromination in C-2 and C-14 position were performed with TMPAP (trimethylphenylammonium perbromide) and NBS (N-Bromosuccinimide) respectively to provide products **4** and **5**. Reduction of carbonyl group at C-3 with $NaBH_4$ gave the hydroxylated compounds **6** while reduction of the double bond C1-C10 with $H_2$ in presence of Pd-C 10% as catalyst gave compound **8**. Oxime ether derivatives of **1**, **7a-c**, were synthesized by treatment of the natural product with substituted hydroxylamine. Relative configurations of **2b**, **3b**, **4-6** and **8** were determined by 2D NOESY

experiments. Relative configurations of **2a**, **3a**, **7a** and **7b** were established by X-ray crystallography (**Figure 3)**.

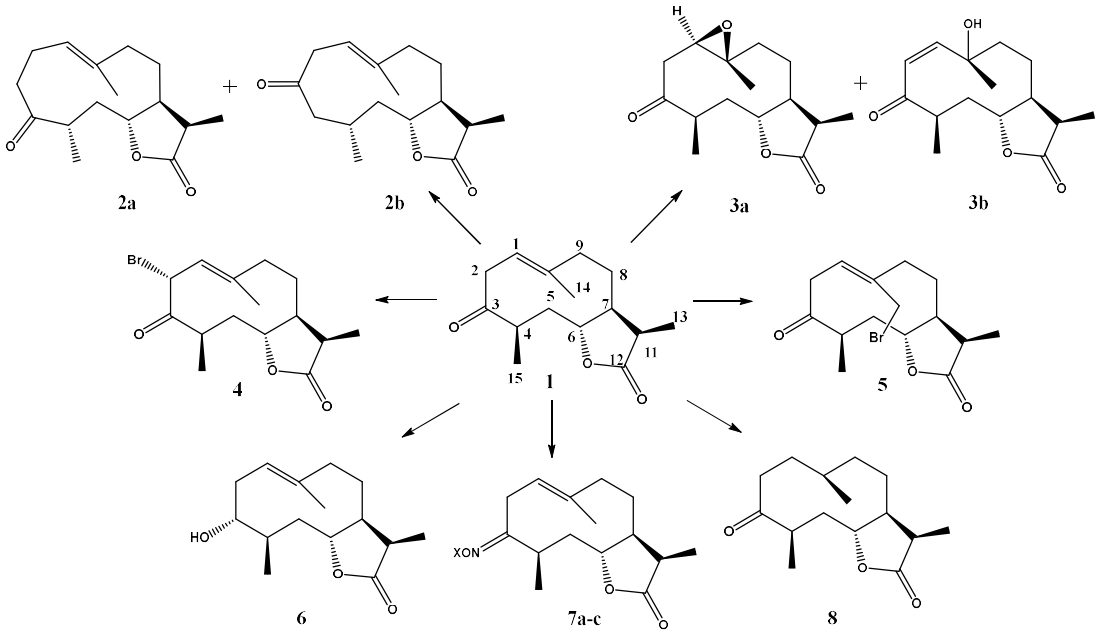


**Figure 2**: Structural modifications of new ketopelenolide B analogues **1**-**8**.

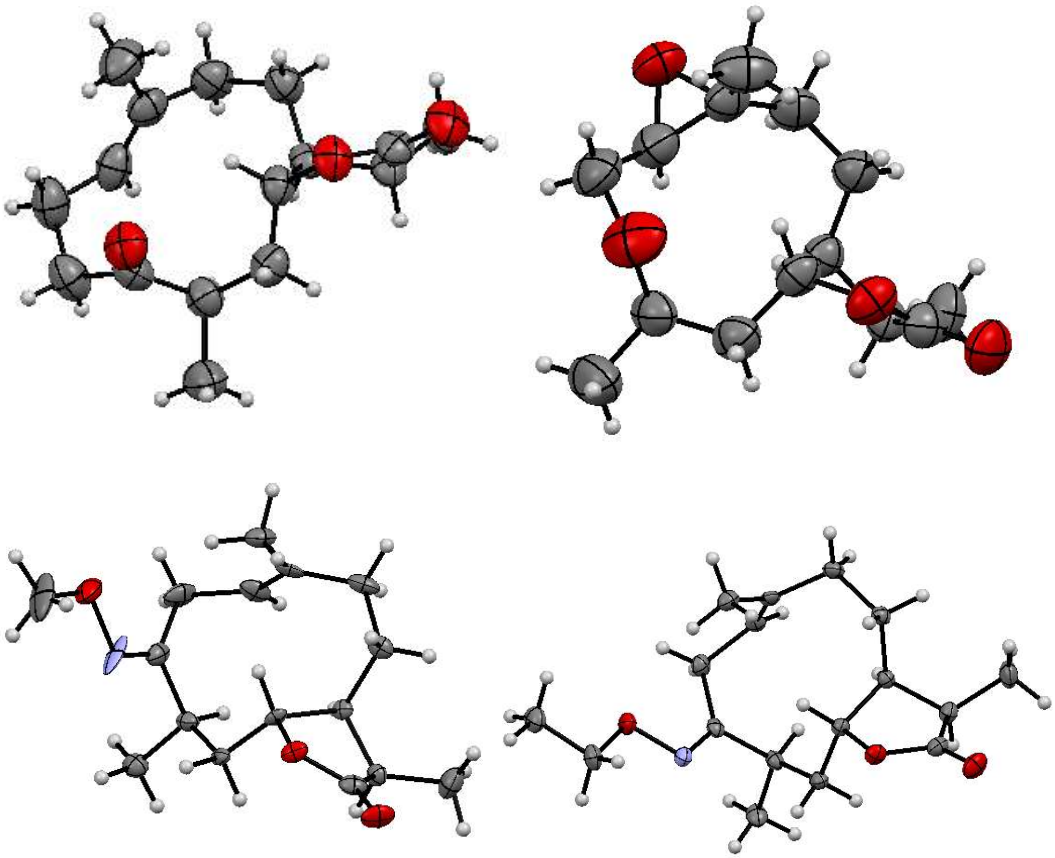

**Figure 3**: ORTEP representation of **2a**, **3a**, **7a** and **7b**.

2.3 *In vitro* and *in silico* inhibition of Arf1-Sec7 interaction

The *in vitro* cell-free nucleotide exchange assay and *in silico* function score and ligand efficiency were performed for all of the derivatives. *In vitro* assay (Table 1) at compound concentration of 50 μM showed a loss of the nucleotide exchange inhibition for derivatives **2a-5** with 3.3 to 8.5 % of inhibition, lower than compound **1** (12.5%). In contrast, oximes derivatives **7a-c** showed stronger inhibition values of 21.6, 32.0 and 28.4, respectively, as in the case of reduced derivatives **6** and **8** ( 20.5 and 24.0%, respectively). Kinetic nucleotide exchange action of more active compound **7b** and of the positive control brefeldin A with the respective controls are displayed in **Figure 4**.

| Nucleotide exchange inhibition for Arf1-Big1Sec7 complex by semisynthetized derivatives | | |
|---|---|---|
| Code | Score *In silico* | % Inhibition at 50 μM |
| **1** | -10,2 | 12,5 |
| **2a** | -10,7 | 5,8 |
| **2b** | | 5,8 |
| **3a** | n.e. | 5,1 |
| **3b** | | 3,3 |
| **4** | -10.5 | 7,3 |
| **5** | -9.6 | 8,5 |
| **6** | | **20,5** |
| **7a** | n.e. | **21,6** |
| **7b** | | **32,0** |
| **7c** | | **28,4** |
| **8** | -10.4 | **24,0** |

**Table 1**: Inhibitory activity for the Arf1-Big1Sec7 complex formation by the ketopelenolide B analogues **1-8**. n.e.: Not evaluated.

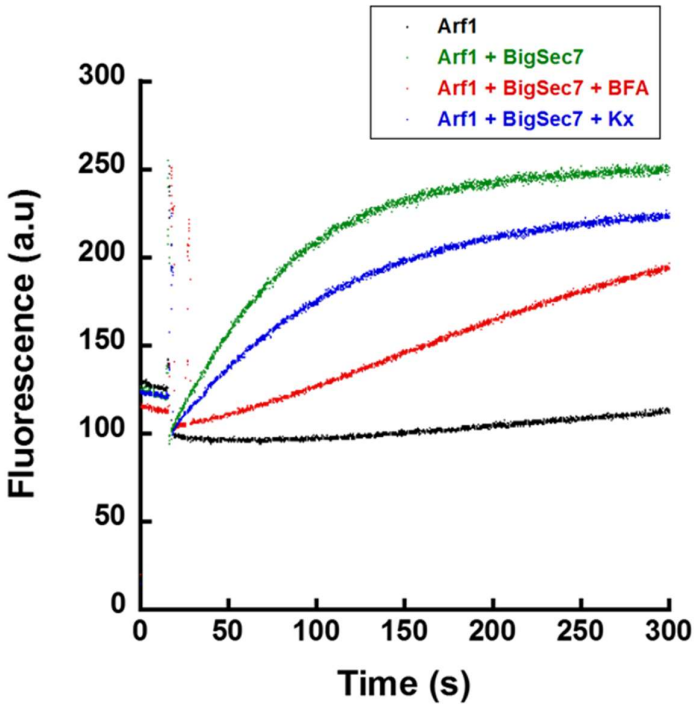


**Figure 4**: Kinetic profiles of compound at 50 µM **7b**, brefeldin A (BFA) and DMSO control.

In addition, the selectivity of the most active compound **7b** was evaluated against other Arf1GEFs, which also comprises a Sec7 domain. Derivative **7b** demonstrated inhibitory nucleotide exchange activity only against the Arf1-Big1Sec7 complex and was inactive against complex formed of Arf1 with ARNOSec7 and Brag1Sec7 GEFs. This nucleotide exchange inhibitory profile is similar than those of brefeldin A (**Table 2**).

| **Arf1GEFs** | **BFA** | **7b** |
|---|---|---|
| BIG1Sec7 | +++ | ++ |
| ARNOSec7 | 0 | 0 |
| Brag1Sec7 | 0 | 0 |

**Table 2**: Nucleotide exchange inhibitory selectivity profile of brefeldin A and compound 7b. 0, < 10% inhibition; +, 10-20% inhibition; ++ 20-40% inhibition; +++, > 40% of inhibition.

Docking simulation in the binding site of target of Arf1-Sec7 structure was performed in parallel of the *in vitro* screening assay. Opposite to the *in vitro* results, oximes derivatives **7a-c** showed poor score functions (> -8.0). Higher scores were recorded for derivatives **2a**, **4**, **8**, **1** and **5** with

values of -10.7, -10.5, -10.4, -10.2 and -9.6 respectively (**Table 1**). Among them, only **8** showed significant *in vitro* nucleotide exchange inhibitory properties (24%). Analysis of docking simulation of **8** showed hydrogen bond formation between amine group of VAL 65 residue and the ketone group of **8**. Similar interaction could be observed in the case of brefeldin A, where one hydrogen bond is also established between the same amino acid residue with the hydroxyl group at position 4. In contrast, this hydrogen bond is not observed in the case of the natural starting product **1**.

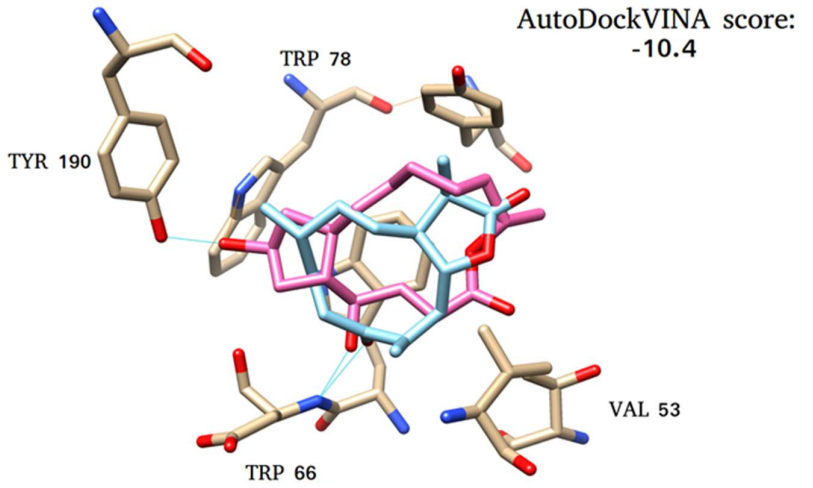


**Figure 5**: Docking with Arf1-Sec7 (ID 1S9D). Illustrate of resemblance between the brefeldin A (red) and **8** (blue). The hydrogen bonds are illustrated with sticks.

