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CICLO XXX

COORDINATORE Prof. Paolo Pinton

CHEMICAL FINGERPRINTING AND BIOACTIVITY PROFILE OF ECUADORIAN ESSENTIAL OILS

Settore Scientifico Disciplinare BIO/15 – Biologia Farmaceutica

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DEDICATION

This work is dedicated to my parents Galo and Ruth and my sister, Diana. These were my very first teachers in life and everything that I have learnt is thanks to the good foundation they helped to establish in me.

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LIST OF ABBREVIATIONS

ABTS	2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
CA	*Chenopodium ambrosioides* L.
DNA	Deoxyribonucleic acid
DP	*Dacryodes peruviana* (Loes.) H.J. Lam.
DPPH	2, 2-diphenyl-1-picrylhydrazyl
EF	*Eryngium foetidum* L.
EO	Essential oil
EOs	Essential oils
FRO	Free radicals of oxygen
PA	*Persea americana* Mill.
PC	*Piper carpunya* Ruiz & Pav.
RNS	Reactive nitrogen species
SM	*Schinus molle* L.
TK	Traditional Knowledge
TM	*Tagetes minuta* L.
TROLOX	6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid
N/B.	Other abbreviations are standard according to IUPAC

ABSTRACT

EOs have been largely employed as natural remedies and about 300 of which are commercially important (Bakkaly et al., 2008). Since the most of the plant species used as traditional health remedies are characterized by flavor and aromatic smell, the study of essential oils is one of the best strategy to draw a research profile matching biodiversity and phytomedicine (Guerrini et al., 2009). In the present study we investigated the chemical composition (GC-MS) and biological activity (antioxidant, anti-yeast, antifungal, antibacterial and mutagenic properties) of seven EOs from Ecuadorian amazon: *Chenopodium ambrosiodes* (Chenopodiaceae), *Dacryodes peruviana* (Burseraceaae), *Eryngium foetidum (*Apiaceae), *Persea americana* (Lauraceae), *Piper carpunya* (Piperaceae), *Schinus molle* (Anacardiaceae) and *Tagetes minuta* (Asteraceae). The principal constituents were limonene, δ-3-carene, *trans*-2-dodecenal, estragole, α-phellandrene and *cis*-tagetone respectively. Interesting bioactivity of *E. foetidum* and its main compound *trans-2-dodecenal* were demonstrated against bacterial strains. While against *C. albicans* the best results were of EF and PA. In the antioxidant assays (DPPH and ABTS) PC and TM showed very interesting radical scavenging ability (IC_{50}) similar to the control *Thymus vulgaris*. Almost all EOs were effective against the fungus *Nannizia gypsea*. These EOs do not prove to be mutagenic so they can be promising natural products for humans uses.

Keywords: Essential oil, chemical characterisation, antioxidant activity, antibacterial activity, antifungal activity, mutagenic activity.

INTRODUCTION

PhD Background and preliminary consideration

The present PhD research has its background in Cooperation and Development projects organised and sponsored by the University of Ferrara. The Centre for the International Development Cooperation of the University of Ferrara is linked with non-profit associations and universities such as Salesian Polytechnic University of Quito, Ecuador. The aim of this Centre and its collaborating institutions is to promote partnerships based on the mutual utilisation of natural and intellectual resources and technical strategies, promoting, coordinating and providing products and services inherent to topics relevant to developing countries.

Medicinal resources of the Amazon rainforest

Tropical plant biodiversity has figured prominently in the news of the past few years as a potential wonder source of new treatments, which, in turn, might contribute to the conservation of biodiversity.

Tropical forests contain a significant percentage of the world's plant species, including those with unique and more varied biochemical modes of defence and survival than their temperate counterparts (Figure 1). Consequently, tropical forests can provide natural products with invaluable compounds that are starting points for the development of new drugs. Although relatively few tropical species have been studied for their pharmaceutical potential (generally accepted to be less than 1%), the tropics have yielded numerous invaluable pharmaceutical compounds, including the anticancer agents vincristine and vinblastine from *Cantharantus roseus*; the muscle relaxant d-tubocurarine from *Chondodendron* and *Strychnos* species, which were originally used in the Amazon for arrow poisons; and steroids from *Discorea* spp. These examples grew out of research programs started in the early or middle parts of this century, programs that had largely been abandoned by the late 1970s. After decades of decline, however, research into natural products is once again on the rise, and a number of pharmaceutical companies and university and government research institutes are actively pursuing the structurally diverse compounds contained within tropical species for their mechanism-based screening programs. A number of promising compounds have resulted from this recent round of natural products, including four species with anti-HIV activity under study by the National Cancer Institute; these are found in the genera of *Ancistrocladus* in Cameroon, *Calophyllum* in Malasya, *Conospermum* in Australia, and *Homolanthus* in Western Samoa.

However, pharmaceutical products are a small component of the medical history and potential of tropical biodiversity. Traditional, largely plant-based, medical systems continue to provide primary health care to more than 75% of the world's population. These systems, of varying scale and formality, produce effective, affordable medicines stemming from long histories of local use. A number of programs are currently underway to research and record the traditional uses of plants as a starting point for pharmaceutical drug discovery. This information can provide an invaluable head start for industry research efforts, and many pharmaceutical companies incorporate ethnobotanical information into their research and development (R&D) programs.

In many cases throughout the tropics, the erosion of traditional cultures has led to an impoverishment of local medicine. A number of programs have been established to record and distribute medicines based on traditional uses of medicinal plants and on their scientific evidences. These programs shore up and build upon local knowledge of medicinal plants and existing medical systems to develop more effective health care systems within the context of local health, economic and social conditions.

Plant-based medicines are also processed and traded extensively in the regional and international herbal medicine markets that serve consumers in both tropical and temperate regions of the world. Commercialised herbal medicine, distinct from traditional medicine, is marketed and consumed outside the cultures and geographic regions from which the plants and their use originated. A growing recognition of the limits of isolated compounds in treating many chronic conditions and diseases, and of the potential for plant-based medicines to provide a more affordable and often more effective alternative, has led to a rapid growth in the herbal medicine industry over the past decade.

It is important to make a distinction between different types of medicine and to recognise that each has very different cultural, economic and social implications and tends to involve very different players. It is not possible to speak about tropical biodiversity and medicine and produce the same a single image in the minds of people. For example, a single plant might provide a lead compound for a pharmaceutical product, which then goes through many derivations and might in the end be manufactured by synthesis. The same plant could be cultivated and processed in the Amazon into formulations of herbal medicines sold in Germany or could be mixed with a variety of other species to be consumed as a medicinal tea by members of a community living in the area where the plant species is endemic. Clearly, tropical biodiversity is manifested as medicine in many very different ways. However, these

medical systems play complementary roles in health care and cannot substitute for one another in any way. Medicine is not a linear progression; there is no ideal system into which all others, given the required economic, social, and cultural conditions, will eventually transform.

Traditional, herbal and pharmaceutical medicines can also play very different but complementary roles in conservation programs in the tropics. The process by which pharmaceutical products are identified and developed has the most tenuous link with local communities and conservation but has the potential to generate revenues can, in turn, be channelled to conservation programs, the bulk of which are chronically underfunded. The sheer scale of pharmaceutical revenues can also support policy arguments for conservation based on the enormous option values held within biologically different areas. Because of the small number of products developed per samples collected, however, the chances for this strategy to provide a wide spectrum of conservation programs are small, and the type of benefits returned to conservation and local communities during the research phase of drug development become of greater importance. At this stage, benefits usually take the form of training, supply of equipment, infrastructure-building, education, health care, and other, more informal services. It is important that relationships between tropical country institutions or communities and commercial collectors be well structured to allow for significant advance benefits, owing to the small likelihood of commercial product development from each collection. Additionally, because the vast majority of pharmaceutical R&D takes place outside the most biologically and culturally diverse regions of the world, these relationships should include steps to increase the capacity for tropical country institutions and communities to better research and utilize their indigenous biological and intellectual resources.

Commercial herbal products generate far less in revenues than pharmaceutical products but also require far less time and money to develop. As with pharmaceutical products, herbal medicines developed from endemic species or traditional knowledge can provide revenues for conservation programs and local communities in the form of royalties. Generally, however, economic benefits are restricted to the cultivation or harvesting of commercial species and, in some cases, the processing of these species into extracts. These activities have the potential to provide an important source of income for local communities and can make the sustainable use of the forest resource a realistic option. Commercialised herbal medicine products can also be sold locally as a more affordable and, in some cases, more effective alternative to pharmaceutical

compounds. Similarly, programs to standardise and distribute medicines based on traditional systems can create economic opportunities for local communities and provide a reliable and affordable form of health care in the short and long term. Overharvesting of species in the wild for pharmaceutical, herbal, and traditional medicines has often resulted in the depletion of valuable species, and any program that attempts to promote the use of these species must incorporate strategies for the sustainable sourcing of raw materials.

The relationship among tropical biodiversity, conservation, and human health is clearly complex and should not be oversimplified. The most effective way in which health and conservation can be combined to serve the needs of local and international communities is by incorporating this complexity into a package of complementary activities among which, could be the development of natural products (Batish et al., 2008).

Figure 1. Amazon rainforest of southern Ecuador

Source: Jose Ballesteros, 2017.

The Area of Study: Ecuadorian's rainforest

Ecuador was recognised by the World Conservation Monitoring Centre as one of the countries with the highest levels of biodiversity in the world; the westernmost part of the Amazon basin is one of the richest regions of the world in terms of plant diversity (Abell et al., 2008; Hoorn et al., 2010). Alpha-diversity of trees and palms (Vormisto et

al., 2004; Hoorn et al., 2010) and lianas (Nabe-Nielsen, 2001) is the highest ever recorded. It represents an interesting subject of study and in this context the collaboration between the University of Ferrara (UNIFE, Ferrara, Italy) and the Politecnica Salesiana University (UPS, Quito, Ecuador) has been developed.

A variety of hypotheses have been proposed to account for the high diversity (Hubbell et al. 2001; Wright 2002), and one explanation seems to be related to local variation in soil resources caused by topography. There evidence that individual species partition topographic ridge-valley gradients, or 'chains' as they are sometimes called, and tree species distributions are affected by underlying geological variation as well (Valencia et al., 2004; Punchi-Manage et al., 2013, 2014; Queenborough et al., 2007; Endara & Jaramillo, 2011). Few studies, however, quantify the abundance of species strongly affected by soil/geological characteristics, and it is higly possible that multiple variables may play an important role in community composition (Hubbell, 2001). For example, many neotropical tree species are generalists, occurring across many soil types (Iverson & Prasad, 1998; Pitman et al. 1999, 2001); for these species, factors controlling abundance remain unknown.

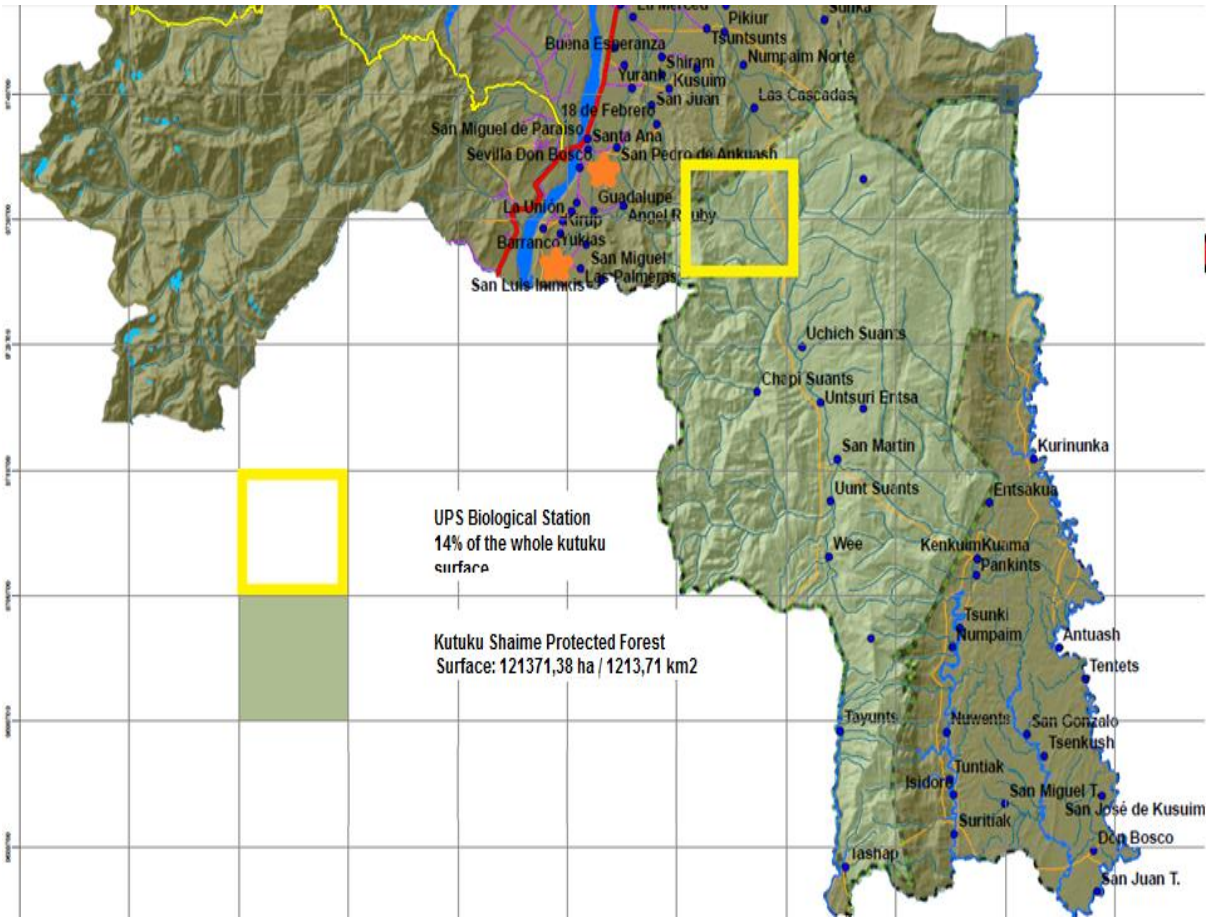
The flora of the Kutuku region in Ecuador can be considered relatively rarely investigated compared with other parts of Amazonia (Herrera-MacBryde & Neill, 1997; Pitman, 2000), and the biogeographical and floristic relationships (plant biodiversity) on a larger scale have not been studied in detail. The present study represents part of a larger scale research project joined between University of Ferrara and Universidad Politecnica Salesiana (Quito, Ecuador) aimed to collect chemical and biological information about plants of medicinal interest for natives and belonging to Amazonian plant biodiversity. With this aim, EOs and aromatic phytocomplex represent a good research paradigm.

In this context, cooperation projects were implemented through collaboration between the University of Ferrara (UNIFE) and Salesian Polytechnic University (UPS), which were made official in 2007 with the Framework Agreement of Cooperation UNIFE-UPS, the VIS NGOs and the participation of local partners involved in international cooperation. These included the local NGO Chankuap Foundation, and people living in the Amazon area of Ecuador, particularly the Shuar ethnic group. The Shuar are a tribe of people from the Ecuadorian and Peruvian Amazon (Descola, 1996). Their history as great warriors goes back to the days of the expansion of the Inca Empire when the Shuar fought to remain free of Inca control (Furneaux, 1975). They also battled the Spanish during the Spanish Conquest. In the centuries following the

conquest, the Shuar continued to fight modern society, resisting successive waves of missionaries (Weyer, 1961). Once known for their practice of shrinking human heads, some Shuar are quickly adapting to contemporary life. No longer isolated from society, their traditional lifestyle is fading as their villages adopt modern ways. Most Shuar, however, remain isolated and continue to live a traditional way of life (Harner, 1973). As previously stated, the Shuar have survived a century of colonisation, unconquered by the Incas and Spanish the Shuar now face a more subtle threat-that of cultural erosion. Despite the onslaught of deforestation and acculturation, the Shuar have maintained many aspects of their traditional practices, especially the use of wild and cultivated plants. They have also begun to record their traditions (Bennet et al., 2002). In 1996 the NGO VIS started a project of cooperation and development in Ecuador with the aim to make the Shuar and Achuar ethnic groups directly and actively aware of the exploitation of their own cultural and biological heritage, thus creating a source of income that does not cause any alterations in this heritage. As part of this project, UNIFE provided expertise and facilities in collaboration with UPS and later the establishment of the University Centre for International Cooperation for Development, and also launched three PhDs which have made active contributions through research and publications subsequent to the enhancement and protection of the Amazonian biodiversity and socio-economic development of the local population (Buso, 2015). Figure 2 shows the exact locations from where the plants were collected for this thesis.

Figure 2. a) Localization of the Amazon rainforest of southern Ecuador, b) Localization of the UPS station in Morona-Santiago province

Source: Google maps, 2017.



Source: IGM / SENPLADES, Ecuador, 2016.

The focus of the investigation: The EOs

The World Health Organisation in recent years has paid more attention to the importance and effectiveness of traditional medical practices, describing a strategy to increase the potential contribution of traditional medicine to health. Under a chemical point of view, EO is a concentrated hydrophobic liquid containing volatile aromatic compounds from plants. EOs are also known as volatile oils, ethereal oils or *aetherolea*, or simply as the "oil of" the plant from which they were extracted, such as oil of clove. The latter way to name EOs could however lead to misunderstandings since “oils” are commonly identified as “fixed oils” i.e. glycerides mixtures. For this reason, the better name to avoid misunderstandings is just EOs. EOs are mainly considered the mixtures of odourous and volatile compounds of aromatic plants. As they have a tendency to undergo evaporation following exposure to air, even at environment temperatures, they are invariably termed volatile oils, EOs or ethereal oils. They mostly contribute to the essences of the aromatic plants that are used abundantly in enhancing the scent by food. Oil is "essential" in the sense that it carries

a distinctive scent, or essence, of the plant. Arabs were the first to develop techniques for obtaining EOs from the naturally occurring organic materials (Saeed, 1989). The Arab physician, Avicenna, designed a protocol to extract the essential oil from the flowers by distillation in the tenth century (Poucher, 1959; Poucher, 1974). He isolated the perfume in the form of oil or attar from the rose flowers and produced rose water. Therefore, the first description of rose water was reported by an Arab historian, Ibn-e-Khulduae.

On the other hand it is important to remember that the term “essential oil” is a contraction of the original “quintessential oil.” This stems from the Aristotelian idea that matter is composed of four elements: fire, air, earth, and water. The fifth element or quintessence, was then considered to be spirit or life force. Distillation and evaporation were thought to be processes of removing the spirit from the plant, and this is also reflected in our language since the term “spirits” is used to describe distilled alcoholic beverages such as brandy, whiskey, and eau de vie (Baser & Buchbauer, 2015).

These EOs can be produced in almost all plant organs such as flowers, buds, stems, leaves, fruits, seeds and roots etc. These are accumulated in secretory cells, cavities, channels, and epidermic cells (Burt, 2004; Chalchat and Ozcan, 2008; Hussain et al., 2008; Anwar et al., 2009a). The extracted oils can vary in quality, quantity and in the chemical composition depending upon the agro climate, plant organ, age and vegetative cycle stage (Masotti et al., 2003; Angioni et al., 2006)

The chemistry of EOs

EOs are mixtures of low boiling substances derived from the secondary metabolism of plants. They are characterized mainly by the following chemical compounds: terpenic hydrocarbons, alcohols, aldehydes, ketones, phenols, sulphur compounds, esters and alcohols, aldehydes and phenols, peroxides. Considering the chemical-physical characteristics, EOs are characterized by low molecular weight hydrocarbons and thus have poor reactivity, liquid physical state and volatility. They can be divided into 2 additional categories according to anatomical localization of the secretory tissue in the plant: 1) EOs that are produced, stored and released in the environment by specialised structures; 2) EOs includes produced and stored in specialized parenchymatic structures allowing the release in the environment in case of mechanical stress. These tissues have a peculiar classification, also in relation to the kind of secretion: Laticiferous tissues (Latex cell and Latex vessels) and Glandular

tissues (Internal glands like: Oil-gland secreting EOs, as in the fruits and leaves of orange and lemon; Mucilage secreting glands, as in the betel leaf; Glands secreting gum, resin, tannin, etc.; Digestive glands secreting enzymes; and Special water secreting glands at the tip of veins; External glands like water-secreting hairs or glands; Glandular hairs secreting gum like substances as in tobacco, plumbago, etc.; and Glandular hairs secreting irritating, poisonous substances, as in nettles and Honey glands, as in carnivorous plants) (Figure 3).

Figure 3. Secretory tissues in plants

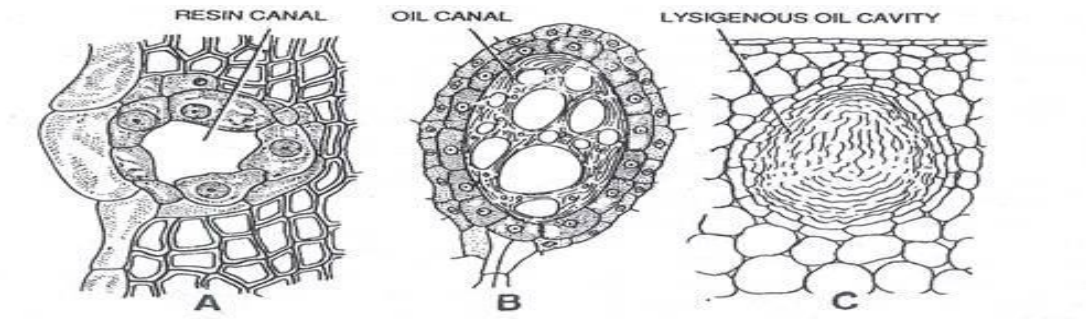


Image Courtesy: http://www.yourarticlelibrary.com/biology/chemical-compounds-that-occurs-as-non-living-inclusions-in-cytoplasm/6754/

The functions of EOs/essences in plants can be summarized in the ecological one that means the relationship between the external release of EO with the ecological role of signal for pollinators for example, and in the protective one – for e.g. from herbivorous. Typical of the internal secretory structures producing EOs; as for the first group, the essences carry out environmental control such as: plant-to-competition competition (deterrents against growth of other plants), pollinator attraction, nutrition cycle mediators, and action as solvent/vector of other compounds. Essences with protective function have two main purposes: protection against herbivorous predators and antibiotic activity against pathogens. It is good to point out that all plants have the capacity to produce volatile compounds, but, in most cases, in small quantities; EO plants are those that produce an EO of commercial interest (Franz, 2010; Buso, 2015).

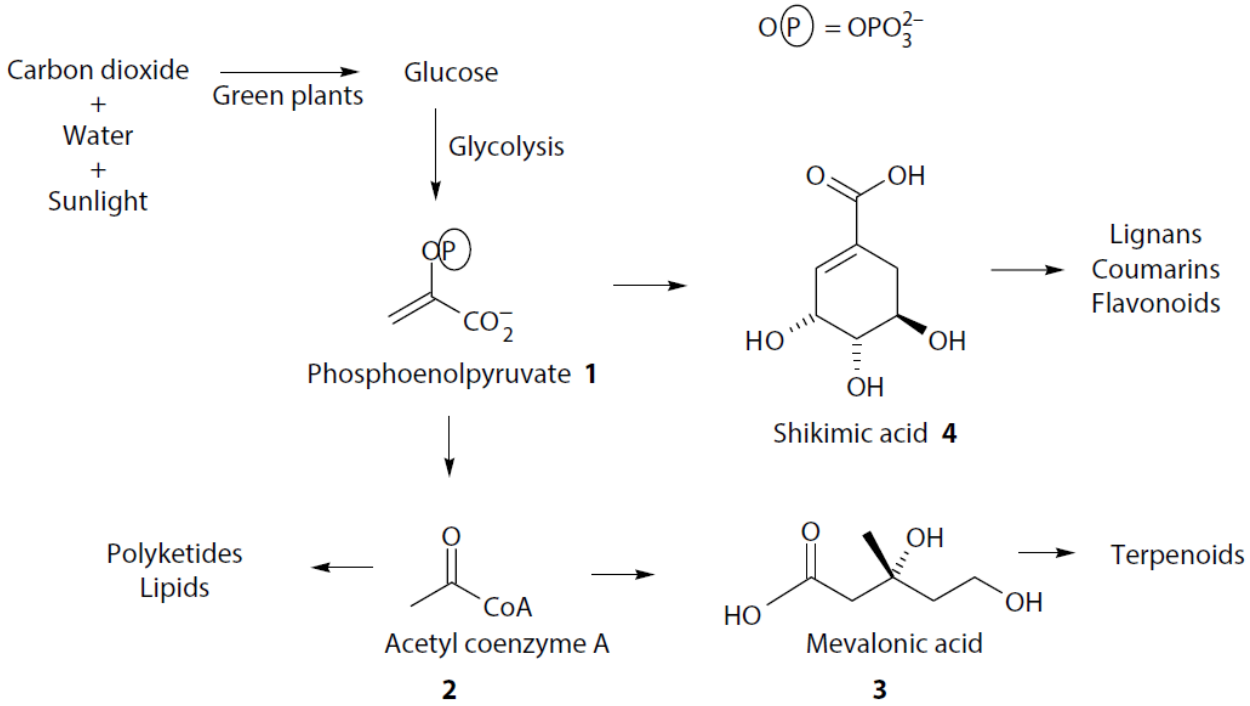
In most cases, EOs are liquid at room temperature, volatile, they have oily consistency, more or less fluid (their density is very often lower than that of water and varies from 0,759 to 1,187), with an aromatic smell, colourless or coloured (for example, the

Chamomilla essence is blue). They are poorly soluble in water, they transmit their aroma because they are slightly hydrophilic, while they are soluble in alcohol, ether, chloroform, and in most organic solvents. They are also soluble in fixed oils. In some cases, the main constituent of the oil is solid at room temperature, as occurs for thymol (*Thymus vulgaris* EO), menthol (*Mentha piperita*), and anethole (*Pimpinella anisum*). These parameters, specific to each essence, are used for recognition and quality control (Noriega, 2014).

