Eunice Sofia da Silva Fernandes

IDENTIFICATION OF ACTIVE COMPOUNDS IN EXTRACTS OF *ERYNGIUM* SPECIES FROM THE IBERIA

Supervisor

Professor Carlos Manuel Freire Cavaleiro, PhD

Faculty of Pharmacy of University of Coimbra

In the present form, this document configures a dissertation proposed to accomplish the degree of Master of Science in "Química Farmacêutica Industrial" by the Faculty of Pharmacy of the University of Coimbra.

Faculty of Pharmacy

University of Coimbra

Coimbra, 2012/2013

This work was carried out in the Center of Pharmaceutical Studies, Faculty of Pharmacy, University of Coimbra under the supervision of the Professor Carlos Manuel Freire Cavaleiro. It includes experiments and results conducted in collaboration with the *Equipe Chimie et Biomasse, Université Pascal Paoli, UMR* CNRS 6134, under the kind direction of the Professors Ange Bighelli and Félix Tomi.

“The study of essential oils had an exciting past.

Now, with improved analytical techniques, it has an equally exciting future.”

By Haagen-Smit

Acknowledgements

I would like to express my gratitude to all those who gave me the possibility to complete this thesis.

First and foremost, I would like to express my sincere gratitude to my supervisor Professor Carlos Manuel Freire Cavaleiro for the continuous support of my MSc study and research, for his patience, motivation, enthusiasm, kindness and immense knowledge. His guidance helped me in all the time of research and writing of this thesis.

Besides my supervisor, I would like to thank the rest lab equip, Professor Lígia Salgueiro and others investigators for sympathy and motivation in the work.

I am thankful to my master friends, Emanuel Sá Miranda, Fátima Nunes, Filipa Oliveira, Tiago Valadeiro and specially Hélia Jeremias e Lígia Mesquita for the help, support, patience, courage, fun time and precious friendship. I would like special thanks yet to my friends Pedro Bastos, Gonçalo Clemente, Humberto Ferreira and André Gomes for unconditional support, courage and friendship.

I would like to thank the research group *UMR* CNRS 6134, Equipe *Chimie et Biomasse, Université de Corse*: Professor Ange Bighelli, Professor Felix Tomi and MSc Mathieu Paoli for their comprehension, patience, knowledge and internship opportunity. I thank also my friends in Corse, Amel Bouzabata, Camille Grousjean and Professor João Batista for the stimulating conversations, fellowship and friendship during my stay in Corse.

Particularly, I would like to give thanks to my family: mother, brother, nephews, brother-in-law for their patience love, comprehension and reliability that enabled me to complete this work. Especially I would like to give my special thanks to my sister and niece Maria João for their help on thesis writing.

I also thank those who contributed directly or indirectly to achieving this step.

Resumo

Ao longo do processo de evolução as espécies, as plantas desenvolveram vias metabólicas que lhes permitem sintetizar uma elevada variedade de metabolitos secundários com importantes funções biológicas. Entre eles, destacam-se alguns compostos de baixo peso molecular, voláteis e dotados de aroma, habitualmente designados como aromáticos. Desde os tempos mais remotos o homem aprendeu a extrair esses compostos, particularmente através do uso de destilação, obtendo misturas, usualmente designadas por óleos essenciais.

O uso de óleos essenciais, tal como os seus constituintes, na saúde e bem-estar é igualmente uma prática já seguida na Antiguidade, por exemplo, como medicamentos tradicionais. Nas últimas décadas foi notório o crescimento da importância e do uso de óleos essenciais, com aplicações em vários domínios industriais, com particular relevância para a indústria alimentar, de produtos cosméticos e de higiene corporal, e de medicamentos.

O género *Eryngium L.* é provavelmente o género taxonomicamente mais extenso e complexo da família Apiaceae, incluindo cerca de 250 espécies distribuídas em todo o mundo. A maioria dessas espécies são consideradas aromáticas e com potencial para a produção de óleos essenciais. Cerca de uma dúzia de espécies de *Eryngium*, incluindo alguns endemismos, crescem no estado espontâneo em Portugal e têm sido objeto de atenção do Grupo *Drug Discovery* do Centro de Estudos Farmacêuticos, no que respeita à caracterização da composição química e potencial biológico. No presente trabalho, reportamos a composição dos óleos essenciais de espécies de *Eryngium*: *E. dilatatum*, *E. pandanifolium*, *E. campestre* e *E. duriaei* subsp. *juresianum.*

Estes óleos essenciais foram preparados por hidrodestilação a partir das partes aéreas das plantas, de acordo com a metodologia descrita na Farmacopeia Europeia. A análise foi realizada por combinação de dados de CG (índices de retenção em diferentes fases estacionárias) e de GC-MS. De forma complementar, uma metodologia analítica especial de ^{13}C-RMN, sem recorrer a isolamento prévio de compostos deu um contributo crucial na identificação de alguns compostos difíceis de identificar por outras técnicas, tais como:

compostos termolábeis (germacreno A e β-elemeno); compostos com espectro de massa semelhante (β-selineno e *E*-β-bergamoteno) e compostos para os quais os dados de CG e CG-MS foram insuficientes para identificações coerentes (selina-11-en-4-α-ol, *E*-dec-2-en-al, α-eudesmol e 4,5-diepi-aristoloqueno). Deste modo, foi reconfirmado o potencial da combinação de CG, CG-MS e ^{13}C-RMN na identificação de componentes de misturas complexas, em particular de óleos essenciais.

Pela primeira vez, é reportada a composição do óleo essencial de *E. dilatatum*, tendo sido identificados 32 compostos que representam 82,6% da composição do óleo. De igual modo, foram identificados 39 compostos no óleo de *E. pandanifolium* (representando 86,7%) e 32 compostos (81% a 98%) do óleo essencial de *E. campestre*. Foi evidenciada a variabilidade química dos óleos de *E. campestre*. Adicionalmente, os nossos estudos permitiram identificar 13 novos constituintes minoritários na composição do óleo essencial de *E. duriaei* subsp. *juresianum,* complementando os resultados anteriormente publicados

De acordo com a estratégia de trabalho do Grupo *Drug Discovery* do Centro de Estudos Farmacêuticos, a caracterização da composição química de óleos essenciais constitui o primeiro e importante passo para a inclusão destas fontes de compostos naturais em programas de avaliação de atividade biológica. Assim, este trabalho é, também, uma contribuição para a valorização das quatro espécies de *Eryngium* da flora portuguesa, que foram objeto de estudo, enquanto fontes de compostos valiosos para programas de screening de novos *leads* e novos fármacos.

Palavras-chave: óleo essencial; *Eryngium*; CG; CG-MS, ^{13}C-RMN

Abstract

Along the evolution, plant species developed metabolic pathways which allow them to synthesize a wide range of secondary metabolites that accomplish important biological functions. Among them are some low-molecular-weight compounds, volatile and endowed of aroma, typically designated as volatile aroma compounds. Since ancient times humans learned to extract such compounds from plants, particularly by the use of distillation rendering mixtures of such compounds known as essential oils. The use of essential oils or some of their constituents for healthcare and human welfare is also a practice that came from the antiquity. Its relevance is steadily growing, specially in some industrial domains, such as, the alimentary, hygiene and cosmetic products or the pharmaceutical.

The genus *Eryngium* L. is probably the most extensive and taxonomically complex genus of Apiaceae family, including about 250 species distributed all around the world, some of them considered aromatic plants, able to produce essential oils. A dozen of *Eryngium* species, including some endemisms, grow wild in Portugal and have been object of attention of the Group of Drug Discovery of the Center of Pharmaceutical Sciences. In present work, we report on the composition of the volatile oils of *Eryngium* species: *E. dilatatum, E. pandanifolium, E. campestre* and *E. duriaei* subp. *juresianum*.

Volatile isolates, essential oils, were prepared by hydrodistillation from the aerial parts of the plants, according the method described in the European Pharmacopoeia. A suited methodology, combining data from GC (retention indices on different stationary phases) and GC-MS was the basis of the analysis of those complex mixtures. However, the methodology of ^{13}C-NMR without previous isolation of compounds gave a crucial input for the identification of compounds that are not easily identified by the other techniques, such as: heat-sensitive compounds (germacrene A and β-elemene); compounds with similar MS spectrum (β-selinene and *E*-β-bergamotene) and compounds for which GC and GC-MS (selina-11-en-4-α-ol, *E*-dec-2-en-al, α-eudesmol and 4,5-diepi-aristolochene) could not attain unambiguous identifications. So, the potential of combination of GC, GC-MS and ^{13}C-NMR for the identification of compounds in complex mixtures was also confirmed.

We report for the first time on the composition of this essential oiln the oil of *E. dilatatum,* in which 32 compounds that represents 82.6% of the whole oil were identified. Similarly 39 compounds were identified in the oil of *E. pandanifolium* (representing 86.7%) and 32 compounds (81 to 98%) in the oil of *E. campestre*. The variability of the oils of *E. campestre* oils was also evidenced. Thirteen new minor constituents were identified in the essential oil of *E. duriaei* subsp. *juresianum* complementing data previously reported.

In accordance with the strategy of the Group Drug Discovery of The Center of Pharmaceutical Studies, the characterization of the composition of essential oils constitutes the first and an important step for the inclusion of such products in screening programs for biological activities. Thus, this work is also a contribution for the valorization of the four *Eryngium* species object of study as sources of valuable compounds for screening of new leads and new drugs.

Keywords: Essential oil; *Eryngium*; GC; GC-MS; ^{13}C-NMR

List of abbreviations

^{1}H-NMR – Proton magnetic resonance

^{13}C-NMR – ^{13}C-Nuclear magnetic resonance

Al – Alentejo

ATP – Adenosine triphosphate

BA – Beira Alta

BL – Beira Litoral

CEF – Centro de Estudos Farmacêuticos

CGC – Capillary gas chromatography

CGC-IRFT – Capillary gas chromatography - Fourier transform infrared spectrometry

CGC-MS – Capillary gas chromatography

COI – Herbarium of the Botanic Institute of Coimbra University

COSY – Correlation spectroscopy

DEPT – Distortionless enhancement by polarization transfer

DMAPP – Dimethylallyl diphosphate

E. – *Eryngium*

EI – Electron impact

FID – Flame ionization detector

FPP – Farnesyl diphosphate

GC – Gas-Chromatography

GC-MS – Gas chromatography - Mass spectroscopy

GPP – Geranyl diphosphate

HMBC – Heteronuclear multiple bond correlation

HMQC – Heteronuclear multiple quantum coherence

HPGC – High performance gas chromatography

HPLC – High performance liquid chromatography

I_{φ} – Linear retention index

I_{φ}^{T} – Kovats Index

IPP – Isopentenyl diphosphate

IR-FT – Fourier transform infrared spectrometry

MIC – Minimal inhibitory concentration

MRSA – Methicillin-resistant *Staphylococcus aureus*

MS – Mass spectroscopy

NAD^+/NADH – Nicotin amide adenine dinucleotide

NMR – Nuclear magnetic resonance

P-GC – Preparative gas chromatography

P-LC – Preparative liquid chromatography

PP – Diphosphate

P-TLC – Preparative thin layer chromatography

PAL – Phenylalanine ammonia lyase

RI – Retention indices

TMS – Tetramethylsilane

Contents

List of tables

List of figures

1. Introduction

1. Introduction

The study of the diversity and complexity of chemical structures found in nature provides important tools for molecular biology, biochemistry and ecological chemistry. These structures play a pivotal role in the complex relationship of organisms on a given ecosystem.

Along the years, with increased of agricultural productivity and diseases, the Man seeks to know the plants and their medicinal properties, leading to their investigation and discovery of substances and plants useful in the healing of various diseases.

Secondary metabolites are compounds that generally exhibit complex structures, relativity low concentrations and occurring in different groups of plants. Among them stand out the essential oils, produced and stored in specialized secretor structures (trichomes, osmophores, ducts and cavities). Essential oils are complex mixtures, containing over one hundred organic compounds. These compounds are volatile, lipophilic and have low molecular weight, which give the characteristic odour plant.

Volatile metabolites are not essential to the organism life that biosynthesizes, but, are essential in communication and survival of the species, defense against predators and attracting pollinators, contributing for the chemical dialogue between plant and ecosystem. These compounds are mediators of ecological interactions that ensure the survivorship of organisms in harsh environments, where they compete with each other.

The art and science of using essential oils in a treatment is named Aromatherapy. René Maurice Gattefossé, a French chemist, first coined the term “aromatherapy” in the 1920’s. Nevertheless, the knowledge of how to extract and apply essential oils is a very ancient art indeed. The ancient Egyptian, Persian, Indian, Chinese and Arab cultures made use of many aromatic oils.

The essential oil composition is mainly determined by genetic factors. However, environmental factors such as seasonality, temperature, hydro availability, soils, etc., can also cause significant variations in the chemical composition.

1. Introduction

For its attractive chemical characteristics, essential oils are a wide group of compounds having antimicrobial properties, fungicides, analgesics, sedatives, expectorants, antitumor agents, among others. Apart from its therapeutic properties, the essential oils are highly sought by perfumes, cosmetics and food industries.

Chemically, essential oils, like all organic compounds, are made up of hydrocarbon molecules and can further be classified as terpenes, alcohols, esters, aldehydes, ketones and phenols. In view of its lipophilic characteristics and low molecular weight, the identification of these compounds allows the drawing up collections of compounds for screening programs of biological activity.

Eryngium (ancient Greek word) is a botanical genus of over two hundred herbaceous species, including annuals, biennials, and perennials and distributed extensively all around the world in temperate regions. *Eryngium* is probably the largest and the most taxonomically complex genus in the Apiaceae family and nearly a dozen species grow wild in Portugal.

Some properties (chemical, genetic and pharmacology) of numerous species of *Eryngium* have been previously studied. However, the essential oil composition and the activities of volatile compounds have been reported only for a few of the species.

In result of these studies, the power of *Eryngium'*s species to produce bioactive compounds with therapeutic potential and the potential for using them as natural food or cosmetic preservatives was confirmed. The identification of these compounds opens alternative therapeutic pathways and enables to elect new leads to design new drugs.

Therefore, we propose in this work the Identification of active compounds in volatile extracts of *Eryngium* species from the Iberia. This project, involves a phytochemical and biological investigation, aims to establish the full composition of the volatile extracts from Iberian *Eryngium* species and identify active compounds on specific biological targets and other disease models.

To carry out the proposed were used extraction methods, phytochemical techniques, bio-guided fractioning, isolation and identification of natural chemical entities and then, screening of biological activities by use of biological techniques.

The work was conducted integrated in the research of the Group of Drug Discovery of the Center of Pharmaceutical Sciences, Faculty of Pharmacy of the University of Coimbra benefiting of the collaboration and expertise in NMR analysis of group *UMR* CNRS 6134, Equipe *Chimie et Biomasse, Université Pascal Paoli, France*.

1. Introduction

2. Chapter 1. *General Aspects – Essential oil*

2.1 Essential oils

2.1.1 History of essential oils

Years ago, aromatic plants supplied important ingredients used for perfume and cosmetics manufacture, in cooking, ritual purposes and medicine. Such ingredients are typically named essential oils, products that continue to be abundantly used in daily life.

The term *"essential oil"* was used for the first time by Paracelsus, an alchemist, who referred them as the effective component of a drug: the *Quinta Essentia* (Guenther 1948). Several aspects of these volatile substances have been studied by chemists, plant physiologists, and pharmacologists, as well as, by historians. Essential oils were viewed as the active constituent of drugs with great pharmacological importance (Haagen-Smit 1961). However, past the mid-20th century, the medicinal use of essential oils has been reduced, their use in pharmaceutical preparations had declined and, beyond aromatherapy, the consumption of essential oils is almost restricted to the flavour and fragrances industries (Edris 2007).

The first report of the use of essential oils comes from the ancient countries of the Orient. The Egyptians employed them for a variety of purposes including embalming the dead in religious ceremonies, and medicinal and cosmetic applications (Worwood 1991).

During same period, India's Ayurvedic literature described medicinal properties and other uses for inumerous herbs and aromatic plant extracts. Their procedures inspired the Chinese herbalists to use and enhance the curative properties and the royalty of their fragrances.

Following the fall of the Egyptian empire, the Greeks assumed much of the Egyptian knowledge and essential oils were used by Hippocrates in aromatherapy. Afterwards, the Romans were impressed by Greek medicine and also used the power of fragrances in aromatherapy.

After the dissolution of the Roman Empire and the renaissance of the Arabian empire, the use of essential oils was continued by the Romans. They was developed an efficient process of their extraction, the distillation (Haagen-Smit 1961).

During the industrial revolution, many compounds of essential oils were identified and therefore allowed to synthesize new components for use in perfume and flavour industries. However, during this time, the use of essential oils decreased but would reappear with aromatherapy in Europe in the next century.

The term "aromatherapy" was introduced by the French physician Jean Valnet Hestarted the scientific study of therapeutic properties of essential oils (1920s) after testifying the full recovery of his friend, the cosmetic chemist Jean Marie Gattefossé, from an accident in the laboratory while preparing fragrances. Accidently Gattefossé burned his arm and hand. He quickly picked up a tube of lavender oil rinsing the wounds and surprisingly pain decreased and infection was controlled and extinguished (Worwood 1991).

Nowadays, the knowledge of health benefits from natural products has been growing. Therefore the use of essential oils in the industry and new applications for human welfare increases continuously.

2.1.2 **Definition**

During the course of evolution, plants developed metabolic pathways that enabled them to synthesize a multitude of secondary metabolites. Among these metabolites are aromatic compounds, whose designation is mainly for its low molecular weight, volatility and odour. These volatile and lipophilic products resulting from secondary metabolism can be found in a considerable number of plants. They are produced and accumulated in specialized secretor structures, externally, in trichomes and osmophores, as well as, internally in idioblasts, ducts and cavities (Proença da Cunha *et al.* 2010). Some of these volatile substances play special physiological and ecological roles: protection against predators, attraction of pollinators; protection for against water loss and temperature control. These metabolites can be found in the glandular hairs, glands, veins or sacs of a plant, grass or tree. They can be extracted from plants

by specific extraction processes, as distillation or expression, leading natural isolates called essential oils.

The designation of "oil" is due to physical and chemical characteristics such as the oily appearance, viscous liquid at room temperature. Its main characteristic is the volatility, differentiating them from the fixed oils, which are lipid mixtures usually obtained from seeds. And the designation "essential" is derived from the Latin *Essentia* which means volatile. As a general rule, an essential oil is identified with a name of the plant origin.

According to the International Organization for Standardization (ISO) in their Vocabulary of Natural Materials (ISO 9235/1997) defines an essential oil as follows: *"An essential oil is a product made by distillation with either water or steam or by mechanical processing of citrus rinds or by dry distillation of natural materials. Following the distillation, the essential oil is physically separated from the water phase*."(ISO 1997).

Essential oils display a set of universal physical characteristics that give them their identity (Table 1).

Table 1 - Physical characteristic of essential oils.

Generally, the essential oils are:
• Volatile substances; • Lipophilic substances; • Highly concentrated, with a pleasant and intense aroma; • Mostly clear in color, usually color less or yellowish. Exceptionally exhibit color due to the presence of certain substances, such as azulenes; • Low stability, especially in the presence of light, heat, and humidity and metals; • Solubility in apolar organic solvents (ex. pentane) and low solubility in water; • Optically active and have a refractive index; • Highly chemically complex.

2.1.3 The chemistry of essential oils

Essential oils can be isolated from the most varied parts of the plant anatomy. The yield of essential oil in plants is normally very low, under 2.5%.

Volatile oils are very complex natural mixtures which can contain more than a hundred compounds at quite different concentrations, including terpenes and their corresponding aldehydes, ketones, alcohols, hydrocarbons, esters, oxides. In general, they are characterized by two or three major compounds in high concentrations (20-70%). The most of the other compounds are present in trace amounts. Normally, these major components determine the biological properties of the essential oils (Bakkali *et al.* 2008). However, some minor compounds can contribute decisively for peculiar organoleptic characteristics or specific biological activities.

The components of the essential oils belong chiefly to class of organic compounds built of "isoprene units." Such compounds are called terpenes or terpenoids (Proença da Cunha *et al.* 2010). Others types of compounds can also be found in essential oils, such as phenylpropanoids, aliphatics, sulphur or nitrogen containing compounds (Betts 2001; Pichersky *et al.* 2006).

2.1.3.1 Terpenes

The class of terpenes includes structurally and functionally different compounds. However all terpenes result from polymerization of isoprene (2-methylbutadiene, C_5H_8), figure 1. They may be classified according to the number of isoprene units incorporated. Monoterpenes resulting from the dimerization of isoprene have 10 carbons, sesquiterpenes resulting from polymerization of three units have skeletons of 15 carbons, diterpenes (20 carbons), triterpenes (30 carbons) and so on.

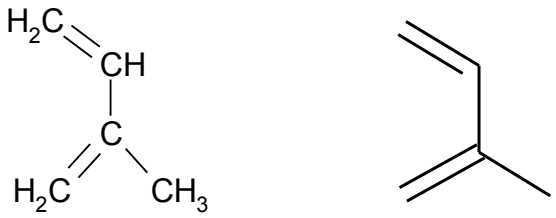


Figure 1 - Representation of the structure of 2-methyl butadiene (isoprene unit).

Isoprene is produced naturally although is not directly involved in the catabolic pathways of these compounds. The biochemically active isoprene units are isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Terpenes biosynthesis starts with the condensation of these two products (Dewick 2002b).

In plants, DMAPP and IPP can be produced by two pathways leading to terpenes: the mevalonate pathway, common to all eukaryotes, taking place in the cytosol, and the deoxyxylulose phosphate pathway in the plastids (Croteau *et al.* 1972; Dewick 1997, 2002a; Eisenreich *et al.* 1998; Kuzuyama 2002).

Table2- Classification of terpenes according to the number of isoprene units.

Class	Isoprene units	C atoms
Monoterpene	2	10
Sesquiterpene	3	15
Diterpene	4	20
Triterpene	6	30
Tetraterpene	8	40

The condensation of DMAPP and IPP leads an intermediate compound with 10 carbons, geranyl diphosphate (GPP), immediate precursor of monoterpenes. GPP, in turn, can condense with another unit of IPP and form a new intermediate with 15 carbons, the farnesyl diphosphate (FPP), precursor of sesquiterpenes. Successively, a

new condensation with IPP originates a precursor of diterpenes with 20 carbons, geranyl geranyldiphosphate (GGPP), Figure 2 (Bakkali *et al.* 2008).

Terpenes represent a diverse class of molecules that provide a wealth of opportunities to address many human health and societal issues. The expansive array of structures and functionalities that have been evolved in nature provide an excellent pool of molecules for use in human therapeutics (Ajikumar *et al.* 2008).

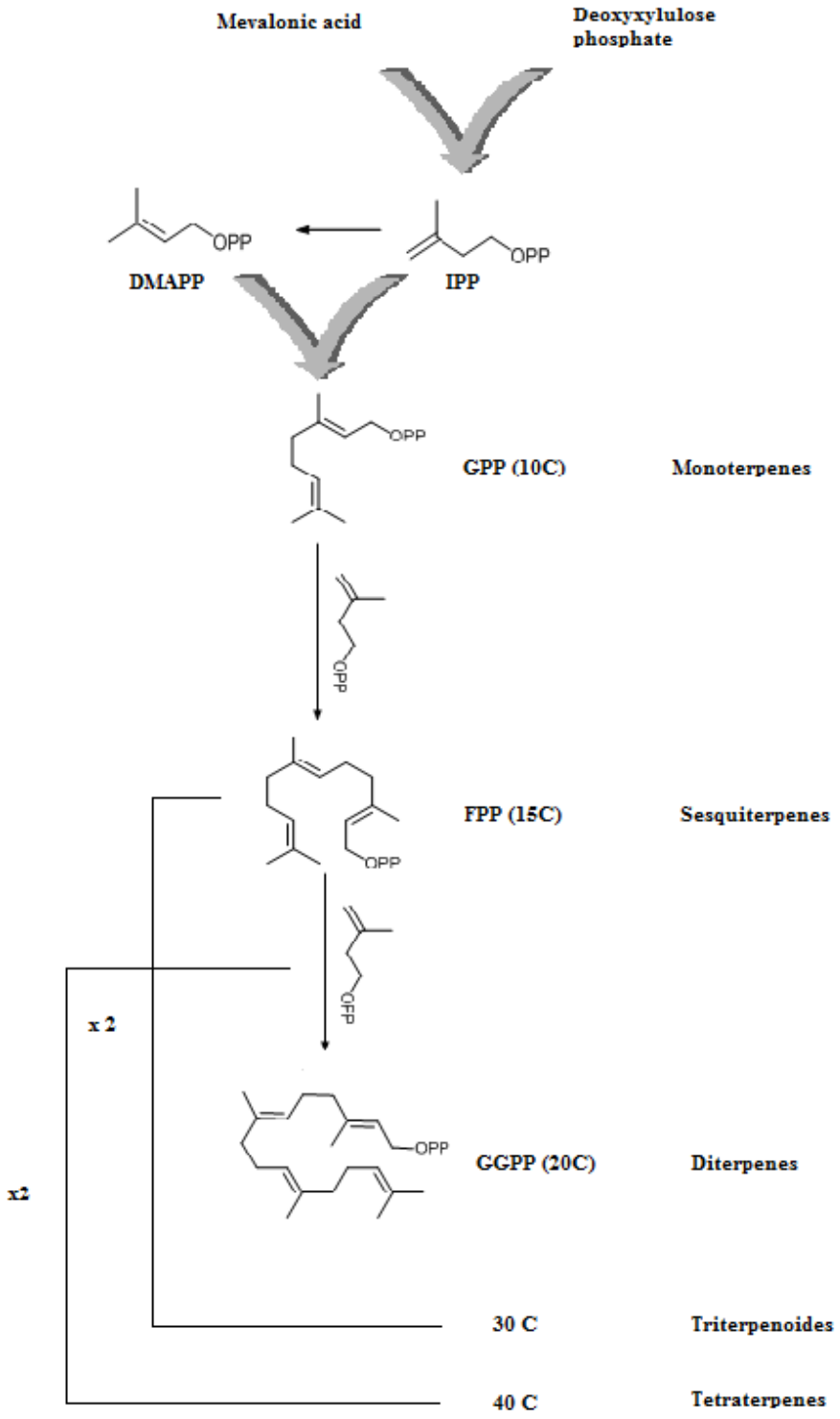


Figure 2 - Biosynthesis of terpenes. Adapted from references (Dewick 2002b; Proença da Cunha *et al.* 2010).