3. Discussion

The nucleotide exchange activity by original ketopelenolide B derivatives (**1**-**8**) has been investigated *in vitro* and *in silico*. By *in silico* docking simulation, compounds with side chains showed no interaction with the protein target. Indeed, higher hindrance of compounds **7a**-**7c** may have hampered their access into the binding site of the Arf1 structure. On the contrary, these compounds showed the highest *in vitro* nucleotide exchange inhibitory capacity. These opposite behaviors could be explained by a hypothetical conformation change of the binding site of Arf1-Sec7 protein systems. Hydrophobic vicinal amino-acid residues in the active site could play a more important role in the stabilization of ketopelenolide B derivatives, interacting with the side chains by hydrophobic interactions.

In addition, **7b** showed a similar inhibitory profil against other ArfGEFs in comparison with the reference inhibitor brefeldin A. ArfGEFs as ARNO and BRAG1 contains the Sec7 domain, essential in the interaction with Arf1 but differ in thie amino-acid sequence and structure. One of the advantage of the inhibitory activity of brefeldin A is its selectivity for Arf1-Big1Sec7

complex, presenting molecular interaction with both main proteins, Arf1 and Big1Sec7. Analogue **7b** has a similar profile, interacting also with both proteins. This fact may explain its selectivity towards ArfGEFs proteins.

## 4 Conclusion

Semisynthesis starting from ketopelenolide B has led to original sesquiterpene lactone derivatives with improved *in vitro* nucleotide exchange cell-free activity. Nevertheless further studies must be performed in order to better understand the molecular interactions of ramified derivatives in the Arf1-Sec7 complex, as well as mechanisms involved in limitation of the cytotoxicity of these compounds.

## 5. Experimental

### 5.1 General experimental chemical procedures.

$^{1}H$ and $^{13}C$-NMR experiments were performed in deuterated chloroform. Spectra were recorded on a Bruker AC 300 MHz or a Bruker 400 MHz spectrometer (Wissembourg, France). Automatized pressurized extractions were performed on a Speed Extractor E-914 apparatus provided by Büchi (Fawil, Switzerland) equipped with four cells (120 mL) and a collector with four flat bottom vials (220 mL). Maximum pressure and temperature can be adjusted from 50 to 150 bar and from 30 to 200 °C, respectively. Mass spectrometry was performed on a mass spectrometer ZQ 2000 Waters (Saint-Quentin, France). Silica gel 60 0.025-0.04 mm (Macherey-Nagel GmbH & Co.) was used for column chromatography (CC). Merck silica-gel 60 aluminium sheets were used for TLC and visualized under UV at 254 and 366 nm and at white light after spray of vanilline/sulfuric acid. All the reagents and solvents were purchased from Carlo Erba-SDS. Cyclohexane, dichloromethane and ethyl acetate were distilled before used. GDP, GTP and N-methylanthraniloyl GTP (mGTP) is from Jena Bioscience. Brefaldin A (BFA) was purchased in Sigma-Aldrich.

### 5.2 Isolation of ketopelenolide B (**1**)

Ketopelenolide B was isolated from *Pentzia monodiana* ethyl acetate extract reported before [1]. Briefly, 557 g of aerial part of *P. monodiana* was extracted at 100 bar and 50 °C in a Speed Extractor apparatus. Ten grams of extract were subject by silica-gel column chromatography with cylclohexane/AcOEt mixtures of increasing polarity, given 562 mg (5.6 % of yield) of ketopelenolide B (**1**).

### 5.3 Synthesis of ketopelenolide B derivatives (**2a**-**8**)

5.3.1 Synthesis of (3R,3aS,11S,12aR,E)-3,6,11-trimethyl-3,3a,4,5,8,9,12,12a-octahydro-2H-cycloundeca[b]furan-2,10(11H)-dione (**2a**) and (3R,3aS,11S,12aR,E)-3,6,11-trimethyl-3,3a,4,5,10,11,12,12a-octahydro-2H-cycloundeca[b]furan-2,9(8H)-dione (**2b**)

To a starring solution at 0°C of 100 mg (0.4 mmol) of **1** in 2 mL of DCM were added 0.25mL of $BF_3 \cdot OEt_2$ (2.0mmol) and 1.1 mL of $TMSCHN_2$ (2.0 mmol) drop by drop. After 18 hrs of starring at 0°C, the crude was neutralized with 3 mL of $NaHCO_3$ (aq) satured, and separated with dichloromethane (3x10mL). The organic layer was concentrated and dry under $N_2$. The crude resulting was purified by silica gel column (cyclohexane/ethyl acetate – 3/7), to give 33.8 mg of white crystals (**2a**), 68 % and 4.5 mg of a white powder (**2b**), 4,3 %. For **2a**, IR νmax(film)/$cm^{-1}$ 2968, 2922, 2850, 1763, 1702, 1455, 1378, 1164 and 986; $^1$H-NMR ($CDCl_3$) δ 5.24-5.23 [m, 1H, H-1], 3.89 [d, *J* = 8.0 Hz, 1H, H-6], 2.83 [q, *J* = 8.1 Hz, 1H, H-11], 2.68-2.65 [m, 3H, H-2a, H-2a', H-4], 2.31 [d, *J* = 11.3 Hz, 1H, H-9a], 2.23 [d, *J* = 8.0 Hz, 1H, H-2b'], 2.20-2.16 [m, 1H, H-5a], 2.13 [d, *J* = 7.4 Hz, 1H, H-2b], 2.06-1.95 [m, 2H, H-7, H-9b], 1.63-1.58 [m, 1H, H-8a], 1.56 [s, 3H, H-14], 1.53-1.43 [m, 1H, H-5b], 1.39-1.32 [m, 1H, H-8b], 1.14 [d, *J* = 8.2 Hz, 3H, H-13], 1.08 [d, *J* = 8.5 Hz, 3H, H-15]; $^{13}$C-NMR ($CDCl_3$) δ 213.7 (C-3), 179.2 (C-12), 135.3 (C-10), 125.2 (C-1), 81.0 (C-6), 45.2 (C-4), 43.0 (C-7), 40.8 (C-9), 39.5 (C-2'), 36.0 (C-11), 35.1 (C-5), 26.0 (C-2), 22.8 (C-8), 16.9 (C-13), 15.1 (C-14), 10.4 (C-15). HRMS *m/z* 287.1618 $[M+Na]^+$ calcld for $C_{16}H_{24}O_3Na$ *m/z* 287.1623. For **2b** IR νmax(film)/$cm^{-1}$ 2942, 2927, 2852, 1764, 1764, 1705, 1455, 1379, 1246, 1171, 1015 and 837; $^1$H-NMR δ 5.24-5.23 [m, 1H, H-1], 4.19 [t, *J* = 6.6 Hz, 1H, H-6], 3.32 [t, *J* = 12.7, 11.1 Hz, 1H, H-2a], 2.90-2.81 [m, 2H, H-2b, H-11], 2.59 [dd, *J* = 13.3, 6.7 Hz, 1H, H-3a'], 2.42 [dt, *J* = 12.7, 3.5 Hz, 1H, H-8a], 2.15-2.14 [m, 1H, H-8b] 2.10-2.02 [m, 2H, H-4, H-7], 1.95 [dd, *J* = 13.3, 6.7 Hz, 1H, H-3b'], 1.85 [s, 3H, H-14], 1.71 [dt, *J* = 14.0, 4.5 Hz, 1H, H-9a], 1.57 [t, *J* = 6.1 Hz, 2H, H-5a, H-5b], 1.50 [d, *J* = 14.0 Hz, 1H, H-9b], 1.17 [d, *J* = 7.2 Hz, 3H, H-13], 1.01 [d, *J* = 6.7 Hz, 3H, H-15]; $^{13}$C-NMR ($CDCl_3$) δ 208.4 (C-3), 179.36 (C-12), 139.3 (C-10), 119.2 (C-1), 80.6 (C-6), 46.4 (C-3'), 46.0 (C-2), 44.9 (C-4), 40.8 (C-8), 39.3 (C-5), 36.3 (C-11), 24.5 (C-9), 21.0 (C-15), 15.9 (C-14), 10.4 (C-13). HRMS *m/z* 287.1618 $[M+Na]^+$ calcld for $C_{16}H_{24}O_3Na$ *m/z* 287.1623.

5.3.2 Synthesis of (1aR,4R,5aR,8R,8aS,10aR)-4,8,10a-trimethyloctahydrooxireno[2',3':5,6]cyclodeca[1,2-b]furan-3,7(4H,8H)-dione (**3a**) and (3R,3aS,6R,10R,11aR,Z)-6-hydroxy-3,6,10-trimethyl-3a,4,5,6,11,11a-hexahydrocyclodeca[b]furan-2,9(3H,10H)-dione (**3b**)

To a starring solution of **1** (80 mg, 0,32 mmol) in 4 mL of DCM were added 69.0 mg of *m*-CPBA. After 20 h of sterring at room temperature, the solvent was removed by under reduced pressure and the residue was purified by silica gel column chromatography (cyclohexane/ethyl acetate – 1/1) to give the products **3a** (64.0 mg, 76%) and **3b** (6.8 mg, 8%). For **3a** IR νmax(film)/$cm^{-1}$

2978, 2934, 2867, 1765, 111, 1454, 1373, 1260, 1167 and 1110; $^{1}$H-NMR ($CDCl_3$) δ 4.00 [dd, *J* = 12.3, 2.5 Hz, 1H, H-6], 3.29 [dd, *J* = 9.8, 5.4 Hz, 1H, H-1], 3.22 [dd, *J* = 14.1, 5.4 Hz, 1H, H-2a], 2.95 [m, 1H, H-4], 2.88 [q, *J* = 7.6 Hz, 1H, H-11], 2.31 [dt, *J* = 13.8, 3.7 Hz, 1H, H-9a], 2.24 [dd, *J* = 8.3, 2.5 Hz, 1H, H-7], 2.19 [dd, *J* = 14.1, 5.4 Hz, 1H, H-2b], 2.11 [dd, *J* = 13.0, 3.0 Hz, 1H, H-5a], 1.82 [dt, *J* = 14.9, 4.0 Hz, 1H, H-8a], 1.66 [m, 1H, H-5b], 1.34 [dt, *J* = 14.5, 3.0 Hz, 1H, H-8b], 1.28 [s, 3H, H-14], 1.17 [d, *J* = 6.9 Hz, 3H, H-13], 1.15 [d, *J* = 6.2 Hz, 3H, H-15], 1.06 [dd, *J* = 13.8, 4.3 Hz, 1H, H-9b]. $^{13}$C NMR ($CDCl_3$) δ 210.6 (C-3), 178.1 (C-12), 81.3 (C-6), 59.2 (C-10), 59.0 (C-1), 44.1 (C-2), 42.0 (C-7), 40.3 (C-4), 39.4 (C-9), 37.6 (C-5), 36.0 (C-11), 22.9 (C-8), 18.4 (C-15), 15.6 (C-14), 10.2 (C-13). HRMS *m/z* 289.1408 $[M+Na]^+$ calcld for $C_{15}H_{22}O_4Na$ *m/z* 289.1416. For **3b**: IR νmax(film)/cm$^{-1}$ 3396, 2972, 2925, 1760, 1703, 1455, 1379, 1164, 1077 and 1053; $^{1}$H-NMR ($CDCl_3$) δ 7.00 [d, *J* = 17.2 Hz, 1H, H-1], 5.97 [d, *J* = 17.5 Hz, 1H, H-2], 4.26 [dd, *J* = 12.7, 4.2 Hz, 1H, H-6], 3.32 [q, *J* = 5.8 Hz, 1H, H-4], 2.90 [q, *J* = 7.4 Hz, 1H, H-11], 2.76 [m, 1H, H-7], 1.92 [m, 1H, H-8a], 1.87 [t, *J* = 4.2 Hz, 2H, H-9], 1.76 [dd, *J* = 12.5, 5.8 Hz, 1H, H-5a], 1.64 [dd, *J* = 13.1, 4.1 Hz, 1H, H-5b], 1.49 [s, 3H, H-14], 1.18 [d, *J* = 7.3 Hz, 3H, H-13], 1.17 [m, 1H, H-8b], 1.12 [d, *J* = 6.5 Hz, 1H, H-15]. $^{13}$C-NMR ($CDCl_3$) δ 203.4 (C-3), 178.9 (C-12), 155.5 (C-1), 128.5 (C-2), 79.3 (C-6), 74.1 (C-10), 43.5 (C-9), 39.7 (C-4), 39.1 (C-7), 37.8 (C-5), 36.8 (C-11), 27.9 (C-14), 22.6 (C-8), 16.0 (C-15), 9.3 (C-13). HRMS *m/z* 289.1402 $[M+Na]^+$ calcld for $C_{15}H_{22}O_4Na$ *m/z* 289.1416.