EOs are complex mixtures of substances belonging to: Terpenes, composed almost exclusively of mono- and sesquiterpenes; aromatic molecules, which are generally derived from phenylpropane.

It can be said that the biosynthesis of the compounds characterizing EOs comes from phosphoenolpyruvate (PEP), from which begins the bioprocessing of the phenylpropanoids and the acetyl coenzyme A (AcCoA) from which the biogenetic chain of the terpenes begins, as seen in Figure 4.

Figure 4. General pattern of biosynthesis of secondary metabolites



Source: *(Baser & Buchbauer, 2015).*

Aromatic products derived from phenylpropane form a particularly important group, which is generally responsible for the organoleptic characteristics of EOs. These aromatic compounds are formed from phosphono-pyruvic acid, such as terpenes, but the biosynthesis then proceeds following the phenylpropane acid pathway. Various types of aromatic compounds, such as eugenol and anethole, are derived from this molecule.

The composition of EOs is very complex, but in general, it is possible to subdivide into two distinct groups of chemical constituents; the hydrocarbons which are made up almost exclusively of terpenes, and the oxygenated compounds which are mainly esters, aldehydes, ketones, alcohols, phenols, and oxides (Table 1).

Table 1. Composition of EOs

Constituents		**Examples**
Terpenes	Inhibit the accumulation of toxins and help discharge existing toxins from the liver and kidneys	Sesquiterpenes Farnesene Limonene Pinene
Esters	Are the compounds resulting from the reaction of an alcohol with an acid (known as esterification). Esters are very common and are found in a large number of essential oils. They are anti-fungal, calming and relaxing.	Linalyl acetate Geraniol acetate Bornyl acetate Eugenol acetate Lavendulyl acetate.
Aldehydes	Are highly reactive and characterized by the group C-H-O (Carbon, Hydrogen, Oxygen). In general, they are anti-infectious with a sedative effect on the central nervous system.	Citral Citronellal Benzaldehyde Cinnamic aldehyde Cuminic aldehyde Perillaldehyde
Ketones	Are sometimes mucolytic and neuro-toxic when isolated from other constituents.	Thujone Jasmone

	Ketones stimulate cell regeneration, promote the formation of tissue, and liquefy mucous.	Camphor Carvone Menthone Pinacamphone
Alcohols	Are commonly recognized for their antiseptic and anti-viral activities. They create an uplifting quality and are regarded as non-toxic.	Terpene Alcohols (Linalol – Citronellol – Geraniol – Farnesol – Borneol – Menthol – Nerol – Terpineol) Sesquiterpene Alcohols (Bisabolol)
Phenols	Are responsible for the fragrance of an oil. They are antiseptic, anti-bacterial, and strongly stimulating but can also be quite caustic to the skin.	Eugenol Thymol Carvacrol Methyl eugenol Methyl chavicol Anethole Safrole Myristicin Apiol
Oxides	Is a binary compound of an element or a radical with oxygen.	Cineol Eucalyptol Linalol oxide Acaridol Bisabolol oxide Bisabolone oxide

Isolation of EO

(Extraction Methods)

EOs are generally extracted by steam distillation. They are used in perfumes, cosmetics, soaps and other products, for flavouring food and drink, and for adding scents to incense and household cleaning products. In general, it has been observed that a single volatile oil invariably comprises more than 200 different chemical components, and the trace constituents are solely responsible for attributing its characteristic flavour and smell.

Another type of aromatic products available in the market are supercritical, CO_2 extracts, referred to simply as that, CO_2 extracts. They slightly differ in chemistry from their related distilled EOs, they cannot be named as EOs but are becoming increasingly available in the market because of their similar flavour.

Distillation appears to have been practiced throughout ancient times. Based upon the current interpretation of Paolo Rovesti's discovery of an earthenware distillation apparatus, the production or extraction of aromatic oils by means of steam distillation has been known for 5000 years. During the fifth century AD, Zosimus of Panopolis, referred to the distilling of a divine water and panacea. Throughout the early Middle Ages and beyond, a crude form of distillation was known, which was used primarily to prepare floral waters or distilled aromatic waters. These appear to have been used in perfumery, as digestive tonics, in cooking, and for trading.

In 900 AD, Avicenna, was accredited with refining the process of distillation by improving the cooling system.

Distillation is still the most common process of extracting EOs from plants. The advantage of distillation is that the volatile components can be distilled at temperatures lower than the boiling points of their individual constituents and are easily separated from condensed water. During distillation the plant material is placed upon a grid inside the still. Once inside, the still is sealed, and, depending upon the above methods, steam or water/steam slowly breaks through the plant material to remove its volatile constituents. These volatile constituents rise upward through a connecting pipe that leads them into a condenser. The condenser cools the rising vapour back into liquid form. The liquid is then collected in a vehicle below the condenser. Since water and EO do not mix, the EO will be found on the surface of the water where it is siphoned off. Occasionally, an EO is heavier than water and is found on the bottom rather than

the top, such as with clove EO. In this study we have used hydro-distillation by Clevenger apparatus.

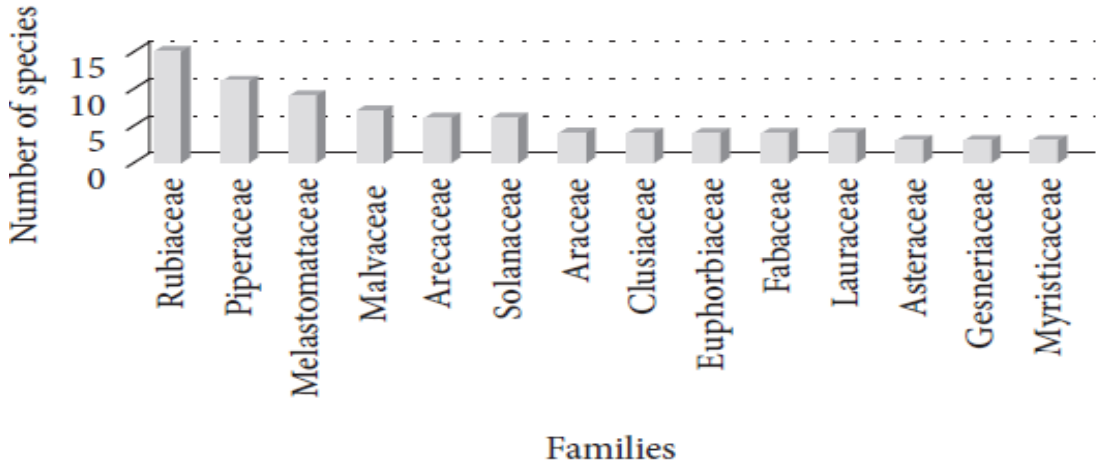
Uses of EOs

EOs have been used medicinally in history. Medical applications proposed by those who sell medicinal oils range from skin treatments to remedies for cancer and are often based solely on historical accounts of the use of EOs for these purposes. Claims of the efficacy of medical treatments, and the treatment of cancers in particular, are now subject to regulation in several Countries. EOs have received renew interest in recent decades. They are used in aromatherapy as they are believed to exhibit certain medicinal benefits for curing organ dysfunction or systemic disorder (Perry et al., 1999; Hajhashemi et al., 2003).

Recent scientific reports have also focused on the antioxidant principles and other different biological activities of EOs (Skocibusic et al., 2006; Yuenyongsawad and Tewtrakul, 2005; Hussain et al., 2008; Anwar et al., 2009b). These EOs have shown potential as anti-bacterial agents, disinfectants, anti-fungal agents, insecticides and herbicides (Skocibusic et al., 2006; Bozin et al., 2006; Maksimovic et al., 2007; Van Vuuren et al., 2007). EOs of some spices and herbs such as sage, oregano, thyme, and satureja etc. have shown antioxidant potential (Ruberto & Baratta, 2000; Rota et al., 2004; Rota et al., 2008) and can therefore be used as natural antioxidants for the protection of fats/oils and related products (Burt, 2004; Sacchetti et al., 2005).

Recently, the use of natural antioxidants are becoming very popular in food and preventive medicine due to the claims that they are safer and have disease-preventing and health promoting attributes. Research is now underway to explore the applications of some EOs for therapeutic uses and the management of infectious diseases as an alternative to standard drug remedies (Bozin et al., 2006; Celiktas et al., 2007; Politeo et al., 2007). Table 2 shows the families used in phytotherapy in the Ecuadorian Kutuku rainforest. It clear that the Shuar community are ancient consumers of medicinal plants. Rubiacea and Pipereacea families have a traditional use in Ecuador.

Table 2. The most frequently used families of plants in the area of Kutuku Scientific Station in Ecuador



Source: *Ballesteros et al, 2016.*

Variables that influence the quality and yield of EOs

The essences can be different from the point of view of the chemical composition and product yield from the fresh plant. These variations are determined by factors that can be classified as natural (genetic, ecological) and artificial (harvest, preparation, conservation, alteration). Endogenous natural factors, ontogenetic cycle of the plant, age, balsamic period, and genetic factors such as hybridisation, polyploidy, selection and mutation are distinguished. Among other factors, we also find naturally exogenous natural variables, such as climatic factors, soil (composition, granulometry, etc.), allelopathy (proximity to other plant species to the essence), light, temperature, degree of rainfall, etc. Finally, artificial factors linked to the agronomic choices to be made in the cultivation of aromatic plants should not be overlooked in order to direct the cultivation technique towards obtaining good productions both qualitatively and quantitatively (example: seedling density, fertilization, irrigation, defence treatments, etc.) (Noriega, 2014).

As said before, EOs may be produced from an endemic population; there can be several reasons why the composition and thus, the EO quality from aromatic plants might differ greatly. Genetic, physiological and environmental factors as well as processing conditions, which are presented in Table 3, may play important roles (Hay, 1993; Hay and Svoboda, 1993; Rohloff, 1999 and 2002; Lawrence, 2002).

Table 3. Factors influencing the composition of EOs

Factor		**Description**
Genetics	Taxon	Clone, hybrid, cultivar, population
	Infraspecific	Chemotypes (distinct populations within a species)
Physiology	Ontogenetic	Developmental changes (vegetative <> generative)
	Plant organ	Morphological differences (root, leaf, flower, seed)
Environment	Climate	Light, temperature, edaphic factors
	Origin	Latitude, height above MSL, country, continent
	Agriculture	Cultivation technique, fertilizer, irrigation, harvest time
Processing	EO isolation	distillation, extraction, maceration, pressing, enfleurage
	Storage	Effects of aging, ΔT, rH etc. on raw material or EO
	Adulteration/Standardization	EO blending prior to distillation/ directly into the EO.

Infraspecific and intervarietal differences can be observed in both morphology and chemical structures, which establish the basis for determining important chemically defined populations or chemotypes (Hay and Svoboda, 1993). Despite

aromatherapeutic demands, which require that the medicinal value of an EO be based on its complete composition rather than its constituent parts (Franchomme et al., 1990), one still has to consider the chemical specification of EOs with regard to toxic concentrations of single constituents (Tisserand and Balacs, 1995).

Quality controls of EOs

Usually, in the quality control of a substance, reference is made to its purity considering the percentage of the main component or the most interesting component from the point of view of the application. In the field of EOs, as complex mixtures of natural origin and of variable composition, the concept of purity is understood in the broad sense; in fact, more than the percentage of the individual components looks at the product as a whole. There are several types of controls on EOs, some based on the classic techniques and dictated by the Pharmacopoeia, others developed more recently and successfully applied to EOs (Noriega, 2014).

Physical Determinations

- Organoleptic examination: control colour, smell, and taste. The latter two are subjective, and considerable experience is needed. For smell, indicate the perceived note at the opening of the container and sum up the impressions perceived by slow evaporation on paper. For this purpose, a strip of absorbent paper is used which is about 15 cm long and 7/8 mm wide; the end of the strip is injected into the oil for about 2 cm and begins to smell to perceive the head notes. The subsequent smells are repeated at fifteen minute intervals, recording the smell variations for a time varying from one to several hours. The test should preferably be performed in parallel with a reference sample. The taste test, on the other hand, cannot be carried out on any EO, but it is reserved, for example, for citrus oils, mint, and anise, after dilution (2000 to 5000 times).
- Relative density: measured to the third or fourth digit, and specifying the temperature to which it is measured.
- Rotating Power: Many EOs contain constituents that have one or more carbon atom asymmetric. These substances, when crossed by polarized light, are able to rotate the plane either to the right or to the left.

- Refractive index: is the value of the ratio between the incidence and refraction angles of a beam passing from the air to the substance under consideration (Noriega, 2014).

Chemical Determinations

- Determination of the esters index: the index of esters is given by the number in milligrams of KOH (potassium hydroxide) needed to saponify the esters contained in 1 gram of the substance under consideration.
- Determination of the residue on the evaporation of the essences: constitutes the percentage by weight after evaporation at the bain-marie.
- Determination of the essence water: 10 drops of essence are mixed with 1 mL of carbon disulphide. The solution you get must remain clear.
- Determination of esters in the essences: heat 1 mL of essence with 3 mL of potassium hydroxide solution (100 g/L) in alcohol prepared at the time of use for 2 minutes. No crystals should be formed in the next 30 minutes, even after cooling.
- Determination of fatty oils: One drop of essence on filter paper is dropped; the drop must evaporate completely over the course of 24 hours without leaving any translucent or greasy stains.
- Determination of the solubility of the essences in alcohol: it is used to determine the quality of the essences and their components.
- Determination of 1.8-cineole in the essences: This is based on the determination of the crystallisation temperature that the orthochrosol assumes in the presence of a greater or lesser amount of eucalyptol. The 1.8-cineole values obtained are compared with those of the monograph of the EO being examined. If the values obtained in relation to the essays fall into those of the pharmacopoeia, it is possible to associate an official character with essence (Noriega, 2014).

Traditional Knowledge and Biopiracy

According to UNESCO definition, Traditional Knowledge (TK) is “the cumulative and dynamic body of knowledge, knowhow and representations possessed by peoples with long histories of interaction with their natural milieu. It is intimately tied to language, social relations, spirituality and worldview, and is generally held collectively”. As defined by the World Intellectual Property Organization (WIPO), it is

knowledge, know-how, skills and practices that are developed, sustained and passed on from generation to generation within a community, often forming part of its cultural or spiritual identity (WIPO, 2013). TK includes indigenous knowledge, folklore, and traditional medical knowledge and often used to develop commercial products such as new pharmaceuticals, herbal medicines, seeds, cosmetics, personal care and crop protection products, e.g. traditional medicine may be used to guide the screening of plants for medically active compounds. Knowledge about characteristics of plants having healing properties and technology of its use gives medicinal plants their social and economic value. This technology of use has been acquired through thousands of years of experience, trial and error and generation to generation refinement. As a result of this, age-old communities have developed their knowledge of the plant, animal and mineral resources to a grown-up and scientifically-sound technology, which reflects in old traditions of healing science like Ayurveda and Siddha. In addition to this, tribal, island and local ethnic communities have developed their own knowledge base about the flora, fauna and mineral wealth of their region (Sahai et al., 2007).

Biopirates, are pillaging a new kind of wealth, that of biodiversity and the TK and techniques of rural and indigenous peoples. Biopiracy can be defined as, “the misappropriation and commercialization of genetic resources and traditional knowledge of rural and indigenous people” (Mgbeoji, 2014). Pharmaceutical biopiracy is a term used generally to describe the legal practice by pharmaceutical companies exploiting the indigenous people’s TK of medicine. Ecuador and other developing countries are rich in bio-resources and TK are favourite targets and victims of biopiracy. Turmeric, neem and basmati rice were well known examples of biopiracy. Biopirates are mainly pharmaceutical, cosmetic and agrifood firms. Biopiracy of genetic resources and genetic materials are also noticed. They draw on biodiversity hotspots in order to create supposedly “innovative” products and guarantee their monopoly on them through the patent system. Such misappropriation of TK results in grant of patent for the invention to the “first–to–file” (the pharmaceutical company) rather than to the “first–to–invent” (the indigenous community). It involves making profit from freely available natural products (plants, spikes, leaves, etc.), by copying techniques used daily for generations by local peoples in order to feed or take care of themselves. Biopirates do not give any profit or proper benefit to local communities and TK holders (Sahai et al., 2007).

Conservation and sustainable use of medicinal plants

Medicinal plants are globally valuable sources of new drugs (Nalawade et al., 2003; Hamilton, 2004). There are over 1300 medicinal plants used in Europe, of which 90 % are harvested from wild resources; in the United States, about 118 of the top 150 prescription drugs are based on natural sources (Balunas & Kinghorn, 2005). Furthermore, up to 80 % of people in developing countries are totally dependent on herbal drugs for their primary healthcare, and over 25 % of prescribed medicines in developed countries are derived from wild plant species (Hamilton, 2004). With the increasing demand for herbal drugs, natural health products, and secondary metabolites of medicinal plants, the use of medicinal plants is growing rapidly throughout the world (Nalawade et al., 2003; Cole 2007).

A highly conservative estimate states that the current loss of plant species is between 100 and 1000 times higher than the expected natural extinction rate and that the Earth is losing at least one potential major drug every 2 years (Pimm et al., 1995). According to the International Union for Conservation of Nature and the World Wildlife Fund, there are between 50,000 and 80,000 flowering plant species used for medicinal purposes worldwide. Among these, about 15,000 species are threatened with extinction from overharvesting and habitat destruction and 20 % of their wild resources have already been nearly exhausted with the increasing human population and plant consumption (Ross, 2005). Although this threat has been known for decades, the accelerated loss of species and habitat destruction worldwide has increased the risk of extinction of medicinal plants, especially in China, India, Kenya, Nepal, Tanzania and Uganda (Hamilton, 2008; Heywood & Iriondo, 2003; Zerabruck & Yirga, 2012).

The conservation and sustainable use of medicinal plants have been studied extensively (Larsen & Olsen, 2007; Uprety et al., 2012). Various sets of recommendations have been compiled regarding their conservation, including the establishment of systems for species inventorying and status monitoring, and the need for coordinated conservation practices based on both in situ and ex situ strategies (Hamilton, 2004). For medicinal plants with increasingly limited supplies, sustainable use of wild resources can be an effective conservation alternative.

For medicinal plants with limited abundance and slow growth, destructive harvesting generally results in resource exhaustion and even species extinction (Larsen & Olsen, 2007; Baker et al., 2007). Therefore, the sustainable use of medicinal plants should be considered, and good harvesting practices must be formulated. Root and whole-plant harvesting is more destructive to medicinal plants (e.g. herbs, shrubs and trees) than collecting their leaves and flowers or buds (Table 4).

Table 4. Susceptibility of species to overharvesting regarding life forms and plant parts used

Life form	Percent (%)	Leave	Flower / Bud	Fruit / Seed	Bark	Root	Whole plant
Herb	52	Medium	Medium	High	None	High	High
Shurb	16	Low	Low	Low	High	High	High
Tree	22	Low	Low	Low	High	High	High

Source: Schippmann et al., 2002; Teklehaymanot & Giday, 2007.

For herbal drugs made of whole plants or roots, using their leaves as a remedy can be a benign alternative. For example, Wang et al., 2009, discovered that extracts from ginseng leaf-stems and roots have similar pharmacological activities, but ginseng leaf-stem has the advantage of being a more sustainable resource.

STRATEGY AND GOALS

EOs are the product of the secondary metabolism of plants and are generally a complex mixture of volatile mono and sesquiterpene hydrocarbons, and biosynthetically derived oxygenated materials. Other common constituents include phenylpropanoids derived from the shikimic acid pathway and their transformation products, and other fatty acid compounds and amino acid metabolites. Apart from these groups of major compounds, there are many other molecules that include nitrogen and sulphur compounds. For many years, our research team has been dedicated to the study and application of EOs, both for the particular biosynthetic path from which they derive and for the notable applications in the field of health (pharmaceutical, cosmetic, food) (Sacchetti et al. al., 2005).

The Amazon forest represents an invaluable source of aromatic plants that are rich in EOs. The purpose of this work is to select and evaluate medicinal aromatic plants, used in traditional practice (ethno-pharmacy) by the indigenous people of the Amazon in southern Ecuador. In this research, the following departments have taken part: the Department of Life Sciences of the University of Ferrara, the Salesian Polytechnic University (Ecuador) and the Centre for International Cooperation for promoting development of the University of Ferrara, Italy.

The PhD thesis was focused on the optimisation of extraction processes, the study of chemical components (GC-MS, NMR) and the evaluation of the biological activity (antioxidant, antimicrobial, antifungal and mutagenic) of EOs obtained from the following species of plants:

1. *Chenopodium ambrosiodes* (Chenopodiaceae)
2. *Dacryodes peruviana* (Burseraceaae)
3. *Eryngium foetidum (*Apiaceae)
4. *Persea americana* (Lauraceae)
5. *Piper carpunya* (Piperaceae)
6. *Schinus molle* (Anacardiaceae)
7. *Tagetes minuta* (Asteraceae)

The choice of species is the result of an “in progress study”, of which the present PhD is part having the overall objective to map the Amazonian biodiversity, guided by ethnopharmaceutical uses that characterise the tradition of the indigenous population of the Ecuadorian southern amazonic region.

The important thing regarding EOs is the fact that the aromaticity of plants and parts used in tradition is the first element of choice for health uses and therefore probably has a central role in the efficacy and safety of derived preparations.

The chemical characteristics of EOs make them a particularly effective tool in characterising biodiversity through a chemical fingerprint of Amazonian plant species.

The general research profile is reported below (Table 5), the plants collection was carried out in the Kutukù Mountain Range Forest, Ecuador.

Table 5. Diagram of the lines of work done during the PhD

In Ecuador	• Ethnobotanical research • Collection of plants • Extraction of EOs
In Italy	• Chemical characterisation (GC-MS, NMR) • Identification and isolation of major and unknown compounds • Biological activity (Antioxidant, antimicrobial, antifungal, mutagenic and cytotoxic)

MATERIALS AND METHODS

Materials

Chemical and standard compounds

Reagents: Hexan, methanol, ether, 2, 2,-diphenyl-1-picrylhydrazyl, anhydrous sodium, DMS (dymethylsulphoxide). All reagents and solvents were chromatographic grade and from Sigma-Aldrich.

Reference chemicals: (piperitone, limonene, estragole and α-Phellandrene) used to identify the constituents were obtained from Sigma-Aldrich Chemie GMBH Munich, Germany. While the compounds δ-3-carene, *trans*-2-dodecenal and *cis*-tagetone were isolated using silica gel column chromatography.

Positive controls

Doxorubicin
Chloramphenicol
Clotrimazole
Fluconazole
Thymus vulgaris EO
Thymol
Trolox

All culture media and positive controls were purchased from Biochek Comp and Sigma-Aldrich.

Instruments

The instruments used for different analyses are listed in Table 6 below.

Table 6. Instruments used for different analyses

Apparatus	**Model**	**Company**
GC-MS	MS-4000	VARIAN
Microplate reader	680 XR	BIO RAD
Digital balance	L.A 100	LABOR ALLIANCE
Autoclave	Steristeam 108	CDL

Laminar flow cabinet	Antares 48	STERIL
Colony counter	560	SUNTEX

Strains of microorganisms utilized to access the antimicrobial and antifungal activity of EOs

Bacterial strains

Gram (-)

Klebsiella oxytoca (ATCC 29516)

Pseudomonas aeruginosa (ATCC 17934)

Proteus vulgaris (ATCC 6361)

Gram (+)

Enterococcus faecalis (ATCC 29212)

Listeria grayi (ATCC 19120)

Micrococcus luteus (ATCC 9622)

Staphylococcus aureus (ATCC 29213)

Yeast strain

Candida albicans (ATCC 48274)

Fungal strains

Botrytis cinerea (kindly provided by Dr. Marina Collina, University of Bologna, Italy)

Pythium ultimum (CBS 29131)

Fusarium oxysporum (kindly provided by Sumitomo Chemical Italia S.r.l.)

Nannizea gypsea (CBS 286.63)

Trichophytum mentagrophytes (CBS 160.66)

Collection of Herbal samples

Fresh leaves and aerial parts of *Chenopodium ambrosiodes* (Chenopodiaceae), *Dacryodes peruviana* (Burseraceaae), *Eryngium foetidum* (Apiaceae), *Persea americana* (Lauraceae), *Piper carpunya* (Piperaceae), *Schinus molle* (Anacardiaceae)

and *Tagetes minuta* (Asteraceae) were collected at balsamic period (before flowering) in the Kutuku mountain range in the province of Morona Santiago near to the city of Macas in the southern amazon region in Ecuador and they were air dried in a room (in the shade). Finally the plants were identified by our botanist Dr. Carlos Ceron at the National Herbarium of Ecuador in Quito.