2.1.3.1.1 Monoterpenes

The reaction between DMAPP and IPP via the enzyme prenyltransferase yields GPP, with the formation of a new double bond *trans* (*E*). By ionization to the allyliccation, are formed isomers of GPP, linalyl-PP and neryl-PP, which can thus allow a change in attachment of the diphosphate group (to the tertiary carbon in linalyl-PP) or a change in stereochemistry at the double bond (to *Z*-in neryl-PP), figure 3.

Starting from these three compounds, with small changes, it can give rise to a range of monoterpenes (Dewick 2002b; Stolle *et al.* 2009).

The monoterpenes are substances with a strong aroma and sometimes spicy. Most of them have boiling points between 140^{o}C and 180^{o}C. Some of these compounds are liquid state at normal temperature and pressure. Others are crystalline solids, especially those which contain oxygen (eg, menthol, camphor ...). In general, monoterpene hydrocarbons are colourless substances while oxygenated derivatives exhibit yellowish, pale to strong.

According to the structural arrangements, monoterpenes can be classified in acyclic (myrcene, linalool, geraniol), monocyclic (α-terpineol), bicyclic (α-pinene), as well as, irregular monoterpenes (chrysanthemic acid). All that classes includes saturated hydrocarbons, as well as, functionalized compounds: unsaturated hydrocarbons, alcohols, aldehydes or ketones, esters, ethers, phenols (Epstein *et al.* 1991; Proença da Cunha *et al.* 2010).

2.1.3.1.2 Sesquiterpenes

Adding a further IPP unit to GPP leads to the fundamental sesquiterpene precursor, farnesyl diphosphate (FPP). Increasing the chain length and adding double bond, the number of possible cyclization modes is also increased, and a vast range of mono-, bi-, and tri-cyclic structures can result. FPP can then give rise to sesquiterpenes (Degenhardt *et al.* 2009).

The sesquiterpenes, compounds with 15 carbon atoms, exhibit organoleptic characteristics variable, but always with intense aroma. They have boiling points ranging between 160°C and 200°C.

Likewise, the sesquiterpenes can be classified according to their cyclization: acyclic (farnesol, nerolidol), monocyclic (γ-bisabolene), bicyclic (β-bisabolene, β-caryophyllene) and tricyclic (longifolene) (Proença da Cunha *et al.* 2010). From the 80s up to today, Fraga has published systematic reviews about natural sesquiterpenoids in Natural Produt Reports (Fraga 1987, 1994, 2005, 2011).

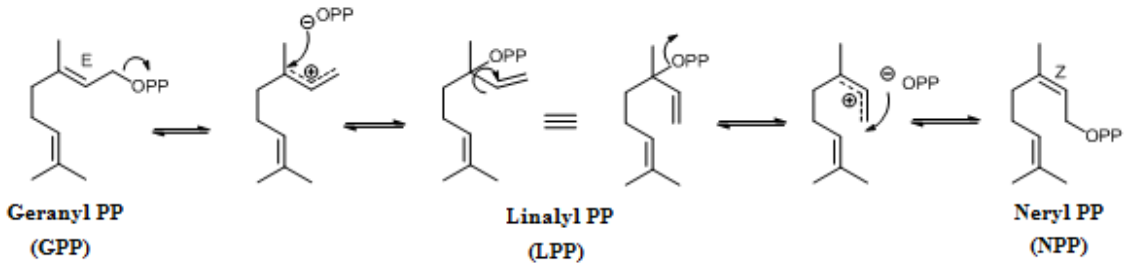


Figure 3 - Formation of isomers of GPP, Linalyl PP and Neryl PP. Adapted of reference (Dewick 2002b).

2.1.3.1.3 Diterpenes

The diterpenes are structures of 20 carbons with molecular weights greater than 280 Da. Comparing to monoterpenes and sesquiterpenes, the increase of the number of carbons results in the increase of the structural diversity and complexity, as well as, in the increase of boiling temperatures and reduction of volatility. For this reason, diterpenoids are less frequent in essential oil.

2.1.3.2 Phenylpropanoids

Although terpenes are the predominant constituents of essential oils, compounds from other classes can occur in variable abundances. The class of phenylpropanoids, which includes compounds derived from a specific biosynthetic pathway is particularly

important. Phenylpropanoids predominate in certain essential oils, especially those from Apiaceae and Orchidaceae families.

The structural feature of phenylpropanoids is the benzene ring attached to a chain of three carbon atoms. They arise from a common precursor, the cinnamic acid, a product of the shikimic acid pathway. The structural diversity of phenylpropanoids is much less than terpenes. However, in essential oils can be found functionalized phenylpropanoids, particularly phenols (eg. eugenol), phenolic ethers (eg. apiole) and aldehydes (eg. cinnam aldehyde and vaniline) (Sangwan *et al.* 2001).

The shikimate pathway produces aromatic amino acids such as phenylalanine. Deamination through the phenylalanine ammonia lyase (PAL) forms cinnamic acid the precursor of phenylpropanoids.

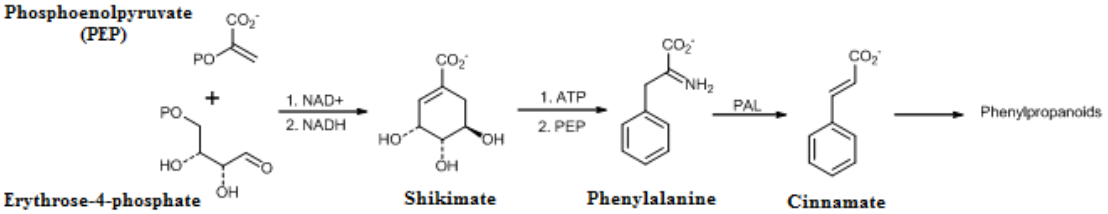


Figure 4 - Biosynthesis of phenylpropanoids. Shikimic acid pathway. Adapted of reference (Proença da Cunha *et al*. 2010). NAD^+/NADH – Nicotin amide adenine dinucleotide**; ATP –** Adenosine triphosphate**; PAL -** Phenylalanine ammonia-lyase

2.1.4 **Biological activities of essential oils**

Essential oils have a long tradition of use in folk medicine, being widely used as bactericidal, virucidal, fungicidal, parasiticidal, insecticidal, for diverse medicinal and cosmetic applications, and recently as pharmaceutical, cosmetic and food ingredients (Edris 2007).

The biological properties of various essential oils have been proved by a number of studies. The most biological activity assigned to aromatic drugs, both plants and essential oils, is the antimicrobial activity, namely antibacterial and antifungal (Bassole

et al. 2011; Bassole and Juliani 2012; Burt 2004; Kalemba and Kunicka 2003; Lang and Buchbauer 2012; Luangnarumitchai *et al.* 2007; Proença da Cunha *et al.* 2010). The essential oils have been proved its antimicrobial power against multiple organisms from the respiratory tract (eg., *M. pneumonia*) (Reichling *et al.* 2009); gastrointestinal tract (eg., *H. pylori; Salmonella spp*., *E. coli O157:H7* and *L. monocytogenes*) (Miguel *et al.* 2008); skin (eg., *P. acnes; Candida species; M. canis and S. epidermidis*) (Cavaleiro *et al.* 2011; Oh *et al.* 2009); oral tract (eg., *S. pyogenes and S. mutans)* (Rasooli *et al.* 2008) and others (Lang and Buchbauer 2012). The difference in antimicrobial activity of the essential oils may be due to the difference in chemical compositions.

However, their use in the treatment of pain, inflammation, viral diseases and cancer and their potential to enhance the penetration of other drugs, their insect repellent activity and their antioxidative effects were also confirmed. In the last few years, many scientific studies were conducted to investigate the effect and the mechanisms of action of these compounds. An update of the latter topics was recently discussed in detail by Adorjan and Buchbauer, where they highlight the therapeutic potential anti-nociceptive, anticancer, anti-inflammatory, penetration-enhancing, insect repellent, antiviral and antioxidative properties of diverse essential oils (Adorjan and Buchbauer 2010).

In addition to the anti-nociceptive activity, the essential oils have other actions on the central nervous system. Dobetsberger and Buchbauer described in depth the analgesic effects, anxiolytic effects, effects on the treatment of stress, effects on learning, memory, attention and arousal, effects on relaxation, sedation and sleep, effects on mood, behaviour and perception, anticonvulsive effects, and finally effects on the treatment of epilepsy, Alzheimer's disease and Parkinson's disease (Dobetsberger and Buchbauer 2011).

Cardiovascular effects of essential oils were also studied by Lahlou *et al.*. They tested, *in vivo,* the treatment of diverse essential oils or isolated compounds of essential oils and confirm the ability to induce hypotension and bradycardia, constituting promissory drugs in hypertension (Lahlou *et al.* 2002; Lahlou *et al.* 2004; Lahlou *et al.* 2005).

Nonetheless, more studies are necessary to analyse the biological properties of other essential oils or to prove their mechanism of action.

2.1.5 *Eryngium* Species

Eryngium (ancient Greek word) is a botanical genus of over two hundred herbaceous species, including annuals, biennials, and perennials and distributed extensively all around the world in temperate regions. It is the largest genus in the Apiaceae family and accounts for around three-quarters of the species diversity within Saniculoideae, the subfamily to which it belongs. *Eryngium* is probably the largest and the most taxonomically complex genus in the Apiaceae family (Calvino *et al.* 2008). However, it is easily distinguished from other members of Apiaceae by its capitates inflorescences and single bract per flower. *Eryngium*, are morphologically extremely variable (Wörz 2011).

In the 1st century, *Eryngium* was indicated by Dioscorides as a vegetable for its young leaves. He also refers the medical uses of some species, as a diuretic, effective in intestinal and liver crisis and epilepsy. In the 9th century, François Delaroche presented a first reviewing monograph of *Eryngium*. His monograph includes excellent illustrations and describes numerous species of this genus. In the following decades, various species were described (Wörz 2011).

Nine of the twenty six *Eryngium* species described in Flora Europaea grow wild in Portugal. Other *Eryngium* species are cultivated as ornamental, vegetable, or medicinal crops for folk uses. With increasing chemical and biological investigations, *Eryngium* has shown potential as a pharmaceutical crop (P. Wang *et al.* 2012).

Although the medicinal usefulness of several species employed in traditional medicines for preparation of diuretic, appetite stimulant, laxative or anti-inflammatory remedies, only few of them were object of bioactivity surveys or chemical investigations (Cavaleiro *et al.* 2011; P. Wang *et al.* 2012).

Some properties (chemical, genetic and pharmacology) of numerous species of *Eryngium* have been previously studied. However, the essential oil composition and

the activities of volatile compounds have been reported only for a few of the species, table 3.

The first report of the composition of the essential oil dates from 1932 by Koolhaas. He analysed the stem and leaves oils of *E. foetidum* and reported dodecenal as the main constituent of the oil (Chowdhury *et al.* 2007). *E. foetidum* is the oil that has aroused more curiosity in the scientific community. To date, its chemical composition has been studied by different researchers in different countries (Cardozo *et al.* 2004; Chowdhury *et al.* 2007; Leclercqa *et al.* 1992; A. Martins *et al.* 2003; Paul *et al.* 2011; Pino *et al.* 1997a, 1997b; N. Thi *et al.* 2008a; Wong *et al.* 1994).

Nevertheless, is in the last decade, in an attempt to find potential drugs, happened a boom in phytochemical and biological studies of this kind of oils.

Pála-Paúl and co-authors have devoted much of their research to the *Eryngium*'s oils of Spain and Australia. In 2003, they studied the chemical composition of essential oil of *E. vesiculosum* by analytical gas chromatography and gas chromatography/mass spectrometry. In this study, they found quantitative but not qualitative differences between the two samples (gathered during summer and winter). Years later, they studied the chemical composition of other species of the genus *Eryngium*: *E. vesiculosum* (Pala-Paul *et al.* 2003)*, E. glaciale* (Pala-Paul *et al.* 2005a), *E. bourgatii* (Pala-Paul *et al.* 2005b), *E. rosulatum* (Pala-Paul *et al.* 2006), *E. corniculatum* (Pala-Paul *et al.* 2007), *E. paludosum* (Pala-Paul *et al.* 2008b), a preliminary study of *E. campestre* oil composition (Pala-Paul *et al.* 2008a) and more recently *E. aquifolium* (Pala-Paul *et al.* 2010).

Lately, after the discovery of interesting phytochemical compounds in essential oils, studies have been made focusing on biological and pharmacological activity.

Natural compounds like terpenes have been suggested as promising non-toxic, non-irritating transdermal penetration enhancers. Saeedi *et al.* depicts the effect of different concentrations of two essential oils (*E. caruleum and E. bungei*) on the transdermal absorption of piroxicam, an anti-inflammatory drug. The results of this

study showed that the essential oils increase the penetration of piroxicam across rat skin. Given that this genus has anti-inflammatory and analgesic effects, the enhancer effects of essential oils will be emphasized (Saeedi, 2008; Saeedi, 2009).

In 2011, the inhibitory activity of nine different methicillin-resistant Staphylococcus aureus (MRSA) strains was also tested by the Kirby-Bauer disk-diffusion method. The anti-MRSA activity of *E. thorifolium* oil, the most active of the three oils tested, was shown equivalent with those of the reference antibiotic vancomycin and or egano oil (Celik, 2011).

Another study, from Cavaleiro, shows the antifungal activity of the essential oil of *Eryngium duriaei subsp. Juresianum* in dermatophytes species (*Trichophyton mentagrophytes, T. rubrum, Epidermophyt onfloccosum; T. verrucosum, T. mentagrophytes varinterdigitale, Microsporumcanis and M. gypseum*), with MIC values between 0.16 and 0.32$\mu L mL^{-1}$. Consequently, an additional study was made to elucidate the mechanism of action of that essential oil in human chondrocytes, as part of a strategy to identify natural compounds with potential disease modifying anti-osteoarthritic activity (Cavaleiro *et al.* 2011).

Table 3 - Different studies of chemical composition and biological activities of *Eryngium*'s essential oils.

Species	Study	Reference
E. foetidum	Composition of essential oil	Koolhaas, 1932 in (Paul *et al.* 2011)
E. foetidum	Composition of essential oil	(Yea 1974)
E. foetidum	Composition of essential oil from Vietham	(Leclercqa *et al*. 1992)
E. foetidum	Composition of leaf and root oils	(Wong *et al.* 1994)
E. foetidum	Composition of leaf oil from Cuba Composition of seed oil from Cuba	(Pino *et al.* 1997a) (Pino *et al.* 1997b)
E. paniculatum	Composition of essential oil	(Cobos *et al.* 2002)
E. foetidum	Composition of essential oil from S.Tomé e Príncipe	(A. Martins *et al.* 2003)
E. expansum, E. pandanifolium, E. rostratum, E. vesiculosum	Composition of essential oil from New South Wales (Australia)	(Brophy *et al.* 2003)
E. vesiculosum	Composition of essential oil from Australia	(Pala-Paul *et al.* 2003)
E. billardieri	Composition of essential oil from Iran	(Sefidkon *et al.* 2004)
E. foetidum	Composition of essential oil from Venezuelan Andes	(Cardozo *et al.* 2004)
E. bungei	Composition of essential oil	(Morteza-Semnani 2005)
E. caeruleum	Composition of essential oil from Iran	(Assadian *et al.* 2005)
E. glaciale	Composition of essential oil from Spain	(Pala-Paul *et al.* 2005a)
E. bourgatii	Composition of essential oil from Spain	(Pala-Paul *et al.* 2005b)
E. rosulatum	Composition of essential oil from Australia	(Pala-Paul *et al.* 2006)
E. yuccifolium	Composition of essential oil	(Ayoub *et al.* 2006)
E. serbicum *E. palmstum*	Composition of essential oil from Serbia	(Capetanos *et al.* 2007)
E. corniculatum	Composition of essential oil from Spain	(Pala-Paul *et al.* 2007)
E. paludosum	Composition of essential oil from Eastern Australia	(Pala-Paul et al. 2008b)
E. caeruleum	Study of drug permeation of piroxicam	(Saeedi and Morteza-Semnani 2008)
E. campestre	Preliminary study of essential oil composition	(Pala-Paul et al. 2008a)
E. foetidum	Composition of essential oil from Vietnam	(N. Thi *et al*. 2008b)
E. amethystium	Composition of essential oil from Italy	(Flamini *et al.* 2008)
E. bungei	Study of drug permeation of piroxicam	(Saeedi and Morteza-Semnani 2009)
E. aquifolium	Composition of essential oil from Spain	(Pala-Paul *et al*. 2010)
E.foeticum	Effect of solar drying on the composition of essential oil	(Banout *et al.* 2010)
E. caucasicum	Composition of essential oil	(Hashemabadi *et al.* 2010)
E. durei subsp. juresianum	Composition of essential oil from Portugal and antifungal activity	(Cavaleiro *et al.* 2011)
E. campestre, E. thorifoliumand, E. creticum	Phytochemical constituents and anti-MRSA activity	(Celik *et al.* 2011)
E. planum	Composition of essential oil	(Thiem *et al.* 2011)
E. maritimum	New oxygenated sesquiterpenes and antimicrobial activity	(Darriet *et al.* 2012)
E. durei subsp. juresianum	Anti-osteoarthritic activity of essential oil	(Rufino *et al.* 2012)

In sum, the results of these investigations confirmed the power of *Eryngium'*s species to produce bioactive compounds with therapeutic potential and the potential for using them as natural food or cosmetic preservatives.

3. Chapter 2 *Analytical methodologies*

The complex composition of many essential oils requires high resolution methodologies to characterise each one of their multiple components:

- Essential oils are complex mixtures of up to hundreds of volatile components with different structures and functional groups. Furthermore, in these mixtures may coexist geometric, optical or positional isomers, with similar physical and chemical properties;
- Essential oils exhibit a significant number of components that occur at low concentrations, frequently in trace concentrations. However, these components may be responsible for important biological properties or relevant sensory notes. Therefore, we can't ignore their detection, identification and quantification.

Because of the unbelievable complexity and hundreds of different chemical compounds contained in one single oil, it becomes clear that analysis of essential oil is a hard task. In addition to the complexity of these volatile oils, the moment of harvest, climate, the soil and the mode of essential oil extraction influences the oil composition and consequently the amount of biologically active compounds.

The design of the analytical strategy should enable the identification of the highest number of components in the complex mixture and allow for their quantification. Thus, it becomes possible to study the chemical and genetic differences between essential oils and the identification of active biological compounds that have some medicinal purpose.

Due to the complexity of essential oils, most of analytical strategies involve two sequential steps:

1. Isolation of each component of the oil that presupposes (or not) sequential fractionation;
2. Acquisition of analytical, chemical or spectroscopic information of each component.

For studies on plant material, previously to these two steps it must be considered the suitable method for isolation of the volatile metabolites.

3.1 Isolation of essential oil from plant

Two main methodologies are considered to isolate the essential oils from plant materials: distillation and expression.

Distillation of plants has a long and diverse history. Egypt, Persia, and India are some countries where distillation was first carried out. Distillation is easily transposed from laboratorial to the industrial scale, obtaining essential oils with similar characteristics. The distillates are free of fixed compounds, simplifying the procedures for sample preparation for analytical purposes (Proença da Cunha *et al.* 2010).

There are different types of distillation, which, while basically the same process, differ in detail to suit specific types of raw material and specific types of oil: hydrodistillation, steam distillation and hydrodiffusion (Worsfold *et al.* 2005).

In the hydrodistillation, the raw material is totally immersed in water and the temperature elevated to boiling point. On cooling the distillate, the oil and water separate and the oil is selectively removed. One advantage of this methodology is that the oil is never raised above a temperature of 100 degrees Celsius and, thus, thermal decomposition is minimized. For thermally labile oils, the temperature can further be reduced by distilling under reduced pressure (Worsfold *et al.* 2005). Hydrodistillation is also the basis of the technique approved by major pharmacopoeias for the isolation of essential oils, viewing its quantification in aromatic plants. This methodology is adopted by most of the researchers due to the simplicity and functionality of the distillation head, the modified Clevenger apparatus (Pharmacopoeia 2011; Proença da Cunha *et al.* 2010).

In steam distillation, hot steam is forced through the matrix of raw material, opening the cavities in which the oil is held, and volatilizing the oils. The oil separates from the

water in the distillate and is collected. The steam is produced at greater pressure than the atmosphere and therefore boils at above 100 degrees Celsius which facilitates the removal of the essential oil from the plant material at a faster rate and in so doing prevents damage to the oil. As the steam is under pressure, the temperature can be carefully adjusted to provide the maximum rate of extraction with minimum thermal decomposition. On a lab-scale steam distillations are carried out using also a Clevenger-type apparatus (Worsfold *et al.* 2005).

Hydrodiffusion is basically a modification of steam distillation. It differs from the conventional steam distillation in that the steam is passed into the top of the container, passes through a bed of botanical material supported on a grill. The condensation of the oil containing steam mixture occurs below the area in which the botanical material is held in place by a grill. The main advantage of this method is that less steam is used, shorter processing time and a higher oil yield (Worsfold *et al.* 2005).

Solvent and supercritical fluids extractions are often used to recover plant volatiles, leading aromatic extracts suitable for analysis, however, facing regulatory guidelines, they can to be not considered essential oils.

3.2 Acquisition of analytical, chemical or spectroscopic information

The analytical methodologies applied to the study of the composition of essential oils are mainly dependent on the purpose of the scientific-technical analysis and previous knowledge of the oil composition.

The chemical analysis of essential oils remains an important step for their development and/or marketing. It is necessary to have an optimized methodology (fast, reliable and personalized analytical tools) to identify and quantify the components of these different complex mixtures.

In general, the study of the chemical composition of a natural mixture can be followed by three different ways:

Via A, also known as the *classical via*. It is the most suitable methodology for the identification of new molecules. It involves the purification of components by different physicochemical and chromatographic techniques, prior the spectroscopic study of each one of them. This methodology is required for unequivocal identifications of unknown components.

Via B. It turns out to be very standard for routine analysis or identification of the constituents of essential oils already described (eg. quality control). This methodology involves the coupling of one or more high resolution chromatographic techniques (Capillary Gas Chromatography - CGC, High Performance Liquid Chromatography - HPLC) with spectroscopic detectors (Mass Spectrometry-MS, Fourier Transform Infrared Spectrometry - IR-FT). The coupling of these techniques allows us to identify the constituents by comparing their spectral data with those of known products contained in computerized spectral libraries.

Via C. It is an alternative approach, intermediate to the previous ones, uses the ^{13}C-Nuclear Magnetic Resonance (^{13}C-NMR) as a technique for identifying the components of a complex mixture without previous separation or fractionation. Moreover, this technique can be used for the identification or quantification of compounds difficult to quantify by traditional techniques.

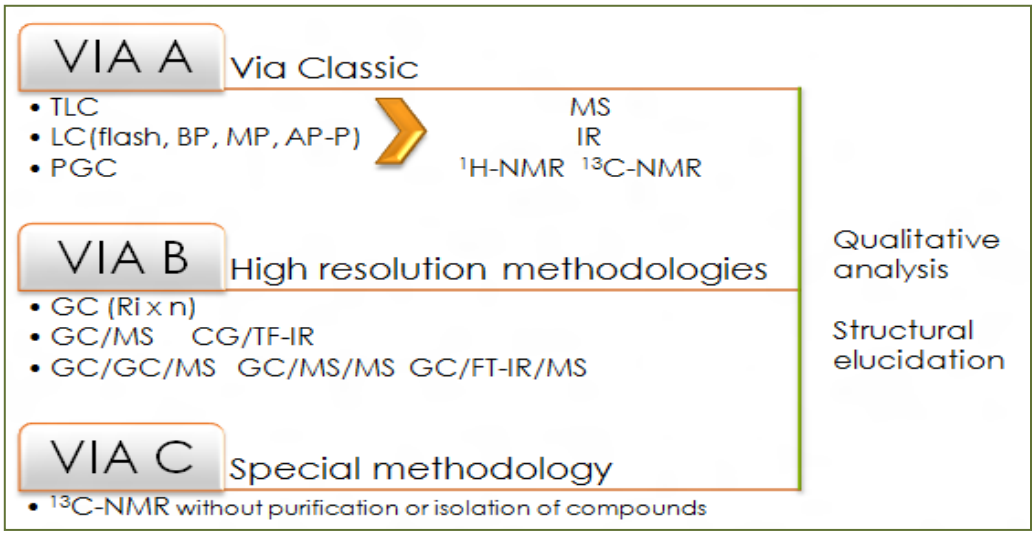


Figure 5 - Schematic representation of the three Vias of compositional analysis of essential oils.

3.2.1 **Via A**

The operational sequence performed in this via is analogous to the study of a new molecule (first isolation/purification and second spectroscopic characterization).

In this case, the essential oil is first fractionated by different preparative chromatographic techniques, such as Liquid Chromatography (P-LC), Thin Layer Chromatography (P-TLC), or preparative Gas Chromatography (P-GC), preceded or not by fractional distillation. Latter, the different isolated components are identified by comparison or interpretation of spectral data: mass spectra, IR spectra, ^{1}H-NMR and ^{13}C-NMR spectra (when amount of product isolated is sufficient).

This methodology is extremely reliable and allows us the identification of novel compounds present in natural mixtures. However, it is a very time consuming and costly due the various stages of fractionation and purification that require a large investment in time.

But in some cases its great reliability compensates investment time required, for example when we have essential oils composed of unknown compounds.

3.2.2 **Via B**

Great efforts have been directed towards improving methods in order to obtain high resolution separations at lower cost and faster speed, especially required for complex mixtures analysis.

Capillary gas chromatography (CGC) has been found to be the most selective technique for the chemical analysis of essential oils. This technique is now recommended in the European Pharmacopoeia as the standard method to analyze essential oils.

CGC is the most efficient chromatography technique for separating volatile mixtures, because of its high resolving power and the availability of universal detection using the flame ionization detector (FID).

Chemical analysis of volatile samples as we know it in our days, couldn't exist without capillary CGC: it is present in the our analytical activities applied to the most diverse areas, as food, flavours and fragrance, petrochemicals, pharmaceutical and environmental analysis. CGC has strongly contributed for the development of essential oil science from equally the academic research (eg. study of new compounds) and the industrial point view (eg. quality control).