5.3.3 Synthesis of (3R,3aS,8R,10R,11aR,E)-8-bromo-3,6,10-trimethyl-3,3a,4,5,11,11a-hexahydrocyclodeca[b]furan-2,9(8H,10H)-dione (**4**)

To a stirred reaction of **1** (20 mg, 0.08 mmol) in 2 ml of 1,4-dioxane were added 45 trimethylphenylammonium perbromide, TMPAP, (0,15 mmol). After 3 h of stirring to room temperature, the solvent was removed under reduced pressure and the residue was purified by silica-gel column chromatography (cyclohexane/ethyl acetate – 1/1) given 14.9 mg (56.6%) of **4** as brown oil. IR νmax(film)/cm$^{-1}$ 2974, 2919, 2849, 1762, 1706, 1450, 1383, 1175, 1094 and 978; $^{1}$H-NMR ($CDCl_3$) δ 5.54 [d, *J* = 8.1 Hz, 1H, H-1], 4.75 [d, *J* = 8.1 Hz, 1H, H-2], 3.45 [d, *J* = 11.8 Hz, 1H, H-6], 3.06 [m, 1H, H-4], 2.83 [q, *J* = 7.4 Hz, 1H, H-11], 2.33 [d, *J* = 11.5 Hz, 1H, H-9a], 2.22 [m. 1H, H-7], 2.21 [m, 1H, H-9b], 1.99 [m, 1H, H-5a], 1.77 [d, *J* = 12.4 Hz, 1H, H-8a], 1.75 [s, 3H, H-14], 1.63 [m, 1H, H-5b], 1.34 [m, 1H, H-8b], 1.34 [d, *J* = 6.8 Hz, 3H, H-15], 1.17 [d, *J* = 6.9 Hz, 3H, H-13]; $^{13}$C-NMR ($CDCl_3$) δ 202.8 (C-3), 178.5 (C-12), 143.5 (C-10), 122.2 (C-1), 81.9 (C-6), 48.8 (C-2), 41.7 (C-7), 39.9 (C-9), 38.3 (C-5), 37.4 (C-4), 35.6 (C-14), 24.2 (C-11), 21.9 (C-8), 16.6 (C-15), 10.4 (C-13). HRMS *m/z* 351.0570 $[M+Na]^+$, calcld for $C_{15}H_{21}O_3BrNa$ *m/z* 351.0572.

5.3.4 Synthesis of (3R,3aS,10R,11aR,Z)-6-(bromomethyl)-3,10-dimethyl-3,3a,4,5,11,11a-hexahydrocyclodeca[b]furan-2,9(8H,10H)-dione (**5**)

To a stirred solution of **1** (20 mg, 0,08 mmol) in 5 mL of $H_2O$/THF (1:5) were added 16 mg of NBS (0,088 mmol).After 2 h of stirring at 0 °C, the solutions was neutralized with a solution of $Na_2S0_3$ and extracted with DCM (3x20 mL). The organic layers were put together, the solvent removed under pressure and the residue was purified by silica-gel column chromatography (cyclohexane/ethyl acetate – 1/1) given 14.6 mg (55.0 %) of **5** as brown oil. IR νmax(film)/$cm^{-1}$ 2928, 2875, 1730, 1699, 1455, 1378, 1183, 1042 and 983; $^1$H-NMR ($CDCl_3$) δ 6.01 [t, *J* = 8.2 Hz, 1H, H-1], 4.19 [dd, *J* = 12.2, 3.5 Hz, 1H, H-6], 4.06 [d, *J* = 10.3 Hz, 1H, H-14a], 3.95 [d, *J* = 10.3 Hz, 1H, H-14b], 3.22 [dd, *J* = 17.4, 7,6 Hz, 1H, H-2a], 3,13 [d, *J* = 17.4, 8.6 Hz, 1H, H-2b], 3.01-2.93 [m, 1H, H-4], 2.81 [q, *J* = 7.6 Hz, 1H, H-11], 2.42 [t, *J* = 4.2 Hz, 1H, H-9a], 2.36 [dd, *J* = 12.8, 4.1 Hz, 1H, H-9b], 2.22-2.17 [m, 1H, H-7], 2.08 [td, *J* = 12.5, 3.8 Hz, 1H, H-5a], 1.84-1.82 [m 1H, H-8a], 1.58 [td, *J* = 12.4, 3.5 Hz, 1H, H-5b], 1.34 [t, *J* = 3.7 Hz, 1H, H-8b], 1.17 [d, *J* = 7.4 Hz, 3H, H-13], 1.10 [d, *J* = 6.8 Hz, 3H, H-15]; $^{13}$C-NMR ($CDCl_3$) δ 213.4 (C-3), 178.8 (C-12), 140.0 (C-10), 125.7 (C-1), 78.8 (C-6), 42.6 (C-2), 39.7 (C-4), 38.1 (C-7), 38.0 (C-5), 36.0 (C-14), 35.7 (C-11), 24.7 (C-9), 24.0 (C-8), 18.3 (C-15), 10.1 (C-13). HRMS *m*/*z* 351.0570 $[M+Na]^+$, calcld for $C_{15}H_{21}O_3BrNa$ *m*/*z* 351.0572.

5.3.5 Synthesis of (3R,3aS,9R,10R,11aR,E)-9-hydroxy-3,6,10-trimethyl-3,3a,4,5,9,10,11,11a-octahydrocyclodeca[b]furan-2(8H)-one (**6**)

To a stirred solution of **1** (20 mg, 0.08 mmol, 1 eq) in 3 mL of MeOH/$CHCl_3$ (2:1) at 0°C were added 60 mg of $CeCl_3 \cdot 6H_2O$ (0.16 mmol, 2 eq) and 3.7 mg of $NaBH_4$. After 6 h at room temperature, the reaction was neutralized with AcOH 1%. The solvent was removed under pressure and the residue was purified by silica-gel column chromatography (cyclohexane/ethyl acetate – 3/2) given 6.3 mg (31.3 % of yield) of **6** as white powder. IR νmax(film)/$cm^{-1}$ 3417, 2910, 2851, 1761, 1455, 1379, 1164, 1090, 1027 and 972; $^1$H-NMR ($CDCl_3$) δ 5.42 [br, 1H, H-1], 4.05 [br, 1H, H-6], 3.76 [br, 1H, H-3], 2.79 [q, *J* = 7.8 Hz, 1H, H-11], 2.27 [d, *J* = 12.5 Hz, 1H, H-2a], 2.19 [t, *J* = 11.4 Hz, 1H, H-7], 2.12-1.94 [m, 3H, H-2b, H-5a, H-5b], 1.86 [br, 1H, H-4], 1.72 [s, 3H, H-14], 1.68-1.65 [m, 1H, H-8a], 1.42 [t, *J* = 5.8 Hz, 1H, H-8b], 1.28-1.21 [m, 2H, H-9a, H-9b], 1.16 [d, *J* = 7.6 Hz, 1H, H-16], 1.05 [d, *J* = 7.0 Hz, 3H, H-15]. HRMS *m*/*z* 275.1634 $[M+Na]^+$, calcld for $C_{15}H_{24}O_3Na^+$ *m*/*z* 275.1623.

5.3.6 Synthesis of (3R,3aS,6E,9E,10R,11aR)-9-(methoxyimino)-3,6,10-trimethyl-3,3a,4,5,9,10,11,11a-octahydrocyclodeca[b]furan-2(8H)-one (**7a**), (3R,3aS,6E,9E,10R,11aR)-9-(ethoxyimino)-3,6,10-trimethyl-3,3a,4,5,9,10,11,11a-octahydrocyclodeca[b]furan-2(8H)-one (**7b**) and (3R,3aS,6E,9E,10R,11aR)-9-((benzyloxy)imino)-3,6,10-trimethyl-3,3a,4,5,9,10,11,11a-octahydrocyclodeca[b]furan-2(8H)-one (**7c**)

To a stirred solution of **1** (20 mg, 0.08 mmol) in 3 mL of pyridine, were added the respective hydroxylamine (0.16 mmol). The reaction mixture was stirred at room temperature overnight.

The solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography (cyclohexane/ethyl acetate – 3/7) to give the respective oxime. For **7a**, were obtained 16.3 mg, 73 %. IR νmax(film)/$cm^{-1}$ 2961, 2943, 2854, 1758, 1632, 1664, 1444, 1376, 1355, 1168, 1043, 965 and 871; $^{1}$H NMR ($CDCl_3$) δ 5.41 [t, *J* = 9.1 Hz, 1H, H-1], 3.91 [dd, *J* = 14.0, 9.1 Hz, 1H, H-2a], 3.82 [s, 3H, H-1'], 3.80-3.76 [m, 1H, H-6], 2.82 [q, *J* = 8.1 Hz, 1H, H-11], 2.42-2.33 [m, 1H, H-4], 2.32-2.26 [m, 1H, H-2b], 2.24 [ dd, *J* = 14.3, 7.5 Hz, 1H, H-9a], 2.14 [dd, *J* = 10.4, 8.3 Hz, 1H, H-7], 2.06-1.95 [m, 2H, H-5a, H-9b], 1.68-1.66 [m, 1H, H-5b], 1.63 [dt, *J* = 10.3, 3.0 Hz, 1H, H-8b], 1.35 [dt, *J* = 14.3, 3.0 Hz, 1H, H-8a], 1.59 [s, 3H, H-14], 1.17 [d, *J* = 8.0 Hz, 3H, H-13], 1.15 [d, *J* = 8.0 Hz, 3H, H-15]; $^{13}$C NMR ($CDCl_3$) δ 179.3 (C-12), 159.3 (C-3), 139.6 (C-10), 120.2 (C-1), 82.6 (C-6), 61.6 (C-1'), 41.9 (C-7), 39,8 (C-9), 39.4 (C-5), 35.9 (C-11), 34.8 (C-4), 25.3 (C-2), 23.8 (C-8), 21.9 (C-15), 16.0 (C-14), 10.6 (C-13). HRMS *m*/*z* 302.1739 $[M+Na]^+$, calcld for $C_{16}H_{25}NO_3Na$ m/z 302.1732. For **7b**, were obtained 16.4 mg, 70 %. IR νmax(film)/$cm^{-1}$ 2977, 2931, 2857, 1757, 1633, 1664, 1444, 1377, 1169, 1047 and 966; $^{1}$H-NMR ($CDCl_3$) δ 5.46-5.36 [m, 1H, H-1], 4.06 [q, *J* = 7.4, 2H, H-1'], 3.94 [dd, *J* = 14.1, 9,1 Hz, 1H, H-2a], 3.76 [d, *J* = 11.0 Hz, 1H, H-6], 2.86 [p, *J* = 8.1 Hz, 1H, H-11], 2.41-2.20 [m, 3H, H-2b, H-4, H-9b], 2.17-1.97 [m, 3H, H-5a, H-7, H-9b], 1.68-1.62 [m, 1H, H-8a], 1.57-1.50 [m, 1H, H-5b], 1.41-1.31 [m, 1H, H-8a], 1.59 [s, 3H, H-14], 1.26 [t, *J* = 7.0 Hz, 3H, H-2'], 1.17 [d, *J* = 7.3 Hz, 3H, H-13], 1.15 [d, *J* = 7.3 Hz, 3H, H-15]; $^{13}$C-NMR ($CDCl_3$) δ 179.4 (C-12), 158.8 (C-3), 139.6 (C-10), 120.3 (C-1), 82.7 (C-6), 69.2 (C-1'), 42.5 (C-7), 39.8 (C-9), 35.9 (C-11), 32.8 (C-4), 25.4 (C-2), 23.8 (C-5), 23.0 (C-8), 21.9 (C-15), 16.0 (C-2'), 14.8 (C-14), 10.5 (C-13). HRMS *m*/*z* 31 6.1880 $[M+Na]^+$, calcd for $C_{17}H_{27}NO_3Na$ *m*/*z* 316.1889. For **7c**, were obtained 15.6 mg, 55 %. IR νmax(film)/$cm^{-1}$ 3065, 3036, 2972, 2926, 2873, 1758, 1656, 1633, 1450, 1378, 1360, 1174, 1033 and 967; $^{1}$H-NMR ($CDCl_3$) δ 7.39-7.32 [m, 5H, H-ar], 5.36 [m, 1H, H-1], 5.05 [s, 2H, H-1']3.92 [dd, *J* = 14.1, 9.0 Hz, 1H, H-2a], 3.67 [d, *J* = 11 Hz, 1H, H-6], 2.83 [q, *J* = 8.0 Hz, 1H, H-11], 2.36 [m, 1H, H-4], 2.24-2.23 [m, 2H, H-2b, H-9a], 2.12 [m, 1H, H-7], 2.07-2.03 [m, 2H, H-5a, H-9b], 1.65-1.63 [m, 2H, H-5b, H-8b], 1.26 [m, 1H, H-8a], 1.43 [s, 3H, H-14], 1.17 [d, *J* = 7.1 Hz, 3H, H-13], 1.15 [d, *J* = 7.1 Hz, 3H, H-15]; $^{13}$C-NMR ($CDCl_3$) δ 179.4 (C-12), 159.9 (C-3), 139.7 (C-10), 128.8 – 127.7 (6Car), 82.6 (C-6), 76.1 (C-1'), 41.8 (C-7), 39.7 (C-5), 39.5 (C-9), 35.9 (C-11), 33.3 (C-4), 25.5 (C-2), 23.7 (C-8), 21.9 (C-15), 15.8 (C-14), 10.6 (C-13). HRMS *m*/*z* 378.2035 $[M+Na]^+$ calcd for $C_{22}H_{29}NO_3Na$ m/z 378.2045.