Distillation of fresh leaves and aerial parts to obtain EOs

The fundamental nature of steam distillation is that it enables a compound or mixture of compounds to be distilled at a temperature substantially below that of the boiling point of the individual constituent. EOs contain substances with boiling points up to 200°C or higher temperatures. In the presence of steam or boiling water, however, these substances are volatilized at a temperature close to 100°C, at atmospheric pressure.

The hydrodistillation was carried out with a Clevenger-type apparatus at Polytechnic Salesian University in Quito, Ecuador. The EOs were extracted by a 6 h steam distillation of fresh leaves and aerial parts (approximately 8 Kilograms) using 8 L of deionised water. Fresh leaves are placed in the plant chamber of the still and the steam is allows to pass through the herb material under pressure which softens the cells and allows the EO to escape in vapor form. The temperature of the steam must be high enough to vaporize the oil present, yet not so high that it destroys the plants or burns the EOs. Besides the steam tiny droplets of EO evaporates and travel through a tube into the still's condensation chamber. Here EO vapors condense with the steam. The EO forms a film on the surface of the water. To separate the EO from the water, the film is then decanted or skimmed off the top. The remaining water, a byproduct of distillation, is called floral water, distillate, or hydrosol. Finally the refractive index of EOs was measured at 68°F using the refractometer Abbe 60 and their density using a density metre DCE-DB 600. EO yield was determined on a volume to dry weight basis. The values for EO yield of three distinct distillations corresponding to the three different samplings were averaged. EO samples were then stored in airtight glass vials at 18 ± 0.5°C in the dark to prevent degradations prior to analyses. The EOs were deposited in the Department of Life Sciences and Biotechnology, University of Ferrara, Italy.

Gas Chromatographic analyses coupled with mass spectrometry detection (GC-FID)

The chemical characterisation of EOs was performed by gas chromatography coupled to mass spectrometry using a Varian CP-3800 gas chromatograph equipped with a capillary column Varian FactorFour VF-5ms (5% -phenyl- 95% -dimethyl-polysiloxane, length 30 m, 0.25 mm in diameter and film thickness 0.25 μm) coiled on a metallic support coupled to a Varian MS-4000 mass spectrometer with electronic impact ioniser, ion trap analyser and software provided by the NIST (National Institute of Standards and Technology) for component identification.

The experimental conditions were as follows: helium gas carrier (1 mL/min), ioniser voltage 70 eV, emission current 10 μA, sampling rate 1 scan/sec, m/z ratio range 40-400 Da, temperature of the trap 150°C, transfer-line temperature 300°C.

Samples were prepared with 10 μL of EO and dissolving in 1 mL of solvent, CH_3OH or CH_2Cl_2. Then 1 μL of each solution was injected into the injection chamber, maintained at 250°C.

The program used starts at a temperature of 55°C, rises up to 100°C in increments of 1 degree per minute, reaching 100°C, through a second ramp reaches 250°C at intervals of 5 degree per minute: at this temperature it remains constant for 15 minutes (Rossi et al., 2011).

They will first elute, and then reveal themselves as peaks, the lowest substances boiling and less related to the stationary phase. The result of the GC/MS analysis is a chromatogram showing the number of ions generated during the fragmentation, and the retention time expressed in minutes. Integrating the peaks corresponding to the ions of each identified analyte, a percentage area value is obtained with respect to the total area of the peaks, which is used as a semi-quantitative expression. Quantitative analysis was performed by gas-chromatographic analysis with flame ionisation detectors (FID, maintained at 300°C), to obtain a better proportionality between area and concentration.

The compounds were identified by comparing their n-alkane retention times containing 8-32 carbon atoms with those of the reference compounds, and comparing the spectra

produced by the fragmentation of molecules with known spectra present in literature and/or contained in specific databases published by NIST (National Institute of Standards and Technology). For the calculation of the relative retention time (Kovats index), a mixture of C_8 to C_{32} hydrocarbons (Sigma-Aldrich), previously injected into the system using the same method used to analyse EOs. The specific equation proposed for elution with temperature programmed is:

Equation 1. Elution with temperature programmed in GC-FID

$$I\ t = 100\left(\frac{t\ Ri^T - t\ Rz^T}{t\ R(z+1)^T - t\ Rz^T}\right) + Z$$

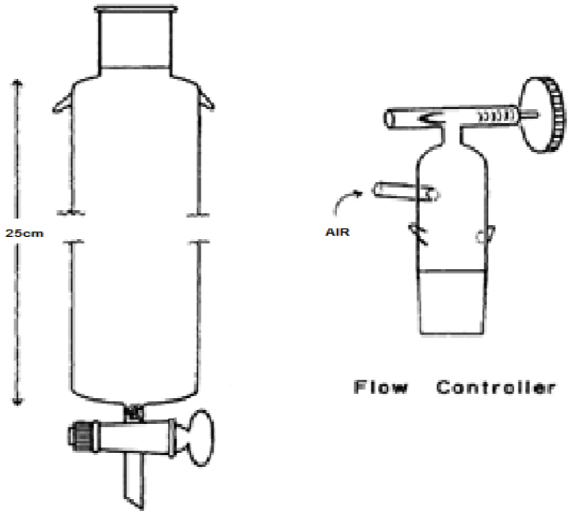
Where Ri is the retention time of the peak of interest, Rz is the retention time of the hydrocarbon which elutes immediately before the compound that gave the peak considered, R (z + 1) is the retention time of the hydrocarbon which elutes shortly after the compound that gave the peak considered and Z equals the number of hydrocarbon carbons related to the peak of interest.

Isolation of main and unknown compounds of EOs from Ecuador by flash column chromatography

The EOs were subjected to column chromatography in a silica gel (60–120 mesh) glass column. Approximately 350 mg of *T.minuta* EO and 450 mg of *E. foetidum* EO and 700 mg of *S. molle* EO were mixed with 8 g of silica gel and loaded onto the column of 25 × 3 cm and eluted with hexane: ethyl acetate (98:2) for the first two EOs and hexane: ethyl acetate (9:1) for the last EO. All the collected fractions were subjected to a TLC silica gel 60 F254 plate using hexane: ethyl acetate (98:2) as the developing solvent system and detected with an oxidant solution of phosphomolybdic acid in ethanol (1g/10 ml), as blue spots on yellow background. Fractions with similar Rf values were pooled and the organic solvent was removed by a rotary evaporator. The apparatus required for this technique consists of a set of chromatography columns and a flow controller valve (Figure 5). The column is a flattened bottom 18-in glass tube fitted with a Teflon stopcock and topped with a 24/40 glass joint. Columns without

fritted glass bed supports are generally preferred because they have significantly less dead volume than the standard fritted round-bottom variety. The flow controller valve is a simple variable bleed device for precise regulation of the elution rate and is constructed from a glass/Teflon needle valve and a standard 24/40 joint (Figure 5).

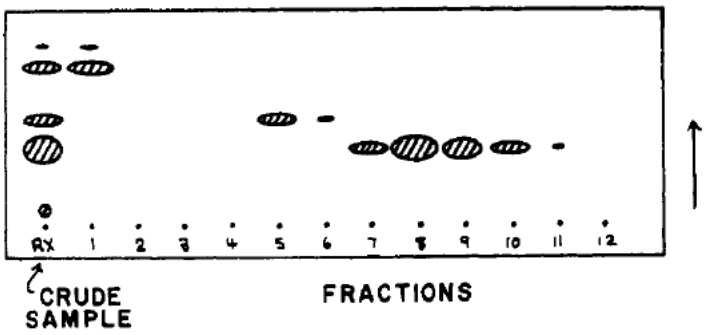
Figure 5. Flash chromatography apparatus



Source: Still et al, 1978

The time required to elute the desired components from the column is generally so fast (5–10 min) that we have abandoned automatic fraction collectors in favour of a simple rack holding forty 20 X 150 mm test tubes. Small fractions are typically collected early in the elution with larger ones being collected toward the end of the chromatography. Separated components are conveniently detected by spotting ~ 5 μL of each fraction along the long side of 7 X 2.5 cm TLC plates and then by developing the plate sideways. Heavier spotting may be required for small samples or highly retentive components such as the one shown in Figure 6. Finally, the collected samples were analysed by gas-chromatographic coupled with a mass spectrometry detection (GC-FID) analyses and nuclear magnetic resonance spectroscopy (NMR).

Figure 6. Typical separation on TLC plate



Source: Still et al, 1978

Antoxidant activity

DPPH test

An assay based on 1,1-diphenyl-2-picrylhydrazine radical (DPPH) spectrophotometry was used to evaluate the antioxidant activity of EOs using a variant of the method classic (Kedare and Singh, 2011). The experiment was conducted in 96-well microplates and a microplate reader (Microplate Reader 680 XR, Biorad), set at a wavelength of 515 nm, the closest to the peak of absorption of the radical form (Cheng et al., 2006).

Each well was charged with a 50% ethanol solution. Subsequently, EOs were added and serially diluted (with factor 1: 1) until 7 different concentrations were reached in a range of 15.63-1000 µg/mL. Similarly, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was placed as a positive control in a range of 1.25 to 79.9 mmol/L. Finally, a solution of DPPH (0.104 mM final) in 50% ethanol was added to each well. As a negative control, DPPH was used, with 50% ethanol used as an instrumental white.

The microplates were incubated at room temperature for 30 minutes and then analysed in the microplate reader. Values obtained were calculated as the equivalent milliliter in trolox per gram of EO and the IC 50 (EO concentration capable of eliminating 50% of the radical DPPH) by determining the percentage of racemic DPPH elimination according to the following formula:

Equation 2. Percentage of racemic DPPH elimination

$$\%\ DPPH\ eliminated = \left(1 - \frac{EOA - Ablank}{Acontrol - Ablank}\right) x\ 100$$

Where EOA is the absorbance of the well with the sample, Acontrol is the absorbance of wells with only DPPH and Ablank is the absorbance of the wells with 50% ethanol. In the same way, the main compounds of each EO were analysed, to verify if the antioxidant capacity of the oil is due to its majority compound.

ABTS test

The radical scavenging capacity of the samples for the ABTS (*2,2'-azino-bis*(*3-ethylbenzothiazoline-6-sulphonic* acid) radical cation was determined by the modified method proposed by Turoli et al. (2004). ABTS was generated by dissolving 0.0348 g of 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) in 10 ml of a sodium persulfate solution (2.45 mM), followed by storage in the dark at room temperature for 16 h before use. The mixture was diluted with ethanol to give an absorbance of 0.70 ± 0.02 units at 734 nm using a spectrophotometer (Thermo Spectronic Helios c, Cambridge, U.K.). For samples, solutions of the EOs from Ecuador in ethanol (100 µL) were allowed to react with fresh ABTS solution (900 µL), and then the absorbance was measured 6 min after initial mixing. *Thymus vulgaris* EO and trolox were used as a standard, and the capacity of free radical scavenging was expressed by IC_{50} (mg/L). IC_{50} values were calculated as the concentration required for scavenging 50% of ABTS radicals. The capacity of free radical scavenging (IC_{50}) was determined by the equation previously used for the DPPH method (Equation 2). In the same way, the main compounds of each EO were analysed, to verify if the antioxidant capacity of the oil is due to its majority compound.

Antibacterial Activity

Microdilution method

Antibacterial activity was analysed using the microdilution method in 96-well plates on Gram-positive and Gram-negative bacterial strains as reported in previous pages.

Starting from a broth culture solution (Tryptone Sota Broth - TSB-Oxoid LTD, Basinstoke, Hampshire, England, serial dilutions were made with 1: 1 dilution factor of the EOs, obtaining seven different concentrations between 31.25 and 2000 µg/mL (Fouotsa et al., 2013). To facilitate the solubilisation of EOs in aqueous environment, dimethylsulphoxide (DMSO, Fluka, Sigma-Aldrich) was added. For bacteria, however, *Thymus vulgaris* EO was used as a positive control (Sacchetti et al., 2005), while DMSO was used as a negative control. The bacteria were inoculated at a final concentration of $1x10^7$ UFC/mL in volumetric volume of 200 µL/well incubated for 8 hours at 37°C under constant stirring (110 rpm). Subsequently the 2,3,5-triphenyltetrazole chloride (TTC, Sigma-Aldrich) dye was added to a final concentration of 0.3 mg/mL. After one hour, the cultures were analysed to verify bacterial growth (red wells) or inhibition (yellow or transparent wells) and determine the minimum inhibitory concentration (MIC).

MIC was defined as the lowest concentration of EO and main compound of EO when tested as pure that causes growth inhibition (Rolli et al., 2016). The inhibitory activities were subdivided into strong, moderate and weak on the basis of the table, as reported on Table 7.

Table 7. MIC by microdilution method of EOs and their main components

Activity	MIC
Strong	≤ 500 μg/mL
Moderate	500 μg/mL < MIC < 5000 μg/mL
Weak	5000 μg/mL < MIC < 20000 μg/mL
Absent	> 20000 μg/mL

Source: Baser and Buchbauer, 2015.

Antifungal Activity

Agar vapour method

The analysis of EO vapours on fungal grown in agarised soil was used. Three phytopathogenic species (*Botrytis cinerea, Fusarium oxysporum* and *Pythium ultimum*) and two dermatophytic species (*Trichophyton mentagrophytes* and *Nannizzia gypsea*). Fungal cultures were obtained from mycelial disks taken from a

mother culture during stationary growth, then transferred to Petri dishes with a 90 mm diameter ground-based agarised soil and potato extract (PDA, Difco, Ditroit, MI, USA) for phytopathogens and sabouraud for dermatophytes (SDA, Oxoid LTD, Basinstoke, Hampshire, England).

At the centre of the lid of the plate, a sterile cellulose flask (6 mm diameter) impregnated with 10 µL of EO or principal compound of EO (1 mg/mL solution in DMSO). The plates were then sealed hermetically and incubated for 7-10 days in the dark at 26°C (Kumar et al., 2014; Romagnoli et al., 2016). As a negative control, a plate containing a DMSO impregnated disk was used while as a positive control, a plate containing a clotrimazole was used. The growth inhibition percentage was evaluated by measuring the diameter of the mycelium and comparing the negative control (considered as 100% growth). All samples were tested in triplicate.

Antifungal activity against *Candida albicans*

Microdilution assay

To determine the minimum inhibitory concentration (MIC), the microdilution method was used in accordance with "Clinical and Laboratory Standards Institute / National Committee for Clinical Laboratory Standards" (CLSI / NCCLS). EOs and fluconazole were serially diluted in 96-well wells. All samples were cultured in RPMI-1640 supplemented with 3- (N-morpholino) propane sulfonic acid (MOPS) at pH 7.0 and added Tween® 20 (Sigma-Aldrich, St. Louis, MO, USA) to improve solubility. EOs were diluted in a range of 48.8 to 6250 µg / mL and fluconazole in a range of 0.5 to 64 µg / mL. In each microwell, *Candida albicans* was inoculated at a final concentration of $2.5x10^3$ UFC / mL and incubated for 48 hours at 30 ° C (Pietrella et al., 2011).

The MIC was determined by direct observation of the turbidity of the culture medium. The MIC values were represented by the lowest concentration at which turbidity did not occur. Subsequently, the minimum concentration of fungicidal activity (MCF) was determined by transferring 10 µL of the culture medium of each well into a new well with saboraud right agar and incubating at 30 ° C for 48 hours. MFC was considered as the lowest concentration to which no cell growth was observed.

Mutagenic Activity

AMES Test

In order to investigate the possible mutagenic activity of EOs, an agar inclusion test was used, which is known as the Ames test (from its inventor, Maron and Ames, 1983). To this end, 4 different strains of *Salmonella typhimurium* (TA97a, TA98, TA100 and TA1535) were specifically engineered for this type of analysis, each with different sensitivity to potential mutagens and containing a mutation that makes them auxotrophic for histidine and biotin. All the samples were tested in petri plates (diameter of 90 mm) containing an agar base and low histidine and biotin concentrations, sufficient to keep the bacteria alive but not to allow replication. 100 mL of fresh bacterial culture grown overnight in liquid medium, 100 mL of dilute DMSO (5 dilutions in a range of 0.1 to 10 µg/plate) and 0.5 mL of phosphate buffer at pH 7.4 were contained in each plate or not a metabolic activator. The metabolic activator, added in half the total plates, consists of an exogenous metabolic system represented by a microsomal lysate of male rat liver, called S9, dissolved in a 0.154 mM KCl solution with the addition of NADPH and glucose-6-phosphate. This S9 mix is commonly used for the activation of mutagenic pro-mutagenic metabolites (purchased from Molecular Toxicology, Inc. Boone, NC, USA). Prior to its use, the S9 mix was filtered with a 0.45 µm porous filter from Millipore (Guerrini et al., 2009). As a negative control, 100 µL of DMSO was used with or without S9 mix; As a positive control, 2 µg/2-nitrofluorene platelets for TA97a, TA98 and TA1535 strains and 1 µg/sodium azide plate for TA100 strain were used for plates with activation from S9 2 µg/2-aminoantracene platelets were used for all species.

The plates were incubated for three days at 37°C and the colonies were counted manually (Colony Counter 560 Suntex, Antibioticos, Italy). The results were considered positive when the number of reverting colonies was at least twice that of the negative control (Maron and Ames, 1983).

In vitro cytotoxic activity

Cytotoxic activity is perform on HaCaT (human keratinocytes) purchased at “Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia-Romagna” (Brescia, Italy) and it is expressed as the concentration of sample that inhibits 50% of cell growth

(IC_{50}). The cell lines are grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 U/mL penicillin/streptomycin. They are grown in 75 cm^2 flasks in an air atmosphere characterized by 5% of humidity, 95% of CO_2 at 37 °C, until 80% confluence. Cytotoxic activity is determined by MTT colorimetric assay (Mosmann, 1983) as reflected by the activity of succinate dehydrogenase. Cells are seeded in 96-well plates at a density of 2 x 10^4 cells/well in 200 μL DMEM (Dulbecco's Modified Eagle Medium) complete medium; a period of 24 h is given for ensuring the cell attachment. Then the culture medium is replaced with 200 μL medium containing different concentrations (from 1 to 50 μg/mL) of 6-oxo-germacra-1(10)E,4(15)-diene, molecule present in *E. foetidum* EO. Negative control is exposed to vehicle only, corresponding to medium containing 2% FBS. As a positive control is used doxorubicin. After 24 h, the culture medium is removed and washed with PBS (phosphate-buffered saline) for twice. Lather 20 μL of MTT (5 mg/mL in PBS) is added in each well and the plates are incubated for 4 h at 37 °C. The medium is removed and replaced with 100 μL dimethylsulphoxide to dissolve the formazan crystals. The extent of MTT reduction is measured spectrophotometrically at 570 nm using a microplate reader. The experiments are performed in triplicate. IC_{50} values of anti-proliferative is assessed by logarithmic regression curves with 95% confident limits. Relative standard deviations and statistical significance (Student's t test; $p \leq 0.05$) are calculated using software STATISTICA 6.0 (StatSoft Italia srl).

RESULTS AND DISCUSSION

Botanical and pharmacognostic description of the investigated plant species

Section 1. Botanical and pharmacognostic description of the plant species, crude drugs, parts used and EOs

Piper carpunya

Image Courtesy: Royal Botanic Gardens Kew, 2017

Common name: Guaviduca

Scientific name: *Piper carpunya* Ruiz & Pav.

Taxonomy:

Class: Equisetopsida

Subclass: Magnoliidae.

Superorder: Magnolianae

Order: Piperales

Family: Piperaceae

Genus: *Piper*

Distribution: Costa Rica, Colombia, Ecuador and Peru

Description

P. carpunya, commonly known in Ecuador as "guaviduca", is part of the family of Piperaceae, a family distributed in tropical regions of both hemispheres including herbaceous plants and woody plants. The stem has vascular bundles arranged in multiple rings or distributed as in Monocotyledons. Leaves are simple and generally alternate. The bare flowers are gathered in dense axillary spines. The fruit is represented by a berry or a drupa. The piperaceae family includes 13 genera, including the best-known Piper genus, where the *Piper nigrum* species is one of the oldest drugs used in India, by Greeks and Romans in Europe.

It prefers sandy loam soils and altitudes between 1000 and 2000 m. It is an average height of two or three meters in length from the tree trunk of about three centimeters in diameter. The bark of the trunk is green, of the same colour as the young branches, while, if cut, inside it is brown and releases a very pleasant sweet odour. The leaves are simple, alternate, with the accented apex and the acute base of dark green colour. The flowers come in inflorescences of colour ranging from white to green 7-11 cm long and 3 mm in diameter. Unlike other Piper species, *P. carpunya* is used not only for fruits but also for its leaves - predominantly in decoction and infused, or as such - on which our attention has focused on the study of EOs.

Traditional/Ethnobotanical uses and efficacy

The leaves of *Piper carpunya* Ruiz & Pav. (Piperaceae), known by the popular name of “guaviduca” in Ecuador, are widely used in folk medicine in tropical and subtropical countries of South America, as an anti-inflammatory, anti-ulcer, anti-diarrheal and anti-parasitical remedy as well as an ailment for skin irritations (Díaz and Dorado, 1986).

P. carpunya is a plant that has been studied for a long time and there are several literature data that show the chemical characterisation of both EO and extracts with various organic solvents (Calle and Ferreira, 1973; Diaz and Dorado, 1986; Quílez et al., 2010 and Vargas et al., 2004).

Several biological activity studies have been conducted from traditional medical knowledge to verify their effectiveness. For example, anti-inflammatory properties

were confirmed using an animal model in experiments performed on rats where *P. carpunya* - hydrocarbon extracts and EO were extracted - was able to reduce the onset of carrageenin-induced oedema (De Las Heras et al., 1998). Gastroprotective activity has also been demonstrated in rats experiments, counteracting the onset of ulcers due to the administration of non-steroidal anti-inflammatory drugs (Trabadela et al., 2008). Other recent studies have investigated both anti-inflammatory and gastroprotective activity on various fractions of *P. carpunya* ethanol extracts as well as antibacterial activity against *Helicobacter pylori* (Quílez et al., 2010).

Toxicology

Phytochemical characterisation studies of EO have identified safrole as the main component, present at a level of 14.9% in leaves and 32.0% in the tops (Vargas et al., 2004). This substance is found in small quantities also in other food-producing plants, for example in *P. nigrum* in the preparation of black pepper. However, high doses are considered to be toxic to humans and have also been shown to have a potential carcinogenic effects (National Toxicology Program, 2011).

Distillation and chemical characterisation

Extraction of the EO of *P. carpunya* was carried out by steam distillation of the leaves, using optimised parameters to achieve maximum quantitative and qualitative yield. With regard to the chemical characterisation, this was achieved by gas chromatography coupled to flame ionization (GC-FID) and gas chromatography associated with mass spectrometry (GC-MS). In total, 42 compounds were identified for a total of 95.62% of total phytocomplex. As shown in the table 8, the most present compounds were piperitone (26.22%), limonene (9.48%), elemicine (7.22%) and β-phellandrene (5.62%) respectively. In the chemical characterisation of the EO derived from leaves carried out by the research group of Vargas (2004), a totally different composition was found, where the compounds present at a percentage greater than 5% are in the order: safrole (14.9%), 1,8-cineole (13.0%), α-terpinene (12.1%), p-cymene (10.9%), spathulenol (9.8%) and bicyclo-germacrene (6.7%). In the

characterisation performed in our laboratories, safrole was present only at 2.19%. The differences found are most likely due to environmental conditions that have differently influenced the quality of the two EOs examined and partly probably due to intraspecific genetic variability.

Table 8. Chemical composition of *Piper carpunya* EO

RT (min)	Area	%[a]	Compound	KI[b]
36,419	50300000	26,22	piperitone	1250
13,688	18190000	9,48	limonene	1023
56,424	13860000	7,22	elemicin	1551
13,78	10790000	5,62	β-phellandrene	1024
50,731	8670000	4,52	methyleugenol	1401
13,887	7659000	3,99	1,8-cineole	1026
12,771	7488000	3,90	α-terpinene	1012
13,369	6478000	3,38	p-cymene	1019
7,887	6459000	3,37	α-pinene	927
15,98	5748000	3,00	γ-terpinene	1051
27,14	4596000	2,40	3-thujen-2-ol	1168
40,39	4211000	2,19	Safrole	1283
51,641	2943000	1,53	p-cymen-7-ol acetate	1422
19,886	2811000	1,47	linalool	1099
10,971	2760000	1,44	myrcene	985
53,809	2611000	1,36	germacrene D	1474
9,95	2578000	1,34	sabinene	965
46,846	2543000	1,33	α-terpinyl acetate	1354
39,932	2453000	1,28	α-terpinen-7-al	1279
54,381	2262000	1,18	bicyclogermacrene	1488
10,214	2190000	1,14	β-pinene	970
12,035	2024000	1,05	α-phellandrene	1003
41,5	1720000	0,90	thymol	1293
47,414	1623000	0,85	eugenol	1361
42,323	1250000	0,65	carvacrol	1300
57,137	1242000	0,65	spathulenol	1574
34,411	1133000	0,59	neoisodihydrocarveol	1233
57,397	916522	0,48	globulol	1583
48,57	732350	0,38	α-copaene	1374
34,965	551844	0,29	cumin aldehyde	1237
51,018	507894	0,26	E-caryophyllene	1408
20,907	492581	0,26	1,3,8-p-menthatriene	1109
27,852	479442	0,25	terpinen-4-ol	1174
12,107	471223	0,25	δ-3-carene	1004
15,135	461688	0,24	*trans*-E-ocimene	1041
11,542	456512	0,24	δ-2-carene	995
56,725	356049	0,19	*trans*-nerolidol	1561
17,088	338949	0,18	*cis*-sabinene hydrate	1065
7,55	322477	0,17	α-thujene	920
49,588	296355	0,15	β-elemene	1387

50,414	283311	0,15	β-longipinene	1397
57,666	203417	0,11	viridiflorol	1592
		95.62%		

[a] Relative area percentage (peak area relative to total peak area %).