3.2.2.1 CGC (RI x n)

The CGC is a process merely separative. Nevertheless, the high efficiency separations, the capacity to distinguish each component from a complex mixture, the low gas' flow of mobile phase and the possibility of using different types of detectors, make it an important analytical tool for identification and quantification of volatile components of complex mixtures, such as essential oils.

Whatever the detector's type used, CGC gives useful information to analyzing the composition of complex mixtures, particularly on the retention performance of the volatile components in certain conditions. Although, CGC is one of the analytical techniques most used because the retention time provide information about the nature of the molecules and peak areas provide a relative quantification, the identification of essential oil constituents is hardly achievable only by CGC.

In this methodology, the identification of compounds based on only one parameter, typically retention time has become inadequate for complex mixtures. A different strategy for obtaining unequivocal identification of compounds is to increase the number of parameters that can be used simultaneously in detection.

Kowats (1958) has found a solution to surpass this limitation by verifying that there is a linear relationship between the number of carbon atoms of the linear alkanes (*n*-alkanes) and the logarithm of its retention time. Thereby, he suggested that in the chromatographic analysis of a mixture (eg: essential oil), adding to the sample a mixture of *n*-alkanes to serve as "markers" of retention of the components. This correlation, resulted the Kowats expression (Kovats Index, I_{φ}^{T}), which relates the

retention of each component of a mixture, with the adjusted retention time of the two *n*-alkanes with previous and subsequent elution (Kováts 1958).

$$I_{\varphi}^{T} = 100z + 100\left[\frac{\log T'_{r(x)} - \log T'_{r(z)}}{\log T_{r(z+1)} - \log T'_{r(z)}}\right]$$

Figure 6 - Kowats Equation.

$I_{,}^{T}$ - Kowats Index; X - compound "X"; Z - number of carbon atoms of the linear alkane which elutes just before the X; Z+1 - number of carbon atoms of the alkane linear eluting immediately up on X; T_{r}' - adjusted retention time.

Following a chromatographic analysis and calculation of the Index Kowats in two chromatographic columns with different polarity becomes possible to assign the retention of each component by comparison with a database (from the laboratories or published in the scientific and technical literature).

However, the Kowats equation has an inconvenience that limits the value of the Kowats Index in the analysis of essential oils. It requires that the chromatography temperature is constant (isothermal conditions).

In order to overcome this limitation, Van Den Dool and Kratz, 1963, proposed a variant (not logarithmic) of the Kowats Equation applicable to linear temperature programmed operation (chromatographic thermal gradient). This proposal opened the possibility of exploiting all advantages thermal gradient in CGC and maximizes the efficiency of the technique, especially important in the analysis of complex mixtures (Herent *et al.* 2007; Vandendool and Kratz 1963).

$$I_{\varphi} = 100z + 100\left[\frac{T_{(x)} - T_{(z)}}{T_{(z+1)} - T_{(z)}}\right]$$

Figure 7 - Linear interpolation equation of Kowats Equation. (Vandendool and Kratz 1963)

I_{φ} -Linear Retention Index for stationary phaseφ; X - compound "X"; Z - number of carbon atoms of the linear alkane which elutes just before the X; Z+1 - number of carbon atoms of the alkane linear eluting immediately upon X; T_Z-Retention time or elution temperature (in Kelvins) when the temperature gradient is not constant..

Widely used in the technical-scientific community dedicated to the analysis of essential oils, this equation makes thus possible to compare the Retention Index of components of the complex mixtures, in a gradient constant temperature.

Thus, in order to make a reliable identification of the compounds of essential oils, diverse couplings between CGC and spectroscopic detectors were developed. These detectors, apart from fulfilling the function of detection, they also allow us to acquire precious spectroscopic information for identifying the constituents of complex mixtures. In these hyphenated techniques, after the chromatographic separation, occurs *on-line*, the acquisition of spectroscopic information of the eluate at a given moment. It is the combination of chromatographic and spectral methodologies to exploit the advantages of both (Marriott *et al.* 2001; Ragunathan *et al.* 1999).

3.2.2.2 CGC-MS

As already stated, of the analytical point of view, important advances have been carried out by the coupling CGC with detectors, such as mass spectrometry (MS) (Marriott *et al.* 2001).

In effect, GC-MS in electron impact (EI), known as GC-MS/EI, is routinely used in chemical analysis of essential oils. The molecules enter into the MS detector, where they are bombarded with free electrons leading to the ionization and fragmentation of the analyte in a characteristic and reproducible way. The resulting

ions (positively charged molecular ion and fragment ions) and respective abundance, define the mass spectrum of the molecule.

The molecular fragmentation depend the electron energy applied to the system, in general 70 eV, leading reproducible spectra making possible the comparison of the acquired spectra with reference spectra banked in laboratory or commercial libraries, like NIST, Wiley or Adams (Adams 2001, 2007; Wiley/NIST 2006).

Thus, the hyphenation these techniques allow acquiring continuous scans almost over GC effluent and consequently obtain one or more spectra about each of the resolved peaks, figure 8.

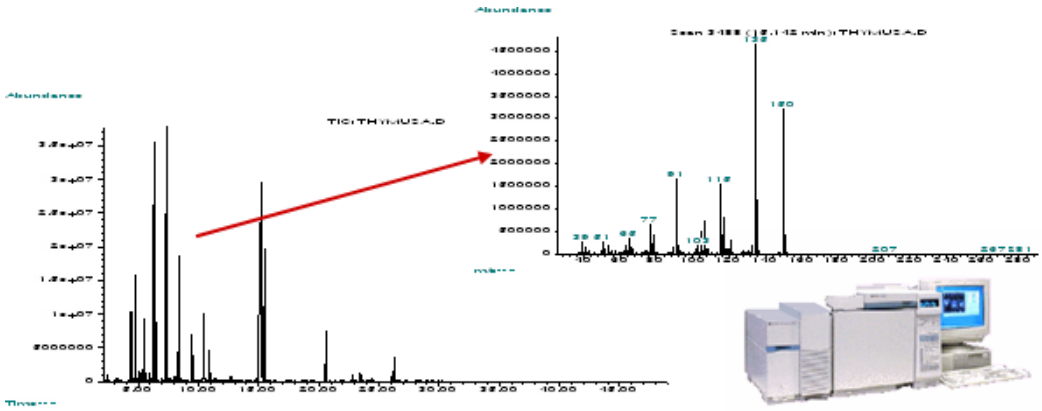


Figure 8 - GC-MS methodology. MS detectors coupled to CGC, enables not only obtaining the elution profile of the compounds in the sample (chromatogram) across graph registration of total ion current, but also the acquisition of mass spectra about each of the resolved peaks.

The sole use of mass spectra may lead to erroneous identifications. Two or more compounds may produce similar mass spectra, making impossible a decision on the correct identity. The combination of the linear retention indices (RI) and mass spectral data is a common approach to avoid such limitation. Identities should result, not only from matching of mass spectra, but also from matching RI. An improved methodology considers a set of two retention indices (from two GC columns with different polarity phases) together with mass spectra.

3.2.2.3 CGC-IRFT

The CGC-IRFT methodology is mostly useful in essential oil analysis to acquire information on functional groups.

Such as in CGC-MS, the spectres acquired may, due to reproducibility, be compared with spectra libraries. However, the difficulty of interpretation of spectra acquired about poorly resolved peaks and lower availability of reference spectra, determine the most successful analysis by CGC-MS than by CGC-IRFT.

If the individually CGC-MS and CGC-IRFT techniques are valuable in the analysis of complex mixtures, the combination of the two is a valuable tool, where the limitations of each of them can be minimized. The potential of coupled systems, like CGC - IRFT-MS, already demonstrated in the essential oils analysis, have the disadvantage in the acquisition and equipment cost (Ragunathan *et al.* 1999).

3.2.2.4 GC-GC (Two-dimensional gas chromatography)

It has been over a decade since the first description in chromatographic literature of the technique now called comprehensive two-dimensional (2D) gas chromatography or *comprehensive GC-GC*. Afterwards, the number of publications in this field has grown quickly as researchers have explored GC-GC, its variants and related techniques (Chin *et al.* 2012; Marriott *et al.* 2003; Marriott *et al.* 2012; Ong and Marriott 2002; Phillips and Beens 1999; Ryan and Marriott 2003).

The GC-GC results of the combination *on-line* of two chromatographic columns subjected to independent thermal conditioning. Thus, the sample components eluted from the first column (*first dimension*) are sequentially re-chromatographed in the second dimension (*comprehensive GC-GC*).
This process results in an exponential increase of resolution and acquisition of two sets of chromatographic information. After, this information can be represented in a two-dimensional referential. Wherein each chromatographic peak is then referenced by two coordinates, allowing more reliability in the identification of compounds based in retention parameter, and even its quantification.

Therefore, by effecting a heartcut event during a given region of a chromatogram, the desired components are transferred to a second column (more selective), whereupon the components are better resolved. A valve system ensures the transfer of flow in preselected time interval. It's mostly appropriate for studies of enantiomeric composition. The separation of enantiomers depends of the use of columns with suitable selectivity (usually cyclodextrin derivatives) but which are ineffective for the separation of complex components of essential oils (Shellie and Marriott 2002).

Thus, in a two-dimensional system, a conventional column installed in the first dimension, provides the separation of constituents; and in the second dimension, the flow corresponding to a selected peak (eg., mixture of enantiomers) can be individually re-chromatographed on an enantioselective column. This methodology offers, directly, the study of chiral compounds in essential oils.

Comprehensive two-dimensional gas chromatography is an emerging technology for chemical separation that provides an order-of-magnitude increase in separation capacity over traditional gas chromatography and is capable of resolving several thousands of chemical compounds (Dalluge *et al.* 2003).

3.2.2.5 Other couplings

Over recent decades, there has been a great investigation in search of analytical methodologies ever more efficient, fast and optimized in the essential oils analysis, particularly in the hyphenation of several analytical techniques.

Hyphenation refers to the coupling of spectroscopic detection methods or other specific types of detection techniques to improve the separation performance or quality of data from analysis. We can highlight a few examples of its strategy described by Marriott *et al.*: HPLC-HRGC; SFE-GC; GC-O and GC-TOF-MS (Chin *et al.* 2012; Marriott *et al.* 2001).

In conclusion, the CGC is, from among all chromatographic methods, the most efficient fractionation of complex volatile mixtures, like essential oils, enabling in most cases the individualization of majority of its components.

If the Via B, thanks to its rapidity is very functional in routine chemical analysis, the Via A assures high reliability in the identification of compounds. However, the various stages of fractionation and purification often require a large time investment. Thence arises a third via (Via C), complementary and intermediate to the previous ones, which bases its identification and quantification of the main compounds of a natural mixture by the study of ^{13}C-NMR spectra of a mixture without prior separation.

3.2.3 **Via C**

The identification of some of the constituents in essential oils may require, in addition to the interpretation of the chromatographic mass spectral or infrared information, further studies by other spectroscopic techniques. Compounds with similar mass or IR spectra and equal or close retention indices often coexist in the same sample. In the particular case of sesquiterpenoids, more than 230 compounds are described that elute in a range of retention indices of only 300 units of retention indices, with a molecular mass of 204Da, and whose mass spectra have the same ion fragmentation peaks.

The proton (^{1}H-NMR) or carbon-13 (^{13}C-NMR) Nuclear Magnetic Resonance techniques (mono or bidimensional) are usually used for structure elucidation of natural organic compounds. Nevertheless, their application in the analysis of complex mixtures has the limitation of requiring the isolation and purification, or at least a high enrichment, to identify each constituent.

The work required to enrich or isolate and purify the complex mixtures isn't compatible with the need to investigate the large number of constituents present in complex samples. In order to overcome this limitation appears the pioneering work of Formácek and Kubeczka opened a world of possibilities for the analysis of essential oils by ^{13}C-NMR, without prior isolation or purification of its constituents (Formácek and Kubeczka 1982; Kubeczka and Formácek 2002). The analysis technique is based on the assignment of resonance signals to the carbons of each compound from a unique spectrum, recorded for the mixture.

Many of the molecules studied by NMR contain carbon. The carbon-13 nucleus, despite its low isotopic abundance (1%), is appropriate for the analysis of complex mixtures without prior isolation of its constituents because the carbon constitutes the skeleton of all organic molecules, so the slightest structural variation involves a measurable change (of higher or smaller magnitude) of the chemical shifts of all carbons of the structure. Thus, the ^{13}C-NMR spectra where proton decoupled originate only one resonance signal for carbon, allowing a signal resolution of 0.01ppm or less; the carbons' resonance domain extends over 240ppm, when compared to the 12ppm area of the proton spectra.

To make feasible the analysis of a complex mixture without prior isolation of constituents, are crucial the resolution and sensitivity of the equipment, factors which depend, respectively, the individualization of the different resonance signals and registration of resonance peaks of minor compounds.

By the diversity of compounds presents in composition of essential oils, since unsaturated hydrocarbons to functionalized compounds, the resonance signals of the carbons are distributed all over the spectral window, minimizing the need of optimization of resolution.

In early studies of application the ^{13}C-NMR analysis in essential oils without prior isolation of constituent, the acquired spectra with practically no dilution of the sample, showed significant differences in chemical shifts of the carbons of the constituents, compared to the reference substances. Over the past years, the group *Chimie et Biomasse-CNRS / Université de Corse* has been optimized the conditions for acquisition of spectra of complex mixtures so as to avoid the variations previously described and ensure the reproducibility in the values of chemical shifts.

So with the optimized conditions and having a spectrometer with enough resolution allows us to solve different closed signals and minimize the overlap of carbons' signals of different compounds.

In sum, this method is based on the comparison of the chemical shifts in the spectra of the sample with those reference compounds contained in a spectra database. The acquisition of reference and sample spectra is realized under the same experimental conditions (solvent, concentration, parameters of NMR-spectra acquisition). The originally of this methodology in the processing information of spectroscopic data, thanks to a computer-assisted identification specially developed. This software can help identify preciously information to the characterization of components of a mixture, namely: the number of peaks observed in relation to the expected number of peaks per molecule; the number of overlapping peaks that can occur when two different carbons of two molecules have the same chemical shift; variations in chemical shifts of the carbons compared with its reference values.

The major limitation of this method for the analysis of complex mixtures puts up on the ability to spectra interpretation and assignment of the signals recorded. This limitation was solved by developing the computer-assisted methodology that, making use of a database of reference spectra of compounds acquired under the same experimental conditions and/or a spectra database published, enabling the processing of spectroscopic information.

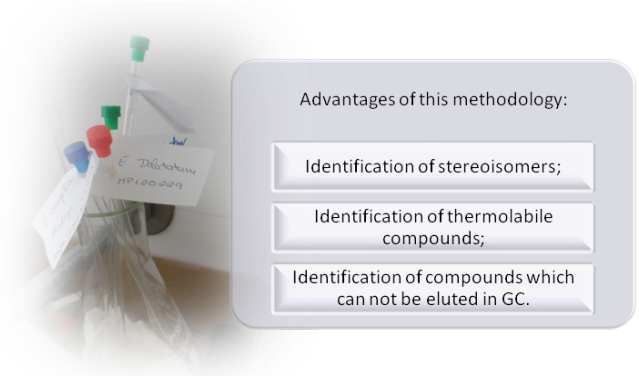


Figure 9 - Advantages of ^{13}C-NMR methodology, without a previous purification.

4. Chapter 3. *Experimental*

4.1 Overview

Among the *Eryngium* species spontaneous in Portugal we proposed to study the essential oils of *E. dilatatum*, *E. pandanifolium*, *E. campestre* and *E. duriaei* subsp. *juresianum* in order to establish their compositions and conclude on their significance.

E. dilatatum is a species with limited occurrence, native from the Iberia and North-Africa (Morocco) that grows wild in Portugal in dry and stony soils. As our knowledge the composition of the essential oil of *E. dilatatum* was never object of any investigation. Then we report, for the first time, on the composition of the essential oil of this species.

Although the wider distribution of *E. pandanifolium*, with occurrence described from southern and central South America, south west Europe and Australia, a single note was published on the composition of the essential oil isolated from plant material from Australia. In Portugal this species grows in sunny and well-drained soils and can be found in the low lands of the center-west of Portugal (Baixo Mondego). Due the little information available on the oil of this *taxon* we proposed to characterize that from Portuguese populations.

E. campestre is an Iberian endemism. Pála-Paul *et al*. investigated the composition of the essential oils from two populations of different locations of Castilla, Spain, and concluded on the chemical variability of the essential oil, maybe due to differences of soil composition and acidity (Pala-Paul *et al.* 2008a). Attending that no information is available on the essential oils from plants growing in Portuguese soils, we proposed to study populations from different location in Portugal to characterize their essential oils to evaluate and expand information on the chemical variability.

Eryngium duriaei subsp. *juresianum* essential oil was previously studied by Cavaleiro *et al.* (2011). The oil exhibits noteworthy antifungal proprieties against dermatophytes, probably due to caryophyllene derivatives. In the mentioned paper, 25 compounds representing 84.6% of the oil were identified, among them, caryophyllene derivatives, *E*-caryophyllene, caryophyllene oxide, 14-β-hydroxy-caryophyllene and

isocaryophyllen-14-al (β-betulenal). However, three other sesquiterpenes, occurring in relevant concentration (> 1.0%) remained unidentified. For this reason we proposed to acquire complimentary information in order to contribute for the identification of these compounds.

4.2 Material and Methods

4.2.1 Plant material

4.2.1.1 Eryngium dilatatum

Eryngium dilatatum is a hemicryptophyte with erect 5 to 40 cm stalks and 2 to 10 cm basal subcoriaceous, obovade evergreen leaves with indistinct petiole, more or less winged till the base. Inflorescence is bluish with twelve globous and pedunculate capitulum.

It grows wild in the Iberia and North Africa (Morocco). In Portugal it can be found in dry and stony places of the center and north. Samples for study were collected at Rio de Galinhas, near Marco de Canaveses, Minho.

4.2.1.2 Eryngium pandanifolium

Eryngium pandanifolium is a hemicryptophyte with 150 to 400 cm erect stalks and 150 to 250 cm basal leaves with thin marginal spikes. Inflorescence is paniculate with numerous 6-10 x 4-8 oval globous white-greenish capitulum. It grows wild in the wetlands of the edges of lagoons. In Portugal it can be found in sunny and well-drained soils of the edges of Baixo Mondego rice fields. Samples for study were collected in Baixo Mondego, near Maiorca, Montemor-o-Velho.

4.2.1.3 Eryngium campestre

Eryngium campestre is a hemicryptophyte with 20 to 70 cm erect stalks and ovade coriaceous basal leaves with 5-20 cm limb, usually evergreen. Pale-greenish inflorescence, corymbiformis, with numerous 5-10 x 15-25 oval pedunculate capitulum. It grows in dry uncultivated lands. In Portugal it can be found from the north to the south except in Algarve. Samples for study were collected from populations growing wild in Alandroal, Alentejo, Mira, Beira Litoral and Porto da Carne, Beira Alta.

4.2.1.4 Eryngium duriaei subsp. *juresianum*

E. duriaei is an erect, spinosous and perennial herb, measuring 30-100 cm. The leaves of this plant show variable features: whereas plants from lower altitudes have the basal leaves more or less linear-spathulate and not undulate with a denticulate margin, those of higher altitudes have the basal leaves narrower, linear-oblanceolate, undulate, pinnatifite, showing a regularly sinuate-dentate margin. According these different morphologies Laínz considered two different taxa first at specific level *E. juresianum* (M. Laínz) M. Laínz and *E. duriaei* J. Gay ex Boiss., latter defining two subspecies *E. duriaei* subsp. *juresianum* and *E. duriaei* subsp. *duriaei*. Our work concerns to the first subspecies, *E. duriaei* subsp. *juresianum*. In Portugal it can be found wild in the rocky places of the lower altitudes of Serra da Estrela and Serra-do-Açor. Due to deforestation and recurrent fires conservation status of this subspecies is considered vulnerable.

Samples for study were collected at Serra-do-Açor, Colcurinho (800-1000 m), near the village of Piodão. Voucher specimens (20.09.2008, A.C. Tavares, 113 and 19.06.2010, A.C. Tavares, 136) were deposited at the Herbarium of the Botanic Institute of Coimbra University (COI).

4.2.2 **Isolation of essential oils**

The isolation of the essential oils was made by hydrodistillation, during 3 hours, in a Clevenger type apparatus according the procedure described in the European Pharmacopoeia (2011), however without the use of any retention solvent. Oils were stored in glass vials at 4^{o}C prior analysis (Pharmacopoeia 2011).

4.2.3 **Analysis**

The analysis of the volatile oils was initiated using a combination of two methods: capillary gas chromatography (CGC) with determination of retention indices on two stationary phases and gas chromatography-mass spectroscopy (GC–MS). Complementarily, ^{13}C-NRM, without previous isolation or purification of essential oils components, was also used.

4.2.3.1 Gas chromatography

Analytical CGC was carried out in a Hewlett-Packard 6890 (Agilent Technologies, Palo Alto, CA, USA) chromatograph with a HPGC ChemStation Rev. A.05.04 data handling system, equipped with a single injector and two flame ionization detectors (FID).

A graphpak divider (Agilent Technologies, part no. 5021-7148) was used for simultaneous sampling to two Supelco (Supelco, Bellefonte, PA, USA) fused silica capillary columns with different stationary phases: SPB-1 (polydimethylsiloxane 30m×0.20mmi.d., film thickness 0.20μm), and SupelcoWax-10 (polyethyleneglycol 30m×0.20mmi.d., film thickness 0.20μm). Oven temperature program: 70–220ºC (3◦Cmin^{-1}), 220ºC (15 min); injector temperature: 250ºC; carrier gas: helium, adjusted to a linear velocity of 30cms^{-1}; splitting ratio 1:40; detectors temperature: 250ºC. Samples were diluted in *n*-pentane (1:8) and injected (0.2 μL) through autosampler.

Table 4 - Experimental conditions of CGC methodology.

Capillary Columns	Column Characteristics	Temperature Program	Injector Temp.	Carrier Gas	Splitting Ratio	Detectors	Detectors Temp.
SPB-1	polydimethylsiloxane 30m×0.20mm i.d., film thickness 0.20μm	70–220°C (3°C min^{-1}); 220°C (15 min)	250°C	He (30cms^{-1})	1:40	FID	250°C
SupelcoWax-10	polyethyleneglycol 30m×0.20mmi.d., film thickness 0.20μm					FID	250°C

4.2.4 **Gas-Chromatography - Mass spectroscopy (GC-MS)**

GC–MS was carried out in a Hewlett-Packard 6890 gas chromatograph fitted with a HP1 fused silica column (polydimethylsiloxane 30m×0.25mm i.d., film thickness 0.25μm), interfaced with an Hewlett-Packard mass selective detector 5973 (Agilent Technologies) operated by HP Enhanced ChemStation software, version A.03.00. GC parameters as described above; interface temperature: 250°C; MS source temperature: 230°C; MS quadrupole temperature: 150°C; ionization energy: 70 eV; ionization current: 60μA; scan range: 35–350 units; scans s^{-1}: 4.51.

Table 5 - Experimental conditions of GC-MS.

Capillary Columns	Column Characteristics	Interface Temp.	Detector MS	MS source temp.	MS quadrupole temp.	Ionization
HP1	polydimethylsiloxane 30m×0.25mm i.d., film thickness 0.25μm	250°C	5973 (Agilent Technologies)	230°C	150°C	70 eV 60μA

4.2.4.1 Qualitative analysis:

The methodology of identification of compounds was based on two steps:

1. Assessment of their retention indices on both SPB-1 and SupelcoWax-10 columns, through linear interpolation relative to retention times of C8–

C24 of *n*-alkanes, according Van den Dool & Katz (1963) and compared with reference data included in CEF laboratory database or literature;

2. Acquisition and matching of mass spectra with reference data from the CEF spectral database, Wiley / NIST database or literature (Wiley/NIST 2006).

4.2.4.2 Quantitative analysis:

Relative amounts of individual components were calculated based on GC raw data areas without FID response factor correction. Chromatograms produced from the SPB-1 column were customarily used. However, quantification of compounds co-eluted in the SPB-1 column used the chromatograms produced from the SupelcoWax-10 column.

4.2.5 ^{13}C-NMR, without previous isolation of compounds

NMR spectra were acquired on a Bruker400, AVANCE, 9.4 Tesla, operating a 100.623MHz to carbone-13, equipped with a 5mm probe.

The solvent used was deuteriochloroform, $CDCl_3$, which was added to tetramethylsilane, TMS. The chemical shifts (δ) are given in ppm compared to TMS internal reference.

The 13C-NRM spectra were registered with the following parameters: flip angle 45°; acquisition time: 2.7s corresponding to an acquisition of 128K with a spectral width (SW) 240000Hz (about 240ppm); period of relaxation 0.1s; resolution digital 0.183Hz/pt. For acquisition of 13C-NMR spectra, a mass of 40 to 70mg essential oil was dissolved in 0.5ml of $CDCl_3$. The number of accumulation is between 2000 and 5000 for each record (50mg of the oil in 0.5mL $CDCl_3$). The signals obtained are multiplied before application of the Fourier transform by an exponential function.

For recording the NMR spectra involving 2D heteronuclear correlations (HSQC, HMBC), we used the pulse sequences defined by programs Brucker.

The identification of constituents based on ^{13}C-NMR spectra of the mixture was performed according to a computer-assisted methodology, which through the use of a database of reference compounds' spectra, acquired under the same experimental conditions, allows us process and conjugate the following information with rapidity:

- number of peaks observed for the number of peaks expected. Generally, they are observed resonance signals of all carbons present, except for quaternary carbons of minor compounds, which sometimes are not observed. This reason is explained by slower relaxation of quaternary carbons, the respective signal is less intense than that of the remaining carbons;
- chemical shift variations for each carbon of each compound of the mixture compared to the values of the chemical shifts of the reference compounds;
- number of overlaps, i.e., the number of signals that can be attributed simultaneously to carbons of more than one compound. Stereo and electronic effects, or when they coexist compounds with similar parts of the carbon skeleton, may imply that two or more carbons of compounds have the same chemical shift.