5.3.7 Synthesis of (3R,3aS,6R,10R,11aR)-3,6,10-trimethyloctahydrocyclodeca[b]furan-2,9(3H,10H)-dione (**8**)

To a stirred solution of **1** (10 mg, 0,04 mmol) in 1 mL of absolute EtOH, were added 2 mg of Pd-C 10 % as catalyst. The reaction mixture was stirred under $H_2$ atmosphere during 24 h at room temperature and after, was filtered on silice and the solvent was removed under pressure. The residue was purified by silica-gel column chromatography (cyclohexane/ethyl acetate – 3/7) given

6.4 mg (53 % of yield) of **8**. IR νmax(film)/$cm^{-1}$ 2966, 2925, 1759, 1698, 1443, 1386, 1185 and 1070; $^{1}$H-NMR ($CDCl_3$) δ 4.40 [q, *J* = 4.5 Hz, 1H, H-6], 2.97-2.87 [m, 1H, H-4], 2.75 [q, *J* = 7.3 Hz, 1H, H-11], 2.53 [td, *J* = 7.8, 4.3 Hz, 2H, H-2a, H-2b], 2.40-2.34 [m, 1H, H-7], 2.34-2.25 [m, 1H, H-5a], 1.84-1.71 [m, 2H, H-1a, H-5b], 1.68-1.58 [m, 2H, H-1b, H-10], 1.45-1.34 [m, 2H, H-8a, H,9a], 1.33-1.26 [m, 2H, H-8b, H-9b], 1.16 [d, *J* = 7.0 Hz, 3H, H-15], 1.14 [d, *J* = 7.5 Hz, 3H, H-14], 0.95 [d, *J* = 6.7 Hz, 3H, H-14]; $^{13}$C-NMR ($CDCl_3$) δ 217.0 (C3), 179.4 (C12), 79.6 (C6), 44.4 (C4), 39.1 (C2), 38.2 (C7), 37.6 (C11), 36.6 (C5), 33.0 (C1), 31.6 (C8), 30.5 (C10), 25.1 (C9), 23.3 (C14), 18.6 (C15), 10.4 (C13). HRMS *m*/*z* 275.1631 $[M+Na]^+$ calcd for $C_{15}H_{24}O_3Na$ *m*/*z* 275.1623.

5.4 Arf1-Sec7 inhibition assay

5.4.1 Protein expression and purification

Expression and purification of proteins used in this study were similar as described before by Benabdi et al. [12]. Briefly, N-terminally truncated bovine Δ17Arf1 (identical to human) was expressed and purified and loaded with GDP prior to use. Human BRAG2Sec7, ARNO2Sec7 and BIG1Sec7 were produced in *E. coli* and purified as described in [12]. Protein purity was aaaessed by SDS-PAGE. All proteins were flash frozen in liquid nitrogen and stored at -80 °C prior use. Protein concentration were measured using their respective absorption coefficient at 280 nm after centrifugation to remove any precipitate.

5.4.2 Nucleotide exchange assay

Nucleotide exchange kinetics were monitored by the change in tryptophan fluorescence that follows the conformational change from Arf-GDP to Arf-GTP (excitation/emission wavelengths of 292/340 nm) [12]. Fluorescence measurements were done with a Cary Eclipse fluorimeter (Varian) at 37 °C, under continueus stirring in a total volume of 800 µL. Experiments in solution used 1 µM of N-terminally truncated Arf-GDP. Protein and inhibitors (or DMSO for controls) were incubated in HKM buffer (50 mM HEPES pH 7.4 120 nM potassium acetate, 1mM $MgCl_2$ and 1 mM DTT) for 2 min at 37 °C before initiating the reaction with 100 µM GTP. Exchange rates ($k_{obs}$) were determined from monoexponential fits over the entire kinetics and expressed as percentage of control activity. All exchange reactions were done in triplicate and means are given ± SD.

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Arf Guanine Nucleotide Exchange Factors with Small Molecules: Evidence of Unique Inhibitory Profiles, Biochemistry. 56 (2017) 5125–5133. doi:10.1021/acs.biochem.7b00706.

Spectrum of ketopelenolide B Derivatives

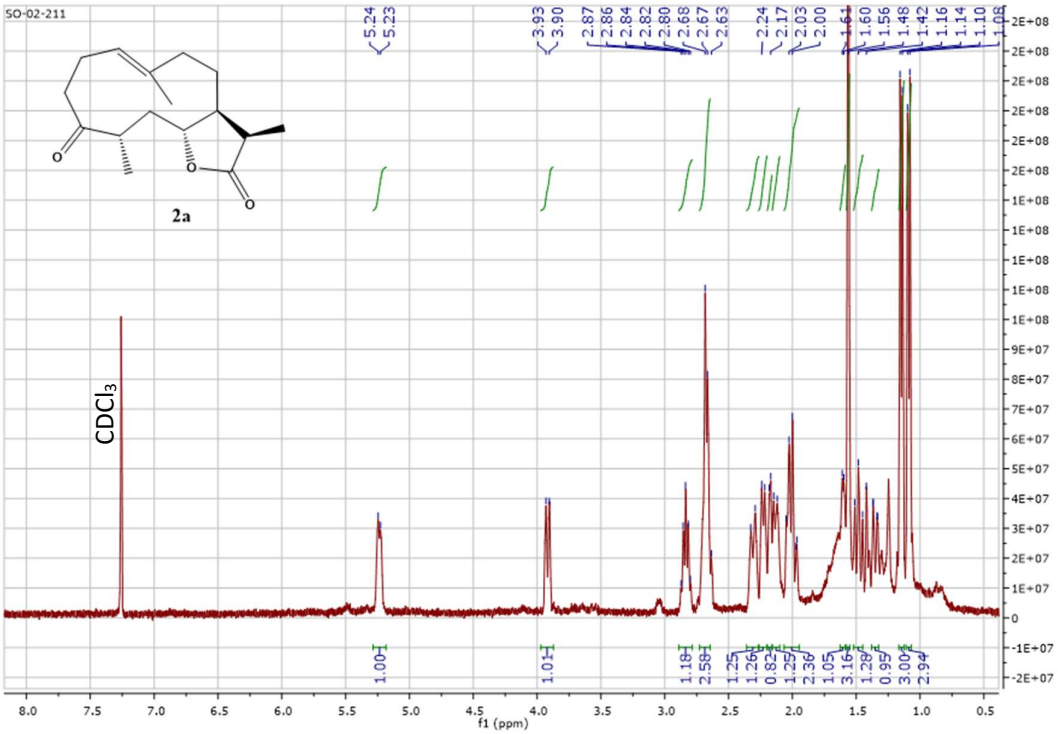


Fig 1: $^{1}H$ spectrum from **2a** ($CDCl_3$, 400 MHz).

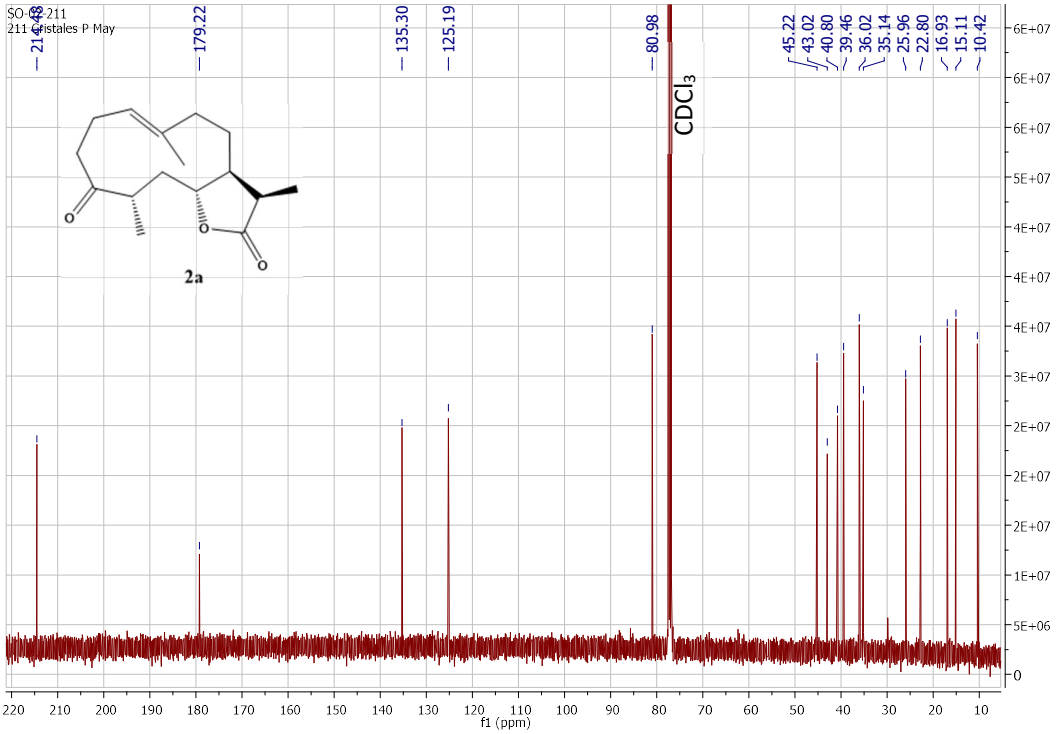


Fig 2: $^{13}C$ spectrum from **2a** ($CDCl_3$, 400 MHz).

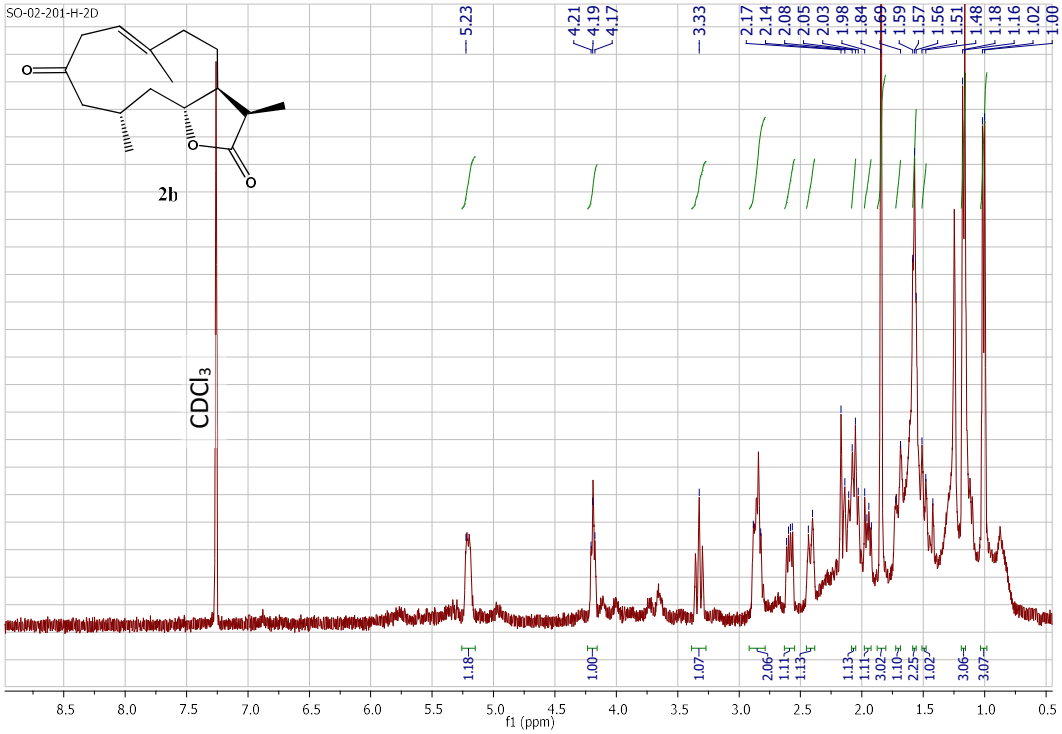


Fig 3: $^{1}H$ spectrum from **2b** ($CDCl_3$, 400 MHz).