[b] Retention indices calculated on a Varian VF-5ms column.

Dacryodes peruviana

Image Courtesy: Royal Botanic Gardens Kew, 2017

Common name: Copal

Scientific name: *Dacryodes peruviana* (Loes.) H.J. Lam.

Taxonomy:

Class: Equisetopsida

Subclass: Magnoliidae

Superorder: Rosanae

Order: Sapindales

Family: Burseraceae

Genus: *Dacryodes*

Distribution: Brazil, Colombia, Ecuador, French Guiana, Guyana, Peru and Venezuela.

Description

D. peruviana is an evergreen tree that prefers high humidity environments and altitudes between 800 and 1000 m. It can grow up to 25 metres high and the trunk can reach a diameter of half a meter. It has a brownish-reddish bark and rounded

branches. The size of the leaves varies from 8 to 28 cm in length and 4 to 10 cm in width. Flowering inflorescence forms that can reach a length of over 20 cm and has oval petals of about 1.7x2 mm in size. Finally, the fruits are oval which are brown in maturation, have a single large seed inside them and average 2.5 cm in length. Fruits are edible and consumed abundantly by indigenous peoples (Grandtner and Chevrette, 2013).

Traditional/Ethnobotanical uses, efficacy and toxicology

The traditional main use of *D. peruviana* is certainly a food. In fact, it has sweet delicious fruits with a very pleasant taste. The Amazonian populations of Ecuador also burn the resin inside the houses because of its ability to act as a good repellent against insects, as well as for its pleasing perfume. There is also a second type of use of the resin and plant stem, which in a certain way can be associated with an ethnomedic use because it is used in the treatment of various disorders like the female reproductive system, jaundice, spleen diseases and liver problems (Ballesteros et al., 2016).

From a thorough search on databases and search engines at our disposal it was found that the *D. peruviana* scientific data in the literature are very small. The information contained herein is derived mainly from traditional Latin texts from Latin America and by orally reported information from locally-based indigenous people about the uses and customs that are passed on from generation to generation (Raffauf, 1990 and Rawcliffe, 1997). No information was found regarding phytochemistry, pharmacology and toxicology or biological activity in general. Hence, the complete originality of all the results we have achieved in this study.

Distillation and Chemical characterisation

As with other EOs, the extraction of *D. peruviana* was performed by steam distillation using optimised parameters for maximum quantitative and qualitative yield. As for the chemical characterisation of individual components, it was developed by gas chromatography coupled to flame ionization (GC-FID) and gas chromatography

associated with mass spectrometry (GC-MS). This led to identify 35 different compounds for 97.18% of the whole phytocomplex.

There is no chemical characterisation of *D. peruviana* EO in the literature. The only comparison that can be suggested is that of the EO of another member of the same genus, such as *D. edulis*, a plant grown in Africa in the wetlands of mountain for edible fruits and for the production of fixed oil. Even in this case, there have not been many studies conducted; only two have been found. Among these, the first scientific article reported as main compounds: α-pinene (fruits/seeds: 22.3/21.5%), β-pinene (13.7/19.7%), limonene (7.2/27.5%) and α-phellandrene (10.8/12.1%). The second one, however, contains sabinene (21.8%), terpinene-4-ol (19.8%), α-pinene (17.5%) and p-cymene (11.3%) extracted from the resin (Jirovetz et al.., 2008). From the analyses performed in our laboratories in Italy, the major compound was δ-3-carene, which was less than 0.3% in *D. edulis* studies. Less apparent discrepancies refer to the other main compounds found, such as α-pinene (6.03%), limonene (3.13%) and p-cymene (3.03%). The table 9 shows the identified products.

Table 9. Chemical composition of *Dacryodes peruviana* EO

RT (min)	Area	%[a]	Compound	KI[b]
12,277	87610000	70,01	δ-3-carene	1006
7,903	7550000	6,03	α-pinene	927
13,666	3916000	3,13	limonene	1023
13,37	3787000	3,03	p-cymene	1019
29,607	1884000	1,51	α-terpineol	1191
18,286	1564000	1,25	p-mentha-2,4(8)-diene	1080
10,235	1208000	0,97	β-Pinene	971
9,952	1183000	0,95	sabinene	965
27,163	1110000	0,89	p-mentha-1-5-dien-1ol	1168
9,889	1035000	0,83	verbenene	964
28,249	988806	0,79	m-cymen-8-ol	1178
31,191	857013	0,68	safranal	1205
50,091	744933	0,60	α-gurjunene	1393
17,958	737059	0,59	p-mentha-3,8-diene	1075
13,741	704833	0,56	1,8-cineole	1024
57,269	615989	0,49	caryophyllene oxide	1578
28,903	587916	0,47	p-cymen-8-ol	1184
27,854	549475	0,44	terpinen-4-ol	1174
51,032	529846	0,42	E-caryophyllene	1408
51,908	513173	0,41	*trans*-α-Bergamotene	1429
12,792	498064	0,40	α-terpinene	1012
8,637	405104	0,32	camphene	941
16,008	341441	0,27	γ-terpinene	1052
22,844	334189	0,27	*cis*-Limonene oxide	1127

53,55	287161	0,23	allo-aromadendrene	1468
18,919	265572	0,21	4-isopropenyltoluene	1087
26,761	259499	0,21	borneol	1164
57,154	242870	0,19	spathulenol	1575
13,036	240993	0,19	4-isopropyltoluene	1015
11,562	222438	0,18	δ-2-carene	996
52,73	202468	0,16	α-caryophyllene	1448
7,561	189925	0,15	α-thujene	921
48,585	164445	0,13	α-copaene	1375
30,512	153054	0,12	*trans*-isocarveol	1200
24,225	128806	0,10	camphor	1140
		97,18%		

[a] Relative area percentage (peak area relative to total peak area %).

[b] Retention indices calculated on a Varian VF-5ms column.

Eryngium foetidum

Image Courtesy: Royal Botanic Gardens Kew, 2017

Common name: Culantrillo

Scientific name: *Eryngium foetidum* L.

Taxonomy:

Class: Equisetopsida

Subclass: Magnoliidae

Superorder: Asteranae

Order: Apiales

Family: Apiaceae

Genus: *Eryngium* L.

Distribution: Belize, Bolivia, Canada, Cuba, Dominican Republic, China, Colombia, Costa Rica, Ecuador, El Salvador, French Guiana, Guatemala, Guyana, Haiti, Honduras, Jamaica, Mexico, Nicaragua, Puerto Rico and Panama.

Description

Eryngium foetidum L. is a perennial, erect, glabrous herb, 10-80 cm high with a taproot, a stem which is branched at the top, and leaves that are oblong-oblanceolate, serrate, 5-25 cm long and 1-4 cm wide. It is native to South America, but is also grown in China, India and Southeast Asian countries (Wong et al., 1994). The leaves can be eaten as a vegetable, raw or cooked, or used as food flavouring. This plant is called culantrillo in Ecuador, but culantro in most South American countries where it is widely used as food.

Traditional/Ethnobotanical uses and efficacy

The roots of the plant are reputed to be medicinal, given as a stomachic (Chopra & Chopra, 1956). Also, it is used as anti-convulsant, hypotensive, fungicide, antiseptic, germicide and viricide (Albornoz, 1980). It has been used to treat malaria, pock, gonorrhoea (Garca-Barriga, 1975), vomit, fever and flatulence (Lagos-Witte, 1997). In the Amazonian south of Ecuador, this plant is used as an abortive, slimming, aphrodisiac, diabetes and cholesterol lowering (Ballesteros et al., 2016).

A decoction from the leaves of *E. foetidum* has been evaluated for anti-inflammatory and analgesic properties (Saenz et al., 1997). The dry residue from the decoction of the plant was given orally and it inhibited the carrageenan-induced oedema in rat paw. *E. foetidum* also showed a topical anti-inflammatory effect because it inhibited swelling of mouse ear caused by 12-O-tetradecanoylphorbol acetate (TPA). Myeloperoxidase activity (MPO) was also assessed in the inflamed tissue.

The antibacterial activity of *E. foetidum* aerial part oil was tested by Thi & Thach in 2008, using a paper dish method on *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Shigella flexneri*. Interestingly, oil obtained from the microwave method exhibited stronger antibacterial activities than that generated from the conventional method.

Toxicology

The most common anti-nutrients in leafy vegetables are oxalic acid, phytic acid, nitrate, polyphenol, tannins and hydrocyanic acid (Akwaowo et al., 2000). In 100 g fresh leaves of *E. foetidum*, the nitrate, phytate, oxalate and saponin contents were found to be 71.5, 41.4, 43.2 and 60.0 mg, respectively (Singh et al., 2013). Gupta et al. (1986) also reported green leafy vegetables as rich in anti-nutritional factors like oxalates, tannins, dietary fibre and saponin. Phytate forms stable complexes with Cu^{2+}, Zn^{2+} Co^{2+}, Mn^{2+}, Fe^{2+} and Ca^{2+}. The level of oxalates observed in *E. foetidum* is less toxic to human health and is unlikely to pose any toxicity problems since phytate content is below the toxic levels of 2–5 g. Saponins reduce the uptake of certain nutrients including glucose and have hypocholesterolaemic effects (Price et al., 1987). According to Singh (2013), saponin content in this plant is estimated as 60.0 mg/100 g. A daily intake of 450 mg of oxalic acid has been reported to interfere with metabolism (Akwaowo et al., 2000) while intake of phytic acid of 4.0–9.0 mg per day reduces iron absorption by 4–5-fold in humans. Total oxalate content in leaf portion was estimated to be more than the safe limit of 2–5 mg.

Distillation and chemical characterisation

As with other EOs, the extraction of *E. foetidum* was performed by steam distillation of fresh leaves using optimised parameters for maximum quantitative and qualitative yield. As for the chemical characterisation of individual components, it was developed by gas chromatography coupled to flame ionization (GC-FID) and gas chromatography associated with mass spectrometry (GC-MS). This made it possible to identify 35 different compounds for 96.49% of the whole phytocomplex.

As shown in the table 10, the most present compounds were *trans*-2-dodecenal (20.40%), 2,3,6-trimethyl benzaldehyde (16.67%), α-pinene (11.86%) and dodecanal (6.27%) respectively. In the chemical characterisation of the EO derived from leaves

carried out by the research group of Cardozo (2004), a similar composition was found, where the main compounds present are in the order: 2,4,5-trimethylbenzaldehyde (27.7%), (E)-2-dodecenal (27.5%), and carotol (8.8%). The same thing happens in the works carried out Wong et al (2004), who had found (E)-S-dodecenal (59.72%), 2, 3, 6-trimethylbenzaldehyde (9.61%), and dodecanal (6.70%) while the group of Pino (1997) had found 2,4,5 trimethylbenzaldehyde (20.53%), hexadecanoic acid (12.05%), carotol (9.94%) and (E)-2-dodecenal (5.67%). As can be seen, the data found in this thesis show a great similarity with those found in other studies. It is important to mention that in the characterisation performed in our laboratories, carotol was present only at 2.86%.

Table 10. Chemical composition of *Eryngium foetidum* EO

RT (min)	Area	%[a]	Compound	KI[b]
53.867	6.80E+07	20,40	*trans*-2-dodecenal	1476
47.547	5.56E+07	16,67	2,3,6-trimethyl benzaldehyde	1362
8.040	3.96E+07	11,86	α-pinene	930
51.257	2.09E+07	6,27	dodecanal	1413
16.195	1.83E+07	5,50	γ-terpinene	1054
59.915	1.26E+07	3,78	*trans*-2-tetradecenal	1677
57.983	9.55E+06	2,86	carotol	1602
10.110	7.41E+06	2,22	sabinene	968
56.853	6.99E+06	2,10	spathulenol	1565
31.455	6.92E+06	2,08	decanal	1208
49.724	6.19E+06	1,85	β-elemene	1388
43.529	5.54E+06	1,66	2,3,4-trimethyl benzaldehyde	1314
11.142	4.93E+06	1,48	myrcene	988
53.095	4.51E+06	1,35	*cis*-2-dodecanal	1457
50.712	3.99E+06	1,20	*cis*-4-dodecenal	1400
53.028	3.83E+06	1,15	*trans*-β-farnesene	1456
13.844	3.37E+06	1.01	limonene	1025
20.111	3.16E+06	0,95	linalool	1101
51.176	2.83E+06	0,85	E-caryophyllene	1411
58.341	2.16E+06	0,65	tetradecanal	1616
58.626	2.13E+06	0,64	ledene oxide (II)	1627
13.558	2.11E+06	0,63	p-cymene	1022
54.671	2.06E+06	0,62	viridiflorene	1495
28.100	2.05E+06	0,61	terpinen-4-ol	1177
11.446	1.92E+06	0,58	mesitylene	993
50.841	1.85E+06	0,55	methyl-n-methyl anthranilate	1403
48.970	1.85E+06	0,55	daucene	1379
58.829	1.80E+06	0,54	ledene oxide (II) isomer	1635

41.368	1.63E+06	0,49	2-n-octylfuran	1292
50.496	1.54E+06	0,46	*trans*-2-undecenal	1398
15.306	1.37E+06	0,41	*trans*-ocimene	1043
49.286	1.32E+06	0,40	β-cubebene	1383
12.349	1.29E+06	0,39	δ-3-Carene	1007
55.100	1.20E+06	0,36	β-himachalene	1507
27.428	1.05E+06	0,32	p-mentha-1,5-dien-8-ol	1170
60.434	1.02E+06	0,31	β-sinensal	1697
10.389	1.02E+06	0,30	β-pinene	974
52.834	886498	0,27	α-humullene	1451
55.054	859839	0,26	α-E-E-farnesene	1506
19.517	780032	0,23	6-camphenone	1095
52.014	776605	0,23	*trans*-α-bergamotene	1431
53.563	663760	0,20	cis-muurola-4(14),5-diene	1468
54.045	611104	0,18	1-dodecanol	1480
54.509	587217	0,18	β-selinene	1491
19.956	534029	0,16	undecane	1100
18.506	530230	0,16	terpinolene	1082
53.331	521379	0,16	α-acoradiene	1463
9.080	427505	0,13	thuja-2,4(10)-diene	949
12.963	388133	0,12	α-terpinene	1014
24.530	315402	0,09	*cis*-verbenol	1143
54.989	294235	0,09	α-farnesene	1503
		96,49%		

[a] Relative area percentage (peak area relative to total peak area %).

[b] Retention indices calculated on a Varian VF-5ms column.

Chenopodium ambrosioides

Image Courtesy: Royal Botanic Gardens Kew, 2017

Common name: Paico

Scientific name: *Chenopodium ambrosioides* L.

Taxonomy:

Class: Equisetopsida

Subclass: Magnoliidae

Superorder: Caryophyllanae

Order: Caryophyllales

Family: Amaranthaceae

Genus: *Chenopodium*

Distribution: Argentina, Australia, Belize, Bolivia, Brazil, Chile, Costa Rica, Ecuador, Equatorial Guinea, French Guiana, Gabon, Guatemala, Guayana, Honduras, Kenya, Madagascar and Mexico.

Description

C. ambrosioides is an herbaceous annual plant or perennial shrub that can reach 1 m in height, characterised by a strong aromatic fragrance (Trivellato Grassi et al., 2013). It is a native plant from Central and South America and is capable of adapting to

various environmental conditions. It is considered a weed plant and is also spread in several African countries and in some areas of New Zealand (Bieski et al., 2015; Trivellato Grassi et al., 2013).

From the systematic point of view, *C. ambrosioides* belongs to the family of the Amaranthaceae, and Chenopodioideae subfamily. The grouping is known to be represented by species of agri-food interest, such as *Beta vulgaris* which, depending on the variety, is used for different purposes, from the production of sugar to the food use of beetroot, red (beetroot) and from feed for livestock feed. Another species of Chenopodioideae is *Spinach oleracea*, commonly known as spinach and *Chenopodium quinoa* (quinoa). The latter plays a key role in feeding the Andean populations, cultivated in the Andes for about 7000 years; following the Spanish conquest, its food use was denied, which was then rediscovered in the second half of the 20th century and since then it was exported and cultivated also in countries outside the Andean territory (Bazile et al., 2016).

The used part of *C. ambrosioides* consists of flowers and flowering summits, particularly aromatic, to which traditional ethnomedic uses are associated, which make the reason for the interest accrued in this study.

Traditional / Ethnobotanical uses and efficacy

Unlike the species with which *C. ambrosioides* shares the botanical family, this species does not have particular nutritional uses but is rather known for its ethnomedicine. *C. ambrosioides* is widely used in the ethnomedia environment, it is known to be mainly used in the form of teas, wraps and infusions for the treatment of inflammatory disorders, bruises, pulmonary infections and anti-inflammatory and antifungal (TrivellatoGrassi et al., 2013). Other uses relate to healing from bronchitis, tuberculosis and rheumatism (Kumar et al., 2007) and is also widely used as an aid in healing lesions, distortions, bone fractures and oedema, soothing localised pains, gastritis and disorder stomach, hepatitis, and intestinal infections (Bieski et al., 2015). Some less well known use of the plant involves the treatment of amenorrhea, dysmenorrhoea and bites of insects or bites of snakes (Song et al., 2015).

For *C. ambrosioides* there are numerous scientific evidence regarding different biological activities, which are particularly indicative for possible health uses consistent with modern disorders and pathologies. For example, the potential antimalarial effect

of the hydroalcoholic leaf extract was studied (Cysne et al., 2016) and the activity against *Helicobacter pylori* both in vitro and in vivo (Ye et al., 2015). For a long time, the activities of EO against dermatophytic fungi such as *Trychophyton mentagrophytes* and *Micosporum audouinii* (Kishore & Dubey, 1996), and also against other filamentous fungi such as *Aspergillus* sp., *Fusarium* sp. and *Colletotrichum* sp. (Jardim et al., 2008).

Toxicology

Toxicological studies conducted on hydrothermal extracts of *C. ambrosioides* in a murine model showed that there is no toxic effect at normal doses (comparable to those employed in traditional plant uses) (Pereira et al., 2010). Studies on methanol extracts also showed no toxicity either in rats or in saline Artemia (a small saltwater crustacean very sensitive to environmental pollutants and toxic substances) (Garcıa et al., 1997). However, considering the EO as a type of extract, there is some effect of potential probabilistic toxicity due to the presence of carvacrol. From analyses conducted using the three main components of EO, namely carvacrol, caryophyllene oxide and ascaridol, a potent toxic effect on mitochondria has emerged. This toxicity is associated with the anti-mythical properties of the plant known in the traditional medicine (Monzote et al., 2009) or those demonstrated at *Leishmania* sp. (Pastor et al., 2015).

Distillation and chemical characterisation

Extraction of the EO of *C. ambrosioides* from fresh leaves was done by steam distillation as provided by the pharmacopoeia, where the distillation parameters were optimised to obtain the greatest yield. Subsequently, its chemical characterisation was determined by gas chromatography coupled to flame ionization GC-FID and gas chromatography-mass spectrometry GC-MS. In total, 28 different components were identified representing 95.15% of total EO.

The most represented compounds are limonene (41.48%), *trans*-p-mentha-2,8-dien-1-ol (7.68%), *trans*-isocarveol (7.09%), *cis*-p-mentha-1(7), 8-dien-2-ol (6.04%) and p-cymene (5.36%). Limonene, which represents the major component, is a cyclic

monoterpene highly represented in the *Citrus* genus, where it is abundant in the fruit pericarp and gives it a characteristic citrus fragrance.

The chemical composition found in our laboratories is not in line with literature data, where the major composition is usually represented by ascaridole (Bossou et al., 2013; Harraz et al., 2015; Hu et al., 2015; Monzote et al., 2014; Pastor et al., 2015), which was not found in our sample. However, some studies point to the presence of limonene as a major component (Sagrero-Nieves and Bartley, 1995). *Trans*-p-mentha-2, 8-dien-1-ol and *cis*-p-mentha-1(7) 8-dien-2-ol have been frequently detected as minor components in EO in the above-mentioned literature. Table 11 shows the identified products.

Table 11. Chemical characterisation of *Chenopodium ambrosiodes* EO

RT (min)	Area	%[a]	Compound	KI[b]
13,687	61120000	41,48	limonene	1023
21,818	11310000	7,68	*trans*-p-mentha-2,8-dien-1-ol	1118
28,774	10450000	7,09	*trans*-isocarveol	1183
33,587	8895000	6,04	*cis*-p-mentha-1(7),8-dien-2-ol	1226
13,312	7897000	5,36	p-cymene	1019
12,715	7226000	4,90	α-terpinene	1011
23,293	4582000	3,11	*cis*-p-mentha-2,8-dien-1-ol	1131
53,801	4456000	3,02	γ-muurolene	1474
35,098	3390000	2,30	carvone	1238
34,368	2598000	1,76	neoiso-dihydrocarveol	1232
42,144	2282000	1,55	isoascaridole	1298
7,84	2154000	1,46	α-pinene	926
32,39	1906000	1,29	*trans*-carveol	1215
11,981	1817000	1,23	α-phellandrene	1002
30,027	1771000	1,20	*cis*-dyhydrocarvone	1195
55,254	1279000	0,87	γ-cadinene	1512
59,383	925465	0,63	α-cadinol	1656
22,829	857214	0,58	*cis*-limonene oxide	1127
13,845	728764	0,49	1,8-cineole	1025
8,657	645089	0,44	camphene	941
59,082	607169	0,41	tau-cadinol	1645
33,927	540317	0,37	cis-carveol	1229
51,01	478966	0,33	E-caryophyllene	1407
53,969	461066	0,31	ar-curcumene	1478
10,163	429100	0,29	β-pinene	969
54,376	235550	0,16	bicyclogermacrene	1488
6,607	174033	0,12	santene	903
25,821	146634	0,10	pinocarvone	1155
		95,15%		

[a] Relative area percentage (peak area relative to total peak area %).

[b] Retention indices calculated on a Varian VF-5ms column.

Persea americana

Image Courtesy: Royal Botanic Gardens Kew, 2017

Common name: Aguacate

Scientific name: *Persea americana* Mill.

Taxonomy:

Class: Equisetopsida

Subclass: Magnoliidae

Superorder: Magnolianae

Order: Laurales

Family: Lauraceae

Genus: *Persea*

Distribution: Argentina, Belize, Bolivia, Brazil, China, Colombia, Costa Rica, Ecuador, El Salvador, French Guiana, Gabon, Guatemala, Guayana, Honduras, Madagascar, Mexico. Panama, Paraguay, Peru, Philippines, Suriname, Trinidad & Tobago, United States and Venezuela.

Description

P. americana is a medium to large tree, 9-20 m in height. The avocado is classified as an evergreen, although some varieties lose their leaves for a short time before flowering. The tree canopy ranges from low, dense, and symmetrical to upright and

asymmetrical. Leaves are 7-41 cm in length and variable in shape (elliptic, oval, and lanceolate). They are often pubescent and reddish when young, becoming smooth, leathery, and dark green when mature. Flowers are yellowish green and 1-1.3 cm in diameter. The many flowered inflorescences are borne in a pseudoterminal position. The central axis of the inflorescence terminates in a shoot. The fruit is a berry, consisting of a single large seed, surrounded by a buttery pulp. It contains 3-30% oil (Florida varieties range from 3% to 15%). The skin is variable in thickness and texture. Fruit colour at maturity is green, black, purple, or reddish, depending on the variety. Fruit shape ranges from spherical to pyriform, and weighs up to 2.3 kg (Yasir & Kharia, 2010).

Traditional / Ethnobotanical uses and efficacy

The leaves of *P. americana* are used in the traditional medicine for an uric acid control, diarrhoea, digestion, liver, respiratory system (Gião et al., 2007). From the perspective of ethnopharmacy of the Aztec culture, the use of avocado seeds as sources of phytotherapeutic agents, they have traditionally been used to treat mycoses and parasitic infections. Furthermore, avocado seed preparations are known to have local anaesthetic effects that decrease muscle pain (del Refugio Ramos et al., 2004). Avocado, its English common name, also has medicinal uses for wound healing and to stimulate hair growth (fruit pulp), as an aphrodisiac and emmenagogue (fruits), and to treat dysentery and diarrhoea (DerMarderosian & Beutler, 2002). Finally, another use but in this case in the African ethnomedicine is to treat skin ulcers using an infusion of its leaves (Pesewu & Humber, 2008) and to treat infectious diseases, including sexually transmitted diseases but in this case, using a decoction of its leaves (Magasoouba, 2007).