5. Chapter 4. *Composition of the essential oil of Eryngium dilatatum*

Contents of this chapter was presented and discussed in scientific events in the form of communications:

- Combination of capillary GC, GC/MS and ^{13}C-NMR for the analysis of the volatile extracts of E. dilatatum and E. pandanifolium. Conference CEF2012. Coimbra, 25 September 2012. Oral Communication.
- Composition of the volatile oils of *E. dilatatum Lam.* and *E. pandanifolium* Cham. & Schltdl. 43rd International Symposium on Essential Oils (ISEO2012), Lisbon, 5-8 September 2012, Abstract Book, p. 155. Poster.
- Composition of the volatile oil of *Eryngium dilatatum* Lam. 3PYCHEM, Porto, 9-11 May 2012, Abstract Book, p.108. Poster.

As our knowledge *Eryngium dilatatum* was never object of any phytochemical study. Here, we report for the first time, on the volatile metabolites of this species.

The distillation of the aerial parts yields a pale yellow liquid [0.3% (v/m), fresh weight] with a peculiar soft odour.

The composition of the essential oil was investigated using chromatographic retention data, mass spectra acquired by GC-MS and ^{13}C-NMR without prior isolation of components. The latter technique received the contribution of the research group *UMR* CNRS 6134, Equipe *Chimie et Biomasse, Université de Corse*.

Thirty-one compounds were identified representing 82.6% of the whole composition. Sesquiterpene hydrocarbons (35.7%) and oxygen containing sesquiterpenes (23.6%) are dominant. The full composition is summarized in Table 6 where compounds are listed in order to their elution from the SPB1 column. Several sesquiterpenes occur in concentrations over 5%, namely germacrene D (10.3%), bicyclogermacrene (8.1%), spathulenol (5.9%) and α-cadinol (5.7%).

However, two of the three major compounds are the oxygen containing monoterpene, *Z*-α-chrysanthenyl acetate (11.1%) and the monoterpene hydrocarbon, α-pinene (9.2%), Figure 10.

Table 6 - Chemical composition of essential oil *Eryngium dilatatum*.

RI[a]	*RI*[b]	*Compouds*	*%*	*Identification*
928	1028	α-Pinene	9.2	IR; MS; NMR
964	1125	Sabinene	0.2	IR; MS
969	1116	β-Pinene	0.3	IR; MS; NMR
981	1160	Myrcene	1.3	IR; MS
981	1291	Octanal	0.3	IR; MS
1020	1204	Limonene	1.0	IR; MS; NMR
1241	1565	*Z*-Chrysantenylacetate	11.1	IR; MS; NMR
1368	1488	α-Copaene	0.3	IR; MS
1375	1515	β-Bourbunene	0.3	IR; MS
1407	1591	*E*-β-Caryophyllene	4.5	IR; MS; NMR
1423	1579	*E*-α-Bergamotene	0.5	IR; MS; NMR
1440	1661	α-Humulene	0.8	IR; MS; NMR
1466	1700	Germacrene D	10.3	IR; MS; NMR
1475	1675	*E*-β-bergamotene	2.8	NMR; IR; MS
1481	1724	Bicyclogermacrene	8.1	IR; MS; NMR
1486	1724	α-Muurolene	0.8	IR; MS; NMR
1496	1740	*E,E*-Farnesene	1.7	IR; MS; NMR
1501	1747	γ -Cadinene	0.4	IR; MS; NMR
1501	n.d.	*Z*-γ-Bisabolene	0.4	IR; MS
1507	1747	δ-Cadinene	4.4	IR; MS; NMR
1515	n.d.	*E*-γ-Bisabolene	0.4	IR; MS; NMR
1552	2110	Spathulenol	5.9	IR; MS; NMR
1557	1970	Caryophyllene oxide	3.0	IR; MS; NMR
1560	2061	Globulol	0.4	IR; MS; NMR
1564	1995	Salvial-4(14)-en-1-one	0.3	IR; MS
1566	2069	Viridiflorol	0.5	IR; MS; NMR
1611	2212	Isospathulenol	1.0	IR; MS
1616	2158	T-Cadinol	1.8	IR; MS; NMR
1616	2173	T-Muurolol	2.3	IR; MS; NMR
1620	2186	α-Muurolol	1.2	IR; MS
1623	2218	β-Eudesmol	1.4	IR; MS; NMR
1629	2218	α-Cadinol	5.7	IR; MS; NMR
		Monoterpene hydrocarbons	11.9	
		Oxygen containing monoterpenes	11.1	
		Sesquiterpene hydrocarbons	35.7	
		Oxygen containing sesquiterpenes	23.6	
		Other compounds	0.3	
		Total identified	82.6	

Compounds listed in order to their elution on the SPB-1 column; t: traces; n.d.: not detected; [a] Retention indices on the SPB-1 column relative to C8-C24 *n*-alkanes; [b] Retention indices on the SipelcoWax-10 column relative to C8-C24 *n*-alkanes

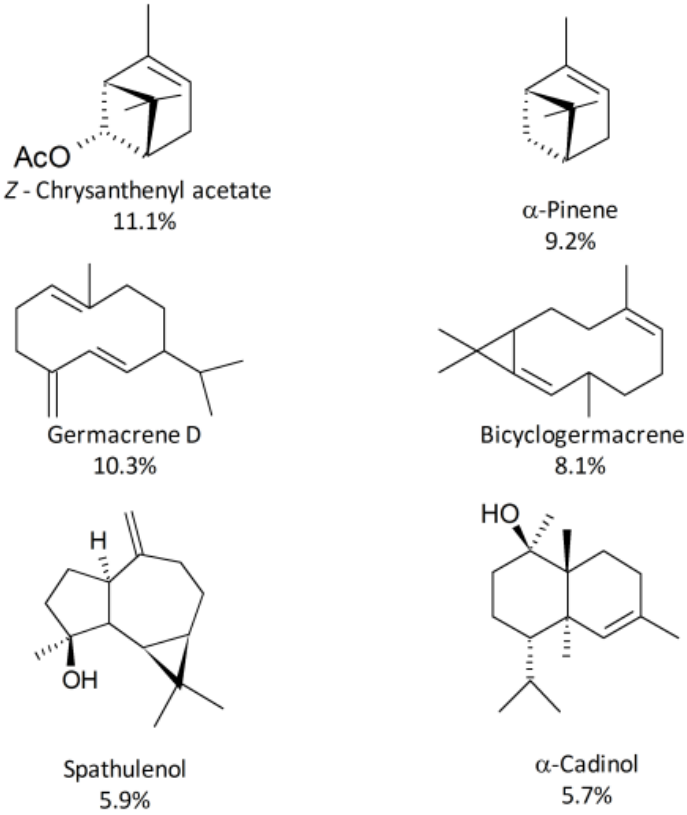


Figure 10 - Chemical structures of major compounds present in *E. dilatatum* oil.

5.1 ^{13}C-NMR contribution to the identification of minor compounds, without prior isolation

^{13}C-NMR analysis allowed confirming the identity of the most abundant components as proposed by retention indices and mass spectra. Additionally, it allowed to identify the sesquiterpene hydrocarbon E-β-bergamotene, amounting 2.8%.

5.1.1 Identification of *E*-β-bergamotene

Mass spectra acquired on a relevant peak on SPB-1 column, with relative area of 2.8% and retention indice, RI_{SPB-1}= 1472, suggested, when matched with reference data

(Wiley / Nist database), the identity of the sesquiterpene hydrocarbon, β-selinene (m/z 204). Reference retention indice for β-selinene at a SPB-1 column was also in accordance (RI_{SPB-1} =1470), Figure 11.

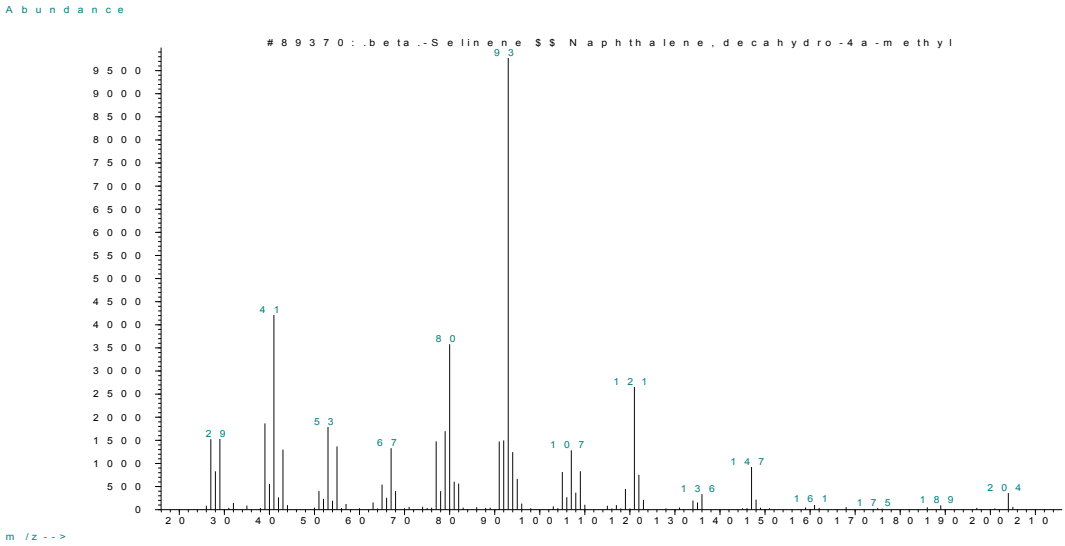
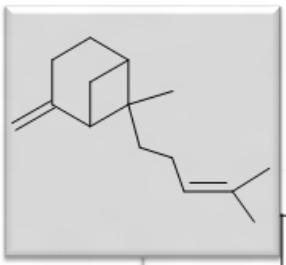


Figure 11 - Reference mass spectrum 70eV of β-selinene. In Wiley/Nist database.

However, in the ^{13}C-NMR spectra of the whole oil, the resonance signals of the β-selinene carbons were absent, contradicting the existence of β-selinene. Instead, some signals of the spectrum, with intensities compatible with such concentration remain unassigned. When matching those signals with those of reference spectra from database, the identity of *E*-β-bergamotene (m/z 204) was proposed.

Table 7 - Resonance signals and chemical structure of *E*-β-bergamotene.



E-β-bergamotene (1472; 1675)

Chemical shift (δ ppm)		Relative abundance	Overlap
Reference	Experimental		
152.08	152.12	3.13	
131.04	131.06	2.75	
125.04	125.00	25.33	α-Humulene + n.i.
106.05	106.04	11.66	
50.21	50.20	11.97	
43.81	43.80	4.91	
38.70	38.68	12.44	
38.19	38.24	13.07	
31.52	31.46	58.02	α-Pinene and β-bisabolene
27.14	27.12	19.57	α-Humulene
25.71	25.71	40.86	*E*-α-Farnesene
23.77	23.78	19.35	T-Cadinol, T-muurolol and *E*-α-bergamotene
23.54	23.53	15.99	β-Eudesmol
18.61	18.60	12.71	
17.58	17.58	9.52	*E*-α-bergamotene

This new hypothesis was then subjected to confirmation: in a first step, mass spectrum was re-examined leading an alternative, but consistent, identification proposal of *E*-β-bergamotene. Spectra of *E*-β-bergamotene, Figure 12, is fortuitously similar to that of β-selinene.

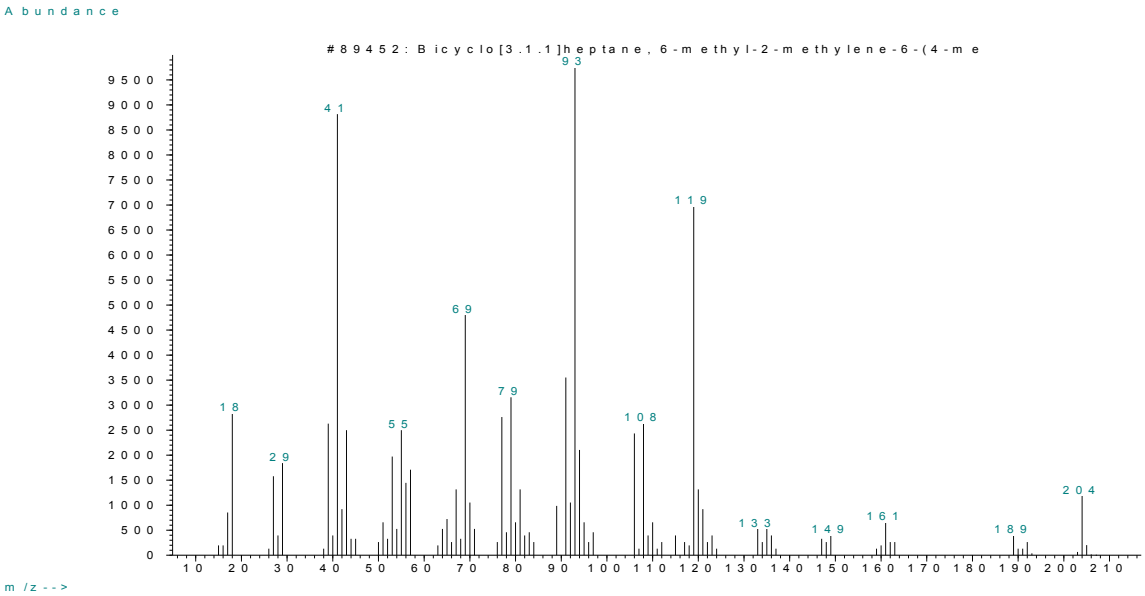


Figure 12 - Reference mass spectrum 70eV of *E*-β-bergamotene. In Wiley/Nist database.

Afterwards, retention indices were also re-examined. In view that the laboratory database did not include reference data of *E*-β-bergamotene other databases were enquired. Retention on SPB-1 (RI_{SPB-1}=1472) is in accordance with reference data (RI_{DB5}=1480) from Joulain & Koenig (1998) and from the *Equipe Chimie et Biomasse*. Retention on SupelcoWax-10 column (RI_{Swax10} =1675) agrees with reference data from the *Equipe Chimie et Biomasse* (RI_{DBwax} =1682).

5.2 Relevance of the composition of the *E. dilatatum* essential oil

This composition is different from any other of the *Eryngium* oils. The occurrence of *Z*-chrysanthenyl acetate at high concentration is not frequent, making this essential oil a valuable natural source for this compound.

Essential oils with *Z*-chrysanthenyl acetate were associated to relevant biological activities: antioxidant, angiotensin I converting enzyme inhibition, antimicrobial (Zouari

et al. 2010), anti-inflammatory (Cabral *et al.* 2012) and antifungal activity against dermatophyte strains (Cabral *et al.* 2013).

The second component in high percentage, α-pinene, is common in essential oil composition to several plants. Essentially, it was associated to stronger antimicrobial and antifungal activities (Rivas da Silva *et al.* 2012); anti-tumoral activity (Leite et al. 2007) and anti-inflammatory activity (Martin *et al.* 1993; Neves *et al.* 2010).

In this essential oil other relevant compounds are associated to interesting biological activities: are the cases of germacrene D, bicyclogermacrene, spathulenol and α-cadinol.

It has been proposed that germacrene D plays a pivotal role as a precursor of various sesquiterpenes such as cadinenes and selinenes (Davis and Croteau 2000). Such metabolic relationships are evident when looking to the composition of *E. dilatatum* oil. Furthermore, it also suggested that germacrene D may have antibacterial activity (Laouer *et al.* 2009; Montanari *et al.* 2011; Simões *et al.* 2008), cytotoxic activity against human breast tumor cells (Palazzo *et al.* 2009), insecticidal activity (Noge and Becerra 2009) and antioxidant activity (Laouer *et al.* 2009).

Bicyclogermacrene is a well known sesquiterpene to which are assigned several biological proprieties: antiprotozoal activity against promastigotes (Siqueira *et al*. 2011), antibacterial activities (Costa *et al.* 2011; de Abreu Gonzaga et al. 2003; Montanari *et al.* 2011), larvicidal activity (Costa *et al*. 2011) and gastric antiulcer and anti-inflammatory (Esteves *et al*. 2005).

Spathulenol is an oxygenated sesquiterpene present in numerous natural sources. Only a few studies deal about its biological activity. Martins *et al.* (2010) suggests that spathulenol is a good candidate to be used in combination chemotherapy of multidrug resistance cancer (A. Martins *et al.* 2010). (Cantrell et al. 2005) and (Ziaei et al. 2011) reported, respectively the repellent and the immunoinhibitory activities of spathulenol.

Other oxygenated sesquiterpene, α-cadinol, is associated to antimycobacterial activity against drug resistant-tuberculosis and nontuberculous mycobacteria strains (Bueno *et al.* 2011), as well as, antimite activity (Chang *et al.* 2001) and antifungal activity (Ho *et al.* 2011).

6. Chapter 5. *Composition of the essential oil of Eryngium pandanifolium from Portugal*

Contents of this chapter was presented and discussed in scientific events in the form of communications:

- Combination of capillary GC, GC/MS and 13C-NMR for the analysis of the volatile extracts of *E. dilatatum* and *E. pandanif*olium. Conference CEF2012. Coimbra, 25 September 2012. Oral Communication.
- Composition of the volatile oils of *E. dilatatum* Lam. And *E. pandanifolium* Cham. & Schltdl. 43rd International Symposium on Essential Oils (ISEO2012), Lisbon, 5-8 September 2012, Abstract Book, p. 155. Poster.

Till date, a single study was published regarding the *E. pandanifolium* Cham. et Schlecht essential oil (Brophy *et al.* 2003). This study led to the identification of the components of the essential oil from aerial parts (leaf and fruits) of Australian *E. pandanifolium* by means of GC and GC-MS analysis. The leaf oil of the Australian *E. pandanifolium* contained bornyl acetate (20.8%), β-selinene (13.8%), α-selinene (11.3%) and α-muurolene (8.0%) as the main compounds, while the fruit oil was characterized by heptanol (11.5%) and β-selinene (9.2%).

We report here on the composition of essential oil isolated by hydrodistillation from the aerial parts of *E. pandanifolium* growing wild in Portugal.

The aerial parts of the plant were distilled in a Clevenger apparatus yielding a pale yellow liquid [0.10% (v/m), fresh weight] with a peculiar odour. The composition was then investigated using chromatographic retention data (GC), mass spectra acquired by GC-MS and ^{13}C-NMR without prior isolation of components. The latter technique received the contribution of the research group *UMR* CNRS 6134, Equipe *Chimie et Biomasse, Université de Corse*.

The composition of essential oil of *E. pandanifolium* from Portugal is summarised in Table 8 where compounds are arranged in order of their elution on the SPB1 column.

In sum, thirty-five compounds were identified, representing 86.7% of the total. As demonstrated in the table 8 the essential oil is constituted predominantly by monoterpenes hydrocarbons (41.8%) and sesquiterpene hydrocarbons (29.7%). The oil has α-pinene (20.8%), β-elemene (10.6%), limonene (5.8%), myrcene (4.6%) and germacrene A (4.7%). as major constituents. Remaining compounds are present at concentrations under 3%.

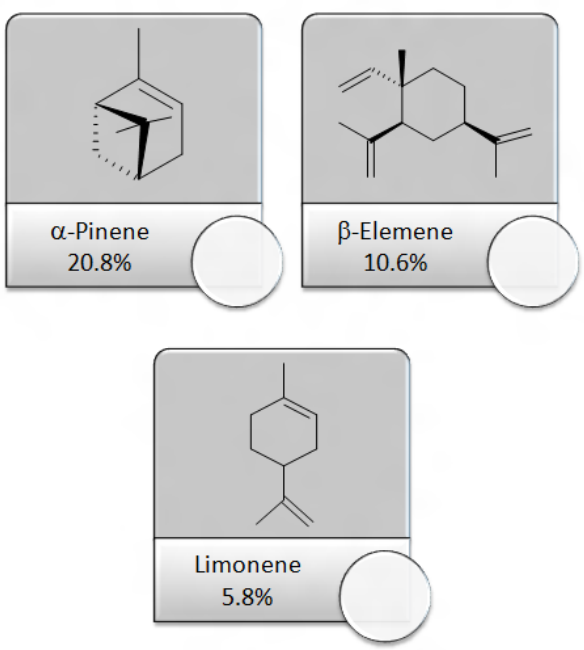


Figure 13 - Chemical structures of main monoterpenes hydrocarbons (α-pinene and limonene) and sesquiterpene hydrocarbons (β-elemene) present in *E. pandanifolium* oil.

The core terpinene is present in various compounds which constitute the essential oil, as can be seen in the table 8. α-Terpinene, γ-terpinene, terpinolene, terpine-4-ol, α-thujene and sabinene derive from the same metabolic pathway, as show by figure 14.

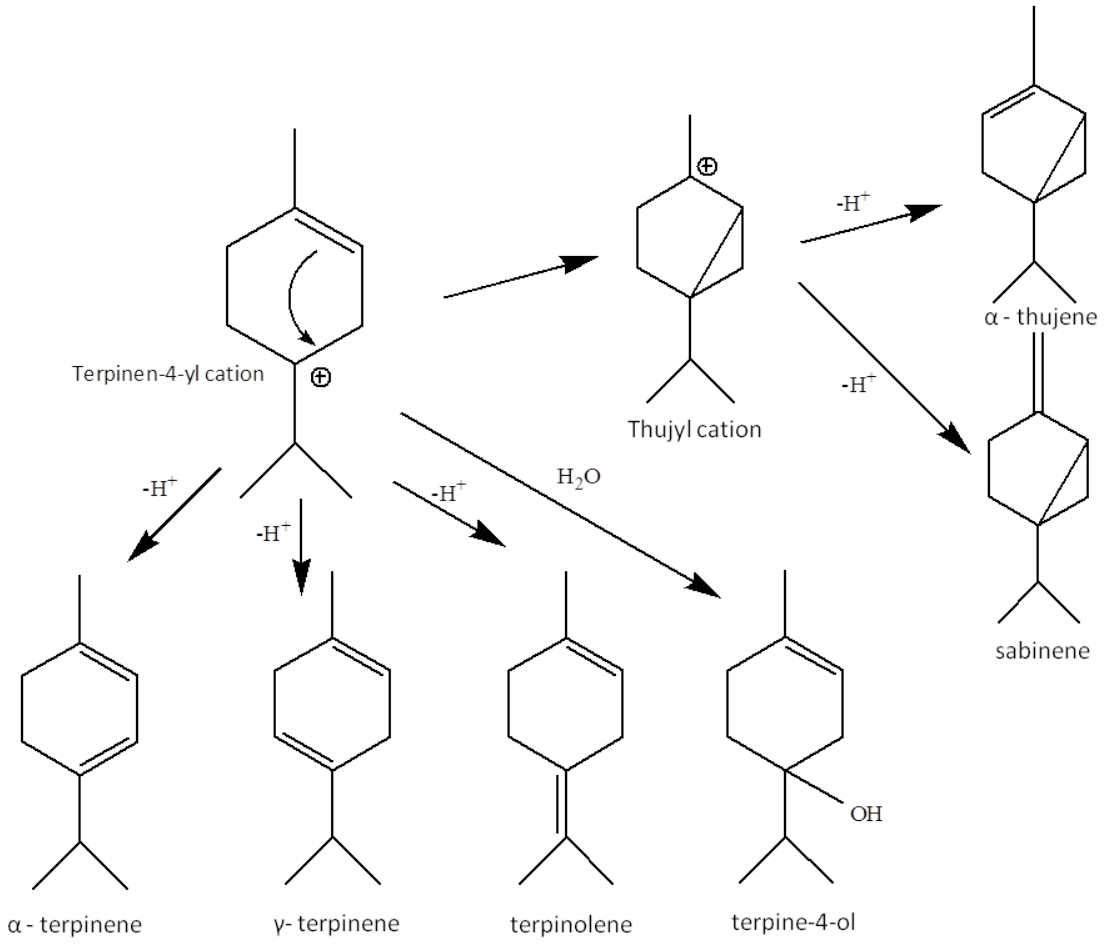


Figure 14 - Synthetic pathway of terpinenes proposed by (Dewick 2002b).

The presence and abundance of β-elemene may be related with germacrene A. In fact, at high temperatures, the Cope rearrangement of germacrene A can lead β-elemene.

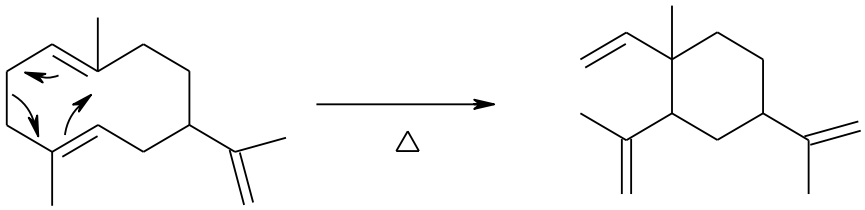


Figure 15 - Cope rearrangement of germacrene A to β-elemene.

This can occur at any stage of the analysis involving thermal conditions, such as the sample vaporization in the GC inlet (Adewale 2009). Thus, the relative amount of germacrene A on essential oil can be greater than the measured. Conversely, the relative amount of β-elemene can be lower than the measured. These artefacts are well known by those who work with essential oils, however analytical results rarely are unambiguous in what concerns to the real content of β-elemene and germacrene A in the oils or in biological raw materials. Differently our results allowed us to conclude that, in the *E. pandanifolium* essential oil, β-elemene is not a thermal artefact. It was identified, as a major constituent, by ^{13}C-NMR analysis (see section 6.1.3.).

The composition of the oil was found to be different from that studied by Brophy *et al.* (2003). Major constituent in the Australian oil is the oxygenated monoterpene, bornyl acetate, whereas the monoterpene hydrocarbon, α-pinene, dominates, at similar concentration, the composition of the Portuguese oil.

Table 8 - Chemical composition of essential oil *Eryngium pandanifolium*.