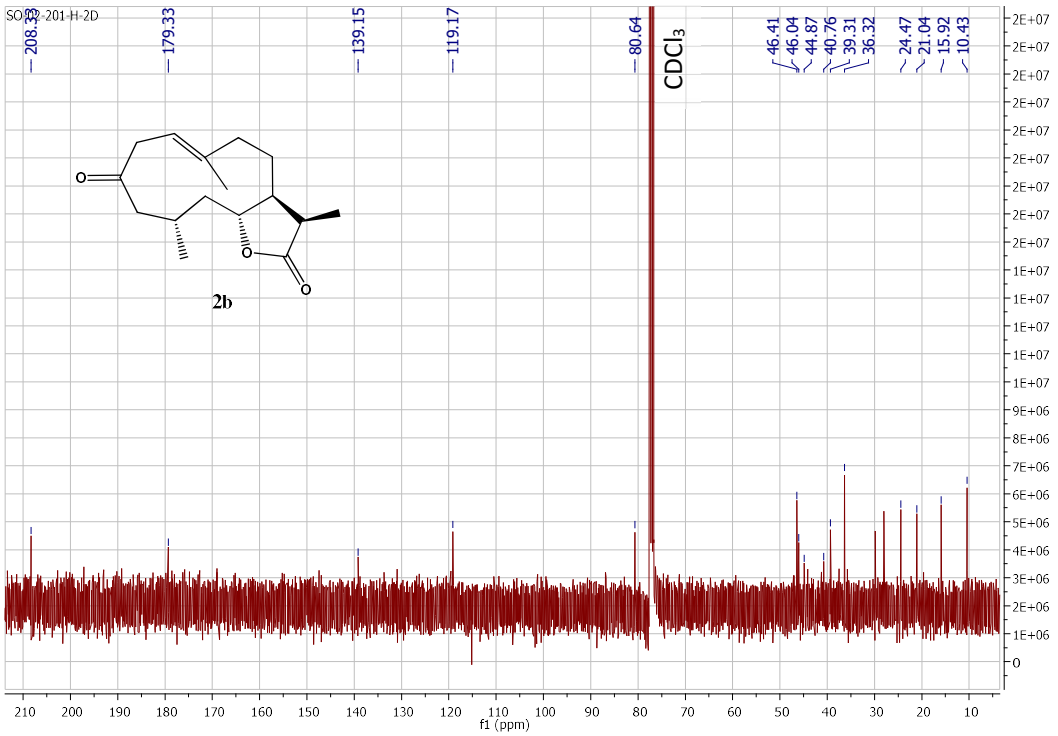


Fig 4: $^{13}C$ spectrum of **2b** ($CDCl_3$, 400 MHz).

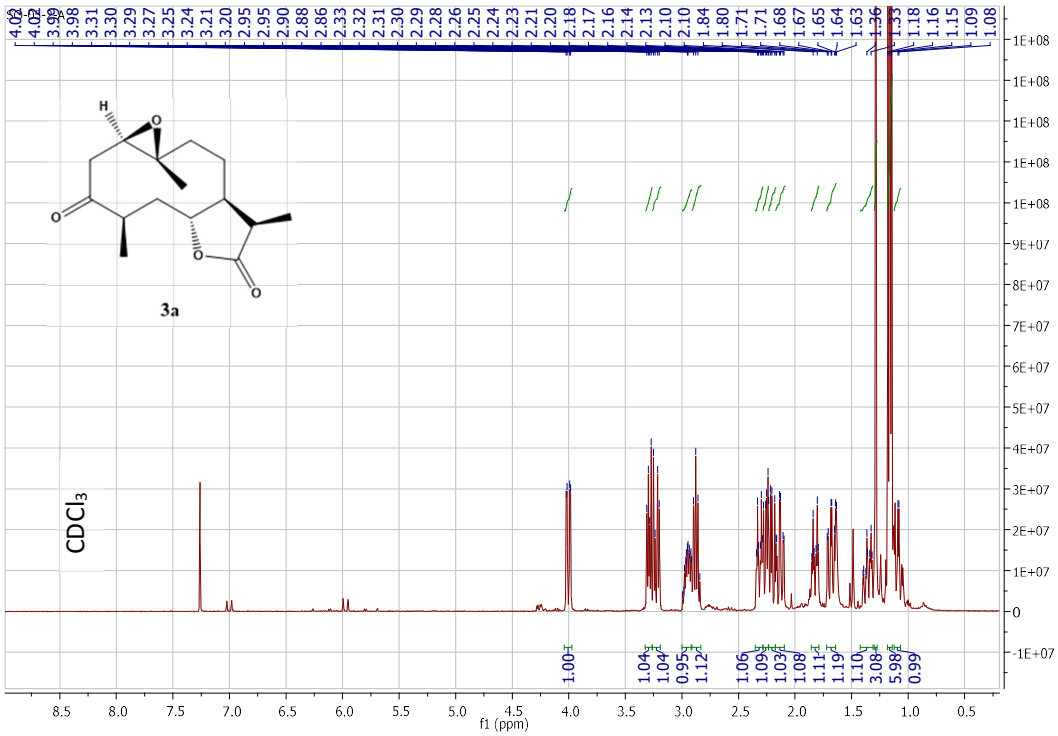


Fig 5: $^{1}H$ spectrum of **3a** ($CDCl_3$, 400 MHz).

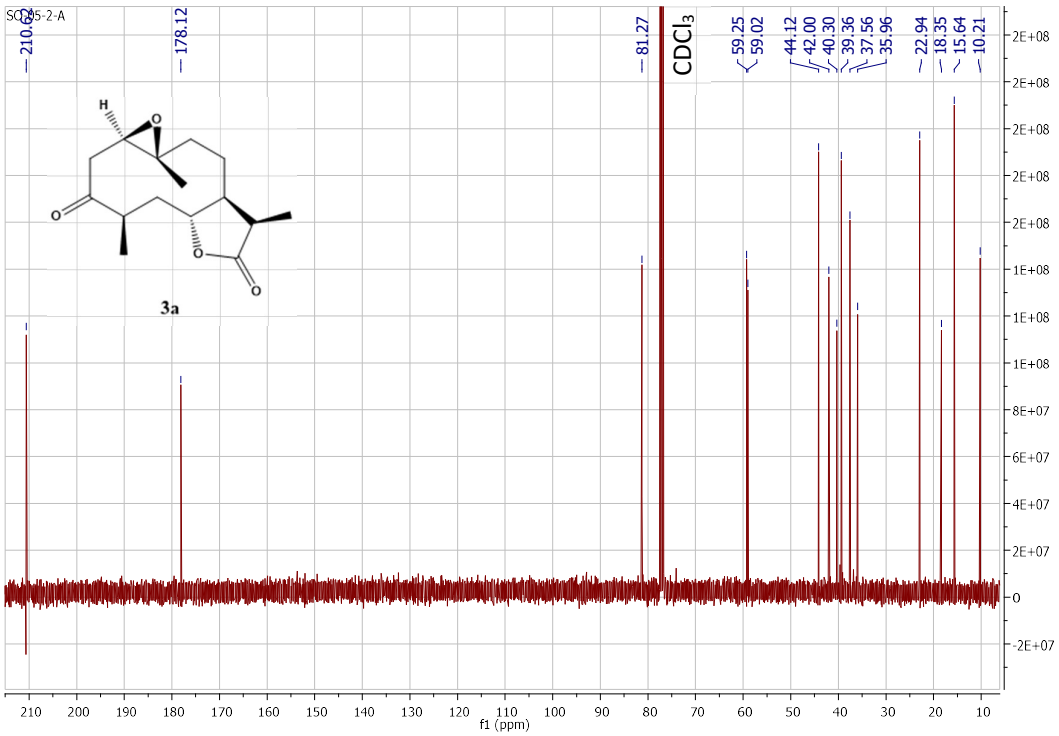


**Fig 6**: $^{13}C$ spectrum of **3a** ($CDCl_3$, 400 MHz).

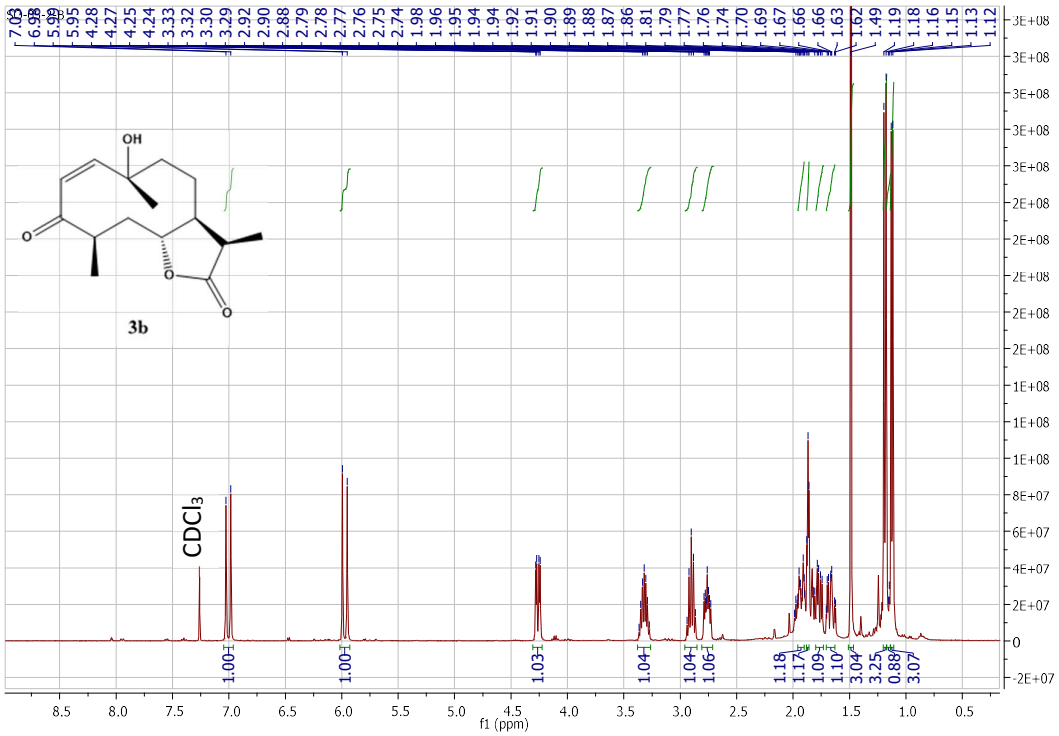


**Fig 7**: $^{1}H$ spectrum of **3b** ($CDCl_3$, 400 MHz).

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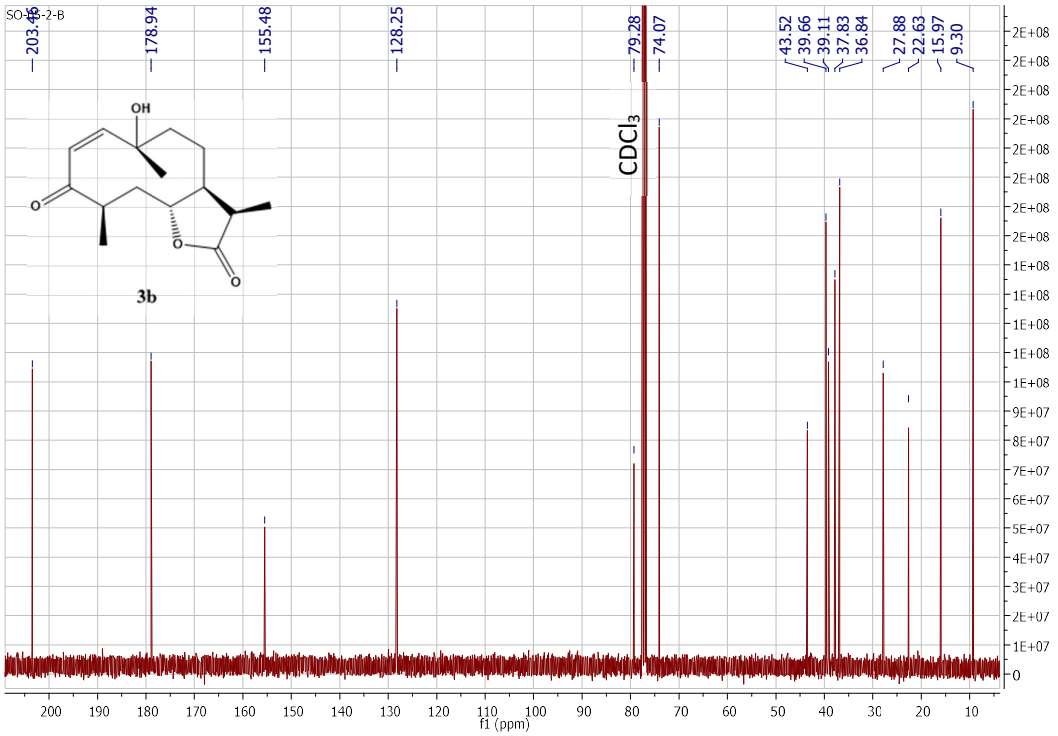