The aqueous extract of *Persea americana* leaves produced a dose-dependent inhibition of both phases of formalin pain test in mice, a reduction in mouse writhing induced by acetic acid and an elevation of pain threshold in the hot plate test in mice. The extract also produced a dose-dependent inhibition of carrageenan-induced rat paw oedema. The results obtained indicate that the extract possesses analgesic and anti-inflammatory effects (Adeyemi & Ogunti, 2002). In addition, *P. americana* leaves have been reported to possess antifungal activity (Prusky, 1991). In addition, this plant, is a widely grown and consumed fruit that is high in nutrients and low in calories,

sodium, and fats. Studies have shown that phytochemicals extracted from the avocado fruit selectively induce cell cycle arrest, inhibit growth, and induce apoptosis in precancerous and cancer cell lines. Recent studies indicate that phytochemicals extracted with chloroform from avocado fruits target multiple signalling pathways and increase intracellular reactive oxygen leading to apoptosis (Ding et al., 2007).

Their antimicrobial potential was also studied by another investigation group; peels and seeds had higher amounts of phenolics and a more intense in vitro antioxidant potential than the pulp. Peels and seeds were rich in catechins, procyanidins, and hydroxycinnamic acids, whereas the pulp was particularly rich in hydroxybenzoic and hydroxycinnamic acids and procyanidins. The avocado materials also displayed moderate antimicrobial effects against Gram-positive bacteria. Extracts (70% acetone) from avocado peels and seeds were tested as inhibitors of oxidative reactions in meat patties. Avocado extracts protected meat lipids and proteins against oxidation with the effect on lipids being dependent on the avocado variety (Rodriguez-Carpena et al., 2011).

Toxicology

Different works have been done with *P. americana*, for example, the aqueous seed extract of *P. americana* is safe on sub-acute basis in rats but extremely high doses may not be advisable (Ozolua et al., 2009). In another investigation, in toxicity tests with *Artemia salina*, the hexane and methanol extracts from avocado seeds showed LC_{50} values of 2.37 and 24.13mg mL^{-1} respectively (Leite et al., 2009). Similarly, when they used aqueous extract with fresh lives, the administration of the extract up to 1 g/kg (intraperitoneally) and 10 g/kg (orally) did not produce signs and symptoms of toxicity in mice (Adeyemi & Ogunti, 2002). As far as EO is concerned, no toxicity data are recorded for *P. americana.*

Distillation and chemical characterisation

As with other EO, the extraction of *P. americana* was done by steam distillation using optimised parameters for maximum quantitative and qualitative yield. As for the chemical characterisation of individual components, it was developed by gas chromatography coupled to flame ionization (GC-FID) and gas chromatography

associated with mass spectrometry (GC-MS). This made it possible to identify 51 different compounds for 98.24% of the whole phytocomplex.

As shown in the table 12, the most present compounds were estragole (40.34%), isocaryophyllene (13.87%), β-pinene (6.32%) and sabinene (4.88%), respectively. In the chemical characterisation of the EO derived from leaves carried out by the research group of Ogunbino (2007), a different composition was found, where the main compounds present are E-caryophyllene (43.9%), valencene (16.0%), germacrene D (5.9%) and α-humulene (5.0%). In the characterisation performed in our laboratories, germacrene D was present at 5.15%. The differences found are most likely due to environmental conditions: Africa and South America, the first one for its dry characteristics and the second for having a more humid environment, as a result we have differently influenced the quality of the two EOs examined and partly probably by intraspecific genetic variability.

However, when we compare the compounds with a Mexican avocado, the group of Sagrero-Nieves & Bartley (1995), they were found a similar composition where the main compounds were: estragol (78.12%), α-cubenene (3.58%), methyl eugenol (3.37%) and β-caryophyllene (2.10%). The fact that the estragole is in a greater percentage in the Mexican avocado than in the Ecuadorian one, can be due to the fact of the different climatic conditions between these countries, mainly in Ecuador where the amazon, influence changes in all the genetic variability.

Table 12. Chemical characterisation of *Persea americana* EO

RT (min)	Area	%[a]	Compound	KI[b]
30.443	1.51E+07	40,34	estragole	1199
51.206	5.20E+06	13,87	isocaryophyllene	1412
10.431	2.37E+06	6,32	β-pinene	974
10.162	1.83E+06	4,88	sabinene	969
8.071	1.66E+06	4,43	α-pinene	930
53.948	1.18E+06	3,15	germacrene D	1478
55.374	1.00E+06	2,67	δ-cadinene	1516
52.868	928331	2,48	α-humulene	1452
57.393	668999	1,78	caryophyllene oxide	1583
50.932	569088	1,52	methyleugenol	1406
55.285	527385	1,41	cubebol	1513
14.129	522791	1,39	1,8-cineole	1029
15.370	506911	1,35	*trans*-ocimene	1044
54.581	378995	1,01	epi-cubebol	1493

48.794	282571	0,75	α-copaene	1377
11.198	282017	0,75	myrcene	989
13.917	243259	0,65	limonene	1026
49.639	227681	0,61	β-cubebene	1387
28.211	209035	0,56	terpinen-4-ol	1178
14.563	190955	0,51	*cis*-ocimene	1034
49.786	167455	0,45	β-elemene	1389
54.349	162082	0,43	*trans*-muurola-4(14),5-diene	1487
38.907	155539	0,41	geranial	1271
57.247	155462	0,41	germacrene D-4-ol	1578
59.499	140201	0,37	α-cadinol	1661
56.861	132570	0,35	*trans*-nerolidol	1565
59.138	130919	0,35	cubenol	1647
54.697	128720	0,34	α-muurolene	1495
16.270	128699	0,34	γ-terpinene	1055
46.757	118961	0,32	α-cubebene	1353
58.739	107191	0,29	1-epi-cubenol	1631
55.182	103743	0,28	γ-cadinene	1510
53.642	102331	0,27	*trans*-cadina-1(6),4-diene	1470
12.293	99501	0,27	α-phellandrene	1006
14.010	99216	0,26	β-phellandrene	1027
35.267	91647	0,24	neral	1240
55.507	83345	0,22	*trans*-calamenene	1520
41.876	79024	0,21	2-undecanone	1296
52.639	73252	0,20	*cis*-muurola-3,5-diene	1446
59.198	73217	0,20	tau-muurolol	1649
18.608	70703	0,19	terpinolene	1083
55.853	70096	0,19	*trans*-cadina-1(2),4-diene	1532
13.033	69774	0,19	α-terpinene	1015
53.771	64991	0,17	γ-muurolene	1473
7.728	64804	0,17	α-thujene	924
13.646	48001	0,13	p-cymene	1023
54.251	45914	0,12	β-selinene	1485
48.238	45292	0,12	cyclosativene	1370
53.057	40311	0,11	allo-aromadendrene	1456
59.271	38822	0,10	α-muurolol	1652
12.404	37716	0,10	δ-3-Carene	1007
		98,24%		

[a] Relative area percentage (peak area relative to total peak area %).

[b] Retention indices calculated on a Varian VF-5ms column.

Schinus molle

Image Courtesy: Royal Botanic Gardens Kew, 2017

Common name: Molle

Scientific name: *Schinus molle* L.

Taxonomy:

Class: Equisetopsida

Subclass: Magnoliidae

Superorder: Rosanae

Order: Sapindales

Family: Anacardiaceae

Genus: *Schinus*

Distribution: Argentina, Bolivia, Brazil, Chile, Colombia, Costa Rica, Ecuador, El Salvador, Guatemala, Honduras, Mexico, Nicaragua, Paraguay, Peru, South Africa, United States and Uruguay.

Description

Among the most popular plants known as source of the spice, Schinus species are characterised by pungent-smell EOs concentrated especially in fruits. EOs are produced and stored in the structures of the plant such as secretary cells, tissue, and glandular trichomes (Dell and McComb, 1979). The genus Schinus L. (Anacardiaceae) is native to South America particularly to the coast of Brazil (Barbosa and others 2007) and includes approximately 29 species (Barkley, 1957). Two species (*S. molle* L. and

S. terebinthifolius Raddi) were introduced to North Africa, in particular to Tunisia. They are dioecious and female trees that produce large crops of small bright pink berries arranged in bunches on pendulous stems. *S. molle* is an evergreen medieval sized tree that reaches approximately 8 m in height. The trunk can reach a diameter of 40 cm and the bark, when sliced, leaves a collapsed latex. It has leaves and pendulum branches and the composite leaves have a length of about 25 cm, consisting of numerous linear-lanceolate leaves with serrated margins. The flowering develops small flowers arranged in much branched flowering inflorescences. The fruits finally have a diameter of 7-10 mm (Orwa et al., 2009).

Traditional / Ethnobotanical uses and efficacy

S. molle are widely used in traditional medicine. In popular medicine, it is used for its astringent, diuretic, antispasmodic properties among others. In pest control, it is used as a fumigant, repellent, and ovicide (Ruffínengo et al., 2005; Ferrero et al., 2006). In addition, *S. molle* was used as antibacterial, topical antiseptic, digestive, and purgative diuretic (Duke, 1992), for toothache, wound healer, rheumatism, and menstrual disorders, also as stimulant and antidepressant (Machado et al., 2007), for respiratory and urinary infections (Perez & Anesini, 1994), and as analgesic and central depressant (Barrachina et al., 1997).

In Costa Rica, *S. molle* is occasionally used as an ornamental garden tree but in other countries this plant is utilised popularly for many ailments. It is used as anti-rheumatic, antiseptic, anti-inflammatory, antifungal, antibacterial, cicatrizant and also for the treatment of skin disorders (Gupta, 1995; Ruffa et al., 2002). Finally, thanks to the pleasant fragrance, the fruit is also used to prepare syrups or aromatic drinks (Taylor, 2005).

S. molle is a well-studied plant, given its wide use in the ethnomedic and culinary field and it is wide spread. Starting from the traditional information, recent studies have demonstrated an antidepressant effect of hexane extracts of *S. molle* leaves by tail suspension tests. These extracts had effects common to those of the drugs used as controls and demonstrated a possible mechanism of action involving interactions with serotoninergic, noradrenergic and dopaminergic systems (Machado et al., 2007).

Fruit juice extracts of *S. molle* have proved to be excellent against numerous parasitic plant insects, so several studies have been carried out to investigate the safety of herbivorous mammals and humans. For example, rats studied potentially toxic effects both acute (giving 2 g/kg body weight) and subacute (1 g/kg body weight) observing both behavioural changes and physiological changes: these studies have shown only a stimulating effect, increasing excitement. No morphological changes were observed at histological level. At the state of art of knowledge, *S. molle* extracted for their possible application in agriculture as foetal substances were found to be substantially safe with respect to possible environmental and human impact (Ferrero et al., 2007).
Other studies have shown a moderate or strong antioxidant activity of certain substances present in leaves methanol extracts, hyperin, 2-α-L-ramnopyranosyl-iperin and 6'-O-gallatin hyperamine (Marzouk et al. 2006). Methanol extracts have also confirmed the antimicrobial effect provided by traditional medicine. Extracts of leaves, flowers and bark of *S. molle* have been shown to be effective on clinical and commercial strains of *Staphylococcus aureus* and several species of *Candida* spp., As well as reconfirming antioxidant activity (Salazar-Aranda et al., 2011).

The EO obtained from the leaves has also showed to be effective on filamentous fungi; for example, it shows a strong activity against fungi responsible for dermatomyces in animals (*Cladiosporum* sp., *Epidermophyton floccosum*, *Fonsecaea pedrosi, Geotrichum candidum, Histoplasma capsulatum, Micosporum* sp., *Nocardia* sp., *Phialophora jeanselmei, Sporotrichum schenckii* and *Trichophyton* sp.) and in mushrooms that affect plants and foods (*Cladiosporum* sp., *Lunar curvularia* and *Fusarium moniliforme*) (Dikshit et al., 1986).

Toxicology

Investigations on tumour cell lines revealed a cytotoxic effect of *S. molle*'s EO, which induces cell apoptosis, indicating it as a possible candidate for anti-tumoral action (Diaz & Dorado, 1986). To confirm this, other recent studies have shown efficacy on several tumour cell lines, particularly those of breast cancer and leukemic lines where there was a cytotoxic effect linked to an apoptotic mechanism (Díaz et al., 2008).
Toxicological studies conducted on rats have shown that prolonged contact with ethanol and hexane extracts causes reversible skin irritation, which disappears after 48 hours, as well as causing transient excitatory effect (Bras et al., 2011). If the

extracts were administered on the diet (1 g/kg body weight) for 90 days, a decrease in lymphocyte count and total cholesterol levels was observed, while no morphological changes were observed in various organs; this suggested promising aspects of using these extracts in the treatment of dyslipidaemia (Bras et al., 2010). Further studies have shown a strong toxicity of aqueous and alcoholic extracts against a plant parasitic insect such as *Xanthogaleruca luteola*, as it further emphasises the important potentialities of this species, even in the context of agricultural treatments, today more than ever in research of eco-compatible, eco-sustainable and at the same time effective (Huerta et al., 2010).

Distillation and chemical characterisation

Extraction of the EO of *S. molle* was done by steam distillation using optimised parameters to obtain maximum quantitative and qualitative yield. As for the chemical characterisation of individual components, it was developed by gas chromatography coupled to flame ionization (GC-FID) and gas chromatography associated with mass spectrometry (GC-MS). 53 compounds were identified for a total of 97.47% of total phytocomplex. As shown in the table 13, the most present compounds were α-phellandrene (13.62%), δ-cadinene (9.95%), D-limonene (6.51%), β-phellandrene (5.90%) and α-cadinol (5.62%).

In the literature there are many chemical characterisation data of EO of *S. molle*, in some cases quite overlapping, in other completely different cases. Often in this EO a high content of myrcene, α- and β-phellandrene and limonene have been found (Baser et al., 1997; Bendaoud et al., 2010; Bernhard et al., 1983; Díaz et al., 2008 Hayouni et al., 2008; Huaman et al., 2004; Maffei and Chialva, 1990). In contrast, a study in which the major compound appears to be p-cymene, present for as much as 69.39% of the total (Abdel-Sattar et al., 2010).

The composition of the EO we have studied is in line with what has been reported in most of the literature regarding α- and β-phellandrene and limonene content, while myrcene is present in smaller quantities (1.95%).

Table 13. Chemical characterisation of *Schinus molle* EO

RT (min)	Area	%[a]	Compound	KI[b]
12.190	2.42E+07	13,62	α-phellandrene	1005
55.345	1.77E+07	9,95	δ-cadinene	1515
13.811	1.16E+07	6,51	D-limonene	1025
13.903	1.05E+07	5,90	β-phellandrene	1026
59.444	1.00E+07	5,62	α-cadinol	1659
58.073	8.89E+06	5,00	6-oxo-germacra- 1(10)E,4(15)-diene	1606
54.453	6.73E+06	3,78	bicyclogermacrene	1490
56.349	6.17E+06	3,47	elemol	1548
59.143	5.57E+06	3,13	tau-muurolol	1647
8.821	5.28E+06	2,97	camphene	944
53.877	5.04E+06	2,83	germacrene D	1476
60.443	4.92E+06	2,77	eudesm-7(11)-en-4-ol	1697
51.108	4.74E+06	2,67	E-caryophyllene	1410
13.487	4.43E+06	2,49	p-cymene	1021
8.002	4.26E+06	2,40	α-pinene	929
59.082	4.15E+06	2,33	tau-cadinol	1645
54.632	4.14E+06	2,33	α-muurolene	1494
55.108	3.59E+06	2,02	γ-cadinene	1507
11.081	3.48E+06	1,95	myrcene	987
58.801	3.15E+06	1,77	γ-eudesmol	1634
57.183	2.82E+06	1,58	germacrene D-4-ol	1576
54.276	1.77E+06	1,00	γ-amorphene	1485
57.723	1.72E+06	0,97	viridiflorol	1593
49.675	1.58E+06	0,89	β-elemene	1388
52.792	1.47E+06	0,83	α-humulene	1450
53.711	1.38E+06	0,78	γ-muurolene	1472
45.069	1.24E+06	0,70	δ-elemene	1333
50.498	1.07E+06	0,60	longifolene	1398
55.920	903178	0,51	α-cadinene	1534
56.957	893965	0,50	ledol	1568
53.573	838477	0,47	*trans*-cadina-1(6),4-diene	1469
7.514	777932	0,44	α-thujene	920
58.676	667381	0,38	1-epi-cubenol	1629
39.846	636350	0,36	bornyl acetate	1279
10.342	600478	0,34	β-pinene	973
57.467	595100	0,33	globulol	1585
53.102	578650	0,33	*cis*-muurola-4(14),5-diene	1457
52.977	574842	0,32	*allo*-aromadendrene	1454
52.558	525904	0,30	*cis*-muurola-3,5-diene	1444
55.783	524179	0,29	*trans*-cadina-1(2),4-diene	1530
54.168	372580	0,21	*cis*-β-guaiene	1483
59.229	370781	0,21	α-muurolol	1650
55.455	3.61E+05	0,20	zonarene	1519
58.324	350550	0,20	1,10-di-epi-cubenol	1615
48.664	267684	0,15	α-copaene	1376

56.761	256230	0,14	β-calacorene	1562
55.219	254783	0,14	cubebol	1511
52.019	244924	0,14	aromadendrene	1431
56.080	226183	0,13	α-calacorene	1539
62.224	208110	0,12	γ-eudesmol acetate	1774
57.513	198489	0,11	gleenol	1586
57.333	195459	0,11	caryophyllene oxide	1581
18.418	191140	0,11	terpinolene	1081
12.902	174276	0,10	α-terpinene	1014
		97,47%		

[a] Relative area percentage (peak area relative to total peak area %).

[b] Retention indices calculated on a Varian VF-5ms column.

Tagetes minuta

Image Courtesy: Royal Botanic Gardens Kew, 2017

Common name: Tsinzu

Scientific name: *Tagetes minuta* L

Taxonomy:

Class: Equisetopsida

Subclass: Magnoliidae

Superorder: Asteranae

Order: Asterales

Family: Asteraceae

Genus: *Tagetes*

Distribution: Argentina, Bolivia, Brazil, Chile, China, Ecuador, Mexico, South Africa, and United States.

Description

Tagetes minuta is an annual herbaceous plant belonging to the Asteraceae, the richest family among the Angiosperms as a number of genus (around 1600) and species (around 23,000) spread all over the world. This family is composed of herbaceous and shrubby plants, very rarely arboreal. Leaves, generally alternate, are simple, whole or toothed. The flowers are typically assembled in end caps or axillaries and can be very different or very similar. In many species, peripheral flowers, called rayon flowers, are feminine or sterile, while central flowers, are hermaphrodites. The fruit is an achene, often topped by a flying organ.

Tagetes minuta has been used as a source of EO for the flavouring in the food industries. The powders and extracts of *Tagetes* are rich in the orange-yellow carotenoid and are used as a food colourant in foods such as pasta, vegetable oil, margarine, mayonnaises, salad dressing, baked goods, confectionery, dairy products, ice cream, yogurt, citrus juice, mustard and as a colourant in poultry feed (Zhang et al. 2009; Nerio et al. 2010).

Traditional / Ethnobotanical uses and efficacy

T. minuta has many uses in traditional medicine. This plant grows in temperate regions of South America (Chamorro et al., 2008). Depending on the country, its uses may vary. In Argentina and Ecuador, and with leaves, a decoction is prepared to treat gastric and intestinal disorders (Zardini, 1984). *T. minuta* is also used as a condiment especially in Chile and Argentina, which is very typical in rice and stew salads, or is used in the preparation of refreshing drinks (Parodi, 1959). Commercial crops, especially for the preparation of perfumes and for the extraction of EO, are very common, the "Tagete oil", of which Brazil is the world's first producer (Meshkatalsadat et al., 2010).

T. minuta is also extensively used medicinally as a condiment and herbal tea in a wide variety of fields in its native region and as a popular traditional folk remedies or in the complementary and medical therapy. *T. minuta* has several medical benefits such as remedy for colds, respiratory inflammations, stomach problem, anti-spasmodic, anti-

parasitic, anti-septic, insecticide, and sedative. It is used for chest infections, coughs and catarrh, dilating the bronchi, facilitating the flow of mucus and dislodging congestion and can be used in cases of skin infections. It also has a healing effect on wounds, cuts, calluses and bunions (Gillij et al. 2008; Rahimi et al. 2010; Maity et al. 2011). *T. minuta* also are reported to possess anticancer and anti-ageing properties (Block et al., 1992).

Tagetes minuta L. (Asteraceae) has been effective in controlling Coleoptera (8), mosquitoes and other insects (Weaver et al., 1997), helminth (Oduor-Owino & Waudo, 1994), bacteria (Tereschuk et al., 1997) and fungi (Zygadlo et al., 1994).
Another work showed a certain degree of toxicity in relation to *Varroa destructor* when mites are pulverised with solutions of this oil in low concentrations (Ruffinengo et al., 2001). Finally an additional work shown that *T. minuta* the Asteraceae family, has fungitoxic effects (Rozwalka et al., 2008), activity against *Anopheles stephensi* larvae (Hadjjiakhoondi et al., 2005), antibacterial activity (De Souza et al., 2000) and effect on *H. contortus* egg and larvae (Macedo et al., 2012).

Toxicology

There are no medical studies of *T. minuta* EO on humans but some have been conducted with other plants of the *Tagetes* genera, showing efficacy and being safe for health (Meshkatalsadat et al., 2010). Recently, however, by the regulations introduced by the Scientific Committee on Consumer Safety, the use of EO of *T. minuta* in quantities of less than 0.01% in cosmetic and perfume products was imposed except for rinse products such as shampoo and foam bath, due to the phototoxicity found. However, it is totally excluded from its use in solar products (SCCS) and Coenraads, 2016.

Extraction and Chemical characterisation

Extraction of the EO of *T. minuta* was done by steam distillation using optimised parameters to obtain maximum quantitative and qualitative yield. As for the chemical characterisation of individual components, it was developed by gas chromatography coupled to flame ionization (GC-FID) and gas chromatography associated with mass spectrometry (GC-MS). 17 compounds were identified for a total of 98.43% of the total

phytocomplex. As shown in the table 14, the compound with a clear abundance is *cis*-tagetone, present at 62.19%, followed by *cis*-ocimene (20.70%) and dihydrotagetone (8.05%) which together with *trans*-tagetone (3.64%) account for almost 95% of the total phytocomplex.

T. minuta EO as well as other oils can vary drastically in the composition depending on the environmental conditions in which the plant is developed, as well as the period of plant growth and genetic factors. From studies in which samples from different regions of the world have been compared, some compounds have been identified that may prevail in concentration compared to others.

In many cases, the major component is *cis*-β-ocimene for example in 62,8% (Eguaras et al., 2005), 38,6% (Hetlelyi et al., 1986) especially when considering flowers as a source of extraction, with regard to leaf rather often the presence of dihydrotagetone (Shirazi et al., 2014; Baser & Malyer, 1996). Our EO sample is basically in line with the descriptions in the literature because our main compound, *cis*-tagetone, 62.19%, is very similar to that found by Marotti et al (2004) that is, *cis*-tagetone at 58%.

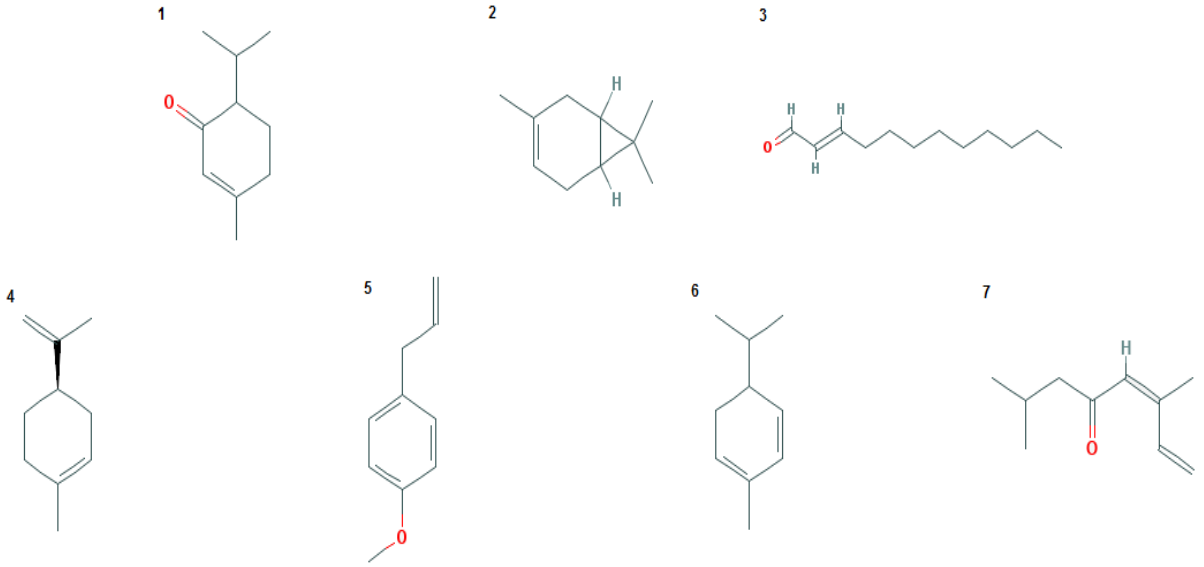
Table 14. Chemical characterisation of *Tagetes minuta* EO

RT (min)	Area	%[a]	Compound	KI[b]
25,255	24900000	62,19	*cis*-tagetone	1150
14,38	8289000	20,70	*cis*-Z-ocimene	1032
15,663	3225000	8,05	dihydrotagetone	1047
24,517	1458000	3,64	*trans*-tagetone	1143
51,078	381069	0,95	E-caryophyllene	1409
22,659	272740	0,68	allo-ocimene	1125
4,878	148715	0,37	2-methyl-, butanoic acid, ethyl ester	843
5,007	140405	0,35	ethyl isovalerate	848
54,986	91619	0,23	α-bisabolene	1503
54,419	82445	0,21	bicyclogermacrene	1489
15,221	74850	0,19	*trans*-E-ocimene	1042
22,972	70837	0,18	epoxyocimene	1128
52,763	68917	0,17	α-caryophyllene	1449
12,131	68881	0,17	α-phellandrene	1004
27,058	51195	0,13	Borneol	1167
48,836	44928	0,11	β-maaliene	1378
34,174	41553	0,10	*cis*-ocimenone	1231
		98.43%		

[a] Relative area percentage (peak area relative to total peak area %).