RI[a]	RI[b]	Compouds	%	*Identification*
922	1028	α-Thujene	2.2	IR; MS; NMR
928	1028	α-Pinene	20.8	IR; MS; NMR
942	1075	Camphene	0.5	IR; MS
963	1125	Sabinene	2.2	IR; MS; NMR
968	1116	β-Pinene	2.7	IR; MS; NMR
977	n.d.	Butylbutyrate	0.7	IR; MS
979	1160	Myrcene	4.6	IR; MS; NMR
979	1290	Octanal	1.6	IR; MS
1009	1184	α-Terpinene	0.2	IR; MS
1012	1272	p-Cymene	0.9	IR; MS; NMR
1019	1204	Limonene	5.8	IR; MS; NMR
1035	1249	E-β-Ocimene	0.5	IR; MS; NMR
1041	1263	3-Methybutylbuturate	0.4	IR; MS
1046	1246	γ-Terpinene	1.2	IR; MS; NMR
1074	n.d.	Terpinolene	0.2	IR; MS
1077	1392	Nonanal	0.4	IR; MS
1085	1541	Linalool	0.6	IR; MS; NMR
1160	1594	Terpinen-4-ol	0.8	IR; MS; NMR
1175	1412	Hexylbutirate	2.1	IR; MS; NMR
1185	1495	Decanal	2.0	IR; MS; NMR
1234	1639	*E*-dec-2-en-al	0.7	NMR; IR
1381	1585	β-Elemene	10.6	IR; MS; NMR
1407	1592	E-Caryophyllene	2.3	IR; MS; NMR
1428	n.d.	α-Guaiene	0.9	IR; MS
1440	1661	α-Humulene	0.9	IR; MS; NMR
1466	1700	Germacrene D	1.9	IR; MS; NMR
1471	1714	β-Selinene	2.5	IR; MS; NMR
1473	1671	4,5-diepi-aristolochene	0.6	NMR; IR
1481	1705	α-Selinene	3.1	IR; MS; NMR
1486	1724	α-Muurolene	0.4	IR; MS
1489	1753	Germacrene A	4.7	IR; MS; NMR
1491	1709	δ-Guaiene	1.1	IR; MS; NMR
1501	n.d.	γ-Cadinene	0.3	IR; MS
1507	n.d	δ-Cadinene	0.4	IR; MS
1553	2109	Spathulenol	0.2	IR; MS
1558	1970	Caryophyllene oxide	0.5	IR; MS; NMR
1617	2173	T-Muurolol	0.6	IR; MS; NMR
1625	2239	Selina-11-en-4-a-ol	4.0	NMR; IR
1644	2219	α-Eudesmol	0.6	NMR; IR
		Monoterpene hydrocarbons	41,8	
		Oxygen containing monoterpenes	1,4	
		Sesquiterpene hydrocarbons	29,7	
		Oxygen containing sesquiterpenes	5,9	
		Other compounds	7,9	
		Total identified	86,7	

Compounds listed in order to their elution on the SPB-1 column; t: traces; n.d.: not detected; [a] Retention indices on the SPB-1 column relative to C8-C24 *n*-alkanes; [b] Retention indices on the SipelcoWax-10 column relative to C8-C24 *n*-alkanes

6.1 Contribution of the ^{13}C-NMR technique without prior isolation

^{13}C-NMR analysis on the total oil allowed us to confirm the most components and, additionally, allowed us to identify four other compounds: selina-11-en-4-α-ol (4%), *E*-dec-2-en-al (0.7%), α-eudesmol (0.6%) and 4,5-diepi-aristolochene (0.6%). This methodology contributes so with an addition at 5.9% in whole identification essential oil.

6.1.1 Identification of selina-11-en-4-α-ol, *E*-dec-2-en-al, α-eudesmol

For each of them, the values of chemical shifts observed in ^{13}C-NMR spectrum of whole oil correspond perfectly to chemical shifts described in the database of the Equipe *Chimie et Biomasse*. (Table 9, 10, 11)

Table 9 - Chemical structure and resonance signals of selina-11-en-4-α-ol.

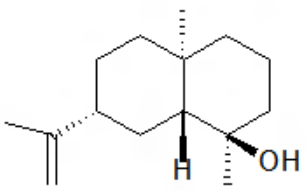
Selina-11-en-4-α-ol	Chemical shift (δ ppm)		Relative abundance	Overlap
	Reference	Experimental		
	150.66	150.98	5.01	
	108.22	108.22	5.42	
	72.29	72.27	3.71	
	54.84	54.90	8.19	
	46.38	46.37	8.29	
	44.72	44.70	8.49	
	43.35	43.38	8.47	
	41.14	41.11	27.37	n.d.
	34.64	34.62	5.99	Terpinen-4-ol
	26.84	26.82	19.93	α- Selinene
(RI_{SPB-1}=1625; RI_{Swax10} =2239)	26.04	26.06	9.65	
	22.7	22.72	13.82	n.d.
	21.14	21.17	12.05	α- Selinene
	20.17	20.16	10.51	
	18.73	18.72	8.67	α-Guaiene

Table 10 - Chemical structure and resonance signals of *E*-dec-2-en-al.

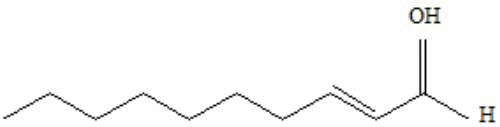
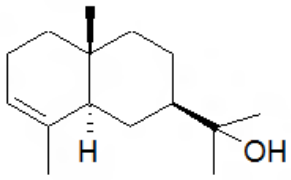
E-dec-2-en-al	Chemical shift (δ ppm)		Relative abundance	Overlap
	Reference	Experimental		
	194.21	194.14	2.67	
	159.12	159.03	2.17	
	132.99	133.01	2.97	
	32.76	32.76	6.41	Germacrene D; δ-guaiene
	31.73	31.76	6.77	δ-guaiene
	29.12	29.15	11.32	
	29.04	29.05	11.79	Sabinene
(RI_{SPB-1}=1234; RI_{Swax10} =1639)	27.87	27.88	4.50	Linalool
	22.63	22.61	11.53	
	14.08	14.09	10.94	

Table 11 - Chemical structure and resonance signals of α-eudesmol.

α-Eudesmol	Chemical shift (δ ppm)		Relative abundance	Overlap
	Reference	Experimental		
	135.18	135.12	3.63	α-Selinene; p-cymene
	121.01	120.97	8.55	α-Thujene
	72.85	n.d	--	
	50.03	50.01	2.55	
	46.69	46.68	5.05	α-Guaiene
	40.22	40.27	11.84	α-Selinene
	37.90	37.86	1.65	
	32.21	32.25	1.94	4,5 – diepi - aristolochene
	27.35	27.34	2.72	*E*-β-Ocimene
(RI_{SPB-1}=1644; RI_{Swax10}=2219)	26.80	26.82	19.93	α-Selinene
	24.39	24.34	3.26	
	22.99	23.00	100.0	α-Pinene; α-selinene; γ-terpinene
	22.56	22.56	12.37	n.d.
	21.18	21.17	12.05	α-Selinene; selina-11-en-4a-ol
	15.58	15.64	10.81	α-Selinene

Such as, the retention indices (apolar and polar) of the compounds were assigned by comparison with the Equipe *Chimie et Biomasse* database:

- Selina-11-en-4-α-ol retention on SPB-1, RI_{SPB-1}=1625, is in accordance with reference data (RI_{DB1}=1628 and RI_{DB5}=1642) from *Equipe Chimie et Biomasse* database. Retention on SupelcoWax-10 column, RI_{Swax10} =2239, agrees with reference data from the *Equipe Chimie et Biomasse* (RI_{DBwax} =2240);
- Regarding to dec-2-en-al, the index retention on SPB-1, RI_{SPB-1}=1234, is in agreement with reference data (RI_{DB5}=1239) from *Equipe Chimie et Biomasse* database. The index retention on SupelcoWax-10 column, RI_{Swax10} =1639, is consistent with reference data from the *Equipe Chimie et Biomasse* (RI_{DBwax}=1643);
- Likewise, α-eudesmol apolar retention, RI_{SPB-1}=1644; and polar retention, RI_{Swax10}=2219, is in accordance with reference data from *Equipe Chimie et Biomasse* database, (RI_{DB5}=1649) and (RI_{DBwax} =2224), respectively.

6.1.2 **Identification of 4,5-diepi-aristolochene**

Mass spectra acquired on a peak on SPB-1 column, with relative area of 0.6% and retention indice, RI_{SPB-1}=1473, suggested, when matched with reference data (Wiley/Nist database), the identity of the sesquiterpene hydrocarbon, eremophilene (m/z 204). Reference retention indice for eremophilene at a SPB-1 column was also in accordance (RI_{SPB-1} =1474).

Nevertheless, in the ^{13}C-NMR spectra of the whole oil, the resonance signals of the eremophilene carbons were absent, contradicting its existence. As an alternative, the resonance signals propose an isomer of eremophilene, 4,5-diepi-aristolochene (m/z 204), that presents an equally mass spectrum and fortuitously similar retention index, Table 12.

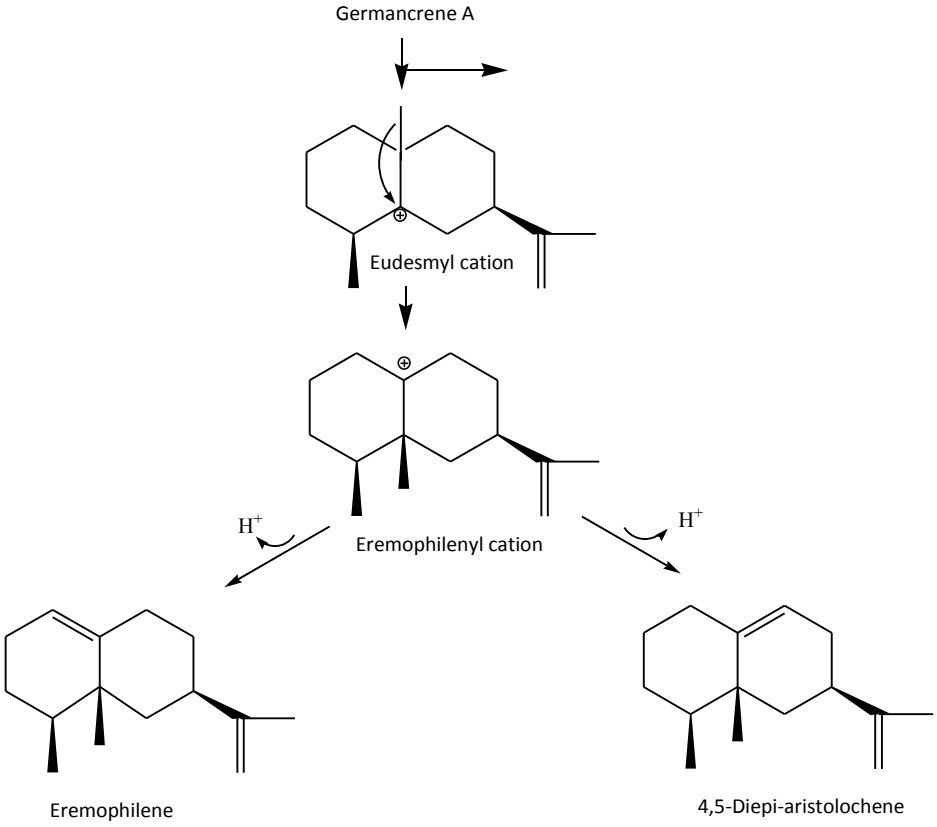


Figure 16 - Proposed reaction mechanisms for eremophilene and 4,5-diepi-aristolochene. Adapted of the mechanism proposed by (Greenhagen *et al.* 2006; O'Maille *et al.* 2006).

Afterwards, retention indices were also re-examined. In view that the laboratory database did not include reference data of 4,5-diepi-aristolochene other databases were enquired.

4,5-Diepi-aristolochene retention on SPB-1 (RI_{SPB-1}=1473) is in accordance with reference data (RI_{DB5}=1470) described in Joulain & Koenig (1998) and *Equipe Chimie et Biomasse* database. Retention on SupelcoWax-10 column (RI_{Swax10}=1671) agrees with reference data from the *Equipe Chimie et Biomasse* (RI_{DBwax} =1672).

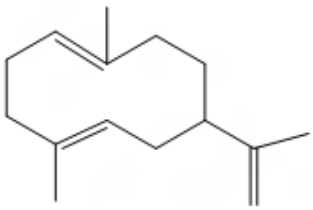
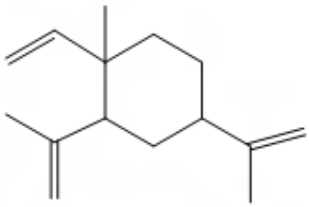
Table 12 - Chemical structure and resonance signals of 4,5-diepi-aristolochene.

4,5-diepi-Aristolochene	Chemical shift (δ ppm)		Relative abundance	Overlap
	Reference	Experimental		
	150.27	150.24	7.75	β-Elemene
	146.35	n.d.	--	
	117.98	117.94	1.94	
	108.31	108.39	35.09	Limonene
	39.72	39.76	4.22	α-Humulene
	39.50	39.51	2.44	
	38.75	38.76	3.52	
	37.75	37.74	2.28	
	32.32	32.25	1.94	α-Eudesmol
(RI_{SPB-1}=1473; RI_{Swax10}=1671)	31.62	31.65	12.21	n.d.
	31.52	31.57	5.20	
	29.38	29.39	18.31	Decanal
	21.05	21.01	6.44	
	20.80	20.75	6.97	Germacrene D
	15.86	15.78	2.51	

6.1.3 **Confirmation of the presence of β-elemene**

This methodology allowed us yet confirm that the presence of β-elemene as a natural constituent of the oil and its prevalence regarding germacrene A. Acquisition of NMR spectrum is carried on at low temperature, conditions unsuited for the Cope rearrangement. The intensity of the ^{13}C-NMR signals of carbons of β-elemene, greater than those of germacrene A, testifies the natural prevalence of the first over the last, as determined by GC analysis (Table 13).

Table 13 - Chemical structures of germacrene A and β-elemene. Experimental resonance signals and relative abundance in ^{13}C-NMR spectra of whole oil.

			
Germacrene A		**β-Elemene**	
Chemical shift (δ ppm) Experimental	**Relative abundance**	**Chemical shift (δ ppm) Experimental**	**Relative abundance**
153.70	1.57	150.31	17.91
138.14	1.87	150.24	7.75
131.71	5.11	147.70	8.61
128.99	8.94	112.10	25.16
126.42	5.14	109.85	22.59
107.32	7.53	108.27	29.55
51.43	5.05	52.76	22.33
41.74	6.33	45.73	23.79
39.57	7.19	39.92	27.27
34.87	8.13	39.81	9.83
33.68	7.80	32.91	26.68
26.54	7.67	26.75	26.05
20.30	5.81	24.80	23.11
16.71	5.62	21.08	24.78
16.22	3.79	16.62	27.33

6.2 Relevance of the composition of *E. pandanifolium* essential oil

α-Pinene is the major component of the *E. pandanifolium* essential oil. α-Pinene is a monoterpene hydrocarbon found in high concentrations the volatile oils of many species, especially in pine tree oil. However, it is naturally present in over 400 other natural oils in small quantities. As previously described it is associated to antimicrobial, anti-tumoral and anti-inflammatory activity (Leite *et al*. 2007; Martin *et al.* 1993; Neves *et al.* 2010; Rivas da Silva *et al.* 2012).

The second compound present at high percentage in essential oil, β-elemene, is a promissory drug. β-Elemene is described as the new plant-derived anticancer agent

with low toxicity in a few human cellular lines: lung tumor cells (Li *et al.* 2009; G. Wang *et al.* 2005; Zhang *et al.* 2011) and prostate cancer and other types of solid tumor cells (Li *et al.* 2010). However, the mechanism of action of β-elemene as an anticancer drug remains unknown. Recently, many efforts have been made and, is proposed its mechanism of action by mitochondria-mediated intrinsic apoptosis pathway (Li *et al.* 2009). Notwithstanding to the non-elucidation of the mechanism of action, arises patented their synthesis with the intention of co-adjuvant therapeutic in different cancers (Huang 2006) even as biodistribution studies to determine its pharmacokinetic (Sun *et al.* 2009).

Limonene, monocyclic monoterpene, is associated to a wide spectrum of biologic activities including chemotherapeutic and chemopreventive effects in animal models (Crowell *et al.* 1992; Crowell and Gould 1994; Miller *et al.* 2011). Oral limonene administration in humans is well tolerated even at high doses supporting its research as a potential bioactive compound for cancer prevention (Vigushin *et al.* 1998). Although the exact mechanisms of action of limonene are unclear, immune modulation and anti-proliferative effects are commonly reported.

7. Chapter 6. *Composition and variability of the essential oil of Eryngium campestre*

Contents of this chapter was presented and discussed in scientific events in the form of communications:

- Composition and variability of the volatile oils of *Eryngium campestre. L*. Conference CEF2012. Coimbra, 25 September 2012. Poster.

Essential oils of *E. campestre* growing wild in Castilla, Spain, were studied by Pála-Paul *et al.* (2008). These authors found chemical variability in the oils, mainly expressed in clear quantitative differences for the major compounds: germacrene D (from 30.3% to 40.3%), β-curcumene (from 0.7% to 22.2%), myrcene (from 3.0 to 21.7%) and *E*-β-farnesene (from 0.1 to 19.0%). In such composition sesquiterpenes predominate over monoterpenes (Pala-Paul *et al.* 2008a).

The composition and variability of three representative samples of *E. campestre* from three populations growing wild at Alentejo (Al), Beira Litoral (BL) and Beira Alta (BA) are reported.

These volatile oils were isolated by hydrodistillation from the aerial parts of *E. campestre,* yielding 0.10 % [(v/m) fresh weight] of pale yellow oil.

The composition was investigated using chromatographic retention data (GC), mass spectra acquired by GC-MS. Two of three samples were also studied by ^{13}C-NMR without prior isolation of components.

The compositions of three Portuguese populations of *E. campestre* are summarised in Table 14, where compounds are listed in order of their elution from the SPB1 column.

Generally, the oils are predominantly composed by sesquiterpene hydrocarbons, 75.1%$_{Al}$, 67.5%$_{BL}$ and 51.4%$_{BA}$; followed by the oxygen containing sesquiterpenes, 12.2%$_{Al}$, 10.2%$_{BL}$ and 28%$_{BA}$; monoterpene hydrocarbons, 10.2%$_{Al}$, 9.5%$_{BL}$ and 1.3%$_{BA}$; and oxygen containing monoterpenes 0.6%$_{Al}$, 2.3%$_{BL}$ and 0.3%$_{BA}$.

Although the comparable amounts of each group of components, important compositional differences were registered: *E*-β-farnesene, the major component of the oil of sample Al (31.3%), attains only 1.9% in the oil of sample BL and 6.9% in the oil of sample BA. Similarly, β-elemene the major component of the oil of sample BL (19.7%) is, in sample Al and BA, at the concentration of 0.6%. In oil sample BA, the main compound is β-bisabolene, at concentration of 15.6% very similar to sample Al (17.8%), but only 4.8% in other sample (BL).

Together with other worthy differences among three samples (Al, BL and BA): myrcene (9.1% vs. 8.6% vs. 1.3%), germacrene D (6.8% vs. 18.4% vs. 12.3%), germacrene A (5.0% vs. 9.2% vs. not detected), β-sesquiphellandrene and δ-guaiene are present only in sample Al and BA (respectively) – sustain the evidence of chemical variability affecting the volatile composition of *E. campestre,* table 14.

Thereby, Portuguese wild *E. campestre* expresses three chemical phenotypes concerning volatile metabolites (Figure 17):

- Phenotype Al is characterized by the prevalence of *E*-β-farnesene (31.3%) and β-bisabolene (17.8%);

- Phenotype BL is characterized by germacrene skeleton derivatives, germacrene D (18.4%), germacrene A (9.2%) and β-elemene (19.7%) (possible thermal artefact);

- Phenotype BA is characterized predominantly by of β-bisabolene (15.6%), germacrene D (12.3%) and α-cadinol (8.2%).

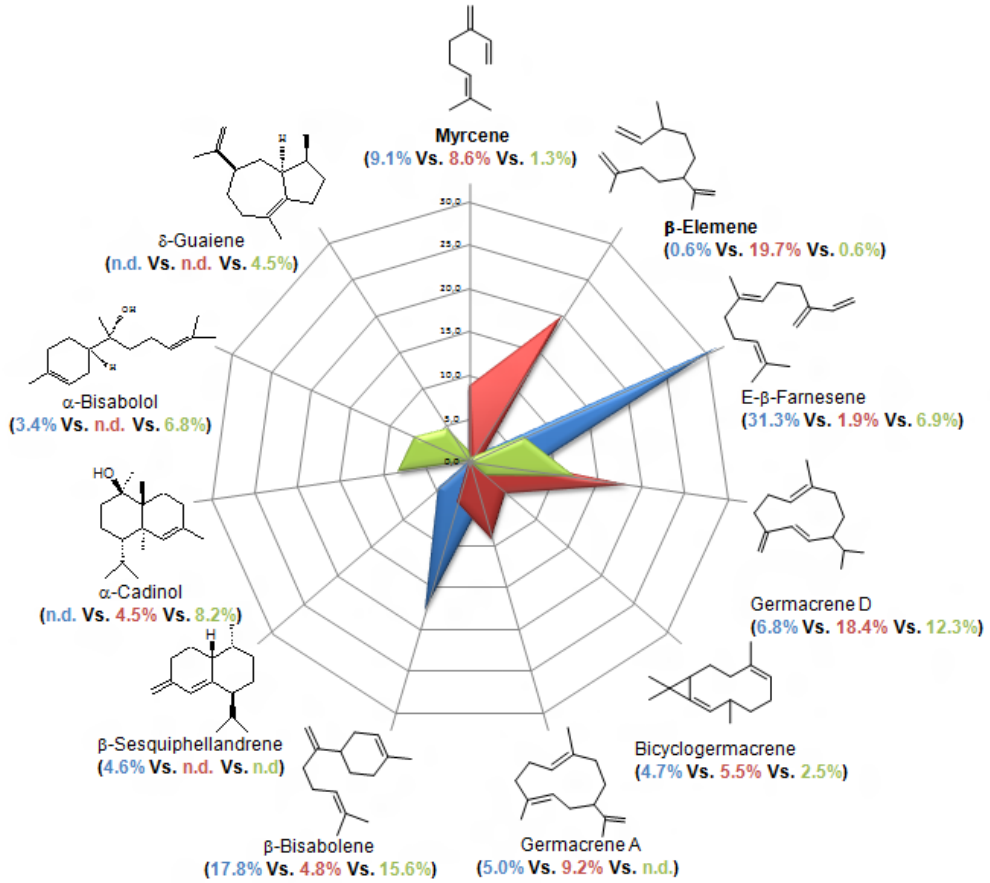


Figure 17 - Discriminative scatter plot of the compositions of *E. campestre* volatile isolates, regarding major components.

Major metabolites are driven from the three alternative pathways initiated by cyclization arrangements (or not) of a common substrate, figure 18.

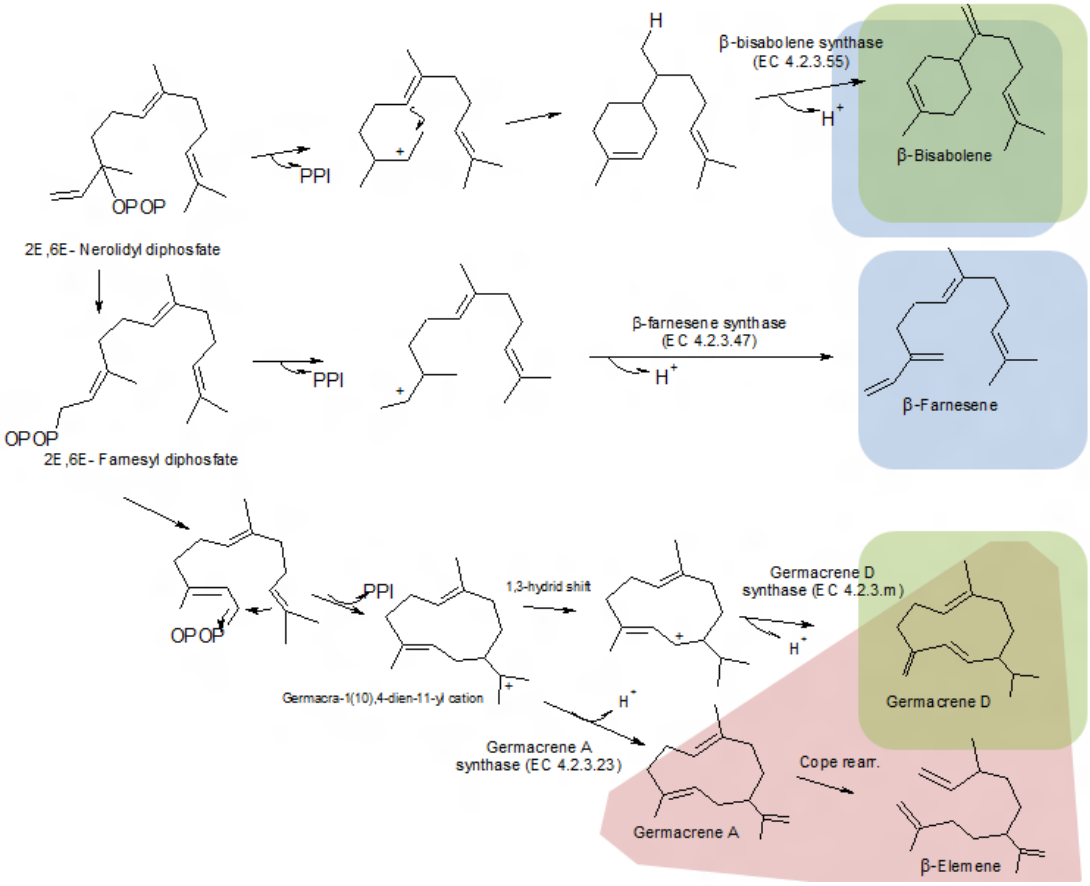


Figure 18 - Biosynthetic pathways of the major volatile metabolites of the three *E. campestre* chemical phenotypes.

Table 14 - Detailed compositions of the volatile isolates from the aerial parts of *E. campestre* (populations Al, BL and BA).