**Fig 8**: $^{13}C$ spectrum of **3b** ($CDCl_3$, 400 MHz).

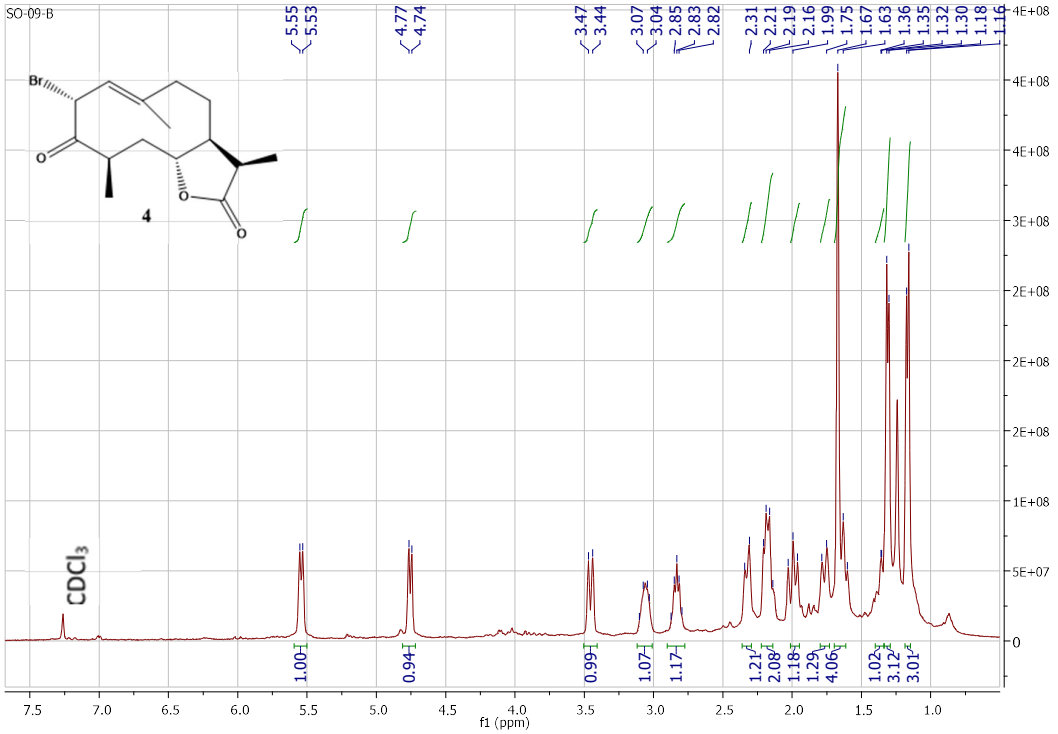


**Fig 9**: $^{1}H$ spectrum of **4** ($CDCl_3$, 400 MHz).

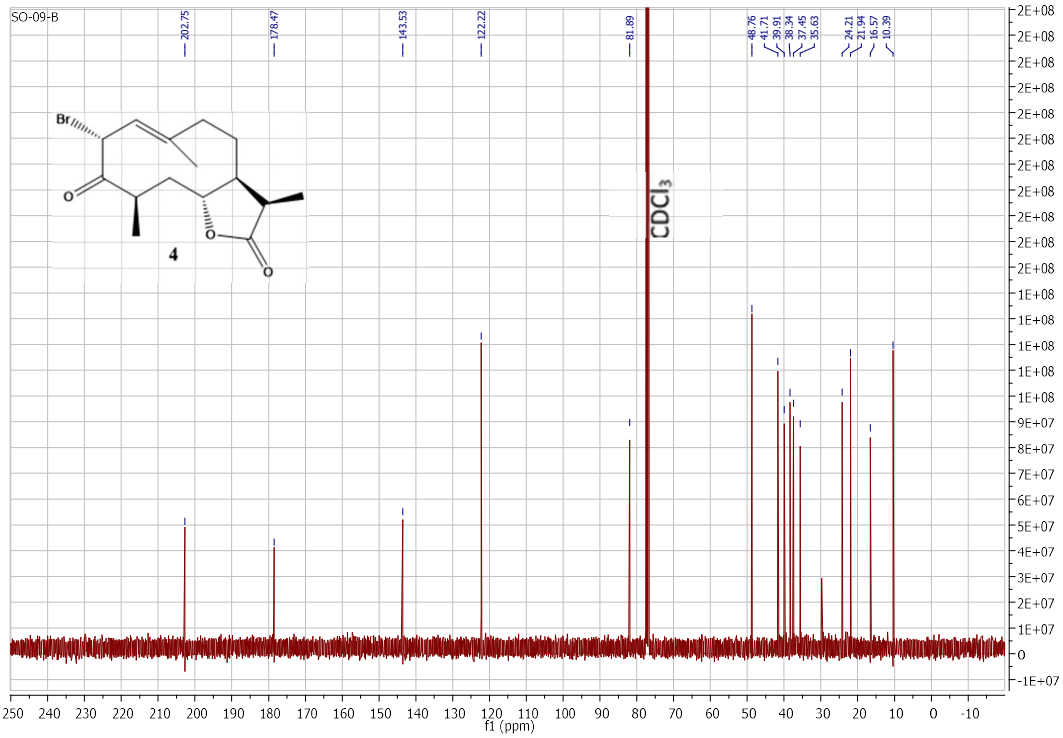


**Fig 10**: $^{13}C$ spectrum of **4** ($CDCl_3$, 400 MHz).

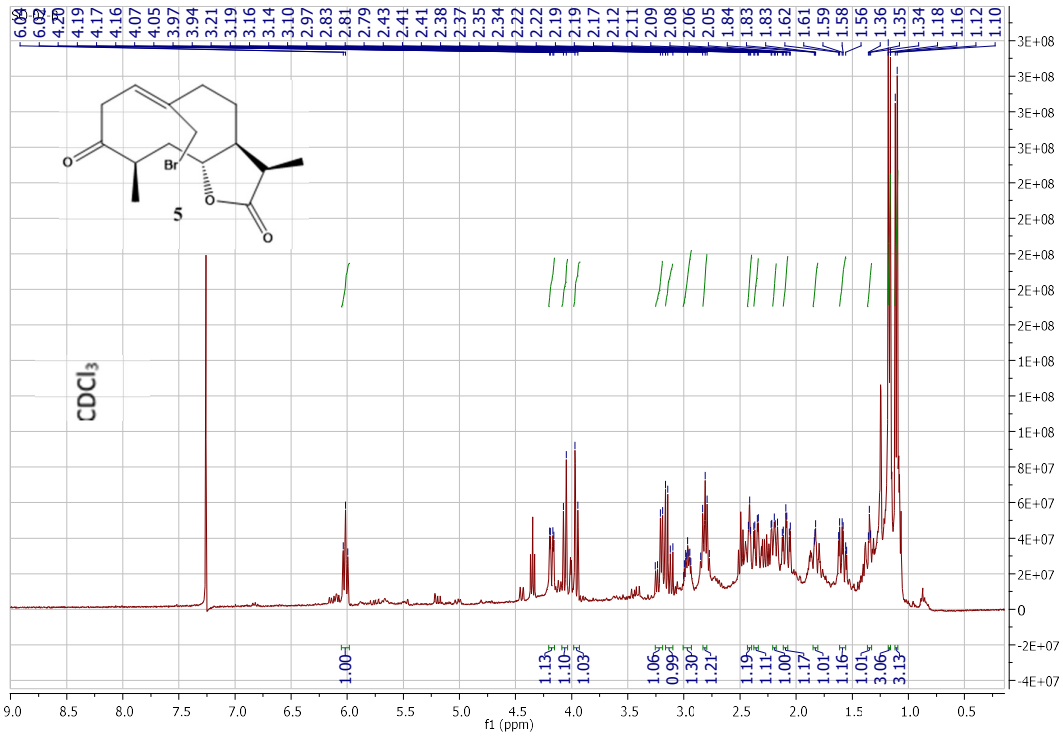


**Fig 11**: $^{1}H$ spectrum of **5** ($CDCl_3$, 400 MHz).

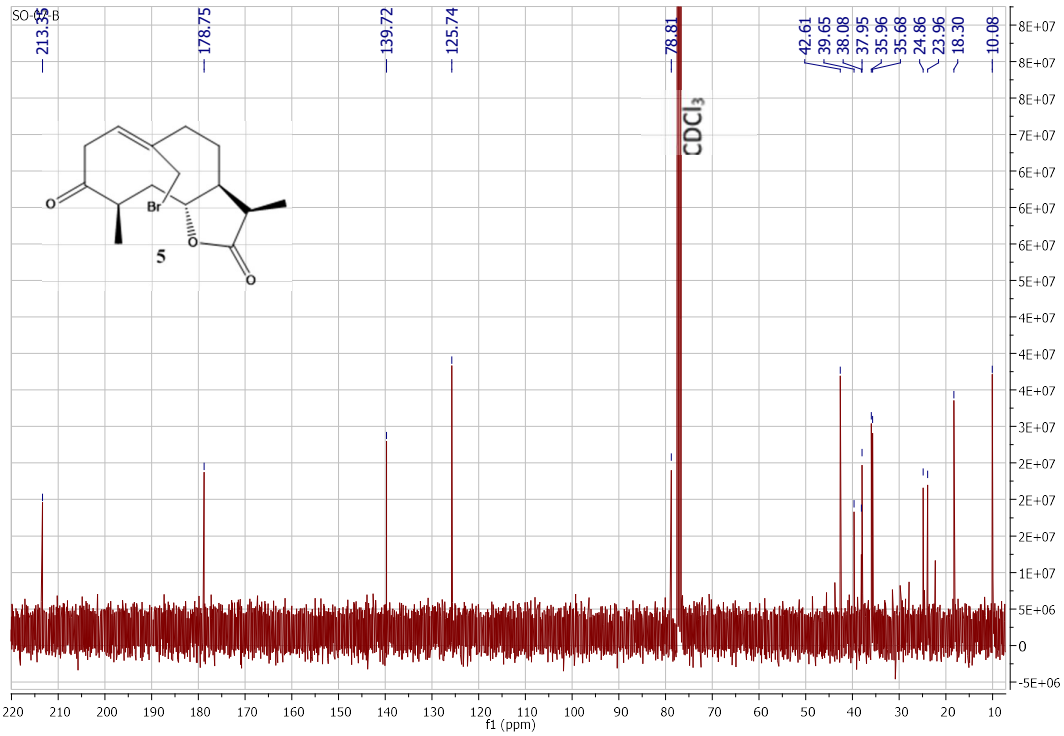


**Fig 12**: $^{13}C$ spectrum of **5** ($CDCl_3$, 400 MHz).

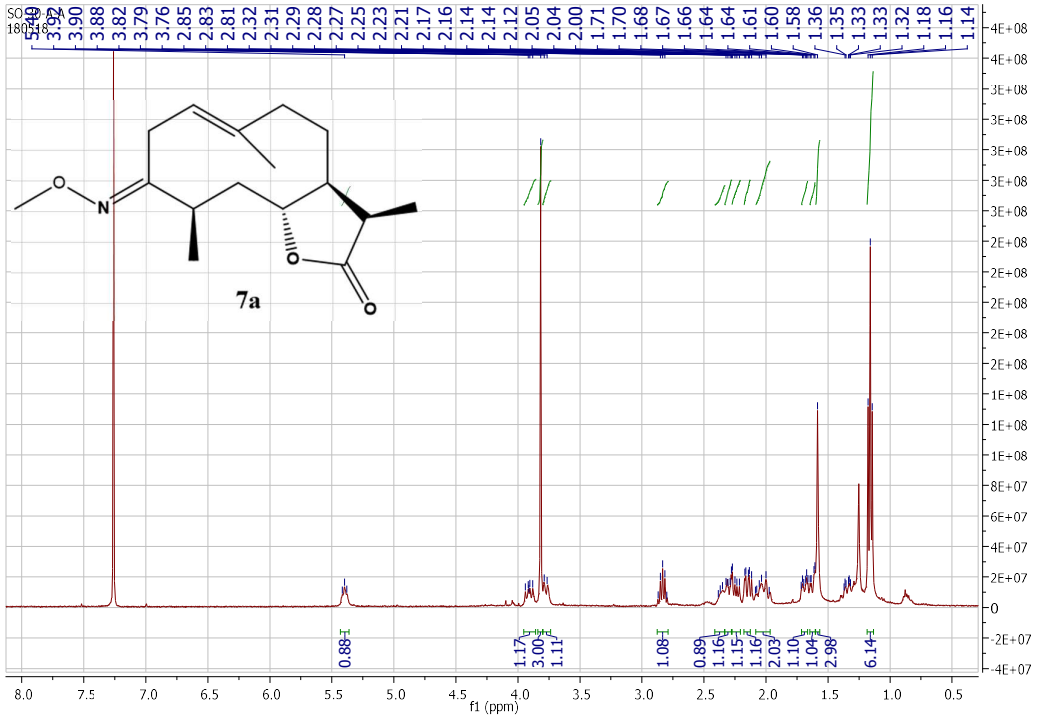


**Fig 13**: $^{1}H$ spectrum of **7a** ($CDCl_3$, 400 MHz).

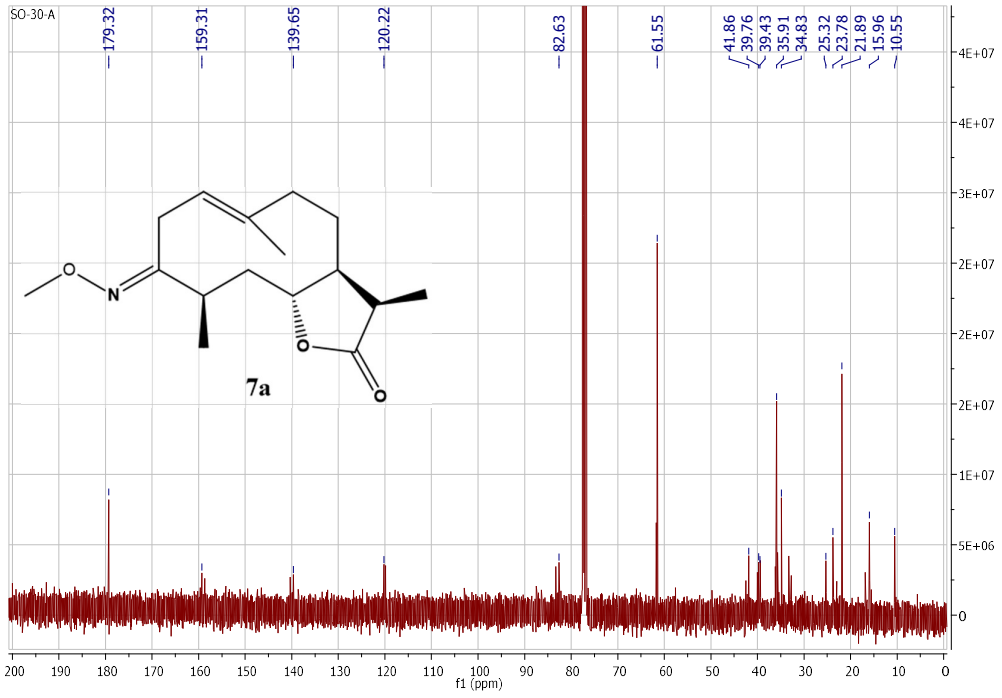


**Fig 14**: $^{13}$C-NMR spectrum of **7a** ($CDCl_3$, 400 MHz).

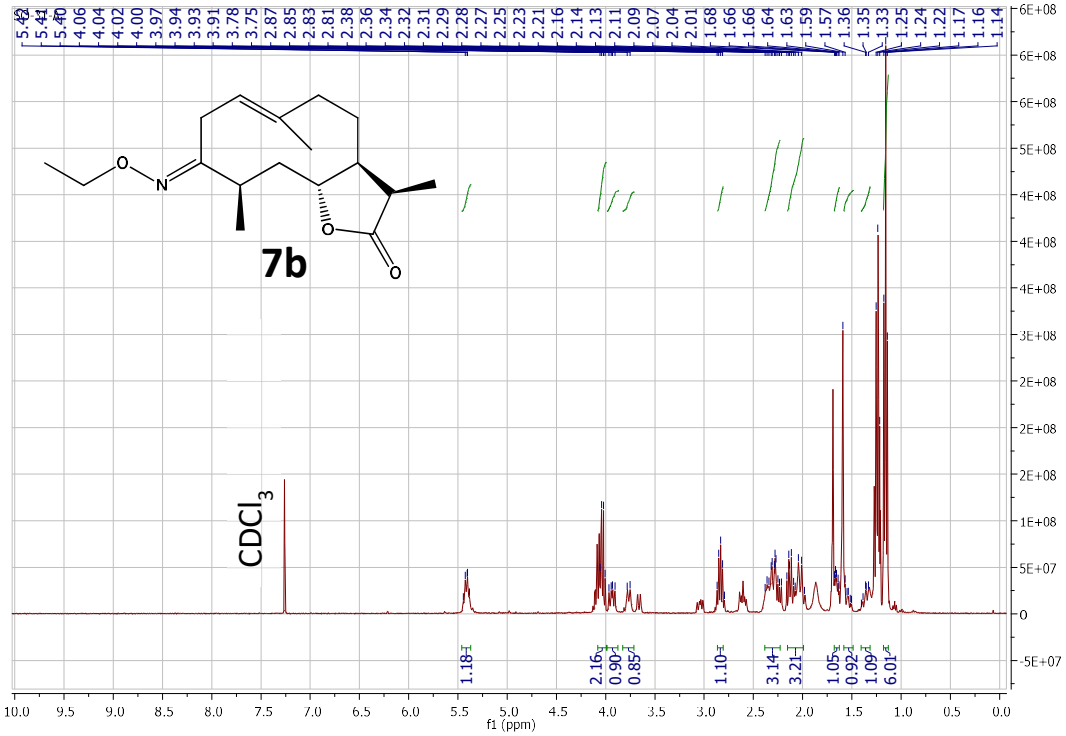


**Fig 15**: $^{1}$H-NMR spectrum of **7b** ($CDCl_3$, 400 MHz).

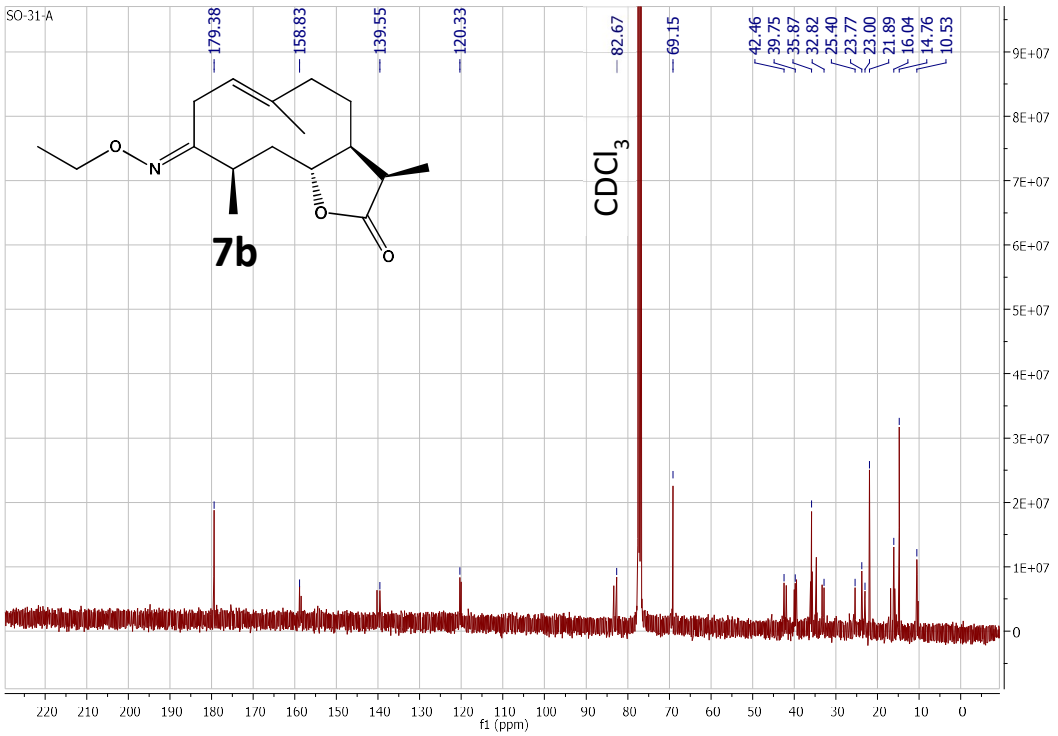


**Fig 16**: $^{13}$C-NMR spectrum of **7b** ($CDCl_3$, 400 MHz).

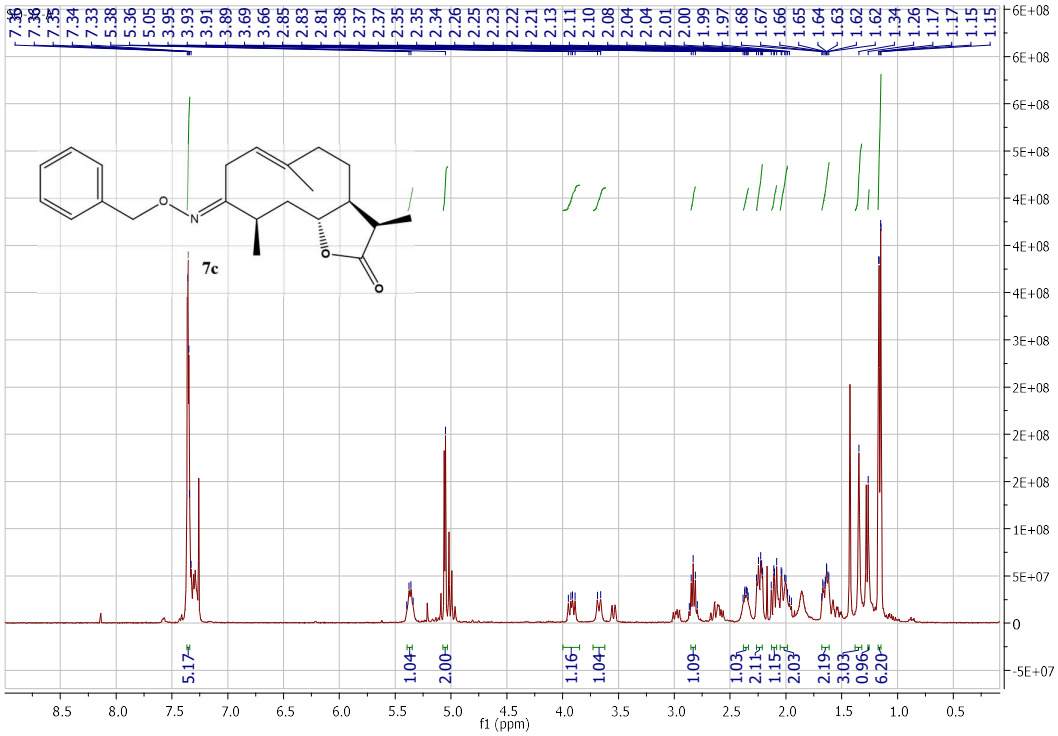


**Fig 17**: $^{1}$H-NMR spectrum of **7c** ($CDCl_3$, 400 MHz).

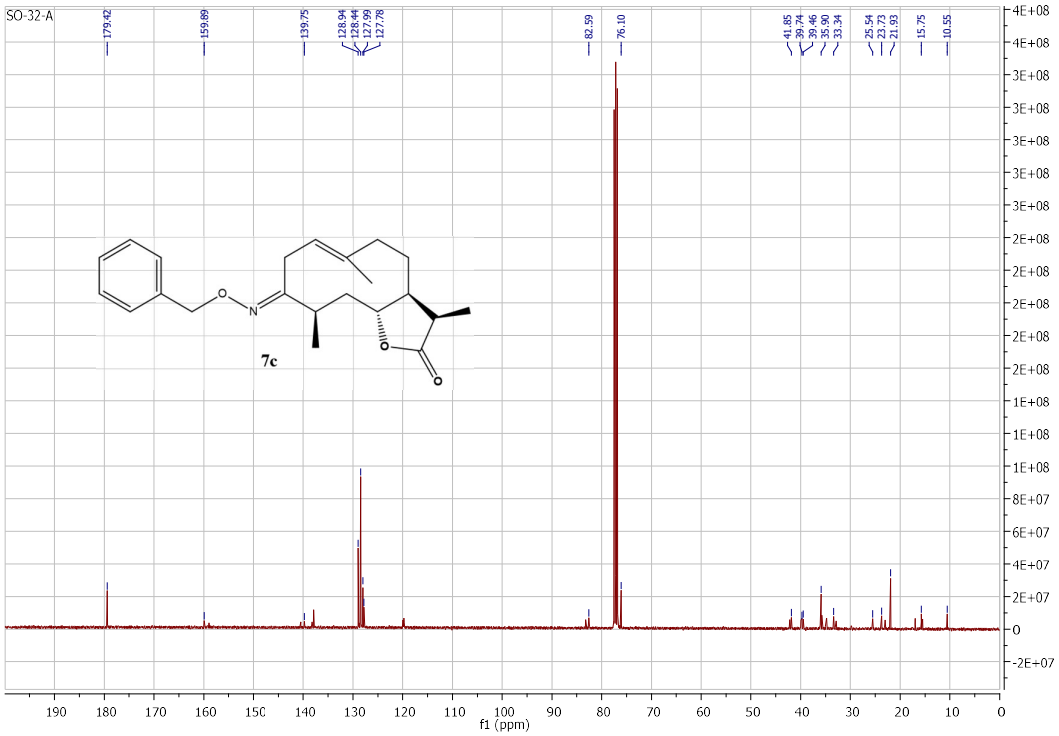


**Fig 18**: $^{13}$C-NMR spectrum of **7c** ($CDCl_3$, 400 MHz).

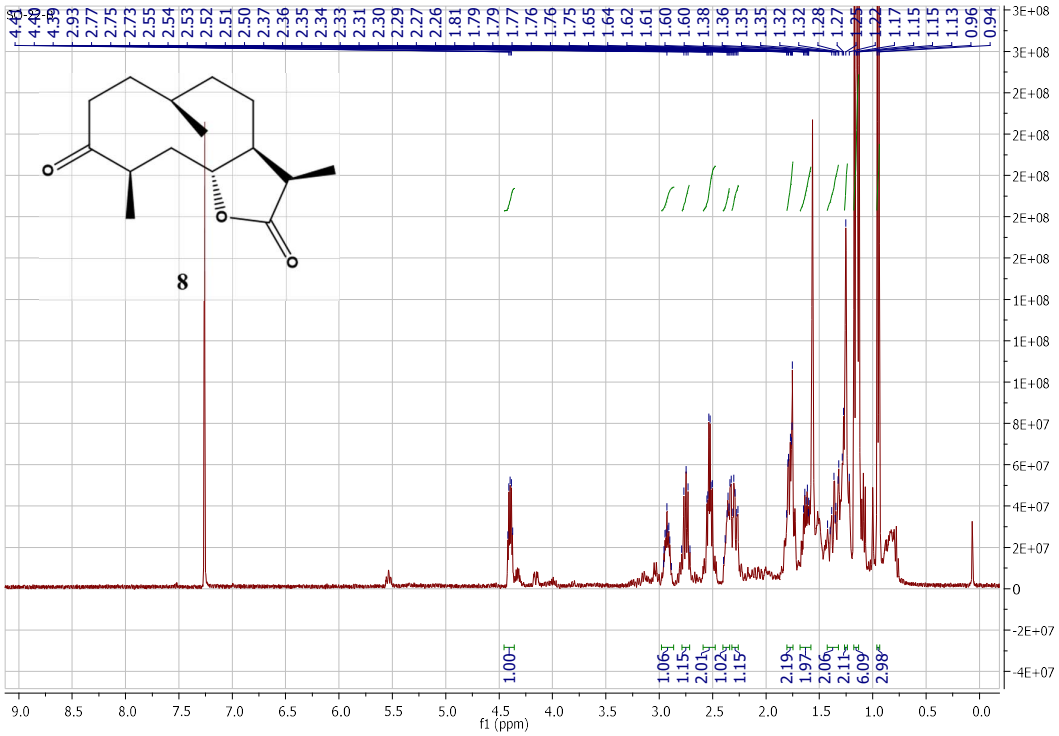


**Fig 19**: $^{1}$H-NMR spectrum of **8** ($CDCl_3$, 400 MHz).

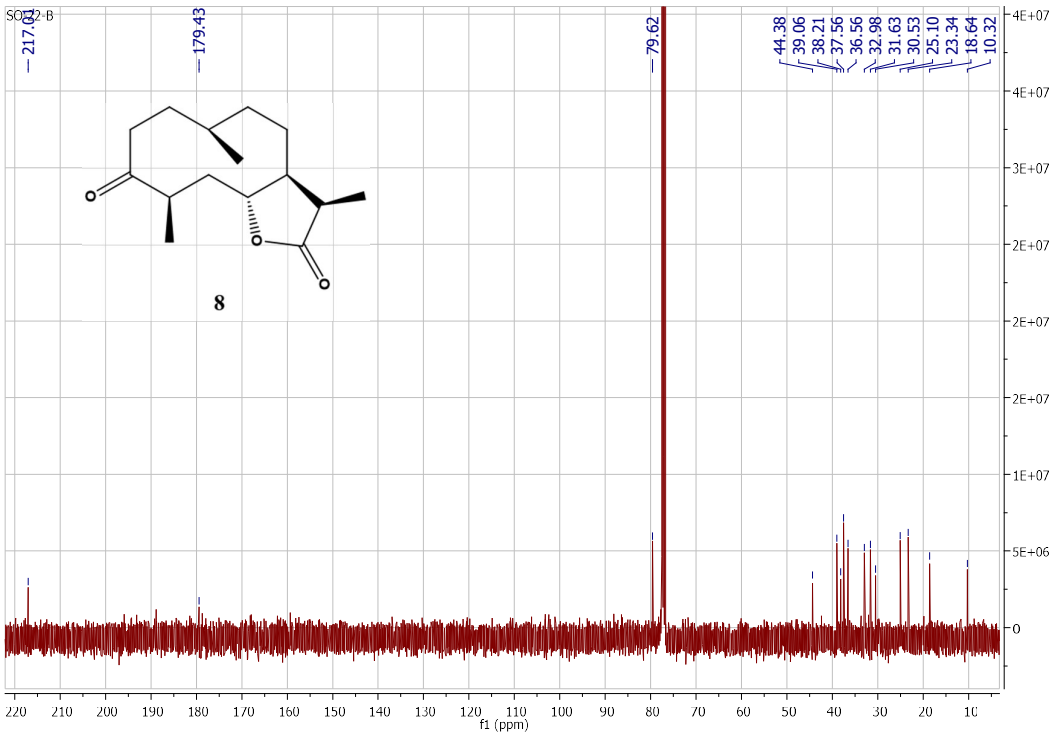


**Fig 20**: $^{13}$C-NMR spectrum of **8** ($CDCl_3$, 400 MHz).

## Conclusion, perspectives and contribution

*In silico* approach was applied aiming to find potential pharmacological interactions. Arf1-Sec7 protein complex was identified as target of ketopelenolide B, showing a weak *in vitro* inhibitory effect. After structure modification of ketopelenolide B, the biological activity has strongly increased.

Two perspectives may be proposed from this work:

- Cytotoxic activity:
    - *In silico* evaluation, in order to evaluate the putative hydrophobic interactions that could be involved in the inhibitory activity of Arf1-Sec7 by oxime derivatives.
    - Evaluation of physical properties of the semisynthetic derivatives.