[b] Retention indices calculated on a Varian VF-5ms column.

Figure 7. Chemical structures of main components from Ecuadorian EO.



Note: (1) piperitone, (2) δ-3-carene, (3) *trans*-2-dodecenal, (4) limonene, (5) estragole, (6) α-phellandrene, and (7) *cis*-tagetone

Isolation of main and unknown compounds of EOs from Ecuador

Some of the main compounds (Figure 7) were purchased (piperitone, δ-3-carene, limonene, estragole and α-phellandrene) while c*is*-tagetone and *trans*-2-dodecenal were isolated to analyse the biological properties of individual compounds. In our laboratory, we have used flash chromatography as a faster technique for the routine purification of complex mixture of EO. After the flash chromatography of *E. foetidum* and *T. minuta* EO we collected 30 mg of *trans*-2-dodecenal in the fractions 96 – 108 and 9 mg of *cis*-tagetone in the fractions 65 – 88. GS-MS and H^1NMR characterisation are shown in figure 8 and figure 9 compared with literature data (William et al., 1985). The *trans*-2-dodecenal was identified by Forbes et al., in 2014 as an anthelmintic compound in *E. foetidum* extract while for *cis*-tagetone there have been no studies about its bioactivity.

Finally, after the flash chromatography of *S. molle* we separated 15 mg of an unknown GC-MS compound in the fraction 15. ^{1}H, ^{13}C NMR, DEPT and COSY, HMQC and HMBC characterization elucidated its chemical structure (Figure 10): 6-oxo-germacra-1(10)E,4(15)-diene, a compound described in literature, but no studied for its biological properties (Zdero et al., 1989).

Figure 8. *trans*-2-dodecenal by flash chromatography: GC-MS and NMR characterisation

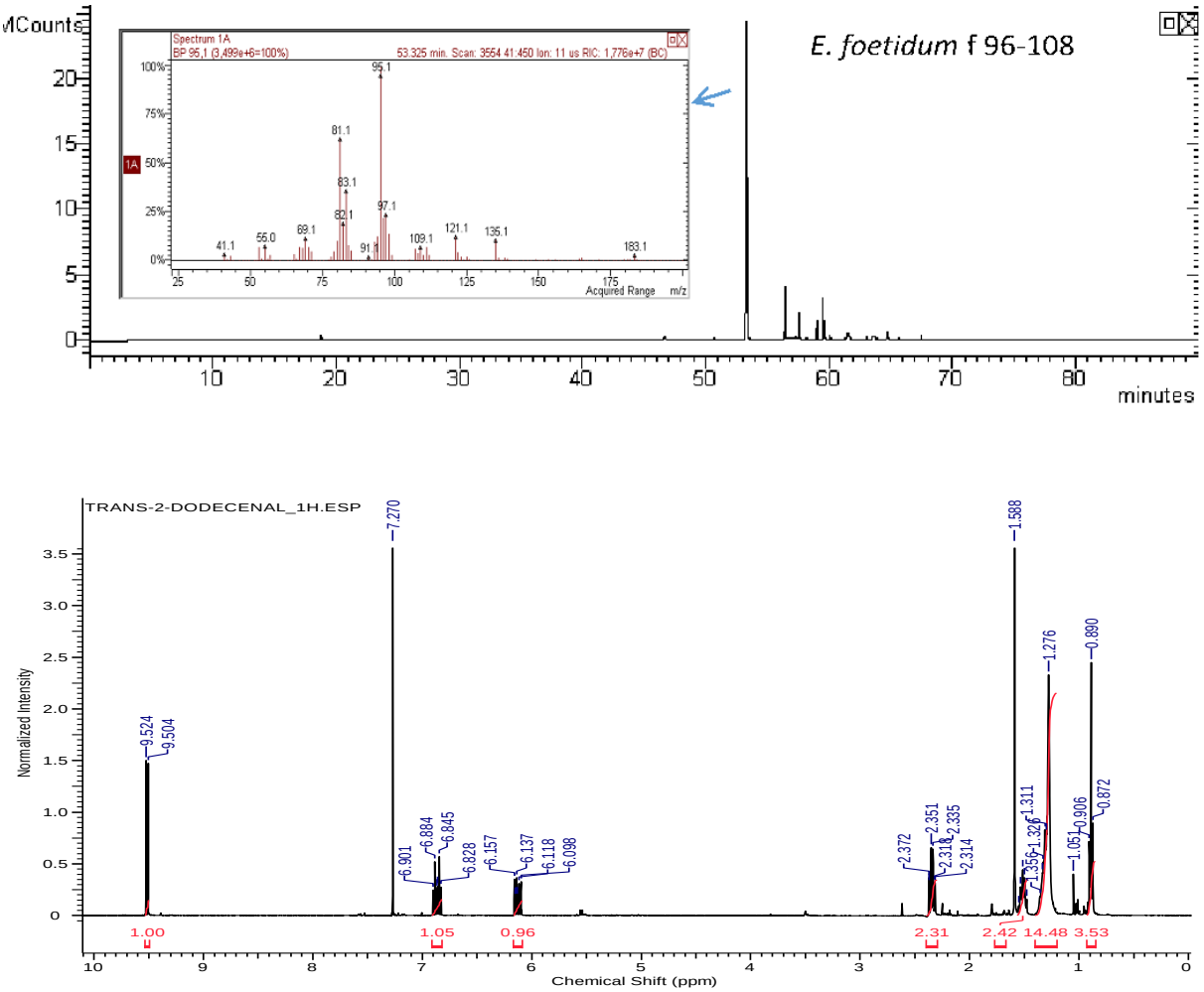
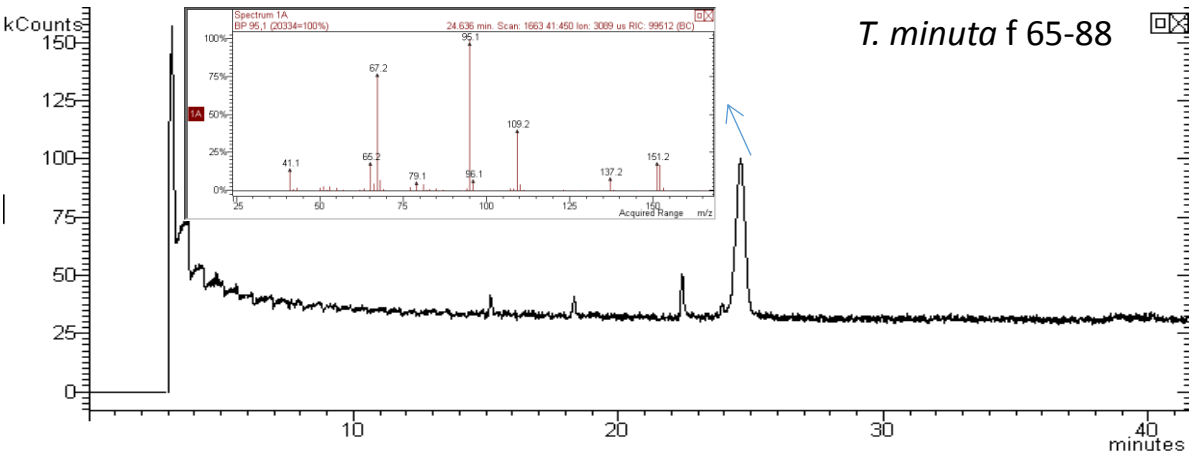


Figure 9. *cis*-tagetone by flash chromatography: GC-MS and NMR characterisation



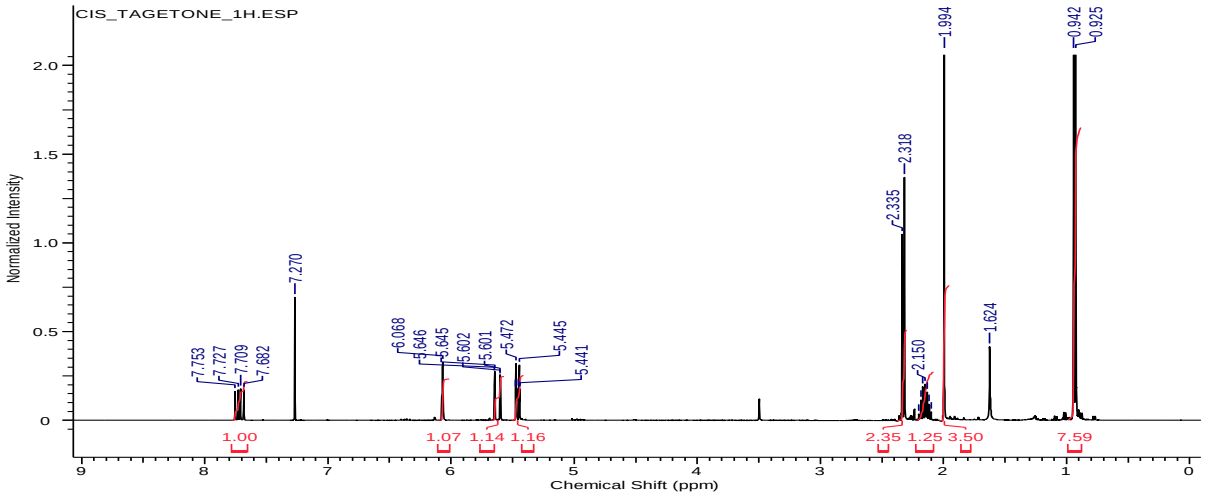
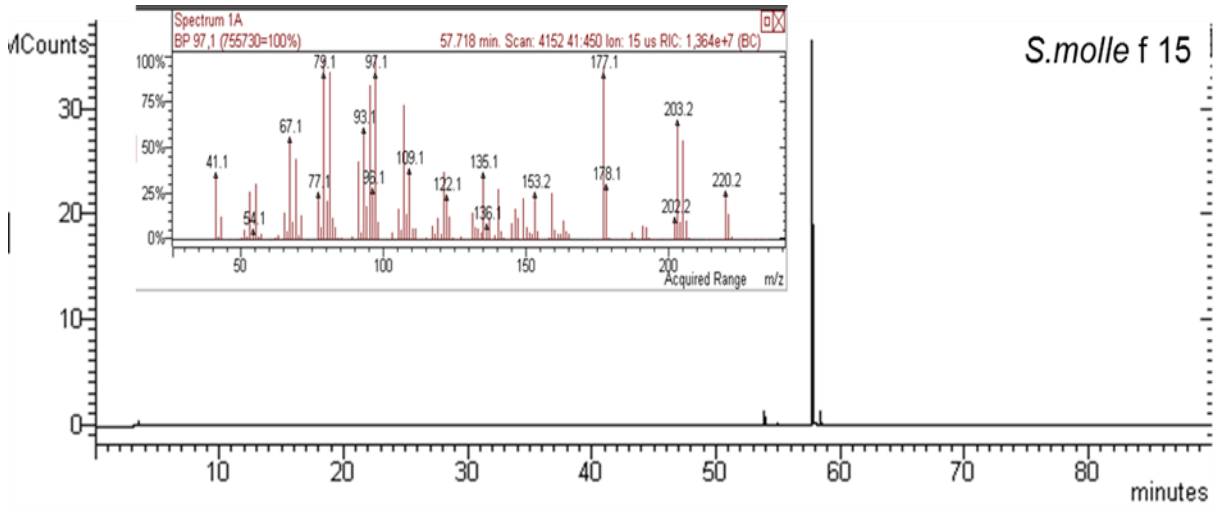
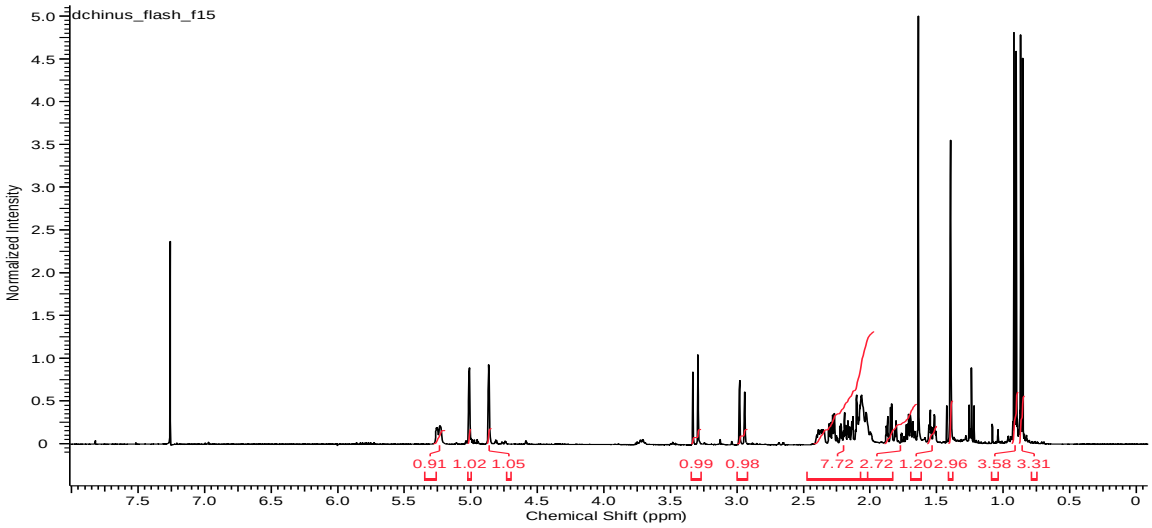


Figure 10. 6-oxo-germacra- 1(10)E,4(15)-diene by flash chromatography: GC-MS and NMR characterisation





Section 2. Results and discussion of the biological activity evaluated in vitro of all EOs compared

Antioxidant activity

Antioxidant activity is the ability to counteract the oxidation process generated by free radicals, compounded by more electron dissipation. Antioxidant activity is known to be the cause of numerous degenerative diseases due to mutagenic processes, carcinogenesis, cardiovascular disorders and aging (Singh and Singh, 2008). The destructive action of free radicals is directed mainly towards cells, in particular the lipids that form cellular membranes, sugars and phosphates, proteins, enzymes, and DNA to alter the genetic information. Free radicals of oxygen (FRO), as well as reactive nitrogen species (RNS), are produced by normal cell metabolism, but can also be introduced exogenously. FRO and RNS play a dual role, may be deleterious or have a beneficial effect (Valko et al., 2006). The beneficial effects of free oxygen radicals appear at low concentrations and involve physiological roles in cellular responses including defence against infectious agents, signal function and induction of a mutagenic response. The harmful effect of free radicals, responsible for potential biological damage called oxidative stress and nitrosative stress (Ridnour et al., 2005), occurs when, on the one hand, there is an overproduction of FRO / RNS and on the other a deficiency of enzymatic and non-enzymatic antioxidants. The balance between the beneficial and harmful effects of free radicals is achieved through a process called "redox regulation", which aims to maintain redox homeostasis (Dröge, 2002). Antioxidants are compounds that act by neutralizing free radicals by acting on one of the three steps of the oxidative process: initiation, propagation and termination (Cui et al., 2004). Antioxidants are also important from a food standpoint as they naturally occur in many foods, preserving or protecting against oxidative damage, avoiding deleterious changes and loss of commercial and nutritional value. There are many EOs that have antioxidant activity, such as EO of thyme, oregano, rosemary, basil, bergamot, cinnamon, clove, tea tree, melissa, mint, sage and black cumin. This protective action is exerted by some of their constituents, including thymol, carvacol, 1,8-cineole, citral, citronellal, terpinene and thymoquinone, which are very effective agents in neutralizing free radicals.

Antioxidants can be classified according to their mechanism of action. Preventive antioxidants are distinguished, able to inhibit or counteract the formation of radicals by anticipating the oxidative phenomenon in the "initiation" phase and "chain-breaking" antioxidants, which can prevent propagation to one or more levels by trapping free radicals formed during the oxidation reaction. Preventive antioxidants have the advantage of having a higher rate of reaction than chain propagation radicals with their substrate; originate from stable radicals and act in hydrogen or electronic transfer (Giovannini et al., 2006).

Due to the high importance of antioxidants, it is necessary to have methods for their detection. Two of the most commonly used methods are the "DPPH" and “ABTS” assays, developed by Blois (1958), with the aim of determining antioxidant activity considering the stable free radical 1,1-diphenyl-2-picryl-hydrazide (DPPH) and *2,2'-azino-bis*(*3-ethylbenzthiazoline-6-sulfonic acid*) (ABTS), respectively. The assays are based on the ability to neutralize the radical DPPH and ABTS by any antioxidant compound. These methods are quick, simple, economical, and widely used to measure the ability of compounds to act as free radicals or hydrogen donors and lends itself to the examination of both hydrophilic and lipophilic substances (Prior et al., 2005). A variant of the classical method of the "DPPH and ABTS” assays was used or the evaluation of the antioxidant activity of EOs considered in this thesis. This altered assay involves the use of 96-well microplates for an even faster and more accurate analysis (Cheng et al., 2006; Kedare and Singh, 2011). Table 15 shows the data of antioxidant activity of EOs in terms of IC_{50} (sample quantity needed to eliminate 50% of radical DPPH and radical ABTS).

Table 15. Antioxidant activity of Ecuadorian EOs

EO	**DPPH test**	**ABTS test**
	IC_{50} (mg / mL)	**IC_{50} (mg / mL)**
Chenopodium ambrosioides	>10 (inactive)	>10 (inactive)
Dacryodes peruviana	>10 (inactive)	>10 (inactive)
Schinus molle	>10 (inactive)	>10 (inactive)
Tagetes minuta	4.98	0.381
Eryngium foetidum	>10 (inactive)	>10 (inactive)
Persea Americana	>10 (inactive)	>10 (inactive)

Piper carpunya	0.5	2.07
Thymus vulgaris	0.5	0.014
thymol	0.071	0.023
trolox	0.003	0.002

Note: Free radical-scavenging activity percentage of 7 EOs evaluated by the DPPH and ABTS assays and comparison with that of the references (trolox, timolo and *Thymus vulgaris* esential oil). Standard deviations are between 1% and 15%.

Three different positive controls included a strong synthetic antioxidant trolox and EO of *Thymus vulgaris* (chemotype thymol) that is known for its strong antioxidant activity among EO, but no comparable to trolox and finally, its main compound timolo. The results showed that *P. carpunya's* EO was the most active with an antioxidant capacity equal that of the *T. vulgaris* control with DPPH method. In addition, *T. minuta* showed antioxidant activity consistent with the results of Ruiz (2011), while it was much less active than in the experiments conducted by Ali (2014), although the chemical composition of the EO is significantly different. The results obtained for *P. carpunya* are very interesting because their ability to eliminate free radicals with such efficiency could be useful and efficient also for the protection of humans against oxidative stress damage, making it particularly applicable to the fields of cosmetic and phytotherapeutic nutraceuticals.

The DPPH and ABTS tests were repeated on the main compounds of EOs to better understand the efficacy of the EO of *P. carpunya* and *T. minuta* mainly. The antioxidant activities of two main compounds, piperitone (*P. carpunya*) and *cis*-tagetone (*T. minuta*) were assessed, as shown in Table 16.

Table 16. Antioxidant activity of main compounds from active EOs.

	DPPH	**ABTS**
	IC_{50} (mg / mL)	**IC_{50} (mg / mL)**
piperitone (*Piper carpunya)*	>10 (inactive)	>10 (inactive)
***cis*-tagetone (*Tagetes minuta*)**	0.53	0.335

Note: Standard deviations are between 1% and 15%

With the results obtained with piperitone, we can rule out that the antioxidant activity shown by *P. carpunya* corresponds to the activity of its major compound. Instead, such activity could be provided by a synergy between *P. carpunya's* various components. In contrast, the main component of *T. minuta* (*cis*-tagetone) may be responsible for the antioxidant activity of this EO. In summary, the activity presented by piperitone and above all by *cis*-tagetone are not described in the literature. Therefore, this research is very interesting and may be a starting point for possible new nutraceuticals and pharmaceutical products.

Antimicrobial activity

Antibacterial activity

Antibacterial activity, evaluated in vitro, varies from one microorganism to another and one EO to the other, but is always dose dependent. The effectiveness of EOs in counteracting the proliferation of microorganisms is directly proportional to the potential toxicity (low therapeutic index), therefore, it is necessary to have a minimum inhibitory concentration (MIC) that is as low as possible to avoid side effects. The mechanism of action of EO with respect to microorganisms is complex and has not yet been clarified because it depends on various factors. The chemical composition is most important followed by a possible synergy between the different compounds, and then by the type of microorganisms considered with respect to the structure of their cell wall.

Due to the significant variety of components present in EOs (most or even small), it is very likely that their antimicrobial activity results from different modes of action at the cellular level. Hydrophobicity allows EOs to be partitioned between the lipids of the bacterial or fungal cell membrane, altering the cellular structures and making them more permeable. Excessive loss of ions and molecules from the microbial cell will inevitably lead to death. Gram-positive bacteria generally appear to be more sensitive to antimicrobials than Gram-negative bacteria, as shown in the following Table 17.

Table 17. Minimum inhibitory concentration (μg / mL) of EOs from Ecuador

	KO	PA	PV	EF	LG	ML	SA
Chenopodium ambrosiodes	>2000	2000	2000	1000	1000	2000	1000
Dacryodes peruviana	2000	2000	1000	500	500	2000	1000
Piper carpunya	>2000	2000	2000	2000	2000	2000	1000
Schinus molle	1000	250	500	500	250	500	500
Tegetes minuta	>2000	500	2000	2000	2000	>2000	2000
Eryngium foetidum	2000	>2000	>2000	500	1000	2000	125
Persea americana	>2000	>2000	>2000	>2000	>2000	>2000	>2000
Thymus vulgaris	>2000	>2000	1000	2000	125	500	1000
Thymol	500	>2000	1000	500	250	500	500
chloramphenicol	250	500	250	<125	250	250	125

Note: [1]KO = *Klebsiella oxytoca*; PA = *Pseudomonas aureginosa*; PV = *Proteus vulgaris*; EF = *Enterococcus faecalis*; LG = *Listeria grayi*, MC = *Micrococcus luteus* and SA = *Staphylococcus aureus*. [2]Standard deviations are between 1% and 15%.

Considering the "strong activity" antibacterial values of MIC ≤ 500 μg / mL (Baser and Buchbauer, 2015), *S. molle* EO was the most effective and showed significant activity against almost all bacteria. Other EOs with less antibacterial activity were *D. peruviana*, *T. minuta* and *E. foetidum.* The lowest MIC was obtained for *Staphylococcus aureus* (MIC = 125 μg / mL), which is a common cause of skin infections including abscesses, respiratory infections such as sinusitis, and food poisoning. Pathogenic strains often promote infections by producing virulence factors such as potent protein toxins, and the expression of a cell-surface protein that binds and inactivates antibodies. Low MIC values were also found for *Pseudomonas aeureginosa* and *Listeria grayi* (MIC = 250 μg / mL). *Pseudomonas aureginosa* can cause disease in plants and animals, including humans, while *L. grayi* is a non-pathogenic and non-haemolytic bacterium.

S. molle from Ecuador, contrary to that studied in Ethiopia (Abrha & Unnithan, 2014), has shown a strong activity against *Staphylococcus aureus*. This result confirms once again that the geographic location of the plant and its epigenetics, regardless of its chemical composition, has a direct influence on its biological activity. Research in Brazil (Deveci et al., 2010) confirms the antibacterial activity of *S. molle*.

In this thesis, *D. peruviana* showed antibacterial activity against *E. faecalis* and *L. grayi*, which provides novel and interesting results for this plant. *T. minuta* from Iran previously showed antibacterial activity against *Salmonella typhii* and *Escherichia coli*

(Shirazi et al., 2014), unlike in this study where *T. minuta* showed activity against *Pseudomonas aeruginosa*. This discrepancy may be due to the fact that the main component of the Iranian plant was methylchavicol, while our main component is *cis*-tagetone.

Finally and one of the key points of this research, was to understand the biological activity of these EOs. For the reason, the antimicrobial tests were replicated on the main components of each oil as shown in the following Table 18.

Table 18. Minimum inhibitory concentration (μg / mL) of main compounds from active EOs

	KO	PA	PV	EF	LG	ML	SA
δ-3-carene (*Dacryodes peruviana)*	1000	1000	1000	1000	1000	1000	1000
α-phellandrene *(Schinus molle)*	1000	1000	1000	1000	1000	1000	1000
cis*-tagetone (*Tegetes minuta)	1000	1000	1000	1000	1000	1000	1000
trans*-2-dodecenal (*Eryngium foetidum)	500	1000	1000	250	250	1000	250

Note: [1]KO = *Klebsiella oxytoca*; PA = *Pseudomonas aureginosa*; PV = *Proteus vulgaris*; EF = *Enterococcus faecalis*; LG = *Listeria grayi*, MC = *Micrococcus luteus*; SA = *Staphylococcus aureus*. [2]Standard deviations are between 1% and 15%.