RI[a]	RI[b]	Compound	Al	BL	BA
			%	%	%
930	1029	α-Pinene	0.3	0.9	t
969	1116	β-Pinene	0.5	t	t
980	1160	Myrcene	9.1	8.6	1.3
1020	1204	Limonene	0.3	t	n.d.
1085	1539	Linalool	0.6	0.5	0.3
1361	1763	Geranyl acetate	n.d.	1.7	n.d
1368	1489	α-Copaene	n.d.	n.d.	0.4
1380	1584	β-Elemene	0.6	19.7	0.6
1406	1592	*E*-Caryophyllene	n.d.	1.3	1.0
1439	1667	α-Humulene	n.d.	0.4	t
1445	1638	*allo*-Aromadendrene	n.d.	2.3	n.d.
1445	1661	*E*-β-Farnesene	31.3	1.9	6.9
1465	1701	Germacrene D	6.8	18.4	12.3
1480	1726	Bicyclogermacrene	4.7	5.5	2.5
1484	1726	α-Muurolene	n.d.	n.d.	1.5
1488	1755	Germacrene A	5.0	9.2	n.d.
1490	1706	δ-Guaiene	n.d.	n.d.	4.5
1495	1719	β-Bisabolene	17.8	4.8	15.6
1506	1749	δ-Cadinene	2.1	2.8	4.8
1507	1762	β-Sesquiphellandrene	4.6	n.d.	n.d.
1527	1766	*E*-α- Bisabolene	1.1	n.d.	0.5
1539	1820	Germacrene B	1.1	1.2	0.5
1551	2113	Spathulenol	2.8	1.9	2.4
1580	n.d.	Globulol	T	1.5	n.d.
1579	2017	Ledol	0.4	n.d.	n.d.
1552	2040	Germacrene-1(19),5-diene-4-ol	n.d.	n.d.	2.8
1563	n.d.	Salvial-4(14)-en-1-one	n.d.	n.d.	1.7
1609	2216	Isospathulenol	0.7	n.d.	0.3
1615	n.d.	T-Cadinol	1.5	2.3	4.5
1627	2176	T-Muurolol	3.3	n.d.	1.3
1627	2222	α-Cadinol	n.d.	4.5	8.2
1661	2210	α-Bisabolol	3.4	n.d.	6.8
		Monoterpene hydrocarbons	10.2	9.5	1.3
		Oxygen containing monoterpenes	0.6	2.2	0.3
		Sesquiterpene hydrocarbons	75.1	67.5	51.4
		Oxygen containing sesquiterpenes	12.1	10.2	28
		Total identified	98.0	89.4	81

Compounds listed in order to their elution on the SPB-1 column; t: traces; n.d.: not detected; [a] Retention indices on the SPB-1 column relative to C8-C24 *n*-alkanes; [b] Retention indices on the SipelcoWax-10 column relative to C8-C24 *n*-alkanes

The results bring intricacy to the understanding of the chemical variability of *E. campestre*:

- β-Bisabolene a major metabolite of two of the Portuguese phenotypes was not detected in any Spanish *E. campestre* population. Instead, Spanish *E. campestre* growing in alkaline soils synthesise β-curcumene, structurally and biochemically related to β-bisabolene;
- Biosynthesis pathways of β-curcumene and β-bisabolene differ only in the last catalysis step, demanding specific synthases (EC 4.2.3.94 and EC 4.2.3.55) which are highly dependent of Mg^{2+}. Metabolic drifting towards β-curcumene or β-bisabolene can not merely be justified by differences of edaphic conditions, the reason previously pointed to explain the variability of the volatile metabolites of *E. campestre*.

7.1 ^{13}C-NMR contribution to the identification of minor compounds, without prior isolation

In this case, the contribution of the ^{13}C-NMR methodology, only allowed us confirming the majority compounds in essential oils of two *E. campestre* species (Al and BL), not contributing to any additional identification.

7.2 Relevance of the composition of *E. campestre* essential oils

The variability of the volatile oils of *E. campestre* was already reported by Pála-Paul *et al.* (2008) that hypothesised on a possible influence of the soil Ca^{2+} availability on the composition of the volatile oils of *E. campestre* (Pala-Paul *et al*. 2008a). The compositions here reported are different from those reported Pála-Paul *et al.* and any other *Eryngium* oil, with exclusive compounds prevailing in the composition.

The chemical composition of the *E. campestre* oils also offer compounds with interesting biological activity.

E-β-Farnesene, a sesquiterpene, has a stronger insecticidal activity because it is the main component of the alarm pheromone of many aphid species (Kunert *et al*. 2010). However, the use of *E*-β-Farnesene is difficult due its relatively high volatility, susceptibility to oxidation and complex and expensive synthesis. In order to overcome these inconveniences have been to develop (design and synthesis) new analogues of *E*-β-Farnesene (Sun *et al*. 2011a; Sun *et al.* 2011b).

Despite their high occurrence in essential oils, β-bisabolene has not been choice for studies of biological activity. This makes the compound interest for future research on biological activity.

8. Chapter 7. *Contribution for the identification of minor constituents of the essential oil of Eryngium juresianum*

Cavaleiro *et al.* (2011) reported for the first time the composition of the essential oil of *E. duriaei subsp. Juresianum* (Cavaleiro *et al.* 2011). These authors identified twenty-five components accounting 84.6% of the total composition. However, they signalize some minor constituents that could not identify. In order to investigate the remaining unidentified constituents we undertook new analysis of the essential oil of *E. duriaei* subsp. *juresianum*. The composition previously reported was confirmed and complimentary studies by GC, GC-MS and ^{13}C-NMR were made on fractions.

8.1 Composition of the essential oil of *E. duriaei subsp. Juresianum*

The new analysis of the oil of *E duriaei* subsp. *juresianum* confirmed a composition dominated by sesquiterpenes, particularly caryophyllane derived compounds: α-neocallitropsene (31.9% vs. 26%), *E*-β-caryophyllene (20.2% vs. 6.3%); isocaryophyllen-14-al (8.0% vs. 16.2%); 14-hydroxy-β-caryophyllene (6.9% vs. 13.4%); caryophyllene oxide (5.0% vs. 7.6%) and bicyclogermacrene (4.7% vs. 3.8%) are the major constituents, as can be seen in table 15.

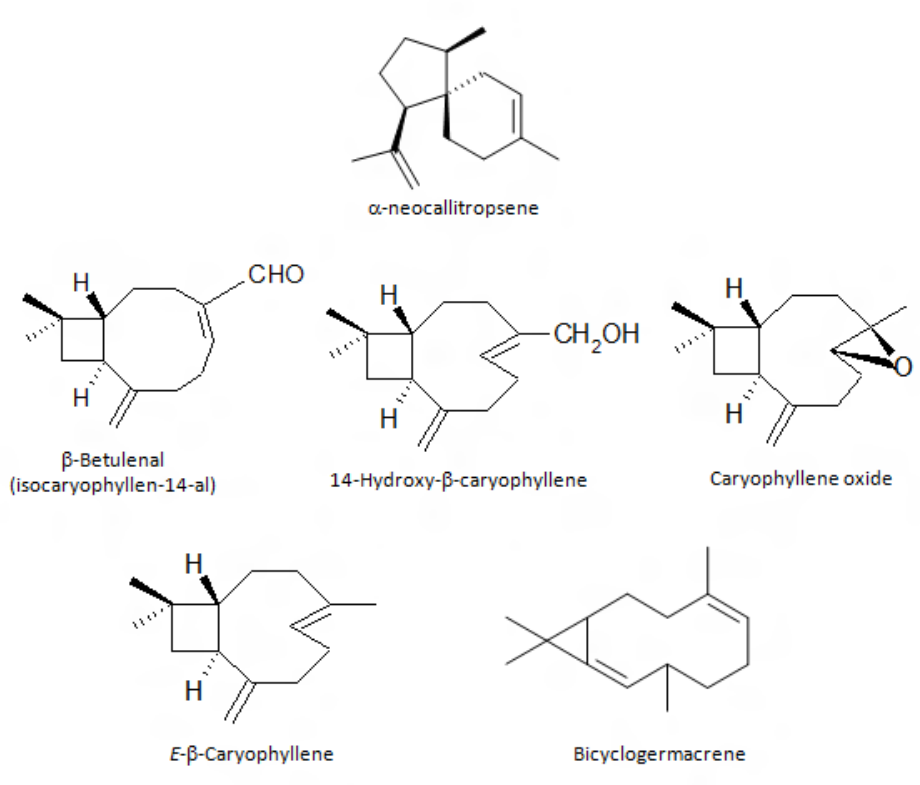


Figure 19 - Major compounds of the volatile oil of *E. duriaei* subsp. *jurasianum*.

Table 15- Chemical composition of essential oil *E. duriaei* subsp. *jurasianum*.

RI [a]	RI [b]	Compounds	% (Cavaleiro et al. 2011)	% Experimental	Notes
929	1030	α-Pinene	0.7	0.3	
978	1290	*n*-Octanal	0.4	0.4	
978	1161	Myrcene	T	t	
1019	1206	Limonene	T	0.1	
1024	1237	*Z*-β-Ocimene	0.3	0.2	
1034	1255	*E*-β-Ocimene	0.9	0.5	
1048	n.d.	*n*-Octanol	-	0.1	
1079	1393	*n*-Nonanal	T	t	
1098	n.d.	*n*-Undecane	-	0.1	
1160	n.d.	Terpinen-4-ol	-	t	
1181	1495	*n*-Decanal	0.6	0.3	
1235	n.d	2-Decenal	0.2	0.2	
1327	n.d.	Bicycloelemene	T	0.2	
1373	n.d.	β-Bourbonene	-	0.1	
1380	1585	β-Elemene	0.7	0.4	
1386	n.d.	*n*-Dodecanal	T	0.3	
1408	1595	*E*-β-Caryophyllene	6.3	20.2	

1440	1665	α-Humulene	0.4	0.9	
1444	1665	*E*-β-Farnesene	0.4	0.8	
1461	1685	α-Neocallitropsene	26.0	31.9	
1468	1676	γ-Curcumene	0.8	-	
1471	1711	β-Selinene	3.0	1.8	
1481	1727	Bicyclogermacrene	3.8	4.7	
1495	n.d.	β-Bisabolene	-	0.5	
1499	1749	γ-Cadinene	-	0.2	
1506	1761	β-Sesquiphellandrene	-	0.3	
1515	n.d.	*E*-γ-Bisabolene	-	0.2	
1545	n.d.	Unknown 1	1.5	2.1	
1549	n.d.	Unknown 2	-	3.6	Co-eluded with spathulenol
1551	2112	Spathulenol	1.4	1.3	
1556	1969	Caryophyllene oxide	7.6	5.0	
1560	2063	Globulol	0.5	0.3	
1563	1991	Salvial-4(14)-en-1-one	-	0.2	
1560	n.d.	Viridiflorol	-	0.3	
1586	2025	Humulene epoxide II	1.0	0.4	
1615	2145	Isocaryophyllen-14-al (β-Betulenal)	16.2	8.0	
1621	n.d.	Unknown 2	1.0	-	
1637	2344	14-Hydroxy-β-caryophyllene	13.4	6.9	
1655	n.d.	Unknown 3	1.2	1.1	
		Monoterpene hydrocarbons	1.9	1.1	
		Sesquiterpene hydrocarbons	41.4	62.2	
		Oxygen containing sesquiterpenes	40.1	22.4	
		Other	1.2	1.4	
		Total identified	84.6	87,1	

Compounds listed in order to their elution on the SPB-1 column; t: traces; n.d.: not detected; [a] Retention indices on the SPB-1 column relative to C8-C24 *n*-alkanes; [b] Retention indices on the SipelcoWax-10 column relative to C8-C24 *n*-alkanes

Other compounds, not reported by Cavaleiro *et al.* (2011) with relative amounts ranging between 0.1% and 0.5% were identified: *n*-octanol, *n*-undecane, β-bourbonene, β-bisabolene, γ-cadinene, β-sesquiphellandrene, *E*-γ-bisabolene, globulol, salvial-4(14)-en-1-one and viridiflorol. The contribution of these identifications increases 1.8% the knowledge on the composition of the oil.

However, other constituents with retention indice in SPB-1 column, RI_{SPB-1} = 1545, RI_{SPB-1} = 1549 and RI_{SPB-1} = 1655, remain unknown (Unknown 1-3). Hence, we proposed to acquire complimentary information to contribute for their identification. Sample was submitted to flash chromatography and fractions with unknown compounds were subjected to ^{13}C-NMR analysis.

8.2 Fractionation of *E. juresianum*

Liquid-solid flash chromatography is a commonly used technique for fractionating essential oils in preparative scale. The stationary phases of alumina and especially of silica gel (particle size 0.063 to 0.5 mm) enable effective fractionations.

8.2.1. Fractionation conditions:

A pre-fractionation of 4 g of essential oil was performed using an Omnifit glass column (400 mm x 20 mm) filled with 40g of silica gel 0.2-0.5 mm and two fractions were collected:

- *Fraction F_1* (2.15 g), corresponding to the elution with *n*-pentane, colorless with terebintinic odour;
- *Fraction F_2* (1.83 g), corresponding to the elution with diethyl oxide, yellowish and odour resembling the original oil.

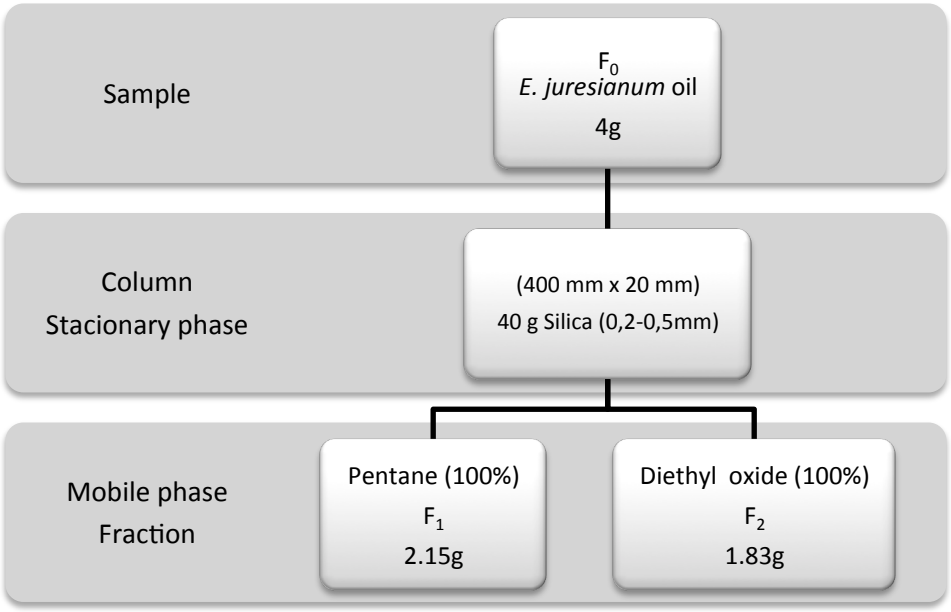


Figure 20 - Experimental conditions of fractionation of *E. juresianum* oil.

After a CG-MS monitoring, unknown compounds were recognised in fraction F_2. Elution with 100% diethyl oxide and mass spectra are consistent with the hypothesis of oxygen containing sesquiterpenes.

A new fractionation starting from fraction F_2 was then performed, using an Omnifit glass column (400 mm x 10 mm) filled with 18g of silica gel 0.2-0.5 mm. Elution was made in step gradient starting with 100% *n*-pentane, then *n*-pentane / diethyl oxide at different proportions (95:5, 90:10, 50:50) and finally 100% diethyl oxide. Sixty fractions of 10 mL were collected using a Gilson automated fraction collector and then monitoring by GC-MS. Mobil phase composition changed each time successive fractions had not solutes.

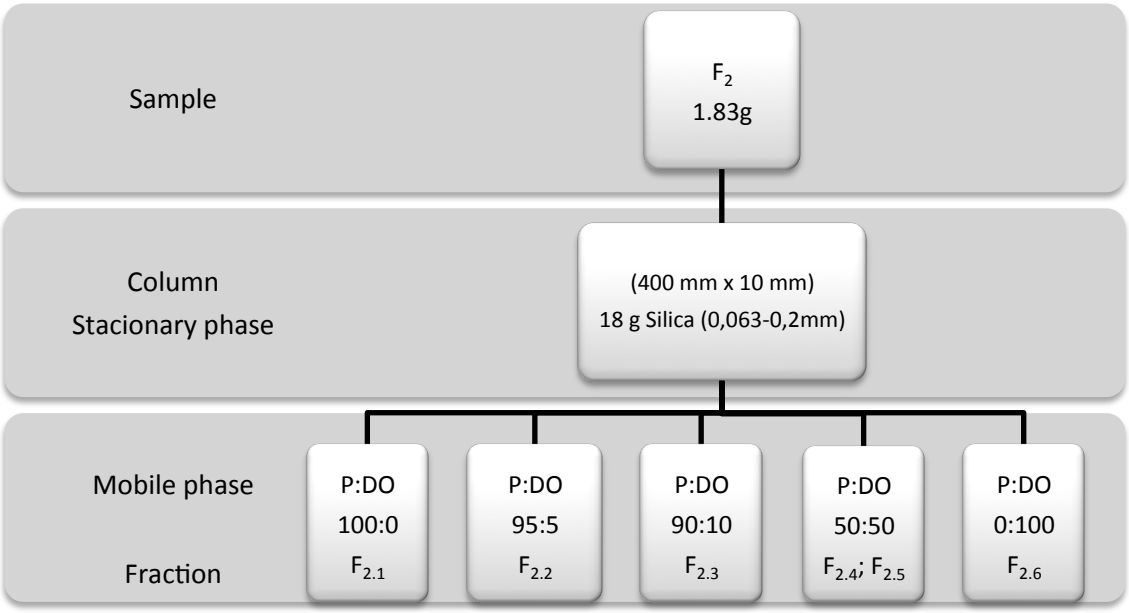


Figure 21 - Experimental conditions of fractionation of fraction F2.
P – *n*-Pentane; **DO –** Diethyl oxide.

After the final elution with diethyl oxide, fractions with similar composition were gathered, rending seven samples (2.1 to 2.6) which were analyzed by GC and GC-MS. Two of them, samples $F_{2.3}$ and $F_{2.4}$ include in their compositions the unknown compounds, at relevant concentrations (Figure 22). These samples were submitted to ^{13}C-NMR analysis without previous isolation of components.

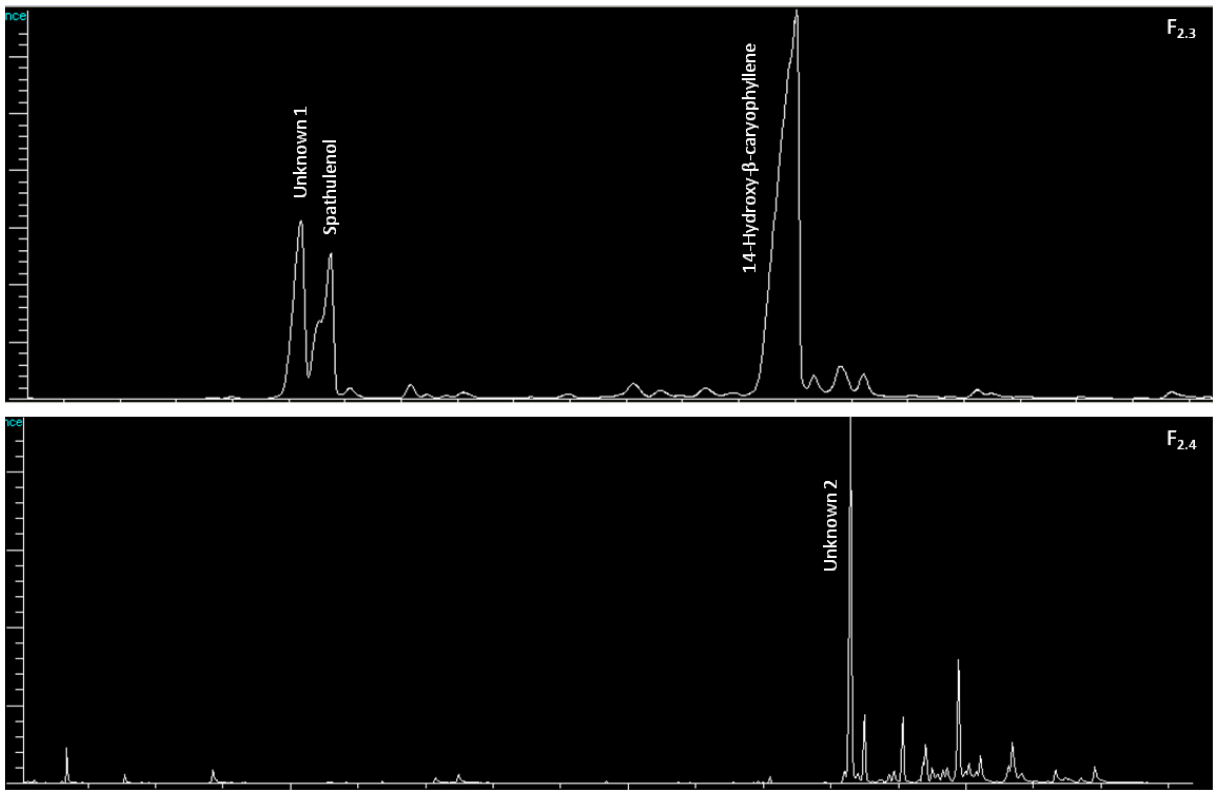


Figure 22 - GC-chromatogram acquired in apolar column of samples $F_{2.3}$ and $F_{2.4}$.

8.2.1 **Analysis of sample $F_{2.3}$**

^{13}C NMR spectra of sample $F_{2.3}$ was composed of 364 resonance peaks, figure 23.

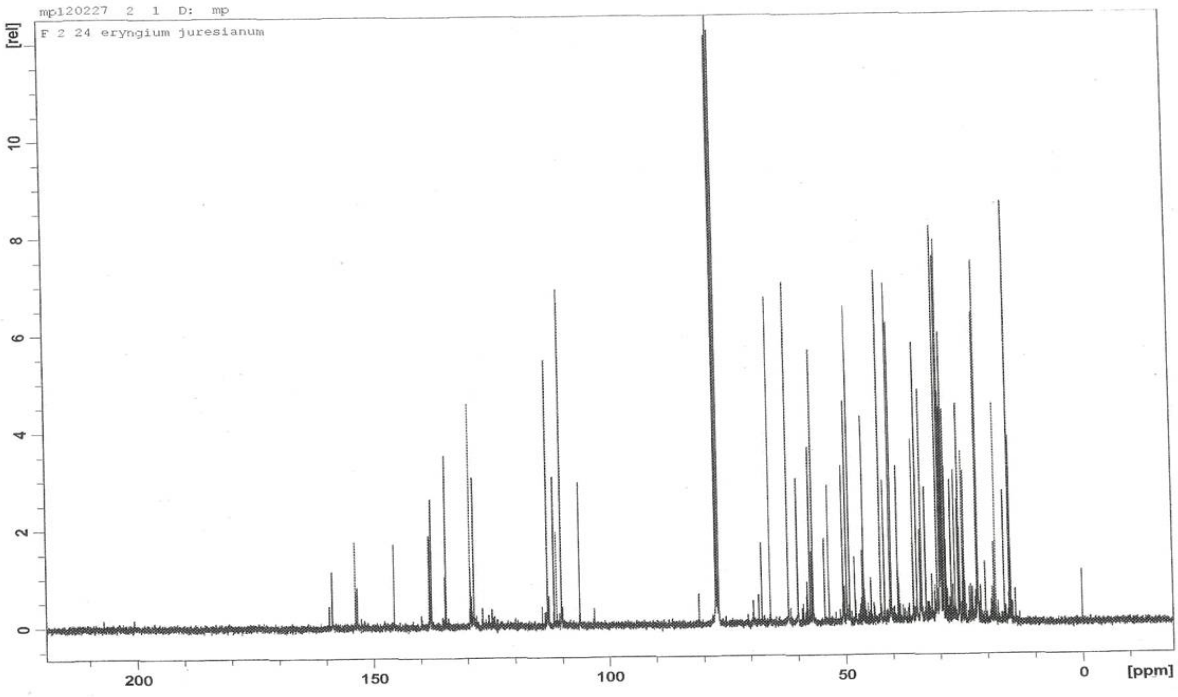


Figure 23 - ^{13}C-NMR spectra of fraction $F_{2.3}$ of *E.* subsp. *juresianum*.

A computer assisted query within the "Terpene" and the "Literature" databases of the *Equipe Chimie et Biomasse* assigned some of these signals (45) to compounds previously identified by GC and GC-MS: spathulenol (12.9%) and 14-hydroxy-β-caryophyllene (67.3%), in this case revealing the existence of the two conformers β,β- and β,α- of 14-β-hydroxy-caryophyllene (Figure 24).

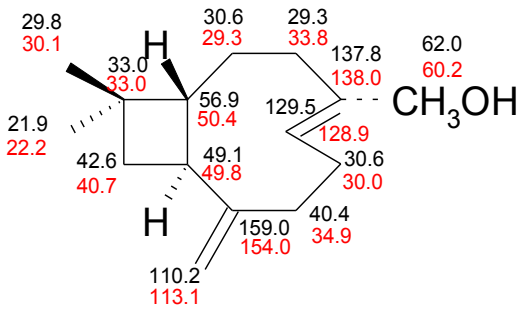
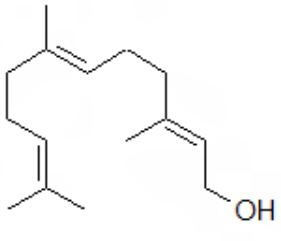


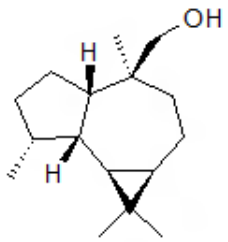
Figure 24 - ^{13}C-NMR resonance of βα- (in red) and ββ- conformers of 14-hydroxy-β-caryophyllene in accordance with (Barero *et al.* 1995).

Additionally 26 other signals allowed to identify *E,E*- farnesol (0.6%) and viridiflorol (0.8%), in table 16. The low concentration of these compounds in the sample hinders the viewing of all of resonance signals, namely quaternary carbons signals.

Table 16 - Chemical structure and resonance signals of *E,E*-farnesol and viridiflorol.