    - *In vitro* cytotoxic evaluation on invasive cancer cell lines known to overproduce Arf1 protein.
- Antiparasitic activity:
    - Identification and production of trypanosomal ArfGEFs.
    - *In vitro* cell-free nucleotide exchange assay with Arf1 and trypanosomal ArfGEFs by ketopelenolide B derivatives.
    - *In vitro* evaluation of antitrypanosomal activity by ketopelenolide B derivatives.

## Personal contribution

My contribution to **Chapter IV** and **V** includes both the chemical study of extracts i.e. extraction, fractionation, purification and structure elucidation of isolated compounds, the structural modifications by semisynthesis of natural and commercial derivatives and the biological evaluations of samples, as described below:.

- Extraction of aerial parts of *P. monodiana*.

- Isolation of major metabolites from ethyl acetate extract of *P. monodiana* by silica-gel chromatography, preparative HPLC and centrifugal partition chromatography.
- Semisynthesis by mono and polymethoxylation from isolated and commercially available flavones: generation of a library of 33 derivatives.
- Semisynthesis of 10 derivatives from ketopelenolide B.

- *In silico* prediction of physical proprieties of the flavones.

- Implementation and accomplishment of *in vitro* hemozoin measurement on erythrocytes infected by 3D7 *P. falciparum* in co-incubation with tested compounds
- Structural elucidation of original derivatives by spectroscopic (1D and 2D-NMR) and spectrometric (HRMS) techniques.
- *In vitro* cell-free nucleotide exchange factor activity of derivatives against Arf1-Sec7 complex.

- Redaction of drafts and modification after reviewers comments.

# CONCLUSION

During these investigations, ethnopharmacological and chemotaxonomic approaches were followed with the main purpose to identify bioactive compounds with pharmacological, nutraceutical and/or cosmetical interest.