The results clearly showed the lack of direct relationship between the main component of each EO and its antibacterial activity. The exception was *E. foetidum* for which its major compound, *trans*-2-dodecenal, showed a strong antibacterial activity. This high antimicrobial activity of *E. foetidum* EO and its *trans*-2-dodecenal ingredient supports the results reported by other researchers (Erdem et al., 2015) and Forbes et al. (2014) who published an article on the biological activity of *trans*-2-dodecenal and its comparison with ivermectin in vitro.

Antifungal activity

As with bacteria, EOs are often effective in fighting fungi, such as pathogenic yeast, dermatophytes and phytopathogenic fungi. Visible signs of their action can be observed with morphological and functional micro and macroscopic changes. Most studies on the antifungal action mechanism of EOs were conducted on *Candida albicans*, human fungal pathogens, responsible for mucocutaneous pathologies and,

in some cases, severe systemic infections, especially in nosocomial immunocompromised patients. EOs, given the mainly lipophilic nature of their constituents, seem to primarily affect structural and functional changes in the fungal membranes, destroying the strong impermeability of protons and large ions and subsequent dispersion of the cytoplasm and alteration of the cellular enzymes until they reach cell death (Stringaro et al., 2014). There is often a blockage in membrane synthesis, inhibition of germination, reproduction, and cellular respiration. Studies conducted on dermatophytic fungi are very interesting, where growth-inhibiting EOs could be used in topical products. Finally, in the agricultural field, the study of EOs effective on phytopathogenic fungi is useful in the development of pesticides with less environmental impact. For example, the use of natural parasites for the benefit of integrated treatments is increasingly sought after if not totally organic.

In this study, the activity of EOs was also tested on three phytopathogenic fungi of great interest, such as *Botrytis cinerea* (responsible for grey grain of pear, vine and numerous other fruit plants) *Fusarium oxysporum* (responsible for rooted tomato rotting) and *Pythium ultimum* (caused by root rot and numerous other crops, including cabbage, carrot, cucumber, melon, etc.). Two strains of dermatophytic fungi were also investigated including *Trichophyton mentagrophytes* (causes dermatitis in the face and on the limbs, onychomycosis, or may lead to the appearance of deep, if neglected ulcers) and *Nannizzia gypsea* (reported as ringworm of man, dog, cat, monkey, horse, daman, guinea-pig, mouse and other rodents, rat, rabbit, tiger and chicken), which has been isolated from the fur of wild animals showing no signs of infection. Ajello (1953) provides an extensive review of the distribution of *N. gypseum*.

A total inhibition of growth was observed in many cases at concentrations used in the test, which were significantly lower than the limit allowed for normal formulations. The EOs of *C. ambrosiodes*, *P. carpunya*, and *T. minuta* totally inhibited the growth only of *N. gypsea* (Table 19). This underscores the different specificity of action that EOs present in relation to different species of microorganisms. *Chenopodium ambrosiodes* has been tested as an excellent growth inhibitor for diversal fungi (Jardim et al., 2008), however, our data on *N. gypsea* are new and interesting for the scientific community. Similarly, *T. minuta*, has been tested on *Sclerotium cepivorum*, *Colletotrichum coccodes* and *Alternaria solani* with interesting results (Zygadlo, 1994). Among the phytopathogens, the best result was the effect of *E. foetidum* EO against *B. cinerea*, with an inhibition of growth of 63.5%. A study conducted in the same genus but

different species, *Eryngium duriaei*, showed activity against dermatophyte fungi, however, *N. gypsea* was not tested (Cavaleiro et al., 2011).

Table 19. Antifungal activity of Ecuadorian EOs (growth inhibition %)

	Phytopathogens			**Dermatophytes**	
	Botrytis cinerea	***Pythium ultimum***	***Fusarium oxysporum***	***Nannizea gypsea***	***Trycophitum mentagrophytes***
Chenopodium ambrosiodes	34.5 ± 2.8	43.9 ± 2.5	20.4 ± 2.4	100.0 ± 0.0	10.8 ± 0.6
Dacryodes peruviana	0.9 ± 4.1	10.1 ± 4.6	17.3 ± 3.2	0.0 ± 3.6	1.9 ± 0.4
Piper carpunya	52.6 ± 3.0	56.1 ± 2.9	28.6 ± 3.0	100.0 ± 0.0	9.6 ± 1.0
Schinus molle	0.9 ± 5.2	22.3 ± 3.0	14.3 ± 3.9	18.2 ± 2.9	2.7 ± 0.4
Tagetes minuta	37.1 ± 2.0	18.0 ± 3.1	19.4 ± 3.2	100.0 ± 0.0	7.7 ± 1.3
Eryngium foetidum	63.5 ± 3.6	47.0 ± 2.6	41.3 ± 2.3	62.3 ± 3.5	8.1 ± 0.5
Persea Americana	39.3 ± 2.2	40.5 ± 2.2	27.8 ± 1.5	59.3 ± 3.2	10.0 ± 0.5
thymol	96.5 ± 4.8	96.5 ± 4.8	92.2 ± 4.8	96.5 ± 4.8	100 ± 0.5
clotrimazole	100.0 ± 0.0	98.9 ± 0.6	97.5 ± 0.5	100.0 ± 0.0	100.0 ± 0.0

Note: Data expressed as percentage of inhibition of growth diametrically greater than negative control and relative standard deviation.

Table 20. Antifungal activity of main compounds from active EOs (growth inhibition %)

	Phytopathogens			**Dermatophytes**	
	Botrytis cinerea	***Pythium ultimum***	***Fusarium oxysporum***	***Nannizea gypsea***	***Trycophitum mentagrophytes***
limonene (*C. ambrosiodes)*	41.2 ± 1.0	15.8 ± 2.5	35.6 ± 2.9	44.1 ± 0.9	16.2 ± 1.1
piperitone (*P. carpunya*)	21.5 ± 3.0	14.0 ± 2.9	17.8 ± 1.4	38.8 ± 0.9	12.9 ± 1.8
cis*-tagetone *(T. minuta)	15.9 ± 2.0	1.5 ± 3.1	7.3 ± 3.2	45.6 ± 0.0	2.6 ± 1.3

Note: Data expressed as percentage of inhibition of growth diametrically greater than negative control and relative standard deviation.

Table 20 shows the antifungal activity of the main compounds of the active EOs. These did not prove to have an interesting activity against the fungi tested. With *N. gypsea* a decrease of 50% inhibition with respect to its oils can be appreciated. D-limonene has been tested as a *B. cinerea* inhibitor (Wilson et al., 1997), a fact that corroborates in part in our work, since limonene only has an inhibition close to 50%. The same happens in a work carried out by Marei et al., 2012, who demonstrate that limonene has a 70% inhibition in the *F. oxysporum* strain. The other compounds have also been investigated by other authors, but in those cases, with other strains of fungi like the piperitone that has a strong activity against the *Penicillium citrinum* (Saleh et al., 2006). Finally, it should be noted that antifungal tests with cis-tagetone have not been carried out.

Anticandidal activity

Yeasts are widely distributed in nature and are able to spoil many foods such as wines, cheese, vinegar, beverages, juices, fruits, salads, sugar and meat, causing changes in odour, colour, taste and texture (Ray & Bhunia, 1996). *Candida* spp.*, Pichia* spp.*, Rhodotorula* spp.*, Torulopsis* spp.*, Saccharomyces spp., Zygossacharomyces spp., Hansenula* spp. and *Trichosporon* spp. are documented food spoiling yeasts

(Forsythe, 2013; Wojtatowicz et al., 2001). Microbial spoilage has been an important factor influencing both the cost and food availability (Graham, 1980; Riedel, 2005). Consumers have demanded more natural foods with low levels of chemical additives and less processing while retaining a long shelf life. In addition, food legislation has restricted the use of some synthetic antimicrobials based on a possible toxicity for consumers (Burt, 2004). In this panorama, EOs have emerged as effective compounds to provide the microbiological safety of foods. EOs are composed of many compounds (eugenol, citral, pinene, thymol, cinnamic acid and carvacrol) that are characterized by a prominent antimicrobial activity (Konning et al., 2004). Table 21 shows the MIC of Ecuadorian EOs on one yeast, *Candida albicans,* using the microdilution technique.

Table 21. Anticandidal activity expressed by minimum inhibitory concentration (MIC) and minimum concentration of fungicidal activity (MCF) of Ecuadorian EOs

	***Candida albicans* (ATCC 48274)**	
	MIC (μg / mL)	**MCF (μg / mL)**
Chenopodium ambrosiodes	2500	2500
Dacryodes peruviana	1250	3135
Piper carpunya	312.5	1563
Schinus molle	2500	> 6250
Tagetes minuta	5000	> 6250
Eryngium foetidum	250	> 2000
Persea Americana	>2000	> 2000
Thymus vulgaris	125	125
thymol	62.5	62.5
fluconazole	6.26	6.26

Note: [2]Standard deviations are between 1% and 15%.

Piper carpunya and *E. foetidum* EOs demonstrated novel antifungal activities against *Candida albicans*, with MIC values of 312.5 and 250 µg / mL, respectively. Only investigations of the same genera such as *Piper hispidum* from Venezuela and *Eryngium tricospidatum* from Algeria, showed strong activity against *C. albicans* with MIC values of 200 and 4.6 µg / mL, respectively (Morales et al., 2013; Merghache et al., 2014) .Considering literature defition of "strong activity" (Baser and Buchbauer, 2015) and antibacterial experimental MIC values of ≤ 500 µg / mL, two EOs from *P. carpunya* and *E. foetidum* were the most effective against *C. albicans.* The results obtained with *P. carpunya* were confirmed by previous results in South America (Ruiz Quiroz, 2013) demonstrating a similar anticandidal activity of the *Piper* genus.
Finally, and as previously mentioned, the aim of this research was to understand the biological activity of these EOs. Therefore, the anticandidal test was replicated using the main component of each oil and resulting in the MIC values shown in Table 22.

Table 22. Minimum inhibitory concentration (MIC) and minimum concentration of fungicidal activity (MFC) of main compounds from active EOs from Ecuador against *Candida albicans*

	***Candida albicans* (ATCC 48274)**	
	MIC (µg / mL)	**MCF (µg / mL)**
piperitone (*Piper carpunya)*	2000	2000
trans*-2-dodecenal (*Eryngium foetidum)	500	> 500

Note: [2]Standard deviations are between 1% and 15%.

In the case of *P. carpunya* there is no direct relationship between the main component of each EO and its anticandidal activity. Therefore, the activity shown by *P. carpunya* EO may be due to a synergy between its various components and not only to its major component. However, there are no results on this subject reported in literature. In contrast, the major component (*trans*-2-dodecenal) of *Eryngium foetidum* EO was effective against *C. albicans*, suggesting that it is the primary ingredient responsible for the anticandidal activity of *Eryngium foetidum EO*. These data are consistent with

those of a previous study where *trans*-2-dodecenal showed significant anticandidal biological activity (Janssen et al., 1985).

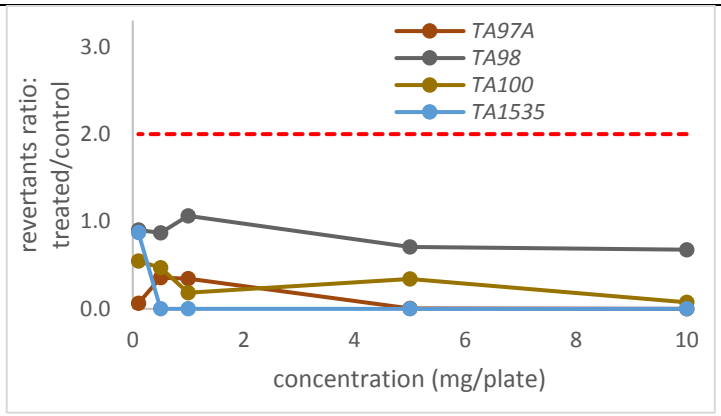
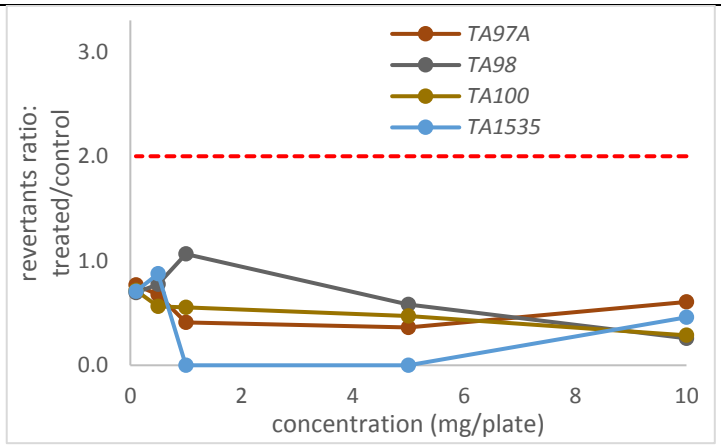
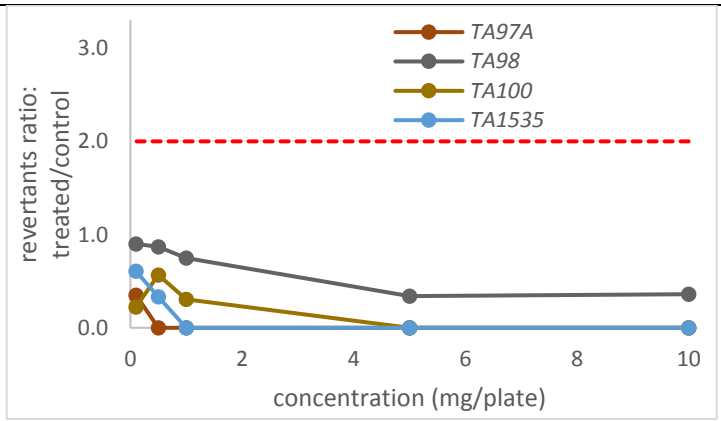
Mutagenic activity

Mutagenic activity was tested using the widespread and consolidated Ames test to verify the genotoxic safety of Ecuadorian EOs. It envisages the use of engineered *Salmonella typhimurium* strains, auxotrophic yields for histidine and biotin and highly susceptible to potential mutagenic substances. The interpretation of results is rather simple and is based on the fact that for each strain a mutation reversion occurs, making it auxotrophic for histidine and biotin by restoring the wild-type status at a characteristic frequency. By placing these bacteria in contact with the substance to be tested and growing on a non-histidine-free soil, biotin will assist in the development of colonies derived exclusively from reverting bacteria. The substance is considered safe if the number of revertent counts remains within a range that does not exceed at least twice that of the revertents found in the negative control (in which only the solvent used to dissolve the substances to be tested is added); the substance is considered a potential mutagen if the number of revertents is twice that of the negative control (Maron and Ames, 1983). It is also important to observe a dose-dependent pattern to exclude false positives, such as isolated reversing peaks, called by Ames "spikes". There are numerous strains of salmonella produced for mutagenic activity tests, each possessing a different mechanism and sensitivity. We have decided to test EOs on four different salmonella strains that can detect almost all potential mutagens; the most stable strains and most commonly used in this type of test are TA97a, TA98, TA100 and TA1535. The TA97a and TA98 strains are useful for detecting mutations that cause frameshifts, deletions or genomic insertions, it only changes the mutation site, which makes them complementary and most sensitive to some substances rather than others. TA1535 and TA100 mainly detect substances that cause base-pair replacement and have a different genome repair system, TA1535 is less efficient and therefore more susceptible to mutations (Thorne et al., 2015). Figures 10 and 11 shows the results obtained for each EO on several strains of *S. typhimurium*.

The results showed that none of the tested EOs were potentially mutagenic. Only three "spikes" were observed in *P. carpunya* for TA1535, *S. molle* for TA97a and *P. Americana* TA1535 but given their isolated character as outlined by the protocol, they

exclude the hypothesis of mutagenicity and are to be considered false positives. A cytotoxic effect was occasionally observed at higher concentrations, particularly in the TA1535 strain, which is attributed to the antibacterial activity of EOs, in accordance with the data observed in the antimicrobial activity tests and consistent with those in the literature (Ipek et al., 2005, Evandri et al., 2005).

Figure 11. AMES test on *Salmonella typhimurium* strains without activator S9

Chenopodium ambrosioides	
Dacryodes peruviana	
Eryngium foetidum	

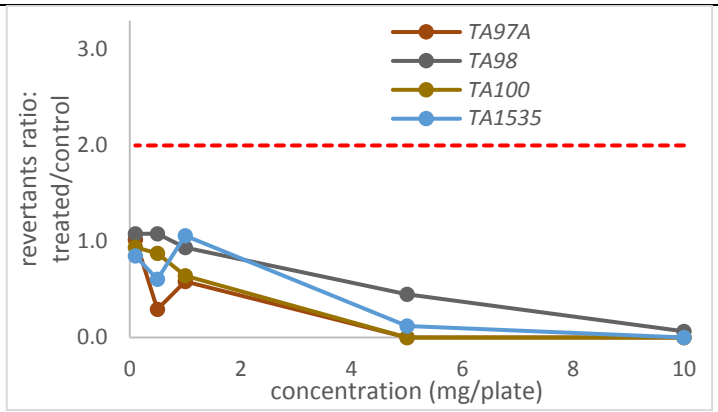
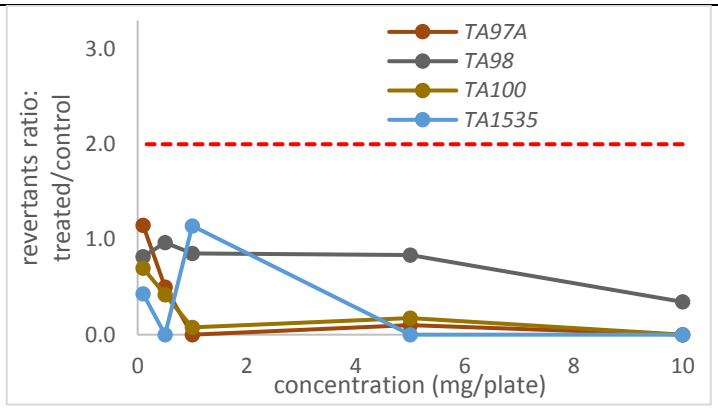
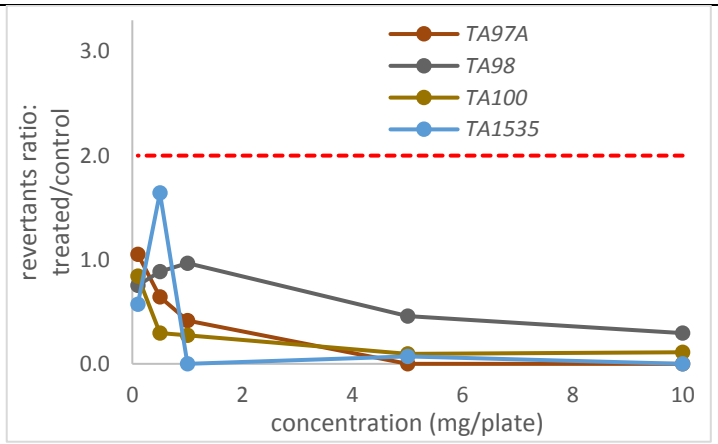
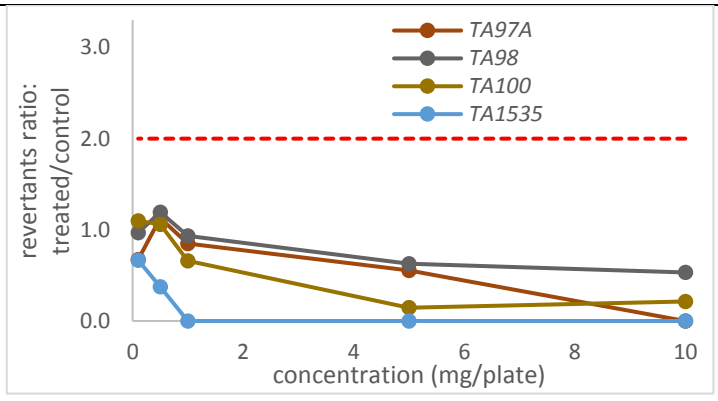
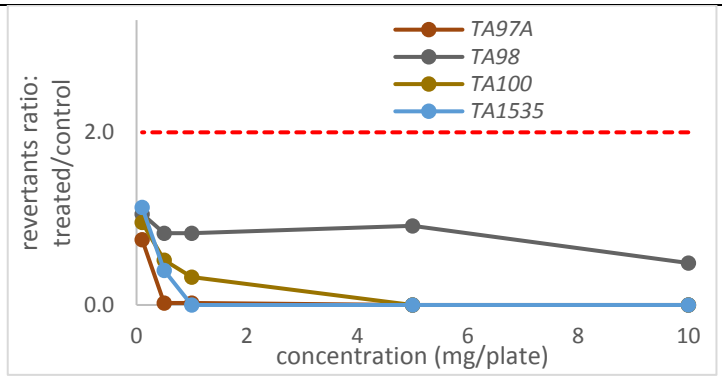
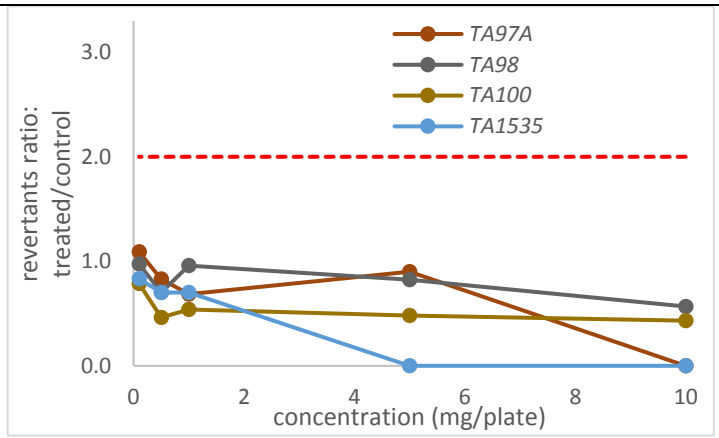
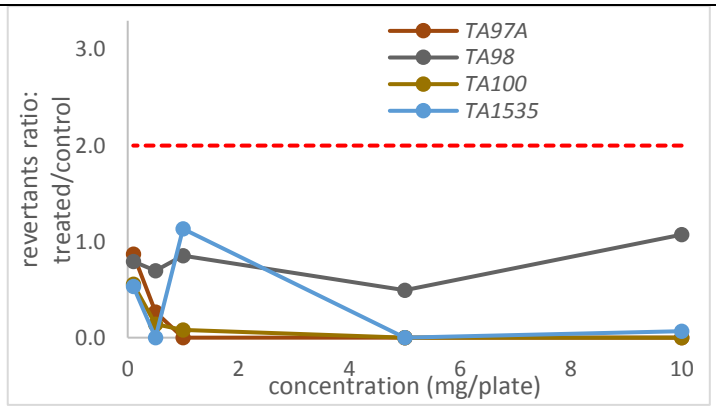
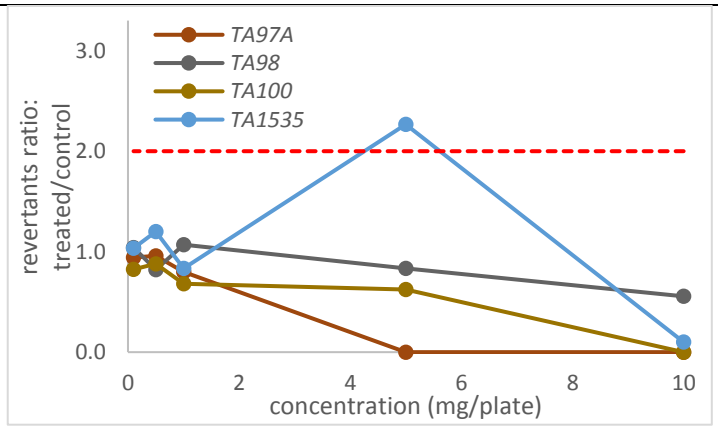
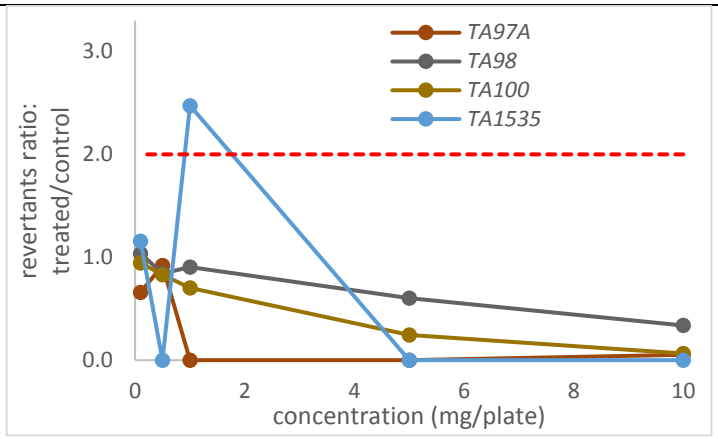
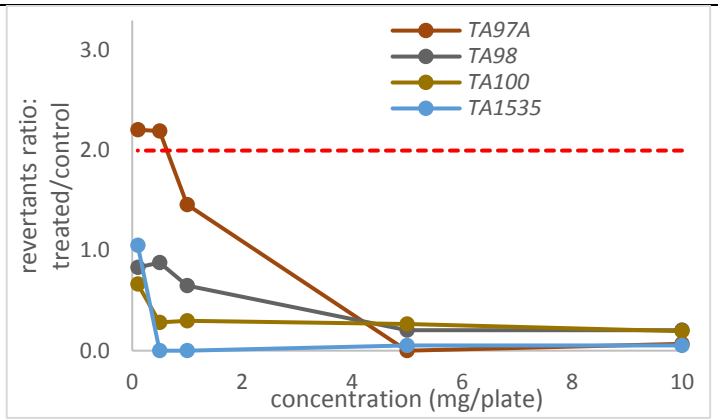
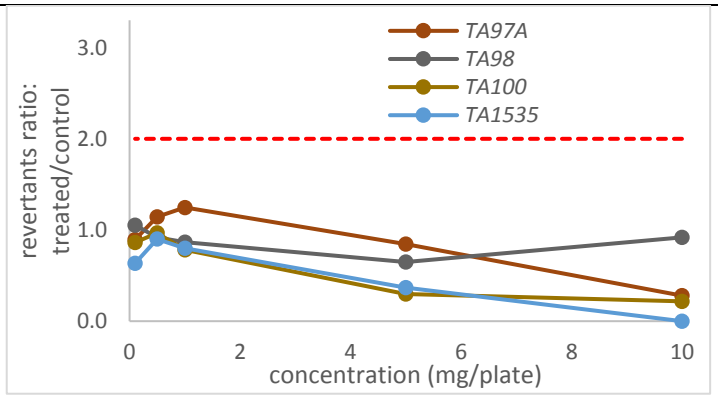
Persea americana	
Piper carpunya	
Schinus molle	
Tagetes minuta	

Figure 12. AMES test on *Salmonella typhimurium* strains with activator S9

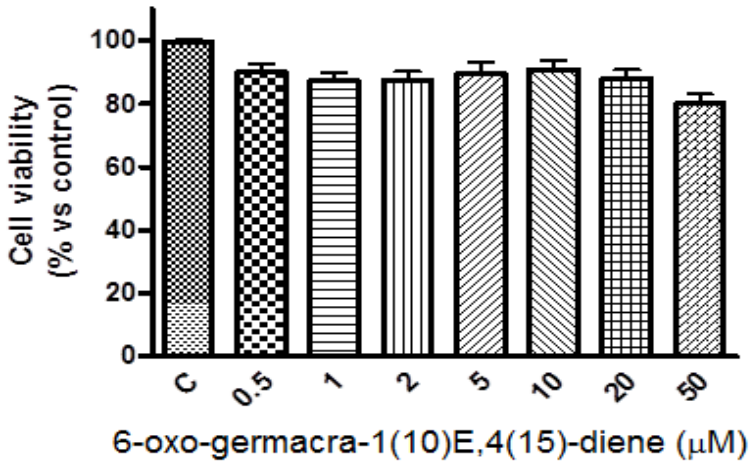
Chenopodium ambrosioides	
Dacryodes peruviana	
Eryngium foetidum	
Persea americana	

Piper carpunya	
Schinus molle	
Tagetes minuta	

In vitro cytotoxic activity

The molecule we have isolated from *E. foetidum* EO, 6-oxo-germacra-1(10)E,4(15)-diene is a derivative of germacrene D, a compound that has been shown to be cytotoxic (Palazzo et al., 2009). As shown in the figure 13, the treatment with 6-oxo-germacra-1(10)E,4(15)-diene did not show cytotoxicity at any of the concentrations tested on the HaCaT cells, this being a new result in the scientific literature. Experiments in progress, on these range of concentrations on tumor cell lines, should allow the potentialities of this molecule as possible anticancer agent.