***E,E*- Farnesol**



Viridiflorol

δppm experimental	Relative abundance	Overlap
139.78	2.24	
124.51	1.71	x2
124.23	2.05	
58.97	1.38	
31.91	1.68	
26.65	3.43	
26.40	1.72	
25.72	22.50	n.i.
23.42	5.02	x2
17.69	4.93	

δppm experimental	Relative abundance	Overlap
58.18	2.59	
39.72	3.32	
38.46	4.09	
37.77	3.86	
32.07	3.61	
29.09	8.17	
28.67	36.85	Spathulenol
28.57	4.99	
25.77	6.20	
22.29	5.44	
18.81	4.54	
18.46	10.07	n.i.
16.34	31.17	Spathulenol
16.13	3.22	

Among the remaining non-assigned resonance peaks, fifty of them have intensities compatible with a component at relevant concentration in the sample:

Table 17 - Remaining relevant chemical shifts in ^{13}C-NMR spectra of sample $F_{2.3.}$

δppm experimental	Relative abundance
145.54	19.43
138.11	21.39
134.53	40.59
111.16	21.99
57.39	41.61
46.04	48.82

35.55	43.37
34.05	21.86
30.02	78.29
29.64	54.58
29.43	24.41
28.77	50.24
25.72	22.50
25.07	41.33
18.15	51.69
145.54	19.43
138.11	21.39
134.53	40.59
111.16	21.99

In the computer database and in the literature data we could not find any identity proposal compatible with such spectrum. In order to identity this component further monodimensional and bidimensional NMR analysis, such as Distortionless Enhancement by Polarization Transfer (DEPT), Correlation Spectroscopy (COSY), Heteronuclear Multiple Quantum Coherence (HMQC) and/or Heteronuclear Multiple Bond Correlation (HMBC), are required, however demanding the isolation and purification of the component. Such studies were not concluded in time to be part in this work.

8.2.2 **Analysis of sample $F_{2.4}$**

Chromatogram of sample F2.4 revealed four major components with relative abundances of 38.4%, 6.0%, 6.5% and 16.0%, Figure 21. For the first component retention indices and mass spectrum suggested α-copaene-8-ol. For the last component retention indices and mass spectrum suggested 14-hidroxy-β-caryophyllene. Other peaks had not identity proposals.

^{13}C NMR spectra of the whole sample is composed of 325 resonance peaks (Figure 25).

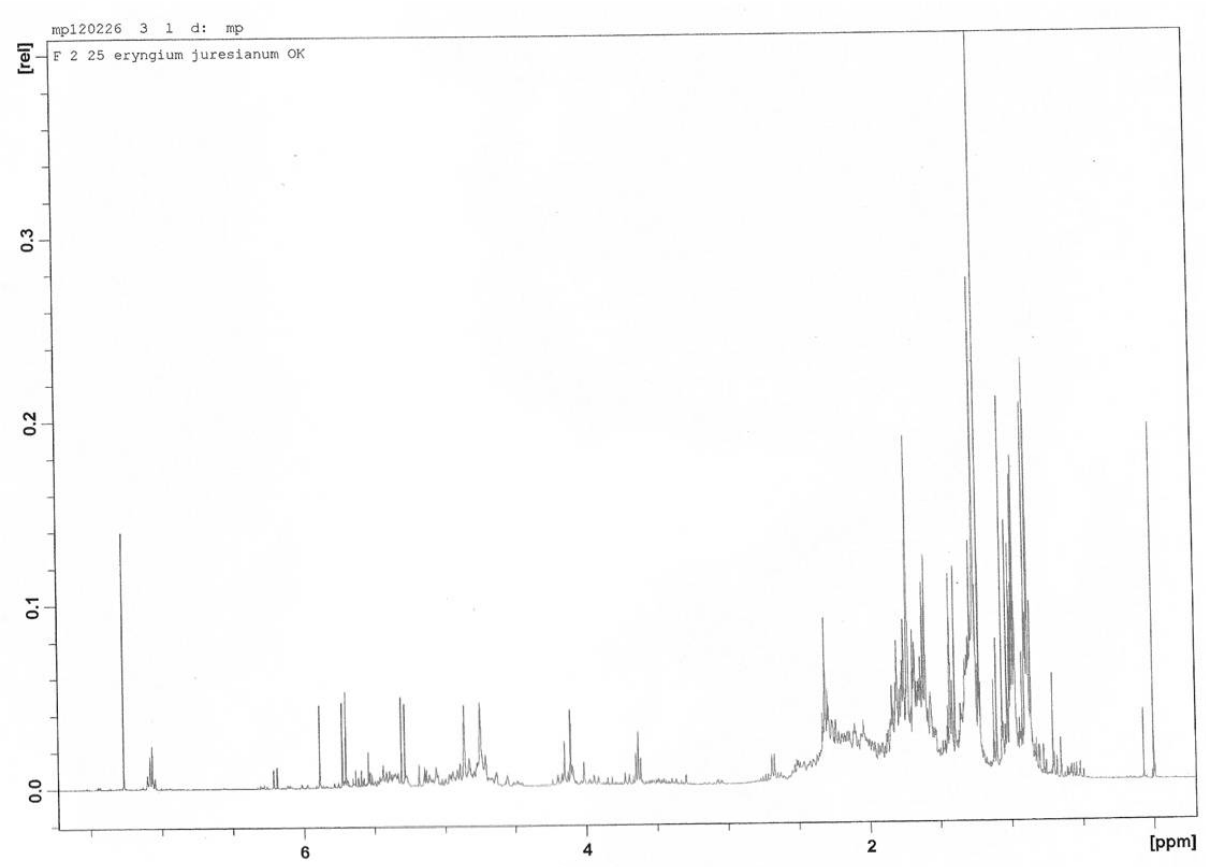
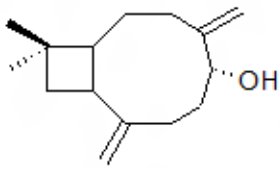


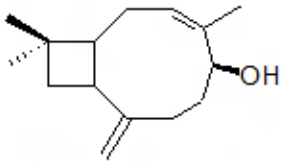
Figure 25 - ^{13}C-NMR spectra of fraction $F_{2.4}$ of *E.* subsp. *juresianum*.

The computer assisted query of the resonance peaks, within the "Terpene" and the "Literature" databases of the *Equipe Chimie et Biomasse*, did not confirm the existence of α-copaene-8-ol nor 14-hidroxy-β-caryophyllene, but proposed the identity of two minor compounds, caryophylla-4(14),8(15)-5α-ol and caryophylla-3,8(15)-diene-5β-ol, table 18. The low concentration of these compounds in the sample hinders the viewing of quaternary carbons signal in both compounds.

Table 18 - Chemical structure and resonance signals of caryophylla-4(14),8(15)-5α-ol and caryophylla-3,8(15)-diene-5β-ol in ^{13}C-NMR spectra.



caryophylla-4(14),8(15)-5α-ol



caryophylla-3,8(15)-diene-5β-ol

δppm Experimental	Relative abundance	δppm Experimental	Relative abundance
155.50	3.87	137.59	4.71
113.52	8.34	125.89	3.94
109.15	7.85	109.68	3.93
75.25	8.70	69.61	4.46
54.26	7.26	50.36	10.63
43.83	10.52	42.53	17.15
37.02	9.43	39.67	27.08
33.46	14.17	34.17	9.71
32.85	9.77	33.24	6.05
32.74	12.39	32.45	9.83
32.62	12.17	29.47	38.72
30.67	10.28	28.46	19.52
30.04	19.10	22.70	39.10
21.99	12.45	15.61	5.49

Two sets of peaks with high intensities are obvious: a set includes 13 peaks with intensities compatible with the most concentrated component (~40%); other set includes 53 peaks, which intensities suggest to belong to components with relative amount around 10-15%. Table 19, reports the chemical shifts and intensities of the 13 peaks apparently belonging to the major component.

Table 19 - Remaining resonance signals with intensity over 40% presents in sample $F_{2.4}$ of *E.* subsp. *juresianum*.

δppm Experimental	Relative abundance
138.00	76.16
134.67	74.91
111.16	88.93

57.04	75.83
35.64	85.97
29.70	81.37
29.66	69.14
29.44	99.93
28.52	87.04
25.72	100.00
24.88	92.70
18.42	88.55
14.94	87.93

Following table (Table 20), reports the chemical shifts and intensities of the remaining fifty seven peaks that are in agreement with the existence three or four sesquiterpenic compounds.

Table 20 - Remaining fifty seven resonance signals presents in sample $F_{2.4}$ of *E.* subsp. *juresianum*.

δppm experimental	Relative abundance	Overlap	δppm experimental	Relative abundance	Overlap
145.55	28.06		30.51	31.09	
141.57	23.82		29.94	29.52	
141.50	22.65		29.61	50.35	
139.81	20.37		29.56	35.82	
139.35	22.59		29.47	38.72	caryophylla-3,8(15)-dien-5β-ol
131.20	20.84		29.37	37.30	
129.50	32.96		29.28	46.76	
126.87	30.00		29.12	45.05	
125.47	21.75		28.67	28.20	
109.92	27.66		28.35	25.29	
68.35	33.15		28.12	27.73	
63.02	28.65		27.83	27.43	
61.35	21.45		26.73	27.51	
54.00	26.02		26.15	26.26	
54.00	26.02		25.82	27.66	
54.00	26.02		24.82	39.31	
49.04	21.15		23.44	34.92	
47.76	27.71		23.05	24.83	
46.61	20.99		22.70	39.10	caryophylla-3,8(15)-dien-5β-ol
44.56	24.06		21.47	26.42	
44.36	24.56		20.53	27.68	
41.95	25.26		20.20	26.44	
40.51	28.82		20.17	30.00	
39.67	27.08	caryophylla-3,8(15)-dien-5β - ol	16.04	27.88	
34.91	23.48		15.83	21.72	
33.90	30.87		15.23	25.75	
32.78	46.84		14.22	27.63	
30.75	27.88		14.13	39.30	
30.63	27.94				

HMBC and HMQC spectra (Figures 26 and 27), were acquired; however interpretation could not be concluded in time to be part in this work.

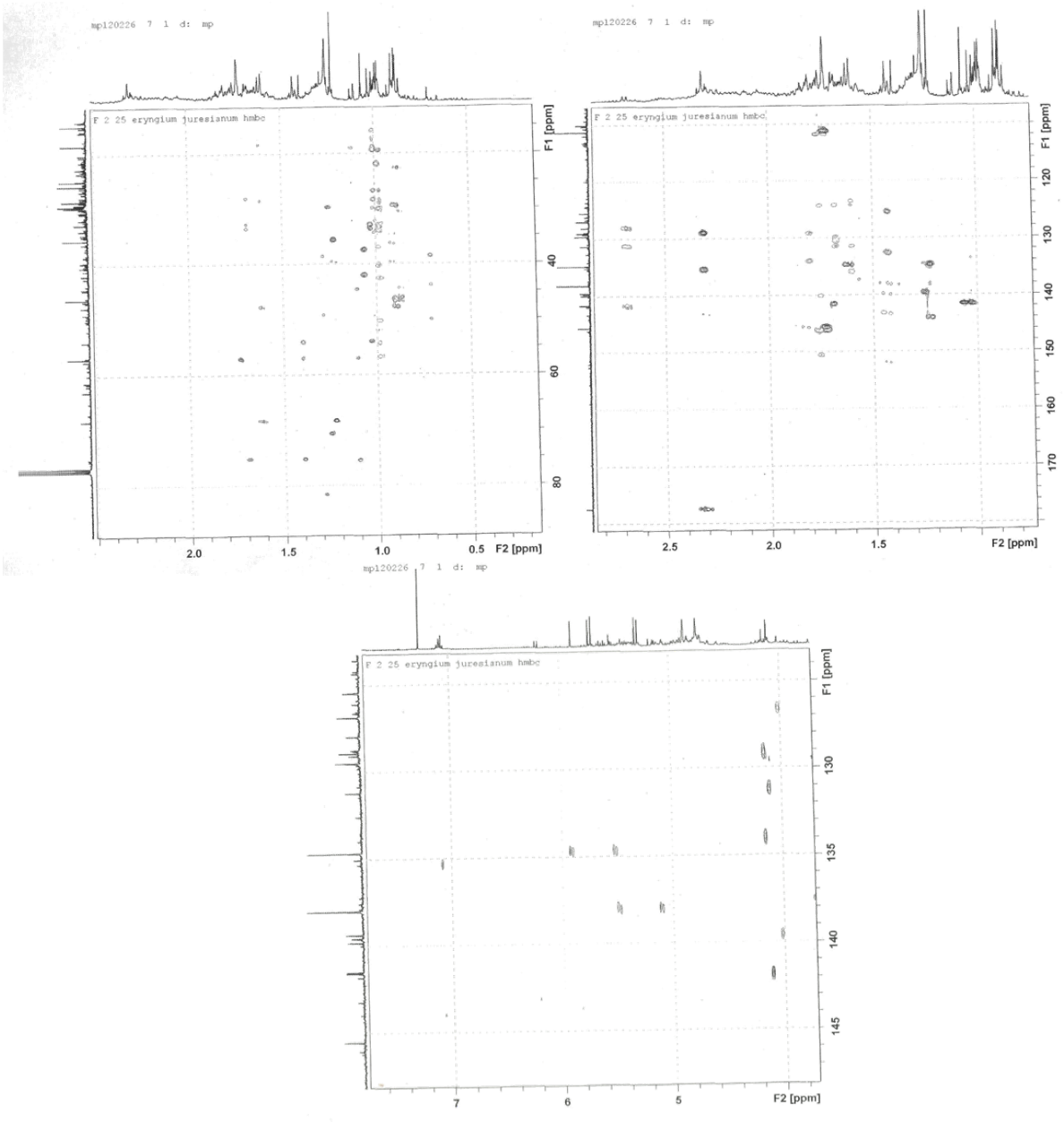


Figure 26 - HMBC spectra of principal peaks of sample $F_{2.4}$.

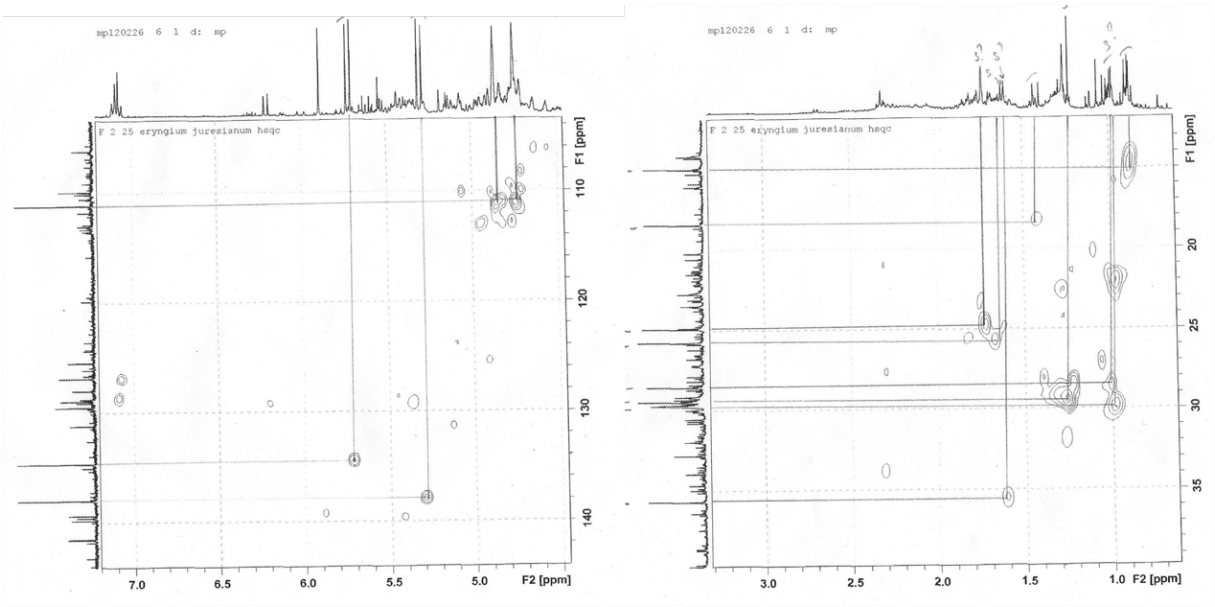


Figure 27 - HMQC spectra of some relevant peaks in the sample.

In conclusion, the study of the new samples of *E. duriaei* subsp. *juresianum* allowed to confirm previous identifications and also to propose new components. From GC and mass spectra, *n*-octanol, *n*-undecane, β-bourbonene, β-bisabolene, γ-cadinene, β-sesquiphellandrene, *E*-γ-bisabolene, globulol, salvial-4(14)-en-1-one and viridiflorol, amounting together 1.8%, were included in the composition.

From the analysis of fractions by ^{13}C-NMR without previous isolation of constituents of three additional components resulted identified: *E*-*E*-farnesol, caryophylla-4(14),8(15)-5α-ol, caryophylla-3,8(15)-diene-5β-ol. The two conformers of 14-hydroxy-β-caryophyllene were also disclosed. From these results the number of compounds identified in the oil of *E. duriaei* subsp. *juresianum* raised from 25 to 38.

9. Conclusion

Conclusion

In general, the purpose of this work, i.e., the identification of active compounds in volatile extracts of *Eryngium* species from the Iberia was successful. The combination of different methodologies of analysis, GC-Ri, GC-MS and ^{13}C-NMR without previous purification, was crucial input in the chemical identification of essential oil.

In study of *E. dilatatum* oil, thirty-one compounds were identified representing 82.6% of the whole composition. Sesquiterpene hydrocarbons (35.7%) and oxygen containing sesquiterpenes (23.6%) are dominant. The major compound is the oxygen containing monoterpene, *Z*-α-chrysanthenyl acetate (11.1%), and after several sesquiterpenes occur in concentrations over 5%, germacrene D (10.3%), bicyclogermacrene (8.1%), spathulenol (5.9%) and α-cadinol (5.7%). The ^{13}C-NMR methodology allowed us to clarify a chromatographic peak, where the mass spectra proposed β-selinene, but in reality was *E*-β-bergamotene, with similar mass spectra but different resonance signals.

The analysis of *E. pandanifolium* enabled the identification of thirty-five compounds, representing 86.7% of the total. The essential oil is constituted predominantly by monoterpenes hydrocarbons (41.8%) and sesquiterpene hydrocarbons (29.7%). The oil has α-pinene (20.8%), β-elemene (10.6%), limonene (5.8%), myrcene (4.6%) and germacrene A (4.7%) as major constituents. The confirmation of the presence of β-elemene, a heat-sensitive molecule, in the essential oil and the identification of selina-11-en-4-α-ol, *E*-dec-2-en-al, α-eudesmol and 4,5-diepi-aristolochene was possible through ^{13}C-NMR methodology. Chemical variability was found between this essential oil and Australian oil studied by Brophy *et al.*. Major constituent in the Australian oil is the oxygenated monoterpene, bornyl acetate, whereas the monoterpene hydrocarbon, α-pinene, dominates the composition of the Portuguese oil. The compounds identified in this oil are of great interest therapeutic.

The oil of aerial parts of *E. campestre* demonstrated the occurrence of three different compositions: *E*-β-farnesene/β-bisabolene/myrcene (Al), β-elemene/germacrene D/germacrene A (BL) and β-bisabolene/germacrene A/α-cadinol (BA). Nonetheless, in all of them, the oils are predominantly composed by sesquiterpene hydrocarbons,

followed by the oxygen containing sesquiterpenes, monoterpene hydrocarbons and oxygen containing monoterpenes. In both cases, a chemical variability was observed enabling the establishment of three different phenotypes. Some of the identified compounds still lack of biological activity studies, so become crucially important targets for future study.

The study of the *E. duriaei* subsp. *juresianum* oil allowed to confirm previous identifications and also to propose new components. From GC and mass spectra, *n*-octanol, *n*-undecane, β-bourbonene, β-bisabolene, γ-cadinene, β-sesquiphellandrene, *E*-γ-bisabolene, globulol, salvial-4(14)-en-1-one and viridiflorol, amounting together 1.8%, were included in the composition. The analysis of fractions by ^{13}C-NMR without previous isolation of constituents allowed to identify three additional components: *E*-*E*-farnesol, caryophylla-4(14),8(15)-5α-ol, caryophylla-3,8(15)-diene-5β-ol. The two conformers of 14-hydroxy-β-caryophyllene were also disclosed. In result, the number of compounds identified in the oil of *E. duriaei* subsp. *juresianum* raised from 25 to 38.

The combination of GC, GC-MS and ^{13}C-NMR proved to be efficient in the identification of compounds in complex mixtures and allowed us to enrich our RI and MS laboratory libraries. Particularly, the methodology of ^{13}C-NMR without previous fractionation or isolation of compounds showed important for the identification of molecules that are not easily identified by conventional techniques, such as: heat-sensitive molecules and molecules with similar MS spectrum.

One of the initial objectives, screening of biological activities by use of biological techniques was not possible to realize due to lack of time.

In sum, chemical and therapeutic potential of the essential oils was confirmed. The compounds identified are of great interest as chemical tools and lead compounds for drug discovery and development.

Personally, this work gave me contact with different methods of chemical analysis, particularly the newness of NMR analysis of complex mixtures and contact with an excellent spirit of equip in research.

Future challenges

- Improve the analytical techniques to permit identification of unknown compounds;
- Testing compounds identified *in vitro, in vivo* and *in situ* models;
- Draw analogous of lead compounds;
- Project lab syntheses of compounds that are present in the essential oils in low concentrations and showing therapeutic or diagnostic interest.

10. References

References

Adams, R. P. (2001), *Identification of essential oil components by gas chromatography/quadrupole mass spectroscopy* ([3rd edn.; Carol Stream, Ill.: Allured Pub. Corporation) iii, 456 p.

Adams, R. P. (2007), *Identification of essential oil components by gas chromatography/mass spectorscopy* (4th edn.; Carol Stream, Ill.: Allured Pub. Corp.) viii, 804 p.

Adewale, M. A. (2009), '(−)-trans-β-Elemene and related compounds: occurrence, synthesis, and anticancer activity', *Tetrahedron,* 65 (27), 5145-59.

Adorjan, B. and Buchbauer, G. (2010), 'Biological properties of essential oils: an updated review', *Flavour and Fragrance Journal,* 25 (6), 407-26.

Ajikumar, P. K., Tyo, K., and Carlsen, S.; *et al.* (2008), 'Terpenoids: opportunities for biosynthesis of natural product drugs using engineered microorganisms', *Mol Pharm,* 5 (2), 167-90.

Assadian, F., Masoudi, S., and Nematollahi, F.; *et al.* (2005), 'Volatile Constituents of Xanthogalum purpurascens Ave-Lall., Eryngium caeruleum M.B. and Pimpinella aurea DC. Three Umbelliferae Herbs Growing in Iran', *Journal of Essential Oil Research,* 17 (3), 243-45.

Ayoub, N., Al-Azizi, M., and König, W.; *et al.* (2006), 'Essential oils and a novel polyacetylene from Eryngium yuccifolium Michaux. (Apiaceae)', *Flavour and Fragrance Journal,* 21 (6), 864-68.

Bakkali, F., Averbeck, S., and Averbeck, D.; *et al.* (2008), 'Biological effects of essential oils--a review', *Food Chem Toxicol,* 46 (2), 446-75.

Banout, J., Havlik, J., and Kulik, M.; *et al.* (2010), 'Effect of Solar Drying on the Composition of Essential Oil of Sacha Culantro (Eryngium Foetidum L.) Grown in the Peruvian Amazon', *Journal of Food Process Engineering,* 33 (1), 83-103.

Barero, Alejandro F., *et al.* (1995), 'Stereochemistry of 14-Hydroxy-β-caryophyllene and related compunds', *Tetrahedron,* 51 (13), 3813-22.

Bassole, I. H. and Juliani, H. R. (2012), 'Essential oils in combination and their antimicrobial properties', *Molecules,* 17 (4), 3989-4006.

Bassole, I. H., Lamien-Meda, A., and Bayala, B.; *et al.* (2011), 'Chemical composition and antimicrobial activity of Cymbopogon citratus and Cymbopogon giganteus essential oils alone and in combination', *Phytomedicine,* 18 (12), 1070-4.

Betts, T. J. (2001), 'Chemical characterisation of the different types of volatile oil constituents by various solute retention ratios with the use of conventional and novel commercial gas chromatographic stationary phases', *J Chromatogr A,* 936 (1-2), 33-46.

Brophy, J. J., Goldsack, R. J., and Copeland, L. M.; Palá-Paúl, J. (2003), 'Essential Oil of Eryngium L. Species from New South Wales (Australia)', *Journal of Essential Oil Research,* 15 (6), 392-97.

Bueno, J., Escobar, P., and Martinez, J. R.; *et al.* (2011), 'Composition of three essential oils, and their mammalian cell toxicity and antimycobacterial activity against drug resistant-tuberculosis and nontuberculous mycobacteria strains', *Nat Prod Commun,* 6 (11), 1743-8.

Burt, S. (2004), 'Essential oils: their antibacterial properties and potential applications in foods--a review', *Int J Food Microbiol,* 94 (3), 223-53.

References

Cabral, C., Cavaleiro, C., and Goncalves, M.J.; *et al.* (2012), 'Chemical composition, anti-inflammatory activity and citotoxicity of Otanthus maritimus (L.) Hoffmanns. & Link from Portugal', (Granada Congress and Exhibitions Centre 6th European Congress of Pharmacology (EPHAR 2012)).

Cabral, C., Cavaleiro, C., and Goncalves, M.J.; *et al.* (2013), 'Otanthus maritimus (L.) Hoffmanns. & Link as a source of a bioactive and fragrant oil.', *Industrial Crops & Products* 43, 484-89.

Calvino, C. I., Martinez, S. G., and Downie, S. R. (2008), 'The evolutionary history of Eryngium (Apiaceae, Saniculoideae): rapid radiations, long distance dispersals, and hybridizations', *Mol Phylogenet Evol,* 46 (3), 1129-50.

Cantrell, C. L. , Klun, J. A., and Bryson, C. T.; *et al.* (2005), 'Isolation and identification of mosquito bite deterrent terpenoids from leaves of American (Callicarpa americana) and Japanese (Callicarpa japonica) beautyberry', *J Agric Food Chem,* 53 (15), 5948-53.

Capetanos, C., Saroglour, V., and Marinz, P. D.; *et al.* (2007), 'Essential oil analysis of two endemic Eryngium species from Serbia', *Journal of the Serbian Chemical Society,* 72 (10), 961-65.

Cardozo, E., Rubio, M., and Rojas, L. B.; Usubillaga, A. (2004), 'Composition of the Essential Oil from the Leaves of Eryngium foetidum L. from the Venezuelan Andes', *Journal of Essential Oil Research,* 16 (1), 33-34.