Bioguided approach in the study of several traditional medicines has led to describe the activity of several kinds of metabolites such as the benzofuran (+)-conocarpan (active against the growth of aerobic and facultative anaerobic Gram-positive bacteria), the endoperoxide-containing diterpene mulinic acid (that inhibits the growth of *Plasmodium falciparum* strains at the same level than chloroquine) and ginkgolic acid (C17:1) (anti-virulence efficacy on *Pseudomonas aeruginosa* infections).

Modern techniques, such as dereplication, metabolomic analysis and molecular networking, were carried out to analyze the anti-inflammatory activity of European medicinal plants and of the fruits of *Pistacia lentiscus*:

- In the case of European medicina plants, dereplication by LC-MS and metabolomic analysis allowed to identify glycoside iridoids (harpagoside and harpagide) as potent antagonists of P2X7 receptor, blocking the signaling pathway of the inflammatory response.
- By using molecular networking main compounds and biomarkers (gallic acid esters) of *Pistacia lentiscus* fruit extracts obtained from ripe and unripe fruits were characterized.

Through chemotaxonomic approach, polymethoxyflavonoids from *Pentzia monodiana* have demonstrated moderate antiplasmodial activty with potential to form heme-adducts in a LC-MS assay. After pharmacomodulation, semisynthetic derivatives were more active than the natural compounds, stromgly reducing *in vitro* production of hemozoin on a *P. falciparum* infected erythrocytes model.

The sesquiterpene lactone ketopelenolide B, isolated from the same plant species presents structural similarities with brefeldin A, a natural macrolide inhibitor of the Arf1-Big1Sec7 complex system. Eleven derivatives were obtained by semisynthesis, allowing to upgrade the initial antagonist effect against Arf1-Big1Sec7 proteins complex. The most active compound showed similar inhibition profile against other ArfGEFs than brefeldin A.

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