Figure 13. Cytotoxic activity of 6-oxo-germacra-1(10)E,4(15)-diene on HaCaT cells



In the same way, the main components of the EOs do not show cytotoxic activity according to the current literature (Yoon et al., 2010; Villarini et al., 2014; Shirazi et al., 2014 and Mejia-Barajas et al., 2012**)**. The only exceptions were Piperitone that was reported to be cytotoxic against two human cancer cell lines ANC3A (Uterine), and Hela (Cervical) (Medina-Holguin et al., 2008) and *trans*-2-dodecenal that was toxic on L6 muscle cells (Pillon et al., 2010)**.**

Discussion

Chemical and Biological Activities

The activity of an EO would be expected to relate to the respective composition of the plant volatile oil, the structural conformation of the chemical components of the oil, their functional groups and possible synergistic interactions of the constituents.

The chemical complexity of EOs, often a mixture of dozens of compounds with different functional groups, polarity and chemical behaviour, could lead to scattered results, depending on the test employed. Taking this into account, the *in vitro* antioxidant activity of the 7 EOs tested, compared to that of *Thymus vulgaris* EO oil,

was assessed by two different tests: the DPPH and ABTS. *P. carpunya* and *T. minuta* reduced the concentration of free radical, with an efficacy slightly similar than that of reference oil *T. vulgaris*. Other EOs performed poorly, with an average inhibition percentage lower than 25%. Oils with a higher monoterpenic abundance, such as *E. foetidum* and *S. molle*, were practically ineffective. This result is in agreement with the poor performance given by other oils with similar patterns and by single monoterpenic hydrocarbons (Ruberto & Baratta, 2000). A special mention deserves the compound *cis*-tagetone, which in this work, showed that it could be the compound responsible for the antioxidant activity of *T. minuta* EO.

A correlation of the anti-microbial activity of EO, percentage composition, chemical structure of the components, functional groups and configuration has been done, and a number of observations were suggested (Knobloch et al., 1989). The solubility of EOs and their terpenoid compounds in water is directly related to their anti-microbial activity (Griffin et al., 1999). The variations in the fungicidal and bactericidal action of EO components seem to rely on their solubility and lipophilic properties (Suppakul et al., 2003).

The EO obtained from *D. peruviana* had a high percentage of monoterpenes (73.04%), *S. molle* (22.49%), *T. minuta* (20.70%) and *E. foetidum* (3.7%), that accounts for the good activity against bacterial strains. Presence of an acetate moiety in the structure increases the activity of parent compound (Knobloch et al., 1989). *S. molle* oil had bornyl acetate (0.36%) and γ-eudesmol acetate (0.12%) in minor quantity, in *D. peruviana* borneol (0.21%), and in *T. minuta* borneol (0.13%), which could have contributed to the pronounced activity of the oil. Borneol has been found to be less active than its acetate but active against *Bacillus subtilis*, *Aermonas hydrophila*, *Beneckea natriegens*, *Escherechia coli*, *Flavobacterium suaveolens* and *Serratia marcescens* (Tabanca et al., 2001).

S. molle, D. peruviana, and *E. foetidum* have a number of alcohols, among the most abundant: α-cadinol, α-terpineol and carotol, respectively. Alcohols are known to possess bactericidal rather than bacteriostatic activity against vegetative cells (Knobloch et al., 1989). The alcohols exhibit activity against micro-organism by potentially acting as either protein denaturing agents or solvent dehydrating agents (Nazzaro et al., 2013).

Research has shown that gram-negative micro-organisms are less susceptible to EOs than gram–positive ones because they posses outer membrane surrounding the cell membrane (Ratledge & Wilkinson, 1988) which restricts diffusion of hydrophobic compounds through its lipopolysacharide covering (Vaara, 1992). This explains why the MIC for gram-positive was lower than the MIC for gram-negative bacteria.

Limonene, which was found in appreciable amounts in the EOs of *C. ambrosioides* and *P. carpunya*, has been reported to exhibit anti-fungal activity (Chee et al., 2009). Therefore the remarkable antifungal activity of these oils could be due to the presence of limonene. *T. minuta* EO and its main component, *cis*-tagetone, showed poor anti-bacterial effects, but good anti-fungal effects. The *T. minuta* EO had a high percentage of acyclic monoterpenes. Acyclic monoterpenes possess significant antifungal activity (Hammer et al., 2003).

From literature study, the many biological properties, which have been reported to be exhibited by the differente EOs, may be as a result of the synergistic effects of the chemical constituents combined but not attributed to a single chemical compound (Odalo et al., 2005). This pheno-menon is nevertheless widespread in phyto-chemicals (Nascimento et al., 2000). Further, these chemical constituents may change their biological properties depending on the concentrations in use or composition proportion in which they occur in a given sample (Kametani et al., 2007).

No mutagenic or co-mutagenic potential of EO was detected in the range of concentrations applied; there was no increase in the number of spontaneous revertants compared with the corresponding solvent controls. Moreover, the number of spontaneous revertants decreased in a concentration dependent manner on plates with EO. Safrole and eugenol at high level are known carcinogens in animals and methyleugenol is a suspected carcinogen compound (Maralhas et al., 2006). *P. carpunya* contains these compounds but in an amount less than 4,5% while *P. americana* barely contains 1,52% of methyleugenol. For this reason, and due to the absence of these phenylpropenes in the other EOs, these results confirm their no mutagenic character.

Variability of EO

Organ development

The stage of development of the plant organ (leaf, flower and fruit ontogeny) can be a determinant for the composition of the volatiles compounds (Figueiredo et al., 2008). In several cases, there is an increase in the yield of the volatiles compounds from the flower bud to the mature flower. Concomitantly, the composition can undergo major changes, some components varying from traces to 10% in the initial stages, to 50–70% in the full flowering stage. In other cases, the volatiles are largely accumulated before the organ is fully expanded. According to Máñez et al., 1991, the changes in the composition of the volatiles with the maturation of the organ are directly related to higher rates of cyclization and dehydration of the compounds.

It is also important to mention that differences in the yield and composition with organ development can be partly explained by the different types of secretory structures. Plants with external secretory structures as in the case of *P. carpunya, E. foetidum* and *C. ambrosiodes* can release secretions with maturation of the organ because of trichome cuticle disruption, whereas plants with internal secretory structures more often maintain a more stable yield and composition.

Pollinator activity cycle

The scents of plants, together with nectar availability or pollen maturation, are thus in most cases related to the activity of the pollinator. So, in plants with diurnal pollinators, volatiles emission attains its maximum during the day, whereas those plants having night pollinators, such as bats, mice or nocturnal moths, show a maximum emission during night time. Indeed, the Amazon forest of Ecuador there is an immense amount of pollinators, the reason is obvious, and the Ecuadorian amazon rainforest is in the world one of the most important biodiversity locations. The zoogeographic district is known to protect some 200 mammal species, there are well over 550 bird species, 30% of all of Ecuador's birds. In terms of fish around 630 species. Finally, over 200 reptile and amphibian species have been recorded in the region. There is not enough information about the insects, but the number must range in the thousands (Tylianakis et al., 2004).

Type of plant material

Although quite a few species yield a similar EO composition for their different organs, the composition can also be largely dependent on the plant part used: flowers, green parts (leaves and stems), bark, wood, whole fruits, pericarp or seed only, or roots (Figueiredo et al., 2008; Olawore et al., 2005; Novak et al., 2005). In our case, we used fresh leaves and aerial parts. It is important to mention that the variability can be particularly evident in entomophilous flowers, where compounds can function as orientation clues, and thus the compounds emitted by the flowers or flower parts are distinct from those of the other plant parts. It is also frequent that the higher levels of secondary metabolites are found in young organs rather than in old ones (Máñez et al., 1991; Kuropka et al., 1991).

Type of secretory structure

The differences found in the composition of EOs obtained from diverse plant parts can be partly explained by the existence of distinct secretory structures that are heterogeneously distributed over the plant body. Plant volatiles compounds are produced in specialized secretory structures that minimize the risk of autotoxicity and simultaneously allow the presence of high levels of metabolic components at sites where their defensive and/or attractive role may be vital. The type and location of these structures is, mostly, characteristic of the plant family (Table 23).

Table 23. Examples of different types of secretory structures occurring in some plant families.

Secretory structures	**Families***
External secretory structures	
Trichomes	**Asteraceae**, Cannabaceae, Geraniaceae, Lamiaceae, Plumbaginaceae, Rubiaceae, Rutaceae, Solanaceae, Verbenaceae
Osmophores	Araceae, Orchidaceae, **Piperaceae**
Internal secretory structures	
Idioblasts	Araceae, Aristolochiaceae, Calycanthaceae, **Lauraceae**, Magnoliaceae, **Piperaceae**, Saururaceae
Cavities	Hypericaceae, Leguminosae, Myrtaceae, Myoporaceae, Rutaceae
Ducts / Canals	**Anacardiaceae**, **Apiaceae**, **Asteraceae**, Fabaceae, Hypericaceae, Myrtaceae, Pinaceae

Source: Adapted from Figueiredo et al., 2008. *Apiaceae / Umbelliferae; Asteraceae / Compositae; Hypericaceae / Guttiferae; Lamiaceae / Labiatae; Fabaceae / Leguminosae

In addition, these secretory structures do not always develop in a synchronous way, do not always secrete the same types of compound and can have different secretory processes. Moreover, in some cases, i.e. in the taxa of a genus with internal secretory structures, the chemical composition of the secretion can be similar but the position of the secretory structure may define its final function for the plant, defensive or pollinator-attractive (Armbruster et al., 1997).

Seasonal variation

In some species, the composition of the EO also changes with the time of year, and thus the right time of harvest may be of major importance from an agronomic and economic point of view. For our *P. carpunya* collected before the flowering period the dominant component was piperitone and in the chemical characterisation of the EO derived from leaves collected during the flowering carried out by the research group of Vargas (2004), the major component was safrole.

Apart from the monthly and annual fluctuations, or the changes according to the vegetative or flowering period of the plant, there may also be diurnal fluctuations that seem to be related to the activity of the pollinator, as previously mentioned. In other cases, the variations in the yield and composition of the oils could be correlated with weather parameters (day length, temperature and humidity) and to the attack of fungal pathogens, particularly in the months of rainfall that in the Amazonian Ecuador are very abundant throughout the year.

Mechanical or chemical injuries

The emission of volatiles compounds not only has a stimulant and/or attractive role but also works in a direct or indirect defensive way. Nevertheless, the effect of mechanical or chemical injuries, e.g. wounds, infestation by predators or treatment with herbicides, on the yield and composition of EOs has been little studied.

The plants produce, under normal conditions a bulk of secondary metabolites, which is considered, in all, a constitutive production. When subjected to any type of traumatic injury, new compounds not previously present in the plant can be produced, which is considered an induced production. The difference between constitutive and induced production can be ambiguous, since, for instance, the majority of the volatiles compounds usually released by healthy plants become induced after any kind of injury. In most cases, the compounds are then produced in larger amounts and/or in different proportions. The induced response is not only a function of the species, but depends also on its developmental stage, the availability of water, the amount of light, etc. These are particularly important in our study because the Amazon forest in Ecuador has a high humidity, too many species per square meter and a constant amount of

light, which enriches and provides a particularity with respect to other parts of the world.

Climate

EO production and that of secondary metabolites in general is extremely dependent on the weather conditions. Turtola et al., 2003, showed that under induced stress conditions of drought the total amount of terpenes and resin acids increased, simultaneously with a decrease in the growth of *Pinus sylvestris* and *Picea abies* seedlings. In our research, the influence of this factor is visible in a country close to Ecuador. Our *D. peruviana* collected near to Macas city (940 m.s.l) that has a climate hot-humid with high temperatures throughout the year meanwhile the same *D. peruviana* in Merida, Venezuela (1500 m.s.l) has tropical monsoon climate, with cooler temperatures than other cities in Venezuela, because of its high altitude. The differences in their chemical compositions are described in this work.

Among other effects, drought stress can limit photosynthesis in plants and alter nutrient uptake and carbon, sugar, amino acid and inorganic ion fluxes. In addition, a plant under stress due to adverse weather conditions and/or limited nutrients is more prone to attacks by pathogens and herbivores.

During the months of lowest temperature and fewest hours of sunlight, there is an obvious decrease in secondary metabolites (Tingey et al., 1980). A tropical climate is said to favour the formation of oxidized components in the oils (Badoc & Lamarti, 1991). It is evident then that our plants from a humid tropical climate will be constitutively different to plants from other regions such as Africa that has less quantity of water or Europe with variations in the amount of light.

Pollution

Although known to occur, the detrimental effect of air pollution on secondary metabolites production, and particularly EO components, is difficult to access, since the consequences can be confused with the response to other types of stress. Ozone

is considered one of the major plant pollutants. In addition, the effect of other pollutant agents, such as gases released by vehicles and fires, can be increased by factors such as wind, rain and temperature, among other things. Only a few studies have addressed the effect of air pollutants on EO components, and the results are sometimes contradictory (Bucher, 1984) and differ according to the polluting agent (Judzentiene et al., 2007).

Dust pollution, such as near roadsides, cement factories and quarry works, causes gloom, stoma closure and diminished CO_2 flux causing constitutional changes inevitably. In fact, the point of collection of the plants of our research, is close to a road and near to the Upano River, which brings the waste of the nearest city that in this case is Macas, capital of this region (Figure 2).

Edaphic factors

Several authors considered the type and composition of the soil as one of the determinant factors in secondary metabolites composition and that of volatiles compounds in particular, in addition to other explanations for the differences found in oils of the same species. The growth and survival of many plant species is severely depressed in poorly drained soils, thereby greatly reducing crop and EO yields as well as affecting the volatiles composition. In fact, the Amazonian rainforest can be considered "wet-desert" because its soil is clay-like laterite soils which are acidic and low in nutrients. Many tropical forest soils are very old and impoverished, especially in regions like the Amazon basin where there has been no recent volcanic activity to bring up new nutrients. Amazonian soils are so weathered that they are largely devoid of minerals like phosphorus, potassium, calcium, and magnesium, which come from "rock" sources, but are rich with aluminum oxide and iron oxide, which give tropical soils their distinctive reddish or yellowish coloration (De Moraes et al., 1996). According to Hornok, 1988, the supplementation of the soil with three of the most important nutrients (nitrogen, phosphorus and potassium) has generally shown an increase in the oil yields, although the separate addition of the same nutrients gave different results in the yield and composition of the same oils.

Geographic Variation

There are countless examples of the occurrence of geographic variations of the yield and composition of volatiles compounds, determining, for several species, the existence of distinct chemotypes/chemical races. The different EO compositions of a species found for different origins reflect the different environmental conditions of each particular location and culture conditions (different altitudes, different solar exposition, different soil types, etc.). In addition, we can not forget that all these things mix together, so the differences in oil composition found for different geographical origins are also due to genetic differences.

Genetic Factors and Evolution

Genetic and hybridization studies have shown that the composition of EOs is under genetic control. Natural selection determines changes in the production and composition of the EOs. The mechanisms that lead to the evolution of volatiles formation in plants include: (a) gene duplication followed by divergence, which retains the original enzymatic function, while a new function evolves from the duplicated gene; (b) convergent evolution, where new functions have arisen independently multiple times; (c) evolution of an existing gene, without duplication, resulting in a new enzymatic function, with loss of the original one; and (d) loss of enzymatic activity, caused by several factors, such as hybrid formation, mutations and/or chromosomal rearrangements (Gang, 2005). In any of these cases, these processes lead to changes in gene expression. Changes in the expression may not necessarily lead to loss of the enzymatic activity, but may, for instance, cause the production of secondary metabolites to occur in a different cell, tissue or organ.

Storage

The relative amounts of secondary metabolites may also be affected by the storage method. Although drying can give rise to a number of negative physical and chemical

modifications influencing the quality of the plant, such as changes in appearance and aroma, due to the possible loss of volatile compounds, it may also reduce the growth of microorganisms and prevent some negative biochemical reactions. Light, humidity, temperature, age of the material, contaminations, oxidation, resinification, etc., affect the yield and composition of the volatile fraction to a certain extent. As previously mentioned, the structural part, in our case leaves and aerial parts are also of importance in this respect. These parts are more vulnerable to mechanical damage resulting from handwork, transport and/or crushing.

Other reasons that explain the variability can be the extraction methods. A hydrodistilled material contains a significantly larger proportion of the lower boiling point hydrocarbon and oxygenated terpenes. Different milling techniques might induce modification in the composition of the vegetable matrix and may have an adverse effect on the content of thermally labile compounds (Reverchon et al., 1992).

CONCLUSIONS AND PERSPECTIVES

Conclusions

- This PhD research is a part of a large scale project aimed to value the plant biodiversity that characterizes the Amazon rainforest specifically targeted to aromatic specied belonging to ethnomedicine.
- The selection criterion for plant species to be considered was based primarily on the etnobotanical traditions available bibliographically or locally by oral polls.
- Aromatic plants are best known for traditional medicine as their aroma has always captured the attention of natives. This feature is due to the accumulation of important quantities of EOs, i.e. a complex mixture of highly volatile molecules with predominantly mono- and sesquiterpene hydrocarbons and oxygenated derivatives.
- The composition of EOs, in addition to being dependent on genetic factors, is particularly affected by environmental factors, further extending the biological diversity and providing a wealth of molecules with high potential for industrial applications.
- The antioxidant capacity of *P. carpunya* EO is distinguished by an activity comparable to that of the synthetic control. This activity is not related to the EO main component. It could be instead the result of a synergistic interaction among all its components.
- *T. minuta* EO also has an interesting antioxidant activity, which may be due to the fact that its main component, *cis*-tagetone, demonstrated an antioxidant capacity similar to the positive controls.
- Several antimicrobial tests were peformed in which *S. molle* and *E. foetidum* showed a strong activity against pathogenic bacteria for humans, such as *E. faecalis, P. vulgaris, M. luteus* and *K. oxytoca*.
- *trans*-2-dodecenal, the main compound of *E. foetidum* EO, is an active compound against several bacteria strains. This compound seems to be directly responsible for the biological activity of the EO. This does not happen with *S. molle* since its main component, α-phellandrene, does not show biological activity.
- Almost all EOs had good anti-fungal properties, particularly against the dermatophytic *N. gypsea*. Therefore, the EO of these plants could be used in the development of a drug for the treatment of fungal infection.

- Noteworthy was the anti-candida activity where still *E. foetidum* was the most active. This opens new perspectives on the use of natural substances to support drug therapies.
- From the mutagenic activity test, none of the EOs proved to be positive, guaranteeing their genotoxic safety for humans, even if at *in vitro* stage.
- The bioactivity results confirm the traditional empirical knowledge belonging to the plant species.
- The chemical composition of the EOs of the plants *P. carpunya* and *E. foetidum* contain compounds, which are known to relieve stress (like linalool). Therefore communities should be advised to use the EO to provide cheap aromatherapy for themselves. This could be done by boiling the leaves from the aromatic medicinal plants and inhaling the steam which contains the volatile EOs.
- One of the most significant findings of this study was the isolation of two compounds: *trans*-2-dodecenal and *cis*-tagetone. The remaining main compounds (piperitone, δ-3-carene, limonene, estragole and α-phellandrene) were tested as a mixture. The results evidenced that there is no direct relationship between the activity of EO and the activity of it main compound. Except *trans*-2-dodecenal that even in previous studies, shows to have an interesting biological activity.
- The research finding adds value to the seven aromatic, medicinal plants and lays down significant groundwork for a more comprehensive study on the potential application of EOs from Amazonia in the development of novel, affordable and eco-friendly bioproducts.

In conclusion, this PhD research gave to me the possibility to contribute to enrich the knowledge about medicinal and aromatic plants from Amazonian Ecuador, connecting modern research to traditional knowledge. However, the main research topic that I have learned is to plan a research profile about medicinal and aromatic plants thorugh analytical and biological tools, valorizing and qualifying crude drugs/parts used and their derivates for possible industrial application, with particular reference to cosmetic and food supplement field.

Perspectives

- Other compounds could be separated and chemical characterised to evaluate their biological activities alone and in mixture.
- Antimicrobial activity against the tested phytopathogens under greenhouse and field conditions.
- Studies about the effect of seasons, plant drying, laboratory procedures, environmental variables (for e.g. soil composition, pH, etc.), agronomic conditions aimed to cultivation, and plant onthogenic stage on the yields and chemical composition of EOs.
- Specific studies aimed to prove the safety of the EOs as natural ingredient of cosmetics.
- Synergistic studies starting from mixing the most interesting compounds (for e.g., *cis-tagetone* and *trans*-2-dodecenal).
- Cytotoxic activity of *trans*-2-dodecenal on HaCaT cells to corroborate its toxicity with muscle cells.
- The development of genetic engineering has led to the feasibility of large-scale biosynthesis of natural products, and advancements in tissue culture and fermentation of medicinal plants have opened new avenues for the largescale and highly efficient production of desirable bioactive compounds.
- Tissue culture is a promising alternative for the production of rare and high-value secondary metabolites of medical importance
- Improvements in medicinal plants can be carried out using molecular marker-based approaches applied at the genetic level, and the time required for breeding may be significantly shortened

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CONGRESSES AND PUBLICATIONS

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- Ballesteros J, Spagnoletti A, Grandini A, Tacchini M, Maresca I, Guerrini A, Sacchetti G. (2016). Chemical fingerprinting and bioactivity of essential oils from Ecuadorian's amazon: *Chenopodium ambrosiodes* (Amaranthaceae), *Schinus molle* (Anacardiaceae) and *Dacryodes peruviana* (Burseraceae). International Congress of Biotechnology and Biodiversity. Guayaquil, Ecuador.
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