Cavaleiro, C., Goncalves, M. J., and Serra, D.; *et al.* (2011), 'Composition of a volatile extract of Eryngium duriaei subsp. juresianum (M. Lainz) M. Lainz, signalised by the antifungal activity', *J Pharm Biomed Anal,* 54 (3), 619-22.

Celik, A., Aydinlik, N., and Arslan, I. (2011), 'Phytochemical Constituents and Inhibitory Activity towards Methicillin-Resistant Staphylococcus aureus Strains of Eryngium Species (Apiaceae)', *Chemistry & Biodiversity,* 8 (3), 454-59.

Chang, S. T., Chen, P. F., and Wang, S. Y.; Wu, H. H. (2001), 'Antimite activity of essential oils and their constituents from Taiwania cryptomerioides', *J Med Entomol,* 38 (3), 455-7.

Chin, S. T., Eyres, G. T., and Marriott, P. J. (2012), 'System design for integrated comprehensive and multidimensional gas chromatography with mass spectrometry and olfactometry', *Anal Chem,* 84 (21), 9154-62.

Chowdhury, J. U., Nandi, N. C., and Mohammed, Y. (2007), 'Chemical Constituents of Essential Oil of the Leaves of *Eryngium foetidum* from Bangladesh', *Bangladesh Journal of Scientific and Industrial Research,* 42 (3), 347-52.

Cobos, M. I., Rodriguez, J. L., and de Petre, A.; *et al*. (2002), 'Composition of the Essential Oil of Eryngium paniculatum Cav', *Journal of Essential Oil Research,* 14 (2), 82-83.

Costa, E. V., Dutra, L. M., and de Jesus, H. C.; *et al.* (2011), 'Chemical composition and antioxidant, antimicrobial, and larvicidal activities of the essential oils of Annona salzmannii and A. pickelii (Annonaceae)', *Nat Prod Commun,* 6 (6), 907-12.

Croteau, R., Burbott, A. J., and Loomis, W.D. (1972), 'Biosynthesis of mono- and sesqui-terpenes in peppermint from glucose-^{14}C and $^{14}CO_2$', *Phytochemistry,* 11 (8), 2459–67.

References

Crowell, P. L. and Gould, M. N. (1994), 'Chemoprevention and therapy of cancer by d-limonene', *Crit Rev Oncog,* 5 (1), 1-22.

Crowell, P. L., Lin, S., and Vedejs, E.; Gould, M. N. (1992), 'Identification of metabolites of the antitumor agent d-limonene capable of inhibiting protein isoprenylation and cell growth', *Cancer Chemother Pharmacol,* 31 (3), 205-12.

Dalluge, J., Beens, J., and Brinkman, U. A. (2003), 'Comprehensive two-dimensional gas chromatography: a powerful and versatile analytical tool', *J Chromatogr A,* 1000 (1-2), 69-108.

Darriet, F., Bendahou, M., and Desjobert, J. M.; *et al.* (2012), 'Bicyclo[4.4.0]decane oxygenated sesquiterpenes from Eryngium maritimum essential oil', *Planta Med,* 78 (4), 386-9.

Davis, E. M. and Croteau, R. (2000) *Biosynthesis: Aromatic Polyketides, Isoprenoids, Alkaloids* [online text],

de Abreu Gonzaga, W., Weber, A. D., and Giacomelli, S. R.; *et al.* (2003), 'Composition and antibacterial activity of the essential oils from Zanthoxylum rhoifolium', *Planta Med,* 69 (8), 773-5.

Degenhardt, J., Kollner, T. G., and Gershenzon, J. (2009), 'Monoterpene and sesquiterpene synthases and the origin of terpene skeletal diversity in plants', *Phytochemistry,* 70 (15-16), 1621-37.

Dewick, P. M. (1997), 'The biosynthesis of C5-C25 terpenoid compounds', *Nat Prod Rep,* 14 (2), 111-44.

Dewick, P. M. (2002a), 'The biosynthesis of C5-C25 terpenoid compounds', *Nat Prod Rep,* 19 (2), 181-222.

Dewick, P. M. (2002b), 'The Mevalonate and Deoxyxylulose phosphate pathways: terpenoids and steroids', in John Wiley & Sons Ltd (ed.), *Medicinal Natural Products. A Biosynthetic Approach* (England: Second Edition).

Dobetsberger, C. and *Buchbauer*, G. (2011), 'Actions of essential oils on the central nervous system: An updated review', *Flavour and Fragrance Journal,* 26 (5), 300-16.

Edris, A. E. (2007), 'Pharmaceutical and therapeutic potentials of essential oils and their individual volatile constituents: a review', *Phytother Res,* 21 (4), 308-23.

Eisenreich, W., Schwarz, M., and Cartayrade, A.; *et al.* (1998), 'The deoxyxylulose phosphate pathway of terpenoid biosynthesis in plants and microorganisms', *Chem Biol,* 5 (9), R221-33.

Epstein, W.W. , Klobus, M. A., and Edison, A. S. (1991), 'Irregular monoterpene constituents of Artemisia tridentata cana. The isolation, characterization, and synthesis of two new chrysanthemyl derivatives', *J. Org. Chem.,* 54 (14), 4451-56.

Esteves, I., Souza, I. R., and Rodrigues, M.; *et al.* (2005), 'Gastric antiulcer and anti-inflammatory activities of the essential oil from Casearia sylvestris Sw', *J Ethnopharmacol,* 101 (1-3), 191-6.

Flamini, G., Tebano, M., and Cioni, P. L. (2008), 'Composition of the essential oils from leafy parts of the shoots, flowers and fruits of Eryngium amethystinum from Amiata Mount (Tuscany, Italy)', *Food Chemistry,* 107 (2), 671-74.

References

Formácek, V. and Kubeczka, K. H. (1982), *Essential oils analysis by capillary gas chromatography and carbon-13 NMR spectroscopy* (Chichester ; New York: John Wiley) xiii, 373 p.

Fraga, B. M. (1987), 'Natural sesquiterpenoids', *Nat Prod Rep,* 4 (5), 473-98.

Fraga, B. M. (1994), 'Natural sesquiterpenoids', *Nat Prod Rep,* 11 (5), 533-54.

Fraga, B. M. (2005), 'Natural sesquiterpenoids', *Nat Prod Rep,* 22 (4), 465-86.

Fraga, B. M. (2011), 'Natural sesquiterpenoids', *Nat Prod Rep,* 28 (9), 1580-610.

Greenhagen, B. T., O'Maille, P. E., and Noel, J. P.; Chappell, J. (2006), 'Identifying and manipulating structural determinates linking catalytic specificities in terpene synthases', *Proceedings of the National Academy of Sciences,* 103 (26), 9826-31.

Guenther, E. (1948), *The essential oils* (New York,: D. Van Nostrand Co.) v.

Haagen-Smit, A. J. (1961), 'Essential oils', *Engineering and Science,* XXIV (8).

Hashemabadi, D., Kaviani, B., and Erfatpour, M.; Larijani, K. (2010), 'Comparison of essential oils compositions of eryngo (Eryngium caucasicum Trautv.) at different growth phases by hydrodistillation method', *Plant Omics,* 3 (4), 135-39.

Herent, M. F., De Bie, V., and Tilquin, B. (2007), 'Determination of new retention indices for quick identification of essential oils compounds', *J Pharm Biomed Anal,* 43 (3), 886-92.

Ho, C. L., Liao, P. C., and Wang, E. I.; Su, Y. C. (2011), 'Composition and antifungal activities of the leaf essential oil of Neolitsea parvigemma from Taiwan', *Nat Prod Commun,* 6 (9), 1357-60.

Huang, L. (2006), 'Synthesis of (-)-beta-elemene, (-)-beta-elemenal, (-)-beta-elemenol, (-)-beta-elemene fluoride and their analogues, intermediates and composition and uses thereof', in Inc. Long Range International USA Limited (ed.), *European Patent Register* (EP1773741; USA).

ISO (1997), 'ISO 9235', *Aromatic Natural Raw Materials - Vocabulary* (Geneve).

Kalemba, D. and Kunicka, A. (2003), 'Antibacterial and antifungal properties of essential oils', *Curr Med Chem,* 10 (10), 813-29.

Kováts, E. (1958), 'Gas-chromatographische Charakterisierung organischer Verbindungen. Teil 1: Retentionsindices aliphatischer Halogenide, Alkohole, Aldehyde und Ketone', *Helvetica Chimica Acta,* 41 (7), 1915-32.

Kubeczka, K. H. and Formácek, V. (2002), *Essential oils analysis : by capillary gas chromatography and carbon-13 NMR spectroscopy* (2nd completely rev. edn.; New York: Wiley) xviii, 461 p.

Kunert, G., Reinhold, C., and Gershenzon, J. (2010), 'Constitutive emission of the aphid alarm pheromone, (E)-beta-farnesene, from plants does not serve as a direct defense against aphids', *BMC Ecol,* 10, 23.

Kuzuyama, T. (2002), 'Mevalonate and nonmevalonate pathways for the biosynthesis of isoprene units', *Biosci Biotechnol Biochem,* 66 (8), 1619-27.

Lahlou, S., Carneiro-Leao, R. F., and Leal-Cardoso, J. H. (2002), 'Cardiovascular effects of the essential oil of Mentha x villosa in DOCA-salt-hypertensive rats', *Phytomedicine,* 9 (8), 715-20.

Lahlou, S., Interaminense, L. F., and Magalhaes, P. J.; *et al.* (2004), 'Cardiovascular effects of eugenol, a phenolic compound present in many plant essential oils, in normotensive rats', *J Cardiovasc Pharmacol,* 43 (2), 250-7.

References

Lahlou, S., Magalhaes, P. J., and de Siqueira, R. J.; *et al.* (2005), 'Cardiovascular effects of the essential oil of Aniba canelilla bark in normotensive rats', *J Cardiovasc Pharmacol,* 46 (4), 412-21.

Lang, G. and Buchbauer, G. (2012), 'A review on recent research results (2008–2010) on essential oils as antimicrobials and antifungals. A review', *Flavour and Fragrance Journal,* 27 (1), 13-39.

Laouer, H., Yabrir, B., and Djeridane, A.; *et al.* (2009), 'Composition, antioxidant and antimicrobial activities of the essential oil of Marrubium deserti', *Nat Prod Commun,* 4 (8), 1133-8.

Leclercqa, P. A., Duñgb, N. X., and Lôb, V. N.; Toanhb, N. V. (1992), 'Composition of the Essential Oil of Eryngium foetidum L. from Vietnam', *Journal of Essential Oil Research,* 4 (4).

Leite, A. M., Lima, E. O., and Souza, E. L; *et al.* (2007), 'Inhibitory effect of b-pinene, a-pinene and eugenol on the growth of potential infectious endocarditis causing Gram-positive bacteria', *Revista Brasileira de Ciências Farmacêuticas,* 43, 121-26.

Li, Q. Q., Wang, G., and Zhang, M.; *et al.* (2009), 'beta-Elemene, a novel plant-derived antineoplastic agent, increases cisplatin chemosensitivity of lung tumor cells by triggering apoptosis', *Oncol Rep,* 22 (1), 161-70.

Li, Q. Q., Wang, G., and Huang, F.; *et al*. (2010), 'Antineoplastic effect of beta-elemene on prostate cancer cells and other types of solid tumour cells', *J Pharm Pharmacol,* 62 (8), 1018-27.

Luangnarumitchai, S., Lamlertthon, S., and Tiyaboonchai, W. (2007), 'Antimicrobial Activity of Essential Oils Against Five Strains of Propionibacterium acnes', *Mahidol University Journal of Pharmaceutical Sciences,* 34 (1-7), 60-64.

Marriott, P. J., Shellie, R., and Cornwell, C. (2001), 'Gas chromatographic technologies for the analysis of essential oils', *J Chromatogr A,* 936 (1-2), 1-22.

Marriott, P. J., Haglund, P., and Ong, R. C. (2003), 'A review of environmental toxicant analysis by using multidimensional gas chromatography and comprehensive GC', *Clin Chim Acta,* 328 (1-2), 1-19.

Marriott, P. J., Chin, S., and Maikhunthod, B.; *et al.* (2012), 'Multidimensional gas chromatography', *TrAC Trends in Analytical Chemistry,* 34 (0), 1-21.

Martin, S., Padilla, E., and Ocete, M. A.; *et al.* (1993), 'Anti-inflammatory activity of the essential oil of Bupleurum fruticescens', *Planta Med,* 59 (6), 533-6.

Martins, A., Hajdú, Z., and Vasas, A.; *et al.* (2010), 'Spathulenol inhibit the human ABCB1 efflux pump', *Planta Med,* 76 (12), P608.

Martins, A. , Salgueiro, L. , and Proença da Cunha, A.; *et al.* (2003), 'Essential oil composition of Eryngium foetidum from S. Tome e Principe', *Journal of essential oil research : JEOR.,* 15 (2), 93-95.

Miguel, G., Faleiro, L., and Cavaleiro, C.; *et al.* (2008), 'Susceptibility of Helicobacter pylori to essential oil of Dittrichia viscosa subsp. revoluta', *Phytother Res,* 22 (2), 259-63.

Miller, J.A., Thompson, P.A., and Hakim, I.A.; *et al.* (2011), 'd-Limonene: a bioactive food component from citrus and evidence for a potential role in breast cancer prevention and treatment', *Oncology Reviews,* 5 (1), 31-42.

References

Montanari, R. M., Barbosa, L., and Demuner, A. J.; *et al.* (2011), 'Chemical composition and antibacterial activity of essential oils from verbenaceae species: alternative sources of (E)-caryophyllene and germacrene-D', *Quimica Nova,* 34, 1550-55.

Morteza-Semnani, K. (2005), 'Essential Oil Composition of Eryngium bungei Boiss', *Journal of Essential Oil Research,* 17 (5), 485-86.

Neves, A., Rosa, S., and Goncalves, J.; *et al.* (2010), 'Screening of five essential oils for identification of potential inhibitors of IL-1-induced Nf-kappaB activation and NO production in human chondrocytes: characterization of the inhibitory activity of alpha-pinene', *Planta Med,* 76 (3), 303-8.

Noge, K. and Becerra, J. X. (2009), 'Germacrene D, a common sesquiterpene in the genus Bursera (Burseraceae)', *Molecules,* 14 (12), 5289-97.

O'Maille, P. E., Chappell, J., and Noel, J. P. (2006), 'Biosynthetic potential of sesquiterpene synthases: alternative products of tobacco 5-epi-aristolochene synthase', *Arch Biochem Biophys,* 448 (1-2), 73-82.

Oh, T. H., Kim, S. S., and Yoon, W. J.; *et al.* (2009), 'Chemical composition and biological activities of Jeju Thymus quinquecostatus essential oils against Propionibacterium species inducing acne', *J Gen Appl Microbiol,* 55 (1), 63-8.

Ong, R. C. and Marriott, P. J. (2002), 'A review of basic concepts in comprehensive two-dimensional gas chromatography', *J Chromatogr Sci,* 40 (5), 276-91.

Pala-Paul, J., Brophy, J. J., and Goldsack, R. J.; *et al.* (2003), 'Essential oil composition of the seasonal heterophyllous leaves of *Eryngium vesiculosum* from Australia', *Australian Journal of Botany,* 51 (5), 497-501.

Pala-Paul, J., Perez-Alonso, M. J., and Velasco-Negueruela, A.; *et al.* (2005a), 'Analysis of the essential oil composition from the different parts of Eryngium glaciale Boiss. from Spain', *J Chromatogr A,* 1094 (1-2), 179-82.

Pala-Paul, J., Perez-Alonsoa, M. J., and Velasco-Negueruela, A.; *et al.* (2005b), 'Essential oil composition of the different parts of Eryngium bourgatii Gouan from Spain', *J Chromatogr A,* 1074 (1-2), 235-9.

Pala-Paul, J., Copeland, L. M., and Brophy, J. J.; Goldsack, R. J. (2006), 'Essential oil composition of Eryngium rosulatum P.W. Michael ined.: A new undescribed species from eastern Australia', *Biochem Syst Ecol,* 34, 796-801.

Pala-Paul, J., Brophy, J. J., and Perez-Alonso, M. J.; *et al.* (2007), 'Essential oil composition of the different parts of Eryngium corniculatum Lam. (Apiaceae) from Spain', *J Chromatogr A,* 1175 (2), 289-93.

Pala-Paul, J., Usano-Alemany, J., and Soria, A. C.; *et al.* (2008a), 'Essential oil composition of Eryngium campestre L. growing in different soil types. A preliminary study', *Natural Product Communications,* 3 (7), 1121-26.

Pala-Paul, J., Copeland, L. M., and Brophy, J. J.; Goldsack, R. J. (2008b), 'Essential oil composition of Eryngium paludosum (Moore et betche) P.W. Michael: An endemic species from eastern Australia', *Journal of Essential Oil Research,* 20 (5), 416-19.

Pala-Paul, J., Usano-Alemany, J., and Brophy, J. J.; *et al.* (2010), 'Essential oil composition of the different parts of Eryngium aquifolium from Spain', *Nat Prod Commun,* 5 (5), 817-21.

References

Palazzo, M. C., Wright, H. L., and Agius, B. R.; *et al.* (2009), 'Chemical compositions and biological activities of leaf essential oils of six species of Annonaceae from Monteverde, Costa Rica', v. 3, no. 3, p. 153-160.

Paul, J. H., Seaforth, C. E., and Tikasingh, T. (2011), 'Eryngium foetidum L.: a review', *Fitoterapia,* 82 (3), 302-8.

Pharmacopoeia, European (2011), ed. Council of Europe - European Directorate for the Quality of Medicines (7th Edition edn.; Strasbourg).

Phillips, John B. and Beens, Jan (1999), 'Comprehensive two-dimensional gas chromatography: a hyphenated method with strong coupling between the two dimensions', *Journal of Chromatography A,* 856 (1–2), 331-47.

Pichersky, E., Noel, J. P., and Dudareva, N. (2006), 'Biosynthesis of plant volatiles: nature's diversity and ingenuity', *Science,* 311 (5762), 808-11.

Pino, J. A., Rosado, A., and Fuentes, V. (1997a), 'Composition of the Leaf Oil of Eryngium foetidum L. from Cuba', *Journal of Essential Oil Research,* 9 (4), 467-68.

Pino, J. A., Rosado, A., and Fuentes, V. (1997b), 'Chemical Composition of the Seed Oil of Eryngium foetidum L. from Cuba', *Journal of Essential Oil Research,* 9 (1), 123-24.

Proença da Cunha, A., Cavaleiro, C., and Salgueiro, L. (2010), 'Fármacos Aromáticos (Plantas aromáticas e Óleos Essenciais)', in Fundação Calouste Gulbenkian (ed.), *Farmacognosia e Fitoquímica* (3ª Edição), 339-401.

Ragunathan, N., Krock, K. A., and Klawun, C.; *et al.* (1999), 'Gas chromatography with spectroscopic detectors', *J Chromatogr A,* 856 (1-2), 349-97.

Rasooli, I., Shayegh, S., and Taghizadeh, M.; Astaneh, S. D. (2008), 'Phytotherapeutic prevention of dental biofilm formation', *Phytother Res,* 22 (9), 1162-7.

Reichling, J., Schnitzler, P., and Suschke, U.; Saller, R. (2009), 'Essential oils of aromatic plants with antibacterial, antifungal, antiviral, and cytotoxic properties--an overview', *Forsch Komplementmed,* 16 (2), 79-90.

Rivas da Silva, A. C., Lopes, P. M., and Barros de Azevedo, M. M.; *et al.* (2012), 'Biological activities of alpha-pinene and beta-pinene enantiomers', *Molecules,* 17 (6), 6305-16.

Rufino, A. T., Cavaleiro, C., and Judas, F.; *et al.* (2012), 'The Essential Oil of Eryngium Duriaei Subsp Juresianum Inhibits Il-1 Beta Induced Nf-Kb and Mapk Activation in Human Chondrocytes', *Osteoarthritis and Cartilage,* 20, S290-S90.

Ryan, D. and Marriott, P. (2003), 'Comprehensive two-dimensional gas chromatography', *Anal Bioanal Chem,* 376 (3), 295-7.

Saeedi, M. and Morteza-Semnani, K. (2008), 'Effect of the Essential Oil of Eryngium caeruleum on Percutaneous Absorption of Piroxicam through Rat Skin', *Journal of Essential Oil Bearing Plants,* 11 (5), 485-95.

Saeedi, M. and Morteza-Semnani, K. (2009), 'Penetration-Enhancing Effect of the Essential Oil and Methanolic Extract of Eryngium bungei on Percutaneous Absorption of Piroxicam through Rat Skin', *Journal of Essential Oil Bearing Plants,* 12 (6), 728-41.

Sangwan, N.S., Farooqi, A., and Shabih, F.; Sangwan R.S. (2001), 'Regulation of essential oil production in plants ', *Plant Growth Regulation,* 34 (1), 3-21.

References

Sefidkon, F., Dabiri, M., and Alamshahi, A. (2004), 'Chemical Composition of the Essential Oil of Eryngium billardieri F. Delaroche from Iran', *Journal of Essential Oil Research,* 16 (1), 42-43.

Shellie, R. and Marriott, P. J. (2002), 'Comprehensive two-dimensional gas chromatography with fast enantioseparation', *Anal Chem,* 74 (20), 5426-30.

Simões, M., Rocha, S., and Coimbra, M. A.; Vieira, M. J. (2008), 'Enhancement of Escherichia coli and Staphylococcus aureus antibiotic susceptibility using sesquiterpenoids', *Med Chem,* 4 (6), 616-23.

Siqueira, C., Oliani, J., and Sartoratto, A.; *et al.* (2011), 'Chemical constituents of the volatile oil from leaves of Annona coriacea and in vitro antiprotozoal activity', *Revista Brasileira de Farmacognosia,* 21, 0-0.

Stolle, A., Ondruschkaa, B., and Hopf, H. (2009), 'Thermal Rearrangements of Monoterpenes and Monoterpenoids', *Helvetica Chimica Acta,* 92.

Sun, Y., Ren, Y., and Zhu, H.; et.al (2009), 'Radioactive synthesis and biodistribution study of beta-elemene-99mTc(CO)3 conjugates', *J Biol Inorg Chem,* 14 (6), 899-904.

Sun, Y., Li, Y., and Ling, Y.; *et al.* (2011a), 'Design, Synthesis and Biological Activity of E-beta-Farnesene Analogues Containing Pyra-zole-carboxamide', *Chin. J. Org. Chem.,* 31 (09), 1425-32.

Sun, Y., Qiao, H., and Ling, Y.; *et al.* (2011b), 'New analogues of (E)-β-farnesene with insecticidal activity and binding affinity to aphid odorant-binding proteins', *Journal of agricultural and food chemistry,* 59 (6), 2456-61.

Thi, N., Anh, T., and Thach, L. N. (2008a), 'The essential oil composition of Eryngium foetidum L. in South Vietnam extracted by hydrodistillation under conventional heating and microwave irradiation', *Journal of Essential Oil Bearing Plants,* 11 (2), 154-61.

Thi, N. , Anh, T. H., and Thach, L. N. (2008b), 'The essential oil composition of Eryngium foetidum L. in South Vietnam extracted by hydrodistillation under conventional heating and microwave irradiation', *Journal of Essential Oil Bearing Plants,* 11 (2), 154-61.

Thiem, B., Kikowska, M., and Kurowska, A.; Kalemba, D. (2011), 'Essential Oil Composition of the Different Parts and In Vitro Shoot Culture of Eryngium planum L.', *Molecules,* 16 (8), 7115-24.

Vandendool, H. and Kratz, P. D. (1963), 'A Generalization of the Retention Index System Including Linear Temperature Programmed Gas-Liquid Partition Chromatography', *J Chromatogr,* 11, 463-71.

Vigushin, D. M., Poon, G. K., and Boddy, A.; *et al.* (1998), 'Phase I and pharmacokinetic study of D-limonene in patients with advanced cancer. Cancer Research Campaign Phase I/II Clinical Trials Committee', *Cancer Chemother Pharmacol,* 42 (2), 111-7.

Wang, G., Li, X., and Huang, F.; *et al.* (2005), 'Antitumor effect of beta-elemene in non-small-cell lung cancer cells is mediated via induction of cell cycle arrest and apoptotic cell death', *Cell Mol Life Sci,* 62 (7-8), 881-93.

Wang, P., Su, Z., and Yuan, W.; *et al.* (2012), 'Phytochemical Constituents and Pharmacological Activities of *Eryngium L.* (Apiaceae) ', *Pharmaceutical Crops,* 3, 99-120.

References

Wiley/NIST (2006), *Wiley Registry/NIST 05 Mass Espectral Library*.

Wong, K. C., Feng, M. C., and Sam, T. W.; Tan, G. L. (1994), 'Composition of the Leaf and Root Oils of Eryngium foetidum L.', *Journal of Essential Oil Research,* 6 (4), 369-74.

Worsfold, P., Townshend, A., and Poole, C. F. (2005), *Encyclopedia of analytical science*, 10 vols. (2nd edn.; Amsterdam ; Boston: Elsevier Academic Press).

Worwood, Valerie Ann (1991), 'Medicines Out of the Earth', in New World Library (ed.), *The Complete Book of Essential Oils and Aromatherapy*.

Wörz, A. (2011), 'Revision of Eryngium L. (Apiaceae-Saniculoideae): General part and Palaearctic species', *Bibliotheca Botanica,* 159.

Yea, P.H. (1974), 'Essential oils - XVI - Oil of Eryngium Foetidum, Linn', *Journal of Chenise Chemical Society,* 21 (3), 139-47.

Zhang, F., Xu, L., and Qu, X.; *et al.* (2011), 'Synergistic antitumor effect of beta-elemene and etoposide is mediated via induction of cell apoptosis and cell cycle arrest in non-small cell lung carcinoma cells', *Mol Med Report,* 4 (6), 1189-93.

Ziaei, A., Ramezani, M., and Wright, L.; *et al.* (2011), 'Identification of spathulenol in Salvia mirzayanii and the immunomodulatory effects', *Phytother Res,* 25 (4), 557-62.

Zouari, S., Zouari, N., and Fakhfakh, N.; *et al.* (2010), 'Chemical composition and biological activities of a new essential oil chemotype of Tunisian Artemisia herba alba Asso', *Journal of Medicinal Plants Research,* 4 (10), 871-80.